Acquisition Protocol for Cytek® 25-Color Immunoprofiling Assay

Contents

Introduction

This acquisition protocol provides step-by-step instructions to set up your Cytek Aurora® system (4-laser V-B-YG-R configuration or higher) for data acquisition of the Cytek® 25-Color Immunoprofiling Assay. This protocol provides instructions on 1) preparing SpectroFlo® software, 2) setting up the instrument, and 3) acquiring controls and samples.

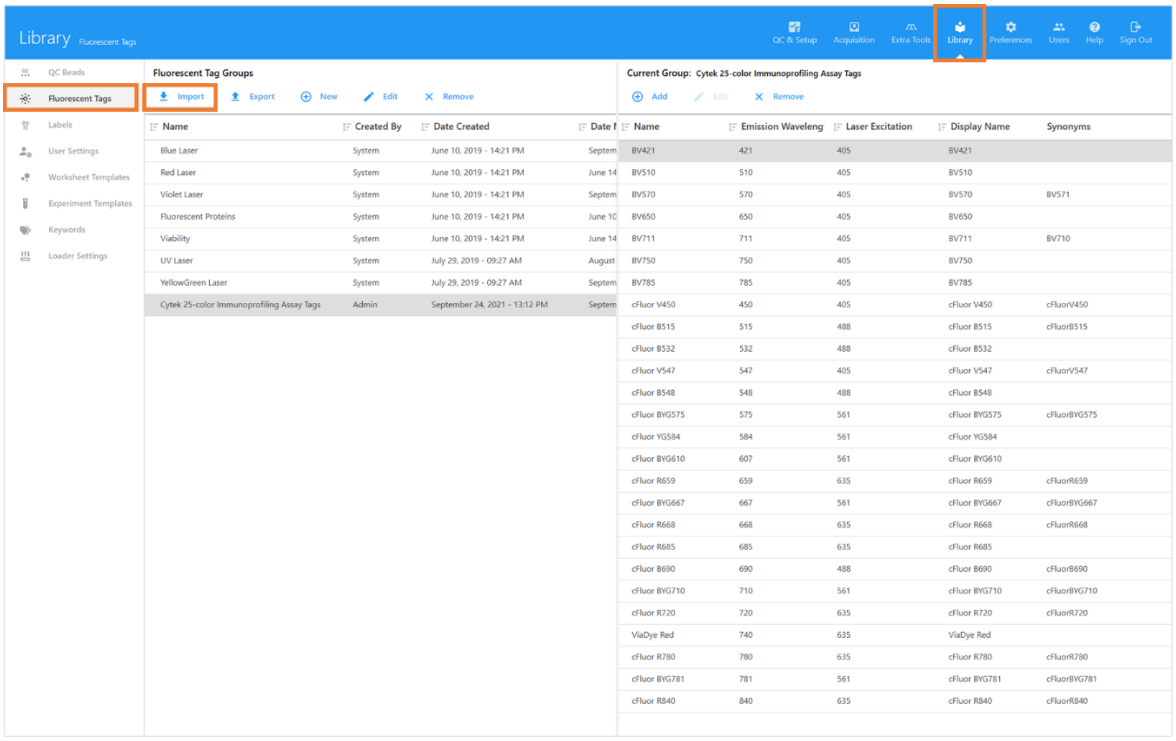
This kit contains 25 tests.

NOTE: *For suggestions on how to prepare human peripheral mononuclear cells and whole blood, see* ***Cytek 25-Color Immunoprofiling Assay_Sample Prep_PBMC*** *and* ***Cytek 25-Color Immunoprofiling Assay_Sample Prep_Whole Blood.***

Preparing SpectroFlo® Software

Add fluorochromes to the library

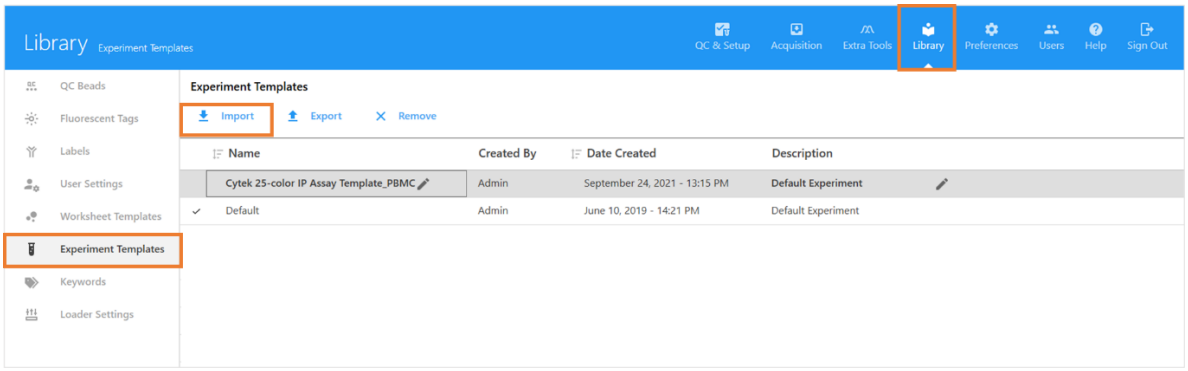
1. Import **Cytek® 25-Color Immunoprofiling Assay Tags**.csv file into the **Library** module, under **Fluorescent Tags** to add the fluorochromes used in this kit.



NOTE: *If any fluors 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.*

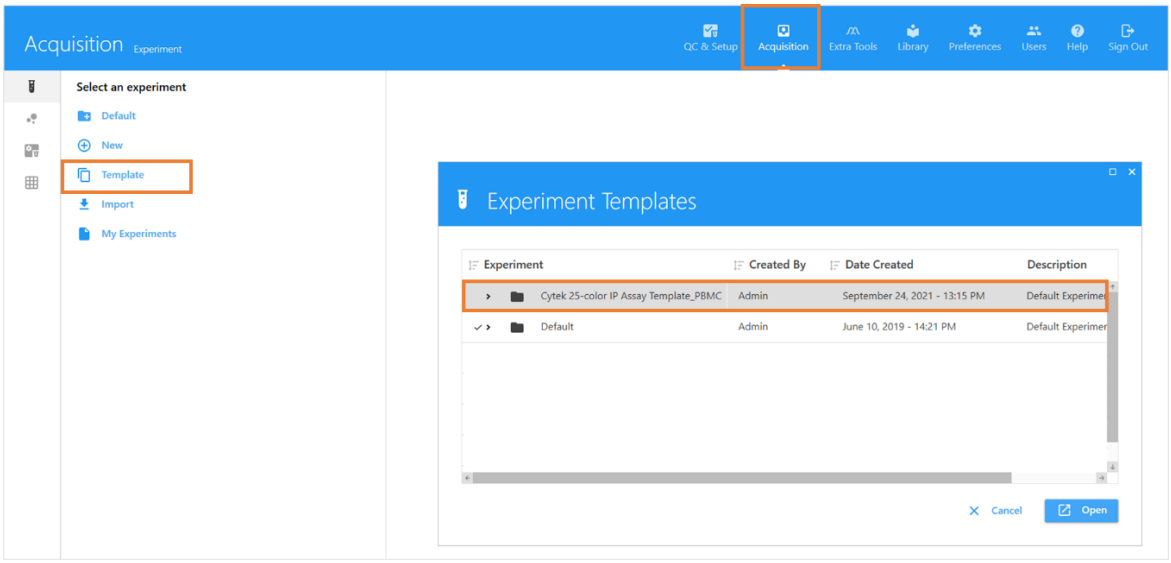
Import the experiment template

1. **Cytek 25-Color IP Assay Templates** include 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 either "Cytek 25-Color IP Assay template_PBMC" or "Cytek 25-Color IP Assay template_Whole Blood"



3. For best results, we recommend running cells for single color controls.
   ***NOTE**: See Table 1. In page 4 for sample 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.

