All FACS staining reagents need to be titrated by the user!

Even if a titer is supplied by the manufacturer.

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Titration of FACS staining reagents

- Fluorochrome-conjugated mAb reagents (and other dyes) need to be titrated before use
- Must be titrated using same staining protocol under the same conditions used for experiment
- Same reagent may have different titers depending on staining protocol
  - Surface staining
  - Intracellular staining
  - Staining temperature (RT vs. 0deg)
  - Cell type or source or tissue (e.g., macaque vs. human)

Standardization

- Standardization of staining protocols allows titration information to be recorded in a database to be shared between individuals and groups
- Titration data should be recorded for a **standard staining volume** or as a **dilution**
  - E.g., use 5 ul per 100 ul final staining volume
  - Equivalent to 1:20 dilution
- Recording titers per volume has an advantage
  - For staining panels with multiple reagents, titers for each reagent simply need to be added together

Titration procedure

- 2-fold dilutions over range of expected titer
  (if suggested titer not supplied by manufacturer, then estimate starting titer as 1 to 2ug per 100ul staining volume)
- Optimally test 8 to 12 dilutions (to generate a saturation curve)
- Can titer multiple reagents at same time on the same cells
  - Must be different fluorochromes detected in different channels
  - Cannot use same Ab on different colors, or different Ab's directed to same Ag
  - May require compensation (collect single-stained compensation samples)
- Counter-staining may be beneficial (for markers expressed on rare cell populations or to confirm specificity of staining)

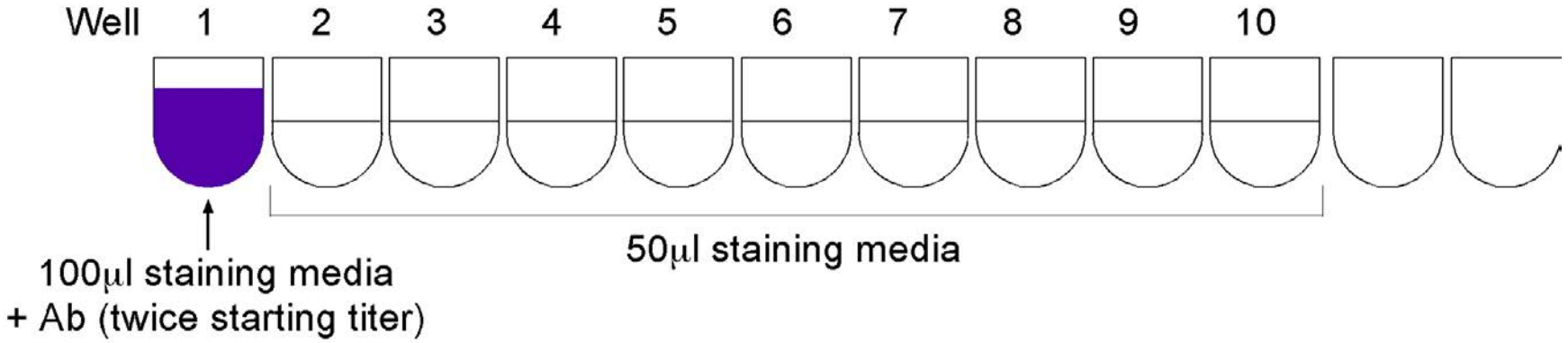
Example of titration protocol

- Use your standard staining protocol, e.g., 50ul of cell suspension mixed with 50ul of Ab solution (100ul final staining volume)
- Prepare 2-fold dilutions in FACS tubes or 96-well plates (round-bottom or V-bottom)
  - Add 50ul of staining buffer* in each well or tube except first
  - For first tube or well, add twice the Ab amount for the first dilution and enough staining buffer to bring final volume to 100ul
- Gently mix first well, take 50ul and add to second well
- Gently mix second well, take 50ul and add to third well
- Repeat for all wells or tubes - discard last 50ul

