THE UNIVERSITY OF CHICAGO BIOLOGICAL SCIENCES

Flow Basics 2.2: Optimizing the Staining Protocol

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The Flow Basics 2.0 Series

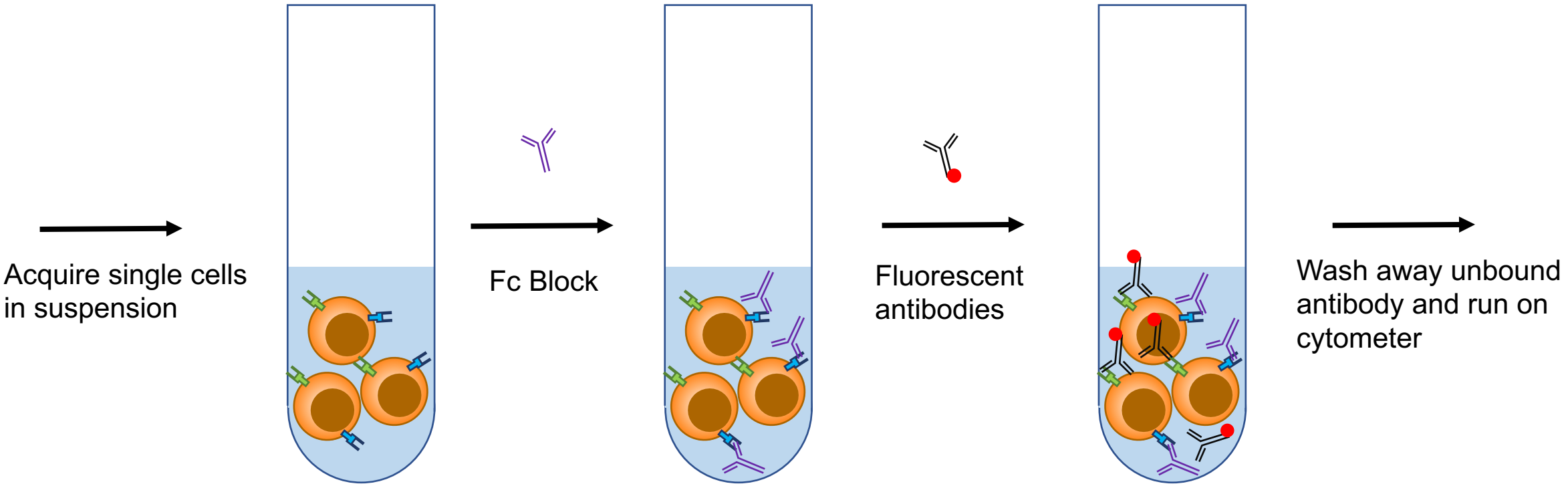
Understanding Flow Cytometry Experiments to Get Better Results

- For all scientific experiments the best data is achieved by optimization and consistency!

- This course will go over:
  - An in depth breakdown of each step of the basic staining protocol and the effect on the final data
  - How to improve consistency in sample staining
  - How to optimize sample sample staining

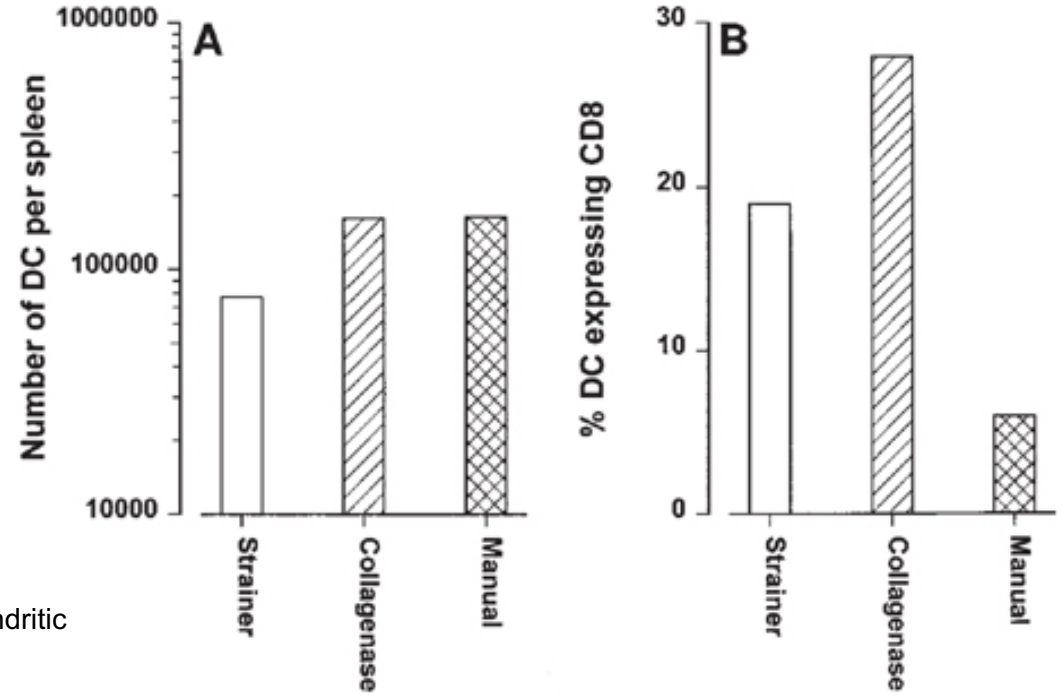
Optimizing the Staining Protocol

Basic staining protocol



Why is the tissue digestion important?

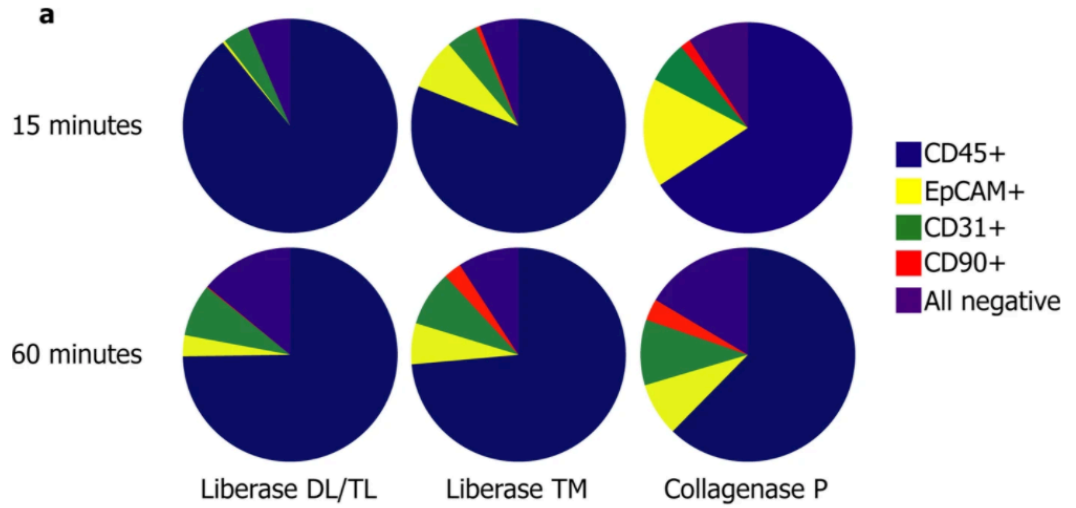
- Tissue digestion can give different results. Consider running flow cytometry on a mouse spleen:



AJ Stagg et al., Isolation of mouse spleen dendritic cells. Methods Mol Med. 2001;64:9-22. doi: 10.1385/1-59259-150-7:9.

