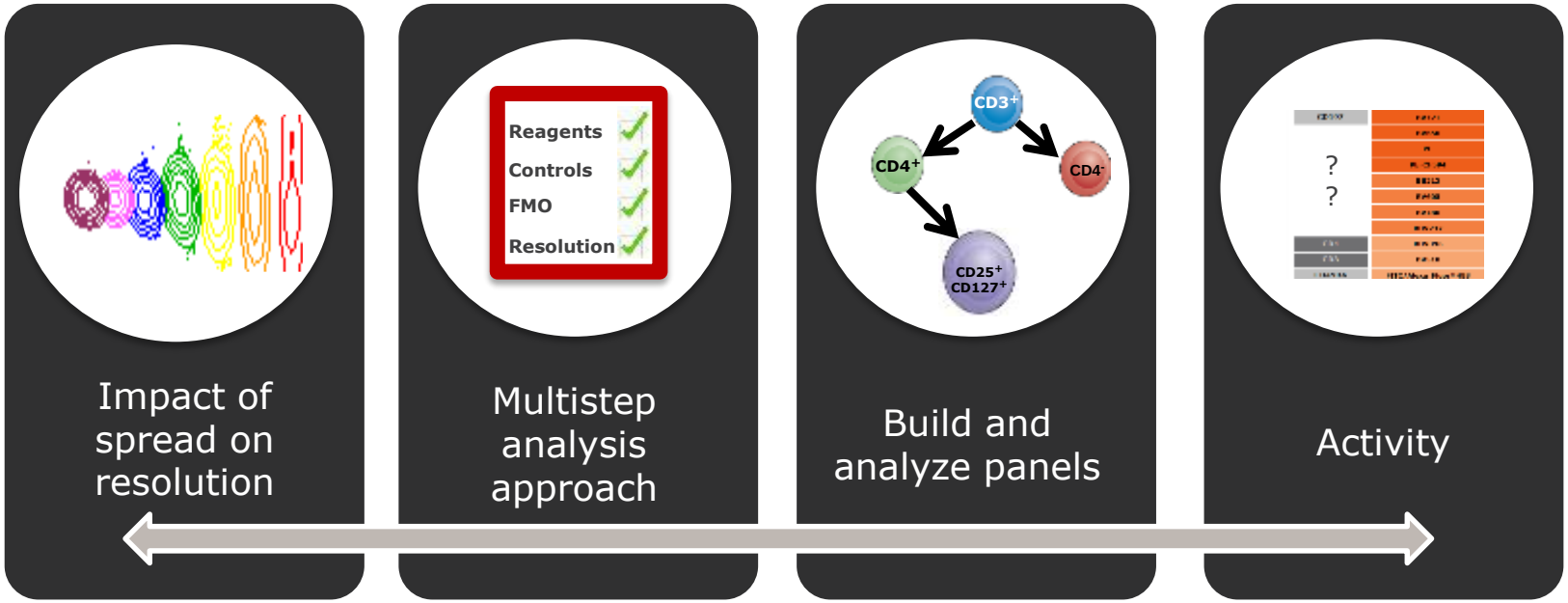
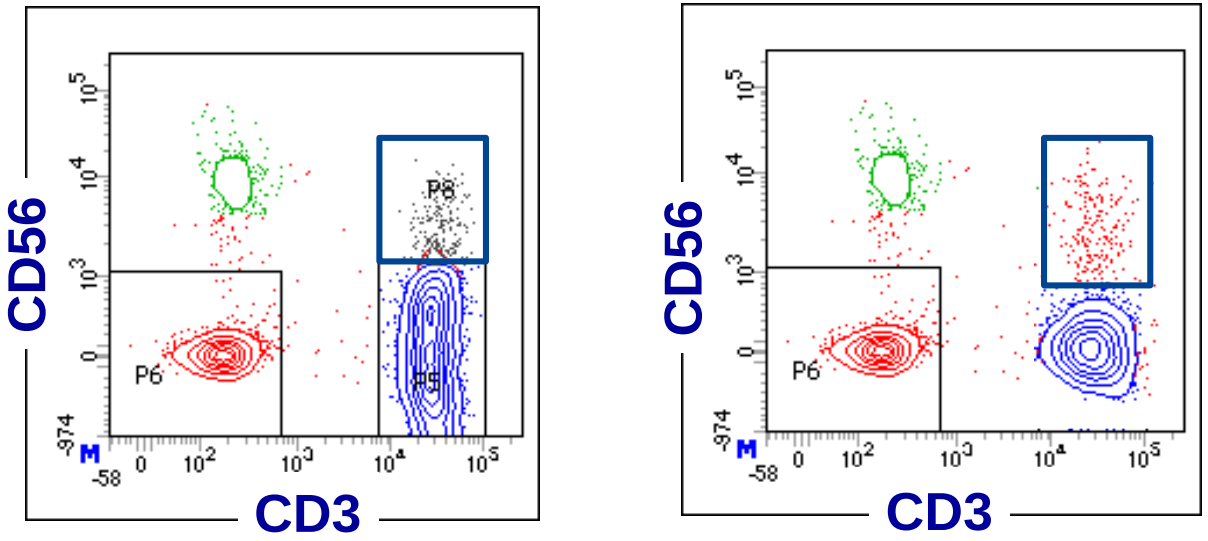
Analyze

A step-by-step approach to build and analyze a multicolor panel

Best practices for building and analyzing panels



Fluorescence spillover and spread impact resolution



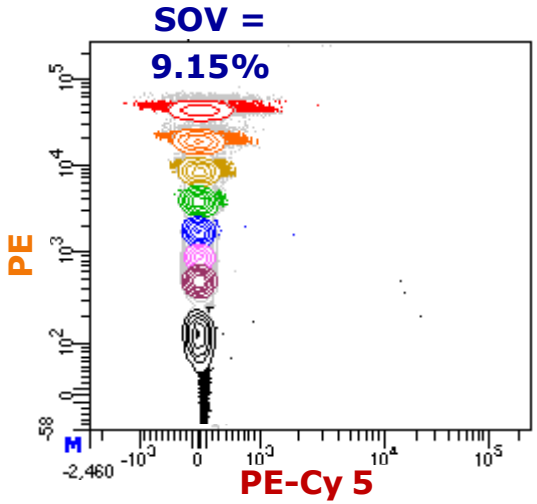
- Understanding the impact of fluorescence spillover and spread is the key to good panel design.

BD

Spread impacts resolution

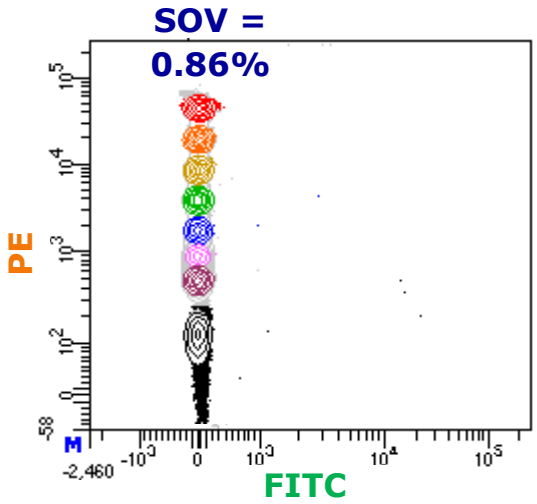
Spillover introduces spread and background

Spillover introduces background and spread



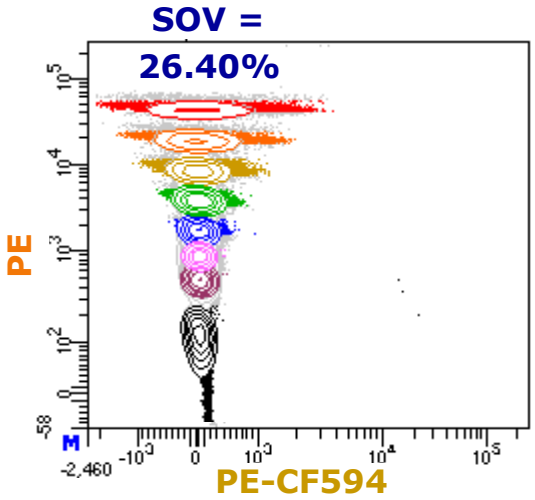
The amount of the spread $\propto$ **Amount of spillover**

Spillover introduces background and spread



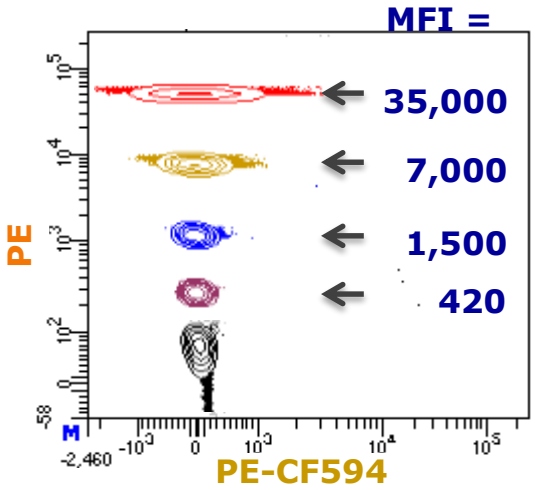
The amount of the spread $\propto$ **Amount of spillover**

Spillover introduces background and spread



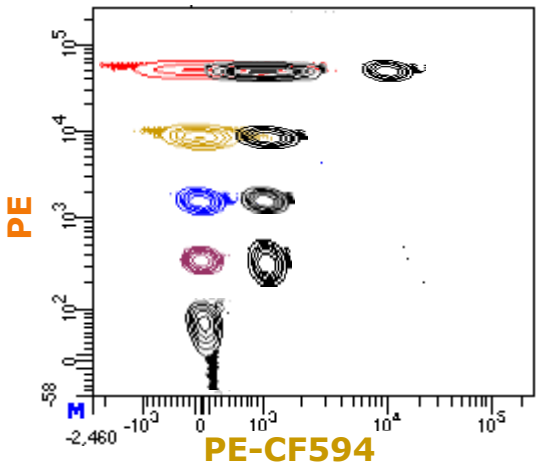
The amount of the spread $\propto$ **Amount of spillover**

Spillover introduces background and spread



The amount of the spread ∝ **Amount of spillover** × **Reagent brightness**

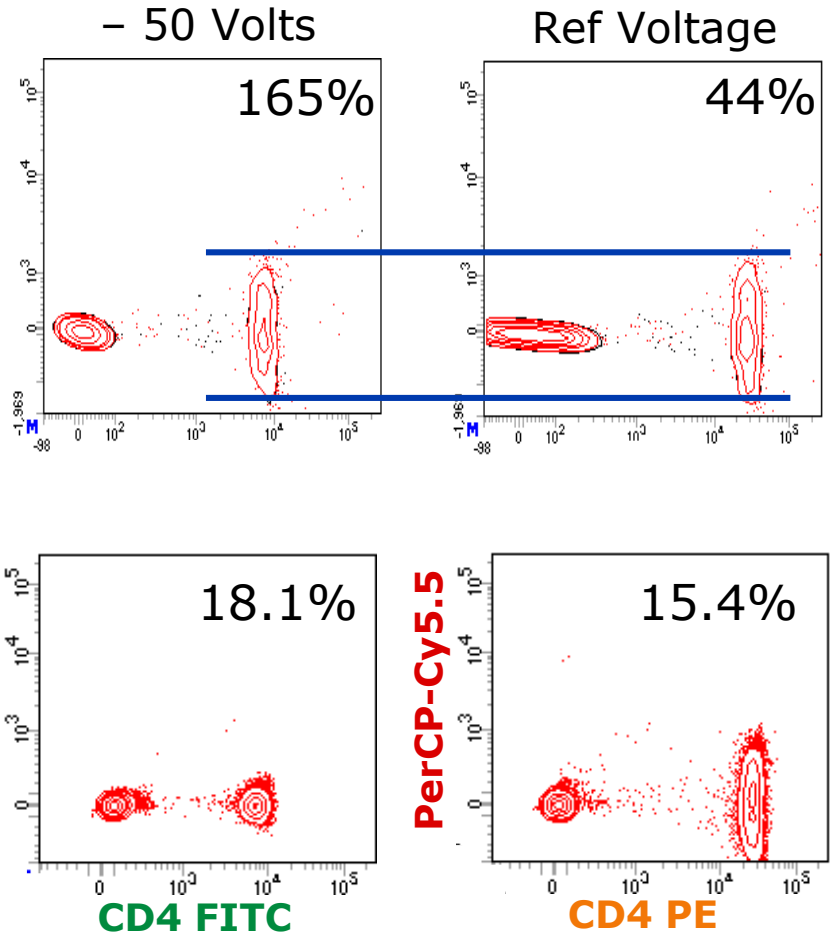
Spillover introduces background and spread



- Population resolution for the PE-CF594 fluorescence parameter is decreased by increased spread due to PE spillover from another fluorescence parameter.
- To maximize the resolution of a given double-positive subpopulation:
  - Minimize fluorescence spread into the detector that defines that population

$$\textbf{The amount of the spread} \propto \textbf{Amount of spillover} \times \left(\textbf{Antigen density} \times \textbf{Fluorochrome brightness} \right)$$

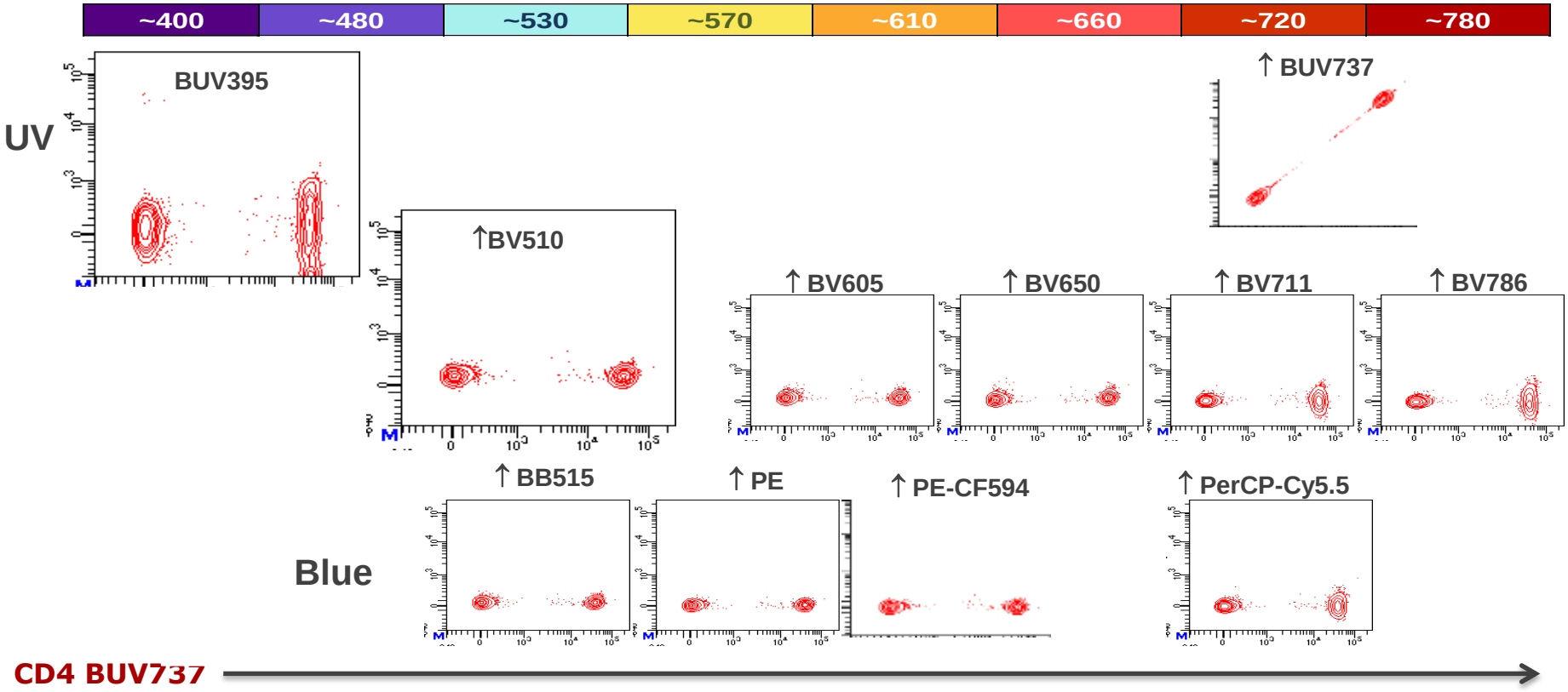
Quantifying the impact of fluorescence spillover



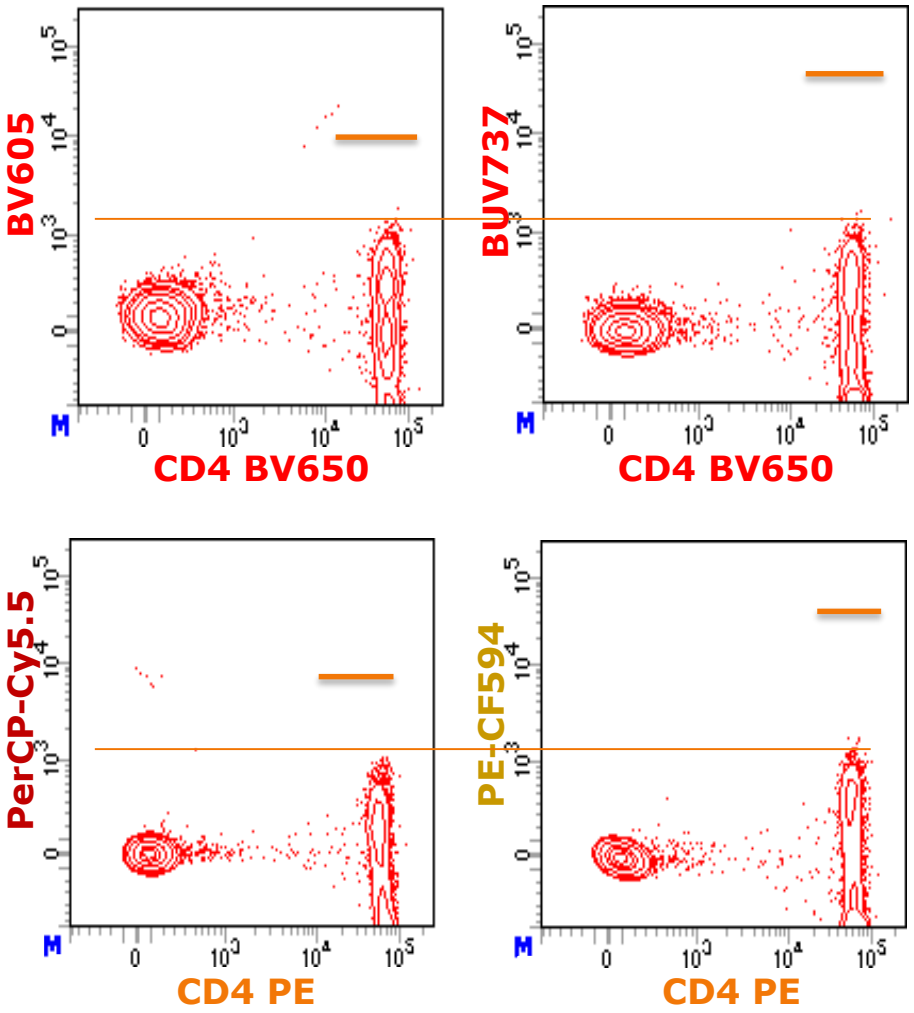
Spillover Values (SOVs)

- Are totally dependent upon gain settings (PMTVs).
- Does not always accurately reflect the impact of spread.

