Principles of Multicolor Panel Design

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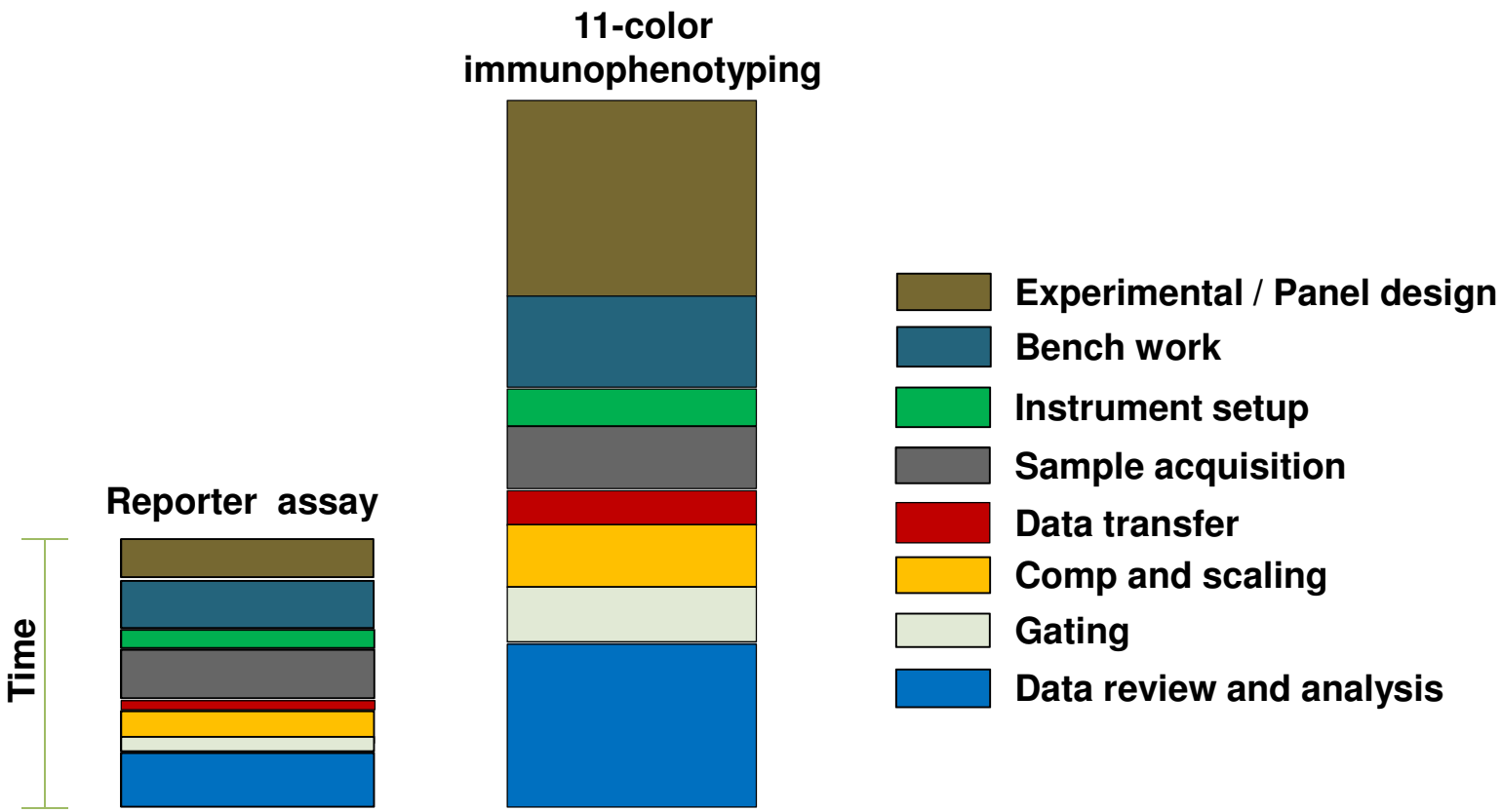
Common Multicolor Research Applications

- Intracellular cytokine staining
- Regulatory T cells (Tregs)
- Protein phosphorylation (BD Phosflow)
- Leukemia and lymphoma phenotyping
- Stem cell research

Increasing Complexity



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Challenges of Multicolor Applications

- Loss of sensitivity due to spillover
- Cytometer optimization and experimental design are critical
- Complexity of analysis

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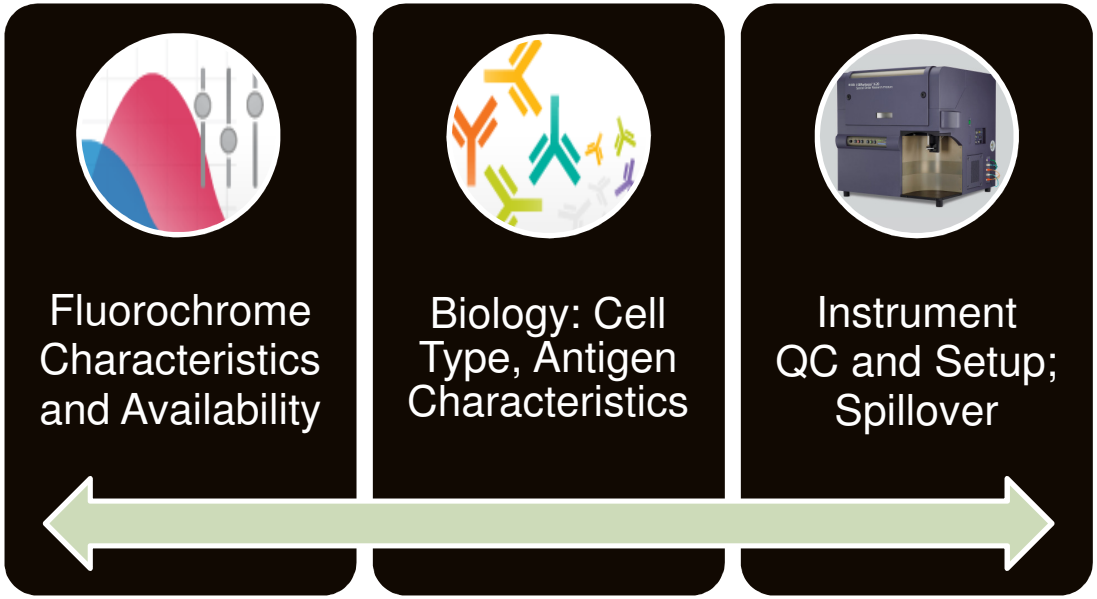
Elements of Multicolor Flow Cytometry



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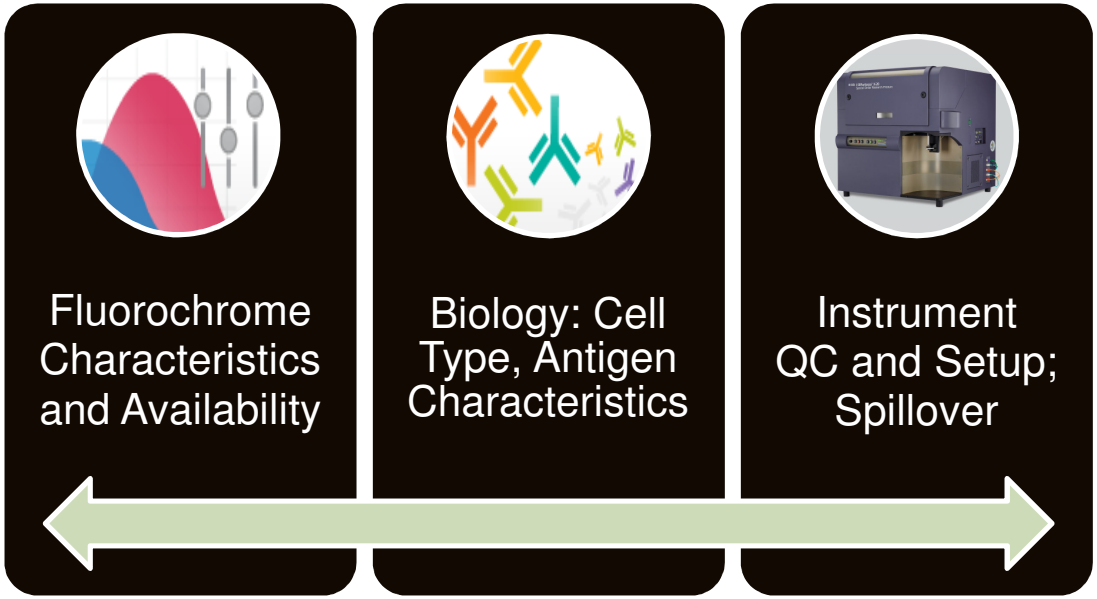
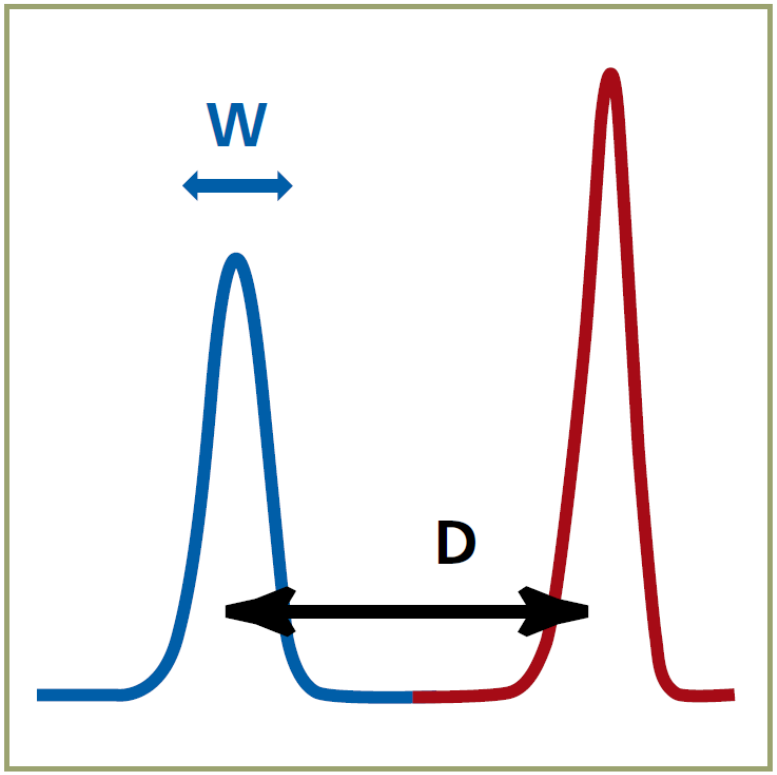
Elements of Multicolor Flow Cytometry



荧光强度
Stain Index

抗原密度
Antigen Density

系统设置



$$\text{Stain Index} = \frac{D}{W}$$

Stain Index = D/W

Resolution sensitivity (the ability to resolve a dim positive signal from background) is a function of the difference between positive and background peak means (D) and the spread of the background peak (W). The stain index is a metric that captures both of these factors.

$$\text{stain index} = \frac{(\text{positive population median} - \text{negative population median})}{2 \times \text{negative population rSD}}$$

BD Accuri™ C6	BD FACSCalibur™	BD FACSVerse™	BD FACSCanto™ II	BD LSRFortessa™ X-20	BD FACSAria™ Product Family	BD Influx™	BD FACSJazz™	Excitation Laser Line	Filter	Relative Brightness 488 / 561		Blue Laser (488 nm) / Yellow-Green Laser (561 nm)
●	●	●	●	●	●	●	●	488 nm	530/30	■		BD Horizon Brilliant™ Blue 515 (BB515) (Ex_{max} 490 nm/Em_{max} 515 nm) is a dye that was exclusively developed by BD Biosciences as a brighter alternative to FITC. This dye is up to seven times brighter than FITC and has less spillover into the PE channel. Due to similar excitation and emission properties, BD Horizon BB515 and FITC/ Alexa Fluor® 488 cannot be used simultaneously.
●	●	●	●	●	●	●	●	488 nm	530/30	■		Alexa Fluor® 488 (Ex_{max} 495 nm/Em_{max} 519 nm) conjugates are highly photostable and remain fluorescent over a broad pH range. Alexa Fluor® 488 tends to be brighter than FITC and more optimal for intracellular applications. Due to nearly identical excitation and emission properties, FITC and Alexa Fluor® 488 cannot be used simultaneously. Alexa Fluor® 488 exhibits extraordinary photostability, which makes it highly suitable for fluorescence microscopy.
●	●	●	●	●	●	●	●	488 nm	530/30	■		FITC (Ex_{max} 494 nm/Em_{max} 520 nm) Fluorescein isothiocyanate (FITC) is a fluorochrome with a molecular weight of 389 Da. FITC is sensitive to pH changes and photobleaching. Due to nearly identical excitation and emission properties, FITC and Alexa Fluor® 488 cannot be used simultaneously. FITC is relatively dim and should be reserved for highly expressed markers whenever possible.
●	●	●	●	●	●	●	●	488 nm 532 nm 561 nm	575/26	■	■	PE (Ex_{max} 496 nm/Em_{max} 578 nm) R-phycoerythrin (PE) is an accessory photosynthetic pigment found in red algae. It exists in vitro as a 240-kDa protein with 23 phycoerythrobilin chromophores per molecule. This makes PE the brightest fluorochrome for flow cytometry applications, but its photobleaching properties make it unsuitable for fluorescence microscopy.
		●	●	●	●	●		488 nm 532 nm 561 nm	610/20	■	■	BD Horizon™ PE-CF594 (Ex_{max} 496 nm/Em_{max} 612 nm) is a tandem conjugate, developed exclusively by BD Biosciences, that combines PE and CF594. PE-CF594 is a brighter alternative to PE-Texas Red® with improved spectral characteristics.
	●	●	●	●	●	●		488 nm 532 nm 561 nm	670/14	■	■	PE-Cy™5 (Ex_{max} 496 nm/Em_{max} 667 nm) is a tandem conjugate that combines phycoerythrin and the cyanine dye Cy5. Because of its broad absorption range and the fact that its emission spectra are equivalent to APC, PE-Cy5 is not recommended for simultaneous use with APC. The Cy5 molecule has been shown to exhibit nonspecific binding to Fc receptors, which is most apparent on monocyte populations.
●	●	●	●	●	●	●		488 nm 532 nm	695/40	■		PerCP (Ex_{max} 482 nm/Em_{max} 678 nm) is a component of the photosynthetic apparatus found in the dinoflagellate *Glenodinium*. PerCP is a protein complex with a molecular weight of ~35 kDa. Due to its photobleaching characteristics, PerCP conjugates are not recommended for use on flow cytometers with high-power lasers (>25 mW).
●	●	●	●	●	●	●	●	488 nm 532 nm	695/40	■		PerCP-Cy™5.5 (Ex_{max} 482 nm/Em_{max} 695 nm) is a tandem conjugate that combines PerCP with the cyanine dye Cy5.5. PerCP-Cy5.5 is not subject to photobleaching like PerCP and can be used with stream-in-air flow cytometers. Additionally, the PerCP-Cy5.5 tandem conjugate is not as susceptible to fixative or light instability compared to APC-Cy™7 and PE-Cy7.
●	●	●	●	●	●	●	●	488 nm 532 nm 561 nm	780/60	■	■	PE-Cy™7 (Ex_{max} 496 nm/Em_{max} 785 nm) is a tandem fluorochrome that combines PE and the cyanine dye Cy7. PE-Cy7 is sensitive to photo-induced degradation, resulting in loss of fluorescence and changes in spillover. Extreme caution must be taken to avoid light exposure and prolonged exposure to paraformaldehyde fixative. Fixed cells should be analyzed within 4 hours of fixation in paraformaldehyde or transferred to a paraformaldehyde-free buffer for overnight storage.

Red Laser (640 nm)

BD Accuri™ C6	BD FACSCalibur™	BD FACSVerse™*	BD FACSCanto™ II	BD LSRFortessa™ X-20	BD FACSAria™ Product family	BD Influx™	BD FACSJazz™	Excitation Laser Line	Filter	Relative Brightness	
●	●	●	●	●	●	●	●	633 nm 635 nm 640 nm	660/20	□ ■ ■ ■	**APC (Ex_{max} 650 nm/Em_{max} 660 nm)**, Allophycocyanin (APC), is an accessory photosynthetic pigment found in blue-green algae. Its molecular weight is approximately 105 kDa. Due to nearly identical excitation and emission properties, APC and Alexa Fluor® 647 cannot be used simultaneously.
●	●	●	●	●	●	●	●	633 nm 635 nm 640 nm	660/20	□ ■ ■ ■	**Alexa Fluor® 647 (Ex_{max} 650 nm/Em_{max} 668 nm)** conjugates are highly photostable and remain fluorescent over a broad pH range. Due to nearly identical excitation and emission properties, APC and Alexa Fluor® 647 cannot be used simultaneously. APC tends to be brighter while Alexa Fluor® 647 is more optimal for intracellular applications. This fluorochrome exhibits uncommon photostability, making it an ideal choice for use in fluorescence microscopy.
		●	●	●	●	●		633 nm 635 nm 640 nm	730/45	□ ■ ■ ■	**BD Horizon™ APC-R700 (Ex_{max} 652 nm/Em_{max} 704 nm)** is a tandem fluorochrome that combines APC with R700, a proprietary organic dye. This dye has been developed exclusively by BD Biosciences as a brighter alternative to Alexa Fluor® 700. Due to similar excitation and emission properties, APC-R700 and Alexa Fluor® 700 cannot be used simultaneously.
		●	●	●	●	●		633 nm 635 nm 640 nm	730/45	□ □ □ ■	**Alexa Fluor® 700 (Ex_{max} 696 nm/Em_{max} 719 nm)** is a far-red dye that can be excited with a 633–640-nm laser. This enables multicolor analysis in conjunction with APC or Alexa Fluor® 647 and APC-H7 or APC-Cy7 reagents.
		●	●	●	●	●	●	633 nm 635 nm 640 nm	780/60	□ □ □ ■	**APC-Cy™7 (Ex_{max} 650 nm/Em_{max} 785 nm)** is a tandem fluorochrome that combines APC and the cyanine dye Cy7. Special precautions must be taken with APC-Cy7 conjugates, and cells stained with them, to protect the fluorochrome from long-term exposure to light. Fixed cells should be analyzed within 4 hours of fixation in paraformaldehyde or transferred to a paraformaldehyde-free buffer for overnight storage. Due to nearly identical excitation and emission properties, APC-Cy7 and APC-H7 cannot be used simultaneously.
		●	●	●	●	●	●	633 nm 635 nm 640 nm	780/60	□ □ □ ■	**APC-H7 (Ex_{max} 650 nm/Em_{max} 785 nm)** is an APC-cyanine tandem fluorochrome which uses an analog of Cy7 and has similar spectral properties to APC-Cy7. APC-H7 conjugates provide greater stability in light and paraformaldehyde fixatives and have less spillover into the APC channel than APC-Cy7 conjugates. Due to nearly identical excitation and emission properties, APC-Cy7 and APC-H7 cannot be used simultaneously.

