Basic Quality Control

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Basic Quality Control

Standardizing or monitoring your cytometer:

Track day to day sensitivity and reproducibility

Ensure valid and consistent data on that cytometer

When to QC?

- Run beads at least once a day
- When clog, bubbles or weird data.
- Even if the instrument is QC’d in the morning, it doesn’t mean somebody hasn’t messed it up in the meantime.
- Some laboratories insist that QC beads are run with every experiment. This is ideal.

QC: How to

Individual lab procedure you develop yourself:

- Cross platform
- Hard dyed beads, many available
- Manual procedure
- Track daily

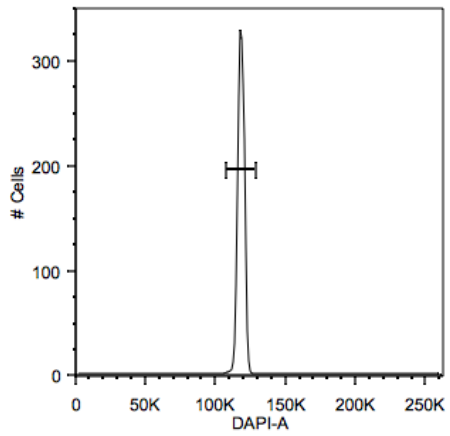
Manufacturer's procedure:

- Hard dyed beads supplied by company
- Automated procedure
- Track daily

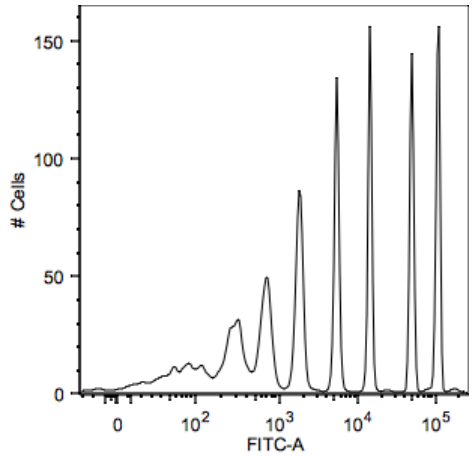
Hard dyed Beads

Fluorochromes are incorporated into beads

- Not the standard fluorochromes used for staining cells
- Multiple excitations and emissions across lasers
- Do not fluoresce uniformly in all channels
- Single peak or multipeak



single peak (UltraRainbow)



Multipeak beads (Rainbow 8 peak)

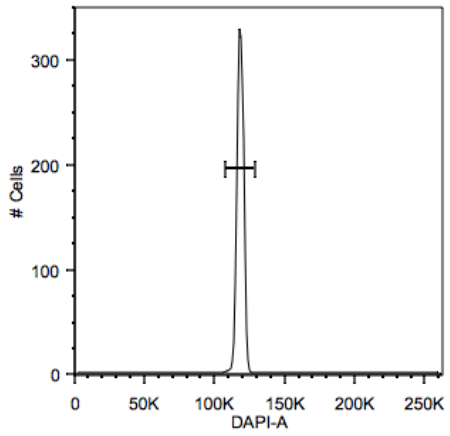
Single peak beads

monitor day to day stability

evaluate subtle fluidics problems

Pick up some problems early

good for DNA cell cycle applications where tight CV is essential



Single peak beads, linear display

Multipeak beads

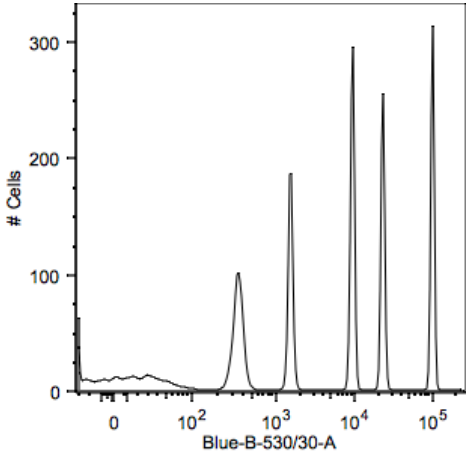
Mixture of beads with different amounts of fluorochrome

Allow evaluation of

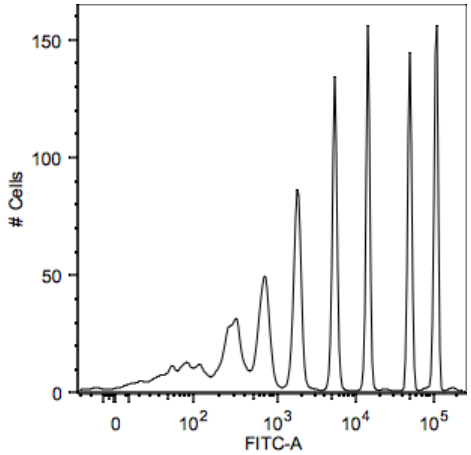
resolution of dim vs negative staining

linearity

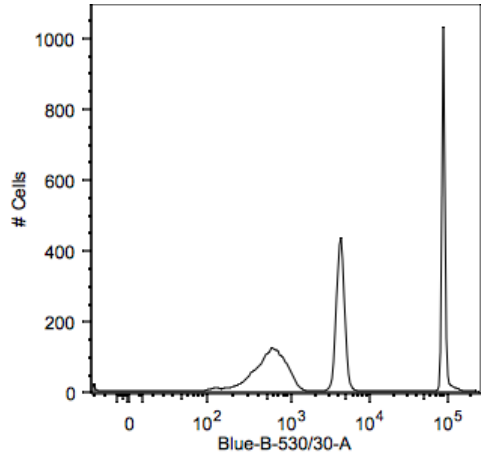
Some have calibrated amounts of fluorescence (MESF, ABD)



Cytocal



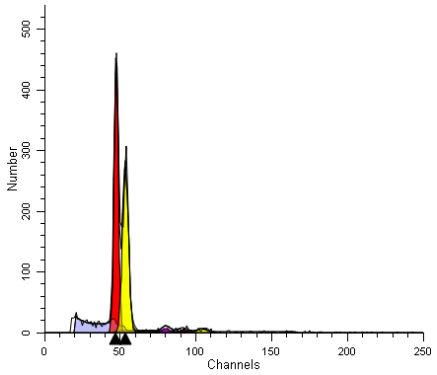
8 peak rainbows

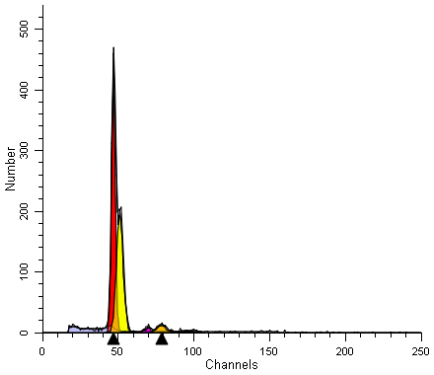


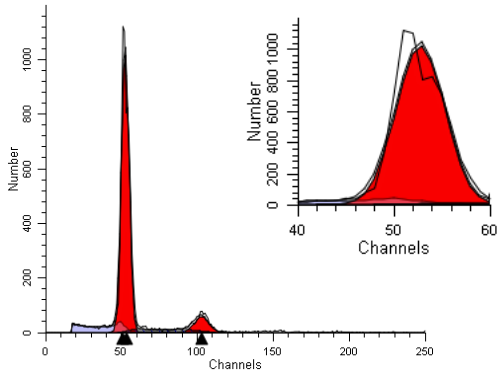
CS&T, BD's QC beads

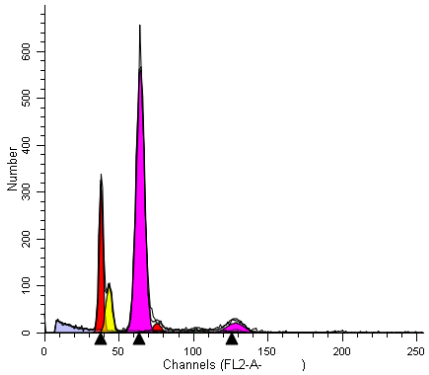
QC Impact on DNA histograms

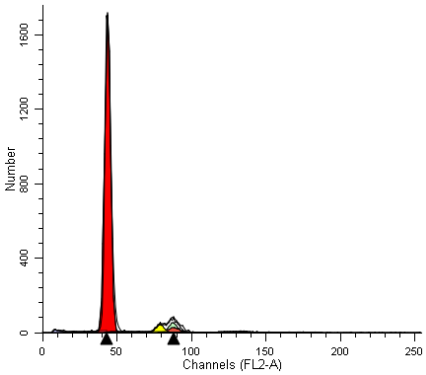
- Human breast cancer samples
- If your QC bead CVs are high,
- you will not detect near diploid or small aneuploid populations











Individual lab QC: daily check

- Open template
- Run beads
- 2 options for tracking peaks

  either

  1) simply record peak channel numbers and save file

  or

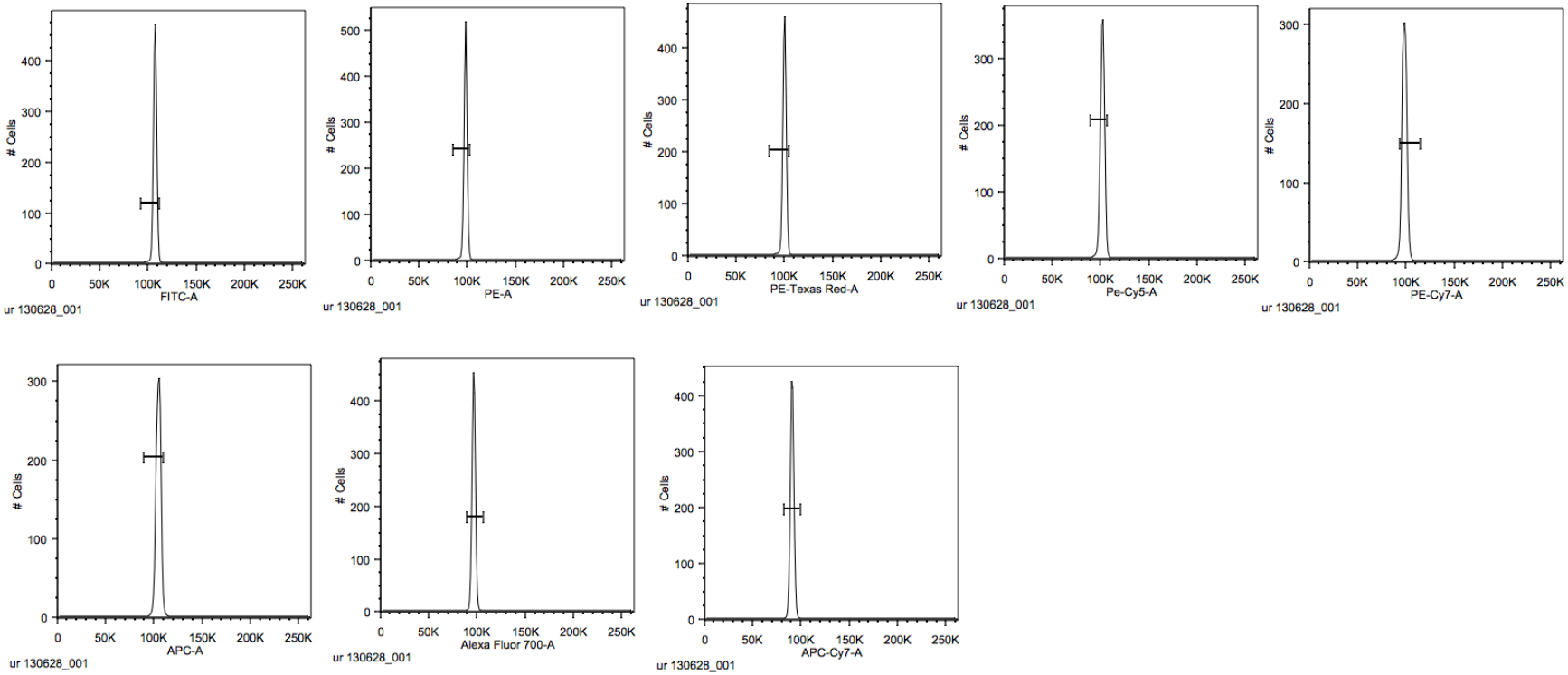
  2) change PMT voltages so beads are in target channel and record PMT voltage changes
- Record peak CVs

