Cells Sorting:
How to keep your cells and sorter happy

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Outline

- Optimize your sorter
- Optimize your cell preparation
- Experiment and sort setup
- Keep your cells happy
- How good was your sort?

Optimizing your sorter

- Daily QC
- Keep your sorter clean!
- Sterility
- Sheath fluid
- Temperature

Daily Quality Control (QC)

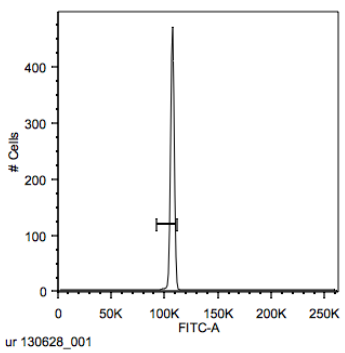
It is essential to run QC beads every day

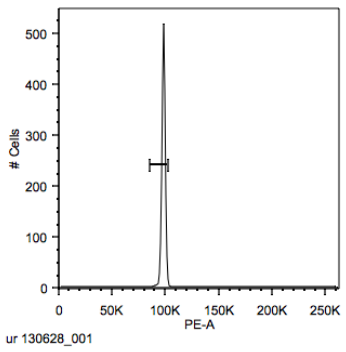
You should have an easy protocol to QC your cytometer

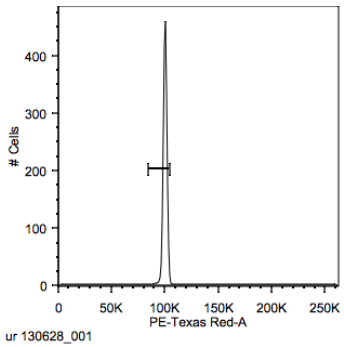
This verifies the cytometer performance
- that fluidics, lasers and detectors are ok

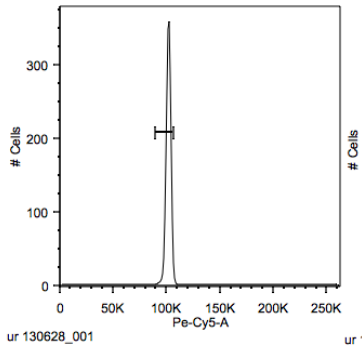
Rerun QC beads as needed during the day:
- after a clog
- if air in sample line
- if your data looks weird

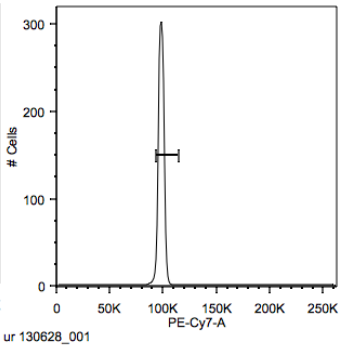
Daily check: A typical run in our lab

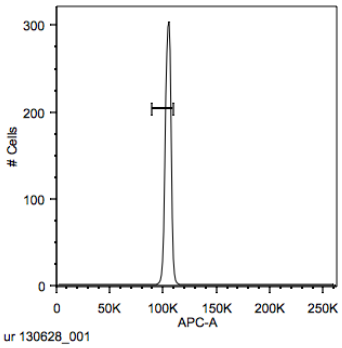


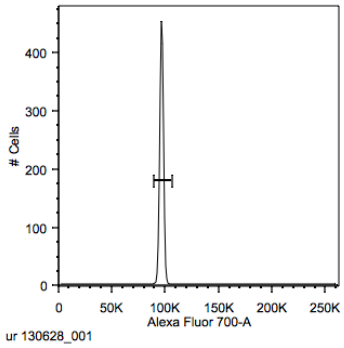


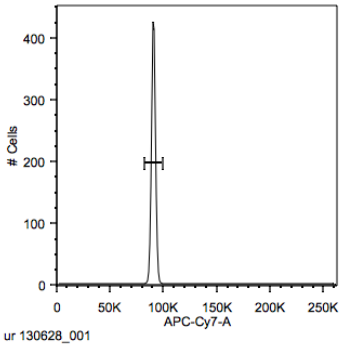


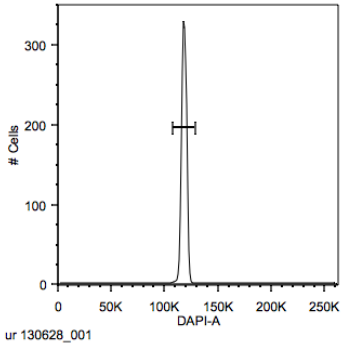


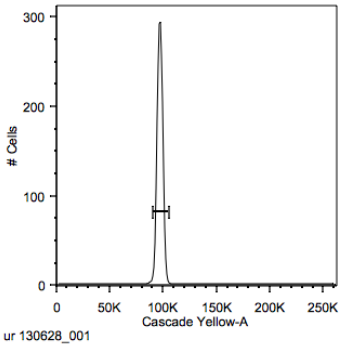












UltraRainbow beads

Keep your sorter clean!

A clean sorter is a happy (and stable) sorter

- Good daily maintenance is essential
- Clean sample lines well at the end of the day:
  bleach, contrad, ethanol or detergent
- You don't want to have to run strong cleaning solutions just before or in the middle of a sort. Bleach will kill cells
- Keep the fluidics running:
  Sorter should be run up every few days, even if not used

Keep your sorter sterile

- Daily cleaning!

