Flow Cytometry Quiz

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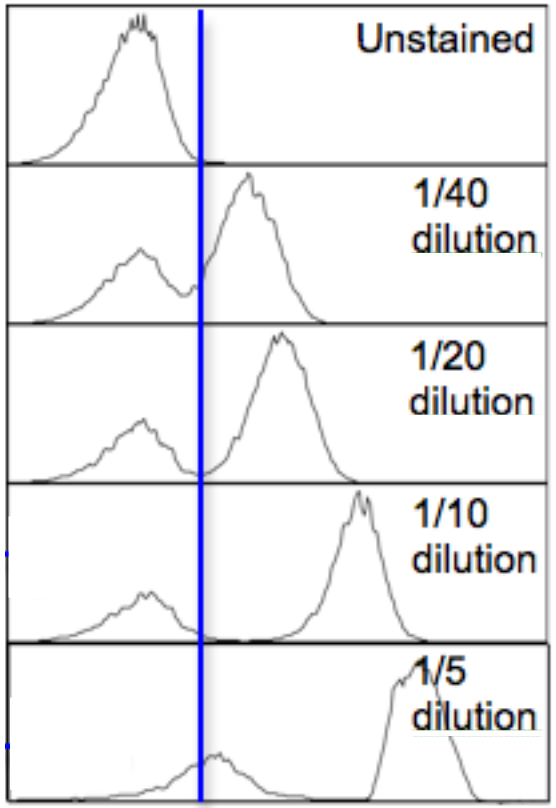
Titration! Titration! Titration!

Titration

A) What is the goal of a titration?

-particularly with respect to the positive and negative populations?



B) Which concentration of antibody is correct?

Answer: Titration

A) What is the goal of a titration?

-particularly with respect to the positive and negative populations?

To chose the correct concentration of antibody to stain with for future experiments, making sure the:

-positives are as bright as possible (saturating stain)

-the negatives stay unstained

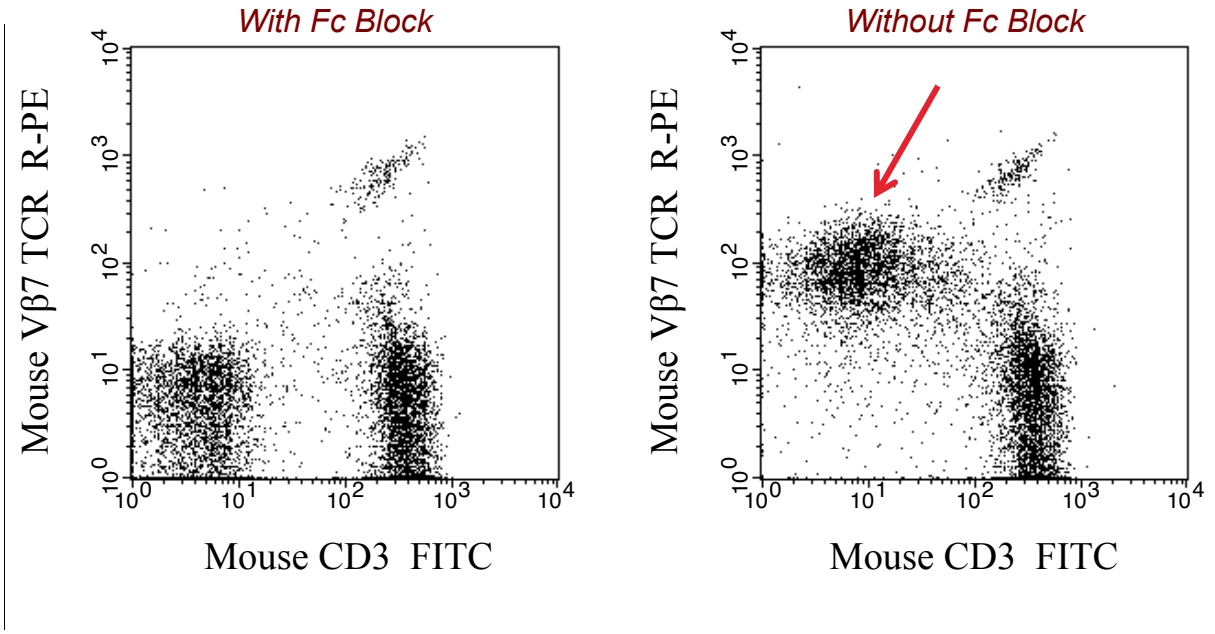
B) Which concentration of antibody is correct?

1/10 dilution

Block Fc Receptors

Fc Block

Lymph node cells



Data from Invitrogen

A) Are the cells the red arrow is pointing to expressing VB7 TCR?

B) Why do they have a significant amount of PE fluorescence?

Answer: Fc Receptors

A) Are the cells pointed to with the red arrow expressing VB7 TCR?

