

# Fluorescence Compensation

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# Outline

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## 1. Compensation Basics

## 2. Practical Applications

- selecting comp controls (cells, stains?)

## 3. Multicolor Issues

- spreading due to measurement error

- Bi-Exponential scaling

- FMO controls

# Acknowledgements

*Mario Roederer* - Vaccine Research Center, NIH

*Alan Saluk* - Scripps Research Institute

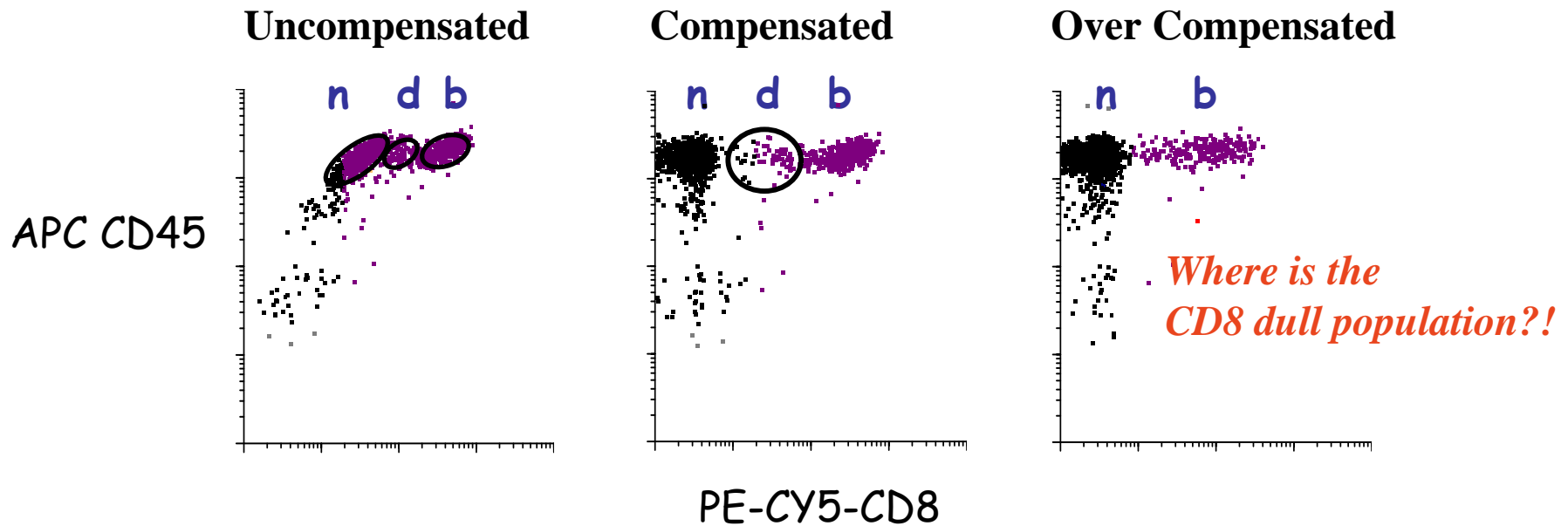
*Carleton Stewart* - Roswell Park Cancer Institute

