Design of Multicolor Flow Cytometry Panels Incorporating BD Horizon™ Brilliant Violet™ Dyes

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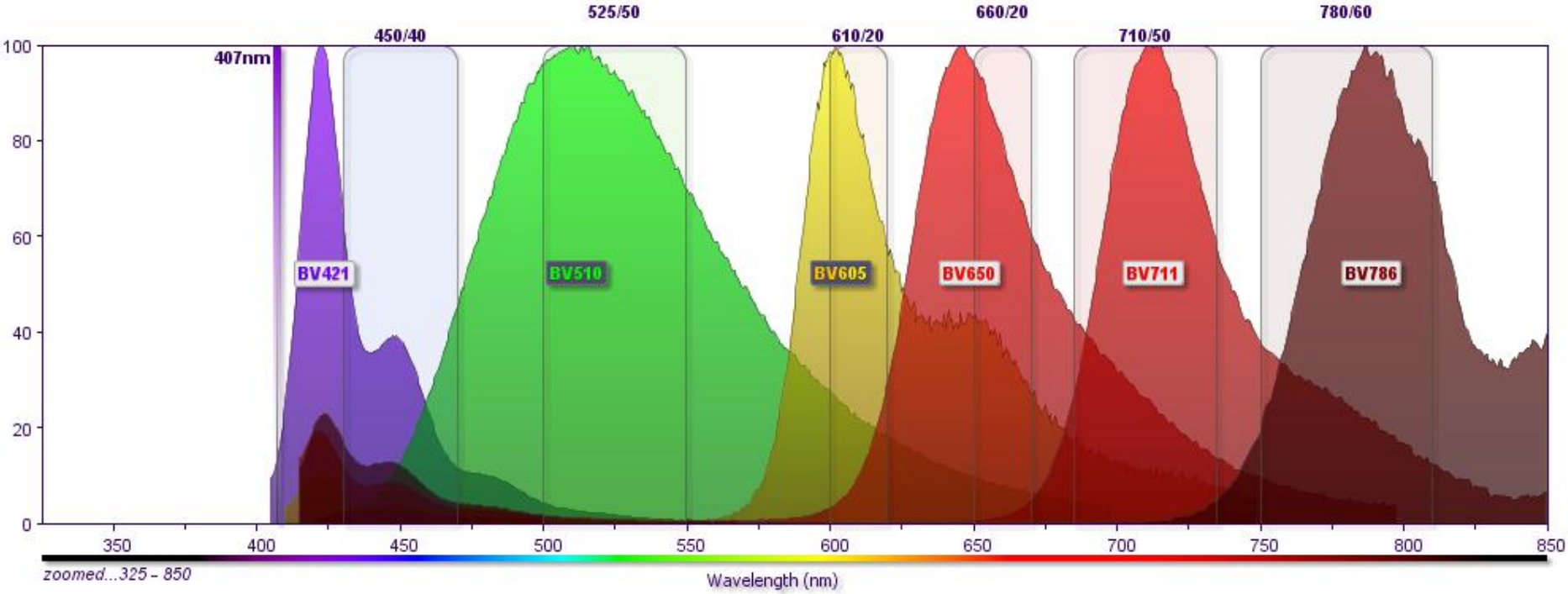
Outline

- BD Horizon™ Brilliant Violet™ fluorochrome characteristics:
  - Excitation/emission
  - Brightness
  - Spillover
  - Buffer compatibility

- New BD Horizon™ Brilliant UltraViolet™ fluorochrome
- Panel design considerations and examples

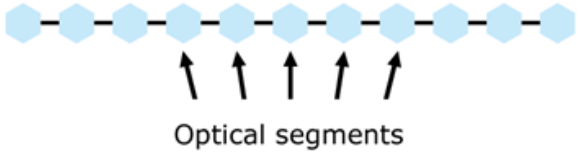
Brilliant Violet™ fluorochromes

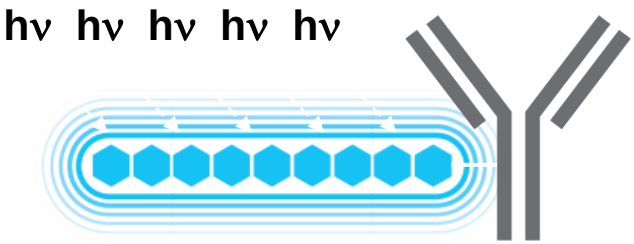


BV421 **BV510** **BV605** **BV650** **BV711** **BV786**

Sirigen polymers overview

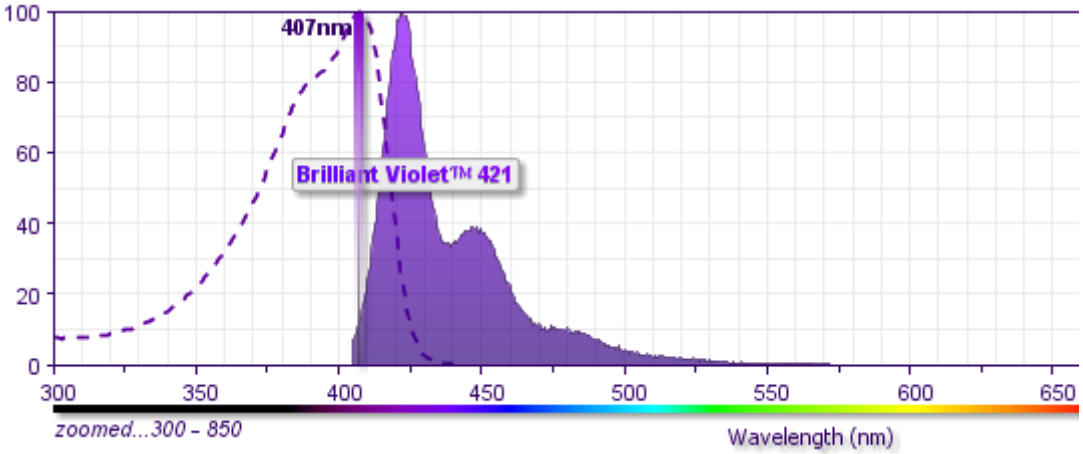
Brightness of QDot® and fluorescent proteins… with the well-defined and tunable features of dye chemistry





Direct reporters

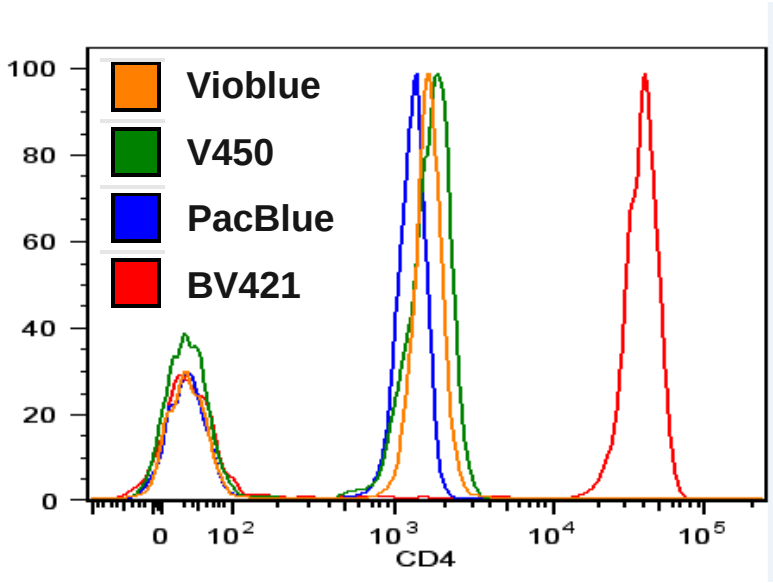
- **Many units = efficient light harvesting**
- **Properties controlled by size and composition**
- **Replaces poor performing reporters**



BD Helping all people live healthy lives

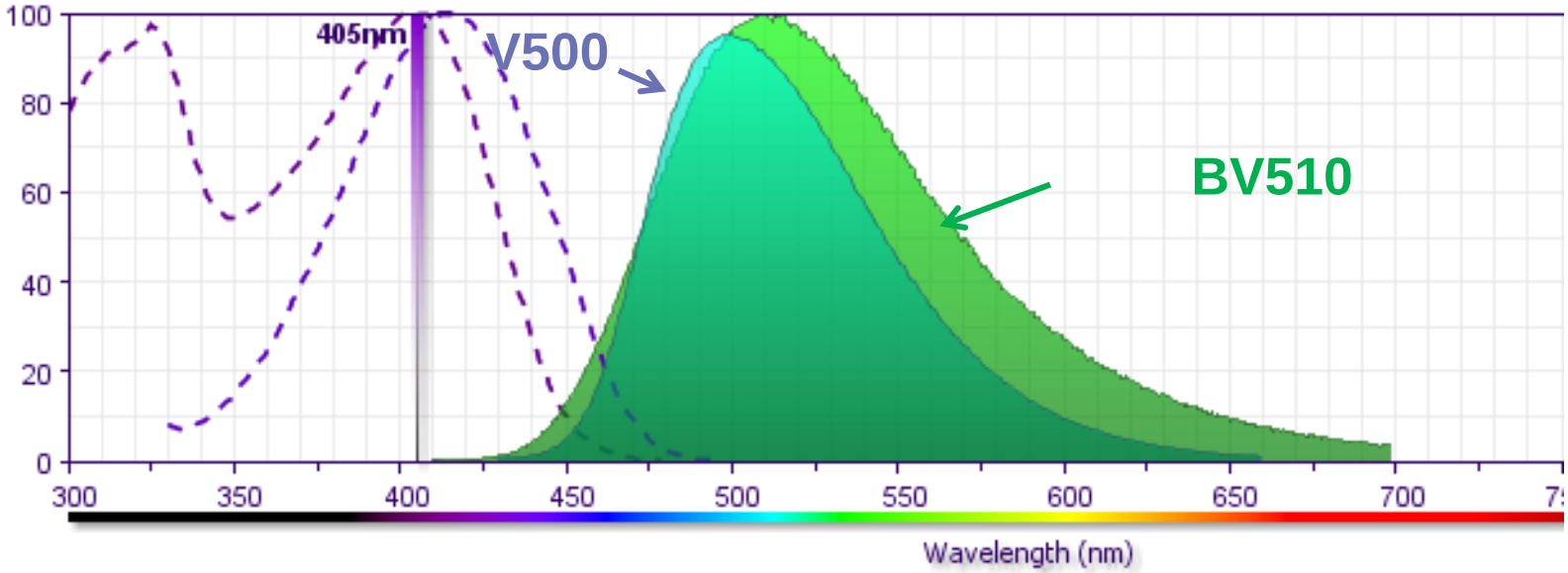
BD Horizon™ BV421: The new standard for brightness

- PE: Has been the brightest fluor available
- BV421: The new standard for brightness



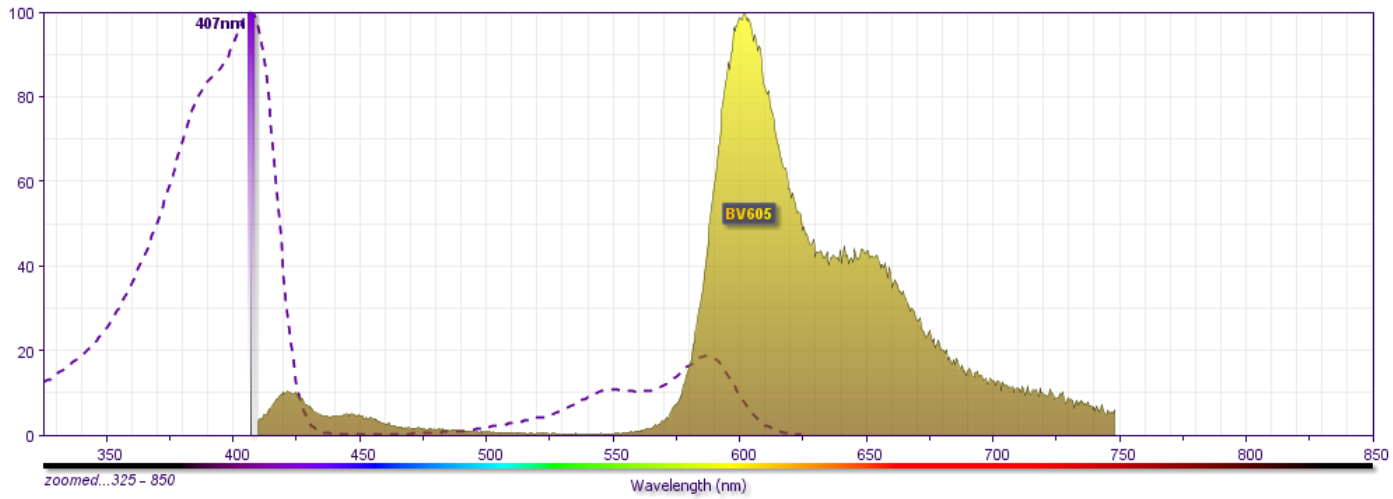
		Stain index			X-fold increase compared to:	
Spec.	µg/test	BV421	V450	PE	V450	PE
CD3	0.125	759	71	544	11	1
CD4	0.125	520	51	248	10	2
CD8	0.125	924	34	445	27	2
CD19	0.125	400	33	119	12	3
CD25	0.125	52	27	7	2	7
CD38	0.125	30	4	8	8	4
CD56	0.125	76	8	22	9	3
CD73	0.125	50		19		3
CD117	0.25	30		8		4
CD184	0.25	20		3		7
				Average	11.3	3.6

