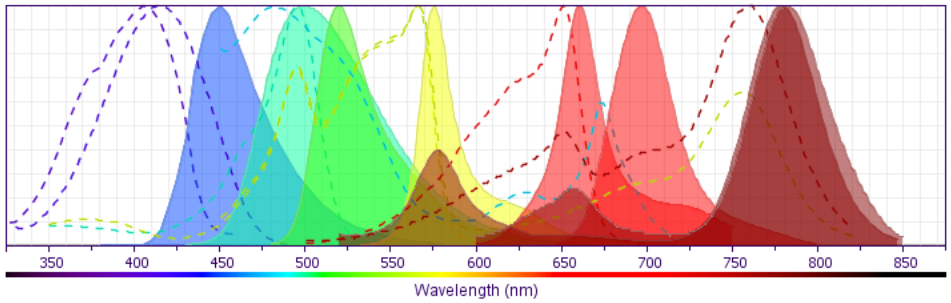
Multicolor Panel Design

Zosia Maciorowski

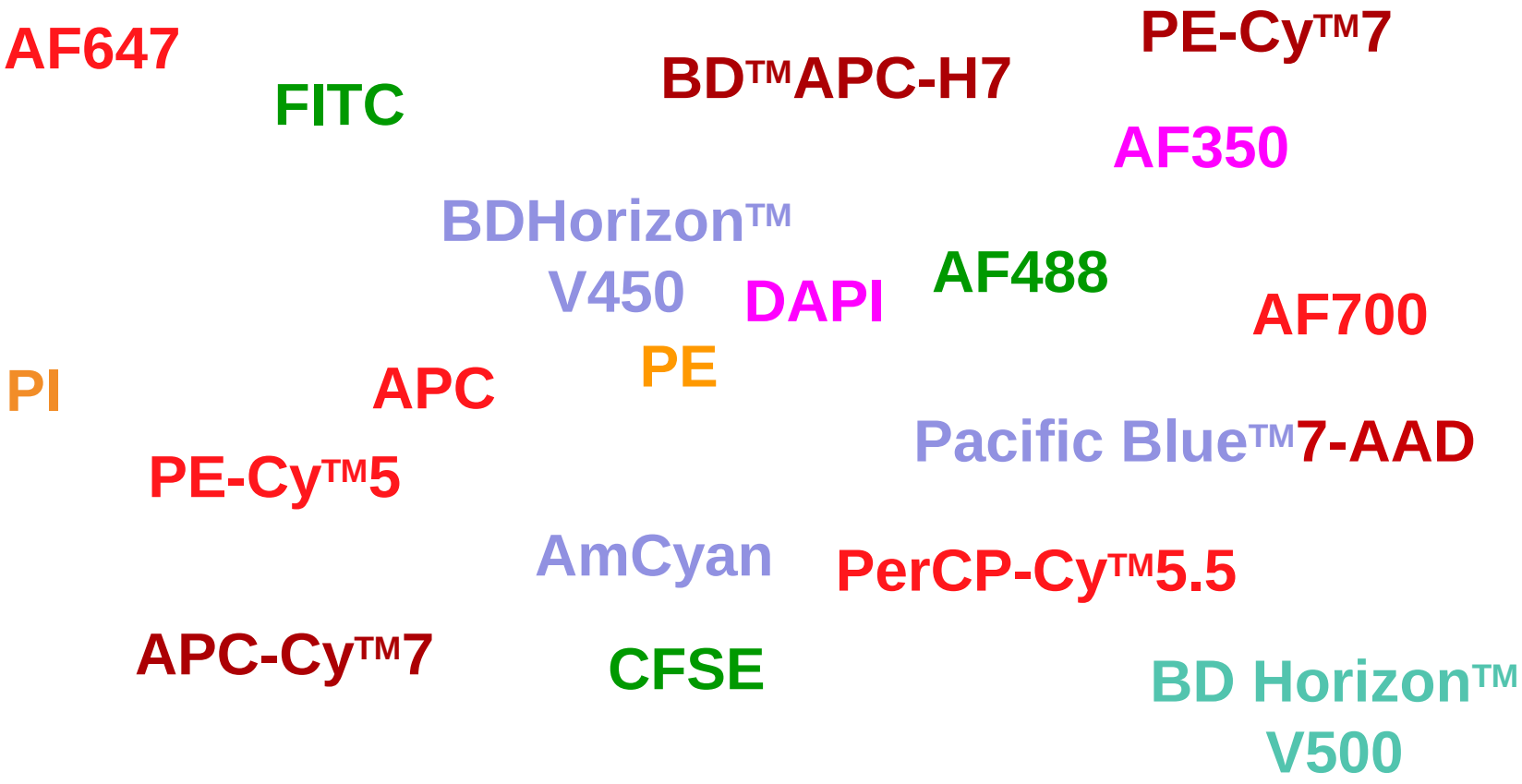
Institut Curie

Paris, France

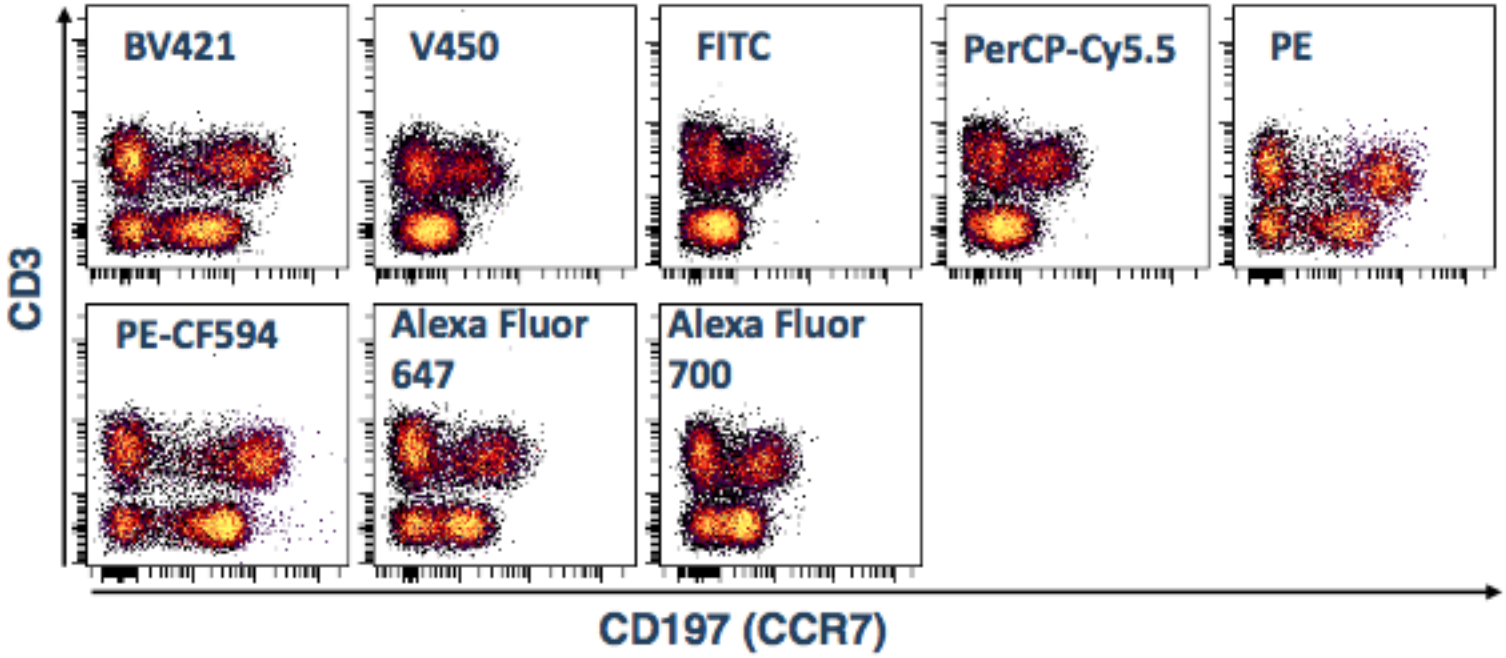


DESIGNING A MULTICOLOR PANEL: Fluorochromes Galore!

But how to combine them for multicolor experiments?



Fluorochrome Choice is Key



- Choosing the correct combination of fluorochromes is key to answer biological questions

BD

How to choose fluorochromes

1. Know your cytometer: lasers and filters, what is possible

2. Know your antigens: priority, expression and density

3. Match fluorochrome brightness with antigen density

   low density antigens need bright fluorochromes
   high density antigens are ok with dim fluorochromes

4. Look at coexpression of 2 or more antigens on same cell

5. If there is coexpression:

   choose fluorochromes very carefully!
   avoid high spread situation from a high expressor into a low expressor.

Cytometer Configuration

Your fluorochrome choice will be determined by your cytometer

- Know what colors you can use before you start

You may have a choice of different cytometers

- Lots of lasers are good even if you don't need all the colors
- High laser power is good; you will get better resolution of weak staining
- Often filters can be easily changed to improve your sensitivity

Know your cytometers

- Characterize stain index and SSM (your core facility may have done this)
- These will be different from machine to machine

