# Acquisition Protocol for the Cytek® cFluor® Human Pan Leukocyte Kit, LNW

## Contents



## Introduction

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

This kit contains 50 tests.

***NOTE:*** *For suggestions on how to prepare human peripheral whole blood, see* ***Sample Preparation (Whole Blood) Guidelines for the Cytek® cFluor® Human Pan Leukocyte Kit, LNW****.*

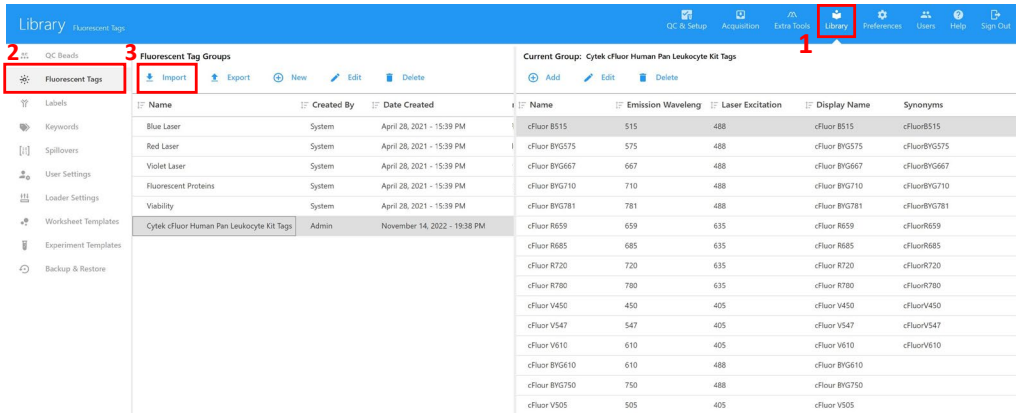
## Preparing SpectroFlo® Software

### Add fluorochromes to the library

1. Import **cFluor® Pan Leukocyte Kit, Tags**.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 for the existing fluorescent tag information.*

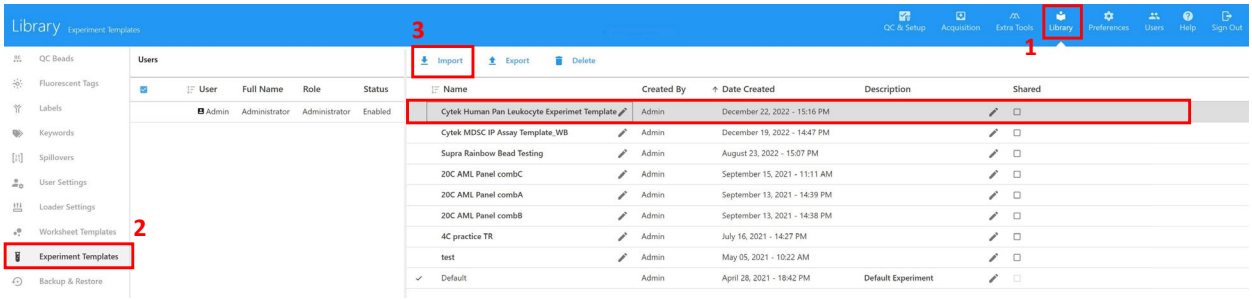
Importing Fluorescent Tags:




**Import the experiment template**

1. **Cytek Human Pan Leukocyte Experiment Template** 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**.

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 **Cytek Human Pan Leukocyte Experiment Template**, click **Open** and give a name to your experiment.
3. For best results, we recommend running cells or beads for single color controls as shown in **Table 1**.

**Table 1.** Reference Control Type Recommendations for Single Color Reference Controls

| Laser | Target | Fluorochrome | Peak | Recommended Control Type |
|---|---|---|---|---|
| Violet | CD8 | cFluor® V450 | V3 | Cells or Beads |
| | HLA-DR | cFluor® V505 | V5 | Cells or Beads |
| | CD45 | cFluor® V547 | V8 | Cells or Beads |
| | CD4 | cFluor® V610 | V10 | Cells or Beads |
| Blue | CD16 | cFluor® B515 | B1 | Cells |
| | CD34 | cFluor® BYG575 | B4 (YG1) | Beads |
| | CD123 | cFluor® BYG610 | B6 (YG3) | Beads |
| | CD193 (CCR3) | cFluor® BYG667 | B8 (YG5) | Beads |
| | CD56 | cFluor® BYG710 | B10 (YG7) | Beads |
| | CD19 | cFluor® BYG750 | B12 (YG8) | Cells or Beads |
| | CD14 | cFluor® BYG781 | B13 (YG9) | Cells or Beads |
| Red | CD7 | cFluor® R659 | R1 | Cells or Beads |
| | CD20 | cFluor® R685 | R3 | Cells or Beads |
| | CD66b | cFluor® R720 | R4 | Cells or Beads |
| | CD3 | cFluor® R780 | R7 | Cells or Beads |

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

4. 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. To acquire unstained and single-color samples, **Pan Leukocyte Single & Unstained Raw** worksheet and the **Pan Leuko Single & Unstained** user settings should be set to collect in the template (See **Appendix A**).
2. Preview unstained cell control at low flow rate to minimize wasted sample volume and, optimize the FSC and SSC gains, as well as the FSC threshold to fully visualize the cells of interest including granulocytes (**see Figure 1**).
3. Preview unstained beads at low flow rate and optimize the FSC gain setting if needed.

***NOTE:*** *Instrument settings can be saved/overwritten as* **Pan Leuko Single & Unstained** *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 FSC, SSC, and threshold need to be optimized for specific sample types.*

4. Acquire unstained and single-color controls in the Reference Control group at high flow rate within 6 hours post staining. Click **Start** to preview for at least 10 seconds until the event rate stabilizes, then click **Record** to record each sample.

