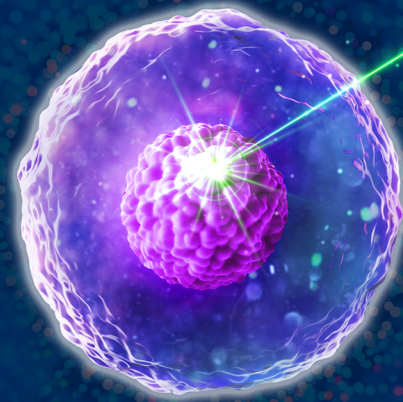


Fluorochrome Performance Guide



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 (SJ) ↑	④	BV421 BB515 RB744 RB780 RY586	RB613 RB670 RB705 PE RY703	RY743 RY775 PE-Cy7 RR688	BV711 BB700 PE-CF594 RY655	BV650 PE-Cy5
	③	RB824 RY610 Alexa Fluor™ 647 R718	BUV615 BV480 BV786 APC APC-R700	BUV563 BUV737 BV605		BUV661
	②	BUV395 RV828 FITC RB545	BUV496 BV510 BV750	BV570		PerCP-Cy5.5
	①	BUV805 V450 V500 Alexa Fluor™ 488 Alexa Fluor™ 700	APC-Cy7 APC-H7			PerCP
		①	②	③	④	
		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.

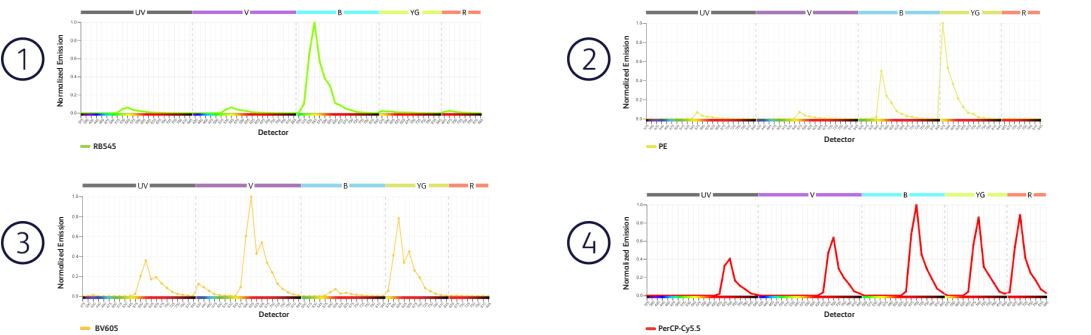


Generating the Fluorochrome Performance Chart

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

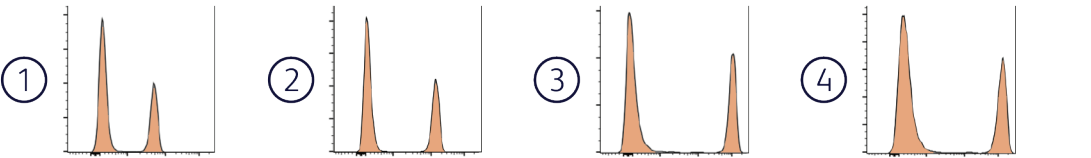
Fluorescence spillover defines the spectral overlap between the emission profile of two fluorochromes. Spectral overlap can be managed through compensation or spectral unmixing to prevent data artifacts. However, these two processes do not eliminate spillover spread, the main source of background and 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.

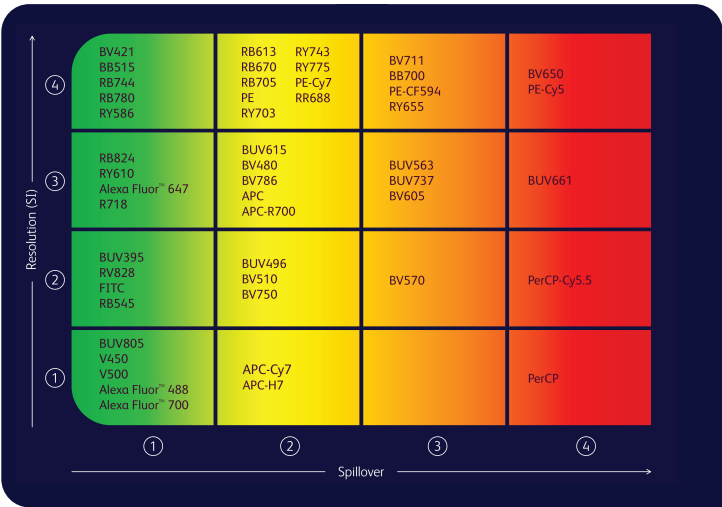
Resolution is determined by comparing the stain index of fluorochromes conjugated to several antibody clones on a variety of flow cytometers to capture variation in configurations. A ranking of 1 identifies dim fluorochromes with relatively low stain index, and 4 identifies brighter fluorochromes with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



