Research Flow Cytometry Core

Multicolor Panel Design on Spectral Instruments

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General overview

- Define your markers
- Know your fluorochromes
- Match the markers and the fluorochromes
- Plan your experiment

Define your markers

- Understanding your experiment and what you want to examine
  - What is the question you are trying to answer?
  - How will you analyze the data to answer the question?

- Which markers will you need to characterize your cells
  - What is already known in the field?
  - Is there a published panel for the subset of cells you are interested in? (OMIP)
  - Which clones have been used by others in publications?
  - Will you need common markers to normalize your data between samples? (CD45)
  - Always add a Live/Dead stain to remove non-specific binding from dead cells.
  - Will it help to have a dump channel to remove all the subsets of cells you are not interested in?

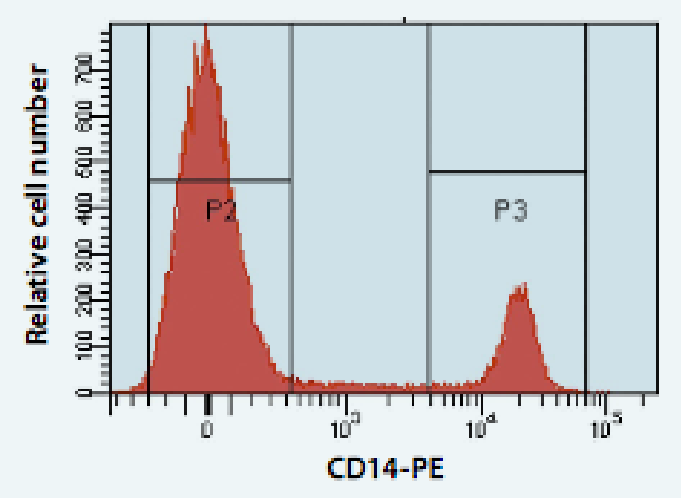
Define your markers

- How are these antigens expressed (primary, secondary and tertiary)?
  - What is already known in the field?
  - Will your experiment affect expression of the molecules that you are assessing? (CD3 surface expression will decrease when T cells are activated)

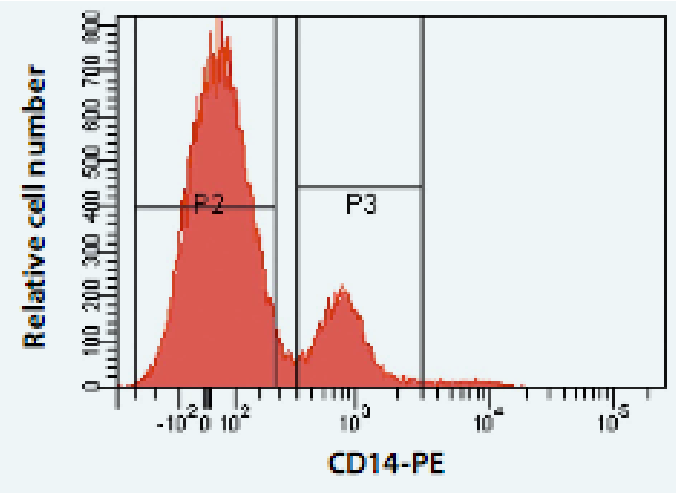
- Are some antigens co-expressed on a subset of cells?
  - What is already known in the field? BD Human and Mouse CD marker handbook
  - What are you expecting?
  - Don’t forget your fluorescent proteins/probes

Define your markers – choice of clone

Clone: TÜK4



Clone: MφP9



Source: Miltenyi Biotech

- Difference could be due to:
  - Differential binding affinity of Abs?
  - Conformation or position of epitope as to how well it is accessible for the Ab to bind?
- Find the information in a paper… (BenchSci)

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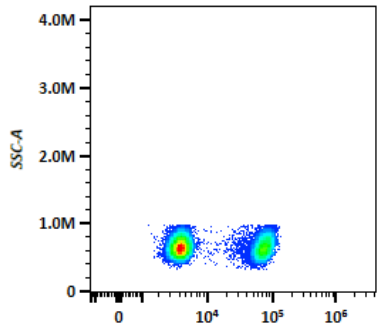
Define your markers – antigen density

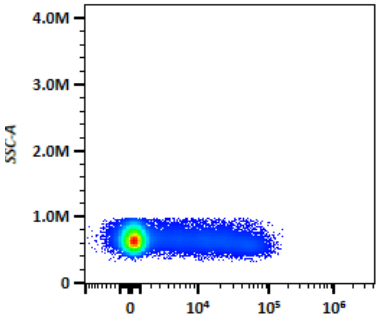
- ***Primary:*** high density, on and off expression
- ***Secondary:*** relatively high density, continuous expression
- ***Tertiary:*** uncharacterized or expressed at low levels

Y. Mahnke and M. Roederer. Clin Lab Med:2007. 27:469

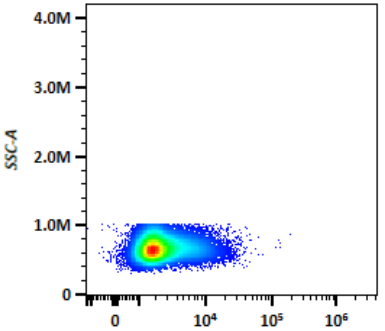
Primary
e.g. CD4



Secondary
e.g. CCR7



Tertiary
e.g. PD-1



PRIMARY

CD3	CD19	CD56	CD14
CD4		CD16	CD16
CD8			CD11b
			HLA-DR

SECONDARY

CD45RA	IgD	CD8
CCR7	CD27	CD57
CD27	CD38	
CD28	CD1c	
CD127	CD95	
HLA-DR		

TERTIARY

PD-1	CD1c	CD123
CD25		CD11c
TCR *g/d*		

Level of Antigen Expression

Define your markers – antigen density

Where to find the Antigen expression information?

- Reagent companies host good resources: (BioLegend, BD Biosciences, ebioscience)
- Literature search
- www.genecards.org for tissue specific information

Common surface markers on blood cells (BioLegend)