BD FACSCelesta™ flow cytometer B/V/UV – BUV737 spread

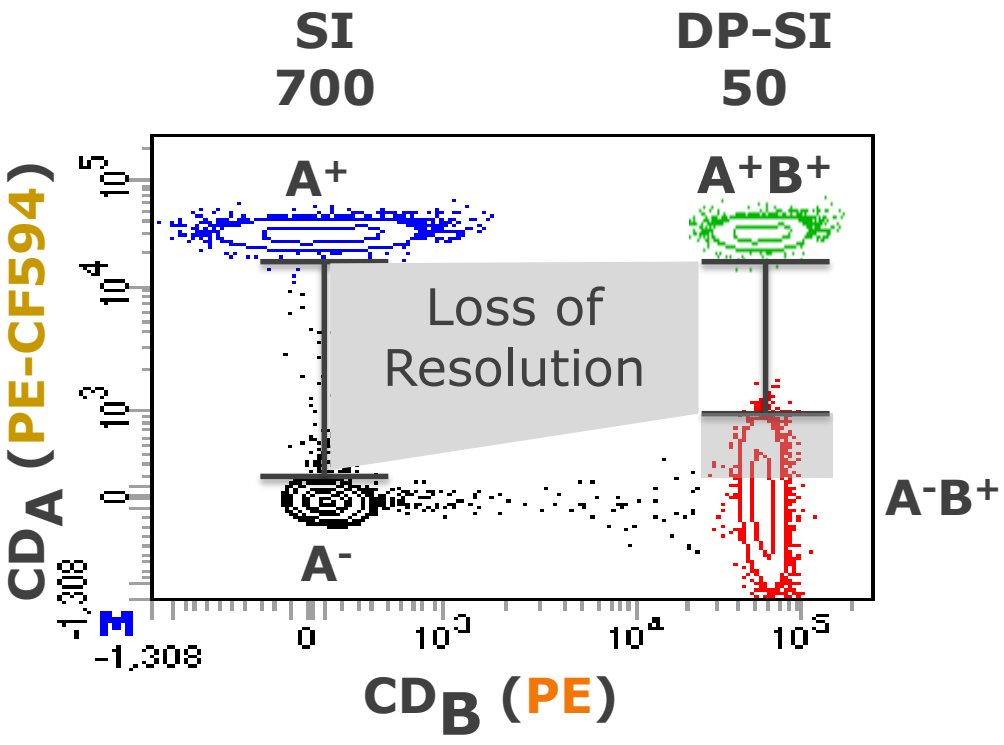


Spread impacts the resolution of double-positive populations



- Spread is most important when considering reagents for antigens co-expressed on a subpopulation.
  - Double-positive populations need to be resolved from the single-positive populations.
- Although the spread of BV650 is equivalent into BV605 & BUV737, it has less of an impact on the resolution of a marker stained with BUV737.
- Although the spread of PE is equivalent into PerCP-Cy™5.5 and PE-CF594, it has less of an impact on the resolution of a marker stained with PE-CF594.

Resolution of double-positive populations



- Here is a classic resolution of a single-positive (A^+) population being resolved from a negative (A^-) population.
  - Resolution measured in Stain Index (SI)
- Adding a second co-expressed marker, we now have to resolve a double-positive (A^+B^+) population from a single (A^-B^+) population.
  - Resolution measured in a double-positive Stain Index (DP-SI)
- The spread of fluorochrome B into the A detector reduces the resolution of a double-positive (A^+B^+) population from the A^-B^+ population.

Double-positive stain index matrix

Double-Positive Stain Index*						
	Co-expressed Fluor					
Primary Fluor	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
FITC	68	61	70	74	67	72
PE	170	340	350	216	358	356
PerCP-Cy5.5	73	15	131	60	131	124
PE-Cy7	454	132	63	522	375	265
APC	522	471	169	444	486	287
APC-H7	120	127	42	15	18	116

* Run on a BD FACSVerse™ flow cytometer

- This table shows double-positive Stain Index values
  - A double-positive Stain Index less than the single-positive Stain Index indicates that the spread of the secondary co-expressed fluorochrome has reduced resolution.

BD

Double-positive stain index matrix

Double-Positive Stain Index*						
	Co-expressed Fluor					
Primary Fluor	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
FITC	68	61	70	74	67	72
PE	170	340	350	216	358	356
PerCP-Cy5.5	73	15	131	60	131	124
PE-Cy7	454	132	63	522	375	265
APC	522	471	169	444	486	287
APC-H7	120	127	42	15	18	116

* Run on a BD FACSVerse

- This table shows double-positive Stain Index values
  - A double-positive Stain Index less than the single-positive Stain Index indicates that the spread of the secondary co-expressed fluorochrome has reduced resolution.
- This table shows the relative loss of resolution which can be evaluated as the percent difference between the Stain Index and the double-positive Stain Index
  - **1- (50/700) x 100%.**

BD

The resolution impact matrix

Resolution Impact Matrix*						
	Co-expressed Fluor					
Primary Fluor	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
FITC	68					
PE		340				
PerCP-Cy5.5			131			
PE-Cy7				522		
APC					486	
APC-H7						116

* Run on a BD FACSVerse

% loss of SP-SI				
<20%	20-40%	40-60%	60-80%	>80%

SI (Primary)

- This table shows double-positive Stain Index values
  - A double-positive Stain Index less than the single-positive Stain Index indicates that the spread of the secondary co-expressed fluorochrome has reduced resolution.
- This table shows the relative loss of resolution which can be evaluated as the percent difference between the Stain Index and the double-positive Stain Index
  - **1- (50/700) x 100%.**
- Color coding the percent loss makes it easy to visualize which fluorochromes have the greatest impact on each other.

Using the resolution impact matrix

- The resolution impact matrix provides a quick visual tool to help assess potential problems with spread when evaluating potential use of two fluorochromes for co-expressed markers on a population of cells.

Resolution Impact Matrix*

Primary Fluor	Co-expressed Fluor: FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
FITC	68					
PE		320				
PerCP-Cy5.5			131			
PE-Cy7				522		
APC					486	
APC-H7						116

* Run on a BD FACSVerse

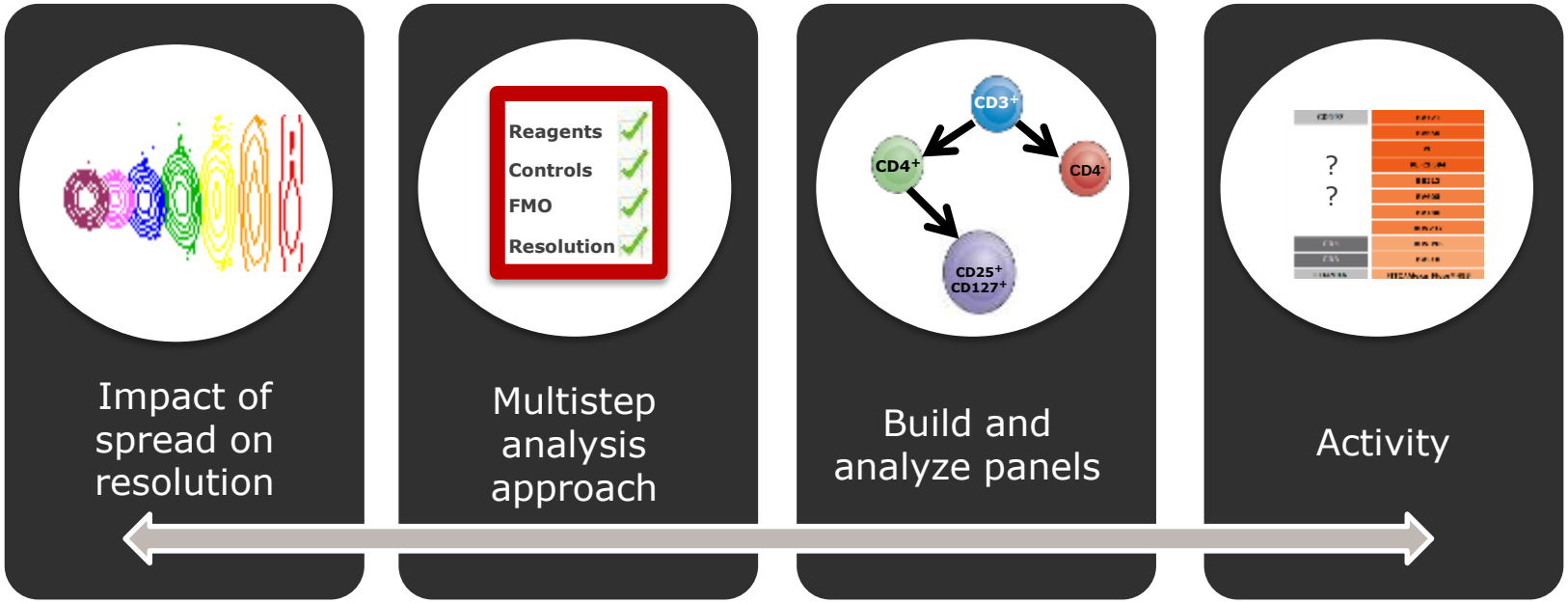
% loss of SP-SI				
<20%	20-40%	40-60%	60-80%	>80%

SI (Primary)

The table shows:

- Adding a PE reagent to a co-expressed marker will have significant spread into and a major negative impact on the resolution of the double-positive cells in the PerCP-Cy5.5 detector.
- No fluorochromes have a significant negative impact on the resolution of FITC$^+$ cells.
- FITC has minimal impact on any other fluorochrome.

Best practices for building and analyzing panels



Multistep analysis approach

A systematic process for the correct analysis of data

The panel design process

Goal

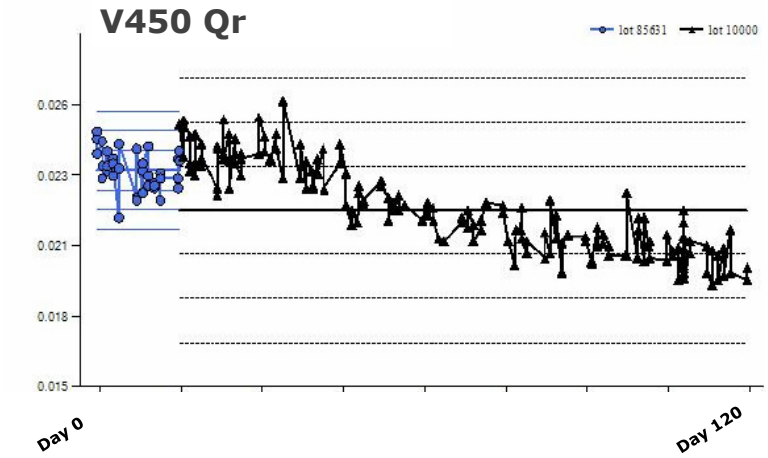
- To assess if data from a flow cytometry assay is "optimal"
- What does optimal mean?
  - Can I resolve each of the critical populations in the panel?
  - Can I easily draw gates for each of the populations identified with this assay?
  - If answers to both of these questions are YES, then panel can be considered optimal.
  - If answers to either of the questions is NO, then you should try another iteration of the panel design.

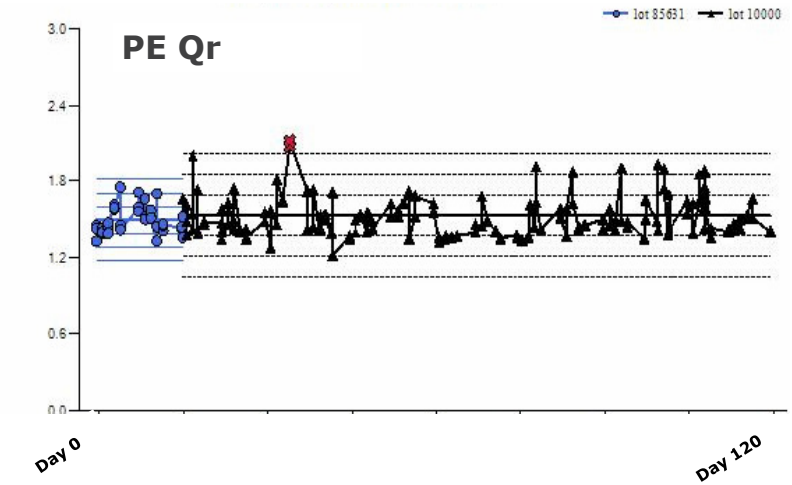
BD

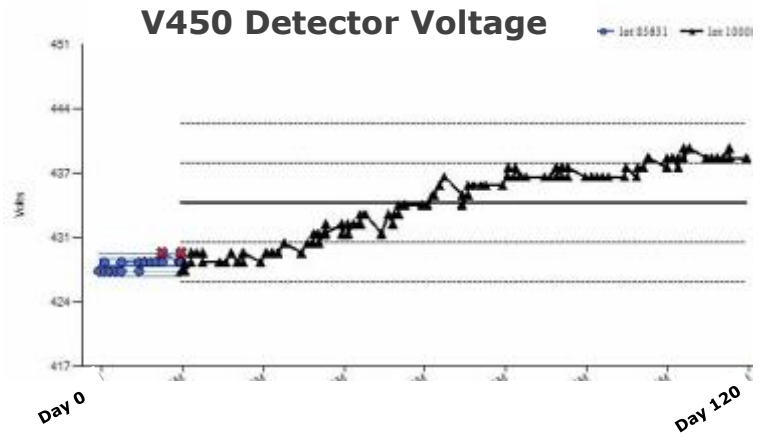
Step-by-step analysis

		Controls used
1.	**Assess adequacy of instrument performance and setup**	CST Reports / L-J Plots Unstained
	↓	
2.	**Assess reagent performance**	Single Stain
	↓	
3.	**Assess compensation accuracy**	Single Stain / Comp
	↓	
4.	**Assess protocol impacts (when necessary)**	Biological
	↓	
5.	**Assess level of resolution for each marker**	Single Stain
	↓	
6.	**Identify any sources of loss resolution**	FMO

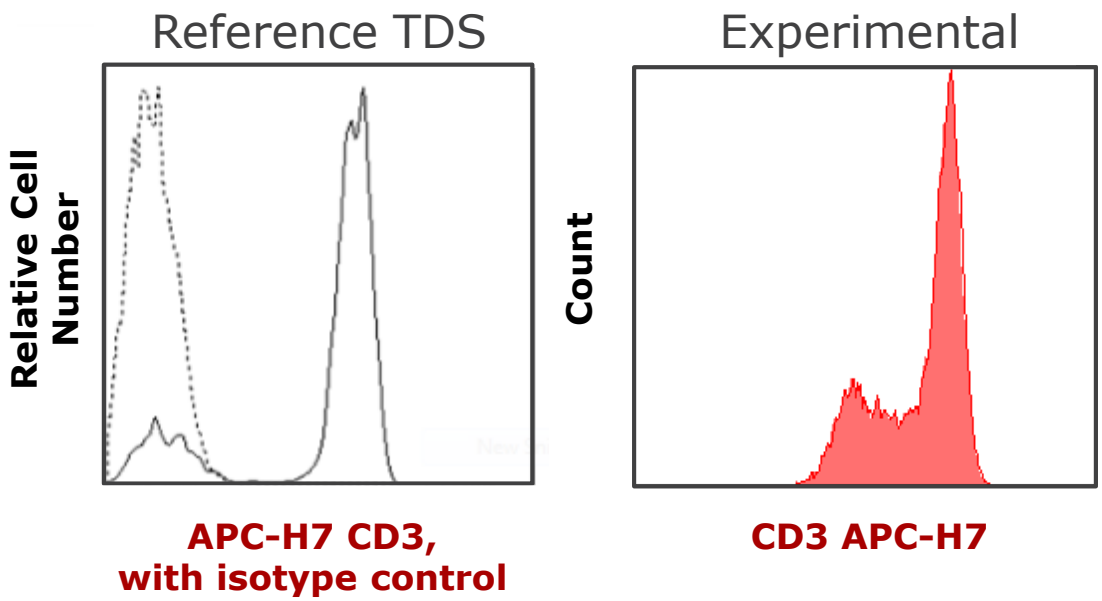
Step 1: assess adequacy of instrument performance and setup







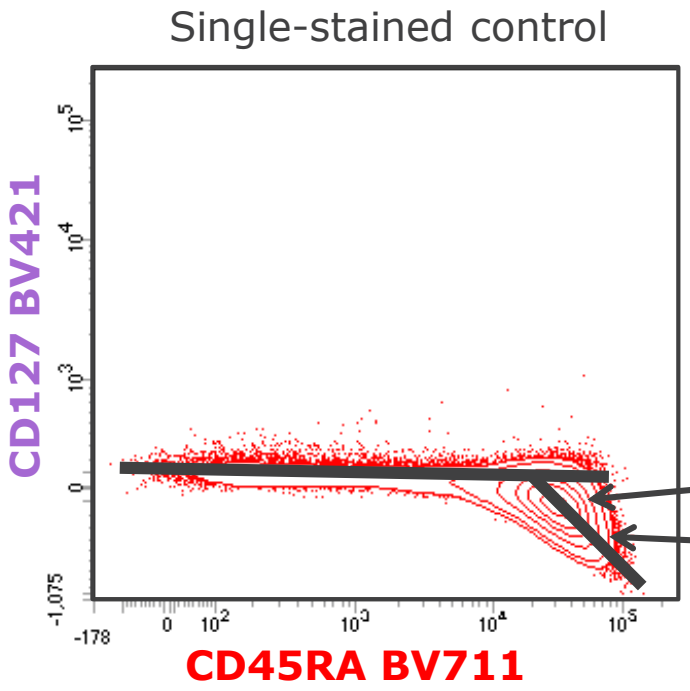
Step 2: assess reagent performance



TDS shows surface staining. Experimental is IC protocol.

