THE UNIVERSITY OF CHICAGO BIOLOGICAL SCIENCES

Flow Basics: Sample Preparation and Experimental Design

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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:
  - Factors of sample preparation can affect data
  - How to improve consistency in sample staining
  - How to optimize sample sample staining
  - How to design experiments to achieve consistent results

Many Factors Affect Flow Cytometry Data

Staining Protocol

- Nonspecific staining
- Staining with antibodies
  1. Time
  2. Temperature
  3. Volume
  4. Cell number
  5. Antibody amount
- Washing
- Buffers and Fixatives
- Pipetting error

Panel Design

- Number of markers
- Antigen expression level
- Antigen co-expression
- Fluorophore brightness
- Gating strategy

Instrument Setup

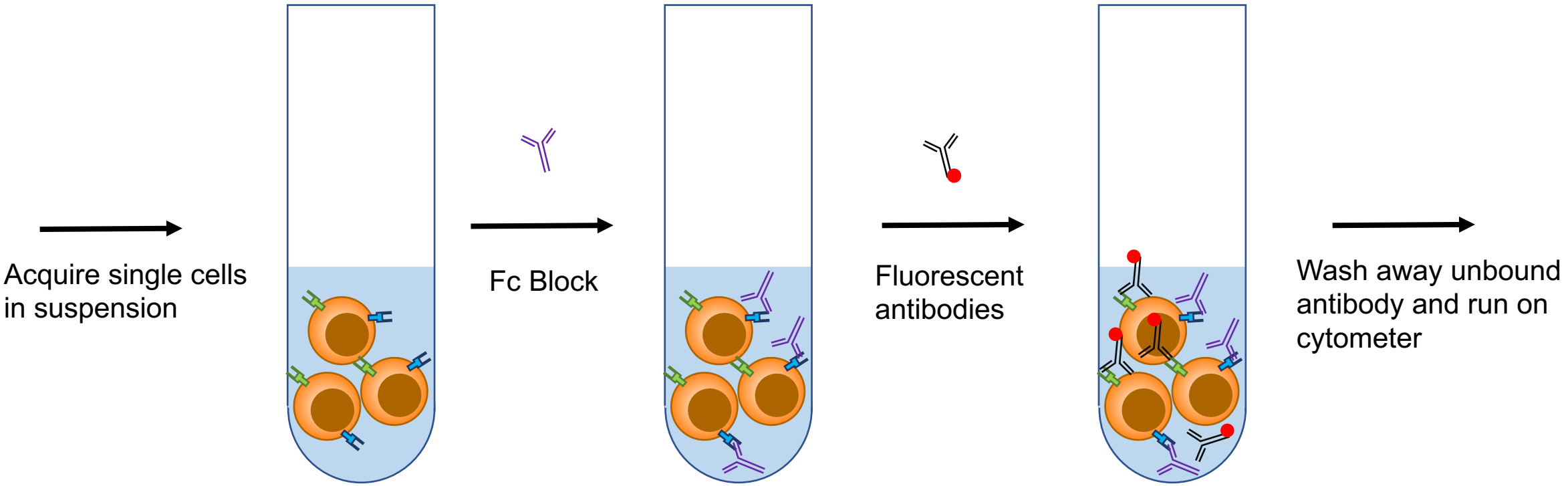
- Voltage Settings
- Compensation controls

Other Considerations

- Digestion of tissue into single cells
- Controls
  - Gating controls for data analysis
  - Staining controls for compensation
- Deciding on number of cells to stain
- Repeating experiments

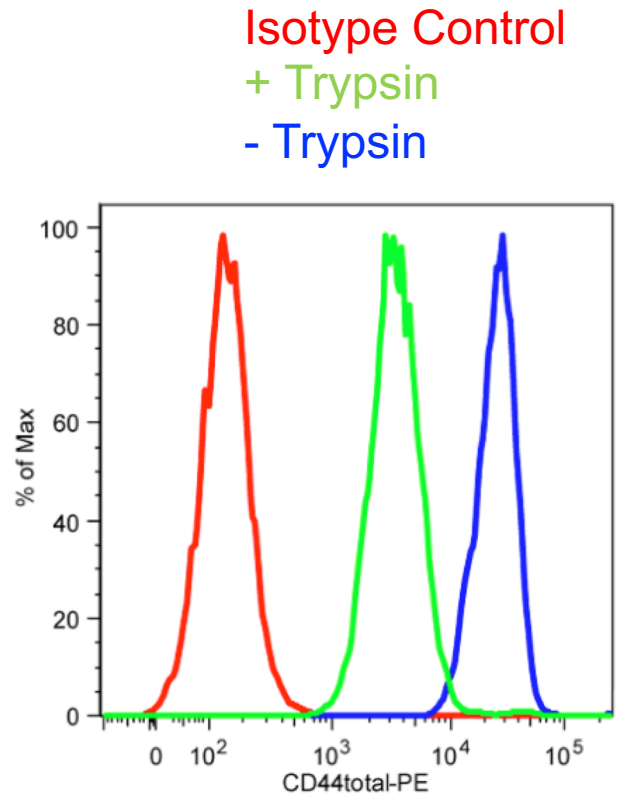
Staining Protocol

Basic staining protocol



Getting single cells in suspension

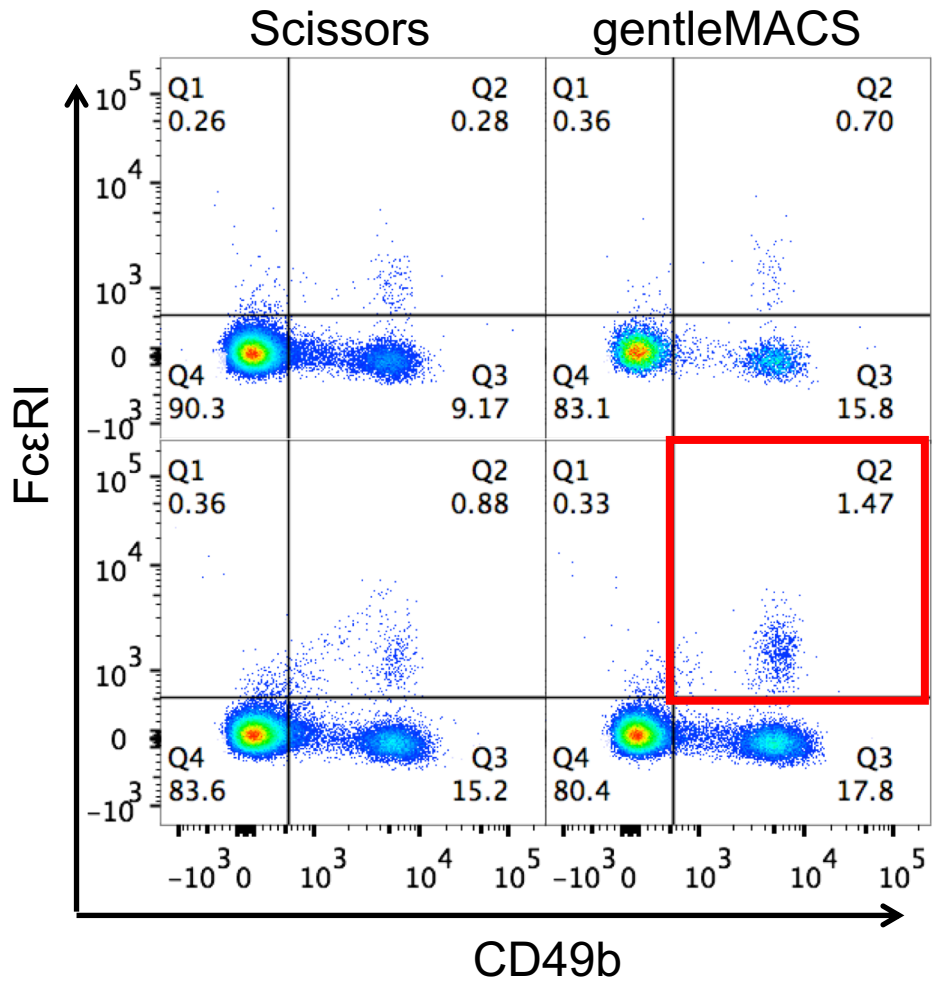
- Depends on tissue and cells of interest
  - Example: squishing a spleen between slides is sufficient to get lymphocytes, but a enzyme digestion will improve recovery of dendritic cells
- Consider using a digestion enzyme
  - https://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center/cell-detachment.html
  - Caution: Digestion enzymes might cleave surface proteins



