BD FACService™ TECHNOTES

Contributors:

Burt Houtz
Joe Trotter
Dennis Sasaki

Special Sorting Issue

Increasingly, investigators need to isolate unique cell populations based on multiple phenotypic and functional characteristics that can be measured by flow cytometry. This special issue of BD FACService TECHNOTES addresses many important technical requirements for successful cell sorting, using instruments with either analog or digital electronic systems.* This issue assumes the reader is familiar with the principles of cell sorting by flow cytometry. The first article identifies factors related to cell preparation that affect flow cytometric analysis and sorting. This article includes a discussion of factors that affect resolution and maintenance of cell viability, and offers suggestions on sample preparation. The second article describes how digital sorting with the BD FACSAria can be optimized with appropriate sort gates, taking into account fluorescence resolution and cell aggregates.
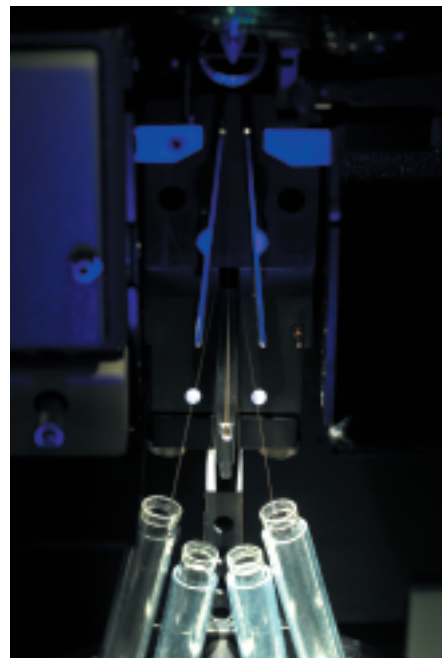
* More fundamental information can be obtained from the BD FACSAria™ or BD FACSVantage™ SE user's guides or operator training courses, and from textbooks on this topic.[1,2]

Tips on Cell Preparation for Flow Cytometric Analysis and Sorting

Introduction

Multiple factors need to be considered when analyzing flow cytometry data, especially when sorting cells. Many problems come up not related to instrumentation. Based on customer feedback, the following tips have been compiled to help flow cytometrists resolve common problems with protein concentration, buffering, cell conditions, viability, and autofluorescence.

BD Biosciences

Sample Identification and Resolution

Protein Concentration and Refractive Index

Gustav Mie described light scattering of particles in 1908. His theory describes how light scatter is related to the magnitude of the refractive mismatch of the particle to the surrounding medium coupled with the surface size of the refractive mismatch. Any refractive mismatches between sample buffer and surrounding sheath will play a role in scatter measurements as well.

The maintenance of healthy cell populations in culture requires the presence of protein such as bovine serum albumin (BSA) or fetal calf serum (FCS). Cells differing in their protein requirements and labile cell populations may require higher concentrations of protein to survive. Conversely, any sample buffer hydrodynamically focused into an optical pathway consisting of sheath fluid may create optical interference. A particle surrounded by the sample core stream requires minimal optical interference for maximal resolution. Sufficient protein concentrations in a sample buffer can create a Schlieren Effect and act as a dynamic fluid lens at the sheath/sample boundary.[3] Protein in saline creates a refractive bias with surrounding protein-free sheath buffer because of the increased optical density and refractive index of the buffer containing protein. As light passes through media possessing different indices of refraction, the light is bent, ultimately resulting in dynamic distortions in the light-scatter signals.

If you need to sort cells in sample buffer containing protein and you require the maximum resolution possible, it may be necessary to use a different buffer. Use the least amount of protein necessary to keep the cells viable, to minimize FSC and SSC signal distortion. In other words, if a protocol calls for 5% FCS in the sample buffer, yet the cells will survive and perform well using anywhere from 0.5% to 2.0% BSA instead, the data quality will often be significantly better using a lower concentration of BSA.

Autofluorescence and High Background

Fluorescence sensitivity is an important consideration when evaluating flow cytometric data. In addition to instrumentation factors, cell sample autofluorescence can reduce fluorescence sensitivity by increasing relative background "noise." NADH and flavins are intracellular co-enzymes that are also known to increase cellular autofluorescence. The presence of unbound antibody and phenol red, a cell culture media pH indicator, will increase background fluorescence. Although the system baseline restore algorithm will correct the signal levels in the presence of a high background such as fluorescent buffer,

the standard deviation contributions will remain from the measurement and ultimately decrease sensitivity. The 488 nm (blue) laser line will excite each of these sources and the fluorescence emission is generally green (530 nm). The signal from autofluorescence can be reduced with the addition of Trypan Blue.[4] Since Trypan Blue will fluoresce red, it is necessary to avoid using red detectors when using this dye as a counterstain. Wash cells and avoid phenol red to minimize background fluorescence. Use red-excitable fluorochromes, such as APC or APC-Cy7, when autofluorescence is an issue, since cells exhibit little autofluorescence when excited by a red laser and the emission is measured above 650 nm.

Sheath Fluids and Cell Buffers

For applications that require extremely good resolution, investigators have found that using the same buffer for the sample and sheath fluid improves resolution. This is the case for chromosome analysis and sorting whereby minimizing fluorescence signal CVs is a critical feature. Large-scale chromosome sorting has been successfully performed by using a chromosome-stabilizing polyamine buffer as the sheath fluid.[5] If you use a protein-containing sheath fluid, avoid high concentrations of protein to minimize foaming.