Conclusion: need to improve staining conditions.

Step 3: assess compensation accuracy



BV711 into BV421 appears to be over compensated.

Population center is below zero

Population is on a diagonal

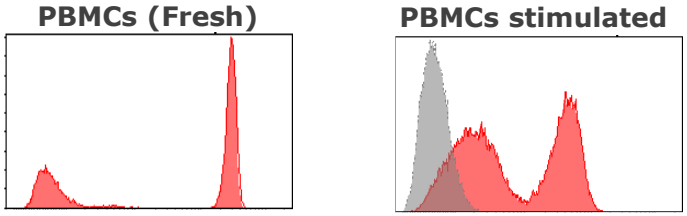
Both are indications that there is an error in the compensation.

Step 4: assess protocol impacts

Single stain control vs Biological control

Impact of culturing / activation on antigen resolution

CD4 PE-Cy7

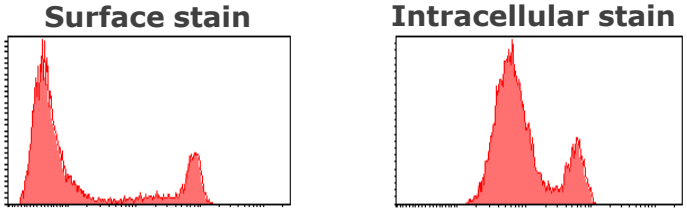


Antigen resolution is reduced because:

- Stimulation increased the background staining and reduced antigen expression.

Impact of intracellular staining

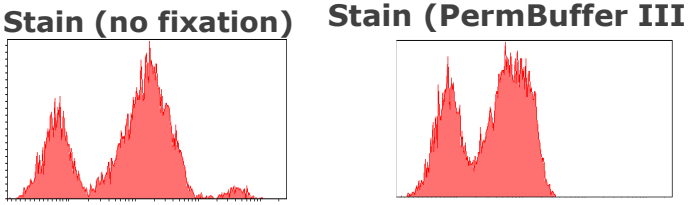
CD8 APC-H7



- Intracellular staining increased the background staining.

Impact of fixation on surface staining

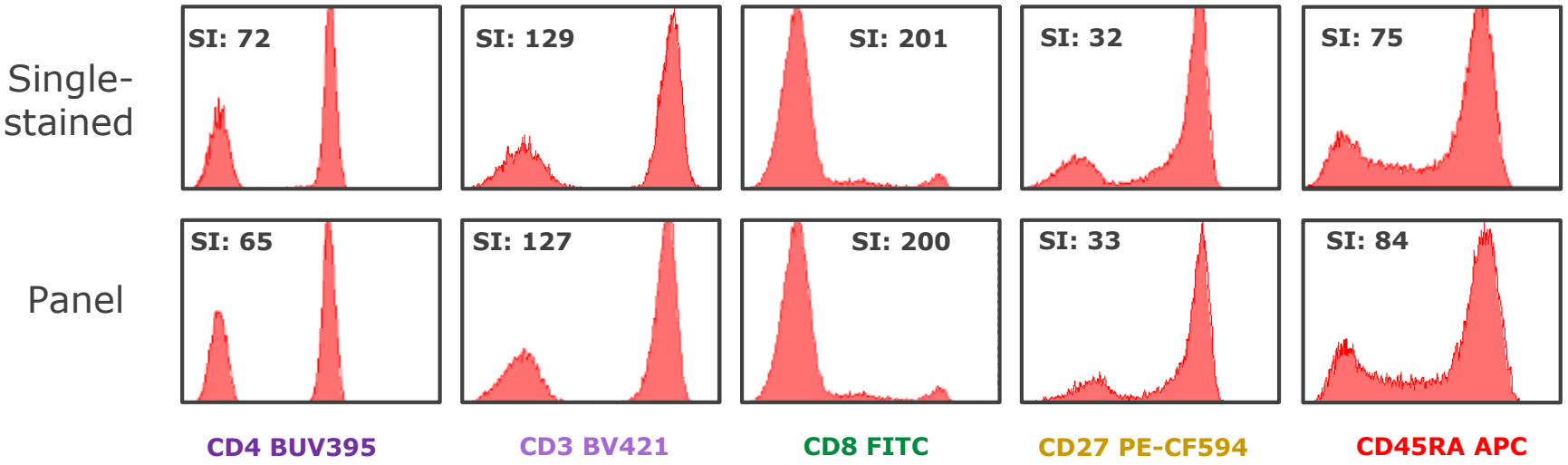
CD127 BV421



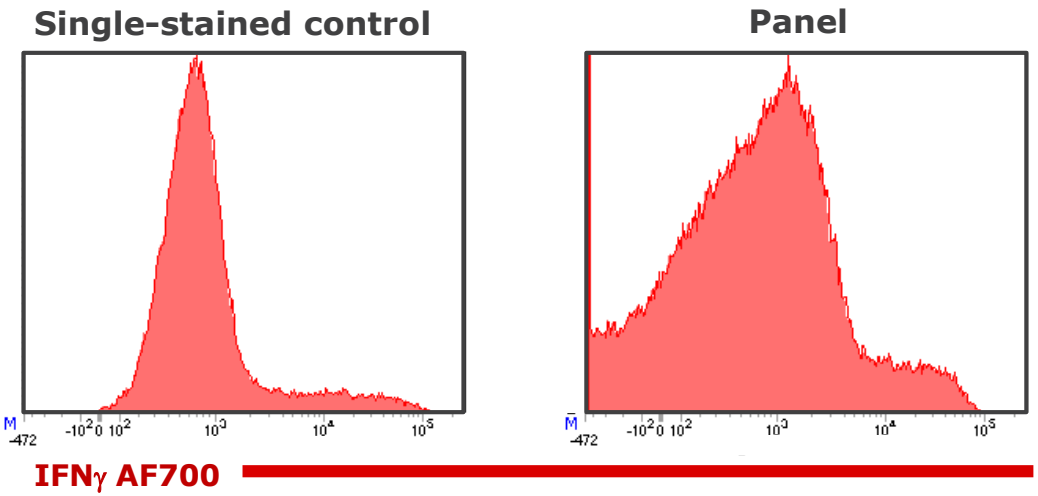
- The fixation reduced the fluorescence of the BV421.

Step 5: assess level of resolution for each marker

- Here there is little or no difference in the profiles of the single stained reagents and the multicolor tube.
- Marker resolution is not affected by the panel design.

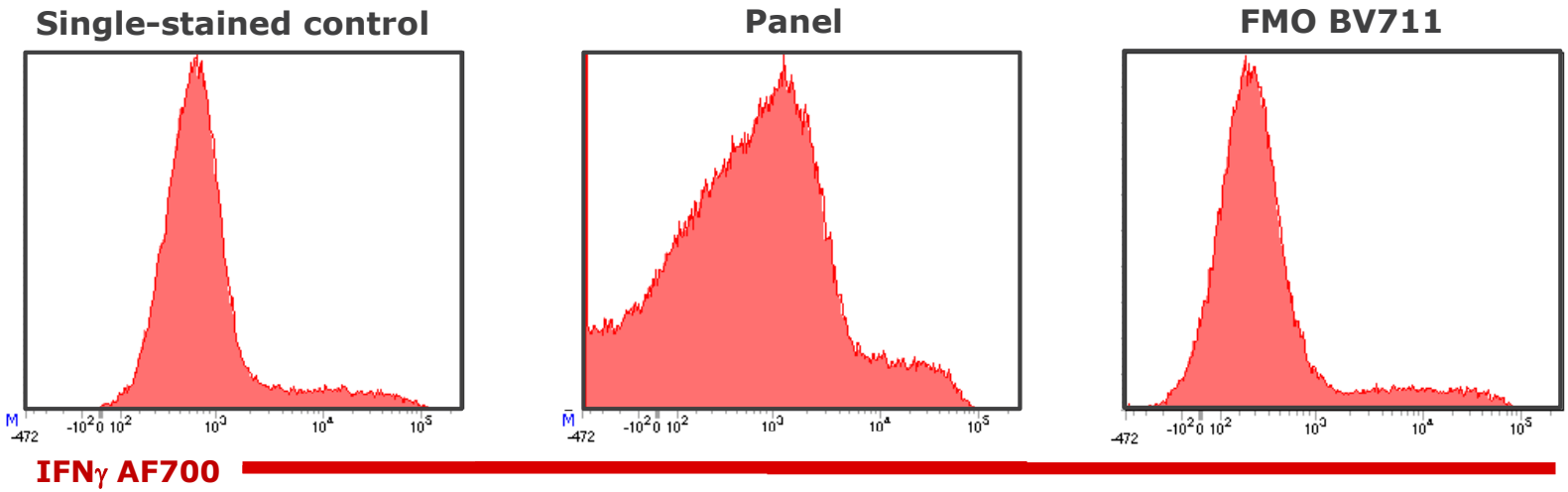


Step 5: assess level of resolution for each marker



- Here, there is a clear loss of resolution of the dim IFNγ (Alexa Fluor® 700) cells.

Step 6:identify sources of resolution loss



- Here, there is a clear loss of resolution of the dim IFNγ (Alexa Fluor® 700) cells.
- The BV711 FMO control confirms that the loss of resolution in the Alexa Fluor® 700 detector is due to the BV711 reagent.

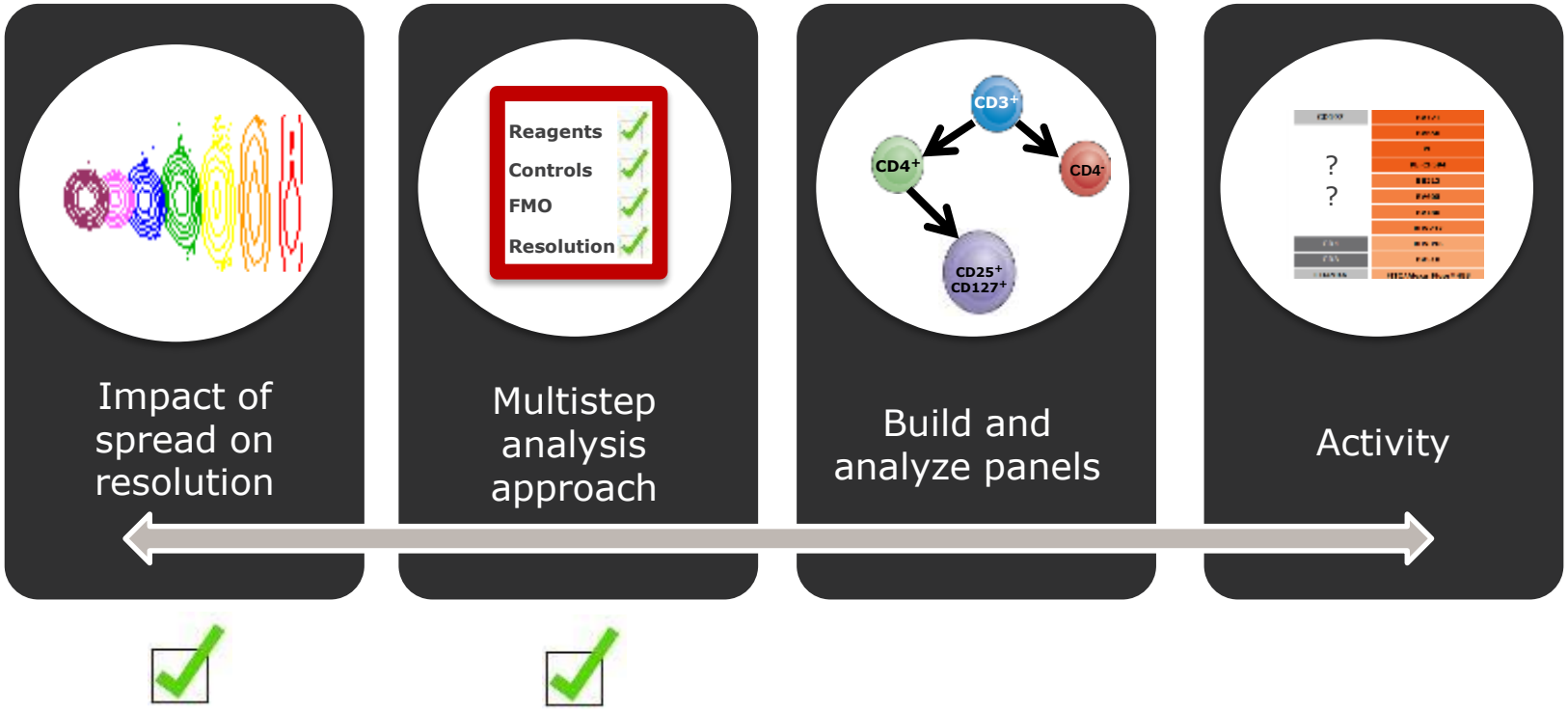
Review - experimental controls

Controls are used to identify and resolve issues when optimizing a new multicolor panel

- Unstained controls
  - to highlight the background or autofluorescence of the biological system
  - to optimize instrument setup
- Single stained controls
  - to QC the compensation
  - to assess any impact of marker resolution in the panel
- Fluorescence Minus One (FMO) controls
  - to help identify potential impact of spillover/spread impacting resolution in the panel

BD

Best practices for building and analyzing panels



Build and analyze panels

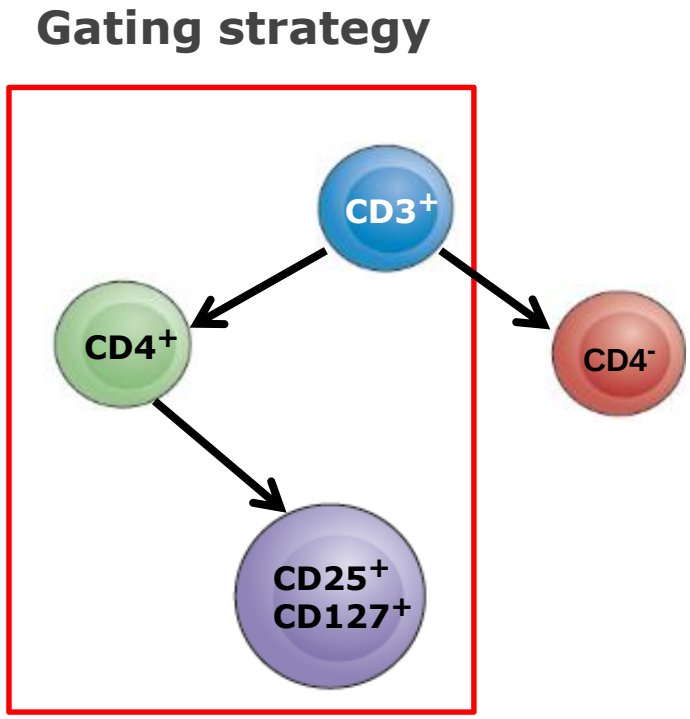
Apply the panel design rules to achieve the best resolution for your population of interest

Building a 4-color panel to identify regulatory T-cells (Tregs)

Experimental goal:

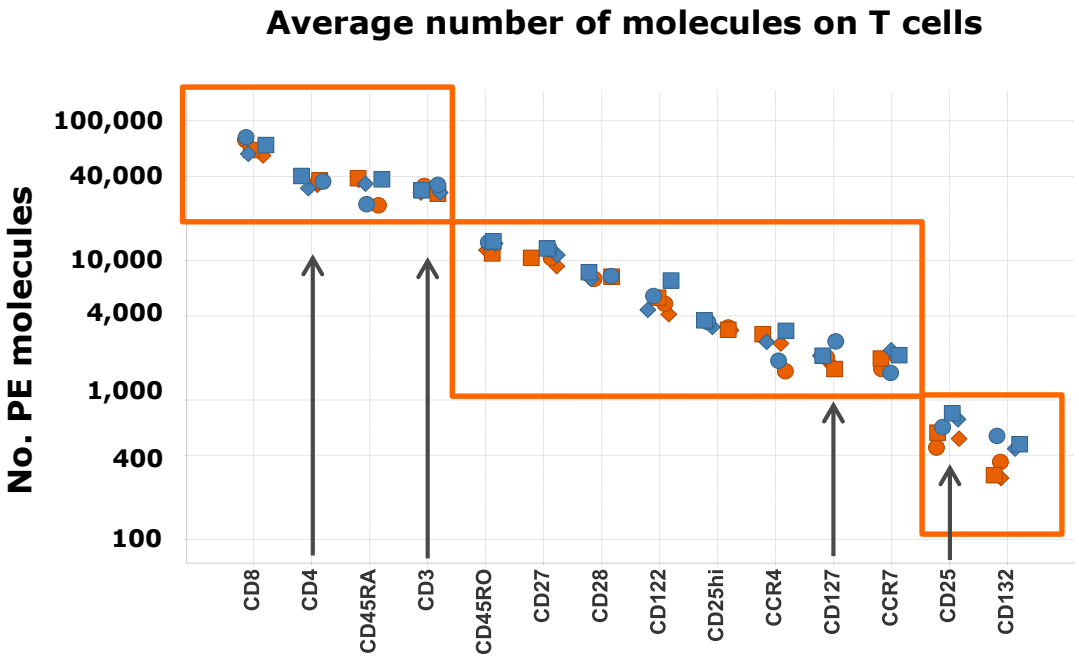
- Identify Treg cells
- Design the best panels for different instrument configurations

