Compensation, Negative Controls and the Optimized Choice of Reagents for Multicolor Flow Cytometry

Overview

- Compensation
  - Introduction
  - Prevention of compensation related artifacts
- Negative Controls
  - Fluorescence Minus One (FMO)
  - Isotype controls
  - Transfection controls
- Combination of Reagents for Multicolor Flow Experiments
- Characteristics of Fluorochromes

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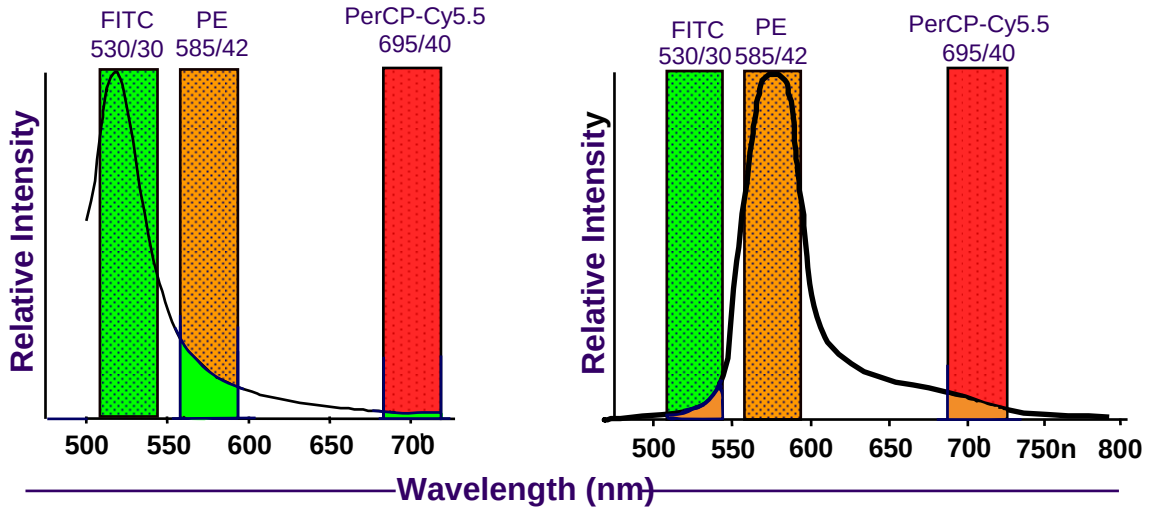
Compensation:
Introduction

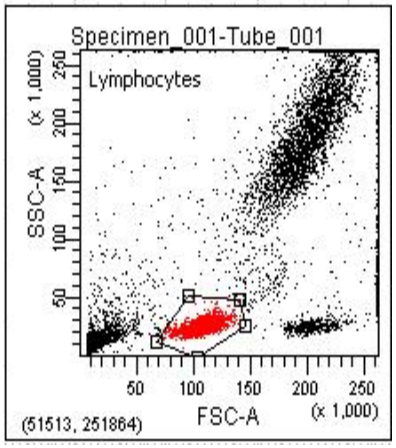
- Photons reaching a specific detector are coming from
  - the fluorochrome specific for this detector
  - optical background (especially cell-type specific auto-fluorescence)
  - photon spill over from all fluorochromes present in experiment

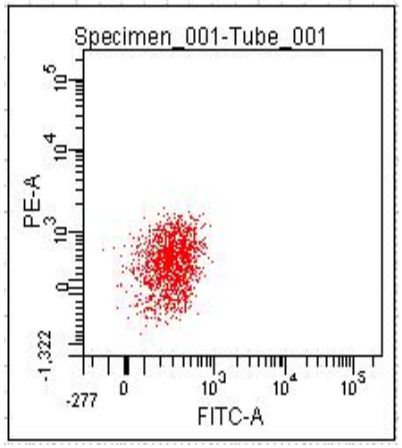


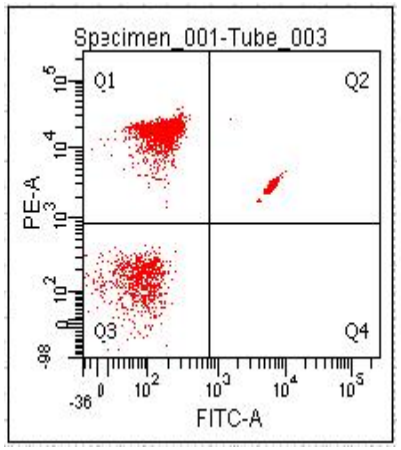
Compensation:
Introduction

- Compensation is a procedure to subtracts from all photons that reach a detector the
  - 1) nonspecific electronic signal from cellular autofluorescence

    Application of “Instrument Settings” for one specific (type of) sample
  - 2) nonspecific electronic signal from fluorochrome spill over







Population	FITC-A Mean	PE-A Mean
Lymphocytes	2,476	11,679
Q1	170	19,532
Q2	6,287	2,849
Q3	78	159
Q4	####	####

