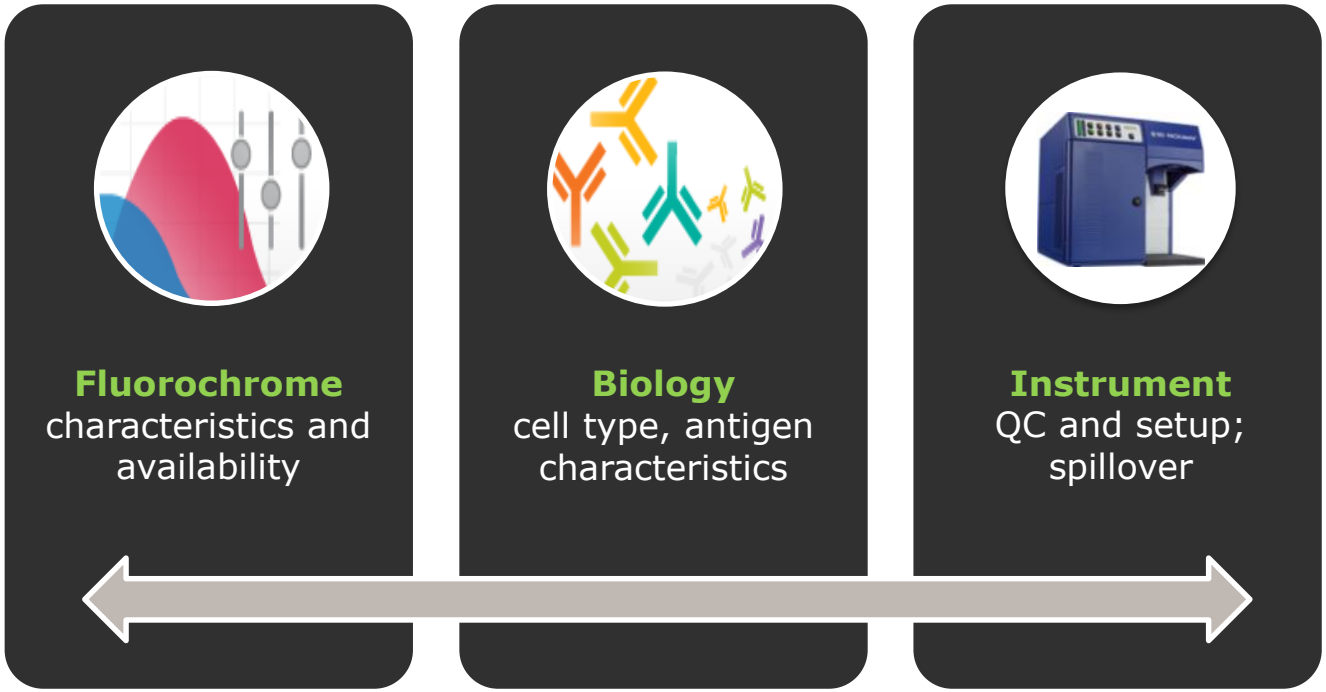
Design

What you need to know before designing a panel

Elements of multicolor flow cytometry

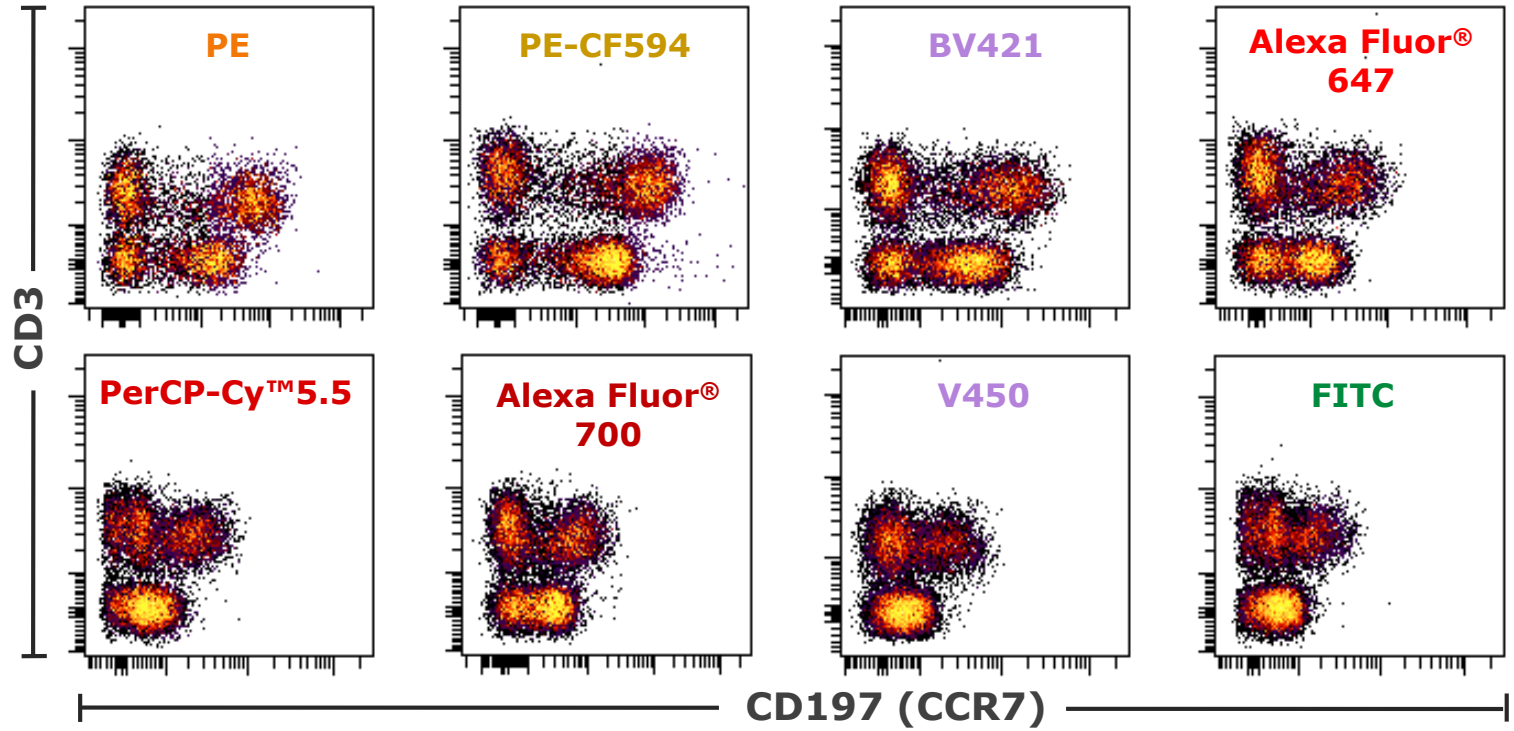
Considerations in designing panels:



Fluorochromes

Expanding the range of choices to reveal biological context

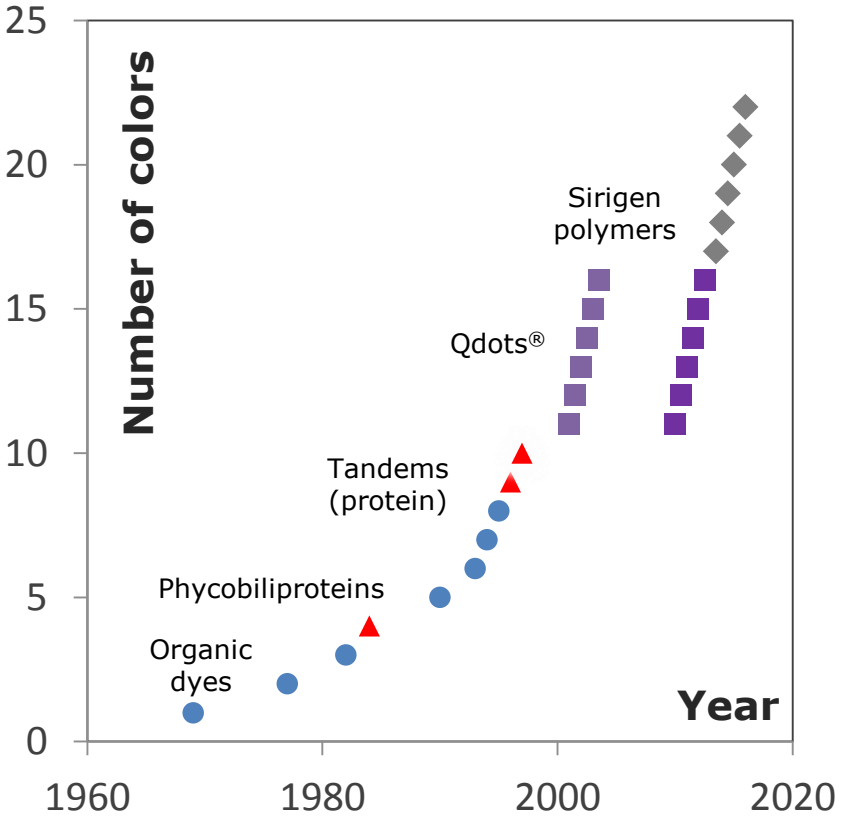
Fluorochromes reveal biology



- The proper choice of fluorochrome helps us understand more about the biology of the experiment.
- Bright dyes are important when looking at dim antigens.

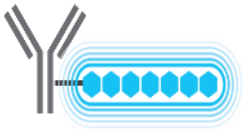
Evolution of fluorochromes

- 380 CD markers
- Intracellular proteins
  - Cytokines
  - Cell signaling
  - Transcription factors
  - Phosphoproteins
- The availability of fluorochromes has driven major advances in flow cytometry.

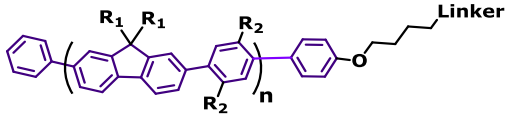


CD Nomenclature 2015: Human Leukocyte Differentiation Antigen Workshops as a Driving Force in Immunology. Engel P, Boumsell L, Balderas R, et al. *J Immunol.* 2015 Nov 15;195(10):4555-4563.