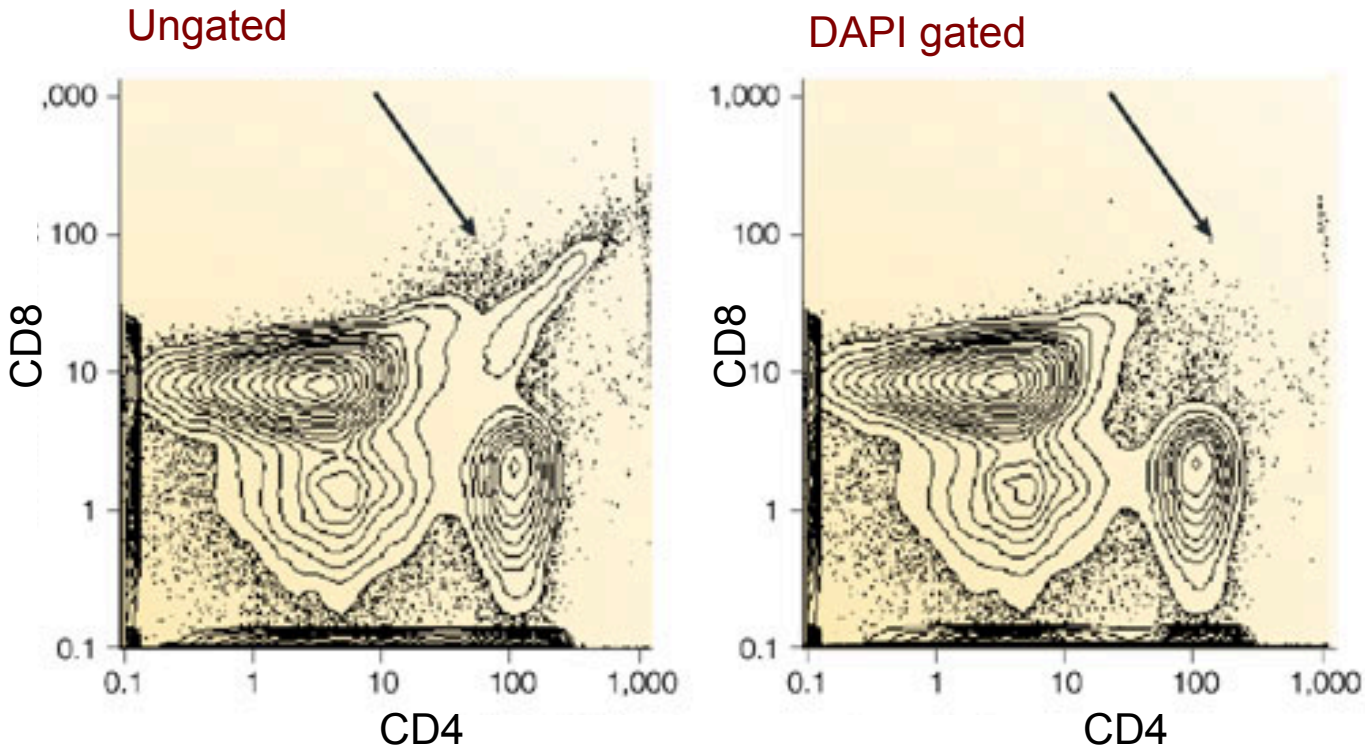
No.

B) Why do they have a significant amount of PE fluorescence?

The anti-VB7 antibody is binding to the monocytes via the Fc receptor.

Dead Cells Kill Your Data

Dead Cells



A) What are the double-positive cells in the left figure (indicated by arrow)? Are they expressing both CD4 and CD8?

From: Nature Reviews Immunology 4, 648-655 (August 2004)

Answer: Dead Cells

A) What are the double-positive cells in the left figure (indicated by arrow)?
Are they expressing both CD4 and CD8?

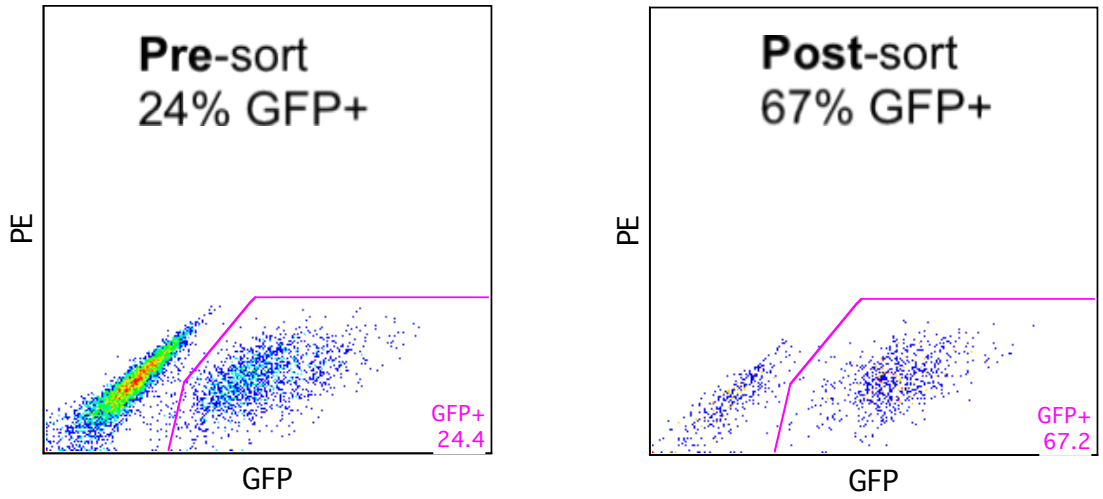
They are dead cells that are picking up antibody non-discriminately.
These dead cells are not expressing CD4 and CD8.
The staining is non-specific!