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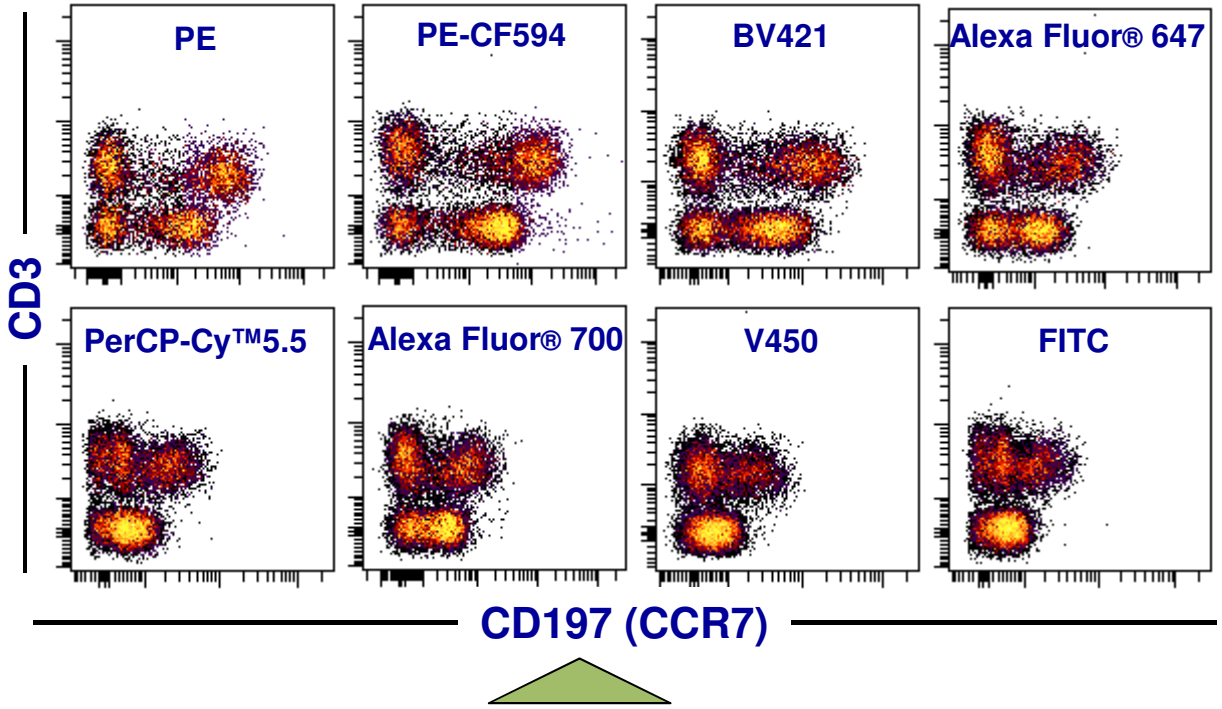
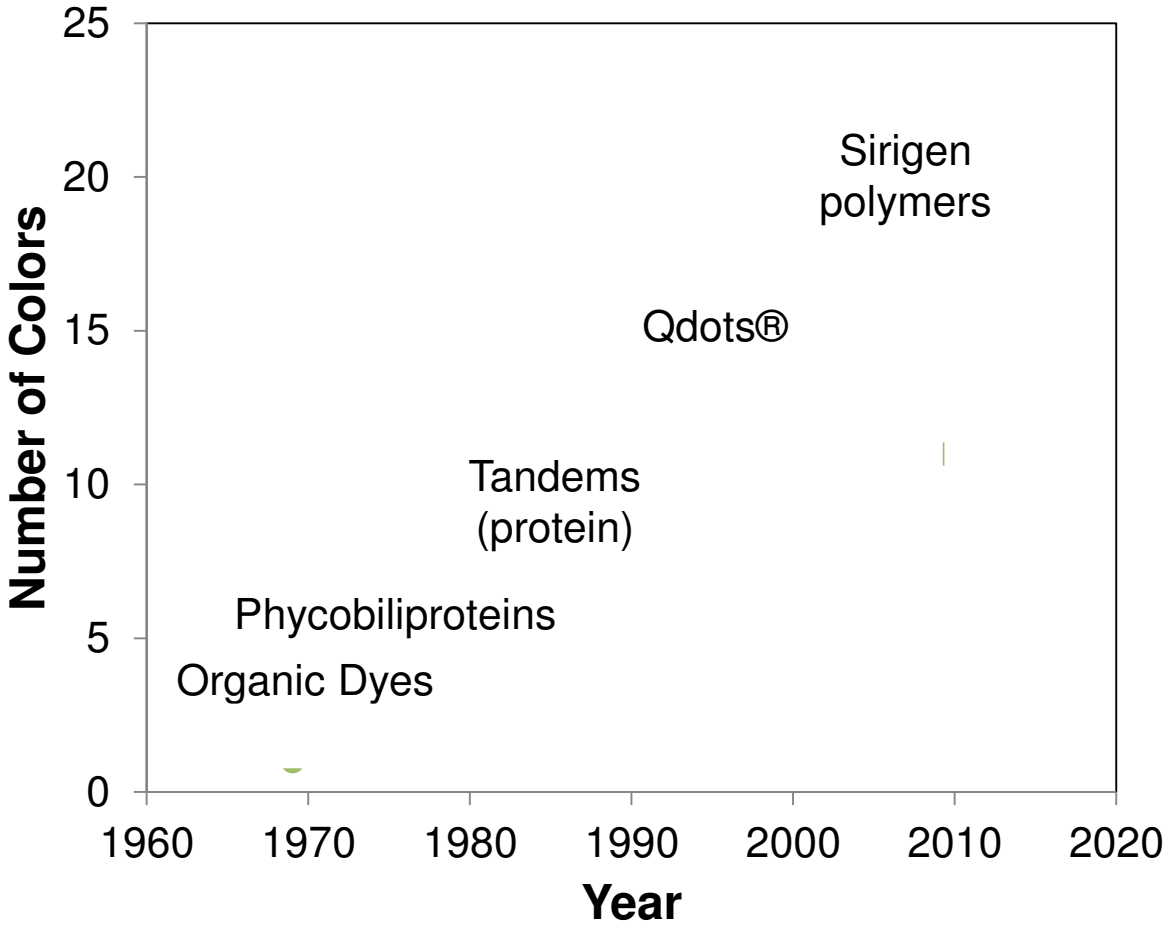
Fluorochromes Reveal Biology



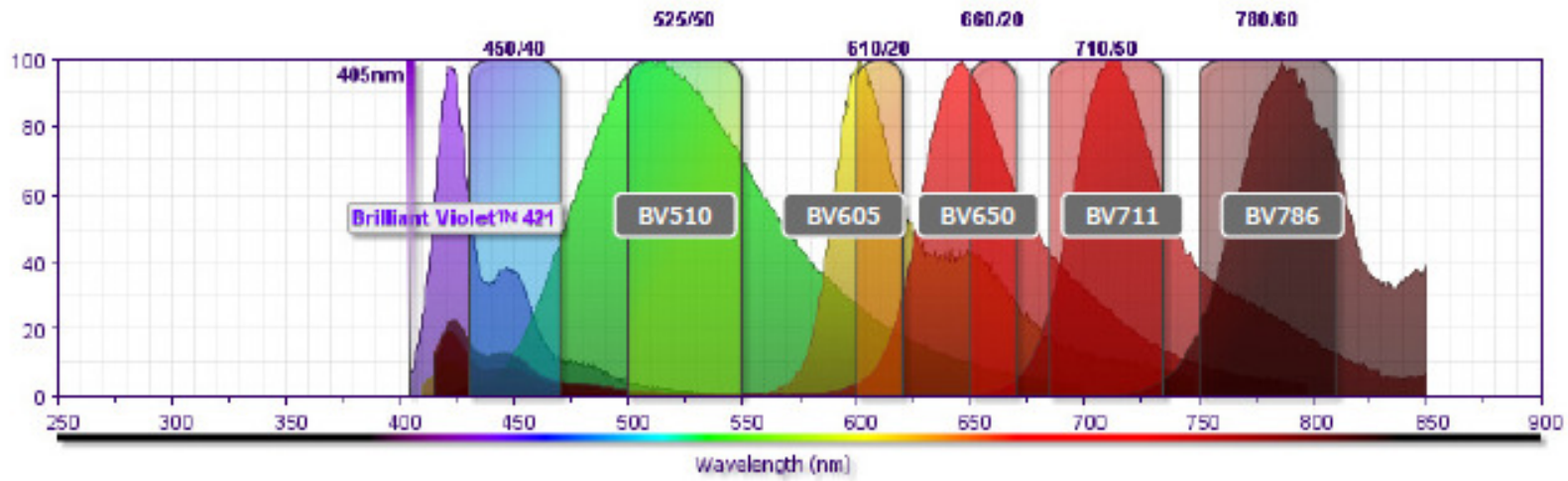
Bright dyes are important when looking at dim antigens.
Proper choice of fluorochrome helps us understand more about
The biology of the experiment.

Evolution of Fluorochromes



- 367 CD markers
- Intracellular proteins
  - Cytokines
  - Cell signaling
  - Transcription factors
  - Phosphoproteins
- Availability of fluorochromes has driven major advances in flow cytometry

BD Horizon Family-- Brilliant Violet

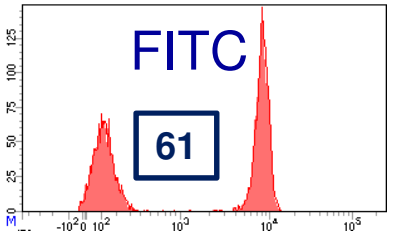


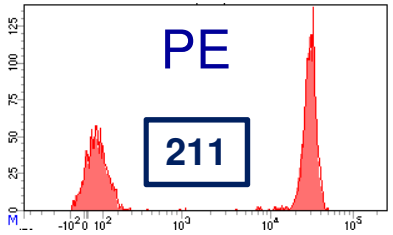
BV家族：BV421 BV510 BV605 BV650 BV711 BV786

- Six dyes excited by the violet laser
  - Base polymers: BV421 and BV510
  - Tandems: BV605, BV650, BV711, and BV786
- Bright dyes
- Limited cross laser excitation
- Compatible with surface and intracellular targets

BD Horizon Brilliant Violet

CD3





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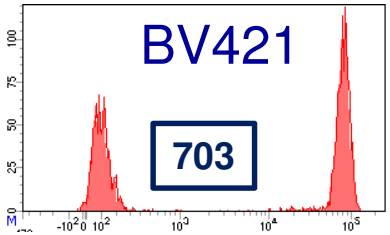
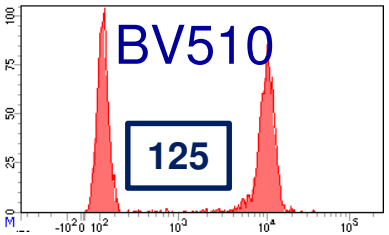
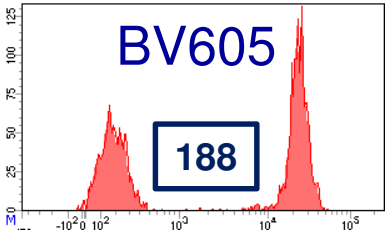
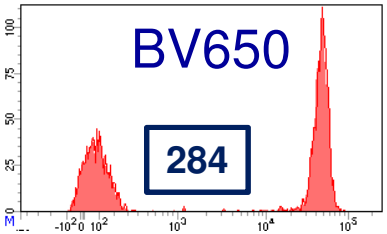
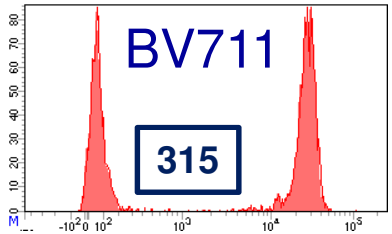
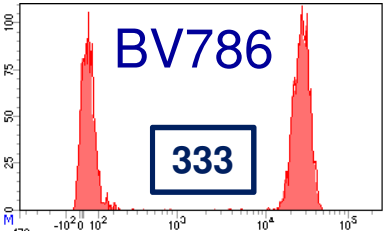






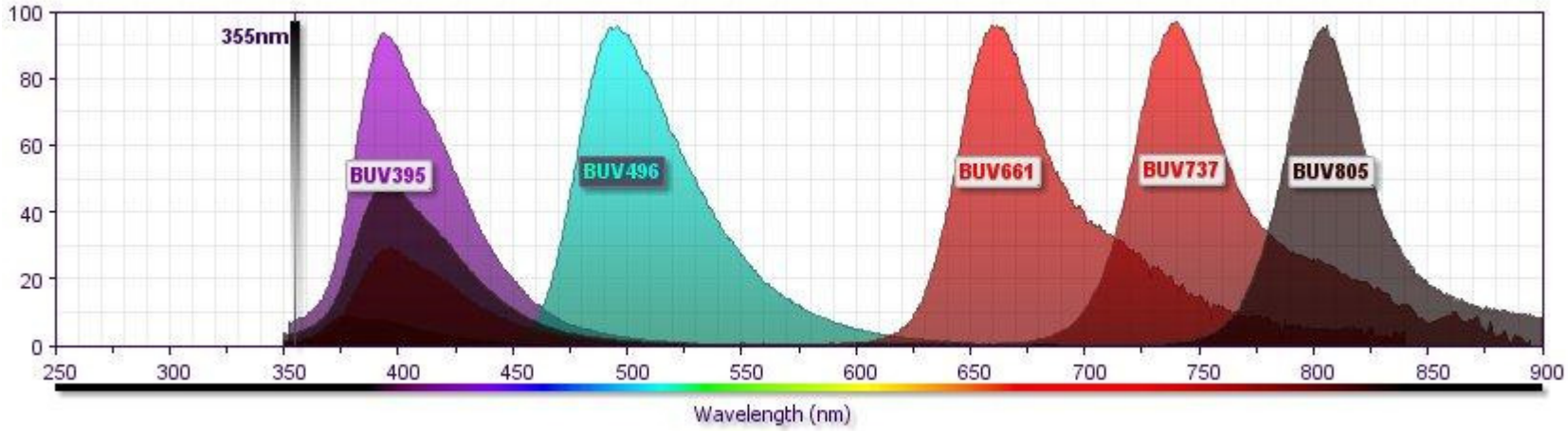






Very Bright	Bright	Moderate	Dim
BD Horizon BV421	BD Horizon BV605	BD Horizon BV510	BD Horizon V450
BD Horizon BV650	BD Horizon BV786		BD Horizon V500
BD Horizon BV711			

BD Horizon Family--Brilliant UltraViolet

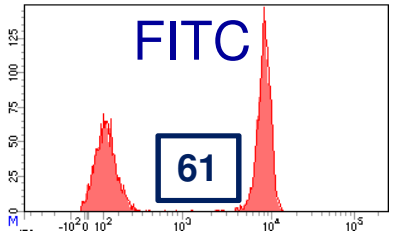


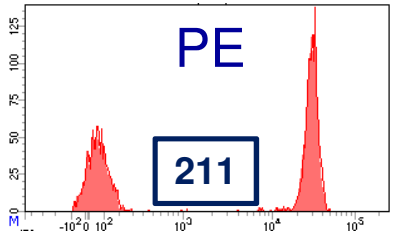
BUV家族：BUV395, BUV496, BUV661, BUV737, BUV805

