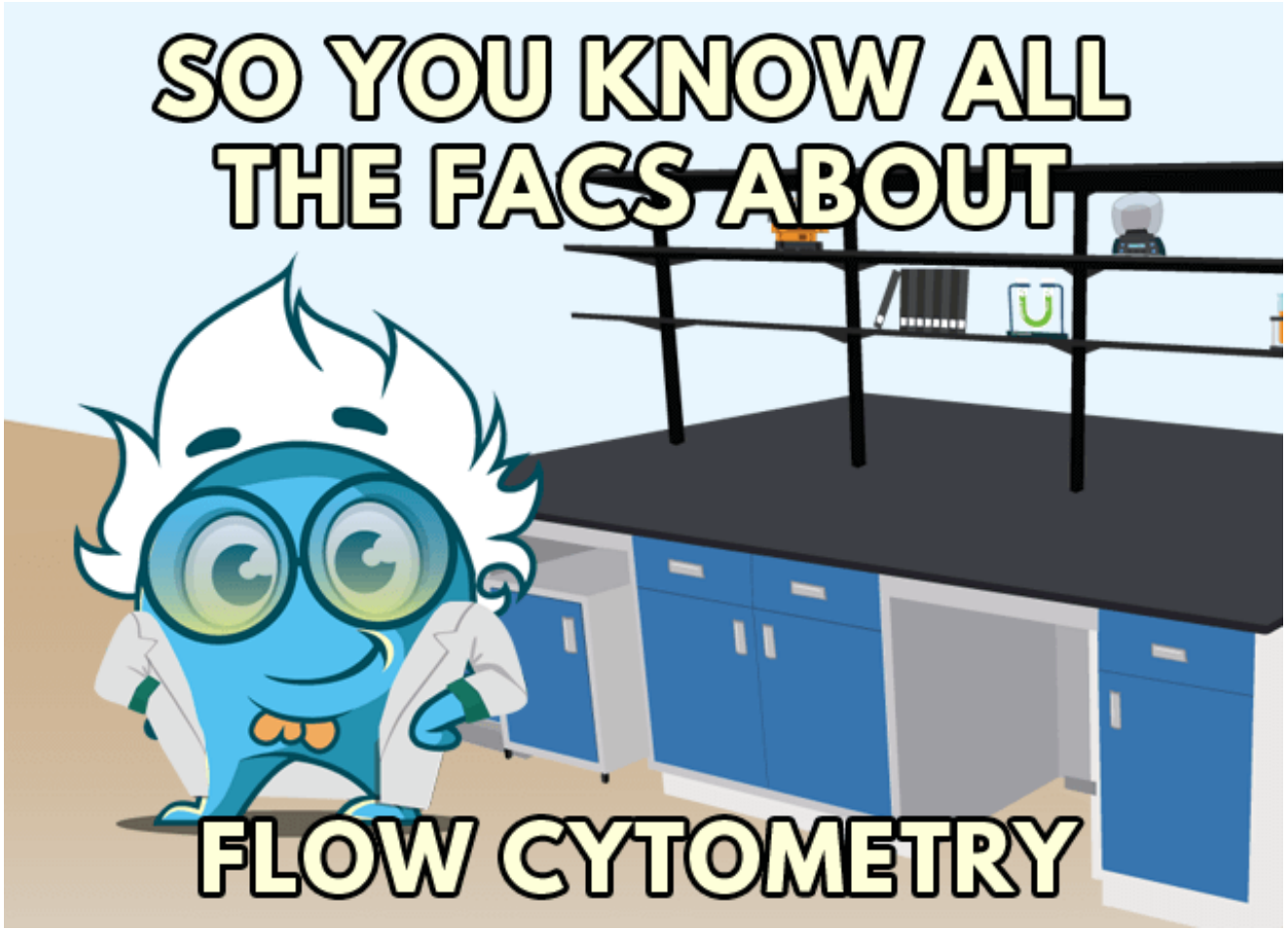
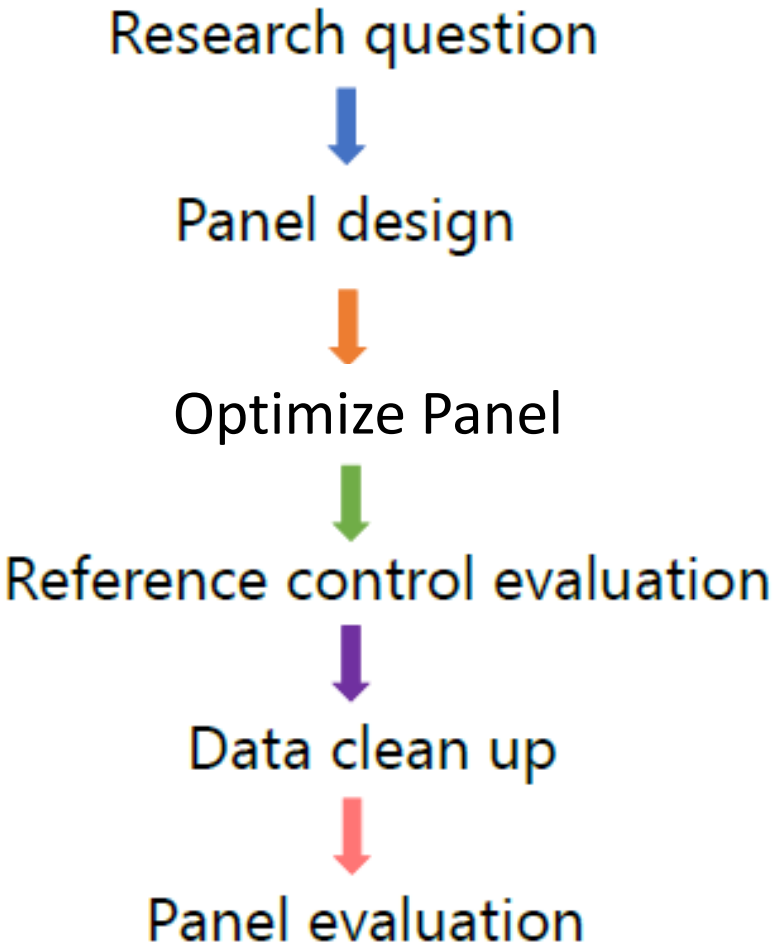
Experimental design



Experimental design steps



Research question

- Know your biological hypothesis
  - Which populations need to be identified in which tissue
- What are the available instrument configurations/ fluorochromes

BD FACS Canto

Laser	PMT	LP	BP	Fluorchromes
488	A	735	780/60	PE-Cy7
	B	685	710/50	PE-Cy5.5 PerCp
	D	556	585/42	PE
	E	520	530/30	FITC A488
633	A	735	780/60	APC-Cy7 APC-H7
	B	-	660/20	APC A647
405	A	750	510/50	V500 BV510
	B	-	450/40	Pacific blue Hoechst Dapi BV421

Most standard applications

Cytek Aurora

UV laser (355): 7 channels

Violet laser (405): 18 channels

Red laser (635): 6 channels

Blue laser (488): 7 channels

https://spectrum.cytekbio.com

High autofluorescence
Big panels (>8 markers)
Highly similar fluorophores

BD Fusion sorter

Laser	PMT	LP	BP	Fluorchromes
488	A	655	695/40	PerCp-Cy5.5 PerCp
	B	502	530/30	FITC
	C	-	488/10	SSC
561	A	735	780/60	PE-Cy7
	B	685	710/50	PE-Cy5.5
	C	630	670/14	PE-Cy5
	D	600	610/20	PE-Cy594 PI mCherry PE-TxRed
	E	-	582/15	PE DsRed
640	A	755	780/60	APC-Cy7 APC-H7
	B	-	670/30	APC A647
	C	690	730/45	Alexa700

To sort out populations for further analysis:

- RNA/protein extraction
- Cell culture
- …

Research question

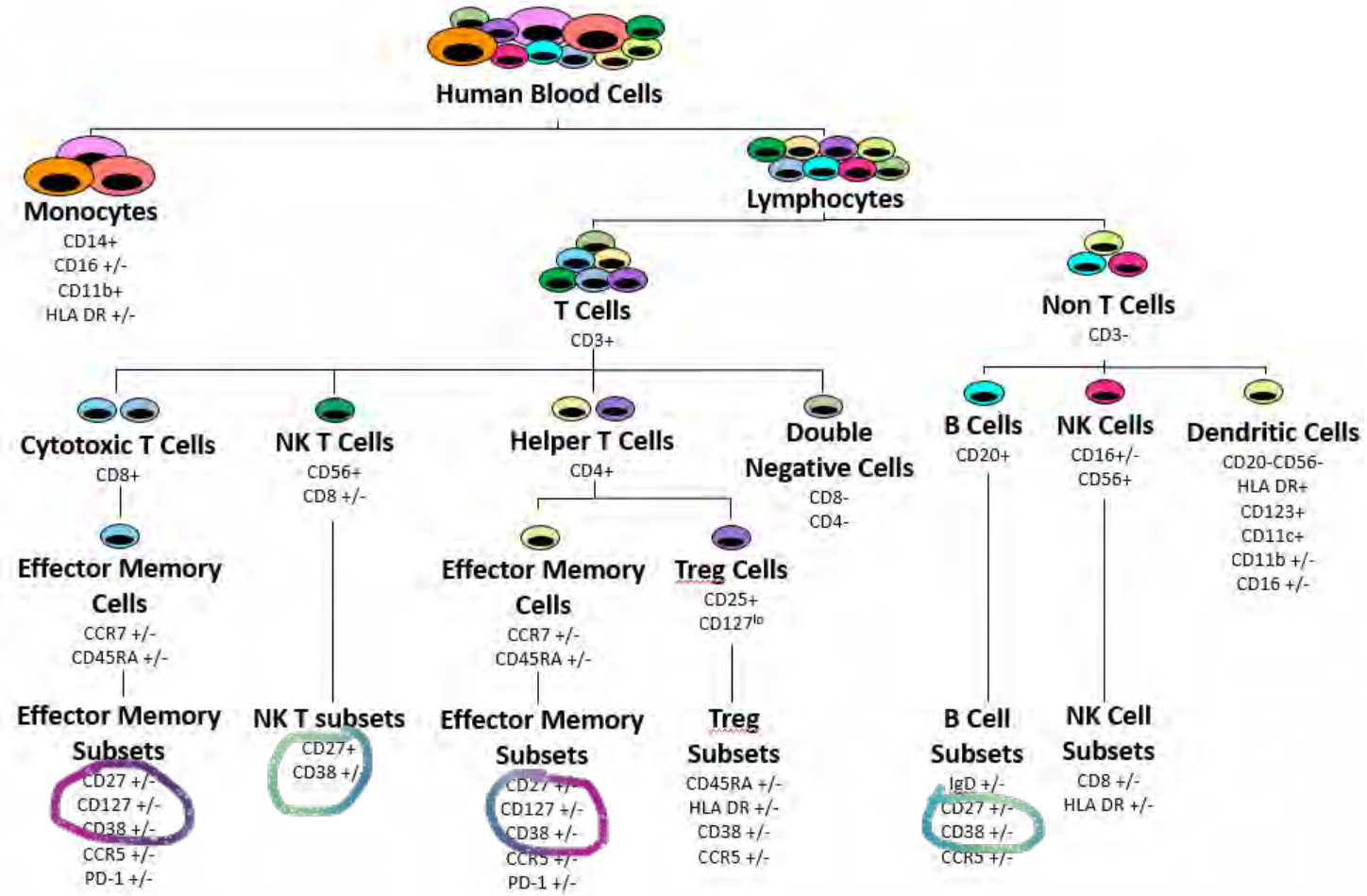
Select antigens

- Know your biological hypothesis
- Select antigens: identify markers of interest
  - Expression level?
  - Co-expressed?
  - Gating strategy

  - Check literature!

