# Bigfoot Spectral Cell Sorter

## Options for light scatter and new opportunities for research

### Introduction

The field of flow cytometry is rapidly maturing, and instrumentation is constantly improving. However, many researchers are frustrated by instrument limitations that make it difficult or impossible to observe small particles that are likely present in samples, yet are undetectable. Fortunately, as manufacturers continue to improve offerings, scientists continue to make new discoveries. During the last decade, investigators have had the means and imagination to explore particles smaller than 1 µm in diameter (Figure 1). While older flow cytometers were limited to detecting particles >500 nm in size, recent instrumentation advancements have improved the detection range to <100 nm, leading to exciting discoveries in cell signaling, tumor metastases, coagulation, inflammation, drug development, disease progression, response to therapeutics, and more. Unfortunately, signals of very small particles are often indistinguishable from background noise caused by instrument operation, buffer composition, sample preparation, sheath particulates, and issues with instrument cleanliness. The Invitrogen™ Bigfoot Spectral Cell Sorter was developed to help scientists overcome these obstacles.

### Light scatter design and architecture

Light scatter parameters are essential for robust flow cytometry studies. The Bigfoot Spectral Cell Sorter is equipped with up to 5 highly sensitive light scatter detectors, including forward scatter (FSC) and side scatter (SSC) on the 488 nm laser path, FSC on the 405 nm laser path, and depolarized light scatter detectors for both FSC and SSC on the 488 nm path (Figure 2). All light scatter detectors on the Bigfoot Spectral Cell Sorter are photomultiplier tubes (PMTs) rather than the less sensitive photodiode detectors that are used for FSC detection on many flow cytometers.

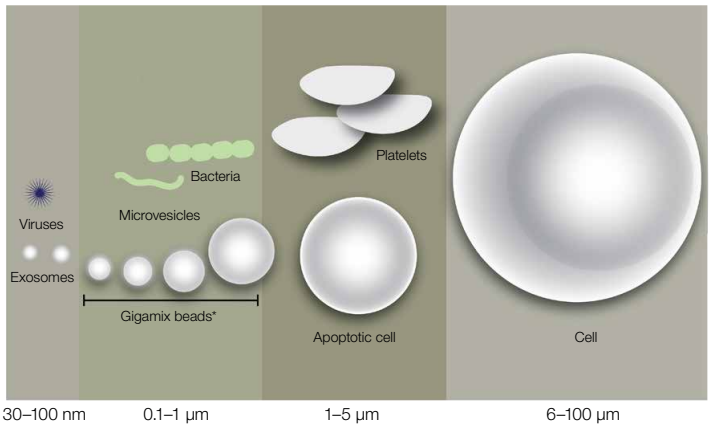


**Figure 1. Size range of Gigamix beads as compared to cells, microvesicles, bacteria, viruses, and exosomes.**

* Gigamix beads are a combination of Megamix FSC and Megamix SSC standardization beads from BioCytex (Marseille, France).

Recent scientific interest in particles smaller than 1 µm in diameter has prompted manufacturers to incorporate small particle detection (SPD) into flow cytometers. The detectors are based on FSC or SSC, depending on the architecture of the instrument. Traditionally, the 488 nm laser has been used for FSC and SSC detection in flow cytometry. Over time, manufacturers included additional lasers to accommodate more fluorophores, and investigators discovered that shorter wavelengths are better for detecting scattered light from smaller particles. For this reason, the Bigfoot Spectral Cell Sorter has an optional FSC detector on the 405 nm laser dedicated as a

