

Acquisition Protocol for Cytek® 25-Color Immunoprofiling Assay

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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.

Lik	orary Fluorescent Tags						ि QC & Setup	C /A Acquisition Extra Tools	Library Preferences	📫 🕑 🕞 Users Help Sign Out
QC	QC Beads	Fluorescent Tag Groups				Current Group: Cytek	25-color Immunoprofiling A	ssay Tags		
	Fluorescent Tags	🛓 Import 🏦 Export 💮 New	🧨 Edit	× Remove		🕀 Add 🧪 Ed	it 🗙 Remove			
Ϋ́	Labels	I≓ Name	IF Created By	E Date Created	:≓ Date I	IF Name	Emission Waveleng	E Laser Excitation	17 Display Name	Synonyms
<u>.</u>	User Settings	Blue Laser	System	June 10, 2019 - 14:21 PM	Septem	BV421	421	405	BV421	
.9	Worksheet Templates	Red Laser	System	June 10, 2019 - 14:21 PM	June 14	BV510	510	405	BV510	
н	Experiment Templates	Violet Laser	System	June 10, 2019 - 14:21 PM	Septem	BV570	570	405	BV570	BV571
		Fluorescent Proteins	System	June 10, 2019 - 14:21 PM	June 10	BV650	650	405	BV650	
-	Keywords	Viability	System	June 10, 2019 - 14:21 PM	June 14	BV711	711	405	BV711	BV710
111	Loader Settings	UV Laser	System	July 29, 2019 - 09:27 AM	August	BV750	750	405	BV750	
		YellowGreen Laser	System	July 29, 2019 - 09:27 AM	Septem	BV785	785	405	BV785	
		Cytek 25-color Immunoprofiling Assay Tags	Admin	September 24, 2021 - 13:12 PM	Septem	cFluor V450	450	405	cFluor V450	cFluorV450
						cFluor B515	515	488	cFluor BS15	cFluorB515
						cFluor B532	532	488	cFluor B532	
						cFluor V547	547	405	cFluor VS47	cFluorV547
						cFluor B548	548	488	cFluor B548	
						cFluor BYG575	575	561	cFluor BYG575	cFluorBYG575
						cFluor YG584	584	561	cFluor YG584	
						cFluor BYG610	607	561	cFluor BYG610	
						cFluor R659	659	635	cFluor R659	cFluorR659
						cFluor BYG667	667	561	cFluor BYG667	cFluorBYG667
						cFluor R668	668	635	cFluor R668	cFluorR668
						cFluor R685	685	635	cFluor R685	
						cFluor B690	690	488	cFluor B690	cFluorB690
						cFluor BYG710	710	561	cFluor BYG710	cFluorBYG710
						chuor K720	720	635	CHUOT K720	cHuork720
						viabye ned	740	035	viauye kėd	-FL0700
						criuor R/G701	701	621	chuor R/80	chuor@VG701
						childr BTG/81	040	626	CHUOT BTG/81	chuor040
						criuor ka40	040	030	criuor no40	criuomono

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.

Lib	rary Experiment Templa			QC & Setup	Acquisition Extra Tools	Library	🗢 Preferences	XX Users	? Help	
	QC Beads	Experiment Templates								
÷¢÷	Fluorescent Tags	🛓 Import 🏦 Export 🗙 Remove								
Ϋ́	Labels	t≓ Name	Created By	17 Date Created	Description					
20	User Settings	Cytek 25-color IP Assay Template_PBMC 🧨	Admin	September 24, 2021 - 13:15 PM	Default Experiment	1				
••	Worksheet Templates	✓ Default	Admin	June 10, 2019 - 14:21 PM	Default Experiment					
Ŭ	Experiment Templates									
\square	Keywords									
<u>++</u>	Loader Settings									



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"

	lisition Experiment			ୁଙ୍କ QC & Se	tup Acquisition		ib Library	🔅 Preferences	users	? Help
	Select an experiment									
	Default									
	New									
ſ	Template									
	1 Import	6	Expe	eriment Templates						
	My Experiments									
		1F	Experim	ent	17 Created By	1= Date Cr	eated		Desci	iption
			› 🖿	Cytek 25-color IP Assay Template_PBMC	Admin	Septemb	er 24, 2021	- 13:15 PM	Defau	t Experime
		~	› 🖿	Default	Admin	June 10, 2	2019 - 14:21	PM	Defau	t Experime

- 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.

