Introduction to Panel Design and Sample Preparation

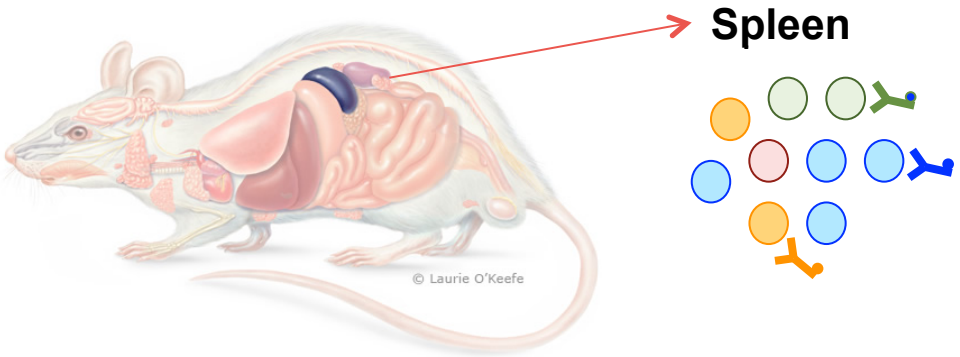
Diana Ordonez

April 18, 2016.

EMBL

Definition

Panel is a combination of reagents, usually antibodies, that recognize surface and intracellular molecules allowing the identification of cell populations in a given sample/tissue.



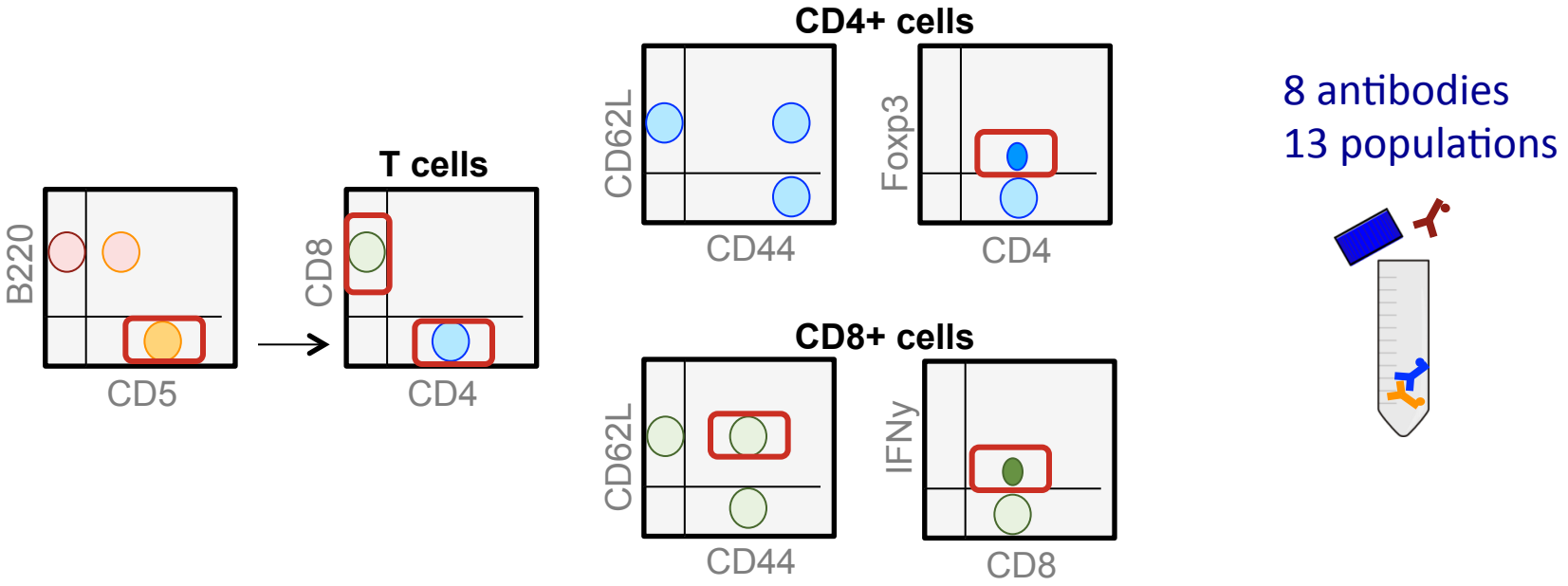
Before starting your panel, define:

-Which cells are the target of the experiment
-Find out how these cells are identified in the literature, or by past experience
-Define a consecutive gating strategy in order to obtain clear and interpretable data.

Gating strategy

The process of gating is simply selecting an area on the scatter plots or histograms to identify and define subsets of populations for further analysis.

Subsets defined by a gate are exactly like subsets in biology: they represent a fraction of cells with specified properties.



8 antibodies
13 populations

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How to prepare an Antibody Panel?

Golden Rule

“Be patient in developing/optimizing your panel”

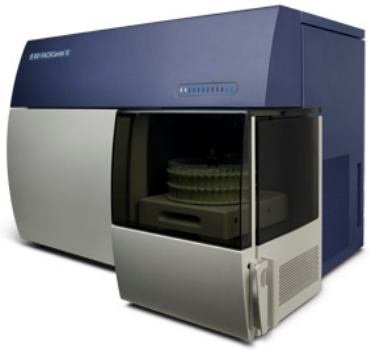
Principles of Panel Design

- Know your instrument
- Determine antigen expression and density
- Determine Antigen-Fluorochrome Combos
- Minimize spillover
- Spread antigens across lasers
- Consider technical limitations of tandem dyes
- Use appropriate controls
- Tips to improve your panel

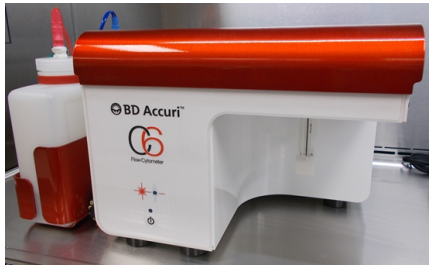
- **Multi-Parameter Flow Cytometry Experiment**

Know your instrument

- **Laser and detectors:** the type and number of laser and detectors dictate whether the optical system can excite and detect a given fluorochrome.



BD FACScanto II:
405-488-633
Fluorescence detectors: 8



BD Accuri C6:
488-633
Fluorescence detectors: 4

BD LSRFortessa:
355-405-488-561-633
Fluorescence detectors: 16

BD LSRFortessa

Laser	Detector Name	Fluorochromes
355nM	355-450/50	**DAPI, Hoechst 33258 and 33342**, Indo-1 violet, Calcein Blue.
	355-530/30	**Indo-1 Blue**, AlexaF 430, Qdot 525.
405nM	405-450/50	**Pacific Blue**, AlexaF 405, BV421, V450, BFP, CFP, Cell Trace Violet.
	405-525/50	**Sytox Blue, Aqua Dead**, Amcyan, V500, AlexaF 430, Pacific O, Qdot 525.
	405-540/30	**Qdot 565**, Pacific Orange, Krome Orange
	405-586/15	**BV570**, Fixable Yellow Dead Cell Stain, Qdot 585, Qdot 605.
	405-610/20	**BV605**, Qdot 605, eFluor605NC , Fixable Yellow Dead, Pacific O.
	405-670/30	**BV650, Qdot 655.**
488nM	488-530/30	**FITC, GFP, YFP**, Alexa F488, mCitrine, Syto9, SYTOX Green.
	488-710/50	PerCP-Cy5.5, DRAQ5, PerCP, eFluor710.
561nM	561-586/15	**PE, DsRed, RFP**, tdTomato, SYTOX Orange.
	561-610/20	**PI, mCherry**, PE-Texas Red, Texas Red, PE-CF594.
	561-670/30	**7AAD**, Pe-Cyc5.
	561-710/50	**Pe-Cy5.5**, 7-AAD.
	561-780/60	**PE-Cy7**, APC-Vio770, PE-Vio 770.
633nM	640-670/14	**APC, Alexa F647**, DRAQ5, DRAQ7, Fixable far red,TO-PRO-3.
	640-730/45	**Alexa Fluor 700**, APC-Cy5.5, DRAQ7, Dye Cycle Ruby.
	640-780/60	**APC-Cy7, Alexa F750**, APC-eFluor 780, APC-H7, Fixable near-IR.

BD Biosciences Relative Fluorochrome Brightness

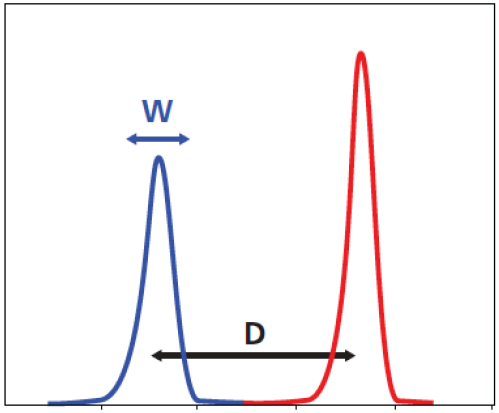
Rankings were determined by comparing the staining index of cells stained with several antibody clones run on a variety of flow cytometers.

Laser	Fluorochrome: Very Bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon™ BUV661 BD Horizon™ BUV737	BD Horizon™ BUV395 BD Horizon™ BUV496	BD Horizon™ BUV805
Violet (405 nm)	BD Horizon™ BV421 BD Horizon™ BV650 BD Horizon™ BV711	BD Horizon™ BV605 BD Horizon™ BV786	BD Horizon™ BV510	BD Horizon™ V450 BD Horizon™ V500
Blue (488 nm)	BD Horizon™ BB515 BD Horizon™ PE-CF594 PE-Cy™5	PE PE-Cy™7	FITC Alexa Fluor® 488 PerCP-Cy™5.5	PerCP
Yellow/Green (561 nm)	PE BD Horizon PE-CF594 PE-Cy5 PE-Cy7			
Red (640 nm)		APC Alexa Fluor® 647 BD Horizon™ APC-R700		Alexa Fluor® 700 APC-H7 APC-Cy7

https://www.bdbiosciences.com/documents/Fluorochrome-Chart-Relative-Brightness.pdf

- **Fluorochromes Brightness:** Available dyes could be rank according to their brightness on a given instrument.

