THE UNIVERSITY OF CHICAGO BIOLOGICAL SCIENCES

Flow Basics 2.4: Controls and Experimental Design

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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:
  - Discuss types of controls and when to use them
  - What to do if you don't have extra cells for controls

Controls

Types of Controls

Single Stains

- Serve two purposes: setting voltages on the instrument and calculating compensation
- Single stains are required for accurate compensation

Gating

- Determine which cells are stained and antibody
- Not required but very beneficial

Staining

- Used for troubleshooting
- Determine if there is nonspecific staining

Other Controls

- Unstained control
- Experimental Controls
- Absolute cell counts

Single Stain/Compensation Controls

- Controls stained with a single fluorophore
  - Example: for a 6-color panel, 6 individual single stain controls are needed
- The single stains can be the cells of interest, any other cells type that expresses the marker, or compensation beads

Tips for Staining Single Stain/Compensation Controls

- Avoid pipetting small volumes of antibody
  - If you must pipette 0.5 µL, make a 1:10 dilution and pipette 5 µL
- Sometimes you need to “manufacture” controls to get good staining
  - E.g. kill some cells for the viability dye control

- 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.

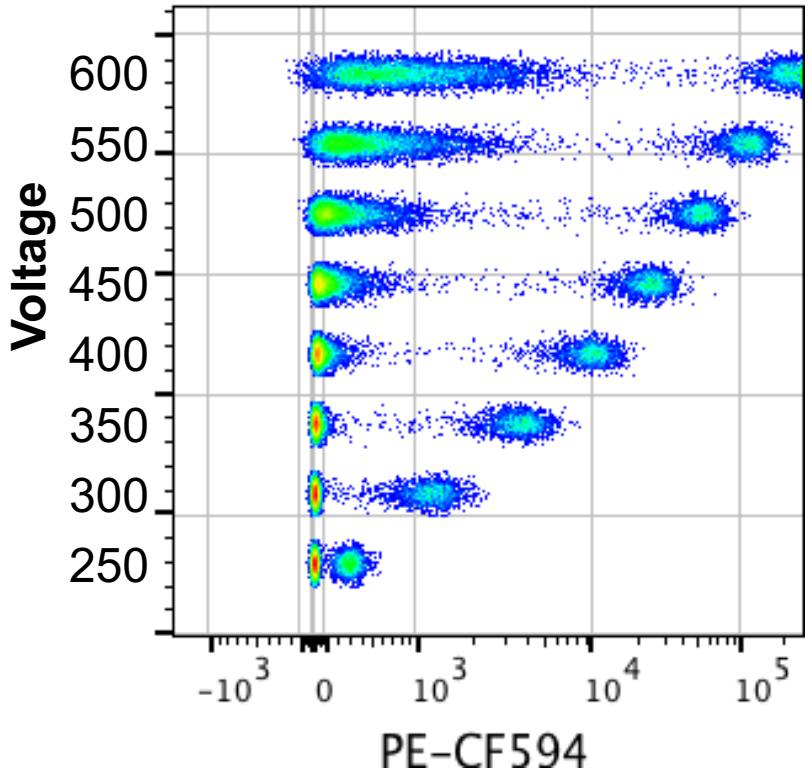
Single Stain Controls Serve Two Purposes

1. Setting voltages/gains on the cytometer

2. Calculating compensation (or spectral unmixing)

Single Stains are Used to Set Voltage

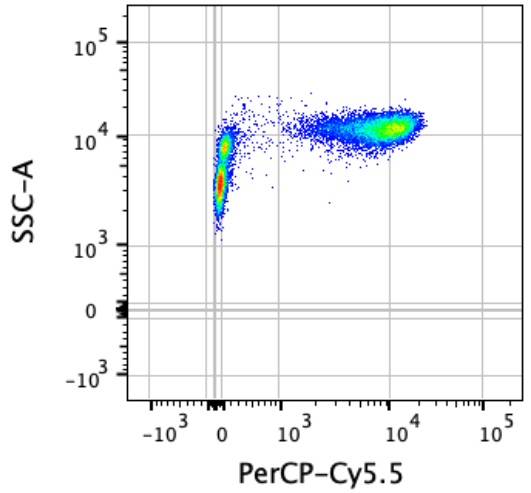
- Similar to antibody concentration, instrument voltage is another factor that can affect the separation of your positive and negative populations
  - Too high and your positive population could be off the plot (saturating the detector)
  - Too low and you may not be able to visualize the positive population
- Optimal separation of the positive population is achieved when BOTH antibody concentration and voltage are optimized



