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What is wrong with my flow cytometry data?

Hints, tricks and pitfalls

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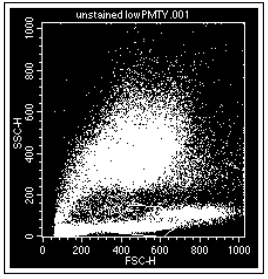
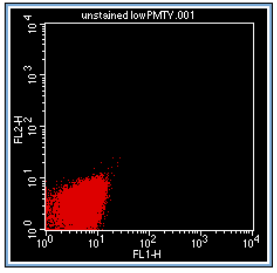
Core Facility für Zellsortierung und Zellanalyse Universitätsmedizin Rostock

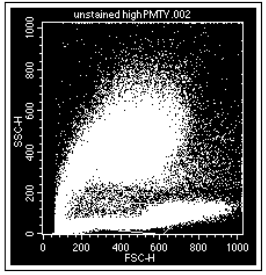
Webinar 2
07.03.2023

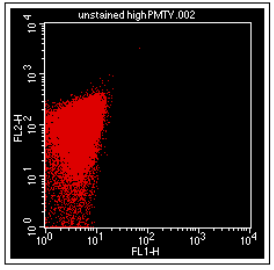
(The) perfect control(s) - how and what to use?

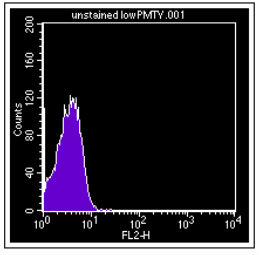
The need for controls … an example.

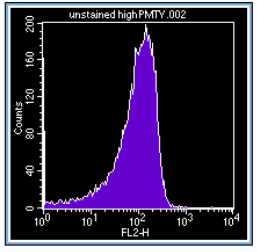


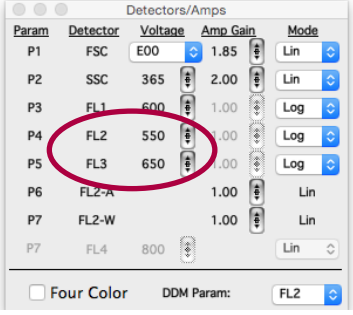


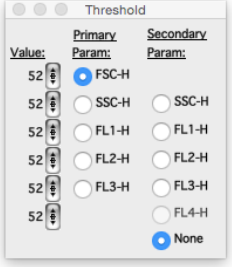


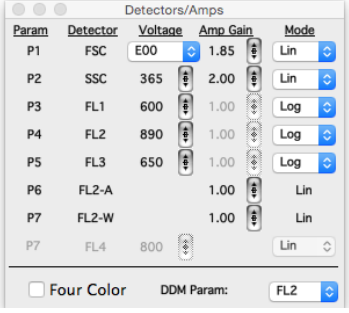


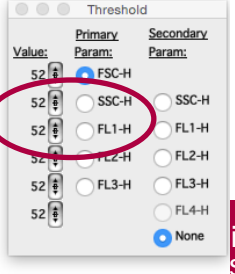












unstained?