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

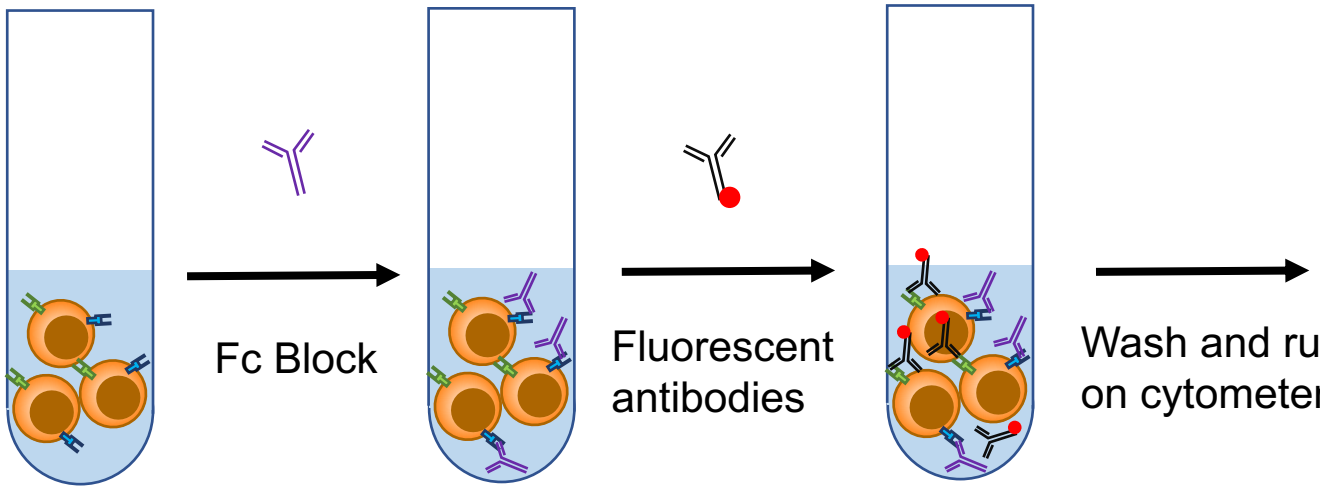
Optimize digestion protocols

- **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

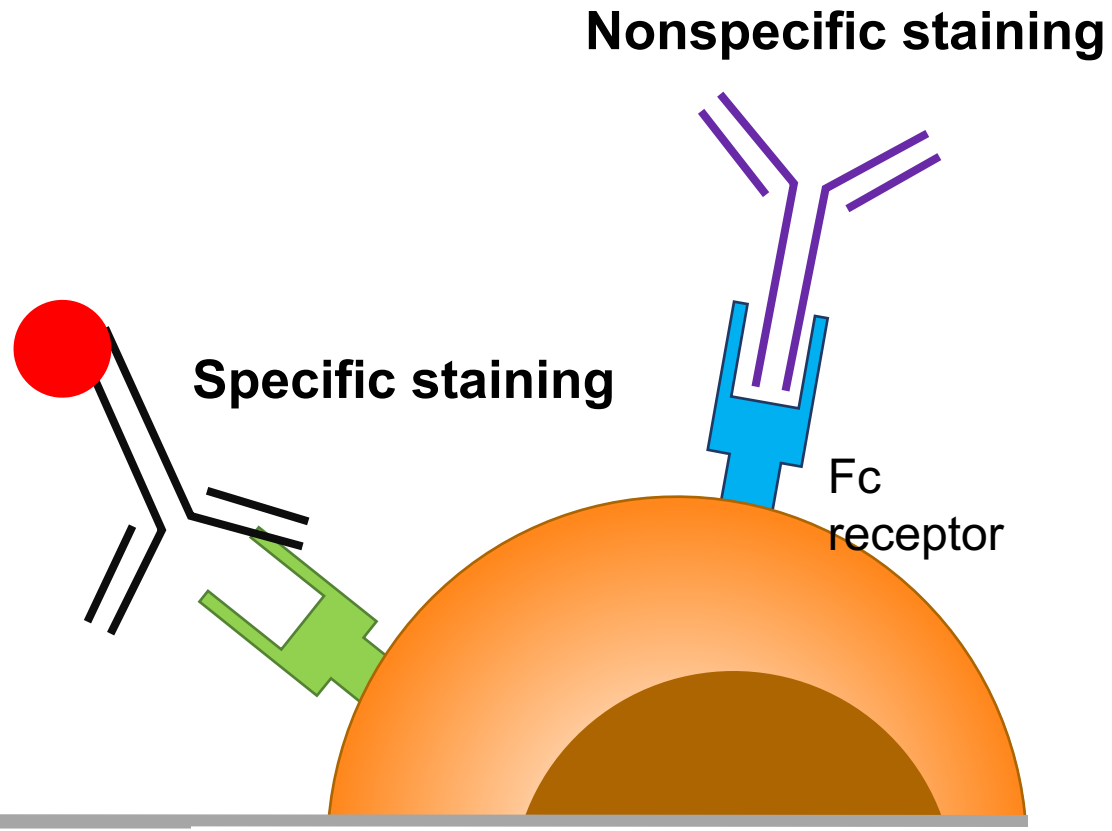


1. Staining tube: Eppendorf, 5 mL FACS tube, or 96-well round bottom plate
   - Choice depends on preference. Main difference is wash volume

