Research Flow Cytometry Facility (RFCF) Cell Sorting Guidelines

The purpose of this document is to provide general guidelines and best practices for preparing samples for cell sorting. Be mindful that every sample type is different and modifications may be needed. This document contains hyperlinks, so it is best read electronically. New clients must:

- Complete “RFCC course 10” on ELM.
- Complete “RFCC course 1” if they have not received training on an analytical cytometer.
- Confirm their IBC protocol include “Flow cytometry sorting of unfixed samples.”
- Submit the “Cell sorter training/consultation” form on Stratocore under the “Request” tab.
- Schedule a consultation with RFCF staff to discuss experimental objectives.

A successful sort results in good purity and yield of the target population and depends on these five things:

1. ***Cell harvesting and preparation***
2. ***Cell staining and fluorochrome choice***
3. ***Proper controls***
4. ***Sample and sorting conditions***
5. ***Sample collection conditions***

1. CELL HARVESTING AND PREPARATION:

A successful sort depends on good sample preparation.

When harvesting cells prior to staining and/or cell sorting, cell death and aggregation need to be minimized to obtain the best purity and yield. The following are preparation tips to achieve this goal:

Preparing Cells from Tissues:

The goal of preparing tissues for cell sorting is to maximize the yield of functionally viable, dissociated cells. Unfortunately, these types of samples often contain a high percentage of dead cells and debris as a byproduct of the dissociation process, which interferes with the quality of the sort and the resolution of the target population. Good resources for tissue dissociation can be found from Worthington Biochemical or Sigma-Aldrich. The RFCF has a GentleMACS Dissociator, which has built-in protocols or can be used with a user’s established protocol. It can be checked out by all RFCF clients.

Preparing Adherent Cultured Cells:

Adherent cultured cells are typically removed from the culture substrate by treatment with trypsin. Trypsin formulations and conditions vary depending on the cell type, but incubating cells with a trypsin concentration too high and/or for too long will damage cell membranes and kill the cells. In addition, trypsin can alter cell surface antigens and therefore alter binding of detection antibodies used to identify target populations. To inactivate trypsin, use of a trypsin inhibitor such as Soybean trypsin inhibitor is better than serum, as serum adds back divalent cations that facilitate cell adhesion/aggregation. To inhibit cell aggregation, EDTA can be added as a divalent cation chelator as described in Section 4 (sample and sorting

conditions). An alternative for dissociation of adherent cultured cells is Accutase or Accumax, which can aid in dissociating cell aggregates especially for use in flow cytometry applications.
Cells can also be released from their culture substrate by scraping; however, this mechanic dissociation may increase cell death and not separate cells from each other.

Cell Enrichment/Depletion and Red Blood Cell (RBC Lysis):
Whether to perform enrichment or depletion depends on the frequency of the target population and the specimen type. Depletion of unwanted cells that constitute a larger population in your sample cut down the sorting time and increase the efficiency of rare event sorting. Depletion/enrichment can be done by using either magnetic bead-based technology (such as MicroBeads from Miltenyi, Dynabeads from Thermo, MojoSort Nanobeads from biolegend,), buoyancy-based technology (Akadeum) or by using density gradient centrifugation (Ficoll, Percoll, Lympholyte).
Samples abundant with RBCs should be treated with ACK (Ammonium-Chloride-Potassium) lysis buffer that can be made or purchased from a variety of companies.

Cell Filtration:
Cell aggregation can also be reduced by filtering samples during processing and just prior to analysis or cell sorting. During processing of the samples, a 70 µm cell strainer insert (Falcon 352350) or a 35 µm cell strainer tube (Falcon 352235) can be used to collect the filtered sample. You will later need to filter the sample again through a 35 µm cell strainer tube (Falcon 352235) immediately prior to sorting or analysis (even if you filtered before staining).

Dead Cells:
Dead cells typically have high autofluorescence and bind non-specifically to antibodies resulting in high background and/or false positives. Dead cells release nucleic acids and other intracellular material that cause intact cells to aggregate. Cell death should be minimized at every step of sample preparation.

