Bigfoot Spectral Cell Sorter

High-dimensional cell sorting utilizing real-time spectral unmixing

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Introduction

The rapid advance of flow cytometry over the past two decades has been an incredible asset to the cell research community. The convergent evolution of digital electronic acquisition, solid-state laser excitation, refined emissions collection optics, advanced hardware automation, and fluorophore development has increased capabilities from 4 to 40 colors, allowing more data from each sample. Analytical flow cytometry and multicolor phenotyping panels have been helpful in establishing the presence of cell subsets. However, segregation of specific cell subsets for molecular profiling and sequencing has been problematic because cell sorters have lacked the technical sophistication to isolate cell types found in complex multicolor spectral panels and sort them in real time. The Invitrogen™ Bigfoot Spectral Cell Sorter was designed to address this challenge by pairing the power of full-spectrum flow cytometry with real-time sorting to extend the frontiers of cell type discovery. Recent studies have shown that interactions between cell surface carbohydrate polymers (glycans) and their protein receptors (lectins) can induce clustering of the latter and result in profound changes to leukocyte phenotype. Of particular importance are the sialic acid–binding immunoglobulin-like lectin (Siglec) family of receptors, expressed by most leukocytes at various stages of development and activation [1]. These surface-exposed transmembrane lectins can be recruited to an immunological synapse by their cognate glycan ligands. In this way, Siglec–sialic acid interactions are immunosuppressive and help immune cells distinguish between self and nonself matter, because pathogens often lack sialic acids. Correlations between cell immunophenotypes and specific Siglec expression promise to provide new insights into immune responses and pathologies.

In this paper we will demonstrate the capability of the Bigfoot Spectral Cell Sorter using a 23-color panel (Figure 1) designed for isolating Siglec-expressing cell subpopulations, which are expressed on most white blood cells of the immune system and play critical roles in immune cell signaling.

Laser	Antigen	Label	Vendor	Cat. No.	Clone
349	Siglec-F	BUV395	BD Biosciences	740280	E50-2440
349	LIVE/DEAD Fixable Blue Dead Cell Stain		Thermo Fisher	L23105	NA
349	CD8a	BUV496	BD Biosciences	750024	53-6.7
349	CD62L	BUV563	BD Biosciences	741230	MEL-14
349	Siglec-E	BUV615	BD Biosciences	752326	750620
349	Siglec-H	BUV661	BD Biosciences	749806	440c
349	CD44	BUV737	BD Biosciences	612799	IM7
405	F4/80	BV421	BD Biosciences	565411	T45-2342
405	CD11b	BV570	BioLegend	101233	M1/70
405	CD80	Super Bright 600	Thermo Fisher	63-0801-82	16-10A1
405	Siglec-G	BV650	BD Biosciences	745364	SH1
405	I-A/I-E	BV711	BioLegend	107643	M5/114.15.2
405	CD25	BV785	BioLegend	102051	PC61
488	Siglec-1	FITC	Bio-Rad	MCA947F	MOMA-1
488	CD11c	Alexa Fluor™ 532	Thermo Fisher	58-0114-82	N418
488	Ly6C	PerCP-Cyanine5.5	Thermo Fisher	45-5932-82	HK1.4
561	Siglec-2	PE	BioLegend	126111	OX-97
561	CD4	PE-Texas Red™	Thermo Fisher	MCD0417	RM4-5
561	NK1.1	PE-Cyanine5	BioLegend	108716	PK136
561	Siglec-3	PE-Cyanine7	Thermo Fisher	25-0331-82	9A11-CD33
561	CD3e	PE-Cyanine5.5	Thermo Fisher	35-0031-82	145-2C11
640	CD19	APC	Thermo Fisher	17-0193-82	eBio1D3 (1D3)
640	Ly6G	APC-eFluor™ 780	Thermo Fisher	47-9668-82	1A8-Ly6g

Figure 1. 23-color fluorophore panel.

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Spectral analysis and cell sorting

Sixteen years ago, the concept of spectral flow cytometry was demonstrated by flow cytometry pioneer and innovator J. Paul Robinson (Cytometry Lab, Purdue University), but it was not incorporated into standard instruments for many years due to limited practical applications [2]. In 2015, commercial spectral flow cytometers appeared on the market, ready for installation in core lab facilities seeking to take on new challenges [3]. These instruments still offer a chance to analyze complex, heterogeneous populations where autofluorescence limits dye options [4]. This type of versatility is reshaping panel design by allowing freedom to combine fluorescent proteins, surface markers, and other dyes while still allowing spectral separation. However, many instruments produced today are uniquely configured to primarily perform spectral analysis, with a limited option to perform the accepted, reliable, and trusted technique of compensation, which some researchers still want due to familiarity or for comparison to other methods. The Bigfoot Spectral Cell Sorter's acquisition and sorting application, Invitrogen™ Sasquatch (SQ) Software, allows the operator to sort and acquire either spectrally unmixed data or traditionally compensated data.

