

# Cell Sorting- Things to Consider

## Quick tips

1. The droplet containing the cell is charged and can be attracted to the charge on the plastic. This results in the droplet hitting the side of the tube wall, and the cell dying as the small volume of liquid evaporates. To prevent this, pre-coat the tube with protein (FBS/BSA) containing buffer to neutralize the plastic charge and use polypropylene collection tubes.
2. Cells are going to be traveling in buffered saline. This is not conducive for keeping cells alive for long periods of time. To improve recovery, ensure the catch buffer (1/3 vol. of collection tube) has protein (BSA/FBS) (10-50%).
3. If sorting into media, make sure the media is HEPES buffered. Buffers like RPMI are formulated to buffer in the CO<sub>2</sub> atmosphere of an incubator and don't buffer well in our normal atmosphere. This is especially true for plate sorting!
4. Know how the cells respond to temperature differences. Some cells do not like to be kept cold and will die quickly if sorted into 4-degree Celsius buffer. The collection chamber can be controlled for temperature.
5. Adherent cells are commonly disassociated with trypsin with FBS used to neutralize the trypsin. However FBS can also add back all of the components that cells need to re-adhere causing clumping. Try soybean trypsin inhibitor instead.
6. A clogged nozzle will take a long time to re-stabilize. **Just** before sorting pass the cells through an appropriate sized filter to remove clumps and debris. Also consider looking at the cells under a microscope to ensure that there are no clumps prior to sorting. If a sample contains **visible** clumps it will not pass through the sort nozzle!
7. Include a viability dye to help eliminate dead cells.
8. When trying to define a cell population, include both positive and negative markers in your antibody panel to ensure the cells are what they are supposed to be. *Dump channels*, can also help to identify unwanted populations.
9. Know the cell count *at the time* the cells are going onto the sorter – **NOT** from when you first began preparing them.
10. Since an optimal sort speed is typically  $\frac{1}{4}$  the droplet generation frequency, over concentrating the cells will reduce purity. Bring some dilution buffer with you just in case the cells are too concentrated.
11. The higher the threshold, the easier it is to visualize the specific cell population. But this does not eliminate the fact that debris and junk are still present within the cell population you're visualizing. It just means that the cytometer is ignoring it.

**Whatever the cytometer ignores will end up in the final sorted population.**

For example, a “pure” population of sorted mature B cells may contain a lot of red blood cells if they have not been removed!

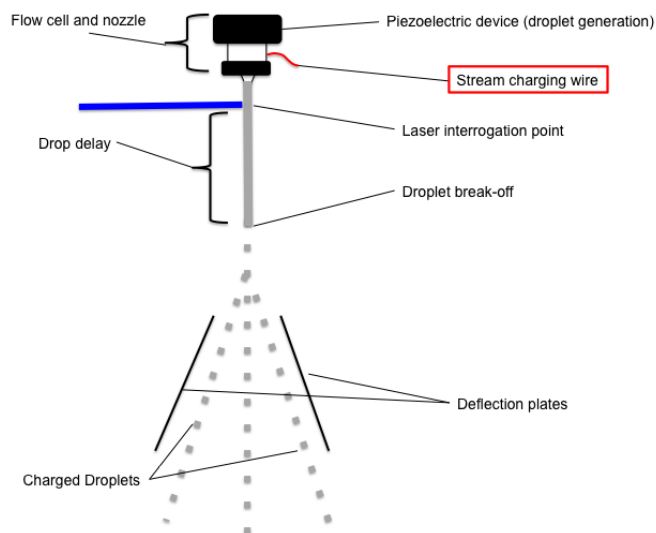
12. Anything can be sorted as long as it's big enough to be detected by the instrument (this depends on the instrument, but  $>0.5 \mu\text{m}$  is reasonable for many instruments) and small enough to fit through the nozzle tip at the end of the flow cell without clogging it or causing side stream instability (usually  $< 1/5$  the size of the nozzle tip). Common nozzle sizes are 70/100/130 $\mu\text{m}$ .
13. Cells that have just come out of the nozzle are fragile. Allow them time to recover. Cell sorters are usually housed in air-conditioned rooms wherein the room temperature hovers between 19-22°C. Your cells are not used to being cold so let them equilibrate to 37°C before manipulating them.
14. Use tight sorting gates with biexponential plots along with low threshold (see point 11).

