Panel Optimization for High-Dimensional Immunophenotyping Assays Using Full-Spectrum Flow Cytometry

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Technological advancements in fluorescence flow cytometry and an ever-expanding understanding of the complexity of the immune system have led to the development of large flow cytometry panels reaching up to 43 colors at the single-cell level. However, as panel size and complexity increase, so too does the detail involved in designing and optimizing successful high-quality panels fit for downstream high-dimensional data analysis. In contrast to conventional flow cytometers, full-spectrum flow cytometers measure the entire emission spectrum of each fluorophore across all lasers. This allows for fluorophores with very similar emission maxima but unique overall spectral fingerprints to be used in conjunction, enabling relatively straightforward design of larger panels. Although a protocol for best practices in full-spectrum flow cytometry panel design has been published, there is still a knowledge gap in going from the theoretically designed panel to the necessary steps required for panel optimization. Here, we aim to guide users through the theory of optimizing a high-dimensional full-spectrum flow cytometry panel for immunophenotyping using comprehensive step-by-step protocols. These protocols can also be used to troubleshoot panels when issues arise. A practical application of this approach is exemplified with a 24-color panel designed for identification of conventional T-cell subsets in human peripheral blood. © 2021 Malaghan Institute of Medical Research, Cytek Biosciences. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Preparation and evaluation of optimal spectral reference controls
Support Protocol 1: Antibody titration
Support Protocol 2: Changing instrument settings
Basic Protocol 2: Unmixing evaluation of fully stained sample
Basic Protocol 3: Evaluation of marker resolution
Support Protocol 3: Managing heterogeneous autofluorescence
Basic Protocol 4: Assessment of data quality using expert gating and dimensionality reduction algorithms

Keywords: assay optimization and troubleshooting • full-spectrum flow cytometry • high-dimensional flow cytometry panel

INTRODUCTION

Technological advancements in fluorescence flow cytometry and an ever-expanding understanding of the complexity of the immune system have led to the development of large panels reaching 40 fluorophores in the Optimized Multicolor Immunofluorescence Panel (OMIP; Park, Lannigan, & Jaimes, 2020) and 43 colors in a technical note (Sahir, Mateo, Steinhoff, & Siveen, 2020). In contrast to conventional flow cytometry, which primarily measures the peak emission of each fluorophore in a target detector, full-spectrum flow cytometry uses a larger number of detectors with narrow band-pass filters. This allows the entire emission spectrum for every fluorophore to be captured across all laser lines, creating a detailed signature of each fluorophore. This makes it possible to distinguish fluorophores with very similar emission maxima but unique overall spectral fingerprints, increasing the flexibility in fluorophore selection. This feature, coupled with an instrument designed to maximize the detection of emitted light and highly efficient avalanche photodiodes, provides improved detection efficiencies that translate to better detection limits and higher signal resolution (Feher et al., 2016). While these features, unique to full-spectrum flow cytometry, provide the high-quality signals and low noise needed for successful high-dimensional panels, many of the same panel design considerations from conventional flow cytometry still apply. Common characteristics, together with those specific to full-spectrum flow cytometry, have been previously described (Ferrer-Font, Pellefigues, et al., 2020; Park et al., 2020). Now that hardware limitations hindering the use of highly overlapping dyes have been overcome, the main limitations for successful large panel design are the spillover-spreading error inherent to the use of fluorescence and the number of fluorophores available with unique spectral signatures. Many companies have recently begun developing spectrally distinct fluorophores, thus advancing the number of markers available to be analyzed in a single experiment.

Increasing the number of markers in a panel consequently increases the probability of compromising the resolution of the markers and populations of interest. The theoretical approach to panel design aims to avoid issues would that prevent resolution of every marker in the panel; however, in practice it is challenging to perfectly predict the impact of co-expression, variations in marker expression levels, and the performance of each specific reagent. Furthermore, it is difficult to anticipate the accuracy of reference controls for optimal unmixing results. For example, certain fluorophores emit slightly different spectra when bound to compensation beads or in the presence of different buffers.

One obstacle that has always impacted panel design and performance is the unique autofluorescence (AF) signatures of different sample and cell types. Cellular AF levels can vary depending on the type and metabolic state of cells (Mayeno, Hamann, & Gleich, 1992; Roederer, 2016; Shi et al., 2017) as well as sample preparation and staining procedures. This translates into different AF brightness levels and distinct spectral signatures in the samples being analyzed. Full-spectrum flow cytometry can resolve cellular AF signatures and ensure that they are not attributed to any of the fluorophores used. This can improve the signal-to-noise ratio and resolution of markers attached to fluorophores that emit closest to AF maxima (Ferrer-Font, Pellefigues, et al., 2020) in highly

autofluorescent tissues such as brain, lung, skin, intestine, and tumor (Schmutz, Valente, Cumano, & Novault, 2016). It is therefore highly recommended to characterize the AF spectrum of an unstained sample from the tissue or cell type of interest prior to panel design. This will provide useful information during fluorophore selection, ensuring that fluorophores are not allocated to areas of the spectrum where AF dominates.

STRATEGIC PLANNING

Once a high-dimensional full-spectrum flow cytometry panel has been optimally designed (Ferrer-Font, Pellefigues, et al., 2020), the step-by-step protocols presented here provide a series of practical steps for full and successful optimization. As outlined in Figure 1, the main procedures include evaluation of spectral reference controls (SRCs; see Basic Protocol 1), evaluation of unmixing of the fully stained (FS) sample (see Basic Protocol 2), evaluation of marker resolution (see Basic Protocol 3), and assessment of data quality (see Basic Protocol 4). Before any of these methods are performed, it is essential to titrate the antibodies used as well as any viability dye used. This is described in Support Protocol 1. An additional protocol describes changes that can be made to the instrument settings (see Support Protocol 2). Methods for evaluating and mitigating autofluorescence are included in Basic Protocol 3 and Support Protocol 3. Together, these protocols can also be used when troubleshooting a panel to identify sources of problems and provide insights into fixing them.

A 24-color panel optimization for identification of conventional T-cell subsets in human peripheral blood is provided to illustrate these procedures. The protocols were developed using the five-laser (5L) Cytek Aurora (Cytek Biosciences), but should be adaptable to any spectral flow cytometer. The protocols were designed for new full-spectrum flow cytometry users. Once familiarity and experience with specific tissue types is achieved, it is possible to modify the steps and order of the protocols to reduce the overall time
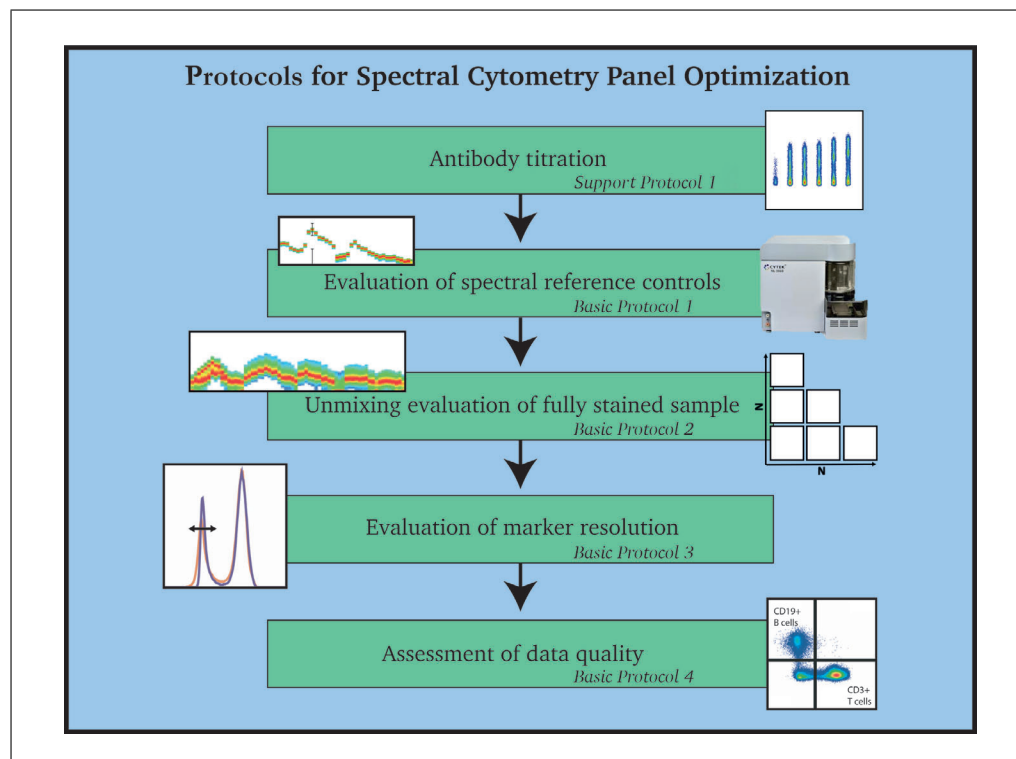

Figure 1 Overview of protocols for successful optimization of a high-dimensional spectral flow cytometry panel.

spent evaluating the panel. If a modified approach is taken, it is recommended that all the overall goals of the protocols (as outlined in Fig. 1) still be carried out.

PREPARATION AND EVALUATION OF OPTIMAL SPECTRAL REFERENCE CONTROLS

This protocol is divided in two sections. The first is for preparation of SRCs and ensures generation of the high-quality controls required for accurate unmixing. The steps describe the staining of polystyrene compensation beads and cryopreserved PBMCs with surface-labeling antibodies. They can be adapted for other tissues or staining procedures such as intracellular staining. It is important to mention that the preparation procedure will also guide users to prepare a fully stained (FS) sample and fluorescence minus one (FMO) controls as the protocols are very similar; these will be used later for evaluating the unmixing of the FS sample (see Basic Protocol 2). If preferred, FS and FMO samples can be stained separately, but treatment of samples should be kept identical. Importantly, to successfully complete this protocol, SS and FS cells should also be treated the same (antibody concentration, incubation time/temperature, fixed/unfixed, etc.).

The second section of the protocol is for evaluation. This aims first to check the quality of the acquired SRCs and then to evaluate whether there are any spectral mismatches between beads and cells for each fluorophore, which is accomplished by assessing how well the beads unmix the cells using $N \times N$ plots for all markers. Unmixing accuracy must be assessed on the actual sample (cells) to be used in the assay. In some cases beads will be acceptable as SRCs and in other cases it may be necessary to use cells. When this protocol is completed, the optimum SRCs (cells or beads) will be determined for future unmixing. If desired, well-characterized and high-quality controls can be stored for future use.

Materials

Cryopreserved PBMCs
Phosphate-buffered saline (PBS; Gibco, cat. no. 14190-250)
Fetal bovine serum (FBS; Gibco, cat. no. 10091-148)
Polystyrene compensation beads (e.g., UltraComp eBeads, Life Technologies, cat. no. 01-222-42)
FACS staining buffer: PBS with 2% bovine serum albumin (BSA; MP Biochemicals, CAS no. 9048-46-8) and 0.2% sodium azide (Sigma-Aldrich, cat. no. S8032)
Zombie NIR Fixable Viability Kit (BioLegend, cat. no. 423106)
Human TruStain FcX (Fc Receptor Blocking Solution; BioLegend, cat. no. 422301)
Antibodies (see Table 1 for list; see Support Protocol 1 for titration)
Brilliant Stain Buffer Plus (BD Biosciences, cat. no. 566385)

37°C water bath (e.g., Julabo Ecotemp TW12)
Hemocytometer (e.g., Hawksley Counting Chamber) and coverslips
96-well U-bottom plate (In Vitro Technologies, cat. no. 353077)
Filters with 0.65-µm or smaller pore size (*optional*)
Spectral cytometer (e.g., Cytek Aurora)
5-ml polypropylene round-bottom flow tubes (In Vitro Technologies, cat. no. 352008)
Cytek SpectroFlo software
Data analysis software for analyzing FCS files (e.g., FlowJo or FCS Express)

Prepare and acquire samples

1. Thaw PBMCs quickly in a 37°C water bath and add to 5 ml PBS with 2% FBS.

Table 1 Antibodies Used in Example Spectral Flow Cytometry Panel

Fluorophore	Marker	Supplier	Clone	Catalog number	Concentration (mg/ml)
BUV395	HLA-DR	BD Biosciences	G46-6	564040	1
BUV496	CD3	BD Biosciences	UCHT1	612940	1
BUV563	CD27	BD Biosciences	M-T271	741336	1
BUV737	CD45RA	BD Biosciences	HI100	612846	1.25
BV421	CD28	BioLegend	CD28.2	302930	2
Pacific Blue	CRTH2 (CD294)	BioLegend	BM16	350130	8
BV480	CD127	BD Biosciences	HIL-7R-M21	566101	2
BV510	TCRgd	BioLegend	B1	331220	15
BV570	CD4	BioLegend	RPA-T4	300534	1
BV605	CCR4 (CD194)	BioLegend	L291H4	359418	0.6
BV650	CCR6 (CD196)	BD Biosciences	11A9	563922	1
BV785	CD45RO	BioLegend	UCHL1	304234	1
BB515	CD25	BD Biosciences	2A3	564467	8.3
AF488	CCR10	R&D Systems	314305	RDSFAB3478G 0100	0.13
AF532	CD8	Thermo Fisher Scientific	RPA-T8	58-0088-42	0.25
PerCP-Cy5.5	CCR7	BioLegend	G043H7	353220	4
PE	Hu CD1d tetramer	NIH Tetramer Core Facility	PBS-57 loaded		0.81
PE-CF594	PLZF	BD Biosciences	R17-809	565738	0.63
PE-Cy7	CTLA4	eBioscience (TFS)	14D3	25-1529-42	0.2
APC	Hu MR1 tetramer	NIH Tetramer Core Facility	5-OP-RU loaded		0.18
AF647	FoxP3	BioLegend	259D	320214	0.6
APC-Cy7	CXCR3 (CD183)	Biolegend	49801.111	353722	0.13
BV711	CD19	BD Biosciences	SJ25C1	563036	0.25

As the thawing procedure can be critical, we recommend user-specific optimizations to ensure high sample viability (>80%) (Disis, dela Rosa, Goodell, & Ling-Yu, 2006).

CAUTION: *Steps 1-2 should be performed in a biosafety hood.*

2. Centrifuge 5 min at 500 × g, room temperature, and carefully flick off the supernatant.

3. Resuspend cells in PBS with 2% FBS and count viable cells on a hemocytometer.

4. Centrifuge as before and carefully flick off the supernatant.

5. Resuspend cells in PBS with 2% FBS to a concentration of 5×10^6 viable cells/ml.

6. Distribute 100 µl suspension per well of a 96-well U-bottom plate, allocating one well per fluorophore in the panel and one well for an unstained sample.

