THE UNIVERSITY OF CHICAGO BIOLOGICAL SCIENCES

Flow Basics 2.5: Instrument setup and Compensation

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Laura K Johnston, PhD
Scientific Associate Director
Cytometry and Antibody Technology Facility
University of Chicago

The Flow Basics 2.0 Series

Basic Workflow – Hands On

- In your lab:
  - Obtain single cell suspension
  - Stain cells with fluorescent antibodies/dyes
- At the cytometer:
  - Create your experiment layout
  - Set flow rate
  - Set voltages for FSC and SSC to get all cells on interest within the plot
  - Set voltages for fluorophores so that the fluorescent intensity is not saturating the detector (no cells on right or top axis)
  - Compensate data (optional, can be done on your own computer)
  - Run samples and controls
- In your lab:
  - Analyze data

Creating an Organized Experiment Layout

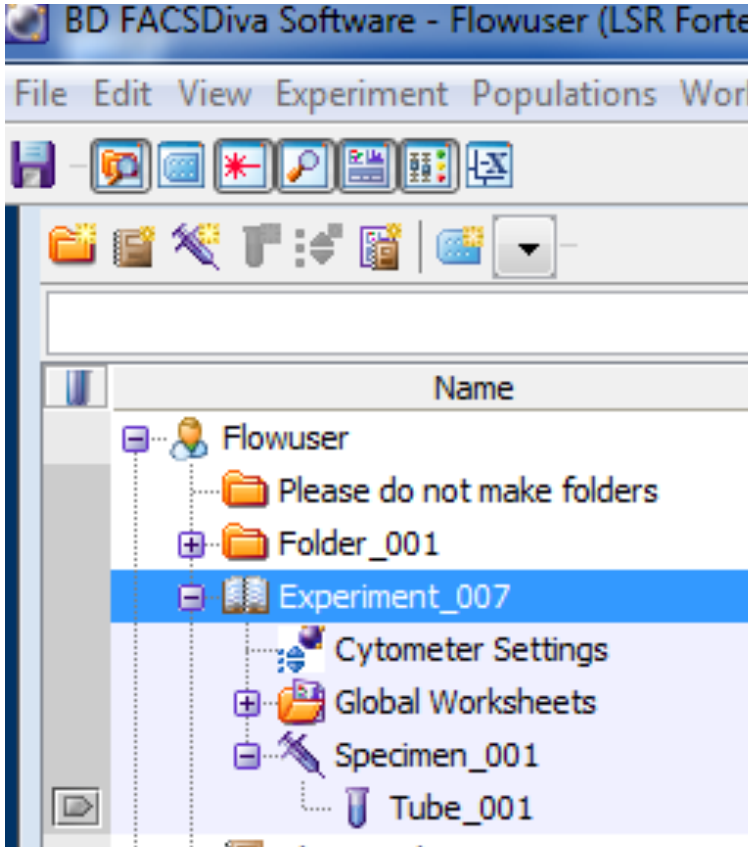
Why bother adding annotations to FCS files?

- Adding annotations to the FCS files will make data analysis easier – especially when generating a manuscript with multiple flow cytometry experiments
  - If you our your PI have to analyze the data months (or years!) after the data was collected, you will be VERY thankful for annotations

- Examples of Information that can be added:
  - Marker/fluorophore used
  - Mouse ID/patient ID, mouse genotype
  - Treatment type
  - Tissue type

File Names (BD FACSDiva)

- The experiment (“Experiment_001”) will be the name of the folder on the server that contain your files
  - We recommend including your name/initials followed by the date
- The specimen and tube make up the file names
  - The default file names would be Specimen_001_Tube_001.FCS, Specimen_001_Tube_002.FCS, etc.
  - We recommend changing the specimen to something that applies to a group of your samples, such as Sample, Spleen, Lung, FMO, Single Stain, Control, or the date



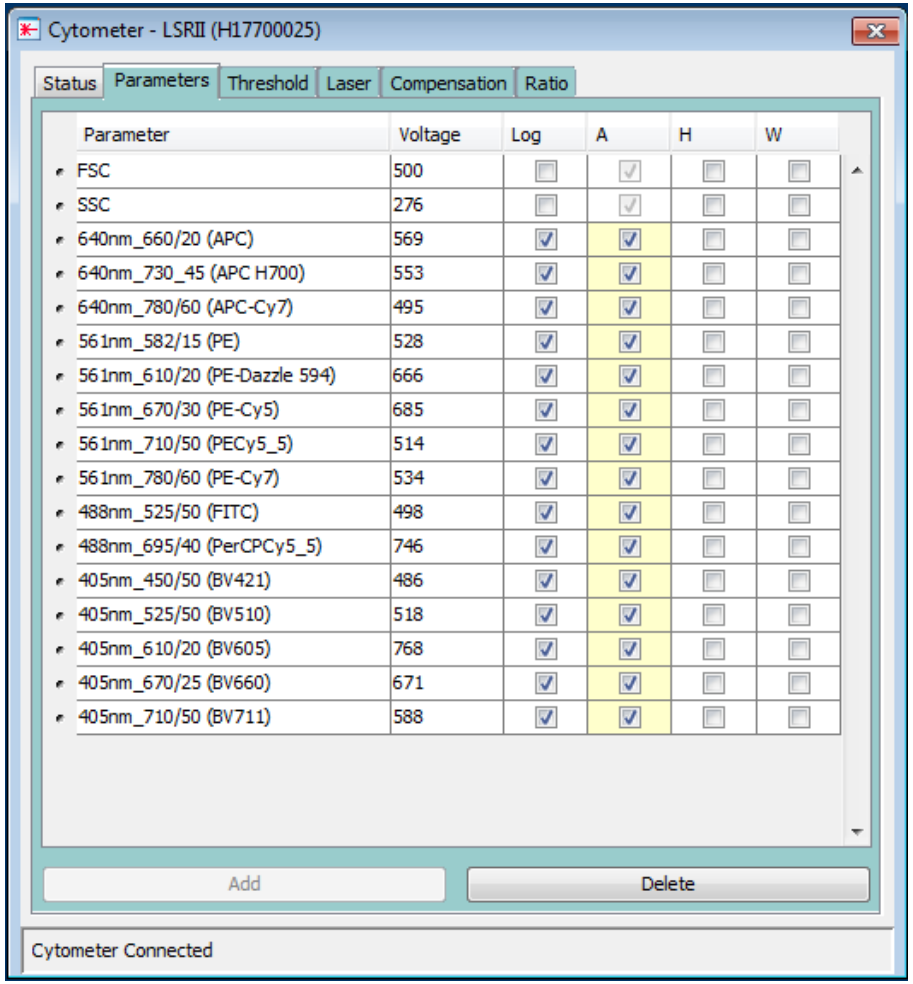
Shortcuts for Creating Filenames

- When the “next tube” button is clicked, the software will add “_001” to the file name
  - Example: label the first tube “ PBS_001” and click “next tube” 4 times to generate “PBS_002, PBS_003, PBS_004, and PBS_005”
  - You can start at any number with the “_xxx” format: clicking “next tube” on “mouse_479” will generate “mouse_480”

- The specimen and all tubes within can be copied
  - Example: Change “specimen_001” to “unstimulated” and label the 5 tubes. Then duplicate at the specimen level and rename to ”stimulated”. Now all of the unstim and stim tubes are labeled in half the time!

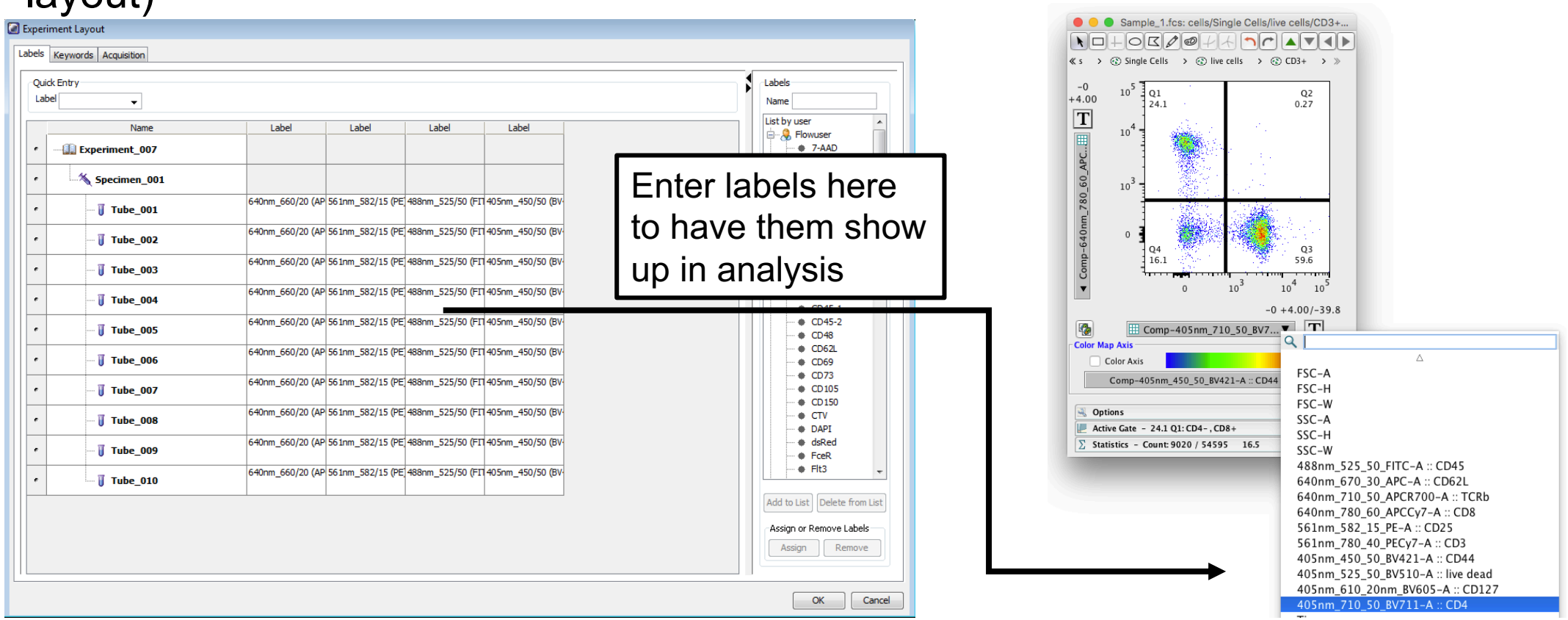
Annotating Fluorophores

- By default, all of the detectors are included in your experiment
- We recommend deleting the detectors that you are not using to simplify and downsize your files
  - Select the unused detectors and click delete
  - These can be added back with the add button
  - Once you have recorded a sample, you cannot add back detectors, so make sure you include all of the ones you need!