DOUBLE Trouble

Sorting

1 million GFP+ cells are sorted into a collection tube.

The customer counts the cells and finds 1.1 million cells in the collection tube and a poor post sort analysis (shown below):



Hint: the cells settled very fast in the sample tube and the customer reports that these cells tend to be “sticky”.

A) What happened?

Answer: Double Trouble

A) What happened?

The cells stuck together to create doublets (which are not removed by filtering). A GFP+/GFP- doublet is sorted because it looks to the machine like a slightly larger GFP+ cell. The doublets fall apart in the collection tube and the post sort analysis shows poor purity.

*Note: the Width (Time of Flight) parameter does not distinguish **all** doublets from singlets cells, therefore it is important to focus on preparing the cells in such a way as to have as few doublets as possible.*

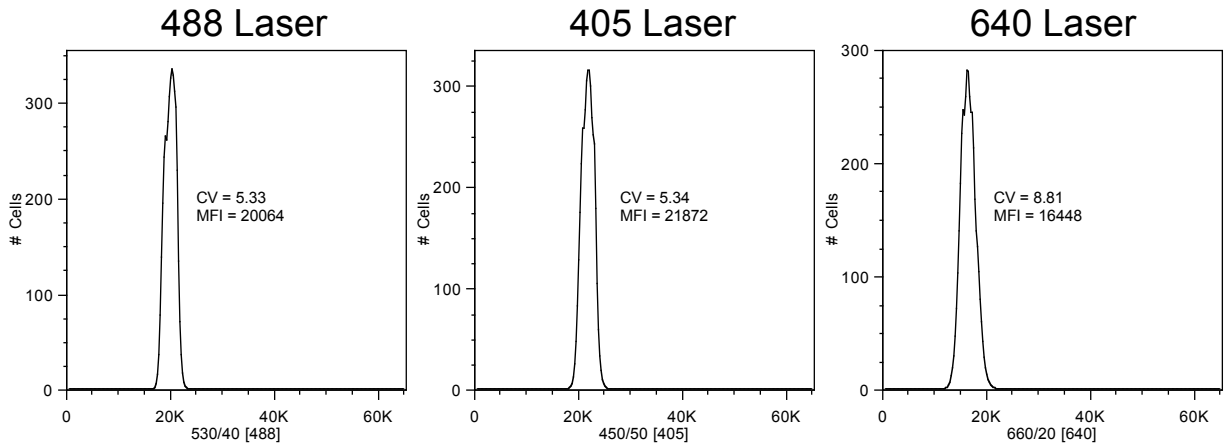
Alignment:

Tight and Bright

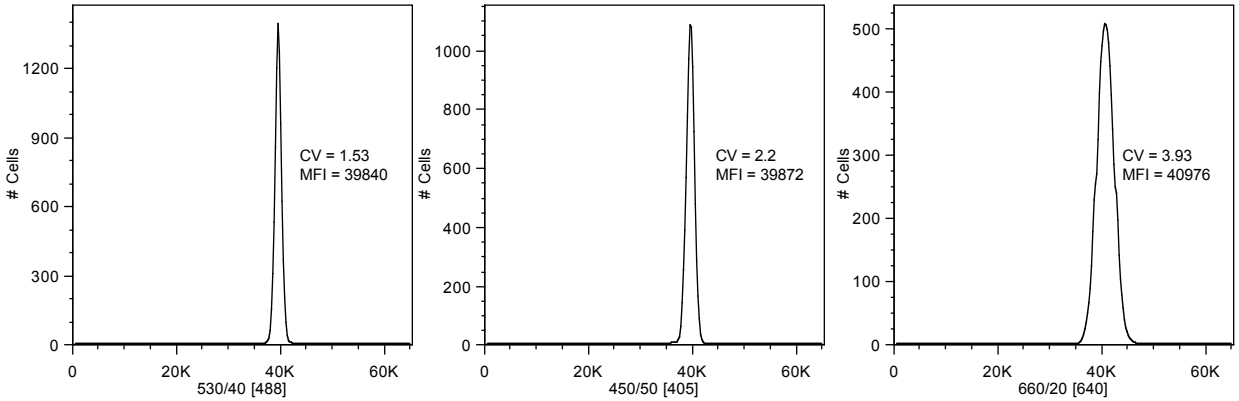
Alignment

QC beads

Instrument Condition #1



Instrument Condition #2



A) Why are the beads broader in condition #1 than #2?

B) What would you do to fix this problem?

Answer: Tight and Bright

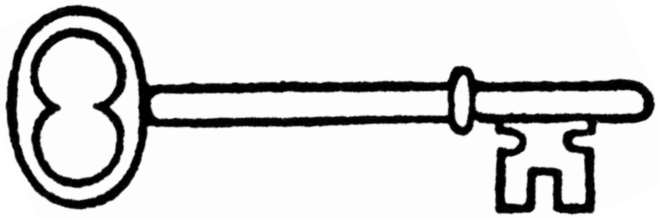
A) Why are the beads broader in condition #1 than #2?

The machine was not aligned optimally. Possibly because the nozzle is partially clogged with a clump of cells.