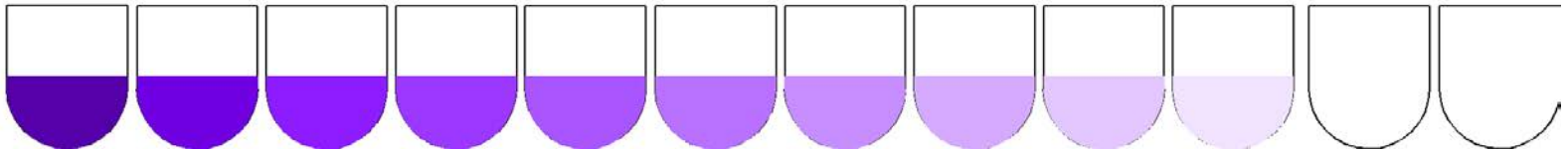
*Use appropriate staining buffer for experiment, e.g., intracellular staining may require saponin-containing buffer

2-fold dilution series for titration

Before dilution:



After 2-fold dilution:



Stain cells

- Prepare cells to use for titration, e.g.:
  - Prepare PBMC from whole blood or thaw frozen PBMC
  - Some reagents, such as cytokines, require cells to be stimulated, etc.
- For intracellular titrations, cells need to be fixed and permeabilized before staining
  - can fix/permeabilize cells in “bulk”
- Prepare cell suspension
  - Typically we use $1x10^6$ cells per test, so we prepare the cell suspension to provide $1x10^6$ cells per 50ul (=$20x10^6$ cells/ml).
  - If cells are limited, 100,000 to 250,000 cells can be used.
- Add 50ul of cell suspension to each well or tube
  - For titrations in plates, a multi-channel pipette can be used
- Follow staining protocol specific for study
- Final step is to re-suspend cells in a volume appropriate for FACS collection (for tubes, typically ≥200ul with staining buffer including 0.5% paraformaldehyde)

