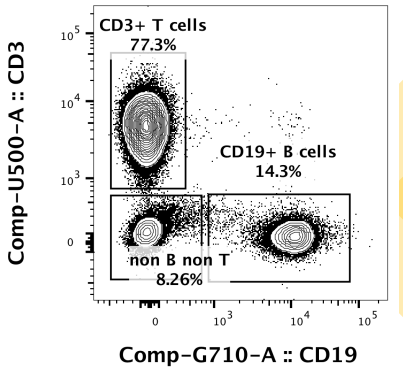
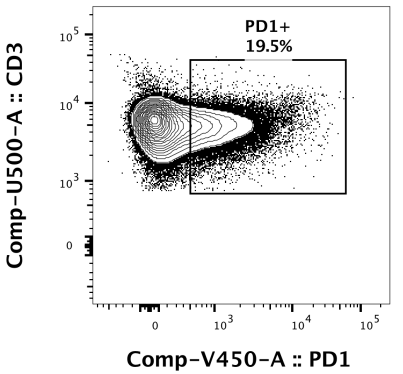
Antigen Categorization

- Marker expression usually comes in three different patterns:
- **On/Off** expression, usually lineage markers (CD3, CD4, CD19)
- **Intermediate** or **continuous** expression patterns (CD45RA, CD38, CD57, cytokines, many more)
- **Dimly** expressed or rare markers
  - Choose detectors that receive low spillover and pair with bright fluorochromes





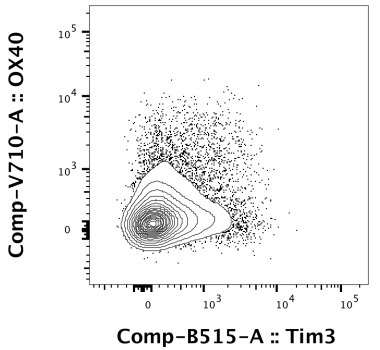
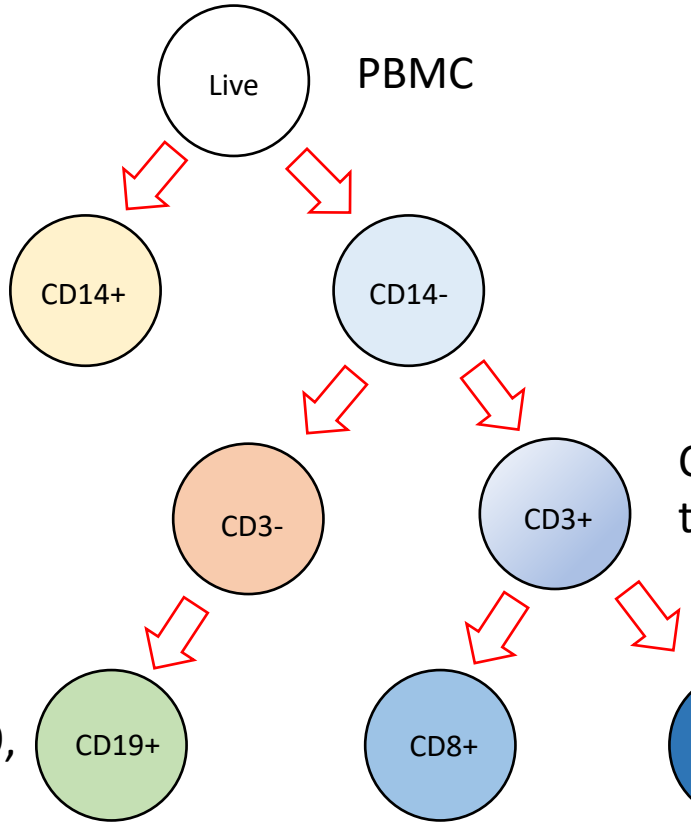


Image courtesy of Florian Mair

USE A GATING TREE TO ASSESS CO-EXPRESSION OF MARKERS



These cells express CD14, but nothing else

CD4 and CD8 T cells will co-express CD3, but they are mutually exclusive for CD4 and CD8

These cells express CD19, but nothing else

These cells will co-express FOXP3, CD4, and CD3

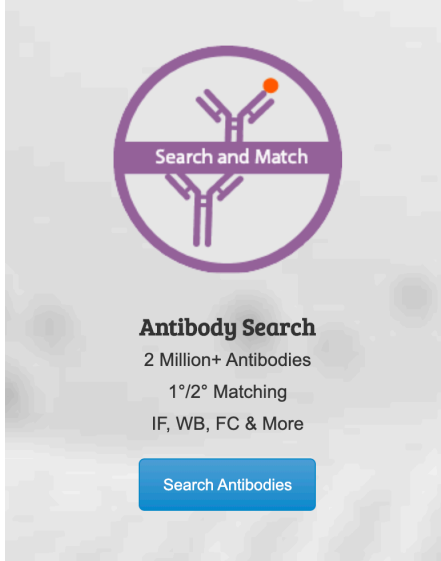
These cells will co-express CD4 and CD3, but not FOXP3