If you're planning on sorting these cells in the future, remember stream in air sorters often have lower sensitivity

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Antigens: Priority and classification

1. Prioritize your markers:

   Which ones are necessary

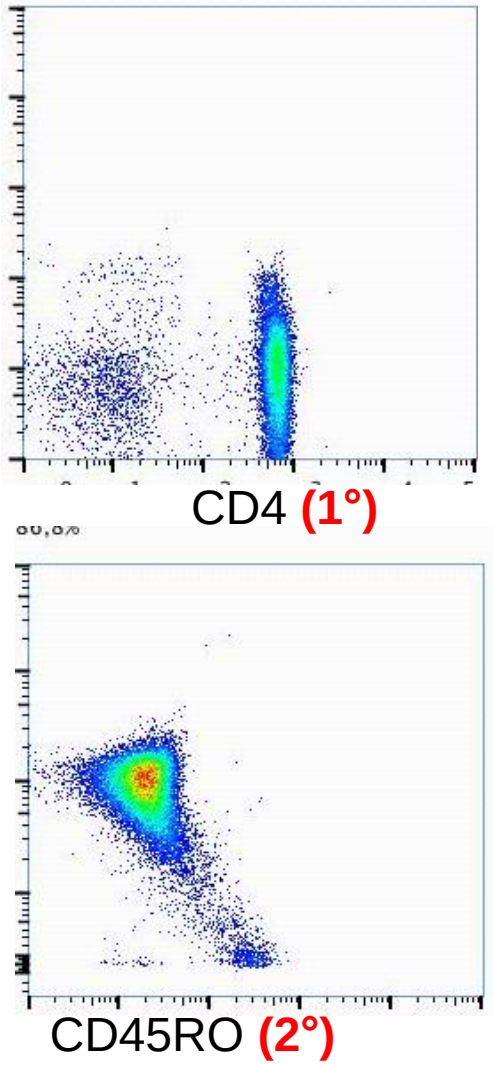
   Which ones are luxury

2. Classify your antigens

   1° high density, on or off

   2° high density, continuum staining

   3° low or unknown density

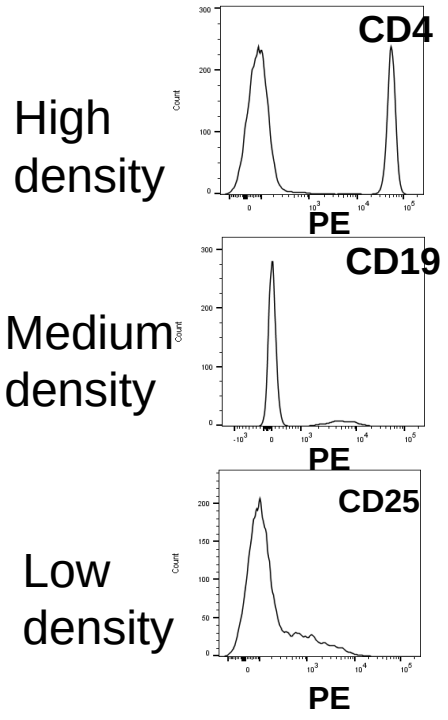


What is your Antigen Density?

Find literature data for density of antigen molecules per cell in **your** system
Low expression antigens will be most difficult to detect

Antigen	Antigen-Density	ExpressionLevel
CD3	90.000	++
CD4	100.000	++
CD8	124.000	+++
CD14	110.000	++
CD19	18.000	+
CD45	200.000	+++
CD56	10.000	+
CD80	2.000	+

Antigen-expression High / Intermediate / Low:
+++ / ++ / +



List of Antigen Densities

A list of antigen density for 300 antigens is being elucidated by BD and will be published in the near future

Subset	Antigen	Density
Lymphocytes	CD3	32,000
	CD4	36,400
	CD8	65,500
	CD19	7,800
T Cells (CD3⁺CD4⁺ Lymphocytes)	CD25	600
	CD25^{hi}	3,400
	CD27	10,900
	CD28	7,700
	CD45RA	33,400
	CD45RO	12,600
	CD122	5,300
	CD127	2,000
	CD132	400
	CD194 (CCR4)	2,500
	CD197 (CCR7)	2,000
B Cells (CD19⁺ Lymphocytes)	CD20	24,600
	CD24^{mid}	3,000
	CD24^{hi}	16,100
	CD27	3,200
	CD38^{mid}	2,800
	CD38^{hi}	15,900
	CD138	400
	IgD^{mid}	4,900
	IgD^{hi}	23,800
	IgG	28,100
	IgM	3,800

