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Sony Biotechnology Inc.

Basics of droplet-based sorting

Droplet-based cell sorting is a powerful technique used in research and clinical applications to separate single cells, or a subset of cells with specific characteristics from a heterogeneous population. Cell sorter instruments first analyze individual cells for light scatter and fluorescence parameters using established flow cytometry techniques, before physically separating them into collection tubes via deflection or redirection. To do this, the cell suspension is passed through the center of a narrow, rapidly flowing stream of liquid, creating a steady cascade of individually spaced cells that is then broken up into individual droplets. Information about the physical characteristics of each droplet-encased cell is provided to the operator to select the target cell population of interest.

Overview of cell sorting systems

There are three main components of a droplet-based cell sorting system: optics to detect light signals, electronics to convert those signals into data, and fluidics to generate droplets.

Optics

Each particle passes through focused laser beams, creating scattered light and fluorescence signals. Optical mirrors and filters (Figure 1) split the emitted light into narrower wavelength ranges that are delivered to specific detectors known as photodiodes or photomultiplier tubes (PMTs). The anode of the detector then emits a stream of electrons that is proportional to the intensity of the scatter or fluorescence signal detected, generating an optical signal or 'pulse'.

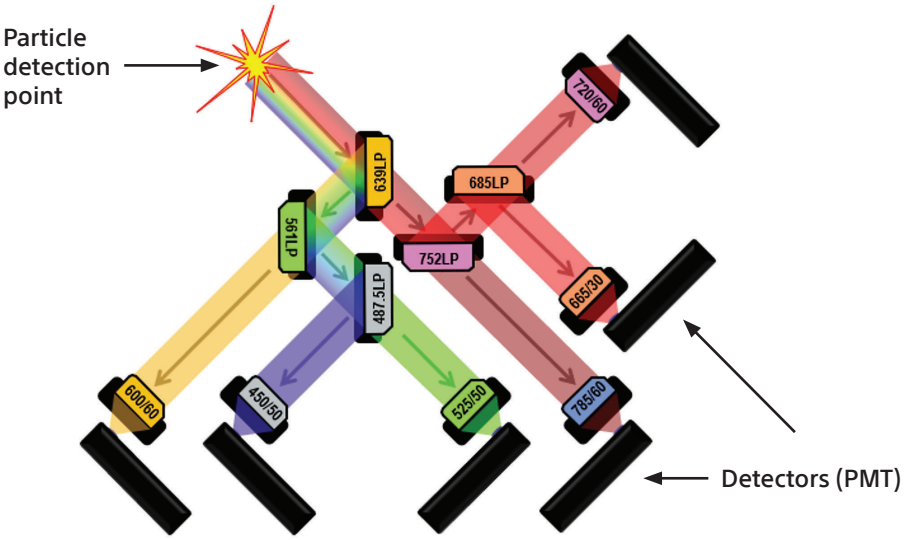

Figure 1: Optical mirrors and filters split emitted light to be read by specific detectors.

Electronics

The electronic system processes optical signals from the detectors by converting the voltage pulses from an analog to a digital format, then sending them to the computer for data processing. The measurements recorded – including forward scatter (FSC), side scatter (SSC), and marker expression – are then used to select the target cells for sorting based on user-defined criteria. The combination of FSC and SSC, which provides information about the particle size and complexity respectively, is often unique to the target cell type, and can be combined with the expression of specific markers to isolate target cell populations.

Fluidics

The fluidic system is critical to droplet formation, and the majority of cell sorters work by using compressed air to drive sheath fluid from a tank into the flow cell and out through a narrower nozzle of a specified size, increasing the fluid velocity to create a laminar flow. The sample is then injected into the center of this stream of sheath fluid and hydrodynamically focused by the Bernoulli effect, causing cells to move in a single-file cascade through the laser beams. The nozzle or entire sorting chip is vibrated at a specific high frequency to generate uniform droplets containing individual cells at a very steady rate and precise distance from the nozzle. The cells are then sorted based on one of two principles – electrostatic ballistics or microfluidics.

SH800



MA900



Types of cell sorting

Electrostatic ballistic cell sorting

The most prevalent commercially available instruments are electrostatic ballistic cell sorters. These sorters work by applying a charge to droplets containing cells that satisfy the gating criteria, then passing droplets through the air between two deflection plates that create an electrostatic field. The charged droplets are deflected into a collection vessel, while uncharged droplets drop into the waste. Standard electrostatic ballistic cell sorters are well suited to isolating and analyzing small particles such as lymphocytes. However, larger particles can be marginally more difficult to sort due to their size and susceptibility to shear stress. More recently, large-particle cell sorters or specialty modules, including wider nozzle heads, have become available. These sorters are specifically designed to isolate larger targets that cannot be accommodated by conventional systems, however, they may offer lower throughput.[1]

Sony Biotechnology offers two electrostatic ballistic cell sorters that use microfluidic chips for cell interrogation and droplet formation: the SH800S Cell Sorter and the MA900 Multi-Application Cell Sorter. These automated systems are designed to simplify and standardize flow cytometry workflows. The SH800S is a compact, user-friendly system with up to four collinear excitation lasers (405, 488, 561 and 638 nm) and six fluorescence detectors, making it well suited to routine sorting across a range of cell sizes. The MA900 is a multi-application platform that allows the detection of up to 12 fluorescence parameters (14 total parameters) and four-way sorting, enabling complex, high-dimensional experiments.

Practical considerations – how to set up a sort

When setting up a sort, it is important to understand the properties of the cells that are being sorted, and their intended downstream use. This chapter discusses these factors, as well as the practical considerations for preparing the cells and the sorting equipment that will lead to success.

Considering cell type, cell number and downstream applications

The cells of interest and their use will influence cell preparation and the sorting parameters, including mode, nozzle selection and the choice of collection vessel. Table 1 summarizes the properties of the sort that should be considered and the decisions required. These factors are discussed in more detail further on in the chapter.

