Flow Cytometry Guidance for Single Cell Protocols

Introduction

10x Genomics Single Cell assays require a suspension of high-quality single cells or nuclei as input. Cell sorting using flow cytometry enables the enrichment of specific cell types as well as removal of dead cells, which can be especially useful in sample preparation. This Technical Note provides general guidance on using cell sorting to prepare samples for use in various 10x Genomics assays. For additional information on using cell sorting for specific 10x Genomics products, consult the Demonstrated Protocols available on the 10x Genomics Support website: support.10xgenomics.com.

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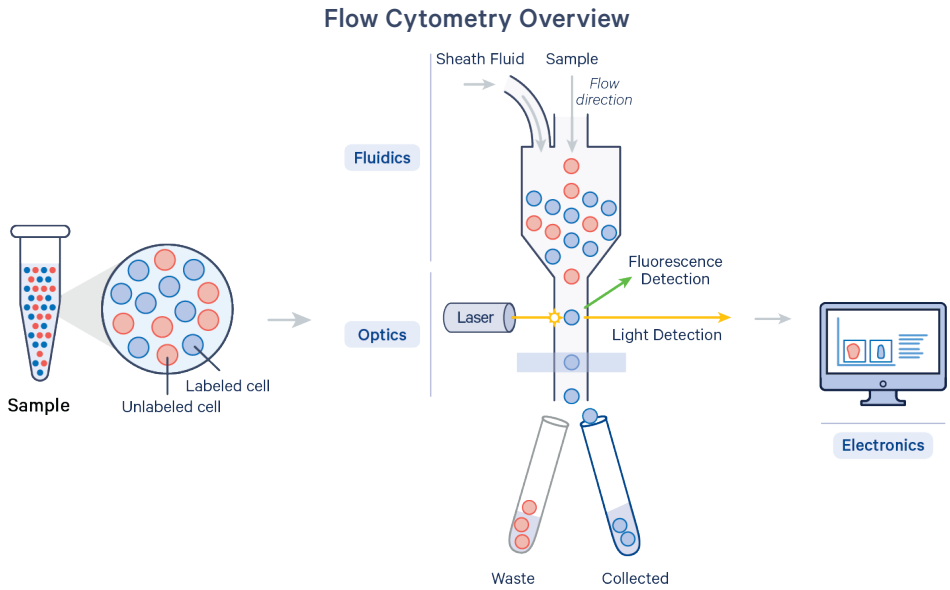
Flow cytometry is a technology that enables multiparametric analysis of single cells or nuclei in a solution and facilitates the exploration of physical and biological details at the subcellular level. In this technology, a flow cytometer instrument generates multiparametric data from the single cell/nuclei suspensions (cell analyzer) and utilizes that data to sort and purify the populations of interest (cell sorter).

10x Genomics Single Cell assays benefit from cell sorting to either characterize and purify a rare population of interest or to clean up a sample that cannot be used as is for generating high-quality data. The success of a flow cytometry/cell sorting experiment depends on the experimental design, sample quality, instrument settings, and choices made during cell sorting and postsorting sample collection. This document provides guidance on using cell sorting to obtain good quality single cell data from sorted cells.

Flow Cytometry Overview



A cell suspension is loaded onto the cell sorter/analyzer, where it is aspirated into a rapidly flowing liquid stream. Vibrations within the machine cause the liquid stream to break into individual droplets. These droplets are passed through a laser, which excites any fluorophores associated with the cell in the droplet. The light scattered by the cells (forward scatter, FSC and side scatter, SSC) and the fluorescence emitted is detected and converted to electrical signals to generate multivariate data. Additionally, cell sorters can also direct the cells into different collection tubes based on the analyzed parameters.

Flow Cytometry System

A flow cytometer includes three main systems that work in alignment:

- **The fluidics system** enables the flow of cells/particles in a single stream to an interrogation point, where each event interacts with a laser beam.
- **The optics system** includes a laser beam (comprising multiple spatially separated lasers) to illuminate each cell/particle, which in turn scatters and emits light/fluorescence. Optical filters direct the emitted signals to detectors (see Signal Detection section for details).
- **The electronics system** converts the emitted signals to numerical values to generate data (see Signal Processing & Data Readout section for details).