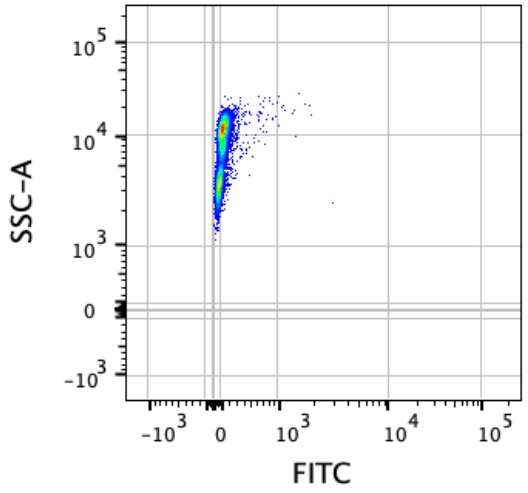
Single Stains are Used to Calculate Compensation

- Since only one fluorophore is in the tube, it can be assumed that signals in other detectors is due to spillover, which will be removed with compensation

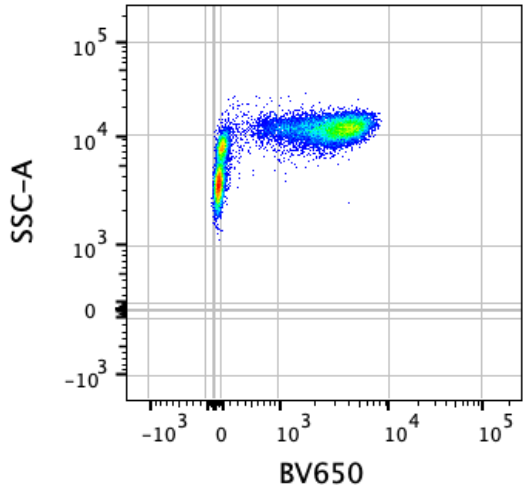
Positive signal from PerCP-Cy5.5



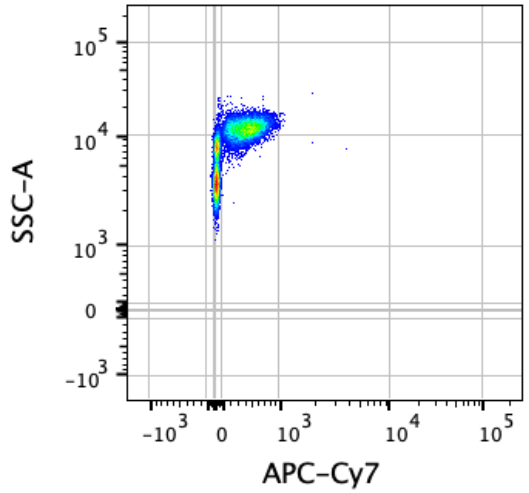
PerCP-Cy5.5 doesn't spill into FITC



PerCP-Cy5.5 spills into BV650

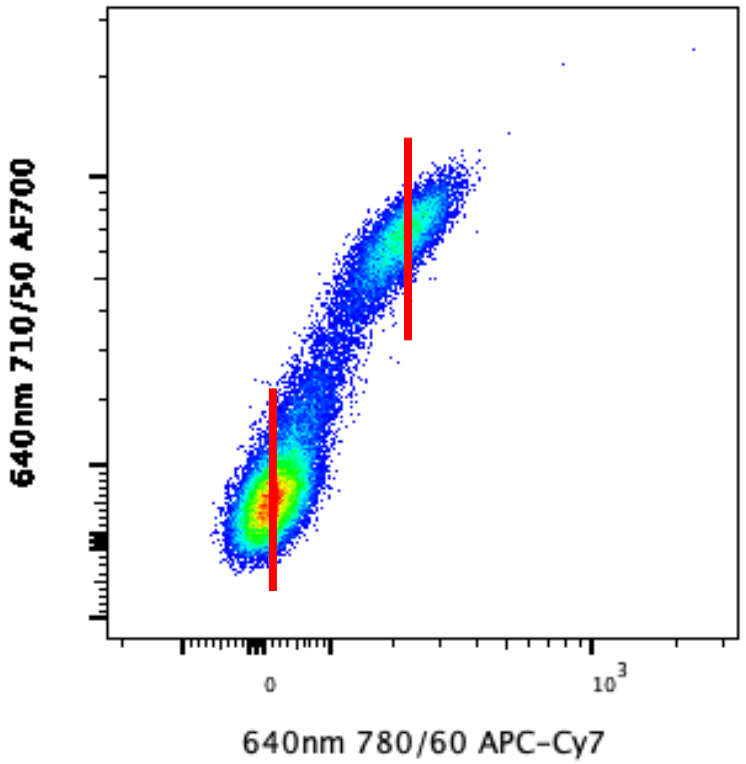


PerCP-Cy5.5 spills into APC-Cy7

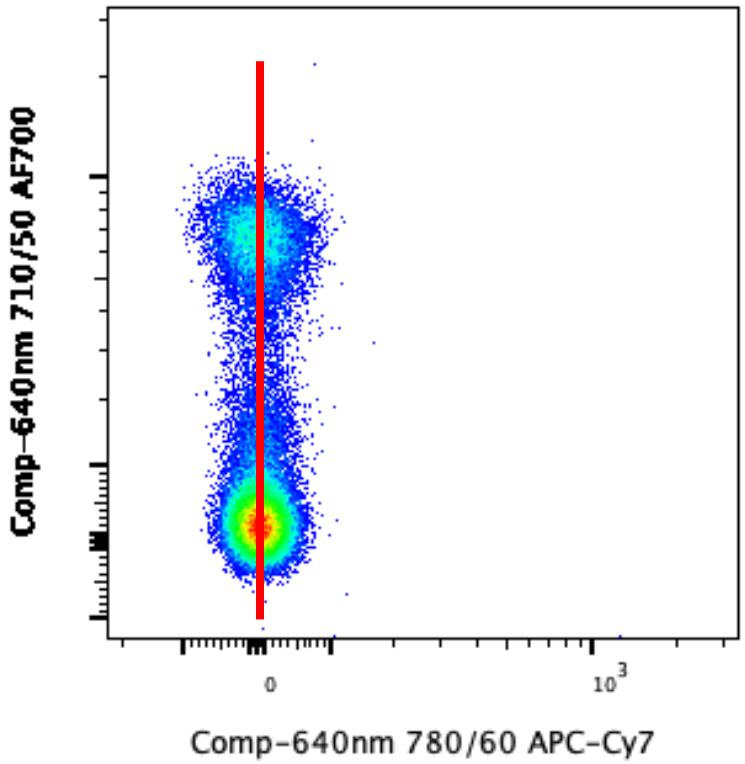


Compensation will align the median of the positive and negative populations

