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MEDICAL DISCOVERY INSTITUTE

Guide to Cell Sorting

Flow Cytometry Shared Resource

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1 Introduction

This document has been developed to address the questions that are most frequently asked prior to sorting. Many of these recommendations are generic, and it should be understood that some cell types would require optimization to achieve the best possible results.

2 Location

The Sanford Burnham Prebys Flow Cytometry Shared Resource cell sorting lab is **not** on the main SBP campus. You can find the sorting lab at this address:

10905 Road to the Cure, San Diego, CA 92121

Visitor parking is available in marked stalls in the parking lot. The building is access controlled. If you do not have cardkey access to the building call core staff at **858-646-3143**. We will come escort you to the lab.

2.1 Contact

Email: amyc@sbpdiscovery.org, yoav@sbpdiscovery.org
Phone: Lab 858-646-3143, Amy 858-646-3128, Yoav: 858-6446-3106

3 Sort Scheduling

3.1 Requesting a sort appointment via iLab

We schedule sorts in the order that we receive iLab sort requests. If you do not have an iLab account, register for one now at ilab.sbpdiscovery.org.

To create an iLab sort request, navigate to the SBP Flow Cytometry Shared Resource iLab page, click on the **Request Services** tab, then click on the **Sort Request** link that's highlighted in blue. On the right side of the page, click on the **Initiate Request** button.

Please complete all required fields in the sort request. This provides core staff with the information we need to determine how much time to allocate, and what settings to use for you proposed sort.

You can specify your scheduling needs in the sort request and we will do our best to accommodate them. We do not have an online sorting calendar.

3.2 How many cells do I need?

The number of cells that you should bring depends upon how many cells you want to recover for your downstream application. For instance, if you need to recover 100,000 GFP-positive cells for a western blot and 10% of your cells are GFP-positive, then of course you would need to start with no fewer than 1,000,000 cells.

Since sorting is not a perfectly efficient process, and sorting gates are usually more stringent than analysis gates, we recommend doing your calculations and then *doubling* the resulting number. Using the example above, only 1,000,000 cells are needed theoretically, but in practice it's better to bring at least 2,000,000 cells.

3.3 How long will my sort take?

Under ideal conditions we can pass $5x10^6$ to $3x10^7$ cells per hour through the system. The actual throughput will depend upon many things including the type and size of the cells, quality of the cell suspension, cell concentration, and rarity of the population to be sorted.

To reduce the likelihood of cross-contamination between samples, we wash with 10% bleach, followed by DI water after each sample. The wash steps add about 10 minutes per sample to the sort time. Thus if you have 6 samples we'll need to factor in about 50 minutes of wash time into your appointment.

Based upon the information you provide in your iLab sort request, core staff will estimate how much time we need to book for you sort. We generally add in a little bit of buffer time in case things take a little bit longer than expected.

If on the day of your sort you bring far fewer cells or samples than was indicated in your sort request, *you may be billed for the entire scheduled time*. Similarly if you bring more cells than indicated, or if there is something about the samples that requires us to run slower than expected *we may not be able to run all of your samples to completion in the allotted time*. Your sort operator will let you know if this seems likely so you can decide how to allocate the remaining sort time.

4 Sample Preparation

Good sample preparation plays a large role in the success of a sorting experiment. The best sorts will be achieved with cells that are ***highly viable***, in a ***single-cell suspension without clumps***, and in a buffer that maintains the cells for the duration of the sorting experiment. Samples that have many dead cells prior to sorting are not likely to survive the sorting process. Samples with many clumps are likely to clog the sorter, slow down sorting, and reduce final sort purity.

The guidelines in this section provide a good starting point for your sample preparation protocol. You are encouraged to practice your sample prep prior to the day of your sort so that you can determine whether it's necessary to optimize conditions to improve cell recovery and viability, or reduce clumping before the big day.

4.1 Tubes

Our FACSAria sorters can accept cells in 1.5 ml eppendorf tubes, 5 ml FACS tubes, or 15 mL conical tubes.

In general, it is best to use ***round-bottom polypropylene*** tubes throughout sample preparation. Polypropylene tubes are less sticky than polystyrene tubes and reduce cell loss during staining and washing steps. Regardless of which tubes you use for staining and washing, we recommend pre-rinsing the tube walls with a serum-containing buffer prior to adding your cells.

All samples must be brought to the sort lab in sealed, airtight tubes. See the section 6 for our policy on sample transportation.

4.2 Buffers

During sorting your cells will be outside of the incubator for many minutes up to several hours, and will be exposed to pressures from 20 to 70 psi. Thus, cells that will be sorted should be placed in a buffer that can maintain pH at high pressure and in normal atmospheric carbon dioxide concentration. Carbonate-based cell culture media is not recommended without modification.

The autofluorescence of phenol red can reduce sensitivity in some of the fluorescence channels. **Phenol red should be avoided** and most media are available in a phenol-free formulation.

