Cell Stem Cell
Protocol Review

Critical Appraisal of the Side Population Assay in Stem Cell and Cancer Stem Cell Research

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The "Side Population" (SP) discrimination assay is a flow cytometry method used to detect stem cells based on the dye efflux properties of ABC transporters. We discuss the SP assay and its applications in stem cell biology, with an emphasis on the technical challenges related to sample preparation, data acquisition, analysis, and interpretation. We highlight the value of multicolor phenotyping, the impact of DNA ploidy, and the importance of distinguishing graft versus host cells for an appropriate SP discrimination. To improve the consistency and reliability of data between laboratories, we propose a set of recommendations for SP assay data reporting.

The Side Population (SP) discrimination assay is based on the differential potential of cells to efflux the Hoechst dye via the ATP-binding cassette (ABC) family of transporter proteins expressed within the cell membrane. ABC transporters belong to the superfamily of membrane pumps that catalyze ATP-dependent transport of various endogenous compounds and xenobiotics out of the cell. Following sequencing of the human genome, it has been estimated that between 500 and 1200 genes encode drug transporters (Venter et al., 2001). ABCB1 (P-glycoprotein, MDR1), ABCC1-5 (multidrug-resistant proteins, MRP1-5), and ABCG2 (breast cancer resistance protein, BRCP1) are the best-characterized transporter proteins that function to establish the SP phenotype (Robey et al., 2009; Schinkel and Jonker, 2003; Sun et al., 2003; Zhou et al., 2001).

The ability of ABC transporters to rapidly efflux lipophilic fluorescent dyes in vitro serves as the basis of the SP assay, which was first described using mouse bone marrow cells (Goodell et al., 1996). The bone marrow SP has been shown to be highly enriched for functional hematopoietic stem cells (HSCs) and also overlaps with the phenotypically defined $CD117^{+}Sca\text{-}1^{+}Lin^{-}Thy1^{lo}$ HSC population (Camargo et al., 2006; Challen et al., 2010). Indeed, combining SP determination with cell-surface marker phenotyping leads to efficient, reliable characterization of the HSC subset, and HSCs isolated according to these combined traits are one of the most pure and potent adult stem cell populations available.

Since its development more than 15 years ago, the SP assay has emerged as a promising method for identifying stem cell and progenitor populations in different tissues, including umbilical cord blood (Storms et al., 2000), skeletal muscle (Asakura et al., 2002; Gussoni et al., 1999), kidney (Iwatani et al., 2004), liver (Shimano et al., 2003), mammary glands (Clayton et al., 2004), lung (Summer et al., 2003), and forebrain (Kim and Morshead, 2003). Importantly, stem cells that exhibit SP properties are rare in most tissues and often constitute a heterogeneous population, differing with organ type and stage of development. Although SPs are clearly enriched in stem cells, several reports caution that dye efflux is not a common property of all stem cell populations (Terunuma et al., 2003; Triel et al., 2004; Zeng et al., 2009). Moreover, the SP phenomenon is not restricted to the stem cell phenotype because it has also been described in certain differentiated cells in adult tissues. Indeed, ABC transporters are expressed by specialized cells in several organs, including small intestine (Mayer et al., 1996), liver, kidney (Smit et al., 1998), brain microvessel endothelial cells (Schinkel, 1999), epithelial cells of blood-cerebrospinal fluid barrier (Segal, 2000), blood-testis barrier (Choo et al., 2000), and placenta (Lankas et al., 1998). In these tissues, the transporters are thought to play a role in protection against the cytotoxic effects of toxins and xenobiotics (multiple drug resistance) by limiting toxin/drug entry into certain tissues and promoting their elimination into the bile and urine (Fromm, 2000).

The identification of cancer stem-like cells has further raised the interest in the SP technique, and the SP phenotype might explain the resistance of a subpopulation of tumor cells to chemotherapy (Chua et al., 2008; Dean et al., 2005; Hirschmann-Jax et al., 2004; Ling, 1995; Szotek et al., 2006; Wu et al., 2007). SP cells have been identified in a number of cancers where they have been shown to display increased capacity of self-renewal and tumorigenicity when transplanted into immunocompromised mice (Bleau et al., 2009; Chiba et al., 2006; Chua et al., 2008; Haraguchi et al., 2006; Ho et al., 2007; Mitsutake et al., 2007; Patrawala et al., 2005; Wu and Alman, 2008; Wu et al., 2007). The percentage of SP cells in primary mesenchymal tumors appears to correlate with tumor grade and has been proposed as a predictor of patient outcome (Wu et al., 2007). SP cells from colon and breast carcinoma display higher expression of stem-cell-related genes compared to non-SP cells (Haraguchi et al., 2006; Zhou et al., 2007). However, it should be emphasized that, similar to normal tissues, not every cancer contains SP cells. Therefore, SP cells may only represent one of the putative cancer stem cell populations.

Thus, the SP assay constitutes a highly valuable primary purification strategy for isolating potential stem/progenitor cells from

Table 1. Overview of the SP Protocol

SP Protocol Steps	Critical Parameters	Proposed Data Reporting Details
(1) Cell/tissue extraction	stable in vitro cell line culture conditions; fresh primary tissue	type and origin of cell line/tissue; inbred animal strains
(2) Single-cell suspension	optimized dissociation protocol; stable cell concentration (nucleated cells)	type of dissociation protocol (type of instruments and enzymes); dissociation conditions and time
(3) Hoechst dye efflux	appropriate dye concentration; stable incubation conditions (time, temperature, darkness); inhibition controls	Hoechst or other dye concentration in SP test (if other than recommended justification should be provided); cell concentration in SP test; type and concentration of efflux inhibitors
(4) Cell-surface phenotyping	inhibition of cell metabolic activity (cold incubation conditions); antibody type and concentration; dead cell discrimination marker (see Table 2 for published examples and references)	antibody clone and fluorochrome; antibody concentration/dilution and staining conditions (time, temperature)
(5) Data acquisition	flow cytometer with a high power UV laser (50–100 mV); sensitive 'Hoechst red' channel detector; low coefficients of variation (CVs); low sample pressure (Hoechst profile in linear mode); high cell concentration	flow cytometer model used, shear pressure; laser types and power used; emission wavelength, specification of filters
(6) Data analysis	gating strategy including: single-cell analysis with debris exclusion; erythrocyte exclusion; dead cell discrimination; inhibition controls; cell surface phenotyping; cell ploidy	software package(s) used for analysis: display of gating strategy, including percentage of events in each gate; display of examples of gating strategy on dot plots applied before SPgate, including scale used (linear/log) and specification of the population displayed on the plot; display of SP test and inhibition controls to validate the SP gate; for SP cell membrane phenotyping display of SP on phenotyping plot and phenotyped population on Hoechst plot (back-gating)

Chronological steps in the multicolor SP assay (left column) and associated critical parameters (middle column), as discussed in text. Right column: recommendations of parameters to be included for data reporting of SP assay.

various tissues, particularly in the absence of specific cell-surface markers. Nevertheless it is important to keep in mind that in most tissues, the SP phenotype is not exclusive to stem cells. While a number of detailed SP method protocols (Goodell, 2005; Goodell et al., 2005; Lin and Goodell, 2006; Petriz, 2007) and reviews summarizing our knowledge of SP cells in different tissues (e.g., Challen and Little, 2006; Schroeder, 2010; Wan et al., 2010; Wu and Alman, 2008) have been published, here we provide an overview of the principle and potential of the SP assay and focus on the critical parameters that can challenge the experimental design, conduct, and interpretation of results when performing this assay. We highlight the value of combining the SP assay with multicolor phenotyping, as well as the impact of DNA ploidy on SP analysis, particularly in the context of cancer stem-like cells. We also provide suggestions on how to improve reproducibility of the SP analysis between laboratories and how to standardize data reporting from SP assays. An overview of the SP protocol and the associated critical parameters is provided in Table 1.

