BD FACSymphony™ S6 Cell Sorter
User's Guide

23-20080(04)
2022-07

CE For Research Use Only

Regulatory information

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

Laser safety information

Class 1 Laser Product.

FCC information

WARNING: Changes or modifications to this unit not expressly approved by the party responsible for compliance could void the user's authority to operate the equipment.

NOTICE: This equipment has been tested and found to comply with the limits for a Class A digital device, pursuant to Part 15 of the FCC Rules. These limits are designed to provide reasonable protection against harmful interference when the equipment is operated in a commercial environment. This equipment generates, uses, and can radiate radio frequency energy and, if not installed and used in accordance with the instruction manual, may cause harmful interference to radio communications. Operation of this equipment in a residential area is likely to cause harmful interference in which case the user will be required to correct the interference at his or her own expense. Shielded cables must be used with this unit to ensure compliance with the Class A FCC limits. This Class A digital apparatus meets all requirements of the Canadian Interference-Causing Equipment Regulations. Cet appareil numérique de la classe A respecte toutes les exigences du Réglement sur le matériel brouilleur du Canada.

History

Revision	Date	Change made
23-20080-00	2018-10	Initial release
23-20080-01	2020-03	Updates to 6-way sorting and troubleshooting. Specific features added to Symphony S6
23-20080-02	2020-10	Removed references to BD FACSRinse™ and added BD® Detergent Solution Concentrate in place of BD FACSRinse™.
23-20080(03)	2021-07	Added waste tank baffle, amber collection devices, laser shutter, sort collection hanger, assignment of multiple fluorochromes to a channel, error message in the troubleshooting section, and CST page.
23-20080(04)	2022-07	Added 6-way 1.5 mL tube holder option. Added support for USB digital cameras. Added sort conflict support for 6-tube sort layouts. Updated part numbers for BD FACSDiva™ CS&T Research Beads.

Contents

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1

About this guide

This user guide contains the instructions necessary to operate and maintain your BD® FACSymphony™ S6 cell sorter. Because many instrument functions are controlled by BD FACSDiva™ software, this guide also contains basic software information needed for instrument setup. For detailed information on software functions, see the *BD FACSDiva Software Reference Manual*.

This user guide assumes you have a working knowledge of basic Microsoft® Windows® operation. If you are not familiar with the Windows operating system, see the documentation provided with your computer.

Before using the BD FACSymphony™ S6 cell sorter for the first time, review the following information:

- System components (page 11) to become familiar with system components
- Theory of operation (page 33) to understand how the instrument works and to learn about the software components used to control different subsystems
- Using BD FACSDiva™ software (page 55) to see where software controls are located

Instructions for routine acquisition, analysis, and sorting can be found in Chapters 4 and 5.

Conventions

The following tables list conventions used throughout this guide. The first of the following tables list the symbols that are used in this guide or on safety labels to alert you to a potential hazard. Text and keyboard conventions are shown in the second table.

Symbol[1]	Meaning
	Caution: hazard or unsafe practice that could result in material damage, data loss, minor or severe injury, or death
	Electrical danger
	Laser radiation
	Biological risk
	Pinch hazard

[1] Although these symbols appear in color on the instrument, they may be in black and white throughout the printed user guide; their meaning remains unchanged.

Technical assistance

For technical questions or assistance in solving a problem:

- Read the section of the user guide specific to the operation you are performing.
- See Troubleshooting (page 169)

If additional assistance is required, contact your local BD Biosciences technical support representative or supplier. Visit our website, bdbiosciences.com, for up-to-date contact information.

When contacting BD Biosciences, have the following information available:

- Product name, part number, and serial number
- Any error messages
- Details of recent system performance

Limitations

This instrument is for Research Use Only. Not for use in diagnostic or therapeutic procedures.

BD Biosciences is providing software without warranty of any kind on an as-is basis. The software and workstations are intended for running the instruments supplied by BD Biosciences. It is the responsibility of the buyer/user to ensure that all added electronic files including software and transport media are virus-free. If the workstation is used for internet access or purposes other than those specified by BD Biosciences, it is the buyer/user's responsibility to install and maintain up-to-date virus protection software. BD Biosciences does not make any warranty with respect to the workstation remaining virus-free after installation. BD Biosciences is not liable for any claims related to or resulting from the buyer/user's failure to install and maintain virus protection.

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2

System components

The following topics are covered in this chapter:

BD FACSymphony™ S6 Cell Sorter system

The BD FACSymphony™ S6 cell sorter is a high-speed fixed-alignment benchtop cell sorter. With its fixed-optics design and digital electronics, the BD FACSymphony™ S6 cell sorter enables multicolor analysis of up to 60 parameters, including two scatter parameters.

The system consists of three main components: the BD FACSymphony™ S6 cell sorter, the optional Biosafety Cabinet (BSC), and the workstation, as shown in the following figure.

Workstation

Data acquisition and analysis, as well as most BD FACSymphony™ S6 cell sorter functions, are controlled by BD FACSDiva™ software on a PC workstation. The workstation is equipped with the following applications:

- Microsoft® Windows® operating system
- BD FACSDiva™ software, version 9.5 or later
  - Data acquisition and analysis
  - Automatic fluidics startup, shutdown, and cleaning modes
  - Sort setup, stream monitoring, clog detection, and recovery
  - BD® Cytometer Setup and Tracking (CS&T) module for setup and tracking performance
- Supporting documentation for the software

For information about software features specific to the BD FACSymphony™ S6 cell sorter, see Theory of operation (page 33) and Using BD FACSDiva™ software (page 55). For general software information, see the BD FACSDiva™ Software Reference Manual.

BD FACSDiva™ software includes a BD® CS&T Research Beads module. For information on running the BD® CS&T Research Beads module with the BD FACSymphony™ S6 cell sorter, see Checking cytometer performance (page 99). For information about the BD® CS&T Research Beads module, see the *BD® Cytometer Setup and Tracking Application Guide*.

Note: For easy access to the online *BD FACSDiva™ Software Reference Manual*, select **Help > Documentation > Reference Manual**.

Biosafety cabinet

The optional BSC for the BD FACSymphony™ S6 cell sorter has been verified to meet personnel and product protection standards for a Class II Type A2 biosafety cabinet, the National Sanitation Foundation International Standard 49, and the Australian Standard® AS 2252.2–2009. The BSC is suitable for work with Biosafety Level (BSL) 1, 2, and 3 (low to moderate risk) infectious agents.

The BSC has an integrated Aerosol Management System (AMS). See Aerosol Management System (page 210) for information.

Flow cytometer

The BD FACSymphony™ S6 cell sorter consists of three subsystems (fluidics, optics, and electronics). The cytometer requires only a 20-A electrical outlet and an in-house air source (or optional compressor) for the fluidics. The following figure shows a view of the BD FACSymphony™ S6 cell sorter without the optional BSC.

See the following sections for more information on each subsytem:

- Fluidics system (page 14)
- Optics system (page 25)
- Cytometer electronics (page 29)

Fluidics system

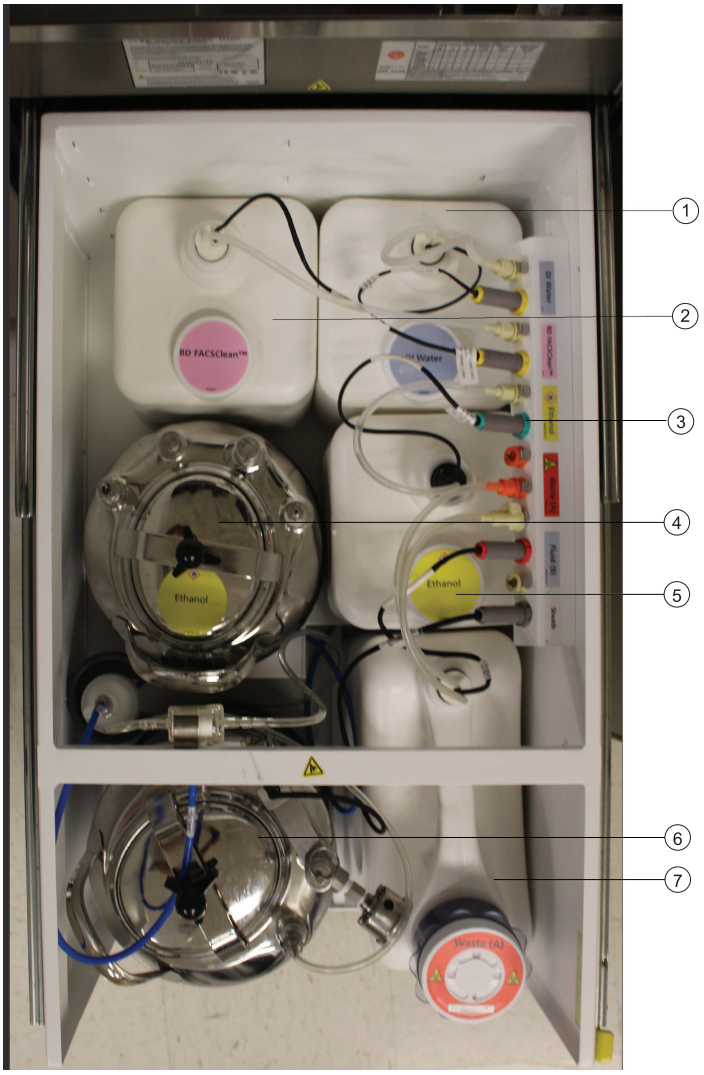
The fluidics system supplies sheath and cleaning fluids, and collects waste from the cytometer. The contents of the fluidics drawer and the fluidics components located inside the cytometer make up the fluidics system.

Fluidics drawer

The fluid containers, lines, and filters, and the connectors panel are located inside the fluidics drawer. The fluidics drawer slides out for easy access to its contents. Press down the yellow lever on the top right side of the drawer to release the lock and slide the drawer out or in.

Containers

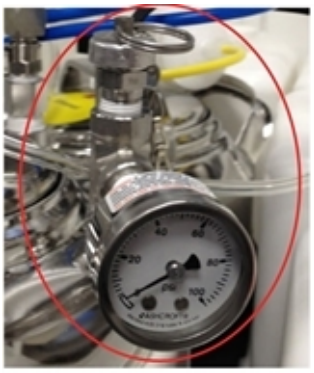
The fluidics drawer holds a 10-L stainless steel sheath tank, a 5-L stainless steel ethanol shutdown tank, a 10-L waste container, and three 5-L auxiliary cleaning fluid containers.



Item number	Component
1	DI water tank
2	BD FACSClean™ solution tank
3	Connector panel
4	Ethanol shutdown tank
5	Ethanol tank
6	Sheath tank with pressure gauge
7	Waste tank

Pressure gauge

A pressure gauge is located on the sheath fluid tank to confirm that the tank is pressurized.

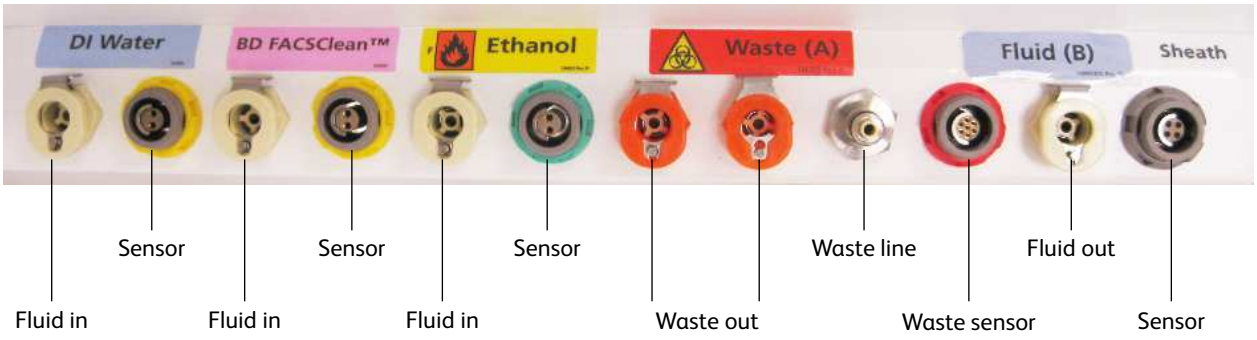


Fluid filters

The fluid filters for DI water, BD FACSClean™ cleaning solution, and ethanol are located in the outside left panel of the fluidics drawer.

Connectors panel

The connectors panel is located in the top right section of the fluidics drawer. A fluid line and a sensor connect each container to the panel.



Fluidics components

When the fluidics system is activated, the sheath fluid from the pressurized sheath tank is forced from the fluidics drawer up into the cuvette flow cell where hydrodynamic focusing forces particles from the sample injection chamber through the cuvette in a single-file stream.

Within the cuvette flow cell, laser light is focused on the sample core stream. Fluorescent molecules excited by the different laser wavelengths are detected by the optics and analyzed by the electronics. Particles are then either transported to waste reservoirs via the waste aspirator, or sorted into a collection device within the sort collection chamber.

The following fluidics components are described in this section. For more information about fluidics, see Fluid movement (page 34).

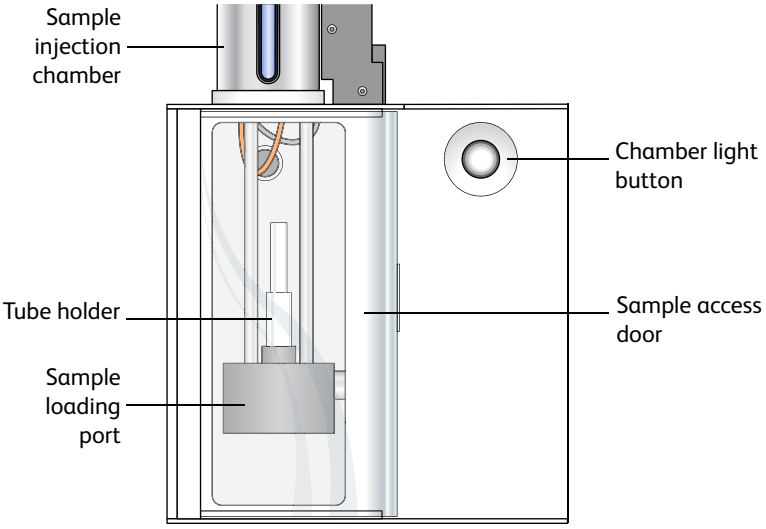
- Sample injection chamber (page 17)
- Tube holders (page 18)
- Cuvette flow cell (page 19)
- Integrated nozzle (page 19)
- Sort block (page 20)
- Sort collection devices for 2- and 4-way sorting (page 22)

Sample injection chamber

The sample injection chamber is where sample is introduced into the flow cytometer. During acquisition, the chamber is pressurized to force sample toward the cuvette flow cell.

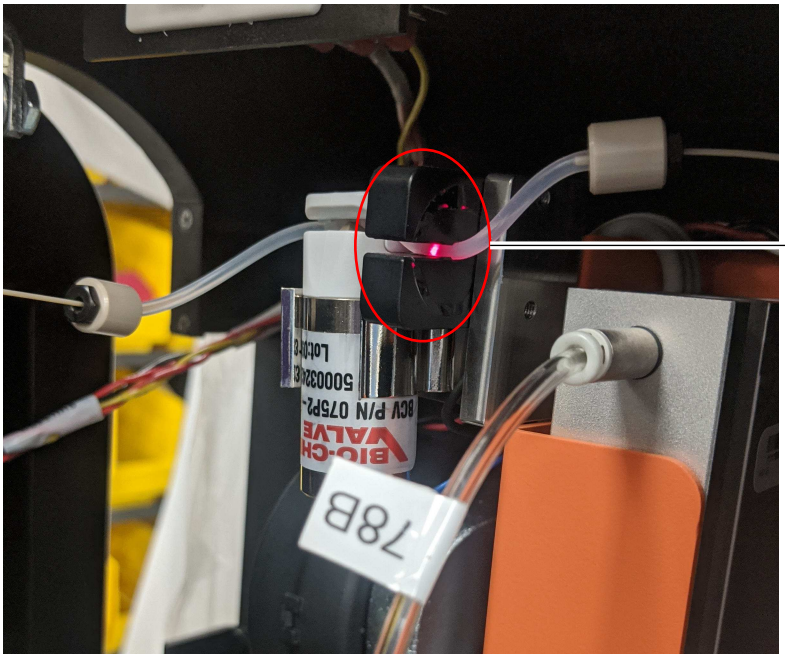
Samples can be agitated and temperature-controlled within the sample injection chamber using controls in the software (see Fluidics controls (page 56)). You can view the amount of fluid remaining in your sample tube by pressing the chamber light button shown in the following figure.

Do not use the chamber light for long periods with samples stained with light-sensitive reagents.



Bubble detector

The bubble detector is an optical sensor that detects air bubbles from the sample tube. Located on the sample line just before the pinch valve, it triggers the pinch valve to close, cutting off sample flow and preventing bubbles from entering the flow cell. When a bubble is detected, the bulk injection chamber light and LED in the button blink to alert the operator.

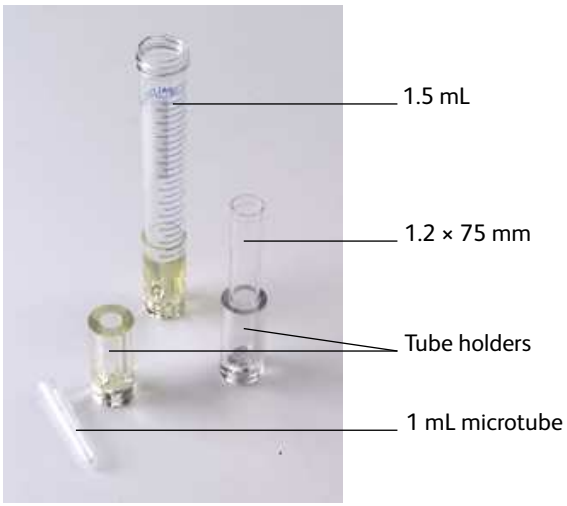


Tube holders

A variety of tube holders are provided with the cytometer to accommodate tubes from 15-mL centrifuge tubes to 1.0-mL microtubes.

To load a tube, install the appropriate-size tube holder onto the loading port, and place a tube in the holder. Make sure to press the tube holder down firmly onto the metal rod in the loading port, so the tube holder is seated correctly each time a tube is installed.

When the Load button is clicked in the software (see Acquisition controls (page 68)), the loading port rises to enclose the tube within the chamber. The chamber is then pressurized so that sample will flow to the flow cell.



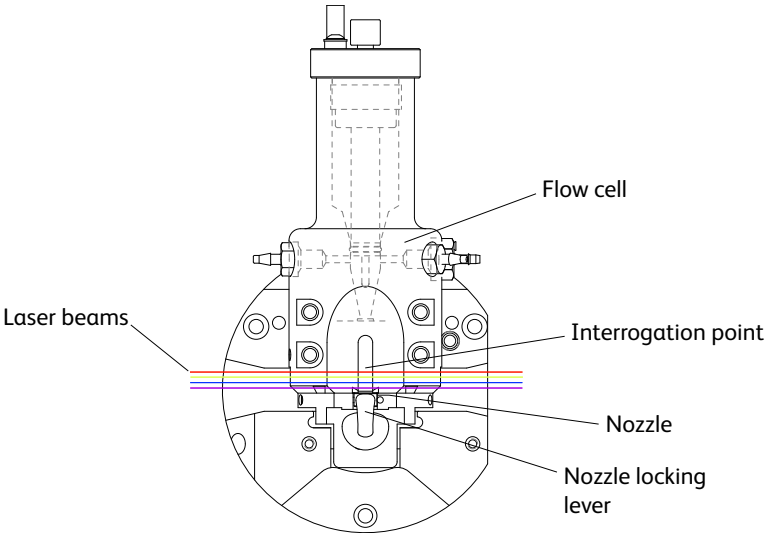
After a tube is loaded, the Load button changes to Unload. Click the Unload button to lower the loading port after data has been recorded. After each tube is unloaded, sheath fluid flushes the sample tubing inside and out to reduce potential sample carryover.

To prevent injury from moving parts, keep your hands and clothing away from the loading port when a tube is loading or unloading. Do not place objects under the loading port.

Cuvette flow cell

The BD FACSymphony™ S6 cell sorter system includes a next-generation flow cell. The cuvette flow cell is the heart of the BD FACSymphony™ S6 cell sorter. Within the flow cell, hydrodynamic focusing forces particles through the cuvette in a single-file stream, where laser light intercepts the stream at the sample interrogation point.

As a precautionary measure, prior to opening the Flow Cell access door, use the **Stop** button in the laser control software to put the lasers in standby and then close the laser control software. This will turn off the laser diode, but will keep the diode temperature constant.

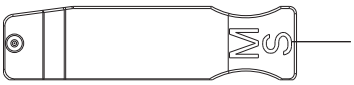


The unique flow cell design permits particles to flow through the cuvette at a low velocity (approximately 6 meters per second for the 70-micron sort setup), allowing longer exposure to laser energy. The cuvette is gel-coupled to the fluorescence objective lens to transmit the greatest amount of emitted light from the interrogation point to the collection optics (see Optics system (page 25)). After passing through the cuvette, the stream is accelerated (to approximately 30 meters per second with the 70-micron sort setup) as it enters the nozzle tip, where the drop drive breaks the stream into droplets for sorting.

Integrated nozzle

The BD FACSymphony™ S6 cell sorter next-generation flow cell requires integrated nozzles that have been optimized for it.

The integrated nozzles are available in four sizes (70, 85, 100, and an optional 130 µm) to accommodate a variety of particle sizes, plus a closed-loop nozzle for use in cleaning and shutdown procedures. The nozzle is keyed to a fixed position at the lower end of the cuvette. Because the nozzle is below the interrogation point, optical alignment is not affected when the nozzle is changed.



MS indicates integrated nozzle for the BD FACSymphony™ S6 cell sorter flow cell

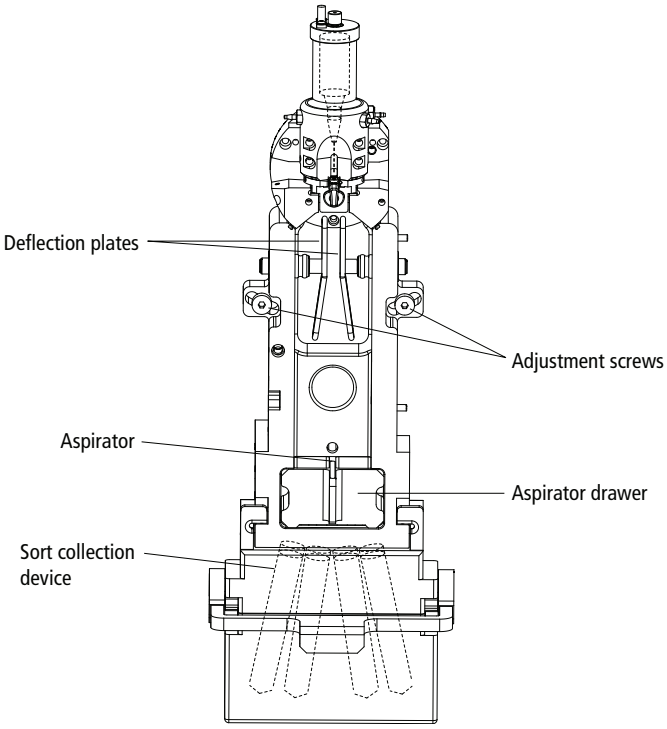
Bottom view of nozzle

See these sections for more information on the nozzle:

- Changing the integrated nozzle (page 155)
- Cleaning the integrated nozzle (page 156)
- Handling the integrated nozzle (page 158)

Sort block

After leaving the nozzle, particles pass through the sort block where they are either transported to waste via the waste aspirator, or sorted into a collection device in the sort collection chamber. The sort block houses the high-voltage deflection plates, along with the aspirator and aspirator drawer.



As a precautionary measure, prior to opening the Flow Cell access door, use the **Stop** button in the laser control software to put the lasers in standby and then close the laser control software. This will turn off the laser diode, but will keep the diode temperature constant.

The entire sort block assembly can be rotated on a fixed pivot point to adjust the position of the stream in the waste aspirator. If the keyed stream position differs between different nozzles, the stream might not hit the center of the aspirator after the nozzle is changed. In this case, you can change the angle of the sort block by loosening the adjustment screws on both sides of the deflection plates and rotating the sort block. An Allen wrench is provided in the accessory kit. Tighten the screws when the stream is re-centered in the aspirator.

Deflection plates

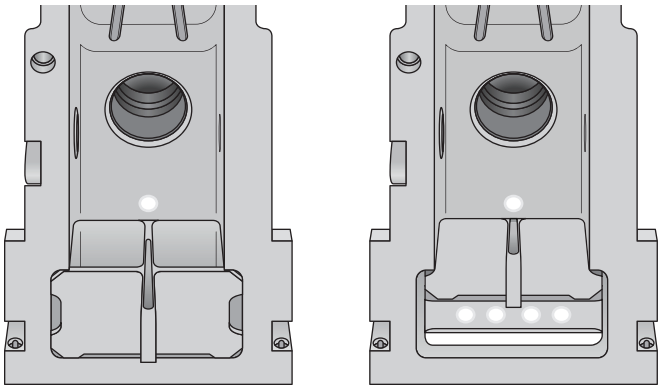
The high-voltage deflection plates are used to deflect side streams during sorting. The plates are turned on and off using the Voltage control in the Side Stream window (see Side Stream Formation (page 47)). A red warning light is illuminated whenever the plate voltage is on.

A 12,000-volt potential exists between the deflection plates when they are on. Contact with the charged plates results in serious electrical shock. Do not touch the deflection plates when the voltage warning light is illuminated, or when the software indicates that the plate voltage is on. The plates remain energized even when the sort block door is open.

Aspirator drawer

The aspirator drawer keeps the sort collection tubes covered until sorting begins. You can open and close the drawer using a control in the Sort Layout or Side Stream window (see Using Sorting Controls (page 74)). When the Sweet Spot is on and a clog is detected during sorting, the drawer automatically closes to protect the sort collection tubes. For information on how to handle a clog, see Sorting (page 127).

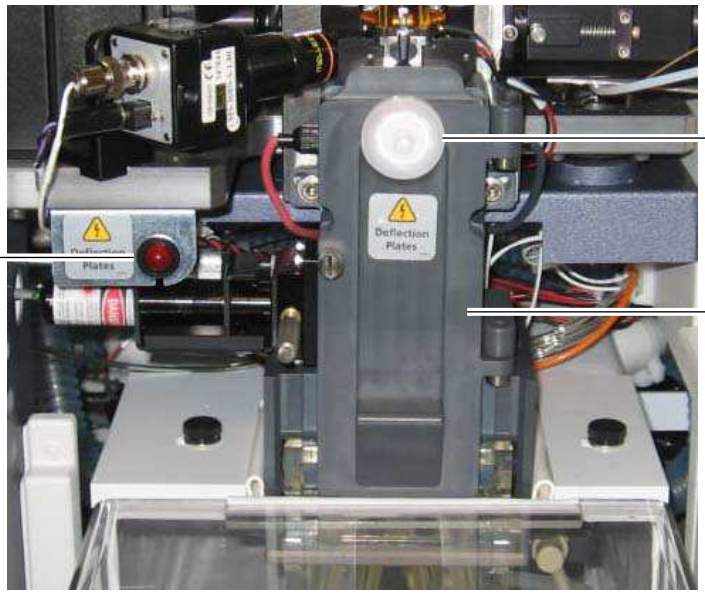
To avoid pinching your hands or fingers in the drawer, keep your hands away from the sort block during sorting.



Aerosol management

During sample acquisition and sorting, the sort block door and flow cell access door should be kept closed to help contain potential aerosols.

Cell sorters that use droplet generation methods, such as the BD FACSymphony™ S6 cell sorter, can produce aerosols around the sample stream. When acquiring biohazardous samples, follow universal precautions at all times. Keep the sort block door closed during sorting. If you need to access the sort block and you are working with highly infectious samples, consider turning off the stream before opening the sort block door.



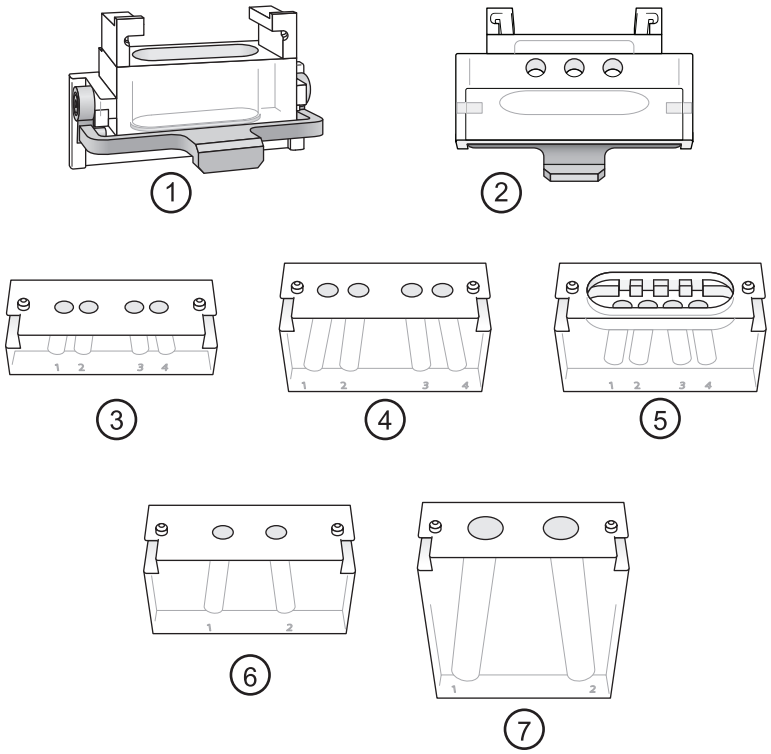
Additional aerosol removal is provided by either the AMS or the BD® Aerosol Management Option (AMO).

The AMS is integrated into the BSC and contains a high efficiency particulate air (HEPA) filter. See Operating the AMS (page 211) for instructions.

The AMO is an optional device for systems without a BSC. The AMO evacuates the sort collection chamber during sorting and is equipped with an ultra-low penetrating air (ULPA) filter to trap aerosolized particles. See BD® Aerosol Management Option (page 191) for complete information on using this option.

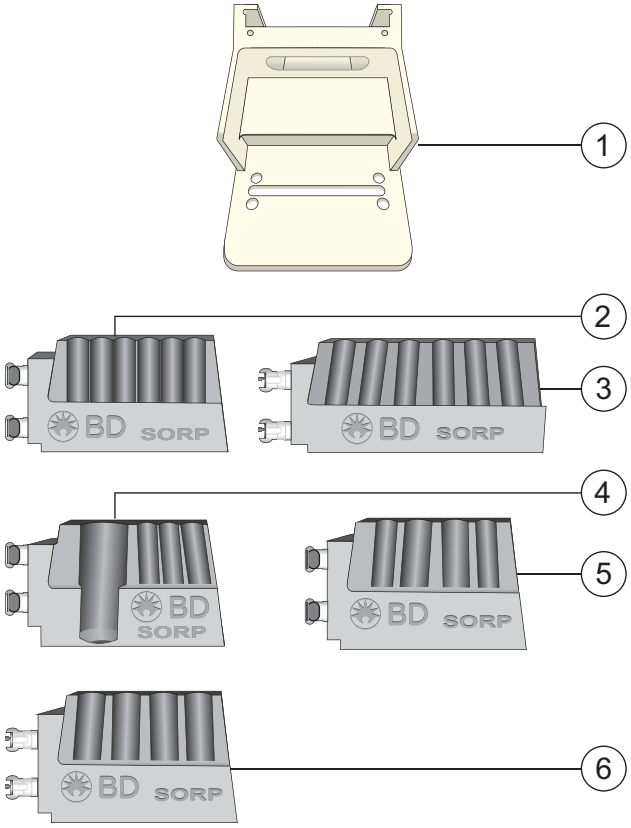
Sort collection devices for 2- and 4-way sorting

Collection devices are installed in the sort collection chamber to collect sorted samples. The collection devices are designed in two pieces with a universal top that can be used with different tube configurations. The universal top has three holes on the back side that help with aerosol evacuation when an AMO or AMS is installed.



