Cell Sorting Considerations

Successful cell sorting mainly depends on the quality of the input sample and there are many factors to take into consideration during sample preparation. Please familiarize yourself with the following recommendations in case you are a new user.

Safety Issues

The high instrument operating pressures during cell sorting can produce particle aerosols, and the lack of any form of containment means that both the operator and surrounding environment are exposed to these aerosols. We will need information such as from which species your cells originate; into what biohazard category do they fall or whether the sample contains a chemical hazard. Prior to sorting, it is therefore mandatory to provide both chemical and biological hazard information of your sample. This you do by filling in the booking form in the PPMS software.

Cell Type

During sorting, cells are passed under high pressure through a narrow orifice. Larger cells will require a larger orifice, and particularly fragile cells may not survive higher pressures. Both orifice size and pressure affect the rate at which cells can be sorted, and hence the duration of your sort.

Table I: Approximate cell sorting values correlated with the FACSAria Nozzle size.

	Harsh	→	Gentle	
	Fast	←	Slow	
NOZZLE SIZE	**70 µm**	**85 µm**	**100 µm***	**Notes and explanation of correlations between different parameters**
Pressure	70 psi	45 psi	20 psi	Provide stable stream conditions for the certain nozzle size
Frequency	87 kHz	42 kHz	22 kHz	
Maximum cell size permitted for sorting	14 µm	17 µm	20 µm	~ 1/5 of the nozzle size (to prevent clogging, control sheer forces, enhance stream stability)
Cell Type	Non-activated T-cells, B-cells, platelets, bacteria, yeast	Activated T-cells, NK T cells, NK cells, Monocytes, mDCs, pDCs	Cell culture cells	Corresponding to cell size
Number of drops generated (per second)	87 000	42 000	22 000	Equals frequency
Maximum cell throughput (per second)	17 400	8 400	4 400	~ 1/5 of number drops (to accomplish reasonably high sorting yield)
Maximum cells throughput (per hour)	79×10^6	38×10^6	20×10^6	Maximum cell throughput (cells/sec) x 3600 sec/hour
Cell concentration of the sample needed to reach the maximum throughput	20-30 x 10^6/ml	10-15 x 10^6/ml	5-7 x 10^6/ml	Practical observation
Approximate size of the drop	1 nl	2 nl	4 nl	Practical observation (depends on both - nozzle size and frequency)
Cell concentration of the post-sort	1 x 10^6/ml	0.5 x 10^6/ml	0.25 x 10^6/ml	Equals number of drops to collect one milliliter of the post-sort sample

* To be used for single cell sort into plates

Time Considerations

The speed at which cell sorting can take place depends on the type of cells to be sorted. As a general rule: the larger and more fragile a cell the slower the sort rate. The following table outlines approximately how long it will take to collect a given number of cells in relation to how frequent the cell of interest occurs in the total population.

Table II: Calculation of time to reach the required number of particles.

Time needed to sort required number of desired particles (10.000 cells per second)					
Required number of desired particles	**Desired particles as percent of total particles**				
	0,1%	1%	5%	10%	50%
10^3	2 min	10 s	2 s	1 s	0,2 s
10^4	17 min	2 min	20 s	10 s	2 s
10^5	47 min	17 min	3 min	2 min	20 s
10^6	2 h	47 min	33 min	17 min	3 min
10^7	11d	27 h	5 h	47 min	33 min
10^8	115 d	11 d	2 d	1 d	5 h

How Many Cells Do You Need?

Before performing an experiment you need to calculate how many target cells you need and therefore how many cells you have to bring. After having measured the percentage of your cells of interest within the total cell population at any cytometer or other detection system, you need to take further factors into consideration.

Cell loss during preparation and sorting

During sorting there is a process termed 'sample aborts' which is necessary to achieve good sort purity, but causes cells of interest to be lost. Sample aborts usually result in loss of 10% of the total cell number in well prepared samples, but can be as high as 20% in samples which contain more cell doublets, where the cell population to be sorted is low (<5%), or at higher sort speeds. Cells will be lost during the labelling/staining process. What you start off with is not what you will have after labelling, no matter how careful you are. Assume that at each washing step you will lose 10% of your total cells. Once all labelling steps are complete, samples will need to be filtered immediately before sorting to remove clumps.

Sample Preparation

It is essential that a single cell suspension is maintained at all times during sample preparation. Please avoid any mechanical detachment of cells; if possible, use an enzymatic detachment method or incubate in Ca^{2+}/Mg^{2+} free media. Re-suspending cells in Ca^{2+}/Mg^{2+} free media containing 0.1 mm EDTA during sample preparation can help prevent the formation of clumps.