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 as "Cytek 25-Color IP Assay" 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.*

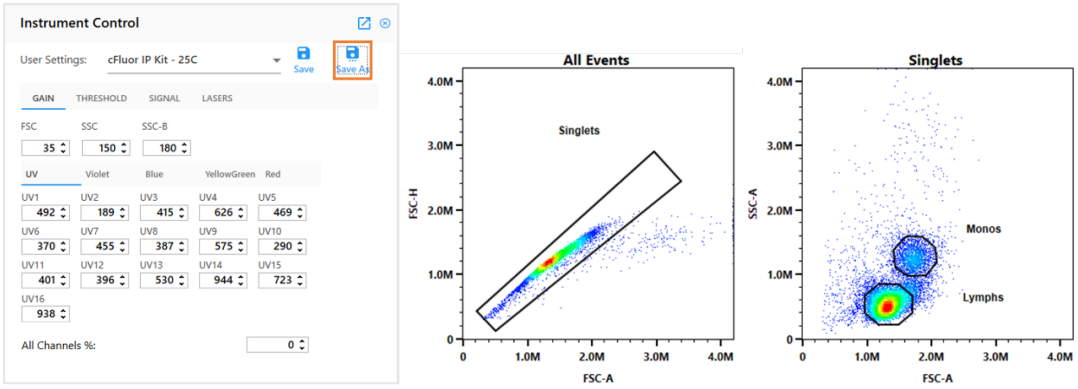


Figure 1: *Example cell resolution of human PBMC samples after FSC and SSC adjustment with threshold set at FSC 300,000.*

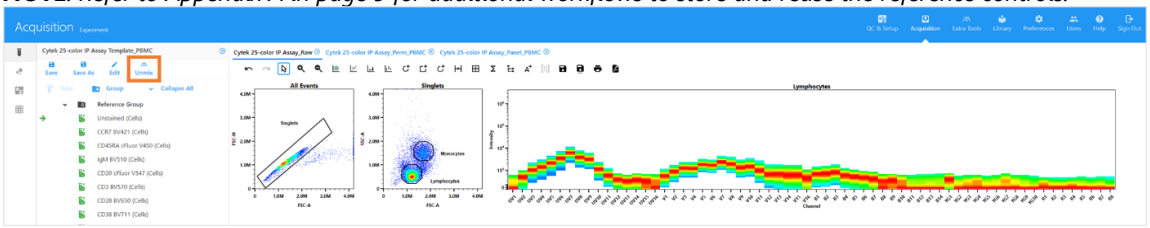
2. Acquire unstained and single color controls in the Reference Control group. Click **Start** to preview for 5 to 10 seconds until the event rate stabilizes, then click **Record** to record each sample.

Table 1. Sample Type Recommendations

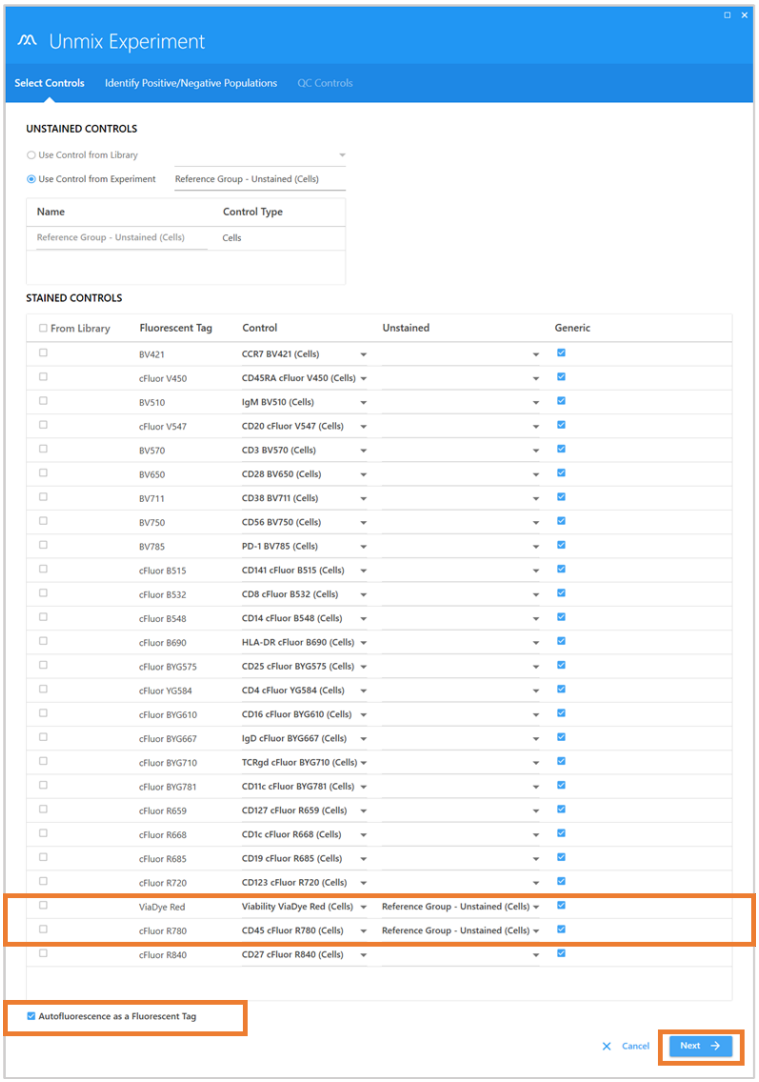
Laser	Target	Fluorochrome	Recommended Sample Type
Violet	CCR7	Brilliant Violet 421™	Cells or Beads
	CD45RA	cFluor® V450	Cells or Beads
	IgM	Brilliant Violet 510™	Cells or Beads
	CD20	cFluor® V547	Cells Only
	CD3	Brilliant Violet 570™	Cells Only
	CD28	Brilliant Violet 650™	Cells Only
	CD38	Brilliant Violet 711™	Cells Only
	CD56	Brilliant Violet 750™	Cells or Beads
	PD-1	Brilliant Violet 785™	Cells or Beads
Blue	CD141	cFluor® B515	Cells or Beads
	CD8	cFluor® B532	Cells or Beads
	CD14	cFluor® B548	Cells or Beads
	HLA-DR	cFluor® B690*	Cells Only
Yellow/Green	CD25	cFluor® BYG575*	Cells or Beads
	CD4	cFluor® YG584	Cells or Beads
	CD16	cFluor® BYG610	Cells or Beads
	IgD	cFluor® BYG667*	Cells Only
	TCRγδ	cFluor® BYG710	Cells or Beads
	CD11c	cFluor® BYG781*	Cells or Beads
Red	CD127	cFluor® R659*	Cells or Beads
	CD1c	cFluor® R668	Cells or Beads
	CD19	cFluor® R685	Cells Only
	CD123	cFluor® R720	Cells or Beads
	CD45	cFluor® R780*	Cells or Beads
	CD27	cFluor® R840	Cells or Beads