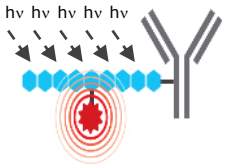
Sirigen polymer technology
– High-sensitivity fluorescence



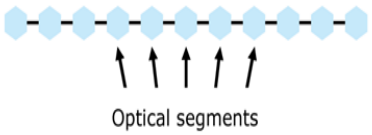
Direct reporters

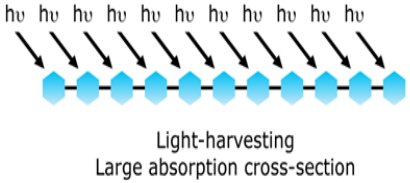


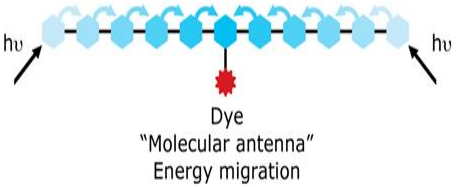
π-conjugated polymers

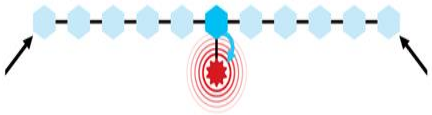


Tandem reporters









Intense dye emission

- Bright fluorescent materials
- Large collective optical response

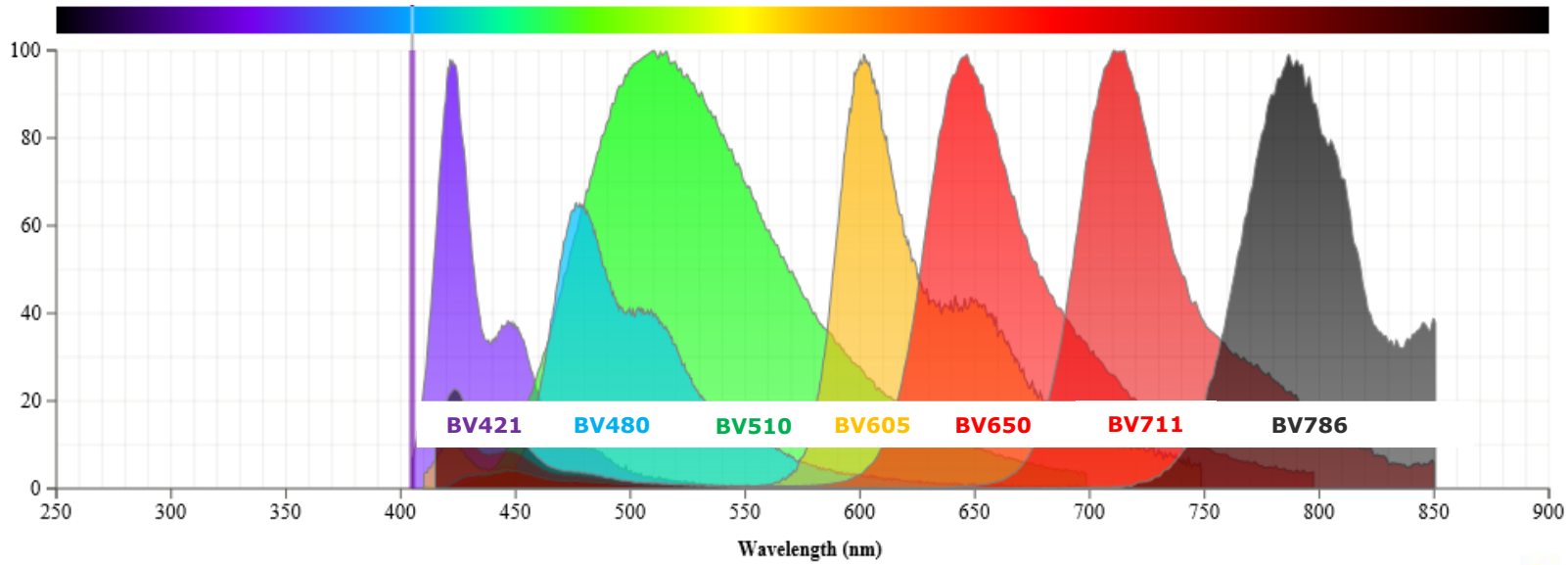
- Efficient energy donors
- Amplified dye emission
- Reproducible synthetic framework

BD

BD Horizon Brilliant™ Violet dyes

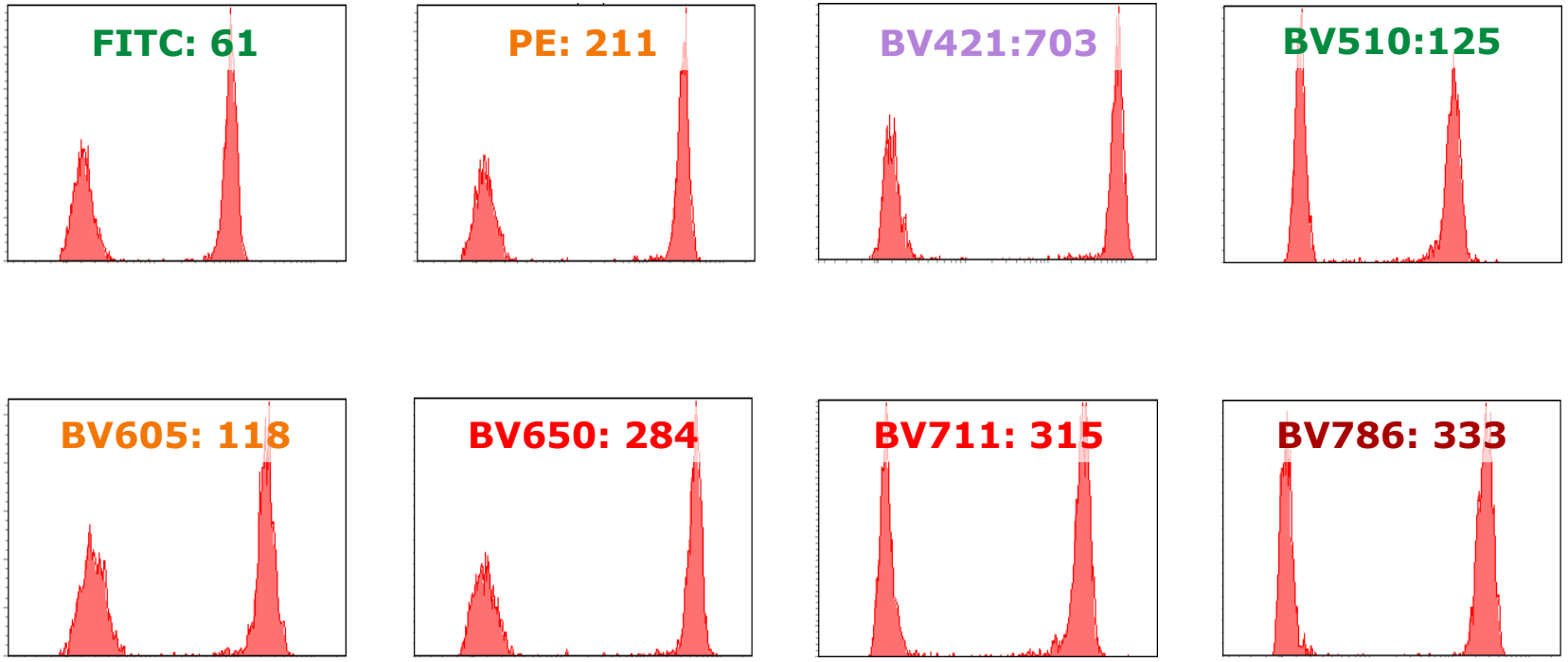
- Seven dyes excited by the violet laser
  - Base polymers: BV421, BV510 and BV480[new]
  - Tandems: BV605, BV650, BV711 and BV786

- Bright dyes
- Limited cross laser excitation
- Compatible with surface and intracellular targets