- Markers used: CD3, CD4, CD25, CD127



Grouping antigen density: T-cells

	Ag density	Classification
CD3	High	Primary
CD4	High	Primary
CD127	Medium	Secondary
CD25	Low	Tertiary



Approaches to panel design on BD Accuri™ C6 Plus

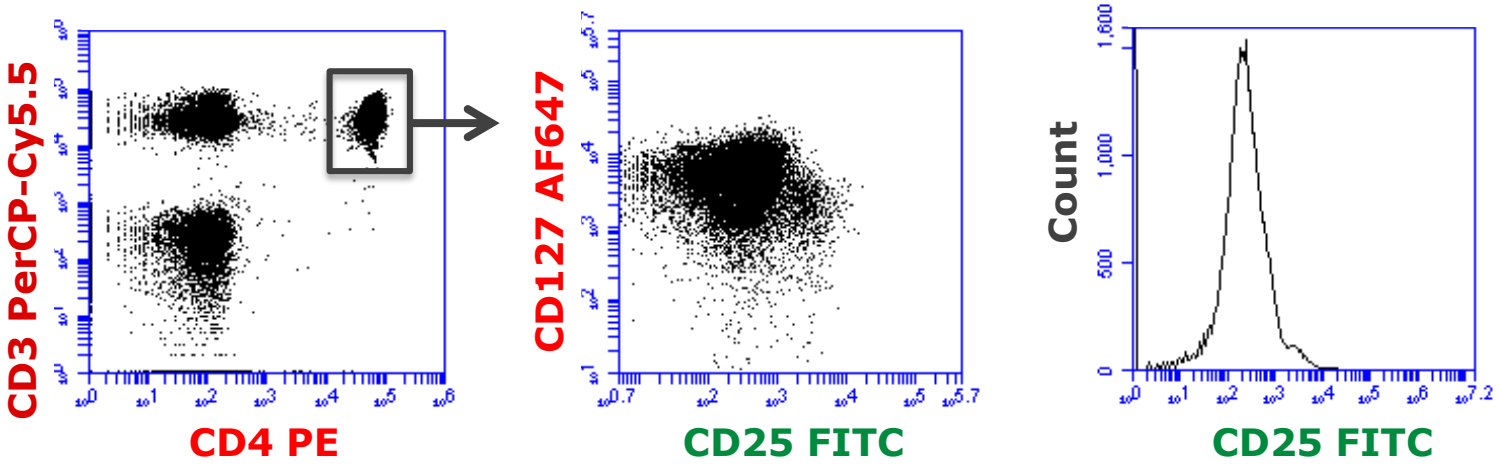
Laser	Fluorochrome	
Blue (488 nm)	FITC	BB515
	PE	
	PerCP-Cy™5.5	
Red (640 nm)	APC	

- 3 detectors off the blue and 1 off the red laser: possible spillover issues.
- The BD Horizon Brilliant dye BB515 offers an additional choice for a bright fluorochrome.

BD Accuri C6 Plus – panel 1

Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
CD4	PE	●
CD3	APC/Alexa Fluor® 647	●
CD127	FITC/Alexa Fluor® 488	●
CD25	PerCP-Cy5.5	●

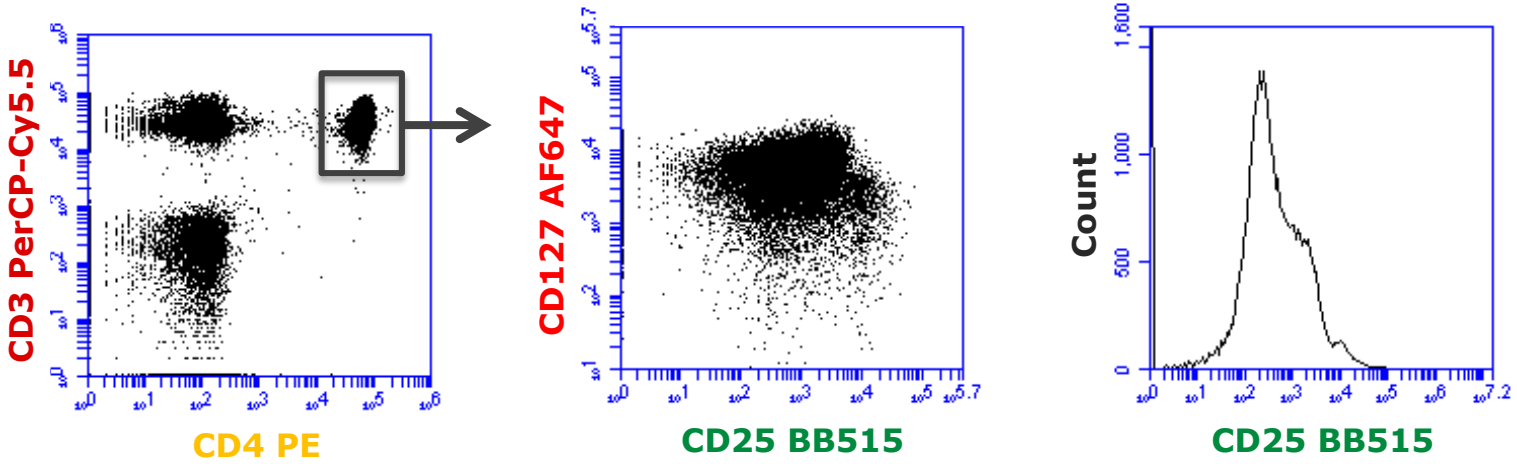
BD Accuri C6 Plus – panel 1



BD Accuri C6 Plus – panel 2

Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
CD4	PE	●
CD3	BB515	●
CD127	APC/Alexa Fluor® 647	●
CD25	PerCP-Cy5.5	●

BD Accuri C6 Plus – panel 2



Approaches to panel design on BD FACSCelesta Blue/Violet (B/V)

Laser	Fluorochrome	
Blue (488 nm)	FITC	BB515
	PE	
	PE-CF594	
	PerCP-Cy5.5	
Violet (405 nm)	V450	
	V500	

- Four detectors off the blue and five off the violet laser:
  - reduced spillover issues.
- Before the BD Horizon Brilliant dyes, only two dim violet dyes were available.

Approaches to panel design on BD FACSCelesta Blue/Violet (B/V)

Laser	Fluorochrome	
Blue (488 nm)	FITC	BB515
	PE	
	PE-CF594	
	PerCP-Cy5.5	
Violet (405 nm)	BV421	
	BV480	BV510
	BV605	
	BV650	
	BV711	
	BV786	

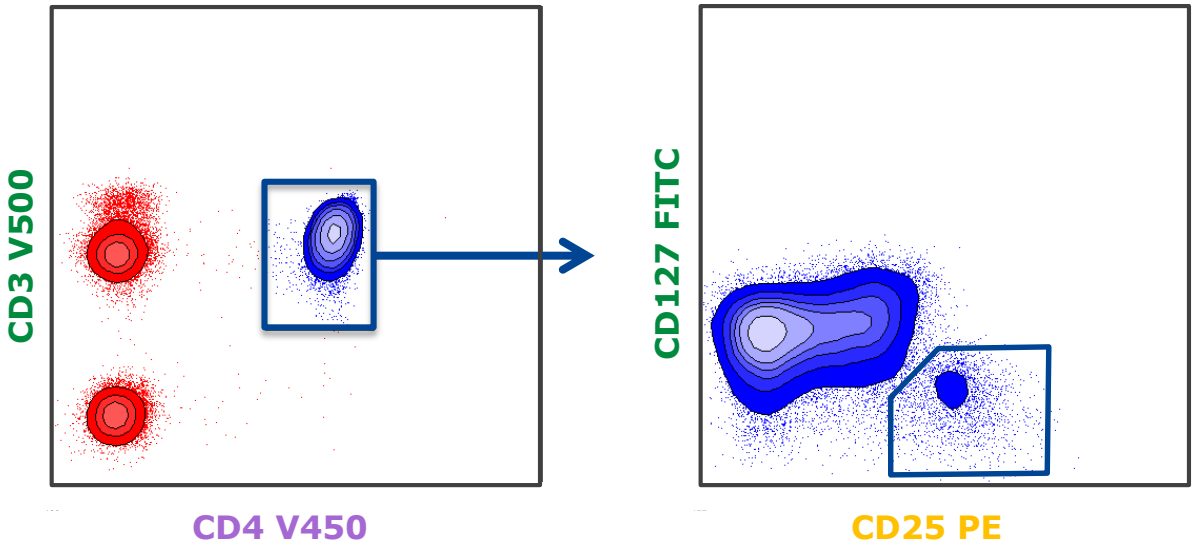
- Four detectors off the blue and five off the violet laser:
  - reduced spillover issues.
- Before the BD Horizon Brilliant dyes, only two dim violet dyes were available.
- Seven moderate/bright BD Horizon Brilliant dyes are now available.

BD

Building a panel on BD FACSCelesta B/V – panel 1

Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
CD4	PE	●
CD3	PE-CF594	
CD127	PerCP-Cy5.5	
CD25	FITC/Alexa Fluor® 488	●
	V450	●
	V500	●

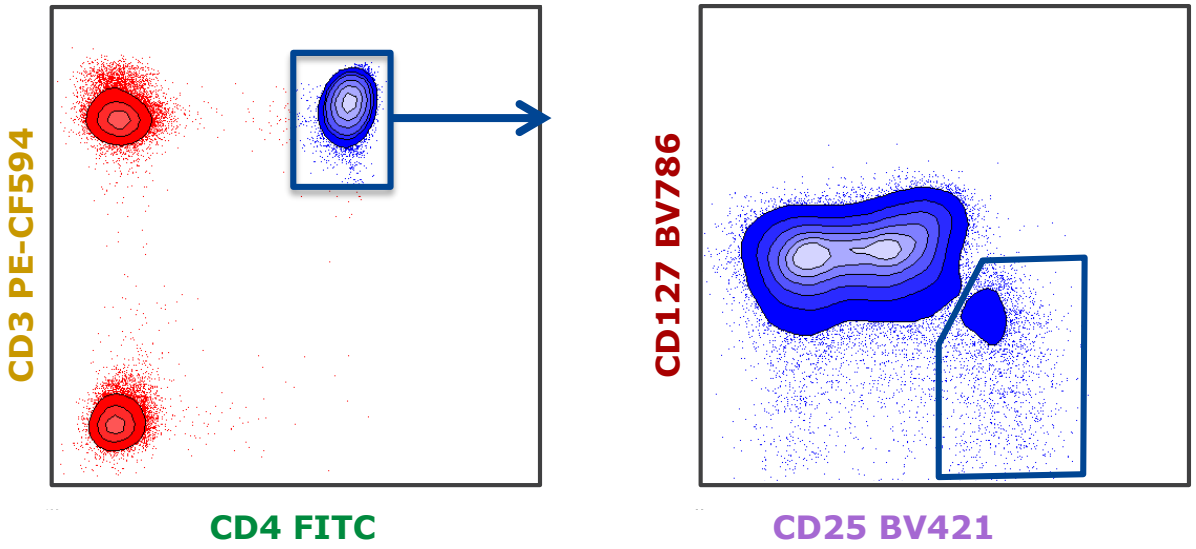
BD FACSCelesta B/V – panel 1



Building a panel on BD FACSCelesta B/V - panel 2

Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
CD4	BV421	●
CD3	BV650	
CD127	BV711	
CD25	PE	
	PE-CF594	●
	BB515	
	BV786	●
	BV605	
	BV510	
	FITC	●
	PerCP-Cy5.5	
	V450	
	V500	

BD FACSCelesta B/V – panel 2



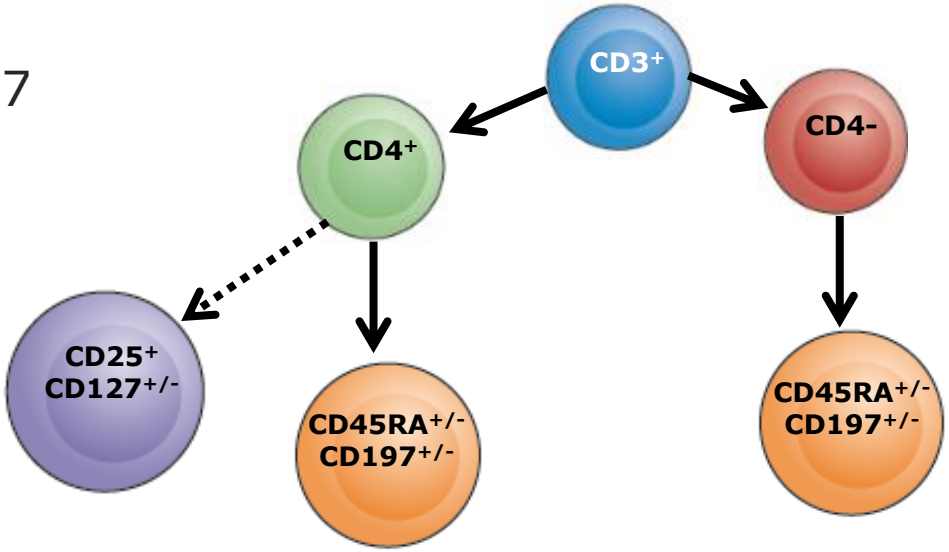
Building a 6-color panel to identify T-cell subsets

Experimental goal:

- Drop in 2 markers (CD45RA and CD197) to identify Tregs and memory/effector T-cells
- Markers used: CD3, CD4, CD25, CD127, CD45RA, CD197
- Assign antigen expression levels

	Ag density	Classification
CD3	**High**	**Primary**
CD4	**High**	**Primary**
CD45RA	**High**	**Secondary**
CD127	**Medium**	**Secondary**
CD197	**Medium**	**Secondary**
CD25	**Low**	**Tertiary**

Gating strategy



Approaches to panel design on BD FACSCelesta Blue/Violet/Red (BVR)

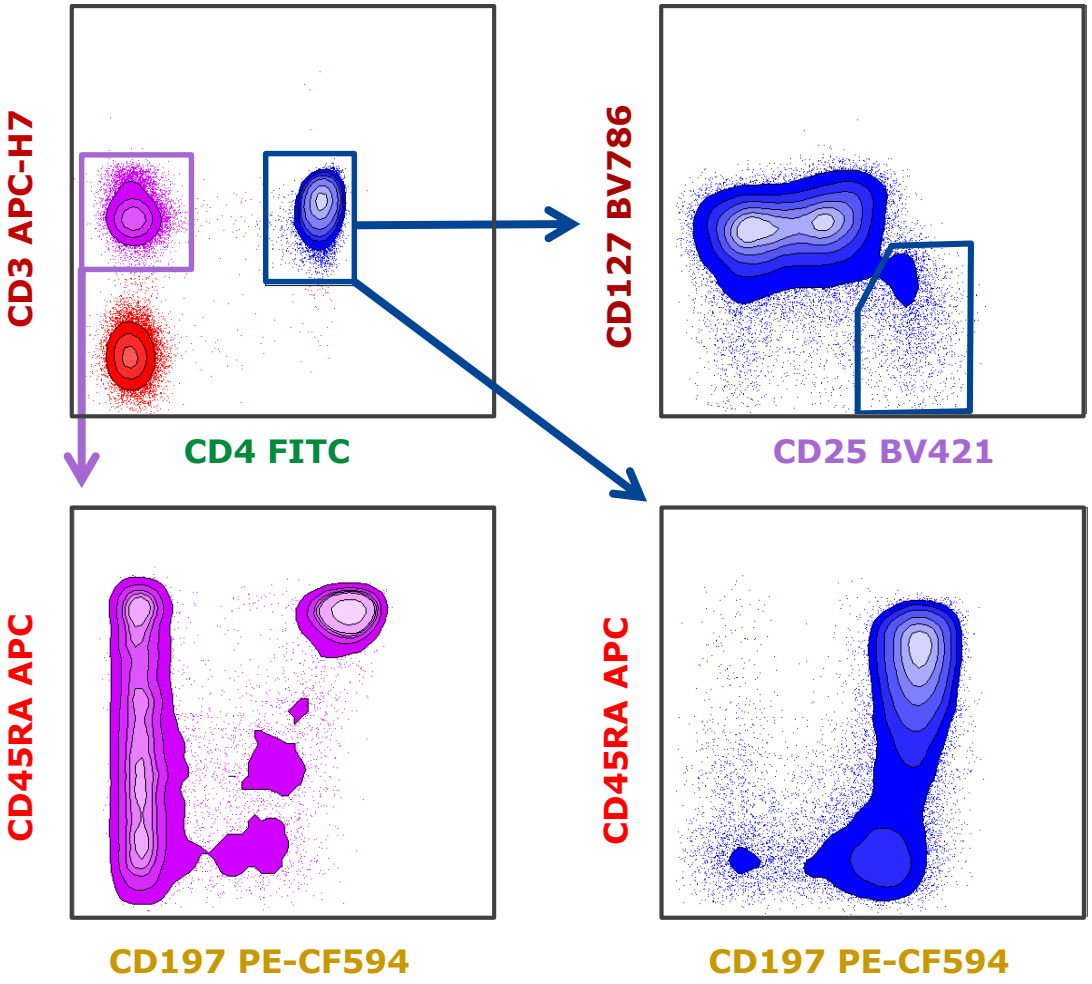
Laser	Fluorochrome	
Blue (488 nm)	FITC	BB515
	PE	
	PE-CF594	
	PerCP-Cy™5.5	
Violet (405 nm)	BV421	
	BV480	BV510
	BV605	
	BV650	
	BV786	
Red (640nm)	APC	
	APC-R700	
	APC-H7	

- The addition of the red laser allows fluorochromes to be spread across 3 lasers.
- Minimize spillover and maximize resolution.