Unmix reference controls

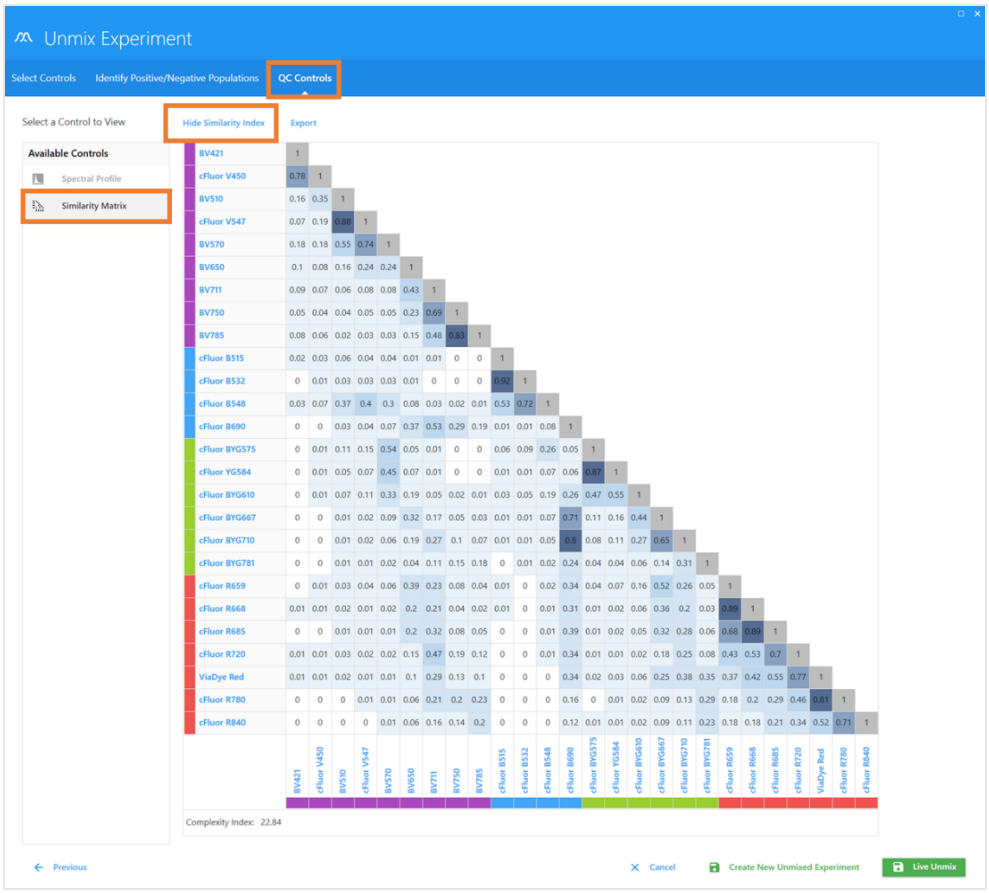
1. Once all controls have been acquired, Click **Unmix**.
   NOTE*: Refer to Appendix A in page 9 for additional workflows to store and reuse the reference controls.*



2. Under **Select Controls** tab in the Unmix Experiment wizard, ensure 1) the Unstained is selected as **Reference Group – Unstained** for ViaDye Red and cFluor® R780 CD45, and 2) **Autofluorescence as a Fluorescence Tag** is selected for 5 laser instruments and unselected for 4 laser instruments. Click **Next**.
Note: *Autofluorescence extraction is ALWAYS recommended for data generated in 5-laser systems. For data generated in 4-laser systems (V-B-YG-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 page12, autofluorescence extraction is NOT recommended.*



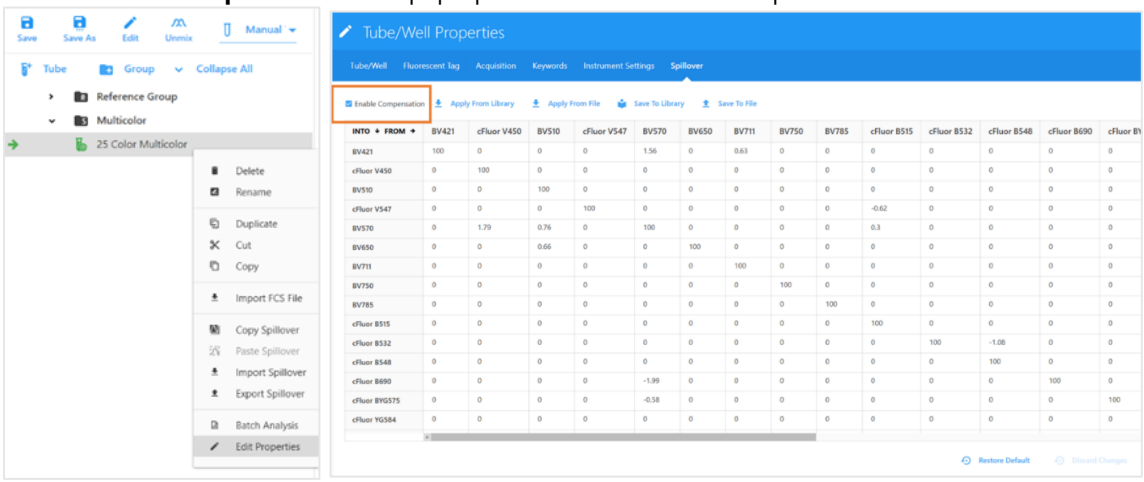
3. Under **Identify Positive/Negative Populations** tab, ensure for all single color controls that the 1) scatter plot is gated on the appropriate population, 2) black bar is on the peak channel, 3) signature of each fluor matches the expected spectrum, and 4) negative and positive gates in the histogram are correctly positioned.
Note: *Refer to Appendix B in page 10 for the correct gate positioning, expected spectra and peak channels of each fluor, 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 in page 13.



4. Click **Live Unmixing**.

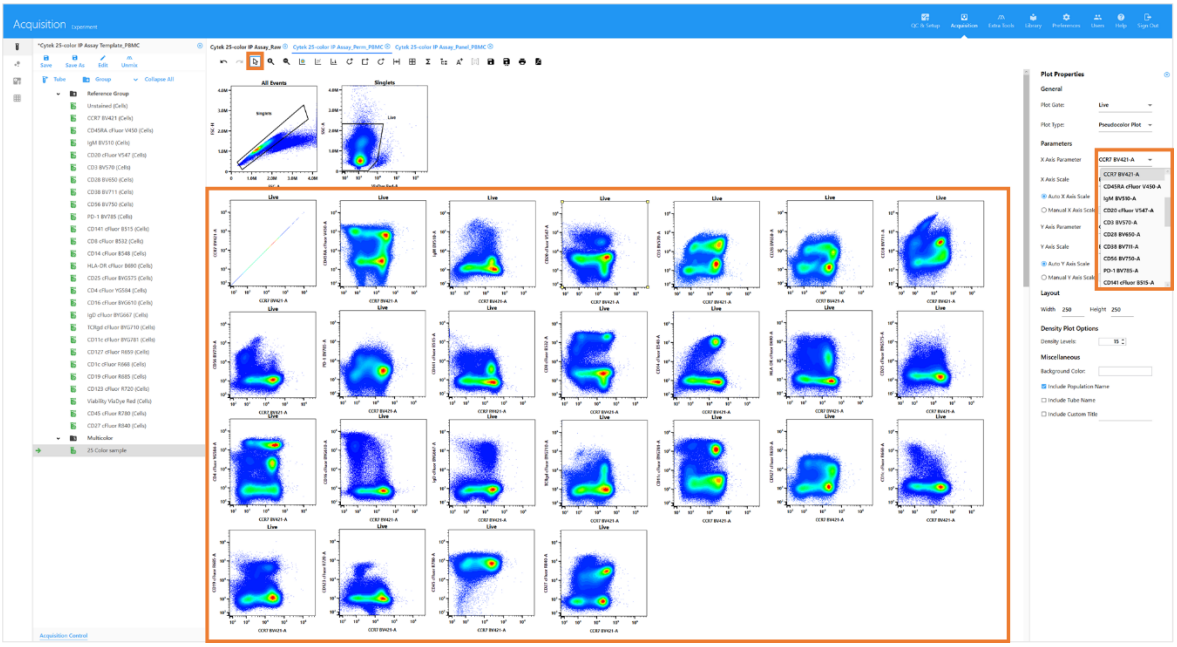
Acquire and analyze multicolor samples

1. Acquire multicolor samples.
2. Import and open **Cytek 25-Color IP Assay_Perm** worksheet to view the multicolor sample in the worksheet.
3. Right click on the multicolor tube. 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.



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 fluor under **X Axis Parameter**, and 4)

Check all 25 permutation plots against the first fluor for any unmixing errors and adjust spillover (compensation) as needed. Do this for all 25 fluors by selecting each fluor one by one under **X Axis Parameter**.



6. 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).

