



Acquisition Protocol for Cytek® cFluor® MDSC Kit on Whole Blood

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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 (Whole Blood) Protocol for Cytek® cFluor® MDSC Kit**

Preparing SpectroFlo® Software

Add fluorochromes to the library

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

Name	Created By	Date Created	Date Modified	Emission Wavelength	Laser Excitation	Display Name	Synonyms
Blue Laser	System	May 12, 2021 - 12:36 PM	March 02, 2023	cFluor B515	488	cFluor B515	cFluorB515
Red Laser	System	May 12, 2021 - 12:36 PM	March 02, 2023	cFluor B548	488	cFluor B548	cFluorB548
Violet Laser	System	May 12, 2021 - 12:36 PM	March 02, 2023	cFluor BYG575	488	cFluor BYG575	cFluorBYG575
Fluorescent Proteins	System	May 12, 2021 - 12:36 PM	May 12, 2022	cFluor BYG610	488	cFluor BYG610	cFluorBYG610
Viability	System	May 12, 2021 - 12:36 PM	May 12, 2022	cFluor BYG667	488	cFluor BYG667	cFluorBYG667
cFluor Immunoprofiling Kit Tags, 25 Color	Admin	June 24, 2021 - 10:42 AM	June 24, 2022	cFluor BYG710	488	cFluor BYG710	cFluorBYG710
MDSC Kit Tags_Blood	Admin	March 17, 2023 - 11:09 AM	March 17, 2023	cFluor BYG781	488	cFluor BYG781	cFluorBYG781
MDSC Kit Tags_PBMC	Admin	March 17, 2023 - 11:09 AM	March 17, 2023	cFluor R659	635	cFluor R659	cFluorR659
				cFluor R685	635	cFluor R685	cFluorR685
				cFluor R720	635	cFluor R720	cFluorR720
				cFluor R840	635	cFluor R840	cFluorR840
				cFluor V450	405	cFluor V450	cFluorV450
				cFluor V505	405	cFluor V505	cFluorV505, V505

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.

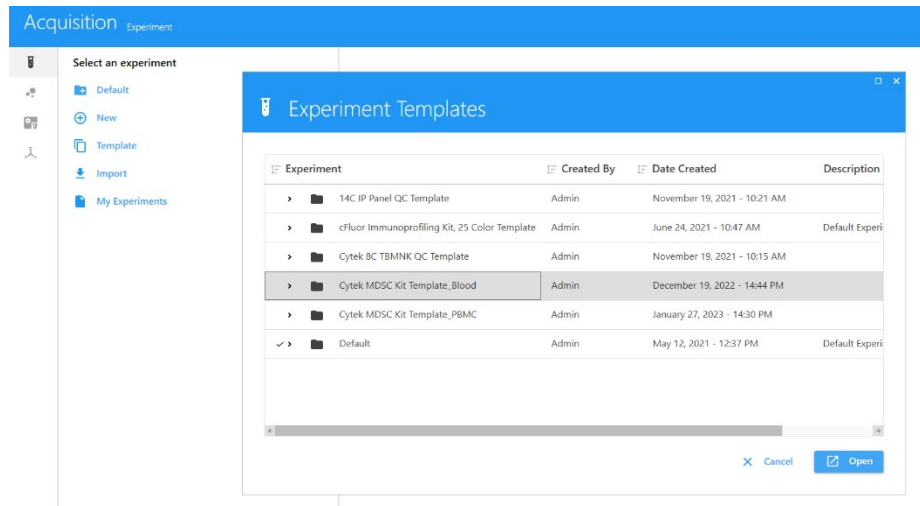
Import the experiment template

1. **Cytek® MDSC Kit Template_Blood** 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**.

Name	Created By	Date Created	Description	Shared
Cytek MDSC Kit Template_Blood	Admin	March 07, 2023 - 15:58 PM		<input type="checkbox"/>
Cytek MDSC Kit Template_PBMC	Admin	March 07, 2023 - 15:58 PM		<input type="checkbox"/>
Default	Admin	March 02, 2023 - 16:47 PM	Default Experiment	<input type="checkbox"/>
14C IP Panel QC Template	Admin	November 19, 2021 - 10:21 AM		<input type="checkbox"/>
Cytek 8C TBMNK QC Template	Admin	November 19, 2021 - 10:15 AM		<input type="checkbox"/>
cFluor Immunoprofiling Kit, 25 Color Template	Admin	June 24, 2021 - 10:47 AM	Default Experiment	<input type="checkbox"/>

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_Blood**". 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 a certain number of cells. This can be changed by clicking **Edit**, then changing the Stopping Gate and Stopping Criteria under **Acquisition**.

