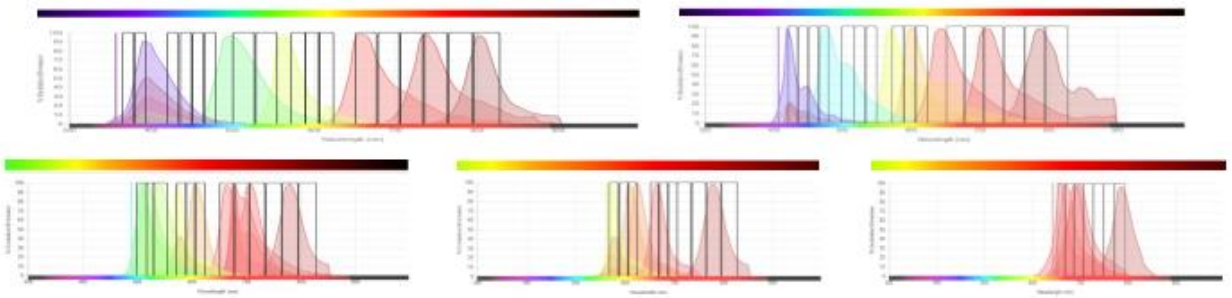
Universität Rostock Traditio et Innovatio

Universitätsmedizin Rostock

# 6.) „The golden rules of panel design“

# 6.) „Die goldenen Regeln des Paneldesigns“

# more parameters – better panel design needed

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

# panel design – question before you start

- what cells you want to look at? (which markers do you need, gating strategy)
- what is my sample (living cells, fixed cells, small amount)
- which analyzer do you want to use →
  - what's the configuration and
  - how many parameter are possible?

# 3 types of antigens

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

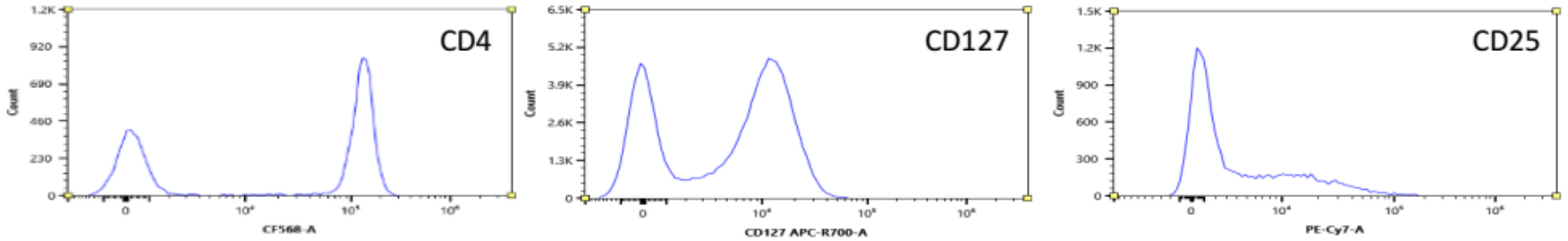
**Primary**
High density
on or off expression
CD3
CD4
CD8