- *Before staining*, dead cells can be removed using a Ficoll gradient or a dead cell removal kit (Miltenyi, StemCell, BioLegend, Akadeum…).
- *During staining*, Hank's Balanced Salt Solution (HBSS) is a better choice to maintain live cells than PBS. The addition of 2% BSA or 2% FBS and 25 mM HEPES buffer pH 7.0 comprises a basic cell sorting buffer that has been found to maintain pH and cell viability. A fluorescent dead cell discriminator such as 7-AAD or DAPI should be included to eliminate dead cells from the sorted population. For an extensive listing of viability dyes, visit the Molecular Probes Handbook.
- *After staining*, aggregation due to dead cells can be minimized by DNase I treatment for 15 to 30 minutes in a solution of 100 µg/mL DNase and 5 mM $MgCl_2$ in HBSS at room temperature. Wash the cells once in the presence of 5 mM $MgCl_2$ in HBSS. Gently suspend the cells in Staining Buffer (or PBS) containing $MgCl_2$ and 25-50 µg/mL DNase (as a maintenance dose) prior to and during the sort.

Prepare Enough Cells:
Cell loss occurs during every manipulation at each stage of preparation. A realistic estimate of the number of cells needed before sorting will be based on these losses and on sorting efficiency. Sorting efficiency is an electronic calculation made by the cell sorter. Sorter-specific algorithms and rate of flow interact with sample characteristics such as cell size, type, cell death, cell population distribution, and cell concentration to affect sorting efficiency. Empirically, dim and/or cultured cells tend to have lower sorting efficiencies than bright,

primary cells such as mouse bone marrow cells. Typical sorting efficiencies for cultured fibroblasts may be as low as 60%, while a clean, bright preparation of primary lymphocytes may sort at 99% depending on relative abundance of target population and gating strategies. Preliminary experiments with the same cell preparation and staining are recommended on an analytical cytometer in order to know the approximate number of input cells needed to achieve the desired yield after sorting.

2. CELL STAINING AND FLUOROCHROME CHOICE:

A successful sort depends on good resolution of sorted populations.

If there are any questions about fluorochrome choice or cell staining procedures, please consult our staff. You can find information about the laser and filter configuration, ranking of fluorochrome brightness, and spillover spread matrix for each instrument on our website.

Fluorochrome Selection:

Consider doing a preliminary pilot experiment before bringing your cells for sorting. Generally, “positive” populations that are dim and only minimally separated from a slightly dimmer "negative" population will lead to a poor analysis and/or a poor sort. A thoughtful balance of fluorochrome brightness with cellular marker abundance is important for optimal resolution of cell populations. Please refer to the brightness charts on our website for each sorter, and refer to the "Related Resources" section for more details on multicolor panel design.

Spectral Overlap:

Another important consideration in panel design is the amount of spectral overlap between fluorochromes. Spectral spillover from a very brightly stained channel into a detector that requires high sensitivity can significantly decrease the resolution and sensitivity in a flow experiment. The amount of overlap between emission spectra can be visualized using one of one of the many “Spectra Viewers” available online. Please see the links for panel design software and tools in the “Related Resources” section on our website.

Blocking Fc receptors aka non-specific binding:

An ideal antibody would have affinity to only one specific cellular epitope. Unfortunately, off-target binding to Fc receptors can occur even when using a correctly titrated antibody. In these cases, a blocking reagent is needed. Usually, a blocking reagent contains a high concentration of species-specific immunoglobulin that can bind to the Fc receptors. Unconjugated, nonfluorescent antibodies specific for Fc receptors (CD16 and CD32) can also be purchased from a variety of vendors (BioLegend, ThermoFisher, BD, etc).

