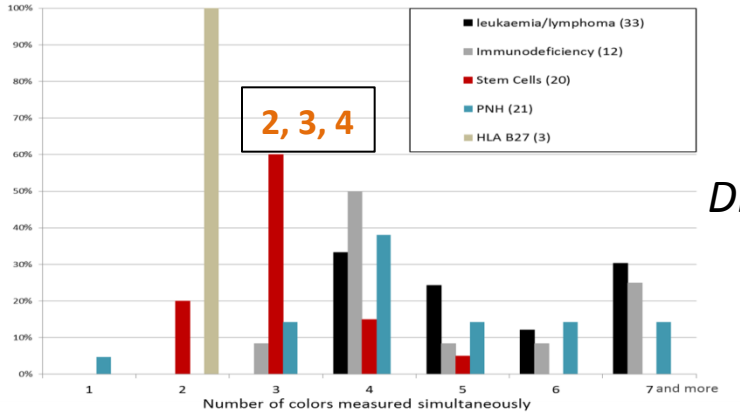
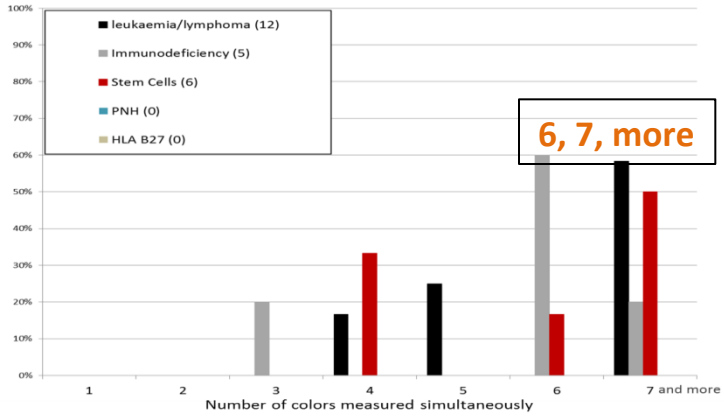

FLOW CYTOMETRY:
ANALYSIS, SAMPLE PREP,
INSTRUMENT AND REAGENTS

- Survey Results

- “Background”
  - analysis, sample prep, instrument and reagents



DIAGNOSTICS



RESEARCH

7-MARKER ANALYSIS

7-color assay

- 1x sample:
  - CD1/CD2/CD3/CD4/CD5/CD6/CD7
- 1x unstained control
- 7x singly labeled controls
- 7x FMO controls

Total **16** Tubes

2-color assay

- 6x sample:
  - CD1/CD2
  - CD1/CD3
  - CD1/CD4
  - CD1/CD5
  - CD1/CD6
  - CD1/CD7
- 1x unstained control
- 7x singly labeled controls

Total **14** Tubes

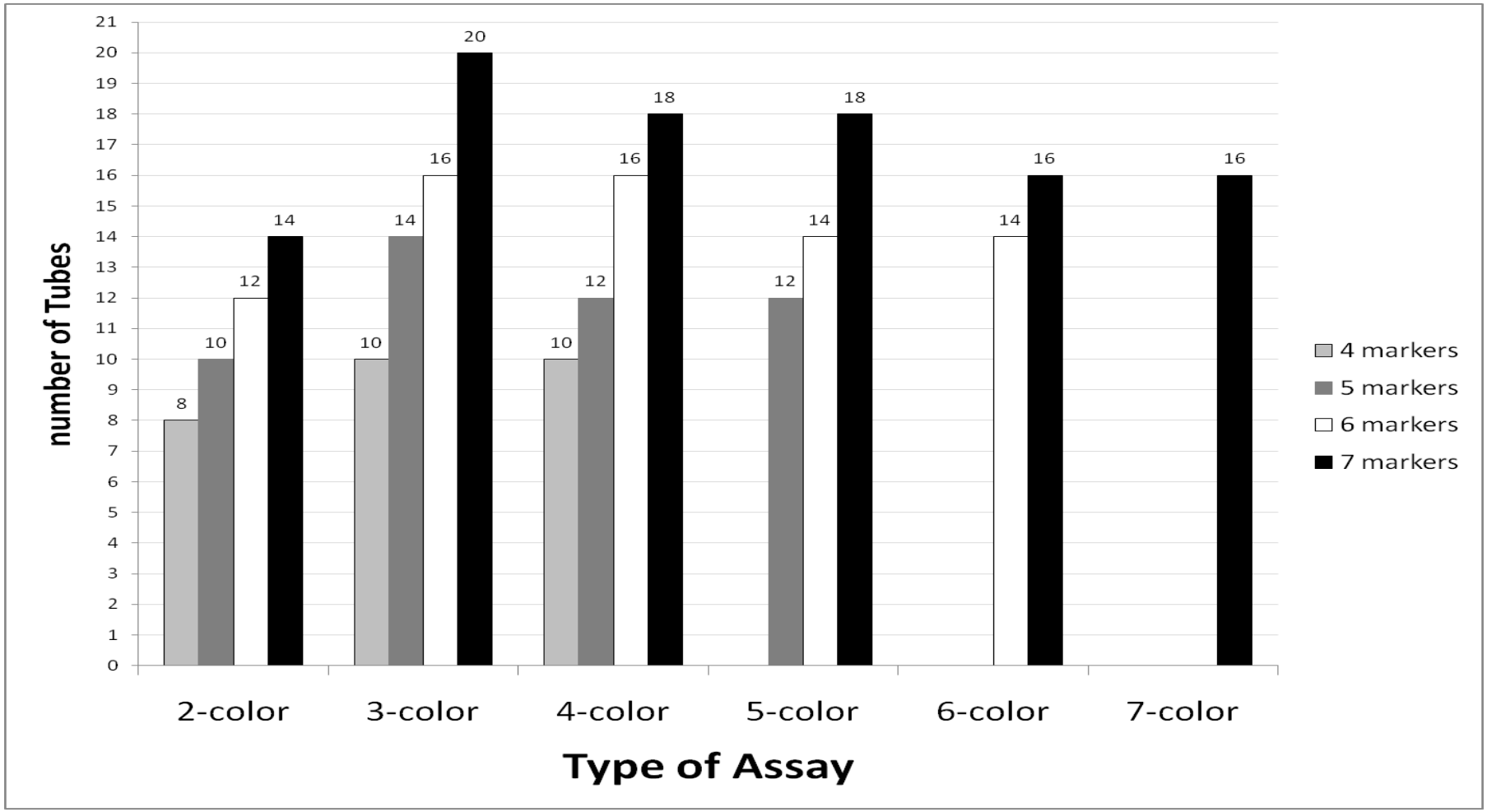
7-MARKER ANALYSIS

3-color assay

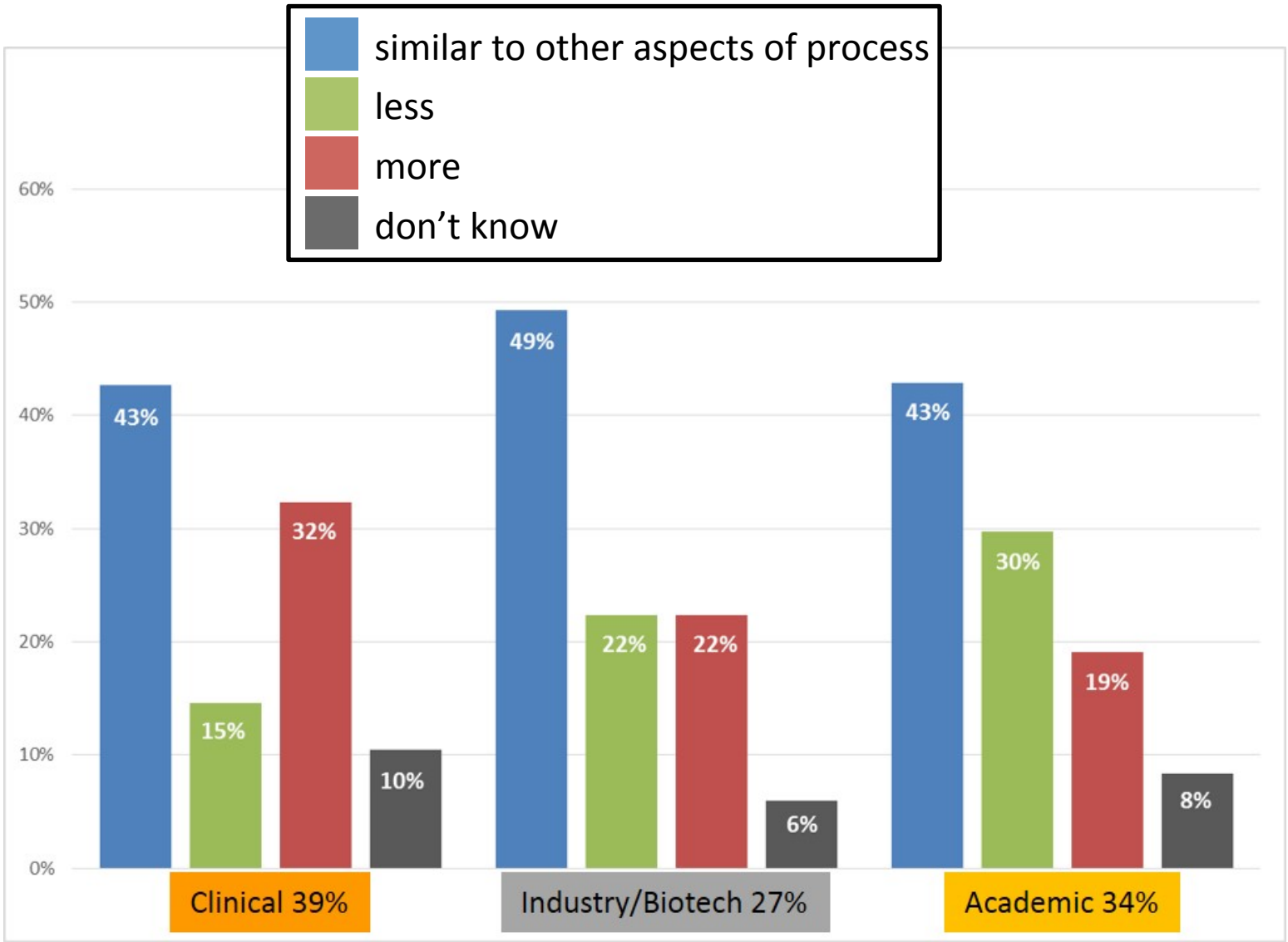
- 3x sample:
  - CD1/CD2/CD3
  - CD1/CD4/CD5
  - CD1/CD6/CD7
- 1x unstained control
- 7x singly labeled controls
- 9x FMO controls

