Flow cytometry of the Side Population: Tips & tricks

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Abstract. *Background*: The Side Population (SP) has become an important hallmark for the definition of the stem cell compartment, especially in the detection of these cells and in their physical isolation by fluorescence-activated cell sorting (FACS). SP cells are CD34^{neg} and were discovered using ultraviolet excitation based on the efflux of Hoechst 33342 (Ho342). Although the method works as originally described, we believe that this method is difficult for most 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 the analysis of the acquired data requires an extensive expertise on flow cytometry to accurately detect the SP events. *Methods*: First of all and mainly for the SP application, the laser beam paths were exhaustively checked to ensure the lowest coefficients of variation. Blood suspensions were prepared by erythrocyte lysis with ammonium chloride and hematopoietic cells were labeled with Ho342. *Results*: The Ho342 concentration and the staining procedure are critical for the optimal resolution of the SP cells. Although UV laser alignment is very important to resolve the dim tail that outlines the SP, the problem with Ho342 excitation is not the Hoechst Blue emission, but rather the Hoechst Red's (because of the weak emission). *Conclusions*: Each laboratory must establish its own expected ranges based on its instrument and results may vary slightly due to instrument differences such as the narrowness of the band pass filters, laser power, laser emission wavelength, nozzle type, differential of pressure, light collection system (cuvette versus jet-in-air) and beam shaping optics.

Keywords: Side Population, stem cells, CD34-negative, Hoechst 33342, flow cytometry

1. Introduction

In 1996, Goodell et al. described for the first time a very rare subpopulation of murine bone marrow (BM) stem cells [3]. This population was termed Side Population (SP), and was found to be enriched more than 1000 times in hematopoietic repopulation ability, in comparison with non-SP cells. The detection of this population is carried out by flow cytometry and ultraviolet (UV) excitation of the BM cells pre-incubated with Ho342, which has two emission wavelengths, in blue and red. Based on the Ho342 dual (or double) emission, the SP profile appears as a small fraction of cells forming a dim tail extending from the vast majority population of cells, which are positive for both Ho342 emissions. This profile was reversed when staining was performed in the presence of verapamil, and so it was attributed to a multidrug resistance (mdr) or mdr-like protein mediated efflux of Ho342 [4]. Zhou et al. identified this protein as ABCG2, a half-transporter that belongs to the ATP binding cassette transporter superfamily [12].

SP cells are present in a wide variety of tissues in addition to BM, including skeletal [1] and cardiac muscle, endothelial tissue [5], nervous system [8], fetal liver [11], pancreas [6], skin [7] and umbilical cord blood [9], and can be found in all mammal species

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studied so far, which include humans, mice, rats, pigs and non-human primates. SP cells are present at a very low number in adult tissues, usually representing less than 0.1% of the total cell content. ABCG2 expression is believed to represent a common molecular mechanism for stem cells possessing multi-organ plasticity. Recent studies suggest an evolving concept of the stem cells as a function instead of a phenotype, based on cell fate transitions following tissue injury (reviewed by Blau [2]).

The SP has become an important hallmark for the definition of the stem cell compartment, especially in the detection of these cells and in their physical isolation by fluorescence-activated cell sorting (FACS). A comprehensive protocol for Ho342 staining and isolation of hematopoietic stem cells (HSC) can be found online and downloaded to be reproduced by different laboratories (http://www.bcm.edu/labs/goodell/ protocols.html). However, analysis of Ho342 fluorescence requires a UV laser providing excitation at ~350 nm, which is relatively uncommon in standard flow cytometers. We have performed Ho342 analyses as described by the authors. Although the method works as described, we believe that this method is difficult for most 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 the analysis of the acquired data requires an extensive expertise on flow cytometry to accurately detect the SP events.

Although some authors claim that Ho342 SP analysis can be carried out on flow cytometers equipped with near-UV laser diodes, cell sorters with gas lasers are still required for physical sorting of SP cells [10]. Here we describe how apparently minor steps of the protocol can be important and we outline their relevance for the identification of the SP. In this regard, we show that analysis can be heavily affected by laser power settings and alignment, cell viability, the batch of Ho342 used, sample incubation and sample origin. The aim of this work is to provide specific guidelines for the SP cell analysis and isolation by FACS.

2. Materials and methods

2.1. Chemicals and drugs

Ho342 and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vera-

pamil (Vpl) was obtained from Knoll Laboratories (Mt Olive, NJ, USA). The ABCG2-specific inhibitor fumitremorgin C (FTC) was a generous gift from Dr. S.E. Bates (National Cancer Institute, Bethesda, MD, USA).

2.2. Hematopoietic cells

Bone marrow (BM) and apheresis (AP) samples were obtained from healthy donors and patients undergoing stem cell transplantation. They gave their written informed consent according to the protocol approved by the Ethical Committee of the Hospital Clinic of Barcelona. Samples were processed during the first two hours after stem cell harvest. Cord blood (CB) units were obtained from the Cord Blood Bank of Barcelona. Murine C57BL6/J BM samples were obtained from 8–12 week old mice by flushing their femora.

2.3. Ammonium chloride lysing procedure

All samples were lysed with NH_4Cl and centrifuged at 483 rcf for 5 min. This solution is used to lyse erythrocytes and consists of 1.5 M NH_4Cl, 100 mM $NaHCO_3$, 1 mM disodium EDTA and H_2O to 900 ml (adjust pH to 7.4 with 1 N HCl or 1 N NaOH). Then H_2O was added to 1 litter and stored up to 6 months at $4°C$. Stock solution was diluted $1:10$ to make working lysing solution fresh before use. If BM processing and sorting could not be performed on the same day, the sample was lysed, stained and kept at $4°C$ overnight prior to cell sorting. Red cells were lysed and the stained BM sample was kept at $4°C$ to maintain cell viability. Cord blood specimens were stored at $4°C$ at the Cord Blood Bank, and then they were collected and processed within the first 24 hours likewise the BM samples. For the density gradient experiments, a Ficoll Separation Solution (Biochrom, Berlin, Germany) with a density of 1.077 g/ml was used.

2.4. Flow cytometry and UV setup

Data were collected on a MoFlo® (DakoCytomation, CO, USA) jet-in-air high-speed sorter. Dual-laser analyses used a water cooled Enterprise II ion laser (Coherent, CA, USA) with a multiple output operating at 351 nm and 488 nm wavelengths. Beam Shaping Optics were installed, giving an elliptical focal spot of $20 \times 60\ \mu$m. Three-laser analyses were carried out us-

ing an air cooled helium–neon laser (Spectra-Physics, CA, USA) operating at 633 nm. Hamamatsu H957-15 photomultiplier tube types mounted in the MoFlo® cytometer have a spectral range of 185–900 nm and provide extremely high sensitivity, ultra-fast response and improved far red sensitivity. In order to obtain the lowest coefficients of variation (CVs), both laser and mirror alignment were optimized using fluorescent beads, prior to analysis. We used Fluoresbrite BB Carboxylate 4.5 μm microspheres (Polysciences inc., WA, USA), Flow-Check^{TM} fluorospheres (Beckman-Coulter, FL, USA) and SpectraComp Particles (Dako-Cytomation) for 351, 488 and 633 nm laser alignment respectively. CVs below 3.5% were considered acceptable. Hoechst blue and red signals were detected through a 405/30 and 670/40 nm band-pass filters respectively. A 440 nm long-pass dichroic mirror was used to split the emission wavelengths and the signals measured in linear scale. Ho342 uptake and retention depend on cell membrane integrity. Therefore, this integrity must be assessed in Ho342 staining experiments by parameters other than light scatter (lifegate). Simultaneous staining with PI, a non-permeant dye that does not stain living cells, is used to overcome artifactual retention phenomena. PI was excited at 488 nm and its emission was measured in logarithmic scale through a band pass filter of 630/30. Optimal resolution of the SP cells was found using 30 mW of power with the UV laser.