Name	Worksheet	Stopping Gate	Storage Gate	Events To Record	Stopping Volume (uL)	Stopping Criteria	Stopping Time (sec)
Experiment_001							
Reference Group	MDSC_References_Blood (Raw)	Cells or Beads	All Events	1 - 20,000,000	250	<input type="checkbox"/> Count & Volume	1,200
Unstained (C-19)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	1 - 20,000,000	250	<input type="checkbox"/> Count & Volume	1,200
Unstained #445 (Beads)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	25,000	250	<input type="checkbox"/> Count & Volume	1,200
CD15 eFluor V450 (Cells)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	5,000	250	<input type="checkbox"/> Count & Volume	1,200
CD15 eFluor V355 (Cells)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	20,000	250	<input type="checkbox"/> Count & Volume	1,200
CD14 eFluor R515 (Cells)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	20,000	250	<input type="checkbox"/> Count & Volume	1,200
CD44 eFluor R548 (Cells)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	20,000	250	<input type="checkbox"/> Count & Volume	1,200
CD94 eFluor R5275 (Cells)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	20,000	250	<input type="checkbox"/> Count & Volume	1,200
CD11b eFluor R5210 (Cells)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	20,000	250	<input type="checkbox"/> Count & Volume	1,200
CD3 (CD19)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	20,000	250	<input type="checkbox"/> Count & Volume	1,200
CD11b eFluor R519 (Cells)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	20,000	250	<input type="checkbox"/> Count & Volume	1,200
CD33 eFluor R5278 (Cells)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	20,000	250	<input type="checkbox"/> Count & Volume	1,200
CD3_CD19_CD58 eFluor R685 (Cells)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	20,000	250	<input type="checkbox"/> Count & Volume	1,200
CD68b eFluor R720 (Cells)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	20,000	250	<input type="checkbox"/> Count & Volume	1,200
Hi A (K eFluor R640 (Cells)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	20,000	250	<input type="checkbox"/> Count & Volume	1,200
LOK-1 eFluor R659 (Beads)	MDSC_References_Blood (Raw)	Cells or Beads	All Events	5,000	250	<input type="checkbox"/> Count & Volume	1,200
MultiColor Samples	MDSC_Acquisition_Blood (Unmixed)	Cells	All Events	500,000	250	<input type="checkbox"/> Count & Volume	1,200
Tube_001	MDSC_Acquisition_Blood (Unmixed)	Cells	All Events	500,000	250	<input type="checkbox"/> Count & Volume	1,200

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	Cells or Beads
	CD15	cFluor® V505	Cells or Beads
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	Cells or Beads
	CD33	cFluor® BYG781	Cells or Beads
Red	LOX-1	cFluor® R659	Beads
	CD3	cFluor® R685	Cells or Beads
	CD19		
	CD56		
	CD66b	cFluor® R720	Cells or 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, adjust 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.

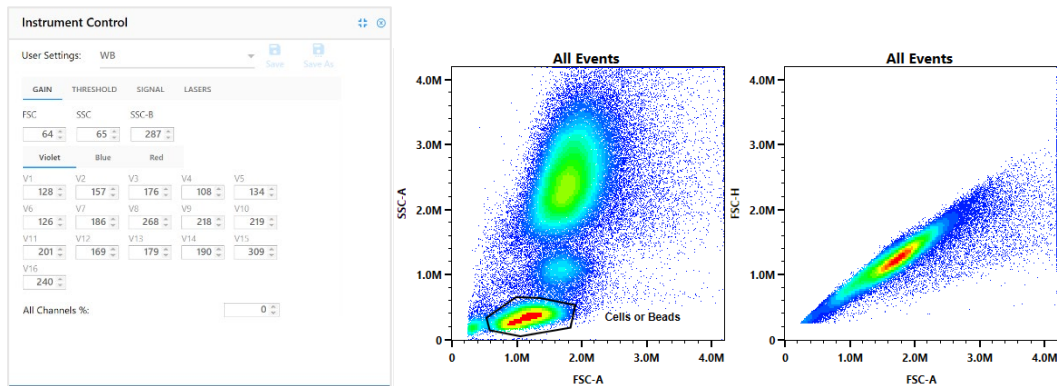
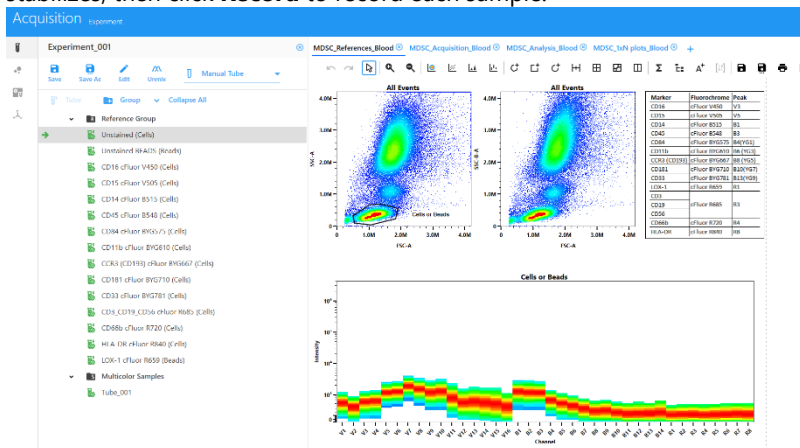


Figure 1: Example cell resolution of human blood samples after FSC and SSC adjustment with threshold set at FSC 250,000.

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

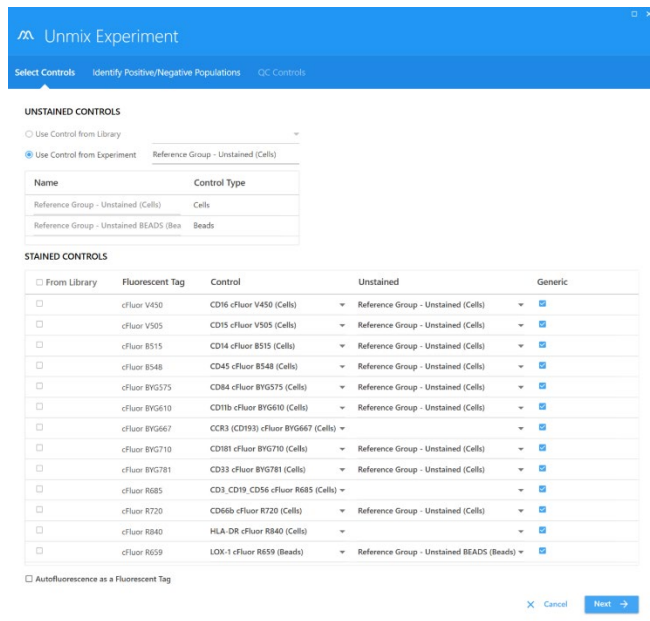


Unmix reference controls

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: CD84, CD3/CD19/CD56, HLA-DR, and CCR3 (CD193).
- 3) For marker LOX-1, 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.



