User Guidelines for Cell Sorting

Booking regulations
An account in the PPMS booking portal is mandatory. Before your very first sorting, you should fill out the link: User Sheet Sorter and come to see us for a project discussion in order to ensure correct sample preparation, sample concentration, sorting devices, sterility, etc. (also test sorts can be agreed on). After clearance of the project you are free to book the FACS AriaIIIu.

Test your expression level or transfection efficiency prior to sorting (highly recommended)
You should test your transfection efficiency or expression on an analyzer first and please bare in mind that fluorescence microscopy is often not comparable to flow cytometry. It takes a lot of time to set up the sorter and it's a waste of your time and our time and your money if you realize on the sorter that your transfection did not work out.

Sample preparation
Please avoid any mechanical detachment. If possible, use an enzymatic detachment method or incubate in Calcium/Magnesium-free media. It is essential that the cells do not clump, as this will immediately clog the cell sorter. Therefore be sure to add EDTA to your sorting buffer as mentioned below. The cells need to be filtered, either with cell strainers for 50 ml tubes or a cell strainer cap, which are suitable for standard FACS tubes.
Bring your cells in 15 ml tubes or 12x75 mm standard FACS tubes (preferably polypropylene tubes if your cells attach easily).

Filtering
Falcon® 12x75mm, 5 ml polystyrene tube with cell strainer cap (35 µm)
sterile, 25/pack, 500/case
Product #352235

If you do not bring filtered cells, we will filter them for you at the facility and bill the cell strainer tubes after the sorting.

Sorting buffer
Usage of 1x PBS alone is not recommended. If pH-stability is crucial, supplement the PBS with HEPES. If you use cell culture media, it should not contain any phenol red. The sample solution should also contain some kind of protein like BSA or heat-inactivated fetal calf serum (FCS) to a final concentration of 1-5% (v/v).

Some basic recipes for sorting buffers are given below. However, they may need further optimization dependent on the cell type.
1x PBS (Ca^{2+}/Mg^{2+} free), 0.5 - 2 mM EDTA, 1-2% FCS, 0.2 µm filtered, store at 4 °C
1x PBS (Ca^{2+}/Mg^{2+} free), 1 mM EDTA, 25 mM HEPES pH 7.0, 1% FCS (heat-inactivated) or BSA, 0.2 µm filtered, store at 4 °C.

If you have extensive cell death in the sample, add DNAse. It will not only decrease clump formation in the sample, but also will decrease viscosity of the single cell suspension buffer, by digestion of the free DNA. Sort purity and yield could go substantially up after DNAase treatment.

Add 10-25mM of HEPES, pH range 6.8 - 8.2 (for example: HEPES buffer solution 1M in H_2O, Sigma-Aldrich Co., Cat# 83264-100ML-F) to your buffer. Addition of HEPES will significantly increase the buffer capacity of the original sample buffer. Buffer capacity of the common phosphate and carbonate buffers gets compromised by high pressure within the instrument during the cell sorting procedure.

Buffer Suggestions for the Cell Sorting

Purpose and Details	Components	Basic Cell Sorting Buffer	Variations of the Basic Cell Sorting			Cell Sorting Buffer for Clean Lymphoid Cells
			For Sticky Cells	For Adherent Cells	For Samples with a Lot of Dead Cells	
Increase buffer capacity and stability	1x Phosphate Buffered Saline (DPBS, Ca/Mg++ free), pH 7.0-7.4	√	√	√	√	
	HEPES pH 7.0 (10-25 mM)	√	√	√	√	
	HBSS, with Calcium/Magnesium, no Phenol Red					√
Chelating agent to prevent cell aggregation	EDTA (~ 5mM or 0.5%)		√	√		
Protein to maintain cell viability	BSA (Bovine Serum Albumin, 0.1-1%) or cation-free-FBS or FCS (dialyzed against Ca/Mg++ free DPBS, 1-5%)	√	√	√	√	
	FBS-HI (Fetal Bovine Serum Heat-Inactivated, 1%)					√
Digestion of free DNA	DNAse-I (RNAse free; 10U/ml or 25-50ug/ml); add $MgCl_2$ (1-5mM)				√	
Sterility	Sterilize by 0.2um filtration, store at 4°C	√	√	√	√	√
Additional Notes				Use cation-free FBS in order to stop the trypsin reaction	Keep DNAse in all the buffers through the sample preparation	

Controls

Negative control, mandatory

Please always bring along cells that do not express your antigen, e.g. non-transfected or better mock-transfected cells. Concerning surface stainings, always bring along unstained cells otherwise we are not able to set the cutoff appropriately.