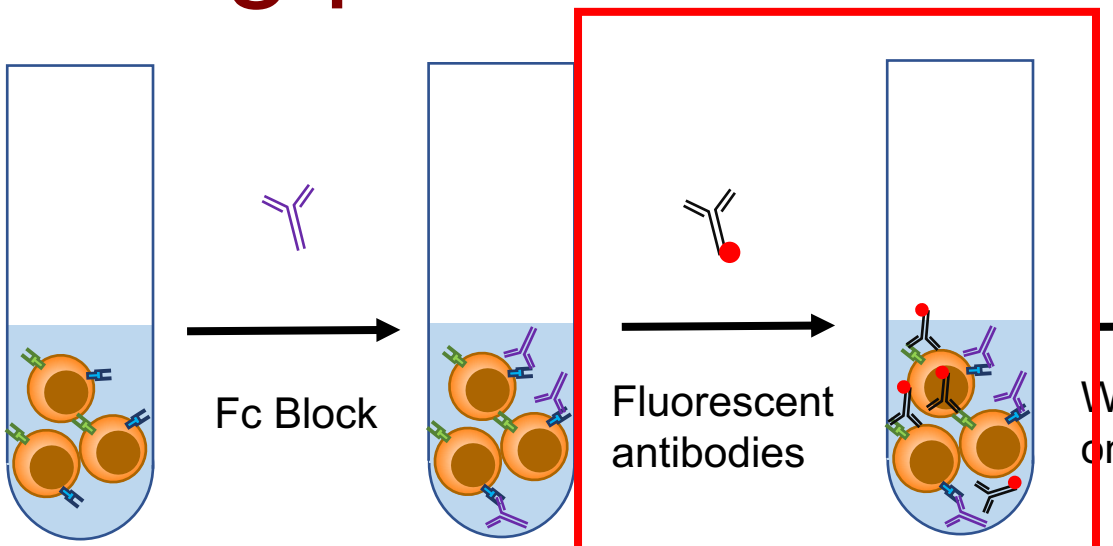
2. Buffer
   - Choice depends on your cell type
   - FACS buffer – can be cell dependent
     - 1X Ca/Mg2+ free PBS (No phenol red!)
     - Either FBS (1-10%) or BSA (0.1-1%)
     - EDTA (0.5-5mM) if you have very sticky cells
     - Commonly used: 1% FBS in 1X PBS (consider filtering FBS to remove debris, especially for cell sorting)

Blocking to reduce nonspecific staining

- Immune cells have Fc receptors that bind antibodies. Most antibodies used for flow cytometry are IgG antibodies
  - Fc Block inhibits IgG antibodies from binding to Fc receptors nonspecifically
    - In mice, Fc block is anti-CD16/CD32
  - Serum is used less often
    - E.g. Rabbit serum for rabbit antibodies, goat serum for goat antibodies
    - Also BSA/FBS in staining buffer helps
  - Heparin - uncommon, but useful for tricky cells. See my blog post about heparin blocking here



Basic staining protocol



Staining Surface Markers with Fluorescent Antibodies

1. Staining tube: Eppendorf, 5 mL FACS tube, or 96-well round bottom plate
   - Choice depends on preference. Main difference is wash volume

2. Buffer
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   - FACS buffer – can be cell dependent
     - 1X Ca/Mg2+ free PBS (No phenol red!)
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     - Commonly used: 1% FBS in 1X PBS (consider filtering FBS to remove debris, especially for cell sorting)

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!

Antibody Staining is Affected by Five Factors

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

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?

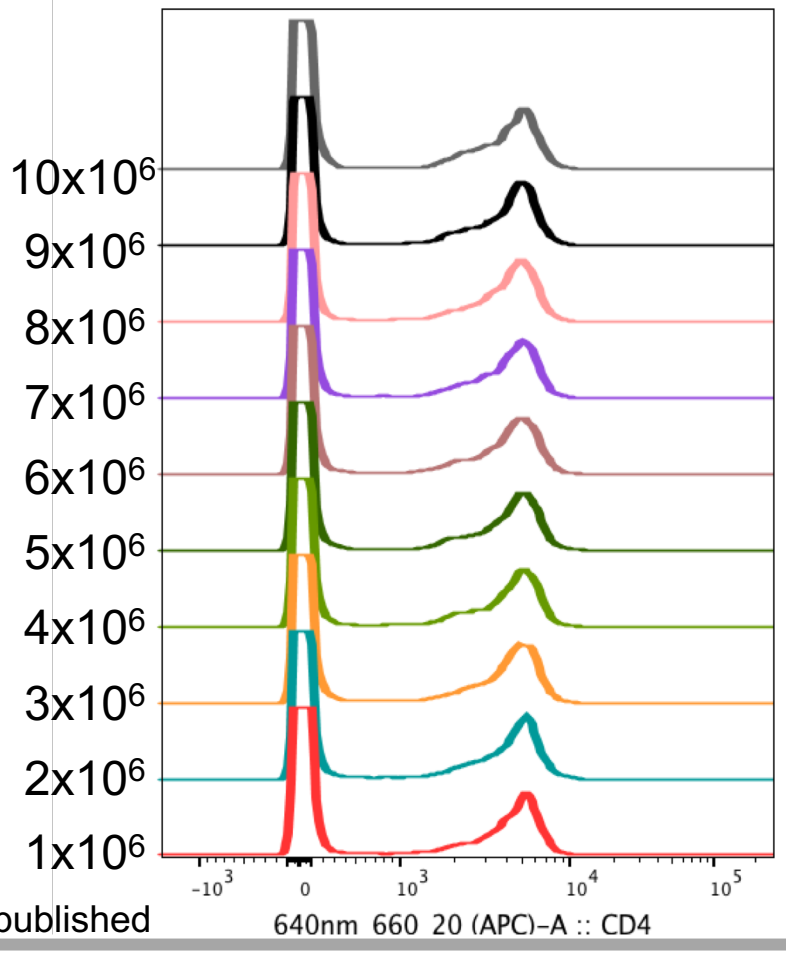
Cell Concentration

Antibody Concentration

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

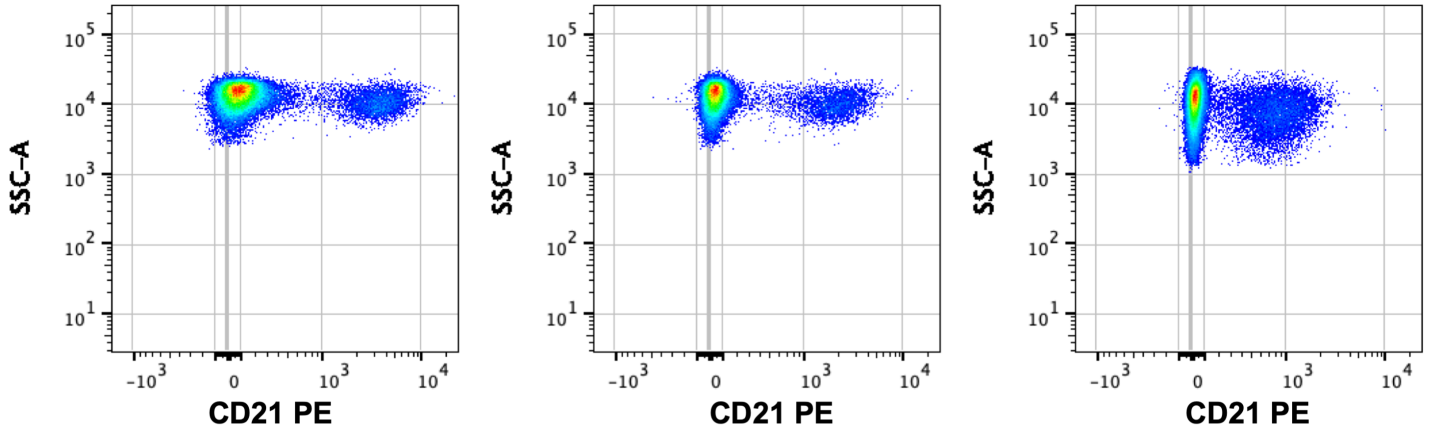
Mouse spleen cells were stained with 0.1 µg anti-CD4 (clone GK1.5) in 100 µL. Cell number was varied between 1-10x10^6.