	Property	Considerations for cell preparation	Considerations for cell sorting
Upstream	Cell format (solid tissue, cell culture etc.)	Preparation method to achieve single-cell suspension	
	Cell size	Appropriate mesh for filtering cells	Nozzle size and pressure/drop frequency
	Cell type (morphology, fragility, stickiness)	Sample buffer recipe; optimal sample concentration	Nozzle size and pressure/drop frequency
	Markers to identify target cells	Appropriate color panel; single-color compensation controls	Laser and filter selection
	Specialized labeling requirements	Fix/perm for intracellular markers; FoxP3 for nuclear markers	
Downstream	Purity requirements	Optimal sample concentration	Sort mode selection – purity or enrichment; requirements for purity check; need for doublet exclusion
	Number of cells required	Number of cells to prepare	Time to complete sort; how long to book cell sorter
	Format required	Collection media recipe; coating of receptacles; appropriate collection buffer volume	Collection vessel – plate or tube; calibration of cell sorter; alignment and collection height

Table 1: Factors to consider during cell and equipment preparation for a successful sort.

Cell prep optimization

Optimal sample preparation is arguably the most critical factor for successful cell sorting. Regardless of the cell source, there are some common key considerations for keeping cells viable and ready for sorting.

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Prepare a single-cell suspension

Cells should be harvested as a single-cell suspension. For adherent cells, this can be achieved by:

- preparing cells for detachment by washing them with a neutral buffer like phosphate-buffered saline (PBS);
- detaching cells using trypsin, Accutase™ or other enzymatic methods;
- halting enzymatic digestion using serum-containing culture media;
- mechanically dissociating cells through tapping or scraping;
- collecting and concentrating cells through pipetting and centrifugation;
- washing cells properly in calcium- and magnesium-free buffer to reduce clumping;
- keeping the final sample on ice prior to sorting.

Sample concentration

The sample concentration should be adjusted based on the cell type and the nozzle that will be used. A guide to the ideal concentration is shown in Table 2. 'Sticky' cells should be run slightly more diluted.

Nozzle size	Example cell types	Concentration (cells/ml)
70 microns	Lymphocytes, thymocytes	10-20 x 10^6
100 microns	Myeloid cells, larger adherent cells	5-10 x 10^6
130 microns	Larger adherent fragile samples	1-5 x 10^6

Table 2: Cell suspension concentrations depending on nozzle size.

Maintain single cells

Once the cells are prepared, they should be maintained as single cells by:

- filtering through a 35-50 micron mesh, depending on the cell size, prior to sorting;
- using an onboard agitator and cooling element in the cell sorter.

Setting up a sorter

The cell sorter should be prepared according to the type and size of the cells that are being sorted, and what receptacle they are being collected in.

Nozzle selection

Nozzle selection involves a balance between sorting speed and the size of the cells. Smaller nozzles generate more droplets per unit of time than the larger nozzles, so the sort can run faster. However, small droplets can only contain small cells, and smaller nozzles result in higher pressures, which can stress fragile cells. Bigger nozzles can handle larger cell types, but will take longer to sort an equal number of cells, due to the slower droplet rate.

Generally, the nozzle diameter should be about four to five times larger than the cell size, otherwise purity and recovery may be reduced. The 100 micron nozzle works for the majority of common cell types, including any of the smaller cells, so is a good starting point if it is not clear which nozzle to choose. For very large, fragile or unevenly shaped cells, the 130 micron nozzle is likely to give the best viability, as the large droplet size and low pressure minimize stress on the cells.

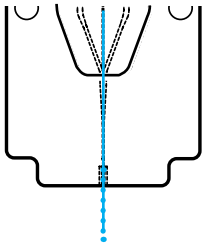
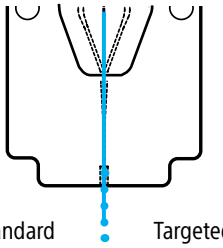
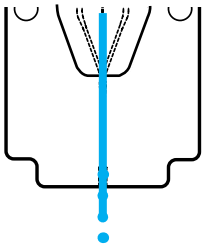
Part number	LE-C3207	LE-C3210		LE-C3213
Nozzle size	**70 µm**	**100 µm**		**130 µm**
Mode		 Standard	Targeted	
Drop frequency (Empty drops/second)	~50 K	~30 K	~22 K	~12 K
Max suggested* event rate (Total events/second)	12 K	6 K	<4 K	2.5 K
	*When sorting for purity, do not exceed the indicated event rate.			
Drop volume	1.5 nL	3 nL	4 nL	7.9 nL
Sorted events/1 mL (of sorted volume)	~700 K	~333 K	~250 K	~40–125 K
Application	• Fastest sort speed • Limited to small particles • Runs at highest pressure	• Most common nozzle for various cell types • Targeted mode improves targeting precision of larger cells with uneven geometry into tubes or multiwell plates		• Runs at lowest pressure • Slowest sort speed • Can sort any cell size under 25 µm
Cell type examples	*E. Coli* RBC Yeast Spleen Lymphocytes	Bone Marrow CHO Macrophages Cultured Stem Cells		ES cells K562 Fibroblasts Neurons Hybridoma HeLa HEK-293T

Figure 2: Suggested nozzles for different applications and cell types on the MA900 Multi-Application Cell Sorter.