*Sue DeMaggio* - FloCyte Training Course

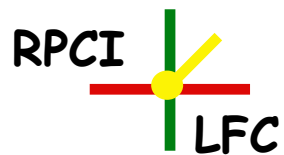
*Purdue Cytometry Resource*

# Importance of ACCURATE Compensation

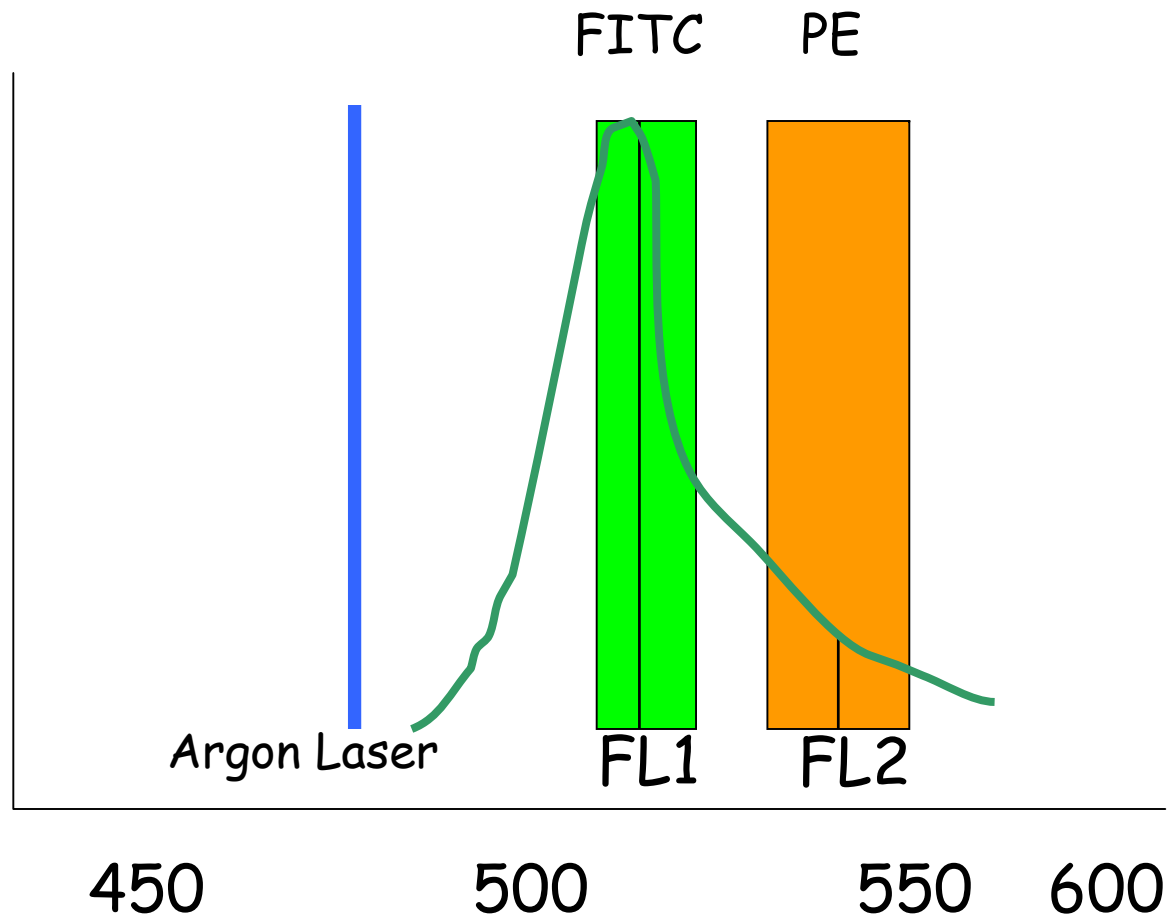
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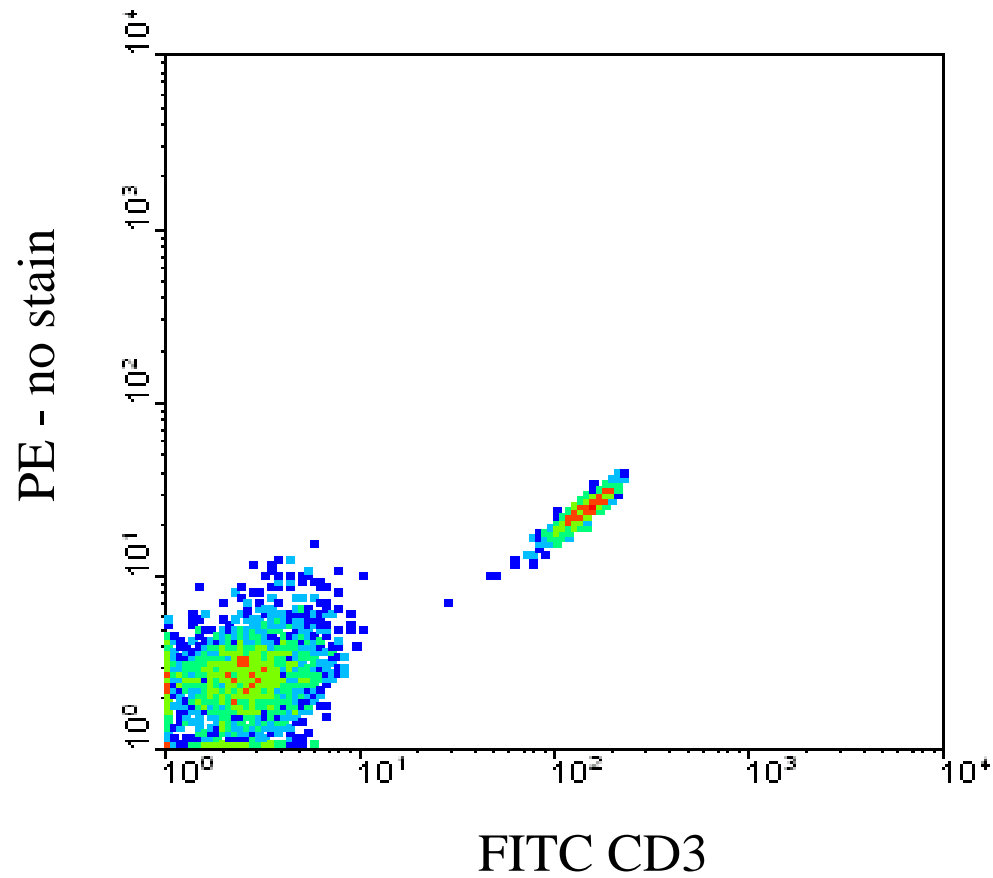
n = negatives  
d = dim positives  
b = bright positives



