Sorting 101

How to prepare for a successful cell sorting experience

Research Flow Cytometry Core

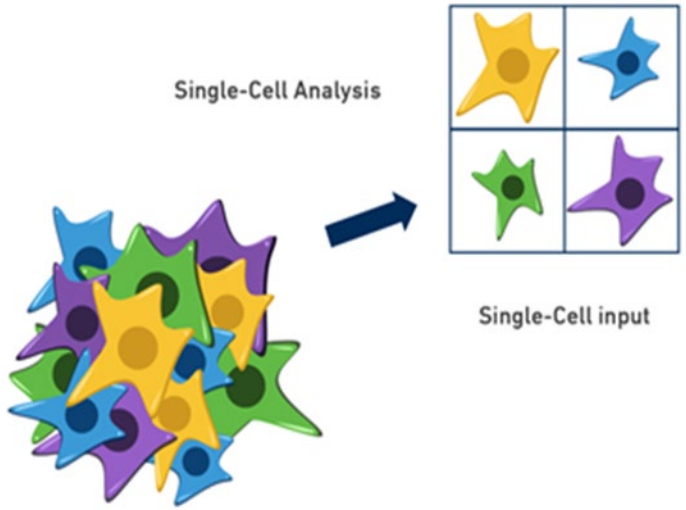
Cincinnati Children's
changing the outcome together

Things to think about

1. What is the aim of the sort?

2. Preparation of sample and staining?

3. What sorter to use and parameters?



What are you trying to accomplish?

a. Collect cells for culture?
b. Collect cells for down stream assay?
c. Collect for transplantation?
d. Collect cells for transfection?
e. Collect nuclei?
f. Collect for cloning?

This determines how to prepare for a successful sort.

How many cells are needed?

What is the goal?

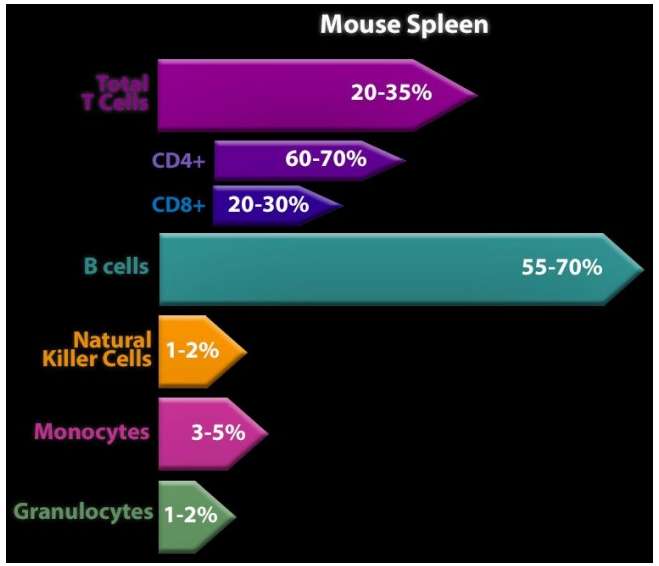
- E.g. Goal: Plate 100k CD8 T-cells from mouse spleen into each well of a 6 well plates.

How many target cells are needed at the end of the sort?

- Need 0.6 x 10^6 CD8 T-cells.

How does the source affect the number of cells needed?

- Consider that CD8 T-cells are ~10% of mouse spleen. Therefore, need to sort a minimum of 6 x 10^6 total cells.
- However, sorting is a lossy process, so double the number of total spleen cells. 12 x 10^6 cells should be prepared to ensure 0.6 x 10^6 CD8 T-cells.

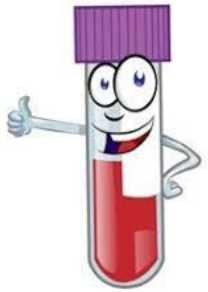


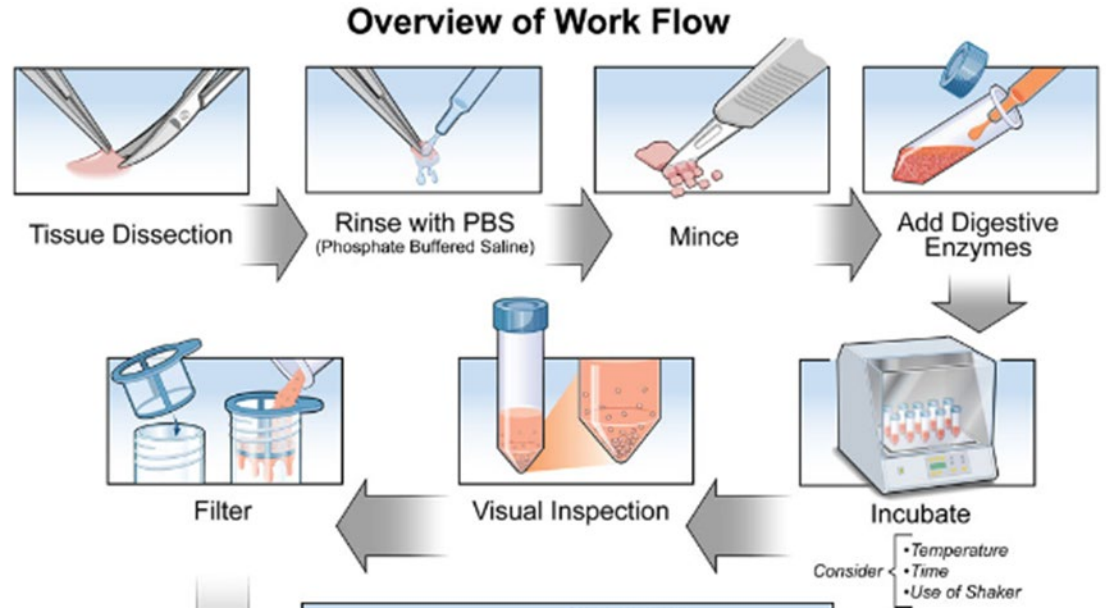
Cell loss due to presence of dead cells, doublets, sort mode, sort efficiency, and abort rate related to sample condition