## Principles of cell sorting

Droplet charging occurs in the nozzle and is applied to the entire connected stream rather than individual droplets. The point at which the droplet is charged (droplet break-off) is downstream of where the charge is applied.

By calculating the “drop delay” (time between laser interrogation point and droplet break-off.) the system can determine how long the system waits before applying a charge as a particle is detected.

The application of positive and negative charge at varying amounts will allow the charge plates to deflect the droplet containing the cell into the appropriate tube.



## Cell preparation – in depth

Depending on the cell preparation method there could be significant cell death and debris which will impact the quality of the sort. For cells grown on plastic try to avoid mechanical scraping which usually results in a large proportion of dead cells, debris and clumps. “Difficult to remove cells” can be grown on low adherence tissue culture plates and polypropylene tubes should be used instead of polystyrene with round instead of conical bottoms. Adherent cells are commonly treated with trypsin but trypsin at high concentrations or over too long a period can damage cell membranes, impair antibody detection and kill cells. Trypsin inhibitors like Soybean trypsin inhibitor are better than serum since serum adds back divalent cations that facilitate cell adhesion and aggregation. EDTA can be added as a divalent cation chelator to reduce clumping. Furthermore, use of Accutase or Accumax can help reduce clumps.

Centrifugation should be of minimum duration and cells should not remain in pelleted state. Use swinging-bucket rotors as opposed to “fixed angle” rotors to prevent cell loss due to cells being deposited on the sides of the tube rather than the bottom. Remove supernatant by pouring rather than through suction to minimize chance of cell loss and disaggregate cells in pellet before re-suspending.

Clumps will clog the cell sorter; therefore filter the cells just prior to sorting since cells can re-clump over time. Options include a 70um cell strainer (Falcon 352350) for larger cells or a 45 um strainer (Falcon 352235).

Dead cells can have high autofluorescence and bind antibodies non-specifically therefore a viability dye should be included in the staining panel to gate out dead cells during sorting. Dead cells can also release DNA which enhances cell clumping. Consider DNase 1 treatment- 200ug/ml DNase + 5mM MgCl<sub>2</sub> in HBSS 15 – 30 min RT. Cells should be washed in the presence of 5mM MgCl<sub>2</sub>/HBSS and re-suspend in staining buffer containing 5mM MgCl<sub>2</sub> with 25-50ug/ml DNase. Furthermore, to minimize cell death consider HBSS or culture media without phenol red. Addition of 2%BSA or FBS and 25mM HEPES buffer can also help.

Consider using magnetic bead enrichment or density gradient centrifugation to increase purity and decrease sort times and improve rare cell sorting.

Be realistic about how many cells will be lost during preparation to estimate the number of cells required to sort the target population(s). This will also be impacted by sort efficiency which is affected by many factors such as cell size, type, death and concentration. In practice dim and or cultured cells tend to have lower efficiency than bright primary cells such as lymphocytes. Preliminary analysis in advance of sorting can help establish sort efficiency and potential yield. Consider running the cells on

the ImageStream Imaging flow cytometer to evaluate the cell preparation with respect to cell aggregates as well as specificity of stain prior to sorting.

Culture media is not an ideal sort buffer since the high pressure increases the partial pressure of CO<sub>2</sub> resulting in lower pH. Use a buffer with strong pH buffering capacity like HEPES (25mM). This is especially important for sorts into micro-well plates. Furthermore, the phenol red in media can introduce a fluorescent background while the high protein content can adversely affect light scatter. Re-suspend the cells in a low protein buffer such as Ca<sup>++</sup>/Mg<sup>++</sup> Free PBS or HBSS (0.5-2% BSA or up to 2% FBS) 25mM HEPES pH7.0.