Annotating Fluorophores: Experimental Layout Window

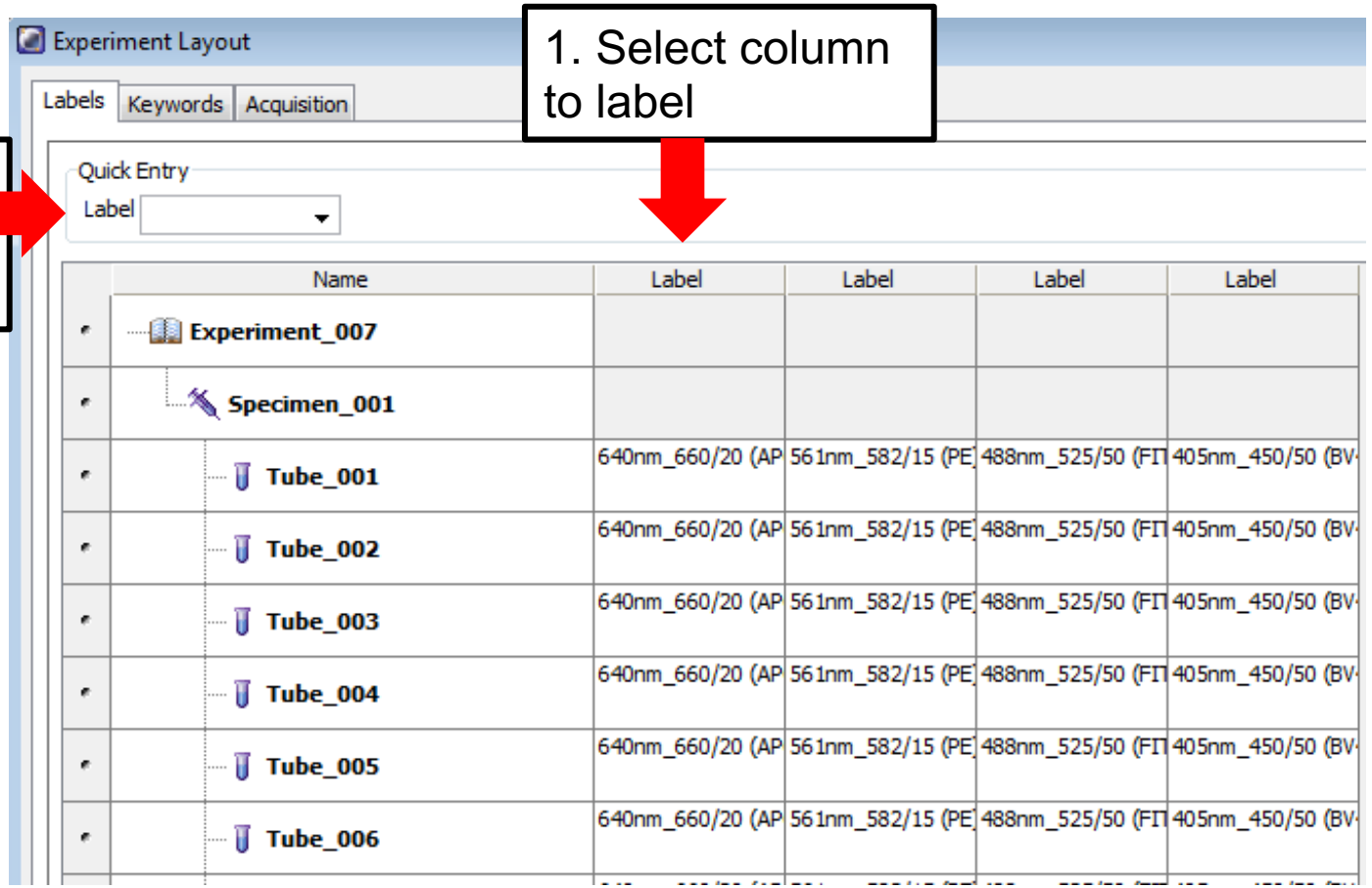
- You can use the experiment layout window to label your fluorophores with their respective markers and avoid future confusion (Experiment menu-> experiment layout)



Annotating Fluorophores: Experimental Layout Window

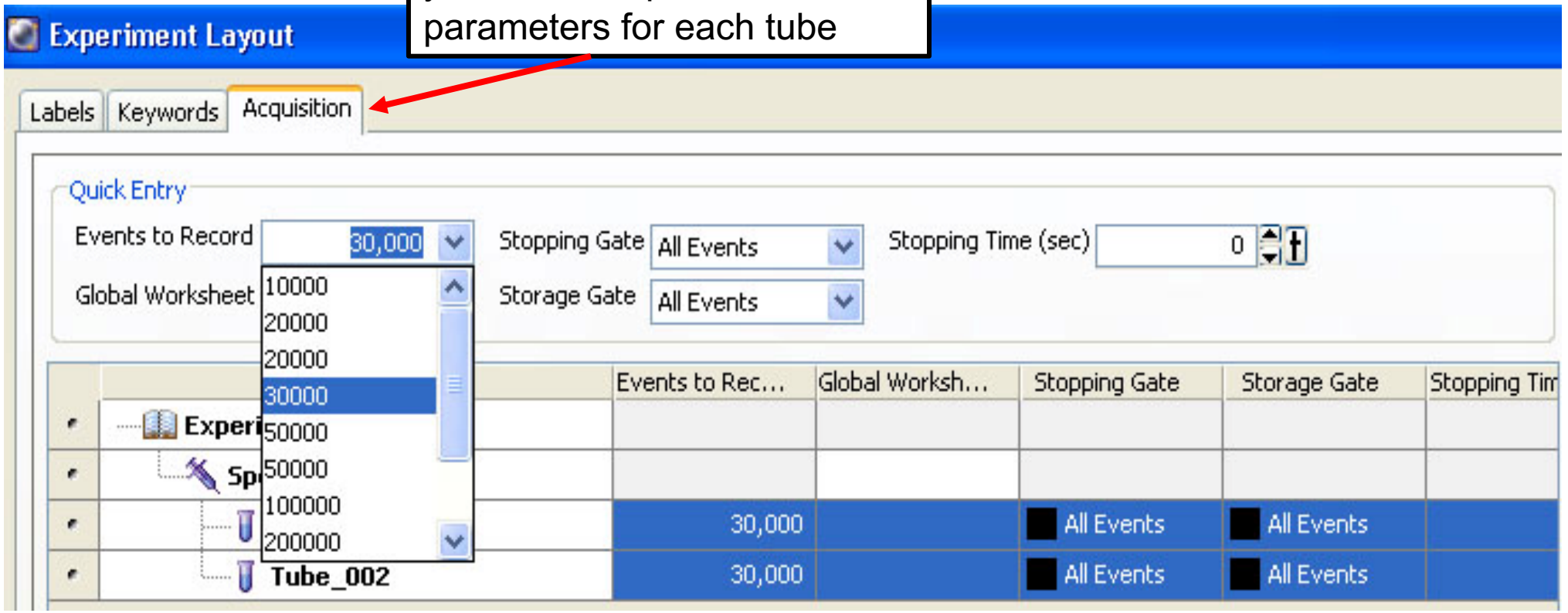
2. Type marker name here, then hit enter/return

1. Select column to label



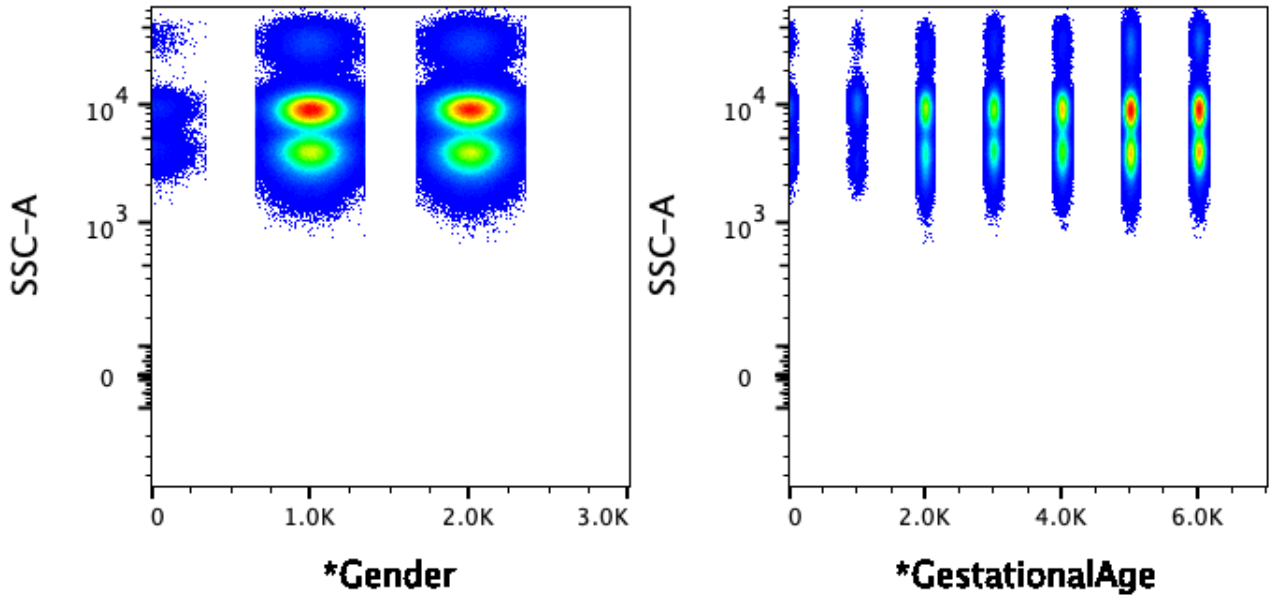
Annotating Fluorophores: Experimental Layout Window

The acquisition tab will allow you to set acquisition parameters for each tube



Keywords are useful for analysis with algorithms

- Add keywords to FCS files:
  - Gender, age, treatment, etc. (useful for clinical information)
- Convert information to integers
  - Example:
    - 1=yes, 2=no
    - 1=male, 2=female
- Keywords can be entered on the cytometer or analysis software

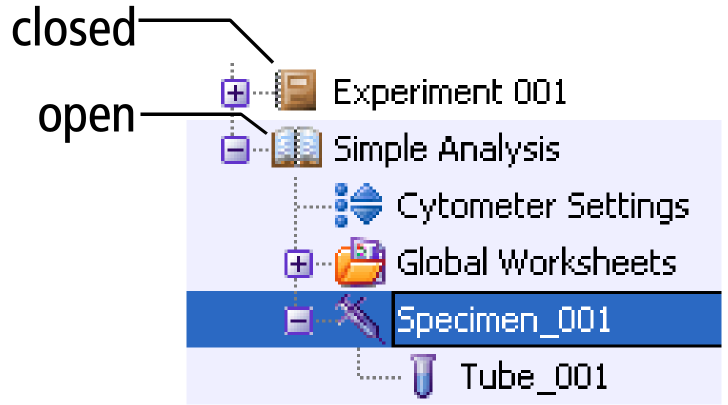


Experiment Templates can save time

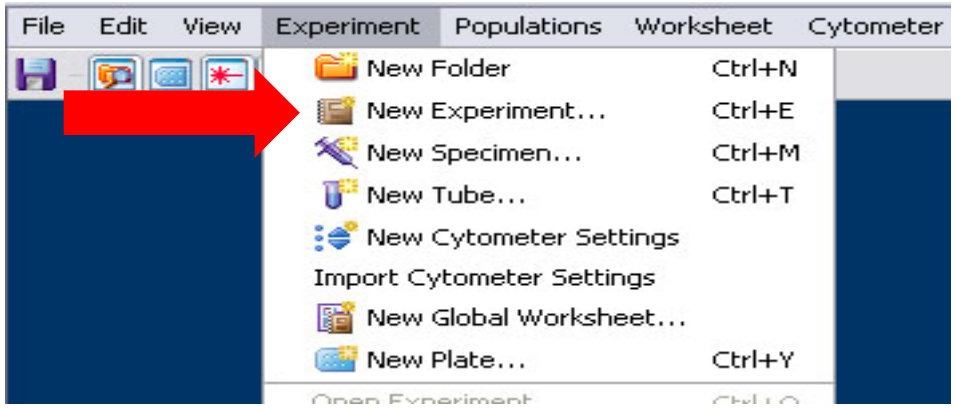
- If you are planning on running the same experiment over several months, consider creating a template to save time!
- Experiment templates include specimens, tubes, keywords, cytometer settings, labels, worksheet elements, and worksheets, but do not include recorded data.