  Sample lines : Contrad or bleach clean at the end of the day

  Sheath tank and lines: shutdown in ethanol

- Check that it is sterile

  Do daily sterility checks on sample line and sort stream

- Decontaminate if you have problems

  bleach or H_2O_2/periacetic acid

Be careful! bleach residues will kill your cells, so rinse well!

Drop Delay

Always verify drop delay just before the sort

Reverify drop delay in case of problems during a sort

If there is an error on the drop delay, the purity and especially the recovery of the sorted cells will be poor!

Sheath fluid

Fluid must have ions or the droplets won't charge (so not DI water)

No preservatives or bacterial inhibitors to damage cells

Maintain physiological pH

Dulbecco's PBS recommended: should be Ca^{++} Mg^{++} free

There are concentrated sheath fluids available on the market (cheaper!)

You can adapt the sheath fluid to unusual cell types if necessary

Keep the lab cold!

low and constant room temperature

essential for sorter fluidics and drop breakoff stability

Make sure you have a good air conditioning system!

Optimize your cell preparation

- You must have single cells!
- Minimize:
  clumps
  debris
  dead cells
  - Optimize cell concentration for different cell types
- Always filter your cells just before sort

Cell size, nozzle size and clumps

Shapiro's First Law of Flow Cytometry:

A 51 µm Particle CLOGS a 50 µm Orifice!

Cells should be smaller that 1/5 the diameter of the nozzle

So for a 100u nozzle the cells should be no larger than 20 u

Clumps are bad

Clumps cause:

- Nozzle clogs
- Sample line clogs
- Fluctuating event rates
- breakoff instability
- side stream fanning

How to minimize clumps

Cell adhesion requires Ca^{++} and Mg^{++}

- Use Ca^{++} Mg^{++} free cell buffer to resuspend cells
- Include 1 mM EDTA to remove Ca^{++} Mg^{++}
- Up to 5 mM for sticky samples
- Make sure that your cells are happy in EDTA
- Use BSA instead of serum. Serum contains Ca^{++} Mg^{++}

DNA released from dead cells causes clumps

- Especially in solid tissues or tumors
- Include DNAse 20-200 µg/mL

Filter, Filter and Refilter!!

Always filter samples **just before sorting**

Refilter if sticky cells reaggregate during the sort

If your nozzle clogs, you could be fighting with fluidics stabililty for the rest of the day

35 µm mesh is most common

filters available in many mesh sizes (Partec Celltrics)

BD Falcon tubes with cell strainer cap

Cut-your-own mesh (various suppliers)

Debris is Deadly!

Reduces sort efficiency due to high sort aborts

Causes clogs in the nozzle tip or sample line

Decreases purity

If debris is below threshold, it is not seen by sorter,
not excluded from sorted drop
so will be found in sorted tube

Dead Cells

- Dead cells often mask as false positives
- Generate debris
- Release cytoplasmic constituents that may affect cell function or downsteam analysis (RNases)
- Release genomic DNA, which may lead to clumping
- Increased cell stickiness

Minimize debris and dead cells

Solid tissues, tumors and frozen cells are the most problematic

Optimize the disaggregation procedure for your tumor or tissue

It is **NOT** the same for all cell types

Use a live dead marker

Will help eliminate dead cells

Will help find the live cells amid the debris (not always easy!)

Pre-sort enrichment :

Centrifugation gradients, eg Ficoll Hypaque

Magnetic bead enrichment or depletion

Optimize cell dissociation for your system

Disaggregation procedures need to be tailored
to each tissue or tumor type

For best yield and quality of difficult cell preparations
maximize viable cells
minimize debris
ensure representation of the different cell subpopulations

Find the best enzymatic or non enzymatic combination for your tissue. Lots of options
Trypsin, Collagenase, Dispase, Accutase
Worthington Biochemical is an excellent resource
http://www.worthington-biochem.com/default.html

Cell Temperature

Most (but not all) cells do better if kept cold (4° C)

During cell preparation
During the sort
Both the sample and the collect tubes

This keeps the cells metabolically inactive
Helps keep them viable
Reduces capping or antigen internalisation

Sort Efficiency

Watch your sort statistics carefully

Efficiency, sorts/second, aborts/second

If aborts are high and efficiency is low, you have a problem!

Low efficiency (high sort abort rate) can be due to

too much debris in sample (dilute sample)

Event rate too high (slow down or dilute!)

Nozzle size not adapted to cell size

Gating error (watch out for conflicting gates)

Dilute Sticky Cells

In large or sticky cells:

more variation in cell dispersion in droplets.

This causes

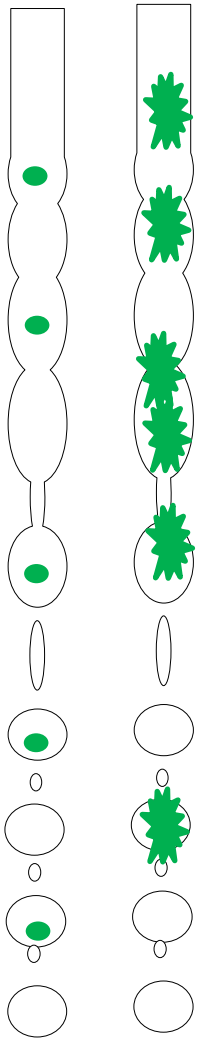
more sort aborts

lower efficiency

To minimize this

dilute sticky cells

optimize buffers to reduce stickiness



Keep your cells happy: before the sort

pH is important!