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

Antibody Staining is Affected by Five Factors

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

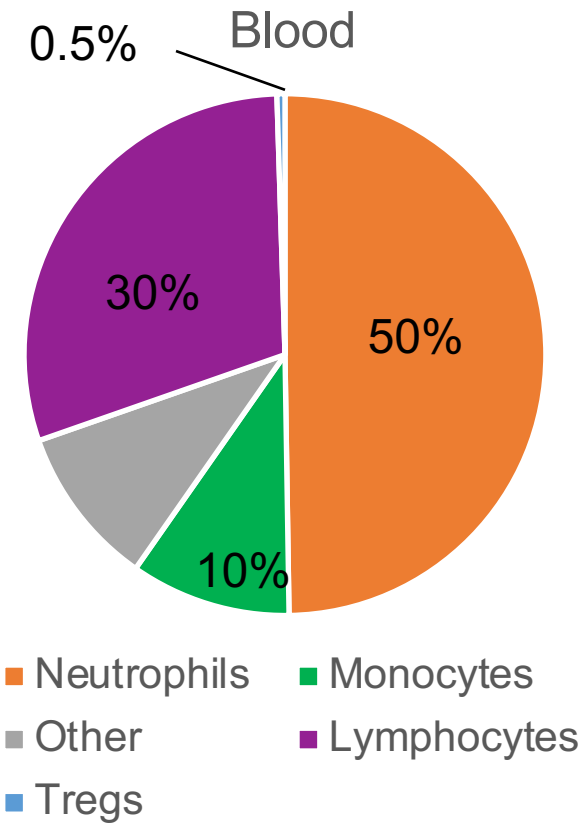
- Time, temperature, and volume are relatively easy to keep constant – usually 30 minutes at 4°C in 100 µL
- Cell number and antibody amount require more thought, but antibody concentration is much more important than cell concentration

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.

Optimizing Staining Protocol

- Antibody staining time, temperature, volume, and cell number are often parameters that you can decide. Choose these parameters and stay consistent from experiment to experiment.
  - Always read manufacturer protocols to determine if specific antibodies or dyes need a specific staining time or temp!!

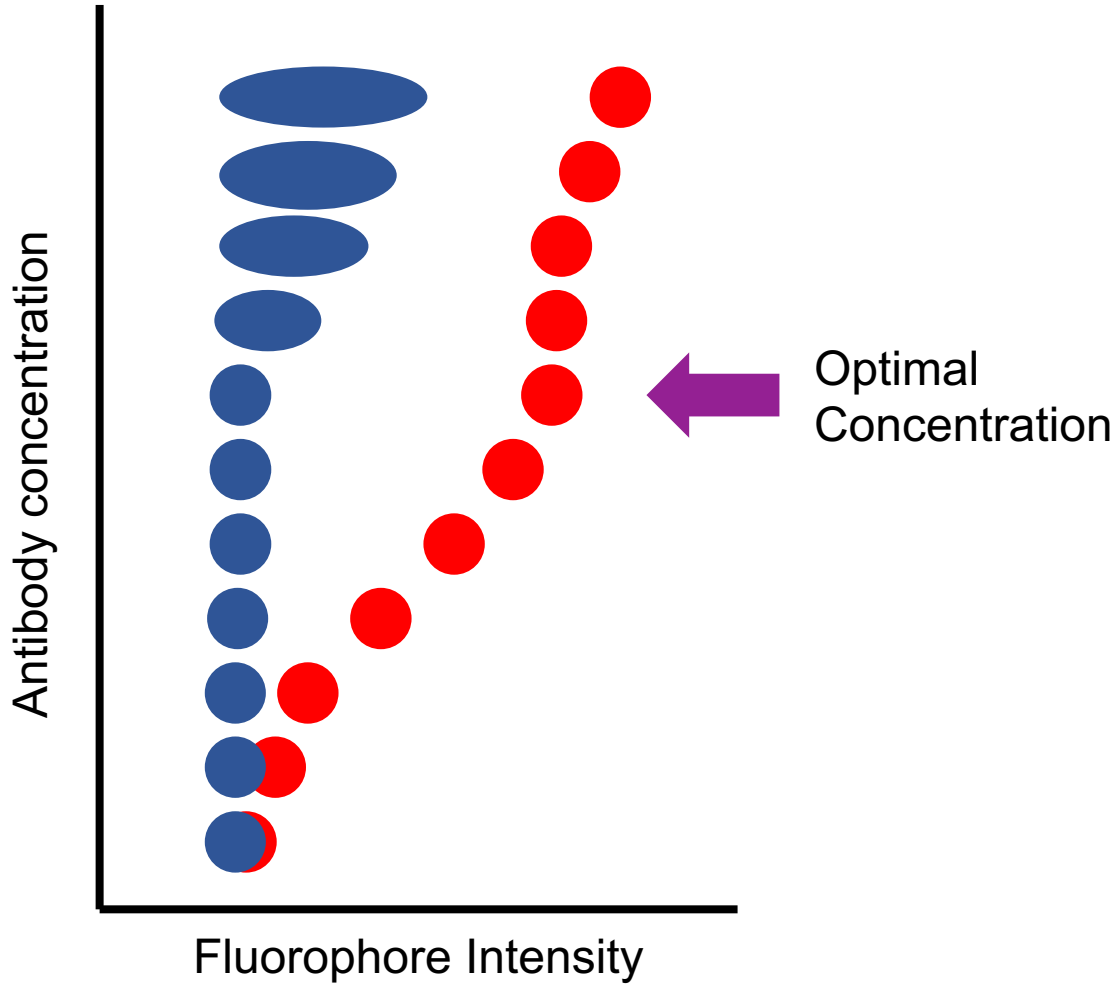
- Antibody concentration should always be optimized using an antibody titration experiment