Laser	Target	Fluorochrome	Recommended Sample Type
	CCR7	Brilliant Violet 421™	Cells or Beads
	CD45RA	cFluor® V450	Cells or Beads
	lgM	Brilliant Violet 510™	Cells or Beads
	CD20	cFluor® V547	Cells Only
Violet	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
	CD25	cFluor [®] BYG575*	Cells or Beads
	CD4	cFluor® YG584	Cells or Beads
	CD16	cFluor® BYG610	Cells or Beads
rellow/Green	lgD	cFluor® BYG667*	Cells Only
	ΤCRγδ	cFluor® BYG710	Cells or Beads
	CD11c	cFluor [®] BYG781*	Cells or Beads
	CD127	cFluor® R659*	Cells or Beads
	CD1c	cFluor® R668	Cells or Beads
Ded	CD19	cFluor® R685	Cells Only
Red	CD123	cFluor® R720	Cells or Beads
	CD45	cFluor® R780*	Cells or Beads
	CD27	cFluor® R840	Cells or Beads

Table 1. Sample	Type Recommendations
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Unmix reference controls

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.

Acc		😰 U A 🛶 🗘 🕰 🥹 O2.5.5exp Aquidato Latura Note Usary Pedereces Uses Help Sig	n Out
¥.	Cytek 25-color IP Assay Template_PBMC	Centra Scatter P Aug, Ram 3 Centr # Aug, Frem, FREC 8 Centra # Aug, Frem,	
	Save As Edit Unnix		
=	Reference Group Urstained (Cells)	1.000 Sugin A 100 UV	
	CCR7 BV421 (Cells) CD45RA cFluor V450 (Cells)	2 200 1 2 200 1 1 1 1 1 1 1 1 1 1 1 1 1	
	IgM EV510 (Cells) CD20 cfluor V547 (Cells)		
	CO28 8V650 (C88)	их им им на	



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 page 12, autofluorescence extraction is NOT recommended.

JNSTAINED CONTRO	LS				
O Use Control from Libra	агу	~			
Use Control from Expe	eriment Reference Gr	oup - Unstained (Cells)			
Name		Control Type			
Reference Group - Un	stained (Cells)	Cells			
TAINED CONTROLS					
From Library	Fluorescent Tag	Control		Unstained	Generic
	BV421	CCR7 BV421 (Cells)	٣	•	
	cFluor V450	CD45RA cFluor V450 (Cells)	٣	*	
	BV510	IgM BV510 (Cells)	Ŧ	·	
	cFluor V547	CD20 cFluor V547 (Cells)	*	•	
	BV570	CD3 BV570 (Cells)	٣	*	
	BV650	CD28 BV650 (Cells)	Ŧ	Ψ	
	BV711	CD38 BV711 (Cells)	Ŧ	Ť	
	BV750	CD56 BV750 (Cells)	Ť	-	
	cEluor P515	CD141 cEluor B515 (Cells)	-		
	cFluor B532	CD8 cFluor B532 (Cells)	*	· · · · · · · · · · · · · · · · · · ·	
	cFluor B548	CD14 cFluor B548 (Cells)	*	•	
	cFluor B690	HLA-DR cFluor B690 (Cells)	Ŧ		Z
	cFluor BYG575	CD25 cFluor BYG575 (Cells)	Ŧ	*	
	cFluor YG584	CD4 cFluor YG584 (Cells)	Ŧ	*	2
	cFluor BYG610	CD16 cFluor BYG610 (Cells)	¥	*	
	cFluor BYG667	IgD cFluor BYG667 (Cells)	Ŧ	~	
	cFluor BYG710	TCRgd cFluor BYG710 (Cells)	Ŧ	•	
	cFluor BYG781	CD11c cFluor BYG781 (Cells)	Ŧ	•	
	cFluor R659	CD127 cFluor R659 (Cells)	Ŧ	•	
	cFluor R668	CD1c cFluor R668 (Cells)	*	•	
	cFluor R685	CD19 cFluor R685 (Cells)	*	*	
	cFluor R720	CD123 cFluor R720 (Cells)	Ŧ	Ŧ	
	ViaDye Red	Viability ViaDye Red (Cells)	٣	Reference Group - Unstained (Cells) 👻	
	cFluor R780	CD45 cFluor R780 (Cells)	Ŧ	Reference Group - Unstained (Cells) 👻	
	cFluor R840	CD27 cFluor R840 (Cells)	٣	· · · · · · · · · · · · · · · · · · ·	

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.

 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.