As with most technological tools, the quality of data produced depends on proper use. Successful high-speed sorting based on spectrally unmixed data requires a precise combination of concepts from the disciplines of biology, mathematics, and high-speed computing. Some researchers may find it overwhelming to balance numerous fields of expertise where even a slight knowledge gap can result in suboptimal experiment design and results. For the Bigfoot Spectral Cell Sorter, the underlying mathematical theory of spectral data handling was used to develop tools that provide researchers with valuable guidance, starting with panel design and continuing throughout all phases of the experiment. Not only does this impart mathematical rigor and eliminate subjectivity, but also it frees the researcher to focus on his or her scientific specialty. SQ Software includes extensive user-support tools to identify fluorophores that, when paired, are expected to either unmix properly or be challenging due to high levels of signal overlap. To assist with experiment design, geometric and statistical comparisons are performed to assess the likelihood of successful unmixing of the chosen panel. Similar comparisons are used to identify and account for autofluorescence. The spectral unmixing process is performed on specialized, state-of-the-art hardware that allows for remarkably fast real-time processing and high-purity spectral sorting.

Methods

The following methods were used for instrument setup, sample preparation, spectral setup, and gating.

Panel design

Fluorophores were selected to detect leukocyte phenotyping markers, activation markers, and Siglec proteins. Single cells were gated using scatter parameters (FSC vs. SSC), and dead cells were excluded via a UV laser–compatible viability dye. Gating schemes are presented in Figures 5–9 and are based on well-established expression profiles for each leukocyte population.

Instrument setup

The Bigfoot Spectral Cell Sorter was started and allowed 10 minutes to warm up and stabilize, after which the automated QC process was initiated. The comprehensive three-bead QC protocol set the optimal nozzle position, calibrated the droplet and stream settings, adjusted the stream positions, and calculated the system's drop delay value. The resulting CVs, voltages, and separation factor, as well as sort settings, are tracked day to day and can be viewed in a trending report. PMT voltages can be applied as application references in future experiments.

Sample preparation

Samples were prepared and stained according to the following flow cytometry staining protocol:

1. Prepare staining buffer: HBSS, 2 mM EDTA, 1% BSA.
2. Mash spleen tissue in a nylon filter with a rubber-capped syringe plunger in 5 mL of staining buffer. Transfer the suspension to a 15 mL conical tube.
3. Centrifuge at 350 x *g* at 4°C for 5 min.
4. Remove the supernatant and resuspend the cells. Add 3 mL of erythrocyte lysis buffer. Incubate at room temperature for 3 min.
5. Add 7 mL of staining buffer, shake, and centrifuge at 350 x *g* at 4°C for 5 min. Remove the supernatant.
6. Wash cells with 10 mL of protein-free PBS. Centrifuge at 350 x *g* at 4°C for 5 min.
7. Prepare a stock solution of Invitrogen™ LIVE/DEAD™ Fixable Blue Dead Cell Stain according to the manufacturer's instructions (reconstitute in DMSO). Prepare a solution of approximately 1:1,000 dye stock in protein-free PBS. Remove the supernatant from step 6, and add 500–1,000 µL of 1:1,000 dye stock to the cells and thoroughly suspend them.
8. Cover the sample with foil and incubate for 15 min at room temperature.
9. Add 10 mL of staining buffer and mix. Incubate the sample briefly at room temperature. Centrifuge at 350 x *g* at 4°C for 5 min. Remove the supernatant.
10. Deposit the cells in a 96-well U-bottom plate. Add 50 µL of Fc-block solution (1:100 dilution of unlabeled mouse Fc-block antibody (anti-CD16/32)). Incubate for 10 min on ice.
11. Dilute the sample with 150 µL of staining buffer and centrifuge at 350 x *g* at 4°C for 5 min. Discard the supernatant.
12. Make the antibody cocktail in staining buffer (1:200–1:500 dilution is typical for each reagent).
13. Resuspend cells in the antibody staining cocktail (typical staining cocktail volume is 50 µL).
14. Keep the sample covered with foil and incubate it for 20 min on ice.
15. Dilute the samples with staining buffer (150 µL) and centrifuge at 350 x *g* at 4°C for 5 min. Discard the supernatant.
16. Wash the samples twice with 200 µL of staining buffer.
17. Reconstitute the cells in staining buffer, filter them through a 40 µm nylon mesh, and run the samples on a flow cytometer.
18. Use a small volume of unstained cells as a negative control to generate an autofluorescence reference signal.
19. Use antibody capture beads for single-color reference spectra controls.

Spectral workflow and unmixing

In SQ Software, a new sorting workflow was selected and designated as a spectral experiment. The fluorophores required for the experiment were selected, and the software provided a set of three analysis tools used to determine the suitability of the selected fluorophores for a spectral unmixing experiment. Figures 2–4 give examples of the graph of the predicted spectral signature, the spectral similarity matrix, and the breadcrumb display of the complexity index of the panel, respectively.

