Acquisition Protocol for Cytek® cFluor® MDSC Kit on PBMCs

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Introduction

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

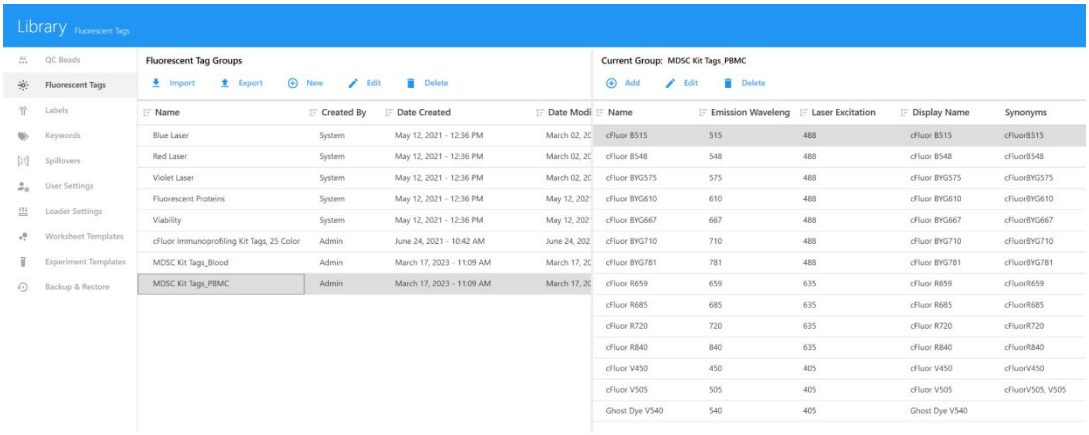
This kit contains 25 tests.

NOTE: *For suggestions on how to prepare human peripheral mononuclear cells and whole blood, see* ***Sample Preparation (PBMCs) Protocol for Cytek® cFluor® MDSC Kit***

Preparing SpectroFlo® Software

Add fluorochromes to the library

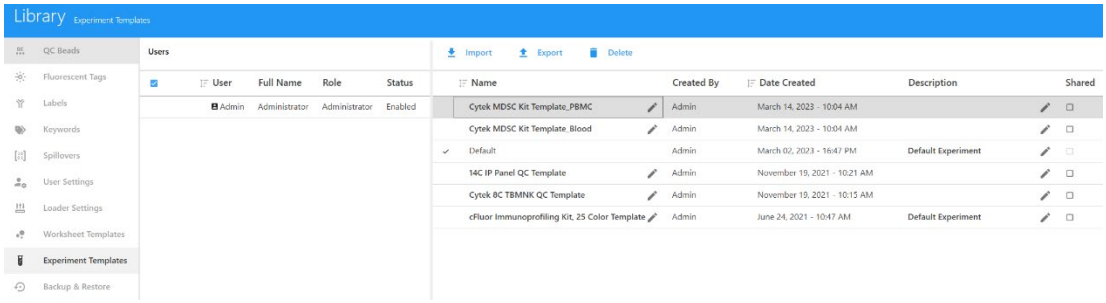
1. Import ***Cytek® MDSC Kit Tags_PBMC***.csv file into the **Library** module, under **Fluorescent Tags** to add the fluorochromes used in this kit.



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

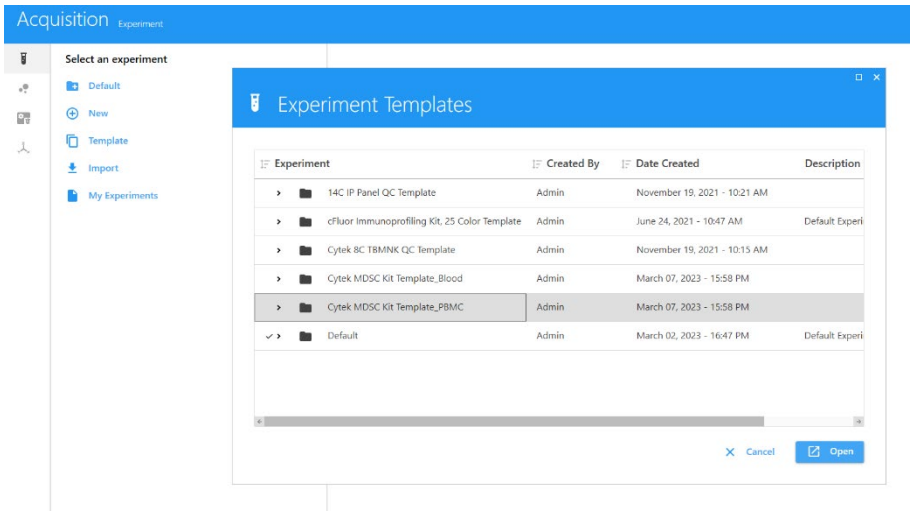
Import the experiment template

1. ***Cytek® MDSC Kit Template_PBMC*** includes a reference group with predefined stopping criteria, assigned marker names, as well as recommended acquisition and analysis worksheets.
2. Import the template into the **Library module**, under **Experiment Templates**.