ASSESS COMMERCIAL AVAILABILITY OF CONJUGATED FLUOROCHROMES

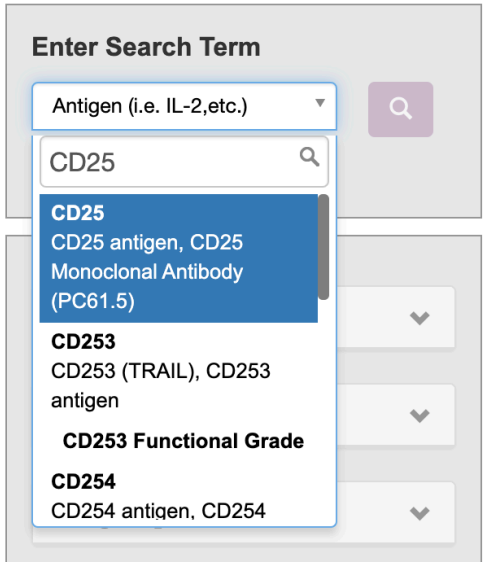
- Fluorofinder – online resource/database that shows all commercially available antibody-fluorochrome conjugates
  - www.fluorofinder.com
  - Also has an online panel builder and spectra viewer

- Use filter to narrow results for
  - Target species
  - Company (or you will get false positives from Biorbyt)
  - Fluorochrome (if you want)
  - Clone (if you want)

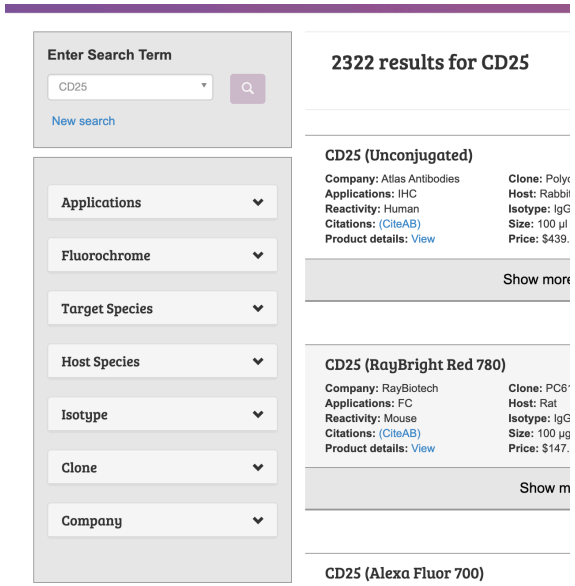
USING FLUORFINDER



Click on “Search Antibodies”

