Acquisition Protocol for Cytek® cFluor® Human B Cell Monitoring Kit

Contents

Introduction

This acquisition protocol provides step-by-step instructions to set up your Cytek® Northern Lights® system (2-laser B-R configuration or higher) or Aurora® system for data acquisition of the Cytek® cFluor® Human B Cell Monitoring Kit. This protocol provides instructions on 1) preparing SpectroFlo® software, 2) setting up the instrument, and 3) acquiring controls and samples.

This kit contains 25 tests.

NOTE: *For suggestions on how to prepare human peripheral mononuclear cells and whole blood, see* ***Sample Preparation (PBMCs) Guidelines for Cytek® cFluor® Human B Cell Monitoring Kit*** *and* ***Sample Preparation (Whole Blood) Guidelines for Cytek® cFluor® Human B Cell Monitoring Kit.***

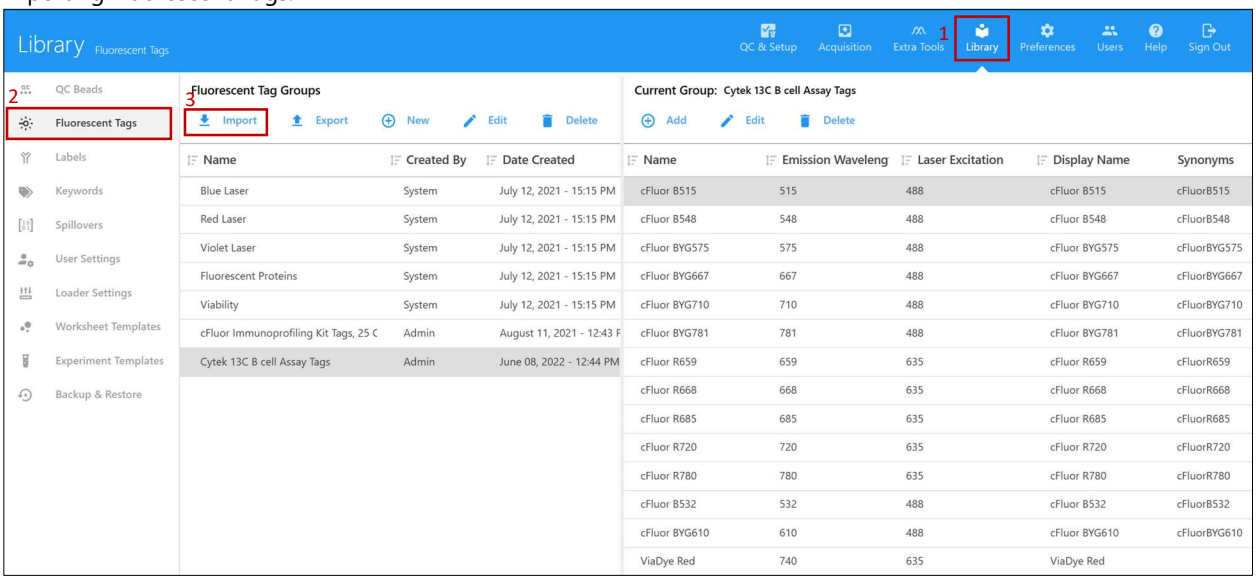
Preparing SpectroFlo® Software

Add fluorochromes to the library

1. Add the kit fluorochromes into the **Library**. Select **Fluorescent Tags** in the **Library** and import the ***Cytek® 13C B cell Assay Tags***.csv file.

NOTE: *If any fluorochromes were previously entered into SpectroFlo® Library, a warning message will appear. Click* ***Replace*** *to overwrite the information in the library with the new fluorescent tag information.*

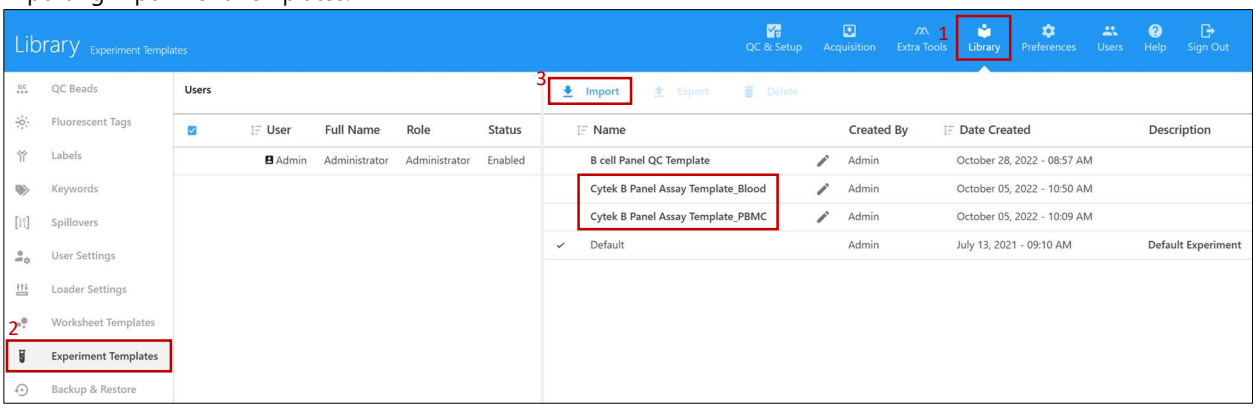
Importing Fluorescent Tags:



Import the experiment template

1. The ***Cytek® B Panel Assay Templates*** include a reference group with predefined stopping criteria, assigned marker names, as well as recommended acquisition and analysis worksheets. The Template for PBMCs is setup for ViaDye Red, while the Blood Template is not. The use of viability dyes is not needed with fresh blood.
2. Add Templates into the **Library**. Select **Experiment Templates** and import the templates.

Importing Experiment Templates:

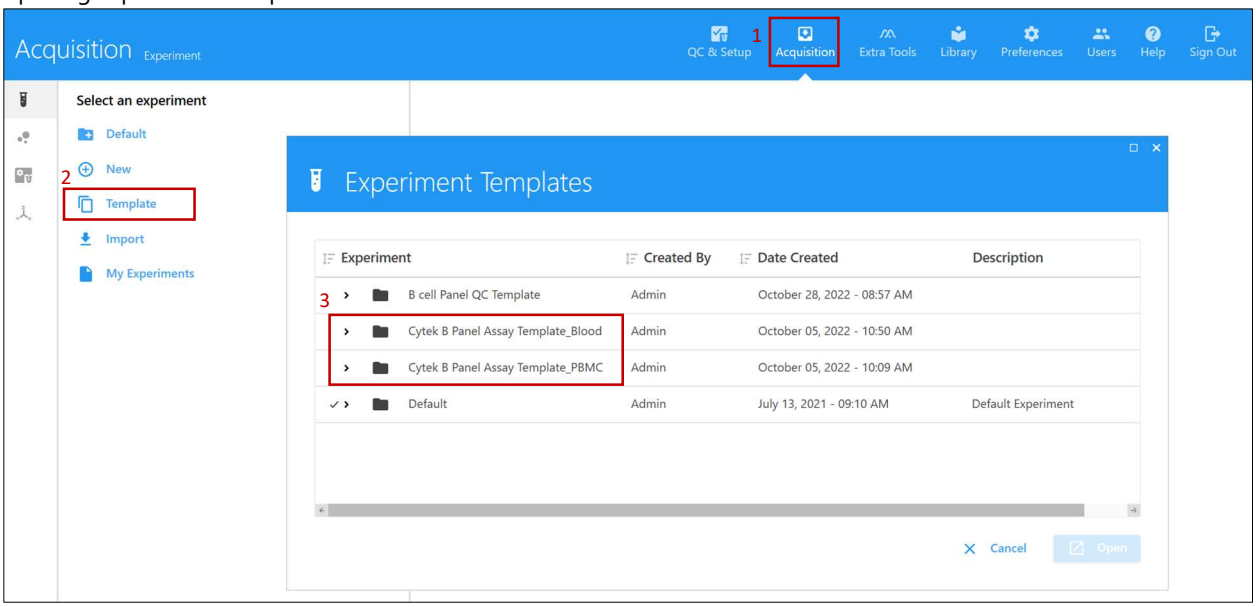


Setting up the Instrument

1. Follow the instructions for instrument setup and **Performing Daily QC** as outlined in the User's Guide.

2. From the **Acquisition** module, create a new experiment by clicking on **Template**, then choose either "*Cytek® B Panel Assay Template_PBMC*" or "*Cytek® B Panel Assay Template_Blood*"

Opening Experiment Template:



3. Either beads or cells can be used for single color controls except cFluor® R668 IgG for which only beads should be used. See below (Table 1) for reference control sample type recommendations for each marker.

Table 1. Reference Control Type Recommendations

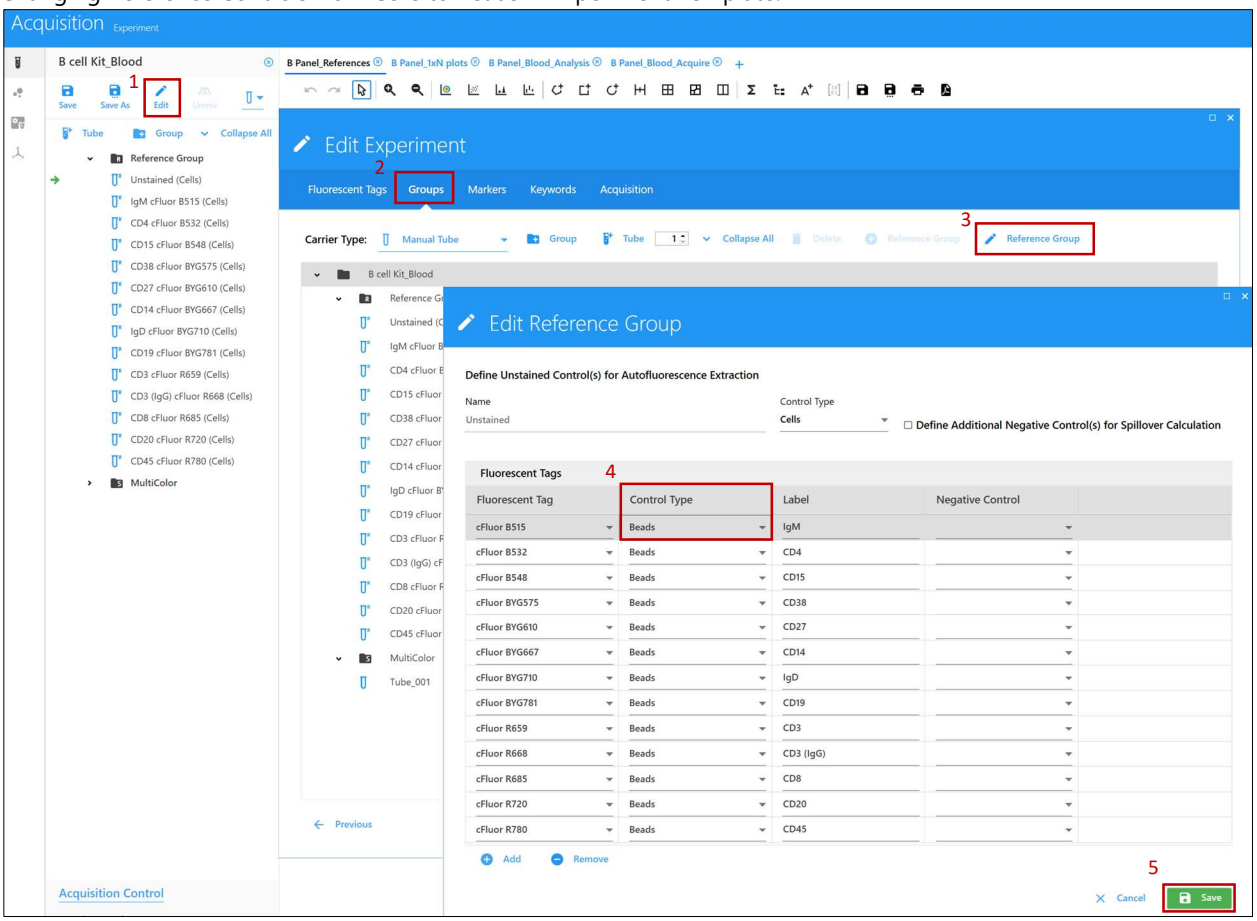
Laser	Target	Fluorochrome	Recommended Control Type
Blue	IgM	cFluor® B515	Cells or Beads
	CD4	cFluor® B532	Cells or Beads
	CD15	cFluor® B548	Cells or Beads
	CD38	cFluor® BYG575	Cells or Beads
	CD27	cFluor® BYG610	Cells or Beads
	CD14	cFluor® BYG667	Cells or Beads
	IgD	cFluor® BYG710	Cells or Beads
	CD19	cFluor® BYG781	Cells or Beads
Red	CD3	cFluor® R659	Cells or Beads
	IgG	cFluor® R668	Beads*
	CD8	cFluor® R685	Cells or Beads
	CD20	cFluor® R720	Cells or Beads
	CD45	cFluor® R780	Cells or Beads