#### To help reduce clumping

1mM EDTA up to a maximum of 5mM to prevent macrophages and monocytes from sticking to tubing. Note serum will negate function of EDTA.

**or**

10u/ml DNase especially if there are a lot of dead cells present.

**DNase should not be used with EDTA because EDTA chelates the ions required for DNase function!**

Cell concentration should be in the range of 10-20 X10<sup>6</sup> cells/ml and if you have fewer than 5X10<sup>6</sup> cells re-suspend the cells in a volume of 300ul. These are general guidelines and the concentration may need to be adjusted so bring extra buffer.

## **Fluorochrome choice**

Generally speaking weak separation between populations will impact sort efficiency so aim to design a panel with the best separation possible. Use titrated reagents to add sufficient antibody to saturate all of the epitopes while avoiding excess antibody that will increase the potential for non-specific binding. For more information on how to perform antibody titrations consult LTRI flow cytometry core staff or visit the resource section of our website. Consult our website for guidelines on panel design or talk to the LTRI core staff for options.

#### Panel design tips

1. The biological question dictates panel design.
2. Assess antigens required and fluorochrome combinations available.
3. Lasers, detectors and filters available

Which detectors are most sensitive – reserve for important, unknown or low expression

Least sensitive – highly expressed markers

#### 4. Classify antigens

Primary/major markers –highly expressed major subsets like CD4

Secondary markers – variable expression - subsets like CD45

Tertiary or unknown markers like CD25, EpCAM

Detectors for viability dye and a “dump” channel (for e.g. a lin neg subset) if necessary

#### 5. Reduce the amount of spectral overlap especially into detectors reserved for dim markers

#### 6. Use FC blocker (anti-CD32/16, Human FC block) if appropriate

7. Include a viability dye in your panel to allow for dead cell exclusion even if you are only interested in RNA. Many choices are available to work with your panel. Good sources are Invitrogen and Biolegend.

8. Panel design tools like Flurofinder have links to vendor websites which will aid you in design of a panel. FluroFinder <https://flurofinder.com>. Look for our institution and identify the instrument you want to use. Check your panel spectrum viewers to see if it can be improved and adjust panel if necessary.

Spectrum viewers - BD biosciences, Thermo fisher/Invitrogen, Biolegend, Chromocyte

## Controls

Controls are necessary for setting PMT voltages, compensation, and regions to identify the population of interest.

### Unstained and single stained controls

Unstained controls help identify the auto-fluorescent background while single stained controls are necessary for establishing the compensation values for your panel. Rules of thumb for single stained controls are:

1. One single stained control for each fluorochrome.
2. Each single stained control must be as bright as or brighter than the same stain within the sample.
3. Each single stained control must have a negatively stained component that is spectrally matched (i.e. same autofluorescence).
4. Compensation is a property of the fluorochrome not the carrier. Therefore consider antibody capture beads or a different cell type with good expression of the epitope(s) in question for performing compensation.

### Fluorescence minus One Control(s) (FMO)

FMO controls are gating controls used to establish gates between positive and negative staining for a fluorochrome of interest. They contain all of the fluorochromes from a panel except the one of interest. Combining all of the fluorochromes except the one being measured will allow regions to be drawn that take into account of a phenomenon referred to as measurement error. This can be critical for sorting populations with small changes in fluorescence especially if using a panel for the first time! Ask the LTRI flow core staff for more details about this important control.

### Biological Controls

Positive and negative biological controls if available are also very useful for establishing sort regions.

### Collection media

Options

PBS-/- 10-50%FBS

Culture media

PBS

Lysis buffer from RNA isolation kit

Collection tubes should be 1/3 full with collection media

Tubes should be sterile and RNase free for RNA collection. For recovery of live cells pre-coat the tubes with 1%BSA to keep cells from sticking to the sides of the tube by filling the tubes with PBS 1%BSA for 30 minutes prior to sorting. For western blotting you may want to avoid adding an external protein.

Transport the samples in an ice container with a lid. BSLII transport follows “transportation of dangerous goods guidelines” Essentially this means that samples are carried in a sealed primary tube such as a microfuge tube within a sealed secondary container such as a Styrofoam container.