Sample Preparation

Goal: keep cells alive and in single cell suspension

- The method for sample preparation will depend on the starting material and the nature of the epitope.
- A liquid sample (blood, suspension cell culture) is pretty easy to obtain a single cell suspension.
- A solid sample (tissue) need to go through various workflow.





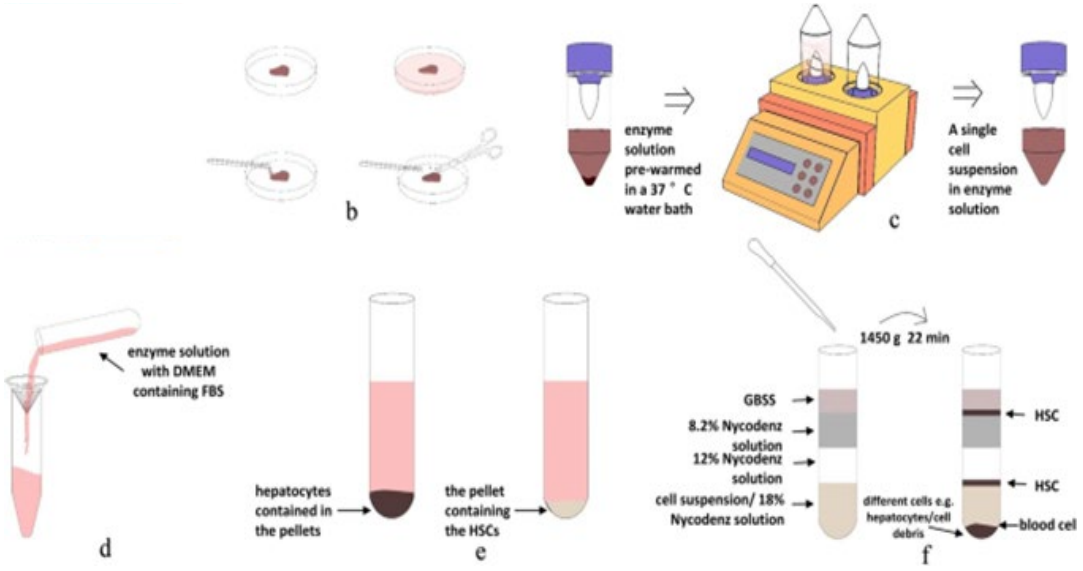
Research Flow Cytometry Core

Cincinnati Children's
changing the outcome together

Sample Preparation

For solid tissues, dissociation techniques become critical.

- References and vendors very helpful.
- Gentle MACS Dissociator in the core from Miltenyi with suggested protocols.
- *Best Practices for Preparing a Single Cell Suspension from Solid Tissue for Flow Cytometry*. Reichard & Asosingh. Cytometry A. 2019 February ; 95(2): 219–226. doi:10.1002/cyto.a.23690.



Research Flow Cytometry Core

Cincinnati Children's changing the outcome together

Sample Preparation

If a lot of debris are in the sample:

- Adjust enzymatic digestion time. e.g. If cells remain in trypsin too long, they will die.
- Try a different dissociating enzyme.
- Consider a gentler dissociation process: enzymatic vs. dissociator.
- Use a dead cell removal kits.