**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

# fluorophore brightness

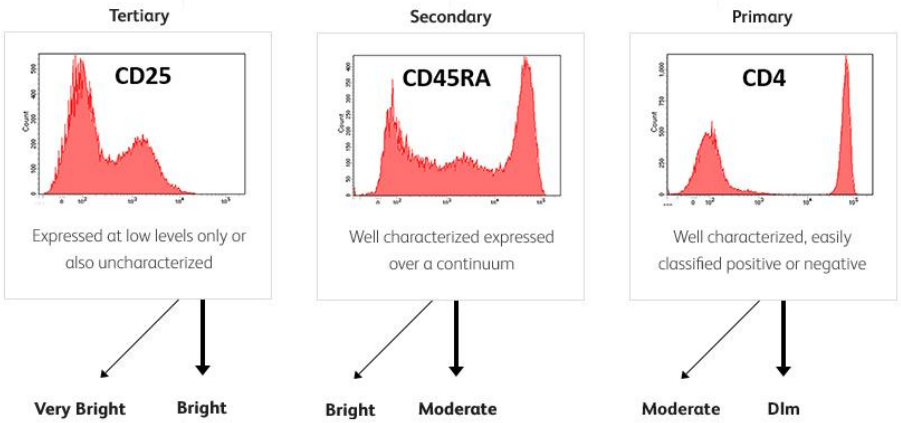
| Laser | Fluorochrome: Very Bright | Bright | Moderate | Dim |
|---|---|---|---|---|
| Ultraviolet (355 nm) | | BD Horizon™ BUV563<br>BD Horizon™ BUV615<br>BD Horizon™ BUV661<br>BD Horizon™ BUV737 | BD Horizon™ BUV395<br>BD Horizon™ BUV496 | BD Horizon™ BUV805 |
| Violet (405 nm) | BD Horizon™ BV421<br>BD Horizon™ BV650<br>BD Horizon™ BV711 | BD Horizon™ BV480<br>BD Horizon™ BV605<br>BD Horizon™ BV786 | BD Horizon™ BV510<br>BD Horizon™ BV750 | BD Horizon™ V450<br>BD Horizon™ V500 |
| Blue (488 nm) | BD Horizon™ BB515<br>BD Horizon™ BB700<br>BD Horizon™ PE-CF594<br>PE-Cy™5 | PE<br>PE-Cy™7 | FITC<br>Alexa Fluor® 488<br>PerCP-Cy™5.5 | PerCP |
| Yellow/Green (561 nm) | PE<br>BD Horizon™ PE-CF594<br>PE-Cy™5<br>PE-Cy™7 | | | |
| Red (640 nm) | | APC<br>Alexa Fluor® 647<br>BD Horizon™ APC-R700<br>BD Horizon™ R718 | | Alexa Fluor® 700<br>APC-H7<br>APC-Cy™7 |

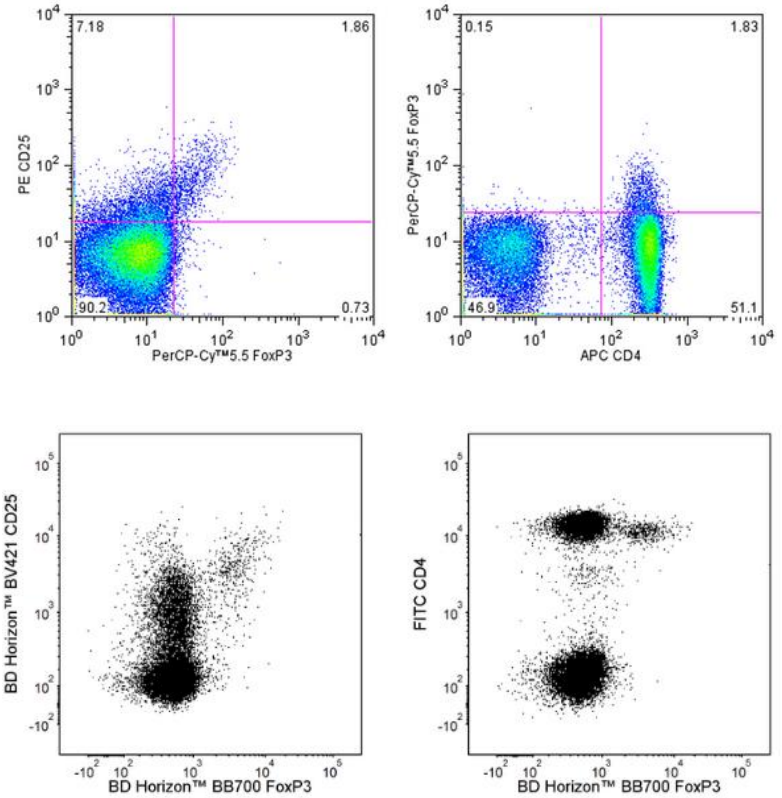
https://www.bdj.co.jp/biosciences/support/pdf/23-16181_Post_Card_BD_Life_Sciences_Relative_Fluorochrome_Brightness.pdf