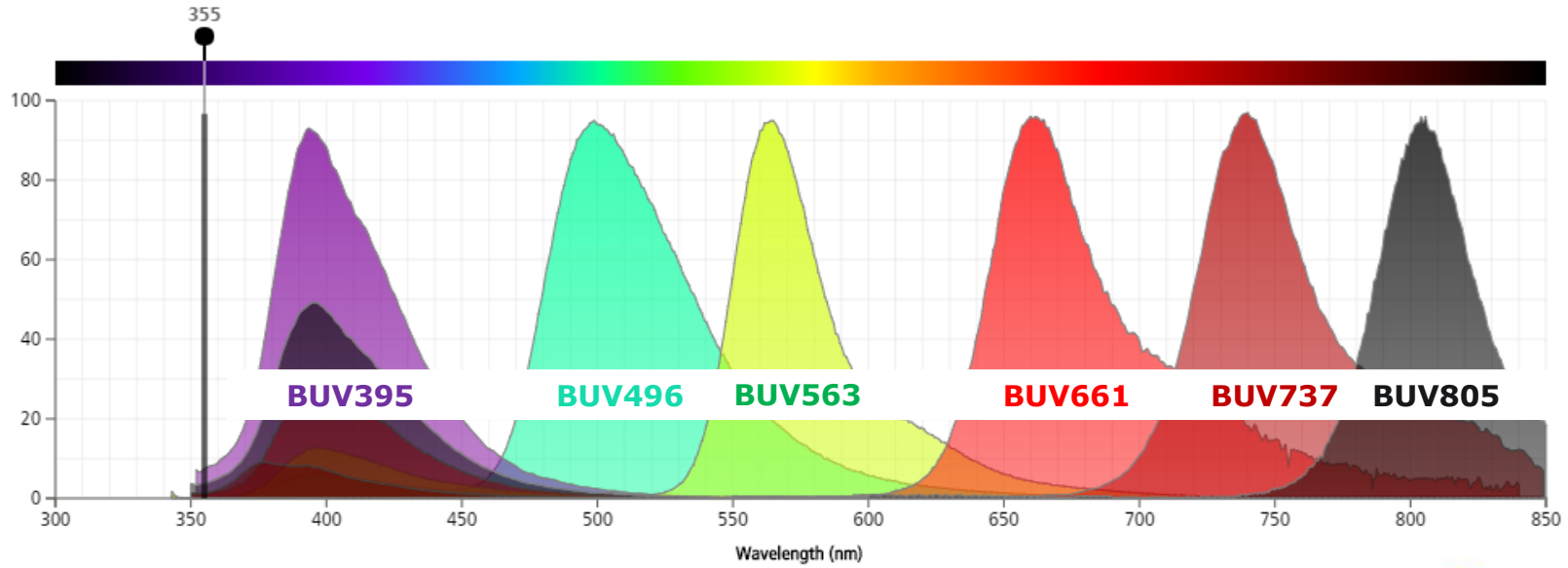
CD4 resolution comparison

Stain index

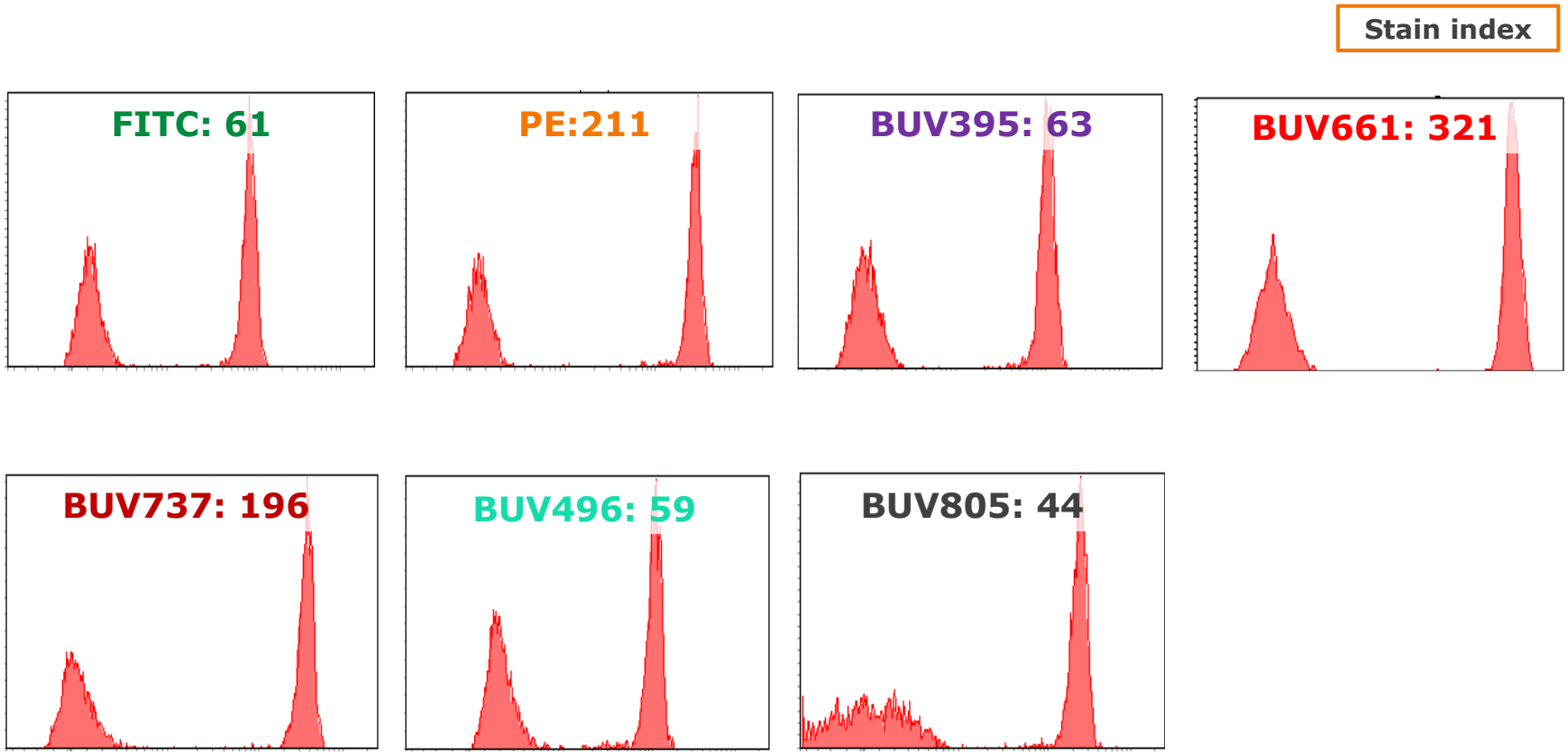


BD Horizon Brilliant™ Ultraviolet dyes

- Six fluorochromes excited by the 355-nm UV laser
  - Base polymer: BUV395
  - Tandems: BUV496, BUV563, BUV661, BUV737, BUV805
- Designed for reduced spillover into violet channels
- Bring phenotyping to the UV-laser line



CD4 resolution comparison



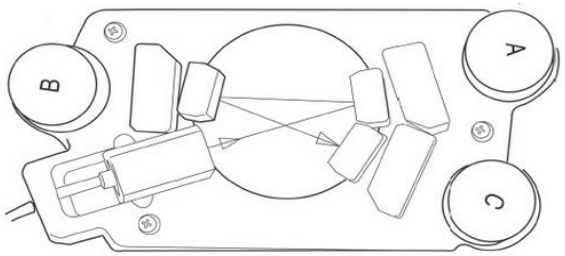
Many fluorochrome choices

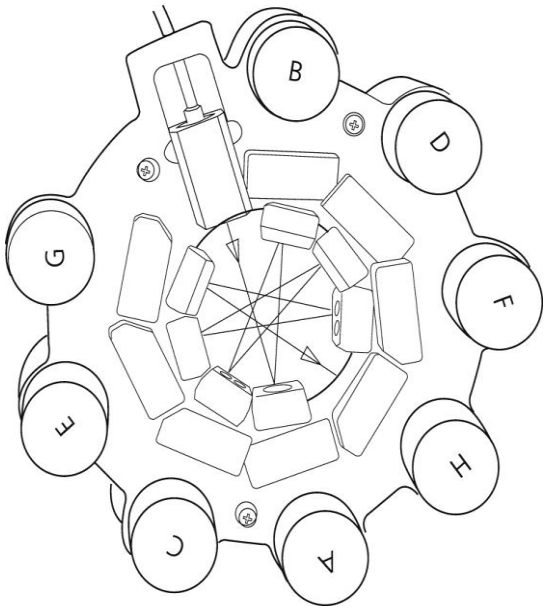
Laser \ Emission								
Ultraviolet (355 nm)	BUV395		BUV496	BUV563		BUV661	BUV737	BUV805
Violet (405 nm)		BV421 V450	BV480 BV510 V500		BV605	BV650	BV711	BV786
Blue (488 nm)			BB515 FITC Alexa Fluor® 488	PE	PE-CF594	PE-Cy™5	PerCP PerCP-Cy5.5	PE-Cy™7
Yellow/Green (561 nm)				PE	PE-CF594	PE-Cy5	PE-Cy5.5	PE-Cy7
Red (640 nm)						APC Alexa Fluor® 647	APC-R700 Alexa Fluor® 700	APC-H7 APC-Cy7

Choice of fluorochromes depends on the available instrument configuration and the total number of markers being used in an experiment.

Understand instrument configuration

- The fluorochrome choice must be compatible with the instrument being used.
- Reconfiguration might be necessary to take full advantage of the BD Horizon Brilliant Violet and Ultraviolet portfolio.
- Reconfiguration allows for expansion of the instruments' capability.





Choose fluorochromes based on configuration

	BD Accuri C6	BD FACSVerse BD FACSCanto II	BD FACSVerse BD FACSCanto II	BD LSRFortessa BD LSRFortessa X-20	BD LSRFortessa BD LSRFortessa X-20
Blue (488 nm)	BB515/FITC PE PerCP-Cy5.5	BB515/FITC PE PerCP-Cy5.5 PE-Cy7	BB515/FITC PE PerCP-Cy5.5 PE-Cy7	BB515/FITC PE PE-CF594 PerCP-Cy5.5 PE-Cy7	BB515/FITC PerCP-Cy5.5
Red (640 nm)	APC	APC APC-H7/APC-Cy7	APC APC-H7/APC-Cy7	APC APC-R700 APC-H7/APC-Cy7	APC APC-R700 APC-H7/APC-Cy7
Violet (405 nm)			BV421/V450 BV510/V500	BV421/V450 BV510/V500 BV605 BV650 BV711 BV786	BV421/V450 BV510/V500 BV605 BV650 BV711 BV786
Yellow/Green (561 nm)					PE PE-CF594 PE-Cy5 PE-Cy7
Ultra-violet (355 nm)				BUV395 BUV496 BUV661 BUV737 BUV805	BUV395 BUV496 BUV661 BUV737 BUV805
# Lasers	**2**	**2**	**3**	**4**	**5**
# Colors	**4**	**6**	**8**	**18**	**18**

BD FACSCelesta™ configurations

- Enabling new bright fluorochrome choices for assay design

Blue/Violet

Laser	Fluorochromes
405 nm	BD Horizon™ BV421, V450, Pacific Blue
	BD Horizon™ BV510, V500
	BD Horizon™ BV605
	BD Horizon™ BV650
	BD Horizon™ BV711
	BD Horizon™ BV786
488 nm	BD Horizon™ BB515, FITC, Alexa Fluor® 488
	PE
	BD Horizon™ PE-CF594, PI
	PerCP, PerCP-Cy™5.5, 7-AAD