Total **20** Tubes

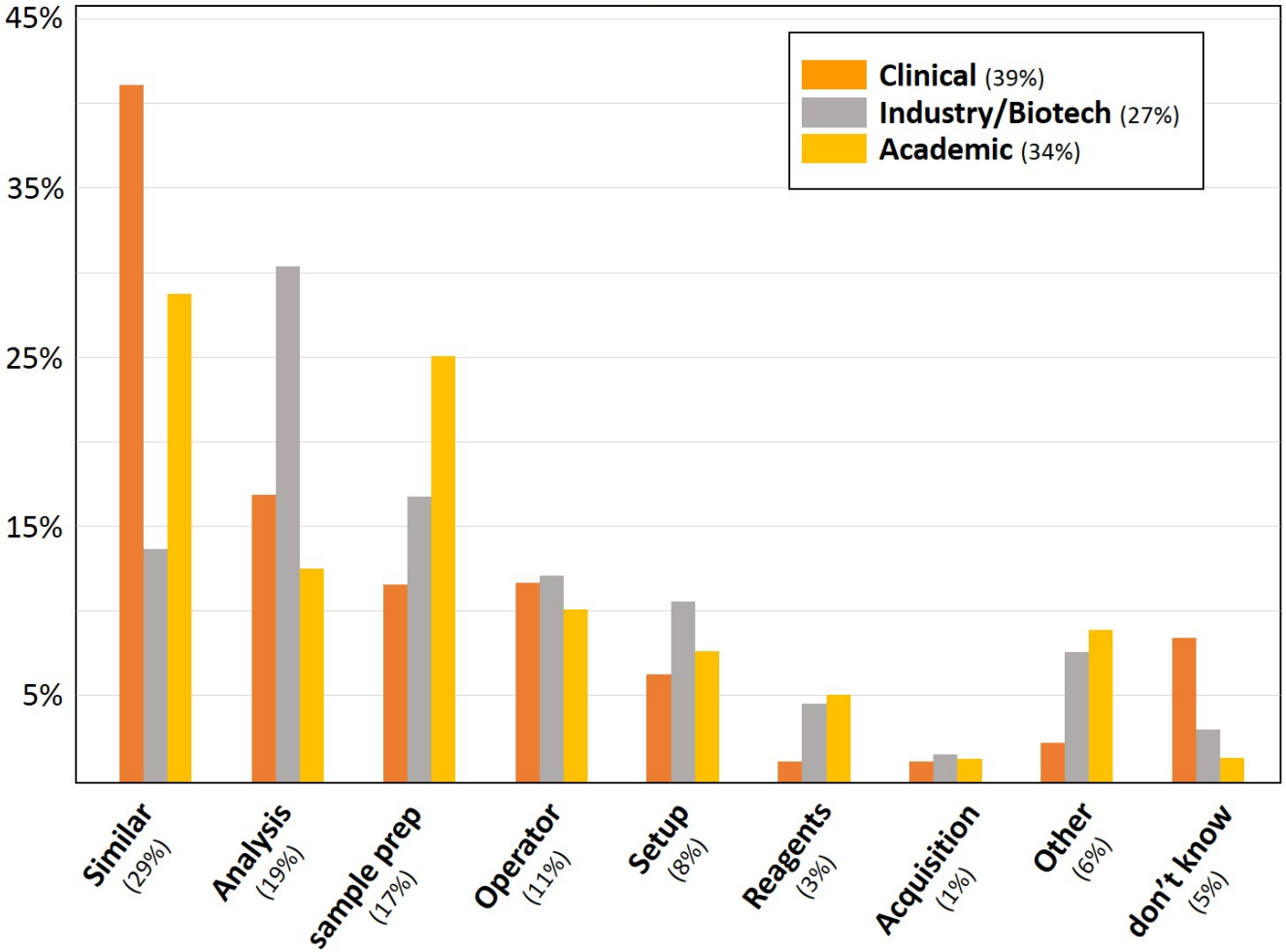
HOW MANY ‘TUBES’ IN AN ASSAY ?



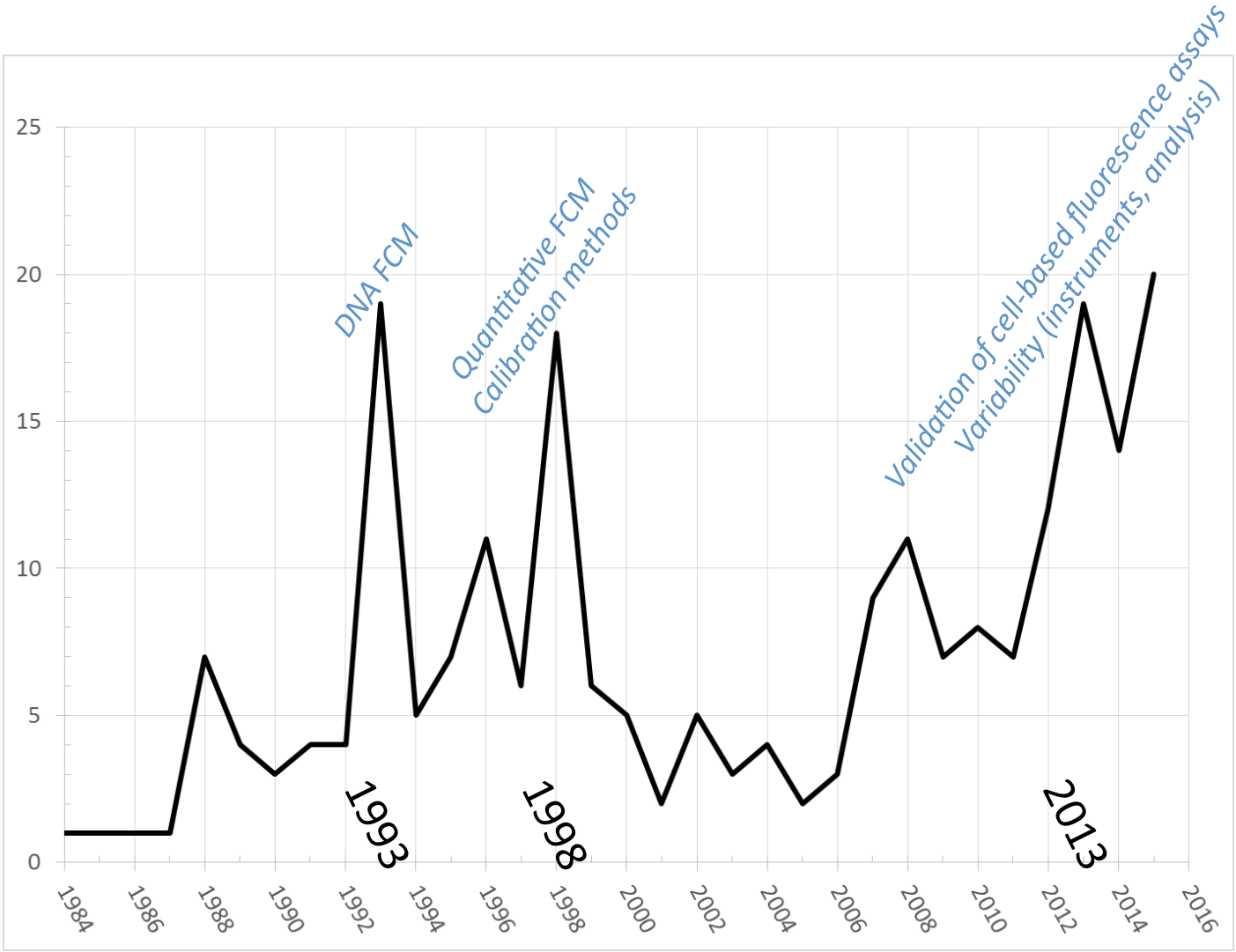
How much does flow cytometry contribute to variability in your data ?



What aspect of flow cytometry contributes most to variability?



Variability-related FCM publications



Background Fluorescence

AUTOFLUORESCENCE

- optical configuration
- excitation source
- biological conditions
  - cell type
  - cell activation/cell death
- physiological conditions
  - labeling conditions
  - sample prep conditions

SPECTRAL OVERLAP

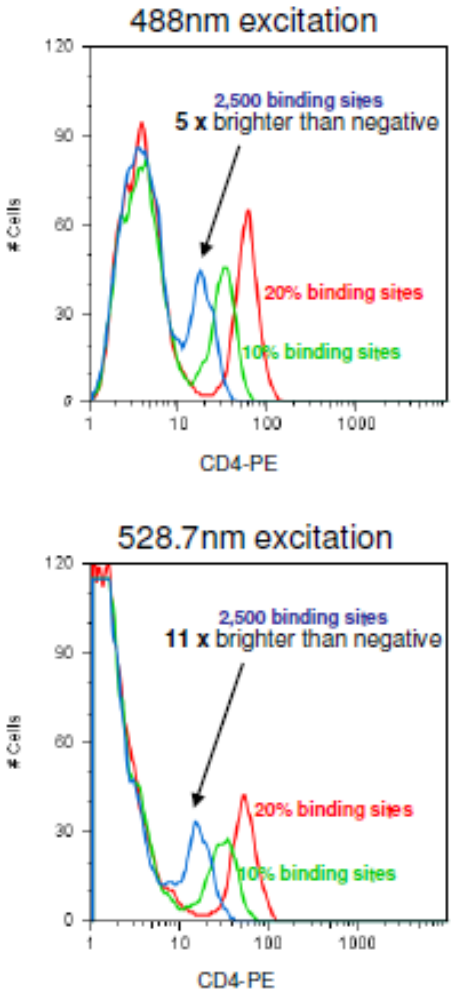
- optical configuration
- excitation source
- spectral compensation
- fluorescence intensity
  - antigen expression level
  - choice of fluorochrome

UNDESIRED Ab BINDING

- antibody specificity
  - cell type
  - cell activation/death
  - physiological conditions
  - clone/affinity
- binding through fluorochrome
  - choice of fluorochrome
- binding through Fc region
- labeling conditions
  - Ab amount
  - Ab concentration

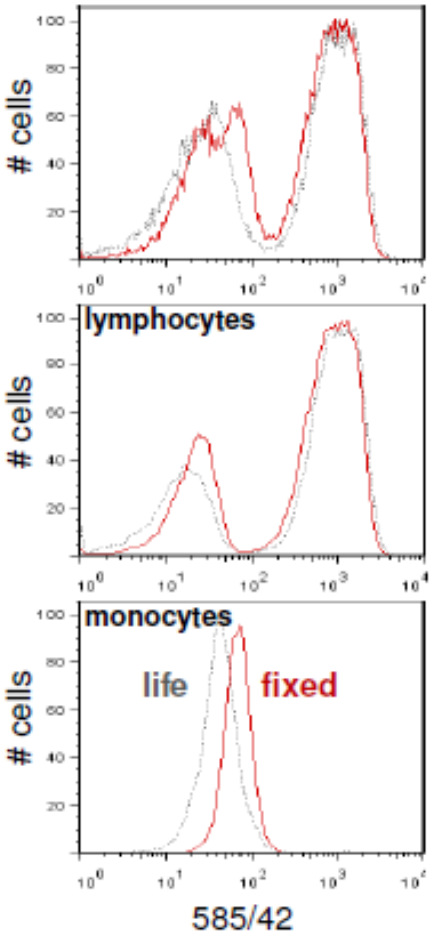
Hulspas R, O'Gorman MRG, Wood BL, Gratama, JW, Sutherland DR.
Considerations for the control of background fluorescence in clinical flow cytometry.
Cytometry Part B 2009;76B:355–364

Autofluorescence



- Different excitation wavelengths result in different levels of autofluorescence.
- Naturally occurring cellular components (Flavins, NADPH).
- Biological and physiological conditions.