***NOTE****: Samples may be stored at 4°C for up to 24 hours prior to acquiring on a cytometer.*

Example of Single stain cells (CD8-V450)

Example of single stain beads (CD34-BYG575)

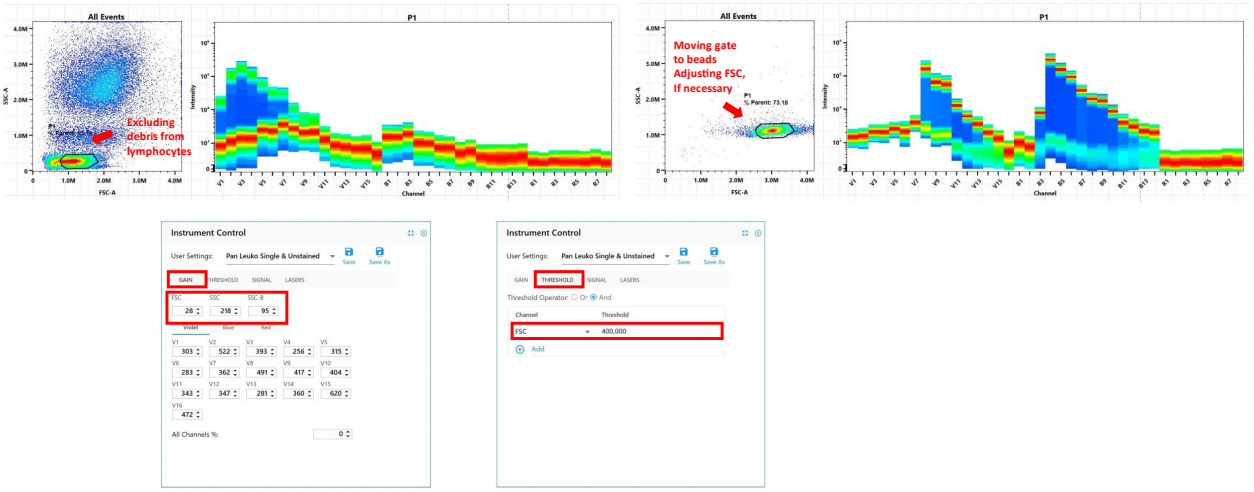
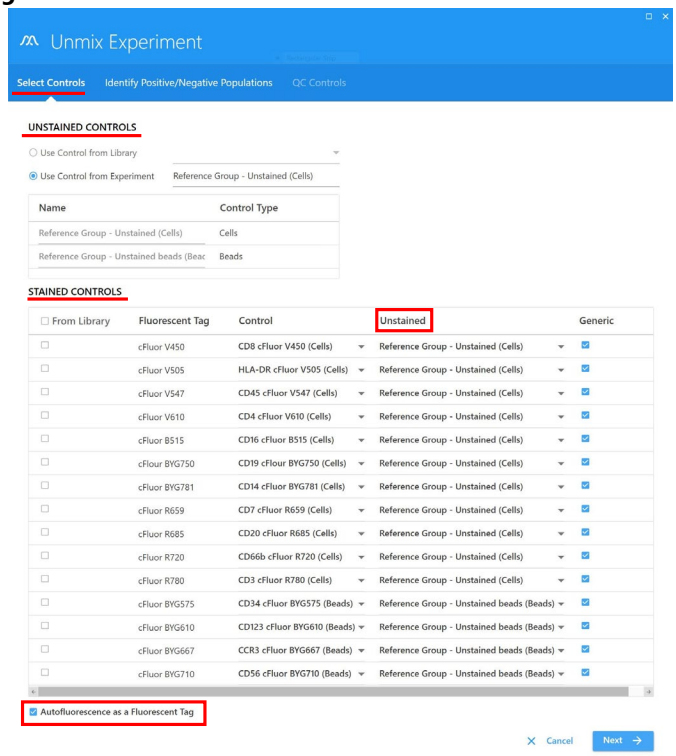



***Figure 1:*** *Example cells and the* FSP™ CompBeads *beads data after FSC and SSC adjustment with threshold set at FSC 400,000.*

## Unmix reference controls

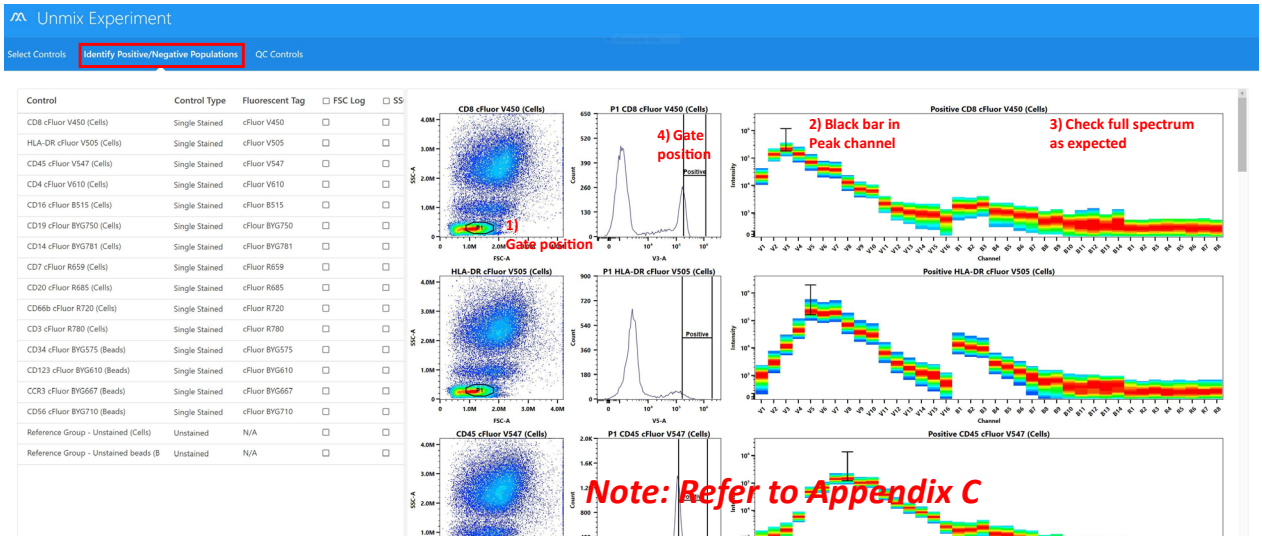
1. Once all controls have been acquired, Click **Unmix**.
   ***NOTE****: Refer to* ***Appendix B*** *for additional workflows to reuse the reference controls.*
2. Under **Select Controls** tab in the Unmix Experiment wizard, ensure:
   1) Under **Unstained Controls** check that "Use Control from Experiment" is selected and Reference Group-Unstained (Cells) and Unstained beads (Beads) are present.
   2) Under **Stained Controls** that Unstained (Cells) and Unstained beads (Beads) are selected for all single stained cells and beads, respectively, in the Unstained column
   3) ***Note:*** *Unmix will not be available until the Unstained tube for each sample group has been acquired*. Group specific unstained cell controls can be added to sample folders by clicking **Edit** the experiment template in the Acquisition module. Select **Groups** tab and right click on the sample folder(s) and select **+ Unstained control**.

4) **Autofluorescence as a Fluorescence Tag** is selected. Click **Next**.