*If cells must be used for the single-color control of cFluor R668 anti-IgG, since the IgG+ B cell population is small, it is recommended to use either cFluor R668 conjugated anti-human CD3 or CD4, or other antibodies for highly expressed markers.

4. The Experiment Template is setup for using cells as references. If using beads, go to **Edit**, select the **Groups** tab, and click on **Reference Group**. In **Edit Reference Group**, for the Fluorescent Tags that will be using beads, change the **Control Type** to "Beads." Be sure to save all changes.

5. Each sample tube is set to acquire a certain number of cells (see Appendix A). This can be changed by clicking **Edit**, then changing the Stopping Gate and Stopping Criteria under **Acquisition**. If using beads as references, the stopping criteria should be changed to collect 5,000 beads for all reference tubes. After making any changes to the acquisition criteria be sure to save all changes.
6. Add tubes and groups as needed for acquiring all samples. To preserve the predefined acquisition conditions, duplicate the existing tubes or groups.

Changing Reference Controls from Cells to Beads in Experiment Template:



Acquiring Controls and Samples

Acquire controls in the Reference Group

1. Preview unstained cell control at low flow rate to minimize wasted sample volume. Starting from the default CytekAssaySetting, optimize the FSC and SSC gains, as well as the threshold to fully visualize the cells of interest (see Figure 1).

NOTE: *Instrument settings can be saved as "Cytek® B cell Kit" for future use by clicking the* ***Save As*** *button in the Instrument Control window. The gains for all fluorescent parameters are set up with CytekAssaySetting in the instrument and only FCS, SSC and threshold need to be optimized for specific sample types.*

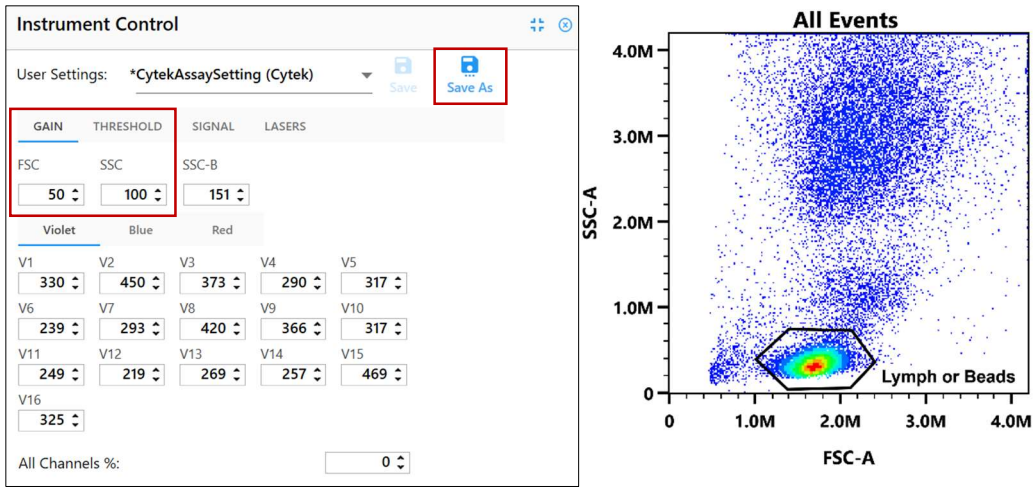


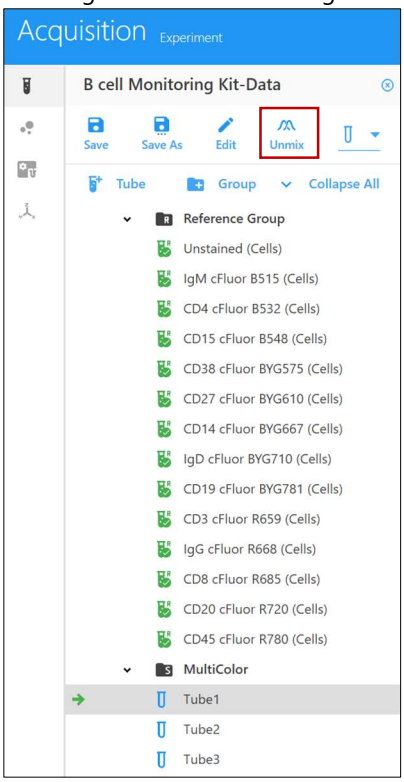
Figure 1: *Example cell resolution of human blood samples after FSC and SSC adjustment with threshold set at FSC 350,000.*

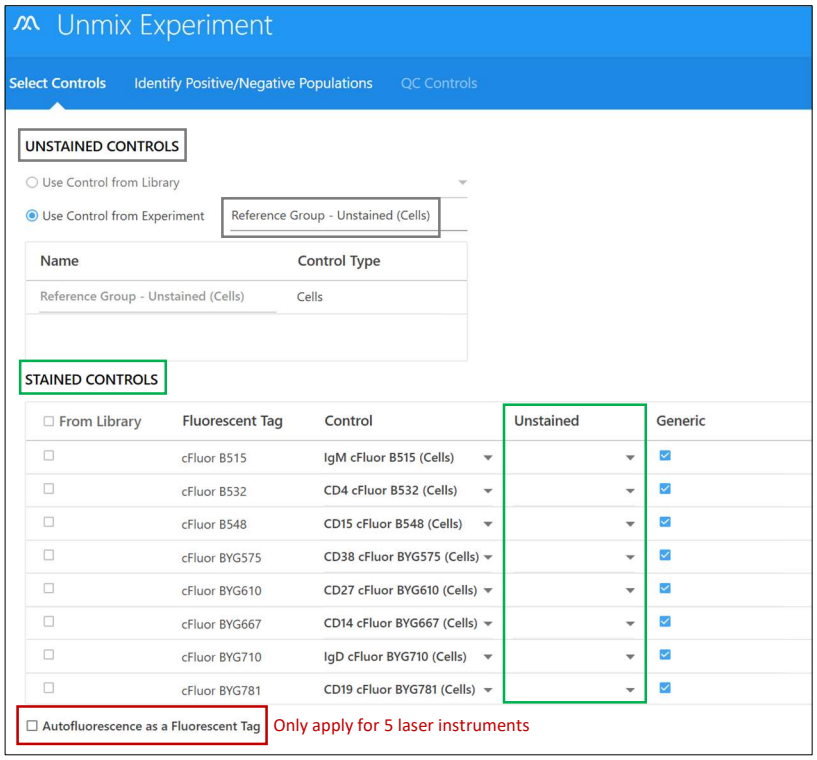
2. Acquire unstained and single-color controls in the Reference Control group. Click **Start** to preview for 5 to 10 seconds until the event rate stabilizes, then click **Record** to record each sample.

Unmix reference controls

1. Once all controls have been acquired, Click **Unmix**.
   NOTE*: Refer to Appendix B for additional workflows to reuse the reference controls.*
2. Under **Select Controls** tab in the Unmix Experiment wizard, ensure:
   1) Under **Unstained Controls** that Unstained (Cells) is selected as the Reference Group
   2) Under **Stained Controls** that the Unstained column is blank, which will enable selecting the negative population in the **Identify Positive/Negative Populations** section
   3) **Autofluorescence as a Fluorescence Tag** is selected for 5 laser instruments and unselected for 2-4 laser instruments. Click **Next**.

Selecting Controls for Unmixing:





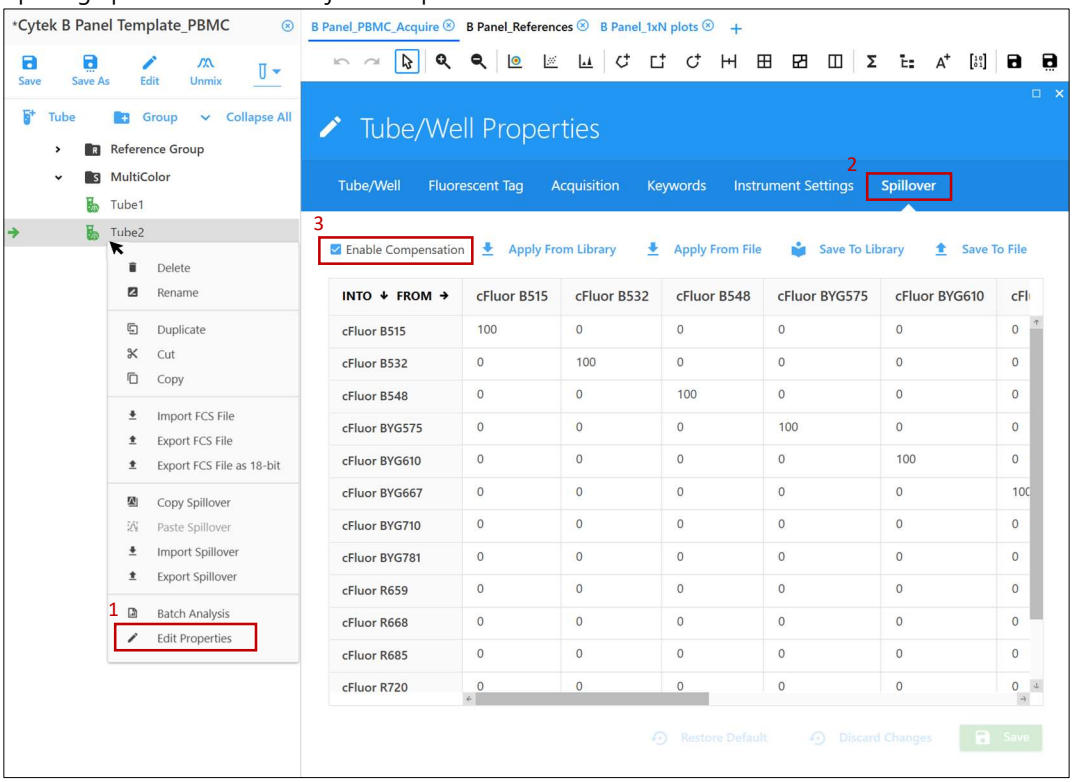
3. Under **Identify Positive/Negative Populations** tab, ensure for all single color controls that:
   1) Scatter plot is gated on the appropriate populations,
   2) Black bar is on the peak channel,
   3) Signature of each fluorochrome matches the expected spectrum, and
   4) Negative and positive gates in the histogram are correctly positioned.