- best is HBSS or PBS with low glucose
- if use medium, CO_2 independent medium is preferred
- regular CO_2 medium is alkaline in air, not good for cells

Add protein 1-2%

- Serum or BSA (BSA better for sticky cells)
- Use a trypsin inhibitor to stop trypsin. Serum causes clumps.

HEPES 25mM important

- pH regulation
- protects the cells from decompression during sorting

EDTA 1 mM is good to reduce clumps, but some cells don't like it. Really clumpy might need 5mM.

Keep your cells happy: collecting the sorted cells

Sort into tubes with 1-2 mls PBS with protein

NOT into dry tubes!!!

Use polypropylene tubes for collection
less sticking, less static

Coated tubes for fragile cells:
coat tubes overnight with serum
pour excess off leaving 1-2 ml serum

For very fragile cells,
leave the coated tube filled almost to the top

More hints for collecting the sorted cells

Cells often fall on the side wall of the collection tube.

rinse down sides of tubes immediately after sort

Or cap and invert tube

Dont mix up your tube caps

keep caps for unsorted cells and collect tubes separate.

For cloning into wells:

Fussy cells may be happier in conditioned medium

Keep your cells happy: sheath pressure

Adapt nozzle size and sheath pressure to cell size and fragility

Many cells don’t survive well at high sheath pressure

If functionality of the cells post sort is essential,
you may have to sort at lower pressure.

Experiment setup

- Threshold
- Single cells (again)
- Rare or weakly stained populations
- sort gates

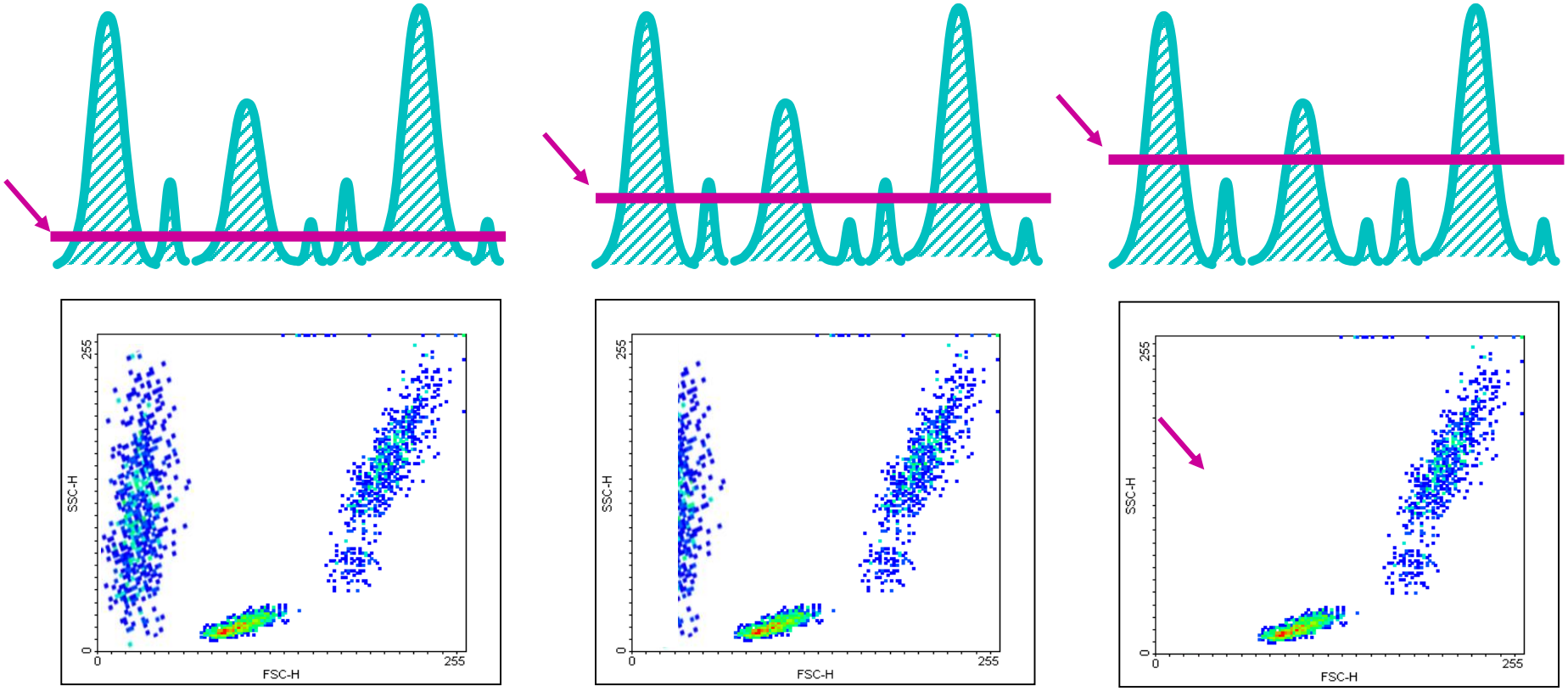
Experiment setup: threshold

How you set your threshold can affect post sort purity

Cells and debris below threshold not seen by sorter

Therefore sorter can’t exclude them from sorted drops

Threshold: Impact on purity



Increasing the threshold removes smaller pulses thus smaller events from analysis

