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TECHNICAL NOTE

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Fluorochrome-dependent specific changes in spectral profiles using different compensation beads or primary cells in full spectrum cytometry

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Abstract

Full spectrum flow cytometry is a powerful tool for immune monitoring on a single-cell level and with currently available machines, panels of 40 or more markers per sample are possible. However, with an increased panel size, spectral unmixing issues arise, and appropriate single stain reference controls are required for accurate experimental results and to avoid unmixing errors. In contrast to conventional flow cytometry, full spectrum flow cytometry takes into account even minor differences in spectral signatures and requires the full spectrum of each fluorochrome to be identical in the reference control and the fully stained sample to ensure accurate and reliable results. In general, using the cells of interest is considered optimal, but certain markers may not be expressed at sufficient levels to generate a reliable positive control. In this case, compensation beads show some significant advantages as they bind a consistent amount of antibody independent of its specificity. In this study, we evaluated two types of manufactured compensation beads for use as reference controls for 30 of the most commonly used and commercially available fluorochromes in full spectrum cytometry and compared them to human and murine primary leukocytes. While most fluorochromes show the same spectral profile on beads and cells, we demonstrate that specific fluorochromes show a significantly different spectral profile depending on which type of compensation beads is used, and some fluorochromes should be used on cells exclusively. Here, we provide a list of important considerations when selecting optimal reference controls for full spectrum flow cytometry.

KEYWORDS
compensation beads, full spectrum flow cytometry, reference controls, spectrum overlay

1 | STATEMENT OF PURPOSE

Full spectrum flow cytometry is a powerful tool for immune monitoring on a single-cell level with extensive use in cancer immunotherapy research as well as in investigation of various immunological diseases [1, 2]. With the availability of 5-laser instruments and novel fluorophore families, panels of 40 or more markers became possible [3, 4]. However, with an increase of panel size, spectral unmixing issues arise, and panel optimization is becoming increasingly important to maximize the potential data outcome from each specimen [5].

Appropriate single stain reference controls are required for accurate experimental results and to avoid unmixing errors [6]. Most commonly, reference controls are compensation beads or cell samples stained with a single fluorescently-labeled antibody used in the experiment, which provides the spectral signature of each dye for the unmixing algorithm and establishes a baseline for comparison to the studied sample [5, 7].

In contrast to conventional flow cytometry, which solely focuses on the signal within the specific wavelength bandwidth of interest, full spectrum flow cytometry takes into account even minor differences in spectral signatures that can impact the reliability of reference controls.

To ensure accurate and reliable results the full spectrum of each fluorochrome must be identical in the reference control and the fully stained sample and signal intensity of the reference control should be as high or higher than the fully stained sample [5]. Otherwise, the differences between reference controls and samples may lead to increases in the calculated spreading error (i.e., the spreading of a fluorochrome-positive signal into a secondary detector) and result in incorrect or insufficient unmixing [8, 9]. As a result, interpretation of the data might be incorrect due to inappropriate gating, variability in data obtained from different samples and reduced sensitivity in detecting low fluorescence levels. It is also important to employ quality control measures that monitor instrument performance in order to ensure low day-to-day variability and early detection of issues that influence data quality (e.g., detector efficiency or laser power) [9, 10].

When establishing reference populations both beads and cells have proven to be valuable options for single stain controls. In general, using the same type of cells that will also be analyzed in the experiment is considered optimal, since this takes into account the background fluorescence present in the biological sample, which might be caused by autofluorescence, spectral overlap or undesirable antibody binding [11]. This is especially important for spectral unmixing, which treats the cellular autofluorescence as an additional fluorochrome in order to correct for it. However, certain markers may not be expressed at sufficient levels or on enough cells to generate a reliable positive control (e.g., lineage markers for dendritic cells, which represent less than 1% of leukocytes in the peripheral blood [12]). In this case, compensation beads show some significant advantages as they bind a consistent amount of antibody independent of its specificity [13]. In addition, they are commercially available and relatively inexpensive ready-to-use products, while the availability of biological specimens, especially when working with human samples, might be limited.