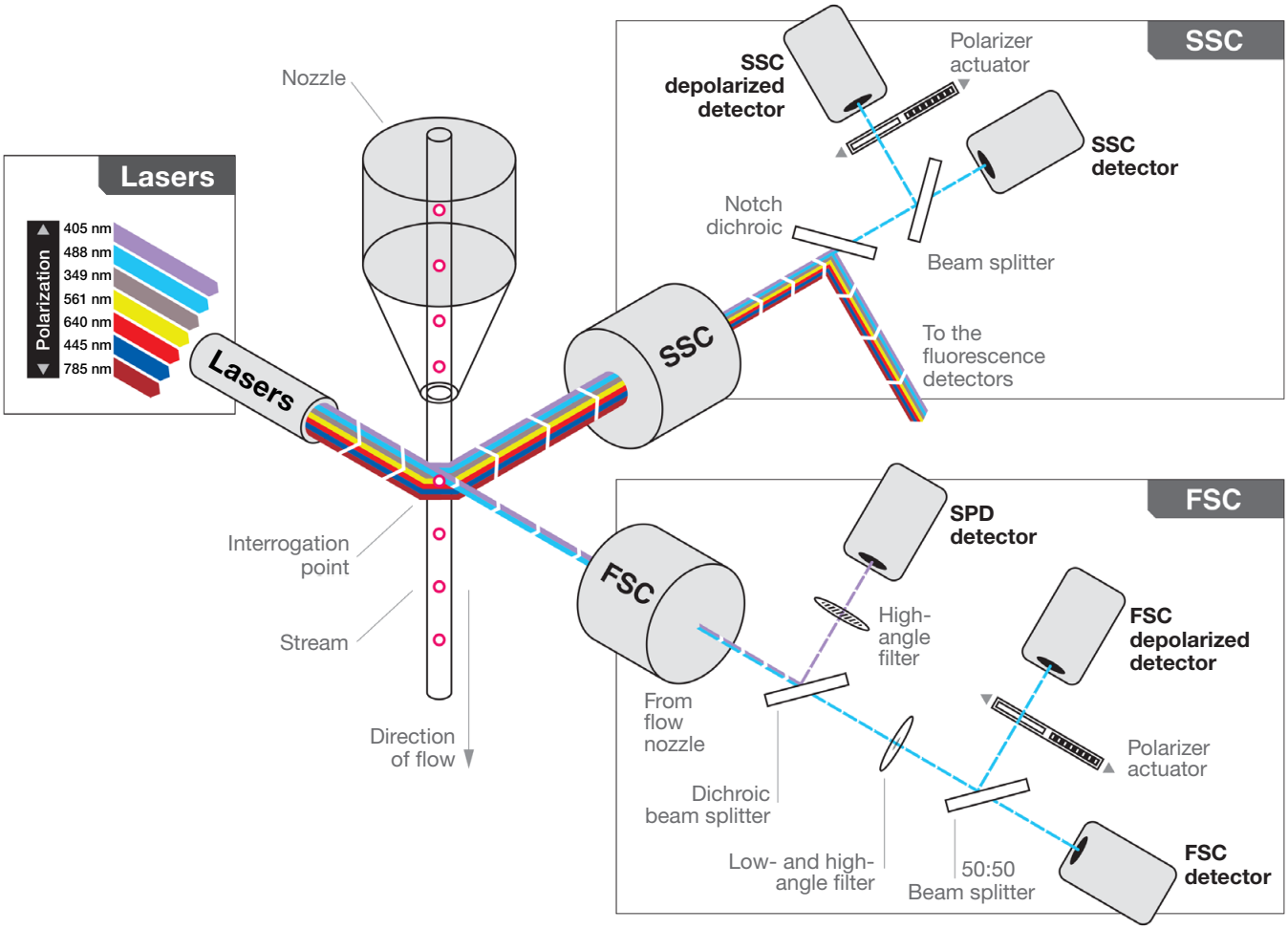


**Figure 2. Light scatter and optical design architecture.**

small particle detector (405 SPD). Because the optical path for this detector is split from 488 nm scatter collection, the angles of collection are controlled independently. The standard configuration collects emitted FSC light to 20 degrees outside of the laser path. A custom high-angle filter on the SPD path blocks all low-angle scatter rays and allows angles greater than 10 degrees from the laser path to pass through to the detector.

The FSC and SSC detection paths are both equipped with polarized filters and additional detectors to differentiate polarized and nonpolarized emissions from the 488 nm laser. Polarization is a property of laser light that is often ignored in flow cytometry. Since 1987, it has been known that the polarization of the incoming light is sometimes changed due to the interaction with a cell, and the amount of change is specific to various cell types, such as eosinophil granulocytes [1]. This discernment does not require staining, so money is saved on fluorophores and cells remain clean. Due to the layout of the optical detection area of the Bigfoot Spectral Cell Sorter, it was possible to add another detector onto the FSC light path (FSC-POLAR) and the SSC light path (SSC-POLAR) for additional detection. There is a 50:50 beam-splitting optic that sends half the light from the FSC (or SSC) path to the polar detector. The polarizer is placed just in front of the polar detectors and is precisely oriented such that it blocks the maximum of vertically polarized laser light (Figure 2). In addition, the polarizer is on an actuator and can be automatically moved in and out of the light path for the user to see the effect, with and without the polarizing filter, on their data plot.