Cell	Antigen	Molecules per Cell	Reference
T cell	TCR	100,000	Cho, B. et al. 2000. *PNAS.* 98:1723.
	CD2	55,000	Ginaldi, L. et al. 1996. *J Clin Pathol.* 49:539.
	CD3	124,000	Ginaldi, L. *et al. 1996. Br J Haematol. 93:921.*
	CD5	90,000	Ginaldi, L. et al. 1996. J Clin Pathol. 49:539.
	CD7	20,000	Ginaldi, L. *et al. 1996. Br J Haematol. 93:921.*
	CD45	>200,000	Glatting, G. *et al. 2006. J Nucl Med. 47:1335.*
CD4+ T cell	CD4	100,000	Davis, K. *et al. 1998. Cytometry. 33:197.*
	CD28	20,000	Bryl, E. *et al. 2005. Arthritis Rheum. 52:2996.*
	CCR5	4,000-24,000	Reynes, J. *et al. 2006. J Infect Dis. 181:927.*
CD8+ T cell	CD8	90,000	Takada, S. *et al. 1987. J Immunol. 139:3231.*
	CD28	15,000	Bryl, E. *et al. 2005. Arthritis Rheum. 52:2996.*
B cell	CD19	18,000	Ginaldi, L. *et al.* 1998. *Pathobiology.* 66:17.
	CD20	109,000	Ginaldi, L. *et al.* 1998. *Pathobiology.* 66:17.
	CD21	210,000	Ginaldi, L. *et al.* 1998. *Pathobiology.* 66:17.
	CD22	14,000	Ginaldi, L. *et al.* 1998. *Pathobiology.* 66:17.
	HLA-DR	85,000	Ginaldi, L. *et al.* 1998. *Pathobiology.* 66:17.
	CD11a	10,000	Unternaehrer, J. *et al.* 2007. *PNAS.* 104:234.
	CD40	2,000	Unternaehrer, J. *et al.* 2007. *PNAS.* 104:234.
	CD86	16,000	Unternaehrer, J. *et al.* 2007. *PNAS.* 104:234.
	CD80	2,000	Unternaehrer, J. *et al.* 2007. *PNAS.* 104:234.
Dendritic cell	CD11a	27,000	Unternaehrer, J. *et al.* 2007. *PNAS.* 104:234.
	CD40	17,000	Unternaehrer, J. *et al.* 2007. *PNAS.* 104:234.
	CD80	132,000	Unternaehrer, J. *et al.* 2007. *PNAS.* 104:234.
	CD86	208,000	Unternaehrer, J. *et al.* 2007. *PNAS.* 104:234.
Monocyte	CD14	110,000	Antal-Szalmas, P. *et al. 1997. J. Leukoc. Biol. 61:721.*
	CD32	21,000	Antal-Szalmas, P. *et al. 1997. J. Leukoc. Biol. 61:721.*
	CD64	13,000	Antal-Szalmas, P. *et al. 1997. J. Leukoc. Biol. 61:721.*
Neutrophil	CD14	3,500	Antal-Szalmas, P. *et al. 1997. J. Leukoc. Biol. 61:721.*
	CD16	225,000	Antal-Szalmas, P. *et al. 1997. J. Leukoc. Biol. 61:721.*
NK cell	CD56	10,000	Ginaldi, L. *et al.* 1996. *J Clin Pathol.* 49:539.
Red Blood Cell	Glycophorin A	340,000	Antal-Szalmas, P. *et al. 1997. J. Leukoc. Biol. 61:721.*
Basophil	CD23	15,000	MacGlashan, D. *et al. 2000. J Leuk Biol. 68:479.*

Disclaimer: While these numbers are published data, actual numbers can vary significantly depending on factors such as: antibody clone used, patients, method of molecule number calculation, flow cytometer and fluorochromes used. BioLegend recommends that these numbers only be used as relative indications of high, intermediate, or low expression of proteins on certain cell types. We also recommend viewing our product data sheets to view actual fluorescence data for fluorochrome-conjugated antibodies.

Know your fluorochromes

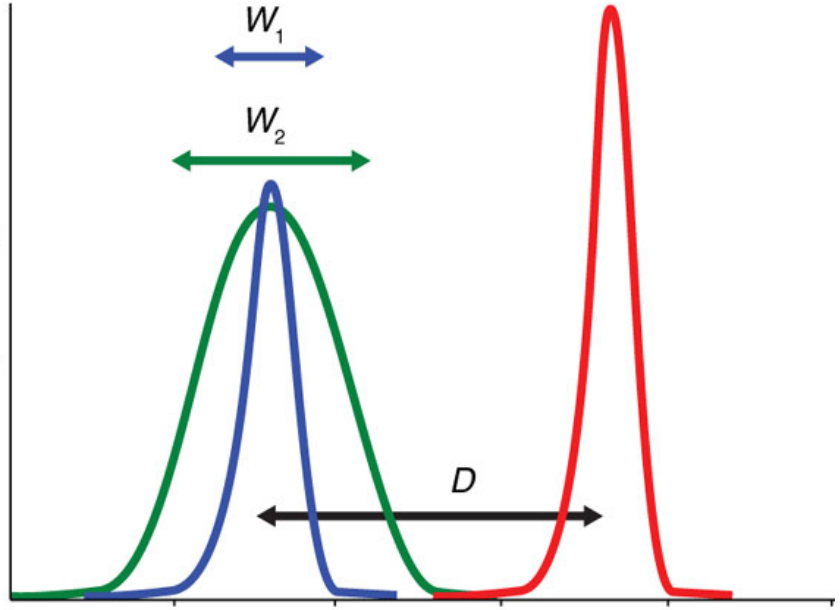
- Which instrument are you going to use?
  - Instrument Configuration, setup and QC
- How bright are the fluorochromes?
  - Have an idea of the stain index on the specific instrument you are going to use
- Which fluorochromes can’t be discriminated efficiently?
  - Look at the similarity matrix (specific to the instrument) and consider any fluorescent proteins
  - Have an idea of the spread matrix

Know your fluors – stain Index (SI)

- The SI calculation is used to quantify the effective brightness of a fluorochrome accounting for the:
  - Reagent (Antigen density, fluorochrome used)
  - Instrument (PMT gain settings, laser power)

$$SI = D/W$$

$$SI = \frac{MFI^{\text{positive pop}} - MFI^{\text{negative pop}}}{2rSD^{\text{negative pop}}}$$



Holden Maecker & Joe Trotter. Nature Methods 5, (2008).