Maintenance of Cell Viability and Functionality

Buffering Capacity and Sorting

Common phosphate and carbonate buffers are frequently used in culture media and saline solutions to maintain a pH optimal for cells in solution. As these cells are exposed to increased pressure prior to and during a sort (10 to 100 psi), the partial pressure of CO_2 also increases and will reduce the pH of the solution unless it is adequately buffered.

According to Henry's Law, the concentration of a solute gas in a solution is directly proportional to the partial pressure of that gas above the solution. Additionally, the presence of dissolved CO_2 readily equilibrates with water to form H_2CO_3, carbonic acid. Quantitatively, buffering capacity is defined as the number of moles of a strong acid or strong base required to change the pH of 1 liter of the solution by 1 pH unit.[6,7] Therefore, increasing the partial pressure of CO_2 will require a buffer with a strong pH-buffering capacity to minimize the effects of acid in solution.[6,8] What buffers are best at physiological pH? We have found that HEPES buffer has an optimal buffering capacity with a useful pH range from 6.8 – 8.2, and a pKa of 7.5. A final concentration of 25 mM of HEPES in an

appropriate sample buffer (PBS, HBSS, or phenol-free culture media) with a neutral pH has been found to minimize these effects.

## UV Irradiation of Cell Lines and Cell Viability

For many flow cytometric applications it is necessary to excite cells with UV light. This includes monitoring expression of blue fluorescent protein, identifying "side population" stem cells, or measuring DNA content of viable cells using Hoescht 33342. Some investigators have also reported reduced viability of sorted cells after these cells were excited with UV light.[1] In these cases, it is necessary to determine the cause of the low cell viability, which may not be due to UV excitation. Although some cells may be more sensitive to the effects of high-speed sorting and high-energy wavelengths of light, these factors, independently, will not necessarily be the cause of cell loss. Investigators testing the effect of UV radiation on both human skin fibroblasts and CHO cells have cultured cells after exposure to laser powers ranging from 25 mW to 500 mW, with no resulting loss of survival.[9]

Sheath Pressure and Sheath Formulation

Sasaki has also reported using sheath pressures as high as 60 psi while sorting peripheral blood mononuclear cells with no significant loss in cell viability. Other possible sources of accelerated cell death include high sheath pressures used on fragile or aged samples, and collection tube conditions, such as media and temperature.[10] It is also important to ensure that cells are sorted to areas on tubes or slides that have liquid protein–containing media. It is not uncommon for the cell yield to be reduced because the trajectory of the sort streams caused the cells to land on dry plastic or glass.

Use care when selecting commercial sheath fluids, as some contain preservatives such as ethanolamine, and may affect results from some applications. Sterile 25 mM HEPES in the sample buffer will help keep cells viable both during sorting and afterwards (when mixed and diluted with sheath). Once the cells are sorted and are no longer under high pressure, the extra buffering capacity of the HEPES is no longer as critical.

Sample Preparation
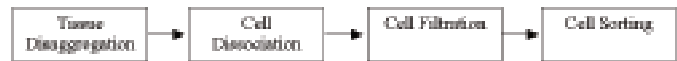
Mechanical and enzymatic procedures have been used to disperse cells from tissues, a necessary requirement for sorting cells derived from solid tissue. It is important to process cells as quickly as possible after dissection to minimize cell death. The following steps help maximize cell recovery and minimize cell loss prior to sorting.

Tissue Disaggregation

Organs such as spleen can be minced, teased, and pushed through stainless steel mesh filters in the presence of culture media. Rubbing tissue between frosted glass slides can similarly release cells into media. This process can be laborious and lacks standardization. Alternatively, the Medimachine™ sample preparation system is for automated, mechanical disaggregation of solid tissues for flow cytometric analysis and sorting. This system has three components: the Medimachine, Medicons™ (tissue holders), and Filcons™ (filters). Various pore sizes of disposable, sterile, and non-sterile Medicons and Filcons allow an operator to optimize experimental conditions to suit the tissue under study,* ensuring clean, viable preparations of single cells, cell clusters, or cell nuclei.

Cell Dissociation

Cell clumping can result in poor sort purity when sorted target cells are attached to nontarget cells, and poor recovery when coincident aborts exclude all clumped cells. DNA from lysed cells in the medium can cause cells to clump. DNAse I (Sigma Cat. No. D-4513) in the presence of magnesium chloride (Sigma Cat. No. M-2670) will help reduce cellular aggregation. Treat cells for 15 to 30 minutes in a solution of 100 µg/mL DNAse and 5 mM $MgCl_2$ in HBSS at room temperature. Wash the cells once in the presence of 5 mM $MgCl_2$ in HBSS. Gently suspend the cells in BD Pharmingen™ Stain Buffer (BSA) containing $MgCl_2$ and 25-50 µg/mL DNAse (as a maintenance dose) prior to and during the sort.[†] DNAse I requires a concentration of at least 1 mM magnesium to work effectively, although 5 mM is optimal. Note: It is important to minimize the presence of dead cells during this procedure, since actin released from dead cells irreversibly inhibits DNAse I.[11]

* Medimachine Filcon:
  • 50 µm Sterile, Syringe-Type, Pkg of 100, Cat. No. 340601
  • 100 µm Sterile, Syringe-Type, Pkg of 100 Cat. No. 340609
† BD Pharmingen Stain Buffer (BSA) Cat. No. 554657

Cell Filtration

Cell clumping can also be reduced by filtering samples just prior to analysis or cell sorting. In addition to the Medimachine Filcon, the 12 x 75-mm tube with cell strainer cap offers a convenient way to filter laboratory samples.* A 35-µm nylon mesh is incorporated into the tube cap, which can be used to collect the filtered sample for sorting.† As the 35-µm nylon mesh size is suitable for leucocytes, it is important to choose an appropriate mesh size for larger cell types.