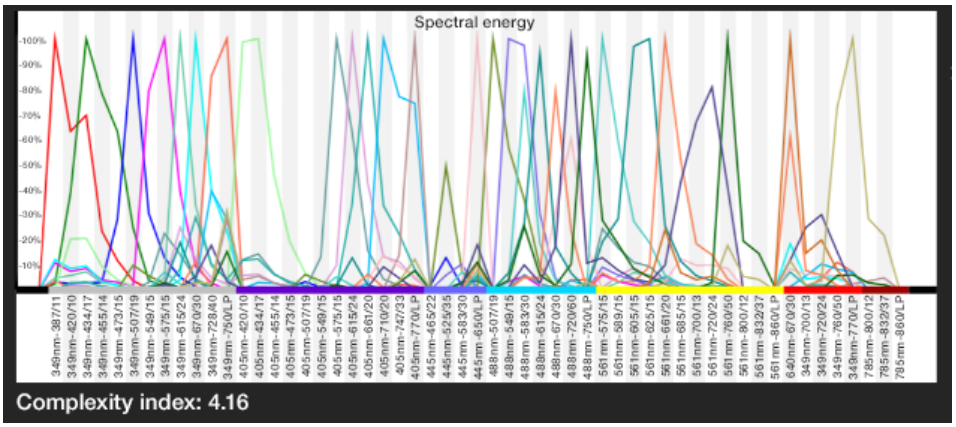

Figure 2. Graph of the predicted spectral signature. SQ Software presents a visual representation of the selected panel of fluorophores. This graph is helpful because it provides a clear picture of where additional fluorophores can be added without overlapping with the current selections. A specific emission curve can be shaded by highlighting it in the “Selected Fluorophores” list.

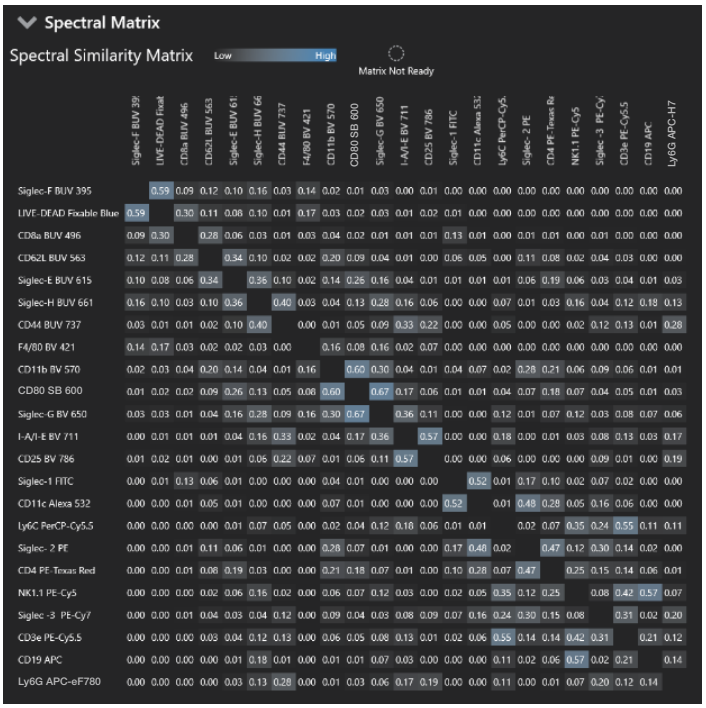

Figure 3. Spectral similarity matrix. The spectral similarity matrix (with values of 0–1.0) of SQ Software allows the user to identify fluorophore combinations that cause an increase in complexity and may be more difficult to spectrally unmix.