In this study, we evaluated two types of manufactured compensation beads for use as reference controls for full spectrum cytometry and compared them to primary leukocytes from both human and mouse. We provide a list of optimal reference controls for 30 of the most commonly used and commercially available fluorochromes when working with peripheral leukocytes. While most fluorochromes show the same spectral profile on beads and cells, we demonstrate that specific fluorochromes show a significantly different spectral profile depending on which type of compensation beads is used, including some that should be used on cells exclusively.

2 | MATERIALS AND METHODS

2.1 | Single stains on compensation beads

One drop of ThermoFisher Ultracomp eBeads Plus™ (Thermo Fisher Scientific, Waltham, USA) or one drop each of positive and negative Biolegend compensation beads (Biolegend, San Diego, USA) were diluted in 1 mL of staining buffer (HBSS + 2 mM EDTA). After thorough vortexing 100 µL of the diluted compensation beads were used for each reference control. Fluorochrome-conjugated antibodies were added at the indicated concentrations (Supplementary Table 1) and incubated for 15 min at room temperature. A washing step was performed with 2 mL of staining buffer (HBSS + 2 mM EDTA) and samples were resuspended in 200 µL of staining buffer and acquired immediately (within 20 min).

2.2 | Single stains on human peripheral blood leukocytes

Anti-coagulated (EDTA) whole blood was obtained from volunteers (ethical approval number EA2/065/21) and processed immediately. 200 µL of whole blood were incubated with indicated concentrations (Supplementary Table 1) of fluorochrome-conjugated antibodies for 20 min at room temperature. Lysis of red blood cells was performed by adding 2 mL of 1× BD Pharm Lyse™ Lysing buffer and incubating the samples for 10 min at room temperature. Cells were washed in staining buffer (HBSS + 2 mM EDTA) and incubated with lysis buffer a second time for 5 min at room temperature. After washing, cells were fixed in 2% formaldehyde (in 1× PBS) for 10 min. After the last washing step, cells were resuspended in 200 µL staining buffer and acquired immediately (within 20 min).

2.3 | Single stains on mouse splenocytes

Mouse splenocytes were harvested by forcing a spleen from a wild-type mouse through a 70 µm nylon mesh to achieve a single-cell suspension. Pelleted cells were resuspended in 5 mL of 1× BD Pharm Lyse™ Lysing buffer and incubated for 5 min at room temperature to remove red blood cells. Cells were washed with HBSS and resuspended in an appropriate volume of staining buffer. After filtering through a 70 µm mesh again, 10% of the single cell suspension were used for each reference control. Fluorochrome-conjugated antibodies were added directly to the cells at the indicated concentration (Supplementary Table 1) and incubated for 20 min at room temperature. A washing step was performed with 2 mL of staining buffer (HBSS + 2 mM EDTA) and samples were resuspended in 200 µL of staining buffer and acquired immediately (within 20 min).

2.4 | Data acquisition and analysis

Sample analysis was performed on a standard issue Cytek® Aurora Cytometer equipped with 3 lasers and 38 detectors (Supplementary Table 2) and running Spectroflo® v3.0.3 (Cytek Biosciences, Inc.). QC was performed daily, and samples were acquired at medium speed using Cytek Assay Settings. The stopping gate was set at 2500 events for beads or 100,000 events for cells. FSC and SSC were adjusted individually depending on the nature of the single stain control: compensation beads, human or mouse immune cells.

Histogram and dot plot overlays of single stain controls were generated with FlowJo 10.8.0 (BD Biosciences).