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.*

Marker Co-expression



Gating strategy

Size/shape
FSC vs SSC

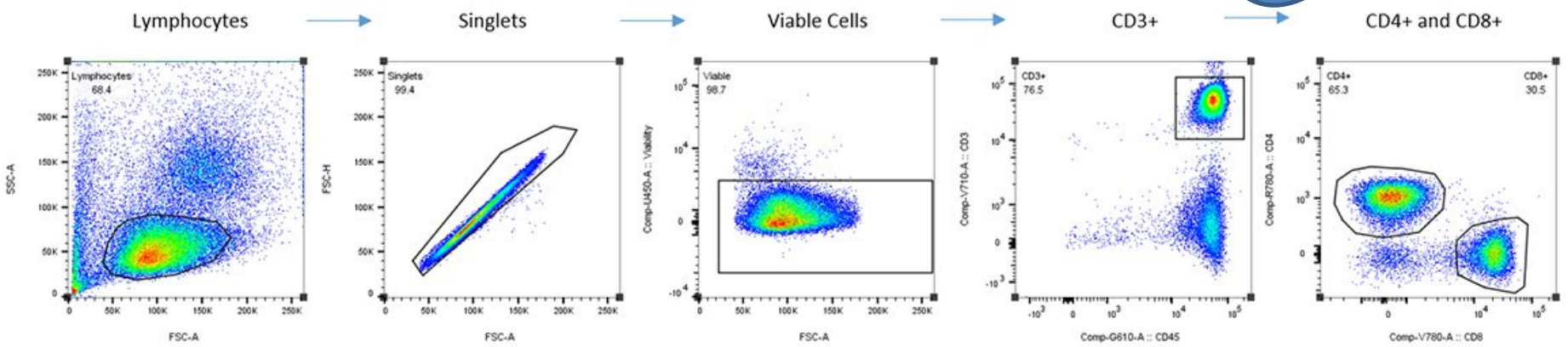
Peak uniformity
Area vs Height

Dead cell dye
negative

CD45+
CD3hi

CD4+

CD8+



Think about how you want to “arrive” at your population

FlowMetric.com

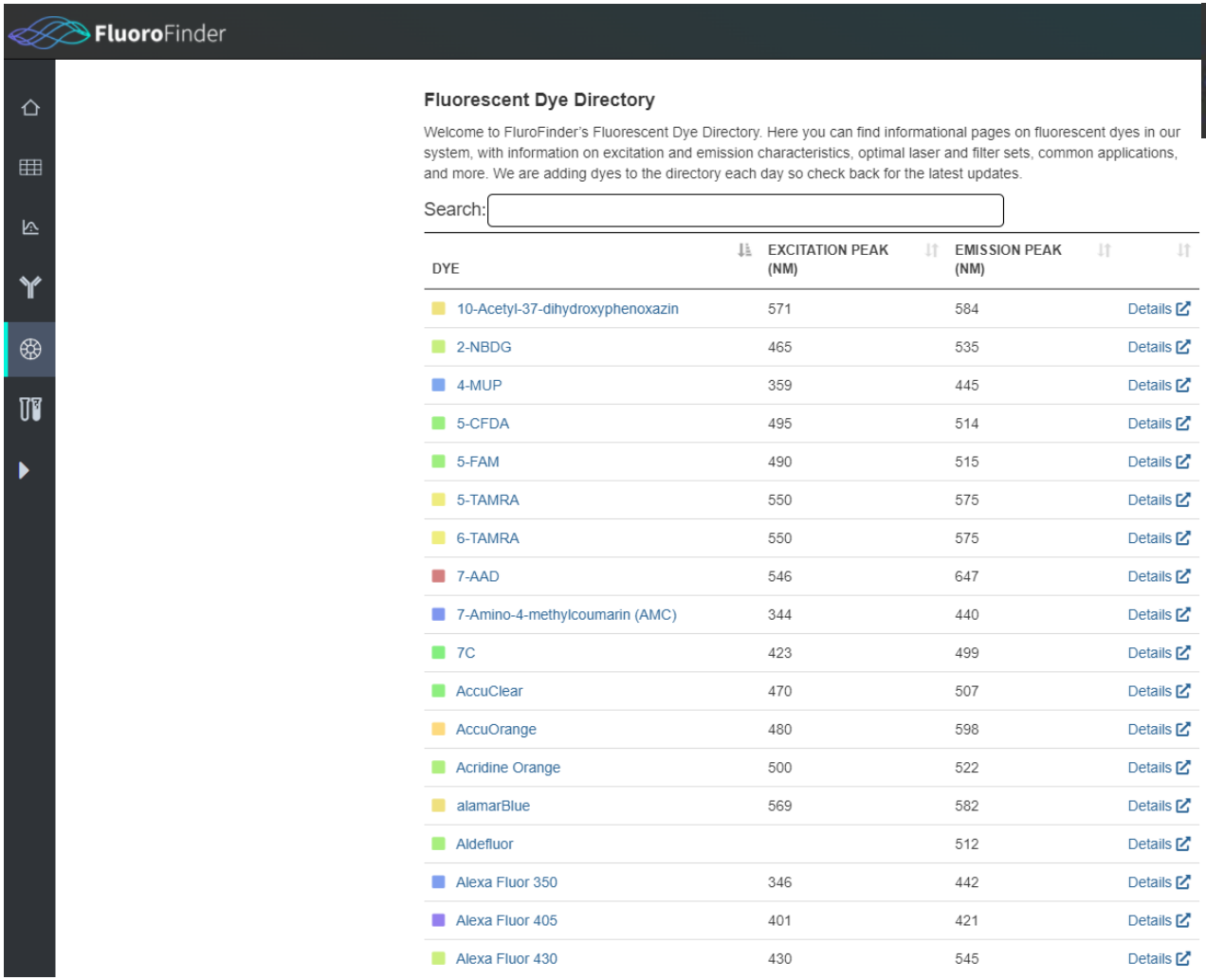
Panel design

Basic rules

- Make sure you know the limitations of your machine
- Start with your “rare” antigens and try to match them with fluorophore-labeled antibodies
- Match low expressed antigens with bright fluorophores and high expressed antigens with dimmer fluorophores
- Avoid similar fluorophores on co-expressed markers
- Avoid fluorophores with high similarity to autofluorescence of your cells of interest

Panel design

Fluorochrome types:



FluoroFinder

Fluorescent Dye Database

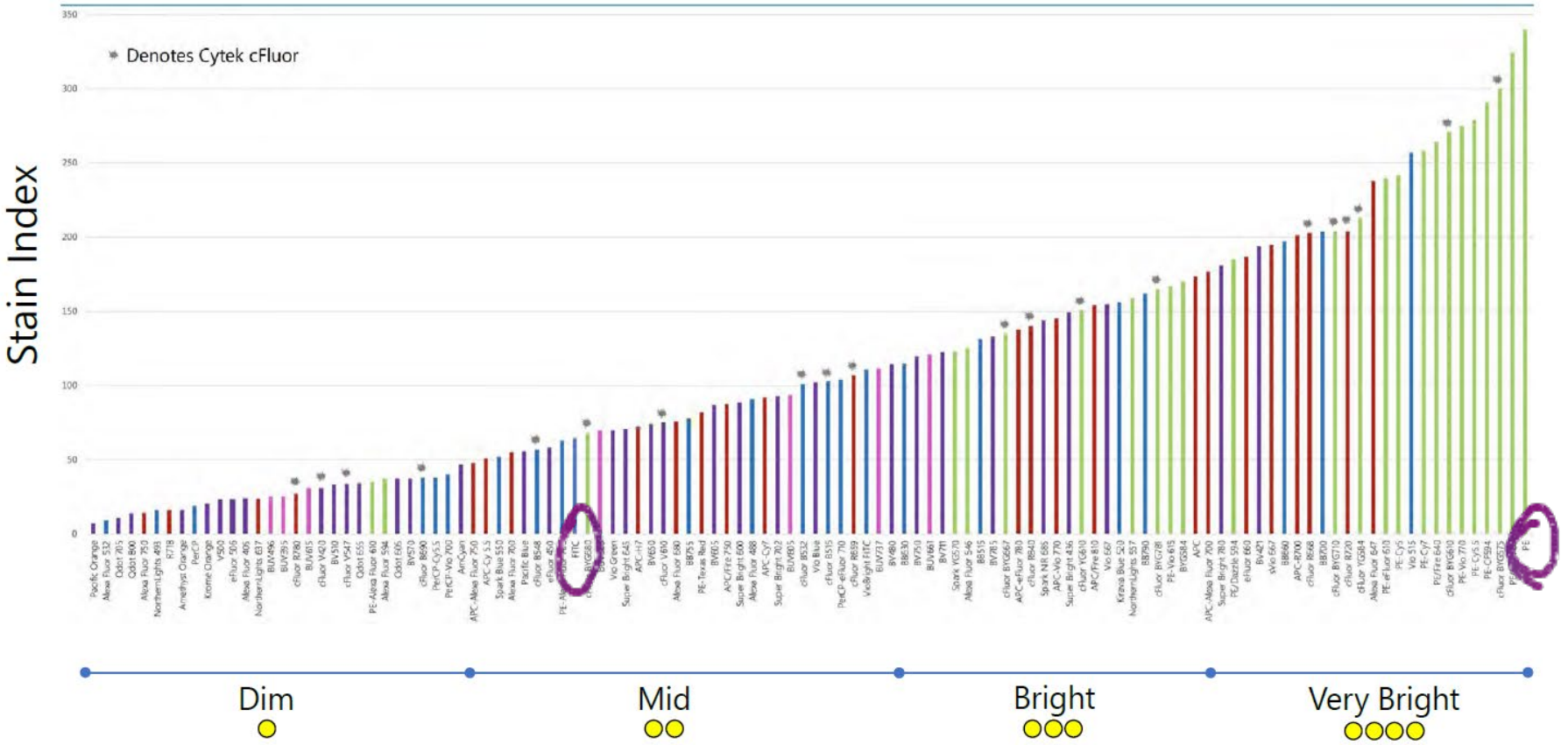
Database with over 1000 fluorochromes and their characteristics

app.fluorofinder.com/dyes

Panel design

Select fluorochromes

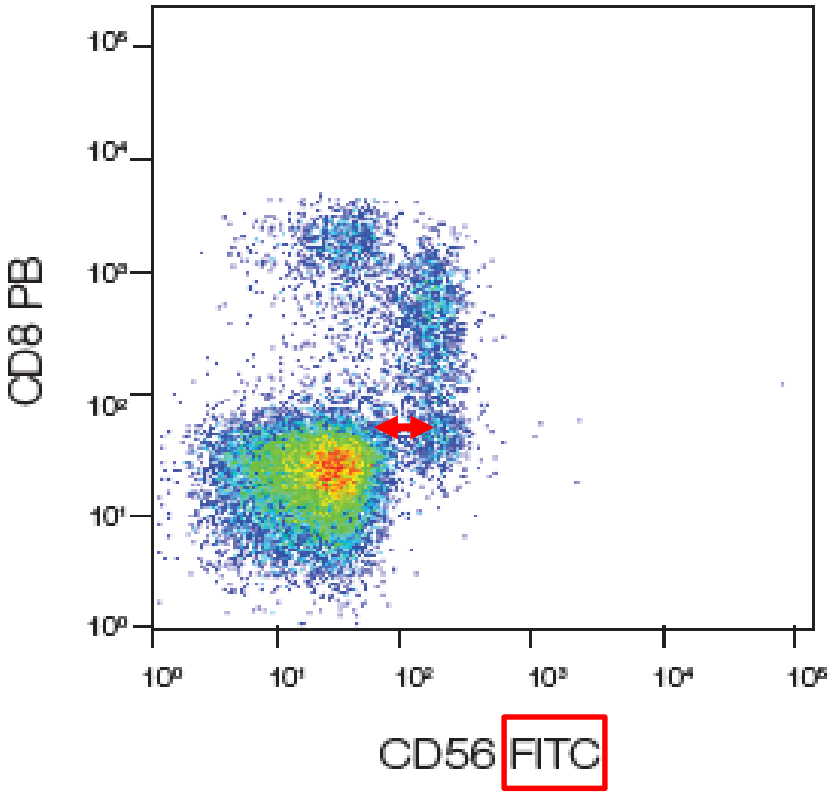
- Staining index: measurement of brightness

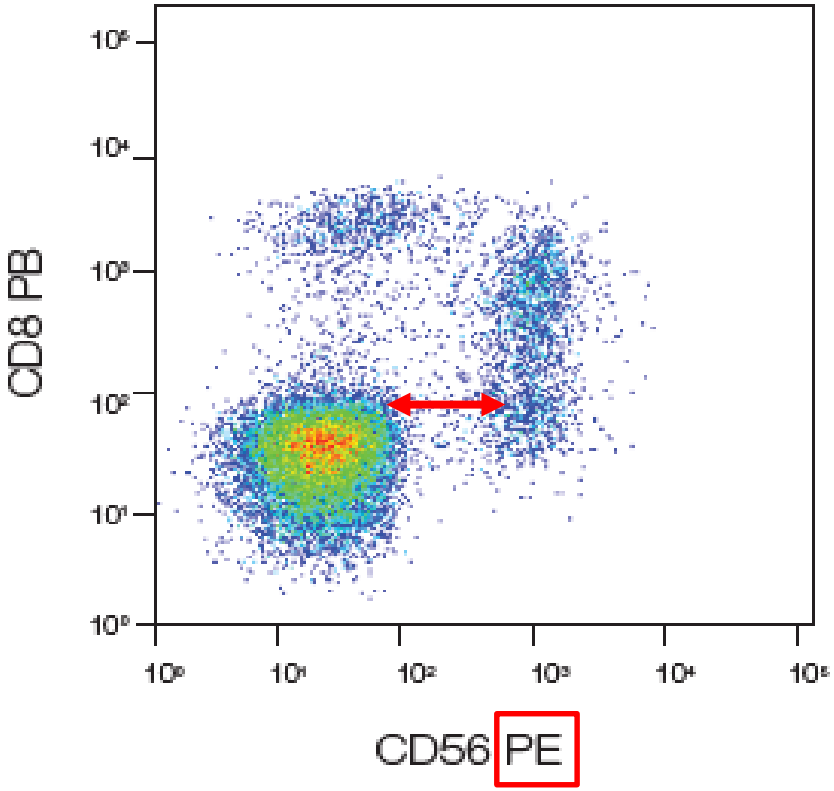


Cytek

Panel design

Select fluorochromes



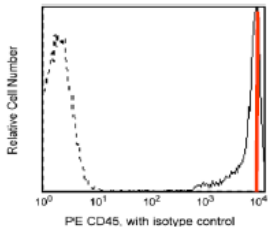


Look at dot plots of specific antibody clone/fluorophore combinations in literature, on company websites etc

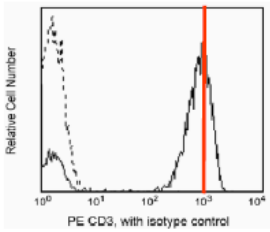
BioRad

Panel design

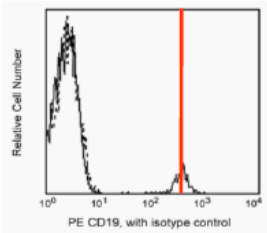
Select fluorochromes



CD45 PE

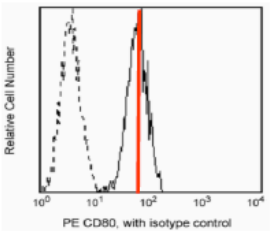


CD3 PE



CD19 PE

CD3, CD45 and CD19 are all high density antigens which don‘t need a bright fluorochrome
Don‘t waste your PE here, use a dimmer fluorochrome.