Compensation:
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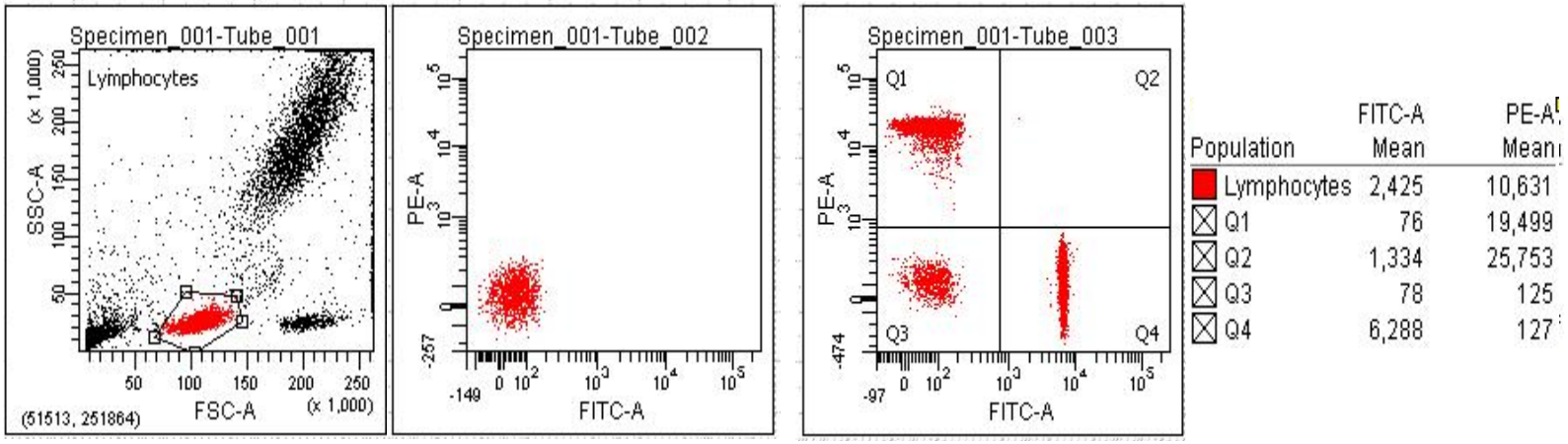


Compensation:
Introduction

- 1) Generate sample-specific “Application Settings” according to SD_{EN} given in CS&T Baseline Report
  - Refer to Handout #3: Generation of Application Settings
  - Refer to the Presentation: QC for Digital Instruments: BD CS&T™
- 2) Perform an Automated Compensation using the BD FACS Diva™ software
  - Refer to Handout #4: Automated Compensation

Compensation:
Prevention of compensation related artifacts

- To avoid errors in compensation

1) never change the PMT V of fluorescent parameters (Instrument Settings) after a compensation has been calculated

2) only the “median-related” = “statistic-based” compensation will result in accurate compensation values: Never compensate “by eye”

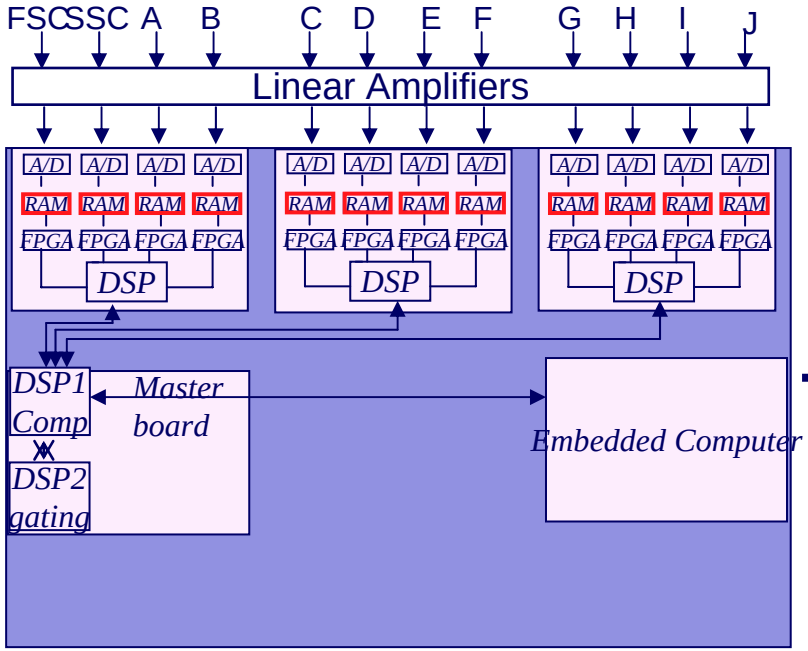
3) use only compensation controls that show in minimum equally high signal intensities than the highest signal you expect in your sample

4) BUT, the signal intensities of the compensation controls have to lie within the maximum linearity range of the scales (refer to the CS&T baseline report)

5) it is not recommended to re-use compensations if a tandem dye is included in the panel

Compensation:
Prevention of compensation related artifacts

- Digital Compensation is a software-based compensation in that linear numbers are subtracted



Uncompensated List-Mode Data

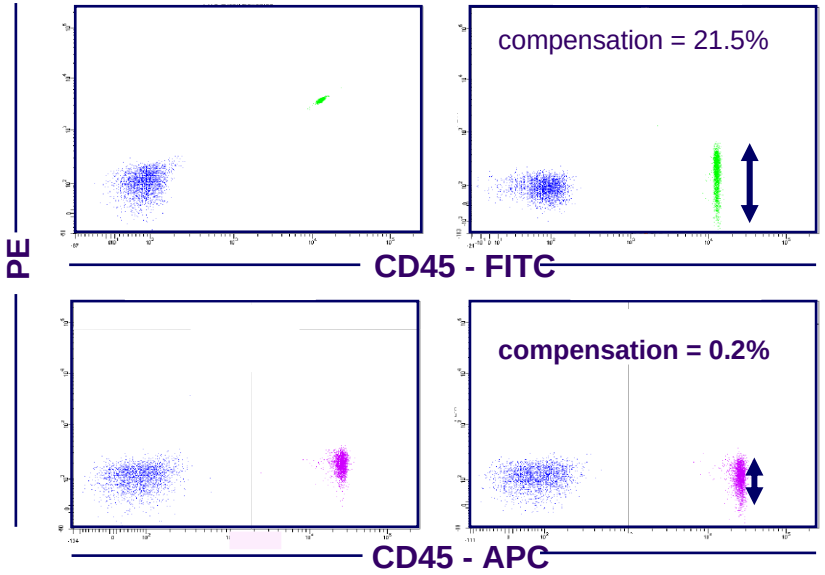
	Time	FSC	SSC	FITC	PE
Event 1	0	60	120	5	23
Event 2	10	160	65	89	675
Event 3	30	650	160	39.271	30.621