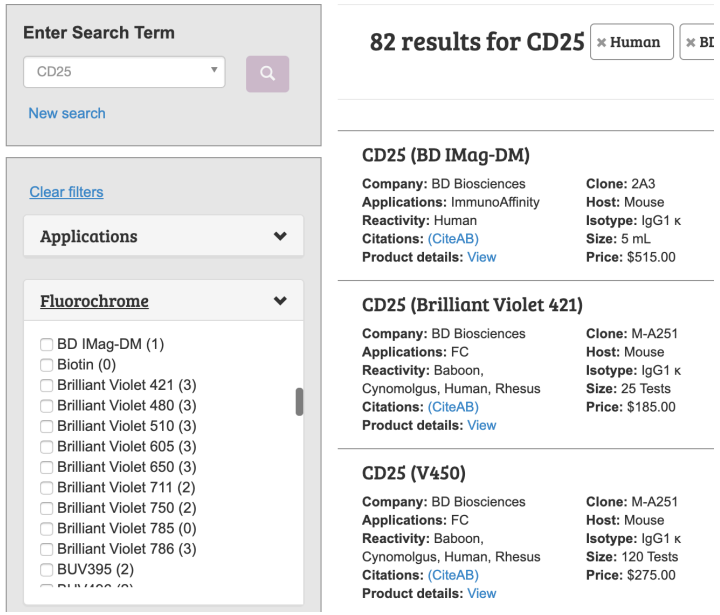


Enter antigen of interest



Narrow down results using the filters

Scroll through fluorochromes to check availability

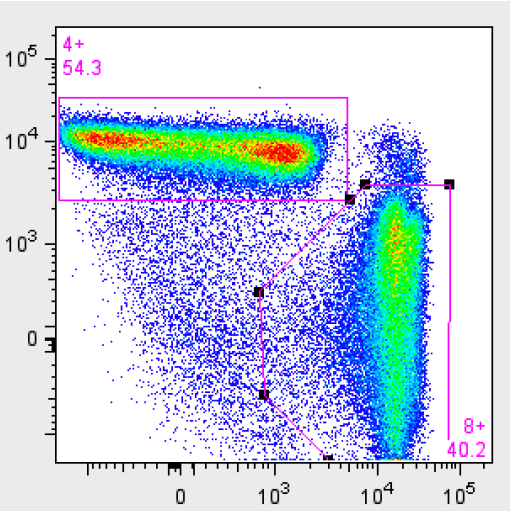


USE THE SSM AND GATING TREE TO GUIDE ANTIBODY-FLUOROCHROME PAIRINGS

- Assign bright markers (or highly/broadly expressed antigens) to channels that contribute little spillover
- Assign critical or dimly expressing makers to channels that accept little spillover
- Place mutually exclusive combinations on channels with high spillover/spread values
- Use the spillover/spread matrix and gating tree to guide placement of co-expressing markers

	B515	B610	B660	B710	B780	G575	G610	G660	G710	G780
B515	0	0.668	0.638	0.763	0.319	0.237	0.236	0	0.649	0.0928
B610	0.251	0	4.66	5.11	1.41	1.35	3.71	2.08	4.76	0.659
B660	0.918	2.02	0	7.09	1.98	2.59	1.04	3.25	6.1	0.977
B710	0.848	0.677	2.73	0	4.34	2.05	0.205	1.53	10.1	3.62
B780	0.713	0.538	0.637	1.34	0	0.537	0.335	0.342	1.22	2.38
G575	0.203	4.1	2.3	2.87	0.676	0	2.1	2.01	2.95	0.55
G610	0.162	5.08	4.33	6.28	1.54	2.17	0	3.71	6.55	1.39
G660	0	0.439	4.38	6.35	2.35	1.95	0.506	0	7.69	2.06
G710	0.362	1.38	3.54	17.9	5.04	5.27	0.953	4.41	0	6.3
G780	0	0.304	0.383	0.598	6.64	0.476	0.331	0.43	0.824	0
R660	0	0.218	1.19	1.49	0.62	0.622	0.376	1.1	1.76	0.561
R710	0	0	0.465	1.74	0.844	0.511	0.14	0	2.07	0.884

Ex: CD4 and CD8 on G710 and B710 are still distinguishable as both markers are mutually exclusive



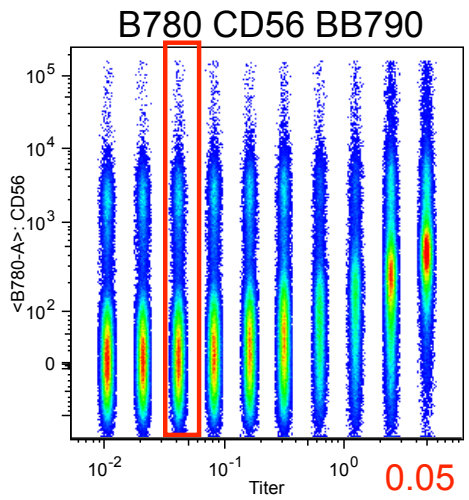
ANTIBODY TITRATION(1)

- ALWAYS TITRATE!!!!
  - Every clone will behave differently
  - Manufacturers vial at different concentrations

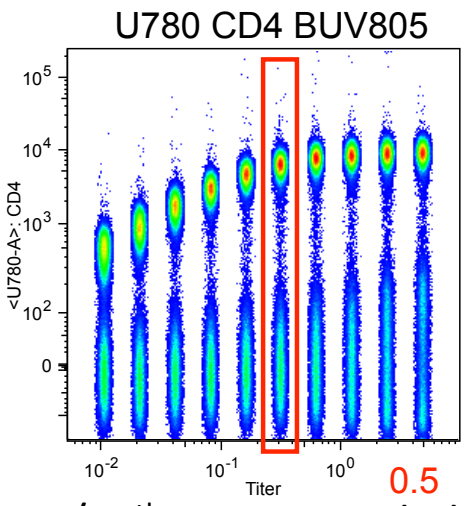
- Titrate under the conditions in which the antibody will be used in the full panel
  - i.e. surface antibodies that are part of an intracellular assay must be fixed/permed

