Cancer Center
NCI-DESIGNATED COMPREHENSIVE CANCER CENTER

Flow Cytometry Shared Resource
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Sorting with the Flow Core Shared Resource

Biosafety Requirements

Regulatory standards require that cell sorting laboratories comply with Biosafety Level 2 (BSL-2) requirements at a minimum. The Flow Cytometry Shared Resource (FCSR) facility complies with these standards up to the level of BSL-2 with enhanced precautions. The FCSR is **not** currently equipped to provide sorting for samples that are radioactive, or require BSL3 containment such as specimens containing, or potentially containing, BSL-3 or BSL-4 organisms. Organisms requiring BSL3 containment include influenza (1918, Avian, H1N1), Monkeypox, Mycobacterium tuberculosis, Mycobacterium bovis, Neisseria meningitides, Treponema pallidum, measles virus, Coxiella burnetii (Q fever), or any other BSL-3 organism which may be transmitted by aerosols. Pathogen Safety Data Sheets on many infectious microorganisms are available at the Public Health Agency of Canada website: http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/index-eng.php.

Scheduling

All appointments for sorting are scheduled through the iLab Solutions online system. A staff member is required for the Astrios and MoFlo XDP sorters. Customers are allowed to operate the Sony MA900 sorter after training.

Sorting Instruments

Astrios EQ

Sorter tip size:	70 µm
Acquisition speed:	25,000 cells/sec (90 million cells/hr)
Collection concentration:	1 million cells/mL
Populations:	Up to 6-way sorting
Five Lasers:	405, 488, 532, 561, 640
Sample types:	Primary human samples, enhanced forward scatter for better small particle detection

Astrios EQ

MoFlo XDP70

Sorter tip size:	70 µm
Acquisition speed:	25,000 cells/sec (90 million cells/hr)
Collection concentration:	1 million cells/mL
Populations:	Up to 4-way sorting
Five Lasers:	UV, 405, 488, 552, 635
Sample types:	Mouse cells, small cell types, UV applications such as side populations

MoFlo XDP70 & 100

MoFlo XDP100

Sorter tip size:	100 µm
Acquisition speed:	10,000-12,000 cells/sec (36-43.2 million cells/hr)
Collection concentration:	250,000 cells/mL
Populations:	Up to 4-way sorting
Lasers:	Four including 405, 488, 561, 641
Sample types:	Cultured cells, cell lines, “large” or “sticky/clumpy” cell types

Sony MA900

Sorter chip size:	70 & 100 µm
Acquisition speed:	4000 cells/sec (100 chip); 12,000 cells/sec (70 chip)
Collection concentration:	250,000 cells/mL (100); 1 million cells/mL (70)
Populations:	Up to 4-way sorting
Four Lasers:	405, 488, 561, 641
Sample types:	

If you are not sure which instrument is appropriate for your samples, please email the details of your experiment and cell type to the FCSR staff at cc.flowcyto@ucdenver.edu. We will advise you on which instrument to schedule.

Sample Requirements

Sample Volume and Concentration

Resuspend cells to these ideal sample concentrations (if possible):

- Cultured cells: 20 x 10^6/mL
- Fresh cells: 75 x 10^6/mL

If you do not have that many cells then resuspend samples in a minimum volume of 400 µL.

Samples should be in buffer no more than 2% serum.

Bring extra buffer along so that we may dilute the sample, if needed.

Filtering Samples

We filter all samples using 30 or 50 micron filters before sorting to remove cell clumps and prevent sorter clogs.

Sorting Buffer

Suspend cells in a basic sorting buffer, such as:

- 1x PBS (Ca/Mg++ free)
- 1mM EDTA
- 25mM HEPES pH 7.0
- 1% FBS or BSA

Gating and Compensation Controls

The following controls are needed.

- **Unstained cells** to evaluate autofluorescence.
- **Compensation controls** also known as **Single Stains** - Beads or cell samples stained individually with each fluorochrome used in the experiment.
    - If using a **live-dead marker**, do not include it in your compensation controls.
    - If your cells have **fluorescent proteins**, use beads or non-fluorescent cells for compensation controls.
- **Fluorescence Minus One (FMO)** controls are recommended to determine accurate gating of positive events.

Collection Requirements

Collection Tubes

Collection tubes must be polypropylene (polystyrene tubes can accumulate a charge and should not be used). Acceptable tubes include:

- 5ml 12x75 round bottom tubes (preferred vessel)
- 1.5ml snap-top tubes
- 15 or 50ml conical tubes (limited to two populations for collection and incompatible with sort rescue)

Plate Sorts

We can also sort into a variety of plate types, such as 96-well, 6-well, etc., and can sort single or multiple cells/per well. Sorting into plates uses one sort stream to sort only one population at a time; however, different populations can be sorted into one plate. Please load plates with media.