Experiment Templates can save time

- With your experiment open and selected, choose File > Export > Experiment Template. Name the template and click finish



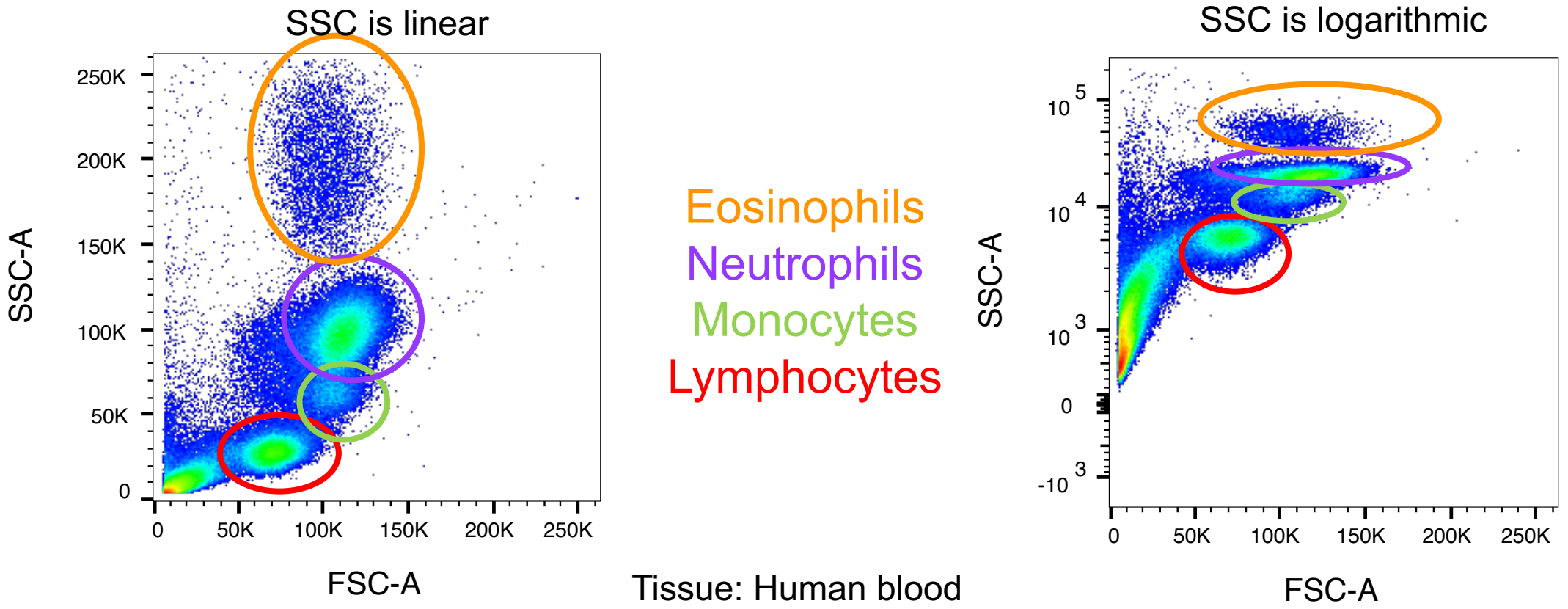
- To create a new experiment from the template, select “New Experiment” from the Experiment menu, then select your template from the list



Setting Voltages

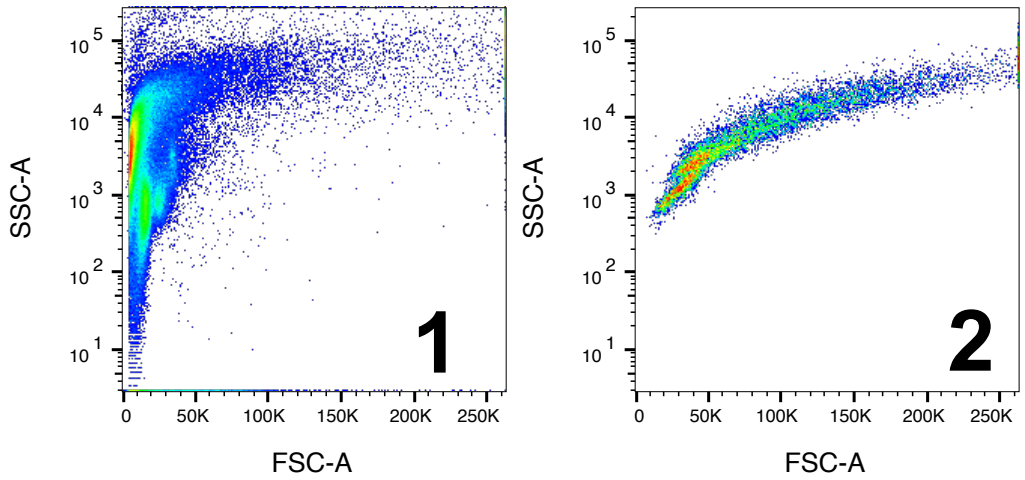
Always set the voltages for FSC and SSC for each tissue to make sure all cells are within the plot

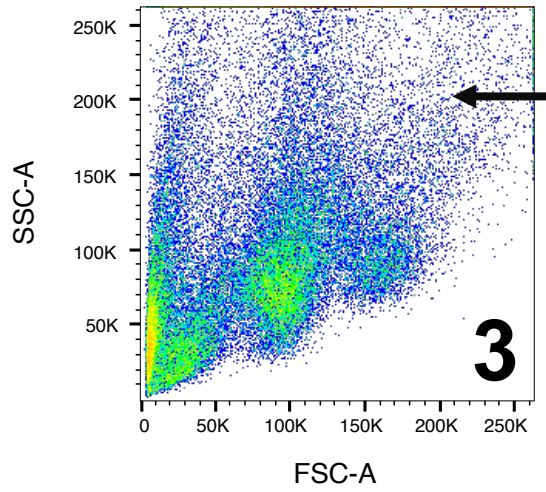
- First decide if you want your SSC to be in linear or log, then set the voltages



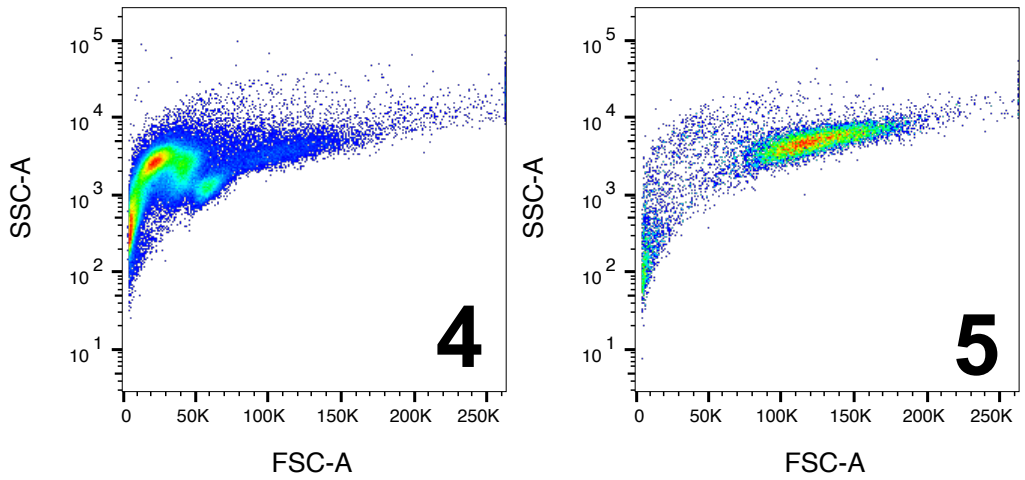
Tissue: Human blood

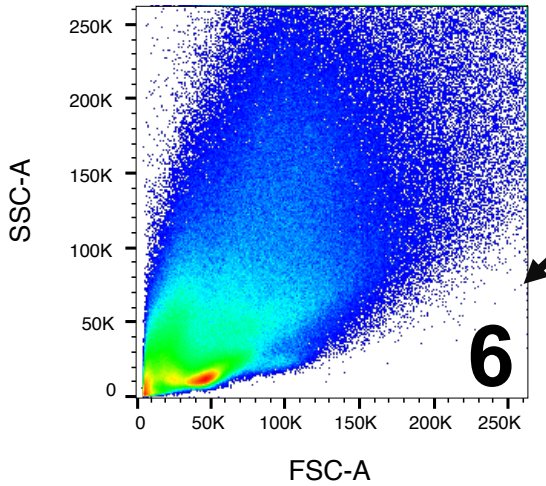
Exercise: How do these FSC and SSC voltages look?