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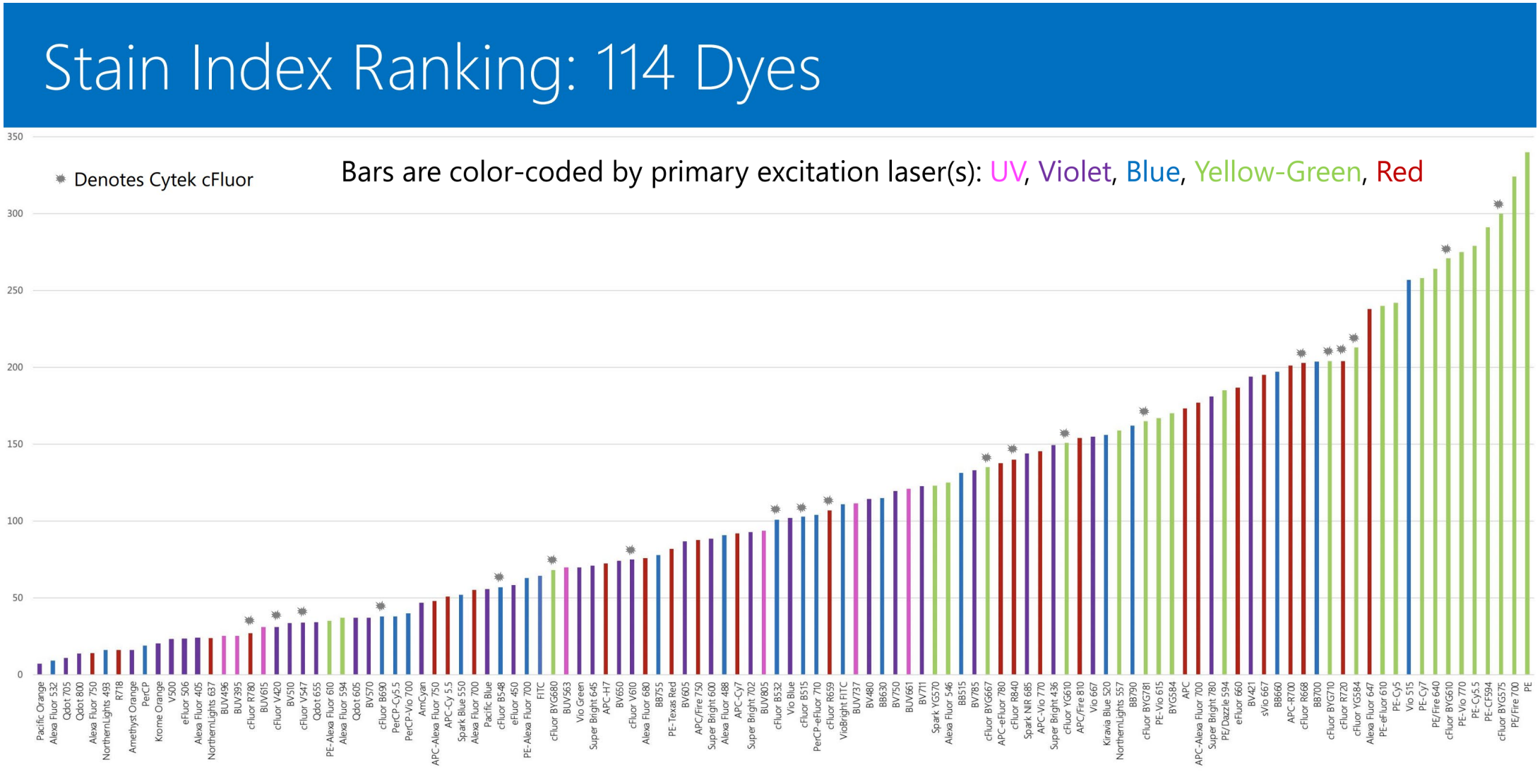
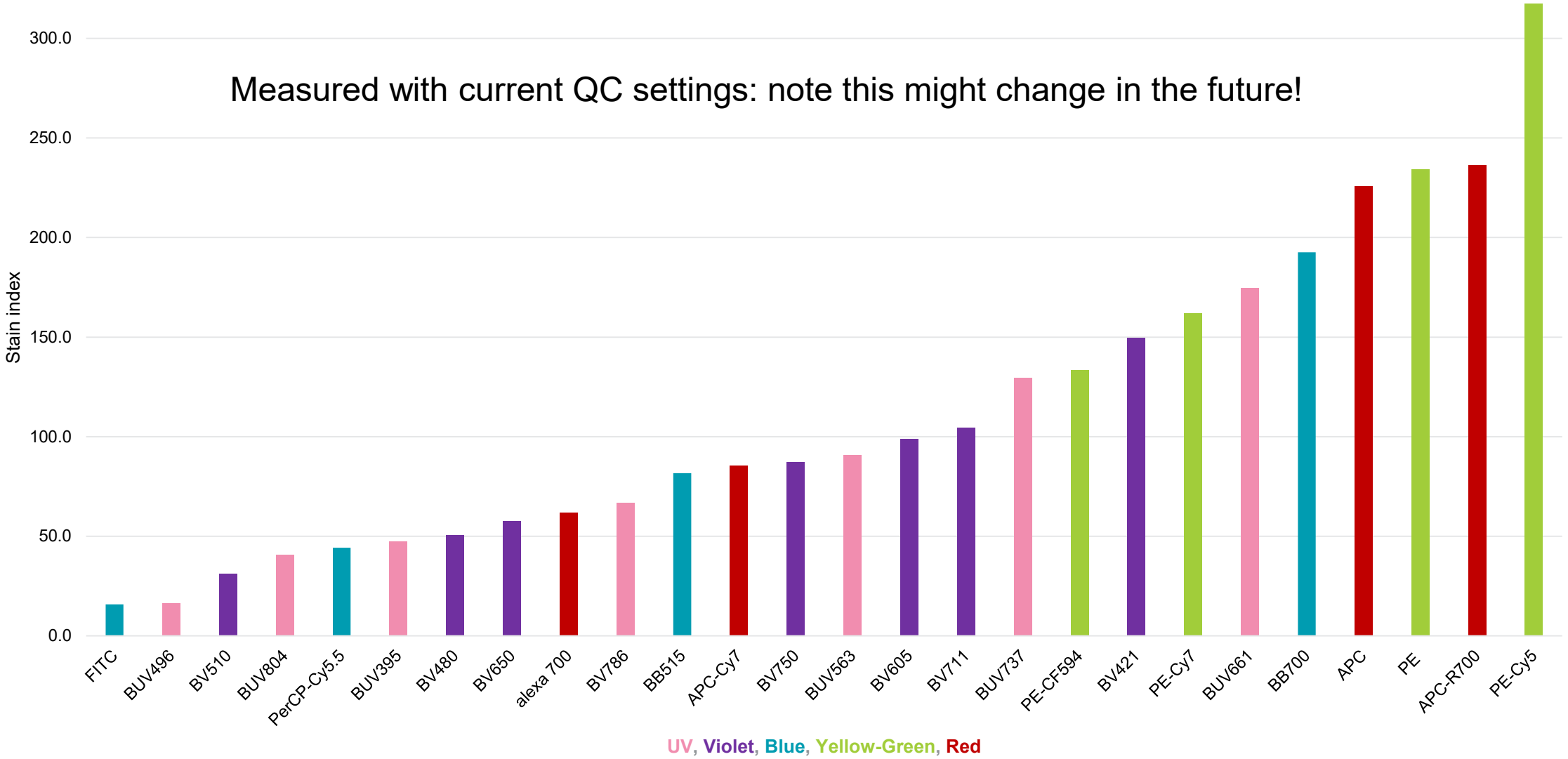
Know your fluors – Aurora brightness ranking

From Cytek [fluorochrome guide]



Know your fluors – Bigfoot brightness ranking



Know your fluors – spectral similarity matrix

Aurora

	BUV395	BV421	BB700	LIVE DEAD NIR	PE	APC	PE-Cy7	Alexa Fluor 488	BV786	BUV737
BUV395	1									
BV421	0.05	1								
BB700	0	0	1							
LIVE DEAD NIR	0	0	0.16	1						
PE	0.01	0	0.06	0	1					
APC	0	0	0.33	0.15	0.04	1				
PE-Cy7	0	0	0.17	0.21	0.02	0.05	1			
Alexa Fluor 488	0.01	0	0.02	0	0.07	0	0	1		
BV786	0	0.2	0.22	0.33	0	0.04	0.17	0	1	
BUV737	0.03	0	0.3	0.34	0	0.24	0.14	0	0.29	1

