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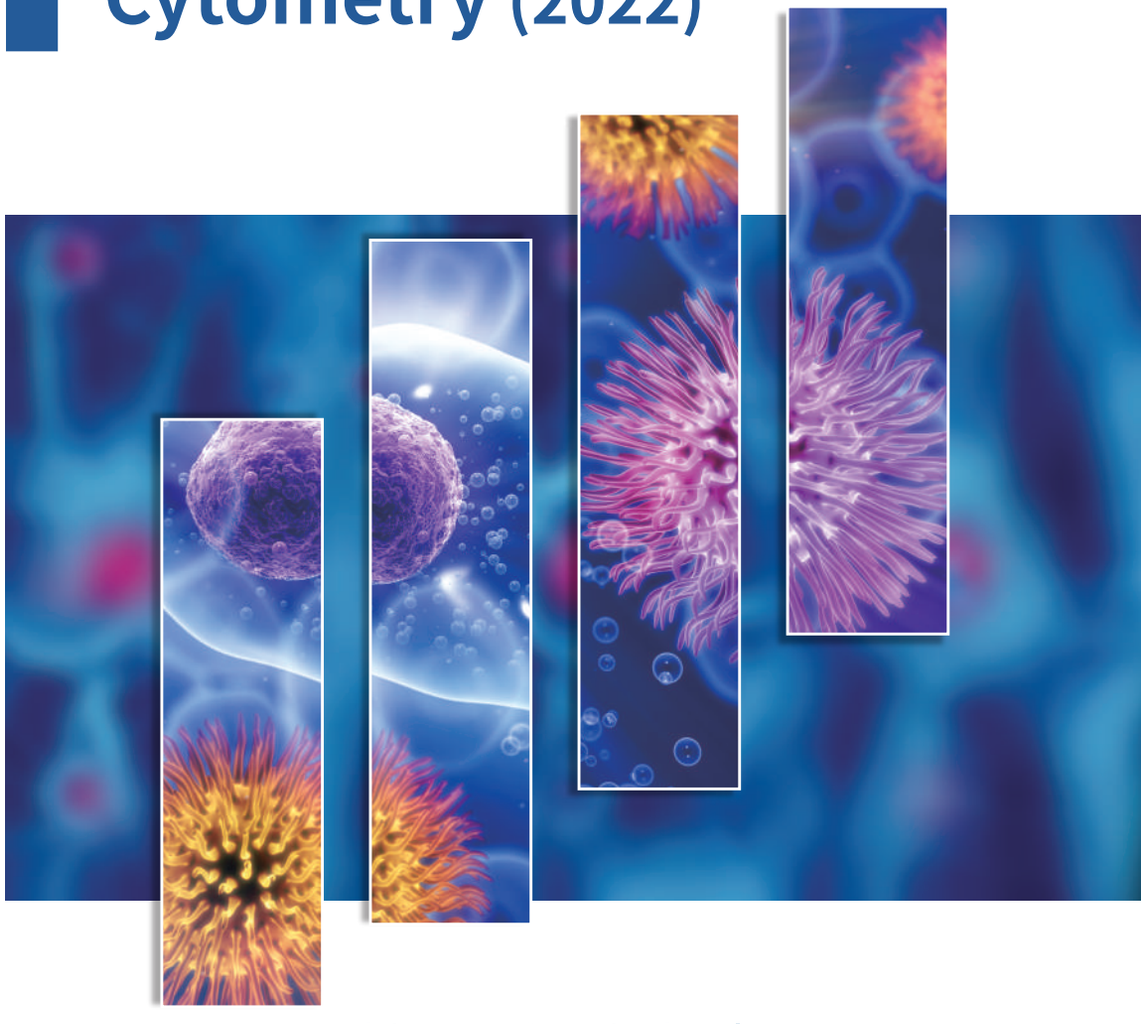
Multi-Color Panel Design in Flow Cytometry (2022)