Summary

Conditions that can adversely impact your cell sample prior to sorting include cell media, sheath fluid, buffering capacity, and protein concentration. Low cellular viability, autofluorescence, and cell aggregates will result in poor sorts. Identification of these factors, and taking the appropriate action to remedy the problem, will improve sort purity, yield, and cell viability. ■

BD FACSAria Sorting

Introduction

The expanding applications used in drug discovery and proteomics, and improvements in flow cytometry technology, have made cell sorting a more demanding task. Increasing use of transfected cell lines, dendritic cells, and other cell types require more sophisticated gating strategies for sorting. Appropriate expectations, an optimal gating strategy, and the proper sort "tools" are essential for successful sorting results. Beam geometry, noise, and proper data interpretation can have a profound effect on gating and resulting sort gate purity. The following factors, case study, and recommendations are presented to assist users of the BD FACSAria on these issues.

Factors Influencing Sort Gates

Fluidics and Particle Orientation

The BD FACSAria is designed to be a sorter that combines the benefits of optical interrogation within a quartz cuvette with jet-in-air sorting performance. To achieve the necessary stability for proper timing throughout the cuvette into the emerging jet, the system design makes use of a long fluidic path within the cuvette and fully developed laminar flow. As a result, nonspherical particles experience torque and may slowly rotate from their orientation from when they enter the cuvette channel to when they pass through the laser beams and emerge within the jet.

This means nonspherical objects in the FACSAria may be in any rotation when analyzed, as opposed to passing through the beams primarily on-axis as in other systems. Previously developed BD cytometers have shorter fluidic paths and the particles are predominately oriented on-axis. Since the BD FACSAria has a longer fluidic path to accommodate the required sort timing precision, other doublet figures are present.

Beam Geometry

The BD FACSAria has highly elliptical beam geometry (9:1) that produces a core interrogation focus spot that is approximately 9 x 81 microns. This optical feature concentrates light energy in the vertical plane and distributes it over the sample stream to a greater degree in the horizontal plane, providing uniform illumination and better resolution of most cell types. The smaller optical interrogation zone of the sample in the vertical plane enables the sort electronics to make more accurate sort decisions while cells are analyzed at higher speeds. In contrast, many previous BD instruments use a more oval beam geometry (3:1) with a spot size that is approximately 25 x 75 microns.‡ As a result, the optical interrogation and subsequent analysis, while still accurate, can be significantly different from other cytometers. See Figure 1a and 1b.

To use an analogy for analysis, if a BD FACSCalibur is a knife, then the BD FACSAria is a scalpel. The dissection of the data is performed differently. The larger spot size in a BD FACSVantage SE or a BD FACSCalibur allows for passive integration of most particles as they pass through the beam because most commonly sorted particles are less
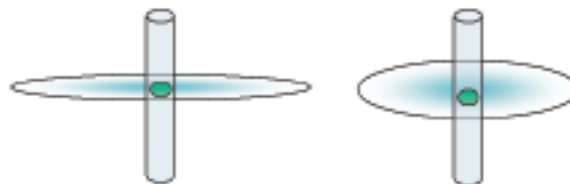

Figure 1a. Single 6.0-micron particles are shown in the BD FACSAria on the left and the BD FACSCalibur (or BD FACSDiva) on the right. The resulting pulses and data will be similar.

Figure 1b. With a 12-micron particle, however, the BD FACSAria acts more as a slit-scanning cytometer because the cell is larger than the beam height.

* BD Falcon™ Round Bottom Test Tubes with Cell Strainer Cap, BD Falcon Cat. No. 352235
† http://www.bdbiosciences.com/discovery_labware/Products/tubes/round_bottom/
‡ The BD LSR II has a beam spot height that is normally intermediate between that of a BD FACSCalibur and BD FACSAria.

than 25 microns in diameter. In contrast, any particle significantly larger than 9 microns will be essentially slit scanned by the BD FACSAria, making use of the pulse area, essential for proper measurement.[12,*]

Because of this optical difference, detecting doublet figures and coincident events are important considerations. Coincident events occur when two (or more) unattached particles are in the beam at the same time. Doublets and aggregates occur when two (or more) particles are stuck together, sometimes weakly, and are seen by the system as a single, larger particle. With a larger beam spot, as in the BD FACSVantage or BD FACSCalibur, cell doublets are often seen as two cells and are relatively easy to distinguish from single cells. In contrast, the cell doublet angle of rotation in the BD FACSAria beam will determine if it is difficult to correctly classify. See Figure 2.