A) What would you do to fix this problem?

Remove the clog by sonicating the nozzle and then run the alignment beads to align the machine by adjusting the nozzle position and the lasers. The lasers should intersect with the stream and this interrogation point should be focused in front of the pinholes. Use alignment particles to make the final adjustments while looking for the beads to be "Tight and Bright".

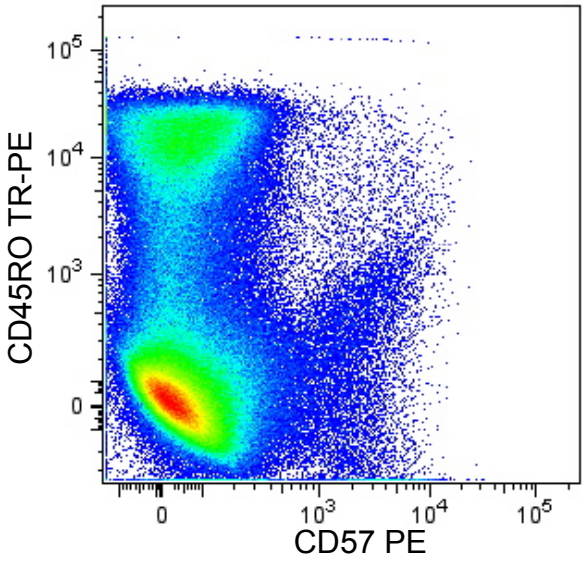
Compensation Controls are KEY



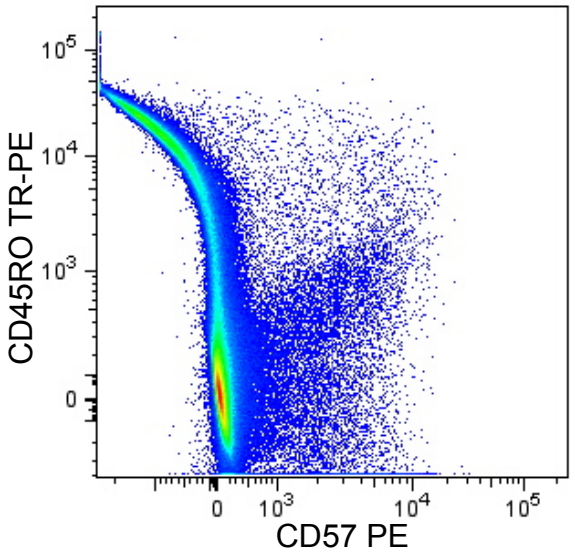
Compensation

The same cells are shown in both plots below, however the compensation applied is different. Different single color controls were used to calculate the compensation.

Single color controls:
-CD45RO TR-PE
-CD57 PE



Single color controls:
-CD3 TR-PE
-CD57 PE



Note: TR-PE = TexasRed-PE (tandem dye)

Data from Mario Roederer

A) Why did the compensation go wrong in the right-hand plot?

Answer: Compensation #1

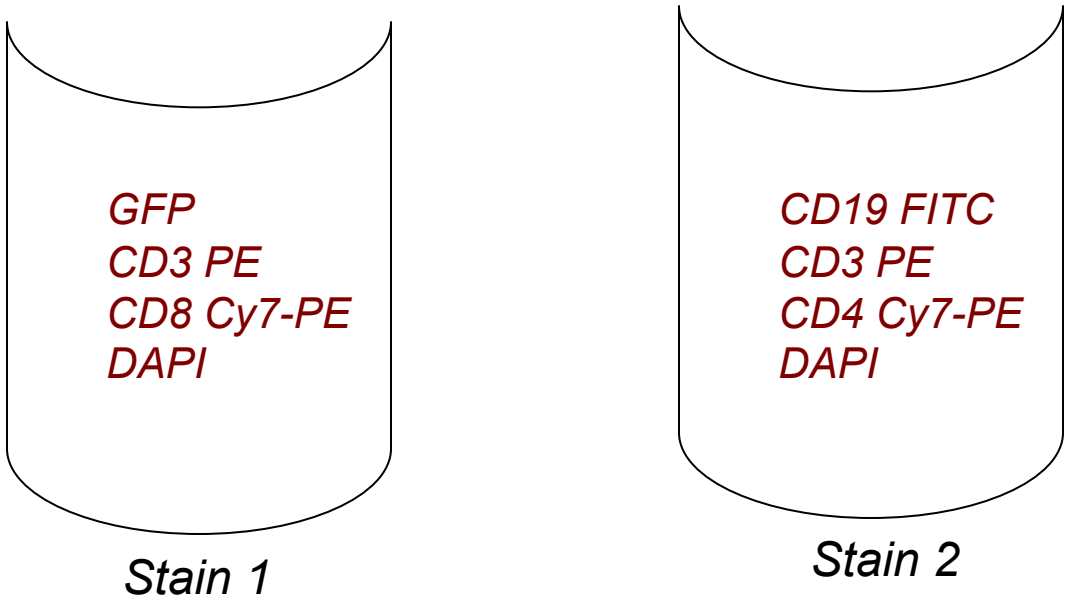
A) Why did the compensation go wrong in the right-hand plot?