# brighter is always better?

## Match antigen expression with fluorochrome brightness

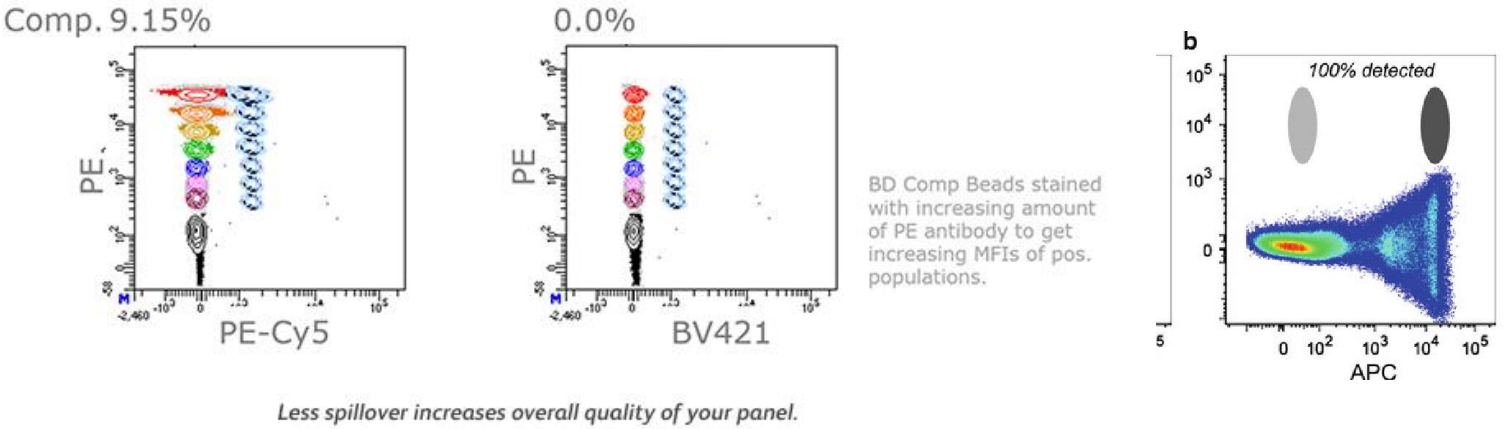
Match tertiary antigens with bright fluorochromes and primary antigens with dim fluorochromes. In the following table, you can find Fluorochrome ranking on brightness which can be used to help you in panel design.




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

# brighter is always better?




*Less spillover increases overall quality of your panel.*

- not always --> (very) bright dyes increase general light emitted by the stained cell
- increase of spillover spread
- can reduce resolution especially for coexpressed markers (double positive)