Building a 6-color panel on BD FACSCelesta B/V/R

Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
CD4	BV421	●
CD3	BV650	
CD127	PE	
CD45RA	PE-CF594	●
CD197	BB515	
CD25	APC/Alexa Fluor® 647	●
	BV786	●
	BV605	
	BV510	
	FITC	●
	PerCP-Cy5.5	
	V450	
	V500	
	Alexa Fluor® 700	
	APC-H7	●

6-color panel on BD FACSCelesta B/V/R

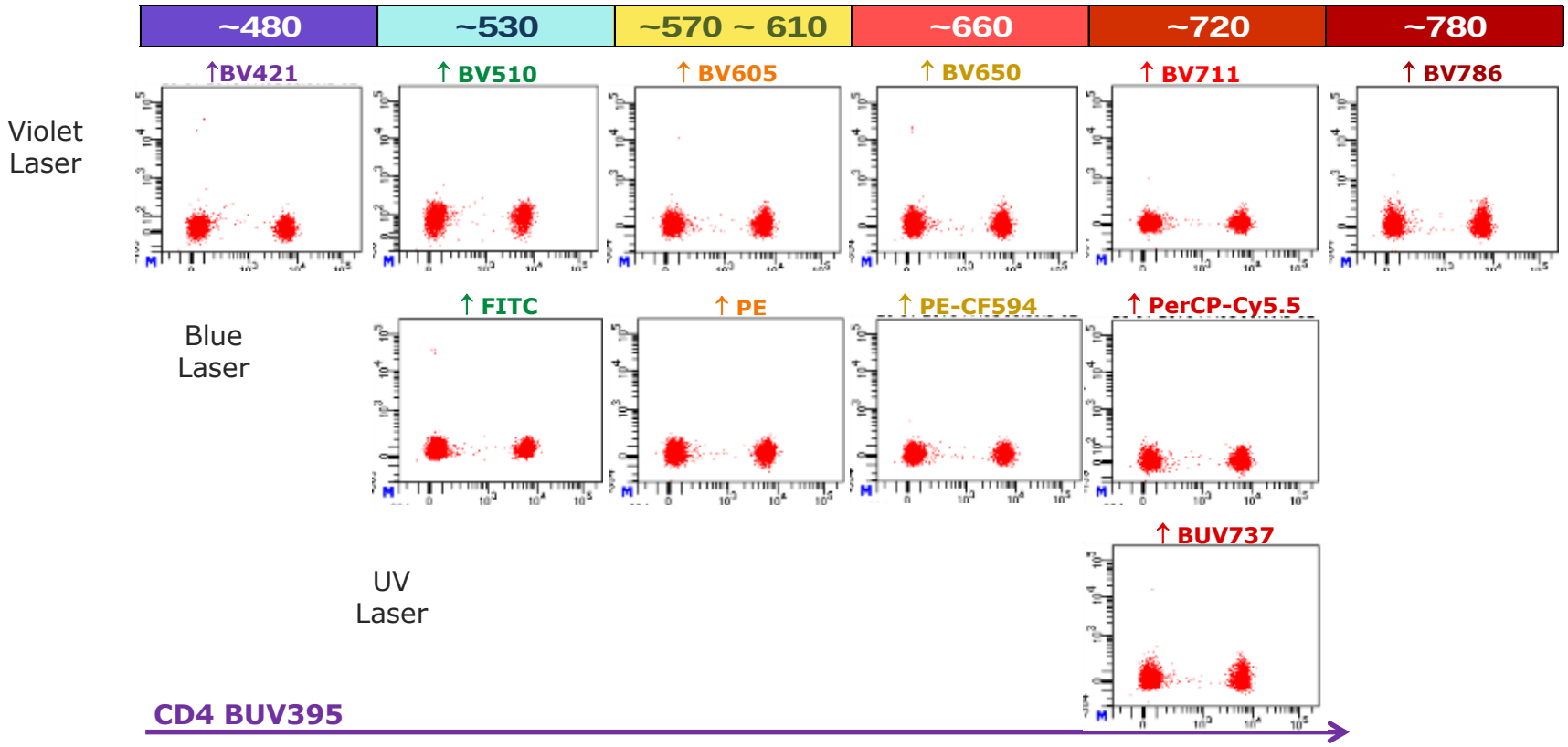


Approaches to panel design on BD FACSCelesta B/V/UV configuration

Laser	Fluorochrome	
Blue (488 nm)	FITC	BB515
	PE	
	PE-CF594	
	PerCP-Cy™5.5	
Violet (405 nm)	BV421	
	BV480	BV510
	BV605	
	BV650	
	BV711	
	BV786	
UV (355nm)	BVUV395	
	BVUV737	

- The addition of the ultraviolet laser allows fluorochromes to be spread across 3 lasers.
- Minimize spillover and maximize resolution.
- The use of BUV395 facilitates panel design
  - Lack of spillover in any other channel.
  - Not impacted by the majority of the other dyes.

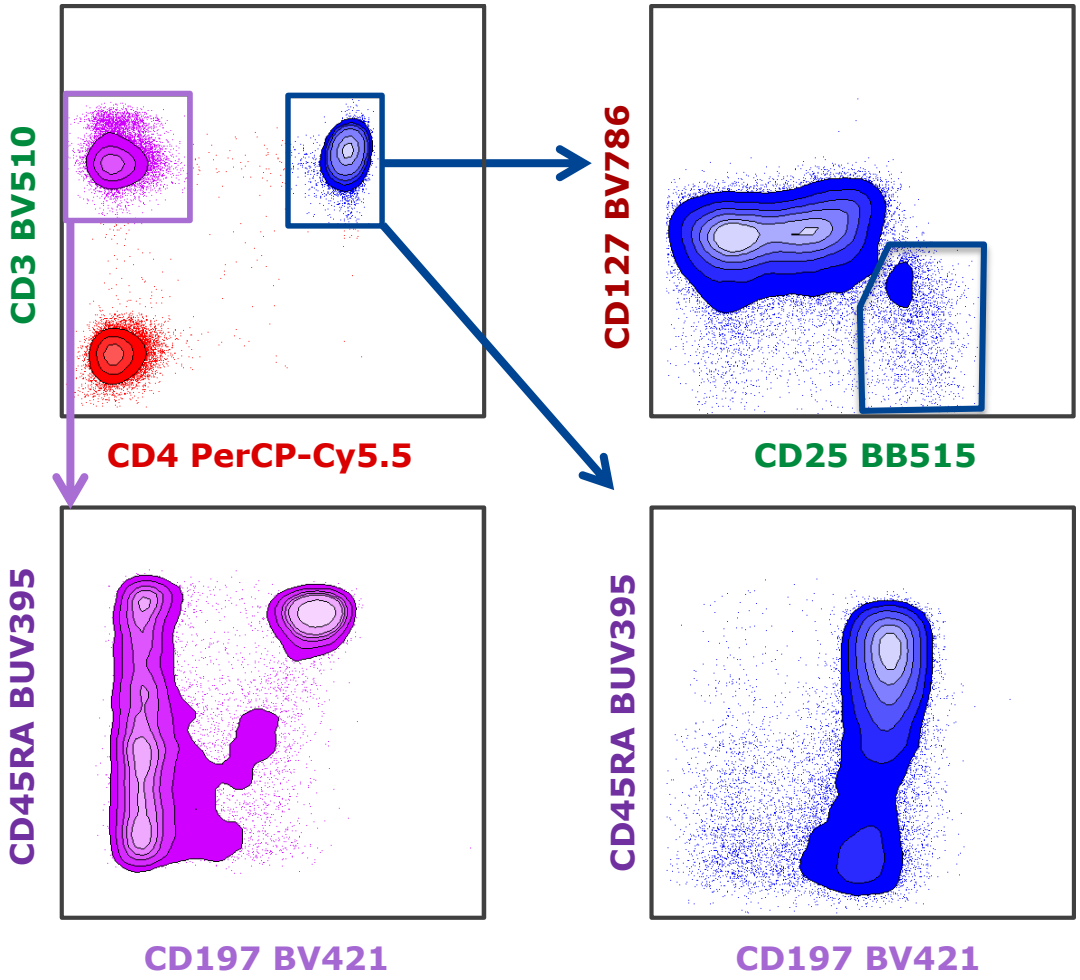
BD FACSCelesta B/V/UV – BUV395 spread



Designing a 6-color panel on BD FACSCelesta B/V/UV

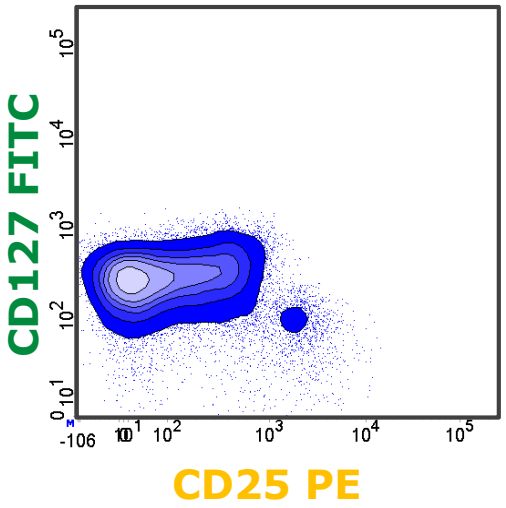
Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
CD4	BV421	●
CD3	BV650	
CD127	BV711	
CD45RA	PE	
CD197	PE-CF594	
CD25	BB515	●
	BV786	●
	BUV737	
	BV605	
	BUV395	●
	BV510	●
	FITC	
	PerCP-Cy5.5	●
	V450	
	V500	

6-color panel on BD FACSCelesta B/V/UV

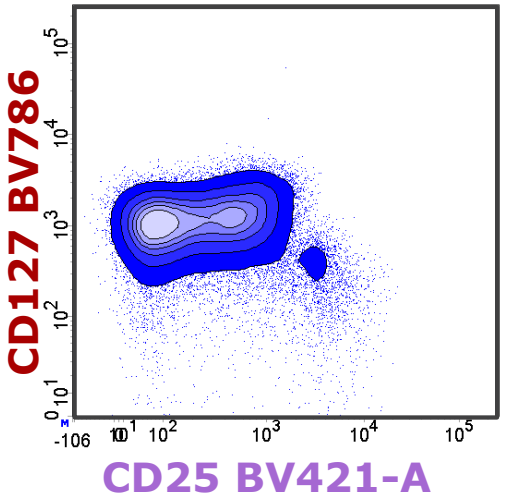


Summary

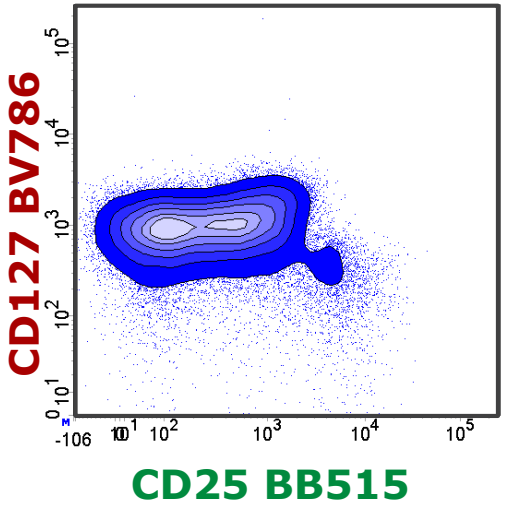
FACSCelesta B/V



FACSCelesta B/V/R



FACSCelesta B/V/UV

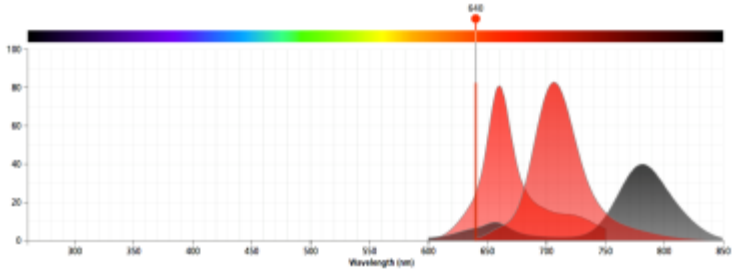


Minimal spectral overlap panels

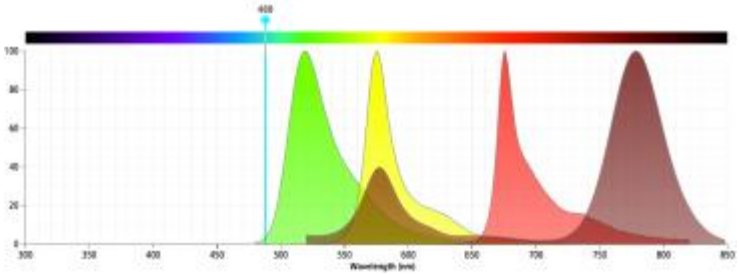
Strategies to maximize resolution by minimizing spillover and compensation

Multiple lasers for multicolor analysis

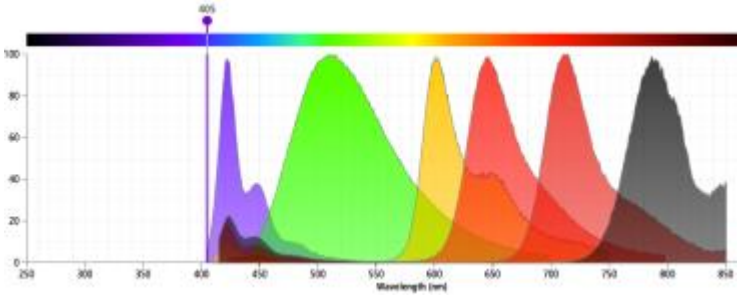
Red (640 nm)



Blue (488 nm)



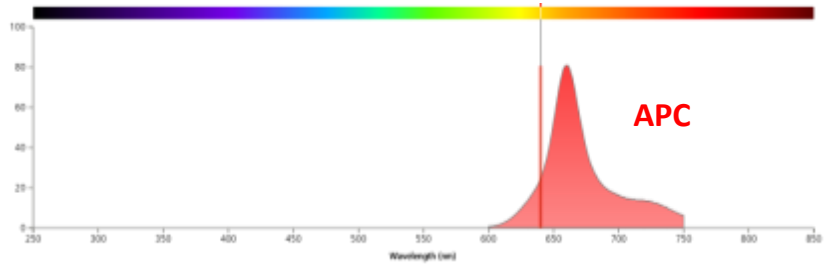
Violet (405 nm)



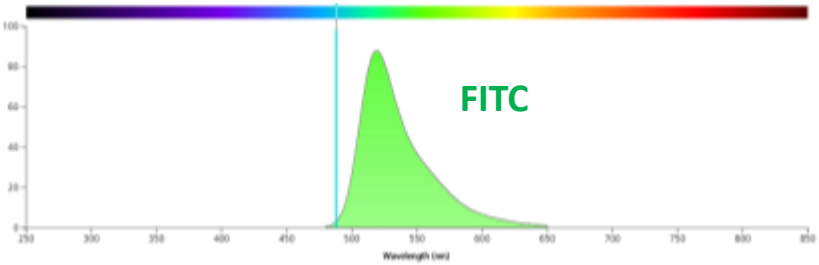
- Three lasers allow for the simultaneous analysis of multiple fluorescent parameters.