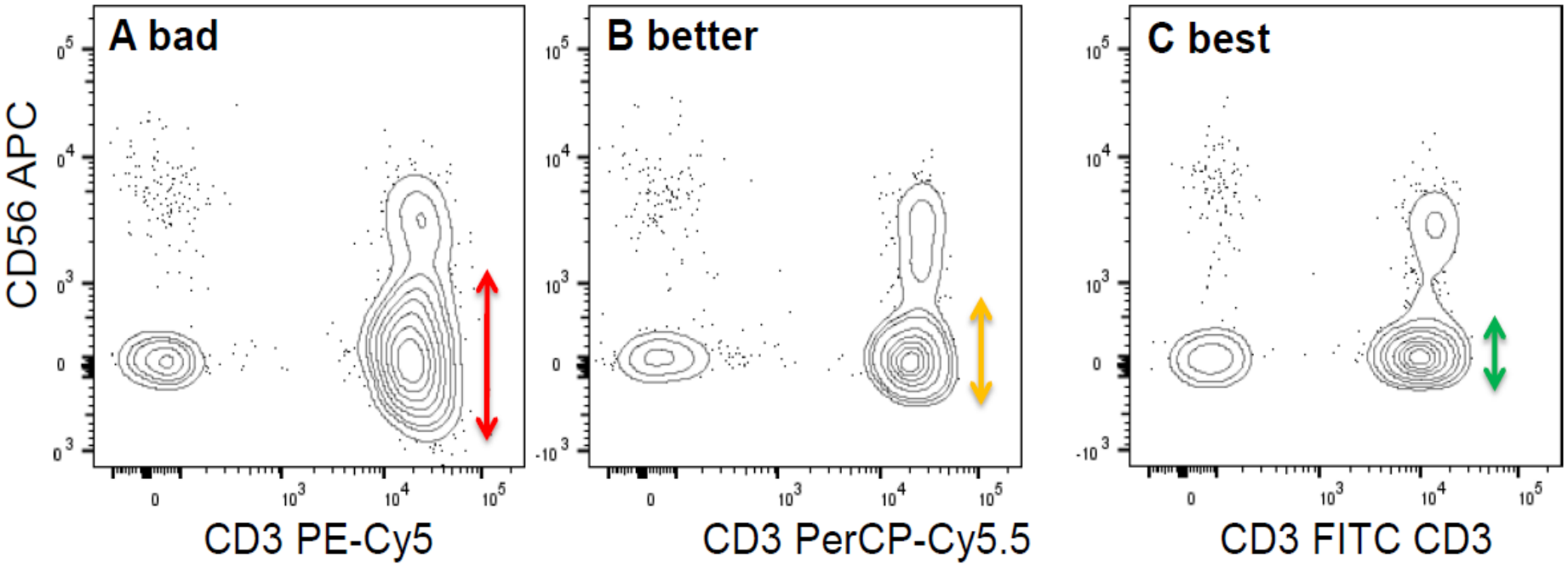
CD80 PE

CD80 is a low density antigen which needs a bright fluorochrome like PE for good resolution of it‘s dim staining

Panel design

Select fluorochromes

- Co-expressed markers: mimimize spectral overlap of fluorochromes and so data spread



You can see the CD3+ CD56-negative population "spreads" into the APC channel, making it hard to identify the CD3+CD56+ cells

ZosiaMaciorowski

Panel design

Spread Quantification Index

	Detector	BB700	BV421	BV605	BV711	PE	PE-CF594	PE-Cy5	PE-Cy7	APC	APC-R700
System	Fluorochrome	SQI	SQI	SQI	SQI	SQI	SQI	SQI	SQI	SQI	SQI
Symphony	BB700		45	68	563	23	18	38	56	523	509
Fusion			29	158	1819	8	17	43	74	329	471
Cytoflex			20	106		5	6	51	38	181	567
Quanteon			54	228	827	30	33	37	55	124	279
Symphony	BV421	13		33	27	22	15	14	9	35	20
Fusion		13		44	26	7	10	10	11	23	17
Cytoflex		7		38		4	3	5	5	38	20
Quanteon		33		46	35	29	30	26	25	37	38
Symphony	BV605	73	39		177	129	220	81	52	83	77
Fusion		110	92		220	61	248	87	61	89	107
Cytoflex		85	70			44	115	29	29	47	35
Quanteon		95	68		145	103	145	77	46	62	78
Symphony	BV711	85	48	23		22	15	17	41	161	604
Fusion		75	85	32		7	9	15	62	92	476
Cytoflex											
Quanteon		97	79	40		29	29	28	54	62	374
Symphony	PE	105	16	128	74		260	97	49	105	31
Fusion		125	17	334	101		197	96	55	109	39
Cytoflex		71	20	277			88	32	27	43	43
Quanteon		91	47	182	53		153	71	38	58	44
Symphony	PE-CF594	215	18	184	150	156		178	103	198	51
Fusion		309	19	656	254	67		183	123	208	68
Cytoflex		201	19	480		42		49	66	64	103
Quanteon		202	47	334	93	124		134	73	94	70
Symphony	PE-Cy5	490	22	28	305	61	41		192	855	319
Fusion		736	29	51	629	83	32		232	935	343
Cytoflex		649	21	39		34	13		156	816	453
Quanteon		435	49	51	370	127	47		141	326	201
Symphony	PE-Cy7	31	16	21	29	43	26	18		36	41
Fusion		23	20	30	27	203	23	46		20	36
Cytoflex		22	20	23		306	7	131		41	25
Quanteon		36	47	39	46	74	37	30		38	47
Symphony	APC	105	18	20	120	22	18	128	52		280
Fusion		121	20	29	145	8	12	136	56		160
Cytoflex		111	22	24		5	10	145	32		264
Quanteon		121	48	39	127	32	33	129	49		165
Symphony	APC-R700	51	16	20	117	22	14	35	85	366	
Fusion		42	17	26	219	7	10	34	114	570	
Cytoflex		105	21	22		5	2	83	60	179	
Quanteon		59	48	36	173	30	29	40	88	267	

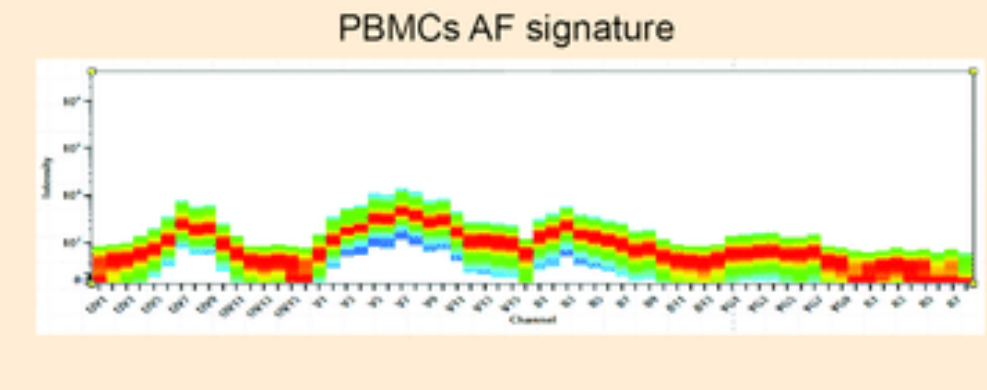
Bhowmick, Scientific Reports (2021) 11:20553

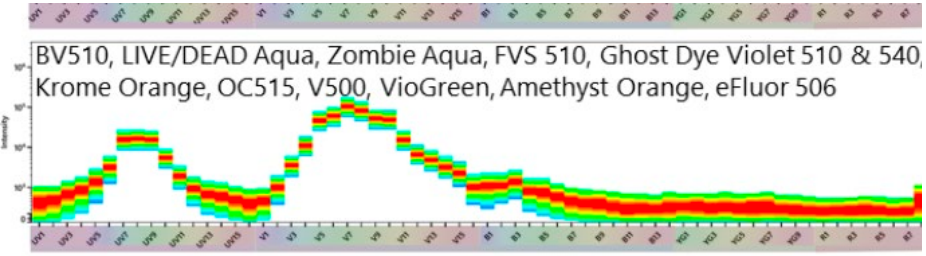
Table 1. Comparison of spread in four different flow cytometers: ten different single stained bead sets were run on four different instruments. SQI was calculated for every combination. For BV711 there was no data available for the Cytoflex S. The SQI values are categorized as follows: 1–120 (Green); 121–199 (Yellow); 200–299 (Orange); and 300 + (Red).

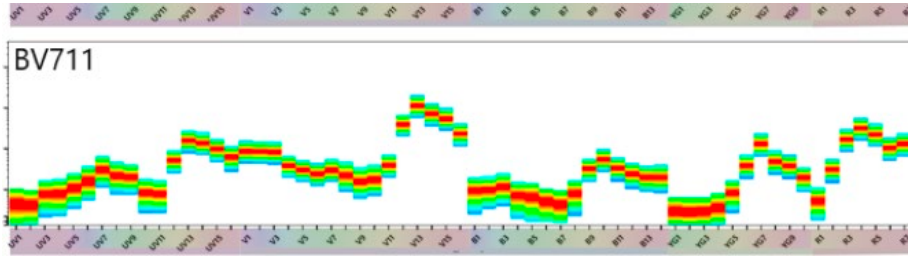
Panel design

Autofluorescence effect

Do not match critical markers to autofluorescent-like fluorophores







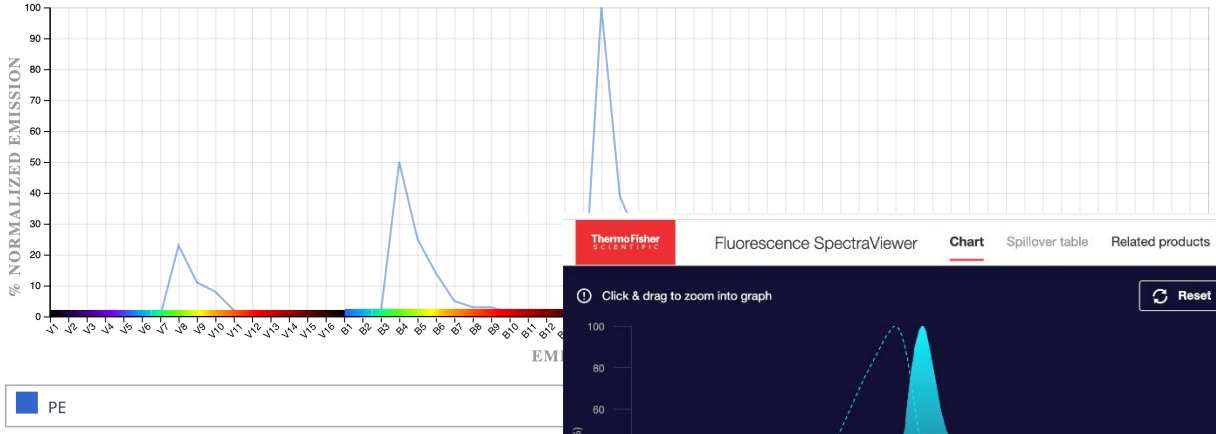
Cytek Unique Signatures Page

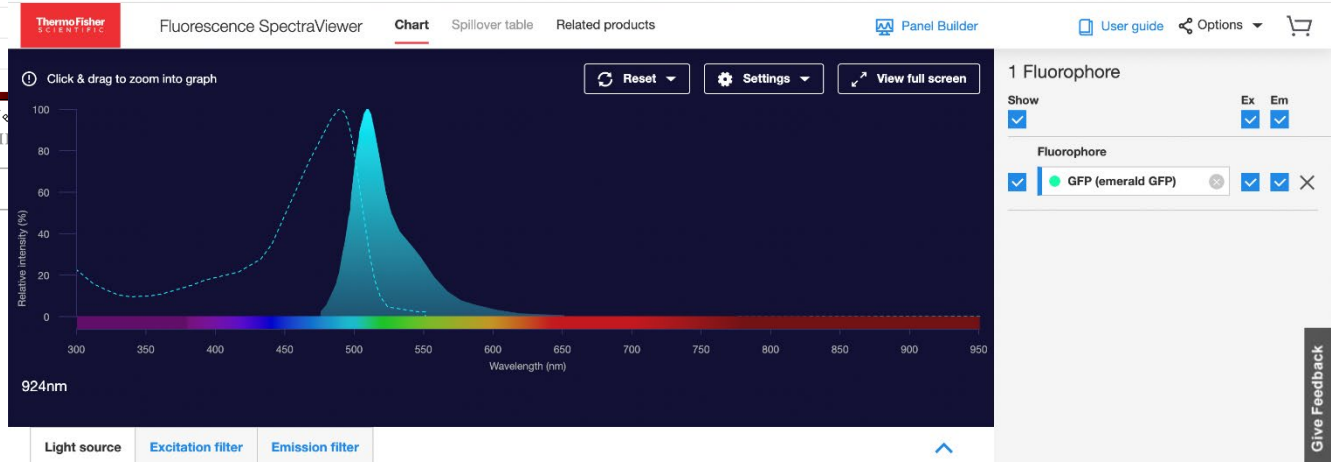
Panel design

- Come to us!
- Use spectrum viewers to match fluorochromes to your machine configuration

https://spectrum.cytekbio.com/

https://www.thermofisher.com/order/fluorescence-spectraviewer





Panel design

- Come to us!
- Use spectrum viewers
- Panel design software:
  - https://fluorofinder.com/
  - https://www.thermofisher.com/order/panel-builder/#!/
  - https://www.biolegend.com/en-us/panel-builder
  - https://cloud.cytekbio.com/panelbuilder
- OMIP: Optimized multicolor immunofluorescence panel: published optimized panels

› Cytometry A. 2020 Oct;97(10):1044-1051. doi: 10.1002/cyto.a.24213. Epub 2020 Aug 31.