ANTIBODY TITRATION(2)

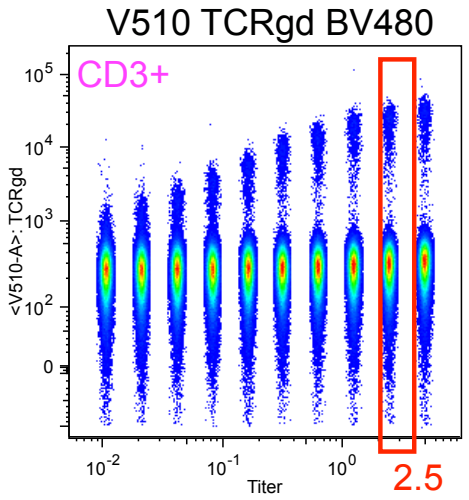
- Titrating will identify the optimal concentration at which to use the antibody
  - ➢ It will (almost always) save reagent (money)



1/100th recommended titer



1/10th recommended titer



1/2 recommended titer

ANTIBODY TITRATION(3)

- Spreading error can be reduced if a saturating concentration is not needed (lineage markers)
  - ➢Spreading is proportional to signal intensity

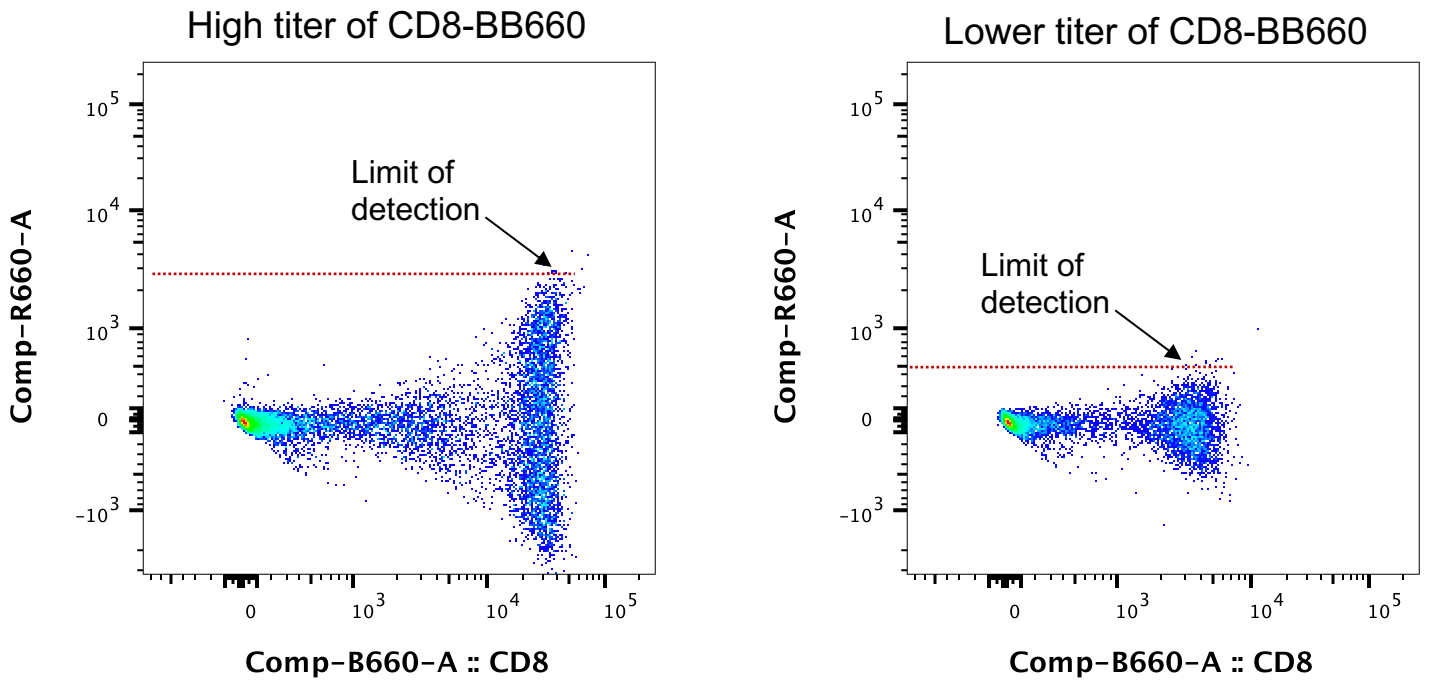
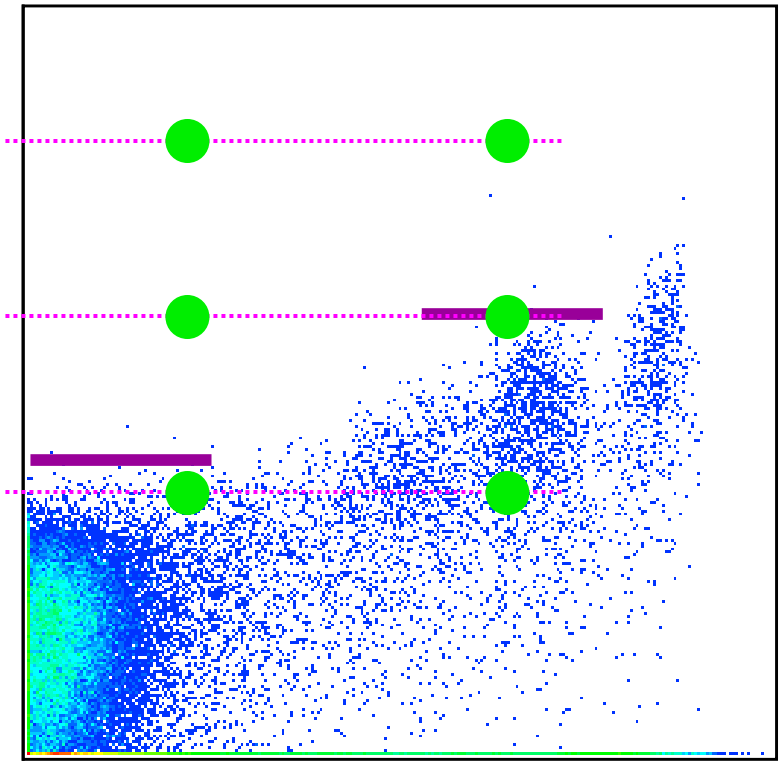