Stain Index: “How resolvable the signal is over the unstained cell population”



Stain Index = D/W

$$SI = \frac{\mu \text{ Positive} - \mu \text{ Background}}{2 \times SD \text{ Background}}$$

When the same antibody is conjugated to various dyes, their stain indexes can be compared to determine the relative brightness of the dyes on a particular instrument.

Maecker and trotter, Selecting reagents for multicolor flow cytometry. BD Application Note

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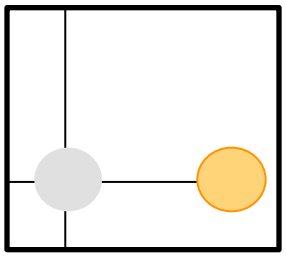
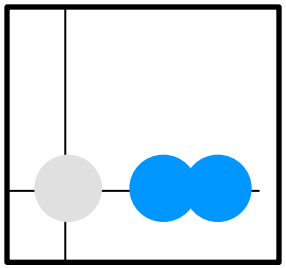
Antigen expression and density

Antigens are classified in one of the following three categories:



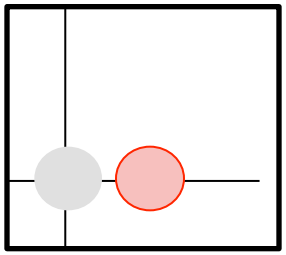
Primary:
Expression: ON/OFF
Used to gate on cellular subsets

Lineage markers



Secondary:
Expression is often a continuum
High molecular density

Activation markers

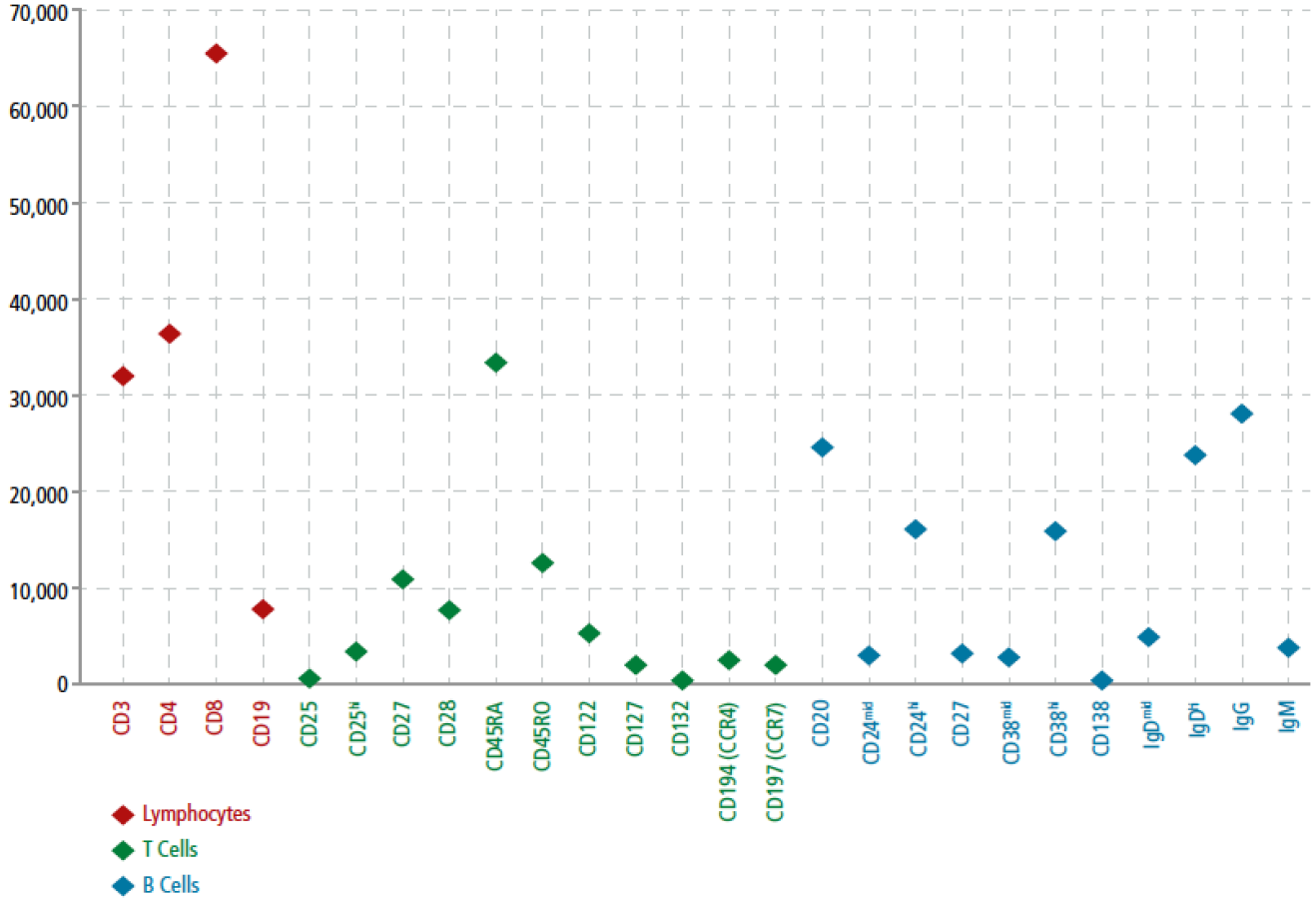


Tertiary:
Expressed at low levels
Or uncharacterized

"Difficult antigens"

Mahnke, Y, Clin Lab Med, 37(2):364-76, Au 2007: "Optimizing a Multi-colour Immunophenotyping Assay."

BD Biosciences Density of Common Human Surface Antigens



BD

Antigen density determined on human blood samples using PE conjugated antibodies to each antigen and BD Quantibrite™ beads for quantitation.

bdbiosciences.com/colors

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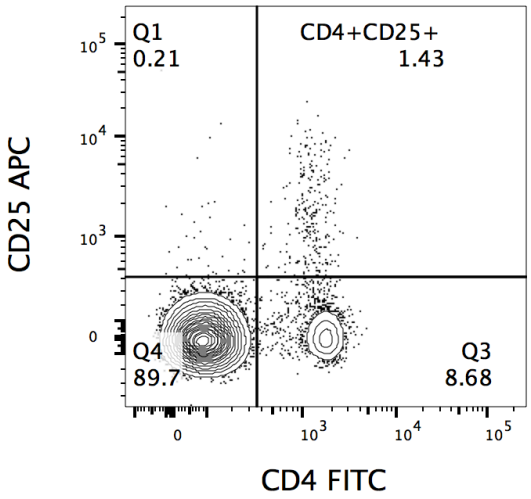
Determine antigen-fluorochrome combos

Define winning combinations matching fluorochromes brightness with antigen density.

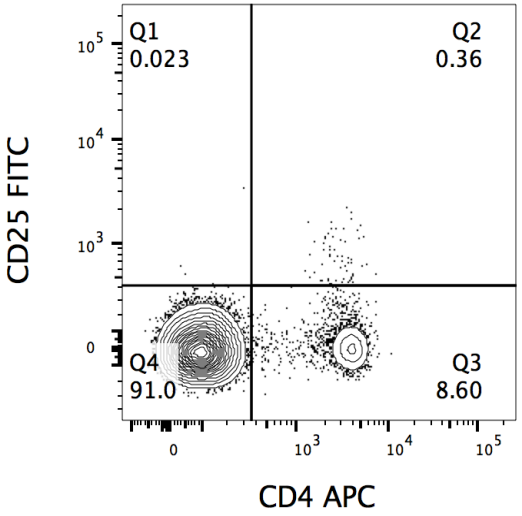
- Reserve the brightest fluorochrome for dim antigens and dim fluorochromes for antigens highly expressed.

CD4 Lineage marker ------- 36,400 m/cell--------Dim fluorochrome
CD25 Low expressed ag ------- 3,400 m/cell--------Bright fluorochrome

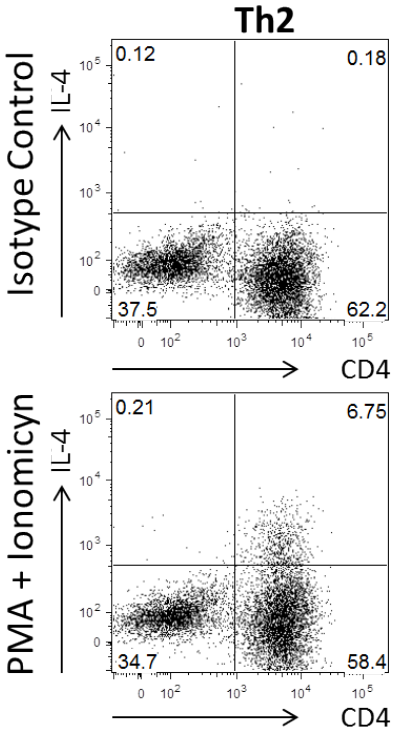


CD4 FITC
CD25 APC

When antigens with low level expression are assigned to “dull” fluorochrome:
Underestimation of the size of the positive population----False negative.



CD4 APC
CD25 FITC



- Start with a “difficult antigen”:

Ag expressed on a small population and or/at low level
Few antibody conjugate options.