How do you choose a digestion enzyme?

- Depends on tissue and cells of interest – search PubMed
- Consider using a digestion enzyme
  - https://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center/cell-detachment.html
  - A Reichard and K Asosingh. Best Practices for Preparing a Single Cell Suspension from Solid Tissues for Flow Cytometry. Cytometry A. 2019 Feb;95(2):219-226. doi: 10.1002/cyto.a.23690.

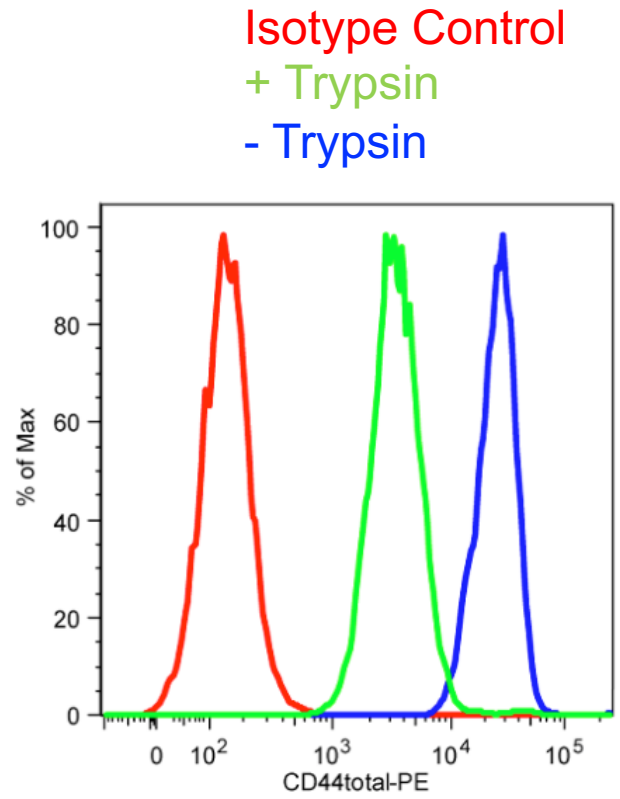


S Waise, et al., *Sci Rep* **9,** 9580 (2019). https://doi.org/10.1038/s41598-019-45842-4

Know how tissue digestion could affect your results

- Some digestion enzymes could cleave surface proteins
- The digestion process could cause cell death/injury
- These are issues you should be aware of when interpreting data, but sometimes there is no way around them

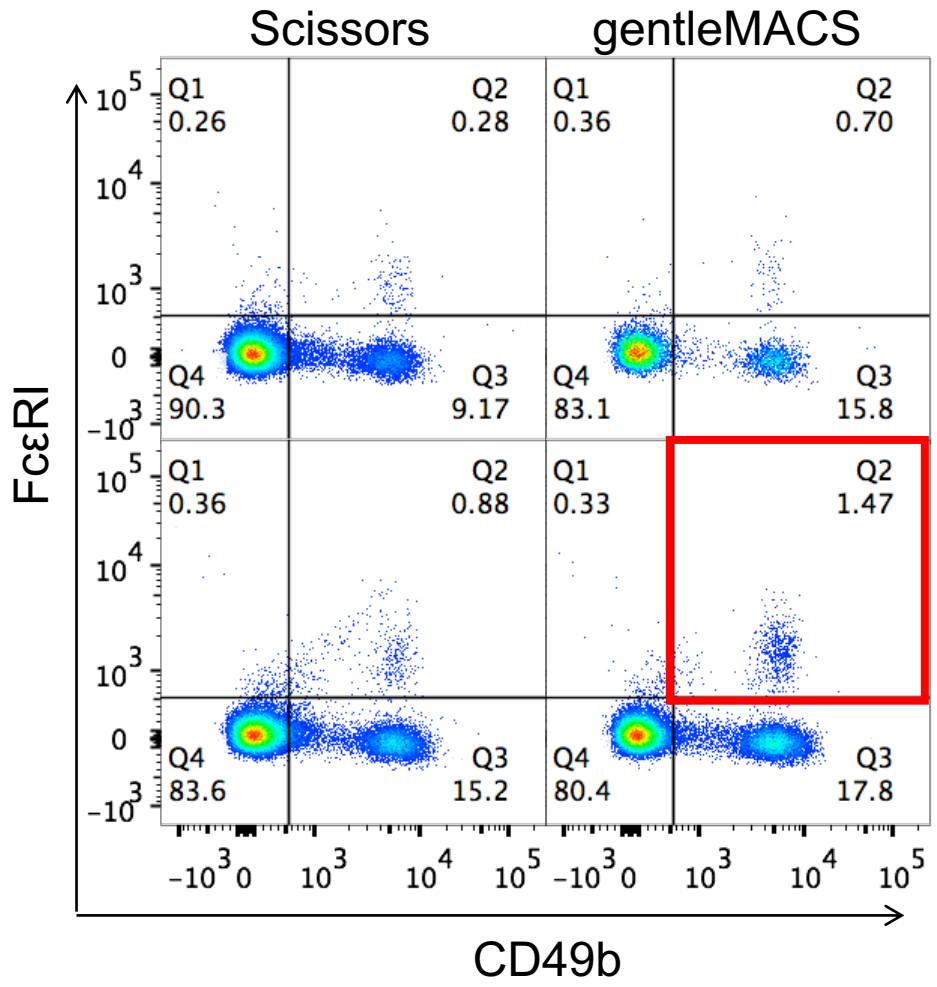
- Remember the importance of being consistent between experiments – all samples should be digested similarly!