The incorrect TR-PE single color control was used to compensate. The sample was stained with CD45RO-TRPE but the single color control used to calculate the compensation was CD3-TRPE. Tandem dyes cannot be substituted!

Note: tandem dyes differ from lot-to-lot and each vial changes over time based on their light exposure and time from manufacture.

Compensation

Your experiment contains two staining combinations:



A) List the single color compensation tubes needed to compensate properly.

B) Should you resuspend ALL the controls in media containing DAPI?

Answer: Compensation #2

A) List the single color compensation tubes needed to compensate properly.

GFP
CD19 FITC
CD3 PE
CD8 Cy7-PE
CD4 Cy7-PE
DAPI

GFP and FITC are both green but not the same fluorochrome!

Tandem dyes must be treated like different fluorochromes.

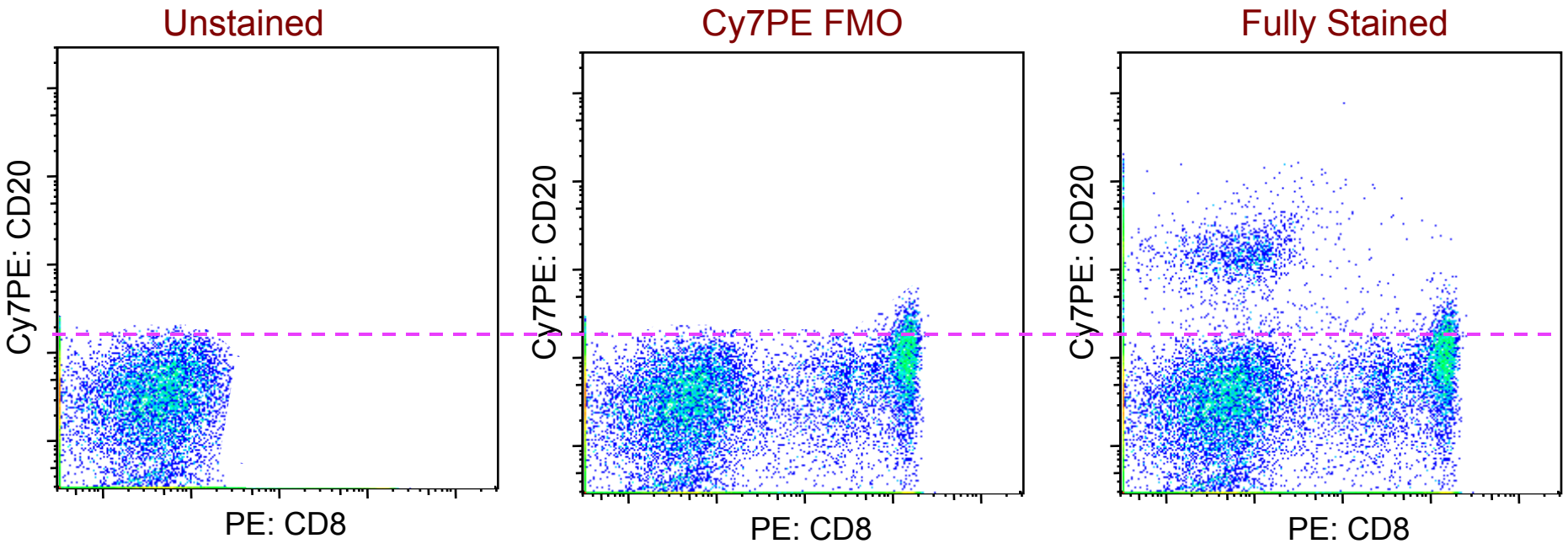
A) Should you resuspend ALL the controls in media containing DAPI?

No. If you add DAPI to all the single color controls they are no longer "Single" and can't be used to compensate.

Gating Controls:

Fluorescence Minus One

Gating Controls: Fluorescence Minus One (FMO)



A) Is the cutoff (dotted line) correctly gating the CD20+ population?
B) Are some of the CD8+ cells expressing CD20?
C) Where would you draw the gate to define the CD20+ population?

Answer: FMO

A) Is the cutoff (dotted line) correctly gating the CD20+ population?

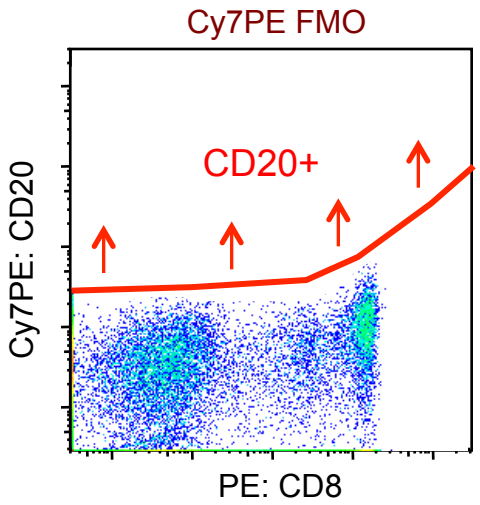
No because the line is based on the unstained and not the FMO gating control.

A) Are some of the CD8+ cells expressing CD20?

No. The FMO shows that the CD8+ population is spread out. This spread is due to measurement error and not due to CD20 expression.