Cytokines
Transcriptional factors

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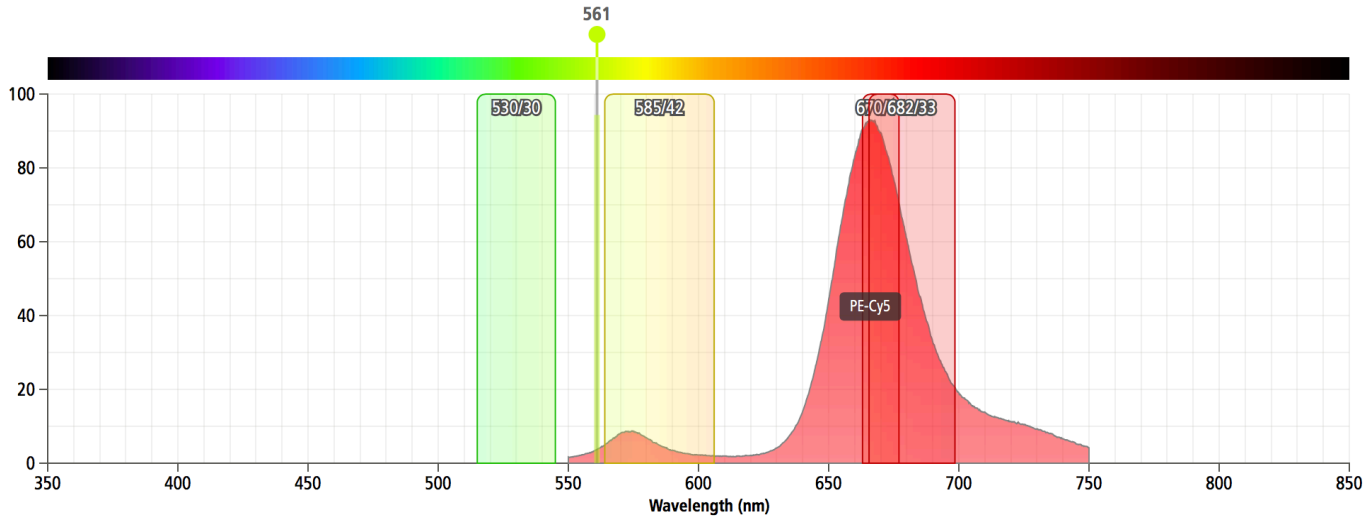
Minimize the spillover

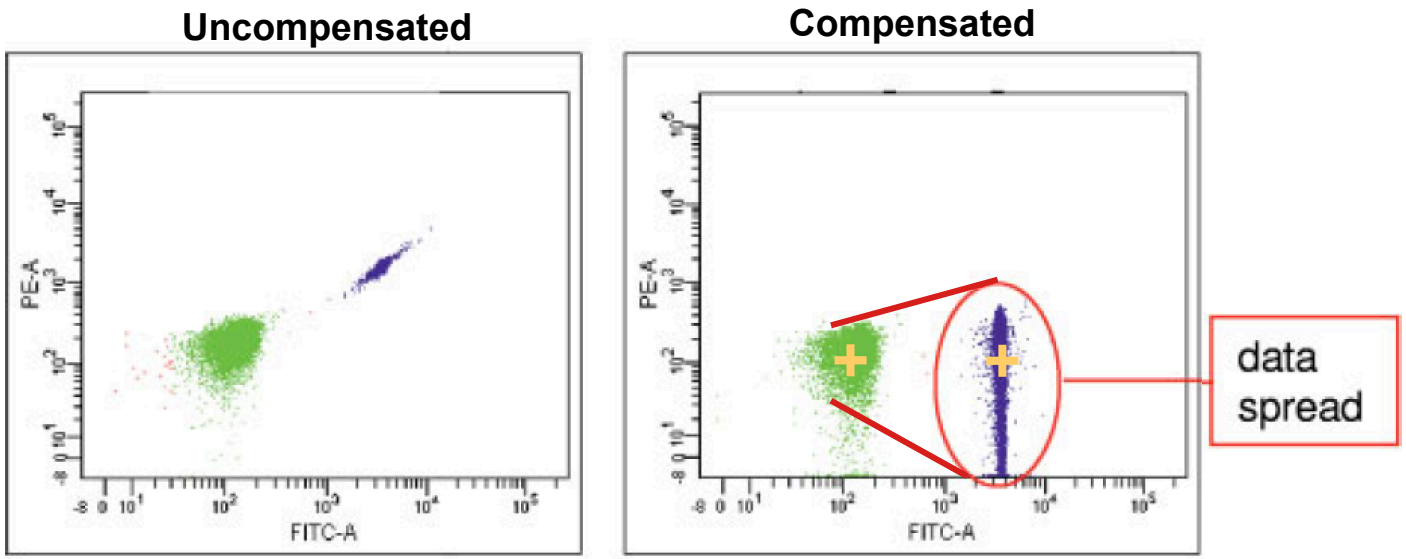
Minimize the potential of spectral overlap when choosing a reagent combination.

Spectral overlap in the APC detector



As soon as more colors are included in the panel, more spillover between those colors that will have to deal with!!

Instruments deal with spillover applying ------- compensation

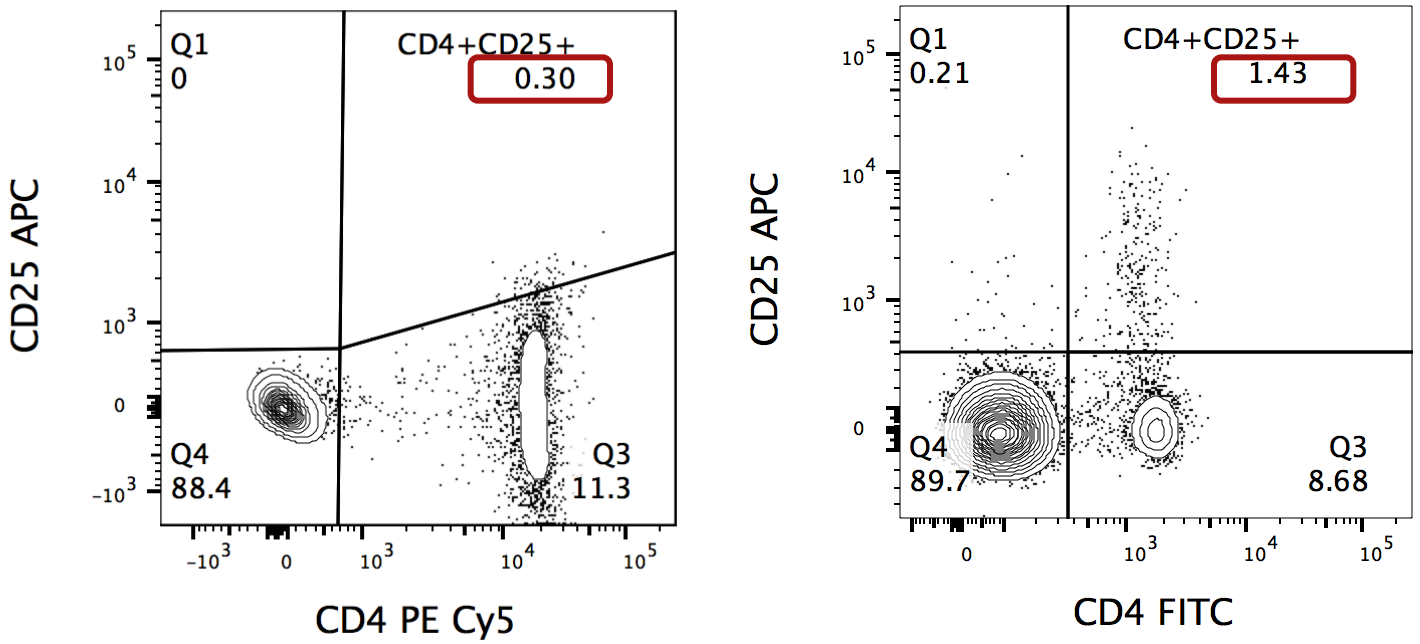


After compensation FITC+ population will show no PE fluorescence

Individual cells can fall above or below the mean, increasing the SD of that population in the PE channel ----- Data Spread.

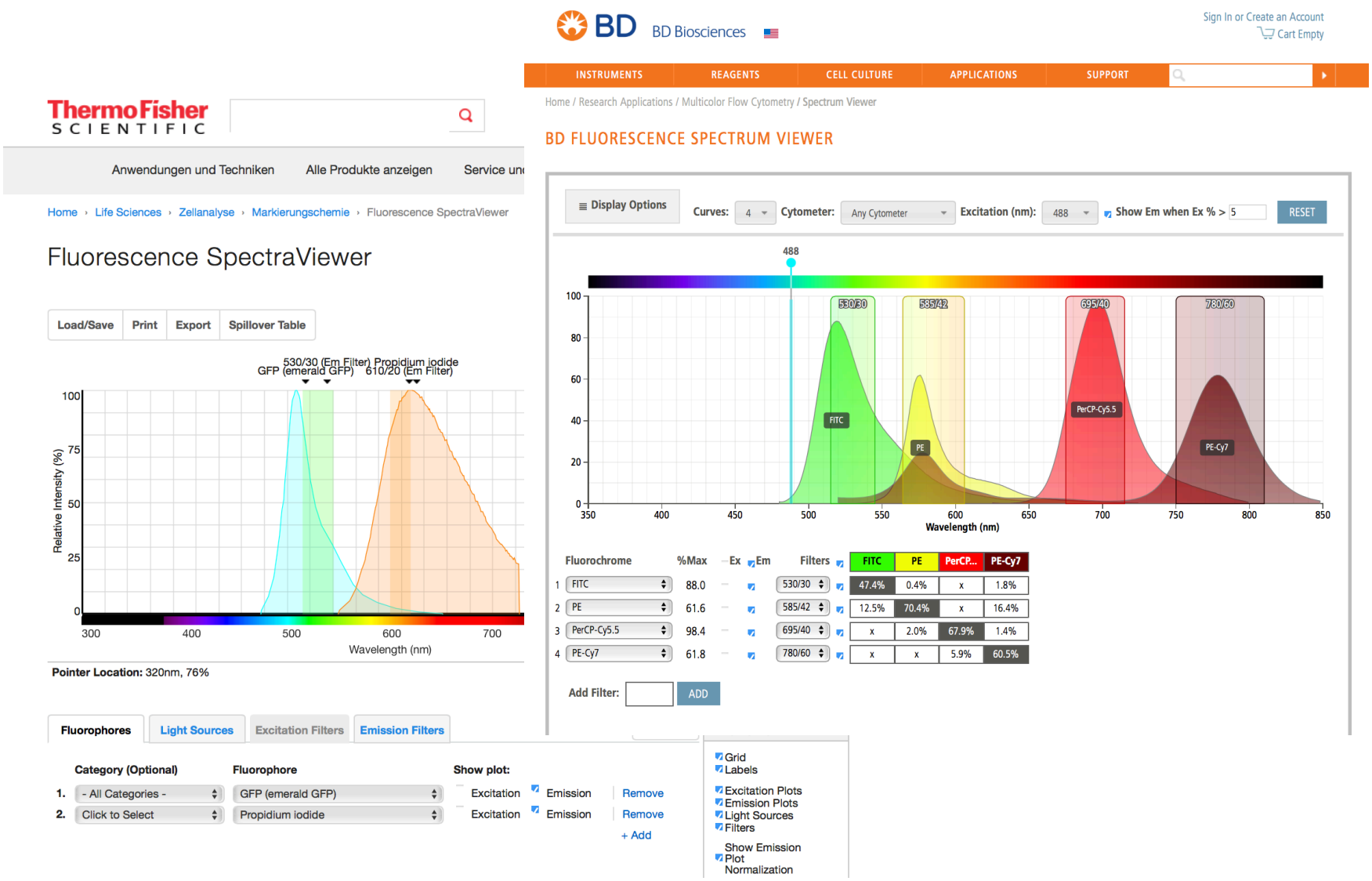
The greater the spillover, greater the spread of the compensated positive population.