This white paper characterizes the use of these unique light scatter parameters and describes additional features such as below-the-trigger-threshold detection, extremely low background signal, and superior electronics that make the Bigfoot Spectral Cell Sorter well suited for micro- and nanoparticle research.

## Polarized light applications

Polarized light detection is useful for the detection of blood eosinophils, malaria diagnosis, and marine phytoplankton studies. Traditionally, FSC by SSC plots are used to identify the basic lymphocyte, monocyte, and granulocyte cell populations in lysed human blood samples. In 1987, de Grooth et al. showed that the eosinophil granulocytes in human blood could be separated using a polarized light filter in the SSC path [1]. Eosinophils were found to show relatively higher levels of depolarization of the polarized laser light in the SSC detector. Further studies have attributed this difference to the birefringent properties of granules present in eosinophils. This characteristic has been used in a commercial hematology instrument to enumerate eosinophils. Figure 3 shows an analysis of lysed normal human whole blood with depolarized side scatter, high autofluorescence, and negative staining for CD16.

Similarly, malaria diagnosis utilizes depolarized SSC to identify the birefringent malarial hemozoin pigments present in the white and red blood cells of malaria patients [2]. The more unique parameter, the depolarized FSC, is useful in the identification of coccoliths of the marine phytoplankton *E. huxleyi*, where birefringent calcium carbonate crystals decorate the outside of the cells [3].

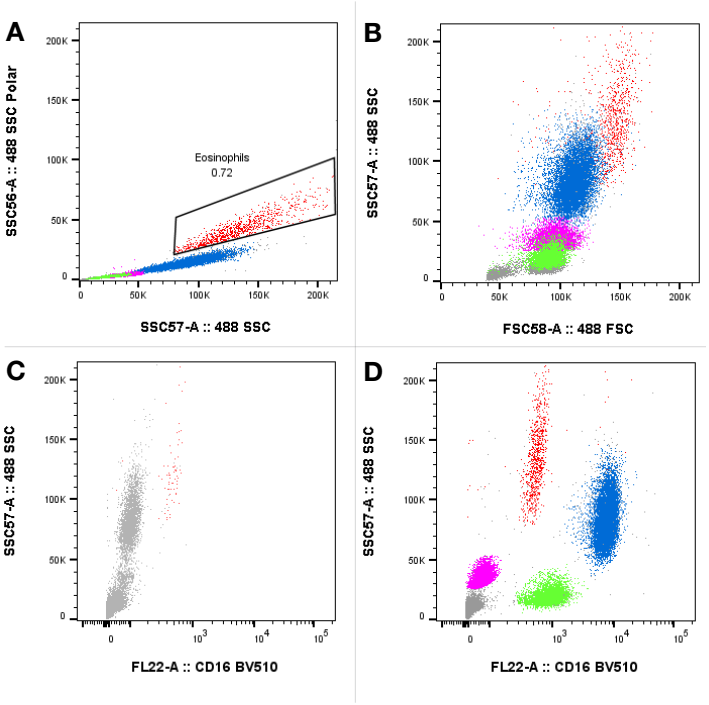


**Figure 3. Analysis of lysed normal human whole blood (red), performed on a Bigfoot Spectral Cell Sorter, showing depolarized side scatter, high autofluorescence, and negative staining for CD16.** **(A)** Eosinophils, with higher depolarized SSC, are highlighted in red. **(B)** FSC by SSC showing eosinophils with high FSC and SSC signal due to their larger size compared to other cells. **(C)** Unstained sample showing the autofluorescence of eosinophils. **(D)** CD16 BV510 stained cells showing CD16-negative eosinophils and highlighting other cell populations based on CD16 expression.

## Small particle detection

As previously discussed, micro- and nanoparticle studies are hindered by challenges inherent to flow cytometry. Such problems include background signal from instrument operation, buffer composition, sample preparation, and lack of instrument cleanliness; these issues need to be addressed and are particularly problematic for cell sorting. The study of micro- and nanoparticles also raises the issue of instrument standardization against event size verification.

