What is wrong with my flow cytometry data?

Hints, tricks and pitfalls

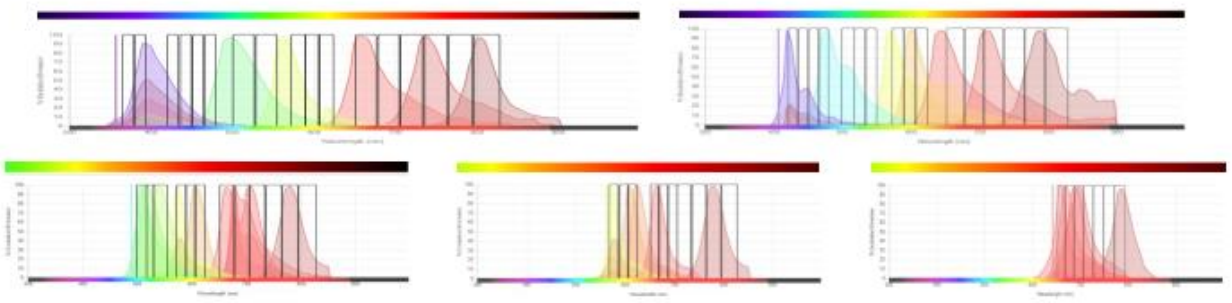
Universität Rostock Traditio et Innovatio

Universitätsmedizin Rostock

Webinar 7
01.08.2023

Golden rules of panel design

The more…the better (the more complicated)



1980 — 1 Laser, 2 Colors

1990 — 2 Lasers, 4 Colors

2000 — 3 Lasers, 8 Colors

2010 — 3 Lasers, 13 Colors

2017 — Aurora: 3 Lasers, 20 Colors

2019 — Aurora: 5 Lasers, 40 Colors

source: Jesus Gil Pulido - Cytek Training Rostock 2020

What do you want to look at…

1. Which type of cells you want to look at?
    1. Mouse/human/…
    2. Origin (blood, tissue,…)
    3. Antigens
    4. Gating strategy
2. What is my sample's nature?
    1. Live or fixed
    2. Rare cells
    3. Small amount
3. What is the configuration of my analyzer?
    1. Number and type of lasers
    2. Number and bandwith of filters

3 types of antigens

If the level of expression of your target protein is unknown then consider antigen as tertiary!

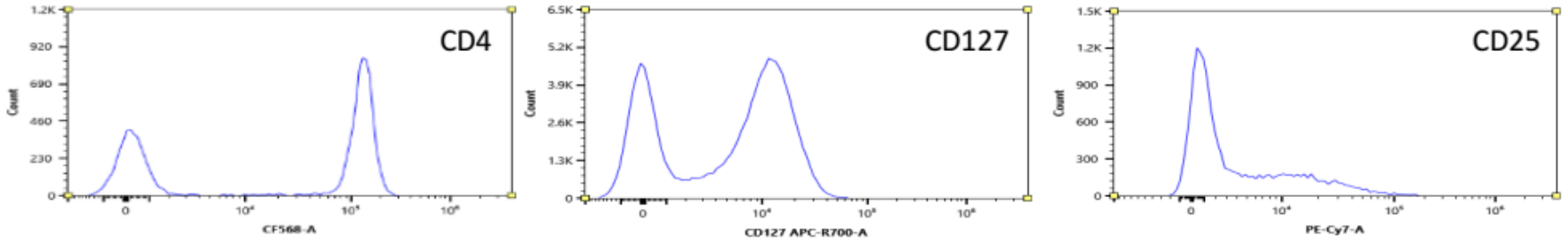
Primary
High density
on or off expression
CD3
CD4
CD8