Antibody Titration Determines the Optimal Antibody Amount

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

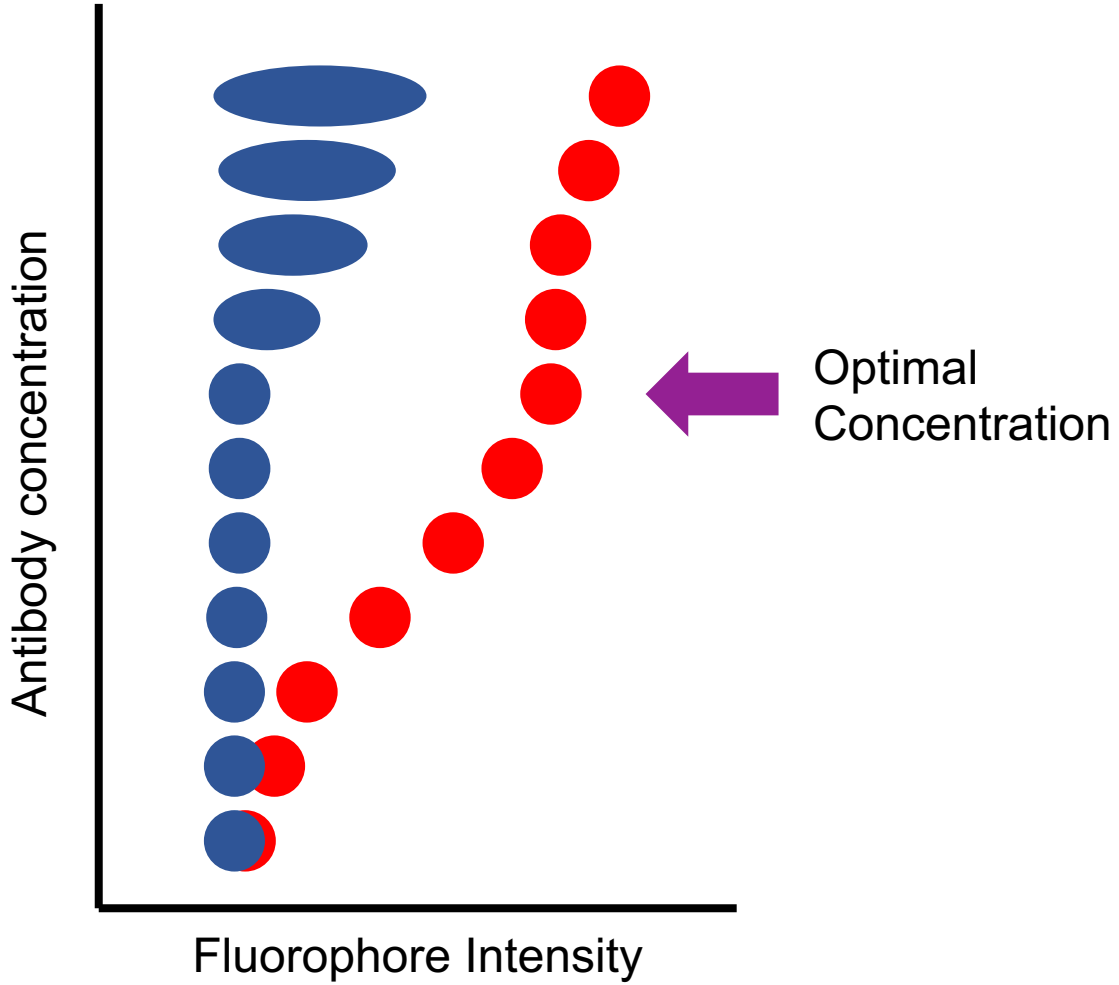
General Effect of Antibody Concentration

- 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



Staining/Separation Index (SI)

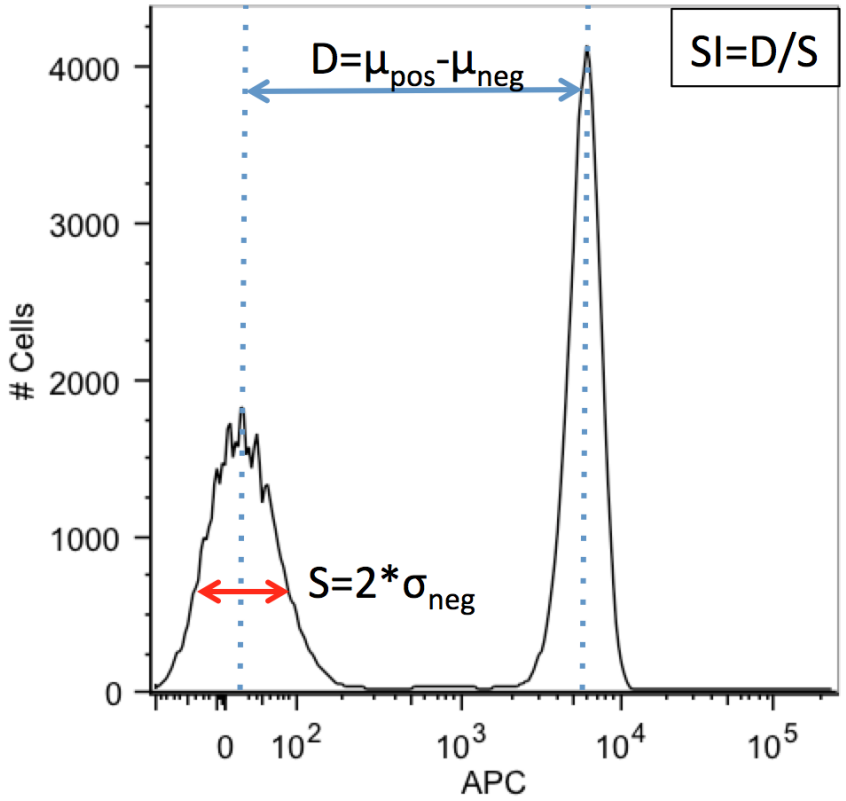
- SI takes into account the distance between the means of the positive and negative populations and the spread of the negative population
- The highest SI value will be the optimal antibody concentration
  - Maximizes the distance between the positive and negative populations
  - Minimizes the spread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



Calculating Staining Index

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

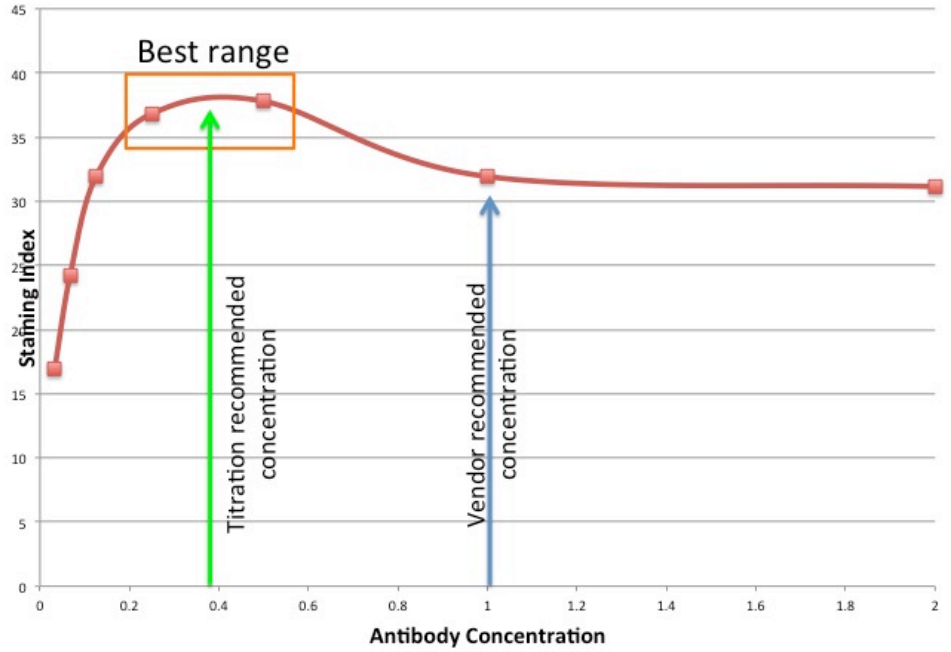
- $\mathrm{SI} = \frac{MFI_{pos} - MFI_{neg}}{2 \times SD_{neg}}$



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
  - If it is a small population, you may need other antibodies to gate the population
  - You should have a population with both a positive peak and a negative peak
  - 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
- If I know that I've titrated a PE antibody to 0.1 µg in 100 µL and I decide to switch the fluorophore to FITC (same antibody clone), then I know that I easily calculate and use 0.1 µg in 100 µL even though the PE antibody has a concentration of 0.2 mg/mL and the FITC antibody has a concentration of 0.5 mg/mL
- If I keep track of my antibody titrations as "1:100" or "2 µL", then it is more work to switch fluorophores on antibodies
- 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

