invitrogen

BIGFOOT SPECTRAL CELL SORTER

User Guide
Rev. F
DM00110
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ThermoFisher SCIENTIFIC

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Chapter 1: Introduction and Safety

Cell Sorting Introduction

Flow cytometry is the characterization and measurement of cells and their properties. Additionally, cell sorting provides the user with the ability to separate specific cells within the sample for further use or study. Samples are aspirated in a single-cell suspension and hydrodynamically focused in a stream as they pass through spatially separated laser beams. Scattered and fluorescent light is collected as cells pass through each interrogation point. The light is then split into different wavelengths and presented to light sensitive detectors. Electronic signals are then correlated for each cell and displayed within the software. Gates and regions can be set to analyze the data produced. These regions can also be defined as sorting criteria to separate single cells into tubes or microtiter plates at high rates of speed.

Bigfoot Cell Sorter Introduction

The Bigfoot Cell Sorter takes cell sorting to the next level of performance with up to nine lasers and 60 detectors, event rates up to 100,000 per second and spectral data handling. The instrument allows up to 6-way sorting into tubes and 4-way sorting into microtiter plates with well-integrated aerosol management and biosafety containment.

Free-space excitation of up to nine lasers (ranging from 349-785nm) makes possible the widest set of applications currently available in a cell sorter. Extra parameters such as small particle detection and depolarized light detection allow for resolution of novel populations. A multi-tube sample loader with built in agitation and temperature control, efficiently delivers cells from a set of samples or controls to compliment the highly polychromatic analysis and sorting capability from the acquisition system.

All acquisition and sorting occur within an integrated biosafety enclosure with an additional aerosol management system (AMS) to maintain safety for both the sample and the operator. Cell sorting modes from ultra-high-speed with high-yield, to multi-way-single-cell deposition, are provided by a modular cell sorting and collection system with integrated temperature control.

The Bigfoot Spectral Cell Sorter configuration provides spectral data unmixing in addition to standard compensation for the removal of fluorophore emission overlap. Spectral unmixing allows the separation of spectral signatures from cell populations. The subsequent data can then be used to set regions required to sort the populations of interest.

Please review all documentation and safety notices before using the instrument.

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System Support

For service and support, please contact the manufacturer at:
345 East Mountain Ave
Fort Collins, CO 80524 USA
+1 970 295 4570
http://www.thermofisher.com

Maintenance and repairs should be carried out only by authorized service personnel.
Please contact technical support for troubleshooting or service needs.

Safety Information

Please review the safety information shown below before operating the Bigfoot. If the Bigfoot system is used in a manner not specified by the manufacturer in this manual, the protection provided by the equipment may be impaired.

Safety Symbols:

	**Risk of Danger!** This symbol draws attention to possible damage to instruments or instrument parts if the associated directions are not followed correctly.
	**Shock Hazard!** This symbol draws attention to a possible injury or danger to life if the associated directions are not followed correctly.
	**Laser Hazard!** Laser irradiation can be hazardous. Please do not remove system covers as they are in place for your safety. Only trained personnel should access the exposed laser beam.
	**Biohazard!** Biosafety is of utmost importance while operating this instrument. Consult with your local safety officer or review local state and federal regulations to ensure proper handling and disposal of biohazardous substances.

Safe Use Specifications and Compliance

The Bigfoot System is designed to operate safely when used in the manner prescribed by the manufacturer. If the Bigfoot System or any associated component is used in a manner other than prescribed, or if modifications to the instrument are not performed by an authorized agent, then the warranty on the system will be void and the protection provided by the equipment might be impaired. Service of the Bigfoot System should be performed only by Thermo Fisher personnel.

This instrument must be operated in accordance with the information provided in these instructions for use.

	**Caution:** If the equipment is used in a manner not specified by the manufacturer, the protection provided by the equipment may be impaired.

 	Only qualified, trained technicians should carry out service work on electronic components due to potential shock hazard. Electronic components are sensitive to electrostatic charges and can be destroyed by a discharge. **Caution:** Use caution when opening and closing the following mechanisms to avoid pinching: biosafety enclosure sash, bulk fluids door, detection door, storage cabinet, sample line cover and nozzle door.

Where motorized systems are utilized in areas such as sort door, output stage, nozzle mover and sample loader the motions are all designed for safety. This involves multiple sensors as well as limiting the forces available. When physically challenged, the motorized assembly will either stop or stop and reverse its movement to avoid any possible pinch injury.

Laser Product Hazard Classification

The intent of the laser hazard classification is to provide clear distinction of the laser, or laser product properties and the hazards to users so that appropriate protective measures can be taken. Bigfoot is a Class 1 laser product that complies with 21 CFR 1040.10 and 1040.11 except for deviations pursuant to Laser Notice No. 50, dated June 24, 2007; meaning operators are not exposed to harmful levels of laser radiation during normal operation, maintenance and/or service. During times of repair and/or major service by a trained technician, laser safety controls for Class 3B must be followed.

	**Warning!** Use of controls, adjustments, or performance of procedures other than those specified herein may result in hazardous laser radiation exposure.

Laser Safety Information

There are four to nine laser sources in the Bigfoot system for the purpose of sample excitation. These lasers vary in wavelength from the UV (349nm) through visible and into the IR range. In addition, the automated drop delay module uses an additional 405nm laser.

Wavelength	Maximum Power (CW)	Location of Labels
349nm	110mW	Optics Plate + Omala Cover
405nm	110mW	Optics Plate + Omala Cover
445nm	220mW	Optics Plate + Omala Cover
488nm	138mW	Optics Plate + Omala Cover
532nm	110mW	Optics Plate + Omala Cover
561nm	138mW	Optics Plate + Omala Cover
594nm	110mW	Optics Plate + Omala Cover
637nm	110mW	Optics Plate + Omala Cover
785nm	110mW	Optics Plate + Omala Cover
405nm	5mW	Drop Delay Module

Figure 1. Optics Plate Laser Safety Covers

Figure 2. Optics Plate under Laser Safety Covers

Figure 3. Drop Delay Module

Nozzle Area Interrogation Point

Under normal operating conditions the instrument protects the user from exposure to laser radiation because the lasers are fully blocked from the user through multiple layers of doors, enclosures and interlocks. Therefore, the Bigfoot is considered a Class 1 laser product. For safe user maintenance, the instrument is equipped with safety interlocks designed to protect the operator from inadvertent exposure to laser radiation. These multiple, redundant interlocks ensure that lasers are disabled prior to user access to the interrogation area for cleaning. In addition, the nozzle can move to the front of the instrument for a more convenient location for the user to access the tip and nozzle.

Figure 4. Behind the Nozzle Access Door, is the Nozzle and Laser Interrogation Door

For the lasers to turn on and enter the interrogation region, the three interlocks must be in their safe state with the green indicator present:

(1) Nozzle Access Door closed all the way to the right
(2) Nozzle is in chamber and in the down position
(3) Laser Interrogation Door closed and latched

When the above conditions are met, the System Health status for the interlocks displays green. Likewise, if any of the conditions are not met, the system health issues a warning and system status turns orange to indicate the lasers are not reaching the sample. The orange block next to the interlock label indicates which of the interlocks needs to be corrected.

Figure 5. System Health, interlocks status green = safe, interlock status orange = not engaged

Drop Delay Module

A 405nm laser is utilized to illuminate micro beads as they pass through the drop delay module. The drop delay module is connected by fluid tubing from the waste collector assembly. This module determines the number of beads that pass through per the delay time to determine the optimum delay time for proper droplet charging.

The drop delay module is mounted inside the instrument. There are no operator accessible parts in the drop delay module. This user guide provides no instructions on how to interact with the internal parts of the drop delay module. Access to the internal parts of the drop delay module, including its laser, is only for trained service personnel.

Mechanical Safety Light Curtain

The Bigfoot system includes an LED light curtain to protect the user from possible mechanical injury while the probe is in motion. When the system is in a safe state during acquisition, the three LEDs will appear green. When an object crosses the light curtain during acquisition and the system is not in a safe state, the hardware stops, the touch panel flashes red, the affected LEDs change to red and a software warning appears on screen. The user must clear the affected area of obstruction, ensure the three LEDs turn green and click **Soft Hardware Reset**. After the system resets the hardware, the user must click **Exit**.

Figure 6. Light Curtain in Safe State and Light Curtain Breached

Electrical Classification

⚠	The Bigfoot system conforms to international regulations encompassing the accessibility of high voltages by the user (EN61010-1). Use all protective housings and shields as identified in this manual. Further information about specific electrical hazards is listed in the hardware description.

AC Fuse Requirements

Remove the power cord before replacing fuses.

⚠	Fuses are 5 mm x 20 mm and must be rated to 250VAC, 10A Slow Blow with a minimum 1.5kA interrupt rating, such as Eaton Electronics BK/S505-10-R.

AC Power Cord Requirements

Power cord must be IEC 60320-1 compliant with a C13 plug on the instrument end. When using the optional UPS, the power cord to the UPS must also be IEC 60320-1 compliant with a C19 plug on the UPS end. If either power cord needs to be replaced, replace it with an NRTL approved power cord, suitable for the application and acceptable for installation per the applicable National Electrical Code requirements. Use of an unapproved, or underrated cords should not be used with the equipment as they will increase the risk of fire.

The unit should be positioned such that it is not difficult to disconnect the power cord from the PDU or the wall outlet to which it attached.

Electrical Safety Information

Under normal operating conditions the instrument protects the user from exposure to any electrical hazard. The instrument is equipped with safety interlocks designed to protect the operator from inadvertent exposure to high voltage. The sort deflection plates are a potential source of high voltage exposure. These plates enable the system to separate charged droplets that are formed during the normal sorting process. For access to the sort collection area where the plates are exposed there is an electrical interlock. This is operated by the access door to the sort area.

Figure 7. Sort door and nozzle access door closed

The large sort door must be closed and the interlock must be engaged before the system will apply high voltage to the deflection plates. The user is informed in several of the status areas of the instrument that the Sort Output Interlock is open, such as the top of the screen in SQ Software.

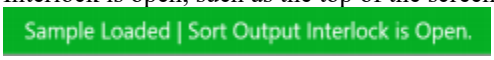


Figure 7. SQ Software status message

Biosafety Information

The Bigfoot system has a fully integrated biosafety containment area with a separate Aerosol Management System (AMS). For detailed information see the Integrated Aerosol Containment section of this publication. If biohazardous samples are present, adhere to the following guidelines and comply with any local guidelines specific to your laboratory and location.

	A responsible body at the customer site must ensure that appropriate decontamination is carried out if hazardous material is spilled onto or into the equipment. They must ensure that no decontamination or cleaning agents are used which could cause a hazard as a result of a reaction with parts of the equipment or with material contained in it. The manufacturer or its agent must be consulted if there is any doubt about the compatibility of decontamination or cleaning agents with parts of the equipment or with material contained in it.

General Precautions

- Always wear laboratory gloves, coats and safety glasses with side shields or goggles.
- Keep your hands away from your mouth, nose and eyes.
- Completely protect any cut or abrasion before working with potentially infectious materials.
- Wash your hands thoroughly with soap and water after working with any potentially infectious material before leaving the laboratory.
- Remove wristwatches and jewelry before working at the bench.
- Store all infectious or potentially infectious material in unbreakable leak-proof containers.
- Before leaving the laboratory, remove protective clothing.
- Do not use a gloved hand to write, answer the telephone, turn on a light switch, or touch anything that other people may touch without gloves.
- Change gloves frequently. Remove gloves immediately when they are visibly contaminated.
- Do not expose materials that cannot be properly decontaminated to potentially infectious material.
- Upon completion of the operation involving bio-hazardous material, decontaminate the work area with an appropriate disinfectant.

Disposal of Biohazardous Material

The Bigfoot includes waste containers that may contain hazardous biological materials, depending on the sample(s) used. Dispose of the following potentially contaminated materials in accordance with laboratory, local, regional and national regulations:

- Content in waste containers
- Reagents
- Used reaction vessels or other consumables that may be contaminated.

Chemical Hazards

The Bigfoot system includes waste containers and cleaner bottles that may potentially contain hazardous chemical materials, depending on the sample(s) used. Handle and dispose of the materials in accordance with laboratory, local, regional, and national regulations.

We recommend the use of a cleaner, such as Coulter Clenz®, in the cleaner bottle. In the decontamination bottle, we recommend Oxivir® Five 16 Concentrate diluted with DI water (1:16), or Oxivir® Plus diluted with DI water (1:40). Both dilutions are per the manufacturer recommendation. While the Bigfoot materials are compatible with other chemicals, such as 70% ethanol, bulk use in the cleaner or decontamination bottles of flammable liquids is specifically prohibited.

Maintenance

Preventative maintenance should be performed by Thermo Fisher personnel every 12 months. Regular user maintenance is not required other than regular cleaning, however, if the system has been unused for two weeks, you should start up and then shut it down to keep it in good operating condition.

Cleaning

The laboratory manager must determine the appropriate cleaning protocol depending on instrument usage. The following schedule can be used as a guideline.

Daily Cleaning

Wipe all surfaces with 70 percent Ethanol using a lint-free cloth to remove visible debris. Fill a sample tube of your laboratory's preferred cleaner in position one on the loader. Access the Maintenance tab in SQ software and click **High Pressure Wash**. Perform instrument shutdown with either Clean, Decon, or Both enabled in Global Settings depending on laboratory needs.

Weekly or Monthly Cleaning

The laboratory manager will determine whether to perform this protocol every week or once per month based on instrument usage. Wipe all surfaces with 70 percent Ethanol using a lint-free cloth to remove visible debris. Access the Maintenance tab in SQ software and click **Decon** to run an hour-long decontamination procedure. After the decontamination procedure has run and instrument shutdown is complete, clean the streams window components, sort plates and waste catcher as directed by the on-screen instructions.

Biannual or Yearly Cleaning

The laboratory manager will determine whether to perform this protocol twice per year or once per year based on instrument usage. Wipe all surfaces with 70 percent Ethanol using a lint-free cloth to remove visible debris. Perform instrument shutdown. Click **Full Fluidics Decon** and follow the on-screen instructions.

Installation Requirements

The following tables list the specifications for the instrument and the environmental requirements for installation. The equipment must be properly connected to ground prior to use.

Dimensions and Weights

Component	Height	Length (depth)	Width	Weight
Bigfoot Cell Sorter	178 cm (70 in.)	99 cm (39 in.)	99 cm (39 in.) instrument only 205 cm (81 in.) configured system with monitor, keyboard and work tray.	Approximately 680 kg (~1500 lbs) Weight per castor 170 kg (~375 lbs)

Environmental Requirements

Condition	Acceptable range
Installation site	Dry, indoor use only
Electromagnetic interference	Do not use this device in close proximity to sources of strong electromagnetic radiation (for example, unshielded intentional RF sources). Strong electromagnetic radiation may interfere with the proper operation of the device.
Altitude	Located between sea level and 2000 m (6500 ft.) above sea level
Humidity (operation and storage)	60% maximum
Temperature (instrument and computer)	18°C to 25°C (64°F to 77°F) Note: The room temperature must not fluctuate more than 2°C over a 2-hour period.
Transient category	Installation category II
Overvoltage category	Installation category II
Vibration	The instrument is not adjacent to strong vibration sources, such as a centrifuge, pump, or compressor. Excessive vibration will affect instrument performance.
Pollution degree	II Install the instrument in an environment that has nonconductive pollutants such as dust particles or wood chips. Typical environments with a Pollution Degree II rating are laboratories and sales and commercial areas.
Liquid waste collection	Dispose of the polymer, buffer, reagents and any liquid waste as hazardous waste in compliance with local and national regulations.
Maximum sound emitted	60.1 dB
Voltages	100-240V~, 50/60Hz, 800W
Other conditions	Ensure the room is away from any vents that could expel particulate material on the components. Avoid placing the instrument and computer adjacent to heaters, cooling ducts, or in direct sunlight.

Optional Fluidic Connection Requirements – External Deionized Water

	Use of house deionized water with the Bigfoot Spectral Cell Sorter is optional. Only qualified, trained technicians are permitted to install and connect the External Deionized Water Supply kit.

Regulatory Compliance

This instrument has been tested and found to comply with all applicable requirements of the following safety and electromagnetic standards:

- IEC 61010-1:2010 (3rd Ed), EN61010-1:2010 (3rd Ed). Electrical Equipment for Measurement, Control and Laboratory Use - Part 1: General Requirements
- UL/CSA 61010-1:2012 (3rd Ed), Standard for Safety Electrical Equipment for Electrical Safety (USA, Canada, NRTL)
- IEC 61010-2-081:2015, EN61010-2-081:2015. Safety requirements for electrical equipment for measurement, control and laboratory use. Part 2-081: Particular requirements for automatic and semiautomatic laboratory equipment for analysis and other purposes (includes Amendment 1)
- IEC 60825-1:2014, EN 60825-1:2014. Safety of laser products - Part 1: Equipment classification and requirements
  Class 1 laser product per IEC 60825-1 and CDRH requirements and regulations EN 61326-1:2013 (Class A) Electrical equipment for measurement, control and laboratory use. EMC requirements, Part 1: General requirements
- IEC 61326-1:2012 (Class A) Electrical equipment for measurement, control and laboratory use. EMC requirements - Part 1: General requirements
- FCC Part 15 Subpart B Emissions (Class A)
- EN55011 (Class A)
- KN11 (Class A)
- This ISM device complies with Canadian ICES-001

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 their own expense.

Transport

Moving the Bigfoot system is not recommended after installation. If the system must be moved, it is highly recommended to contact the manufacturer for assistance to ensure the warranty will not be voided. Prior to moving, follow the decontamination procedure in this guide and remove all bulk fluidics. After a move, you must run the QC process to ensure that the instrument is functioning properly.

	A minimum of two people should move the Bigfoot and shall move it only using the wheeled cart attached to the instrument base. Use caution to keep instrument level and handle it gently. The Bigfoot shall only be moved and installed by trained personnel.

Operating Conditions

The Bigfoot Cell sorter must be operated under the following conditions:

- Temperature range 18–25°C (+/-2°C from setpoint)
- Relative humidity 20–60%

Disposal

The Bigfoot system contains electronic or electrical materials; they should be disposed of as unsorted waste and must be collected separately, according to European Union Directive 2002/96/CE on waste and electronic equipment — WEEE Directive. Before disposal, contact your local representative or safety officer for country-specific instructions.

Warranty

The Bigfoot system and associated accessories are covered by a standard manufacturer warranty. Contact your local office for details of the warranty.

Chapter 2: Hardware Description

System Summary

This chapter gives an overview of the hardware of the Bigfoot system. Understanding the system's hardware is essential for proper operation.

Figure 8. Bigfoot Cell Sorter

1. System Storage	6. Optical Filters
2. Nozzle Area	7. System Monitor showing SQS Software
3. Sort Collection Area	8. Keyboard and Mouse
4. Sample Loader	9. User Worktable
5. Bulk Fluids Cabinet	

⚠	A power cord and a communication cable located at the back of the instrument could pose a tripping hazard. Use caution when walking behind the instrument. In addition to the moving parts in the sample input area, there are three doors which require regular access. Use caution to avoid pinching when interacting with these areas.

Subsystem Overview

The Bigfoot system consists of optics, fluidics, electronics and SQ software. These subsystems are described below.

Fluidics Overview

The Bigfoot utilizes pressurized sheath fluid to deliver sample through a nozzle and past laser beams for excitation, detection and particle sorting. The sorted particles are collected as sorted samples or by the waste collection system.

Sheath and Sample Pressure

Pressurized, filtered air is generated by an internal air pump and stored for system usage. The air then enters the active sheath tank and is pressurized to the correct sheath pressure. This two-stage pressurization system allows Bigfoot to achieve the range of sheath pressure required for small and large nozzle sizes as well as allowing fine control to maintain a stable droplet breakoff. Sheath pressure is maintained with the sample air pressure utilizing a differential pressure gauge allowing controlled sample delivery regardless of sheath pressure. The sheath fluid is delivered to the nozzle via a valve which can be selected by the SQ Software to deliver other fluids such as DI water or cleaner directly to the nozzle for shutdown and cleaning purposes.
Unlike sheath pressure, which is maintained over long periods of time at the same value; sample pressure must react quickly as each sample is loaded and unloaded. On the Bigfoot, moving from one sample to the next takes approximately 20 seconds.

Waste Collection

Waste is collected from the waste trough as well as other points on the system such as the probe wash station and delivered to a centralized waste tank and then delivered to one of two external waste tanks that must be emptied on a regular basis.

Bulk Fluids Tanks

The fluidics system supplies sheath/DI water, cleaner, sheath, decontamination solution and sample to the nozzle, allows for cell sorting and sends waste for disposal. Sheath fluid is typically 1x saline.

Five 4L and two 1L bottles are in the bulk fluids area of the system. Two large bottles are filled with sheath fluid and have white caps. One large bottle is filled with DI water and has a blue cap. Two large bottles collect waste and have red caps. A small decontamination solution bottle with a white cap and a small system cleaner bottle with a blue cap are in the top right of the cabinet. The DI and waste tanks that are in use while the system is running are illuminated.

Figure 9. Bulk Fluids

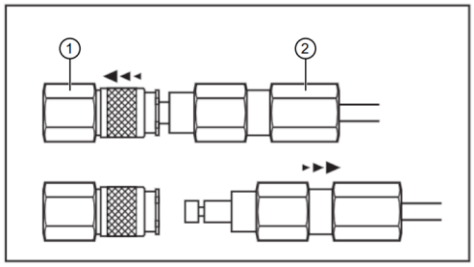
1. Waste Bottle	5. DI Water Bottle
2. Sheath Bottle	6. Sheath bottle (in use)
3. System Decontamination Bottle	7. Waste bottle (in use)
4. System Cleaner Bottle	

Fluidic Connections

The fluidics connections are accessed through the bulk fluidics door and are depicted on page 18. Each waste tank has a set of two connections. One connector allows waste fluid to pass into the tank while the other allows air flow out of the tank when it is displaced. Each DI tank has one connection to the instrument. The bottles that are in use when the system is running, are illuminated in blue. Unused bottles are unlit and may be removed for maintenance. Additionally, the DI water bottle, has one connection to the instrument. The decontamination solution bottle and system cleaner bottle are located at the top right of the fluidics area, and each have one connection to the system.

Disconnecting Bottles

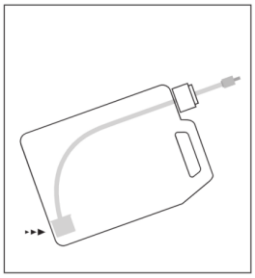
1. To remove bottles, push the sleeve behind the system-side connector toward the instrument to disengage the tubing.
2. Pull the bottle out of the cabinet.



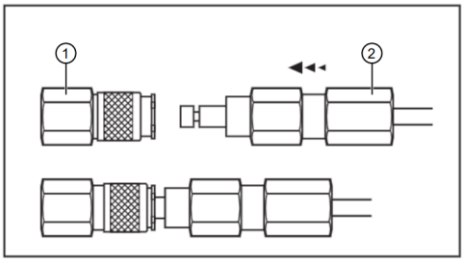
1.	System-side connector
2.	Bottle-side connector

Connecting Bottles

1. Ensure the bottle cap is securely connected.
2. If the cap assembly includes an interior weight, ensure that it is facing down.



3. Slide the bottle into the appropriate compartment in the fluidics cabinet.
4. Push the bottle-side connector into the system-side connector until you hear it click.



1.	System-side connector
2.	Bottle-side connector

5. Pull on the connector to ensure it is securely attached.

DI Bottle

One four-liter bottle in the lower-right corner of the fluidics cabinet holds the deionized water (DI water) for the system. DI water is used for cleaning and rinsing the probe between runs. This container is sealed with a blue cap. The run time between refills depends on the nozzle size and pressure used on the system.

Figure 10. DI Bottle

SQ Software warns the operator one hour, 30 minutes and 5 minutes before the DI bottle is empty. SQ Software allows the operator to snooze the message and/or swap the tank. Shutdown will occur when 5 minutes of fluid remains to prevent running the system dry.

Filling DI Bottles

After disconnecting the bottle unscrew the cap and pull the tubing and filter out. Fill the tank to the fill line on the side of the bottle. The weight that is attached to the fluidics tubing must be in the bottom corner, opposite the cap to ensure full utilization of the fluid contents.

⚠	Avoid touching the interior weight and tubing surfaces. If it is necessary to set the cap assembly down, do so on a sanitized surface. When handling sheath fluid and DI water containers, minimize air exposure to help avoid contamination.


Figure 11. DI Bottle connected

Waste Bottles

The waste bottles in the left column of the fluidics cabinet receive the system fluid once it has run through the nozzle, sort module and waste lines. The two containers are distinguished by red caps and hold four liters each. The run time before maintenance depends on the nozzle size and pressure used on the system. Two bottles on a system running continuously, using a 70-micron nozzle should last for approximately 13 hours and for approximately 9.5 hours when using the 100-micron nozzle. The fluid collected in the waste bottles must be decontaminated in accordance with local state and federal biohazard waste disposal guidelines.

Figure 12. Waste Bottle

SQ Software warns the operator one hour, 30 minutes and 5 minutes before both waste bottles are full. SQ Software allows the operator to snooze the message and/or swap the tanks. If the waste bottles are not changed before they are full, the Bigfoot will automatically shut down to avoid overfilling.

Emptying Waste Bottles

Each waste bottle has two connections to the instrument. One connection is a fluidic connection, while the other is an air vent connection to ensure that air is properly displaced as waste fluid is pumped into the bottle. After disconnecting the bottle unscrew the cap and empty the bottle in accordance with your local institutional guidelines. The connectors on the bottle can be attached to either one of the ports on the instrument when reinstalling the empty waste bottle.

	Caution! These bottles may contain biohazardous material. Refer to your local institution's safety guidelines for handling biohazardous waste.


Figure 13. Waste Bottle connected

Sheath Bottles

Two sheath bottles are sealed with white caps and contain four liters each of a 1x PBS balanced salt solution without antimicrobial/antifungal agents or surfactants. This fluid enables correct droplet charging and allows the viability of sorted cells to be maintained. The bottles are in the middle column of the bulk fluids cabinet.

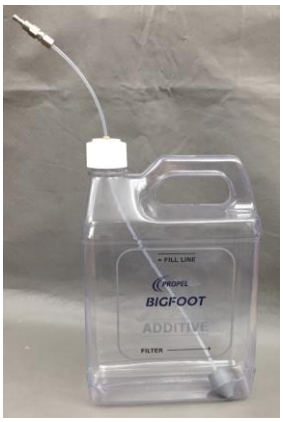


Figure 14. Sheath Concentrate Bottle

After the first pair of sheath and waste bottles has been fully utilized the system will automatically switch to the second pair. Additionally, the operator can force the system to switch to the second set of bottles by clicking the button in the maintenance tab in the control panel flex space in SQ Software.

Replacing the Sheath Bottle

After disconnecting the bottle unscrew the cap and pull the tubing and weight out. Open a new bottle of 1x sheath fluid and install the cap on the new bottle. The weight at the bottom of the fluidics line must be in the bottom corner, opposite the cap to ensure full utilization of the fluid contents.

⚠	Avoid touching the weight and tubing surfaces outside of the containers. If it is necessary to set the cap assembly down, do so on a sanitized surface. When handling sheath fluid and DI water containers, minimize air exposure to help avoid contamination.

System Cleaner Bottle

The system cleaner bottle contains cleaning reagent. During the shutdown process, cleaner is delivered to the portions of the fluidics system that are in contact with sample, such as the nozzle and sample line, to minimize sample build up over time and to preserve the integrity of the system. This can be set in global preferences in SQ Software. Additionally, cleaning cycles can be initiated from the SQ Software by the user.

Figure 15. System Cleaner Bottle

Filling the Cleaner Bottle

After disconnecting the bottle, unscrew cap and fill to the fill line on the bottle to ensure liquid does not leak from the vent in the cap. Fill the cleaner bottle with a laboratory cleaner such as Coulter Clenz® (Beckman Coulter, 8546931). This fluid must be changed regularly.

Decontamination Solution

The system decontamination bottle can be filled with AHP/DI solution for system decontamination. We recommend Oxivir® Five 16 Concentrate diluted with DI water (1:16), or Oxivir® Plus diluted with DI water (1:40). Both dilutions are per manufacturer recommendation. While the instrument materials are compatible with other chemicals, such as 70% ethanol, bulk use in the cleaner or decontamination bottles of flammable liquids is

specifically prohibited. Decontamination fluid can be used during the shutdown process to minimize sample build up over time and to preserve the integrity of the system. The sample line decontamination routine should be run daily/weekly to keep the lines free from debris and crystal formation. This can be set in global preferences in software. Additional cleaning cycles can be initiated from the software.

Figure 16. Decontamination Solution Bottle

Filling the Decontamination Bottle

After disconnecting the bottle, unscrew cap and fill the bottle to the fill line to ensure liquid does not leak from the vent in the cap. This fluid must be changed regularly.

⚠	Caution! Risk of chemical injury from AHP solution. To avoid contact with the AHP solution, use barrier protection, including protective eyewear, gloves and suitable laboratory attire. Refer to the Safety Data Sheet for details about chemical exposure before using the chemical.

Sheath and Cleaner Filters

The onboard reagents are filtered through 0.2µm capsule filters which remove particulates from the fluid before it is circulated through the system. This helps reduce data background noise, especially in the scatter channels and prevents microbes from entering a major portion of the fluidics path. These filters are mounted behind a panel on the left side of the system. Replacement of these cartridges should be performed during the regularly scheduled service visit or by a trained lab manager.

Figure 17. Filter Access Panel and Filters

1. Removable panel to access fluidic filters.	2. Panel removed to show fluidic filters.

Integrated Aerosol Containment

The Invitrogen™ Bigfoot™ Spectral Cell Sorter biosafety enclosure and AMS are designed to be fully integrated parts of the cell sorter. Sample-related subsystems are segregated inside the containment area for optimal safety, sanitation and performance. Sealed optical windows surround the nozzle, defining the barrier between the inside and outside of the containment area. This separation allows lasers, excitation optics and scatter objective lenses to remain outside the contaminated zone yet close to the interrogation point, which maintains the superior performance of a jet-in-air sorter. All other subsystems, such as detection, electronics and fluidics, are also outside the containment area. This allows better service access and temperature regulation as compared to other sorters.

The NSF49 and EN12469 standards require certification while the hood is empty, which is not the normal use case for this application of biocontainment. The Bigfoot sorter is a containment system and AMS, which can be certified while in operation to meet the safety and airflow requirements of these standards and cell sorting guidelines.