Try one of the following as a sort buffer depending upon your cell type:

4.2.1 HBSS Sorting Buffer

HBSS
1% BSA
20 mM HEPES pH 7.2

4.2.2 DPBS Sorting Buffer

1X DPBS (Ca^{++}/Mg^{++} free)
20 mM HEPES pH 7.2
1% BSA

4.2.3 DPBS Sorting Buffer w/ Glucose and Sodium Pyruvate

Try this for cells showing poor viability after sorting.
DPBS
1g/L D-Glucose
36mg/L Sodium Pyruvate
5% Heat Inactivated FBS
(GIBCO #14287-080 is a good starting point, though if your cells are sticky then better to make a buffer without Ca^{++} or Mg^{++})

4.3 Considerations for specific types of cells

4.3.1 Lymphoid Cells

Use HBSS Sorting Buffer for all steps.

4.3.2 Adherent Cells

To achieve single cell suspension, one can use either Trypsin or Accutase for cell detachment. If using Trypsin, deactivate it using a PBS/cation free FBS buffer. Resuspend cells in the PBS sort buffer with a higher concentration of EDTA. If using Accutase, neutralization is not required. Add PBS sort buffer directly and the cells are ready for sorting. If cells are sensitive to detachment, Accutase is recommended but Trypsin is fast acting and better for stronger cells.

4.3.3 Sticky Cells

Some cells that have been activated tend to clump more. But if you find that cells (any type) need to be filtered more than 2X, use the PBS sort buffer with EDTA 1mM up to 5mM. Please practice caution when increasing EDTA. Although a higher concentration of EDTA is very helpful in producing single cell suspension, adding too much EDTA can be deleterious.

4.3.4 Samples with poor viability

Samples with large numbers of nonviable cells often become sticky due to the presence of solubilized DNA released from the dying cells. Add 10 U/ml of DNAse I to the HBSS staining buffer to reduce clumping. Note that EDTA should not be used with DNAse I. In this case it is best to optimize buffers to reduce cell death. Adding glucose and sodium pyruvate can help.

4.4 Cell Concentration

Samples that are too dilute or too concentrated can make the sort take longer than expected. It is much easier for core staff to dilute samples than to concentrate them. Err on the side of making your samples too concentrated and **bring a 50 ml conical tube full of extra sort buffer** to your appointment in case the samples need to be diluted.

4.4.1 Small Cells: 10-20 x 10^6 cells/ml

4.4.2 Large Cells: 5-10 x 10^6 cells/ml

4.5 Viability Dyes

Inclusion of a viability dye is highly recommended for most sort experiments, and the wonderful selection of new viability dyes for flow cytometry means it is almost guaranteed that there is a commercially available reagent that will work with your current staining panel on one of our sorters.

The core stocks a few very common viability dyes (PI, DAPI, 7AAD, ToPro-3) and may add one to your sort samples if you haven't and it seems appropriate.

5 Collecting sorted cells

Cells sorted on the FACSAria can be captured into tubes, standard microtiter plates, or directly onto microscope slides. Each collection device should be brought to the sorting lab preloaded with collection media appropriate for the cell type, downstream application, and collection device.

5.1 Sorting into tubes

To collect 1 or 2 target populations, 15 ml, 5ml, or eppendorf tubes can be used. 3 or more target populations must be sorted into 5ml or eppendorf tubes. Polypropylene tubes are highly recommended.

At our standard sorting conditions, we can deposit approximately 200,000 cells into an eppendorf tube, 1 million cells into a 5 ml tube, or 3 million cells into a 15 ml tube.

5.1.1 Collection media

To increase the viability of sorted cells, we recommend a collection media of either 100% FBS or a media solution with a high percentage of FBS. Recommended volume depends on tube size and should be enough to fully coat tube walls when the tube is vortexed.

Add 2 ml collection media to 15 ml tubes.
Add 1 ml collection media to 5 ml tubes.
Add 500 µl collection media to 1.5 ml tubes.

5.1.2 Sort Purity Check

It is core policy to assess the quality of your sort by running an aliquot of your sorted cells back through the sorter. We do this for all collected populations with more than 50,000 cells unless you specify otherwise. We feel this is an important bit of data to

give us feedback about the progress and quality of your sort and also when it comes time to publish your results.

5.2 Plate sorting

The core's FACSAria sorters can sort into standard 96-well or 384-well format plates, reliably depositing the desired number of cells into each well. Most frequently this mode is used to derive clonal populations by sorting *one cell per well*. For non-clonal populations, if more than a few thousand sorted cells are desired, it's best to first sort into a tube, spin down, and then transfer the cells to a plate.

5.2.1 Sorting into flat-bottom plates for tissue culture

When sorting one cell per well, cells often grow more robustly with ***sterile-filtered conditioned media*** added to the well. If this is your first time sorting into plates, a good starting point is:

1:1 mixture of fresh and sterile-filtered conditioned media
with 25 µg/ml gentamicin
20 mM HEPES
0.2 µm filtered

Use 100-150 µl/well in 96-well plates.
Use 50 µl/well in 384-well plates.