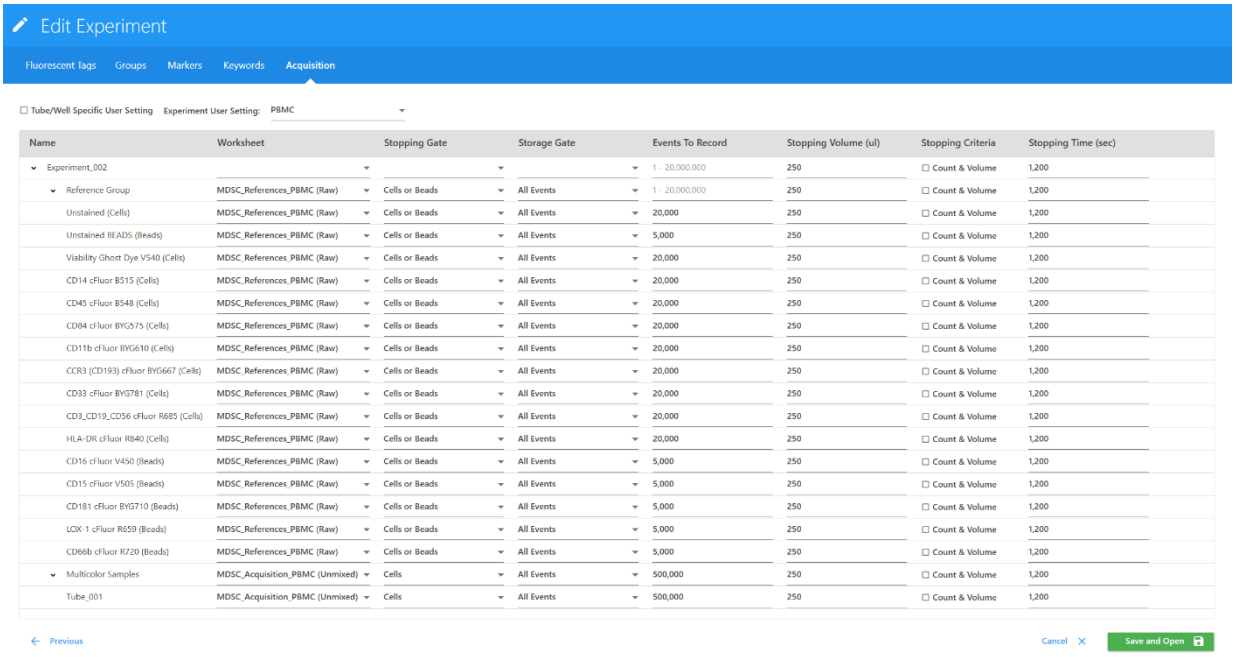


Setting up the Instrument

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 "***Cytek® MDSC Kit Template_PBMC.***" Click ***Open*** and give a name to your experiment.



3. For reference controls it is recommended to run cells for single color controls unless otherwise stated. ***NOTE****: See Table 1 for reference control type recommendations for each marker.*
4. Each sample tube is set to acquire certain number of cells. This can be changed by clicking **Edit**, then changing the Stopping Gate and Stopping Criteria under **Acquisition**.



5. Add tubes and groups as needed. To preserve the predefined acquisition conditions, duplicate the existing tubes or groups.

Table 1. Reference Control Type Recommendations

Laser	Target	Fluorochrome	Recommended Control Type
Violet	CD16	cFluor® V450	Beads
	CD15	cFluor®V505	Beads
	Viability	Ghost Dye™ V540	Cells
Blue	CD14	cFluor® B515	Cells or Beads
	CD45	cFluor® B548	Cells or Beads
	CD84	cFluor® BYG575	Cells or Beads
	CD11b	cFluor® BYG610	Cells or Beads
	CCR3 (CD193)	cFluor® BYG667	Cells or Beads
	CD181	cFluor®BYG710	Beads
	CD33	cFluor® BYG781	Cells or Beads
Red	LOX-1	cFluor® R659	Beads
	CD3	cFluor® R685	Cells or Beads
	CD19		
	CD56		
	CD66b	cFluor® R720	Beads
	HLA-DR	cFluor® R840	Cells or Beads

NOTE: *Recommendations are for use with Cytek® FSP™ CompBeads.*

Acquiring Controls and Samples

Acquire controls in 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 for future use by clicking the* ***Save As*** *button in the Instrument Control window. The gains for all fluorescent parameters are set up with Cytek Assay Setting in the instrument and only FCS, SSC and threshold need to be optimized for specific sample types.*

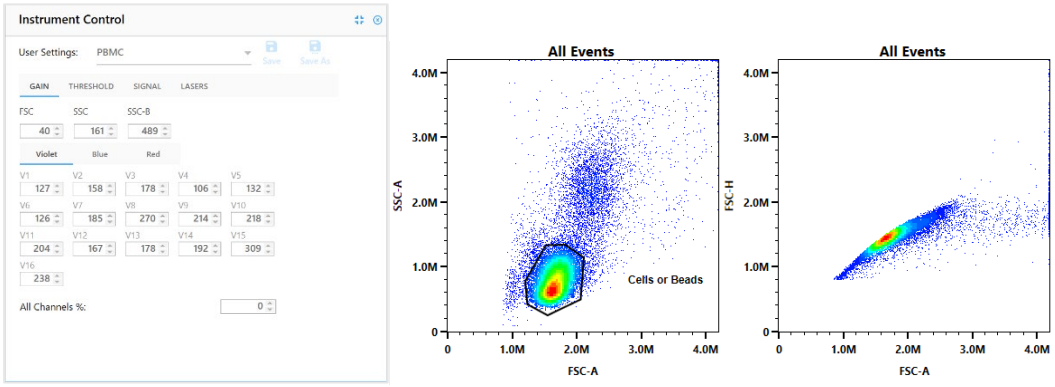
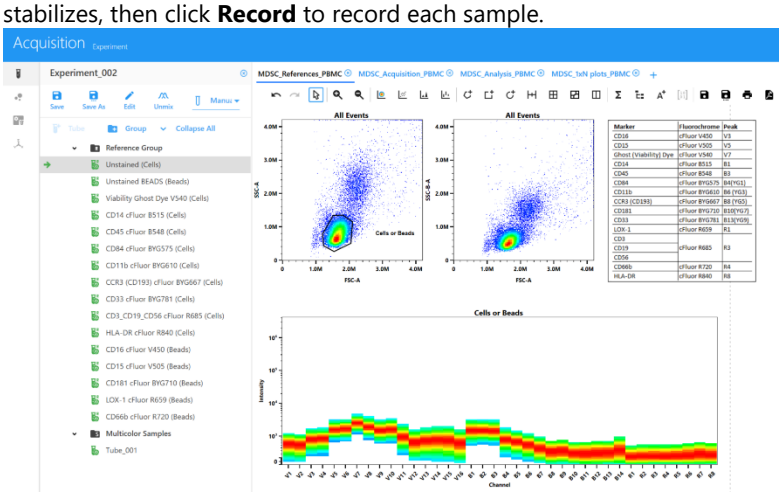


Figure 1: *Example cell resolution of human PBMC samples after FSC and SSC adjustment with threshold set at FSC 50,000.*

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



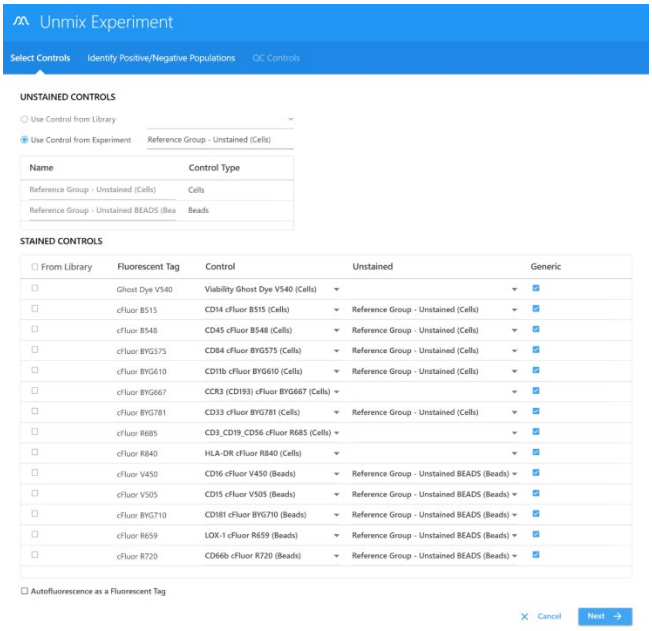
Unmix reference controls

1. Once all controls have been acquired, Click **Unmix**.
   NOTE*: Refer to Appendix A for additional workflows to store and reuse the reference controls.*