**Don't forget the biosafety form.**

### **Determining the number of cells to begin sort with.**

Approximations of the starting cell number to sort 100,00 cells. The frequency of the population, the recovery from the sorter, and recovery from sample processing factor into how many cells are required at the start of the experiment. The time to sort is an estimate of how long it would take to sort the cells at different nozzle sizes (which relates to how fast the sorter can run, and thus the droplet generation frequency).

Cells needed	Frequency of population	Expected recovery (Sorter)	Expected recovery (Sample Processing)	Approximate # cells to stain	Time to Sort (Min)		
					20 KHz	45 KHz	87 KHz
100,000	10%	50%	80%	2,500,000	6.67	2.96	1.53
100,000	1%	50%	80%	25,000,000	66.67	29.63	15.33
100,000	0.10%	50%	80%	250,000,000	666.67	296.30	153.26
100,000	10%	25%	80%	5,000,000	13.33	5.93	3.07
100,000	10%	50%	80%	2,500,000	6.67	2.96	1.53
100,000	10%	75%	80%	1,666,667	4.44	1.98	1.02
100,000	10%	90%	80%	1,388,889	3.70	1.65	0.85
100,000	10%	50%	40%	5,000,000	6.67	2.96	1.53
100,000	10%	50%	60%	3,333,333	6.67	2.96	1.53
100,000	10%	50%	80%	2,500,000	6.67	2.96	1.53

## Annie's recommendations

Here are notes about how to prepare your samples for cell sorting:

1. Cell concentration (before sort) should be about 2 – 5 million per mL. All samples must be filtered (minimum 40 micron pore) before sorting.

2. **Bring extra sort sample buffer.** Optimal buffer in which to bring your cells, although some variation is acceptable:

1 x Phosphate Buffered Saline (PBS) or Hanks Balanced Salt Solution (HBSS) (Ca<sup>2+</sup>/ Mg<sup>2+</sup> Free)

25 mM HEPES pH7.0

1% Fetal Calf Serum (Heat inactivated) or 1% Albumin (for sticky cells)

3. For samples with a high dead cell concentration or primary tissue preps: Dead cells can release their DNA into sorting media which in turn can cause cells to clump together. Adding DNase I in the presence of MgCl<sub>2</sub> will help reduce the aggregation.

a. Treat cells for 15-30 minutes in a sterile solution of 100 ug/mL DNase I and 5 mM MgCl<sub>2</sub> in HBSS at room temp.

b. Wash the cells 1x in HBSS containing 5mM MgCl<sub>2</sub>.

c. Resuspend the cells in HBSS containing 25-50 ug/mL DNase I, plus at least 1mM MgCl<sub>2</sub> prior to and during the sort. (5mM MgCl<sub>2</sub> is optimal)

4. For bulk collection, **bring media with a high FBS content (~50%)** to help cells recover from the sorting process.

a. For direct plate collection, bring plates loaded with the culture media appropriate for the sorted cells.

b. For post sort mRNA analysis, cells can be sorted directly into RNA isolation reagents, such as Trizol-LS, RTL buffer, RNA Later, etc. Please ask for more details.