# FITC Single Stain Control

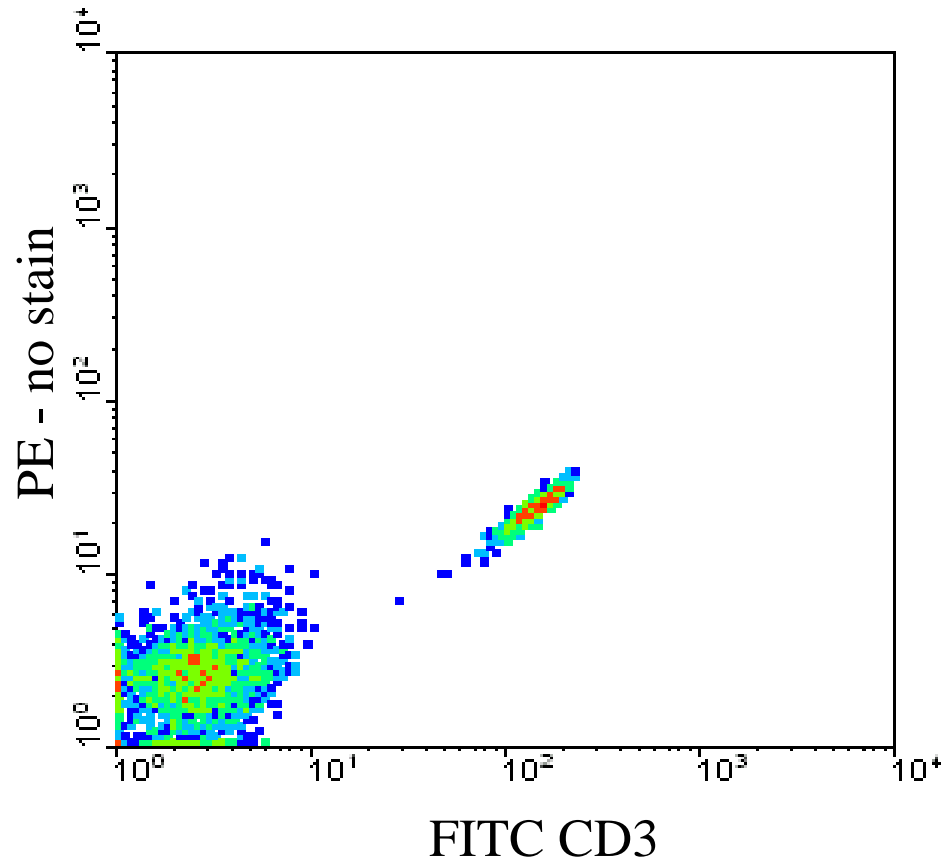


# Uncompensated FITC single stain control



# Uncompensated FITC Single stain Control

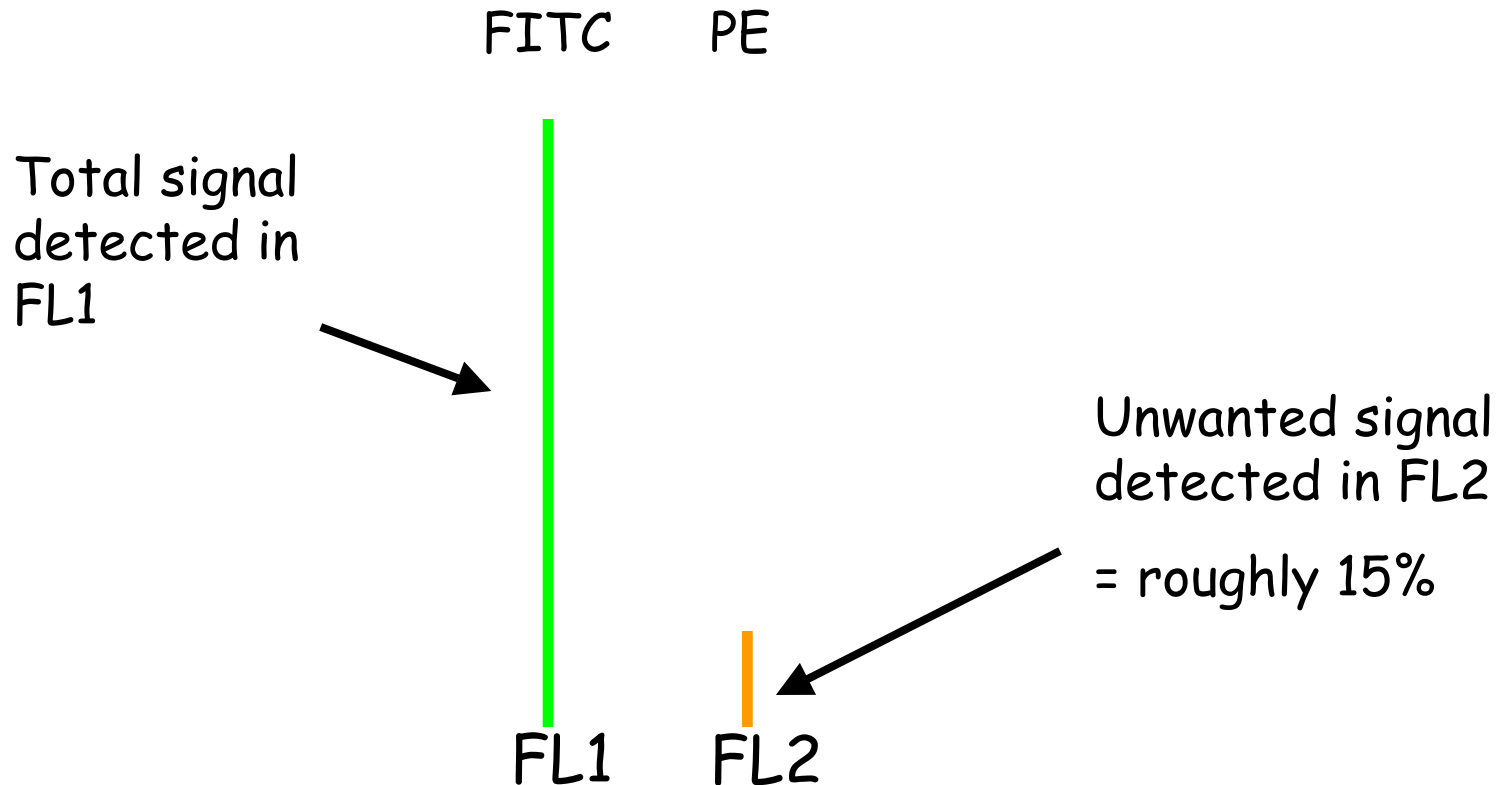
Unwanted  
signal detected  
in FL2  
roughly 15%



FITC CD3

Total signal  
detected in FL1

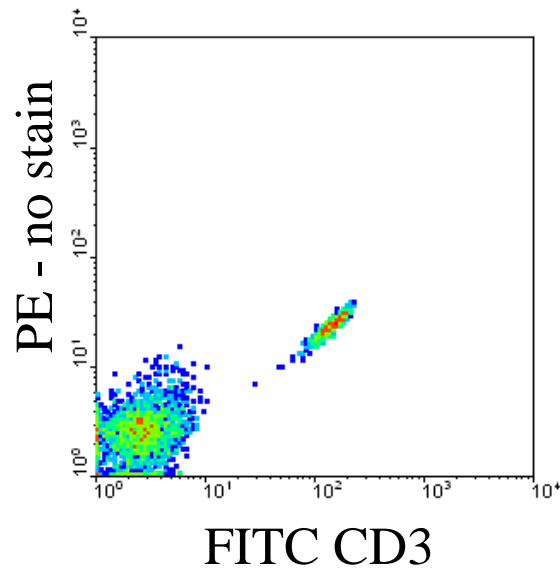