2. Under **Select Controls** tab in the Unmix Experiment wizard, ensure that:
   1) Under **UNSTAINED CONTROLS** "Use Control from Experiment" is selected, and the additional **Unstained BEADS** control is included**.**

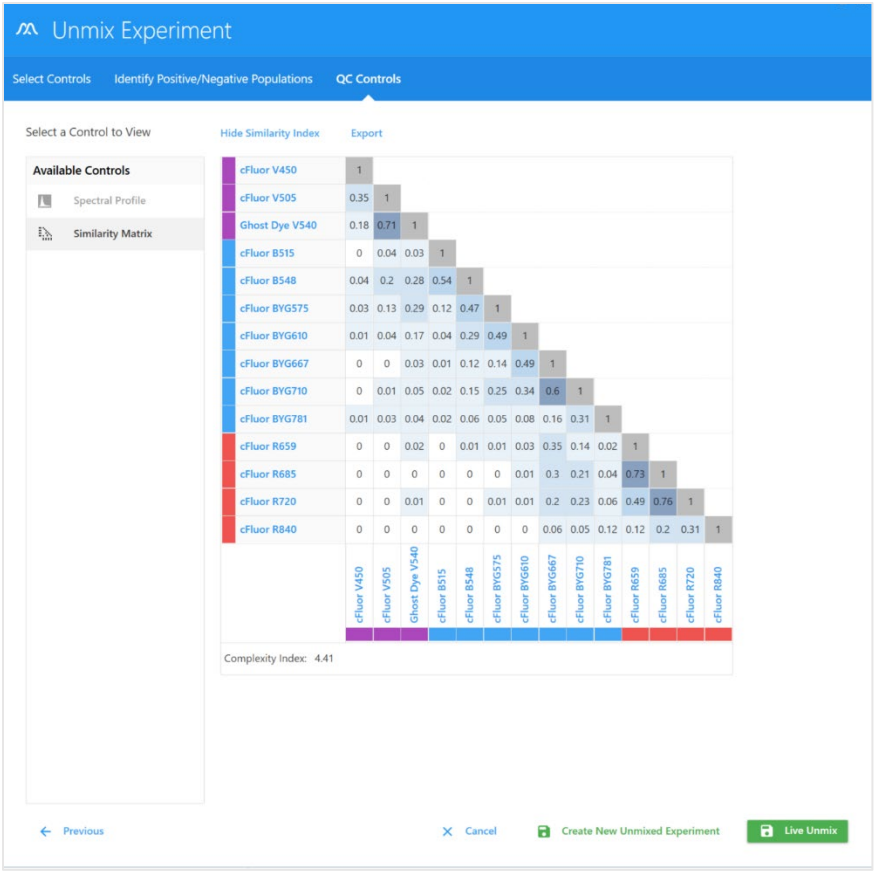
2) Unstained is selected as **Reference Group – Unstained (Cells)** for all markers stained on cells except for the following which should use marker internal negative signal as negative reference: Ghost Dye V540, CD84, CD3/CD19/CD56, HLA-DR, and CCR3 (CD193).
3) For markers LOX-1, CD16, CD15, CD66b, and CD181 Unstained is selected as **Reference Group – Unstained BEADS (Beads)**
4) **Autofluorescence as a Fluorescence Tag** is selected for 5 laser instruments and unselected for 3 laser instruments. Click **Next**.

Note: *Autofluorescence extraction is ALWAYS recommended for data generated in 5-laser systems. For data generated in 3-laser systems (V-B-R), there is a need to assess the level of autofluorescence of the sample by checking the spectrum plot of unstained cells. If autofluorescence is low in unstained cells, as shown in Appendix B, autofluorescence extraction is NOT recommended.*



3. Under **Identify Positive/Negative Populations** tab, ensure for all single color controls that:
   1) Scatter plot is gated on the appropriate population,
   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 B 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 C.



4. Click **Live Unmix**.

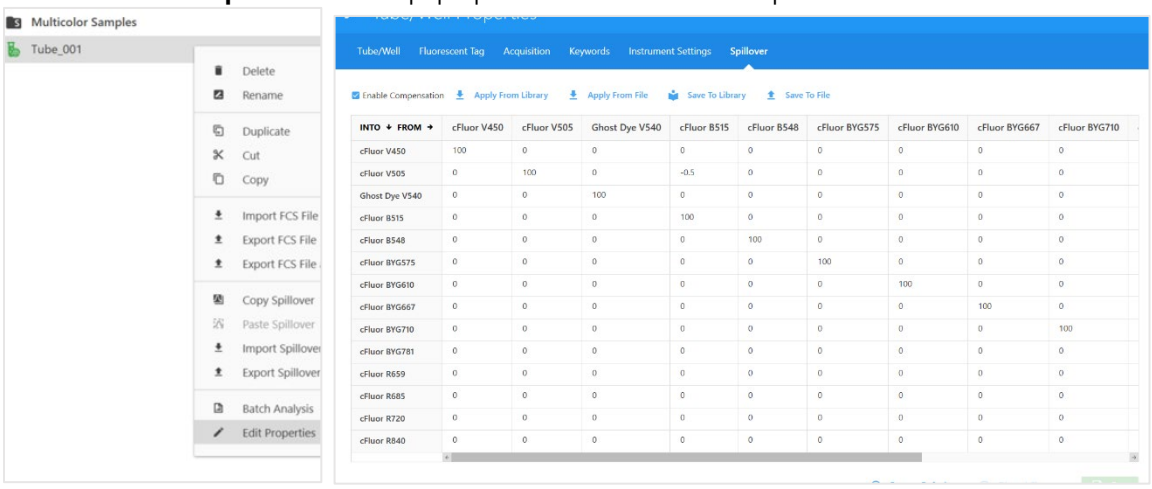
Acquire multicolor samples

1. To acquire multicolor samples, **MDSC_Acquisition_PBMC** worksheet should be selected in the Acquisition tab to collect multicolor samples in the experiment template.
2. Ensure the same settings for the FSC and SSC gains used in the Unstained control are used for the multicolor samples. These FSC and SSC gains can be verified by right clicking on the tube in the experiment, selecting **Edit Properties** and checking on the **Instrument Settings** tab. If needed, the scatter gains can be adjusted.