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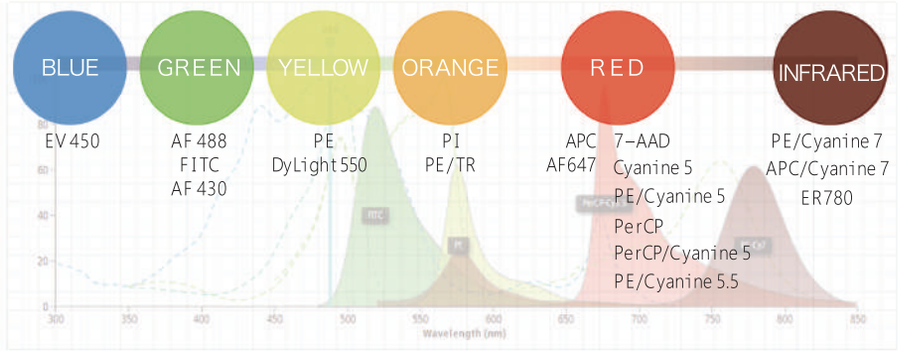


Balance Antigen Density and Fluorescence Brightness

High abundance antigen + Dim Fluorescence.
Low abundance antigen + Bright Fluorescence.

Avoid Spectral Overlap among Fluorescence

Low abundance antigen can be detected in non-interference channel.
High abundance antigen must be detected in channels that do not interfere with other channels.

Minimize the Complexity of Analysis

Allow the spillover of mutually exclusive antigens.
Allow the spillover of co-expressed antigens with highly abundance.
Allow the spillover of offspring to their parents, but not the opposite.

Use Tandem Dyes Carefully

Tandem dyes are necessary in multi-color panel design.
Easily degraded when exposed to light or undergoing fixation.
Follow protocols strictly to avoid tandem dyes degradation.

Cautions with Experiment Working Buffers

The acidic buffer or fixing step may destruct some dyes.
eg: FITC is susceptible to low pH condition
Fixation and extended storage lead to dye degradation.

02 Steps of Multi-Color Panel Design

STEP 01

Select the Target Markers

STEP 02

Check the Flow Cytometery Information

STEP 03

Check Fluorescence Information

STEP 04

Pair Antigen with Fluorescence

STEP 01

Select the Target Markers

Refer to relevant literature and select the target markers

Human	Maker
B Cells	CD19
T Cells	CD3, CD4, CD8
Treg Cells	CD4, CD25, CD127
Th1/Th2/Th17 Cells	CD4, IFN-γ, IL-4, IL-17
Dendritic Cells	CD1c, CD83, CD141, CD209, MHC II
Natural Killer Cells	CD3-, CD16, CD56
Macrophage	CD11b, CD68, CD163
Monocyte	CD14, CD16, CD64
Plasma Cells	CD138
Red Blood Cells	CD235a

Mouse	Maker
B Cells	CD19
T Cells	CD3, CD4, CD8
Treg Cells	CD4, CD25, Foxp3
Th1/Th2/Th17 Cells	CD4, IFN-γ, IL-4, IL-17
Dendritic Cells	CD11c, MHC II
Natural Killer Cells	CD3-, CD49b (clone DX5) or NK1.1
Macrophage	F4/80, CD11b, CD80, CD86, CD206
Monocyte	CD11b, CD115, Gr-1, Ly-6C
Plasma Cells	CD138
Red Blood Cells	TER-119

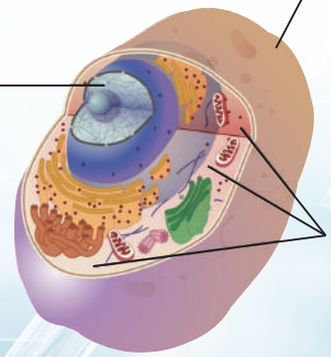
Check the marker locations

Cell membrane

- Direct labeling of living cells
  a. Most of CD markers

Nucleus

- Transcription factors and histone markers
- Severe fixation | Permeable cell and nuclear membrane | Washing after dyeing
  a. Foxp3



Cytoplasm

- Cytokinemarkers
- Mildfixation | Permeable cell membrane | Washing after dyeing
  a. Interleukins
  b. Interferon
  c. Tumor necrosis factor,etc.

Classification of cell markers

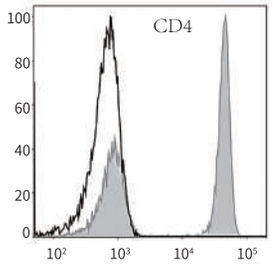
Generally speaking, most CD markers are located on the surface of cytomembrane. Cytokines, such as interleukins and interferon (IFN-α, IFN-β and IFN-γ), tumor necrosis factors (TNF-α, TNF-β) etc., are intracellular markers. And Foxp3 is the most popular intranuclear marker.