Spread data could affects the resolution of dim signals in the second channel!!!.



CD25 positive cells (APC) could no longer be distinguished from the increased background of the the CD4 PECy5 staining into the APC channel.

Use on-line spectral viewer tools to predict spillover !!!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Display Options
Curves: 4
Cytometer: Any Cytometer
Excitation (nm): 488
Show Em when Ex % > 5
RESET
488
530/30
585/42
695/40
780/60
FITC
PE
PerCP-Cy5.5
PE-Cy7
Wavelength (nm)
Fluorochrome
%Max
Ex
Em
Filters
FITC
PE
PerCP...
PE-Cy7
1 FITC 88.0 530/30 47.4% 0.4% x 1.8%
2 PE 61.6 585/42 12.5% 70.4% x 16.4%
3 PerCP-Cy5.5 98.4 695/40 x 2.0% 67.9% 1.4%
4 PE-Cy7 61.8 780/60 x x 5.9% 60.5%
Add Filter:
ADD
ThermoFisher SCIENTIFIC
Anwendungen und Techniken
Alle Produkte anzeigen
Service un
Home › Life Sciences › Zellanalyse › Markierungschemie › Fluorescence SpectraViewer
Fluorescence SpectraViewer
Load/Save
Print
Export
Spillover Table
530/30 (Em Filter) Propidium iodide
GFP (emerald GFP) 610/20 (Em Filter)
Relative Intensity (%)
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Pointer Location: 320nm, 76%
Fluorophores
Light Sources
Excitation Filters
Emission Filters
Category (Optional)
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Show plot:
1. - All Categories - GFP (emerald GFP) Excitation Emission Remove
2. Click to Select Propidium iodide Excitation Emission Remove
+ Add
Grid
Labels
Excitation Plots
Emission Plots
Light Sources
Filters
Show Emission Plot Normalization

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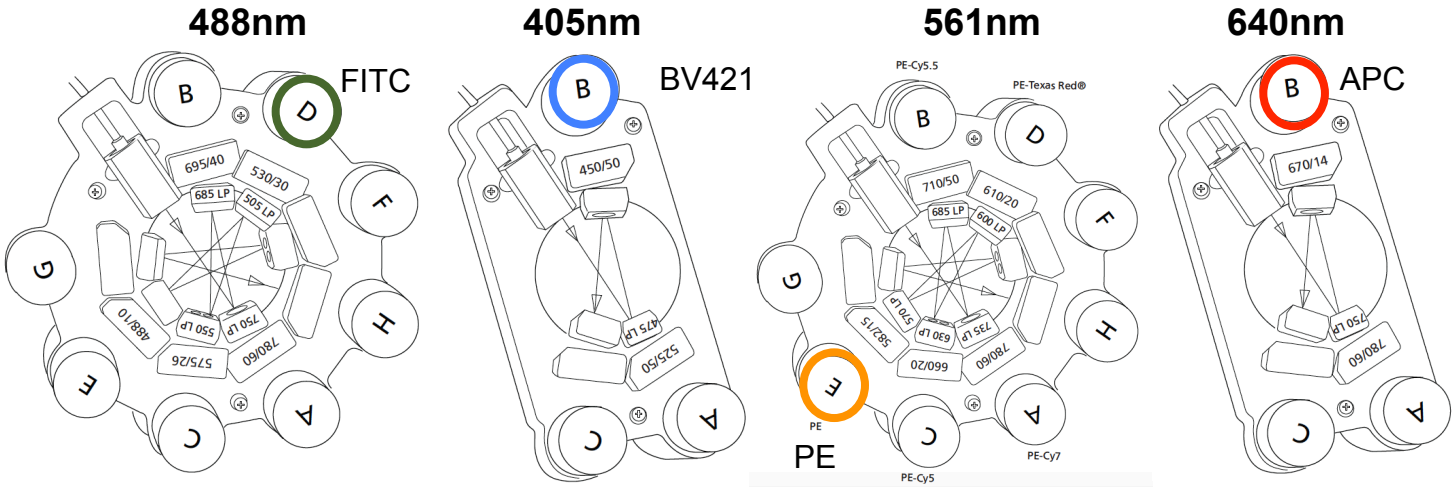
Spread antigens across lasers

If multiple antigens are present on a cell, spread them across as many lasers as possible to minimize compensation and spillover.

Instrument lasers: Pick one color off each laser:

488 ⟶ FITC
405 ⟶ BV421
561 ⟶ PE
633 ⟶ APC



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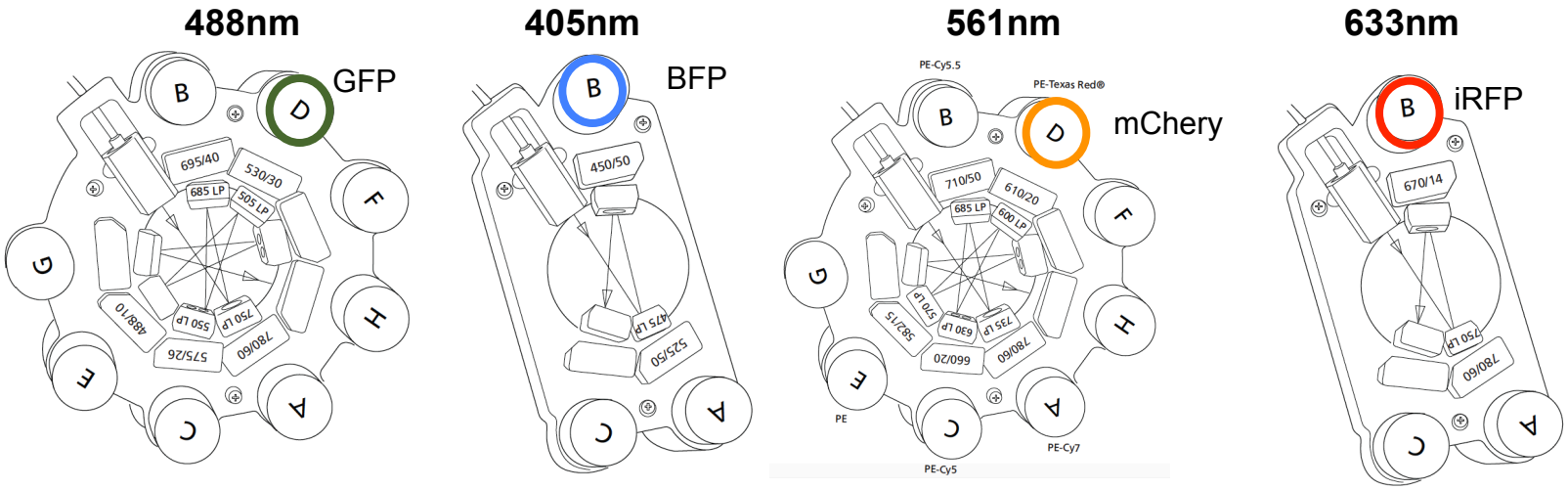
Fluorescent Protein Multi color panel

Instrument laser: pick one color off each laser:

488 ⟶ GFP, YFP, Venus,Citrine
405 ⟶ BFP,CFP
561 ⟶ mCherry, DsRed.
640 ⟶ iRFP



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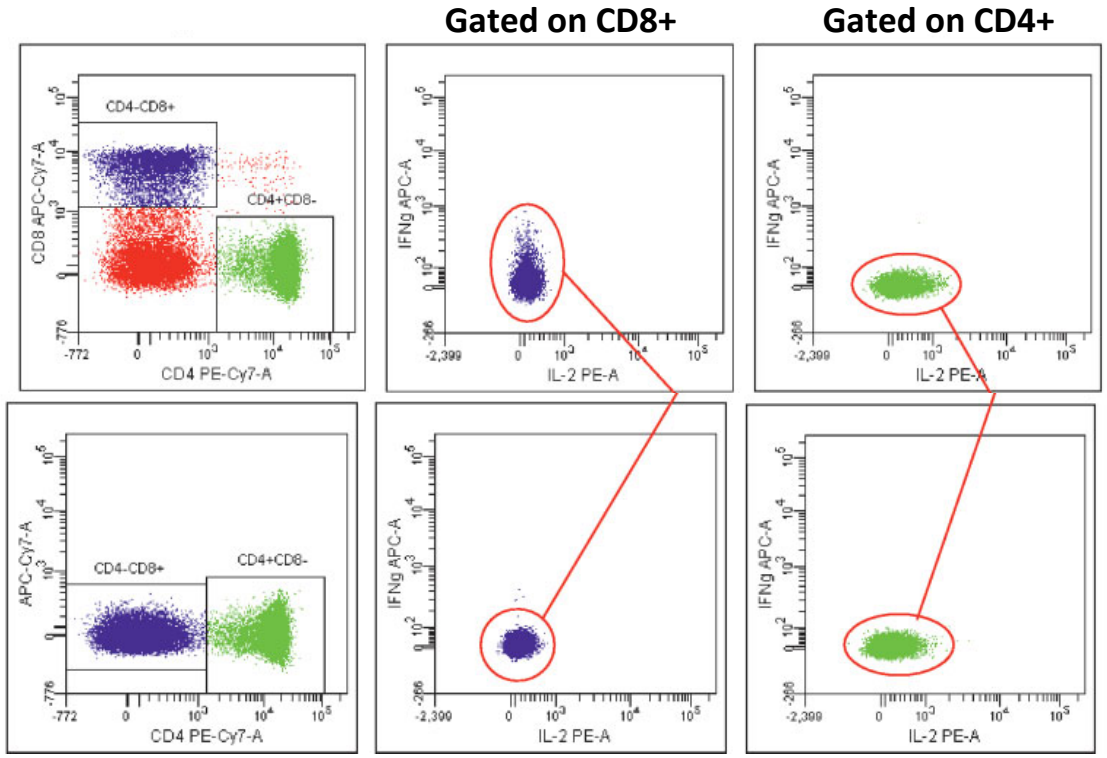
Technical limitations of tandem dyes

Some tandem dyes (APC Cy7, PE Cy7) can degrade in the presence of light, high temperatures and fixation, emitting light in the parent dye detector.



