Considerations when sorting at the Flow Cytometry Facility

Biotechnology Resource Center (BRC) - Flow Cytometry Core Facility

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Initiating a New Sorting Experiment

All requests involving cell sorting are required to go through the risk evaluation process to determine if BSL2 practices are needed. This process will ensure that the proposed unfixed sort specimens have received IBC approval with the corresponding MUA number.

Sorter Sensitivity

The Sony MA900 Cell Sorter has a microfluidic chip-based design and advanced automation that simplifies operation. The internal micro-channel structure of the sorting chip encases the sample within the sheath fluid to ensure high sensitivity and stable optical detection properties. At the heart of the BD FACSAria Fusion and FACSMelody is the patented quartz cuvette flow cell that is in fixed alignment with the laser, and is gel coupled to the collection optics. This design will ensure that lasers are precisely focused on the sample stream and that the maximum amount of emitted light is collected.

System Buffer

All sorters use sterile HyClone Phosphate Buffered Saline (PBS), Calcium and Magnesium-free, for sheath buffer at 1x concentration. Daily maintenance and between-user cleaning include flushing sample lines with a 10% dilution of household bleach and autoclaved ultra-pure Millipore Super-Q water. Weekly cleanings include a full fluidics system flush with 70% Ethanol. Each sorter is checked for contamination weekly and sterilized as necessary using freshly prepared 1% H2O2 diluted in autoclaved ultra-pure Millipore Super-Q water. After sterilization, the system is flushed with 70% Ethanol and autoclaved ultra-pure Millipore Super-Q water. Users are encouraged to bring sterile solutions whenever possible and to only use those cleaning solutions and water provided by staff.

Cell Size

The sorters come with different sized nozzles from which a sorted sample is ejected. At a point, a short distance beneath the sorting chip (Sony MA900) or ceramic nozzles (FACSAria Fusion and FACSMelody), called the breakoff point, the fluid flow breaks into a very regular stream of uniform droplets. The size of the droplets is determined by the

nozzle size, frequency of vibration, and the speed of the fluid flow. Typically, a nozzle size is selected which is 3-5 times larger than the cells to be sorted. Available nozzle sizes range from 70 to 130 µm. The standard nozzle size in the facility is 100 µm.

Single Cell Suspensions

Preparing a single cell suspension is critical for the optimal flow cytometry measurements. Before arriving at the flow cytometry facility, all particles must have been filtered a 35 or 40 µm mesh shortly before running them on the cytometer. This procedure will ensure the mono-dispersed single particles as they pass through a nozzle orifice typically 100

µm in diameter. We recommend Corning Falcon polystyrene FACS tube with 35 µm nylon mesh (cat. # 08-771-23). For additional information go to the Fisher Scientific website. We normally do not supply filters.

Sort Buffers

Sort buffers include the pre- and post-sort solution that is designed in maintaining single-cell suspensions as well as successful sorts. Single-cell suspensions can be prepared from lymphoid tissues, bone marrow, peripheral blood, cultured cells or other sources. It is advised the Pre-Sort buffer contains no phenol red to minimize background. The Pre-Sort buffer should have minimal calcium and magnesium to minimize cell aggregation as these cations are necessary cofactors for many cell adhesion molecules. It should also contain an FBS-based protein to help maintain cells in a viable state during cell sorting. The function of a sort buffer is simply to get your cells through the sorting process, and it is highly recommended to pellet the cells and re-suspend in the buffer of choice.

The following are suggestions for sort buffers to maximize recovery and viability of sorted cells.

Basic Sorting Buffer

- Phosphate Buffered Saline (PBS), Ca/Mg^{++} free, 1 mM EDTA
- 25 mM HEPES, pH 7.0
- 1% Fetal Bovine Serum (FBS), heat-inactivated
- 0.2 um filter sterilize and store at 4^{o}C

It is recommended that a general antibiotic also be added to the collection media to help prevent bacterial contamination.

Lymphoid Cells: The Basic Sorting Buffer can be simplified to PBS with 1% FBS. The lack of EDTA will not be an issue as these cells do not clump.

Sticky Cells: Raise the concentration of EDTA from 1 to 4mM depending on the nature of cell clumpiness. The FBS should be dialyzed against Ca/Mg^{++} free PBS. Some activated cells become clumpy and the chelators (EDTA) can help reduce cation-dependent cell-to-cell adhesion.

Adherent Cells: For sorting experiments that involve adherent cells, it is important to keep cells in single-cell suspension before, during and after the sorting process. If the end game of these cell types is cell sorting, it is critical to choose appropriate reagents. Trypsin/EDTA is the typical detachment buffer used for adherent cultured cells.

Other Sorting Buffers

Trypsin/EDTA

Trypsin/EDTA is quenched with culture media or a PBS/FBS buffer. The disadvantage of using this buffer is that it reintroduces cations that facilitating cells to reattach to the plate or each other. It is important to use a cation-free FBS buffer to stop cells from reattaching. If necessary, the concentration of EDTA can be raised. However, too much EDTA can be damaging. It is highly suggested to coat polypropylene tubes from which the samples are sorted from with cation-free-4% FBS-DPBS for at least 30 minutes thoroughly, ideally overnight at 4^0C.

Accutase

Accutase can be considered as an alternative to Trypsin/EDTA. It is gentler on cells and does not damage surface antigens and does not need neutralization. Viability has been shown to be 97 +/- 3% after 45 minutes in contact with Accutase. Cells can be suspended in Accutase for sorting and the recovery tubes must contain Accutase. Post sorting, the collection buffer can then be spun down and the cell can be re-suspended with media of choice. Accutase can be purchased from Sigma, eBioscience and other companies.