2.5. Laser alignment and quality control

First of all and mainly for the SP application, the laser beam paths were exhaustively checked. The 488 nm beam was good, with no clipping or diffusion of the beam. Pulse shape on 488 nm parameters was uniform with an acceptable width. The UV beam had some clipping with some diffusion. However, the pulse shape on the UV parameters was not uniform and was slightly wide. The UV alignment was adjusted to remove the clipping and the beam shaping optic was cleaned. Beads were then run and the system was aligned. The UV parameter pulse shape was improved, and after fine tuning the UV beam alignment the pulse width was uniform. The overall effect was an improvement in blue and red sensitivity and lower CVs. Beads were then run on the system and data acquired against time to monitor the stability of the UV path over time. The stability of the system was monitored over several hours after the initial work. On subsequent days the stability was monitored from a "cold start up" of the

system, and after a restart after an hour shut down. We found that the Enterprise II laser requires a minimum of 30 min warm up for beam stability. Over this period the beam quality improves giving more homogeneous bead populations and an increased sensitivity. During the warm up period there is a little drift on the system over time, but this means approximately 10 channels (linear scale) in the first 30 minutes. Increasing the power on the UV laser resulted in lower CVs. Following warm up and prior to analysis we check the laser alignment. First, UV power is set to 1 mW (low power) and we seek for the best CV. To maximize the UV fluorescence and scatter signals we routinely use 4.5 μm diameter fluorescent particles with an emission maximum of 407 nm and analyzed through a band pass filter of 405/30 and a 440 DLP filter. With the detector in linear mode, the flow rate is adjusted to less than 300 events per second. Acceptable CVs using a MoFlo® with operating conditions of high pressure (60 psi-70 μm nozzle tip; 0.1 of differential pressure) are ⩽3.5%. However, each laboratory must establish its own expected ranges based on its instrument and results may vary slightly due to instrument differences such as the narrowness of the band pass filters, laser power, laser emission wavelength, nozzle type, differential of pressure, light collection system (cuvette versus jet-in-air) and beam shaping optics.

2.6. Hoechst 33342 labeling

All samples, with the exception of murine BM cells, were stained using the same protocol. After red cell lysis, cells were resuspended in DMEM (Gibco BRL, Grand Island, NY) prewarmed at 37°C supplemented with 2% heat-inactivated fetal calf serum (FCS) (Gibco BRL) and 10 mM HEPES at a density of (1–2) × 10^6 cells/ml. Ho342 was added at a concentration of 5 μg/ml or 10 μg/ml respectively depending on the density of cells per ml. Samples were incubated in a water bath at 37°C for 2 hours (human samples) or 90 min (murine samples) with periodic agitation. Cells were centrifuged at 483 rcf for 6 min at 4°C and resuspended in cold HBSS/2% FCS/10 mM HEPES at a concentration of (1–2) × 10^7 cells/ml. Samples were kept at 4°C until analysis. PI was added at a concentration of 5 μg/ml to exclude dead cells. In order to remove cellular aggregates, cells were filtered through a 50 μm nylon mesh prior to analysis.

2.7. Gating strategy and sample acquisition

We first used a dot plot displaying Forward Scatter (FSC) and PI to exclude dead cells and analyze the remainder live cells. A region representing PI-negative cells was used to exclude all dead (PI positive) cells. A second dot plot region representing Side Scatter (SSC) and Ho342-blue cells was used to include all live DNA-positive cells, excluding erythrocytes and debris. We used a third dot plot displaying Side Scatter (SSC) and Ho342-red cells to set the emission for determining DNA labeling in live cells. Special care was taken to include low SSC and low FSC events, which are enriched in SP cells. All samples were acquired using the maximum differential pressure that gave the best resolution. A minimum of 100,000 live cell events were acquired to resolve specimens with scarcity of SP cells.

2.8. Simultaneous staining with antibodies

Cells were prepared for immunofluorescence by using directly conjugated antibodies. Samples were incubated with 10 μl per 10^6 cells of the following antibodies: FITC-conjugated CD45 (DakoCytomation, A/S Denmark) monoclonal antibody, PE-conjugated CD54, CD90, CD117 (Immunotech, Marseille, France) and CD133 (Miltenyi Biotec Gmbh, Bergisch Gladbach, Germany), PerCP-conjugated CD34 (8G12 clone, BDIS, San Jose, CA) and APC-conjugated anti-CD38 (BDIS, San Jose, CA). Incubations were carried out for 30 min at 4°C in the dark. Cold HBSS/FCS/HEPES was then directly added to the pre-stained whole blood samples without washing.

3. Results and discussion

3.1. Hoechst 33342 staining and quality control

Based on Ho342 emitting properties, a Ho-Blue vs. Ho-Red dotplot was created in order to detect the presence of the SP in BM samples pre-incubated with Ho342. Given that the Ho342 dye is a DNA marker, a double-emitting population (blue and red) placed in the center of the plot represents those cells in the G0/G1 phase of the cell cycle. Extending to the left from this G0/G1 cells, the SP appears as a dim tail as result of the differential expression of the ABCG2 transporter and its capability of recognizing and effluxing the Ho342 dye. Extending to the upper right corner of the dotplot,

we can find cells in the S and G2+M phases of the cell cycle. The SP profile is reversed when staining is performed in the presence of verapamil or fumitremorgin-C (FTC), two ABCG2 inhibitors (Fig. 1).

The Ho342 concentration and the staining procedure are critical for the optimal resolution of the SP cells. Although UV laser alignment is very important to resolve the dim tail that outlines the SP, the problem with Ho342 excitation is rather the Hoechst Red emission than the Hoechst Blue one. 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 the Blue one, and relatively higher laser power is required. We found that using the Enterprise II, 12–30 mW usually gives a good resolution, but depending on the instrument used, a relatively higher power can be required. We first used a filter combination consisting of BP 405/30 (blue), BP 670/40 (red) and 440 DLP filters. Another combination we used consisted of BP 450/65 (blue), BP 670/40 (red) and 510 DLP filters (Fig. 2). However, the first combination was chosen because it gave a better resolution for the dim SP tail analysis. Initial experiments used different power settings and PMT voltages, always using a linear scale. We found that powers below 12 mW were not adequate to resolve the SP (Fig. 3). When the power was increased to 30 mW of UV-laser excitation, a double-emitting population (blue and red) was resolved that is mainly the G0/G1 fraction.

The original protocol developed by Goodell et al. [3] measured the PI emission through the same filter used for the Red-Ho342. However, based on the emission properties of this dye and for dead cell discrimination, we chose an alternative setting. Here we show that PI emission measured in logarithmic scale using a different PMT (BP 630/30) instead of using the same PMT for both PI and Ho342-red emission results in a better resolution of the SP. We routinely use a live-gate based on this condition to better resolve the SP, even if apoptotic/pre-necrotic cells are present in the sample. For that purpose, the use of a different PMT also improves discrimination of debris (Fig. 4).