Collection Media

A small volume of media must be provided in the collection tubes and plates to cushion the sorted cells, ideally 1ml of media per 5ml of collection volume (e.g., 1ml in a 5ml tube). If adding serum, 50% serum is a good initial concentration as it will be diluted by the sorted cells which are delivered in a droplet of sheath fluid. The following media can be used:

- Culture media with antibiotics
- PBS, if collecting cells for RNA or DNA
- Fetal Bovine Serum only

Additional Sorting Tips

Sample Preparation Tips

Sample Volume and Concentration

Cells should be counted after all preparation as it is not uncommon to lose up to 50% of cells during the staining process. At ideal concentrations, we can operate the cell sorters at their most efficient event rates. If the cells are less concentrated, we may not be able to sort your entire sample, or we may require more time to sort your samples and your fees will be much higher than they need to be.

Sorting Buffer

Resuspend cells for sorting in a basic sorting buffer, rather than culture media. Culture media is not ideal for sorting for the following reasons:

- The pH becomes basic under normal atmosphere reducing the cell viability.
- The calcium chloride in most culture media is incompatible with the phosphate component of the instrument sheath buffer causing calcium phosphate crystals to form.
- The phenol red increases the background fluorescence of the cells which may reduce the resolution between negative and positive cells.

Sorting Tips

Sort Efficiency

You should plan on a final yield of about 75% of the starting number of desired cells. For example, $1x10^7$ cells with 30% positive for GFP would yield $2.25x10^6$ GFP positive cells (10^7*0.3*0.75). Keep in mind that "clumpy" cells produce a greater number of doublets which can greatly reduce this yield. Rare event sorts (1% or less) can produce a lower yield, as low as 50%.

Your cell yield may also be affected by cells sticking to the sides of the collection tube. Pre-coating the collection tubes with fetal calf serum can help to prevent this adherence.

Sort Purity

If cell numbers permit, a small amount of cells are reanalyzed to verify the purity of the sort. Factors which negatively affect purity include the following:

- Clumped cells
- Dim fluorescence
- Low percentage of cells in the sort gate

Poor Viability

We recommend adding a viability dye to your stain set to eliminate dead cells. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) is a nucleic acid stain that is predominantly impermeant to live cells, allowing it to be used as a viability dye in unfixed cells. Dissolve DAPI in water to make a 1 mg/ml stock solution, then dilute in PBS to make a 100 mg/ml working solution. Add 10 ul of working solution to 1 ml of stained cell suspension (1 ug/ml). Alternatively, propidium iodide may also be used at a final concentration of 1-2 ug/mL.

If sorting more than one or two samples, it is also recommended to stagger the cell preparation process so that the cells are not stored at less than ideal conditions for any longer than necessary.

Sterile Sorts

While absolutely sterile sorting is not technically possible, most of the sorts we perform are done in an aseptic manner with no resulting contamination. The instrument lines are cleaned between sorts using Dullbecco's PBS. In addition, we use Beckman Coulter Isoflow sheath fluid which contains an anti-fungicidal and anti-bacterial additive. We have not had any problems with cell viability using this sheath fluid.

Troubleshooting Sort Problems

Optimizing Cell Sorting

Sorting can be optimized to provide greater cell recovery at the expense of purity or greater purity at the expense of recovery. It may take several sorts to optimize the sample preparation and sorter operation for your cells, but we find that sort results are highly consistent once optimized.

Staining Large Quantities of Cells

When staining large numbers of cells, the antibody concentration rather than the cell number is the important factor. If you are staining 10 million cells, use the same staining volume and antibody amount that you use when staining 1 million cells. If you are staining 100 million cells, increase the antibody 5-fold.

Sticky Cells

Cell sorting requires cells in a single cell suspension. Clumping cells cause several problems:

- A large clump will clog the cell sorter which may contaminate the collection tubes and causes a delay in sorting.
- Clumped cells will reduce the sort yield as the clumped cells will be excluded in the singlet gating.
- Aggregated cells will cause more coincidence (or software) aborts, and will not be sorted.

Adherent Cell Lines

Adherent cell lines can reaggregate when serum is used to inactivate trypsin. Soybean trypsin inhibitor can be used as an alternative to serum.

Staining Buffer with DNase

Frequently, dead and/or lysed cells cause severe clumping problems (especially neutrophils). If this is a problem, it is helpful to stain on ice and use a DNase cell staining buffer, such as:

- PBS with 1% human serum albumin
- 100 units/ml DNase I
- 1 mM $MgCl_2$