Events below threshold are not seen

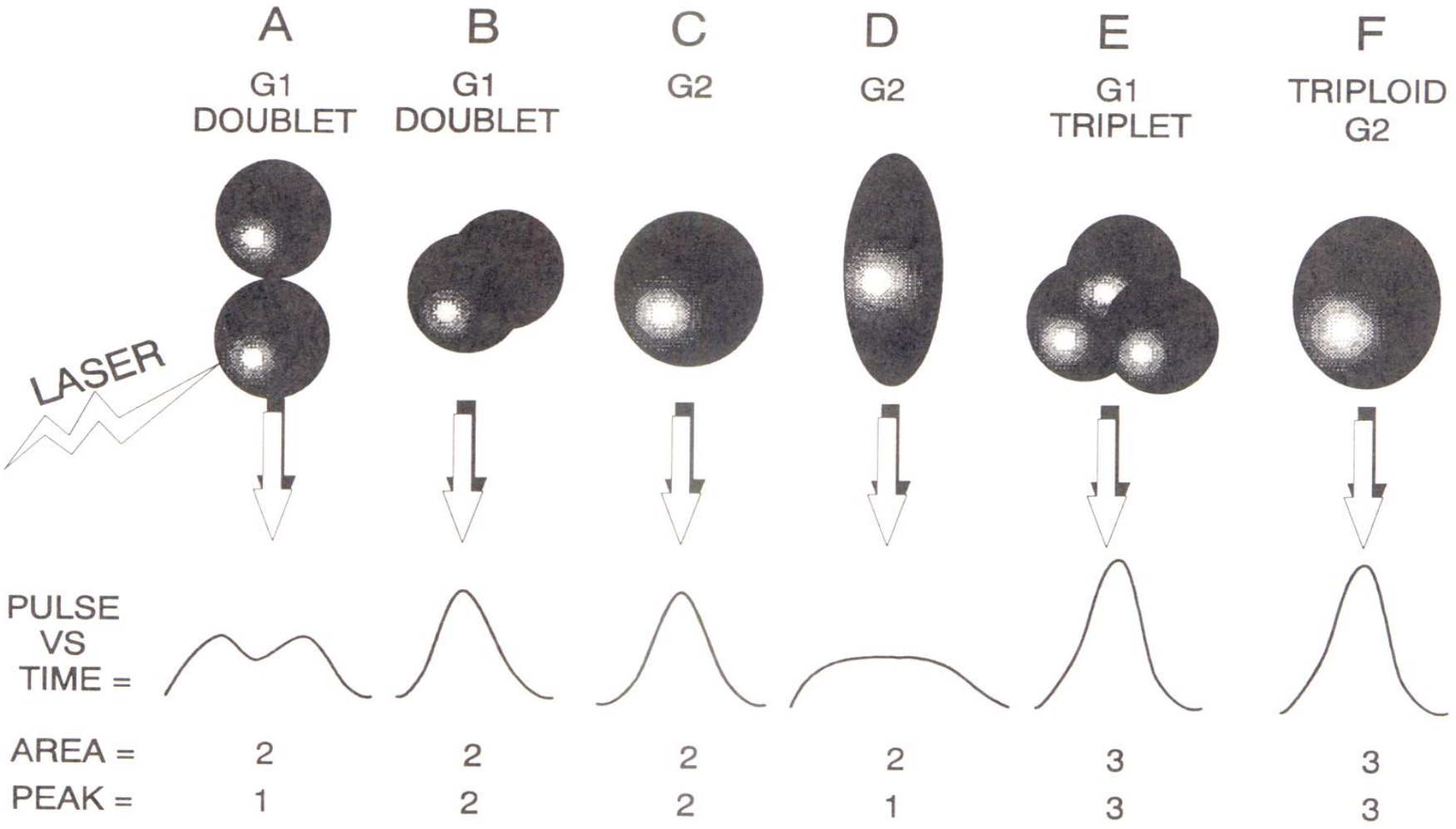
EVENTS BELOW THRESHOLD ARE STILL GOING THROUGH THE CYTOMETER BUT THEY WILL NOT BE EXCLUDED FROM SORTED DROPS .