Blue/Violet/UV

Laser	Fluorochromes
405 nm	BD Horizon BV421, V450, Pacific Blue
	BD Horizon BV510, V500
	BD Horizon BV605
	BD Horizon BV650
	BD Horizon BV711
	BD Horizon BV786
488 nm	BD Horizon BB515, FITC, Alexa Fluor® 488
	PE
	BD Horizon PE-CF594, PI
	PerCP, PerCP-Cy5.5, 7-AAD
355 nm	BD Horizon BUV395
	BD Horizon BUV737

Blue/Violet/Yellow-Green

Laser	Fluorochromes
405 nm	BD Horizon BV421, V450, Pacific Blue
	BD Horizon BV510, V500
	BD Horizon BV605
	BD Horizon BV650
	BD Horizon BV711
	BD Horizon BV786
488 nm	BD Horizon BB515, FITC, Alexa Fluor® 488
	PerCP, PerCP-Cy5.5, 7-AAD
561 nm	PE
	BD Horizon PE-CF594, PI
	PE-Cy™5, 7-AAD
	PE-Cy™7

Blue/Violet/Red

Laser	Fluorochromes
405 nm	BD Horizon BV421, V450, Pacific Blue
	BD Horizon BV510, V500
	BD Horizon BV605
	BD Horizon BV650
	BD Horizon BV786
488 nm	BD Horizon BB515, FITC, Alexa Fluor® 488
	PE
	BD Horizon PE-CF594, PI
	PerCP, PerCP-Cy5.5, 7-AAD
640 nm	APC, Alexa Fluor® 647
	BD Horizon™ APC-R700, Alexa Fluor® 700
	APC-H7

Fluorochrome resolution ranking

Fluorochrome

Laser	Very bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon BUV661 BD Horizon BUV737 BD Horizon BUV563	BD Horizon BUV395 BD Horizon BUV496	BD Horizon BUV805
Violet (405 nm)	BD Horizon BV421 BD Horizon BV650 BD Horizon BV711	BD Horizon BV480 BD Horizon BV605 BD Horizon BV786	BD Horizon BV510	BD Horizon V450 BD Horizon V500
Blue (488 nm)	BD Horizon BB515 BD Horizon PE-CF594 PE-Cy5	PE PE-Cy7	FITC Alexa Fluor® 488 PerCP-Cy5.5	PerCP
Yellow/Green (561 nm)	PE BD Horizon PE-CF594 PE-Cy5 PE-Cy7			
Red (640 nm)		APC Alexa Fluor® 647 BD Horizon APC-R700		Alexa Fluor® 700 APC-H7 APC-Cy7

- Rankings were determined by comparing the resolution of LWB cells stained on several clones run on a variety of flow cytometers.
- Many factors can influence the relative fluorochrome/reagent performance on a given instrument, including laser power, PMT voltage, optical filters, antibody clone, biological sample and staining methodology.

Know your biology

Antigen density and co-expression influence panel design

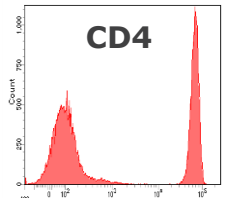
Elements of multicolor flow cytometry

Considerations in designing panels:

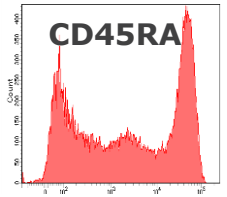
Classification of antigens

Leucocyte antigens can be categorized based upon their patterns of expression:

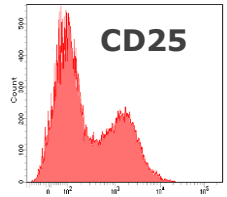
- **Primary**: Well characterized, easily classified as positive or negative, typically define broad subsets or lineages
  - **Examples: CD3, CD4, CD19**



- **Secondary**: Well characterized, typically expressed at a higher density, often over a continuum
  - **Examples: CD27, CD28, CD45RA, CD45RO**



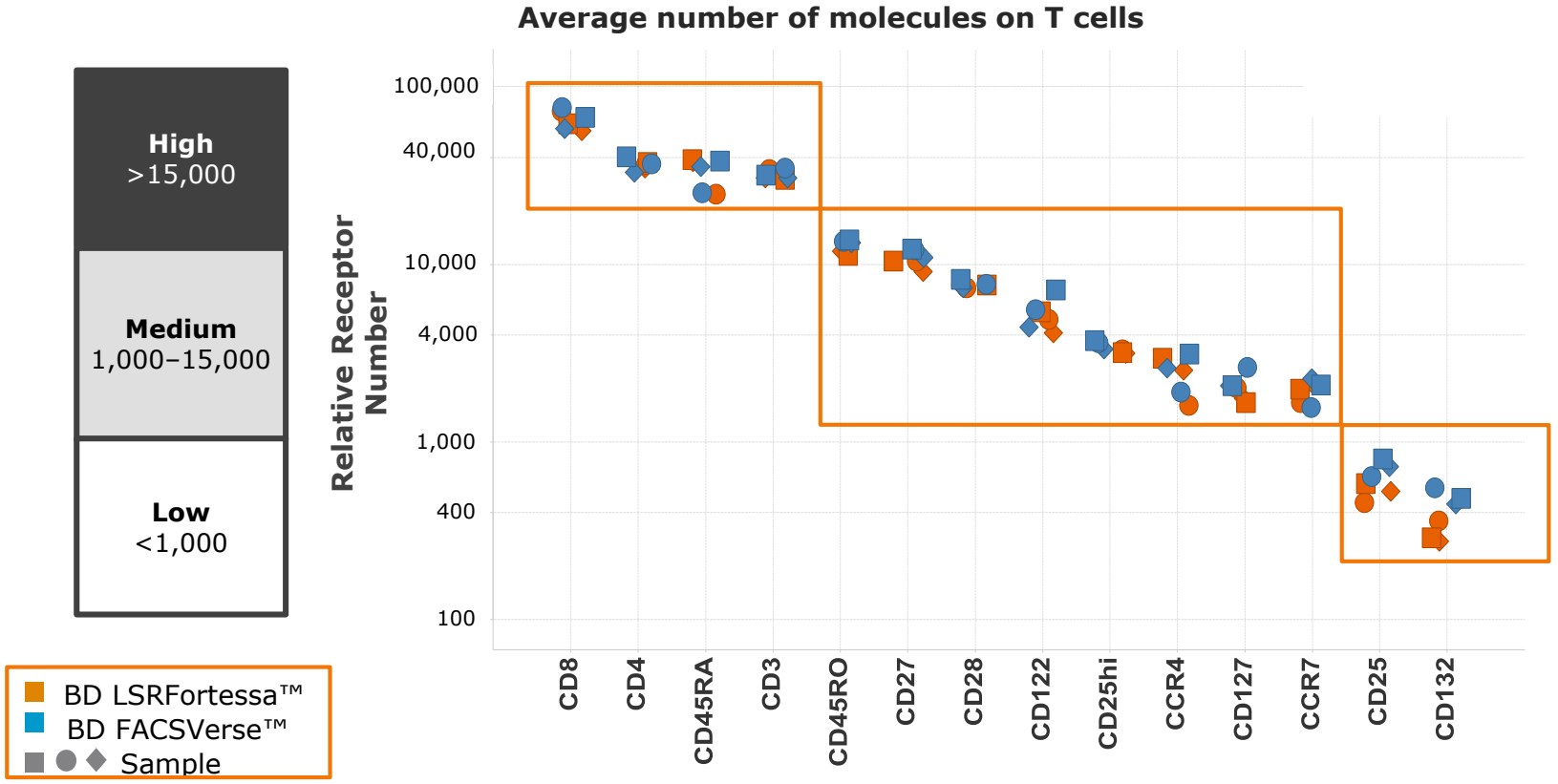
- **Tertiary**: Expressed at low levels, variable upon activation unknown, critical
  - **Examples: CD25, STAT5, FoxP3**



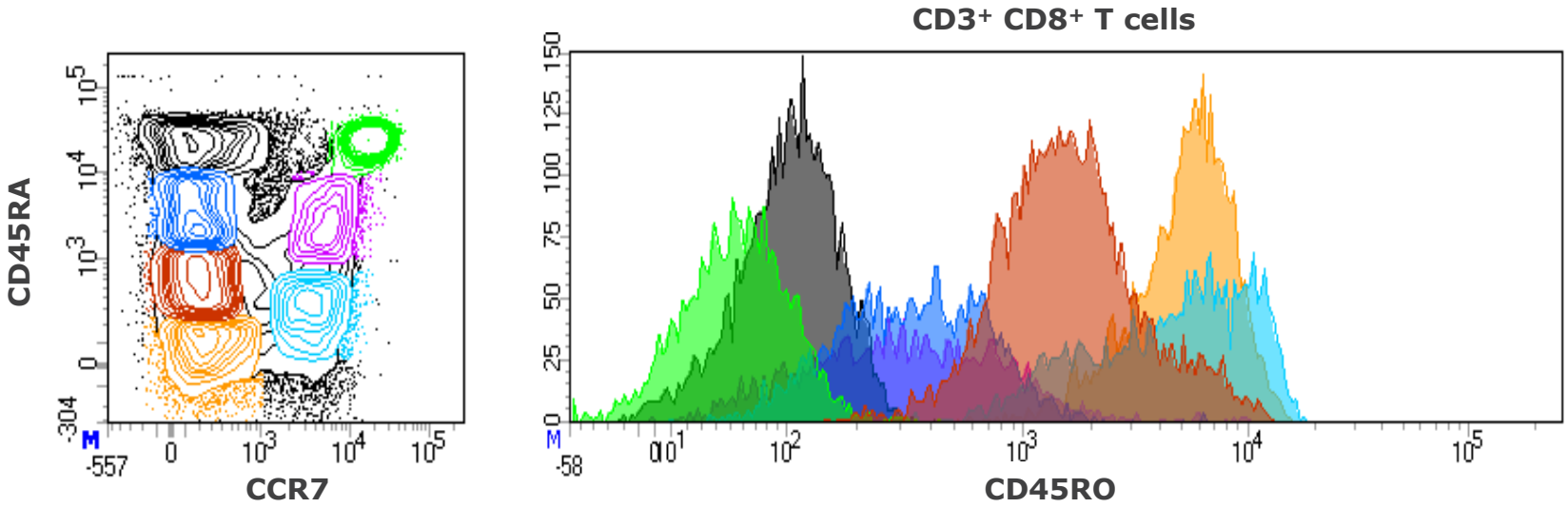
Mahnke YD, Roederer M. Optimizing a multicolor immunophenotyping assay. *Clin Lab Med*. 2007;27:469-485.