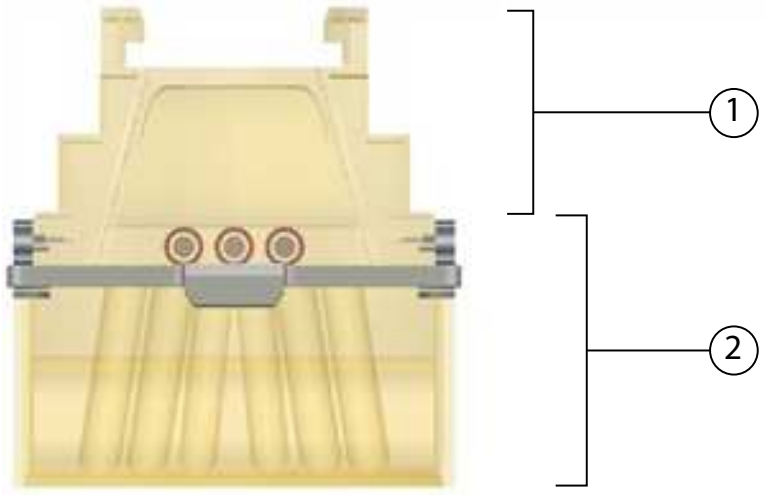
1	Enclosed top (front view)
2	Enclosed top (back view)
3	Four-way 1 mL (optional)
4	Four-way 12 × 75 mm
5	Four-way 1.5 mL Eppendorf
6	Two-way 12 × 75 mm
7	Two-way 15 mL

Sort collection devices for 4- and 6-way sorting



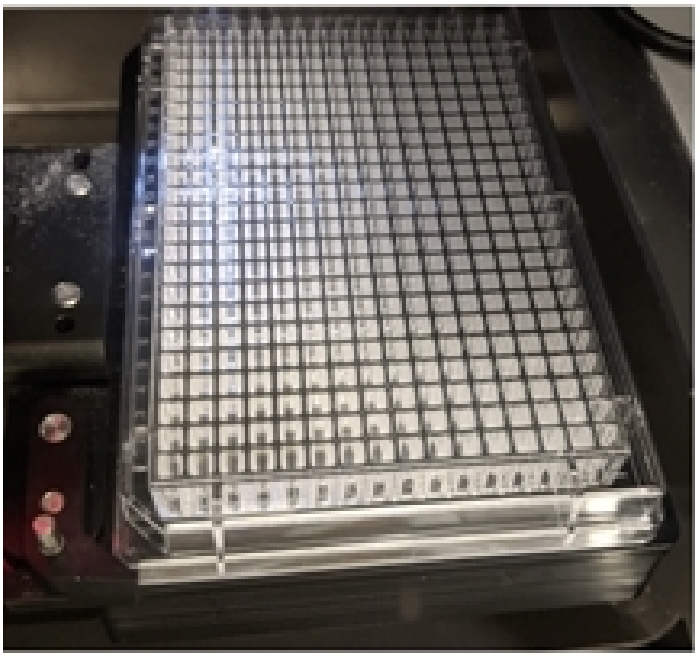
1	Upper tube holder mount
2	6-way 12 × 75 mm
3	6-way 1.5 mL
4	Left: 50 mL; Right: 3-way 12 × 75 mm
5	4-way; Outside: 12 × 75 mm; Inside: 15 mL
6	Four-way 15 mL

A transparent amber 6-way sort collection device with integrated tube holder mount is also available as shown in the following diagram.



1	6-way 5 mL tube holder top
2	6-way 5 mL amber tube holder

An automated cell deposition unit (ACDU) holds multi-well plates and slides for sort collection. The sort collection chamber door should be kept closed when sorting onto a plate. The door keeps the chamber free of dust and other airborne particles, and seals the chamber during aerosol evacuation for cytometers equipped with the AMO.



Optics system

The BD FACSymphony™ S6 cell sorter uses innovative designs for both the excitation and collection optics.

Excitation optics

The excitation optics consist of lasers, beam steering optics, and focusing lenses.

For information about how signals are generated, see Signal generation (page 36).

The BD FACSymphony™ S6 cell sorter uses air-cooled and solid state lasers that do not have special power or cooling requirements.

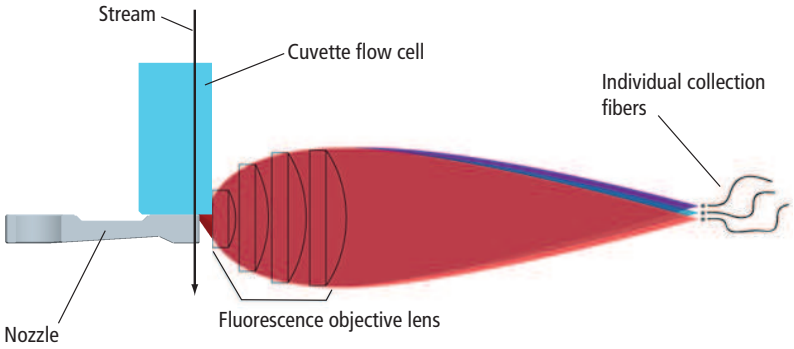
The system also supports up to 9 installed lasers.

Lasers emit intense, coherent electromagnetic radiation that can cause irreparable damage to skin and eyes. To prevent retinal burns and possible blindness, do not remove laser shielding, defeat interlocks, or attempt to service the cytometer in any location where laser warning labels are attached. See the *BD FACSymphony™ S6 cell sorter Safety and Limitations Guide* for the placement of laser warning labels.

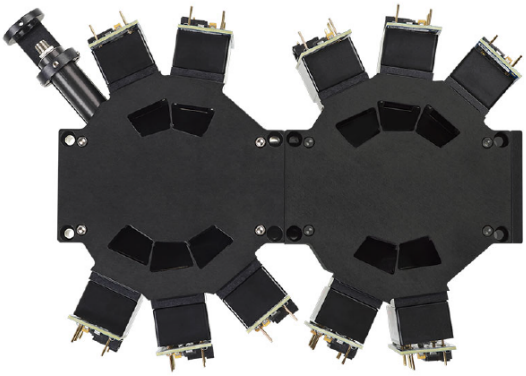
Steering optics direct the laser light in a precise and constant manner onto a focusing lens. The lens focuses the laser light onto the sample core stream within the cuvette flow cell. The lasers are positioned on the sample stream for optimal generation of signals. Since the optical pathway and sample core stream are fixed, optimization is constant from day to day.

Collection optics

From the cuvette flow cell, laser light is collected by a fluorescence objective lens that is gel-coupled to the cuvette to transmit the maximum amount of light. The lens collects and focuses fluorescent light emitted at each of the laser focal points onto individual collection fibers. These fibers transfer the emitted light to the collection optics, as shown in the following figure.



The collection optics are set up in high-parameter cascade (HPC) arrays that are engineered to maximize signal detection from each laser. This is accomplished by transmitting the highest wavelengths to the first photomultiplier tube (PMT), and reflecting lower wavelengths to the next PMT through a series of longpass dichroic mirrors. Bandpass filters in front of each PMT allow fine-tuning of the spectral wavelengths that need to be collected. Since reflection is more efficient than transmission, this design greatly increases the multicolor detection capabilities of the cytometer.



Detectors

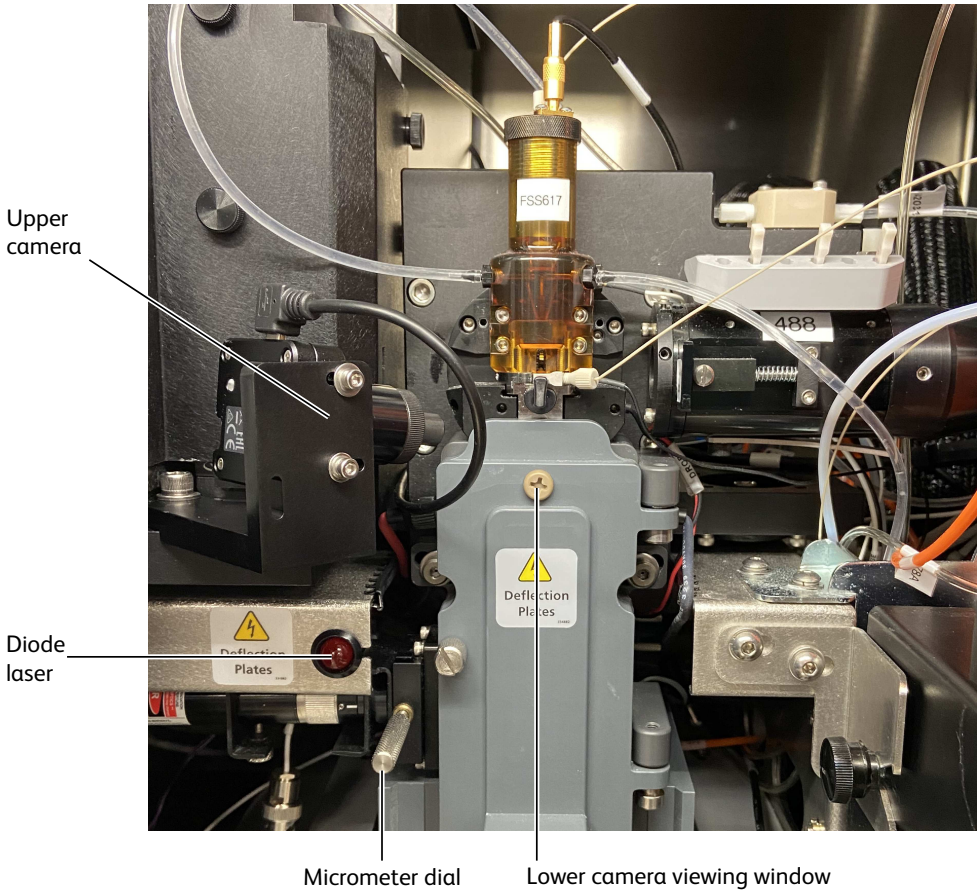
The following figure shows the locations of detector arrays in a 5-laser system on the electronics cabinet.

Stream-viewing optics

The BD FACSymphony™ S6 cell sorter is equipped with optical components that are used to view the stream.

- The upper camera generates an image used to monitor drop formation. It is focused on the stream, just below the nozzle, to provide an image of the drop breakoff.
- The lower camera generates an image used for the BD FACS™ Accudrop option. It enhances the ability to

see the side streams and assists in setting an accurate drop-delay value.



Special image-processing software allows you to view the stream images from each camera within separate windows in BD FACSDiva™ software. See Sorting (page 43) for more information about viewing the streams and to learn how Accudrop components are used to determine the drop delay.

Cytometer electronics

The electronic components consist of the power and air supply panels along with processing boards in the card cage. The power and air supply panels are located on the right side of the cytometer base in the lower left corner. Other electronic components are embedded within the cytometer and do not need adjustment. This section describes only adjustable cytometer electronics. For more information about the electronics, see Signal detection (page 37).

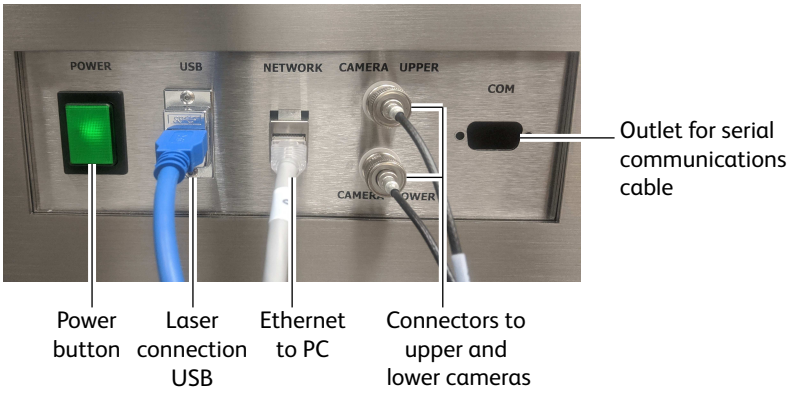
Power and operation

Power to the BD FACSymphony™ S6 cell sorter is supplied by a power cord plugged directly into a standard electrical outlet. The optional BSC is hard-wired to the in-house electrical system.

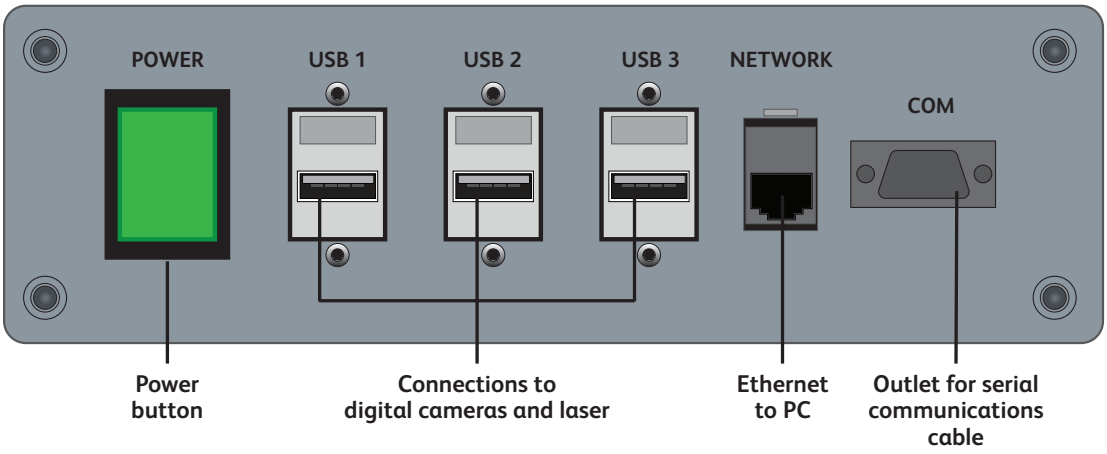
Power panel

The power panel contains the power button, a USB port for BD service, an ethernet connection, connectors for the stream-viewing cameras, and a serial communications cable.

Power panel for analog cameras:

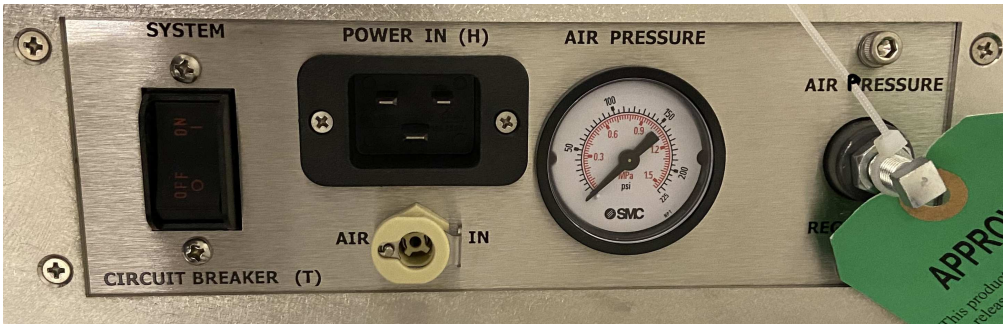


Power panel for digital cameras:



Air supply panel

The air supply panel is located to the right of the power panel. The air supply panel contains the external air regulator, air pressure gauge, power cord, external air input connector, and the cytometer circuit breaker. Air pressure is adjusted using BD FACSDiva™ software.



Connecting to the external air supply

The BD FACSymphony™ S6 cell sorter system requires an external (in-house) air source or optional compressor to operate.

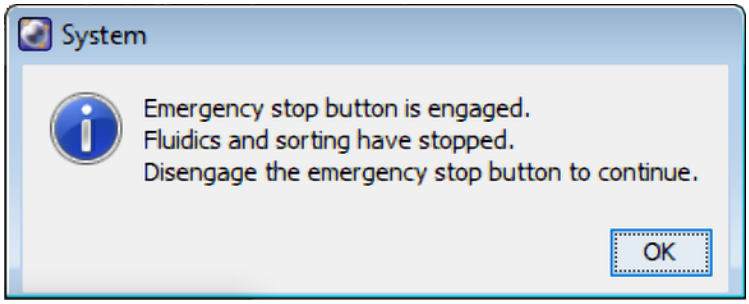
To connect the fluidics to the external air supply, switch on the external air, if necessary. Attach the external air line to the external air input connector. The external air supply must provide 6.6–6.9 Bar (95–100 PSI) regulated. The source of the compressed air must deliver clean (<5 ppm) dry-filtered (oil-free) air at stable pressures.

Emergency stop button

The emergency stop button is a safety feature that can be used to stop the movement of the loading port and ACDU stage. The emergency stop button is located on the front of the optics drawer. The button lights up when a tube is loading to remind you to keep your hands away from the loading port.

The following occur when this button is pushed:

- The software displays the following message:



- The tube is unloaded from the sample injection chamber.
- The ACDU stage (if in use) stops moving.
- The stream is turned off.
- The deflection plate voltage is turned off.
- The aspirator drawer (if open) closes to protect the sort collection tubes.

Note: The emergency stop button does not turn off the lasers or shut down the cytometer main power.

To reset the button, slowly turn the button clockwise until the light turns off and the button returns to its original position.

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3

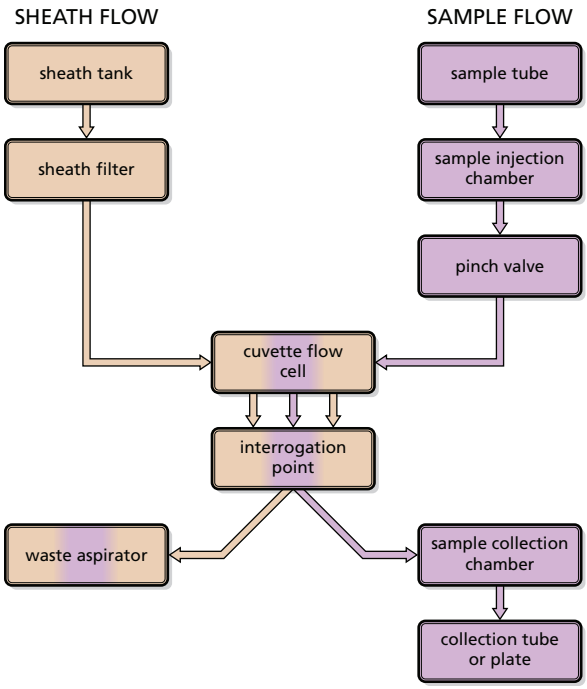
Theory of operation

This chapter describes how the BD FACSymphony™ S6 cell sorter works and how BD FACSDiva™ software controls are used to operate different system components. For a general overview of the software, see Using BD FACSDiva™ software (page 55).

See the following sections for a description of these BD FACSymphony™ S6 cell sorter functions:

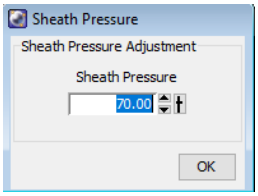
Fluid movement

The fluidics system is responsible for moving particles from the sample injection chamber through the cuvette flow cell for interrogation, and then to waste or into a collection device during sorting. The following sections describe the controls used to move fluids through the BD FACSymphony™ S6 cell sorter fluidics system.

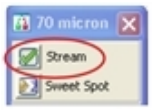


Sheath flow

When you turn on the cytometer, the external air compressor must be turned on or house air must be supplied to the system. The fluidics system is activated when you select the Fluidics Startup command in BD FACSDiva™ software. During fluidics startup, sheath fluid is forced from the pressurized sheath tank through a filter and is delivered to the cuvette flow cell at a constant pressure. You can view the current sheath pressure setting by selecting Cytometer > Sheath Pressure.



After fluidics startup, sheath flow is controlled using the Stream button in the Breakoff window. When clicked, the button changes from a red “X” to a green check mark, and sheath flows through the cuvette flow cell at the rate that is specified in the Sheath Pressure dialog. (See Drop Formation (page 43) for a complete description of the Breakoff window.)

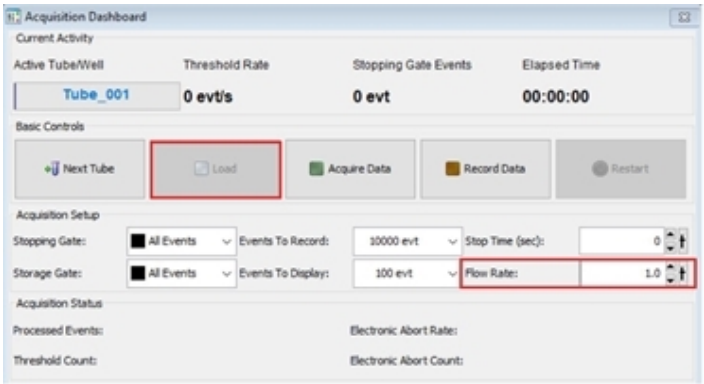


As a general rule, the sheath pressure level is set by selecting a sort setup mode from the Sort menu, rather than by adjusting the sheath pressure control. Each sort setup mode is optimized at a preset sheath pressure. If you change the sheath pressure, a multitude of other values will be affected and need updating, including the drop-drive frequency, drop-delay value, laser delay, area scaling factor, and other values. For more information, see Sort setup (page 70).

Sample flow

Sample is introduced into the cuvette when the Load button is clicked on the Acquisition Dashboard. After Load is clicked, the loading port rises to enclose the tube within the sample injection chamber. The chamber is automatically pressurized and the chamber pressure forces sample through the sample line into the cuvette flow cell. To stop sample flow after a tube is loaded, click the Stop Acquiring button.

The sample flow rate is specified using the Flow Rate field on the Acquisition Dashboard. You can adjust the flow rate from 1–11, which corresponds to approximately 10–80 µL/minute.

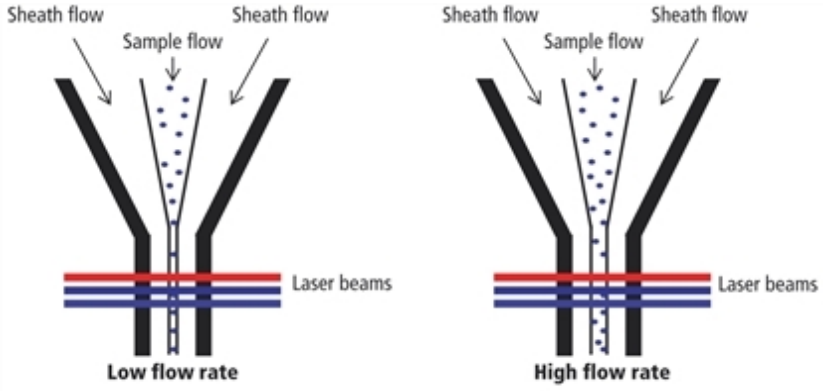


Note: The relatively longer sample tubing on the BD FACSymphony™ S6 cell sorter results in a different flow rate between cells and beads. Thus, absolute counting using BD Trucount™ Beads can yield erroneous results.

Hydrodynamic focusing

In the flow cell, pressurized sheath fluid surrounds the sample fluid to hydrodynamically focus the core stream of suspended cells into the center of the cuvette, where the particles are intercepted by the laser beam.

The difference in pressure between the sheath fluid and the sample fluid can be used to vary the diameter of the sample core. A lower difference results in a relatively narrow core stream, while a higher difference results in a wider sample stream.



Ideally, you want the core stream at its minimum diameter so that cells pass through the laser beam in a single-file stream. However, depending on your application, a lower resolution might be acceptable in order to acquire the data more quickly. For example, with this three-laser configuration, a higher flow rate is generally used for qualitative measurements such as immunophenotyping—the data is less resolved but is acquired more quickly. A lower flow rate is generally used in applications for which greater resolution is critical.

Signal generation

The following sections describe how signals are generated when cells or particles intercept the laser within the cuvette flow cell.

Light scatter

When a cell or particle passes through a focused laser beam, laser light is scattered in all directions. Light that scatters axial to the laser beam is called forward scatter (FSC). Light that scatters perpendicular to the laser beam is called side scatter (SSC). FSC and SSC are related to certain physical properties of cells:

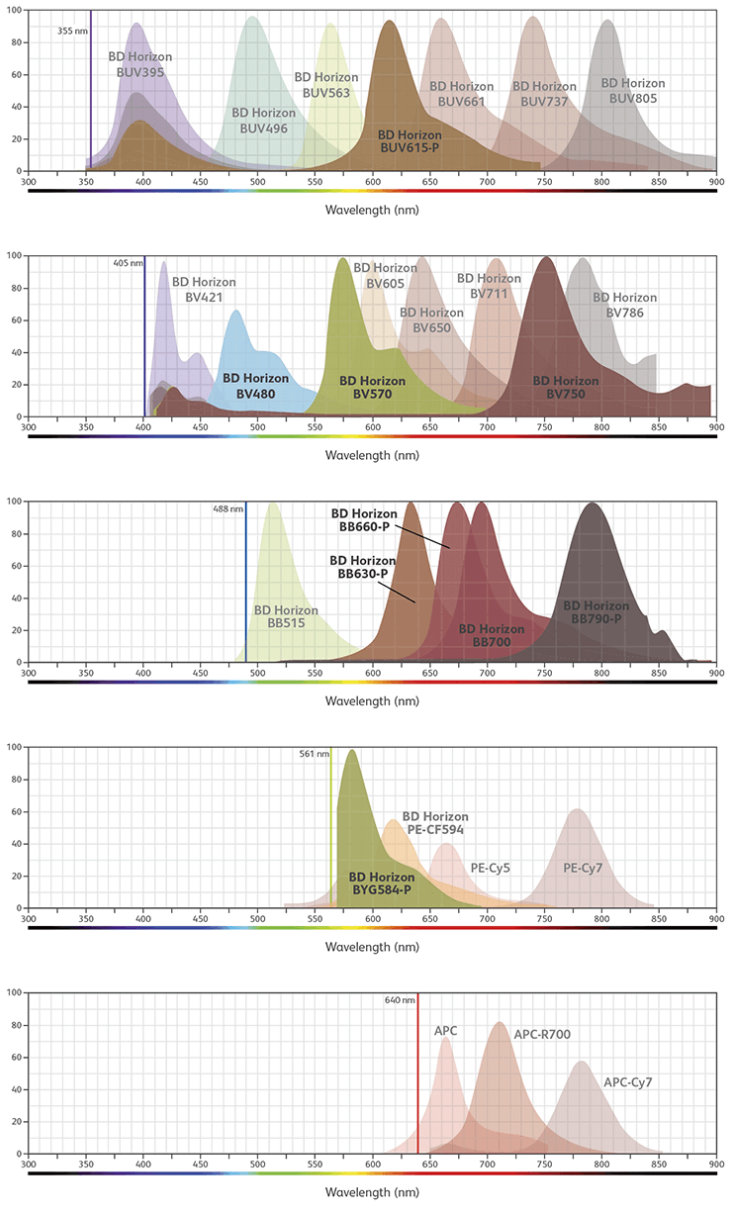
- FSC indicates relative differences in the size of the cells or particles.
- SSC indicates relative differences in the internal complexity or granularity of the cells or particles.

Fluorescence signals

When cells or particles stained with fluorochrome-conjugated antibodies or other dyes pass through a laser beam, the dyes can absorb photons (energy) and be promoted to an excited electronic state. In returning to their ground state, the dyes release energy, most of which is emitted as light. This light emission is known as fluorescence.

Fluorescence is always a longer wavelength (lower-energy photon) than the excitation wavelength. The difference between the excitation wavelength and the emission wavelength is known as the Stokes shift. Some fluorescent compounds such as PerCP exhibit a large Stokes shift, absorbing blue light (488 nm) and emitting red light (675 nm), while other fluorochromes such as FITC have a smaller Stokes shift, absorbing blue light and emitting green light (530 nm).

The emission spectra for some commonly used fluorochromes are shown in the following figure.



Signal detection

From the cuvette flow cell, scattered and fluorescent light is collected by the fluorescence objective lens. The lens collects and focuses fluorescent light emitted at each of the laser focal points onto individual collection fibers. These fibers transfer the emitted light to the individual detector arrays.

The following sections describe how laser light is detected and translated into signals that can be displayed in a plot.

Detector arrays

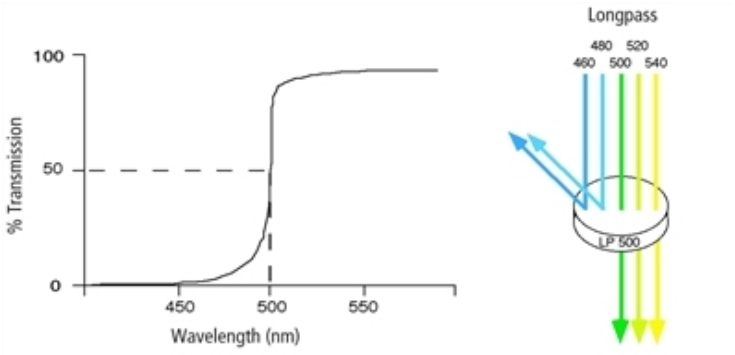
The BD FACSymphony™ S6 cell sorter has HPC detector arrays. Each detector array houses dichroic and bandpass filters, which steer and filter the emitted light, and PMTs, which detect light signals.

Filters

Optical filters modify the spectral distribution of light scatter and fluorescence directed to the detectors. Three kinds of filters are used in the detector arrays. Longpass (LP) filters are used to steer light between the detectors within a detector array, while bandpass (BP) and neutral density (ND) filters allow fine-tuning of the spectral wavelengths that need to be collected.

Longpass filters

LP filters pass wavelengths longer than the filter rating and reflect shorter wavelengths. For example, a 500 LP filter permits wavelengths longer than 500 nm to pass through it and reflects wavelengths shorter than 500 nm.



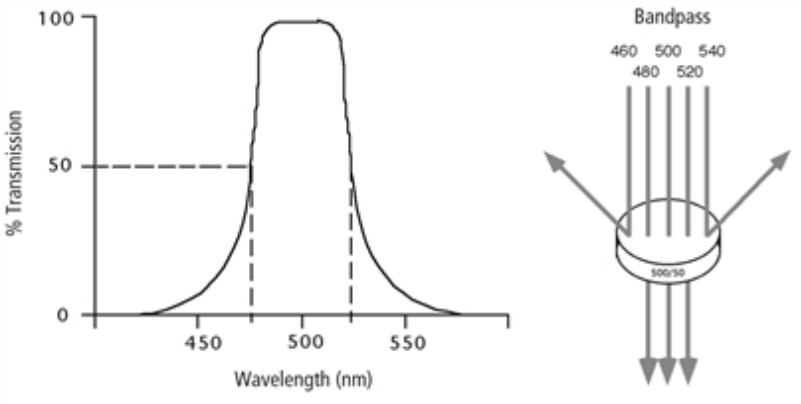
Dichroic filters that are used to direct different color light signals to different detectors are called dichroic mirrors or beam splitters.