We also assayed different Ho342 concentrations and we found that 5 μg/ml was the optimal one (Fig. 5). Lower concentrations of Ho342 make the resolution of the SP more difficult, because the time needed for the dye-efflux is reduced, leading to the underestimation of the size of the SP compartment (Fig. 5a). Moreover, higher concentrations of Ho342 increase the number of dead cells and may lead to the underestimation of the SP cells, because the time required for the dye-efflux is
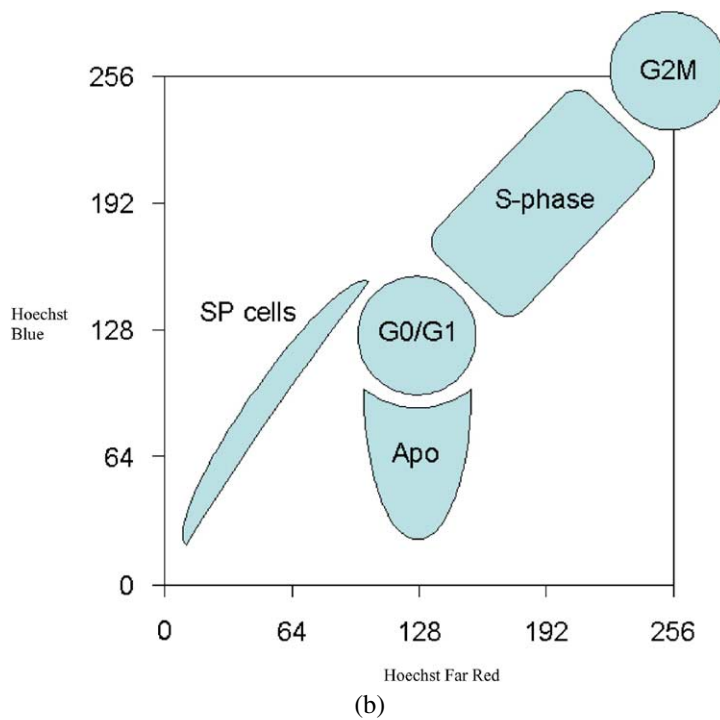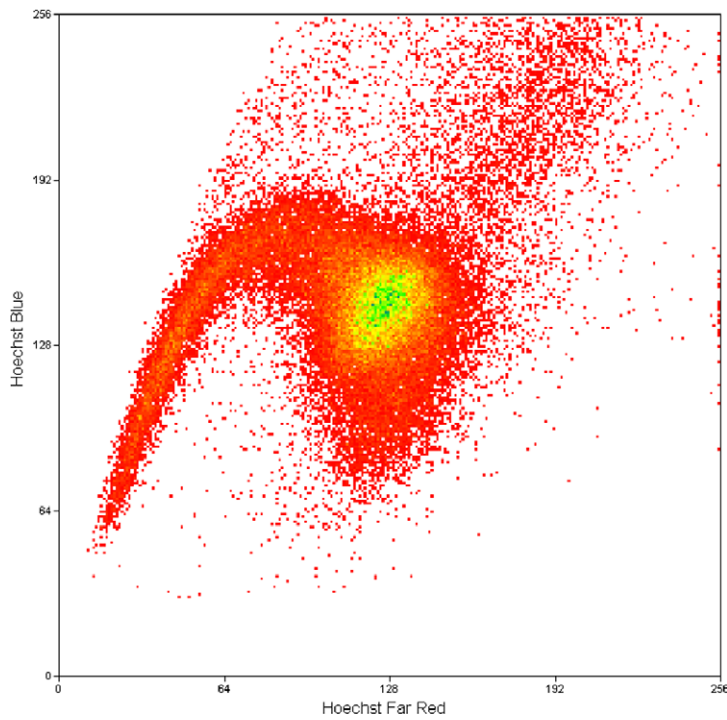
(a)

(b)

Fig. 1. Hoechst 33342 SP analysis of unpurified human BM on a MoFlo® cell sorter equipped with an Enterprise II water-cooled ion laser emitting at 351 nm (30 mW of laser power). Representative dotplot for Hoechst Blue vs. Hoechst Red simultaneous emission of a human BM sample containing SP cells (a). Schematic dotplot showing distribution of events (b). Characteristic Ho342 dye staining profiles in presence of the specific ABCG2 inhibitor, fumitremorgin C (c).
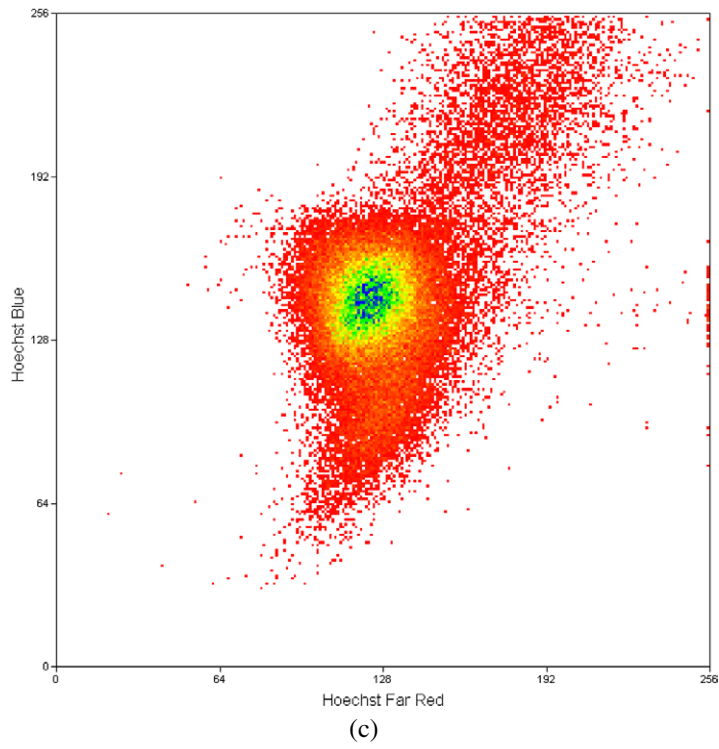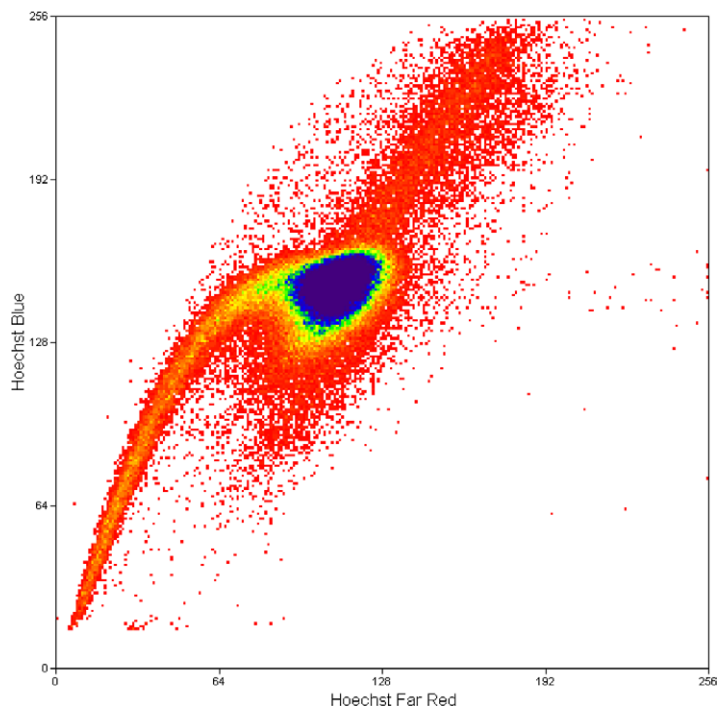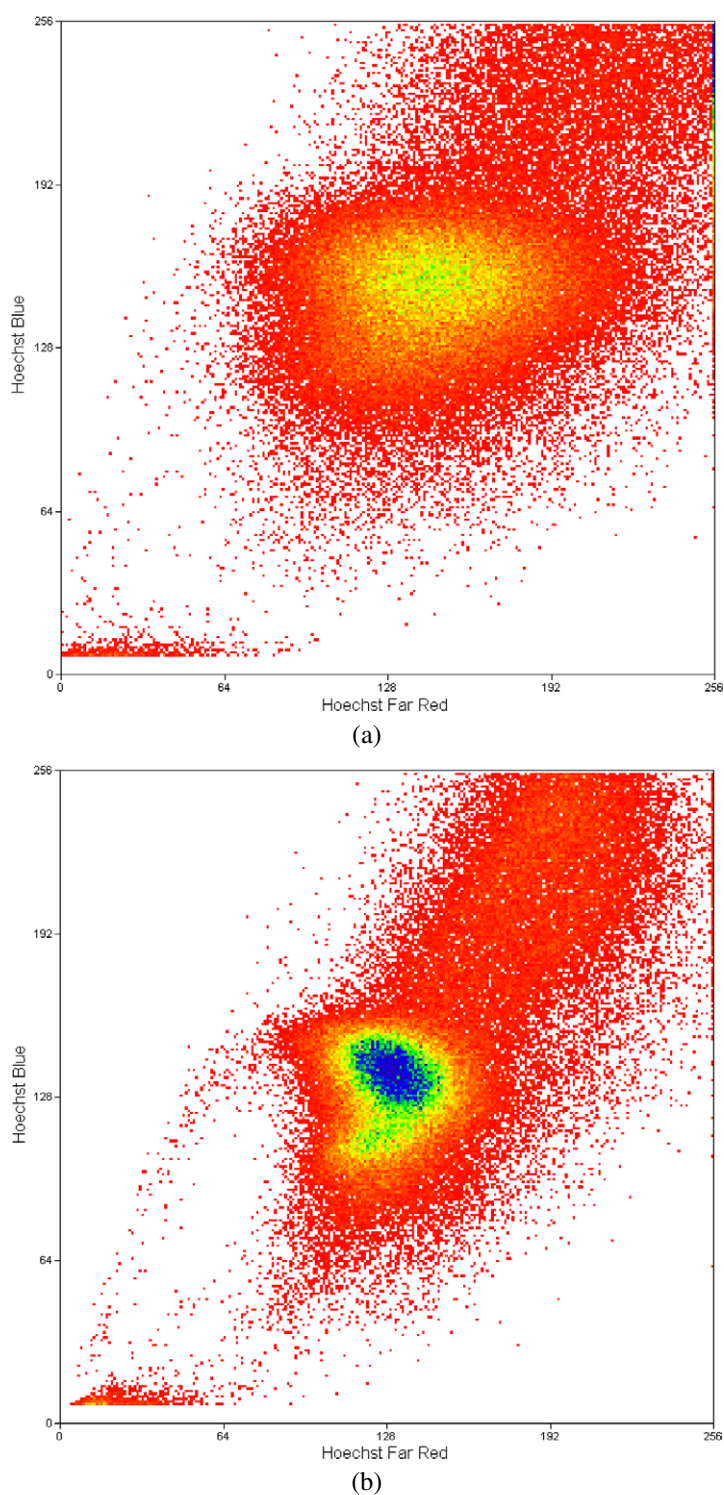
(c)