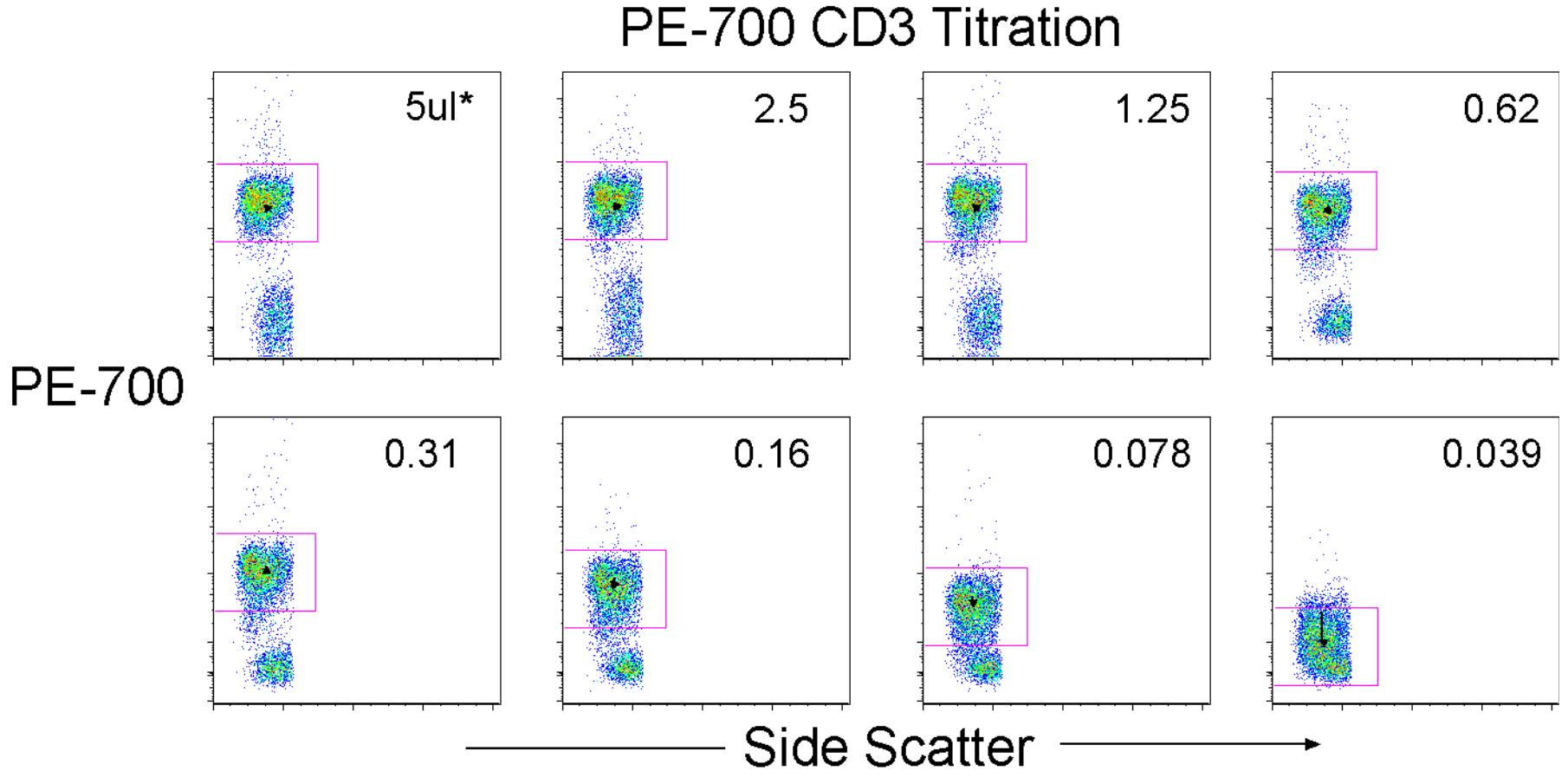
Analysis procedure

- Gate on population of interest, e.g., lymphocytes
- Gate on positive population
  - will need to move this gate for the different dilutions
- Print one (or more) graph from each dilution showing the staining profile for the reagent
  - This can be a histogram, or a bivariate plot
  - It is useful to print all graphs on one page

Statistical analysis

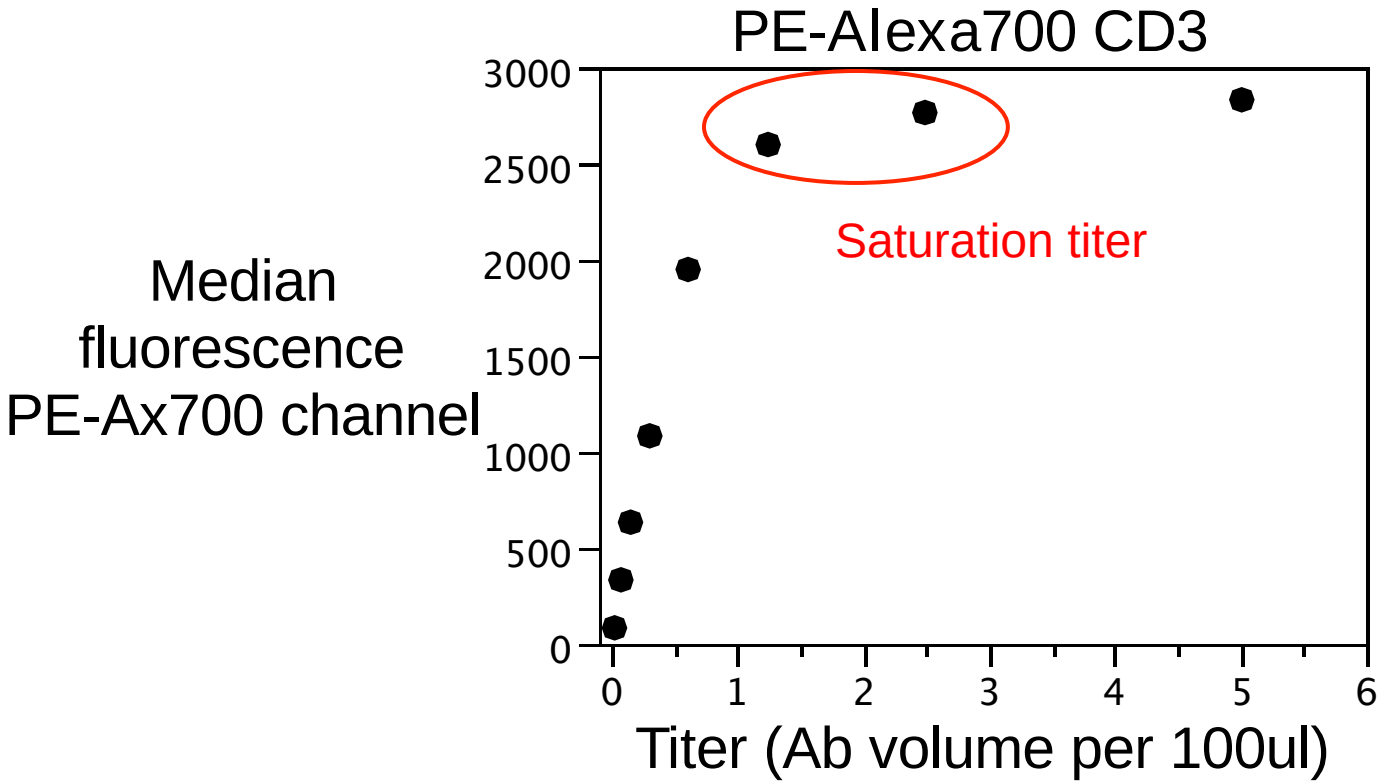
- Determine median fluorescence values for stained cells for each dilution
- Plot these medians vs. the dilution values (needs to be a linear scale)

