SORTING BEST PRACTICES

Flow Cytometry Core Facility
MICHIGAN STATE UNIVERSITY

On average, for ideal/optimized cell preparations, our instruments can obtain a sort purity of >99.0% viable cells. There are many factors that affect sort purity, sort efficiency, yield, and viability. Instrument set-up, cell preparation, and maintaining cell viability are key components to successful sorts.

Contents

BIOSAFETY

Regardless of Biosafety level (BSL), all sample types to be sorted must be approved by EH&S/IBC on a case-by-case basis. A related Cell Sorting Addendum must be submitted through the PI's HURON Click Biosafety Protocol and approved by the IBC/EH&S prior to scheduling a sort.

- **BSL-3 or higher samples are not accepted in the MSU Flow Cytometry Core Facility**.
- **Radioactively labeled samples are prohibited.**
- **All sorters in the MSU Flow Cytometry Core Facility are rated for sorting up to BSL-2+ (sorting with enhanced precautions).**
- For RNA isolation post-sort, please find alternatives to sorting directly into Trizol. Cell sorters in the Core Facility are not set up within externally vented fume hoods.

Why do I have to receive approval from EH&S and the MSU IBC?

Aerosol Risk: High-speed electrostatic cell sorters use high system pressures and higher drop drive frequencies, which produce small droplets. During instrument failure (e.g., partial blockage of the nozzle) the generation of secondary aerosols can occur (*Schmid I, Lambert T, Ambrozak D, Marti GE, Moss DM, Perfetto SP. International Society Cytology and Biosafety Standard for Sorting of unfixed Cells. Cytometry 2007; 71A:414-4371*). The potential exposure to escaped aerosols may be a health risk to sort operators and others in the lab space. All cell sorters in the MSU Flow Cytometry Core Facility have been installed within a Baker Biosafety Cabinets (BSC), specifically designed for each instrument, in order to mitigate exposure to aerosols, when present.

Biological material that pose a higher safety risk through inhalation exposure or viruses such as 2nd generation lentivirus are often characterized as BSL-2+ when using electrostatic sorters and require sorting with enhanced precautions (negative air pressure monitoring, additional PPE [goggles, wrap-around gown, N95], etc.).

At what Biosafety Level (BSL) should my samples be classified?

	BSL-1	BSL-2	BSL-2 + (enhanced precautions)
Examples of cells/samples	Cells from murine or other non-human/non- primate species that have not been exposed to any microbial agent but have been genetically modified using non-viral methods (e.g., cells from transgenic animals or cells treated with nucleic acids). **Or** cells determined by EH&S to be approved as non-recombinant BSL-1 or recombinant BSL-1. *Examples: primary murine splenocytes, fibroblasts, or bone marrow derived macrophages without genetic modification or pathogens present. NIH 3T3, B16-F10, 4T1; S. cerevisiae; genetically modified with non-viral methods.*	Cells of human or non-human primate origin or cells that have been genetically modified using viral methods or cells exposed to microbial agents (e.g., viral, bacterial, fungal, protozoan, or parasitic) and **have** been approved by EH&S for BSL-2 containment and sorting. *Examples: HEK293T, HeLa, RAW264.7; murine and human cell lines transfected with **3rd generation lentivirus or later.***	Cells of human or non- human primate origin or cells that have been genetically modified using viral methods or cells exposed to microbial agents (e.g., viral, bacterial, fungal, protozoan, fungal, parasitic) **that have elevated risk if aerosolized** and have been approved by EH&S for BSL-2+ containment and sorting. *Examples: primary human PBMCs from healthy donors not screened for pathogens, murine or human and murine cell lines transfected with **2nd generation lentivirus.***
PPE Required	Lab attire is required (Closed-toed shoes, long pants and lab coat). When manipulating samples (e.g., loading and unloading) gloves and lab coat are required. Spills (**required**): Lab coat, and nitrile gloves.	Lab attire is required (Closed-toed shoes, long pants, lab coat, and gloves **required** at all times. When manipulating samples (e.g., loading and unloading) eye protection is also required. Spills (**required**): Lab coat, nitrile gloves, and goggles.	Lab attire is required (Closed-toed shoes, long pants, lab coat, and gloves at all times. When manipulating samples (e.g., loading and unloading) eye protection and an N95 respirator is also **required**. Spills (**required**): front-closed gown, nitrile gloves, goggles, and N95 respirator.
Room Restrictions	None	Yes, door to room must be closed during sort and the BSL-2 Biohazard sign posted on the outside.	Door to room must be **locked** during sort and the BSL-2+ Biohazard sign posted on the outside. Room will be monitored for negative pressure prior to and during sort.