BD Horizon™ BV510 spectra



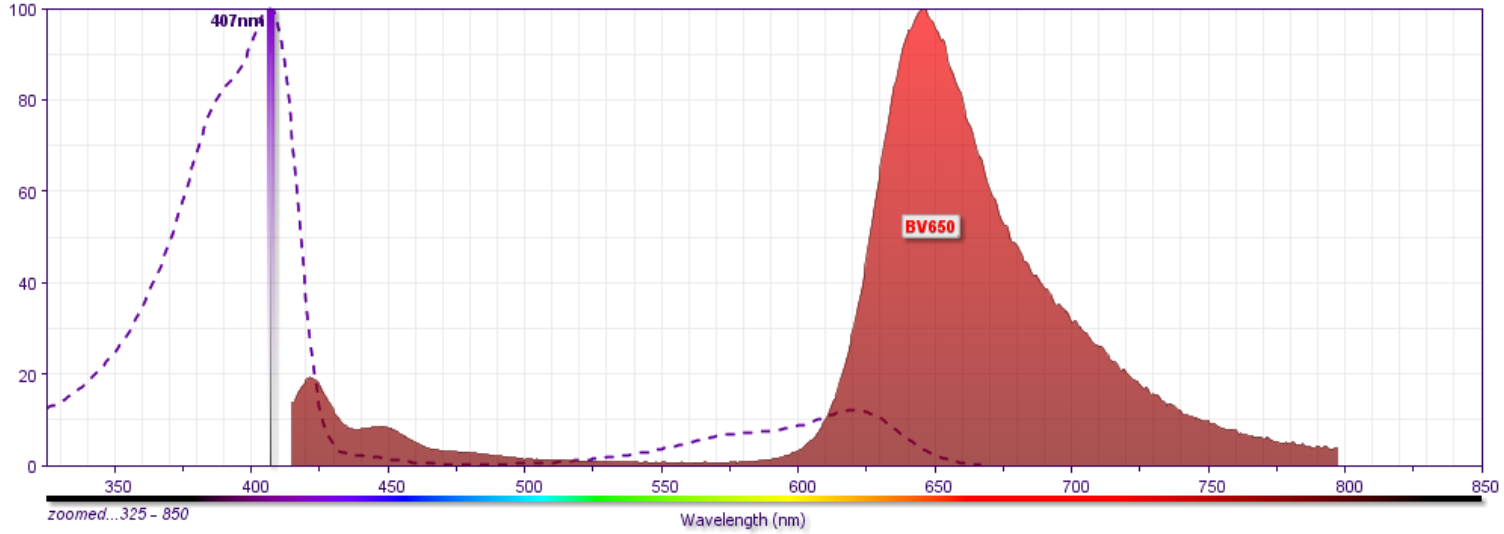
- Base polymer
- Excitation maximum at 405 nm (also has UV excitation)
- Emission maximum at 510 nm
- Use in same filter as BD Horizon™ V500/V500-C/ AmCyan: 525/50 nm

BD Horizon™ BV605 spectra



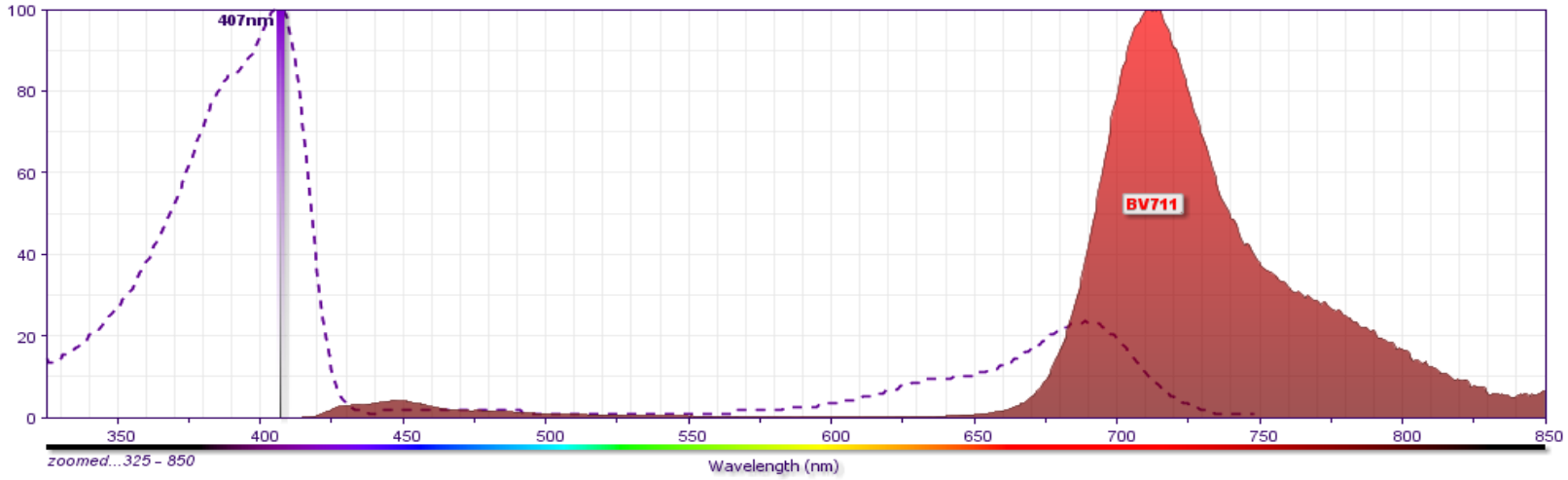
- Polymer-based tandem
  - BV421 + Acceptor Em 605
- Excitation maximum: 407 nm
- Emission maximum: 605 nm

BD Horizon™ BV650 spectra



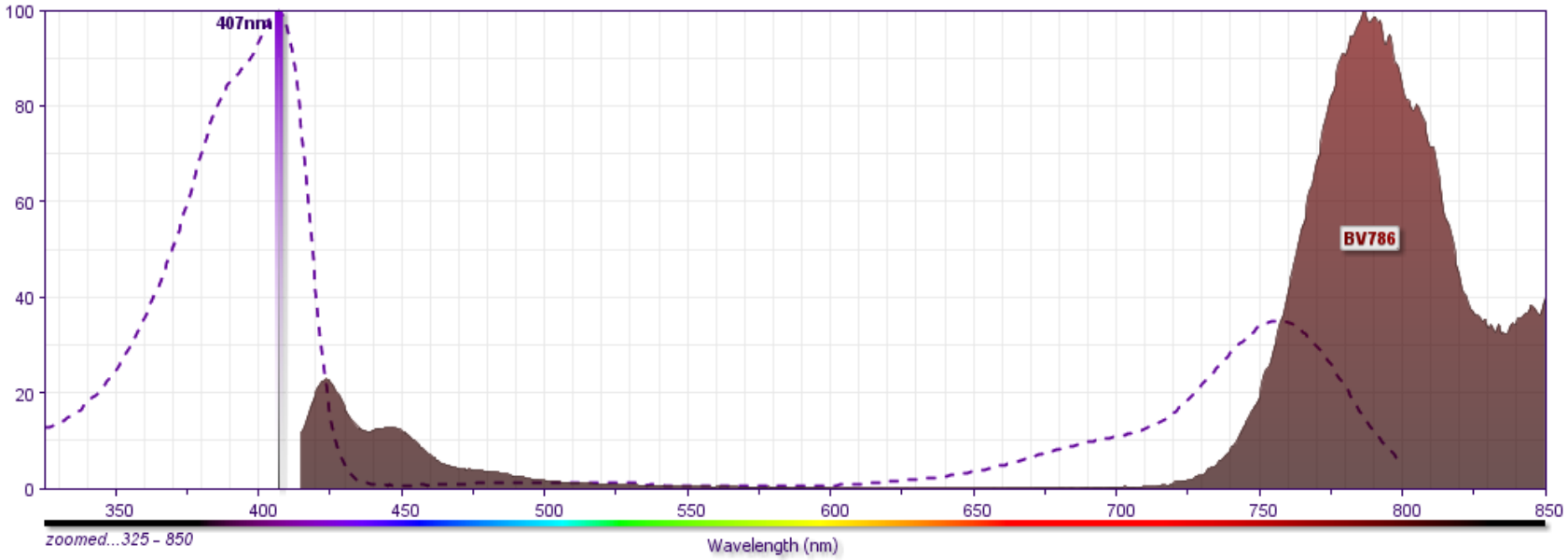
- Polymer-based tandem
  - BV421 + Acceptor Em 650
- Excitation maximum: 407 nm
- Emission maximum: 650 nm
- Recommended filter: 660/20

BD Horizon™ BV711 spectra



- Polymer-based tandem
  - BV421 + Acceptor Em 711
- Excitation maximum: 407 nm
- Emission maximum: 711 nm
- Recommended filter: 710/50

BD Horizon™ BV786 spectra



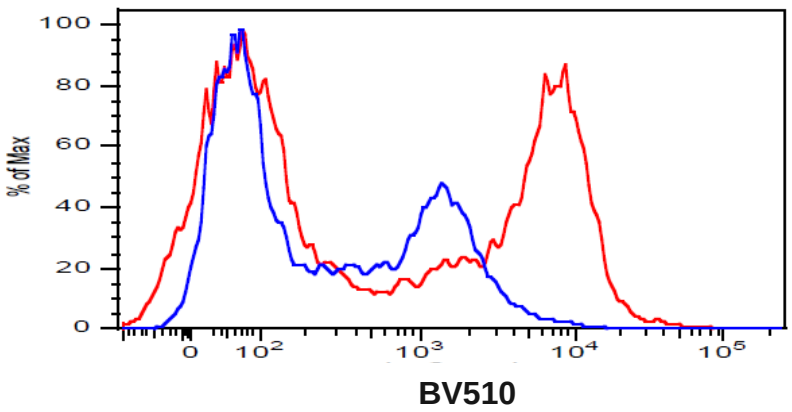
- Polymer-based tandem
  - BV421 + Acceptor Em 785
- Excitation maximum: 407 nm
- Emission maximum: 786 nm

PERFORMANCE COMPARISON TO OTHER EXISTING DYES

BV510 is brighter than V500

Reagent	Fluor	Stain index
Ms CD11b	BV510	25
	V500	10
	FITC	25
Hu CD19	BV510	49
	V500	17

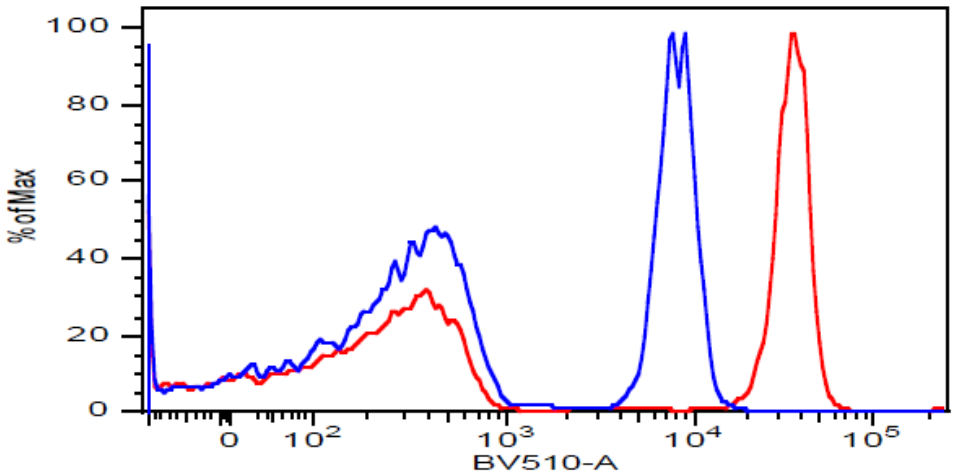
Ms CD11b staining



BV510
V500

BV510 is brighter than Qdot® 525

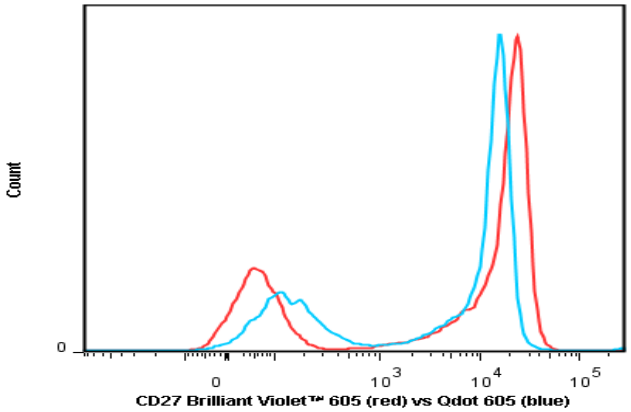
Hu CD4 Biotin + SAV

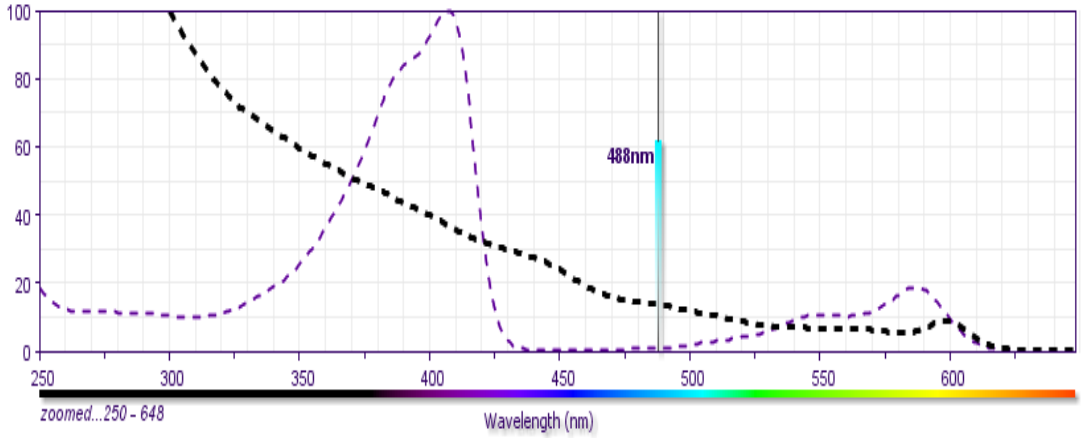


BV510

Qdot® 525

BV605 is brighter and has less spillover than Qdot® 605



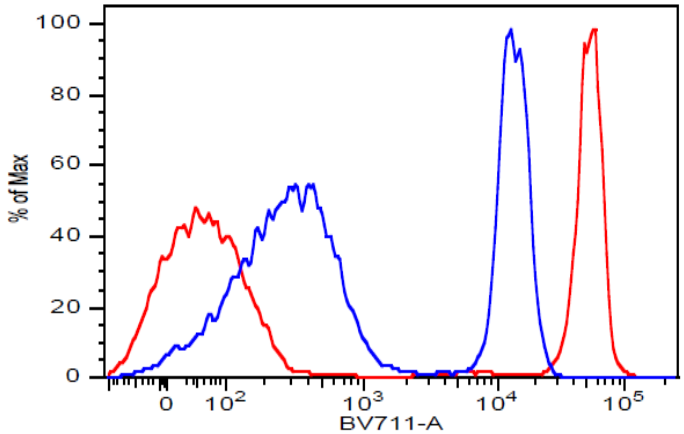


Excitation profile: Qdot® 605 Brilliant Violet™ 605

			% Spillover into Detector			
Specificity	Clone	Fluorochrome	Stain Index	BD Horizon™ 450 (450/50)	BD Horizon™ V500 (525/20)	BD Horizon™ PE-CF594 (610/20)
CD27	L128	Brilliant Violet™ 605	174	2.1%	0.3%	5.7%
	CLB-27/1	Qdot® 605	62	0.0%	0.0%	71.7%