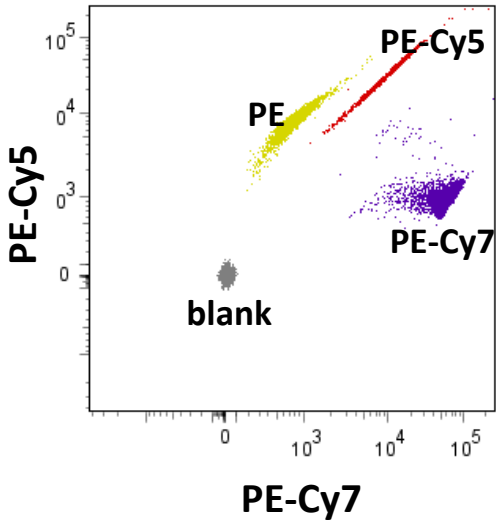
Autofluorescence Due to Sample Preparation Procedure

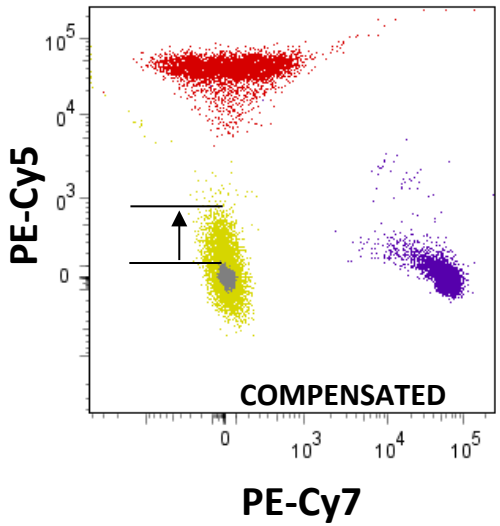


- Fixation procedure can cause increase in autofluorescence
- Increase most pronounced when measured around 530 nm
- Cell type specific

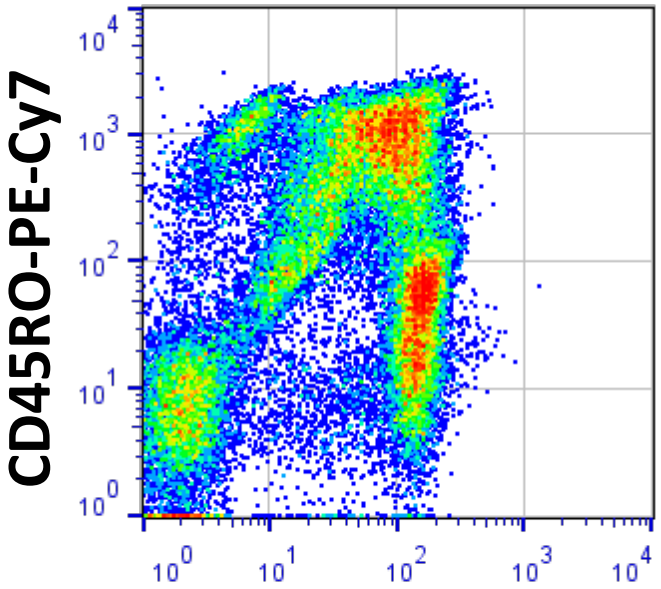
Spectral overlap

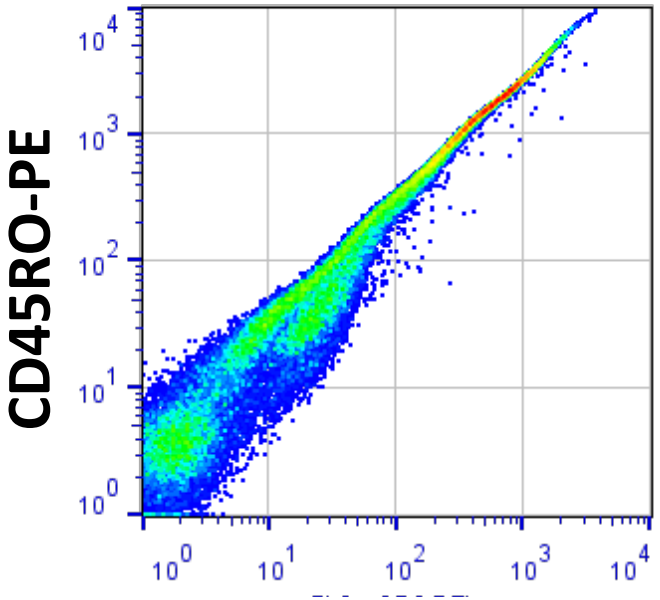
- Minimize spectral overlap
  - Spectrally separated fluorochromes
  - Narrow bandpass filter
  - Separate excitation wavelenghts for individual fluorochromes
  - Feng Shui in panel design