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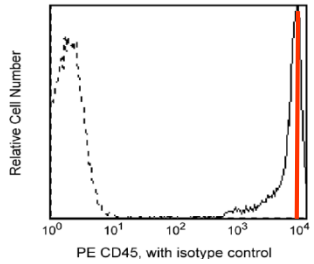
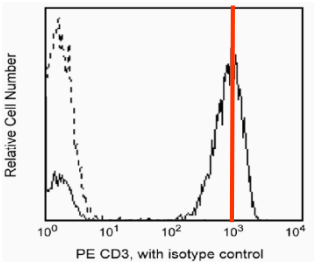
Choose your fluorochromes based on antigen density

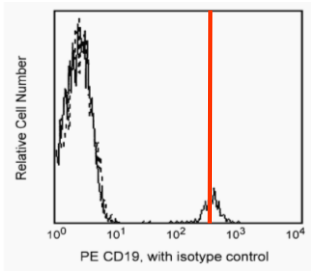
Here we are using PE-coupled antibodies: PE is a very bright fluorochrome



CD45 PE

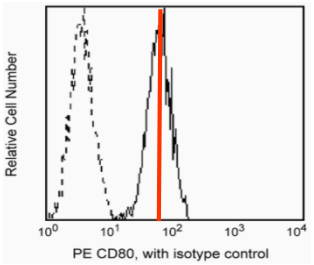


CD3 PE



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

Pairing Fluorochromes with Antigen Density

SI (Stain Index) is a measure of staining intensity on a specific cytometer

Fluorochrome	SI
BD Horizon™ V450	65
BD Horizon™ V500	27
AmCyan	37
Alexa Fluor® 488	68
FITC	43
PE	305
APC	263
Alexa Fluor® 647	184
PE-Cy™5	198
PerCP	30
PerCP-Cy™5.5	99
Alexa Fluor® 700	64
APC-Cy™7	36
BD™ APC-H7	25
PE-Cy™7	122

Use bright fluorochromes to detect weakly expressed antigens

Use intermediate to bright fluorochromes to detect intermediately expressed antigens, or antigens of unknown expression-levels

Use dim fluorochromes to detect highly expressed antigens

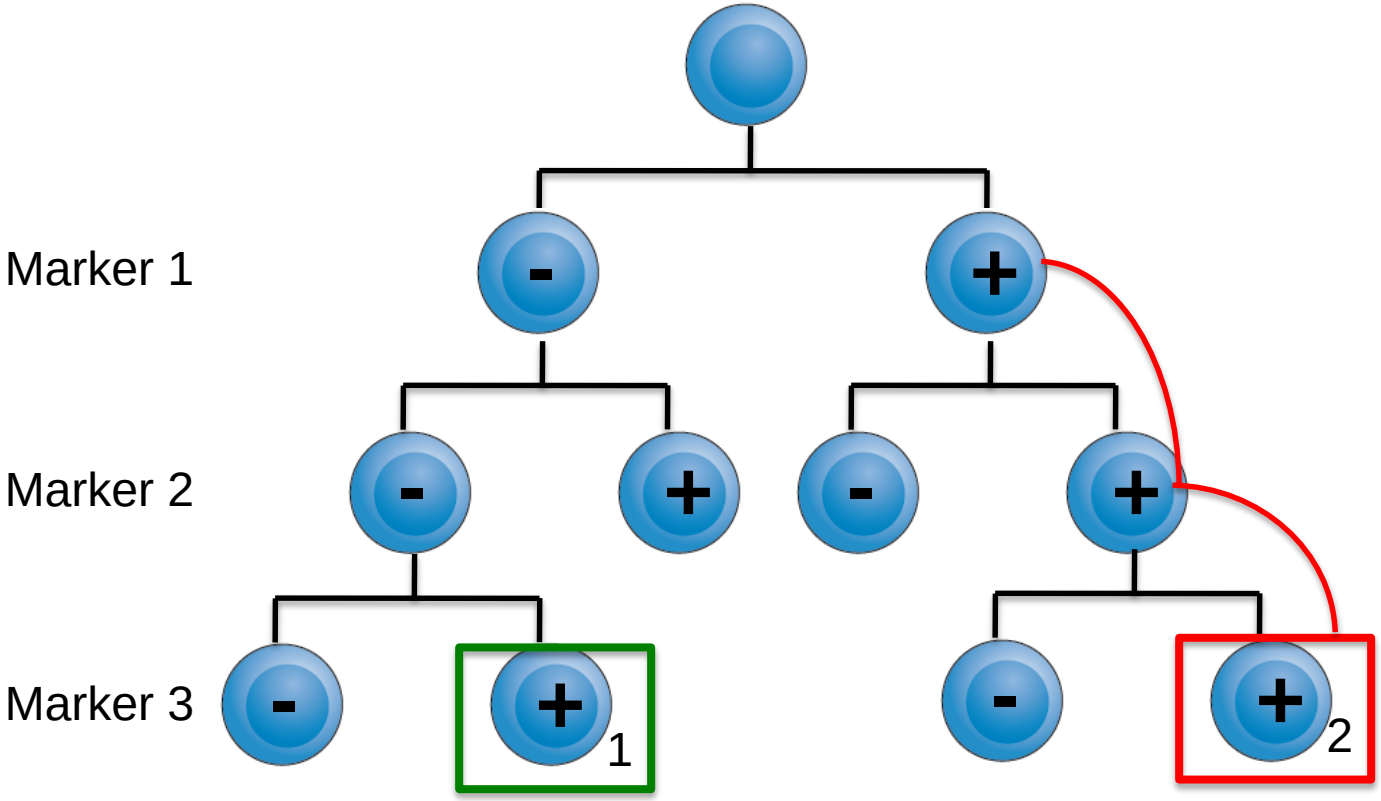
Is there co-expression?

