Cytometry PART A
Journal of the International Society for Advancement of Cytometry

MIFlowCyt

Yellow–Green Laser-Based Flow Cytometry for CD34+ Progenitor Cell Counting

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THE CD34 molecule is a cell surface marker widely used to both identify and isolate hematopoietic progenitor cells. Although this marker was initially identified as an antigen expressed in progenitors cells, it has also been used to detect CD34+ leukemic cells, vascular endothelial cells, muscle satellite cells, and epidermal precursors (1–3). Flow cytometry is a rapid and reproducible method to quantify the numbers of circulating CD34+ cells following stem cell mobilization with cytokines, as well as to predict the total number of CD34+ harvested cells in leukapheresis products. Nowadays, CD34+ cell enumeration is routinely used in clinical transplantation centers to optimize stem cell collections for reconstituting the hematopoietic system following myeloablative therapies (2,3).

First flow cytometry assays for CD34+ cell enumeration used indirect immunofluorescence techniques and red-cell lysing procedures with centrifugation and washing steps. CD34+ counts were initially obtained using a dual-platform technique, also known as the Milan protocol. Since then, different methodologies have been described for improved detection of CD34+ cells. Using mutiparametric flow cytometry and, in agreement with the International Society of Hematotherapy and Graft Engineering (ISHAGE), CD34+ cells are counted in combination with CD45 staining to eliminate nonleukocytes and debris (4). Current flow cytometry-based methods for human hematopoietic progenitor cell counting consist of a series of consensus steps. Most accepted and standardized protocols include a single-platform strategy, absolute counting beads, lyse no-wash procedures and single laser excitation at 488 nm for PE-CD34+/FITC-CD45dim/7-AAD negative cell counting. The single-platform ISHAGE protocol is the most widely accepted method for CD34+ progenitor cell counting. This methodology follows the more recent recommendations for different monoclonal antibody clones and fluorescent conjugates or different viability dyes for excluding dead cells practice. As an example, the UK NEQAS quality control program has determined that this methodology and minor variations of this assay is used by 95% of participating centers in the United Kingdom, 81% of international participants use ISHAGE in the proficiency testing programme (5). Moreover, the Iberian Society for Cytometry Working Group for counting CD34+ cells has highlighted that there is significant subjectivity related to variability in gating, as demonstrated using CD34 in silico studies. Participating laboratories ($n = 50$) analyzed four representative FCS data files, showing unexpectedly high coefficients of variation, from 12.62% to 58.72% for CD34+ events (6).

USING THE ISHAGE METHOD FOR CD34 ENUMERATION

The original ISHAGE protocol used forward scatter (FSC), side scatter (SCC), fluorescein isothiocyanate (FITC)-CD45 and phycoerythrin (PE)-CD34 dual platform analysis, with the requirement of isotype controls with an additional tube combining FITC-CD45 and PE-IgG. Since then, different modifications have been introduced such as removal of isotype controls, the use of single platform methods and improved gating. Commonly, progenitor cell identification is

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Grant sponsor: Obra Social La Caixa-Fundació Josep Carreras and Generalitat de Catalunya (to J.P.)

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Additional Supporting Information can be found in the online version of this article.

Published online 00 Month 2017 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.23299

still based on staining with PE-CD34 monoclonal antibodies in combination with FITC-CD45 for leukocyte-based counts, and viability dyes are used to exclude dead cells (i.e., 7-AAD). This modified ISHAGE protocol uses boolean sequential gating to eliminate the use of an isotype control and is generally still based on single blue-laser excitation for three-color analysis using lyse no-wash cell manipulation in combination with counting beads (single-platform method) for nonvolumetric flow cytometers. General recommendations includes the acquisition of a minimum of 75,000 CD45+ events with a minimum of 100 CD34+ cells (7). The primary reason for using one blue laser is simply that it was generally available on almost all cytometers in the past.