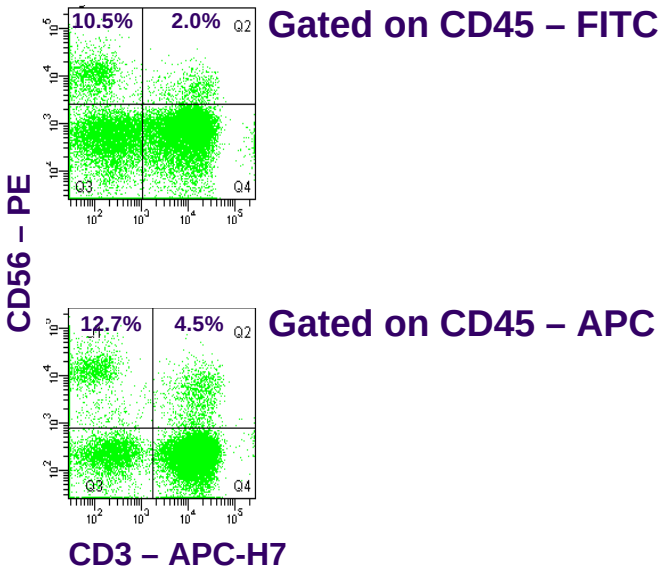
- But if each optimal compensation is a subtraction that results in “0”, why is a compensation value of 20% worse than 0.2%?

Compensation:
Prevention of compensation related artifacts

- High compensation values induce “data-spread” effects that reduce substantially the resolution between populations

Uncompensated | Compensated





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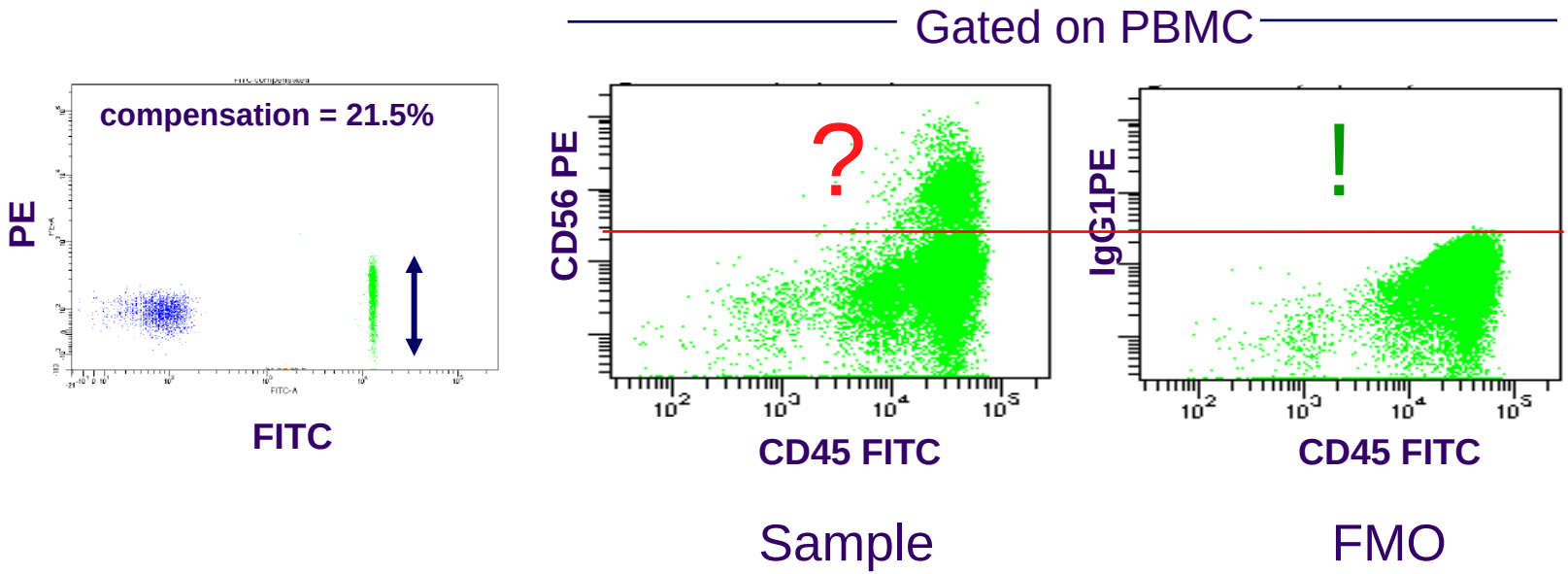
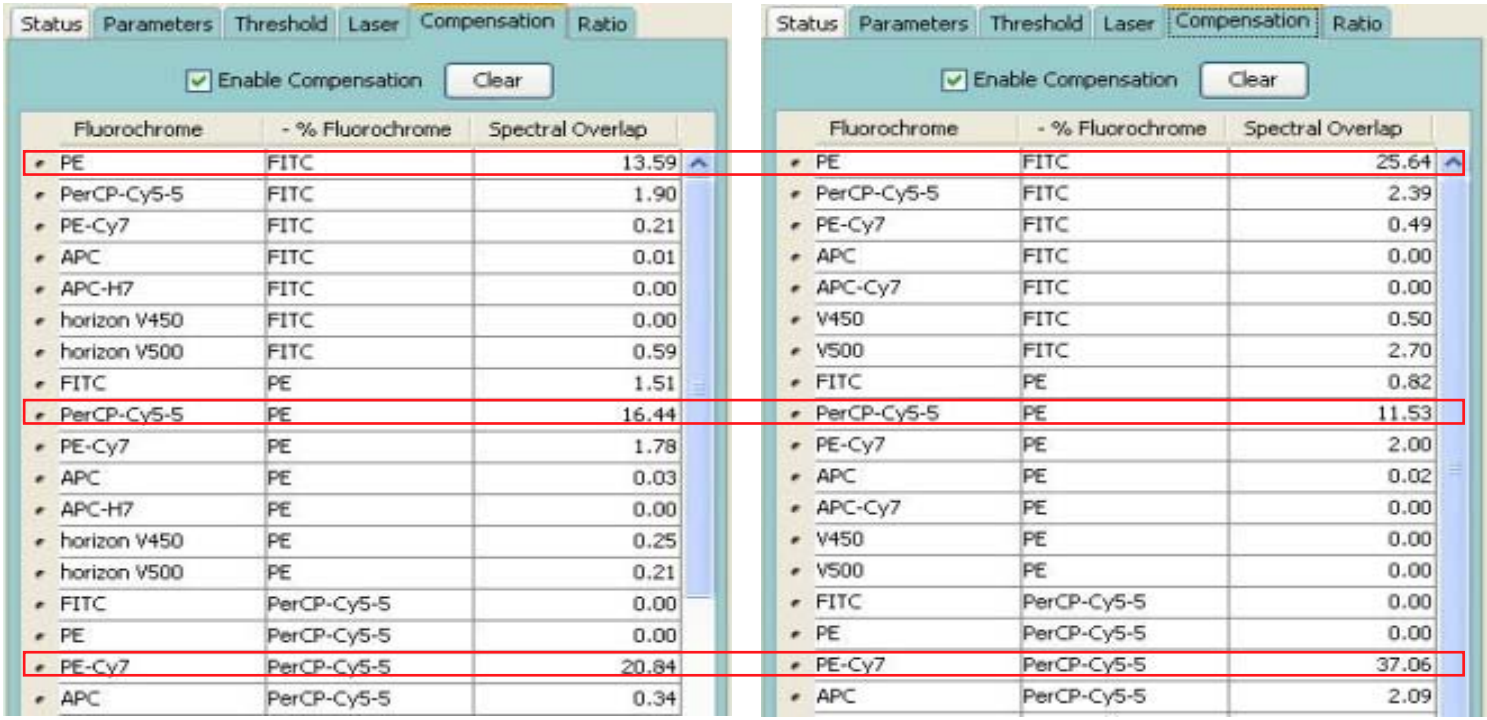
Negative Controls:
Fluorescence Minus One (FMO)