For the intracellular and intranuclear markers, the cell needs to be fixed and broken before staining. If there is any intracellular or intranuclear maker, by conventional method, the first step is to stain the surface markers. Because "fixation" is easy to damage the tandem fluorescein, tandem dyes shall be not used in this step.

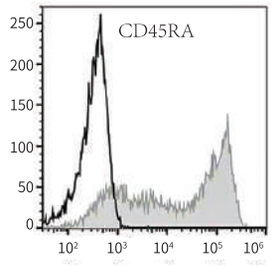
Check the antigen abundance

The antigen abundance can be roughly divided into three categories according to the expression of the corresponding antigen on/ in the cell types:

Easy to identify and antigens with obvious separation of negative-positive groups which can be easily distinguished. eg: CD3, CD4, CD19, etc..



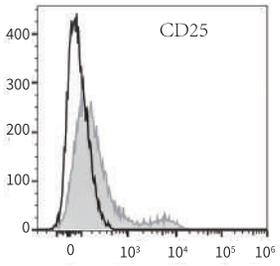
Negative and postive groups can be easily distinguished



Easy to identify, high abundance antigen expresses continuously. eg: CD27, CD28, CD45RA, CD45RO, etc..

High abundance antigen expresses continuously

Low abundance, active marker, or unknown but key markers. eg: CD25, STAT5, Foxp3, etc..



Low expression

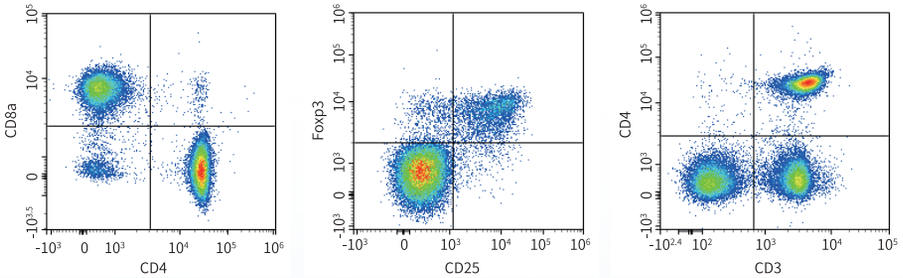
++Common cell surface antigen density ++

Cell Type	Marker	Density
Lymphocyte	CD3	++
	CD4	++
	CD8	++
	CD19	+
T cells	TCR	+++
	CD2	++
	CD3	+++
	CD5	++
	CD7	++
	CD45	++++
CD4+T cells	CD4	+++
	CD28	++
	CCR5	++
CD8+T Cells	CD8	++
	CD28	++
Monocyte	CD14	+++
	CD32	++
	CD64	++

Cell Type	Marker	Density
B cells	CD19	++
	CD20	+++
	CD21	++++
	CD22	++
	HLA-DR	+++
	CD11a	++
	CD40	+
	CD86	++
	CD80	+
Dendritic cells	CD11a	++
	CD40	++
	CD80	+++
	CD86	++++
NK Cells	CD56	++
Red blood cells	Glycophorin A	+++++
Neutrophils	CD14	+
	CD16	++++
Basic granulocyte	CD23	++

“+” means the antigen density is below 10,000.
“++” means the antigen density is 10,000~100,000.
“+++” means the antigen density is 100,000~200,000.
“++++” means the antigen density is 200,000~300,000.
“+++++” means the antigen density is above 300,000.

Check the markers interrelation



Mutual exclusion　　Co-expression　　Offspring and parents

The markers' relationship includes mutual exclusion, co-expression, offspring and parents, etc.

- Mutual exclusion means that two antigens will not be expressed on one cell at the same time, that is, if there is Protein A, there will be no Protein B, or if there is Protein B, there will be no Protein A. And mutually exclusive antigens allow fluorescence spillover. eg: T cells are divided into CD4+ T cells and CD8+ T cells. CD4+ T cells express CD4 but not express CD8, and CD8+ T cells express CD8 rather than CD4.

- Antigen co-expression means that two antigens are expressed on the same cell. eg: Mouse Treg cells express CD25 and Foxp3 at the same time. Co-expressed but highly expressed antigens allow spillover.