Avoid the exposure of stained samples to light/heat/PFA based fixatives
Replace APC Cy7 for APC H7, with enhanced stability to…

Maecker HT, Frey T, Nomura LE, Trotter J. Selecting Fluorochrome conjugates for maximum sensitivity. *Cytometry A* 2004; 62:169-173.

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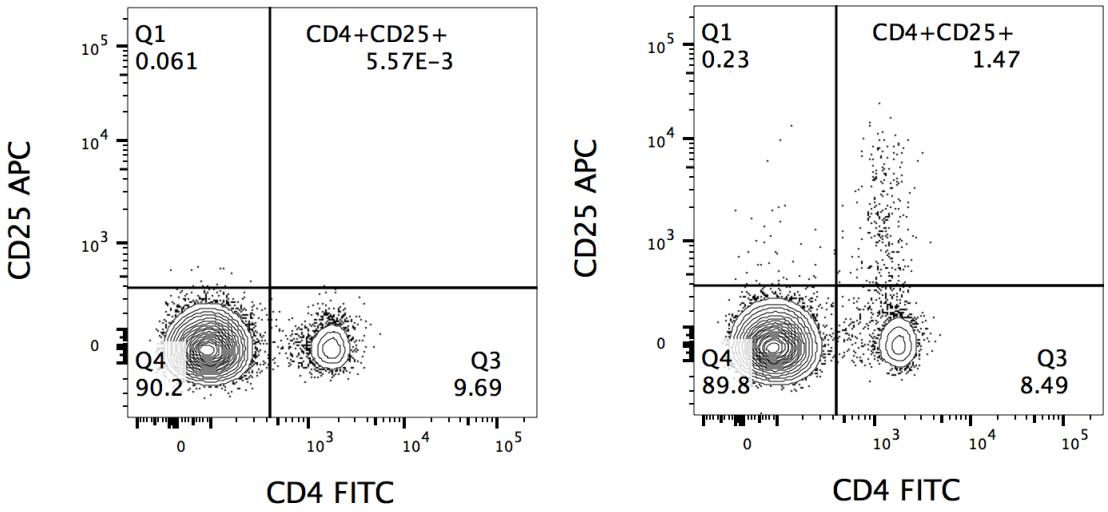
Appropriate controls

- Instrument set up and validation: CST beads, 8 peak beads.
- Biological controls: Unstimulated/stimulated
- Gating controls: are critical for ensuring the proper cells are identified.

"Fluorescence-Minus-One" (FMO): Control sample that has all antibodies except one: Typically directed against a tertiary antigens.



Assess the contribution of spillover into one particular detector, facilitating the precise gating of negative and positive population boundaries.

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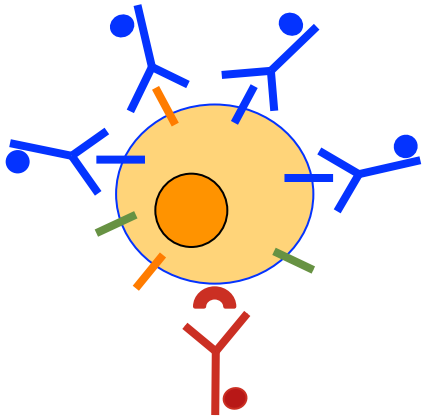
Reduce non-specific binding

In addition to binding to receptors of interest, antibodies may also bind to other sites ⟶ nonspecific binding (NSB).

- Fc receptor mediated

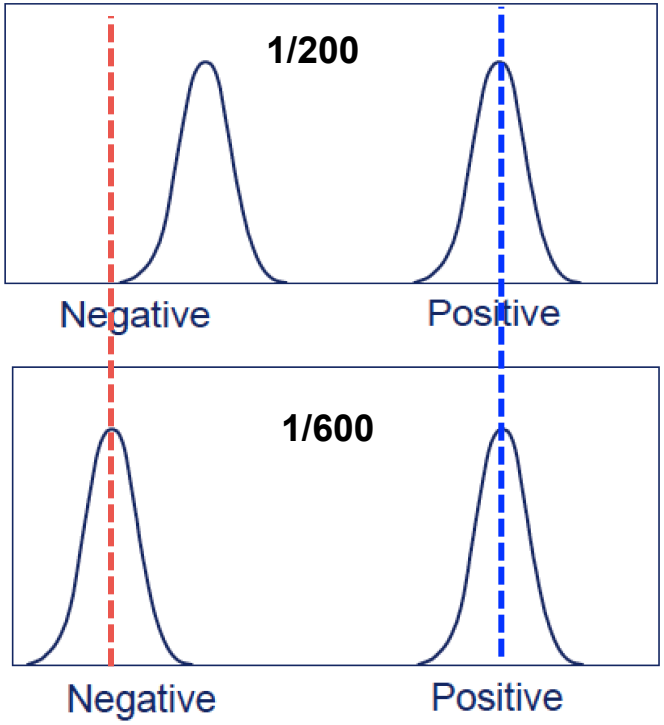
  Mouse: CD16/32 --- Fcy II/III receptors
  Human: Human Ig or 10% autologous serum in PBS.

- Binding to a non-specific target.
- Binding to dead cells

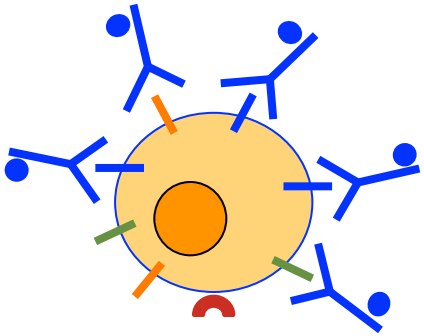


Antibody titration

Determine the antibody concentration resulting in the highest signal of the positive population and the lowest signal of the negative population.

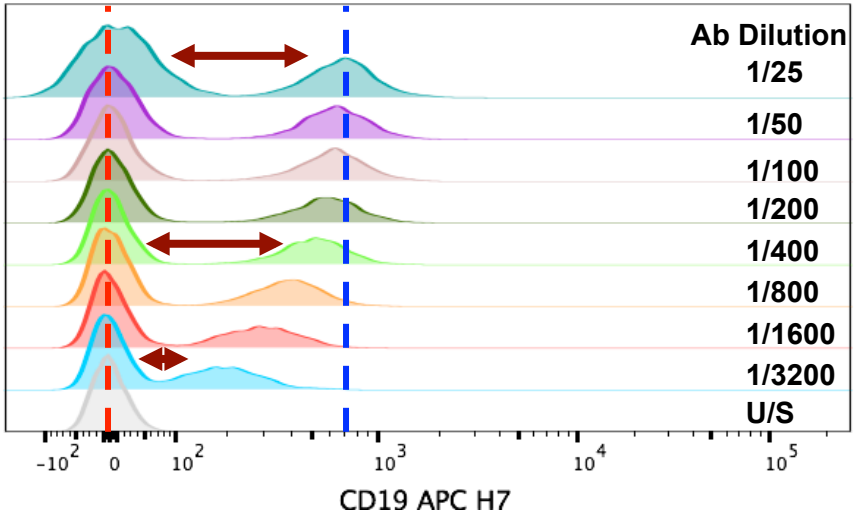


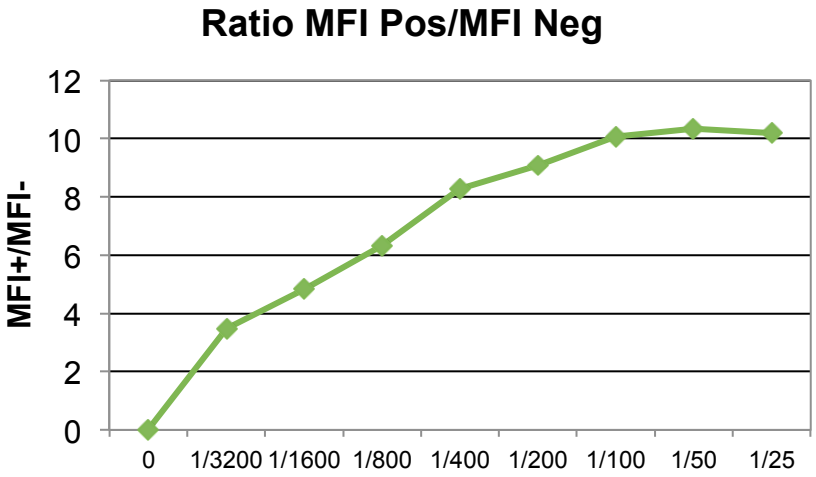
Saturated



Nonspecific binding: The total number of antibody molecules greatly exceeds the number of target antigens.

Signal-to-noise ratio: Choose the dilution that gives you the best discrimination between the positive and the negative cells.





Titrate all your reagents to optimize signal, achieve saturation, minimize background and **economize reagents**.

Antibody titration protocol:

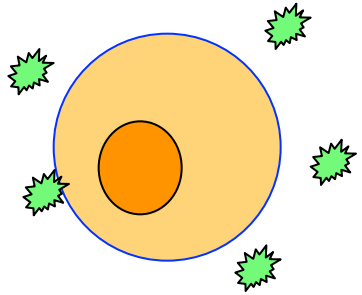
- Refers to the antibody data sheet ---- valuable information.
- Perform the titration in a cell type/stimulation condition in which a max. amount of antigen is available for staining.
- Centrifuge newly acquired antibodies at high speed to eliminate aggregated immunoglobulin --- source nonspecific binding.
- Stain 1x106 cells in 100ul of antibody dilution.
- Evaluate antibody dilutions from 1/25 to 1/3200 (serial dilutions).
- Titration of antibodies that require a secondary antibody: Test different concentrations of the primary antibody, keeping a fix concentration of the secondary (1/200).