Although not an actual biosafety cabinet (BSC,) the Bigfoot Spectral Cell Sorter provides personnel and product protection similar to a Class II BSC. Test procedures and criteria laid out within NSF49 and EN12469 standards can be utilized to demonstrate performance. Specifically, this means the containment system:

- Maintains an average air velocity of 100 ft/min (NSF49) or >79 ft/min (EN12469) through the work access opening Provides high-efficiency particulate air (HEPA)–filtered downflow air that is mixed with the inflow air
- Exhausts HEPA-filtered air into either the laboratory or, via an optional canopy connection, through an external exhaust system
- Holds all biologically contaminated ducts and plenums under negative pressure

The AMS and biosafety enclosure portions of the system can be operated and are monitored independently. The system houses two independent exhaust fans and two independent HEPA filters; one fan and filter for the AMS and

one fan and filter for the containment and product protection. Pressure sensors independently monitor containment in both systems for redundant biosafety containment. Aerosols in the segregated sort chamber are entrained in air that is ducted to a HEPA filter in the AMS. The door to access the sort chamber is inside the greater enclosure. In the unlikely event hazardous aerosols leak out of the sort chamber, the greater enclosure offers a secondary biosafety system to capture aerosols. As with all Class II Type A2 cabinets, a portion of the filtered air is recirculated to deliver uniform downflow air and the remaining filtered air is exhausted to the laboratory environment, or through an external exhaust system connected to the optional cabinet canopy connection.

Product and personnel protection are achieved using H14 HEPA filters and air velocity relationships similar to a BSC. H14 HEPA filters are used with the Bigfoot sorter, which capture 99.995% of the most penetrating particle size (MPPS). A H14 HEPA filter:

- Provides 99.995% collection efficiency at the most penetrating particle size
- Has no localized collection efficiency lower than 99.975%
- When placed in local exhaust ventilation (LEV) and containment devices, has no leaks greater than 0.01% when scanned with an aerosol photometer

During a sort, the separate Bigfoot AMS continuously draws air from the sort chamber and through a HEPA filter. If a clog is detected, software notifies the operator, stops the sort, sample and sheath, and automatically increases the speed of the AMS fan. The fan quickly purges the sort chamber of aerosols before the user can access the affected area. All the while, the greater enclosure continues operating independently to maintain containment.

The Bigfoot system contains on-board cleaner and decontamination fluid reservoirs that can be used to disinfect and clean the sample line, nozzle body and adjacent tubing pathways. Combined with a low carryover sampler system, HEPA filtered downflow air with design similar to a Class II BSC and a contained sort output area with a separate AMS, the Bigfoot system provides best-effort product protection and isolation between samples. However, given the variability in sample type and concentration and varying laboratory conditions, no specific claims can be made as to the absolute system carryover or cleanroom specification inside the cabinet.

Biosafety Enclosure Certification

Periodic testing and certification of the Integrated Aerosol Containment system using test protocol DT00149 is required to assure the continuous operation of the enclosure. The recommended test interval is twelve months.
To certify the Bigfoot biosafety systems using an external certification provider you must provide the following items.

- The current revision of the testing protocol DT00149 to the certification provider for review prior to their visit.
- The Biosafety Certification Accessory Kit shown below to the certification provider when they arrive to test the instrument.

Document DT00149 guides the certification provider on how to verify the biosafety system performance and adjust the instrument if required. Document DT00149 also includes example certification forms to record the test results.

Storage

To the left of the biocontainment cabinet, is a storage area for additional loader adapters, sort output adapters, spare parts such as nozzle tips or sample lines and tubes or other supplies for system operation. The top portion of the cabinet is designed for sample loader tube adapters. Storage for spare optical filter sticks is located inside the detection door below the biocontainment cabinet.

Figure 18. Built-in storage locations

Sample Loader Overview

Cell sorters have historically had single tube input systems as opposed to autoloaders. This has been primarily due to the need for high-pressure sample delivery, continuous sample mixing and sample temperature regulation as well as large samples for long sorts. However, there is often a need to run multiple small samples such as single color and FMO controls to set up the sort. It is also important to reduce sample handling in and out of the biocontainment environment to increase both biosafety and workflow efficiency. The Bigfoot system integrates an autoloader with six sample positions for a variety of tube types, including 1.5, 5 and 15ml tubes. Each tube type has a specialized adapter to ensure each tube is held firmly and presented properly to the uptake probe. The tube holders are color coded to help the operator and the system sensors detect the tubes that are in use.

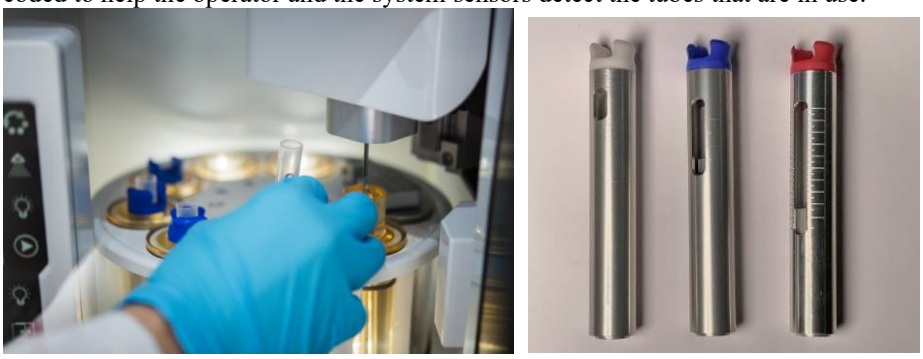


Figure 19. Sample Loader and 1.5 mL, 5 mL and 15 mL tube holders

Vortex Mixer

A vortex mixer, modulated for each sample media type, is built into the sample loader and ensures that samples are adequately mixed. Sample temperature control prior to and during acquisition, facilitates precise temperature control from 4 to 37 degrees Celsius.

QC Beads

The QC beads, a mix of three different calibration beads, are retained onboard the system in a dedicated loader position. This allows scheduled startup, QC and drop delay and allows the operator to perform QC at any time

without interrupting the workflow. The QC bead holder is automatically capped to minimize evaporation from the vial and contains an integrated vortex to keep the beads suspended.

Figure 20. QC Bead Station labeled "B" Wash Station labeled "W"

Sampling Assembly and Wash Station

To reduce sample-to-sample carryover, the loader has an integrated wash station. Between samples the probe is automatically backflushed and washed. All parts of the sampling system exposed to sample are washed thoroughly with DI water. When the sample tube or QC vial is in the sampling position, the sample probe assembly is automatically lowered into position and forms a seal onto the holder. The cell suspension is pressurized at a user determined value to begin sample flow to the nozzle. Adjusting sample pressure allows control of the sample flow rate. The sample pressure can also be automatically adjusted by the system to maintain a selected event rate.

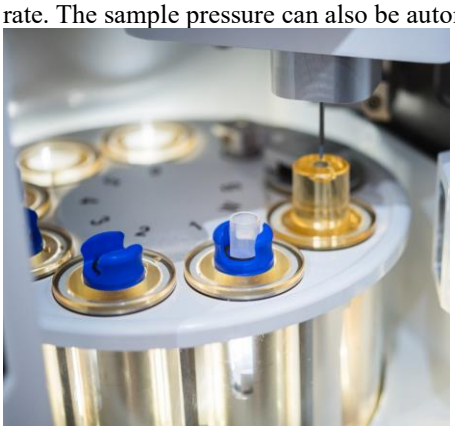


Figure 21. Sampling Assembly with the probe lowered toward the Wash Station

Touch Panel

A touch panel within the biocontainment area allows the user to control elements of the subsystems within the biosafety enclosure, including basic operation of the loader, without needing to leave the containment area. Both lighting for the biocontainment area as well as the sort chamber can be controlled from this panel or from SQ software. Loader operations such as rotate loader, perform an extra probe wash and run sample can be performed.

Figure 22. Touch Panel

Sample Line

An interchangeable sample line within the sample assembly is inserted into the sample fluid. This allows pressurized sample to flow to the nozzle assembly. When the sample tube is empty, air is drawn into the sample line. This air is detected before it can reach the nozzle, thus preventing it from interfering with the stream and droplet formation. The air is flushed out when the sample line is automatically washed by the wash station.

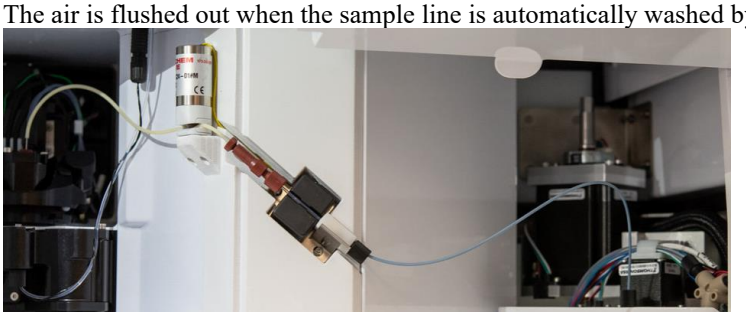


Figure 23. Sample Line

Nozzle

The nozzle provides sheath and sample to the intersection of the fluidics and the lasers, creates the droplets for sorting and charges droplets for deflection into sort media. The nozzle tip is composed of a ceramic material cone terminating in a 70 to150-micron orifice.

Figure 24. Nozzle tip

The nozzle assembly is mounted to an automated 5-axis mount allowing the nozzle to be placed in three positions. Active position is the normal operating location for data acquisition, droplet formation and sorting. The stream position is calibrated by the system during daily QC. The droplets are setup and maintained and drop delay calculated using on-board automation.

Figure 25. Nozzle in active position

The nozzle can also be moved by the system to a nozzle cleaning location for removal of blockages and for automated startup and shutdown operations. This is called the unclog/storage position. While the system is shut down, the nozzle is in this position to reduce the possibility of crystal formation and clogs.

Figure 26. Nozzle in unclog/storage position

The nozzle can be moved to nozzle swap position for easy removal as described in the swap tip wizard integrated into the SQ Software.

Figure 27. Nozzle in swap position

When the nozzle is in the default position for acquisition and sorting, sheath fluid from a pressurized internal reservoir is introduced into the nozzle assembly producing a downward vertical flow. The sheath pressure has a range of 4 to 60 psi, depending on the orifice size of the nozzle. Different nozzle orifice sizes are required for different cell sizes. Nozzle definition and calibration for the 70 µm and 100 µm tips is set up at the factory before the instrument is shipped. However, the first time you use the 120 µm and 150 µm tips, you must contact the support team to setup and finalize the configuration.

The sample is delivered into the nozzle assembly via the sample line. This terminates in the sample introduction needle within the nozzle body. The flow of sheath fluid around the sample needle hydrodynamically focuses the sample into a narrow-diameter core stream allowing cells to flow at a high rate while maintaining the integrity of single-file particle flow out of the nozzle tip orifice and through the laser beams.

Lasers and Optics

The Bigfoot optics include the lasers, mirrors, filters, and lenses that shape and guide the laser light to the interrogation point through which sample fluid is focused and flows. Optical elements collect scattered and fluorescently emitted light for detection.

	**Caution!** Laser irradiation can be hazardous. Please do not remove system covers as they are in place for your safety. Only trained personnel should access the exposed laser beams.

Three laser interlocks are designed to protect the user from laser irradiation:

- Nozzle Access Door
- Nozzle Ready Position
- Laser Interrogation Door

The status of each interlock is reported through the **System Status** and **System Health** areas of the software. All three interlocks must be in a ready condition to allow the system to acquire or sort. The optics are located outside of the biohazardous area, thus eliminating the chance of liquid spray on optical components. This also improves access for serviceability.

The Bigfoot can be configured with a selection of lasers. Each laser or laser module is attached to the optical table with the beam steering optics, nozzle, light collection assemblies and scatter detectors for maximum stability. The lasers are actively temperature regulated to prevent hotspot formation and subsequent optical instability as well as to reduce audible lab noise.

To maximize laser power at the nozzle the lasers travel in air, as opposed to fiber delivery, and are focused to spots on the stream for cell interrogation.

Sample Interrogation

The Bigfoot system directs each laser or pair of lasers to its own interrogation point and can support up to seven spatially separated interrogation points along the core stream. Bigfoot can be configured with nine lasers. Five interrogation points contain the light from a single laser and two interrogation points contain the light from pairs of colinear lasers. At each of these positions, light is scattered around each particle and, if any fluorophores or dyes are present in or on the particle, the particle may also fluoresce.

Pairs of lasers focused together on an interrogation point are known as colinear lasers. In the case of colinear lasers, the light at the interrogation is mixed, therefore detected light is also mixed. Care should be taken in selecting combinations of dyes using colinear lasers and filter selection should be carefully considered.

Once the cell suspension and its surrounding sheath fluid have passed through the interrogation points, they are either sorted or they flow through the waste catcher to the waste bottle.

Light Collection

Light is collected from two angles relative to the laser beam. The forward scatter parameter is collected immediately in front of the laser. The side scatter parameter is collected at approximately 90º relative to the laser. The angle at which light is collected determines the type of signal that is measured. Scattered light collected at both the forward and side angles is generated when individual particles disperse laser light. As such, the wavelength of light collected will be identical to the wavelength of the laser being used to illuminate the particle (typically 488nm or 405nm on the Bigfoot). Additionally, the Bigfoot has optional depolarized light detectors for both forward scatter and side scatter. These parameters can be used to help distinguish different cell types and functional changes. Fluorescent light, which is always collected at 90° relative to the laser beams, refers to the light emitted by the fluorophores/dyes in or on the cell because of laser excitation. This emitted light is primarily of a higher wavelength than the excitation light and therefore can be separated and detected using optical filters. A single collection lens is utilized for the collection of fluorescence signals and light is transmitted through optical fibers to the detection area. In the detection area, light is separated through a system of optical filters and mirrors for wavelength separation and measurement with photomultiplier tubes (PMTs).

Mirrors and Filters

Optical mirrors and filters are coated pieces of glass that are used to partition and direct light through the detection paths. Dichroic mirrors and filters allow light of a range of wavelengths to pass through. Mirrors reflect light that is not permitted to pass through them while filters typically absorb this light. Mirrors, which are used to direct light

around the detection path, are named based on the cutoff wavelength and whether they allow longer or shorter wavelength light to pass and are typically placed at a 45° angle to the incident light. For example, a 560 Dichroic Long Pass (DLP), allows light longer than 560 nm to pass. Light shorter than 560 nm is reflected. Similarly, a 470 Dichroic Short Pass (DSP) allows wavelengths shorter than 470 nm to pass. Any photons with wavelengths longer than 470 nm are reflected.

Filters placed directly in front of detectors determine the specific range of wavelengths that the detector measures and are defined as *bandpass, shortpass*, *or longpass*. Bandpass filters allow only a *band* of light to pass and are named to specify the center of the band as well as the width of the band. A 447/60 filter, for example, allows light from 417 nm to 477 nm through (447 +/-30 nm), absorbing the rest. Longpass and shortpass filters allow light to pass that is only longer or shorter, respectively, than the specific wavelength.

Mirrors and filters permit multi-parametric analysis of each cell. By partitioning the spectrum of collected light into specific ranges of wavelengths, each detector can be dedicated to the measurement of a specific fluorophore or dye.

The Bigfoot detection system and optical filters are arranged in banks, or groups of PMTs dedicated to a distinct interrogation point and laser. The system can be configured with up to seven banks of PMTs, one per laser or pair of lasers.

Optical Response Baselining (ORB)

The Bigfoot system automatically verifies the configuration of the optical filter setup by pulsing eleven different wavelengths of light from eleven LEDs into the optical fibers leading to the detector banks. This is called Optical Response Baselining (ORB). The ORB filter confirmation process is part of automated QC and startup. The system tracks the median of the signals over time.

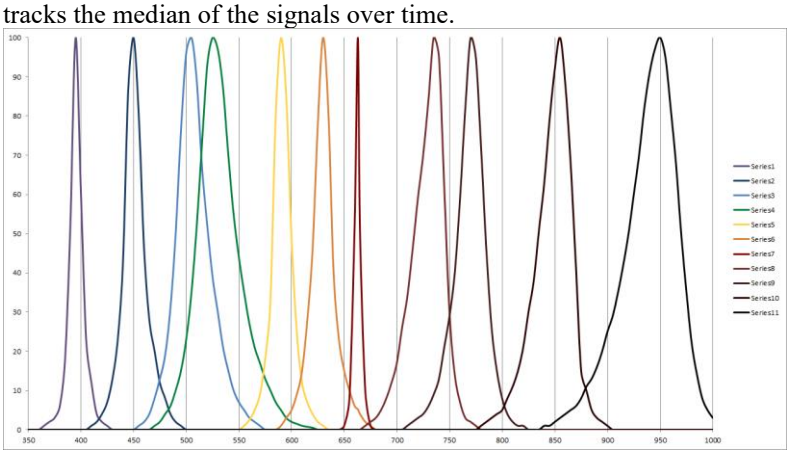


Figure 28. Spectra of LEDs used for filter confirmation

Detection Filter Access

The filter confirmation check runs in the background any time the detection filter door is opened and closed. If any changes to the detection filters are detected, SQ Software will alert the user to the change.

⚠	**Caution!** Use caution when opening and closing the detection door to avoid pinching.

Photomultiplier Tubes (PMTs)

Photomultiplier Tubes (PMTs) are used to detect and amplify the light signals coming from each particle. They are located behind the optical filters and detect specific bands of light based on fluorophores that are attached to the cell. The Bigfoot can be configured with a maximum of 55 fluorescent PMTs – seven banks and five independent FS/SS PMT detectors – can be utilized simultaneously. Modulating the voltage delivered to a PMT changes the PMTs signal amplification. Therefore, assays that require a wide sensitivity range can be carried out on the same instrument. The PMT voltages optimized for a specific application are stored in the SQ Software within a protocol

when an assay is repeated.

Electronics

The instrument electronics process the PMT signals and deliver data to the SQ Software for analysis. The pre-amplifiers are used to boost the signals coming from the PMTs.

	**Caution!** Only qualified, trained technicians should carry out service work on electronic components due to potential shock hazard.

Data Processing

Analog to digital converters (ADCs) convert the electrical signal coming from the pre-amplifier into a digital signal and transfer it to the SQ Software for data visualization. Bigfoot is a fully digital instrument, transforming signals with 24-bit resolution for signal area and height. Simultaneously measured peak, area and width is provided for every channel with true measured width at half-height. Low-noise converters and proprietary digital processing reduce channel noise.

The system utilizes high-speed parallel electronics, such as customized Field Programmable Gate Arrays (FPGA), that are used for real-time data acquisition and sorting. This enables the Bigfoot to simultaneously acquire 60 parameters at over 100,000 signals per second and more than 70,000 sort decisions per second with zero hard aborts. This powerful acquisition system enables the implementation of 60 x 60 parameter real-time color compensation or spectral unmixing and 64 gate cell sorting. The FPGA nature of the system enables field upgrades of electronics capabilities without hardware changes.

Spectral Module

An optional spectral instrument configuration allows collection and display of sample fluorescence data from every fluorescence parameter simultaneously. As an alternative to traditional compensation, the system automatically unmixes the data based on controls presented during setup. The unmixed data can be used for both acquisition and cell sorting without compromising sort speed purity, yield, or recovery.

Sorting Principals and Components

The hardware outlined thus far is common to both acquisitions and sorting. The following principles and hardware are directly associated with cell sorting.

	Motorized systems are utilized in the sort collection area, the motions are all designed for safety. This involves multiple sensors as well as limiting the forces available. When the motorized assembly is physically challenged it will either stop or stop and reverse direction to avoid any possible pinch injury.

	The instrument is equipped with safety interlocks designed to protect the operator from inadvertent exposure to high voltage. A potential source of high voltage is from the Sort Deflection Plates, these enable the system to separate the charged droplets formed during the normal sorting process. For access to the sort collection area where the plates are exposed there is an electrical interlock. This is operated by the access door to the sort area.

Sorting on Bigfoot

As discussed earlier, the nozzle generates a jet of sheath fluid and sample that exits the orifice and is then interrogated with one or more lasers. In parallel to the creation of the jet of fluid, a pressure wave of a given frequency is delivered to the entire stream. This pressure wave is induced by the motion of a bi-morphological piezoelectric crystal attached to the nozzle body. The waves created cause the stream leaving the nozzle to break into uniform droplets. The frequency of oscillation of the crystal is controlled by the system. However, different nozzle sizes require different frequencies to produce stable droplets, these can be predicted and are stored as a reference for each available nozzle size. With a set amplitude and frequency and a constant sheath pressure (and temperature), droplets form at a consistent distance from the nozzle tip exit. This is termed the droplet breakoff point, or last attached drop, as it is still in contact with the rest of the stream.

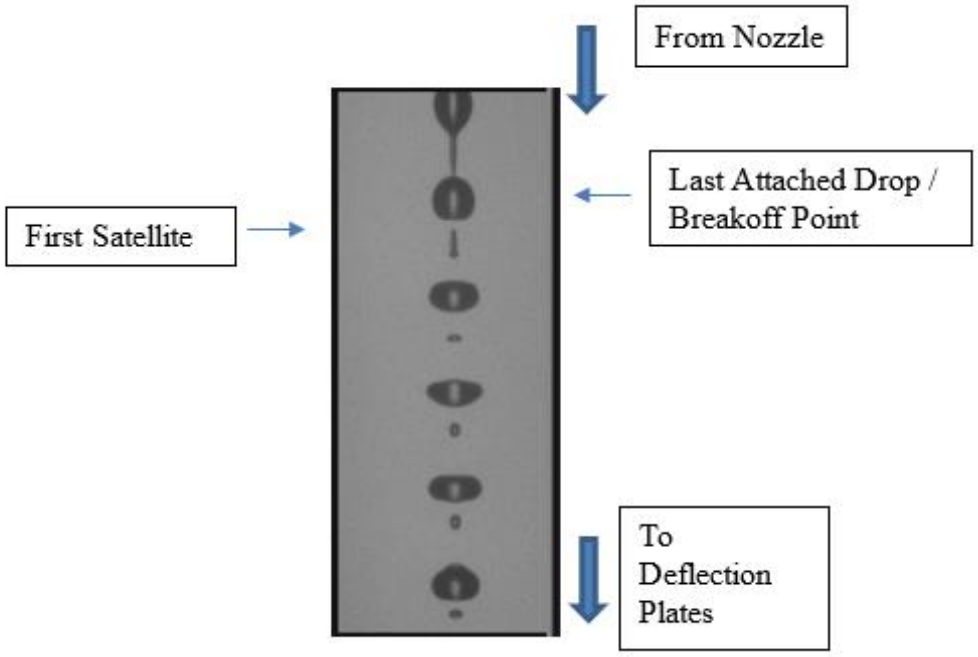


Figure 29. Image from droplet camera

When cells meet the gating criteria selected by the user, a sort decision is made. When that cell reaches the last attached drop, the system charges the stream in phase with the droplet formation creating a charged droplet containing the required cell. As the droplet breaks away from the rest of the stream, the charge is retained on that droplet and removed from the rest of the stream. Cells that meet other sort criteria can be given different charge levels, both positive and negative, to ensure proper deposition into the desired media. The droplet charging time must be precisely measured because it is very important for maximizing sort yield and purity. The exact distance (and therefore time) from the interrogation points to the last attached drop is determined during the daily QC process. This timing is called the drop delay. To ensure maximum yield and purity, after measuring the timing, the breakoff location is maintained by automatic monitoring.

After cell interrogation, droplet formation and droplet charging, sheath and sample droplets pass through the deflection plates. These plates are charged to a high negative and positive static charge. For tube-based cell collection, charged droplets are deflected into up to six sample tubes within the sorting assembly. Droplets not identified for sorting pass into the waste catcher and are pulled to the waste tank for disposal. For microwell plate cell collection, the system can increase the number of different droplet charges to fill each well of the plate quickly and efficiently. The number of droplet charges varies by plate type. For ultra-precise positioning of the droplet the waste stream may be deflected and the sorted cells can pass straight down into the sort collection media.

Sort Output Media

The Bigfoot accommodates a wide range of sort collection media that can be temperature controlled throughout the sort. A collection box and adapters for 1.5, 5, 15 ml and 50mL tubes are available and can be mixed and matched to suit the experiment. Any microwell plates with a standard footprint up to 1536 wells can be loaded directly onto the sort output arm. The most common plates are 96-well, 96-deep-well and 384-well plates. Custom plate setups can be created in the software if a different number or position of wells is desired. Custom output adapters are available for genomics applications. Please contact the manufacturer for more information.

Power and Communication Connections

The power and communication connections are made on the Entry Panel on the side of the instrument. The black connection is for the IEC compliant AC power cord. The power switch is located next to the power cord. The communication connection is next to the fan.

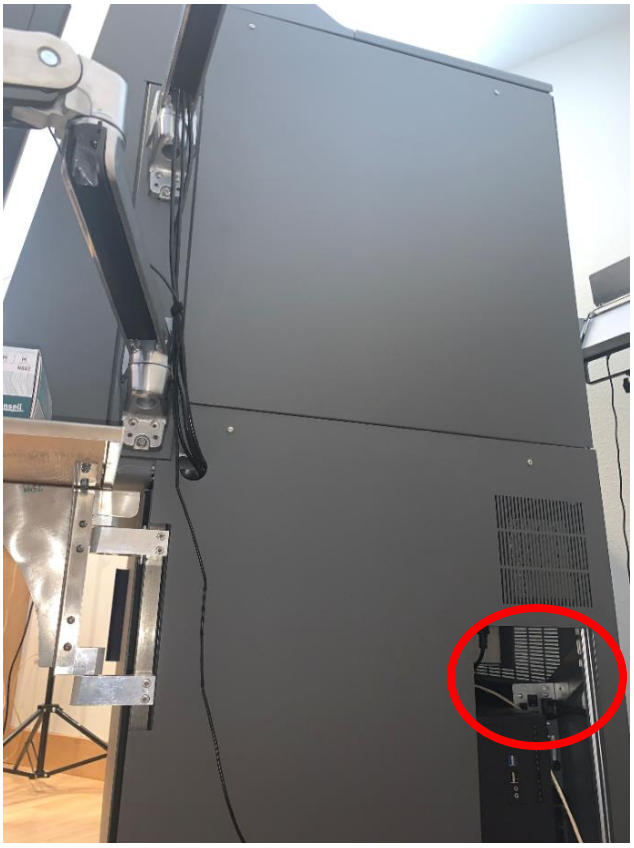


Figure 30. Connections on right side of instrument

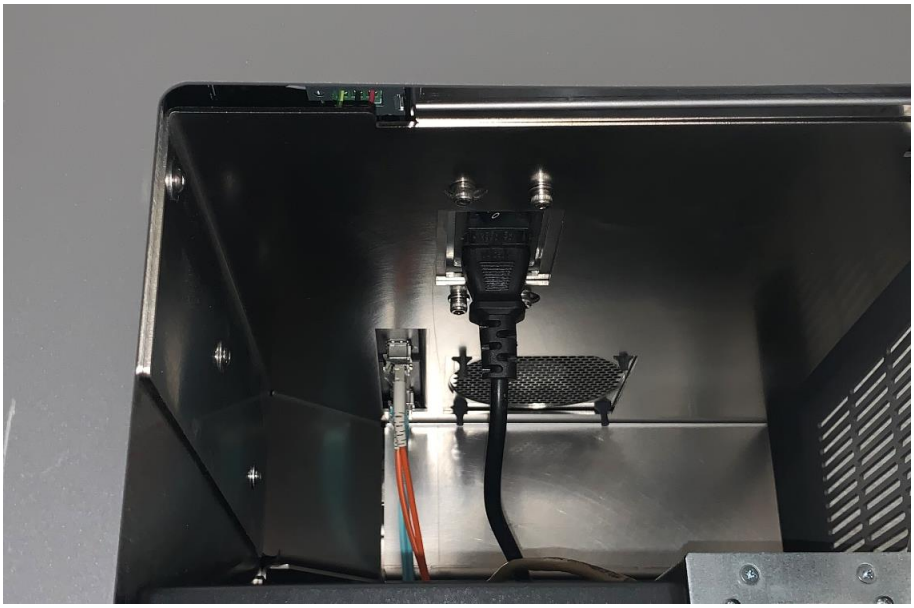


Figure 31 Connections and switch detail

Chapter 3: The Principals of Spectral Acquisition and Sorting

The optional spectral module contains specific hardware configurations and software to allow the collection of data for each event from every laser beam spot and each fluorescence PMT simultaneously. This complex data set is then delivered to a spectral unmixing system for the display of a specific fluorescence signal based on the removal of overlapping spectra from other dyes present as well as cell autofluorescence. The derived signals can be used for both analysis and cell sorting without compromising sort speed purity, yield, or recovery. To achieve this, single-color controls as well as a negative sample should be run to provide the system with the data needed to perform the unmixing.

Spectral Hardware, Detectors and Optics

The spectral option is available on systems with 48 fluorescence parameters or more. The number of detectors and the bandwidths of optical filters have been optimized for each laser to give the maximum fluorochrome resolution and laser blocking.

PMT Voltage Selection

As in compensation experiments, spectral experiments require the correct PMT voltage to be set to represent at least 95% of data within the plot area. PMT voltages can be set for spectral use in multiple ways.

Voltages Imported from QC

The PMT voltages derived by the QC system are useful as application settings. Bigfoot daily QC assays the separation index of a negative bead and a positive bead for each detector. After the QC protocol has run successfully, the values found are displayed on a Levy-Jennings plot for longitudinal comparison. These voltages are automatically used for spectral experiments.

Voltages Set While Running the Negative Control

To cope with major biological differences in some cell populations, the spectral adjustment tool can be used by right-clicking in the scatter region and selecting run for spectral voltage. This automated process will set the MFI of negative populations to ~10^1 allowing ample room for the positive populations.

Voltages Manually Adjusted

Finally, using the spectral plot, each PMT voltage can be manually adjusted manually while running the negative control. These voltages can then be used for the spectral setup panel and sample. Voltages can also be imported from a previous experiment.

Software

After naming the protocol, SQ Software asks if it is a spectral experiment or a compensation experiment. Note: This choice is available only for spectral configurations. If spectral mode is selected, Fluorochromes are designated on the following page.

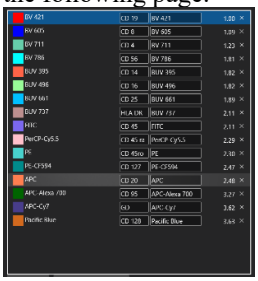


Figure 32. Spectral Fluorophores List

A prefix can be added to each label. This is used on the Spectral Similarity Index Table as well as the groups created for the single-color controls and plot parameters after the data has been unmixed.