   A FS sample and appropriate FMO controls can be added but are not required for assessing the SRCs at this point. They will be used for assessing unmixing in Basic Protocol 2.

   The number of cells required will differ by tissue type; for PBMCs, ~5×10^5 cells is sufficient.

7. Add one drop of vortexed compensation beads plus 150 µl FACs staining buffer to a second set of wells, allocating one well per fluorophore in the panel except the viability dye.

   Figure 2A shows a typical plate layout. At this stage, the plate should contain unstained and FS sample wells (fully stained beads are not needed) plus two wells per fluorophore in the panel: one with cells and the other with compensation beads (except for the live/dead reagent, which is typically only used on cells). FMO controls should also be included for fluorophores where marker expression is very low or where the positive population is dim and gate placement would be subjective.

8. Centrifuge plate 5 min at $500 \times g$, 4°C, and flick off the supernatant.

   It is recommended to centrifuge samples at 4°C to maintain high viability, but room temperature is acceptable if a refrigerated centrifuge is not available.

9. Prepare viability stain in PBS according to the titration result (see Support Protocol 1) and add 100 µl to the live/dead cell control and FS sample, if included. Resuspend remaining wells with 100 µl PBS. Incubate for 15-30 min at room temperature, protected from light.

10. Centrifuge as above and flick off the supernatant.

11. Block Fc receptors of the cell samples using 100 µl of 1:40 Fc block in FACS staining buffer. Add an equal volume of FACS staining buffer to the beads. Incubate for 10 min at 4°C.

    Fc block should not be used on compensation beads, as all antibody binding sites will become occupied.

12. Centrifuge as above and flick off the supernatant.

13. Remove aggregates from antibody stocks by centrifuging 5 min at 16,000-18,000 $\times$ g, 4°C (Aass et al., 2011; Ayers et al., 2011; van der Vlist, Nolte-'t Hoen, Stoorvogel, Arkesteijn, & Wauben, 2012) and/or by filtering using a pore size of 0.1-0.65 µm (Inglis et al., 2015).

14. Prepare SS, FS, and FMO control antibody mixes by diluting stocks in FACS staining buffer according to titration results (see Support Protocol 1). Be sure to pipette from the top of the liquid to avoid centrifuged aggregates.

    Prepare enough volume of each antibody mix that two samples can be stained from the same mix. This will reduce differences due to antibody preparation when comparing bead versus cell SRCs. Allow for pipetting errors by making excess antibody mix (e.g., n + 1). Additionally, when more than one Brilliant polymer dye is used at the same time (FS and FMOs), Brilliant Stain Buffer Plus (or equivalent) should be added per manufacturer's instructions to decrease interaction between the dyes. Do not add Brilliant Stain Buffer Plus to compensation beads, as it is known to alter the spectral profile of some beads (Ferrer-Font, Pellefigues, et al., 2020).
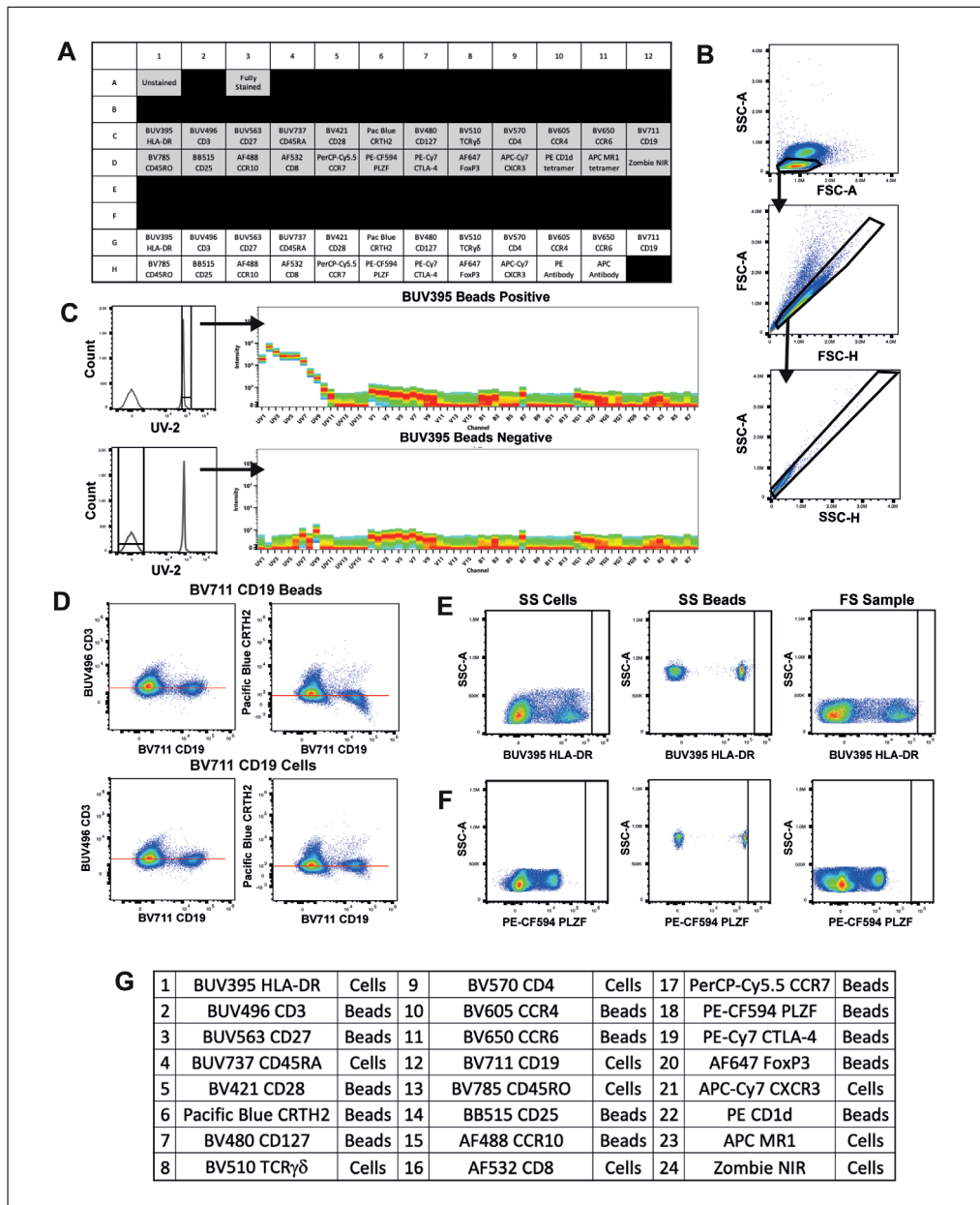
Figure 2 Evaluation of SRCs and selection of optimal control type. (**A**) Example plate plan. Shaded wells contain cells and unshaded wells contain compensation beads. (**B**) Expert gating of cells of interest and doublet exclusion plots. A well-optimized ASF can be visualized in the second pseudoplot. (**C**) BUV395 bead SRC gating of positive and negative populations and spectral signatures of gated events showing clear positive and negative signals. (**D**) SS cells unmixed with either BV711 CD19-stained compensation beads (top) or cells (bottom). Unmixing of BV711 CD19 is incorrect against certain parameters (e.g., Pacific Blue CRTH2) but not others (e.g., BUV496 CD3) when beads are used. This error is corrected through unmixing with cells with no negative impact to the plot with previously correct unmixing. Red line indicates equal median fluorescence intensity (MdFI) between positive and negative populations. (**E**) Comparison of brightness achieved using SS cells versus beads and the FS sample for BUV395 HLA-DR. Black line shows the maximum fluorescence of positive stained cells. In this example, cells should be used for unmixing as they are brighter than the beads. (**F**) Comparison of brightness achieved using SS cells versus beads as compared to the FS sample for PE-CF594 PLZF. In this example, beads are brighter than cells and should be used for unmixing. Black line shows the maximum fluorescence of positive stained beads is brighter than the FS sample. (**G**) Final decision of optimal SRCs (cells or beads) used for unmixing the panel.

15. Stain all cells and beads with 100 µl of the appropriate antibody mix and incubate in the dark using the incubation time and temperature that will be used in the final assay.

16. Centrifuge as above and flick off the supernatant.

17. Wash twice with 200 µl FACS staining buffer, centrifuging again after each wash.

    At this stage, cells may be fixed (e.g., 1% paraformaldehyde) if required for biosafety or sample longevity. If cells are fixed, beads must also be fixed to ensure equal treatment of fluorophores and to mimic any change to the spectral signature caused by fixation. The fixative must be removed by washing twice with FACS staining buffer before acquisition.

18. Resuspend in 200 µl FACS staining buffer.

19. Acquire on a Cytek Aurora, taking care to meet the following acquisition criteria:

    a. Cytek assay settings are used as a starting point for instrument setup.
    b. The scatter profiles of cells and beads are on scale and the FSC area scaling factor (ASF) is optimized (see example in Fig. 2B).
    c. All fluorescence signals are on scale ($<4 \times 10^6$). This can be assessed in the full-spectrum plot or in individual plots for every detector.
    d. All tubes are recorded with the same fluorescence gain settings for each detector.
    e. Sufficient events are recorded to find a clear positive signal.

    A minimum of 300 events is needed for each positive and negative population. A good starting point is 5000 total events for beads and 30,000-50,000 total events for cell controls, although it may be necessary to record >50,000 cells to get at least 300 positive events of similar fluorescence intensity for rare markers.

Evaluate results (see Video 1)

20. In the SpectroFlo software, check the raw reference control data and verify that the acquisition criteria in the previous steps have been met:

    a. The scatter profiles of the cells and beads are on-scale, clean, and easily gated, and the FSC ASF has been optimized (Fig. 2B).
    b. Cytek assay settings or a close alteration (also see Critical Parameters discussion of Complex Samples and see Support Protocol 2) have been used, and all tubes have been recorded with the same settings.
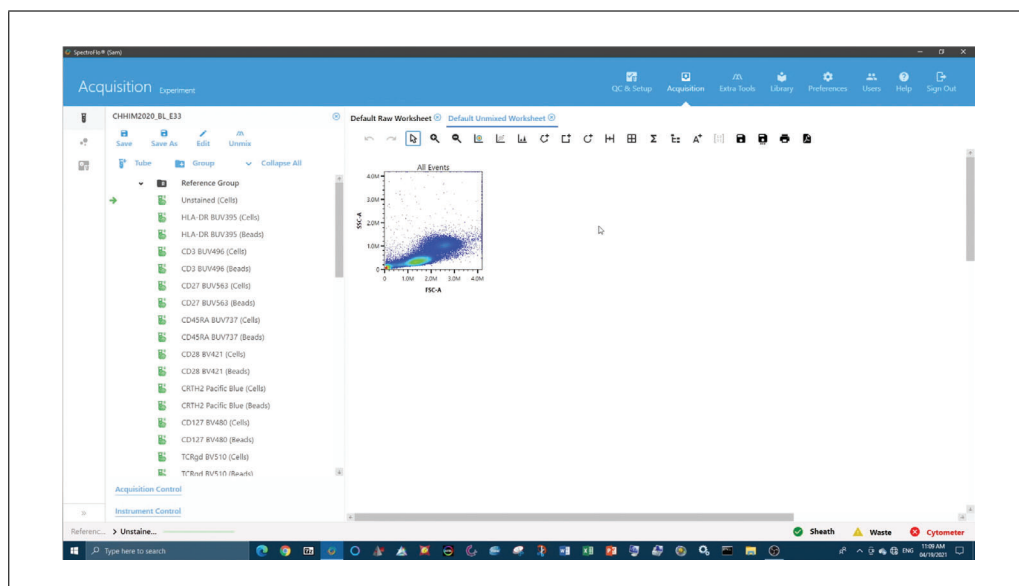
Video 1 Evaluation of optimal spectral reference controls (Basic Protocol 1).

21. Ensure that the unstained sample has no contamination from other fluorophores.

    Unstained cells often have AF signal in the detectors off the UV and violet lasers, which should not be mistaken for contamination.

22. In the SpectroFlo software, step through the Unmixing Wizard and select beads for all SRCs except for unstained and viability controls.

23. Accurately place positive and negative gates for unmixing.

    When setting scatter gates on bead SRCs, it is common to see singlet and doublet populations. Set the scatter gate on the smaller-sized (lower FSC) or more abundant singlet population. Beads should show little variability in staining level when prepared as described above, and thus the positive gate may include the complete positive bead population. If multiple peaks are seen, verify that they are not contaminating fluorophores or sample carryover, and optimize the staining procedure where needed to achieve homogenous staining intensity across all positive beads.

24. Perform quality control (QC) of all spectral signatures by verifying the following criteria:

    a. The peak channel matches that defined in Cytek's Full Spectrum Viewer (*spectrum.cytekbio.com*).
    b. The spectral signature appears as expected based on Cytek's Full Spectrum Viewer, published Cytek fluorophore guidelines, or historical data.
    c. Each channel contains a tight population of events.
    d. All fluorescent signals are on-scale ($<4 \times 10^6$). This can be assessed in the full-spectrum plot or in individual plots for every detector.
    e. Sufficient events have been recorded to find a clear positive signal.

25. Iterating through each fluorophore, place the positive gate over the negative population to verify that there is no fluorophore contamination (Fig. 2C).

26. Under the QC Controls tab, check Similarity Indices to ensure all spectral signatures are unique (i.e., all values within the matrix are ≤0.98).

27. Select the Live Unmix button to generate unmixed FCS files.

28. Create N × N plots on an unmixed worksheet (e.g., using software such as FlowJo or FCS Express).

    a. Create as many pseudocolor or dot plots as there are fluorophores in the panel.
    b. Ensure all *x* axes are set to the same fluorophore.
    c. Set each *y* axis to a different fluorophore in the panel.
    d. Change the *x* and *y* axes from manual scaling to autoscaling in the plot properties.
    e. Save this workspace as a template for use in later protocol steps.

29. Select the first SS cell sample.

30. Select all N × N plots. Then, in the Plot Properties window, change the *x*-axis option to the fluorophore matching the selected sample so all plots change simultaneously.

31. Evaluate the unmixing accuracy of the marker on the *x* axis by visually inspecting whether the positive and negative populations are well aligned horizontally along the *x* axis. If unmixing errors are seen in the N × N matrix plots (Fig. 2D, top), make a note of which control was being viewed.