Principle, Potential, and Pitfalls of the SP Assay

In the early 1990s, it was shown that in vitro cultures of a subpopulation of bone marrow cells are able to efflux fluorescent dyes, such as Hoechst 33342 or Rhodamine 123 applied alone or in combination (Li and Johnson, 1992; McAlister et al., 1990; Wolf et al., 1993). Hoechst is a fluorescent dye that binds all nucleic acids with a preference for the AT-rich regions of the minor groove of DNA (Lalande and Miller, 1979). Hoechst 33342, in contrast to Hoechst 33258, can traverse the intact plasma membrane of living cells. While uptake of the dye occurs uniformly in all cells through passive diffusion, efflux is an active energy-driven process. Only cells expressing a sufficient number of ABC transporters are able to actively efflux the dye out of the cell.

Visualization of the Hoechst profile simultaneously in two distinct channels of the flow cytometer, published first in 1996 by Goodell and colleagues, resulted in a significantly improved resolution of the cell population with efflux properties (Goodell et al., 1996). Optimal SP resolution requires a flow cytometer equipped with an ultraviolet (UV) laser. Hoechst 33342, when excited by UV light, emits fluorescence that can be detected in two distinct channels on the flow cytometer: the "Hoechst Blue" (450/50 nm band-pass filter) and the "Hoechst Red" (675/20 nm long-pass filter) channel, while a dichroic mirror (LP635 nm) is used to split the emission wavelengths. As the "Hoechst Red" channel is more sensitive to small changes in dye concentration, the so called "side population" (SP) cells emerge as a distinct dim "tail" extending first on the left side of G0/G1 cells (often referred to as the main population) toward the lower "Hoechst Blue" signal (for example, see Figure 1), and which is lost upon inhibition of ABC transporter activity. If a UV laser is unavailable on the flow cytometer, Hoechst can also be excited with non-UV wavelengths. However, in our experience, neither Hoechst excitation by the near-UV or violet laser (Telford and Frolova, 2004) nor the use of alternative non-UV excitable DNA dyes (Telford et al., 2007) leads to the sharp SP resolution as observed with traditional UV sources.

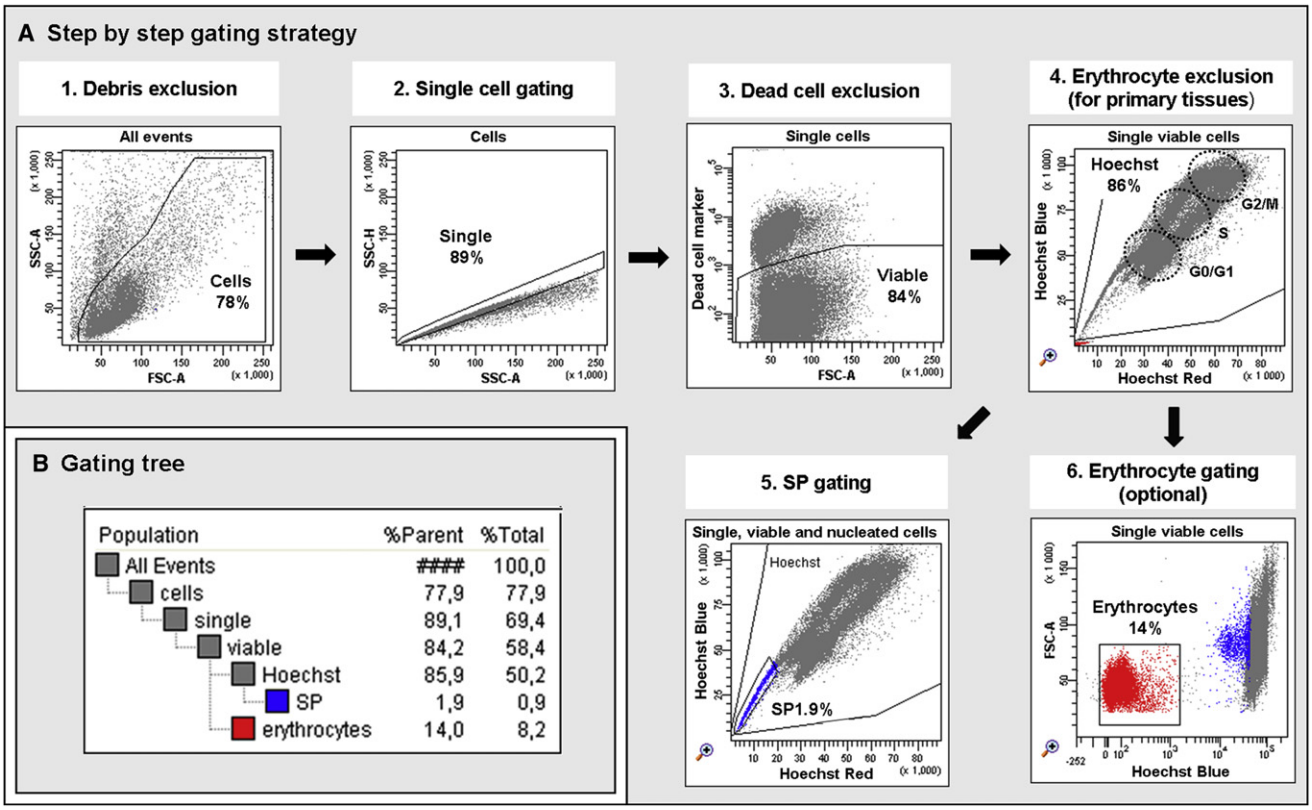

Figure 1. Gating Strategy for SP Data Analysis

An example of a step-by-step gating strategy and the resulting percentage of cell populations is shown for mouse bone marrow, a well-established example of SP-containing tissue (Goodell et al., 1996; Lin and Goodell, 2006). Appropriate discrimination of single, viable, and nucleated cells is crucial for adequate characterization of the SP.

