Application Note 1

Four-Color Analysis of Bone Marrow Transplantation Specimens*

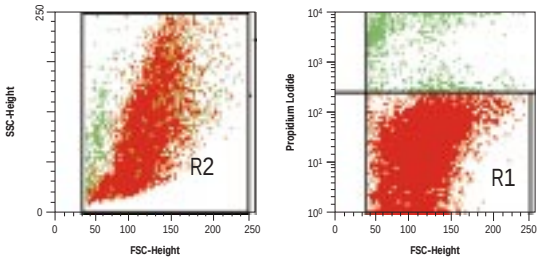
Introduction

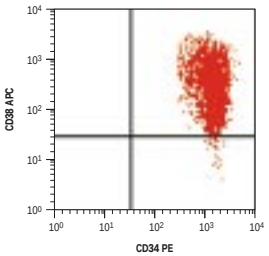
Flow cytometric analysis of bone marrow and stem cell products is commonly used as a research tool to characterize and enumerate populations of stem cells and lymphocytes. With multicolor flow cytometric analysis, it is possible to determine subsets of complex cell populations that include a variety of overlapping phenotypes. $CD34^+$ progenitor cells and $CD3^+$ T cells are two populations that contain many subsets relevant to the engraftment process in bone marrow transplantation. $CD34^+$ progenitor cells, which include hematopoietic stem cells, are responsible for reconstitution of the immune system after myeloablative chemo/radio therapy. $CD3^+$ T cells include cells that mediate graft-versus-host disease (GvHD) and might provide short-term immune reconstitution. With four-color analysis, more complex populations can be analyzed than when limited to three colors. Four-color analysis also allows an increased number of fluorescence-tagged antibodies to be combined into a single tube to create a condensed panel with fewer tubes per sample.

Analysis of $CD34^+$ progenitor cells is of particular interest in understanding engraftment after bone marrow or peripheral blood stem cell transplantation. $CD34^+$ cell content of bone marrow and peripheral blood is the best predictor of the kinetics of hematopoietic engraftment following bone marrow transplantation.[1] High-dose cyclophosphamide chemotherapy followed by GM-CSF or G-CSF mobilizes up to 300 $CD34^+$ cells/µL into the peripheral circulation. These $CD34^+$ cells can be collected by apheresis for subsequent infusion.[2–4] Compared to bone marrow cells, mobilized peripheral blood $CD34^+$ stem cells obtained by apheresis show equal or superior biologic activity in promoting hematological recovery following myeloablative therapy.

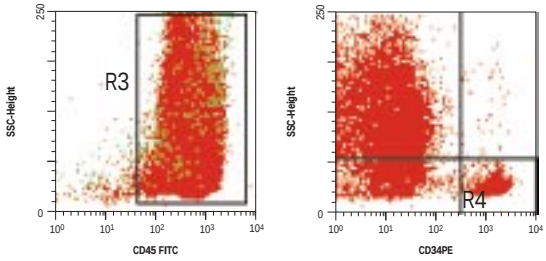
This application note describes four-color staining procedures for use in bone marrow and stem cell research, using a benchtop flow cytometer (FACSCalibur™). We present examples of analyses of relevant $CD34^+$ and $CD3^+$ subsets in bone marrow using combinations of monoclonal antibodies conjugated to FITC, PE, PE-Cy5 or PerCP, and APC .

CD34⁺ Stem Cell Subsets and Viability





Light scatter gate (left) and viability gate (right) give 94% of cells viable.

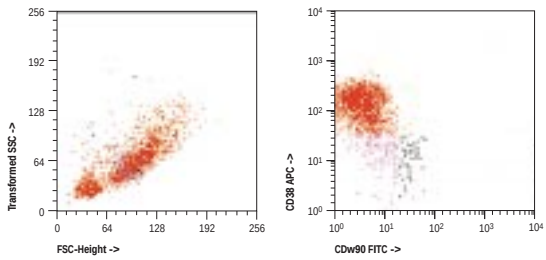


Percent viable CD34⁺ cells (R1 AND R2 AND R3 AND R4) is 8.81.