   NOTE: *Refer to Appendix C for the correct gate positioning, expected spectra and peak channels of each fluorochrome, and the positioning of negative and positive gates in the histograms.*
3. Click **Next**. Under the **QC Controls** tab, click on **Similarity Matrix** to confirm all controls were appropriately stained. Click on **View Similarity Index** to compare the expected complexity index value found in Appendix D.
4. Click **Live Unmixing**.

Acquire and analyze multicolor samples

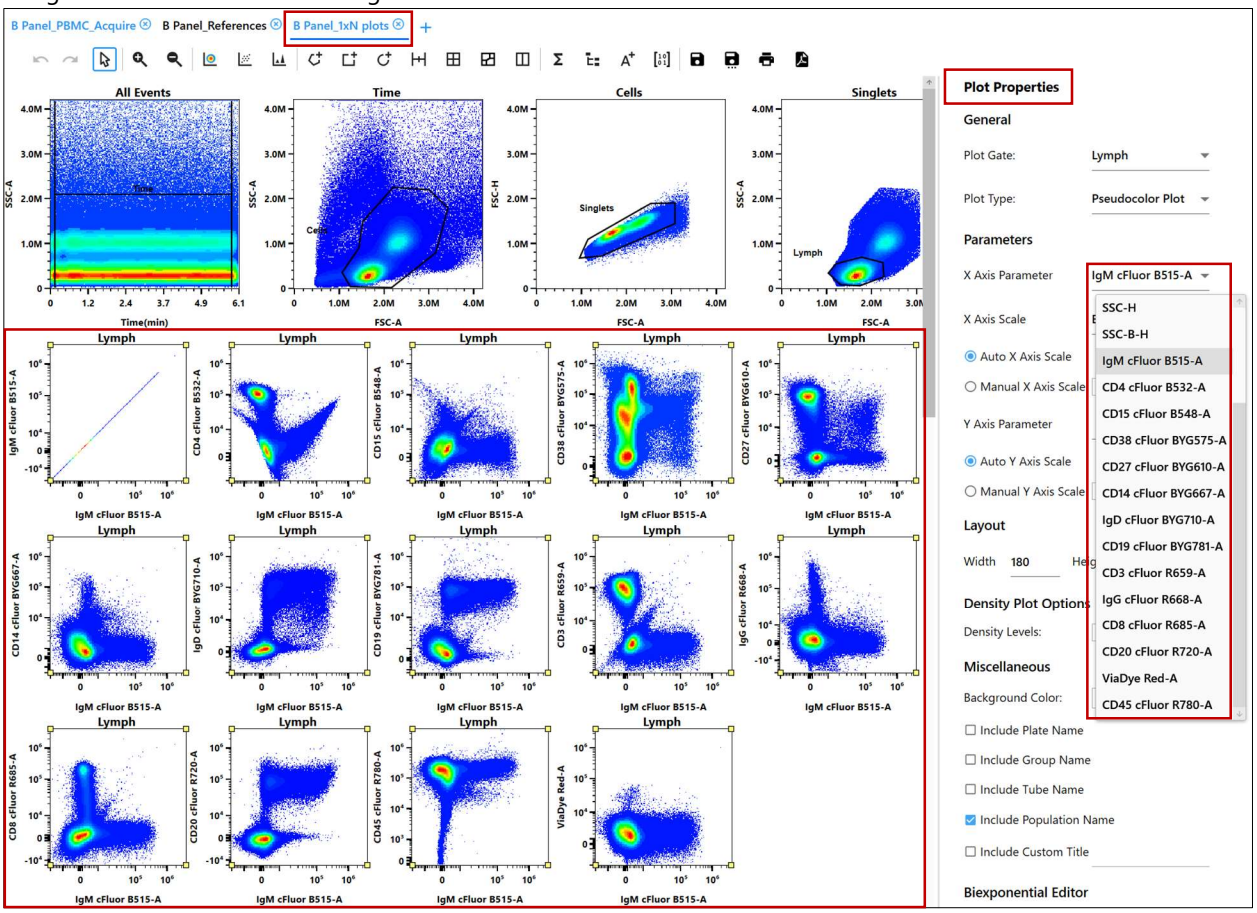
1. Acquire multicolor samples. See Appendix E for example of gating populations.
2. Compensation should not be needed when using cells as references for unmixing on a 2 or 3 laser system. There may be minor unmix errors using beads and in the BYG dyes when using a 5-laser system, which usually does not affect the identification of cell populations of interest. To adjust compensation, open the **B Panel_1xN plot** worksheet.
3. Right click on the multicolor tube needing compensation adjustments. From the drop-down menu, click **Edit Properties**.
4. Click on **Enable Compensation** in the pop-up wizard. Leave the wizard open and move it aside.

Opening Spillover Matrix to Adjust Compensation if Needed:



5. To check and adjust for unmixing error:
   1) Select all permutation plots,
   2) Right click and select **Properties** from the drop-down menu
   3) From **Plot Properties**, select the first fluorochrome under **X Axis Parameter**, and
   4) Check all permutation plots against the first fluorochrome for any unmixing errors and adjust spillover (compensation) as needed. Do this for all fluorochromes by selecting each fluor one by one under X Axis Parameter.

Using 1XN Plots to Assess Unmixing Errors:



6. To adjust unmixing error of a plot, click the **Adjust Spillover** icon in the ribbon menu. Click and drag upward or downward on the plot to make the adjustment (see Figure 2). Alternatively, the spillover values can be typed directly into the Spillover Matrix by double clicking on the cell you want to change the value for.

Adjusting Spillover to Correct Unmix Error:

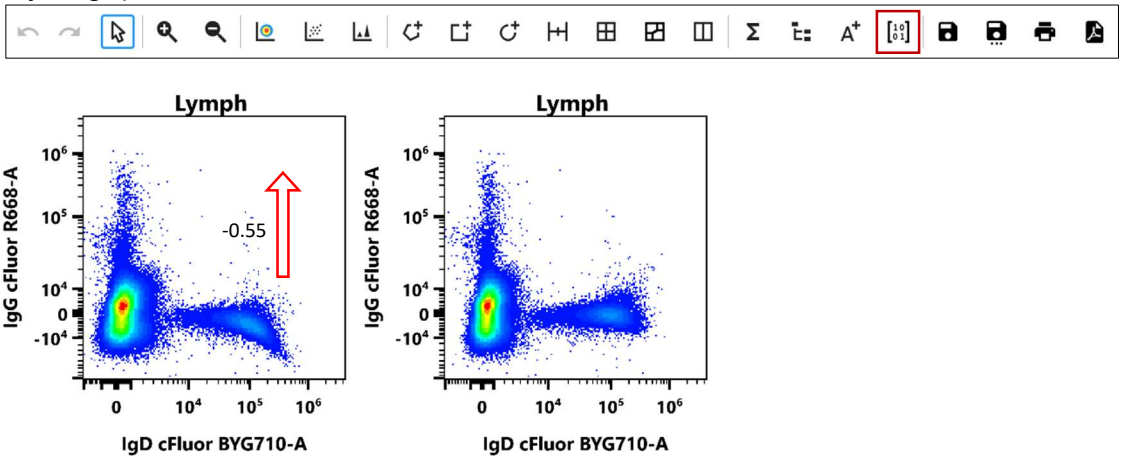
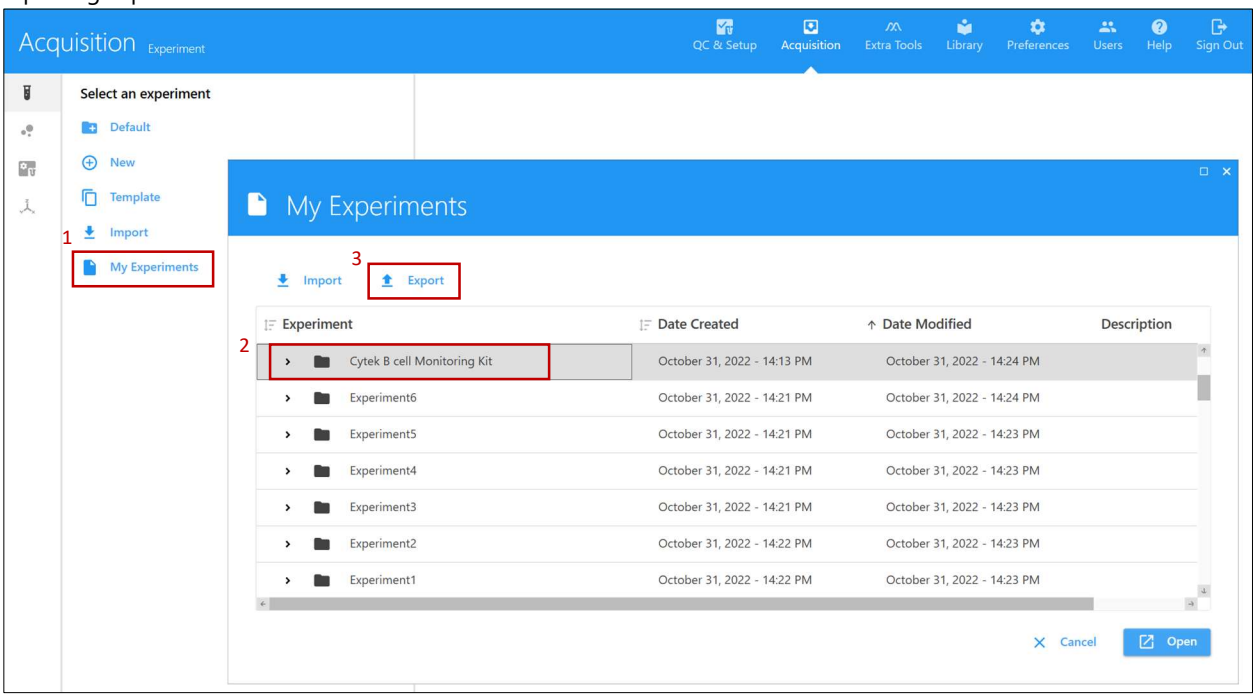


Figure 2: *Example of unmixing error adjustment for a blood sample unmixed with beads.*