## ***In depth***

### ***2.1.1 Choice of buffers***

The most commonly used media/buffers for processing mammalian cells were designed to work at 1 atmosphere pressure either on a laboratory bench or within a CO<sub>2</sub> incubator, yet inside the sample chamber of most cell sorters the pressure can often exceed 2 to 4 atmospheres depending on the conditions and nozzle size chosen for the sort. Sample buffers that historically tend to perform well for sorting such as Dulbecco's Phosphate Buffered Saline or HBSS (minus Ca<sup>2+</sup> and Mg<sup>2+</sup>), both with 10 to 25 mM HEPES and protein (usually 1 to 2% heat inactivated serum or BSA), and more recently BD FACST<sup>™</sup> Pre-Sort Buffer plus from 0.2 to 2% protein (application dependent) are recommended. Bicarbonate media buffers such as Roswell Park Memorial Institute (RPMI) or DMEM usually do not make the best candidates for sample sort buffers or sort collection buffers since they (i) are a different buffer type than the cytometer's sheath buffer (bicarbonate versus phosphate), and (ii) by design require 5% CO<sub>2</sub> to maintain physiological pH, and (iii) usually contain divalent cations (Ca<sup>2+</sup> and Mg<sup>2+</sup>) plus phenol (very fluorescent). If a bicarbonate media is used, one should be wary and use either Ca<sup>2+</sup> or Mg<sup>2+</sup> minus formulas without phenol or mitigate the undesirable divalent cation side effects for sorting (making the cells "sticky") by adding ~1 mM EDTA in addition to 25 mM HEPES and protein. HEPES buffered bicarbonate media has been reported to be light sensitive [109](#), and it is generally a good idea to protect any sample for flow cytometry cell sorting from light.

### ***2.1.2 Considerations for adherent cells and cells isolated from solid tissues***

In preparing adherent cell lines for sorting a common pitfall is often within the protocol to remove the cells from a dish using trypsin or trypsin-EDTA and subsequently inactivate the trypsin by adding back culture media containing a significant amount of serum. This step is



designed to stop the proteolytic activity of the trypsin and make the cells “sticky” to easily adhere to a plastic dish when passaging the cells. The opposite effect is desired for flow cytometry cell sorting, the sample should not be “sticky” with a tendency to adhere to plastic. As a result, good flow cytometry cell sorting protocols for adherent cells will typically either inactivate the trypsin with soybean trypsin inhibitor or use one of the many available non-enzymatic cell disassociation buffers (e.g. Accutase™); in either case, if the cells grow in media with serum, the culture should be gently rinsed twice with Dulbecco's Phosphate Buffered Saline before disassociating and removing the cells from their substrate. Some cell types, when disassociated with non-enzymatic disassociation buffers that rely on chelating agents, may show decreased viability as compared to trypsin disassociation **110**. If there is any doubt, a few simple pilot experiments designed to determine the best preparation method for the specific cells in question is often a very good investment toward successful sorting.

Similarly, isolating cells from any primary tissue for flow cytometry cell sorting can be very challenging, care should be taken to ensure the chosen protocol is optimized and tested to not only provide the intended cells (e.g. regarding isolated dendritic cells from spleen different protocols can enrich for different phenotypes), but helps coerce the cells into a well behaved single-cell suspension. The highest quality reagents should be used, especially when using proteolytic enzymes such as collagenase, pronase, dispase, or trypsin since small amounts of contaminants can have serious undesirable effects resulting in poor sample performance. Collagenase is dependent on calcium for activation, for example, and other divalent cations may be activators ( $Zn^{2+}$ ) or inhibitors ( $Mg^{2+}$ ) **111**, and care should be taken to ensure any additive endotoxin levels are as low as possible.

### ***2.1.3 Stickiness to plastic: The menace of cell sorting***

When performing bulk sorts and collecting a sorted fraction into a plastic tube, it is usually best to pre-coat the tube with serum leaving some at the bottom, or if desired, additionally seed the tube with a small volume of the sample buffer containing 2 to 10% serum. Adding unbuffered bicarbonate media to the collection tube and sorting on top of it runs the risk of high pH conditions causing undesirable salts to form while the phosphate and bicarbonate buffers mix with the cells present, thereby reducing cell viability. When performing single-cell sorts into a microtiter plate, any media pre-added to the wells should be HEPES buffered and conditioned beforehand if possible. Additionally, when sorting onto/into small targets such as microtiter plate wells extra care should be taken to ensure the accuracy of the deflected drops during the sort by choosing an appropriate nozzle size to minimize the effects of cells on drop breakoff **112** (choose a nozzle at least 5–6 times the cell diameter as verified under a microscope).