As fluorochromes are added to the list, the software generates the following:

- **Complexity Index** – A cumulative complexity index score is shown next to the fluorochrome. Complexity index is a score of the overlapping values of the selected fluorochromes. When well separated fluorochromes are added, the complexity index rises slowly. However, if a fluorochrome is added that greatly overlaps with one or more of the fluorochromes already selected, the complexity index rises sharply.
- **Spectral Energy Plot** – For each fluorochrome selected, a stored spectrum is used to show the approximate intensity of the fluorochrome in each detector channel of each laser. The plot is normalized to the laser with the highest emission value for the dye. Selecting a fluorochrome in the list will cause it to be shaded. The colors used for the line and the shading are the same as those in the fluorochrome list.

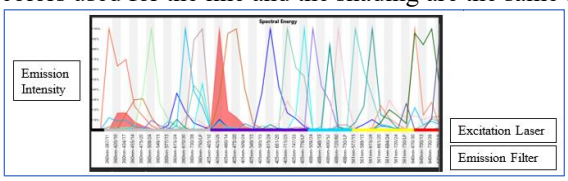


Figure 33. Spectral Energy Plot

- **Spectral Similarity Matrix** – As fluorochromes are added, a similarity matrix is created of each fluorochrome vs. each other fluorochrome. The range of each value is 0 to1; when dyes are relatively dissimilar the value is close to 0 and when dyes are relatively indistinguishable the value is close to 1. This is provided as guidance for experimental design.

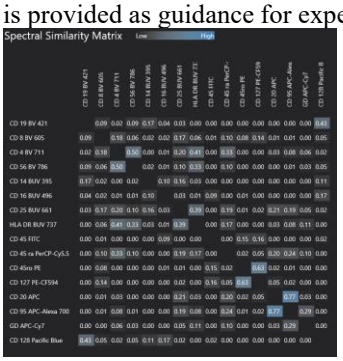


Figure 34. Spectral Similarity Matrix

The Complexity Index and Similarity Index help the user build a panel that will resolve the required populations. The greater the complexity index and subsequent similarity index, the more challenging it will be for the software algorithms to successfully unmix the signals. Keep in mind this is just a guide because fluorophores from different vendors or fluorophores degraded by time can result in different actual spectra.

- **Spectral Plot –** The X-axis is the detector wavelength band of each PMT used with a specific laser. Lasers are shown in wavelength order. The Y-axis is the signal intensity in logarithmic scale. The color denotes the number of cells present. Blue represents a lower number of cells and red represents a higher number of cells. Linking the areas of similar colors can show the emission spectra of a dye in a specific laser beam. Dyes with high intensities across several lasers show the high amount of cross-beam excitation and subsequent emission of a dye.

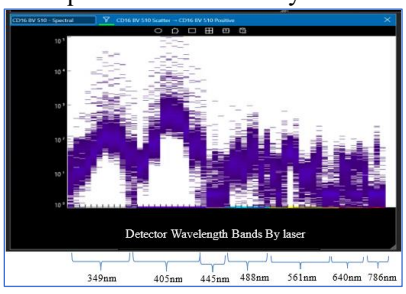


Figure 35. Spectral Plot

Chapter 4: Starting Up and Running

The Bigfoot Cell Sorter must be started up through SQ Software. The instrument should always be powered ON and the shutdown procedure should be performed in SQ Software at the end of each day. This will maintain minimum airflow for constant containment. During the shutdown procedure an automatic startup time and date can be set, or the user can choose to manually start up the next session.

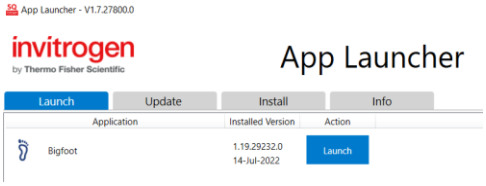
Getting Started

Bigfoot SQ software is launched and updated through the SQS Launcher.

Logging In

1. Open SQS Launcher.

2. Click the **Launch** button.

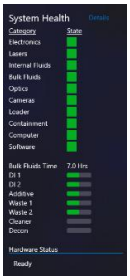


3. Enter your username and password. It is optional to enter session notes. Session notes will be logged with the user session in the user report for administrative purposes.

4. Check the instrument status bar. If **Ready** is displayed, the system is started up and running. If **Off** is displayed, the instrument is shut down and will need to go through the startup process before it can be used.

Maintaining Fluids

1. Click **System Health** and assess the bulk fluids levels.



2. The seven bulk fluidic bottles – two waste bottles, two DI water bottles, one sheath concentrate bottle and two cleaner/decontamination solution bottles- are located behind the fluidic door on the left front of the instrument and require maintenance on a regular basis. See the Bulk Fluids Tanks section for details.

Scheduled Automated Startup

The automated startup process turns on the lasers, pressurizes the internal sheath tank, brings the nozzle assembly into the run position and initiates sheath flow through the nozzle. System cameras are used to verify the primary alignment of the nozzle. After droplets are created, the system initiates a warmup period and notifies the user that this is in progress.

Startup

1. Click **Startup** to start the automated startup process. Note: You can start the system before or after logging in. The **Startup** button is replaced by the **Shutdown** button when the system is running.

Perform Automated Quality Control with Bead Alignment 2.0

Auto Bead Alignment now uses an advanced search pattern of possible nozzle positions and a data-driven algorithm tuned with field statistics to calculate optimal alignment. The new algorithm collects data by moving the nozzle in the X and Y directions to create a grid of data points. This data is analyzed for each laser to measure the optimal alignment point for each laser. The laser-specific results are then combined to determine the optimal alignment position for all lasers.

- Test Measurement (Event Rate 150 EPS)
- Test Adjustment (Event Rate 250 EPS)
- Adjust Voltages
- Tune Laser Delay
- Test Initial Alignment
- Wide or Regular Mesh Grid Sweep
- Lock in Optimum Nozzle X & Y Position

The automated steps are like the manual bead alignment process. The system validates the filter set, tests the measurement event rate and adjusts as needed, adjusts voltages to bring all populations to 256V on a linear scale, tunes laser delay and tests the initial alignment. If the instrument meets the CV and voltage specs at this point the system will not adjust the nozzle. However, if initial alignment reveals the setup does not meet specifications, the system will perform a full X/Y grid sweep that will take approximately 15 minutes while the instrument fully characterizes the optimal signal for each laser. After the full grid sweep is complete, the system performs a sub grid sweep to determine the optimal position for all laser paths. After the instrument has found the optimal nozzle position, it will always return to this optimal point unless a new nozzle position is saved. Note: The event rate during this process is monitored by software to help conserve beads.

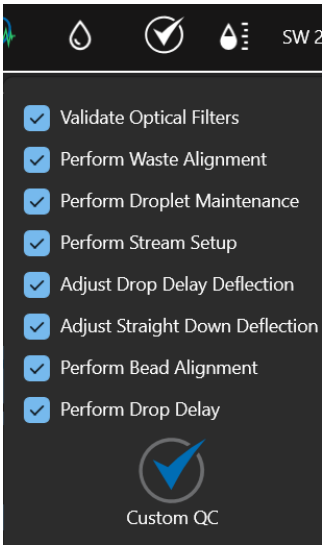
1. Automated QC can be run before or after you log in to the software.
2. Ensure that a bottle of Invitrogen Bigfoot Calibration Beads (PL00287) is in the bead bottle position of the sample loader.
   Click **Full QC**. If all set parameters are within range, QC will pass.

Set Custom Quality Control Options

It is optional to set custom quality control steps after you log in to the software.

1. Perform Startup.
2. Log in to SQ software.
3. Click the **QC** button at the top of the screen.

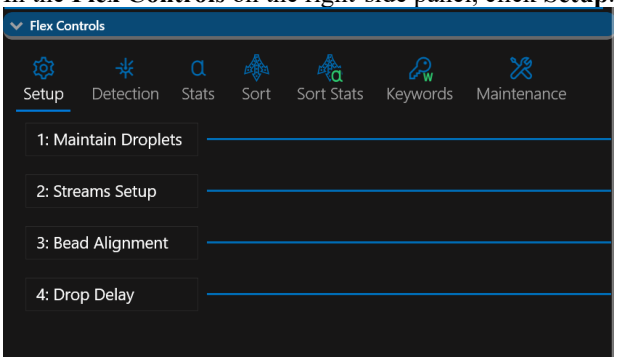
4. An options menu appears. Deselect the steps you want to exclude from the QC protocol and click **Custom QC**.



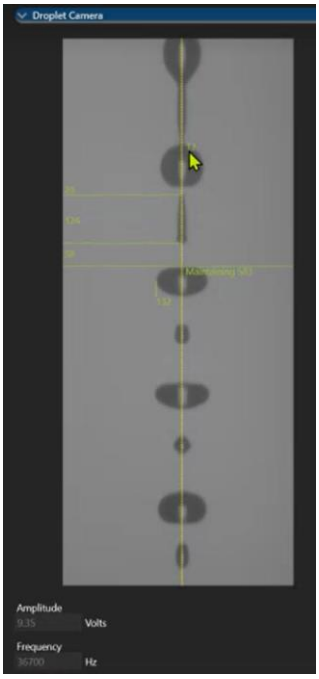
Perform Manual Quality Control Drop Delay Confirmation with Beads

Manual Quality Control allows you to manually adjust the nozzle position, PMT voltages and confirm drop delay results using a microscope and the on-board beads.

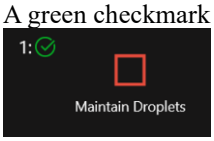
1. From the **Home** screen, click **Quick Run**.

2. In the **Flex Controls** on the right-side panel, click **Setup**.


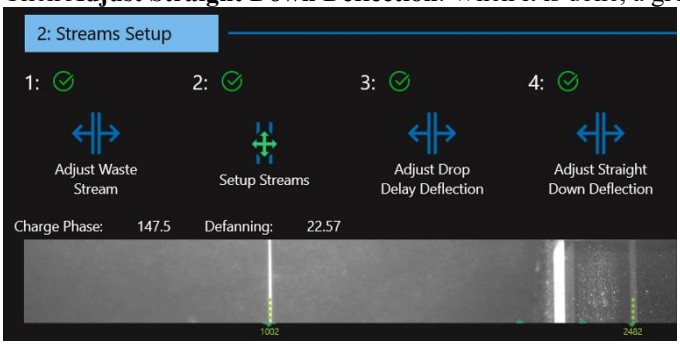
3. Click **1: Maintain Droplets**.



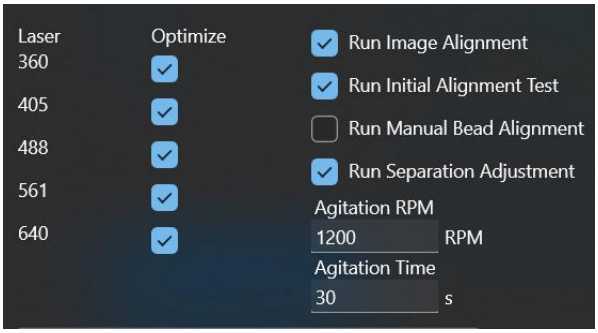
4. A green checkmark appears when droplets are maintained.



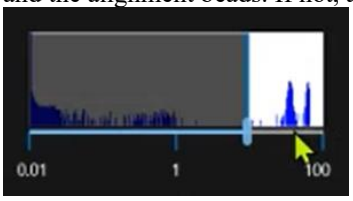
5. Click **2: Streams Setup** and then click **Adjust Waste Stream**. When it is done, a green checkmark appears. Note: If image quality is poor, clean the streams handle and the black window behind the charge plates.
6. Click **Setup Streams**. When it is done, a green checkmark appears.
7. Click **Adjust Drop Delay Deflection**. When it is done, a green checkmark appears.
8. Click **Adjust Straight Down Deflection**. When it is done, a green checkmark appears.



9. Click **3: Bead Alignment**. To select the options for manual alignment, click **Settings**.
   Optimize – When all laser wavelength checkboxes are selected, the Automatic QC process optimizes all lasers equally. Deselecting a laser checkbox does not turn the laser off, but it deprioritizes it for the bead alignment process.
   Run Image Alignment – Select for automated QC to move the nozzle and try to match the saved nozzle position. If you have manually saved a good nozzle position, deselect this box.
   Run Image Initial Alignment Test – Select for automated QC to run 1000 events to quickly check alignment.
   Run Manual Bead Alignment – Select to allow manual bead alignment.
   Run Separation Adjustment – Select for automated QC to adjust voltages to move the blank bead population at 10^1. These will be the starting voltages for new protocols.
   Agitation RPM – Set the RPM at which the QC bead bottle will be agitated before the test is run.
   Agitation Time – Set the seconds that the QC bead bottle will be agitated before the test is run.

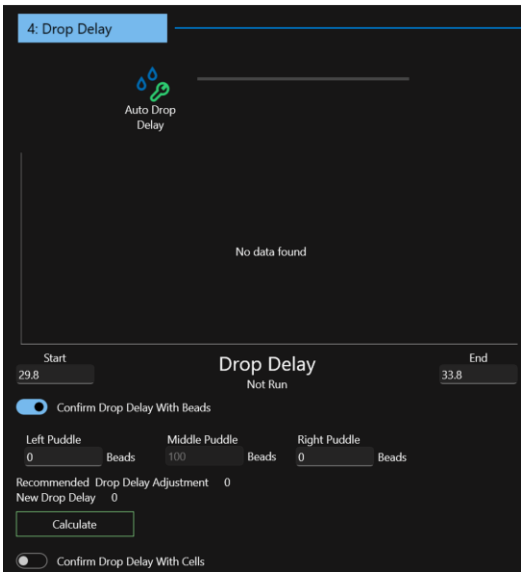


10. Click **Bead Alignment**. Verify that the Threshold plot shows two peaks that represent the drop delay beads and the alignment beads. If not, adjust and save the nozzle position.



11. Plots appear on screen and the initial process will run. If the results do not meet specification, use the controls in the Manual Bead Alignment panel on the left to adjust the nozzle position and voltages until the plots are optimized. Use the **X Controls** first and then click **Adjust Voltages** to bring the populations back on scale then use the **Y Controls** and then click **Adjust Voltages**.

12. When the CVs and voltages look good, click **Accept Bead Alignment**. Note: We recommend that you do not accept bandpass filter CVs above 5, or long pass filter CVs above 9.
13. Wait while the system automatically tests the Measurement Event rate at 150 EPS, the Adjustment Event rate at 250 EPS and then collects 5000 events for the QC Report.
14. Click **4: Drop Delay**. Click **Auto Drop Delay**. Note: To confirm the drop delay result with beads or with sample, click the corresponding toggle button. This example confirms with beads.



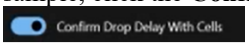
15. Confirm drop delay under a microscope and adjust the positive and negative values if necessary.
16. Click **Calculate**.
17. When complete, the QC status indicators at the top of the screen appear white.

After the system has passed QC, the instrument is ready to acquire and sort. The data and FCS files can be viewed in the QC Report Management screen.

Confirm Drop Delay with Sample

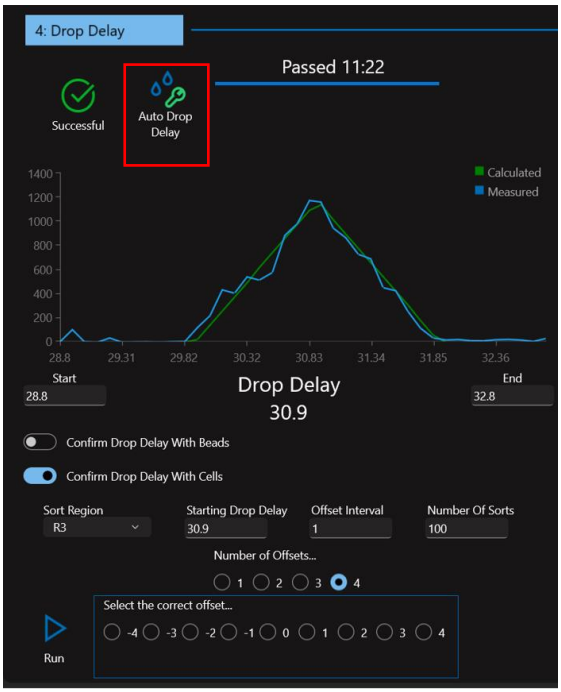
After Drop Delay is automatically calculated with beads, it is now possible to test the sort setup on a slide with a user-provided sample and a user-defined sort region. You can customize the number of drop delay offsets, then sort the sample onto a slide and inspect the results with a microscope. The drop delay setting can then be adjusted to an optimal value for the specific sample.

1. Make sure the sample is stained appropriately so the cells can be seen using a fluorescence microscope.
2. Build the experiment in the workspace and set the sort regions. Note: You can verify drop delay with one or more population.
3. Auto Drop Delay is first determined with the on-board beads. If you intend to confirm the setting using sample, click the **Confirm Drop Delay With Cells** toggle button to on.

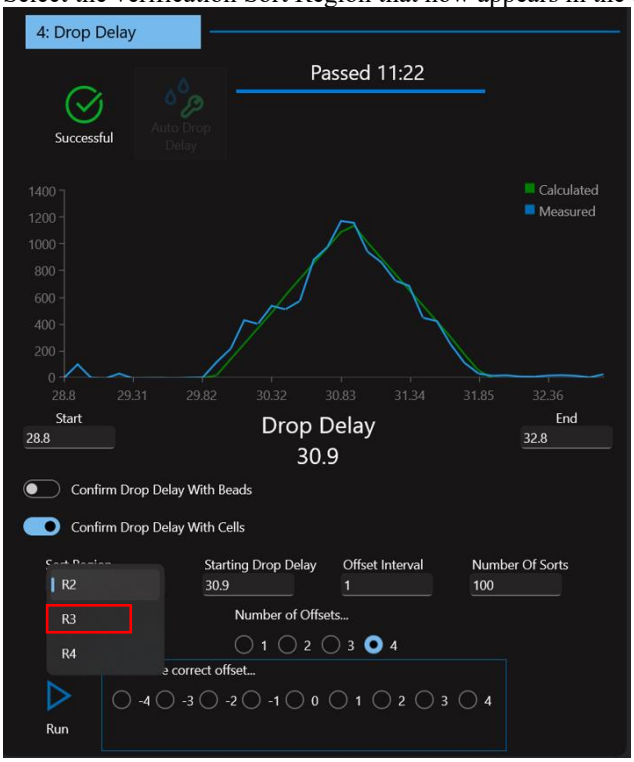


4. Select the number of offsets. The number represents the number of puddles that will be deposited on each side of the primary puddle.

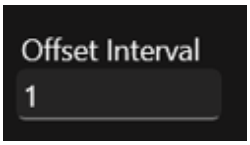
5. Click **Auto Drop Delay**.



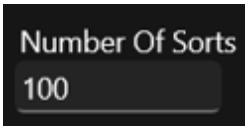
6. The resulting accepted drop delay value appears in the **Starting Drop Delay** field.
7. Run enough sample to set the regions for the populations you intend to use for verification.
8. Right-click in a Region and set up a Sort Region.
9. Stop the sample.
10. Select the verification Sort Region that now appears in the dropdown list.



11. Select an **Offset Interval** that will be applied to each side of the center puddle and between each offset puddle. The offset interval is the distance measured from the calculated starting drop delay value. Note: We recommend that the **Offset Interval** is changed only in increments of 1.

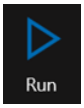


12. Set the **Number of Sorts** that will be applied to each puddle.

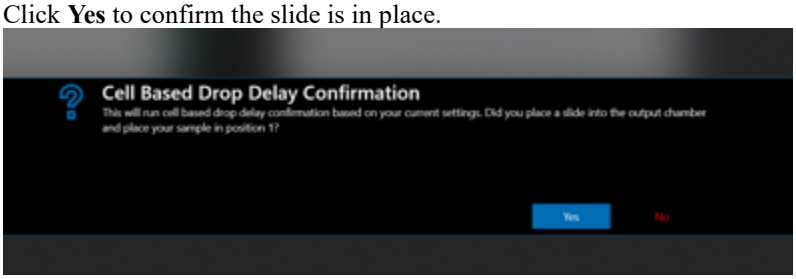


13. Put a slide on top of the 5mL tube adapter in row 1 closest to the sort door. Make sure the frosted edge of the side is on the right.

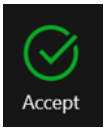
14. Click **Run**.



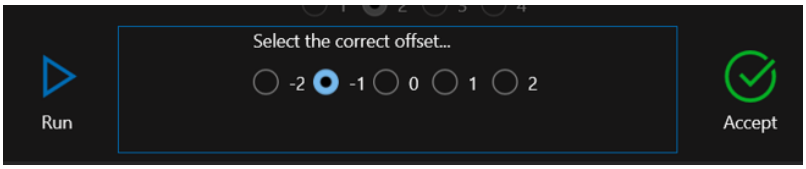
15. Click **Yes** to confirm the slide is in place.



16. Wait for the sample to be sorted onto the slide.
17. View the slide under a microscope to determine the puddle that contains the most cells. Count the cells in each of the offsets. Verify that the offsets do not contain more than 5 cells. If the middle location contains 100 +/- 5 cells, click **Accept**.



18. If any of the offset locations contain more than 5 cells, select the offset location that contains the most cells and click **Accept**.



Sample Preparation Considerations

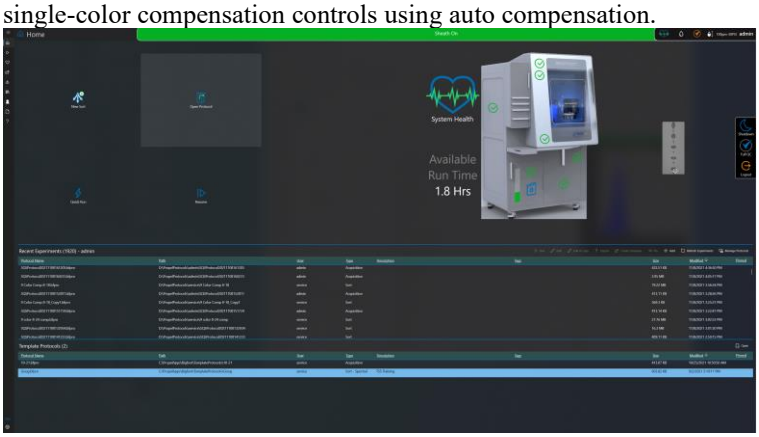
Before building a new experiment, ensure the samples are of high quality. In general, high-quality samples meet the following criteria:

- Viable cells are 85 percent or greater of the total sample.
- Cells are prepared in a single-cell suspension.
- Cells have been passed through a filter with a pore size that is approximately 70% the size of the nozzle tip. Filtering can be done immediately before placing the samples in the loader.
- Samples are of a concentration of 1 million cells per mL for every 1,000 events per second desired, in at least 100 µl.
- Cells are suspended in not greater than 0.1% protein such as FCS or BSA.
- Appropriate controls are available, such as negative controls and single-color controls.

Ensure the selected markers and fluorophores are compatible with your Bigfoot optical configuration. It is also a good idea to anticipate the gating strategy and the kind of data you will collect based on the selected markers.
If you intend to sort, ensure you have suitable collection tubes or plates with a volume of collection buffer to place in the output media prior to sorting.

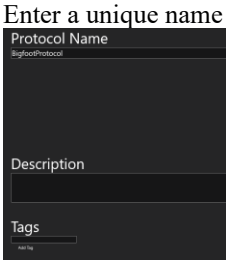
Sort Experiment Setup

After logging in and starting up the instrument, the Home Screen appears. The following section will guide you through the process of creating a new sort experiment without single-color controls and a sort experiment with single-color compensation controls using auto compensation.



1. From the **Home** screen click **New Sort**.

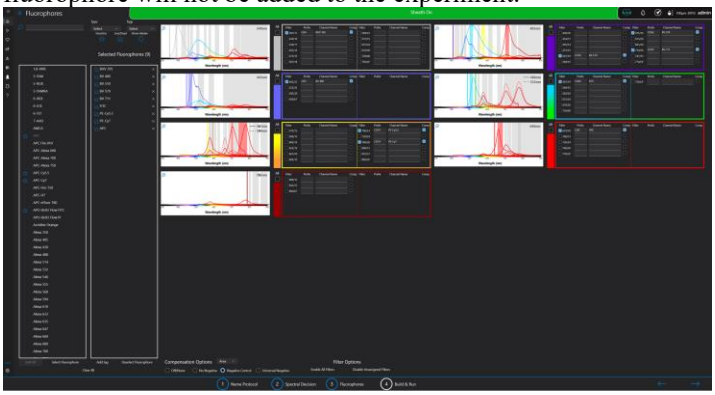
2. Enter a unique name for the experiment. It is optional to enter a description or tags.



3. Click the **Next** arrow to move to the workspace.

4. If Bigfoot is configured for spectral sorting, you must choose between conducting a spectral demixing experiment and standard compensation. For standard compensation click **No**. For a spectral demixing experiment click **Yes**.

5. To select the fluorophores that you have chosen for your experiment, scroll through the fluorophores list on the left of the screen, or type the first few letters or numbers of the fluorophore name in the search box until you see the correct item. Double-click the fluorophores to add them to the experiment. Selected fluorophores appear in the **Selected Fluorophores** list and the emission spectra appear in the plots in the middle of the screen. As fluorophores are added, the optimal detector is automatically selected. However, if the selected fluorophore requires the same detector as a previous selection, a warning appears and the fluorophore will not be added to the experiment.



6. The default parameter name is the name of the fluorophore in the fluorophores list. If desired, the Channel Name field can be changed to include more descriptive information for each detector, as shown. A prefix can be added as well.

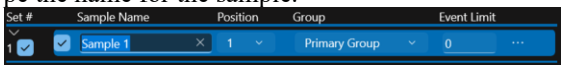
7. Additional detectors can be enabled manually, without using the fluorophore selection tool. Click the filter checkbox next to the additional detector.
8. Rename the detector, in the **Channel Name** field and add a prefix as desired.
9. From this point, there are two workflows that can be followed, one with and one without auto compensation.

Build and Run – Manually

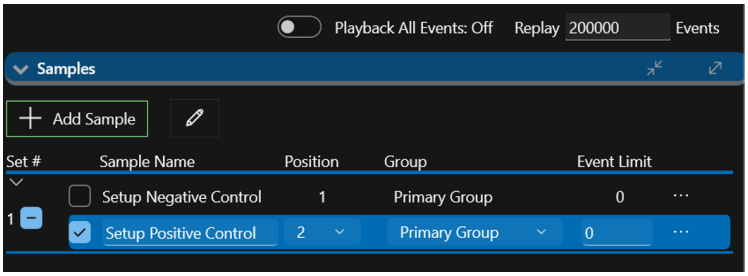
Experiments can be built in real time. If enough sample volume is available, the protocol can be built as samples are run.

1. Follow the steps in Sort Experiment Setup section. After fluorophores are selected, click the **Next** arrow.

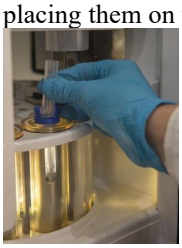
2. By default, the software creates an FSC Area vs. SSC Area plot, which can be modified. Additional plots can be added by double-clicking in the workspace. Plot axes default to linear and can be modified and labeled.
3. The first sample in the sample list is selected by default. It is optional to change Sample 1 to a custom name. Double-click in the **Sample Name** field and type the name for the sample.

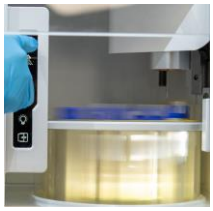


4. Additional samples can be added by clicking the **+Add Sample** button. These samples are assigned to the **Primary Group** by default.
5. If you intend to load samples one at a time, leave the default **Position** as **1**. If you intend to use the multi-sample ability of the loader, place the tubes into the loader and select the corresponding **Position** number from the dropdown list.

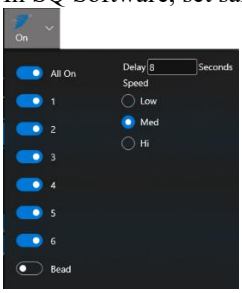


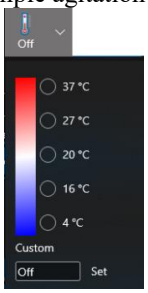
6. Enter a value in the **Event Limit** field. To apply the value to all samples, right-click in the **Event Limit** field and select **Apply Event Limit to Set or Apply Event Limit to All**.
7. Load samples into the sample loader, according to the order shown in the sample list. The loader can be rotated to access the rear tubes using the top touch panel button. If the sample tube holder adapter does not match the tube size, pull it out of the loader and replace it with the appropriate size adapter. When inserting an adapter, rotate until it drops into place with the locator pin. Adapters may be already in the loader or in the storage area on the left side of the instrument. Vortex, uncap and filter the tubes, if necessary, before placing them on the loader.



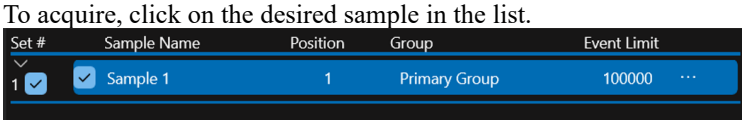



8. While in the biosafety enclosure, the sort collection tubes can be loaded for later use. The sort chamber door can be opened and closed using the bottom button on the touch panel.
9. In SQ Software, set sample agitation and temperature to support cell viability.





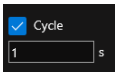
10. To acquire, click on the desired sample in the list.



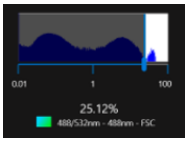
11. Set the sample pressure to Low (30%). Note: This is a good starting place if the sample concentration is not known and before voltage and threshold has been optimized.

12. Click the Run button to acquire sample. Note: In this mode data is shown but not stored.

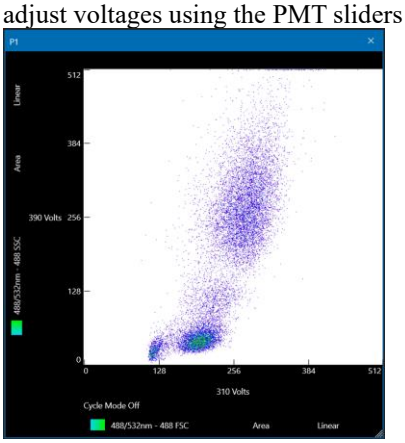
13. Enable cycle mode to quickly see any changes in the data. Remember to disable cycle mode when you want to record data later.