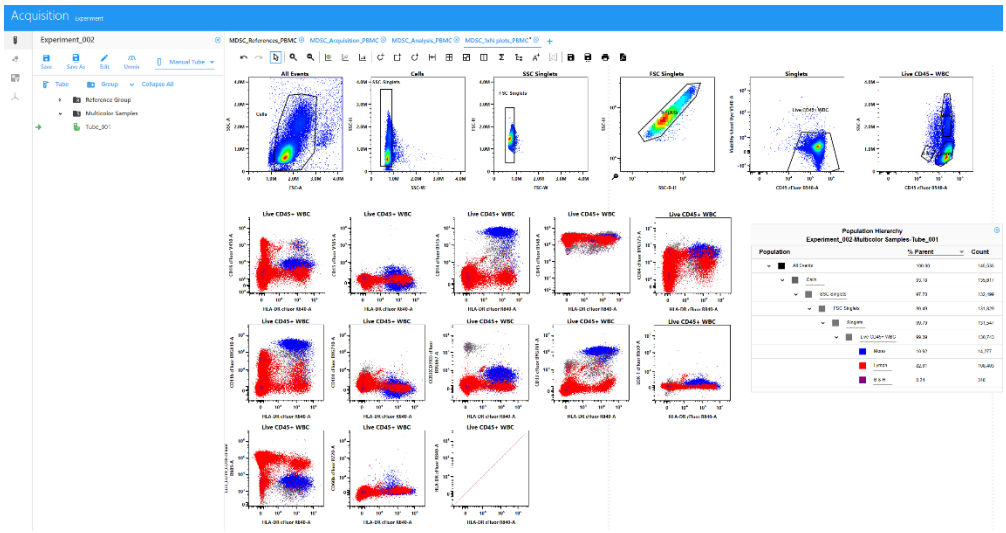
Analyze multicolor samples

3. A **MDSC_Analysis_PBMC** worksheet is included in the experiment template to be used for multicolor sample data analysis. In this worksheet, an additional Time Plot is included as an initial plot to eliminate any abnormal cells (i.e. abnormal cells due to edge effect).
4. Also included in the experiment template is a **MDSC_1xN plots_PBMC** worksheet. Select and acquired multicolor sample and open this worksheet to view the multicolor sample in the worksheet.
5. In case the recorded multicolor samples need spillover adjustment, right click on the multicolor tube. From the drop-down menu, click **Edit Properties**.

6. Click on **Enable Compensation** in the pop-up wizard. Leave the wizard open and move it aside.



7. To check and adjust for unmixing error:
   1) Select all permutation plots in the **MDSC_1xN plots_PBMC** worksheet
   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 13 permutation plots against the first fluorochrome for any unmixing errors and adjust spillover (compensation) as needed. Do this for all 13 fluorochromes by selecting each fluor one by one under **X Axis Parameter**.



NOTE:

Mono: Monocytes;
Lymph: Lymphocytes;
B & H: Basophils & Hematogones

8. To adjust unmixing error of a plot, click **Adjust Spillover** icon in the ribbon menu. Click and drag upward or downward on the plot to make the adjustment (see Figure 2).

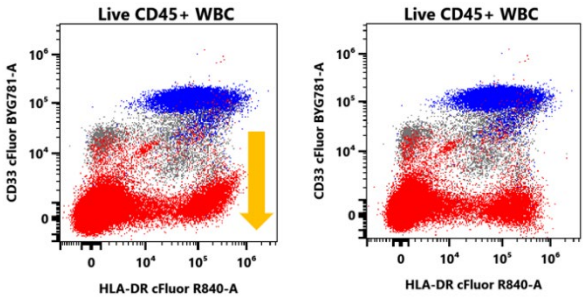


Figure 2: *Example of unmixing error adjustment.*

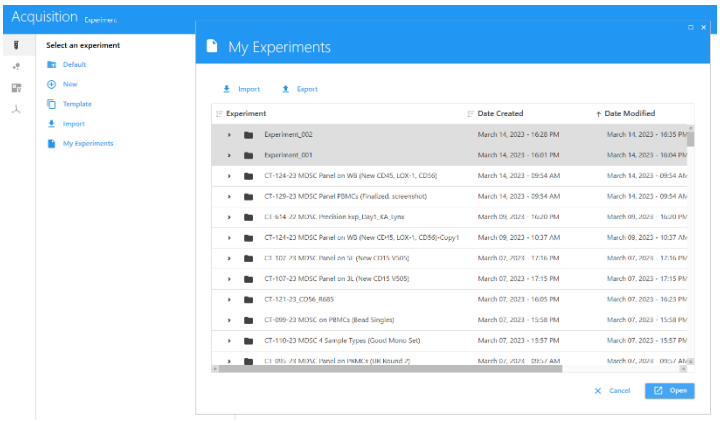
9. Click **Save** and close out of the Tube/Well Properties wizard. The adjusted compensation matrix can be applied to other multicolor samples by copying and pasting Spillover, if similar unmix errors are observed.
10. Repeat the unmixing error adjustment for all multicolor samples as needed
11. Manually adjusted compensation matrix can be saved to library for future use if using SpectroFlo® version 3.0 or higher.

NOTE: *Refer to an example of adjusted spillover matrix in Appendix D.*

12. Double click on the multicolor to open **MDSC_Analysis_PBMC** worksheet to view and adjust gates in the worksheet.

NOTE*: Refer to Appendix E for an example of a PBMC multicolor sample.*

13. 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**.