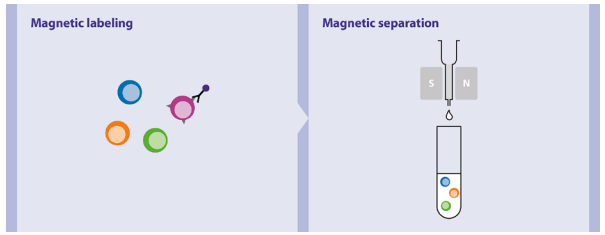
Enzyme	Source	Optimum pH Range	ECM Substrates	Activator(s)	Common Inhibitors
Collagenase type I and type II	Clostridium histolyticum	6.3 - 8.5	Collagen I, II, III and IV	Ca^{2+}, Zn^{2+}	EDTA, EGTA Blood Plasma/Serum
Dipase	Paenibacillus Polymyxa	5.9 - 7.0	Fibronectin, Collagen IV, Collagen I (smal amounts)	Ca^{2+}, Mg^{2+}	EDTA, EGTA
Thermolysin	Geobacillus stearothermophilus, Bacillus thermoproteolyticus	5.0 - 8.5	Non-specific Cell adhesion proteins	Ca^{2+}, Zn^{2+}	EDTA, EGTA
Trypsin	Sus domesticus (porcine) Bos taurus (bovine)	7.0 - 9.0	Non-specific Cell adhesion proteins	Ca^{2+}	EDTA, EGTA Blood Plasma/Serum
Clostripain	Clostridium histolyticum	7.4 - 7.8	Non-specific Cell adhesion proteins	Ca^{2+}	EDTA, EGTA

Sample Preparation

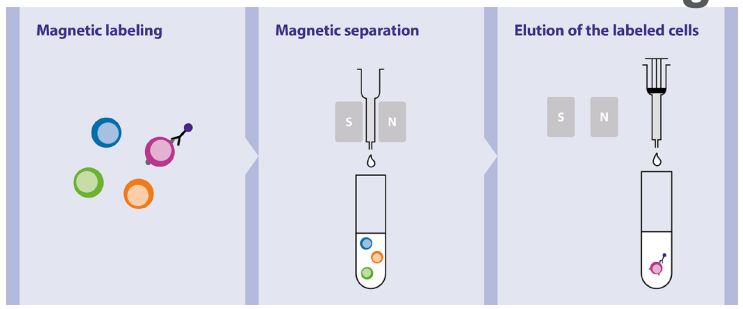
- **Rare target** population:
  - Consider an enrichment step in the sample preparation such as magnetic beads to get rid of some cells (negative selection) or increase the concentration of a population containing the target cells (positive selection).
  - Be Careful doing positive selection, your cell of interest may be activated by the interaction of the Ab with its receptor.
  - After Enrichment: Cells of interest much easier to find. Can record fewer total events to see target cells. This allows very rare cells to be seen.

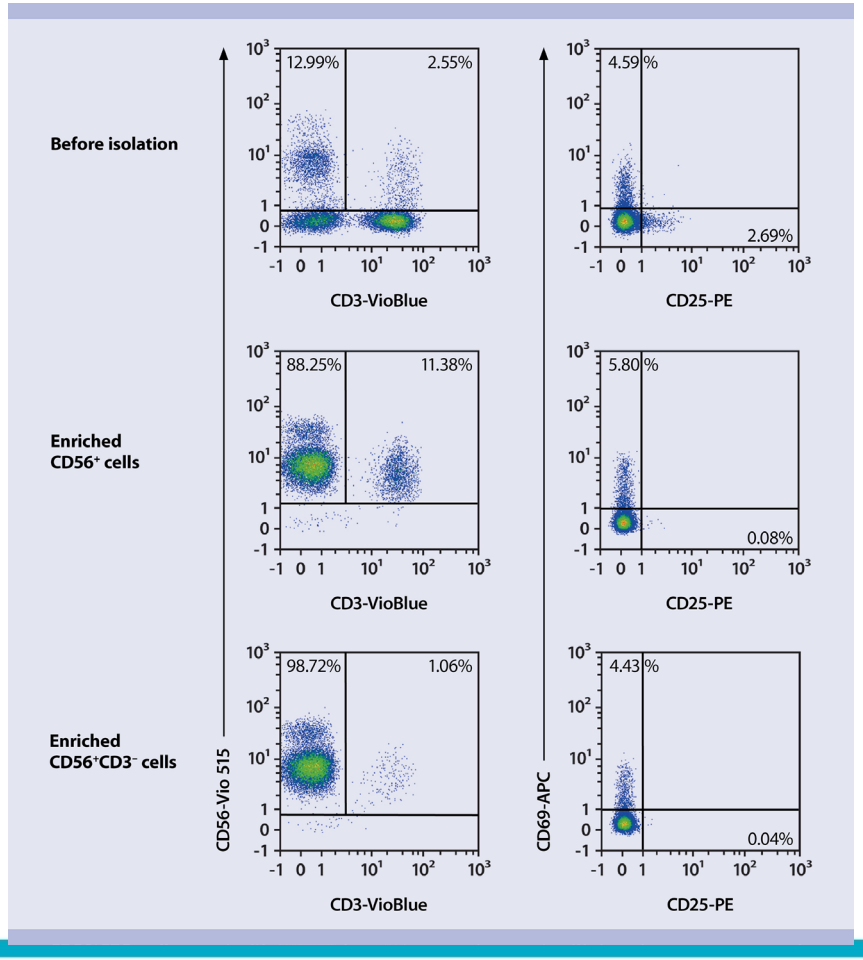
Sample Preparation: Target Enrichment

Negative Enrichment: Unwanted cells removed and target cells flow through.



Positive Enrichment: Target cells removed and unwanted cells flow through. Target cells eluted.