This lung tissue is OK if you are only interested in small cells (lymphocytes), but you are missing out on larger cells





Same tissue, different voltage

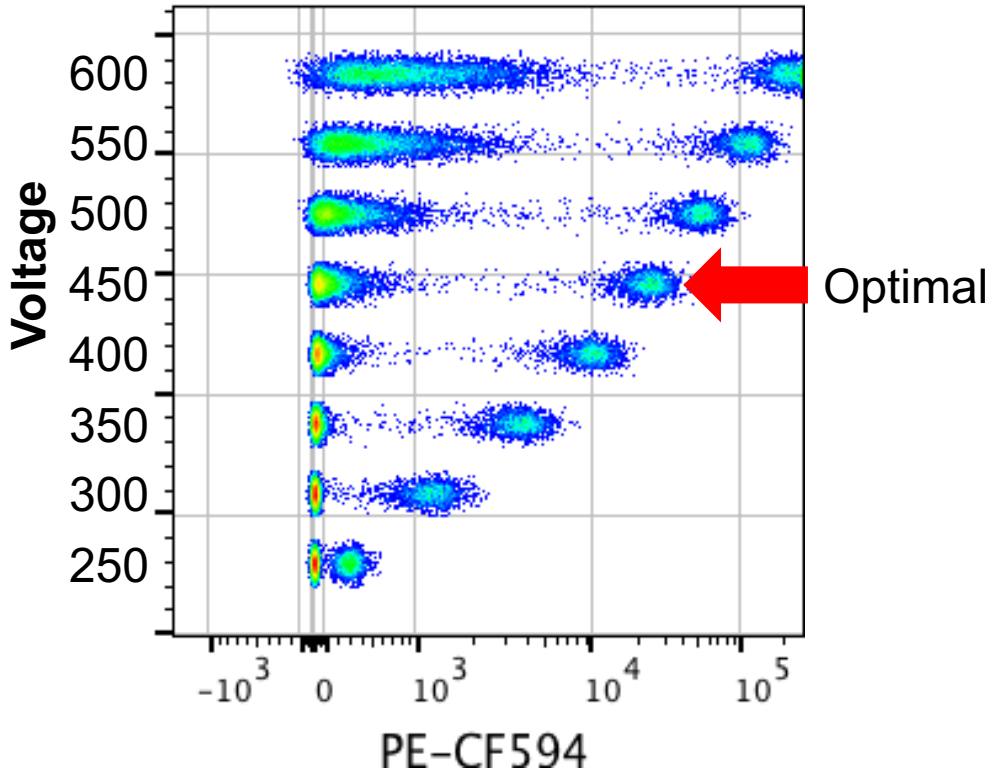
Can you change voltages between samples?

- FSC and SSC can be changed for each sample, though it is recommended that you keep the same voltages for each sample type
- Options:
  - Run all tubes on the same FSC and SSC voltage
  - Run comp beads on one FSC/SSC, run cells on a different FSC/SSC
  - Run tissue 1 on one FSC/SSC, run tissue 2 on a different FSC/SSC

- Do not change FSC and SSC voltages randomly on samples
- Do not change voltages on the fluorophore detectors – every control and sample must be run on the exact same voltages

The CAT Facility has optimized the voltages on the Fortessas for you!

- Setting voltages can be challenging!
- Use the optimized voltages in the default experiment as a starting place
- Read more about voltage optimization here

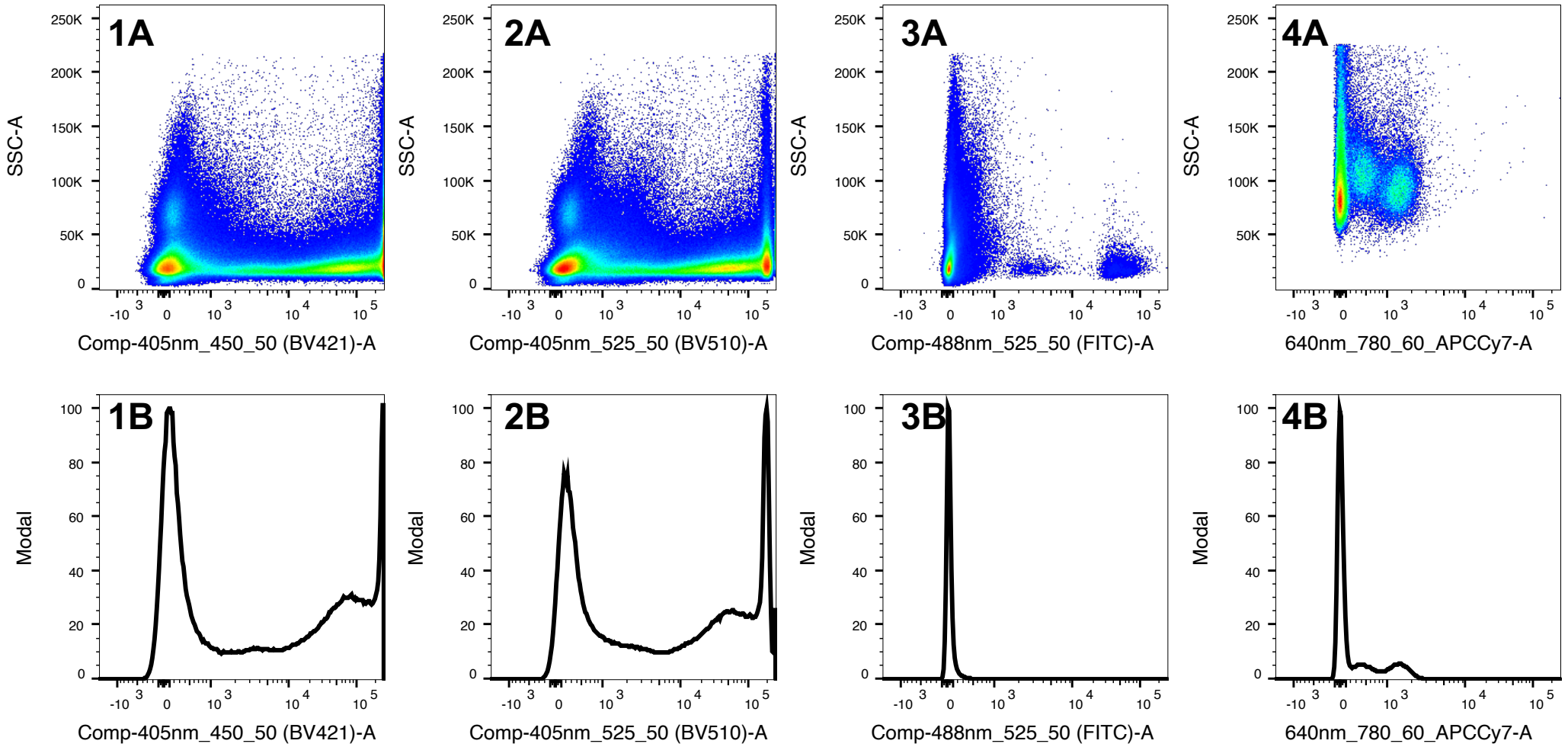


Voltage selection for fluorophores – simplified

- The voltages on the Default Experiment template are the optimized value for cluster resolution
  - In general, there is no advantage to increasing the voltage
  - Voltage should definitely be reduced if the data is off-scale (too bright)

- A good panel design and titrated antibodies will prevent issues of low resolution
- Increasing voltage is not a good way to deal with low resolution problem – switch the marker to a brighter fluorophore instead!

Exercise: How do these fluorophore voltages look?



Can you change voltages after recording data?

- No. Voltage is an instrument setting.
- In contrast, compensation is math that is applied to the data – this can be changed whenever.

- Sometimes new users do not set voltages properly and do not realize their error until analyzing the data. Unfortunately the only way to fix problems with voltages is to run the entire experiment again.

How do you know that your voltage settings are correct?

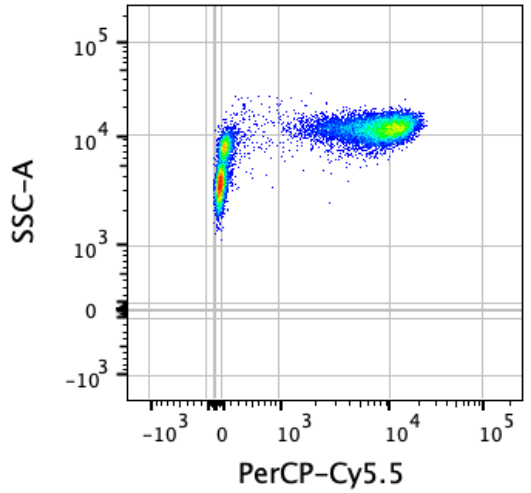
- Compensate the data, check the compensation. Gate your sample.

- If the data doesn't look compensated and it's difficult to gate populations, it's possible the voltage wasn't set correctly

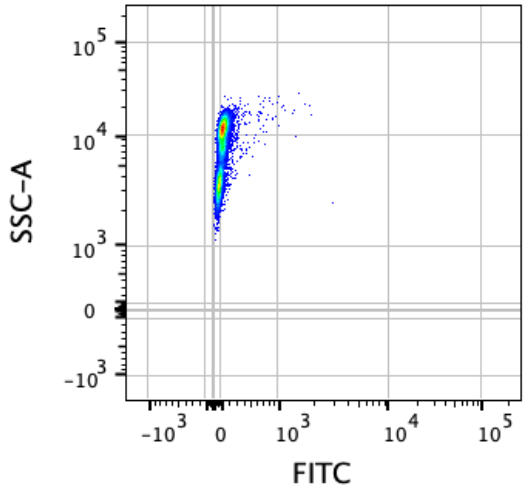
Advanced Voltage Setting

- A popular way of setting voltages is to ensure that the fluorophore in the tube is the brightest in its assigned detector