***Please refer to the NIH Sorter Biosafety Guidelines and 2014 ISAC guidelines for more information pertaining to appropriate classification of cell lines/types and vectors:**

NIH Sorter Biosafety Guidelines: https://policymanual.nih.gov/3038

ISAC Sorter Biosafety Standards: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4117398/

How do I receive approval to sort these materials?

1) Complete a MSU Flow Cytometry Core Cell Sorting Addendum.

   All materials to be sorted must be approved in your lab's Safety Protocol within HURON Click. An addendum for projects involving sorting on a flow cytometer must be completed and approved by EH&S and/or the IBC. This addendum is required due to the increased risk of aerosolization when sorting using an electrostatic-based cell sorter. Our policies are set based upon NIH Sorter Biosafety Guidelines and ISAC (International Society for Advancement of Cytometry) Sorter Biosafety Standards. The Cell Sorting Addendum should be uploaded as an amendment to your lab's existing HURON Click Safety Protocol. If you are a student, postdoc, or staff member, please let your PI know that this submission and approval in the HURON Click system is required prior to any approval of cell sorting by the Core.

2) Following approval, fill out a Biosafety Questionnaire and include the approval letter and submitted Addendum as attachments.

3) Schedule a Project Review Meeting to discuss sorting best practices: Link

4) Schedule cell sorting on iLab on appropriate instrument, as well as concurrent time with a Core staff member: MA900, Aria Ilu, Influx
   *The above links are set to 1-hour durations. If you need more than one hour, please book multiple appointments or email the core at FACS@msu.edu.

What additional precautions are needed when working Lentivirus-transfected cells?

All generations of lentivirus require the following additional sample preparation prior to sorting:

1) Ensure that at least 72 hours or 3 passages have occurred since cells were transfected.
2) Wash cells 3x prior to filtering and bring to Core staff for sorting.

2nd generation lentivirus is classified as BSL-2+ (enhanced precautions) and requires the following additional precautions prior to initiating sorting:

Core Staff are required to:

1) Wear a front-closure gown, face shield and safety glasses and/or goggles, and N95 respirator during the sort
2) Monitor room housing cell sorter for negative air pressure prior to sort initiation using the Aircuity system, Setra pressure monitor, and/or smoke bottle.
3) Periodically evaluate effectiveness of the BSC and Aerosol Management System (AMS) of each sorter through environmental sampling in normal operation and failure mode to ensure compliance to proper safety practices and procedures.
4) Lock the door to the room housing the cell sorter. No personnel other than the operator are allowed in the room while the sort is occurring.
5) Billed time on the instrument is subject to modifications based on additional setup time and stringent cleaning time post-sort.

INSTRUMENT SETTINGS

How do I choose an appropriate cell sorter?
The table below describes the sorter specifications or visit our website:

CELL SORTER SPECIFICATIONS

	SONY MA900	BD FACSAria-IIu	BD Influx
Location	BPS 5115	IQ 2522	BPS 5115
Lasers /# of detectors	405nm|638nm: 7 488nm|561nm: 5 **note: this is a partial co-linear system*	405nm: 4 488nm: 6 633nm: 3	355nm: 2 405nm: 2 488nm: 3 561nm: 4 642nm: 3
Max # of Fluorochromes	12	13	15
Nozzle size Maximum Event Rate (events/s) Pressure (PSI)	70 µm 12,000 events/s | 40 PSI - 100 µm 6,000 events/s | 20 PSI 4,000 events/s | 20 PSI 130 µm 2,500 events/s | 9 PSI -	70 µm 22,500 events/s | 61 PSI 18,750 events/s | 40 PSI 85 µm 13,750 events/s | 45 PSI 100 µm 8,000 events/s | 20 PSI - -	70 µm 25,250 events/s | 65 PSI 18,000 events/s | 33 PSI 14,750 events/s | 22 PSI 86 µm 14,500 events/s | 33 PSI 12,000 events/s | 22 PSI 9,750 events/s | 15 PSI 100 µm 9,000 events/s | 17 PSI 5,750 events/s | 7 PSI 140 µm 3,500 events/s | 5 PSI 200 µm 2,000 events/s | 3 PSI

What nozzle size is appropriate for my cells?
Sorters in the MSU Flow Cytometry Core have range of nozzle sizes (70 µm – 200 µm) that are used at specific pressure settings and drop drive frequencies to accommodate different cell types from different organs/sources. Nozzle sizes are chosen based on cell size, morphology, and fragility, and post-sort application to minimize stress on the cells. A good rule of thumb is to choose a nozzle at least 5× larger than the diameter of the cells to be sorted.

Nozzle sizes: examples of cell types:

- **70 µm:** lymphocytes from spleen, thymus, lymph nodes, PBMCs
- **85 µm:** primary macrophages, b-cell culture, bone marrow
- **100 µm:** dendritic cells, hybridomas, B16, HeLa, U2OS, MCF10a
- **130 µm**: Alveolar macrophages, fibroblasts
- **200 µm**: Cardiomyocytes, megakaryocytes

What types of tubes/plates can my cells be collected in?
***See the cell sorter specification table above for collection vessel capabilities.**

Tubes: 1-6 populations can be sorted simultaneously into tubes depending on the sorter. Sorting can be performed continuously or for a specific # of events. For collection tubes, we recommend **POLYPROPYLENE** over polystyrene to reduce adherence of cells to the tube walls.

- 15 mL conical tubes: 1 - 4 mL collection buffer
- 5 mL tubes: 0.5 - 1 mL collection buffer
- 1.5 or 2 mL Eppendorf tubes: 200 - 400 µL collection buffer

Multi-well plates: 6, 12, 24, 48, 96, and 384 well plates can be used. Only one population at a time can be sorted into an individual well of a plate at one time. For multi-well plates, a specified # of events can be sorted into each well (e.g., single-cell sorting for clones or for single-cell PCR). For single cell sorts, wells of 96-well plates can be filled with 150-200 µL of appropriate media.

CELL SORTER COLLECTION FORMAT

	SONY MA900		**BD FACSAria IIu**		**BD Influx**	
Sorting multiple populations	**Up to 4 populations simultaneously** (*in 5 mL tubes or below*)		**Up to 4 populations simultaneously** (*in 5 mL tubes or below*)		**Up to 6 populations simultaneously** (*in 5 mL tubes or below*)	
Collection Format	Tubes: 1.5mL 2 mL 5 mL 15 mL	Plates: 384-well 96-well 48-well 24-well 12-well 6-well	Tubes: 1.5mL 2 mL 5 mL 15 mL	Plates: 96-well 48-well 24-well 12-well 6-well	Tubes: 1.5mL 2 mL 5 mL 15 mL 50 mL	Plates: 96-well 48-well 24-well 12-well 6-well Custom

How much volume will be added to my tubes during a sort?

Droplet volume for sorted events will vary, depending on the nozzle size (see table below for approximate droplet volumes for each nozzle. The droplet volumes in the table below can be used to estimate total volume based on the number of desired events collected. Keep in mind that sorting in yield mode versus purity mode (see below) will also impact how many droplets are sorted for each detected event.

Approximate volume/droplet for each instrument and nozzle/chip:

Nozzle/Chip	SONY MA900	BD FACSAria IIu	BD Influx
70 µm	*1.5 nL*	*1 nL*	*1 nL*
86 µm		*1.5 nL*	*1.7 nL*
100 µm	*3.5 nL*	*3 nL*	*2.5 nL*
130 µm	*7.9 nL*		
140 µm			*4.5 nL*
200 µm			*7.5 nL*

What Sort Mode should I use?

