UNC Flow Cytometry Core Facility
Practical Cell Sorting

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. How long will it take?
7. Resolution of populations
8. Cell capture Media
9. Sterility – use of antibiotics
10. Consecutive sorts of cell lines
11. Using other sheath fluids
12. Post sort analysis
13. 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 prior to doing this on the more expensive sorters.

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). The sorters are fairly good at detecting the clumps but one presumably does not want to throw away a significant number of cells due to clumps. Cells should be filtered through a nylon mesh (Nytex) 30-100um mesh at each step to continual remove clumps that tend to grow larger otherwise. (Sysmex CellTrics makes single-use, sterile filters in a variety of sizes suitable for cell filtering). 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. Always use round bottom tubes when washing cells - NEVER conical/tapered bottom tubes for centrifugation. 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 (never suck off fluid using a pipette) 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 - 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.

Lymphocytes are probably the easiest to obtain as single cells. Lymph nodes and spleen are best dissociated using frosted (real etched not painted) glass slides. The tissue is placed on the wetted frosted part of the slide and the frosted area of another slide is opposed to the tissue. Gentle pressure and circular rubbing motion will rupture the tissue and release the cells. Gentle up and down pipetting using a glass pasture pipette will release more cells from the clumps. The remaining clumps can be separated by differential settling and the cell suspension - mostly single cells - removed with a pipette.

Other cell types, including adherent cultured cells, can be more difficult. 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**.

A major culprit in cell clumping is DNA released from ruptured cells. **Inclusion of DNase** in the cell media may help substantially in reducing clumping that results from genomic DNA in solution that is released from lysed/dying cells during cell preparation.

When trypsinizing adherent cell lines for flow cytometry it is recommended to quench the trypsin using soybean trypsin inhibitor. Another reagent for preparing adherent cells for flow cytometry which you may find useful - Accutase (for removing adherent cells in culture and solid tissue disaggreagation) and Accumax for breaking up/preventing clumps. Also, if working with solid tissue then refer to Worthington's excellent guide on dissociation.

Pre-sort viability: The media used in the preparation of cells can also make a big difference in the viability of the cells. **We usually recommend that you not use PBS (or PBS with BSA/FBS) as we find cells have limited viability under sorting conditions.** Rather than PBS we recommend HBSS (Hank's Balanced Salt Solution)

or a culture medium e.g. RPMI-1640. If possible get it **without the phenol red**. Using 2% BSA or FBS is also recommended. Cells will generally do best if prepared at 4°C and kept cold during the sort. For mouse lymphocytes we recommend RPMI-1640 but without phenol red, biotin (if using biotinylated antibodies), NADH, and flavins (made by Hyclone).

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 70psi. Note: Some facilities have reported lowered "functional" viability at 70psi and use 45-50psi. We have also had good results sorting murine hematopoeitic 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. At the other end of the spectrum are insect cells. These cells seem to not like to be sorted but we have been successful with several insect cell lines and ex vivo 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. Propidium iodide (PI) is the time honored method for this. PI is included in the final suspension media (for many cell types 0.5ug/ml final concentration works quite well). PI can enter cells with damaged membranes but not those with intact membranes and label the DNA brightly. Dead cells can then be excluded from the analysis/sort. The PI does not generally interfere with measuring markers of a color that overlaps with the PI emission since we are excluding these cells. However, the PI must be brighter than at least one of the detection reagents in the channels in which the PI is detected (PE, PE-TXRed, PerCP, or PE-Cy5/Cy5.5). Other viability dyes are available, e.g. 7-AAD and Live/Dead Violet. An advantage of the Live/Dead Violet (there is also a UV-excited version) is that it is fixable so one can discriminate live and dead cells following fixation.

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, as discussed above, must be sorted using a larger nozzle and to get stable droplet formation these must operate 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. When you arrive and tell us you brought 1 x 10^7 cells this should be the number you determined by counting after all cell preparation including filtration to remove clumps (if you do the filtration) or an estimate if we will do the filtering. The sorters are very good at counting the cells that go through the instrument so if you tell us you brought 1 x 10^7 cells and the sorter

sees only 6.5 x 10^6 you did not delivery us 1 x 10^7. 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 look for and, thus, does not abort sort events that contain unwanted coincident cells).

6. How long will it take?

The sort efficiency table and formula allow you to make an estimate of how long the sort will take. Again keep in mind this is a best case estimate and the actual sort will likely take a bit longer. Also keep in mind there will be setup time required once we have your cells in hand and if compensation is involved this will also add time to run the compensation controls and set the compensation. A half hour is a good estimate for setup except for simple sorting setups, e.g. 1-color GFP sort.

7. 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.

8. Cell-capture media

You must provide tubes for collecting the sorted cells. For collecting cells when sorting with the Aria provide 12 x 75mm 5 ml tubes, 15 x 120mm 15 ml **conical** tubes, or 1.5 ml

tubes. Bring as many cells as needed and have collection media aliquoted into tube prior to arrival. The Aria can also sort into a variety of cell culture plates, such as 96-well, 12-well, 384-well, etc. Usually, the sorted cells will be mostly in PBS 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. 10 - 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 PBS. How many tubes do I need to bring? A rule of thumb is that with 1 ml of capture fluid in a 5 ml tube we can put 3 million sorted drops (when using the 70 um tip). When using the 100 um tip we can put about 1 million sorted droplets in the same tube.

9. 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 2 weeks (sheath fluid goes through an in-line 0.2um filter as well) our operating environment is not well controlled and airborne contaminants, which in our experience are very rare, are possible. The Aria II is operated at all times under containment in a laminar flow hood, and the Aria III may as well. We recommend (essentially require) that when culturing sorted cells you add antibiotics which include 50µg/ml gentamycin (available from the Cancer Center Tissue Culture Facility). 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 rather valuable we suggest prudence.

10. Consecutive sorts of cell lines.

We are frequently asked to do sorts, in a single session, of multiple cell lines - e.g. recombinant DNA transfected cell lines. We make every effort to make sure that subsequent cell lines are not contaminated by prior samples. We will vigorously backflush and sterilize the sample line to prevent this, however, investigators should recognize that the possibility of cross contamination cannot be eliminated. It is the investigator's responsibility to confirm that contamination has not occurred. However, we have never had a documented case of this happening. We will require about 10 minutes between consecutive sorts to complete this process - i.e. 50 minutes for 6 cell lines. Please allow for this time in scheduling sorts like this.

11. Using other sheath fluids.

If you have an application where cells are extremely sensitive to PBS, we can operate the instrument using special sheath fluid. We will ask you to provide the fluid and will consult with you about what is permissible. We will have to charge an additional fee to perform this service as we must setup the instrument and remove the fluid completely following the sort, especially if it contains a carbohydrate e.g. glucose, so that microbial growth in the system does not occur.

12. 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!

13. 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. All sorters can accommodate 6, 12, 24, 48, 96, and 384 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.

Updated, July 2018