Coexpression of 2 or more antigens on the same cell

can make detection of dim double positives very difficult

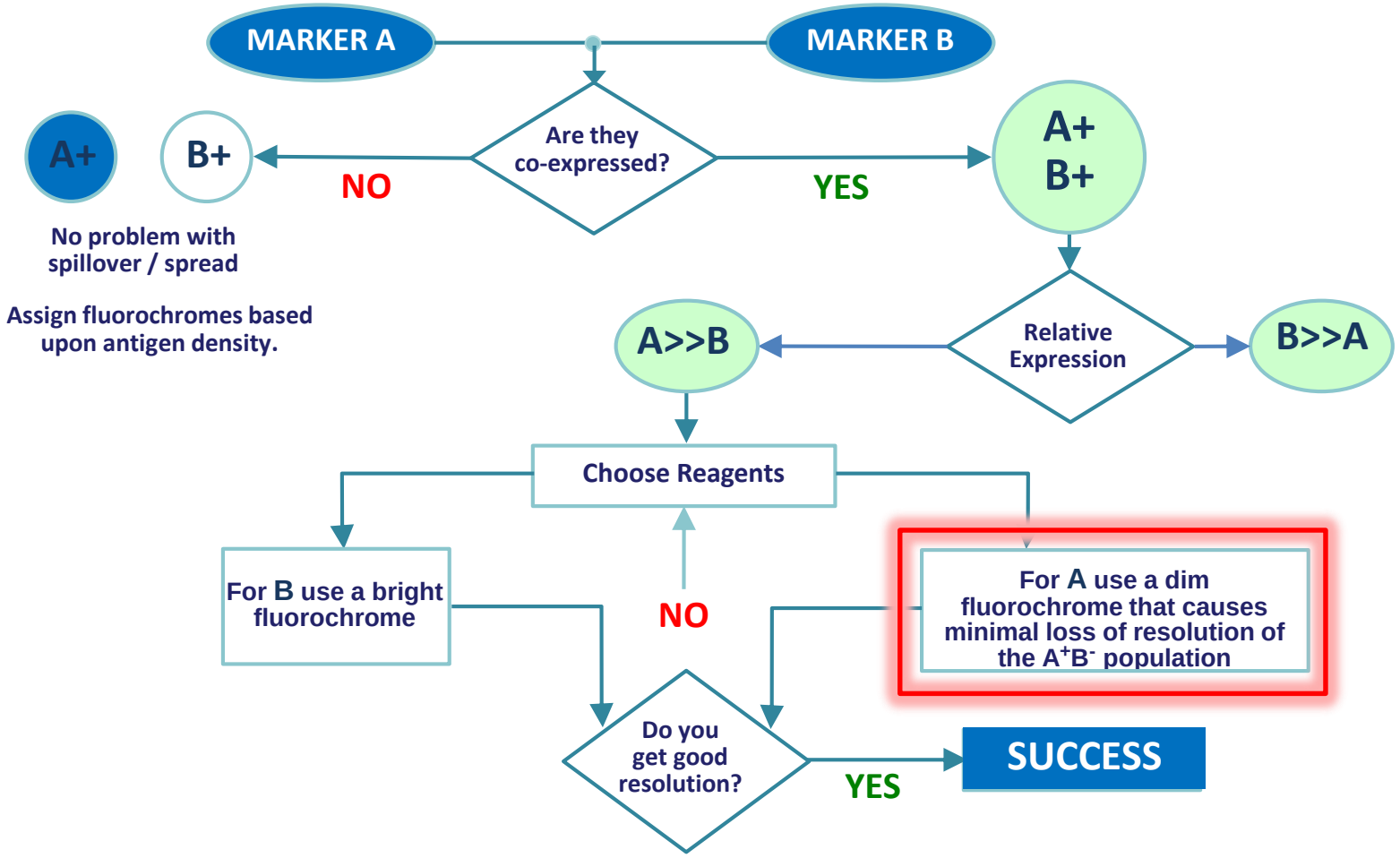
due to spread from multiple fluorochromes

Coexpression



From this experiment strategy, it can be seen that cell 1 is positive only for marker 3, thus has no co-expression. Cell 2 coexpresses all of the other markers, so care must be taken in fluorochrome choice to ensure resolution if some of these markers are dim.

How to Manage Coexpression and Spread



Co-expression and spread

choose your fluorochrome pair carefully

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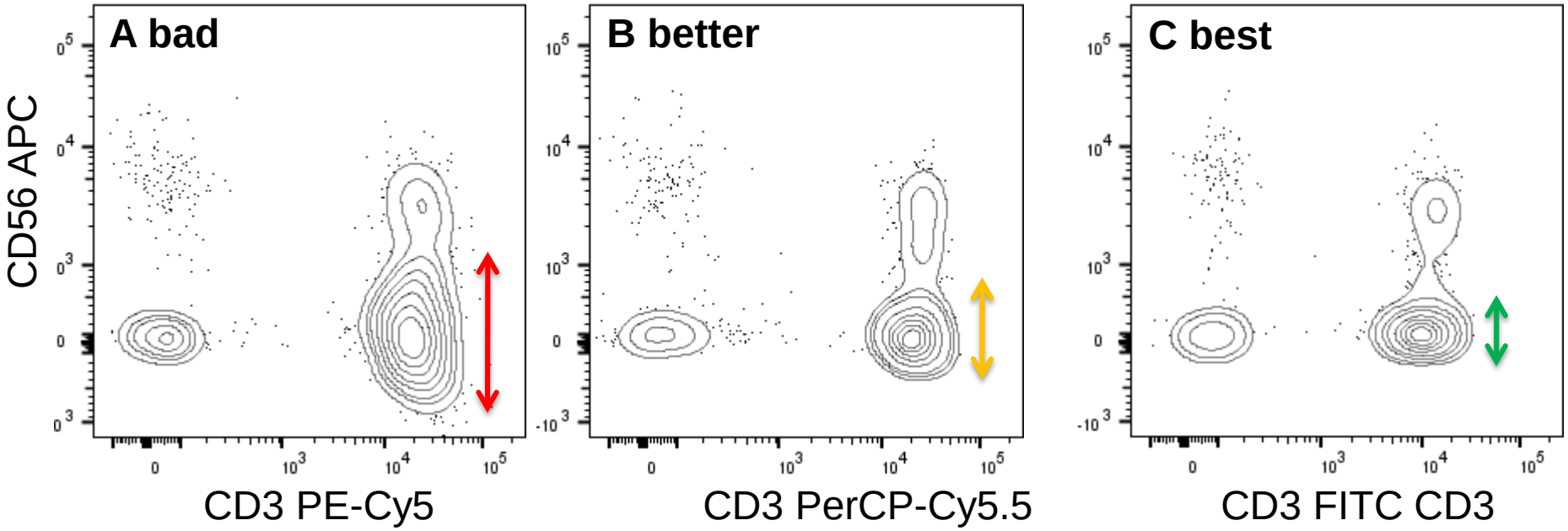
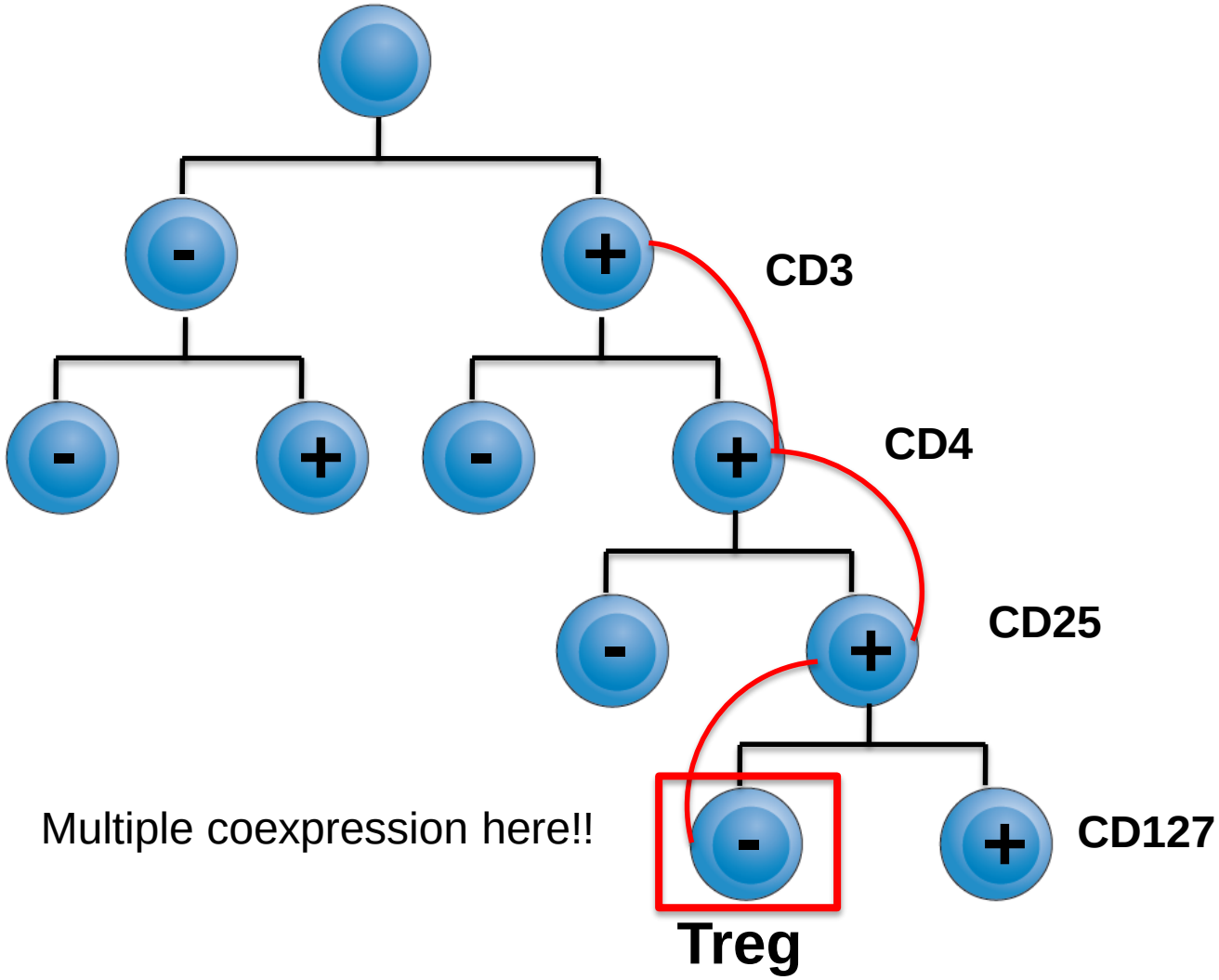
CD56-APC, a low expressor, is shown here paired with 3 different CD3-coupled fluorochromes.