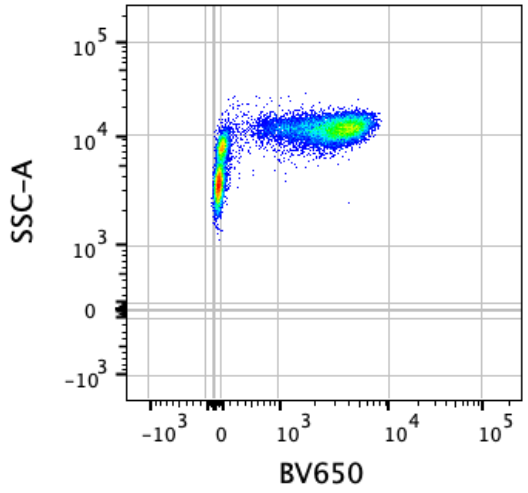
Positive signal from PerCP-Cy5.5



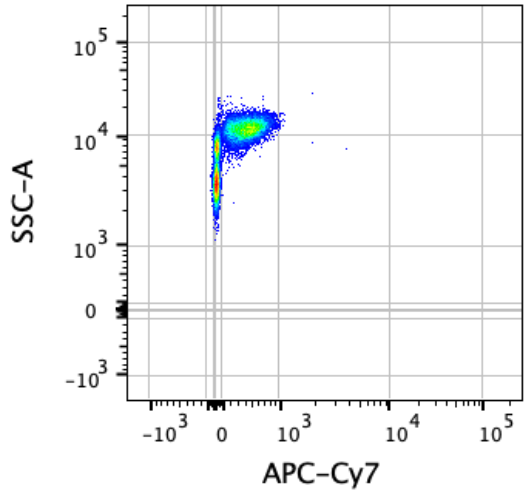
PerCP-Cy5.5 doesn't spill into FITC



PerCP-Cy5.5 spills into BV650



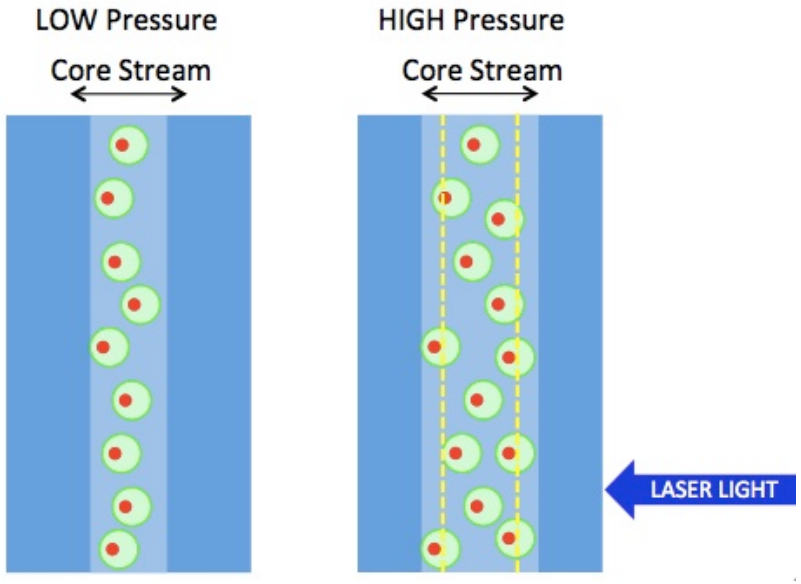
PerCP-Cy5.5 spills into APC-Cy7



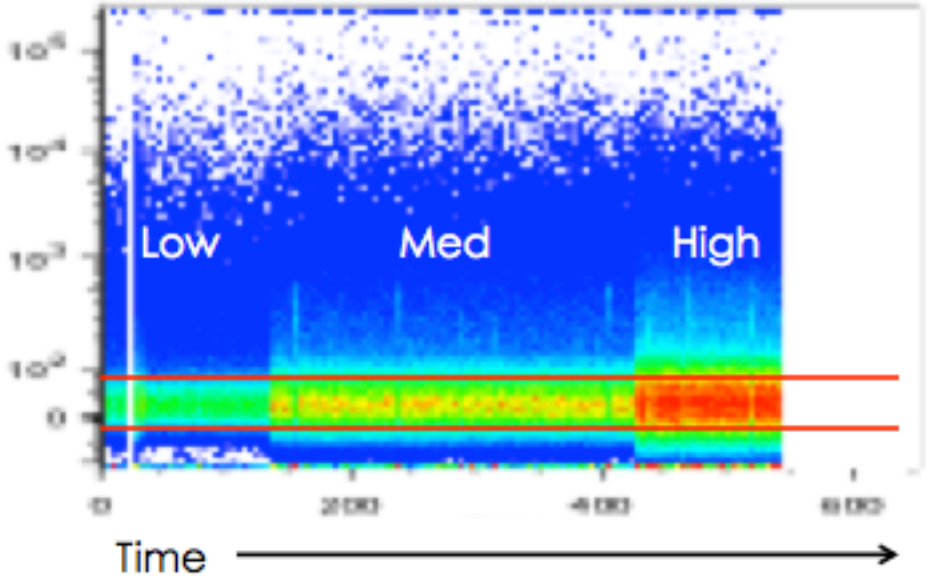
Cytometer Fluidics

The Lowest Cytometer Flow Rate is Best

- For Fortessas: set the flow rate to low
  - The best way to run more cells is to increase concentration of the sample, not the cytometer flow rate



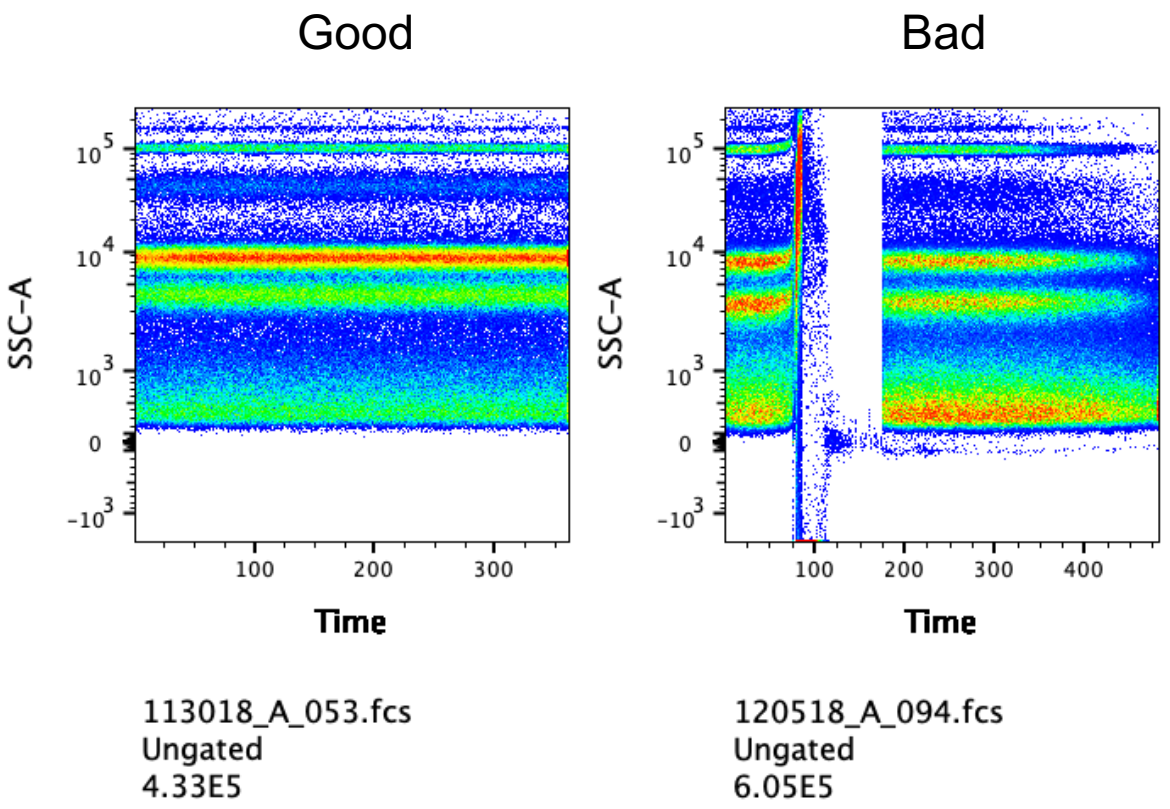
https://expert.cheekyscientist.com/fluidics/



https://expert.cheekyscientist.com/troubleshoot-flow-cytometer-fluidics-system/

Check the time parameter for clogs

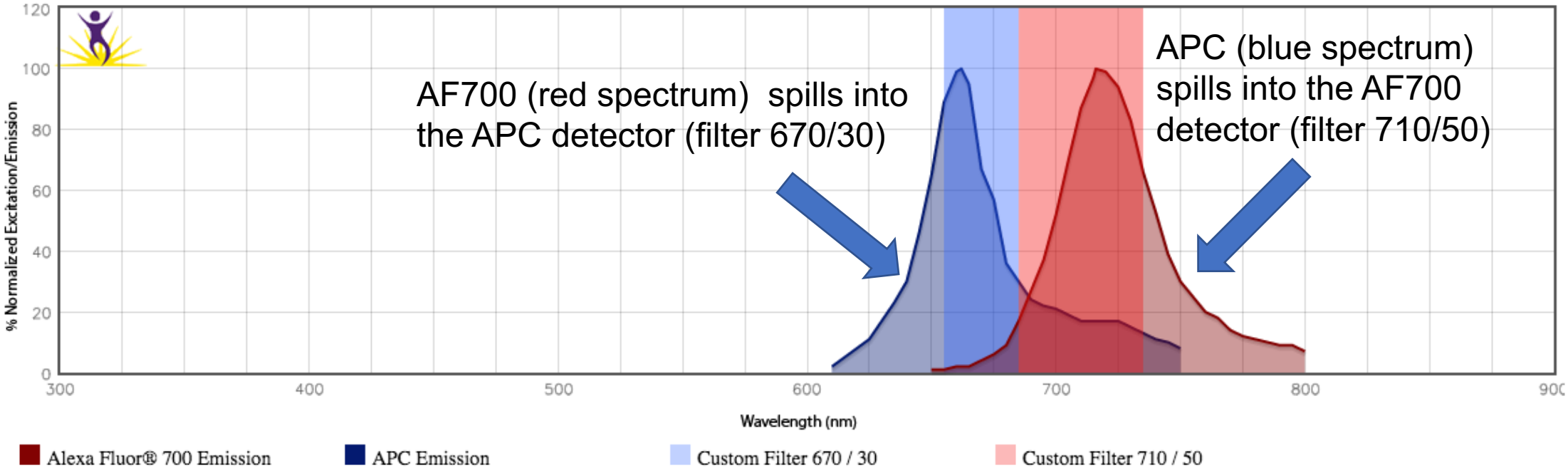
- The signal should be steady over time
- If the signal is not steady, there could be a clog or other issues with the fluidics



Calculating Compensation

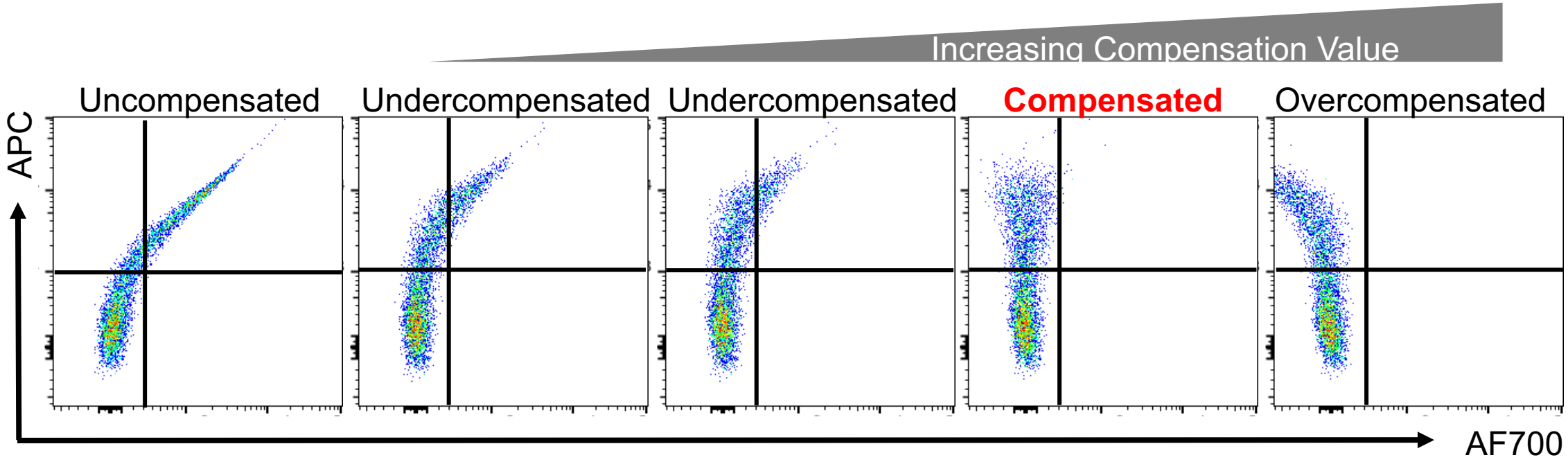
Compensation (conventional flow cytometry)

- In conventional flow cytometry, one detector is assigned to one fluorophore. However, some of the spectra of other fluorophores will spill into the incorrect detector. Compensation “corrects” this mathematically.