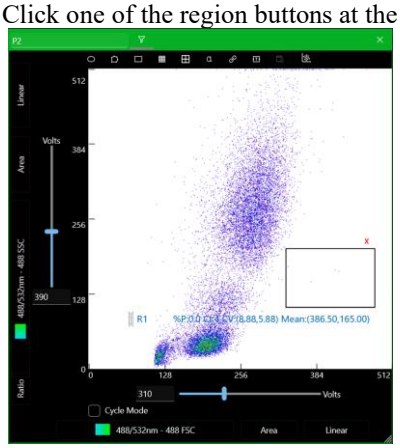
14. View the data plots and adjust the threshold level plot to see the data you require.



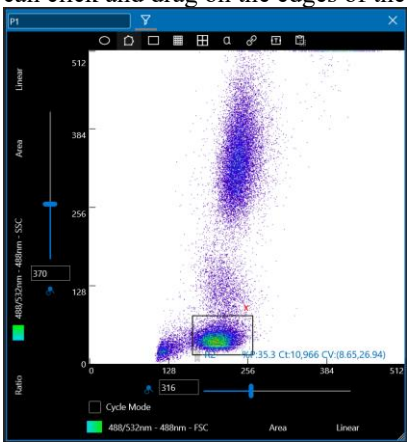
15. Adjust the sample pressure to produce a flow rate of 500 to 2,000 events per second depending on sample concentration. For every 1,000 eps desired, the sample typically should be concentrated to 1 million per ml.
16. Adjust PMT voltages in the FSC vs. SSC plot to fine tune the position of the target population. You can adjust voltages using the PMT sliders on each axis, or by entering values into the fields next to them.



17. Click one of the region buttons at the top of the active plot.



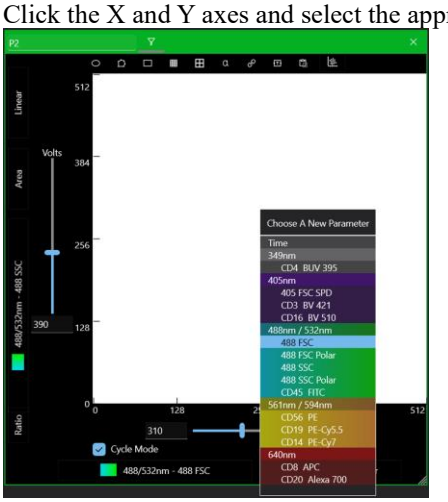
18. Click in the middle of the region and drag it over the population you would like to enumerate. Note: You can click and drag on the edges of the region to change the size and shape of the region.



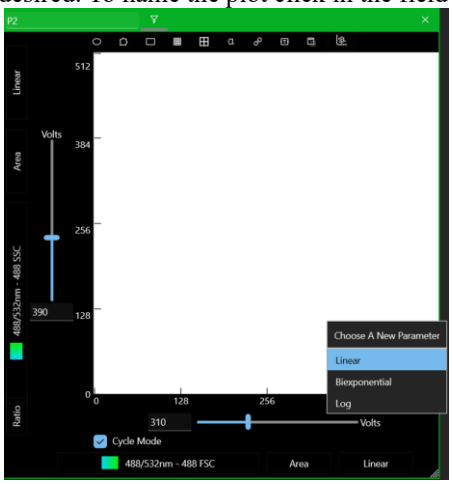
19. Click in the field under the region and enter a name for the region.
20. Use the **Add Plot** and the **Plot Tool** features at the top of the **Workspace** screen to add more plots. Double-click in the workspace to add a new plot or double-click inside a region to create a new plot gated on that

region.

21. Click the X and Y axes and select the appropriate parameters.



22. Click the scaling parameter on the axes to change the scale. The scaling options depend on whether you have selected Biexponential or Hyperlog. Select the **Comp** checkbox and the **Cycle Mode** checkbox if desired. To name the plot click in the field in the upper-left corner of the plot and type a new name.



23. Adjust the PMT voltages to place the data correctly in each plot.
24. Color Compensation: For low complexity or non-immunophenotyping experiments color compensation can be set manually with or without the use of single-color controls. Note: Changing PMT voltages between samples will negate the compensation that was set on previous samples.

Compensating using the Plot Compensation Tool

1. Ensure the **Comp** checkbox on the plot is selected so the plot shows compensated data.
2. Click compensation edit mode.

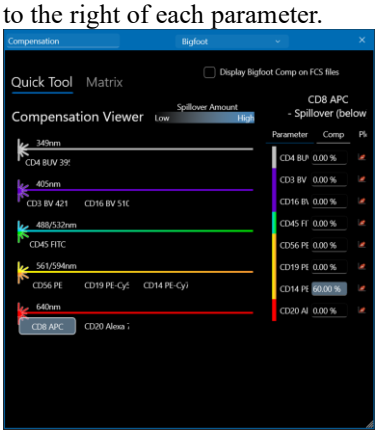
3. The following box appears in the plot.

4. To adjust compensation, put the cursor over the center of the population in the plot and click and drag the population to the desired location on the plot. The compensation value increases as the mouse is moved toward the desired axis and decreases as the mouse is moved away from the axis.

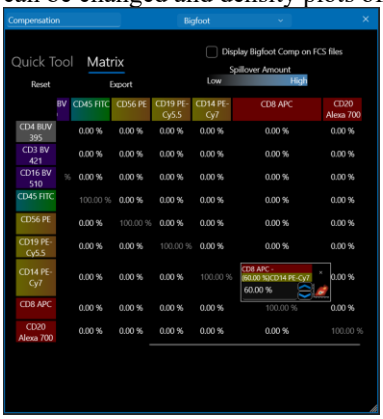
Workspace Compensation Tool

1. Ensure the **Comp** checkbox on the plot is selected so the plot shows compensated data.
2. Click the **Comp** button at the top of the **Workspace** screen.

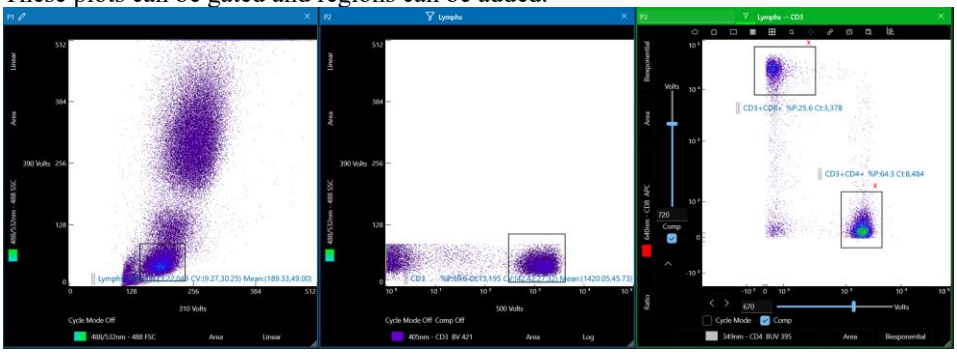
3. The workspace compensation tool has two modes: **Quick Tool** and **Matrix**.
Quick Tool –This tool shows parameters on the left in laser order and the possible combinations of each parameter on the right. The amount of compensation is shown in the **Comp** column. Each compensation value can be adjusted in real time to see its effect. Select the checkbox at the top if you want to display Bigfoot compensation in FCS files. Plots can be created from each combination by clicking the **Plot** button to the right of each parameter.



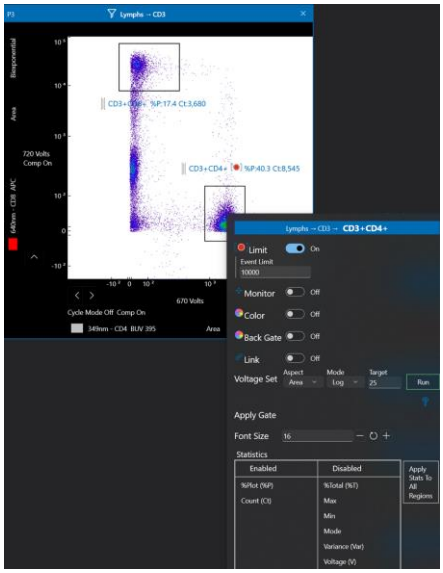
Matrix Tool – The matrix shows a table of each parameter compared against the other with the compensation value at their intersection point. Clicking on the value opens a box where the compensation can be changed and density plots of the selected parameters can be created.



4. These plots can be gated and regions can be added.



5. Double-click in a region to create additional plots or use **Add Plot** in the **Plot Tools** section.
6. In addition to the event limit in the sample list, which is based on all triggered events, a gate-based limit can be set utilizing one of the regions. To do this, right-click in the region, set the **Limit** button to **On** and enter a value.

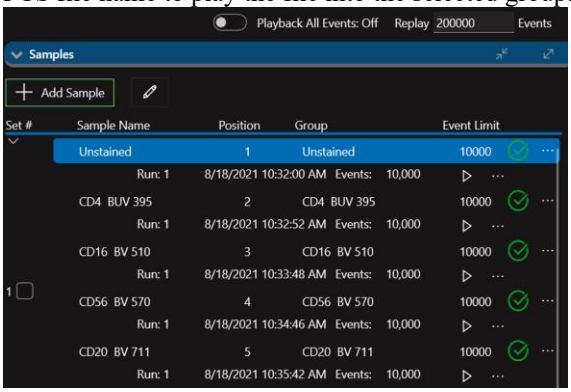


7. Click stop sample.

8. Either set up another sample or click Record to acquire data. Note: The Record button can also be used directly while acquiring. It is not necessary to stop sample before recording.

9. Acquisition will continue until the stop limit is met. This can be a region limit, total event limit, volume limit, or sort limit. If enough data has been acquired, click the stop acquisition button to manually stop. Note: Acquisition will also stop if an air bubble is detected.

10. Once a sample is acquired, any changes in plot definitions or regions result in the system replaying the sample from the FCS file just saved. The data can also be replayed by selecting the play button next to the FCS file name to play the file into the selected group.

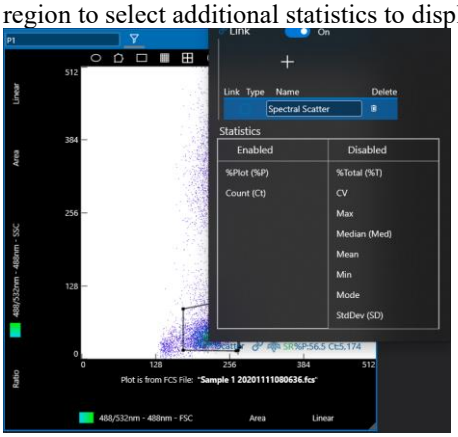


Statistical Analysis

1. Click the Stats Tool at the top of the Workspace screen to view statistics for the gated populations.

2. This information may be exported or shared as a CSV file. It may also be copied as an image and pasted it into another application.

3. Click Plot Stats at the top of the plot to view plot stats directly on the plot. Note: You can right-click in a region to select additional statistics to display.

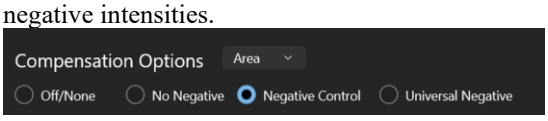


Build and Run using Automated Color Compensation

The auto compensation wizard uses a series of single-color control tubes with populations of negative cells or beads and positive cells or beads within each tube. If no negative events are present, a universal negative can be applied. Furthermore, if few target cells, such as dead cells, are present in a population but still require an exclusion gate to be set, compensation into that parameter can be set without setting compensation of that marker into other detectors.

Tip: If you would like to save the relevant compensation matrix within a Sample's FCS file, make sure compensation has been calculated before the samples are run.

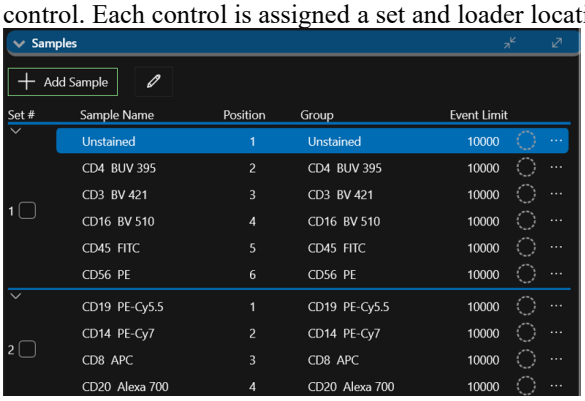
1. From the **Fluorophores Screen** select:
   Off/None – Turns off auto-compensation.
   No Negative – Turns on auto-compensation and specifies no negative control.
   Negative Control – Turns on auto-compensation and specifies a negative control.
   Universal Negative – Turns on auto-compensation and specifies a universal negative control. Note: The universal negative uses the fluorescence distribution from the negative tube in the compensation wizard. Most users prefer not to use a universal negative, particularly where compensation controls have differing negative intensities.



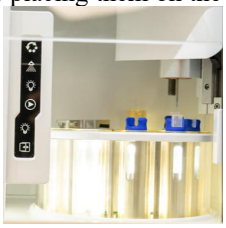
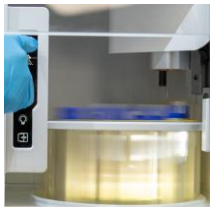
2. After the options in the Fluorophores screen are configured, click the **Next** arrow to move to the **Workspace**. This is the main user interface for visualizing your data and controlling the instrument as well as for setting up and sorting.

3. The system automatically generates Groups to allocate the different parts of the experiment. The Primary Group is designated for the sample that is to be acquired or sorted. Each single-color control is assigned to a group and an Unstained Group is created for the negative control.

4. By default, the groups are shown collapsed in the workspace except for the Unstained group. If required, use **Expand All** or expand each group template individually as needed.
5. SQ Software creates samples in the sample section of the control panel for each single color and unstained control. Each control is assigned a set and loader location. Alternate locations can be assigned as needed.



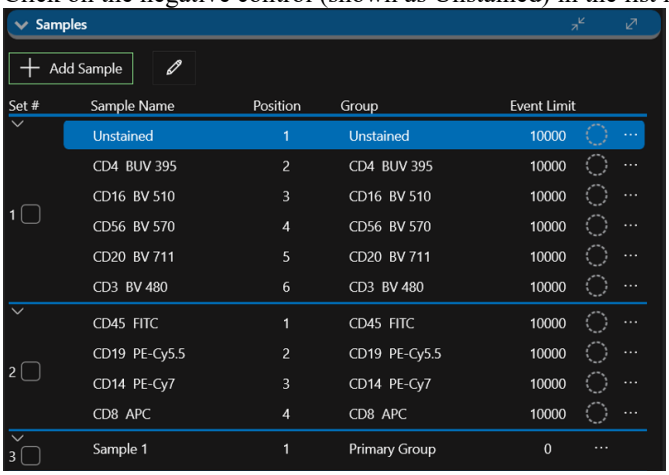
6. Load the controls and samples into the sample loader, according to the order shown in the sample list. The loader can be rotated to access the rear tubes using the top touch panel button. If the sample tube holder adapter does not match the tube size, pull it out of the loader and replace it with the appropriate size adapter. Adapters may be already in the loader or in the storage area on the left side of the instrument. If replacing adapters, rotate until they drop into place with the locating pin. Vortex, uncap and filter the tubes if necessary, before placing them on the loader.


7. While in the biosafety enclosure environment, the sort collection tubes can be loaded for later use. The sort chamber door can be opened and closed using the bottom button on the touch panel.
8. In SQ Software, set sample agitation and temperature to support cell viability. Note: We recommend setting the temperature and waiting for stabilization before loading sensitive samples.

Running the Negative Control

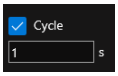
1. Click on the negative control (shown as Unstained) in the list in the lower-right corner of the screen.



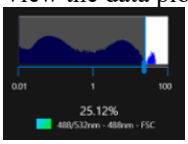
2. If required, edit the event limit for this control. You can also right-click in the **Event Limit** field and set the limit for all samples in the control set.
3. Set the sample pressure to Low (30%). Note: This is a good starting place if the sample concentration is not known and before voltage and threshold are optimized.

4. Click the Run button to acquire sample. Note: In this mode data is shown but not stored.

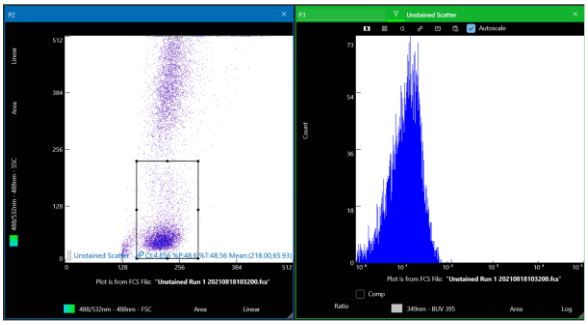
5. Enable cycle mode to quickly see any changes in the data. Remember to disable cycle mode when you want to record data later.



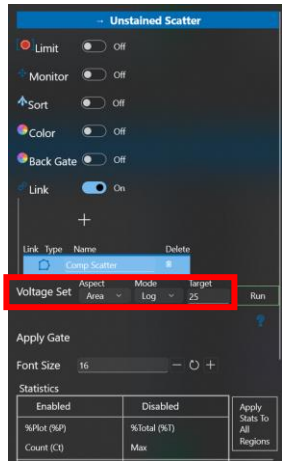
6. View the data plots and adjust the threshold level plot to see the data you require.



7. Adjust the sample pressure to produce a flow rate of 500 to 2,000 events per second depending on sample concentration. For every 1,000 eps desired, the sample typically should be concentrated to 1 million per ml.
8. Adjust PMT voltages in the FSC vs. SSC plot to fine tune the position of the target population. You can adjust voltages using the PMT sliders on each axis or by entering a value into the fields next to them or by using the mouse scroll wheel for fine adjustment.



9. Verify that the scatter gate encompasses the primary cell population.
10. Right-click the scatter region to adjust the PMT voltages for each of the fluorescence plots. Note: Carefully adjust fluorescence PMT voltages until 95% of negative cells are on scale i.e. just to the right of 10^0 in the log plot. Uncompensated data should be viewed in log mode (not hyper log or biexponential), because it is easier to see if most of the events are on scale. Auto voltage adjustment can be done by right-clicking in the scatter region and using the set voltage function. Make sure that most if not all negative populations are above 10^0.

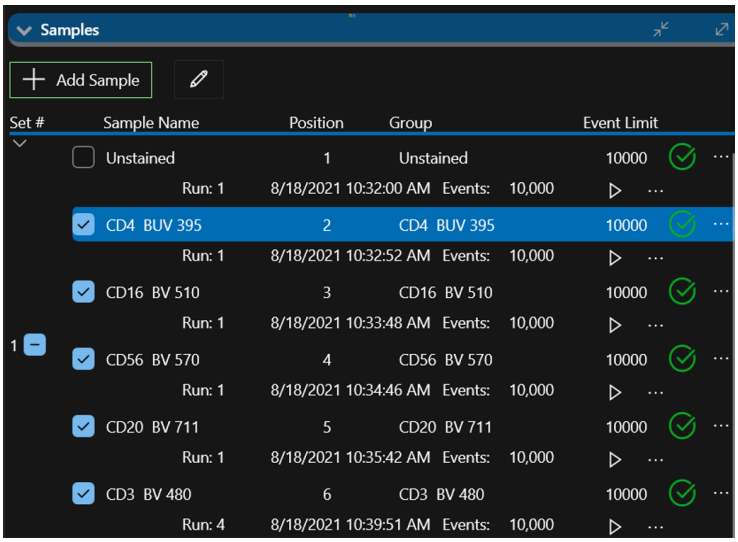


11. Turn off cycle mode.
12. Click Record Sample.

13. If an event limit is set, sample data will be recorded until that limit is reached. If an event limit is not set, stop sample when you have recorded enough events.

Running the Single-Color Controls

1. The single-color controls can be run individually or automatically. To run single-color controls automatically, select the **Set 1** checkbox, deselect the negative control because it has already been run.



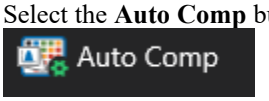
2. Click **Record Sample**.

3. Controls are run in position order and the probe is washed between each control.
4. An FCS file will be recorded for each sample up to the set event limit.
5. Additional single-color controls can be run as additional sets. Unload the controls in the loader that have already been acquired and load the next controls and sample tubes according to the order in the sample list.

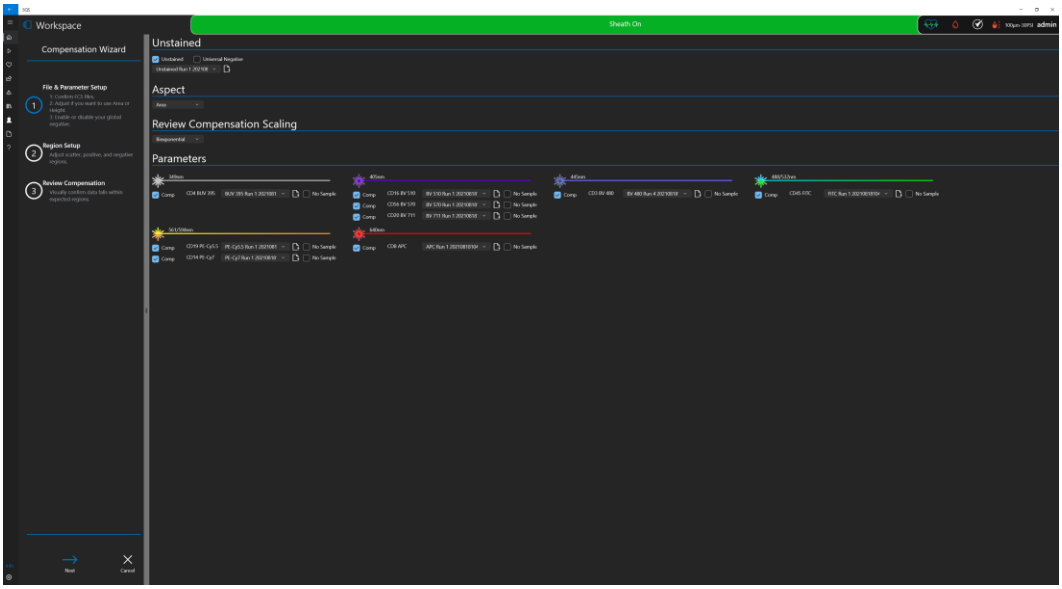
Auto Compensation

After the unstained and single-color controls have been acquired, the compensation wizard can be used.

1. Select the **Auto Comp** button at the top of the workspace.



2. The Compensation Wizard appears. There are three main steps displayed on the left.

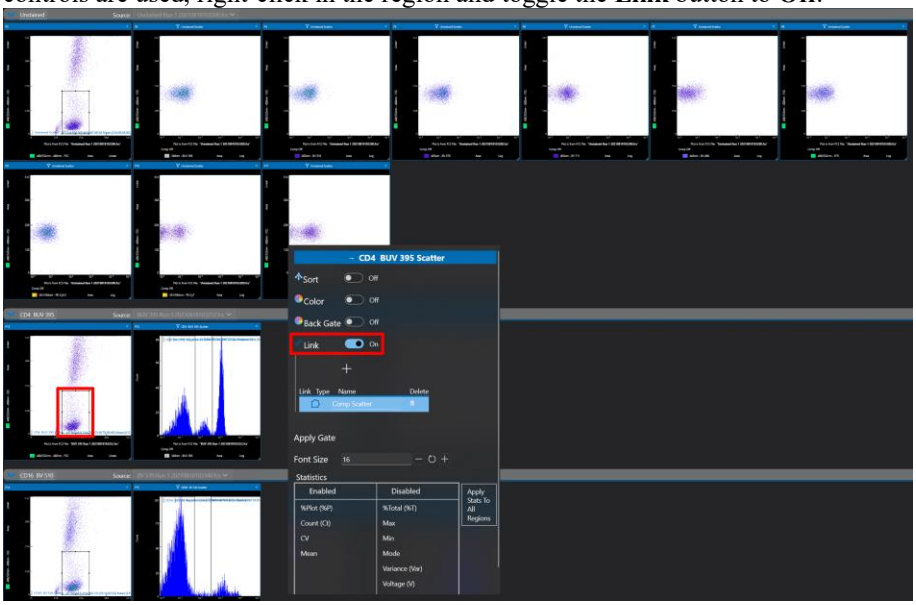


- **Unstained** – If the experiment was designed with a negative control, the **Unstained** checkbox is automatically selected.
- **Universal Negative Control** – If the single-color control does not have a negative population, a universal negative can be used. This option uses the fluorescence distribution from the unstained tube in the compensation calculation. Most researchers do not use a universal negative, particularly where

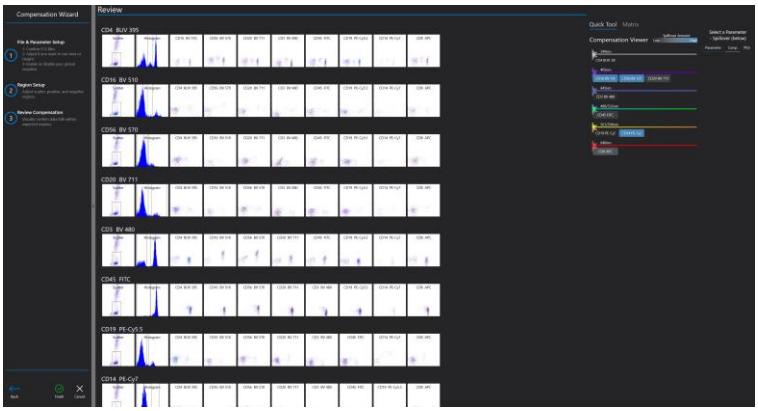
compensation controls have differing negative intensities.

- **Aspect** – Area or Height can be selected for compensation matrix calculations. Area is selected by default.
- **Review Compensation Scaling** – Log or Transformed Scaling can be selected for the Review Compensation screen.
- **Parameters** – The fluorescence parameters to be compensated are shown, grouped by laser excitation. By default, the single-color control data file selection boxes are populated with the files from the current experiment. However, another data file can be selected, if for instance, a control had to be reacquired.
- **Comp** – Select this checkbox to include this parameter in the compensation calculation. Note: All parameters are included by default.
- **No Sample** – Select this checkbox to exclude the parameter from affecting the compensation of other parameters but allow the other parameters to be compensated into it. This is useful for live/dead markers that will be gated out anyway.
- **Cancel** – Click Cancel only to exit the auto compensation workflow and return to the workspace.

3. Select the **Next** arrow to go to the next screen in the wizard. Plots and groups from the experiment are shown with the FCS files loaded into each. Adjust the regions so the rectangular scatter region encompasses the population of interest in the FSC vs. SSC plots. Adjust the fluorescence positive and negative bar regions to encompass the positive and negative populations within each single-color control. Note: The scatter region is automatically linked throughout the groups. Therefore, all scatter gates will move to the same location when adjusted. If individual control of a scatter region is needed, such as when bead and cell controls are used, right-click in the region and toggle the **Link** button to **Off**.



4. Select next to start the calculation.
5. When the compensation calculation is complete click the **OK** button.
6. SQ Software displays the data from the control samples. Review each plot to make sure the results are within expectations.

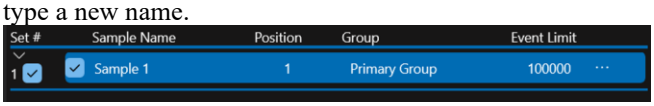


7. If further compensation is required, click the **Comp** button. Use **Quick Mode** or **Matrix Mode** to adjust compensation.
8. When all plots are adjusted, click the **Finish** button. Compensation is applied to all plots where the **Comp** checkbox is selected.

Acquisition

After you have set up the experiment and performed color compensation, the system is ready to acquire test sample data. Plots, regions and gates can be built before or during acquisition.

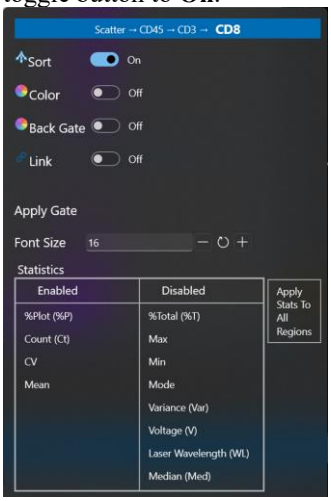
1. Click on **Sample 1** in the sample list. To rename the sample, double-click in the Sample Name field and type a new name.



2. If you intend to acquire sample data but do not intend to sort, click the **Record Sample Data** button and allow the system to reach the event limit.

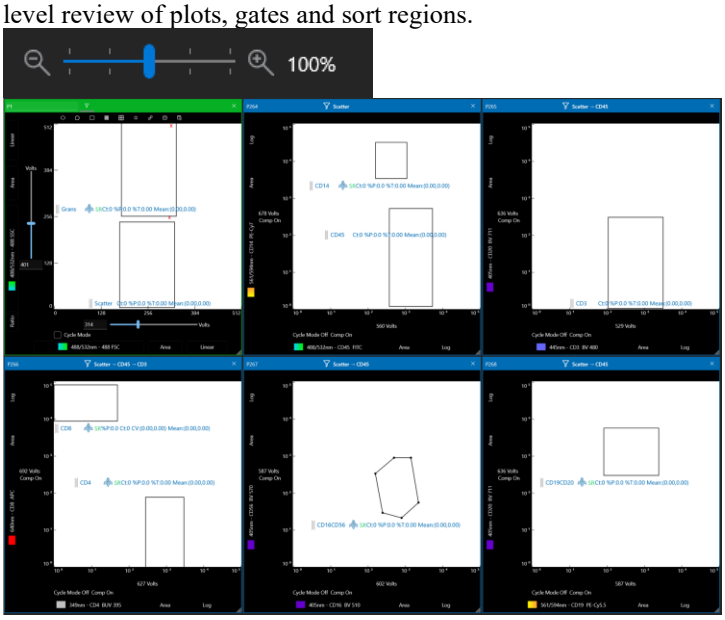
3. The acquisition can be stopped at any time by clicking the **Stop Acquisition** button.

4. If you intend to sort, acquire enough sample to confirm regions and gates. Adjust regions and gates to identify the populations of interest.
5. Stop acquiring sample to set sort regions. Right-click in a region you intend to sort and click the **Sort** toggle button to **On**.

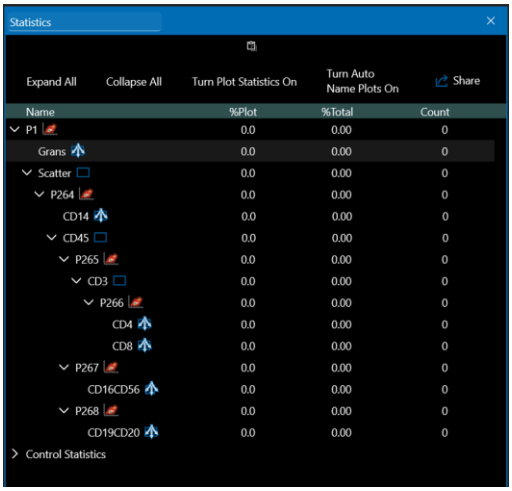


6. A **Sort Region** icon appears on the plot. Additional sort regions can be created. Keep in mind the sort media you intend to use when creating sort regions.

7. The **Zoom Workspace** tool can be used to fit the plots created to the workspace into one view for a high-level review of plots, gates and sort regions.



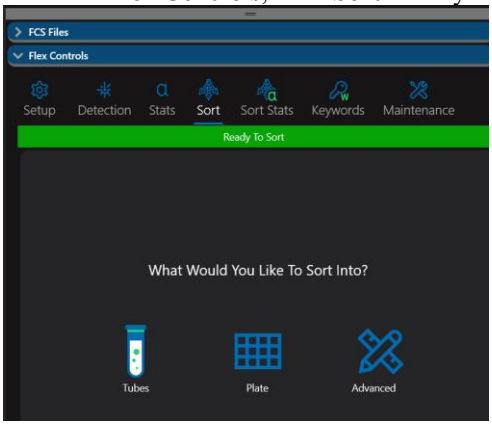
8. Click the **Stats** button to view a summary of region statistics and hierarchy. Note: Each plot has its own **Stats** button for additional information.



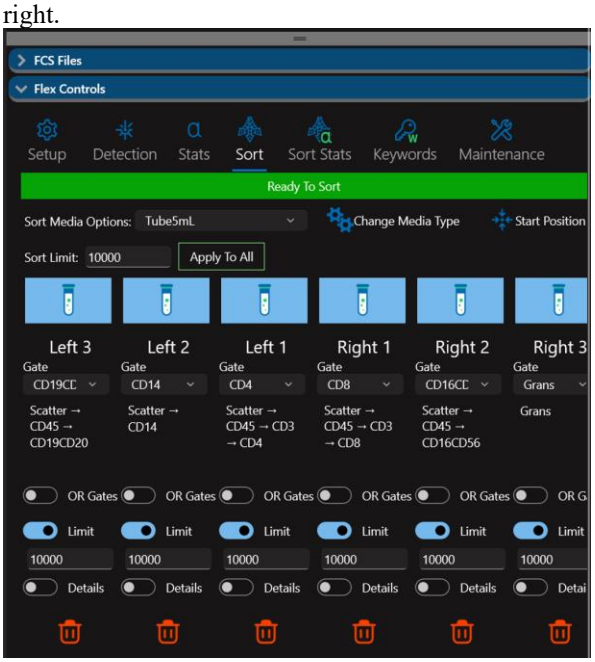
Select Sort Media (Tubes)

After sort regions are created sort media can be selected.

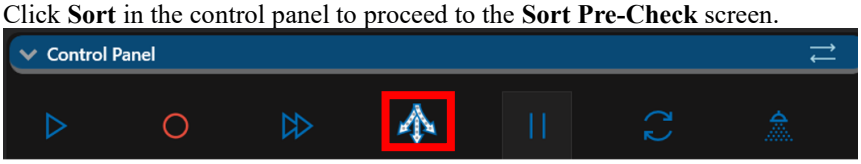
1. From the **Flex Controls**, click **Sort**. The system sort status appears.



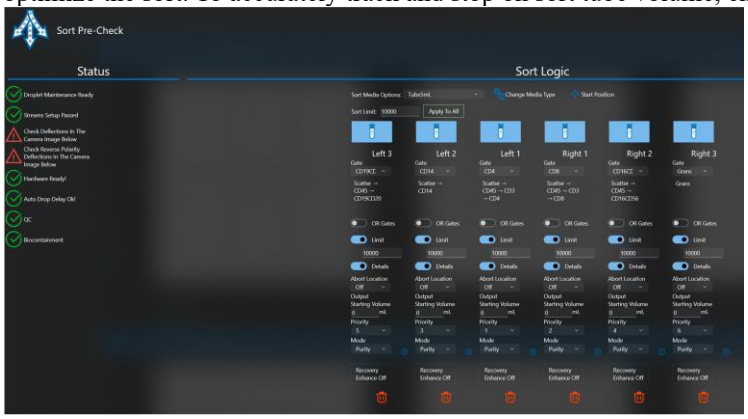
2. Click on the media you would like to sort into. **Tubes** are selected in this example. The system automatically allocates the direction the sort regions will be sorted. Note: When tube sorting is selected, the default is 5 ml tubes with 6-way sorting. This allows up to three tubes on the left and three tubes on the right.



3. If required, set a limit for the number of cells to be sorted for each direction. If all directions require the same limit enter the sort limit and select apply to all. When 0 is set as the limit, it will sort until out of sample or the volume limits are met. The **Details** toggle buttons under each tube can be expanded for more choices such as sort mode and envelope.
4. Click **Sort** in the control panel to proceed to the **Sort Pre-Check** screen.

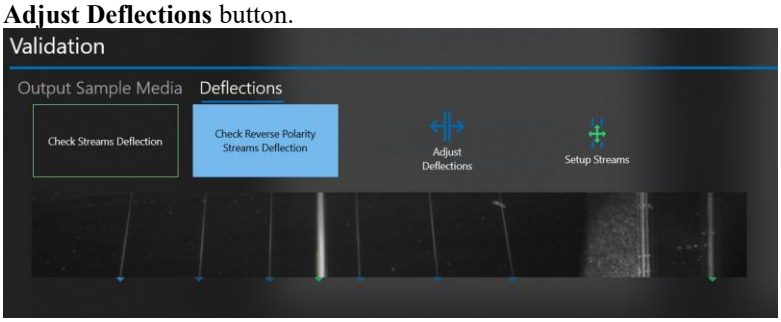


5. The **Sort Pre-Check** screen allows review of the system sort status and all the settings that can be used to optimize the sort. To accurately track and stop on sort tube volume, enter a starting volume for each tube.



6. Toggle the **Check Streams Deflection** button to check stream deflection. Make sure the streams are aligned with the blue markers. If deflection is not correct, click the **Adjust Deflections** button.

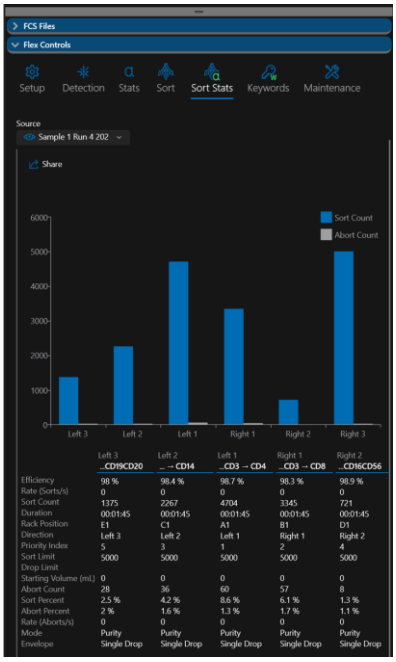
7. Toggle the **Reverse Polarity Streams Deflection** button to check stream deflection with reverse polarity applied. Make sure the streams are aligned with the blue markers. If deflection is not correct, click the **Adjust Deflections** button.



8. The media camera is also shown in this view to visually confirm the proper sort collection media is installed in the sort output area. Click the Move to start position for a better view of the media. Test Sort Targets is only available for plates.

9. Click **Start** to sort. During the sort, the event rate can be adjusted to optimize the efficiency of the sort. If sort regions/gates are adjusted during the sort, the sort will pause and then resume when the region is reset to its new location.

10. The **Control Panel**, **Flex Controls** and **Sort Stats** are shown during the sort by default.

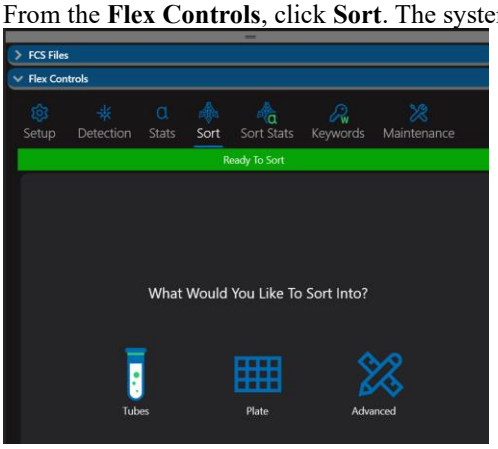


11. In the upper part of **Sort Stats** is a graph that displays the numbers of cells sorted into each sort receptacle and the number of aborts. Below the sort plots are real-time sort statistics showing the on-going details of the sort. Scroll to see additional data if necessary.
12. The sort continues until the sort limits are met, the maximum volume is reached, or the end of sample is detected.
13. When the sort is complete, a detailed sort report appears. This report can be exported by clicking the **Share** button.

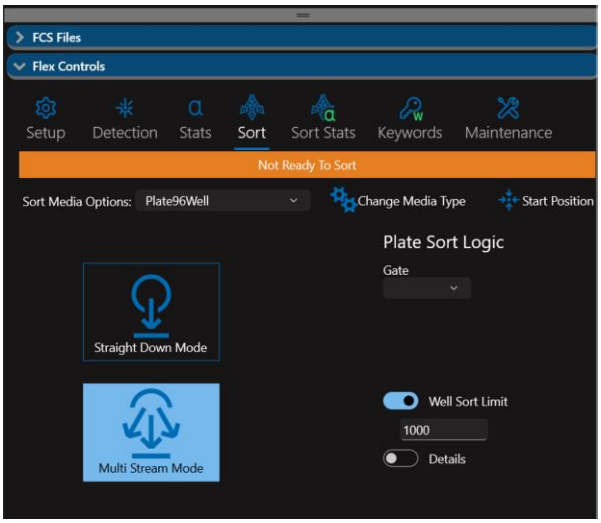
Select Sort Media (Plate)

After sort regions are created sort media can be selected.

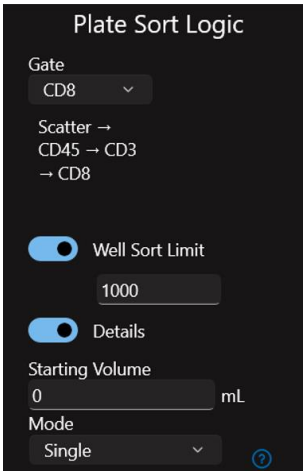
1. From the **Flex Controls**, click **Sort**. The system sort status will be shown.



2. Click on the media you would like to sort into. **Plate** is selected in this example.

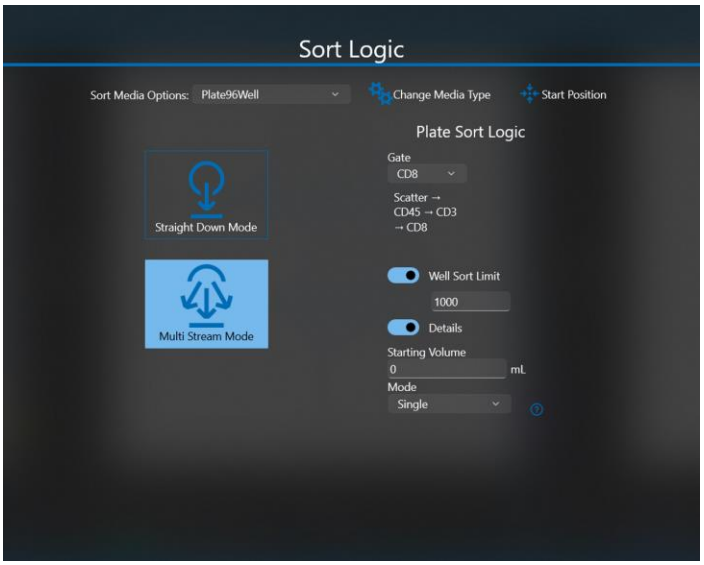


3. Select the plate type from the **Sort Media Options** dropdown list. Enter a value in the **Well Sort Limit** field and click the **Apply To All** button. Note: The **Straight Down Mode** button is an option ideal for plates with very small wells such as a 384-well plate.
4. Under **Plate Sort Logic**, select the gate from which you intend to sort. Details such as starting volume and mode can also be defined.

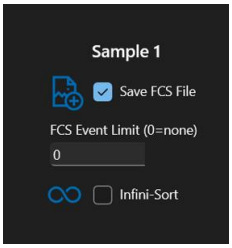


5. Click **Sort** in the control panel to proceed to the **Sort Pre-Check** screen.

6. The **Sort Pre-Check** screen allows review of the system sort status and all the settings that can be used to optimize the sort.

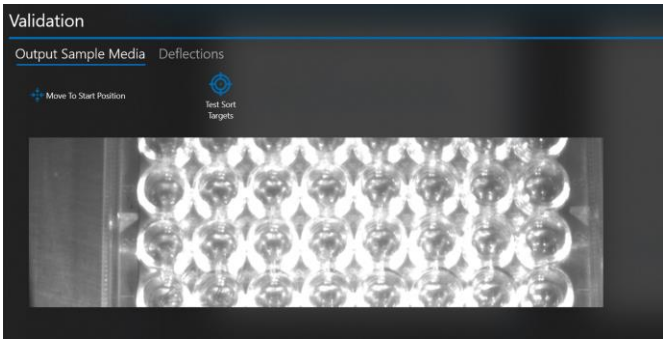


7. If you want to limit the events that are recorded in the FCS file, but you do not want to apply the same limit to the sort, type the limit value in the **FCS Event Limit** field. Note: This option allows you to save the FCS file as a subset of the entire sort and will result in a smaller FCS file without affecting the sort.



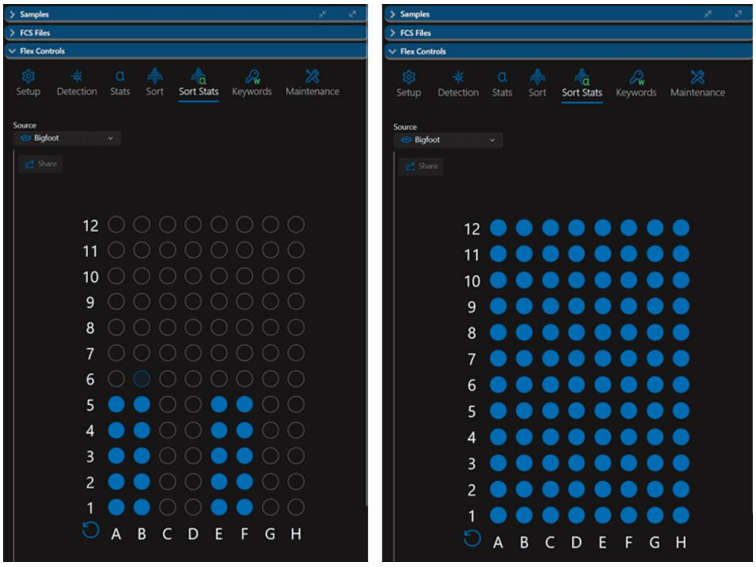
8. Select the **Infini Sort** checkbox to pause sample flow at the end of each plate sort and open the sort door to allow the plate to be exchanged during multi-plate sorting.
9. Click the **Check Streams Deflection** button to check stream deflection. Make sure the streams are aligned with the blue markers. If deflection is not correct, click **Cancel**.

10. Click **Adjust Sort Deflections**. Click **Check Streams Deflection** again. If the streams are aligned with the blue targets, click **Confirm**. Note: If you encounter excessive stream fanning, you can click **Setup Streams** to automatically recalculate charge phase and defanning. If you know that charge phase is already correct, but the streams are still fanning, click **Calculate Defanning** to automatically adjust the streams. The **Defanning** slider allows you to manually adjust the streams.
11. Toggle the **Check Reverse Polarity** button to check streams deflection. Make sure the streams are aligned with the blue targets. If deflection is not correct click **Cancel** and adjust deflections.
12. The media camera is also shown in this view to visually confirm the proper sort collection media is installed in the sort output area. Click the **Move To Start Position** for a better view of the media. It is optional to click **Test Sort Targets** to deposit droplets onto each well.

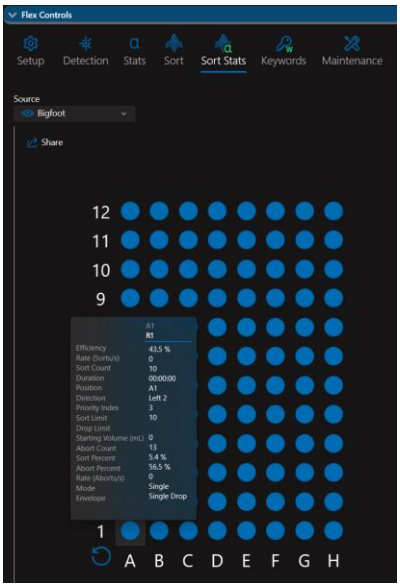


13. Click **Start** to sort. During the sort, the event rate can be adjusted to optimize the efficiency of the sort. If sort regions/gates are adjusted during the sort, the sort will pause and then resume when the region is reset to its new location.

14. The **Control Panel**, **Flex Controls** and **Sort Stats** are shown during the sort by default. The images below show a plate sort in progress and then complete. If the Straight Down Sort option was selected, you will see one row at a time rather than several rows sorted at once.



15. The sort continues until the sort limits are met, the maximum volume is reached, or the end of sample is detected.
16. You can hover the mouse over a completed well to view statistics for the well.



17. When the sort is complete, a detailed sort report can be exported by clicking the **Share** button.

Select Sort Media (Advanced)

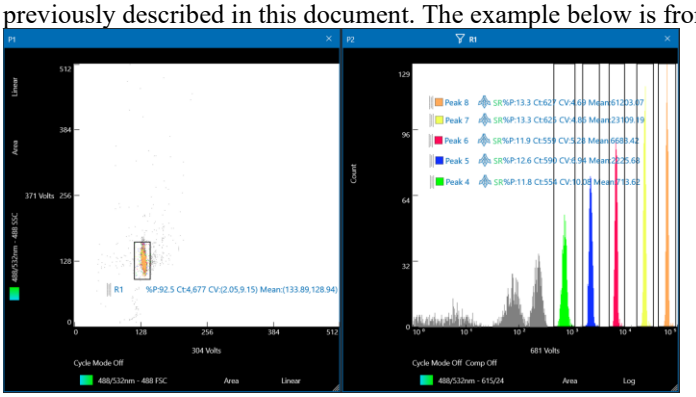
Advanced sorting includes additional options for sorting into tubes and plates such as the ability to use different tube types and the option to allocate different gates, sort counts and sort modes to different tubes or wells.

Advanced Tube Sorting – A single tube type, or mixed tube types can be selected and different gates, sort counts and sort modes can be specified for the various collection tube locations.

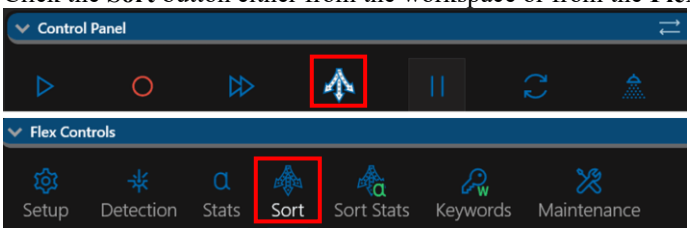
Advanced Plate Sorting – A singe plate type is selected and different gates, sort counts and sort modes can be specified for each well.

Using Advanced Sorting Mode Single Tube Type

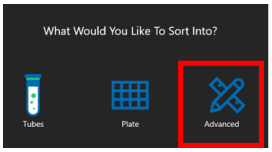
1. Set up acquisition and sort gates for either a spectral experiment or a compensated experiment as previously described in this document. The example below is from an 8-peak bead sample.



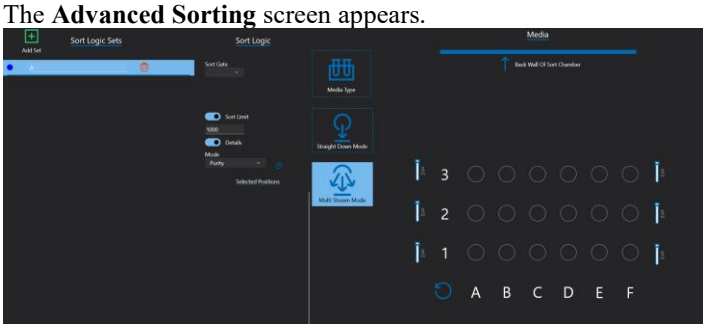
2. Click the **Sort** button either from the workspace or from the **Flex Controls**.



3. Click the **Advanced** button.

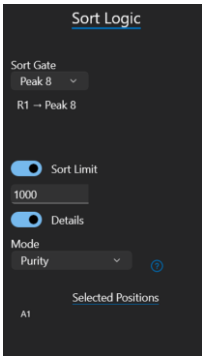


4. The **Advanced Sorting** screen appears.

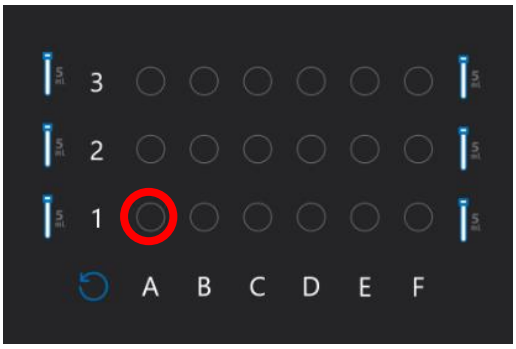


5. Advanced mode utilizes **Sort Logic Sets**. A set is a group of sort instructions such as sort gate, number of cells to be sorted and details like sort mode.
6. Click the **+Add Set** button.

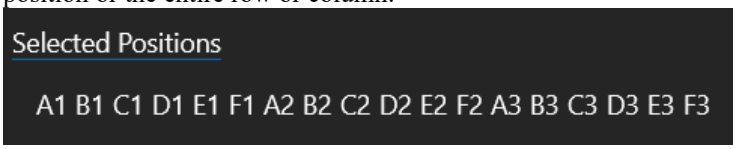
7. Enter the set name and select the Tab or Enter key. Note: if you need to delete a set, click the **Trash** button.
8. Click on the set to highlight it and then select the sort gate, sort limit and sort details for the set.



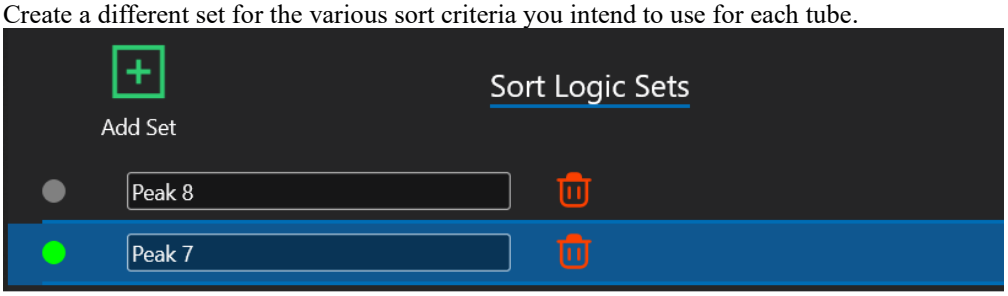
9. The default media is 5mL tubes. Please note the orientation of the tube holder in relation to the representation in the software. The **A1** position for each is shown below.



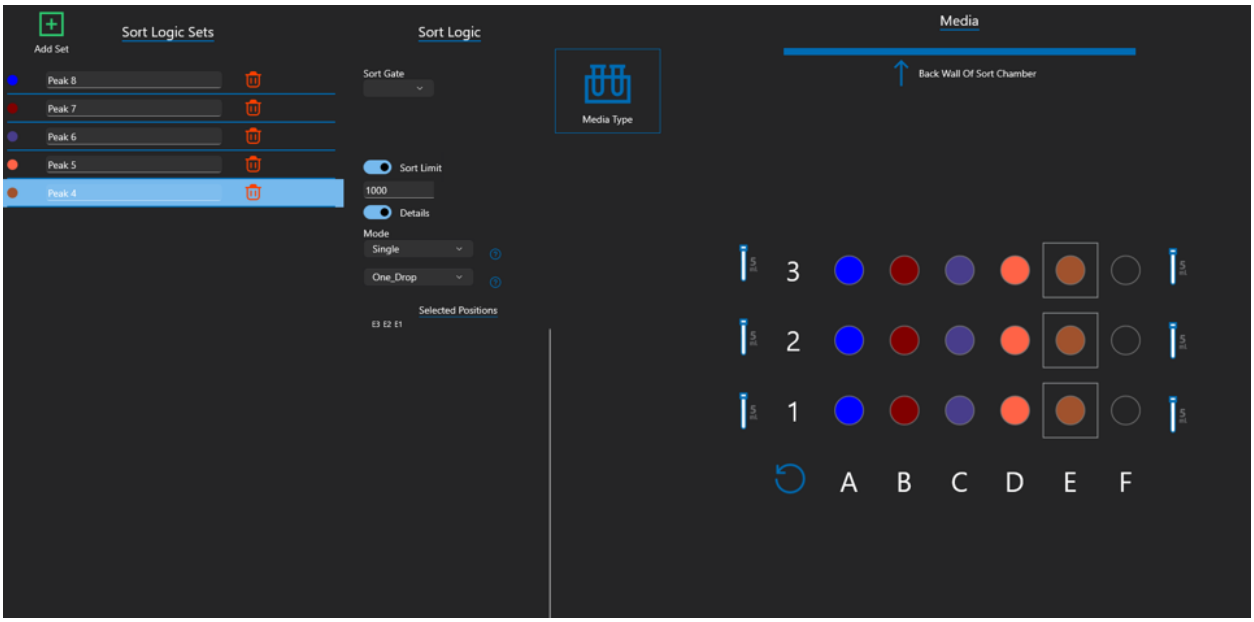
10. To associate a set with a tube, make sure the set is highlighted and then click on the tube position. To select an entire row, click the letter heading. To select an entire column, click the number heading.
11. The selected positions for the set will be shown. Note: To clear a set from a selected position, click on the position or the entire row or column.



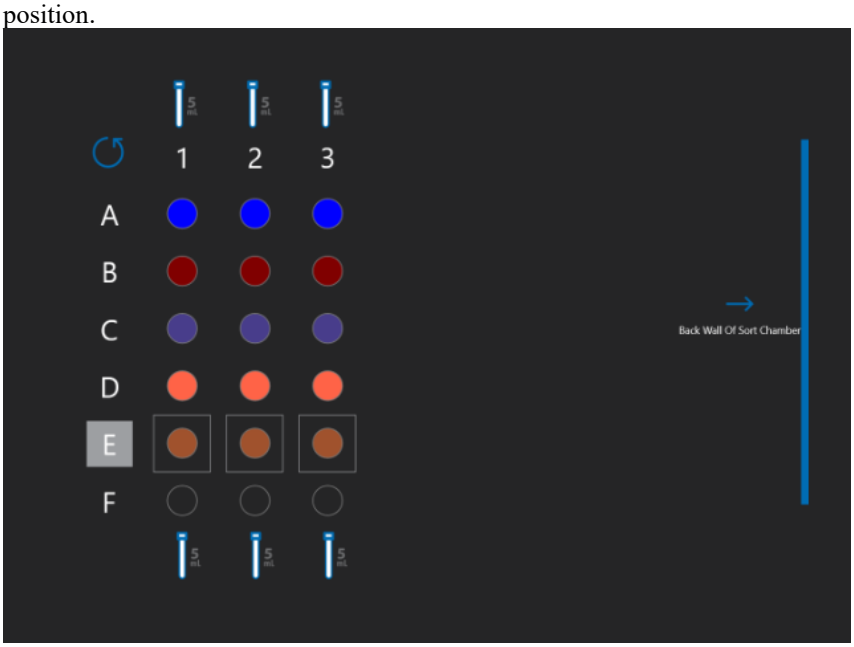
12. Create a different set for the various sort criteria you intend to use for each tube.



13. Name the set and allocate it to the required tube positions.
14. Click **Media Type** and select tubes.



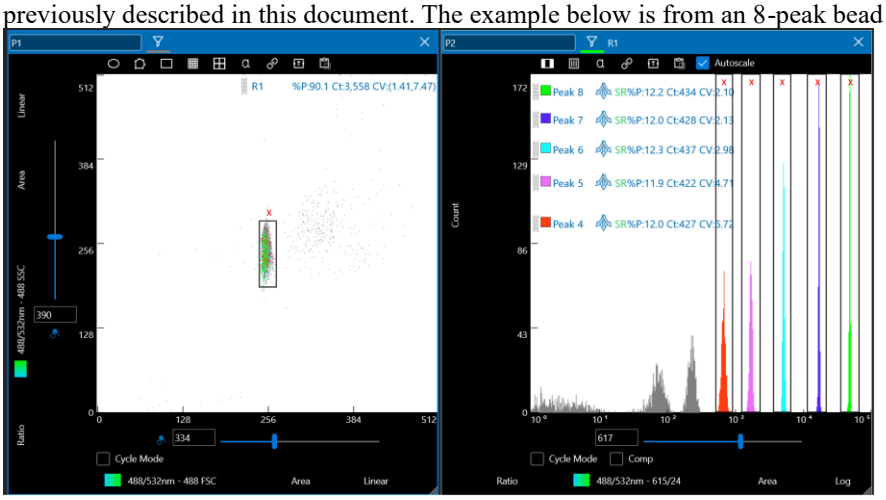
15. If you prefer to view the tube rack graphic in a different orientation, click the rotate button near the A1 position.



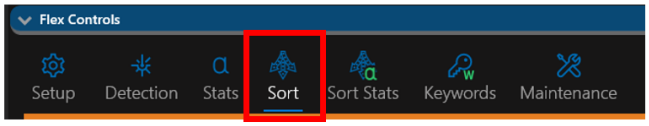
16. Click Sort in the control panel to proceed with sorting.

Using Advanced Sorting Mode Mixed Tube Types

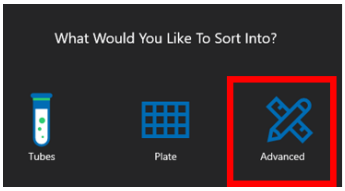
1. Set up acquisition and sort gates for either a spectral experiment or a compensated experiment as previously described in this document. The example below is from an 8-peak bead sample.