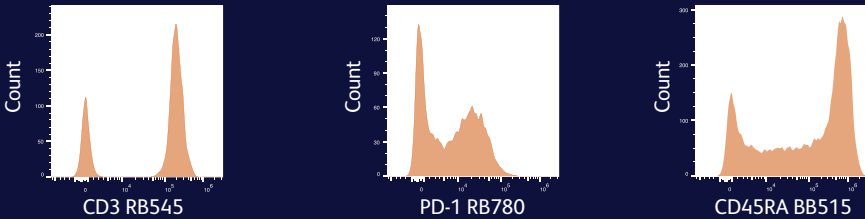
By prioritizing fluorochromes in columns 1 and 2, users can design panels while minimizing resolution loss due to spillover-spreading error (spread). When additional challenges are present, such as limited reagent availability or designing very large panels, the other fluorochromes (columns 3 and 4)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

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

- 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

BUV496
BUV805
V450⁴
BV750
BV786
RV828
FITC or AF488¹
RB545
AF700²
APC-H7 or APC-Cy7

BV421⁴
BV480⁵
BB515¹
RB613
RB670
RB705
RB744
RB780
RB824
PE³
RY586³
RY610
RY703
RY743
RY775
APC or AF647
RR688
R718²

BB515
RB744
RB780
RY586

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.



Relative Fluorochrome Resolution Chart

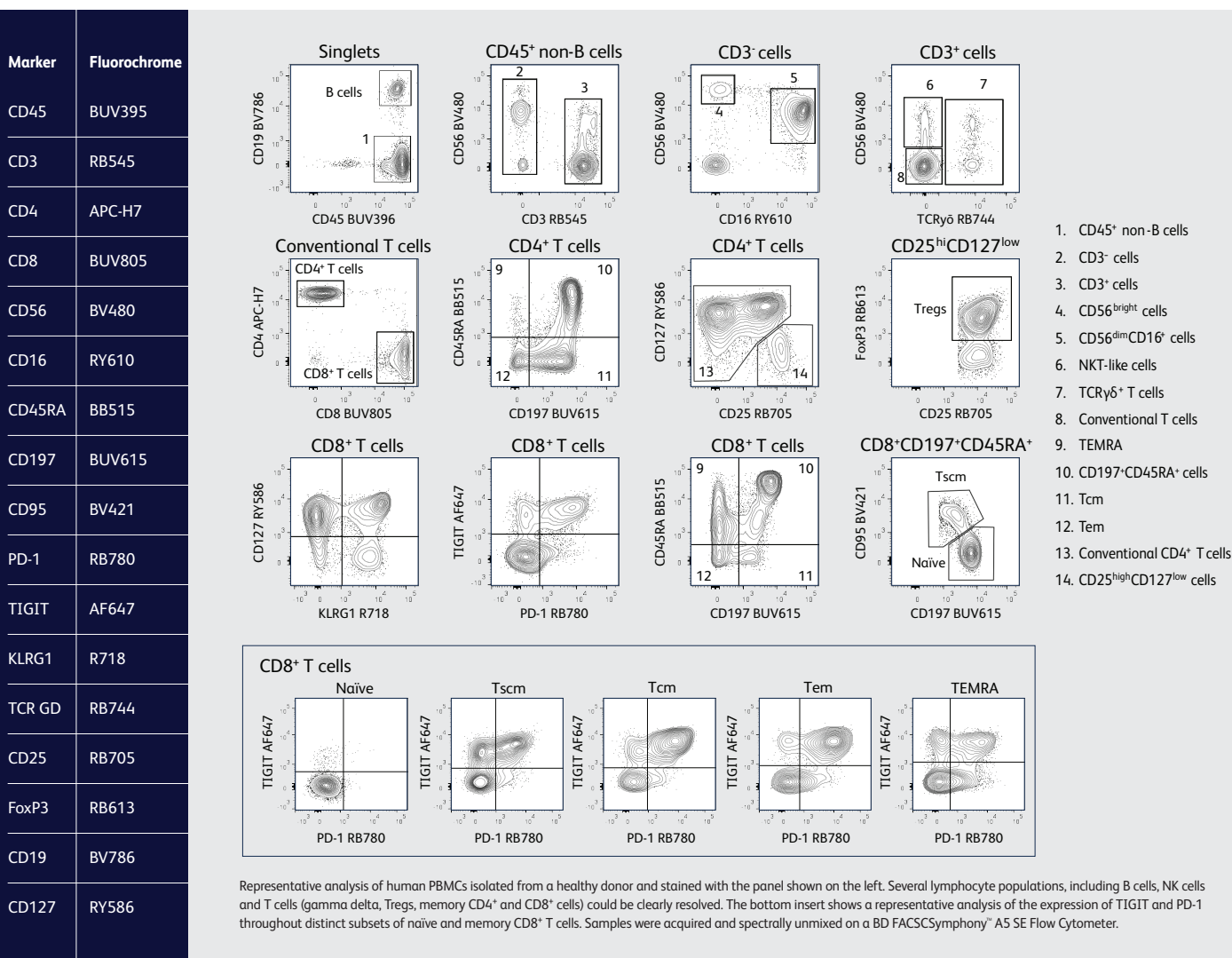
For “Clearly Resolved” and highly expressed markers, resolution is minimally impacted by the spillover spread that may be introduced by fluorochromes with adjacent main emission peaks (e.g., BB515 and RB545), especially if the two markers are not co-expressed. “Not Clearly Resolved Markers” are less 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.



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.

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