BV711 is brighter than Qdot® 705

Hu CD4 Biotin + SAV



SAV Qdot® 705, BV711

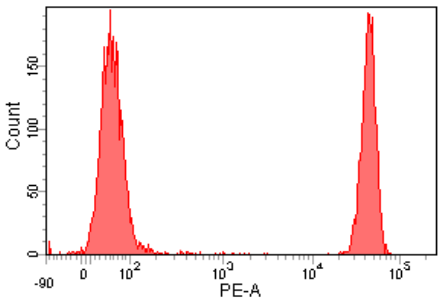
	Stain Index
CD4 BV711	744
CD4 Qdot 705	93
CD4 Bio-SA BV711	316
CD4 Bio-SA Qdot 705	24

NEW FLUOROCHROME BRIGHTNESS RANKING

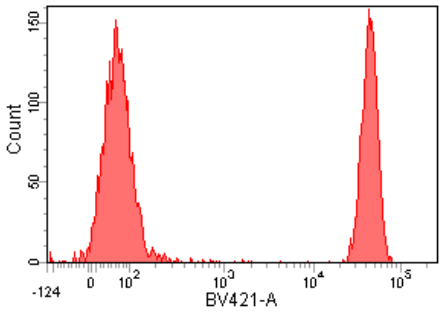
BV reagents are bright

Example: CD4 staining, human LWB

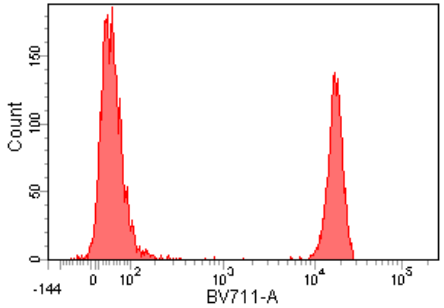
PE



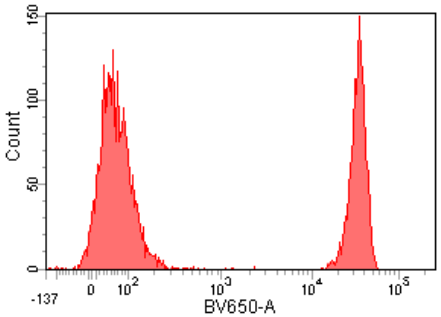
BV421



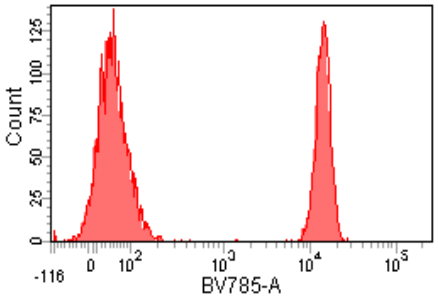
BV711



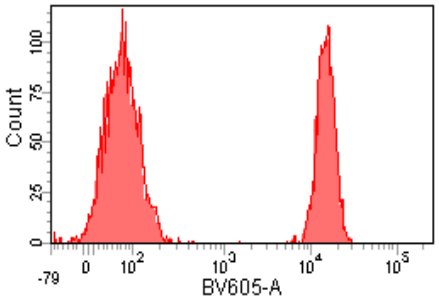
BV650



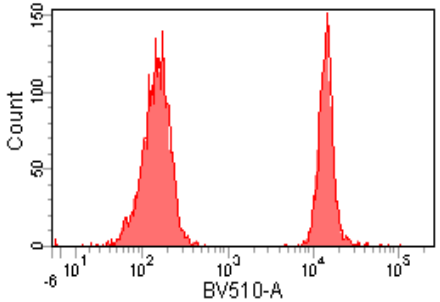
BV786



BV605



BV510



Brightness ranking: Stain index

- Stain index absolute values will depend on:
  - Reagent, clone selection
  - Instrument configuration (laser wavelength, laser power, filters)
  - Instrument setup

- Ideally, ranking should be established for each specific instrument

Brightness ranking

BRIGHT

BV421, BV650, BV711, BV786

PE, PE-CF594, PE-Cy™7

APC

MODERATE

BV605, BV510

FITC, Alexa Fluor® 488,

PerCP-Cy™5.5

Alexa Fluor® 647,

Alexa Fluor® 700

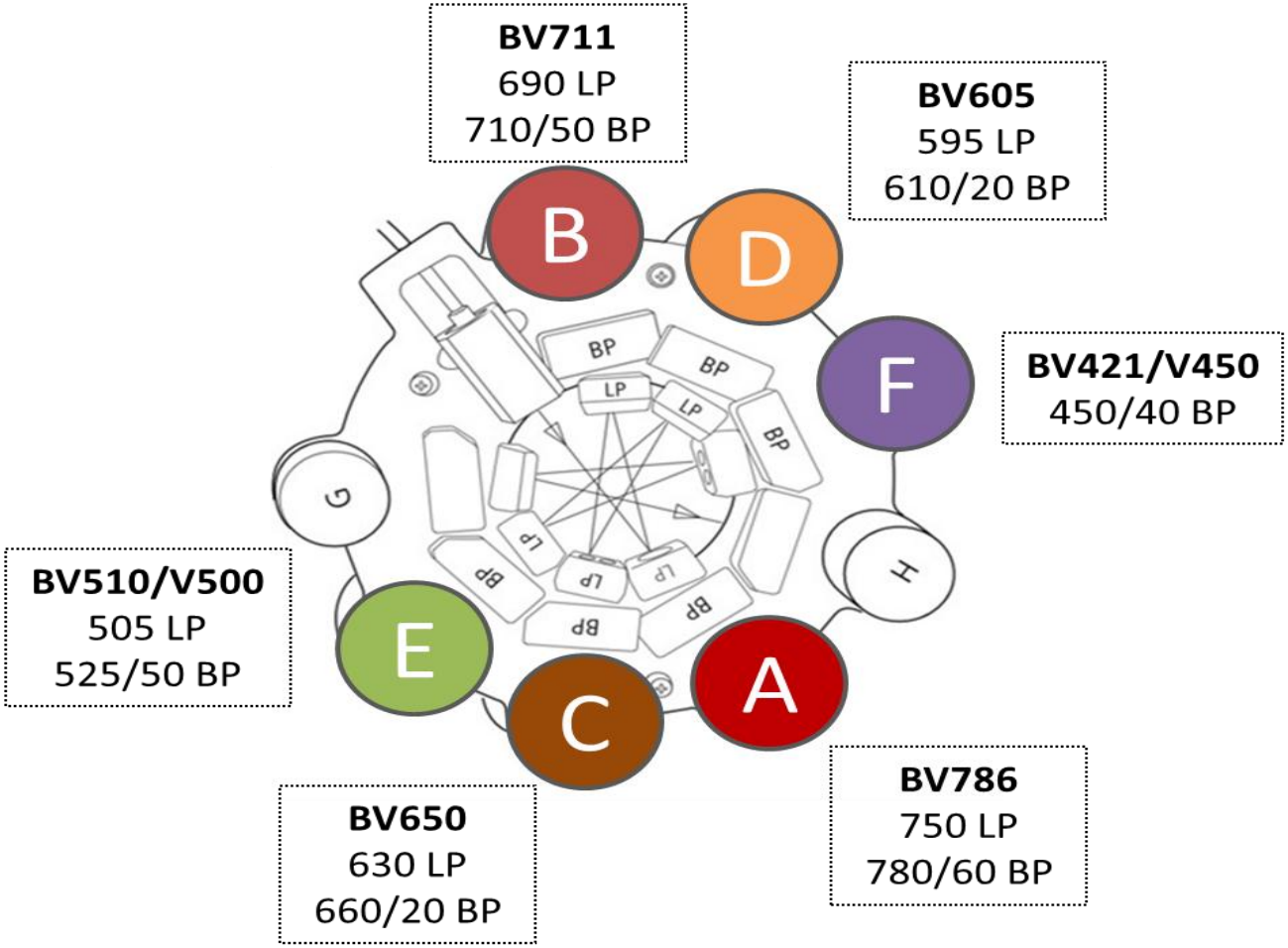
DIM

V450, V500, AmCyan

APC-Cy7/APC-H7

INSTRUMENT CONSIDERATIONS

Violet laser configuration



Example of filter selection: BV711

- Main spillover is from BV650

MFI measurements

Fluor/filter	Into BV711
BV650 695/40	6,850
BV650 710/50	9,933
BV650 712/21	4,096
BV711 695/40	7,786
BV711 710/50	14,438
BV711 712/21	7,286

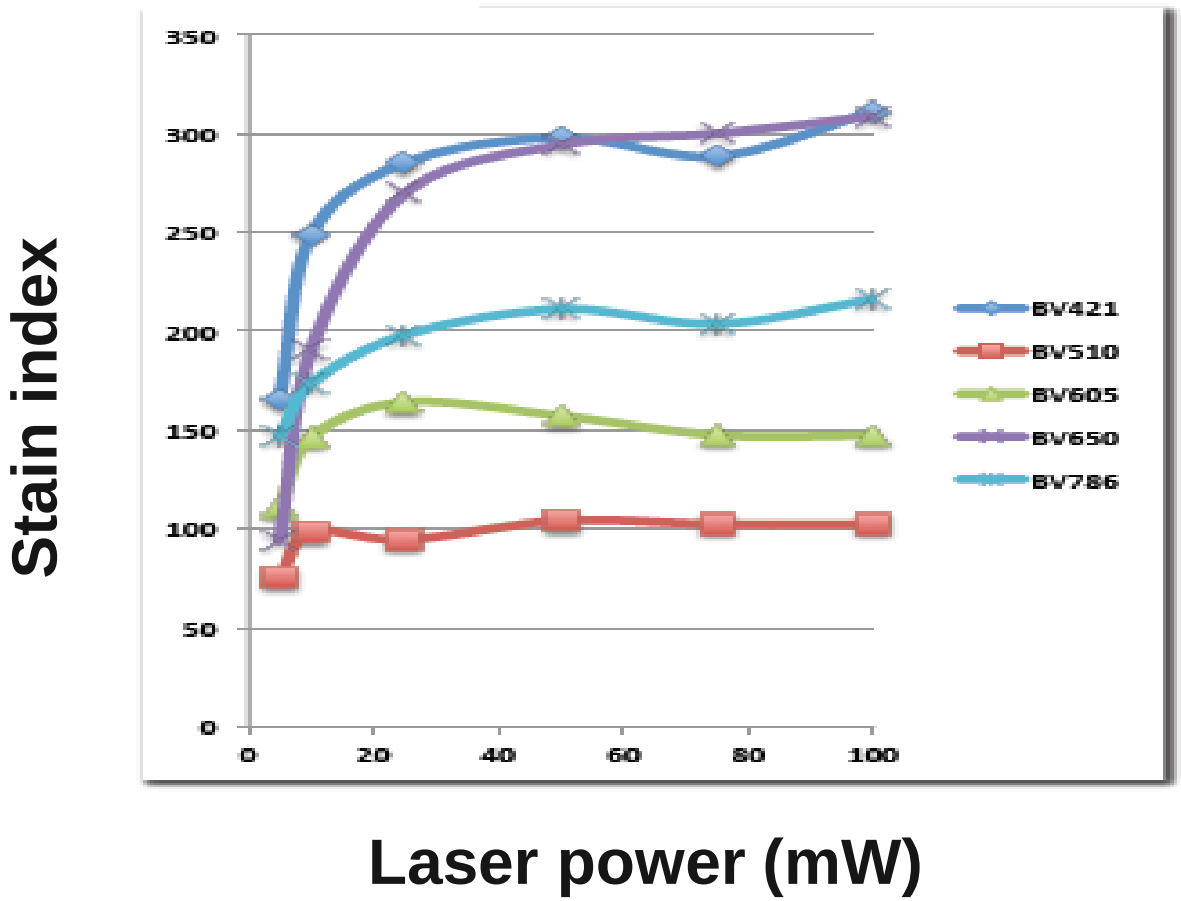
Stain index CD4 SK3 clone

Reagent	Filter	Stain index
CD4 BV711	695/40	228.47
	710/50	313.17
	712/21	242.53

SOV

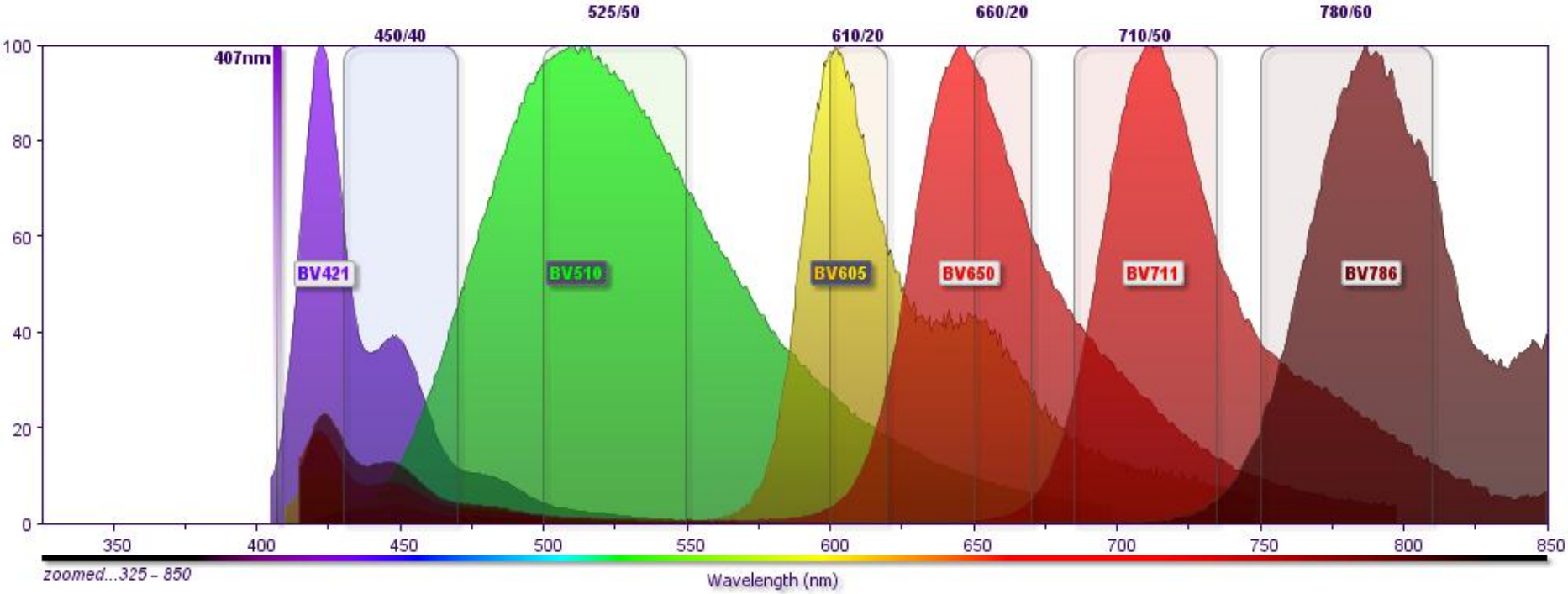
Filter	SOV 650/711
695/40	0.880
710/50	0.688
712/21	0.562