Gate = R1 AND R2 AND R3 AND R4			
Quad	**Events %**	**Gated %**	**Total**
UL	0	0.00	0.00
UR	3032	98.96	6.06
LL	0	0.00	0.00
LR	32	1.04	0.06

Figure 1 *Dot plots showing three surface markers plus propidium iodide to examine CD34⁺ cells, subsets of CD34⁺ cells, and viability. 99% of the viable cells positive for CD34 are also positive for CD38, with the remainder of the CD34⁺ cells negative for CD38. By gating using a combination of regions (R1 + R2 + R3 + R4) it is possible to examine CD34⁺ cells and subsets of CD34⁺ cells, and also viability. Red events indicate viable cells. Nucleated cells are within R3, and R4 includes the CD34⁺ cells.*

CD34⁺ Stem Cell Subsets



83.26 %		$CD34^+, DR^+, CDw90^-, CD38^+$
0.09 %		$CD34^+, DR^-, CDw90^+, CD38^+$
0.47 %		$CD34^+, DR^-, CDw90^-, CD38^-$
1.23 %		$CD34^+, DR^+, CDw90^+, CD38^+$
6.64 %		$CD34^+, DR^+, CDw90^-, CD38^-$
0.19 %		$CD34^+, DR^-, CDw90^-, CD38^+$
2.28 %		$CD34^+, DR^+, CDw90^+, CD38^-$
5.83 %		$CD34^+, DR^-, CDw90^+, CD38^-$

Figure 2 *Four-color analysis of stem-cell subsets using PAINT-A-GATE™ software. CDw90⁺ cells were colored green, HLA-DR⁺ cells were colored red, CD38⁻ cells were colored blue. The putative hematopoietic stem cell CD34⁺/HLA-DR⁺/CDw90⁺/CD38⁻ are therefore black, the overlap color between red (HLA-DR⁺), blue (CD38⁻), and green (CDw90⁺) CD34⁺ subsets.*

Materials and Methods

Surplus samples of bone marrow were obtained from freshly processed buffy coats and prepared using typical procedures for flow cytometric analysis. RBC lysis and cell wash were accomplished using reagents from the R&D Whole Blood Erythrocyte Lysing Kit (R&D Systems, Inc, Minneapolis, MN). Stained cells were resuspended at 1 x 10^6 cells/mL in a 1% final concentration of paraformaldehyde (Sigma Chemicals, St. Louis, MO) in phosphate-buffered saline (PBS).

Acquisition was performed on a FACSCalibur flow cytometer using two excitation beams: primary excitation at 488 nm with an argon-ion laser and secondary excitation at 635 nm with a red diode laser. Amplifiers were set with forward scatter and side scatter in linear mode and all fluorescence (FL) channels in log mode. Fluorochromes used were FITC (in FL1), PE (FL2), PE-Cy5 or PerCP (FL3), and APC (FL4). Propidium iodide fluorescence was collected in FL3. Spectral overlap was subtracted by adjusting compensation appropriately using control samples. T-cell subset data were acquired ungated, with the fluidics control set on HI. For each file 2 x 10^4 events were collected. CD34 subset data were collected using a CD34 PE vs. side scatter gate, and 5 x 10^3 gated events were collected for each file. For viability analysis, 5 x 10^4 ungated events were collected.

Results

The viability of stem cell subsets present in the transplant material can be assessed by flow cytometry. In Figure 1, three surface markers and propidium iodide were used to examine subsets of $CD34^+$ cells for their viability. Using four-color analysis, it is possible to use propidium iodide for viability analysis and CD34 and CD45 for identification of progenitors ($CD34^+$ and $CD45^{dim}$), and still retain the flexibility to identify subsets of the progenitors using the fourth parameter. In the example shown, 99% of the viable cells positive for CD34 are also positive for CD38, with the remainder of the $CD34^+$ cells negative for CD38.

It is also possible to examine more complex phenotypes using a combination of four surface markers (Figure 2). The $CD34^+$ cells were subdivided according to their expression of CD38, CDw90, and HLA-DR. The putative hematopoietic stem cells are $CD34^+HLA\text{-}DR^+CDw90^+CD38^-$.[5–9] The presence or absence of four independent antigens can be correlated on individual cells only when four or more parameters are available for analysis.

Analysis of $CD3^+$ T-cell subsets was also performed. The presence or absence of various subsets might have some correlation with the ability of the graft material to eliminate any residual tumor or to create treatment complications due to graft-versus-host reactions. For example, naive and memory phenotypes of the cytotoxic and suppressor T cells ($CD8^+$) were defined by the expression of the CD45RA and CD45RO antigens, respectively (Figure 3). Other subsets might also be of interest when studying the prediction of graft-versus-host and host-versus-tumor responses.