2.5 | Normalized spectra and similarity

Normalized spectra for each reference control were extracted from Spectroflo® v3.0.3 as follows: After gating on beads or the cells of interest (lymphocytes or myeloid cells, depending on antigen-specificity) according to their FSC/SSC profile, interval gates for positive and negative peaks were defined for each single stain in the respective peak channel (Supplementary Table 3) and statistics tables indicating the median signal intensity of all fluorescent channels were created for both the positive (MFI_{pos}) and the negative (MFI_{neg}) gate. Statistics data was exported to Excel, and the normalized signal intensity for each fluorescent dye in each channel was calculated as $MFI_{pos} - MFI_{neg}/(MFI_{pos} - MFI_{neg})max$. Using normalized medium fluorescence intensity data, spectrum overlays were created in GraphPad Prism 9 and visualized as a histogram overlay graph for comparison.

The similarity between indicated fluorochromes was determined by the unmixing tool of SpectroFlo® and displayed in heat maps, values inside the cells are given in %.

3 | RESULTS

The availability and diversity of manufactured compensation beads have significantly increased in the past years as they are increasingly used as reliable reference controls in flow cytometry. However, not all types of compensation beads bind all antibody species (Supplementary Table 4), which reduces their convenience of use. We compared the species reactivity of commercially available compensation beads (Supplementary Table 4) and decided to test Biolegend Compensation beads as well as ThermoFisher Ultracomp eBeads™ Plus due to their ability to bind the most indicated species. We tested 30 of the most commonly used and commercially available fluorochromes in full spectrum cytometry on both types of beads, as well as human peripheral blood leukocytes and mouse splenocytes. The list of all antibodies used in this study can be found in Supplementary Table 1. In general, we used the same anti-human antibody on both types of beads as well as human peripheral blood leukocytes (Supplementary Table 3), while a different antibody was used on murine splenocytes due to specificity.

Twenty-five out of the 30 tested fluorochromes showed identical emission spectra on cells and either type of beads (Supplementary Figure 1). Consistently, Spectroflo® determined a similarity of 100% for all reference control types for each of these fluorochromes (Supplementary Figure 2). Of note, this included protein-based tandem dyes such as PE-Cy5, PE-Cy7, and APC-Cy7 that are known to be sensitive to PFA fixation, even though we fixed the human peripheral blood leukocytes prior to analysis due to local biosafety regulations. Supplementary Figure 3 shows that the spectral signatures of these fluorochromes on compensation beads do not change with PFA fixation, even after extended storage times (up to 4 h) before analysis. This enables the use of compensation beads as reliable reference controls for each tested color when working with human or murine peripheral leukocytes.

However, five fluorochromes (BV480, BV785, PerCP-Cy5.5, PerCP-eFluor710, and AlexaFluor700) showed different emission spectra on cells and beads (Figure 1). BV480, BV785, and PerCP-eFluor710 showed identical emission spectra on mouse and human primary cells as well as Biolegend Compensation Beads, but different spectra when used on ThermoFisher UltraComp eBeads Plus (Figure 1A,B,D). This is also reflected in the similarity matrices generated by SpectroFlo®. Notably, the spectra for AlexaFluor 700 seemed to differ between murine cells and Biolegend Compensation Beads versus human cells and UltraComp eBeads Plus, but only the difference between human and murine cells is picked up by SpectroFlo® (Figure 1E). PerCP-Cy5.5 showed different emission spectra between all types of primary cells as well as compensation beads (Figure 1C). While primary mouse cells seem to show a spectrum identical to Biolegend Compensation Beads and almost identical to human cells (similarity 99%), UltraComp eBeads™ Plus only reach a similarity of 92% or 94% with mouse and human primary cells, respectively. To determine the impact these reduced similarities have on the quality of the unmixing we unmixed PerCP-Cy5.5 single stains on human and mouse cells with all types of beads and cells and compared the resulting data. To ensure that any observed differences are only due to the different emission spectra we made sure that the single stains on beads were always brighter than the single stains on cells (Supplementary Figure 4A) and that cells were used as unstained controls during unmixing to allow for correct autofluorescence subtraction. Supplementary Figure 4B shows dot plots of PerCP-Cy5.5 versus PerCP-eFluor710—the fluorochrome with the highest similarity to PerCP-Cy5.5—demonstrating that only using the same cell type in the reference control and the sample generates properly unmixed data. Taken together, these results indicate a remarkable advantage of Biolegend compensation beads as a reliable reference control for spectral flow cytometry for all tested fluorochromes except PerCP-Cy5.5.