A. The high spread of PE-Cy5 into APC here prevents resolution of the CD56+CD3+ cells
B. Less spread with CD3-PerCP-Cy5.5: resolution is better
C. Very little spread with CD3-FITC allows good double positive resolution. FITC is not bright, but certainly adequate for a high expressor like CD3

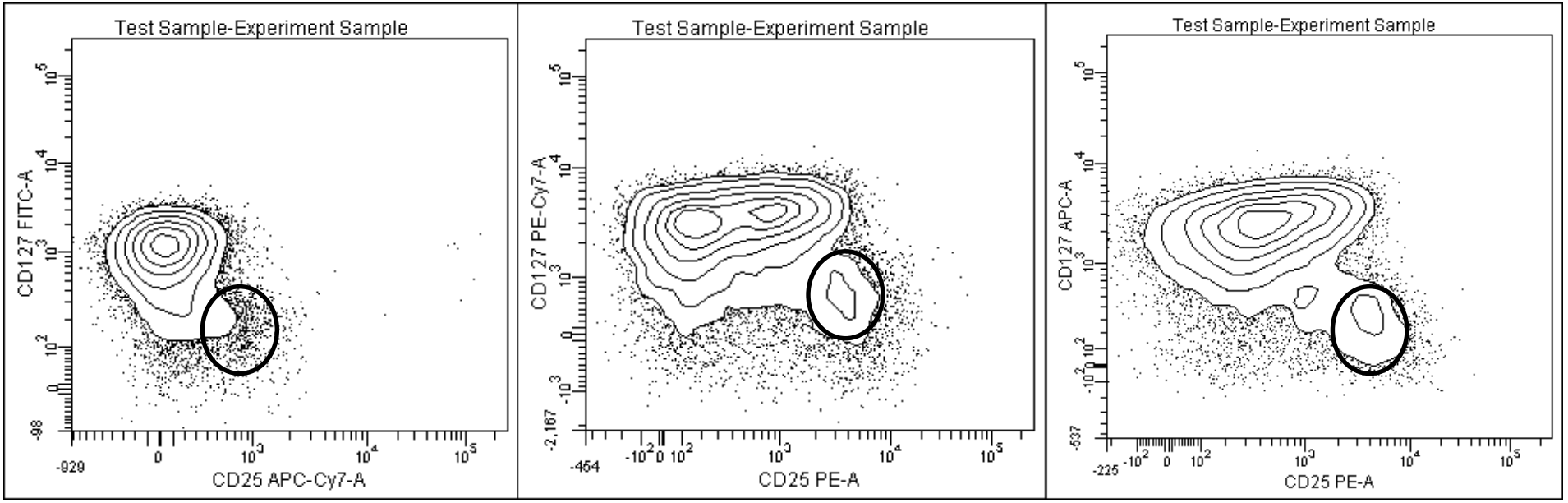
Rule: Choose a dim fluorochrome for high expressor (CD3) which doesn't spread into low expressor (CD56) channel. Choose a bright fluorochrome for low expressor.

Coexpression on Tregs: the real world



3 different fluorochrome pairs for Tregs

CD127 and CD25 are both low density antigens



Problem:
FITC and APC-Cy7 don't have spillover spread but they are **dim** fluorochromes

Problem:
PE and PE-Cy7 are bright fluorochromes, but spillover spread from PE-Cy7 into PE diminishes resolution

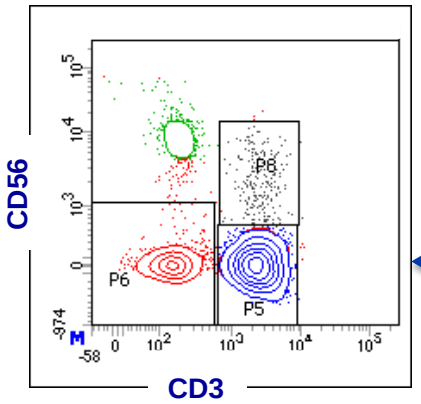
PE and APC are bright fluorochromes with little spread.