- If the markers are offspring and the parents. Parents must be analyzed first. It means that the offspring antigen is analyzed on the basis of the parent antigen. eg: All T cells express CD3, and T cells are divided into CD4+ T cells and CD8+ T cells. In this case, CD3 is the parent, CD4 and CD8 are the offspring. Generally speaking, the spillover of offspring to parents is allowed, but spillover of parents to offspring is forbidden.

STEP 02

Check the Flow Cytometery Information

Channel and optional fluorescence			
Flow cytometery	Excitation	Detector (Filter)	Common fluorescence
Take the flow cytometry with double laser as an example	488nm	530/30	FITC、AF488
		575/26	PE
		610/20	PE/TR、PE/AF594
		695/40	PerCP/Cyanine5.5、PE/Cyanine5、PerCP
		780/60	PE/Cyanine7
	633nm	660/20	APC、AF647
		730/45	AF700
		780/60	APC/Cyanine7、ER780

Different manufacturers or different models have different configurations, even if the same model may have different configurations. When designing the panels, we must check the configuration of flow cytometry before we select appropriate fluorescence. It is suggested to check the information as below:

① Excitation. There are several lasers can be used as excitation wavelength. The common flow cytometry lasers are 405nm, 488nm, 561nm, 633nm, etc..

② Detector. Detectors are used to analysis emission wavelength.

STEP 03

Check Fluorescence Information

- Check the fluorescein excitation and emission wavelength, and confirm which fluorescence can be used on the Flow cytometery according to the information of laser and detector.
- Check the relative brightness of the selected fluorescence.
- Check the spillover among the fluorescence.
- Check the characteristics of different fluorescence, and select the appropriate fluorescein according to the experimental purpose and requirements.

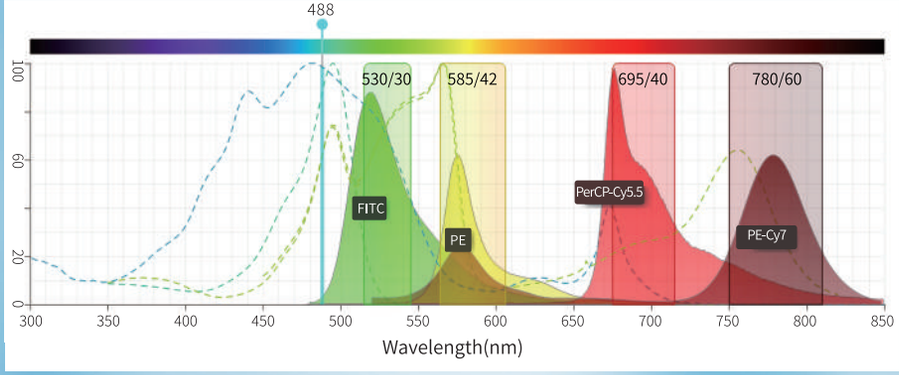
Fluorescence wavelength information

Fluorochrome	Fluorescence Emission Color	Excitation Laser Lines (nm)	Excitation Max(nm)	Emission Max(nm)
EV450	Blue	405	410	450
AF488	Green	488	495	520
FITC	Green	488	490	530
PE	Yellow	488, 532, 561	495, 565	575
PI	Orange	488, 532, 561	536	617
PE/TR	Orange	488, 532, 561	495,565	620
PE/AF594	Orange	488, 532, 561	495,565	615
7-AAD	Red	488, 532, 561	546	650
Cyanine 5	Red	633, 635, 640	650	670
APC	Red	633, 635, 640	650	660
AF647	Red	633, 635, 640	650	670
PE/Cyanine5	Red	488, 532, 561	495, 565, 655	670
PerCP	Red	488	440, 480, 675	675
PerCP/Cyanine5.5	Red	488	440, 480, 675	675
PE/Cyanine5.5	Far Red	488, 532, 561	495, 565, 675	690
PE/Cyanine7	Infrared	488, 532, 561	495, 565, 755	775
ER780	Infrared	633, 635, 640	625	765
APC/Cyanine7	Infrared	633, 635, 640	650, 760	780

Relative brightness of common fluorescence

	Very Bright	Bright	Moderate	Dim
Blue (488 nm)	PE PE/Cyanine7 PE/TR PE/AF594	PE/Cyanine5 PE/Cyanine5.5	FITC AF488 PerCP/Cyanine5.5	PerCP
Red (633 nm)		APC AF647		ER780 APC/Cyanine7
Violet (405 nm)				EV450