Since several of the fluorochromes described in Figure 1 are tandem dyes (BV785, PerCP-Cy5.5, and PerCP-eFuor710), we next investigated the potential impact of different antibodies—with different clones (which come from different production batches) and from different vendors (which might use different variants of fluorescent probes)—on the spectral profiles. To test this, we stained both types

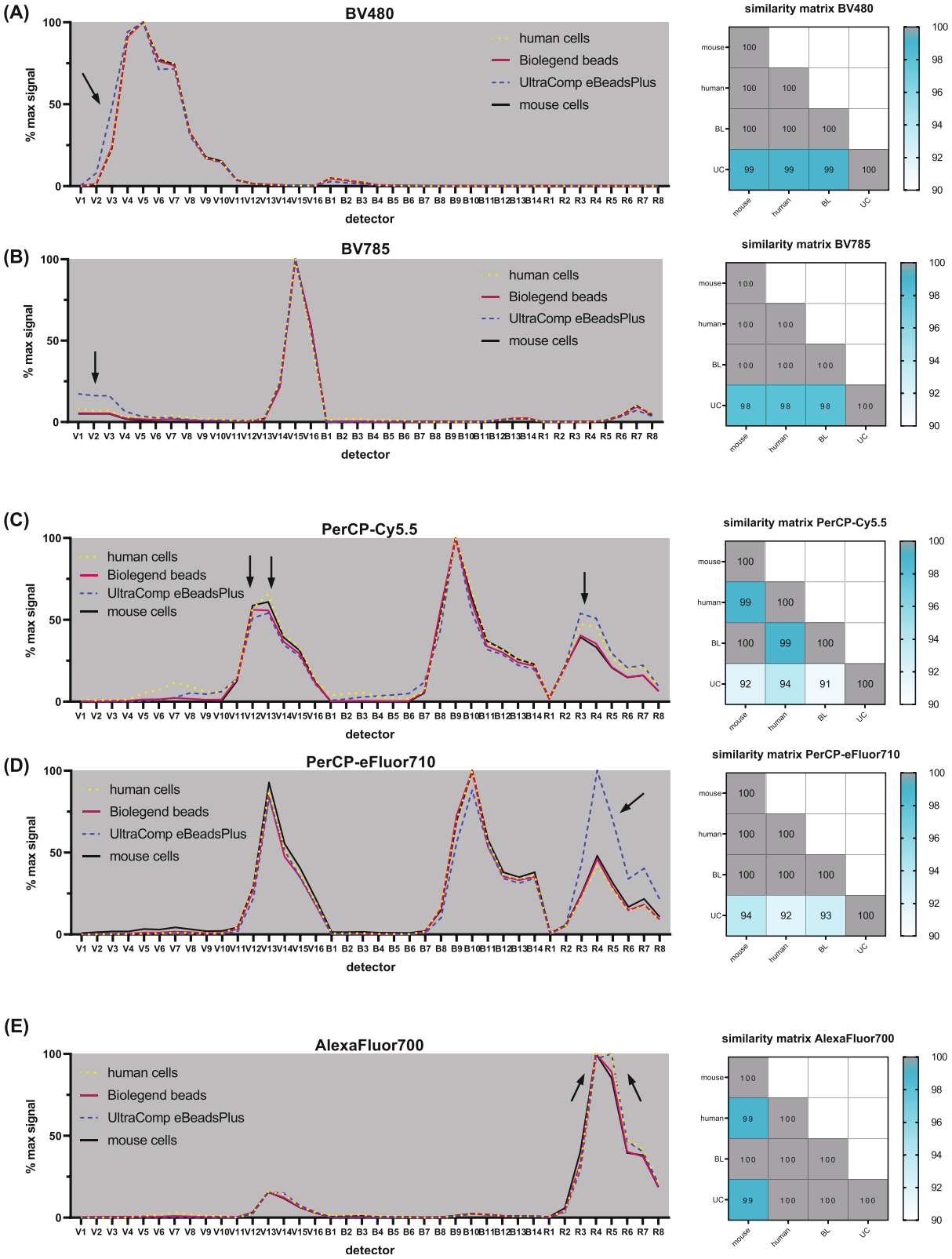

FIGURE 1 Normalized emission spectra and similarity matrizes of fluorochromes showing different spectra on cells and beads. Indicated fluorochromes were stained on cells (human peripheral blood leukocytes or murine splenocytes), Biolegend® Compensation Beads or UltraComp eBeads™ Plus Compensation Beads. Emission spectra were extracted from Spectroflo software, normalized to the maximum signal and overlaid in GraphPad Prism. Similarity was determined in Spectroflo software. (A) BV480, (B) BV785, (C) PerCP-Cy5.5, (D) PerCP-eFluor710, and (E) AlexaFluor 700. BL, Biolegend compensation beads; UC, UltraComp eBeads Plus. [Color figure can be viewed at wileyonlinelibrary.com]

of compensation beads with several antibodies coupled to the same fluorochromes that showed differential emission spectra before. Supplementary Figure 5 provides emission spectra and similarity matrices for each indicated fluorochrome, the spectrum observed on human cells was kept as a reference. When used on Biolegend compensation beads, this analysis confirmed a reproducible pattern of similarity as observed in Figure 1, independent of the antibody used, for all colors except PerCP-Cy5.5. In this specific case, all antibodies showed different emission spectra, even when purchased from the same company. Importantly, on UltraComp eBeads Plus we detected differences between antibodies from different vendors also for BV785 and Alexa-Fluor700, further confirming the advantage of Biolegend compensation beads. Of note, on UltraComp eBeads Plus we also noticed different emission spectra depending on the antibody used for PerCP-eFluor710, even though this fluorochrome is exclusively available from ThermoFisher/Invitrogen.

4 | CONCLUSIONS