Multiple lasers for minimal spectral overlap panels

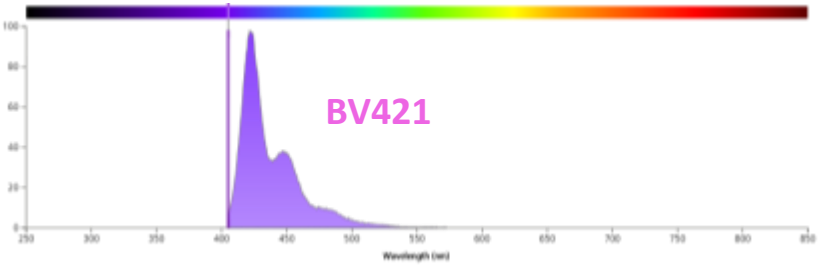
Red (640 nm)



Blue (488 nm)



Violet (405 nm)



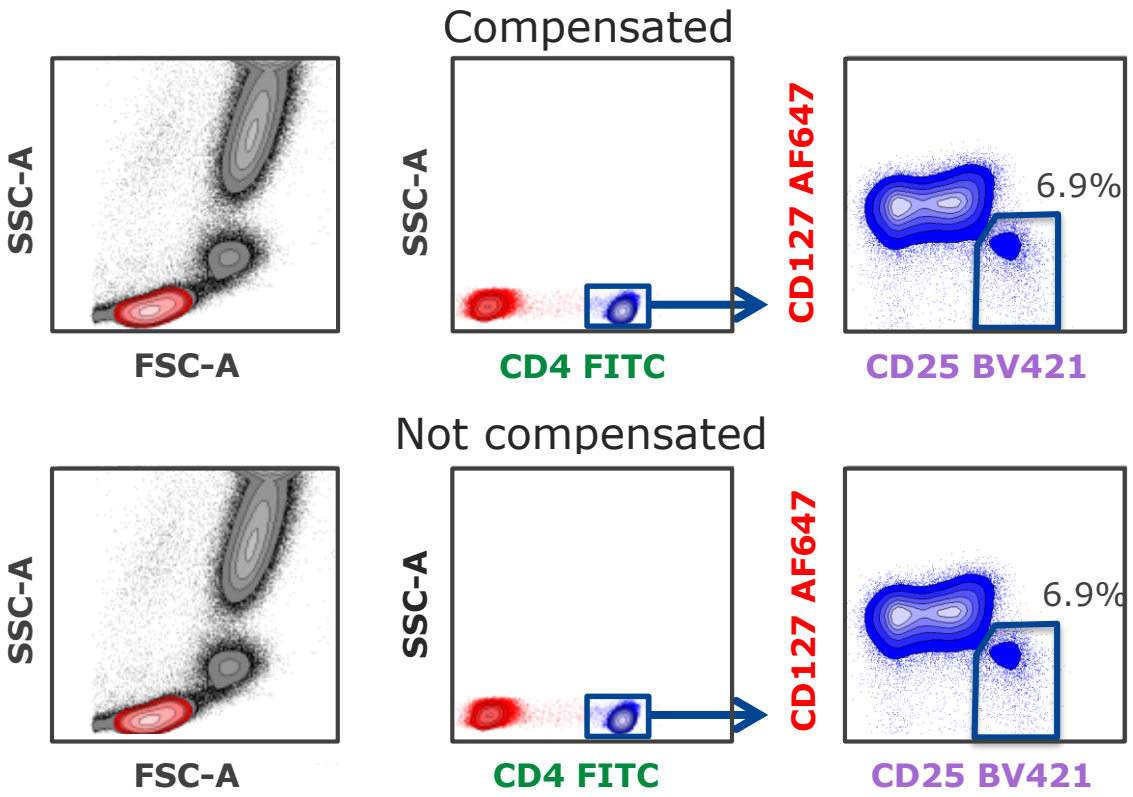
- One color off each laser
- Take into consideration:
  - Residual spillover
  - Cross-laser excitation
  - Antigen density

BD

Designing a 3-color minimal spectral overlap panel on BD FACSCelesta BVR

Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
CD4	BV421	●
CD127	BV650	
CD25	PE	
	PE-CF594	
	BB515	
	APC/Alexa Fluor® 647	●
	BV786	
	BV605	
	BV510	
	FITC/Alexa Fluor® 488	●
	PerCP-Cy5.5	
	V450	
	V500	
	Alexa Fluor® 700	
	APC-Cy7	

3-color minimal compensation panel on BD FACSCelesta B/V/R

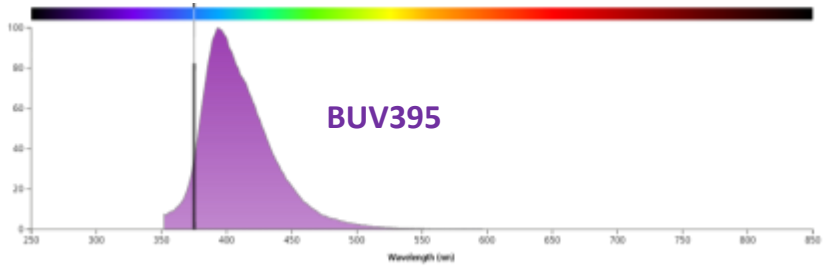


Compensation matrix

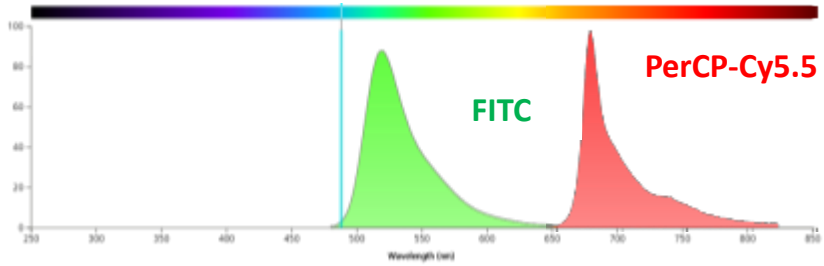
Fluor	-% Fluor	Spectral Overlap
FITC	BV421	0.29
AF647		0.06
BV421	FITC	0.00
AF647		0.00
BV421	AF647	0.00
FITC		0.13

Multiple lasers for minimal spectral overlap panels

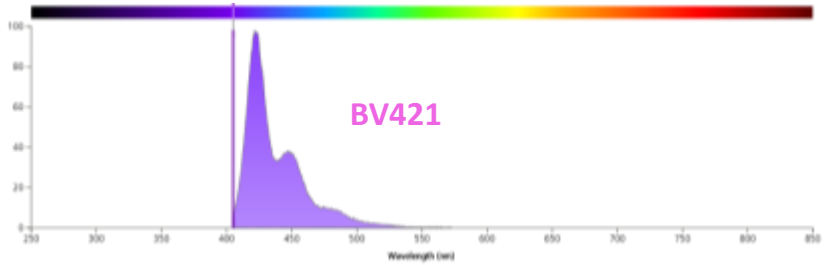
UV (355 nm)



Blue (488 nm)



Violet (405 nm)

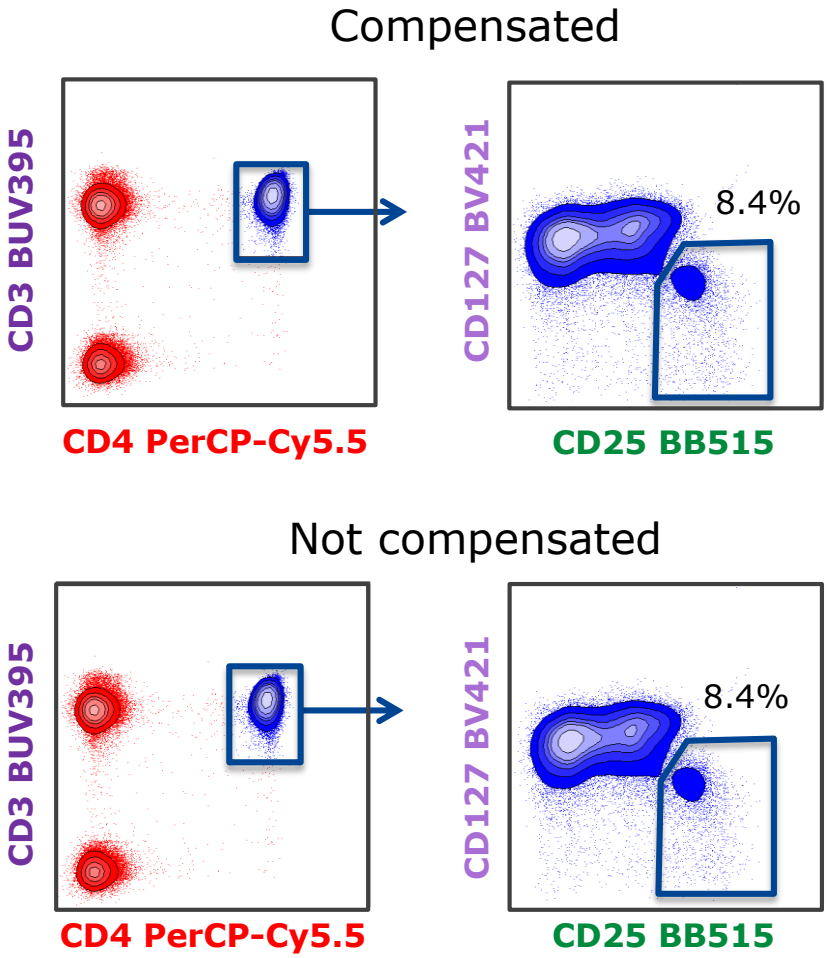


- One color off each laser
- Two colors off one laser if the spectra are well separated
- Take into consideration:
  - Residual spillover
  - Cross-laser excitation
  - Antigen density

4-color minimal compensation panel on BD FACSCelesta B/V/UV

Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
CD4	BV421	●
CD3	BV650	
CD127	BV711	
CD25	PE	
	PE-CF594	
	BB515	●
	BV786	
	BUV737	
	BV605	
	BUV395	●
	BV510	
	FITC/Alexa Fluor® 488	
	PerCP-Cy5.5	●
	V450	
	V500	

4-color minimal spectral overlap panel on BD FACSCelesta B/V/UV



Compensation matrix

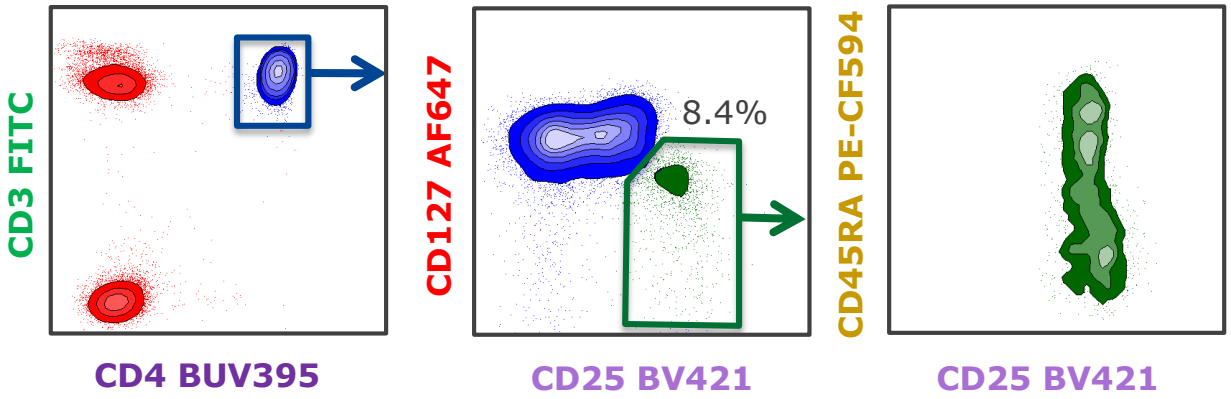
Fluor	-% Fluor	Spectral Overlap
BB515	BV421	0.08
BUV395		0.03
PerCP-Cy5.5		0.12
BV421	BB515	0.00
BUV395		0.5
PerCP-Cy5.5		0.11
BB515	PerCP-Cy5.5	0.00
BV421		0.00
BUV395		0.03
BB515	BUV395	0.01
BV421		0.32
PerCP-Cy5.5		0.00

5-color minimal compensation panel on BD LSRFortessa X20 cell analyzer

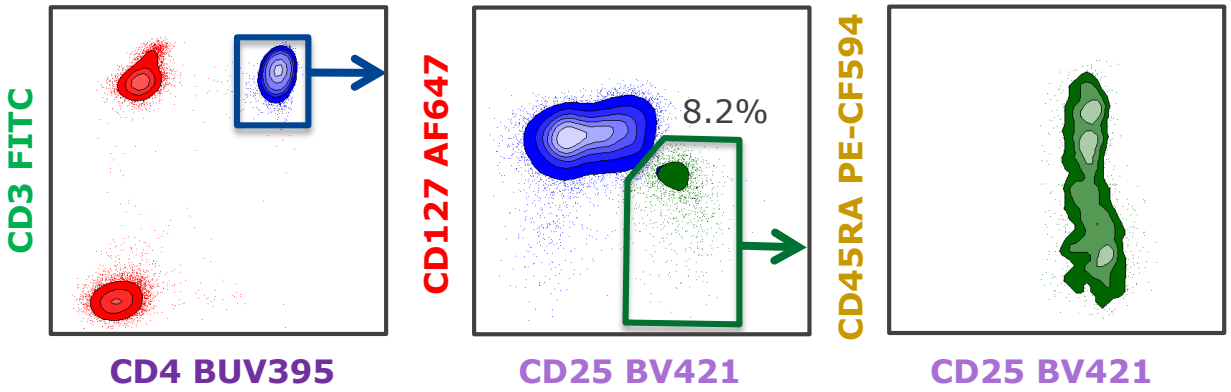
Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
CD4	BV421	●
CD3	BV650	
CD127	BV711	
CD45RA	PE	
CD25	PE-CF594	●
	PE-Cy7	
	BB515	
	APC/Alexa Fluor® 647	●
	BV786	
	BUV737	
	BV605	
	BUV395	●
	BV510	
	FITC/Alexa Fluor® 488	●
	PerCP-Cy5.5	
	Alexa Fluor® 700	
	APC-Cy7	

5-color minimal spectral overlap panel on BD LSRFortessa X20 cell analyzer

Compensated



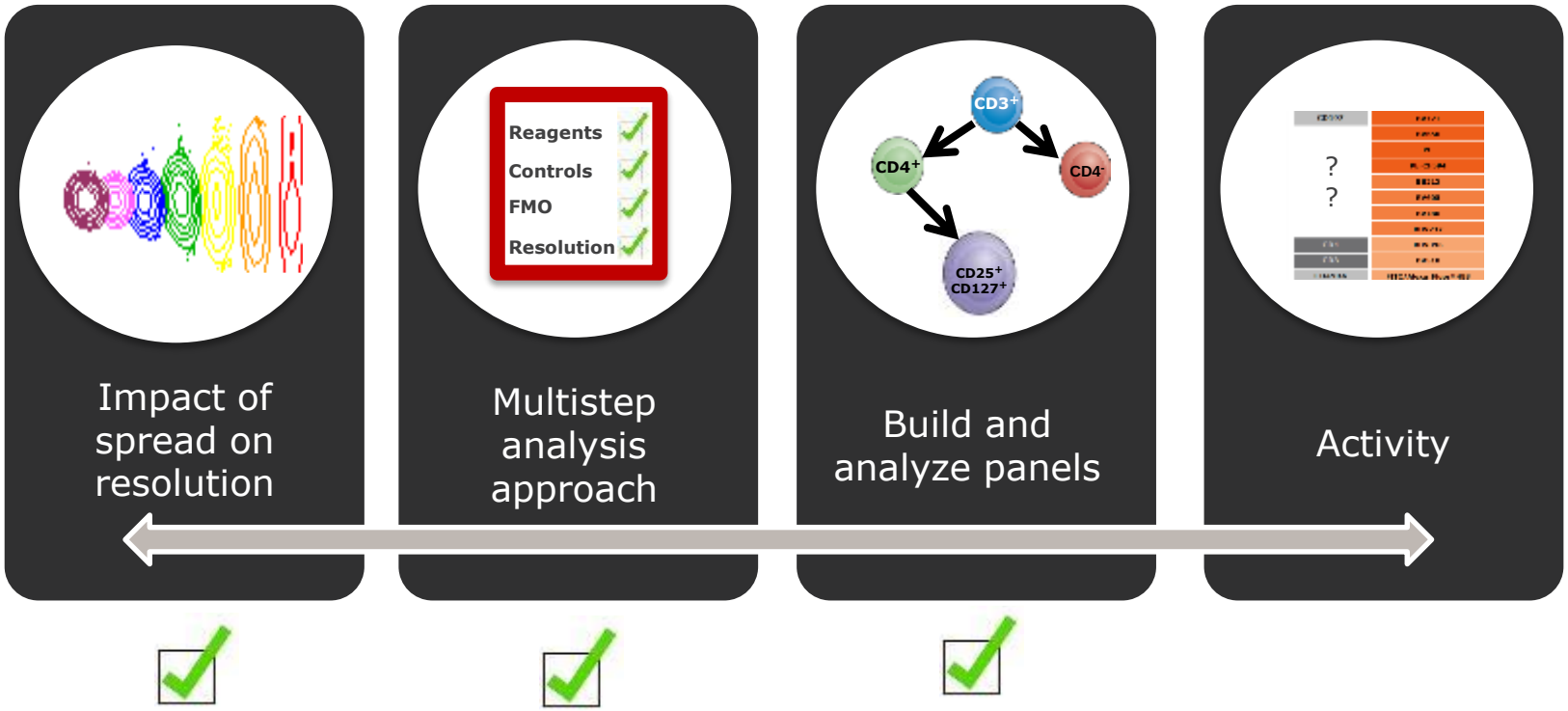
Not compensated



Compensation matrix

Fluor	-% Fluor	Spectral Overlap
BV421	FITC	0.00
BUV395		0.01
AF647		0.00
PE-CF594		0.00
FITC	BV421	0.00
BUV395		0.5
AF647		0.0
PE-CF594		0.00
FITC	BUV395	0.01
BV421		0.27
AF647		0.00
PE-CF594		0.00
FITC	AF647	0.12
BV421		0.00
BUV395		0.07
PE-CF594		0.00
FITC	PE-CF594	0.18
BV421		0.00
BUV395		0.01
AF647		0.64