- Indicates the data-spread induced by “high” compensation values



Negative Controls:
Fluorescence Minus One (FMO)

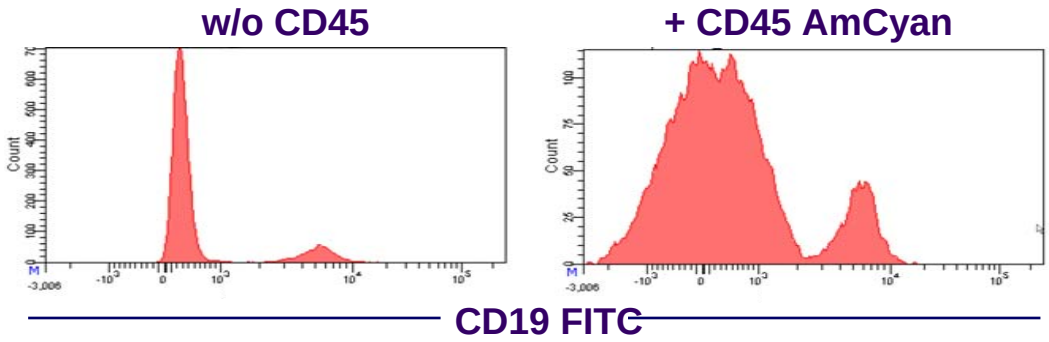
- Fluorochrome-combinations with “high” compensation values?



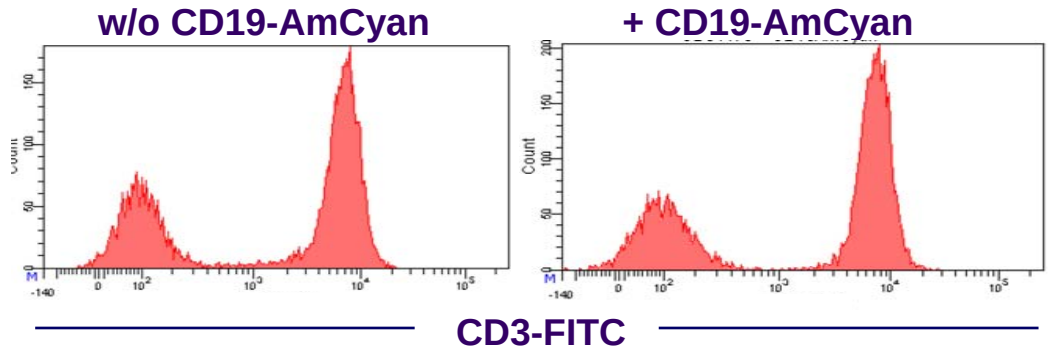
- Get experience: compare of multicolor compensation matrices.

Negative Controls:
Fluorescence Minus One (FMO)

- Does every fluorochrome combination with “high” compensation values requests an FMO Control?