The Bigfoot Spectral Cell Sorter incorporates a unique below-the-threshold visualization window that helps address many of these problems. The threshold visualization window enables the user to see events that fall below the trigger threshold, which are signals that cannot be seen on traditional cell sorters and analyzers (Figure 4). The sample flow can be paused while the sheath is running to view the events present in the sheath fluid. Then the sample line can be checked for contaminants by running a sample of filtered buffer or DI water while watching the threshold visualization window for additional events. By viewing the threshold window under these conditions, the user can easily set one or multiple trigger thresholds and assess the need to modify cleaning protocols and buffer preparation. The Bigfoot system includes a 0.04 µm in-line sheath filter to help reduce background signal.

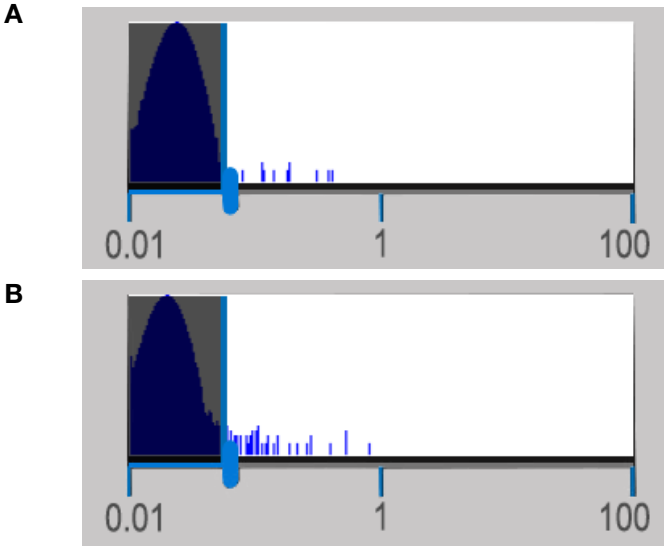


**Figure 4. An SSC trigger and 0.06% threshold were set to produce this threshold visualization. (A)** Threshold window of sheath but no sample running, and **(B)** threshold window of filtered DI water as sample.

## Standardization

Light scatter intensity is dependent on many properties in addition to the actual size of the particle. These properties include the wavelength of the laser, light collection angles, refractive index, and complexity. These variables make it difficult to use flow cytometric measurements to accurately determine the size of particles that have different compositions. However, standardization of signals between instruments or between runs on an individual instrument is essential. To evaluate the performance of the Bigfoot Spectral Cell Sorter, we used Megamix FSC and Megamix SSC bead sets (BioCytex, Marseille, France). Megamix FSC beads were developed for instruments that use enhanced FSC for small particle detection, and Megamix SSC beads were similarly developed for instruments that use enhanced SSC. Either FSC or SSC can be used for small particle detection. Parameter selection is dependent on the design of the instrument, especially the use of PMTs rather than photodiodes, with neither SSC nor FSC being inherently superior to the other [4]. The combination of these two sets is referred to as "Gigamix beads", a mixture of seven different polystyrene bead sets (100, 160, 200, 240, 300, 500, and 900 nm) with varying levels of the fluorophore FITC to aid in the identification of each bead set. Several publications have shown the use of Gigamix beads to evaluate instrument performance, and we used this information in our evaluation of the Bigfoot Spectral Cell Sorter [4,5]. Figure 5 shows a typical Gigamix run with three light scatter parameters showing remarkably clear demarcation of the bead size sets with very low background noise. Note that height signals were used rather than area signals, because they provide better resolution for the smallest particles. Figure 6 shows the superior resolution of height signals compared to area signals. Typical background noise events when running filtered DI water at low speed were less than 25 events per second (EPS). Our researchers used the standard 100 µm nozzle tip at 30 psi with drop drive on and SSC as the trigger. Based on these polystyrene bead data, the lower limit of detection of the Bigfoot Spectral Cell Sorter is in the range of 100–160 nm. This size range compares favorably among leading flow cytometer analyzers in the market and exceeds the reported performance of most cell sorters.