Best practices for building and analyzing panels

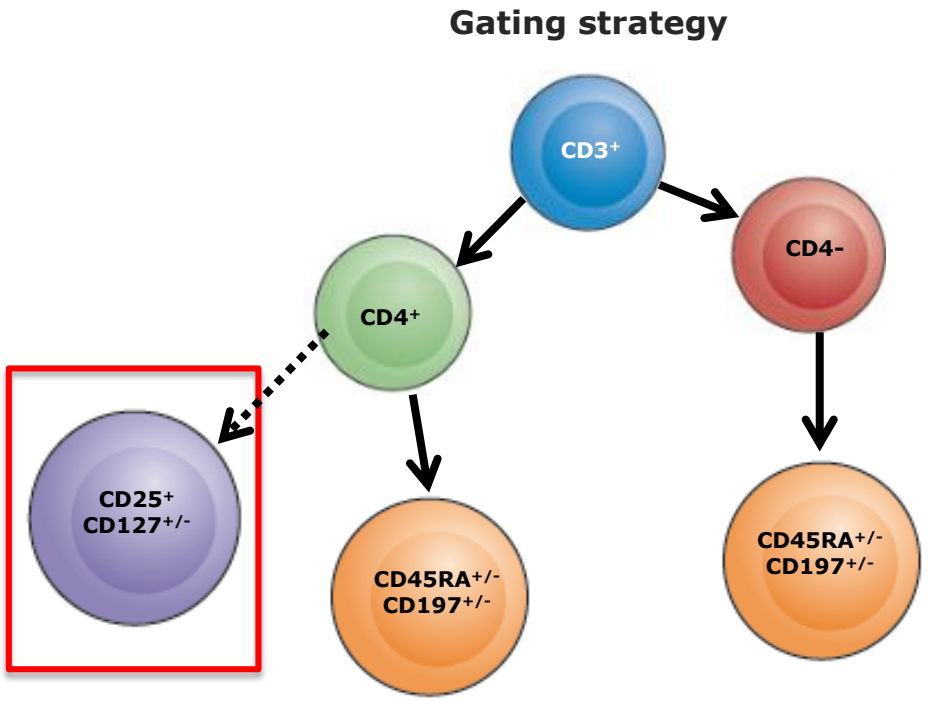


Activity

Complete a 6-color panel for the optimal resolution of Tregs

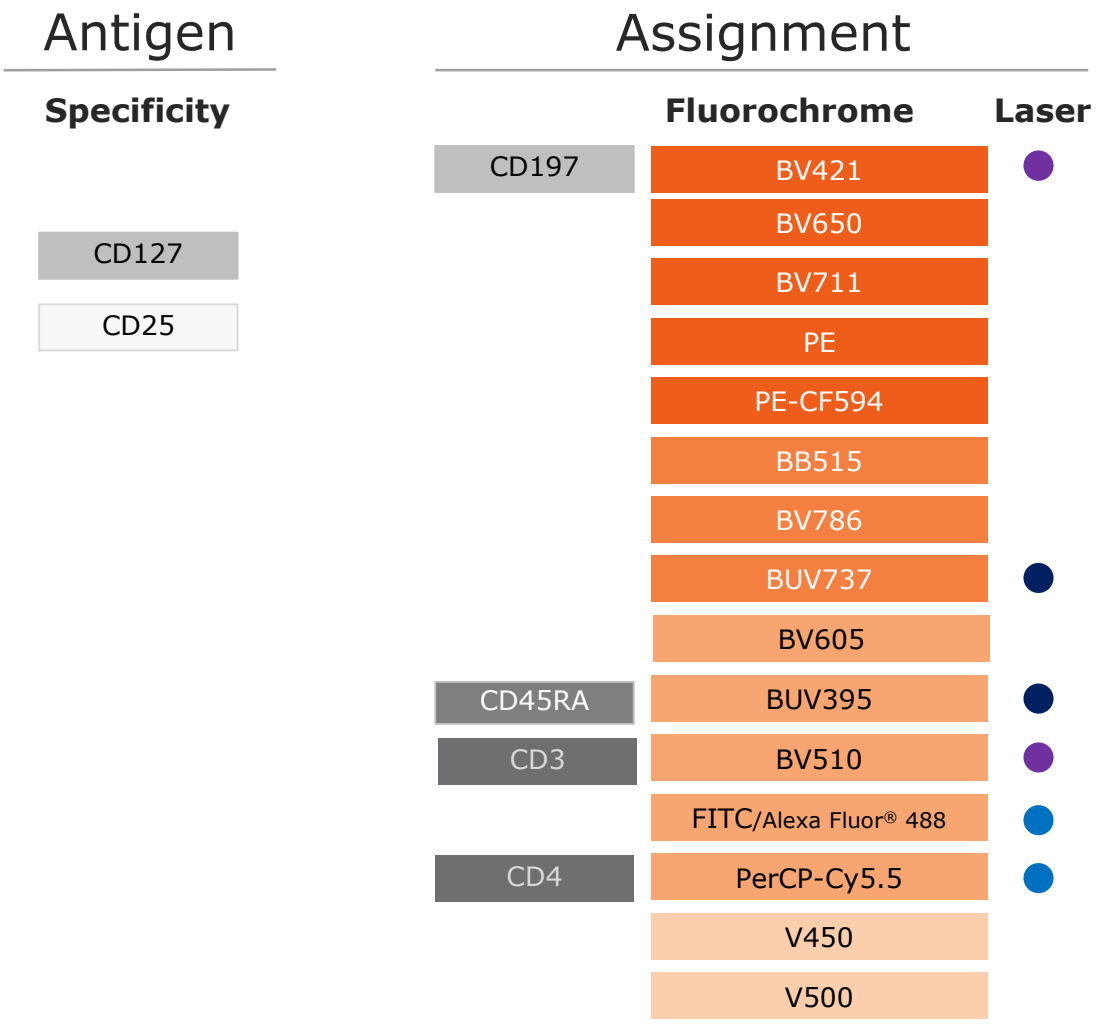
Activity

Marker	Antigen Density	Dye
CD3	High	**BV510**
CD4	High	**PerCP-Cy5.5**
CD25	Low	**?**
CD127	Medium	**?**
CD197	Medium	**BV421**
CD45RA	High	**BUV395**

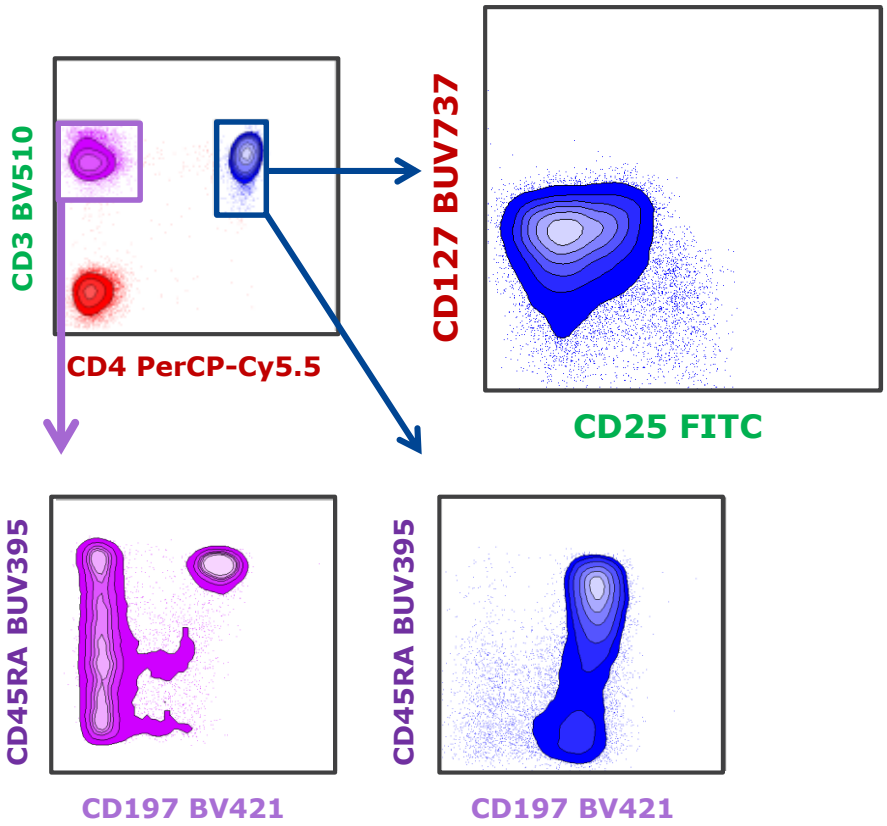


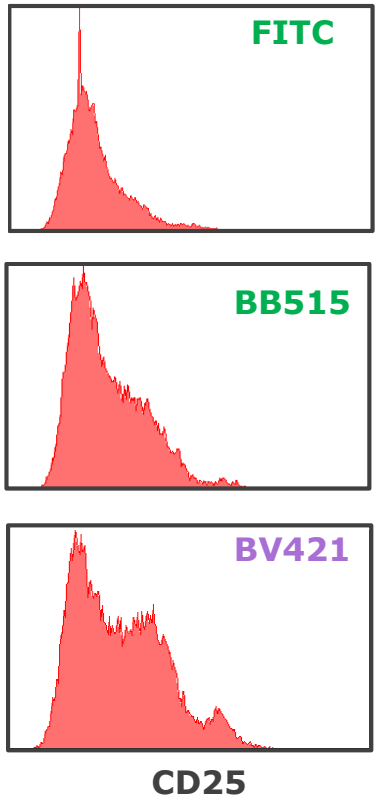
- Complete the panel by assigning fluorochromes for a good resolution of $CD25^{high}$ $CD127^{low}$ Tregs.
- Take into consideration:
  - Co-expression
  - Antigen density
  - Spillover

Panel 1


Antigen
Specificity
CD127
CD25
Assignment
Fluorochrome
Laser
CD197
BV421
BV650
BV711
PE
PE-CF594
BB515
BV786
BUV737
BV605
CD45RA
BUV395
CD3
BV510
FITC/Alexa Fluor® 488
CD4
PerCP-Cy5.5
V450
V500

Panel 1 review – antigen density

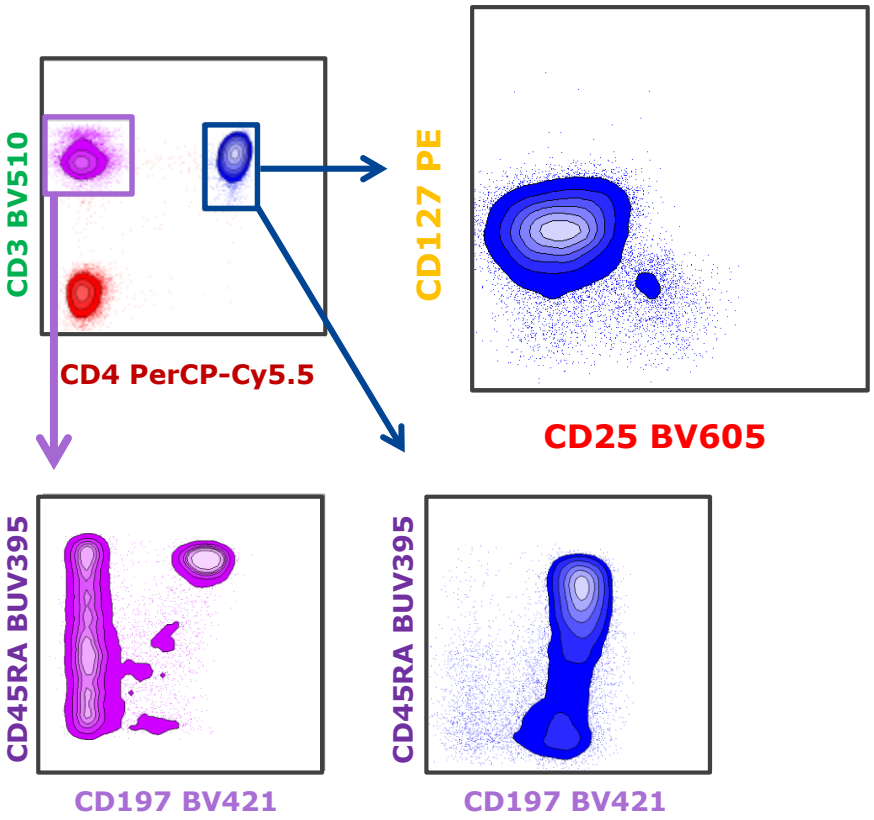




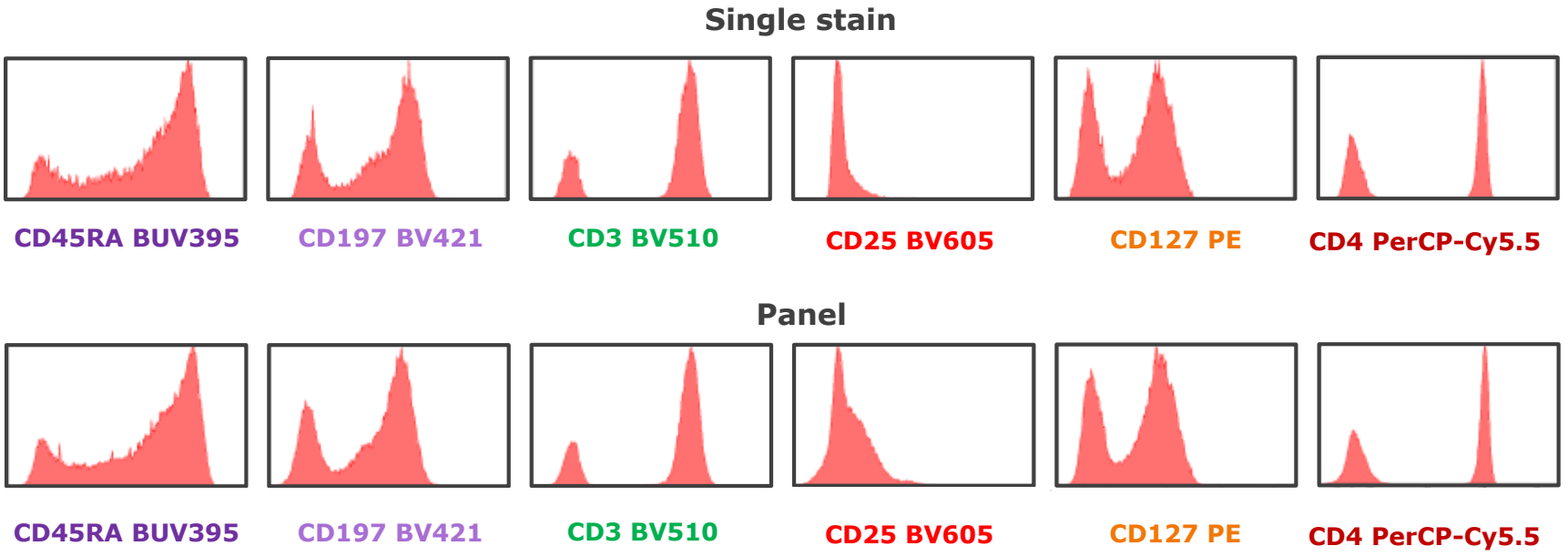
Panel 2

Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
CD197	BV421	●
	BV650	
CD127	BV711	
CD25	PE	●
	PE-CF594	
	BB515	
	BV786	
	BUV737	
	BV605	●
CD45RA	BUV395	●
CD3	BV510	●
	FITC/Alexa Fluor® 488	
CD4	PerCP-Cy5.5	●
	V450	
	V500	