Grouping antigen density: T-cells

- When evaluating antigen density, it can be useful to group antigens based on their relative levels of expression.



Different subpopulations can express the same antigen at different densities



- Antigen density should be evaluated at the level of the subpopulations of interest.
  - Example: for all T-cells, CD45RO has an average density of 15,000.
    - Expression on individual subpopulations can vary 300-fold.
- For novel populations, you might need to do test analyses to assess antigen density on your specific population.
  - Densities can be expressed as ratios of the median fluorescence intensity (MFI) of a known antigen vs the test antigen using the same fluorochrome.

BD antigen expression project

Providing the scientific community information on antigen density and co-expression

Antigen density project

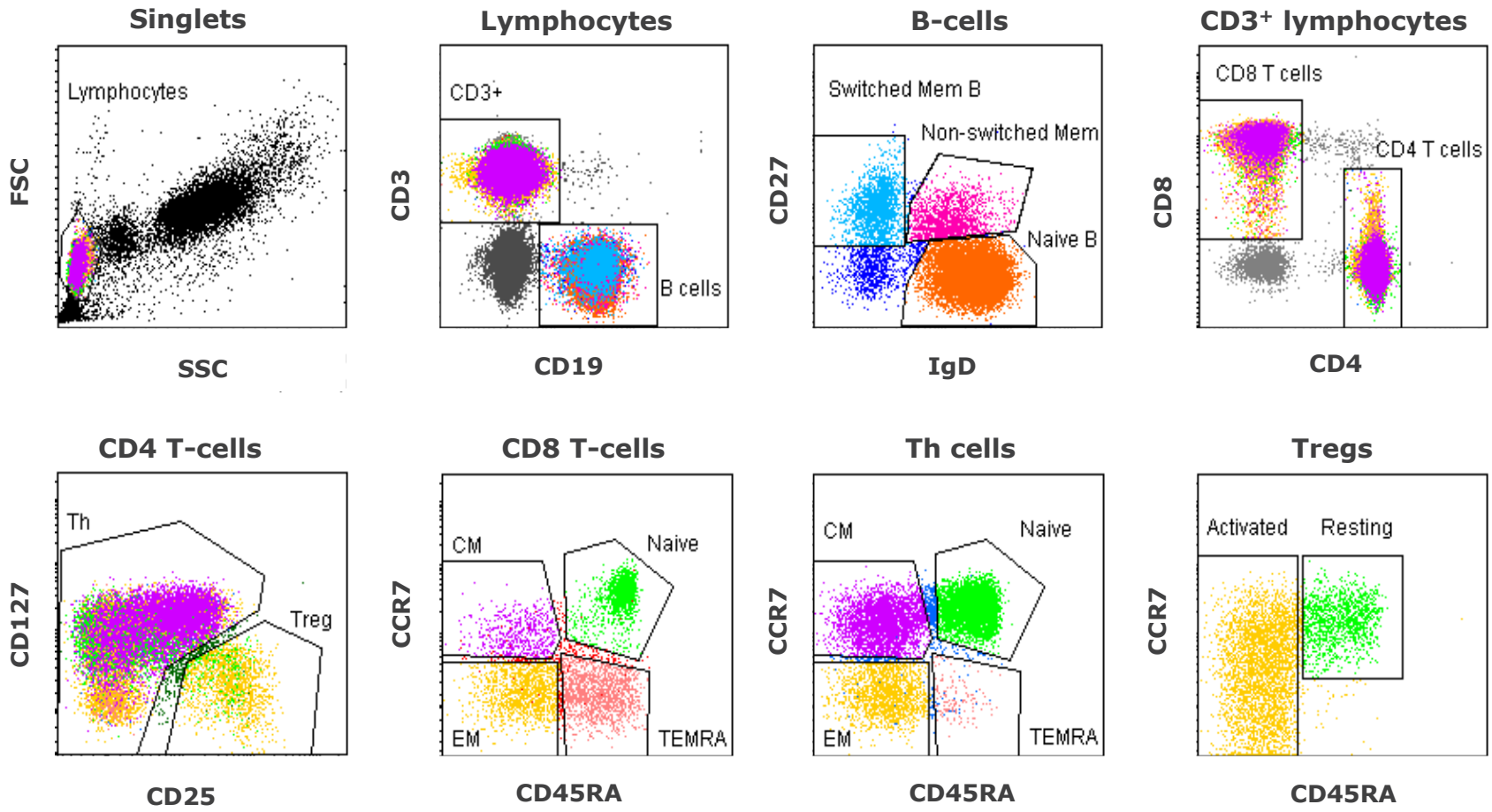
- Antigen density analyses were performed on blood cells from 12 individuals, covering a range of ages and genders (3 male/3 female each from young/old groups).
- Each antigen of interest was measured using a PE-conjugated antibody.

Antibodies in panel		Cell populations identified	
Panel 1 (B/T)	CD3, CD4, CD8, CD25, CD127, CD45RA, CCR7, CD19, IgD, CD27	• Naïve, EM, CM and TEMRA populations (defined by CD45RA and CCR7) from CD8 and Th cell subsets • $CD45RA^{+}$ Tregs • $CD45RA^{-}$ Tregs	• Naïve B-cells • Non-class-switched memory B-cells • Class-switched memory B-cells
Panel 2 (non-B/T)	CD61, CD45, CD3, CD19, CD14, CD16, CD56, HLA-DR CD123, CD11c	• Platelets • Neutrophils • Basophils • Eosinophils • Monocytes (subsets based on CD14 and CD16)	• $CD56^{dim}CD16^{+}$ NK-cells • $CD56^{bright}$ NK-cells • NKT-cells ($CD3^{+}$ $CD56^{+}$) • mDCs • pDCs

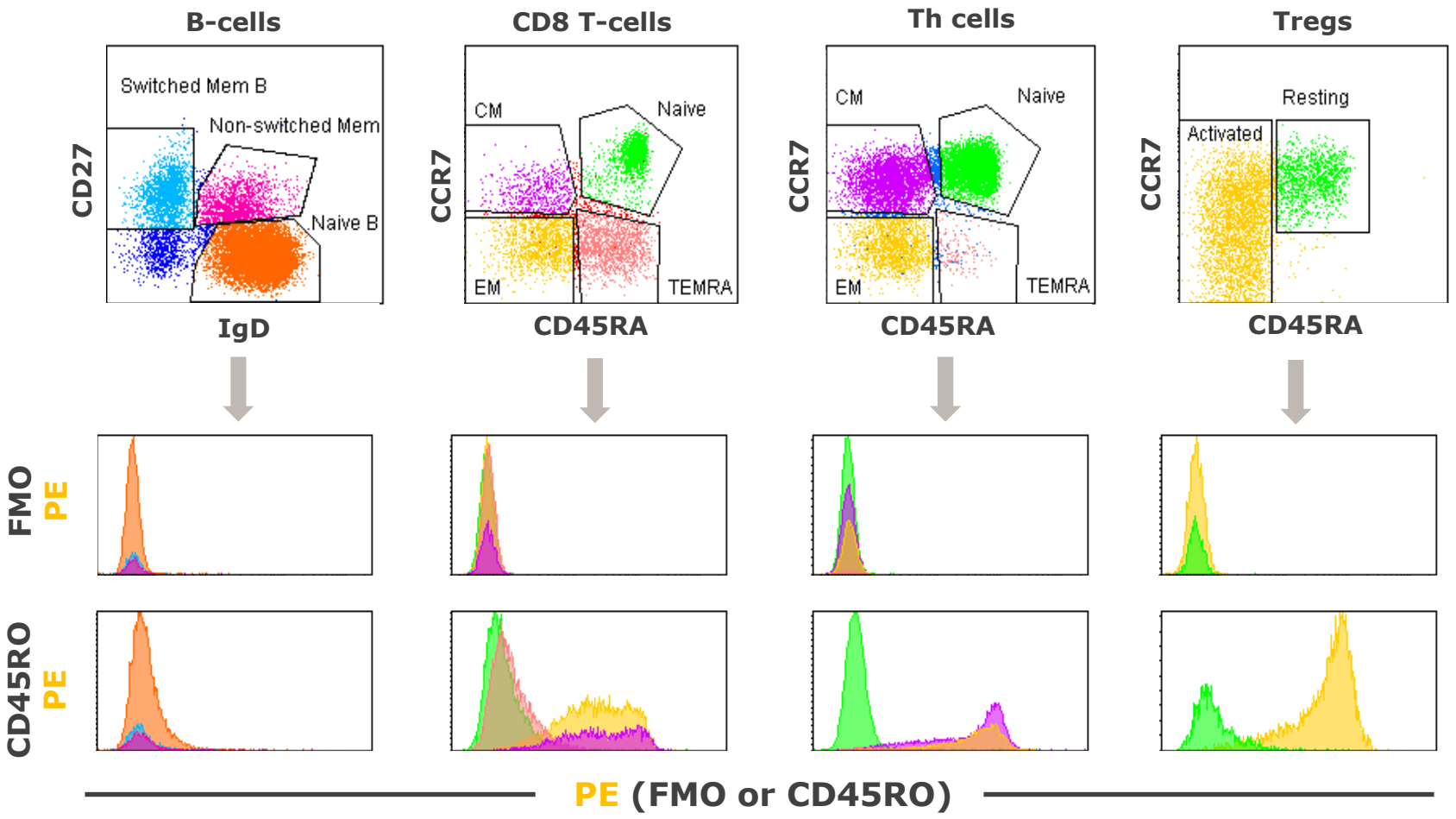
Antigen density: B-cell and T-cell panel