Titrating Antibodies:

The optimal concentration for antibody labeling is when the ratio of antibody to antigen reaches a point of saturation. Too little antibody will not be enough to saturate all the high-affinity binding sites; this will limit brightness, make small pipetting errors significant and make quantitative conclusions about cytometric data suspicious. Too much antibody will waste reagent and lower the signal-to-noise ratio due to high non-specific binding to low-affinity sites. Antibody titration can reveal the proper range where small changes in antibody concentration have little effect, but non-specific binding is minimized. Ideally, titrate the antibodies on a small number of cells to find the optimal concentration before scaling up to stain a larger cell number for sorting. For more specific information about antibody titration see this protocol from Current Protocols in Cytometry.

Using Viability Dyes:
Most investigators are interested in sorting live cells – even when the end application of the sorted cells may be for DNA and RNA analysis. Dead cells may exhibit high non-specific binding and can then contaminate sorted populations and lower RNA quality by releasing RNases. Careful cell preparation to produce single, viable cells and reduce aggregation will help, but nucleic acid stains such as DAPI and 7-AAD, or the various amine reactive live/dead dyes are able to distinguish cells with intact membranes (live) from those with leaky membranes (dead or dying cells). For cell sorting, nucleic acid stains are usually a better choice as the dye is added just before the sorting and kept in the sorting buffer during the sorting. Cells dying during the acquisition will continue to be stained by those dyes and can be excluded from the sorted cells. When staining isolated nuclei to sort for single-cell RNAseq (as opposed to viability staining), 7-AAD is the preferred stain for the 10x Genomics platform. For an extensive listing of viability dyes, visit the Molecular Probes Handbook.

3. PROPER CONTROLS

A successful sort depends on proper controls.

Proper controls are **essential** for adjusting voltages, compensation, and gating the population of interest. This is true for ANY fluorescent marker (e.g. GFP, viability dye).

Unstained and Single-Stained Control(s):
Unstained controls are essential for determining background fluorescence and single-stained controls are used to calculate the correct compensation values before a sort. Incorrect compensation can result in the wrong cells being sorted. Please bring an unstained control AND single-stained controls for each fluorochrome/dye/fluorescent protein being used in your panel for each experiment. The unstained control must be free of all fluorescence, meaning it must not express any fluorescent reporters and must not be stained with any viability stains.

Fluorescence Minus One (FMO) Controls:
An FMO control contains all the fluorochromes/dye/fluorescent protein except one. They represent the combinatorial background fluorescence from other channels into the channel of interest and aid in setting sort gates properly. Many researchers are resistant to using FMO controls on a routine basis because they consume cells, reagents, and time, but in many cases, they are needed to validate gate placement, particularly if you are using the panel for the first time. For more information on FMOs, see Nature Reviews Immunology 4, 648-655 or the Daily Dongle article by TreeStar.

4. SAMPLE AND SORTING CONDITIONS

A successful sort depends on appropriate sample and sorting conditions.

The presorted cells should be in a buffer that maintains a live, single cell suspension for the duration of the sort. In addition, it is vital to select the correct nozzle for the cell type being sorted.

Cell Sorting Buffer:
Culture media is not a good choice for sort buffer because:

- It depends on a 5% CO_2 atmosphere in an incubator to maintain the proper pH. HEPES-based buffering system should be used if culture media is used. HEPES will also help maintain pH for long sorts.

- It contains phenol red dye, increasing fluorescence background.
- It contains high protein concentration, causing excessive light scattering affecting data quality.

Therefore, cells should be resuspended in a buffer that does not depend on 5% CO_2, is free of phenol red and Ca^{2+}/Mg^{2+}, as divalent cations will promote aggregation, and has a low percentage (<2%) of proteins. The addition of HEPES for extra buffer capacity will also help maintain pH for a long sort. The following recipe is a good sorting buffer for most cells, however, be aware that modifications may be necessary for your specific sample.