Cell viability assessment

Antibodies conjugates tends to bind non-specifically to dead cells, creating false positive events.

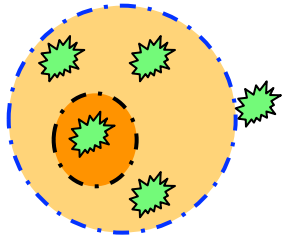
Dead cells could be identify and exclude by the use of viability dyes:
-DNA binding dyes: PI, DAPI, 7AAD, Sytox.
-Cellular amines binding dyes: Live/Dead Fixable Dead Cell Stain

Live cell



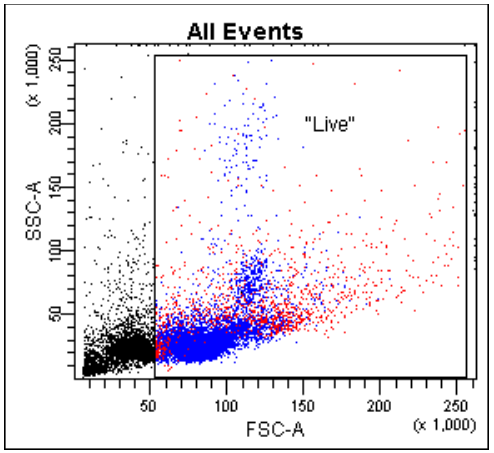
Intact membrane
No entry of the dye

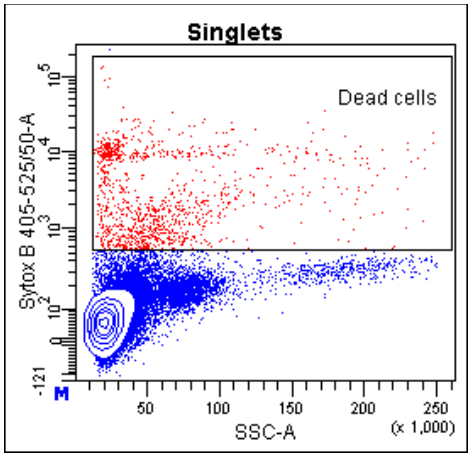
Apoptotitc-Dead cell

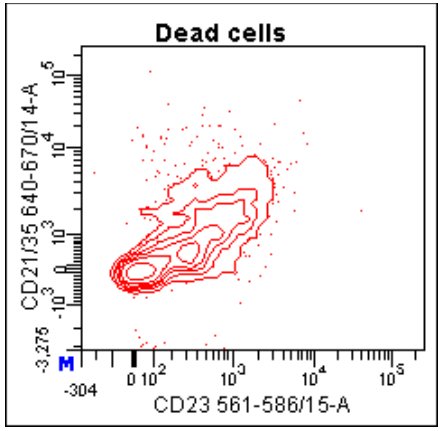


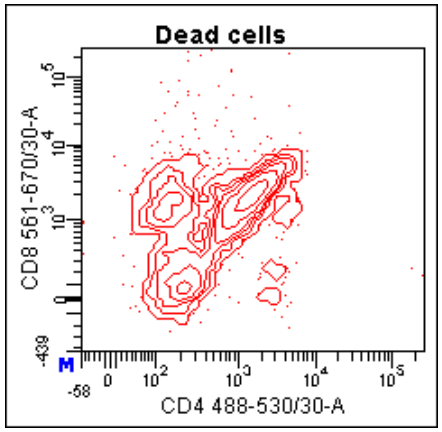
Compromised membrane
Entry of the dye: Positive signal

Morphological gates (FSC/SSC) alone are not sufficient to discriminate live from apoptotic or necrotic cells. Dead cells could actually overlay live cells.







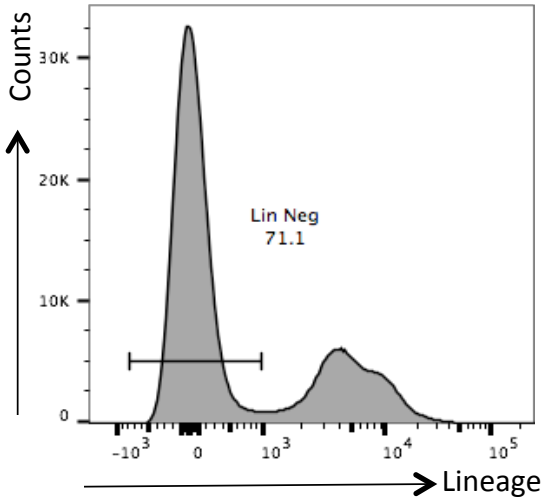


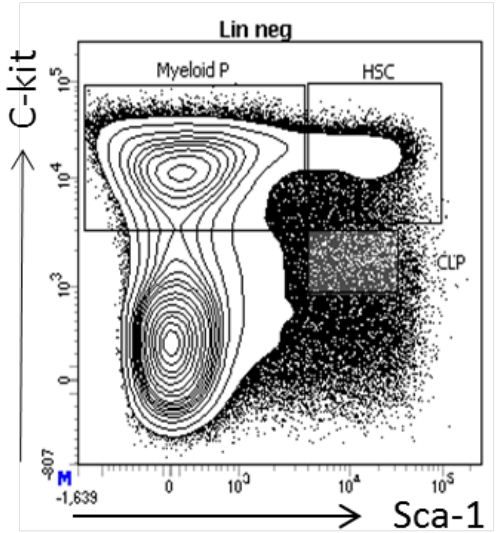
Include always a dye to discriminate live cells.

EMBL

Dump channel

Consider the inclusion of a dump channel, to remove non-desired cell subsets by labelling all with the same fluorochrome then gating and focusing in the negative population.





Lineage +: APC CYC7
CD3: T cells
CD19: B cells
CD11b: Neutro + Monocytes
NK 1.1: NK cells

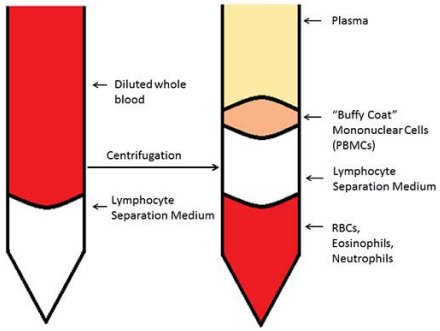
Sample preparation

Bring cells into a single cell suspension

1. **Adherent cell**: Trypsin/Accutase.

2. Enrichment of the target population:

Density gradient : Cells are separated based on their density leaving behind visible layers with distinct cell populations.



3. Solid tissue:

Mechanical dissociation

Enzymatic digestion: Optimal recovery of cell

-Collagenase: Digest native collagen ---- Major fibrous component of the ECM

-DNase I: DNA removal in cell isolation ------ Reduce viscosity

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Skin - Tissue Tables

Species	Species Detail	Cells	Enzyme(s)	Medium	Reference
Canine	Canine	Epidermal	Collagenase: 0.35% Neutral Protease: 0.1%	DMEM	Zheng, Y., Nace, A., Chen, W., Watkins, K., Sergott, L., Homan, Y., Vandeberg, J., Breen, M. and Stenn, K.: Mature Hair Follicles Generated from Dissociated Cells: A Universal Mechanism of Folliculoneogenesis., *Dev Dyn* **239**, 2619-26, 2010
Frog	Frog	Epidermal	Trypsin: 0.18%	Barth's solution, CMF	Nishikawa, A., Shimizu-Nishikawa, K., and Miler, L.: Isolation, characterization, and in vitro culture of larval and adult epidermal cells of the frog Xenopus laevis, *In Vitro Cell Dev Biol* **26**, 1128, 1990
Goat	Goat	Dermis isolated aggrecan sensitive	Neutral Protease: 0.5% Collagenase Type 2: 200 u/ml	DMEM	Deng, Y., Hu, J. and Athanasiou, K.: Isolation and Chondroinduction of a Dermis-Isolated, Aggrecan-Sensitive Subpopulation with High Chondrogenic Potential., *Arthritis Rheum* **56**, 168, 2007
Human	Human	Skin	Neutral Protease: 5 u/ml Collagenase Type 3: 0.3% Deoxyribonuclease I: 0.005%	RPMI 1640	Cheuk, S., Wiken, M., Blomqvist, L., Nylen, S., Talme, T., Stahle, M.and Eidsmo, L.: Epidermal Th22 and Tc17 Cells Form a Localized Disease Memory in Clinically Healed Psoriasis., *J Immunol* **192**, 3111, 2014
Human	Human	Skin Fibroblasts	Collagenase Type 1: 1000 u/ml	DMEM	Douvaras, P., Wang, J., Zimmer, M., Hanchuk, S., O'Bara, M., Sadiq, S., Sim, F., Goldman, J. and Fossati, V.: Efficient Generation of Myelinating Oligodendrocytes from Primary Progressive Multiple Sclerosis Patients by Induced Pluripotent Stem Cells., *Stem Cell Reports* **3**, 250, 2014
Human	Human, adult	Epidermal stem cells	Neutral Protease: 2 u/ml Trypsin: 2.5%	DMEM	Nagel, S., Rohr, F., Weber, C., Kier, J., Siemers, F., Kruse, C., Danner, S., Brandenburger, M. and Matthiessen, A.: Multipotent Nestin-Positive Stem Cells Reside in the Stroma of Human Eccrine and Apocrine Sweat Glands and can be Propagated Robustly In Vitro., *PLoS ONE* **8**, e78365, 2013
Human	Human	Fibroblasts	Collagenase animal free: 2%	HBSS	Karumbayaram, S., Lee, P., Azghadi, S., Cooper, A., Patterson, M., Kohn, D., Pyle, A., Clark, A., Byrne, J., Zack, J., Plath, K. and Lowry, W.: From Skin Biopsy to Neurons Through a Pluripotent Intermediate Under Good Manufacturing Practice Protocols, *Stem Cells Transl Med* **1**, 36, 2012
Human	Human, female, 18-66 yr	Fibroblasts	Trypsin: 0.2%	DMEM	Huschtscha, L., Napier, C., Noble, J., Bower, K., Au, A., Campbell, H., Braithwaite, A. and Reddel, R.: Enhanced Isolation of Fibroblasts From Human Skin Explants., *Biotechniques* **53**, 239, 2012
Human	Human	Epidermal	Collagenase: 0.35% Neutral Protease: 0.1%	DMEM	Zheng, Y., Nace, A., Chen, W., Watkins, K., Sergott, L., Homan, Y., Vandeberg, J., Breen, M. and Stenn, K.: Mature Hair Follicles Generated from Dissociated Cells: A Universal Mechanism of Folliculoneogenesis., *Dev Dyn* **239**, 2619-26, 2010
Human	Human	skin and keloid progenitor cells	Collagenase Type 1: 0.4% Neutral Protease: 0.3%	PBS	Zhang, Q., Yamaza, T., Kelly, AP, Shi, S., Wang, S., Brown, J., Wang, L., French, S., Shi, S. and Le, A.: Tumor-like Stem Cells Derived from Human Keloid are Governed by the Inflammatory Niche Driven by IL-17/IL-6 Axis., *PLoS ONE* **4**, e7798, 2009