(A) Gating strategy. (1) Cells are distinguished from debris on the flow-cytometric profile based on the Forward Scatter (FSC, related to the cell size) and Side Scatter (SSC, related to cell granularity). (2) To assure that a detected signal arises from single cells, cell doublets and aggregates are gated out based on their properties displayed on the SSC area (SSC-A) versus height (SSC-H) dot plot. (3) Dead cells are recognized by their strong positivity for the dead cell discrimination marker. (4) Due to its function as a DNA-binding dye, the Hoechst dye fluorescence signal can be used to visualize cells in a specific phase of the cell cycle (G0/G1, S, and G2/M) by indicating the DNA content per cell. The cell-cycle status of the major cell populations is highlighted by the dotted circles on the dot plot. For cell suspensions from primary tissue, enucleated erythrocytes (marked in red) can be excluded by applying an additional "Hoechst" gate on the "Hoechst Red"/"Hoechst Blue" dot plot in the linear scale. (5) SP cells are recognized as a dim tail extending first on the left side of G0/G1 cells toward the lower "Hoechst Blue" signal. (6) Erythrocytes can also be recognized and gated as the Hoechst negative cell population on the "Hoechst Red" and/or "Hoechst blue" channel displayed in the logarithmic scale.

(B) Gating tree. The gating tree indicates the sequential procedure applied to select out the final population for SP discrimination and the percentage of cells (gated events) resulting from each gating step. "% Parent" indicates the percentage of gated events relative to the preceding gate, while "% Total" indicates the percentage of gated events relative to all events recorded. The SP assay was performed according to the classical SP protocol (Goodell et al., 1996; Lin and Goodell, 2006). Briefly, cells were resuspended in prewarmed DMEM, 2% FBS 10 mM HEPES (pH 7.4) (1×10^6 cells/ml nucleated cells). Samples were incubated in the presence of 5 μg/ml Hoechst 33342 for 90 min at 37°C with agitation in the dark, followed by rinsing in cold HBSS 2% FBS, 10 mM HEPES (pH 7.4) buffer. All subsequent steps were done at 4°C. The Near-IR Live/Dead marker (Invitrogen) was used for dead cell discrimination (30 min at 4°C in the dark). Data acquisition was performed at 4°C with the FACS Aria SORP cytometer (BD Biosciences) using DIVA software. The analysis of bone marrow SP does not differ between mouse strains; however, the exact percentage of SP may vary according to the genetic background of the mouse. Note that bone marrow isolated from NOD/Scid mice (shown here) gives a higher percentage of SP (1.9%) compared to normal bone marrow (0.3%–0.5%), because of the lack of mature lymphocytes in these mice (Niclou et al., 2008).

Importantly, since the SP assay is based on an active metabolic process, it does not only serve as a positive/negative marker for cell phenotyping, but carries additional information about the functional status of the cells. It also has a significant resolution advantage over classical immunostaining with antibodies against ABC transporters. Due to its high sensitivity, even rare SP events (<0.5% of the total cell population) can be detected within heterogeneous samples. The combination of the SP assay with cell surface phenotyping can lead to a further characterization of putative stem cell and cancer stem cell populations. Moreover, the SP assay, being performed on viable cell populations, enables subsequent functional characterization of the cells in vitro and in vivo, which is not possible with many other DNA-binding dyes.

In contrast to classical cell-surface staining protocols for flow-cytometric analysis, the SP assay requires an additional dye incubation step for the appropriate equilibration of the dye between the extracellular and intracellular compartment prior to dye efflux by cells expressing ABC transporters. ABC transporter-mediated dye efflux is an active and dynamic biological process and, thus, is highly sensitive to even slight modifications in the staining procedure. This sensitivity can easily lead to

irreproducible results and/or discrepancies in results between laboratories, which have called into question the reliability of the SP assay. However, we believe that insight into the critical parameters of the assay (discussed in detail in the next paragraphs) will allow the technique to be adopted by a larger research community and to be applied to less well-studied tissues (e.g., solid tissues, including tumors).

Critical Parameters of the Side Population Protocol

Influence of Cell Integrity, Concentration, and Erythrocyte Contamination

The preparation of a viable single cell suspension is a crucial step in the SP assay. This goal is particularly challenging for solid tissues requiring mechanical and/or enzymatic dissociation steps which inevitably affects cell viability and protein integrity at the cell surface. Therefore, dissociation protocols often require adaptation for each tissue type, to achieve single cell suspensions with minimal cellular damage. The culture conditions of established cell lines may also influence the SP analysis. Since the percentage of SP cells depends on cell density, nutrient composition, serum and oxygen levels, it is advisable to standardize the culture conditions between SP assays to obtain reproducible results (Tavaluc et al., 2007).

In addition to viability, the cell concentration of the single-cell suspension is another key parameter to be considered in the SP protocol. Changing the cell number between samples and experiments directly influences the equilibration of Hoechst between the buffer and the intracellular compartment. Although 1×10^6 cells/ml per test is a standard concentration frequently reported, it is important, when working with primary tissues, that only viable and nucleated cells are counted in the reported cell concentration. In highly vascularized tissue, such as bone marrow or brain, the presence of erythrocytes will strongly influence the Hoechst equilibration and the percentage of SP cells recorded and should, therefore, not be included in the cell count. If enucleated cells cannot be properly distinguished at the time of sample analysis, erythrocyte cell lysis can be performed on a small test sample to recalculate the ratio between nucleated and enucleated cells. Erythrocyte lysis on the whole sample preparation is possible using the classical lysis protocol, e.g., with ammonium chloride, but cannot be performed with most of the commercially available lysis solutions, which cause cell fixation. Nevertheless, as cell viability and cell number are already compromised after tissue dissociation, we prefer to avoid the lysis step. Also, any pre-enrichment technique, including magnetic sorting, is not advisable for small-volume samples because it may compromise cell number and viability. A valuable alternative to mechanical or chemical separation techniques is to first exclude erythrocytes from the cell count prior to Hoechst staining and, second, from the analysis by implementing an additional gate to select only nucleated cells (example in Figure 1A, erythrocytes depicted in red). If present, enucleated erythrocytes appear as negative events in the ''Hoechst red'' and ''Hoechst blue'' channels and can properly be visualized if one of the Hoechst channels is displayed in the logarithmic scale (Figure 1A). In this context, it is important to realize that an adequate gating strategy during data analysis, focusing on single, viable, nucleated cell populations, is crucial to reliably determine the presence and the percentage of the SP (Figure 1).

Staining Protocol: Influence of Hoechst Concentration and Hoechst Toxicity

The SP discrimination is also greatly affected by the concentration of Hoechst 33342 (Montanaro et al., 2004). As different dye concentrations (3–20 μg/ml) are reported in the literature, a direct comparison of results is often impossible. While 5 μg/ml Hoechst is considered standard for most tissues, including bone marrow, some tissues, such as muscle and skin, were found to require a higher dye concentration for optimal SP resolution (Gussoni et al., 1999; Montanaro et al., 2004). To increase the chance of SP detection, researchers tend to decrease the concentration of the dye, not realizing that cells with an unsaturated Hoechst staining can be mistaken for SP cells (see example in Figure 2A). Alternatively, in SP-containing tissues, non-SP cells can be inappropriately included in the SP gate. On the other hand, oversaturated Hoechst can lead to increased cell death, poor resolution, or even total loss of the SP. For newly studied tissues, we propose to perform Hoechst saturation and toxicity dose response curves, with the optimal dye concentration lying within a plateau region, where the percentage of SP cells is stable (Figure 2A). Importantly, the Hoechst concentration curve needs to be combined with transporter inhibition tests in order to confirm the specificity of the dye efflux (see below).