# FITC Single Stain Control



$$\text{True PE} = \text{Total FL2} - 15\% \text{ FL1}$$

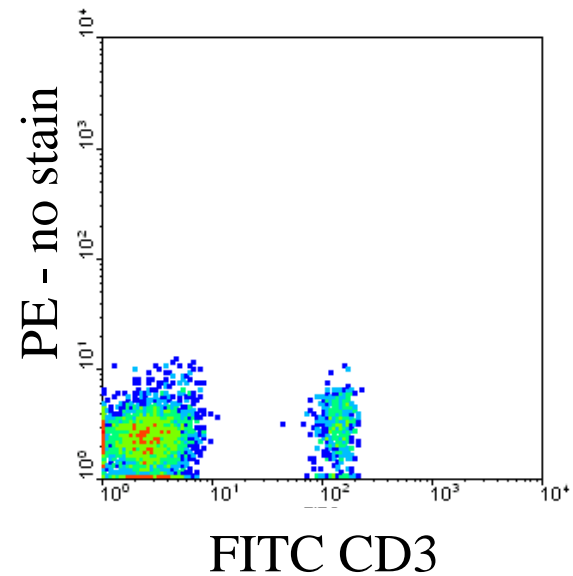
# FITC Single Stain Control

Uncompensated



FL2-15%FL1

Compensated



# **Part 2: Compensation in Practice**

## **Compensation Controls - Single Stains**

- which cells?
- which stains?