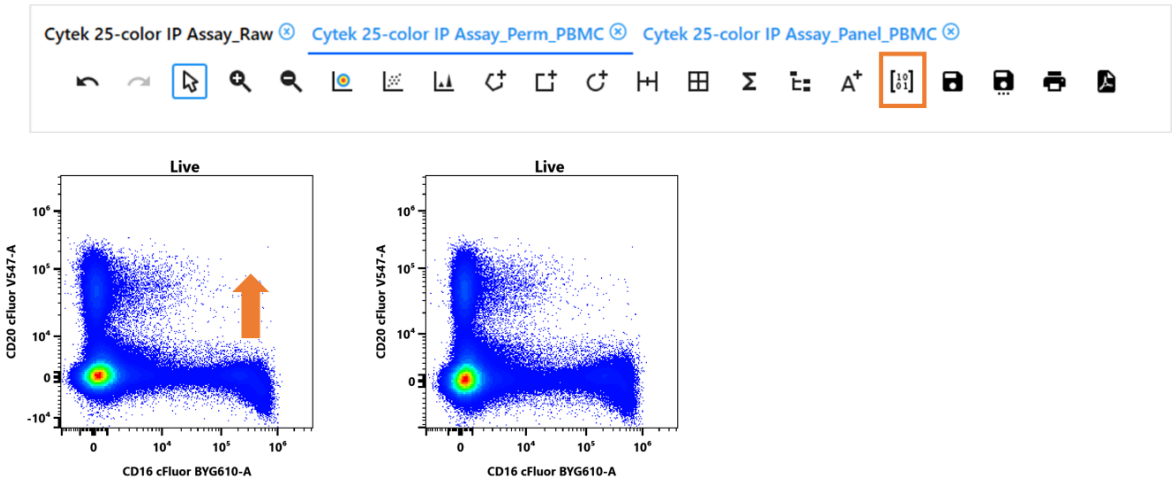
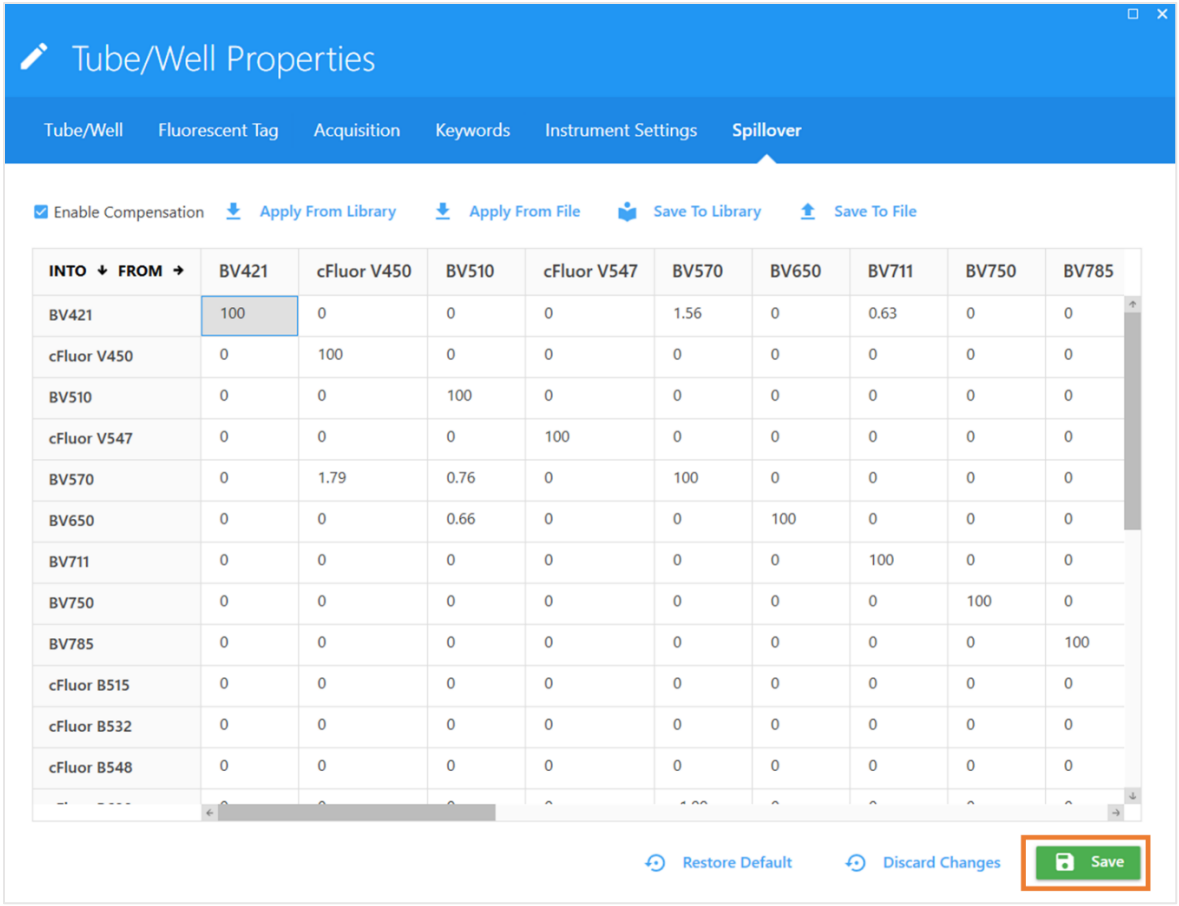


Figure 2: *Example of unmixing error adjustment.*

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.

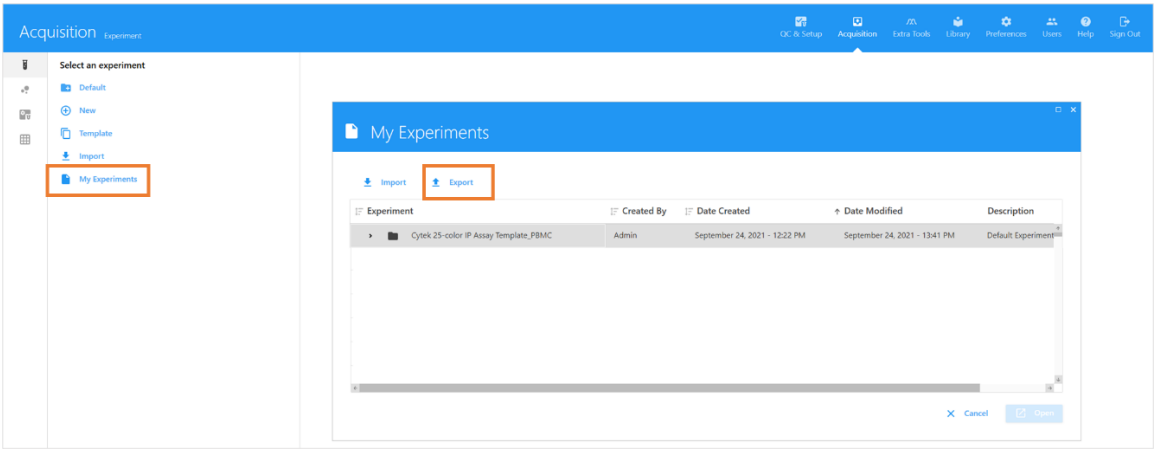


NOTE: *Refer to an example of adjusted spillover matrix in Appendix D in page 14.*

10. Double click on the multicolor to open **Cytek 25-Color IP Assay_Panel** worksheet to view and adjust gates in the worksheet.

NOTE*: Refer to Appendix E in page 15 for an example of a PBMC 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**.