Secondary
Intermediate density
continuous expression
CD45RA
CCR7
CD127
HLA-DR

Tertiary
Low density
unknown expression
PD-1
CD25
TCR γ/δ

Flurochrome Brightness

Level of Antigen Expression



Mahnke, Y. and Roederer, M. Clin Lab Med. 2007 September ; 27(3): 469

direction of choosing fluorophors (searching for products)

source: Jesus Gil Pulido - Cytek Training Rostock 2020

REMINDER: Check fluorophore brightness

Fluorochrome

Laser	Very Bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon™ BUV563 BD Horizon™ BUV615 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™ BV480 BD Horizon™ BV605 BD Horizon™ BV786	BD Horizon™ BV510 BD Horizon™ BV750	BD Horizon™ V450 BD Horizon™ V500
Blue (488 nm)	BD Horizon™ BB515 BD Horizon™ BB700 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-Cy™5 PE-Cy™7			
Red (640 nm)		APC Alexa Fluor® 647 BD Horizon™ APC-R700 BD Horizon™ R718		Alexa Fluor® 700 APC-H7 APC-Cy™7

https://www.bdj.co.jp/biosciences/support/pdf/23-16181_Post_Card_BD_Life_Sciences_Relative_Fluorochrome_Brightness.pdf

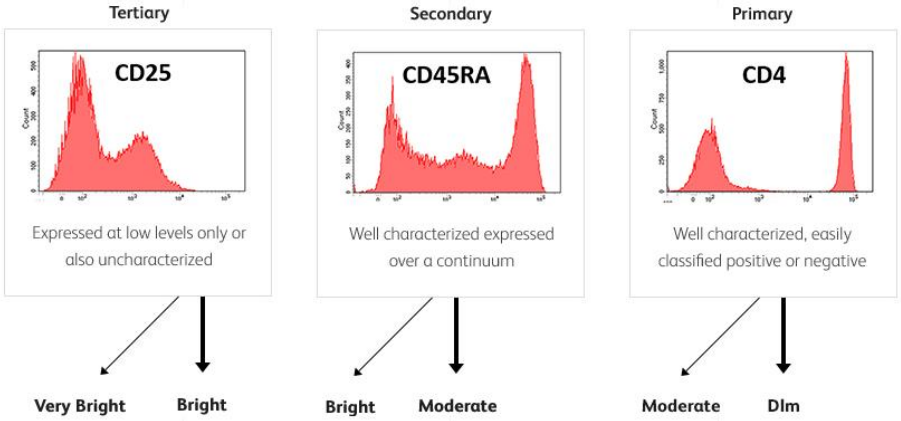
https://www.biolegend.com/en-us/brightness-index

	Em (nm) Max	Filter Used	Brightness
PE	565	575	585/20	5
PE/Cyanine5	565	670	660/20	5
PE/Dazzle™ 594	565	610	610/20	5
PE/Fire™ 700*	565	695	670/30	5
Brilliant Violet 421™	405	421	450/50	5
Brilliant Violet 711™	405	711	710/50	5
Brilliant Violet 605™	405	603	610/20	4
Brilliant Violet 650™	405	645	660/20	4

REMINDER: check spillover-spread

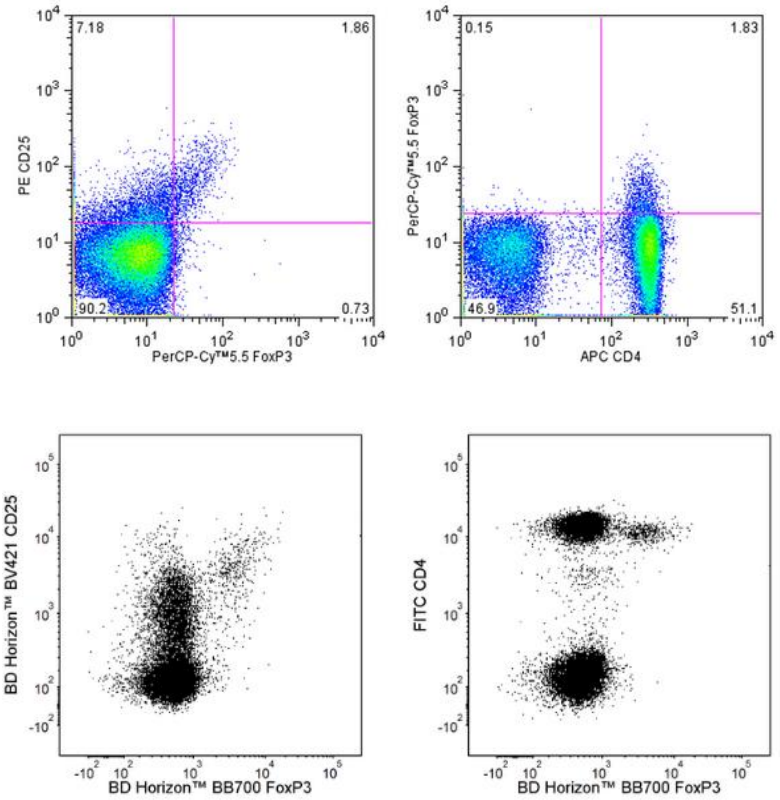
Match antigen expression with fluorochrome brightness