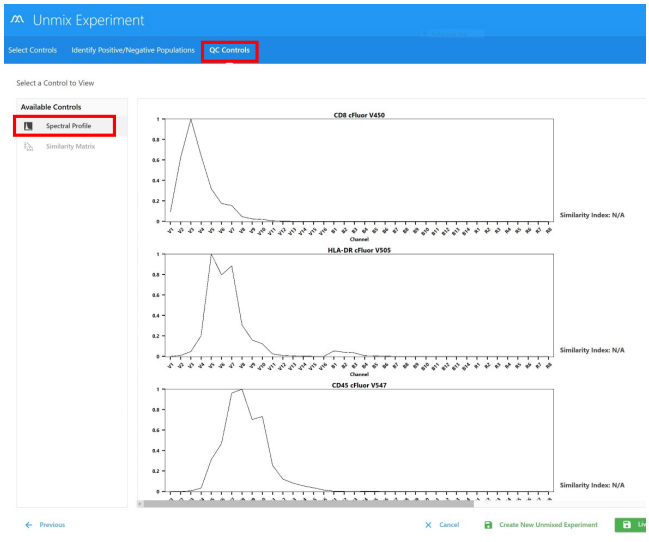


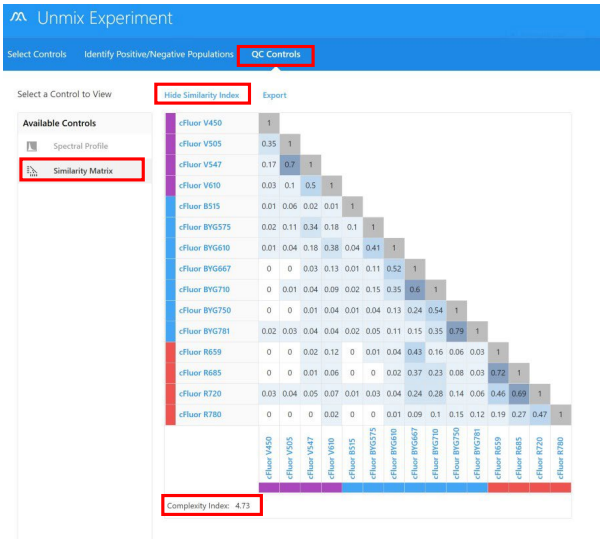

3. Under **Identify Positive/Negative Populations** tab, ensure for all single-color controls that:
   1) The P1 gates on the scatter plots are tightly gated on the appropriate populations
   2) On the full spectral plots, the positive interval gates are on the correct peak channels (see **Table 1**)
   3) Signature of each fluorochrome matches the expected spectrum
   4) 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 positive gates in the histograms.*



4. Click **Next**. Under the **QC Controls** tab, click on **Spectral Profile** to confirm all controls were appropriately stained and match the expected spectrum. click on **Similarity™ Matrix** to confirm the matrix and complexity index are comparable to this example.




5. Click **Live Unmix**.

## Acquire multicolor samples

1. To acquire multicolor samples, **Pan Leukocyte Acquisition** 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.
3. Before running multicolor samples, preview the *CD45-V547 Single Stain Reference Control* tube to set the proper CD45 (V8 channel) threshold to exclude CD45 negative populations and debris.

***Note:*** *Reference the SSC-A vs CD45 cFluor V547-A plot in Appendix D when setting up CD45 (V8) threshold.*

4. Acquire samples in the Multicolor Staining group at high flow rate. Click **Start** to preview for at least 10 seconds until the event rate stabilizes, then click **Record** to record each sample. It will take approximately15 min per multicolor tube.

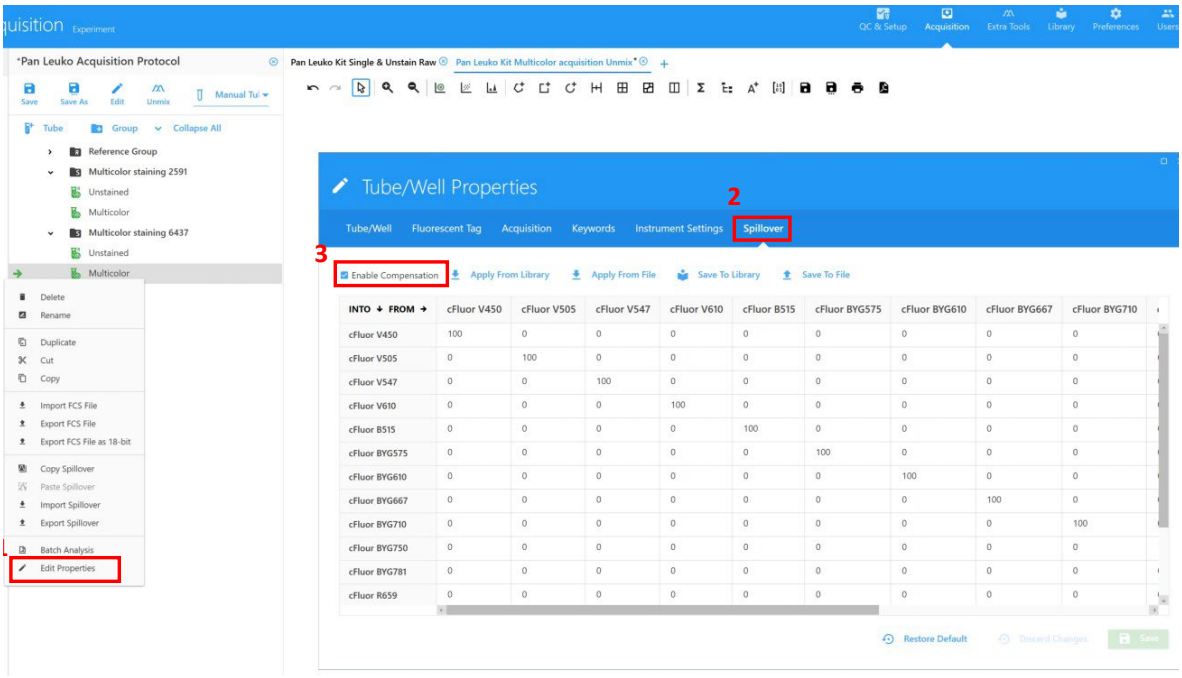
***NOTE****: Samples may be stored at 4°C for up to 24 hours prior to acquiring on a cytometer*.