Sort Modes, including 'Purity' masks or 'Yield' masks can impact the overall purity of the resulting sorted sample. The Sort Mode considers the leading droplet, the droplet containing the interrogated event, and the trailing droplet. Masks define a region within each of these droplets that are monitored for presence of an event of interest. Masks divide each individual droplet into 32 equal segments and the Sort Mode determines which and how many droplets will be sorted based on the location of the event (cell) of interest in the leading, interrogated, and trailing droplet. More information on Sort Modes with detailed diagrams can be found here.

Sort Modes:

- **Purity** – Purity is achieved at the expense of aborting targets of interest if non-target events are present in leading or trailing droplets.
- **Yield** – Enriches target events by sorting droplets containing events of interest even if a non-target event is also present in either the leading, interrogated, and/or trailing droplet; multiple drops are sorted to increase chance of maximum recovery.
- **Single Cell** – Highest purity typically used for single-cell plate sorts; ensures that cells are centered within the interrogated droplet and non-target events are absent from leading and trailing droplets.

Can I control the temperature of my cells during the sort?

Yes! We can control the temperature of the sample being sorted and the collection tubes. Temperature options for each include **4°C, RT, or 37°C**. These preferences can be selected when booking cell sorter time in iLab, using the questionnaire.

SAMPLE PREPARATION

What buffer should I resuspend my cells in for sorting?

Once cells are harvested according to the appropriate protocol, they should then be re-suspended in the proper sorting buffer. Higher protein concentrations in the sorting buffer may create a refractive bias with protein-free sheath buffer due to the increased optical density and refractive index of the sort buffer containing protein. Laser light passing through buffers having different indices of refraction will distort the light scatter signals.

Cells should be prepared in a way that maximizes recovery and optimizes viability. We recommend the following Buffer recipe as a minimum (additional modification and components are described below).

Cell Sorting Buffer Recipe:

1X Phosphate Buffered Saline or HBSS (without Ca/Mg)
1% Fetal Bovine Serum (heat-inactivated)
1 mM EDTA
25 mM HEPES pH 7.0
0.2 um filter sterilize, store at 4°C

The reason for each component is listed below, but keep in mind that not every component is necessary for each sort. Choose the components that are necessary for your assay. Combine 1-4 and 5-6 in separate containers, then combine, as appropriate. Sterile filter through a 0.2µm filter after all the reagents are combined and store at 4°C.

1) PBS (without Ca/Mg): Sigma (D8537)
   Optically clear solution buffered solution that mimics the pH, osmolarity, and ion concentration of the human body.
2) FBS|BSA: 5 -20 mg/mL (0.5% -2%)
   Binds to cell surface proteins which prevents heterophilic antibody interference and reduces cell-surface adhesion. Also mitigates electrostatic attraction between positively charged cells and negatively charged tube walls.
3) EDTA: 0.29 –1.46 mg/mL (1 – 5mM) (ThermoFisher 15576028)
   Chelating agent: removes divalent cations from solution. The proteins involved in cell-cell adhesion are dependent on divalent cations. EDTA prevents cell clumping/aggregation.
4) HEPES: 2.38 – 5.96 mg/mL (10mM -25mM) Sigma (H4034)
   Pressure and CO2 independent pH buffer. Necessary in sorters that use high pressures for sample processing.
5) DNAse I: 25-50 ug/mL (Sigma D-4513)
   Facilitates the degradation of rouge DNA fragments that encourage aggregation. This is particularly a problem when sorting freshly isolated primary cells.
6) $MgCl_2$: 0.476 mg/mL (5 mM)
   Provides Mg ions for DNAse activity, but not enough to encourage cell-cell adhesion.

I am working with an adherent cell line, how should cells be detached from the flask/plate?