UNSTAINED CONTROLS

Use Control from Library

Use Control from Experiment Reference Group - Unstained (Cells)

Name	Control Type
Reference Group - Unstained (Cells)	Cells
Reference Group - Unstained BEADS (Beads)	Beads

STAINED CONTROLS

From Library

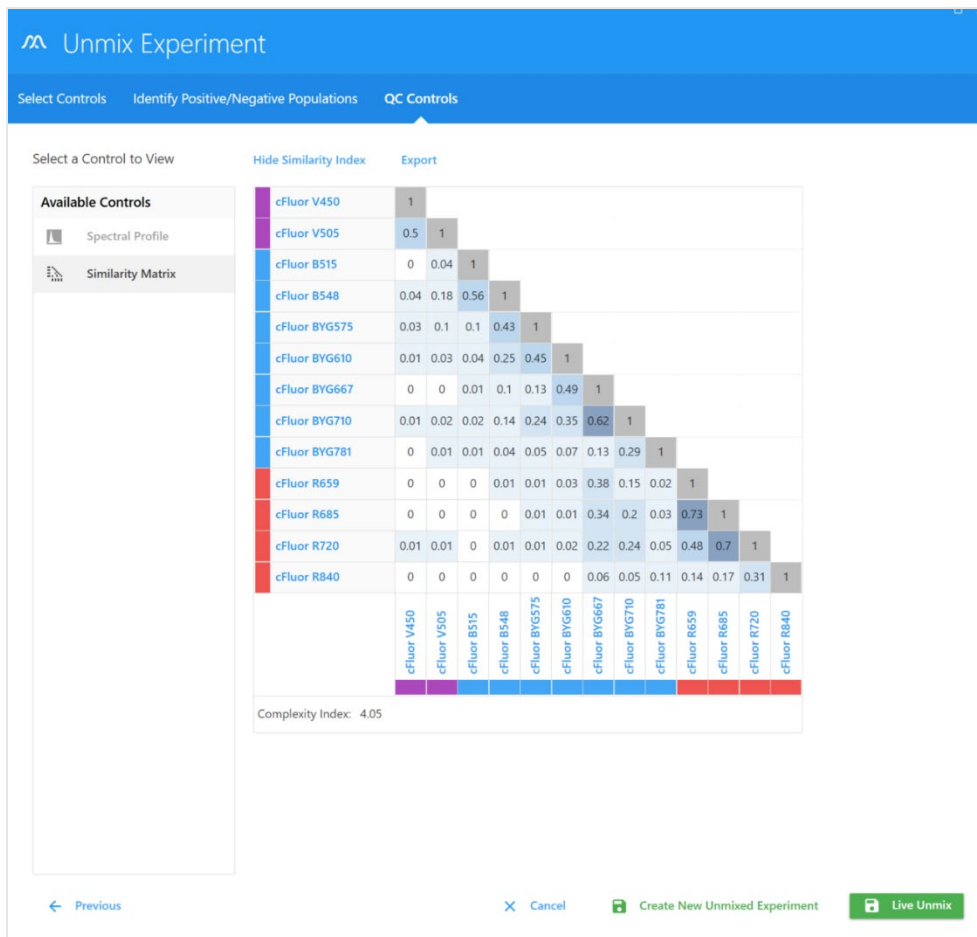
Fluorescent Tag	Control	Unstained	Generic
<input type="checkbox"/> cFluor V450	CD16 cFluor V450 (Cells)	Reference Group - Unstained (Cells)	<input checked="" type="checkbox"/>
<input type="checkbox"/> cFluor V505	CD15 cFluor V505 (Cells)	Reference Group - Unstained (Cells)	<input checked="" type="checkbox"/>
<input type="checkbox"/> cFluor B515	CD14 cFluor B515 (Cells)	Reference Group - Unstained (Cells)	<input checked="" type="checkbox"/>
<input type="checkbox"/> cFluor B548	CD45 cFluor B548 (Cells)	Reference Group - Unstained (Cells)	<input checked="" type="checkbox"/>
<input type="checkbox"/> cFluor BYG575	CD84 cFluor BYG575 (Cells)	Reference Group - Unstained (Cells)	<input checked="" type="checkbox"/>
<input type="checkbox"/> cFluor BYG610	CD11b cFluor BYG610 (Cells)	Reference Group - Unstained (Cells)	<input checked="" type="checkbox"/>
<input type="checkbox"/> cFluor BYG667	CCR3 (CD193) cFluor BYG667 (Cells)		<input checked="" type="checkbox"/>
<input type="checkbox"/> cFluor BYG710	CD181 cFluor BYG710 (Cells)	Reference Group - Unstained (Cells)	<input checked="" type="checkbox"/>
<input type="checkbox"/> cFluor BYG781	CD33 cFluor BYG781 (Cells)	Reference Group - Unstained (Cells)	<input checked="" type="checkbox"/>
<input type="checkbox"/> cFluor R685	CD3, CD19, CD56 cFluor R685 (Cells)		<input checked="" type="checkbox"/>
<input type="checkbox"/> cFluor R720	CD66b cFluor R720 (Cells)	Reference Group - Unstained (Cells)	<input checked="" type="checkbox"/>
<input type="checkbox"/> cFluor R840	HLA-DR cFluor R840 (Cells)		<input checked="" type="checkbox"/>
<input type="checkbox"/> cFluor R659	LOX-1 cFluor R659 (Beads)	Reference Group - Unstained BEADS (Beads)	<input checked="" type="checkbox"/>

Autofluorescence as a Fluorescent Tag

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**.