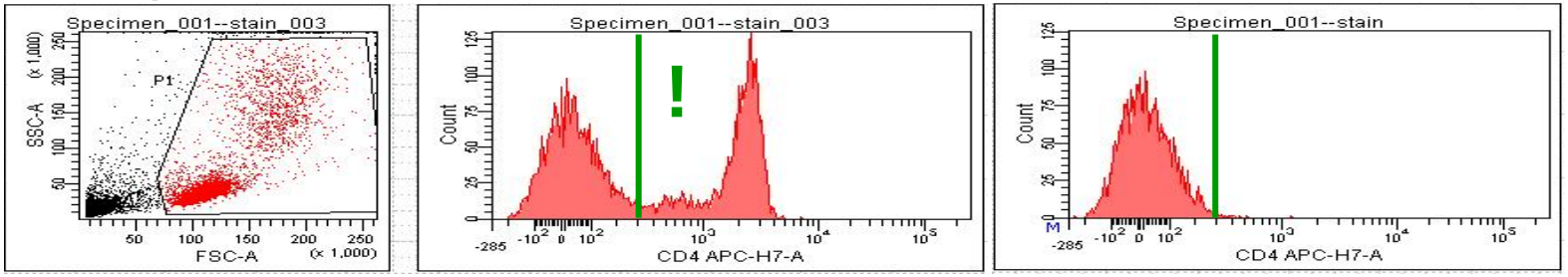
- Not if the fluorochromes bind to different cells!



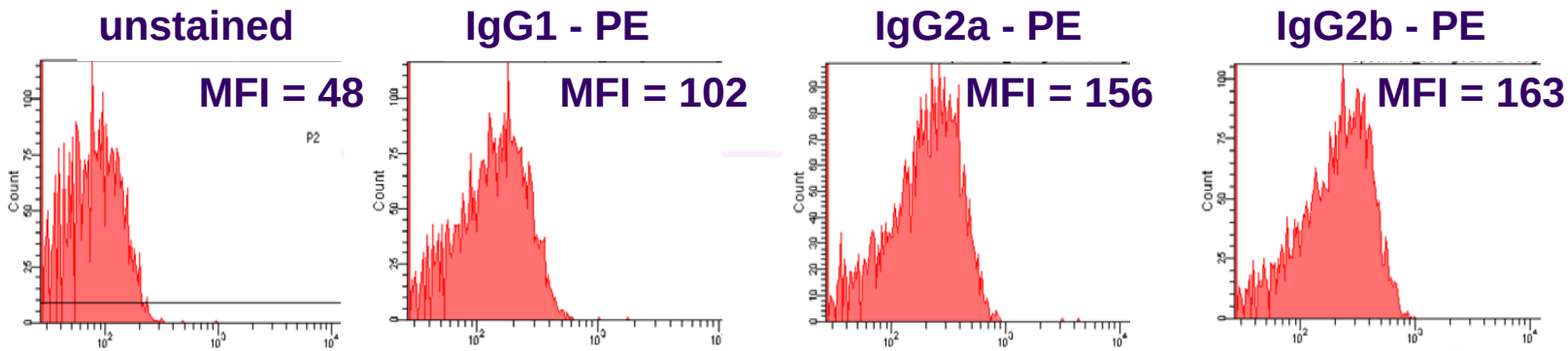
Negative Controls:
Isotype Control

- Indicates specificity of antigen-expression in unknown samples

CD4 is THE T-helper cell marker



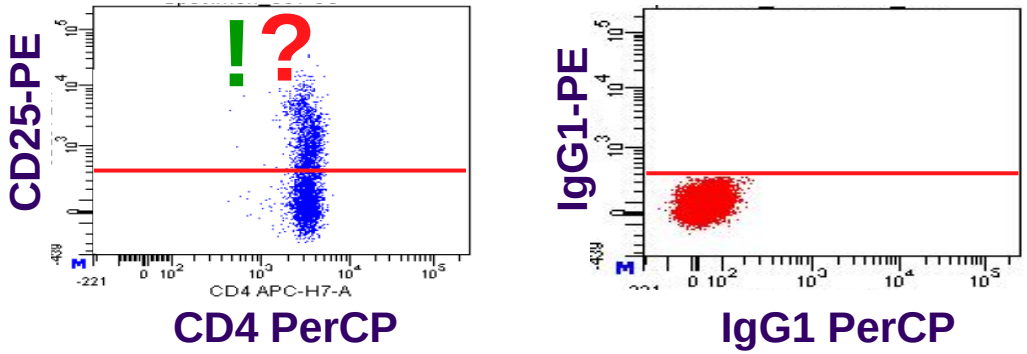
- Indicates the nonspecific binding of antibodies to membranes



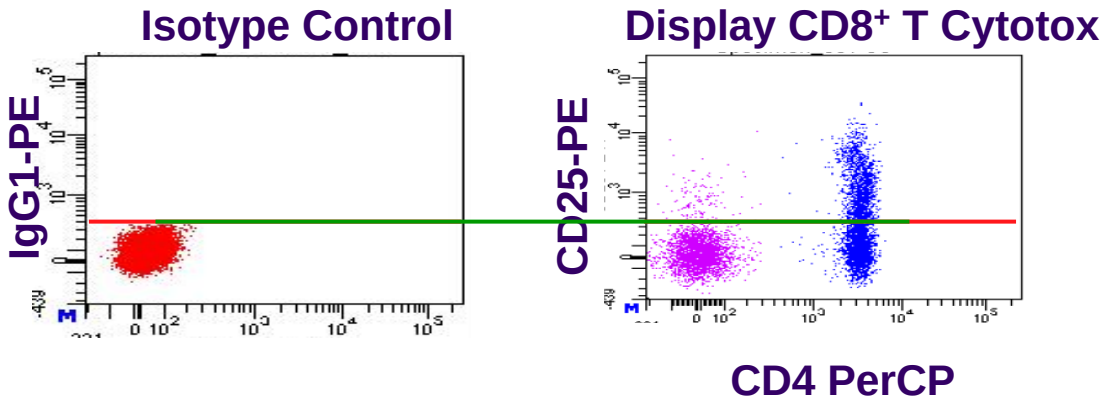
0.025μg antibody per 100 μl whole blood

Negative Controls:
Isotype Control

- Indicates specific binding of antibodies to very low and/or “smeared” expressed antigens