Setting up the sorter

The next stage is to calibrate the instrument for optimal data detection and sorting. The MA900 Multi-Application Cell Sorter and SH800S Cell Sorter automate this process, which takes less than 20 minutes to complete. Calibration involves:

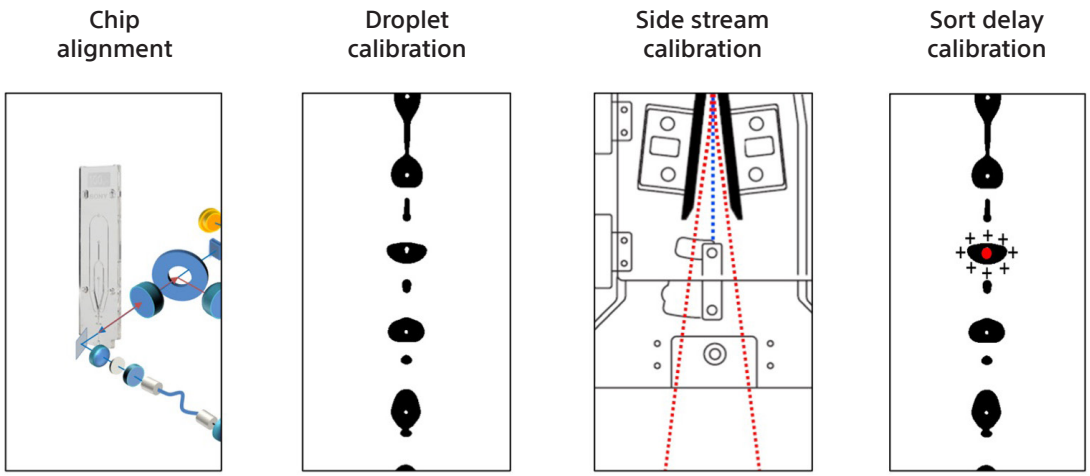


Figure 3: The steps of instrument calibration.

- Chip alignment – aligns the chip's flow cell with the focal point of the lasers.
- Droplet calibration – achieves stable, consistent droplet formation.
- Side stream calibration – determines whether the sorted droplets are deviated uniformly, and accurately hit the bottoms of the collection tubes.
- Sort delay calibration – manages the timing of droplet charging so that only the droplets with target cells are sorted.

Preparing for collection

Before starting the sort, it is important to make sure that drops are being correctly deflected into the collection vessel. This will vary depending on the cells and whether they are being sorted into tubes or plates.

Sorting into tubes

There are a number of factors to consider when sorting cells into tubes to minimize cell loss:

- do not align the stream to the center of the tube;
- watch for droplet formation on tube walls when testing deflection;
- use a splatter shield to prevent contamination of the tubes.

Sorting into plates

When sorting into plates, it is very important to calibrate the plate accordingly, since wells are smaller compared to tubes. Do this by:

- aligning sort stream as vertically as possible using an angled holder or by deflecting the center stream;
- sorting 30-50 drops onto the lid to confirm calibration;
- validating plate calibration using a colorimetric method, such as the horseradish peroxidase (HRP) method.

Sort modes – choosing between purity and yield

The sort mode should be selected to provide the best cell population for the downstream application. Sony cell sorters have a choice of three main sort modes – purity, enrichment, and single cell – depending how the cells will be used, and the purity and yield required. Each mode has its own trade-offs that must be considered.

Purity mode

Purity mode is the most common, and is selected when the focus is to obtain the highest purity possible in the sorted population. This mode will only sort droplets that contain cells of interest, and will abort droplets containing a mix of cells. In this mode, unlike single-cell mode, droplets that contain multiple cells of interest will be sorted. This mode provides the best purity, which should not change with throughput, but some cells of interest will be lost in the mixed droplets.

Yield mode

Yield mode is focused on capturing every cell of interest, even if there is also a contaminating cell in the drop being sorted. As a result, the yield will be high – usually around 99.9% – but the purity will be lower, especially at higher throughputs. The yield mode is suited to precious samples, where recovery of all the cells of interest is more important than purity.

Single-cell mode

Single-cell mode also offers high purity, but only sorts droplets that contain a single cell of interest. This mode is ideally suited for cloning applications, when low yields are not typically a problem.

Choosing sorting conditions

Once the cells and sorter are prepared, it is time to identify the populations of interest and select the target cells to be sorted. Data gating is often performed sequentially, starting with the healthy cells that are singlets, followed by gating on populations expressing specific markers.

Healthy cells

It is helpful to include a viability stain to correctly identify healthy target cells, as gating with only the scatter parameters is not always sufficient. In general, this is good practice to ensure that sorted cells are healthy and representative of their parent populations.

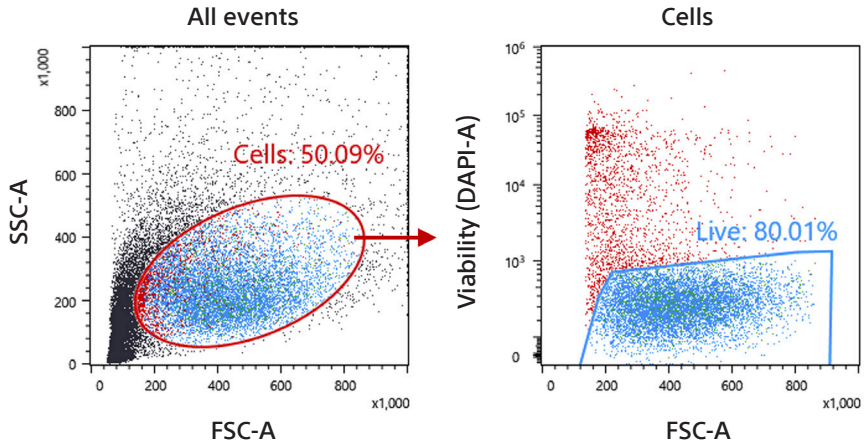


Figure 4: A viability dye allows dead cells to be excluded from a sort.

Compensation

If the sort involves multiple fluorochromes with similar emission maxima, it is important to calculate compensation, as overlap can occur between some fluorochromes. Compensation is a mathematical process that corrects for spectral overlap between detectors, and modern cytometers offer wizards or guided workflows to calculate accurate compensation matrices.

Doublet exclusion

Another significant factor in correctly identifying target cells is doublet exclusion. Doublets are two or more cells stuck together that appear as one particle. Doublet exclusion is critical for all sample types, and especially crucial for single-cell applications.

Each particle passing through the flow cell of a cytometer scatters the laser light onto the detectors. The detectors convert light signals into voltage pulses for each cell, as shown in Figure 5. Each pulse is digitized to give numerical values for its height, area and width. In combination, these parameters can identify if a single cell or doublet is present. In Figure 5, for example, two small cells stuck together generate a wider voltage pulse compared to its height, because they take longer to cross the optical detection point than a small single cell. To identify and gate single particles, pick a detection mode – such as the forward or side scatter – and plot its area, height, or width parameters, one against the other.