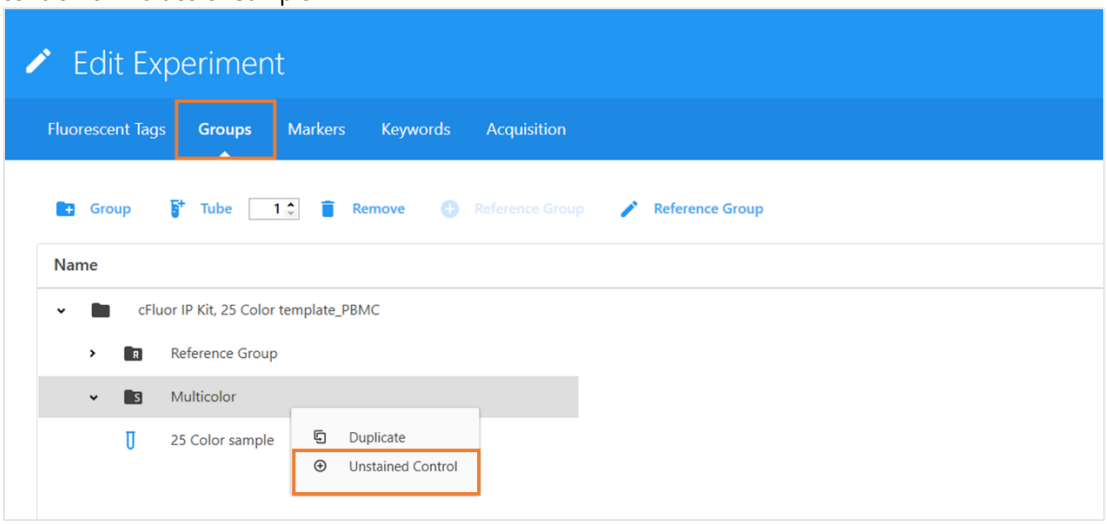
Appendix A: Reusing Single Color Controls

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

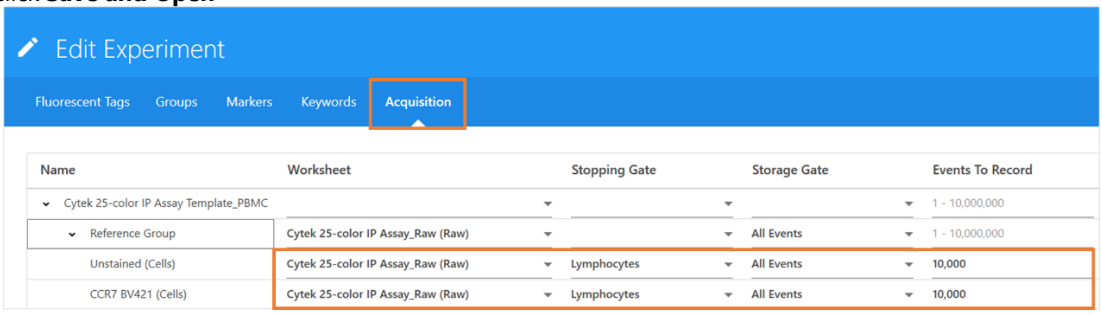
NOTE: *For best results, the instrument should be maintained properly, QC performed daily and the same reagent lot used. The samples need to be collected in the same instrument as the reference controls.*

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

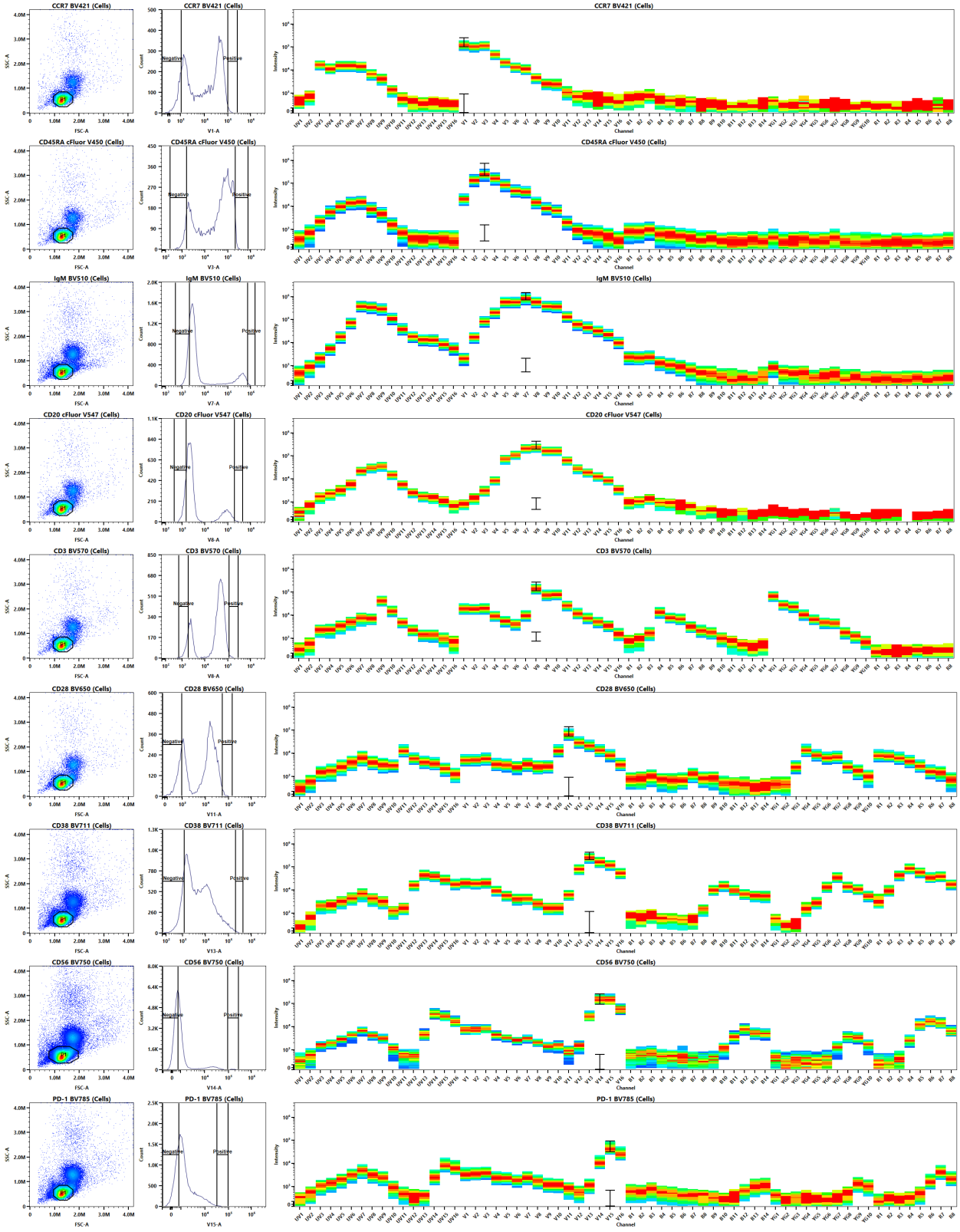


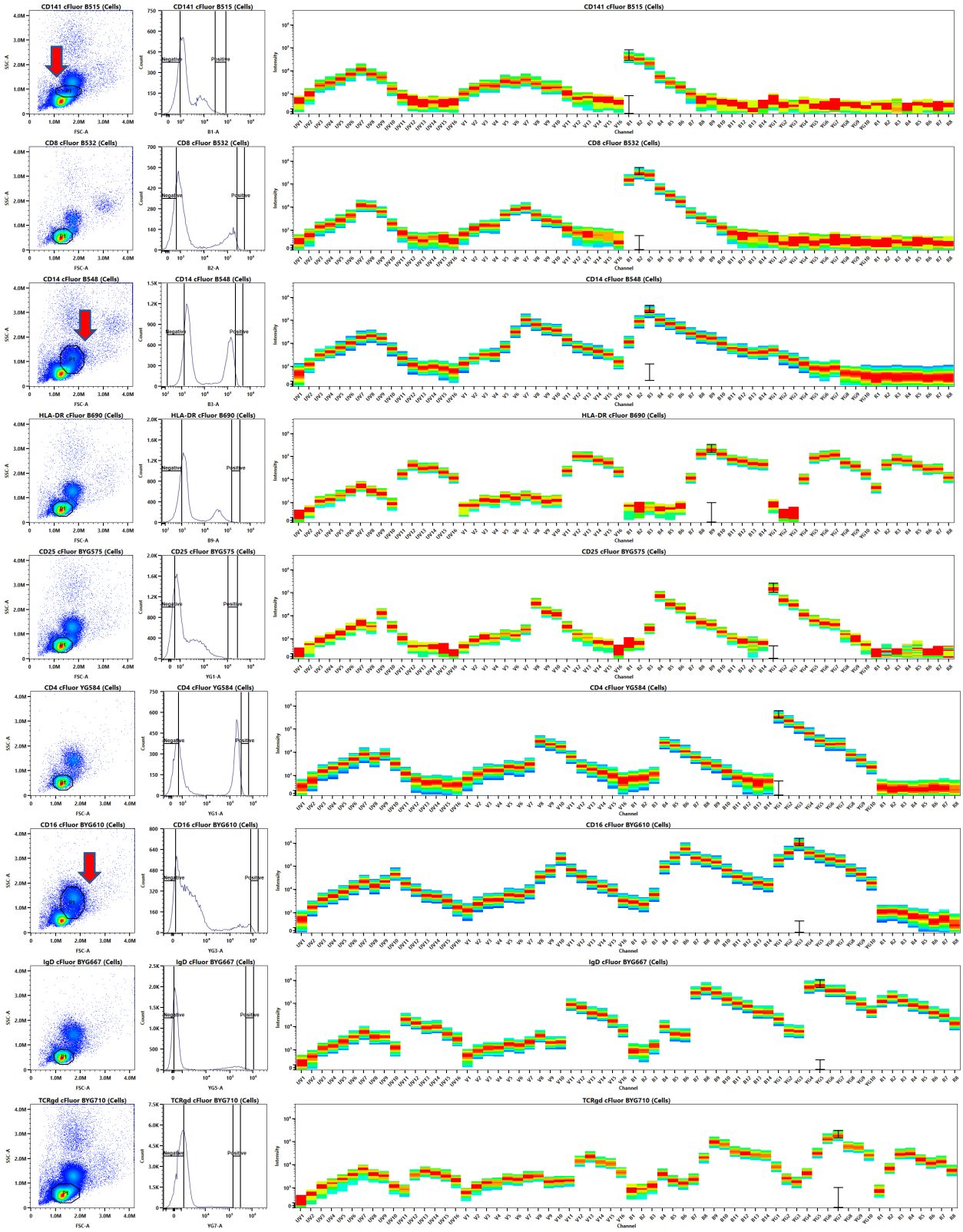
6. Under **Acquisition**, define Worksheet, Stopping Gate, and Events to Record
7. Click **Save and Open**