Match tertiary antigens with bright fluorochromes and primary antigens with dim fluorochromes. In the following table, you can find Fluorochrome ranking on brightness which can be used to help you in panel design.



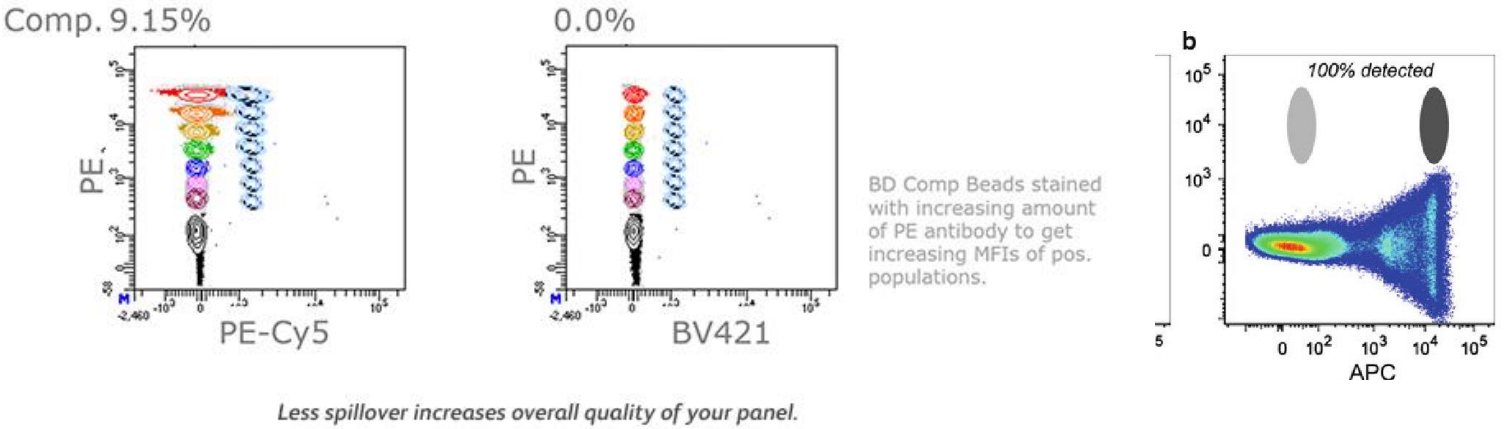
CD25:PE and FoxP3:PerCP/Cy5.5 co-expression / overlap → bad resolution

CD4:APC to brigth → much spread → bad resolution of PerCP/Cy5.5



https://eu.bd.com/panel-design/en/flow-cytometry-fluochrome-brightness-spillover
https://www.bdbiosciences.com/en-de/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/percp-cy-5-5-mouse-anti-human-foxp3.561493
https://www.bdbiosciences.com/en-de/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/bb700-mouse-anti-human-foxp3.566527

REMINDER: check spillover-spread



- (very) bright dyes have increased emitted photon amount
- ➢ increase of spillover spread
- ➢ can reduce resolution especially for co-expressed markers (double positive)

https://eu.bd.com/panel-design/en/flow-cytometry-fluochrome-brightness-spillover

Golden Rules of Panel design – step by step

1. Which type of cells you want to look at?
   - literature search: already described panels → OMIPs
     - OMIPs = Optimized Multicolor Immunofluorescence Panel published e.g. in Cytometry Part A
   - antibodies you already have in the lab OR without alternative

2. Search for antibodies for tertiary antigens (intranuclear, cytoplasmatic proteins)
   - most important targets
   - use the brightest dyes (PE, BV421, APC, PE-Cy7; …)
   - spread the antibodies over all lasers to reduce needed compensation