Overlap information of fluorescence



Fluorescence characteristics

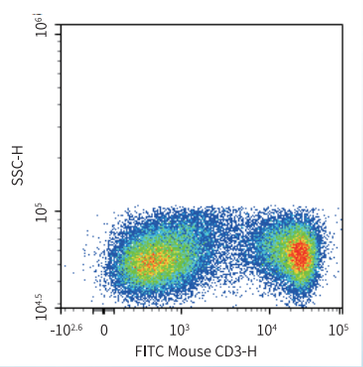
Fluorescence	Characteristics
FITC	Easily affected by pH value. When the pH value decreases, the fluorescence intensity also decreases.
AF488	Resistant to light and remains stable in a wide pH value (pH4~10).
PE	High brightness, relatively stable.
APC	High brightness, less stable than PE.
PerCP/Cyanine5.5	Relatively stable (brightness and fixation) tandem dye.
PE/Cyanine 5	High brightness, easy to quench, not suitable to fixation, no matching with APC.
ER780	Brightness is better than APC/Cyanine 7, which can replace APC/Cyanine 7. Suitable for fixation and has less spillover to APC detector.
APC/Cyanine 7	Weak brightness, not suitable for the analysis of low abundance antigens. Easy to quench and not suitable for fixation.
PE/Cyanine7	High brightness, easy to quench, not suitable for fixation, no overlap with FITC, little interference and spillover with APC.

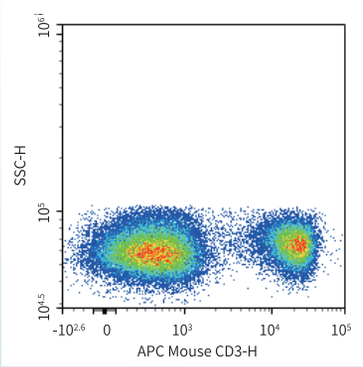
STEP 04

Pair Antigen with Fluorescence

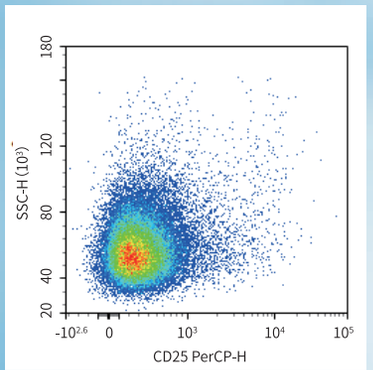
Balance Antigen Density and Fluorescence Brightness

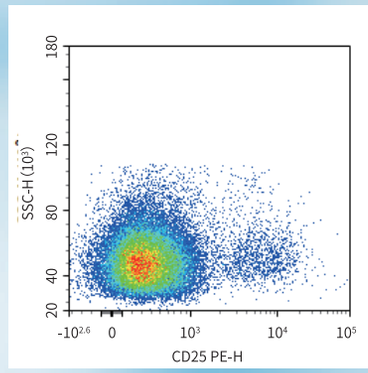
For high abundance antigen, weak or strong fluorescein can be selected. As shown in the figure, high abundance antigen CD3 selects weak fluorescein FITC or strong fluorescein APC, in both situations, the results can be obviously observed.





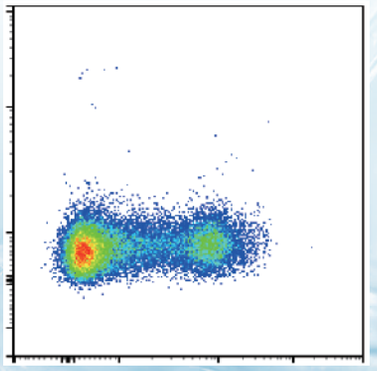
For low abundance antigen, strong fluorescein must be selected. As shown in the figure, weak fluorescein PerCP is selected by low abundance antigen CD25, leading to the inseparability of Negative-Positive cell groups. If strong fluorescein PE is used, positive cell groups can be obvious to observe.