Individual lab QC: protocol setup

Preferably just after a service maintenance

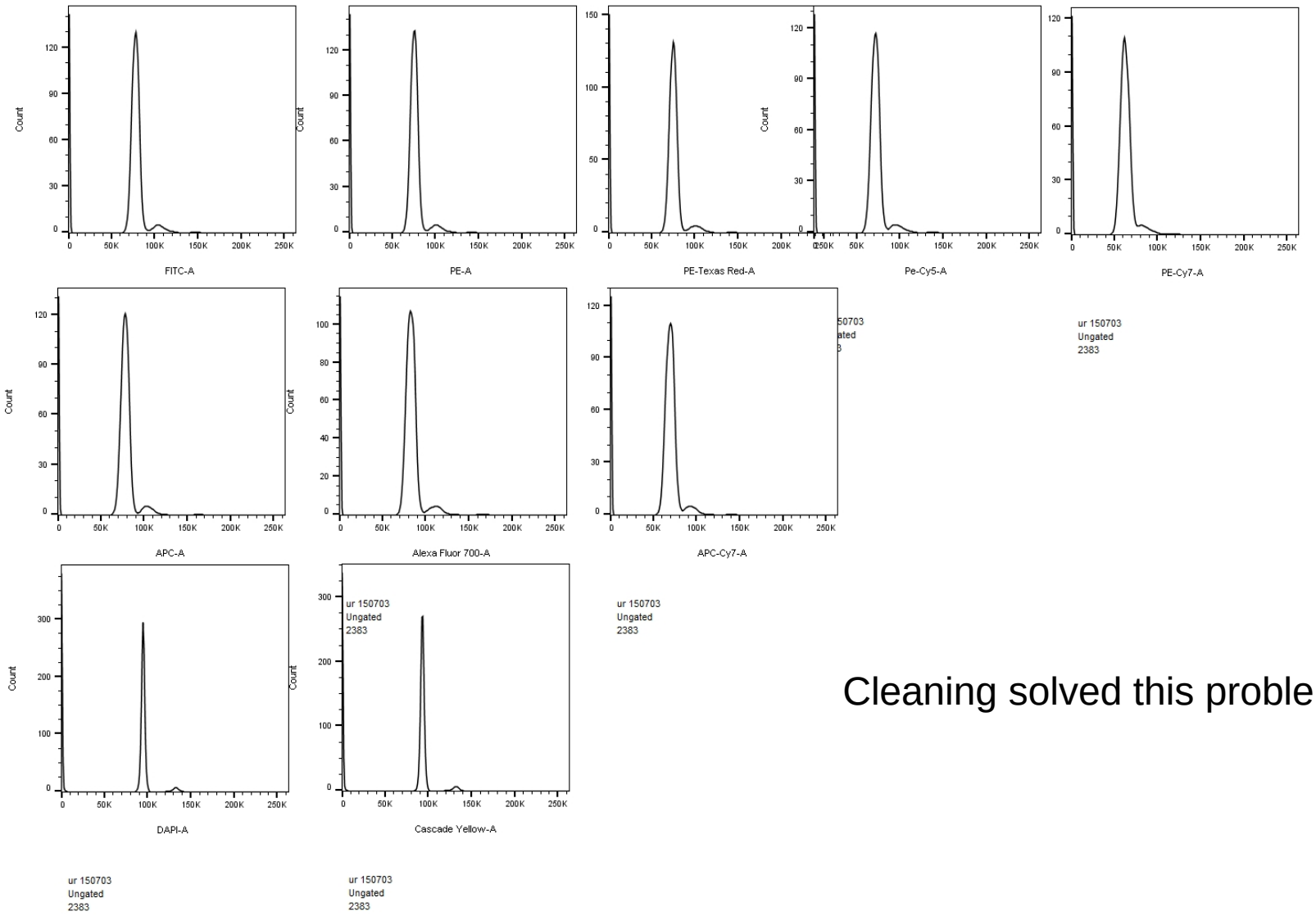
- Run beads on the cytometer (in our lab we use single peak beads)
- Set PMT voltages so beads are in a target channel for all parameters (we set them around midscale)
- Record either peak channel numbers or PMT voltages
- Record peak CVs
- Save a template

Daily check: A typical run: single peak beads



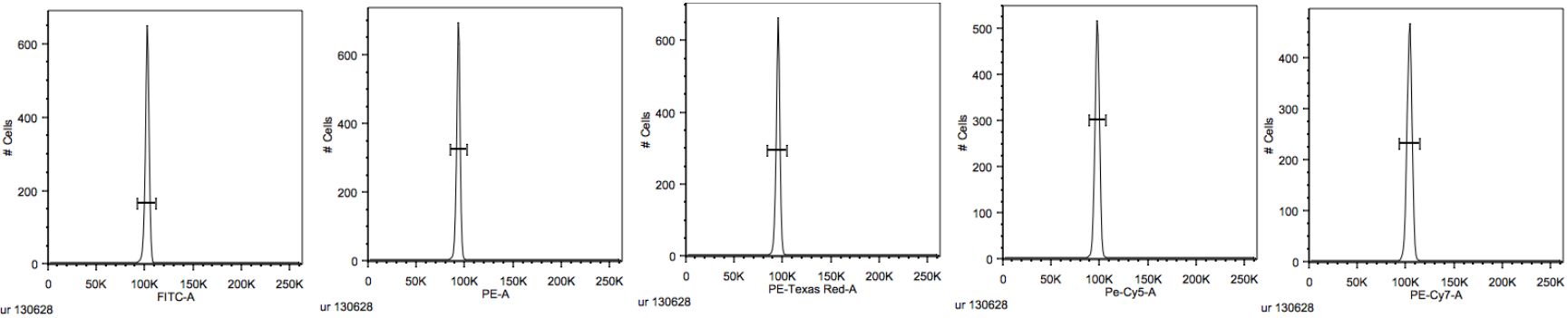
UltraRainbow beads, LSRII

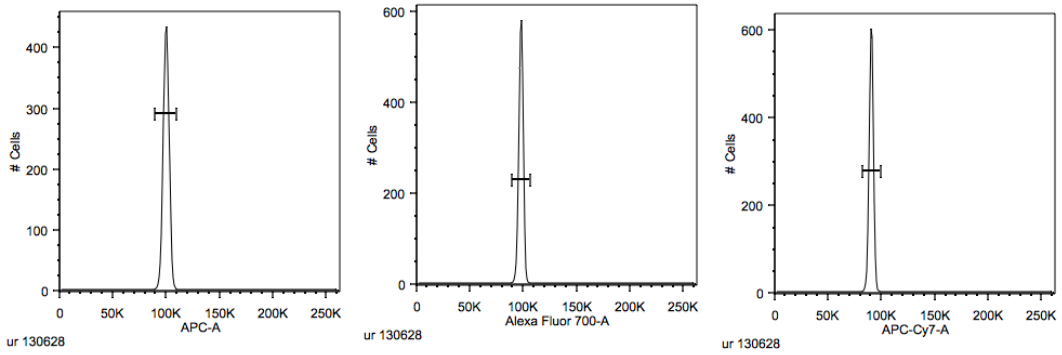
There’s a problem



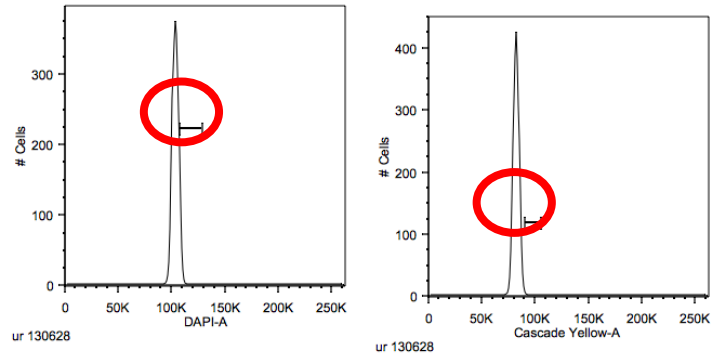
Cleaning solved this problem

Daily check: where's the problem?





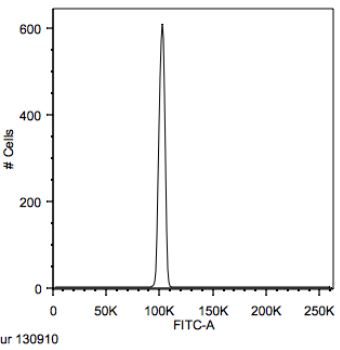
Everything looks good except the violets

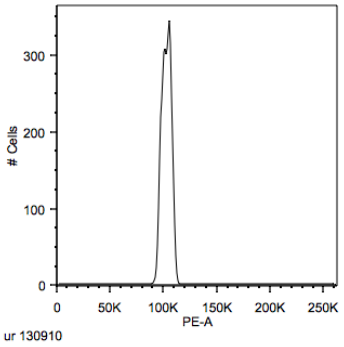


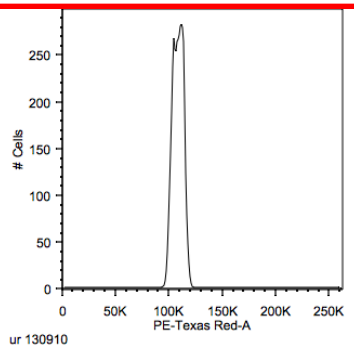
Channel difference from normal: 15,000
PMT voltage difference: 9v