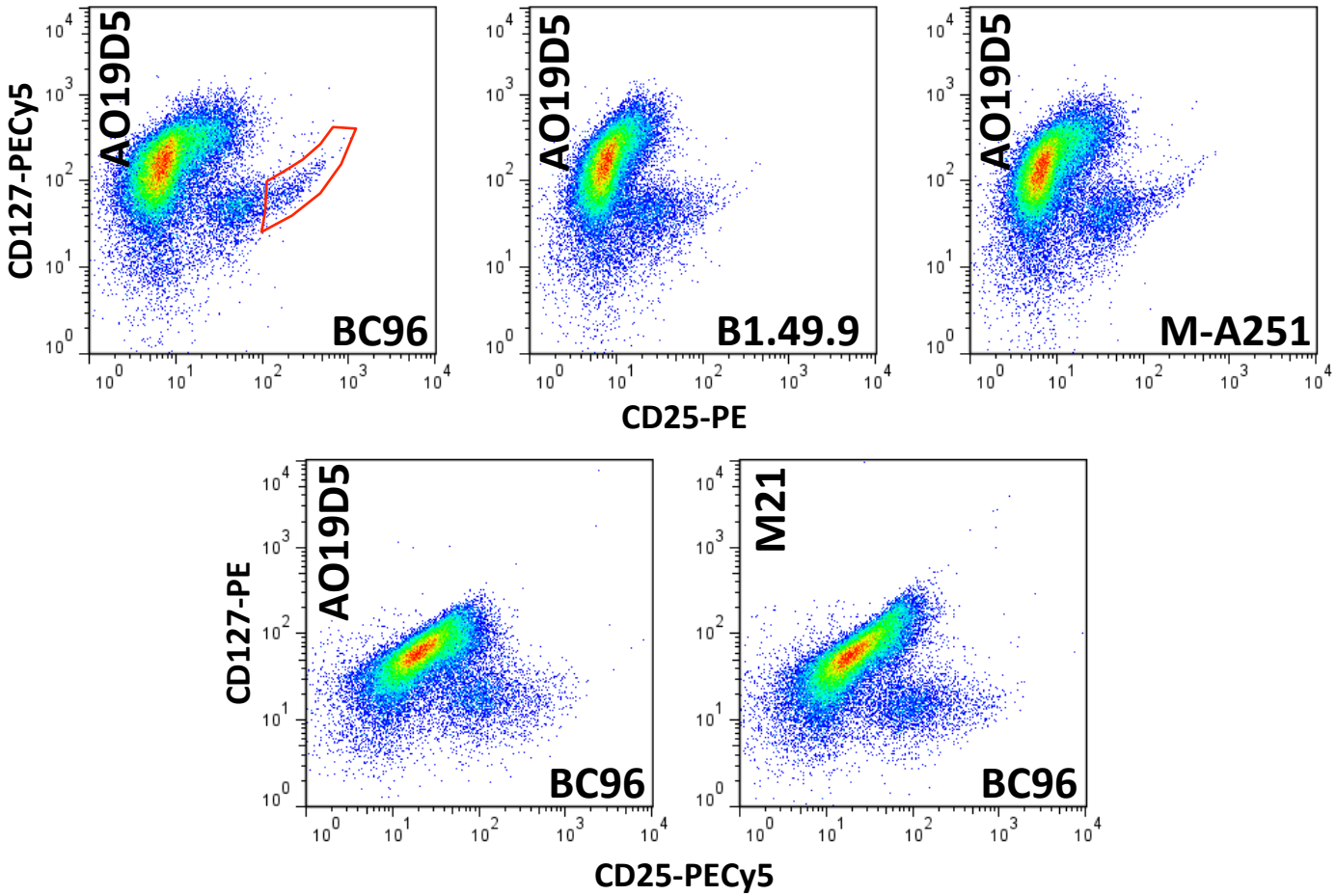
FENG SHUI



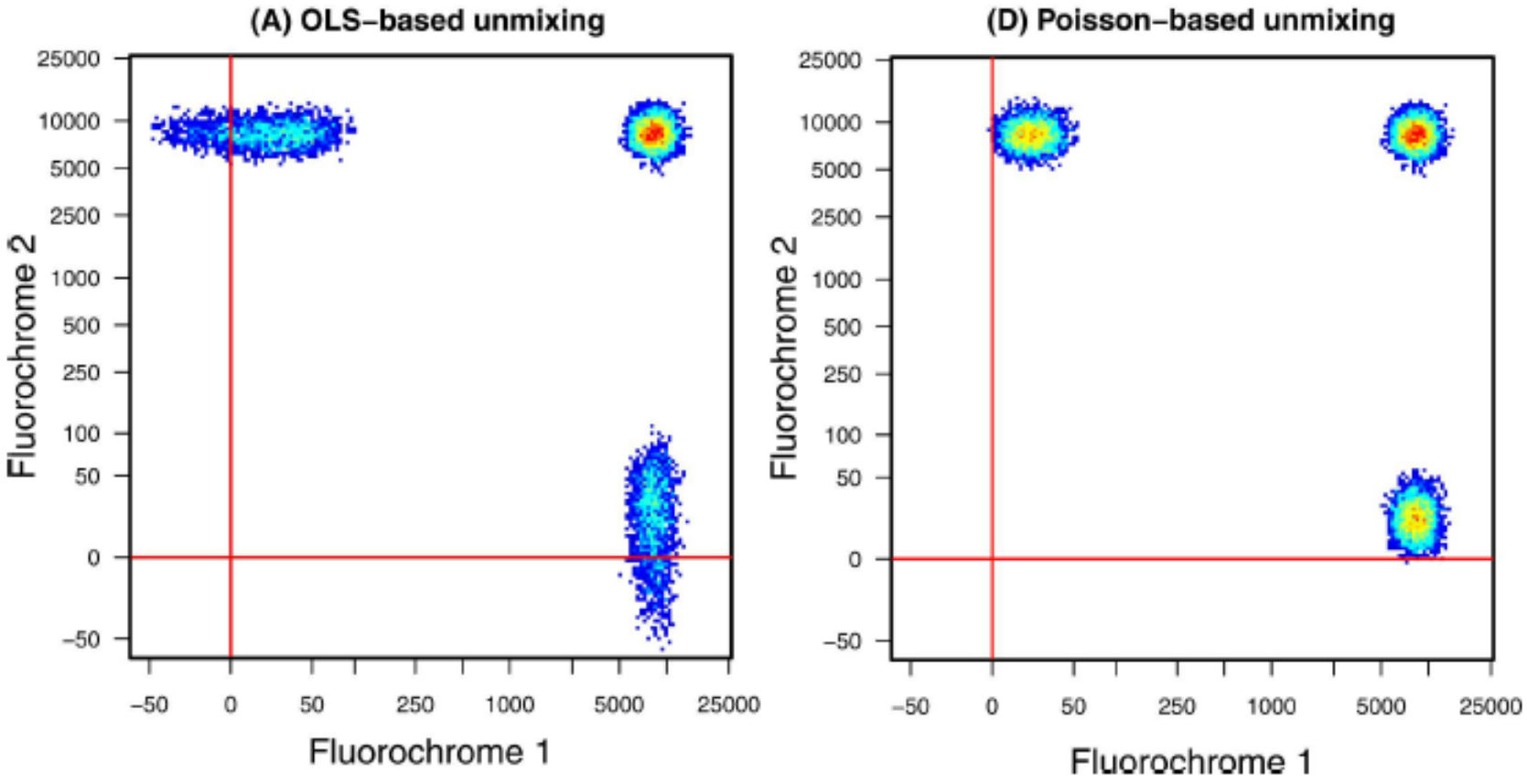


CD45RO	**PC7**	PE
CD27	PE	**PC7**
CD3	ECD	ECD
CD8	PC5	PC5

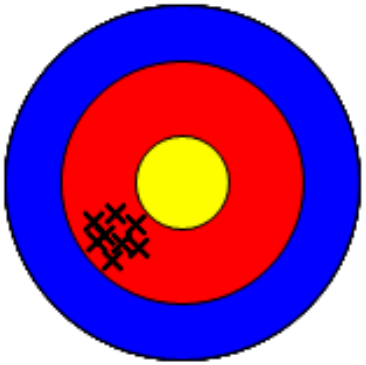
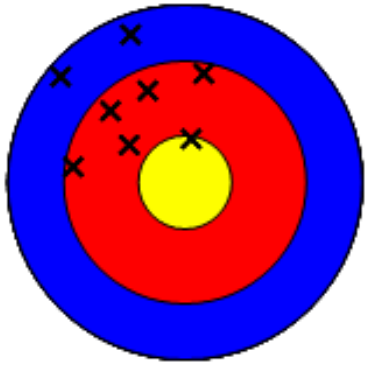
Clones and conjugates



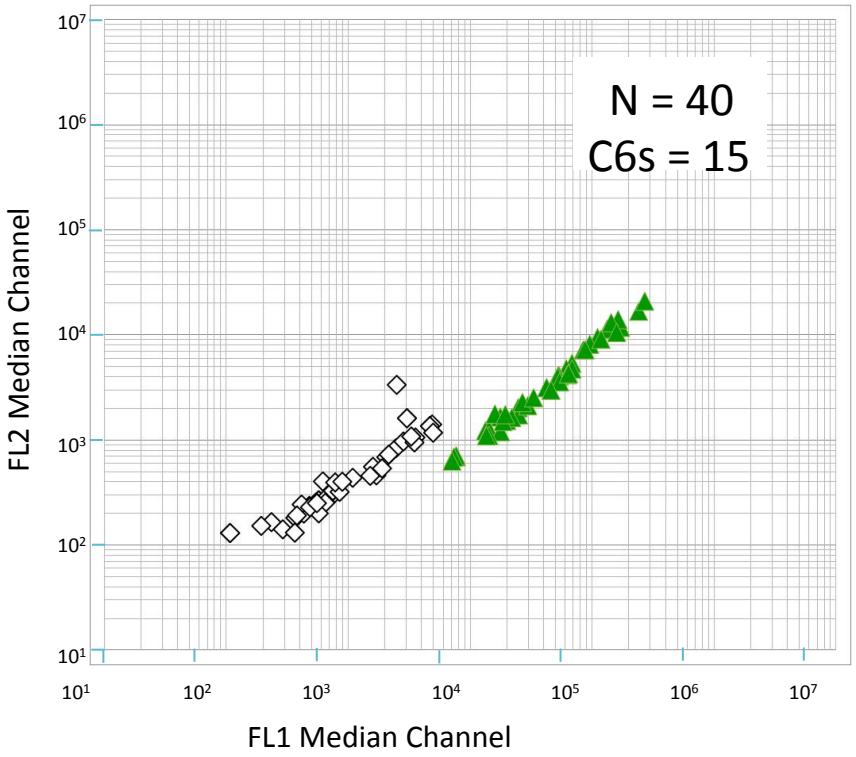
Correcting for spectral overlap



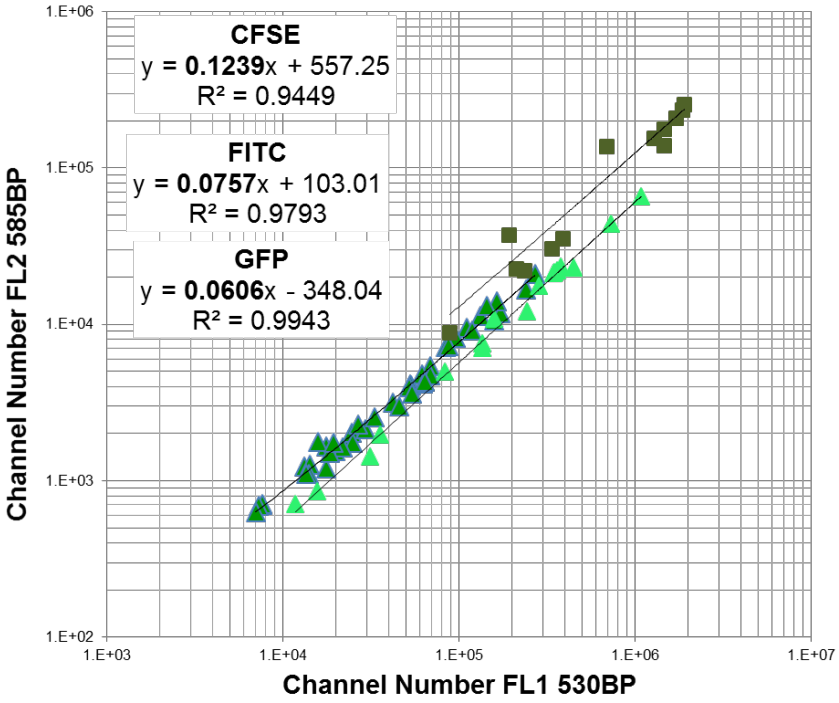
Novo D et al. Cytometry Part A, 2013;83:508–520.
Generalized Unmixing Model for Multispectral Flow Cytometry Utilizing Nonsquare Compensation Matrices.

	Accurate	Inaccurate (systematic error)
Precise		
Imprecise (reproducibility error)		