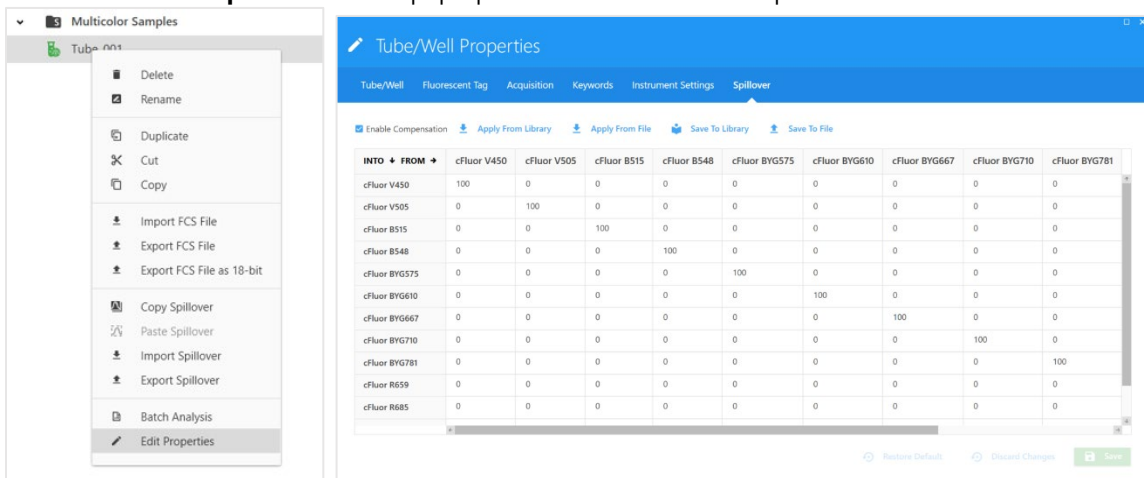
Acquire multicolor samples

1. To acquire multicolor samples, **MDSC_Acquisition_Blood** 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.

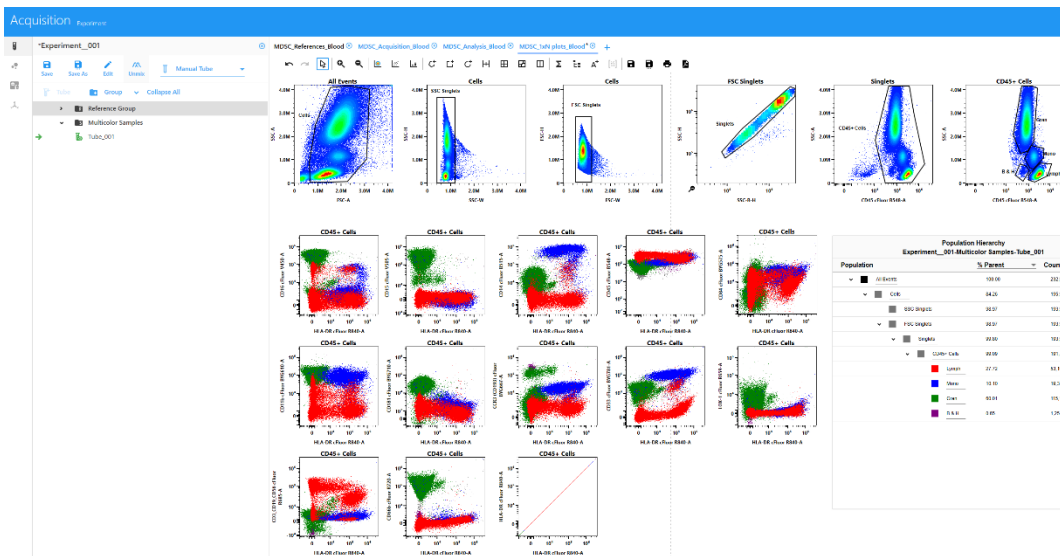
Analyze multicolor samples

1. A **MDSC_Analysis_Blood** 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 exclude areas from the acquisition period where signal instability is observed.
2. Also included in the experiment template is a **MDSC_1xN plots_Blood** worksheet. Select an acquired multicolor sample and open this worksheet to view the multicolor sample in the worksheet.
3. In case the recorded multicolor samples need spillover adjustment, right click on the multicolor tube. From the drop-down menu, click **Edit Properties**.

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



- To check and adjust for unmixing error:
 - Select all permutation plots in the **MDSC_1xN plots_Blood** worksheet,
 - Right click and select **Properties** from the drop-down menu
 - From **Plot Properties**, select the first fluorochrome under **X Axis Parameter**, and
 - 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:

*Gran: Granulocytes;
Mono: Monocytes;
Lymph: Lymphocytes;
B & H: Basophils & Hematogones*

- 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).