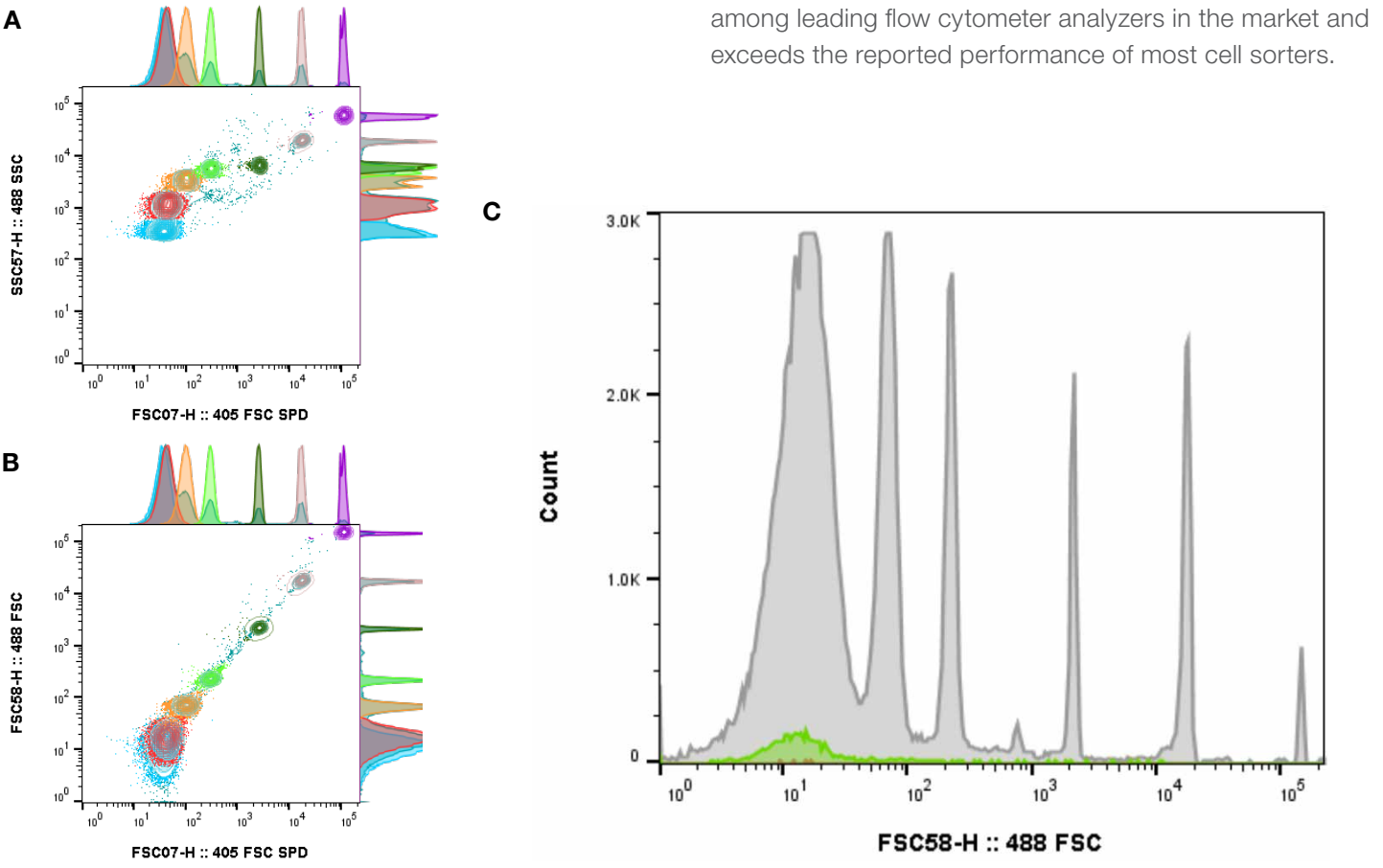


**Figure 5. Plots of Gigamix beads (1:1 mix of Megamix FSC beads and Megamix SSC beads) using the 488 nm SSC trigger.** Megamix FSC includes 100 nm, 300 nm, 500 nm, and 900 nm beads. Megamix SSC includes 160 nm, 200 nm, 240 nm, and 500 nm beads. Note that 500 nm beads are included in both sets. All seven bead sets are resolved in both plots **A** and **B**, but there is better resolution of the larger beads in plot A. 488 nm SSC produced the best resolution between 100 nm and 160 nm. **(C)** Overlay plot of Gigamix beads with sheath-only background events shown in red, and filtered DI water shown in green, illustrating the background event rate of 25 EPS. This is a far lower background signal than those reported for other cytometers, which are typically 400 EPS or higher.

