

Practical Cell Sorting - A Guide for Investigators

Adapted from “Practical issues in high-speed cell sorting”, by Larry Arnold and Joanne Lannigan, Current Protocols in Cytometry. 2010 Jan;Chapter 1:Unit 1.24.1-30.

As you embark on your cell sorting experiments, please review these important considerations

The following is a discussion of best practices for investigators planning experiments that involved flow sorting of cells:

1. Cell preparation
2. Post sort viability
3. Speed
4. How many cells should I bring?
5. Do you need very high purity of cells?
6. Resolution of populations
7. Cell capture Media
8. Sterility – use of antibiotics
9. Post sort analysis
10. Sorting into 96 well plates

First, we strongly recommend that you do some preliminary experiments on the analyzers to work out the staining and cell preparation procedures rather than doing this on the more expensive sorters. A final test run on the sorter is also recommended to confirm that we can see the same population resolution.

1. Cell Preparation:

Sorting, as with all flow cytometry, is only as good as the cell preparation. Of course, the main goal is to prepare a sample that contains as many single cells as possible.

Doublets or higher order clumps will either be detected and thrown out by the cytometer, or will confuse the instrument and result in sort mistakes (appear as reduced purity). Cells should be filtered through a nylon mesh (Nytex) 30-100um mesh at each step to continually remove clumps that tend to grow larger otherwise. Improper procedures following this can lead to gross reformation of clumps. Generally this happens during centrifugations to wash cells and can lead to tremendous loss of cells to mega-clumps. This can be avoided or minimized by following some general procedures. Use of round bottom tubes is preferred over conical/tapered bottom tubes. Use a relatively large diameter tube e.g. 17mm. Polypropylene is the best plastic to use with cells - avoid polystyrene. Centrifuge cells only as fast as necessary to pellet. Do not leave the cells in the pellet for a significant time - it is best to be at the centrifuge when it stops spinning and immediately remove tubes, decant supernatant and re-suspend cells (break pellet up BEFORE adding any additional fluid/media).

Cell viability is a critical issue as most investigators are interested in sorting live cells and even when the end application of the sorted cells may not need live cells - e.g DNA and RNA analysis. Cell preparation to produce single viable cells and reduce clumping and dead cells requires proper methods and depends on the type of cell being studied. Cells which are not independent of each other, not only frankly clumped but non-independent in other ways will decrease cell recovery during sorting.

Cell isolation: Tissue disaggregation and removal of cells from plastic usually requires enzymatic treatment including proteases and collagenase and the best procedure for a tissue may have to be determined by the investigator. For cells that adhere strongly to plastic there are low adhesion types of culture vessels available. Strong proteases such as trypsin can be used but can lead to removal of protein markers from the cell surface

which might destroy antigenic sites for binding of marker antibodies. If using trypsin, it is best to use a trypsin inhibitor instead of neutralizing with serum. Adding the serum back can cause the cells to adhere to one another.

A major culprit in cell clumping is DNA released from ruptured cells. **DNase should be included** in the cell preparation and FACS staining media to reduce cell aggregation and increase purity and yield. Phoenix Flow Systems offer 2 products that you may find useful - Accutase (for removing adherent cells in culture and solid tissue disaggregation) and Accumax for breaking up/preventing clumps.

Pre-sort viability: The media used in the preparation of cells can also make a big difference in the viability of the cells. Using HBSS (Hank's Balanced Salt Solution) or a culture medium e.g. RPMI-1640 without phenol red supplemented with 2% BSA or FBS will increase cell health, but may promote reestablishment of cell to cell interactions with adherent cells, decreasing purity and yield. A FACS staining buffer such as PBS containing EDTA and BSA or FBS will maintain better cell suspension but is not ideal for long term culture. Cells will generally do best if prepared at 4°C and kept cold during the sort.