Adherent cells are typically dissociated using trypsin or EDTA/Versene treatment. Often when cells are trypsinized, the trypsin is inactivated by adding fetal calf serum which contains a protease inhibitor. A solution containing 10% FBS will contain enough protease inhibitor to inactive trypsin. However, the addition of FBS adds back divalent cations enabling the cells to clump. Consider using soybean trypsin inhibitor, in place of serum/media. **Additionally, stringent dissociation buffers can kill cells and/or cleave surface proteins of interest.**

Products like Accutase can gently detach confluent cells from tissue culture plates/flasks and does not have to be neutralized like trypsin. Additionally, it preserves epitopes that that may be sensitive to tryspin.

My cells are sticky and clumping, how should I prepare them?

Remember to look at your cells under a microscope to determine how good your single cell suspension looks prior to bringing cells for sorting. Doublets and higher order aggregates cannot be used for analysis or sorting, because the true status of each fluorescent marker cannot be determined. The presence of aggregates means there are fewer cells available for your analysis or sort. Additionally, aggregates will clog the instrument, shortening the available time to obtain your sorted population of interest. The following can be considered for sticky cells:

- Remove divalent cations. Concentration of EDTA should be raised to 5mM and the use of FBS that has been dialyzed against Ca/Mg free PBS. Not all cells may tolerate this concentration of EDTA, so test prior to using for a sort.
- Cells can often be killed in the enzymatic digestion and the increased frequency of dead cells results in a higher likelihood that more soluble DNA will be released. DNA can coat cells and increase clumping. DNAse I in the presence of magnesium chloride will help reduce cellular aggregation. Also note that DNAse will not work in the absence of divalent cations, so EGTA may be a better option, as it has a lower affinity for Mg++.
    1. Treat cells for 15-30 minutes in a solution of DNAse I or II (100 µg/mL or 10 Units/mL) in the presence of 5 mM $MgCl_2$ in HBSS at room temperature.
    2. Wash the cells once in the presence of 5 mM $MgCl_2$ in HBSS.
    3. Gently resuspend the cells in buffer containing at least 1 mM $MgCl_2$ (although 5 mM may be optimal) and 25-50 µg/mL DNAse.

Do I need to enrich my cell population of interest before sorting?

Enrichment can be helpful, especially for rarer populations. Enriching by positive/negative selection (e.g., StemCell, MojoSort, MACS) or by gradient centrifugation (e.g., Ficoll, Percoll) before the sort usually helps sort efficiency and sort recovery. Starting with a higher percentage of the desired population will improve sort efficiency and decrease the total sorting time.

ex: splenocytes - RBC lysis
= eliminates unwanted RBCs (Ack Lysis Buffer)
ex: tissue digestion with suboptimal viability – dead cell removal
= eliminates dead cells (Annexin V Dead Cell Removal)
ex: rare dendritic cell (DC) population isolation with tissue sample – negative selection of DCs
= remove non-DC cells (Mouse Pan-DC Enrichment)

Can I bring my cells in cell culture media like DMEM or RPMI?

Culture media is not an ideal sort buffer for a number of reasons, including that the pH regulation fails under normal atmosphere (CO_2 evaporates), causing the media to become basic, the calcium chloride in most culture media is not compatible with the phosphate component of the sheath buffer used in the sorters leading to precipitation of calcium phosphate crystals, and also phenol red can increase background auto-fluorescence. Although cell conditions may vary, the suggestions below will help in increasing cell viability and recovery.

For more on this:
http://expertcytometry.com/how-cell-culture-medium-can-decrease-cell-viability-during-a-flow-cytometry-cell-sorting-experiment/

When washing/staining cells, are centrifugation settings important?

Yes! Samples should be centrifuged at an appropriate speed measured as rcf (x g). Using and reporting centrifugation speeds as rpm is not appropriate, as this value can vary wildly across centrifuges due to rotor measurement differences. To prevent cells from clumping, please centrifuge your cells at the slowest speed possible in round bottom tubes.

- **Cell lines typically should be centrifuged at 150 – 300 x g and smaller primary cells like lymphocytes can be centrifuged at 350 – 500 x g.**
- We also recommend using a swing bucket rotor-based centrifuge (not fixed-angle, like an Eppendorf centrifuge), as this will help pellet cells directly to the bottom of the tube/wells and prevent cell loss during staining/washing.