Compensation (conventional flow cytometry)

- Single stain controls are required to determine compensation
  - Single stains should only be positive for one fluorophore! Anything that looks double positive is spillover or autofluorescence

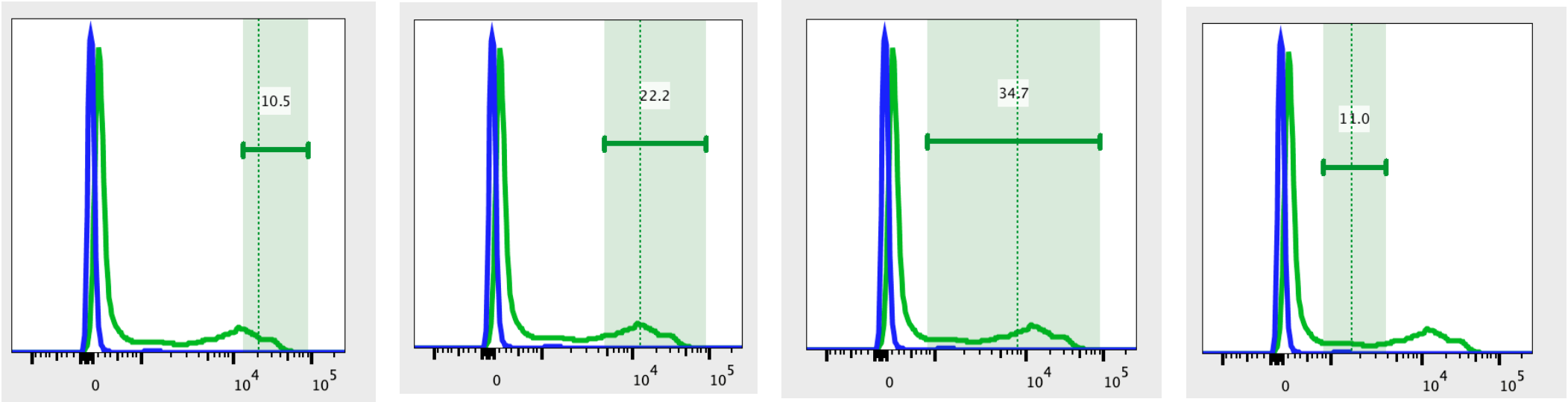


Compensation (conventional flow cytometry)

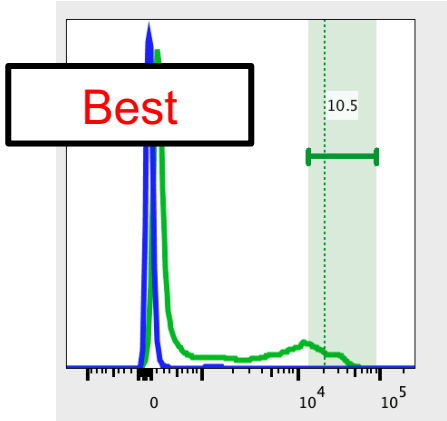
- Compensation can be calculated multiple ways:
  - On the cytometer (pre-acquisition) in FACSDiva
  - Post-acquisition in analysis software (FlowJo/FCS Express)
  - Automatically (compensation wizard)
  - Manually
- It is up to you when/how you want to do compensation
  - Automated methods are quick and great for beginners
  - Everyone should understand how to perform manual compensation

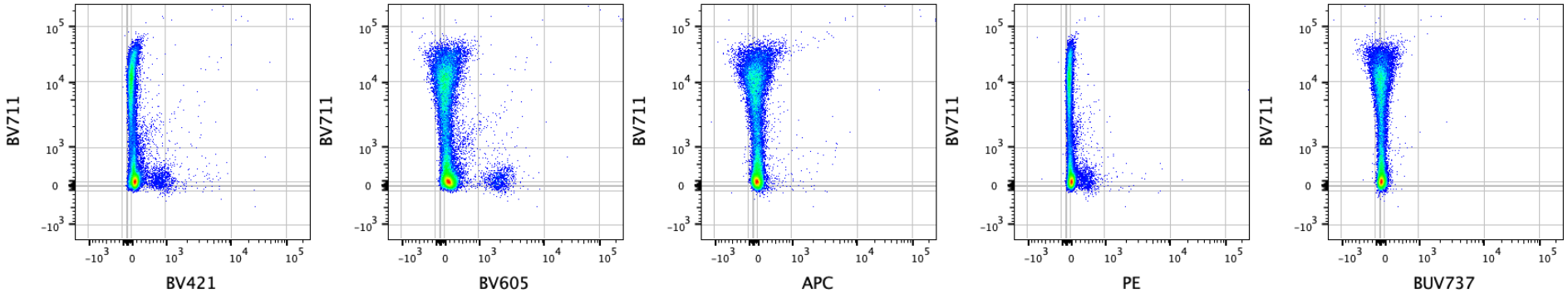
Setting Gates for Automated Compensation

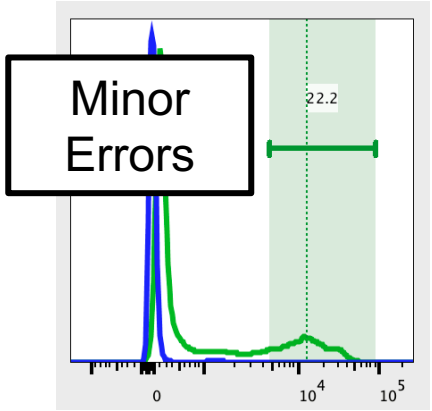
- Gate placement will affect the calculation of the compensation

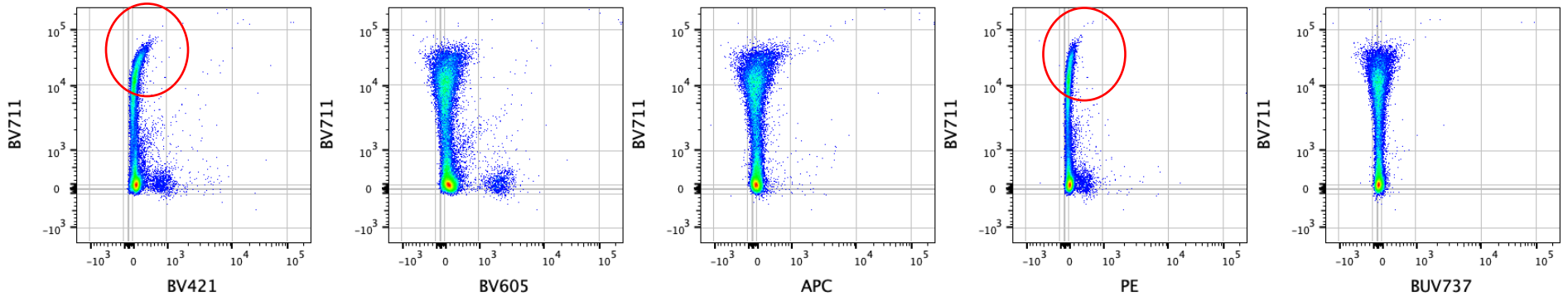


Setting Gates for Automated Compensation



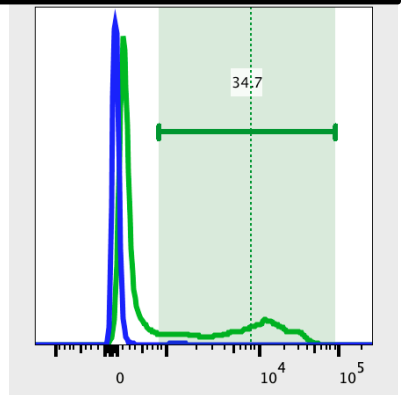


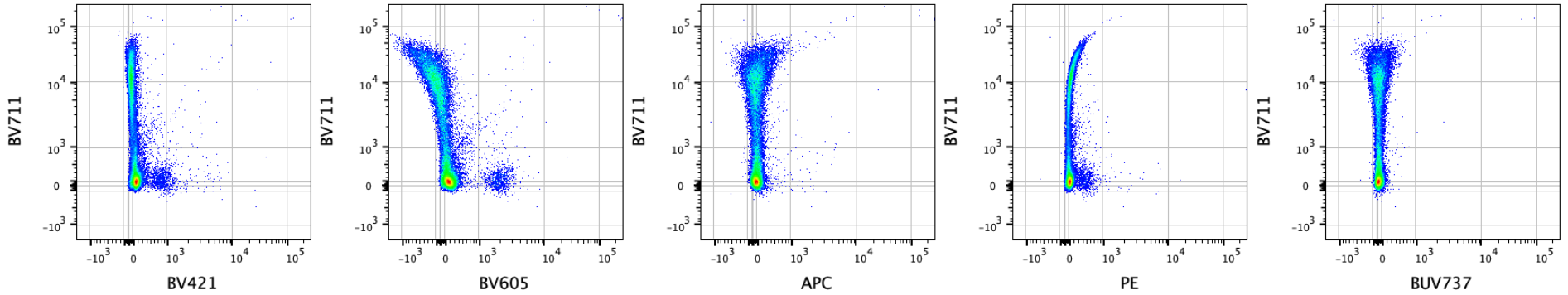




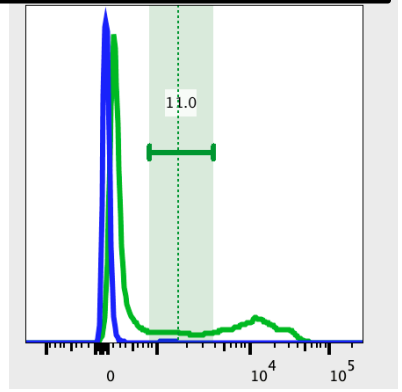
Setting Gates for Automated Compensation