Fig. 1. (Continued).

Fig. 2. Ho342 SP analysis of unpurified human bone marrow. The analysis was carried out using a filter setup consisting of BP 450/65 (blue), BP 670/40 (red) and 510 DLP filters which did not give the appropriate resolution.

(a)

(b)

Fig. 3. Ho342 SP analysis of unpurified human BM on a MoFlo® cell sorter 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 Blue and relatively higher laser power is needed. Effect of UV laser power settings on the resolution of the SP: 1 mW (a) and 12 mW (b).

(a)

(b)
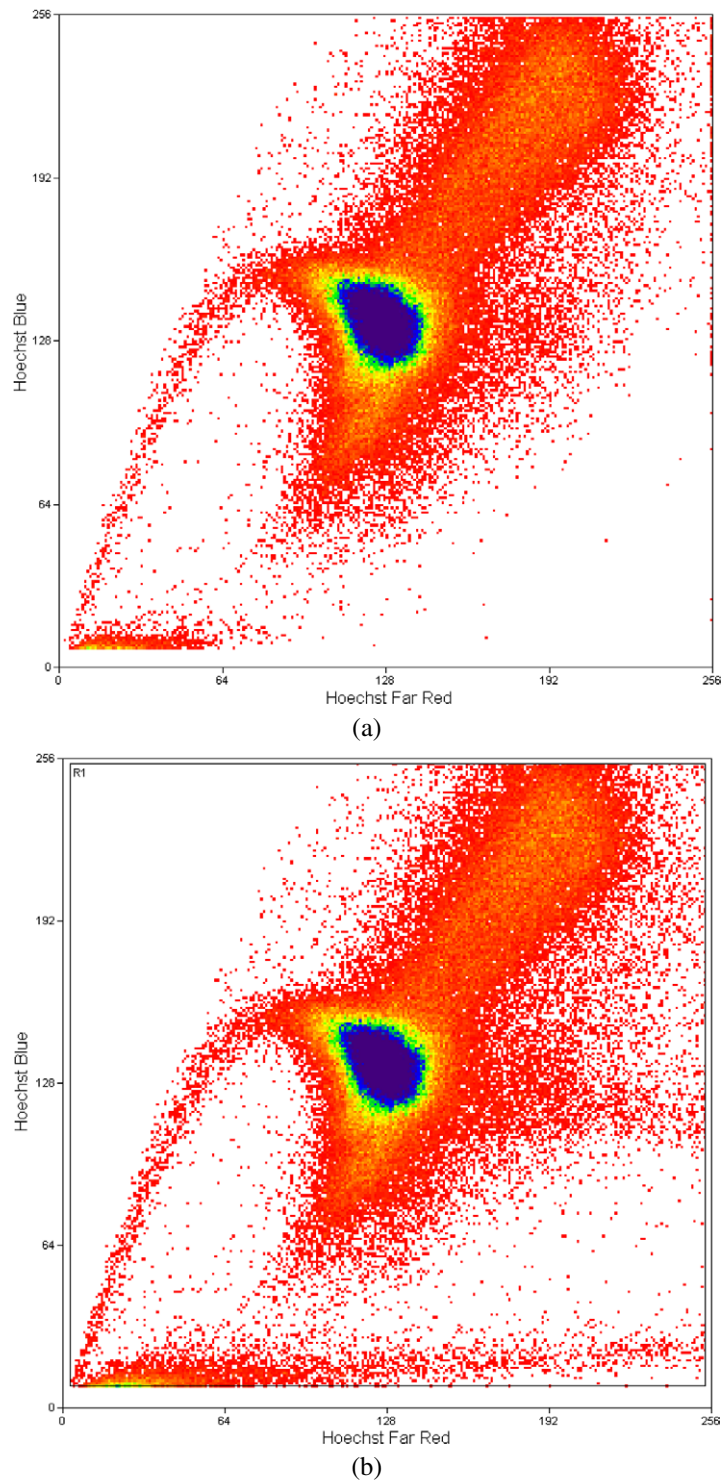
Fig. 4. Representative analysis of the SP using an alternative photomultiplier to discriminate dead cells in human bone marrow samples. Propidium iodide was excited at 488 nm and its emission was measured through the following filter combination: 605 DSP, 718 DSP, 650 DSP and BP 630/30 (a). Side Population analysis using the UV laser for PI excitation, where PI fluorescence was collected using the same photomultiplier as the one used for Red-Ho342 emission (b).
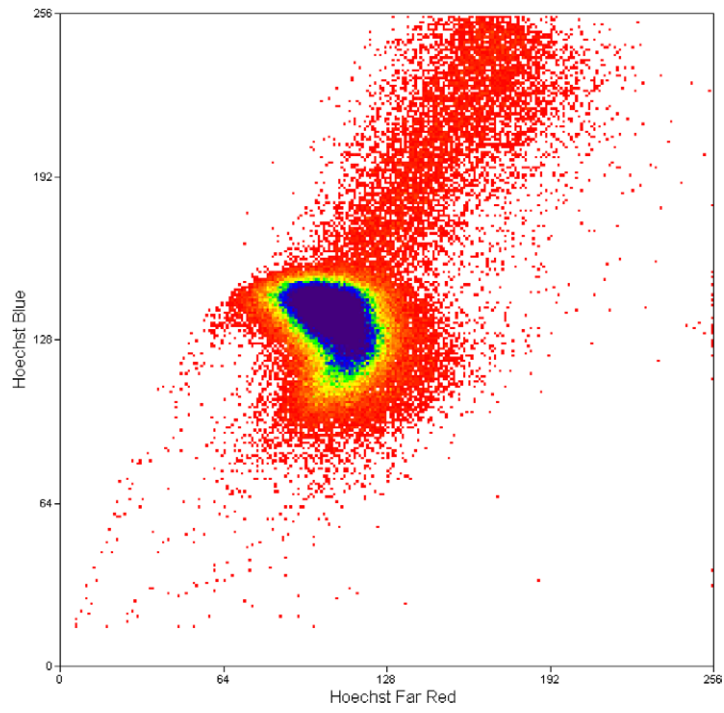
(a)
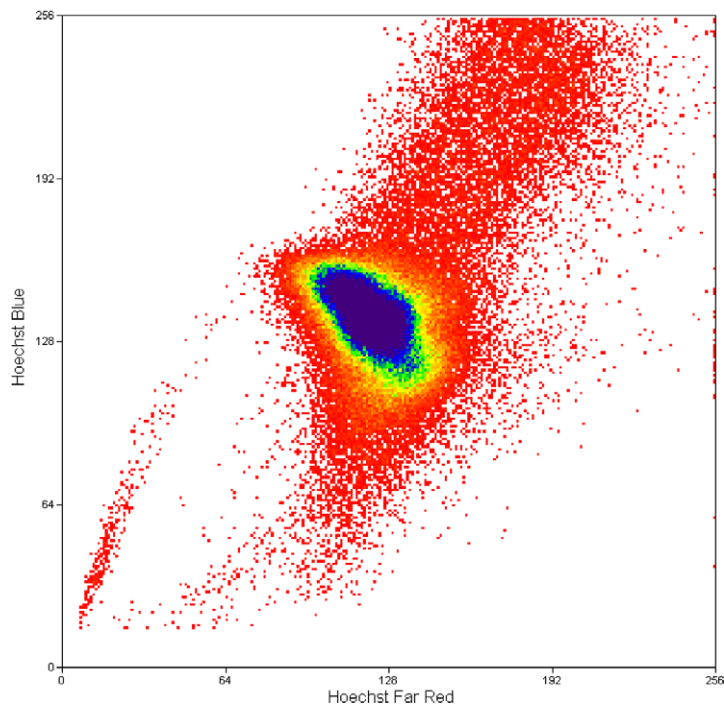
(b)