What is an appropriate cellular concentration for sorting?

Sample concentration will affect a few different variables including abort rate, throughput, and sort efficiency. Cells should be counted **AFTER** staining for a more accurate number. We would prefer you to bring a concentrated sample that can be diluted as needed.

For larger cell lines using a **130 µm+** nozzle/chip (e.g. fibroblasts):	**2 x 10^6/mL**
For cell lines using a **100 µm** nozzle/chip (e.g. cancer cell lines):	**5 - 10 x 10^6/mL**
For smaller cells using a **70 µm** nozzle/chip (e.g. lymphocytes, nuclei):	**20 -30 x 10^6/mL**

What kind of filter should I use to filter my cells prior to bringing them to the Core?

In a pinch, a 70 µm cell strainer will work. However, use sterile cell strainers that are appropriately sized for your cells. We recommend the following options:
https://www.belart.com/flowmi-cell-strainers-for-1000-l-pipette-tips.html
https://us.sysmex-flowcytometry.com/consumables/celltrics-filters/
https://shop.gbo.com/en/usa/products/bioscience/cell-culture-products/easystrainer-cell-strainer/

5 mL 12x75 mm tubes with 35 um filter caps are also useful but should be swapped out for a solid cap in a BSC once filtration has been performed to maintain sterility of cells, where applicable.

Why do I need to use a viability dye?
Viability dyes like nucleic acid dyes (DAPI, PI, 7-AAD) and amine-reactive dyes (Zombie, Ghost, Live/Dead) are useful for staining cells that are apoptotic (late phase) or necrotic, where the cell membrane has become compromised and allows these cell impermeant dyes to enter cells. Cells staining positive for viability dyes can be gated out and excluded from sorting. Importantly, excluding dead cells is important because:

- Dead cells can take up antibodies non-specifically. Eliminating dead cells during acquisition and analysis reduces false positives, preventing us from collecting or analyzing cells that may not be the cell type of interest.
- If your intention is to grow cells *in vitro* or *in vivo* post-sort, dead cells are not going to be useful.
- Dead cells should be excluded as gene expression and subsequent protein translation will be modified in dead/dying cells.

There are many "flavors" of viability dyes and both non-fixable and fixable dyes can be used for live cells:
Non-fixable dyes: DAPI, Sytox, 7-AAD, Helix NPs, DRAQ7
**note: we typically do not recommend Propidium Iodide (PI) for sorting, due to toxicity concerns.*
Fixable dyes: Zombie, Ghost, Live/Dead, FVS, Annexin V, ApoTracker (BioLegend)

I have never sorted these cells before, what SHOULD I do before bringing cells to be sorted?

1. Minimally **look at cells under a fluorescence microscope** before bringing cells to the cell sorter to determine if your cells express your fluorescent protein or are stained with the dye/fluorochrome of interest.
2. **Test the antibody/fluorochrome panel before the sort experiment.** Antibodies should be properly titrated for any flow cytometry experiment. The recommended concentration is usually okay but could be optimized by doing a simple titration experiment. New antibody/fluorochrome combinations or a change in an existing panel should be tested in a pilot experiment first on an analyzer. Changing a few conjugates (marker to fluorochrome) can change the appearance of a population.

COLLECTION OF SORTED CELLS

What type of collection tube should I bring for the cells to be sorted into?
Polypropylene tubes are recommended (rather than polystyrene) and will reduce adherence of cells to the tube walls. Polystyrene 5 mL tubes are the type of tubes that are typically used on analyzers (BD FACS Aria IIu, BD Influx). The SONY MA900 can acquire samples that have been resuspended in 1.5 mL Eppendorf tubes, 5 mL 12x 75 mm tubes, and 15 mL conical tubes.

What buffers/media should I prepare for collecting sorted cells?
Sorted cells are diluted out with sheath buffer, which is sterile phosphate-buffered saline and not conducive to keeping cells healthy for long periods of time. For better viability and recovery, it is best to sort into an appropriate buffer.