## Analyze multicolor samples

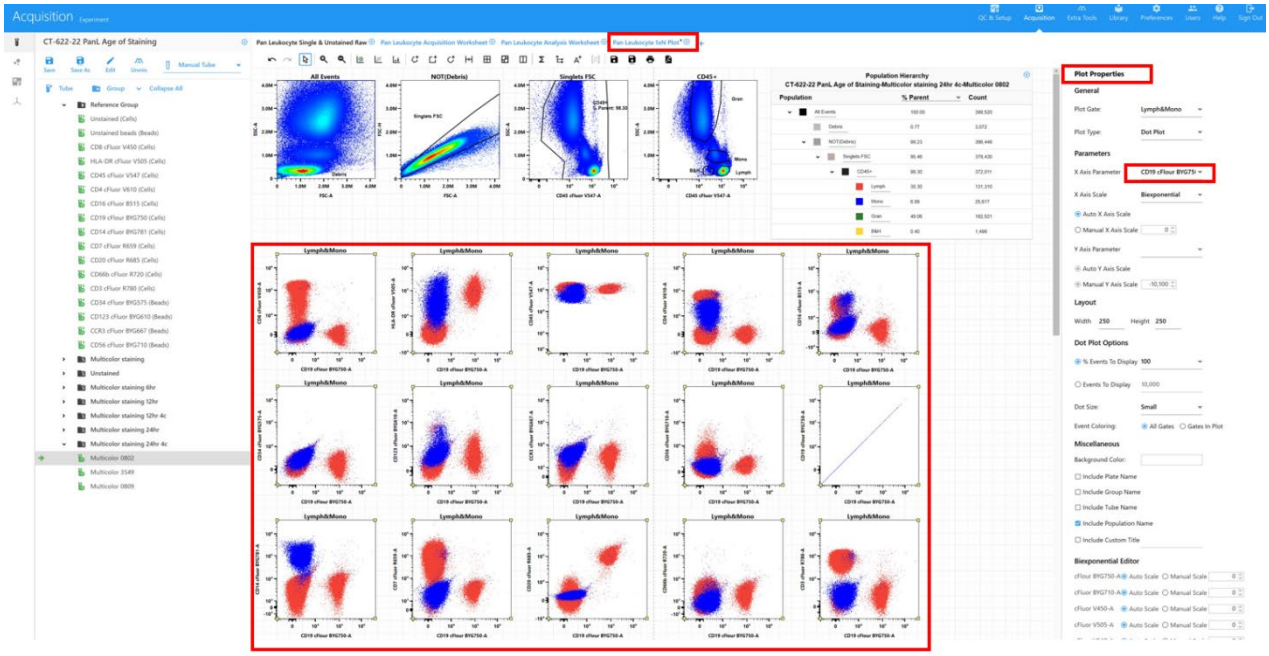
1. A Pan Leukocyte Analysis Worksheet is included in the experiment template to be used for multicolor sample data analysis. In this worksheet, an additional Time Plot is included as an initial plot to eliminate any abnormal cells (i.e. abnormal cells due to edge effect).
2. In case the recorded multicolor samples need spillover adjustment, right click on the recorded multicolor samples. From the drop-down menu, click **Edit Properties**.
3. Select the **Spillover** tab at the top of the pop-up wizard.
4. Click on **Enable Compensation**. Leave the wizard open and move it aside.
5. To check and adjust for unmixing error on **Pan Leukocyte 1xN Plot** worksheet:
    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

Check all 15 permutation plots against the first fluorochrome for any unmixing errors (i.e. the positive and negative populations are well aligned) and adjust spillover (compensation) as needed. Do this for all 15 fluorochromes by selecting each fluor one by one under **X Axis Parameter**.




***Note**: Unmixing issues such as under-unmixing and over-unmixing can be adjusted using compensation in the adjust spillover matrix (Ashhurst, T. M., Smith, A. L., & King, N. J. C. (2017). High-dimensional fluorescence cytometry. Current Protocols in Immunology,2017,5.8.1–5.8.38. doi: 10.1002/cpim.37). Most corrections, if necessary, will be below 5%. If a correction higher than 3% is necessary, please contact your local TAS for further troubleshooting.*



Using 1XN Plots to Assess Unmixing Errors:

***Note:*** *in the 1xN plot, below the permutation plots, there are also SSC vs each individual marker. Check on these plots to make sure the correct staining pattern for each marker is observed.*

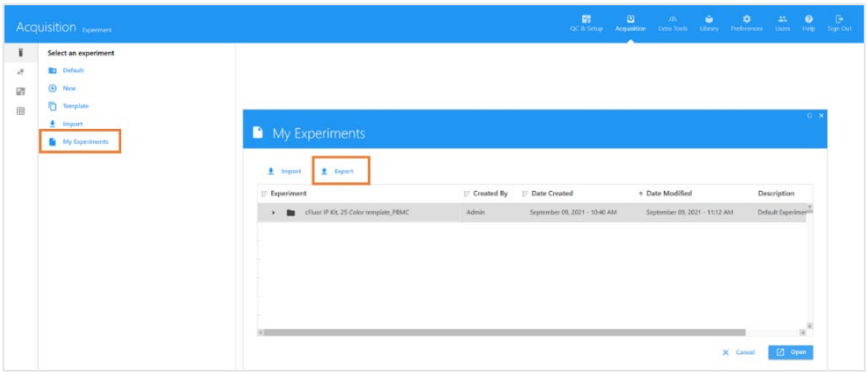
5. Click **Save** and close out of the Tube/Well Properties wizard. The adjusted compensation matrix can be applied to other multicolor samples by right clicking on the adjusted multicolor sample tube and selecting **Copy Spillover** and then right click the other tubes you want to correct and select **Paste Spillover**, if similar unmix errors are observed.
6. Repeat the unmixing error adjustment for all multicolor samples as needed
7. Manually adjusted compensation matrix can be saved to library for future use if using SpectroFlo® Version 3.0.
8. Double click on the multicolor sample tube to open **Pan Leukocyte Acquisition** worksheet to view and adjust gates in the worksheet.

***NOTE****: Refer to* ***Appendix D*** *in page 13 for an example of a multicolor sample.*

9. Statistics analysis table in the **Pan Leukocyte Acquisition** worksheet shows percentage of each leukocyte subset in CD45$^+$ WBC and percentage of each lymphocyte subset in total lymphocytes.