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

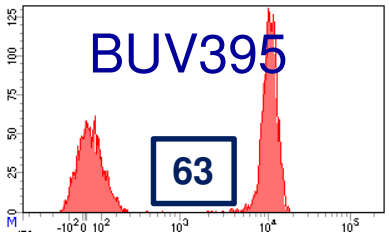
BD Horizon Brilliant Ultraviolet

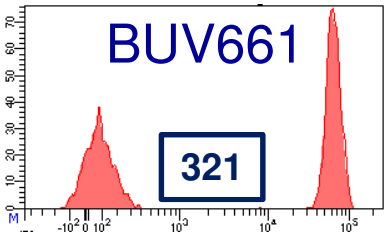
CD3

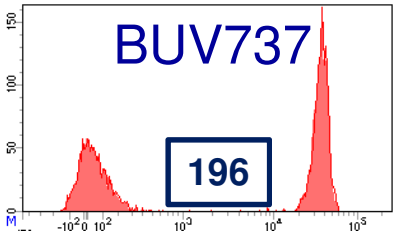


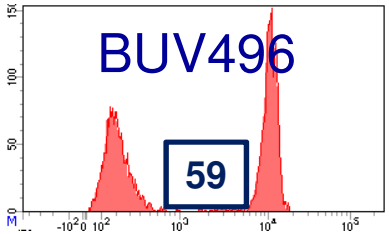


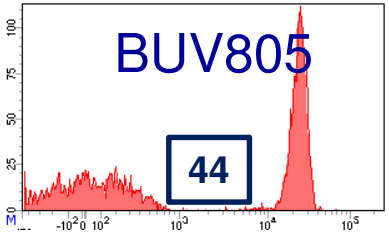
Stain Index





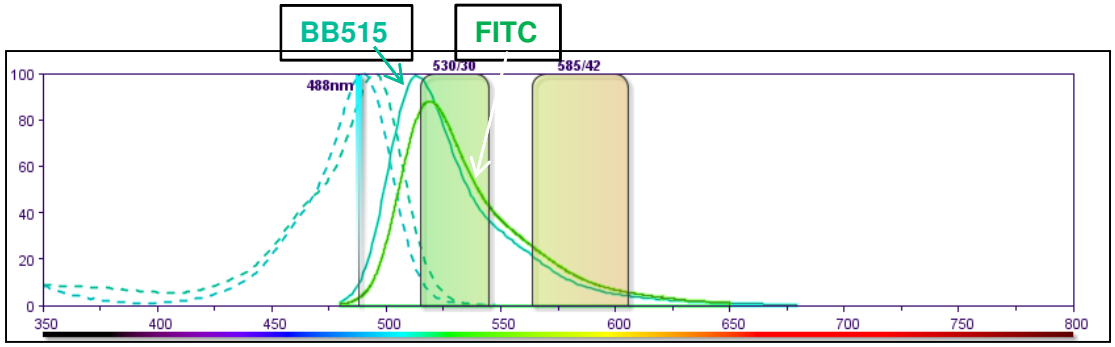




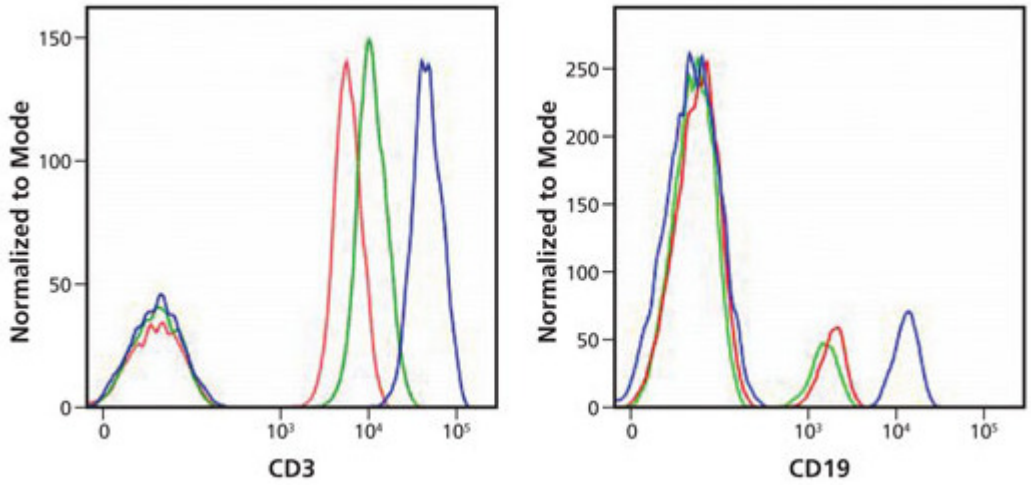


Bright	Moderate	Dim
BD Horizon BUV661	BD Horizon BUV395	BD Horizon BUV805
BD Horizon BUV737	BD Horizon BUV496	

BD Horizon Brilliant Blue515

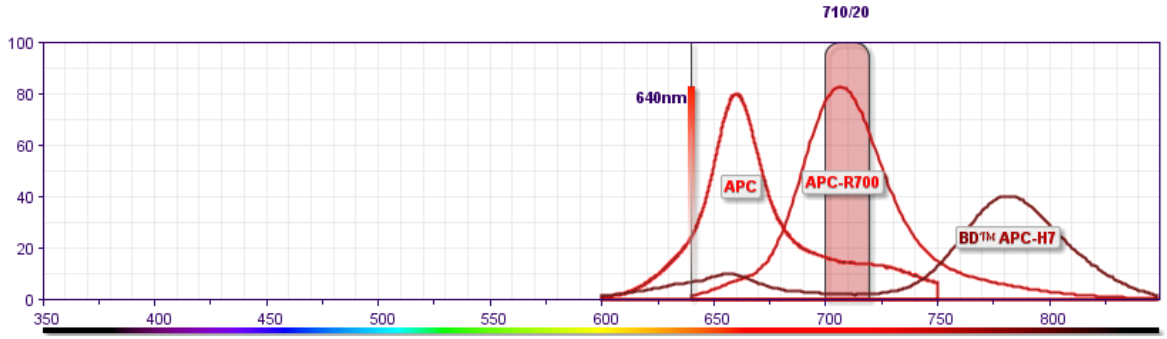


- Blue laser (488 nm) excitation
- Replacement for FITC and Alexa Fluor® 488
- Much brighter alternative to FITC with less spillover into the PE detector

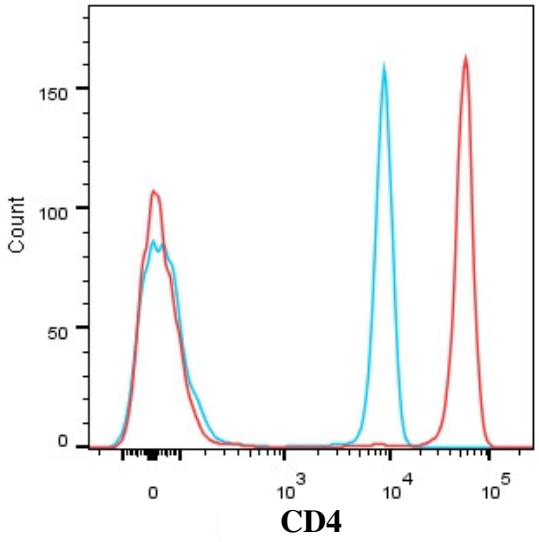


FITC
Alexa Fluor® 488
BB515

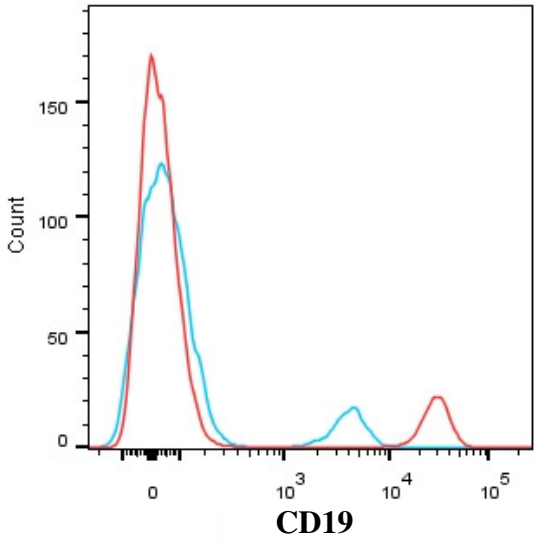
BD Horizon APC-R700



- Red laser (640 nm) excitation
- Replacement for Alexa Fluor® 700
- Much brighter alternative to Alexa Fluor® 700



CD4



CD19

Alexa Fluor® 700

APC-R700

Fluorochrome Resolution Ranking

Laser	Fluorochrome: Very Bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon™ BUV661 BD Horizon™ BUV737	BD Horizon™ BUV395 BD Horizon™ BUV496	BD Horizon™ BUV805
Violet (405 nm)	BD Horizon™ BV421 BD Horizon™ BV650 BD Horizon™ BV711	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-Cy™5	PE PE-Cy™7	FITC Alexa Fluor® 488 PerCP-Cy™5.5	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

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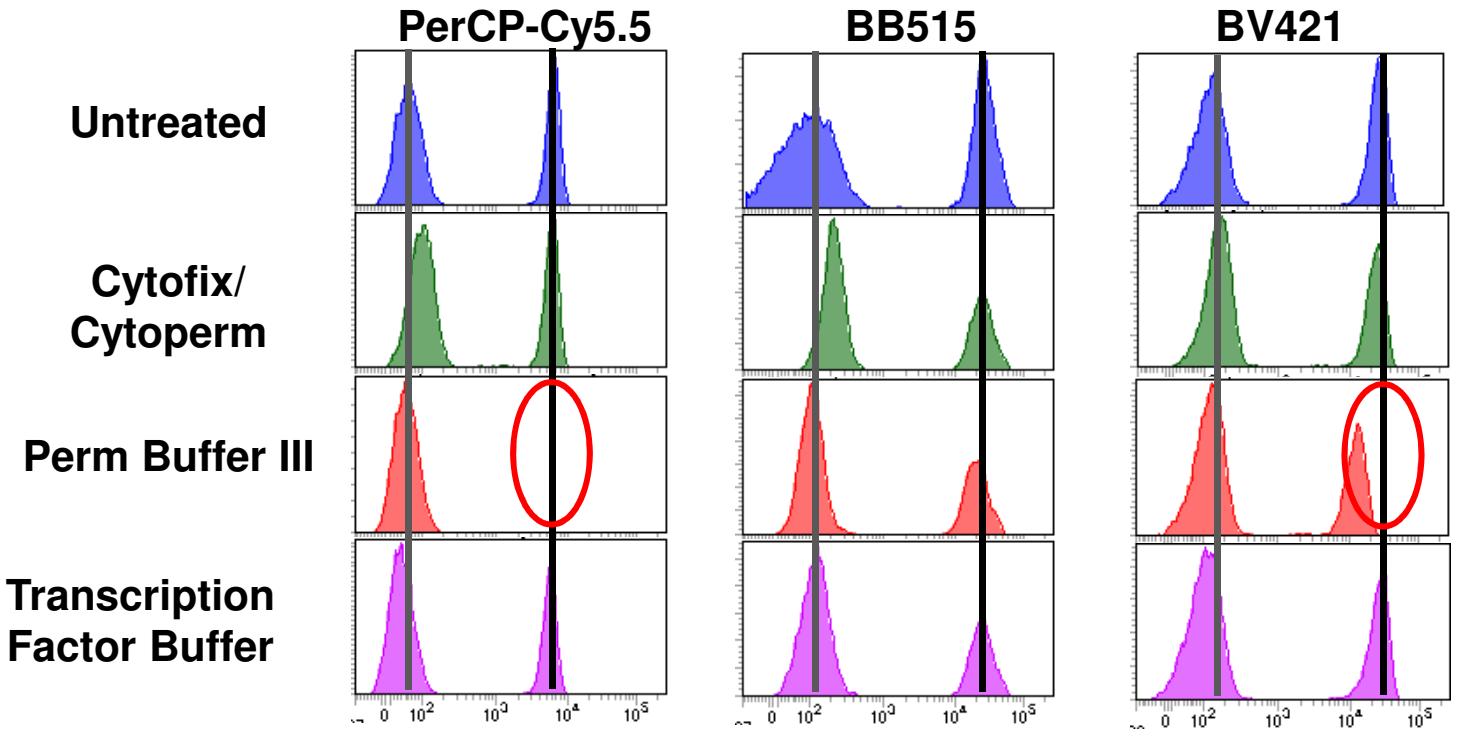
Considerations for Intracellular Panels



Cells were stained with CD4 conjugates of different fluorochromes and then fixed

The fixation and permeabilization process needed for intracellular staining can decrease dye fluorescence intensity.

Impact of Fixation on Fluorescence Intensity

>85%	60-85%	45-60%	20-45%	<20%

Fluor	MFI (% of Control)		
	Cytofix/ Cytoperm	Perm III	TF Buffer
FITC			
Alexa Fluor®488			
BB515			
PerCP			
PerCP-Cy5.5			
PE			
PE-CF594			
PE-Cy5			
PE-Cy7			

Fluor	MFI (% of Control)		
	Cytofix/ Cytoperm	Perm III	TF Buffer
APC			
Alexa Fluor®647			
Alexa Fluor®700			
APC-Cy7			
APC-H7			

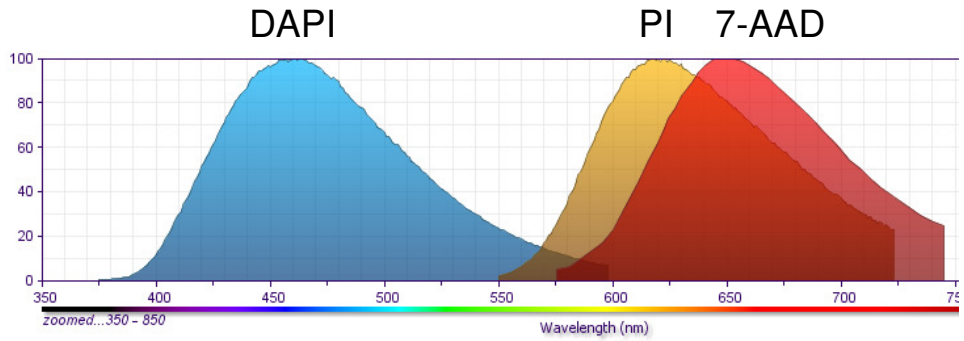
Fluor	MFI (% of Control)		
	Cytofix/ Cytoperm	Perm III	TF Buffer
V450			
BV421			
V500			
BV510			
BV605			
BV650			
BV711			
BV786			
BUV395			
BUV496			
BUV737			

- Cells were stained with CD4 conjugates of different fluorochromes and then fixed
- The fluorescence intensity (MFI) of the $CD4^+$ cells of the fixed sample was compared to unfixed (control).

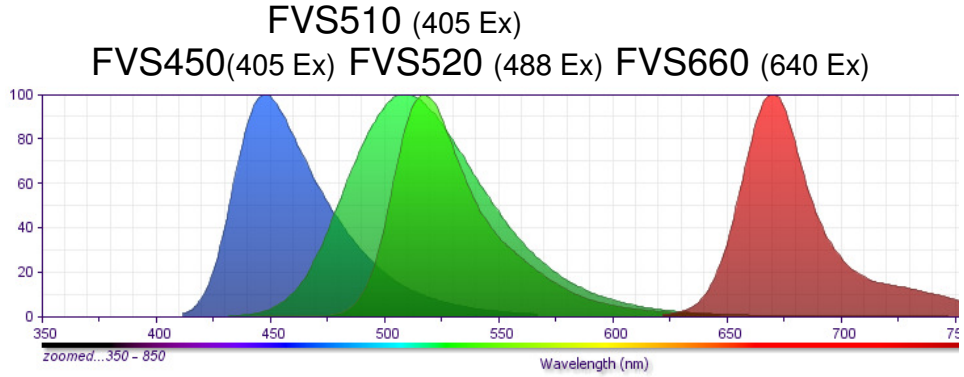
When considering relative fluorochrome ranking you need to take into account the loss of brightness due to fixation and permeabilization.

Assessing Cellular Viability

- DNA intercalation dyes (eg, PI, 7-AAD, DAPI)
  - Broad excitation and emission
  - Incompatible with intracellular protocols
  - Simple staining procedure



- Amine-reactive dyes
  - Discrete excitation and emission
  - Compatible with intracellular protocols
  - Additional step in staining procedure

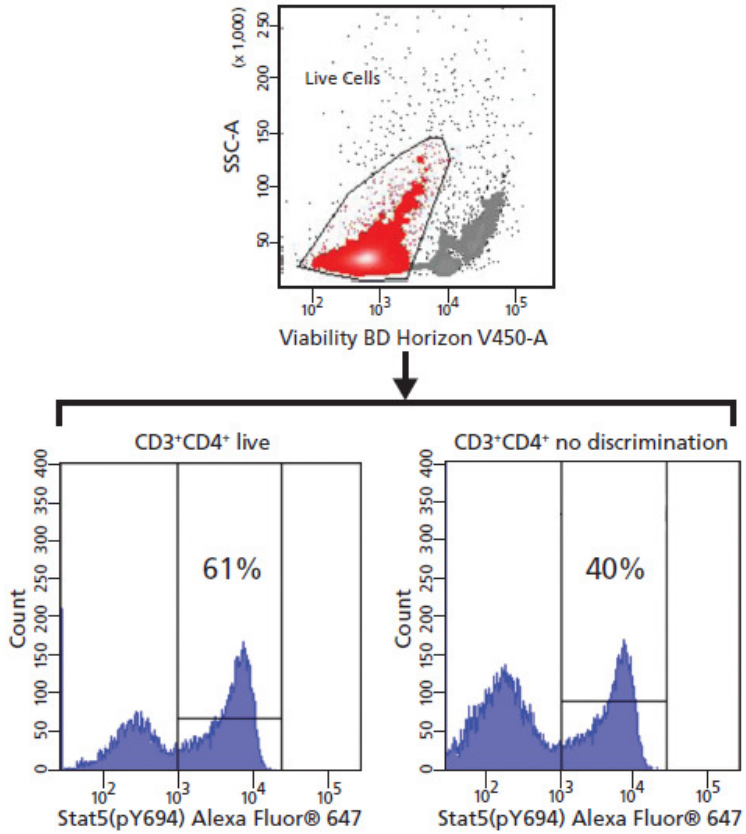


BD Horizon Fixable Viability Stains(FVS)

	FVS450	FVS510	FVS520	FVS570	FVS620	FVS660	FVS700
Excitation Laser	Violet	Violet	Blue	Blue / Yellow-Green	Blue / Yellow-Green	Red	Red
Emission	448 nm	512 nm	521 nm	570 nm	620 nm	660 nm	700 nm
Channel	BV421 V450	BV510 V500	BB515 FITC Alexa Fluor® 488	PE	PE-CF594 PE-Texas Red®	APC Alexa Fluor® 647	APC-R700 Alexa Fluor® 700

- Stain cells in protein- and azide-free buffers for 15 min prior to staining with antibodies.