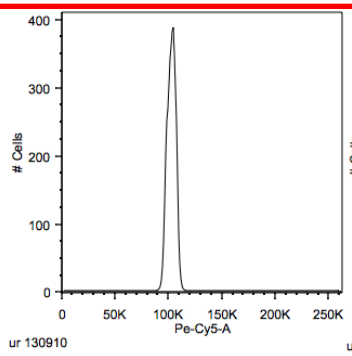
Violet laser alignment drifting

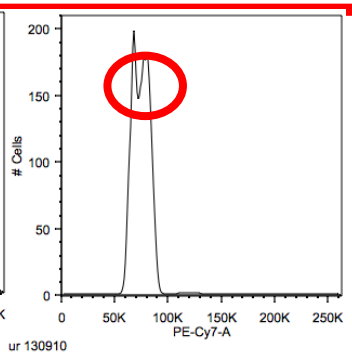
Daily check: there's a problem

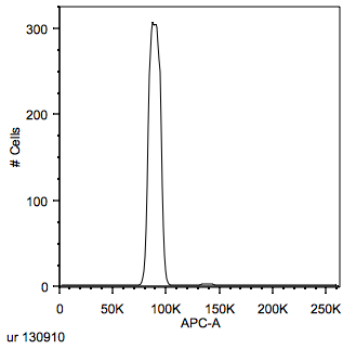


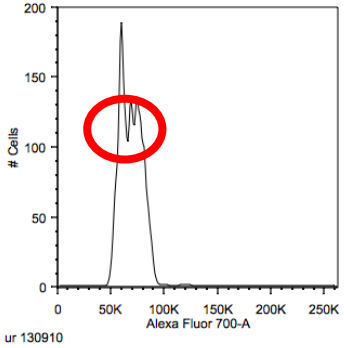


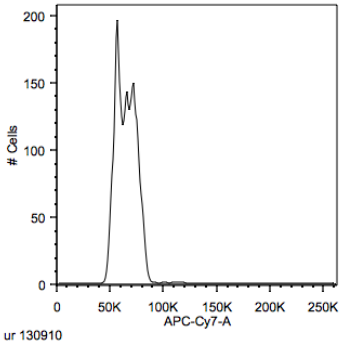


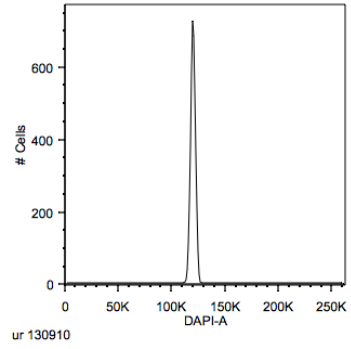


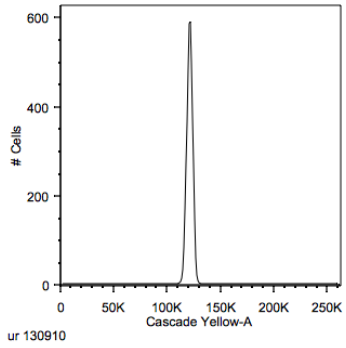












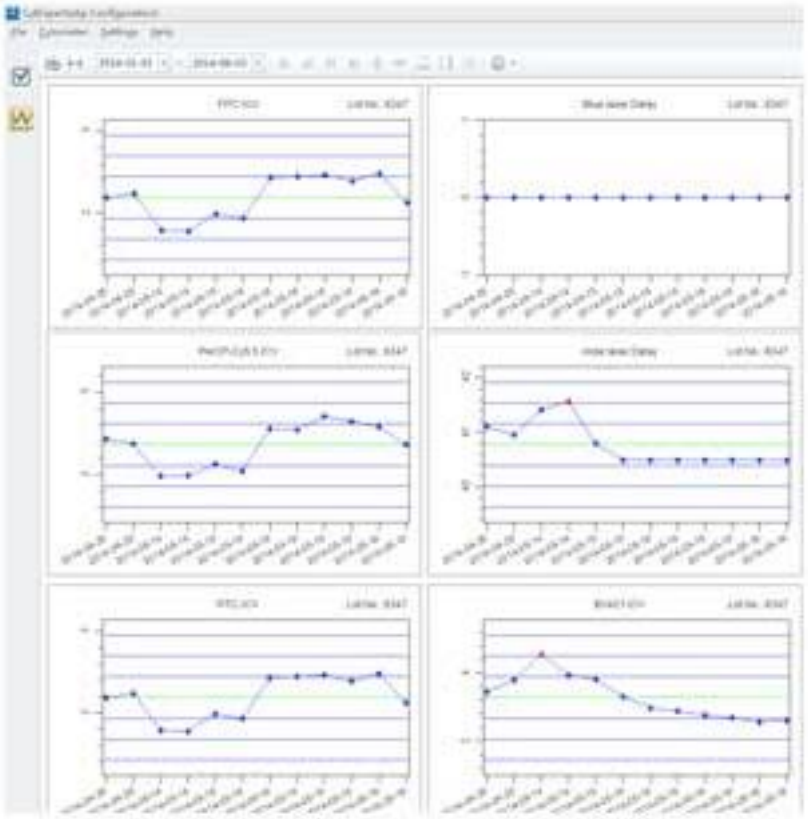
Bead problem:

UR beads fluorescence has degraded in the reds (they don't like contact with bleach)

Tracking

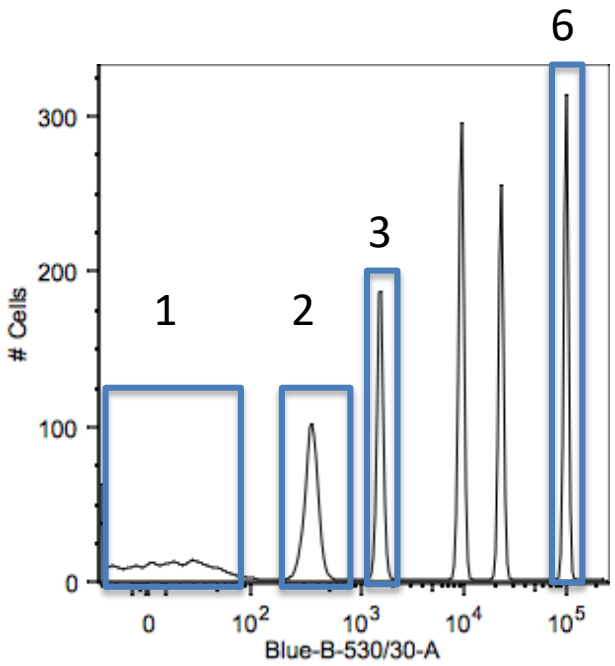
Track data with Levey Jennings plots

Establish acceptable ranges

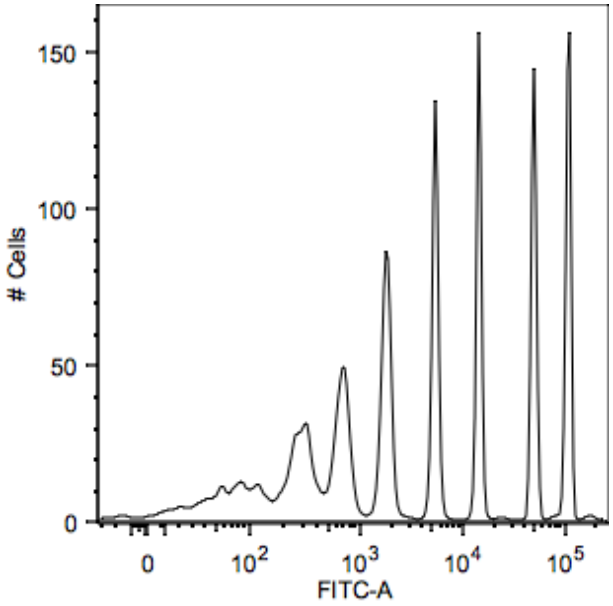


Multipeak beads

Track resolution of dim vs negative staining
Track Linearity



Cytocal



8 peak rainbows

Top peak(6) should be in same channel: decreased peak median indicates a problem
Monitor lower peak medians and cvs for resolution and linearity: Increase in position or width of negatives indicates a problem

Manufacturer Procedures

Use dedicated beads and software supplied by the manufacturer

- Cytoflex beads
- MACSQuant Calibration Beads
- Biorad S3 Proline Beads

Most use single peak beads to track peak channel and CV similar to the manual procedures

Some procedures: BD CS&T Beads monitor in more depth:

- resolution, sensitivity, linearity
- Background (B), Photoelectron efficiency (Q)

Manufacturers Procedure

Generally monitor:

Target peak channels

or PMT voltages

Bead CV

Example:

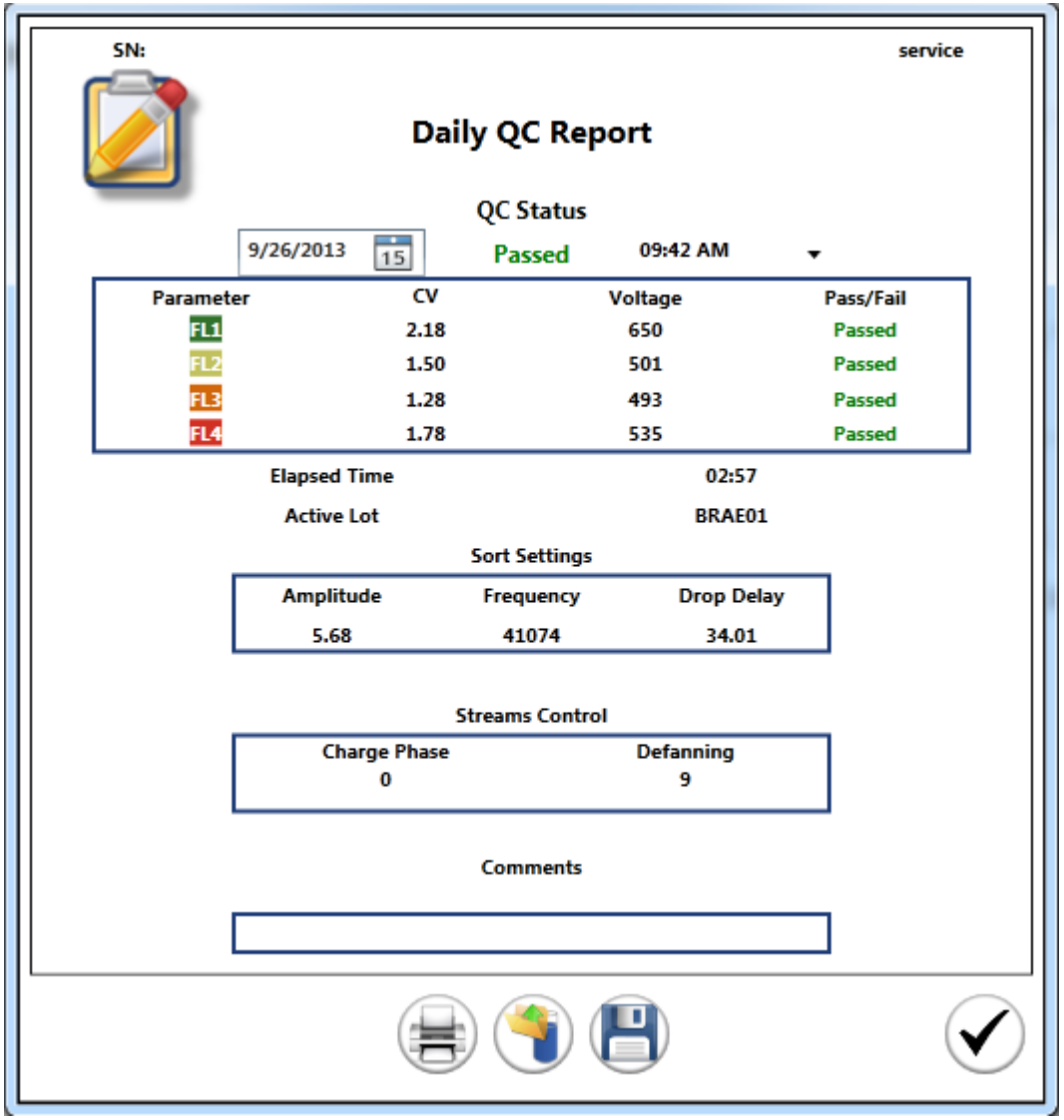
BioRad S3 sorter :