Cell doublet profiles appear differently in a graphical display according to their orientation and the parameters used (height, area, width). Particle rotation occurs on multiple axes, data is represented in *all* rotations. On a properly setup BD FACSAria,[†] sorts that do not meet purity expectations are typically caused by the inclusion of doublets that appear as a single particle and cannot be correctly identified without the appropriate sort gates.

Beam geometry and particle size are key to understanding where doublets are likely to occur. This is a key concept because it is necessary to use one or more gates based on beam geometry to discriminate doublets. This is the case with analog and digital sorters when a "single cell" FSC vs SSC gate region (R3) fails to isolate single cells. See Figures 2 and 3.

Traditionally, pulse width was required to identify single cells. This is shown in the example using Enhanced Green Fluorescent Protein (EGFP)+ CHO cells on the BD FACSVantage SE. Note in Figure 4 that a FSC-W vs EGFP (FITC-H) gate follows a FSC-H vs SSC-H gate. In combination, these gates serve to exclude doublets by using FSC-H, FSC-W, and FL1-H.[‡] By using signal width and height we are taking advantage of beam geometry. Similarly, height, area, and width parameters are important tools to discriminate single cells on the BD FACSAria. The main differences between the BD FACSAria and other sorters in terms of doublet discrimination, are in beam geometry and identification of cell aggregates.[§]
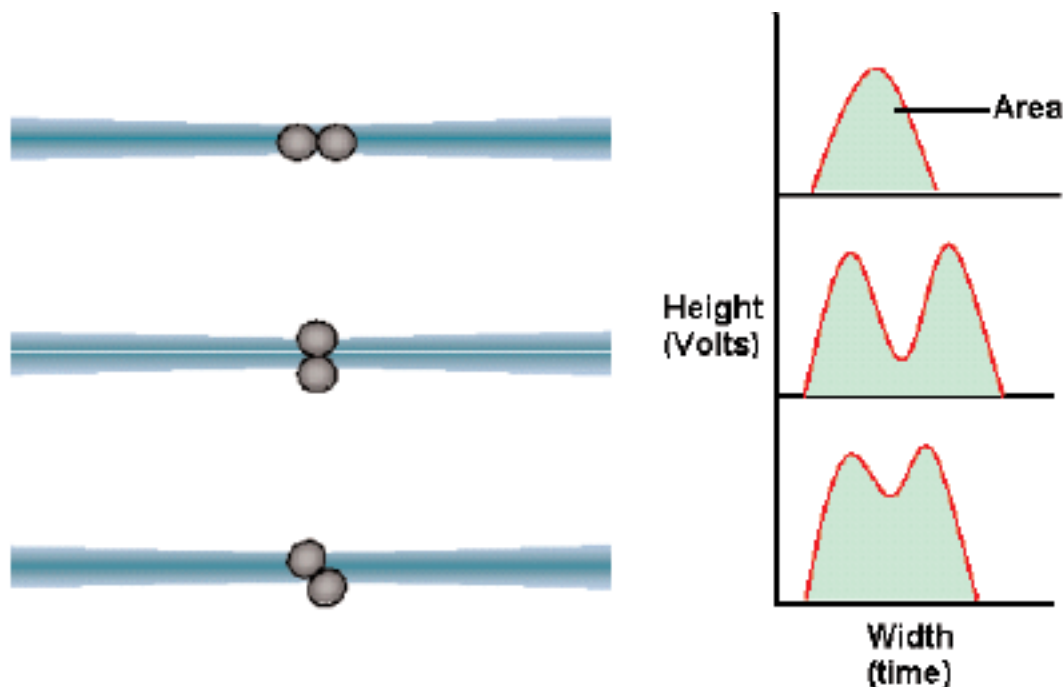

Figure 2. Cell doublets in rotation as diagramed on the laser beam axis and the corresponding voltage pulse.