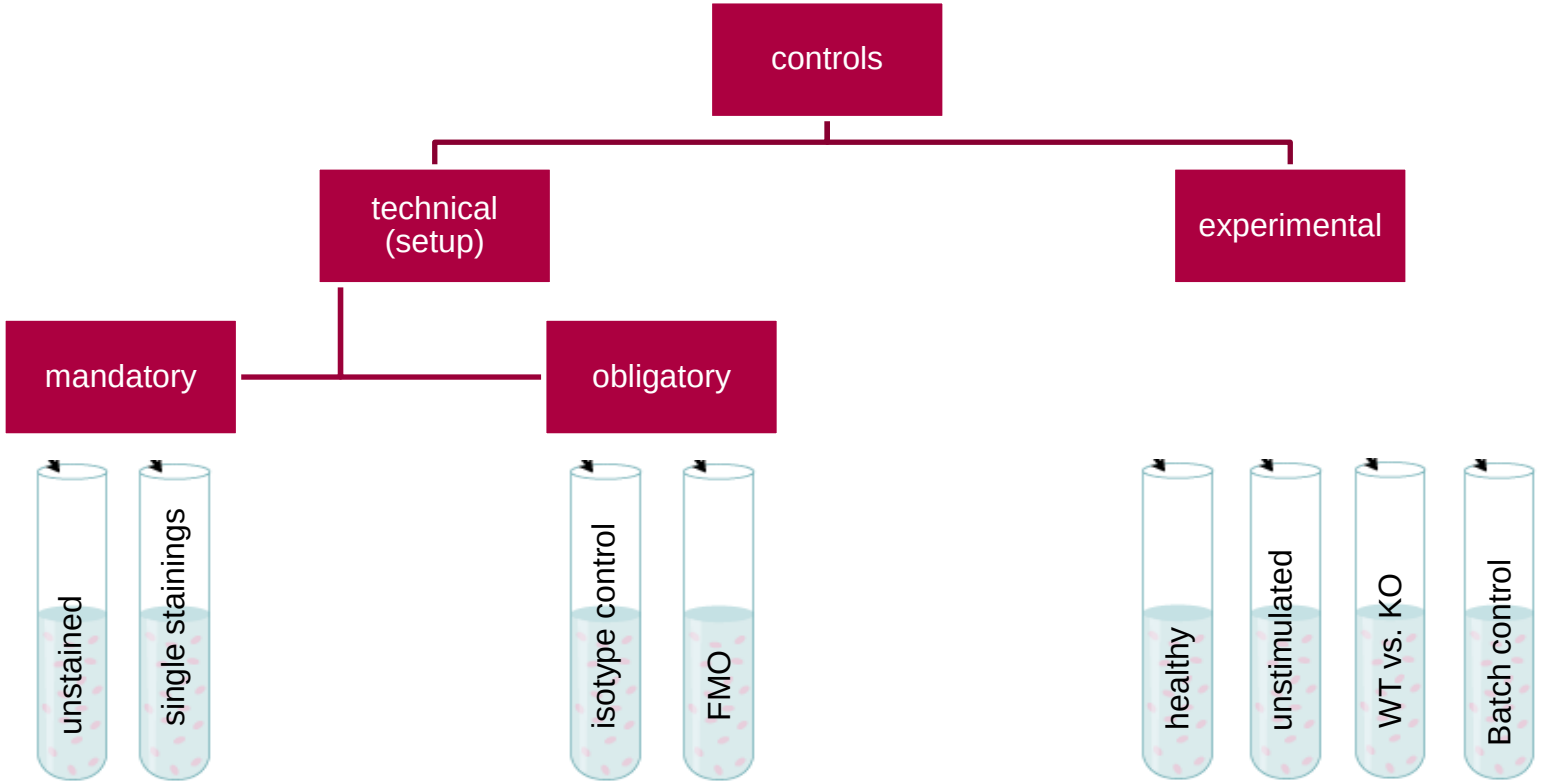
stained?

No! both unstained

Controls: Determining positivity by eliminating false positives

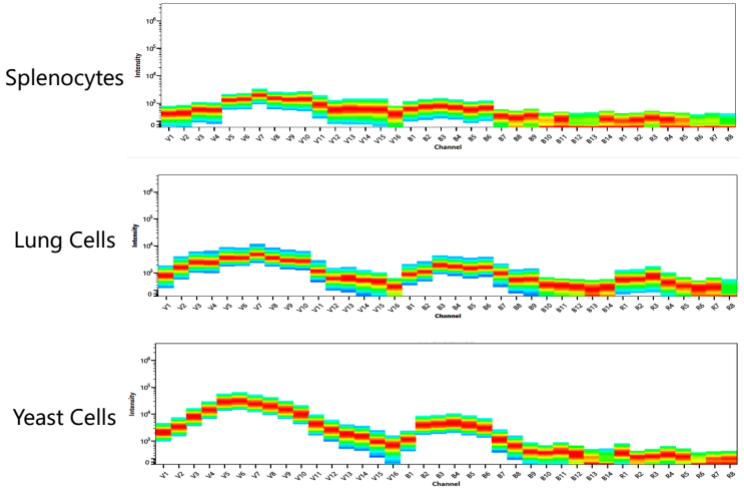
- flow cytometry → multiparametric analysis of single cell suspension
- BUT: relative measurement → results depend on experimental setup, device configuration and settings (e.g. PMT voltages)
- Spectral spillover → compensation & unmixing → single staining are needed
- wrong controls → wrong data and misinterpretation