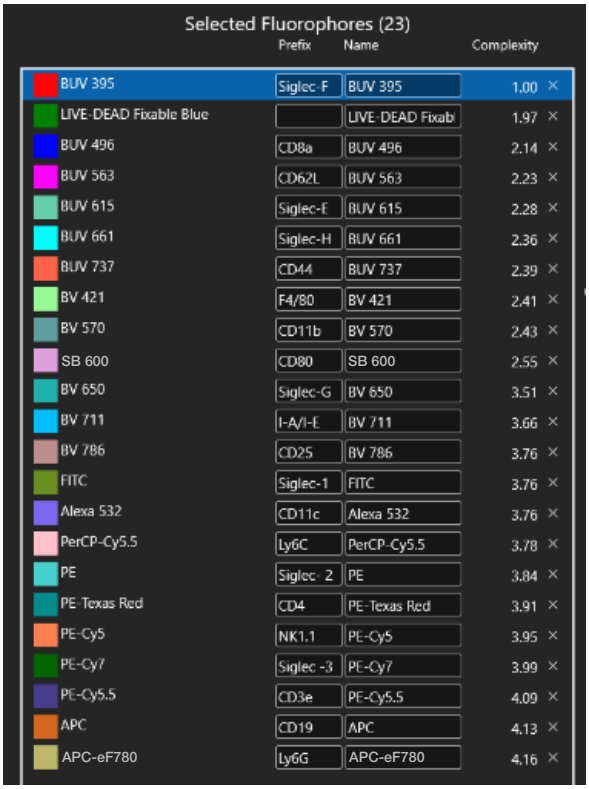

Figure 4. Complexity index of the panel. As the investigator selects fluorophores, the complexity index on the right of this panel tracks the quality of the overall panel. The value increases when there is more predicted overlap between spectral signatures. Fluorophores that minimally increase the index value are ideal choices.

Spectral unmixing

After confirming the fluorophore selections, the single-color control samples and templates were automatically generated in the workspace. Using events from the unstained cell control, the instrument set PMT voltages automatically to be used for the remainder of the experiment. The autofluorescence background signal was captured using the unstained cell control.

The calculation of the unstained signal allows automatic, real-time identification of positive cells during the acquisition of single-color controls. The detection of positive events is based on two separate statistical distances, ensuring a robust positive single-color control signal. Afterward, the positive populations are shown in automatically generated spectral density plots [5].

This process was repeated for each control. Upon successful acquisition of sufficient events for individual controls, the software analyzed the calculated signal for statistically significant separation from the unstained signal and similarity to the predicted signature from the fluorophore selection screen.

Automated mathematical checks were also performed by the software after each single-color control to ensure the unmixing parameters had a high likelihood of successfully separating the spectral signatures. New "spectral parameters" then became selectable in plots and were used for acquisition and sorting in the workspace.

Gating strategy

Initial forward and side scatter plots include all cell types but minimize the inclusion of subcellular debris. The scatter gate is then used to determine cell viability using a DNA stain to identify cells that no longer have membrane integrity and therefore stain positive for the viability dye. The cells that are negative for the viability dye are then assessed via two forms (FSC and SSC) of doublet discrimination using pulse waveform analysis to eliminate potential cell aggregates that were either conjoined or had passed coincidentally through the laser interrogation point (Figure 5). This initial gating strategy allows for only discrete and viable single cells to be identified by subsequent immunophenotyping, as shown in the fluorescence plots in Figures 6–9.

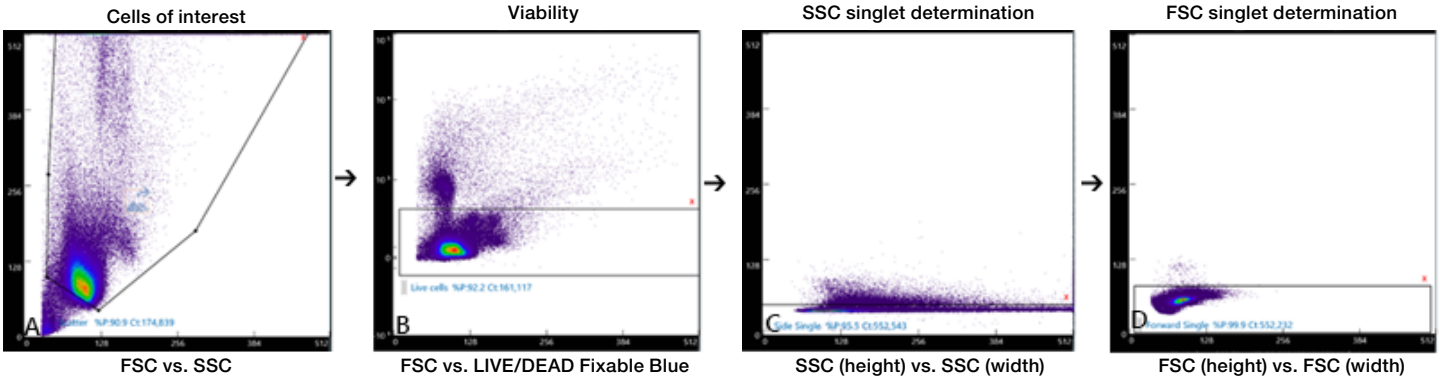

Figure 5. Initial gating strategy to identify single, viable cells. (A) FSC vs. SSC plot with a gate drawn to isolate the cells of interest. **(B)** FSC vs. LIVE/DEAD Fixable Blue Dead Cell Stain with a gate drawn to identify the viable cells. **(C)** SSC (height) vs. SSC (width) with a gate drawn to discriminate doublets. **(D)** FSC (height) vs. FSC (width) to further discriminate doublets.