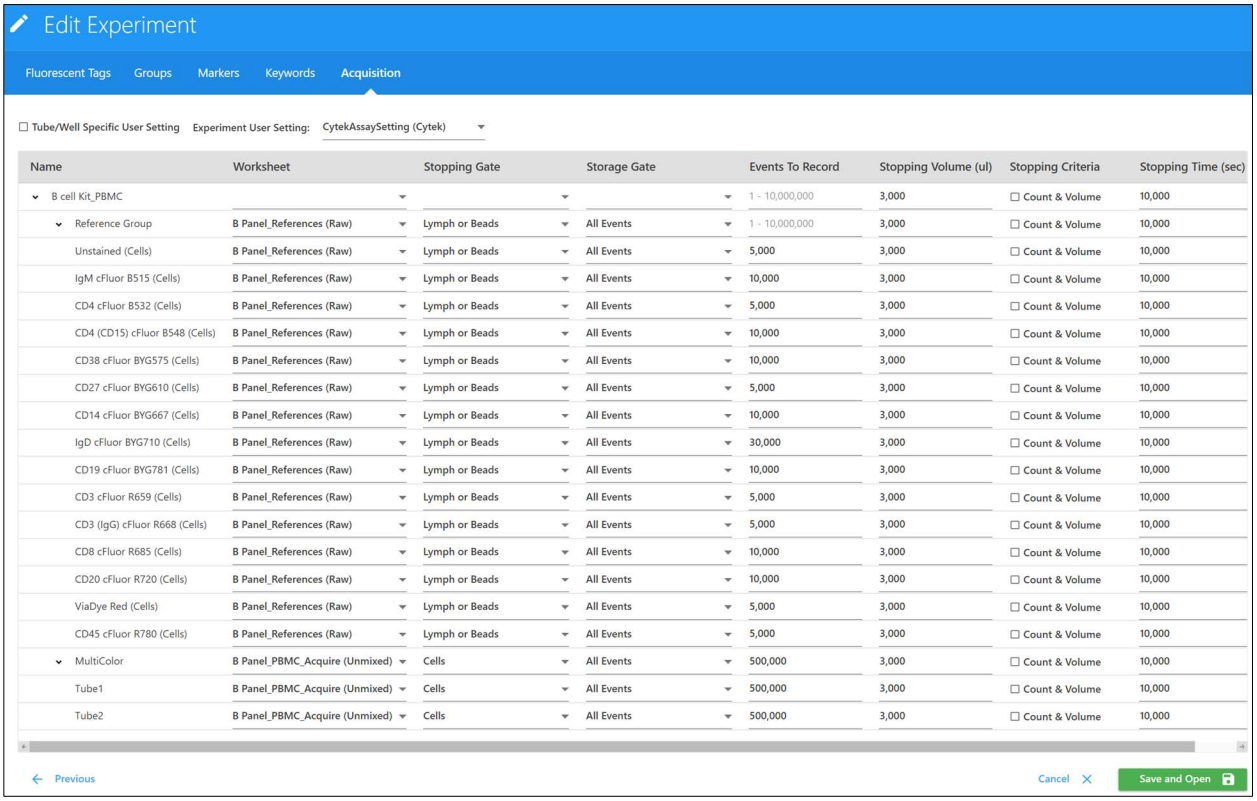
7. Click **Save** and close out of the **Tube/Well Properties** wizard. If similar unmix errors are observed among the multicolor samples, the adjusted Spillover matrix can be applied from one Tube to other Tubes by copying and pasting the Spillover into those Tubes. To do this, right click on Tubes and use the drop down menu to **Copy Spillover** and **Paste Spillover**.
8. Repeat the unmixing error adjustment for all multicolor samples as needed.
9. The manually adjusted compensation matrix can be saved to the **Library** for future use if using SpectroFlo® version 3.0 or higher. To do this, open the Tube**/Well Properties** and in the **Spillover** section click the **Save to Library** icon in the ribbon menu directly above the spillover matrix.
10. For convenience of analyzing samples quickly, we provide an Analysis worksheet that has data plots with gating for populations of interest and a corresponding statistics table with cell counts and percentages.
11. To export an experiment:
    1) Save and close the experiment,
    2) Click **My Experiments**,
    3) Select the experiment and click **Export**, and
    4) Choose a directory and click **Export**.

Exporting Experiment:



Appendix A: Acquisition Setup

Example of Acquisition Setup for PBMCs using cells as References. For Acquiring Blood, the setup does not include ViaDye Red and for acquiring multicolor samples the Events to Record is 1 million Cells. For using Beads as References, the Events To Record can be set for acquiring 5,000 Beads for each control.



Name	Worksheet	Stopping Gate	Storage Gate	Events To Record	Stopping Volume (ul)	Stopping Criteria	Stopping Time (sec)
B cell Kit_PBMC				1 - 10,000,000	3,000	Count & Volume	10,000
Reference Group	B Panel_References (Raw)	Lymph or Beads	All Events	1 - 10,000,000	3,000	Count & Volume	10,000
Unstained (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	5,000	3,000	Count & Volume	10,000
IgM cFluor B515 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	10,000	3,000	Count & Volume	10,000
CD4 cFluor B532 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	5,000	3,000	Count & Volume	10,000
CD4 (CD15) cFluor B548 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	10,000	3,000	Count & Volume	10,000
CD38 cFluor BYG575 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	10,000	3,000	Count & Volume	10,000
CD27 cFluor BYG610 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	5,000	3,000	Count & Volume	10,000
CD14 cFluor BYG667 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	10,000	3,000	Count & Volume	10,000
IgD cFluor BYG710 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	30,000	3,000	Count & Volume	10,000
CD19 cFluor BYG781 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	10,000	3,000	Count & Volume	10,000
CD3 cFluor R659 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	5,000	3,000	Count & Volume	10,000
CD3 (IgG) cFluor R668 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	5,000	3,000	Count & Volume	10,000
CD8 cFluor R685 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	10,000	3,000	Count & Volume	10,000
CD20 cFluor R720 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	10,000	3,000	Count & Volume	10,000
ViaDye Red (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	5,000	3,000	Count & Volume	10,000
CD45 cFluor R780 (Cells)	B Panel_References (Raw)	Lymph or Beads	All Events	5,000	3,000	Count & Volume	10,000
MultiColor	B Panel_PBMC_Acquire (Unmixed)	Cells	All Events	500,000	3,000	Count & Volume	10,000
Tube1	B Panel_PBMC_Acquire (Unmixed)	Cells	All Events	500,000	3,000	Count & Volume	10,000
Tube2	B Panel_PBMC_Acquire (Unmixed)	Cells	All Events	500,000	3,000	Count & Volume	10,000

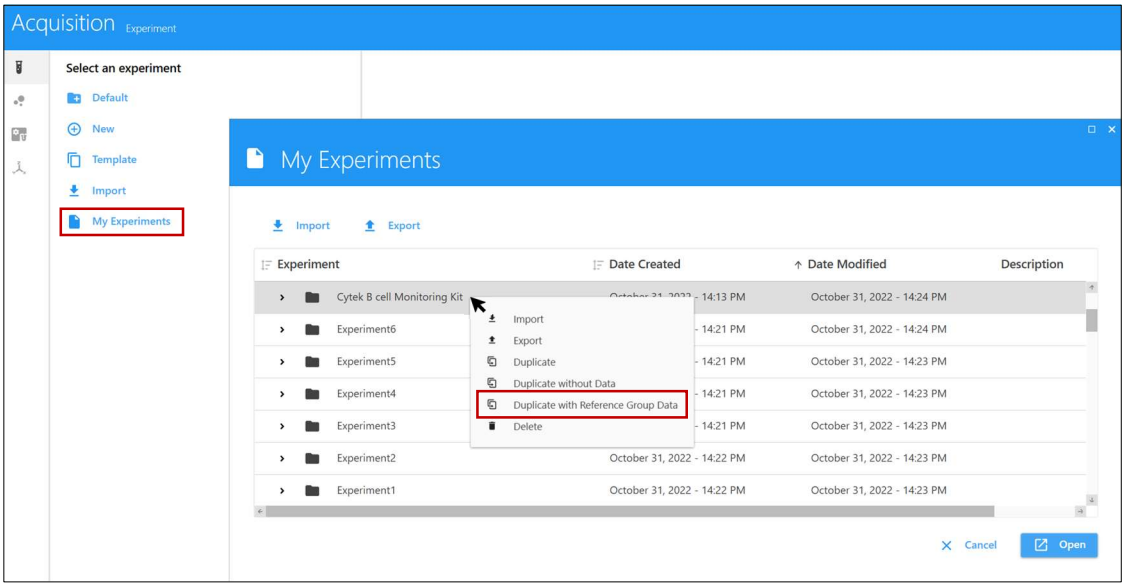
Appendix B: Reusing Single Color Controls

To reuse reference controls in future experiments, follow the instructions below.

NOTE: *For best results, maintain the instrument properly, perform QC daily calibration and use the same reagent lot. The samples need to be collected using the same instrument as the reference controls.*

Reusing single color controls from a previous experiment

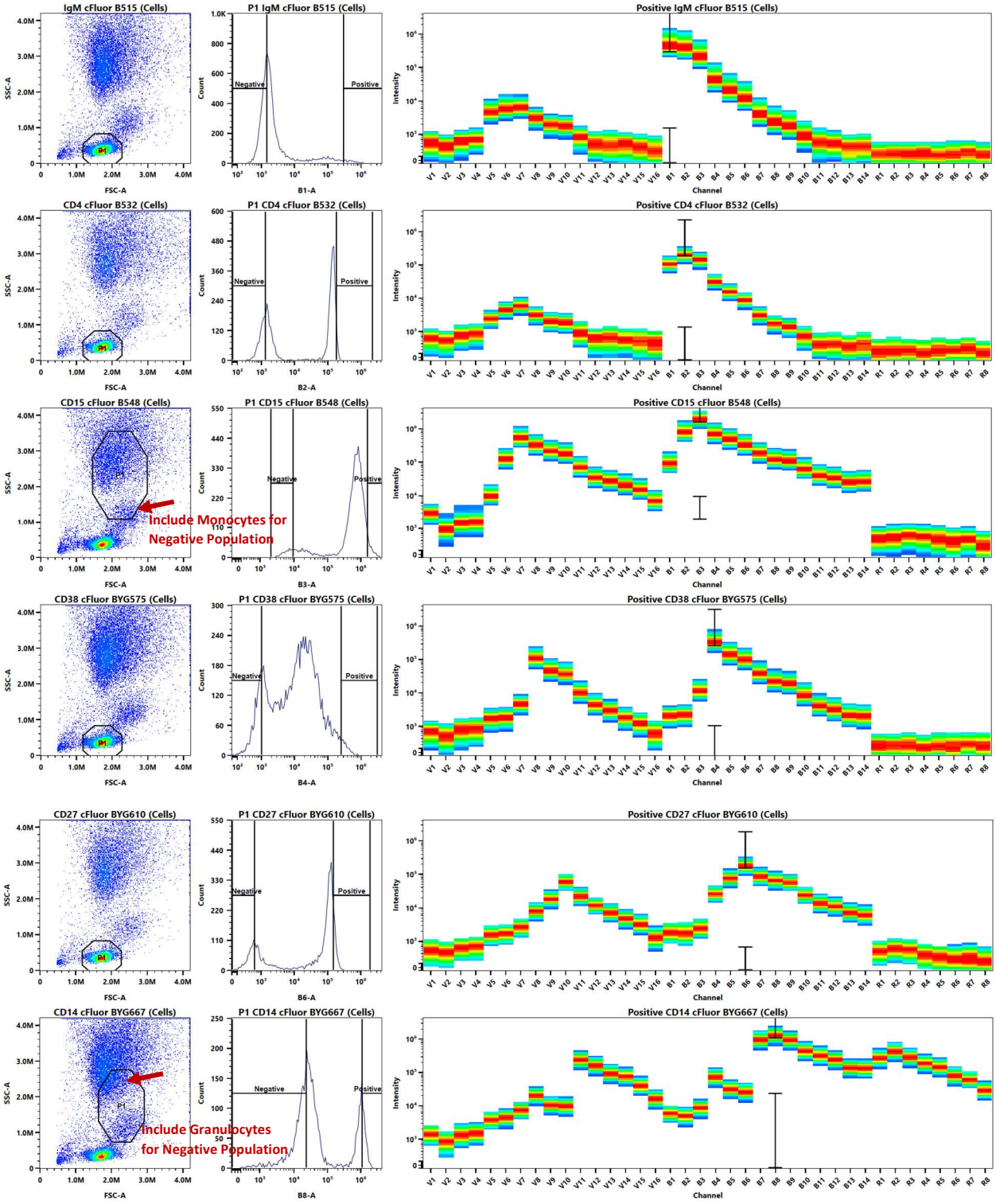
1. From the **Acquisition** module, open **My Experiments**. Right click the saved experiment and select **Duplicate with Reference Group Data**. This will duplicate the experiment with the reference controls and multicolor samples.