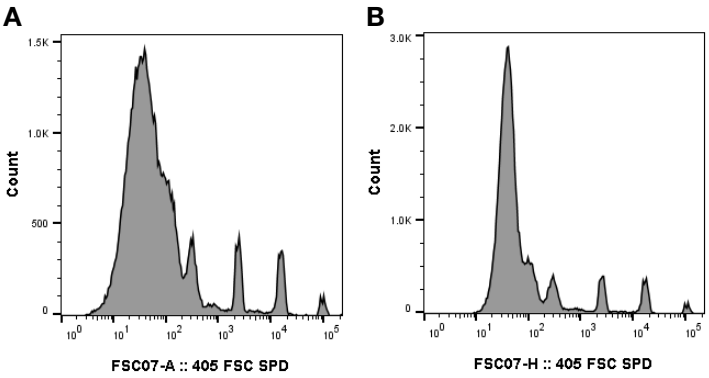


Figure 6. Comparison of Gigamix beads 405 FSC in height signals (A) and area signals (B).

## Sorting small particles

Sorting small particles is complicated by the presence of instrument background noise and buffer debris. Although these events may be inconsequential to the sorting project, these signals are processed by the sorter as valid events and contribute to software aborts and low sort efficiency. Our researchers found that the Bigfoot Spectral Cell Sorter had far less background signal than other instruments, yet the presence of noise caused by buffer debris still made it difficult to evaluate sorting purity. To evaluate the Bigfoot Spectral Cell Sorter's sorting performance with small particles, we sorted the 200 nm microspheres from the Gigamix bead set (Figure 7). The quality of the sorting can be evaluated by the absence of the other six bead-size sets in the sorted sample. Of particular note, the 100 nm and 160 nm beads are engulfed in noise in the pre-sort sample, while the post-sort sample shows only the debris pattern. Additional analysis using a fluorescence gate allowed more specific enumeration of sorting purity.

## Discussion

The Bigfoot Spectral Cell Sorter has several outstanding features that are critical for small particle analysis and sorting. First and foremost, the below-the-trigger-threshold window allows users to visualize the instrument's intrinsic background signal, the cleanliness of the instrument, and the background quality of any buffers used in the analysis.

The remarkably low background signal of the Bigfoot Spectral Cell Sorter makes it possible to select a trigger setting far lower than possible on other leading cell sorters. Cell sorters with high background signals recover a lower percentage of target events when sorting, because background events cannot be distinguished from unwanted events, leading to software aborts. The Bigfoot