Fluorochrome	Marker
BD Horizon™ V450	CD45RA
BD Horizon™ V500	CD3
FITC	CD4 + IgD
PerCP-Cy™5.5	CD19
PE	Drop-in
PE-Cy™5	CD25
PE-Cy™7	CD127
Alexa Fluor® 647	CCR7
Alexa Fluor® 700	CD27
APC-H7	CD8

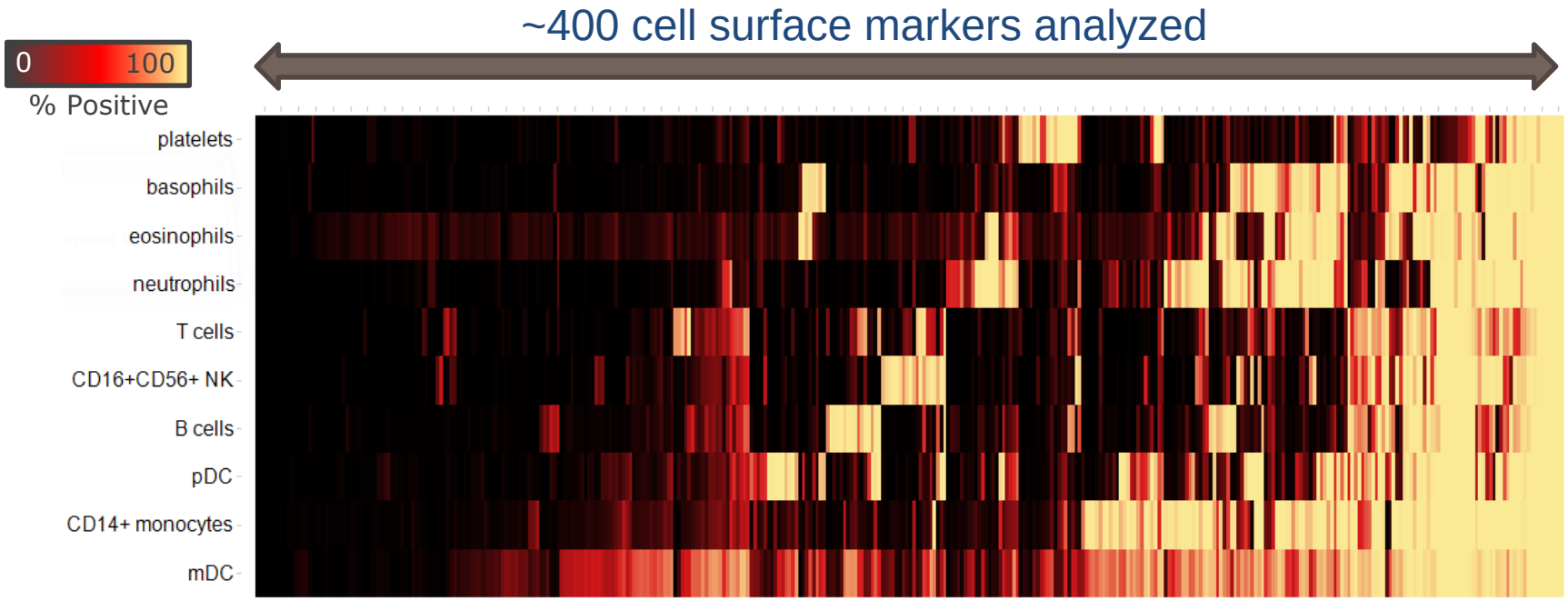
Antigen density: B-cell and T-cell panel



Antigen density: B-cell and T-cell panel



Summary: antigen density study



- Complements the information provided by the BD Biosciences Human CD Marker Chart (additional specificities from other vendors to increase specificities to >350).
- Provides information on antigen expression in common lymphocyte cell subpopulations.
- Enables optimal panel design by guiding the selection of antigen-fluorochrome combinations.

Antigen expression

Defining the biology of your assay

Antigen expression

- Conjugated antibodies used to define specific cell types should be selected with spectrally distinct fluorochrome labels.

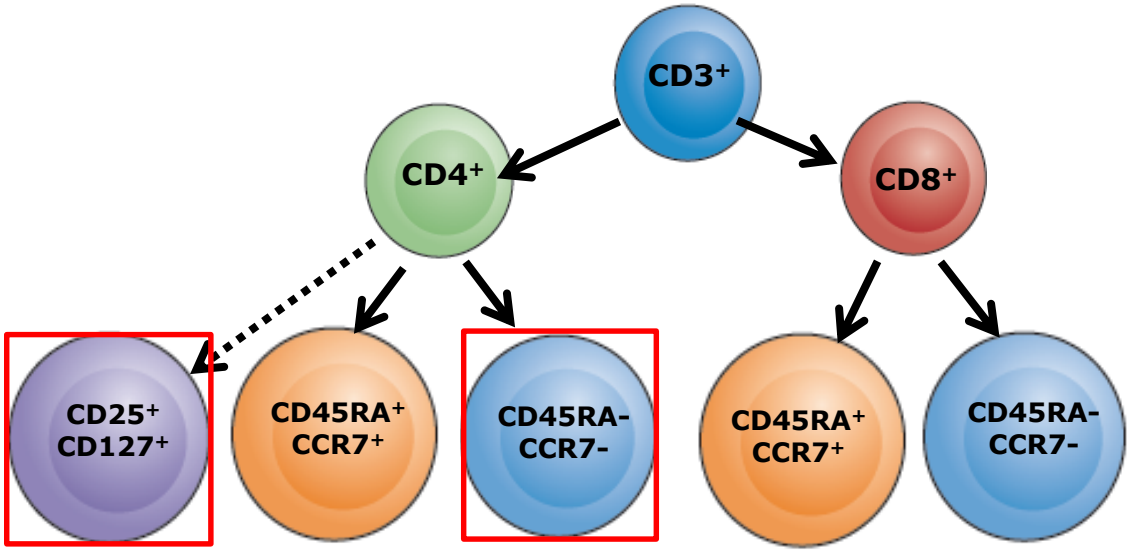
- Basic concept of panel design:
  - "for low expressed antigens use brightest available fluorochrome".

- What does this mean for the possible markers for a T-cell panel?
  - CD3, CD4, CD8, CD45RA, CD27, CCR7, CD25, CD127

BD

Defining the biology of your assay

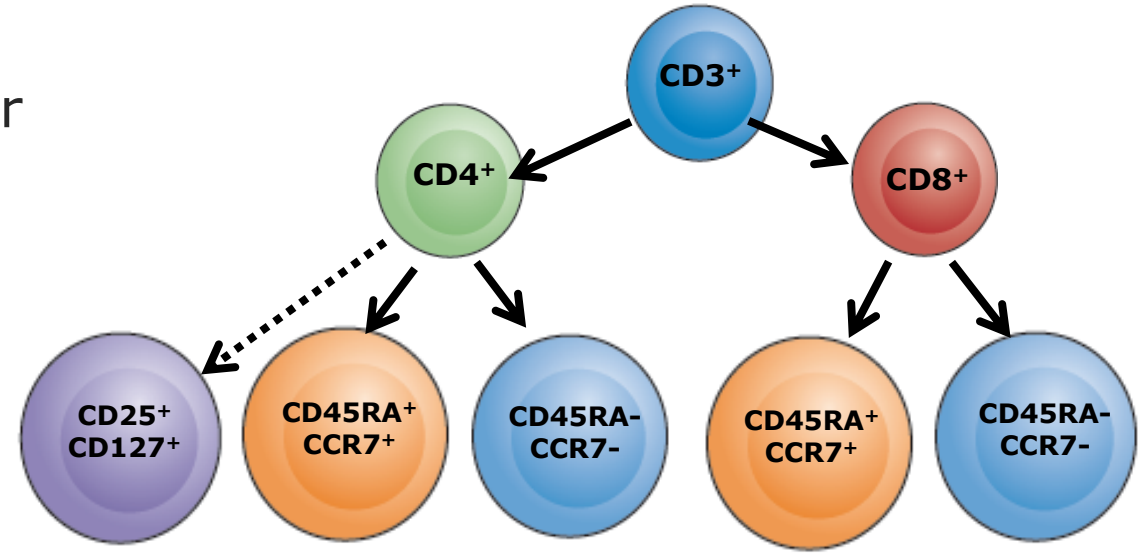
- Define a population tree based on the goals of the assay.
- Identify the critical populations.
- Determine which antigens are co-expressed and at what levels.