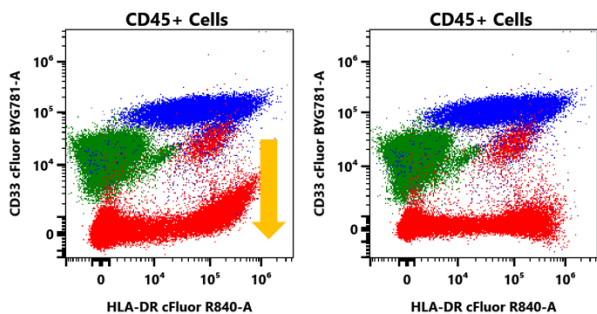


Figure 2: Example of unmixing error adjustment.

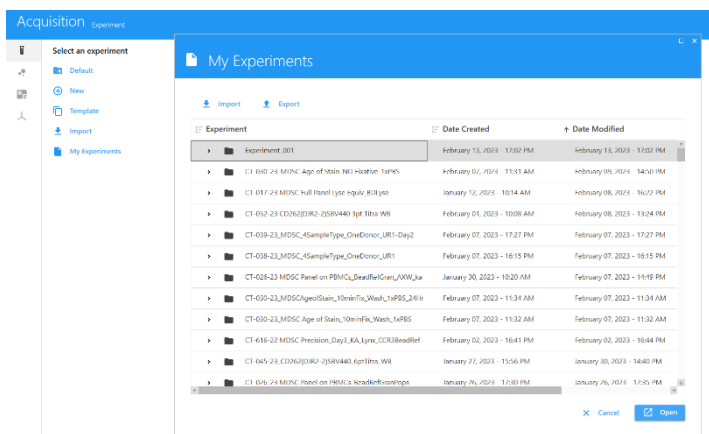
7. Click **Save** and close out of the Tube/Well Properties wizard. The adjusted compensation matrix can be applied to other multicolor samples by copying and pasting Spillover, if similar unmix errors are observed.
8. Repeat the unmixing error adjustment for all multicolor samples as needed
9. Manually 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.

10. Double click on the multicolor to open **MDSC_Acquisition_Blood** worksheet to view and adjust gates in the worksheet.

NOTE: Refer to Appendix E for an example of a human blood multicolor sample.

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**.



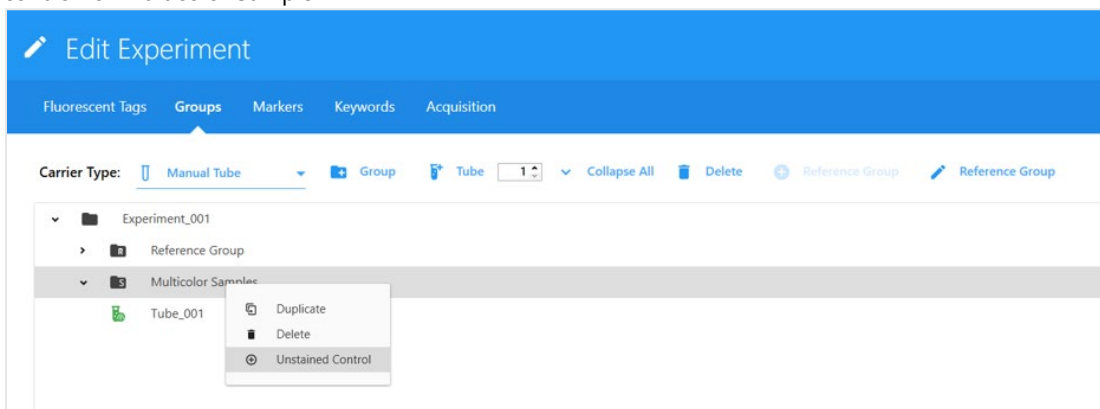
Appendix A: Reusing Single Color Controls