Filtering

In order to ensure removal of any larger clumps and to minimize the possibility of nozzle clogs, sample need to be filtered through a special filter of 35-50 µm pore size. If this is not performed, and your sample contains clumps, it will block the machine and it will not be possible to sort the sample. After filtering, the cells should be kept on ice and protected from light.

Cell Concentration

Samples should be provided at a total cell concentration of approximately 1-2 x 10^7 cells/ml. If only low cell numbers are available (fewer than 5 x 10^6 cells) a minimum volume of 300 µl should be used.

Staining Controls

Staining controls are important to define the background fluorescence of the experimental samples and to set the baseline PMT (photomultiplier tube) voltages of the instrument. Please always bring along cells which do not express your antigen, e.g. non transfected or better mock-transfected cells. Concerning surface staining's, always bring along unstained cells. In case of a multicolor panel (up to 18 colors) or spectral overlap of fluorochromes, please bring compensation controls: Single stainings for every labelled antibody and an unstained control.

Pre-Sort Buffer

A basic Pre-Sort buffer recipe, which has been formulated to minimize cell clumping and enables consistent drop formation, is given below. This Pre-Sort buffer contains no phenol red and is an optically clear buffer to help minimize background. The buffer has minimal calcium and magnesium to minimize cell aggregation as these cations are necessary cofactors for many cell adhesion molecules. It contains FBS or BSA to help maintain cells in a viable state during cell sorting applications. The addition of 25 mM HEPES helps to maintain the pH of the sample at 7.0 - 8.0 during the sorting process.

1x PBS (Ca^{2+}/Mg^{2+} free)
0.5 - 1 mM EDTA
25 mM HEPES pH 7.0
1% Fetal Bovine Serum (heat-inactivated) or BSA
0.2 µm filtered, store at 4 °C

The buffer recipe may need further optimization depending on the cell type and the aim of your experiment. Suggestions for cell type specific buffer modifications are listed below.

Sticky Cells

If dealing with sticky (e.g. macrophages/monocytes/activated cells) raise the concentration of EDTA to 5 mM. The addition of the EDTA acts to chelate Ca/Mg ions and helps to reduce cation-dependent cell adhesion. Use FBS that has been dialyzed against Ca/Mg free PBS or use 1% BSA instead of non-dialyzed FBS, as it facilitates cell-cell adhesion by replacing Ca and Mg, respectively. This can also be used to prevent adherent cells from sticking to tubing.

Adherent Cells

Typically, trypsin (or other detachment enzyme) reaction is stopped by the addition of culture media or PBS containing FBS. This is problematic because it reintroduces the cations that facilitate the cells reattaching to plastic and can cause cells to re-aggregate before sorting. Better use a cation-free FBS buffer in order to stop the enzyme reaction. Additionally, the level of EDTA can be increased to 5mM.

NOTE: Too much EDTA can kill your cells. Accutase and Accumax are cell dissociation products that can aid in maintaining single cell suspensions.

Samples with a High Dead Cell concentration

Samples with large amounts of dead cells and/or debris must be cleaned before use as a large number of dead cells in the preparation will lead to an increase in the amount of soluble DNA in the solution which will coat cells, leading to profound clumping.

The addition of DNAase II (10 U/ml) to the buffer solution can help eliminate the clumping due to free DNA.

Adding DNAse I in the presence of $MgCl_2$ will help reduce the aggregation. Treat cells for 15-30 minutes in a sterile solution of 100 µg/mL DNAse and 5 mM $MgCl_2$ in HBSS at room temp. Wash the cells 1x in HBSS containing 5mM $MgCl_2$. Re-suspend the cells in HBSS containing 25-50 µg/mL DNAse, plus at least 1 mM $MgCl_2$ prior to and during the sort.

Sample Tubes and Collection Media

It is highly recommended that tubes used as sample tubes are made out of polystyrene PS (clear), while those for sample collection be made out of polypropylene PP (opaque). These tubes differ in their electrostatic properties. PP tubes are recommended when handling fast adhering cells.

Collection Tubes

Your cells can be sorted in 15 ml conical tubes (2 way sort), 12x75mm standard FACS tubes (2 way or 4 way sort) or 1.5 ml microtubes (4 way sort). The ACDU enables sorting into standard multiwell plates or microscopy slides. Alternatively, the sorting device (plate or slide) can be customized by the software.

Collection Media

Your tubes or plates should contain some media containing 10-20% FCS in order to keep the cells vital. You can also use 1x PBS supplemented with FCS. For single cell sorting into 96-well plates (cell line generation), fill each well with 200 µl of complete media or as discussed during project consultation. The volume of the droplet containing the cell will not affect the total volume in the well.