Spinning the plates at 300xG for 1 minute after sorting can help the cells attach to the plate.

5.2.2 Sorting into conical-bottom PCR plates

Delivering cells into the bottom of conical-bottom microwell plates with a small amount of buffer requires very precise alignment of the plate stage. Some PCR style plates lack the rigidity needed to clamp securely into the fixture. Consult with core staff to verify that your intended plate is compatible with our plate holder. **Bring a sample of your plate to the core well in advance of your sort** so that we can calibrate the stage to your plate. We may not be able to guarantee deposition of the cell at the bottom of a conical bottom 384-well plate with only a few microliters of buffer. That is pushing the limits of the Aria's precision.

6 Sorting Controls

Two classes of controls, ***compensation*** control and ***gating*** controls are necessary for a successful multicolor sort. Compensation controls have *one color only*, and gating controls usually have *all colors except one*.

6.1 Compensation controls

Compensation controls are required for all sorting experiments that involve 2+ colors. Compensation controls must match the experimental colors by means of brightness, autofluorescence, and tags. Antibody capture beads are preferred but cells can be used if they have low autofluorescence.

6.2 Gating Controls

Fluorescence Minus One (FMO) controls are critical if accurate determination of a target population is essential, especially when antigen expression is low or variable. An FMO control mimics the sample with the exception of one fluorescent reagent that is absent. For instance a FITC FMO gating control would lack the FITC-conjugate reagent but otherwise match the sort sample exactly. These controls permit accurate gating in the presence of spillover spreading.

6.3 Example: 4-color Experiment with FITC, PE, APC, Pacific Blue

6.3.1 5 compensation controls needed. May use either capture beads or cells.

1) Unstained control
2) FITC+ only control
3) PE+ only control
4) APC+ only control
5) Pacific Blue+ only control

6.3.2 4 FMO controls needed which must be made from cells.

7) FITC FMO control: FITC-, PE+, APC+, PACIFIC BLUE+
8) PE FMO control: FITC+, PE-, APC+, PACIFIC BLUE+
9) APC FMO control: FITC+, PE+, APC-, PACIFIC BLUE+
10) PB FMO control: FITC+, PE+, APC+, PACIFIC BLUE-

7 Sample Transportation

7.1 All tubes must be capped during transport to and from the flow cytometry core facility

7.2 All human, non-human primate and materials infected with a human pathogen must be transported to and from the flow cytometry core facility using a secondary containment that is clearly marked as a biohazard

7.3 All tubes must be capped tightly during vortexing within the core facility

7.4 The use of flow cytometry for sorting and analysis of cells infected with a human pathogen or transduced cells must be described on a rDNA or Infectious Material protocol and approved by the IBC prior to use. The IBC may require specific conditions based on the nature of the transgene being sought.

8 After sorting

8.1 Cells sorted for culture

For cells sorted into tubes, gently spin the cells down and decant the supernatant, and resuspend in culture media before transferring to the culture flask or dish.

For samples single-cell sorted into microwell plates, gently spin the plates for 1 minute at 300xG and then place into incubator and leave undisturbed overnight so that the cells can attach.

8.2 Sterility

We routinely run 70% ethanol through our sorters and have a very low incidence of sample contamination. That said, the sorters are considered aseptic, not sterile. We strongly recommend culturing cells in 25 µg/ml gentamicin until the first passage after sorting. The core will not be responsible for contamination of cells that were grown without gentamicin.

8.3 Cross-contamination

We wash with 10% bleach followed by water in between sort samples to reduce the likelihood of cross contamination. This does not fully eliminate the possibility of this occurrence and it is up to you, the investigator, to verify that cross-contamination has not occurred, particularly in cells maintained in culture after sorting.

8.4 Sorting for RNA recovery

We can sort cells directly into lysis or stabilization buffer if you prefer. There are special considerations when sorting cells for subsequent RNA recovery that are beyond the scope of this document. See **ISOLATION OF TOTAL RNA FROM TRANSGENIC MOUSE MELANOMA SUBSETS USING FLUORESCENCE-ACTIVATED CELL SORTING**. Tighe and Held, 2010.

9 Sorting Checklist

Print out this checklist and review it immediately prior to leaving your lab for your sort appointment to make sure you have all needed supplies.

9.1 Cells in appropriate buffer at 2x107 cells/mL

9.2 Cells placed in tubes with airtight caps firmly secured

9.3 Tubes placed in appropriate leakproof transport container

9.4 Extra buffer in a 50 mL conical in case we need to dilute cells

9.5 Collection tubes or plates pre-loaded with collection media

9.6 Extra collection media in a 50 mL conical in case we need to make more collection tubes or plates

9.7 Single-color controls

9.8 FMO (gating) controls

9.9 Viability dye (PI, DAPI, 7AAD etc.) if needed for your experiment

9.10 Labcoat

9.11 A few pairs of gloves