2. Post-sort Cell viability:

Cells will differ in their ability to survive sorting. Lymphocytes are at one extreme and easily handle being sorted with a 70um nozzle and a sheath pressure of 60psi. We have also had good results sorting murine hematopoietic stem cells under these conditions. However, investigators should consider this in planning experiments and may need to do preliminary experiments to establish optimal conditions for their cells. To counter the viability effects of sorting we can use larger diameter nozzle tips and/or lower pressures. Investigators should discuss these issues with the Facility staff. Of course, to have good viability of the sorted cells one needs good viability of the input cells. This will be affected by the cell preparation discussed above. It is highly recommended to include a **viability marker** so that we do not attempt to sort dead cells. A wide range of viability dyes are available with emission spectra comparable to most fluorochromes; EMA, 7-AAD, LiveDead etc. and we can work with you to select the optimal reagent for your needs.

3. Speed - How fast can I go?

Even though all our sorters are considered "high speed", as are all sorters now manufactured, the actual number of cells that can be sorted per second (actually number of input cells per sec) depends on a number of factors. High speed sorters are not high speed because we can put more cell volume through per second. They are high speed because of the characteristics of the electronics and because they can use higher sheath pressure to attain higher droplet rates which permit a higher number of cells per second to be processed. However, we cannot achieve the higher cell throughput rates by pushing a higher volume of sample through per second. To achieve higher cell input rates the cells must be at higher cell concentrations. Not all cells can handle these concentrations and so cannot be sorted as fast. Also larger cells must be sorted using a larger nozzle at lower sheath pressures and, thus, generate fewer drops /sec. The nature of the experiment can also dictate the speed. If sorting rare cells and 'enrich mode' will suffice, then higher speeds may be attainable. If high purity is the goal then lower speeds must be used.

4. How many cells should I bring?

The number of cells you need to bring is primarily determined by your experimental needs of how many sorted cells you need to obtain. Keep in mind there are always losses in any purification - be it sorting or other. Also keep in mind purity. If you want high purity the recovery will be less or the sort slower. A good estimate is to determine the number of cells you need to end up with, divide this by the estimated sort efficiency, and multiply by at least 2. Also remember you must actually bring us this number of cells. There are often losses in the cell preparation and these should also be factored in. Also remember that we will consume some cells in setting up the sort. If this is the first time you are sorting this particular sample we may need more cells initially than we will in subsequent sorts. As always we strongly recommend that you do some preliminary experiments on the

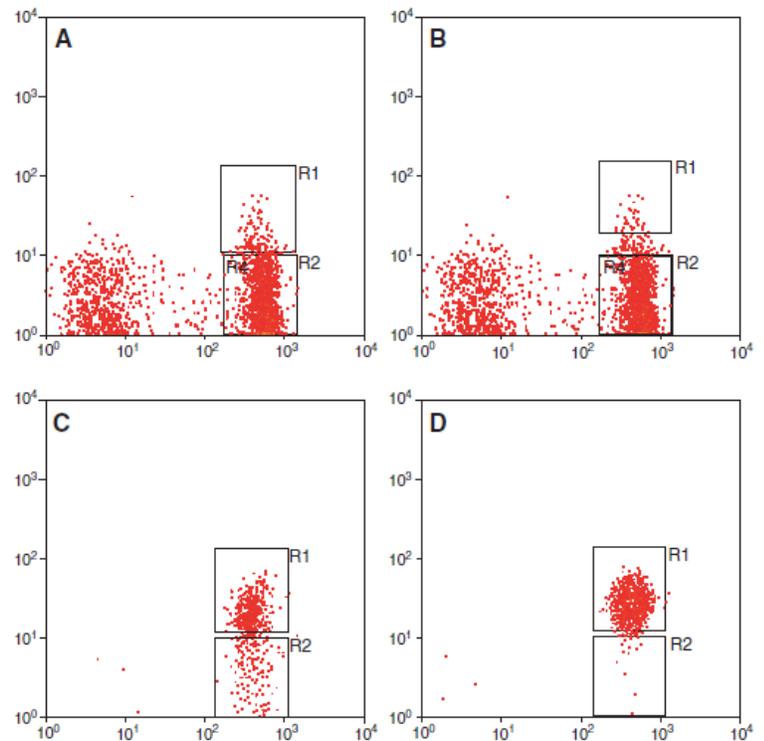
analyzers to work out the staining and cell preparation procedures rather than doing this on the more expensive sorters. A final test run on the sorter is also recommended to confirm that we can see, on the slightly less sensitive sorters, the same population resolution.