Fig. 5. Effect of Hoechst concentration on the SP cells counting. The same bone marrow sample was stained using different concentrations of Ho342: 2.5 μg/ml (a), 5 μg/ml (b) and 10 μg/ml (c).
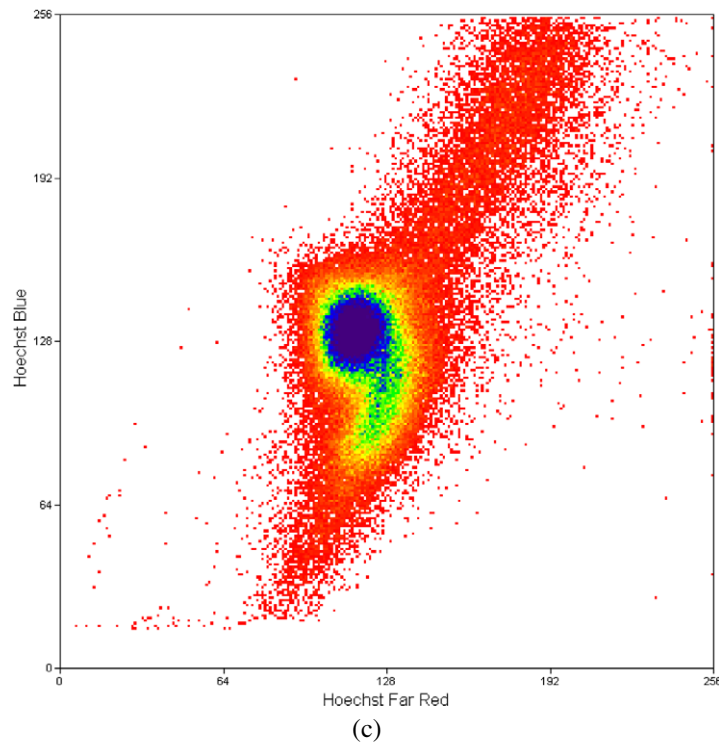
(c)

Fig. 5. (Continued.)

higher (Fig. 5c). In order to achieve an optimal staining, incubation times can be shortened or lengthened respectively. The percentage of dead cells was higher using a concentration of 10 μg/ml ($37.12 \pm 12.45\%$; $n = 3$) in comparison with a concentration of 5 μg/ml ($25.14 \pm 12.49\%$; $n = 3$).

However, we modified the Goodell protocol resuspending 2×10^6 cells per ml of DMEM. Therefore, Ho342 concentration was increased up to 10 μg/ml, that halved the final volume used. Special care should be taken during the preparation of the Ho342 stock solution and in particular when a new batch is used. We have observed that specific sources (different batches) may be suboptimal for the SP staining using our experimental settings. As a result, both blue and red linear CVs are higher and of poorer quality for the far-red emission (data not shown). In this case, we do not recommend to increase the PMTs voltages nor switch the linear to logarithmic scale, because this may result not only in the reduction of the CVs but also in compressing the signal obtained from the SP compartment. Then, check for the laser alignment, and consider checking different Ho342 sources and to discard Ho342 preparations that give a weak emission signal. In addition, for new batch preparation, we recommend to store the dye in its original powder presentation. For
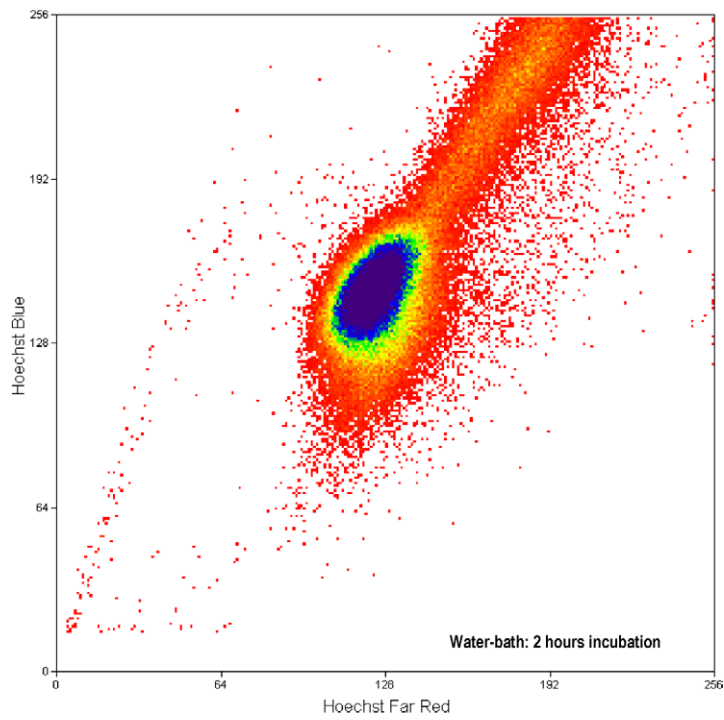
Ho342 staining, a water-bath was also compared with a CO_2 cell-incubator. We observed a more homogeneous staining when the water-bath was used (Fig. 6). We also assayed the Ho342 influx in both conditions. A decreased influx was observed when Ho342 staining was carried out using a cell-incubator, which may lead to an overestimation of the size of SP (Fig. 6) and Red-Ho342 fluorescence was lower when compared with bath staining conditions.