# Compensation Controls

Single Stain Controls

# Single Stain Controls - Which cells?

Does not matter as long as:

- The autofluorescence is the same in the negative and positive populations you are lining up.
  - eg, Pre-gate on lymphocytes if you are using CD8 FITC as a single stain control
- The compensation values will be valid for ALL cell types, regardless of which type of cell is used to calculate the values.
  - The compensation is specific for the fluorochrome, not the cell type

# Single Stain Controls - which reagents?

Use the same reagent (Ab-fluorochrome conjugate) as used in the experimental sample...

OR

A different antibody may be substituted, as long as it is conjugated to the same fluorochrome.

However...

# Single Stain Controls - which reagents?

Caveats for substituting reagents:

- With tandem dyes (Cy5PE/Cy7PE etc.) it is necessary to use the exact same reagent
  - spillover varies from reagent to reagent
- GFP, CFSE, and FITC are NOT the same fluorochrome
  - even though they are all green!
- Controls should be as bright as possible
  - As bright or brighter than the experimental stains

## Using Beads to Compensate

- Antibody-capture beads
- Use reagent in use
- Lots positive
- Small CV, bright
- Some reagents won't work (IgL, EMA/PI)
  - mix with regular comps

# Part 3: Multicolor Issues

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## **Spreading due to measurement error**

-why do these populations look funny?