32. Repeat steps 29-31 until the unmixing of all fluorophores has been evaluated.

33. Note which fluorophores require cells to be used for the SRC (i.e., those with identified unmixing errors in step 31). All other controls can remain as beads.

The most likely explanation for bead SRCs not unmixing the cell SRCs correctly is that the signature of the fluorophore on the beads did not match that of the cells. This phenomenon is known to happen but cannot be easily predicted. Another possible cause is that the bead SRC is dimmer than the cell SRC (Fig. 2E,F). If all recommendations for treatment of SS cells have been followed (i.e., identical treatment to FS cells, use of the same number of cells), then the cell SRC should have identical brightness to the FS cells and thus be an appropriate control.

34. Go through the Unmixing Wizard a second time using the controls identified in step 33 as the optimal controls. Unmix again using live unmixing.

35. Follow steps 23-26 for the modified controls to ensure the best unmixing outcome is achieved.

    When setting gates using cells as SRCs, the scatter gate should be placed only on the cells expressing the marker of interest for each control. In cases where there is no negative staining in this population, a universal negative may be used as a surrogate. To ensure the observed negative signal is due to AF rather than contamination, compare it to the matching unstained tube.

36. Repeat steps 29-32 to evaluate the new unmixing for the fluorophores that did not unmix optimally before (Fig. 2D, bottom) and note which fluorophores require cells or beads to be used for the SRC (Fig. 2G).

    Even with high-quality reference controls, unmixing errors may arise if incorrect gates are used in the Unmixing Wizard or if the spectral signatures have mismatches (see troubleshooting Tables 2-5 for more potential issues and how to fix them).

ANTIBODY TITRATION

Antibody titration is the crucial first step in developing high-dimensional flow cytometry panels. Using the incorrect antibody concentration can increase spread, decrease resolution, increase aggregation of reagents, and give rise to nonspecific binding (Stewart & Stewart, 1997), all of which result in poor panel performance and/or inaccurate results. Ideally, titrations should be carried out in the tissue that will be used in the assay. This is not always feasible, however, if the tissue in question is rare or difficult to work with, or the cells of interest are found at low frequencies within the tissue. In such cases, it is suggested to first add a lineage marker to the mix to help identify the cells that express the rarer marker or to use a surrogate tissue in which the marker is more abundant and simpler to process. Results from this type of titration should always be validated using the tissue of interest.

It is important that readout functional markers are titrated under the maximally activated conditions that will be used in the assay. Staining conditions should also be identical between titration and experiment to prevent spectral pattern mismatches and poor unmixing results.

This protocol describes the preparation of cryopreserved peripheral blood mononuclear cells (PBMCs) for titration of extracellular antibodies. It can be easily adapted and used as a general guideline for antibody titration using other tissues or staining procedures.

Materials

Cryopreserved PBMCs
Phosphate-buffered saline (PBS; Gibco, cat. no. 14190-250)
Fetal bovine serum (FBS; Gibco, cat. no. 10091-148)
Zombie NIR Fixable Viability Kit (BioLegend, cat. no. 423106)
Human TruStain FcX (Fc Receptor Blocking Solution; BioLegend, cat. no. 422301)

Polystyrene compensation beads (e.g., UltraComp eBeads, Life Technologies, cat. no. 01-222-42)

Antibodies (see Table 1)

FACS staining buffer: PBS with 2% bovine serum albumin (BSA; MP Biochemicals, CAS no. 9048-46-8) and 0.2% sodium azide (Sigma-Aldrich, cat. no. S8032)

37°C water bath (e.g., Julabo Ecotemp TW12)

Hemocytometer (e.g., Hawksley Counting Chamber) and coverslips

96-well U-bottom plate (In Vitro Technologies, cat. no. 353077)

Filters with 0.65-μm or smaller pore size (*optional*)

Spectral cytometer (e.g., Cytek Aurora)

5-ml polypropylene round-bottom flow tubes (In Vitro Technologies, cat. no. 352008)

Data analysis software for analyzing FCS files (e.g., FlowJo or FCS Express)

Prepare and acquire samples

1. Thaw PBMCs quickly in a 37°C water bath and add to 5 ml PBS with 2% FBS.

   CAUTION: Steps 1-2 should be performed in a biosafety hood.

2. Centrifuge 5 min at $500 \times g$, room temperature, and carefully flick off the supernatant.

3. Resuspend cells in PBS with 2% FBS and count on a hemocytometer.

4. Centrifuge as before and carefully flick off the supernatant.

5. Resuspend cells in PBS with 2% FBS to a concentration of 5×10^6 cells/ml.

6. Distribute 100 μl suspension per well of a 96-well U-bottom plate, allocating six wells to each antibody being titrated. Include additional wells for unstained and live/dead controls (see example plate plan in Fig. 3A).

   The number of titer points is based on the range of concentrations being tested. The number of cells required will differ by tissue type; for PBMCs, $\sim 5 \times 10^5$ cells is sufficient.

7. Centrifuge plate 5 min at $500 \times g$, 4°C, and flick off the supernatant.

8. Stain titration samples and live/dead controls with a viability dye that will not cause significant spillover into the fluorophore being titrated.

   Viability dyes should also be titrated for use in high-dimensional spectral cytometry panels. When titrating a viability dye, skip steps 8-12 for that sample only (adding PBS instead if other samples are being processed simultaneously). If the viability dye has not yet been titrated, follow manufacturer guidelines for concentration and incubation procedures.

9. Centrifuge as above and flick off the supernatant.

10. Block Fc receptors by applying 100 μl of a 1:40 dilution of Human TruStain FcX and incubating 10 min at 4°C.

11. Add one drop of vortexed compensation beads per well for the appropriate single stain (SS) controls.

12. Centrifuge as above and flick off the supernatant.

13. Remove aggregates from antibody stocks by centrifuging the vials 5 min at 16,000-18,000 $\times g$, 4°C, or by filtering using a pore size of 0.65 μm or smaller.
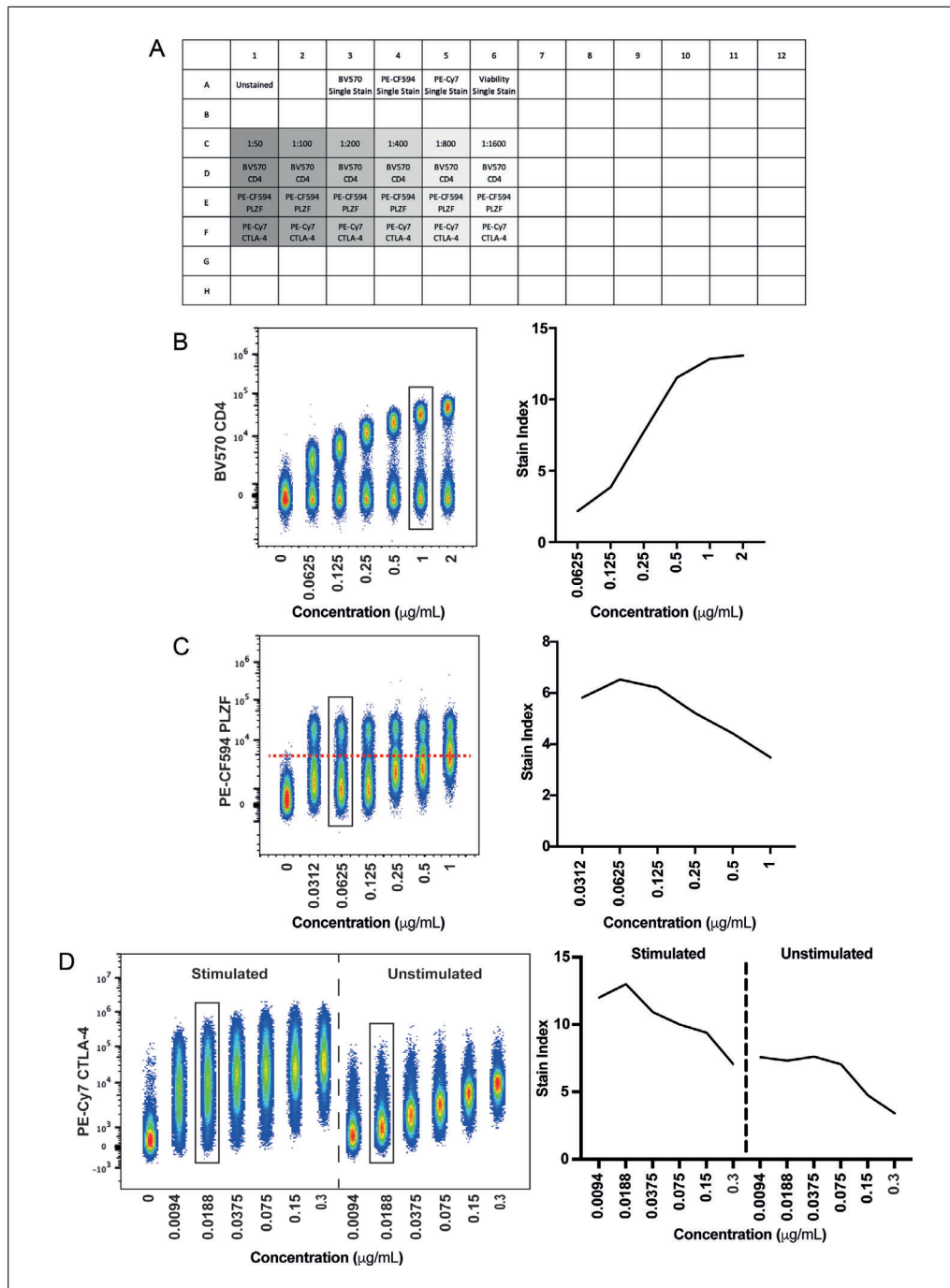
Figure 3 Antibody titration. Example titration of 1:2 serial dilutions for a range of marker subtypes, showing both concatenated flow cytometry data files and the calculated SI. The final dilution selected for the panel is indicated by black boxes. (**A**) Example plate layout. (**B**) T-cell co-receptor CD4 conjugated to BV570. (**C**) Transcription factor PLZF conjugated to PE-CF594. (**D**) Activation marker CTLA-4 conjugated to PE-Cy7 on cells with and without PHA stimulation (5 µg/ml, 2 days).

14. Create a dilution series for each antibody being titrated by diluting stock solutions in FACS staining buffer. Be sure to pipette from the top of the liquid to avoid the spun-down aggregates.

   A suggested dilution series is 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600. This series is broad enough to ensure the likelihood of finding the optimal titer for a range of antibodies. For ease of preparation, concentrations are listed here only in terms of their dilution from stock; actual concentrations (in mg/ml) should be calculated once a titer is selected.

15. Add 100 µl of each antibody dilution to the corresponding sample in the plate. Add FACS staining buffer to unstained and live/dead controls. For compensation beads, use 100 µl of the 1:100 dilution.

    The dilution used for compensation beads should not fall off-scale when using Cytek assay settings, but should be brighter than the samples. A 1:100 dilution is a good starting point, but a lower dilution can be used if some antibodies are too bright.

16. Incubate using the time and temperature that will be used in the final assay.

17. Centrifuge as above and flick off the supernatant.

18. Wash twice with 200 µl FACS staining buffer.

    If fixation will be used for the final samples, titrations should also be determined using fixed samples. For additional details, see Basic Protocol 1, step 17.

19. Resuspend in 200 µl FACS staining buffer and acquire on a Cytek Aurora using Cytek assay settings.

Analyze data
20. Open titration samples in a data analysis software used for analyzing FCS files.

21. Generate median fluorescence intensity (MdFI) values for the positive and negative populations for each concentration.

22. Generate standard deviation (SD) values for the negative population for each concentration.

23. Calculate the stain index (SI) using the equation $SI = (MdnFI_{pos} - MdnFI_{neg}) / (2 \times SD_{neg})$.

    The stain index is a very useful metric of resolution, with a higher value indicating greater resolution. If a lower concentration of antibody produces a reduced positive population MdnFI compared to a higher concentration with no change to the negative population, its stain index is going to decrease, as the separation between the positive and negative populations will be reduced (see example in Fig. 3B). If a higher concentration of antibody gives rise to a similar positive population MdnFI compared to a lower concentration, but has a larger SD of the negative population, its stain index is going to be decreased, as spreading of the negative population reduces the resolution from the positive (see example in Fig. 3C).

24. Select the concentration that gives rise to the best stain index without giving rise to a positive shift of the negative population.

    Such a shift is best visualized by concatenating all FCS files and displaying them on a single plot (as shown in in Fig. 3B-D).

CHANGING INSTRUMENT SETTINGS

In some cases an adjustment of fluorescent gain settings away from the optimized settings cannot be avoided, for instance, if a bright reporter protein (e.g., eGFP) is off-scale. Such adjustments must be carried out carefully to minimize impact on all spectral signatures within the panel. Adjustments can be made detector by detector or to a whole detector array at one time, where all detectors of a given laser line are changed simultaneously. Reduction of the whole array is the recommended approach, as manual alteration of single detectors is prone to distortion of spectral signatures, where the ratio of brightness from detector to detector is not maintained. Regardless, reducing whole detector arrays can lead to peak emissions of spectral signatures being shifted to an incorrect detector (potentially on a different laser line). Therefore, all changes must be checked to ensure

that signatures remain as expected based on Cytek's Full Spectrum Viewer, published fluorophore guidelines, or historical data.

1. Observe which fluorescent channel(s) has an off-scale signal.

2. Use trial and error to determine the gain setting that is appropriate to bring the emission peak on-scale (i.e., $<4 \times 10^6$ on the Aurora).

   Remember that gain and fluorescence have a linear relationship, so that a 50% reduction in gain will give rise to a 50% reduction in fluorescence signal.

3. Reduce secondary detector arrays with off-scale signals by the same percentage as the reduction made in step 2.

4. Use Cytek's Full Spectrum Viewer, published fluorophore guidelines, or historical data to check that the altered spectral signature retains the correct overall pattern and that the primary emission peak has not been reduced below any secondary emission peaks.

   If the overall pattern is correct, no further steps are required. Record all samples at these adjusted settings. If the overall pattern is not correct, continue to step 5.

5. If adjustments to other laser lines are required, continue reducing the respective detector arrays by the same percentage as in step 2.

6. Repeat steps 4 and 5 until the spectral signature appears as expected or all arrays have been reduced by the same percentage.