Biddle A, et al. doi: 10.1371/journal.pone.0057314

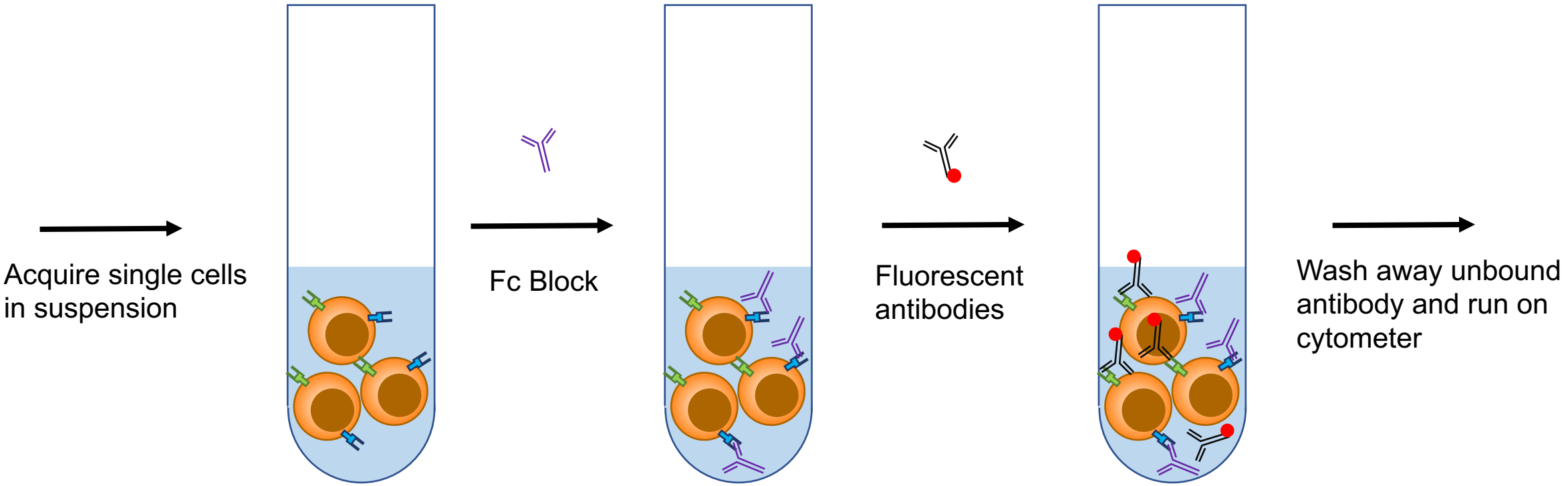
Optimize digestion protocols

- **Example:** Testing digestion protocols to look at basophils in the lung
- Scissors vs. gentleMACS to cut up tissue
- Two different digestion enzyme cocktails