Viability Staining Improves Results



- Gating out dead cells leads to more accurate statistics

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Choosing 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
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
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
405 nm			BV421/V450 BV510/V500	BV421/V450 BV510/V500 BV605 BV650 BV711 BV786	BV421/V450 BV510/V500 BV605 BV650 BV711 BV786
561 nm					PE PE-CF594 PE-Cy5 PE-Cy7
355 nm				BUV395 BUV496 BUV661 BUV737 BUV805	BUV395 BUV496 BUV661 BUV737 BUV805
# Lasers	**2**	**2**	**3**	**4**	**5**
# Colors	**4**	**6**	**8**	**18**	**18**

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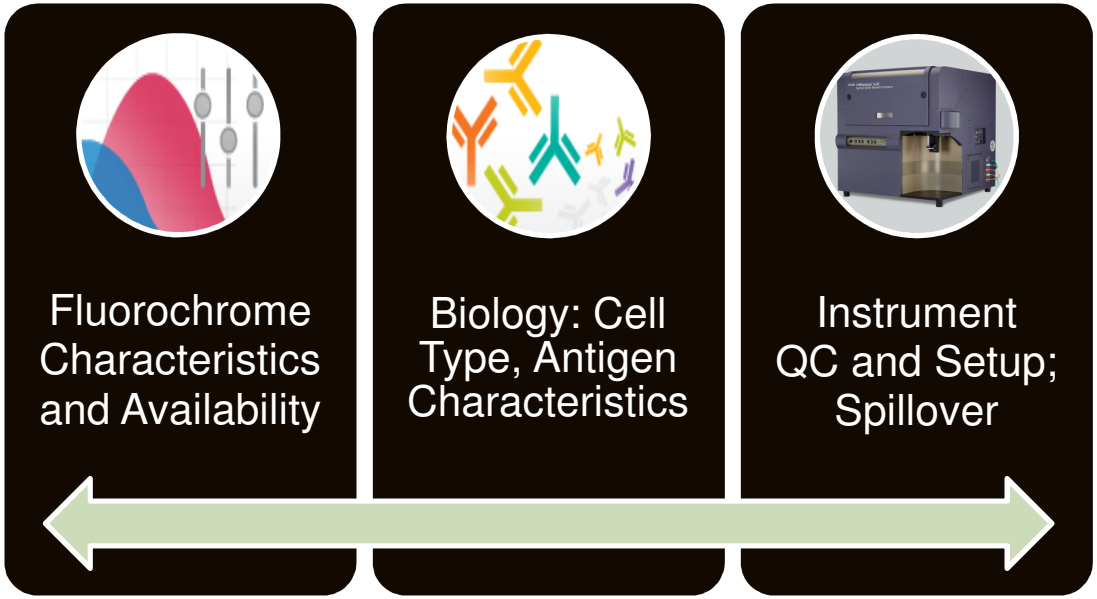
Elements of Multicolor Flow Cytometry



荧光强度
Stain Index

抗原密度
Antigen Density

系统设置

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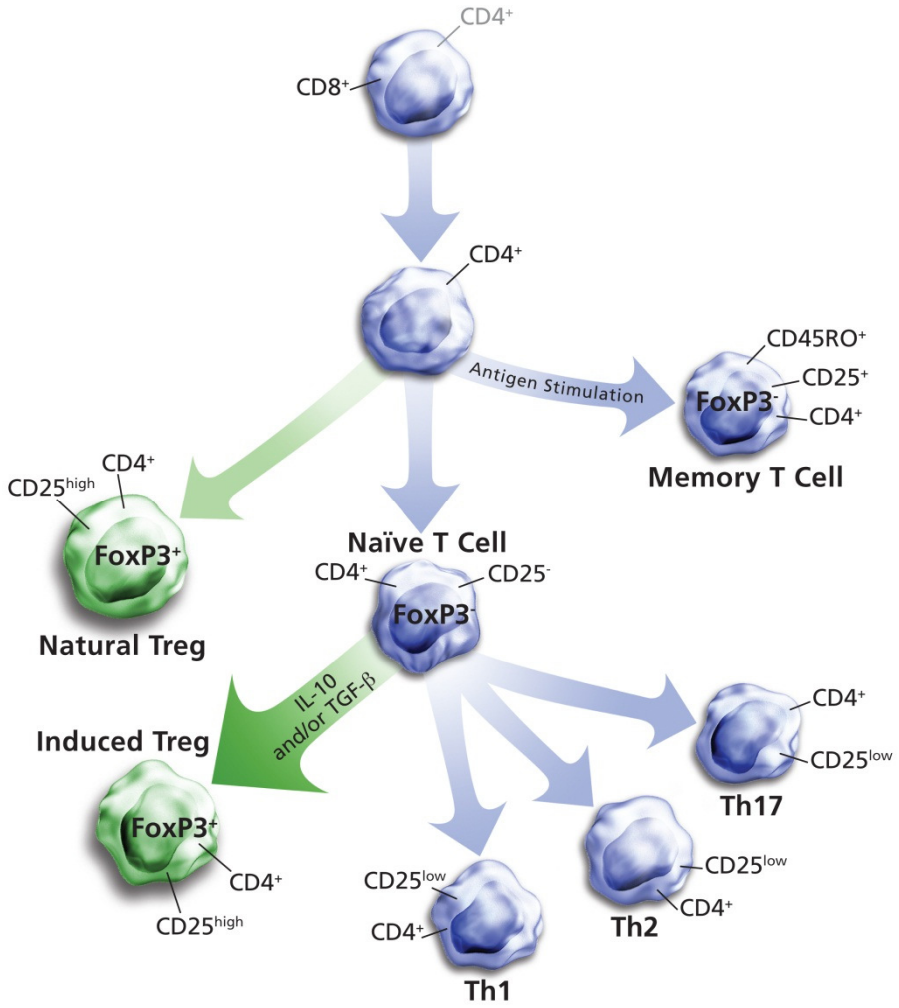
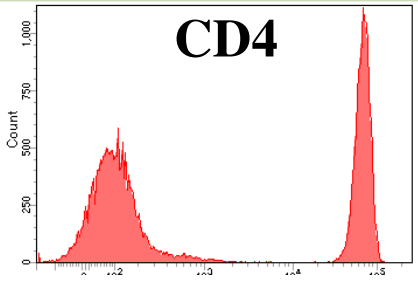
Understanding expression patterns



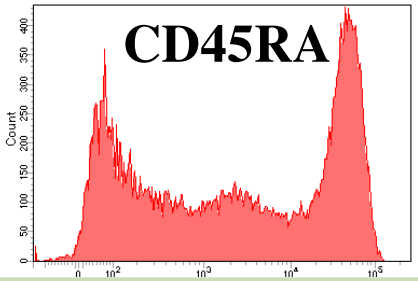
Classification of Antigens

Leucocyte antigens can be categorized based upon their patterns of expression

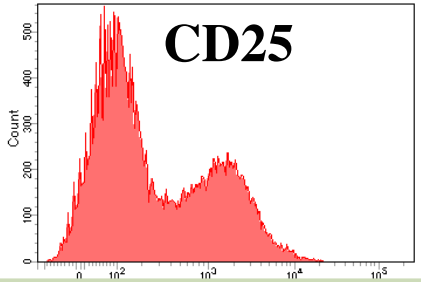
- **Primary:** Well characterized,
  easily classified as positive or negative,
  typically define broad subsets or lineages
  - Examples: CD3, CD4, CD19



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

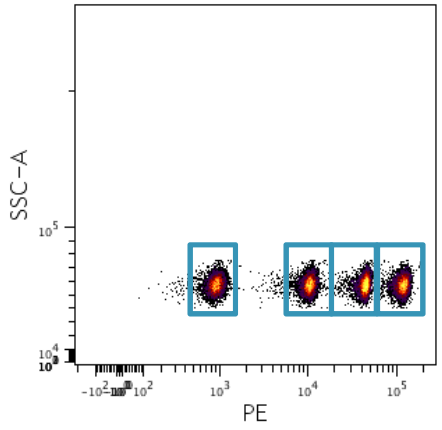


- **Tertiary**: Expressed at low levels,
  variable upon activation
  unknown, critical
  - Examples: CD25, STAT5, FoxP3

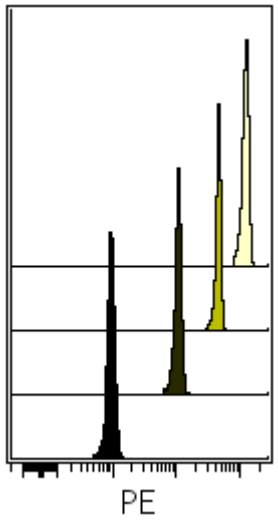


Using BD Quantibrite Beads for antigen enumeration

BD Quantibrite Beads

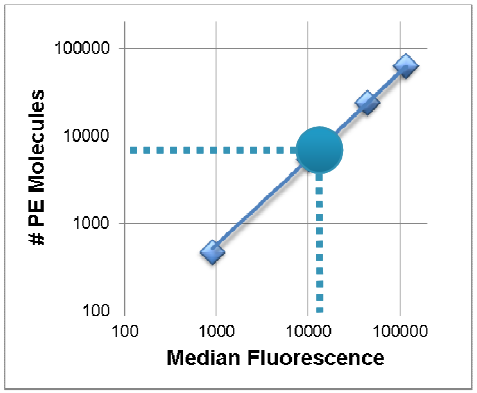


Gate four populations

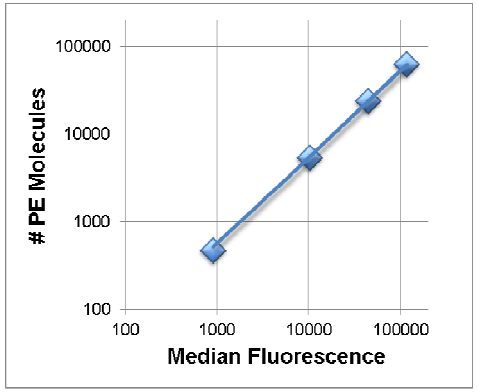


# PE Molecules	Median Fluorescence
474	913
5,359	10,367
23,843	44,342
62,336	117,857

Determine relationship of # PE molecules to MFI



Use MFI of stained sample to calculate Ag density



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Q

1. Are you currently using antigen density information to help you build your panels?

2. How are you currently getting information about antigen expression characteristics to inform multicolor panel design?

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List of Antigen Density

Subset	Antigen	Density
Lymphocytes	CD3	32,000
	CD4	36,400
	CD8	65,500
	CD19	7,800
T Cells ($CD3^+CD4^+$ Lymphocytes)	CD25	600
	$CD25^{hi}$	3,400
	CD27	10,900
	CD28	7,700
	CD45RA	33,400
	CD45RO	12,600
	CD122	5,300
	CD127	2,000
	CD132	400
	CD194 (CCR4)	2,500
	CD197 (CCR7)	2,000

Subset	Antigen	Density
B Cells ($CD19^+$ Lymphocytes)	CD20	24,600
	$CD24^{mid}$	3,000
	$CD24^{hi}$	16,100
	CD27	3,200
	$CD38^{mid}$	2,800
	$CD38^{hi}$	15,900
	CD138	400
	IgD^{mid}	4,900
	IgD^{hi}	23,800
	IgG	28,100
	IgM	3,800

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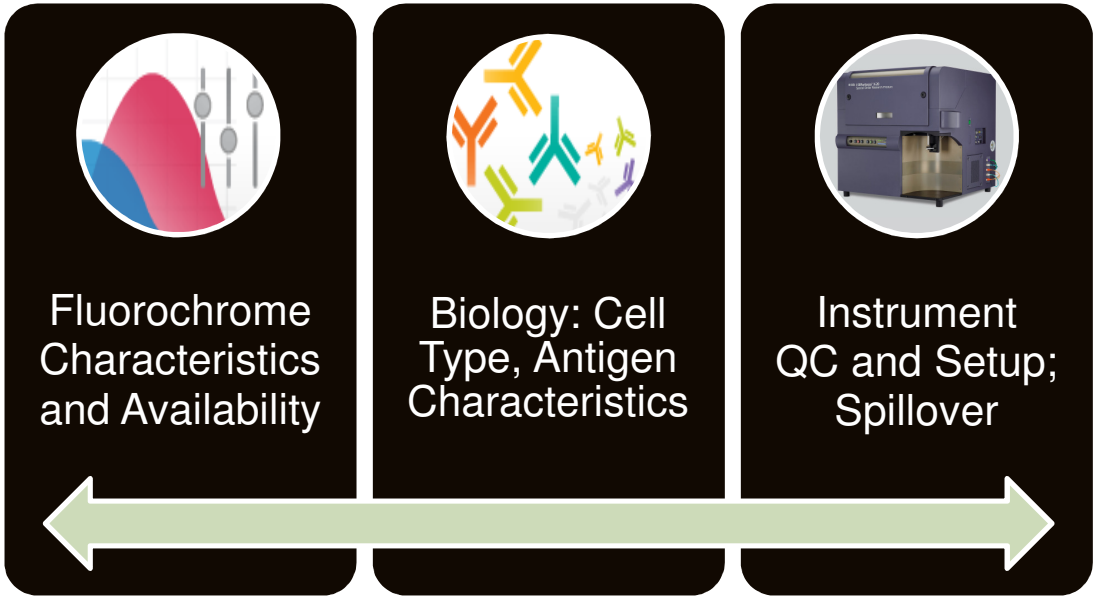
Elements of Multicolor Flow Cytometry



荧光强度
Stain Index

抗原密度
Antigen Density

系统设置

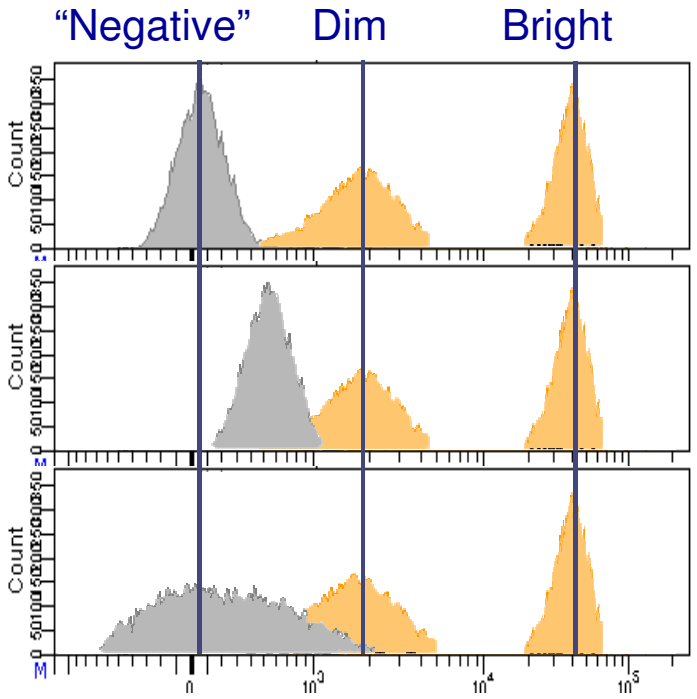
Resolution vs. Background

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.

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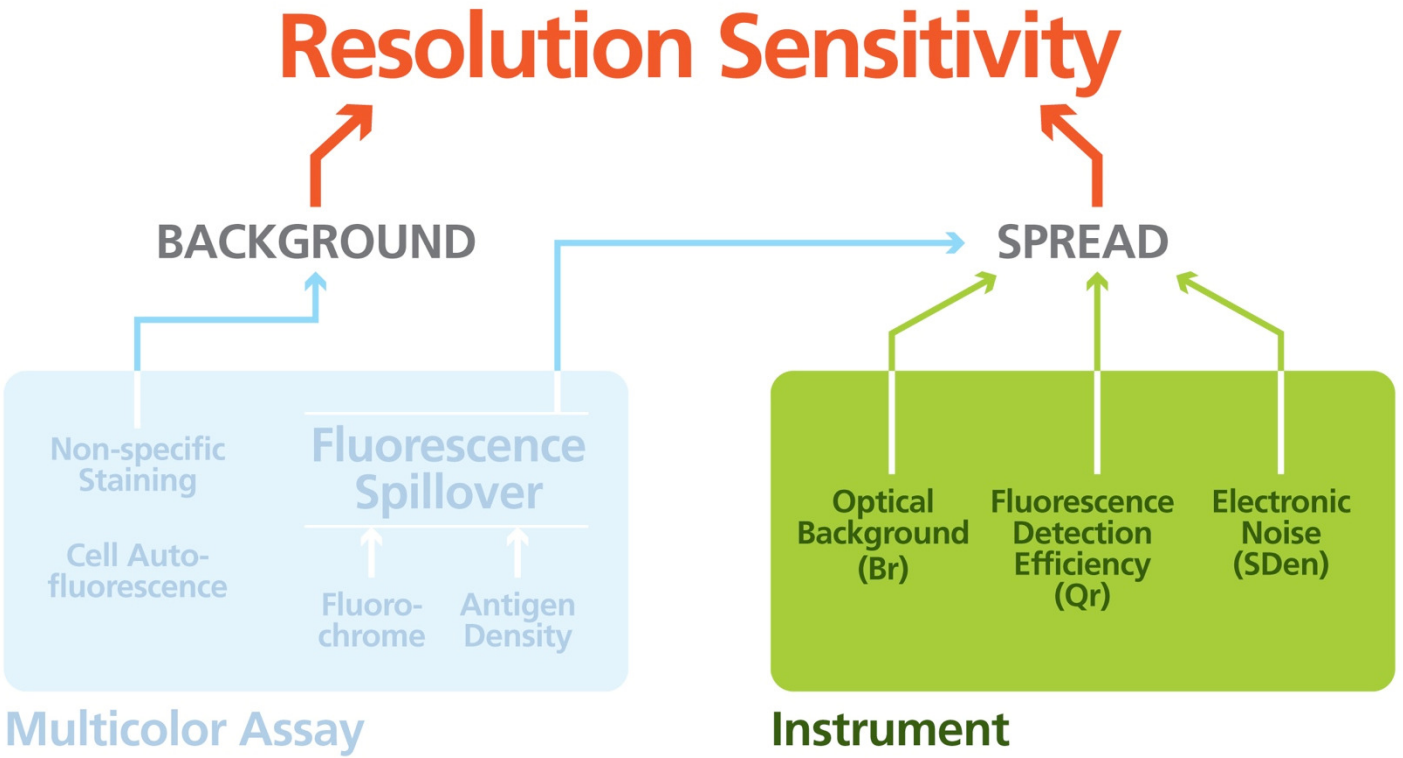
Factors impacting resolution



Cytometer Setup and Tracking(CS&T)

- CS&T is a fully integrated system of software and reagents:
  - BD FACSDiva™ and FACSuite™ software
  - BD™ CS&T Beads
- Functions of the CS&T system:
  - Define and characterize instrument performance factors which can impact sensitivity and population resolution
    - The relative fluorescence detection efficiency (Qr)
    - The relative optical background (Br)
    - The electronic background noise in the system (SD_{EN})
  - Track cytometer performance
  - Standardize and automate cytometer setup
    - Application/Tube Settings

Adjusting PMTVs to Maximize Dim Cell Resolution

- Historically, users would just place the “negative” population in the middle of the first decade.
- Information from the CS&T reports (SD_{EN}, Linearity) allows for rational adjustment of PMTs to ensure maximum resolution.
  1. For each fluorescence detector, run a sample of stained cells.
     - Preferably with a distinct bright positive population (for example, CD4, CD8, CD19)
  2. Adjust the PMTVs such that the negative population meets this condition:

$$\mathrm{rSD}_{\mathrm{Neg\,Cells}} > 2.5 \times SD_{\mathrm{EN}}$$

  3. Make sure that the positive cells are well on scale.
     - Make sure that the brightest cells that will be analyzed are within the linear range.
  4. If the positive cells are off scale or outside the linearity range, lower the PMTV until they are on scale.
  5. This detector is now set up to provide maximize resolution.

