

Optimizing Sorting Experiments

There are several factors that need to be considered in order to prepare cells properly for a successful sort. Since there is a wide range of cell types used and experimental end-points, there is no single preparation methodology that is appropriate for all experiments. The following will attempt to elucidate some of the issues and allow for the researcher to determine what is required for their particular experiment.

Cell Size and Morphology

Researchers frequently sort cell types ranging from resting lymphocytes (small size, spheroid morphology, and robust) to aged microglia (large size, dendritic morphology and very fragile) and everything in between. The drop drive frequency (ddf) and stream velocity (pressure) combinations are optimally matched to a particular nozzle size (50-400um) depending on the cells being sorted. It is important to be familiar with these issues relevant to the particular cell type being sorted.

There is a simple rule we follow that helps govern nozzle selection based on cell size: The cell size should not exceed one-fifth of the nozzle diameter. This rule helps ensure that stream stability can be maintained during the sort. If larger particles are entering the stream, there is a deleterious effect on the droplet breakoff. A drifting breakoff can disrupt the careful calibration of the droplet delay and lead to problems ranging from fanning of the side streams (desired sample ends up missing the tube) to changing of the breakoff (severely compromises sample purity) to ultimately clogging the nozzle.

Morphology is a trickier subject due to the variation between cell types. In general, the more the morphology deviates from an ideal spheroid shape, the more susceptible the cells become to shear induced damage. We typically find that the greater the deviation from the ideal sphere, the larger the nozzle required by the instrument. It is important that you communicate information relative to cell morphology prior to scheduling a sorting experiment.

Sample Preparation

The single most important issue for a successful sort would have to be proper sample preparation. This can be broken down into four separate components:

1. Single Cell Suspensions
2. Optimized Sample Concentration
3. Proper Sort Buffer Recipe
4. Expedient Sample Processing

Single Cell Suspensions

In order for the sorter to function properly and to be able to deliver the proper results, the cells must be in a single cell suspension, and remain that way for the duration of the sort. This becomes a more important factor when working with adherent cell lines or tissues. Achieving a single cell suspension is the goal for a perfect sample preparation. One of the easiest tricks is to remove any large aggregates is by filtration (typically through fine 30-50um nylon mesh). There are two options. The first option, and the easiest, is to use a 12x75 mm Falcon test tube with a cell strainer cap (Falcon 352235). Simply pipet the cell suspension through the top of the cap. The second option is a little more involved. To minimize sample loss, use the techniques described below. The following tools are required (sterilize as necessary):

- * 1 syringe sufficient to hold sample and one additional mL (retain cap)
- * 1 Luer-Lock needle (typically 18 gauge)
- * 1 15mm x 15mm piece of nylon mesh (with a 30-50um mesh)
- * 1 razor blade

The next step is to aspirate the sample up through the syringe needle such that your sample is loaded along with at least 1.0mL of air as a void volume.

Once the sample has been aspirated along with the 1.0mL of void volume, carefully dispose of the needle. Maintain the syringe in an upright position so that the void bubble remains at the tip of the syringe.

The next step is to sandwich the piece of nylon mesh over the tip of the syringe using the original syringe tip. The idea is to use the tip to hold the filter mesh in place. Once the tip is in place, carefully cut off the first few mm of the tip. This will allow a hole for the filtered sample to pass through. Additionally, take care to maintain the void bubble towards the tip so no sample is accidentally lost during this step.

At this point you need to invert the needle so the void bubble rises to the plunger. Now the sample is ready to be passed through the filter mesh and the void bubble should ensure no sample remains in the syringe.

Optimized Sample Concentration

Cells must be at the proper concentration in order for the sorter to function optimally. Simply put, cells that are too concentrated will have a lower recovery due to coincidence aborts (two cell that are too close together will be rejected by the machine in order to ensure purity) and cells that are too dilute will have a longer processing time (or if they are processed faster, an increased signal CV).

Ideal Cell Suspension Concentrations

Having the sample too concentrated, or too diluted can be problematic. There is no ideal concentration that works for all cell types and sort set-ups. It is a matter of understanding some of the issues and deciding what factors are most relevant to a given cell type and experimental design.

- **Coincidence Aborts**

While the sorter is evaluating which cell to sort, it must also determine whether it can do so in a manner that ensures the sorted material remains pure. The sorter makes this decision based on the proximity of events in time. If the desired event is too close to a potential undesired event, the machine will abort (not sort) the desired event to ensure purity. There are other modes of sorting that favor recovery over purity, so if total cell numbers are more important than purity, we can accommodate this. This should explain why we would not want the sample so concentrated that it becomes difficult to space the cells far enough apart while they are going through the sorter. If recovery is a prime concern, aborts tend to be the issue.

- **Signal CVs and Sensitivity**

If the sample is too dilute, to get them to run at a reasonable rate can cause another set of problems. To get them to run at the appropriate rate, the sample differential must be increased. If the differential is too high, the CV's (coefficients of variation) start to become higher. This ultimately leads to less resolution and lower sensitivity. If you are trying to separate two very close populations, the CV becomes more important.

- **Cell Adhesion and Clumping**

Adherent cells are trickier to sort than suspension cell types. Adherent cells typically like to stick to each other given the opportunity. Careful cell preparation and media can help avert this problem, but so can cell concentration. These sticky cell types typically like to be slightly more dilute to mediate clump formation. There is no absolute formula for this, it is determined by trial and error for a given cell type. The take home message is to consider running very sticky samples slightly more dilute than the below suggestions.