OMIP-069: Forty-Color Full Spectrum Flow Cytometry Panel for Deep Immunophenotyping of Major Cell Subsets in Human Peripheral Blood

Lily M Park [1], Joanne Lannigan [2], Maria C Jaimes [1]

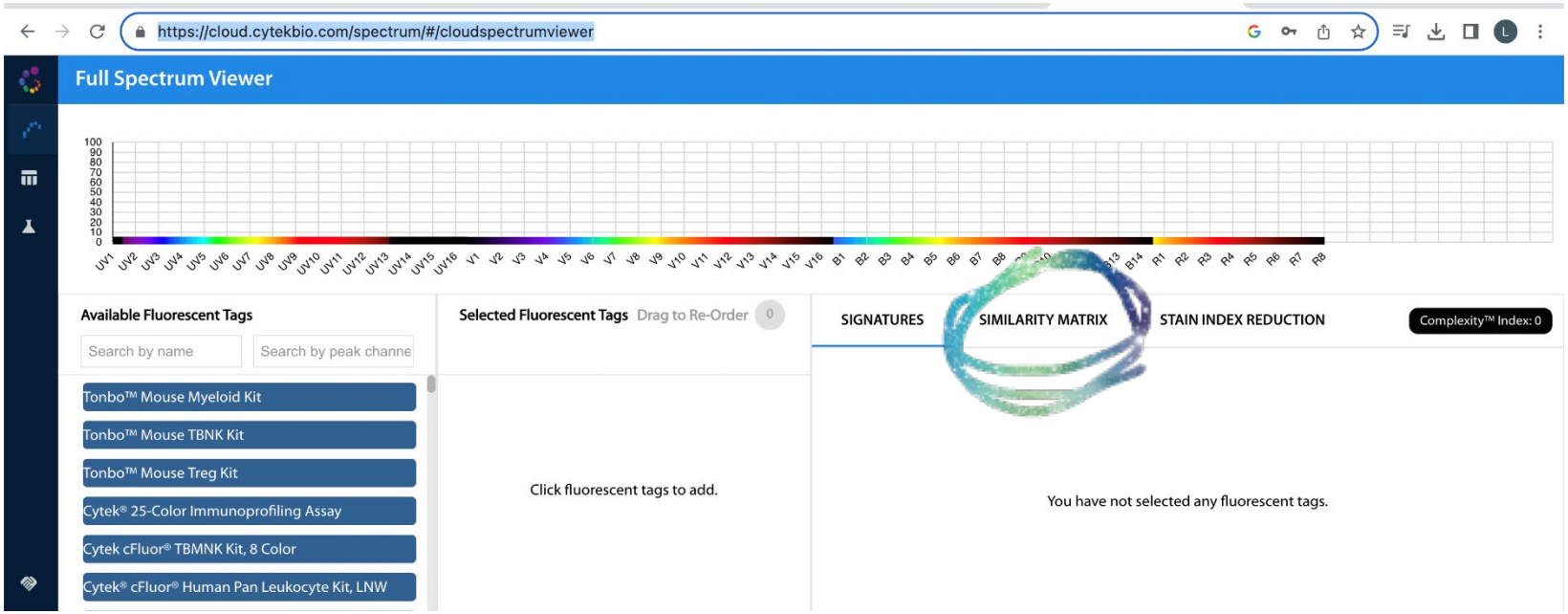
Affiliations + expand

PMID: 32830910 PMCID: PMC8132182 DOI: 10.1002/cyto.a.24213

Panel design

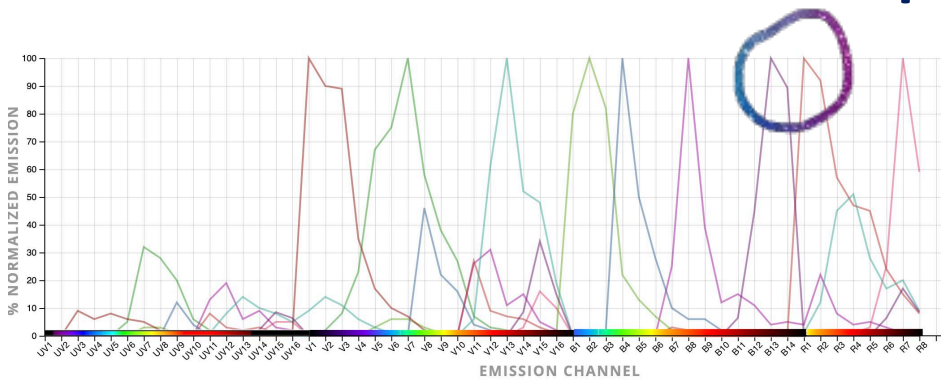
Full spectrum

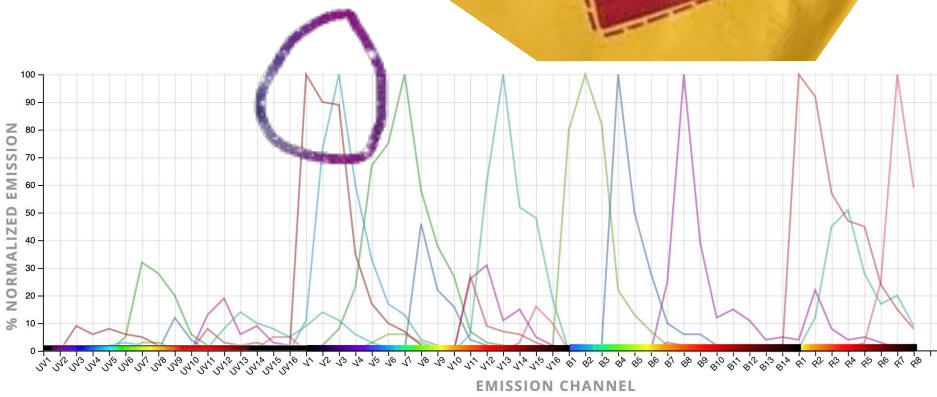
- Similarity and complexity index to evaluate panels
- Also useful to design panels for conventional flow cytometry
  - https://cloud.cytekbio.com/spectrum/#/cloudspectrumviewer



Panel design

Full spectrum





Configuration:4L 16UV-16V-14B-8R

	LIVE DEAD Aqua	PerCP	APC-H7	FITC	APC	PE	Super Bright 702	BV421	PE-Cy7
LIVE DEAD Aqua	1								
PerCP	0	1							
APC-H7	0.01	0.03	1						
FITC	0.06	0.01	0	1					
APC	0.01	0.24	0.15	0	1				
PE	0.19	0.07	0	0.17	0.01	1			
Super Bright 702	0.04	0.27	0.22	0	0.38	0.01	1		
BV421	0.17	0.01	0	0.01	0	0.01	0.13	1	
PE-Cy7	0	0.1	0.19	0	0.03	0.01	0.12	0	1

Complexity™ Index: 1.92

CD3 on PE-Cy7:

CI is 1.92

Configuration:4L 16UV-16V-14B-8R

	LIVE DEAD Aqua	PerCP	V450	APC-H7	FITC	APC	PE	Super Bright 702	BV421
LIVE DEAD Aqua	1								
PerCP	0	1							
V450	0.31	0.02	1						
APC-H7	0.01	0.03	0	1					
FITC	0.06	0.01	0.01	0	1				
APC	0.01	0.24	0	0.15	0	1			
PE	0.19	0.07	0.01	0	0.17	0.01	1		
Super Bright 702	0.04	0.27	0.12	0.22	0	0.38	0.01	1	
BV421	0.17	0.01	0.81	0	0.01	0	0.01	0.13	1

Complexity™ Index: 3.5

CD3 on V450:

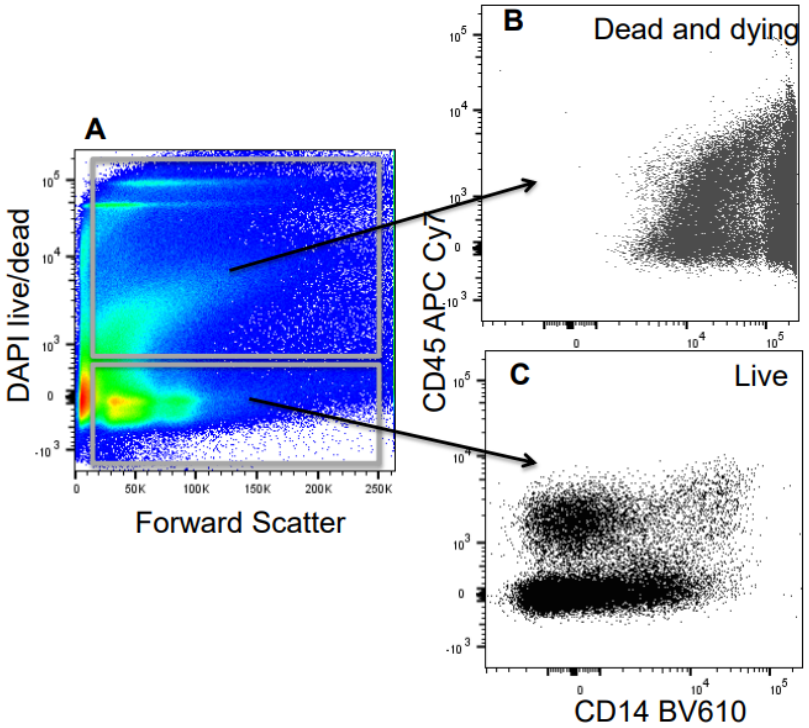
CI is 3.50

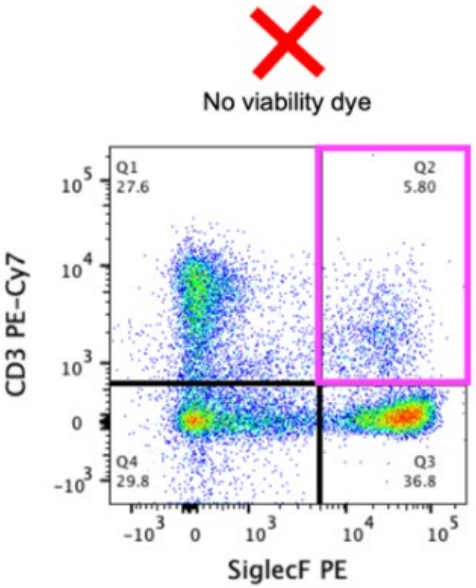
Complexity index is a measurement of overall spectral overlap and reflects the “doability” of your panel

Panel design

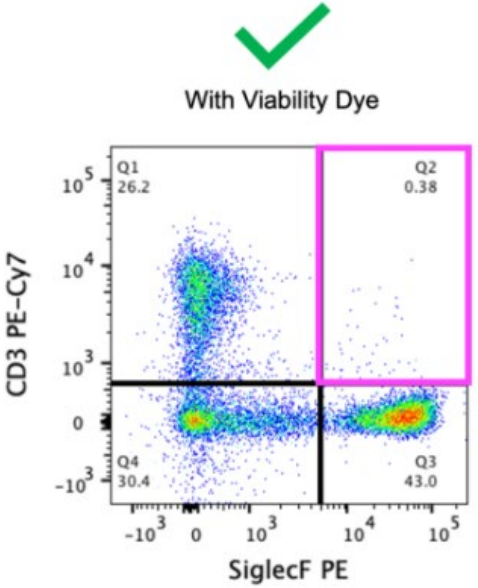
Dead exclusion dyes

- dead cells become sticky & autofluorescent





Gated on single CD45+ cells



Gated on single CD45+ live cells

Dead cells kill your data

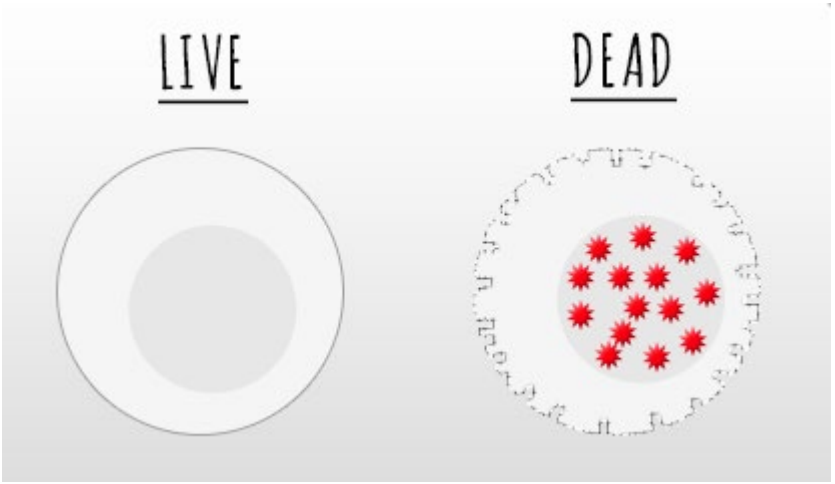
UCFlow

Panel design

Live/Dead stain

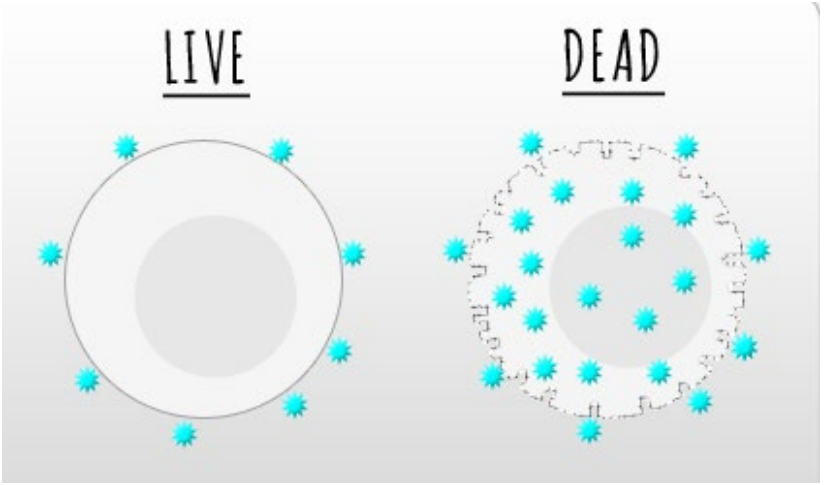
- dead cells become sticky & autofluorescent!