Uncompensated

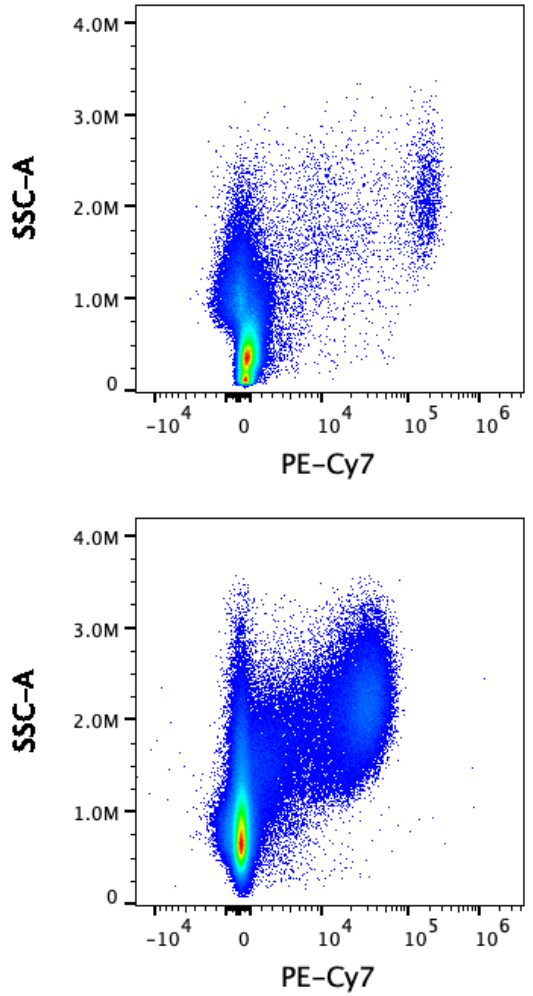


Compensated



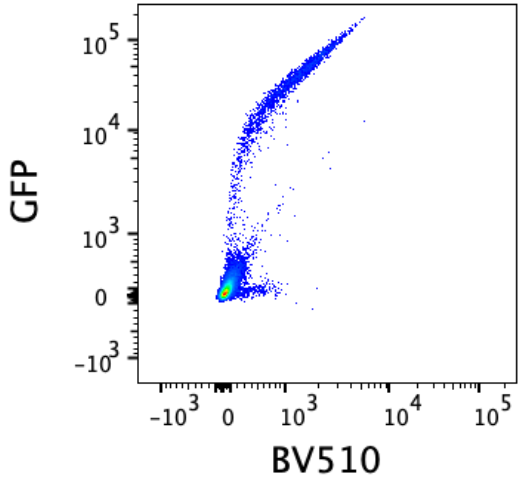
Rules for Compensation Controls

1. The positive population must be as bright or brighter than the multicolor sample
   - A compensation control that is dimmer than the sample can lead to compensation errors

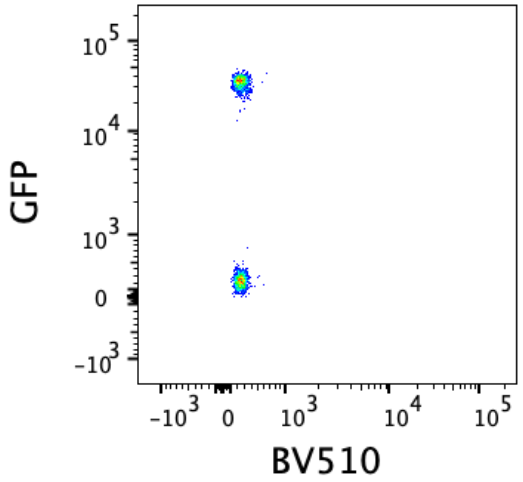


Rules for Compensation Controls

2. The control should ideally be exactly the same fluorophore as the one in the sample
   - E.g. compensating GFP using a FITC single stain control can cause compensation errors