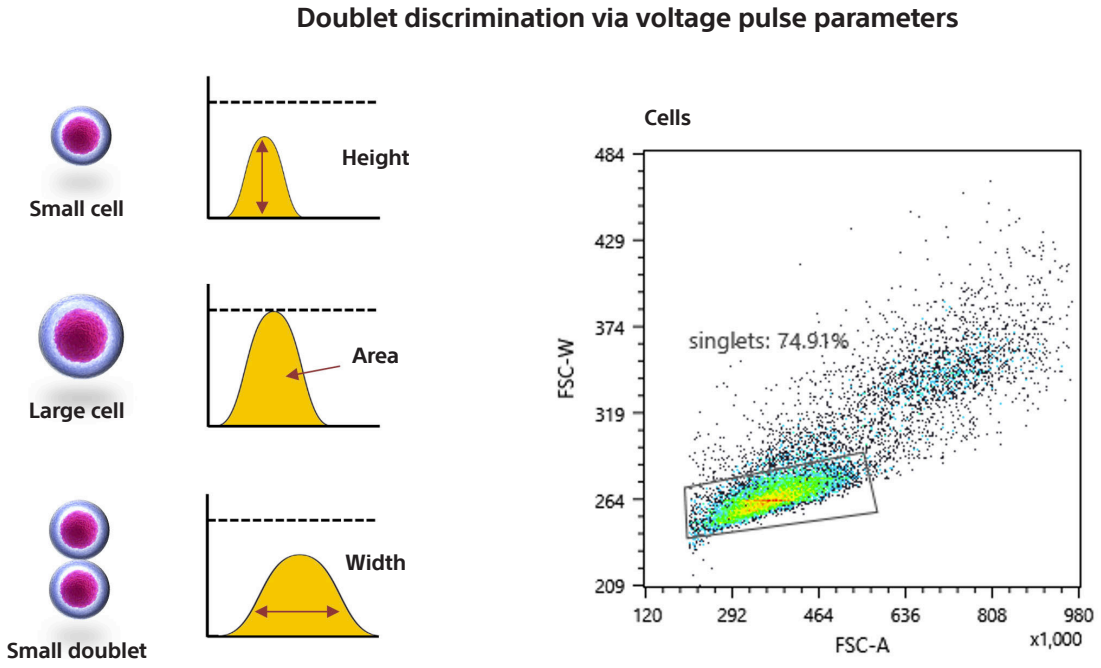


Figure 5: Identifying doublets through voltage pulse parameters.

Understanding sort metrics

Three key metrics – purity, speed and efficiency – are reported for a sort. These are inter-related and present a trilemma, as only two can be prioritized, at the expense of the third. Understanding the relative importance of each of these metrics is crucial when deciding which to prioritize.

Purity

Purity is the percentage of total sorted cells in the collection tube that are the target. This can only be calculated by re-running some of the sorted cells to check for contaminants, and is discussed in more detail in Chapter 3. Purity is dependent on the sort mode selected and, the goal should be to achieve 99.9% in purity mode.

Speed

Speed is the number of cells, or events, processed by the instrument per second. Speed can be adjusted by the user during the sort, and is calculated and displayed in the instrument software. Higher speeds complete the sort quicker, but have a higher chance of having multiple cells in the same droplet, which is more wasteful.

Efficiency

Efficiency is the percentage of cell sorting opportunities where a sort occurs. The efficiency is updated by the software in real time, and is affected by the speed and sort mode selected.

Cell sorting applications – best practices

Effective cell sorting depends on several key technical factors, including careful sample preparation, correct nozzle selection for the cell size and sort requirements, and determining effective gating and sorting parameters. But there are other practical considerations to ensure success, such as knowing how to deal with sample clogs, understanding where target loss may occur, and establishing post-sort processing requirements. This chapter considers best practices for sorting popular cell types with Sony instruments, including some specific applications.

Sorting cancer cell lines and other large cells

Sorting large cells – such as cancer cell lines, primary fibroblasts, stromal cells, cardiomyocytes, epithelial cells, neurons, and plant cells – is challenging, as their size makes them fragile and sensitive to sheer stresses during droplet generation and sorting. There are therefore a number of key considerations for preparing and sorting these large cell types successfully.

Sample preparation

It is important to minimize damage when isolating large cells. Buffers should include serum to prevent cell death, and care should be taken not to over-trypsinize or vigorously vortex the samples. Once cells have been prepared, the samples should be filtered and kept cold, both during preparation and for the duration of the sort. Additionally, use of a viability dye is recommended to exclude dead cells from affecting yields, especially for primary cells.

Nozzle selection

Large cells are particularly sensitive to the shear forces generated at high system pressures, resulting in cell damage or death. Larger nozzles reduce the sheer forces experienced by the cells, as they operate at lower pressures. Sony's 130 micron nozzle can generate eight nanoliter droplets using only 0.6 bar (9 PSI) of pressure, whereas a smaller nozzle would need to run at 2.75 bar (40 PSI). If starting with an unknown sample, approximating the cell size is advisable to ensure correct nozzle selection. This can be achieved by mixing a droplet of beads of known diameter with an aliquot of the sample, and comparing the sizes under a microscope, for example.