Experiment setup:doublet exclusion

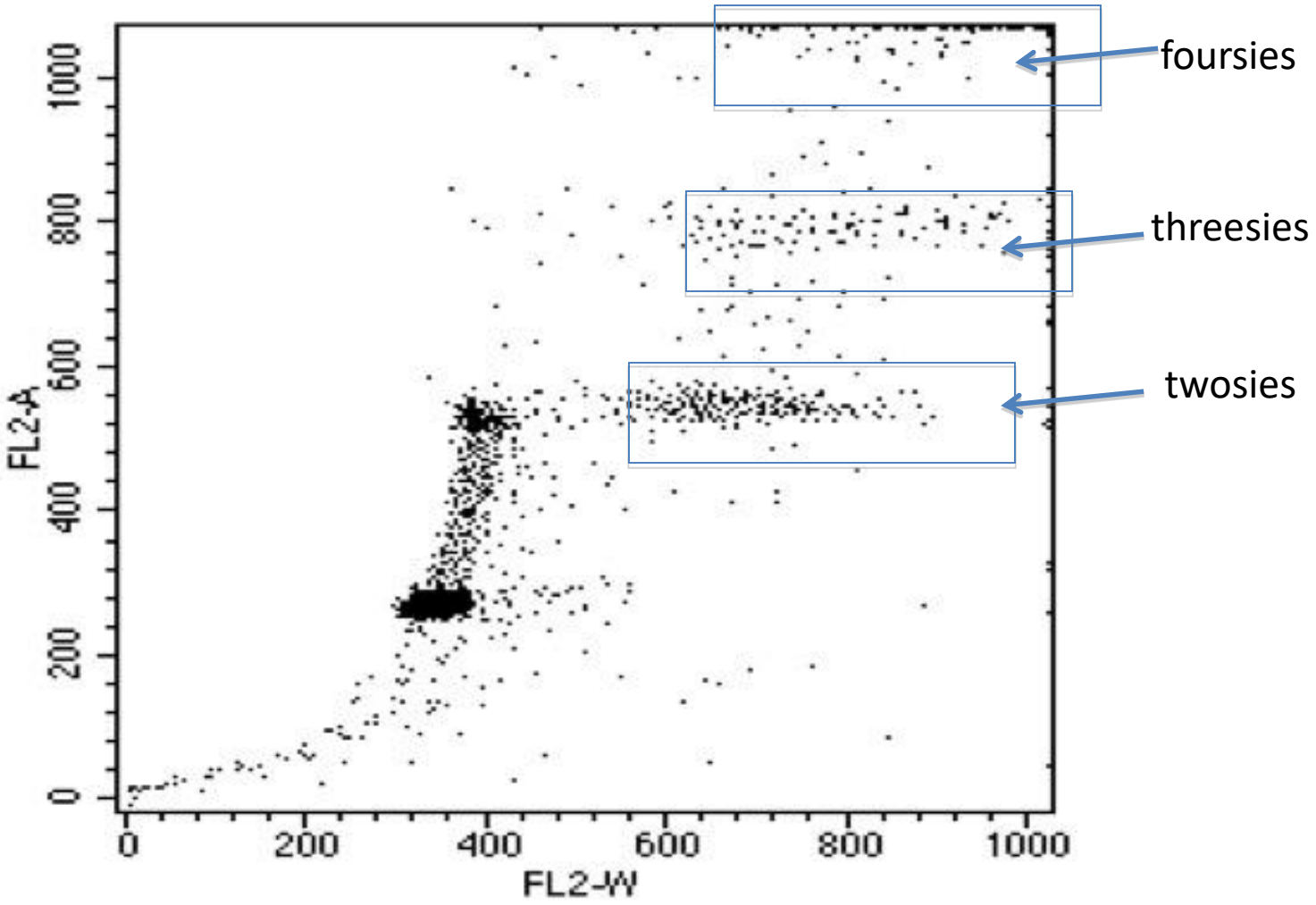
Single cells again!
this time to increase purity of sorted cells

- Doublets are a major source of poor sort purity
- Use pulse height or width gate to exclude doublets
- It helps to tighten down fsc/ssc gate
- But you can never get them all

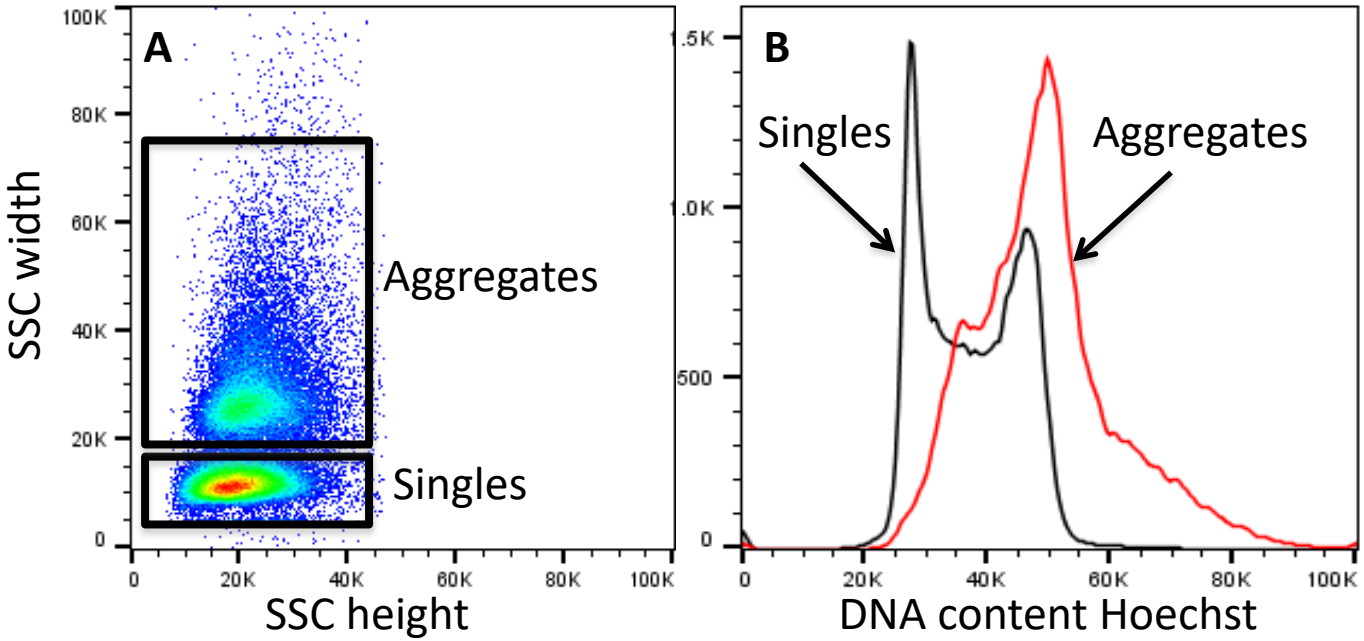
Experiment setup:doublet exclusion



doublet exclusion



Use Pulse shape to Eliminate Aggregates



Experiment setup: small populations

Rare populations

- dont just sort the top of a Gaussian on a single color
- Look at cells on a dotplot vs autofluorescence
- Add more markers if possible to exclude unwanted cells or identify target cells