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

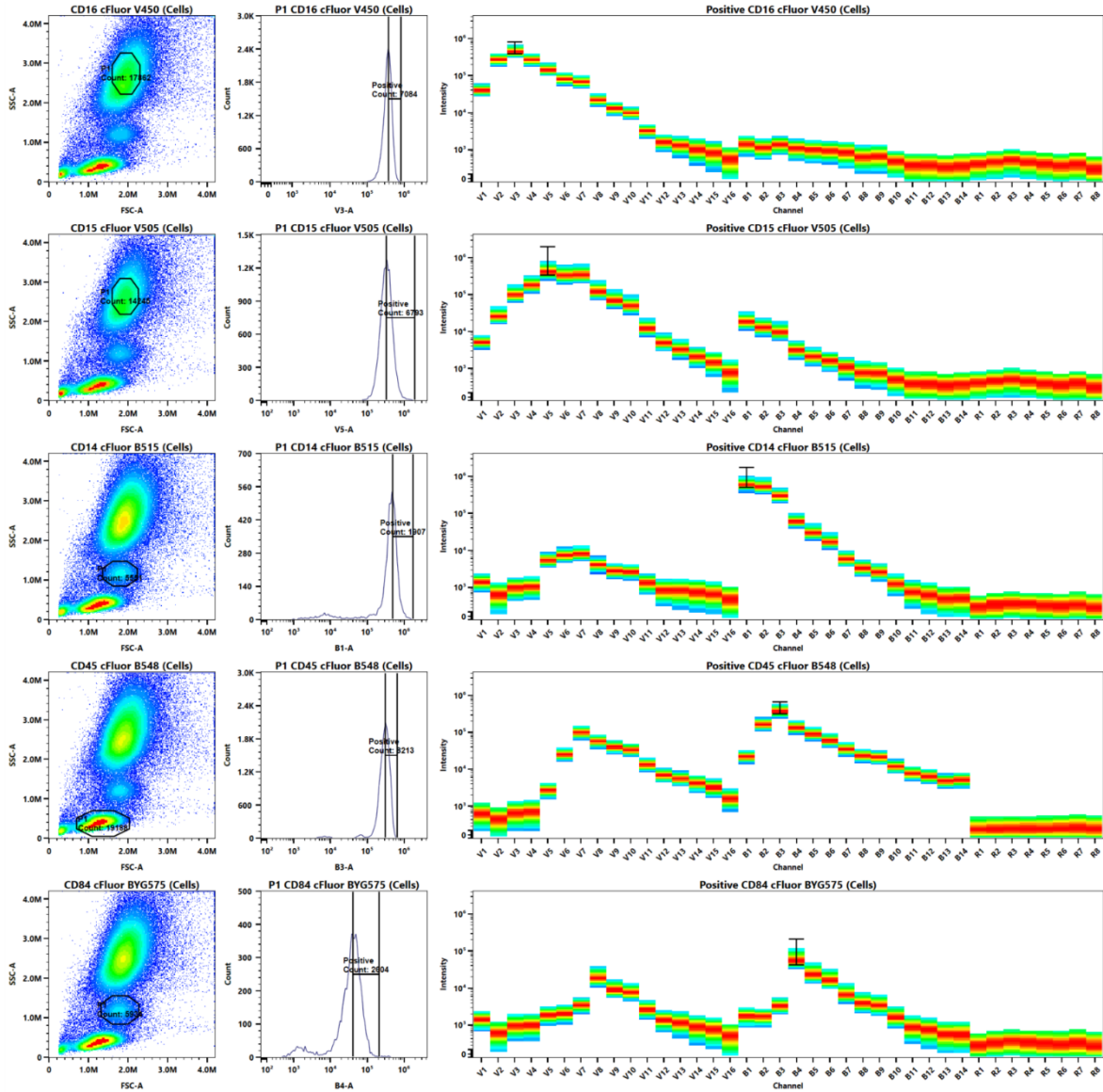


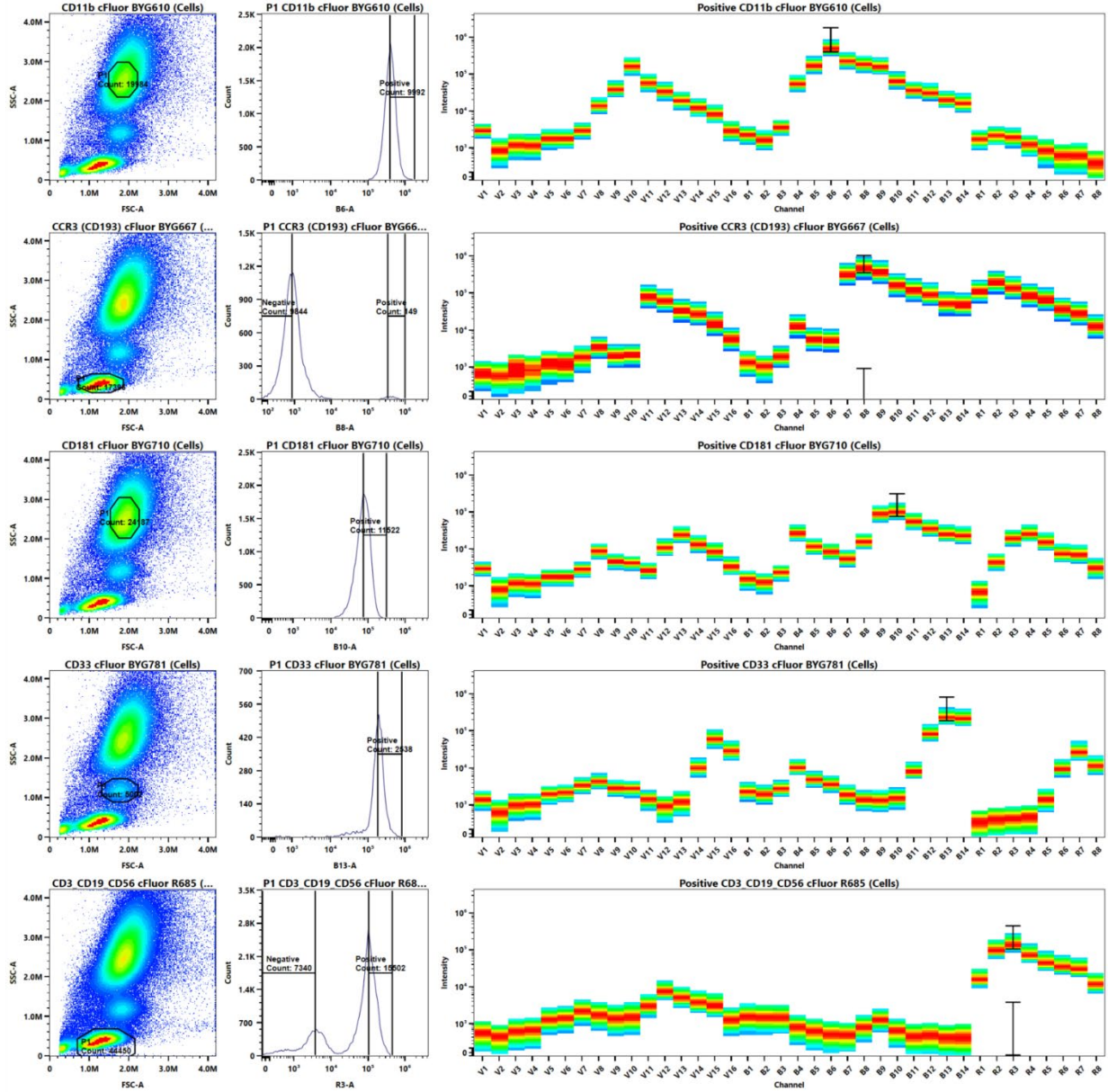
6. Under **Acquisition**, define Worksheet, Stopping Gate, and Events to Record
7. Click **Save and Open**
8. Preview and record the unstained control
9. Live Unmix with **Autofluorescence as a Fluorescence Tag** selected for 5 laser instruments and unselected for 3 laser instruments. Click **Next**.