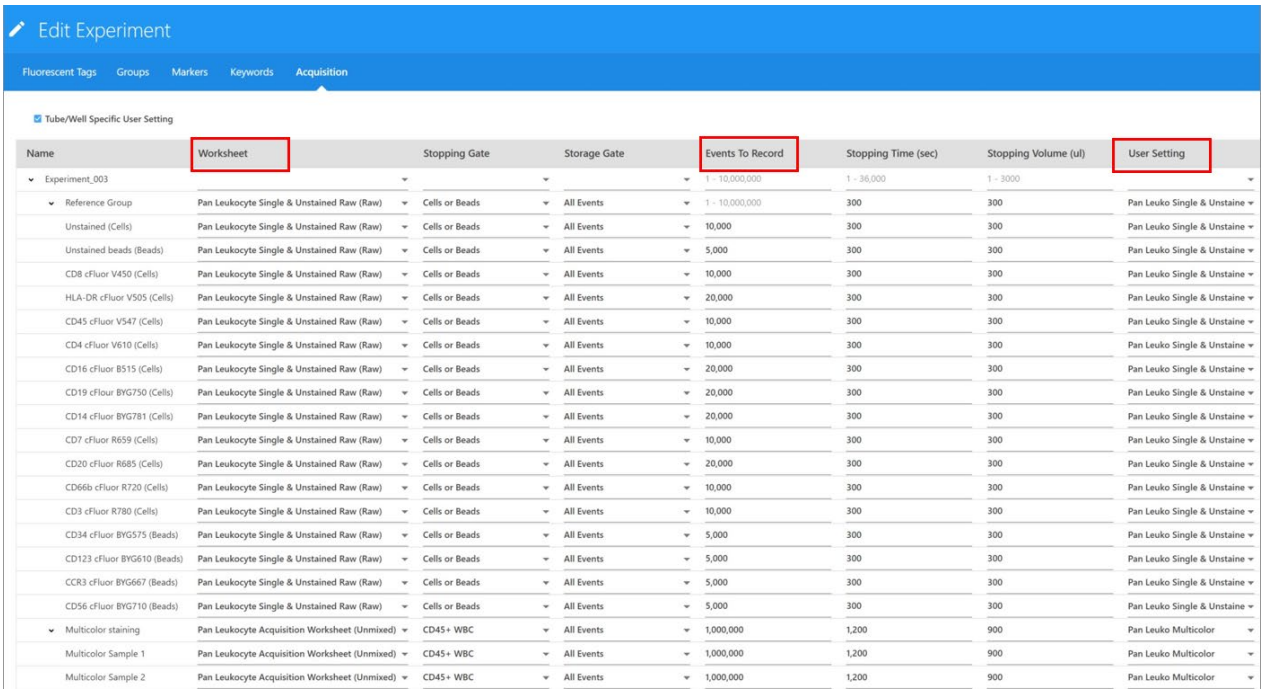
***NOTE****: Refer to* ***Appendix E*** *in page 14 for an example of a statistics table.*

10. 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: Acquisition Setup

Example of Acquisition Setup using cells or beads as References. For single color reference or unstained sample, the specific Worksheet **Pan Leukocyte Single & Unstained Raw** and User setting **Pan Leuko Single & Unstained** should be selected. The Multicolor sample has its own specific Worksheet and User setting, **Pan Leukocyte Acquisition** and **Pan Leuko Multicolor** as shown below.


Edit Experiment
Fluorescent Tags
Groups
Markers
Keywords
Acquisition
Tube/Well Specific User Setting
Name
Worksheet
Stopping Gate
Storage Gate
Events To Record
Stopping Time (sec)
Stopping Volume (ul)
User Setting
Experiment_003
1 - 10,000,000
1 - 36,000
1 - 3000
Reference Group
Pan Leukocyte Single & Unstained Raw (Raw)
Cells or Beads
All Events
1 - 10,000,000
300
300
Pan Leuko Single & Unstaine
Unstained (Cells)
10,000
Unstained beads (Beads)
5,000
CD8 cFluor V450 (Cells)
HLA-DR cFluor V505 (Cells)
20,000
CD45 cFluor V547 (Cells)
CD4 cFluor V610 (Cells)
CD16 cFluor B515 (Cells)
CD19 cFluor BYG750 (Cells)
CD14 cFluor BYG781 (Cells)
CD7 cFluor R659 (Cells)
CD20 cFluor R685 (Cells)
CD66b cFluor R720 (Cells)
CD3 cFluor R780 (Cells)
CD34 cFluor BYG575 (Beads)
CD123 cFluor BYG610 (Beads)
CCR3 cFluor BYG667 (Beads)
CD56 cFluor BYG710 (Beads)
Multicolor staining
Pan Leukocyte Acquisition Worksheet (Unmixed)
CD45+ WBC
1,000,000
1,200
900
Pan Leuko Multicolor
Multicolor Sample 1
Multicolor Sample 2

## Appendix B: Reusing Single Color Controls

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

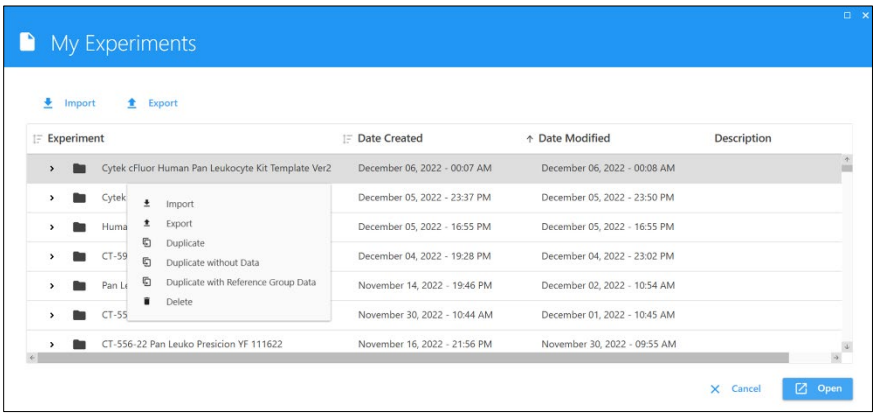
***NOTE:*** *For best results, maintain the instrument properly, perform QC daily and use the same reagent lot. The samples need to be collected using the same instrument as the reference controls.*

### 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 gain at low flow rate, change threshold to **V8** (the peak channel for cFluor® V547 anti-human CD45, the experimental template is pre-set), and adjust threshold to exclude CD45 negative debris in **Pan Leukocyte Acquisition** worksheet