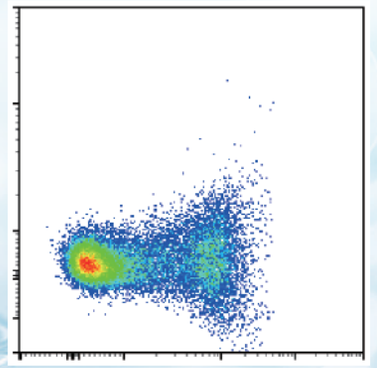


Avoid Spectral Overlap between Fluorescence

Different fluorescence may have spectral overlap. Try to use the fluorescence combination with less spectral overlap in color matching, which can reduce the complexity of data analysis. When the overlap occurs, fluorescence compensation can only eliminate the background. For the reduced sensitivity of the disturbed detectors, it does not work.



No interference

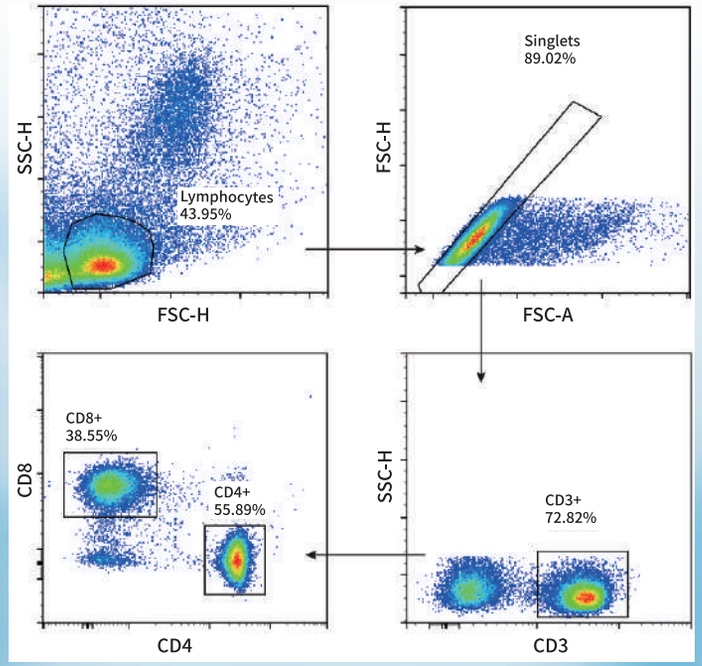


Serious interference

03 Cases of Multi-Color Panel Design

Case1: Mouse Spleen T cells (3 Panels)

Marker	Fluorescence	Clone No.	Cat. No.
CD3	EV450	17A2	E-AB-F1013Q
CD4	APC	GK1.5	E-AB-F1097E
CD8	ER780	53-6.7	E-AB-F1104S

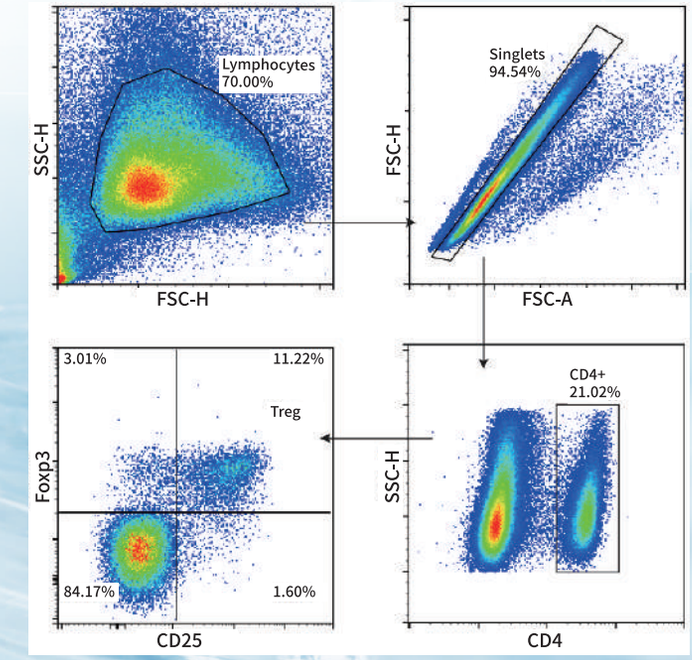


TIPS:

1.Easy to distinguish the Negative-Positive cell groups, and there is no need for single staining tubes for compensation.
2.CD3 / 4 / 8 cells are easily distinguished, and generally speaking, isotype control is unnecessary.
3.The key factor of this experiment is the lysis of red blood cells. Excessive or insufficient lysis of red blood cells will lead to the unclear lymphocyte groups.