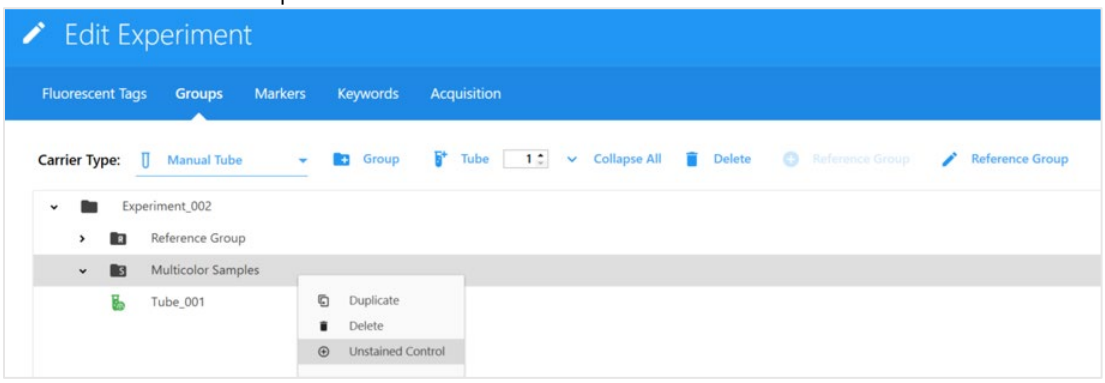
Appendix A: Reusing Single Color Controls

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

NOTE: *For best results, the instrument should be maintained properly, QC performed daily and the same reagent lot used. The samples need to be collected in the same instrument as the reference controls.*

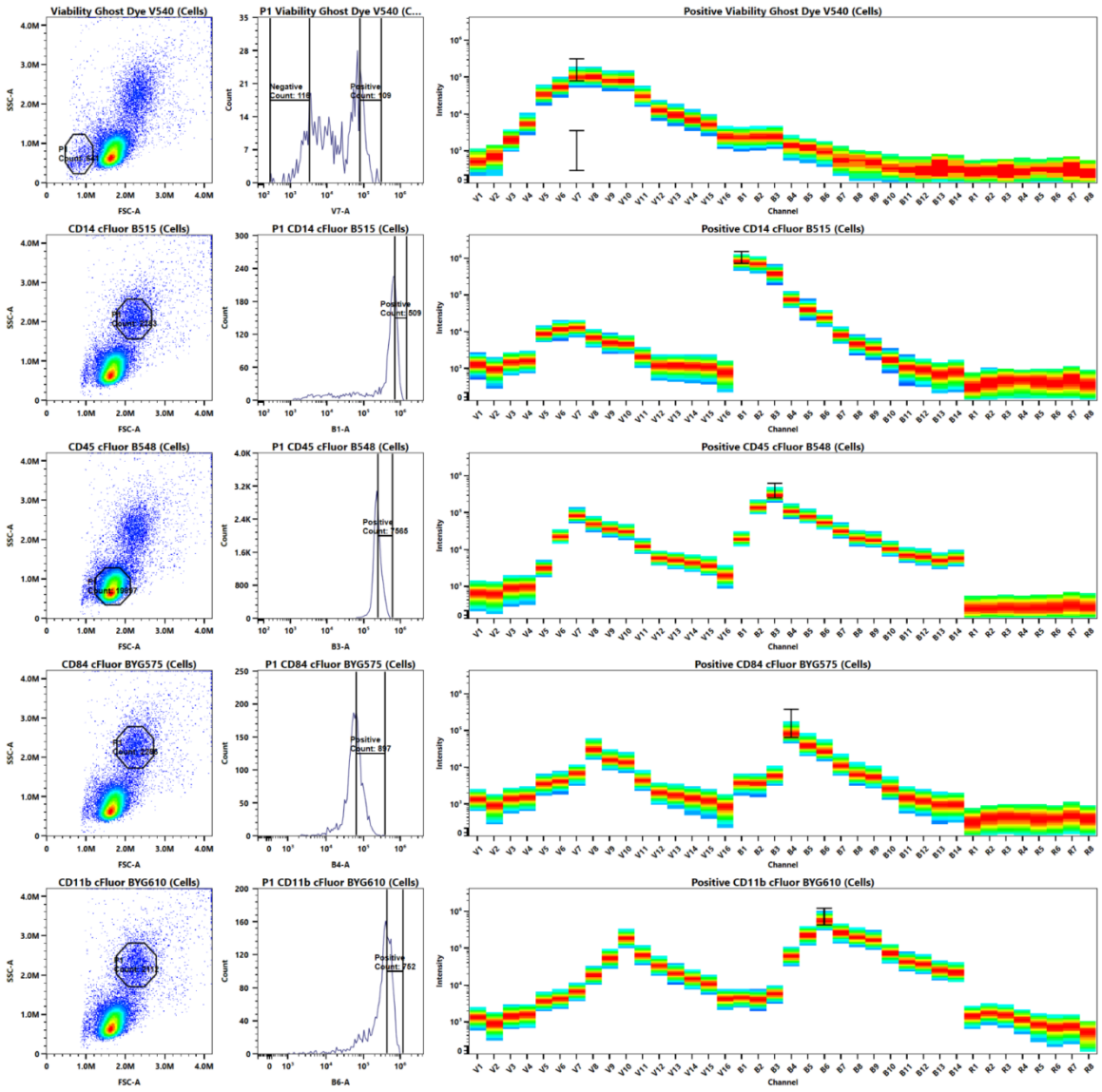
Reusing single color controls from a previous experiment