Laser alignment

PMT voltage

peak CV

drop delay for sorting



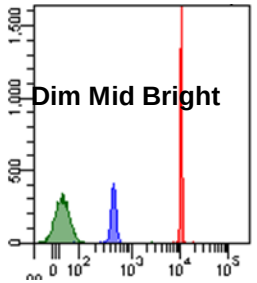
BD's Cytometer Setup & Tracking (CST) System:

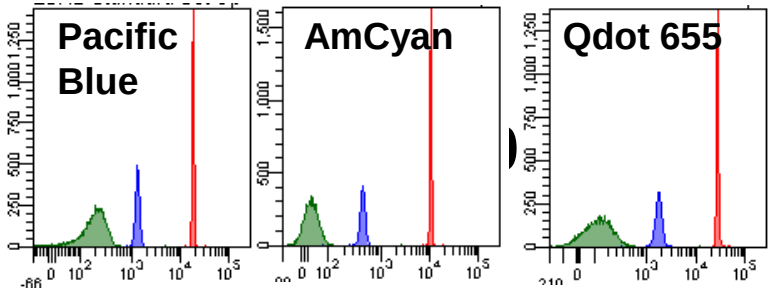
Monitors:

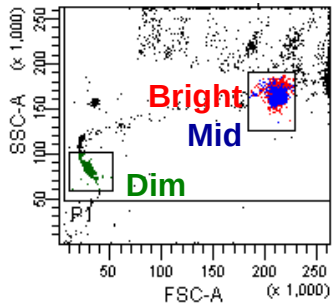
- Linearity
- Detector efficiency (Qr)
- Background fluorescence (Br)
- Electronic noise (sDEN)
- Alignment (rCV)

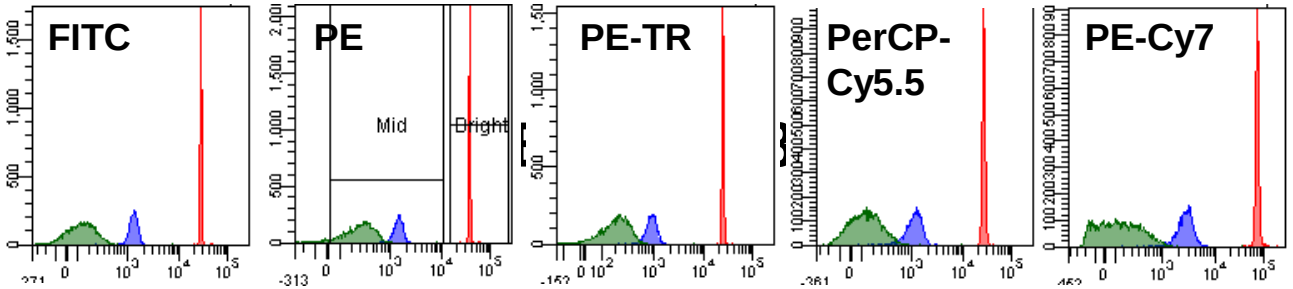
Optimizes:

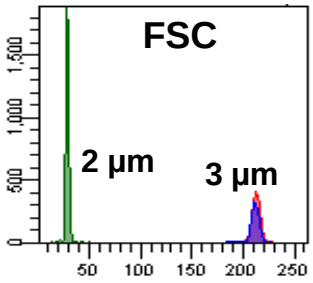
- Laser delays
- Area scaling factors
- PMT voltages

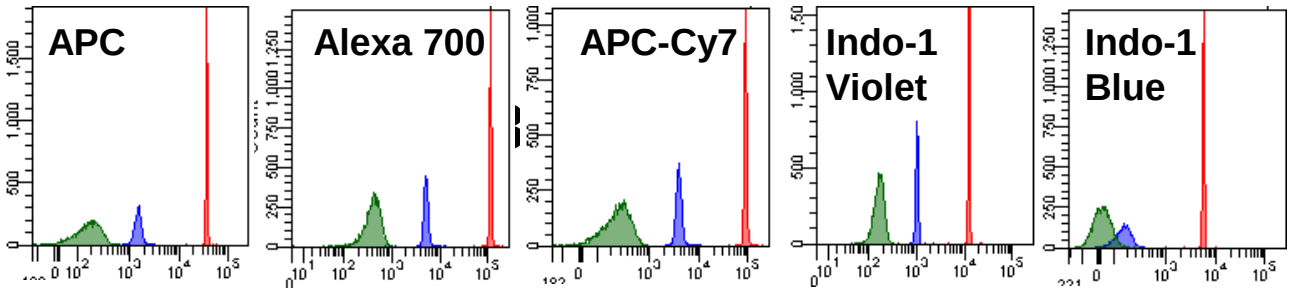












Cross Platform QC systems: single peak

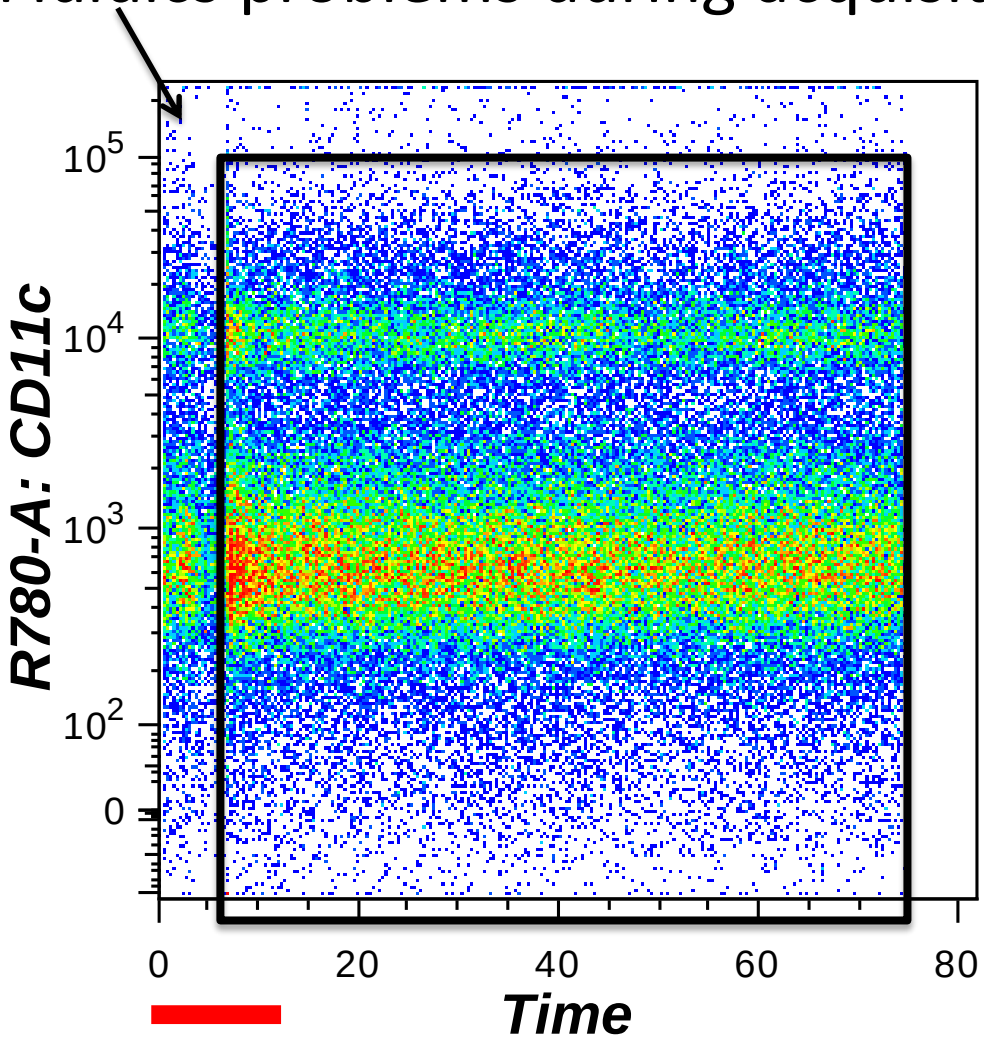
- Pros
  - Fast: Takes less time to record than the manufacturer's system
  - Can be easily run throughout the day, just before each experiment or to verify after machine problems
  - Fluidics problems immediately visible
- Cons
  - Not as comprehensive as multipeak manufacturers QC
  - does not usually monitor linearity, signal to noise ratios, area scaling and laser delays
  - Should use multipeak beads to monitor linearity and signal to noise regularly.

Automated QC systems: multipeak

- Pros
  - Automatic and comprehensive
    - verifies sensitivity, linearity, signal to noise ratios,
    - adjusts area scaling and laser delays
    - Adjusts PMT voltages to adjust for small changes in peak medians
    - Records and tracks all information
- Cons
  - Have to go through all steps, takes time (however can spot check beads to assess before running procedure)
  - If there are minor fluidics problems, laser delay and scaling might be changed unnecessarily
  - Not immediately visual, pass/fail then must read through data to see why

Use a time gate to eliminate artifacts

Fluidics problems during acquisition cause artifacts in the data



To visualize:

look at data vs time

Can gate out the bad data
BUT you need to fix the problem!

Cytometer settings

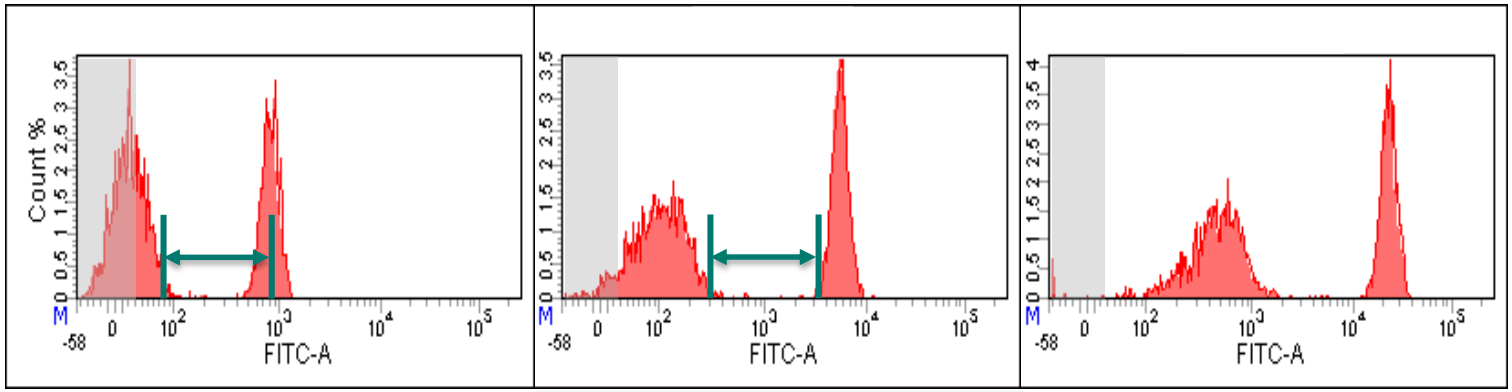
Detector gains (PMT voltages) will affect resolution

- Gains should be optimized for each detector
  - 3-4 x SD electronic noise
  - **Voltration**
- Need to be above the background noise and within the linearity of each detector

Voltration using stain index (SI)