Laser power and BV reagents



SPILLOVER

Spillover between BV reagents (1)



BV421 **BV510** **BV605** **BV650** **BV711** **BV786**

Spillover between BV reagents (2)

Fluor/detector	BV421	BV510	BV605	BV650	BV711	BV786
BV421	100.00	**23.69**	1.78	0.28	0.26	0.28
BV510	3.52	100.00	**29.99**	5.60	3.45	1.50
BV605	3.02	1.96	100.00	**24.66**	13.31	4.97
BV650	4.11	2.06	**25.41**	100.00	**57.93**	16.29
BV711	4.08	1.93	0.80	1.10	100.00	**47.95**
BV786	3.72	1.98	1.05	0.31	2.31	100.00

Spillover calculated as ratio of MFI of secondary fluor in detector x to MFI of primary fluor in its own detector

Spillover to other detectors

- BV605 into PE, PE-CF594 (if using the yellow-green laser)
- BV650 and BV711 into PerCP-Cy5.5, Alexa Fluor® 700
- BV786 into APC-H7

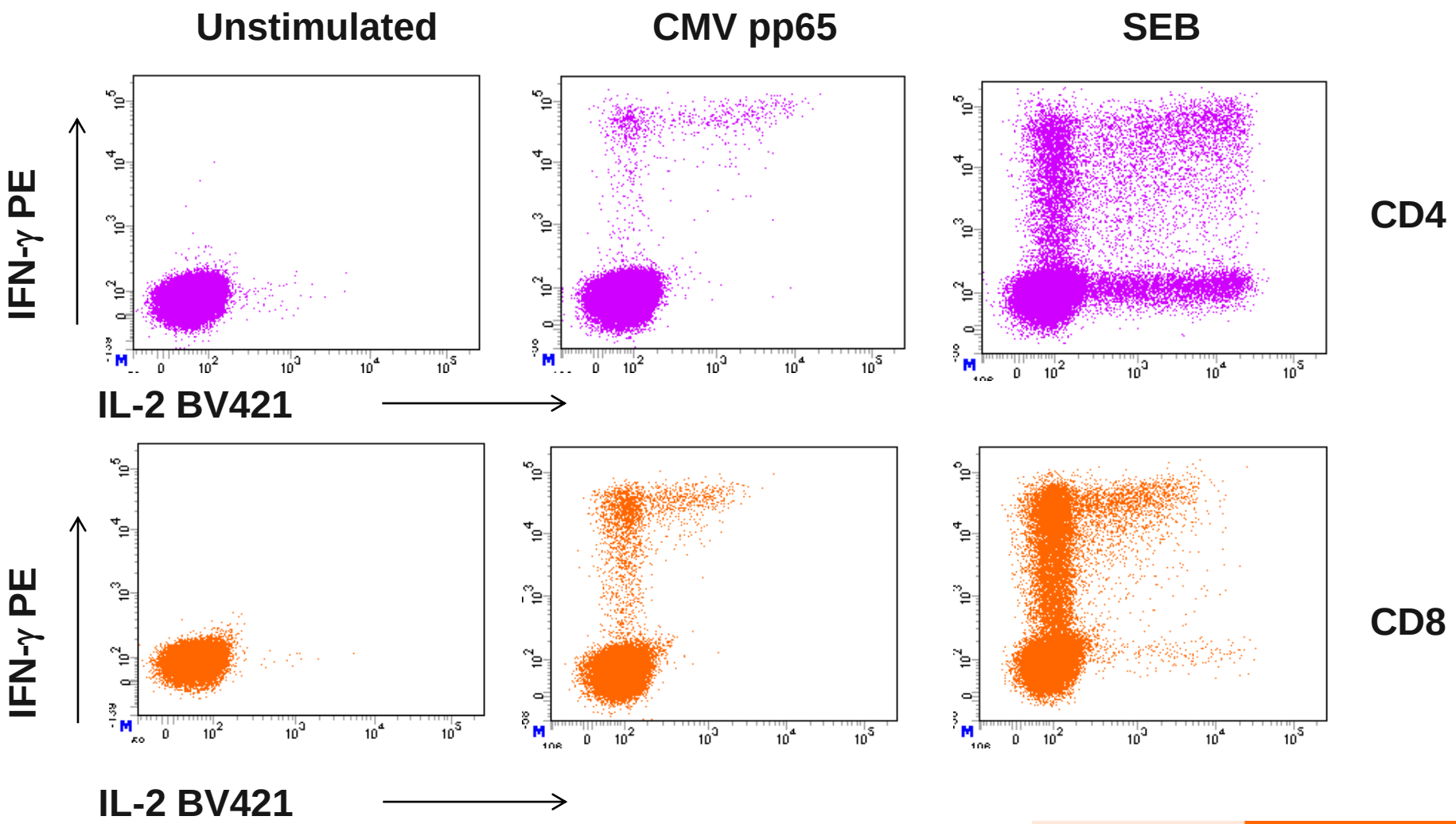
BUFFER COMPATIBILITY

Buffer compatibility (1)

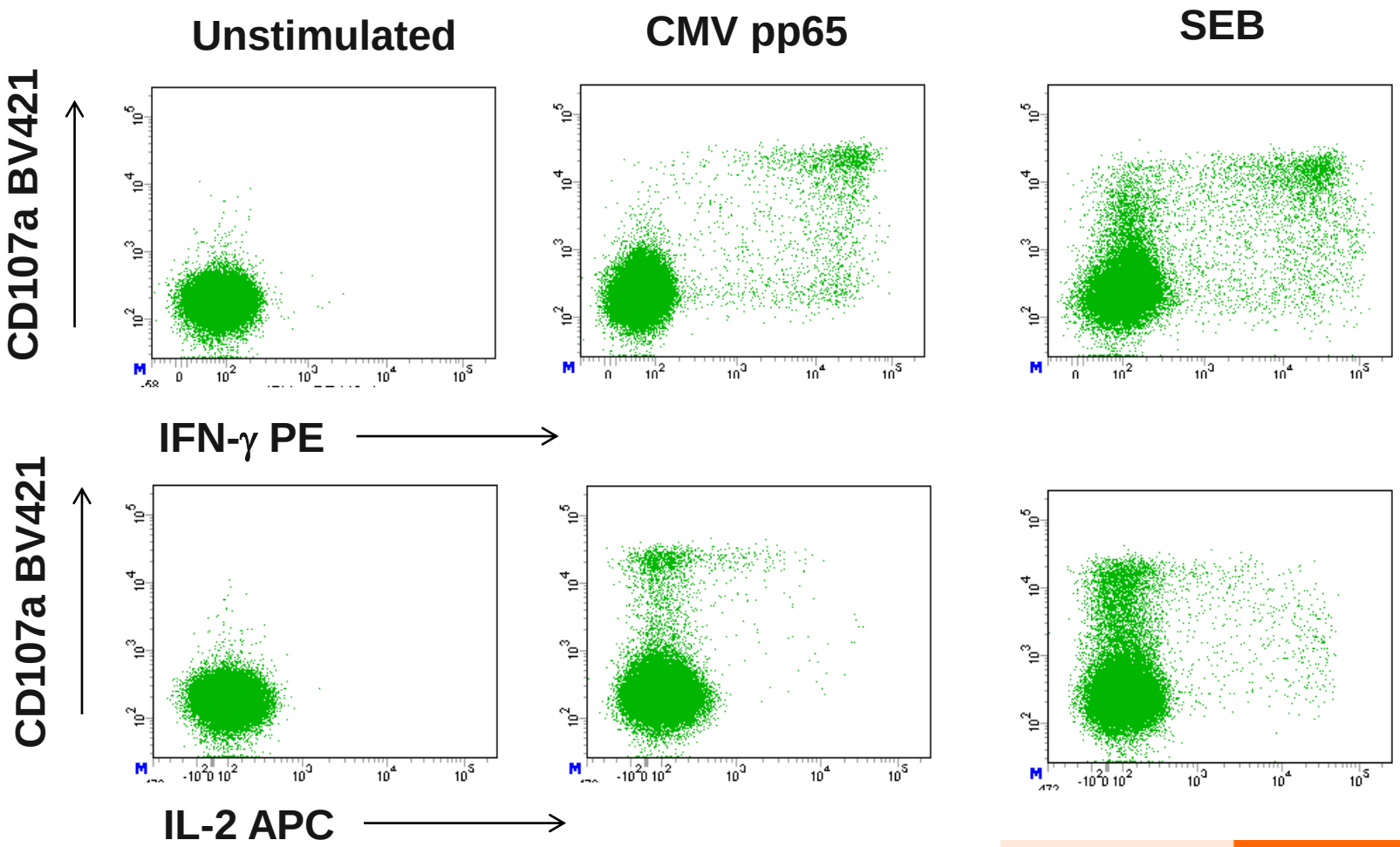
Brilliant Violet reagents are compatible with:

- EDTA and heparin blood collection tubes
- BD Pharm Lyse™ lysing buffer and BD FACS™ lysing solution
- PFA-based fixatives
- BD Cytofix™ fixation buffer/BD Perm/Wash™ buffer
- BD Pharmingen™ Transcription Factor Buffer Set
- BD Phosflow™ Perm Buffer III

Buffer compatibility (2): Intracellular staining

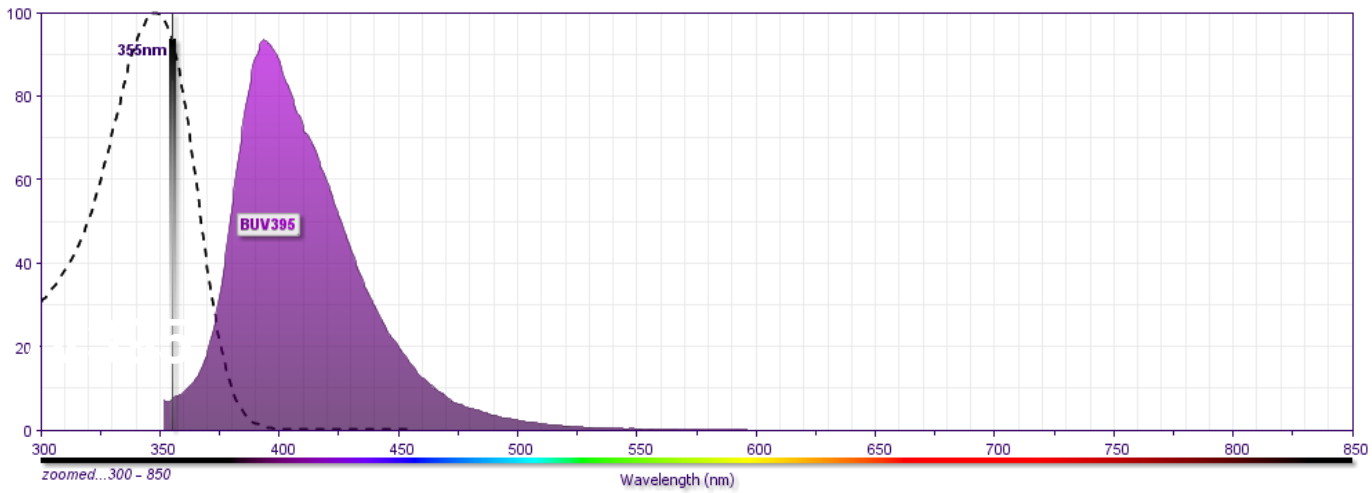


BV421 does not alter cell functionality: CD107a staining



NEW UV LASER FLUOROCHROME

BUV395 spectra

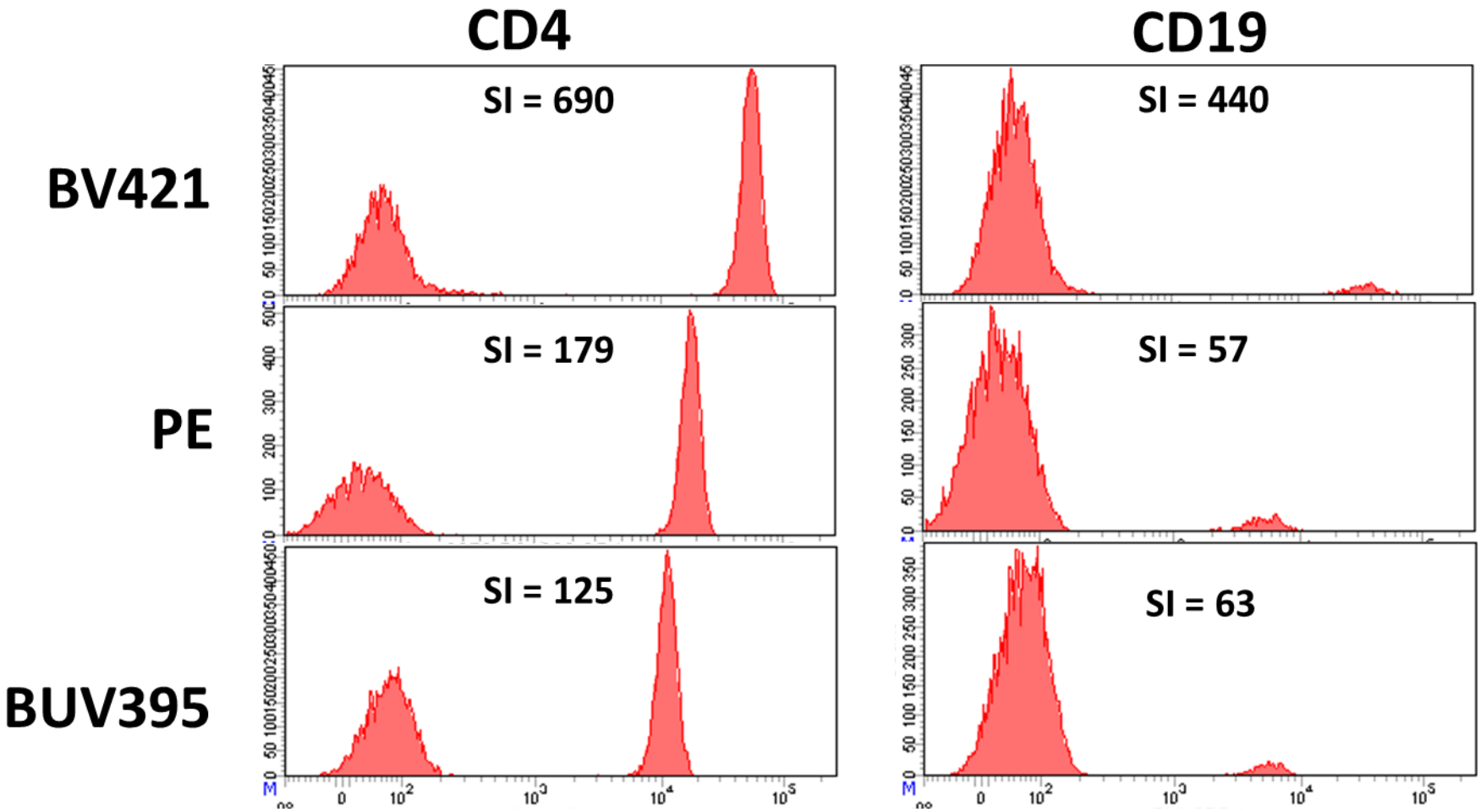


BUV395

BUV395

- Excitation maximum of 347 nm, optimum for a 355-nm ultraviolet laser.
- No significant excitation by a 405, 488, 532, 561, or 640-nm laser.
- Emission maximum of 395 nm.

