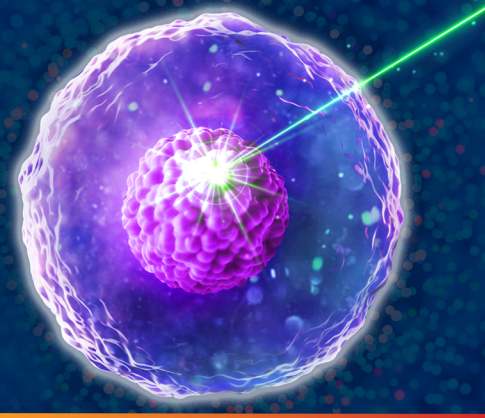
Fluorochrome Performance Guide



Prioritize clean fluorochromes and simplify panel design

Flow cytometry users choose from hundreds of fluorochromes for their conventional and spectral flow cytometry assays. The physical properties of all fluorochromes are not the same, and differences in resolution and spillover can significantly impact panel resolution and data interpretation. The process of learning every fluorochrome's properties can seem overwhelming and intimidating. As a result, flow cytometry users feel more comfortable using familiar fluorochromes, such as PerCP-Cy5.5 or PE tandems, which may present challenges and even limit or compromise the quality of data.

This guide is intended to help simplify panel design and minimize loss of data quality and resolution. By using the Fluorochrome Performance Chart and the Fluorochrome and Antigen Pairing Guide presented here, you can easily prioritize fluorochromes with minimal spillover and appropriate resolution.

Fluorochrome Performance Chart

Resolution (SI)	1	2	3	4
4	BV421 BB515 RB744 RB780 RY586	RB705 RB613 PE PE-Cy7 RY703 RY775	BV711 BB700 PE-CF594	BV650 PE-Cy5
3	RY610 Alexa Fluor™ 647 R718	BUV615 BV480 BV786 APC APC-R700	BUV563 BUV737 BV605 BB630-P2 BB755-P	BUV661 BB660-P2
2	BUV395 FITC RB545	BUV496 BV510 BV750	BV570	PerCP-Cy5.5
1	BUV805 V450 V500 Alexa Fluor™ 488 Alexa Fluor™ 700	APC-H7		PerCP

Spillover

Chart contains representative fluorochromes compatible with a 5-laser spectral flow cytometer. Table may differ based on instrument configuration and settings. Spillover ranking is based on cross-laser excitation and does not take into account spillover into adjacent detectors.

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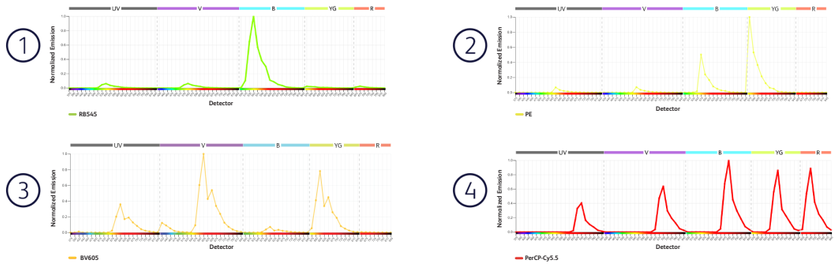
Generating the Fluorochrome Performance Chart

The Fluorochrome Performance Chart organizes and ranks fluorochromes based on spillover and resolution, two of the most critical factors in fluorochrome selection.

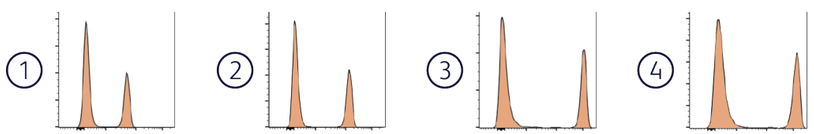
Fluorescence spillover defines the spectral overlap between the emission profile of two fluorochromes. Spectral overlap can be managed through compensation or spectral unmixing to prevent data artifacts. However, these two processes do not eliminate spillover spread, the main source of background and loss of resolution in multiparameter flow cytometry assays. Spread is directly correlated with spillover (the level to which two fluorochrome profiles overlap) and signal intensity (antigen density and fluorochrome brightness).

Spillover is evaluated and ranked based on the analysis of a given fluorochrome's full emission profile across five lasers. Fluorochromes with a single emission peak are ranked as 1 and fluorochromes excited by multiple lasers are ranked as 2, 3 or 4 (additional peaks were counted if the spillover value was greater than 15% of the main peak signal). Adjacent spillover is not taken into consideration for this ranking.