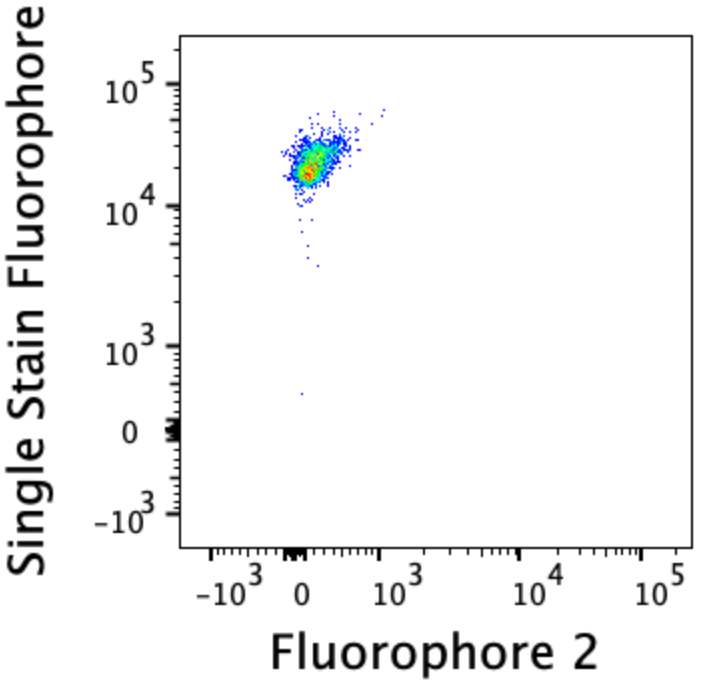
GFP Single stain



FITC Single stain

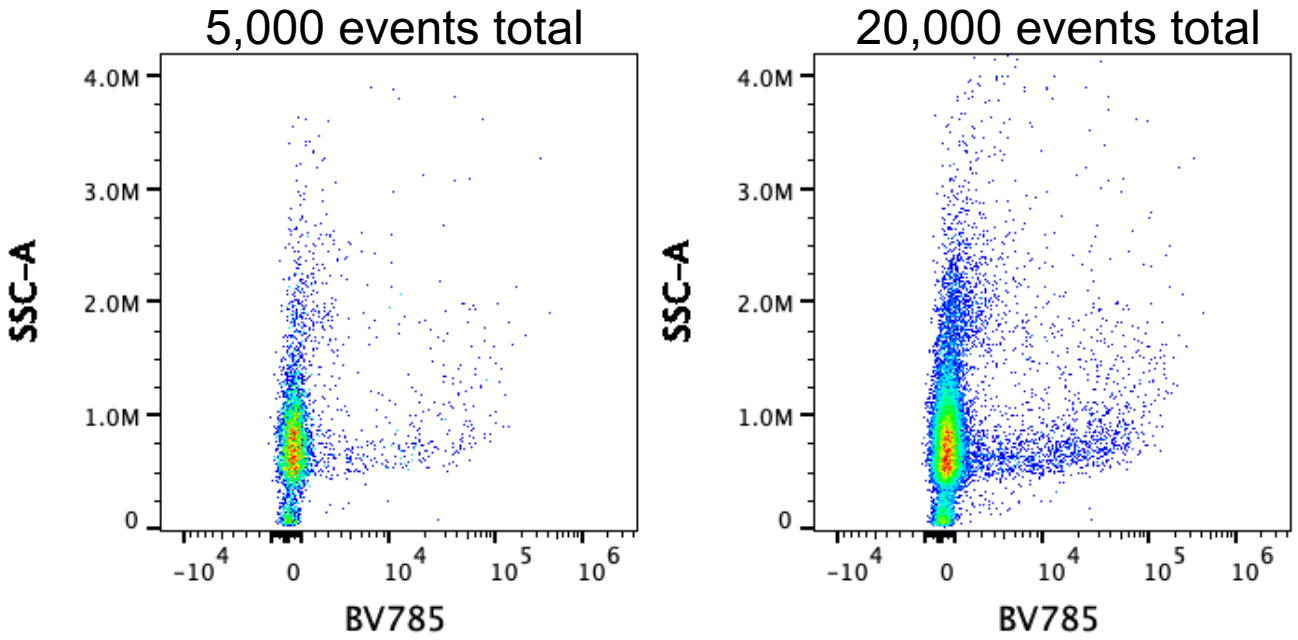
Rules for Compensation Controls

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



Rules for Compensation Controls

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 and/or calculating compensation