A main pitfall of the SP assay is its high sensitivity to slight variations in staining conditions. Stable incubation conditions such as temperature (37°C), duration, and light conditions (darkness) are essential for proper dye equilibration and to minimize cellular toxicity. Although a range of incubation times have been reported for different species, in our hands, no significant difference between SP discrimination and frequency was detected for mouse and human cells (using incubation times between 90–120 min). In case of doubt, a time-course experiment can be performed to assess the minimal incubation time required for best SP discrimination. For adequate temperature control, the Hoechst incubation step is classically carried out in a water bath. In order to avoid manual mixing of the tubes, dye incubation can also be successfully performed in a 37°C incubator (e.g., tissue culture incubator) equipped with a gentle shaker. Importantly, each step following the Hoechst staining, including washing, centrifugation, antibody staining, and data acquisition, should be performed in the cold (4°C) and in the dark, thus minimizing metabolic activity of the cells and preserving the Hoechst profile.

As a DNA-binding molecule, Hoechst is toxic for cells, particularly at high concentrations. The toxic effect is even more profound upon exposure to UV light. Therefore, another major caveat of the SP assay is the effect of Hoechst staining on cell survival after SP analysis and cell sorting. There is, at present, a strong controversy regarding the clonogenic and tumorigenic potential of stem cell/cancer stem cell SP and non-SP populations within cell lines, primary tissues, and tumor biopsies (Adamski et al., 2007; Camargo et al., 2006; Wu et al., 2007; Zheng et al., 2007; Zhou et al., 2007). The possibility that increased clonogenicity of SP cells is based on their efflux capacity leading to decreased Hoechst toxicity is difficult to exclude. It has also been suggested that cell viability is minimally affected by Hoechst at the concentration used but may, rather,

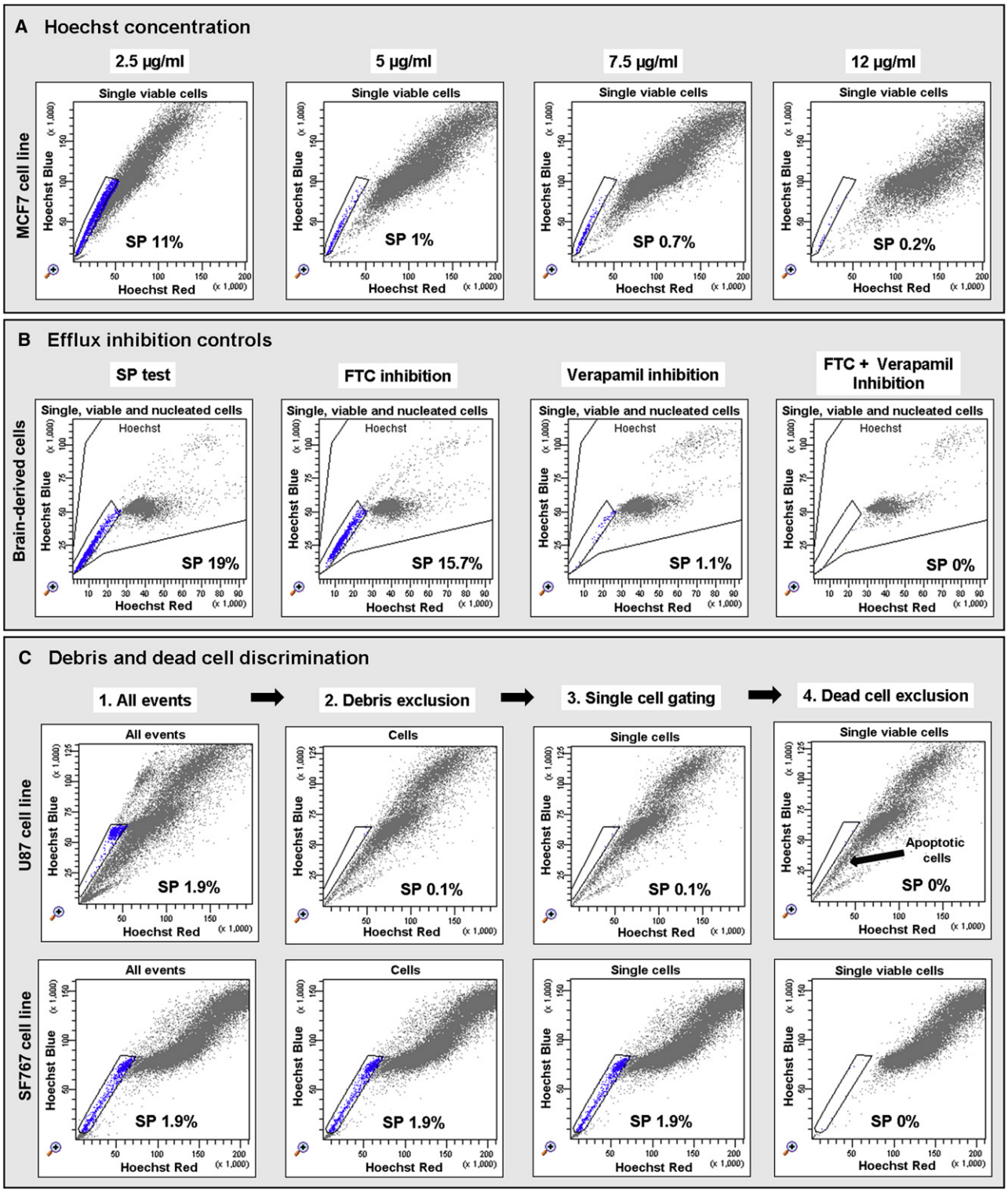

Figure 2. Critical Parameters in the SP Protocol

(A) Hoechst concentration. The importance of the appropriate Hoechst concentration for the reliable detection of the SP is shown for the MCF7 cells, a well-established SP-containing cell line (Patrawala et al., 2005; Yin et al., 2008). Since the optimal dye concentration for best SP resolution may vary according to the cell type analyzed (Montanaro et al., 2004), a Hoechst concentration curve can be performed (e.g., 2.5–12 µg/ml). Based on efflux inhibition controls (data not shown), 5µg/ml of Hoechst is sufficient for dye saturation in these cells (SP∼1%). Decreasing the Hoechst concentration below 5 µg/ml leads to an unsaturated Hoechst profile where non-SP cells are introduced in the SP gate (SP ≈ 11%), while elevated Hoechst concentrations lead to reduced SP resolution (SP < 0.2%). Only single viable cells are displayed on the dot plots.

be compromised by the cell-sorting step (Camargo et al., 2006; Goodell et al., 1997). In view of this unresolved issue, we recommend including functional viability assays in vitro or in vivo of any isolated fractions in order to detect any confounding factors related to dye toxicity and cell sorting.

Significance of ABC Inhibition Controls

A significant number of ABC transporter inhibitors is available, which are characterized by different specificities toward distinct ABC family members. Verapamil, cyclosporine A, and probenecid target the ABCB1 protein (Potschka and Loscher, 2001), while Fumitremorgin C (FTC) is highly specific in its ability to block the ABCG2 protein (Rabindran et al., 2000; Yin et al., 2008) because it exhibits minimal effect on ABCB1 and ABCC1-5 transporters. Nevertheless, some of the inhibitors of ABCB1 and ABCC1, such as imatinib, also inhibit ABCG2 activity (Robey et al., 2009). Indeed, no selective inhibitor has been identified thus far that blocks the ABCC subfamily or its individual members (Loscher and Potschka, 2005b).