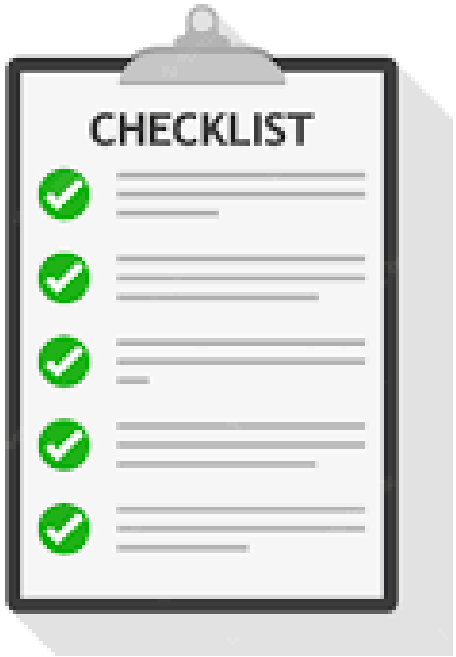


Research Flow Cytometry Core

Cincinnati Children's
changing the outcome together

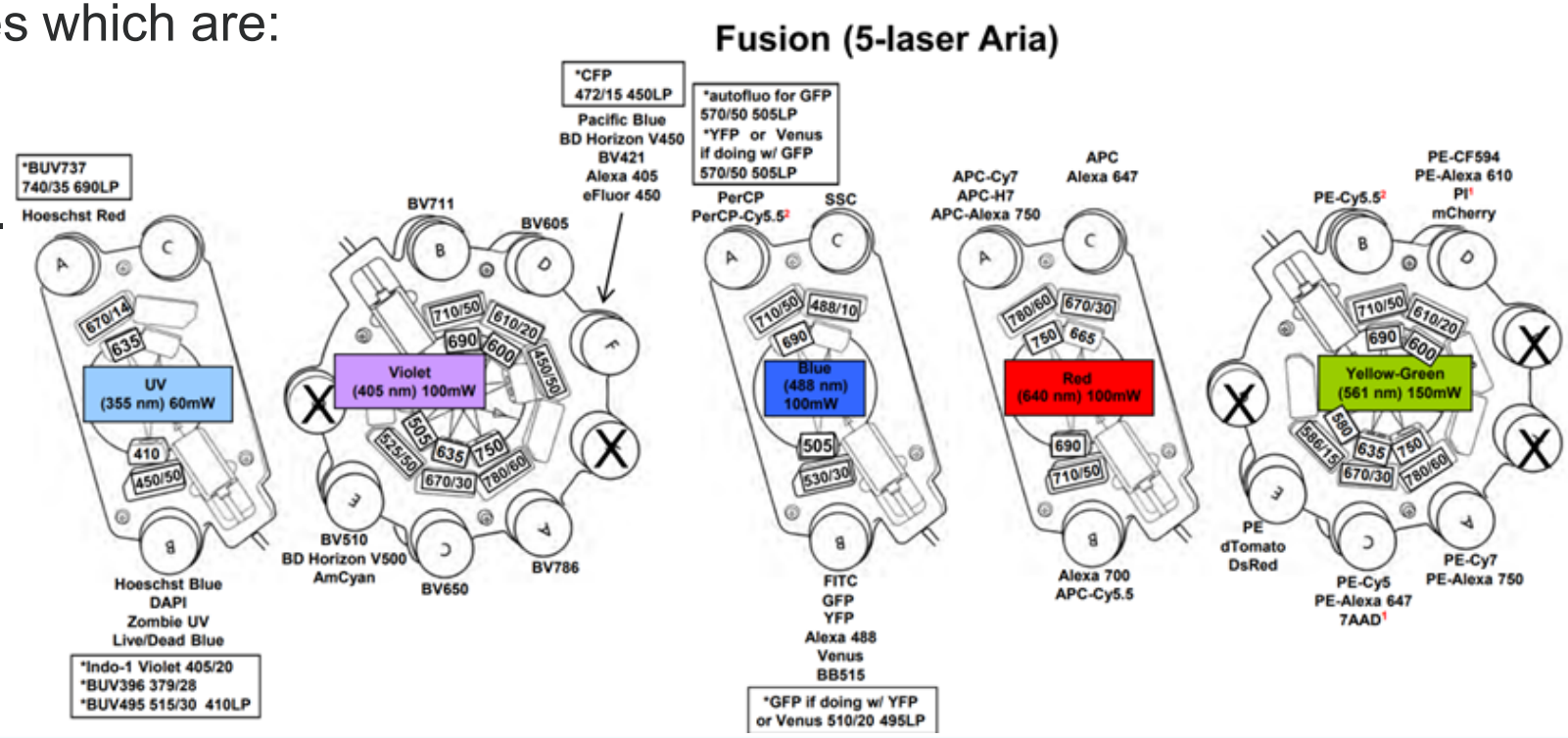
Sample Preparation

- Make a **detailed work list of your protocol**. As the name implies, this is a step-by-step recipe of how to execute the protocol. Include the step, duration, volume of reagent, temperature, etc… This will help with reproducibility.