Types of Compensation Beads

- Not all compensation beads are the same – vary in binding to isotypes and dyes, negative beads mixed in or separate
- Note: not all beads bind to polymer fluorophores (brilliant violet, BUV, BB)
- Popular compensation beads from:
  - ThermoFisher: https://www.thermofisher.com/us/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-calibration/flow-cytometry-compensation-tools.html
  - BD: https://www.bdbiosciences.com/us/reagents/research/instrument-setup-maintenance/compensation-particles/c/2046008

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Other Controls

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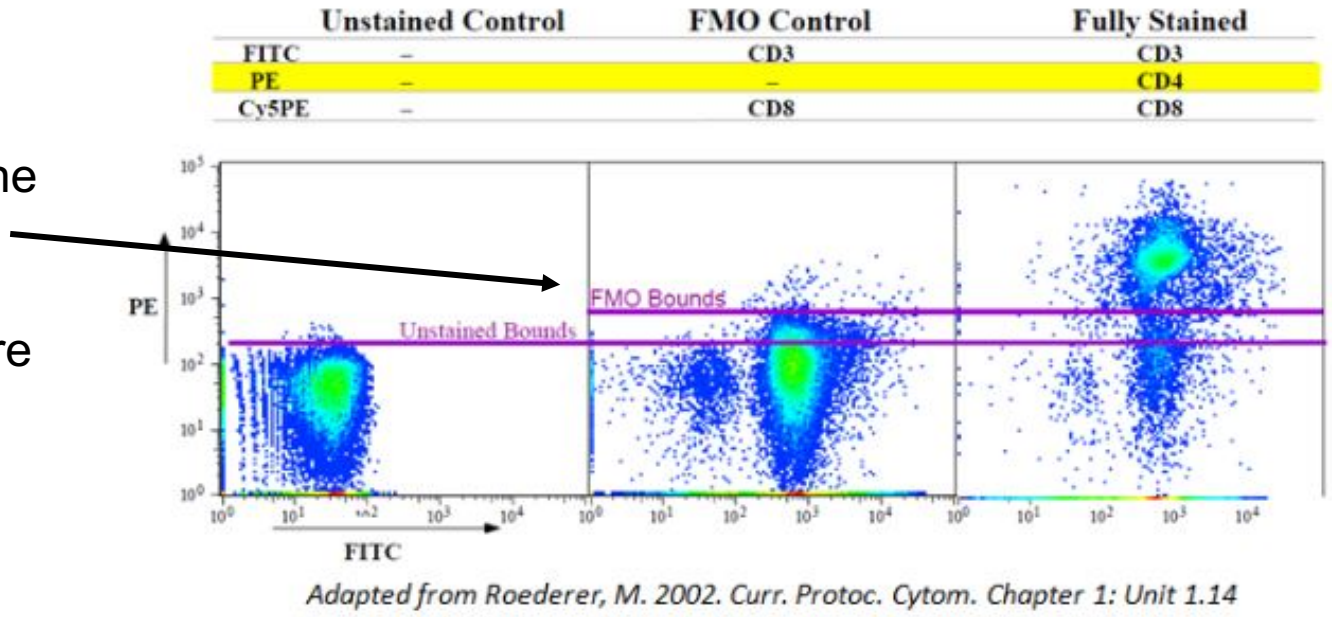
- Absolute cell counts

Gating Controls – Fluorescence Minus One (FMO)