Example - surface staining PBMC



*Antibody titer, amount of antibody per 100ul

Example of saturation curve surface titration

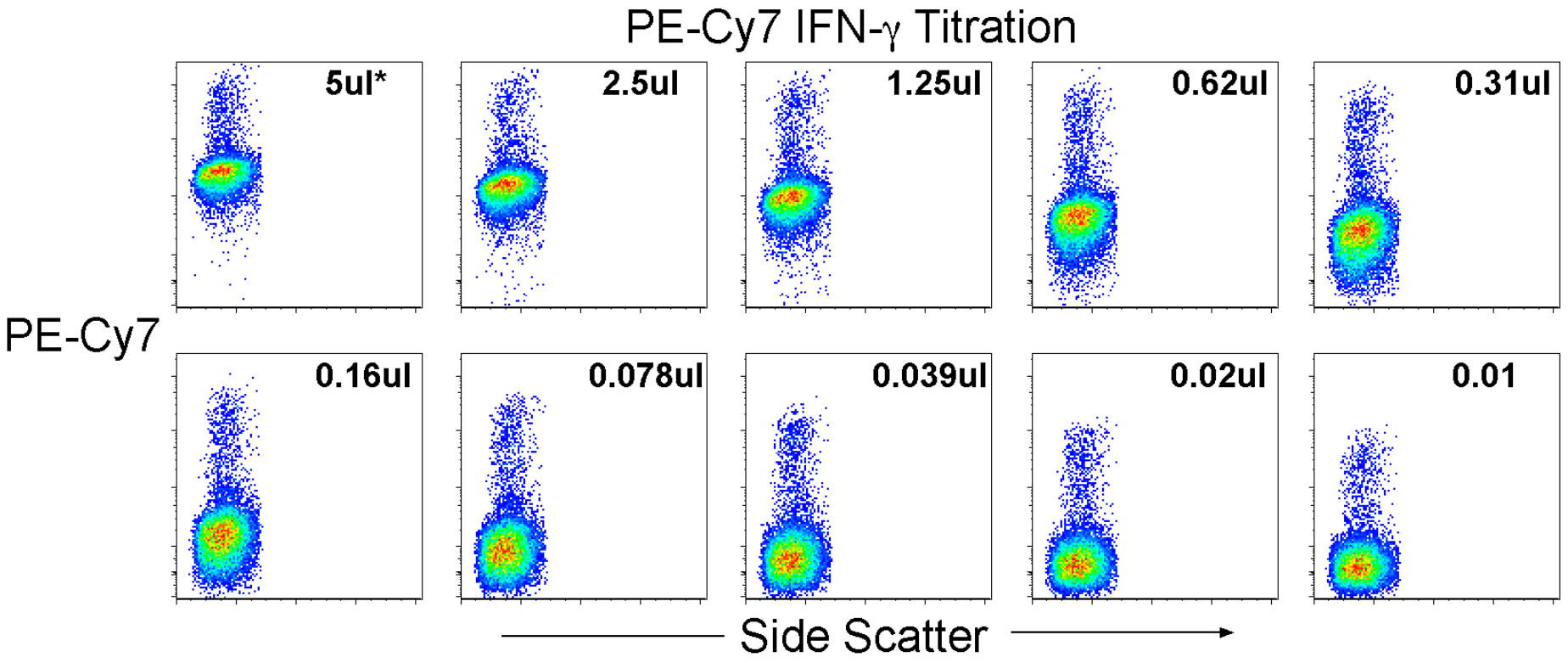


PE-Alexa700 is collected in the PE-Cy5.5 channel

Determine titration value at saturation

- For many reagents the saturation titer is the optimal titer
  - when background staining is minimal
  - for most surface staining
- Using reagents at saturating titers has advantages:
  - Variations in the staining conditions will have minimal effects on staining intensity (the reagent curve “flattens out” at saturation)