In vitro and *ex vivo* measurements for SP cell analysis have to mimic the physiological state. In this study, human peripheral blood samples were collected in either citrate or heparin tubes and assayed for SP cells. PBS-EDTA or heparinized blood was preferred because HBSS solution can neutralize citrate, resulting in leukocyte activation and/or sample clotting (unpublished observations). Our efforts were also aimed to minimize the sample processing time. Times spent on isolation and preparation of cells, as well as temperature and time incubation with antibodies can result in suboptimal SP analysis. For this reason, freshly drawn blood samples were processed immediately. Following blood extraction, SP analyses were performed in less than 2 hours.

We also adapted the methodology for investigating the presence of SP cells in low volume samples, such
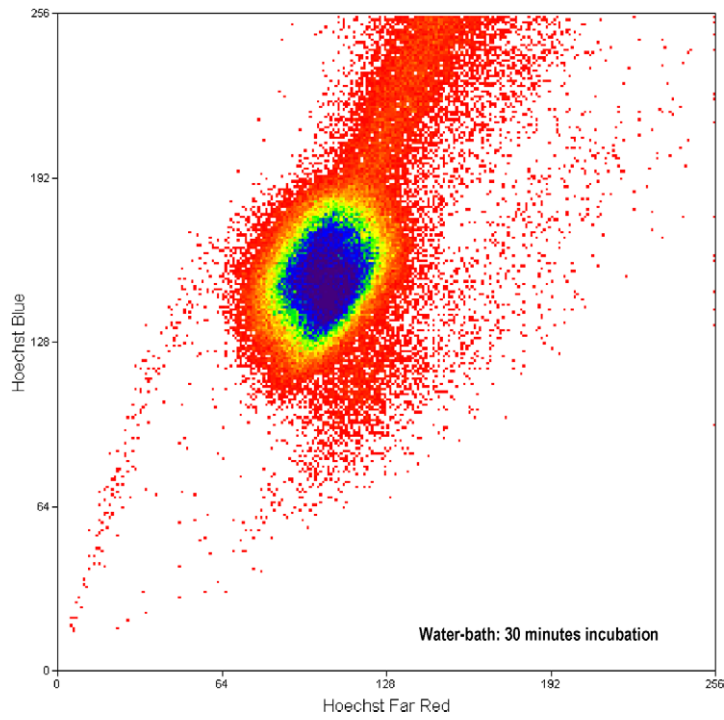
(a)

(b)

Fig. 6. Ho342 staining of a human bone marrow sample using a water-bath and its comparison with a CO_2 cell-incubator. A more accurate staining was observed when a water-bath was used (a) and (b). The influx of Ho342 is slower using a cell-incubator (c) and (d), giving an inappropriate SP profile leading to an overestimation of the SP frequency.
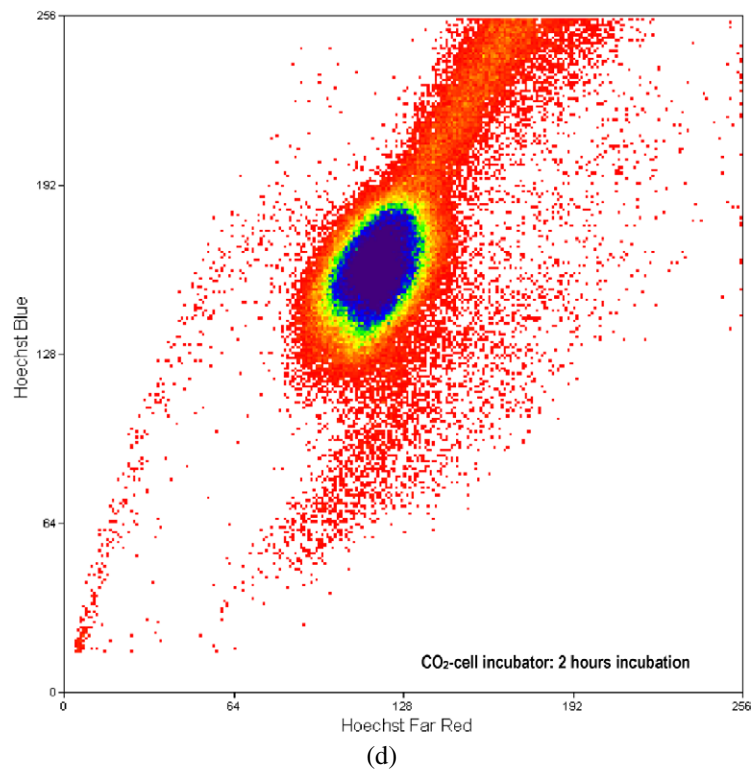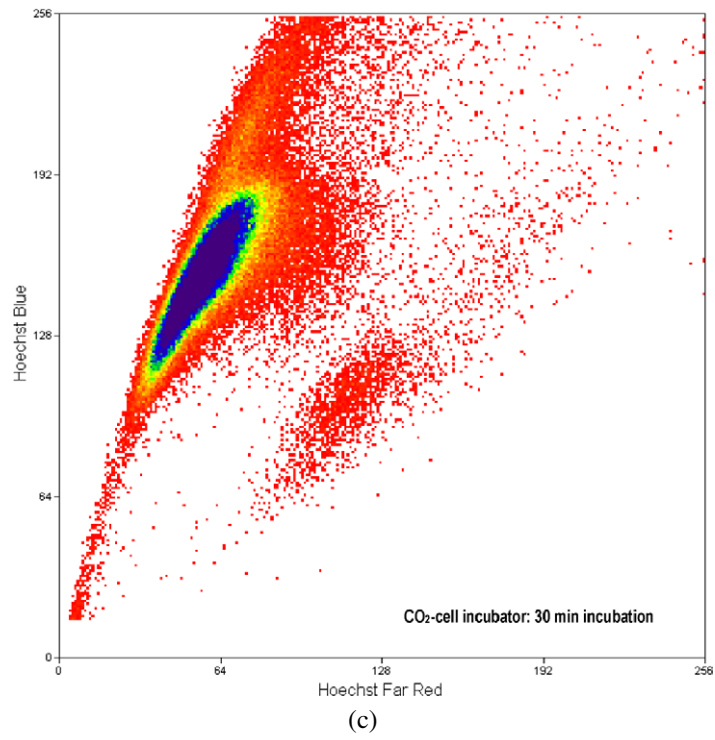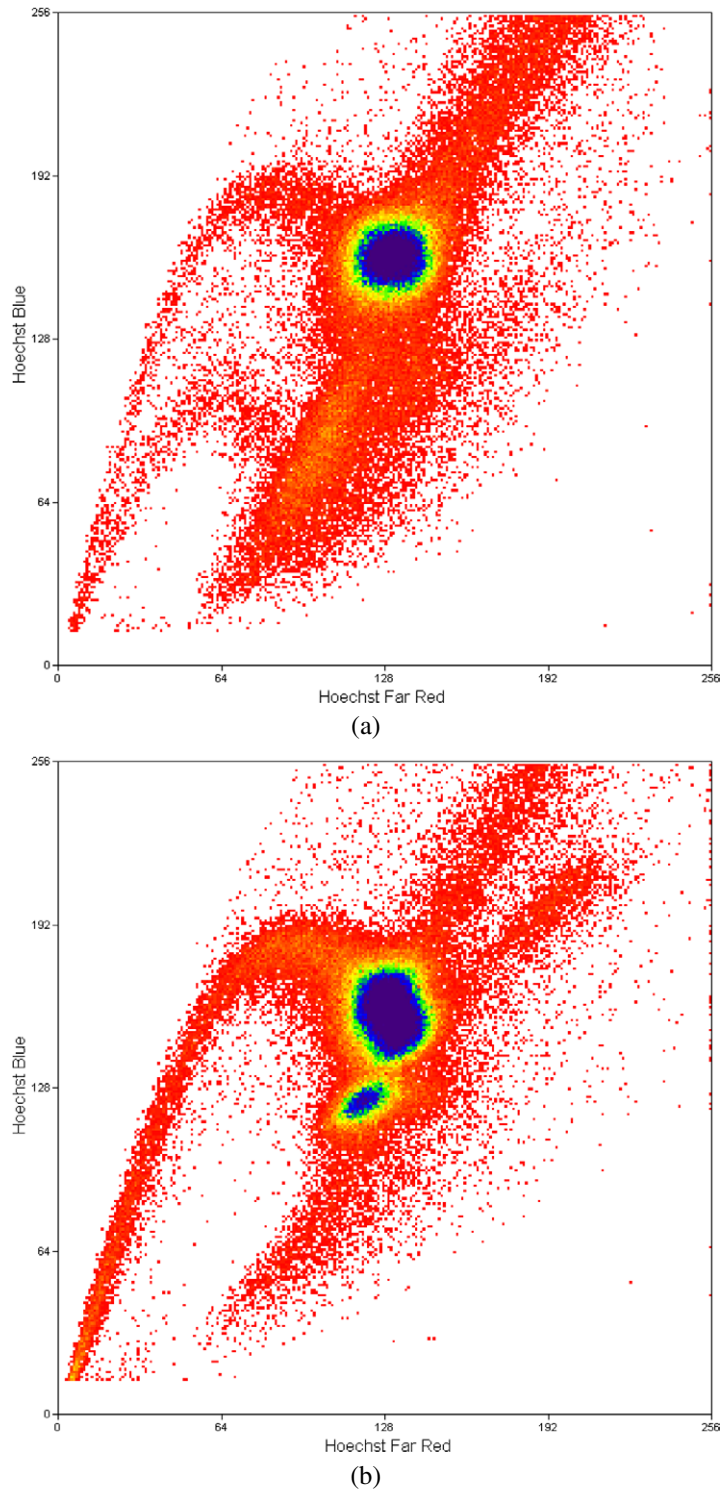
(c)