When CD34+ are present at low frequency such as in poor mobilizers, analysis of hematopoietic progenitor cells can be difficult on a clinically routine basis. Rare cell analysis recommendations include exclusion of nonviable cells in combination with doublet discrimination (8). In addition, flow stability monitoring is crucial to ensure the linearity and accuracy of measurements. Microspheres can be used to monitor stable sample acquisition over time, which allows the use of the time parameter for gating, and most strategies are based on bead counting over time (8). All of the aforementioned dyes, including FITC, PE, and 7-AAD are commonly excited at 488 nm with emission collected in the green, orange, and red channels, respectively. There is overlapping emission of this fluorophore combination when excited by the 488 nm laser. Therefore, correct color compensation should be verified for accurate counting of CD34+ viable cells.

Minimizing and Eliminating Impacts from Color Compensation

PE is the most preferred fluorochrome for CD34 monoclonal antibody conjugation for the identification of human hematopoietic progenitor cells. PE is an intensely bright phycobiliprotein, its maximum absorption peak is at 565 nm with a secondary absorption peak at 496 nm. Yellow–green lasers with the emission wavelength of 561 nm can be used to better resolve PE-CD34 populations. Compared to 488 nm lasers, 561 nm lasers, typically produce approximate fourfold increased separation between the PE-positive and the PE-negative population as measured by the stain index (9). In practical applications at 488 nm excitation, average stain indices for PE (BD PE-labeled CD4 Clone RPA-T4) and for FITC are 302 and 56, respectively (http://www.nature.com/nmeth/journal/v5/n12/full/nmeth.f.229.html). However, the need of compensations does not come from the brightness of PE conjugates. Major overlap between fluorescence emissions when excited with 488 lasers comes from FITC into PE. This means that FITC and PE color compensation needs to be validated on a regular basis. In addition, when more fluorochromes are added, additional fluorochrome interactions can occur that cannot be fully corrected by pair-wise compensations. An added advantage to using the 561 nm laser to excite PE is that spillover from FITC is reduced to 0%, a consequence of the lack of excitation of FITC at this wavelength, which simplifies detecting dim or rare populations without color compensation between FITC and PE (10). This facilitates further strategies aimed at fully automating CD34+ counting methods. In this brief communication, we have followed the ISHAGE guidelines to examine the advantages of 561 nm laser equipped cytometers in enumerating CD34+ cells with better resolution and with no color compensation when PE is excited with the 561 nm laser. The availability of yellow–green laser equipped flow cytometers has enabled us to evaluate the ISHAGE-based method to propose a series of recommendations aimed at CD34+ progenitor cell counting using no color compensation. FITC was detected with 488 nm excitation. PE was detected using 488 nm excitation and compared with the fluorescence of the same fluorochrome simultaneously excited at 561 nm. When the yellow–green laser excites PE, there is virtually no spectral overlap between FITC and PE, eliminating the need to perform compensation and simplifying the assay. When PE was detected using blue excitation however, color compensation is needed to separate the overlapping FITC and PE emission spectra.

Is 561 nm Excitation an Alternative Improvement for CD34 Counting?

The improved PE signal using 561 nm excitation together with the fact that there is no need for color compensation between FITC and PE under 488 nm and 561 nm excitation, results in the improved resolution of CD34+ cells (Figs. 1A and 1B). The experimental evidence of no compensation is needed is also supported by Figures 1C and 1D, showing FITC-CD45 versus PE-CD34 (488 nm excitation) compared with FITC-CD45 versus PE-CD34 (561 nm excitation), both uncompensated. An additional explanation for these differences is the increased mean autofluorescence of leukocytes in the PE channel with 488 nm excitation versus 561 nm excitation (MFI = 920 vs. 707), affecting the signal-to-noise ratio of the antibody staining in each dot plot displaying CD45 versus CD34+ events. Interestingly, 7-AAD background noise was also reduced with PE-CD34 excitation at 561 nm, as compared with PE-CD34 excitation at 488 nm (Figs. 1E and 1F). When binding to DNA, 7-AAD shifts its maximum spectral absorbance from 506 nm to 552 nm and shifts its spectral emission maximum from 675 nm to 655 nm (11). This has the net effect of a more specific signal with greater relative DNA bound signal versus unbound dye signal for 561 nm excitation light. Additionally, the narrow 585/16 filter bandpass used for 561 nm excited PE emission collects less spectral bleed from both bound and unbound dye than the 590/40 filter used for 488 nm excited PE emission. These spectral properties of 7-AAD would also suggest that dead and apoptotic cell discrimination would be easier and would require less compensation with 561 nm laser excitation. Alternatively, a broader bandpass filter could be used for PE emission from the 561 nm laser in order to further improve the stain index improvement at the price of greater 7-AAD bleed. Note that the stain index improvement observed with the filter combination used is < twofold (Figs. 1A and 1B). Minimizing fluorescence spillover between PE and 7-AAD with