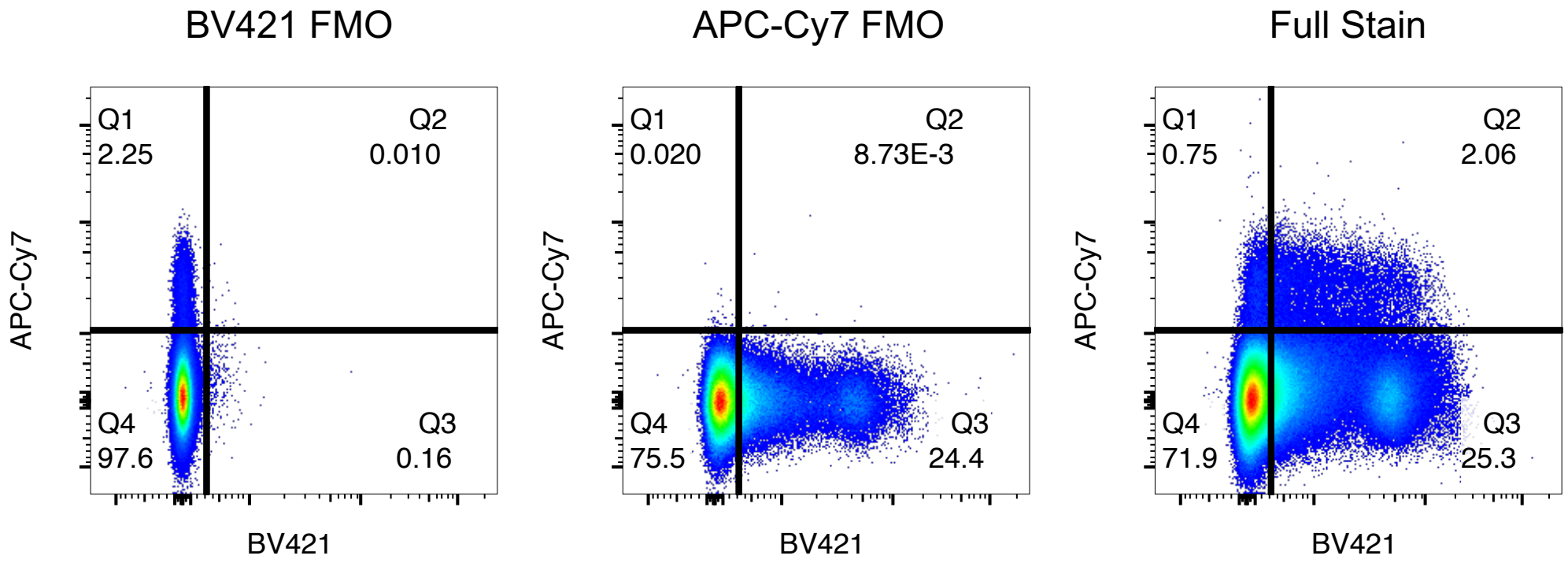
- FMO controls contain all fluorophores in the panel except for one
  - Ex: The PE FMO contains all 4 fluorophores except PE. A 5-color panel can have 5 individual FMOs
- FMOs allow us to draw gates to define positive or negative populations

The FMO is often slightly brighter than the unstained control because of spillover-spreading error (a more complicated topic not discussed here)



Adapted from Roederer, M. 2002. Curr. Protoc. Cytom. Chapter 1: Unit 1.14

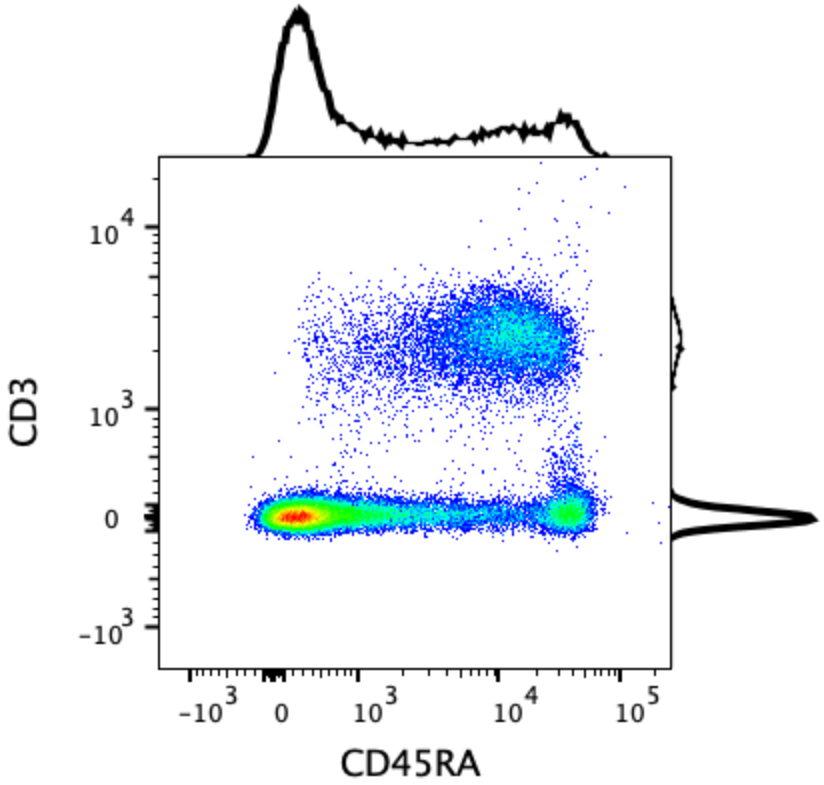
Use FMOs to Draw Gates



- Since all of the other fluorophores are in the FMO, you can gate on a specific subset before using the FMO (e.g. $CD3^{+}CD4^{+}FoxP3^{+}CD127^{lo}$ cells before looking at ICOS)

When to use FMO controls

- *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 (e.g. CD45RA but not CD3)
- The larger the panel, the more important it is to use FMOs



How to pipette FMOs

- Don’t forget to dilute antibodies to avoid pipetting less than 1-2 microliters!
- Watch this video for my process: https://youtu.be/9HXIRZf6rzw