Staining / Panel Design

- Check the **laser and filter configuration** making sure instrument can detect the color fluors you want to use
- If possible, choose fluorophores which are:
  1. excited by different lasers.
  2. if excited by the same laser emit as far apart as possible.



Research Flow Cytometry Core

Cincinnati Children's
changing the outcome together

Staining / Panel Design

- Staining intensity ideally similar between different fluorescent channels. Very unequal staining intensities can make compensation difficult or impossible. To help with this, choose dim antibodies for abundant sites and bright antibodies for sparse binding sites.
- Try out some of the new utilities designed to help with this process such as FluoroFinder.
- **Consult the core:** they will help design or test your panel on the sorter before your important sort.

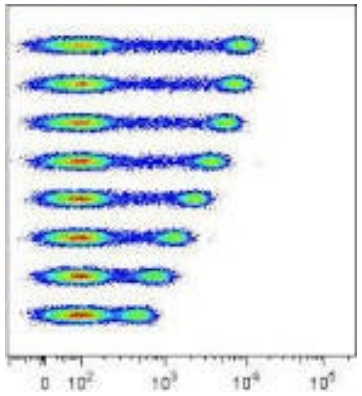
FluoroFinder

Research Flow Cytometry Core

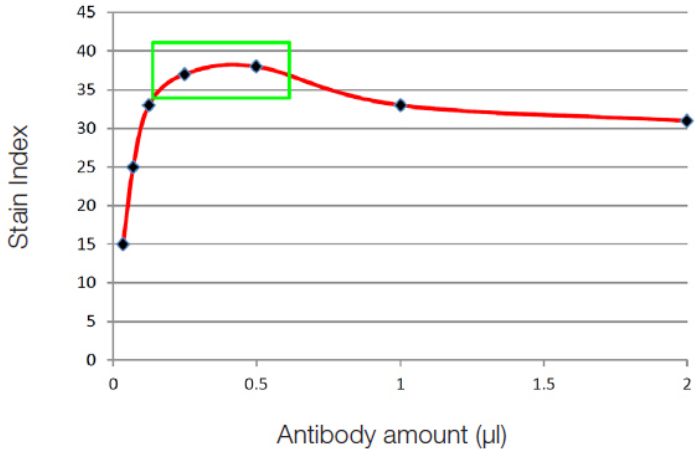
Cincinnati Children's
changing the outcome together

Staining / Panel Design

- It is important to **titrate your fluorescent antibodies and stains**. This includes nuclei staining with DAPI and PI which are often too bright and make it difficult to compensate with other fluorochromes or your fluorescent proteins. Excess antibodies cause **background and artifacts**. The dilution provided on the datasheet can be a starting point. Manufacturers suggested dilution is often too concentrated.



$$\text{Stain index } (\Delta) = \frac{\text{MFI pos} - \text{MFI neg}}{2\text{x SD}}$$

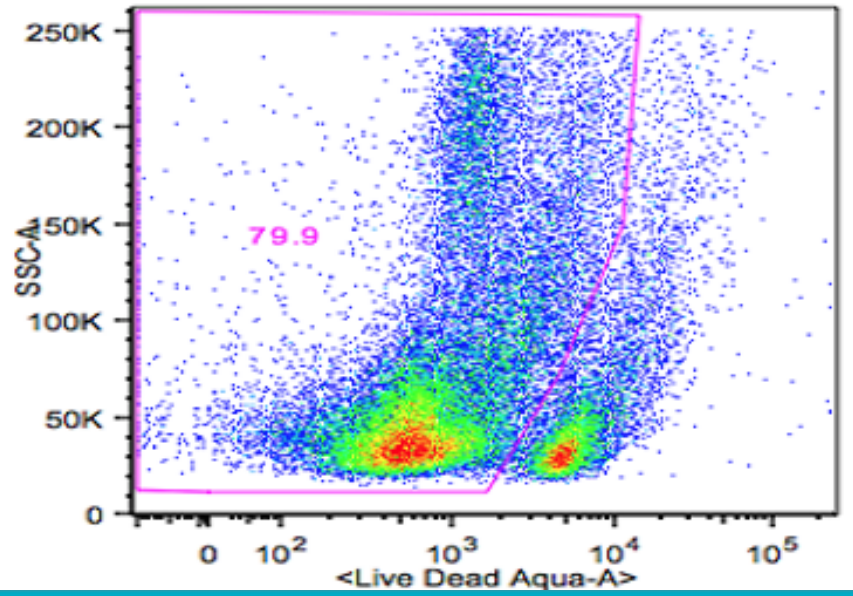


Research Flow Cytometry Core

Cincinnati Children's
changing the outcome together

Staining / Panel Design

- **Negative control** needed.
- Include **single color controls**. Needed for compensation. Not having a control makes the downstream analysis harder or even impossible. Forgetting one could be the difference between success or failure.
- Include a **live/dead stained** sample. FSC/SSC is not enough to determine if the cells are dead. For your control, make sure you have live and dead cells (e.g. remove half of your cells and kill them with heat before adding them back).