The following is a list of concentration ranges based on machine set-up which typically correlates with cell type:

Nozzle Size	Cell Types	Concentration (per mL)
70um	lymphocytes, thymocytes	8-15 x 10⁶
80um	activated subsets, smaller cell lines	7-10 x 10⁶
100um	larger adherent cells	5-9 x 10⁶

These guidelines are in place to suggest where to start, but it might be easier to tend towards a little too concentrated (towards the high end of the recommended range), but bring some additional sample buffer with you so things can be diluted as necessary.

Proper Sort Buffer Recipe

This is probably one of the most important factors to achieve an ideal sort. A properly designed buffer recipe will help maintain a single cell suspension as well as keep the cells in a good physiological state. Culture media is typically a poor sort buffer (although it can be modified).

The proper design of sort buffer for both your pre-sort sample and your collected sample is crucial for a successful sort. The following will be a basic recipe and some suggestions for modifications that might be relevant to your particular experiment. Culture media is not an ideal sort buffer for two reasons: the pH regulation fails under normal atmosphere causing the media to become basic and the calcium chloride in most culture medias is not compatible with the phosphate component of the instrument sheath buffer (the Basic Sorting Buffer without additional protein) leading to precipitation of calcium phosphate crystals. Following the suggested recipes below will help maximize the recovery and viability of your sorted cells.

Basic Sorting Buffer:

- **1x Phosphate Buffered Saline (Ca/Mg++ free)**
- **1mM EDTA**
- **25mM HEPES pH 7.0**
- **up to 1% Fetal Bovine Serum (Heat-Inactivated)**
- **0.2um filter sterilize, store a 40C**
- **antibiotics (pen and strep)**

Optimal Collection Media:

- **Fetal Bovine Serum only**
- or
- **Your own culture media with antibiotics**
- or
- **PBS if collecting cells for RNA or DNA**
- or
- **Lysis buffer from RNA isolation kit (e.g. buffer RLT from Qiagen kit) (however, we do not recommend sorting into TriZol)**

For Clean Lymphoid Cells

The buffer can be simplified to HBSS with 1% FBS. The additional cations in the recipe promote better viability. Since these cells are not prone to clump, the lack of EDTA is not a problem.

For Sticky Cells

Raise the concentration of the EDTA to 5mM and use FBS that has been dialyzed against Ca/Mg++ free PBS. Some activated cells become clumpy and the chelators (EDTA) help reduce cation-dependent cell to cell adhesion.

For Adherent Cells

In order achieve good single cell preparations, one must start at the moment of detaching your cells from the plate. Typically, the trypsin (or other detachment buffer) is quenched with culture media or a PBS/FBS buffer. This is problematic because it reintroduces the cations that facilitate

the cells reattaching to the plate (or each other). One must use a cation-free FBS buffer in order to stop the detachment. Additionally, the level of EDTA can be increased if necessary (but too much EDTA can be deleterious).

[For Samples with High Percentage of Dead Cells](#)

If there are a large number of dead cells in the prep, it is likely that there is soluble DNA from the dead cells that will come out of solution. This DNA will start to coat the cells and lead to severe clumping. The addition of 10U/mL DNAase II to the buffer recipe will help reduce DNA associated clumpiness.

Extremely Important: After cells are sorted into collection tubes, centrifuge cells to remove diluted buffer and replenish with fresh culture media.

These suggestions should help to optimize sample preparation for both enhanced viability and enhanced recovery. It may require some more comprehensive modifications evolving from these simplistic guidelines.

[Expedient Sample Processing](#)

The sample must be prepared in as short a time as feasible to minimize stress on the cells as sorting is a relatively harsh process. Much of this can be achieved by simplifying the staining process and staggering the sample preps if more than one sample is being sorted.

[Cell Physiology](#)

Resting cells are typically very easy to sort, but most researchers have manipulated the system such that the cells are no longer in the most ideal state for processing. This can be addressed by setting up the instrumentation to run at lower pressures to minimize the stress on the cells. It is important for the researcher to convey any of these potential physiological issues to ensure the sort is properly configured.

[End Point Requirements](#)

The desired use for the sorted material can have a role in how the instrument is configured and how the sample can most efficiently be processed. Whether cells need to be viable, sterile or are used for DNA/RNA isolation can also have a role in instrument set-up.

[Specimen Preparation](#)

Samples must be filtered through (40 μ mesh) before sorting. For example, you can use the following products: B-CMN-40 (by sq. feet) from Small Parts, Inc or Falcon 2235 tubes with the mesh in the caps from Becton Dickinson. The optimal concentration of material in the sample tubes (15-20) x10⁶ cells /ml. Cells bigger than lymphocytes might need to be at lower concentrations. See Ideal Cell Suspension Concentrations (above).

The following measures are important to prevent sorting material from clumping:

1. Low protein concentration (FBS or BSA), protein promotes clumping.
2. Use filtered buffer that is Ca²⁺ and Mg²⁺ free unless measurement depends on these ions
3. Add EDTA (see Basic Sorting Buffer recipe above)
4. Use anti-clumping agents, such as Accumax.(Innovative Cell Technologies, Inc., A Phoenix Flow Systems, Inc. Company)

It is highly recommended that unstained and single color stained controls be provided for optimal set-up.

Specimen Collection

Sufficient amount of collecting tubes (of 5ml or 15ml capacity) with media (up to 1ml or 3 ml per tube respectively) is necessary. To sort populations of low percentage, higher volume of media (up to 3 ml in 5ml tubes) is recommended for better cell recovery.

Media in collection tubes should contain at least antibiotics and 10% serum to increase viability of the cells. If many cells are expected to be collected, the serum concentration can be adjusted to 100% for dilution with the sheath fluid. Wash all cells after sort and replenish with fresh media prior to placing back into culture or other experimental post sort design.