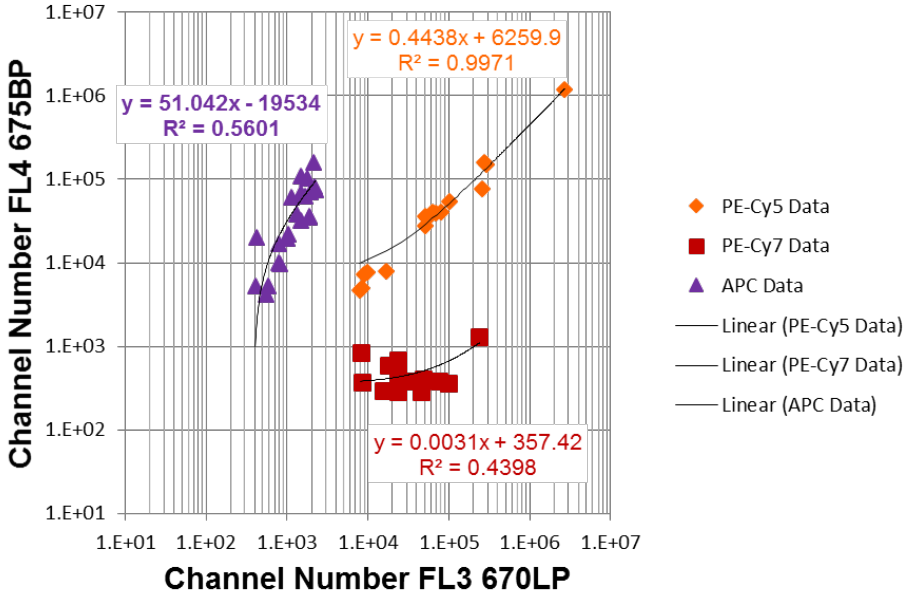
Increasing reproducibility



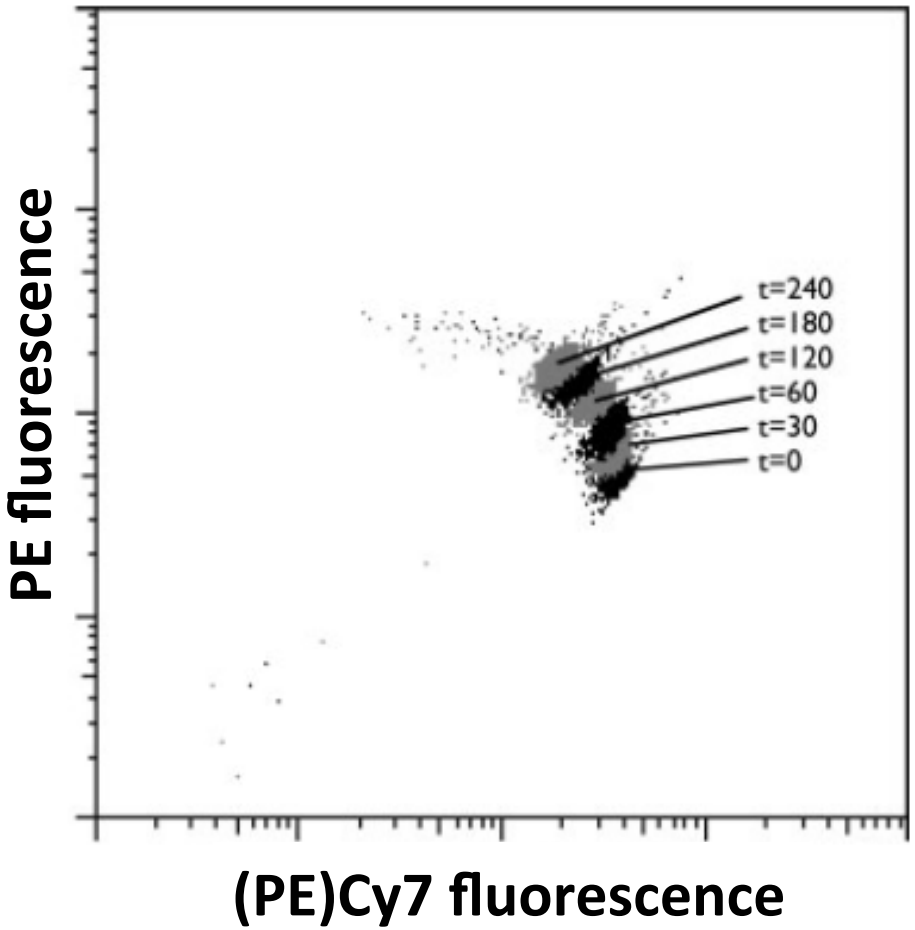
Increasing reproducibility



SpC:
CFSE = 12.4%
FITC = 7.5%
GFP = 6.0%

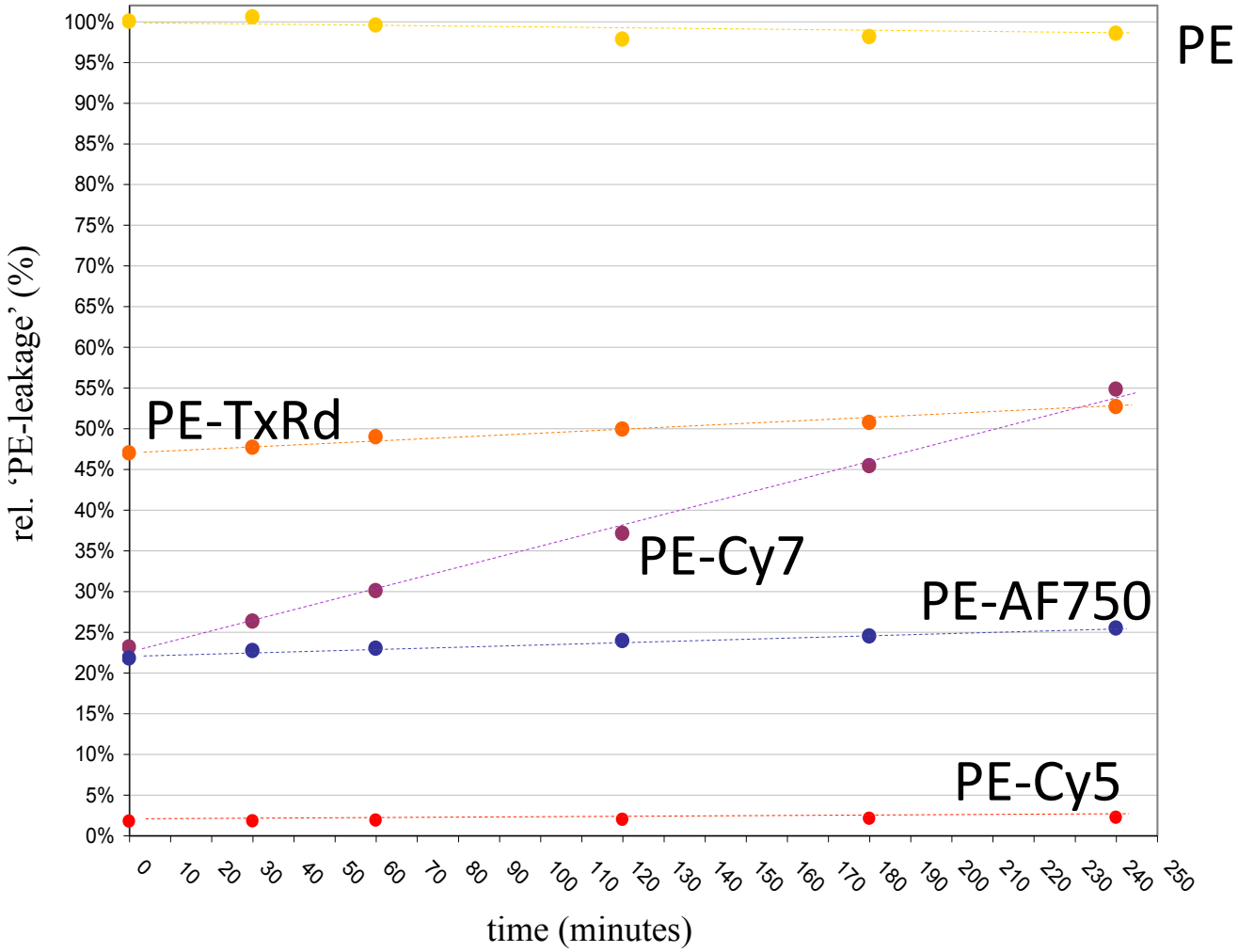


Emission leakage in tandem dyes



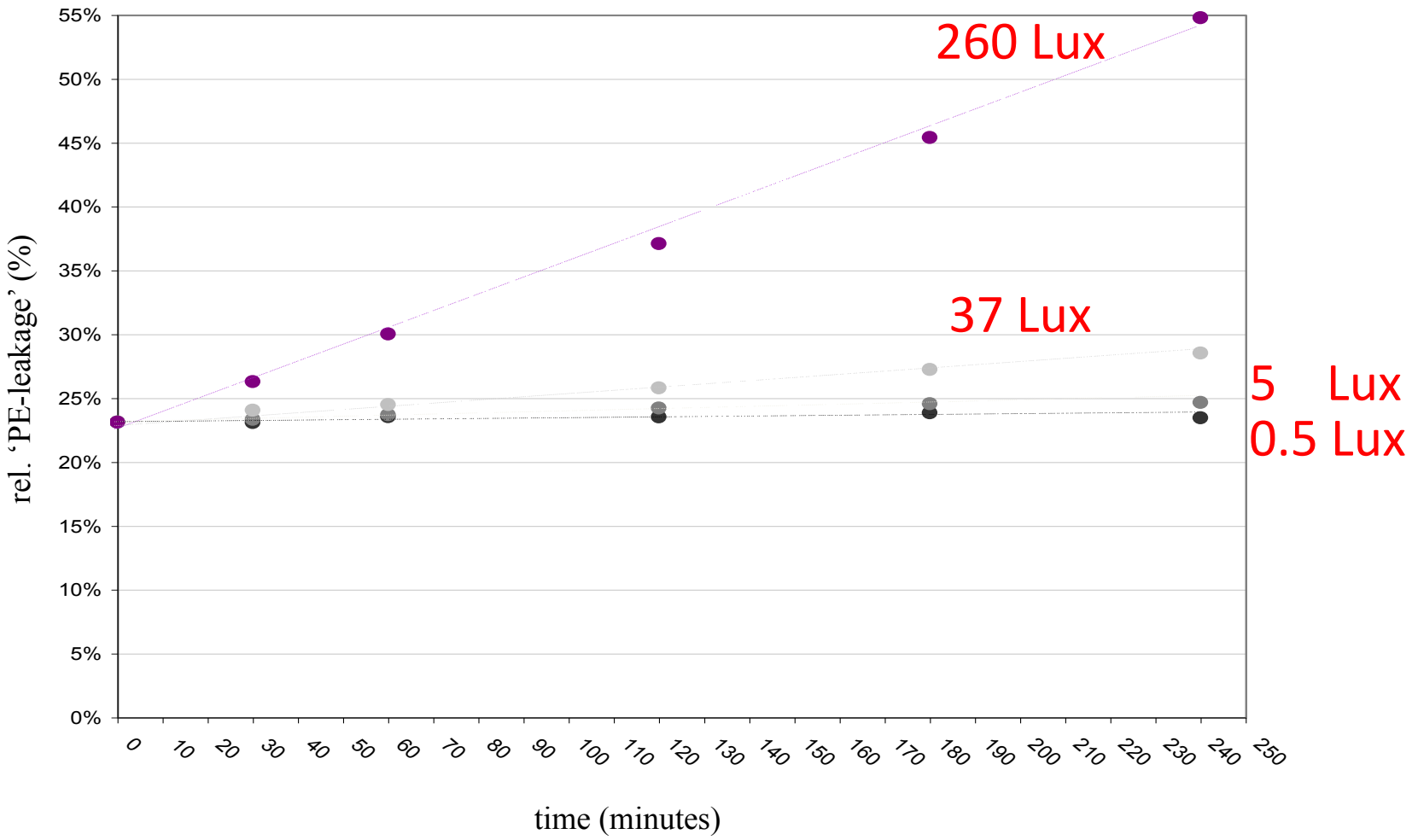
Bayer J et al. Cytometry Part B, 2007;72B:8–13
Thematic workshop on fluorescence compensation settings in multicolor flow cytometry.

PE-tandem dyes in ambient light



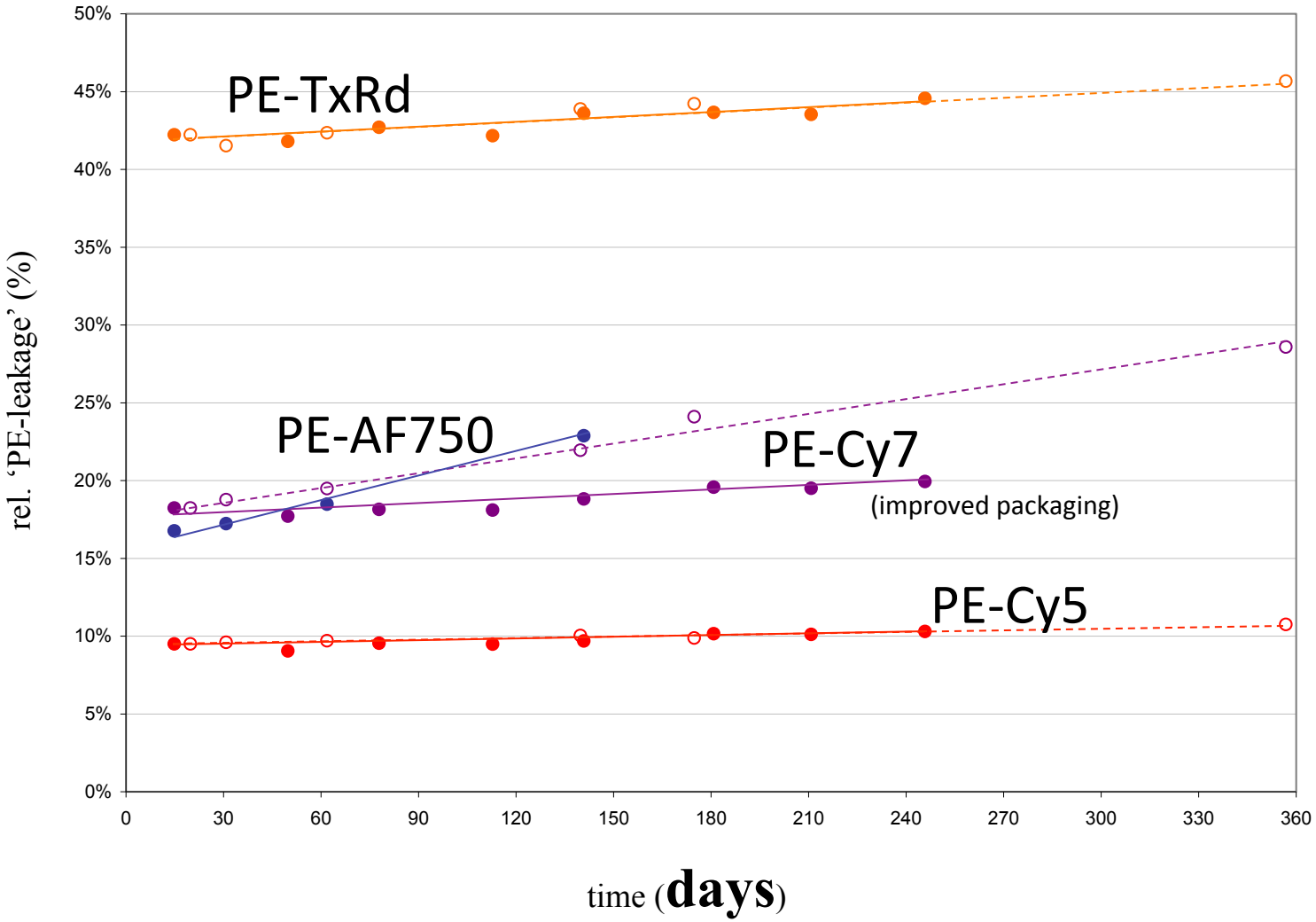
Hulspas et al. Cytometry Part A 75A: 966-972, 2009