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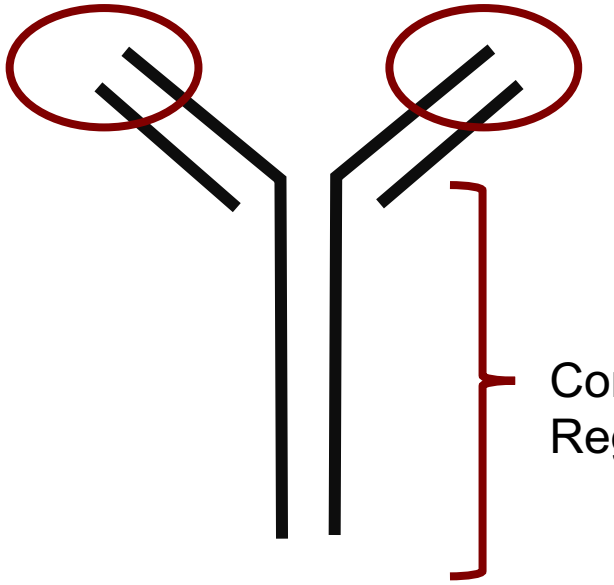
Staining Controls – Isotype Controls

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

Variable Region = binds to marker of interest (epitope)

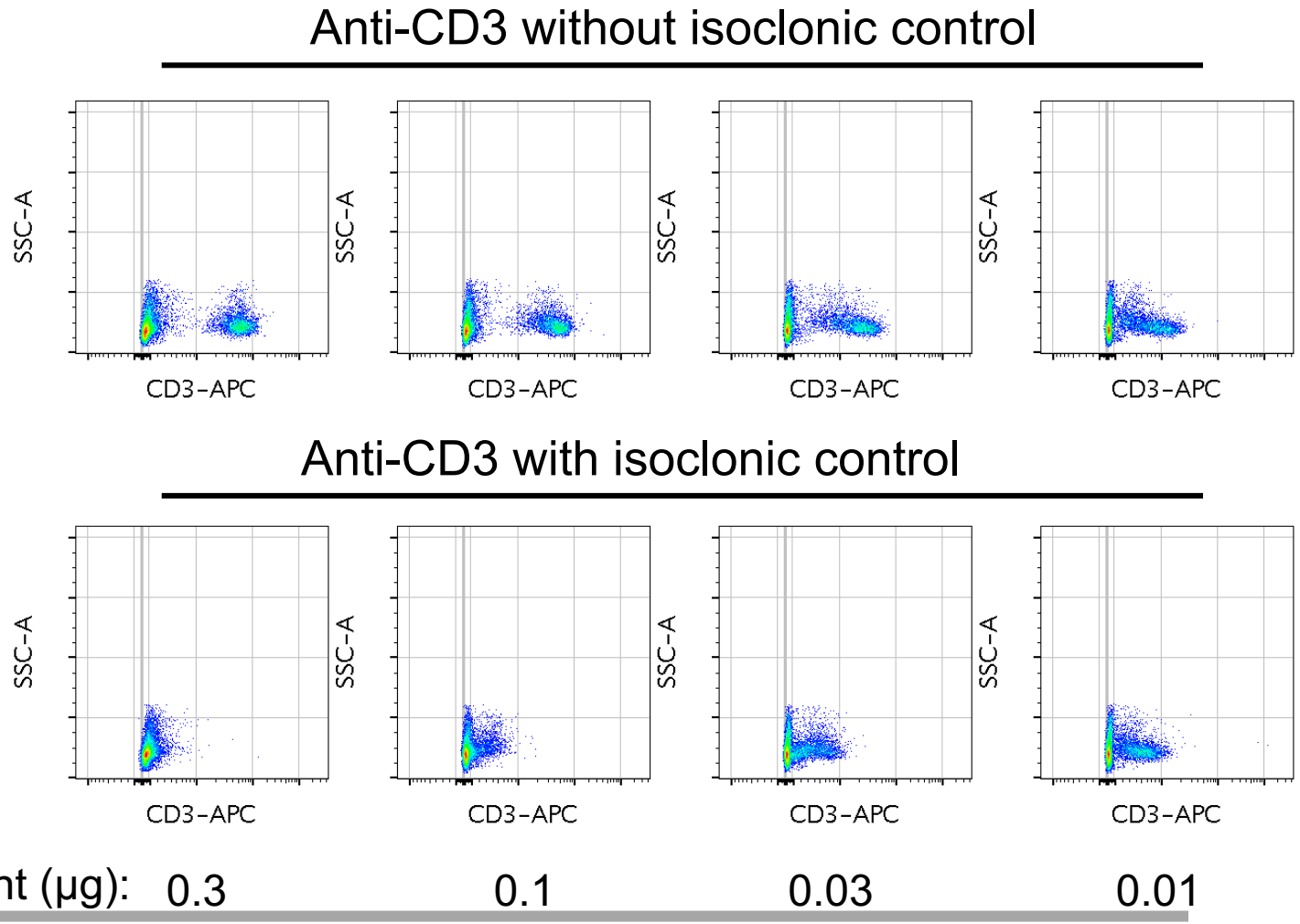


Constant Region

Isotype control: same constant region, different variable region

Staining Controls - Isoclonic Control

- An isoclonic control is the same antibody clone without a fluorophore conjugated
  - Cells are first incubated with unconjugated antibody, then stained with fluorophore-conjugated antibody
- Purpose – to identify nonspecific staining