Save Save As Edit	M Unmix Unmix	🖍 Tube/We	ll Prop	erties												
🐉 Tube 📑 Group	✓ Collapse All	Tube/Well Fluore						pillover								
Reference Gro Multicolor	up	Enable Compensation	🛨 Apply	From Library	👲 Apply P	rom File 🛛 📦	Save To Libra	ry ≜ s	iave To File							
A R DE Color Multi	enlos	INTO + FROM +	BV421	cFluor V450	BV510	cFluor V547	BV570	BV650	BV711	BV750	BV785	cFluor B515	cFluor B532	cFluor B548	cFluor B690	cFluor B
	COLOR	BV421	100	0	0	0	1.56	0	0.63	0	0	0	0	0	0	0
	Delete	cFluor V450	0	100	0	0	0	0	0	0	0	0	0	0	0	0
	Rename	BV510	0	0	100	0	0	0	0	0	0	0	0	0	0	0
		cFluor V547	0	0	0	100	0	0	0	0	0	-0.62	0	0	0	0
	Duplicate	BV570	0	1.79	0.76	0	100	0	0	0	0	0.3	0	0	0	0
	🛠 Cut	BV650	0	0	0.66	0	0	100	0	0	0	0	0	0	0	0
	🗈 Сору	BV711	0	0	0	0	0	0	100	0	0	0	0	0	0	0
		BV750	0	0	0	0	0	0	0	100	0	0	0	0	0	0
	Import FCS File	8V785	0	0	0	0	0	0	0	0	100	0	0	0	0	0
	Copy Spillover	cFluor B515	0	0	0	0	0	0	0	0	0	100	0	0	0	0
	26 Paste Spillover	cFluor B532	0	0	0	0	0	0	0	0	0	0	100	-1.08	0	0
	A Impact Spilleurs	cFluor B548	0	0	0	0	0	0	0	0	0	0	0	100	0	0
	± import spillover	cFluor B690	0	0	0	0	-1.99	0	0	0	0	0	0	0	100	0
	Export Spillover	cFluor BYG575	0	0	0	0	-0.58	0	0	0	0	0	0	0	0	100
	B Batch Analysis	cFluor YG584	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	 Edit Properties 												Ð	Restore Default		

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.



ube/Well Fluor	escent Tag	Acquisition	Keywords	Instrument Se	ttings S	pillover			
Enable Compensatior	n 🛨 Appl	y From Library	🛃 Apply	From File 🛛 📦	Save To Libra	ary 🟦 S	ave To File		
NTO ↓ FROM →	BV421	cFluor V450	BV510	cFluor V547	BV570	BV650	BV711	BV750	BV785
3V421	100	0	0	0	1.56	0	0.63	0	0
Fluor V450	0	100	0	0	0	0	0	0	0
3V510	0	0	100	0	0	0	0	0	0
Fluor V547	0	0	0	100	0	0	0	0	0
3V570	0	1.79	0.76	0	100	0	0	0	0
3V650	0	0	0.66	0	0	100	0	0	0
3V711	0	0	0	0	0	0	100	0	0
3V750	0	0	0	0	0	0	0	100	0
3V785	0	0	0	0	0	0	0	0	100
Fluor B515	0	0	0	0	0	0	0	0	0
Fluor B532	0	0	0	0	0	0	0	0	0
Fluor B548	0	0	0	0	0	0	0	0	0
	4	^	^	~	4 00	^	^	~	~

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**.

			ा QC & Setup	Acquisition Extra Tools L	🐞 💠 🏔 O Jbrary Preferences Users H
Select an experiment					
Default					
New					□ ×
Template	My Experiments				
Import My Experiments	🔮 Import 🚊 Export				
	IF Experiment	IF Created By	17 Date Created	↑ Date Modified	Description
	Cytek 25-color IP Assay Template_PBMC	Admin	September 24, 2021 - 12:22 PM	September 24, 2021 - 13:41 PM	M Default Experiment
	¢				
				\$	X Cancel 🛛 Open



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

	periment	t			
Fluorescent Tags	Groups	Markers Keyword	s Acquisition		
💽 Group	🗗 Tube 🚺	Remove	Reference Group	Reference Group	
Name					
🗸 🖿 cFluc	or IP Kit, 25 Color	template_PBMC			
> R	Reference Group				
√ S	Multicolor				
	25 Color sample	Duplicate			

6. Under Acquisition, define Worksheet, Stopping Gate, and Events to Record

7. Click Save and Open

/	Edit Experiment				
	Fluorescent Tags Groups M	Aarkers Keywords Acquisition			
	Name	Worksheet	Stopping Gate	Storage Gate	Events To Record
	 Cytek 25-color IP Assay Template 	_PBMC	*	.	✓ 1 - 10,000,000
	✓ Reference Group	Cytek 25-color IP Assay_Raw (Raw)	*	→ All Events	▼ 1 - 10,000,000
	Unstained (Cells)	Cytek 25-color IP Assay_Raw (Raw)	- Lymphocytes	→ All Events	✓ 10,000
	CCR7 BV421 (Cells)	Cytek 25-color IP Assay_Raw (Raw)	- Lymphocytes	✓ All Events	▼ 10,000