Enrichment sorting for rare populations

2 sequential sorts:

1st sort

Sort in enrich mode to get all cells,

Can put multiple high thresholds on positive cells of interest

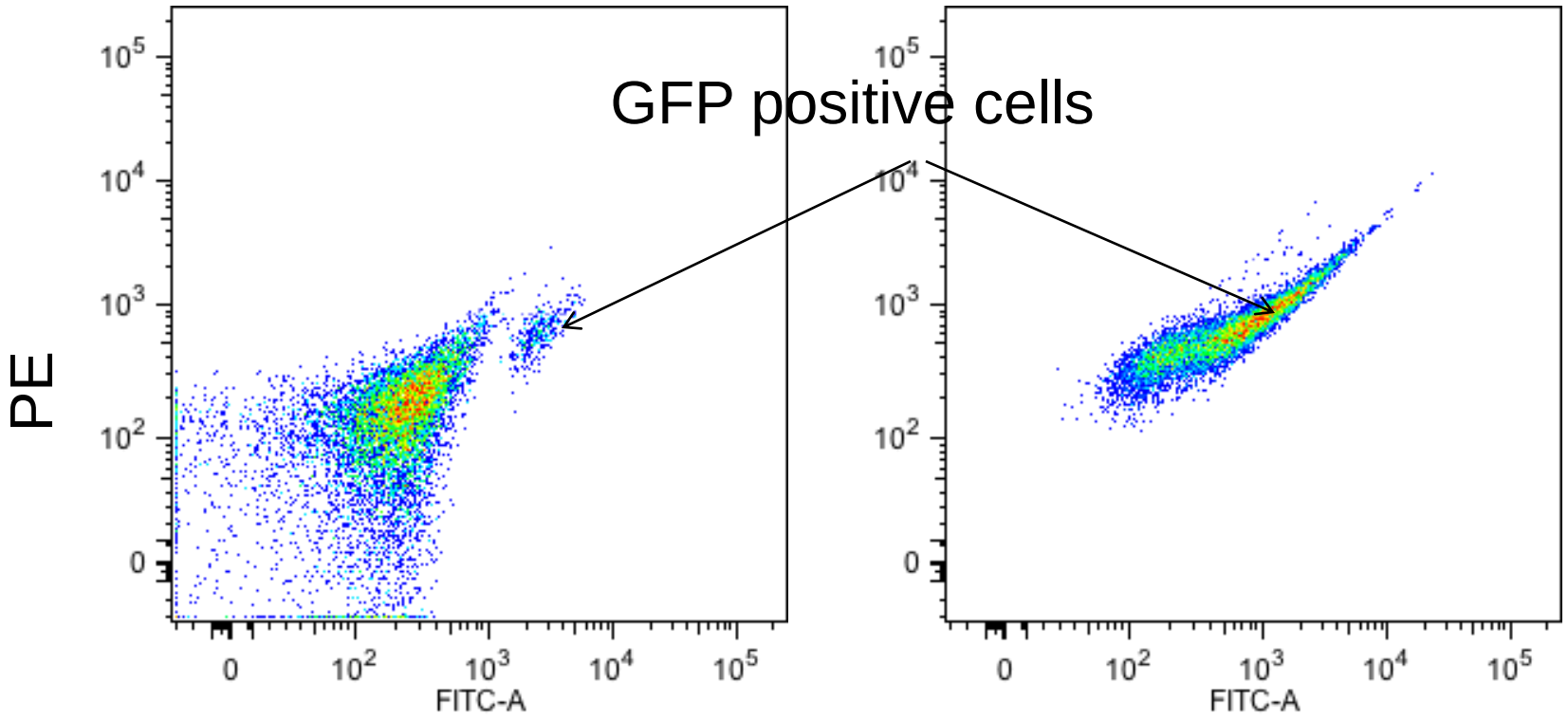
2nd sort

then resort in purity mode with normal threshold

You will lose cells on resorting