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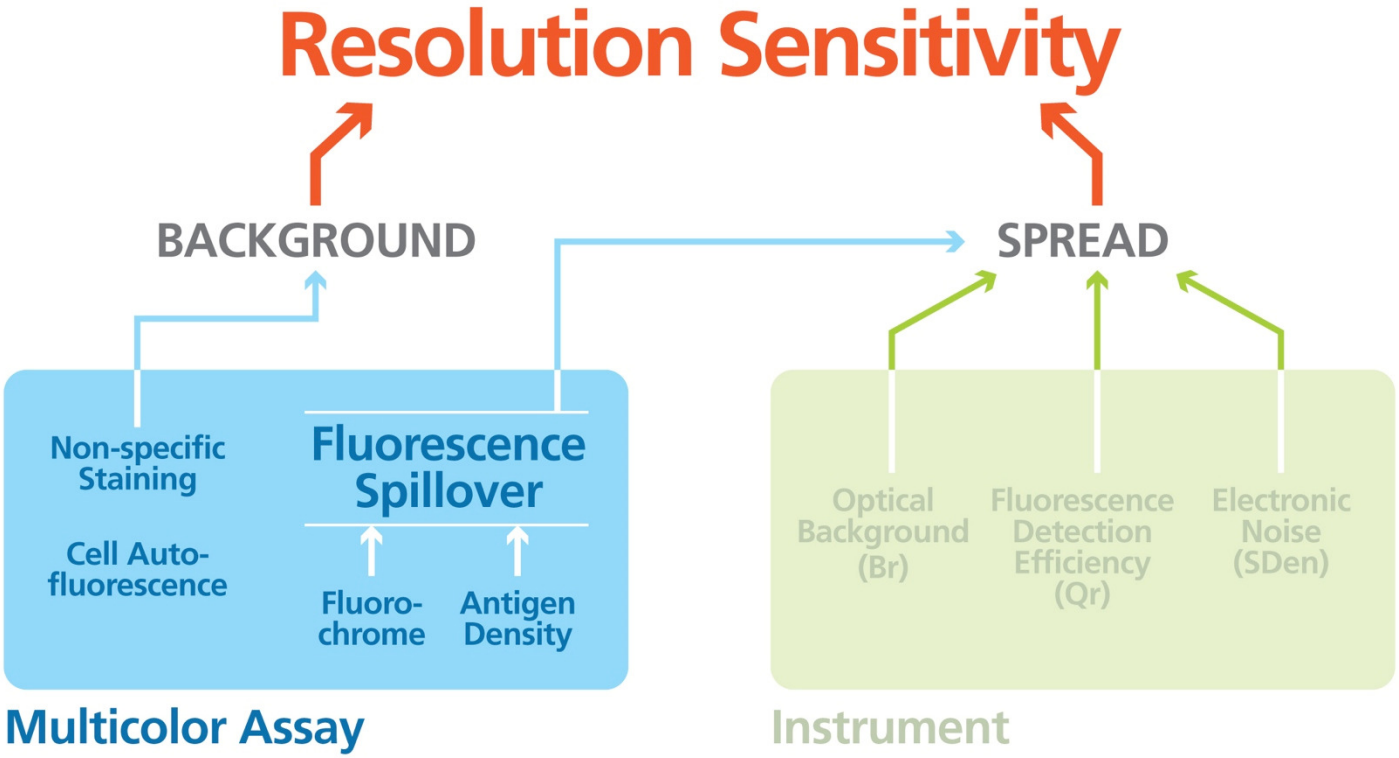
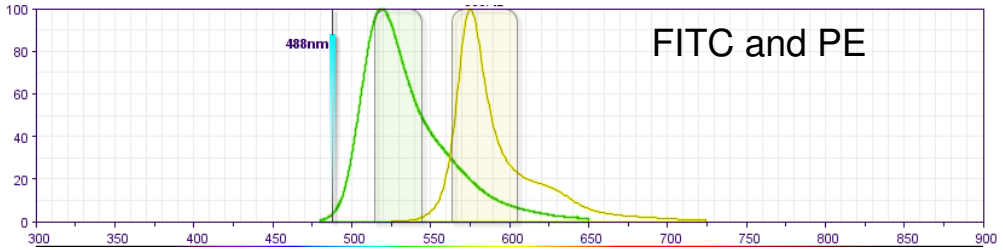
Factors impacting resolution

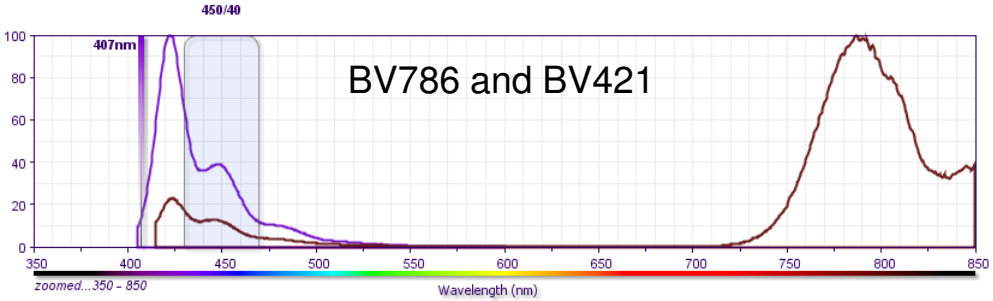


Sources of Spillover

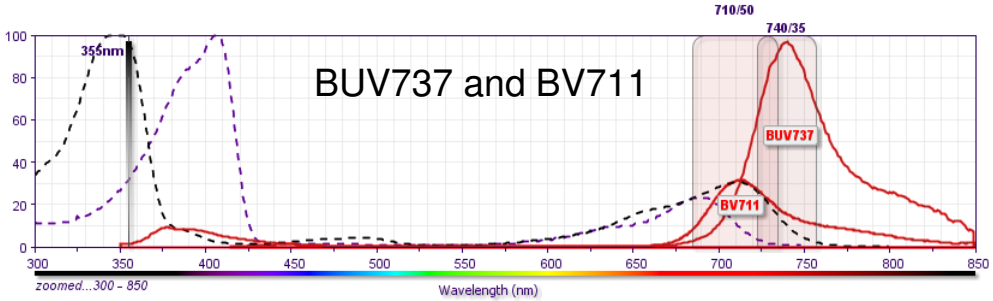
Adjacent Detectors



Residual Base Fluorescence



Similar Emission Spectra (cross-laser)



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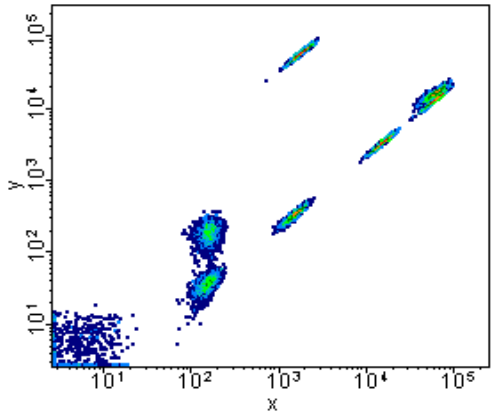
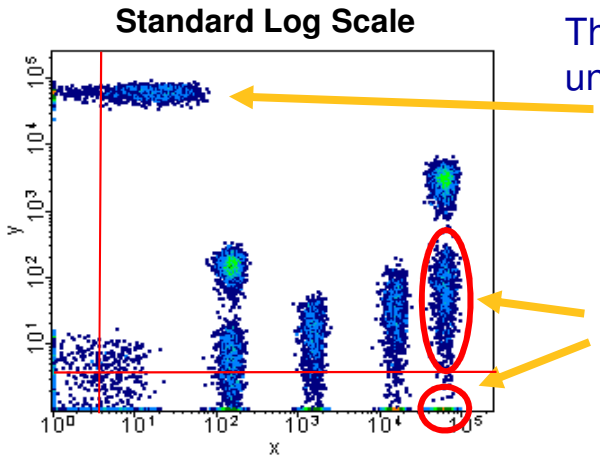
Bi-exponential scale: the best way to look at compensated data



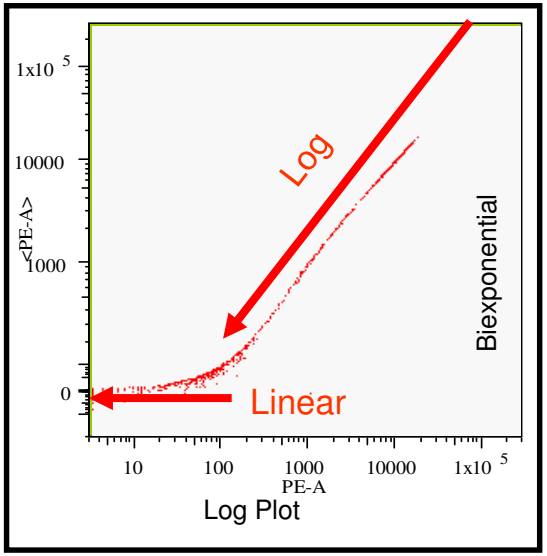
Bi-exponential scale: the best way to look at compensated data



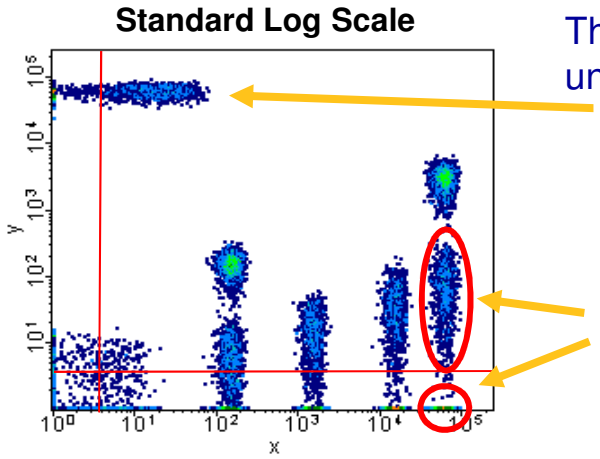
This population "looks" under compensated.

These look like two separate populations.

Visualization of compensated data is greatly improved using the bi-exponential scale.



Bi-exponential scale: the best way to look at compensated data



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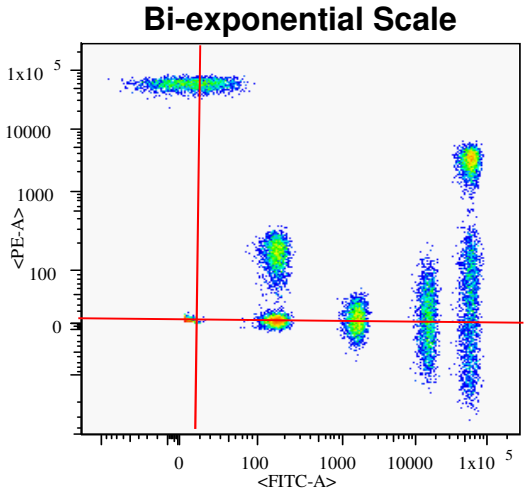
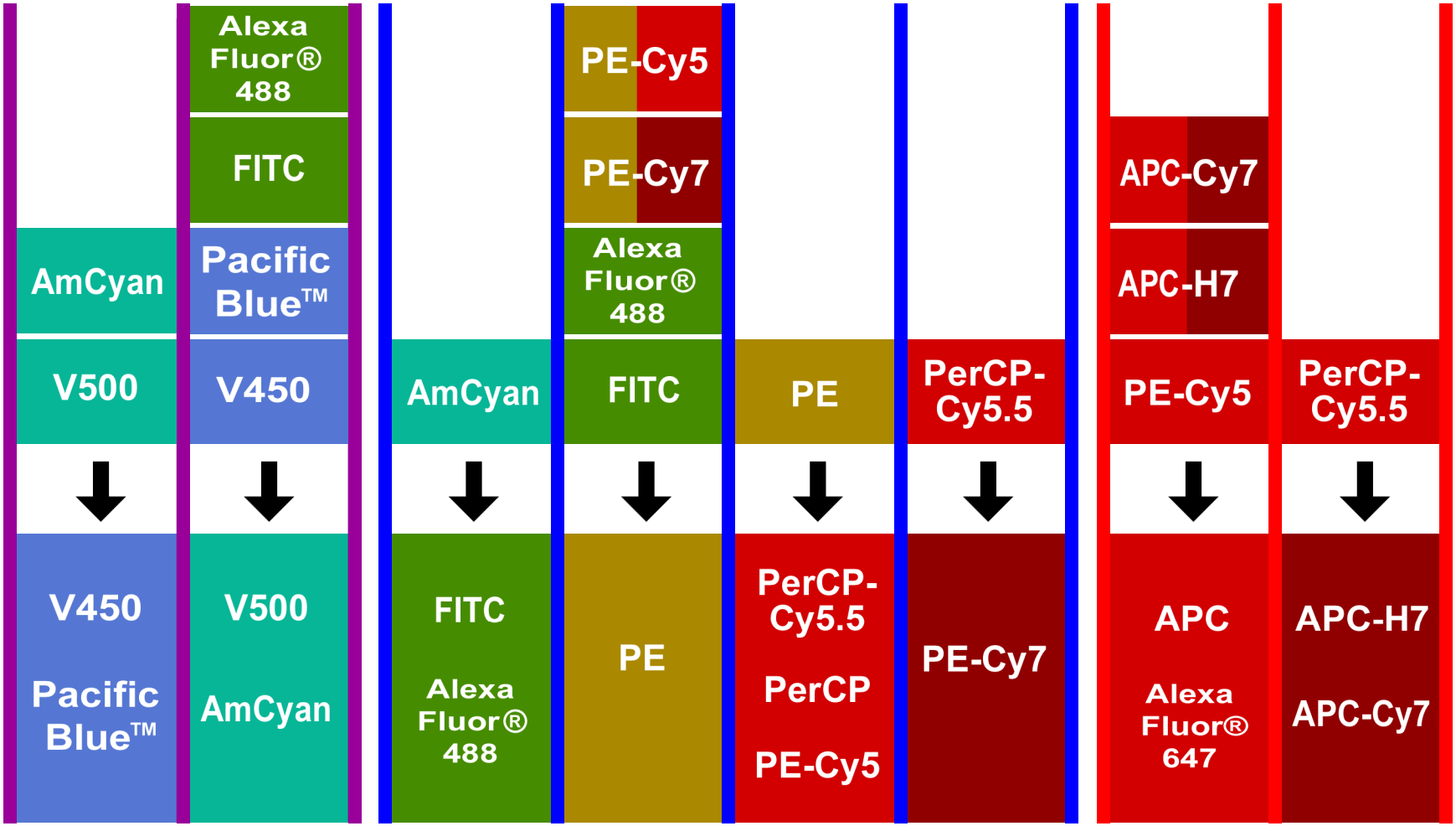
Visualization of compensated data is greatly improved using the bi-exponential scale.



This example of the same data shows the value of the bi-exponential scale, a mostly logarithmic scale on the upper end, linear at the low end and symmetrical about the negatives.

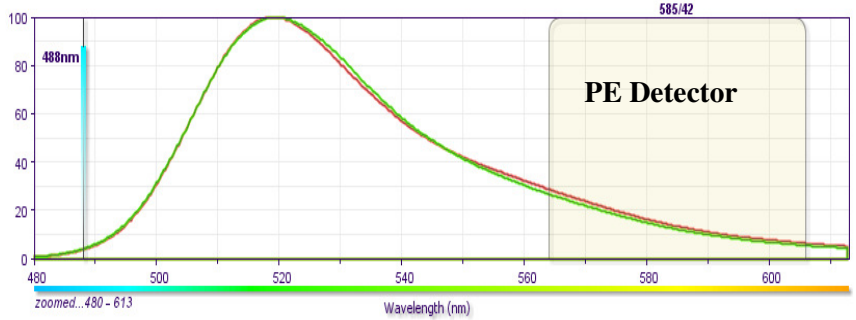
- Compensated single positives are continuous.
- All populations are visible.

Significant Spillovers on 4-2-2 Configuration



Four Principles for Single-color Compensation Controls

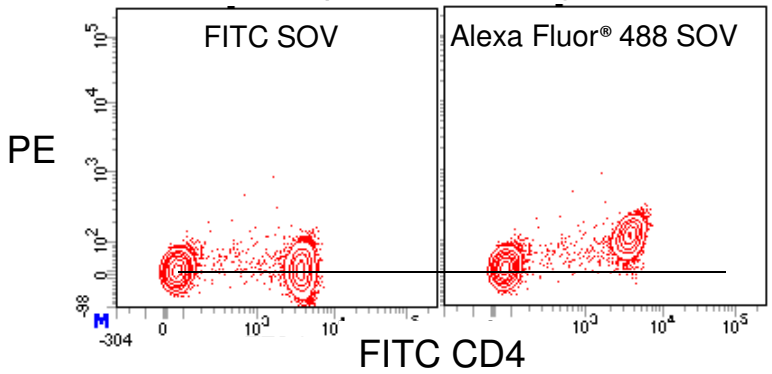
1. The fluorescence spectrum (% spillover) of the compensation control reagent should be *identical* to the reagent used in the experiment.



Spillover Values into PE

FITC = 17.72
Alexa Fluor® 488 = 14.70

Compensated using:



Four Principles for Single-color Compensation Controls

2. The negative and positive populations should have equivalent auto-fluorescence.
   - Compare $CD3^+$ lymphocytes to $CD3^-$ lymphocytes.
   - Don't use $CD3^+$ lymphocytes and $CD3^-$ monocytes or granulocytes.
   - Don't use unstained beads and stained cells to compensate a single fluorochrome.