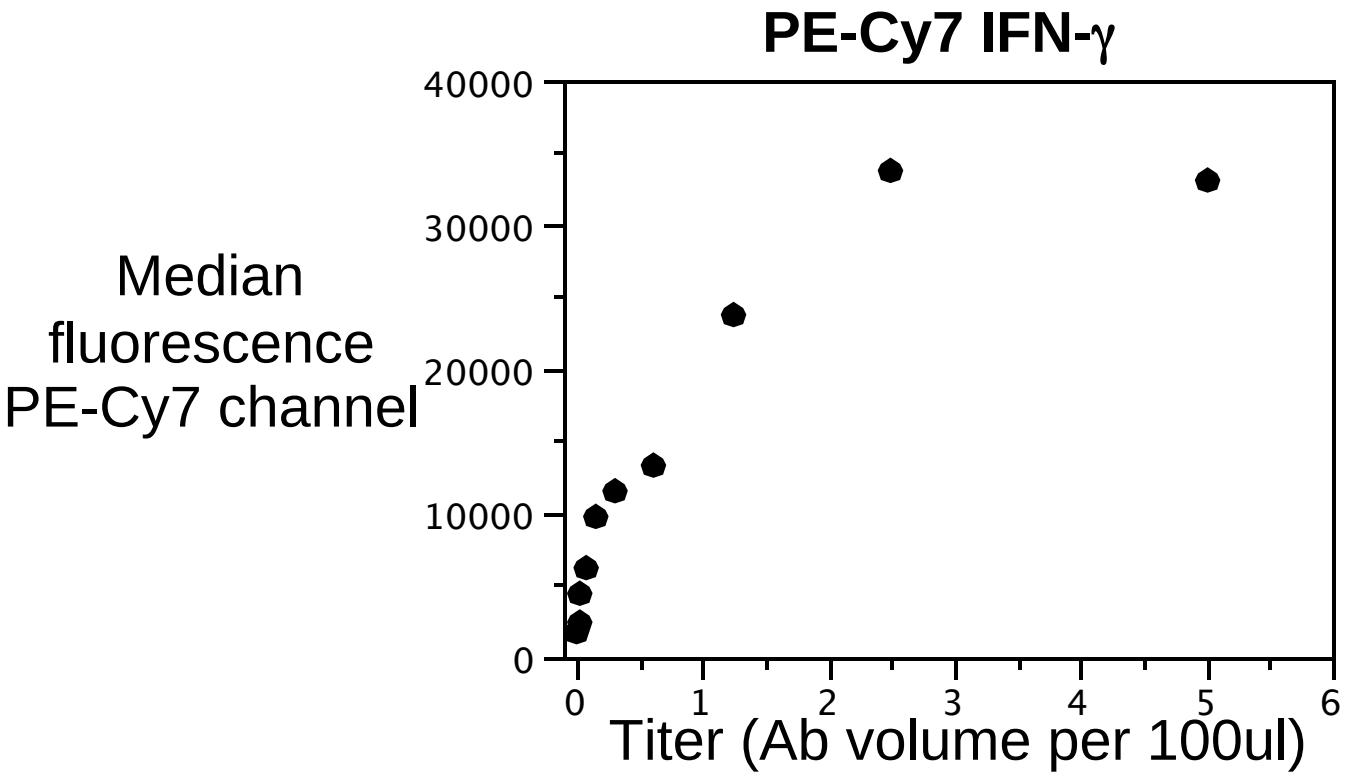
Example - intracellular reagent



*Antibody titer, amount of antibody per 100ul

Cells were stimulated in bulk with SEB overnight before titration.