Live cell impermeant DNA-dyes



- Propidiumiodide
- 7-AAD
- DRAQ7

Amine reactive (fixable) dyes



- LIVE/ DEAD Fixable (ThermoFisher)
- Zombie Dyes (Biolegend)
- …

Dead exclusion dyes

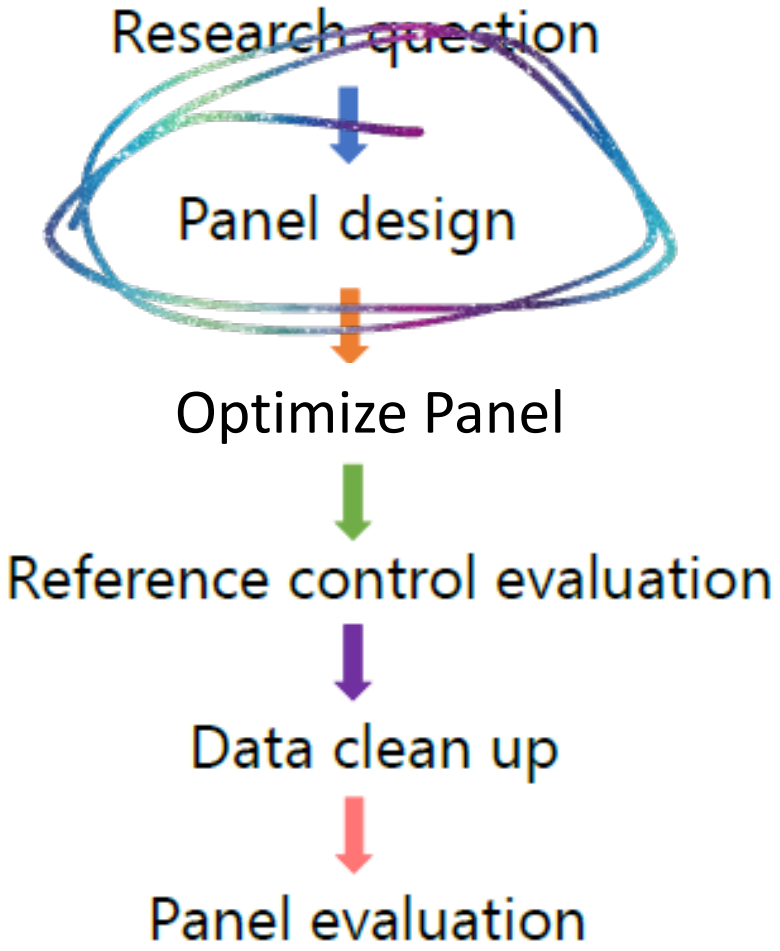
Amine dyes

Dye	Fixed Cells	Ex	Em
Zombie™ UV	Yes	362	459
Zombie™ Violet	Yes	400	423
Zombie™ Aqua	Yes	382	510
Zombie™ Green	Yes	491	515
Zombie™ Yellow	Yes	396	572
Zombie™ Red	Yes	600	624
Zombie™ NIR	Yes	719	746
eFluor™ 780	Yes	633	780
eFluor™ 450	Yes	405	450
eFluor™ 506	Yes	405	506
eFluor™ 520	Yes	488	522
eFluor™ 660	Yes	633	660
eFluor™ 455UV	Yes	350	455
LIVE/DEAD™ Blue	Yes	350	450
LIVE/DEAD™ Violet	Yes	416	451
LIVE/DEAD™ Aqua	Yes	367	526
LIVE/DEAD™ Yellow	Yes	400	575
LIVE/DEAD™ Green	Yes	495	520
LIVE/DEAD™ Red	Yes	595	615
LIVE/DEAD™ Far Red	Yes	650	665
LIVE/DEAD™ Near IR	Yes	750	775
Viobility™ 405/452	Yes	405	452
Viobility™ 405/520	Yes	405	520
Viobility™ 488/520	Yes	488	520
Horizon™ FVS450	Yes	406	450
Horizon™ FVS510	Yes	408	512
Horizon™ FVS520	Yes	498	521
Horizon™ FVS570	Yes	547	573
Horizon™ FVS575V	Yes	396	572
Horizon™ FVS620	Yes	523	617
Horizon™ FVS660	Yes	649	660
Horizon™ FVS700	Yes	657	700

DNA binding dyes

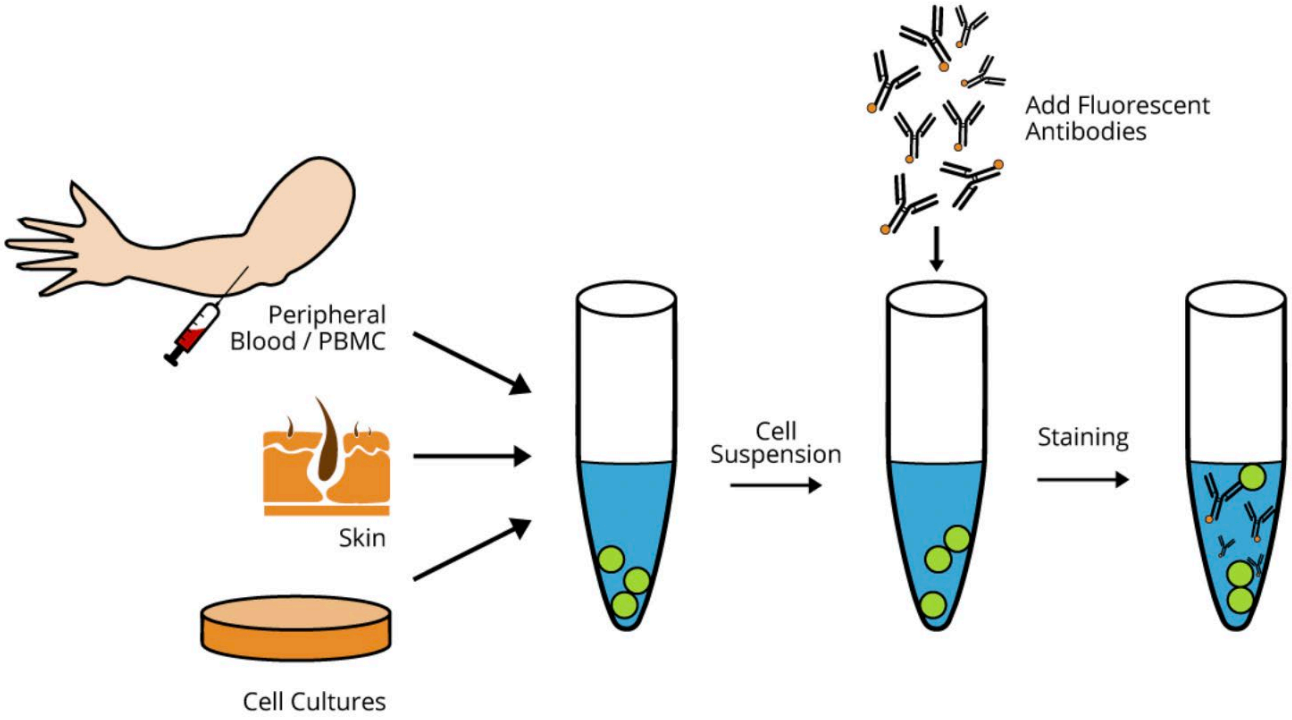
Dye	Fixed Cells	Ex	Em
DAPI	No	358	461
Hoechst (33258)	No	352	461
Hoechst (33342)	No	350	461
SYTOX® Blue	No	444	480
SYTOX® Green	No	504	523
SYTOX® Orange	No	547	570
SYTOX® AAdvanced	No	546	647
SYTOX® Red	No	640	658
TO-PRO®-1	No	515	531
TO-PRO®-3	No	642	661
TOTO®-1	No	514	533
TOTO®-3	No	642	660
Ethidium Monoazide Bromide	No	504	600
Ethidium Bromide	No	210/285	605
Propidium Iodide	No	488	617
7-AAD	No	543	647
DRAQ5™	No	647	681
DRAQ7™	No	633	695
Helix NP™ NIR	No	640	660
RedDot™1	No	662	694
RedDot™2	No	665	695
YO-PRO™-1	No	491	509
YO-PRO™-3	No	612	631
LDS 751	Yes	543	712

Experimental design steps



Experimental design

Typical FACS workflow



Experimental design

- How many cells to stain?

Cell sorting

- e.g. 1% cells of interest in your total sample
- 50% recovery after sorting
- Preparation:
  - 10% sticks to tube
  - 10% loss at filtering
  - 10% loss every centrifugation
- Calculate back to estimate how many cells to prep

Analysis

- STATISTICS
- % population of importance (rare events)
- Which p-value to obtain
- Amount of samples
- Power calculation
- Rule of thumb: Measure minimal 100-200 events to be able to define a population

Experimental design

Sample preparation

- ❑ Add EDTA (2-5mM) to prevent aggregation
- ❑ Filter your samples -> prevent clogging!

- ❑ DNA released from dead cells is sticky
  - → Add Dnase
  - → stop killing your cells

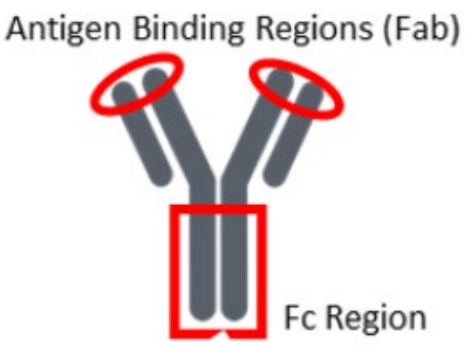
Be gentle with pipetting/vortexing/cell dissociating

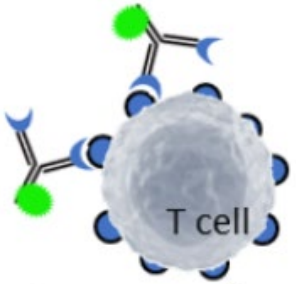
- ❑ Keep samples in the dark during measurements

- ❑ Some adhesion molecules require Ca++/Mg++
  - → do not add EDTA in this case

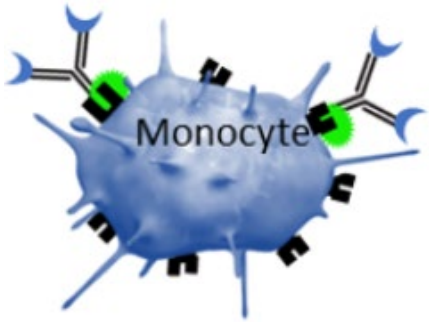
Optimize Panel

- Staining protocol: ask us for help
  - Use BSA/FBS as a blocking agent to minimize non-specific binding
  - FcR blocking
    - Human : 10% homologous serum or commercial Fc block
    - Mouse: anti CD16/32
  - Myeloid cells bind specifically to certain dyes
    - add TrueStain Monocyte blocker