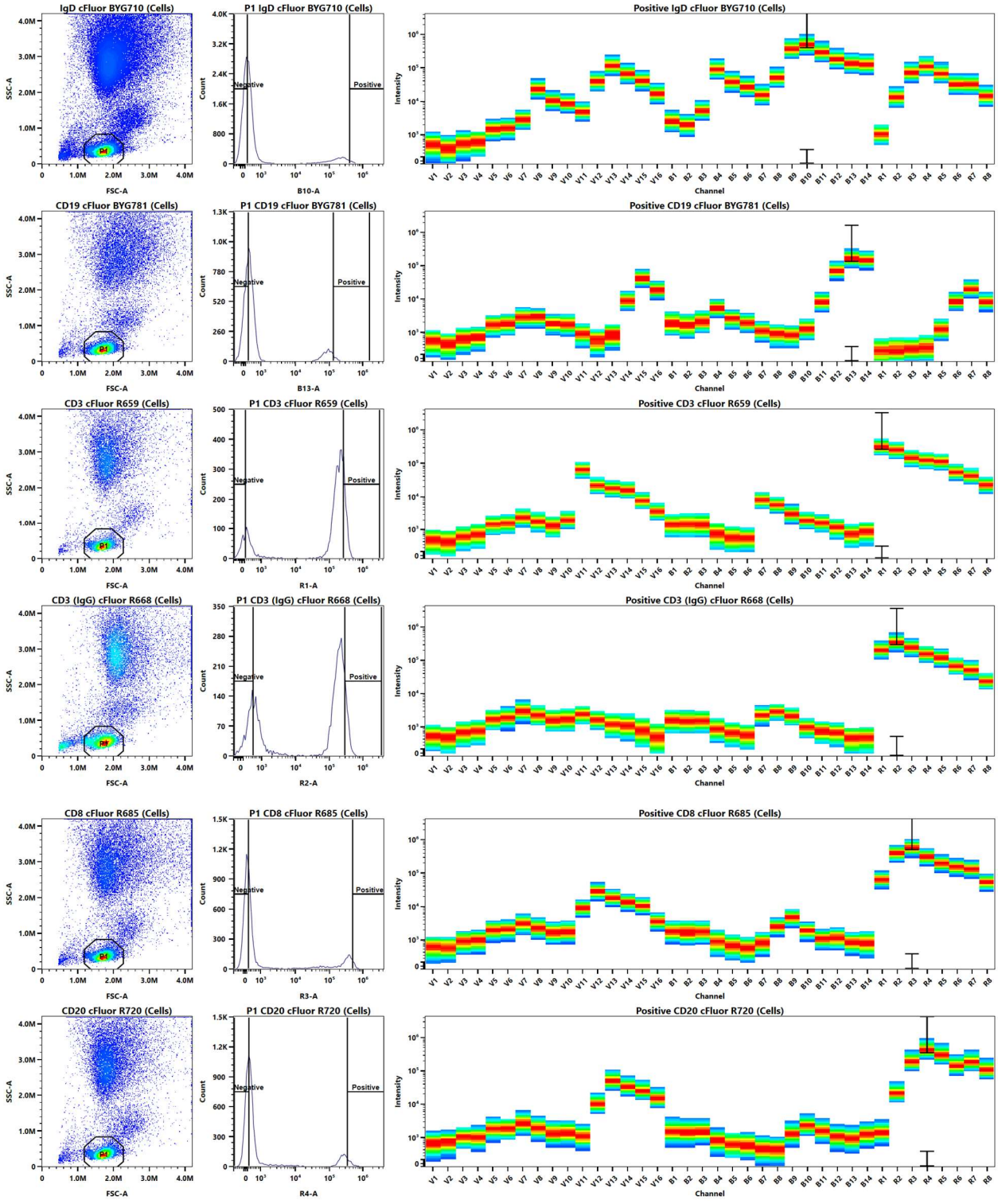


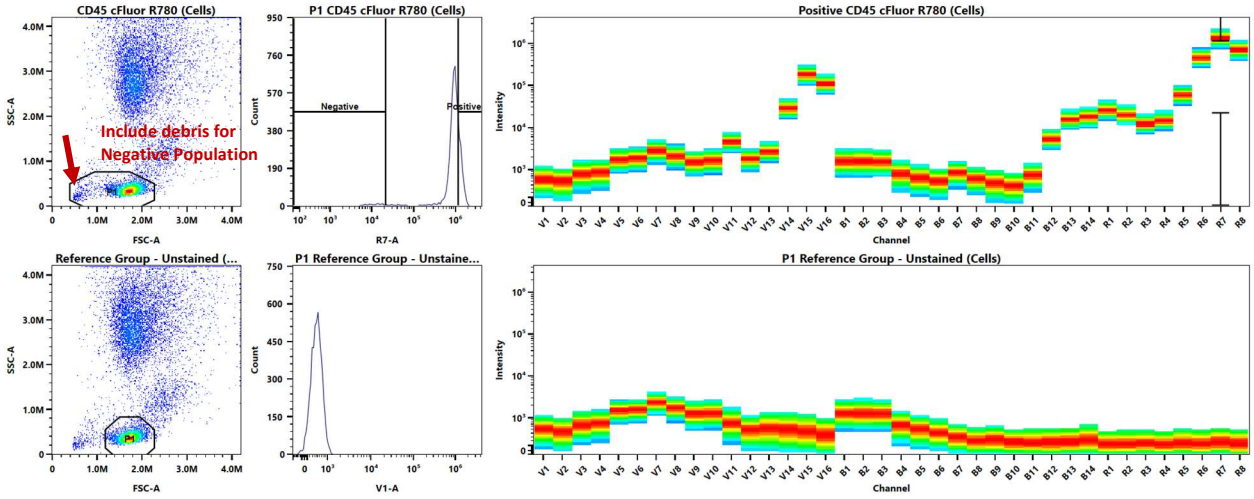
2. Open the newly created Experiment
3. Under Multicolor Group, add tubes for the new multicolor samples as needed
4. Click **Edit** to open Edit Experiment wizard
5. Under **Acquisition**, check to make sure the settings have the correct Worksheets, Stopping Gates, and Events to Record
6. Click **Save and Open**
7. Preview a sample to set FSC and SSC gains and FSC threshold
8. Acquire multicolor samples
9. Adjust the gates in the analysis worksheet as needed.

Appendix C: Single Color Control Gating and Signatures for 3-Laser (V-B-R) Cytek® Cytometers

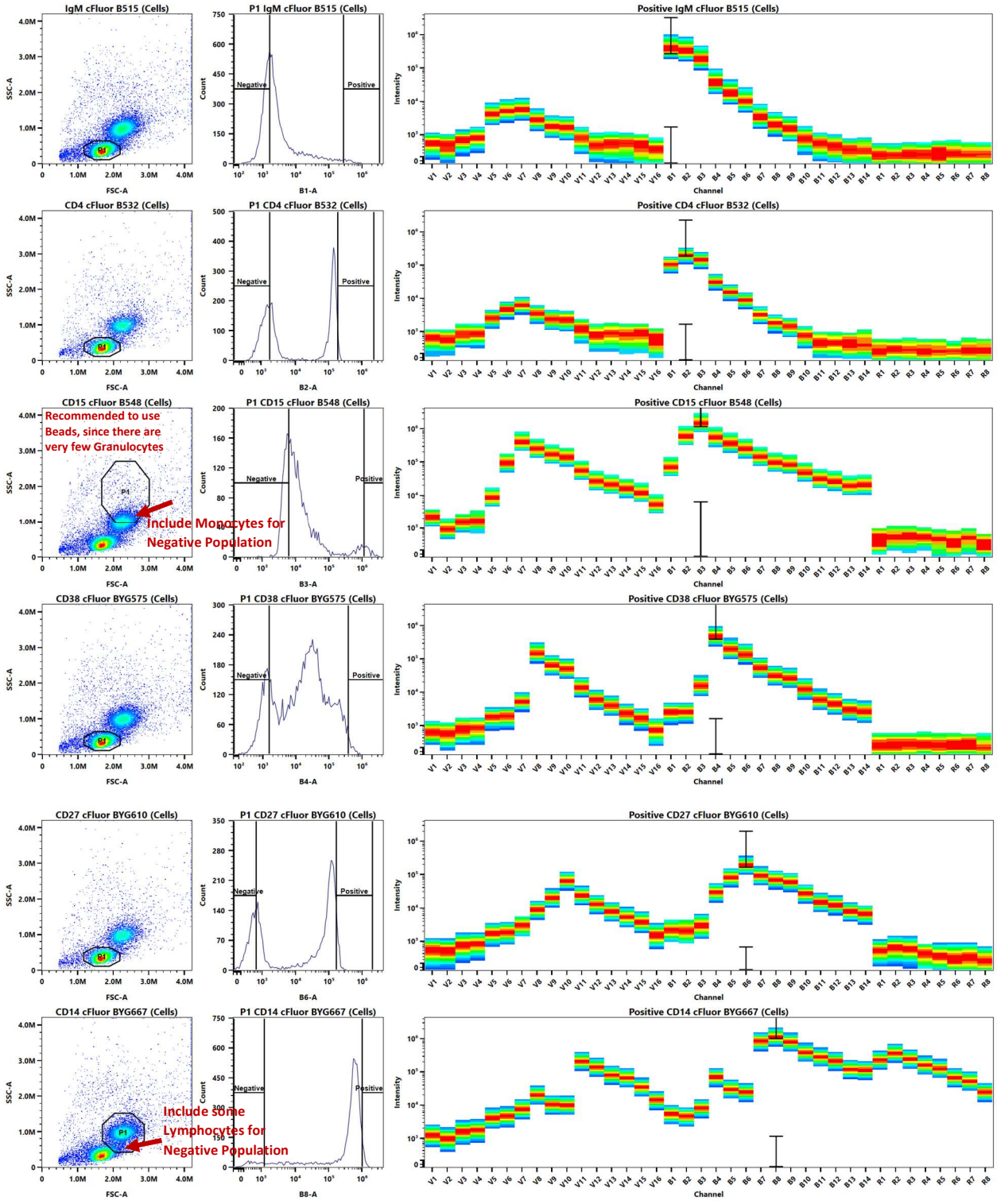
Example of gating and spectrum signatures for single color controls using blood.