561 nm lasers represents an additional advantage for development of new methods for clinical applications, especially for more complicated polychromatic cell analysis.

This new approach shows the feasibility of CD34+ counting without color compensation for the 561 nm laser (Fig. 2). While signal-to-noise of bona fide CD34+ cells

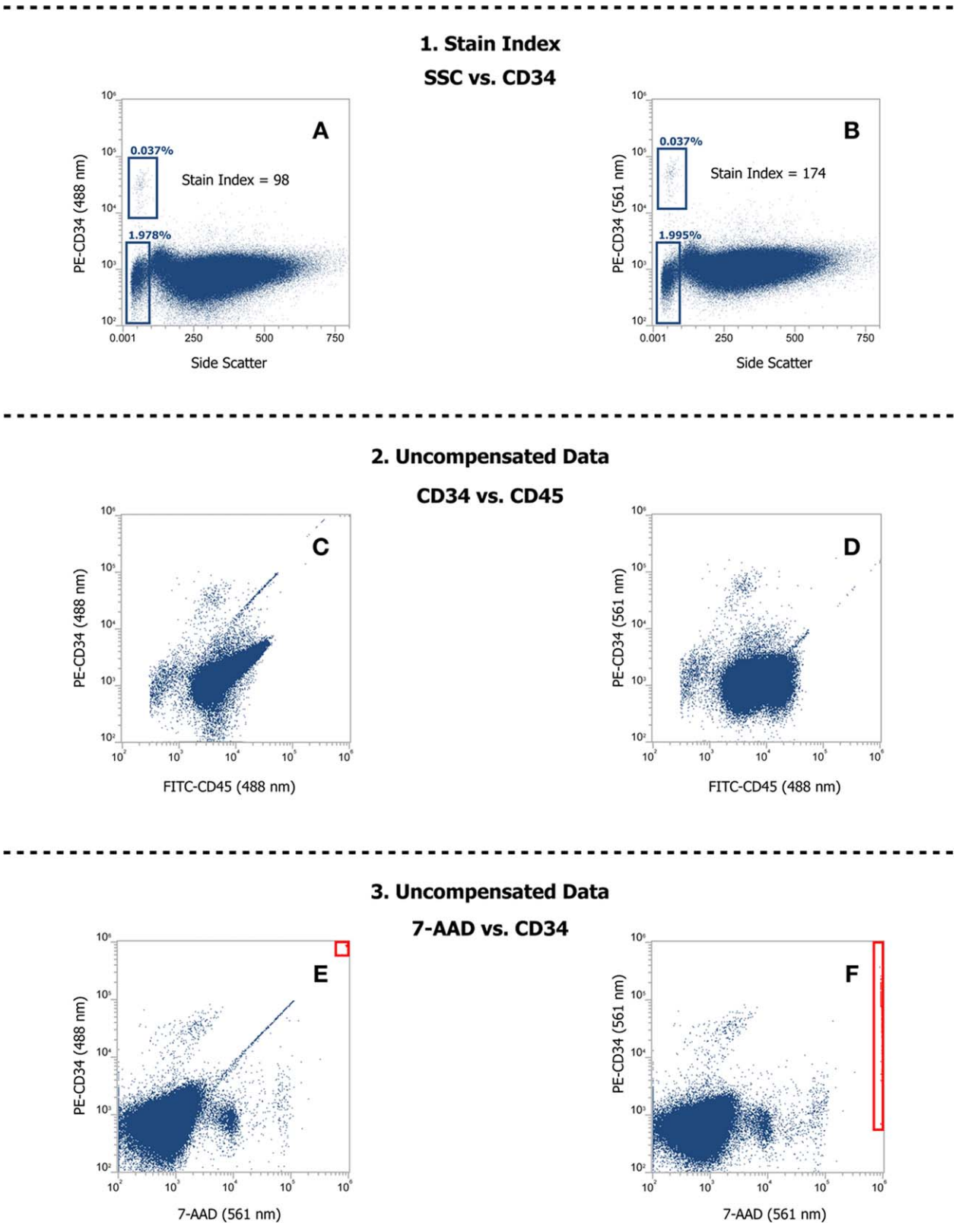

Figure 1.

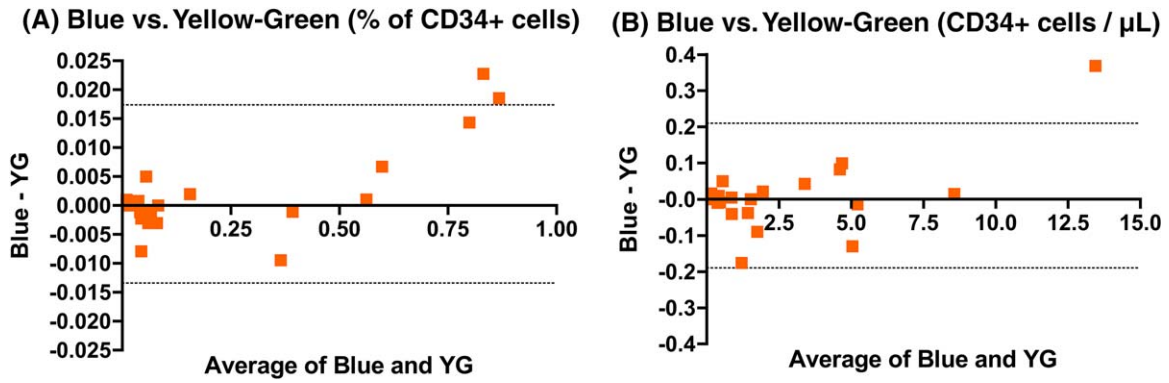

Figure 2. Bland–Altman plots for method comparisons for hematopoietic samples. For each sample ($n = 21$), the counting of CD34+ cells was represented against the difference between CD34+ counts obtained with the compared lasers. Differences between blue and yellow–green lasers calculated as percentage of CD34+ cells A). Differences between blue and yellow–green lasers calculated as CD34+ cells/µL B). The average ± 1.96 standard deviation of the difference value (dotted line) describes the 95% confidence interval for the difference between two methods. The differences between blue laser and yellow–green laser measurements were calculated according to the Bland and Altman statistical method (12) ($n = 21$). Human blood samples were obtained from patients receiving G-CSF for stem cell mobilization and collection. All procedures were performed in accordance with the internal protocols of our laboratory, which were authorized by the HGTiP's Ethical Committee, in accordance with current Spanish legislation, by the Departament de Medi Ambient i Habitatge (file #1899) of the Autonomous Government of Catalonia (Generalitat de Catalunya). [Color figure can be viewed at wileyonlinelibrary.com]

might benefit from detecting PE-CD34 conjugates using 561 nm lasers, the conventional methodology is still capable of generating accurate results regardless of instrument platform or reagent kit used. Nevertheless, as some very poor examples of the protocol's deployment persist, continuous education and support to improve current usage of the ISHAGE protocol are still needed. Development of more simplified automated protocols and analysis could help reduce this need. Bland–Altman shows discrepancy of the methods for high-abundant CD34 samples. While true CD34+ values remain unknown, absolute counting provides accurate comparisons and more agreement when compared with percentage comparisons. A caveat for applying limits of agreement is that the differences between the two methods of measurement should follow an approximate normal distribution and that the mean and standard deviation of the differences should be constant at different points in the measurement scale. Percentages may not fit agreement as absolute counts, as in general the range of absolute values (concentration) is narrow and the difference is small.