Keep tandems in the dark



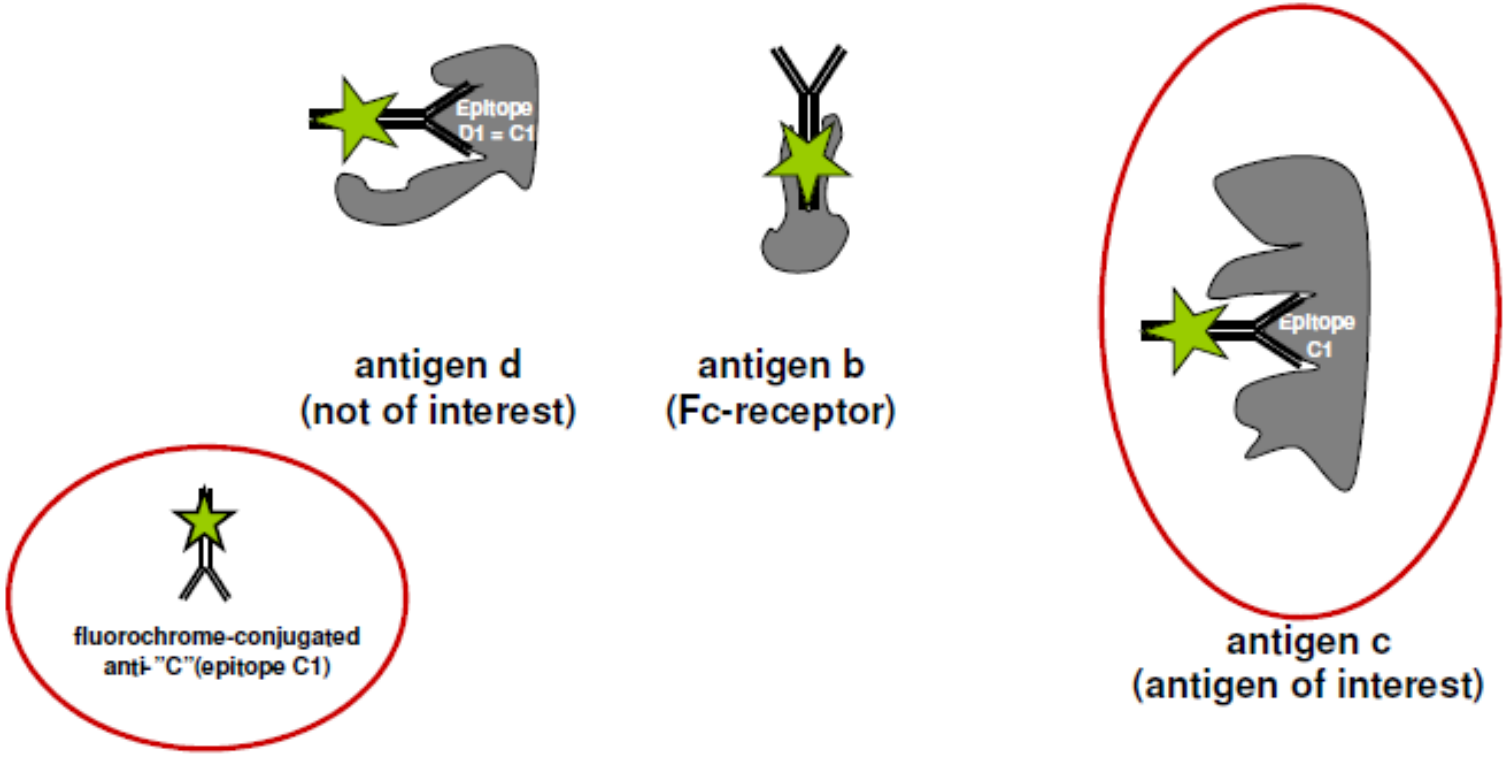
Hulspas et al. Cytometry Part A 75A: 966-972, 2009

Degradation of tandem dyes



Undesired Ab Binding

SPECIFIC BINDING



Undesired Ab Binding

NON-SPECIFIC BINDING

antigen a
(not of interest)

antigen b
(Fc-receptor)

antigen c
(antigen of interest)

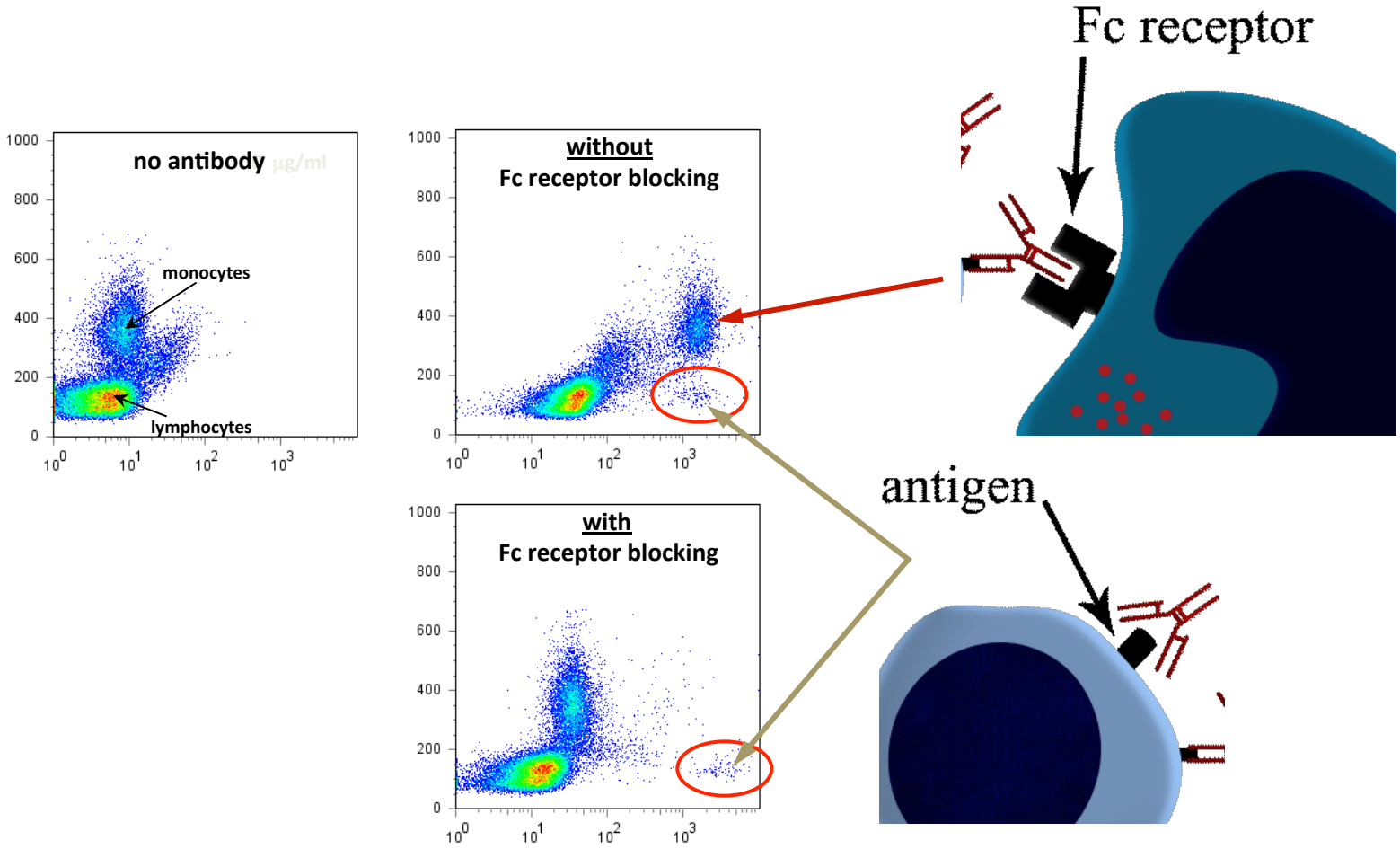
fluorochrome-conjugated
anti-"C"(epitope C1)

The IgG isotype as ligand

FcγRI	(CD64)	high
FcγRIIA	(CD32)	low
FcγRIIB1	(CD32)	low
FcγRIIB2	(CD32)	low
FcγRIIIA	(CD16a)	low
FcγRIIIB	(CD16b)	low
FcRn		

- Monocytes
- Dendritic cells
- Macrophages
- Neutrophils
- Eosinophils
- Mast cells
- Platelets
- B cells
- NK cells