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

# panel design – step by step

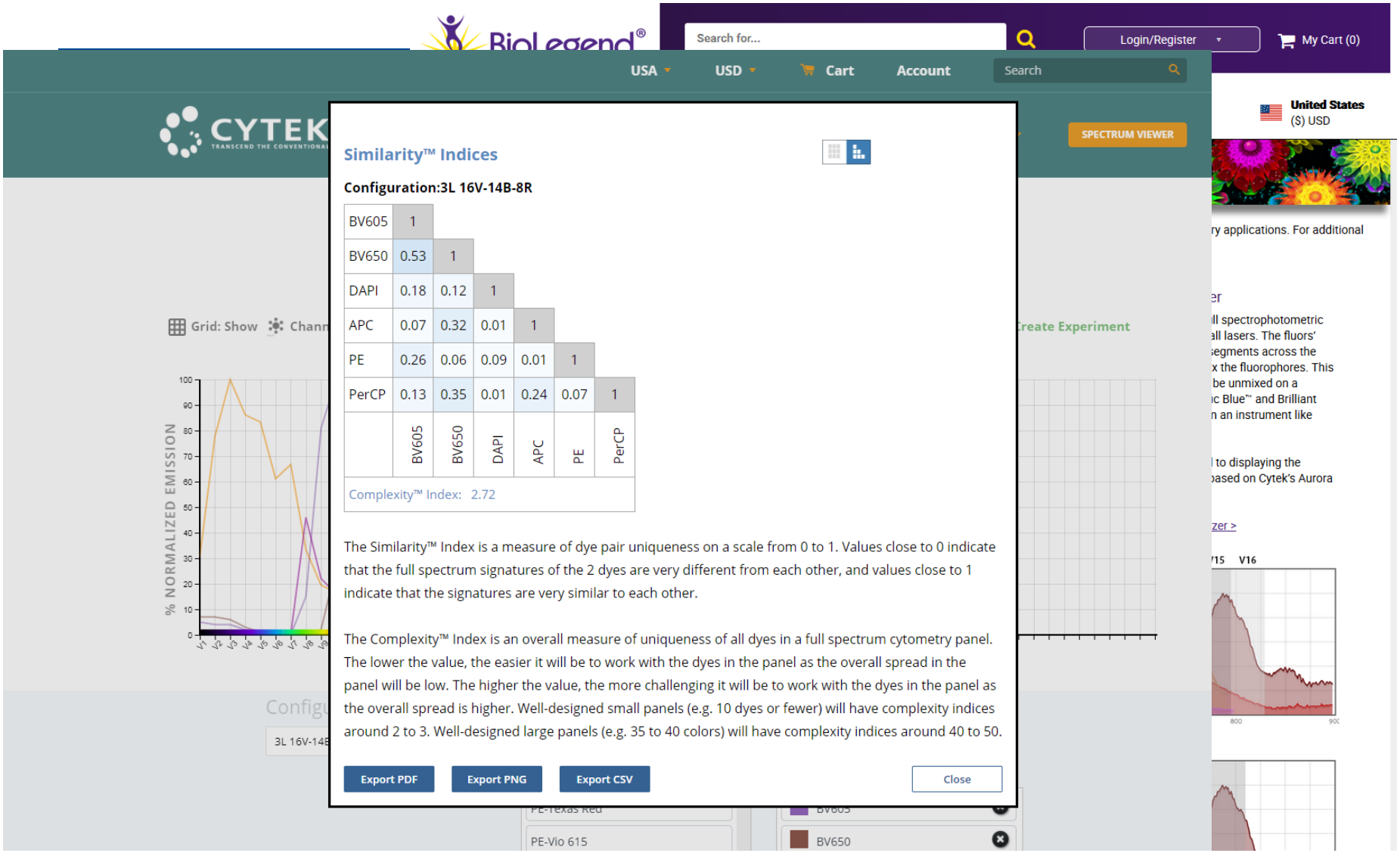
- 1.) answer questions (slide 3): what you want to look at
  - literature search: already described panels → OMIPs

    OMIPs = Optimized Multicolor Immunofluorescence Panel published e.g. in Cytometry Part A
  - are there antibodies you have to use (already in the lab) or without alternative
  - fill in these in the configuration
- 2.) search for antibodies for tertiary antigens, most important targets, use the brightest dyes (PE, BV421, APC, PE-Cy7)
- 3.) spread the antibodies over different lasers to reduce needed compensation

# panel design – step by step

- 4.) fill the gaps with additional markers (dim and moderate)
  (CD3-FITC, CD45-PerCP, …)
- 5.) include live-dead stain: fixable (Zombie, FVS…) or non-fixable (PI, 7-AAD, DAPI)
- 6.) optimize your panel:
  - live-dead stain and ubiquitous markers at the edges of the spectrum (less compensation)
  - search for better clones, better (tandem-) dyes with less spread (e.g. PerCP → PerCP-Cy5.5 or BB700 or PerCP-Vio700)
  - look for fixability of the dyes

# Webtools


BioLegend®
Search for...
Login/Register
My Cart (0)
USA
USD
Cart
Account
Search
CYTEK
SPECTRUM VIEWER
United States
($) USD
Similarity™ Indices
Configuration:3L 16V-14B-8R
BV605 1
BV650 0.53 1
DAPI 0.18 0.12 1
APC 0.07 0.32 0.01 1
PE 0.26 0.06 0.09 0.01 1
PerCP 0.13 0.35 0.01 0.24 0.07 1
BV605 BV650 DAPI APC PE PerCP
Complexity™ Index: 2.72
The Similarity™ Index is a measure of dye pair uniqueness on a scale from 0 to 1. Values close to 0 indicate that the full spectrum signatures of the 2 dyes are very different from each other, and values close to 1 indicate that the signatures are very similar to each other.
The Complexity™ Index is an overall measure of uniqueness of all dyes in a full spectrum cytometry panel. The lower the value, the easier it will be to work with the dyes in the panel as the overall spread in the panel will be low. The higher the value, the more challenging it will be to work with the dyes in the panel as the overall spread is higher. Well-designed small panels (e.g. 10 dyes or fewer) will have complexity indices around 2 to 3. Well-designed large panels (e.g. 35 to 40 colors) will have complexity indices around 40 to 50.
Export PDF
Export PNG
Export CSV
Close
Grid: Show
% NORMALIZED EMISSION
PE-Vio 615
BV650