5. Do you need very high purity sorted cells?

If you need very high purity sorted cells and especially if the frequency of the wanted cell in the starting sample is low we recommend you consider a two-step sorting strategy. In this approach we will sort the cells at as high a speed as possible but will use an enrich mode approach. This will enable us to capture as many wanted cells as possible as quickly as possible but with a somewhat lower purity (enrich mode does not abort sort events that contain unwanted coincident cells).

6. Resolution of populations.

Populations that are dim and only minimally separated from a slightly dimmer (or "negative") population present problems. Measurements in flow cytometry are based essentially on probabilities that derive at a number of points - number of photons released from the fluorochromes, number of photons captured into the PMT, and especially photoelectron statistics (number of photoelectrons generated when the photocathode is illuminated by photons). When all of the above are at low levels the errors (standard deviations) involved can be relatively large and, thus, what the user sees as a negative population when a few cells are analyzed can blur and extend into the dim positive population (or vice versa) when millions are collected. Thus, when a cell goes through the detector and is measured in the sort region that defines the dim "positive" population it may actually be a cell that is at the high end of the negative population distribution. When that cell is reanalyzed after the sort it may be in the negative distribution and, thus, would appear as a sort error. In order to sort dim populations from negatives at high purity one must compromise yield and set very conservative sort regions. If your sort falls in the category described here, we will discuss with you the best approach for sorting.



The dot plots demonstrate the issue with sorting dim populations from negative populations. Panels A and B show the data from the starting sample. Panel A shows the position of R1 that tries to encompass the majority of what appears to be the positive population. Panel B shows a more conservative position for sort region R1 to emphasize purity. Panel C shows the sorting result using panel A R1 sort region and panel D shows the sort result using the panel B R1 sort region. In panel C, the sort result is heavily contaminated with negatives while in panel D the sort result is much more pure for the positive population. Arnold LW, et al. *Current Protocols in Cytometry*, January 2010

7. Cell-capture media

You must provide tubes for collecting the sorted cells. Depending on the number of sorted cells expected, provide either 1ml eppendorf, 5ml FACS or 12 x 75mm round bottom polypropylene tubes. Also bring media to place into the capture tubes. Usually, the sorted cells will be mostly in sheath fluid (PBS-like) so a more suitable media to collect into is desirable depending on the application. The media should be buffered with a non-CO₂ based buffer (e.g. HEPES) to maintain pH. The media should contain serum or BSA at an initially high concentration (e.g. 50%) that will get diluted as the tube fills with sorted cells. The volume of media

placed in the tube depends on the sensitivity of the cells to being in sheath fluid. How many tubes do I need to bring? A rule of thumb is that with 1 ml of capture fluid we can put 3 million sorted drops into a 12 x 75mm tube (70um tip at 60psi). For a 100um tip we can put about 1 million sorted droplets in the same tube.

8. Sterility - use of antibiotics.

While we thoroughly sterilize the sample delivery part of the sorter before each sort and sterilize the entire sheath fluid path once every week (sheath fluid goes through an in-line 0.2um filter as well) in our operating environment the potential for airborne contamination, which in our experience is very rare, is possible. We recommend that when culturing sorted cells you add antibiotics which include 50µg/ml gentamycin. You may leave pen-strep etc. in as well. If a long term culture is desired the gentamycin can be discontinued after about a week. We have done many successful sterile sorts where the investigators used no antibiotics following the sort but since your sorted cells are valuable we suggest prudence.

9. Post sort analysis.

We recommend that whenever possible, you permit us to analyze the sorted cells to determine the effectiveness of the sort. In cases, where the yield is extremely low we may waive this. This post-sort analysis is essential data if you expect to publish the results of your sort experiment.

10. Sorting into microwell plates.

Sorting into microwell plates for cloning or PCR analysis is frequently requested. We will work with you to optimize your system for this. Both the biocontained FACS Aria and the MoFlo XDP system can accommodate 6, 12, 24, 48, and 96 well plates. When sorting cells for subsequent culture we recommend that the media you place in the well to capture the cell be buffered with a non-CO₂ based buffer e.g. HEPES. If not the media will become very alkaline and this may be detrimental to your cells.