BUV395 brightness



BUV395 spillover

Laser		BUV395 SOV into other chanels					
Violet		BV421	BV510	BV605	BV660	BV711	BV785
	BUV395	0.2%	0.1%	0.0%	0.0%	0.0%	0.0%
Blue		FITC	PE	PE-CF594	PE-Cy5	PerCP-Cy5-5	PE-Cy7
	BUV395	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Red					APC	AlexaFluor 700	APC-Cy7
	BUV395				0.0%	0.0%	0.0%

PANEL DESIGN

Requisites for successful multicolor applications

A. Careful reagent selection

B. Optimal sample preparation and staining conditions

C. Proper cytometer performance, setup, and data collection

D. Proper data analysis

Typical challenges

- Some markers are highly expressed, others are expressed at low levels.
- Some dyes are much brighter than others.
- Significant emission spillover from non-primary fluorescent reagents contributes to optical background, which can often diminish the resolution of dim markers (due to spread after compensation).
- Some markers may be available only in certain colors.

Principles of panel design

1. Check for reagent availability/clone selection.

2. **Match fluorochromes by brightness (values from stain index) according to antigen density and distribution (published values or TDS).**

3. **Evaluate co-expression. Minimize spectral overlap impact on sensitivity/resolution.**

4. Run appropriate controls.

Antigen/fluorochrome combos (1)

- Classify the antigens you would like to measure[1]

  Primary: Well characterized, easily classified positive or negative (CD45, CD3, CD4, etc).

  Secondary: Well characterized, also expressed at a higher density, often over a continuum (CD27, CD28, CD45RA/RO).

  Tertiary: Expressed at low levels only (CD25), also uncharacterized antigens.

- Use the brighter fluorochromes for dimly expressed markers.
- Use the dimmer fluorochromes for more highly expressed markers.

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

Antigen/fluorochrome combos (2)

Antigen Group	Tertiary	Secondary	Primary
	BRIGHT	**MODERATE**	**DIM**
	BV421, BV650, BV711, BV786	BUV395	V450, V500, AmCyan
	PE, PE-CF594, PE-Cy7	BV605, BV510	APC-Cy7/APC-H7
	APC	FITC, Alexa Fluor® 488, PerCP-Cy5.5	
		Alexa Fluor® 647, Alexa Fluor® 700	

Spillover

- Characterize spillovers in your own instrument (differences if using blue vs yellow-green laser, filter set, etc.)

- Think in terms of spillover categories:
  - Fluors excited by the same laser
  - Cross-laser (fluor excited by more than one laser)
  - Tandem dyes into primary detector (eg, PE-Cy7 into PE)

Example spillover matrix: 14 colors

Detector			Fluorochrome (spillover into)													
Laser	Filter	Parameter	BUV395	BV421	BV510	BV605	BV711	BV786	FITC	PerCP-Cy5.5	PE	PE-CF594	PE-Cy7	APC	Alexa 700	APC-H7
UV		BUV395		0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Violet		BV421	0.6		4.1	3.8	2.5	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		BV510	0.0	20.4		0.9	1.1	1.9	5.2	0.0	0.1	0.1	0.0	0.0	0.0	0.0
		BV605	0.1	1.0	12.0		0.1	0.3	0.6	0.0	2.5	12.0	0.1	0.0	0.0	0.0
		BV711	0.0	0.1	1.7	24.6		2.6	0.1	75.0	0.4	3.4	0.1	1.0	7.0	0.2
		BV786	0.1	0.0	0.3	4.7	27.0		0.0	10.0	0.0	0.6	4.9	0.6	2.2	11.0
Blue		FITC	0.1	0.0	4.0	0.0	0.0	0.0		0.0	0.8	0.2	0.1	0.0	0.1	0.0
		PerCP-Cy5.5	0.0	0.0	0.2	3.4	0.0	0.0	3.5		7.9	64.0	0.2	1.8	0.1	0.1
Yellow-Green		PE	2.0	0.1	0.4	10.9	0.1	0.1	0.2	1.0		28.0	2.1	0.1	0.4	0.5
		PE-CF594	0.5	0.0	0.1	16.1	0.0	0.0	0.0	0.3	19.2		0.5	0.1	0.1	0.2
		PE-Cy7	0.0	0.0	0.0	2.1	1.4	2.2	0.0	14.7	1.0	11.6		3.1	5.5	52.3
Red		APC	0.0	0.0	0.0	0.2	1.3	0.1	0.0	20.4	0.0	0.4	0.0		2.9	5.3
		Alexa 700	0.0	0.0	0.0	0.1	24.0	1.0	0.0	22.0	0.0	0.1	0.4	34.4		10.6
		APC-H7	0.0	0.0	0.0	0.0	4.0	4.8	0.0	4.4	0.0	0.0	2.5	4.8	13.9	

Spillover is					
< 0.5%	< 3%	< 10%	< 20%	< 30%	> 30%

5 FLUOROCHROMES, MINIMAL COMPENSATION

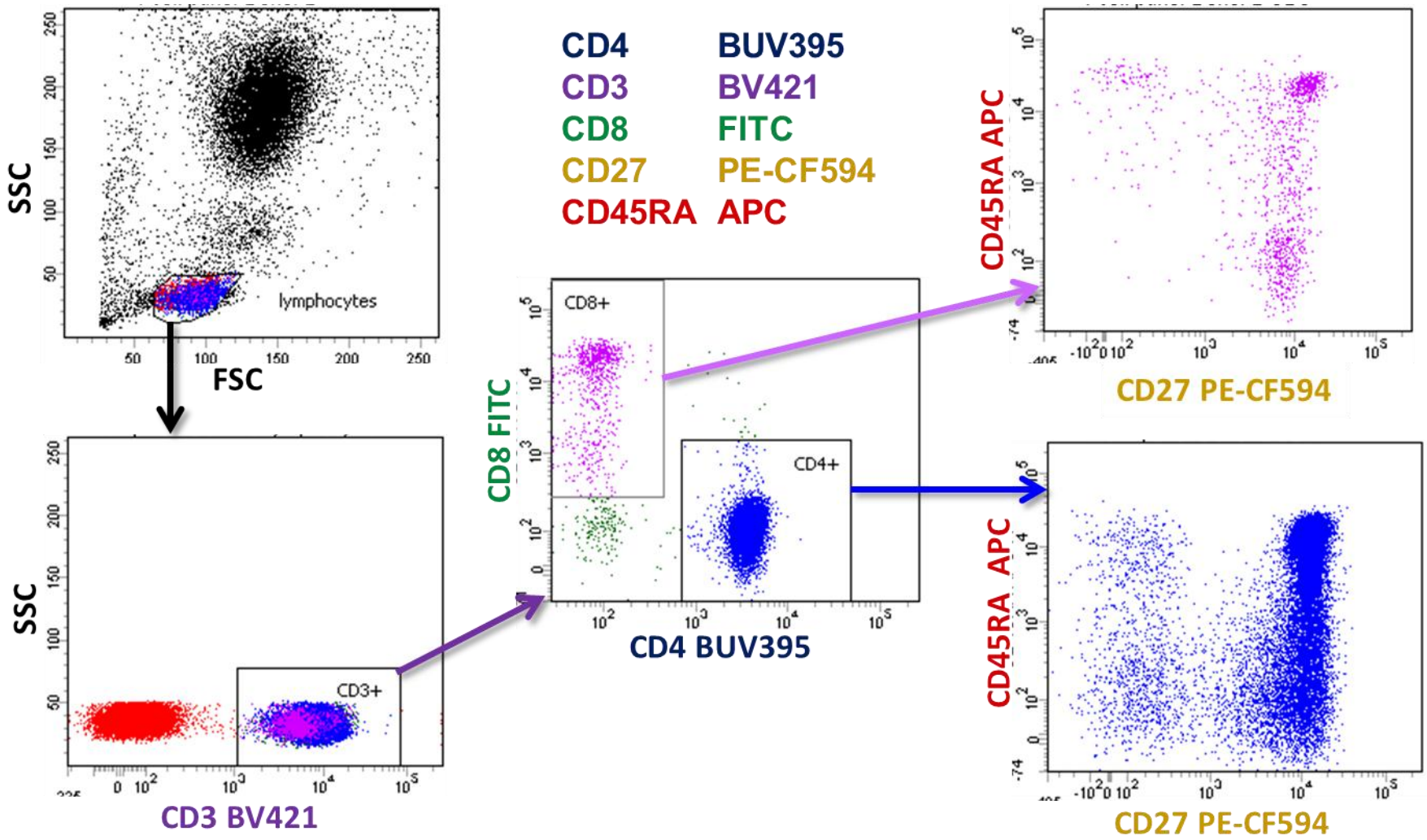
5 fluors, minimal compensation

- We have now developed 5 fluorochromes with:
  - Moderate-to-high brightness
  - Minimal or NO spillover between them

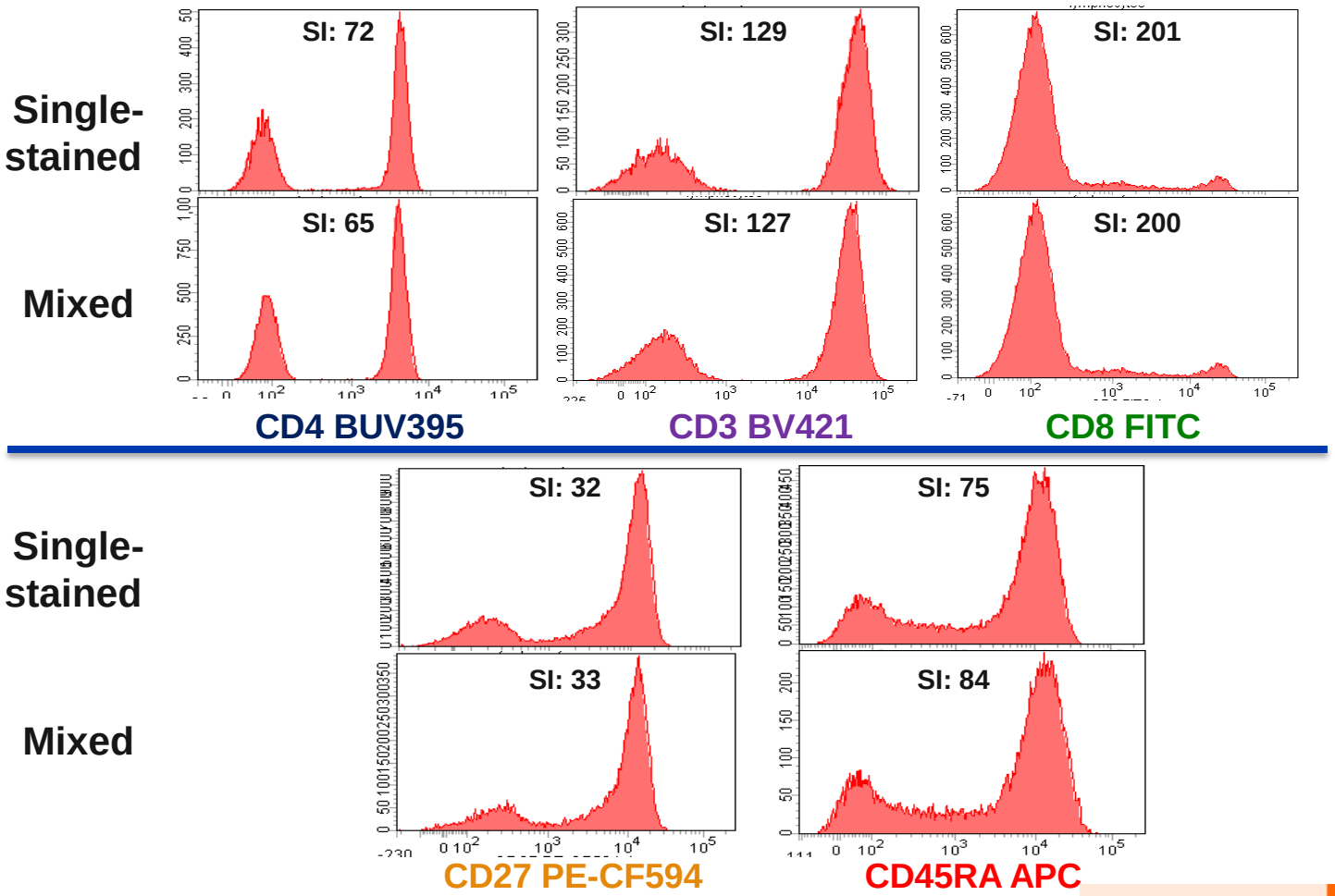
- Prerequisite: 5-laser flow cytometer

		Fluor (Spillover into)				
		BUV395	BV421	FITC	PE-CF594	APC
Channel	BUV395		0.16	0.01	0.00	0.00
	BV421	0.29		0.00	0.03	0.00
	FITC	0.00	0.11		0.23	0.00
	PE-CF594	0.00	0.04	0.00		0.16
	APC	0.57	0.00	0.00	0.32	