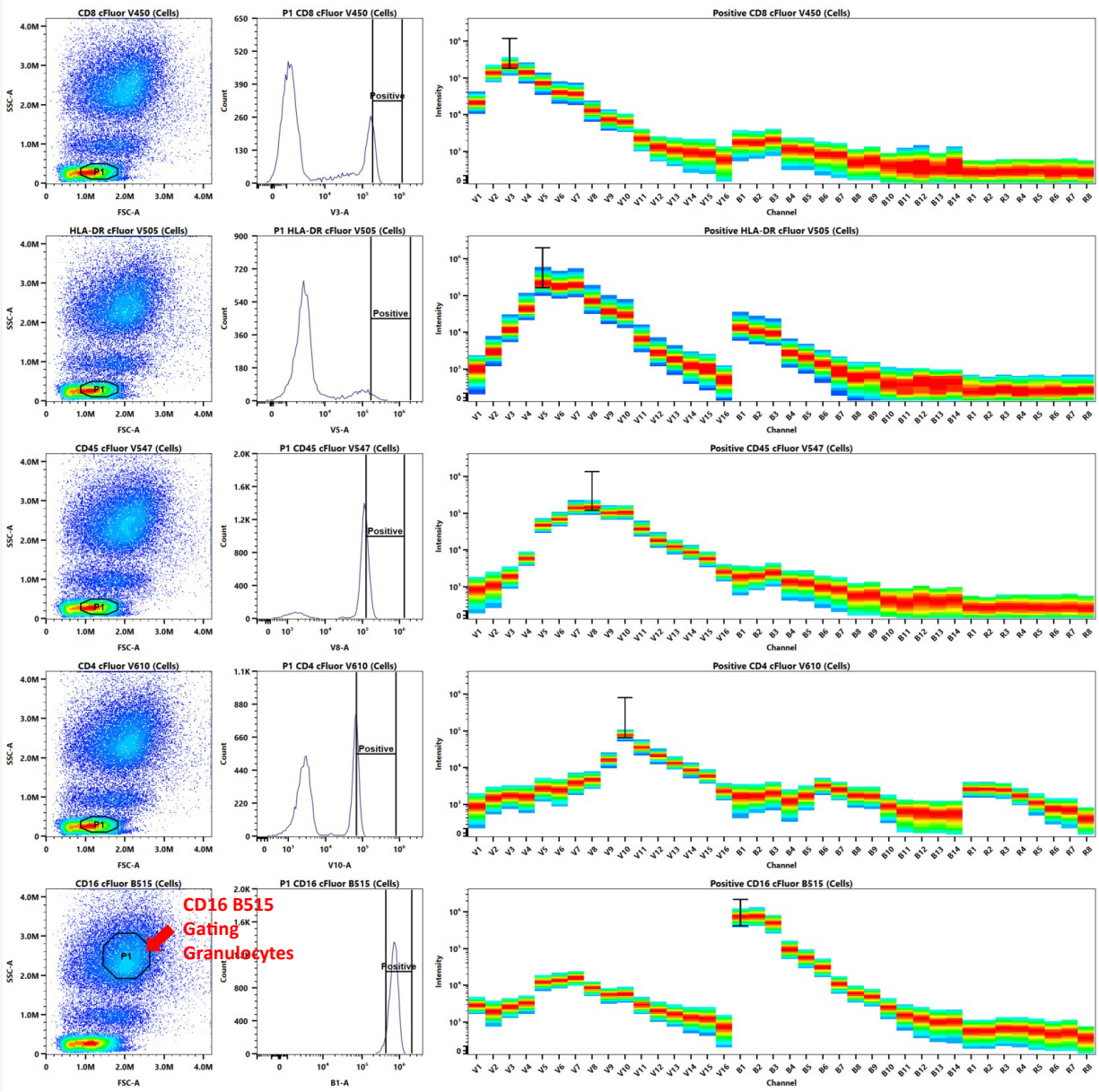
***Note:*** *confirm the adjustment of V8 threshold in* ***Pan Leukocyte Single & Unstained Raw*** *worksheet*

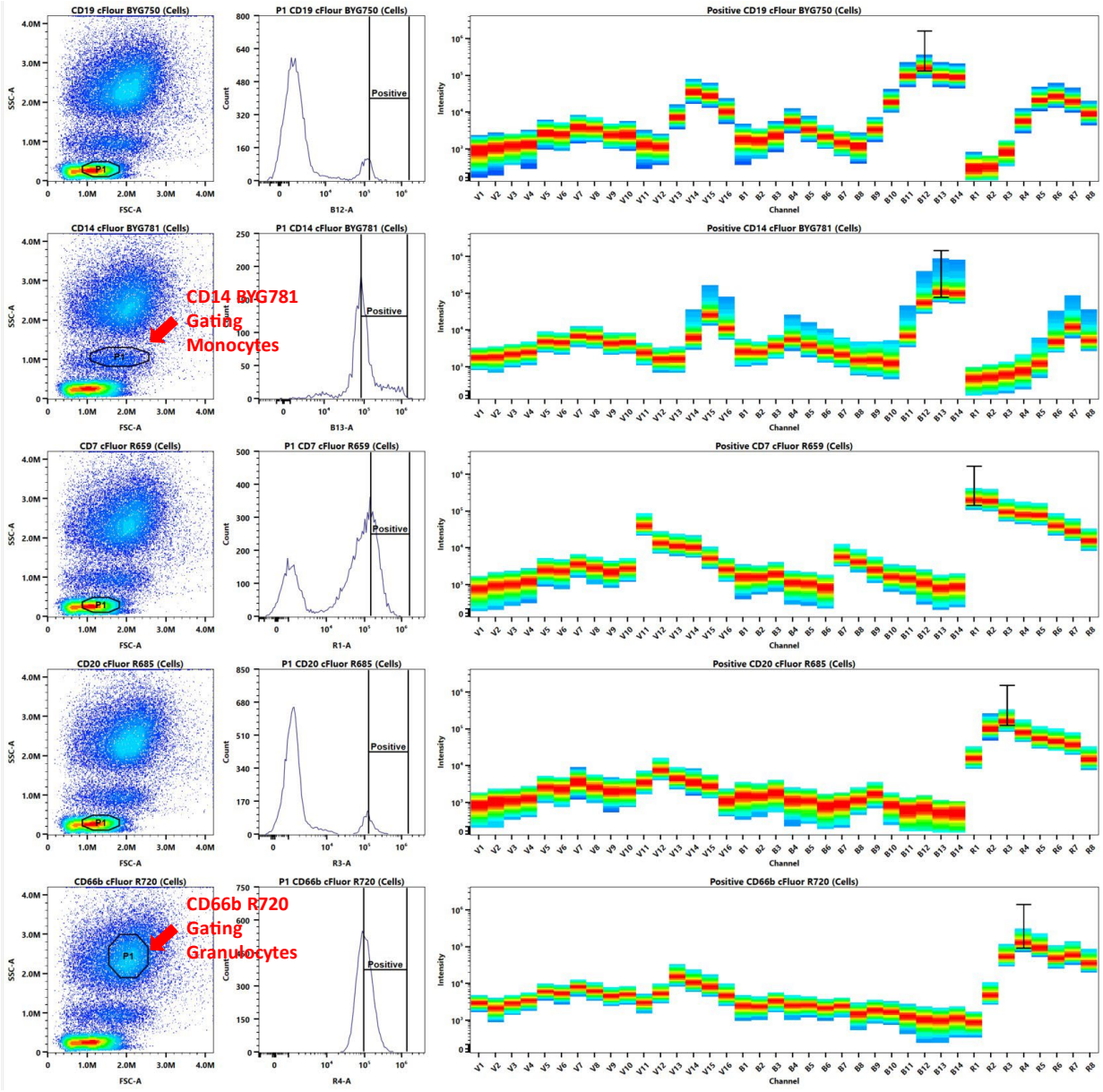
8. Acquire at high flow rate and preview for at least 10 seconds until the event rate stabilizes, then click **Record** to record each sample.
9. Acquire the stained samples within 6 hours post staining. It will take approximately 15 min per multicolor tube.
10. Adjust the gates in the analysis worksheet as needed.