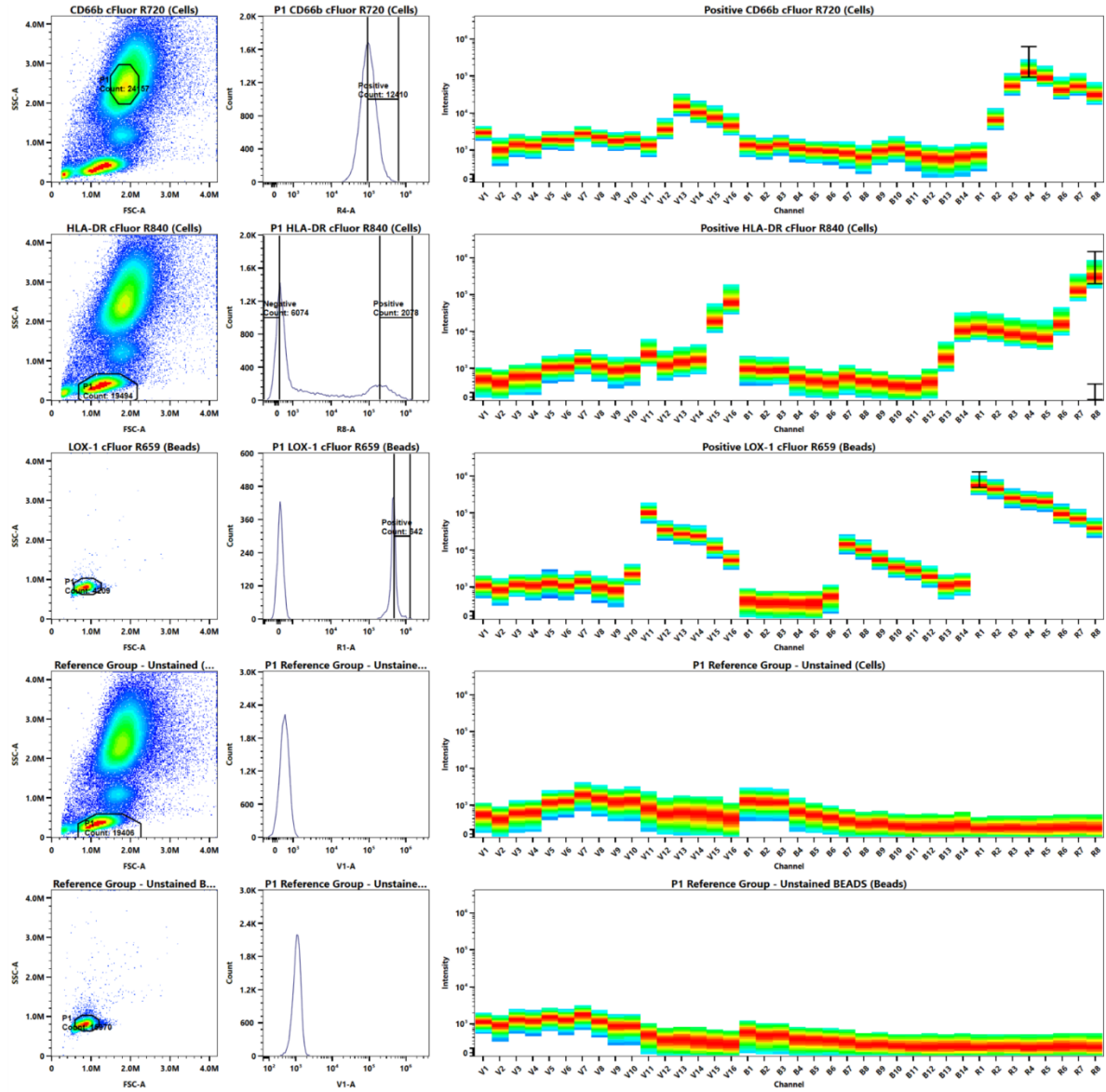
Note: Autofluorescence extraction is ALWAYS recommended for data generated in 5-laser systems. For data generated in 3-laser systems (V-B-R), there is a need to assess the level of autofluorescence of the sample by checking the spectrum plot of unstained cells. If autofluorescence is low in unstained cells, as shown on page 13, autofluorescence extraction is NOT recommended.

10. Acquire multicolor samples
11. Adjust the gates in the **MDSC_Analysis_Blood** worksheet as needed.

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







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

cFluor V450	1																					
cFluor V505	0.5	1																				
cFluor B515	0	0.04	1																			
cFluor B548	0.04	0.18	0.56	1																		
cFluor BYG575	0.03	0.1	0.1	0.43	1																	
cFluor BYG610	0.01	0.03	0.04	0.25	0.45	1																
cFluor BYG667	0	0	0.01	0.1	0.13	0.49	1															
cFluor BYG710	0.01	0.02	0.02	0.14	0.24	0.35	0.62	1														
cFluor BYG781	0	0.01	0.01	0.04	0.05	0.07	0.13	0.29	1													
cFluor R659	0	0	0	0.01	0.01	0.03	0.38	0.15	0.02	1												
cFluor R685	0	0	0	0	0.01	0.01	0.34	0.2	0.03	0.73	1											
cFluor R720	0.01	0.01	0	0.01	0.01	0.02	0.22	0.24	0.05	0.48	0.7	1										
cFluor R840	0	0	0	0	0	0	0.06	0.05	0.11	0.14	0.17	0.31	1									
		cFluor V450	cFluor V505	cFluor B515	cFluor B548	cFluor BYG575	cFluor BYG610	cFluor BYG667	cFluor BYG710	cFluor BYG781	cFluor R659	cFluor R685	cFluor R720	cFluor R840								

Complexity Index: 4.05

Figure 3: Expected Similarity™ matrix and Complexity™ indices 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.