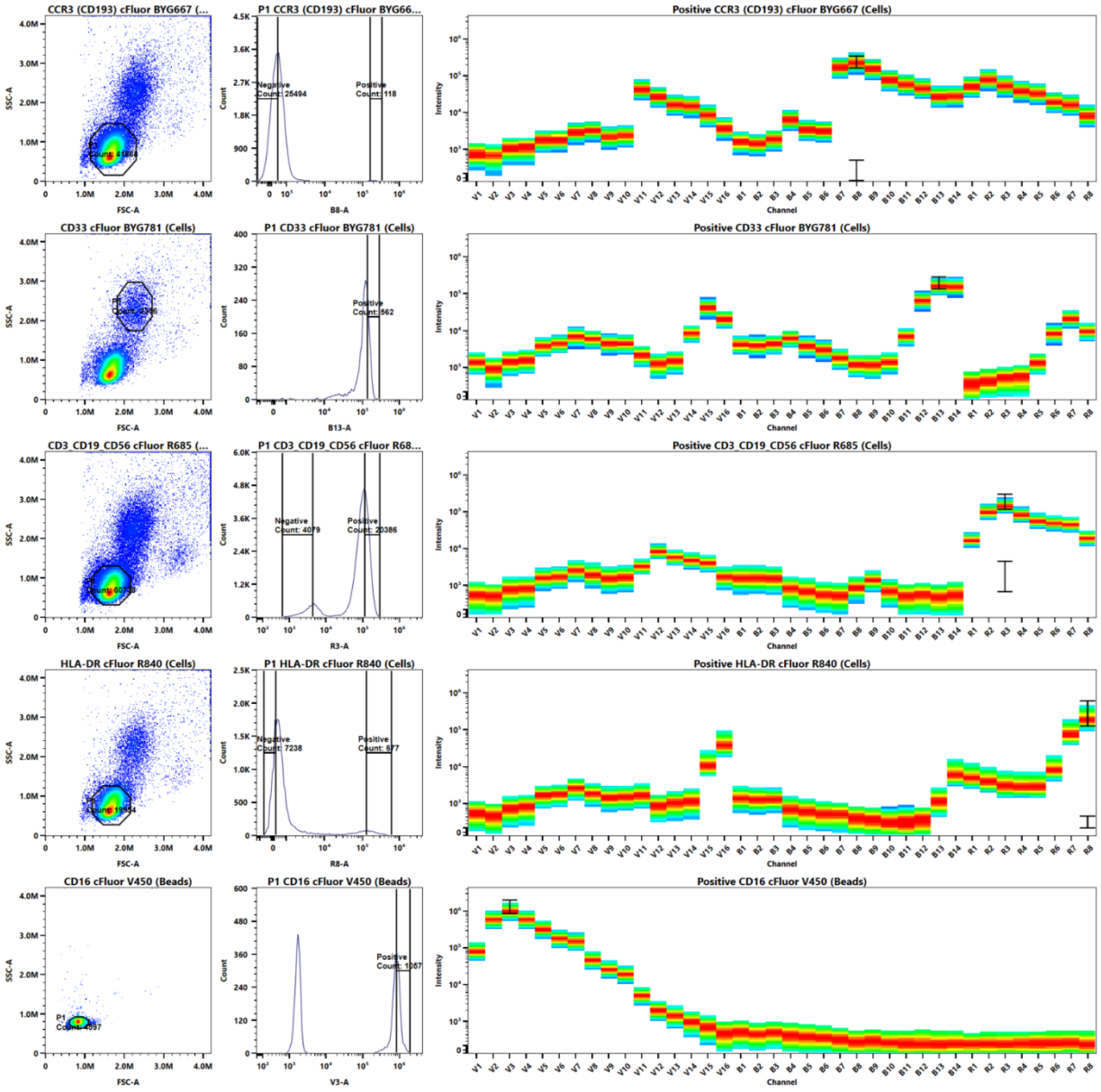
1. From the **Acquisition** module, open **My Experiments**. Right click the saved experiment and select **Duplicate**. This will duplicate the experiment with the reference controls and multicolor samples.
2. Under Multicolor Group, right click on the sample to duplicate or add tubes for the new multicolor samples
3. Delete any old multicolor samples that were carried over with data
4. Click **Edit** to open Edit Experiment wizard
5. Under **Groups**, right click on Multicolor and select **Unstained Control** to add a group specific unstained control for Multicolor Sample

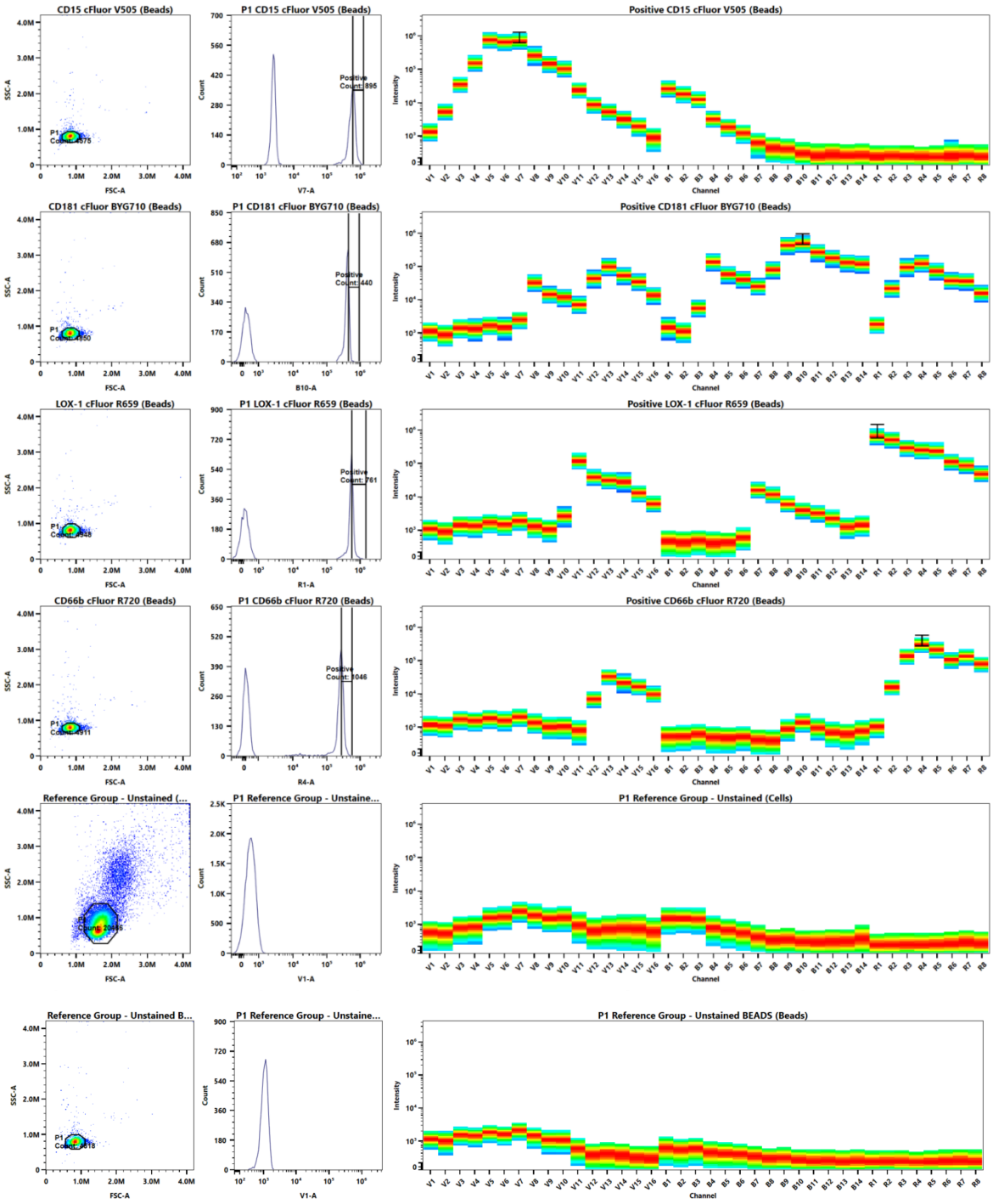


6. Under **Acquisition**, define Worksheet, Stopping Gate, and Events to Record
7. Click **Save and Open**
8. Preview and record the unstained control
9. Live Unmix with **Autofluorescence as a Fluorescence Tag** selected for 5 laser instruments and unselected for 3 laser instruments. Click **Next**.
   Note: *Autofluorescence extraction is ALWAYS recommended for data generated in 5-laser systems. For data generated in 3-laser systems (V-B-R), there is a need to assess the level of autofluorescence of the sample by checking the spectrum plot of unstained cells. If autofluorescence is low in unstained cells, as shown on page 13, autofluorescence extraction is NOT recommended.*
10. Acquire multicolor samples
11. Adjust the gates in the **MDSC_Analysis_PBMC** worksheet as needed.