- **For most cell types, we recommend sorting into tubes pre-coated with 100% FBS, particularly for long sorts**. Pre-incubating tubes with a solution that contains protein will help reduce charge on the plastic (particularly important for polystyrene, which is why use of polypropylene is recommended) and will reduce the attraction of charged sort droplets to the sides of the tube. If tubes are not pre-coated, droplets can dry on the sides of the tube during the sort causing the cell to die if the liquid evaporates.

- **Sorting into a media or buffer:** Buffers or media with at least 10% FBS can be used (up to 100% FBS, especially for primary cells). Conditioned media (see below) can also be beneficial to improving post-sort viability.
- **Sorting into a lysis buffer:** Cells can also be sorted directly into lysis buffer (depending on sort population/sort volume). For RNA, please look at other alternatives to sorting directly into Trizol, as Trizol should be used with sorters that vent externally or within a fume hood.
- **If cells are being sorted into multi-well culture plates, media with antibiotics should be used.**
- Centrifuge plates for 30-60 seconds at 150 x g to settle the cells in the wells immediately after sorting, as some cells can be deposited on the sides of the wells.

I am concerned about low post-sort viability, what optimization should I consider?

The pre-sort health of the cells dictates how well they will withstand the sorting process. Cells that are fragile or already in a compromised state may have lower viability after the sort. Some cell types experience higher cell death, decreased yield, and decreased functionality after sorting. Pressure settings can be lowered to minimize these negative effects.

- Prepare **conditioned media** for single-cell sorts – Collect cell culture media from cells during exponential growth phase (~30-70% confluency) and sterile filter. Add this 50/50 with fresh media to appropriate wells or tubes. This is particularly helpful for single cell sorts.
- Consider sorting **primary cells into 100% FBS**.
- **Expedite sample processing.** Sample preps can be staggered to minimize the amount of time that the cells are sitting. If sorting is taking a long time (e.g., one sort sample – 4+ hours), the post-sorted sample tubes can be taken and processed in increments (e.g., sorted tube processed and changed out with a new collection tube).
- **FBS concentration can be increased up to 5%.** Any higher concentration of FBS will cause a refractive mismatch and change the scatter profile.
- **Discuss modifying pressure settings with a Core Staff member.** When using a 70 µm nozzle your cells are being exposed to pressures up to 70 psi, being accelerated rapidly to ~20 m/sec, rapidly returning to atmospheric pressure, passed through laser beams, exposed to a charge of significant voltage, and hitting a liquid surface traveling at ~20 m/sec. All cells may not survive this trip. Larger nozzle sizes or custom pressure settings may be appropriate for certain cell types.

Do I need to use a dump gate channel?

Good panel design is key to a good experiment and sort. In addition to using a viability dye to exclude dead cells, multiple markers with the same fluorochrome can be used in the same panel to exclude unwanted cells. This will free up other detectors to be used with markers/fluorochromes you are interested in using. For example, multiple markers may be detected using antibodies conjugated to FITC. FITC+ events can then be excluded in the gating strategy to clean up populations of interest.

How do I assess post-sort purity?

We recommend reserving a small aliquot of the sorted material that can be analyzed on the cell sorter to establish post-sort purity. This will determine if the sorting was successful and proved a clear assessment of the purity of the sample to be used in any downstream experiment or functional assay. Additionally, post-sort purity assessment will assist in troubleshooting any issues that may need to be resolved with regard to cell preparation, staining, or instrument settings in order to improve purity.

What do I need to consider after my cells have been sorted?

- After sorting, invert tube and/or wash sides with extra buffer to recover all sorted cells. Sorted cells should be centrifuged and buffer discarded. Please re-suspend cells in the appropriate buffer for your endpoint.
- **Count your cells.** Sorting is not 100% efficient and sorters are not exact cell counters. Keep in mind that there is cell loss before, during, and after the sorting process. Cells should be counted **AFTER** staining (before re-suspension in sort buffer). This will give an accurate estimate of how long the sort will take and how many cells to expect back. Additionally, counting cells after sorting will allow for a more accurate assessment of cell numbers to be used in your downstream endpoint assay/evaluation.