Ag	Tregs	CD4 naïve T cells	CD4 memory T cells	CD8 naïve T cells	CD8 memory T cells
CD3					
CD4					
CD8					
CD45RA					
CD127					
CD25					
CCR7					

Review antigen expression levels

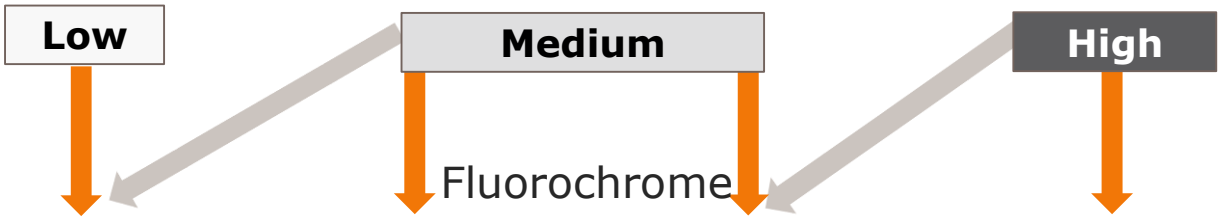
- Assign antigen expression levels for each subpopulation using data from:
  - Antigen density study
  - Technical Data Sheet (TDS)
  - Literature
  - Colleagues
  - Pre-testing



Ag	Tregs	CD4 naïve T-cells	CD4 memory T-cells	CD8 naïve T-cells	CD8 memory T-cells
CD3					
CD4				X	X
CD8	X	X	X		
CD45RA					
CD127					X
CD25				X	X
CCR7				X	

Hi expression	
Med expression	
Lo expression	
No applicable in panel	X

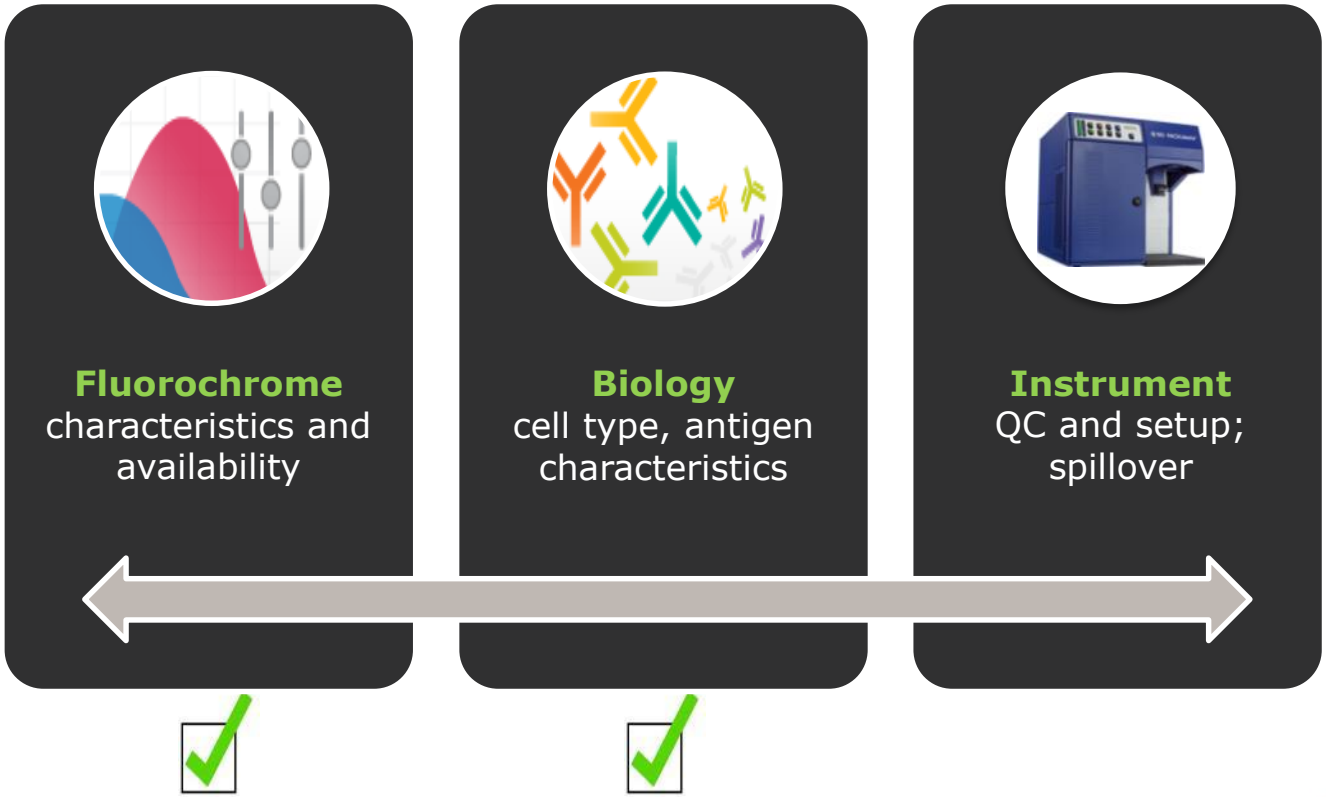
Antigen/fluorochrome combinations



Laser	Very Bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon BUV661 BD Horizon BUV737 BD Horizon BUV563	BD Horizon BUV395 BD Horizon BUV496	BD Horizon BUV805
Violet (405 nm)	BD Horizon BV421 BD Horizon BV650 BD Horizon BV711	BD Horizon BV480 BD Horizon BV605 BD Horizon BV786	BD Horizon BV510	BD Horizon V450 BD Horizon V500
Blue (488 nm)	BD Horizon BB515 BD Horizon PE-CF594 PE-Cy5	PE PE-Cy7	FITC Alexa Fluor® 488 PerCP-Cy5.5	PerCP
Yellow/Green (561 nm)	PE BD Horizon PE-CF594 PE-Cy5 PE-Cy7			
Red (640 nm)		APC Alexa Fluor® 647 BD Horizon APC-R700		Alexa Fluor® 700 APC-H7 APC-Cy7

Elements of multicolor flow cytometry

Considerations in designing panels:



Instrument

Setting up your instrument to maximize resolution and consistency

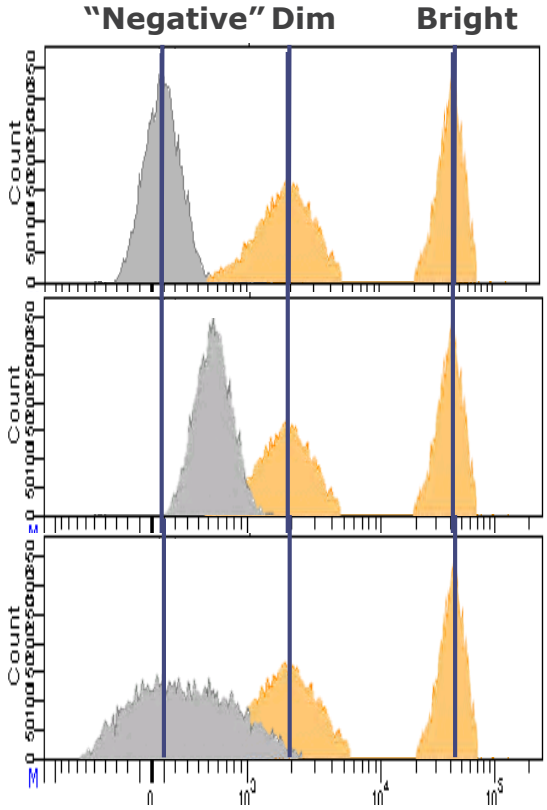
Resolution vs background

- Resolution: The degree to which a flow cytometer can distinguish dimly stained cells from unstained cells.
- This can be challenging in a polychromatic scenario.

Negative population has low background; populations well resolved.

Negative population has high background; populations not resolved.

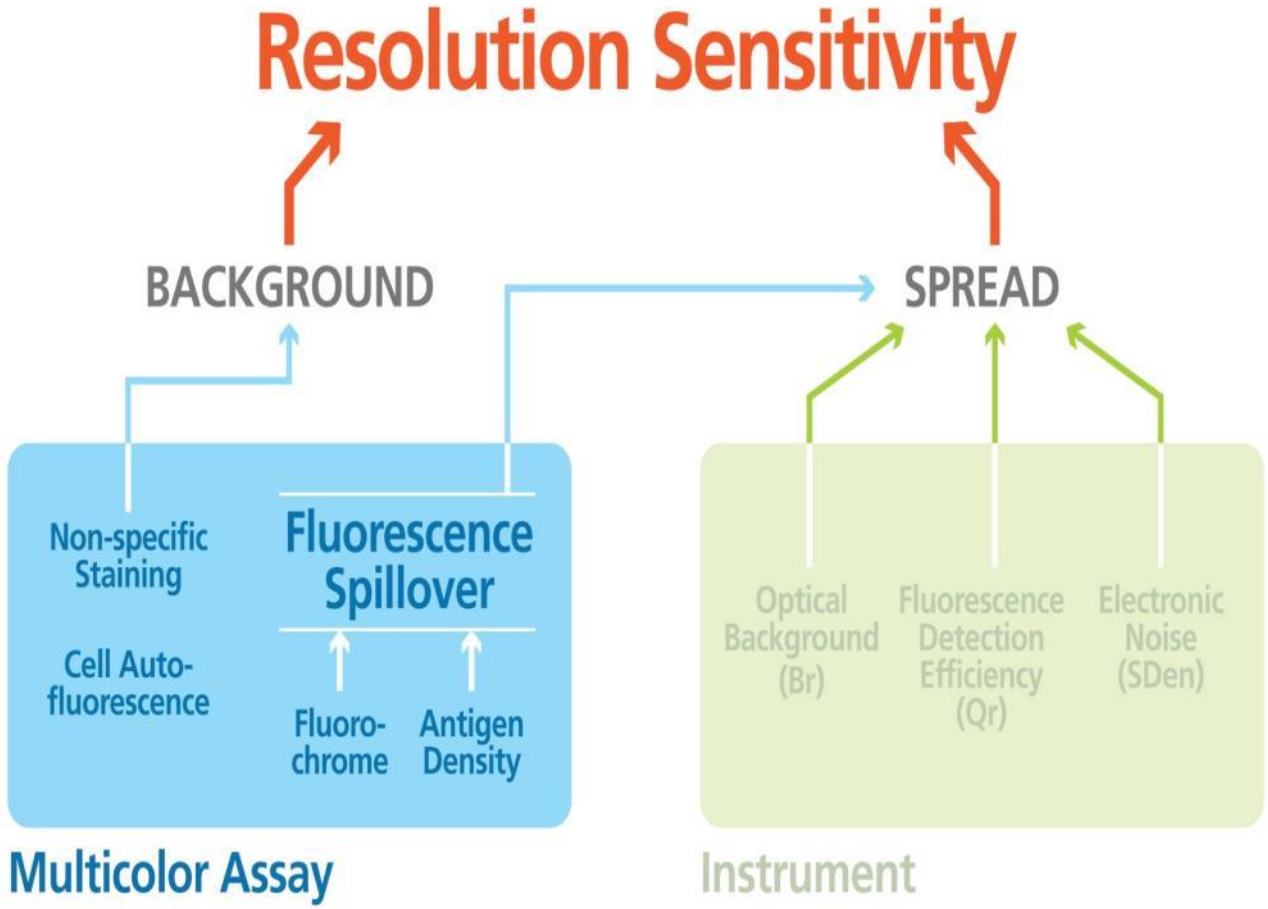
Negative population has low background but high rSD (spread); populations not resolved.