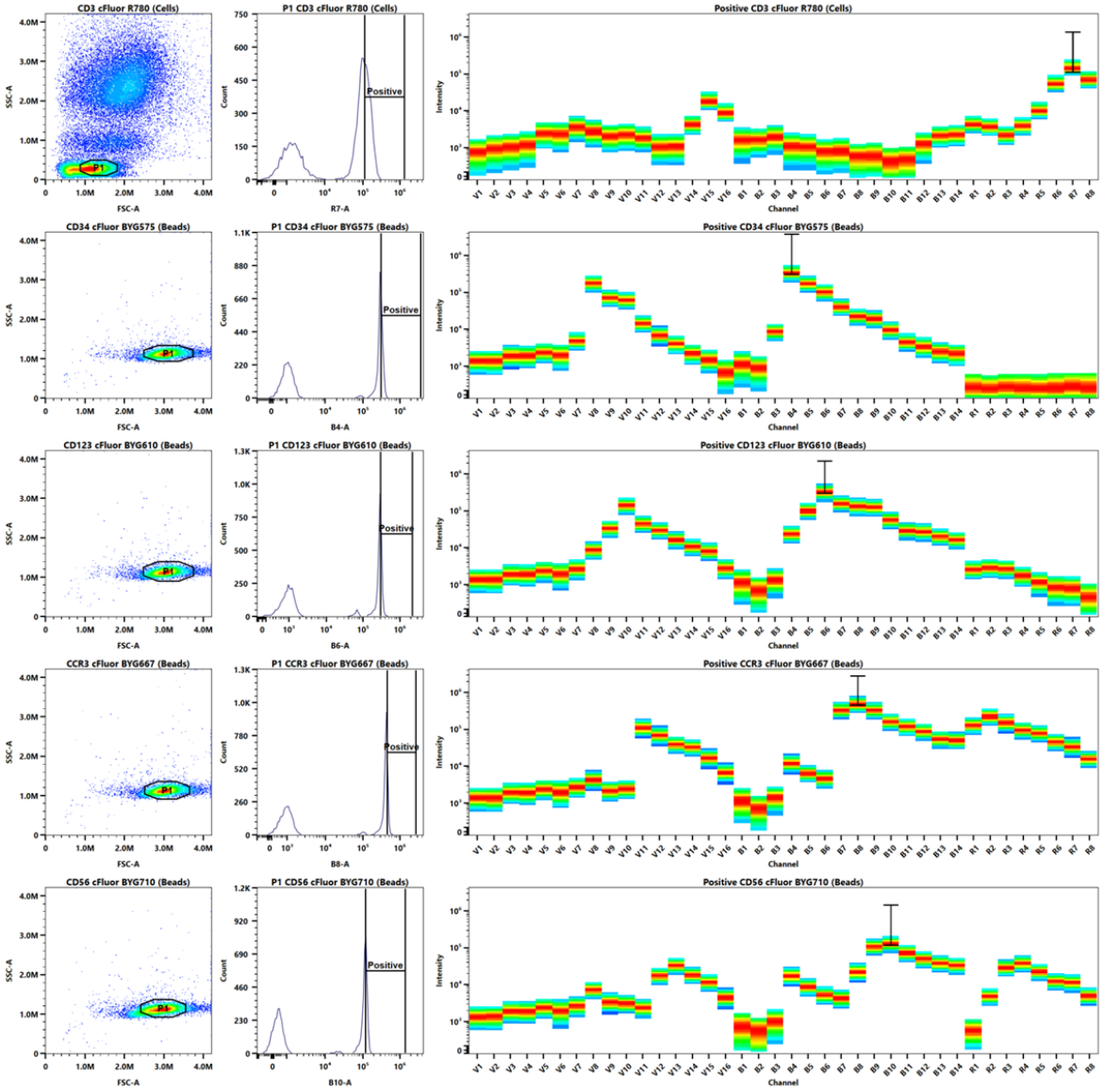


## Appendix C: Single-Color Control Gating and Signatures for 3-Laser (V-B-R) Cytek Cytometers

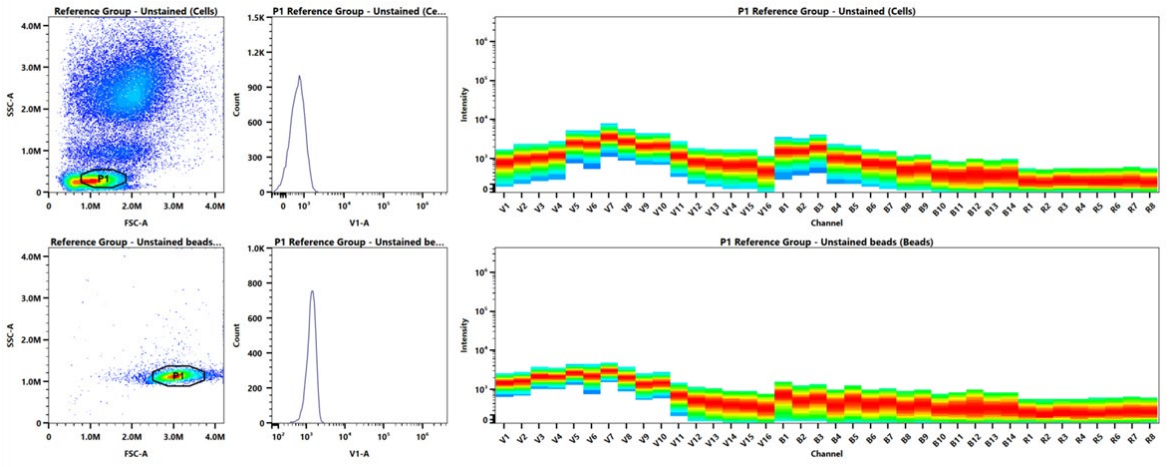
Example of gating and spectrum signatures for single color controls.