DESIRED vs UNDESIRED SPECIFIC BINDING



Some vendors already include blocking reagents in product

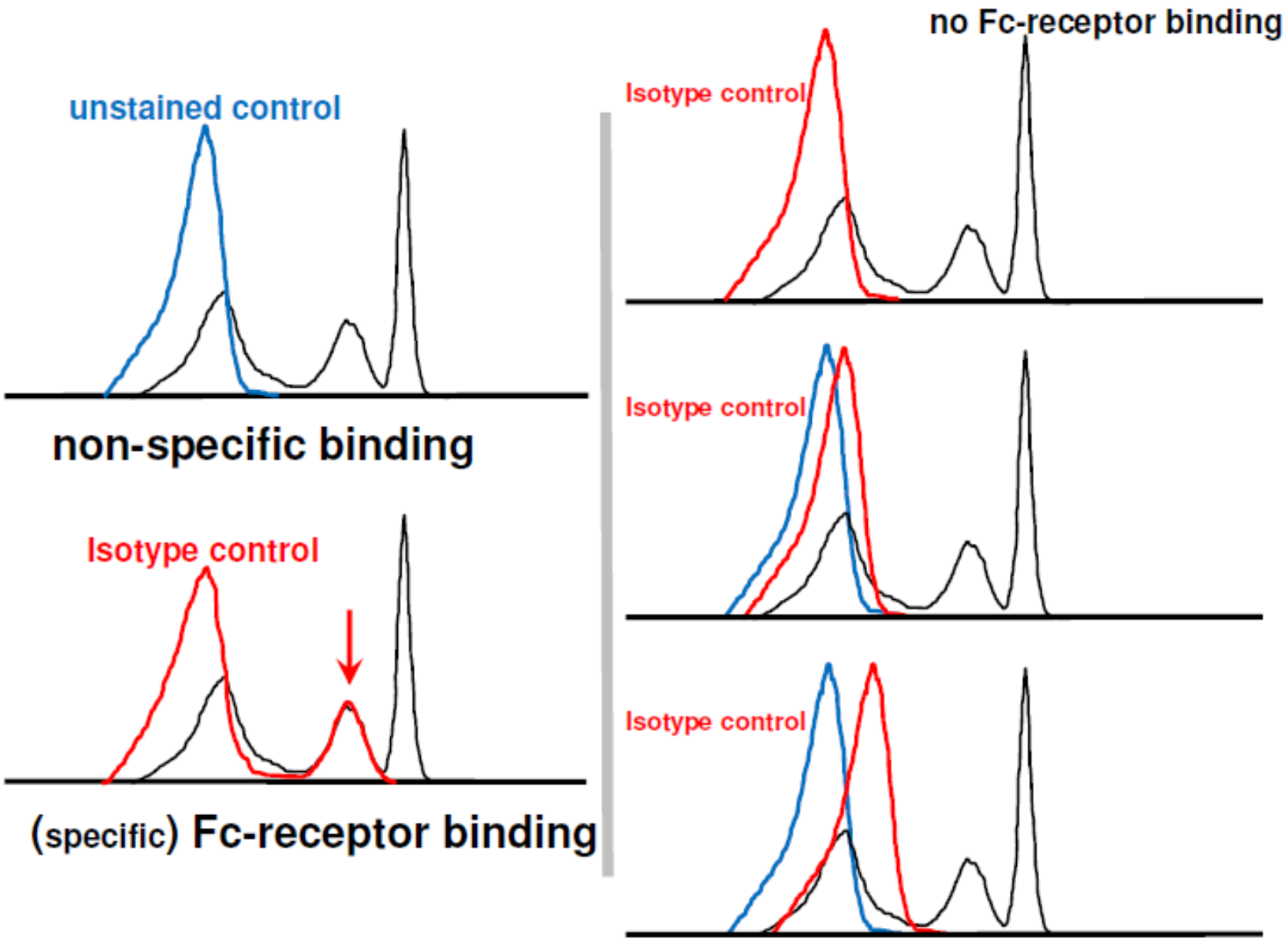
An **isotype control** is an antibody of the same **isotype** as a primary antibody with no relevant specificity to the target antigen. ~~**Isotype controls** are used as negative **controls** to help differentiate non-specific background signal from specific antibody signal~~.

An **isotype control** is another primary antibody !

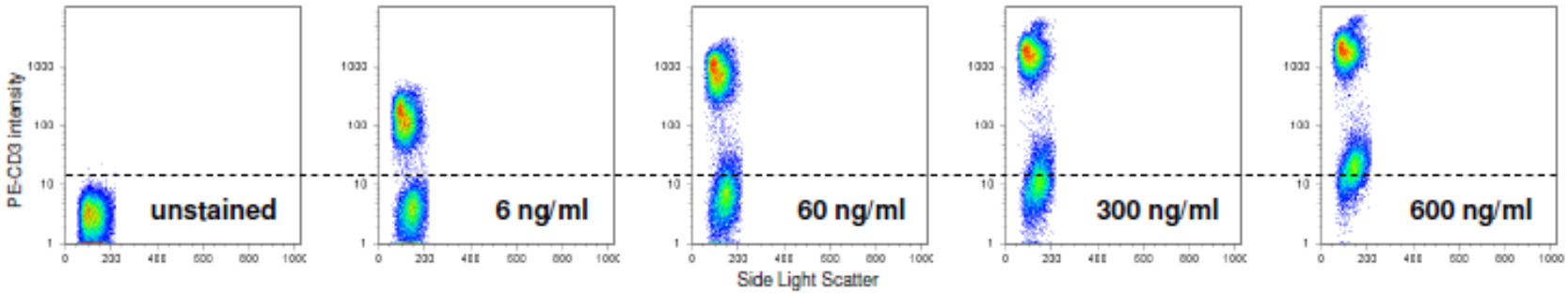
– …. raised against an epitope generally not present on the target cells

(e.g. against keyhole limpet hemocyanin)

Megathura crenulata


unstained control
non-specific binding
Isotype control
(specific) Fc-receptor binding
no Fc-receptor binding
Isotype control
Isotype control
Isotype control

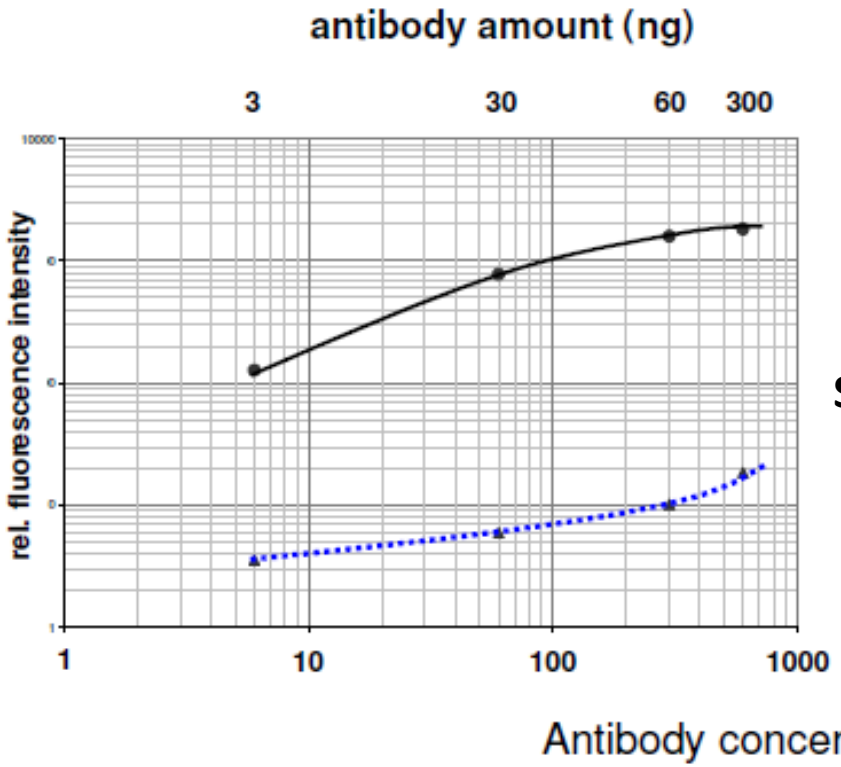
Antibody titration



- Typical manufacturer's recommendations:

  `X µL per 1E6 cells (in 0.2 mL) .`

Antibody titration



Staining Index = $\frac{\text{Mean}_{\text{positive}} - \text{Mean}_{\text{background}}}{2 \times \text{S.D.}_{\text{background}}}$

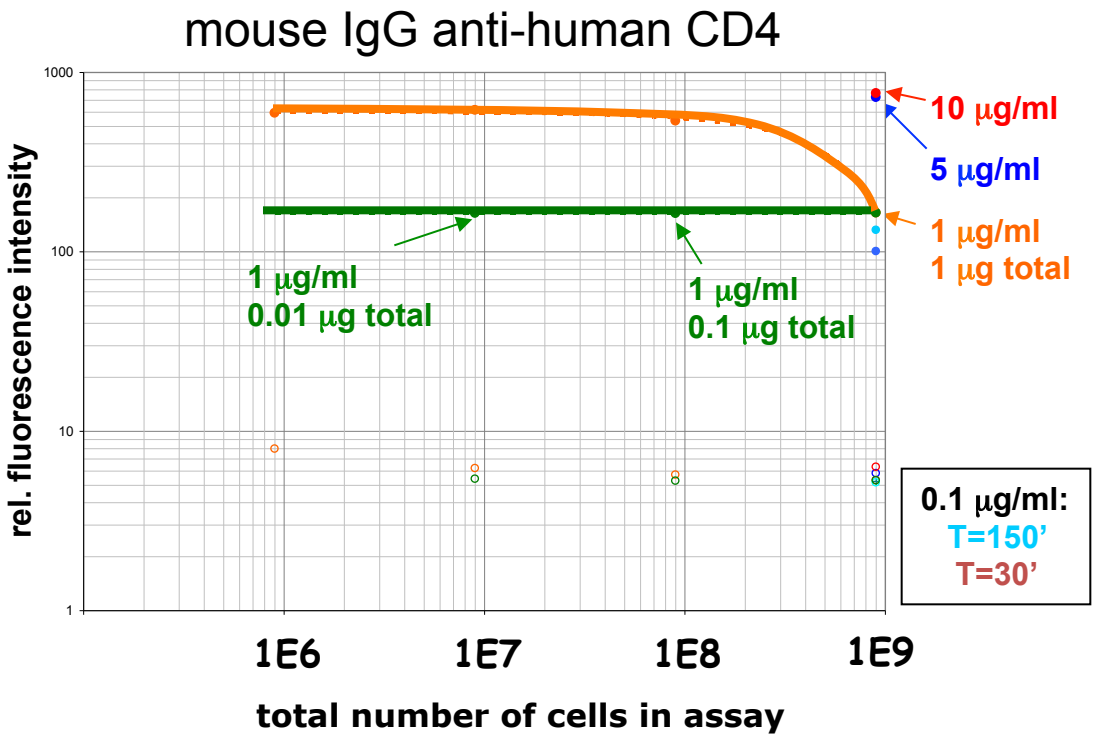
Ab concentration vs Ab amount

- 200 μg/mL
  - 198 μL sample
  - 2 μL Ab
  - **2.0 μg/mL**
  - 400 ng Ab
    - (~1.6E+12 molecules)

- 200 μg/mL
  - 19.8 μL sample
  - 0.2 μL Ab
  - **2.0 μg/mL**
  - 40 ng Ab
    - (~1.6E+11 molecules)

Note: a human T lymphocyte contains about 1.2E+05 CD3 molecules.