## **Bi-Exponential scaling**

-new display options

## **FMO controls**

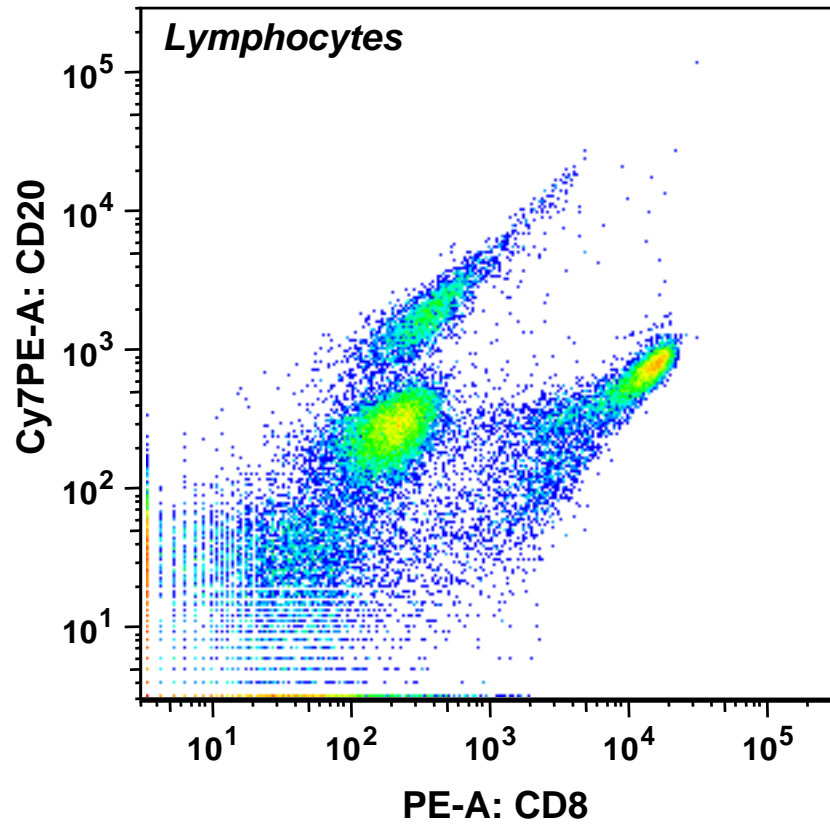
-determine where to set your gates

# Spreading due to Measurement Error

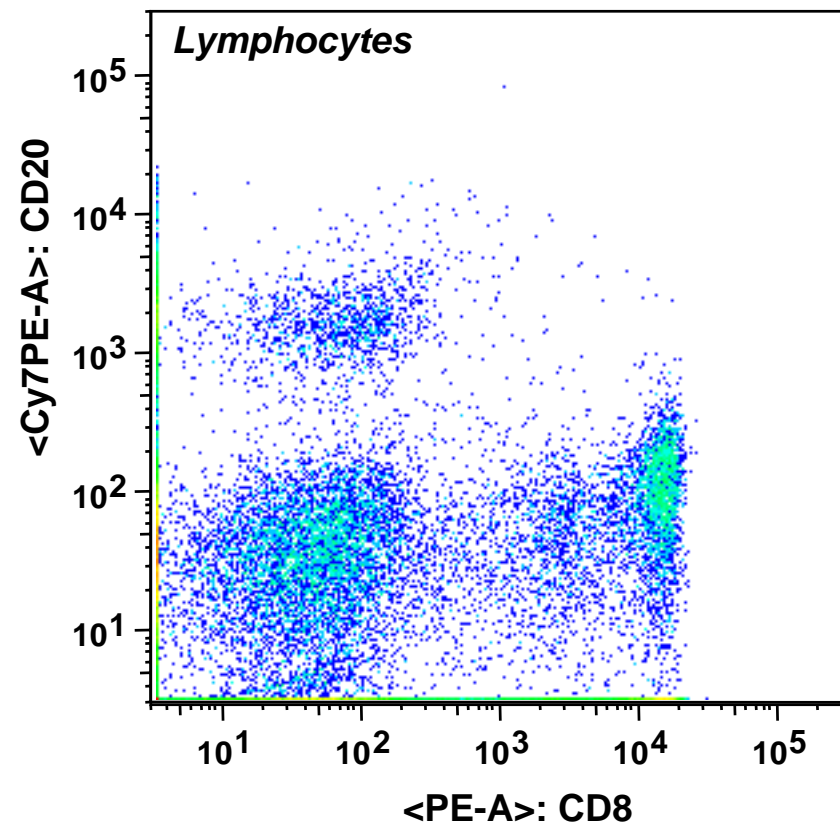
Why do these populations look funny?