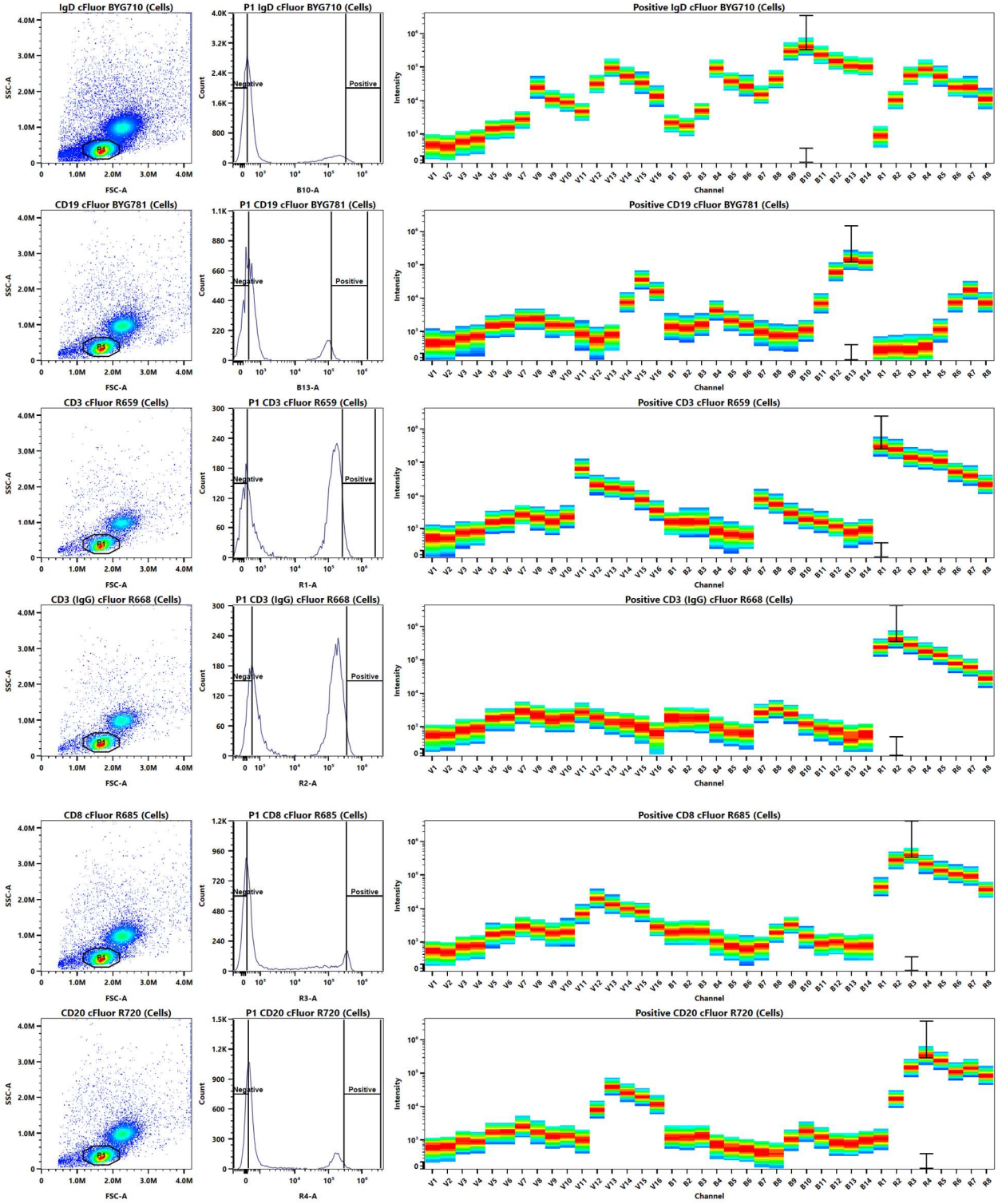


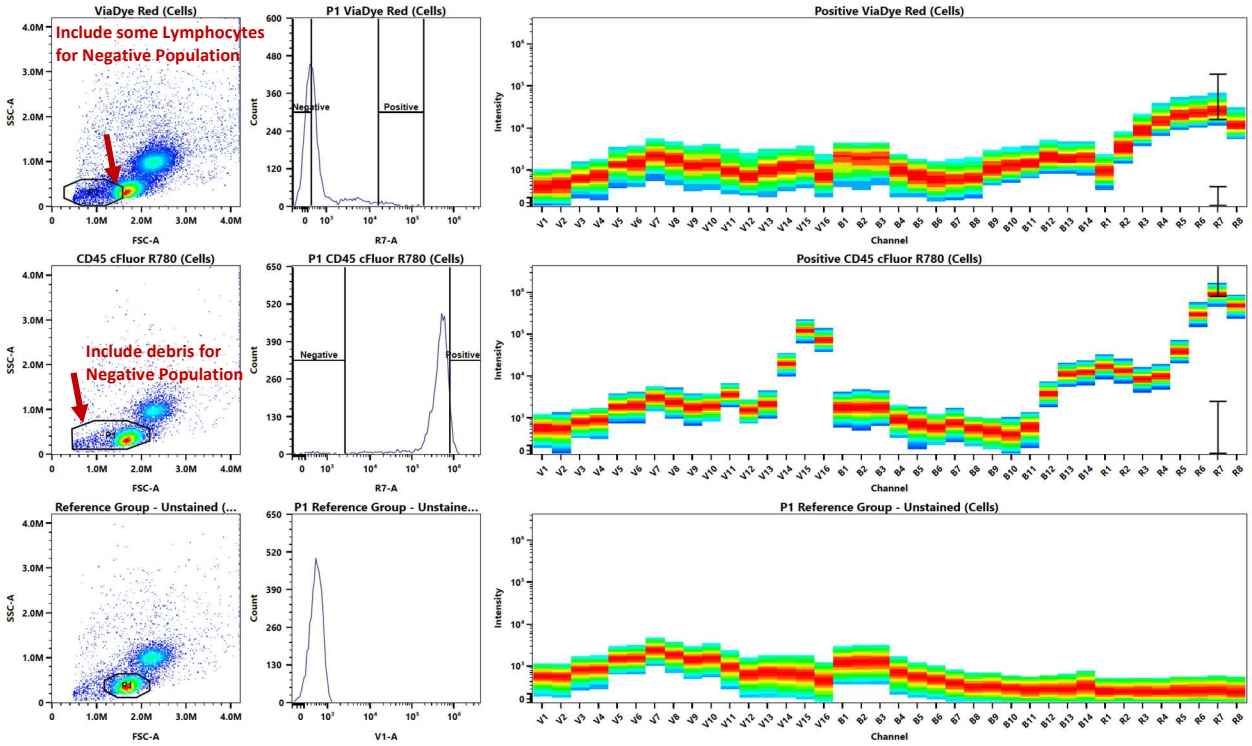

IgD cFluor BYG710 (Cells)
P1 IgD cFluor BYG710 (Cells)
Positive IgD cFluor BYG710 (Cells)
CD19 cFluor BYG781 (Cells)
P1 CD19 cFluor BYG781 (Cells)
Positive CD19 cFluor BYG781 (Cells)
CD3 cFluor R659 (Cells)
P1 CD3 cFluor R659 (Cells)
Positive CD3 cFluor R659 (Cells)
CD3 (IgG) cFluor R668 (Cells)
P1 CD3 (IgG) cFluor R668 (Cells)
Positive CD3 (IgG) cFluor R668 (Cells)
CD8 cFluor R685 (Cells)
P1 CD8 cFluor R685 (Cells)
Positive CD8 cFluor R685 (Cells)
CD20 cFluor R720 (Cells)
P1 CD20 cFluor R720 (Cells)
Positive CD20 cFluor R720 (Cells)
SSC-A
FSC-A
Count
Intensity
Channel
Negative
Positive


CD45 cFluor R780 (Cells)
SSC-A
FSC-A
Include debris for
Negative Population
P1 CD45 cFluor R780 (Cells)
Count
R7-A
Negative
Positive
Positive CD45 cFluor R780 (Cells)
Intensity
Channel
Reference Group - Unstained (...
P1 Reference Group - Unstaine...
V1-A
P1 Reference Group - Unstained (Cells)

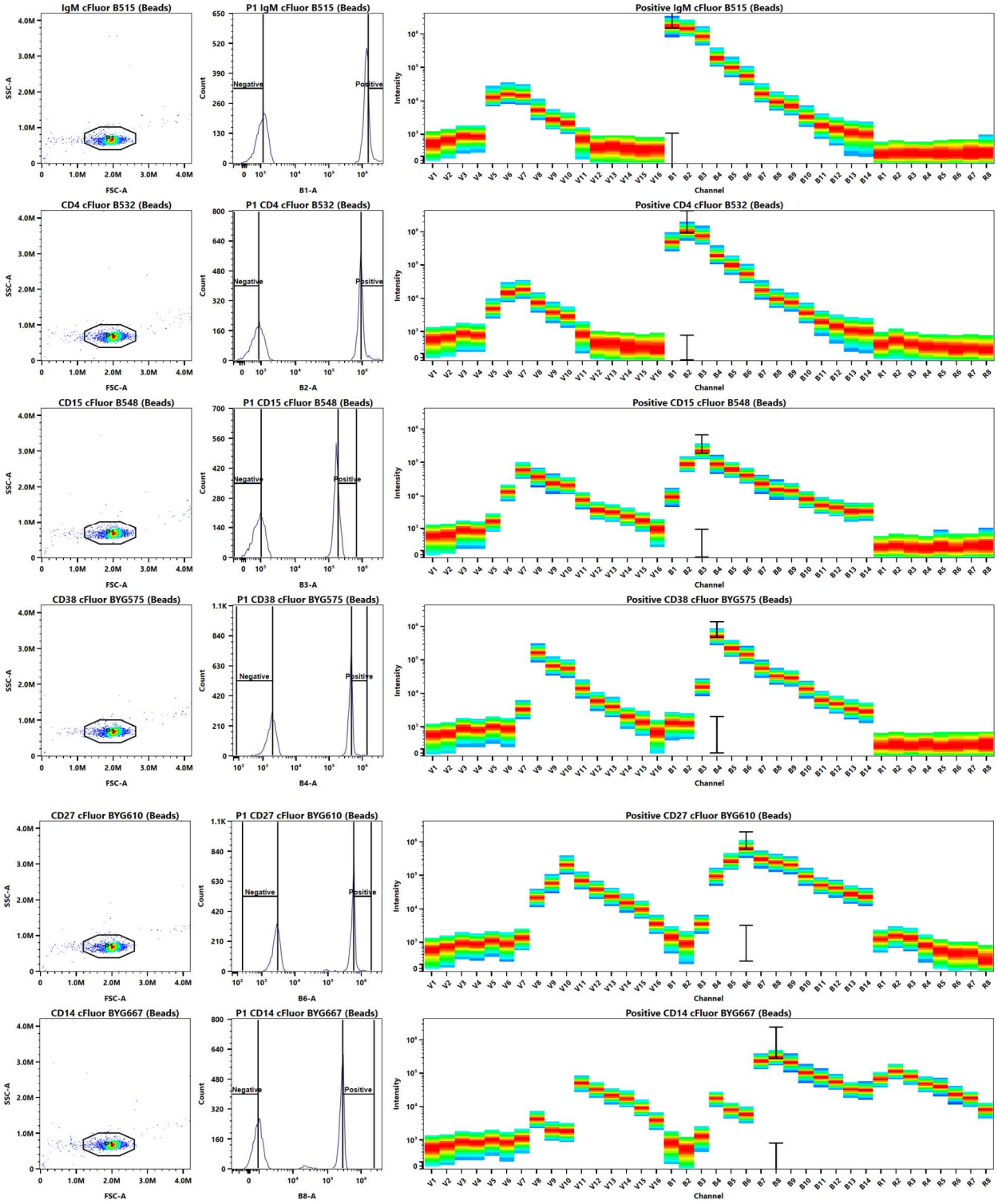
Example of gating and spectrum signatures for single color controls using PBMCs.