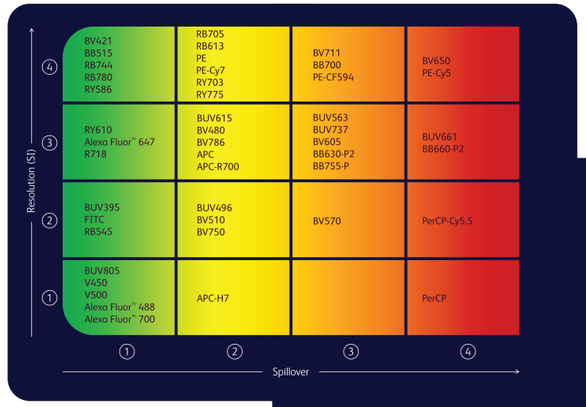


Fluorochrome resolution defines the degree of separation between the negative and positive populations. Signal intensity also contributes to the total amount of spread, where cells expressing antigens at higher density will introduce higher spread.

Resolution is determined by comparing the stain index of fluorochromes conjugated to several antibody clones on a variety of flow cytometers to capture variation in configurations. A ranking of 1 identifies dim fluorochromes with relatively low stain index, and 4 identifies brighter fluorochromes with higher stain index. Scan the QR code for a list of fluorochrome resolution rankings by primary excitation laser line.



By prioritizing fluorochromes in columns 1 and 2, users can design panels while minimizing resolution loss due to spillover-spreading error (spread). When additional challenges are present, such as limited reagent availability or designing very large panels, the other fluorochromes (columns 3 and 4) can be carefully incorporated into the panel.

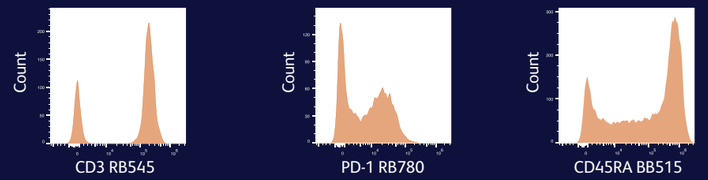


Relative Fluorochrome Resolution Chart

Pairing clean fluorochromes and markers

While the Fluorochrome Performance Chart provides guidance for the prioritization of fluorochromes with minimal impact to resolution, fundamental panel design principles then need to be followed to build a panel. The Fluorochrome and Antigen Paring Guide provides recommendations for the appropriate use of fluorochromes based on target antigen profile and density. Depending on the panel markers and instrument configuration, the total number of minimally overlapping fluorochromes that may be used together will vary.

Fluorochrome and Antigen Pairing Guide



Antigen profile **Antigen density**	**Clearly resolved** **High** Use dim fluorochromes with minimal spillover	**Not clearly resolved** **Low/Medium** Use bright fluorochromes	**Variable** **Low-to-high/Unknown** Use bright fluorochromes with lowest spillover
Recommended fluorochromes	BUV395	BUV615	BV421[4]
	BUV496	BV421[4]	BB515
	BUV805	BV480[5]	RB744
	V450[4]	BB515[1]	RB780
	V500 or BV510[5]	RB613	RY586
	BV750	RB705	
	BV786	RB744	
	FITC or AF488[1]	RB780	
	RB545	PE[3]	
	AF700[2]	RY586[3]	
	APC-H7	RY610	
		RY703	
		RY775	
		APC or AF647	
		R718[2]	

[1] Use either FITC, Alexa Fluor™ 488 or BB515 in the same panel

[2] Use either R718, Alexa Fluor™ 700 or APC-R700 in the same panel

[3] Use either RY586 or PE in the same panel

[4] V450 and BV421 can be used together in spectral flow cytometry with minimal resolution impact

[5] BV480 and either BV510 or V500 can be used together in spectral flow cytometry with minimal resolution impact

Note: Fluorochromes with a single emission peak may still impact resolution of other neighboring fluorochromes with an adjacent main emission peak (e.g., RY586 and RY610, BB515 and RB545, RB744 and RB780). If possible, avoid pairing these adjacent fluorochromes with co-expressed markers with high antigen density.

For "Clearly Resolved" and highly expressed markers, resolution is minimally impacted by the spillover spread that may be introduced by fluorochromes with adjacent main emission peaks (e.g., BB515 and RB545), especially if the two markers are not co-expressed. "Not Clearly Resolved Markers" are less likely to introduce spread due to low antigen density. For variable markers and markers with unknown expression levels, bright fluorochromes with minimal spillover will help ensure resolution of the populations at the low end of expression range, while minimizing any spread from the population at the high end of expression range.