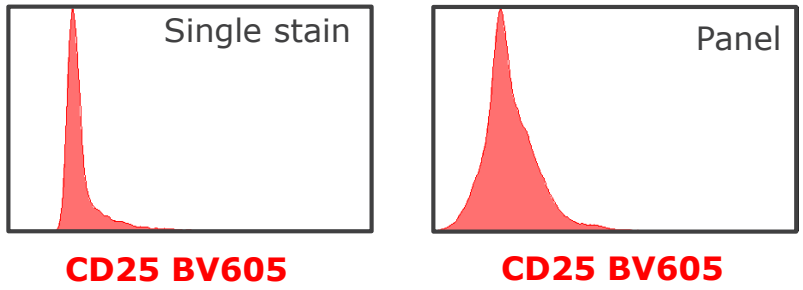
Panel 2 – adjacent spillover

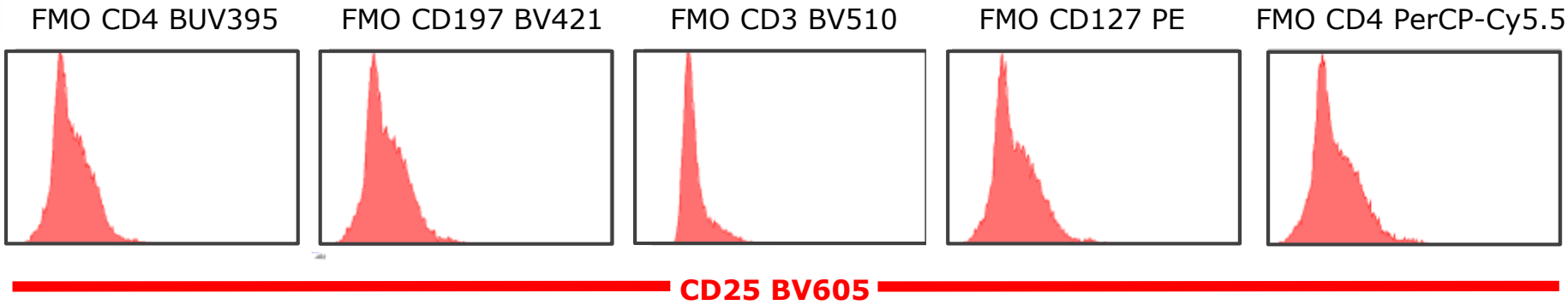


Full panel review



Identify sources of resolution loss

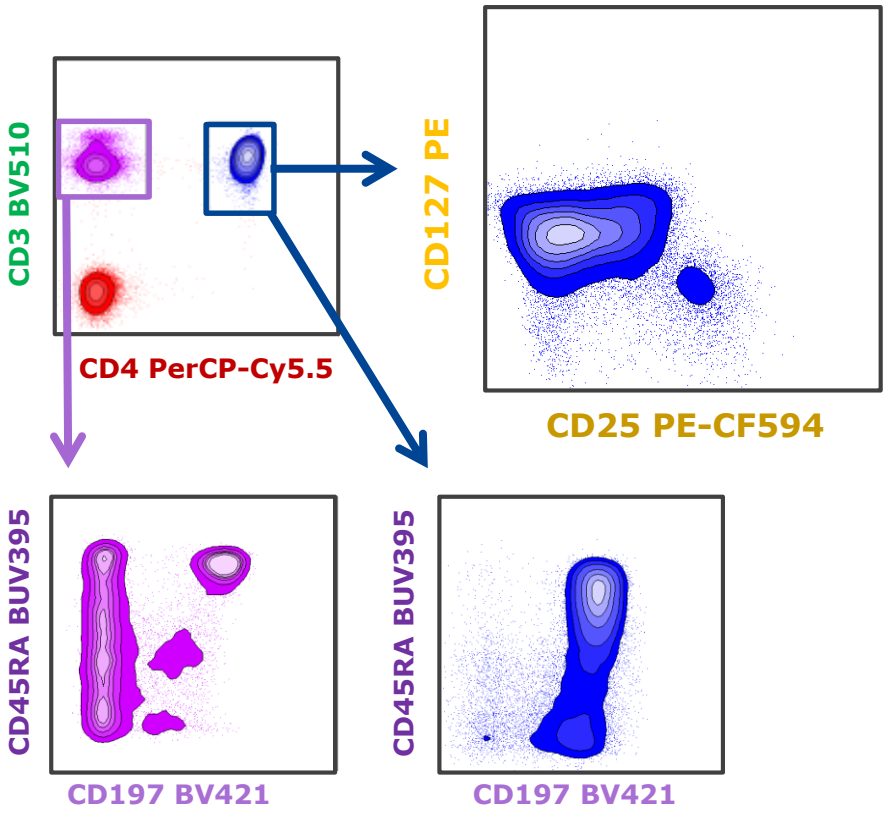


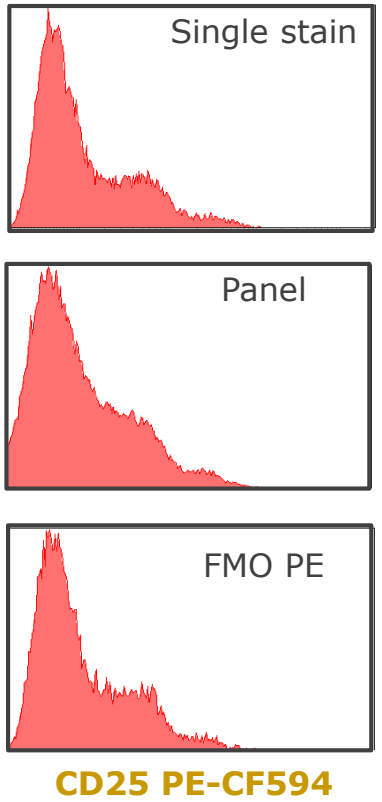


Panel 3

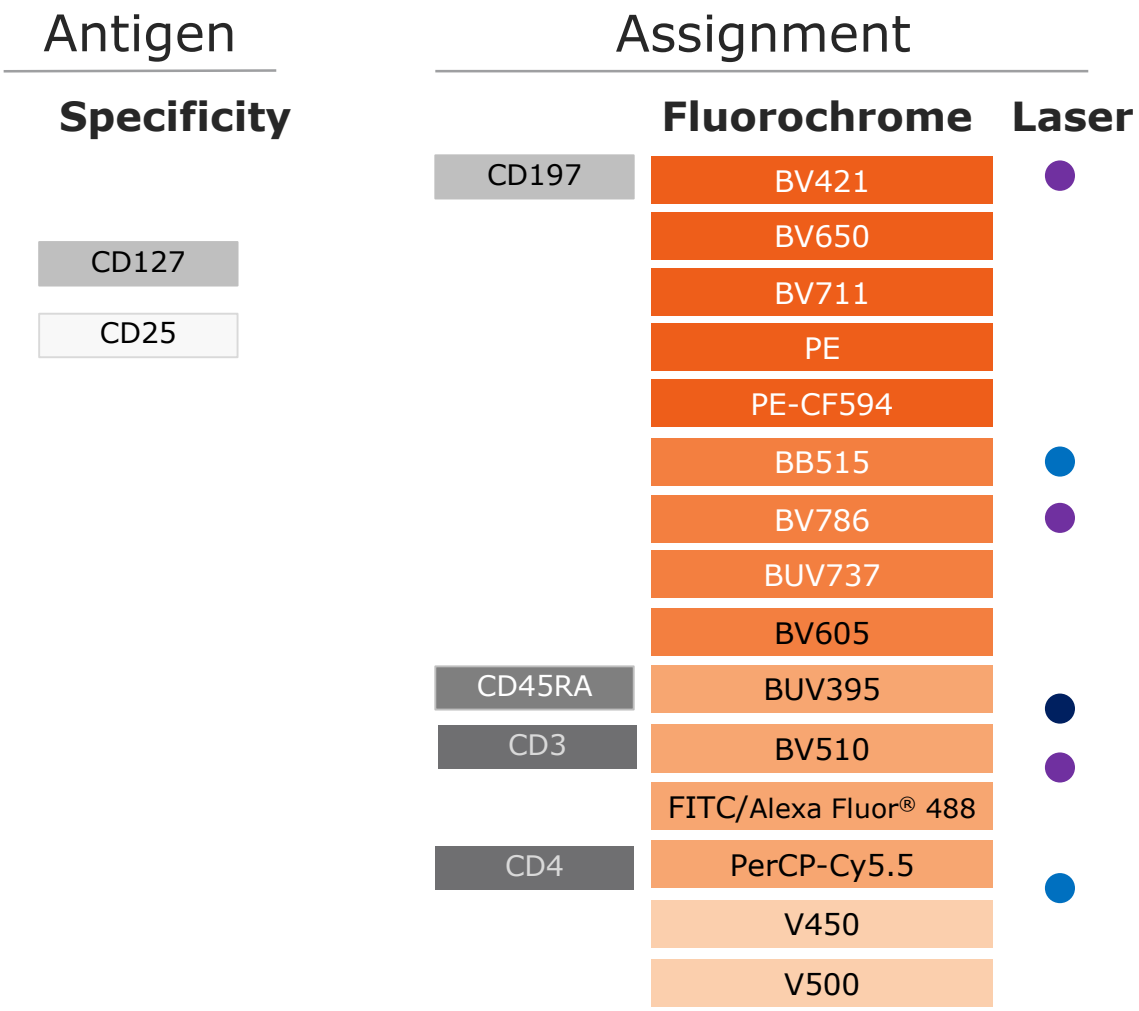
Antigen	Assignment	
Specificity	**Fluorochrome**	**Laser**
CD197	BV421	●
	BV650	
CD127	BV711	
CD25	PE	●
	PE-CF594	●
	BB515	
	BV786	
	BUV737	
	BV605	
CD45RA	BUV395	●
CD3	BV510	●
	FITC / Alexa Fluor® 488	
CD4	PerCP-Cy5.5	●
	V450	
	V500	

Panel 3 review – residual spillover

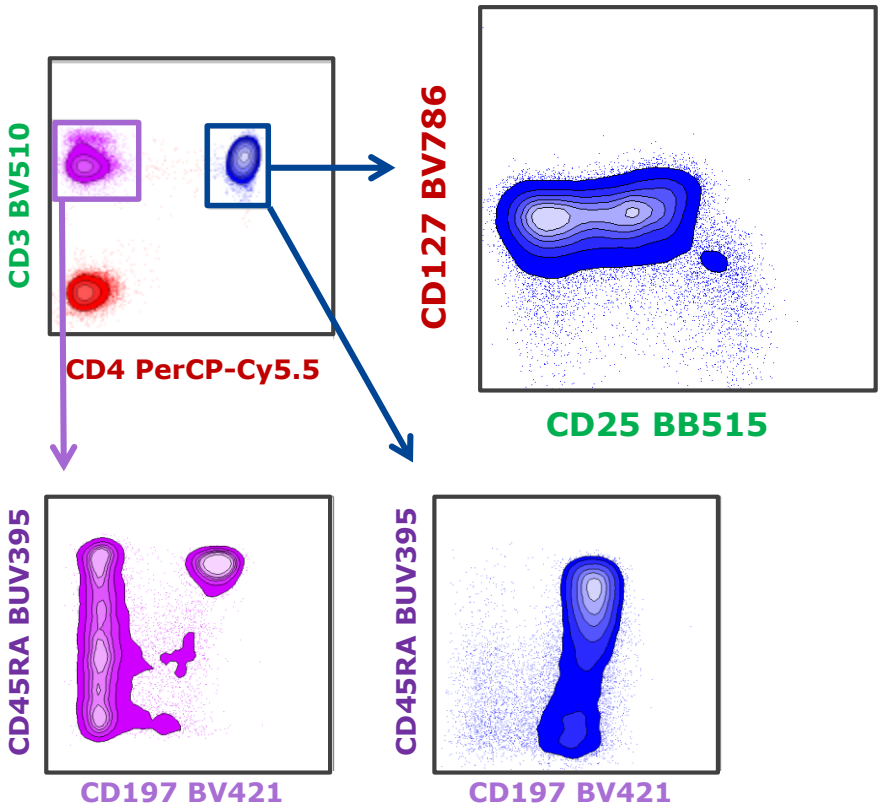


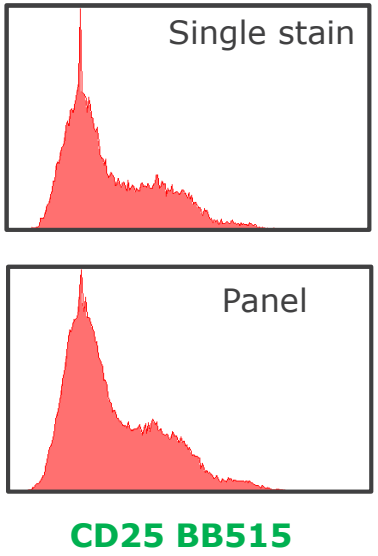


Panel 4

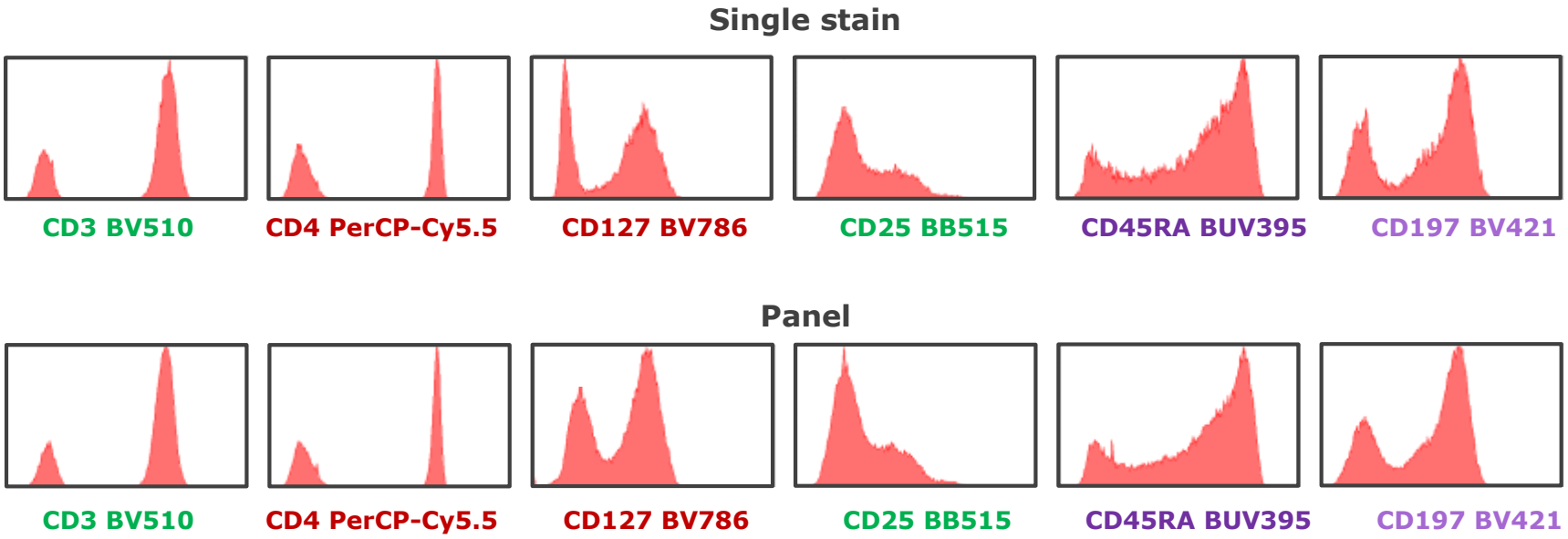

Antigen
Assignment
Specificity
Fluorochrome
Laser
CD197
BV421
BV650
CD127
BV711
CD25
PE
PE-CF594
BB515
BV786
BUV737
BV605
CD45RA
BUV395
CD3
BV510
FITC/Alexa Fluor® 488
CD4
PerCP-Cy5.5
V450
V500

Panel 4 – good choice!

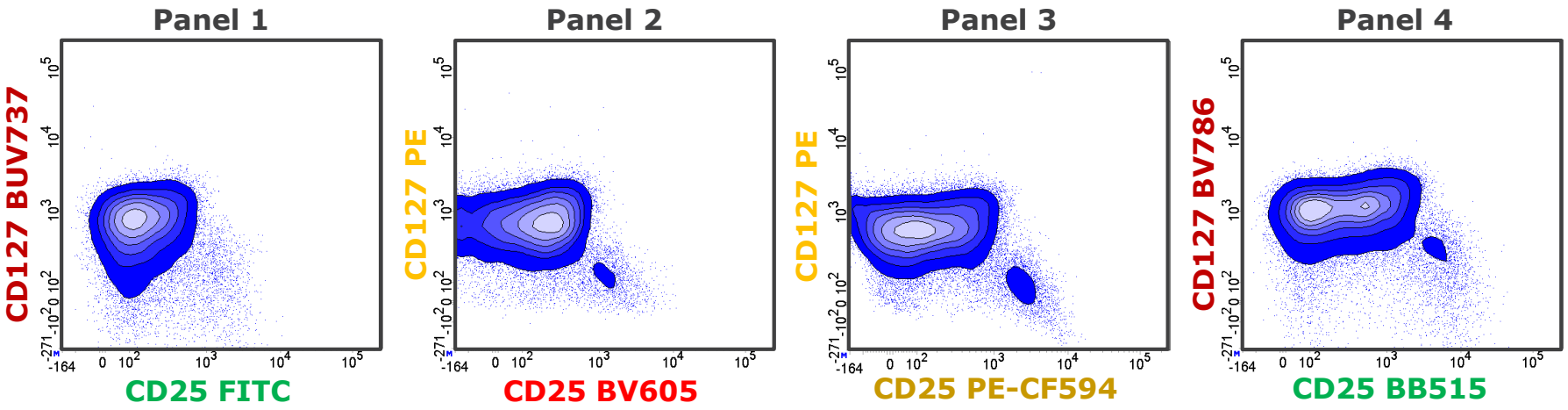




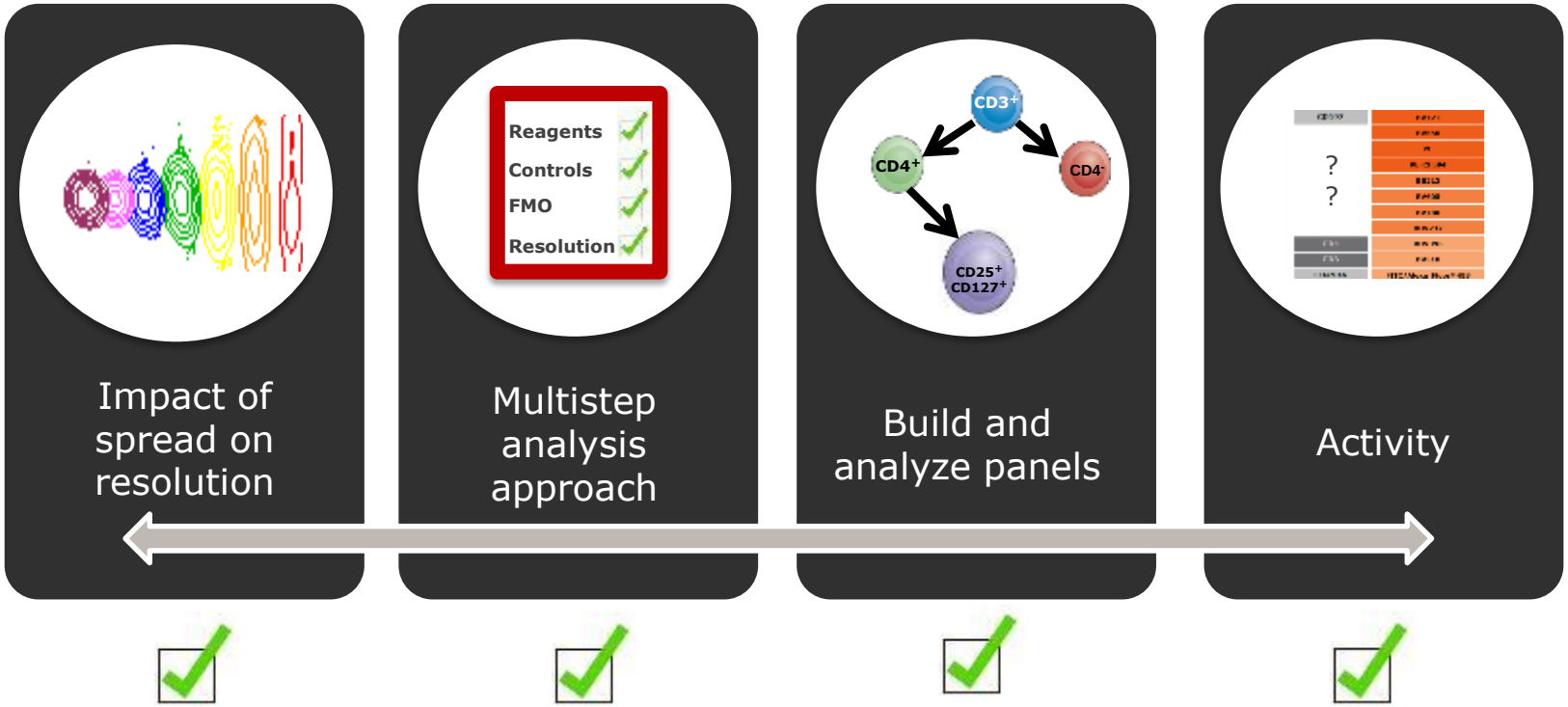
Full panel review



Activity summary



Conclusion


Reagents
Controls
FMO
Resolution
CD3+
CD4+
CD4-
CD25+
CD127+
?
?
Impact of spread on resolution
Multistep analysis approach
Build and analyze panels
Activity