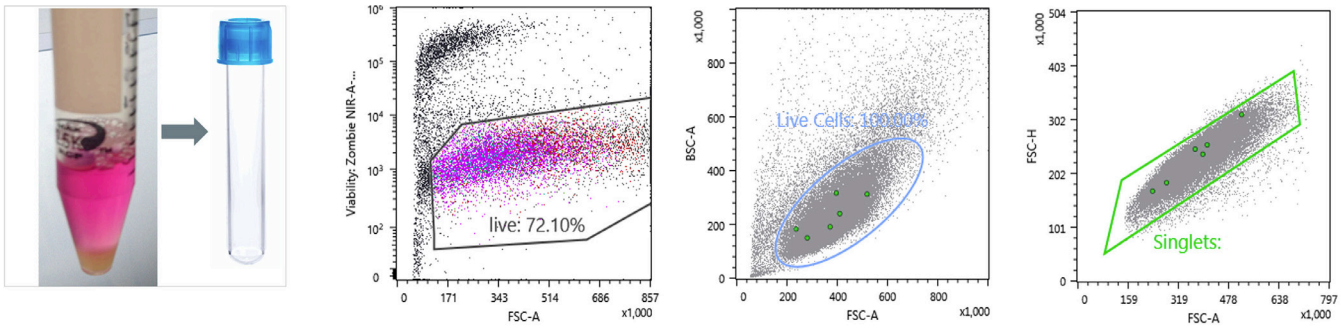
Sample clogs

Sample clogs can be common, especially when sorting large cells. If a sample contains a lot of live cells, they can release DNA and cause re-aggregation, but adding EDTA to the sample will help to prevent this. Samples should also be filtered immediately before sorting, and refiltered whenever the event rate begins to fluctuate. Running more dilute concentrations, or at slightly higher sample pressures, can help to prevent clogs. If a clog occurs, a back flush should clear the sample line. Maintaining and regularly replacing the sample line is particularly important when working with large cells, as old lines may provide lower flow rates, and be more prone to clogging.

Target identification

To sort the cells, the previewing gate should be set to live cells, with the forward and side scatter detector voltages set appropriately, so as to not miss the primary population. This is best achieved by starting with a very low forward scatter setting, and incrementally increasing it until the main population outline appears. A quick sort onto a glass slide will help to ensure that the cells, and not debris, have been correctly gated. For single-cell cloning, a final gate should be set to identify singlets.

Sample setup



Filter cells to remove clumps

Exclude dead cells

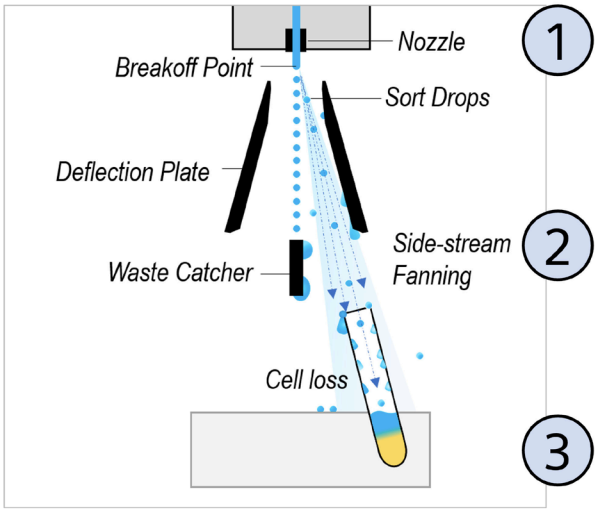
Ensure the cells are not off-scale on FSC and SSC

Gate on singlets

Figure 6: Gating strategy for sorting large cells.

Target loss in sorting

Large cells are particularly prone to target loss during sorting. Target cell loss may happen for three reasons, as shown in Figure 7.



1. Due to direct damage by the nozzle.
2. Due to side-stream fanning, where the droplets are deposited on the sides of the tube or other surfaces.
3. Due to cell death in the collection tube, from impact or exposure to sheath fluid without serum for a prolonged length of time.

Figure 7: Sources of target loss when sorting large cells.

Side-stream fanning, as demonstrated in Figure 8, is a common challenge when working with large cells, and refers to widening side streams that result from inconsistency in charge magnitude on droplets. This can be due to improper droplet setup, cells that are too large for the nozzle, or debris in the sample, and is particularly an issue at high sorting rates, resulting in dripping and puddles forming in the sort chamber.

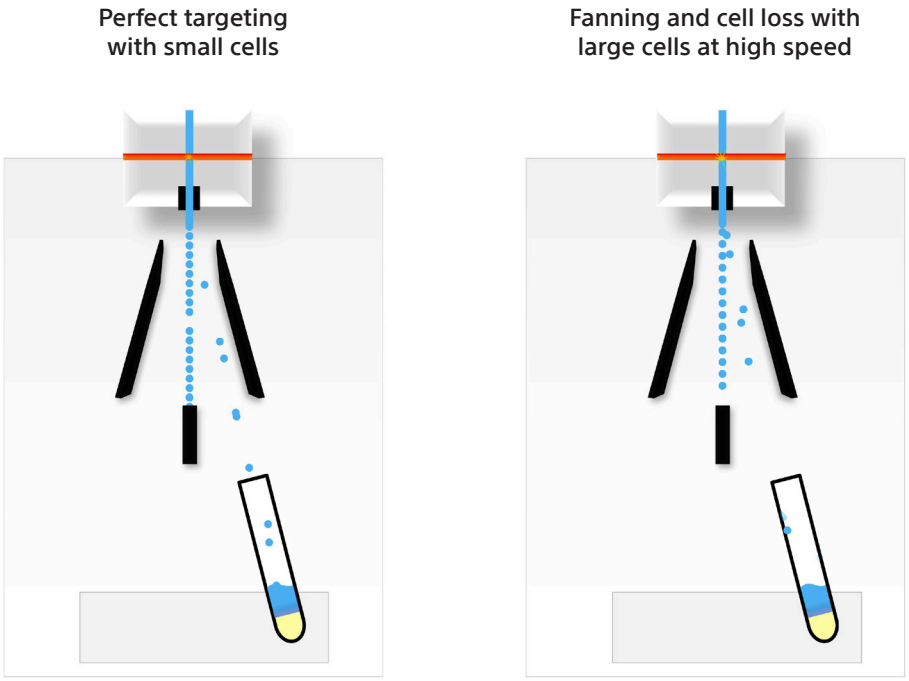


Figure 8: Side-stream fanning with large cells results in target loss.

Sample loss will also be affected by the collection vessel for the cell sort, and mitigating actions can be taken depending on whether the cells are being sorted into tubes or plates.