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* Slit-scanning cytometry makes high-resolution measurements, whereby fluorescence and light scatter measurements can be collected from a narrow strip of a cell in a given instant in time.
† Where particle size does not exceed approximately 1/6 the nozzle diameter and fluidics are stable.
‡ Where two cells are stuck together and behave as a single particle as opposed to two single cells that are coincidently in the laser beam at the same time.
§ The BD FACSAria uses fully developed laminar flow, and particles can experience torque, which will cause them to rotate within the sample core stream.
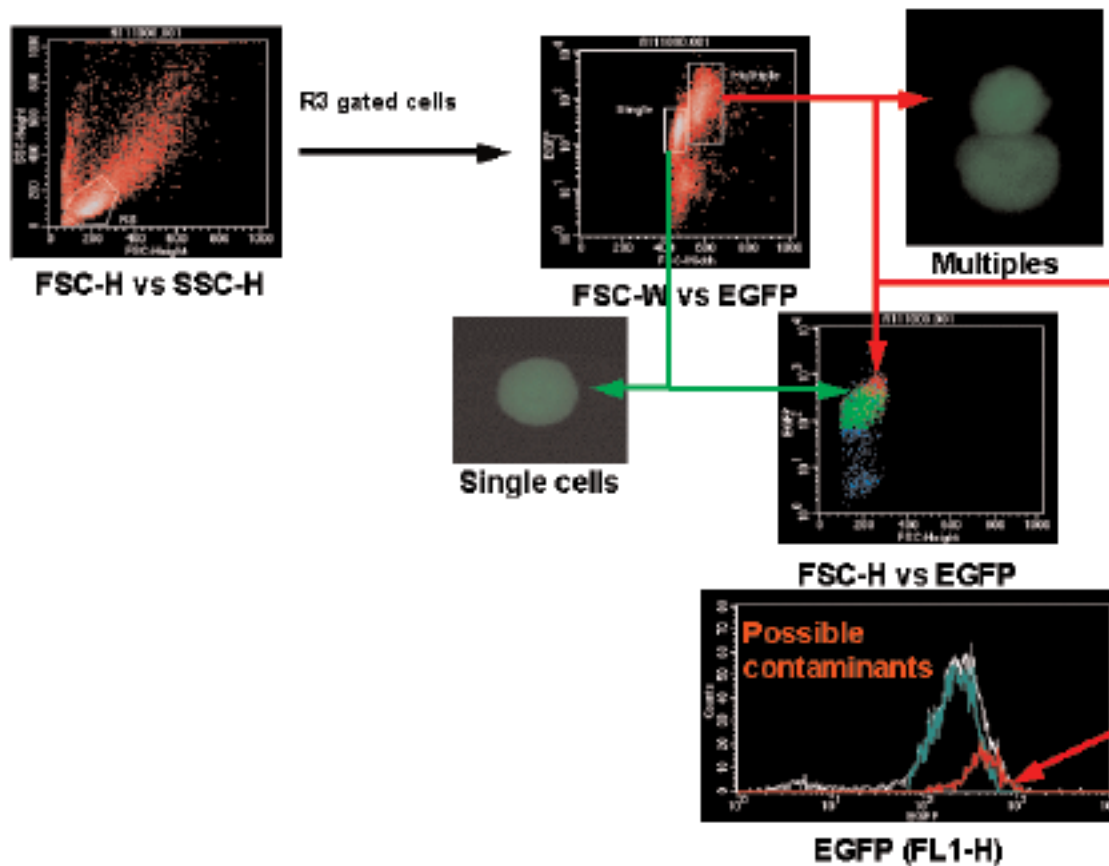
Figure 3. CHO cells transfected with BD Clontech EGFP. Pulse width as well as FSC and SSC are used to discriminate cell aggregates from singlets. Note that cells gated only on R3 do not eliminate cell multiplets, and will result in a larger FSC-W signal.

Noise

Multiple factors, including background light, poor optical efficiency, Raman light scatter, dark current, and "photon counting statistics" will impact *resolution* (the ability to resolve dim cell populations). Collectively, these factors are commonly referred to as noise. The primary sources of error in measuring dim fluorescence signals are 1) high background noise due to autofluorescence, unbound antibody, and/or spectral overlap, and 2) the total number of photoelectron signals measured and the optical efficiency of the system. Error associated with photon-counting statistics refers to the inherent inaccuracies in measuring the relative brightness of a dim population. Statistically, a small sampling of photons will produce greater variation in number than a larger sampling. All these factors will impact sort purity when sorting dim populations from background.

Fluorescence resolution can be measured on a relative basis using "hard-dyed" beads. Cell or bead population CV

broadening at the lower end of the fluorescence scale is due primarily to variance in the number of photons striking the photocathode of the PMT. See Figure 4. We can use this to predict how well some sorts will successfully isolate the cells of interest and attempt to optimize the sort gate location.
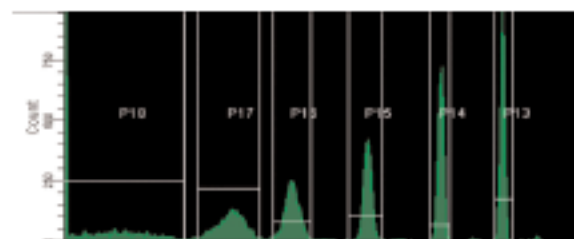

Figure 4. SPHERO™ Rainbow Calibration Particles. Note broadening of the bead peaks at the lower end of the fluorescence range.

Dendritic Cell Case Study—Optimizing Your Sort Gates

Background

Dendritic cells (DCs) are highly efficient antigen-presenting cells that have an essential function in the development of various kinds of immune responses. These cells interact in vivo with T cells in a very dynamic way, physically interacting with hundreds of T cells per hour, and with up to 10 or more simultaneously.[13] These cell "clusters" are often difficult to break apart, and even the best disaggregation procedures may still result in a high number of cluster figures. Additionally, unless you are working with purified preparations, DCs are rare cell populations, which can adversely impact sort yield. DCs are also sticky—they tend to clump to each other and stick to plastic. It is also not uncommon for investigators to use proteolytic enzymes such as collagenase to attempt to increase dendritic cell yield by digesting the clusters for a period of time to help break cells apart. Poor enzyme purity and other factors can easily cause the preparation to become stickier, and result in a larger fraction of dendritic–T cell clusters. See Figure 5.