Complexity™ Index: 2.36

Bigfoot

	BUV 395	BV 421	BB 700	LIVE-DEAD Fixat	PE	APC	PE-Cy7	Alexa 488	BV 786	BUV 737
BUV 395		0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.03
BV 421	0.14		0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00
BB 700	0.00	0.00		0.13	0.04	0.14	0.31	0.02	0.12	0.22
LIVE-DEAD Fixable Near-	0.00	0.00	0.13		0.00	0.05	0.12	0.00	0.12	0.10
PE	0.00	0.00	0.04	0.00		0.02	0.30	0.17	0.00	0.00
APC	0.00	0.00	0.14	0.05	0.02		0.03	0.00	0.00	0.01
PE-Cy7	0.00	0.00	0.31	0.12	0.30	0.03		0.07	0.09	0.12
Alexa 488	0.00	0.00	0.02	0.00	0.17	0.00	0.07		0.00	0.00
BV 786	0.01	0.07	0.12	0.12	0.00	0.00	0.09	0.00		0.22
BUV 737	0.03	0.00	0.22	0.10	0.00	0.01	0.12	0.00	0.22	

Complexity Index: 1.73

Larger numbers = more similar spectra

From Cytek full spectrum viewer under Similarity & Complexity

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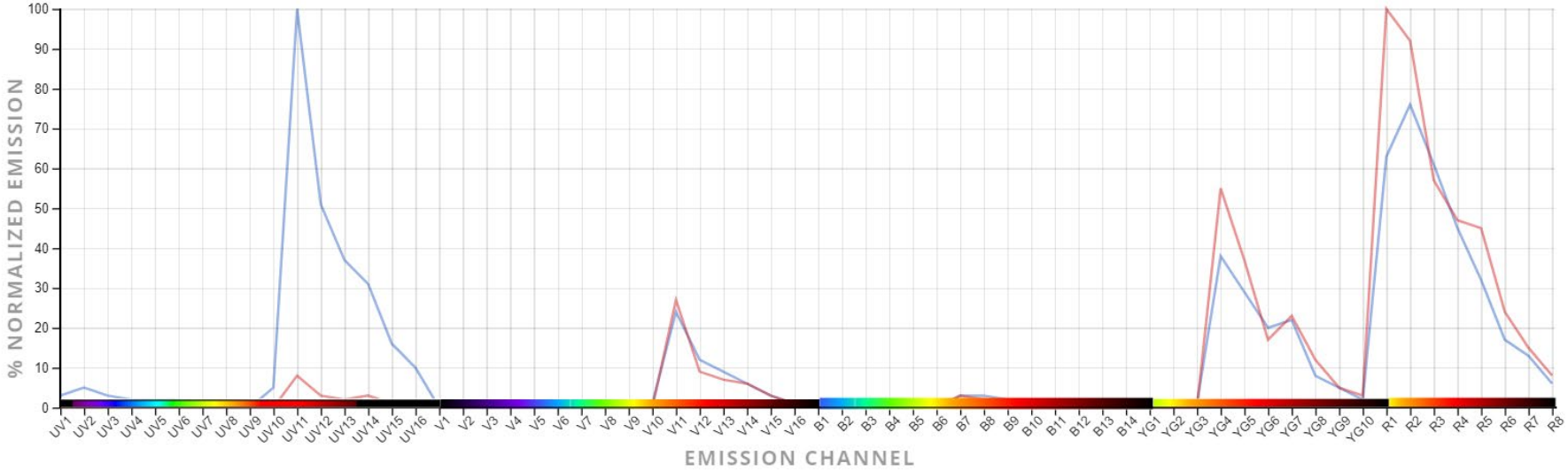
Know your fluors – similarity matrix doesn't always predict spread!



BUV661	1	
APC	0.78	1
	BUV661	APC

Complexity™ Index: 2.84

BUV661

APC

BUV661+ cells will spread APC ***more*** than APC+ cells will spread BUV661!

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Know your fluors – Aurora spread matrix