- 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













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

BV421	1																									
cFluor V450	0.78	1																								
BV510	0.16	0.35	1																							
cFluor V547	0.07	0.19	0.88	1																						
BV570	0.18	0.18	0.55	0.74	1																					
BV650	0.1	0.08	0.16	0.24	0.24	1																				
BV711	0.09	0.07	0.06	0.08	0.08	0.43	1																			
BV750	0.05	0.04	0.04	0.05	0.05	0.23	0.69	1																		
BV785	0.08	0.06	0.02	0.03	0.03	0.15	0.48	0.83	1																	
cFluor B515	0.02	0.03	0.06	0.04	0.04	0.01	0.01	0	0	1																
cFluor B532	0	0.01	0.03	0.03	0.03	0.01	0	0	0	0.92	1															
cFluor B548	0.03	0.07	0.37	0.4	0.3	0.08	0.03	0.02	0.01	0.53	0.72	1														
cFluor B690	0	0	0.03	0.04	0.07	0.37	0.53	0.29	0.19	0.01	0.01	0.08	1													
cFluor BYG575	0	0.01	0.11	0.15	0.54	0.05	0.01	0	0	0.06	0.09	0.26	0.05	1												
cFluor YG584	0	0.01	0.05	0.07	0.45	0.07	0.01	0	0	0.01	0.01	0.07	0.06	0.87	1											
cFluor BYG610	0	0.01	0.07	0.11	0.33	0.19	0.05	0.02	0.01	0.03	0.05	0.19	0.26	0.47	0.55	1										
cFluor BYG667	0	0	0.01	0.02	0.09	0.32	0.17	0.05	0.03	0.01	0.01	0.07	0.71	0.11	0.16	0.44	1									
cFluor BYG710	0	0	0.01	0.02	0.06	0.19	0.27	0.1	0.07	0.01	0.01	0.05	0.8	0.08	0.11	0.27	0.65	1								
cFluor BYG781	0	0	0.01	0.01	0.02	0.04	0.11	0.15	0.18	0	0.01	0.02	0.24	0.04	0.04	0.06	0.14	0.31	1							
cFluor R659	0	0.01	0.03	0.04	0.06	0.39	0.23	0.08	0.04	0.01	0	0.02	0.34	0.04	0.07	0.16	0.52	0.26	0.05	1						
cFluor R668	0.01	0.01	0.02	0.01	0.02	0.2	0.21	0.04	0.02	0.01	0	0.01	0.31	0.01	0.02	0.06	0.36	0.2	0.03	0.89	1					
cFluor R685	0	0	0.01	0.01	0.01	0.2	0.32	0.08	0.05	0	0	0.01	0.39	0.01	0.02	0.05	0.32	0.28	0.06	0.68	0.89	1				
cFluor R720	0.01	0.01	0.03	0.02	0.02	0.15	0.47	0.19	0.12	0	0	0.01	0.34	0.01	0.01	0.02	0.18	0.25	0.08	0.43	0.53	0.7	1			
ViaDye Red	0.01	0.01	0.02	0.01	0.01	0.1	0.29	0.13	0.1	0	0	0	0.34	0.02	0.03	0.06	0.25	0.38	0.35	0.37	0.42	0.55	0.77	1		
cFluor R780	0	0	0	0.01	0.01	0.06	0.21	0.2	0.23	0	0	0	0.16	0	0.01	0.02	0.09	0.13	0.29	0.18	0.2	0.29	0.46	0.81	1	
cFluor R840	0	0	0	0	0.01	0.06	0.16	0.14	0.2	0	0	0	0.12	0.01	0.01	0.02	0.09	0.11	0.23	0.18	0.18	0.21	0.34	0.52	0.71	1
	BV421	cFluor V450	BV510	cFluor V547	BV570	BV650	BV711	BV750	BV785	cFluor B515	cFluor B532	cFluor B548	cFluor B690	cFluor BYG575	cFluor YG584	cFluor BYG610	cFluor BYG667	cFluor BYG710	cFluor BYG781	cFluor R659	cFluor R668	cFluor R685	cFluor R720	ViaDye Red	cFluor R780	cFluor R840
Complexity Index: 22.84																										

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

Note	er R685 Cfluor R72 0 0 0 0 0 0 0 1.27 0 0 0 0 0 0	cFlaor 8720 0 0 0 0 -1.21 0	 ViaDye Red 0 0 0 0 0 	 cFluor R780 0 0 0 0 	0 cFluor 18840 0 0 0 0	AF 0 0
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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









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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