➢ Fill your panel

Golden Rules of Panel design – step by step

3. Combine and add highly and moderate markers with dim dyes
   - CD3-FITC, CD45-PerCP, …

➢ Fill your panel

3. Include live-dead stain
   - fixable (Zombie, FVS…)
   - non-fixable (PI, 7-AAD, DAPI)

➢ Fill your panel

3. Check your panel:
   - put markers on highly autofluorescent cells in red laser module
   - live-dead stain and ubiquitous markers at the edges of the spectrum (less compensation)
   - compare antibody clones (resolution)
   - use better (tandem-) dyes with less spread (e.g. PerCP → PerCP-Cy5.5 or BB700 or PerCP-Vio700)
   - check for fixability of dyes (use sensitive ones for ICS)

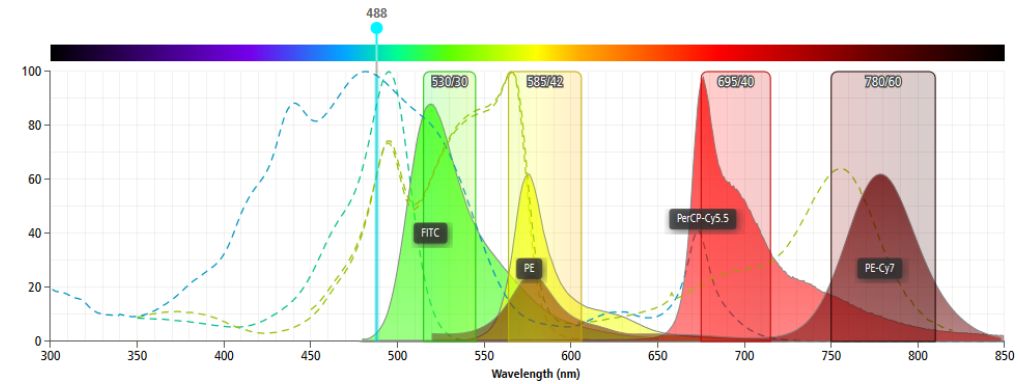
Useful webtools: spillover, brightness, …

BD Biosciences | Germany (English) | Sign-in/Register

BD Spectrum Viewer

The BD Spectrum Viewer is a tool that depicts the excitation and emission curves of fluorochromes common to flow cytometry. This tool can be used to determine appropriate filters to detect a fluorochrome as well as fluorochrome compatibility and fluorescent spillover. Unlike a static image showing excitation and emission histograms, this tool will demonstrate how a fluorochrome will have the same emission profile—but have varying brightness—based on the excitation wavelength of the laser. Choosing different laser wavelengths will transform the emission curve based on the percent excitation at that wavelength.

Display Options | Curves: 4 | Cytometer: Any Cytometer | Excitation (nm): 488 | Show Em when Ex % > 5 | RESET



	Fluorochrome	%Max	Ex	Em	Filters	FITC	PE	PerCP...	PE-Cy7
1	FITC	88.0	☑	☑	530/30	47.4%	0.4%	0.3%	1.8%
2	PE	61.6	☑	☑	585/42	12.5%	70.4%	0.5%	16.4%
3	PerCP-Cy5.5	98.4	☑	☑	695/40	x	2.0%	48.4%	1.4%
4	PE-Cy7	61.8	☑	☑	780/60	x	x	11.9%	60.5%

Add Filter: | ADD

Useful webtools: spillover, brightness, …

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United States ($) USD

Flow Cytometry Tools

We offer over a dozen web tools to educate researchers on fluorophores, build the ideal multicolor panel, and choose the right reagents for flow cytometry applications. For additional inquiries, contact our Technical Services team.

- Spectra Analyzers
- Panel Design Guidance
- Fluorophore Information
- Pre-Experiment Planning
- Post-Experiment Help
- Products

Conventional Spectra Analyzer

"Conventional/traditional" flow cytometers use a series of mirrors and filters in order to capture the emission wavelengths from excited fluorophores. Fluors that have emission profiles that fall into the same filter cannot be separated or identified from one another.

Our spectra analyzer can display the excitation and emission spectra for dozens of fluorophores. This allows you to compare the excitation and emission profiles of fluors, custom filters, and laser lines to see what best fits your cytometer.