Related Pages

- Tissue Dissociation Guide
- Collagenase
- Deoxyribonuclease I
- Neutral Protease (Dispase)
- Trypsin
- Trypsin Inhibitors

Jump to Species

- Canine
- Frog
- Goat
- Human
- Mouse
- Porcine
- Rat

Principles of Panel Design

- Know your instrument
- Determine antigen expression and density
- Determine Antigen-Fluorochrome Combos
- Minimize spillover
- Spread antigens across lasers
- Consider technical limitations of tandem dyes
- Use appropriate controls
- Tips to improve your panel

- **Multi-Parameter Flow Cytometry Experiment**

Multi-Parameter Experiment

Aim: "Identify Lymphocyte subsets in the spleen"

1. Biology of the sample:

- Tissue: Spleen ------- Mechanical disaggregation ------ Viability
- Define the populations to study and the antigens that they expressed

Cell tpe	Antigens
B cells	CD19
Follicular B	CD23
Marginal B	CD21/35
T cells	CD3/CD5
CD4 T cells	CD4
CD4+CD25+	**CD4+CD25+**
CD8 T cells	CD8

2. Reagent selection

Antigen	Category	Fluorochrome	Considerations
CD19	1	APC H7	
CD21/35	2	APC	
CD23	2	PE Cy7	
CD4	1	FITC	
CD8	1	PE Cy5	
CD25	3	BV421	Avoid spillover from CD4

3. Analyze combination of ab conjugates in every cell type

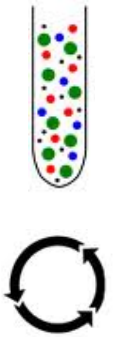
Cell tpe	Antigens
Follicular B cells	CD19 APC H7-CD23 PE Cy7
Marginal B cells	CD19 APC H7-CD21 APC
CD4 T cells	CD4 FITC
CD4+CD25+	CD4 FITC-CD25 BV421
CD8 T cells	CD8 PE Cy5

CD25 BV421

CD4 FITC

CD8 PE Cy5

CD23 PE Cy7



CD19 AC H7

CD21 APC

Use fluorochromes excited by different lasers to reduce compensation requirements.

4. Staining protocol

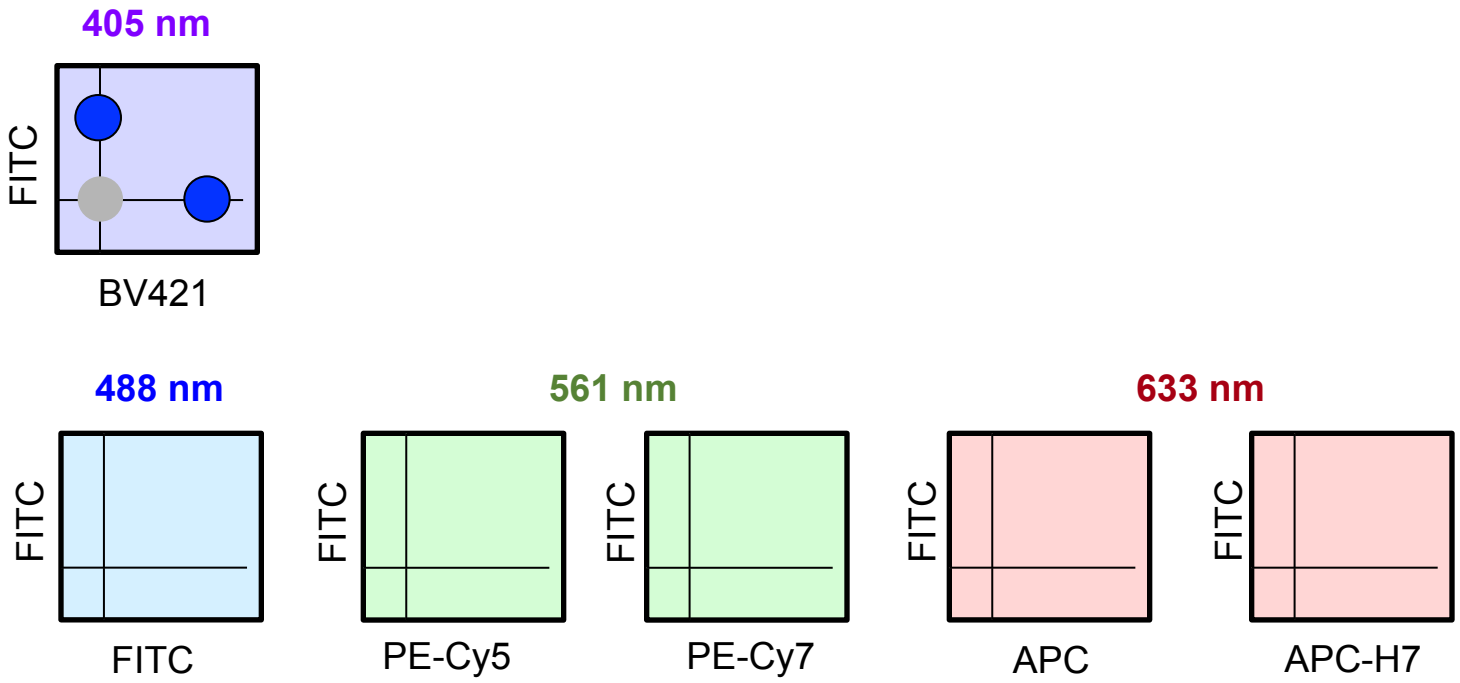
- Staining buffer: Add EDTA to avoid cell aggregation
- Stain on ice to prevent internalization of antigen:antibody complexes
- Keep samples protected from light and heat to avoid tandem degradation

5. Run appropriate controls:

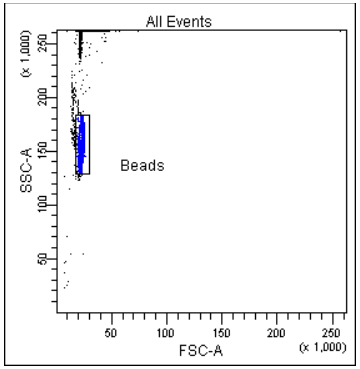
- Unstained and single stained controls: Compensation Matrix
- Gating controls: Fluorescence minus one
- Biological controls: unstimulated-stimulated

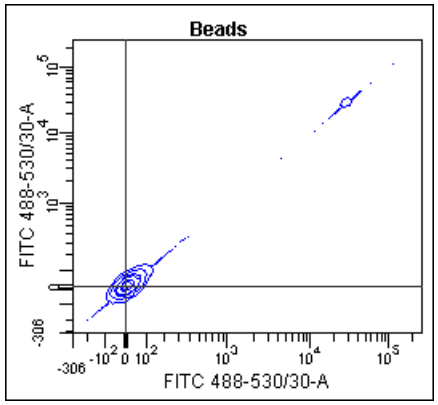
Fine-tune the compensation matrix

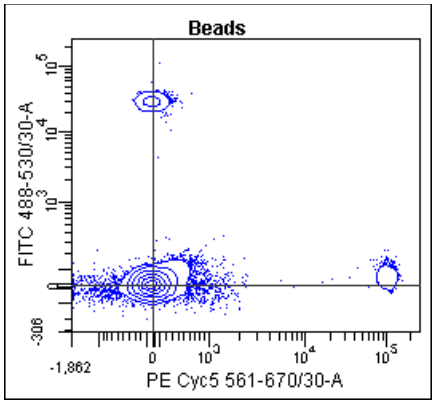
Verification of every parameter against the another ones. Mix of single stained controls.