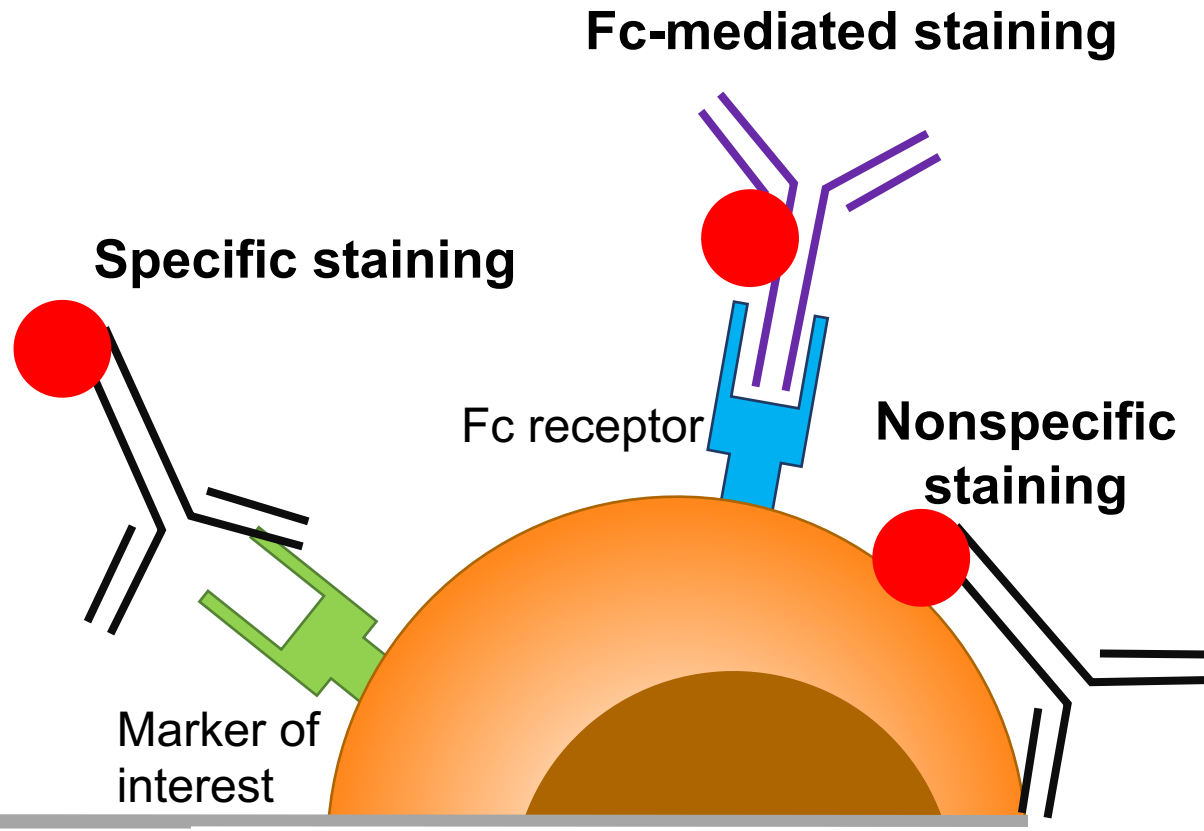
Johnston LK, unpublished

Basic staining protocol

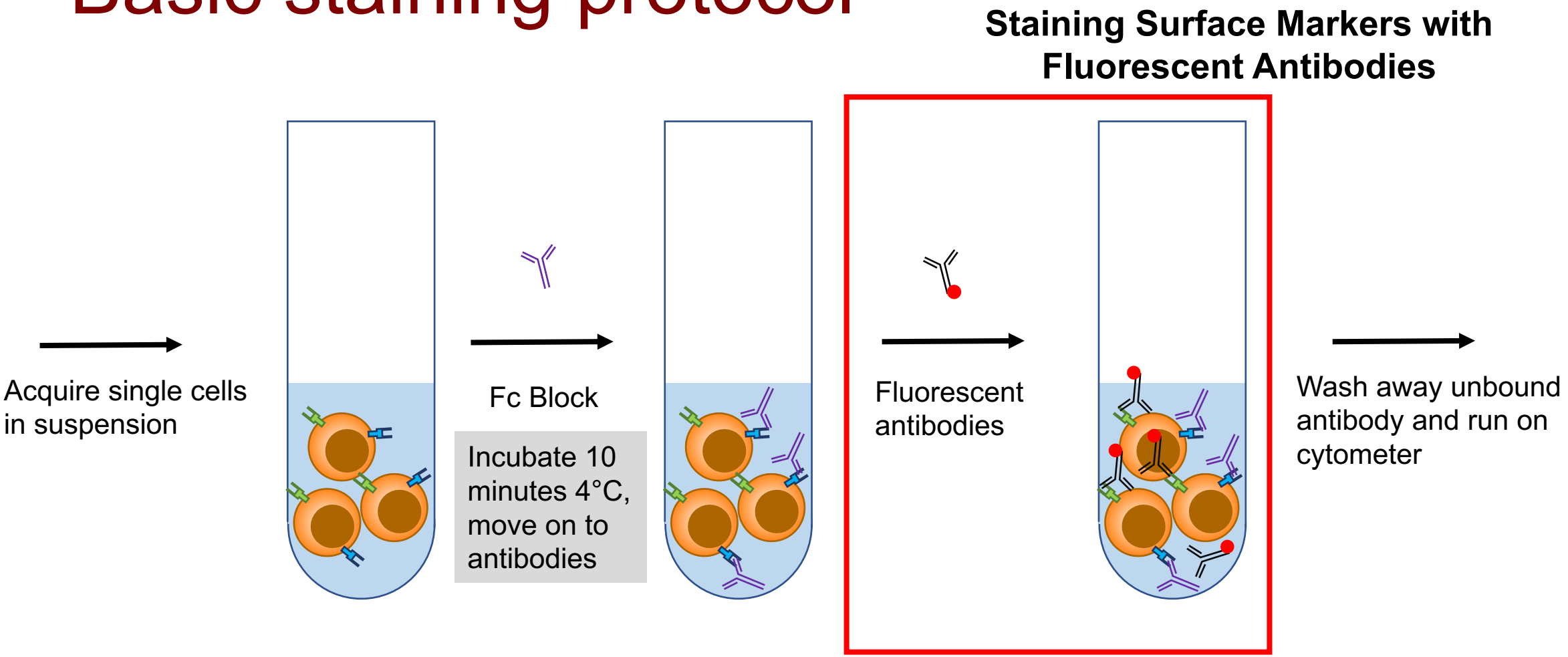


Reduce nonspecific and Fc-mediated staining and cell clumping

- Basic blocking:
  - Fc block and/or serum
- Additional blocking
  - Heparin – charge based block
  - Monocyte blocker – block nonspecific binding to fluorophores



Basic staining protocol



Antibody Staining is Affected by Five Factors

1. Time
2. Temperature
3. Volume
4. Cell number
5. Antibody amount

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Time and temperature

- Typically 30 minutes at 4°C
- Time is often between 15-60 minutes, sometimes overnight
- Temperature is often 4°C or room temp
- Some antibodies/dyes require something else – read manufacturer protocols!

Pick a time and temperature and don't vary the protocol between experiments!

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Staining Volume

- Typically 100 µL
- Some antibodies/dyes require something else
- We will discuss later when changing the staining volume should be considered

Which is most important?

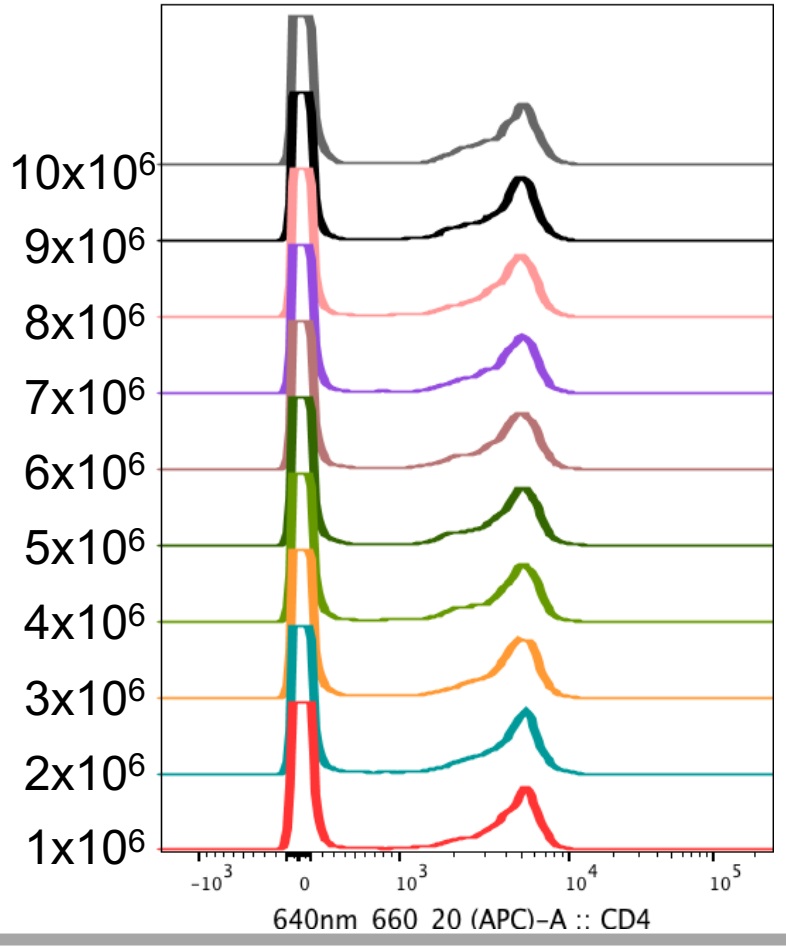
Cell Concentration

Antibody Concentration

Many (but not all!) antibodies are not severely affected by changing cell number

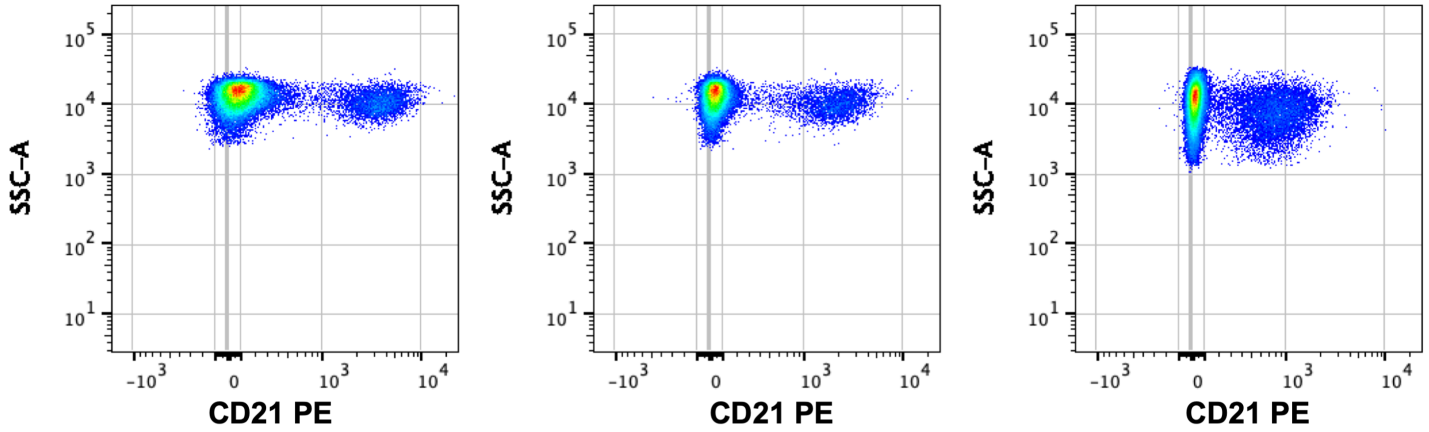
Mouse spleen cells were stained with 0.1 µg anti-CD4 (clone GK1.5) in 100 µL. Cell number was varied between 1-10x10^6.