Problem

Incompletely disaggregated cell clusters will often slip through simple sort gates and decrease sort purity, or result in statistical errors in population estimates. A customer wanted to sort DCs from mouse spleen, had fluidic stability problems, and had a sticky cell preparation with lots of clusters. This is the worst-case scenario for "passenger cell" contamination. Additionally, a region for one of the population boundaries did not provide sufficient separation between negative and dim populations. This increased the probability that a sorted cell did not have the desired characteristics due to photon counting error. As mentioned previously, background light and photon statistics can cause impure sorts and will result in suboptimal isolation of the population of interest. If the sort gate is too close to the negative population, reanalysis of the sorted cells may result in the presence of "negative" and "dim" cells within the positive fraction.
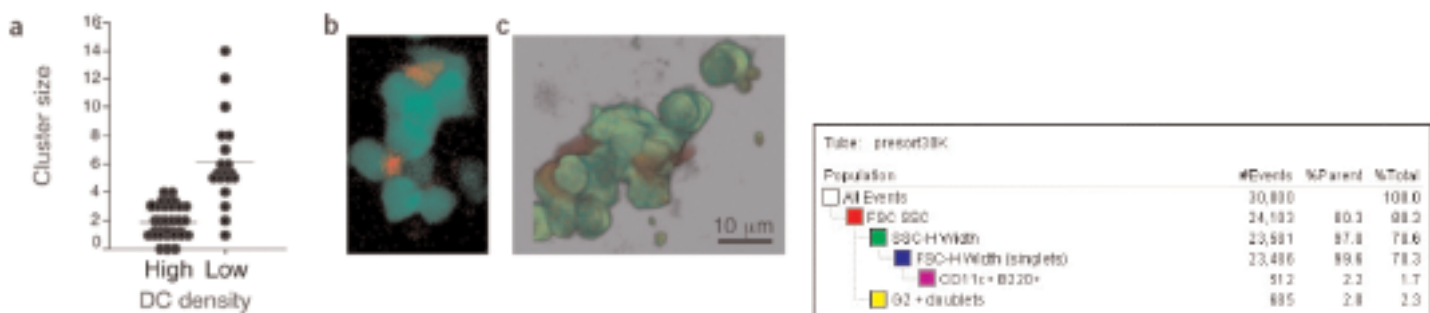

Figure 5. Dendritic–T cell interactions result in cell aggregates, which make sorting difficult.
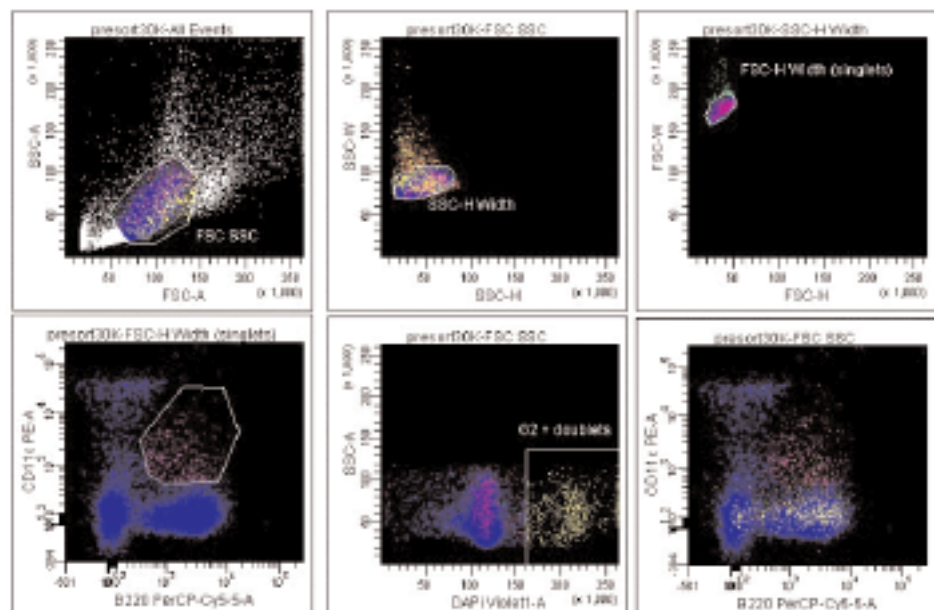

Figure 6. Using a DNA-binding dye such as DAPI can help identify doublets and verify that hierarchical scatter gates eliminate doublet contaminants.

Solution

To check for cell clusters, we counterstained DNA with DAPI to allow "G2/M" level (doublet) events to be identified. By using a series of hierarchical scatter gates to isolate single particles, it was possible to increase the CD11c+ B220+ purity to over 90%. By tightening the gate regions further, it was possible to increase purity to over 95%. Additionally, it was clear the majority of contaminants were passenger cells were doublets that expressed B220, and were included in the original sort gates. Some of these contaminants were most likely a result of measurement error.* This sort became quite pure by using a scatter gate strategy as shown in Figure 6, and by ensuring region boundaries were separated from ambiguous parts of the distribution.

Recommendations

As suggested previously, height, area, and width parameters for FSC and SSC will help discriminate single cells on the BD FACSAria. Specifically, these are particularly useful since the particles tend to rotate, and these measurements are impacted by beam geometry, particle size, and shape.

Take the following steps to improve your sort results:

1. Adjust the FSC-H and SSC-H detector gains so the signals are on scale (as much as possible).

2. Check the Area Scaling Factor (ASF), a secondary gain adjustment, to ensure the Area and Height intensities match reasonably for the particles to be sorted, using the current instrument setup (nozzle, pressure/velocity, detector gain, etc.). Since large cells require more time to pass through the laser, it is important to get an accurate Area measurement. Refer to the BD FACSAria User's Guide or training manual for more information on these adjustments.