Receptor mediated, antigen-specific binding

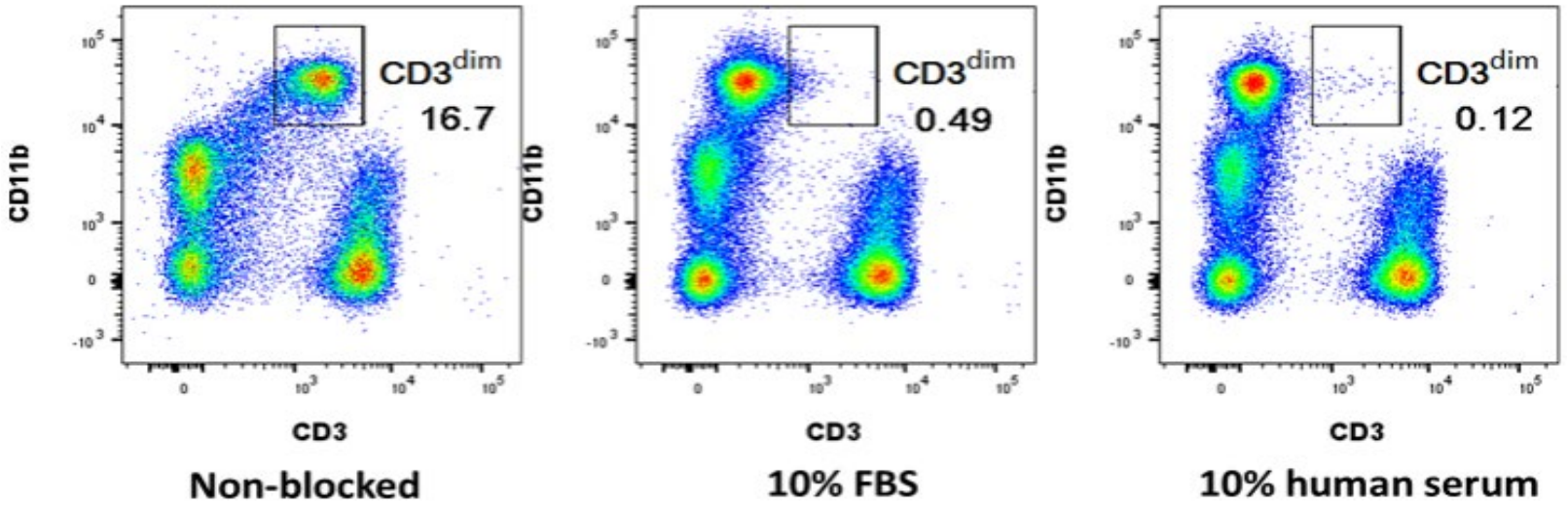


Fc receptor mediated, off-target, specific binding

Optimize panel

- Staining protocol: use blocking!
  - non-specific binding + Fc-receptor

Human PBMC's gated on lymfocytes + monocytes, doublets excluded

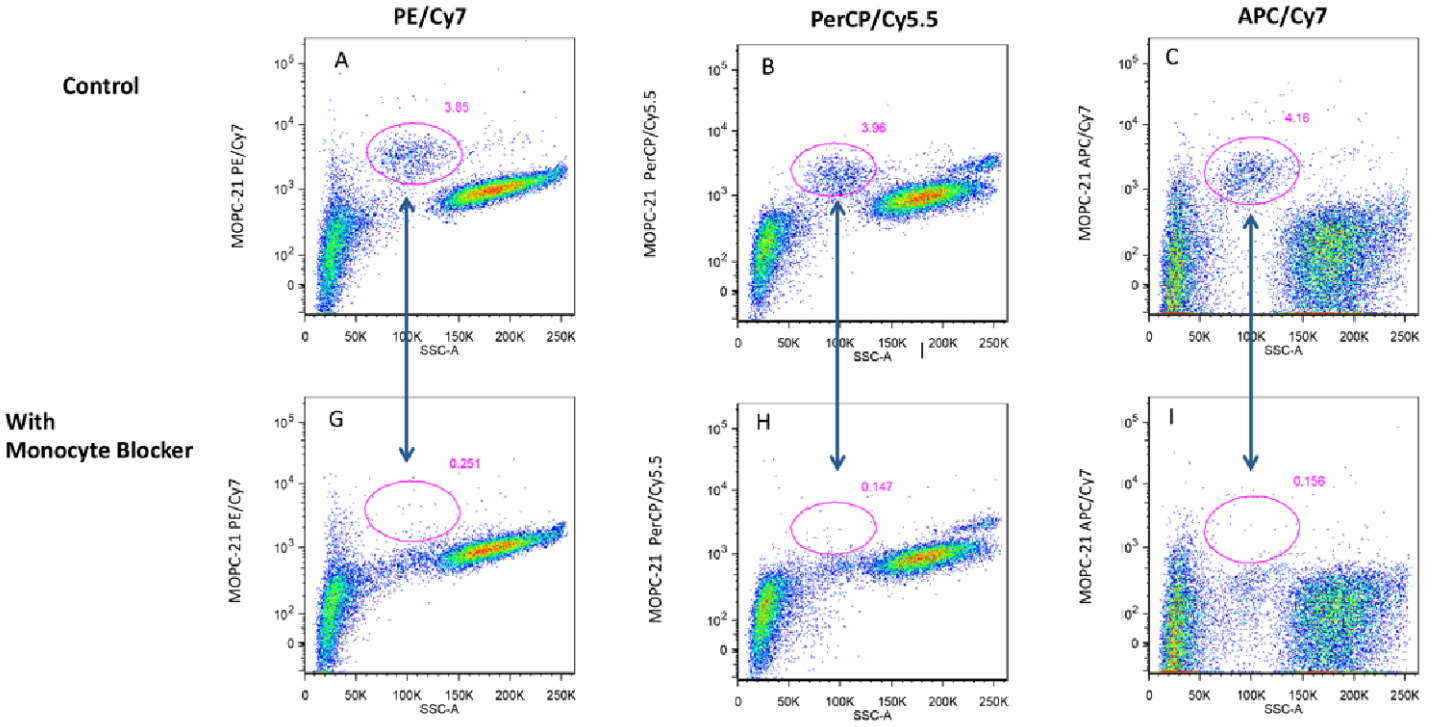


FcR Block to make your data rock

Wushouer Ouerkaxi

Optimize panel

- Staining protocol: use blocking!
  - Some dyes directly bind monocytes/myeloid cells
  - Use True-stain monocyte Blocker (Biolegend)

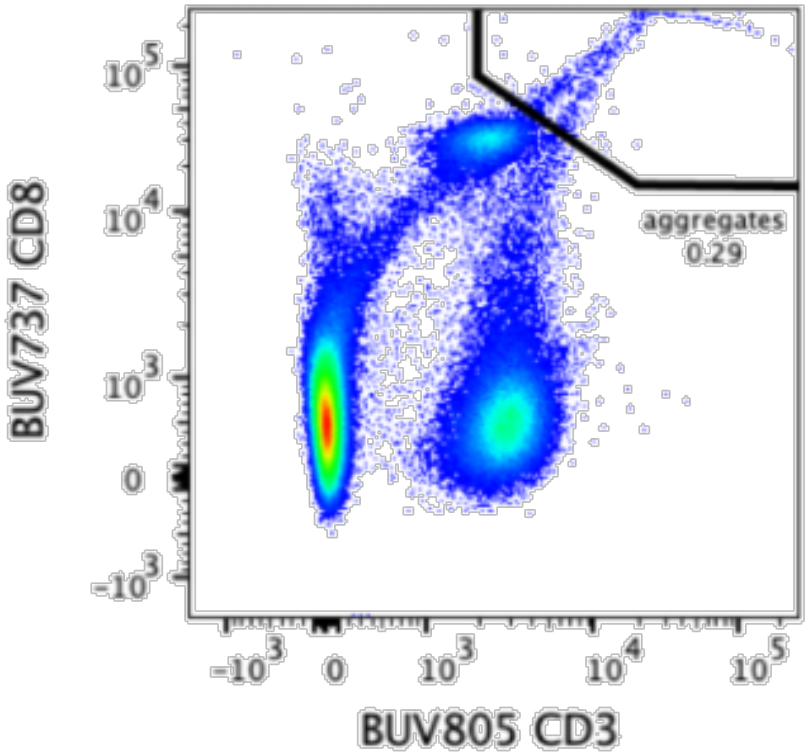


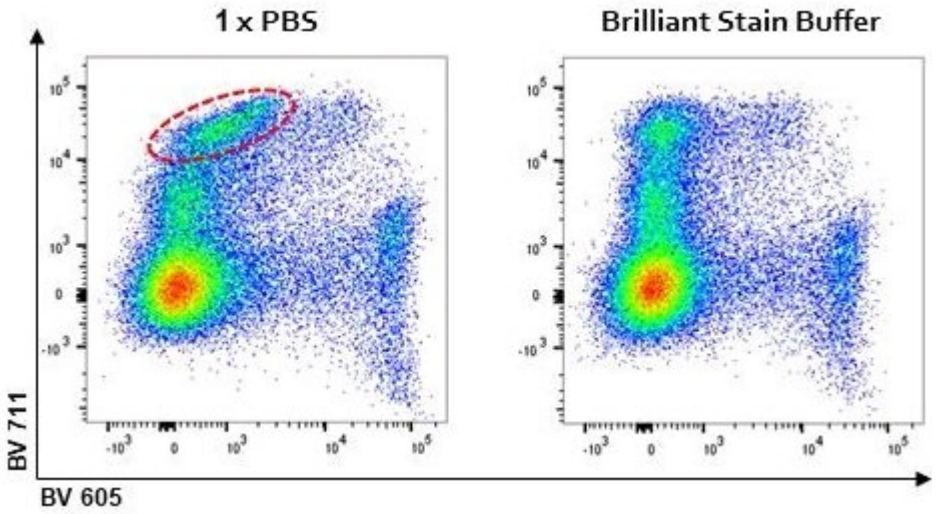
Fluorophore conjugates recommended for use with True-Stain Monocyte Blocker™
PE/Cy5
PE/Cy7
PE/Dazzle™ 594
APC/Fire™ 750
APC/Cy7
PerCP/Cy5.5
PerCP/Cyanine5.5

Optimize panel

Avoid Fluorochrome aggregates

- Brilliant Violet dyes:



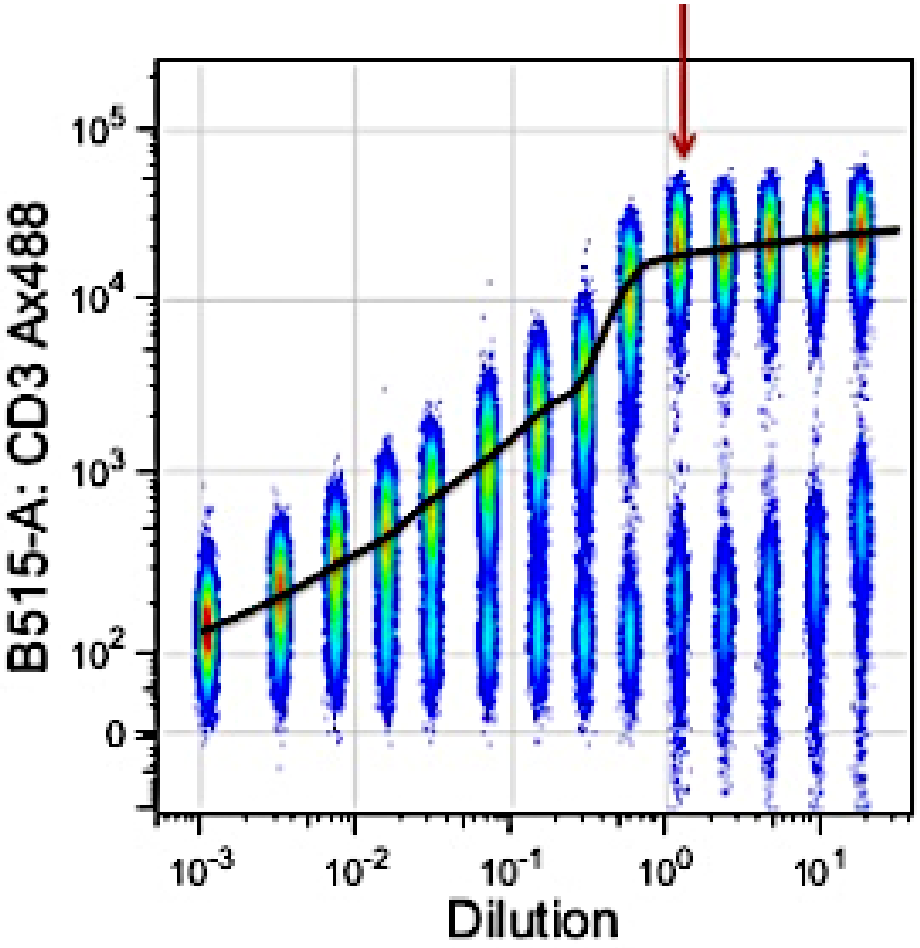


Antibody/ BV aggregates:

- use BV staining buffer
- Spin antibody vial 10,000 RPM for 3 min prior to using

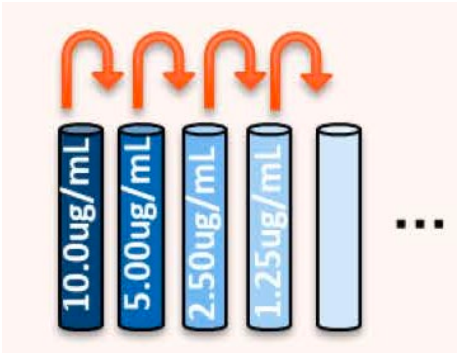
Optimize panel

Antibody/dye titration



Excess antibody binds aspecifically

Find the condition with the largest distance between the positive and negative populations: optimal bandwidth/resolution