	MFI	
	FITC	PE
Positive Cells	3,135	903
Negative Cells	95	78
Negative Bead	107	228

Spillover Calculated Using Negative:

Cells = (903-78) / (3,135-95) = **27.1%**

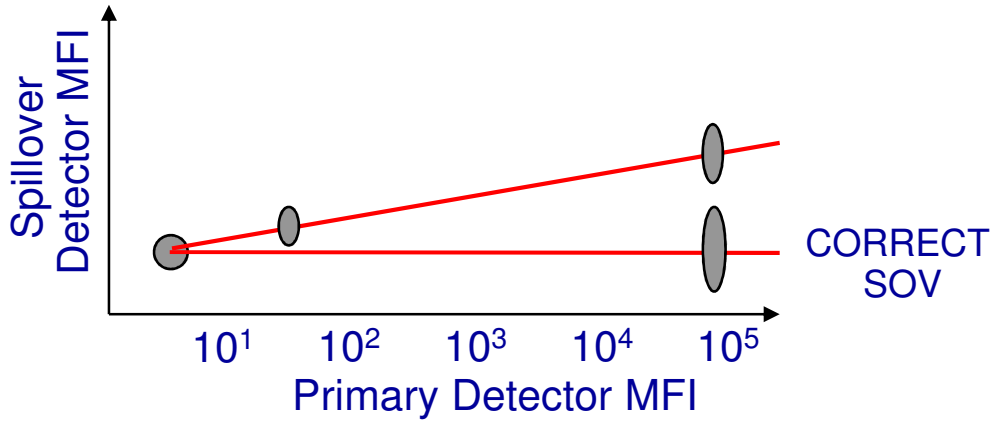
Beads = (903-228) / (3,135-107) = **22.3%**

Difference = 4.8%

Four Principles for Single-color Compensation Controls

3. The positive population should be as bright as possible.

4. Take enough events to get statistically accurate numbers.



Compensation Controls: Cells

- Cells stained with a fluorochrome-conjugated antibody
  - Advantages
    - Can be used for any fluorochrome
      - Run as fluorochrome specific (for example, one control for all FITC reagents)
      - Run as lot specific (for example, separate controls for each PE-Cy7 reagent)
        - » Best possible match of spectra
  - Disadvantages
    - Have to stain cells
    - For lot-specific controls:
      - There may be very few cells.
      - The antigen expression might be low.

Compensation Controls: Beads

- Advantages of compensation beads:
  - Easy to use
  - Do not require cells
  - For most fluorochromes, give SOVs suitable for use with cells
- Considerations when using compensation beads:
  - Beads are a surrogate for cells.
    - Not a perfect match to cells.
    - There can be minor differences in spillover.
      - These differences can vary from provider to provider.
  - Compensation beads do not provide sufficiently accurate spillover values when used with some fluorochromes.
    - For example, V500 and BUV737

	BUV737 Spillover into					
HuCD4	BUV395	BV711	BV786	APC	AF700	APC-H7
Cells	2%	4%	3%	0%	47%	12%
Beads	2%	5%	4%	1%	56%	14%

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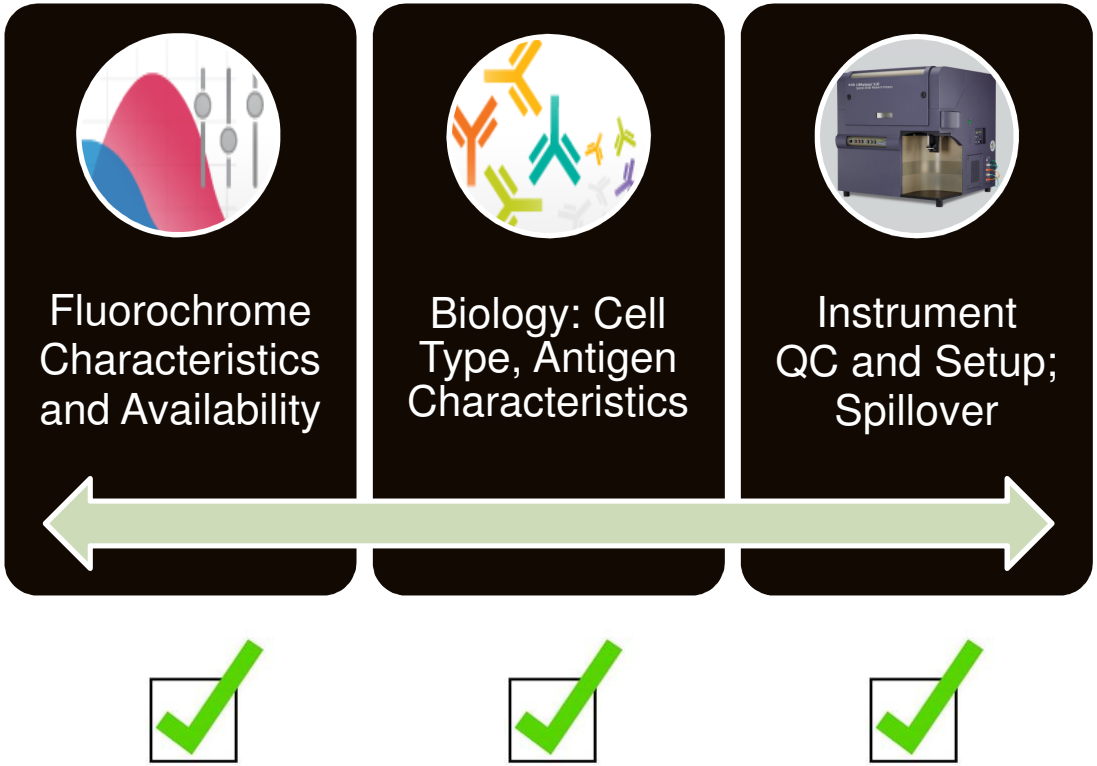
Elements of Multicolor Flow Cytometry



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Top Considerations

What are the top three considerations for optimal panel design?

- Fluorochrome brightness
- Antigen density and co-expression
- Spread due to spillover

6-Color Panel of Treg

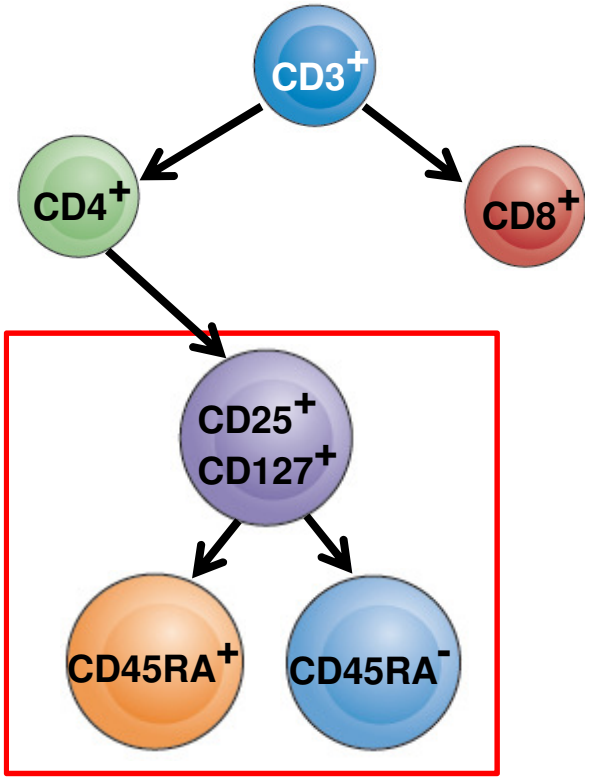
Experimental Goal:
Identify Treg subsets

Markers Used: CD3, CD4, CD8, CD25, CD127, CD45RA

Assign antigen expression levels:

	Tregs	Naïve Tregs	Memory Tregs
CD3			
CD4			
CD8	-	-	-
CD45RA	-		-
CD127			
CD25			

Gating Strategy



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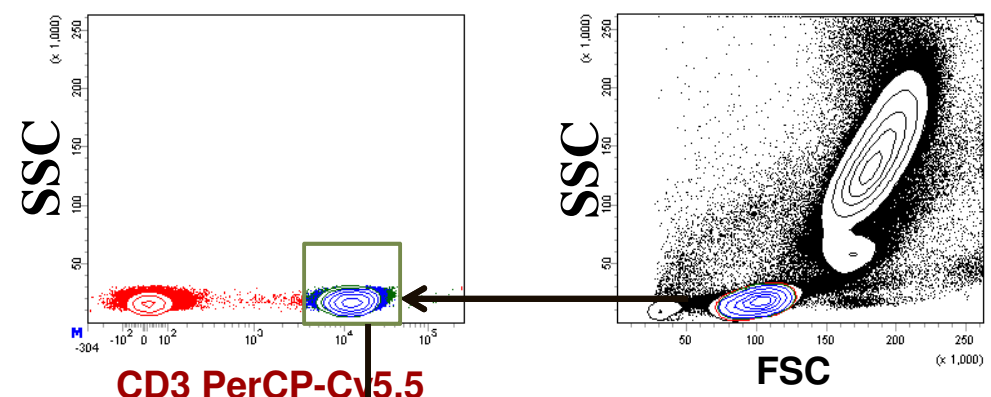
First Example: No Rules

Use of available reagents:
Minimal attention to fluorochrome brightness or antigen density

6-color panel for a 2-laser system:

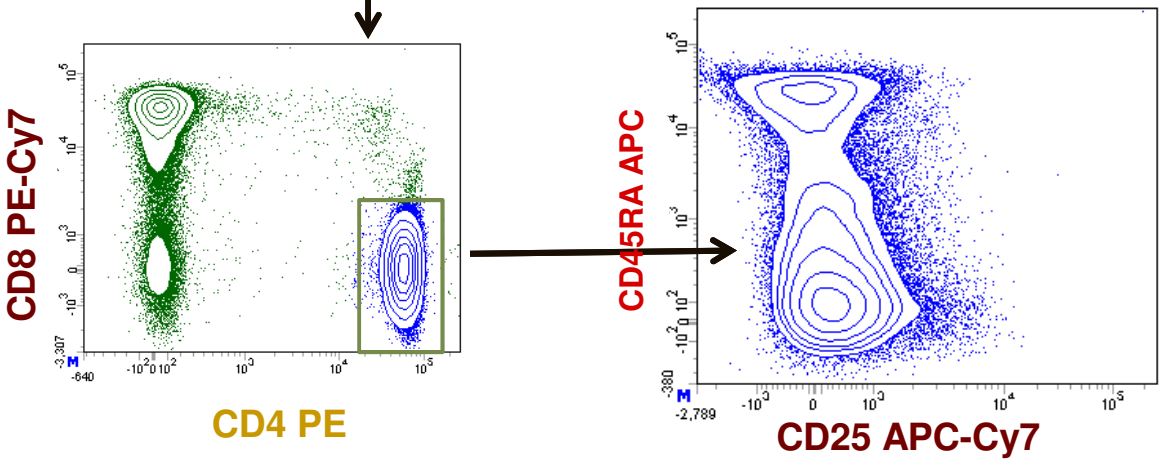
Blue (488 nm)	FITC, Alexa Fluor® 488
	PE
	PerCP-Cy™5.5, PerCP
	PE-Cy™7
Red (640 nm)	APC, Alexa Fluor® 647
	APC-H7, APC-Cy7

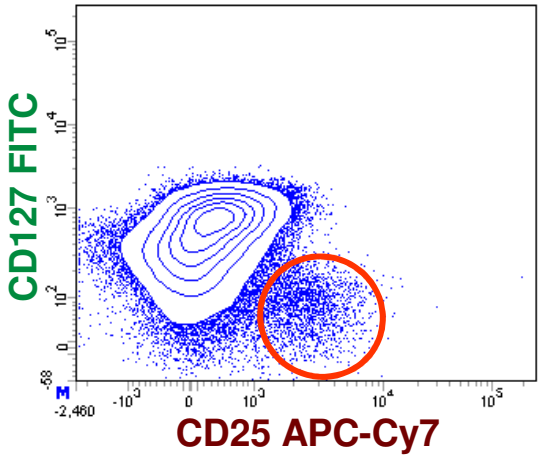
First Example: No Rules



6 color panel using available reagents

FITC	CD127
PE	CD4
PerCP-Cy5.5	CD3
PE-Cy7	CD8
APC	CD45RA
APC-Cy7	CD25



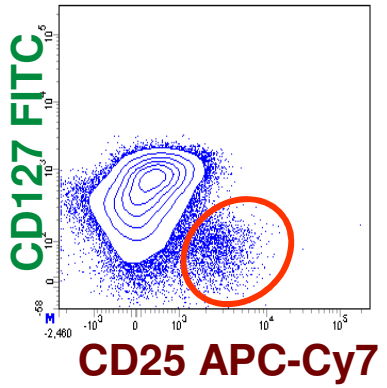


Data acquired on a BD FACSCanto™ II flow cytometer.

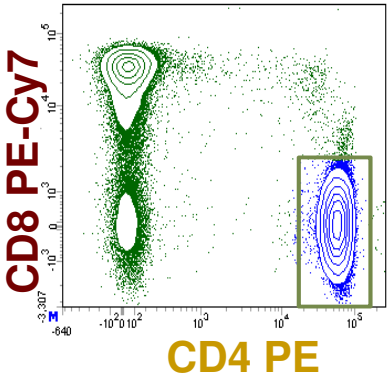
Review of data from First Example

Not following panel design rules results in:

- Poorly resolved $CD127^{dim}/CD25^{++}$ Tregs
- Merged $CD25^{++}$ and $CD25^{+}$ populations



- High-density antigens (CD4, CD8) incorrectly paired with bright fluorochromes (PE, PE-Cy7)
- Lower density antigens (CD127, CD25) incorrectly paired with dimmer fluorochromes (FITC, APC-Cy7)



Second Example: Some Rules

Refined panel with focus on fluorochrome assignment based on expression of antigens

6-color panel for a 2-laser system:

Blue (488 nm)	FITC, Alexa Fluor® 488
	PE
	PerCP-Cy5.5, PerCP
	PE-Cy7
Red (640 nm)	APC, Alexa Fluor® 647
	APC-H7, APC-Cy7

Second Example: Some Rules

Antigen Expression

Marker	Antigen Density
CD3 CD25 CD4 CD127	32,000 3,400 36,400 2,000
CD45RA CD8	33,400 65,500

Fluorochrome Rankings

	BRIGHT	MODERATE	DIM
Blue (488 nm)	PE PE-Cy7	FITC Alexa Fluor® 488 PerCP-Cy5.5	PerCP
Red (640 nm)	APC Alexa Fluor® 647		APC-H7 APC-Cy7

Second Example: Some Rules

Commercial Availability

● – available

	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC Alexa Fluor® 647	APC-H7
CD3	●	●	●	●	●	●
CD4	●	●	●	●	●	●
CD8	●	●	●	●	●	●
CD25	●	●	●	●	●	●
CD45RA	●	●		●	●	●
CD127	●	●	●	●	●	

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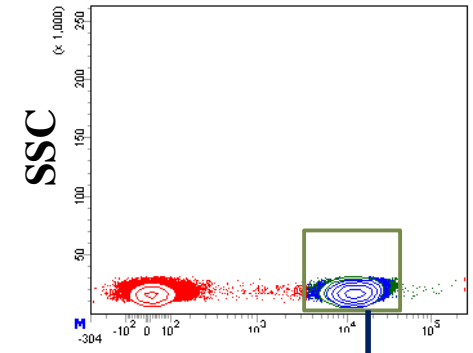
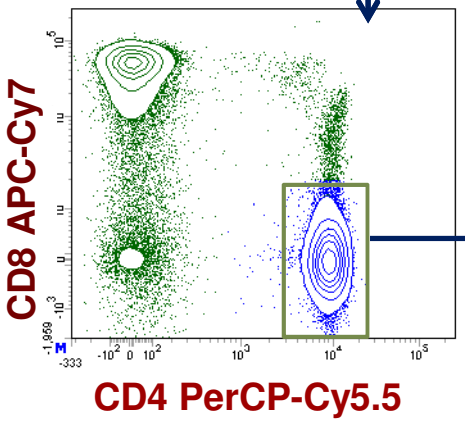
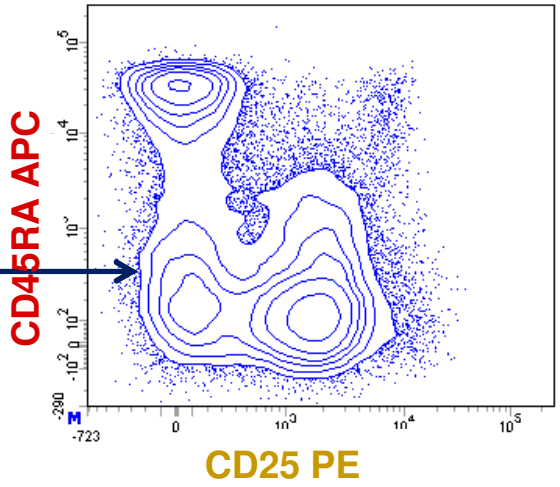
Second Example: Some Rules

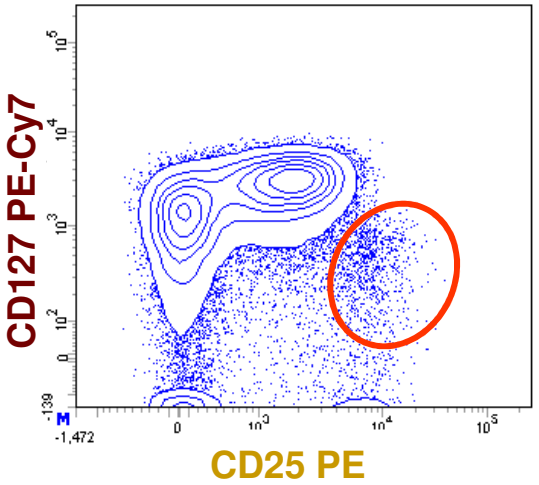
ANTIGEN	ASSIGNMENT	
Specificity	Fluorochrome	Laser
CD25	PE	●
CD127	APC	●
CD45RA	PE-Cy7	●
CD4	FITC	●
CD8	PerCP-Cy5.5	●
CD3	APC-Cy7	●

Second Example: Some Rules









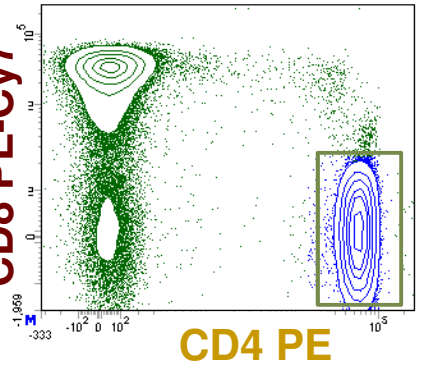
6-color panel using **antigen density** and **fluorochrome brightness** information to choose reagents

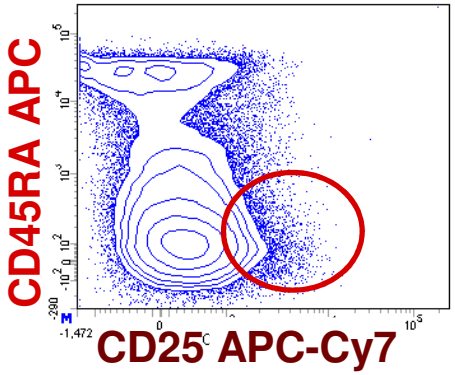
FITC	**CD3**
PE	**CD25**
PerCP-Cy5.5	**CD4**
PE-Cy7	**CD127**
APC	**CD45RA**
APC-Cy7	**CD8**

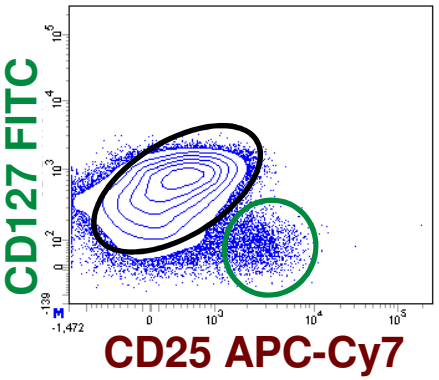
Data acquired on a BD FACSCanto™ II flow cytometer.