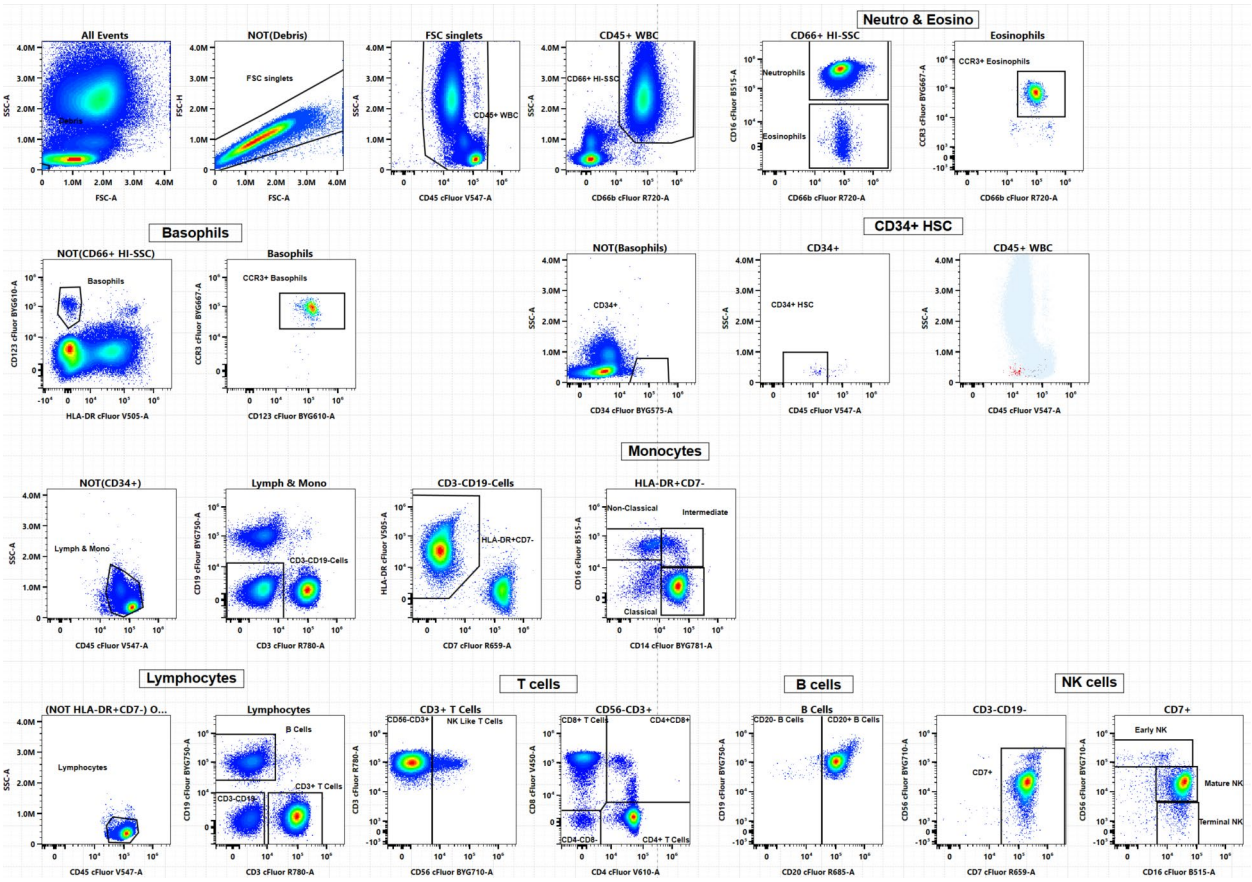

CD19 cFlour BYG750 (Cells)
P1 CD19 cFlour BYG750 (Cells)
Positive CD19 cFlour BYG750 (Cells)
CD14 cFlour BYG781 (Cells)
P1 CD14 cFlour BYG781 (Cells)
Positive CD14 cFlour BYG781 (Cells)
CD14 BYG781
Gating
Monocytes
CD7 cFlour R659 (Cells)
P1 CD7 cFlour R659 (Cells)
Positive CD7 cFlour R659 (Cells)
CD20 cFlour R685 (Cells)
P1 CD20 cFlour R685 (Cells)
Positive CD20 cFlour R685 (Cells)
CD66b cFlour R720 (Cells)
P1 CD66b cFlour R720 (Cells)
Positive CD66b cFlour R720 (Cells)
CD66b R720
Gating
Granulocytes
Positive
P1
SSC-A
FSC-A
Count
Intensity
Channel




Example of gating and spectrum signatures for Unstained



# Appendix D: Example of Gating in a Multicolor Sample



## Appendix E: Example of Statistics Table

| Statistics Table 1<br>CT-547-22 PL CCR3 vs Sig8-3067-Full CCR3 | | |
|---|---|---|
| **Gate** | **% CD45+ WBC** | **% Lymphocytes** |
| CD45+ WBC | N/A | 317.29 |
| Neutrophils | 58.36 | 185.16 |
| Eosinophils | 1.15 | 3.64 |
| Basophils | 0.38 | 1.20 |
| CD34+ HSC | 0.02 | 0.07 |
| Non-Classical | 0.80 | 2.54 |
| Intermediate | 0.52 | 1.65 |
| Classical | 6.11 | 19.38 |
| Total Monocytes | 7.40 | 23.49 |
| Lymphocytes | 31.52 | N/A |
| B Cells | 4.55 | 14.43 |
| Early NK | 0.12 | 0.39 |
| Mature NK | 2.81 | 8.93 |
| Terminal NK | 0.27 | 0.86 |
| Total NK Cells | 3.20 | 10.16 |
| CD3+ T Cells | 23.47 | 74.46 |
| CD8+ T Cells | 6.59 | 20.89 |
| CD4+ T Cells | 14.84 | 47.07 |
| NK Like T Cells | 0.99 | 3.13 |

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

cFluor® V547, cFluor® B515, cFluor® R685 and cFluor® R720 are equivalent to CF®405L, CF®488A, CF®660C 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.

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

cFluor® BYG610, cFluor® BYG667, cFluor® BYG710, cFluor® BYG750, and cFluor® BYG781 are tandem dyes made with R-PE. cFluor® R780 is a tandem dye made with APC. Caution – Tandem dyes may show changes in their emission spectra with prolonged exposure to light or fixatives.