…will increase spread of this parameter

Staining with this fluorochrome…

	BUV395	LIVE/DEAD Blue	BUV496	BUV563	BUV615	BUV661	BUV737	BUV805	BV421	Super Bright 436	eFluor 450	BV480	BV510	Pacific Orange	BV570	BV605	BV650	BV711	BV750	BV785	BB515	FITC	Spark Blue 550	PerCP	PerCP-Cy5.5	PerCP-eFluor 710	PE	cFluor YG584	PE/Dazzle 594	PE-Alexa Fluor 610	PE-Cy5	PE-Alexa Fluor 700	PE-Cy7	PE/Fire 810	APC	Alexa Fluor 647	Spark NIR 685	APC-R700	APC-H7	APC/Fire 810
BUV395		1.97	5.06	3.27	0.44	0.21	0.17	0.65	1.18	2.75	1.22	1.10	3.78	2.76	1.08	0.78	0.67	1.35	1.05	0.36	5.46	5.89	0.83	0.67	1.79	0.70	1.27	0.90	1.05	1.32	1.58	0.87	0.40	0.45	1.23	1.22	2.03	0.76	0.47	0.15
LIVE/DEAD Blue	5.01		9.17	0.61	0.15	1.00	0.27	0.44	3.14	4.29	5.68	2.13	10.94	7.55	2.62	1.05	1.34	0.61	0.07	0.54	2.23	2.19	2.40	0.81	0.38	0.28	0.92	0.37	2.98	0.44	1.14	0.44	0.43	0.18	0.87	1.61	0.85	0.62	0.27	0.41
BUV496	0.83	0.94		1.44	0.05	0.45	0.32	0.40	0.43	1.70	0.66	1.44	6.21	4.87	2.10	0.71	0.39	0.25	0.12	0.37	4.89	4.13	0.64	0.62	0.73	0.40	0.67	0.38	0.41	0.57	0.91	0.39	0.31	0.04	0.57	1.13	0.50	0.17	0.32	0.08
BUV563	0.47	0.17	1.29		0.42	0.70	0.44	0.64	0.55	0.89	0.44	0.89	3.04	3.25	1.76	0.96	0.42	0.23	0.23	0.49	1.22	2.52	1.23	2.09	1.22	0.71	7.79	2.56	4.81	4.27	2.85	0.61	0.31	0.16	1.00	0.99	0.81	0.32	0.28	0.12
BUV615	0.85	0.74	3.82	3.00		3.07	2.39	3.40	0.99	1.18	0.61	1.23	4.08	4.43	1.62	1.21	1.13	1.03	1.22	1.15	2.27	6.08	2.23	1.92	2.06	1.47	3.32	2.54	3.04	2.97	3.17	1.84	0.99	0.32	1.38	2.59	1.18	1.52	0.48	0.50
BUV661	0.55	0.55	1.34	0.87	0.23		3.40	3.22	0.67	0.99	0.40	0.32	1.84	4.32	0.78	0.43	2.69	1.42	1.46	1.31	3.98	5.07	1.69	3.18	2.53	1.99	1.07	0.76	4.80	4.92	5.52	2.50	1.27	0.53	4.47	8.66	5.33	2.88	2.85	0.68
BUV737	0.83	0.90	1.68	1.07	0.09	0.71		6.24	0.95	0.97	0.34	0.20	1.41	3.85	1.04	0.40	1.41	1.85	3.14	2.54	0.62	4.05	0.90	2.86	3.30	3.97	0.13	0.41	0.75	1.22	1.61	2.20	1.16	1.02	3.11	5.60	4.13	3.20	2.37	1.10
BUV805	1.16	1.00	1.66	1.20	0.16	0.13	1.27		0.94	0.50	0.41	0.21	1.15	3.00	0.37	0.74	0.87	0.68	0.30	1.26	1.30	1.66	0.31	1.17	0.44	0.83	0.15	0.03	1.28	0.50	0.43	0.19	0.58	0.62	1.05	2.39	2.01	0.31	2.18	1.18
BV421	0.24	0.89	1.35	0.75	0.11	0.15	0.15	0.08		9.62	5.31	2.47	6.39	5.35	1.58	0.76	0.04	0.27	0.39	0.21	0.71	0.02	0.30	0.44	0.50	0.36	0.26	0.21	0.99	0.57	0.62	0.18	0.08	0.17	0.38	0.44	0.19	0.40	0.09	0.05
Super Bright 436	0.14	0.73	0.37	0.44	0.09	0.15	0.15	0.11	5.17		4.66	2.64	6.88	5.83	1.82	0.91	0.30	0.20	0.32	0.17	1.04	1.85	0.83	0.62	0.76	0.41	0.27	0.25	0.69	0.92	0.62	0.25	0.27	0.16	0.60	0.80	0.71	0.32	0.34	0.13
eFluor 450	0.59	0.95	2.27	0.95	0.08	0.36	0.24	0.44	5.34	10.54		2.89	7.24	5.93	1.78	0.92	0.38	0.45	0.07	0.61	1.25	3.87	0.40	1.09	0.55	0.62	0.17	0.19	0.98	1.03	0.44	0.64	0.12	0.08	0.98	1.48	0.89	0.24	0.58	0.45
BV480	0.34	0.80	3.44	1.92	0.39	0.22	0.07	0.26	1.99	3.82	2.50		12.68	11.33	4.71	2.50	0.65	0.62	0.50	0.49	6.62	7.16	1.89	0.78	0.38	0.27	1.29	0.51	1.21	0.54	0.40	0.15	0.29	0.18	0.41	0.71	0.55	0.33	0.39	0.15
BV510	0.24	0.78	2.22	2.35	0.63	0.54	0.07	0.65	1.30	2.55	1.76	2.36		9.87	5.17	3.37	1.37	0.83	0.89	0.98	5.56	5.49	2.14	1.31	1.06	0.30	1.39	0.49	2.52	1.36	0.17	0.34	0.35	0.28	0.23	0.94	1.17	0.60	0.34	0.39
Pacific Orange	0.18	2.13	2.89	1.24	0.57	0.32	0.81	0.62	1.85	2.81	0.31	2.40	2.67		5.13	3.77	2.12	1.01	0.95	1.99	5.31	2.92	2.45	3.06	2.15	0.90	1.41	0.68	2.71	3.50	0.71	0.78	0.61	0.18	1.17	1.08	1.96	0.77	0.11	1.56
BV570	0.48	0.95	1.49	1.63	0.71	0.42	0.38	0.58	2.00	2.89	1.75	1.26	3.67	4.99		3.30	1.61	0.46	1.01	0.66	4.92	6.49	2.24	1.77	1.27	0.37	3.44	1.51	4.23	3.34	2.20	1.03	0.10	0.32	0.77	2.16	0.31	0.72	0.12	0.29
BV605	0.17	0.75	0.99	0.59	0.84	1.00	0.85	1.04	1.16	1.67	0.86	0.78	4.05	5.73	2.21		2.28	1.23	1.49	1.60	6.23	7.50	2.69	1.86	1.51	1.25	2.94	2.44	5.30	3.94	3.15	1.53	0.87	0.37	1.43	2.66	1.65	0.89	1.01	0.30
BV650	0.14	0.95	1.72	1.14	0.34	1.20	0.96	1.18	1.14	2.52	1.44	0.78	3.50	1.93	0.76	0.83		1.87	2.18	2.01	2.87	3.58	1.23	1.53	1.39	0.93	0.97	0.59	3.43	3.14	2.97	1.42	0.78	0.34	2.34	4.38	2.98	1.54	1.54	0.53
BV711	0.22	0.79	1.06	0.67	0.17	0.46	1.83	2.28	1.42	2.53	1.21	0.79	2.02	1.13	0.93	0.81	0.95		4.05	4.16	2.16	2.37	1.07	1.27	1.90	1.96	0.49	0.09	0.15	0.99	1.24	2.96	1.16	0.53	2.00	2.89	3.44	3.08	1.92	1.18
BV750	0.21	0.90	1.70	0.89	0.03	0.15	2.47	2.80	1.29	2.12	0.96	0.72	2.85	1.84	0.85	0.22	0.48	1.65		6.14	2.75	2.10	0.70	0.52	0.88	1.15	0.44	0.23	0.99	0.82	0.81	0.56	0.69	0.62	1.16	1.99	1.78	0.97	1.70	1.54
BV785	0.29	0.72	0.85	0.83	0.07	0.12	1.17	2.99	1.49	2.05	1.19	0.56	0.78	0.41	0.65	0.30	0.53	0.81	2.93		1.52	0.90	0.64	1.06	0.61	0.39	0.23	0.09	0.96	0.74	0.86	0.30	0.71	0.50	0.83	1.58	1.37	0.05	1.88	2.11