7. Record all samples at the adjusted settings.

UNMIXING EVALUATION OF FULLY STAINED SAMPLE

Before any analysis can be undertaken, the FS sample must be checked to ensure that clean data can be obtained through removal of artefacts such as doublets, dead cells, and aggregates. It is also necessary to verify that there is positive staining for all markers in the panel, taking into consideration the biology of each marker. Once this has been asserted, it must be determined whether the SRCs selected (either beads or cells) successfully unmix the SS cells in Basic Protocol 1 can also be used to successfully unmix the FS sample (Video 2).
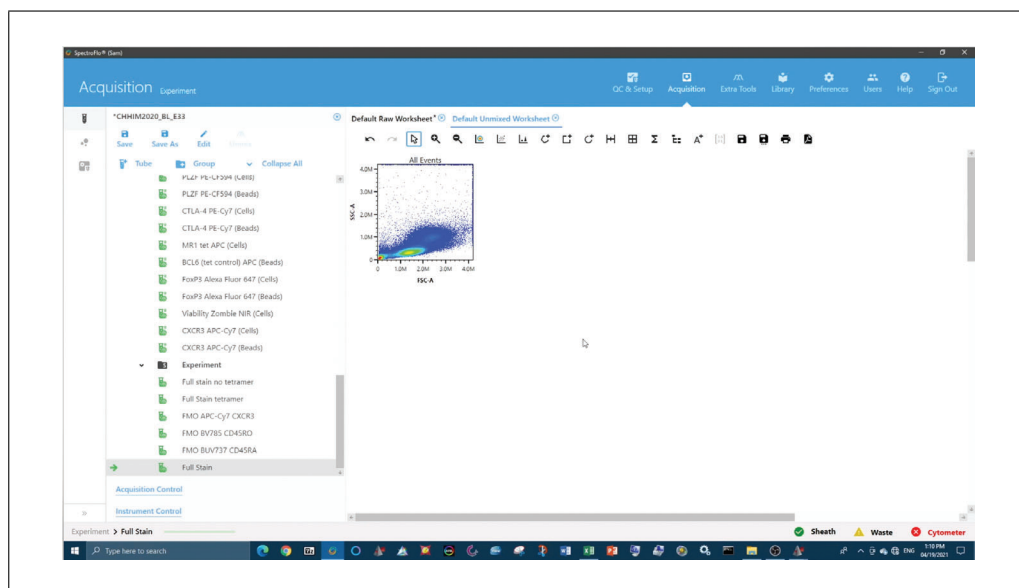
Video 2 Unmixing evaluation of fully stained sample (Basic Protocol 2).

Materials

Cytek SpectroFlo software
FCS files generated in Basic Protocol 1
N × N worksheet template

1. In the SpectroFlo software, work through the Unmixing Wizard and select the SRCs chosen from Basic Protocol 1.

2. In the unmixed workspace, gate on time to remove any events collected during an unstable flow period by plotting time vs. scatter (e.g., FSC-A or FSC-H; Fig. 4A).

3. Gate out doublets by sequentially plotting FSC-H vs. FSC-A and SSC-H vs. SSC-A, and include only the events found in the diagonal population (Fig. 4A).

   If working with whole blood or PBMC samples where incomplete RBC lysis was achieved, unlysed erythrocytes need to be excluded. One method is the blue/violet SSC gating method described by Petriz, Bradford, & Ward (2018).

4. Exclude aggregates through inspection of N × N plots for incorrectly unmixed super-bright events and gate these out of all further analyses (Fig. 4B).

5. Gate out dead cells by including only viability dye negative events (Fig. 4A).

   Viability dyes also need to be titrated (see Support Protocol 1).

6. Gate on cells of interest using FSC-A vs. SSC-A, including only the events required for analysis (Fig. 4A).

   If working with a sample that contains multiple cell types (such as PBMCs), include only the cells that will be involved in downstream analysis. For example, when analyzing a panel that contains only lymphocyte markers, gate out monocytes (validating that T cells are not excluded).

7. Create as many pseudocolor or dot plots as there are fluorophores in the panel. Set the *y* axis to SSC-A and each *x* axis to a different fluorophore (Fig. 4C).

8. Verify that a positive signal can be found for all markers (Fig. 4C).

   For rare or dim markers, FMOs can assist in determining the gating boundaries (Fig. 4D). If no clear signal is observed, take note of that reagent and check the titer and staining protocol. For additional troubleshooting, see Table 3.

9. Open the N × N worksheet template created previously and evaluate how well unmixing was performed for each marker. Inspect the FS sample by assessing whether super-negative events are present (example in Fig. 4E), which can be an indication of unmixing issues.

10. Select all plots making up the matrix. In the Plot Properties window, change the *x* axis option to the next fluorophore down the list so that all plots change simultaneously.

11. Repeat steps 9-10 until all fluorophores have been checked.

12. If there are no major unmixing errors (i.e., the positive and negative populations of each marker in the N × N plots are well aligned), continue to evaluation of marker resolution (see Basic Protocol 3).

13. If there are unmixing issues, even once the unmixing has been performed correctly and optimally, it is possible to make small adjustments (<3%) to the compensation matrix (found in the Tube Properties window).
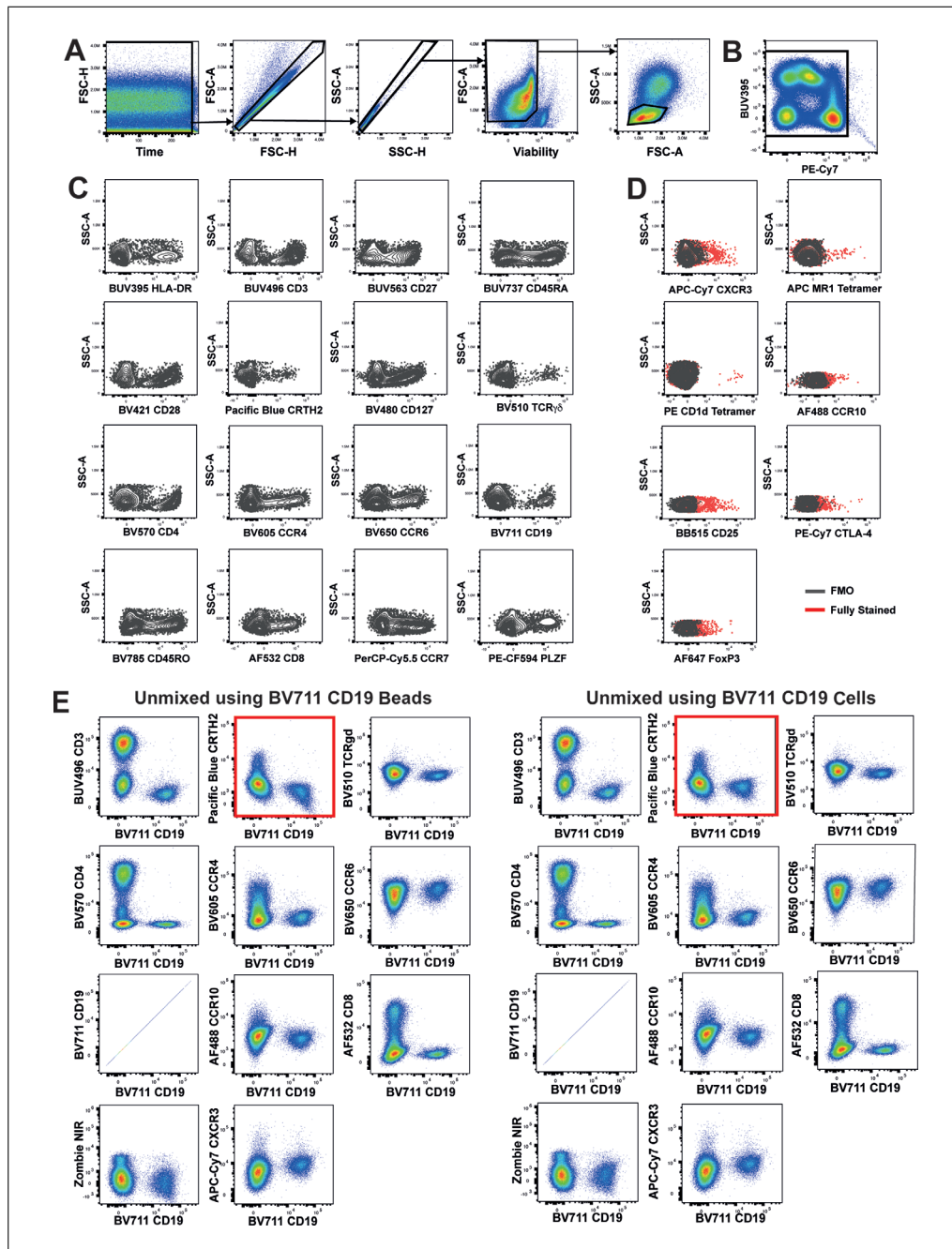
Figure 4 Inspection of the FS sample. (**A**) Gating strategy to gate out inconsistent flow rate events (time gate), doublets, and dead cells and include only cells of interest (lymphocytes). (**B**) Example of aggregates and how to gate them out. (**C**) Dot plots of SSC-A vs. each marker in the panel. (**D**) Dot plots of SSC-A vs. rare/dim markers in the panel, overlaid with FMO controls to show true positive events. (**E**) N × N matrix used to evaluate marker positivity from core panel iteration unmixed using beads (left) or cells (right).

Adjustments to the spillover matrix should be fully justified and the integrity of the data must not be impacted (made with clear knowledge of the biology, such as expression and pattern characteristics). Under-unmixing and over-unmixing issues can be manually adjusted (Ashhurst, Smith, & King, 2017). It is unlikely that there would be a need for more than a 2% to 3% correction. If greater correction is needed, the unmixing accuracy needs to be re-evaluated.

If the samples are something other than PBMCs and significant unmixing issues are still present, more information on how to proceed can be found in the Commentary (see Critical Parameters, Complex Samples).

EVALUATION OF MARKER RESOLUTION

Once the best possible unmixing has been achieved using Basic Protocols 1 and 2, the resolution of each marker in the FS sample must be compared to the SS cell controls. Assessing the spread of the negative population and/or shifts in the positive signal will provide an indication of whether there is any loss of resolution of markers in the panel when fully stained. This assessment is best achieved by overlaying each marker in the SS cell sample onto the FS sample. If any reduction in resolution is seen, the impact of this can be further investigated to identify if it will impact the ability to identify populations of interest using an established gating strategy. Ideally, the same number of cells from the same tissue type should be stained and acquired for all samples. In practice, however, this is not often feasible, and downsampling can be used to achieve identical cell numbers across samples during analysis. In theory, the only difference between the SS and FS cells should be the number of antibodies in the tube (Video 3). Additionally, AF extraction should be tested to determine if marker resolution can be improved (Video 4).
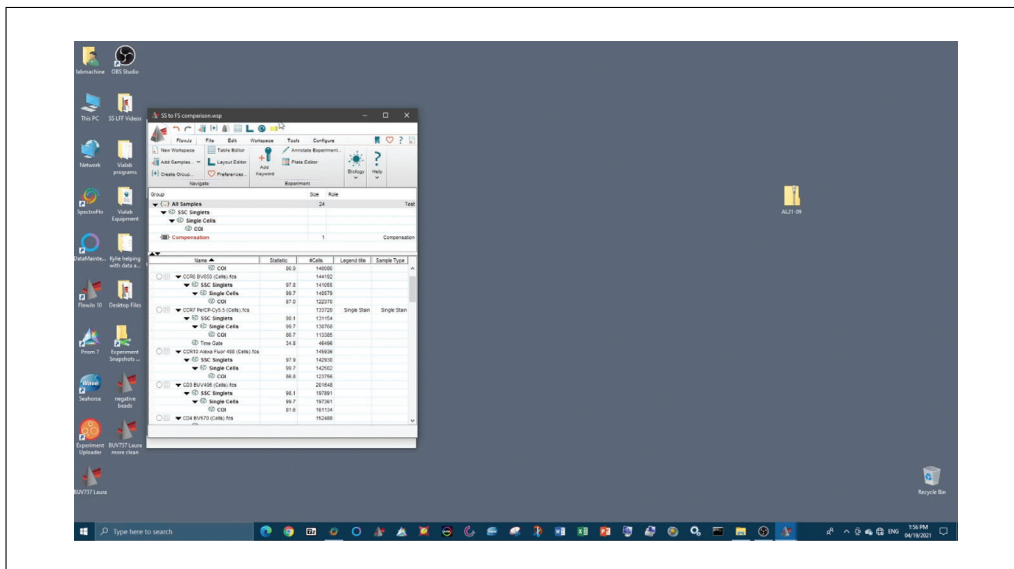
Video 3   Evaluation of marker resolution (Basic Protocol 3).

Video 4   Deciding on an approach for mitigating autofluorescence (Basic Protocol 3) and Support Protocol 3.

Materials

Data analysis software (e.g., FlowJo or FCS Express)
Cytek SpectroFlo software
FCS files generated in Basic Protocol 2
N × N worksheet template

Evaluate marker resolution (Video 3)

1. Open the unmixed FS and SS cell samples in a data analysis software package for analyzing FCS files (e.g., FlowJo or FCS Express).

2. Follow steps 2-6 of Basic Protocol 2.

3. Create a histogram for each fluorophore in the panel (Fig. 5A). If the positive population is rare, use a dot plot for visualization purposes instead of a histogram (Fig. 5B).