Image courtesy of Florian Mair

STAINING CONTROLS

- Necessary to identify cells which do or do not express a given antigen
- Threshold for positivity may depend on the amount of fluorescence in other channels
- Unstained cells or isotype controls stains are improper controls

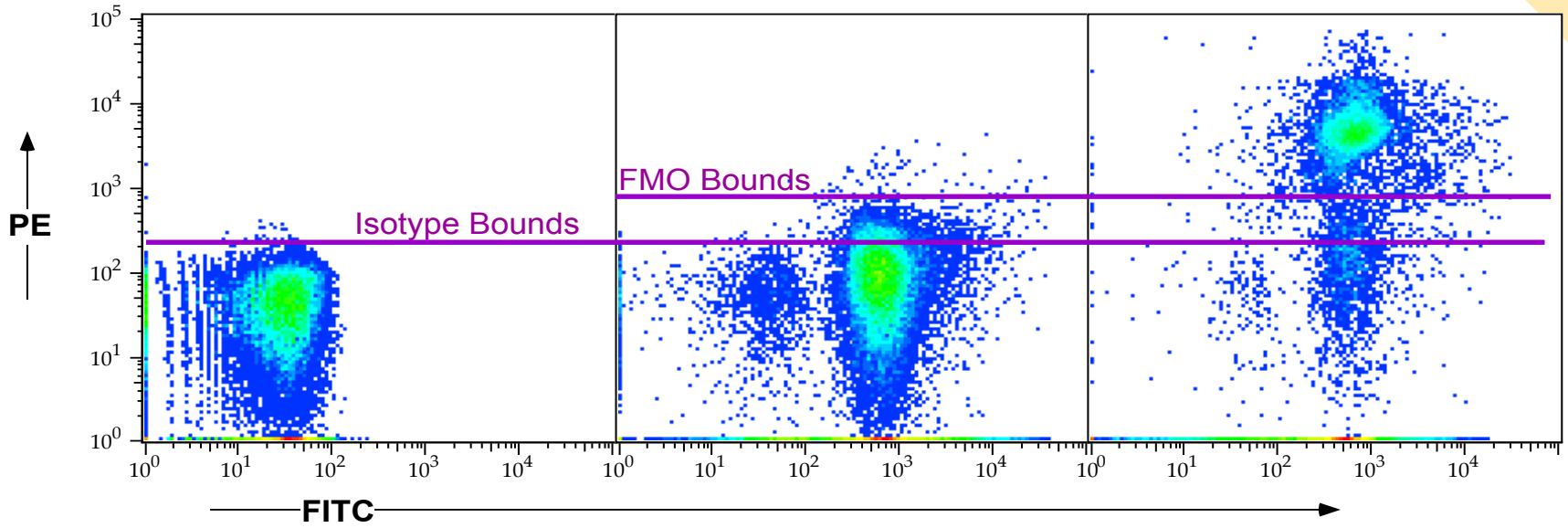


Slide provided by M. Roederer, NIH

FMO Controls

- FMO = Fluorescence Minus One
  - ➢ Cells are stained with all reagents EXCEPT the one of interest
- Essential for complex panels
- Reveal unnoticed or unexpected issues with spreading
- Should be used for setting correct gates