Although dichroic mirrors have the properties of LP optical filters, you cannot necessarily use any type of LP filter as a beam splitter. A beam splitter must have a surface coating that reflects certain wavelengths, but many types of LP filters are absorbance filters that do not have any specific reflective characteristics. Also, optical filters and beam splitters are rated at a specific angle of incidence. When used as a beam splitter, they are placed at an angle relative to the light source. Their optical properties are therefore designed for that angle of incidence.

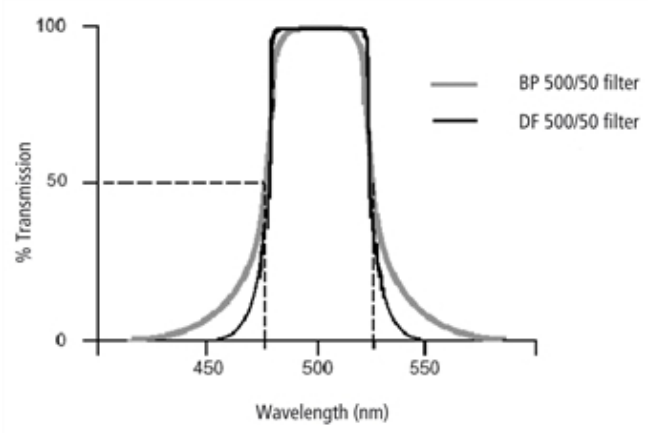
The detector arrays use LP dichroic mirrors to steer progressively shorter wavelengths of light to the next PMT in the array. For example, in the HPC array, light first passes through a 735 LP filter in the A position, followed by a 655 LP filter in the B position. Thus, wavelengths longer than 735 nm are detected at PMT-A. All wavelengths <735 nm are reflected to PMT-B. Wavelengths between 655 nm and 735 nm are detected at PMT-B. All wavelengths <655 nm are reflected to PMT-C, and so on.

Bandpass filters

BP filters transmit a relatively narrow range or band of light. Bandpass filters are typically designated by two numbers. The first number indicates the center wavelength and the second refers to the width of the band of light that is passed. For example, a 500/50 BP filter transmits light that is centered at 500 nm and has a total bandwidth of 50 nm. Therefore, this filter transmits light between 475 and 525 nm.



Discriminating (DF) filters have the same general function—they transmit a relatively narrow band of light. The principal difference between BP and DF filters is their construction. DF filters have more cavities or layers of optical coatings, resulting in a steeper transmission curve than the curve for a BP filter. This steep slope means that a DF filter is better at blocking light outside the rated bandwidth of the filter.

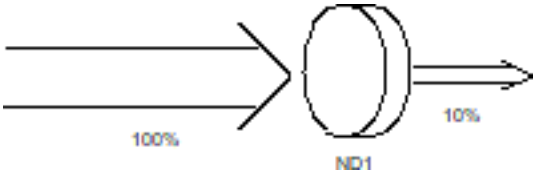


In the detector arrays, DF filters block high-intensity laser light and filter the remaining light to ensure that only the required wavelengths reach their intended detector. For example, in the HPC array, PMT-A has a 780/60 DF filter in front of it, which transmits light of 750–810 nm. Thus, the only wavelengths that will reach the A detector are those between 750 and 810 nm.

For optimal detection of fluorescent light, a bandpass filter must always be installed in front of each detector.

Neutral density filters

ND filters transmit a fixed percentage of light, reducing the transmitted intensity of all wavelengths equally. ND filters are neutral with respect to wavelength.



The ND1 filter on the BD FACSymphony™ S6 cell sorter allows approximately 10% of the light to be transmitted. The FSC detector has an ND filter placed in front of the 488/10 BP filter. The percentage of light needed for detection is based on particle size. For applications involving small particles (for example, bacteria or platelets),

you might need to remove the FSC ND filter. For applications involving large particles for which events appear off scale on the FSC axis with a gain of zero, a higher value ND filter is needed to decrease the FSC signal and keep the events on scale.

The system comes with three ND filters: 1.0, 1.5, and 2.0, where the transmission percentage is 10%, 3%, and 1%, respectively. See Removing or installing the FSC ND filter (page 168).

Detectors

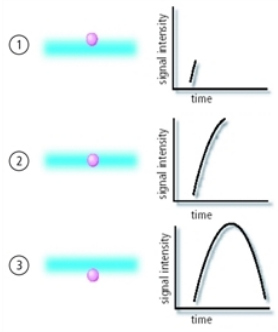
Detectors within each detector array convert light signals into electrical signals that can be processed by the electronics system.

There are two types of signal detectors in the BD FACSymphony™ S6 cell sorter flow cytometer: the photodiode and PMTs. The photodiode is less sensitive to light signals than the PMTs, and is used to detect the stronger FSC signal. The photodiode detects FSC light from the blue laser, and is stored outside the detector arrays. PMTs are used to detect the weaker signals generated by SSC and all fluorescence channels.

In BD FACSDiva™ software, the Cytometer Configuration window lets you define which fluorochromes or cell parameters will be measured at each PMT detector. If more than one fluorochrome is measured using the same PMT, you can add additional parameters to your configuration and select the appropriate fluorochrome within your software experiment. See Cytometer configuration (page 59) for more information.

Electronic processing

As cells or other particles pass through the focused laser beams, they scatter the laser light and can emit fluorescence. Because each laser beam is focused on a small spot and particles move rapidly through the flow cell, the scatter or fluorescence emission has a very brief duration—only a few microseconds. The PMTs convert this brief flash of light into an electrical signal called a pulse.

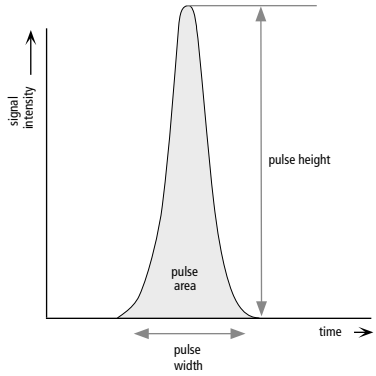


No.	Description
1	A pulse begins when a particle enters the laser beam. At this point, both the beam intensity and signal intensity are low.
2	The pulse reaches a maximum intensity or height when the particle reaches the middle of the beam, where the beam and signal intensity are the brightest. The peak intensity, or height of the pulse, is measured at this point.
3	As the particle leaves the beam, the pulse trails off.

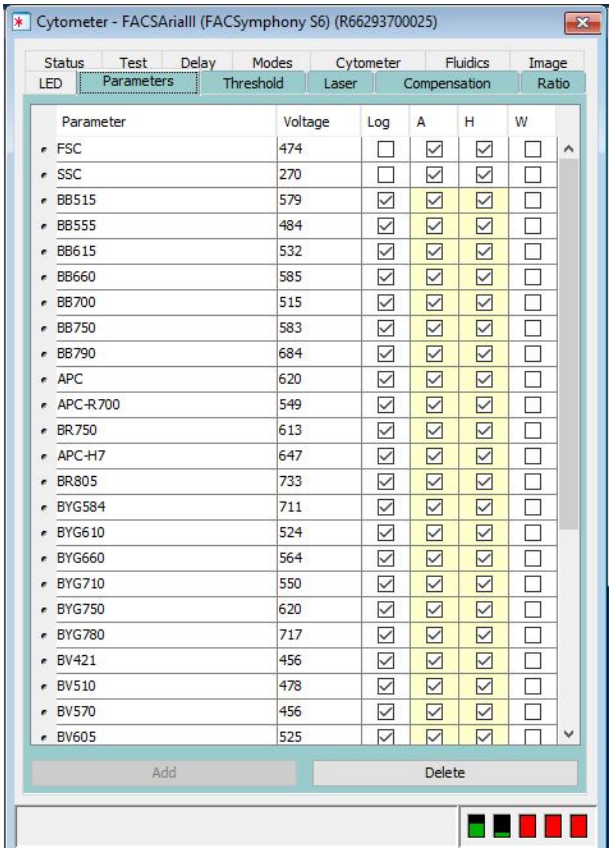
Pulse parameters

A parameter is a pulse property that is generated by a single PMT or photodiode, measuring fluorescent or scattered light. You can measure three characteristics of a pulse: area, height, and width. The pulse height

measures the maximum digitized value for the pulse, the pulse area calculates the sum of all height areas for the pulse, and the pulse width measures the time that the signal is above the threshold.



You can select which pulse characteristics you want to measure for each parameter using the Parameters tab. Pulse area (A) is measured by default. The Parameters tab also contains voltage controls that allow you to amplify signals by applying a voltage to PMTs or an electronic gain to the FSC signal. As the voltage is increased, the detector sensitivity increases, resulting in increased signal. As the voltage is decreased, the detector sensitivity decreases, resulting in decreased signal.

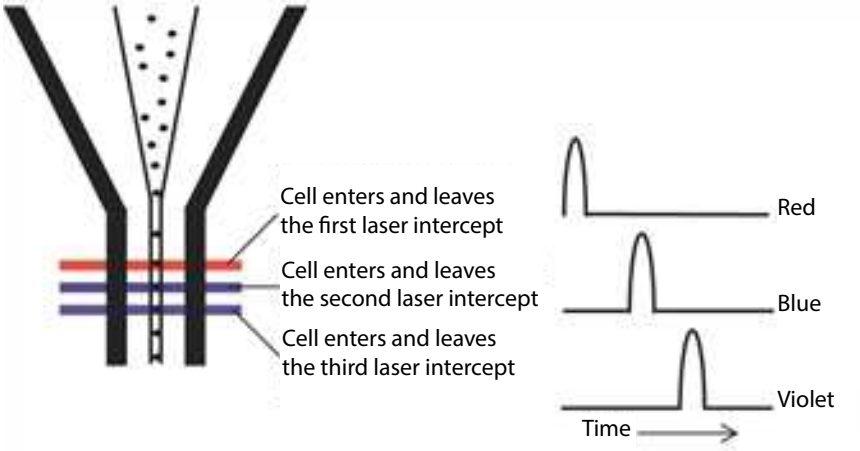


Digital data is displayed on an 25-bit linear scale, from 2.6–33,554,432. Select the Log checkbox to convert the display to a log scale. The Experiment Inspector contains an option to display log data on a four- or five-decade scale. (See the BD FACSDiva™ Software Reference Manual for more information.)

Laser delay

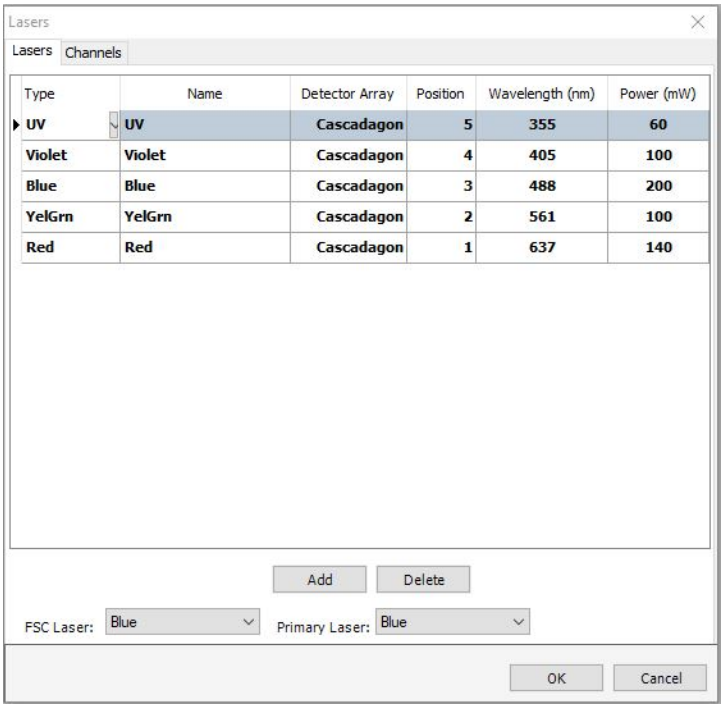
Sample interrogation takes place within the cuvette flow cell. Fiber optic cables are used to direct laser light through a series of prisms that focus each laser on the stream at a separate position. This allows optimal detection of fluorescence signals from each laser with minimal cross-contamination from the other beams.

In a three-laser system, the red laser intercepts the stream first, followed by the blue and then the violet laser. Because the laser signals are spatially separated, there is a slight delay between the detection of each laser's signal.



The delay factor in BD FACSDiva™ software is used to realign the signals so they can be measured and displayed on the same time scale. Signals are aligned with respect to the blue laser, so some lasers may have a negative delay value.

Laser delay is set automatically when you use the CS&T module. For information about manually adjusting the laser delay, see Manual adjustment of laser delay (page 116).

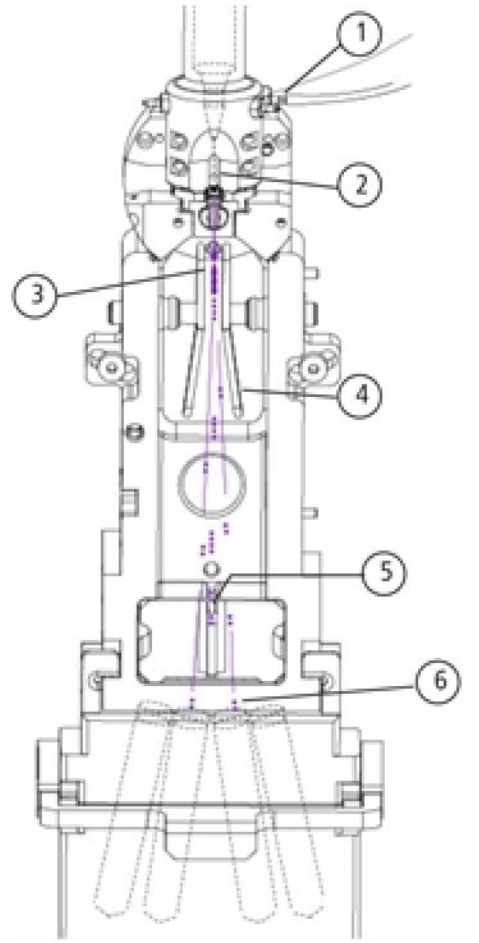


Sorting

While sorting, drop-drive energy is applied to the stream to break it into highly uniform droplets. Droplets detach from the stream a few millimeters downstream from the nozzle. The time between when a particle intercepts the laser and when it reaches the droplet breakoff point is determined using BD FACS™ Accudrop technology (see Drop-Delay Overview (page 49)).

When a particle is detected and meets the predefined sorting criteria, an electrical charge is applied to the stream just as the droplet containing that particle breaks off from the stream. Once broken off from the stream, the droplet—now surrounded by air—still retains its charge. The charged droplet passes by two strongly charged deflection plates. Electrostatic attraction and repulsion cause each charged droplet to be deflected to the left or right, depending on the droplet's charge polarity. Uncharged droplets are not affected by the electric field and pass down the center to the waste aspirator.

The following sections describe how the BD FACSymphony™ S6 cell sorter creates and charges drops, how the drops are deflected, and how sorting populations are identified.



No.	Description
1	Charge is applied via the stream-charging wire in the barb.
2	The sample generates light scatter and fluorescence signal. The signal is analyzed.
3	The charged droplet breaks off.
4	Deflection plates attract or repel the charged droplet.
5	Uncharged droplets pass to waste.
6	Charged drops containing particles of interest are collected.

Drop Formation

The BD FACSymphony™ S6 cell sorter constantly applies drop-drive energy to the stream. Droplets form as soon as you turn on the stream. Sample interrogation takes place upstream of the stream vibration so that analysis

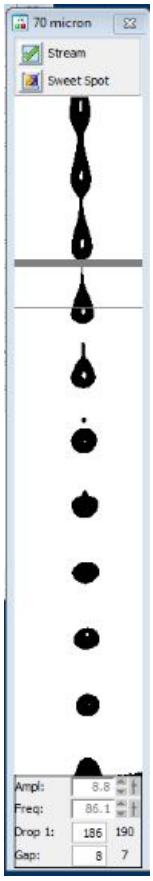
is not affected by the drop drive.

A drop breakoff image is created using an LED strobe and a video camera. The image is displayed in the Breakoff window (see Breakoff Window (page 45)). Patented Sweet Spot technology analyzes the drop breakoff image and provides feedback to the appropriate controls.

Breakoff Window

Use the Breakoff window to control a number of aspects related to drop formation.

Control	Description
Stream button 	Turns the stream on and off.
Sweet Spot button 	Enables automatic adjustment of the drop-drive amplitude to maintain the stability of the breakoff point. When the Sweet Spot is on, the Amplitude and Frequency fields are disabled. The amplitude is automatically adjusted by the software. To enable the fields, turn off the Sweet Spot.
Amplitude field	Adjusts the amplitude or intensity of the drop drive, from 1.0–80.0 volts. The drop-drive amplitude determines the breakoff point. A higher amplitude value results in a shorter stream breakoff. A lower amplitude results in a longer stream breakoff. Typically, the amplitude is set once, at the beginning of a sorting experiment, and then maintained using the Sweet Spot.
Frequency field	Determines the number of drops formed per second and the size of the drops. (Drop size is also influenced by the nozzle size.) The drop-drive frequency can be adjusted from 1.0–102.0 kHz. The higher the frequency, the more drops are generated per second and the smaller the drops. The lower the frequency, the fewer drops generated per second and the larger the drops.
Frequency field (continued)	In general, the drop-drive frequency should not need adjustment. We recommend using the default values that are entered with each Sort Setup mode.
Drop 1 field	The distance between the top of the image and the center of the first broken-off drop, from 100–600 pixels. When you enter a value and turn on the Sweet Spot, the cytometer automatically adjusts the amplitude to attain your target value. **NOTE** Not all Drop 1 targets are attainable. The Drop 1 value jumps in whole increments of approximately 57 pixels according to your drop spacing. The same Drop 1 setting can be used from day to day. A difference of up to 10 pixels between the target value and the actual value is acceptable.
Gap field	This represents the gap between the stream breakoff and the top of the first drop. The default Gap setting for a 70-micron nozzle is 6 pixels.

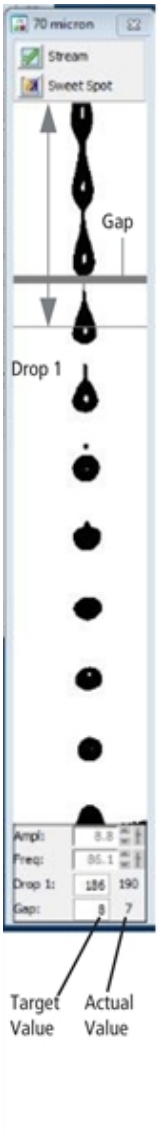


Breakoff Functionality

The upper camera transmits an image of the drop breakoff to the Breakoff window, where video image processing software converts the visual characteristics of the image into numerical properties. The drop breakoff is analyzed for two key features: Drop 1 and Gap.

Drop 1 is defined as the number of pixels from the top of the image to the center of gravity of the first broken-off drop. A thin gray line on the image is used to identify this drop. The number shown to the right of the Drop 1 field at the bottom of the window is the actual pixel location of the gray line. The number entered into the Drop 1 field is the user-defined Drop 1 target.

The Gap is defined as the number of pixels from the first discontinuity in the stream to the next stream object, generally the first broken-off drop. A gray line of varying thickness represents the Gap. The number shown to the right of the Gap field is the pixel width of the gray line. The number entered into the Gap field is a user-defined target.



When the Sweet Spot is turned on, the drop-drive amplitude (Ampl) is automatically adjusted to approximately match the target Drop 1 and Gap values. The amplitude is initially adjusted in larger increments until Drop 1 is achieved. The amplitude is then adjusted in smaller increments until the cytometer attains the target Gap. The Sweet Spot function will make adjustments as necessary to maintain the required breakoff conditions throughout the day.

The Sweet Spot performs two other functions during sorting. When sorting, if the Drop 1 or Gap is out of range, sorting is paused until the values are back within range. This ensures that sorting occurs only under the proper breakoff conditions. If a more severe problem such as a clog is detected by the Sweet Spot, the stream is shut off and sorting is stopped, the deflection plates shut off, the aspirator drawer closes, and the sample tube is unloaded.

Pre-programmed values can be downloaded to the Breakoff window by selecting one of the nozzle sizes (70, 85, 100, or 130 micron) from the Sort > Sort Setup menu.

Changes to values in the Sort Setup windows (Breakoff and Side Stream) are automatically saved. At startup, the last settings used on the cytometer are restored, except the Stream and Sweet Spot controls, which always default to off. For more information, see Sort setup (page 70).

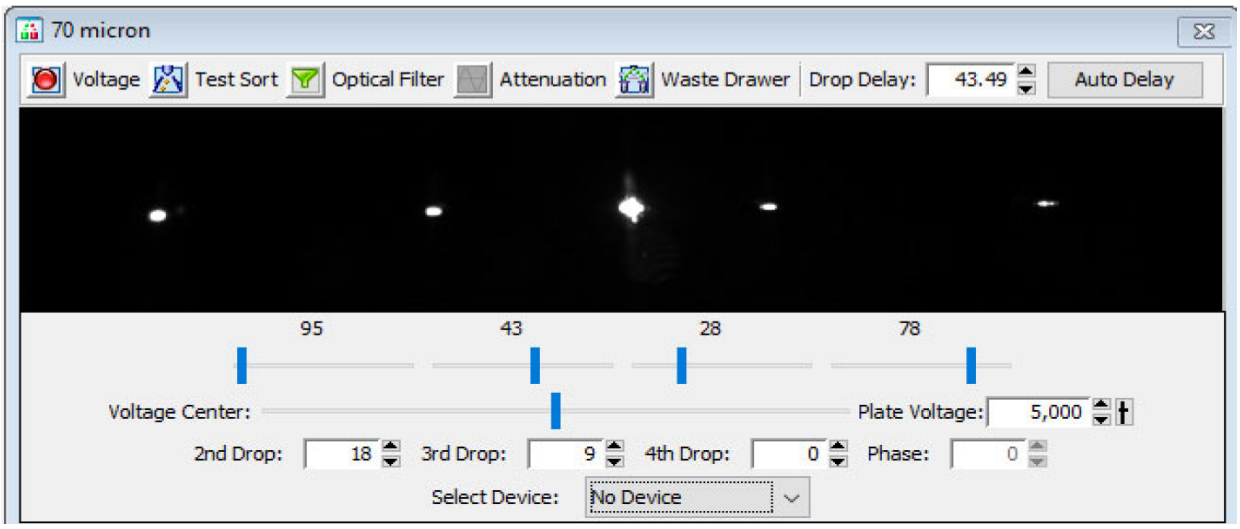
Typically, when setting up for sorting, use the Amplitude to set the required drop breakoff, and copy the generated Drop 1 value to the target field. Then, turn on the Sweet Spot to maintain the drop breakoff. When the Sweet Spot detects a >1-pixel difference between the target Gap setting and the actual Gap, it adjusts the amplitude to reduce the Gap. When a >2-pixel difference is detected, the Sweet Spot stops sorting temporarily (stops charging the stream) until the Gap is restored. Note that when the sort is paused, the sample continues to flow. Once the Gap is back within range, sorting automatically resumes.

For information on setting the breakoff, see Setting up the breakoff (page 92).

Side Stream Formation

Side streams are formed when the voltage is on and a sort is in progress, or when you click Voltage, then Test Sort in the Side Stream window.

The Side Stream window displays an image of the side streams as transmitted by the lower camera. In addition to the stream image, the Side Stream window contains the controls used to adjust electrical charges and to determine the drop delay using Accudrop, as described in Drop-Delay Overview (page 49).



Controls in the Side Stream window are described in the following table. You can send different values to the Side Stream window by selecting a nozzle size from the Sort > Sort Setup menu.

Note: Changes to values in the Sort Setup windows (Side Stream and Breakoff) are linked to the sort setup and are automatically saved. At startup, the last settings used on the cytometer are restored, except the states of the Voltage, Test Sort, and Optical Filter buttons, which always default to off. For more information, see Sort setup (page 70).

Control	Description
Voltage button 	Turns the plate voltage on and off.
Test Sort button 	Generates test side streams based on test sort pulses.
Optical Filter button 	Controls the position of the optical filter in front of the lower (Accudrop) camera.
Attenuation button 	Decreases the amplitude of the drop drive. At lower pressures, you may need to turn on attenuation to dampen the amplitude.
Waste Drawer button 	Opens or closes the aspirator drawer depending on its current state. The default state is closed. For more information, see Aspirator drawer (page 21).
Drop Delay field	Sets the amount of time between when an event is measured and the breakoff point, from 10–140 drops. The drop-delay value determines which drop will be deflected. The drop-delay value is set in an experiment using BD FACS Accudrop technology.
Auto Delay	Opens the Auto Drop Delay dialog.
Voltage sliders (far left, left, right, far right)	Set the percentage of charge to be applied to the corresponding stream (as a percentage of maximum).
Voltage Center slider	Adjusts the relative voltage between the left and right plates which moves the streams slightly to the left or right as a whole. In general, this slider rarely needs adjusting.
Plate Voltage field	Adjusts the total voltage difference between the plates, which determines the angle of stream deflection. The voltage values change with different nozzle sizes.
2nd, 3rd, 4th Drop fields	Apply a correction factor for the drop charge as a percentage of the previous drop, from –100% to 100%.
Phase field	Adjusts the phase between drop generation and charging of the droplets from 0–360°. The selected value is sent to both the drop-charging electrode and the drop strobe. In general, the Phase does not need adjusting. You can keep the default value of zero.
Select Device	Collection device menu for setup of aspirator digital reference lines linked to a specific collection device.

Drop-Delay Overview

The BD FACSymphony™ S6 cell sorter includes integrated Accudrop technology to assist in setting an accurate drop delay value. Accudrop components consist of the following:

- A diode laser, mounted to the left of the sort block
- A camera that provides an image of the side streams
- An emission filter for viewing the fluorescence from BD FACS™ Accudrop Beads

The emission filter is installed in front of the lower camera and can be moved in and out by clicking the Optical Filter control. When the button is green ( ), the filter is out. This position is used to view the center and side streams.

Click the button to move the filter in front of the camera when you are determining the drop delay. The button changes to red ( ). To determine the drop delay, the streams are illuminated by the diode laser just below the point of deflection. Specialized fluorescent particles (Accudrop Beads) can be viewed in the center and left side streams as the delay is adjusted. The best delay yields the most particles in the left stream and the fewest in the center stream.

See Determining the drop delay – manual method (page 123) for more information.

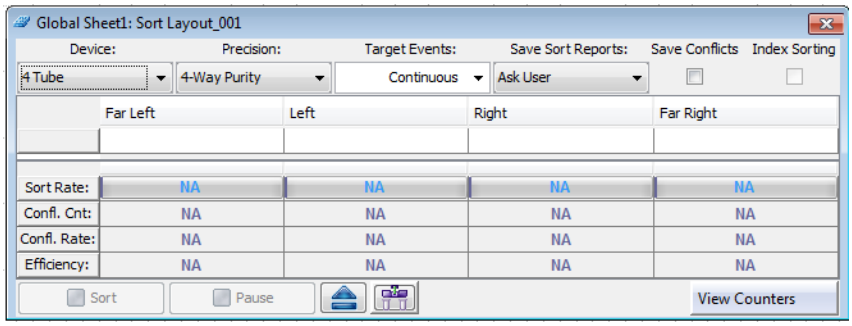
Auto Drop Delay

The Auto Drop Delay feature enables the system to optimize the drop delay automatically. See Determining the drop delay – automatic method (page 126).

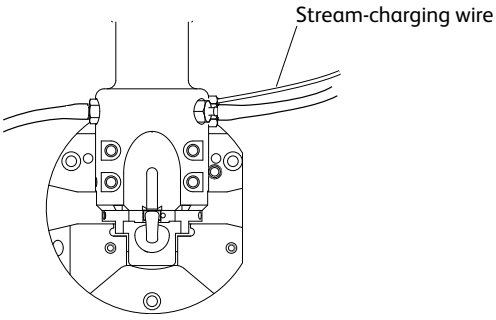
Drop Charging

Drops are charged when an event is detected and meets the defined sorting criteria, as specified in the Sort Layout window.

The Sort Layout window contains all sorting instructions and controls. The sort layout designates which device will be used to collect sorted particles and which particles will be sorted into each sort location. Up to six sort counters can be displayed in the window to give ongoing status during a sort. Depending on your system's configuration, your setup values may vary.



Target events are identified by drawing gates around populations of interest in plots. The Sort Layout window specifies which gated population should be sorted into each sort collection tube or spot in a plate or on a slide. During sorting, when an event is identified within one of the sort gates, the drop containing the particle of interest is charged by the stream-charging wire attached to the flow cell.

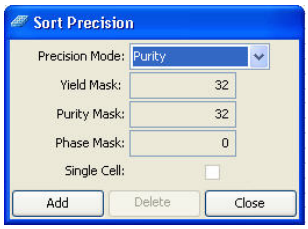


The amount and type of charge determine where the drop will be sorted. For example, in a four-way sort, drops with the most charge will be deflected into the outer streams, while drops with less charge will be deflected into the inner streams.

Conflict Resolution During Sorting

During sorting, the cytometer deflects drops based on the characteristics of the particles in each drop and where the user wants to deflect them. Conflicts can occur depending on the type of target particle, where the particle is located within a drop, or whether the drop is free of contaminating particles. BD FACSDiva™ software accurately measures particle position to within 1/32 of a drop.

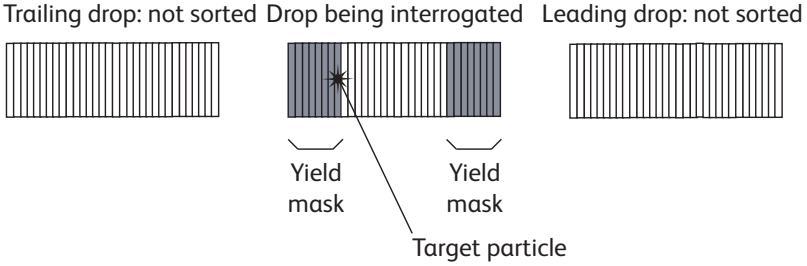
Mask settings determine how drops are deflected when sorting conflicts occur. There are three mask settings, each of which addresses a different type of conflict. These settings are combined to define sort precision modes. Each mode is made up of a set of masks. Precision modes are defined in the Sort Precision dialog, accessed from the Sort menu.



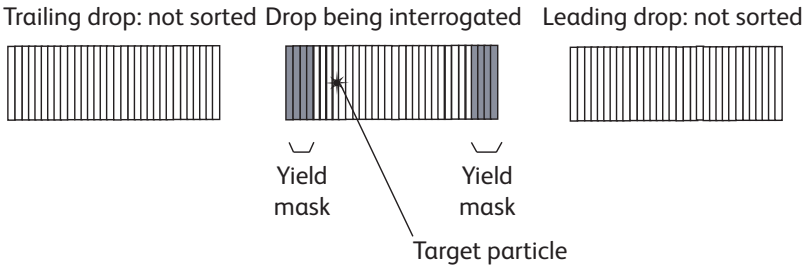
Yield Mask

The yield mask setting defines how close to the edge of the drop, in 1/32-drop increments, a particle of interest can be located before sorting an additional drop. Half of each yield mask setting defines an equal area at each end of the drop.

For example, when the yield mask is set to 16 and an event is within 8/32 from the beginning of a drop, the previous (leading) drop will be sorted. If an event is within 8/32 from the end of a drop, the following (trailing) drop will be sorted.