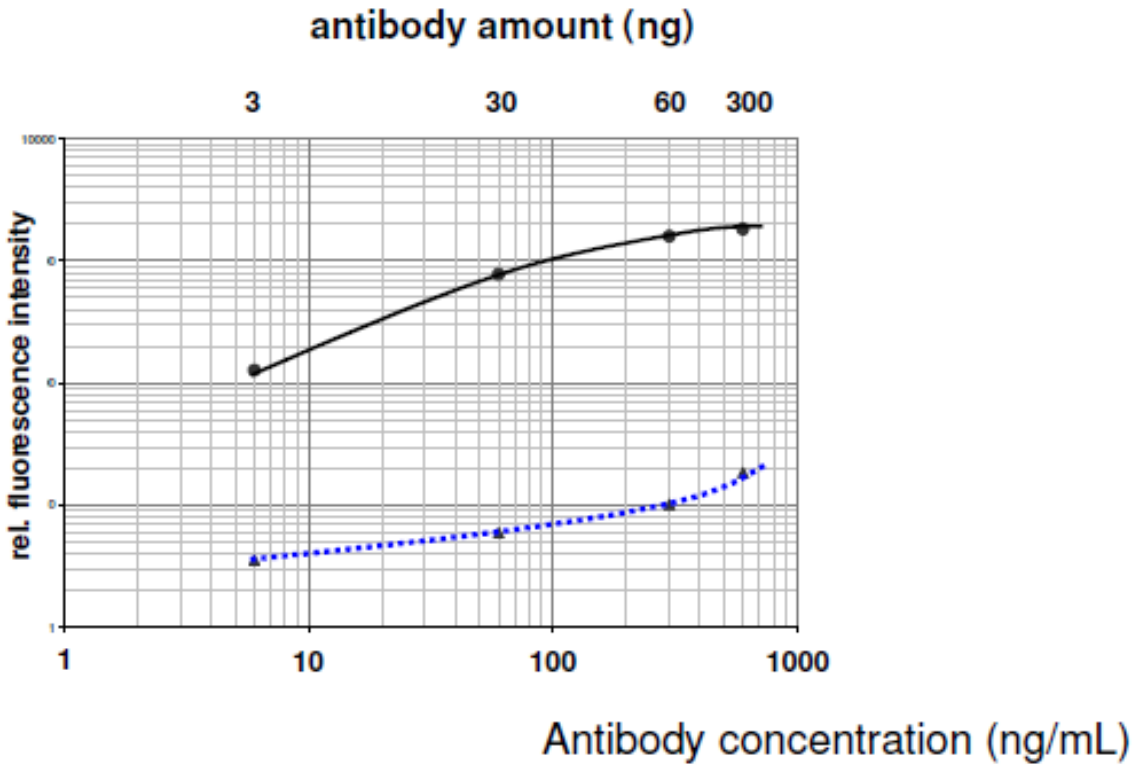
- Many more cells can be labeled with ‘standard’ amount of antibody



$$Ag + Ab \underset{k2}{\overset{k1}{\rightleftharpoons}} Ag\text{-}Ab$$

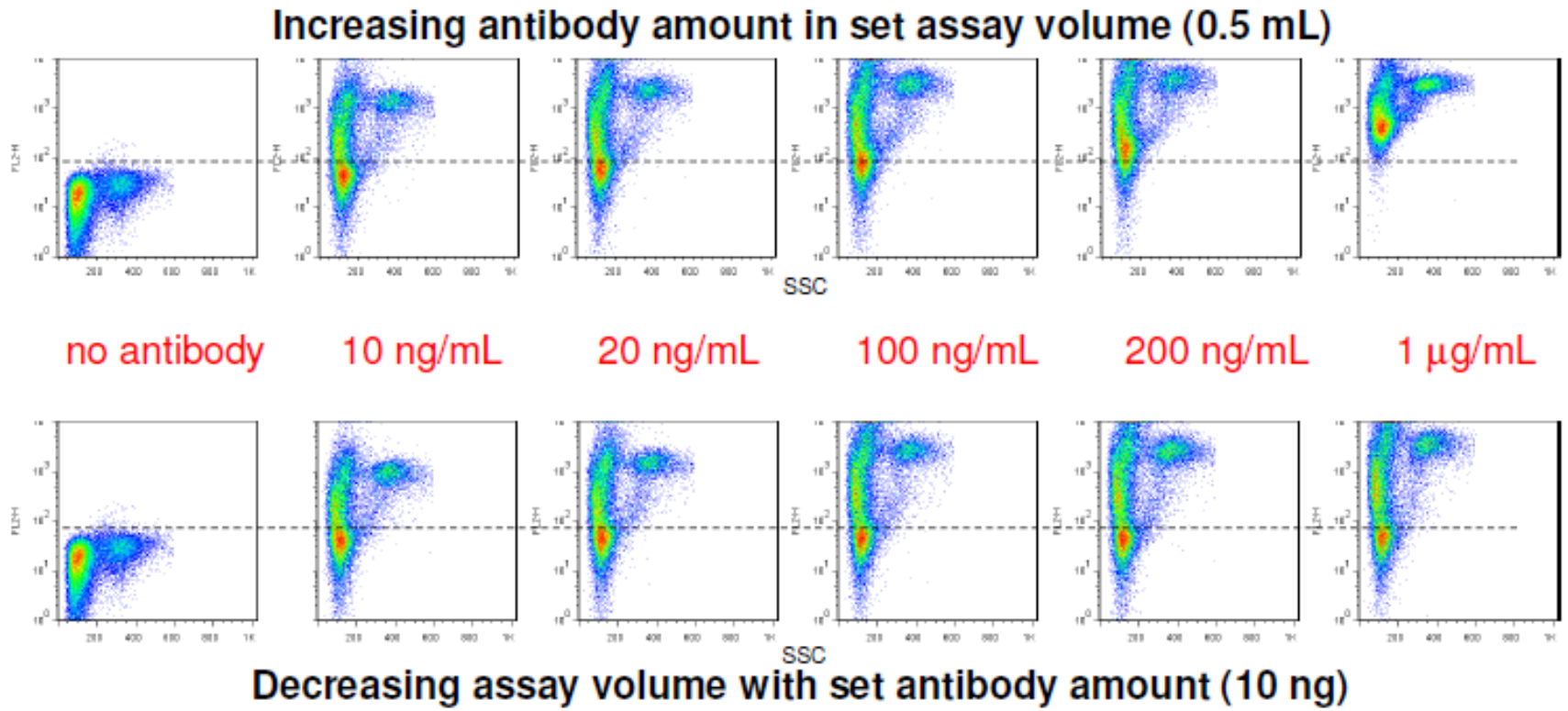
free antigen + free antibody ⇌ antigen-antibody complex

Minimizing non-Specific Binding



Hulspas R. Curr Protoc Cytom, 2010, Chapter 6: Unit 6.29
Titration of fluorochrome-conjugated antibodies for labeling cell surface markers on live cells.

Non-specific binding of low affinity antibody



Hulspas R. Curr Protoc Cytom, 2010, Chapter 6: Unit 6.29
Titration of fluorochrome-conjugated antibodies for labeling cell surface markers on live cells.

Background Fluorescence

AUTOFLUORESCENCE

- optical configuration
- excitation source
- biological conditions
  - cell type
  - cell activation/cell death
- physiological conditions
  - labeling conditions
  - sample prep conditions

- *unlabeled controls*

SPECTRAL OVERLAP

- optical configuration
- excitation source
- spectral compensation
- fluorescence intensity
  - antigen expression level
  - choice of fluorochrome

- *unlabeled controls*
- *FMO controls*
- *(single) positive controls*

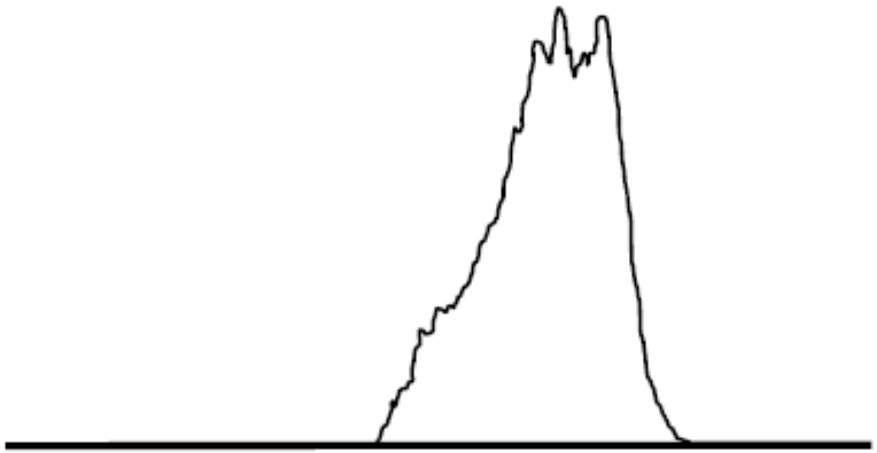
UNDESIRED Ab BINDING

- antibody specificity
  - cell type
  - cell activation/death
  - physiological conditions
  - clone/affinity
- binding through fluorochrome
  - choice of fluorochrome
- binding through Fc region
- labeling conditions
  - Ab amount
  - Ab concentration

- *unlabeled controls*
- *internal negative controls*
- *isotype controls*
- *isoclonic controls*

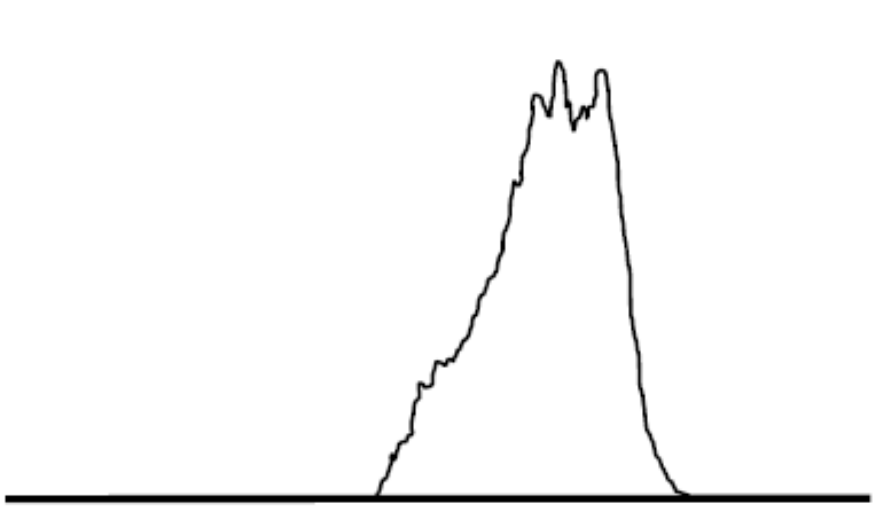
Hulspas R, O'Gorman MRG, Wood BL, Gratama, JW, Sutherland DR. Considerations for the control of background fluorescence in clinical flow cytometry. Cytometry Part B 2009;76B:355–364

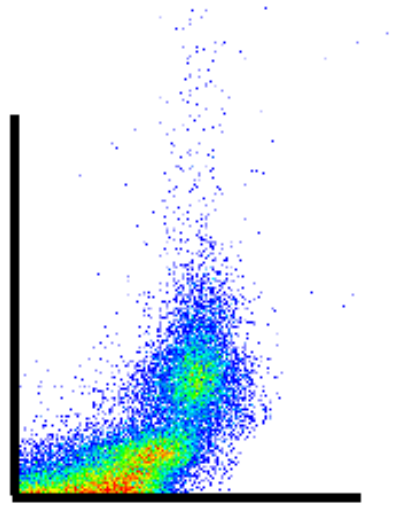
INTERPRETATION OF FLOW CYTOMETRIC DATA



- Three positive ?
- Two positive ?
- One positive ?
- No positive ?

Multi-parameter analysis





unstained human PBMCs