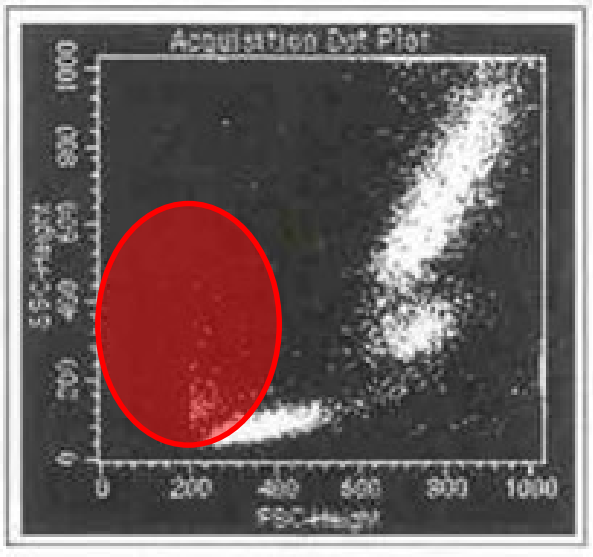
Optimize panel

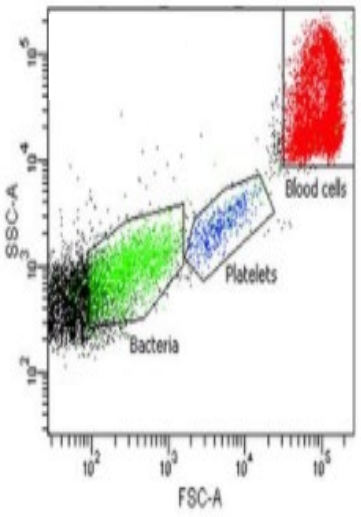
Antibody/dye titration

Optimize panel

- Erylysis: use erylysis buffer on samples with high amounts of erythrocytes

Bulk erythrocytes disturb leukocyte pattern

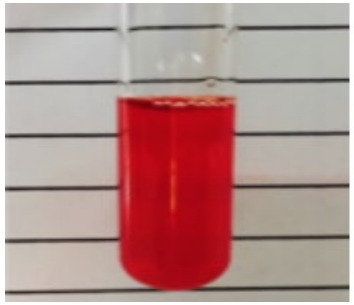




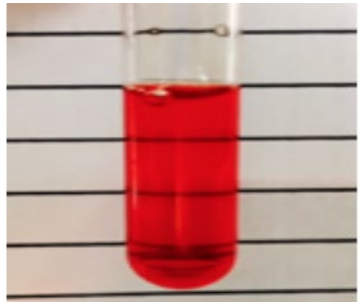
Before erylysis

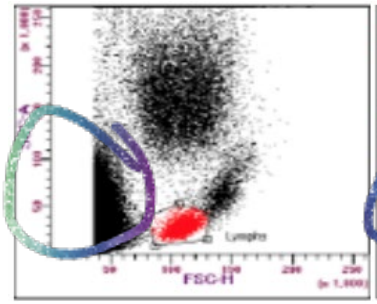
after erylysis

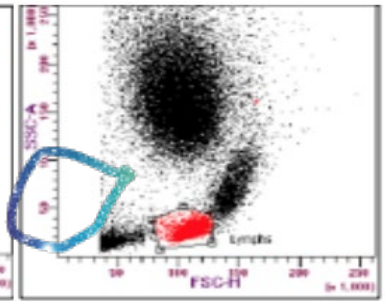
partly erylysis



fully erylysis

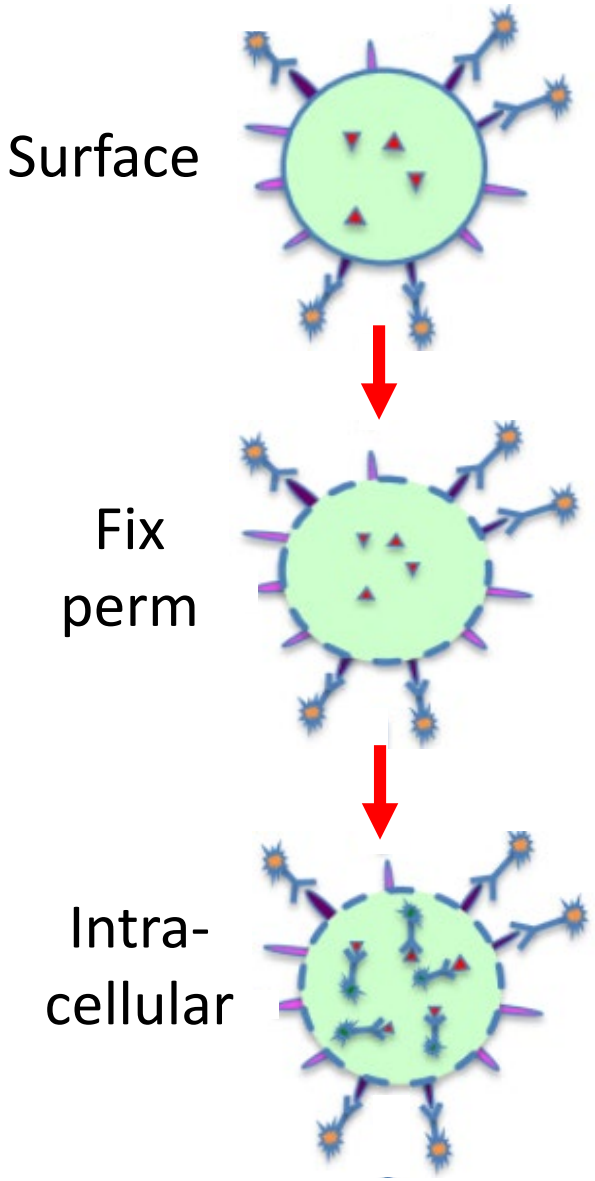






Optimize panel

- Staining protocol
  - Surface stain
  - Intracellulair stain
- Use appropriate fixation and permeabilization buffer system:
  - Cytoplasmic targets
  - Nuclear targets
  - Phosphorylated targets
- Test the effect on your other antibodies: epitopes might be damaged!

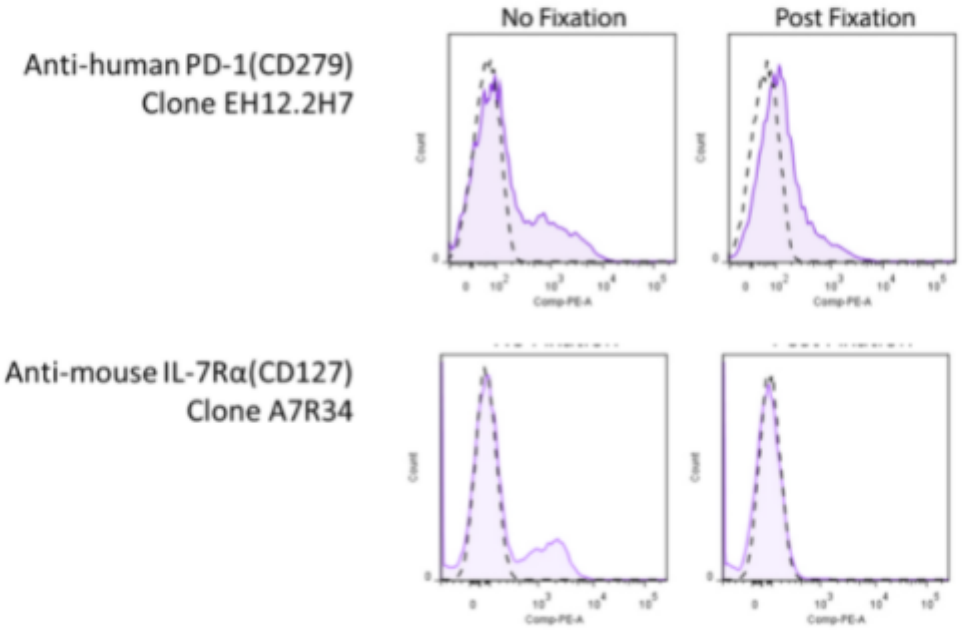


Optimize panel

- Check the effect of the fixative on your staining!

Fixation BEFORE staining - epitope alteration:

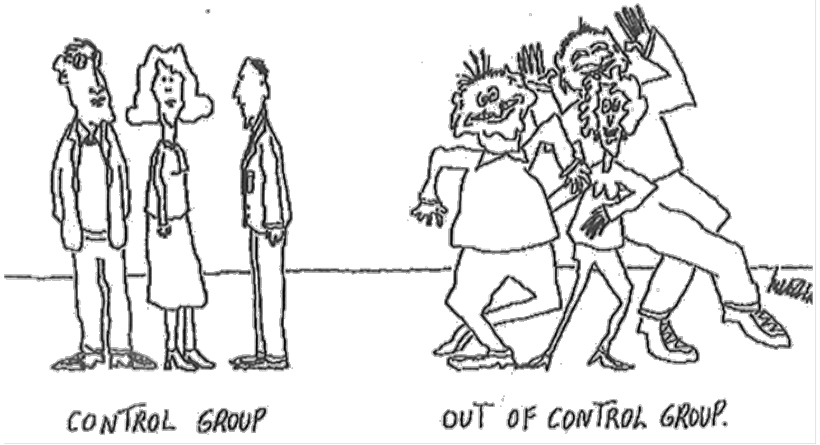
Due to the nature of fixatives, they can cause antigen epitope structures to be altered, which might render the antibodies unable to bind to their targets. Below are some examples of antibodies demonstrating loss of signal when stained on fixed cells:



Representative plots for target cells stained with (Post-Fixation) or without 4% PFA fixation.

https://www.biolegend.com/en-us/blog/fix-now-fix-later-considerations-for-the-use-of-paraformaldehyde-fixation-in-flow-cytometry

Controls



- Unstained controls
  - For every condition

  -> autofluorescence might change

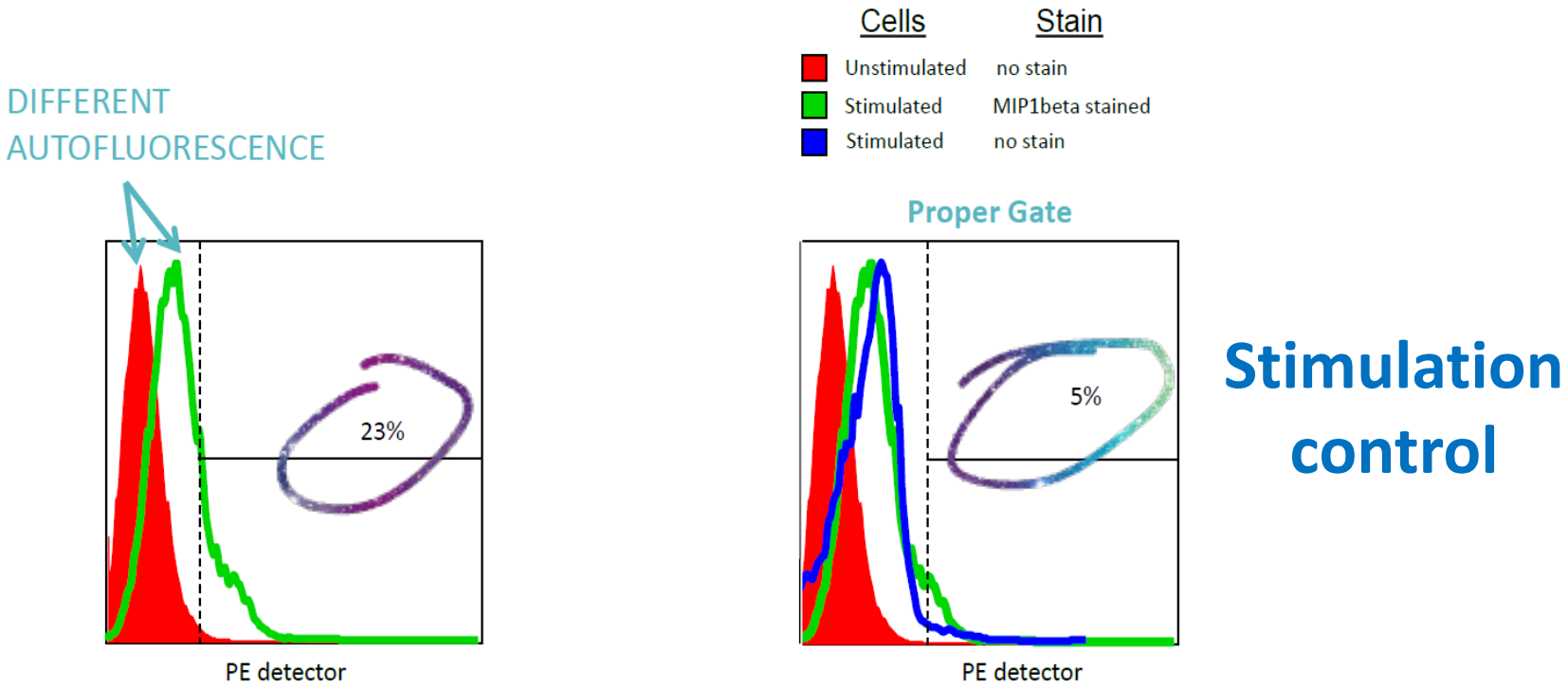
  -> measure enough events! 50-100K
  - Secondary antibody alone

- Compensation controls/ reference single stains
  - The control should be at least as bright as the sample (beads)
  - Kill cells for your life/dead single stain

- Use same reagents as in experiment
  - Fixatives, ….