The ability to resolve populations is a function of both **background** *and* **spread** of the negative population.

Fluorescence spillover

Factors impacting resolution



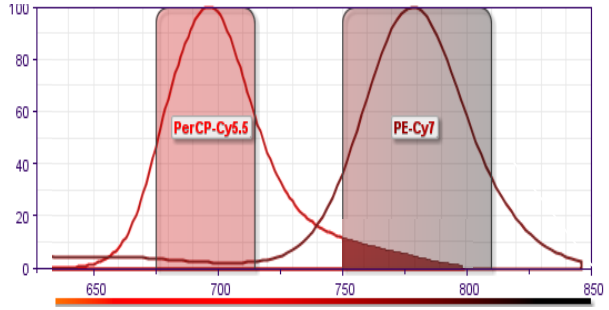
Why do we care about fluorescence spillover?

- Resolution of populations in multicolor panels
  - Fluorescence spillover is an important factor in creating a panel design with good resolution of populations of interest.

- Visualization of multicolor data
  - Incorrect or poor calculation of spillover values (SOVs) negatively impacts the quality of data obtained from an assay.

BD

Fluorescence spillover introduces background and spread into other detectors

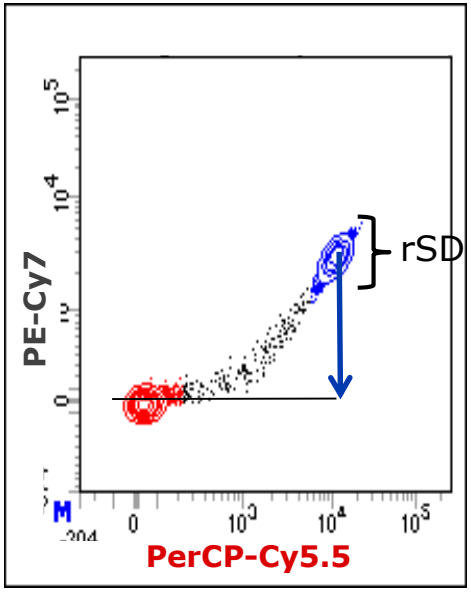


Fluorochromes spill over into other detectors; for example, PerCP-Cy5.5 spills into the PE-Cy7 detector.

This fluorescence spillover contributes to:

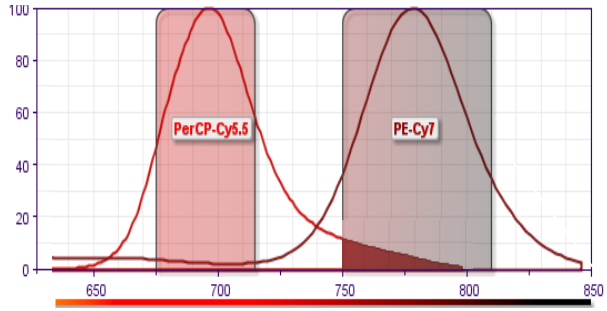
- Increased background (MFI)
- Spread (measured as rSD)

	Negative		Positive	
	MFI	**rSD**	**MFI**	**rSD**
No comp	12	29	3,098	291
Comp				



This "background" is subtracted in the process called compensation.

Fluorescence spillover introduces background and spread into other detectors

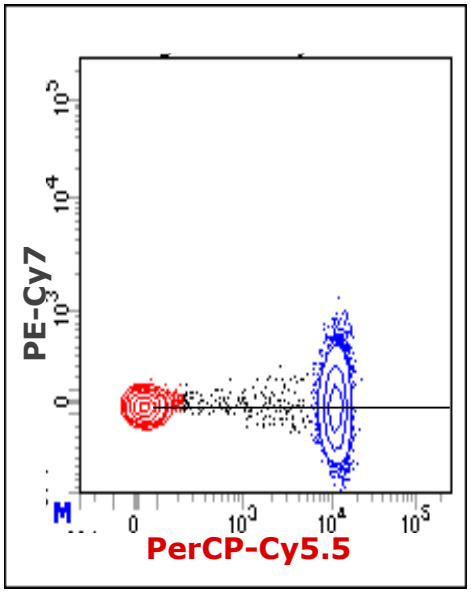


Fluorochromes spill over into other detectors; for example, PerCP-Cy5.5 spills into the PE-Cy7 detector.

This fluorescence spillover contributes to:

- Increased background (MFI)
- Spread (measures as rSD)

	Negative		Positive	
	MFI	**rSD**	**MFI**	**rSD**
No comp	12	29	3,098	291
Comp	4	29	3	289



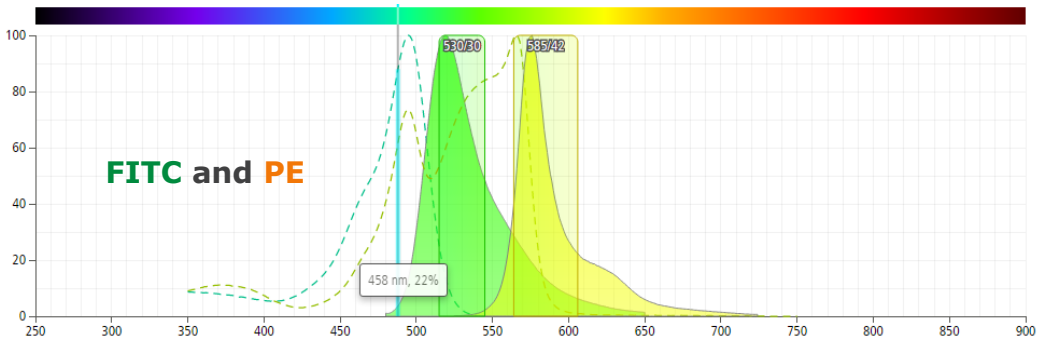
This "background" is subtracted in the process called compensation.

A sample is correctly compensated when, in the spillover detector (PE-Cy7), the MFI of the positive population is equivalent to that of the negative population.

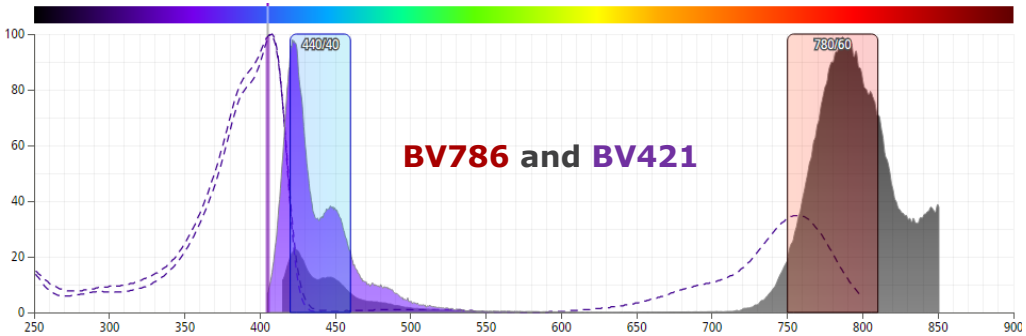
However, the spread introduced by the spillover is not removed by the compensation and reduces the resolution (SI) of any double-positive cells.

What are some sources of spillover?

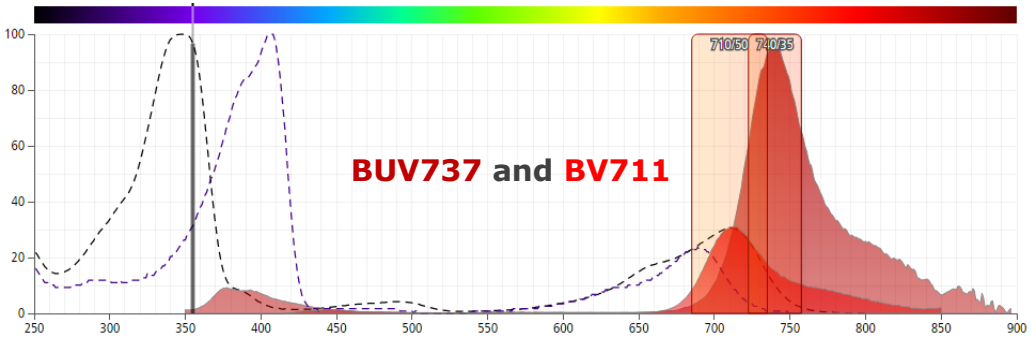
Adjacent detectors



Residual base fluorescence



Similar emission spectra (cross-laser)



A guide to spillover

	BD Biosciences fluorochromes									
	~380	~480	~530	~575	~610	~660	~685	~710	~740	~780
Ultraviolet (355 nm)	BUV395	BUV496				BUV661			BUV737	BUV805
Violet (405 nm)		BV421 V450	BV510 V500		BV605	BV650		BV711		BV786
Blue (488 nm)			FITC BB515	PE	PE-CF594	PE-Cy5	PerCP PerCP-Cy5.5			PE-Cy7
Yellow/Green (561 nm)				PE	PE-CF594	PE-Cy5	PE-Cy5.5			PE-Cy7
Red (640 nm)						APC		APC-R700		APC-H7 APC-Cy7

- Fluorochromes with similar emission spectra will have the greatest potential for cross-laser spillovers.
- Residual spillover between tandems and their base
- Spillover into adjacent detectors

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Conclusion

Considerations in designing panels: