Fred Hutch Cancer Center

# Flow Cytometry

**Research Administration**
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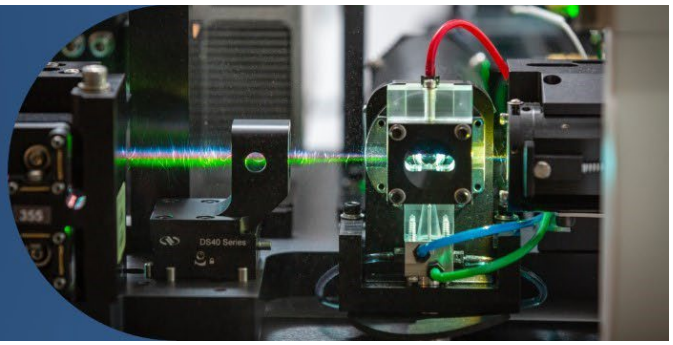

Fred Hutch's Shared Resources are catalysts for lifesaving discoveries. This uniquely centralized program of 15 specialized core facilities and scientific services drives advances by integrating dedicated experts and cutting-edge technologies across the entire research pipeline, from basic science to clinical trial.

## Cell Sorting and Sample Preparation

Cell sorting has revolutionized cytometric analysis, enabling the isolation of specific cell populations with unparalleled precision from complex, heterogeneous samples. This technology has undergone a remarkable evolution, transitioning from rudimentary 3-4 color panels and 2-way sorting to sophisticated platforms capable of simultaneously resolving and sorting 6 distinct populations based on > 30 unique fluorochromes with exceptional purity. While the increasing complexity of modern sorters necessitates meticulous pre-sorting planning and control preparation, even the simplest sorts rely on fundamental sample preparation principles for optimal performance.

### The Importance of Sample Preparation

Regardless of the sorter's capabilities, successful isolation hinges on proper sample preparation. Several factors can significantly impact sort purity, yield, and downstream analysis:

**Media and Buffering Capacity:** Choosing a cell-specific medium that maintains optimal post sort conditions and minimizes the impact of sorting crucial. Buffers help maintain physiological conditions throughout the sorting process, by minimizing cell stress. Insufficient buffering capacity can disrupt cell health, leading to decreased viability and altered metabolic function, negatively impacting sort purity and yield and downstream assays.

**Protein Concentration:** Excess protein can hinder antibody binding and fluorescent detection. Optimizing protein concentration ensures efficient antibody binding and accurate signal interpretation.

**Cellular Viability:** Dead cells can nonspecifically bind antibodies impacting purity, increase the stickiness of cells that can lead to nozzle clogs or aggregates the impact sort yield. Implementing protocols that maximize cell health, and the use of a viability dye is essential.

**Autofluorescence (AF):** Intrinsic cellular fluorescence can mask specific labeling signals, leading to inaccurate data interpretation. Panel design is key to minimize the impact of autofluorescence especially on dim markers. Choosing fluorochromes that do not emit in the same spectral range as high AF should be a consideration when designing a panel. AF is also a consideration in compensation strategies to account for background fluorescence and ensure accurate signal interpretation in single stained controls.

**Cell Aggregates:** Clumps of cells pose significant challenges for accurate sorting and downstream analysis. Implementing appropriate techniques, such as enzymatic dissociation or mechanical disaggregation, to effectively disperse cell aggregates before sorting is essential. Equally important is the gating strategy to eliminate doublets and aggregates while sorting

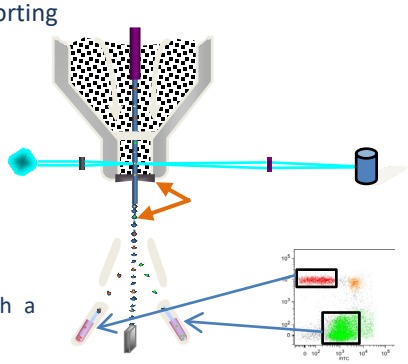

Diagram of a cell sorter with a cuvette style flow cell

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# Generalized Staining protocol

Protocols for staining vary depending on the assay. This is a *generalized* cell surface staining protocol for live cell sorting to use as a guide.

**Sorting Buffers:**

Hank's Balanced Salt Solution (HBSS) and Phosphate Buffered Saline (PBS) at the two most common sorting buffers. They differ in their composition PBS works well for staining and washing cells but lacks ingredients like sodium bicarbonate and glucose which are in HBSS as essential components for cells to maintain functionality. The use of PBS vs HBSS is dependent on the cells and assay.

- 1X HBSS or 1X PBS (Ca/Mg++ free)
- 10mM HEPES (pH= 7.2)*
- 0.5-2% FBS (heat inactivated)
- 1mM EDTA
- 1-10ug/ml DNAse I**
- 0.2 mm sterile filtered

*HEPES helps to maintain a neutral pH to protect the cells from the buffer becoming more basic under high pressure.

**DNAse I is optional, cells that are adherent or if there are dying cells releasing DNA causing clumping DNAse will help minimize aggregation. Concentration is cell type dependent and should be tested on your specific cells.

**Controls:**

Controls are essential for setting up a cytometry experiment. Controls for determining spectral spillover; compensation on traditional cytometers, and unmixing for spectral cytometers follow the same best practices and principles. Spectral instruments can resolve fluorochromes with similar emission maxima and correcting for autofluorescence while traditional instruments require better separation of the colors used in a panel.

**Unstained cells** are required for spectral unmixing and are very helpful for setting up a sorting experiment on both conventional and spectral instruments. Unstained cells need to be the same type that are being sorted for unmixing. If you are sorting primary cells avoid bringing a cell line as the negative control, the auto-fluorescence may not be equivalent.

**Single color positive controls** for each fluorochrome used for compensating spectral overlap between fluorochromes. The positive population needs to be as bright or brighter than the target cells. Beads or cell can be used, the important constraint is that the negative control needs to match the AF of the positive (negative beads for bead controls, negative cells of the same type for cell controls). Tandem dyes need to be the exact one used in the experiment due to variability in spectral signatures from lot to lot. If the control is too bright, titrate it instead of lowering the detector to get it on scale. This will result in lower sensitivity to dim signal that can impact the ability to resolve positive cells.

**Titrate antibodies** and test new lots of antibodies. Tandem dyes do have lot to lot variation and need to be titrated every time a new vial is opened (i.e. PE-Cy5 or PE-Cy7). Poorly titrated antibodies lead to more background, lower resolution and wasted money.

**Fluorescence minus one (FMO's)** are important for determining the overall spillover spreading of fluor's into a specific channel or marker. This is used to apply gates to populations that are dim and not well resolved from the background. In sorting this can be a critical control for proper gating of true positive populations.

Cultured cells:

Split the cells 1-2 days prior to sorting when possible. This will improve the health of the cells and reduce overgrown and dead cells. For adherent tissue culture cells, trypsinize to release from the growing surface. Stop with serum. Wash cells before staining and keep cells on ice to help preserve viability and avoid internalization of antibodies.

Primary cells:

Dissociate cells from tissue using the method that is most suitable for the cells type of interest. Process whole blood and spleen removing the RBC's when possible. Keep cells on ice to help preserve viability.

If the cells are sticky resuspend in a final buffer containing DNAse I** to keep the cells from clumping during the sorting process.

**Live cell staining SOP**

1. Cells must be in a single cell suspension.
2. Wash cells with staining buffer.
3. Centrifuge cells to pellet. Remove supernatant.
4. Resuspend in blocking agent (serum or Fc block). This is optional, determined by cell type and FC binding.
5. Add antibody ~50µl/1E6cells (concentration is dependent on the specific antibody) Titration of antibodies is highly recommended for any flow cytometry experiment.
6. Incubate cell in the dark at 4$^{o}$C for 30-60 min.
7. Wash cells with staining buffer 3 times.
8. Add Secondary antibody, if necessary, repeat steps 5-7.
9. Resuspend cells to a final concentration of ~20E6/ml (see notes/FAQ below) in sorting buffer.
10. Cells must be sent through a 40µm filter to remove large clumps prior to sorting. (Falcon snap cap cell strainer cat# 352235) If your cells are larger than 30µm the standard 70µm nozzle will not be appropriate for sorting. Please contact staff for further assistance.
11. Total starting cell #'s is dependent on how many cells are needed from each population to be sorted. (See notes/FAQ below)
12. Four (4) populations (BD Aria and Sony MA900) or six (6) populations (BD S6) can be sorted simultaneously and deposited into the following tube types; microcentrifuge tubes, 12X75 tubes. 15ml conical tubes can be used for 2- way sorts only.
13. One (1) population can be sorted down to one cell/well of a 96 well plate, other plate configurations available, 96 well PCR can also be used.
14. Cells should be sorted into growth medium with serum if going into culture. Sorting into a dry tube should be avoided.

## Notes and FAQ

Test your antibodies and fluorochrome combinations before embarking on a large or critical sort, but really this is how you should approach every sort to avoid wasting time and money.

**Transfected cells:** If you are trying to isolate cells from a transfection using EGFP, EYFP or other fluorescent proteins, include a mock transfection as a control. This can be very valuable when the transfection efficiency and /or the expression of the protein is very low.

**Serum Concentration: S**erum in the sorting should contain less than 2% FCS or .5% BSA. Excess serum causes optical interference resulting in dynamic distortion of the light scatter signals this ultimately means decreased resolution of your data. Too much serum also interferes with the stability of the stream and increases the potential clumping of cells, these factors affect purity and cell yield.

**Cell size:** plays an important roll in how the sorter is set up. If you are sorting activated cells that are much larger than their non-activated counterparts, the set up will need to take that into consideration for the FSC Area Scaling and the gating scheme. Cell should not exceed 1/3 the size of the nozzle, e.g., a 70um nozzle can sort cell less than 22um effectively. Larger cells may not clog the nozzle but can distort the droplets leading to low yields.

**Cell viability:** The quality of your sorted cells depends largely on the pre-sorted population. Using a viability marker such as PI, 7AAD or DAPI or amine reactive dyes like Zombies or FVS can provide useful data on the original health of the sample. The use of these dyes eliminates sorting or analyzing non-specifically stained events as wells as dead cells that can give misleading total cell yield of a sorted population and analyzed data. Make sure that a viability stain does not interfere with any fluorochrome you are using for sorting before adding to your staining protocol. Sorting does stress the cells, some types more than others. Determine the frequency of your target populations and calculate how much starting material you will need to reach final cell counts needed for the experiment. This will also help estimate the amount of time to signup for.

**Sorting is not 100% efficient!** There is cell loss before, during and after the sorting process. The healthier the cells at the beginning the better the results of the sort will be. There are key values that the sorter will display once the sort begins, soon after the sort has commenced, these values should be looked at and the forecasted yield can be estimated. **Counting cells just prior to final resuspension will aid in accurately determining how many cells you are starting with, what volume to resuspend in, and estimate the maximum yield for each sorted population.

Sorters can theoretically sort at rates up to 25,000 events/sec using the 70um nozzle (this includes debris, dead cells, platelets, and RBC's etc.). Realistically, sort rates are dependent on cell size, stickiness and general health, a good estimator is for lymphocytes is 10,000events/second. This translates to 36E6 cells/hour that can run through the sorter. Events/hour X % of target = theoretical yield/ hour. Example: to calculate how many cells can be sorted per hour of a 2% target population at a rate of 10,000 cells/sec.

36E6/hour X .02= 720,000 cells this assumes no loss due to the following factors:

1. Abort rate = events that the cytometer can not identify, these events are essentially ignored and can potentially be sorted into your sample even if they are not a target event lowering purity. Since these events are not included in the data file high abort rates can also contribute to misleading data.

2. Conflict events / efficiency = This is calculated for each target population being sorted, target cells that have another event leading or following too closely for the sort mask (yield, purity, single cell, etc.) will not be sorted lowering yield.

Possible reasons: Possible resolutions:

| | |
|---|---|
| • cell clumping | • filter cells |
| • high percentage of debris | • DNAse and filter |
| • cell concentration too high | • Dilute cells to lower concentration |
| | • Decrease sample flow rate |

3. General cell population health = your starting population dictates how well the sorted population will be. The healthier the cells are to start with the better they will hold up after the sorting process. Cells that are fragile or already in a compromised state may have lower viability after sorting. Some cell types experience higher cell death, decreased yield and decreased functionality post sort. If these are the type of cells you are sorting, keeping the sample pressure low helps to minimize theses effects. Using a larger nozzle will significantly lower the pressure. Some cell types need to sort at lower concentration and pressure resulting in flow rates of less than 5000cells/sec.

4. Sticky or dry tubes = Some cells are more likely to stick to polystyrene tubes meaning some cells will not get sorted and remain in the starting tube. If your cells are sticky use polypropylene tubes instead. Sorting low % populations into tubes with inadequate media or dry sided tubes can lower yield. The cells end up stuck to the side of the tubes and the sheath fluid dries out resulting in loss of cells.

5. Sort Volume/droplet estimation = For each nozzle size the amount of sheath that is deposited in the collection tube can be estimated keeping in mind sort precision will dictate how many drops are included in a sort event packet e.g. single cell mode will contain one (1) droplet/sorted cell, purity will contain three (3) droplets/sorted cell.

## Droplet volume estimator by instrument

| BD Discover S8 | | |
|---|---|---|
| Nozzle Size | Sheath Pressure | sort droplet volume |
| 85um | 38 psi | 1.8nL |
| 100um | 20 psi | 3.5nL |
| 130um | 7 psi | 8.0nL |

| BD Symphony S6 | | |
|---|---|---|
| Nozzle Size | Sheath Pressure | sort droplet volume |
| 70um | 70 psi | 1.85nL |
| 85um | 45 psi | 3.43nL |
| 100um | 23 psi | 5.39nL |
| 130um | 13 psi | 8.1nL |

| Sony MA900 | | |
|---|---|---|
| Nozzle Size | Sheath Pressure | sort droplet volume |
| 70um | 40 psi | 1.5nL |
| 100um | 20 psi | 3.5nL |
| 130um | 9 psi | 7.9nL |

*sort mode will determine total sort volume. Single cell modes will be a single droplet while yield and purity can include a 3-droplet packet for each sorted cell.