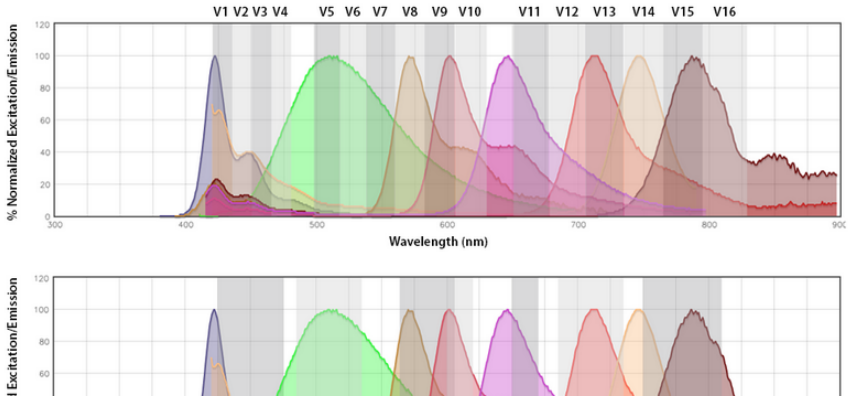
View the Conventional Spectra Analyzer >

Spectral Cytometry Analyzer

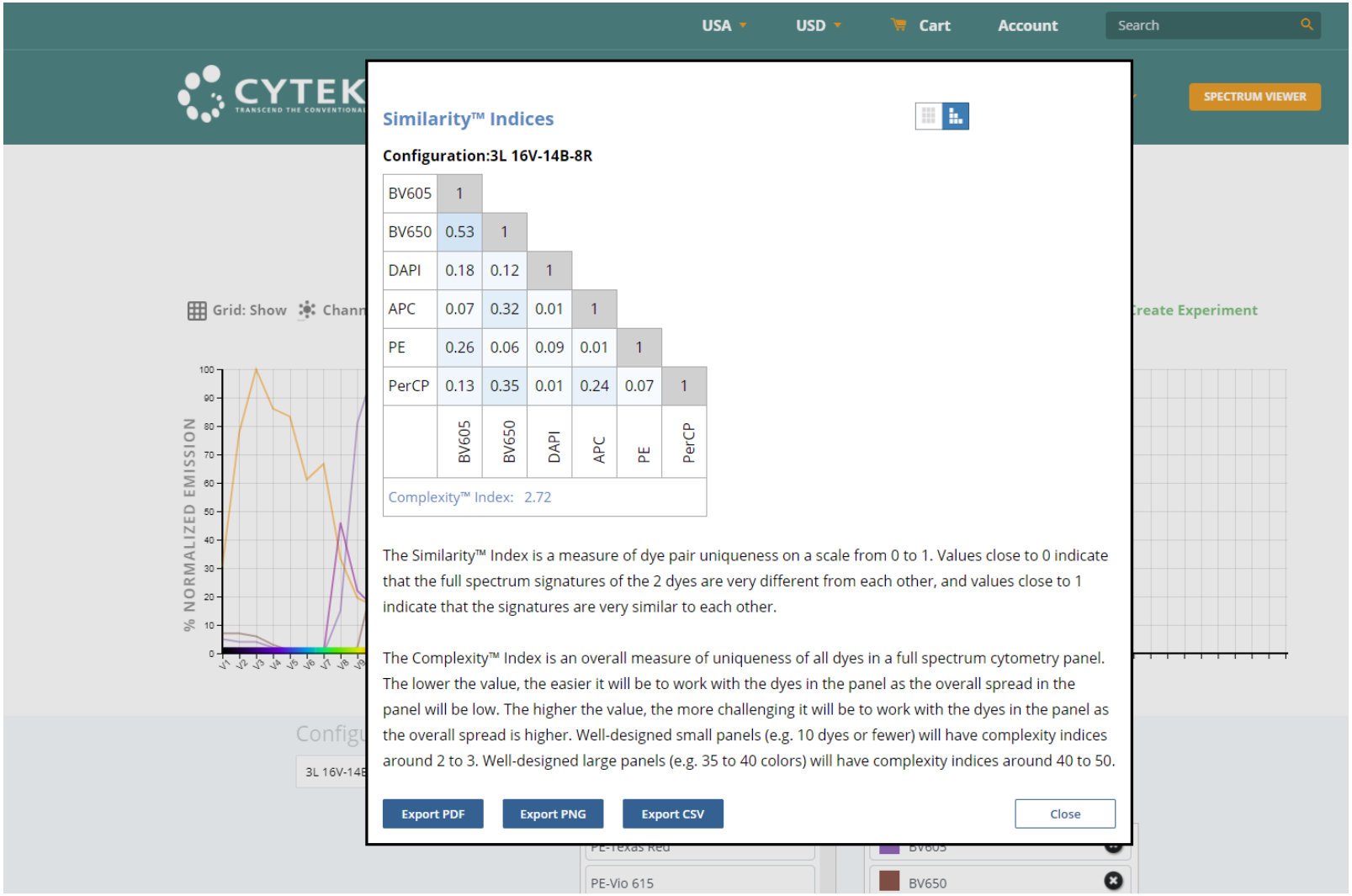
Spectral cytometers capture the full spectrophotometric profile of the fluorophores across all lasers. The fluors' profiles are captured in 10-30 nm segments across the emission range to accurately unmix the fluorophores. This allows fluors that normally cannot be unmixed on a conventional cytometer (*e.g.* Pacific Blue™ and Brilliant Violet 421™) to be used together on an instrument like Cytek's Aurora.