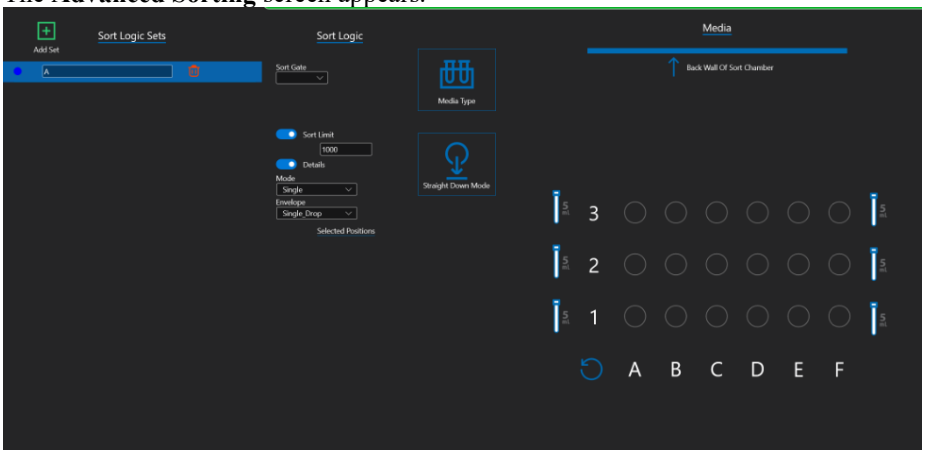
2. Click the **Sort** button either from the workspace or from the **Flex Controls**.



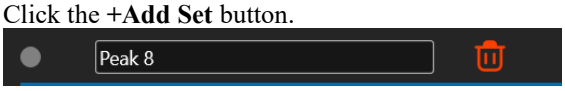
3. Click the **Advanced** button.



4. The **Advanced Sorting** screen appears.

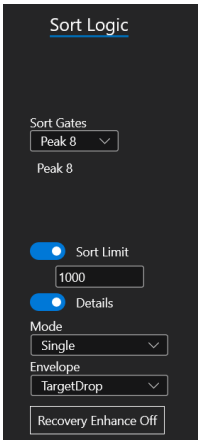


5. Advanced mode utilizes Sort Logic Sets. A set is a group of sort instructions such as sort gate, number of cells to be sorted and details like sort mode.
6. Click the +**Add Set** button.



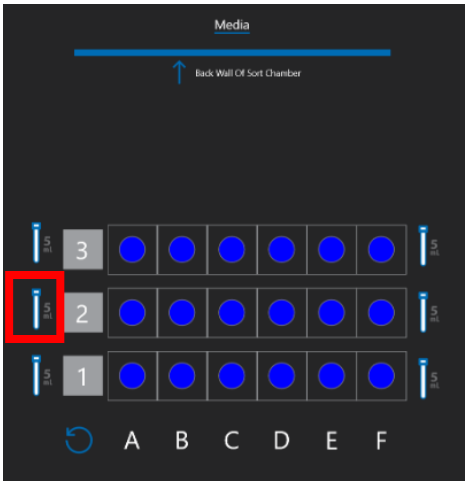
7. Enter the set name and select the Tab or Enter key. Note: if you need to delete a set, click the **Trash** button.

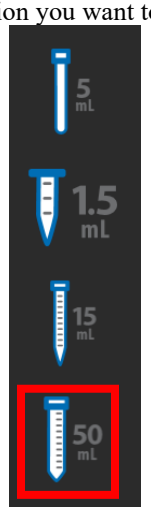
8. Click on the set to highlight it and then select the sort gate, sort limit and sort details for the set.



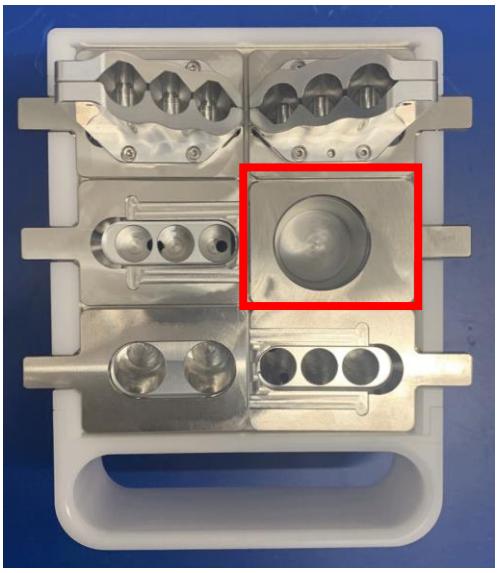
9. The default media is 5mL tubes. However, the tube holder can be set up for a variety of tube types as shown below and the software can be set to match the setup. (Get new from Manufacturing)

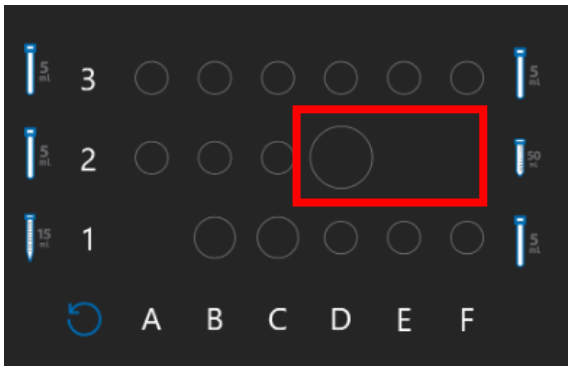
10. Click the tube media button next to the location you want to change. An options menu appears.



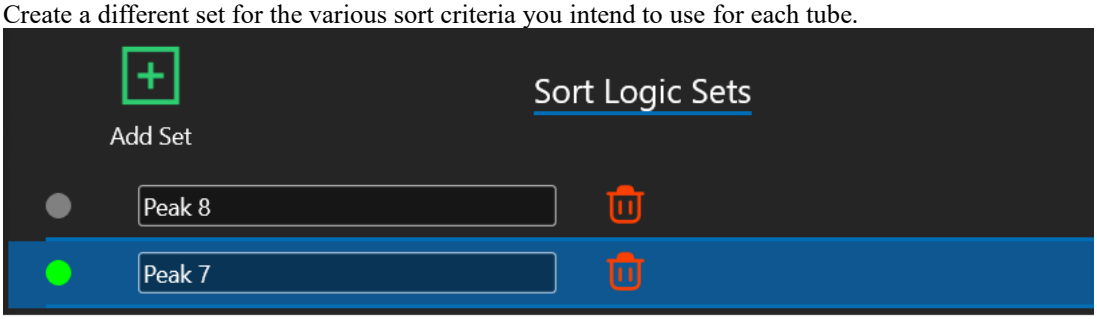


11. Click the tube type that matches the tube type in physical location of the output holder. Do this for all tube types until the software representation of the tube holder is identical to the tube holder you intend to use for sorting.

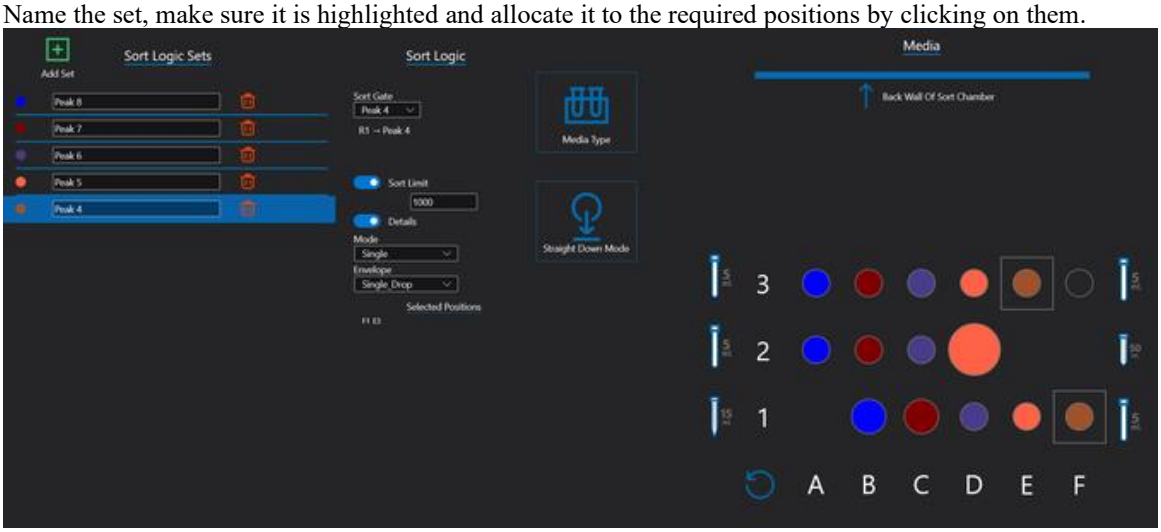




12. To associate a set with a tube, make sure the set is highlighted and then click on the tube position.
13. The selected positions for the set will be shown. Note: To clear a set from a selected position, click on the position or the entire row or column.
14. Create a different set for the various sort criteria you intend to use for each tube.



15. Name the set, make sure it is highlighted and allocate it to the required positions by clicking on them.

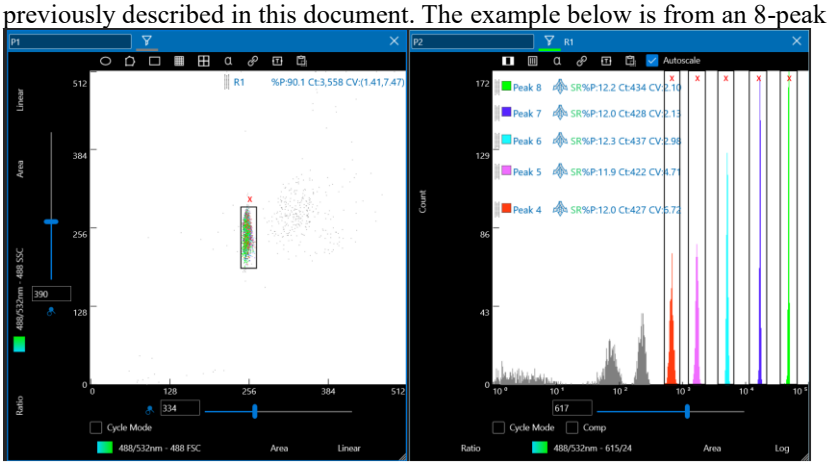


16. Click Sort in the control panel to proceed with sorting.

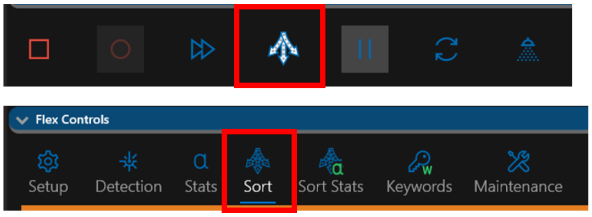
Using Advanced Sorting Mode for Plates

The Bigfoot advanced sort options allow you to select different gates, sort counts, or sort modes for each well of the plate providing optimal flexibility for complex sorting experiments. The Infinisort feature is particularly helpful when sorting into multiple plates. Within the biosafety containment area, Infinisort pauses sample flow at the end of each plate sort and then opens the sort output chamber for rapid plate removal and insertion. The user can then resume the sort from within the biosafety containment area without additional risk to sample or operator safety.
Note: Multi-way plate sorting is used by default.

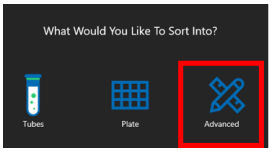
1. Set up acquisition and sort gates for either a spectral experiment or a compensated experiment as previously described in this document. The example below is from an 8-peak bead sample.



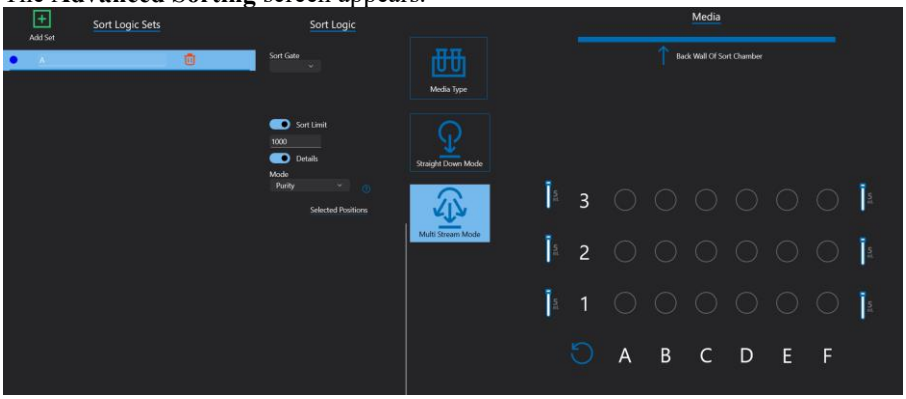
2. Click the **Sort** button either from the workspace or from the **Flex Controls**.



3. Click the **Advanced** button.

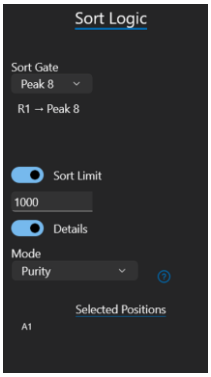


4. The **Advanced Sorting** screen appears.

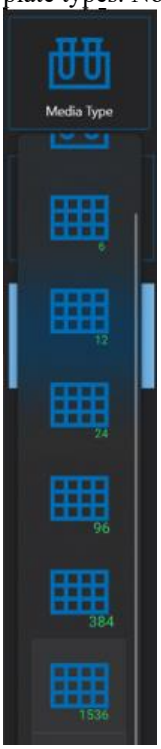


5. Advanced mode utilizes **Sort Logic Sets**. A set is a group of sort instructions such as sort gate, number of cells to be sorted and details like sort mode.
6. Click the **+Add Set** button.

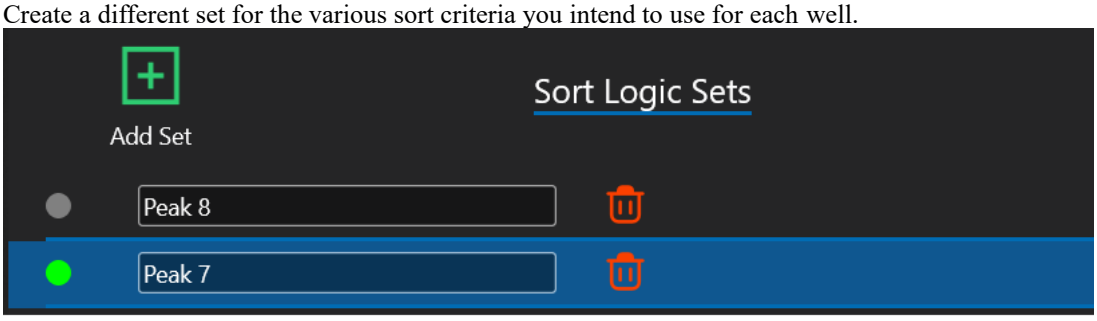
7. Enter the set name and select the Tab or Enter key. Note: if you need to delete a set, click the **Trash** button.
8. Click on the set to highlight it and then select the sort gate, sort limit and sort details for the set.



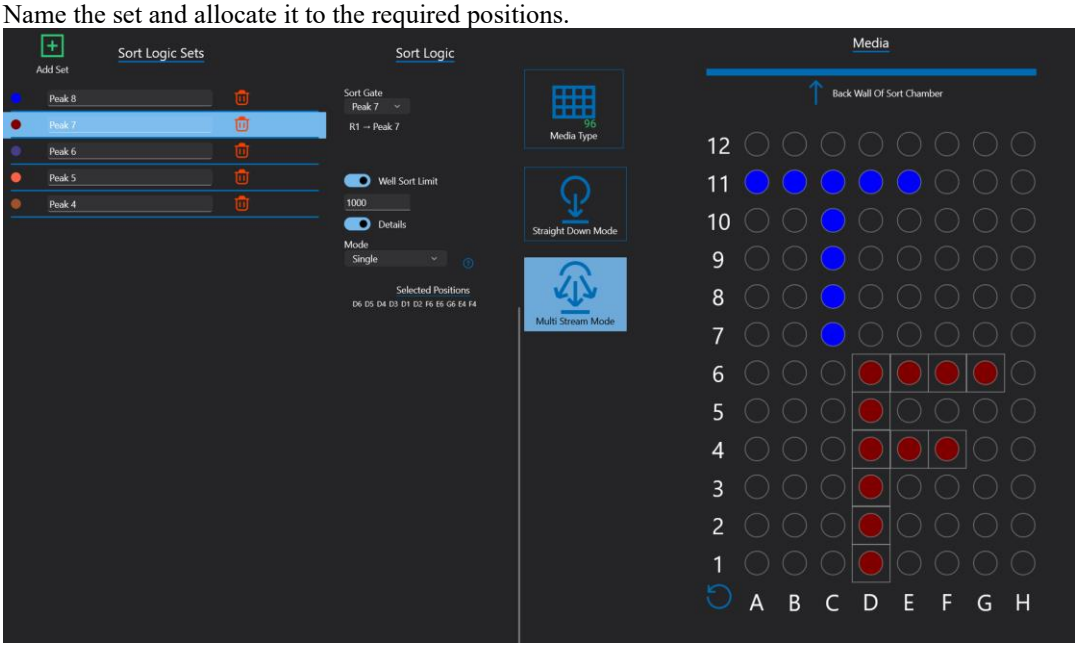
9. The default media is 5mL tubes. To switch to plates, click the **Media Type** button and select from the list of plate types. Note: Currently 1536 and 10x plates are not available for advanced sorting.



10. To associate a set with a well, make sure the set is highlighted and then click on the position.
11. The selected positions for the set will be shown. Note: To clear a set from a selected position, click on the position or the entire row or column.
12. Create a different set for the various sort criteria you intend to use for each well.



13. Name the set and allocate it to the required positions.



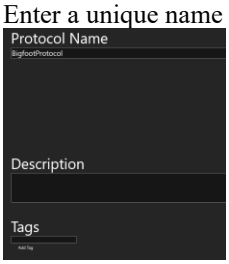
14. Click **Sort** in the control panel to proceed with sorting.

Spectral Sort Experiment Setup

After logging in and starting up the instrument, the Home Screen appears. The following section will guide you through the process of creating a new sort experiment with spectral analysis and sorting. You must have the correct instrument configuration to access spectral analysis and sorting. **Note:** This section will be updated to reflect SQS 1.19 changes in a future release.

1. From the **Home** screen click **New Sort**.

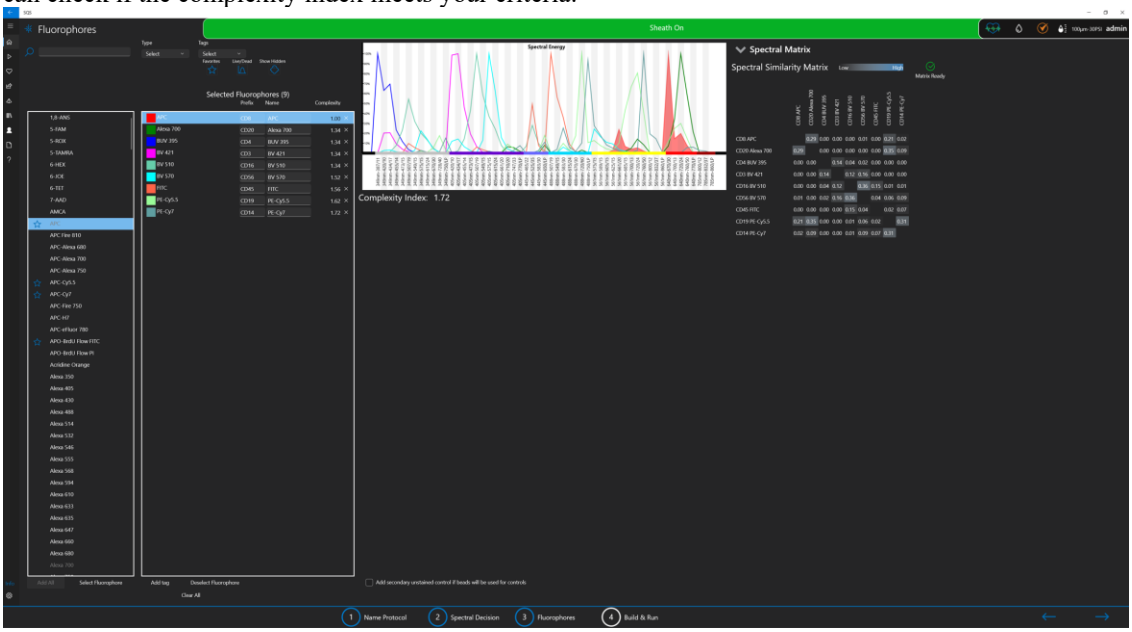
2. Enter a unique name for the experiment. It is optional to enter a description or tags.



3. Click the **Next** arrow.

4. If Bigfoot is configured for spectral sorting, you must choose between conducting a spectral demix experiment and standard compensation. For spectral analysis and sorting select **Yes**.

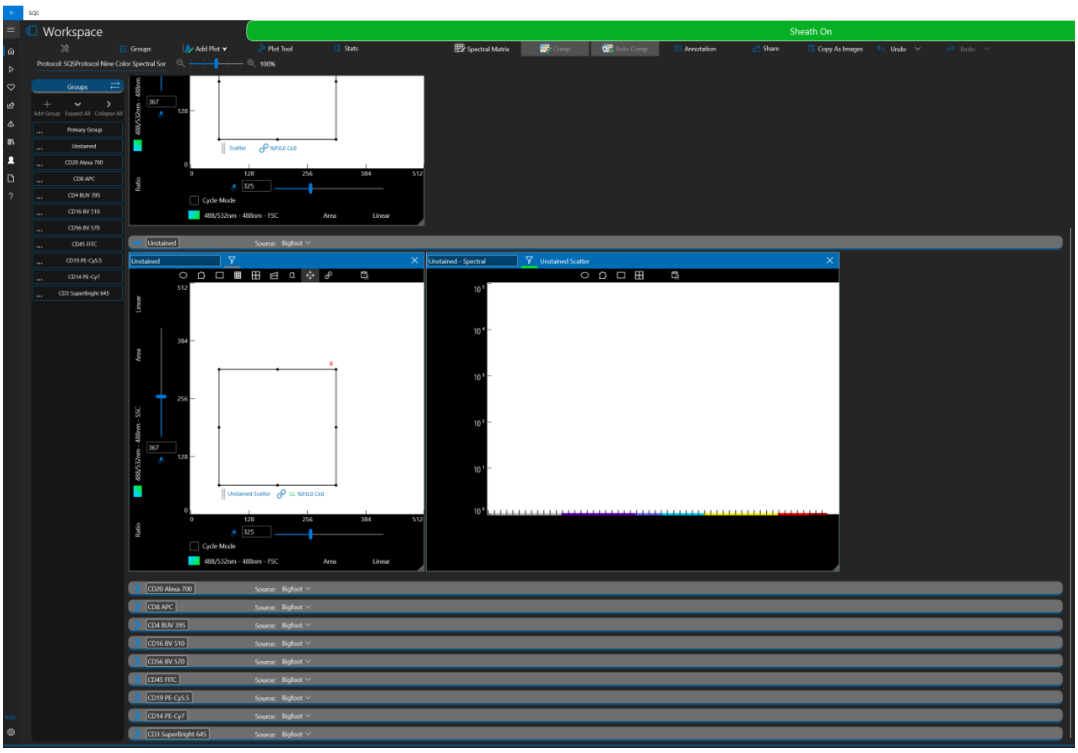
5. To select the fluorophores that you have chosen for your experiment, scroll through the fluorophores list on the left of the screen or type the first few letters of the fluorophore name in the search box until you see the correct item. Double-click the fluorophores to add them to the experiment. Selected fluorophores appear in the **Selected Fluorophores** list and the emission spectra appear on screen. As fluorophores are added you can check if the complexity index meets your criteria.



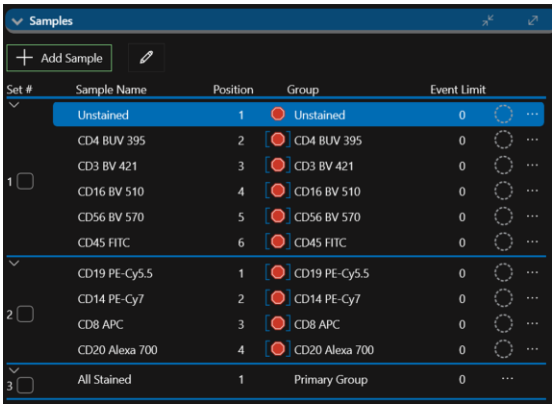
6. The default parameter name is the name of the fluorophore in the fluorophores list. If desired, the Channel Name field can be changed to include more descriptive information for each detector, as shown. A prefix can be added as well.
7. It is optional to select a secondary unstained control if using beads for single-color controls. See Spectral Setup Using Secondary Unstained Control on page 86.

8. After you have selected the fluorophores for the experiment click the **Next** arrow.

9. The software builds the groups required to run the negative and single-color controls you have prepared.

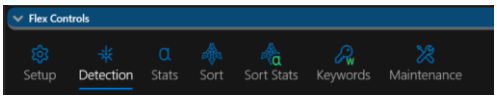


10. A sample list is assembled that allocates each tube to a loader position, these can be run in sets of six or fewer tubes.



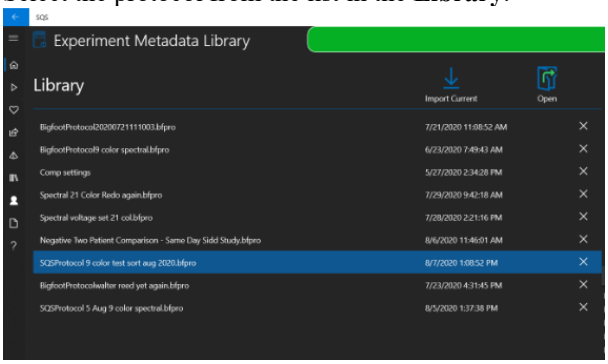
11. In SQ Software, set sample agitation and temperature to support cell viability. Allow temperature changes to stabilize prior to acquisition.

12. You can set detector values while running or import them from a previously run protocol. Note: Default detector values are set from a successfully run QC process.
13. To import voltages from a previously run protocol, click **Detection** in the **Flex Controls** section.

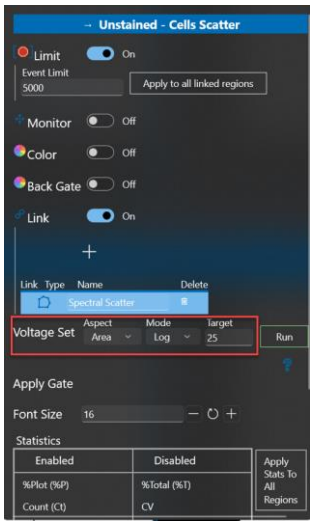


14. Click **Import**.

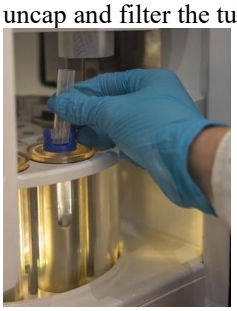
15. Select the protocol from the list in the **Library**.

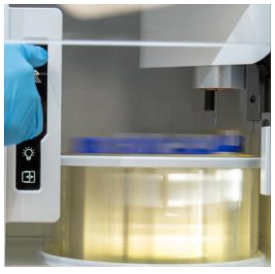


16. Click **Apply**. This will apply voltages from the selected protocol to the current experiment.
17. To set voltages in real time, right-click in the scatter plot region and click in the **Spectral Voltage Target** field.

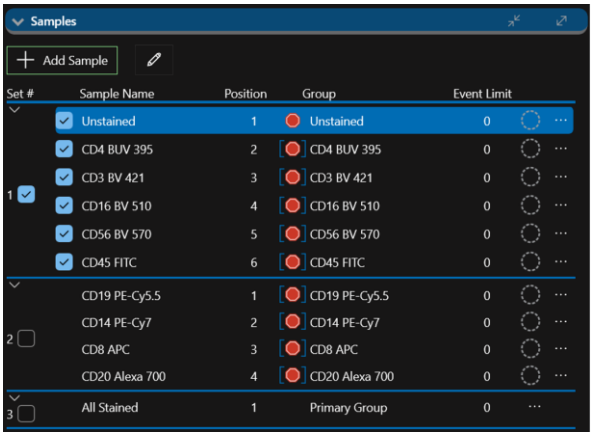


18. Click the **Run** button. The system will automatically set the voltages.
19. Refer to the run order in the sample list and load control samples onto the loader.
20. Load the controls and samples into the sample loader, according to the order shown in the sample list. The loader can be rotated to access the rear tubes using the top touch panel button. If the sample tube holder adapter does not match the tube size, pull it out of the loader and replace it with the appropriate size adapter. Adapters may be already in the loader or in the storage area on the left side of the instrument. Important: Make sure to rotate the tube adapter until the locating pin clicks into place on the loader. Vortex, uncap and filter the tubes if necessary, before placing them on the loader.





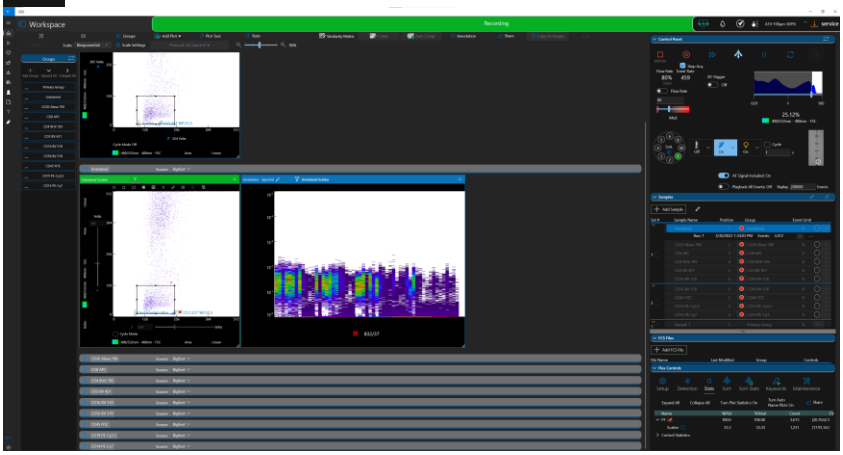

21. Click on the first sample in the list, normally the negative control.



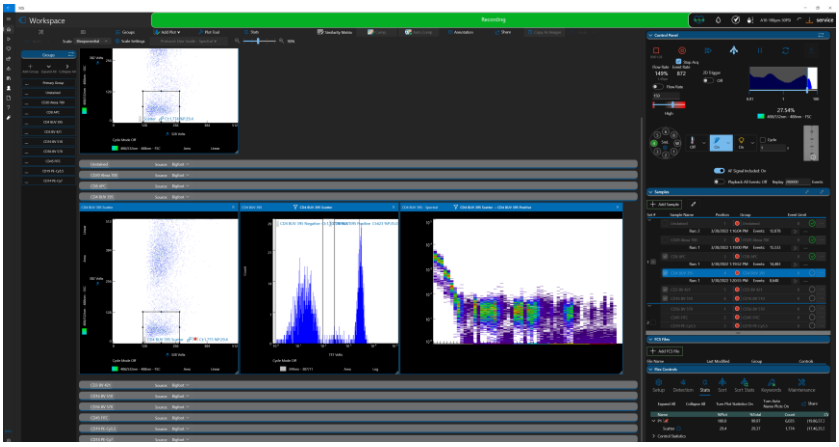
22. Set the **Flow Rate** to **Low**.
23. Click the **Run** button.