Appropriate SP discrimination relies heavily on transporter inhibition control experiments, particularly for very rare SP events (0.1%–0.5%). The observed loss of SP following transporter inhibition allows a confirmation of SP identity and excludes any erroneous SP events. The high variability observed in the published SP gating strategies and the lack of justification thereof represents a major problem in the literature: in some reports, the SP gate is placed close or even within the G0/G1 region (and/or apoptotic cells) while in others it is limited to the low side of the SP. Transporter inhibition controls allow an unequivocal definition of the boundary between the SP and non-SP cell population, thereby justifying the gating strategy (Figure 2B).

It should also be noted that dye efflux can be triggered by several types of transporters expressed within the same cell or within distinct subpopulations of the SP. For instance, the tissue distribution of ABCG2 overlaps extensively with that of ABCB1, and expression of different transporters has been detected within the same tissues (reviewed in Robey et al., 2009; Schinkel and Jonker, 2003; Sun et al., 2003; Zhou et al., 2001). Furthermore, ABC transporter expression changes during organ development. For example, SP cells in the fetal brain are sensitive to the ABCG2 inhibitor Ko143, whereas in the adult forebrain, the SP is inhibited by the ABCB1 inhibitor verapamil, despite the fact that both SP populations comprise mainly cells of endothelial origin (Mouthon et al., 2006).

To ensure maximal transporter inhibition leading to a precise adjustment of the SP gate, it is preferable to establish a dose-response curve for several inhibitors. If necessary, e.g., in poorly defined tissues, this optimization step may even be conducted with different Hoechst concentrations. Only those populations showing decreased efflux properties upon inhibition can be considered as a valid SP. If SP cells show sensitivity to several inhibitors, controls containing a combination of two or more chemicals can be applied (for example see Figure 2B). In the presence of a heterogeneous SP, where cell subpopulations react differently to varying inhibitors, the SP gate may be optionally divided into several subgates according to the separation profile and the SP behavior in inhibition tests. In summary, because of the high sensitivity of the SP protocol, it is highly advisable to include inhibition controls in each experiment, thus validating the specificity of the Hoechst profile obtained. In addition, it is useful to include a well-defined SP model as a positive control and validate the inhibition controls simultaneously in the known model and in the specific population of interest.

Significance of Dead Cell Discrimination Prior to SP Analysis

Adequate exclusion of dead cells is crucial for any flow-cytometric data analysis. Dead cells exhibit properties distinct from their viable counterparts, such as unspecific antibody binding. The Hoechst dye binds to all DNA, whether associated with live, dead, or apoptotic cells or debris. Dead cells can inappropriately appear as SP and, thus, an appropriate gating strategy plays an essential role in defining the Hoechst profile of single, viable, and nucleated cells (Figure 1). In many cases, dead-cell particles, which could be mistaken for SP cells, can be gated out by debris exclusion, as shown for the U87 cell line (Figure 2C) that does not contain SP cells (Bleau et al., 2009). However, for other SP-negative cell lines, additional dead-cell discrimination is indispensable (e.g., SF767 cell line; Figure 2C) and can be easily integrated in the SP analysis by application of an adequate dead-cell discrimination marker.

Furthermore, during apoptosis, the cellular DNA content is diminished, as degraded DNA leaks out during cell rinsing and staining (Compton, 1992). Because some cell lines show a high proportion of apoptotic cells, which display a uniform decrease of the signal in both Hoechst channels, apoptotic cells can easily be mistaken for SP cells (see, e.g., U87 cells in Figure 2C, arrow).

The original protocol developed by Goodell and colleagues for bone marrow employed propidium iodide (PI) for dead-cell discrimination. Although successfully used as a dead-cell marker, PI is excited by the UV laser and is detected in the ''Hoechst red'' channel, giving rise to an additional PI signal in

(B) Effux inhibition controls. An appropriate SP discrimination and gating requires control experiments with one or more ABC transporter inhibitors. An example of inhibition tests with Fumitremorgin C (FTC) and verapamil is shown for brain tissue (see e.g., (Loscher and Potschka, 2005a)). Cells were incubated with FTC and verapamil (2μM and 100μM respectively) for 20 min prior to and during Hoechst staining. FTC leads only to a partial inhibition of brain-derived SP, while significant inhibition is observed with verapamil. Co-incubation with the two inhibitors results in full SP efflux inhibition, indicating that the efflux potential in brain SP is determined by more than one transporter. Importantly, the control with the strongest inhibition effect is used for the final set up of the SP gate. It is advisable to include a 'positive control' by performing the inhibition tests simultaneously on a well defined SP model.

(C) Debris and dead cell discrimination. To illustrate the importance of adequate debris and dead cell discrimination on SP detection, two examples of SP analysis in cell lines that do not contain SP cells (U87 and SF767 glioma cells) are shown. The SP profile is displayed for (1) all events recorded, (2) cell events after debris exclusion, (3) single-cell events after aggregate exclusion, and (4) single viable cells after dead-cell exclusion. The percentage of events displayed in the SP gate for each population is displayed on each dot plot. Most of the putative SP events in U87 cells can already be eliminated after appropriate debris exclusion, whereas in SF767 cells, dead-cell discrimination is required to abolish inappropriate SP events. Inadequate Hoechst concentrations and gating strategies may explain controversial results for U87 and other glioma cell lines reported in the literature (Bleau et al., 2009; Chua et al., 2008; Hirschmann-Jax et al., 2004; Patrawala et al., 2005). A uniform decrease of the signal in both Hoechst channels represents apoptotic cells which should not be mistaken for SP (see U87, arrow on dot plot 4). SF767 cells were obtained from the UCSF Tissue Bank.

Table 2. Examples of SP Phenotyping in Different Tissues

Tissue Type	SP Cell Type Characterized	Phenotyping Markers	References
Bone marrow	hematopoietic stem cells	Lin^- $Sca\text{-}1^+$ $CD117^+$ $CD34^{low}$ $Flk2^-$ $CD45^+$ $CD44^+$	Goodell et al. (1996); Camargo et al. (2006); Lin and Goodell (2006); Challen et al. (2010)
Lung	bone marrow-derived cells	$CD45^+$ $CD34^+$ $CD31^+$; $CD45^-$ $CD34^-$ $CD31^{+/-}$	Summer et al. (2003); Summer et al. (2004)
Liver	very heterogeneous bone marrow-derived population	$CD45^+$ $CD34^+$ $CD117^+$ $Sca\text{-}1^+$ $Thy1^+$; $CD45^-$ $CD34^+$ $CD117^+$ $Sca\text{-}1^+$ $Thy1^+$	Wulf et al. (2003)
Skeletal muscle	muscle stem cells; bone marrow-derived cells	Lin^- $Sca\text{-}1^+$ $CD45^-$; Lin^- $Sca\text{-}1^+$ $CD45^+$	Asakura et al. (2002); Gussoni et al. (1999)
Testis	testis stem cells	$CD45^-$ $CD34^-$ $Sca\text{-}1^+$ $CD117^{+/-}$	Lassalle et al. (2004); Falciatori et al. (2004)
Brain	endothelial cells; astroglial cells; microglia; fetal neural stem cells	$CD31^+$ $CD133^+$ $CD45^-$; $A2B5^+$; $CD11b^+$; $CD15^+$ $CD133^+$ $CD31^-$	Mouthon et al. (2006)