If the yield mask were set to 8 for the same target particle, the target particle would fall outside the yield mask. Therefore, no additional drops would be sorted.



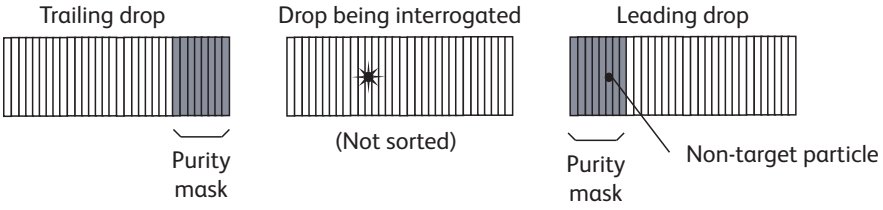
When the yield mask is set to zero, only one drop (the drop containing the target particle) will be deflected. When the mask is set to 32, two drops will always be deflected. Yield masks between 0 and 32 will sort either one or two drops.

Yield masks cannot be used in conjunction with phase masks. Therefore, when the yield mask is greater than zero, the phase mask automatically reverts to zero.

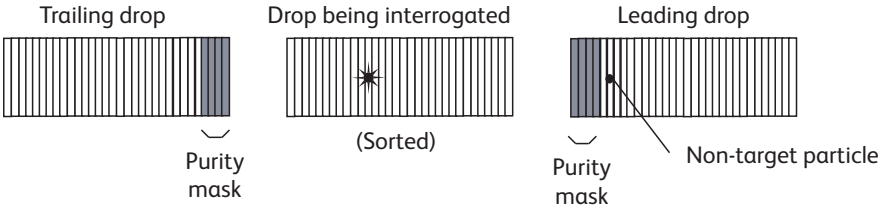
Purity Mask

The purity mask setting defines how close, in 1/32-drop increments, a contaminating drop can be located before ignoring the drop being interrogated.

For example, when the purity mask is set to 16, the drop being interrogated will not be sorted if a non-target particle falls within the first or last 8/32 of the leading or trailing drop. In the following example, a non-target particle falls within the first 8/32, so the interrogated drop will not be sorted.



If the purity mask were set to 8 for the same target particle, the non-target particle would fall outside the purity mask, so the interrogated drop would be sorted.



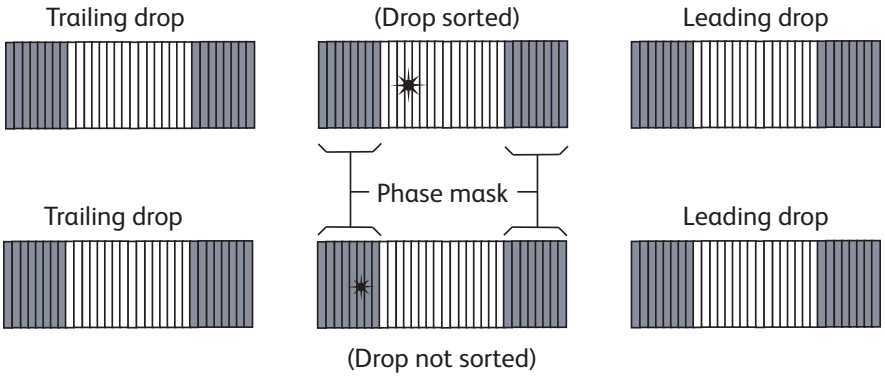
With any purity mask greater than zero, the drop being interrogated must be free of contaminating particles or the drop will not be sorted. If the purity mask is set to zero, a droplet containing the event of interest will be sorted regardless of contaminating particles.

Phase Mask

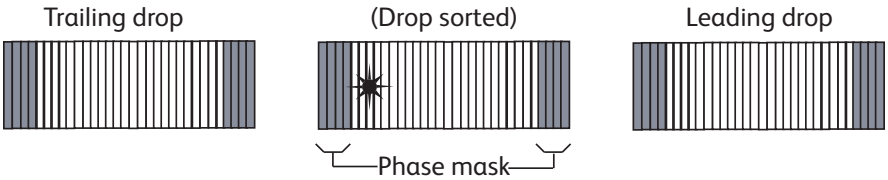
Particles near the drop edge can affect the breakoff and alter the trajectory of the deflected drop. The phase mask restricts drop deflection when an event is too close to the edge of a drop or when there are events close to

the edge of adjacent drops. A phase mask is used to improve counting accuracy and side-stream quality at the expense of yield.

For example, when the phase mask is set to 16, the drop being interrogated will be sorted only if the target particle falls outside the phase mask.



Decreasing the phase mask to 8 allows more drops to be sorted. However, because the target particle is closer to the edge of the drop, there is more variability in drop trajectory.

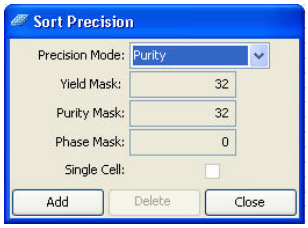


Note: We recommend using a phase mask of at least 8 when sorting single cells.

Phase masks cannot be used in conjunction with yield masks. Therefore, when the phase mask is greater than zero, the yield mask automatically reverts to zero.

Sort Precision Modes

Mask values can be combined in many different ways. By default, six sort precision modes are already defined: Purity, 4-Way Purity, Yield, Single Cell, Initial, and Fine Tune.



	Precision Mode					
	Purity	4-Way Purity	Yield	Single Cell	Initial	Fine Tune
Yield Mask	32	0	32	0	32	0
Purity Mask	32	32	0	32	0	0
Phase Mask	0	0	0	16	0	0
Single Cell	–	–	–	X		–

- **Purity mode**. The yield mask is set to the maximum to obtain the greatest number of particles. Because the purity mask is also set to the maximum, only drops free of contaminating particles will be sorted. Sorting in Purity mode results in a sorted sample that is highly pure, at the expense of recovery and yield.
- **4-Way Purity mode**. The purity mask is set to the maximum, so only drops free of contaminating particles will be sorted. The yield mask is set to zero to ensure that residual charges from adjoining drops do not degrade the quality of side streams. The 4-Way Purity mode is recommended for 4-way sorting and 6-way sorting, when precise deflection is required.
- **Yield mode**. Only the yield mask is used at its maximum value. Recovery and yield are optimized at the expense of purity. The yield mode could be used as a first round sort for enrichment of target particles, followed by a sort for purity.
- **Single Cell mode**. The purity mask is set to the maximum, so only drops free of contaminating particles will be sorted. The phase mask is set to half the maximum, so only particles centered within the sorted drop are deflected. Drop trajectory and count accuracy are optimized at the expense of yield. This mode is recommended for plate sorting or situations when precise counting is required.

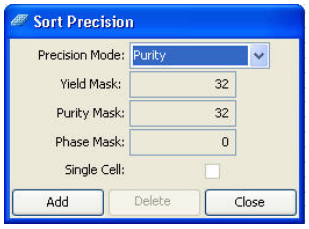
The remaining modes are used mainly during drop-delay determination. Initial mode is equivalent to Yield mode, but it is named differently as a reminder to use this as the initial mode when using Accudrop to set the drop delay.

- **Initial mode**. Only the yield mask is used at its maximum value. Recovery and yield are optimized at the expense of purity.
- **Fine Tune mode**. All masks are set to zero to deflect the maximum number of drops. This mode is used to fine-tune the drop-delay value. See Determining the drop delay – manual method (page 123) for more information.

Defining New Precision Modes

Default precision modes cannot be edited or deleted. However, you can create new modes and then select them from the Precision Mode menu.

1. Select **Sort > Sort Precision**.



2. Click **Add**.

   The current sort mode is duplicated and the Mask fields are enabled.

3. (Optional) Change the name of the mode in the Precision Mode field.

4. Enter values for Yield Mask, Purity Mask, and Phase Mask.
5. Select the **Single Cell** checkbox, if needed.
6. Click **Close**.

   The new mode is added to the Precision Mode menu.

To delete a mode, select it from the **Precision Mode** menu and click **Delete**.

4

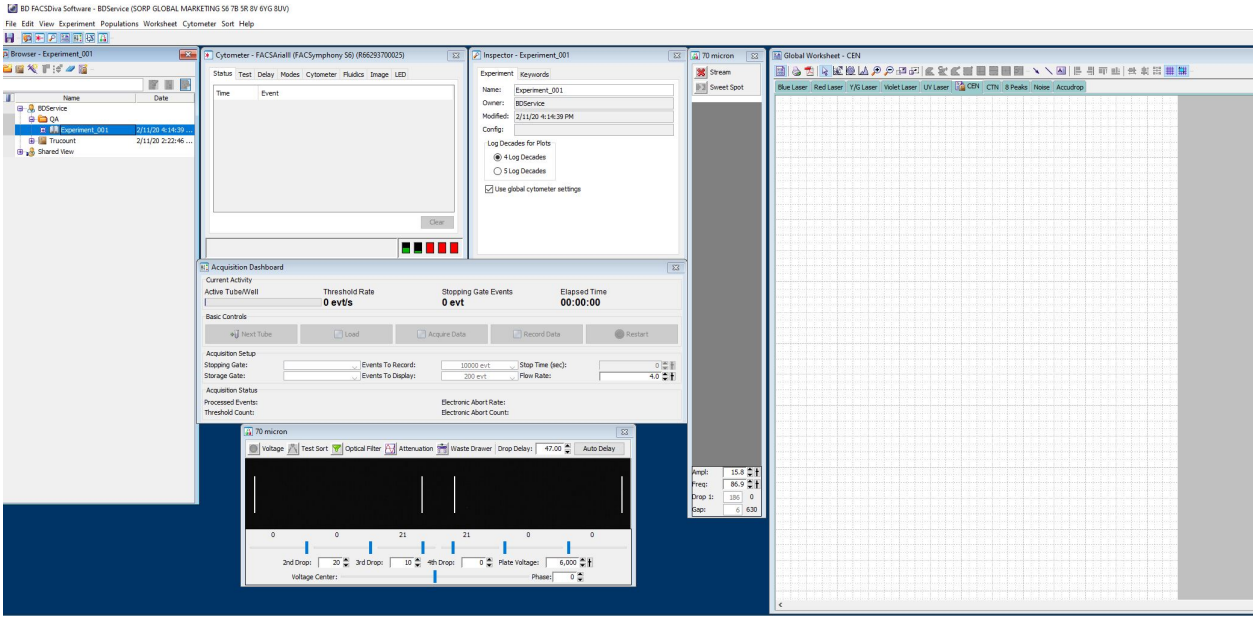
Using BD FACSDiva™ software

Many BD FACSymphony™ S6 cell sorter functions are controlled using BD FACSDiva™ software. This chapter provides a general overview of the workspace components and describes software controls that are unique to the BD FACSymphony™ S6 cell sorter cytometer. For an in-depth description of software components not described in this chapter, see the *BD FACSDiva™ Software Reference Manual*.

The following topics are covered in this chapter:

Workspace components

When you start BD FACSDiva™ software, the workspace opens. For a general overview of the workspace and to get started using the software, see the tutorials and quick reference guides located in the Resource Library on the BD Biosciences website (bdbiosciences.com). When running BD FACSDiva™ software with the BD FACSymphony™ S6 cell sorter, two additional windows can be displayed by clicking the Sorting button on the Workspace toolbar. See Sorting controls (page 69) for a description.



Cytometer controls

Most BD FACSymphony™ S6 cell sorter–specific cytometer controls are accessed through the Cytometer menu. Controls on the Cytometer menu are described in the following sections:

- Fluidics controls (page 56)
- Cytometer configuration (page 59)
- Cytometer status report (page 60)

The Cytometer Details and the Catalogs menu commands are described in the *BD FACSDiva™ Software Reference Manual*. Additional cytometer controls are located on the Acquisition Dashboard. See Acquisition controls (page 68).

Fluidics controls

Fluidics control of the BD FACSymphony™ S6 cell sorter is partially automated by BD FACSDiva™ software. The software contains pre-programmed fluidics protocols that are activated by selecting the corresponding menu command from the Cytometer menu. In addition, fluidics level indicators are available in the Cytometer window. See Fluidics level indicators (page 58).

Fluidics Startup

During fluidics startup, waste and sheath levels are verified and the fluidics system is primed with sheath solution. The fluidics status is displayed at the bottom of the main window.

See Cytometer startup (page 88) for more information.

Fluidics shutdown

Fluidics shutdown removes sheath fluid from the lines, replaces it with ethanol, and cleans the cuvette with a cleaning solution. See Fluidics Shutdown (Weekly or As Needed) (page 142) for more details.

Change sample filter

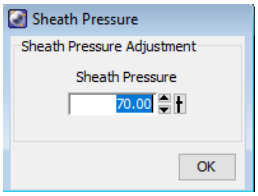
Sample line filters can be installed to filter a sample before sorting. This selection opens the Sample Filter Change wizard, which guides you through the process. See Installing or removing a sample line filter (page 159) for the instructions.

Cleaning modes

BD FACSDiva™ software contains pre-programmed cleaning modes that are activated by selecting the corresponding menu command from the Cytometer > Cleaning Modes menu. See Internal cleaning (page 144) for more information.

Sheath pressure

The sheath pressure determines how quickly particles pass through the laser beam. Select Cytometer > Sheath Pressure to view the current sheath pressure and change the pressure for custom sort setups.

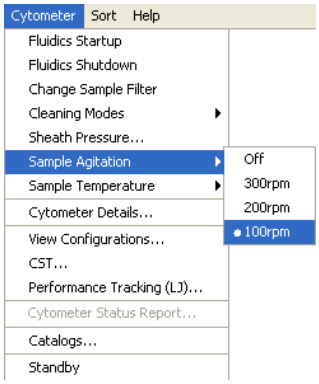


Each sort setup option is optimized at a preset sheath pressure. If you change the sheath pressure, many other values will be affected and need updating, including the drop-drive frequency, drop-delay value, laser delay, and area scaling factor. For proper cytometer operation, change the sheath pressure by selecting an option from the Sort > Sort Setup menu. Do not adjust the pressure using controls in the Sheath Pressure dialog.

Note: The Sheath Pressure command is disabled when the Sweet Spot is on.

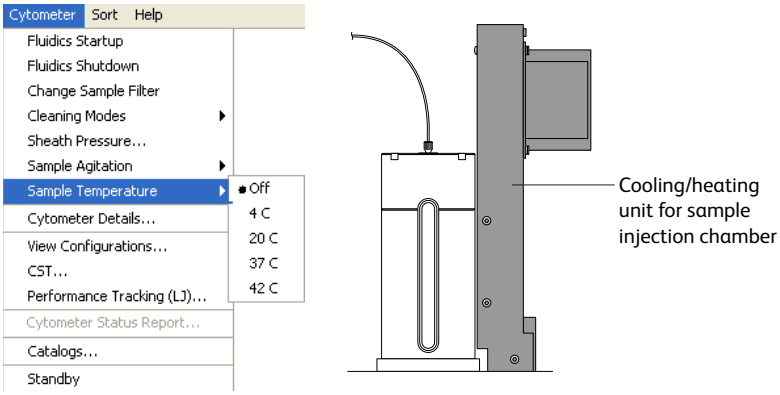
Sample agitation

Select Cytometer > Sample Agitation to specify the speed at which samples are agitated. You can select from one of the specified values, or select Off to turn off agitation.



Sample temperature

Use the Sample Temperature command to set the temperature inside the sample injection chamber. You can select from one of the specified values, or select Off to turn off temperature control.



The cooling/heating unit is designed to maintain the temperature of a sample tube. It is not designed to cool or heat the sample. It takes approximately 45 minutes to reach the required temperature inside the chamber, during which time the chamber must be kept closed.

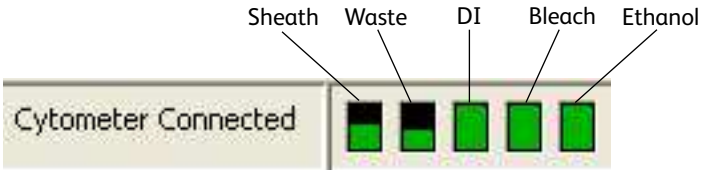
To cool or heat the chamber, install a sample tube and click Load. Click Stop Acquiring to keep the chamber closed and stop running the sample. To maintain the temperature, do not leave the chamber open for extended periods while changing sample tubes.

The sample temperature retains the last setting after startup. For example, if it was set to 20 °C the last time the system was used, then it will return to that setting the next time the system is started up.

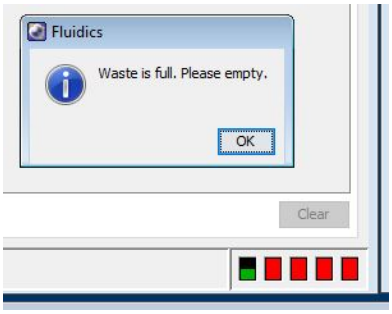
Fluidics level indicators

BD FACSDiva™ software provides fluidics level indicators in the Cytometer window. The sheath and waste indicators provide an approximate indication of the fluid levels in each tank. The DI, bleach, and ethanol tank indicators appear full until the fluid level is below 20% of the tank capacity. When this occurs, the corresponding level indicator changes to black.

Note: The stainless steel ethanol shutdown tank does not have a level sensor. It must be checked manually.



When the sheath is low or the waste is full while the stream is running, the corresponding indicator turns red and the following warning message is displayed every 5 minutes until the stream is turned off. If the warning message is not closed, the system automatically turns off the stream after 15 minutes.



Do not close the warning message without refilling the sheath or emptying the waste. If you continue to run the cytometer after closing the message, the system could shut down.

Cytometer configuration

The menu selections shown in the following figure open the CS&T module in a separate window. The CS&T module enables you to perform multiple functions related to cytometer configuration.

Note: When the CS&T module is open, BD FACSDiva™ software goes into a holding mode and does not accept any user input. When CS&T is closed, BD FACSDiva™ software becomes active again.

- **View Configurations**. Opens the Cytometer Configuration window within the main CS&T window. See the following section for an overview of this function.
- **CST**. Opens the CS&T module. See the *BD® Cytometer Setup and Tracking Application Guide* for complete information on using BD® CS&T Research Beads.
- **Performance Tracking (LJ)**. Opens the performance tracking feature within the main CS&T window. See the *BD® Cytometer Setup and Tracking Application Guide* for complete information on using this feature.

Cytometer configuration window

The BD FACSymphony™ S6 cell sorter cytometer is equipped with a specific set of lasers, filters, and dichroic mirrors. The Cytometer Configuration window lets you define which fluorochromes or cell parameters will be measured at each PMT detector. Within this window, you can define the filters and assign the fluorochromes to the detectors.

Select **Cytometer > View Configurations** to open the window. Click the **Parameter** tabs for a list of parameters. Click the **Filters and Mirrors** tab for the list of optics.

The Cytometer Configuration window also displays the following settings:

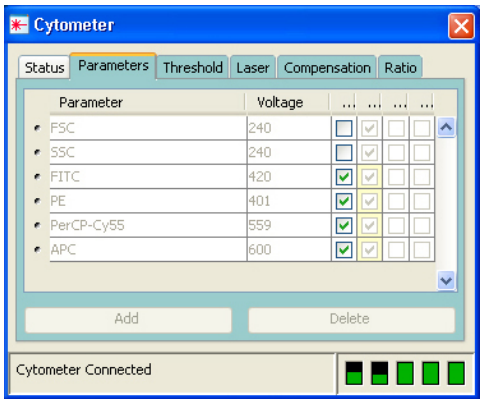
- Sheath pressure
- Nozzle size
- Window extension

See Custom configurations (page 61) for more information on editing configurations, including adding parameters, mirrors, and filters.

Before you start any experiment, verify that the cytometer configuration contains the appropriate parameters for the samples you are running and that the cytometer optics match the current configuration. You cannot modify an existing configuration once it has been baselined. However, you can copy, paste, or modify a configuration as described in Custom configurations (page 61).

For accurate data results, the cytometer optics must match the current cytometer configuration.

Selections in the Cytometer Configuration window determine which parameters are available for your experiment.



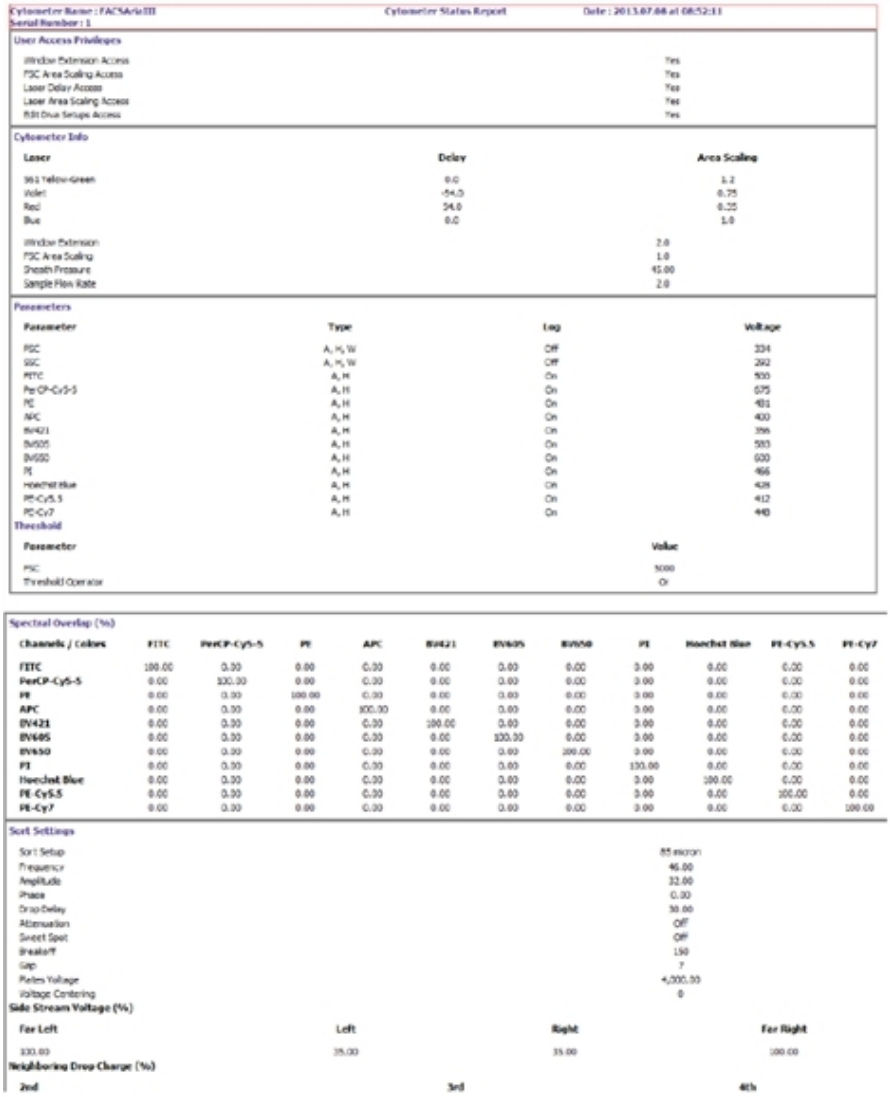
Cytometer status report

The Cytometer Status Report provides a list of all cytometer settings at the time the report was created. The cytometer must be connected to create the report. In an open experiment, click to set the current tube pointer and select **Cytometer > Cytometer Status Report**. The report is displayed in a separate window with a menu bar above the report header. The header lists the cytometer name, type, serial number, and the date and time the report was generated.

For a full description of the Cytometer Status Report, see the *BD FACSDiva™ Software Reference Manual*. A BD FACSymphony™ S6 cell sorter cytometer report includes the following additional information: user access privileges, cytometer information, cytometer settings, and sorting settings. The report can be printed or exported.

- The User Access Privileges section lists access settings for the current user.
- The Cytometer Info section lists values for laser delay, area scaling, window extension, FSC area scaling, sheath pressure, and sample flow rate.
- The Parameters section displays settings for the current acquisition tube.

- The Sort Settings section lists all sort setup values, along with the Plate Voltage and Voltage Center values. If the Sweet Spot is off, Breakoff and Gap values are shown. If the Sweet Spot is on, Drop 1 and target Gap values are shown.



Custom configurations

Before you can record data, you must first ensure that the cytometer configuration is appropriate for the experiment. If needed, you can create custom configurations to add parameters or filters and mirrors that are not listed in the base configuration. You will also need to create custom configurations for each sheath pressure you would like to run.

When creating custom configurations, use descriptive names that make it easy to identify the configuration. See the *BD FACSDiva™ Software Reference Manual* and the *BD Cytometer Setup and Tracking Application Guide* for more information.

Preparing for custom configurations

Custom configurations can be created only by users with administrator access. The easiest way to create a custom configuration is to copy and edit a base configuration. You can also create a new, blank configuration.

1. Log in to the software as an administrator.
2. Select **Sort > Sort Setup** and select the setup mode that matches the nozzle size you are using.
3. Verify that the correct nozzle is installed, then start the stream.
4. Select **Cytometer > CST**.

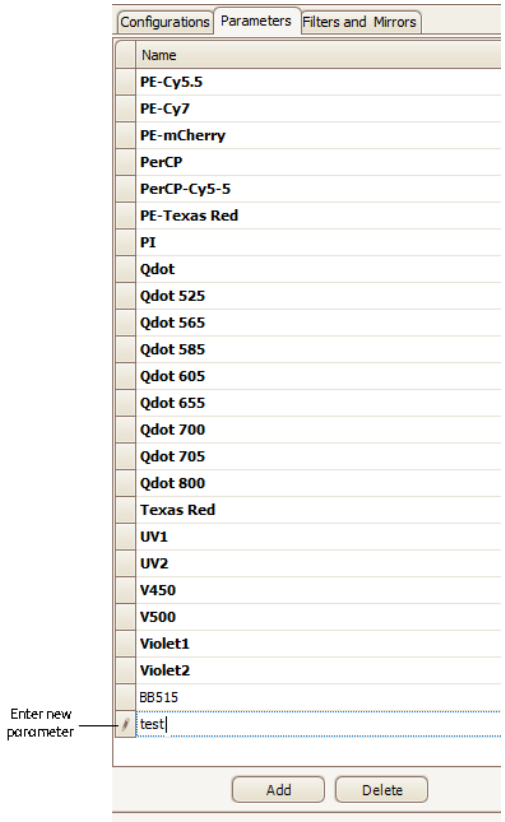
   The **Cytometer Configuration** window opens (see Cytometer configuration (page 59)). For users with administrator access, the window displays the Parameters and Filters and Mirrors tabs, which are not visible to users with operator access.

   When you create a new configuration in CS&T, you need to run a new baseline for that configuration. Once the baseline is established, you cannot edit any of the settings in the configuration.

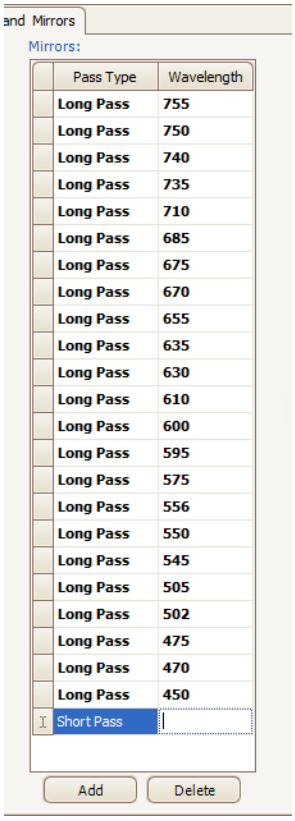
Adding parameters, filters, and mirrors

Before creating a custom configuration, verify that the necessary parameters, filters, and mirrors required for the custom configuration are defined.

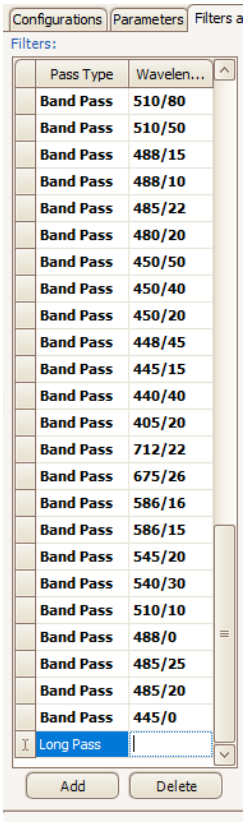
1. To add new parameters, do the following in the **Parameters** tab:
   a. Click **Add**.
   b. Enter a parameter name in the field provided.



2. To add filters or mirrors, do the following in the **Filters and Mirrors** tab:
   a. To add a filter, click **Add** under the Filter list. To add a mirror, click **Add** under the Mirror list.



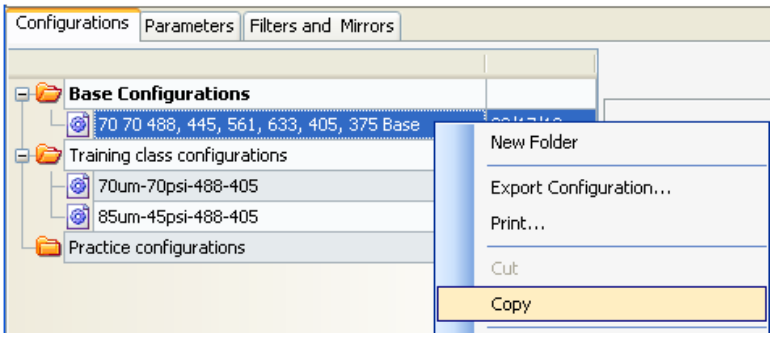
b. Select a pass type and enter a wavelength in the field provided.



Copying a base configuration

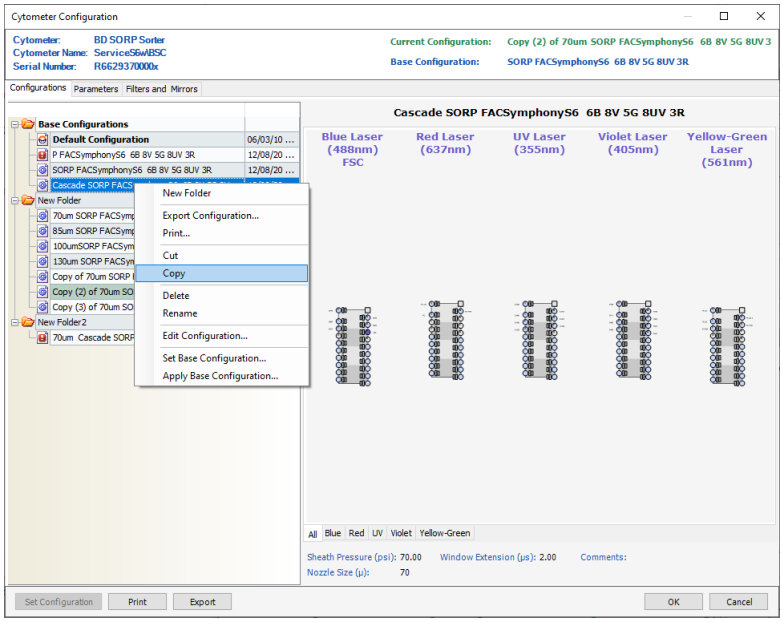
You cannot edit or delete your base configuration. However, you can use it as a starting point to create a custom configuration.

1. In the **Configurations** tab, right-click the **Base Configurations** folder and select **New Folder**.
2. Rename the new folder *Custom Configurations*.
3. Right-click the base configuration and select **Copy**.



4. Right-click the **Custom Configurations** folder and select **Paste**.

   A copy of the base configuration is added to the Custom Configurations folder.



5. Enter a descriptive name and press **Enter**.

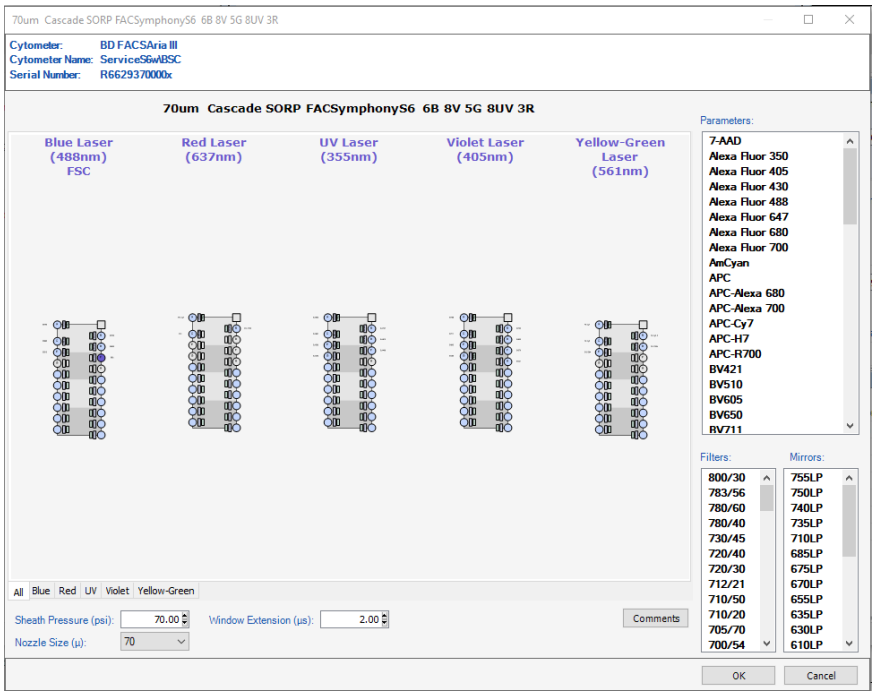
   For example, use the name *70-70*, meaning 70-micron nozzle at 70 PSI. If you need to rename the configuration, right-click the new configuration and select **Rename**.

Editing the copied configuration

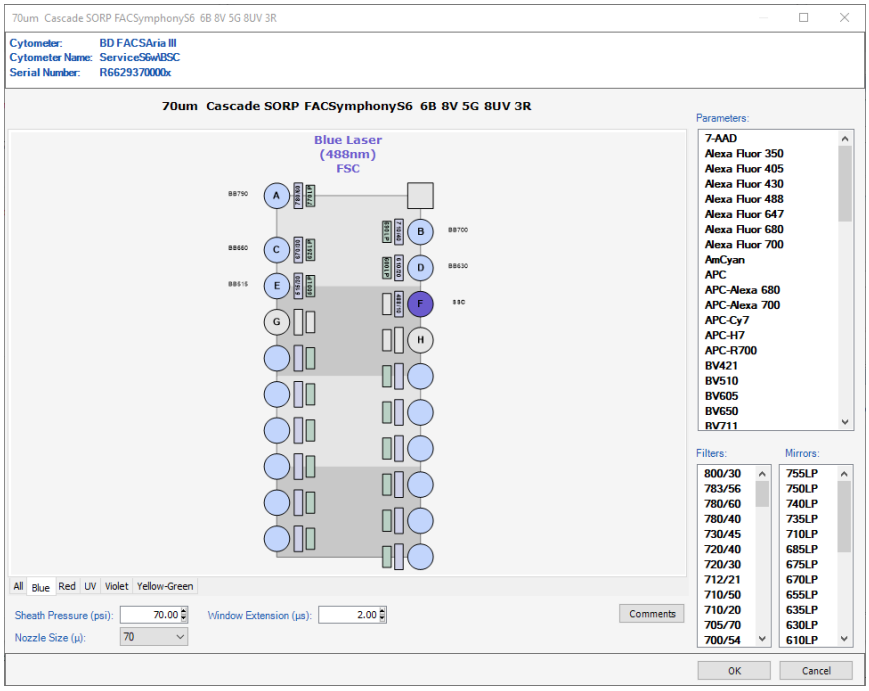
Once the base configuration is copied, you can customize the parameters, filters, and mirrors in the configuration.

1. In the configuration list, right-click the new configuration and select **Edit Configuration**.

   The following window opens.

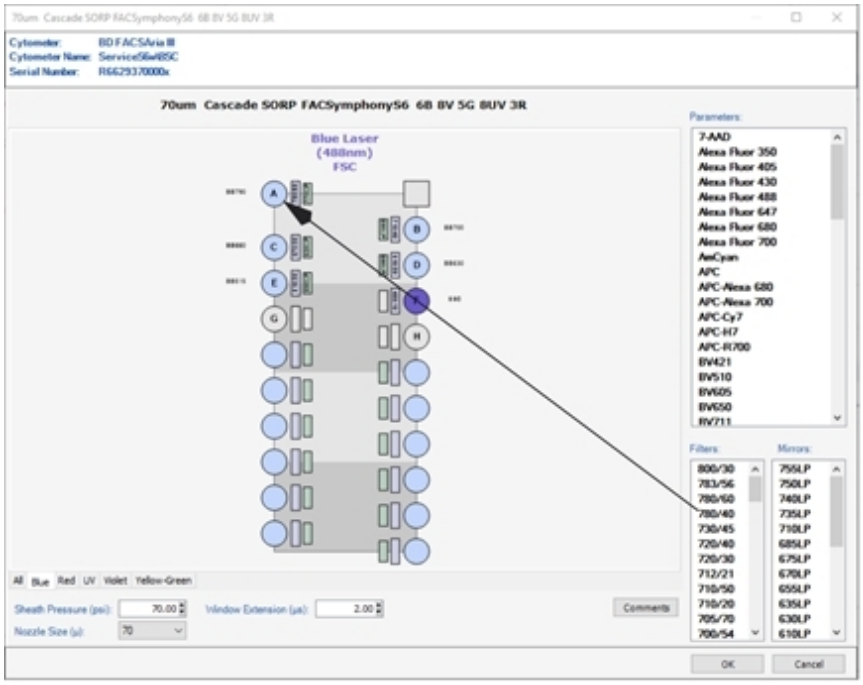


Use the tabs at the bottom of the window to view each laser's detectors separately.The following is an example when the **Blue** tab is selected:



2. To edit the nozzle size and sheath pressure for this configuration:
   a. Enter the appropriate sheath pressure value at the bottom of the configuration window.
   b. Select the appropriate nozzle size.

3. To change the parameter label for a detector, select a parameter from the **Parameters** list and then drag and drop the parameter onto a detector (one of the discs labeled A-H) for the appropriate laser. You can assign multiple parameters by using **Ctrl+click** to select individual parameters or **Shift+click** to select a contiguous range.
4. To change a filter, drag and drop a filter in the **Filter** list menu onto a detector, as in step 3.



5. To change a mirror, drag and drop an item in the **Mirror** list for that detector onto a detector, as in step 3.

To verify that you have selected the correct configuration, do the following.

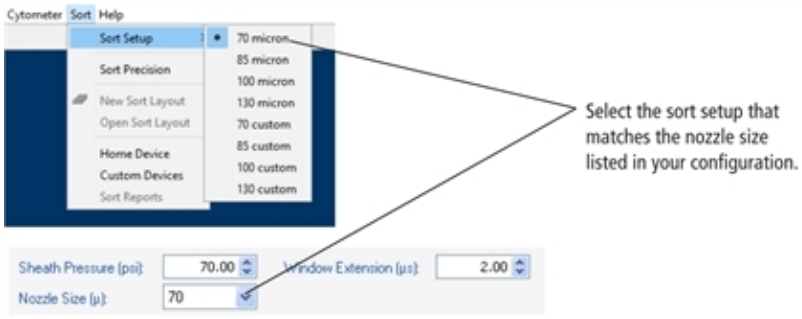
1. Select **Cytometer > CST**.
2. Select the appropriate configuration and select **Set Configuration**.
3. Select **File > Exit** to exit CS&T.

   A mismatch dialog is displayed.
4. Select **Details**, and then select **Use CST Settings**.

Verifying that the configuration matches the sort setup

To verify that the sheath pressure in the sort setup matches the sheath pressure in the configuration:

1. Select **File > Exit** to exit CS&T.
2. Select **Sort > Sort Setup**, and select the sort setup that matches the nozzle size listed in your configuration.



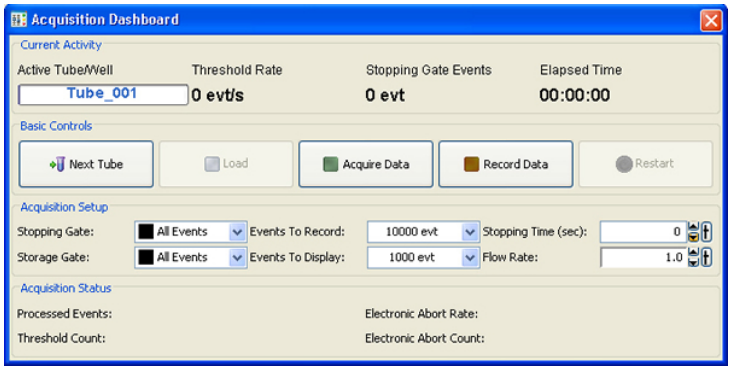
3. Select **Cytometer > Sheath Pressure** and verify that the pressure matches the pressure in your configuration. Enter a new sheath pressure, if needed.

   The new sheath pressure is automatically saved with the current sort setup mode.

The name of the configuration is displayed at the top of the BD FACSDiva™ workspace. If you use a descriptive name for each configuration (such as *100-15-Custom*, for nozzle size-sheath pressure-sort setup mode), it will be easier to verify that the appropriate settings match without going back to view the configuration.

Acquisition controls

Along with the controls described in the *BD FACSDiva™ Software Reference Manual*, the following acquisition controls are available for the BD FACSymphony™ S6 cell sorter cytometer.



- **Load**. Lifts a tube into the sample injection chamber, starts sample agitation (if agitation is turned on), and starts acquisition of the sample.

  When a tube is already loaded, the button changes to Unload. Clicking Unload stops acquisition of the sample, turns off the agitator, and lowers the tube from the sample injection chamber.

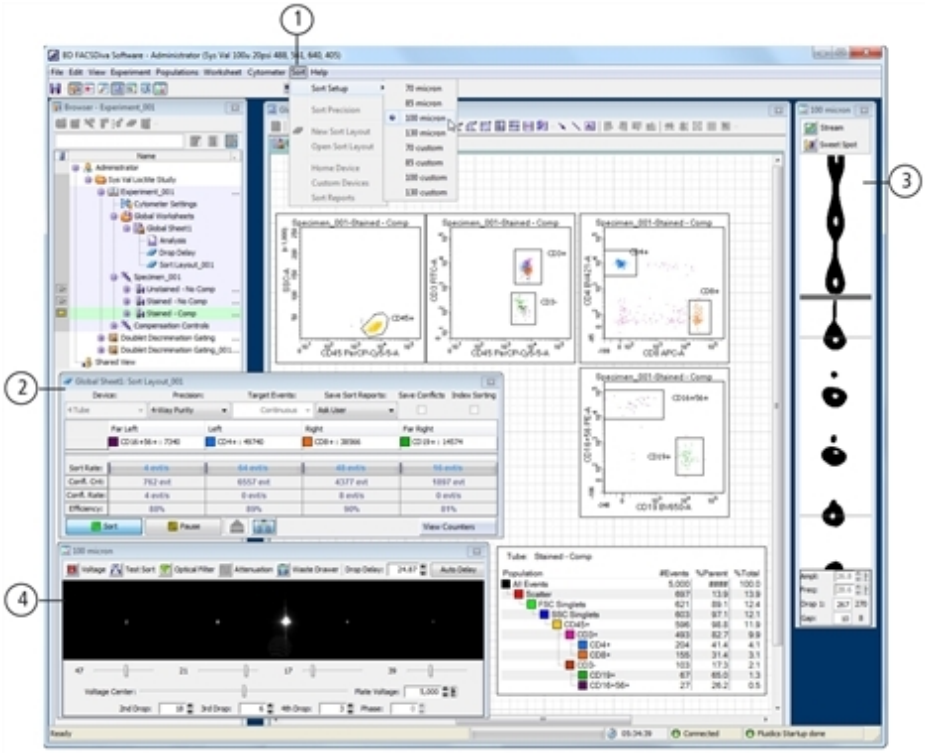
  Note: The Load button is enabled only when the workstation is connected to the cytometer, an experiment is open, the stream is turned on, and the current tube pointer is set to a tube.
- **Stop Acquiring**. Stops sample acquisition without unloading the sample tube.

  The Acquire Data button functions only when a tube is loaded.
- **Flow Rate**. Controls sample flow rate, from 1.0–11.0 (10–80 µL/min).

  Do one of the following to change the flow rate:
  - Select the value in the field and enter a new value.
  - Click the pointer in the slider bar and drag it to a new value.
  - Use the mouse to click the up and down arrows or press the arrow keys on your keyboard to increase or decrease the values in small increments.

Sorting controls

All sorting on the BD FACSymphony™ S6 cell sorter cytometer is controlled by BD FACSDiva™ software. See the table after the following figure for explanations of each area.



No.	Description
①	The **Sort** menu provides access to the sort setup and sort precision modes, sort layouts, sort reports, and sort devices used with the ACDU unit. See Sort menu (page 69).
②	The **Sort Layout** window contains controls to turn on index sorting and designates which device will be used to collect sorted particles and which particles will be sorted into each sort location. Depending on your system's configuration, your setup values may vary.
③	The **Breakoff** window displays an image of the stream and contains controls to adjust drop formation. See Drop Formation (page 43).
④	The **Side Stream** window displays an image of the side streams, and contains controls to adjust electrical charges and the drop delay. See Side Stream Formation (page 47).

Sort menu

Select commands in the Sort menu for the following:

- **Sort Setup**. Downloads the most recently used settings for the selected nozzle size. See Sort setup (page 70).
- **Sort Precision**. Opens a dialog where you can select or define a sort precision mode for handling sorting conflicts. See Conflict Resolution During Sorting (page 50).

- **New Sort Layout**. Opens the default 2-Tube Sort Layout window where other sort layouts can be selected. (Clicking the **Sort Layout** button on the **Browser** toolbar performs the same function.)
- **Open Sort Layout**. Opens an existing sort layout. A sort layout must be selected in the Browser for this menu command to be enabled. Alternatively, double-click any sort layout to open it.
- **Home Device**. Opens a dialog containing commands to move the tray support arm, either manually or to the home position. See Setting up the stream (page 133).
- **Custom Devices**. Opens a dialog where custom devices can be defined. See Creating a custom device (page 135).
- **Sort Report**. Displays a report showing the sort settings, acquisition counters, and sort layout information from the current sort. See Sort report (page 75).

Sort setup

Sort setup values for four different nozzle sizes can be downloaded using the **Sort > Sort Setup** command. Default settings are provided for each sorting option, along with a custom setting for each option.

As a general rule, for optimal results when sorting large or fragile cells, use a larger nozzle size and lower pressure. To increase throughput and yield when sorting smaller or less fragile cells, use a smaller nozzle size and higher pressure.

Sort Setup Name	Nozzle (microns)	Default Pressure (PSI)
70 micron	70	70
85 micron	85	45
100 micron	100	20
130 micron	130	10

If you make changes to any of the settings, the changes are automatically saved when you exit BD FACSDiva™ software or when you switch to a different sort setup mode. When you restart, the most recently used set of values is restored.

In addition, the Sort Setup menu has following menu items: 70 Custom, 85 Custom, 100 Custom, and 130 Custom. Initially, all of these new setups are exact copies of the corresponding original setups with the same defaults. This allows you to create two sets of sort values (pressure, for example) for the same nozzle size.

Do not use a nozzle size that is different from the sort setup setting. The gap values and sensitivity algorithms are optimized for particular nozzles and are not suitable for other nozzle sizes. The system performance could be unstable.

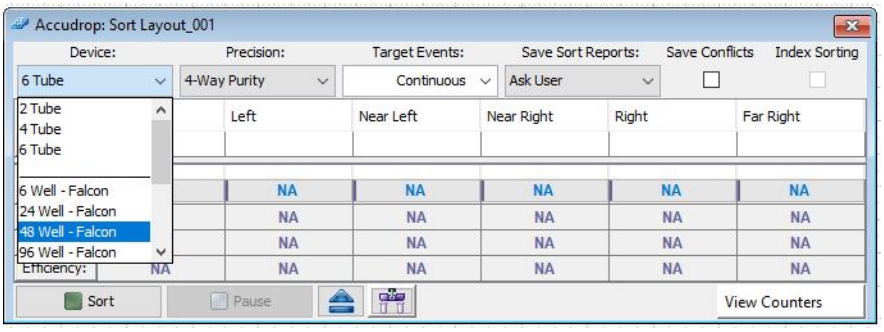
The following table shows default sort setup values. Depending on your system's configurations, your setup values may vary. We recommend optimizing these settings for your system's configurations.

Setting	70 micron	85 micron	100 micron	130 micron
Sheath pressure	70	45	20	10
Amplitude	60	32	12	24
Frequency	87	47	30	12
Drop 1	150	150	150	150
Gap (upper limit)	6 (14)	7 (17)	10 (21)	12 (21)
Attenuation	Off	Off	Off	Off
Drop delay	47.00	30.00	27.00	16.00
Far left voltage	100*	100*	80	60
Left voltage	70	35	30	20
Near left voltage	30	35	30	20
Near right voltage	30	35	30	20
Right voltage	70	35	30	20
Far right voltage	100*	100*	80	60
Plate voltage	6,000	6,000	4,500	4,500
2nd drop	20	20	10	0
3rd drop	10	10	5	0
4th drop	0	0	0	0
Laser delay (blue)	0.00	0.00	0.00	0.00
Laser delay (red)	-40	-35	-40	-40
Laser delay (violet)	40	35	40	40
Laser delay (UV)	78	71	80	78
Laser delay (yellow green)	-80	-80	-80	-80
Area scaling (blue)	0.60	0.60	0.60	0.60
Area scaling (red)	0.60	0.60	0.60	0.60
Area scaling (violet)	0.60	0.60	0.60	0.60
Area scaling (UV)	0.60	0.60	0.60	0.60
Area scaling (yellow green)	0.80	0.80	0.90	0.95
Window extension	2.00	2.00	2.00	4.00
* For 3.5-inch deflection plates, the value needs to be set to 85 or less. Otherwise the plates may get wet, which could cause arcing.				

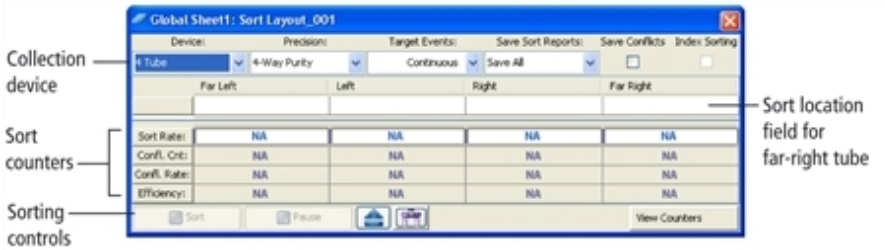
The Sort Layout window contains all sorting instructions and controls. The sort layout designates which device will be used to collect sorted particles and which particles will be sorted into each sort location. Up to four sort counters can be displayed in the window to give ongoing status during a sort.

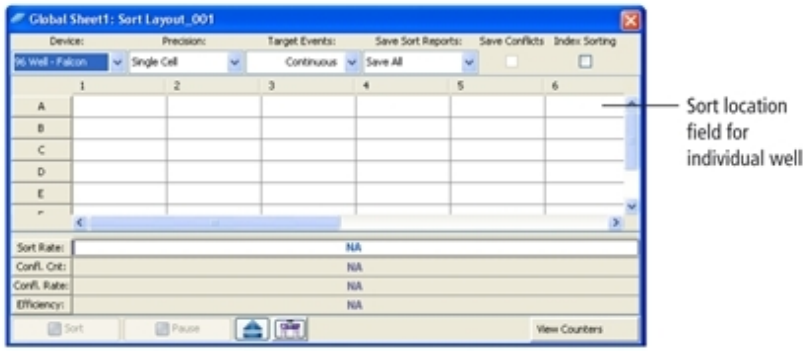
Only one sort layout can be open at a time, but you can create several layouts for a single tube, as long as each sort layout has a different name. Sort layouts can also be added to global worksheets.

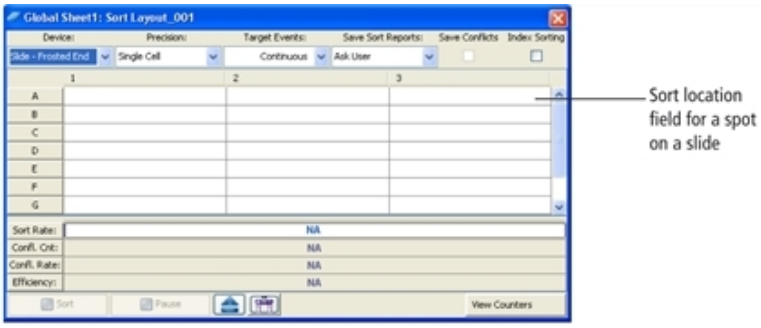
Sort layouts are available for up to 12 default collection devices (shown in the following figure). Additional custom devices can be defined. See Creating a custom device (page 135).



Examples of sort layouts for different devices are shown in the following figures. Instructions for setting up a sort layout can be found in Setting up a sort layout (page 73).







Setting up a sort layout

Sort layouts can be added to tubes or global worksheets.

- Create tube-specific layouts if you are sorting different populations or using different sort devices for each tube in the experiment.
- Create global worksheet–specific layouts if you are sorting the same populations into the same sort device for all tubes in the experiment.

Note: To create a tube-specific sort layout, make sure a normal worksheet (white tab) is shown in the Worksheet window before you create the layout.

To set up a sort layout:

1. Select the icon for a tube or global worksheet in an open experiment and click the **New Sort Layout** button on the **Browser** toolbar.
2. In the **Sort Layout** window, select the type of device from the **Device** menu.

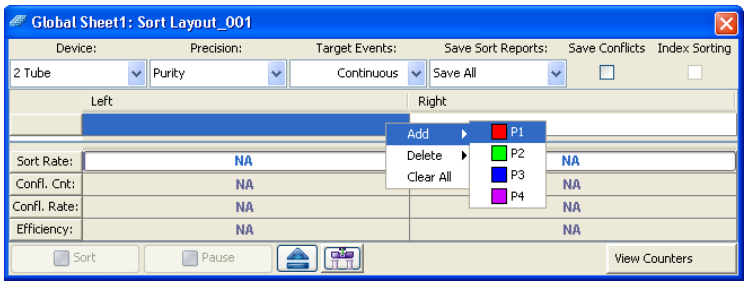
   Default sorting devices are listed along with any defined custom devices. The Sort Layout window changes depending on the selected device. The number of rows and columns in the window matches the number of tubes, wells, or spots in the collection device.
3. Select the sort precision mode from the **Precision** menu.

   For more information, see Sort Precision Modes (page 52).
4. Enter the number of events to be sorted in the **Target Events** field.

   Once entered, the number of events can be selected from the Target Events menu. For continuous sorting, select **Continuous** from the **Target Events** menu.
5. Select one of the following options from the **Save Sort Reports** menu:
   - **Save None**. Sort reports are not saved.
   - **Save All**. Automatically saves a sort report each time the sort is stopped.
   - **Ask User**. Prompts the user each time the sort is stopped to select whether or not to save the sort report. This is the default option.

   This setting is saved with the sort layout.
6. Select the field(s) corresponding to the tube(s), well(s), or spot(s) where the population will be sorted and select a defined population from the **Add** menu.

   When you click in a sort location field, a menu opens allowing you to add, delete, or clear all populations in the field.



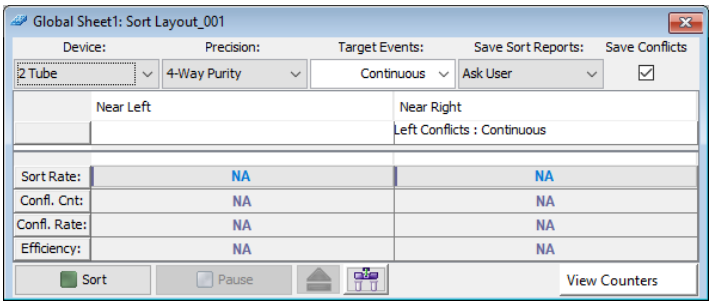
After you add a population, the population and the number of target events are added to the corresponding sort location field.

Note: Select a row or column header to select all fields in that row or column. When you add a population, it will be added to all selected fields at once.

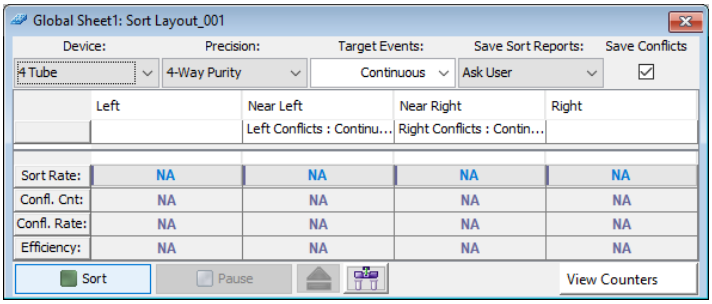
7. Specify whether to save sort conflicts by selecting the **Save Conflicts** checkbox.

   When selected, all sort conflicts are sorted into a default location.

   - For a two-tube layout, conflicts are sorted to the right.



   - For a four-tube layout, conflicts for the far left tube are sorted to the left, and conflicts for the far right tube are sorted to the right.



   - Six-tube layouts do not support conflict sorting.

Editing a sort layout

- To change the number of events for any population, click in the **Sort Location** field(s) containing the population, then select a number from the **Target Events** menu or enter a new number.
- To remove a population from a sort location field, select the field, then select the corresponding population from the **Delete** menu.
- To clear all populations from a field, select the field, then select **Clear All**.

Using Sorting Controls

Sorting controls are located at the bottom of the Sort Layout window. Use these controls for the following functions.

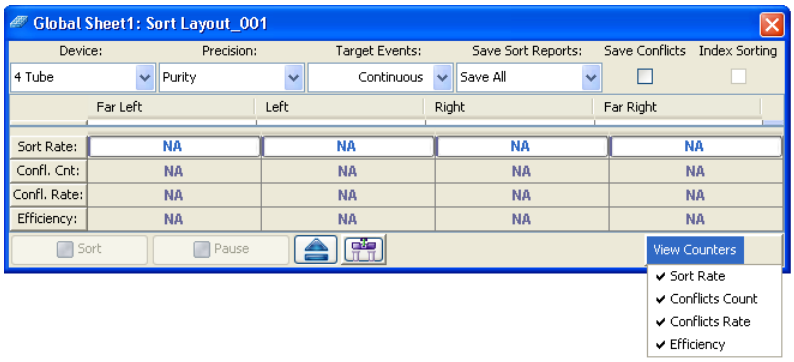
- **Sort**. Starts sorting events for the current acquisition tube. All counters reset to zero when this button is clicked. Events are sorted until the requested number of sorted events has been reached.

  Click the **Sort** button again to stop sorting before reaching the requested number of events. The counters stop at the number of sorted events. If you click **Sort** to restart sorting, the counters reset to zero.
- **Pause/Resume**. Stops sorting, but not acquisition. Sort counters and sort timers freeze when the Pause button is clicked. Click the **Resume** button to continue sorting and to continue incrementing the sort counters and timers. See Pausing and resuming a sort (page 130).

- **Access Stage**. When the ACDU stage is in the back, the stage is moved forward to put a plate on or take a plate off the stage. When the stage is in the front, the stage is moved to the back and out of the way so you can install a collection tube holder.
- **Move Drawer**. Moves the aspirator drawer in (closed) or out (open) depending on its current state. The default state is in. For more information, see Aspirator drawer (page 21).

Using counters

Counters provide ongoing status during sorting. Counter fields cannot be edited. To display fewer counters in the Sort Layout window, click the **View Counters** button and select a menu option. The corresponding counter is hidden. (Only counters with a checkmark next to the name are displayed.)



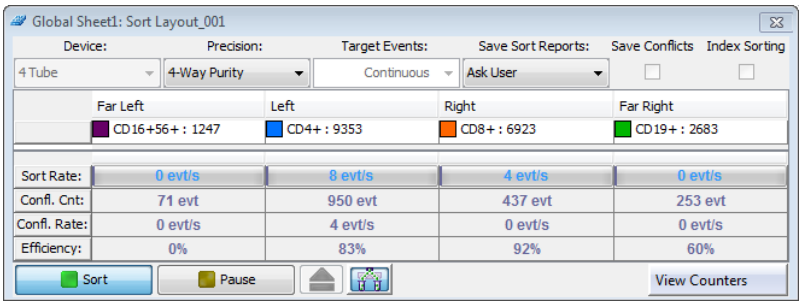
Counters display the following information:

- **Sort Rate**. Number of events per second that met the sort criteria and were sorted.
- **Conflict Count**. Number of events that met the sort criteria but were not sorted because of conflicts.
- **Conflict Rate**. Number of conflicts per second.
- **Efficiency**. Number of sorted events/(sort conflicts + sorted events) × 100.

Monitoring a sort

During sorting, each sort location field displays the number of actual sorted events. When a target number is specified, the field displays the actual number of events along with the number of target events.

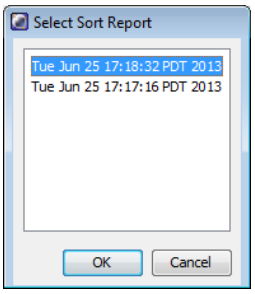
A progress bar opens behind the sort rate counter field showing the progress of the sort.



Sort report

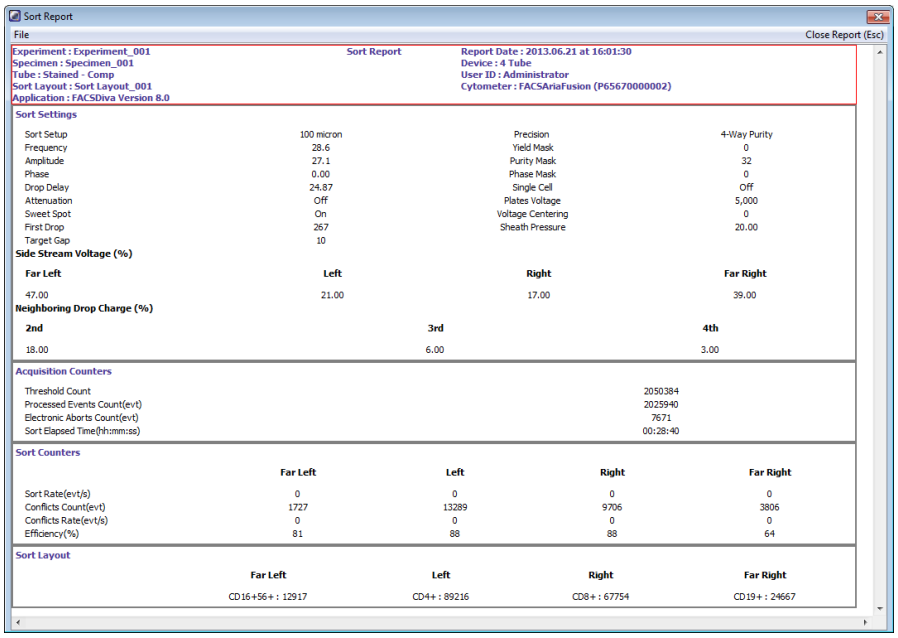
Select **Sort > Sort Reports** to view all of the saved reports for the current sort layout. A sort report can be printed or exported. The **Sort Reports** menu option is enabled if either of the following is true: a sort layout is

selected in the Browser (even if experiment is closed), or a sort layout is open. If both conditions are true, the Browser selection takes precedence.



A sort report contains the following:

- **Header information**. Experiment name, specimen name, tube name, sort layout name, software name and version, the date and time of printing, type of collection device, user ID, and cytometer.
- **Sort Settings**. Sort setup values, precision mode, mask definitions, etc.
- **Acquisition Counters**. Threshold count, processed events count, electronic conflicts count, and elapsed time.
- **Sort Counters**. Counter values per sort destination, or total sort count if sorting sequentially.
- **Sort Layout**. Population(s), sort count, and target event count for each sort location field.



The Sort Report window contains a File menu where you can select to print or export the report. Exported comma-separated value (CSV) files can be opened with a spreadsheet application such as Microsoft® Excel®.

Reference line setup for digital cameras

For digital cameras, reference line adjustment is performed using the Reference Line Setup tab in the Cytometer window. This tab adds reference lines to the BD FACSDiva™ Side Stream window, providing a point of reference to center and align the sort streams into the collection devices.

Note: The Reference Line Setup tab is only present if the user is logged in as an Administrator.

Preliminary operations

Set up the cytometer for sorting with the appropriate nozzle configuration, breakoff, and BD FACSDiva™ software settings. Use the instructions given in the following sections:

1. Starting the stream (page 91)
2. Setting up the breakoff (page 92)
3. Setting up for sorting (page 120)

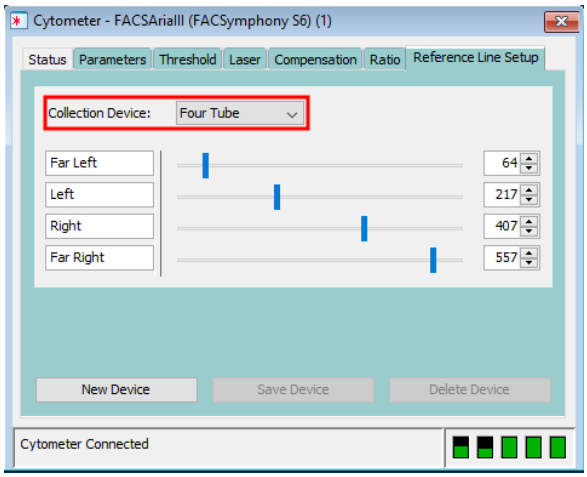
Selecting an existing sort device

The following procedure can only be performed by a user with Administrator privileges.

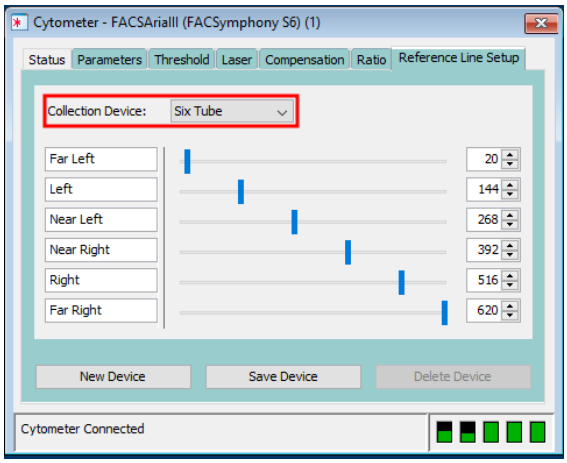
1. In the Cytometer window, select the **Reference Line Setup** tab.
2. Select a device from the Collection Device dropdown list.

   The number of sliders displayed is device-dependent. For example, four slider controls for the **Four Tube** device, similar to the following :

Four Tube device



Six Tube device



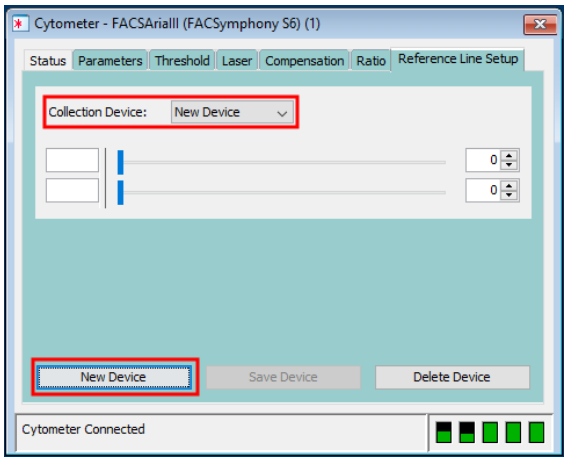
3. Set up the reference lines as described in Setting the reference lines (page 78).

Creating a new sort device

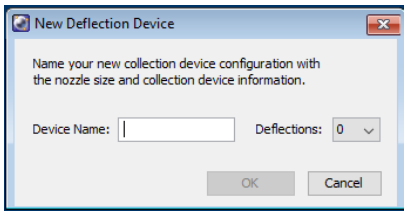
The following procedure can only be performed by a user with Administrator privileges.

1. In the Cytometer window, select the **Reference Line Setup** tab.
2. From the Collection Device dropdown list, select **New Device**.

3. Click the **New Device** button at the bottom of the window.



The software displays a New Deflection Device dialog:



4. In the Device Name text box, type a system-unique device name as prompted.

   If the device name already exists, the software will display a warning dialog, which can only be dismissed by clicking **OK**.

5. In the Deflections dropdown list, select the number of sidestreams corresponding to the sort device.
6. Click **OK** to close the dialog.
7. Set up the reference lines and save the device settings as described in Setting the reference lines (page 78).

Setting the reference lines

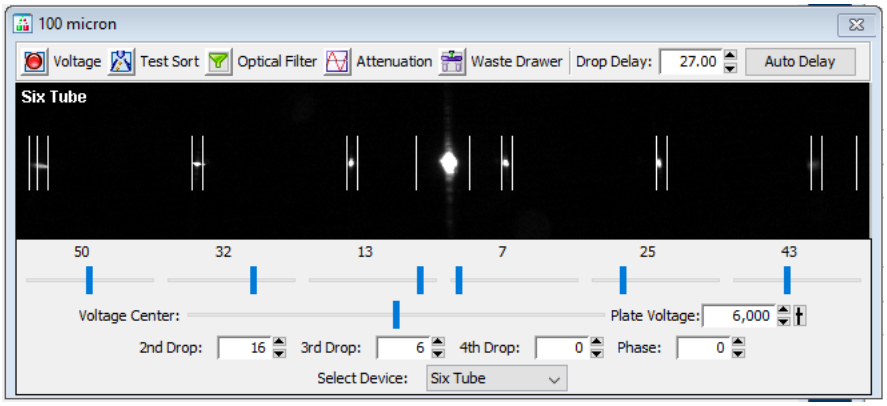
Your BD Field Service Engineer (FSE) positioned the reference lines around the centered streams for BD-provided sort devices. Use this procedure if you want to readjust the reference lines in an existing device or to initialize the values in a new device.

Note: For existing devices, this correction is only necessary if aligning the streams to the reference lines places the streams off center of the actual sort collection device. The procedure can only be performed by a user with Administrator privileges.

Cell sorters that use droplet generation methods, such as the BD FACSymphony™ S6 cell sorter, can produce aerosols around the sample stream. When acquiring biohazardous samples, follow universal precautions at all times. Keep the sort block door and the sort collection chamber door closed during sorting.

You might need to raise the sash of the biosafety cabinet (BSC) above the safe-access opening of 8 inches to do this procedure.

1. Ensure that the appropriate collection device is selected on the **Reference Line Setup** tab of the Cytometer window—see Selecting an existing sort device (page 77) or Creating a new sort device (page 77). Reference lines corresponding to the selected collection device will display in the Side Stream window.
2. Before aligning the Side Stream reference lines, verify that the aspirator reference lines are centered around the stream. If necessary, align the stream to the aspirator reference lines by adjusting the sort block position.
3. Insert the sort collection device under the sort block with tape or lids covering tubes.
4. Perform a one-drop test sort:
   a. Turn on the high-voltage plates.
   b. Open the waste drawer.
   c. Click **Test Sort** once to simultaneously deposit a drop for each side stream and then quickly click the button again to turn it off.



5. Remove the sort collection device and check whether each drop deposited is centered on the collection tube. If not, adjust the voltage slider for each stream to the appropriate setting. Re-insert the sort collection device and repeat the preceding step until all side streams are centered.
6. Close the waste drawer and click **Test Sort** to start the test-sort operation..
7. On the **Reference Line Setup** tab of the Cytometer window, adjust the sliders to center the collection device reference lines around each corresponding side stream.
8. On the **Reference Line Setup** tab of the Cytometer window, click **Save Device**.

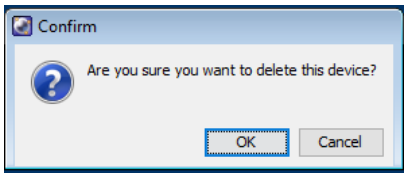
   The software displays a Save Deflection Device dialog.
9. To confirm the save operation, click **Yes** or cancel the operation by clicking **No** or **Cancel**.
10. Click **Test Sort** to stop the test-sort operation.
11. Turn off the high-voltage plates.

Deleting a sort device

The following procedure can only be performed by a user with Administrator privileges.

1. In the Cytometer window, select the **Reference Line Setup** tab.
2. From the Collection Device dropdown list, select the sort device to be deleted.
3. Click **Delete Device**.

The software displays a Confirm dialog:



4. Click **OK** to complete the operation.

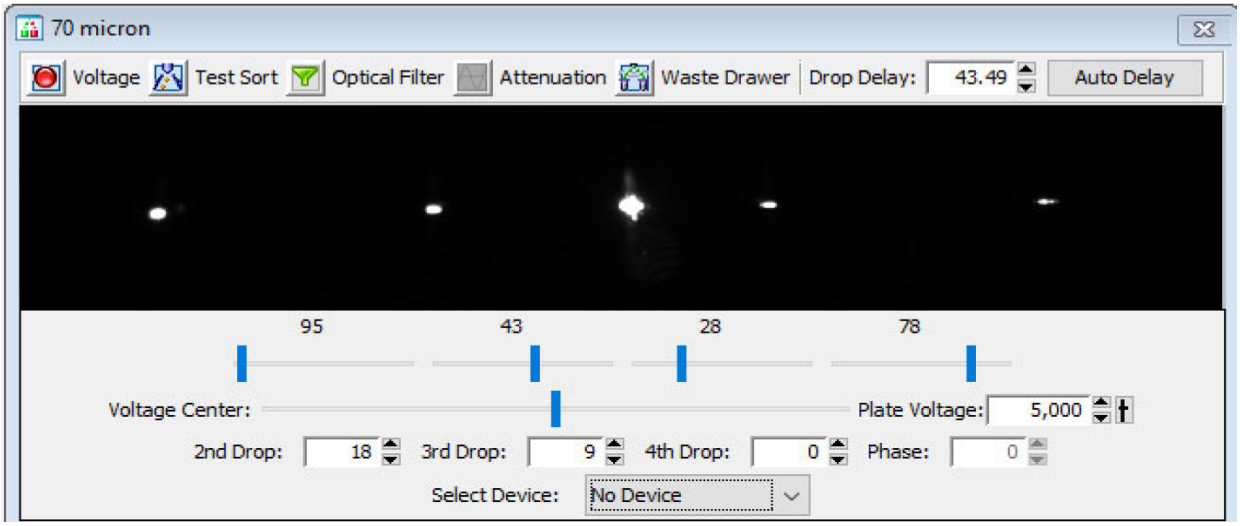
Sort alignment software for analog cameras

The Sort Alignment software adds functionality to BD FACSDiva™ software version 8.0 or later.

Note: The Sort Alignment software is only applicable to analog cameras.

Overview

The Sort Alignment software adds reference lines to the BD FACSDiva™ Side Stream window, providing a point of reference to center and align the sort streams into the collection devices. Your BD FSE installed the software and positioned the reference lines around the centered streams. The following figure shows the reference lines in the Side Stream window.



Aligning the streams

Cell sorters that use droplet generation methods, such as the BD FACSymphony™ S6 cell sorter, can produce aerosols around the sample stream. When acquiring biohazardous samples, follow universal precautions at all times. Keep the sort block door and the sort collection chamber door closed during sorting.

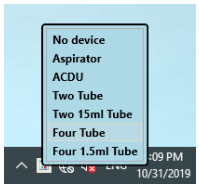
1. Set up the cytometer for sorting with the appropriate nozzle configuration, Breakoff, and BD FACSDiva™ software settings. Use the instructions given in the following sections:

- Starting the stream (page 91)
- Setting up the breakoff (page 92)
- Setting up for sorting (page 120)

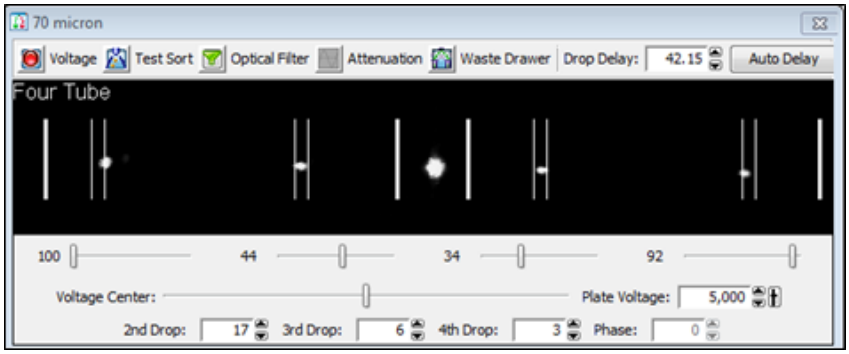
2. Start the Sort Alignment software by clicking the icon in the Microsoft Windows taskbar located in the bottom left corner of the computer monitor.

This adds the Sort Alignment icon to the Notification area.

3. Click the Sort Alignment icon in the Notification area located in the bottom right corner of the computer monitor, and select your collection device from the menu that opens.



4. With the sort block door closed, use the Side Stream window to verify that the aspirator reference lines are centered around the stream. If necessary, align the stream to the aspirator reference lines by adjusting the sort block position.



5. Align the side streams to the center of each of the collection device's reference lines by moving the voltage slider for each stream to the appropriate setting.
6. For more information on setting up the side streams, see the instructions given in Setting up for bulk sorting (page 120).

Setting the reference lines

Your BD FSE positioned the reference lines around the centered streams. If you want to readjust the reference lines, use this procedure.

Note: This correction is only necessary if aligning the streams to the reference lines places the streams off center of the actual sort collection device.

Cell sorters that use droplet generation methods, such as the BD FACSymphony™ S6 cell sorter, can produce aerosols around the sample stream. When acquiring biohazardous samples, follow universal precautions at all times. Keep the sort block door and the sort collection chamber door closed during sorting.

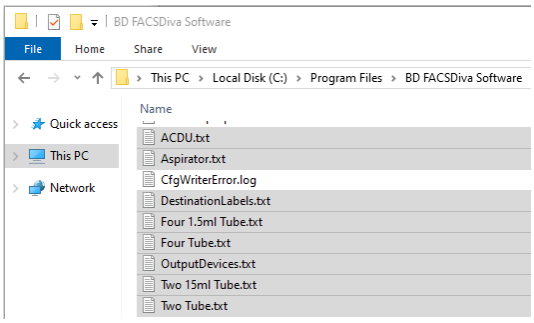
You might need to raise the sash of the biosafety cabinet (BSC) above the safe-access opening of 8 inches to do this procedure.

1. Make a backup copy of the existing BD FACSDiva™ sort alignment settings.

   You can re-import them later, for example, after re-installing BD FACSDiva™ software. (Re-installing BD FACSDiva™ software deletes the settings.)

   a. Navigate to C:\Program Files\BD FACSDiva Software.

   b. Select the following .txt files:



   c. Copy the files and paste them into C:\Libraries\Documents\BD Sort Alignment Tool.

   Note: Create the directory if it does not exist.

2. Set up the cytometer for sorting.
3. Once the stream is centered to the aspirator drawer and the breakoff is set, turn on the Sweet Spot.
4. Navigate to C:\Program Files\BD FACSDiva Software\jarfiles, and double-click **SortDeviceControl.exe** to start the Sort Alignment software.

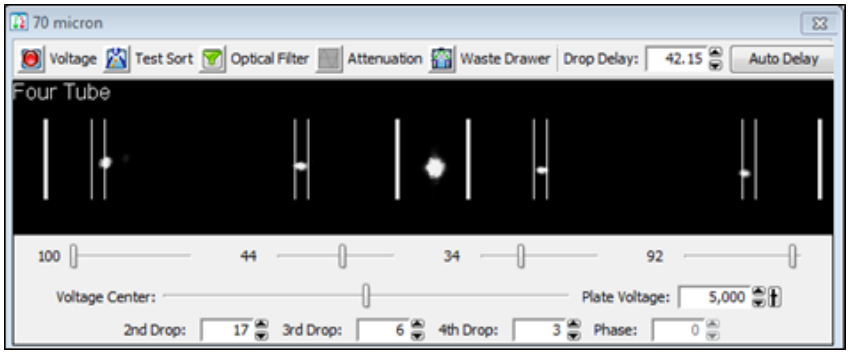
   The Stream Aiming Device Setup window opens.

5. Select the appropriate collection device from the Device selection menu. Reference lines corresponding to the selected collection device will display in the Side Stream window.
6. Before aligning the Side Stream reference lines, verify that the aspirator reference lines are centered around the stream. If necessary, align the stream to the aspirator reference lines by adjusting the sort block position.
7. Insert the sort collection device under the sort block with tape or lids covering tubes.
8. Perform a one-drop test sort:

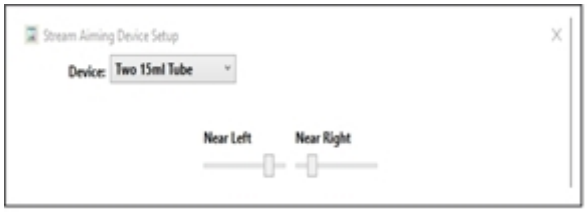
   a. Turn on the high-voltage plates.

   b. Open the waste drawer.

   c. Click **Test Sort** once to simultaneously deposit a drop for each side stream and then quickly click the button again to turn it off.



9. Remove the sort collection device and check whether each drop deposited is centered on the collection tube. If not, adjust the voltage slider for each stream to the appropriate setting. Re-insert the sort collection device and repeat the preceding step until all side streams are centered.
10. Close the waste drawer and click **Test Sort** to start the test-sort operation.
11. Use the Reference Line sliders to center the collection device reference lines around each corresponding side stream.



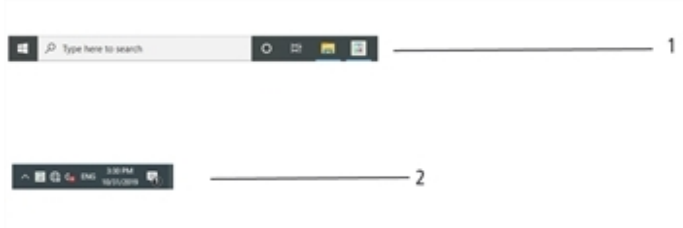
12. Click **Test Sort** to stop the test-sort operation.
13. Turn off the high-voltage plates.
14. Repeat steps 5–13 for each collection device configuration to be used.
15. Close the Sort Alignment software.

Reinstalling the Sort Alignment software

The Sort Alignment software is installed by your FSE. If you need to re-install the software, use this procedure.

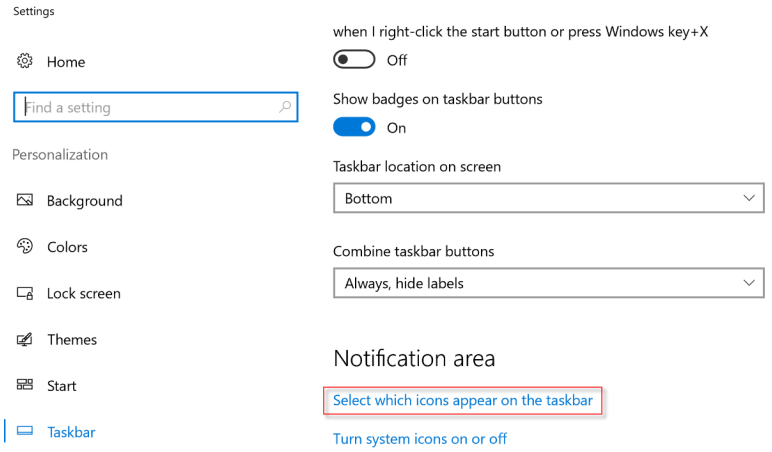
1. Close BD FACSDiva™ software if it is open.
2. Navigate to C:\Program Files\BD FACSDiva Software\jarfiles.
3. Double-click **IconSortDeviceControl.exe** to start the application.

   The Sort Alignment icon is displayed in the Windows taskbar and the systems tray.

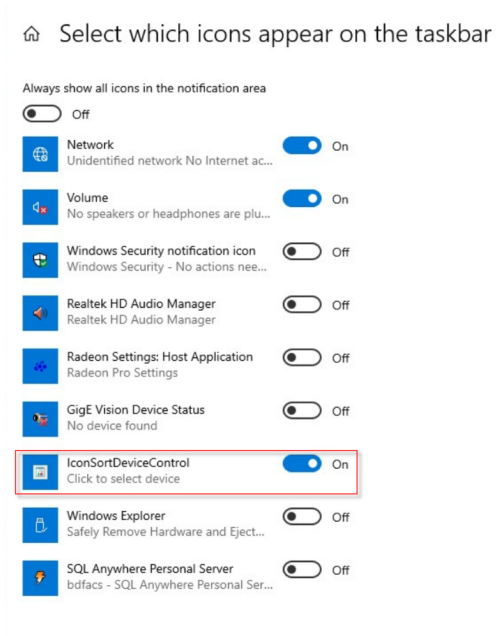


   Note: When starting the Sort Alignment software for the first time, the Sort Alignment icon might be displayed in the hidden area of the systems tray. If this occurs, drag the icon onto the systems tray.
4. Right-click the Sort Alignment icon in the Windows taskbar, and select Pin this program to taskbar from the menu that opens.

5. From the Windows Start menu, select **Settings**. On the Windows Settings screen select **Personalization** -> **Taskbar**. On the right pane, in the Notification area section, click **Select which icons appear on the taskbar**.



6. Ensure that you turn OFF the button for “Always show all icons in the notification area” and turn ON the button for “IconSortDeviceControl”.



7. Navigate to Aligning the streams (page 80) for how to use the software.

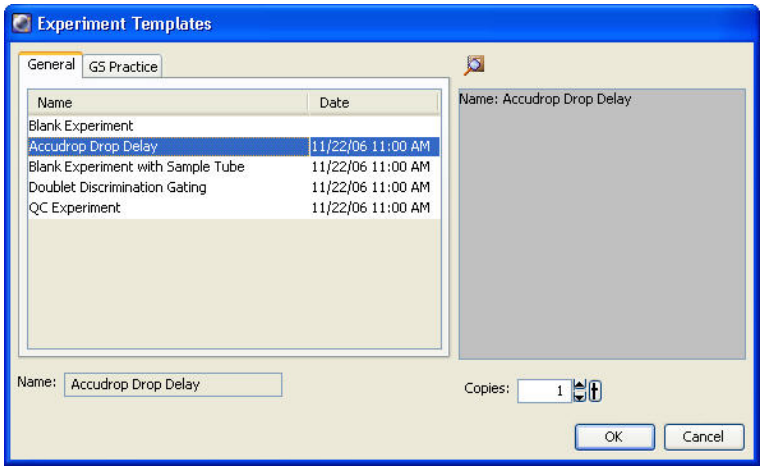
Troubleshooting

Observation	Possible cause	Recommended solution
The aspirator reference lines are off-center	The side stream window camera needs realignment	Call BD customer service.
The sort alignment settings are gone	BD FACSDiva™ software was reinstalled	Reposition the digital reference lines. See Setting the reference lines (page 81)

Templates

When you install BD FACSDiva™ software for the BD FACSymphony™ S6 cell sorter cytometer, the following additional experiment templates are installed in the BD Export\Templates directory:

- **Accudrop Drop Delay template**. Contains a single specimen and tube, a gated plot on a standard worksheet, and a predefined sort layout. This experiment is used for setting the drop delay during sorting as described in Determining the drop delay – manual method (page 123).



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5

Startup and running samples

The following topics are covered in this chapter:

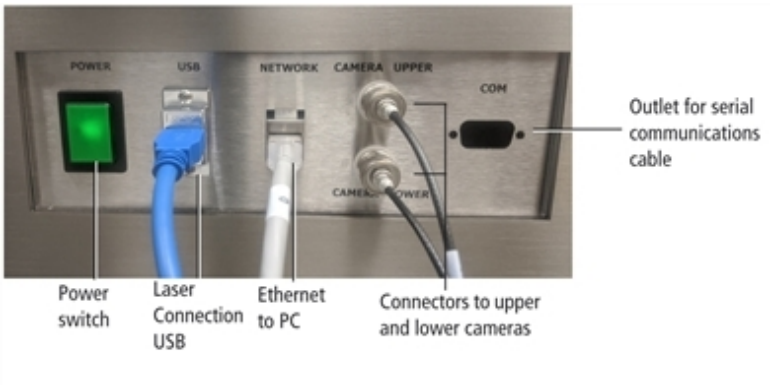
Cytometer startup

Follow these steps to start up your BD FACSymphony™ S6 cell sorter.

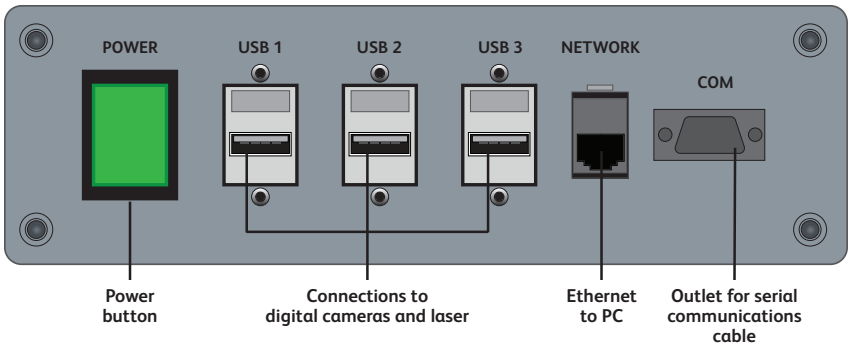
Note: Ensure that the instrument is plugged into the provided power conditioner and select proper voltage setting before turning on the main power.

1. Turn on the cytometer main power.

   Power panel for cytometers with analog cameras:



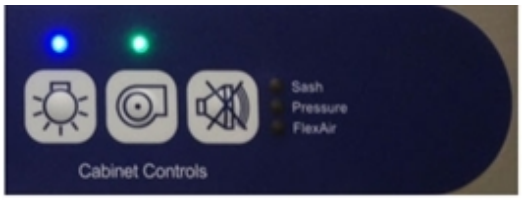
   Power panel for cytometers with digital cameras:



   If the cytometer was just shut down, wait until the system is fully depressurized (stops hissing) before you turn on the main power.

2. Switch on the house air or the optional compressor.
3. Start up the workstation.
4. Press the LASER MAIN button on the front of the optics drawer to turn on the lasers you will be using. Wait 30 minutes for the lasers to warm up.
5. If your system includes the BSC and it is not already on, turn on the blower by pressing the power button on the Cabinet Controls panel.

   A green light indicates the power is on.



6. (Optional) If you are using the temperature control option, turn it on. See BD FACSymphony™ S6 Temperature Control Option (page 217).
7. Log in to Windows 10.
   a. Select **BDOperator login** or **BDAdmin login**.
   b. Type the password. On initial installation, the BDOperator password is **Welcome#1** and the **BDAdmin** password is **BDIS#1$$**. After logging in with the initial password, you will be prompted to change it immediately, and every 90 days thereafter.
8. Open the Coherent Connection laser control software, wait for all lasers to populate.
9. If the **Auto-on** laser option is selected, wait for all lasers controlled by Coherent Connection to power on. Otherwise, for each laser, select the **Laser Start** mode.

   Note: The UV Laser does not turn on automatically, and will display in Coherent Connection in a "fault" state with power set to "0.00 mW". If the laser is already in the **Laser Start** mode, select **Laser Stop**. Set the desired laser power, and then select **Laser Start**.
10. Ensure all lasers controlled by Coherent Connection are powered up and the power levels are stable. The laser power levels should automatically set to the previously used power levels. If not, adjust the power as needed.
11. Once all the lasers are at optimal power, close the Coherent Connection software to avoid laser connection issues with the BD FACSDiva™ software. Closing the Coherent Connection software will not affect the laser power levels.
12. For lasers not controlled through coherent connections software:
    a. Open the GUI-VFL Laser-control software.

    b. Click the image of the serial (COM) port to view or set the port number.

    c. Enter the desired power level in the **Power Set** field, and select **Activate**.
    d. Verify that the power level rises to the desired level.
    e. Set the On/Off control to **On**.
13. Open BD FACSDiva™ software by double-clicking the application shortcut on the desktop.

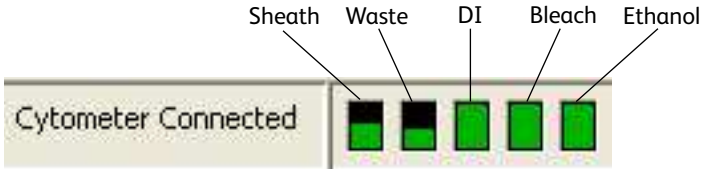
    

14. Log in with your user name and password.

If your login name is not displayed, contact your BD FACSDiva™ software administrator to create a login name for you. For more information, see the *BD FACSDiva™ Software Reference Manual*.

15. Check the fluid levels in the **Cytometer** window. Replenish fluids or empty the waste, if needed.

    To display the **Cytometer** window, click the **Cytometer** button on the **Workspace** toolbar. Check the fluid levels at the bottom of the window. Roll the mouse pointer over fluid indicators to show the fluid type.



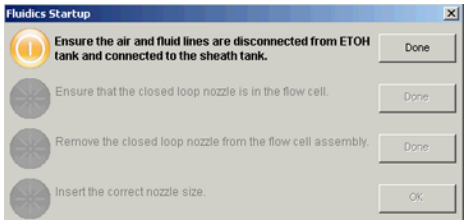
Note: The ethanol level in the Cytometer window indicates the volume of fluid in the white auxiliary ethanol tank. There is no sensor for the stainless steel ethanol shutdown tank.

To service the fluid containers, see Emptying the waste tank (page 97).

Performing fluidics startup

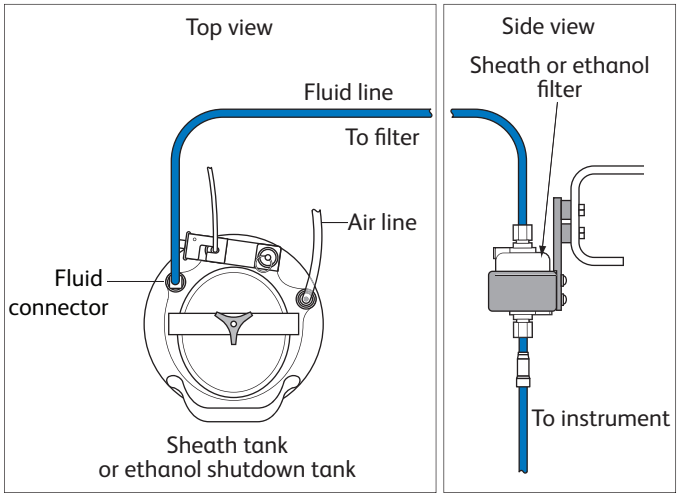
This section describes the fluidics startup procedure. The status of the fluidics system is displayed in the bottom right corner of the main window.

1. From the **BD FACSDiva™ Cytometer** menu, select **Fluidics Startup**. The following window opens.



2. Verify that the air and fluid lines are disconnected from the ethanol tank and connected to the sheath tank, then click **Done**.

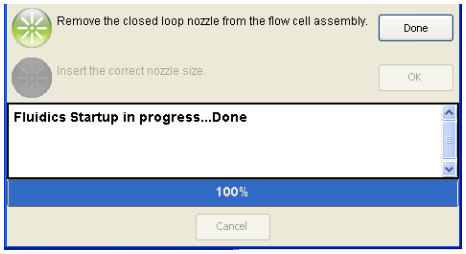
Do not run fluidics startup with the air and fluid lines connected to the ethanol shutdown tank. This can cause damage to the system. Always verify that the lines are connected to the sheath tank.



Note: The flow rate is calibrated with the sheath tank. If the location or elevation of the sheath tank is changed, it could affect the flow rate calibration.

3. As a precautionary measure, prior to opening the Flow Cell access door, use the **Stop** button in the laser control software to put the lasers in standby and then close the laser control software. This will turn off the laser diode, but will keep the diode temperature constant.
4. Open the Flow Cell door and verify that a closed-loop nozzle is installed in the flow cell, then click **Done**.

   The fluidics startup process starts and the progress is displayed at the bottom of the dialog.



5. Turn the nozzle-locking lever counter-clockwise to the 9:00 position, pull the closed-loop nozzle out of the cuvette flow cell, then click **Done**.
6. Insert the correct nozzle size in the flow cell.
   a. Verify that the O-ring is present and that it is installed in the nozzle groove. For more information, see Integrated nozzle (page 19).
   b. Make sure the top side of the nozzle is facing up as you insert the nozzle.
   c. Push the nozzle all the way back into the flow cell.
   d. Turn the nozzle-locking lever clockwise to the 12:00 position.
7. Click **OK** to complete the process.
8. Set the appropriate optical configuration. See Cytometer configuration (page 59).
9. When fluidics startup is complete, select **Sort > Sort Setup** and verify that the setup mode matches the nozzle size.

   For information about Sort Setup modes, see Sort setup (page 70).

Starting the stream

1. Confirm that the in-house air pressure, or the compressor, is on.

   When the stream is on, air pressure fluctuates between 80–100 PSI. A pressure reading of less than 80 PSI or greater than 100 PSI indicates that the fluidics are not functioning properly. If this occurs, contact your BD Biosciences service representative for assistance. Do not operate the cytometer outside the normal air pressure range.

2. Start the stream.
3. a. Click the **Sorting** button on the **Workspace** toolbar to display the **Breakoff** and **Side Stream** windows.
   b. Click the **Stream** button in the **Breakoff** window to turn on the stream.
4. Ensure that the lasers are off or in standby and the laser control software is closed.
5. Open the sort block door and check the stream.

   The stream should flow smoothly from the nozzle into the center of the waste aspirator. If the stream is flowing but is unsteady, check for bubbles in the flow cell. If you see bubbles, turn off the stream, wait for 10 seconds, and turn on the stream again. If you see any dripping or spraying, or the stream image appears abnormal, turn off the stream and see Troubleshooting the stream (page 170).

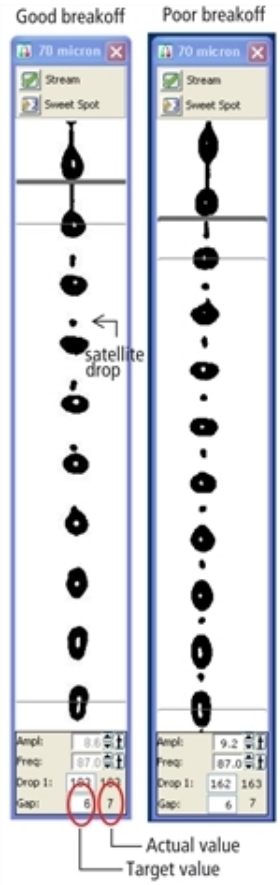
6. Close the sort block door.

   Note: If you encounter bubbles in the sheath fluid stream at the 130-micron sort setting, perform a sample line backflush for approximately 1 minute. Select **Cytometer > Cleaning Modes > Sample Line Backflush**. To prevent bubbles, the sheath tank should be depressurized while not in use to prevent air from dissolving into the sheath solution.
7. Close the Flow Cell access door and use the **Start** button in the laser control software to return the lasers to the Ready state.

Setting up the breakoff

Establishing a stable drop pattern in the breakoff window is an important step in getting optimal results from the system. See Breakoff Window (page 45) for more information on the parameters and controls in the breakoff window.

The following figure shows good versus poor breakoff patterns.



1. Adjust the **Ampl** slider until the drop breakoff is approximately in the top third of the **Breakoff** window.

   Do not exceed 70 volts. If you cannot achieve a drop breakoff at <70 volts, do the following:

   - As a precautionary measure, prior to opening the Flow Cell access door, use the **Stop** button in the laser control software to put the lasers in standby and then close the laser control software. This will turn off the laser diode, but will keep the diode temperature constant.
   - Check the flow cell for air bubbles. If you see bubbles, turn the stream off and back on.

- Make sure the sheath pressure and drop-drive frequency are appropriate.
- If the amplitude is <10 volts, turn on attenuation in the **Side Stream** window.

2. Verify that the small satellite droplets are merging with the large droplets.

   If the satellites are not merging, you might need to clean the nozzle by sonicating. The satellite drops should merge into the drops in 6 satellites or fewer.

3. Enter the actual Drop 1 value as the target in the **Drop 1** field.

   The actual value is displayed in the gray background next to the Drop 1 field. Once a valid target has been established, you do not need to reset it unless you change the nozzle or your sort setup option.

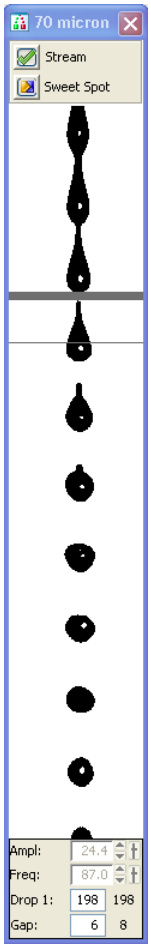
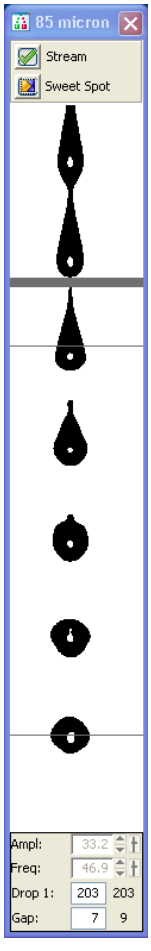
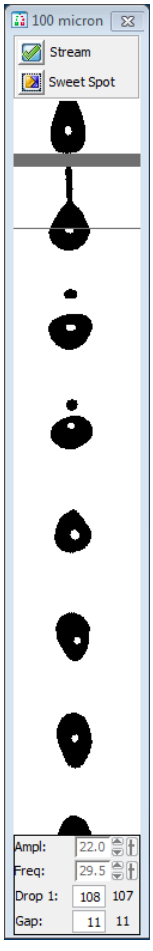
   Note: If the target value causes the amplitude to exceed 70 volts, perform the recommendations under step 1 and re-enter the Drop 1 target value.

4. Turn on the Sweet Spot when the drop pattern is stable.

   The Sweet Spot is designed to automatically adjust the drop-drive amplitude to maintain the stability of the breakoff point. When the Sweet Spot is on, the Amplitude and Frequency fields are disabled. The values are automatically adjusted by the software. For more information, see Breakoff Window (page 45).

   .

The breakoff patterns shown in the following table are intended as examples. The patterns will not always look exactly like these.

70 micron	85 micron	100 micron	130 micron
			

Setting up the fluidics

Check the fluid levels in the sheath tank and waste containers every time you use the cytometer. This ensures that you will not run out of sheath fluid during an experiment or have to service the containers during a sort. Fluid level indicators are shown in the Cytometer window in BD FACSDiva™ software.

If a fluid container is running low, refill it with the fluid indicated on the container label. For best results, fill the containers only with the fluids shown in the following table. For ordering information, see Reagents (page 189).

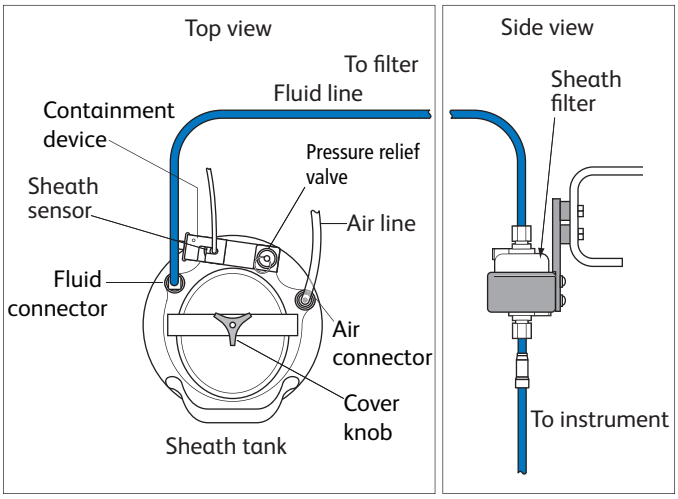
For instructions on emptying the waste, see Emptying the waste tank (page 97).

Container	Compatible Fluids	Capacity
Sheath[a]	• BD FACSFlow™ sheath fluid (non-sterile) • 1X phosphate-buffered saline (PBS), with or without preservatives	One 10-L stainless steel container
Ethanol Shutdown	70% solution diluted in deionized (DI) or laboratory-grade water	One 5-L stainless steel container
Bleach	• 10% household bleach (0.5% sodium hypochlorite) • BD FACSClean™ solution	5 L
DI	Deionized water (Milli-Q or equivalent). Add 3 mL of bleach per liter of DI water.	5 L
Ethanol	70% solution diluted in DI or laboratory-grade water	5 L

Note: Make sure the blue sheath fluid line between the sheath tank and the cytometer does not come into contact with anything that could introduce vibration that might affect the stability of the stream.

Refilling the sheath tank

The fluid level in the pressurized stainless steel sheath tank should be checked often and refilled when low. The startup times for the breakoff stream will increase when the tank is low. If the cytometer is run until the sheath supply is too low, the system will turn off the stream.



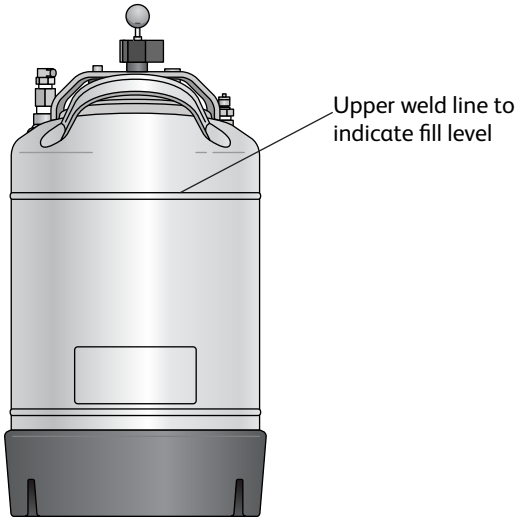
[a]Users should select solutions that are compatible with their specific samples and experiments.

To refill the sheath tank:

1. Turn off the stream.
2. Disconnect the air line.
3. Pull up on the ring of the pressure relief valve to release pressure from the tank.

   Make sure the tank is fully vented.
4. Unscrew the sheath tank cover knob and remove the cover.
5. Fill the tank with sheath fluid up to the upper weld line on the inside of the tank. See the following figure.

   Note: Do not overfill the sheath tank because this can cause incorrect sample flow rates.



6. Replace the cover and tighten the knob.

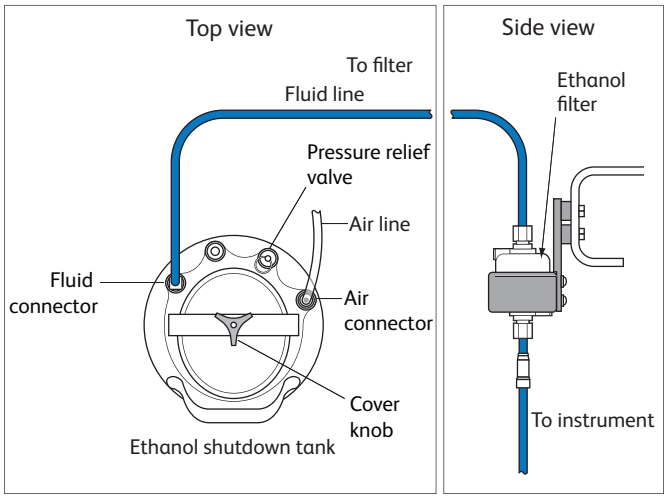
   Make sure the large O-ring on the inside lip of the cover is seated correctly and has not slipped out of position. The tank can leak if the cover is not secured properly.
7. If you removed the sheath tank to refill it, place the tank back in its original position.

   The flow rate is calibrated with the sheath tank in the fluidics drawer. If the location or elevation of the sheath tank is changed, it could affect the flow rate calibration.
8. Connect the air line.
9. Close the vent release valve and use the sheath tank gauge to ensure the tank is pressurizing properly.

   The system is ready to run again.

Refilling the ethanol shutdown tank

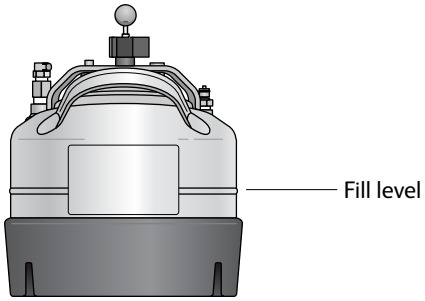
The fluid level in the stainless steel ethanol shutdown tank should be checked before starting the fluidics shutdown procedure and refilled when low. This tank does not have a sensor, so it must be checked manually.



1. Turn off the stream.
2. If the air line on the ethanol shutdown tank is connected, disconnect it.
3. Pull up on the ring of the pressure relief valve to release pressure from the tank.

   Make sure the tank is fully vented.
4. Unscrew the tank cover knob and remove the cover.
5. Fill the tank with ethanol to the level shown in the figure following this procedure.
6. Replace the cover and tighten the knob.

   Make sure the large O-ring on the inside of the cover is seated correctly and has not slipped out of position. The tank can leak if the cover is not secured properly.
7. If you are going to perform fluidics shutdown, connect the air line.



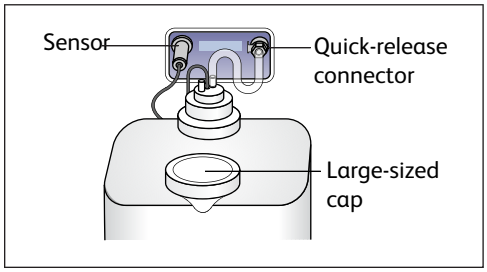
Refilling the plastic containers

You can refill the plastic containers directly in the fluidics drawer without detaching any lines, or you can remove the container for refilling.

Note: During operation, you can add fluid to a container through the large cap without any interruption to your experiment, but if you detach any lines, you will need to prime the system.

1. (Optional) Disconnect the container's sensor and quick-release connector from the connectors panel if you need to move the container.

   If you do not need to move the container, skip to step 2.



2. Remove the large-sized cap from the container.
3. Fill the container with the fluid indicated on the container label.
4. Replace the container cap and hand-tighten it until it is fully closed.
5. If you disconnected the sensor and quick-release connectors in step 1:
   a. Reconnect the sensor and quick-release connectors to their respective ports.

      To ensure that the appropriate solutions are dispensed, do not switch the tank positions. Make sure the label on each container matches the labeled port on the fluidics drawer.

   b. Prime the fluidics system.

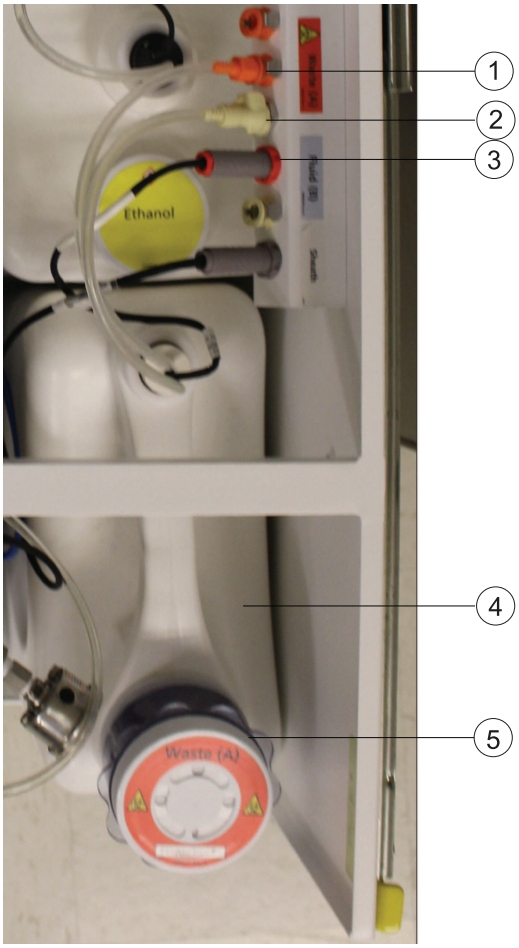
      From the BD FACSDiva™ menu bar, select **Cytometer > Cleaning Modes > Prime after Tank Refill**. In the dialog that opens, select the fluid to prime, then click **OK**.

Emptying the waste tank

Empty the waste tank daily and when the fluid indicator shows the waste is getting full. To prolong the life of the tank, we recommend that you switch to an alternate tank each time the waste is emptied.

All biological specimens and materials coming into contact with them can transmit potentially fatal disease. To prevent exposure to biohazardous agents, expose waste tank contents to bleach (10% of total volume) before disposal. Dispose of waste in accordance with local regulations. Use proper precautions and wear suitable protective clothing, eyewear, and gloves.

Change the waste tank cap and baffle every month, or when it has gotten wet, to prevent container pressurization. To order new replacement caps and baffles, contact your local BD Biosciences representative.



Item Number	Component
1	Waste fluid line
2	Waste air line
3	Waste sensor
4	Waste tank
5	Waste cap and baffle

1. Disconnect the waste tank's sensor and fluid line connectors from the connector panel in the fluidics drawer.

The waste tank can become pressurized when the cytometer is running. Always disconnect the tank from the connector panel before you empty it. Wait at least 1 minute for pressure to dissipate before you open the tank.

2. Remove the disposable waste cap (large-sized cap) and attached baffle from the container. Place the assembly on the bench label-side up.

Do not wet the cap on top of the baffle. If you see liquid inside the baffle, replace the cap an baffle with new components.

3. Empty the waste tank according to your standard laboratory procedures for biohazardous waste.
4. Add approximately 1 L of bleach to the waste tank (10-L container).
   Add a sufficient amount so that 10% of the total waste volume is bleach.
5. Replace the waste baffle and attached filter cap. Hand-tighten the trap and cap until they are fully closed.

To prevent over-pressurization during fluidics startup, do not overtighten the baffle or attached filter cap. Tighten each component only until it is hand-tight. Do not use sealants such as Teflon® tape or other adhesives.

6. If one month has passed since you last changed the cap, replace the filter cap and baffle with new ones. Write the date on the new cap as a reminder.

7. Reconnect the sensor and fluid line connectors to their respective ports.

Checking cytometer performance

Before setting up an experiment, you should first run a performance check. A performance check ensures that the cytometer is performing consistently over time. It also generates default cytometer settings that places each PMT within an optimal range.

To run a performance check, start the BD® CS&T Research Beads application within BD FACSDiva™ software and select the appropriate cytometer configuration. Each configuration must have a valid baseline. See the *BD® Cytometer Setup and Tracking Application Guide* for more information on running a baseline.

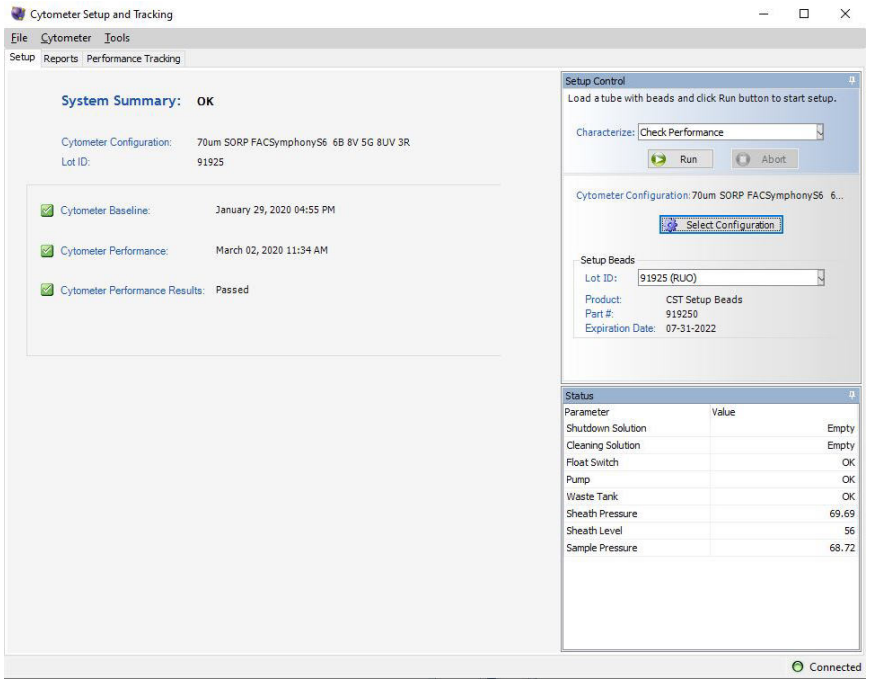
Preparing the BD® CS&T Research Beads workspace

To ensure that your cytometer is performing consistently over time, it's important to keep as many variables constant as possible, such as bead type, sheath pressure, and cytometer settings. For this reason, you should run the daily performance check using the same cytometer configuration each day.

1. Turn off the Sweet Spot (if it is on), then select **Cytometer > CST**.

   The cytometer disconnects from the BD FACSDiva™ interface and connects to the BD® CS&T Research Beads interface. A window similar to the following figure opens.

   Note: Do not run the CST program in a maximized window.



2. Verify that the bead lot information under **Setup Beads** matches the BD® CS&T Research Beads lot.

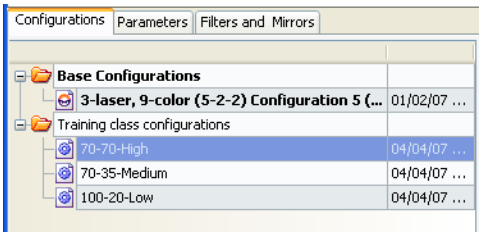
   Select the correct lot ID from the menu. The bead lot ID number is located on the BD® CS&T Research Beads vial.

3. Verify that the cytometer configuration is correct for your experiment.

   If the cytometer is not set to the correct configuration:

a. Click **Select Configuration** in the **Setup Control** window.

b. Select the correct configuration from the list.



c. Click **Set Configuration**. Click **OK**.

4. Verify that the current configuration has a valid baseline defined.

   If not, see the *BD® Cytometer Setup and Tracking Application Guide* for more information on running a baseline.

Preparing the BD FACSDiva™ BD® CS&T Research Beads

BD FACSDiva™ CS&T Research Beads consist of bright, mid, and dim beads dyed with a mixture of fluorochromes. Use the beads to define a baseline and to check cytometer performance using the CS&T application.

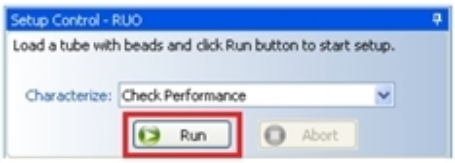
See the BD FACSDiva™ BD® CS&T Research Beads technical data sheet for instructions about preparing the bead suspension. Instructions vary based on the task you are performing.

Running a performance check

The performance check function of BD® CS&T Research Beads will check the cytometer's daily performance.

1. Confirm the lasers are in the Ready state using the laser control software.
2. 

   Movement of mechanical parts within the instrument can pinch or injure your hands or fingers. Keep your hands and clothing away from the loading port when a tube is loading or unloading. Do not place objects underneath the loading port.
3. In the **Setup Control** window, select **Check Performance** from the **Characterize** menu.
4. Click **Run**.



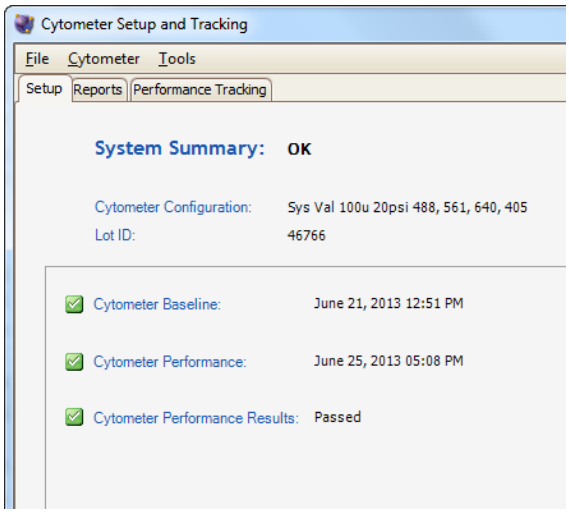
Plots are displayed under the Setup tab and the performance check is run. The performance check takes approximately 5 minutes to complete.

Reviewing the results

1. Once the performance check is complete, click **View Report**.
2. Print the report.
3. Verify that the performance check passed.

   In the Setup tab, the Cytometer Performance Results should have a green checkmark and the word *Passed* next to it.

   If any parameters did not pass, see the *BD® Cytometer Setup and Tracking Application Guide* for troubleshooting information.



4. Select **File > Exit** to close the BD® CS&T Research Beads window and connect back to the BD FACSDiva™ interface.

5. Click **Use CST Settings** in the **CST Mismatch** dialog that opens.

   See CST Mismatch dialog (page 103) for more information.

   When CS&T settings are applied to new experiments, the settings applied include PMT voltages, threshold, laser delays, and area scaling factors. (The window extension values are generated from the cytometer configuration.)

   When CS&T settings are applied to existing experiments, the settings applied include PMT voltages, laser delays, area scaling factors, and window extensions. Threshold values remain the same.

   There is a system preference to always use current BD® CS&T Research Beads settings that can be set by the administrator. For BD FACSymphony™ S6 cell sorter users, we recommend that you do not enable this preference.

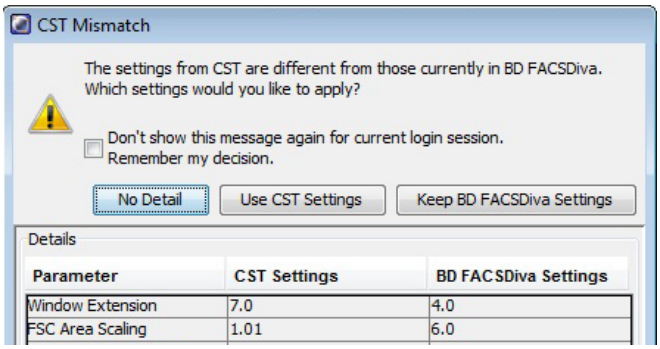
At this point you can do one of the following:

- Create application settings for an experiment you will use frequently. See Application settings (page 103).
- Collect data using existing application settings. See Data collection (page 108).

CST Mismatch dialog

If there is a mismatch between the BD® CS&T Research Beads settings and those in BD FACSDiva™ software, a dialog opens to indicate what the differences are, and gives you the following options:

- **Use CST Settings**. Select this option if you want to use the settings generated from the most recent performance check.
- **Keep BD FACSDiva Settings**. Select this option if you want to keep the settings that are associated with your cytometer.
- **Details**. Click this button to view the differences between the two settings to help with your selection.



- See Data collection (page 108).

Application settings

Application settings are associated with a cytometer configuration and include the parameters needed for the application, area scaling values, PMT voltages, and threshold values, but not compensation. Each time a performance check is run for a configuration, the application settings associated with that configuration are updated in the catalog. To update the application settings in an existing experiment, right-click the application settings in the Browser and select **Application Settings > Apply**.

Using application settings provides an easy, consistent, and reproducible way to reuse cytometer settings for your commonly used applications. See the *BD® Cytometer Setup and Tracking Application Guide* for information on running a baseline and for other details on using the CS&T application.

Before creating application settings you must:

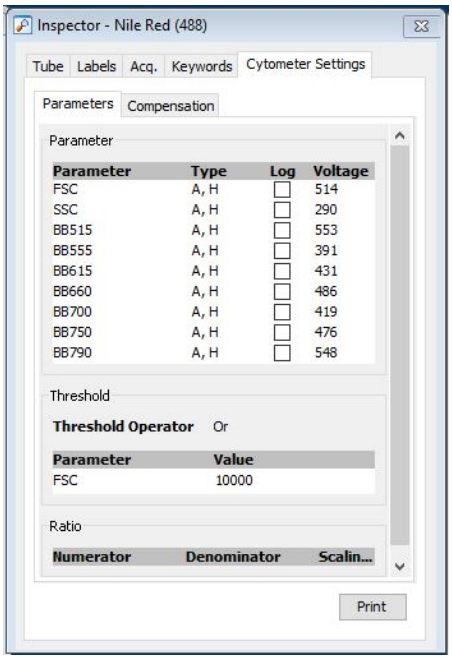
- Perform the cytometer startup procedure described in Cytometer startup (page 88).
- Run a performance check for the cytometer configuration that will be used for the application.

Creating application settings

This section describes how to create and save application settings for a multi-color immunophenotyping sample.

Setting up the workspace

1. Select **Cytometer > View Configurations**, and verify that the current configuration is appropriate for the type of sample you are running.
2. Verify that there is a valid performance check for the configuration.
3. Create a new folder.
4. Create a new experiment.
5. Select the **Cytometer Settings** in the Browser.
6. In the **Inspector** window, click the **Parameters** tab and delete any unneeded parameters.
7. Select the **H** check box to select height for each parameter.



Adjusting area scaling

The required area scaling factor changes based on sheath pressure and particle size. The area scaling factors should be verified for each experiment performed on the cytometer.