4. Overlay the same number of FS and SS cell sample events onto a histogram or dot plot (Fig. 5A,B).
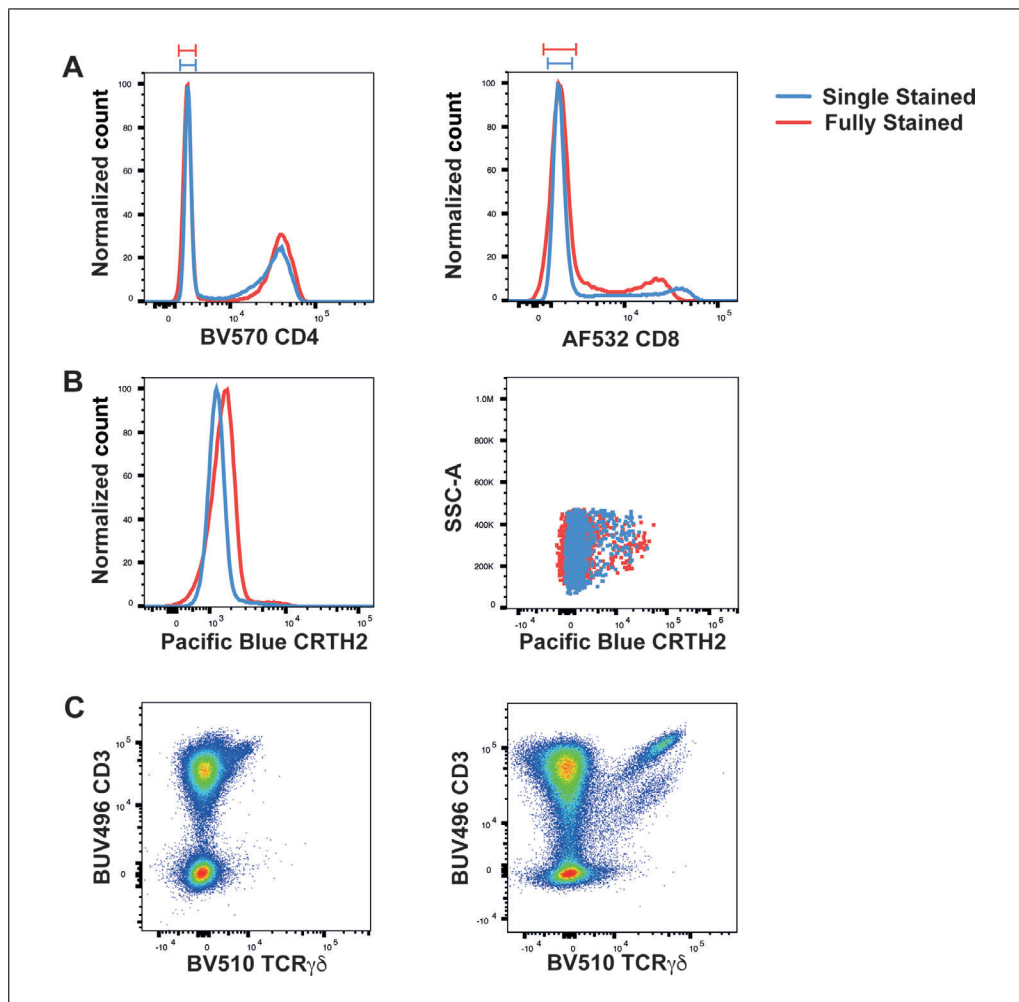
Figure 5 Evaluation of marker resolution. (**A**) A histogram overlay of BV570 CD4 SS cells vs. FS sample (left) provides an example with no additional spread in the negative population, whereas a histogram overlay of AF532 CD8 (right) depicts spreading of the negative population in the FS sample (red) compared to SS cells (blue). (**B**) Histogram and dot plot overlays of Pacific Blue CRTH2. (**C**) Dot plot of BUV496 CD3 vs. BV510 TCR$\gamma\delta$ before (left) and after (right) implementation of sequential staining (Park et al., 2020).

	APC-Cy7-A CXCR3	Alexa Fluor 488-A CCR10	Alexa Fluor 532-A CD8	Alexa Fluor 647-A FoxP3	BB515-A CD25	BUV395-A HLA-DR	BUV496-A CD3	BUV563-A CD27	BUV737-A CD45RA	BV421-A CD28	BV480-A CD127	BV510-A TCRgd	BV570-A CD4	BV605-A CCR4	BV650-A CCR6	BV711-A CD19	BV785-A CD45RO	PE-CF594-A PLZF	PE-Cy7-A CTLA-4	Pacific Blue-A CRTH2	PerCP-Cy5.5-A CCR7	Zombie NIR-A Viability
APC-Cy7-A CXCR3	0	3.57	1.83	23.68	3.33	0.31	7.51	4.04	1.51	0.47	1.34	3.74	0.37	3.00	6.80	1.90	7.39	2.91	5.23	1.11	0.88	6.56
Alexa Fluor 488-A CCR10	0	0	4.87	0.75	22.47	0.62	30.62	7.48	1.20	1.34	3.09	8.22	2.67	2.03	2.41	0.03	1.67	1.45	0.99	2.68	1.26	0.96
Alexa Fluor 532-A CD8	0	5.56	0	0	4.00	0.37	0	0	0	0.39	0	4.88	1.76	1.02	1.34	1.10	1.39	0.38	0.47	0	0.47	0
Alexa Fluor 647-A FoxP3	2.29	0	0	0	0	0	7.48	0	0	0	0	0	0	0	0	0	0	0	0	0.11	0.02	0.07
BB515-A CD25	0.71	14.35	3.27	0	0	0.83	0	0	0	0	0	3.85	1.03	0	0.70	0	0	0	0	2.14	0.75	1.29
BUV395-A HLA-DR	0	1.90	3.64	0.29	3.43	0	0	1.72	0.45	0	1.70	10.54	0	2.20	1.94	0.55	1.57	0.71	0.31	3.08	1.31	0
BUV496-A CD3	0.51	3.18	1.87	0.36	4.92	0.97	0	3.34	0.28	0.86	2.28	6.67	1.41	0.83	1.20	0.94	1.27	1.10	0.47	0.90	0.47	0.33
BUV563-A CD27	0.41	2.53	2.35	0.40	3.88	0.68	9.29	0	0.45	0.62	1.22	5.33	3.28	1.11	1.54	1.13	1.45	1.25	0.53	1.02	0.75	0.37
BUV737-A CD45RA	2.75	0	0	0	0	0	0	0	0	0	0	3.53	0.37	0.47	0	0	0.97	0	1.45	0	0	0
BV421-A CD28	0.42	0.70	1.88	0.29	3.06	1.15	7.93	2.63	0.32	0	2.32	6.60	1.03	0.85	1.16	0.86	1.22	0.72	0.31	5.33	0.54	0.38
BV480-A CD127	1.25	4.28	2.87	0.50	7.26	1.19	20.55	6.43	0.55	1.27	0	8.73	2.50	2.10	2.15	1.62	2.33	1.67	0.53	1.83	0.76	0
BV510-A TCRgd	0	2.10	1.34	0.41	1.51	1.35	0	2.40	0.64	0.52	2.68	0	2.46	3.01	1.02	0.96	0.82	1.86	0.44	4.21	0.62	0
BV570-A CD4	0	2.19	2.14	0.27	3.71	0.64	13.75	6.03	0.43	1.13	1.46	4.25	0	2.64	1.78	0.72	1.02	1.44	0.50	1.45	0.65	0
BV605-A CCR4	0.59	0	2.13	0.53	3.39	0.49	12.30	4.01	0.89	0.93	1.13	5.56	2.13	0	3.04	1.15	1.27	2.92	0.65	1.32	0.85	0.66
BV650-A CCR6	1.19	0.27	0	0.73	0	0	0	0	0.94	0.52	0	0	0	1.02	0	1.13	0	0.70	0.44	1.12	0.43	0.75
BV711-A CD19	2.10	0	0.85	0	0	0	0	0	2.11	0.60	0	0	0	0	0	0	2.22	0	0.35	0	0.63	1.43
BV785-A CD45RO	2.02	0.71	1.09	0.24	1.85	0.44	5.27	1.64	2.01	1.29	0.75	2.84	0.58	0.42	0.48	0.97	0	0.29	0.71	1.07	0.40	1.27
PE-CF594-A PLZF	0.64	3.38	1.65	0.46	3.05	0	0	0	0.51	0.58	0	7.63	0.66	1.96	0.64	0.87	0	0	0.70	0	1.36	0
PE-Cy7-A CTLA-4	2.95	3.82	4.43	0	10.60	0.80	0	4.00	0	1.22	1.85	6.08	4.83	1.69	1.64	2.23	1.92	0.83	0	1.05	0.72	3.04
Pacific Blue-A CRTH2	0.83	1.48	3.69	0	3.95	1.58	0	0	0.64	2.17	6.49	18.71	1.70	0	0	0	0	1.99	0.01	0	0.89	1.07
PerCP-Cy5.5-A CCR7	2.87	0.16	0	2.02	0	0	0	0	1.69	0	0	0	0	1.35	2.86	3.60	2.98	0	1.86	0	0	1.93
Zombie NIR-A Viability	12.39	1.51	1.37	1.53	3.68	0.61	4.65	1.58	1.50	0.56	1.15	3.78	0.82	0.64	0.98	1.52	4.51	0.97	2.29	1.30	0.90	0

Figure 6 Spillover spreading matrix for 24 unique-signature fluorophores used in combination on a panel used to illustrate the protocol. Spread is contributed by fluorophores listed in the rows, impacting the fluorophores listed in the columns. Spillover values are color-coded as follows: white, <3; shades of pink, 3–9; red, >9 (for example, BV480 spreads strongly into BUV496).

To ensure that each sample contains equal events, downsample the cells of interest population from all samples to the same number of events based on the sample with the fewest cells of interest. In FlowJo, for example, select the population of interest, then go to the Workspace tab and select Plugins and then DownSample. Specify the number of events to be downsampled such that all samples can meet the criteria.

5. Determine whether any spreading of the negative population has occurred in the FS sample (Fig. 5A, right).

6. Determine whether such spreading has impacted the resolution of positive and negative populations, either visually or by calculating stain index (SI; see Support Protocol 1, step 22).

7. Create a spillover spreading matrix (SSM) to assess which fluorophores are likely to be introducing spread to the fluorophore of interest (in Fig. 6, find the SSM for the panel used to illustrate the protocol).

In FlowJo, for example, using the SS cells SRCs, navigate through the Compensation Wizard defining positive and negative populations for each fluorophore. The SSM will be automatically generated alongside the compensation matrix and can be displayed or exported from the SSM button.

If the spread of the negative population results in significant loss of resolution such that separation of positive and negative populations becomes difficult, and the marker is
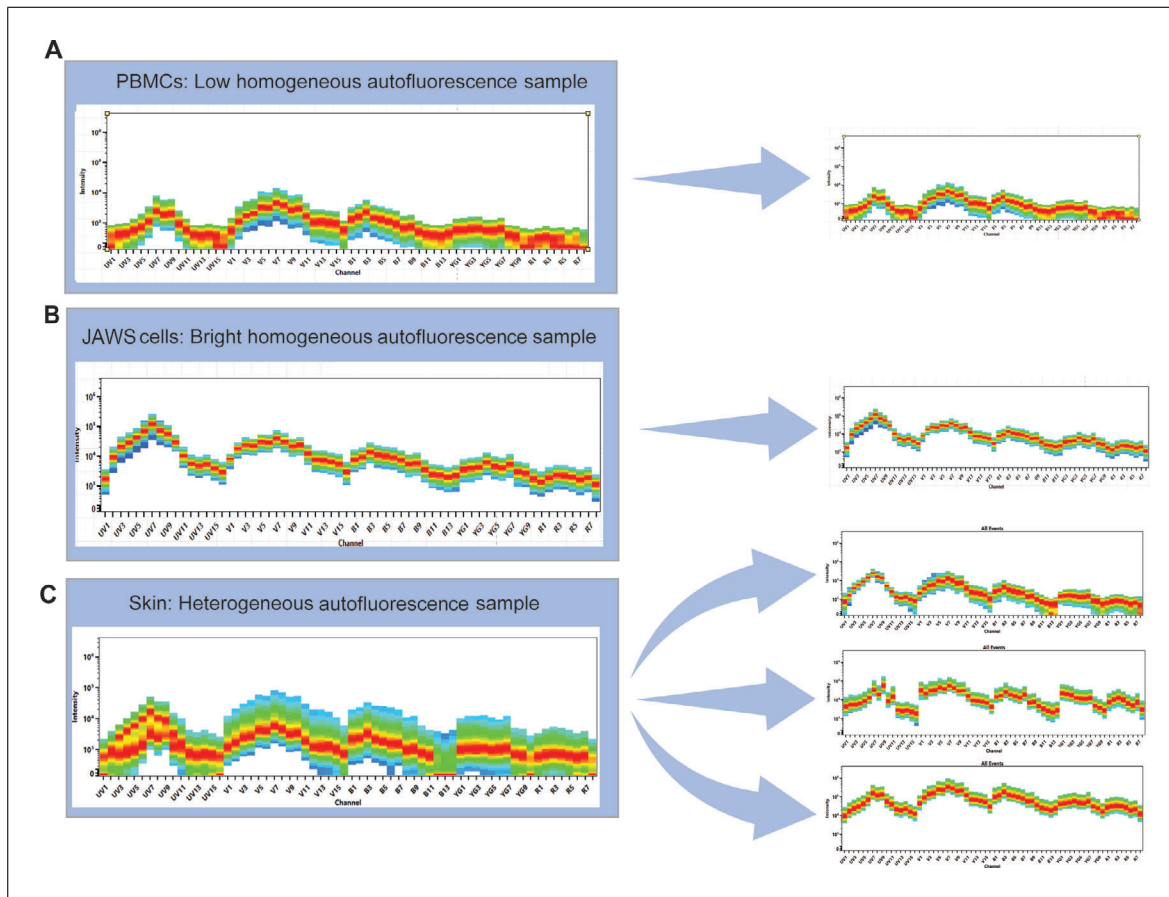
Figure 7 Determination of AF signature type. AF signatures of (**A**) unstained PBMCs, (**B**) unstained JAWS cells (immortalized immature dendritic cell line), and (**C**) unstained skin cells. The latter shows a highly heterogeneous signature that can be split into three unique spectral signatures.

> *co-expressed in the same cell type as the marker causing the spread, the panel design should be reconsidered and a different fluorophore may be needed to improve resolution.*

8. Determine whether any loss in fluorescence intensity of given marker(s) has occurred in the FS when compared to the SS (Fig. 5C).

> *Any reduction in positive signal should be investigated. An explanation is offered in Jalbert, Shikuma, Ndhlovu, & Barbour (2013) and Park et al. (2020), where sequential staining of chemokine receptors was required to achieve positive signal in the FS sample comparable to that of the SS cell controls. Sequential staining can also be applied to non-chemokine receptors (Fig. 5C).*

Choose approach for mitigating AF (Video 4)

Two scenarios should be considered regarding AF: homogeneous AF, where the whole unstained sample has a single low spectral signature (Fig. 7A) or single bright spectral signature (Fig. 7B), or heterogeneous AF, where there are multiple AF signatures (Fig. 7C). For PBMCs, where the unstained sample has a relatively low homogeneous AF signature, the Simple AF Protocol can be followed to assess whether AF extraction improves marker resolution. AF extraction is particularly helpful in resolving low-expressed markers by lowering the background of the negative population with minimal effect on the positive signal. If samples have heterogeneous or homogeneous but very bright AF, it is likely that AF extraction must be used to obtain unmixing that looks correct in Basic Protocols 1 and 2. In this case, the decision to use AF extraction will be based on unmixing quality instead of improvement of marker resolution. The AF protocol for

heterogeneous AF is explained in detail in Critical Parameters (under Working with Complex Samples) and in Support Protocol 3.