### ***2.1.4 Cell concentrations and sorting rates***

Once prepared, the sample should have a final cell concentration that allows the desired event rate to be achieved with only a modest differential pressure on the sample. Increasing the sample rate significantly by simply forcing more through the system is not recommended. The sample should be filtered just prior to being loaded onto the sorter to help ensure no clumps are present and further disperse any weakly adhered cells. After filtering the sample through a Nitex nylon monofilament mesh with an appropriate pore size (~30 to 50  $\mu m$  depending on cell size), any samples that tend to dynamically reaggregate during a sort are best dealt with by installing an in-line nylon sample filter of

the same pore size to help prevent clogs. Generally, since the theoretical sorting efficiency of a single-cell preparation is that of a homogenous Poisson process [10](#), the operational efficiency of the sorter may be estimated by

where rate is total events/second, fraction is percent being sorted, drop packet is the number of drops including any additional temporal purity mask, and frequency is the drop rate in drops per second. Normalizing to sorter drop frequency, this means when sorting a fraction that is 10% of the total at an event rate of one cell to every 4 to 5 drops, it can be expected to sort with an efficiency of 80 to 85% when using a single drop sort.

### **2.1.5 Purity and doublets**

If, after optimizing the sorter during setup, suddenly the application sorting efficiency is low (higher than expected conflict abort rate), it is indicative that the sample is not a monodisperse cell suspension, that cells are likely “sticky,” adhering to one another during entrainment and not arriving into the sensing zone as a homogenous Poisson process. This is a very common scenario with many cell preparations, especially adherent and primary cells, and often the sorter performance is blamed for what is a behavior intrinsic to the sample. Much of the time this can be significantly mitigated by reexamination of the sample preparation protocol to discover what might be improved to help coerce the cells into a well-behaved single-cell suspension. This often involves the addition of EDTA or DNase etc. to the sample sort buffer.

Whenever a sorted sample using a purity sort mode (where system-defined spatial-temporal drop zones in the stream are examined logically for potential contaminants for each sort event) is not as highly sorted as desired, the most common reasons are that either the classification scheme for single cells is not robust enough and hidden passenger cells are occasionally sorted, or that there are particles in the stream that are disturbing the droplet breakoff stability and, as a result, the wrong drops will occasionally appear in the collection tube, or a combination of the two. Sorters certainly cannot read the operator's mind and will attempt to do exactly what they are set up to do so, if a positive selection from the sorter suffers from disappointing purity, one simple performance check is enough to sort a completely negative cell fraction for comparison. If that sorted negative fraction is 99% pure or higher, yet the positive fraction is only 80 to 95% pure, then the likely cause is undetected “doublets” due to an insufficiently constrained single-cell gating strategy. In many flow systems, doublets tend to align with the doublet figure's major axis in line with the partially developed laminar flow and the pulse width becomes a very useful parameter to help distinguish singlets from doublets. Other systems, such as the BD FACSAria™ family that use fully developed laminar flow in their fluidics design can have those same doublet figures rotate off axis after entrainment in flow such that Forward Scatter (FSC) pulse width alone will not detect enough doublets, and in such cases using both FSC and Side Scatter (SSC) looking at plots of Height versus Width (or Height versus Area—but that usually leaves less screen real estate for drawing gates) will help reveal many more doublets, boosting the purity to a more acceptable level with careful gating. Figure [12](#) (reproduced with permission from [113](#)) is an example of such a strategy where pulse geometry gates on both FSC and SSC detect an additional 9% of doublets that would pass through a standard scatter gate.

Matching nozzle size to particle size is key, and the general rule of thumb is that the nozzle should be 4 to 5 times that of the particles for bulk sorting and 5 to 6 times that of the particles for plate deposition where accuracy is more critical. Ensure that the actual cell size is what you expect it to be when choosing a nozzle, and whenever there is doubt it is very useful to quickly compare to known bead size standards by simply putting small drops of each on a microscope slide and checking, not only the size(s) within the sample but also the quality as the amount of debris should be low, the number of single cells high, and clumps/aggregates should be the rare exception rather than the rule. Electrostatic cell sorters tend to perform very well with monodisperse samples and struggle with poorly dispersed ones so, as with many other applications, sample preparation can be the limiting or enabling step.