Example of saturation curve intracellular titration



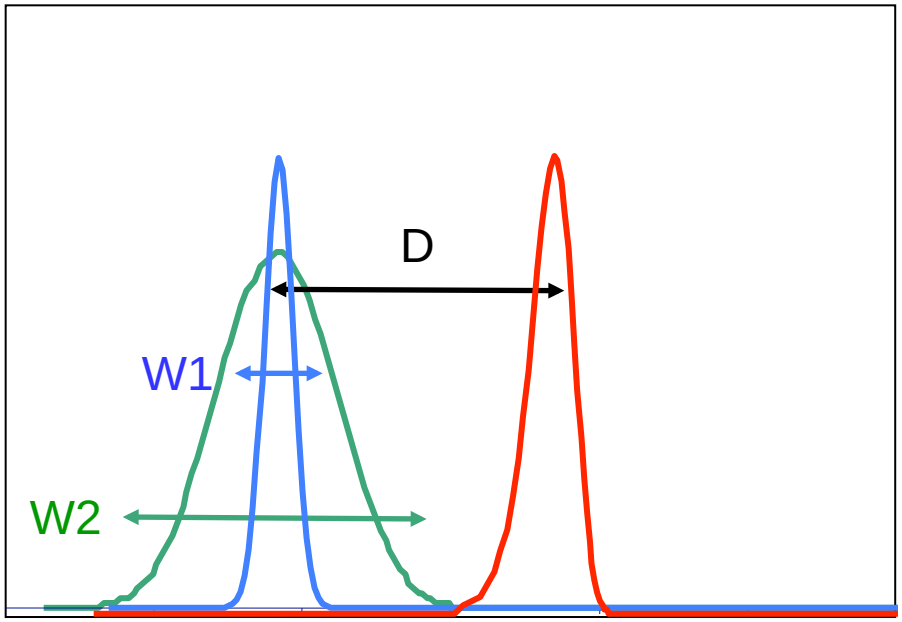
For this reagent, the optimal titer cannot be determined using the saturation curve because the staining intensity of the negative cells increases with titer. Either determine the optimal titer by visual inspection of flow plots or by using the stain index (see below).

Determine titration value

- For many reagents, the saturating titer is not appropriate
- For these reagents the optimal titration value must be determined as:
  - Brightest staining (at or below saturation)
  - Lowest background
- This is often the case for intracellular reagents
  - Reagents saturate at a titer where background is very high
  - Examine FACS profiles to determine titer
- Other reagents do not saturate (while on-scale)
  - Can be doped with unconjugated Ab
- Reagents used at titers below saturation are subject to more variability in staining intensity (the reagent curve is steeper below saturation)
- Stain index is a useful metric to determine optimal separation

Stain Index

Resolution Sensitivity:



Stain Index = D / W

Where D = difference between positive and negative peak medians, and
W (of background) = 2 x rSD (robust standard deviation)

Robust CV

Below is the relationship between robust CV and robust SD. This is useful if the software you are using only provides robust CV and you need the robust SD for the calculation of stain index.