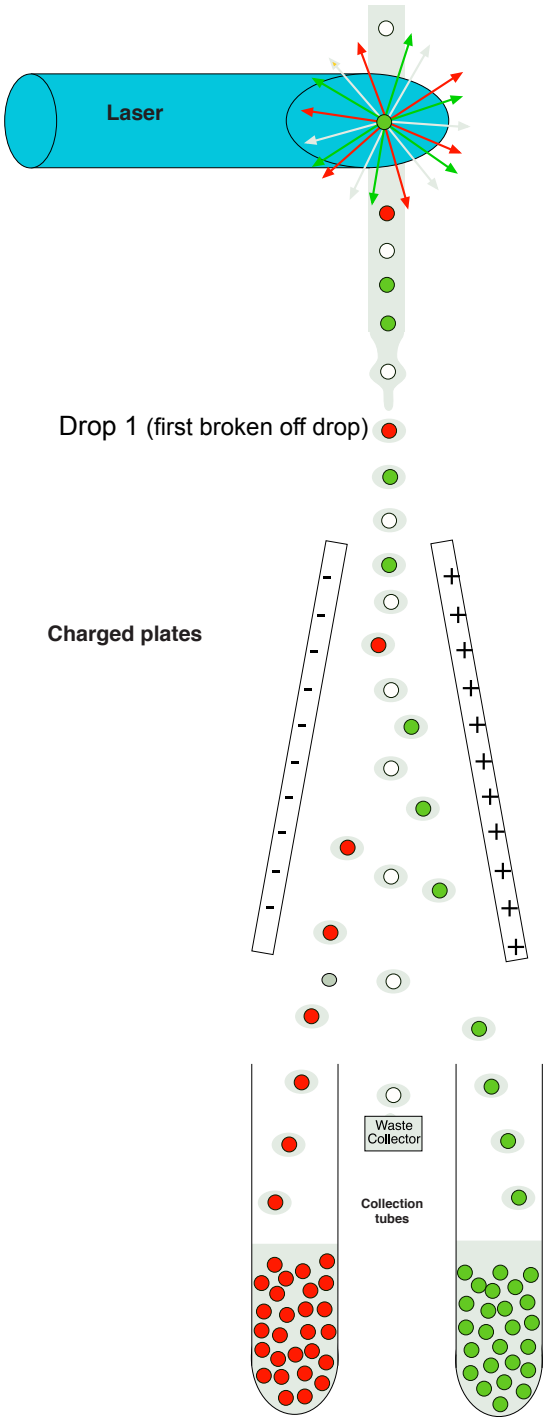
A) Where would you draw the gate to define the CD20+ population?

Use the FMO to draw a curved line above which is CD20+.



Don’t delay the DROP DELAY

Drop Delay



A) Draw a line between two elements on the picture to identify the drop delay.

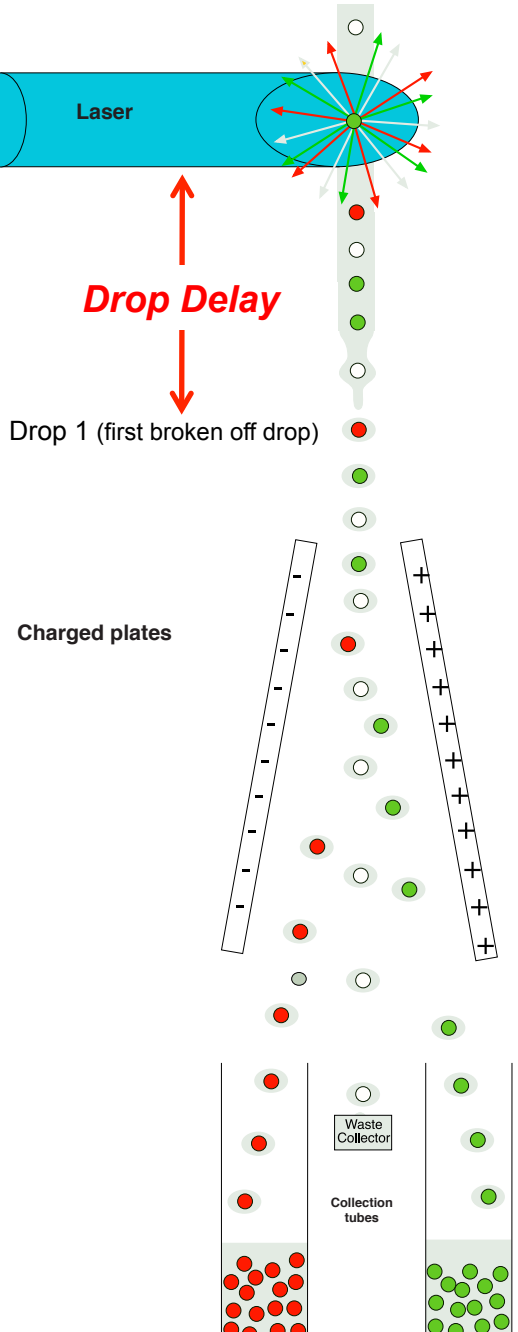
B) What can cause the drop delay to change while sorting?

Answer: Drop Delay #1

A) Draw a line between two elements on the picture to identify the drop delay.

B) What can cause the drop delay to change while sorting?