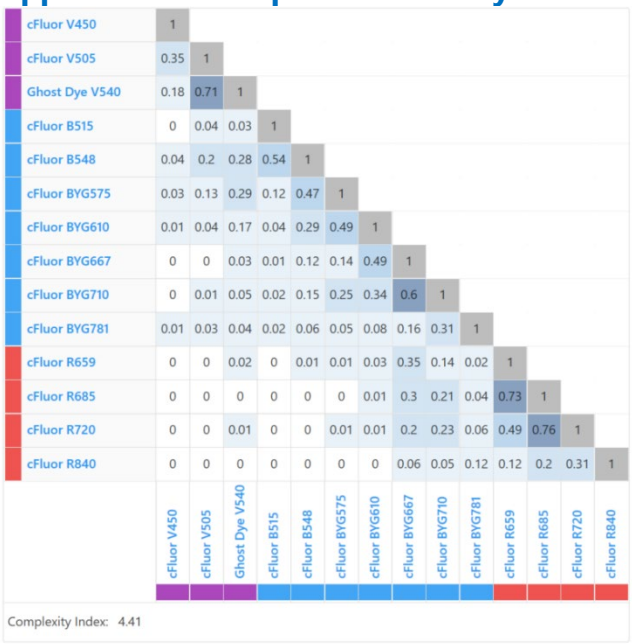
Appendix B: Single Color Control Signatures for 3-Laser (V-B-R) Cytek® Northern Lights™ Suggested Gating When Using Cells as Controls




CCR3 (CD193) cFluor BYG667 (...
P1 CCR3 (CD193) cFluor BYG66...
Negative Count: 25494
Positive Count: 118
Positive CCR3 (CD193) cFluor BYG667 (Cells)
CD33 cFluor BYG781 (Cells)
P1 CD33 cFluor BYG781 (Cells)
Positive Count: 562
Positive CD33 cFluor BYG781 (Cells)
CD3_CD19_CD56 cFluor R685 (...
P1 CD3_CD19_CD56 cFluor R68...
Negative Count: 4079
Positive Count: 20386
Positive CD3_CD19_CD56 cFluor R685 (Cells)
HLA-DR cFluor R840 (Cells)
P1 HLA-DR cFluor R840 (Cells)
Negative Count: 7238
Positive Count: 677
Positive HLA-DR cFluor R840 (Cells)
CD16 cFluor V450 (Beads)
P1 CD16 cFluor V450 (Beads)
Positive Count: 1057
Positive CD16 cFluor V450 (Beads)
SSC-A
FSC-A
Count
Intensity
Channel


CD15 cFluor V505 (Beads)
P1 CD15 cFluor V505 (Beads)
Positive CD15 cFluor V505 (Beads)
CD181 cFluor BYG710 (Beads)
P1 CD181 cFluor BYG710 (Beads)
Positive CD181 cFluor BYG710 (Beads)
LOX-1 cFluor R659 (Beads)
P1 LOX-1 cFluor R659 (Beads)
Positive LOX-1 cFluor R659 (Beads)
CD66b cFluor R720 (Beads)
P1 CD66b cFluor R720 (Beads)
Positive CD66b cFluor R720 (Beads)
Reference Group - Unstained (...
P1 Reference Group - Unstaine...
P1 Reference Group - Unstained (Cells)
Reference Group - Unstained B...
P1 Reference Group - Unstaine...
P1 Reference Group - Unstained BEADS (Beads)
SSC-A
FSC-A
Count
Intensity
Channel

Appendix C: Example of Similarity™ Matrix for Cytek® cFluor® MDSC Kit

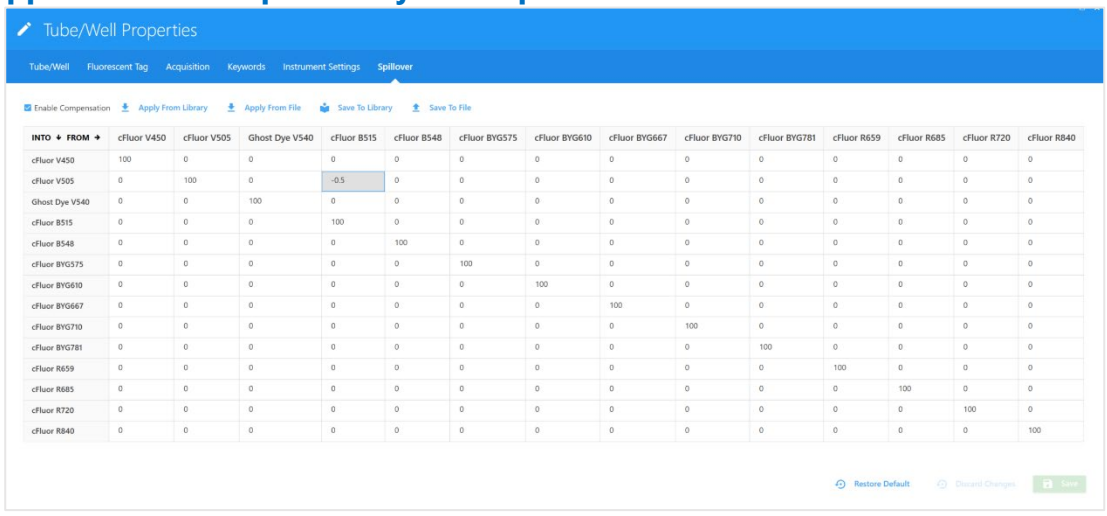


	cFluor V450	cFluor V505	Ghost Dye V540	cFluor B515	cFluor B548	cFluor BYG575	cFluor BYG610	cFluor BYG667	cFluor BYG710	cFluor BYG781	cFluor R659	cFluor R685	cFluor R720	cFluor R840
cFluor V450	1													
cFluor V505	0.35	1												
Ghost Dye V540	0.18	0.71	1											
cFluor B515	0	0.04	0.03	1										
cFluor B548	0.04	0.2	0.28	0.54	1									
cFluor BYG575	0.03	0.13	0.29	0.12	0.47	1								
cFluor BYG610	0.01	0.04	0.17	0.04	0.29	0.49	1							
cFluor BYG667	0	0	0.03	0.01	0.12	0.14	0.49	1						
cFluor BYG710	0	0.01	0.05	0.02	0.15	0.25	0.34	0.6	1					
cFluor BYG781	0.01	0.03	0.04	0.02	0.06	0.05	0.08	0.16	0.31	1				
cFluor R659	0	0	0.02	0	0.01	0.01	0.03	0.35	0.14	0.02	1			
cFluor R685	0	0	0	0	0	0	0.01	0.3	0.21	0.04	0.73	1		
cFluor R720	0	0	0.01	0	0	0.01	0.01	0.2	0.23	0.06	0.49	0.76	1	
cFluor R840	0	0	0	0	0	0	0	0.06	0.05	0.12	0.12	0.2	0.31	1