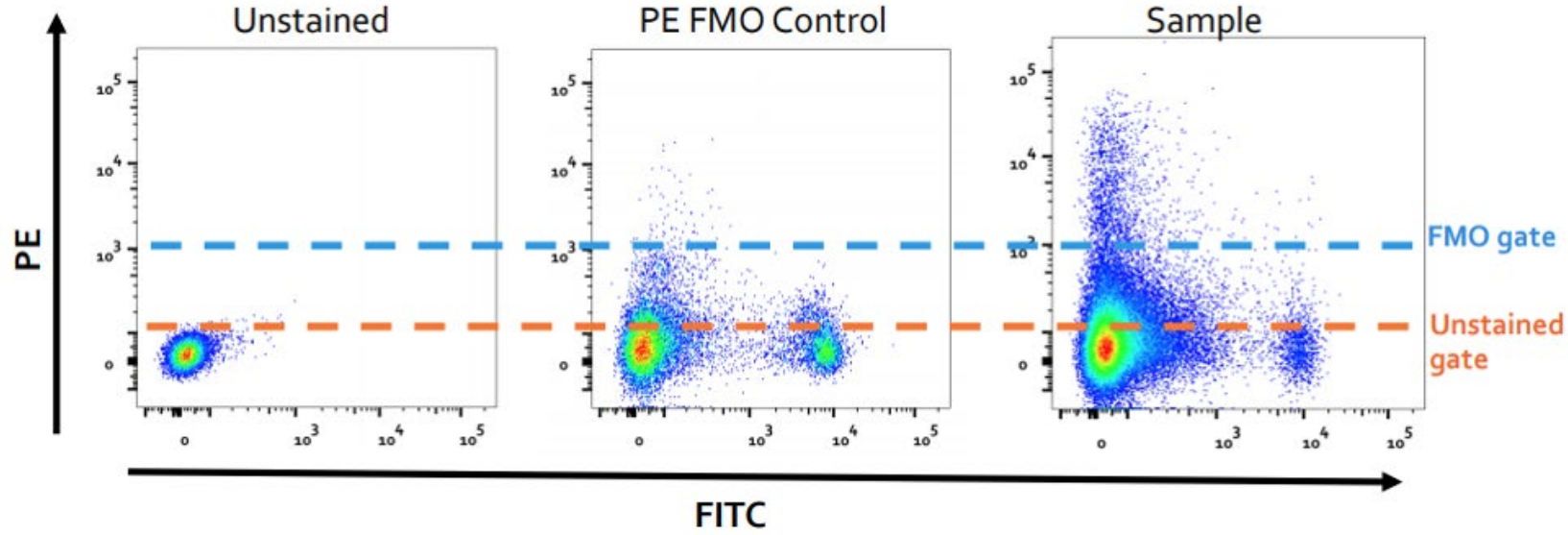


Research Flow Cytometry Core

Cincinnati Children's
changing the outcome together

Staining / Panel Design

- **Fluorescent Minus One Control (FMO): samples stained with all the fluorophores in your panel, minus one of them**. They are used to set the upper boundary for background signal on the omitted label, and thus to identify and gate positive populations in multicolor experiments.



Research Flow Cytometry Core

Cincinnati Children's
changing the outcome together

Final Cell Conditions For Sorting and cell viability

Recommended cell sorting buffer for cell viability:

- 1xPBS or HBSS **without** Ca^{2+}/Mg^{2+}
- No more than 2% BSA or FBS as a source of protein
- 25 mM HEPES pH 7.0 for buffering because it does not require CO_2
- No culture media or bicarbonate buffer which requires CO_2 to buffer (pH will change)
- No phenol red because it may increase the background fluorescence of cells.

Prevent aggregation of sticky cells or preps with cell debris:

- Add 1 mM EDTA ***–OR–*** 10 U/mL DNase

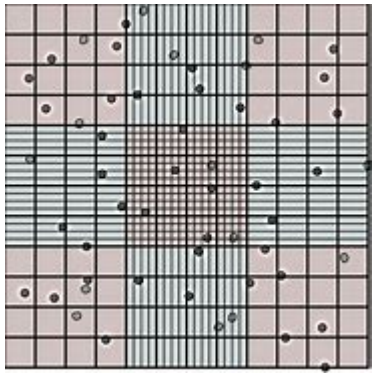
Research Flow Cytometry Core

Cincinnati Children's™ changing the outcome together

Final Cell Conditions For Sorting

Cell concentration

- **Count cells** after staining and various washes.
- 10-20 x 10^6 cells per mL for most cells. This keeps the nozzle from clogging if too concentrated. If concentration is half optimal, sorting will take twice as long.
- 300 µL minimum volume
- Bring extra sorting buffer in case the concentration is too high.
- Cells can be brought in 5mL FACS tube or 15 mL tube



Final Cell Conditions For Sorting

Filter the cell samples immediately prior to transporting them to the core. An additional filtration may be required immediately prior to sorting if you are bringing multiple samples to the lab (the cells may settle and clump while waiting to be sorted), so please bring extra filtration tubes for sticky samples. The RFCC core has a hood and the samples can be filter in the facility.