• Sorting into tubes

When sorting large cells into tubes, it is best to use a larger nozzle and a wider collection vessel, such as a wide-bore centrifuge tube. Precoating the collection tube walls with serum prior to sorting prevents cells from adhering to the surface, and tubes should be mounted higher in the machine or filled with collection buffer, if possible. Slowing down the event rate will also help to reduce droplet stream disruption, minimizing fanning and sample loss.

• Sorting into plates

Large cells are often sorted into plates for cell cloning or single-cell PCR applications. The dynamics of sorting single cells into a plate differs somewhat from bulk tube sorting, and presents some unique challenges: the wells are narrow; sorting is done into a small buffer volume; and surface static can change the droplet trajectory. All of these factors can increase the chances of target loss.

Sorting into plates should be run in single-cell mode – so that only one cell is centered in the droplet – and at a slow event rate, as you typically only need to sort the same number of cells as wells in the plate. It is also essential to check and adjust plate alignment at the time of the sort because, even if previously setup correctly, normal day-to-day variations in the stream and plate mechanism can result in droplets to miss the center of the wells and hit the side walls instead. To align the plate, the plate cover should be kept on, and a test sort of about 30 droplets in each of the four corners carried out. The plate mechanism can then be fine-tuned until the droplets hit the center of those wells. Together, these precautions will help to prevent thinning and accurately target droplets into the center of each well.

Post-sort processing

It is important to sort into a sufficient volume of condition media for the intended downstream application, such as tissue culture. It is also best to keep the stack of tissue culture or 96 well target plates warm until they are needed, to ensure that cells are not damaged when dispensed from the sorter. Cells that have been sorted into PCR plates should be spun down to ensure that all cells have reached the buffer.

Example 1: Sorting yeast

Yeast sorting is commonly required when working with surface-displayed human antibody libraries for therapeutic discovery. Yield mode, which sorts all target cells – even if unwanted cells are present – is popular for this application, as it can enrich the target by hundreds-fold and can be run extremely fast, close to the actual droplet rate. A purity sort may be useful after multiple rounds of yield sorting, to clean up any remaining non-targets.

Sample preparation

Yeast cells will typically clump, clogging sorters and sample lines. Yeast can be strained with a 40 micron filter, and EDTA can be added to prevent clumping. Double discrimination is also necessary due to the tendency of yeast to clump.

Nozzle selection

Yeast sorting should typically be carried out using a 70 micron nozzle, as this offers good sort speed and meets the requirement for cells being at least five to seven times smaller than the nozzle. For diverse or dense cultures, a 100 micron nozzle may improve sort reliability and sample viability.

Sample clogs

Very high concentrations of yeast cells are typically used for sorting, which can result in sample line and nozzle clogging. Ideally, cells should be refiltered as often as necessary throughout the sorting process, as onboard agitation may not prevent cell aggregation at such high concentrations.

Target identification

Properly adjusting the forward and side scatter voltages will place the yeast population in view on the plot and allow gating. Yeast cells typically have two subpopulations: the main population and a smear to the upper right-hand corner, which is most often caused by aggregates. Because yeasts are monoculture, there are no particular rules other than to gate on the majority of the population, away from debris or very large and rare outliers. It is typical to exclude doublets – using voltage pulse area versus pulse height – to achieve high purity. However, if the yeast target is very rare, or contains unusual morphology due to budding, it may require enrichment sorting that includes doublets.

Post-sort processing

It is very important to clean the instrument after completing a yeast sort. If the instrument is only used for yeast, then a simple bleach and water clean should be sufficient. However, in a multi-use scenario, completely isolating the sample path is recommended, allowing a thorough clean of the entire fluidics system using either 1% bleach or 70% ethanol to maintain aseptic conditions. Many labs also keep extra sample lines, and swap them depending on the sample species.

Example 2: Sorting multicolor immunophenotyping panels

Peripheral blood mononuclear cells (PBMCs) – which contain both lymphocytes and monocytes – are a classic example of a complex cell population containing cells with vastly different functions. In this type of mixed sample, target cells need to be identified using several different fluorochrome markers to allow effective sorting.

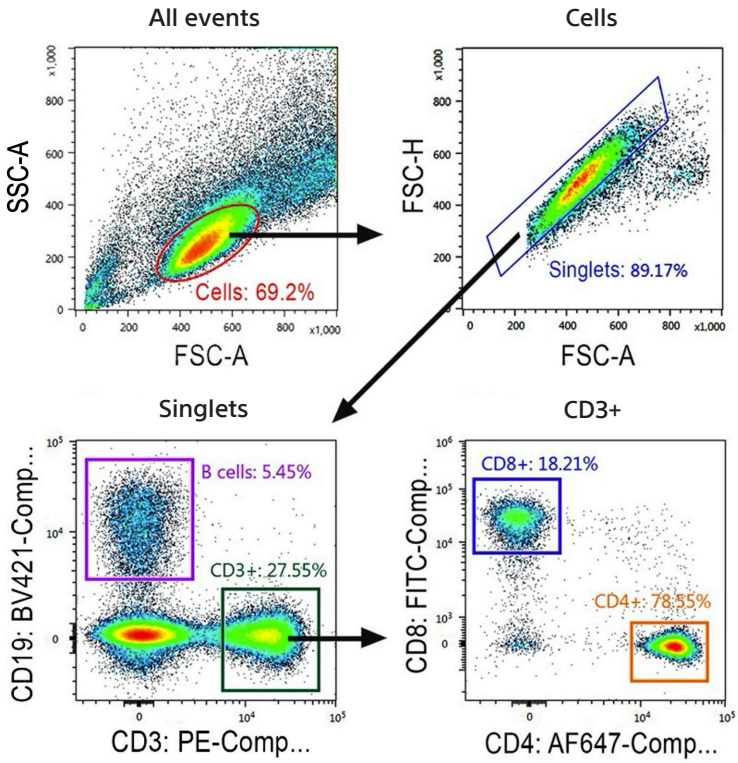


Figure 9: Example of a multicolor cell sort with four fluorochromes, scatter gating on healthy cells that are singlets and contain B-cells and the T-cell subsets, CD4 and CD8.