3. Monitor the system's ability to discriminate doublets or aggregates with 6-micron beads. Large cells such as Raji or Daudi cell lines can also be used as positive controls. These cells are particularly useful for monitoring the ASF based on their size. Look for the "arc" in FSC-A vs FSC-H, and SSC-A vs SSC-H.

4. Note the impact of ASF on SSC-H vs SSC-W. This is not a "set and forget" instrument setting. This setting will need to be checked and optimized for particles if their size is significantly different than the standard setup particles, since the relationship between area and height will differ with different cells. As mentioned previously, cells with greater area require more time to pass through the laser, and detector gain adjustments alone may not give optimal results.
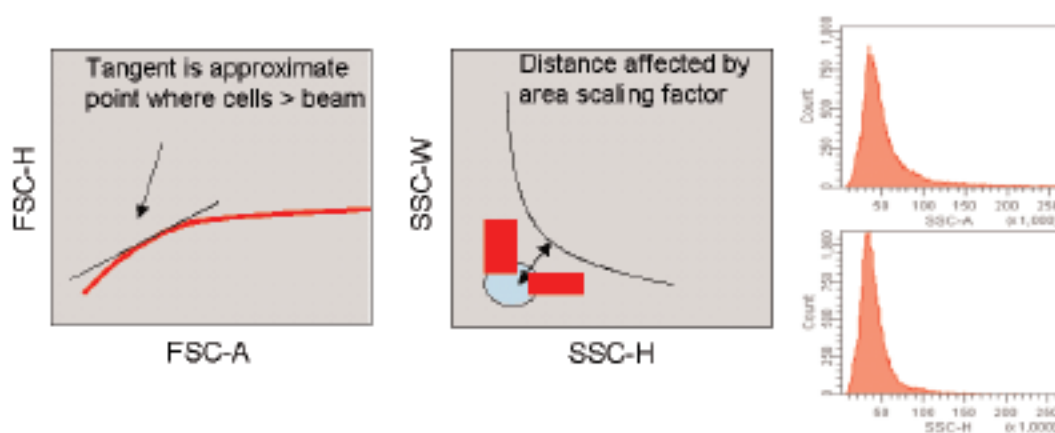

Figure 7. The Area Scaling Factor (ASF) can be adjusted to ensure the signal area and height intensities are both on scale and of equal intensity.

* Note the region on the lower left dot plot in Figure 6. Its lower boundary is dangerously close to B220 single positives.

Conclusion

Design improvements found in the BD FACSAria have resulted in changes that can impact sort results. The most significant change is to the laser beam geometry at the laser–cell interface. Successful cell sorting requires effective discrimination of singlets from aggregates. Effective use of FSC and SSC height, area, and width parameters enable identification of aggregates to eliminate them from a sort. Noise from a variety of sources (electronic noise, photon counting statistics, population variances), as well as aggregates, need to be considered when drawing sort regions for effective singlet isolation. ∎
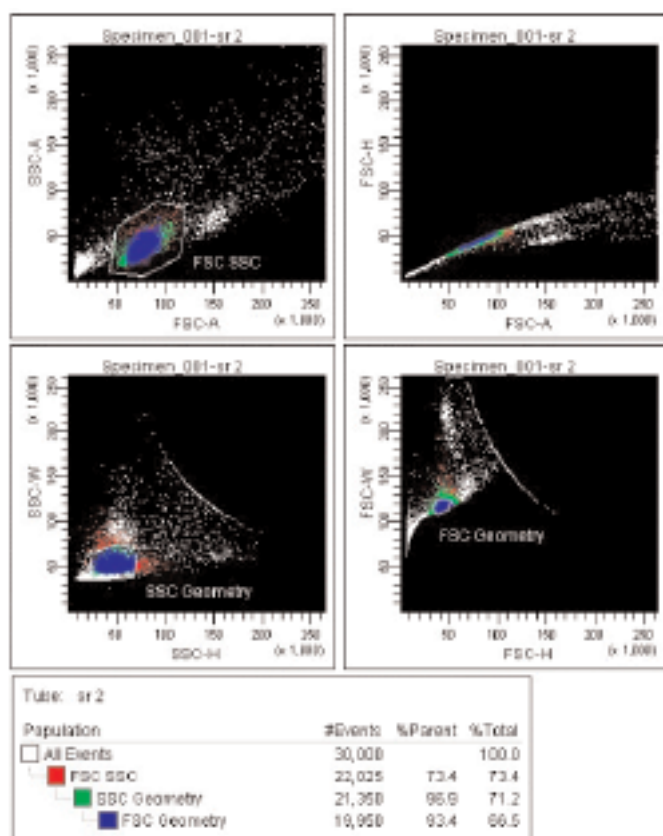
Figure 8. FSC-H vs FSC-W, SSC-H vs SSC-W, and FSC-A vs FSC-H dot plots are used to more effectively eliminate aggregates. Note that reduction of background light with a neutral density (ND) filter in front of the FSC detector improved FSC resolution.*

* FSC ND filter is now standard on all BD FACSAria systems.