As such, this web tool is dedicated to displaying the emission spectra of fluorophores based on Cytek's Aurora spectral cytometers.

View the Spectral Cytometry Analyzer >



Useful webtools: spillover, brightness, …



Exercise – Identification of human Tregs

1. Which type of cells you want to look at?
   - human Tregs in PBMCs
   - CD3, CD4, CD25, FoxP3

2. Search for antibodies for tertiary antigens (intranuclear, cytoplasmatic proteins)
   - FoxP3
   - CD25

➢ Fill your panel

example panel - Tregs

Panel Template BD FACS Aria IIIu					
Laser	Laser Excitation	Parameter	Detector	Filter	Marker:Dye
1	Rot (633 nm)	APC-Cy7, APC-H7, APC-Vio770, APC/Fire750, APC-eFluor780	A	780/60, 735 LP	
		AF700, APC-R700, ZombieNIR	B	730/45, 690 LP	
		APC, AF647, Cy5, eFluor660	C	660/20	
2	Gelbgrün (561 nm)	PE-Cy7, PE-Vio770	A	780/60, 735 LP	
		PE-Cy5, 7-AAD, mPlum	B	670/14, 630 LP	
		PE-TexasRed, PI, mCherry, PE-Dazzle594, ZombieRed, AF594	C	610/20, 600 LP	
		PE, DsRed, Cy3	D	582/15	
3	Blau (488 nm)	PerCP-Cy5.5, PerCP, BB700, PerCP/Vio700, PerCP-eFluor710	A	695/40, 655 LP	
		FITC, GFP/YFP, AlexaFluor488, Zombie Green, VioBright515/FITC, KIRAVIABlue520, AF532	B	530/30, 502 LP	
		SSC	C	488/10	
4	Violet (405 nm)	BV786, BV750, SuperBright780	A	780/60, 750 LP	
		BV650, Qdot655, SuperBright645	B	660/20, 630 LP	
		BV605, Qdot605, SuperBright600	C	616/23, 595 LP	
		BV510, V500, AmCyan, Aqua L/D, eFluor 506,VioGreen	D	530/30, 502 LP	
		DAPI, BV450, BV421, Hoechst, VioBlue, eFluor450, AF430, ZombieViolet	E	450/40	