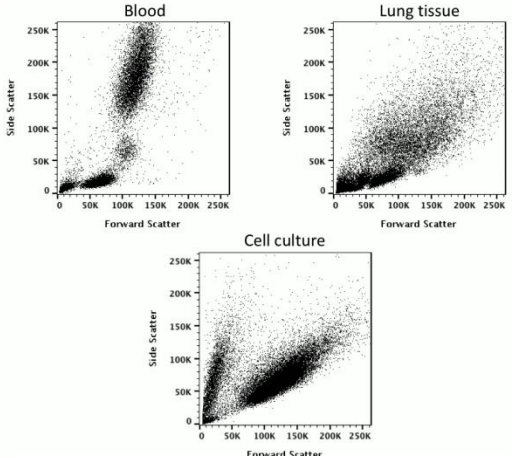
The nature of controls

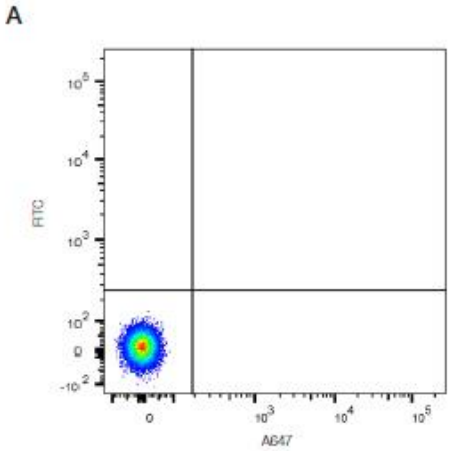


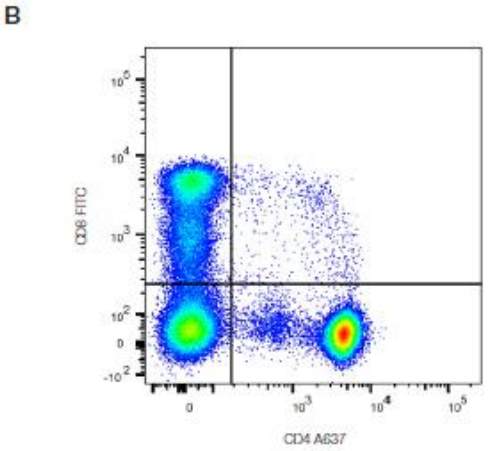
Control types: UNSTAINED

- defines background and autofluorescence
- should be within the first third of the plot/graph
- can be used as a cu off for positive staining
- helpful to find cells in the scatter plots





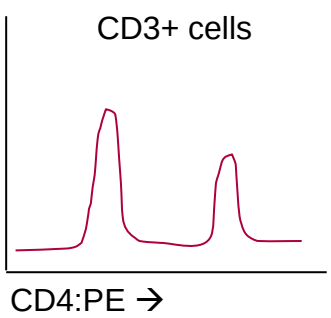


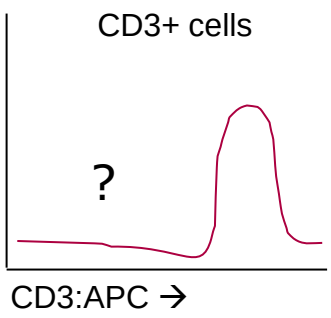


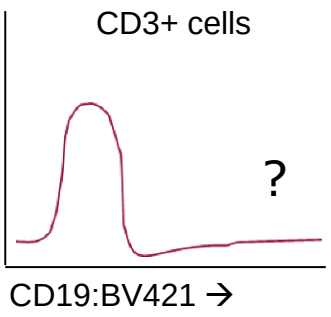
https://www.bio-rad-antibodies.com/flow-cytometry-unstained-controls.html

Control types: SINGLE STAIN

which control is needed?