In summary, 561 nm laser excitation can improve CD34+ cell counting protocols. The expanding technological capabilities of newer flow cytometers will make possible new fluorochrome combinations and panels that will help simplify operation and analysis as well as facilitate more robust flow cytometry results. This will be especially true for clinical applications in which more complex multicolor panels will be required to identify definitive phenotypes.

Figure 1. Uncompensated and ungated data used for side by side comparison of flow cytometry counting of CD34+ cells using 488 and 561 nm laser excitation. 1. Representative Stain Index of PE-CD34+ cells using blue and yellow–green laser excitation. Mobilized peripheral blood was stained and analyzed following the ISHAGE guidelines to display CD34+ cells excited at both 488 nm (**A**) and 561 nm (**B**). PE-CD34 dotplots were used to display equal numbers of progenitor cells with and without color compensation, as shown in A and B plots, respectively. Stain Index (SI) is notably increased under yellow–green laser excitation. The SI was calculated using the median and the robust standard deviation according the following formula: [(median fluorescence intensity of positively stained population—median fluorescence intensity of negative population)/(2 × robust standard deviation of negative population)]. $SI_A = [(31101–767)/(2 \times 309.77)] = 97.92$; $SI_B = [(49277–748)/(2 \times 279.47)] = 173.65$. 2. Advantages of using the 561 nm laser to excite PE. Uncompensated, ungated data used to compare **C**) FITC-CD45 versus PE-CD34 (488 nm excitation) with **D**) FITC-CD45 versus PE-CD34 (561 nm excitation) dot plots. Using 561 lasers, spillover from FITC is reduced to 0%, which simplifies detecting dim or rare populations without color compensation between FITC and PE. PE detection using 488 nm excitation however, needs color compensation to separate the overlapping FITC and PE emission spectra. When the yellow–green laser excites PE, there is virtually no spectral overlap between FITC and PE, eliminating the need to perform compensation and simplifying the assay. This facilitates further strategies aimed at fully automating CD34+ counting methods. 3. Identification of PE-CD34 versus 7-AAD events detected using blue and yellow–green laser excitation. 7-AAD versus PE-CD34 dotplots were used to display equal numbers of progenitor cells with and without color compensation, as shown in **E** and **F** plots, respectively (events in gate illustrate absolute counting beads). Background noise is notably reduced under yellow–green laser excitation. SSC was detected using the 488 nm laser and a 488/10 bandpass filter and FSC was detected using the 488 nm laser (50 mW). FITC was detected with 488 nm excitation and a 530/30 bandpass filter in the BL1 (Blue Laser) detector. PE was detected using 488 nm excitation using a 590/40 bandpass in BL2. The Attune NxT standard four laser configuration was used which has 405 nm, 488 nm, 561 nm, and 637 nm excitation lasers. PE was also excited at 561 nm (50 mW) and its emission was collected using the following filter combination: 577 LP, 600 DLP, and 585/16 BP in the YL1 (Yellow Laser PE) detector. 7-AAD was detected with 488 nm excitation and a 695/40 bandpass filter in the BL3 detector. FITC, PE, and 7-AAD fluorescence are displayed in logarithmic scale. [Color figure can be viewed at wileyonlinelibrary.com]

Acknowledgments

The authors are very grateful to Sara Vergara, Minerva Raya and Silvia Torres for kindly providing samples used in this study. The authors are also very grateful for their advice and technical support to Sergio Ramón, Víctor Querol, Clara Streiff, Paola Paglia, Luca Tiengo and Lluís Sainz from Thermo Fisher Scientific.

Conflict of Interest

M.D.W. and J.B. work for Thermo Fisher Scientific, which is in the business of selling flow cytometers and flow cytometry reagents.

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