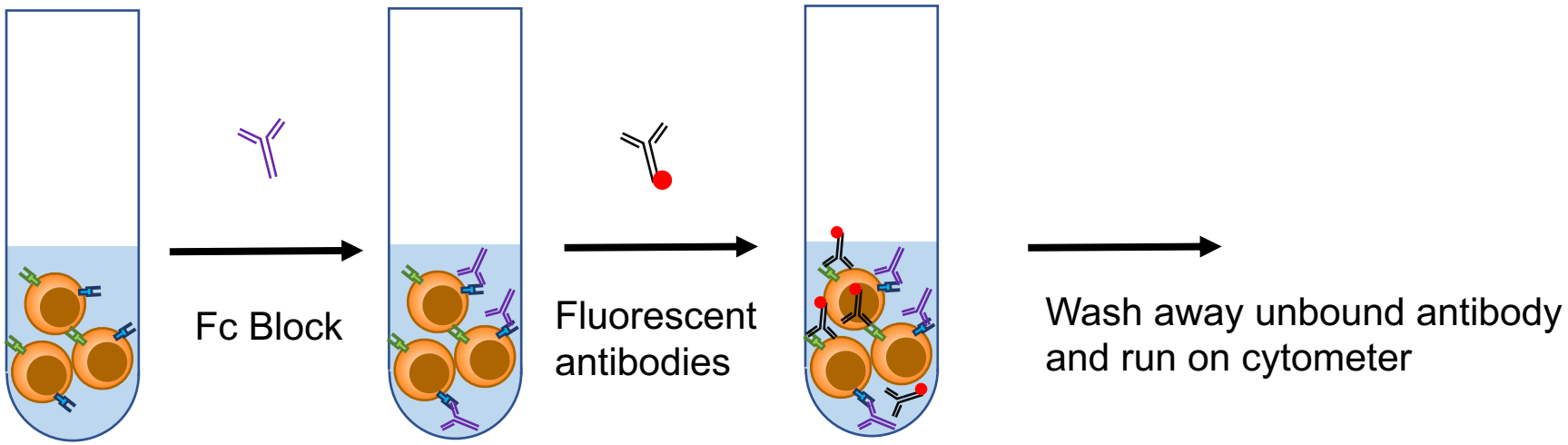
Pipetting – another source of variability

- Avoid pipetting small volumes of antibody
  - If you must pipette less than 1-2 µL, make a 1:10 dilution (pipette 5 µL instead of 0.5 µL)
  - Make a master mix of your antibody cocktail to ensure all of your samples are stained with the same amount of antibody

- Simplify your pipetting
  - You can block your cells in 50 µL
  - Add FACS buffer to master mix to bring up to 50 µL
  - To stain, add 50 µL cells and 50 µL master mix for a total staining volume of 100 µL

	1X	10X
FITC	1	10
APC	5	50
PE	0.5	5
FACS Buffer	43.5	435
TOTAL	50	500

Basic staining protocol



Wash and centrifuge

- Volume depends on tube – 1 mL, 2-3 mL, or 0.2 mL
- Wash buffer with protein (BSA/FBS) helps prevent cell loss compared to PBS without protein
- Aspirate with dumping or pipetting, careful with vacuum

Resuspending Cells to Run on a Cytometer

- If you are new to flow cytometry, it is safer to resuspend your cells (especially controls) in a larger volume. This will allow you to properly set the instrument settings without running out of sample
  - As you become more comfortable with the cytometer, you can use more concentrated controls so that they run faster on the instrument
- The volume to resuspend cells in depends on the instrument
  - Fortessas
    - 150 µL **absolute minimum**, 300-500 µL minimum for new users, 150-300 µL for advanced users
    - In general, run samples at less than 35,000 events/sec
  - Attune: see chart here
  - Sorters
    - 500 µL **absolute minimum**
    - 70um tip (lymphocytes, splenocytes, small cells): 20-25 million cells per mL
    - 100um tip (most cell culture, lung and liver cells, etc): 10-15million cells per mL

Basic Staining Protocol: Additional Notes

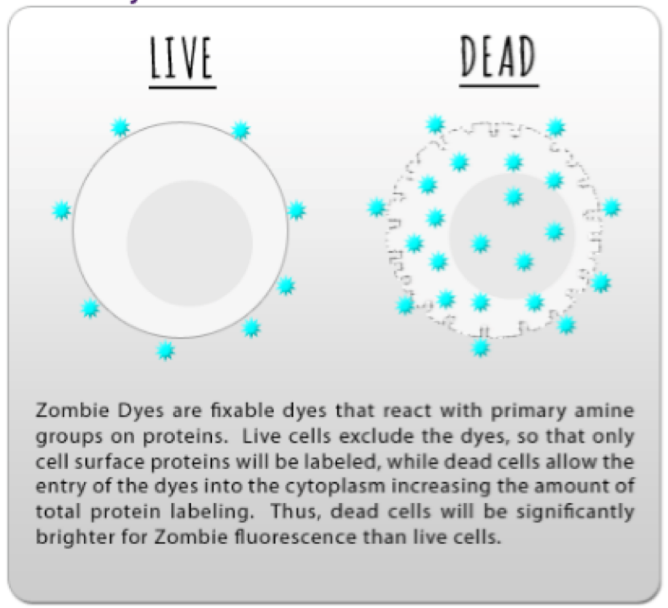
- Choosing a viability staining to remove dead cells from analysis
- Using primary antibodies vs primary and secondary antibodies

Viability markers

- Dead cells will:
  - Bind some antibodies non-specifically
  - Skew statistics
- Amine-binding
  - Fixable (Biolegend Zombie dyes or ThermoFisher Fizable Live/Dead dyes)
  - Stain ***before antibodies*** and **before** fixation (fixation not required)
  - Staining buffer must be free of protein – read the manufacturers protocol!!
- DNA-binding
  - Non-fixable
  - Stain ***after antibodies***, right before acquisition
- More info:

https://www.biolegend.com/en-us/live-dead

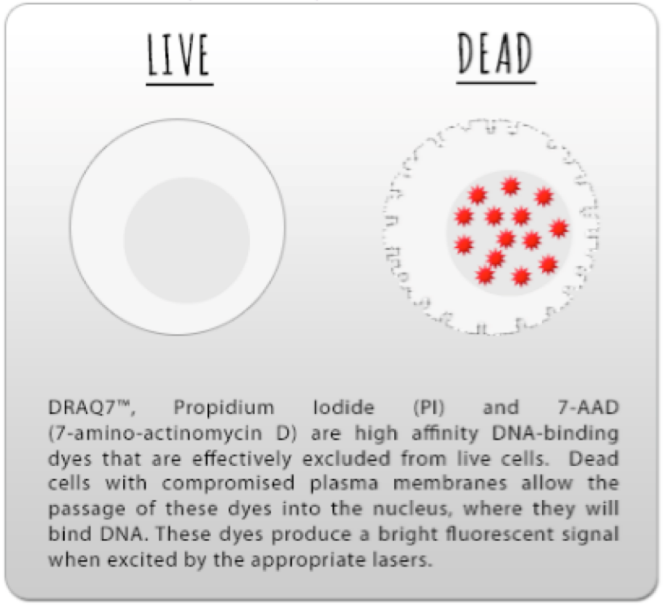
Zombie Dyes



Zombie Dyes are fixable dyes that react with primary amine groups on proteins. Live cells exclude the dyes, so that only cell surface proteins will be labeled, while dead cells allow the entry of the dyes into the cytoplasm increasing the amount of total protein labeling. Thus, dead cells will be significantly brighter for Zombie fluorescence than live cells.