Buffers for samples with high percentage of dead cells

In a sample that has a high proportion of dead cells, the likelihood of free-floating DNA is high. This DNA will coat cells leading to severe clumping. The addition of 10IU/mL DNAse II to the buffer recipe will help minimize DNA-associated clumping. Additionally, dead cells tend to bind to antibodies nonspecifically skewing data interpretation. Light scatter parameters can help exclude dead cells. However, using solely these parameters may not suffice. Supplementing the experiment with a viability dye might be beneficial. Dyes such as DAPI, Propidium Iodide, 7-AAD, TO-PRO-3, fixable viability dyes, among many others, can be used to ensure that only living cells are recovered.

Sample Volume, Concentration, and Number of Cells to Submit

Cells for sorting should be submitted at a concentration of <1.5 x 10^6/mL in a minimum of 500uL. For cloning into a 96 well plate, the cell concentration should be 0.5 x 110^6/mL in a minimum of 500uL.

One should submit double the number of cells. The excess will allow for hard coincidence and/or software aborts of the sorter and still allows for the desired amount of cells to be obtained. For instance, a sample that has 10% of the population to be sorted and one wishes to receive one million sorted cells. Theoretically, 10 million cells will supply this number; however, one must double that number, 20 million cells total, to plan on receiving one's goal of one million positive sorted events.

Sort Collection (Recovery) Containers

It is highly recommended that polypropylene collection tubes be coated with 4% BSA-DPBS or 4% FBS-DPBS for at least 30 minutes, though ideally overnight, at 4°C. Cells may be sorted directly into a variety of collection devices including, among others:

- 12 x 75mm polypropylene test tubes
- 15 or 50mL polypropylene conical tubes
- 24-, 96-, and 383-well plates.

Sort Volume

It is important to know that the sorted volume that 10^6 cells yields using a 70 µm nozzle is about 0.8mL while a 100 µm nozzle will yield a volume of about 2 mL.

Viability and Yields

Normally, the physical aspects of the cell sort do not reduce viability for most cell types. Neutrophils, megakaryocytes and plant protoplasts may be exceptions. In principle, sorted cells should be as viable as the original sample. Sample preparation is paramount in the success of the cell sorting process.

Sort yields are dependent on the ability of the cytometer to clearly distinguish the cell of interest from all others and to establish the proper sort conditions to enable the charged droplet to reach the collection tube. Sort events will be aborted if a cell is too close to its neighbor, and if a full charge cannot be applied to the drop. Thus, the critical factors influencing abort rates are the speed of the sample flow and the rarity of the population of interest. An additional factor affecting cell viability is the environment in which the cell finds itself. Cells may clump together and they may also adhere to the walls of the tubes and hence be unrecoverable. Sorted cells should be harvested at regular intervals to remove excess sheath fluid, which over prolonged periods may reduce viability. The usage of DAPI as a measure of viability in cells to be sorted is highly recommended. This will ensure only live events will be sorted and dead cells will be excluded.

Temperature

Unless cells are damaged by low temperatures (e.g., neutrophils, spermatozoa), samples should be kept on ice when transported to the sorting facility. Researchers using cells damaged by low temperatures must notify the facility staff in advance as the sorters are equipped with sort sample station/sort receptacle chillers that are usually set at 4^{o}C and it takes some time for the recirculating water bath to change temperature.

Definition of Sort Modes

Purity Mode: Purity mode is used when the purity of the sort is most important. It is the most common mode and will sort all positive events only when there are no negatives within the window of the sort droplet envelope. If a negative cell is close to the drop boundary contained by the cell of interest, the sort logic will fail and the drop containing the positive event will be aborted. This results in a high purity of the sorted fraction.

Single Cell Mode: Only one positive must be contained within the sorted droplet envelope to pass the sort logic. If two positive or a negative are within the sort drop envelop, the

sort logic will fail and the positive(s) will be aborted. Single-mode is useful for sorting single positive events into individual wells or when count accuracy is important.

Enrich Mode: The enrich mode is used when recovery is the most important aspect of the sort. With enrich mode, all positive events are sorted except for hard coincidence events.

Rare events

Pre-purification of samples might be a good strategy for sorting rare events, those that are less than 0.5% positive population. These procedures will include antibiotic selection, density gradient centrifugation and/or magnetic-bead antibody pre-enrichment. Pre-purification techniques can significantly decrease the total amount of sort time and user instrument charges.

Post-Sort

A post sort analysis is essential in quality-controlling the entire sort process, which includes the processing of cells as well as the sorting process. After the sample has been sorted, the sample line is flushed with distilled water to clear the sample line of any residual sample that may be remaining and the sorted sample is reanalyzed. The process takes a few minutes per sample. It is important to include this post-sort analysis time in the time scheduled for sorting. Also, the appointment must include the time it takes to run controls, compensate if necessary, sterilize the sample lines, perform the actual sort, and check the post-sort sample. If post-sorted cells will be cultured, then they must be spun-down and washed with antibiotics to remove the sheath buffer.

Instrument downtime

Sorters are complex equipment and issues are bound to. If an instrument goes down, the facility staff will notify those that it affects as soon as possible. It may or may not be possible to fit a missed appointment back into the schedule in a timely manner. The customer will not be billed for scheduled time in instances where the instrument is down.