Cells stained (x10^6)	Freq. CD4$^+$ of Live Cells	Geometric Mean of CD4$^+$
1	13.7	4148
2	13.8	4121
3	14.2	4086
4	13.6	4060
5	14.3	4068
6	14.1	4017
7	13.7	3998
8	14.3	3991
9	14.5	3955
10	13.9	3990



Johnston LK, unpublished

Antibody Concentration Has a Big Impact on Cell Staining



Antibody:	0.3 µg (2 µL)	0.1 µg (0.6 µL)	0.03 µg (0.2 µL)
SI:	15	17	13

Figure 1. The Effect of Antibody Concentration on Cell Staining. All cells were stained in 100 µL of PBS with FBS. The staining index (SI) was calculated for each concentration of antibody. At higher concentrations there is nonspecific staining of the negative population. The spread in the negative population decreases the SI. At lower concentrations the positive population is harder to resolve from the negative population.

Johnston LK, unpublished

How do you determine cell and antibody concentration?

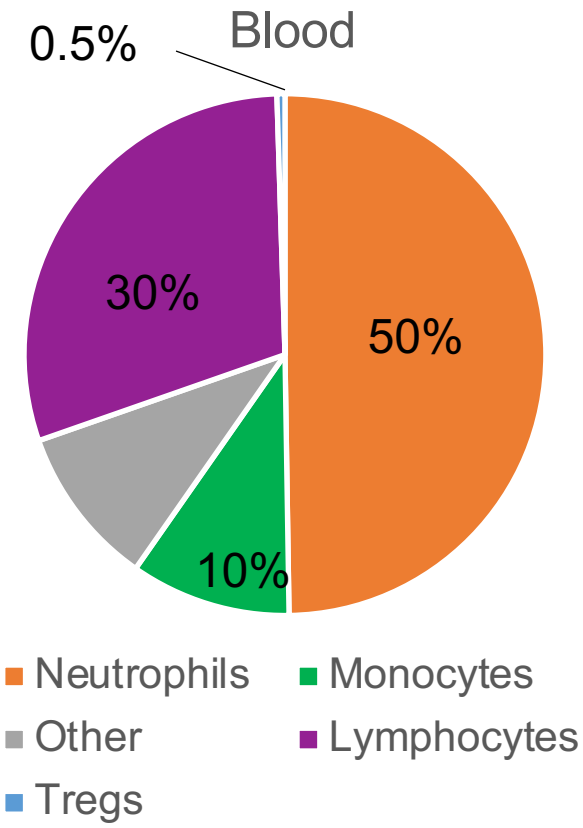
How to decide on how many cells to stain

- Standard protocol is to stain 1×10^6 cells, but really the cell number needed is dependent on the experiment
- The number of total cells is best determined by the frequency of the rarest population of interest
- It is recommended to have at least 100-500 events in the rarest population, depending on how distinct the markers are on the population

- Read my blog post on how many cells to stain here

Example: Determining how many cells to stain

- We have an idea of cell frequencies in the blood
- We should determine how many cells to stain based on our experiment. What is the rarest population of cells that I am interested in for my specific experiment?



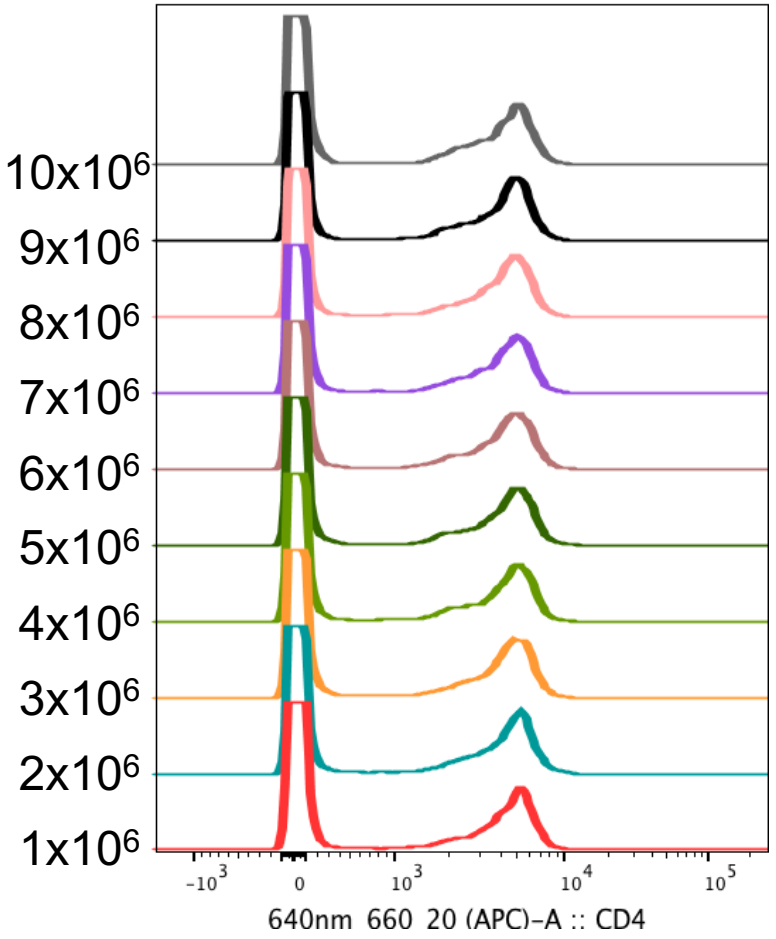
Total cells	Rarest cell of interest	Rarest cell percentage	Rarest cell total number
50,000	Monocyte	10%	5000
50,000	Treg	0.5%	500

Remember to stain more cells to account for loss during staining protocol! Don't expect to stain 50,000 cells and then analyze exactly 50,000 cells. For this example, staining 0.5-1x10^6 would be best, but 0.1-0.2x10^6 would be OK if sample is limited.