*DRAQ7™ is a trademark of Biostatus Limited.

Helix NP™ NIR, DRAQ7™, PI and 7-AAD



DRAQ7™, Propidium Iodide (PI) and 7-AAD (7-amino-actinomycin D) are high affinity DNA-binding dyes that are effectively excluded from live cells. Dead cells with compromised plasma membranes allow the passage of these dyes into the nucleus, where they will bind DNA. These dyes produce a bright fluorescent signal when excited by the appropriate lasers.

Product Links: Helix NP™ NIR, DRAQ7™, PI, 7-AAD

Using Primary and Secondary Antibodies

- Flow cytometrists prefer to use fluorophore-conjugated primary antibodies as opposed to unconjugated primary antibodies and fluorophore-conjugated secondary antibodies
  - Using a primary antibody instead of a primary and secondary:
    - Only requires one incubation time and wash steps instead of two
    - Requires less optimization (one antibody instead of two)
    - Makes the staining protocol easier

- You are welcome to use primary and secondary antibodies if you want – just titrate both antibodies!

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

Many Factors Affect Flow Cytometry Data: Staining Protocol Summary

Staining Protocol

- When staining with antibodies, stay consistent with:
  1. Time
  2. Temperature
  3. Volume
  4. Cell number
  5. Antibody amount
- Titrate antibodies!!
- Beware of pipetting error by using a master mix

Panel Design

- Number of markers
- Antigen expression level
- Antigen co-expression
- Fluorophore brightness
- Gating strategy

Instrument Setup

- Voltage Settings
- Compensation controls

Panel Design

- Full details are covered in a separate course!

- Why spend time on panel design?
  - The larger the panel, the more important panel design becomes
  - Fluorophores are not the same
    - Example: pairing a low expressed marker with a dim fluorophone will not give optimal results

- Check our website for panel design resources: https://voices.uchicago.edu/ucflow/panel-design/

Instrument Setup

- Single stained cells are use to set voltages on the instruments
- Setting voltages can be challenging and we have tried to provide optimized starting voltages that will work for many people
  - Read more about voltage optimization here

Experimental Design

Considerations for Experimental Design

- Controls
  - Compensation
  - Gating controls for data analysis
  - Staining controls
- Deciding on number of cells to stain
- Repeating experiments or splitting large experiments into multiple days

Single Stain/Compensation Controls

- Single stain controls are used to set voltage and determine compensation
- The single stains can be the cells of interest OR you can use other cells or compensation beads
  - It is better to use cells to set voltages, but compensation beads can be used to compensate once voltage is set
- NOTE: if you are using fluorescent proteins (Ex: GFP mice), you must use non-fluorescent cells for your controls so that they contain only one fluorophore. A control containing only the fluorescent protein is needed as well.

Rules for Compensation Controls

1. The positive population must be as bright or brighter than the multicolor sample
2. Fluorophore used for the positive control needs to be the same as to the one in the multicolor sample
   - For example, FITC should not be used to compensate GFP
3. The control should ideally contain a positive and negative population, and these populations must have the same autofluorescence properties
   - Beads and cells have different autofluorescence properties
   - You can combine beads and cells within an experiment, but not the same fluorophore
     - PE can be on cells and FITC can be on beads, but for the PE control, the positive population and negative population must both be cells
4. Controls should have sufficient events in both positive and negative populations

Controls – Cells vs Compensation Beads

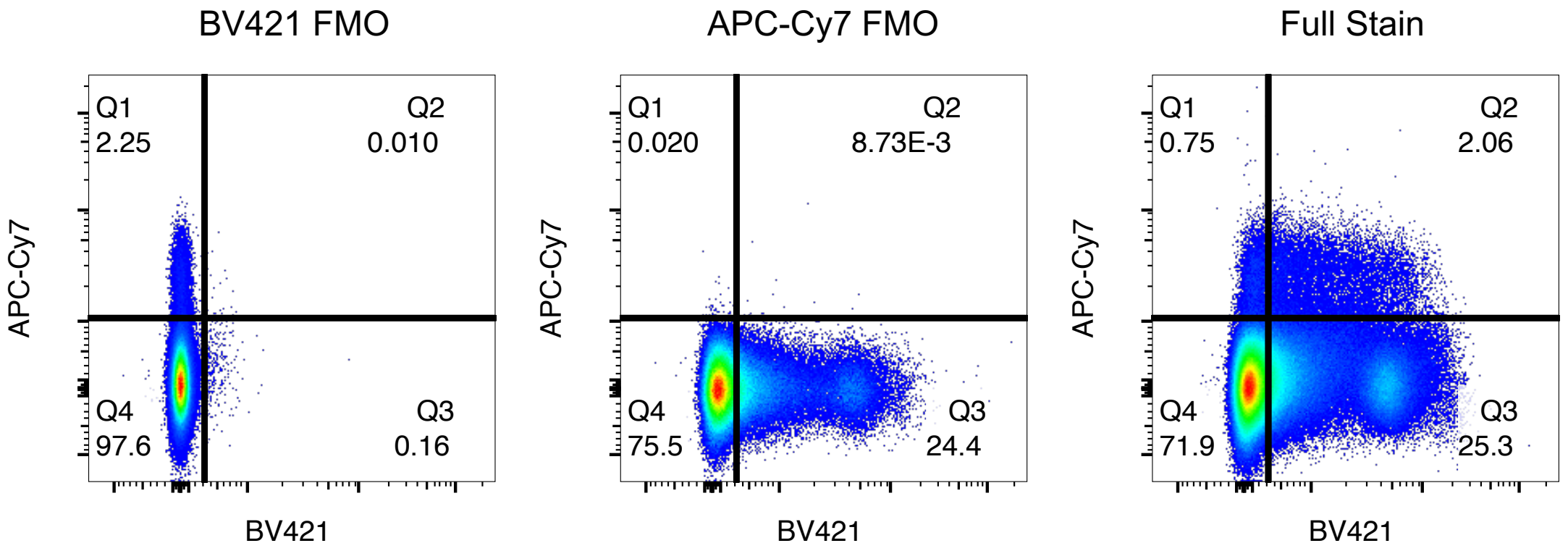
- Cells
  - Pros
    - Same cells as your sample
    - Does not require extra reagents (compensation beads)
  - Cons
    - Difficult when cell number is limited
    - Difficult for poorly expressed or rare antigens

- Compensation Beads
  - Pros
    - Great for poorly expressed and rare antigens
    - Does not use up precious cells
    - Clear positive and negative population
  - Cons
    - Can’t be used for dyes or fluorescent proteins
      - NOTE: GFP beads exist and there are beads for fixable live/dead stains
    - Sometimes not good for setting voltages

Controls – Fluorescence Minus One (FMO)