Strategies to deal with spread

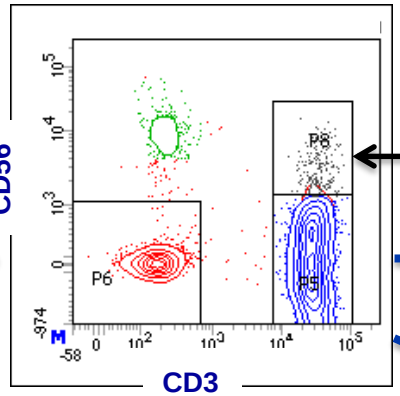
- Use a brighter fluorochrome on the weak antigen
- Use a fluorochromes that don’t overlap
- Dim the staining on the bright coexpressed antigen by diluting the conjugated antibody

How To Minimize the Impact of Fluorescence Spillover to Maximize Resolution

$$\text{Resolution} = \text{SI} = \frac{\text{Brightness}_{\text{MFI}}}{\text{Width of Negative}_{\text{SD}}}$$

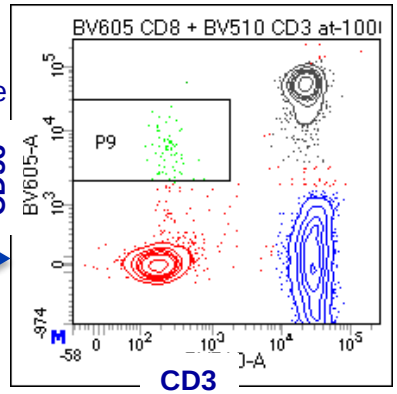


Dilute the CD3 with unconjugated antibody to reduce the spread.



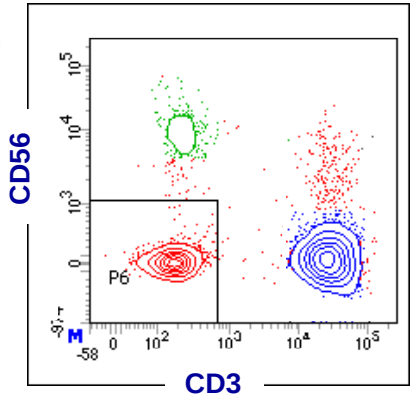
Use a brighter fluor for CD56

How can we improve positive population



Understanding the impact of fluorescence spillover on spread is the key to good panel design.

Use a fluor for CD3 with less spillover into the CD56 detector.



Hints to conserve channels

Use a dump channel to remove unwanted cells:

- In a single channel you can:
  - use several antibodies coupled to the same fluorochrome against multiple markers to eliminate unwanted positive cells.
- Use to gate out positive cells, Only the negs are of interest
- Can use a moderately bright fluorochrome with high spread into other channels

Include a dead cell marker

- Helps eliminate false positives and background

Can combine dump and dead cell channel: PerCP5.5/7AAD, BV510/FVS510

If possible, allow for future expansion:

- plan panel to leave open some channels that are good for dim markers: ideally a high intensity fluorochromes with low spread
- APC, BV421, BUV395, FITC (limited spillover) for example

Other Problems: Background and False Positives

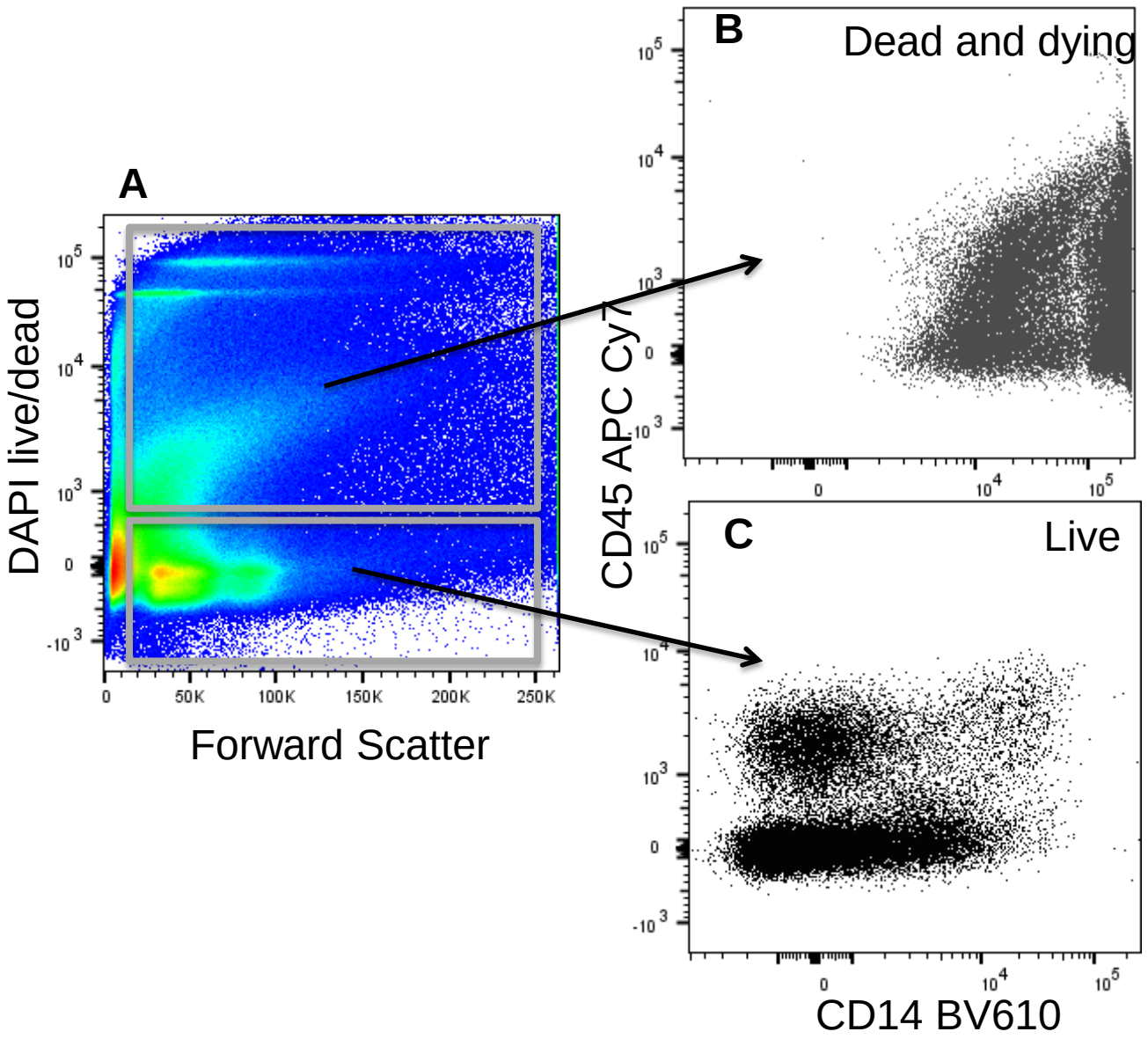
Background:

- Dead cell binding
- High concentrations of reagents (titrate!)
- Non-specific binding of intracellular matrix
- Autofluorescence: varies between populations
  - match red-laser excited fluorochromes with antigens expressed on highly auto-fluorescent cells

False positives:

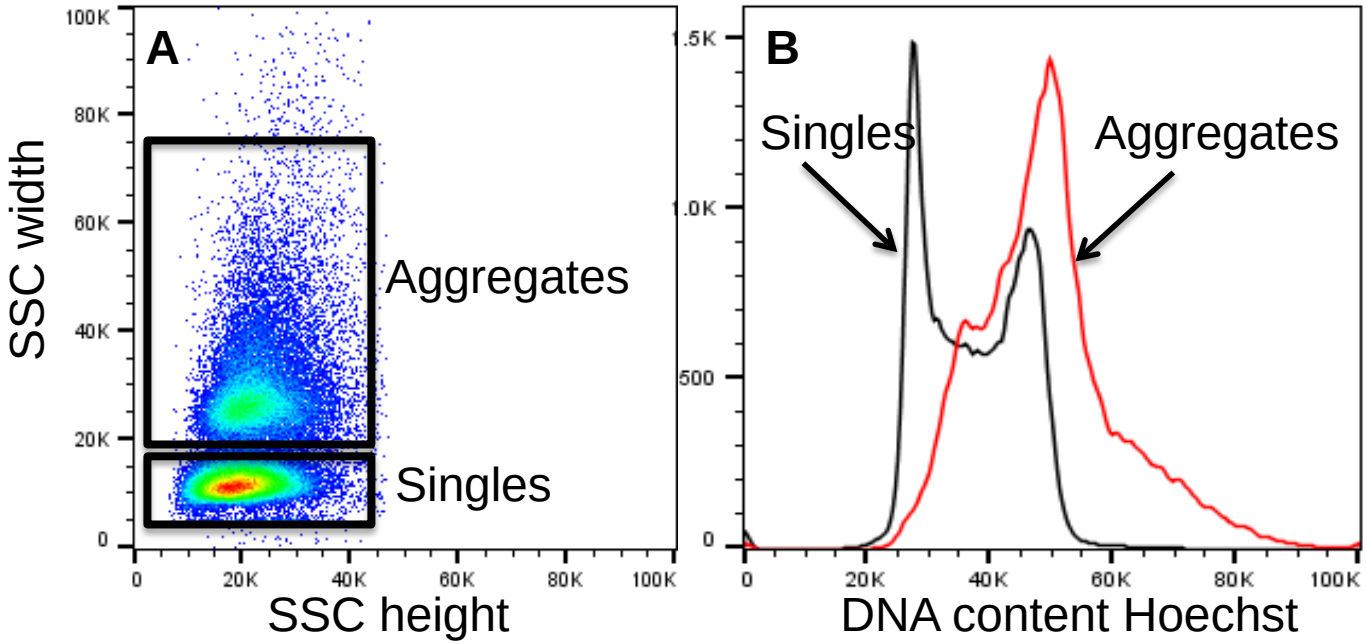
- Aggregates: use doublet discrimination
- Fc receptor binding: use a block
- Beware of Cy dyes (PE-Cy5, PE-Cy7) binding to monocytes and macrophages