- Use a 40 µm mesh filter or smaller, or the 35 µm mesh cap of a 352235 Falcon Tube.

- We will not sort unfiltered samples. We want to ensure a successful sort and once the instrument is clogged, it may take a significant amount of time to bring the instrument back to its original configuration. Time used to unplug the nozzle and bring the instrument back to sorting status may use up your scheduled time.

Collection

Collection media routinely suggested:

- Cell culture media buffered with PBS or HEPES to kept the pH constant
- Use higher % of FBS than usual up to 100%

- PBS or lysis buffer can be used for DNA/RNA extraction:
  - Trizol/phenol not allowed due to lack of chemical hoods

- Note that the final volume in the sort collection vessel will be a mix of collection media plus sorter sheath fluid. The amount of serum and other supplements should reflect the final expected volume.
- Polypropylene tubes preferred over polystyrene. 1.5 mL, 5 mL, 15 mL tube, or plates
- Collection vessel should be ~1/3 full of collection media

Instrument selection

BD FACSAria Fusion – cuvette
- 5 lasers, 18 detectors, 4-way sorting

BD FACSAria II (Arnie) – cuvette
- 5 lasers, 18 detectors, 4-way sorting

BD FACSAria III (cuvette) only 100µm nozzle
- 4 lasers, 13 detectors, 4-way sorting (UC)

BD FACSymphony S6 – cuvette coming soon!
- 5 lasers, 23 detectors, 6-way sorting

ThermoFisher Bigfoot – jet-in-air
- 5 lasers, 48 detectors, spectral option, 6-way sorting

Sony MA900 – chip
- 4 collinear lasers, 12 detectors, 4-way sorting

Sony SH800 – chip
- 4 collinear lasers, 6 detectors, 2-way sorting

Cuvette: ↑ sensitivity | Jet-in-air: ↑ post-sort integrity of fragile cells | Chip: user friendly

Research Flow Cytometry Core

Cincinnati Children's changing the outcome together

Nozzle selection

- Nozzle size should be 5 times larger than the size of the cells being interrogated.

↑ max sorting speed
↓ collected volume
↑ pressure
↑ stress on cells

↓ max sorting speed
↑ collected volume
↓ pressure
↓ stress on cells

70 µm 85 µm (uncommon)

Frequency ~90,000 drops/s
Max sort rate: ~18,000 evts/s
Volume per 10^6 cells sorted: ~ 1 mL

100 µm 130 µm (uncommon)

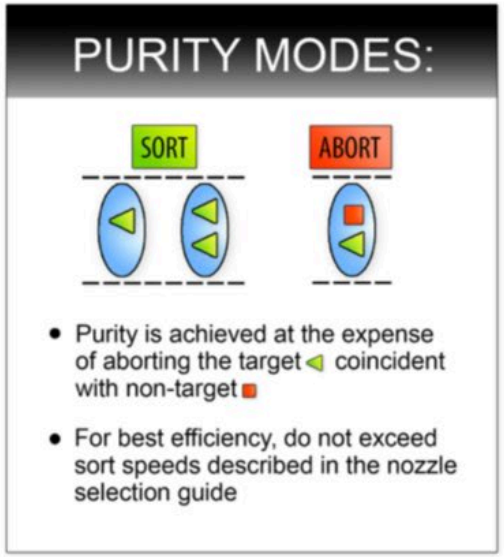
Frequency ~35,000 drops/s
Max sort rate: ~7,000 evts/s
Volume per 10^6 cells sorted: ~ 4 mL