$$rCV = rSD / Median_x$$

$$\%rCV = 100\% * rSD / Median_x$$

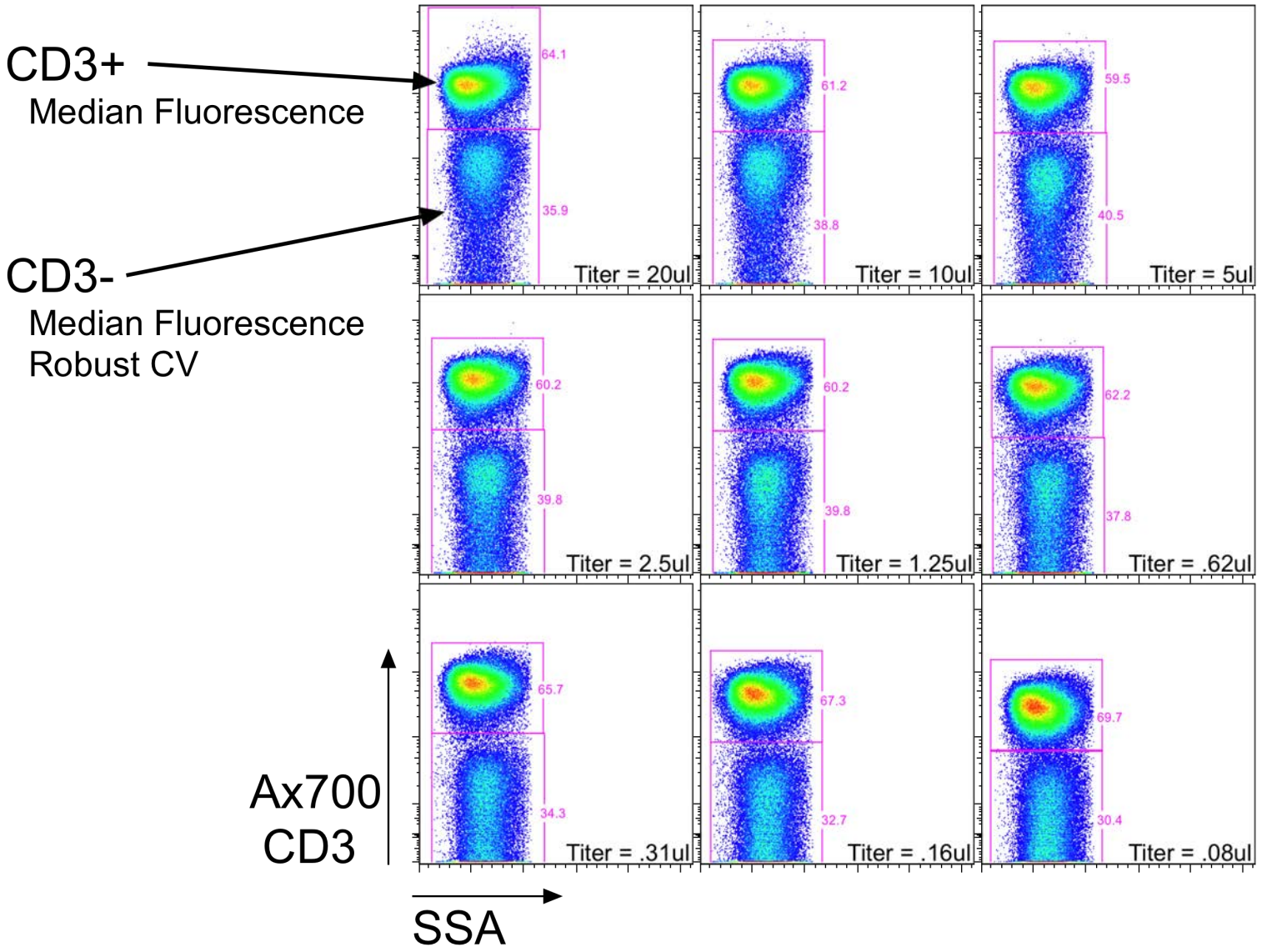
Where rCV = robust CV
rSD = robust Standard Deviation
$Median_x$ = Median Fluorescence of population in question

Formula for calculation of stain index:

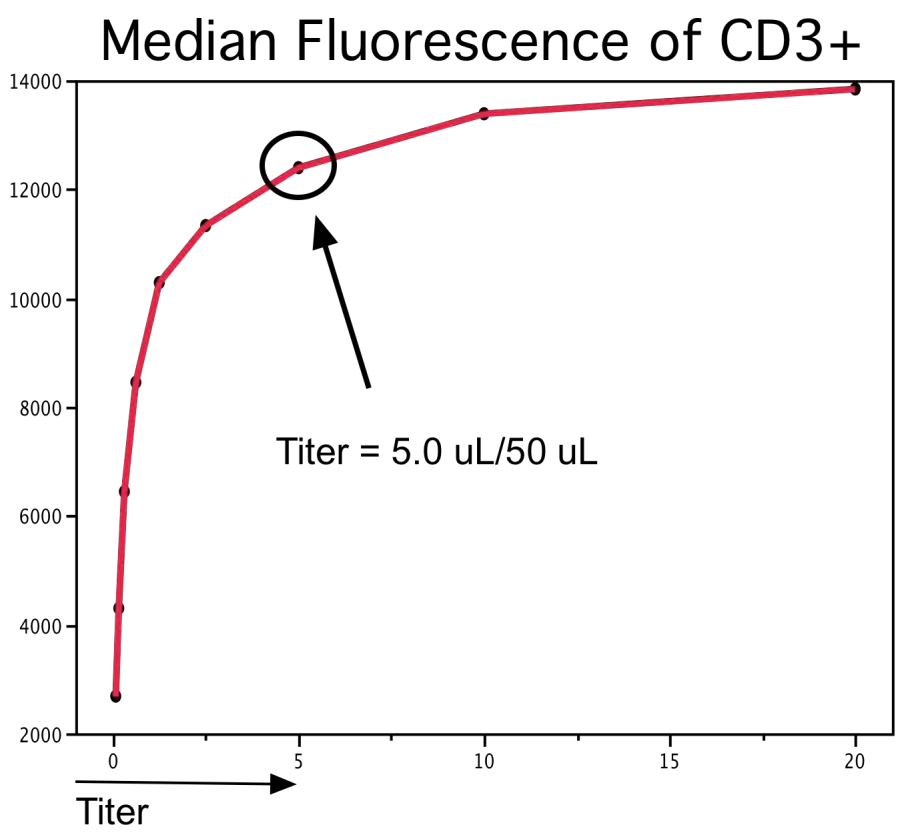
$$\frac{\left(\frac{Med(pos)}{Med(neg)} - 1\right)}{rCV} \times 50$$