Sample preparation

Using the right compensation controls is critical for multicolor sorting. Firstly, there needs to be an unstained control without a viability dye. Secondly, there should be single-color controls with similar or greater brightness than the sample. These could be antibody capture beads, or cells with high levels of antigen expression. Finally, consider other controls such as 'fluorescence minus one' samples, which help with gate placement accuracy.

Nozzle selection

Freshly isolated PBMCs are small, so the nozzle selection is fairly straightforward. A 70 micron nozzle is most commonly used, since this provides the highest sort speed and accuracy.

Sample clogs

Clogs are rare with small cells, but it is always advisable to monitor the event rate and sort efficiency during processing, and refilter the sample if there are any unusual changes. Partial clogs or very high sample pressures may also cause cells to shift out of their gates, so this should be checked throughout sorting.

Target identification

It is critical that all controls and samples are run at the same PMT voltage. Changing voltages after the compensation has been calculated will invalidate the spillover matrix, affecting sample gating.

When using certain fluorochrome combinations or measuring more than four or five colors simultaneously, fluorescence compensation is generally required to ensure successful identification of target cells. This involves correcting for fluorescence spillover – which occurs when fluorophores emit photons of multiple energies and wavelengths – by removing the signal of any given fluorochrome from all detectors except the one devoted to measuring that dye.

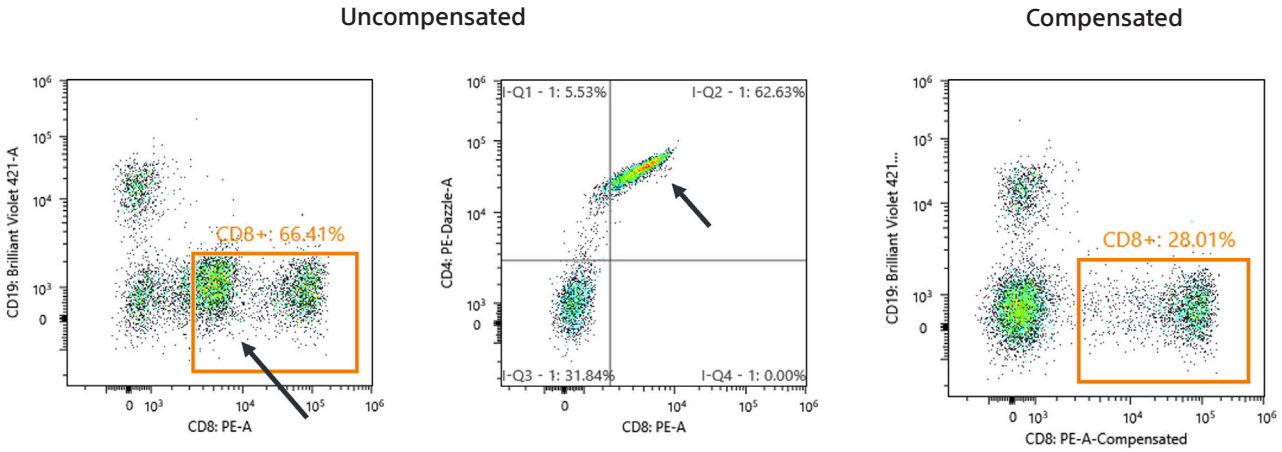


Figure 10: Comparison of uncompensated and compensated plots. The first panel shows a plot of two non-overlapping colors. An intermediate population is present in the CD8 gate (indicated by arrow). A single-color control for a third fluorochrome, PE-dazzle, that identifies CD4 cells, was not compensated for, resulting in the PE-dazzle signal falsely identifying CD4 cells as also positive for CD8. Ignoring this would mean that CD4 cells would be sorted along with CD8s. After recording the single color (second panel) and recalculating the compensation matrix, the light spillover does not interfere with true biological marker expression (panel 3).

Finally, including a viability stain in samples improves efficient target identification, as gating with only the scatter parameters may not be sufficient. Using a viability dye allows identification of healthy cells, especially in a primary tissue with high rates of apoptosis. It is particularly important to use a viability stain for single-cell cloning applications.

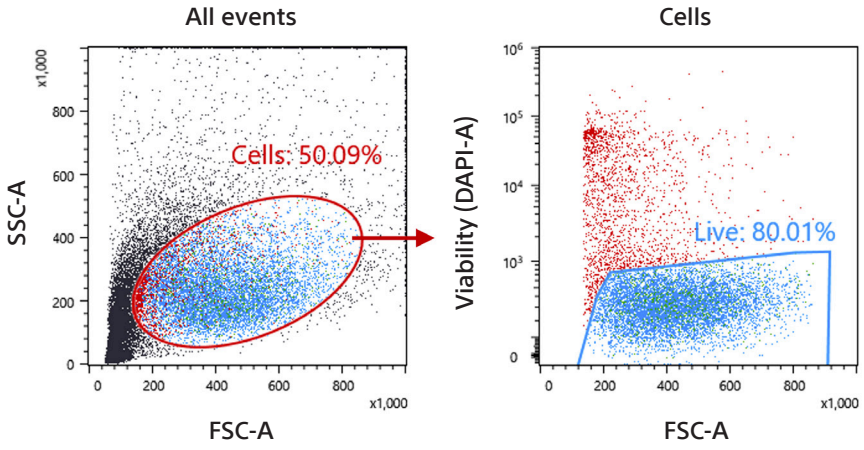


Figure 11: Example plots of viability stain. The plot on the left selects for cells based on morphology. FSC-A: size, SSC-A: granularity. On the right, DAPI is used to label dead cells to exclude them from further analysis.