A partial clog or running a tube dry can cause the drop delay to change. A change in room temperature can also change the drop delay. Check it often!



Drop Delay

1 million GFP+ cells are sorted into a collection tube.

The customer counts the cells and does a post sort analysis and finds:

-100,000 cells in the tube

-only 20% GFP+

A) What happened?

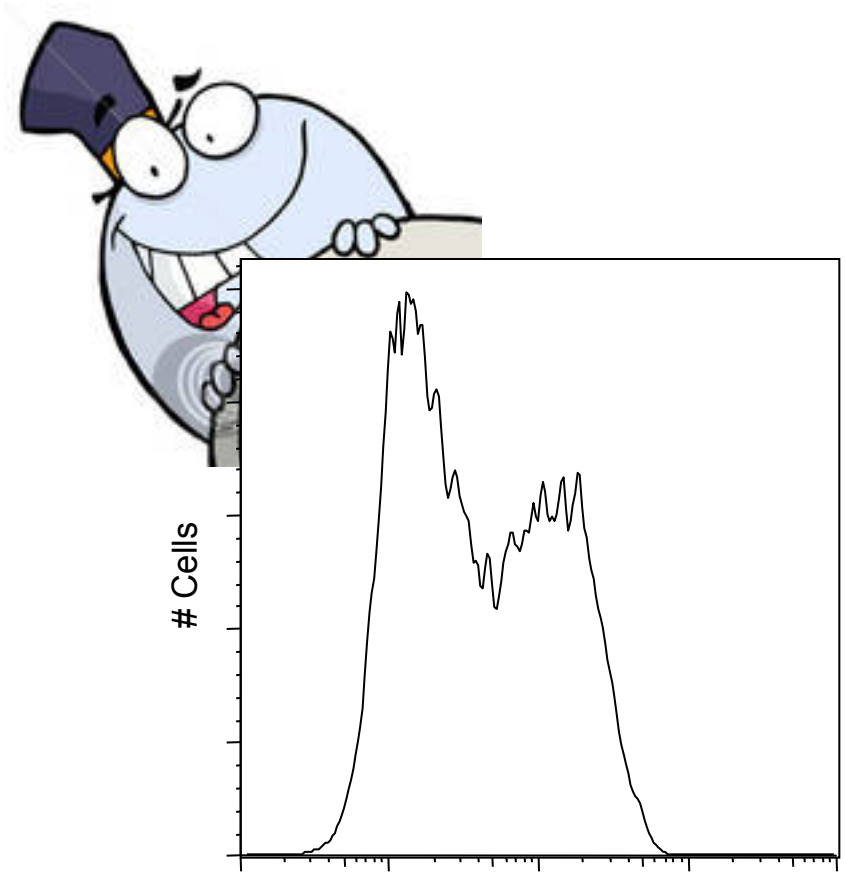
Answer: Drop Delay #2

A) What happened?

The drop delay was incorrect and the drops that were sorted were not the correct ones – they were either empty or contained the wrong type of cell.

Distinguishing Autofluorescence from Specific Fluorescence:

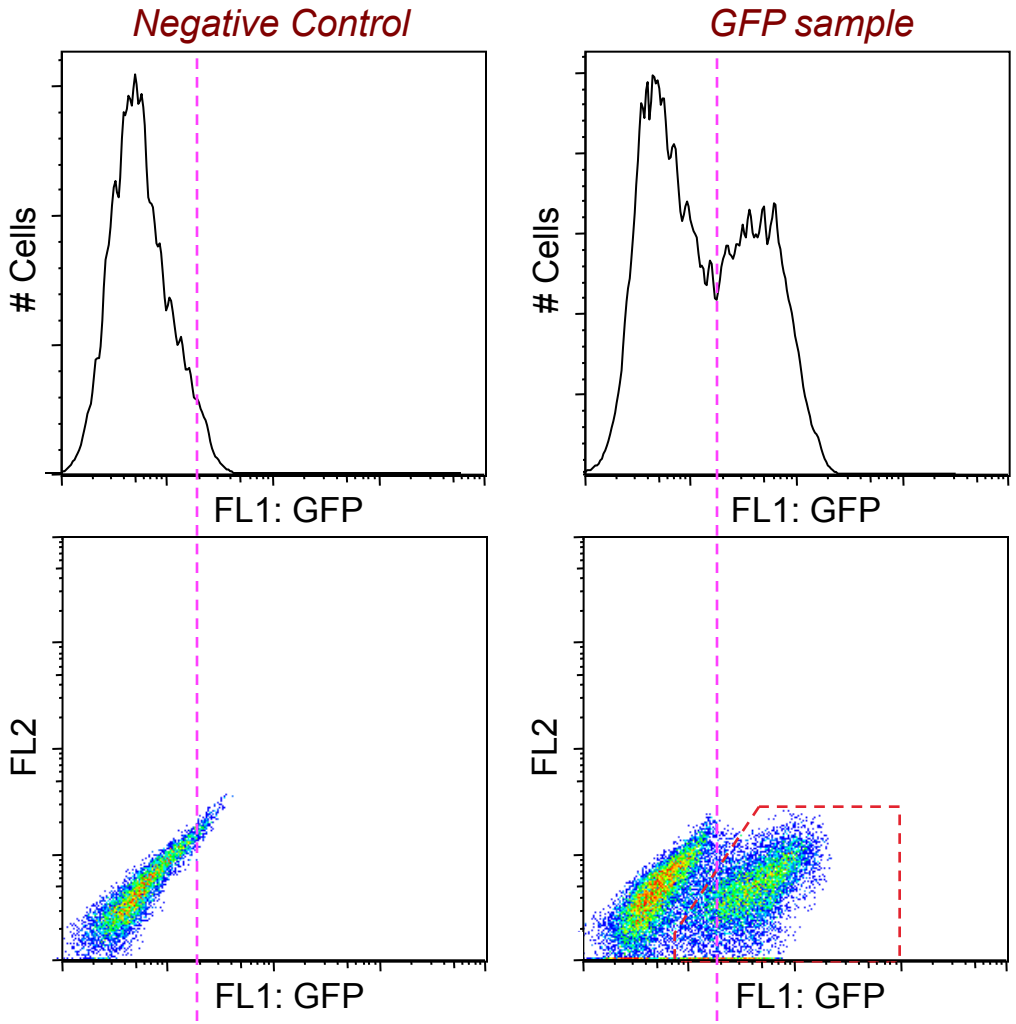
Histograms Hide Data



Distinguishing Autofluorescence from GFP fluorescence

Histogram Plot →

Bivariate Dot Plot →



A) Which plot type is more accurate for setting the GFP+ gate: Histogram or Bivariate Plot? Why?

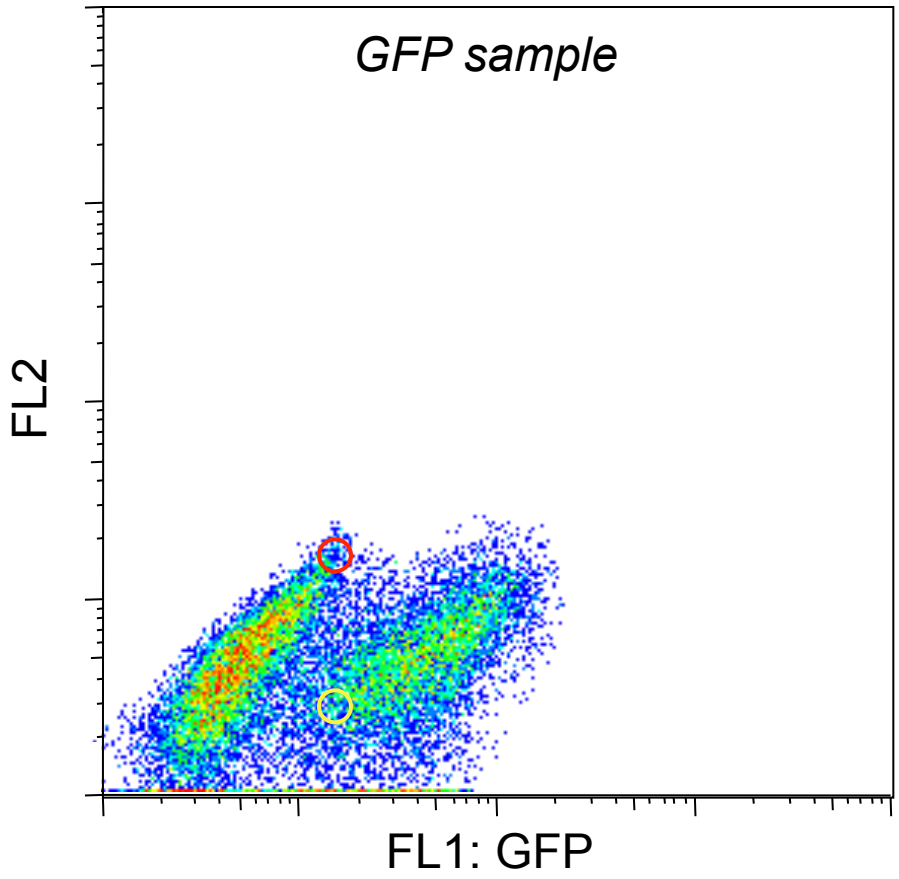
Answer: Histograms Hide Data #1

A) Which plot type is more accurate for setting the GFP+ gate:
Histogram or Bivariate Plot? Why?

The Bivariate Dot Plot is better because it allows you to clearly distinguish the GFP positives and negatives by drawing a gate on a diagonal.

Autofluorescence is fairly even in all parameters and a bivariate plot shows the difference between cells with high autofluorescence (on the diagonal) and those with GFP specific fluorescence (seen only in FL1).

Distinguishing Autofluorescence from GFP fluorescence



B) The cells in the red circle and the yellow circle have the same amount of FL1 (green) fluorescence. But the source of that fluorescence is different.

- What is the origin of the FL1 fluorescence for cells in the red circle?
- What about for the cells in the yellow circle?

Answer: Histograms Hide Data #2

B) The cells in the red circle and the yellow circle have the same amount of FL1 (green) fluorescence. But the source of that fluorescence is different.

- What is the origin of the FL1 fluorescence for cells in the red circle?

Autofluorescence (intrinsic fluorescence)

- What about for the cells in the yellow circle?

Some autofluorescence + GFP specific fluorescence