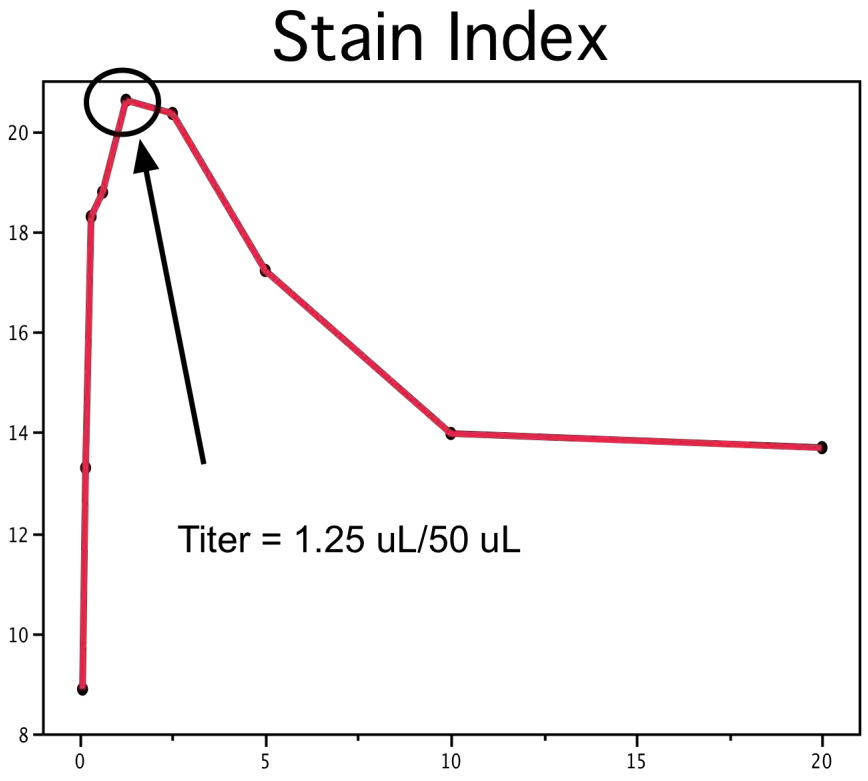
Example of CD3 Ax700 titration

(for calculation of stain index obtain median fluorescence of positive and negative cells and robust CV of negative cells)



Stain index identifies optimal separation





Final Titer = 2.0 uL/50 uL

Reference

For a theoretical discussion concerning Ab affinity and effect on titers:

Kantor, A. and Roederer, M. (1997)
FACS analysis of lymphocytes. In: Handbook of Experimental Immunology (Fifth Edition), Herzenberg, L. A., Weir, D. M., Herzenberg, L. A. and Blackwell, C. (ed.), Blackwell Science, Cambridge, pp 49.1-.13.

Available at:

Herzenberg.Stanford.edu; click on publications

Consequences of non-saturating stains

- Final fluorescence will depend on:
  - Number of cells
  - Concentration of antibody
  - Amount of time of staining incubation
- Reasons for not saturating
  - Not enough antibody (antigen in excess over Ab)
  - Affinity constant very low
- Max amount of Ag that can be stained at saturation (K=binding or affinity constant)

  $[Ag_T] \leq [Ab_T] - 9/K$

Effect of cell number

- Typically the concentration of antibody far exceeds concentration of antigen (100-fold)
  - Larger numbers of cells (10^6 - 10^8 cells) can be stained at same Ab concentration with minimal decrease in fluorescence
  - When staining large number of cells for sorts - may only need to increase antibody concentration minimally (up to 5-fold)
- This can be tested by titrating with different cell numbers
- Note also, that when staining fewer than 10^6 cells, the amount of antibody cannot be decreased