- But (if available and known) “biological controls” are better



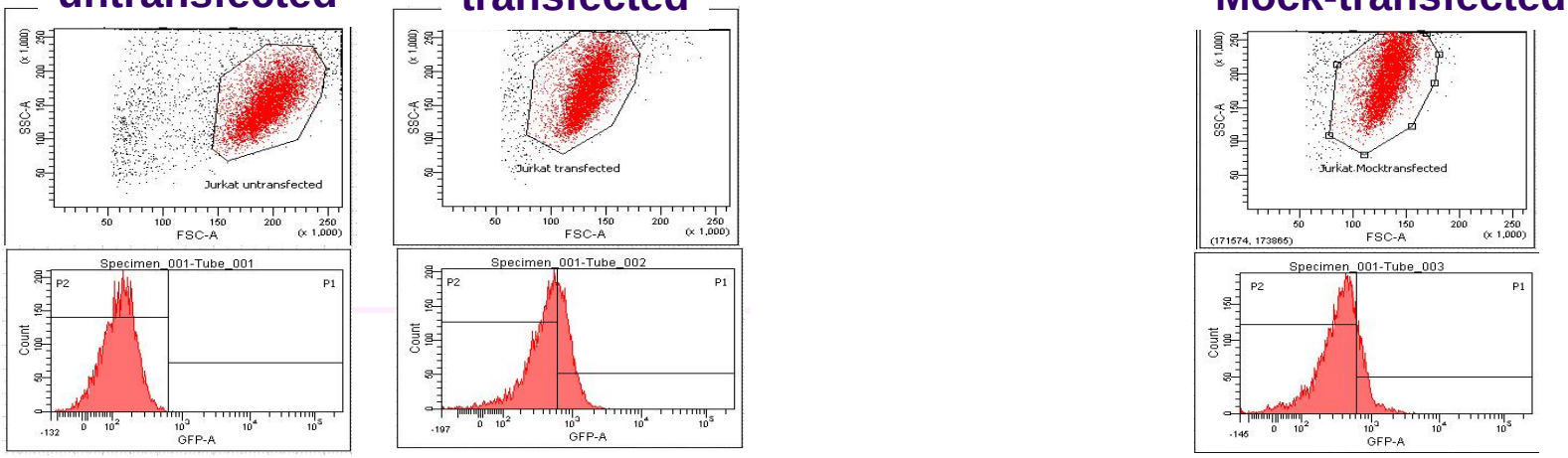
Negative Controls:
Isotype Control

- Isotype controls HAVE to
  - show perfect match in isotype subtype
  - come from the same species
  - be labeled to the same fluorochrome
  - show equalized concentrations between specific antibody and isotype control
  - be specifically purified from free fluorochromes for intra-cellular staining
  - show comparable antibody to fluorochrome ratios

Negative Controls:
Transfection Control (“Mock Control”)

- Transfection methods using Lipofectin and similar substances enhance the autofluorescence due to the binding of plasmid-DNA to cell membranes
- Nontransfected cells are for this reason never a proper control!

untransfected **transfected** **Mock-transfected**



- In this example the transfection-efficiency is 0% as the plasmid-promotor shows a deletion that prevents gene-expression!

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Combination of Reagents for Multicolor Flow Experiments

- Basic rule for the selection of antibody-fluorochrome-combinations:

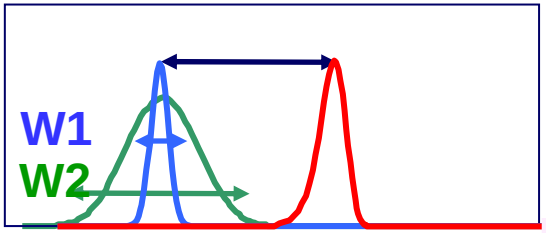
  Match the fluorochrome brightness with the antigen-expression levels

- For further information see in Appendix 4:

  http://www.bdbiosciences.com/documents/Multicolor_AppNote.pdf

Combination of Reagents for Multicolor Flow Experiments

- Match fluorochrome brightness with antigen-expression levels
  - Fluorochrome brightness?
    - The “Stain Index”



$$\text{Stain Index (SI)} = D/W$$

D = Δ between positive and negative peak medians
W = Spread of the background peak

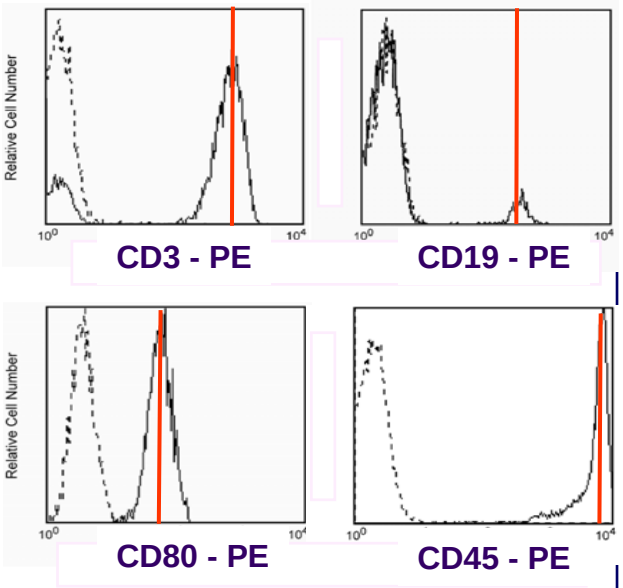
- Stain indices of BD specific fluorochromes obtained on a BD FACSCanto™ II