In this study, we demonstrate the usefulness and reliability of compensation beads as reference controls for full spectrum cytometry, offering the potential to replace primary cells. However, we also show that the spectral signatures of some fluorochromes differ significantly from those observed on cells. It is important to note that for this study we only used human peripheral blood leukocytes and mouse splenocytes and that the observed changes might be different on other types of primary cells, especially those derived from organs known to have a high autofluorescence (such as liver, lung or kidney) or cells with specific metabolic activities that could lead to tandem-dye degradation (such as reactive oxygen species production [14, 15]). Therefore, it is advised to experimentally check that the beads or cells selected for single stained controls yield proper spectral signatures to be applied to the cells of interest for every fluorochrome in the panel. This might need to be tested for every cell type separately. Of note, new types of compensation beads using different materials (e.g., Spectracomp® beads from Slingshot Biosciences, which are hydrogels instead of polystyrene beads) have become available and might exhibit spectral signatures resembling cells even more closely; these could be tested in this context to further improve performance of reference controls. We also did not test ultraviolet or yellow-green excitation in this setup, which needs to be evaluated carefully before using the same type of reference controls on systems with more than three lasers. Furthermore, it is feasible to assume that different sample preparation protocols might have an influence on spectral signatures as well and should therefore be assessed before each experiment.

Importantly, our data also indicate that the spectral signatures of most fluorescent probes are independent of antibody clone/batch or antibody vendor when used on Biolegend compensation beads. Generally, it is advised to test each new antibody batch, at least for tandem dyes, but our data indicate that individual verification of each new antibody panel might be omitted if the fluorochromes and cell populations of interest are the same. For human and murine primary leukocytes, we provide a readily accessible list (Supplementary Table 5) optimized reference controls for 30 of the most commonly used and commercially available fluorochromes in full spectrum cytometry when used on a 3-laser system and under the same conditions described here.