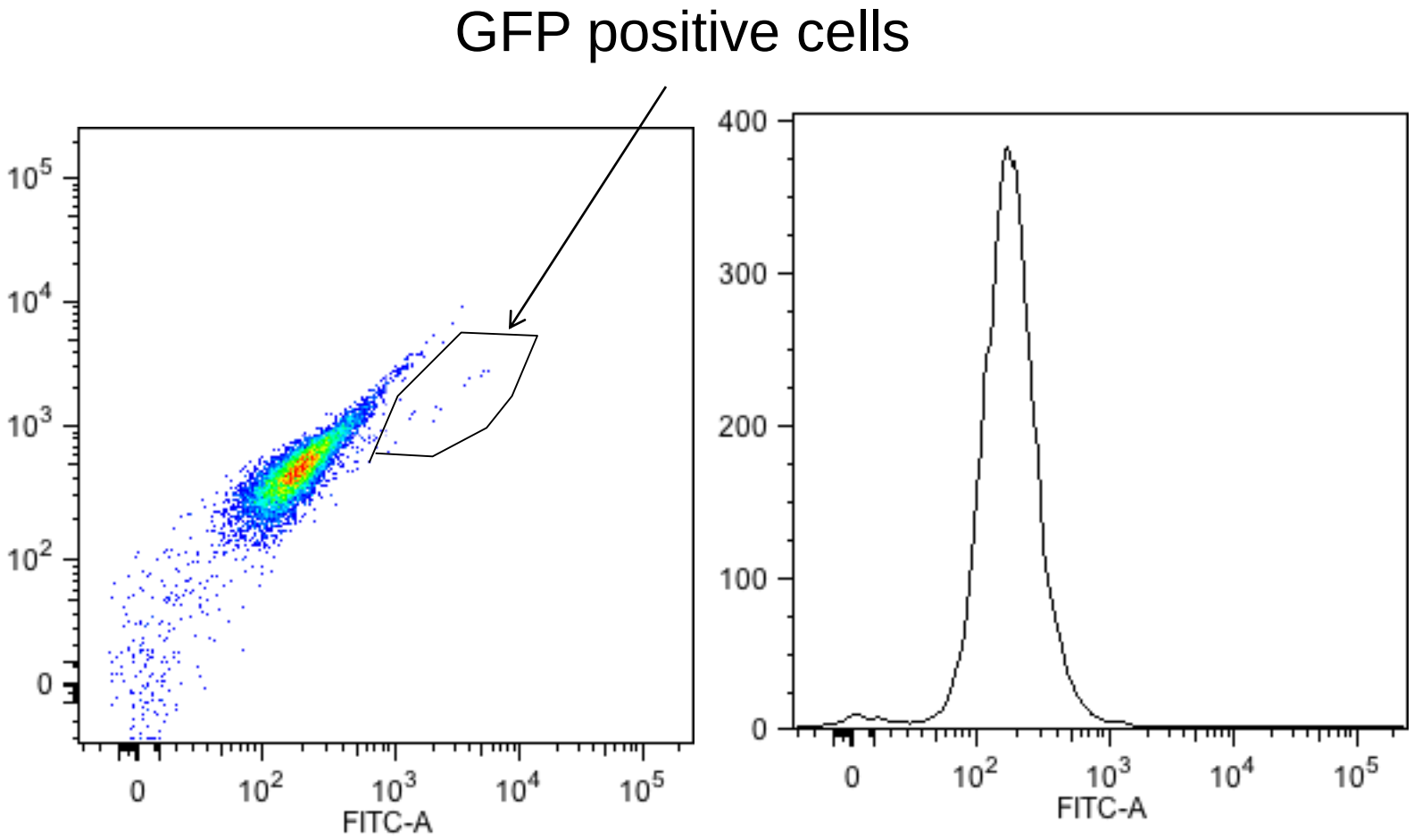
Small populations and autofluorescence

GFP vs PE



Small populations and autofluorescence

GFP vs PE, worst case scenario



Experiment setup: gates

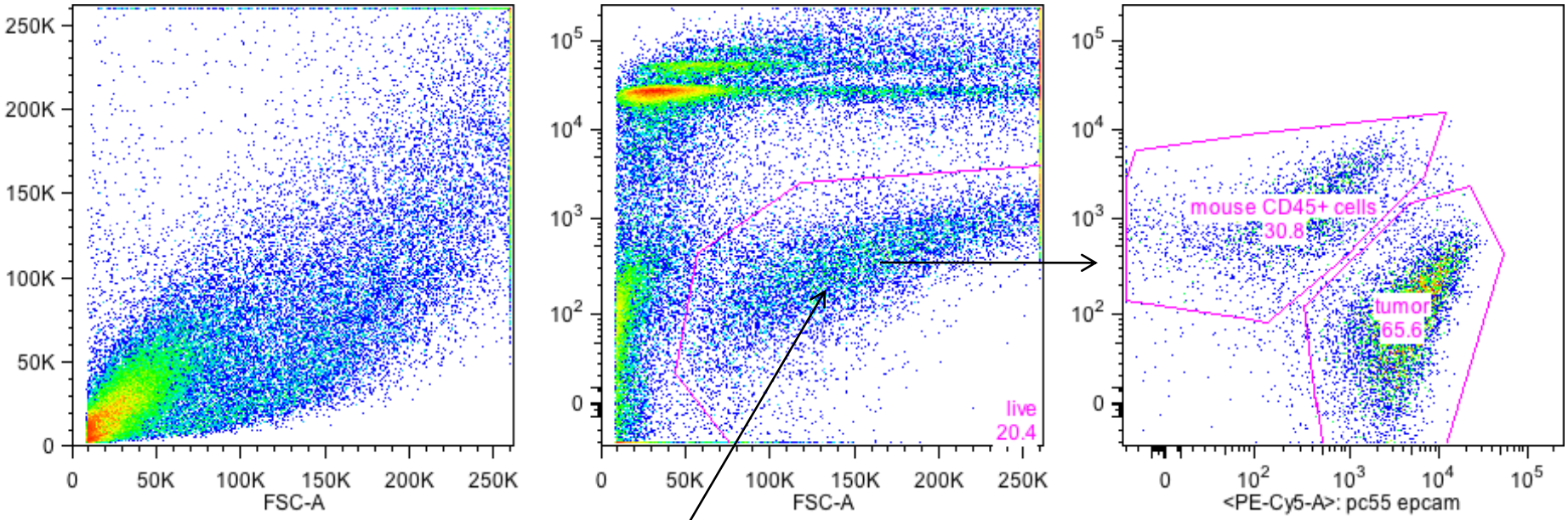
Use biexponential plots to make sure you see everything below the axis. Sometimes there's a whole world down there!