Reports on multicolor phenotyping of SP populations from different tissues. In many tissues (lung, liver, muscle, brain) several cell types with different phenotypic profiles were identified within the SP.

the Hoechst profile, which can be confusing for nonexperienced researchers. Since PI is also excited by a blue laser, measuring PI emission on a logarithmic scale using a different PMT (BP630/30) has been recommended in order to achieve a more precise discrimination of dead cells (Petriz, 2007). Recently, novel dead-cell discrimination markers have been developed, which are not excited by the UV laser and show a narrow range of emission wavelength. In our experience, such markers, including the new DNA-binding reagents (e.g., TO-PRO-3 iodide) or cell membrane dyes (e.g., the LIVE/DEAD Fixable Dead Cell Stains) are of a superior value for SP analysis, particularly in combination with multicolor phenotyping.

Multicolor SP Phenotyping

A major challenge in stem cell biology is the identification of specific cell-surface markers that unambiguously characterize a given stem cell population. Although the dye efflux property has been found to be a valid feature for isolating potential stem/progenitor cells from various sources, the SP populations are often heterogeneous. In several studies the combination of the SP assay with additional phenotyping significantly increased the purity of stem cells (references in Table 2). Multicolor phenotyping is becoming a standard in flow cytometry and can be combined with the SP assay if the antibody staining is carried out in the cold (4°C). Such phenotyping provides essential information as to the cellular subpopulations present within an identified SP. In Figure 3, we provide an example of the potential data display for SP phenotyping in heterogeneous tissues, such as the brain and bone marrow. In order to determine the heterogeneity of the SP, the distribution of SP cells can be shown within the phenotyped cell population (e.g., Figure 3A). In addition, the Hoechst profile can be displayed for the population of interest to indicate the percentage of cells containing the dye efflux property (e.g., Figure 3B). A "back-gating" of the phenotyped populations is useful to validate the gating strategy, as small inadequate shifts in gating can significantly influence final results. Moreover, as the prolonged SP protocol can lead to instability of the Hoechst profile, studies addressing SP subpopulations with multicolor phenotyping require efflux inhibition tests in combination with the cell-surface staining (as in Figure 2B). It should also be noted that within a heterogeneous SP where varying levels of ABC transporters are expressed, different subpopulations can occupy distinct positions within the SP gate, with certain cells exhibiting stronger efflux properties than others (Montanaro et al., 2004). In this case, dividing the SP gate into subgates can be helpful.

Although potentially challenging, multicolor phenotyping of the SP provides important critical information: first, as mentioned earlier, the SP may contain not only a uniform somatic stem cell population, but also early progenitor cells, which may have lost their stem cell potential and phenotypic signature but kept the dye efflux property. For example, it has been shown that cell surface phenotyping in the bone-marrow-derived SP leads to further purification of the HSCs, excluding other precursor cells and multipotential progenitors (Camargo et al., 2006; Lin and Goodell, 2006). Second, the SP can contain more than one stem cell population. In solid tissues, such as lung, liver, or skeletal muscle, the SP has been found to be very heterogeneous and includes not only resident stem cells but also bone marrow derived stem cells (Asakura et al., 2002; Summer et al., 2003, 2004; Wulf et al., 2003). The testis SP population has been found to consist of spermatogonial, germinal, and mesenchymal stem cells (Falciatori et al., 2004; Lassalle et al., 2004). Last but not least, in certain organs, ABC transporter-expressing cells are not associated with stem cell properties. For example, in the brain, ABC transporters are widely expressed in endothelial cells, where they play a major role in the maintenance of the blood-brain barrier (Cooray et al., 2002; Hori et al., 2004; Orford et al., 2009; Zhang et al., 2003). Indeed, within fetal and adult brain, endothelial cells constitute the vast majority of the SP population (Mouthon et al., 2006).

In summary, it is important to emphasize that ABC transporters are not exclusive to stem cells, and the term "side population cell" should not be equated with "stem cell." Cell surface multicolor phenotyping can provide important additional clues on the identity of the SP cells. Also of interest in this respect is the presence of internal markers or the cell-cycle profile of the SP, which can, however, only be examined after sorting of the SP cells. Obviously, in order to confirm the stem cell nature of phenotyped SP cells, downstream characterization at the functional level is indispensable. Thus, the identification of bona fide stem cells/cancer stem-like cells always requires in vitro and in vivo

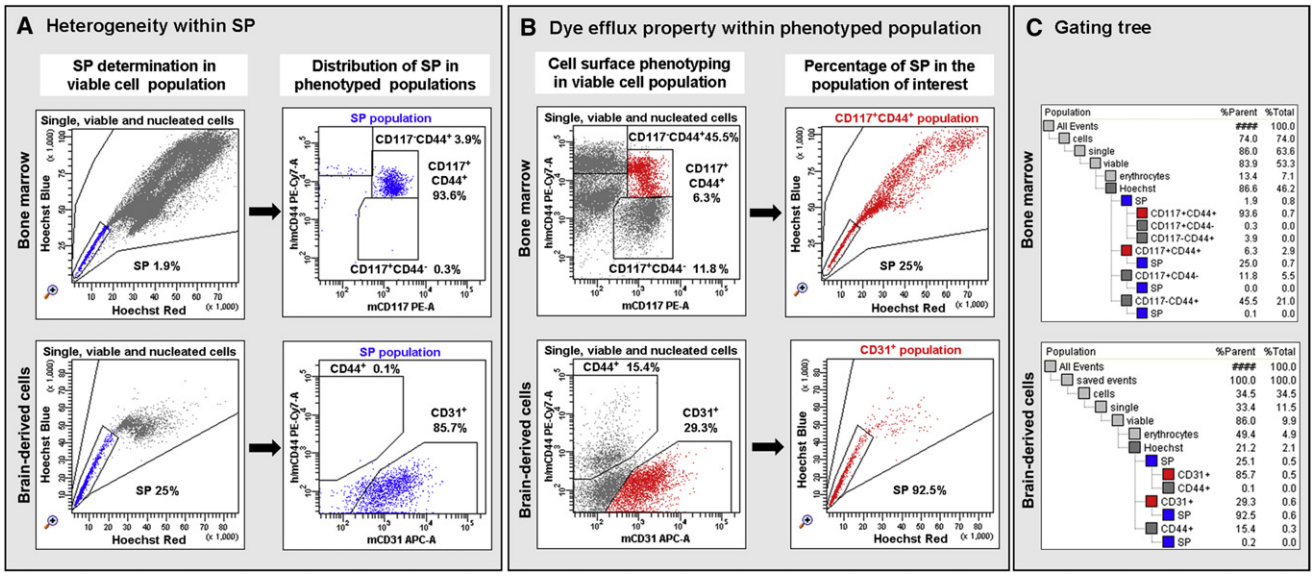

Figure 3. Multicolor SP Phenotyping

Examples of multicolor phenotyping of the SP are displayed for mouse bone marrow and brain as reported, e.g., in Camargo et al. (2006), Lin and Goodell (2006), and (Mouthon et al. (2006). The Hoechst profile (A) and the phenotyped profile (B) of single, viable, and nucleated cells is displayed.