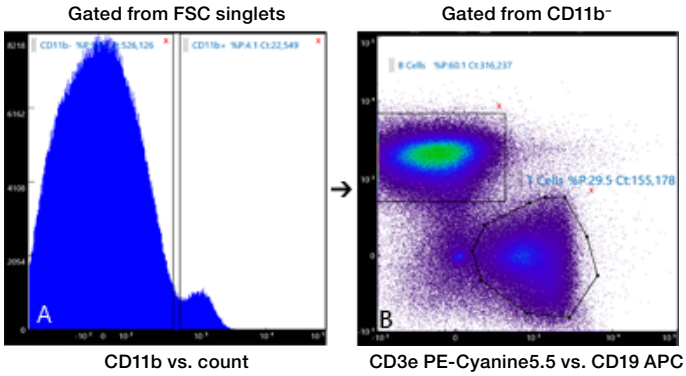

Figure 6. Identification of CD3e⁺ T cells and CD19⁺ B cells. (A) The FSC singlet population was analyzed using a histogram of CD11b vs. count to identify CD11b⁺ and CD11b⁻ populations. **(B)** The CD11b⁻ region was gated and further analyzed using CD3e PE-Cyanine5.5 vs. CD19 APC to identify the CD3e⁺ T cells and CD19⁺ B cells.

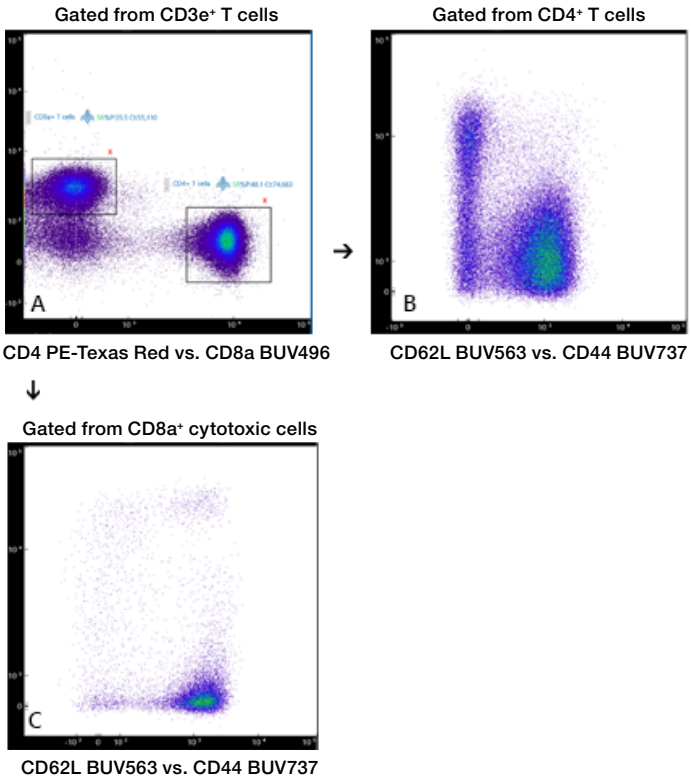

Figure 7. Identification of effector and naive cells from CD3e⁺ T cell subsets. (A) The CD3e⁺ population was analyzed with CD4 PE-Texas Red vs. CD8a BUV496 to identify and sort CD4⁺ T cells and CD8a⁺ cytotoxic cells. The **(B)** CD4⁺ cells and **(C)** CD8a⁺ cells were further analyzed for differential expression of CD62L and CD44 to identify effector and naive cells in each phenotype.

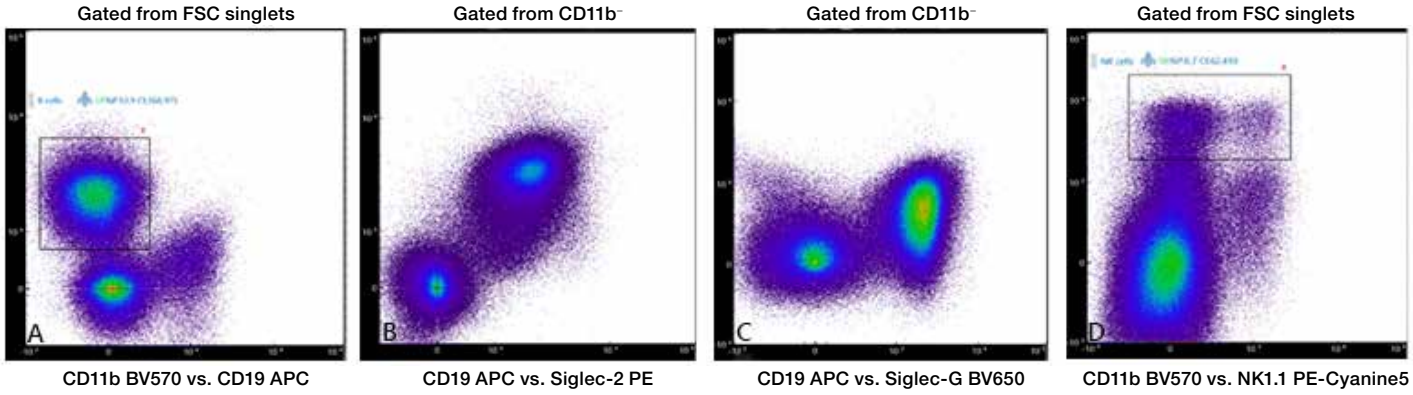

Figure 8. Identification of CD19⁺ B cells and NK cells. (A) The FSC singlet population was analyzed with CD11b BV570 vs. CD19 APC to identify and sort B cells. **(B)** The CD11b⁻ population was analyzed with CD19 APC vs. Siglec-2 PE to identify B cell Siglec expression. **(C)** The CD11b⁻ population was analyzed with CD19 APC vs. Siglec-G BV650 to identify B cell Siglec expression. **(D)** The FSC singlet population was analyzed with CD11b BV570 vs. NK1.1 PE-Cyanine5 to identify and sort NK cells.

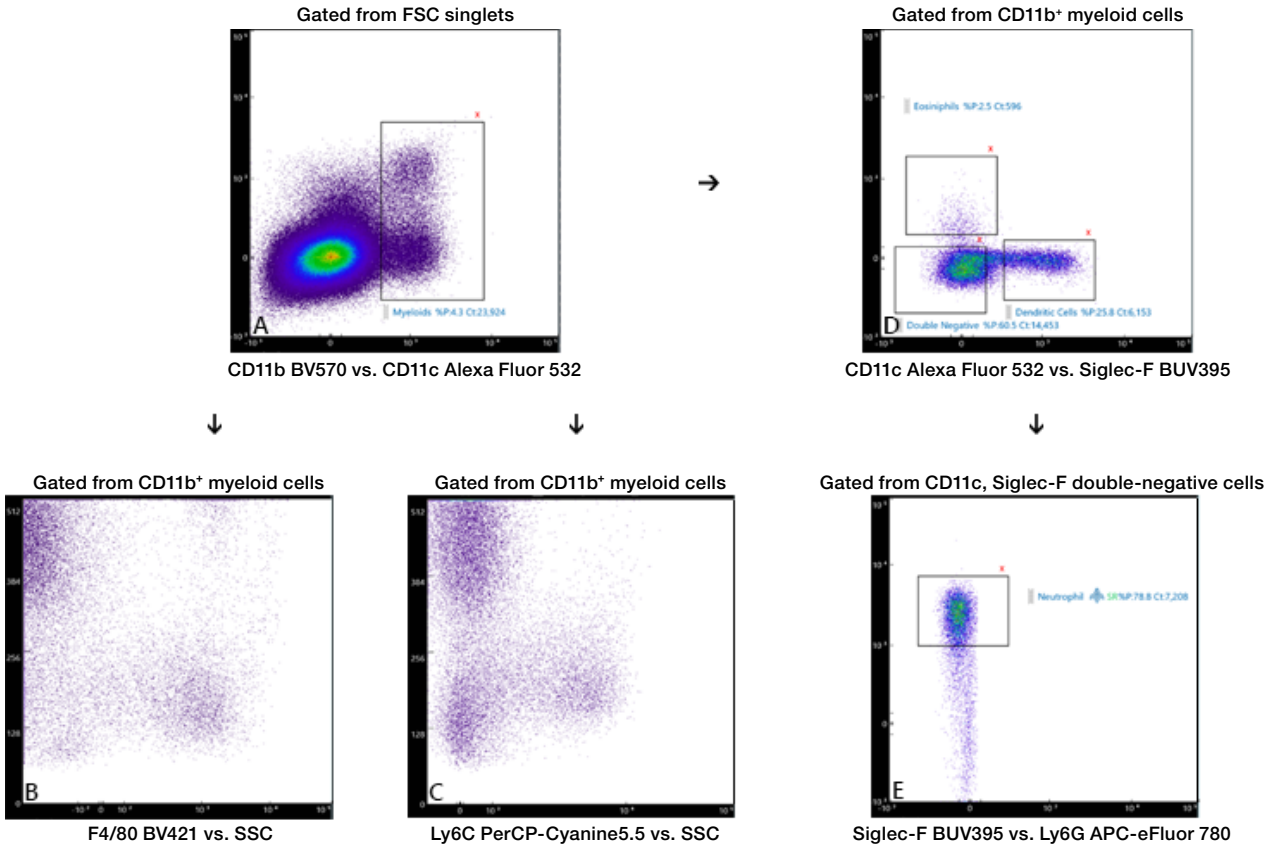

Figure 9. Identification of multiple types of myeloid cells. (A) The FSC singlet population was analyzed with CD11b BV570 vs. CD11c Alexa Fluor 532 to determine the CD11b$^+$ population. **(B)** The CD11b$^+$ population was further analyzed with F4/80 BV421 vs. SSC to identify monocytes and macrophages. **(C)** The CD11b$^+$ population was further analyzed with Ly6C PerCP-Cyanine5.5 vs. SSC to identify monocytes. **(D)** The CD11b$^+$ population was further analyzed with CD11c Alexa Fluor 532 vs. Siglec-F BUV395 to identify dendritic cells and eosinophils. **(E)** The CD11c, Siglec-F double-negative region was further analyzed with Siglec-F BUV395 vs. Ly6G APC-eFluor 780 to identify and sort neutrophils. While the dendritic cells and neutrophils were successfully sorted, the overall counts were extremely low.

Cell sorting setup

Cell subset populations (B cells, dendritic cells, neutrophils, CD8a$^+$ T cells, CD4$^+$ T cells, and NK cells) were selected for sorting. These populations were isolated by the gating strategy described above. The Bigfoot system was configured with a 100 µm nozzle tip, 30 psi sheath pressure, a drop drive amplitude of 13.3 V, and a drop drive frequency of 40,600 Hz. The sort was performed with an event rate of 5,000 events per second on the identified spectrally unmixed populations. Cells were sorted using purity mode into 5 mL tubes with 500 µL filtered HBSS, and reanalyzed for purity.

Results

The most common means of assessing sorter function and sort purity is by conducting a sort check. This is accomplished by collecting sorted cells and verifying that they are indeed the intended target subpopulation as defined by the sort gate logic. Ideally, these sorted populations can be analyzed on a separate instrument to validate that the sorter is configured properly and is sorting the populations as defined. Sort checks are not as useful for defining the overall health and viability of the sorted cells. Recent studies have shown that the process of cell sorting is inherently stressful on cells and several experimental considerations are required to ensure proper cell functionality and viability [6]. The sorted samples presented in this white paper were not intended for further downstream biological applications but solely as proof of principle that subsets could be defined spectrally and sorted successfully from a >20-color panel. In this pilot experiment, the sorted cells were not collected in a proper serum-enhanced collection buffer, and the reanalysis of the cells was delayed due to laboratory constraints. The sort checks for all six populations showed negligible evidence of contamination from other cell subpopulations in the presented data. However, the purely sorted populations exhibited reduced epitope expression because receptor expression is diminished when cells are distressed.

Regardless of the circumstances, each collection tube was reanalyzed for purity using an established five-laser spectral analyzer, and the data were imported into FlowJo™ software for visualization. Examples of the results are shown in Figure 10. The dendritic cell and neutrophil populations were also successfully sorted. However, from this sample, the counts were very low. In future experiments, the overall event count should be greatly increased to provide a statistically relevant population for reanalysis.

The spectral unmixing capability of the Bigfoot Spectral Cell Sorter produced results with high purity and efficiency, and the sorting performance is equivalent to instruments that utilize traditional compensation. This parity will allow users to seamlessly transition between the two methods as experiment complexity increases, thus improving research laboratory workflows.

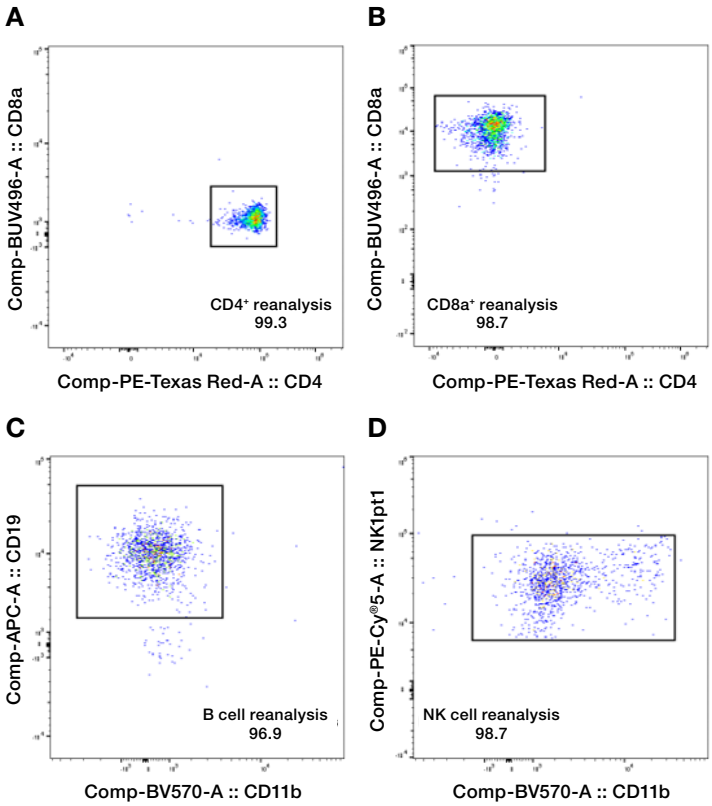

Figure 10. Reanalysis of collection tubes. Following the sort, collection tubes were reanalyzed for purity using a spectral analyzer from an independent facility, and the data were imported into FlowJo software for visualization. **(A)** The CD4 region shows 99.3% purity for the $CD4^+$ PE-Texas Red collection tube. **(B)** The CD8a region shows 98.7% purity for the $CD8a^+$ BUV496 collection tube. **(C)** The CD19 region shows 96.9% purity for the $CD19^+$ APC collection tube. **(D)** The NK1.1 region shows 98.7% purity for the $NK1.1^+$ PE-Cyanine5 collection tube.

Conclusions

Full-spectrum cytometry has been proven to help researchers build complex panels with numerous and novel combinations of markers. The data retrieved from these experiments are well resolved and of higher resolution than possible with traditional methods. However, the Bigfoot Spectral Cell Sorter takes these improvements to the next level by enabling these new combinations of markers to be sorted in real time. Additionally, operators can select up to 18 subpopulations of cells and sort each one to a different tube in a single run, or utilize the index feature to correlate an event in a plot with the physical location of the sorted event in a plate to further support DNA sequencing.

The ability to compare two fluorophores across multiple points on their spectra significantly reduces the correction error compared to compensating two spectral bands. Full-spectrum sorting will produce better population resolution compared to nonspectral sorting, and therefore result in superior sorted populations.

The electronics of the Bigfoot Spectral Cell Sorter include custom-designed, programmable-logic hardware with algorithms developed specifically for the challenges presented by sorting. Multiple innovations and design iterations were required to achieve greater than six terabits per second data processing speed required for successful operation. The resulting architecture allows operators to use either compensation or spectral unmixing in real time at sort rates of over 70,000 events per second.

The Bigfoot Spectral Cell Sorter has up to nine lasers with 3–12 photomultiplier tubes (PMTs) available for each detection path and therefore is ideally suited for the wide range of fluorescence signal intensities found in diverse flow cytometry applications. PMTs provide the best linearity to accurately measure signals and prevent distortions that often necessitate secondary processing such as compensation after unmixing. The Bigfoot Spectral Cell Sorter was designed using PMTs because they provide robust signal strength, which results in a higher signal-to-noise ratio than other collection methods. Furthermore, PMTs paired with selected single-channel filters create improved differentiation of fluorophore spectra and ensure that the system is not limited to a specific application.

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The PMTs of the Bigfoot Spectral Cell Sorter have an 8 mm diameter aperture, which significantly improves signal stability resulting in narrow CVs and consistent results across flow rates for the entire workday. Investigators can set PMT voltages using a control sample of unstained cells or by importing application settings derived from a three-bead QC control. Either way, the system sets PMT voltages automatically for consistent results. The detector and optical configuration, designed into the Bigfoot system, allows investigators to have the option to view data in multidimensional spectral mode or with compensation; both methods achieve outstanding performance. No other instrument can provide these advantages to the researcher.

Flow cytometry and real-time sorting using the Bigfoot Spectral Cell Sorter is an advanced technological tool to isolate cell types obscured in complex multicolor spectra. Isolating Siglecs using spectral cell sorting supports experimental opportunities to further define the roles of these molecules in the hematopoietic, immune, and nervous systems where they are expressed.

The Bigfoot Spectral Cell Sorter is a product of decades of flow cytometry experience, innovative engineering, and constant collaboration. As the first spectral cell sorter, the Bigfoot system will expand research capabilities and provide innovation to serve those in the quest for greater scientific knowledge.

References

1. Duan S, Paulson JC (2020) Siglecs as immune cell checkpoints in disease. *Annu Rev Immunol* 38:365–395. doi: 10.1146/annurev-immunol-102419-035900
2. Robinson JP (2019) Spectral flow cytometry—Quo vadimus? *Cytometry A* 95(8):823–824. doi: 10.1002/cyto.a.23779
3. Futamura K, Sekino M, Hata A et al. (2015) Novel full-spectral flow cytometry with multiple spectrally-adjacent fluorescent proteins and fluorochromes and visualization of *in vivo* cellular movement. *Cytometry* 87:830–842. doi: 10.1002/cyto.a.22725
4. Schmutz S, Valente M, Cumano A et al. (2017) Analysis of cell suspensions isolated from solid tissues by spectral flow cytometry. *J Vis Exp* (123):e55578. doi: 10.3791/55578
5. Thermo Fisher Scientific (2021) Bigfoot Spectral Cell Sorter with Sasquatch Software: The world's first real-time spectral cell sorter. White paper available at: http://assets.thermofisher.com/TFS-Assets/BID/Reference-Materials/bigfoot-spectral-cell-sorter-sasquatch-software-worlds-first-real-time-spectral-cell-sorter-white-paper.pdf
6. Box A, DeLay M, Tighe S (2020) Evaluating the effects of cell sorting on gene expression. *J Biomol Tech* 31(3):100–111. Erratum in: *J Biomol Tech* 31(4):168. doi: 10.7171/jbt.20-3103-004

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