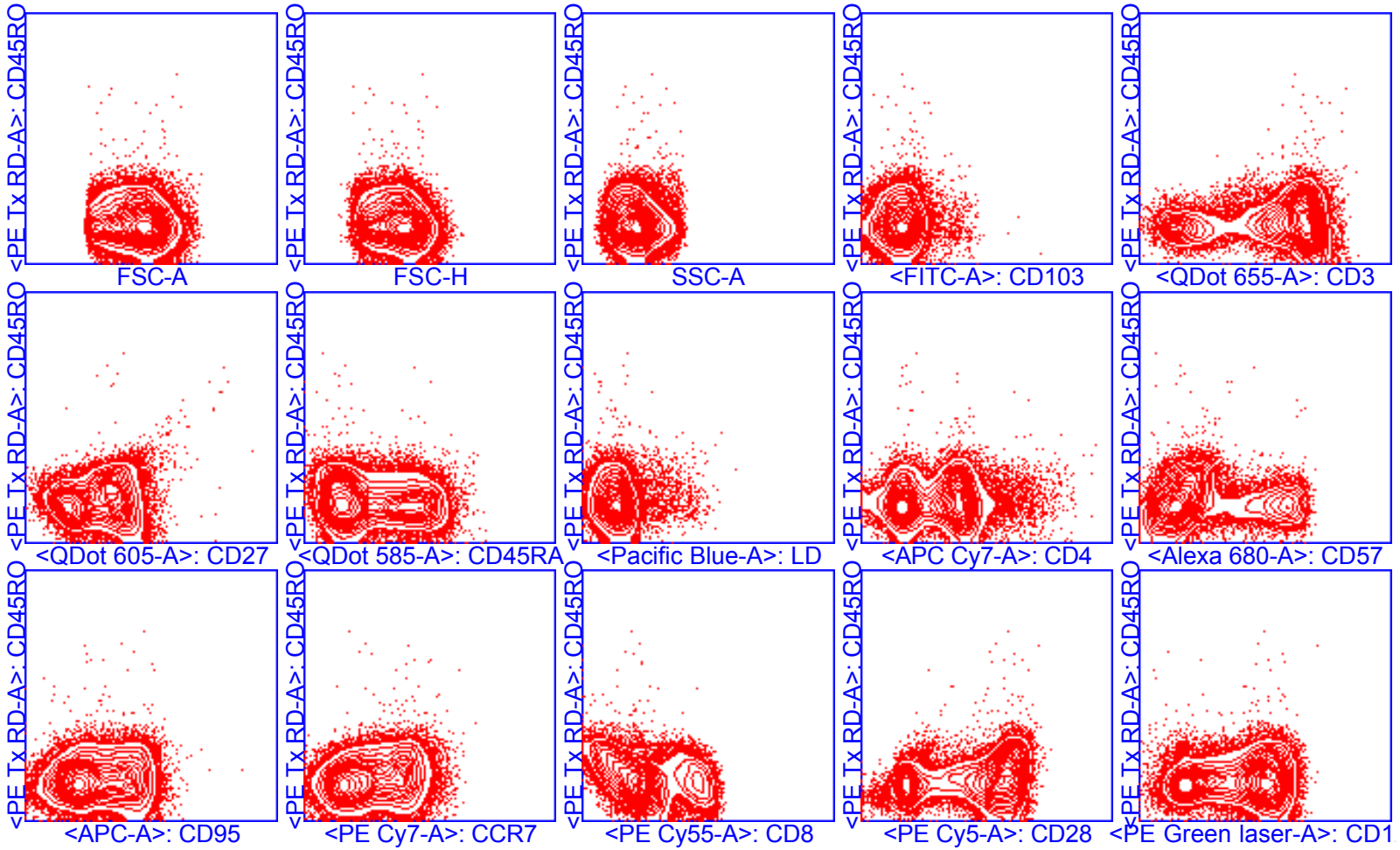
	Unstained Control	FMO Control	Fully Stained
FITC	–	CD3	CD3
PE	–	–	CD4
Cy5PE	–	CD8	CD8
Cy7PE	–	CD45RO	CD45RO



PBMC stained as shown. Compensation properly set.