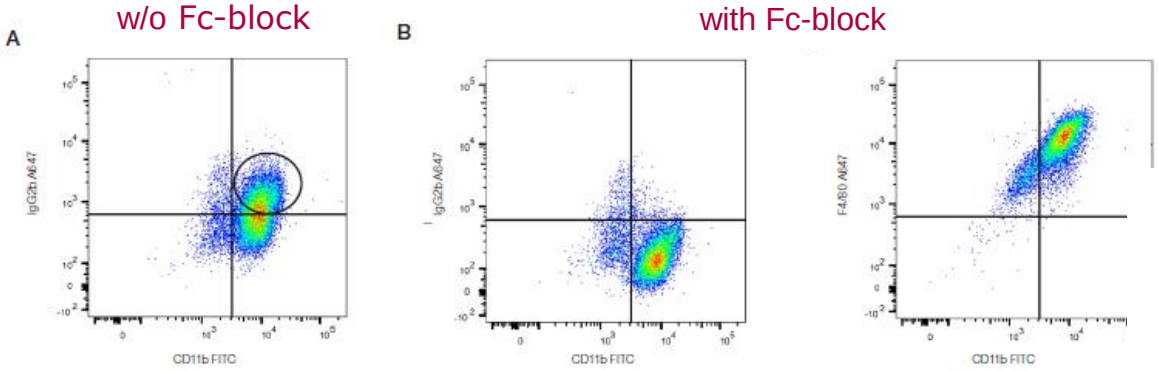




- positive control for each fluorescence dye
- necessary for calculating compensation/unmixing
- **minimum as bright as the real sample**
- **background/autofluorescence of negative and positive control must be the same**
- ! compare beads with beads, cells with cells !

Control types: ISOTYPE

- check for unspecific antibody binding (ionic and hydrophobic interactions between Fc-receptors and antibody)
- need for identical isotype & fluorophore
- e.g. mouse IgG2a (κ) – PE-Cy7 or mouse IgG2b (κ) – PE-Vio770



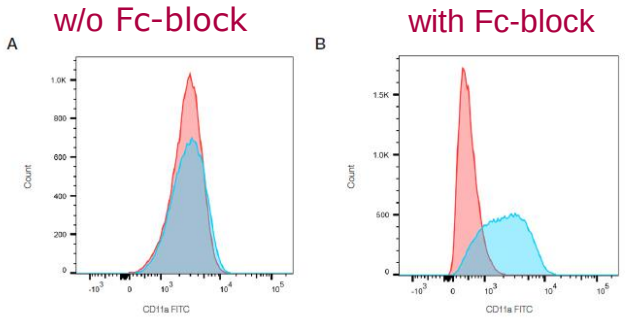


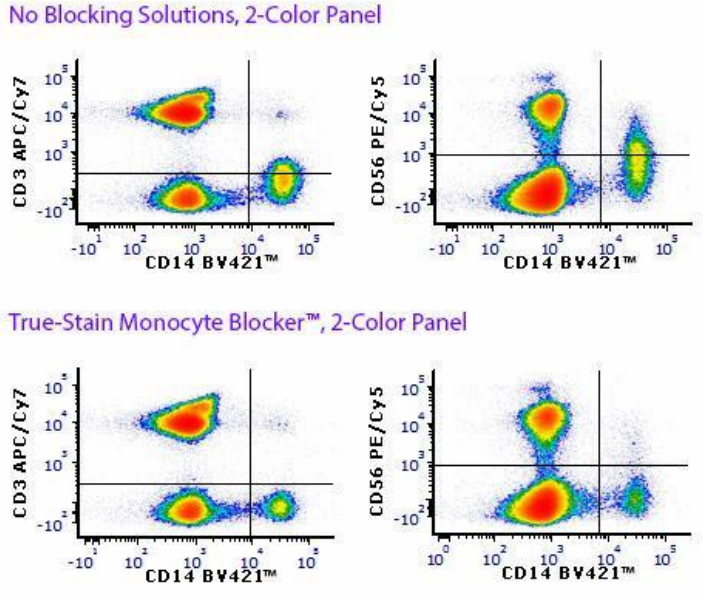
Fig. 21. Fc blocking. THP-1 cells stained with mouse anti human CD11a (blue) or mouse IgG2a isotype control (MCA929F) (red) in the absence (A) and presence (B) of human Seroblock (BUF070A)