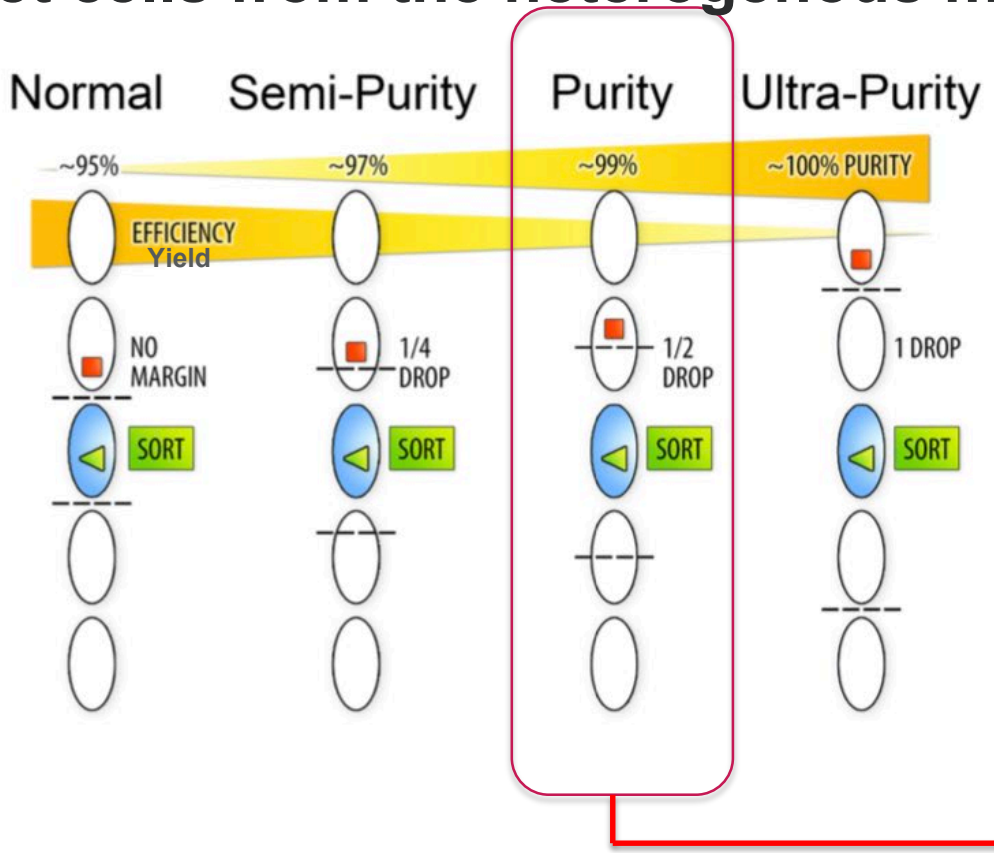
Research Flow Cytometry Core

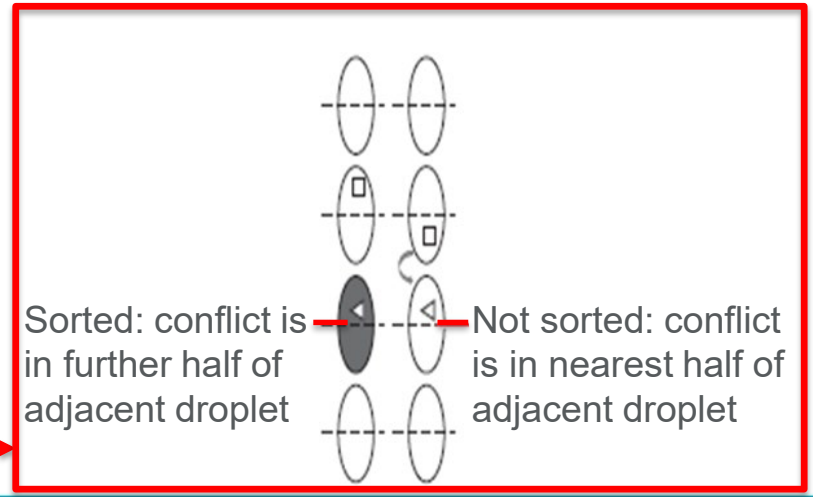
Cincinnati Children's
changing the outcome together

Sort Modes

Separating the target cells from the heterogenous mixture of cells







Research Flow Cytometry Core

Cincinnati Children's
changing the outcome together

Scheduling time for Sort

Time needed to sort 10 x 10^6 cell from each of 8 samples with a 70 um nozzle and a 7-color panel:

Low Speed: 30 min (setup instrument) + 8 x 18 min (acquisition) + 8 x 2 min (changing tubes) + 30 min (clean instrument) = ~4 hr.

High Speed: 30 min + 8 x 12 min + 8 x 2 min + 30 min = ~3 hr.

	70 um Nozzle	100 um Nozzle
Maximum number of events/sec during acquisition LS = low speed; HS = high speed	LS: 10,000 events/sec HS: 15,000 events/sec	LS: 7,500 events/sec HS: 10,000 events/sec
Time to sort **10 x 10^6 cells** (acquisition)	LS: 18 min HS: 12 min	
Time to add for each sample to be sorted. (changing tubes,etc)	2 min	2 min
Time to set up < 8-color panel	30 min	30 min
Time to set up > 8-color panel	1 hr	1 hr
Time to set up 96-well plates	10 min	10 min
Time to set up Terasaki plate	15 min	15 min
Time to post sort 1 sorted tube	5 min	5 min
Time to setup instrument	30 min	30 min
Time to clean instrument	30 min	30 min

Help us help you

Your responsibilities:

- Prepare a quality, filtered single cell suspension at proper concentration.
- Declare any biohazards associated with your samples on form provided when scheduling.
- Provide as much information as possible on the scheduling form. Cell type, size & source.
- Provide the necessary controls.
- Be prepared to communicate your intended gating strategy. Bring template of possible.
- Bring appropriate collection tubes/plates filled with collection solution.
- Schedule enough time to calibrate the instrument, sort your target number of cells, and clean.
- Arrive on time!

Not sure? Ask the RFCC staff!

Mary Mullen

Ken Quayle

Amaya Willemsen

Anastasia Brenn

Alyssa Sproles

Sarah Croswell

Celine Silva-Lages

Sherry Thornton

Research Flow Cytometry Core

Cincinnati Children's™
changing the outcome together