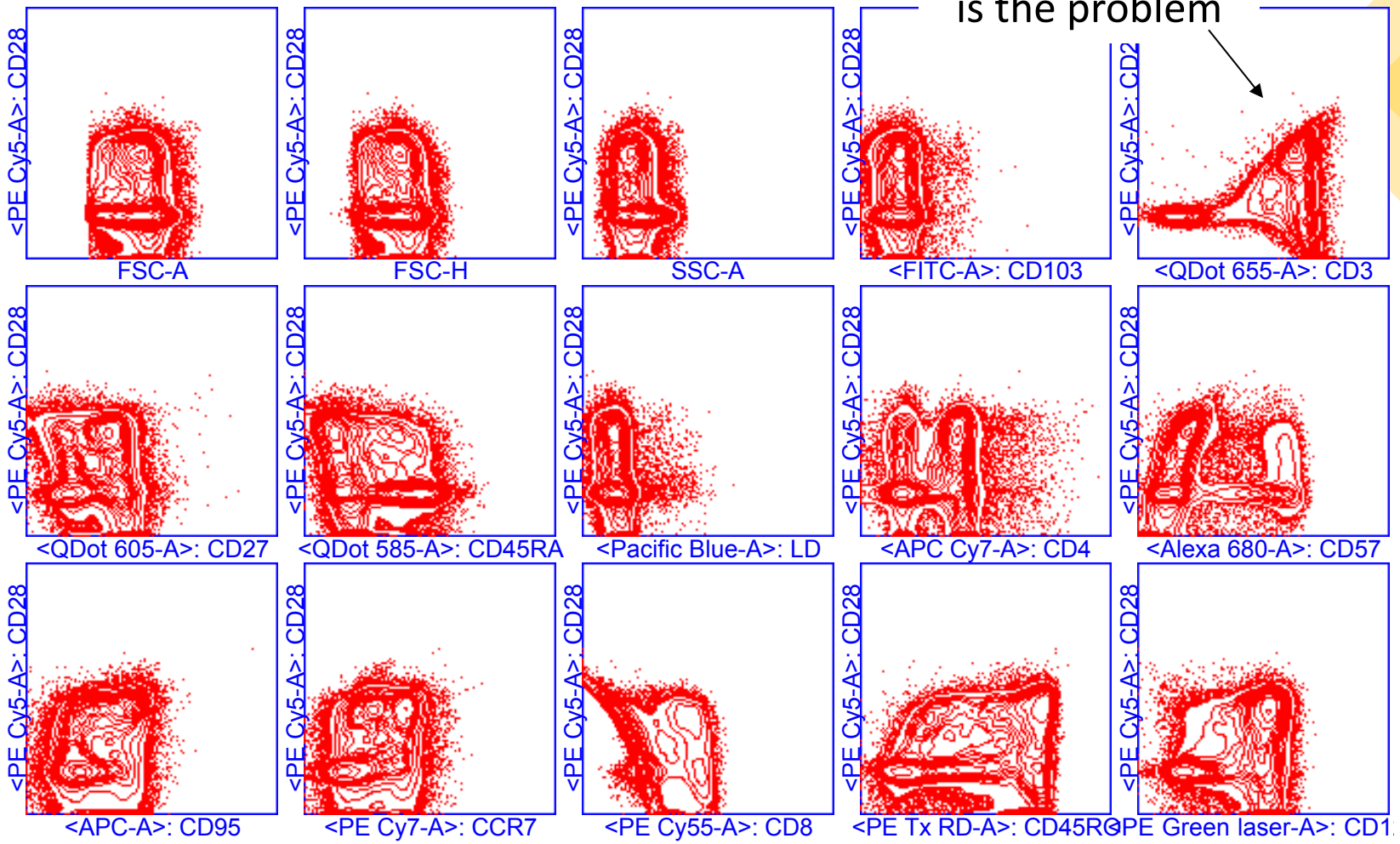
Perfetto et al, Nat Rev Immunol 2004

FMO Example – Missing PE-TR



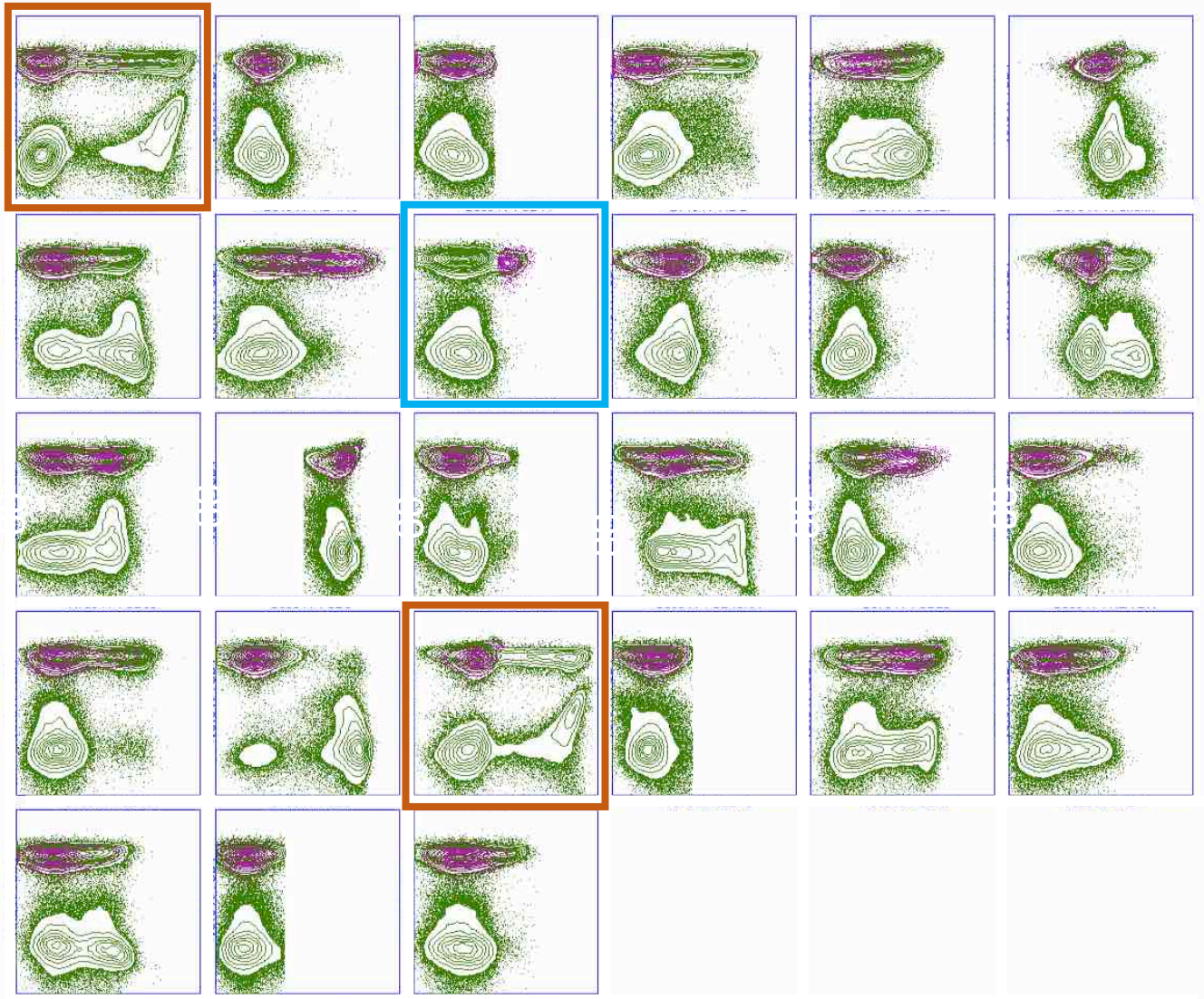
FMO Example – Missing PE-Cy5

- CD28 PE-Cy5 was not added to the staining cocktail but there appears to be a positive signal
- Staining artefact comes from large spreading of Qdot reagent



PANEL ASSESSMENT USING MULTIGRAPH OVERLAYS

- Overlay different populations to see where subsets exist
- Identify potential spreading issues



SUMMARY

- Compensation
  - Compensation values are arbitrary
  - All panels (but especially large) require appropriate compensation controls
  - Analyze properly transformed (and compensated) data
  - Properly compensated data reveals errors, it does not cause them
- Spillover/Spreading
  - Spillover/spreading error is the single most important contributor to background and loss of resolution
  - Spreading error is instruments specific
  - Spreading error is proportional to signal intensity
- Panel Development
  - A standardized, optimized instrument is key to successful panel development
  - Use the SSM matrix and antigen co-expression to guide marker placement
  - Titrate antibodies
  - Use appropriate controls and QC checks when assessing a new panel
  - The process is iterative and sometimes frustrating

Acknowledgments

Mario Roderer, NIH
Stephen Perfetto, NIH
Florian Mair, FH
Stephen De Rosa, FH
Julie McElrath, FH

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