24. Adjust the PMT voltages on the FSC and SSC plot to provide the correct view of the sample, adjust the threshold if necessary, check scatter gate positions and adjust them if necessary.
25. Click the **Stop** button.

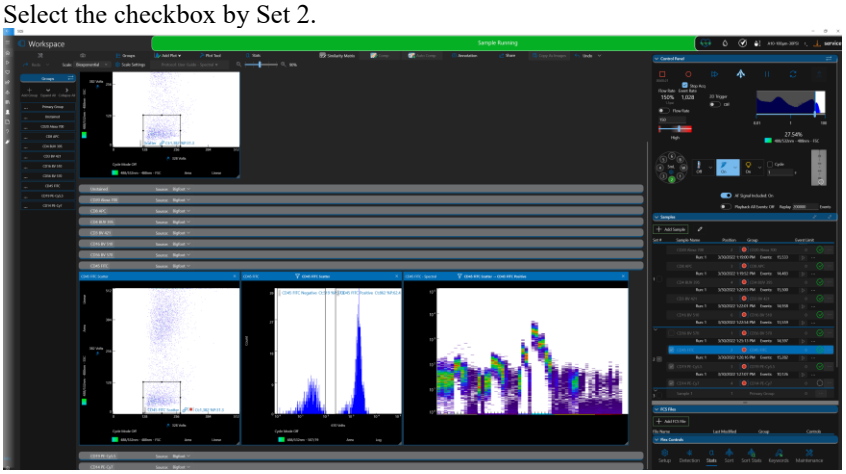
26. Select the **Set 1** checkbox. All the samples in the set will appear checked.
27. Click the **Record Sample Data** button to acquire each of your samples. Note: The spectral acquisition process automatically stops after 5000 Scatter Gate events have been acquired for a sample and moves to the next sample in the set.



28. It is optional to adjust the bar regions in the histograms to display the positive events in the fluorophore graphs. This is just for visualization; the spectral process automatically detects and records all positive events for spectral unmixing. A green checkmark appears next to each sample when it has been recorded and the data has been unmixed.

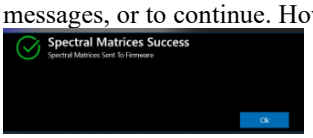


29. When the first set of controls is finished, remove them from the loader.
30. Load the next set of samples.
31. Select the checkbox by Set 2.



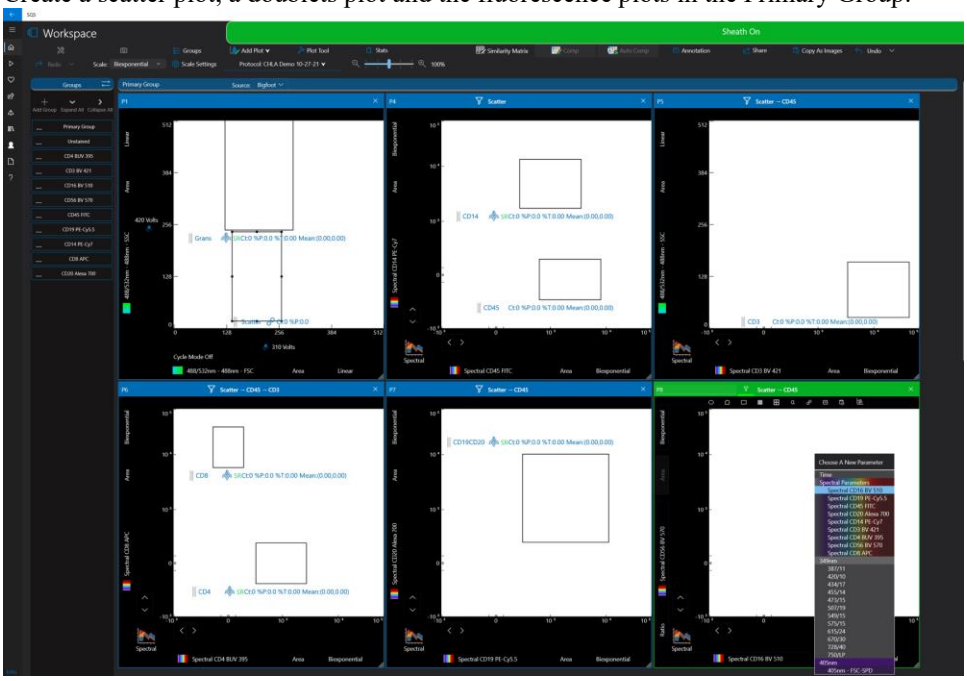
32. Click the **Record Sample** button.

33. After all samples have been acquired, a **Spectral Matrices Success** message appears. Note: Warning messages may appear regarding the quality of the unmixing process. It is optional to address the warning messages, or to continue. However, errors are handled differently.

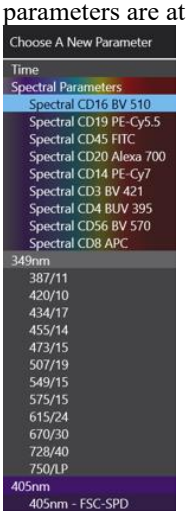


If there were errors during the unmixing process, they will appear in the Samples List and must be resolved before you can continue. Hover the mouse over the orange diamond shape to view the error and a suggestion for resolution. You cannot continue until errors are resolved.

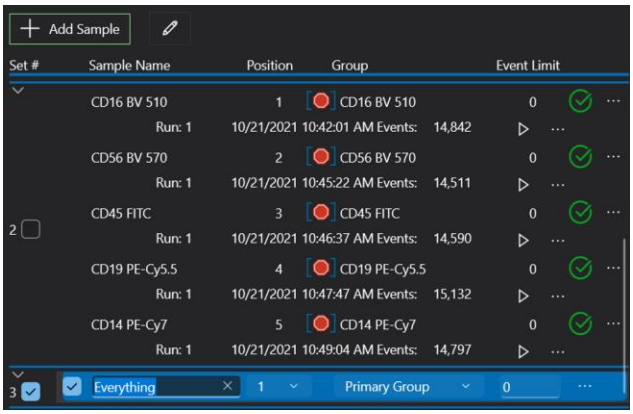
34. If there are no errors and you see the success message, click **Ok**. You are now ready to create new plots, set regions and run sample for sorting.
35. Create a scatter plot, a doublets plot and the fluorescence plots in the Primary Group.



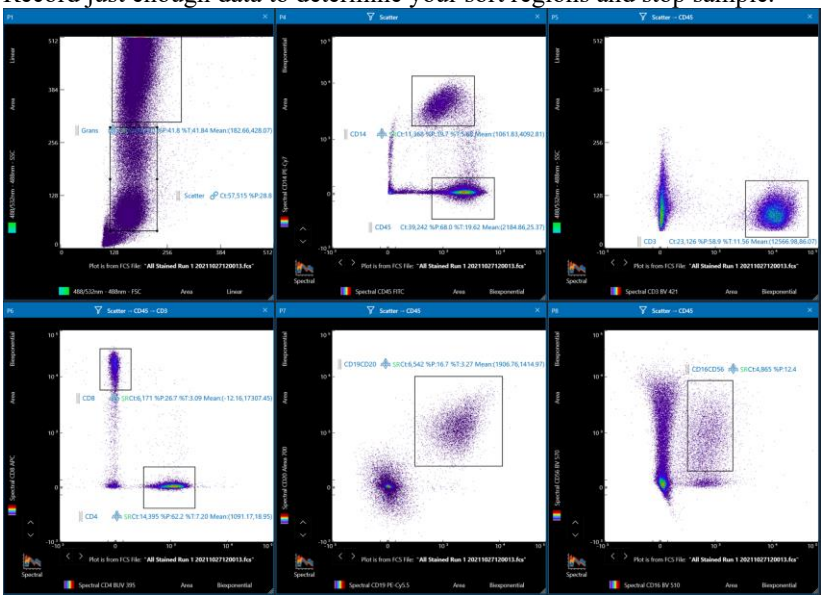
36. Click on the plots' axes to set the spectrally unmixed fluorescence parameters. The unmixed spectral parameters are at the top of the list.



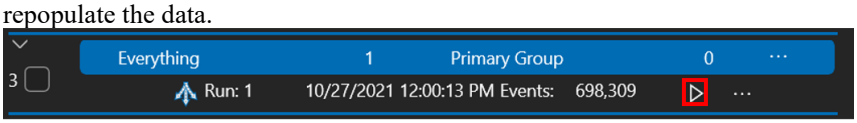
37. Click the plus mark next to the sample naming field to add a sample to the list. It is automatically added to the Primary Group.
38. Click in the sample renaming field and type a name for the sample. In this example "Everything" is the sample name.



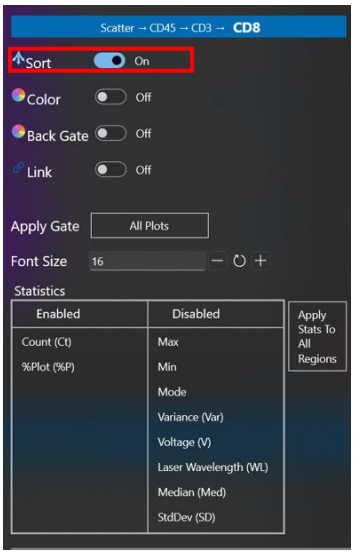
39. Record just enough data to determine your sort regions and stop sample.



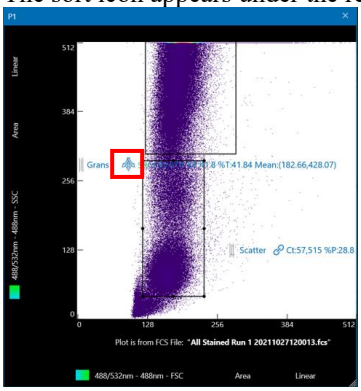
40. Adjust regions to surround populations of interest and rename them if desired. Note: When you adjust regions, the data may clear from the screen. If this happens click the play button next to the sample to repopulate the data.



41. Use the event rate to approximate the EPS displayed during setup. Toggle the Flow Rate button to Event Rate and enter the value to keep the event rate consistent throughout the sort.
42. Right-click in the regions you want to sort and turn the **Sort** toggle button to **On**.

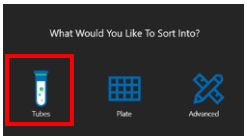


43. The sort icon appears under the region to indicate the population has been selected for sorting.

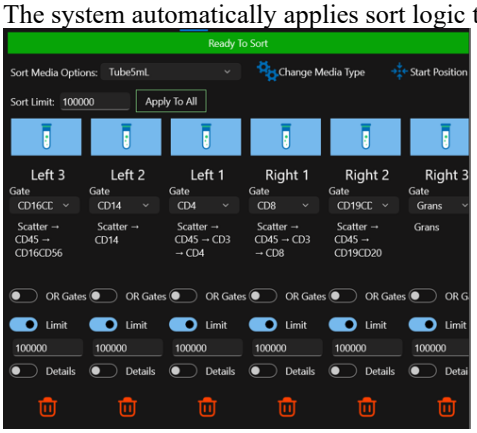


44. After sort regions are assigned, click the **Sort** button in the **Flex Controls**.

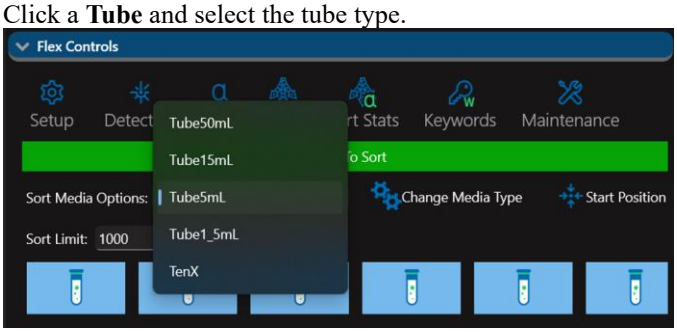
45. Click on the sort medium. This example is sorting into tubes.



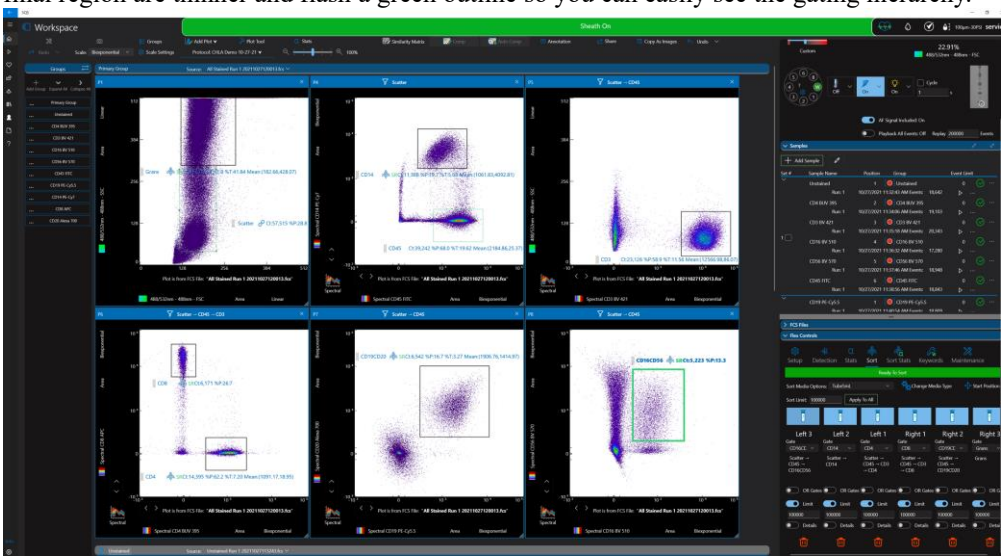
46. The system automatically applies sort logic to output locations.



47. Click a **Tube** and select the tube type.

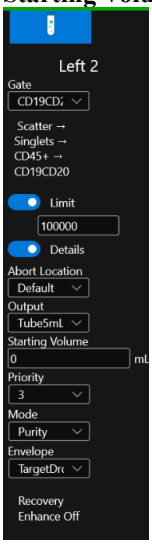


48. It is optional to hover the cursor over a tube to view the associated sort region. The gates leading up to the final region are thinner and flash a green outline so you can easily see the gating hierarchy.

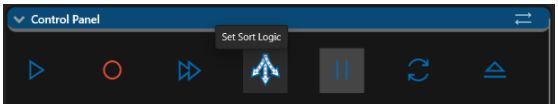


49. Type in a sort limit and click **Apply to All**, or type in individual limits.

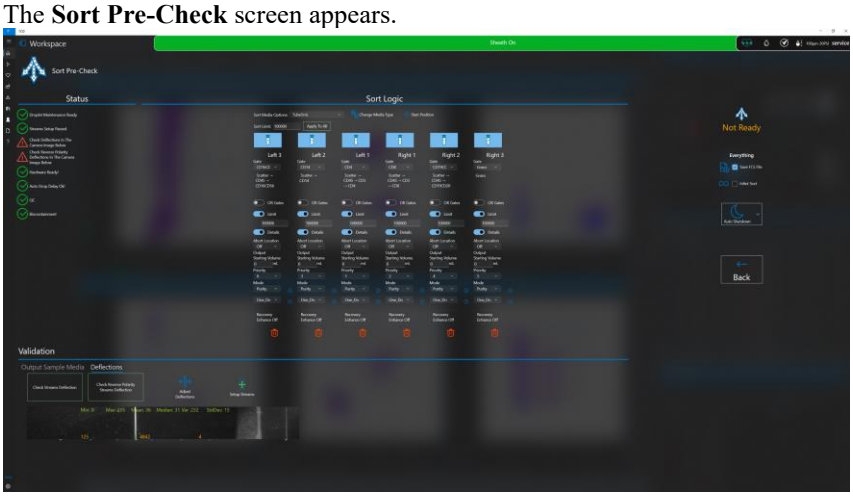
50. You can expand the **Details** section under each tube to apply additional parameters. It is recommended to use a minimum of 0.2 ml of sorting buffer to aid cell viability. Enter the volume of the buffer used in the **Starting Volume** field.



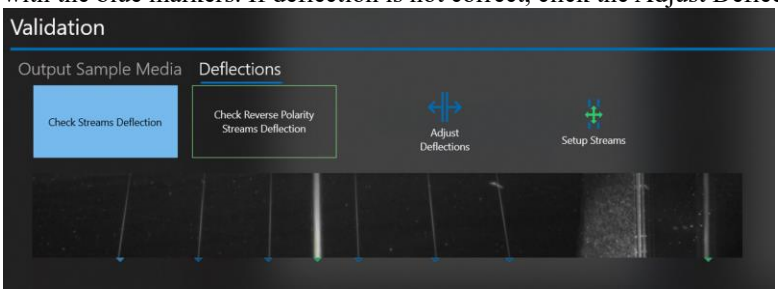
51. Click the **Start Sort** button in the **Control Panel**.



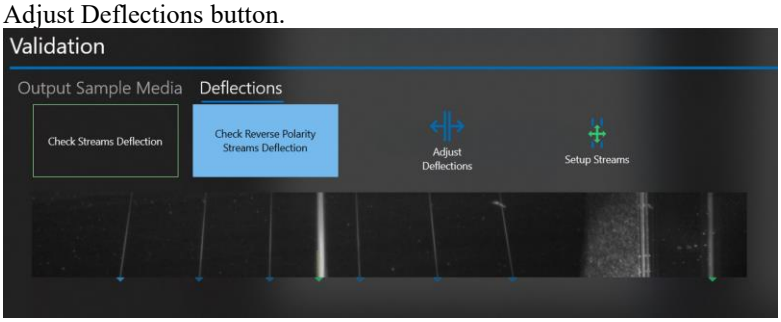
52. The **Sort Pre-Check** screen appears.



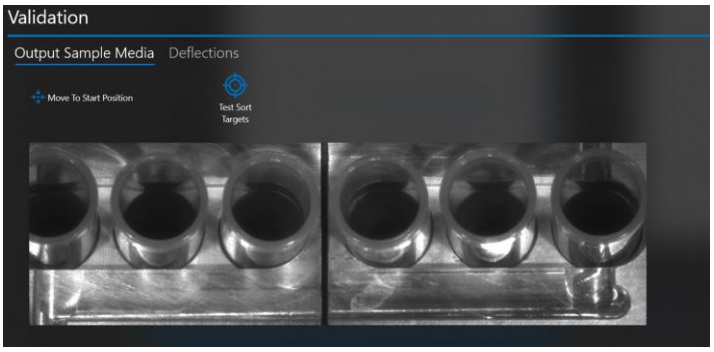
53. Toggle the Check Streams Deflection button to check stream deflection. Make sure the streams are aligned with the blue markers. If deflection is not correct, click the Adjust Deflections button.



54. Toggle the Reverse Polarity Streams Deflection button to check stream deflection with reverse polarity applied. Make sure the streams are aligned with the blue markers. If deflection is not correct, click the Adjust Deflections button.

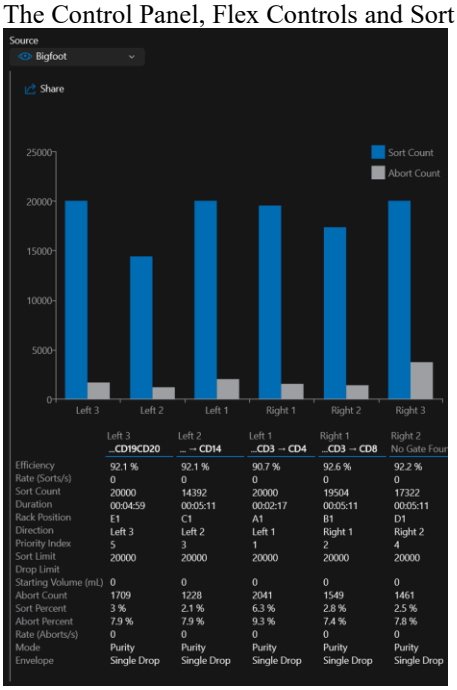


55. The media camera is also shown in this view to visually confirm the proper sort collection media is installed in the sort output area. Click the Move to start position for a better view of the media. It is optional to click Test Sort Targets to deposit droplets onto the selected media type.



56. Click Start to sort. During the sort, the event rate can be adjusted to optimize the efficiency of the sort. If sort regions/gates are adjusted during the sort, the sort will pause and then resume when the region is reset to its new location.

57. The Control Panel, Flex Controls and Sort Stats are shown during the sort.

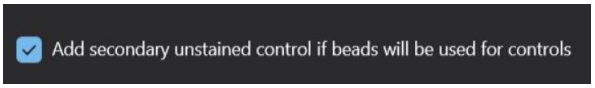


58. In the upper part of Sort Stats is a graph that displays the numbers of cells sorted into each sort receptacle and aborts. Below the sort plots are real-time sort statistics showing the on-going details of the sort. Scroll to see additional data if necessary.
59. The sort continues until the sort limits are met, the maximum volume is reached, or the end of sample is detected.
60. When the sort is complete, a detailed sort report appears. This report can be exported by clicking the Share button.

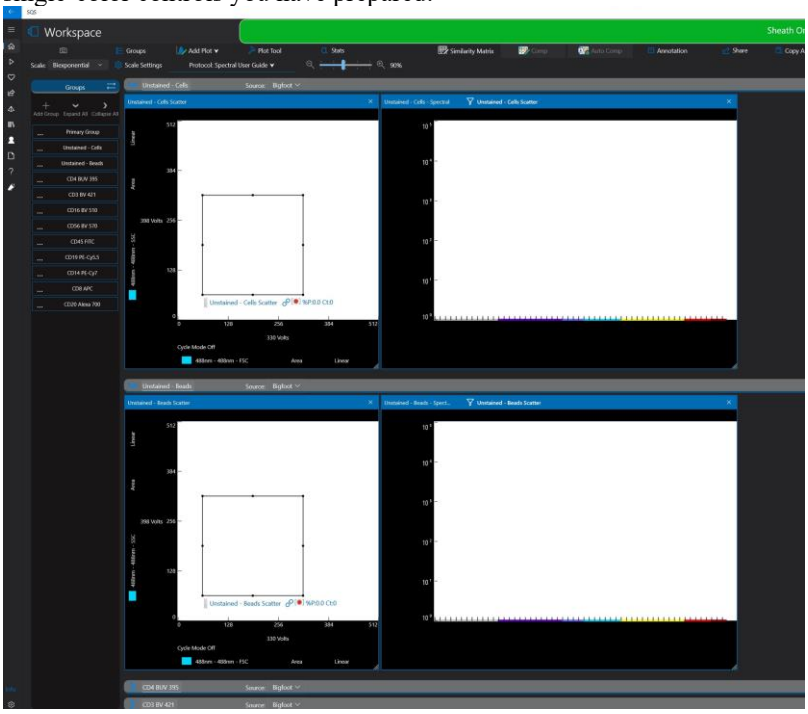
Spectral Setup Using Secondary Unstained Control

A spectral protocol allows you to define a voltage set for unstained beads and cells independently. This helps conserve sample when you intend to examine only a few parameters in a small sample. After the secondary unstained control option is set, you must link the autofluorescence profile of either the unstained beads or the unstained cells to each control in the protocol and then run the controls.

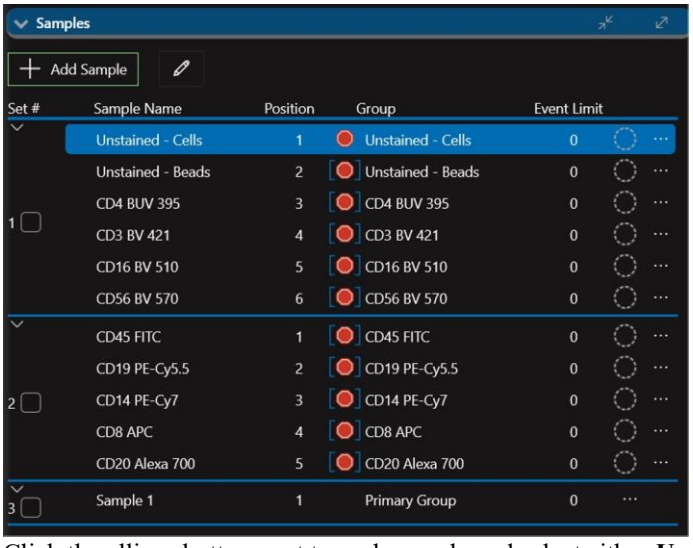
1. Follow the steps to set up a spectral sort experiment, then select the secondary unstained control checkbox.



2. After you have selected the fluorophores for the experiment click **Next**.
3. The software builds the groups required to run the negative beads control, the negative cells control and the single-color controls you have prepared.



4. A sample list is created that allocates each tube to a loader position. These samples can be run in sets of six or fewer tubes.



5. Click the ellipse button next to each sample and select either **Unstained Cells** or **Unstained Beads** depending on the autofluorescence profile you would like to link to each control in the protocol.

6. Return to the steps for setting up a spectral sort experiment to set voltages and run the controls.

Index Sorting

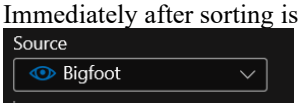
Index sorting is the automatic process of cataloging and displaying sort experiment data so that information from each sorted event is correlated with the physical location in which the event was sorted. A single cell sorted into a single well can be uniquely analyzed and multiple cells sorted into one tube or well can be analyzed as a distinct population.

Sort experiment data that is acquired on the Bigfoot Cell Sorter and saved in an FCS file, is indexed so parameters and destinations of all sorted and aborted events can be tracked. Parameters such as FSC, SSC and fluorescence is displayed with the associated sort media location. The information derived from index sorting can be useful if subsequent processing or analysis of the sorted cells yields novel results because the data from the cells can be traced to their physical locations within the sort media.

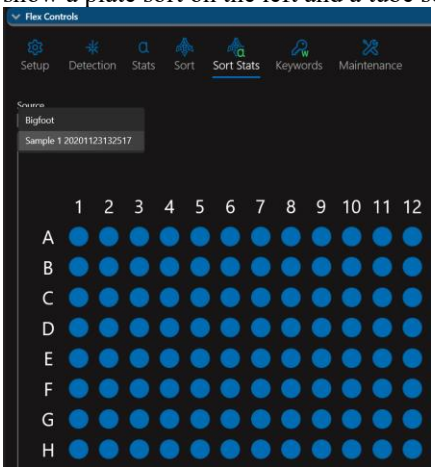
Accessing Index Sort Information

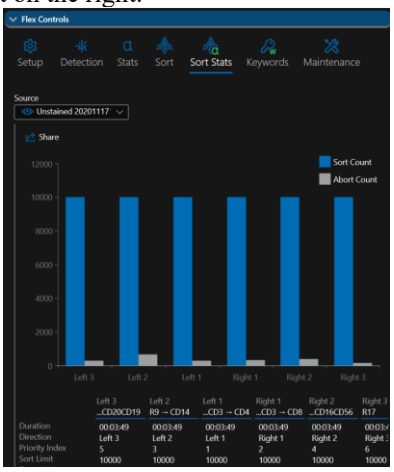
To access index sort information, perform a sort experiment based on compensated or spectrally unmixed parameters.

1. During sorting, a chart of the sort media is displayed in the software while the sort statistics are updated in real time.
2. Immediately after sorting is complete, the **Source** dropdown list will display **Bigfoot**.

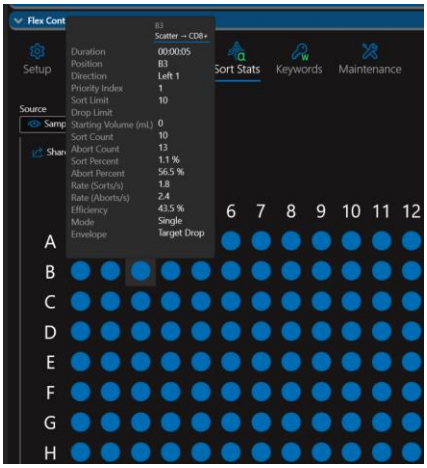


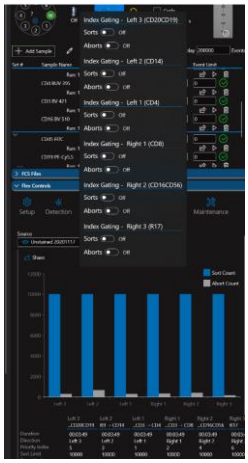
3. To view index sort data, select the saved FCS file from the **Source** dropdown list. The examples below show a plate sort on the left and a tube sort on the right.





4. Hover the mouse over a well, or tube, to view the sort information for that location. You can left click on a well to display the information below the chart.





5. Right-click on a well and select the desired gates. You can select from the Sorts and Aborts that are available depending on the data for each location.

6. A density plot will be added to the workspace and the new gates will be added to the gates list. You can modify the plot parameters if necessary. To apply color gating, turn the **Color Gating** switch **On** and click the color you would like to apply to the gate.

7. Statistics can be included on this data using **Plot Stats**, **Region Stats**, or the **Stats Plot**.

Per-Stream Droplet Spacing and Per-Stream Manual Deflection Adjustment

You can adjust droplet spacing for each stream to ensure all streams are individually optimized for sort rate and efficiency. Per-stream optimization works by controlling the minimum physical spacing between sorted droplets for each sort-stream position. A lower value will decrease the minimum distance between sorted events resulting in a broader physical distribution of sorted droplets and can result in higher efficiency. This is especially important on the outermost streams where closely spaced highly charged droplets contribute to fanning. Similarly spaced particles deflected to the inner streams are not as affected.