Compensation controls for multicolor setup (if you use your panel the first time)

Single stainings for every antibody if you are using fluorophores with overlapping emission spectra

- Provide **compensation controls for multicolor setup:**
  - If **cells** are used as single color controls
    - Unstained cells
    - Titration of the Dead Cell Exclusion dye on the "partially killed" cells
    - All separate single color stained cell controls
      - Preferable concentration of the controls is around 1 x 10^6cells/ml, volume 0.5-1.0 ml
  - If **beads** are used as single color controls
    - Unstained Cells
      - Preferable concentration of unstained cell control is around 1 x 10^6cells/ml, volume 0.5-1.0 ml
    - Unstained beads
    - All separate single color stained bead controls
- Provide **gating controls** - Fluorescent Minus One (FMO) controls, as proper way to

evaluate the background in certain channel and set sorting gates appropriately

FACS AriaIIIu nozzle size (will be chosen by the staff)

Nozzle size	Cell Type	Sheath pressure	Sort cells / s
70 µm	non-activated T cell, B cells, platelets, bacteria, yeast	70 psi	22.000
85 µm	activated T cells, plasma cells, NK T cells, NK cells, monocytes, mDC, pDC, stem cells	45 psi	12.000
100 µm	tissue cells in general, neurons, macrophages, tissue DC, stem cells, cell lines	20 psi	5.000

Cell concentration

Adaption of cell concentration is essential for sortings into tubes and if you aim for higher cell numbers. Otherwise, the sorting will take significantly longer than necessary.

Nozzle size	Cell Type	Concentration
70 µm	Lymphocytes, small cells	7.5 -12 x 10^6 cells/ml
85-100 µm	<15 µm in diameter, cell lines	5 - 7.5 x 10^6 cells/ml

Collection tubes

Your cells can be sorted in 15 ml 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.

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. As an example, if you use a 15 ml tube, prepare a tube with 5 ml media.

For single cell sorting into 96-well plates (cell line generation), fill each well with 200 µl of complete media. The volume of the droplet containing the cell will not affect the total volume in the well.

- Bulk Sort - Provide collection tubes for the sorted cells. Tube size depends on the expected number of post-sorted cells. *Note: Concentration of post-sorted cells is between 0.3 - 1.0 x 10^6cells/ml and depends on the nozzle size*
  - Tube types:
    - 12 x 75 mm Tubes (up to 4-way sort)
      - Polypropylene - best choice
        - sterile with snap cap (Cat#4002053, Corning)
      - Polystyrene - poorer choice, but still possible
    - 15 ml Tubes - any type (up to 2-way sort)
  - Tube pre-treatment:
    - In order to prevent cells sticking to the sides of the tubes, pre-coat the tubes, filling them with 10x BSA solution (1%)
    - Keep filled tubes inverted for at least 30' prior sort
  - Solution to sort into:
    - TRIzol LS reagent (for RNA extraction. Volume of TRIzol LS:Cells=3:1. For example, 750 µl of TRIzol LS + 250 µl of sorted cells)
    - PBS-based buffer
    - PBS/HEPES/BSA (see buffer suggestions)
    - Any specific solution (for example, PCR mix)
    - none
  - Tube special characteristics depend on the sorting purpose:
    - For sterile sort tubes should be also sterile
    - For RNA work tubes should be RNAse free

- For Western blot tubes should not be treated with external protein

Sort using ACDU (automated cell deposition unit):

- Receptacles:
  - Multiwell plates:
    - 96 well plate
    - 24 well plate
  - Microscope slides

- Solution to sort into - depends on the purpose of the sort:
  - Complete medium for cell growth
  - TRIzol LS reagent (for RNA extraction)
  - PCR mix
  - Etc.
- Sort setup specifics:
  - Day before the sort FCF-Berg Staff should be provided with several empty plates (or other receptacles) of the type planned to be used for the sort

Plates (or other receptacles) for the cell sort should be brought to FCF Berg at the time specified by FCF-Berg Staff.

Time considerations

Time needed to sort required number of desired particle (10.000 cells per second using 70 µm nozzle)					
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	27 h	47 min	33 min	17 min	3 min
10^7	11 d	27 h	5 h	47 min	33 min
10^8	115 d	11 d	2 d	1 d	5 h

Note: If using the 100 µm nozzle the time is doubled (max. 5000 cells per second).

Approximate Cell Sorting Values Correlated with the Nozzle Size

Gentle ← *Harsh*

Slow → *Fast*

NOZZLE SIZE	100 um	85 um	70 um	*Notes and explanation of correlations between different parameters*
Pressure	20 psi	45 psi	70 psi	*Provide stable stream conditions for the certain nozzle size*
Frequency	22 kHz	42 kHz	87 kHz	
Maximum cell size permitted for sorting	20 um	17 um	14 um	*~ 1/5 of the nozzle size (to prevent clogging, control sheer forces, enhance stream stability)*
Number of drops generated (per second)	22,000	42,000	87,000	*Equals frequency*
Maximum cell throughput (per second)	5,500	10,500	22,000	*~ 1/4 of number drops (to accomplish reasonably high sorting yeild)*
Maximum cells throughput (per hour)	20×10^6	38×10^6	79×10^6	*Maximum cell throughput (cells/sec) x 3600 sec/hour*
Cell concentration of the sample needed to reach the maximum throughput	$5\text{-}7 \times 10^6$/ml	$10\text{-}15 \times 10^6$/ml	$20\text{-}30 \times 10^6$/ml	*Practical observation*
Approximate size of the drop	4 nl	2 nl	1 nl	*Practical observation (depends on both - nozzle size and frequency)*
Cell concentration of the post-sort	0.25×10^6/ml	0.5×10^6/ml	1×10^6/ml	*Equals number of drops to collect one milliliter of the post-sort sample*