Complexity Index: 4.41

Figure 3: *Expected Similarity™ matrix and Complexity™ index generated on a 3-laser (V-B-R) Cytek® Northern Lights™ system. Generating similar values is a good indication that signatures of your single color controls match those generated by Cytek® Biosciences, Inc.*

Appendix D: Example of Adjusted Spillover Matrix

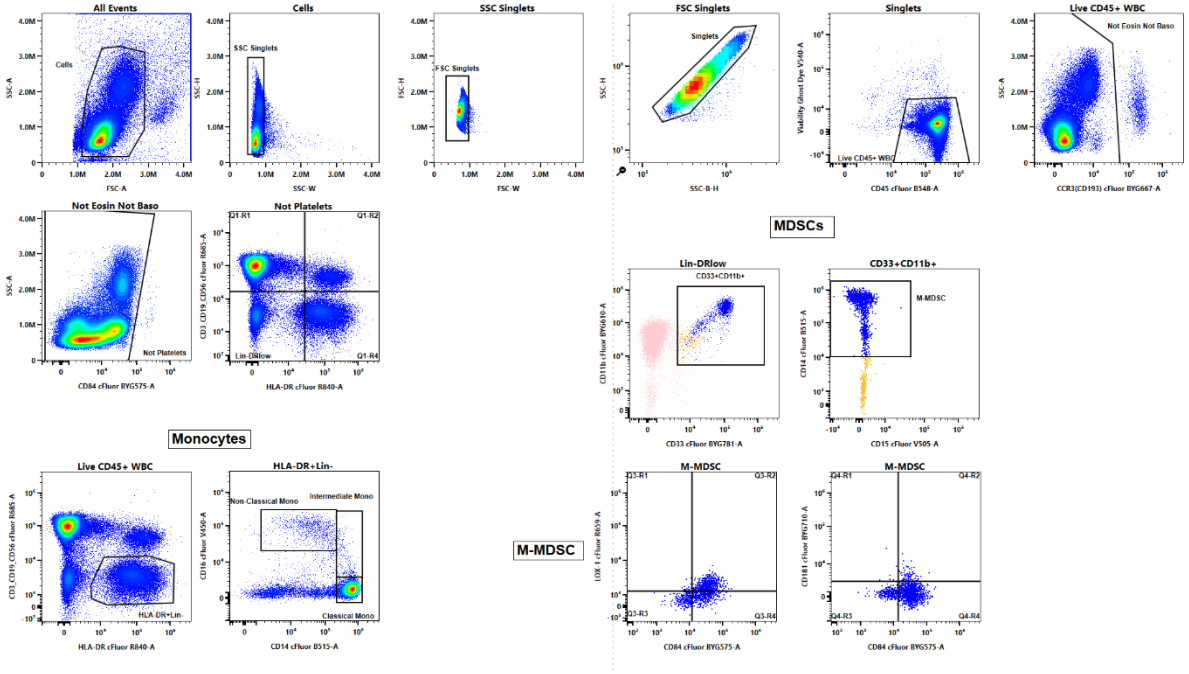


INTO ↓ FROM →	cFluor V450	cFluor V505	Ghost Dye V540	cFluor B515	cFluor B548	cFluor BYG575	cFluor BYG610	cFluor BYG667	cFluor BYG710	cFluor BYG781	cFluor R659	cFluor R685	cFluor R720	cFluor R840
cFluor V450	100	0	0	0	0	0	0	0	0	0	0	0	0	0
cFluor V505	0	100	0	-0.5	0	0	0	0	0	0	0	0	0	0
Ghost Dye V540	0	0	100	0	0	0	0	0	0	0	0	0	0	0
cFluor B515	0	0	0	100	0	0	0	0	0	0	0	0	0	0
cFluor B548	0	0	0	0	100	0	0	0	0	0	0	0	0	0
cFluor BYG575	0	0	0	0	0	100	0	0	0	0	0	0	0	0
cFluor BYG610	0	0	0	0	0	0	100	0	0	0	0	0	0	0
cFluor BYG667	0	0	0	0	0	0	0	100	0	0	0	0	0	0
cFluor BYG710	0	0	0	0	0	0	0	0	100	0	0	0	0	0
cFluor BYG781	0	0	0	0	0	0	0	0	0	100	0	0	0	0
cFluor R659	0	0	0	0	0	0	0	0	0	0	100	0	0	0
cFluor R685	0	0	0	0	0	0	0	0	0	0	0	100	0	0
cFluor R720	0	0	0	0	0	0	0	0	0	0	0	0	100	0
cFluor R840	0	0	0	0	0	0	0	0	0	0	0	0	0	100

+

Figure 4: *Corrections made by checking NxN permutation plots for 14 fluorochromes. Please note that all needed corrections are below 3%. If corrections larger than 5% need to be applied, carefully QC your reference controls, comparing the staining pattern and gating positioning provided in Appendix B.*

Appendix E: Example of Gating in Multicolor Sample on PBMCs



NOTE:

SSC-W vs SSC-H and FSC-W vs FSC-H plots are set to remove aggregates;
SSC-B-H vs SSC-H plot in log scale removes red blood cell lysis debris;
Not Eosin Not Baso: Not Eosinophils Not Basophils;
Non-Classical Mono, Intermediate Mono, Classical Mono: Non-Classical Monocytes, Intermediate Monocytes, Classical Monocytes

cFluor® B515, cFluor® B548, cFluor® R685, cFluor® R720, and cFluor® R840 are equivalent to CF®488A, CF®514, CF®660C, CF®700, and APC-CF®790T respectively, manufactured and provided by Biotium, Inc. under an Agreement between Biotium and Cytek (LICENSEE). The manufacture, use, sale, offer for sale, or import of the product is covered by one or more of the patents or pending applications owned or licensed by Biotium. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim, no right to perform any patented method, and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel.

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