Exercise – Tregs

Panel Template BD FACS Aria IIIu

Laser	Laser Excitation	Parameter	Detector	Filter	Marker:Dye
1	Rot (633 nm)	APC-Cy7, APC-H7, APC-Vio770, APC/Fire750, APC-eFluor780	A	780/60, 735 LP	
		AF700, APC-R700, ZombieNIR	B	730/45, 690 LP	
		APC, AF647, Cy5, eFluor660	C	660/20	**CD25 - APC**
2	Gelbgrün (561 nm)	PE-Cy7, PE-Vio770	A	780/60, 735 LP	
		PE-Cy5, 7-AAD, mPlum	B	670/14, 630 LP	
		PE-TexasRed, PI, mCherry, PE-Dazzle594, ZombieRed, AF594	C	610/20, 600 LP	
		PE, DsRed, Cy3	D	582/15	**FoxP3 - PE**
3	Blau (488 nm)	PerCP-Cy5.5, PerCP, BB700, PerCP/Vio700, PerCP-eFluor710	A	695/40, 655 LP	
		FITC, GFP/YFP, AlexaFluor488, Zombie Green, VioBright515/FITC, KIRAVIABlue520, AF532	B	530/30, 502 LP	
		SSC	C	488/10	
4	Violet (405 nm)	BV786, BV750, SuperBright780	A	780/60, 750 LP	
		BV650, Qdot655, SuperBright645	B	660/20, 630 LP	
		BV605, Qdot605, SuperBright600	C	616/23, 595 LP	
		BV510, V500, AmCyan, Aqua L/D, eFluor 506,VioGreen	D	530/30, 502 LP	
		DAPI, BV450, BV421, Hoechst, VioBlue, eFluor450, AF430, ZombieViolet	E	450/40	

Exercise – Tregs

3. Combine and add highly and moderate expressed markers with dim dyes
   - CD3
   - CD4

4. Include live-dead stain
   - fixable

Exercise – Tregs

Panel Template BD FACS Aria IIIu

Laser	Laser Excitation	Parameter	Detector	Filter	Marker:Dye
1	Rot (633 nm)	APC-Cy7, APC-H7, APC-Vio770, APC/Fire750, APC-eFluor780	A	780/60, 735 LP	
		AF700, APC-R700, ZombieNIR	B	730/45, 690 LP	
		APC, AF647, Cy5, eFluor660	C	660/20	**CD25 - APC**
2	Gelbgrün (561 nm)	PE-Cy7, PE-Vio770	A	780/60, 735 LP	
		PE-Cy5, 7-AAD, mPlum	B	670/14, 630 LP	
		PE-TexasRed, PI, mCherry, PE-Dazzle594, ZombieRed, AF594	C	610/20, 600 LP	
		PE, DsRed, Cy3	D	582/15	**FoxP3 - PE**
3	Blau (488 nm)	PerCP-Cy5.5, PerCP, BB700, PerCP/Vio700, PerCP-eFluor710	A	695/40, 655 LP	
		FITC, GFP/YFP, AlexaFluor488, Zombie Green, VioBright515/FITC, KIRAVIABlue520, AF532	B	530/30, 502 LP	
		SSC	C	488/10	
4	Violet (405 nm)	BV786, BV750, SuperBright780	A	780/60, 750 LP	
		BV650, Qdot655, SuperBright645	B	660/20, 630 LP	
		BV605, Qdot605, SuperBright600	C	616/23, 595 LP	
		BV510, V500, AmCyan, Aqua L/D, eFluor 506,VioGreen	D	530/30, 502 LP	
		DAPI, BV450, BV421, Hoechst, VioBlue, eFluor450, AF430, ZombieViolet	E	450/40	