Extract AF

9. Open SpectroFlo and proceed to the Unmixing Wizard.

10. Test the following options for unmixing:

    a. Unmix without AF extraction first. Select live unmixing.
    b. Repeat steps of the Unmixing Wizard with AF extraction, placing the scatter gate on the population of interest only. Select Unmix, Save & Open to generate a separate set of FCS files from step 10a.

11. If there are other cells of interest in the sample that have a brighter spectral signature, try to unmix with the scatter gate on this population in step 10b.

12. Determine the best unmixing outcome comparing the N × N plots by evaluating changes in resolution for the dyes that overlap with the range of emission of AF (e.g., BUV496, BV510, Pac Orange, BV570, FITC, PE).

MANAGING HETEREOGENEOUS AUTOFLUORESCENCE (VIDEO 5)

The following protocol is divided into three sections: Discover, Distinguish and Designate.

Discover

1. Observe N × N plot permutations of raw channel data.

    a. On the Aurora, draw as many pseudocolor or dot plots as channels.
    b. Leave the *x* axis unchanged.
    c. Set each *y* axis to the different channels, so each plot shows a different raw combination.

    An example of a SpectroFlo N × N raw template is shown in Figure 8.

2. Find a combination that separates the greatest number of populations from one another.
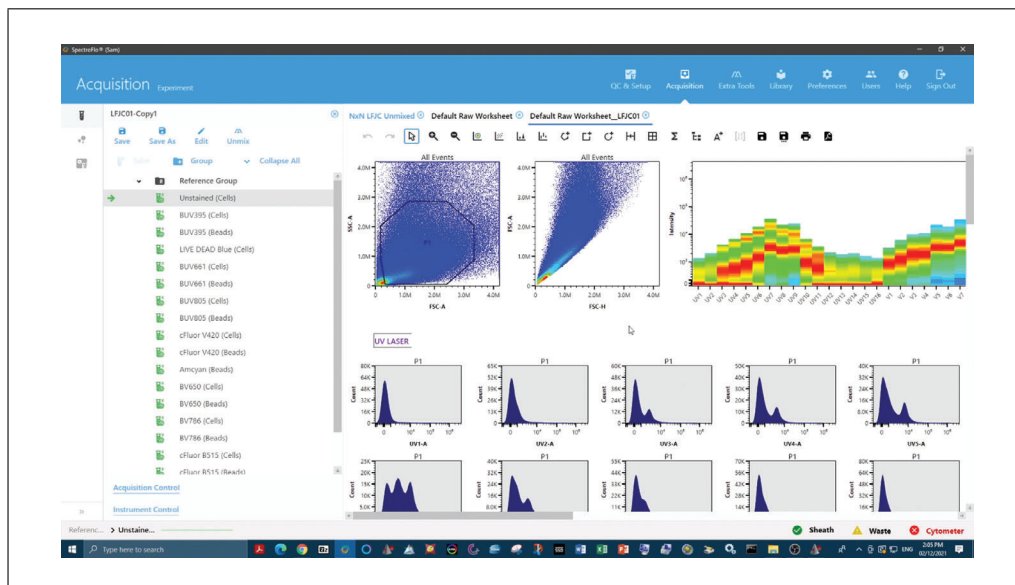
Video 5   Heterogeneous autofluorescence (Support Protocol 3).
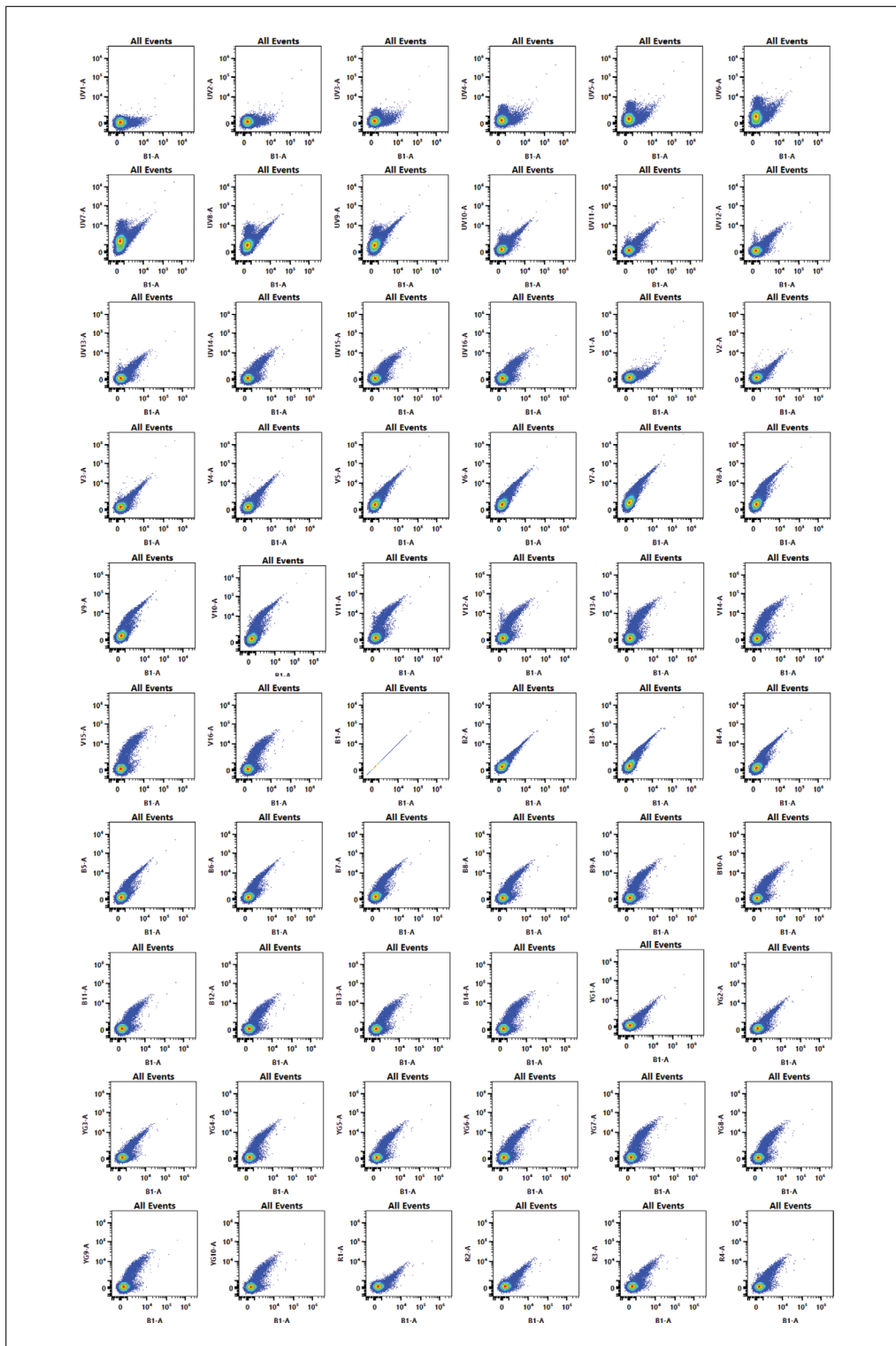
Figure 8 Example of an N × N raw worksheet.

Potential shortcut: Observe spectral plot showing ALL events and find one channel where there is obvious heterogeneity and one channel with similarity. This will immediately pull apart a minimum of two populations. Hint: Mid-range UV channels are most likely to show heterogeneity. If eosinophils are expected, YG1 will assist with isolating eosinophils from other populations.

Distinguish

3. Choose the plot with highest degree of separation.

Aurora 5L Detector Array Configuration

Laser	Channel	Center Wavelength (nm)	Bandwidth (nm)	Wavelength Start (nm)	Wavelength End (nm)	Laser	Channel	Center Wavelength (nm)	Bandwidth (nm)	Wavelength Start (nm)	Wavelength End (nm)
ULTRAVIOLET 355nm	UV1	372	15	365	380	BLUE 488nm	B1	508	20	498	518
	UV2	387	15	380	395		B2	525	17	516	533
	UV3	427	15	420	435		B3	542	17	533	550
	UV4	443	15	435	450		B4	581	19	571	590
	UV5	458	15	450	465		B5	598	20	588	608
	UV6	473	15	465	480		B6	615	20	605	625
	UV7	514	28	500	528		B7	660	17	652	669
	UV8	542	28	528	556		B8	678	18	669	687
	UV9	581	31	566	597		B9	697	19	688	707
	UV10	612	31	597	628		B10	717	20	707	727
	UV11	664	27	650	677		B11	738	21	728	749
	UV12	691	28	677	705		B12	760	23	749	772
	UV13	720	29	705	734		B13	783	23	772	795
	UV14	750	30	735	765		B14	812	34	795	829
	UV15	780	30	765	795						
	UV16	812	34	795	829	YELLOW GREEN 561nm	YG1	577	20	567	587
VIOLET 405nm	V1	428	15	420	435		YG2	598	20	588	608
	V2	443	15	436	451		YG3	615	20	605	625
	V3	458	15	451	466		YG4	660	17	652	669
	V4	473	15	466	481		YG5	678	18	669	687
	V5	508	20	498	518		YG6	697	19	688	707
	V6	525	17	516	533		YG7	720	29	706	735
	V7	542	17	533	550		YG8	750	30	735	765
	V8	581	19	571	590		YG9	780	30	765	795
	V9	598	20	588	608		YG10	812	34	795	829
	V10	615	20	605	625	RED 635nm	R1	660	17	652	669
	V11	664	27	651	678		R2	678	18	669	687
	V12	692	28	678	706		R3	697	19	688	707
	V13	720	29	706	735		R4	717	20	707	727
	V14	750	30	735	765		R5	738	21	728	749
	V15	780	30	765	795		R6	760	23	749	772
	V16	812	34	795	829		R7	783	23	772	795
							R8	812	34	795	829

Figure 9 Aurora 5L detector array configuration.

4. Place gates around all unique populations and observe the scatter properties and spectral signatures on adjacent spectral plots and select only the populations that have unique spectral characteristics.

5. Find the peak fluorescent channel for each unique population and then display said population on a histogram using this channel.

6. Gate the brightest 300-500 cells only, in order to derive the brightest AF SS control.

7. Right-click on the gate and export events as a new FCS file.

8. Repeat for all unique populations, producing an FCS file for each.

9. Export an AF dim population (dimmest AF spectral signature found in the sample) to be used as the negative control for the previously exported populations.

Designate

10. Designate these new AF signatures as fluorescence tags in the SpectroFlo Library.

   a. Create a new AF group.

   This will facilitate filtering and exporting these fluorophores for offline analysis, as well as identification when designing experiments.

   b. Assign the AF tag a name, choose the excitation laser, and assign an emission wavelength (use the optical configuration in Figure 9 to determine wavelength based on peak emission channel).

11. Open the experiment of interest and add the new AF tag(s) as if it were a fluorophore in the experiment.

12. In the reference controls tab, add an additional negative for the AF tags that will be the AF dim.

13. Import the populations exported during the Distinguish steps as SRCs for the corresponding tags, and import the AF dim population as an additional negative for the AF SRCs.

14. Use the Unmixing Wizard QC tools to assess AF signature similarity.

   In our experience, a similarity of 0.96 or less indicates that the AF signatures are generally considered to be unique.

15. Unmix the experiment and evaluate the unmixed N × N matrix.

   If unmixing errors are still present, refer to the troubleshooting guide in Table 3.

ASSESS DATA QUALITY USING EXPERT GATING AND DIMENSIONALITY REDUCTION ALGORITHMS

Informed gating is a useful way to check panel quality. It is necessary to check that all populations of interest can be identified and to investigate how well they can be resolved (Fig. 10A, Video 6). The biology of the sample should be considered to ensure that populations seen in the sample are as expected and that markers are expressed on all the expected cell types. This protocol indicates whether the panel is ready for use on experimental samples. Additionally, dimensionality reduction algorithms can be useful tools for investigating panel quality, as they simplify the data for visualization while also exposing artefacts that may be missed through expert gating. Using *t*-distributed stochastic neighbor embedding (*t*-SNE; van der Maaten & Hinton, 2008; Fig. 10B), markers that are usually co-expressed should be checked to see they are found in similar regions of the *t*-SNE plot (Brummelman et al., 2019).

Materials

   Data analysis software (e.g., FlowJo, FCS Express, or any other software that allows gating of data)
   FCS files generated in Basic Protocol 3

Perform expert gating

1. Open the unmixed FS sample in data analysis software used for analyzing FCS files.

2. Follow steps 2-6 of Basic Protocol 2 to gate on live single cells of interest.

3. Gate the rest of the markers based on panel design, prior knowledge, and published literature.

4. Check the gating strategy for unexpected marker combinations or cell populations (Fig. 10A).

   For example, when analyzing PBMCs, a CD3$^+$CD19$^+$ double-positive population is not expected. If one is found, the source of this staining must be investigated and corrected before the panel is ready for use.

5. Evaluate whether all populations of interest have clear positive signals that can be easily resolved from the negative.