How to proceed if staining controls indicate excessive background staining

- Optimize blocking step to see if background staining can be reduced
  - E.g. increase concentration of FBS/BSA in staining buffer, add in heparin, add in other serum, etc.
- Try a different clone for the antibody

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Other Controls

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Unstained control

- The unstained control identifies sample autofluorescence
- The unstained control is recommended but not absolutely required
  - EXCEPTIONS: the unstained control is required for the autocompensation wizard in FACSDiva (Fortessas) or the Cytek Aurora spectral cytometer. The unstained control is also required if any single stain controls lack a negative population
- 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

Experimental Controls

- Depending on the experiment, experimental controls can be helpful:
  - Unstimulated vs stimulated cells
  - Untreated vs treated cells
  - WT vs KO cells
    - Sometimes staining a KO mouse with an antibody for the same protein can be useful (e.g. anti-IL-5 antibody on IL-5 KO mouse)

Absolute cell counts

- If absolute cell counts are important for the experiment, there are several considerations

- Counting beads can be purchased and added to the sample
  - Will work for any instrument
- Some of our instruments have a volumetric counter that can be used to calculate absolute counts without additional counting beads
  - The ThermoFisher Attune NxT and the Cytek Aurora have a volumetric counter
- If running PBMCs or human blood, consider doing a separate count so that you can calculate the count per µL of blood

Yes, there are often more controls than samples!

- For example, a 6-color experiment:

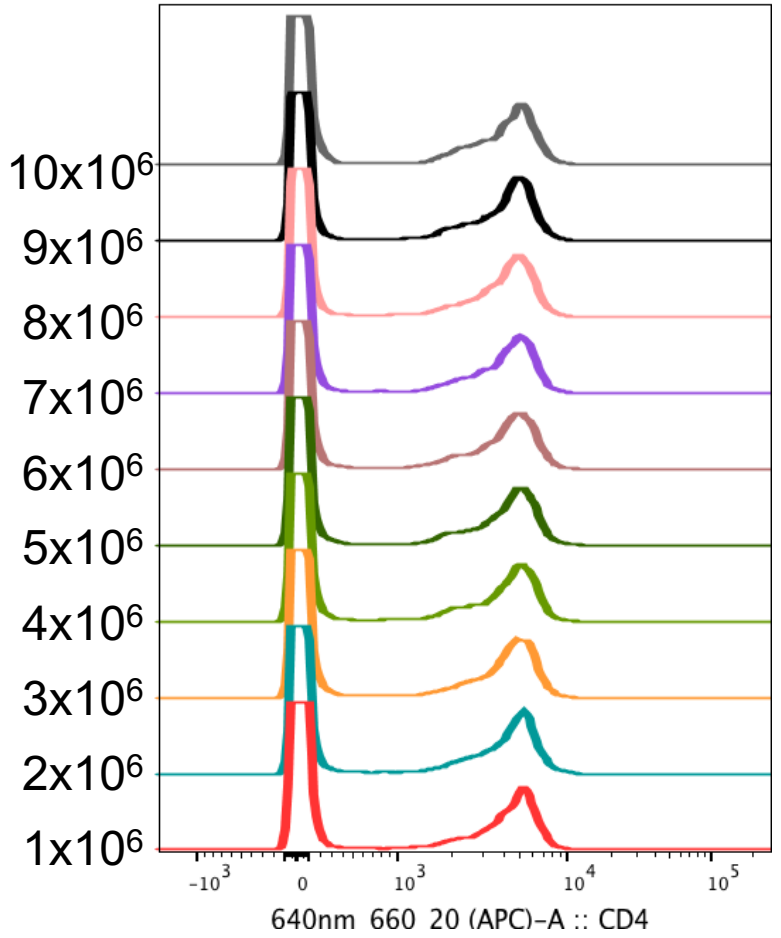
SS FITC	SS PE	SS APC	SS PE-Cy7	SS APC-Cy7	SS DAPI
FMO FITC	FMO PE	FMO APC	FMO PE-Cy7	FMO APC-Cy7	FMO DAPI
Unstained					
PBS 1	PBS 2	PBS 3	LPS 1	LPS 2	LPS 3

13 tubes for controls, 6 tubes for samples

Other Experimental Design Considerations

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



Dividing cells between sample and controls

- 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

Steps for Setting Up a Flow Cytometry Experiment

1. Determine goals of experiment
2. Choose markers and design panel
   - Plan the panel for the instrument you plan on using
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. Plan experiment
   - Decide how many cells are needed
   - Decide what controls needed to properly analyze results
6. Run experiment

Stay Tuned for the Rest of the Flow Basics 2.0 Series