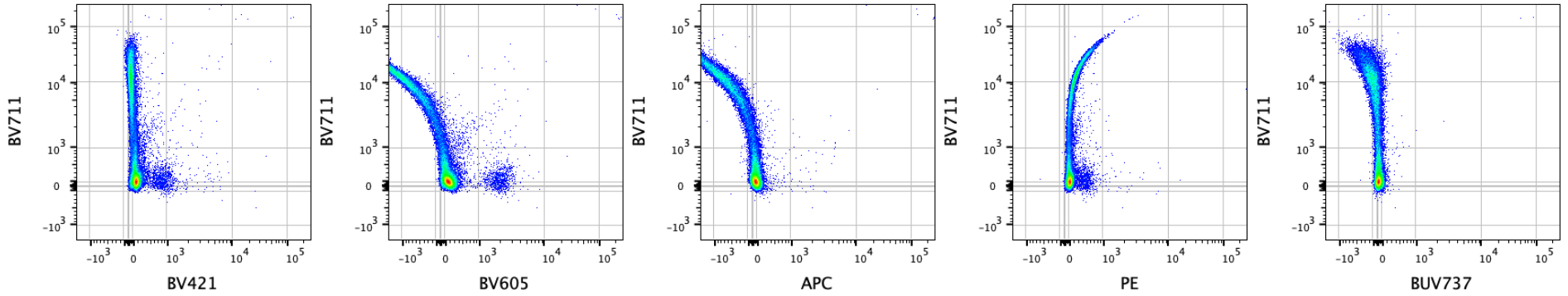
Some Errors





Large Errors

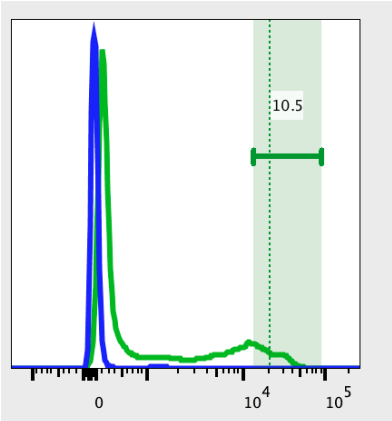


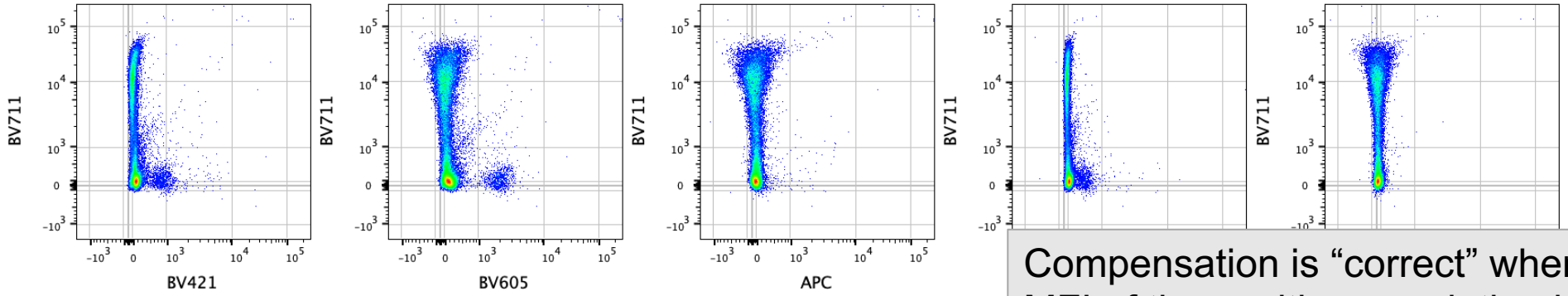


Remember the Rules for Compensation Controls

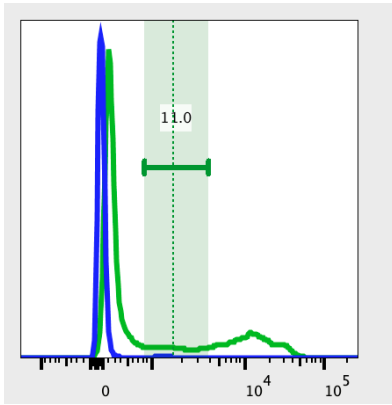
1. **The positive population of the control must be as bright or brighter than the multicolor sample**
2. The control should ideally be exactly the same fluorophore as the one in the sample
3. The control should ideally contain a positive and negative population, and these populations must have the same autofluorescence properties
4. Controls should have sufficient events in both positive and negative populations

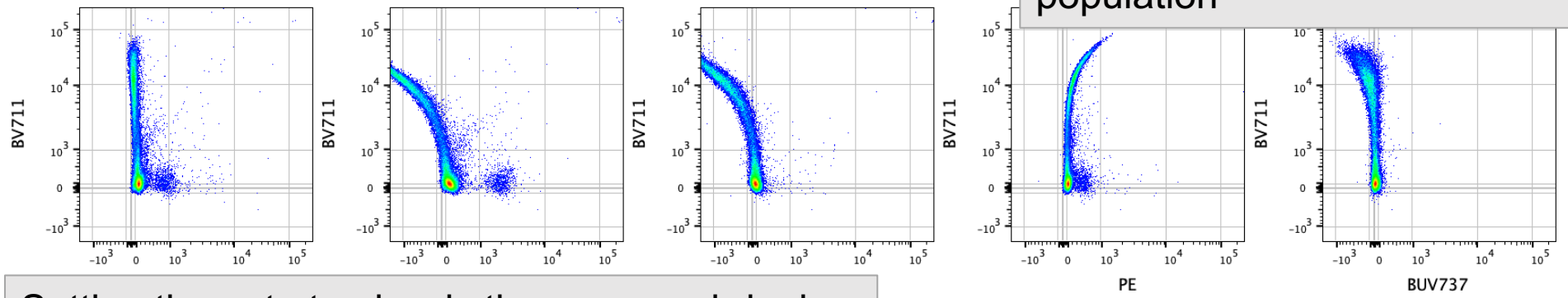
Gate placement determines the MFI





Compensation is “correct” when the MFI of the positive population is aligned with the MFI of the negative population





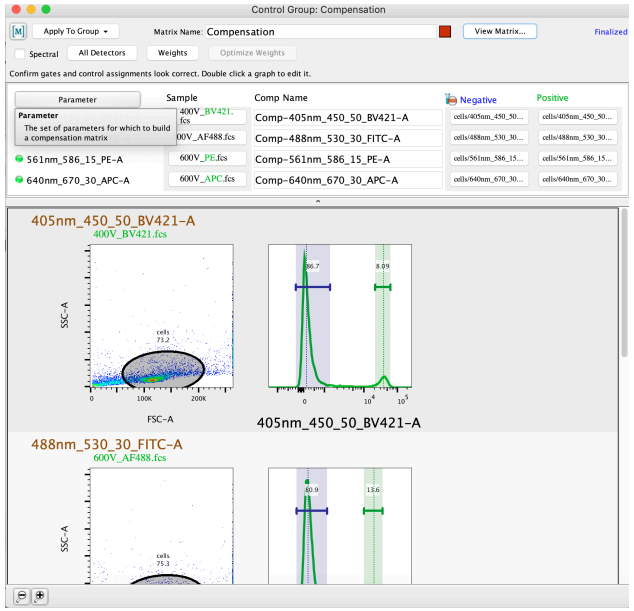
Setting the gate too low is the same as bringing a control that is dimmer than the sample

Remember the Rules for Compensation Controls

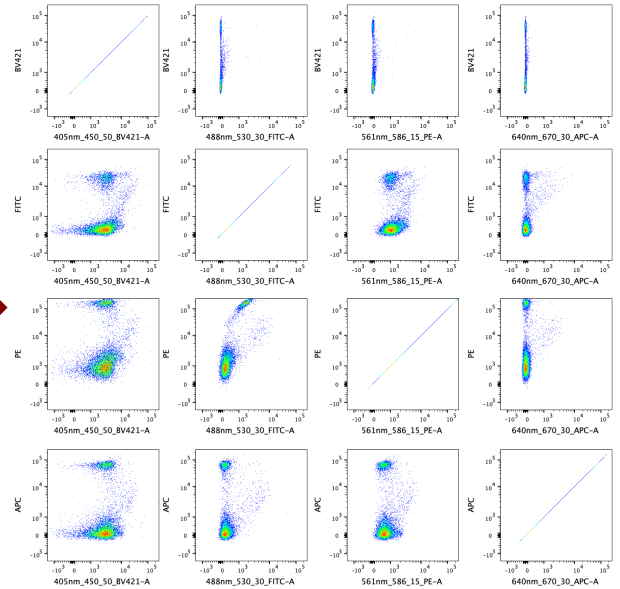
1. The positive population of the control must be as bright or brighter than the multicolor sample
   - When calculating compensation, the MFI of the positive gate is used (dotted line in the examples). In the examples, the compensation is worse as the MFI decreases – cells above the MFI will not be compensated correctly
4. Controls should have sufficient events in both positive and negative populations
   - FlowJo autocompensation: *there are rules regarding the positive population – it must contain more than 100 events and be 5% or greater of the parent population. If the positive gate contains less than 5%, try setting a parent (preceding) gate to ensure that your positive gate meets or exceeds 5%.*