(A) Heterogeneity within SP. To determine the heterogeneity of the SP, the gated SP events can be displayed on the phenotyped dot plot.

(B) Dye efflux property within phenotyped population. Conversely, the stained population of interest can also be shown on the Hoechst plot to indicate the proportion of phenotyped cells that carry the dye efflux property. Importantly, the phenotyped SP subpopulations need to be further confirmed by inhibition controls.

(C) Gating tree: the gating trees of bone marrow and brain cell populations, indicating the appropriate percentage of cells within each gated population relative to parent or total events. SP multicolor phenotyping (CD117/CD44) in bone marrow: ~93.6% of bone marrow SP cells are $CD117^+CD44^+$, while $CD117^-CD44^+$ represent only less than ~3.9% of bone marrow SP (A). Importantly, $CD117^+CD44^+$ cells are heterogeneous, and only 25% of them display the dye efflux property (B). SP multicolor phenotyping (CD31/CD44) in brain tissue: ~85.7% of brain-derived SP cells are $CD31^+CD44^-$ corresponding to endothelial cells (A), and ~92.5% of the $CD31^+CD44^-$ cells display the dye efflux property (B). The SP protocol was as in Figure 1. Cells were subsequently incubated with antibody conjugates for 30 min at 4°C in the dark (mCD117-PE Immunotools no. 22151173 10 μl/test; h/mCD44-PECy7 eBioscience no. 25-0441 1.2 μl/test; mCD31-APC BD Bioscience no. 551262 5 μl/test), in the presence of the Near-IR Live/Dead marker (Invitrogen). Results were confirmed with inhibition controls combined with antibody staining as outlined in Figure 2B.

functional assays, including colony-forming cell assays, cell differentiation assays, engraftment into lethally irradiated mice, or xenografts in immunocompromised animals.

Influence of Tumor/Host Populations and Cell Ploidy in SP Analysis for Cancer Stem Cells

With a growing focus on cancer stem cell populations, the SP discrimination assay has recently been used to assess the presence of putative cancer stem cells in a variety of cancer cell lines and primary tumors. Cancer research models are not perfect, and each carries its own advantages and pitfalls. For patient biopsies or animal models (xenografts or transgenic models), flow-cytometric analysis, including the SP assay, is often hampered by the heterogeneity of the sample and the lack of distinction between tumor and normal cells (Bleau et al., 2009; Harris et al., 2008). In the current literature, putative cancer stem cells within tumor biopsies or xenografts are often characterized with a limited number of phenotypic markers, while neglecting that other differentiated cell populations or stromal cells within a tumor can display similar phenotypic properties. It is worth noting that SP cells within a tumor mass do not always represent tumor cells but can arise from stromal populations carrying dye efflux properties. For example, in brain tumors derived from mouse models, the SP is, similar to the normal brain, largely composed of mouse endothelial cells, and the nonendothelial SP of mouse brain tumors could be tumor and/or stroma derived (Bleau et al., 2009; Mouthon et al., 2006) (see also Figure 3). Therefore, a further characterization of the SP population detected within a tumor mass, based on genotypic and phenotypic profiling, is mandatory in order to confirm their cellular origin. In xenograft models commonly used in cancer research, the discrimination between transplanted tumor cells and normal host cells is possible, though rarely done, either by phenotyping with species-specific and/or cell type-specific antibodies or by marking one population with a fluorescent label. In this respect, the use of a GFP-expressing immunodeficient mouse model is of great value (Niclou et al., 2008) (see also Figure 4). Comparison of a tumor-derived SP with the Hoechst profile from healthy tissue can also facilitate data interpretation, but it should be kept in mind, though, that tumors attract many different cell types, including endogenous stem cell populations from distinct parts of the body. Gliomas, for example, which are highly heterogeneous brain tumors, are known to recruit different types of host cells, such as neural stem cells, mesenchymal stem cells, and endothelial cells (Bjerkvig et al., 2009; Ziu et al., 2006). Moreover, the aberrant composition of the hematopoietic system in immunocompromised mouse models used in cancer research, such as Nude, NOD/Scid, or NOG mice, needs to be taken into account because these mice lack certain differentiated cell types, such as mature lymphocytes (Niclou et al., 2008).