# Multicolor Compensation

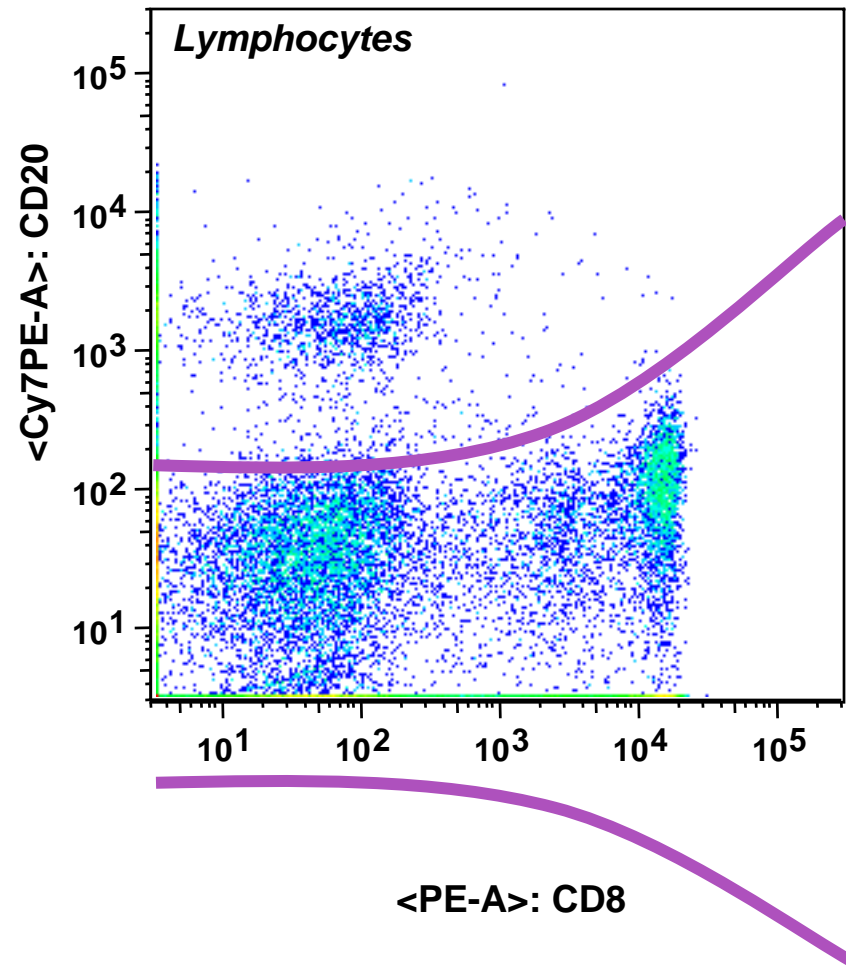
Uncompensated



Compensated



# The Actual Spread

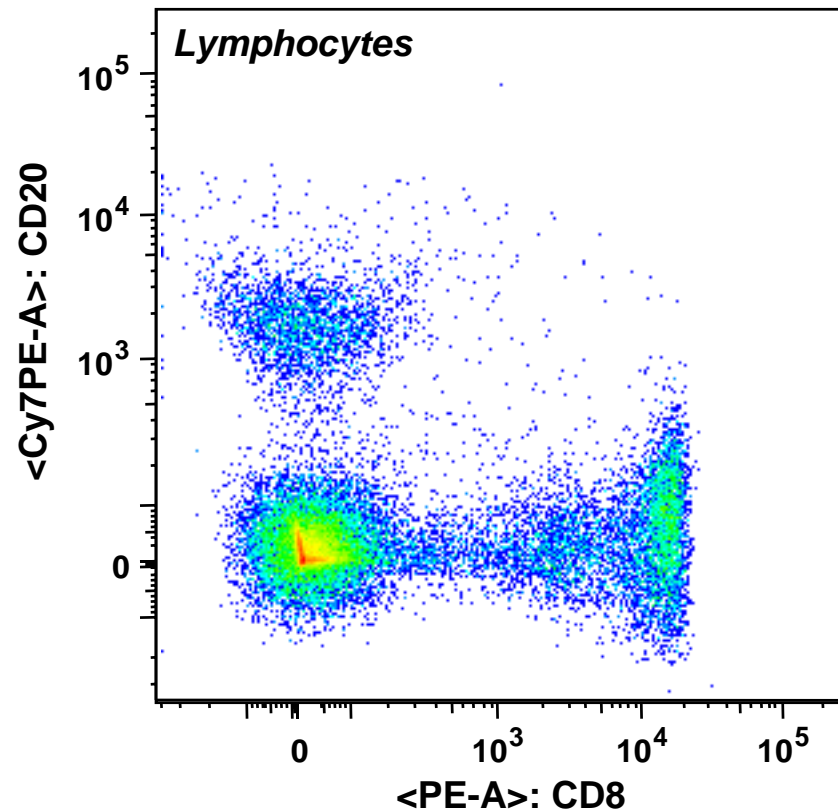


# **Bi-Exponential Scaling**

New Display Options

# Bi-Exponential Scaling

Compensated with New Display



# Gating controls

FMO

(Fluorescence Minus One)

# Gating Controls

- Unstained cells or complete isotype control stains are *improper* controls for determining positive vs. negative expression in multi-color experiments.
- The best control is to stain cells with all reagents *except* the one of interest.

## FMO Control

“Fluorescence Minus One”