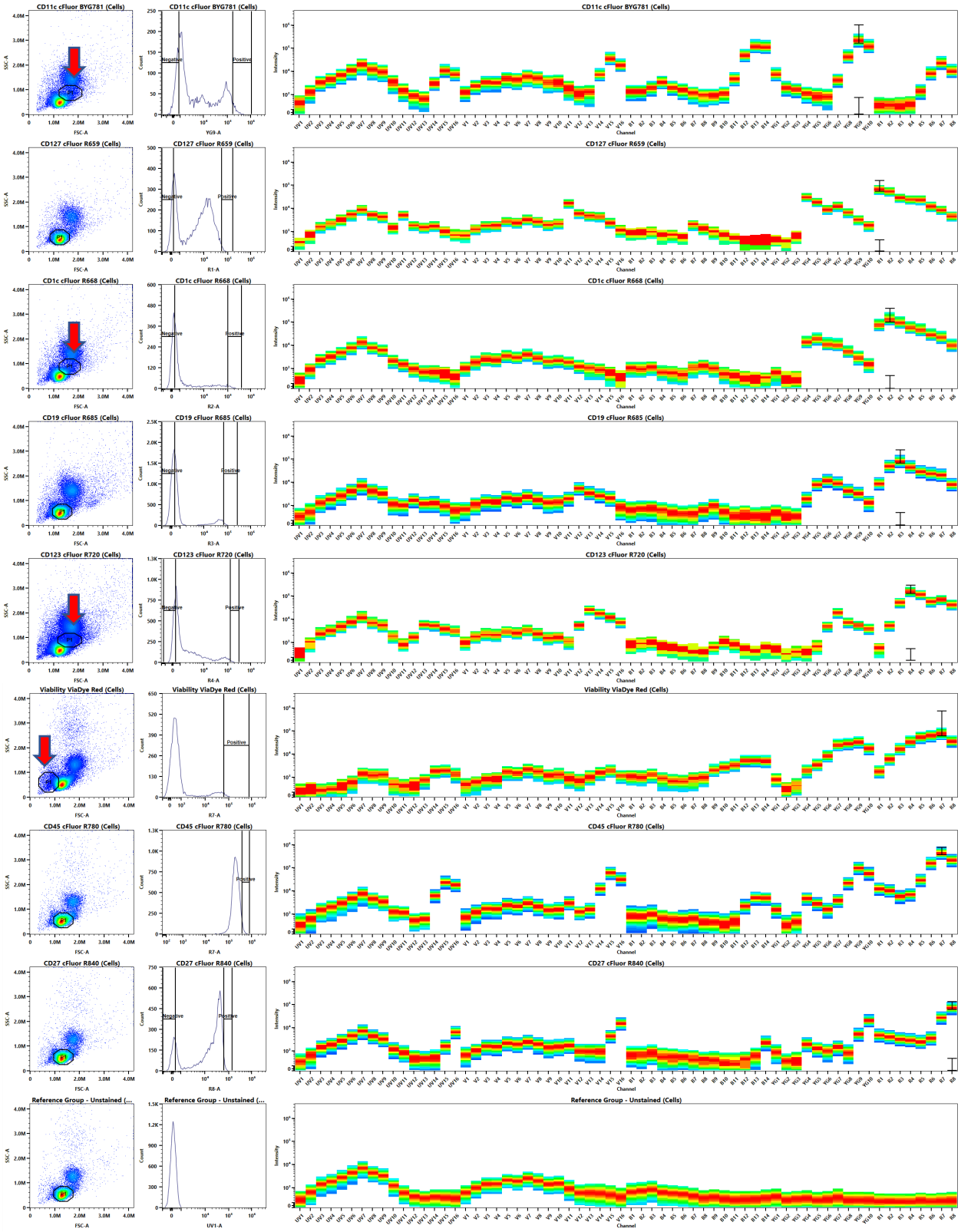


8. Preview and record the unstained control
9. Live Unmix with Autofluorescence Extraction
10. Acquire multicolor samples
11. Adjust the gates in the analysis worksheet as needed.

Appendix B: Single Color Control Signatures for 5-Laser (UV-V-B-YG-R) Cytek Aurora Suggested Gating When Using Cells as Controls




CD141 cFluor B515 (Cells)
CD8 cFluor B532 (Cells)
CD14 cFluor B548 (Cells)
HLA-DR cFluor B690 (Cells)
CD25 cFluor BYG575 (Cells)
CD4 cFluor YG584 (Cells)
CD16 cFluor BYG610 (Cells)
IgD cFluor BYG667 (Cells)
TCRgd cFluor BYG710 (Cells)
Negative
Positive
SSC-A
FSC-A
Count
Intensity
Channel


CD11c cFluor BYG781 (Cells)
CD127 cFluor R659 (Cells)
CD1c cFluor R668 (Cells)
CD19 cFluor R685 (Cells)
CD123 cFluor R720 (Cells)
Viability ViaDye Red (Cells)
CD45 cFluor R780 (Cells)
CD27 cFluor R840 (Cells)
Reference Group - Unstained (Cells)
Negative
Positive
SSC-A
FSC-A
Count
Intensity
Channel

Appendix C: Example of Similarity™ Matrix for Cytek® 25-Color Immunoprofiling Assay