Dead cell markers help distinguish whole cells from debris

Use backgating to find cells of interest

In some systems, multiple conflicting gates on the same parameter can cause high sort aborts.

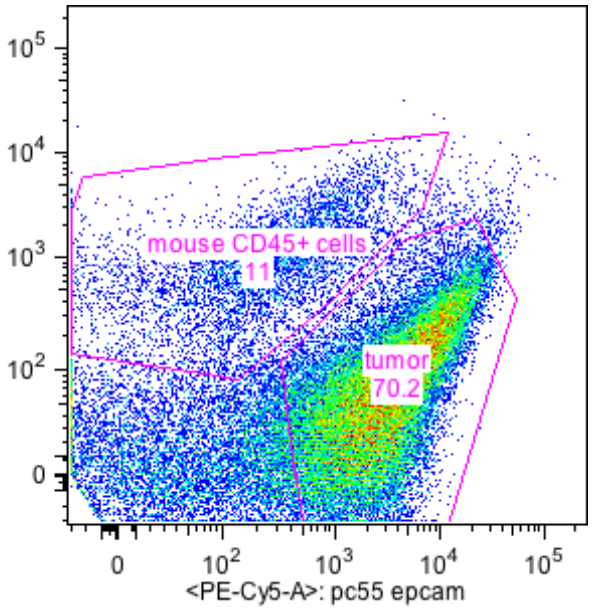
Where are my cells? Use a dead cell marker



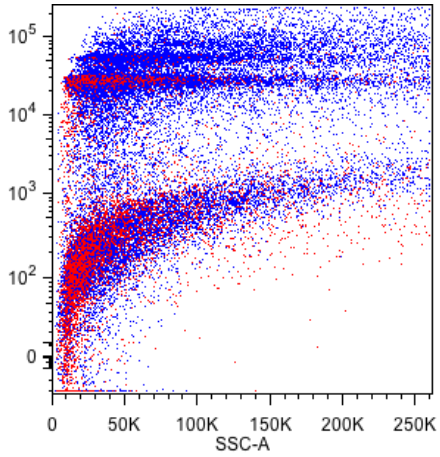
Here they are!

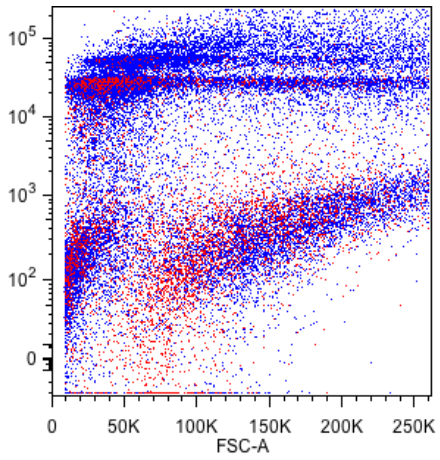
Where are my cells? Gate on the positives

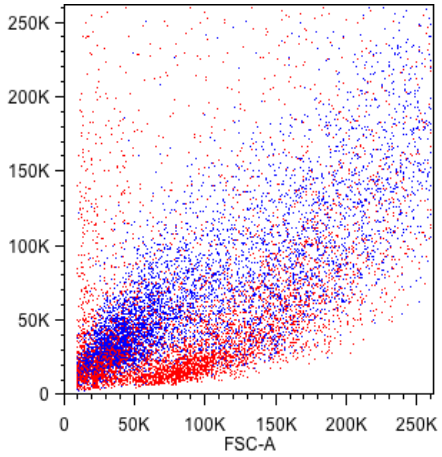
Gate on the cells that are positive for your marker, then look to see where they are on the FSC and SCC to make sure they re above threshold



Look at all cells first







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

Most of the tumor cells are dead!

Metrics: how good was your sort?

Purity

always verify purity if there are enough cells to run

Recovery

are the cells really in the tube?

Viability

Are the cells viable?

If they are not, use larger nozzle, lower sheath pressure

Recovery

How many cells are in the tube compared to what the sorter says are in the tube?

$$\%\ \text{Recovery} = \frac{\text{N}^{o}\ \text{of cells in the tube x Purity}}{\text{N}^{o}\ \text{of cells on the sort counter}} \times 100$$

R max: a way to verify recovery during a sort

What affects Recovery?

Drop Delay!!! If you are slightly off the correct drop delay:

Purity can be good

But recovery will be poor

Side stream fanning, the cells didn’t fall into the tube

Recovery: the cells are there, but!

Cells are on the side of the collection tube

Inaccurate verification by counting of the sorted cells:

difficult to accurately count if there are few cells either by hemacytometer or cell counters.

Count cells before centrifugation

Cells can be in the tube after sorting, but lost during spindown

Thank you!!