6. Ensure readout markers (used for experimental readout) can be quantified in each of the cell types of interest.

Apply dimensionality reduction algorithm (t-SNE)

7. Using the cleaned data from step 2, run a *t*-SNE at the default settings (iterations: 1000; perplexity: 30) utilizing all fluorescence parameters. Exclude non-informative parameters such as those used in the data cleaning steps (e.g., viability and phenotypic markers for identifying cells of interest).
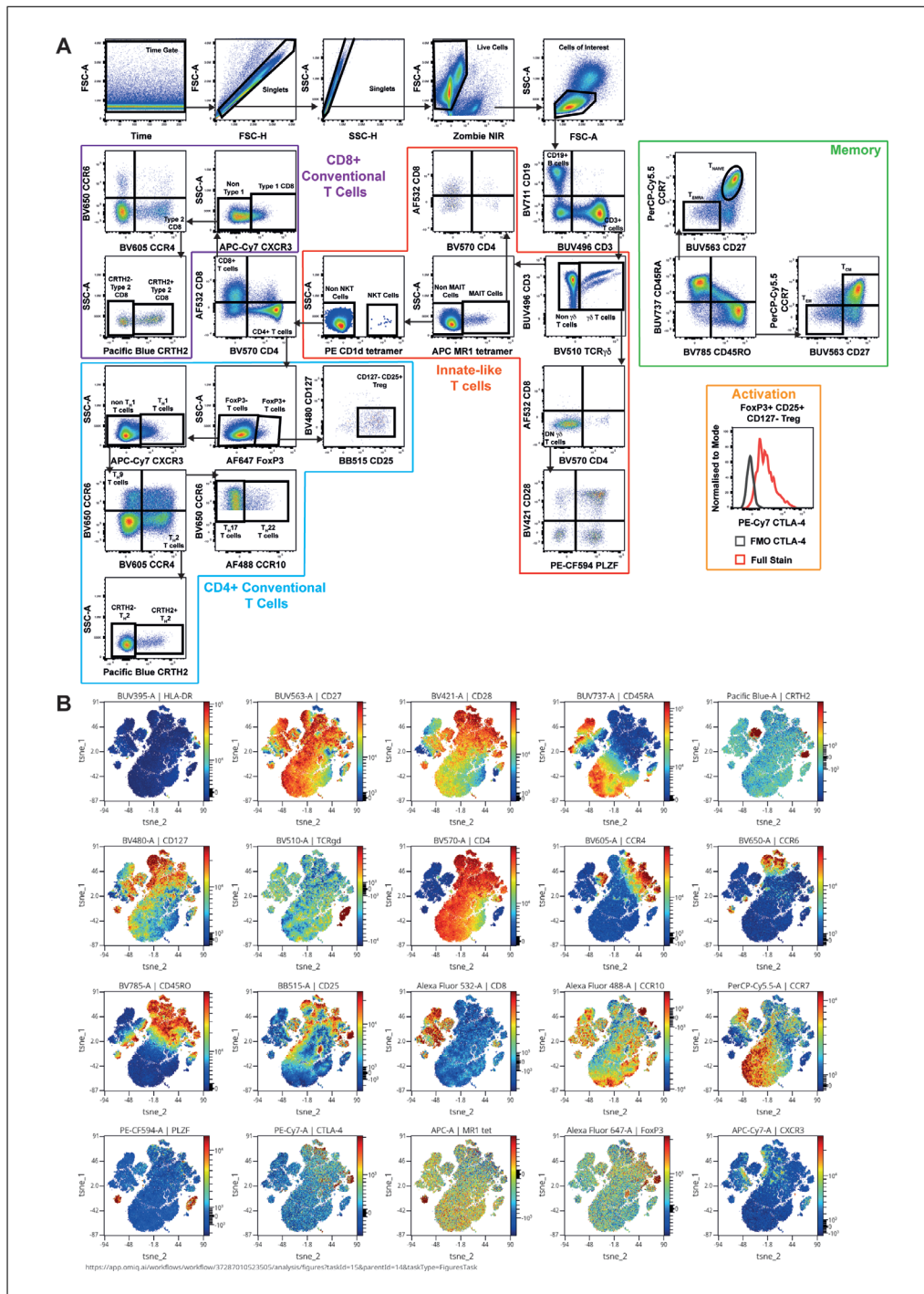
Figure 10 Gating of the FS sample. (**A**) Gating strategy of the panel, including lineage, memory, and activation readouts. (**B**) *t*-SNE as a quality control tool. *t*-SNE analysis was performed with 1000 iterations and perplexity of 30 and displayed in 2D plots using the resultant *t*-SNE 1 and *t*-SNE 2 dimensions according to the per cell expression of 20 proteins. Expression levels of HLA-DR, CD27, CD28, CD45RA, CRTH2, CD127, TCR$\gamma\delta$, CD4, CCR4, CCR6, CD45RO, CD25, CCR10, CD8, CCR7, PLZF, CTLA-4, MR1tet, FoxP3, and CXCR3 are displayed. *t*-SNE scales are shown in each graph and visualized using a rainbow heat scale.

8. Assess marker co-localization one by one in viSNE plots by coloring the *t*-SNE based on the expression of each marker (Fig. 10B).

   viSNE is a visualization tool for high-dimensional single-cell data based on t-SNE.
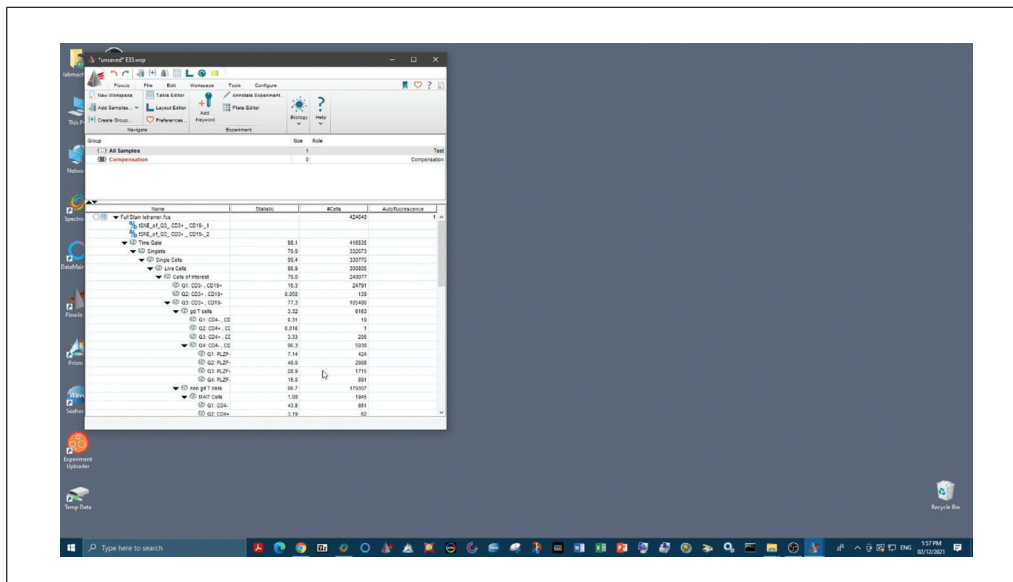
Video 6 Assessment of data quality using expert gating and dimensionality reduction algorithms (Basic Protocol 4).

9. As in step 4, check for the appearance of unexpected marker combinations on specific cell types.

See Figure 11 for a simple checklist to help ensure that all steps have been followed to obtain a high-quality and reliable immunophenotyping panel.

COMMENTARY

Background Information

The first step of building a successful large multicolor panel is good theoretical panel design. Full-spectrum flow cytometry panel design has been previously described (Ferrer-Font, Pellefigues, et al., 2020; Park et al., 2020). In summary, in the context of successful theoretical panel design, it is very important to have a clear experimental question and to know the biology of the markers that are included in the assay, including their expression and co-expression patterns. It is important to understand the instrument configuration (i.e., number of lasers on board) to know what the spectral signatures for each fluorophore will look like with the given configuration, as fluorophore brightness will vary depending on the excitation wavelength available. This information, coupled with the amount of spread (both given and received) of each fluorophore in the panel (detailed in Nguyen, Perfetto, Mahnke, Chattopadhyay, & Roederer, 2013), allows fluorophores to be optimally assigned to the different markers of the immunophenotyping panel.

After the panel has been theoretically designed, performance of QC steps is strongly recommended. This process is necessary to theoretically validate the panel before proceeding to a full experiment in order to reduce preventable issues as much as possible. It is advised to review the panel on a marker-by-marker and population-by-population basis, making sure that fluorophores inducing considerable amounts of spread (which could impair marker resolution) are allocated to non-co-expressed markers, and that dim markers receive a minimal amount of spread, while fluorophore brightness and antigen expression levels are well matched (high-expressing antigens with dim fluorophores and low-expressing antigens with bright fluorophores). To address potential issues, markers that are available with multiple fluorophores can be substituted to see if spillover spread can be minimized. Additionally, fluorophores that create (but do not receive) the most spillover can be designated to dump or viability channels. It is strongly recommended to complete panel optimization before working with an actual biological experiment, as it is a wise investment in terms of time, effort, and cost, without jeopardizing precious experimental samples.

Critical Parameters

Multiple factors influence the success of this protocol. The importance of good sample preparation—addressing sample

Ferrer-Font et al.

Panel Optimization Check List

☐ *Ensure panel has been well designed and optimised following guidelines laid out in "Design and Optimization Protocol for High-Dimensional Immunophenotyping Assays using Spectral Flow Cytometry" (Ferrer-Font et al., 2020)*

Panel Optimization – to be completed in this order

☐ 1. Titrate all antibodies

Core Panel: *Includes only the essential lineage markers required to identify the cell types of interest*

☐ 2. Acquire single stain cells, single stain beads and fully stained core panel samples together in one experiment

☐ 3. Unmix single stained cells using the single stained beads to decide whether beads or cells are the appropriate control for each fluorophore

☐ 4. Compare the single stained cells signal to the multicolour tube to determine whether a clear positive signal can be detected for each marker and that the resolution is sufficient for each marker

☐ 5. Create NxN plots for the fully stained core panel sample and check there are no unmixing issues

Full Panel: *Includes all markers (readout markers, intracellular markers, etc)*

☐ 6. Repeat Steps 2. and 3. above with all the markers of the full panel

☐ 7. Repeat Steps 4. and 5. above with all the markers of the full panel

Important:
If the sample is highly autofluorescent, a plan must be developed regarding how this will be dealt with post sample acquisition.

Figure 11 Panel optimization check list. To ensure the rigor and reproducibility of data acquired by spectral cytometry, the following steps are recommended for optimization and validation of each new panel.

complexity issues and the use of high-quality controls—must be emphasized.

Working with complex samples

When working with more complex samples than PBMCs (e.g., skin, tumor, fat tissue), the steps for panel optimization are the same, but there are some extra considerations.

Quality of single-cell suspension. An important consideration that is sometimes underestimated is the quality of the single-cell suspension that will be used to run the immunophenotyping panel. As different immune cell subsets have varying susceptibility to cell death, the single-cell suspension should have a viability of 80% or more to ensure proportional representation of the original sample (Costantini et al., 2003). Cell death can occur for different reasons, including how the sample has been treated before staining. Cryopreservation and harsh digestion protocols can affect sample quality and these

procedures also need to be optimized. Indeed, when working with digested tissue, epitopes for the markers of interest should be verified to ensure the digestion protocol has not negatively impacted them. For example, if the same epitopes of interest exist in the spleen, one should compare marker staining and epitope preservation between digested and undigested spleen (Ferrer-Font, Mehta, et al., 2020).

Assessing instrument setup. Wherever possible, the instrument settings for fluorescence detector gains should remain unaltered from the Cytek Assay Settings (CAS). When using fluorescently labeled antibodies, this is achieved by carefully pairing expression levels to fluorophore brightness during panel design, and then by antibody titration to ensure fluorescence signals remain on-scale at the optimized settings. In certain applications where fluorescence intensity is not tunable, such as fluorescent reporter protein expression within

the cells of interest, the instrument settings may need to be adjusted to accommodate off-scale fluorescence signal. Any such change will impact not only the spectral signature being accommodated, but also other signatures with emission in the same area. Reducing fluorescence gains may lead to increased similarity between spectral signatures, which can give rise to more spillover spreading error and thus negatively impact marker resolution (Ferrer-Font, Pellefigues, et al., 2020). See Support Protocol 2 for steps required to alter fluorescence gain settings to accommodate off-scale signals. If using something other than the Cytek Aurora, it will be necessary to optimize the settings based on manufacturer's recommendations.

Autofluorescence. Samples with heterogeneous (Fig. 7C) or very bright homogeneous AF signatures (Fig. 7B) can make unmixing challenging. To improve the accuracy of the unmixing and to improve marker resolution in the case of heterogeneous AF samples, multiple AF reference controls can be created for each of the different AF signatures present in the sample (as if they were individual fluorophores included in the panel). The steps required for this process are divided in three sections called Discover, Distinguish and Designate. The goal is to Discover unique spectral signatures within the unstained control through use of a raw N × N plots; Distinguish these unique signatures into separate SRCs by exporting each population as a new FCS file which can then be reimported; and Designate each signature as unique fluorophores within the software to be unmixed as if they were part of the original panel. Care must be taken to ensure only clearly unique signatures (with similarity index <0.98) with at least 300 events of similar fluorescence intensity are distinguished to ensure high quality SRCs are generated (for a detailed protocol, see Support Protocol 3 and Video 4). For very bright homogeneous AF, the steps outlined in Basic Protocol 3 and Video 4 can be followed to improve unmixing and markers resolution outcomes.

Controls

The quality of controls will directly translate to the quality of unmixing and the data obtained. It is therefore worth investing the time to optimize them. It is of utmost importance to include all necessary controls from the beginning of a project. A complete overview about controls can be found in Maecker & Trotter (2006). Below is a summary of the categories of controls that should be considered when optimizing a flow cytometry panel.

Unstained controls. The unstained control is meant for AF assessment and should have a clean signature with no contamination from other fluorophores and match the tissue or sample type being analyzed. If, for example, different tissue types are used in an experiment, multiple unstained sample controls must be used for each tissue type. Similarly, if samples are being treated differently (e.g., fixed/fresh or stimulated/unstimulated), an unstained control should be included for each condition. It is not advised to mix samples from different conditions and collect only one unstained control, as it may not be possible to have enough events to generate clear AF signatures.

Spectral reference controls. Appropriate single-stained SRC samples are required for optimal unmixing of the fully stained sample. The purpose is to provide a signature of each fluorophore to be used by the unmixing algorithm. Briefly, SRCs should have positive and negative populations that are clearly separated or a universal negative; positive populations should be brighter than the fully stained sample; the negative and positive populations should have identical AF characteristics; sufficient events for both populations should be collected; and the fluorescence spectrum of the positive control needs to be identical to the one in the fully stained sample. To fulfill these best practices, cells and beads should be compared and the best option should be used. Special considerations for viability staining are important, as live and dead cells have different AF signatures, which means the positive and negative controls will not have the same AF signature. To overcome this issue, it is possible to kill all cells in the viability SRC (e.g., heat-killing at 55°-70°C for 5-10 min), stain only half of them, and mix these with the unstained cells. In this case, all the cells will be dead and the AF will be the same for the positive and negative controls.

SS cell controls. SS cell controls are used not only as reference controls but also to assess the performance of each marker compared to the FS tube. SS cells are gold-standard for each antibody performance and are used to quantify the spread of the negative population and/or shifts in the positive signal and thus any subsequent loss of resolution of markers in the panel.

Gating controls. A fluorescence minus one (FMO) control is a sample stained with all

fluorophores used in the experiment except one. Analyzing the FMO control for each fluorophore in the panel is not required for panel optimization, but FMOs can be used as a guide to set the boundary between positive and negative events if it is ambiguous. FMOs also aid in the assessment of spread between positive and negative events and are an important tool for assessing panel performance. A good alternative for large 20+ color spectral panels is the use of fluorescence minus multiple (FMM) controls, as recently described by Jensen & Wnek (2020).