CD3⁺ T-Cell Subsets

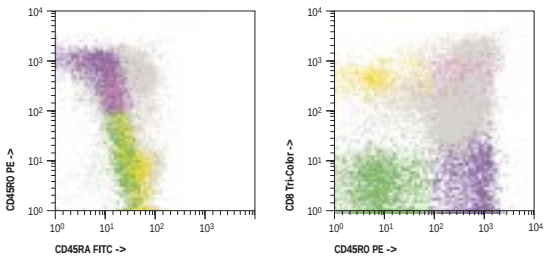
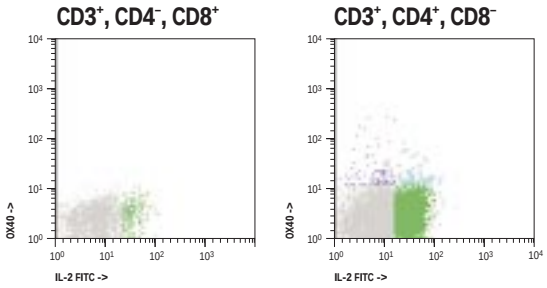


Figure 3 *CD3⁺ T-cell subsets defined by the expression of CD8, CD45RA, and CD45RO. The percentage of CD3⁺ cells in the sample is equal to the sum of all the T-cell subsets (colored events). The red events are CD8⁺CD45RO⁻CD45RA⁻. The green events are CD8⁻CD45RO⁻CD45RA⁺. Blue events are CD8⁻CD45RO⁺CD45RA⁻. Yellow events are CD8⁺CD45RO⁻CD45RA⁺. Violet events are CD8⁺CD45RO⁺CD45RA⁻. Cyan events are CD8⁻CD45RO⁻CD45RA⁻.*

Graft-versus-Host Disease

Normal Control



GvHD Samples

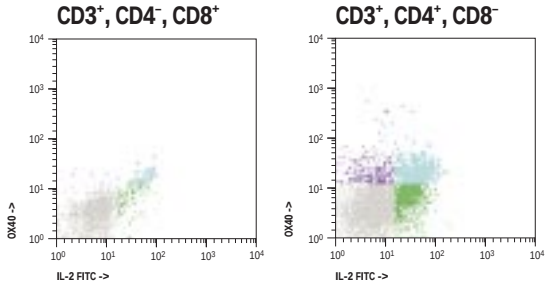


Figure 4 *Four-color analysis of Ox 40 and IL-2 expression during GvHD in an allogeneic BMT recipient. Peripheral blood from a normal volunteer (left panels) and a patient that received an allogeneic bone marrow transplant (right panels) was stimulated with PMA/ionomycin in the presence of Brefeldin A. Following surface staining with CD3, CD8, and Anti-Ox 40 the cells were permeabilized with BD Biosciences FACS™ Permeabilizing Solution and stained with Anti–IL–2. 50,000 events were acquired and CD3⁺/CD8⁺ and CD3⁺/CD8⁻ (CD4⁺) subsets were separately analyzed for Ox 40 and intracytoplasmic IL-2 expression.*

The immune status of post-transplant patients is also of interest. For example, the presence and number of activated T cells might provide information about the onset or severity of GvHD.[10] Ox 40 and cytokine expression during GvHD in an allogeneic bone marrow transplant recipient were studied as markers of activation status (Figure 4). Peripheral blood from a normal volunteer (left panels) was compared to that from a patient that had received an allogeneic bone marrow transplant (right panels). The samples were treated with phorbol myristate acetate (PMA) and ionomycin in the presence of Brefeldin A, then surface stained with CD3, CD8, and Anti-Ox 40 antibodies. The cells were then permeabilized with BD Biosciences FACS Permeabilizing Solution and stained for the presence of intracytoplasmic IL-2. 50,000 events were acquired and $CD3^+CD8^+$ and $CD3^+CD8^-$ ($CD4^+$) subsets were separately analyzed for Ox 40 and intracytoplasmic IL-2 expression.

Discussion

Multicolor analysis has numerous benefits for assessment of $CD34^+$ and $CD3^+$ subsets in bone marrow. A condensed number of sample tubes per panel of antibodies reduces the workload considerably without degrading the reliability or quality of results. Subset analysis of $CD34^+$ and $CD3^+$ cells can be accomplished much more efficiently. Assessment of the viability of bone marrow– or peripheral blood–derived specimens can now be included with an expanded panel of antibodies to reveal more information than could be had in the past, using a single sample tube. Correlated measurements of surface phenotype can reveal populations that cannot be identified using fewer parameters (for example, $CD34^+$HLA-$DR^+CDw90^+CD38^-$ or $CD3^+CD8^+CD45RA^+CD45RO^+$). Reliable fluorochrome-conjugated monoclonal antibodies are readily available, making it now possible to incorporate four-color panels into $CD34^+$ and $CD3^+$ subset analysis.

BD Biosciences publishes this method as a service to investigators. Detailed support for non–flow cytometric aspects of this procedure may not be available from BD Biosciences.

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