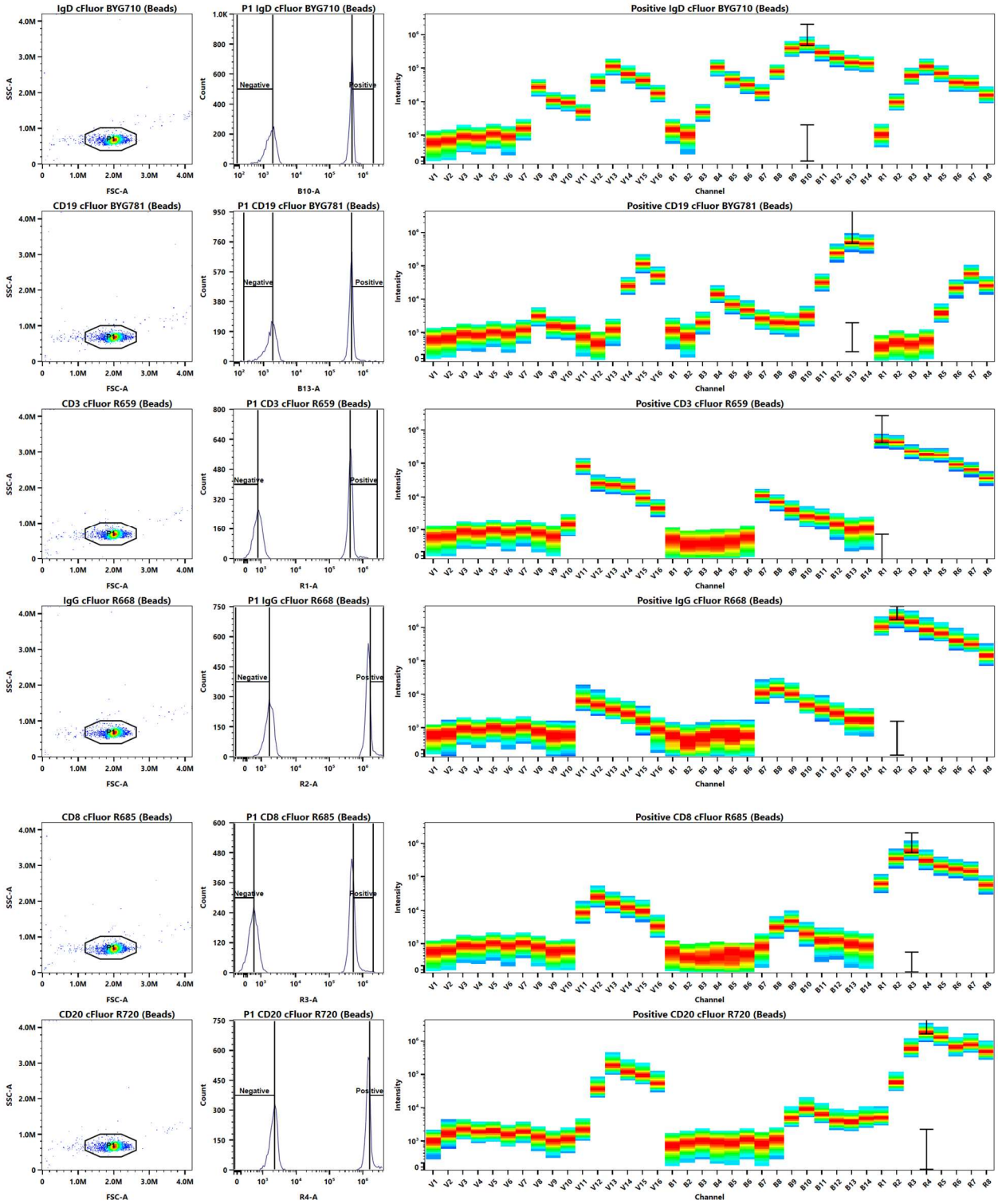


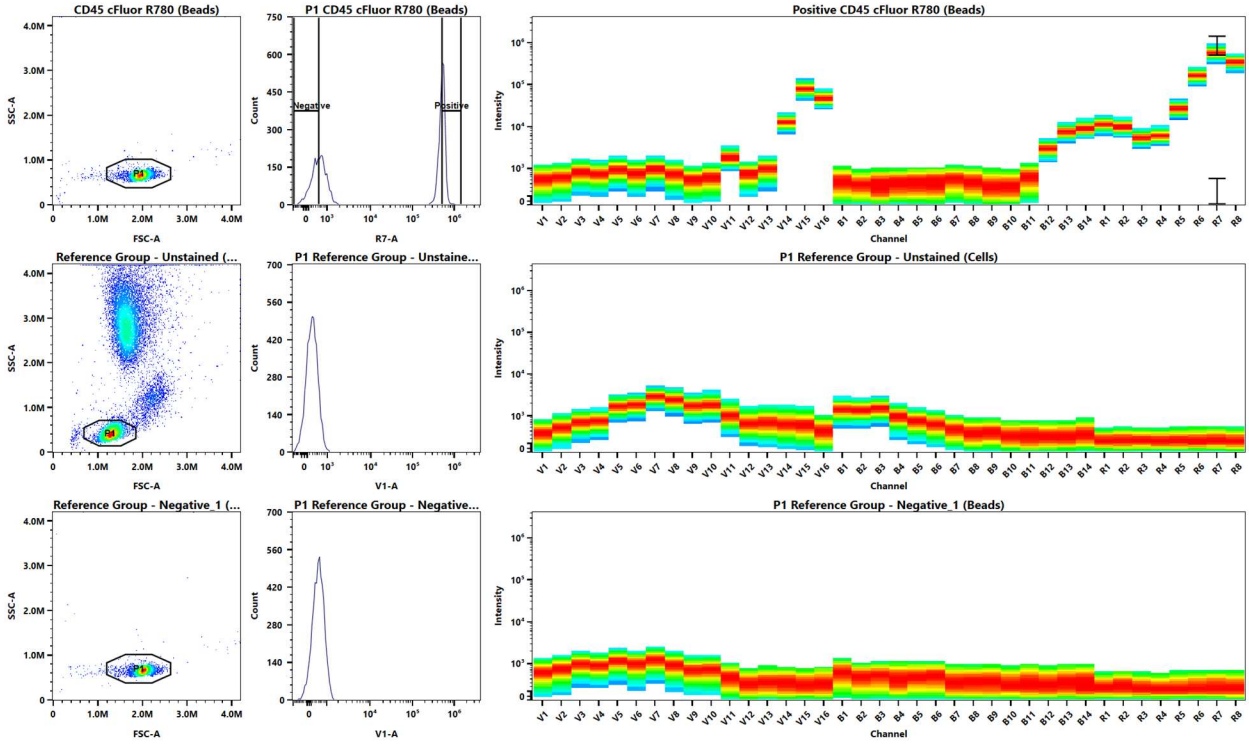

IgD cFluor BYG710 (Cells)
P1 IgD cFluor BYG710 (Cells)
Positive IgD cFluor BYG710 (Cells)
CD19 cFluor BYG781 (Cells)
P1 CD19 cFluor BYG781 (Cells)
Positive CD19 cFluor BYG781 (Cells)
CD3 cFluor R659 (Cells)
P1 CD3 cFluor R659 (Cells)
Positive CD3 cFluor R659 (Cells)
CD3 (IgG) cFluor R668 (Cells)
P1 CD3 (IgG) cFluor R668 (Cells)
Positive CD3 (IgG) cFluor R668 (Cells)
CD8 cFluor R685 (Cells)
P1 CD8 cFluor R685 (Cells)
Positive CD8 cFluor R685 (Cells)
CD20 cFluor R720 (Cells)
P1 CD20 cFluor R720 (Cells)
Positive CD20 cFluor R720 (Cells)
SSC-A
FSC-A
Count
Negative
Positive
Intensity
Channel


ViaDye Red (Cells)
Include some Lymphocytes for Negative Population
SSC-A
FSC-A
P1 ViaDye Red (Cells)
Count
Negative
Positive
R7-A
Positive ViaDye Red (Cells)
Intensity
Channel
CD45 cFluor R780 (Cells)
Include debris for Negative Population
P1
P1 CD45 cFluor R780 (Cells)
Positive CD45 cFluor R780 (Cells)
Reference Group - Unstained (...
P1 Reference Group - Unstaine...
V1-A
P1 Reference Group - Unstained (Cells)

Example of gating and spectrum signatures for Cytek® FSP™ CompBeads.



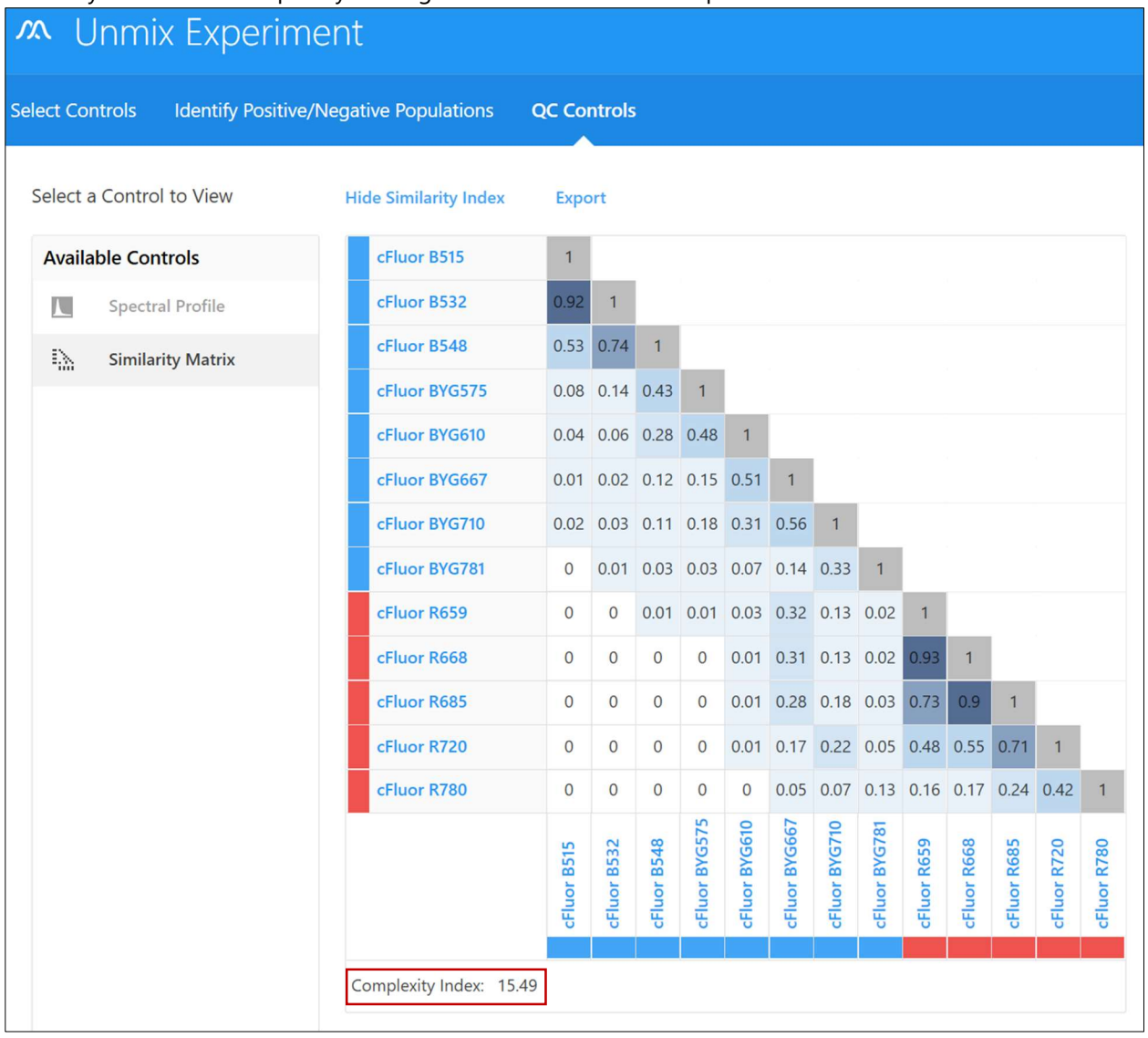

IgD cFluor BYG710 (Beads)
P1 IgD cFluor BYG710 (Beads)
Positive IgD cFluor BYG710 (Beads)
CD19 cFluor BYG781 (Beads)
P1 CD19 cFluor BYG781 (Beads)
Positive CD19 cFluor BYG781 (Beads)
CD3 cFluor R659 (Beads)
P1 CD3 cFluor R659 (Beads)
Positive CD3 cFluor R659 (Beads)
IgG cFluor R668 (Beads)
P1 IgG cFluor R668 (Beads)
Positive IgG cFluor R668 (Beads)
CD8 cFluor R685 (Beads)
P1 CD8 cFluor R685 (Beads)
Positive CD8 cFluor R685 (Beads)
CD20 cFluor R720 (Beads)
P1 CD20 cFluor R720 (Beads)
Positive CD20 cFluor R720 (Beads)
SSC-A
FSC-A
Count
Negative
Positive
Intensity
Channel