BB515	0.15	0.53	0.73	0.51	0.38	0.12	0.08	0.10	0.36	0.84	0.66	0.89	5.22	7.12	2.27	1.67	0.56	0.47	0.49	0.74		21.91	4.37	1.02	0.72	0.73	1.05	0.69	2.46	1.52	1.19	0.54	0.33	0.26	0.55	1.09	0.70	0.17	0.24	0.07
FITC	0.22	0.53	1.25	1.05	0.44	0.22	0.45	0.20	0.60	1.30	0.73	0.66	4.24	7.20	2.21	1.71	0.57	0.67	0.60	1.05	18.20		3.58	0.29	0.47	1.13	1.08	0.97	2.53	2.17	1.23	0.33	0.19	0.26	1.10	0.60	0.71	0.60	0.39	0.44
Spark Blue 550	0.65	0.70	2.29	3.78	2.54	1.33	2.27	0.98	0.33	1.79	2.07	0.17	6.47	8.24	3.85	1.07	1.03	0.42	0.74	0.49	12.41	16.94		2.69	2.09	1.48	3.67	2.47	3.80	2.43	2.41	0.37	0.12	0.61	0.41	3.34	2.04	1.73	0.54	0.13
PerCP	0.58	0.27	5.15	2.60	0.25	1.24	1.22	1.01	1.10	4.52	2.00	1.29	3.74	3.85	0.82	1.54	2.86	1.54	1.03	1.28	1.38	4.87	0.69		5.34	2.82	2.00	1.45	7.45	7.81	7.90	1.65	1.07	0.84	3.21	4.03	2.71	2.12	1.27	0.89
PerCP-Cy5.5	0.69	1.50	3.48	2.23	0.45	1.10	1.86	2.41	0.92	3.31	0.82	0.60	3.44	2.26	0.43	1.52	2.75	2.83	2.79	2.56	0.45	5.08	1.04	9.87		5.90	1.51	1.46	6.44	7.63	7.47	4.91	2.60	1.44	3.03	6.52	6.05	3.34	3.28	1.15
PerCP-eFluor 710	0.36	1.02	1.49	1.03	0.21	0.66	1.78	2.06	0.76	1.31	0.48	0.11	3.00	3.20	1.32	0.23	1.53	4.24	3.60	3.30	4.15	4.85	1.74	6.61	7.36		0.56	0.81	3.36	3.78	3.71	8.49	2.95	1.92	2.48	4.98	5.57	4.18	2.93	1.36
PE	0.17	0.37	0.58	1.16	0.54	0.36	0.13	0.18	0.34	0.45	0.39	0.45	2.44	3.04	2.46	1.62	0.76	0.40	0.36	0.38	3.52	3.91	1.83	2.85	1.72	1.12		3.62	6.60	5.90	3.86	0.94	0.50	0.26	0.92	1.65	0.78	0.37	0.39	0.09
cFluor YG584	0.12	0.22	1.39	1.48	1.75	1.11	0.73	0.22	0.76	1.14	0.46	0.20	2.07	1.92	1.38	0.95	0.60	0.38	0.45	0.16	1.12	1.95	0.41	1.70	1.38	0.99	4.13		2.83	3.55	2.38	1.24	0.71	0.33	1.03	1.78	1.50	0.86	0.19	0.42
PE/Dazzle 594	0.30	0.69	0.60	0.36	1.40	0.59	0.34	0.39	0.22	0.12	0.05	1.01	5.55	7.93	2.51	2.76	1.41	0.90	0.79	0.60	7.69	9.01	3.74	4.10	3.07	2.13	5.29	2.71		6.03	4.87	2.09	1.18	0.60	1.62	2.38	2.07	1.07	0.95	0.30
PE-Alexa Fluor 610	0.51	0.84	2.77	0.68	1.20	0.94	0.48	0.82	0.57	1.08	1.61	0.76	4.98	7.13	2.52	2.05	1.65	0.89	0.26	1.59	6.69	9.31	3.35	6.47	5.45	3.32	4.05	1.81	7.59		7.08	3.80	2.19	1.06	3.77	4.17	3.98	1.77	1.84	0.56
PE-Cy5	0.14	0.48	0.62	0.38	0.82	0.95	0.92	0.68	0.51	0.44	0.57	1.00	5.35	8.02	2.34	0.68	2.60	1.65	1.41	1.09	9.13	11.46	4.51	9.37	5.68	3.80	2.59	2.45	11.49	12.90		5.24	2.22	1.11	4.21	11.07	6.98	3.17	3.13	0.52
PE-Alexa Fluor 700	0.33	0.59	0.86	0.23	0.53	0.86	3.45	2.27	0.66	0.29	0.31	0.16	0.95	1.33	0.08	0.29	0.42	1.81	1.18	0.90	2.12	4.11	1.60	4.90	5.60	9.43	1.44	0.77	1.34	1.53	1.79		3.79	2.34	2.58	5.12	6.00	5.46	1.37	0.79
PE-Cy7	0.10	0.46	0.76	0.41	0.30	0.05	0.94	1.69	0.20	0.37	0.56	1.18	6.38	9.86	3.19	1.60	0.94	2.73	1.00	2.27	11.01	14.65	4.76	1.20	2.52	3.77	1.01	0.68	2.34	1.18	0.66	2.06		3.14	1.89	4.23	3.65	2.43	1.82	1.64
PE/Fire 810	0.58	0.51	1.67	1.63	0.44	1.13	0.74	4.94	0.53	0.79	0.60	0.58	0.98	1.60	1.12	0.36	0.86	0.37	0.40	1.13	3.97	5.60	2.33	2.18	2.25	1.74	3.31	1.93	1.87	1.88	1.50	1.34	4.27		1.46	2.13	2.67	1.65	1.13	1.69
APC	0.42	0.91	1.24	0.74	0.22	0.86	1.14	0.88	0.76	0.90	0.50	0.30	2.05	3.39	1.40	0.04	3.06	1.27	1.29	1.07	5.16	5.49	1.98	2.33	4.03	2.04	1.45	1.02	5.38	6.03	7.34	2.43	1.33	0.58		10.33	6.88	2.97	2.96	0.69
Alexa Fluor 647	0.24	0.64	1.15	0.67	0.21	0.58	0.68	0.38	0.50	0.92	0.28	0.36	0.76	1.60	0.22	0.35	1.60	0.88	0.65	0.67	1.52	2.61	0.79	1.78	1.65	0.97	0.64	0.71	3.17	4.05	4.56	1.62	0.95	0.36	3.95		5.68	2.81	2.71	0.49
Spark NIR 685	0.62	0.93	2.02	1.56	1.23	1.05	1.90	1.66	1.33	1.23	0.75	0.86	1.00	0.69	1.18	0.39	0.93	0.08	1.08	0.59	1.87	2.16	2.39	2.65	1.98	2.08	0.97	0.63	1.16	1.67	1.78	1.83	0.93	0.50	5.44	11.18		4.66	3.01	1.04
APC-R700	0.20	0.55	1.23	0.38	0.11	0.46	1.16	0.88	0.38	0.61	0.08	0.37	1.35	0.20	0.62	0.13	1.28	2.41	1.60	1.37	1.71	2.14	0.87	1.68	1.72	3.14	0.60	0.25	1.44	1.81	1.71	9.56	2.55	0.89	2.66	4.89	5.35		3.48	0.54
APC-H7	0.29	1.36	1.09	0.92	0.13	0.38	0.60	1.74	0.69	1.71	1.19	0.63	3.07	2.02	0.20	0.31	0.74	0.64	0.53	2.38	2.42	1.49	0.22	0.96	1.05	0.52	0.35	0.38	1.39	0.76	0.17	0.58	2.57	1.18	1.29	2.36	2.19	0.92		1.17
APC/Fire 810	0.68	0.47	1.87	0.86	1.11	0.56	1.20	5.68	0.54	1.26	1.30	0.51	1.59	1.22	0.63	0.62	0.62	0.30	0.64	0.95	2.78	2.22	0.35	0.49	0.27	0.52	0.99	0.28	0.90	0.16	0.73	0.51	1.48	1.89	2.17	2.05	2.45	1.23	2.62	
Sum	20.32	31.19	76.56	47.51	19.84	25.73	39.51	57.20	45.07	80.75	47.15	37.25	149.84	170.20	67.69	43.86	46.38	41.72	43.24	51.62	160.22	205.57	67.39	91.77	79.74	66.90	65.30	40.32	116.85	109.88	93.73	68.71	43.41	26.61	69.73	129.21	101.78	61.17	53.49	26.87