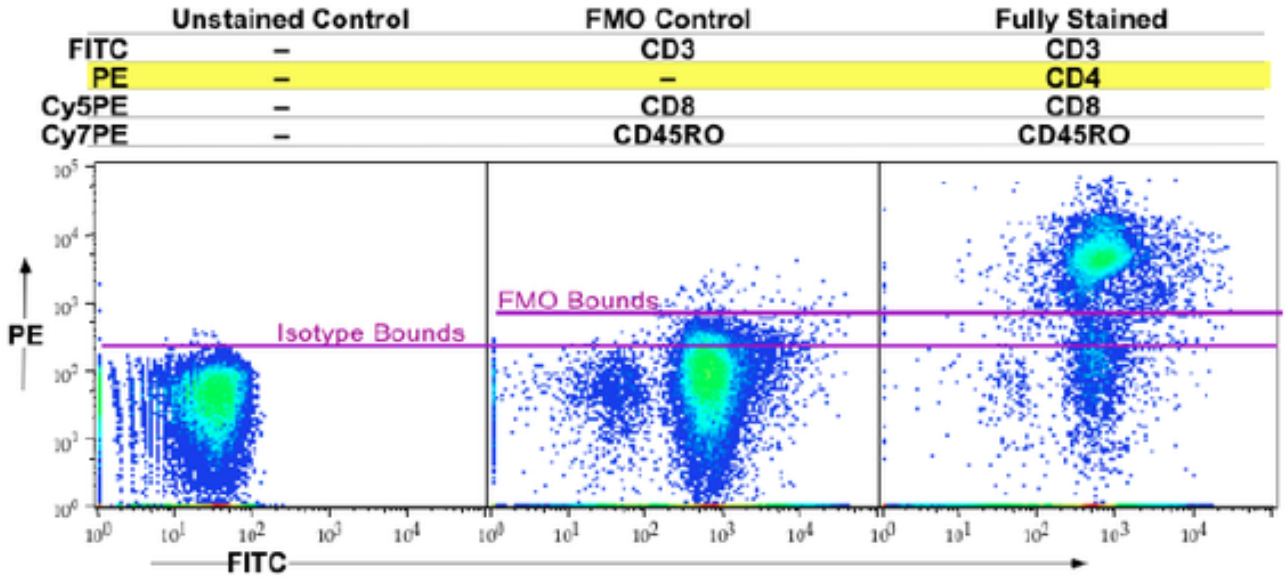
- FMO controls contain all fluorophores except for one
- FMOs allow us to draw gates to define positive or negative populations
- A 5-color panel will have 5 FMOs
  - Ex: The FITC FMO contains all 4 fluorophores except FITC
- Ideally, all FMOs should be used
  - Realistically, FMOs are only required for markers that do not have clear separation between the positive and negative populations
- The larger the panel, the more important it is to use FMOs
- Pipetting FMOs can be tricky! Here's how I avoid mistakes

Use FMOs to Draw Gates



Controls – Isotype Controls

- Unlike FMOs, isotype controls do NOT identify gating boundaries
- Isotype controls identify staining issues, such as excessive background staining
- Isotype controls used to be popular before FMOs, but now they are rarely used

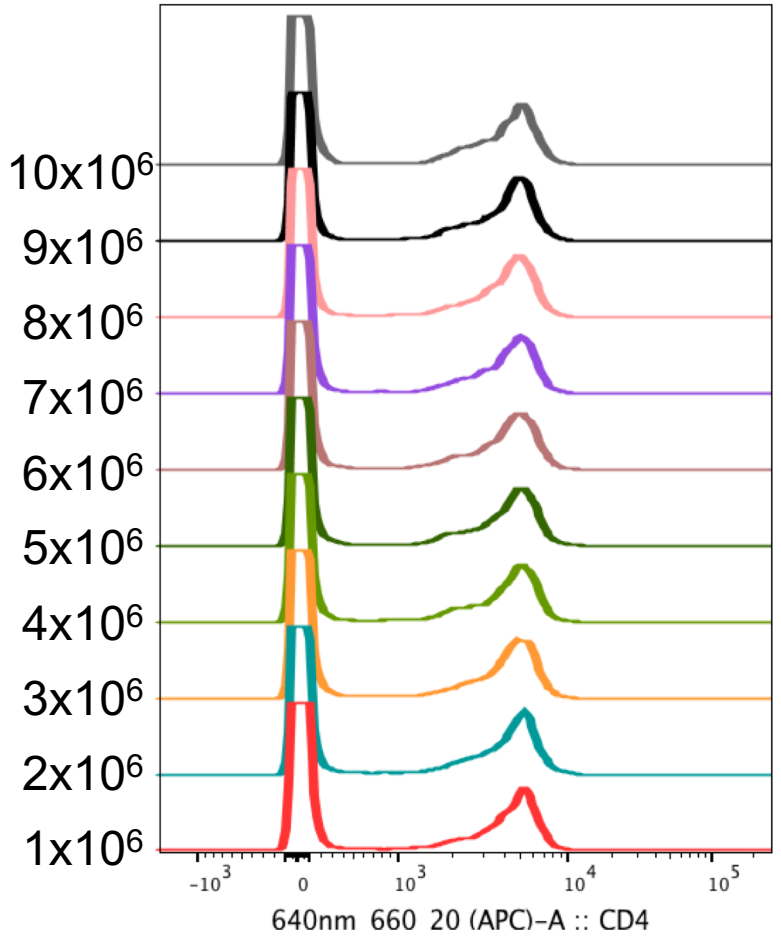


Unstained control

- The unstained control identifies sample autofluorescence
- The unstained control is not absolutely required unless you are using the autocompensation wizard in FACSDiva or the aurora
- The unstained control can be useful for setting FSC and SSC (to avoid wasting sample) or if you have single stained cells that lack a negative population

How many cells to stain – Dividing cells between sample and controls

- Ideally all controls and samples should have the same number of cells – everything should have the same staining protocol (time/temp/volume/cell number/antibody amount)
- Sometimes there just aren't enough cells to do this
- Remember: there is some flexibility in cell number for the staining protocol
- Read my blog post on how many cells to stain here



How many cells to stain – Dividing cells between sample and controls

- Compensation controls
  - I generally assume a titrated antibody is good for up to $5x10^6$ cells (though some antibodies are more sensitive to cell number)
  - Compensation controls need enough cells to see a positive and negative population, and the staining needs to be as bright or brighter than the multicolor sample (but not so bright that the fluorophore is saturating the detector)
- FMO Controls
  - Are all needed?
  - Depending on the marker, some FMOs need lots of cells to define a rare population, whereas others might not need as many cells

How many cells to stain – scaling up staining conditions

- If there is a large discrepancy between the cell numbers in the controls and the sample, staining conditions may need to be scaled up
- This technique can be used when moving from a benchtop analyzer to a sorter

	Cell Number	Staining Volume	Antibody Volume
Control	0.25×10^6	100 µL	1 µL
Sample – exact scale up	5×10^6	2 mL	20 µL
Sample – option 1	5×10^6	1 mL	10 µL
Sample – option 2	5×10^6	0.5 mL	5 µL

20µL is a lot of antibody and unnecessary!!

Note how the antibody concentration is always the same!

Repeating Experiments

- Again – best practice is to keep everything the same, meaning use the **exact same instrument** if you can
- Using the same instrument is more important if you are looking at upregulation/downregulation (change in fluorophore intensity)
- Less important for frequencies of populations

Summary: Steps for Performing a Flow Cytometry Experiment

1. Determine goals of experiment
   - Decide how many cells are needed
   - Decide what controls needed to properly analyze results
2. Choose markers and design panel
3. Titrate antibodies
4. Optional but recommended: test full panel on tissue of interest
   - Strongly recommended if titrations were done on a different tissue
5. Run experiment