CD45 cFluor R780 (Beads)
P1 CD45 cFluor R780 (Beads)
Positive CD45 cFluor R780 (Beads)
Negative
Positive
SSC-A
FSC-A
Count
R7-A
Intensity
Channel
Reference Group - Unstained (...
P1 Reference Group - Unstaine...
P1 Reference Group - Unstained (Cells)
V1-A
Reference Group - Negative_1 (...
P1 Reference Group - Negative...
P1 Reference Group - Negative_1 (Beads)

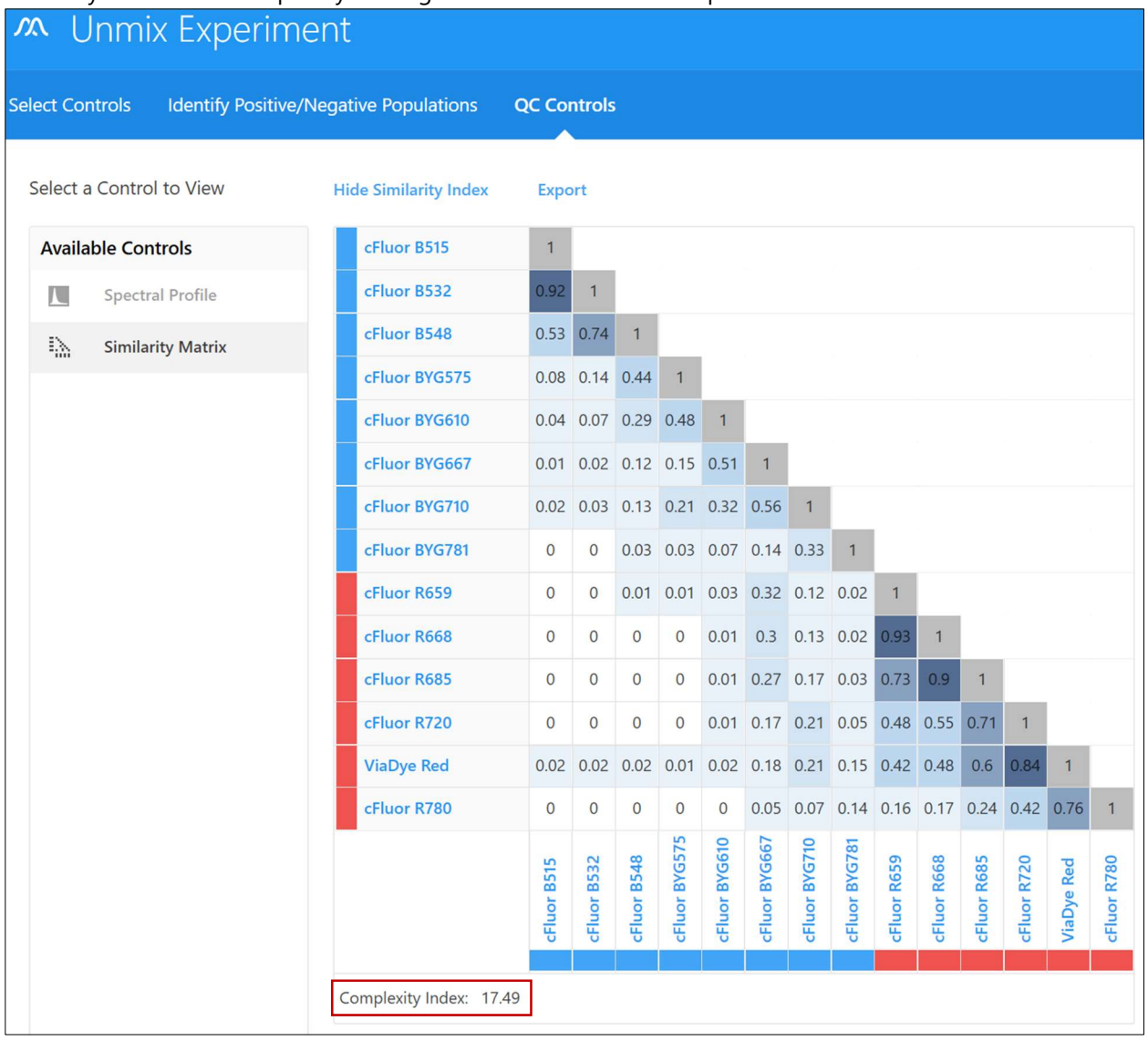
Appendix D: Example of Similarity™ Matrix for Cytek® cFluor® Human B Cell Monitoring Kit

Expected Similarity™ matrix and Complexity™ index generated on a 3-laser (V-B-R) Cytek® Aurora®. Generating similar values is a good indication that signatures of your single color controls match those generated by Cytek®.

Similarity matrix and Complexity index generated from blood sample:

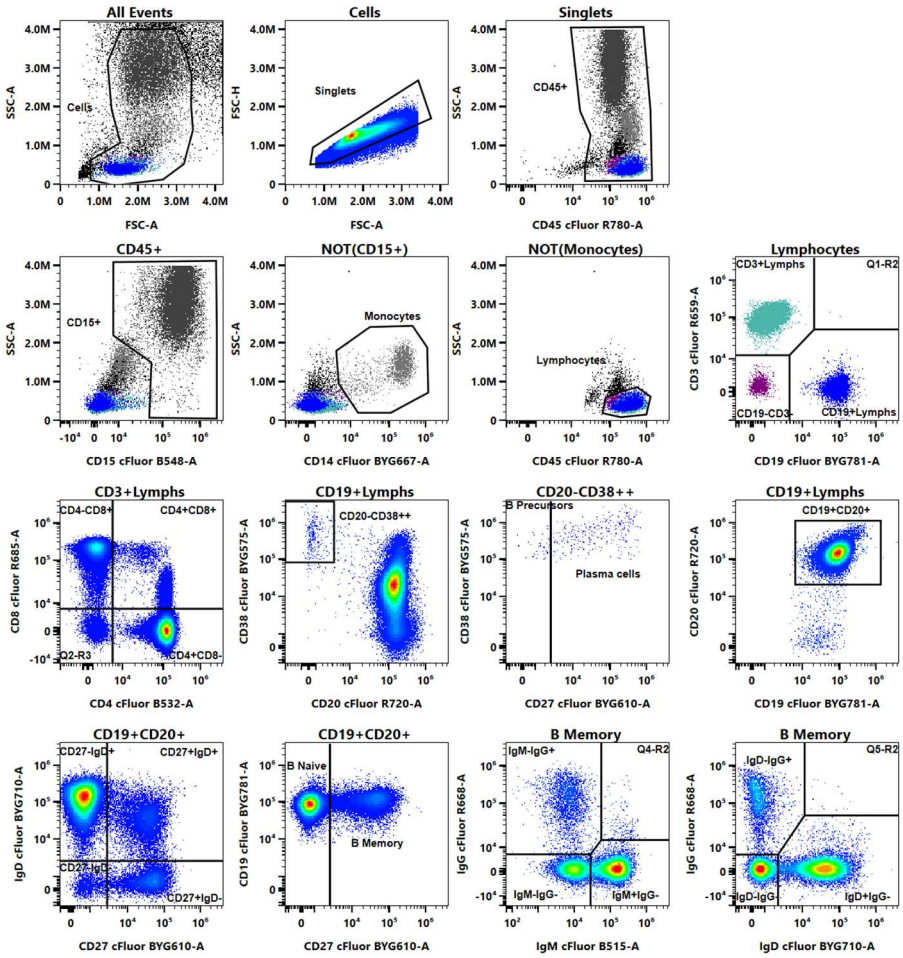


Similarity matrix and Complexity index generated from PBMC sample:

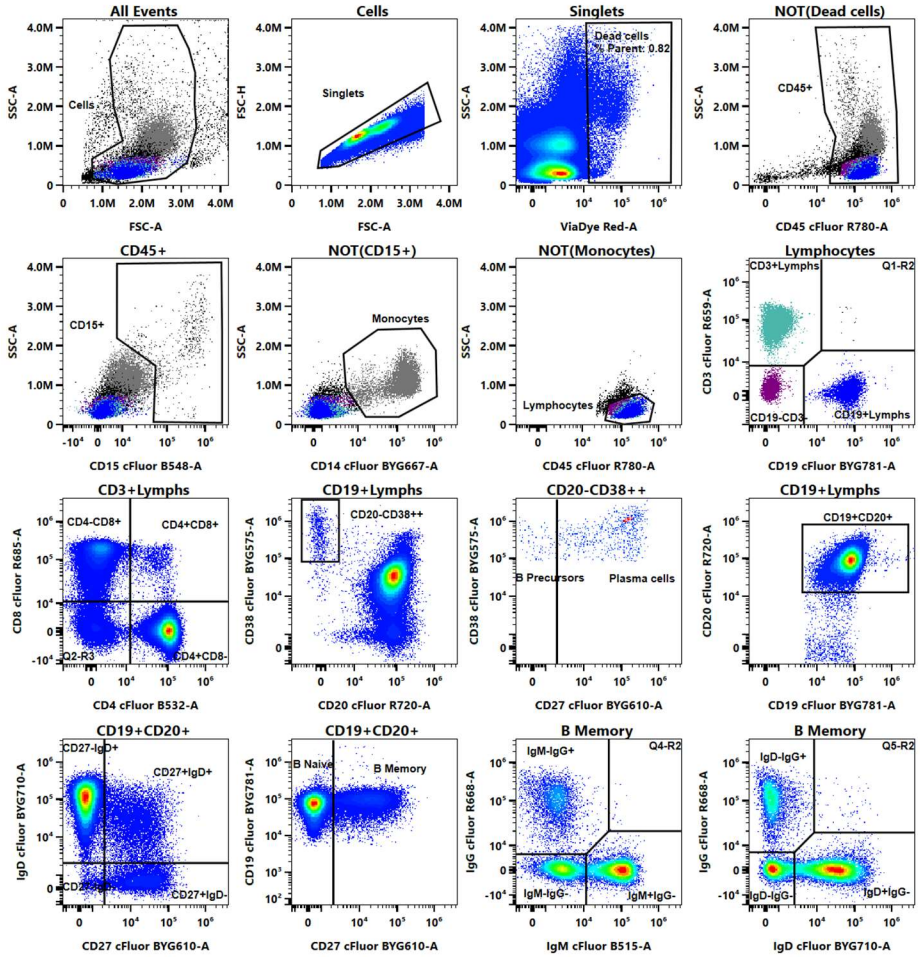


Appendix E: Example of Gating Multicolor Samples

Gating of Populations for Blood acquired on 3 laser Aurora ®



Gating of Populations for PBMC acquired on 3 laser Aurora ®



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