Dear users, we got inspired by our colleagues from cytometric facilities across the world and put together few **recommendations to consider** during sample preparation for cell sorting (but also for cytometric analysis). To avoid cell aggregation, to decrease autofluorescence and to improve cell viability as much as possible are the main goals of these recommendations. All these factors also improve sort purity and especially post-sort cellular viability and resolution in case of cell analysis and protects machines from clogging.

Recommendations:

Buffer Suggestions

Basic Sorting Buffer

1 x Phosphate Buffered Saline (PBS) or Hanks Balanced Salt Solution (HBSS) (Ca2+/ Mg2+ Free)

1mM EDTA

25 mM HEPES pH7.0

1% Fetal Calf Serum (Heat inactivated) or 1% Albumin

- Filter sterilize using a 0.2 µM filter

- Store at 4 degrees.

Note:

Addition of 10-25mM HEPES improves pH stability, especially for long term sorts (over 10min). Sorter is not equipped by controlled atmosphere. Important to consider, higher pressure used during sorting is also changing buffer capacity of used media.

Culture media

Possible to use for sorting, recommended to avoid phenol red since it has quite significant autofluorescence, to avoid higher contain of FBS than 2%, since the FBS re-introduces cations that aid in attachment to plastic and can cause cells to re-aggregate.

In case of sticky cells, recommended to replace FBS by 1% BSA and 5mM EDTA.

To avoid aggregation

- Ca++/Mg++-free buffers are recommended. If needed, include 0.1-1% BSA or FBS at 1-2%. Better to use non-dialyzed FBS, as it facilitates cell-cell adhesion by replacing Ca and Mg.
- Samples based on adherent cells which has to be trypsinized and trypsin neutralized afterwards by FBS - add EDTA at 2-5mM and 1% Accutase into sorting buffer to eliminate FBS introduced cations.
- Samples isolated from tissues which might suffer by increased cell lysis - add 25-50 ug/mL DNAseI with 5mM $MgCl_2$ (NO EDTA than). This digests free DNA released by dead cells and helps to avoid cell 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 1mM MgCl2 prior to and during the sort. (5mM MgCl2 is optimal)

Single Cell Suspension

Filter immediately before sorting. Filters are available in the Flow Lab for charge (48.5CZK per piece) or use own (below are listed recommended and tested commercially available options). When processing tissue samples, pass cells through a 25-gauge needle. Keep the cell suspension at 1-10 million/mL during processing, depending on cell type.

Filter Information

> > 5mL tubes with 35um mesh caps, Fisher Scientific cat. # 0877123
>
> > CellTrics filters 50µm, Sysmex, cat. # 04-004-2327
>
> > nonsterile, recommended for LSRFortessa samples – cat. # UHELON 130T, Silk&Progress, spol.s.r.o. 42um nylon mesh cut in squares.

Dead Cell Discrimination

We recommend using a dead cell exclusion dye with any cell sorting experiment. This will help to reduce autofluorescence and will result in increased population resolution. There is a good selection of dyes for live samples, which will not affect cell physiology for post-sort functional assays (7-AAD, Calcein AM etc.). In our lab is Hoechst 33258 available.

Physical Manipulations

Centrifugation - Use minimal speed to sediment cells. A good starting point for most preparations is 300xg for 10 minutes.

Vortexing - Avoid intense vortexing.

Pelleting - Do not generate a dry pellet at any time during processing.

Air Bubbles - Avoid introducing air bubbles. Surface tension forces can kill cells.

Temperature - Keep samples on ice, unless otherwise required by a specific protocol. Slowing intracellular metabolism helps cells survive longer outside the incubator.

Compensation Controls

If using cells for single color compensation controls, bring unstained cells as the negative control for compensation. If using beads for compensation, include unstained beads for compensation (as an internal population or a separate tube) and bring unstained cells for an additional technical control.

Gating Controls

Always bring negative control with your samples, it's necessary for proper PMT voltage set up.

FMOs are highly recommended for multi-color assays.

Additional biological controls may be necessary for proper gating, including: • Positive Control • Mock Transfected with empty vector • Treated • Untreated Sample.

Collection

For better recovery - small numbers of cells should be collected into small tubes. Consider collecting directly into plates or lysis buffer when cell numbers are low.

For better viability - coat tubes with 100%FBS. Avoid empty collection tubes! In case the cells do not tolerate 100%FBS, use different proteins for coating.

Standard Formats of collection vessels:

- 12x75mm 5mL tubes (1-4 populations possible to sort simultaneously)
- 15mL tubes (1-2 populations simultaneously)
- Multi-well plates (1 population at the time): 6, 24, 48, 96, or 384 wells

Drop volumes depends on used nozzle. Important to consider the volume change when sorting 10^6 or more cells:

70 µm nozzle ⇒ ~1 nL (i.e. 1 x 10^6 cells result in ~1 mL volume change)

85 µm nozzle ⇒ ~2 nL

100 µm nozzle ⇒ ~4.5 nL

Delivery

Samples must be brought in leak-proof tubes and transported in a leakproof container.

Cells are typically sorted at concentration approximately 1-10 million per mL, depending on cell type. If there are fewer than 500 000 cells in a sample, resuspend in 300- 500uL.

The minimum sample volume for cell sorting should exceed 100 µL.

Bring extra sample buffer (5-15mL), FBS, collection buffer, and collection tubes as backups.

Bring extra ice for long sorts, if necessary.

Biosafety Pre-Approval

BD FACSAria Fusion sorter is using jet-in-air type of fluidics which by principle produce aerosol under higher pressure (app. 1.4 ATM – 5.4 ATM) what is very good way how to spread infectious agents into working space of machine and so to expose the operators. According to this, please kindly follow below listed safety instruction!

Prior to any sort requests, all sorting experiments must be discussed with sorter operators (Marziyya Askarzade and Ondrej Honc). **In our sorter (and analyzer also) only samples of BSL1 are allowed!** Any live primary human samples, cells from animals previously exposed to an infectious agent, or cultured cells exposed to potentially infectious agents must be reported to and discussed with cytometry lab operators!

Original resources:

https://cancer.wisc.edu/research/wp-content/uploads/2017/03/Flow_TechNotes_Sample-Preparation-Guidelines-for-Cell-Sorting_20170918.pdf

https://medicine.uiowa.edu/flowcytometry/protocolssample-prep/sample-preparation-sorting

https://www.molgen.mpg.de/4038862/cell-sorting-preparations

https://flowcytometry.utoronto.ca/wp-content/uploads/2016/01/Sorting_Buffers.pdf

The information from these sources were adapted to conditions at our lab.

We recommend to check out also:

https://expert.cheekyscientist.com/how-cell-culture-medium-can-decrease-cell-viability-during-a-flow-cytometry-cell-sorting-experiment/