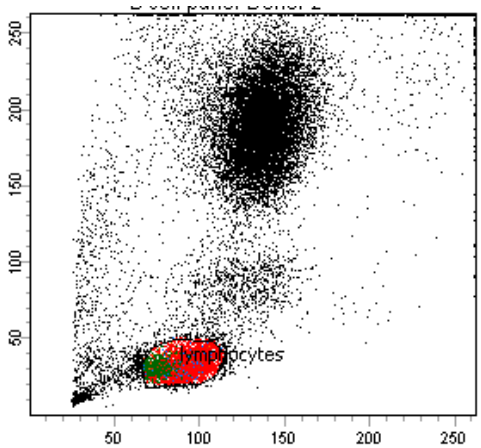
Example: 5-color T-cell panel

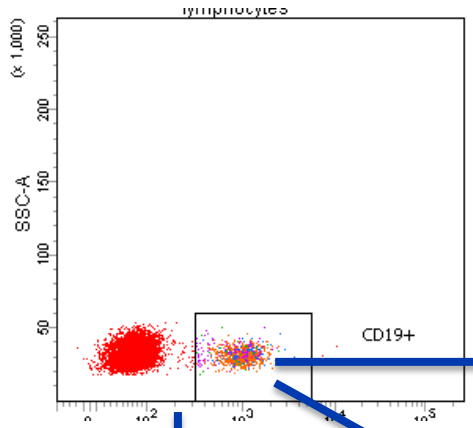


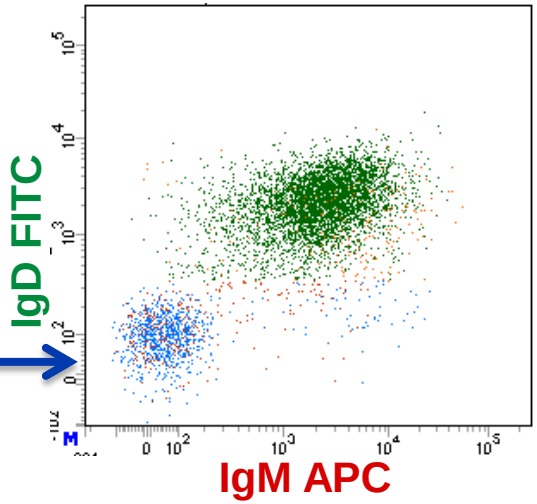
No compensation, optimal resolution



5-color B-cell panel

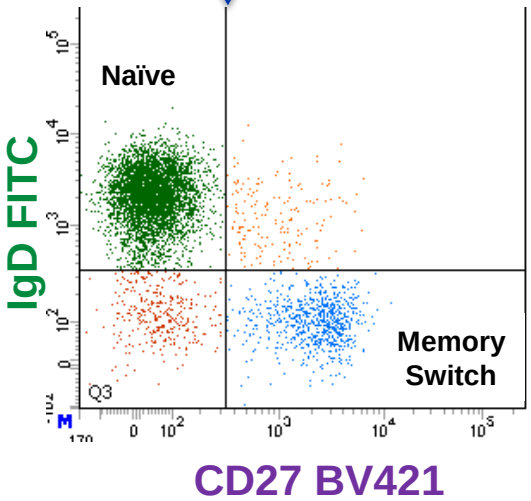


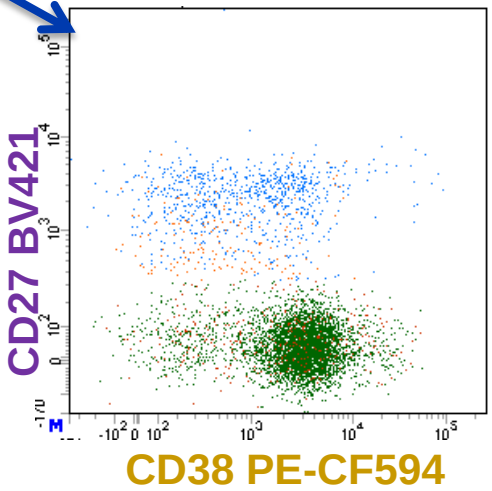




CD19 BUV395

CD19	BUV395
CD27	BV421
IgD	FITC
CD38	PE-CF594
IgM	APC





14-COLOR LEUCOCYTE SUBSETTING PANEL

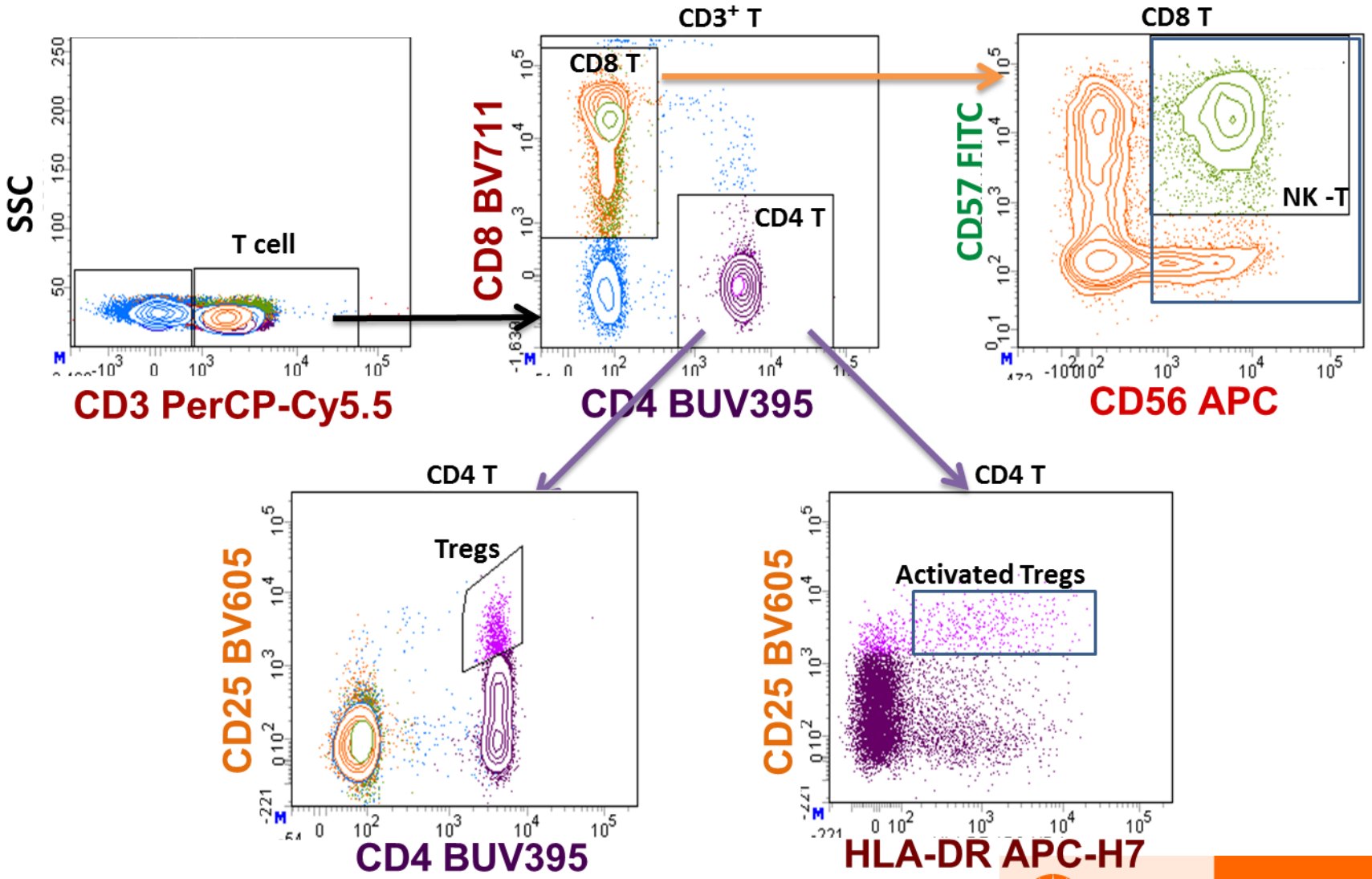
Panel description

- Panel aimed at identifying mayor leucocyte subsets in human peripheral blood:
  - T cells: CD3, CD4, CD8
  - Activated T cells: HLA-DR
  - Regulatory T cells: CD4, CD25
  - B cells: CD19, CD20
  - NK T cells: CD3, CD8, CD56, CD57
  - NK cells: CD56, CD16, CD8, CD57
  - DCs: HLA-DR, CD11c, CD123
  - Monocytes: CD14, CD33, HLA-DR, CD16

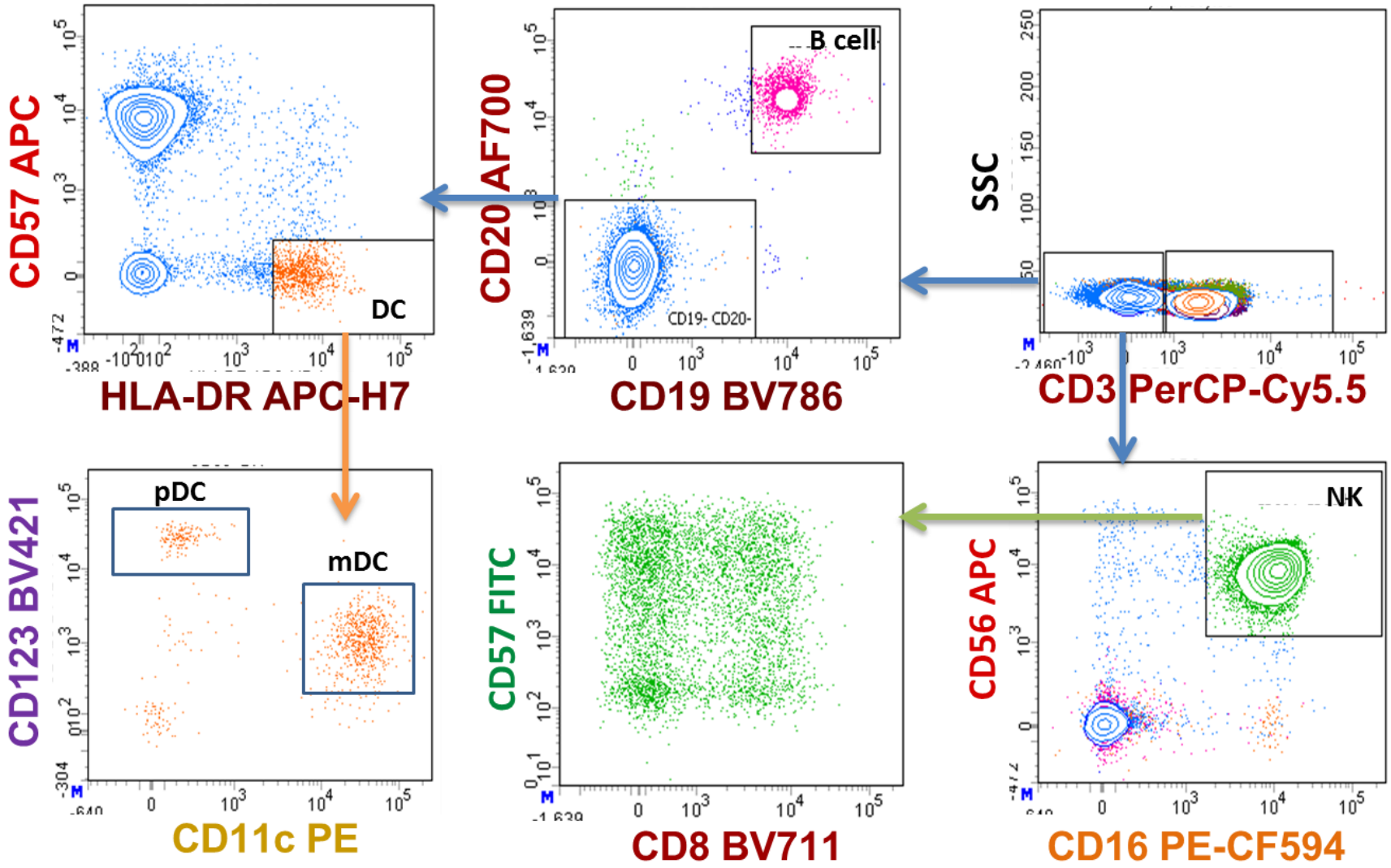
Panel Design

Antigen expression	Co-expression	Fluor assignment
CD3	CD3	CD3 PerCP-Cy5.5
CD4	CD4	CD4 BUV395
CD8	CD8	CD8 BV711
CD56	CD56	CD56 APC
CD16	CD16	CD16 PE-CF594
CD57	CD57	CD57 FITC
CD19	CD19	CD19 BV786
CD20	CD20	CD20 Alexa Fluor® 700
HLA-DR	HLA-DR	HLA-DR APC-H7
CD14	CD14	CD14 V500
CD33	CD33	CD33 PE-Cy7
CD25	CD25	CD25 BV605
CD123	CD123	CD123 BV421
CD11c	CD11c	CD11c PE