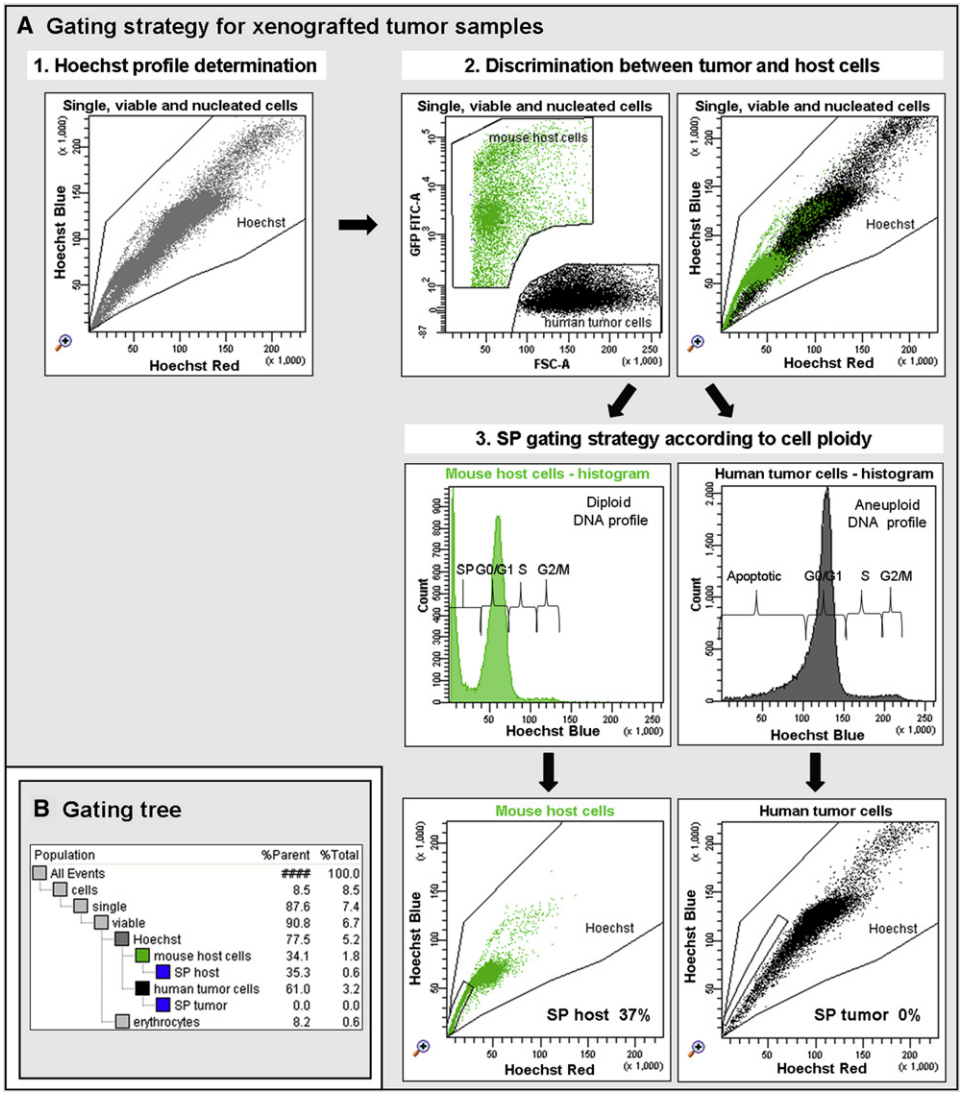

Figure 4. Influence of Graft/Host Populations and DNA Ploidy in the SP Analysis

In xenografted cancer models the discrimination between grafted (tumor) cells and endogenous host (stromal) cells is crucial for SP analysis, since both cell populations can include cells with SP and/or stem cell properties. The heterogeneity of tumor samples (tumor versus stromal cells) needs to be taken into account also in patient biopsies and in transgenic animal models. An example of the SP analysis is shown for an orthotopic glioma xenograft (U87 cells) in the eGFP expressing NOD/Scid mouse (as in (Niclou et al., 2008)). SP cells of gliomas are known to express both endothelial and stem cell markers, whereas U87 cells do not possess SP properties (Bleau et al., 2009).

(A) Gating strategy for xenografted tumor samples. (1) Highly heterogeneous Hoechst profile are displayed within single, viable, and nucleated cells of the tumor mass. (2) Human tumor cells are recognized as the eGFP^{-} population of relatively big cells compared to the eGFP^{+} mouse host cells. Tumor (black) and host (green) populations displayed on the Hoechst dot plot suggest that the two populations display different levels of ploidy. (3) The DNA histograms visualize the aneuploidy of tumor cells (U87; Clark et al., 2010) and the normal diploid profile of mouse stromal cells. The overlap of the tumor G0/G1 cells with the G2/M mouse population is responsible for the overlap on the Hoechst SP profile plot. See also Figure 1 for cell-cycle status visualized on dot plots. After segregation, the appropriate SP gates are adjusted for the tumor and host populations according to their ploidy. The SP should be expected as a distinct tail appearing next to the G0/G1 population, regardless of their ploidy level. The possibility to distinguish tumor (black) from stromal cells (green) clearly shows that in this example all SP cells are derived from the host tissue (37% of host cells).

(B) Gating tree. The gating tree indicates the percentage of cells within each gated population relative to parent or total events.

An additional difficulty in the SP discrimination in cancer research can arise from varying ploidies within tumor cells undergoing clonal selection. Because Hoechst fluorescence intensity correlates with the DNA content of the cell and the chromatin structure, it can distinguish different stages of the cell cycle as well as distinct ploidies within a tumor mass (Arndt-Jovin and Jovin, 1977). Blood and bone marrow consist of mononuclear and polynuclear cells, displaying a characteristic Hoechst profile (see, e.g., Figure 1). Cancer cells often exhibit aneuploid populations, leading to a more heterogeneous Hoechst profile and a misinterpretation of the SP assay results. Therefore, we propose to assess the DNA content of tumor cells within the SP assay and adjust SP gates appropriately according to the ploidy of the cells. An example of such a SP gating strategy

applied to a xenograft tumor is presented in Figure 4, where within the tumor mass normal diploid stromal cells can be segregated from aneuploid tumor cells.

To summarize, tumor samples consist of heterogeneous cell populations, including tumor and stromal cells. Therefore, the discrimination between tumor and stromal cells within an SP analysis may lead to a better understanding of the efflux property within the tumor mass and its role in chemoresistance during treatment. Furthermore, it is advisable to consider the cell ploidy during data analysis of the Hoechst profile, as separate SP gates may need to be applied for diploid and aneuploid cells. If the discrimination is not possible based on marker expression or on ploidy (e.g., diploid tumors), SP cells can be sorted and tested for the presence of genetic aberrations associated with tumor cells, followed by functional assessement with in vitro and in vivo assays confirming their tumorigenic origin.

Data Reporting of SP Assay Results

Flow cytometry, including the SP assay, depends on stringent protocol optimization in order to achieve maximum validity and reproducibility, and efforts have been made recently to standardize the reporting of flow-cytometry-based results in peer-reviewed publications (Alexander et al., 2009; Lee et al., 2008). Since the SP protocol often requires appropriate adaptations for different tissues and species, we believe that standardizing the reporting of data can help to improve comparisons between laboratories. We, therefore, propose a list of parameters related to cell preparation and staining, data acquisition, and analysis that could be included in the methods section of publications (summarized in Table 1). Although there are well-defined populations used in different research centers, the protocol details can vary across laboratories, making results challenging to compare across publications. More specifically, as different enzymatic dissociation procedures can introduce variability to the SP analysis, inclusion of protocol details on the generation of cell suspensions is useful. Because of the high sensitivity of the assay to dye staining conditions, details on the Hoechst staining procedure should be provided. Moreover, as for any antibody-based method, it is advisable to provide precise information on the antibody (as recommended in Alexander et al. [2009]) because a small change, e.g., in the antibody concentration, can potentially lead to a very different outcome. Therefore, we emphasize the importance of providing precise information on Hoechst and antibody staining conditions as well as gating strategies to facilitate the comparison of data between reports. Importantly, in order to standardize the data reported by different laboratories, we also recommend adding essential parameters of the flow cytometer instrument and settings used for data acquisition. In this Protocol Review, we present examples of possible data analysis and display for publications (Figures 1–4). Most importantly, a clear outline of the gating hierarchy during SP data analysis (exemplified in Figure 1) and appropriate justification by inhibition controls can strongly improve the reliability of the assay and the comparison of results between laboratories.

Concluding Remarks

During recent years the SP assay has proven to be an extremely valuable approach for the characterization and isolation of putative stem cell and cancer stem cell populations, particularly in the absence of specific markers. Due to its high sensitivity, it allows the detection of very rare events within heterogeneous cell populations. However, the exponential increase in stem cell and cancer stem cell research in recent years has led to a large amount of reported SP assay data with sometimes questionable interpretations. The dynamic nature of the dye efflux property, relying on an intact cell metabolism, results in greater technical variations in the determination and quantification of SP cells than direct immunopurification procedures. In this Protocol Review, we provide suggestions on how to improve the reliability and reproducibility of the SP assay, with a particular focus on SP protocol optimization, adequate controls for all parameters, and addressing the heterogeneity of SP populations by multicolor phenotyping and functional assays. We emphasize the challenges of SP analysis in cancer stem cell research with regard to discrimination of tumor and stromal cell compartments and diploid versus aneuploid cell populations. Finally, standardizing the reporting of data, rather than standardizing the protocol, may provide significant benefit to the scientific community in the interpretation of SP results.

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