(d)

Fig. 6. (Continued.)

(a)

(b)

Fig. 7. Efflux of Ho342 in human bone marrow SP cells is not impaired by ammonium chloride-based lysing solutions. Cell preparation by density gradient separation (a) and using NH$_4$Cl lysing solution (b).
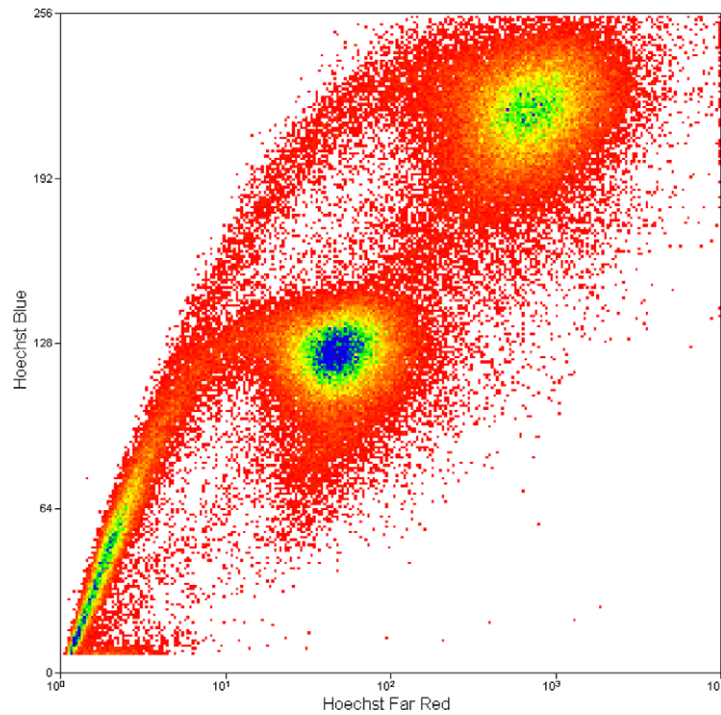
Fig. 8. SP pattern of Ho342 efflux in the BM of a patient with acute myeloid leukaemia (AML) at diagnosis. Figure shows a representative flow cytometry dotplot of diploid and near-tetraploid AML cells stained with Ho342 to identify SP cells (diploid: 13.31% SP cells; near-tetraploid: 1.89% SP cells).

as bone marrow aspirates. Such samples are usually subjected to extensive manipulation that includes centrifugation, washings steps and, in some cases, enrichment of the target cells by density gradients. This manipulation can cause cellular depletion and ABCG2 dysfunction and may result in wrong measurements, especially when target cells are found in a very low frequency. For small sample analyses and to minimize cell loss during density gradient purification, erythrocytes were lysed with the ammonium chloride lysing solution and prepared for Ho342 staining (Fig. 7). We avoided the use of density gradients mainly for three reasons; first, because of toxicity of the gradients; second, because of cell loss as a result of sample manipulation and third, because of small volume samples (i.e. pediatric samples). In addition, density gradients used for the isolation of blood mononuclear cells have specific densities which can be inadequate for SP enrichment. We want to stress that some fixative red cell lysing solutions, such as those containing paraformaldehyde are not useful for functional studies. Here we show that ammonium chloride did not significantly impair the ABCG2 activity for the identification of the SP cells. Since erythrocyte lysis using ammonium chloride does not hamper the resolu-