Case2: Mouse Spleen Treg (3 Panels)

Marker	Fluorescence	Clone No.	Cat. No.
CD4	FITC	GK1.5	E-AB-F1097C
CD25	APC	PC-61.5.3	E-AB-F1102E
Foxp3	PE	3G3	E-AB-F1238D

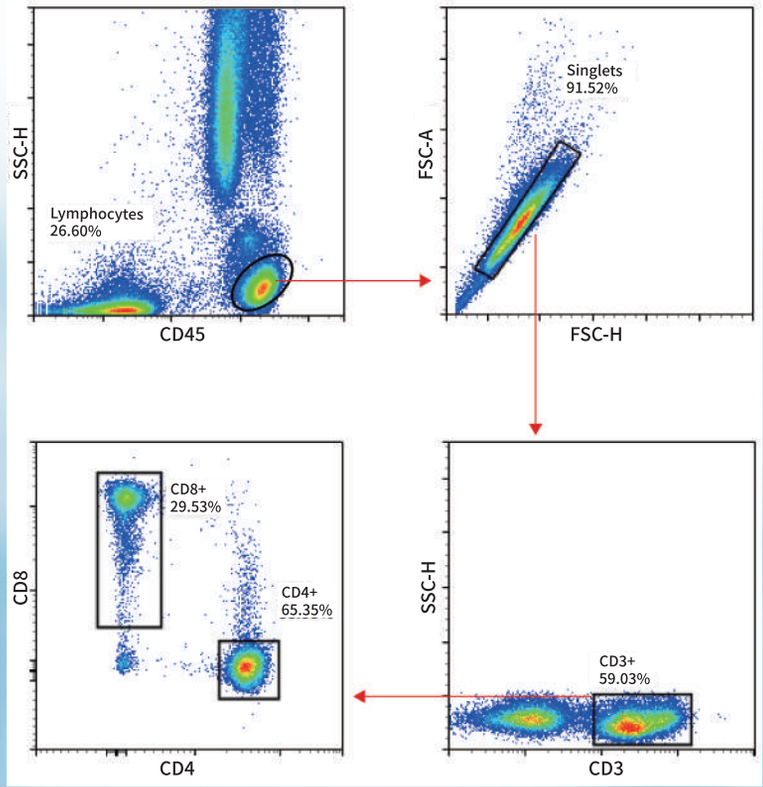


TIPS:

1.Mouse Treg market is CD4+ CD25+ Foxp3+.
2.CD4+ cell group is obvious, and there is no need of isotype control. But CD25 and Foxp3 groups are not obvious, and isotype controls are needed.
3.There is fluorescence spillover, and it is necessary to set single staining tubes for compensation.
4.Inappropriate use of Fixation/Permeabilization buffer may cause high background and unclear cell clustering. Please be careful.

Case3: Human Peripheral Blood T Cells (4 Panels)

Marker	Fluorescence	Clone No.	Cat. No.
CD45	EV450	HI30	E-AB-F1137Q
CD3	APC	OKT3	E-AB-F1001E
CD4	FITC	RPA-T4	E-AB-F1109C
CD8a	PE	OKT-8	E-AB-F1110D

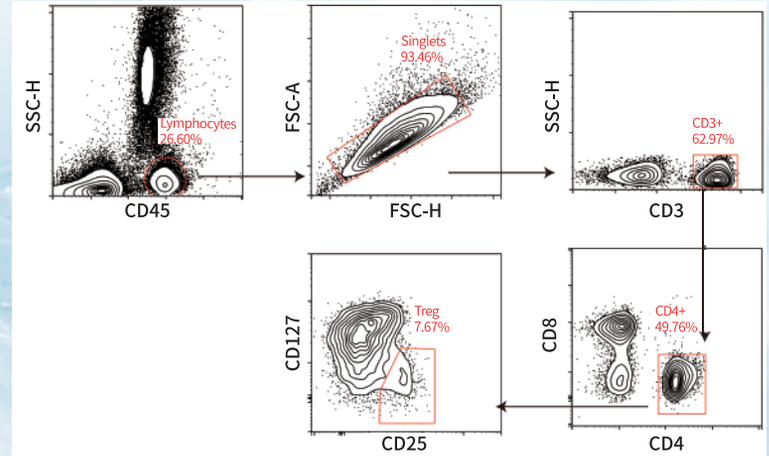


TIPS:

1.For human peripheral blood T cells, it is suggested to use CD45, which can easily gate the lymphocyte group.
2.The cell groups are obvious, and there is no need to set single staining tubes for compensation.