From Cytek fluorochrome guide

Know your fluors – the impact of spread

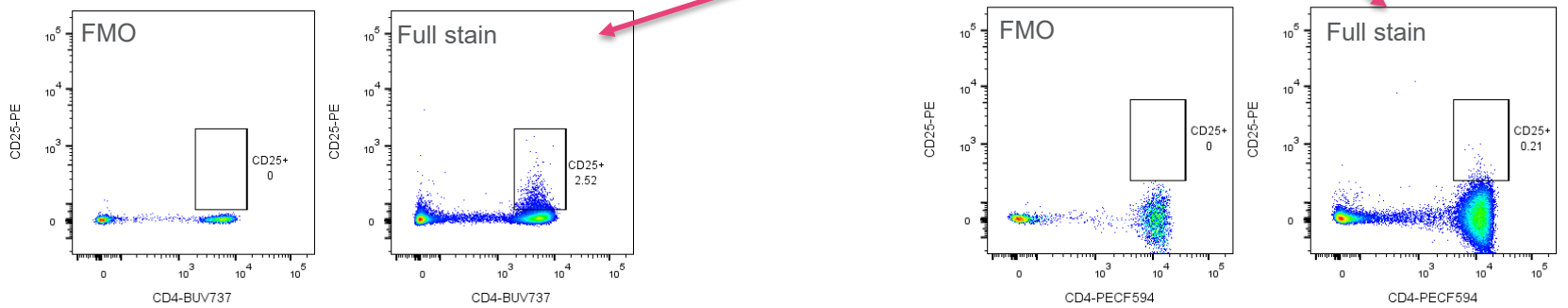
Too much spread will obfuscate a dim population of double positive cells!

…will increase spread of this parameter

Staining with this fluorochrome…

	FITC 530/30	PerCP-Cy5-5 710/50	BV421 450/50	BV510 525/50	BV605 610/20	BV650 670/30	BV711 710/50	BV786 780/60	PE 586/15	PE-CF594 610/20	PE-Cy5 670/30	PE-Cy5-5 710/50	PE-Cy7 780/60	APC 670/30	APC-Alexa 700 710/50	APC-Cy7 780/60	BUV396 378/29	BUV737 740/35
FITC		34.8	16.3	17.4	14.7	14.1	16.8	17.9	11.9	11.9	26.6	25	13.6	18.5	35.9	13.6	21.2	11.4
PerCP-Cy5.5	14.1		16.8	16.3	14.7	27.7	45.6	41.3	11.4	11.9	47.8	48.9	31.5	53.8	113	25	21.7	23.4
BV421	15.2	29.9		20.7	16.8	14.7	16.8	17.9	11.9	13	27.2	25	14.1	19	36.9	14.1	22.3	11.4
BV510	15.8	34.2	17.9		46.2	39.7	35.3	32	20.1	22.8	27.2	25	13.6	28.2	58.2	14.1	21.2	17.4
BV605	14.7	72.3	18.5	17.4		77.2	71.2	56.5	66.3	76.7	88.1	52.2	31.5	52.7	106	14.7	21.2	28.2
BV650	15.2	68	19.6	17.4	48.4		94.7	70.7	22.3	29.9	84.8	50	28.8	108	180	26.6	22.3	33.7
BV711	15.2	144	21.7	16.3	15.7	33.7		137	11.9	11.9	45.1	61.4	49.4	62.5	365	64.7	22.3	63
BV786	14.7	35.9	25.5	17.4	15.2	16.8	30.4		11.9	11.9	27.7	25.5	33.1	22.8	62	38	21.7	28.2
PE	16.8	107	16.3	16.9	36.4	28.8	29.3	21.2		109	99.6	54.9	28.8	32	48.9	14.1	21.7	14.7
PE-CF594	14.7	201	16.8	16.3	45.6	39.7	45.1	27.2	79.4		177	99.6	59.8	47.8	69.6	15.2	21.7	18.5
PE-Cy5	15.2	694	20.1	16.9	23.4	76.7	131	51.1	33.1	26.6		237	137	267	321	52.7	22.8	42.4
NY700	15.2	286	16.8	16.9	15.7	33.1	60.3	23.9	50.5	37.5	212		89.2	212	802	86	21.7	22.8
PE-Cy7	15.2	58.7	17.9	15.8	14.1	14.7	20.1	82.7	28.8	20.6	33.7	35.8		20.6	44	38.6	21.7	22.8
APC	15.2	84.3	15.8	16.3	15.2	30.4	33.7	27.2	11.9	15.2	146	62	40.7		244	41.8	21.7	25
Alexa 700	15.2	42.9	16.8	16.3	13.6	13.6	27.2	28.2	11.4	11.9	32.6	41.3	33.7	31.5		41.8	21.2	22.3
APC-Cy7	14.7	35.9	16.8	16.3	13.6	14.7	18.5	45.6	11.4	11.9	42.9	29.3	90.3	53.2	77.2		21.2	18.5
BUV395	15.2	29.9	16.3	16.9	13.6	13	16.3	17.4	11.4	11.9	26.6	23.9	14.1	17.9	36.4	13.6		11.4
BUV737	15.2	166	17.9	16.9	14.1	22.8	49.4	60.9	11.9	11.9	31.5	52.2	50	33.1	238	68.5	22.8	