Recommended Cell Sorting Buffer:

- **1x PBS (Ca^{2+}/Mg^{2+} free) or HBSS (preferred)**
- **0.5-2% BSA or up to 2% heat-inactivated FBS [dialyzed against Ca^{2+}/Mg^{2+}free PBS]**
- **25 mM HEPES pH 7.0**

If cell aggregation occurs, it is highly recommended to supplement the sorting buffer with:

- **1 mM EDTA (chelates Ca/Mg ions that promote cell aggregation)**. A maximum of 5 mM EDTA could be used to prevent macrophages/monocytes from sticking to tubing. Some activated cells become adherent, and the chelators (EDTA) help reduce cation dependent cell-to-cell adhesion.
  OR
- **10 U/mL DNase (to remove DNA-induced cellular aggregation)**. If there are many dead cells in the prep, it is likely that there is soluble DNA from the dead cells that will come out of solution. This DNA will start to coat the cells and lead to severe aggregation. The addition of 10 U/mL DNase to the sorting buffer recipe will help reduce DNA associated aggregation. DNase should not be used in combination with EDTA, as EDTA chelates the ions required for DNase function.

Cell concentration:

Cell concentration in the above sorting buffer should be in the range of 10 to 20 x 10^6 cells/mL. If you have fewer than 5 x 10^6 cells, use 300 uL as a minimum volume. Cells that are especially prone to aggregation might require a lower concentration. The sample concentration may need to be adjusted by the sorter operator to avoid aggregation of the sample so please bring extra sorting buffer to your appointment.

Temperature:

The temperature for the whole workflow of cell isolation, staining, and sorting should stay as consistent as possible. If the cells were isolated and stained at 4ºC, the samples should be kept on wet ice until the sorter is ready. If they were stained at room temp, they should stay at room temp. All RFCF sorters are equipped with chillers for the sample and collection tubes that are set to 4ºC by default; please let your operator know if you would like it at a different temperature.

Filtering Your Cells:

It is important to filter your cells prior to the sort. Even if you filtered during sample prep, they should be filtered immediately before sorting using a 35 µm cell strainer tube (Falcon 352235).

Selecting Sorting Conditions:

The type of sorter (cuvette vs jet-in-air vs chip), sheath pressure, and nozzle size can greatly impact cellular function and experimental outcome for downstream applications. The correct

choices largely depend on cell size, morphology, and the downstream application. For example, sorting nuclei with a 100 um nozzle increases their integrity. It's not always trivial to change the nozzle/pressure settings in the middle of a sort, so make sure to communicate the appropriate nozzle setting for your sample when scheduling your appointment in Stratocore.

5. SAMPLE COLLECTION CONDITIONS:

A successful sort is dependent on optimal collection conditions.

Optimal Collection Media:

The collection media is the solution that receives the droplets containing the target cells. Keep in mind the collection media will be diluted with sheath fluid as more cells are collected. If you sort 1 x 10^6 cells with a 70 um nozzle, you will have an extra 1 mL in your collection tube. For the same volume, you will have sorted 0.3 x 10^6 cells with a 100 um nozzle. The optimal collection media depends on the downstream experiments planned for the sorted cells, but below are some suggestions:

- **FBS 100% to 50%** in PBS
- **Your own culture media** (doubled the protein amount as sorted droplets will dilute the culture media)
- **PBS** if collecting cells for RNA or DNA
- **Lysis buffer** from RNA isolation kit (e.g., RLT buffer from Qiagen kit, not trizol). Lysis buffer dilution could be a problem depending on the number of cells collected and nozzle size. We recommend sorting a number of cells that will result in no more than 10% dilution of the lysis buffer (for example, if up to 500 uL of lysate can be used in the downstream experiment, start with 450 uL of RLT buffer and sort no more than 150,000 cells with a 100 um nozzle or 500,000 cells with a 70 um nozzle).

Collection options:

All collection tubes should be filled 1/3 with the appropriate collection media. Below is a table indicating the types of collection vessels that can be used on our instruments and how many populations and events can be collected for each.