How to scale up the staining protocol

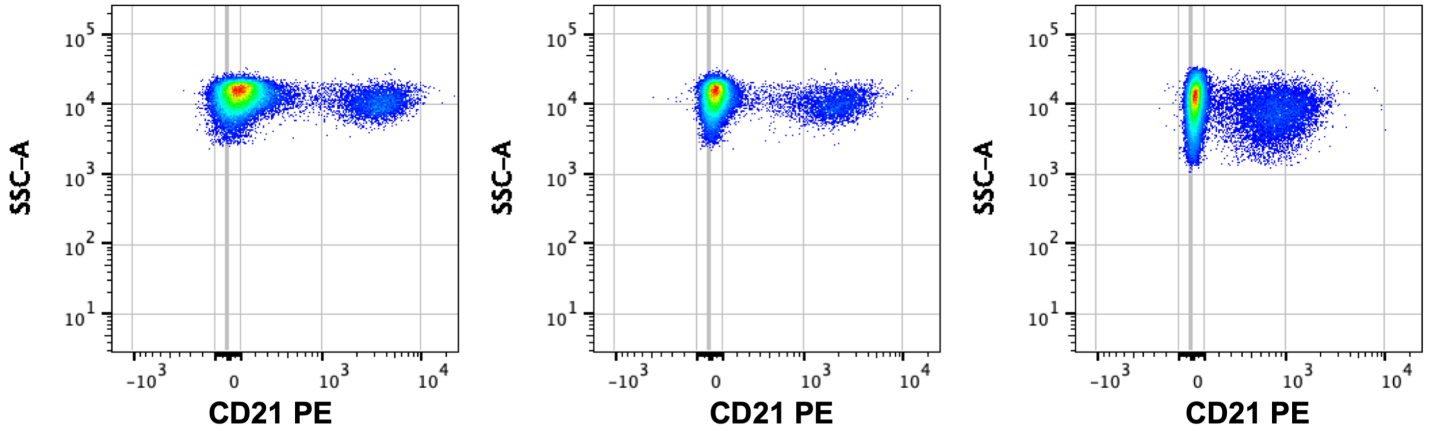
- Example: Used a benchtop analyzer to determine population of interest, next experiment is to sort that population

- If you need to increase the total number of cells, remember that your staining protocol works for a range of cells:
  - Original protocol: $1x10^6$ cells, 100 µL, 0.1 µg antibody
  - New protocol: $10x10^6$ cells, 200 µL, 0.2 µg antibody



Antibody Titration Determines the Optimal Antibody Amount

- Using the same protocol that you plan to use for your experiment, test several different antibody concentrations
  - fix the cell number, time of incubation, and reaction volume and temperature
- Typically titrations are done as single stains (one color per tube)

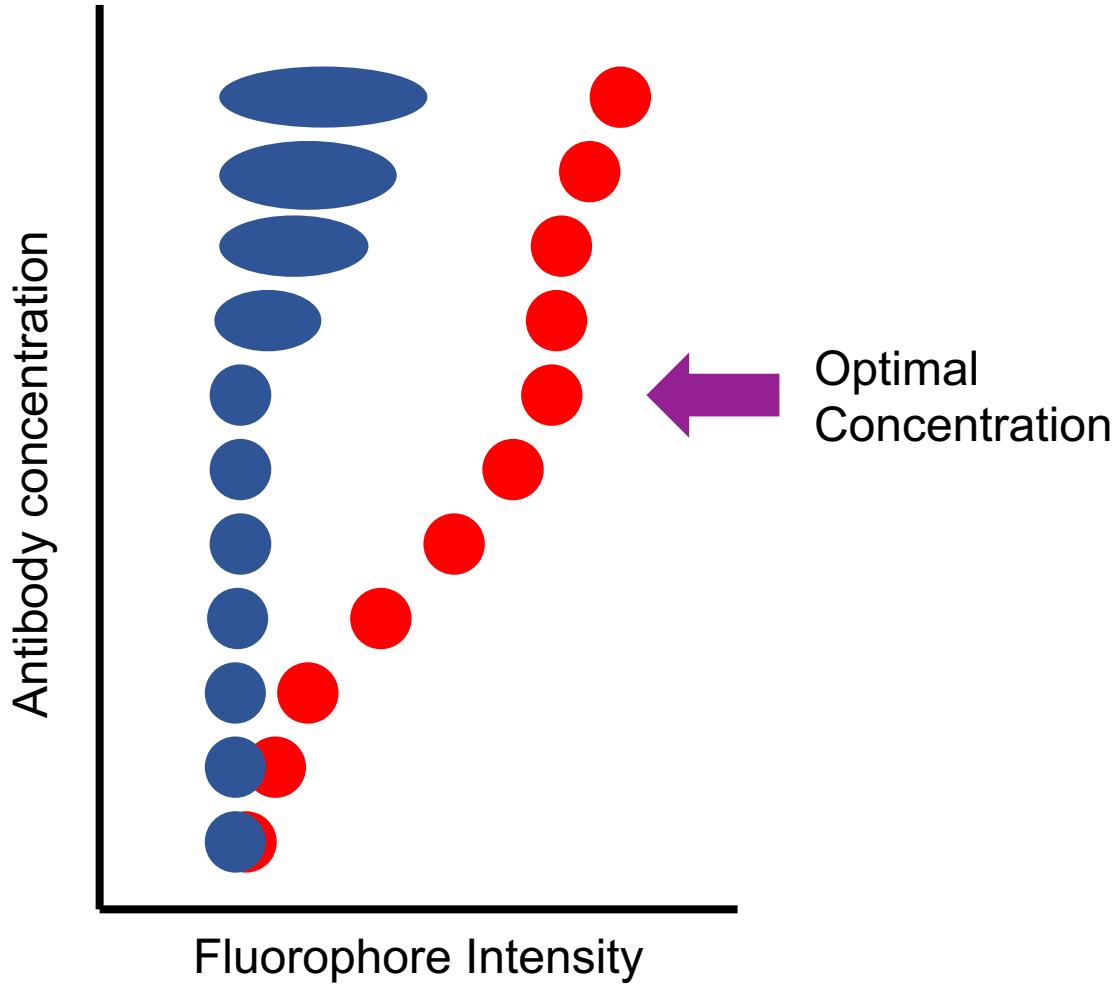


Johnston LK, unpublished

Antibody: 0.3 µg (2 µL) 0.1 µg (0.6 µL) 0.03 µg (0.2 µL)