Reagent	Clone	Stain Index	
PE	RPA-T4	356.3	Bright fluorochromes
Alexa 647	RPA-T4	313.1	
APC	RPA-T4	279.2	
PE-Cy7	RPA-T4	278.5	
PE-Cy5	RPA-T4	222.1	
PerCP-Cy5.5	Leu-3a	92.7	Intermediate-bright fluorochromes
BD Horizon™ V450	RPA-T4	90.0	
Alexa 488	RPA-T4	75.4	
FITC	RPA-T4	68.9	
PerCP	Leu-3a	64.4	
APC-Cy7	RPA-T4	42.2	Dim fluorochromes
Alexa 700	RPA-T4	39.9	
AmCyan	RPA-T4	24.2	
BD™ APC-H7	RPA-T4	18.0	
BD Horizon™ V500	RPA-T4	12.0	

Combination of Reagents for Multicolor Flow Experiments

- Match fluorochrome brightness with antigen-levels
  - Antigen Expression level?

Compare examples of PE-staining given in TDS



Combination of Reagents for Multicolor Flow Experiments

- Match fluorochrome brightness with antigen-levels
  - Antigen Expression level?

Or use literature-data about # of antigen-molecules per cell

Antigen	Antigen-Density	Expression Level
CD3	80.000	++
CD4	100.000	+++
CD14	144.000	+++
CD19	18.000	++
CD25	3.000	+
CD45	200.000	+++
CD56	10.000	+
CD127	2.000	+

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

Example for one proper Antibody-Fluorochrome-Match for a BD FACS Aria™ 405nm / 688nm / 635nm

BD Horizon™ V450
FITC
BD™ APC-H7
PerCP-Cy™5.5
APC
BD Horizon™ V500
PE-Cy™7
PE

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Characteristics of Fluorochromes

- Select fluorochromes according to instrument configuration

Max Emission	Possible fluorochrome-excitation by			
	Violet 405nm	Blue 488nm	Y-G 561nm	Red 635nm
448nm or 452nm	BD Horizon™ V450 or Pacific Blue™			
461nm				
491nm	AmCyan or BD Horizon™ V500			
519nm or 520nm		AF®488 or FITC		
578nm		PE	PE	
660nm or 668nm				APC or AF®647
667nm or 678nm or 695nm		Pe-Cy™5 or PerCP or PerCP-Cy™5.5	Pe-Cy™5 or PerCP or PerCP-Cy™5.5	
723nm				AF®700
785nm or 783nm				APC-Cy™7 or BD™ APC-H7
785nm		Pe-Cy™7	Pe-Cy™7	