Steps for using compensation

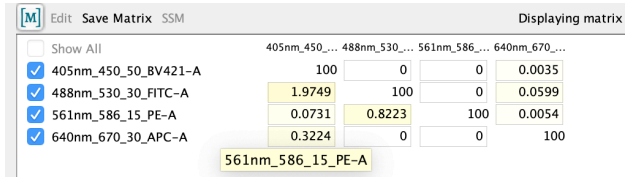
Automated Compensation Tool



Create NxN plots

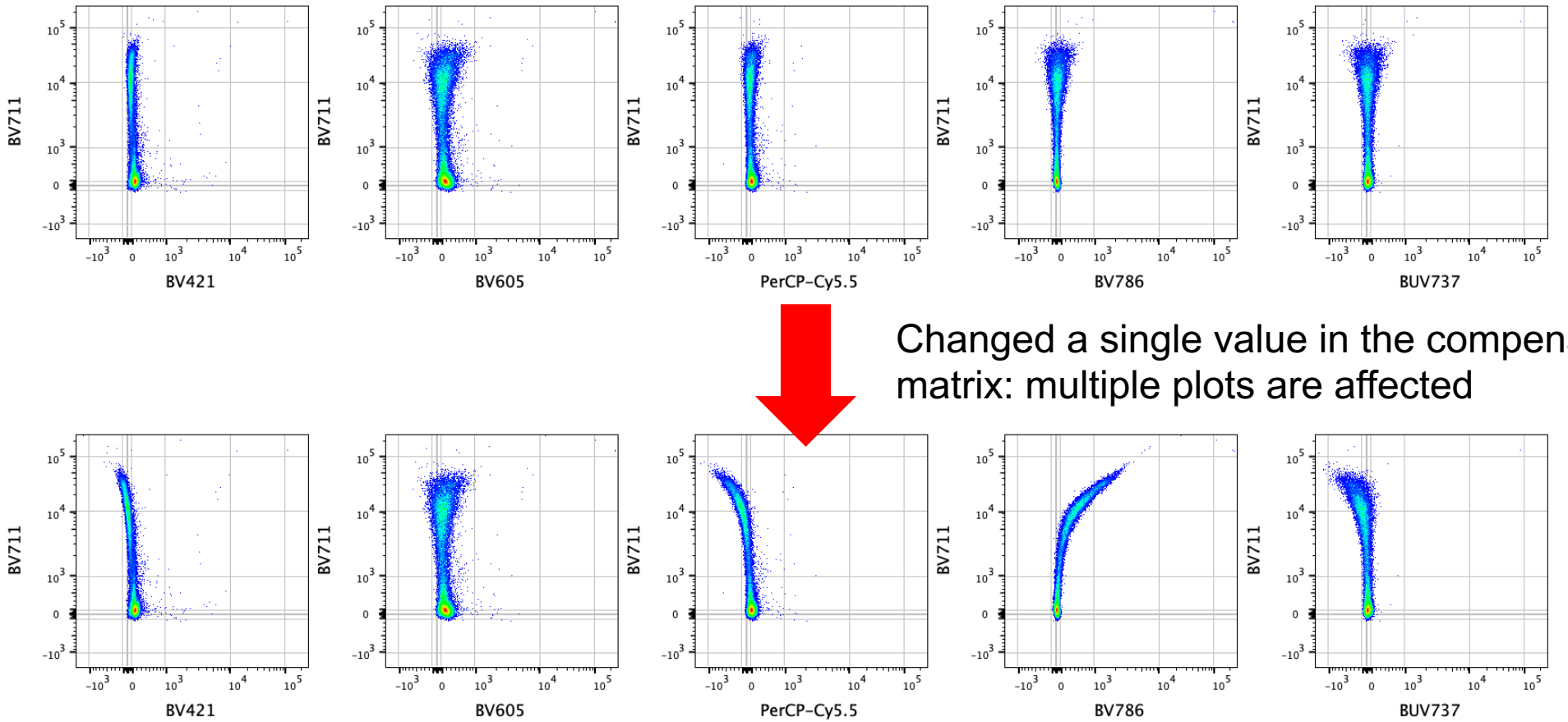


Edit Compensation Matrix

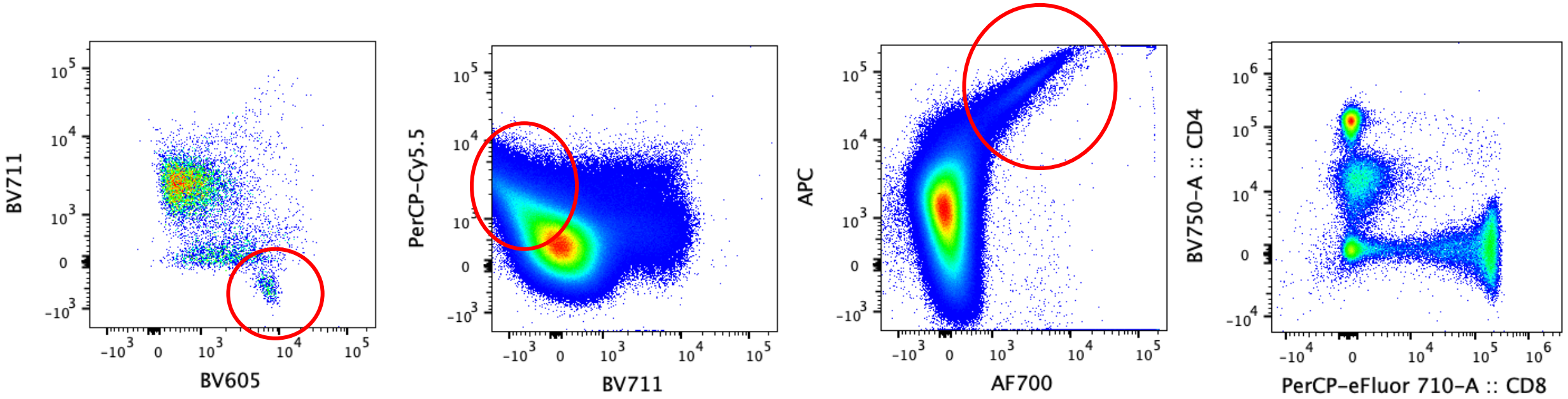


* For manual compensation, skip the automated compensation tool and start with a blank matrix

It’s important to look at multiple graphs when changing compensation



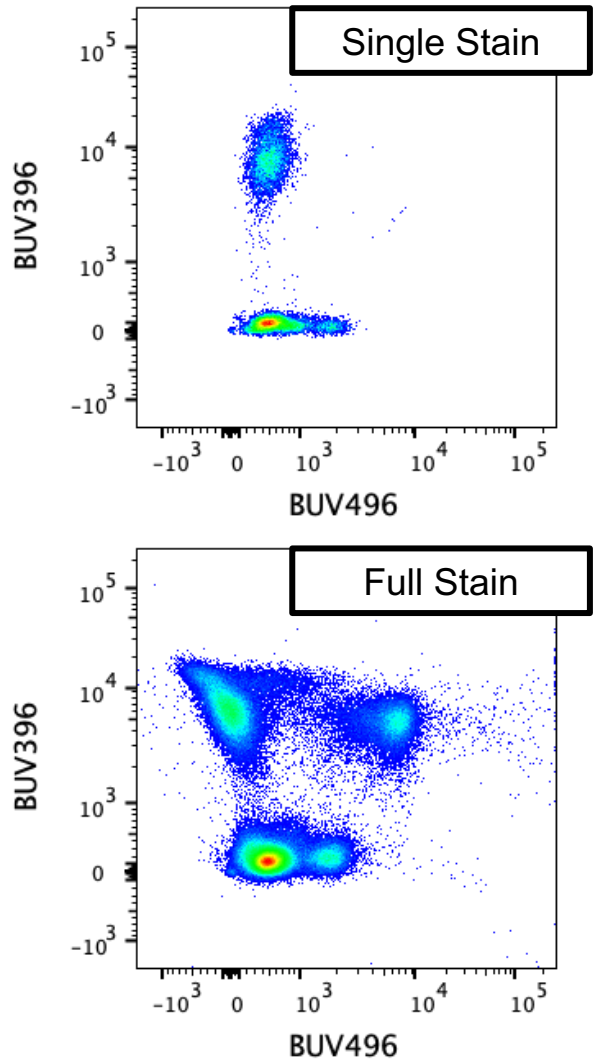
Identifying Compensation Errors in Fully Stained Samples



- Super negative populations and populations with a teardrop shape can indicate compensation errors.
- Symmetrical populations are acceptable

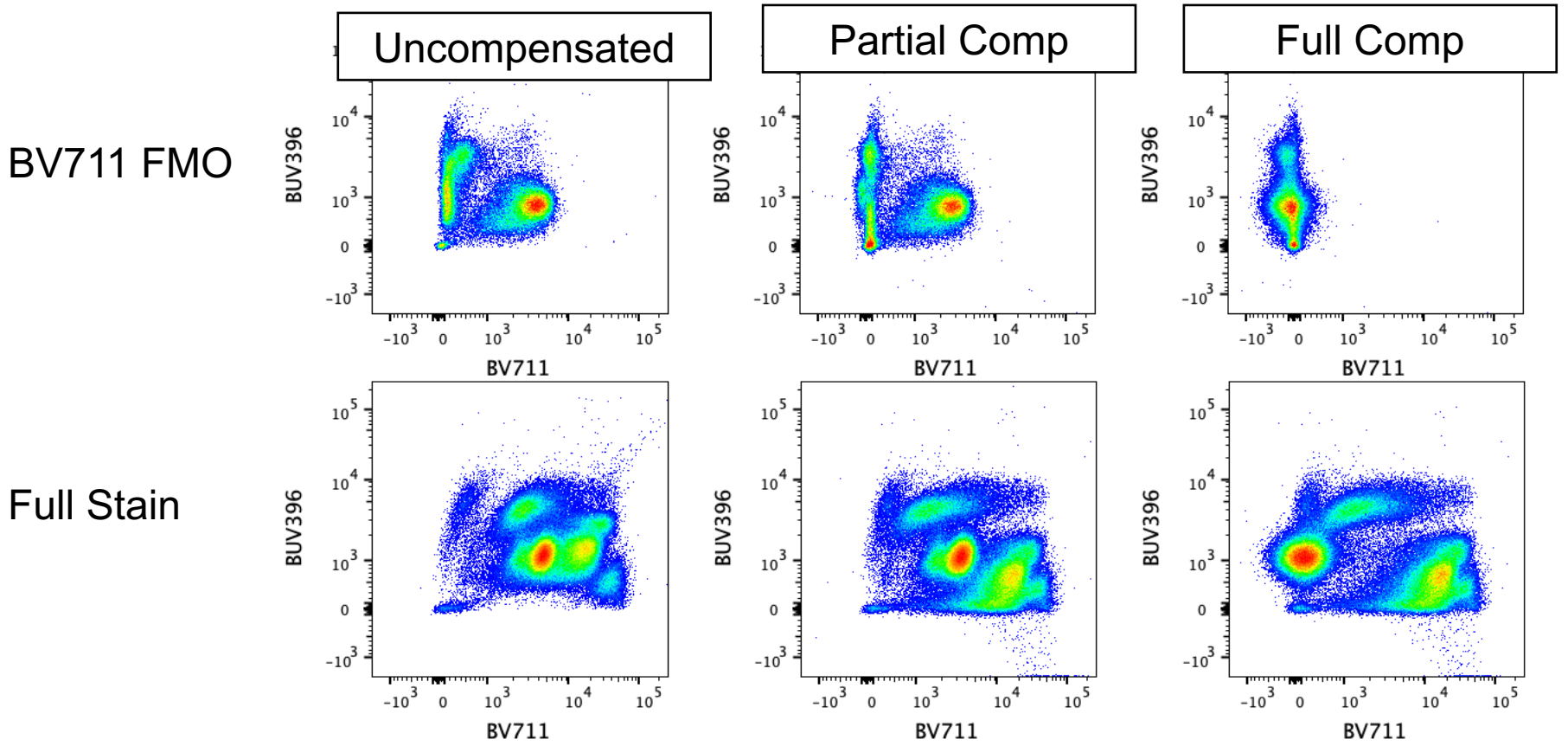
Troubleshooting Single Stain Controls

- I checked my compensation with my single stain controls, but it looks like there are compensation errors in my sample.
  - Are you sure you followed all of the rules for compensation controls?
  - Did you use compensation beads? Sometimes you may not get optimal compensation with comp beads.
  - Do you have a lot of brilliant violet or BUV dyes? Did you use the brilliant stain buffer? These fluorophores are sticky and the brilliant stain buffer prevents this.



What happens if there are compensation errors?

- It's not ideal to have compensation errors, BUT if minor errors exist, the gating controls (FMOs) can be used to ensure that the data is being interpreted correctly



The Flow Basics 2.0 Series