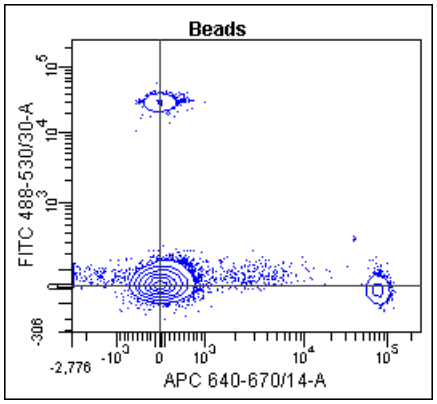


y = look sequentially at all parameters in the spectrum order of light


All Events
Beads
SSC-A
FSC-A
(x 1,000)


Beads
FITC 488-530/30-A
FITC 488-530/30-A


Beads
FITC 488-530/30-A
PE Cyc5 561-670/30-A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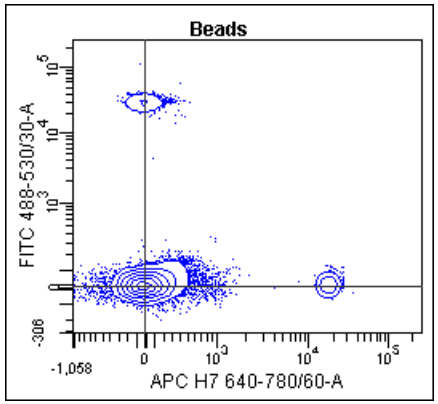
APC 640-670/14-A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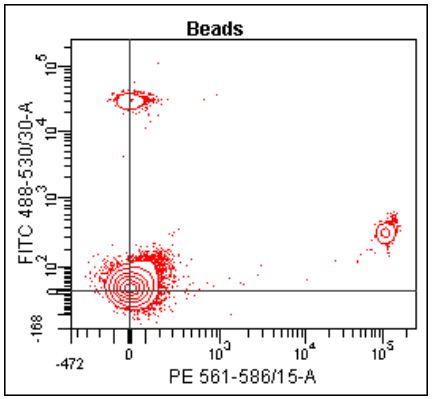
FITC 488-530/30-A
APC H7 640-780/60-A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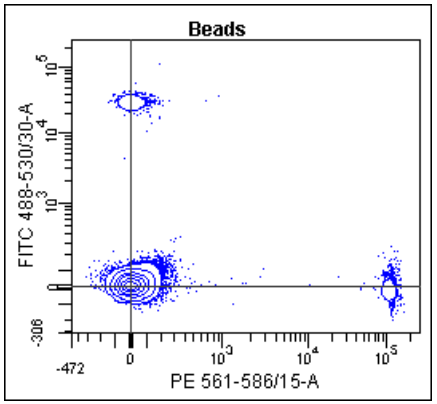
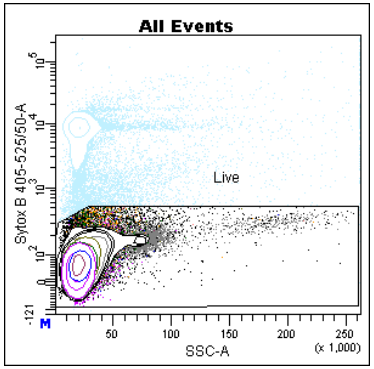
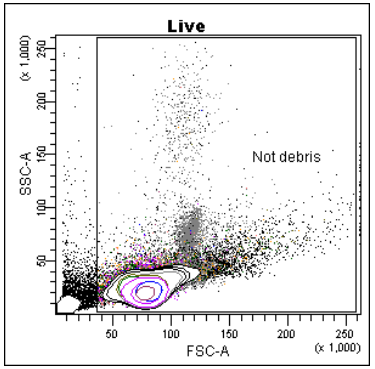
PE 561-586/15-A

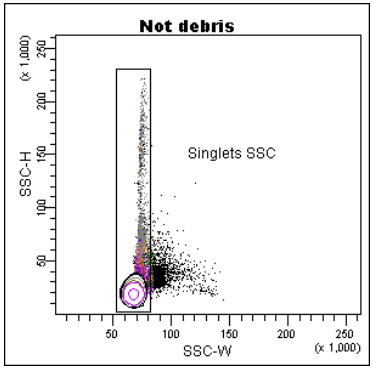

Beads
FITC 488-530/30-A
PE 561-586/15-A

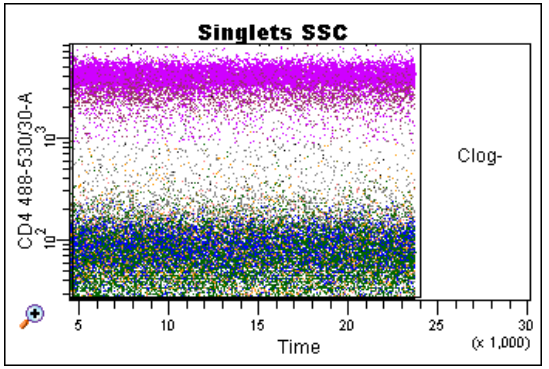
Spleen panel

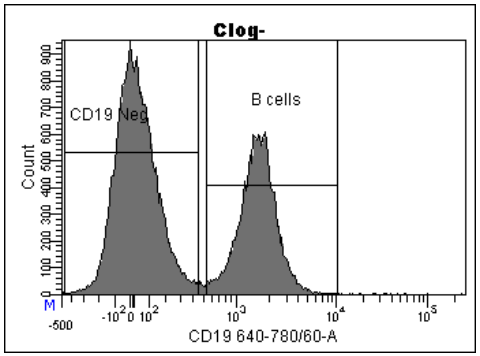
6 antibodies, 1 viability dye, 9 Parameters to determine 6 Populations

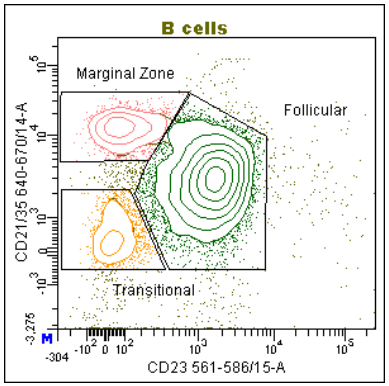


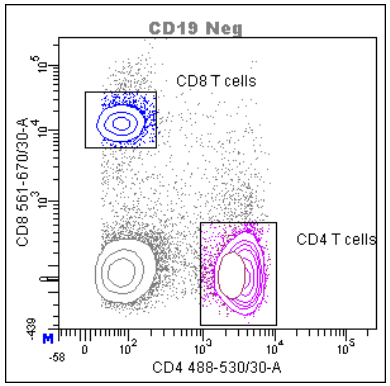


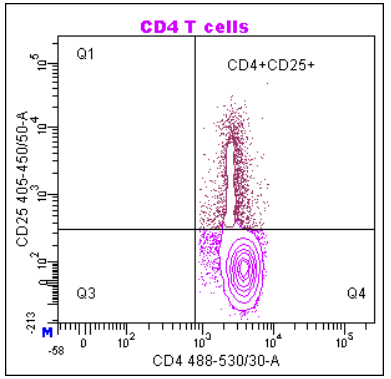










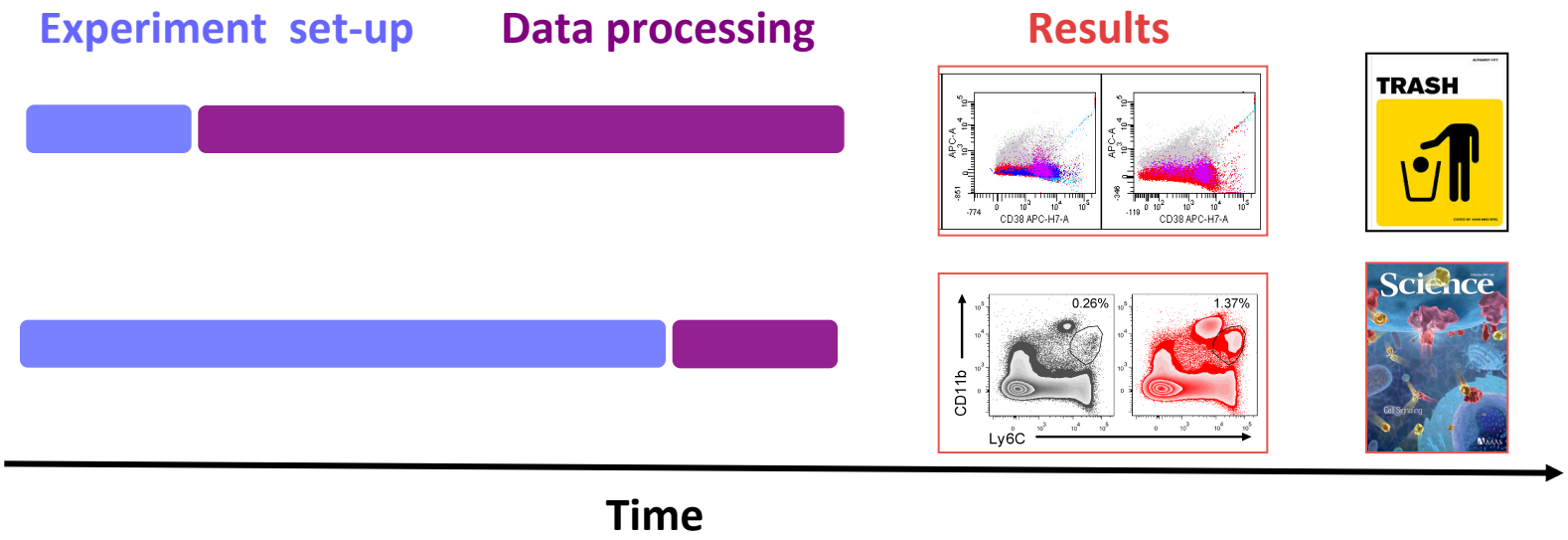


EMBL

Tube: Staining

Population	#Events	%Parent	%Total
All Events	76,433	####	100.0
Live	62,747	82.1	82.1
Not debris	59,096	94.2	77.3
Singlets SSC	56,914	96.3	74.5
Clog-	42,280	74.3	55.3
CD19 Neg	26,507	62.7	34.7
CD4 T cells	15,361	58.0	20.1
Q1	0	0.0	0.0
CD4+CD25+	1,948	12.7	2.5
Q3	0	0.0	0.0
Q4	13,413	87.3	17.5
CD8 T cells	2,921	11.0	3.8
B cells	15,531	36.7	20.3
Marginal Zone	2,008	12.9	2.6
Follicular	11,618	74.8	15.2
Transitional	1,183	7.6	1.5

Summary



"Smart panel design strategy"
Compensation matrix verification
Templates for acquisition/analysis

Thanks a lot

Alexandros!!

- “Titration of Fluorochrome-.-Conjugated Antibodies for Labeling Cell Surface Markers on Live Cells” . Current Protocols Flow Cytometry 6.29.1 Supplement 54
- “Optimizing a Multicolor Immunophenotyping Assay” Clinic Lab Med 27: 269-.-285, 2007 Ag densitiy ☺
- “Nine-.-Color Flow Cytometry for Accurate Measurement of T Cell Subsets and Cytokine Responses. Part I: Panel Design by an Empiric Approach”
- A practical approach to multicolor flow cytometry for immunophenotyping. Journal of Immunological Methods 243 (2000) 77–97
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- Maecker HT, Frey T, Nomura LE, Trotter J. Selecting Fluorochrome conjugates for maximum sensitivity. *Cytometry A* 2004; 62:169-173.