Comparison: First vs Second

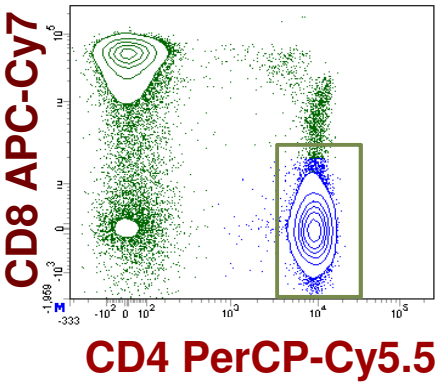
First Example

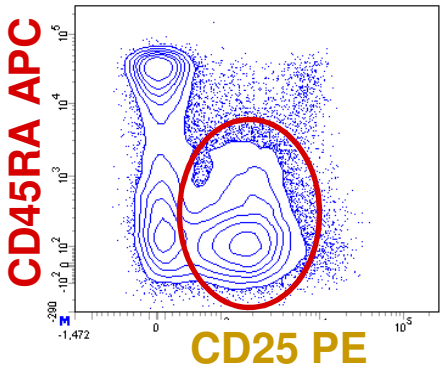


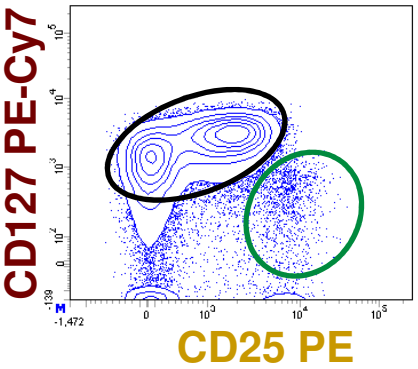




Second Example







Using a dimmer fluorochrome for CD4 did not impact resolution of CD4+ cells

Using brighter fluorochromes for low density CD25 and CD127 markers improved their resolution

Third Example: Best Practices

Use of best practices to further optimize the panel to maximize the resolution of Tregs:

- ✓ **Antigen density and co-expression**
- ✓ **Fluorochrome brightness**
- ✓ **Spread due to spillover**

6-color panel for a 2-laser system:

Blue (488 nm)	FITC, Alexa Fluor® 488
	PE
	PerCP-Cy5.5, PerCP
	PE-Cy7
Red (640 nm)	APC, Alexa Fluor® 647
	APC-H7, APC-Cy7

Third Example: Best Practices

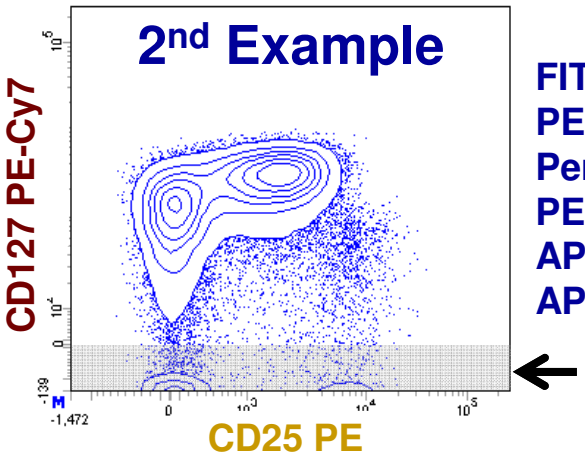
The key to any final panel optimization is to focus on the critical populations of interest

- The goal is to minimize loss of resolution due to spread from the fluorescence spillover of co-expressed antigens

How to avoid spectral spillover

- **When antigens are co-expressed on a cell**
  - Avoid significant spillover of a bright marker into a dim marker
  - Spread the antigens across as many lasers as possible
- **Fluorochromes that are excited by more than one laser cause high spillover**
  - AmCyan excited by the violet and blue lasers spills into the FITC detector
  - PE-Cy5 excited by the blue and red lasers spills into APC detector
- **Considerations for tandem dyes**
  - Take into consideration residual donor emission

Resolution Impact Matrix



FITC	CD3
PE	CD25
PerCP-Cy5.5	CD4
PE-Cy7	CD127
APC	CD45RA
APC-Cy7	CD8

Whenever you have a number of events significantly below zero that indicates that there is spread into that detector

In this panel something is introducing spread into the PE-Cy7 detector

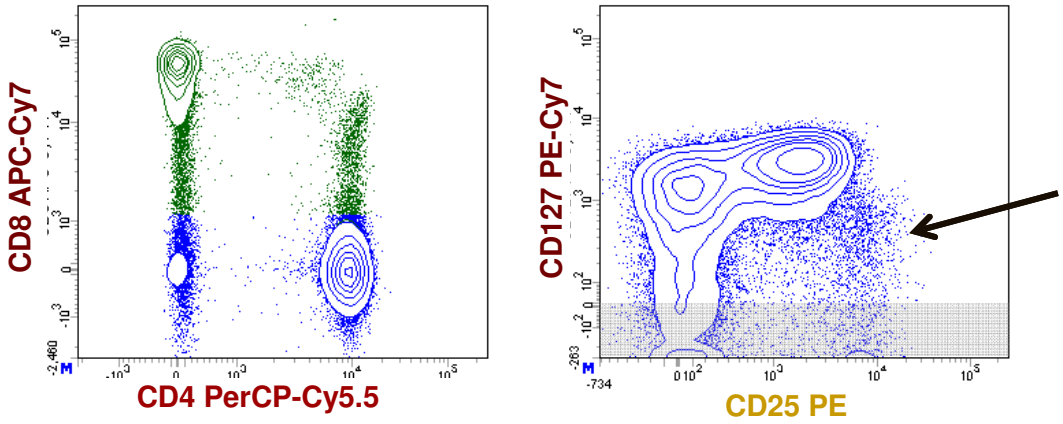
Where is it coming from?

	Resolution Impact Matrix						
Primary Fluor	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	% loss of SP-SI
FITC	75						<20%
PE		422					20-40%
PerCP-Cy5.5			130				40-60%
PE Cy7				761			60-80%
APC					635		>80%
APC-H7						216	SP-SI (Primary)

The resolution impact matrix suggests that spread from the PerCP-Cy5.5 conjugate of the highly co-expressed CD4 will result in a significant loss of resolution in the PE-Cy7 detector (CD127)

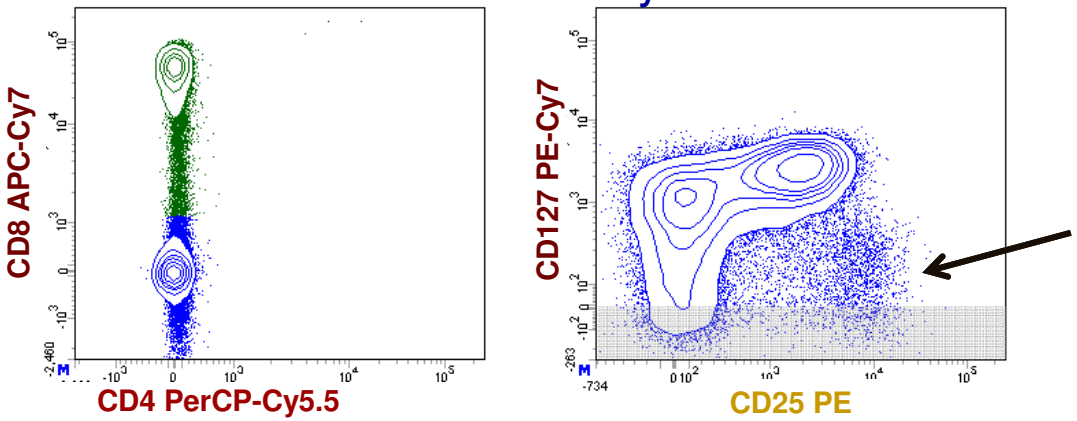
FMO Controls

Full Panel



- FMO controls allows us to assess the effect of spillover and spread of a given fluorochrome into other detectors

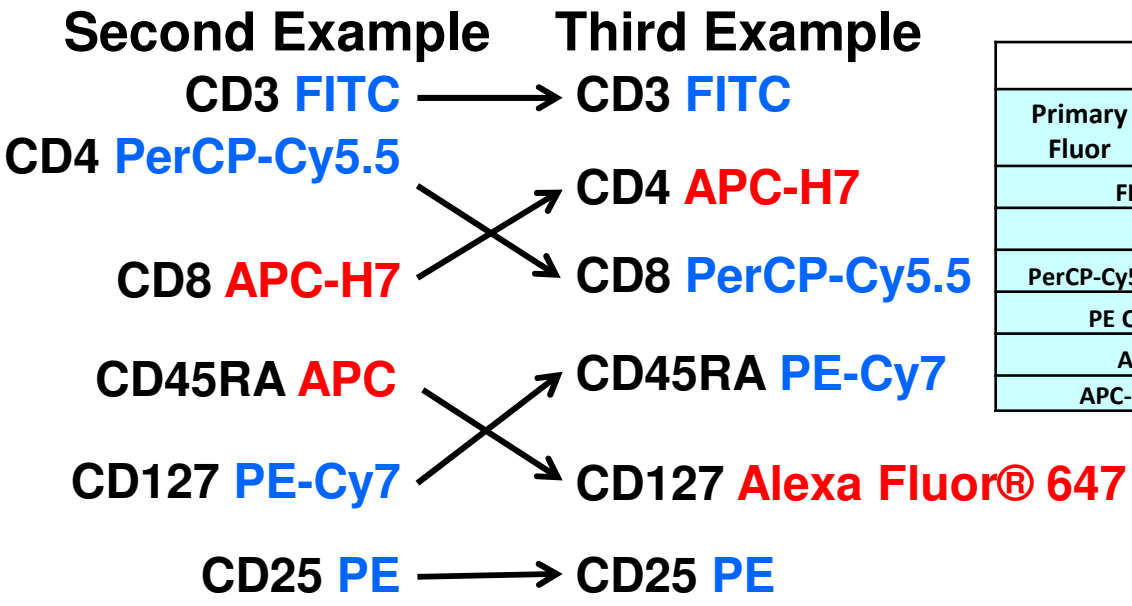
FMO PerCP-Cy5.5



- In this example, the absence of PerCP-Cy5.5 significantly improves CD127 PE-Cy7 resolution
  - To improve CD127 resolution, combinations of reagents with less spillover into the CD127 detector are required

Optimizing Panel Design

- **Minimize impact of PerCP-Cy5.5 spillover into the PE-Cy7 channel by assigning PerCP-Cy5.5 to CD8**
- **Maximize Treg resolution** by spreading CD25 and CD127 fluorochrome assignment across different lasers and by choosing fluorochromes with minimal spillover into each other



	Resolution Impact Matrix						
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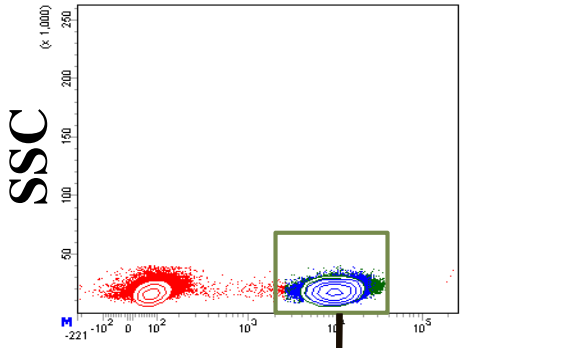
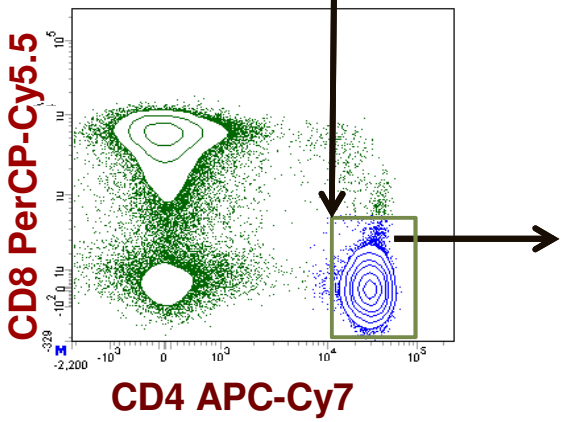
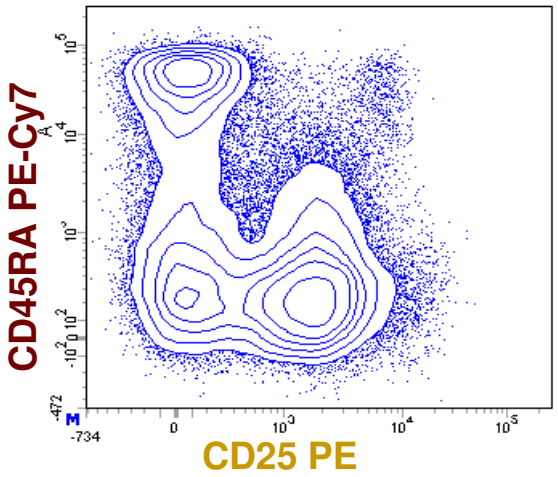
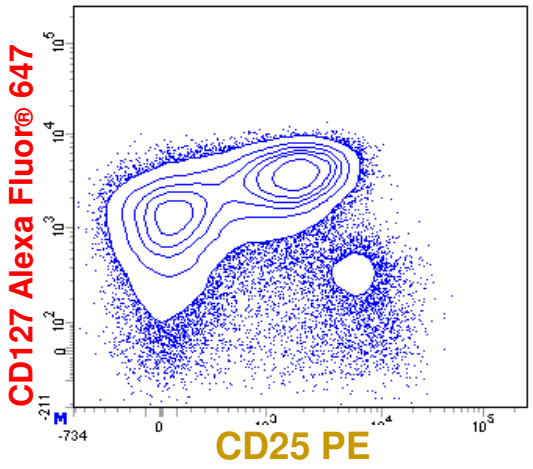
PerCP-Cy5.5			130				40-60%
PE Cy7				761			60-80%
APC					635		>80%
APC-H7						216	SP-SI (Primary)

Third Example: Best Practices









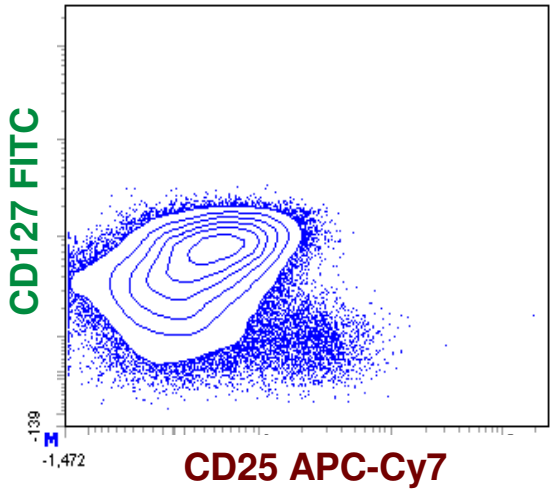
6-color T cell panel optimizing reagent selection to **minimize spread** due to fluorescence spillover

FITC	**CD3**
PE	**CD25**
PerCP-Cy5.5	**CD8**
PE-Cy7	**CD45RA**
Alexa Fluor® 647	**CD127**
APC-Cy7	**CD4**

Data acquired on a BD FACSCanto™ II flow cytometer.

Reviewing three examples

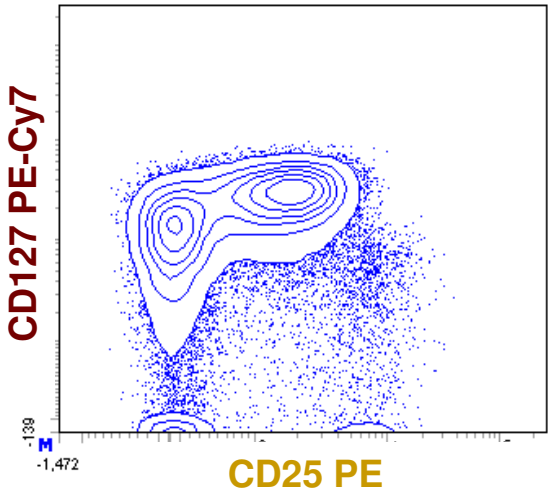
First Example



The basic rules of panel design were not applied.

Treg resolution **Suboptimal**

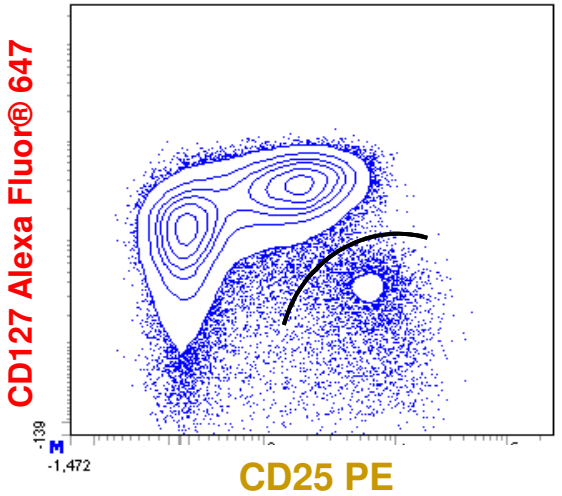
Second Example



Panel optimized to use bright fluorochromes for the low expressors. Resolution of CD127$^+$, CD25$^+$ cells improved.