Collection device	Fusion, Ariall, MA900	S6, Bigfoot	SH800	Max events collected 70 um*	Max events collected 100 um*
1.5 mL or 2 mL microfuge	*Up to 4*	*Up to 6*	*Up to 2*	*1.0 x 10^6*	*0.3 x 10^6*
5 mL tube	*Up to 4*	*Up to 6*	*Up to 2*	*2.5 x 10^6*	*1 x 10^6*
15 mL conical	*Up to 2*	*Up to 4*	*Up to 2*	*10 x 10^6*	*3 x 10^6*
Multiwell plates or slides	*1 at a time*	*1 at a time*	*1 at a time*	*varies*	*varies*

**Assumes single-drop sort mode*

Sterility:

All tubes, plates, slides, and buffers for collection should be sterile irrespective of the sterility requirement of your downstream process (even if it is for RNA/DNA/protein). We require this to maintain an aseptic environment in the instruments and to avoid contamination of the

actual sterile sorts. RNase-free tubes should be considered if collecting the cells directly into RNA lysis buffer.

Tube pre-coating:
If recovery of live cells is the goal, pre-coat the tubes with 1% BSA in PBS or 100% FBS to avoid sorted cells sticking to the sides of the tubes. Fill 1/3 of the tubes with proteins, cap, put on a rotating mixer or an orbital shaker for at least half an hour, and empty the tubes before filling 1/3 with the desired collection media. However, if a Western blot is part of the downstream experiment, tubes should not be treated with external proteins.

Post-sort analysis:
A small sample of the sorted cells can be run through the cytometer to determine the effectiveness of the sort. Post-sort analysis may be required for publication.

Research Flow Cytometry Facility

CELL SORTING CHECKLIST

Contact numbers for RFCF during your sort:
Ariall/Fusion/S6 room 636-2770
Bigfoot room 636-2054

WHAT TO KNOW WHEN SCHEDULING A SORT IN STRATOCORE:

□**CELL TYPE** – Communicate all information about the type of sample you are bringing including cell size, species, source, biosafety information (any infectious agents?).

□**MARKERS/FLUORS** – Be clear about what exact fluors and markers will be detected. Remember to include fluorescent proteins (ie. GFP), viability dyes, functional dyes etc.

□**SAMPLE/CELL NUMBER** – Know the exact number of samples and number of total cells per sample to determine the length of the sort. We cannot always accommodate last minute add-ons.

□**POPULATIONS COLLECTED –** Knowing the expression patterns and frequency of your populations will help determine the length of the sort and will aid in the setup process.

WHAT TO BRING TO YOUR SORT APPOINTMENT:

□**SAMPLES** – Bring your samples in clearly labeled tubes. It is highly recommended to filter samples immediately before the sort.

□**CONTROLS** – Bring unstained and single stained controls in clearly labeled tubes to *every sort*.

□**SAMPLE TRANSPORT** – Bring samples on ice and with a lid. For samples requiring handling at BSL2 or BSL2+, the tubes must be transported in the following manner:

1. Sealed primary tube (snap caps or screw caps)
2. Sealed secondary container
3. Absorbent between primary and secondary containers to absorb the entire contents.
    - This can be accomplished by placing your samples in a tube rack into a sealable plastic container with paper towels or other absorbent. These containers are inexpensive and can be purchased in local retail stores. **Remember to place a biohazard sticker on the outside.**
    - If you need to place many specimens on ice, this can be done by placing the secondary container in an oblong ice pan or Nalgene pan. Or you can place your entire ice bucket with sealed primary tubes and absorbent into a biohazard bag and seal the bag sufficiently so that no liquid can escape.

□**BUFFER** – Bring extra cell sorting buffer to dilute your sample, if necessary.

□**COLLECTION TUBES** – Bring clearly labeled collection tubes of an appropriate size at least 1/3 full of appropriate collection media for your cells.

AFTER THE SORT:

□**COMMUNICATE –** Give us feedback on the endpoint of your sort. This allows us to determine best practices for different cell types.