tion of the dim tail of the SP it can be applied for the simultaneous staining with antibodies. Human and murine SP cells represent about 0.05% of total nucleated BM cells. Most of the specimens analyzed contained a very rare fraction of SP cells ($n = 100$, median = 0.09%; range = 0.004–0.96%). This enrichment in the SP compartment is likely to be associated with the loss of sensitive cells during lysis with ammonium chloride, such as granulocytes, a phenomenon that may help to enrich the SP fraction prior to cell sorting. In normal human BM, the SP constitutes a very rare cell fraction which accounts for approximately 0.03–0.07% of all MNCs. However, patients with acute myeloid leukaemia (AML) can contain a significant fraction of SP cells. Here we report two cases, where SP cells (reported as percentages of viable MNCs in the BM) were found in large amounts (2.15% and 15.2% respectively). Furthermore, in one of the two samples the SP profile was duplicated (Fig. 8). In this sample we identified a near tetraploid secondary population with a DNA index = 2.1 and representing the 36.24% of the total cell count. In our opinion, these cells are likely to represent leukaemic cells and may represent a potential source of cells that challenge in achieving long-term remission.
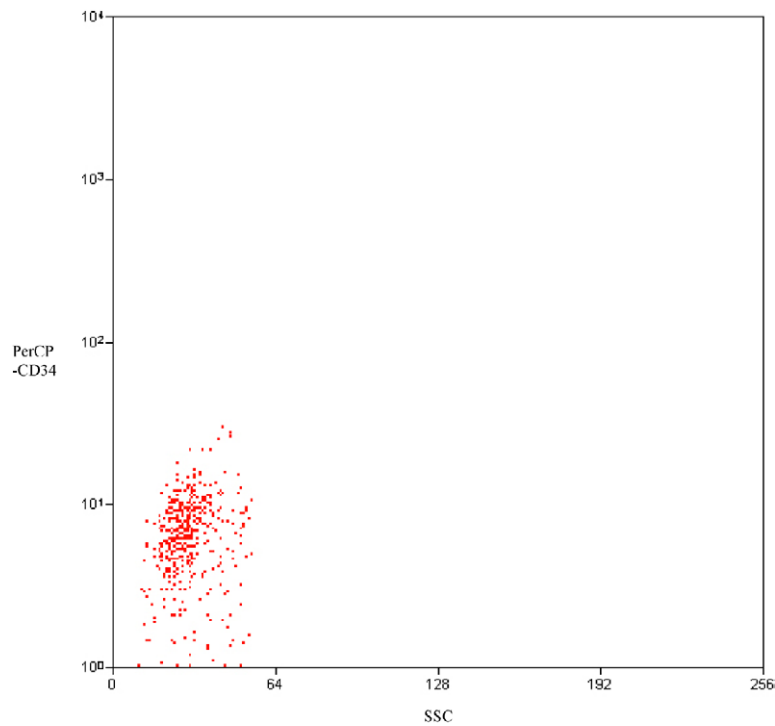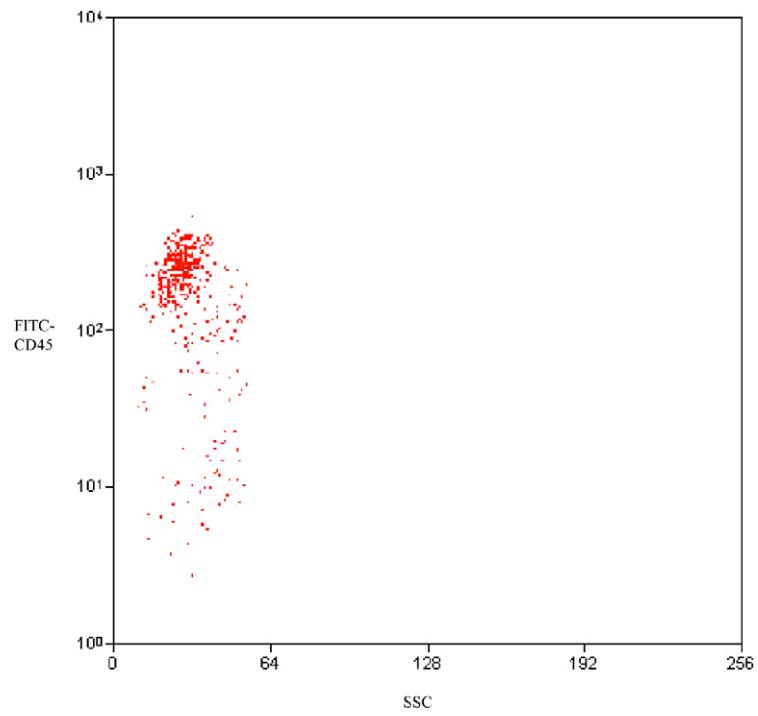
(a)

(b)

Fig. 9. Representative multicolor analysis of the Side Population. Human BM samples containing SP cells were assayed for several antibodies, a gate was established for the SP. Here, we show side scatter vs. fluorescence dotplots showing positive/dim staining for CD45 (a) and negativity for CD34 (b). Side scatter vs. fluorescence dotplot showing dim staining of CD38 events (c).
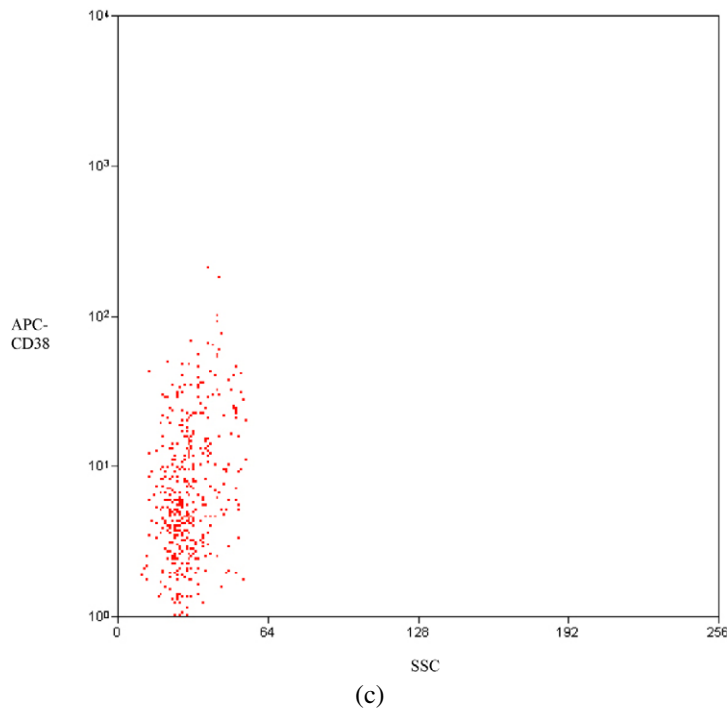
(c)

Fig. 9. (Continued).

The staining with directly conjugated antibodies allows a feasible multicolor analysis, even using high-power laser settings (30 mW). As a control for the identification of the SP cells, we simultaneously measured different fluorescences from individual SP events in a single sample tube using surface expression markers. Optimal multicolor combinations used for immunophenotypic analysis of the SP cells were: Ho-Blue, Ho-Red, PI, FITC-CD45, APC-CD38, PerCP-CD34 (fixed panel) and only PE-conjugated antibodies were replaced by the different markers: CD54, CD90, CD117 and CD133 (data not shown). The SP fraction was CD45 positive/dim and CD38 negative/dim. SP cells were also negative for the CD34 marker, which is in agreement with previous studies and suggest a primitive phenotype for the SP fraction (Fig. 9). The autofluorescences for all markers were adjusted considering that the vast majority of the MNCs are negative for all surface markers assayed, except for CD45, a widespread hematopoietic marker.

The SP compartment can only be assayed using the Ho342 efflux. Conventional flow cytometers are not equipped with lasers capable of exciting Ho342 and they cannot be used to identify these cells. New analyzers and sorters can be equipped with violet or near-violet laser diodes. Although it has been demonstrated that Ho342 SP analysis can be carried out on bench top flow cytometers equipped with near-violet excitation sources, the larger cell sorters are still required for a clear resolution of the SP cell fraction and for the physical sorting of SP cells [10].

Our results suggest a more primitive origin for the SP fraction as compared to other cell populations such as the CD34+, since they are negative for all the markers assayed with the exception of CD45 and CD38. Human samples used here were from healthy donors and from patients with hematological malignancies, which could partially explain the heterogeneity observed in the SP cell compartment. Only a small group of samples were enriched in SP cells (>0.1%), which may be attributed to the cell manipulation. In order to find candidate markers that would help defining the SP phenotype, further studies using a wider panel of surface markers are required. The identification of the SP cells stresses the complexity of the human stem cell compartment which has important clinical applications in fields such as stem cell transplantation (i.e. CD34+ selection) or stem cell gene therapy. SP analysis also represents a field of growing interest with profound implications in the understanding of the stem cell biology, or in the study and characterization of hypothetical cancer stem cells. However, current methodology may require leukocyte isolation by density gradients that can include a significant sample manipulation, which may re-

sult in associated (unwanted) cellular damage to leuko-cytes, and in abnormal ABCG2 activity. Flow cytometry is a rapid multiparametric method that provides a reliable measure of SP cells. However, there is a great heterogeneity among SP measurements using flow cytometry and this makes it difficult to compare results between different laboratories. Further flow cytometric multicolor studies will help characterizing the phenotype and the function of these cells and the relation between the SP cells that can be found in different organs and tissues.

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