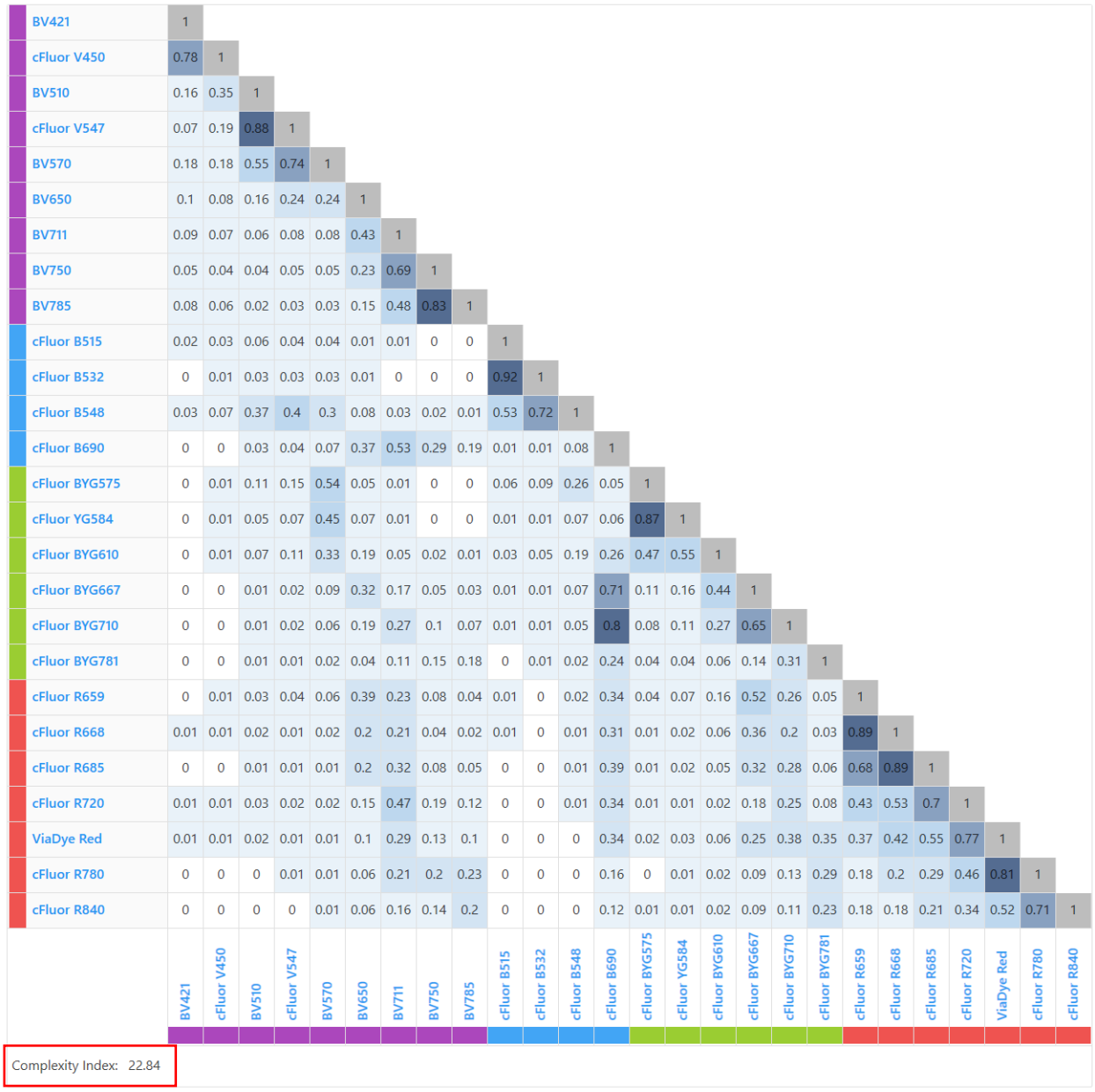


Figure 3: *Expected Similarity™ matrix and Complexity™ index generated on a 5-laser (UV-V-B-YG-R) Cytek® Aurora. Generating similar values is a good indication that signatures of your single-color controls match those generated by Cytek.*

Appendix D: Example of Adjusted Spillover Matrix

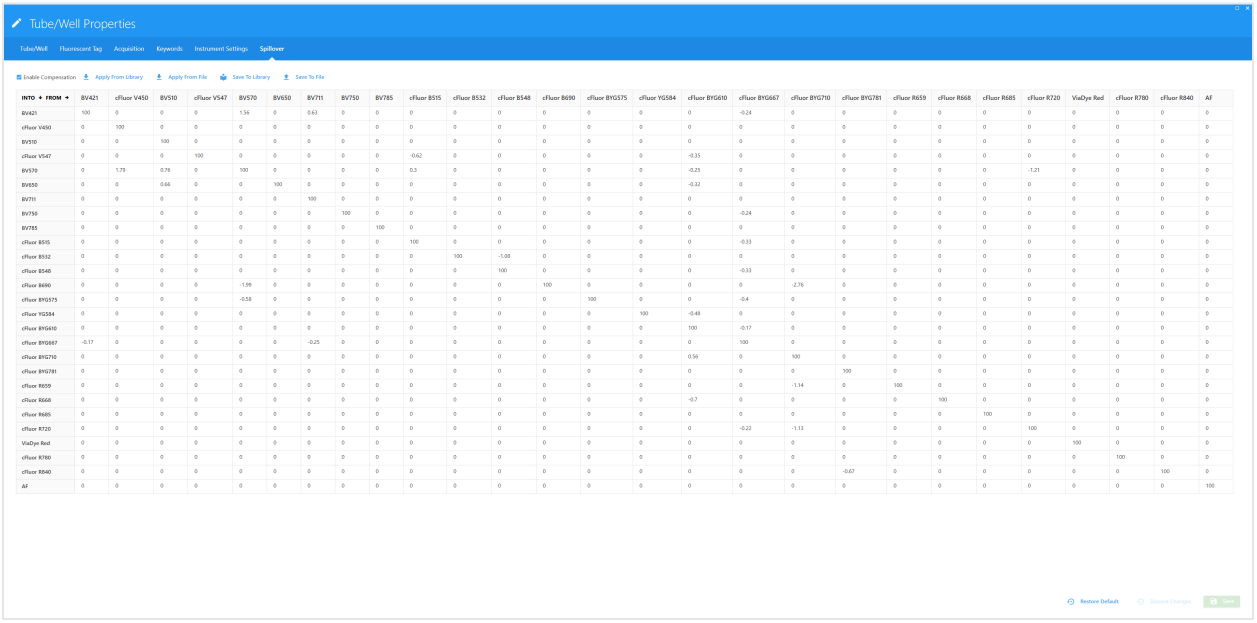
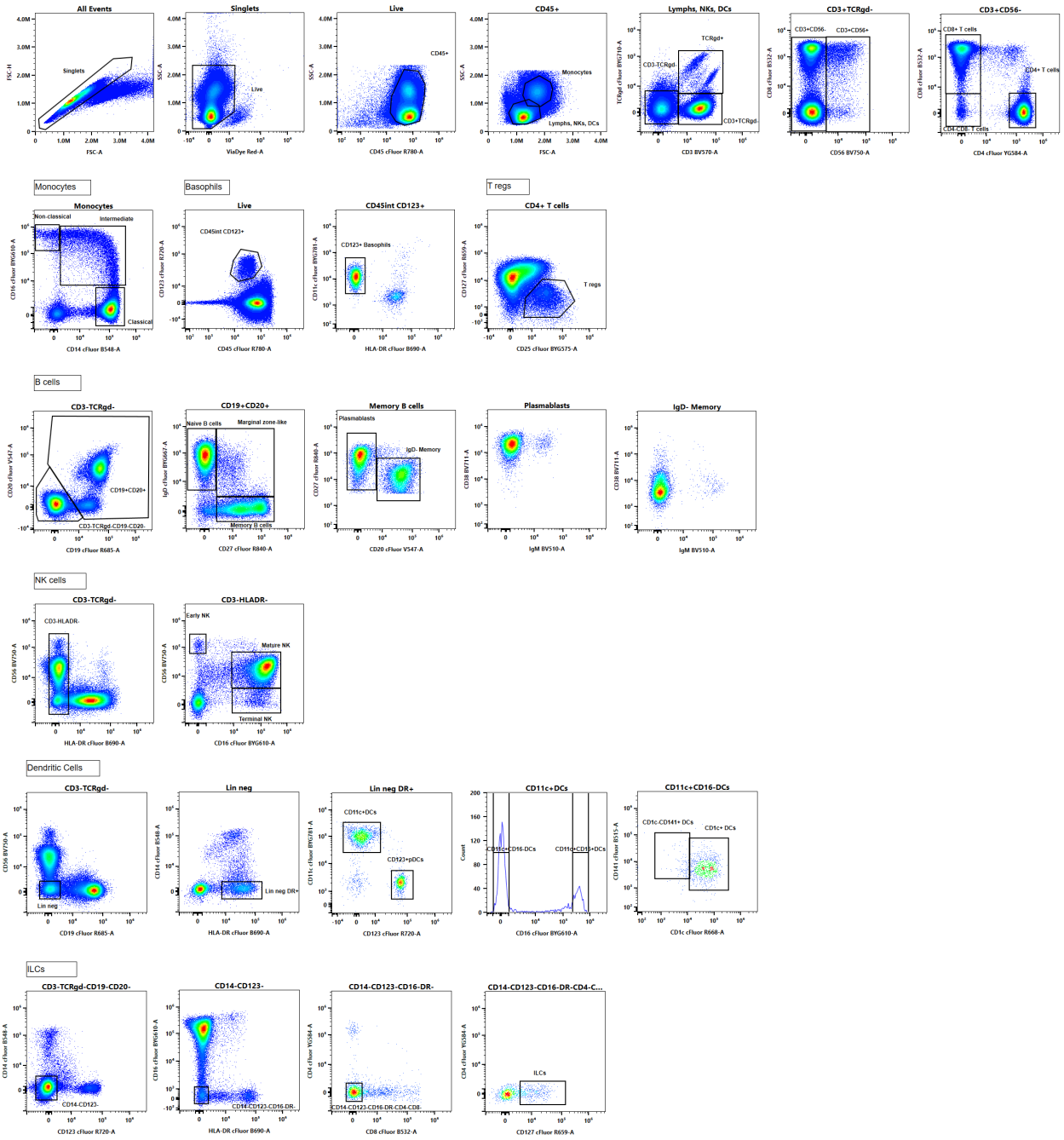
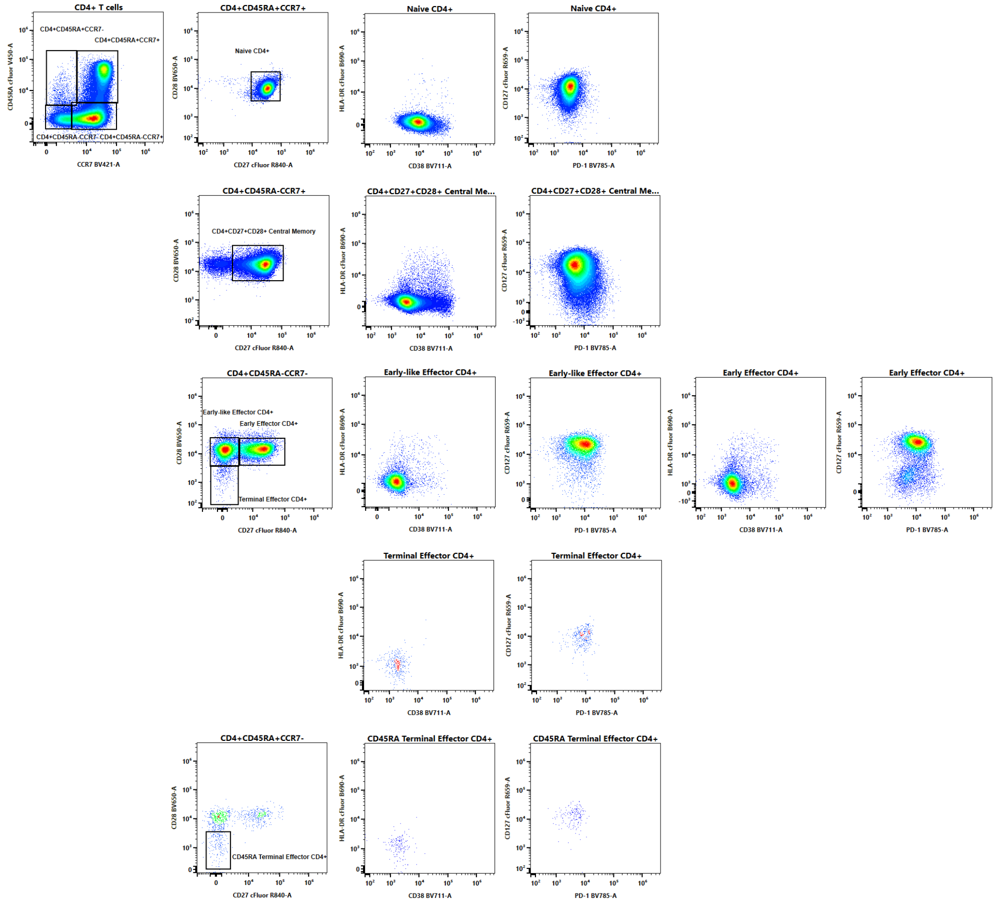