Run stained cells at a series of PMT voltages
Calculate Stain Index
Choose optimal voltage for **each** parameter for **each** instrument

FITC Detector (SD_{EN} = 20) x 2.5 = 50



PMT Voltage	370	470	570
Stain Index	15	39	42
MFI Pos Cells	750	5,072	21,183
MFI Neg Cells	15	94	415
rSD Neg Cells	24	64	245

Longitudinal Standardization

(same cytometer over time)

Standardization over time 1

The first time you set up your experiment:

- Use PMT or gain settings optimized for all detectors using single stained cells or comp beads (voltation)
- Run QC beads
- Put gate around peak for each detector, show MFI
- These are your target channels for the QC beads
- Save the template

Standardization over time 2

The next time, and all future runs:

- Open the template
- Run the QC beads
- The bead peaks should be in the target channels
- If not, change the PMT voltages to put beads in the target channel
- (PMT or gain change should be minor, if they are large you will have to resolve the instrument problem)
- Run your experiment using those PMT voltage settings

Standardization over time

- You should also have control cells for each run, frozen aliquots for example
  - To verify that staining profiles remain stable
  - To control for cell preparation, reagent or staining problems

Inter Cytometer Variations

The fundamental problem is that cytometers differ in sensitivity

Fluorochrome	Brightness x 10^5	Brightness relative to PE	Stain index	
			Cytometer 1	Cytometer 2
PE	16	100%	348	262
PeCy5	NA	NA	180	131
APC	4.7	29%	238	281
FITC	0.4	2%	132	61
BV421	16	100%	264	145

Due to cytometer differences in:

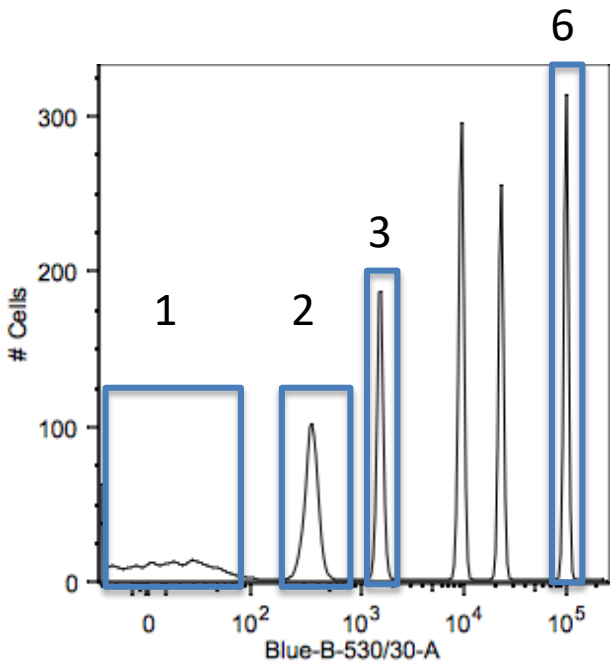
Laser configuration and power
Laser and optical alignment
Dichroic mirrors and filters
PMT sensitivity (Q)
PMT background (B)

Q and B: criteria for cytometer performance

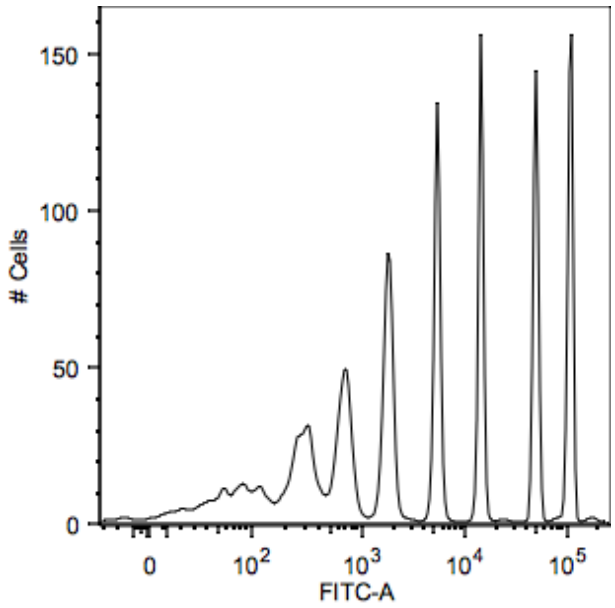
- Used to quantify sensitivity and background for each detector
- Q: number of photoelectrons produced per molecule of fluorochrome: sensitivity
- B: electronic and optical background when no fluorochrome is present
- Big differences between cytometers and between individual PMTs
- Best detection when high Q and low B

Background and sensitivity variations

Resolution of the lower peaks will vary between detectors and between cytometers and over time



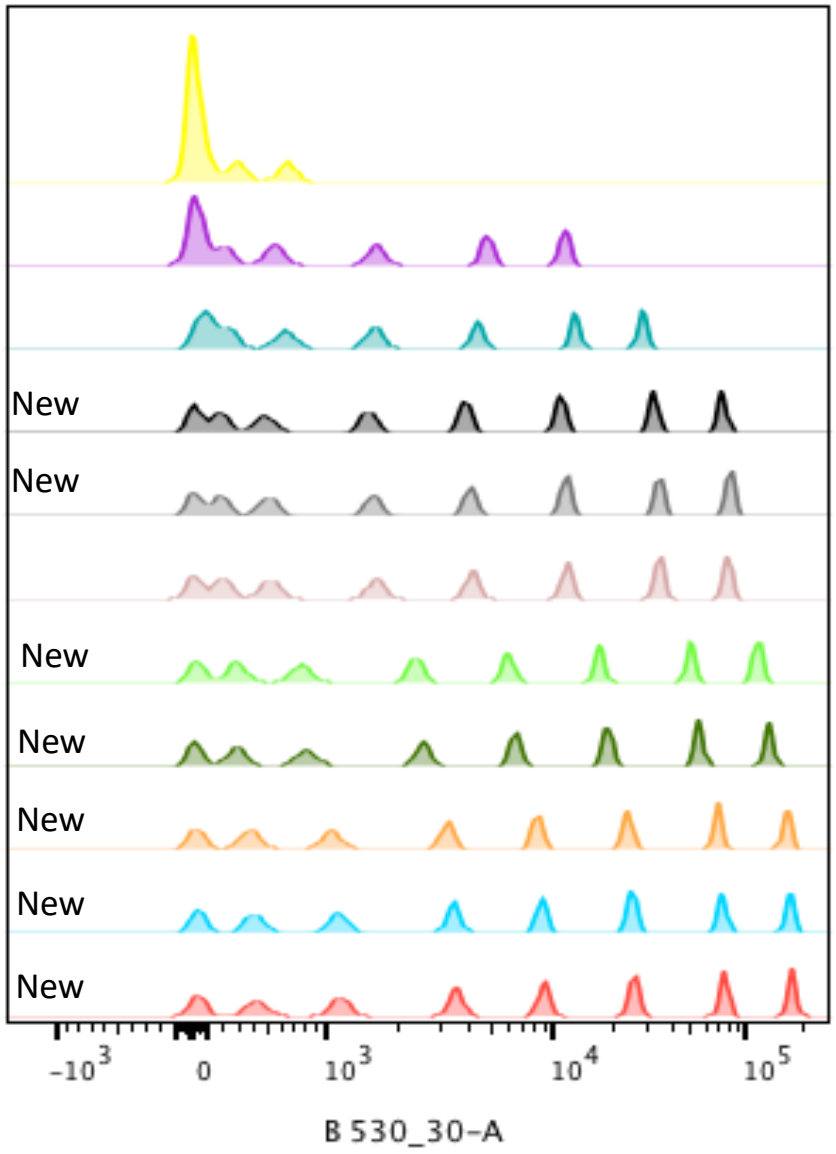
Cytocal



8 peak rainbows

Poor separation due to increased background position or peak width, indicates a problem

Cytometer variations in resolution



Courtesy of Grace Chojnowski

8 Peak Rainbow Beads.

Look at the resolution of the 3 dimmest peaks

Different 530/30 filters were compared on the same instrument FITC channel

Resolution is affected by laser power, wavelength, alignment, detector Q and B

Different across parameters and across instruments

Standardization across cytometers

Standardization across cytometers

Simplest scenarios

Set cell or bead peak to the same target channel across all cytometers **(standardize)**

or

Use best PMT voltages or gains determined for each instrument **(optimize)**

Standardization: set to target channel

single color controls: cells or beads stained with real fluorochromes

In this procedure you will need:

- cells, unstained and fully stained
- cells or comp beads stained with fluorochromes
- Hard dyed QC beads e.g. rainbow, **UR** or CST beads

Standardization across cytometers: step 1

On the first cytometer (cytometer A) you set up your experiment:

- Use best pmt voltage settings for **this** instrument
- Run the stained single colors at these optimized settings
- put gate around the positive population for each color, **note MFI**
- **This are your target channels for the positive population on the other cytometers**
- Then run QC beads, set a gate around peak, these are QC target channels for future runs on this cytometer
- Save the template

Standardization across cytometers: step 2

On cytometer B, the same day,

- Run the **same** comp cells or beads as on cytometer A
- Set the PMT voltages to put positive population at the same target channel as they were in cytometer A
- Then run QC beads, set a gate around peak, these are QC target channels for future runs on this cytometer
- Save the template

Future runs for all cytometers

- Open template
- adjust PMT voltage to put **QC beads in QC target channel**
- Run cells at these settings

Target channels across different types of cytometers

The scale, or maximum number of channels, may not be the same on different kinds of cytometers.
The positive population target channel should be at the same relative position on the scale as cytometer A.

For example: in the FITC channel
On cytometer A, the target channel was 176,400 in a 252,000 channel system

- Thus they are at 70% of maximum scale

On cytometer B, the scale is a 1024 channel system

- positive position should be at 70% maximum scale
- If there are 1024 channels, it should be in channel 717

Cross Cytometer Standardization drawbacks

The PMT voltages are chosen so that the positives are in the same position on the scale for all the cytometers

This was the optimal PMT settings for Cytometer A, but may not be the optimum for resolution of positive and negative on the other cytometers.

You can have standardization or optimization, but not both!

AND even if you put the positive peak in the same target channel, the negative peak position and spread will differ from cytometer to cytometer because the stain index differs. This impacts the discrimination of dim populations. You must verify that you can resolve dim populations on the weakest instrument.

Maria's Virtual Cytometer Standardization

Want acceptable population resolution for each parameter on a "worst" instrument

Build a virtual cytometer with the worst parameters.

Uses electronic noise and linearity criteria to determine worst sensitivity for each parameter on each instrument

Need to know the electronic noise and linearity:

for each parameter
for each instrument

Maria's Standardization: criteria

Electronic noise:

on BD instruments get it from the CST report

on other instruments:

Run blank beads, lasers blocked, optimized voltage setting and with PMT voltage set to zero

Linearity:

on BD instruments get it from the CST report

on other instruments:

Run multipeak beads at different voltage settings

Calculate ratio of MFIs of 2 bead peaks for each setting, high bead should stay on scale for the whole range. The ratio will be the same until the linearity maximum is reached

Maria's Standardization: Virtual Cytometer

For each parameter, choose the instrument that has

the highest background and lowest linearity max

Construct a virtual cytometer with each of these worst parameters

Determine settings

Run unstained cells and brightly stained cells

Set unstained cells so that the peak SD is greater than 2.5x SD of the electronic noise for each parameter on the instrument worst for that parameter

Make sure brightly stained cells are within linearity

Maria's Standardization

Once these settings are determined for the virtual instrument

Run comp controls and set in the target channel across other instruments

What next?