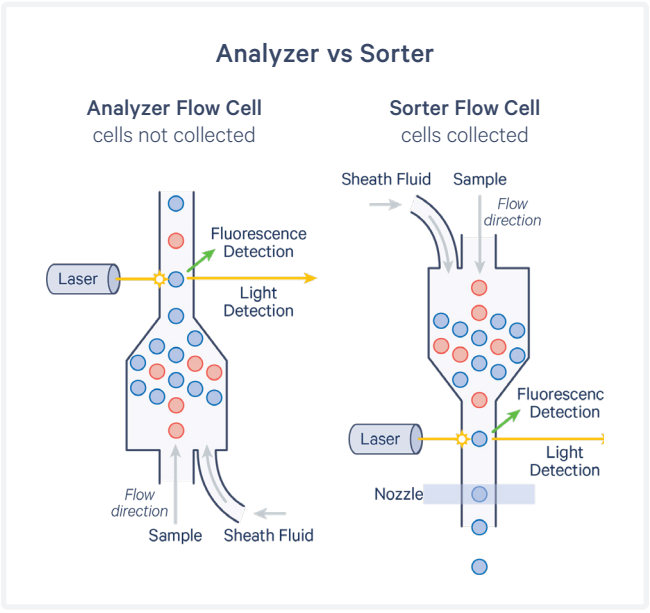
Flow cytometry can be analytical or could include cell sorting. Both use the same underlying principles where the cells/particles are first analyzed. After that, the cell sorters generate droplets and direct cells/particles as per predefined parameters into different collection tubes.

Analyzer vs. Sorter

Flow cytometers can be broadly classified into analyzers and sorters based on their functions. An analyzer provides high-resolution data in a short period of time. A sorter has an added advantage of using this same data and yielding purified/enriched cells as the final product. This in general makes the cell sorter a more complex instrument.

Analyzer & Cell Sorter Common Features
• A cell analyzer/sorter takes cells from the input tube to the flow cell where the lasers interrogate every cell • In the flow cell, lasers of different wavelengths excite any fluorophores associated with the cell, resulting in emission of fluorescence at a different wavelength • Once the cell is interrogated by the laser, a voltage pulse is recorded

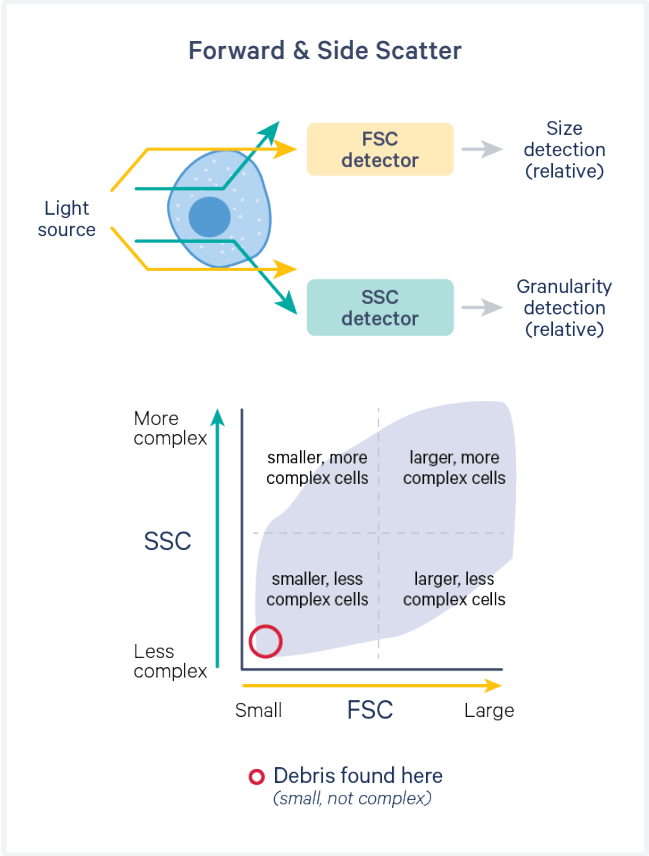
Analyzer	Sorter
• Signal derived from the voltage pulse is converted to data, which is the final output • The cells pass through the analyzer once and there is no cell recovery	• Signal derived from the voltage pulse is converted to data and stored • The cells then reach a nozzle where they are sorted based on predefined parameters



Signal Detection

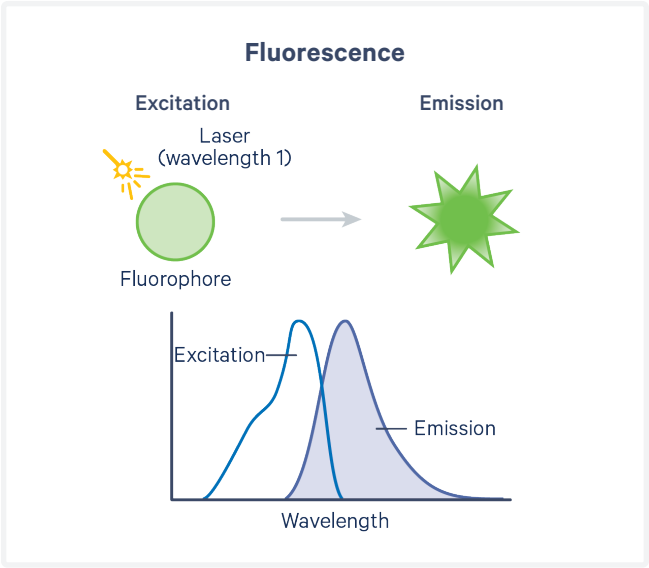
Light Scattering

All cells/nuclei/events passing through the laser beam scatter light and this light is measured by optical detectors. Detectors in front of the light beam measure forward scatter (FSC) and detectors to the side of the light beam measure side scatter (SSC, also known as back scatter, BSC). FSC indicates the size of the cell relative to granularity, while SSC indicates granularity of the cell relative to size of the cell.



Fluorescence Emission

Fluorescence detectors within the flow cytometer measure the fluorescence emitted from labeled cells/nuclei/events. For example, when cells labeled with an antibody conjugated to a fluorophore or cells that autofluoresce are excited by a laser with the corresponding wavelength, they will emit fluorescence at a different wavelength.



Signal Processing & Data Readout

FSC and SSC light and fluorescence emission from cells are split into defined wavelengths and channeled by a set of filters and mirrors towards detectors known as photomultiplier tubes (PMTs). The PMTs convert the energy of a photon into an electrical signal/pulse. The fluorescent light is filtered so that each sensor will only detect fluorescence at a specified wavelength.

As the pulses are generated, the electronics system quantifies each pulse to measure signal intensity (based on pulse height and area) while also distinguishing closely interacting particles and doublets (based on pulse width). A computer then processes the gathered data and displays plots and graphs, which can be used to understand the characteristics of the sample.

Spectral & Conventional Flow Cytometry

Flow sorting can be performed on both the spectral and the conventional flow cytometers. However, there are some key differences during signal processing and data readout when using spectral versus conventional cytometers.

Spectral Cytometers	Conventional Cytometers
• Fluorescence is measured using signals from all the detected channels, irrespective of the number of fluorophores • The fluorescence intensity adds up wherever the spectra overlap	• Fluorescence is only measured within specific defined ranges along the spectrum using one detector for each fluorophore • Any fluorescence signal that is outside the defined detection range is not collected

Antibody Panel Design & Development

Overview

This chapter provides guidance on antibody panel design and development for cell sorting to maximize viability, separation, and isolation of cells for a successful downstream 10x Genomics Single Cell application.

Key Considerations

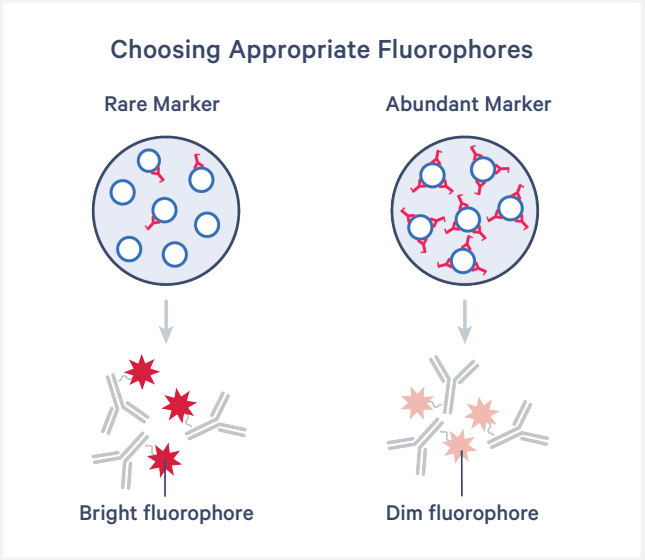
The following factors should be carefully considered before starting antibody panel development for cell sorting.

- Overall quality of the sample type
- Biological background and cellular abundance of the cell type of interest
- Expression level of markers of interest
- Instrument settings including number of lasers and filters

Markers and Fluorophores

Antibody panel development involves defining the cellular markers (see table below) by understanding their expression level and then matching those markers with the appropriate fluorophores.

Marker Type		
Primary	**Secondary**	**Tertiary**
• Identify broad subsets • Parental in nature • High expression • Usually serve as a lineage marker	• Well characterized • Moderate expression	• Uncharacterized • Low expression



Abundant markers (primary and secondary) should be matched with dim fluorophores and rare markers (tertiary) should be matched with bright fluorophores. Consult this relative brightness index chart to determine which fluorophore is bright and which one is dim.

Panel Development

Marker panels for flow sorting should contain the following components:

- **Live Dead Marker**: These markers selectively label dead cells, allowing the sorter to distinguish between live and dead cell populations. Refer to Sample Staining - Viability Dyes for the dead cell markers compatible with 10x Genomics Single Cell workflows.
- **Markers for Desired Cells**: Antibodies specifically labeling the population of interest including the lineage and specific markers for smaller subcellular subsets should be included.

- **Markers for Undesired Cell Lineages:** Additionally, markers for cells that are not desired should also be included so that these cell populations can be excluded during sorting.

Example Antibody Panel for Human CD3+ CD8+ cells

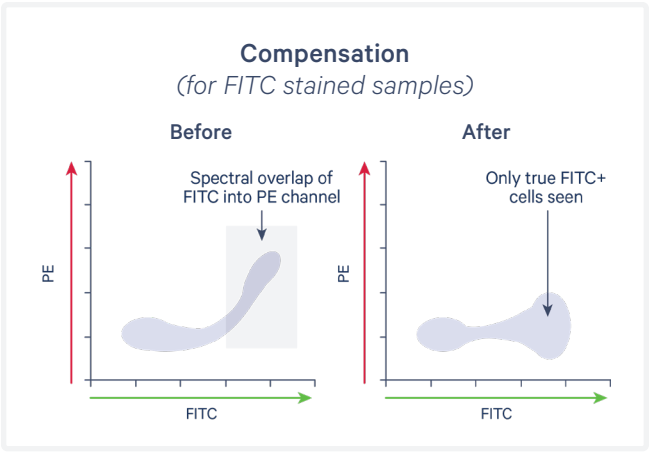
Marker	Target Cell Type
CD3 (+)	Total T cells
CD8 (+)	Cytotoxic T cells
CD56 (-)	Natural killer cells
7-AAD (-)	Live dead marker

Controls

Compensation Controls

When using multiple fluorophores in one experiment, consider that the spectral overlap between fluorophores (sometimes called spillover) will need to be corrected. To determine the true contribution of each fluorophore to the signal in a given detector, compensation must be performed.

- Use compensation beads (i.e. UltraComp eBeads Compensation Beads, 01-2222-41, Thermo Fisher Scientific) labeled separately with each antibody that will be used in the sort.
- For a live/dead control, use a sample rich in dead cells and label with the live/dead dye. A sample rich in dead cells can be generated by heating a sample at 65°C for 5–10 min.
- Compensation controls should be made fresh on the day of the experiment.

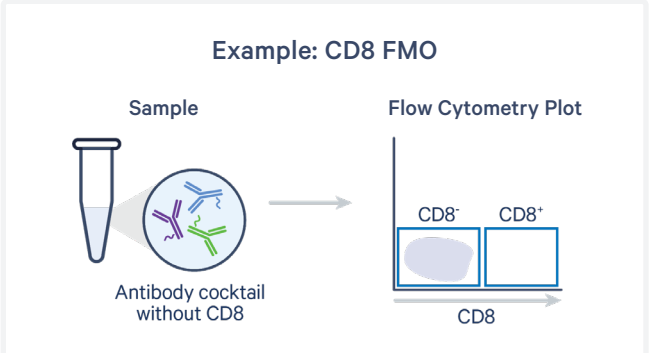


FMO Controls

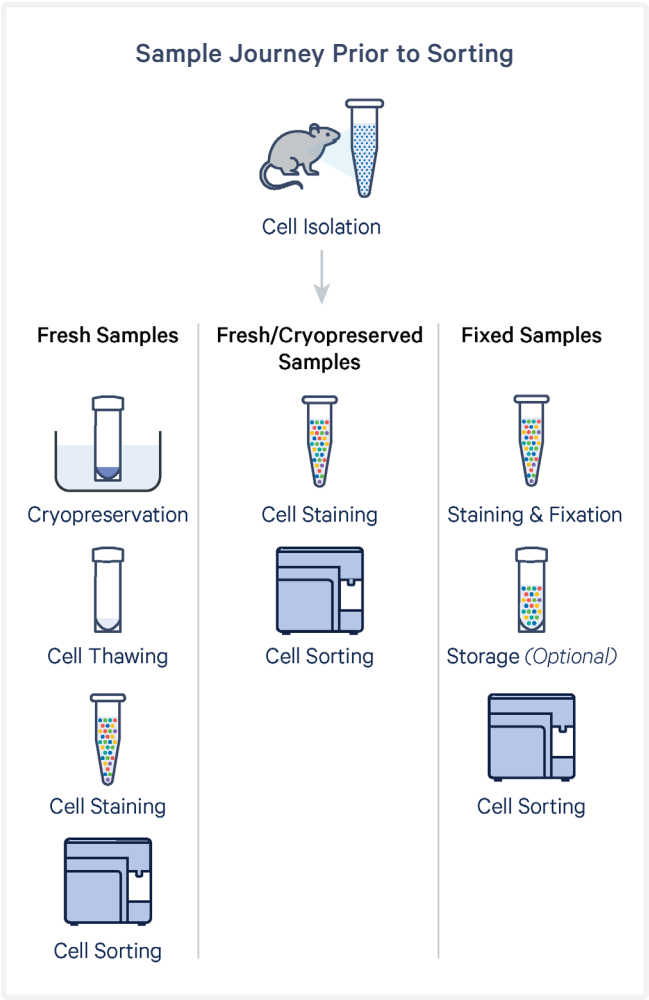
Fluorescence minus one (FMO) controls are samples that are labeled with all the antibody/fluorophores in the panel except for one. These controls are used to determine the maximum background signal for the missing fluorophore and thus can help in accurately identifying and gating the positive populations. A positive gate can be set for a particular fluorophore based on its respective FMO. This is especially useful in rarely expressed markers or in markers that have a smear staining.

For example, a CD8 FMO control in an experiment for sorting CD8+ cells will include all the required antibodies (CD3, CD56, and 7-AAD) except CD8 itself (see below).

Marker	Target Cell Type
CD3	Total T cells
CD56	Natural killer cells
7-AAD	Live dead marker



Sample Preparation for Cell Sorting



Overview

There are many sample preparation steps involved, from tissue/cell procurement to actual sample sorting. Proper preparation during these steps is critical for obtaining a high-quality single cell suspension following sorting. This chapter provides an overview on best practices for preparing samples for flow sorting.

Sample Quality

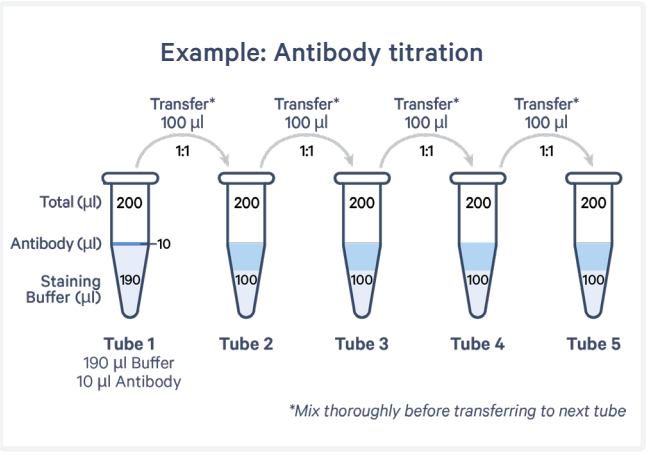
The key considerations for preparing high-quality single cell suspensions are minimal cell debris, low levels of cell aggregates, and high cell viability (at least 70%). Some standard cleanup methods that can be used alone or in combination to remove debris and aggregates include filtration, cell washes, slow centrifugation, and density gradient centrifugation. Consult the Cell Preparation for Single Cell Protocols (Document CG00053) for details.

Sample Staining Best Practices

Antibody Titration

Always perform antibody titration to find:

- The minimum antibody amount required to stain the cells of interest for good quality staining
- The appropriate concentration of antibodies required to achieve the maximum separation between positive and negative populations



Viability Dyes

Dead cells should be excluded from the analysis as they are more autofluorescent than live cells and bind antibodies nonspecifically, leading to false positive data. However, it is difficult to eliminate dead cells based on the forward and side scatter properties alone and it is therefore recommended to include a viability dye while staining the samples for flow analysis.

Viability dyes compatible with 10x Genomics Single Cell workflows include:

Assay Type	Sample Types	Recommended Viability Dye
3' and 5' Gene Expression Assays	Cells/nuclei	• 7-aminoactinomycin D (7-AAD) • DAPI/Hoechst • Other live/dead stains may also be used to enrich viable cell populations of interest in single cell suspensions
Fixed RNA Profiling Assays	Postfixation samples	• 7-AAD • DAPI/Hoechst • Other DNA-based dyes
ATAC and Multiome ATAC + Gene Expression Assays*	Nuclei	• 7-AAD

**The choice of sorting dye is critical for both ATAC and Multiome ATAC + Gene Expression assays. Several intercalating or minor-groove binding dyes can disrupt chromatin structure and negatively impact data quality. The dyes that are NOT recommended for ATAC and Multiome ATAC + Gene Expression assays include PI, DAPI, Ethidium Homodimer, Vybrant DyeCycle Green, and Zombie Violet.*

Sample Filtration

Flow sorting requires homogenous single cell suspensions. The samples left in inappropriate suspension buffers or at suboptimal temperatures for an extended period may exhibit aggregate formation. These aggregates as well as other impurities including debris and fibers can clog the flow cytometer. It is therefore recommended to filter samples prior to sorting.

Additionally, certain sample types, e.g., dissociated tumor cells have lower quality samples and will require filtration using a 40 or 70 µm cell strainer.

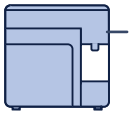
Cell Sorting for Single Cell Protocols

Overview

This chapter provides guidance on cell sorter parameters, gating strategies, and postsorting sample collection.

Cell Sorter Parameters

Cell Sorter Guidelines



- Select a nozzle that provides gentle pressure
- Ensure sheath fluid is compatible with 10x workflow (no EDTA or excessive magnesium)
- Adjust flow rate to maintain linear stream of cells & high sort efficiency
- Choose the appropriate sorting mode

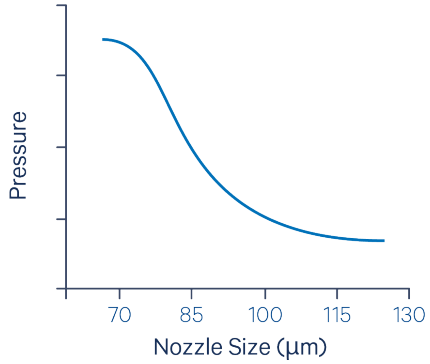
Nozzle Selection

The correct nozzle size and associated pressure play important roles in sample quality postsorting. The typical nozzle sizes available on a cell sorter are generally 70, 85, 100, or 130 µm.

The larger the nozzle size, the lower would be the pressure. Nozzles with mild pressure help preserve cell viability and integrity, enabling optimum sample input for downstream single cell RNA sequencing assays. Typical cell sorting guidance dictates that the nozzle should be at least three times larger than the average cell size.

However, postsort cellular health is preserved better at gentler sort pressure (larger nozzle size). Thus, it is recommended to use a nozzle that provides gentler pressure sort irrespective of the size of the cell to better preserve cells for downstream 10x Genomics Single Cell assays.

Effect of Nozzle Size on Sort Pressure



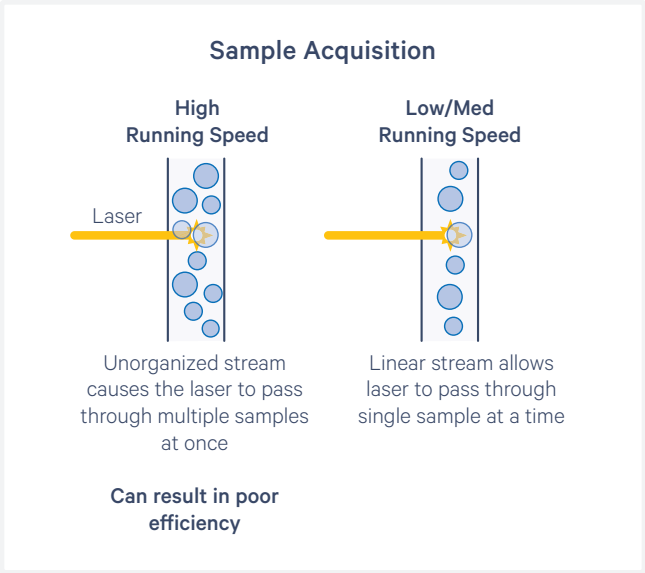
Sheath Fluid

Sheath fluid is the liquid that flows through the cell sorter. Cells are injected into the sheath fluid, which carries it past the laser. Sheath fluids should be compatible with 10x Genomics workflows and should not contain EDTA or excessive amounts of magnesium (less than 3 mM magnesium final concentration postsorting).

If the sheath fluid has EDTA or excessive magnesium, the sample should be concentrated by centrifugation, and the pellet should be resuspended in appropriate buffers postsorting.

Flow Rate

The flow rate, defined as the volume of fluid per second flowing through the sorter, can either have a numerical range or low, medium, or high settings. These settings can be adjusted to influence the sample injection area width. For example, an increase in flow rate will widen the sample injection area and increase the number of droplets containing multiple cells, which will impact data quality.



Sorting Efficiency

Sorting efficiency is a real-time measurement of the number of events out of the total sortable events that the instrument is sorting.

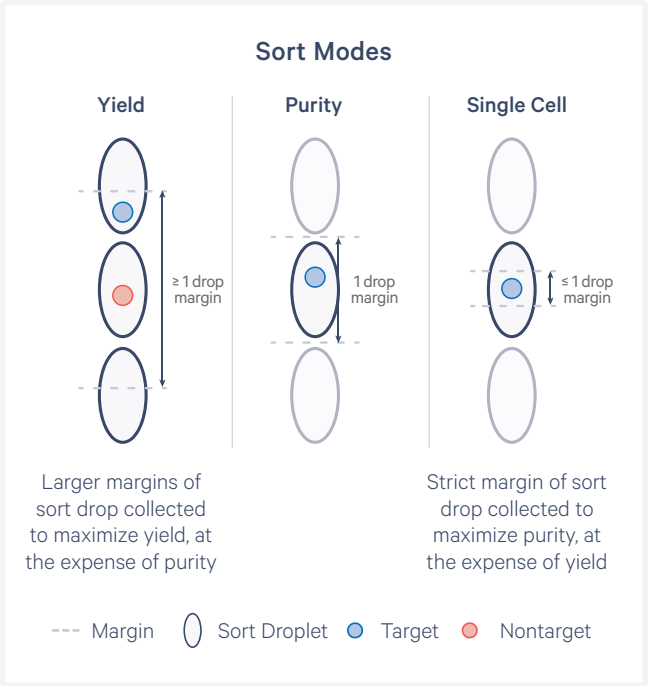
Maintaining a sorting efficiency of above 90% is critical for obtaining a pure target population and reducing contamination. The following recommendations will help improve efficiency:

- The flow rate should be low to medium to reduce the number of collinear events.
- Use high-quality samples to minimize the amount of debris present during the sort.
- Ensure the gating strategy used (discussed in the next section) does not have any logical flaws.

Sorting Modes

Cell sorters typically have a variety of sort precision modes available. It is recommended to use Purity mode for sorting. Purity mode means that if a nontarget drop is 1/2 drop away, sorting of the drop containing a target particle will not occur. It is not necessary to use a Single Cell mode, as this will greatly reduce yield.

The sorting mode nomenclature might vary based on the specific sorter. The figure below explains the Purity mode in a Sony MA900 sorter.



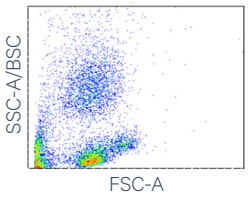
Adjustments during Sorting

During sorting, FSC and SSC/BSC voltages should be adjusted to exclude doublets and dead cells to resolve cells of interest and large particles.

Adjusting Voltage

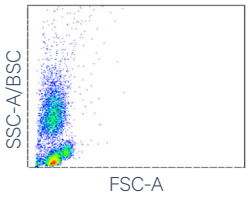
Good

- Clear separation of cells of interest and cell debris, dead cells, etc.



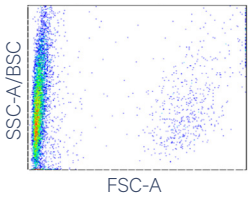
Too Low

- Loss of resolution of small events.



Too High

- Loss of resolution of large events.



FSC-A: forward scatter-area
SSC-A: side scatter-area
BSC: back scatter

Gating Parameters

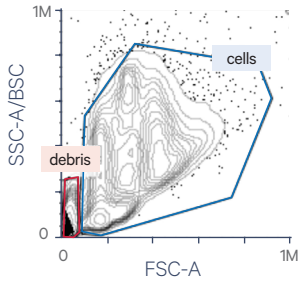
Gating Parameters for Sorting Cells

Gating out unwanted cell populations will help enrich the population of interest.

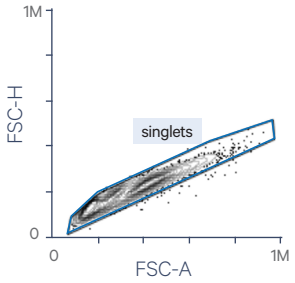
- Gate on forward scatter-area (FSC-A) vs. side scatter-area/back scatter (SSC-A/BSC). Gating for granularity will exclude small debris particles.
- Plot the height (FSC-H) or width against the area for FSC or SSC to identify and exclude doublets.
- Use FSC-A vs. viability dye to gate out positive (dead) cells.

Gating Parameters for Cells

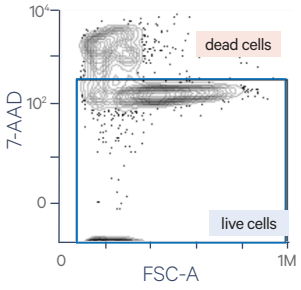
1. Cells



2. Singlets

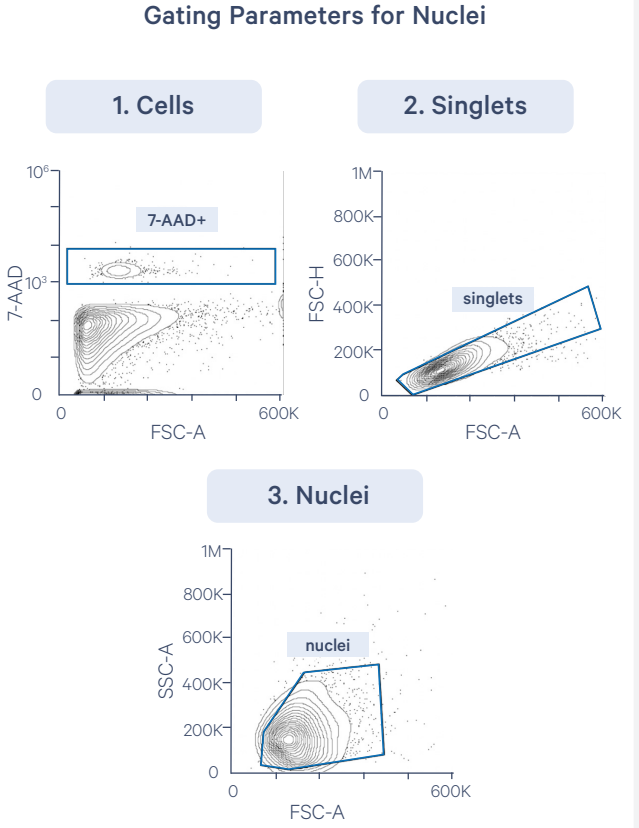


3. Live Cells



Gating Parameters for Sorting Nuclei

- Gate for nuclei dye positive events using 7-AAD+ or other DNA intercalating dye.
- Gate on singlets using FSC-A and FSC-H.
- Gate on FSC and SSC/BSC (back scatter) to select nuclei. Nuclei are smaller than cells, but slightly larger than debris. Carefully set FSC and SSC/BSC to ensure the population is not lost.



Sample Collection

Sample Collection Guidelines

- Tube coated & filled with collection buffer before sorting
- Composition: optimal for sample with increased concentration of serum/ BSA to account for dilution during the sorting process
- Compatible with 10x workflow (no EDTA or excessive magnesium)
- Volume: depends on sorter, nozzle, sort mode, and desired # of sorted cells
- Temperature: 4°C

Droplet Volume

Droplet volume may vary from sorter to sorter and should be determined prior to performing the assay. This may be done by sorting a known number of events into an empty tube using the same sort mode and flow rate parameters as will be performed on the actual experiment. The resulting volume in the tube can be used to determine the per-droplet volume. The per-droplet volume can be used to predict the final volume that will be obtained after sorting a desired number of cells.

Collection Buffer

- Cells/nuclei should always be collected in a suitable buffer/media to preserve their viability.

Sample Type	Collection Buffer
Cells	5-20% FBS in PBS for collecting ≤500,000 cells
	100% FBS for collecting fragile cells, ≥500,000 cells, or if the sorting duration is ≥30 min
Nuclei	PBS + BSA

- A higher percentage of FBS is needed to maintain cell viability when sorting larger cell numbers, as the collection buffer will get diluted during sorting.
- Collection buffer volume should be such that the cells are not being diluted too much. For ≤10,000 cells, sort into low volume of collection buffer.
- For >10,000 cells, the sample can be concentrated by centrifugation and removing the supernatant. If no pellet is visible, leave 20–50 µl supernatant behind. Select a centrifugation speed compatible with the sample type. For low-yield and fragile cells, a longer, slower speed is recommended.
- Cells/nuclei are often more fragile after sorting. Minimize additional sources of cell stress like handling, temperature, and time. Keep samples on ice before, during, and after sorting.
- Count cells/nuclei after sorting or after concentration. Numbers reported by flow cytometers can vary by up to 50% and must be confirmed before loading. If the yield is low, additional counting should be avoided.
- Promptly load cells into 10x Genomics chips after sorting/postsorting concentration to avoid a decrease in viability.

Sorting Temperature

Keep cells on ice and in the dark prior to sorting. During sorting, the collection tube and sample should be chilled. Place the sorted cells on ice immediately after sorting.

This section provides general guidelines on sample collection. For collection of antigen+ cells using Barcode Enabled Antigen Mapping (BEAM) workflow, consult Reagent Assembly, Sample Labeling & Flow Sorting For Barcode Enabled Antigen Mapping (Document CG000595).

Additional Resources

Topic	10x Genomics Resources
Sample preparation guidance	CG000053 Cell Preparation for Single Cell Protocols
Guidance on Barcode Enabled Antigen Mapping (BEAM) workflow	Chromium Single Cell 5' Barcode Enabled Antigen Mapping (BEAM) - Reagent Assembly, Sample Labeling, and Flow Sorting User Guides CG000598 Chromium Single Cell 5' Barcode Enabled Antigen Mapping (BEAM) - Flow Sorting Guidance
Postfixation sample sorting	Refer to 10x Genomics Support website Fixed RNA Profiling Q&A for information regarding postfixation cell sorting
Compatibility with TotalSeq antibody-oligonucleotide conjugates	CG000231 Quality Control of Cell Surface Protein Labeling using Flow Cytometry

Topic	Other Resources
Sort mode guidance	Sony MA900 Sorting Modes Guide
Spectral cytometry	Sony Introduction to Spectral Flow Cytometry

Document Revision Summary

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