Improved

Third Example



Panel optimized to minimize loss of resolution due to spillover of co-expressed markers.

Maximized

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Top Considerations

- What are the top three considerations for optimal panel design?
  - Fluorochrome brightness
  - Antigen density and co-expression
  - Spread due to spillover

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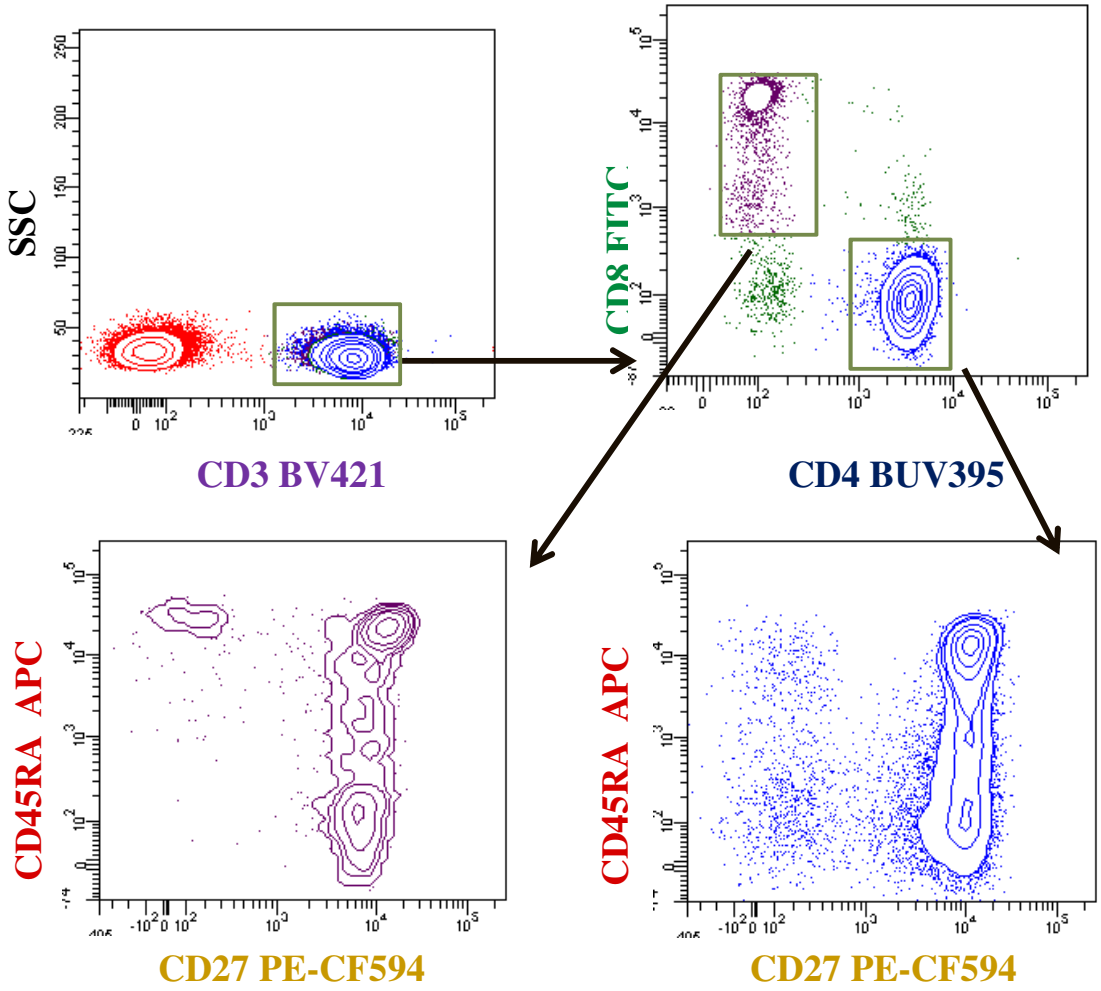
Experimental Controls

- Controls should be used to help resolve issues in staining
  - **Isotype controls** help identify staining issues
  - **Unstained controls** highlight the background or autofluorescence of the system
  - **Single-stained controls** allow you to QC the compensation and to assess the resolution impact
  - **Fluorescence Minus One** (FMO) controls help identify gating boundaries and illustrate the potential impact of spillover

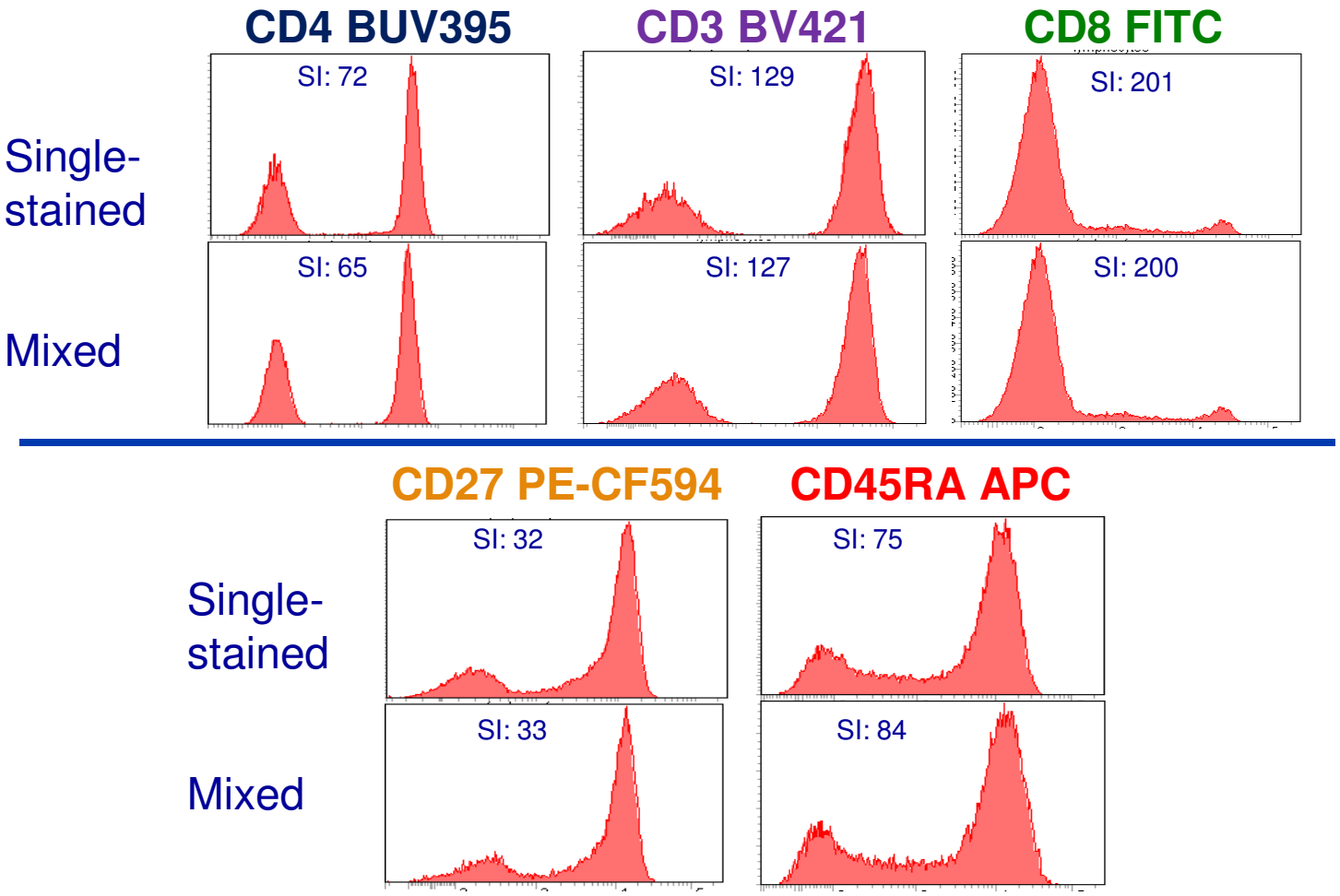
5-Color Panel on 5-Laser System



CD4	BUV395
CD3	BV421
CD8	FITC
CD27	PE-CF594
CD45RA	APC

Data acquired on a BD LSRFortessa X-20 flow cytometer.

5-Color Panel on 5-Laser System



Data acquired on a BD LSRFortessa X-20 flow cytometer.

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Experiment Goal

To enumerate and characterize multiple leucocyte cell subsets in peripheral blood:

- T cells: CD3, CD4, CD8, CD27, CD127, CCR7
- Regulatory T cells: CD4, CD25, CD127, HLA-DR
- B cells: CD19, IgD, CD25, CD27
- NK and NT T cells : CD3, CD56, CD16, CD8
- Dendritic cells: CD3, CD19, CD16, CD56, HLA-DR, CD123, CD11c
- Monocytes: CD14, CD16, HLA-DR

Panel Design

T Cells

- CD3 BV605
- CD4 BUV737
- CD8 BV786
- CD27 BV711
- CCR7 PE-CF594

Tregs

- CD3 BV605
- CD4 BUV737
- HLA-DR APC-H7
- CD127 Alexa Fluor® 647
- CD25 BB515

B Cells

- CD19 BUV395
- IgD BV421
- CD27 BV711
- CD25 BB515

NK/NK-T cells

- CD3 BV605
- CD56 APC-R700
- CD16 PE-Cy7
- CD8 BV786

DCs

- HLA-DR APC-H7
- CD123 PerCP-Cy5.5
- CD11c PE

Monocytes

- CD14 V500
- HLA DR APC-H7
- CD16 PE-Cy7

15-Color Leukocyte Panel

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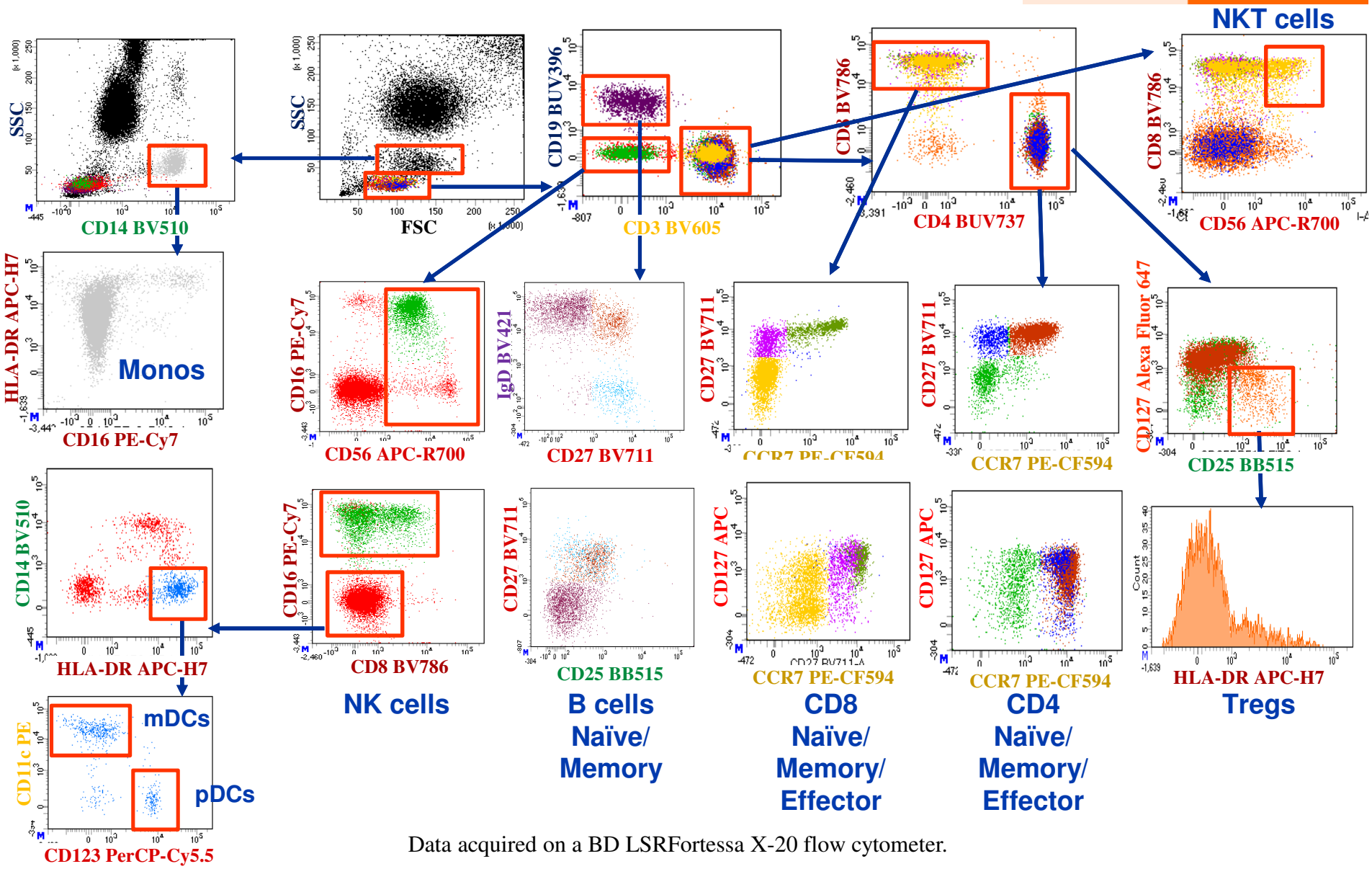

Data acquired on a BD LSRFortessa X-20 flow cytometer.

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Summary

- The key to good panel design is to maximize the resolution of the critical subpopulations.
- Resolution can be improved through:
  - Instrument setup using CS&T
    - Optimize your PMT voltages for application-specific settings.
    - Track the CS&T parameters that impact resolution.
      - Qr, Br, and SD_{EN}
  - Understanding and minimizing the spread of populations due to fluorescence spillover.

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Summary

- The key to getting the best results out of multicolor assays
  - Daily setup and QC
    - Use CS&T daily to reproducibly set up the instrument to obtain consistent results.
    - Confirm the instrument is performing as expected.
  - Single-color fluorescence controls
    - Evaluate and use the appropriate controls for the assay.
      - Fluorochrome-specific controls vs reagent-specific controls
      - Beads vs cells
    - As part of assay development, pre-test the fluorescence controls to confirm they meet the needs of the assay.

You are Ready to Achieve

- Using the information we have learned:
  - How to REVEAL information from dim markers in your assays by using the new classes of ultra-bright fluorochromes
  - How to ELEVATE your understanding of the biology inherent in your assays by using information on the exact expression of cell surface receptors on each of the populations
  - How to RESOLVE each of the populations in your assays by minimizing the impact of fluorescence spillover on spread and resolution

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THANKS

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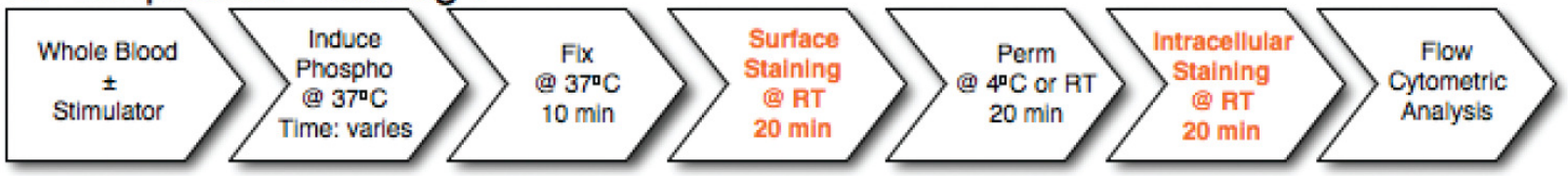
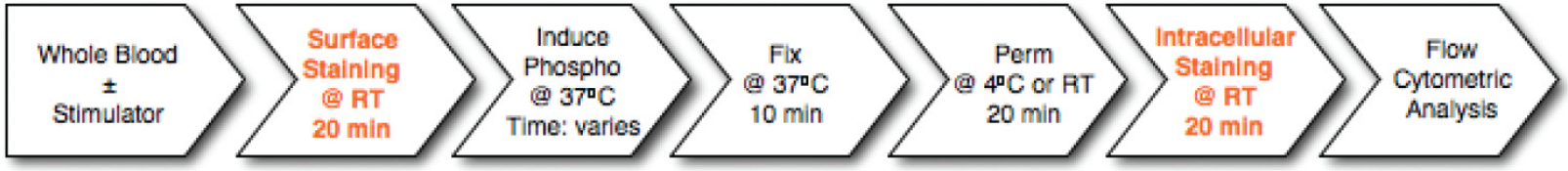
A. Standard Protocol

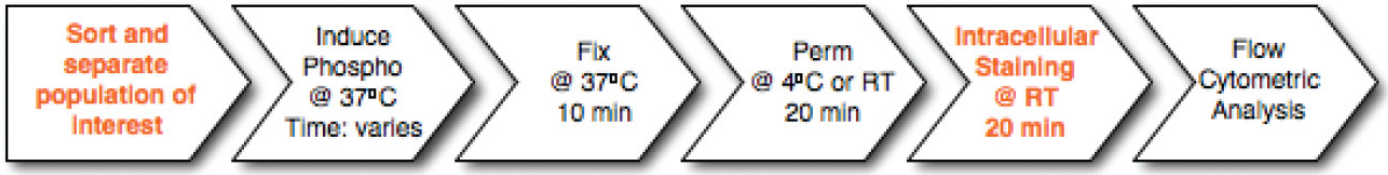
B. Sequential Staining I



C. Sequential Staining II



D. Sort



Resolution of Intracellular Antigens

- The ability to resolve intracellular stained antigens is impacted by:
  - Loss of effective epitopes (lower positive signals) due to fixation
  - Up- or down-regulation of antigen expression following activation
  - Higher backgrounds due to:
    - Increased autofluorescence (especially when cells are activated)
    - Using “surface-titered” concentrations of reagents
      - Reagents need to be titrated specifically for intracellular staining.
  - The ability of fluorochromes to pass through membranes
    - More difficult for nuclear staining (eg, phosphoproteins)