Core versus FS panel

When optimizing a high-dimensional immunophenotyping panel, it is recommended to first complete Basic Protocols 1-4 with a *core panel.* A core panel is limited to only the essential lineage markers required to identify the cell types of interest. This will reduce confounding factors when attempting to understand the source of errors. Once a core panel has been optimized, additional markers can be added (including readout functional markers, intracellular markers, etc.) and optimized with the knowledge that issues are not originating from the core panel.

Fixation/permeabilization buffers

Intracellular staining procedures also require special consideration. It is important to source the fixation/permeabilization reagent that is most appropriate for the markers being detected (e.g., transcription factors, cytokines, or intracellular proteins) while also considering their relative locations (cytosolic or nuclear). The type of fixation buffer can influence the staining of both intracellular and surface markers. The type of fixative can have an impact in many aspects of staining: damaging the epitopes, altering the fluor stability and resulting in a different optimal titer, altering the background fluorescence without altering the positive signal (leading to reduced resolution between positive and negative populations), and altering the fluorescence spectral signatures (causing spectral signature mismatches between FS samples and SRCs, resulting in unmixing issues). It is recommended that antibodies be titrated using the same fixation/permeabilization buffer conditions used for the final staining of FS samples and SRCs.

Troubleshooting

The term troubleshooting is used when one or more issues are found in the panel and the source of these issues needs to be identified and steps included to rectify them. In this regard, the exact steps provided for validating the panel can also be applied for troubleshooting. By following these clear steps, the user will gain a better understanding of the quality of the panel and identify issues prior to acquiring experimental samples.

In general, 80%-90% of unmixing issues that arise can be traced to suboptimal controls. Therefore, it is important to use controls that are well characterized, high quality, and appropriate for the experiment (i.e., matched to the conditions of the experimental sample). This is particularly vital if they are going to be reused.

The checklist provided in Figure 11 aims to help users follow clear steps to optimize and troubleshoot their panels. By following these steps, users should be able to discover issues if they exist and have a pathway and alternatives to address and resolve them. Additional troubleshooting for general issues that may arise during panel optimization can be found in Tables 2-5.

Understanding Results

The example panel optimized using this protocol and presented here aims to identify conventional T cell subsets in peripheral blood of healthy participants. The participants were infected with low-dose human hookworm as part of a longitudinal study. It is well established that during early parasitic infection there is an increase in T-helper Type 2 immune responses (Th2) in the T cell compartments due to the primary role these cells play in host responses to parasites (McSorley & Loukas, 2010). However, the wider effects on the human immune system of long-term chronic parasitic infection with the gut-residing hookworm *Necator americanus (Na)* have not been investigated. Therefore, a panel was designed and optimized to look at the following conventional and unconventional T cell subsets of interest: CD4$^+$ T cells (Th1, Th2, Th17, Th9, Th22, and Tregs), CD8$^+$ T cells (Tc1 and Tc2), and innate-like T cells (γδ T, mucosal associate invariant T [MAIT], and natural killer T [NKT] cells). This panel can assess the frequency of these cell subsets as well as their memory and activation phenotypes, using both expert gating and high-dimensional data analysis.

Some characteristics of the fluorophore selection for a certain panel including the

Table 2 Troubleshooting for Preparation and Evaluation of Optimal SRCs (Basic Protocol 1)

Problem	Cause	Potential solution
Spectral signature of SRC does not match expected signature for fluorophore	Contamination of control with another fluorescent antibody	Preferably prepare new SRC; alternatively, in SpectroFlo software using the raw SRC, exclude the contaminating signature, export the clean file, and import this FCS file as the correct SRC
	Carryover from previous SRCs on the cytometer	Preferably prepare a new SRC; alternatively, in the SpectroFlo software using the raw SRC, exclude the contaminating signature, export the clean file and import this FCS file as the correct SRC
	Tandem dye degradation	Find the cause of degradation (issue with fixative, how long samples are stored in fixative, temperature of incubation, light exposure during protocol, etc.) and make necessary corrections (modifying staining procedure or buy a new vial)
	Brilliant Stain Buffer added to bead controls	Remake bead SRCs without addition of Brilliant Stain Buffer
	Wrong tube was recorded (signature matches different fluorophore)	Read the correct control or import the correct SRC FCS file
More than one spectral signature is visible	Autofluorescence signature	If positive and negative populations have the same secondary signature(s), this may be autofluorescence. See Basic Protocol 3 or Support Protocol 3 to decide on an approach for mitigating AF.
	Contamination of control with another fluorescent antibody	If there are multiple positive populations and gating on each one produces a distinct signature, then it is likely that there are two fluorophores in the SRC. Prepare a new SRC or exclude the signature of the contamination and import this FCS file as the correct SRC, if possible
Fluorescent signal in the negative population of the SRC	Nonspecific binding of antibody to negative beads	Revisit antibody titer to make sure the optimal titration is used; alternatively, use the universal negative feature in the software
	Inadequate sample preparation/wash procedure	Wash SRC controls well in the presence of excess wash solution such as FACS Staining Buffer (see Basic Protocol 1)
	Carryover of samples from previous SRC	Look at time vs. fluorescent signal, export a cleaned FCS file removing the contaminating signal, and import this FCS file as the correct SRC
Unmixing errors in SRCs	Gates were not set correctly in the Unmixing Wizard	Ensure that the P1 gate is set on the population with the highest expression of the marker. Place the positive histogram gate on the brightest signal (this may be different than gating on all positive signals). Better results can be obtained with tighter gates that do not include a side variety of cell sizes and/or fluorescence intensities.
	Contamination of control with another fluorescent antibody	Preferably prepare a new SRC; alternatively, in SpectroFlo software using the raw SRC, exclude the contaminating signature, export the clean file, and import this FCS file as the correct SRC

(Continued)

Table 2 Troubleshooting for Preparation and Evaluation of Optimal SRCs (Basic Protocol 1), *continued*

Problem	Cause	Potential solution
No positive signal can be detected	Gates were not set correctly in the Unmixing Wizard	Move P1 gate to population that expresses marker and/or move histogram to peak detector
	Not enough events were recorded	The unmixing algorithm requires a minimum of 300 positive events; record more total events
	Antibody was not added to SRC	Prepare new SRC

Table 3 Troubleshooting for Unmixing Evaluation of Fully Stained Sample (Basic Protocol 2)

Problem	Potential cause	Potential solution
Unmixing Wizard unmixes SRCs cells correctly, but unmixing errors are present in FS cells	SRCs are dimmer than fully stained sample	Use brighter SRCs; ensure staining protocol is exactly the same for SRCs and FS cells (antibody concentration, incubation time/temperature, use of fixative, stimulation of cells, etc.). Note that the optimal antibody concentration for compensation beads is often different than for cells. Pipetting error can easily occur when pipetting small volumes (<1-2 µl) for SRCs compared to using a master mix for FS samples. In this case, it is recommended to dilute antibodies to avoid pipetting small volumes. Other common mistakes include not using fix/perm buffer on compensation beads or using a tissue for SRCs that has lower marker expression than FS sample.
	Beads were used for all the SRCs and there may be a mismatch in the emission spectra between beads and cells	Use SRC stained cells instead.
	Polymer dyes (more than 2) are included in the panel without using Brilliant Stain Buffer or Super Bright Stain Buffer	Use Brilliant Stain Buffer when more than one polymer dye is added in the same tube following manufacturer recommendations.
	AF signature in FS sample is complex and different to the controls	Use AF extraction and see information regarding complex AF samples. Ensure the unstained control used for AF extraction is treated the same as the FS stained sample. AF signature may change with treatment (fixation, stimulation, timepoint, etc.). With human samples there may be patient-to-patient variability in AF signatures, so if using AF extraction for complex AF samples, an unstained control for each patient may be required. Also ensure that enough events are recorded in the unstained control to assess AF.
	Wrong tube was recorded (signature matches different fluorophore)	Read the correct control or import the correct SRC FCS file
Some FS samples unmixed correctly while others have unmixing errors	Biological variation; marker level of expression greatly changes across donors or across experimental conditions.	Try using beads as SRCs. If they are not optimal, use donor with the highest level of expression as control.

Table 4 Troubleshooting for Evaluation of Marker Resolution (Basic Protocol 3)

Problem	Potential cause	Potential solution
Low signal in certain markers (in FS and SS samples)	Marker expression too low or non-existent	Check that the marker is expected to be expressed on the particular cells or animal model of interest. Compare to Technical Data Sheet (TDS) or published literature.
	Experimental design (i.e., timepoint or stimulation) does not elicit certain markers	Stimulate cells with a positive control (e.g., PMC-ionomycin) to ensure cells are capable of expressing the markers of interest.
	Epitope is damaged by digestion procedure	Test different clone or modify digestion conditions
	Epitope is damaged by staining procedure	Test different clone or modify staining conditions
	Fluorophores chosen were too dim	Choose a brighter fluorophore for the specific marker and optimize concentration used
	Tandem dyes have degraded or decoupled	Find cause of degradation (issue with fixative, how long samples are stored in fixative, temperature of incubation, light exposure during protocol, etc.) and replace tandem dye with a new vial
FS sample stained less (lower MFI) than SS sample for a given marker	Binding site blocked by other reagents in the panel or different Ab/receptor binding kinetics	Test sequential staining, perform experiments to identify antibodies that are interfering with each other or try different clones
	Saturation is not achieved at optimal titer for SRCs	Increase titer to reach saturation
	Pipetting error when pipetting antibody cocktails	Repeat experiment to double-check
	Antibody/antibodies were trapped in the column when filtering the antibody cocktail	Optimize centrifuge spinning time and speed
Unstained, SS, and FS cells have high AF background	Cells of interest are highly autofluorescent	Extract autofluorescence
Suboptimal separation/resolution between negative and positive populations	Antibody concentration is too low or too high	Optimize antibody concentration based on antibody titration results
	Spread	Check SSM and use FMOs to confirm source of spread
	Unbound antibodies were not adequately washed from samples	Add additional centrifugation and FACS Staining Buffer wash step
	Viability dye concentration is too high and live cells are stained with dye	If live cells are stained with viability dye, the spreading error from the viability dye must be accounted for and this may cause problems with marker resolution. Ideally, viability dye should be titrated so that live cells do not stain with viability dye.

Table 5 Troubleshooting for Assessment of Data Quality (Basic Protocol 4)

Problem	Potential cause	Potential solution
Appearance of unexpected biological patterns	Inadequate cleaning gates	Make sure you are using time gates, excluding doublets, dead cells, and antibody aggregates, and gating on cells of interest
	Viability dye concentration is too high and live cells are stained with dye	Titrate viability dye to make sure the optimal concentration is used
	Lack of viability dye in panel and dead cells nonspecifically bind to antibodies	Titrate and add viability dye to FS samples
	Lack of addition of Brilliant Stain Buffer	Use Brilliant Stain Buffer when more than one polymer dye is added in the same tube following manufacturer recommendations

similarity and complexity indices of the panel (Fig. 12A) and the spectral signatures of fluorophores used (Fig. 12B) have been included. Although some fluorophores have a high similarity index, they have been allocated to different cell types and therefore the impact of the expected spreading error should be minimal (Fig. 12). A panel distribution table has also been included (Fig. 12C) to show the peak emission wavelengths for the fluorophores and markers assigned to the panel.

After following all the steps of Basic Protocols 1-4 for our panel shown here, and resolving any issues that arose (such as one tandem degradation or implementing the sequential staining for some of the markers), high-quality full-spectrum flow cytometry data as defined by clear resolution of all expected populations was achieved. The optimized staining protocol for this panel is detailed in the Supplementary Material. Sequential staining was applied, following the order given in the protocol, for markers that showed a reduction in positive signal in the FS compared to the SS control, starting at the marker with the greatest reduction and working toward the one with the least reduction. This approach provided satisfactory results and did not require the testing of other combinations. To reduce staining procedure time, further optimization would be required to determine whether some markers could be added at the same time instead of using all of them sequentially.

A clean and clear positive population can be detected for all markers in the panel, with no signal resolution loss when all antibodies are combined. In some cases, such as for the activation markers, the use of FMOs is necessary to assist in evaluating gates to determine positive staining with confidence. All populations of interest could be found using expert gating, and the populations resemble the expected expression patterns and frequencies. Additionally, high-dimensional data analysis algorithms were successfully used without the appearance of artefacts, confirming the high quality of the data.

Time Considerations

The time needed for designing, optimizing, and analyzing a high-dimensional flow cytometry panel can be highly variable and can depend on the assay complexity, number of markers, wait-time required for reagents (geographically dependent), sample access and frequency of sample delivery (particularly for patient samples), duration of the disease model being investigated, and more. As an example, it took five months to optimize the digestion, design, and optimization of a 23-color spectral flow cytometry panel in gut tissue (Ferret-Font, Mehta, et al., 2020), whereas one month may be sufficient for a PBMC panel where digestion does not need optimization. It is therefore advisable to develop certain core panels that can be applied to several experimental questions and models.

Figure 12 Theoretical panel design. (**A**) Similarity and complexity indices of fluorophores used in the final panel design. (**B**) Cytek Full Spectrum Viewer readout showing the fluorescence signatures of all fluorophores used in the panel, using the 5L Aurora configuration. (**C**) Panel distribution table showing peak emission wavelengths for markers and fluorophores in the panel.

Author Contributions

Laura Ferrer-Font: Conceptualization; investigation; methodology; project administration; writing–original draft; **Sam J. Small:** Investigation; methodology;

writing–original draft; **Brittany Lewer:** Investigation; writing–review & editing; **Katherine R Pilkington:** Resources; writing–review & editing; **Laura K. Johnston:** Writing–review & editing; **Lily M. Park:** Writing–review & editing; **Joanne Lannigan:** Methodology; writing–review & editing; **Maria C. Jaimes:** Conceptualization; methodology; resources; supervision; writing–review & editing; **Kylie M. Price:** Funding acquisition; project administration; supervision; writing–review & editing.

Conflicts of Interest

Kate Pilkington, Lily Park, Maria C. Jaimes are employees of Cytek Biosciences, Inc., the manufacturer of the Aurora full-spectrum flow cytometer used in these studies. Joanne Lannigan is a paid consultant for Cytek Biosciences, Inc.

Data Availability Statement

Data available on request due to privacy/ethical restrictions.

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