Currently:

Too much variability across Instruments to have exactly the same resolution for each parameter, even with the "same" instrument: optical configuration, laser wavelength and power. Filters, PMT sensitivity, Q and B, instrument alignment etc all play a role

PMT balancing to ideal performance?

Try to optimize or equalize performance so that Q and B, resolution/stain index similar for parameters across instruments

? Manufacturer evolution of instruments to guarantee better and more uniform performance

MESF calibration: FITC or PE calibration beads or Simply Cellular Capture Beads
Quantiflash LED pulser: calibrated photons of light to establish a photonic scale across instruments?

References

Mittag, A. and Tarnok A. Basics of standardization and calibration in cytometry – a review. J Biophoton, 2, 2009, 470-481.

Hoffman, RA. Standardization, Calibration, and Control in Flow Cytometry. In: Current Protocols in Cytometry 2005, pp 1.3.1–1.3.21.

Chase, ES and Hoffman, RA. Resolution of dimly fluorescent particles: a practical measure of fluorescence sensitivity. *Cytometry* 1998, 33:267-279.

Hoffman, RA and Wood, JC. Characterization of Flow Cytometer Instrument Sensitivity. In: Current Protocols in Cytometry 2007, 40:1.20.1–1.20.18.

Perfetto S, Ambrozak D, Nguyen R, Chattopadhyay P and Roederer M. Quality assurance for polychromatic flow cytometry. Nature Protocols 2006, 1, 1522 -1530.

Perfetto S, Ambrozak D, Nguyen R, Chattopadhyay P and Roederer M. Quality assurance for polychromatic flow cytometry using a suite of calibration beads. Nature Protocols 2012, 7, 2067-2079.

Troubleshooting

No cells or aberrant data during acquisition?

HELP! Where are my cells?

What do you do when you're in front of your cytometer and there's NOTHING going by on the screen.

Usually it's 10 at night and you can't call your core staff (they won't appreciate it!)

How to Troubleshoot

1. Is it the cytometer?

   First run QC beads to make sure the cytometer is functioning properly

2. Is it the cells? Are there cells there??

   Look at the cell preparation to assess debris and viability

3. Are the cytometer PMT or threshold settings wrong?

   use scatter vs dead cells and marker positivity

   to find your cells

1. Is it the cytometer?

Run your QC beads

If the QC beads show a problem, you must solve it before running your cells.

If there is a problem on all lasers:

In most cases it is a clog, dirty fluidics or bubbles,

- purge your sheath filter
- make sure the sheath pressure is ok
- run a cleaning solution, bleach, facsrinse or contrad
- only do a prime when the fluidics are clean

Keeping the cytometer fluidics clean is essential!!!!!

What to do if there's a problem

- If deviation from normal is greater than acceptable
  - Problem must be resolved
  - Beads must pass QC
  - Before acquiring experimental data

If experimental data looks aberrant, run beads and verify cytometer.

Many things can go wrong: dirty fluidics, old filters, dying lasers

Sheath tanks

Always make sure you have enough sheath fluid in the tank

Some systems don’t have level detectors in the sheath and waste

If the sheath runs dry, there will be bubbles everywhere

It will be a hassle to get them all out and the cytometer functioning correctly again

Your core staff will kill you.

Troubleshooting the machine

If there is a problem on one or several, but not all lasers

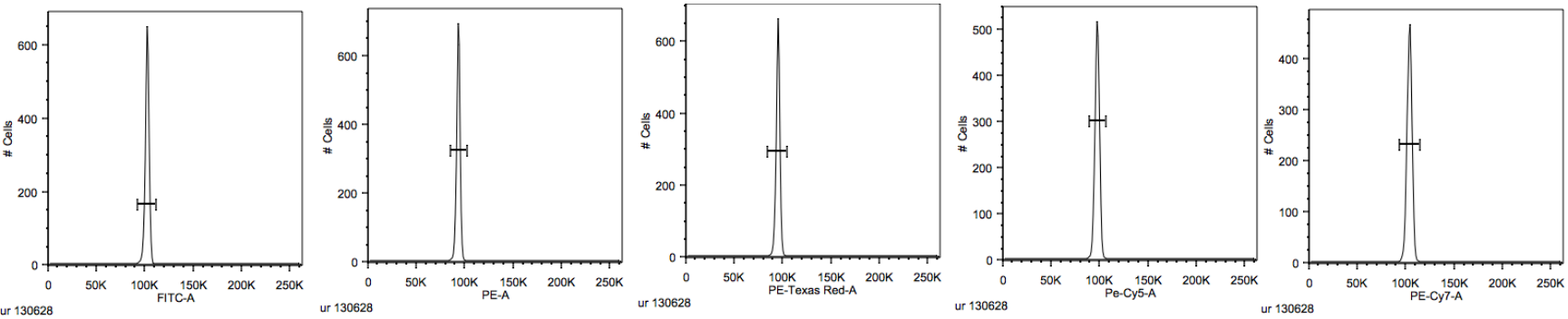
fluidics or sheath pressure (again!)

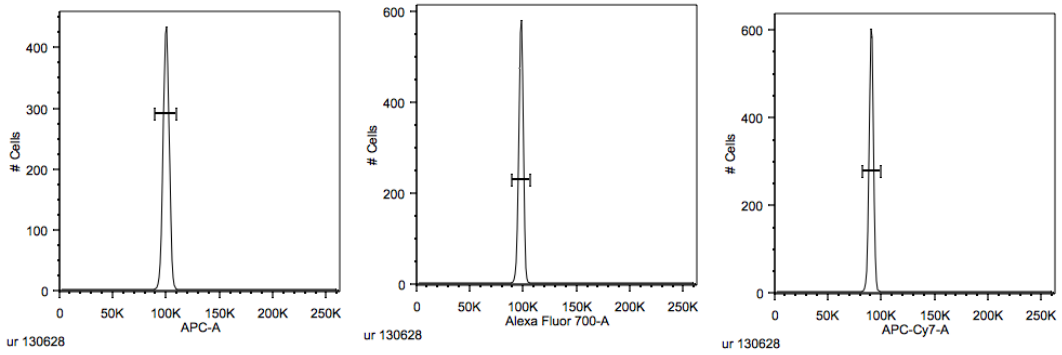
clean and prime if necessary

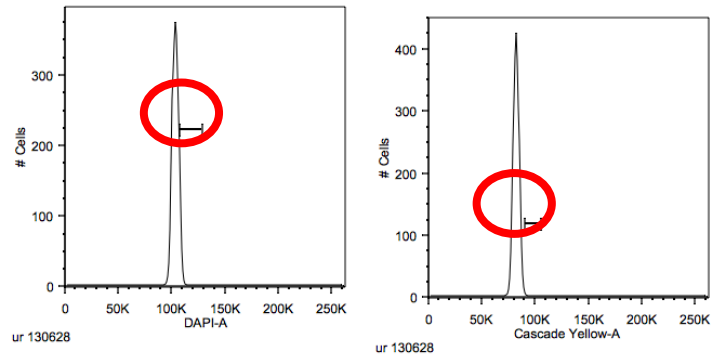
it could be a laser problem, a bad clog, or something else

In which case you'll have to call your service technician

Where's the problem?







If all parameters look bad, it's probably fluidics

If all the colors on a particular laser look bad,

it could be fluidics, laser alignment or laser power

If only 1 parameter looks bad,

it could be wrong or bad filter or pmt

2. Are there really cells in the tube?

It is good to have a microscope, preferably a fluorescent microscope, next to the cytometer

If you run a core facility, you will see all kinds of problems, from no cells to dead cells to unstained cells.

Just because the cells were there before fixation and staining doesn’t mean they are in the tube at the end.

Assess the quality of the preparation with a viability stain, trypan blue or PI.

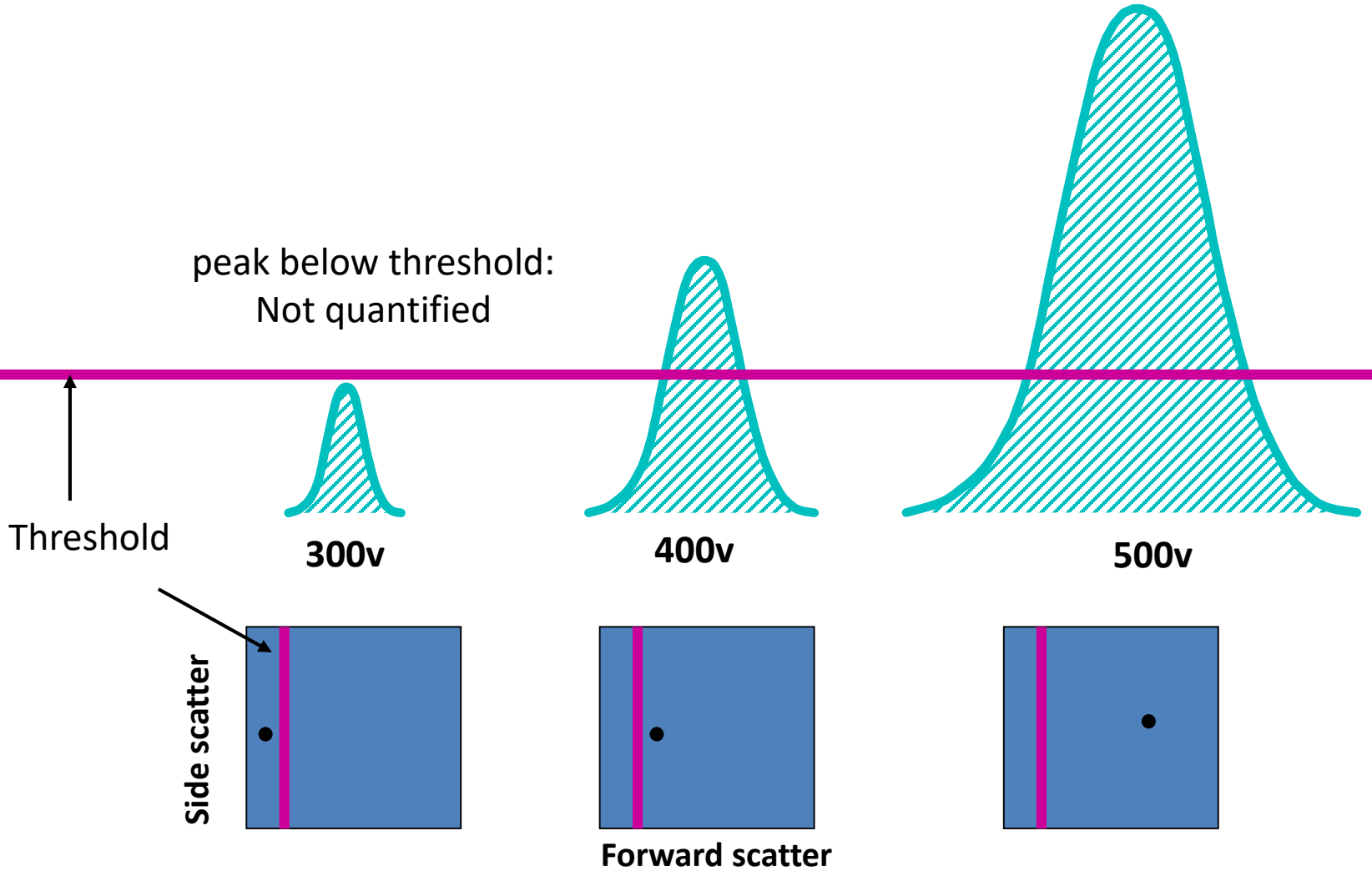
3. Are the cytometer settings correct?