Figure 4: *Corrections made by checking NxN permuation plots for 25 fluors. Please not that all needed corrections are below 3%. If corrections larger than 5% need to be applied, carefully QC your reference controls, comparing the staining pattern and gating positioning provided in AppendixB.*

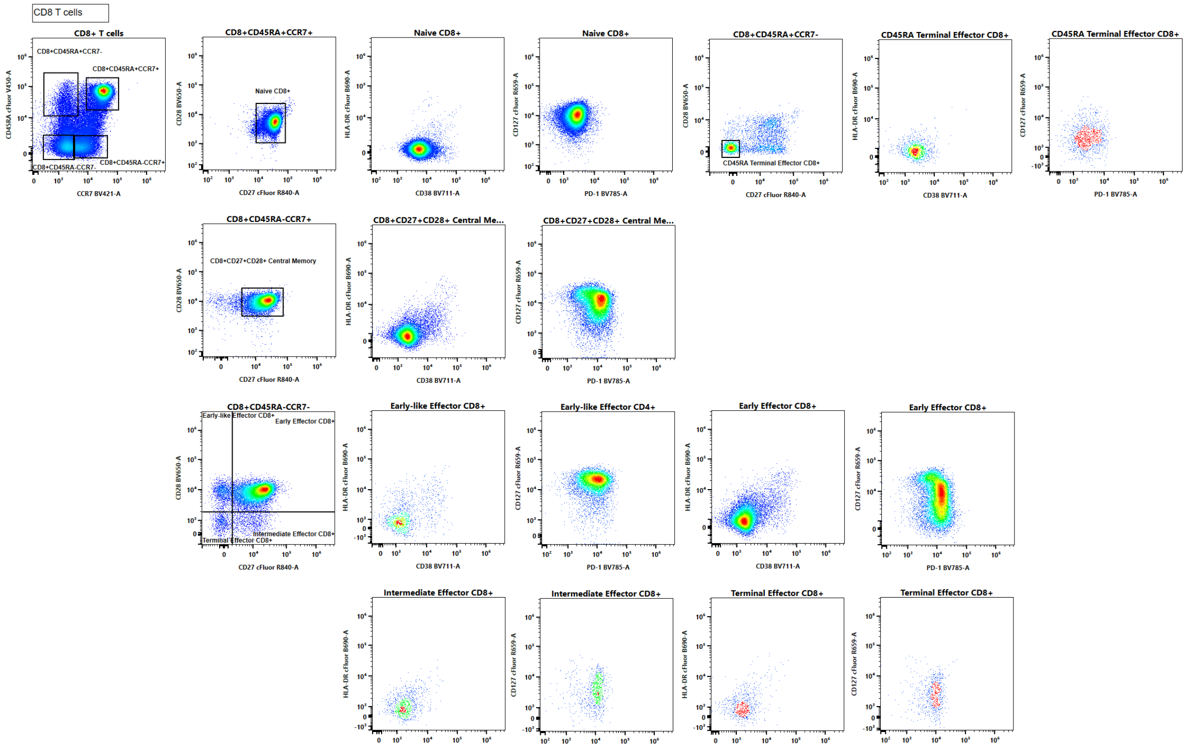
Appendix E: Example of Gating in Multicolor Sample



CD4 T cells



CD8 T cells



cFluor® V547, cFluor® B515, cFluor® B532, cFluor® R668 and cFluor® R720 are equivalent to CF®405L, CF®488A, CF®503, CF®647, and CF®700 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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*Fluor conjugated antibody manufactured and supplied by BioLegend Inc.

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