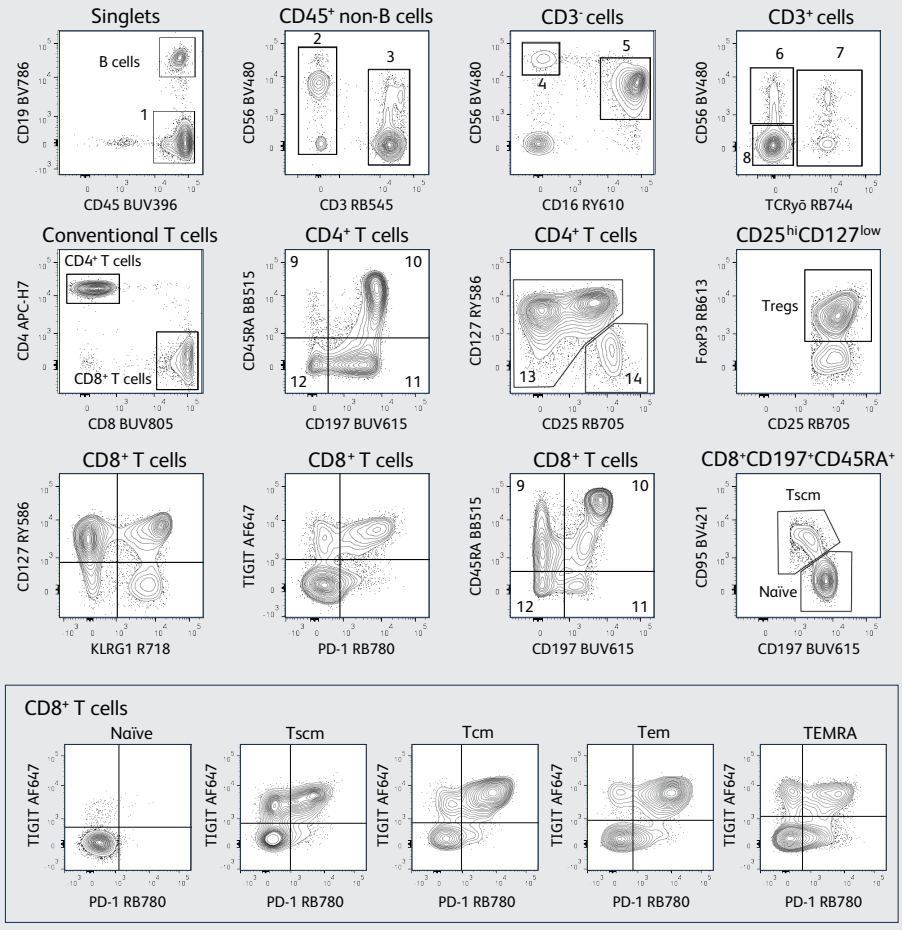
Note that although feasible in spectral flow cytometry, use of very similar fluorochromes in a panel (e.g., FITC and BB515, APC and Alexa Fluor™ 647) should be avoided to prevent high spread.

Putting the Performance Guide to use

A 17-color flow cytometry panel was designed following the strategy provided in this guide. The list of usable fluorochromes was first narrowed down based on low spillover ranking from the Fluorochrome Performance Chart (Figure 1, columns 1 and 2). Fluorochromes were then selected and assigned to markers based on antigen profile, expression profile and reagent availability, as per the Fluorochrome and Antigen Pairing Guide.

The use of overall clean dyes with minimal spillover ensured the clear resolution of several lymphocyte populations and the analysis of inhibitory receptors' expression therein.

Marker	Fluorochrome
CD45	BUV395
CD3	RB545
CD4	APC-H7
CD8	BUV805
CD56	BV480
CD16	RY610
CD45RA	BB515
CD197	BUV615
CD95	BV421
PD-1	RB780
TIGIT	AF647
KLRG1	R718
TCR GD	RB744
CD25	RB705
FoxP3	RB613
CD19	BV786
CD127	RY586



1. CD45+ non-B cells
2. CD3- cells
3. CD3+ cells
4. CD56bright cells
5. CD56dimCD16+ cells
6. NKT-like cells
7. TCRγδ+ T cells
8. Conventional T cells
9. TEMRA
10. CD197+CD45RA+ cells
11. Tcm
12. Tem
13. Conventional CD4+ T cells
14. CD25highCD127low cells

Representative analysis of human PBMCs isolated from a healthy donor and stained with the panel shown on the left. Several lymphocyte populations, including B cells, NK cells and T cells (gamma delta, Tregs, memory CD4+ and CD8+ cells) could be clearly resolved. The bottom insert shows a representative analysis of the expression of TIGIT and PD-1 throughout distinct subsets of naïve and memory CD8+ T cells. Samples were acquired and spectrally unmixed on a BD FACSCSymphony™ A5 SE Flow Cytometer.

The continuous development of fluorochromes with lower cross-laser excitation offers more and new options for the design of flow cytometry panels with reduced spread and higher biological resolution. Combine the information from the Fluorochrome Performance Guide and the Antigen Pairing Guide to simplify the design of high-quality flow cytometry panels.