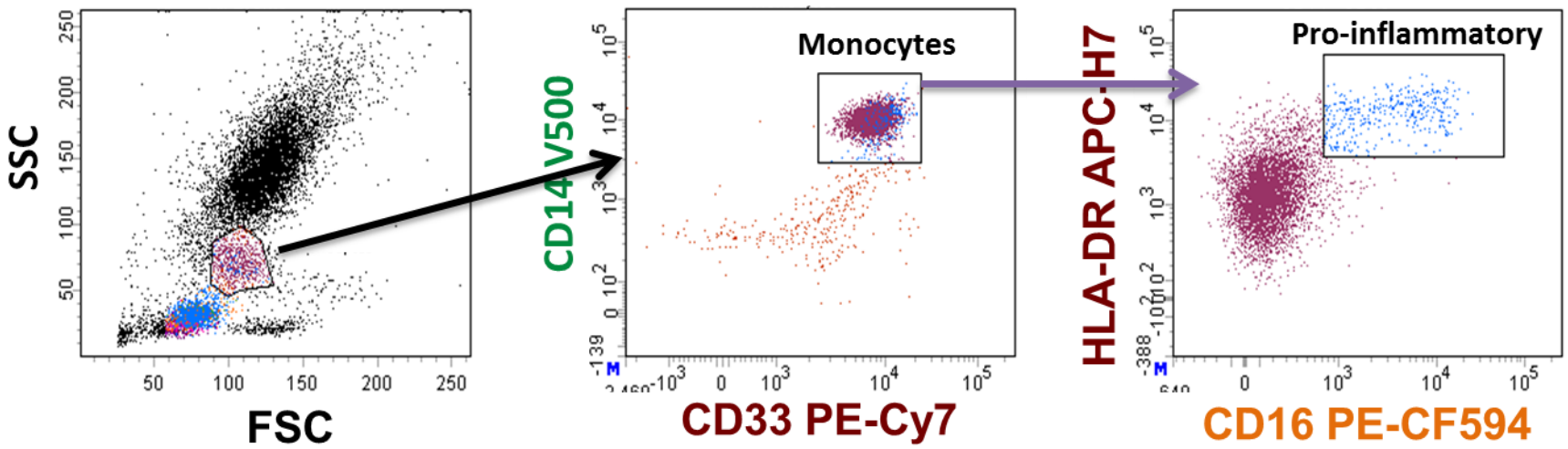
T cell/NK-T cell subsets



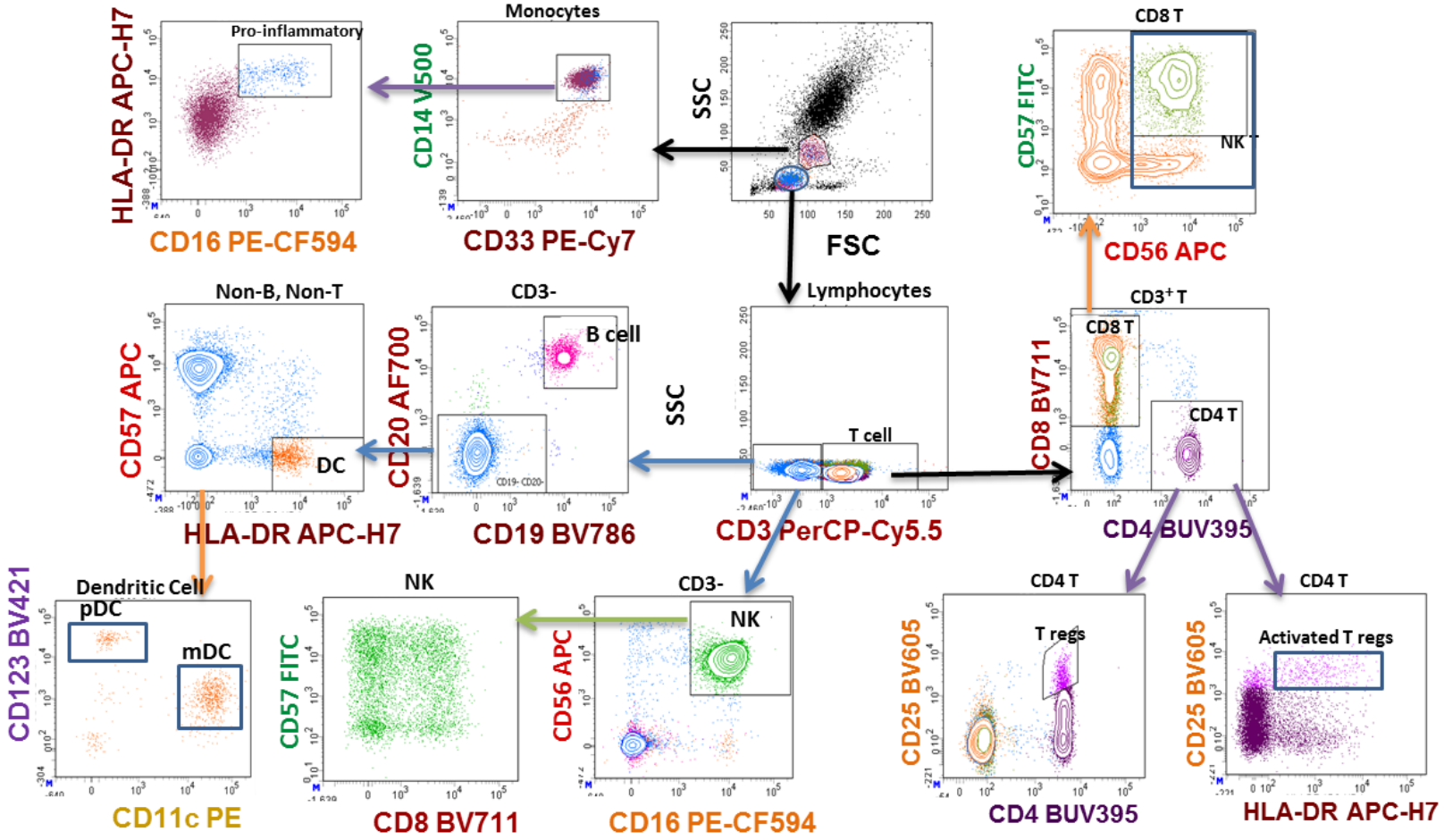
B cell/NK cell/Dendritic cell subsets



Monocyte subsets

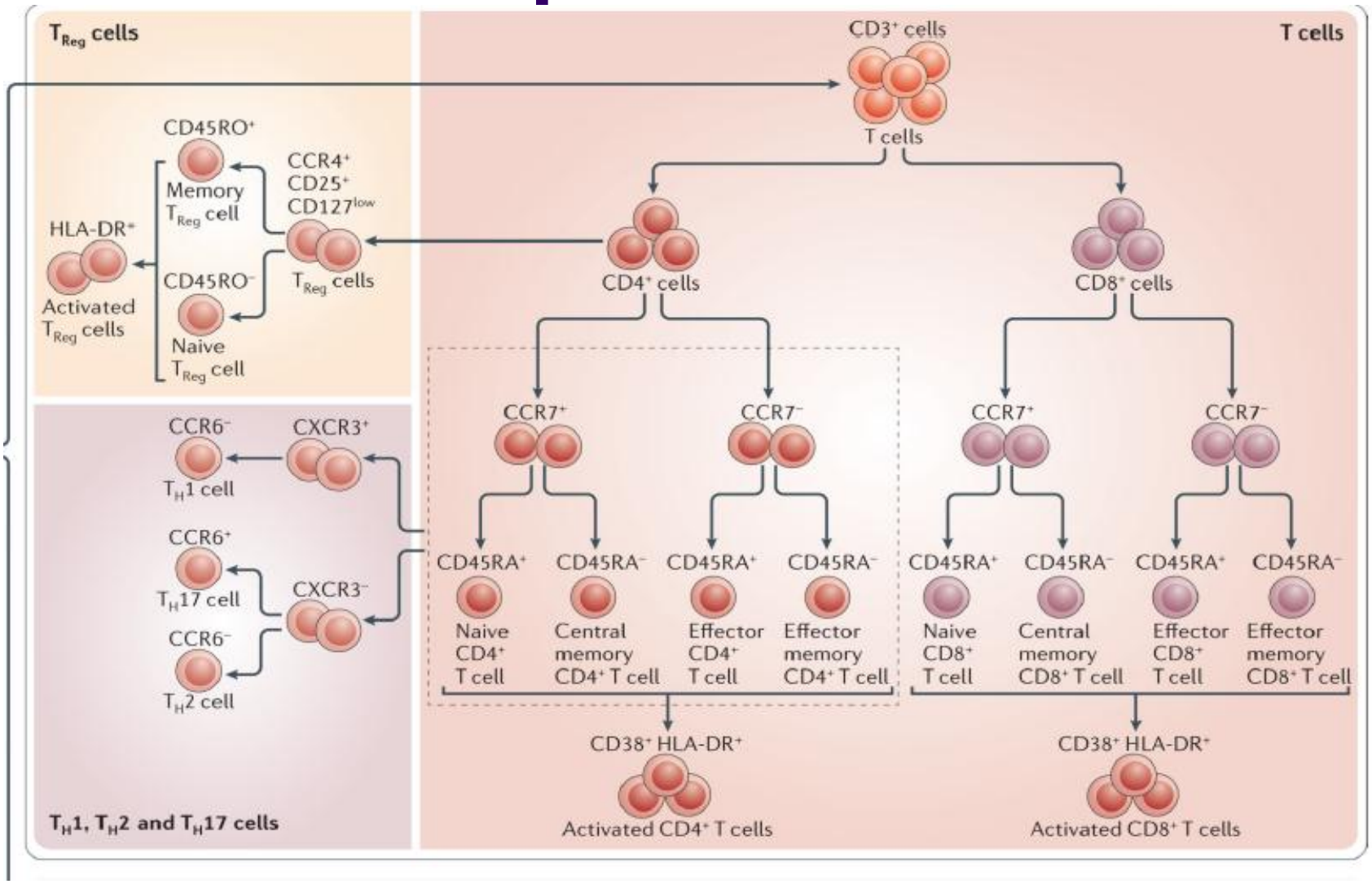


14-Color analysis
T/B/NK/NK-T/Mono/Dendritic cell subsets



13-COLOR T-CELL PANEL

Panel description



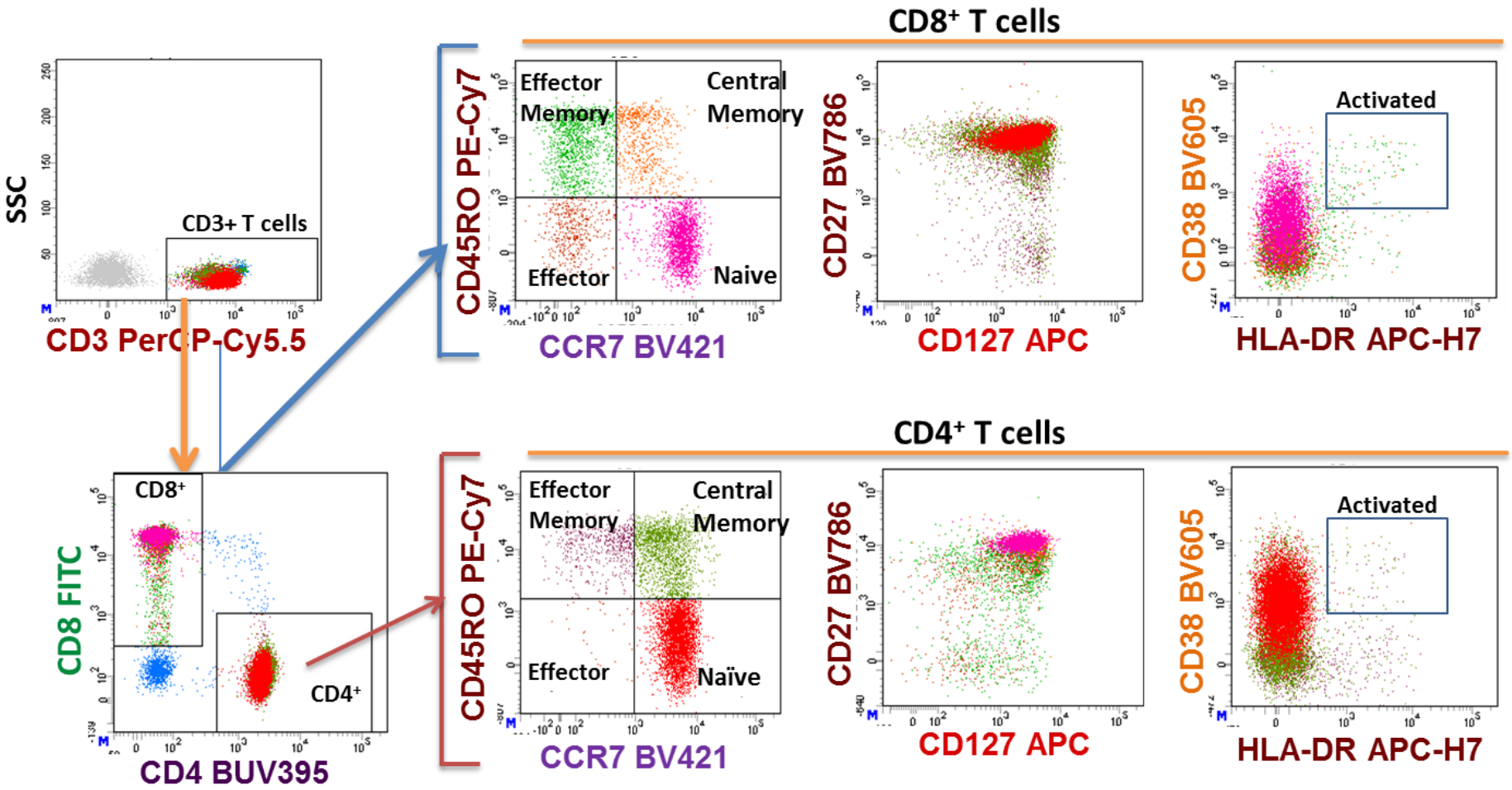
Maecker H, et al. Standardizing immunophenotyping for the Human Immunology Project.
***Nat Rev Immunol. 2012;12:191–200*.**

Panel design

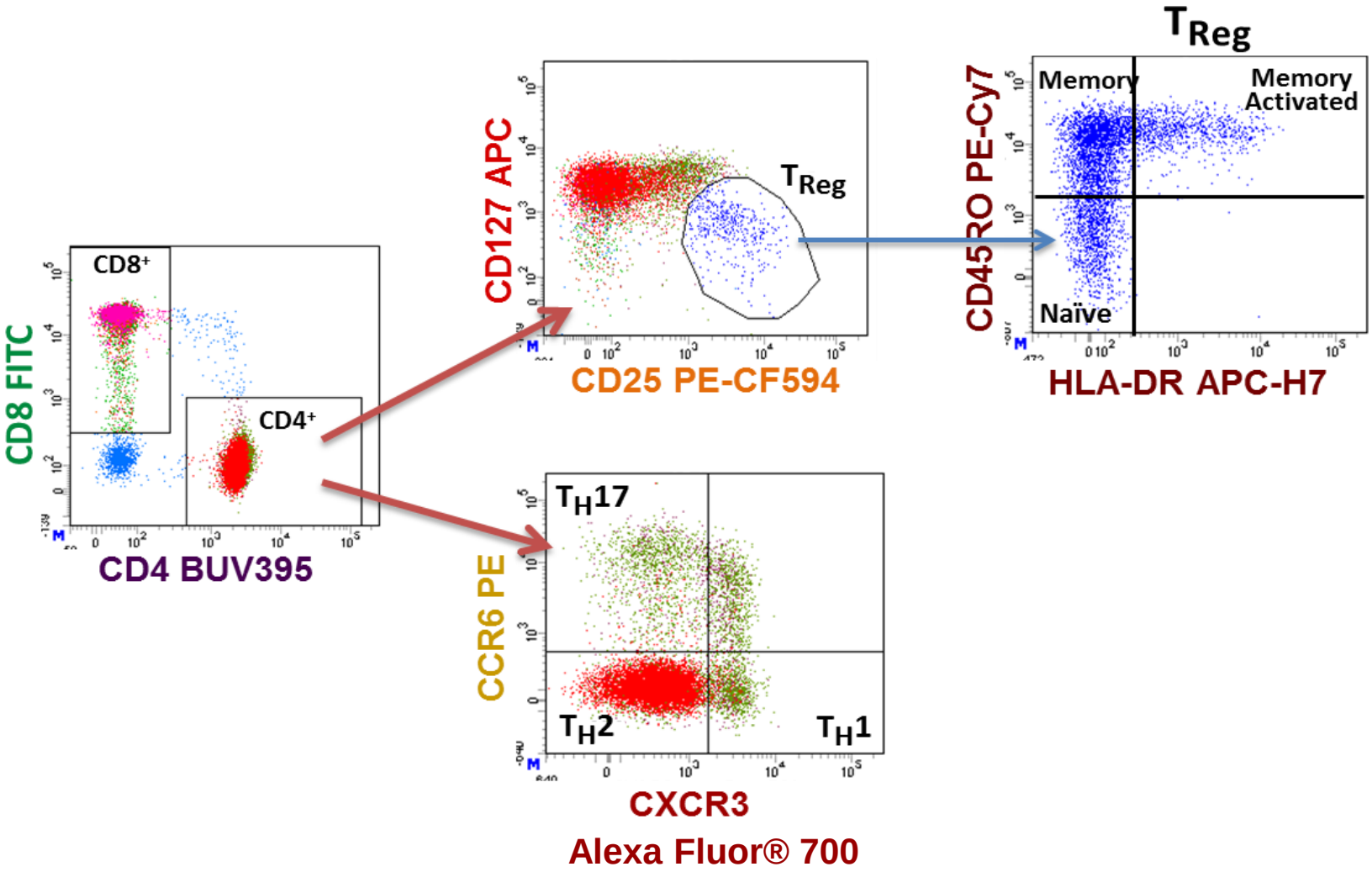
Antigen expression	Co-expression	Fluor assignment
Viability	**Viability**	**Viability (V500)**
CD3	**CD3**	**CD3 PerCP-Cy5.5**
CD4	***CD4***	***CD4 BUV395***
CD8	**CD8**	**CD8 FITC**
CD127	**CD127**	**CD127 Alexa Fluor® 647**
HLA-DR	***HLA-DR**	***HLA-DR APC-H7**
CD45RO	***CD45RO**	***CD45RO PE-Cy7**
CCR7	***CCR7**	***CCR7 BV421**
CD38	***CD38**	***CD38 BV605**
CD27	***CD27**	***CD27 BV786**
CD25	**CD25**	**CD25 PE-CF594**
CCR6	***CCR6***	***CCR6 PE***
CXCR3	***CXCR3***	***CXCR3 Alexa Fluor® 700***