Need to optimize your settings, PMT voltages and threshold

Difficult Samples

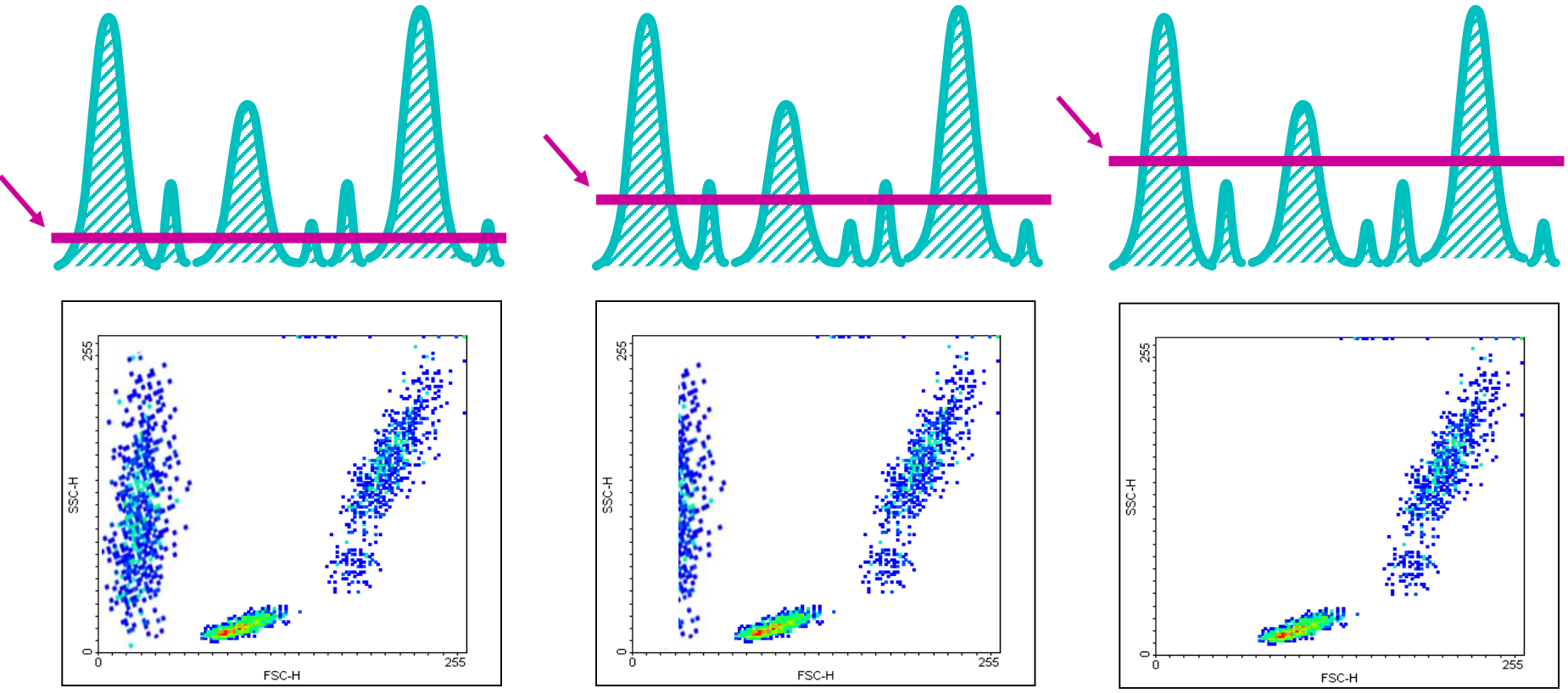
- Lots of debris in the sample: where are the cells?
- Particularly difficult with preparations of solid tumors or tissues
- Very small populations: positive or autofluorescent

Threshold



No events seen!

Threshold



- Increasing the threshold removes smaller pulses thus smaller events from analysis
- Events below threshold are not recorded, thus lost for good.

Where are my cells?

You must be sure that your cells are above threshold

In most cases threshold is on forward scatter

You can use a viability stain to see where your dead cells are

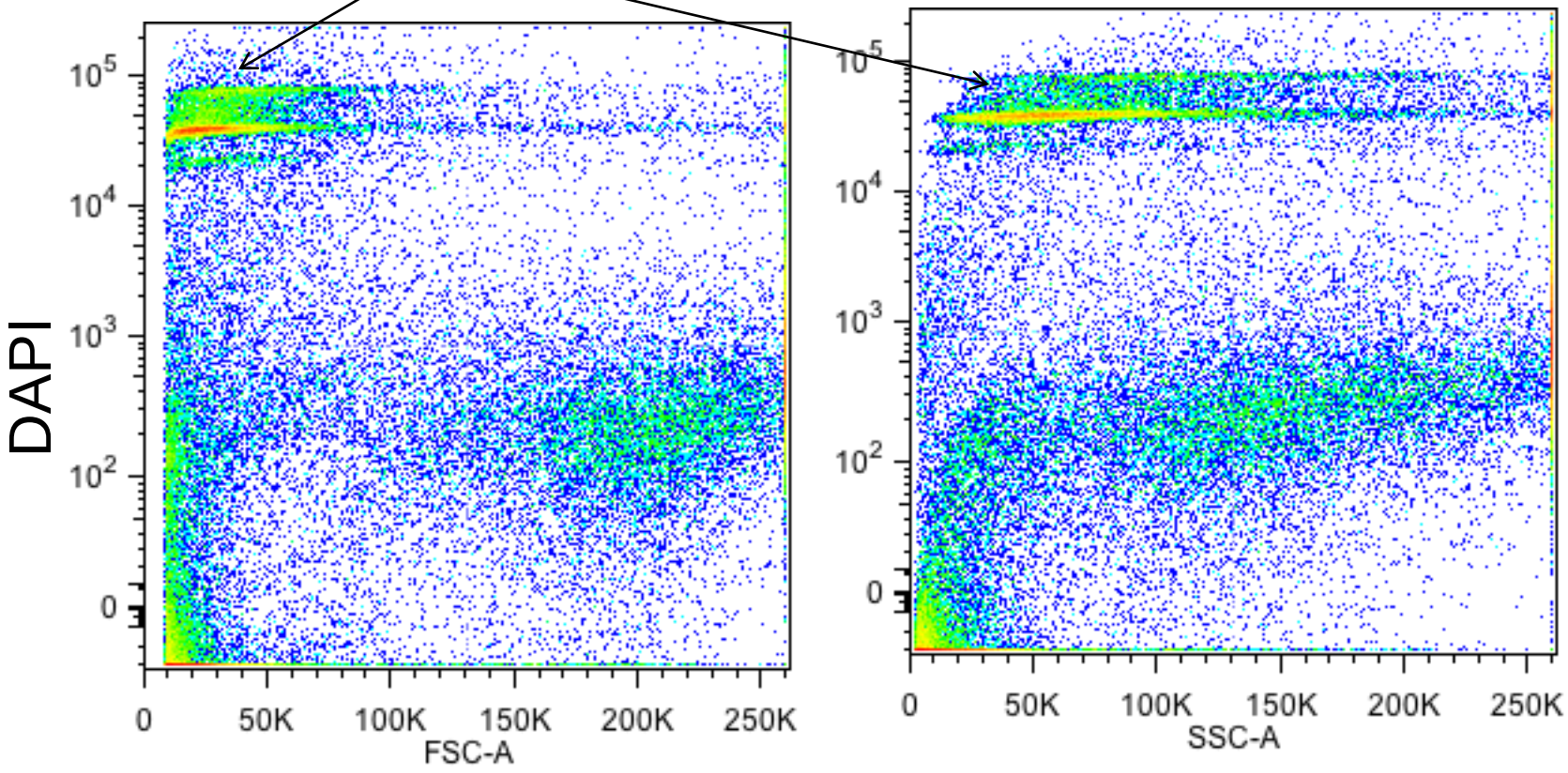
Look at viability vs forward scatter to set your FSC PMT voltage

This will indicate that you have whole cells or nuclei above threshold

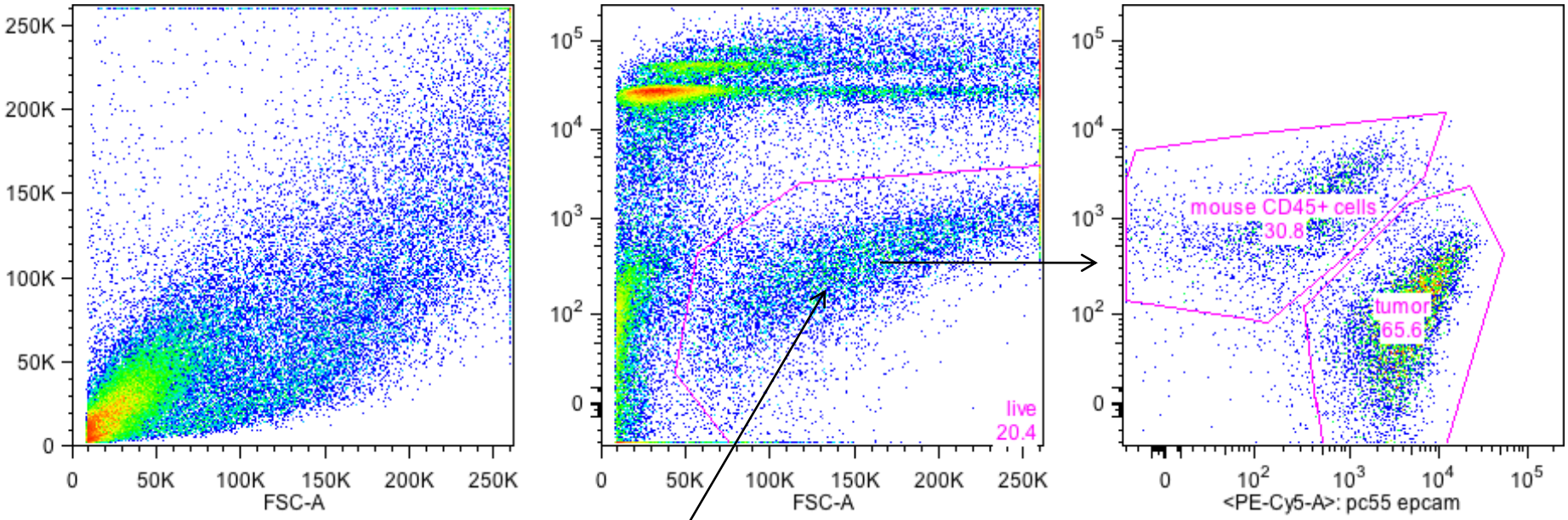
Where are my cells?