Measured on Aria 1 (Fusion) by RFCC

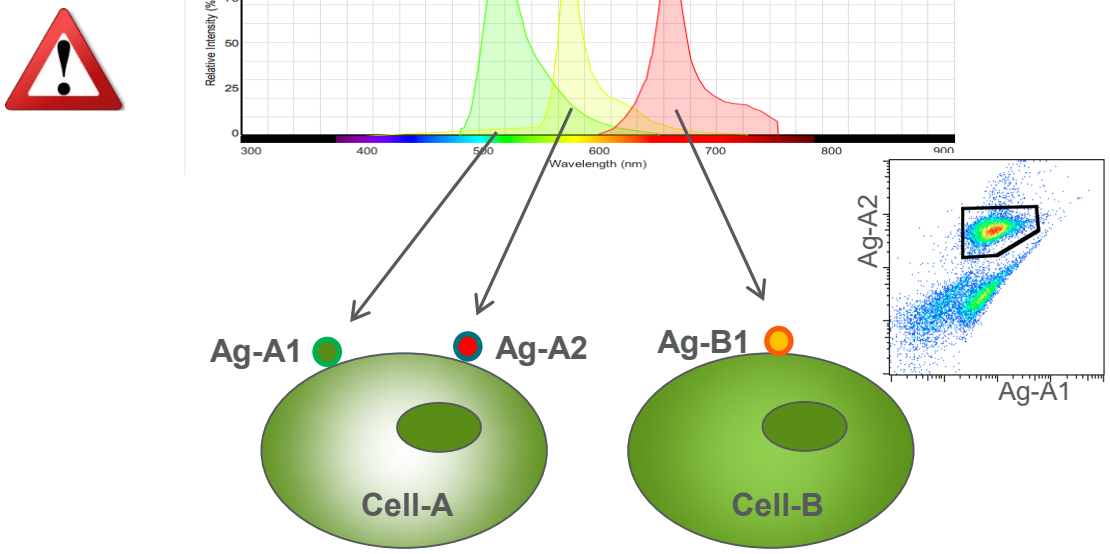


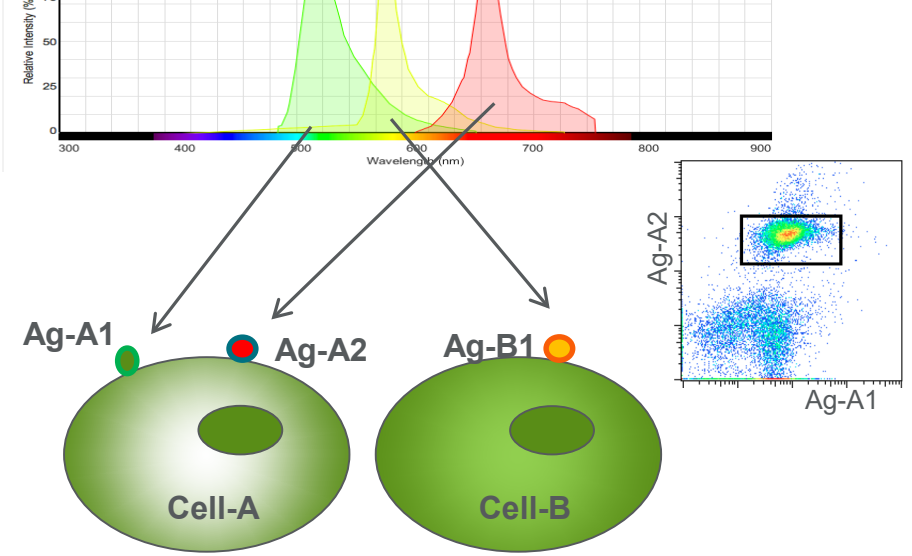
Match markers/fluorchromes

- Is there a kit available for your subset of cells and instrument?
- Which fluorochromes are available for your markers of interest?
  - Look first at the rare/unknown markers as they will have fewer fluorochromes available
  - Tools to search for commercially available reagents:
    - FluoroFinder (www.fluorofinder.com)
    - Chromocyte (www.chromocyte.com)
    - Fluorish (www.fluorish.com)
- Choose an appropriately bright fluorochrome for the antigen of interest:
  - Tertiary antigens (low antigen density) with bright (high stain index) fluorochromes
  - Primary antigens with dim fluors. Brighter = more spread!
  - Minimize spread on co-expressed markers
- What about your fluorescent proteins?

Match markers/fluors – co-expressed markers

Assign fluorochromes that have significant spectral overlap to different cell populations





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Match markers/fluors – the impact of panel design

Reagents pulled out of the fridge:

Marker	Antigen classification	Fluorochrome	Brightness
Live/dead		Zombie UV	
CD45	1	PerCP-Cy5.5	2
CD3	1	Alexa 532	1
CD19	1	BV 711	3
CD4	1	BV 750	3
CD8	1	BV 570	2
CD56	1	BV 650	2
CD14	1	BV 510	2
CD16	1	eFluor 450	2
CCR7	3	BV 480	3
CD45RA	2	Alexa 488	3
CD163	3	PE	5
CD209	3	APC	4
CD40L	3	BV421	4
CD64	2	Alexa 700	2
HLA-DR	2	APC-Fire750	3
CD80	3	PE-Cy7	5
CD69	3	BV 605	2

Complexity™ Index: 7.56

Following panel design best practices:

Marker	Antigen classification	Fluorochrome	Brightness
Live/dead		Live/dead NIR	
CD45	1	BUV 395	1
CD3	1	BV 605	2
CD19	1	BUV 563	2
CD4	1	BUV 496	1
CD8	1	Alexa 700	2
CD56	1	BB 700	4
CD14	1	BUV 737	3
CD16	1	BV510	2
CCR7	3	PE-CF594	5
CD45RA	2	BV421	4
CD163	3	PE	5
CD209	3	APC	4
CD40L	3	PE-Cy5	5
CD64	2	BV 711	3
HLA-DR	2	Alexa 488	3
CD80	3	PE-Cy7	5
CD69	3	BV 786	3

Complexity™ Index: 4.17

Match markers/fluors – Cytek panel building tool

Notes:

	UV			Violet			Blue			Yellow/Green			Red		
Emission	355			405			488			561			640		
	Marker	Rec. Dil.	Fluor	Marker	Rec. Dil.	Fluor	Marker	Rec. Dil.	Fluor	Marker	Rec. Dil.	Fluor	Marker	Rec. Dil.	Fluor
395			BUV395												
420						BV421									
440						Super Bright 436									
450			LIVE DEAD Blue			eFluor 450									
480						BV480									
500			BUV496			BV510			BB515						
520									FITC						
550						Pacific Orange			Spark Blue 550						
570			BUV563			BV570						PE			
580												cFluor YG584			
600			BUV615			BV605						PE/Dazzle594			
660			BUV661			BV650						PE-Alexa Fluor 610			APC
680									PerCP			PE-Cy5			Alexa Fluor 647
690									PerCP-Cy5.5						Spark NIR 685
700						BV711			PerCP-eFluor 710			PE-Alexa Fluor 700			APC-R700
730			BUV737												
750						BV750									
780						BV785						PE-Cy7			APC-H7
800			BUV805									PE-Fire 810			APC/Fire 810

Total Non-Dump:	**40**
Total Dump:	0
Total Markers:	40

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Match markers/fluors – Cytek panel design form

CYTEK
TRANSCEND THE CONVENTIONAL

Panel Design Request Form

Customer Name: **Institution:**

Customer Email: **Customer Phone:**

Assay Goal:

Species (human, mouse,rat, etc):

Type of cells/particles (example PBMCs, splenocytes, etc):

Please provide a schematic of the gating strategy to identify the populations of interest in this assay.

Please provide the staining protocol to be used, including proposed single-color controls.

Marker	Cell Type	Antigen Classification (1, 2, 3)	Fluorochrome (proposed)
1			
2			

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Plan your experiment – garbage in, garbage out

Checklist before acquiring experimental samples:

- ✓ How many samples are needed for statistical power? Consult a biostatistician and plan for loss.
- ✓ Remember to be consistent with nomenclature! HLA-DR ≠ HLADR to a computer.
- ✓ Will samples acquired on different days be compared? If so, need to consider batch effects.
- ✓ Check the autofluorescence of your cells
- ✓ Titrate any new reagents
- ✓ What controls are necessary (in addition to reference controls for unmixing)?
  - Biological controls
  - Gating controls: FMO or isotype
  - Reference controls for batch effects

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In summary

1. Define a population tree based on the goals of the assay
2. Identify the critical populations – gate strategy
3. Determine which antigens are co-expressed and at what levels for each subpopulation
4. Choose the instrument you are going to use
   - Check if a similar panel on the same kind of instrument is published
   - Get the similarity matrix, the spread matrix and the brightness level of fluorochromes for that instrument
5. Check reagent availability
6. Balance fluorochrome brightness based on expression level, expression pattern and marker requirement (bright is not always good)
7. Minimize spread:
   - Choose fluorochromes that have minimal spectral overlap
   - Assign fluorochromes that have significant overlap to different cell populations
8. Use the right reagents:
   - Antibody concentration and clone affect the signal brightness. Use optimal concentration and right clone.