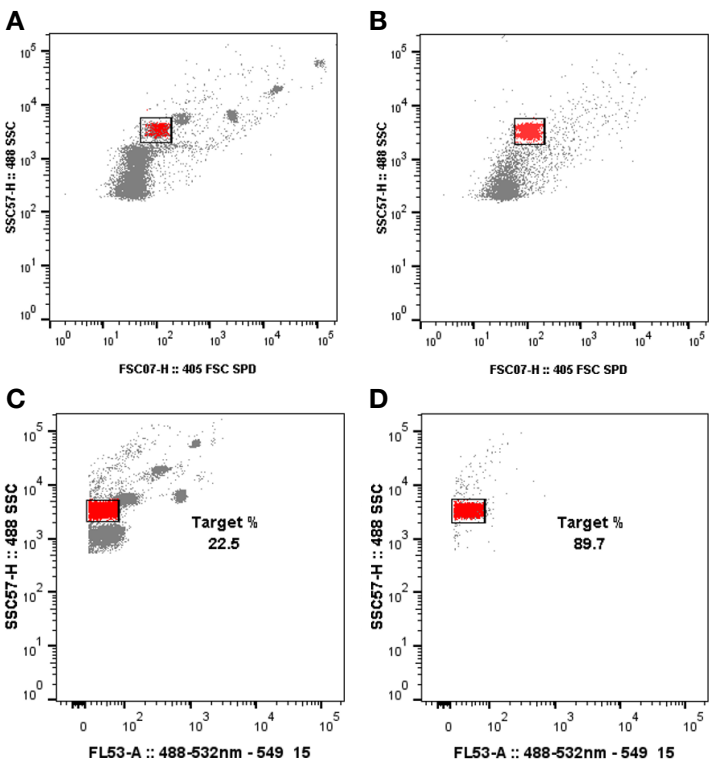


**Figure 7. Evaluating sorting performance of the Bigfoot Spectral Cell Sorter. (A)** The 200 nm bead population (red) in the Gigamix sample was set as the sort target. The sort was performed using only the 405-SPD and 488 nm SSC parameters. **(B)** This plot shows the analysis of the sorted sample. Note that only the 200 nm beads and debris were present in the sorted sample. In particular, the 100 nm and 160 nm beads are not seen in this sorted sample, and only debris is visible in the lower left portion of the plot. **(C)** To confirm the purity of the sorting, the data were gated on bead fluorescence for analysis. The unsorted target population was 22.5% of the plot events. **(D)** The sorted sample target region contained 89.7% of the plot events.

Spectral Cell Sorter's superior electronic speed with high-resolution, responsive pulse width effectively eliminates hardware aborts and produces high-purity results. Sorters with slower electronic processing experience hardware aborts, which occur when the instrument cannot process information in time to make sorting decisions. Slower electronic processing can also allow undetected debris and cells to contaminate results. Setting the instrument to trigger on fluorescence to eliminate background does not solve this problem, because this setting makes it impossible to detect events that do not fluoresce.

During instrument setup, the background signal on the Bigfoot Spectral Cell Sorter was so low that our investigators found the manufacturer's suggestion to use the FITC signal of the Gigamix beads to identify the different bead sets was unnecessary.

invitrogen

Higher background signal was observed when using a 70 µm nozzle tip, due to the increased drop drive magnitude required for droplet formation. This higher background signal necessitated setting the threshold at the level equivalent to the 300 nm polystyrene beads. While larger microparticles can be sorted using this setting with the 70 µm tip, it is a better choice to use the 100 µm tip and set the threshold lower, because the user can then eliminate more of the undesirable debris and small particles.

When sorting with the 100 µm tip, the detection event rate should be limited to approximately 12,000 EPS. Although this event rate is far less than the instrument can achieve in most circumstances, it is important to run at a lower event rate to avoid the coincidence effect known as swarming, when sorting microparticles.

While the 405 SPD parameter on the Bigfoot Spectral Cell Sorter was designed for small particle detection, the standard FSC parameter had equivalent resolution. The combination of five different light scatter parameters enables the detection of a wide range of cell and particle sizes. In addition to the three standard detectors (488 nm FSC, 405 nm FSC SPD, and 488 nm SSC), the two polarized light scatter detectors can also serve as auxiliary scatter detectors, because their polarizing filters can be enabled and disabled by the user.

The five light scatter detectors, below-the-trigger-threshold detection, superior electronic processing, and extremely low background signal provide a unique set of features offering new possibilities for research on small particles and organisms. This will undoubtedly help investigators build on inspiring discoveries in cell signaling, therapeutics and vaccine development, disease progression, response to therapeutics, and more.

### References


1. de Grooth BG, Terstappen LWMM, Puppels GJ et al. (1987) Light-scattering polarization measurements as a new parameter in flow cytometry. *Cytometry* 8:539-544.
2. Hanscheid T, Melo-Cristino J, Pinto BG (2001) Automated detection of malaria pigment in white blood cells for the diagnosis of malaria in Portugal. *Am J Trop Med Hyg* 64(5-6):290-292.
3. Petersen TW, Brent Harrison C, Horner DN et al. (2012) Flow cytometric characterization of marine microbes. *Methods* 57(3):350-358.
4. Poncelet P, Robert S, Bouriche T et al. (2016) Standardized counting of circulating platelet microparticles using currently available flow cytometers and scatter-based triggering: Forward or side scatter? *Cytometry A* 89(2):148-158.
5. LaCroix R, Robert S, Poncelet P et al. (2010) Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative Workshop. *J Thromb Haemost* 8(11):2571-2574.


Find out more at **thermofisher.com/bigfoot**

ThermoFisher SCIENTIFIC