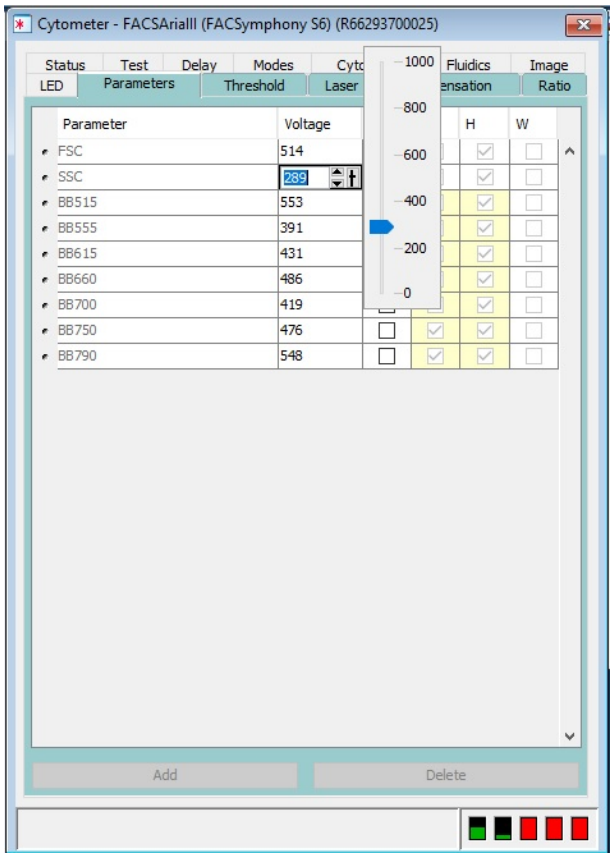
Note: You will need to modify the template to support the lasers installed on your system. Don't forget to save the settings when you are done.

1. In the **Browser**, right-click **Global Sheet1** and select **Apply Analysis Template**.
2. In the **Template** dialog, select the **Area Scaling** worksheet and click **OK**.
3. Create a new specimen by clicking the **New Specimen** button on the **Browser** toolbar.
4. Expand the new specimen, then click to set the current tube pointer to Tube_001.

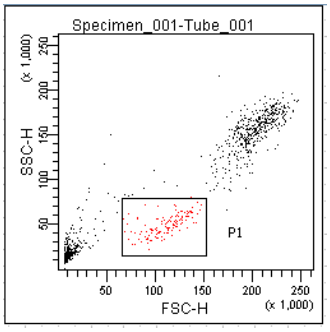
5. Install the FITC-positive control tube onto the loading port and click **Load** on the **Acquisition Dashboard**.

   Movement of mechanical parts within the instrument can pinch or injure your hands or fingers. Keep your hands and clothing away from the loading port when a tube is loading or unloading. Do not place objects underneath the loading port.

6. Adjust the FSC and SSC voltages to place the particles on scale.

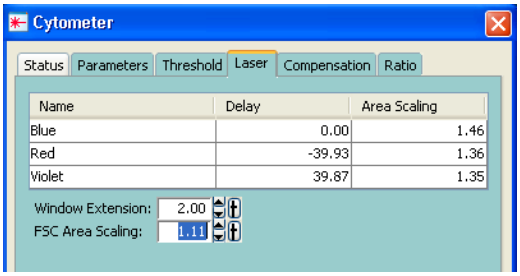


7. Adjust the P1 gate around the population of interest in the FSC vs SSC plot.



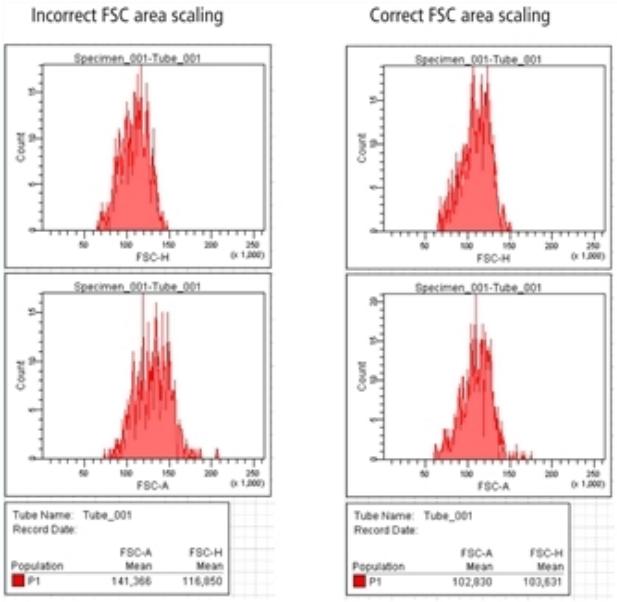
8. Adjust the FSC area scaling.

a. Click the **Laser** tab in the **Cytometer** window.

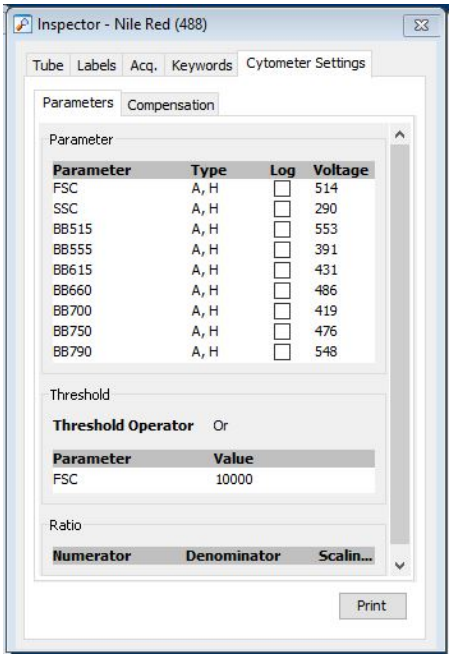


b. Adjust the FSC area scaling factor until the FSC-A signal matches the FSC-H signal.
   - Increase the area scaling factor if the FSC-A signal is lower than FSC-H.
   - Decrease the area scaling factor if the FSC-A signal is higher than FSC-H.

c. View the result of your change in the histograms and statistics views.



9. Adjust the blue laser area scaling until the FITC-A signal matches the FITC-H signal, if needed.
10. Unload the FITC-positive control tube, then load the APC-positive control tube.
11. Adjust the red laser area scaling until the APC-A signal matches the APC-H signal, if needed.
12. Check and adjust, as necessary, the area scaling for the remaining lasers using a single color stained tube. Only one parameter from each laser needs to be used for area scaling.
13. In the **Inspector** window, clear the checkboxes for height for all parameters.

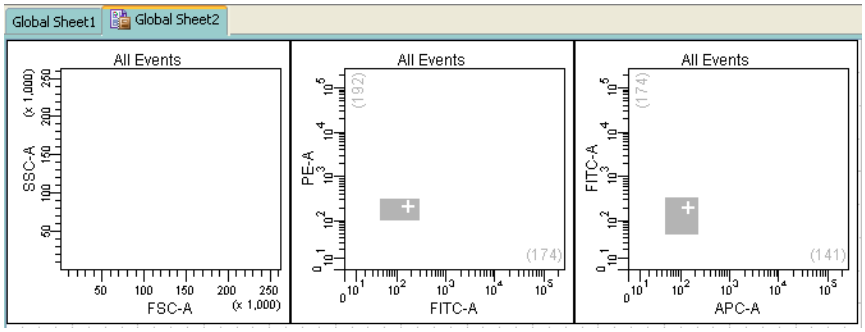


Optimizing PMT voltages

1. Right-click **Cytometer Settings** in the **Browser**, then select **Application Settings > Create Worksheet**.

   A second global sheet is added with the plots created according to your selections in the Parameters tab. Use the gray boxes and crosshairs to guide your optimization.

   Note: The gray boxes and crosshairs are not displayed until acquisition starts.

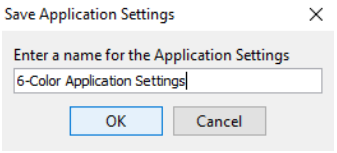


2. Load a multicolor sample tube onto the cytometer.
3. In the **Cytometer** window, optimize the settings for your application.
   a. Optimize the FSC and SSC voltages to place the population of interest on scale.
   b. Optimize the FSC threshold value to eliminate debris without interfering with the population of interest.
4. Verify that the positive populations are on scale.

   If a positive population is off scale, lower the PMT voltage for that parameter until the positive population can be seen entirely on scale.
5. Unload the multicolor sample from the cytometer.

Saving application settings

1. Right-click **Cytometer Settings** in the **Browser**, then select **Application Settings > Save**, to save the values for reuse.



   a. In the **Application Settings** dialog, rename the application settings with a descriptive name.
   b. Click **OK**.

The application settings are saved to the catalog.

Data collection

Before you record data for a sample, cytometer settings should be optimized to position the cells of interest on scale for scatter and fluorescence parameters. In the previous section, application settings were created by taking into consideration the following:

- FSC and fluorescence area scaling
- FSC and SSC voltages
- FSC threshold
- Fluorescence PMT voltages

In this section, the application settings will be applied and compensation will be calculated before collecting test data.

The following sections describe how to use previously optimized application settings for a 4-color experiment. See Application settings (page 103) for more information.

Compensation will be automatically calculated using the compensation setup feature. For more information about this function, see the *BD FACSDiva™ Software Reference Manual*. If you are performing compensation manually, not all steps will apply.

Setting up the workspace

Before you begin optimizing settings, it is important to verify the cytometer configuration and create an experiment containing appropriate parameters for the assay.

1. Select **Cytometer > View Configurations**, and verify that the current configuration is appropriate for the type of sample you are running.
2. Verify that there is a valid performance check for the configuration.
3. Make sure that the HPC(s) contain appropriate filters.

   For assistance, see Cytometer configuration (page 59).

   For accurate data results, the cytometer optics must match the current cytometer configuration.

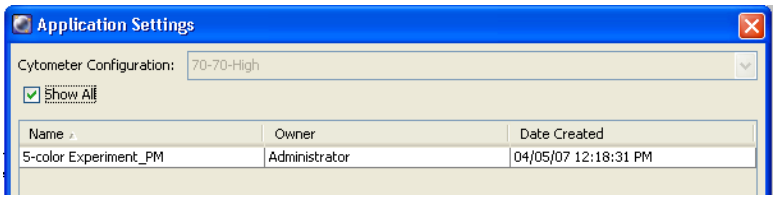
4. Click the corresponding buttons on the Workspace toolbar to display the Browser ( ), Cytometer ( ), Inspector ( ), Worksheet ( ), and Acquisition Dashboard ( ) windows, as needed.
5. (Optional) Create a folder for your experiment.

Select the icon for your database and press Ctrl+N. Rename the folder appropriately. See the *BD FACSDiva™ Software Reference Manual* for ideas on how to organize experiments.

Note: To place an experiment inside a folder, select the folder *before* you create the experiment.

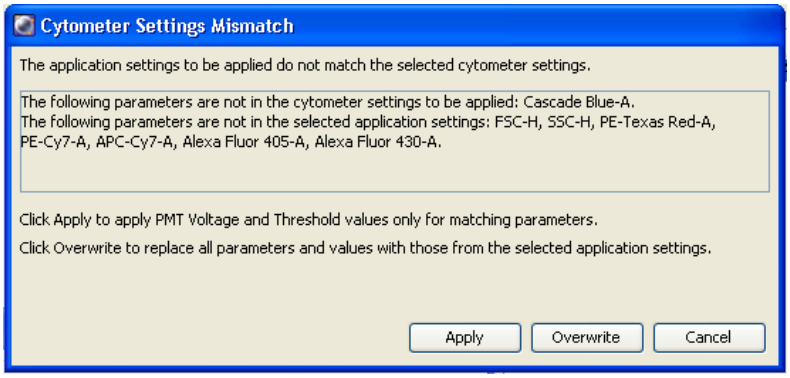
6. Create a new experiment.
7. Rename the experiment appropriately (for example, *4-Color experiment)*.
8. Create the following plots for doublet discrimination in the global worksheet:
   a. FSC-A vs SSC-A, containing a scatter gate
   b. FSC-A vs FSC-H, displaying events from the scatter gate, with an FSC gate drawn on the plot
   c. SSC-A vs SSC-H, displaying events from the FSC gate, with an SSC gate drawn on the plot

   We recommend that you perform doublet discrimination in order to analyze and sort only singlet events. A procedure is given in Recording data (page 113).
9. Right-click the experiment level **Cytometer Settings** in the Browser and select **Apply Application Settings**.



   See Application settings (page 103) for instructions on creating application settings.
10. Select the application setting for your sample and click **Apply**.

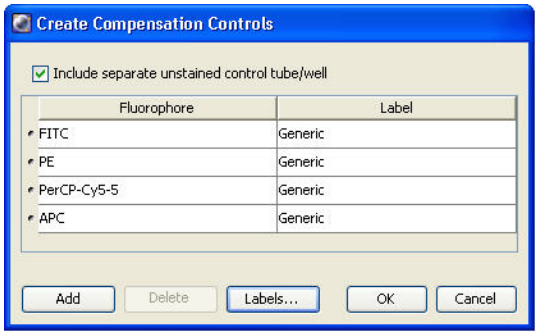
    The following figure shows a typical error message if there are any mismatches between the application and cytometer settings.



11. Select **Experiment > Compensation Setup > Create Compensation Controls**.

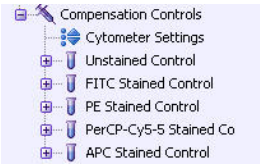
    The Create Compensation Controls dialog opens, listing only those parameters associated with the application settings.
12. Click **OK** to add the specified controls.

    Alternatively, add and define label-specific controls, then click **OK**.



Add label-specific controls when your experiment contains samples stained with the same fluorophore conjugated to different antibodies (labels) that require different compensation values. This is especially useful with tandem conjugates due to lot-to-lot variation. See the *BD FACSDiva™ Software Reference Manual* for more information about this function.

A compensation specimen is added to the experiment, along with a stained control tube for each compensation control. (Expand the specimen to view all tubes.) Worksheets containing appropriate plots are added for each compensation tube.



Calculating compensation

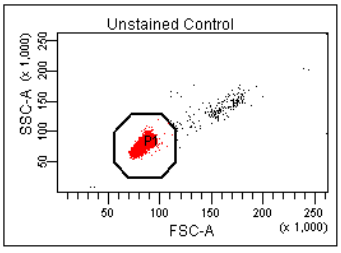
The unstained control will be used to verify the settings for FSC, SSC, and FSC threshold, and to gate the population of interest.

1. Confirm the lasers are in the Ready state using the laser control software.
2. Install the unstained control tube onto the cytometer.
3. Expand the compensation specimen in the **Browser**.
4. Set the current tube pointer to the unstained control tube and click **Load**.

Movement of mechanical parts within the instrument can pinch or injure your hands or fingers. Keep your hands and clothing away from the loading port when a tube is loading or unloading. Do not place objects underneath the loading port.

5. Verify that the population of interest is displayed appropriately on the FSC vs SSC plot. Adjust if needed.

   Since the application settings have already been optimized for your sample, the cytometer settings should require little or no adjustment (unless you are using BD® CompBead particles).



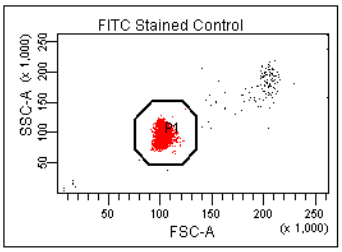
6. Click the **Threshold** tab and adjust the FSC threshold, if needed.

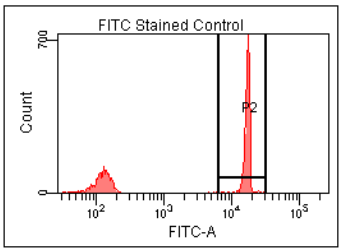
   Set the threshold to remove most of the debris without cutting off the singlet population.

7. Adjust the P1 gate to surround only the singlets.
8. Right-click the P1 gate and select **Apply to All Compensation Controls**.
   The P1 gate on each stained control worksheet is updated with your changes.
9. Click **Record Data**.
10. When recording is finished, click **Unload** and remove the unstained control tube from the cytometer.

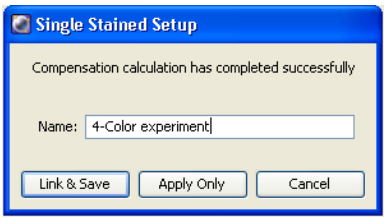
Do not change the PMT voltages after the first compensation control has been recorded. In order to calculate compensation, all controls must be recorded with the same PMT voltage settings. If you need to adjust the PMT voltage for a subsequent compensation control, you will need to record all compensation controls again.

11. Install the next tube onto the cytometer and repeat steps 9 and 10 until data for all stained control tubes has been recorded.
12. Double-click the first stained control tube to display the corresponding worksheet.
13. Verify that the snap-to interval gate encompasses the positive population. Adjust the gate, if needed.





14. Repeat steps 12 and 13 for the remaining compensation tubes.
15. Select **Experiment > Compensation Setup > Calculate Compensation**.
16. Enter the name of your experiment as the setup name, then click **Link & Save**.



To keep track of compensation setups, include the experiment name, date, or both in the setup name.

Note: We recommend that you always visually and statistically inspect automatically calculated overlap values. The means of the positive controls should be aligned with the means of the negative.

Data recording and analysis

Once you have optimized the cytometer electronics for your sample type, you are ready to record and analyze data.

Before you record data, we recommend that you gate out doublets in order to record only singlet events.

During analysis, recorded data is displayed in plots, and gates are used to define populations of interest. BD FACSDiva™ software analyzes the gated data and calculates statistics that you can print or export. With global worksheets, data can be shown for a series of tubes on the same worksheet, manually or in an automated batch analysis.

The following sections describe how to use BD FACSDiva™ software features to record and analyze sample data.

Setting up the experiment

Before you record data, set up an experiment with appropriate tubes, plots, and labels for your assay. This section describes how to add Browser and worksheet elements to the experiment that was started in the previous section, Data collection (page 108).

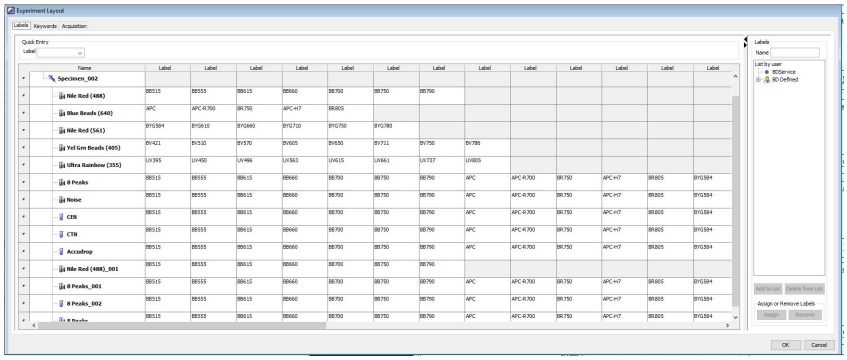
1. Rename **Specimen_001** to a descriptive name.
2. Rename **Tube_001** to *4-color_001*.
3. Set the current tube pointer to the **4-color_001** tube.
4. Click **Next Tube** in the **Acquisition Dashboard** to duplicate the first tube with the name *4-color_002*.
5. Use the experiment layout to specify the number of events to record for each tube.

   The experiment layout can be used to define the events to record as well as parameter labels for all tubes in an experiment. Labels are displayed on the plot axes and in all statistics views.

   a. Select **Experiment > Experiment Layout**.

   b. In the **Acquisition** tab, select the events to record field for all specimen tubes, and select or enter *5,000* events.

   c. Click **OK**.



Setting up the global worksheet

A global worksheet is used to perform doublet discrimination and to set up plots to preview and record data.

1. Click the **Worksheets View** button on the **Worksheet** toolbar.

   The global worksheet is shown.

   The second and third plots are set up to display gated data from the first and second plots, respectively, as shown in the population hierarchy.

2. Create two dot plots for previewing and recording data.

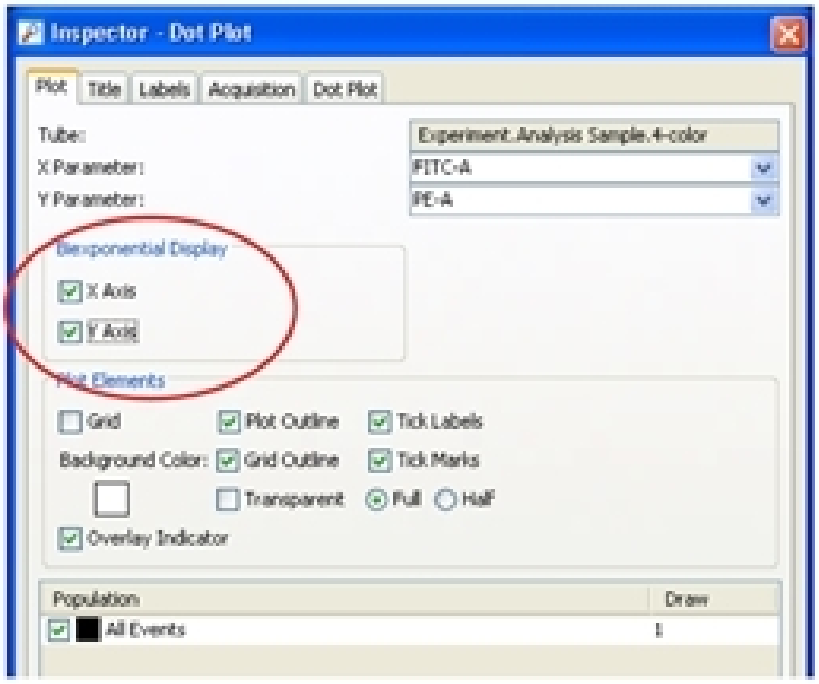
   For this example, create a FITC vs PE plot and an APC vs PerCP-Cy5.5 plot.

   Note: Double-click the **Plot** button to keep it selected until you create all plots. Click any other button to undo the selection.

3. Turn on biexponential display.

   a. Select the two plots.

   b. In the **Inspector**, select the checkbox for **X Axis** and **Y Axis** under **Biexponential Display**.



4. Set up the fluorescence plots to display data from the SSC gate.

   The SSC gate defines singlet events. By gating the fluorescence plots through this population, only singlet events will be used for analysis and sorting.

   a. Select the two plots.

   b. In the **Inspector**, select the **SSC Gate** checkbox.

5. Arrange the fluorescence plots so they fill the page vertically.

Recording data

This section describes how to adjust the gates to eliminate doublets and record singlet events.

1. Confirm the lasers are in the Ready state using the laser control software.
2. Move the current tube pointer to the **4-color_001** tube.
3. Install the first mixed sample tube onto the loading port and click **Load**.

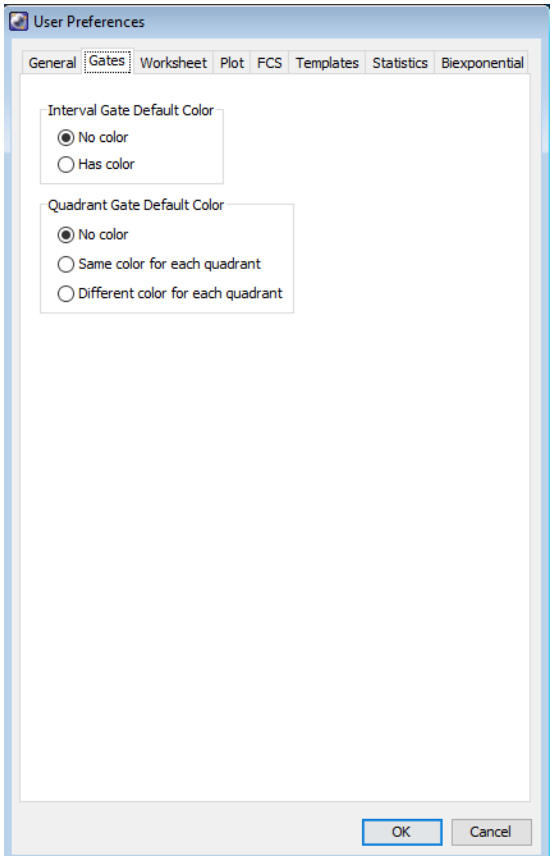
   Movement of mechanical parts within the instrument can pinch or injure your hands or fingers. Keep your hands and clothing away from the loading port when a tube is loading or unloading. Do not place objects underneath the loading port.

4. Change the **Events to Display** to 5,000 events.
5. Adjust the scatter gate to encompass the events of interest.
6. Adjust the FSC gate to encompass the low FSC-H population.
7. Adjust the SSC gate to encompass the low SSC-H population.
8. Click **Record** in the **Acquisition Dashboard** to record data.
9. When all events have been recorded, click **Unload** and remove the tube from the cytometer.
10. Install the next tube, then click **Next Tube** in the **Acquisition Dashboard** to move the current tube pointer to the next tube.
11. Repeat steps 7 through 9 for the remaining tubes.

Analyzing data

This section describes how to set up plots, gates, and a statistics view to analyze the recorded data.

1. Select **Edit > User Preferences**.
2. In the **Gates** tab, set the preferences as follows.



3. Create the following gates:
   - Quadrant gate on the FITC vs PE plot.
   - Interval gate on the APC vs PerCP-Cy5.5 plot to capture the APC beads.
   - Rectangle gate on the APC vs PerCP-Cy5.5 plot to capture the PerCP-Cy5.5 beads.
4. Rename each population in the population hierarchy.

   Note: Press the Enter key twice to move to the next population without using the mouse.
5. Right-click either fluorescence plot and select **Create Statistics View**.

   A statistics view is added to the worksheet.
6. Right-click the statistics view and select **Edit Statistics View**.
7. Edit the statistics view as follows:
   - In the **Header** tab, select the **Use 2 columns for display** checkbox.
   - In the **Populations** tab, clear the checkboxes for all populations except FITC, PE, PerCP-Cy5.5, and APC.
   - In the **Statistics** tab, select the **Mean** checkboxes for the fluorescence-A parameters.
8. Resize the statistics view so it fits on the page.
9. (Optional) Print the analysis.

Performing a batch analysis

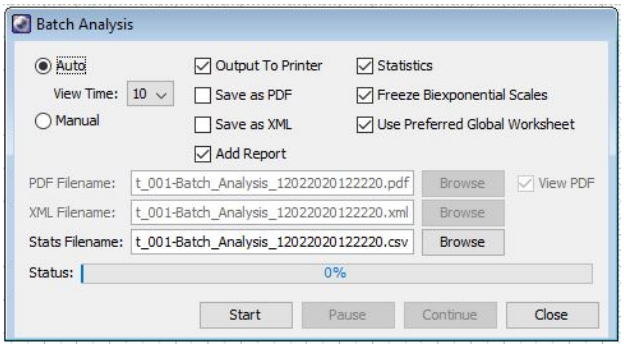
Batch analysis allows you to automatically advance through a selected set of tube data when using a global worksheet.

To perform a batch analysis:

1. Verify that the global worksheet you will be using for analysis is displayed in the worksheet window.
2. Right-click the specimen to analyze in the **Browser** and select **Batch Analysis**.

   The Batch Analysis dialog opens.

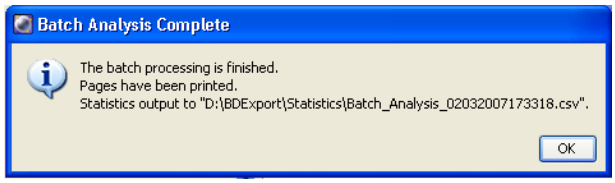
   Only tubes under the selected specimen will be processed. Tubes without data are skipped during a batch analysis.
3. Do the following in the **Batch Analysis** dialog.



   - Select **Auto** to analyze all the files without user intervention.
   - Select **5** from the **View Time** menu to pause the analysis for 5 seconds after each tube is loaded.
   - Select the **Output to Printer** checkbox to print a copy of the analysis after the data for each tube is loaded.
   - Select the **Statistics** checkbox to export the statistics to a separate file, then enter a name for the statistics file. By default, the file is saved at D:\BDExport\Statistics.
   - Select the **Freeze Biexponential Scales** checkbox to process all files with the same biexponential scales.
   - Clear the **Use Preferred Global Worksheet** checkbox to display analyses of tubes within the same global worksheets. This option is useful for analyzing panels that require a separate global worksheet for each tube.

   See the BD FACSDiva™ Software Reference Manual for more information on batch analysis.
4. Click **Start** to begin the analysis.

   When the analysis is finished, a completion dialog opens.



Manual adjustment of laser delay

Laser delay is automatically adjusted during performance checks. However, you may want to adjust laser delay manually, for example, when changing sheath pressure.

The laser-delay factor in BD FACSDiva™ software is used to realign the signals so they can be measured and displayed on the same time scale. Signals are aligned with respect to the blue laser, so the blue laser will have a 0 delay value. The delay values for other lasers will be negative or positive depending on their order in relation to the blue laser.

To manually adjust the delay:

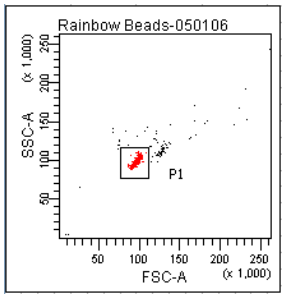
1. Select your experiment folder in the **Browser** and create a new experiment.
2. Rename the experiment *Laser Delay*.
3. Create a specimen and a tube in the experiment and set the current tube pointer next to the tube.
4. On the global worksheet, create a plot for FSC vs SSC and then histograms for each laser.
5. Create a P1 gate on the FSC vs SSC plot and change the histograms to view P1.
6. Prepare a tube of Spherotech™ Rainbow beads.

BD FACSFlow™ Solution	Spherotech Rainbow Particles (3.0–3.4 µm)	Ordering Info
1 mL	2 to 3 drops	Cat. No. 556291

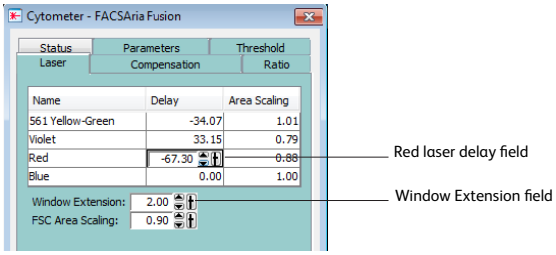
7. Load the tube of Spherotech Rainbow beads and click **Load**.

Movement of mechanical parts within the instrument can pinch or injure your hands or fingers. Keep your hands and clothing away from the loading port when a tube is loading or unloading. Do not place objects underneath the loading port.

8. Place the population on scale in the FSC vs SSC plot.
9. Adjust P1 to encompass the singlet population, if needed.



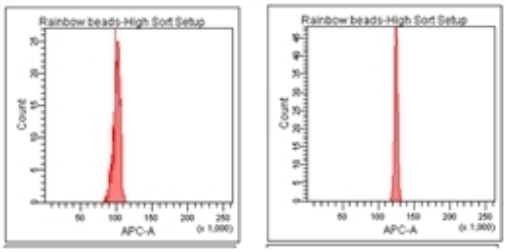
a. Optimize the delay setting for the red laser.



b. Click the **Laser** tab in the **Cytometer** window.

c. While watching the peak in the APC-A histogram, change the window extension to 0.
   - If the APC-A signal stayed the same or had little change, then go to step d.
   - If the APC-A signal decreased, then follow steps c through d.

d. Adjust the laser-delay value until the maximum APC-A signal is achieved.



e. Reset the window extension to the appropriate setting (typically 2).

10. Repeat step 9 for each laser, using the appropriate fluorochrome for each laser.
11. (Optional) To return to the laser delay values determined by CS&T, right-click the cytometer settings in the **Browser** and select **Apply CST Settings**.
12. (Optional) To save this worksheet for future use, export it as an analysis template.
    a. Right-click the global worksheet in the **Browser**.
    b. Select **Export > Analysis Template**.
    c. Name the template *Laser Delay Plots* and click **Save**.

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