Dead cells show as false positive



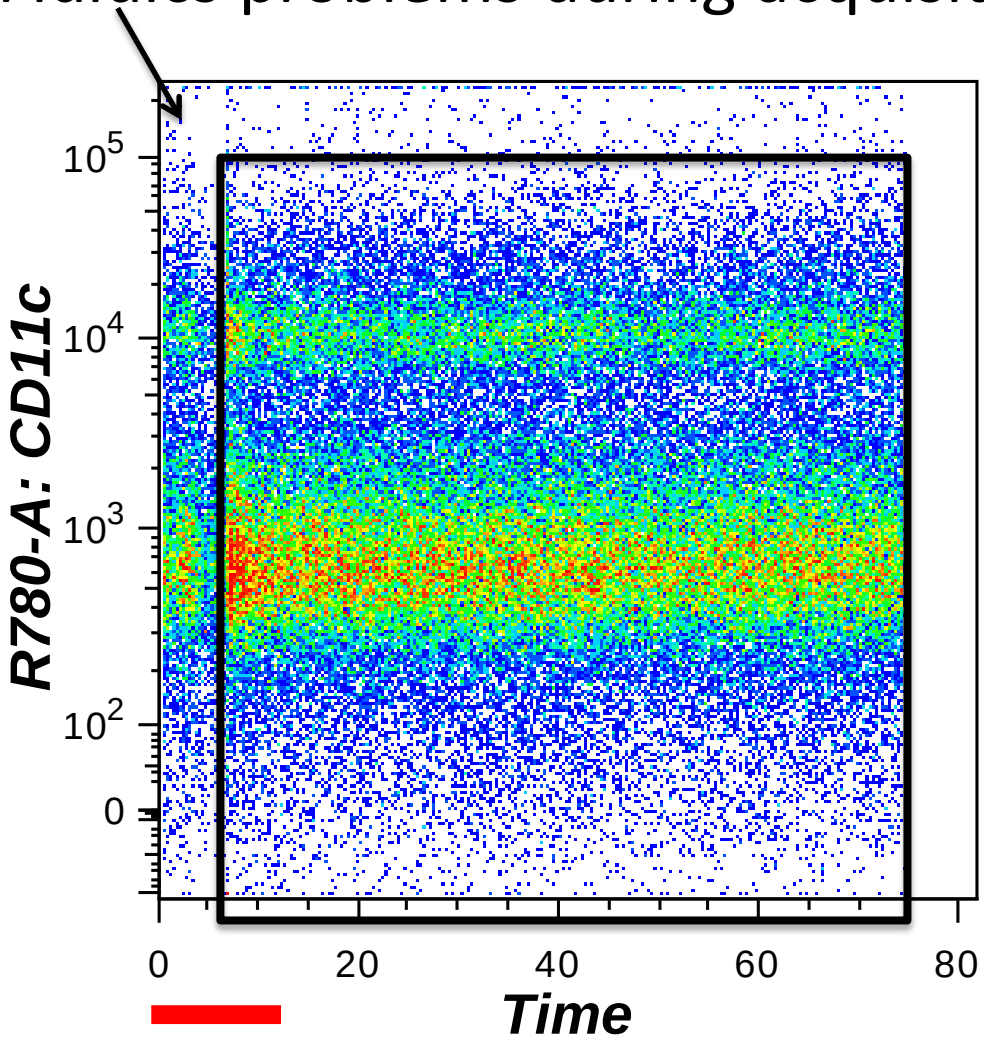
Use Pulse shape to Eliminate Aggregates

Aggregates can appear as false positives



Use a time gate to eliminate artifacts

Fluidics problems during acquisition cause artifacts in the data

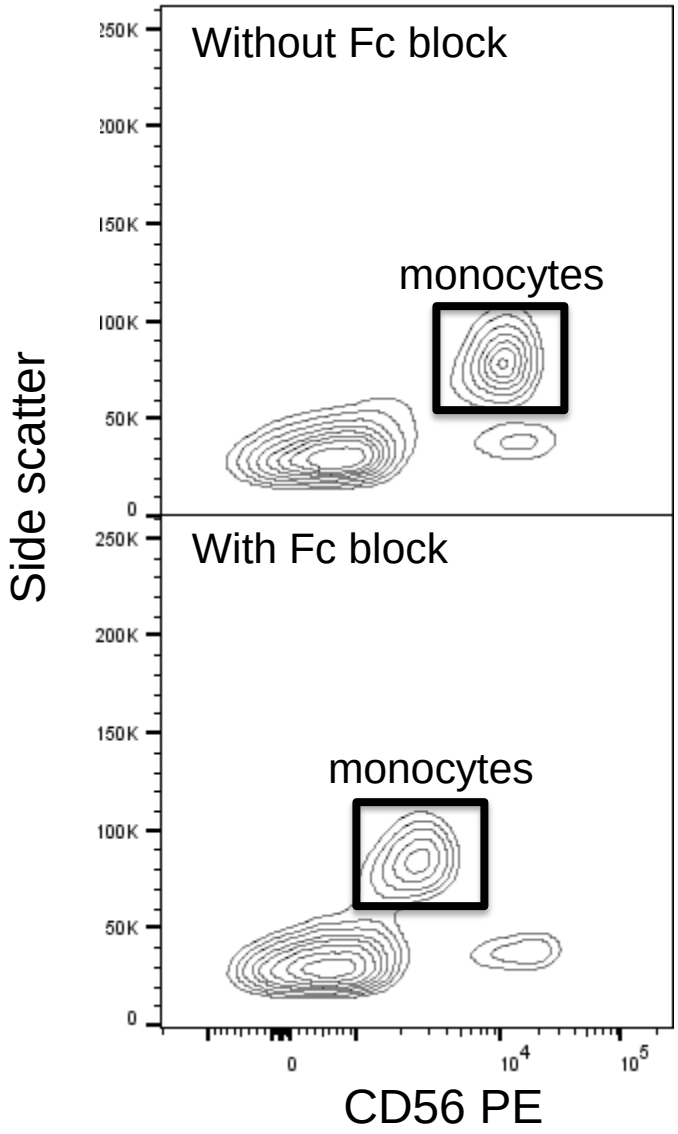


To visualize:

look at data vs time

Then gate out the bad data

Block false positive staining via Fc binding



How to start:Look at your antibody choices

Verify which colors you can run on your cytometer

look at stain index and SSM for your cytometer

Make a table of your possibilities: antigens vs fluorochromes

Often you can ask the manufacturers for samples to test

For rare antigens you may not have much choice of colors

sometimes only fitc or pe

you will have to slot those in first

For 3° antigens you only want to look at bright fluorochromes

Screen the potential antibodies on your cells can to see which ones look best

Make a table of your possibilities

	DR	CD15	CD19	CD123	CD117	CD38	CD34	CD71	CD45	X
PB / V450	●	●	●						●	
FITC	●	●	●	●		●	●	●	●	
PE	●	●	●	●	●	●	●		●	●
PE-TR	●		●				●		●	
P-X	●	●	●	●	●	●	●	●	●	
PE-Cy7	●		●		●	●	●		●	
A594			●			●				
APC	●	●	●	●	●	●	●	●	●	
APC-A700								●		
APC-X7	●		●						●	

Slide courtesy of Dr. Brent Wood

You need lots of controls when you develop your panel

STAINING MATRIX (PANEL 1)									
Tube no.	**BV 421**	**V500**	**FITC**	**PE**	**PerCP-Cy5.5**	**PE-Cy7**	**Alexa 647**	**APC-H7**	
1	-	-	-	-	-	-	-	-	Unstained Control (Cell)
2	CD8	CD4	-	-	-	-	-	CD3	Gating Control (Cell)
3	-	-	-	-	-	-	-	-	Negative Bead Control
4	CD8	-	-	-	-	-	-	-	Compensation Controls Using BD CompBeads (except CD4 Tube for which cells are to be used)
5	-	CD4	-	-	-	-	-	-	
6			CD45RA	-	-	-	-	-	
7			-	CD127	-	-	-	-	
8			-	-	CD45RO	-	-	-	
9			-	-	-	CD25	-	-	
10			-	-	-	-	CD197	-	
11			-	-	-	-	-	CD3	
12	-	CD4	CD45RA	CD127	CD45RO	CD25	CD197	CD3	FMO Controls (Cells)
13	CD8	-	CD45RA	CD127	CD45RO	CD25	CD197	CD3	
14	CD8	CD4	-	CD127	CD45RO	CD25	CD197	CD3	
15	CD8	CD4	CD45RA	-	CD45RO	CD25	CD197	CD3	
16	CD8	CD4	CD45RA	CD127	-	CD25	CD197	CD3	
17	CD8	CD4	CD45RA	CD127	CD45RO	-	CD197	CD3	
18	CD8	CD4	CD45RA	CD127	CD45RO	CD25	-	CD3	
19	CD8	CD4	CD45RA	CD127	CD45RO	CD25	CD197	-	
20	**CD8**	**CD4**	**CD45RA**	**CD127**	**CD45RO**	**CD25**	**CD197**	**CD3**	**Experiment sample**

During panel development:
Run comp controls with both beads and cells, sometimes beads don't give same values
Run FMOs for each color
Once developed you will probably only need 1 or 2 FMOs for difficult markers
During panel development, add antibodies sequentially to identify problems
Compare Stain Index of single stained cells with fully stained cells: should be the same if no problem

Validate your panel

Run many samples

- Under same conditions as to be used
- Positive and negative controls

Slide courtesy of Dr. Brent Wood

Summary

Know your cytometer: lasers and filters

Know your Antigens: priority, expression and density

Match bright fluorochromes with low density antigens and dim fluorochromes with high density antigens

If there is coexpression: avoid high spread from a high density expressor into a low density expressor.

If possible, spread antigens across lasers

Leave room for future expansion:

bright fluorochromes with little spillover, APC, BV421, BUV395

Panel design software

Fluorofinder panel design program

https://fluorofinder.com/

BD Biosciences panel design program

https://www.bdbiosciences.com/sg/paneldesigner/index.jsp

Fluorish panel design program

https://www.fluorish.com/

You can load your cytometer configurations into these programs

Reference:

Maciorowski, Z., Chattopadhyay, P.K., & Jain, P. (2017). Basic multicolor flow cytometry. Current Protocols in Immunology, 117, 5.4.1–5.4.38. doi: 10.1002/cpim.26