General Effect of Antibody Concentration

- If the antibody concentration is too high, it may nonspecifically bind to the negative population
- If the antibody concentration is too low, we may not be able to separate the negative and positive populations



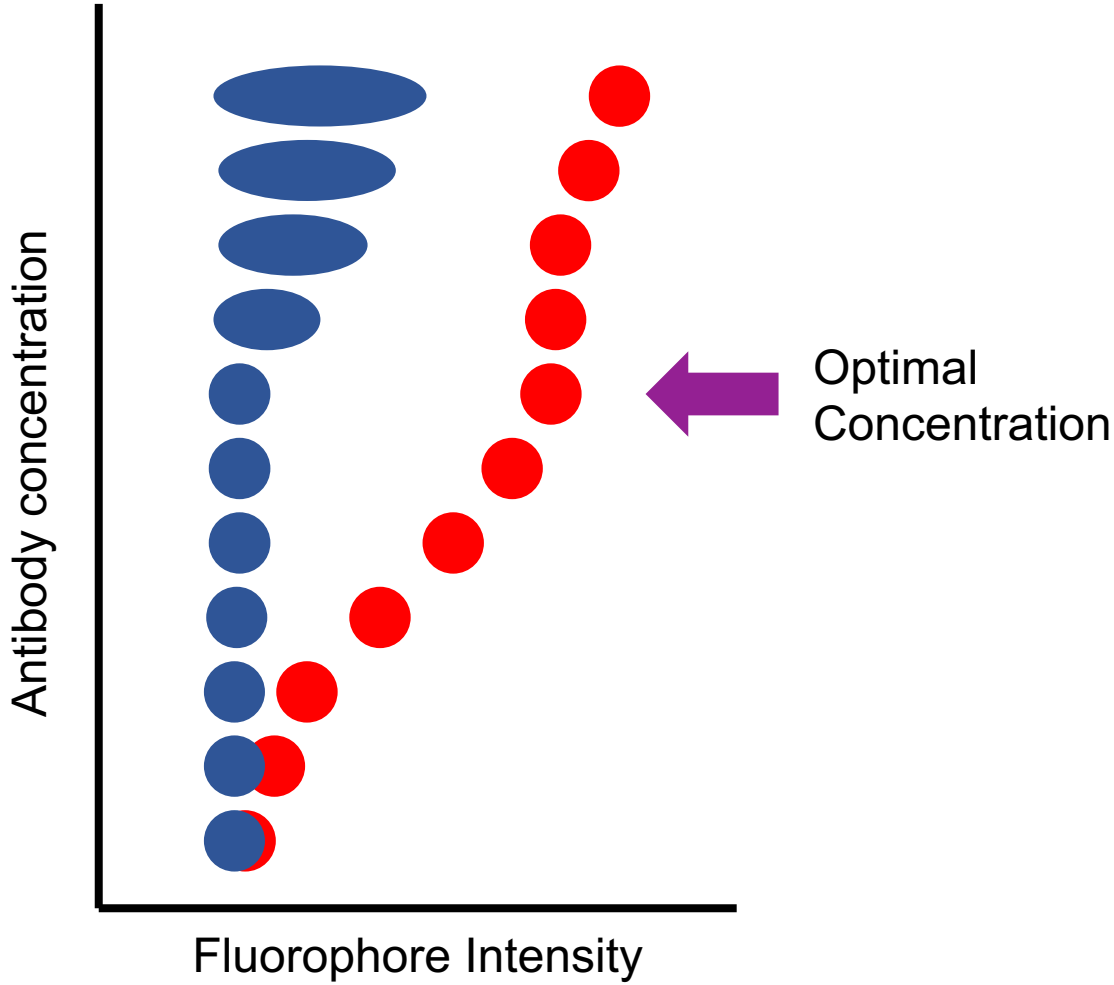
What is needed for an antibody titration experiment?

- It is best to use your cell/tissue of interest, but if the marker is not expressed, another cell type can be used
- Consider using a condition where you expect the marker to be expressed
  - Example: stimulate T cells with PMA/ionomycin to see activation markers

- Once you have collected the data, you will need to analyze it in FlowJo or FCS Express. You may want to calculate the separation/staining index (not everyone does)

Staining/Separation Index (SI)

- SI takes into account the distance between the means of the positive and negative populations and the spread of the negative population
- The highest SI value will be the optimal antibody concentration
  - Maximizes the distance between the positive and negative populations
  - Minimizes the spread of the negative population

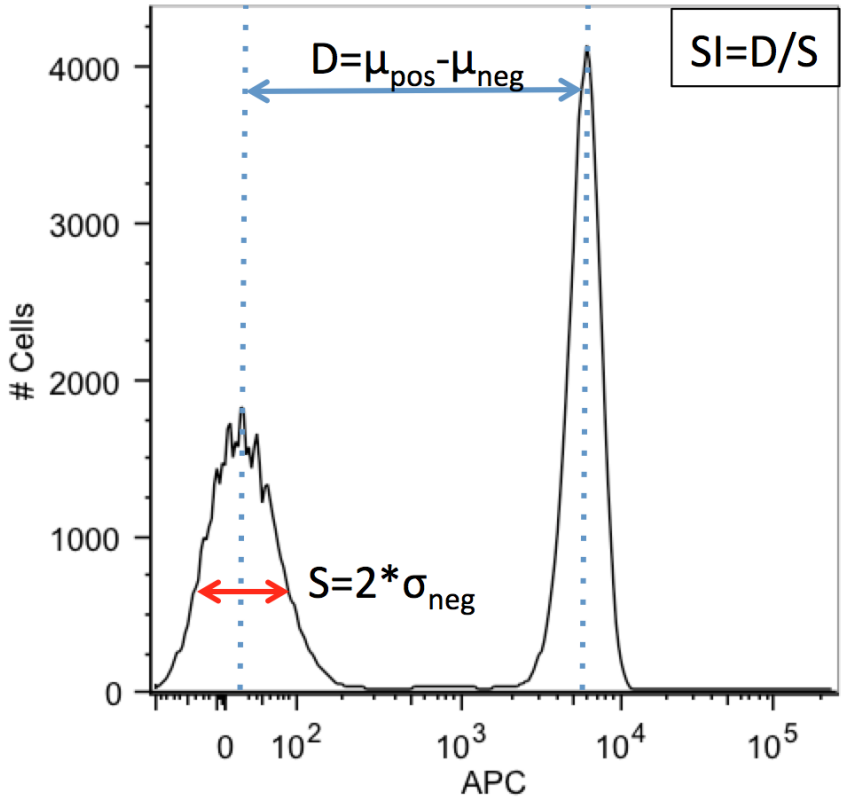


Calculating Staining Index

- Statistics needed:
  - Mean (geometric) fluorescence intensity of positive population
  - Mean (geometric) fluorescence intensity of negative population
  - Standard deviation of negative population