Appendix D: Example of Adjusted Spillover Matrix

Tube/Well Properties

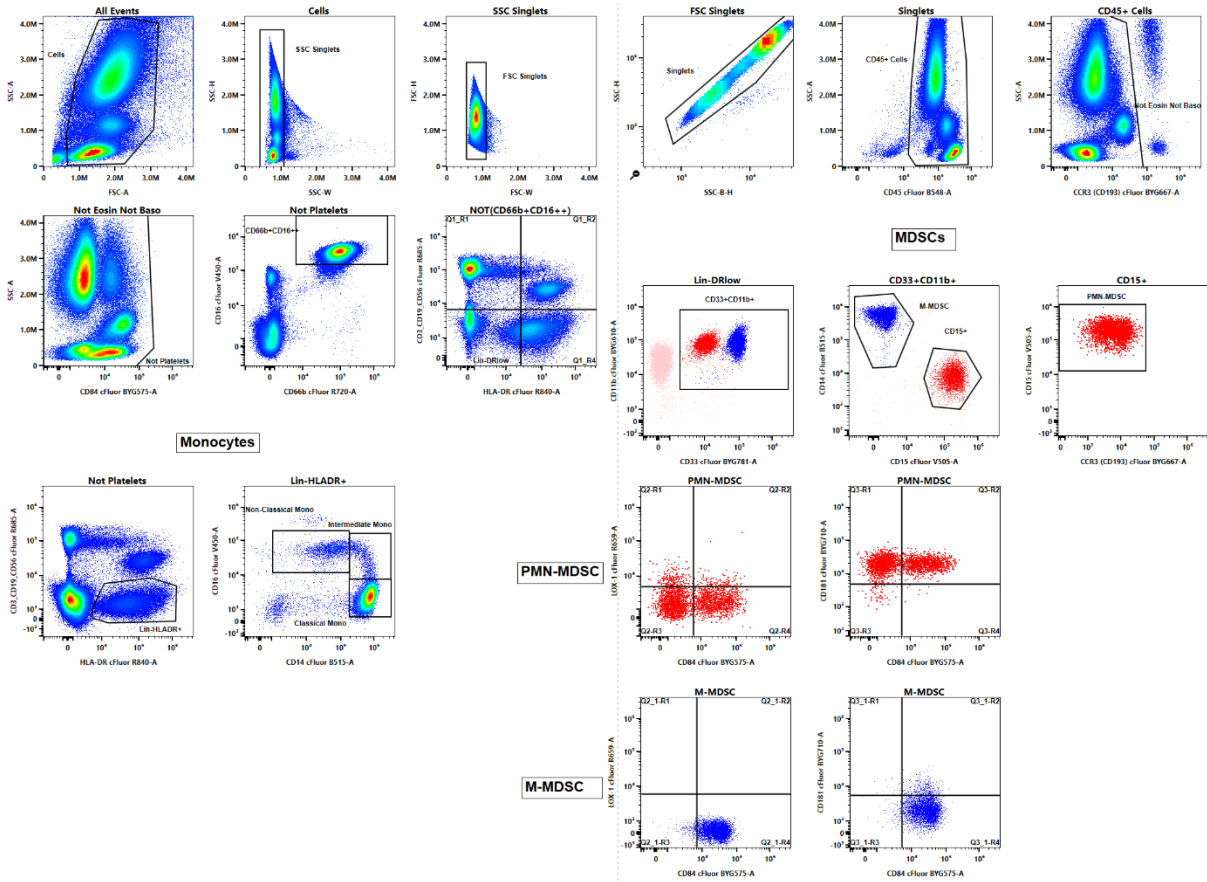
Tube/Well Fluorescent Tag Acquisition Keywords Instrument Settings Spillover

Enable Compensation
 Apply From Library
 Apply From File
 Save To Library
 Save To File

INTO + FROM +	cFluor V450	cFluor V505	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
cFluor V505	0	100	0.5	0	0	0	0	0	0	0	0	0	0
cFluor B515	0	0	100	0	0	0	0	0	0	0	0	0	0
cFluor B548	0	0	0	100	0	0	0	0	0	0	0	0	0
cFluor BYG575	0	0	0	0	100	0	0	0	0	0	0	0	0
cFluor BYG610	0	0	0	0	0	100	0	0	0	0	0	0	0
cFluor BYG667	0	0	0	0	0	0	100	0	0	0	0	0	0
cFluor BYG710	0	0	0	0	0	0	0	100	0	0	0	0	0
cFluor BYG781	0	0	0	0	0	0	0	0	100	0	0	0	0
cFluor R659	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	100	0	0
cFluor R720	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	100

Figure 4: Corrections made by checking 1xN permutation plots for 13 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 Whole Blood



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



For Research Use Only. Not intended for use in diagnostic procedures.

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.

Cytek® FSP™ CompBeads are developed and manufactured by Slingshot Biosciences, Inc., Emeryville, CA.

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