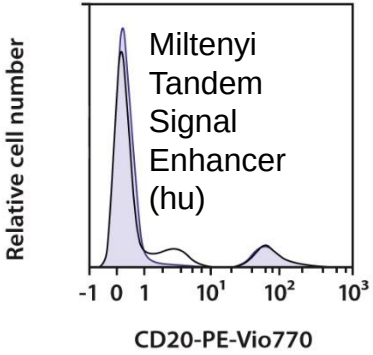
How to get away with an unspecific staining

- particularly B cells and myeloid cells: monocytes, dendritic cells, macrophages
- positive signal with isotype ctrl → blocking!
  - commercial Fc block (BioLegend, BD, …)
    - based on CD16/32
    - for human and mouse
  - self-made: 10% serum in PBS (+ 0,5 % BSA)
  - reducing unspecific binding of tandem conjugates to Fc receptors
    - Monocyte blocker or Tandem Enhancer

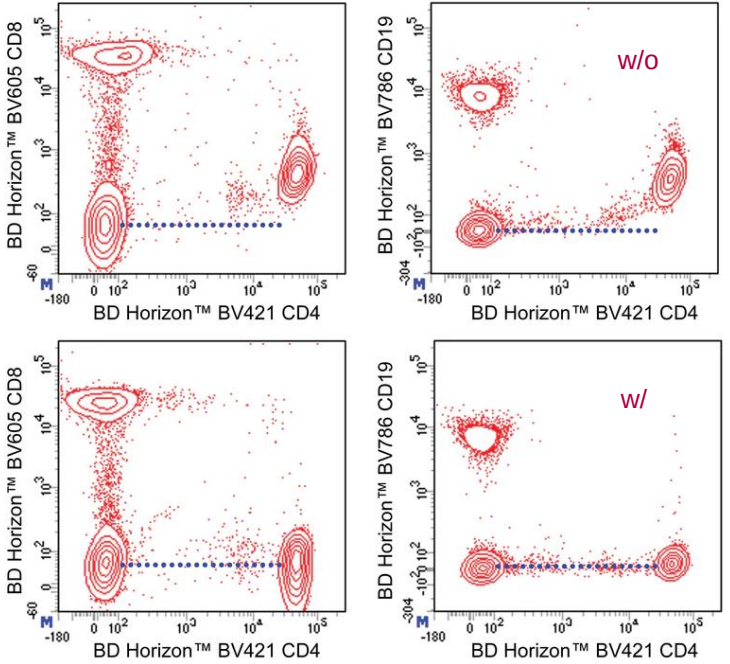
How to get away with an unspecific staining

BioLegend TruStain Monocyte Blocker





BD Brilliant Stain Buffer



Control types: Fluorescence minus one

- control staining without antibody of concern, all others stained
- defines measure of spread of other fluorophors
- gate for positive population
- often necessary for tertiary antigens

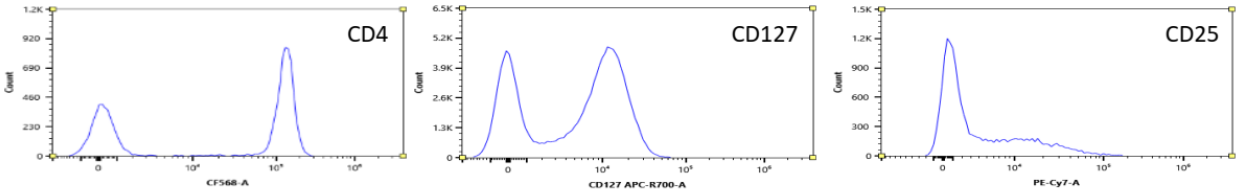
Primary
High density
on or off expression
CD3
CD4
CD8