Case4: Human Peripheral Blood Treg (6 Panels)

Marker	Fluorescence	Clone No.	Cat. No.
CD45	EV450	HI30	E-AB-F1137Q
CD3	ER780	OKT3	E-AB-F1001S
CD4	FITC	RPA-T4	E-AB-F1109C
CD8a	PerCP/Cyanine5.5	OKT-8	E-AB-F1110J
CD25	PE	BC96	E-AB-F1194D
CD127	AF647	A019D5	E-AB-F1152M

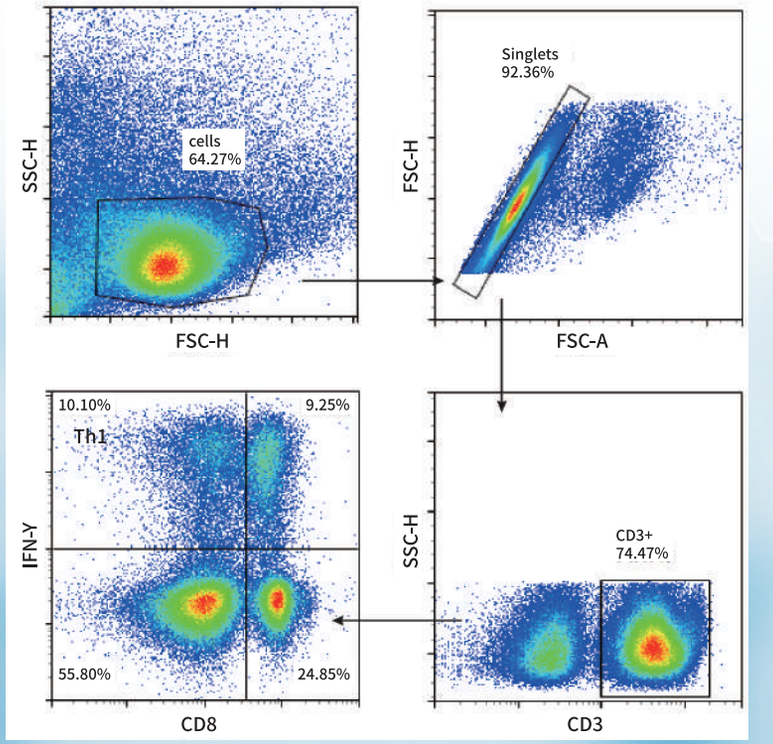


TIPS:

1.Detecting human Treg by CD127 is no need of Fixation/Permeabilization step.
2.Gate the lymphocyte directly through CD45 and SSC, and then analyze the proportion of CD4+ CD25+ CD127-/low cells. Treg cells account is about 3% ~ 10% of lymphocytes in normal human peripheral blood.
3.It is suggested to set single staining tubes for compensation.

Case5: Human Peripheral Blood Th1 (3 Panels)

Marker	Fluorescence	Clone No.	Cat. No.
CD3	EV450	OKT3	E-AB-F1001Q
CD8a	PerCP/Cyanine5.5	OKT-8	E-AB-F1110J
IFN-γ	FITC	B27	E-AB-F1196C



TIPS:

1.PMA stimulation can lead to CD4 expression down on the surface of human T cells. Therefore, we define CD4+ T cells by gating CD3 and CD8, and CD3+ cd8- IFN-γ+ group is Th1 cells.

2.IFN-γ isotype control is necessary, since generally this marker abundance is not high.

3.Inappropriate use of Fixation/Permeabilization buffer may damage the cells. It is suggested to re-suspend the cells after centrifugation, and then add the permeabilization buffer to reduce cell damage.

04 Data Analysis Services

You can also provide the original data of experimental results and logical relationship of markers to technical support. We can provide professional data analysis services for you.

01 Customer provides basic information.

Estimate experimental design and data integrity. 02

03 Data analysis.

Provide analysis results. 04