- $\mathrm{SI} = \frac{MFI_{pos} - MFIneg}{2 \times SDneg}$

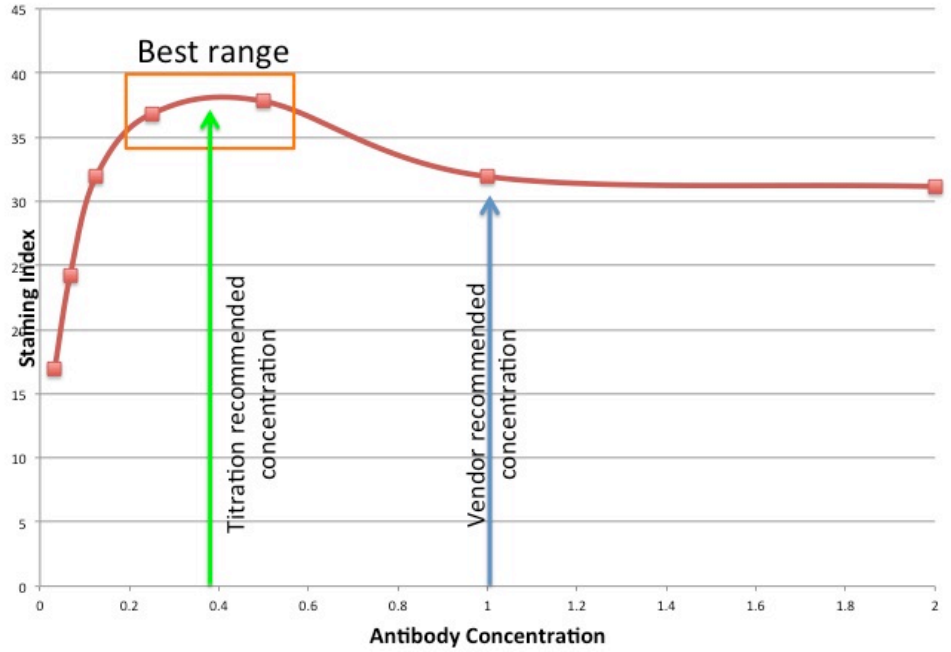
- Note: separation index is a slightly more complex formula, just choose one formula to use



https://expert.cheekyscientist.com/essential-calculations-for-accurate-flow-cytometry-results/

Full Antibody Titration Protocol

- Stain 8 tubes with 8 concentrations of antibodies
  - Detailed protocol: https://www.leinco.com/library/Titration-for-FACS.pdf
- Calculate the staining/separation index https://expertcytometry.com/stain-sensitivity-index/
- Select the antibody concentration with the maximum SI



https://bitesizebio.com/22374/importance-of-antibody-titration-in-flow-cytometry/

Antibody Titration – Abbreviated Protocol

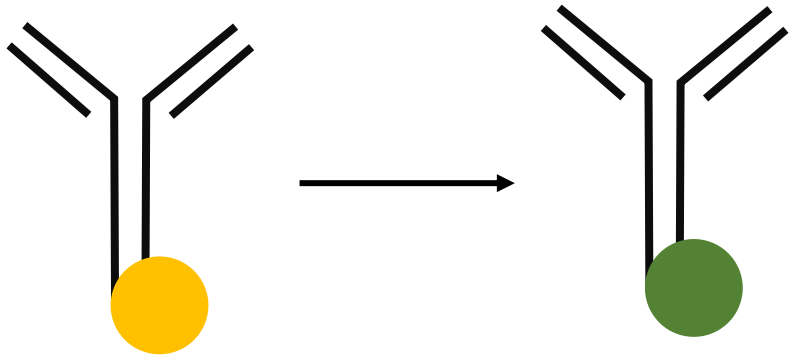
- Many users skip the antibody titration for various reasons – not enough cells, not enough time, etc. I find this abbreviated protocol to be sufficient and practical.

- Using your standard protocol for staining, stain your antibody of interest at 4 concentrations
  - Tube 1 = 0.3 μg
  - Tube 2 = 0.1 μg
  - Tube 3 = 0.03 μg
  - Tube 4 = 0.01 μg
  - You may choose to go higher (3 μg, 1 μg) or lower (0.003 μg, 0.001 μg)
  - Beware: some antibodies are 0.5 mg/mL and others are 0.2 mg/mL, brilliant violet dyes may be any concentration
  - You may calculate SI, but people often just pick the best looking concentration

Chosen concentrations are in half-logs because intensity is measured on a log scale

Notes About Antibody Titration

- It's easiest to keep track of the amount of antibody in mg/mL as opposed to the volume of antibody per volume of staining buffer



- PE conjugated
- Clone X
- 0.2 mg/mL
- Use at 0.1 µg

- FITC conjugated
- Clone X
- 0.5 mg/mL
- Use at 0.1 µg

- If I keep track of my antibody titrations as "1:100" or "2 µL", I have to look up the concentration of the original antibody
- To be clear, the best practice is always to titrate every single antibody, but using the same mg/mL (or µg per 100 µL) often works and the approach of titrating each antibody clone regardless of fluorophore is better than not titrating at all

Beyond the Basic Staining Protocol

- Fixing cells before/after staining
- Staining intracellular markers
  - Cytoplasmic - Cytokines
  - Nuclear – Transcription factors
  - Phosphoproteins
- Dyes to examine cell cycle or proliferation

Resources for Fixation

- https://www.biolegend.com/en-us/blog/fix-now-fix-later-considerations-for-the-use-of-paraformaldehyde-fixation-in-flow-cytometry
- https://bitesizebio.com/22141/fixation-and-flow-cytometry/

Resources for Intracellular Staining

- https://www.bdbiosciences.com/documents/Intracellular_brochure.pdf
- https://www.thermofisher.com/us/en/home/references/protocols/cell-and-tissue-analysis/protocols/staining-intracellular-antigens-flow-cytometry.html
- https://www.biolegend.com/en-us/protocols/intracellular-flow-cytometry-staining-protocol

Resources for Cell Cycle Analysis

- https://expert.cheekyscientist.com/cell-cycle-analysis-details-are-critical-in-flow-cytometry/
- https://www.thermofisher.com/us/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-assays-reagents/cell-cycle-assays-flow-cytometry.html

Resources for Cell Proliferation

- https://www.thermofisher.com/us/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-assays-reagents/cell-proliferation-flow-cytometry.html
- https://bitesizebio.com/21329/using-flow-cytometry-for-cell-proliferation-assays-tips-for-success/
- https://expert.cheekyscientist.com/5-mistakes-scientists-make-when-doing-flow-cytometry-proliferation-experiments/
- http://www.cyto.purdue.edu/cdroms/cyto10a/educationandresearch/flowanalysis.html

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