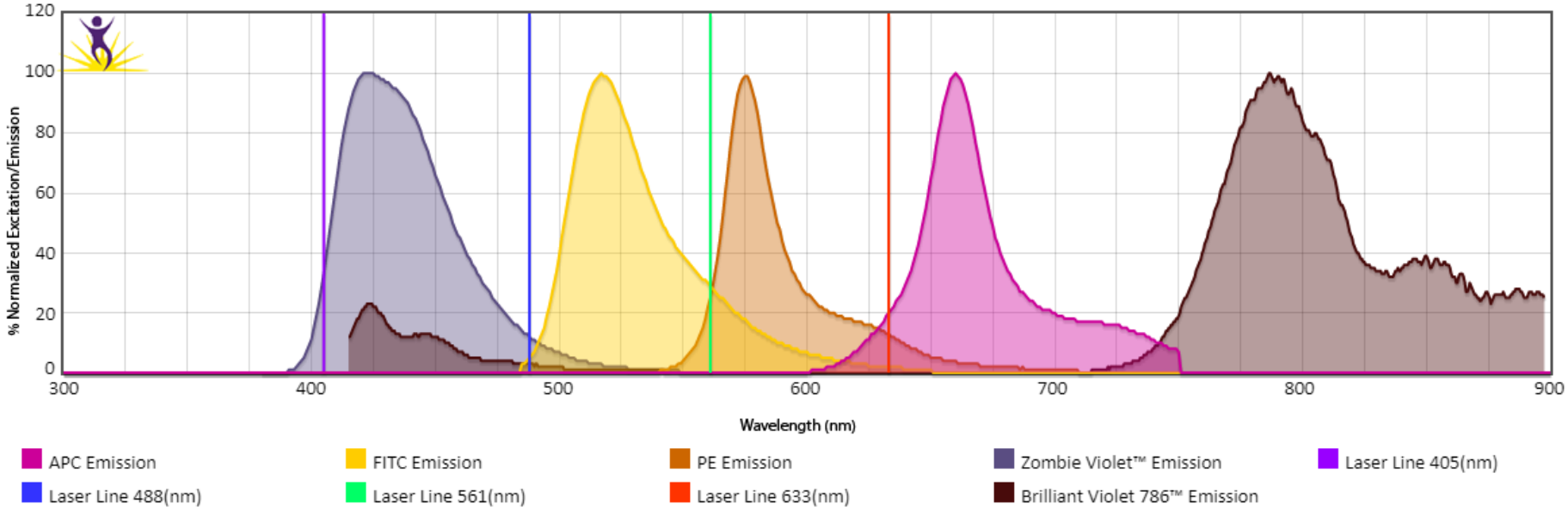
Exercise – Tregs

Panel Template BD FACS Aria IIIu

Laser	Laser Excitation	Parameter	Detector	Filter	Marker:Dye
1	Rot (633 nm)	APC-Cy7, APC-H7, APC-Vio770, APC/Fire750, APC-eFluor780	A	780/60, 735 LP	
		AF700, APC-R700, ZombieNIR	B	730/45, 690 LP	
		APC, AF647, Cy5, eFluor660	C	660/20	**CD25 - APC**
2	Gelbgrün (561 nm)	PE-Cy7, PE-Vio770	A	780/60, 735 LP	
		PE-Cy5, 7-AAD, mPlum	B	670/14, 630 LP	
		PE-TexasRed, PI, mCherry, PE-Dazzle594, ZombieRed, AF594	C	610/20, 600 LP	
		PE, DsRed, Cy3	D	582/15	**FoxP3 - PE**
3	Blau (488 nm)	PerCP-Cy5.5, PerCP, BB700, PerCP/Vio700, PerCP-eFluor710	A	695/40, 655 LP	
		FITC, GFP/YFP, AlexaFluor488, Zombie Green, VioBright515/FITC, KIRAVIABlue520, AF532	B	530/30, 502 LP	**CD3 - FITC**
		SSC	C	488/10	
4	Violet (405 nm)	BV786, BV750, SuperBright780	A	780/60, 750 LP	**CD4 – BV786**
		BV650, Qdot655, SuperBright645	B	660/20, 630 LP	
		BV605, Qdot605, SuperBright600	C	616/23, 595 LP	
		BV510, V500, AmCyan, Aqua L/D, eFluor 506,VioGreen	D	530/30, 502 LP	
		DAPI, BV450, BV421, Hoechst, VioBlue, eFluor450, AF430, ZombieViolet	E	450/40	**Zombie Violet**

Exercise – Tregs

5. Check your panel:
   - put markers on highly autofluorescent cells in red laser module
   - live-dead stain and ubiquitous markers at the edges of the spectrum (less compensation)
   - compare antibody clones (resolution)
   - use better (tandem-) dyes with less spread (e.g. PerCP → PerCP-Cy5.5 or BB700 or PerCP-Vio700)
   - check for fixability of dyes (use sensitive ones for ICS)



TAKE Home Message

- primary antigens → dim dyes
- secondary antigens → moderate to bright dyes
- tertiary (unknown) antigens → bright to very bright dyes
- spillover spread and cross laser excitation reduces resolution → exchange tandems
- use vendor information, published panels and web tools

Always ask for help or assistance with your panel!!!

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Thank you for your attention.

See you next month: **5th September**

Next topic
Cell sorting – why and how?