# example panel - Tregs

- we want to stain human Tregs in PBMCs
- What markers we need:
  - CD45 or CD3, CD4, CD25, FoxP3
  - fixable live dead stain, because of FoxP3-staining

# 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 | |

# example panel - Tregs

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| 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 | |

# example panel - Tregs

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| | | 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 | |

# example panel - Tregs

Panel Template BD FACS Aria IIIu

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| | | 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 | |
| | | 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 | |

# 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 | |
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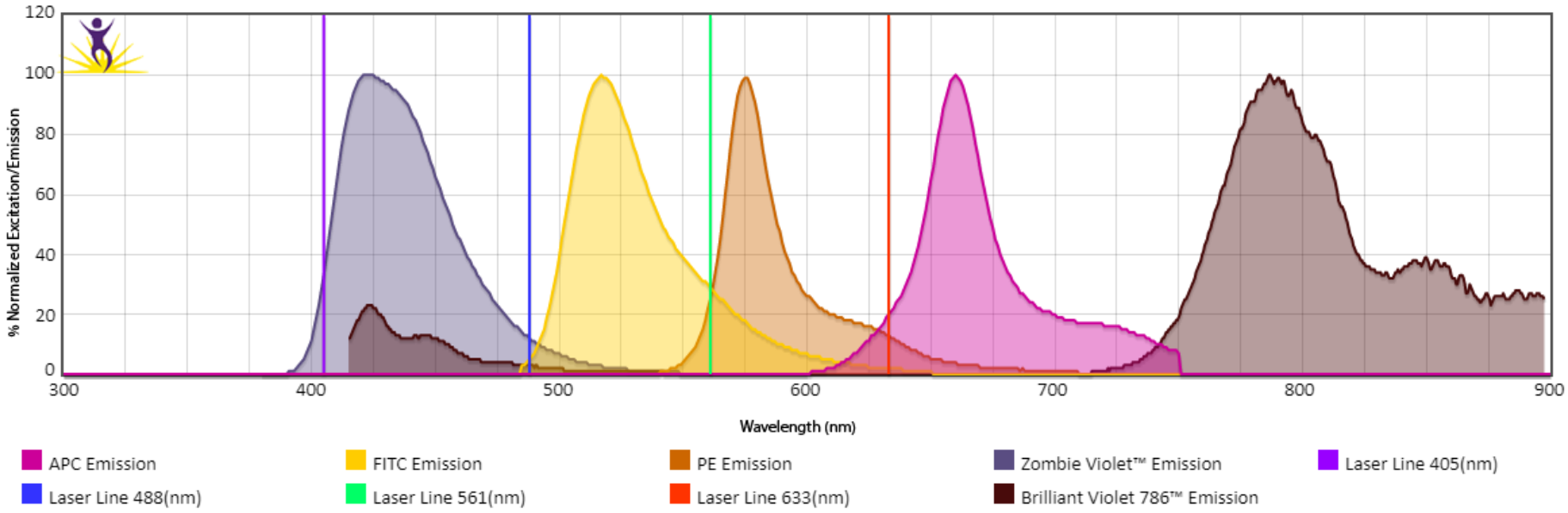
| | | PE-TexasRed, PI, mCherry, PE-Dazzle594, ZombieRed, AF594 | C | 610/20, 600 LP | |
| | | PE, DsRed, Cy3 | D | 582/15 | **FoxP3 - PE** |
| 3 | Blau (488 nm) | PerCP-Cy5.5, PerCP, BB700, PerCP/Vio700, PerCP-eFluor710 | A | 695/40, 655 LP | |
| | | FITC, GFP/YFP, AlexaFluor488, Zombie Green, VioBright515/FITC, 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** |

# check your panel



# TAKE Home Message

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

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Universitätsmedizin Rostock

Thank you for your attention!

Next topic (10th August):
„FACSorting – selecting the fit ones“

Nächstes Mal (am 10. August):
„FACSorting – die Fitten in`s Töpfchen “