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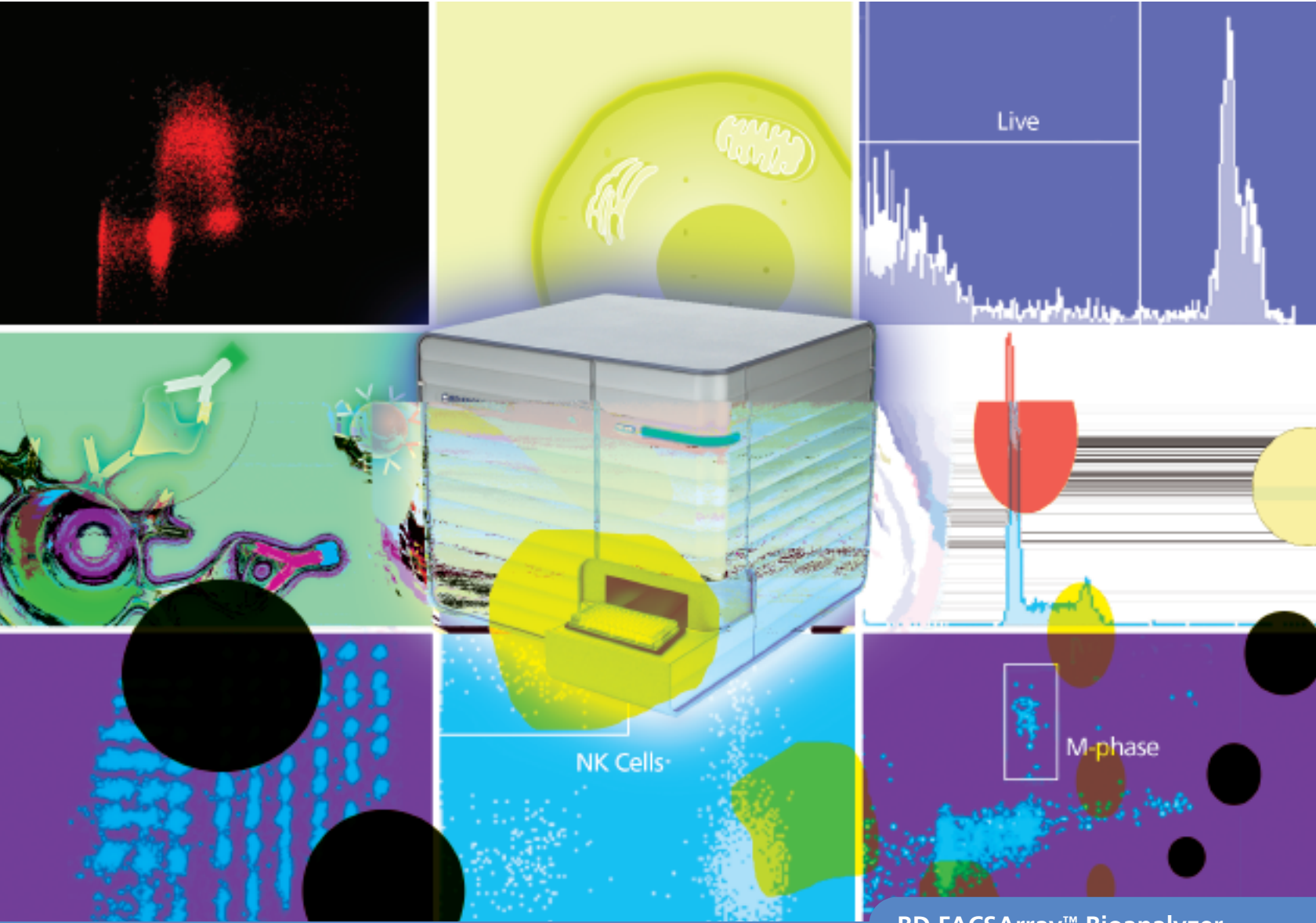
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				• Troubleshooting sample prep, data acquisition, and analysis problems

Prerequisites	A basic understanding of immunology and completion of an operator course, or equivalent experience that includes data acquisition and analysis using BD CellQuest™ software	Completion of an operator course, or equivalent experience that includes BD CellQuest™ and ModFit LT™ software	Basic Macintosh® skills and a basic understanding of flow cytometry, and the analysis of flow cytometry data	A basic understanding and completion of an operator course, or equivalent experience that includes data acquisition and analysis using BD CellQuest software.
Who Should Attend	Users proficient in BD FACSCalibur™/ BD FACScan™/ BD FACSort™ operation and who want to gain experience in preparing and analyzing specimens used in studying immune function	Users proficient in BD FACSCalibur /BD FACScan/BD FACSort operation and who want to expand their knowledge of DNA analysis techniques.	Users who have upgraded to BD FACStation™ system, users who deal mainly with data analysis rather than cytometer operation, or users who are familiar with BD CellQuest software but require more in-depth training	Users proficient in BD FACSCalibur/BD FACScan/ BD FACSort operation and who want to gain experience in quantitative assays.
Duration	1 day	2 days	1 day	1 day
Schedule	October 28, San Jose, CA	October 26 - 27 San Jose, CA	October 25, San Jose, CA	October 29, San Jose, CA
Tuition	$495	$990	$495	$495

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Phycoerythrin (PE) conjugates and allophycocyanin (APC): US Patent No. 4,520,110; 4,859,582; and 5,055,556; European Patent No. 76,695; Canadian Patent No. 1,179,942

APC-Cy7: US Patent No. 5,714,386

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