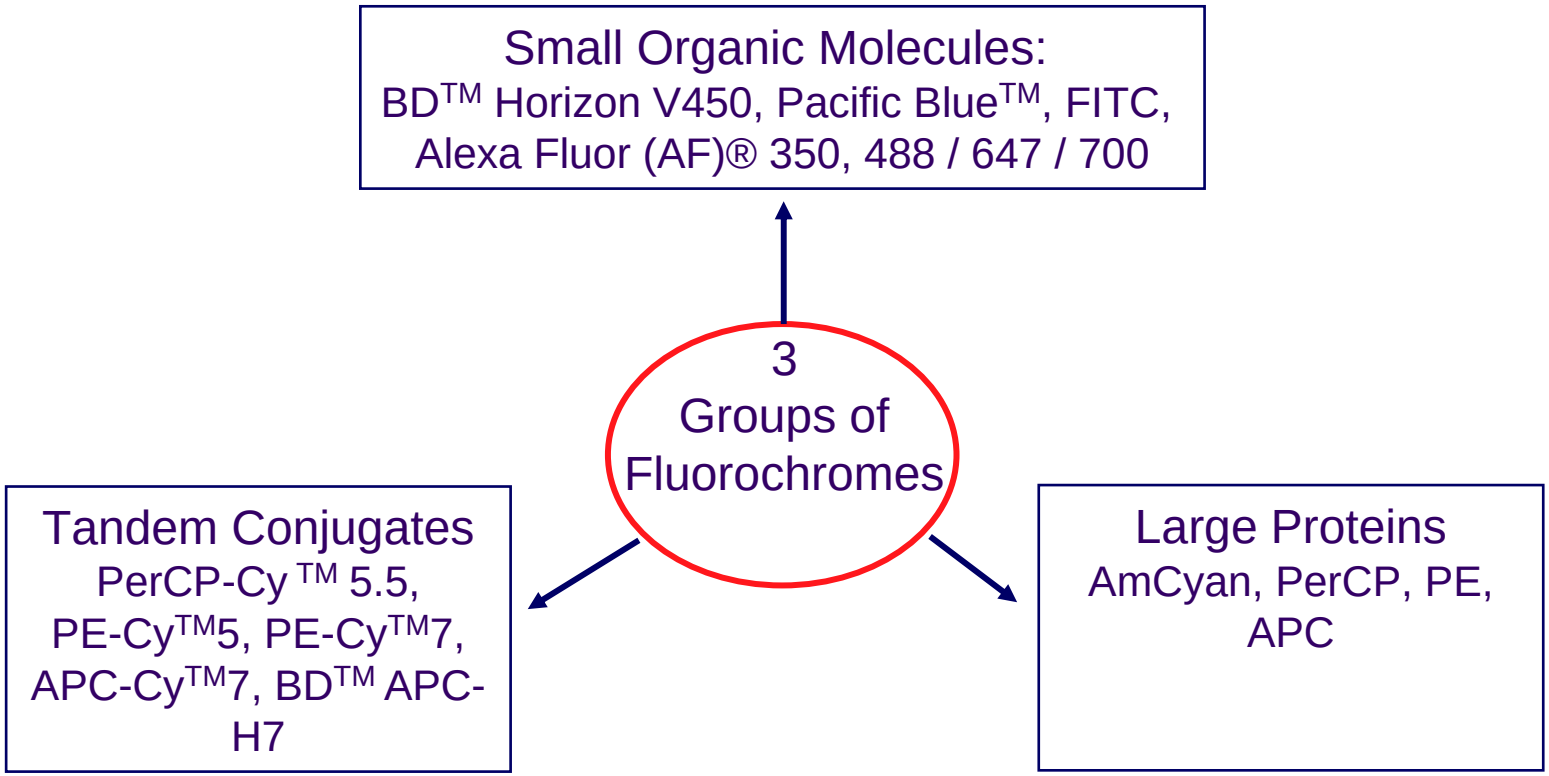
Characteristics of Fluorochromes

- Select fluorescent dyes according to instrument configuration

Max Emission	Possible fluorochrome-excitation by			
	Violet 405nm	Blue 488nm	Y-G 561nm	Red 635nm
461nm or 470nm or 477nm	Dapi or SYTOX-Blue or ECFP			
505 or 510nm		CFSE or EGFP		
522nm or 524nm or 528nm		SYBR-Green or SYTOX-Green or EYFP		
562nm			mOrange	
581nm			mTomato	
586nm		dsRed		
610nm			mCherry	
617nm or 647nm		PI or 7-AAD		
650nm			mPlum	
658nm				SYTOX Red
683nm				DRAQ5

Characteristics of Fluorochromes

- BD-supported fluorochromes



Characteristics of Fluorochromes:
The “blue laser fluorochromes (I)”

	Advantages	Disadvantages
1. FITC	▪ Most widely used ▪ Stable, long-lasting conjugates ▪ Easy conjugation (3-5 per Ig) ▪ Cheapest fluorochrome	▪ One of the dullest fluorochromes ▪ Photobleaching (relevant only for microscopy) ▪ pH sensitive
2. Alexa Fluor® 488	▪ Very photo- and pH-stable (superior for microscopy)	▪ One of the dullest fluorochromes
3. PE	▪ Bright reagents: IC/FCM ▪ Low backgrounds: IC/FCM 1st choice for intracellular protein/cytokine detection! ▪ Good for quantification: 1 PE per Ig (BD™QuantiBrite)	▪ Large size (240.000 D) is NOT a disadvantage!

Characteristics of Fluorochromes:
The “blue laser fluorochromes (III)”

	Advantages	Disadvantages
6. PE-Cy™5	▪ Bright fluorochrome ▪ Useful additional fluorochrome for the LSR II	▪ Unspecific binding to Fc Receptors ▪ Very strong spill over to APC: has to be adjusted often by titration ▪ Lot to lot differences in compensation ▪ Light sensitive (just keep samples in the dark)
7. PE-Cy™7	▪ Bright fluorochrome: Preferable above PE-Cy5	▪ Lot to lot differences in compensation ▪ Light sensitive (just keep samples in the dark)

Characteristics of Fluorochromes:
The “blue laser fluorochromes (II)”

	Advantages	Disadvantages
4. PerCP	▪ Minimal Spill over to PE	▪ Dull (equivalent to FITC) ▪ Sensitive to photo-bleaching (relevant only for microscopy or high energy blue lasers. See BD FACS™ Vantage)
5. PerCP-Cy™5.5	▪ Intermediate bright fluorochrome (PerCP is dim)	▪ Lot to lot differences in compensation ▪ Light sensitive (just keep samples in the dark)

Characteristics of Fluorochromes:
The “red laser fluorochromes (I)”

	Advantages	Disadvantages
1. APC	▪ Bright fluorochrome ▪ Low background: 2nd choice for intracellular protein / cytokine detection!	▪ Large size (106.000 D) is NOT a disadvantage!
2. Alexa Fluor® 647	▪ Equivalent to APC, BUT ▪ Very photo- and pH-stable (superior for microscopy)	/
3. Alexa Fluor® 700	▪ Very photostable (superior for microscopy) ▪ Additional fluorochrome for the red laser	▪ Filter set not included in standard BD FACSAria™ configuration, but easy to obtain additionally

Characteristics of Fluorochromes:
The “red laser fluorochromes (II)”

	Advantages	Disadvantages
4. APC-Cy™7	▪ Additional red laser excited fluorochrome	▪ Dim ▪ Photo-instable ▪ Fixative-sensitive ▪ Lot to lot differences in compensation
5. BD™ APC-H7	▪ Photo-stable ▪ Fixative-stable: Preferable above APC-Cy7	▪ Dim ▪ Lot to lot differences in compensation

Characteristics of Fluorochromes:
The “violet laser fluorochromes (I)”

	Advantages	Disadvantages
1. Pacific Blue™	▪ Little Spill over to FITC	▪ dim
2. BD Horizon™ V450	▪ Little Spill over to FITC ▪ Intermediate bright	/

Characteristics of Fluorochromes:
The “violet laser fluorochromes (II)”

	Advantages	Disadvantages
3.AmCyan	▪ Before BD Horizon™ V500 and BD Horizon™ V450 the only violet-excited fluorochrome from BD	▪ dim ▪ extremely strong spill over to FITC ▪ Fixative-sensitive ▪ Light-sensitive
4. BD Horizon V500	▪ Little spill over to FITC: highly preferable alternative to AmCyan ▪ Fixative-stable	▪ Dim

Characteristics of Fluorochromes

- Summary:
  - There are no “bad” fluorochromes!
  - Considering limitations, all fluorochromes are usefull designing multicolor flow experiments
  - But some fluorochromes are easier to handle for multicolor-combinations than others!