FSC and SSC vs DAPI: human breast tumor xenografts grown in mice

Here are the dead cells



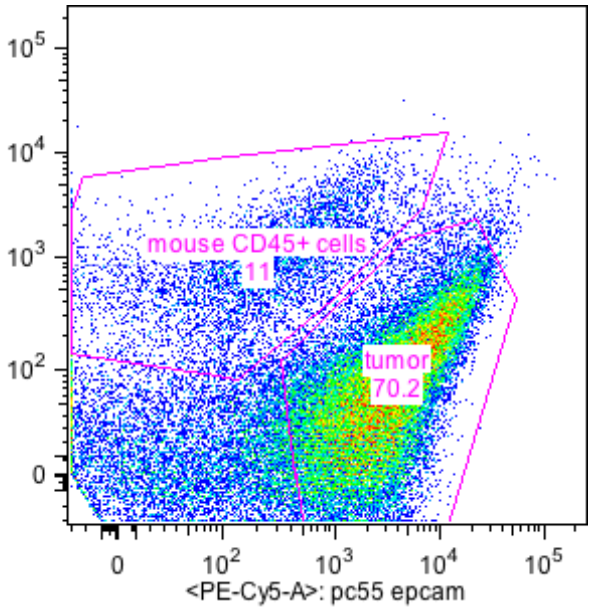
Where are my cells?



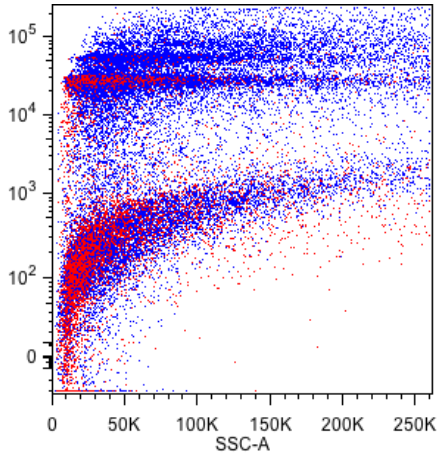
Here they are!

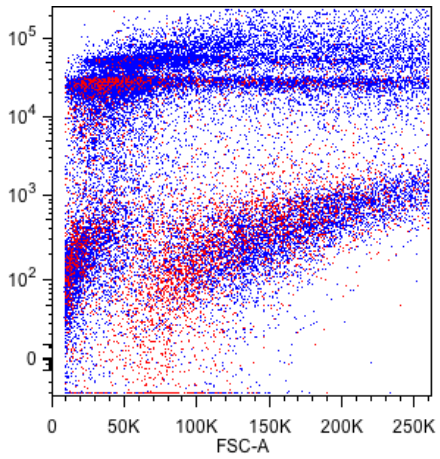
Backgating

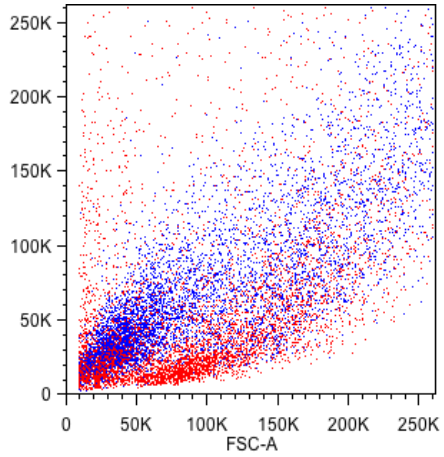
Gate on the cells that are positive for your marker, then look to see where they are on the FSC and SCC to make sure they re above threshold



Look at all cells first







Overlay of tumor cells (blue) and mouse cells (red)

Most of the tumor cells are dead!

Small populations and autofluorescence

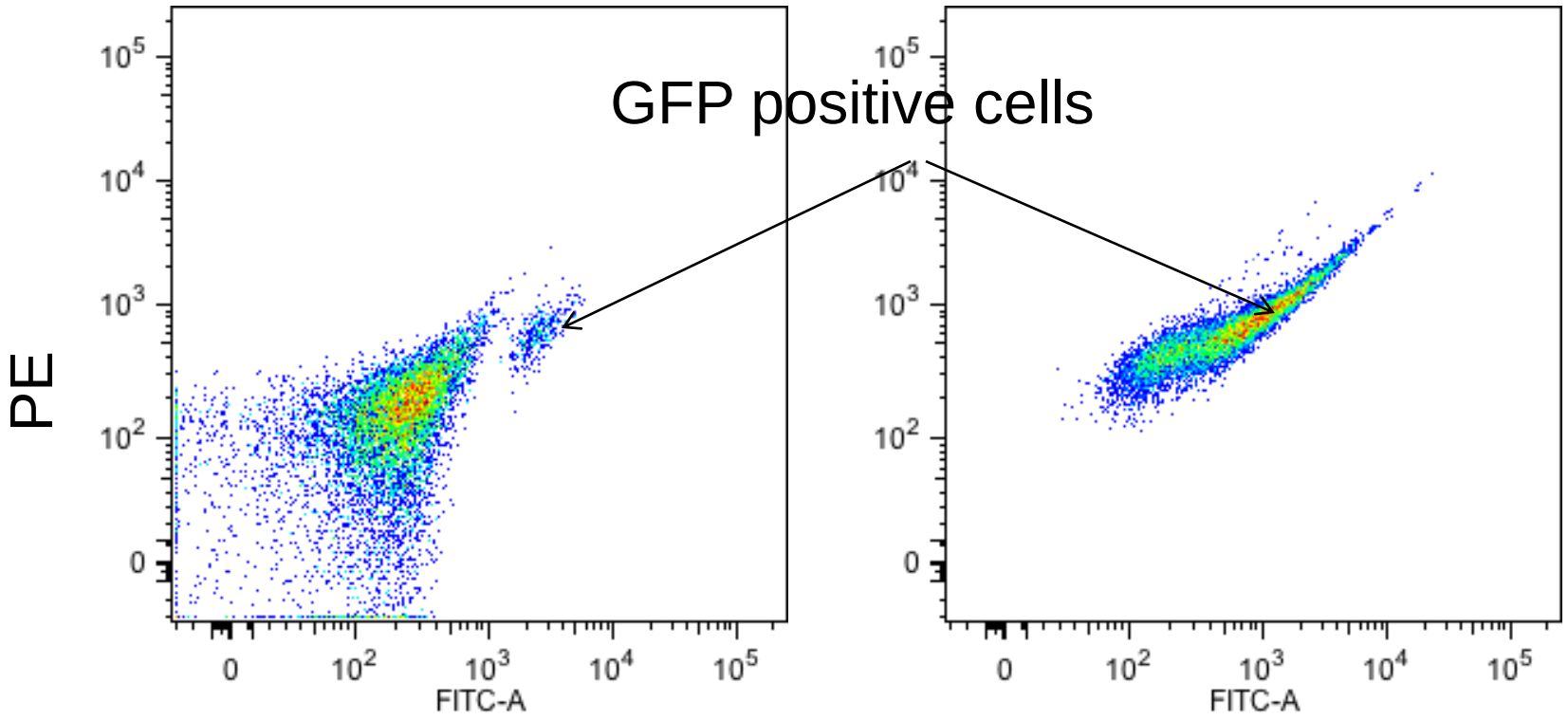
If you have a small population, GFP transfected cells for example, you may have a hard time finding them on a single parameter histogram.

Look at the cells using a dot plot against a parameter where there is no staining (autofluorescence).

The positive cells will be visible separate from the autofluorescence.

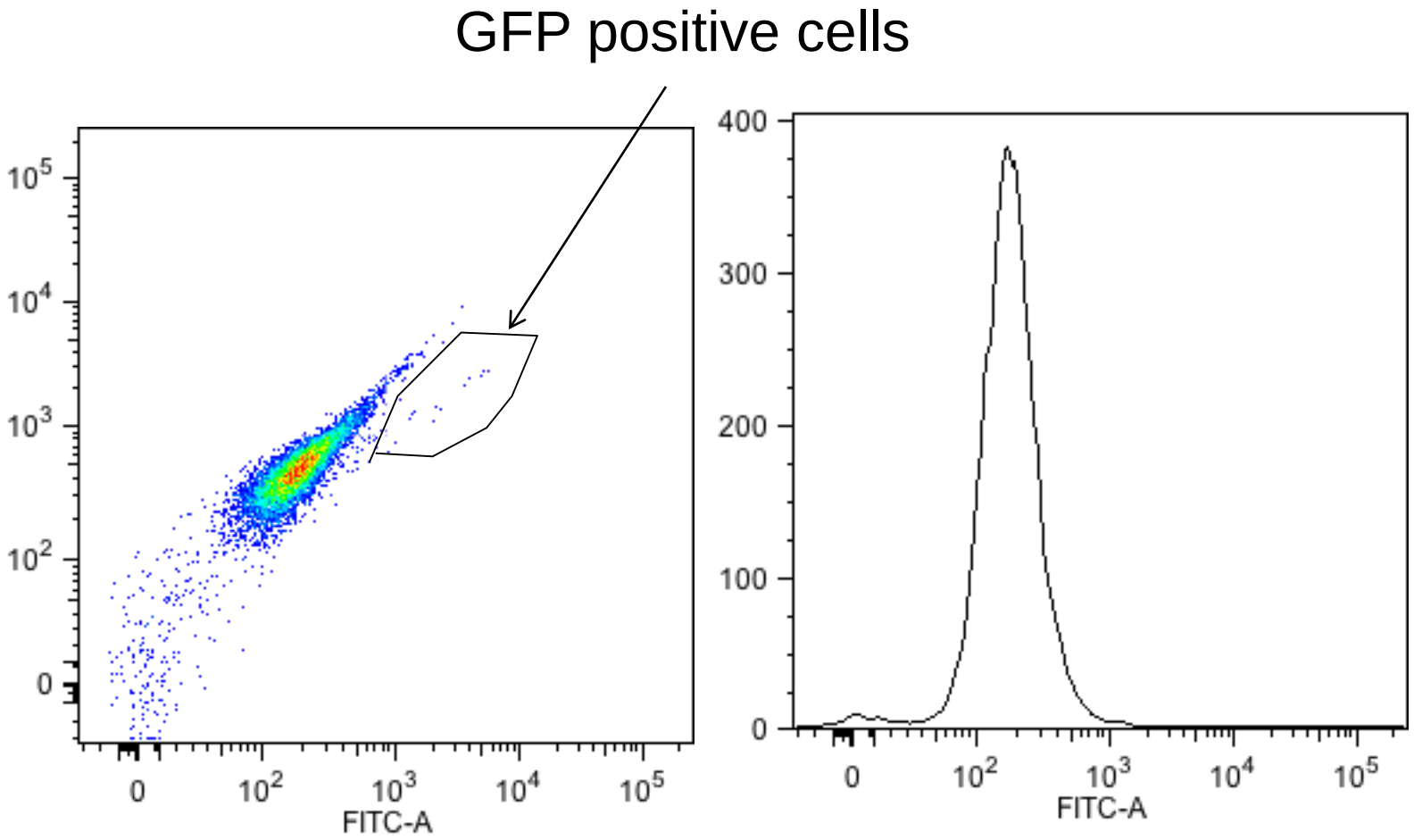
Small populations and autofluorescence

GFP vs PE



Small populations and autofluorescence

GFP vs PE, worst case scenario



Troubleshooting

Determine the source of the problem

- Make sure the machine is running properly (QC beads)
- Look under the microscope
- Make sure your PMT and threshold settings are correct

Also:

Look at live/dead cell markers to determine where cells separate from debris

Look at cells vs autofluorescence to find small truly positive populations

Backgate on positive cells of interest

Good tissue dissociation techniques are crucial for good data