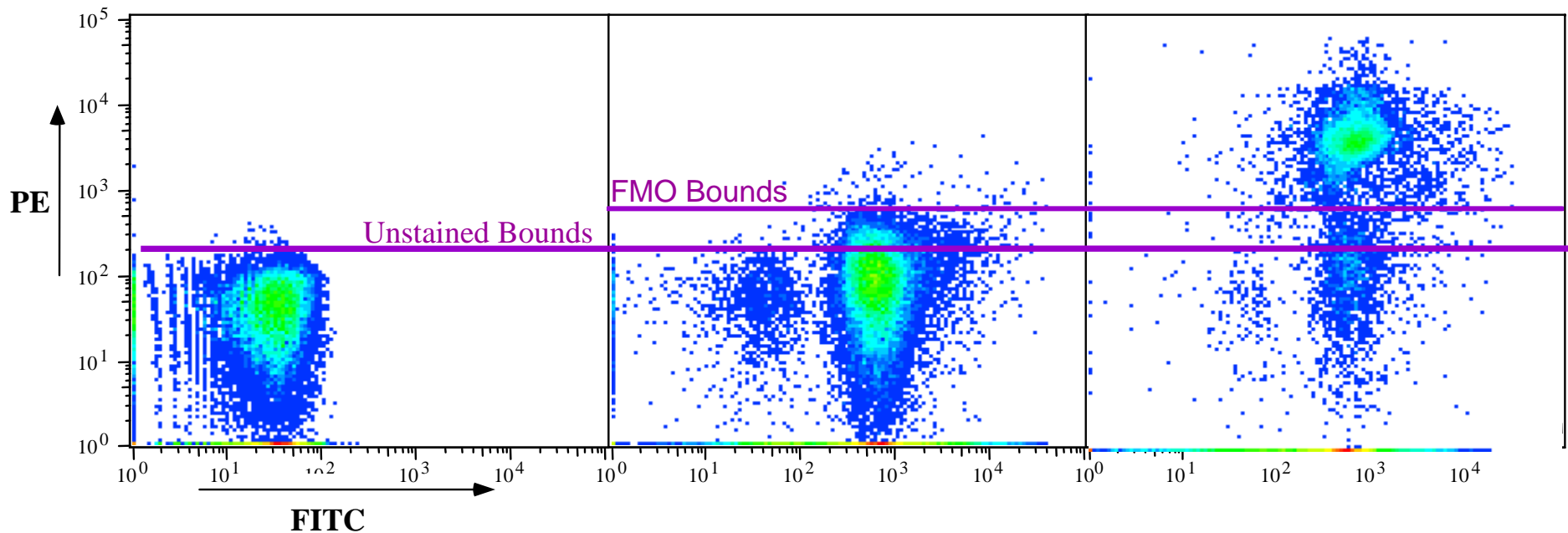
# FMO Controls

- FMO controls are necessary to identify cells which do or do not express a given antigen.
- FMO controls should be used whenever accurate discrimination is essential or when antigen expression is relatively low

# Identifying CD4 cells with 3 colors

- PBMC were stained as shown in a 3-color experiment.
- Compensation was properly set for all spillovers

|              | Unstained Control | FMO Control | Fully Stained |
|--------------|-------------------|-------------|---------------|
| <b>FITC</b>  | –                 | <b>CD3</b>  | <b>CD3</b>    |
| <b>PE</b>    | –                 | –           | <b>CD4</b>    |
| <b>Cy5PE</b> | –                 | <b>CD8</b>  | <b>CD8</b>    |



# 3 Color Experimental Setup

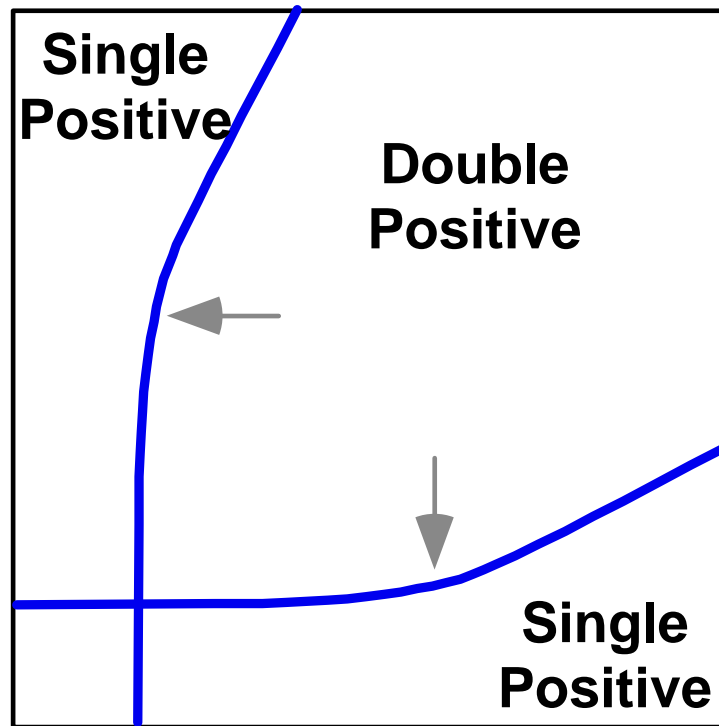
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## Example staining setup of a 3 color experiment:

| Tube # | Description  | FL1      | FL2    | FL3       |
|--------|--|----------|--------|-----------|
| 1      | <i>Experimental Sample</i>   | CD3 FITC | CD4 PE | CD8 Cy5PE |
| 2      | <i>Compensation Controls</i><br>(Single stains – one for each fluorochrome used in the experiment)           | CD3 FITC | -      | -         |
| 3      |  | -        | CD4 PE | -         |
| 4      |  | -        | -      | CD8 Cy5PE |
| 5      | <i>Gating Controls</i><br>(FMO – leave out one fluorochrome at a time)                                       |          |        |           |
| 6      |  | CD3 FITC | -      | CD8 Cy5PE |
| 7      |  | CD3 FITC | CD4 PE | -         |
| 8      | <i>Experimental Controls</i><br>(fully stain healthy or untreated samples to compare to experimental sample) | CD3 FITC | CD4 PE | CD8 Cy5PE |
| 9      |  | CD3 FITC | CD4 PE | CD8 Cy5PE |
| 10     |  | CD3 FITC | CD4 PE | CD8 Cy5PE |

\* no stain added or add isotype matched control stain.

# New Quad-Gates



# Take Away Lessons

- Proper CONTROLS are essential
- DON'T compensate by eye
  - Use Median to adjust the populations if you must do it manually
- New DISPLAYS help view data intuitively