AUTHOR CONTRIBUTIONS

Linda Hammerich: Conceptualization; investigation; writing – original draft; writing – review and editing; project administration; data curation; funding acquisition; formal analysis; visualization. **Yaroslava Shevchenko:** Investigation; methodology; data curation; writing – original draft. **Isabella Lurje:** Data curation; writing – review and editing; investigation; supervision; visualization. **Frank Tacke:** Writing – review and editing; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no financial conflicts of interest.

PEER REVIEW

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REFERENCES

1. Bonilla DL, Reinin G, Chua E. Full spectrum flow cytometry as a powerful technology for cancer immunotherapy research. Front Mol Biosci. 2020;7:612801.
2. Hammerich L, Shevchenko Y, Knorr J, Werner W, Bruneau A, Tacke F. Resolving 31 colors on a standard 3-laser full spectrum flow cytometer for immune monitoring of human blood samples. Cytometry B Clin Cytom. 2023;104:367–73.
3. Park LM, Lannigan J, Jaimes MC. OMIP-069: forty-color full spectrum flow cytometry panel for deep immunophenotyping of major cell subsets in human peripheral blood. Cytometry A. 2020;97(10):1044–51.
4. Sahir F, Mateo JM, Steinhoff M, Siveen KS. Development of a 43 color panel for the characterization of conventional and unconventional T-cell subsets, B cells,NK cells, monocytes, dendritic cells, and innate lymphoid cells using spectral flow cytometry. Cytometry A. 2024;105(5):404–10.
5. Ferrer-Font L, Small SJ, Lewer B, Pilkington KR, Johnston LK, Park LM, et al. Panel optimization for high-dimensional immunophenotyping assays using full-spectrum flow cytometry. Curr Protoc. 2021;1(9):e222.

6. Ferrer-Font L, Pellefigues C, Mayer JU, Small SJ, Jaimes MC, Price KM. Panel design and optimization for high-dimensional immunophenotyping assays using spectral flow cytometry. Curr Protoc Cytom. 2020;92(1):e70.
7. Maecker HT, Trotter J. Flow cytometry controls, instrument setup, and the determination of positivity. Cytometry A. 2006;69(9):1037–42.
8. Farrand K, Holz LE, Ferrer-Font L, Wilson MD, Ganley M, Minnell JJ, et al. Using full-spectrum flow cytometry to phenotype memory T and NKT cell subsets with optimized tissue-specific preparation protocols. Curr Protoc. 2022;2(7):e482.
9. Cossarizza A, Chang HD, Radbruch A, Acs A, Adam D, Adam-Klages S, et al. Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur J Immunol. 2019;49(10):1457–973.
10. Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay PK, Roederer M. Quality assurance for polychromatic flow cytometry using a suite of calibration beads. Nat Protoc. 2012;7(12):2067–79.
11. Hulspas R, O'Gorman MR, Wood BL, Gratama JW, Sutherland DR. Considerations for the control of background fluorescence in clinical flow cytometry. Cytometry B Clin Cytom. 2009;76(6):355–64.
12. Haller Hasskamp J, Zapas JL, Elias EG. Dendritic cell counts in the peripheral blood of healthy adults. Am J Hematol. 2005;78(4):314–5.
13. Roederer M. Compensation in flow cytometry. Curr Protoc Cytom. 2002;1:14. Chapter 1.
14. Le Roy C, Varin-Blank N, Ajchenbaum-Cymbalista F, Letestu R. Flow cytometry APC-tandem dyes are degraded through a cell-dependent mechanism. Cytometry A. 2009;75(10):882–90.
15. Jensen IJ, McGonagill PW, Lefebvre MN, Griffith TS, Harty JT, Badovinac VP. Worry and FRET: ROS production leads to fluorochrome tandem degradation and impairs interpretation of flow cytometric results. Immunity. 2020;52(3):419–21.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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