Markers of the same color are co-expressed. Asterisked markers co-express with their same color and also with markers of their opposite color (either green or purple)

Memory/effector/activated subsets



Tregs/Th1/Th2/Th17



REPLACING QDOT® REAGENTS WITH BV REAGENTS IN A 12-COLOR PANEL

BD

Helping all people live healthy lives

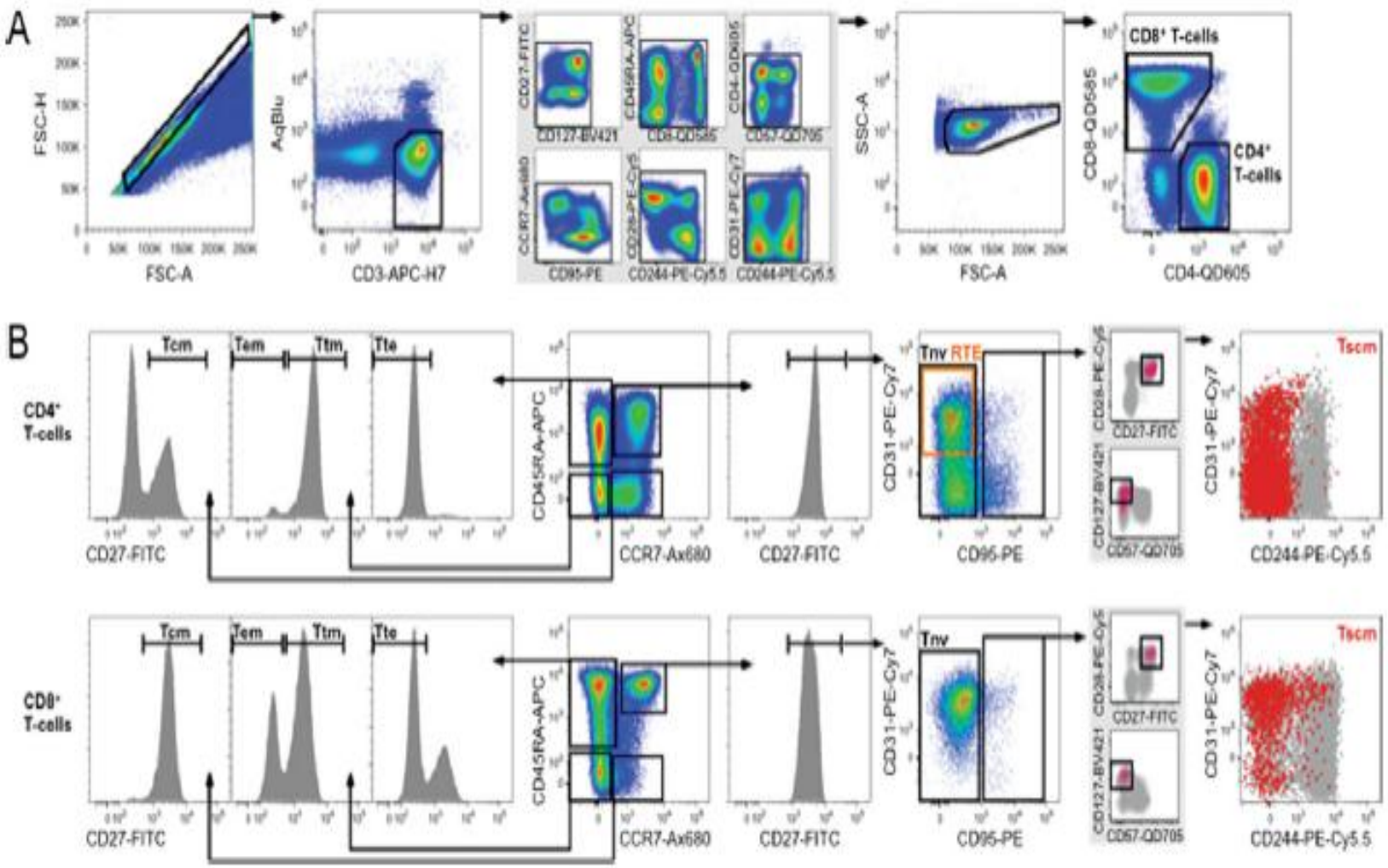
Original panel (1)

Table 2. Reagents used for OMIP-013

SPECIFICITY	CLONE	FLUOROCHROME	PURPOSE
CD3	SK7	APC-H7	lineage
CD4	M-T477	QD605	
CD8	RPA-T8	QD585	
CCR7	150503	Ax680	memory/
CD27	O323	FITC	differentiation
CD28	CD28.2	PE-Cy5	
CD31	WM59	PE-Cy7	
CD45RA	HI100	APC	
CD57	NK-1	QD705	
CD95	DX2	PE	
CD127	A019D5	BV421	
CD244	C1.7	PE-Cy5.5	
Dead cells	–	AqBlu	dump

Mahnke, et al. OMIP-013: Differentiation of human T-cells. *Cytometry Part A*. 2012:81A:935-936.

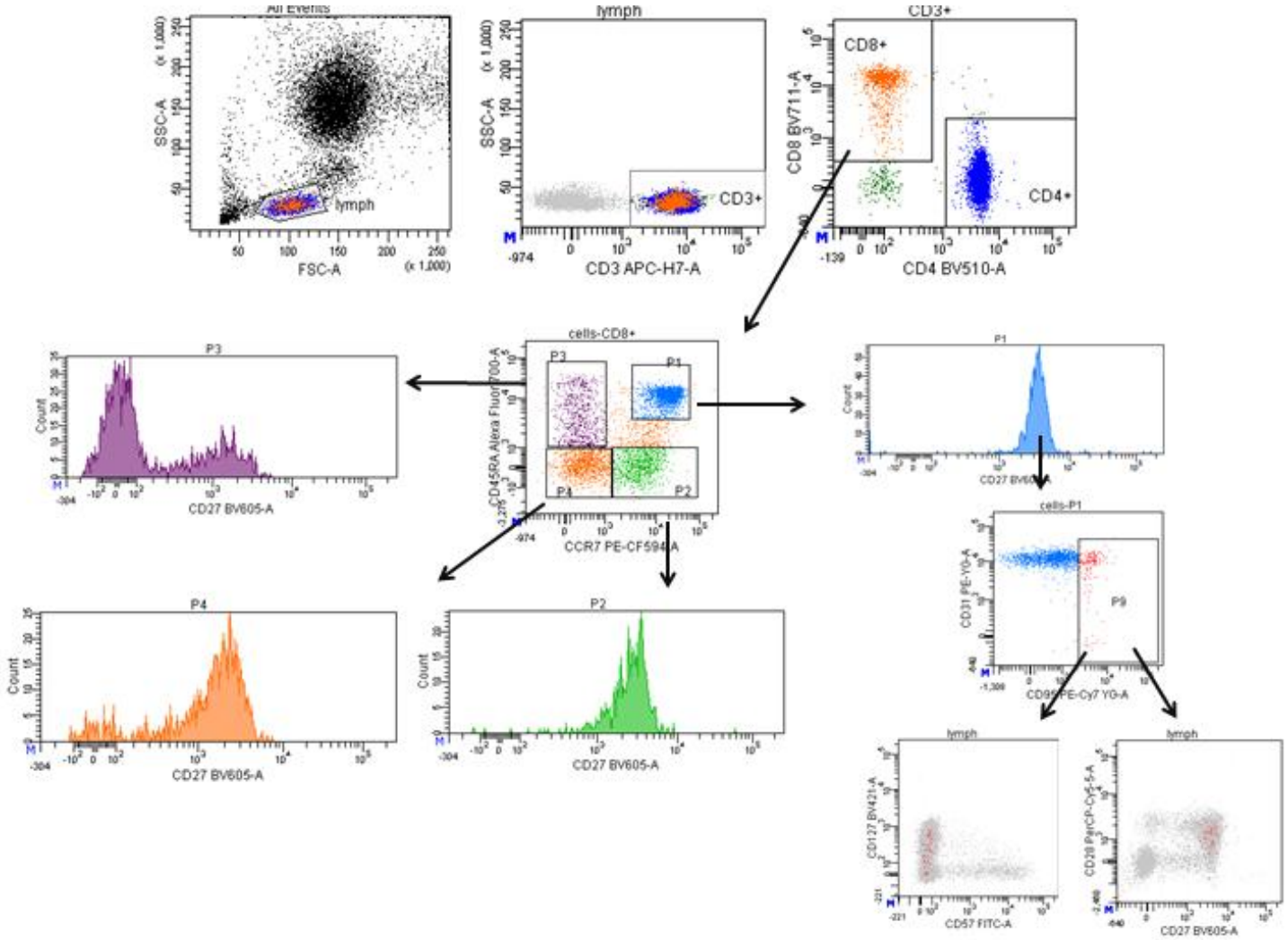
Original panel (2)



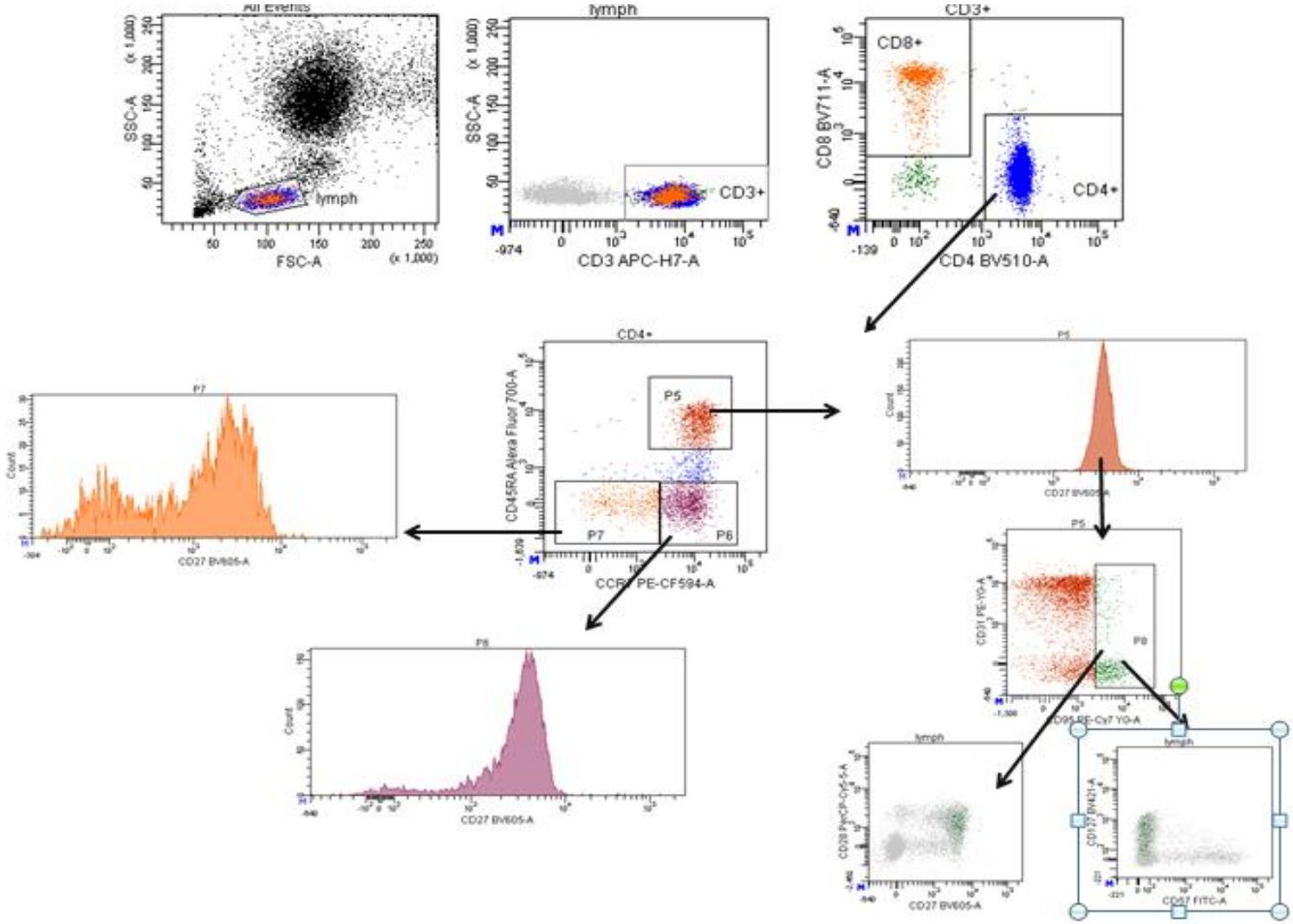
Proposed alternative panel

- CD57 FITC
- CD28 PerCP-Cy5.5
- CD31 PE
- CCR7 PE-CF594
- CD95 PE-Cy7
- CD62L APC
- CD45RA Alexa Fluor® 700
- CD3 APC-H7
- CD127 BV421
- CD4 BV510
- CD27 BV605
- CD8 BV711

Results: CD8 subsets



Results: CD4 subsets



Conclusions

- BV dyes offer a wide array of options for successful multicolor flow cytometry
- Brightness is a key feature of BV dyes that enhances resolution
- Panel design needs to be carefully assessed in order to mitigate spillover issues
- BUV dyes are an exciting and promising new family of dyes

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