Target loss in sorting

Target loss in PBMC sorting can be minimal, but all the common recommendations apply. Sorting cells into polypropylene tubes with sufficient collection buffer helps and, if sorting for long periods of time, periodically pausing the sort and mixing the collected cells avoids cell death. There may also be some losses due to cell damage, side-stream fanning or inaccurate cell counts after sorting, since the collected cells will be quite diluted. Some downstream applications require the cells to be spun down to a pellet after sorting. If this is the case, using a 200 µl pipette to manually remove the supernatant prevents further cell loss, especially when working with very small cell numbers, or cells that do not form a stable pellet.

Post-sort processing

A purity check gives confidence that a sort has gone well, but sacrifices some of the sorted cells in the process. Before performing a purity check, it is advisable to check and, if necessary, clean the sample line to avoid carryover. Once the line is clean, a few hundred cells from the sorted sample can be run to calculate the purity. During this analysis, target cells will mostly appear in the original sort gate. However, it is not uncommon for some to shift out by a small amount – due to the variability in detection, or the dye degrading or washing off – and the sort gate can be expanded to compensate for this effect. Contaminants can be distinguished from the target cells as they will appear as cell populations further away from the sort gate. Finally, it is likely that some damaged or dead cells will be present in the sorted sample. These cells will shift left on the scatter plot during the purity check, or can be identified by staining with a viability marker.

Example 3: Sorting nuclei for RNA-seq experiments

Using nuclei instead of intact cells is increasingly popular for downstream next generation sequencing applications – such as single-nucleus RNA-seq – as it allows the use of cell types that are difficult to isolate as single cells, such as neurons. However, nuclear isolation methods often result in a significant amount of debris that can cause poor sequencing results. It is important to remove this debris, which can be done using a cell sorter.

Sample preparation

Protocols for nuclear isolation should be developed to match the particular cell type and application, but generally involve homogenizing tissue in a nuclear lysis buffer to release the nuclei, which are then filtered, pelleted and washed. Isolation methods can result in nuclei clumping together. Analyzing the sample under a microscope before sorting will identify problematic aggregates, then an extra filtration step can be carried out if necessary to produce an optimized sample.

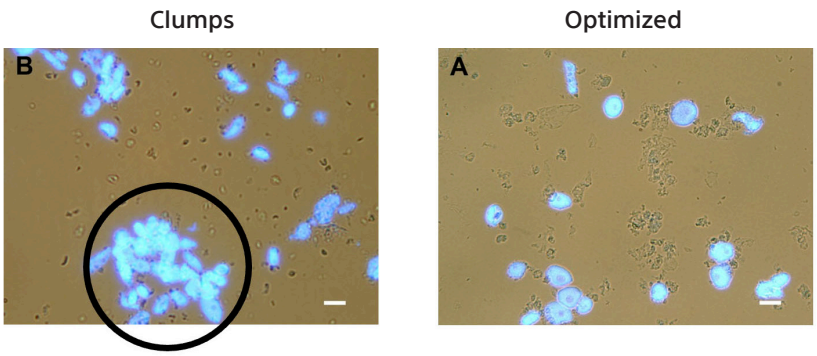


Figure 12: Clumped versus optimized nuclei – clumps will require extra filtration.

Nozzle selection

It is common to use a 70 or 100 micron nozzle for nuclei sorting, because they are generally small, with the diameter of an average mammalian cell nucleus being approximately 6 microns. The 70 micron nozzle is faster, so it is better for longer sorts, but a larger nozzle can help with sorting preparations with many aggregates that may cause clogs.

Sample clogs

Nuclei clumping can lead to clogs that will slow down the sort and, because the nuclei are small, this could affect the laminar flow in the flow cell. This kind of disruption can result in an increase in the population spread, and may result in some sudden shifts in the scatter and gate percentages, and even a reduction in fluorescence signal. If this occurs, the sort should be paused, the sample refiltered, and a back flush run on the cell sorter to unclog the sample path.

Target identification

One of the more challenging aspects of nuclei sorting is gating. A lot of debris can be excluded using forward and side scatter gating, then a DNA dye – such as DAPI – can be used to gate on singlets, and gate out debris with similar scatter parameters. Voltages and detection threshold settings for singlet gating can be uncertain, so it is worth conducting a test sort onto a glass slide, then using microscopy to visually confirm that the correct targets are being sorted. Displaying the DNA dye on a linear scale helps to identify the classical phases of the cell cycle with either a bivariate plot or a histogram. Finally, it is worth using the back-gating function to help visualize where the nuclei may be in the scatter plot or the subsequent gates.

Post-sort processing

It is good practice to check for purity and yield using a microscope after sorting. However, this is not always possible for nuclei sorted directly into reverse transcription buffer, in which case it is advisable to run a preliminary experiment to make sure that sorting gives the correct purity and yield for downstream applications.

Consideration	Yeast	PBMCs	Nuclei for RNA-seq
Primary challenge	Cell clumping at high concentrations	Accurate identification of rare cell populations	Debris and nuclei aggregation
Sample preparation	Filter (40 µm), add EDTA, discriminate doublets	Prepare appropriate compensation controls and viability stain	Optimize nuclei isolation, filter, inspect for aggregates
Recommended nozzle	70 µm (100 µm for dense or diverse cultures)	70 µm	70 or 100 µm depending on aggregation
Managing clogs	Refilter frequently due to high cell density	Monitor event rate and refilter if shifts occur	Pause, refilter and back flush if aggregates disrupt flow
Target identification	Forward and side scatter gating, exclude aggregates unless enriching rare targets	Fluorescence compensation, viability staining	Scatter gating plus DNA dye (e.g. DAPI), verify by microscopy
Post-sort processing	Thorough instrument cleaning	Purity check and viability assessment	Purity and yield check with microscope before sequencing

Table 3: Summary of best practice approaches for common cell sorting applications.

References

1. Kannan S, Miyamoto M, Lin BL, *et al*. Large particle fluorescence-activated cell sorting enables high-quality single-cell RNA sequencing and functional analysis of adult cardiomyocytes. *Circ Res.* 2019;125: 567-569.

2. Rodrigues, O.R. and Monard, S. A rapid method to verify single-cell deposition setup for cell sorters. *Cytometry*, 2016;89: 594-600. https://doi.org/10.1002/cyto.a.22865

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