Controls

- Use appropiate experimental controls
  - Treated/ untreated (stimulated)
  - Biological controls: cells with/without marker expression



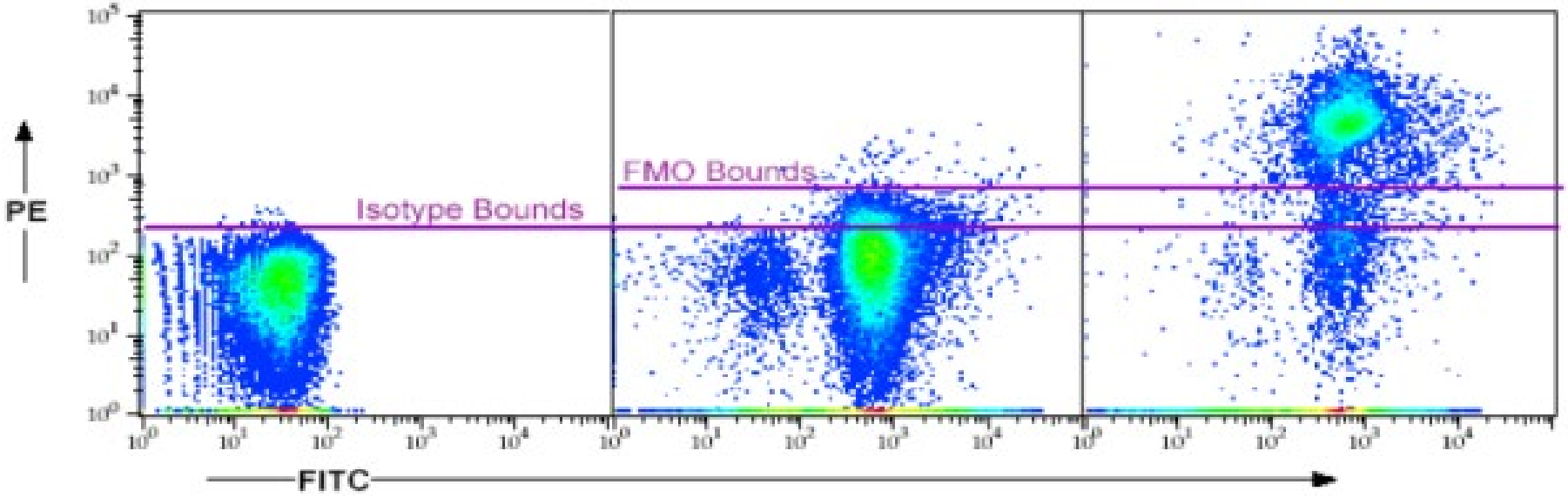
Stimulation control

Janine Bögli

Controls

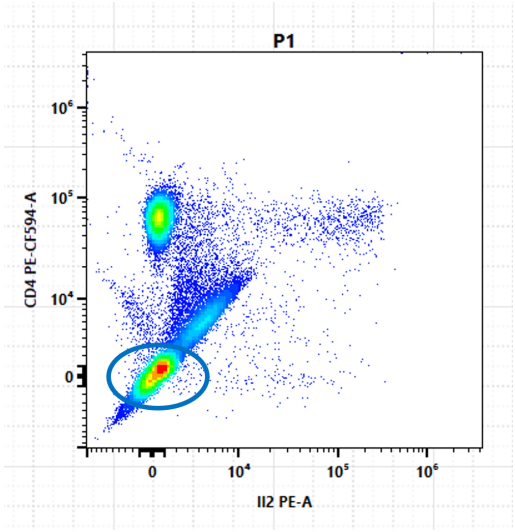
- FMO (Fluorescence Minus One)
  - Contains all the antibodies in the panel, minus one of them
  - Helpfull to define background, spread, autofluorescence and gate setting

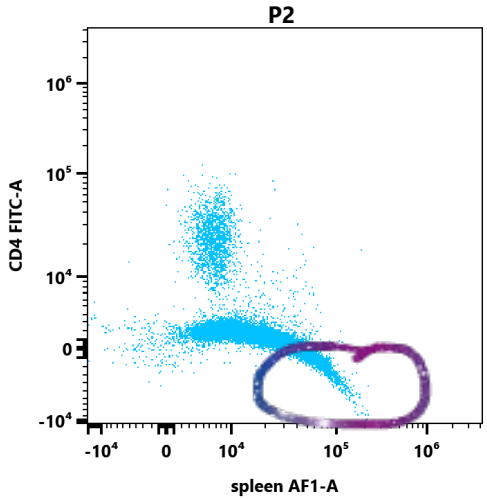
	Unstained Control	FMO Control	Fully Stained
FITC	–	CD3	CD3
PE	–	–	CD4
Cy5PE	–	CD8	CD8
Cy7PE	–	CD45RO	CD45RO

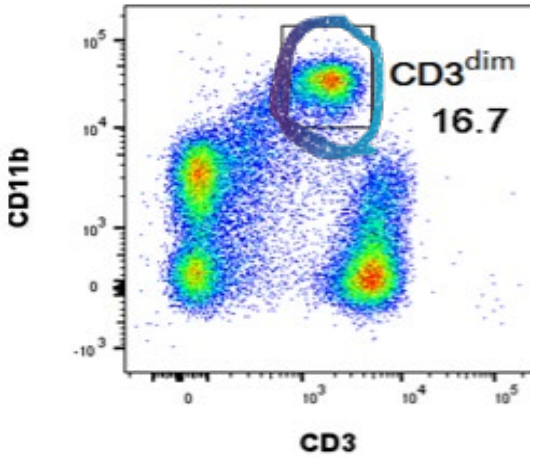


Clean up data

- Spot spillover/compensation/unmixing errors
  - -> weirdly shaped populations -> should be round
  - -> extreme negatives -> a below 0 light signal does not exist
  - -> biological impossibilities -> marker expression







- Come to us for help

Clean up data

Fix common errors

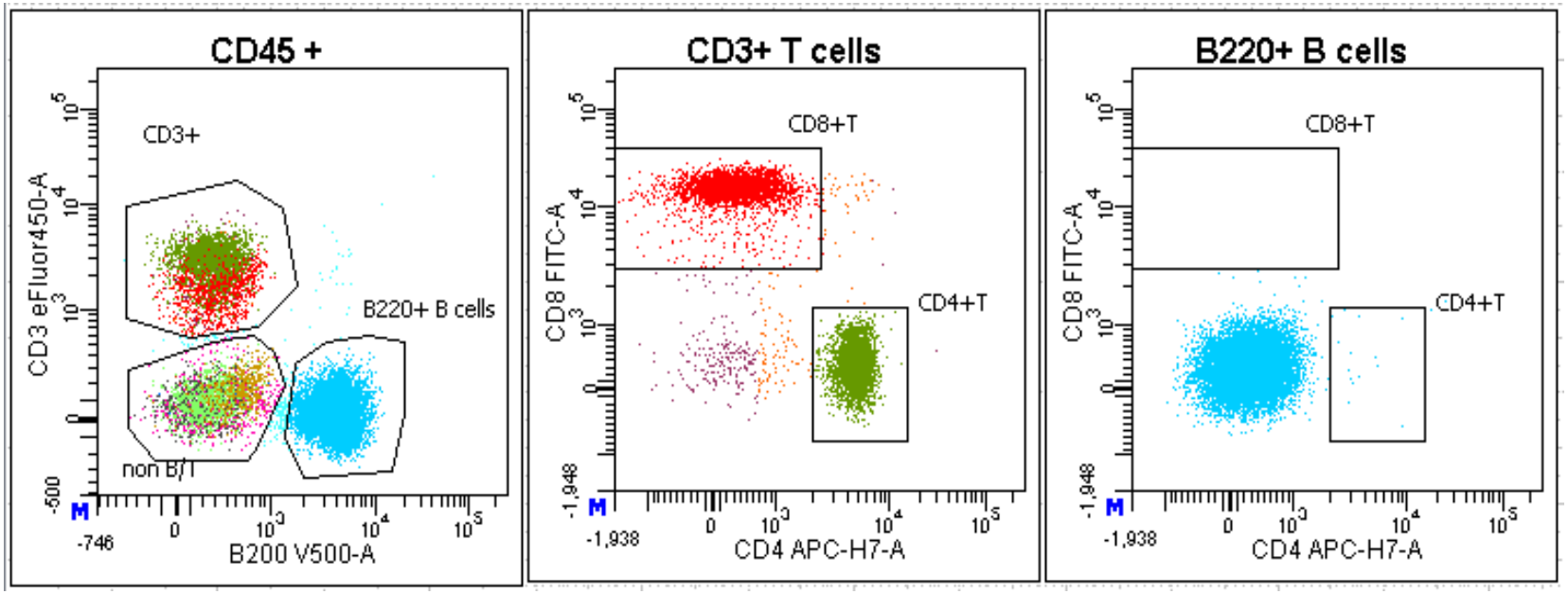
- ❑ Check controls: are they good?
- ❑ Set compensation/unmixing gates better
  - ➢ Bright and narrow
- ❑ Replace bead controls with cells or vice versa
- ❑ Record a new control if needed
- ❑ Come to us for help

Evaluate panel

- Use your biological knowledge to quality-check your data

  -> Check populations for correct marker expression: eg B-cells are negative for Tcell markers

  -> compare with literature (expected % of populations)



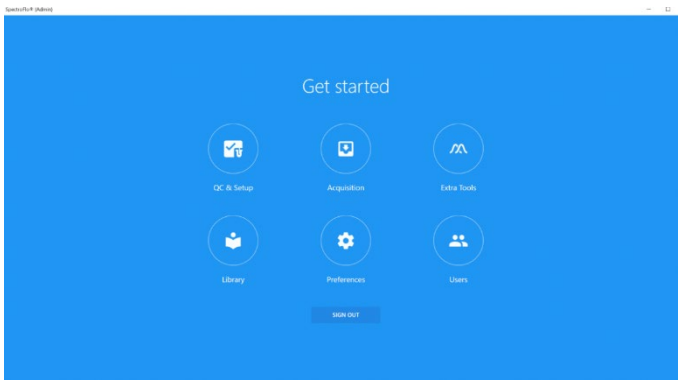
Setup experiment in software

DIVA

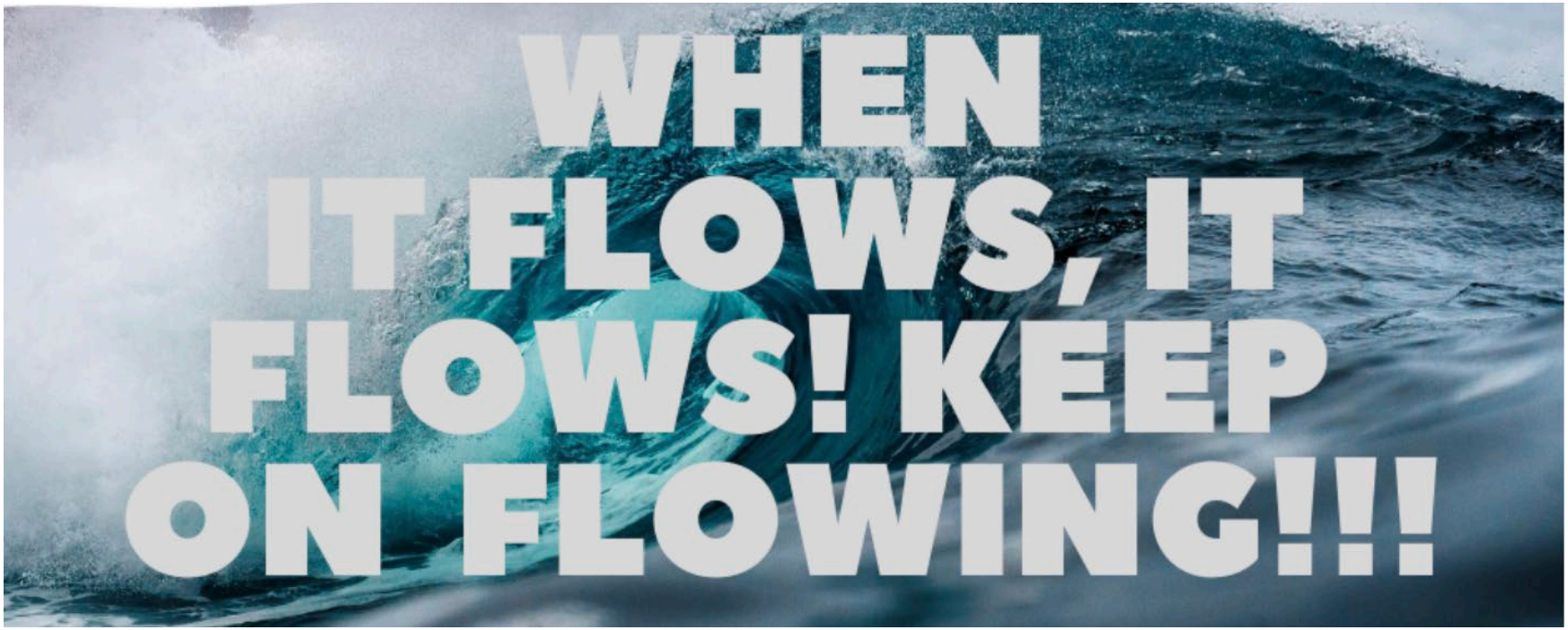
- For CANTO
- Introduction through Erwin
- Excellent SOP available
- BDFacsDIVA manual

SPECTROFLO

- For Cytek Aurora
- Introduction through Lieve/Kristiaan
- SOP in progress
- Excellent on-line tutorials
  - Cytek website
  - University of Chicago Flow: youtube channel



Questions?



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