Secondary
Intermediate density
continuous expression
CD45RA
CCR7
CD127
HLA-DR

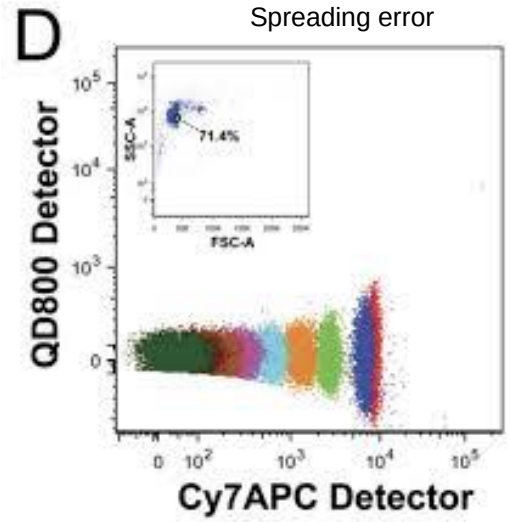
Tertiary
Low density
unknown expression
PD-1
CD25
TCR γ/δ

Flurochrome Brightness

Level of Antigen Expression



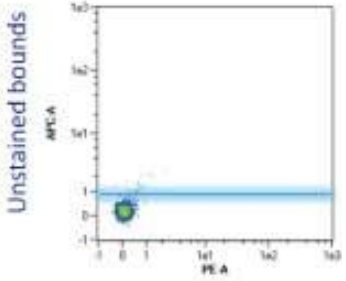
Spreading error



Mahnke, Y. and Roederer, M. Clin Lab Med. 2007 September ; 27(3): 469

Control types: Fluorescence minus one

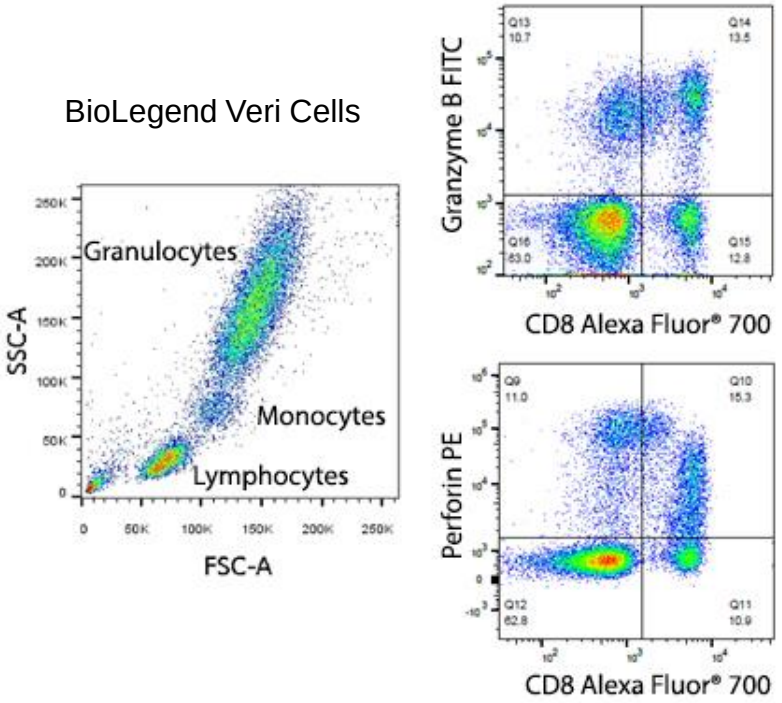
Unstained



Fluorophore	VB FMO	FITC FMO	PE FMO	APC FMO
VioBlue	-	+	+	+
FITC	+	-	+	+
PE	+	+	-	+
APC	+	+	+	-

Control types: Experimental

- depends on experiment
  - ➢ cell lines negative for markers
    - WT vs KO
- healthy vs. disease
- activated vs. non-activated (scatter properties!)
- control sample to rule out batch effects



Perfect control: Take home message

- for setup: unstained and single stained controls
- **single stainings as bright or better brighter as the real sample**
- pos./neg. population: beads go with beads, cells go with cells

- always ask: what additional controls do I need?
  - isotype → unspecific binding → blocking agents
  - FMO → gating and analysis control
  - experimental → healthy, unstimulated, batch control
- antibody producer often provides hints for QC/positive controls
- especially for spectral flow cytometry:
  - use the same treatments/buffers/fixative for controls and samples

- **no staining → search for positive cell line or stimulation**

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Thank you for your attention.

See you next month **4th April.**

Next topic:
Cell cycle analysis – it‘s considered to be simple!