For best results, we recommend gating and sorting the lower sort-rate populations to the outside tubes and optimizing droplet spacing and deflection for each stream. It is also now possible to manually fine tune individual sort stream deflections during an active sort instead of only prior to the sort using a test pattern. This allows users to control streams to adjust sorted stream positions based on real sort conditions and user adjusted per-stream

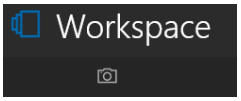
optimization values. Per-stream optimization and stream deflection offset adjustments made during a sort are now saved with the protocol and can be used again when using the same tip serial number, tip size and sheath pressure. If any of these three operating conditions are changed, the settings will return to defaults.

Per-Stream Optimization Procedure

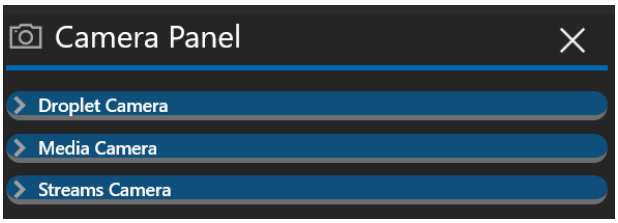
The following example depicts a 6-way sort into 5mL tubes.

- A new sort protocol starts with default values for per-stream optimization based on tip size, media type and sort-stream position. These defaults can be adjusted for individual streams and can be reset to the default values by clicking **Use Default Drop Spacing**.
- The stream deflection offset default value is 0 and can be adjusted on a per-stream basis. Stream deflection defaults can be reset by clicking **Reset Deflection Offsets**.

1. Set up the instrument, run QC and set up the experiment including sample plots and sort regions.
2. Click the camera icon in the Workspace to display the camera panel.

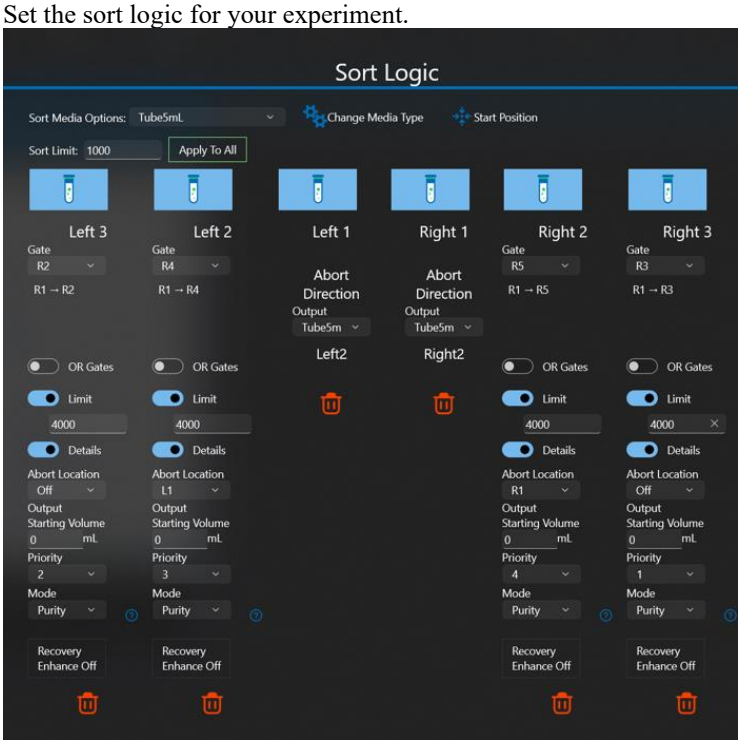


3. Click streams camera. This panel provides access to the Drop Spacing controls and Deflection Offset controls.



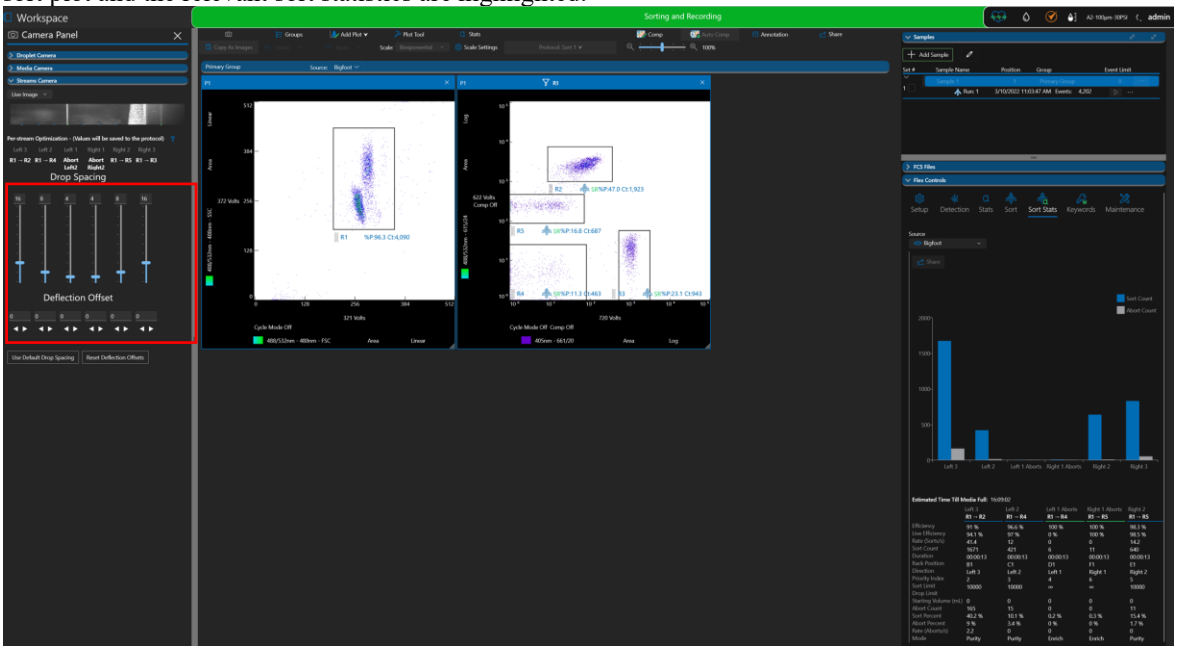
4. Click Set Sort Logic button.

5. Set the sort logic for your experiment.

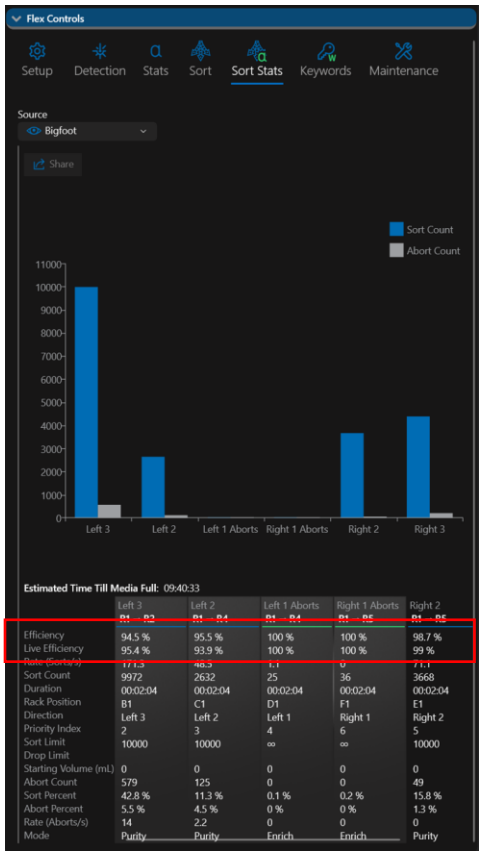


6. Click **Check Streams Deflections**. Verify that the streams align with the targets.

7. Click **Check Reverse Polarity Deflections**. Verify that the streams align with the targets.
    a. If the streams do not align with the targets, click **Adjust Deflections** then check streams deflections and reverse polarity again.
    b. If the streams are very wide, adjust the Defanning slider until the streams appear tighter. Then adjust deflections, check streams and check polarity again.
8. Verify the output media is in place.
9. Click **Start** to start the sort streams.
10. As you hover over the controls for **Droplet Spacing** or **Deflection Offset** the corresponding region in the sort plot and the relevant sort statistics are highlighted.

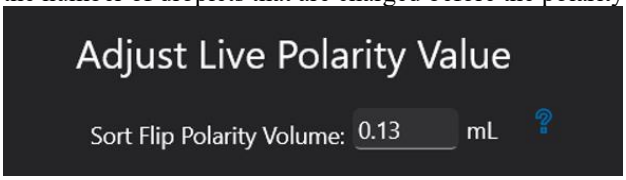


11. To increase sort efficiency for each region, adjust Drop Spacing with the corresponding slider or enter a new number in the field above the slider. Reducing the droplet spacing should increase the **Live Efficiency** value in the Sort Stats panel but will also increase stream fanning. View the Live Efficiency statistic while adjusting Drop Spacing to verify your results.



12. When sort efficiency statistics are optimized for each stream, view the amount of fanning in the streams camera display and confirm the physical collection of the sample in the tubes. Fluid accumulation outside of the tubes, fluid on the rims of the tubes, or many droplets on the interior sides of the tubes may be signs that the deflection offset requires optimization. Note: Inaccurate media calibration can also contribute to droplet splatter.
13. If you notice splatter or the streams are no longer accurately hitting the stream targets you can adjust streams deflection during the sort. To optimize stream deflection, click the **Deflection Offset** left or right arrows or enter a value in the field above the arrows to move the stream. View the camera panel as well as the physical tubes to verify results.

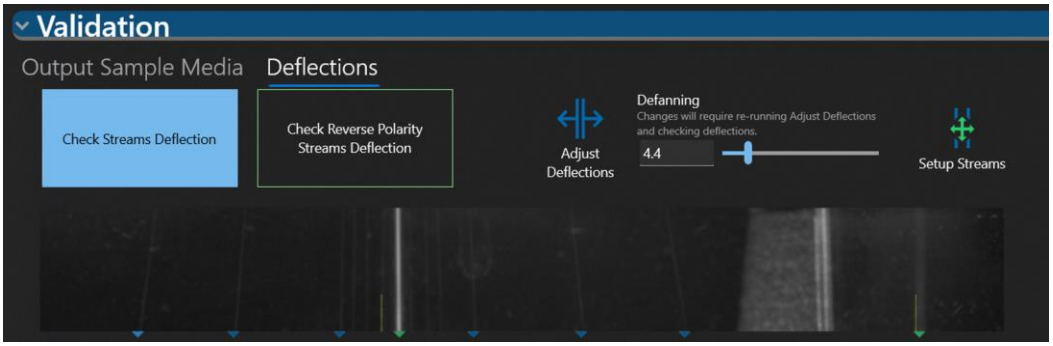
14. If excessive fanning continues, you may want to adjust the **Sort Flip Polarity Volume**. This feature affects the number of droplets that are charged before the polarity is flipped to reduce charge build up.



15. If one or more tubes reach their sort limit, it may be desirable to adjust the Event Rate/Flow Rate to quickly finish the remaining tubes. Keep in mind that adjustments to Event Rate/Flow Rate may require additional stream optimization.

Manual Stream Defanning Adjustment

When using the 70-micron nozzle tip, stream fanning can occasionally be too wide. To address this, we have added a manual control to adjust the streams on the **Sort Pre-Check** screen in the **Validation Deflections** tab. Click in the text field and use keyboard arrows or the scroll wheel to adjust the value while observing the streams. Make the streams as thin as possible.



This is an example of a poorly adjusted waste stream.

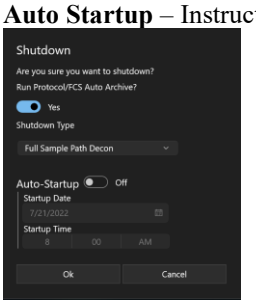
This is an example of a properly adjusted waste stream.
Note: To remediate excessive stream fanning, see the Troubleshooting section for more options.

Shutdown

The automated shutdown process does not require operator intervention. After the **Shutdown** button is clicked and the auto-startup decision has been made, the instrument can be safely left until the next startup.

1. Before shutting down the system make sure the instrument is not acquiring or sorting.
2. Click **Shutdown**. Note: Shutdown is available from the Home screen and from the Login screen.

3. The options that appear have been previously set in **Global Settings**. It is optional to change these selections.
   Run Protocol/FCS Auto Archive – Instructs the software to automatically archive all protocols and FCS files that we created during the last session.
   Shutdown Type – Causes the system to follow the selected cleaning protocol during the shutdown process.
   Auto Startup – Instructs the software whether to automatically perform startup on a specific date and time.



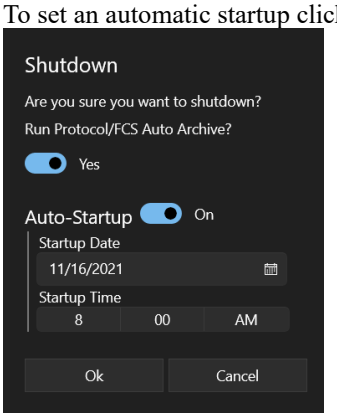
4. The shutdown protocol turns off all lasers, cleans the sample line, runs cleaner if selected in **Global Settings**, depressurizes the fluidics and puts the nozzle in the docking station for storage.

Auto Startup

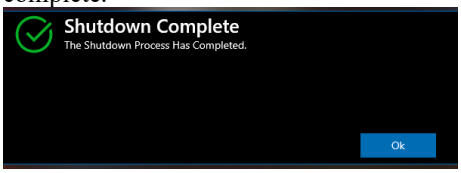
When Shutdown is initiated, the software provides an option to set an automatic startup. Note: If the system is automatically started up and a user does not log in for two hours, the system automatically shuts down.

Set an Automatic Startup

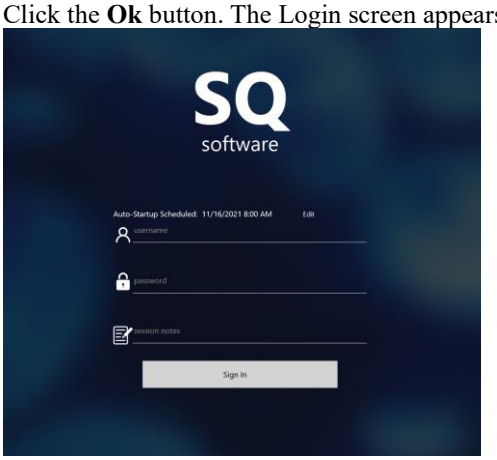
1. To set the software to automatically archive all protocols and FCS files before shutting down, click the **Run Protocol/FCS Auto Archive** button to **On**.
2. To set an automatic startup click the **Auto-Startup** button to **On**.



3. Click in the **Startup Date** field to select the date for the automatic startup.
4. Click in the **Startup Time** field to select the time for the automatic startup.
5. Click the **Ok** button. The system performs the Shutdown protocol, and a message appears when it is complete.



6. Click the **Ok** button. The Login screen appears showing the time and date of the scheduled Auto-Startup.

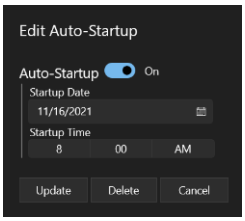


Edit an Auto-Startup

After an automatic startup has been set it can be modified.

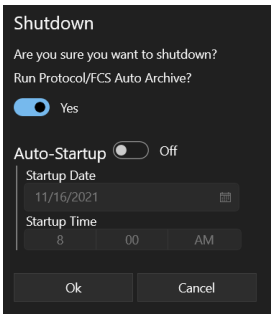
1. Click **Edit** on the Login screen.

2. Make changes to the **Startup Date** and **Startup Time** information and click the **Update** button.
   Or
3. Click the **Delete** button to disable the Auto-Startup.
4. The **Cancel** button closes the menu without changing the previously set Auto-Startup.

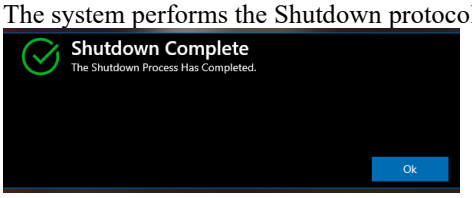


Decline an Automatic Startup

1. Make sure the Auto-Startup button is **Off** and click the **Ok** button.



2. The system performs the Shutdown protocol and a message appears when it is complete.



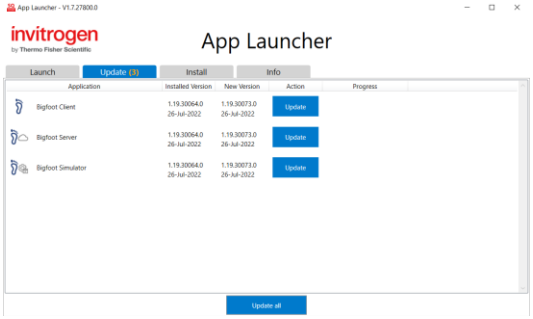
3. Click the **Ok** button. The Login screen appears ready for manual startup.

SQ Software Overview

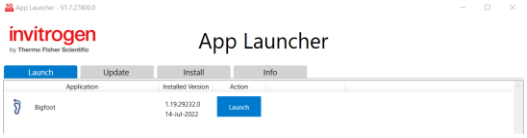
SQ software facilitates operator control, instrument monitoring, data collection, acquisition and sorting. SQ software is opened and maintained through the Launcher. The Launcher will be installed and configured by support personnel. Once it is installed, use the Launcher to start and update SQ Software.

1. Open the SQS App Launcher.

2. The App Launcher opens and checks for software versions that are installed and software versions that are available. The Launcher has four tabs: Launch, Update, Install and Info. If there are updates to installed software the App Launcher will open the Update tab. To update the software, click **Update All**. Note: If you do not want to update the software, click the **Launch** tab.



3. Click **Launch**.

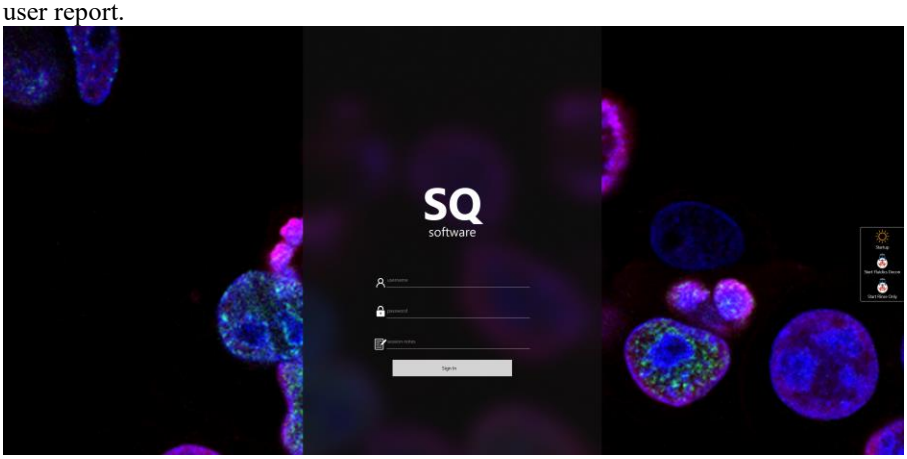


Chapter 5: SQ Software Screen Maps

Refer to this section to become familiar with the screen elements in SQ Software.

Login Screen

After you have opened SQ Software with the PL Launcher you will see the Login screen that displays system status and allows you to log in using a username and password. You can also enter session notes that can be seen in the user report.



Login Access and Control

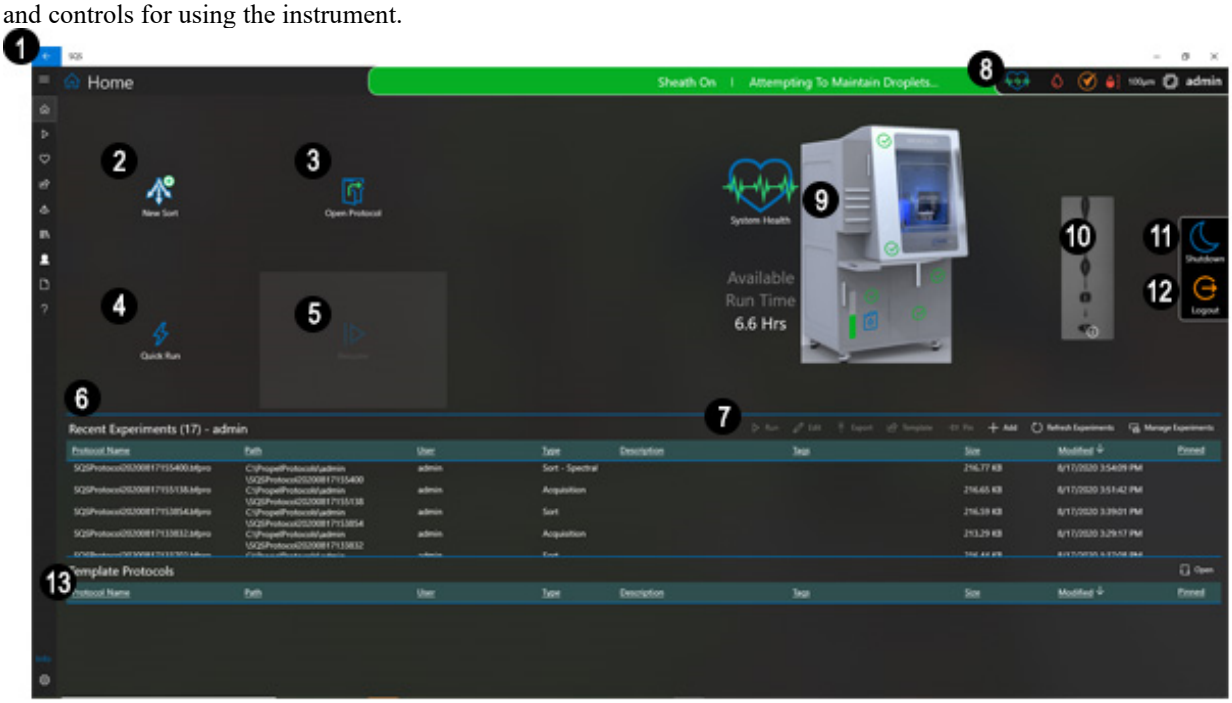
There are two levels of user control in the SQ Software. An Administrator login is granted additional control and access over standard User login. Assign at least two administrators per system. The different privileges for the Administrator and User logins are shown in the table below.

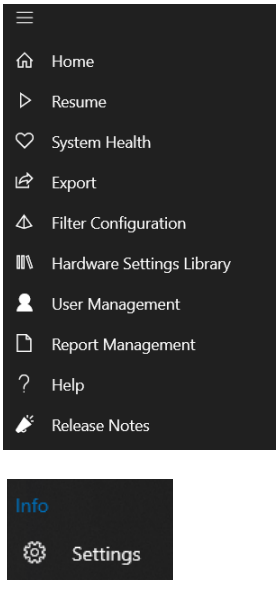
Login Access	Administrator	User
Startup	X	X
Shutdown	X	X
Run QC	X	X
Print QC Reports	X	X
Print QC Trending Reports	X	X
Edit QC criteria	X	
Acquire data	X	X
Print analysis reports	X	X
Sort data	X	X
Print sort reports	X	X
Print user reports	X	
Change User Password	X	X
Change User Rights	X	
Delete Users	X	
Create Users	X	
Edit Users	X	
Reset Other User Passwords	X	

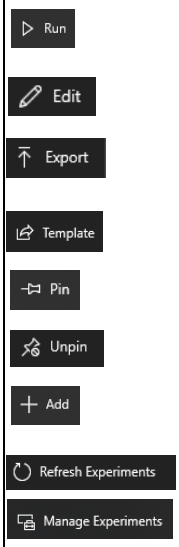
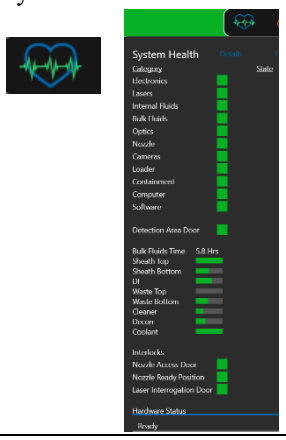
Adjust Global Preferences	X	
Adjust User Preferences	X	X

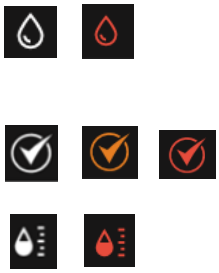
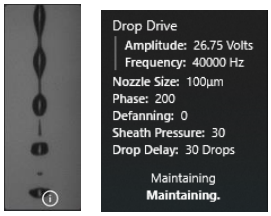
Home Screen

The Home screen appears after you log in to the software. This screen displays system status and provides menus and controls for using the instrument.



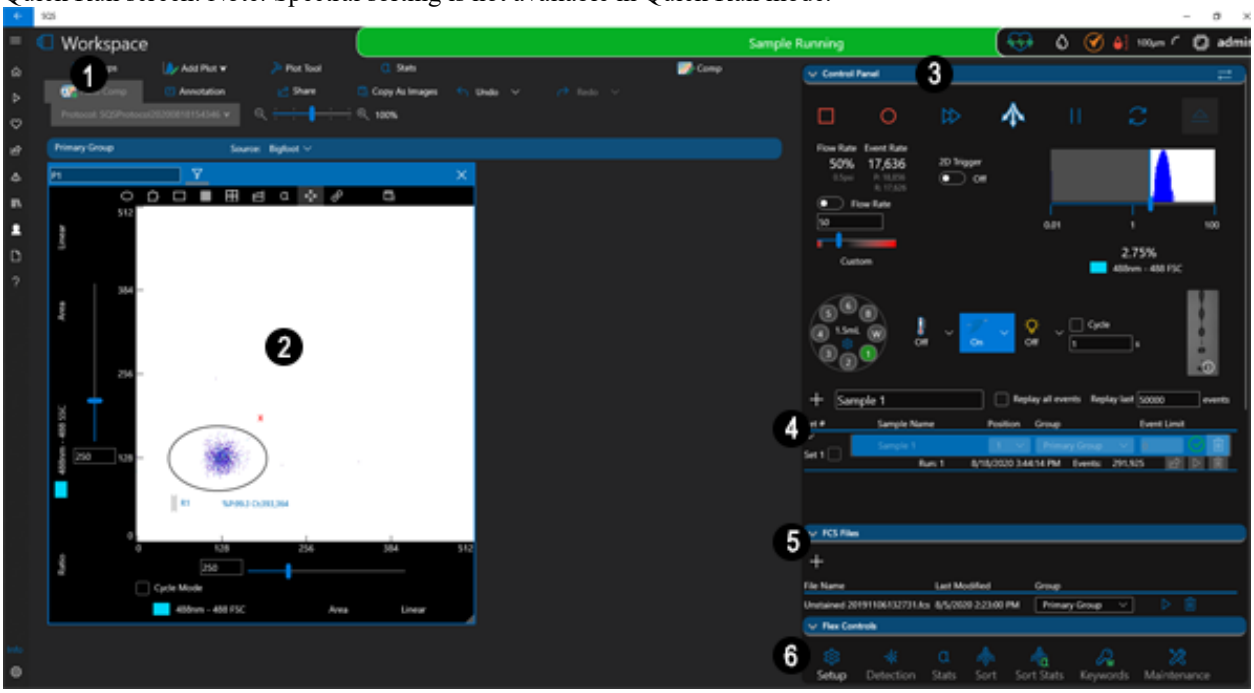
Screen Position	Name	Description
1	Quick Navigation Menu  	**Quick Menu** is found on the left screen and is accessible from any screen within SQ Software. Click the Menu button to see menu item titles. **Home** – Navigates to the Home screen. **Acquisition** – Navigates to workspace if a protocol is loaded. **System Health** – Displays details regarding system health. **Export** – Navigates to the Export FCS Files screen which allows selection of FCS files for export from the selected experiments. **Filter Configuration** – Displays system lasers and filters and allows configuration changes. **Hardware Settings Library** – Allows you to view and/or load settings from an experiment or FCS file. Settings include PMT voltages, trigger, threshold, laser power and compensation. **User Management** – Allows you to create a new user, edit users, or create a CSV formatted report of the total usage per user. **Report Management** – Allows you to view individual QC reports, QC trending reports of selected QC runs and a Drop Delay trending report. **Help** – Provides access to the Bigfoot user guide and quick start guide. **Release Notes** – Provides information regarding SQ Software including new features, bug fixes and known issues. **Info** – Displays system information including serial number, support contact and software version. **Settings** – Allows access to User Settings and Global Settings.

2	New Sort 	**New Sort** leads you through the experiment builder, which provides guidance for detector setup, selection of single-color controls or negative control template and ends with workspace setup. Use this option to streamline and optimize experimental design. Note: Spectral Sorting is only accessible through New Sort mode.
3	Open Protocol 	**Open Protocol** allows you to select a saved protocol to use for a new experiment or to view previously recorded data or sort statistics.
4	Quick Run 	**Quick Run** bypasses the experiment builder and allows sample to be run from any position on the loader. By default, all lasers and all parameters are active in Quick Run mode. The QC protocol is accessed in Quick Run. Note: Spectral sorting is not available in Quick Run mode.
5	Resume 	**Resume** allows you to return to the previous workspace to continue acquiring or sorting from an experiment.
6	Recent Experiments	**Recent Experiments** is a list of experiments used recently or pinned for regular use for the logged in user. Click on the column titles to reorder or filter the list.
7	Recent Experiment Controls 	Click on a specific experiment in the list to make use of the Recent Experiment Controls. Note: This list is specific to login permissions. **Run** – Loads the highlighted experiment into the workspace, double clicking on an experiment also loads the experiment into the workspace. **Edit** – Allows the name and experiment features to be changed in the selected experiment. **Export** – Allows FCS files from the selected experiment to be copied to another location. **Template** – Creates a global template from the selected experiment allowing its usage by any other logged in user. **Pin** – Sends the selected experiment to the top of the list and keeps it there even when new experiments are added to the list. **Unpin** – When new experiments are added to the list the unpinned experiment will no longer be at the top of the list. **Add** – Opens the file manager and allows you to select a previously saved experiment to add to the recent experiments list. **Refresh Experiments –** Updates the experiments list. **Manage Experiments** – Opens a screen where you can search, move, export, or delete protocols from the list. Note: Windows Explorer can also be used to manage experiment files.
8	System Status 	**System Status** – Items are displayed in the upper-right portion of the screen and include: **System Health** – Click to view status details regarding all instrument subsystems. Green icons indicate the subsystem is in a normal state and red icons indicate that maintenance is required.

		**Droplet Maintenance** – A white icon indicates that the system is successfully maintaining droplets. A red icon indicates that the system is not maintaining droplets. **QC Status** – A white icon indicates that QC has been run successfully, an orange icon indicates QC is needed and a red icon indicates an error occurred during the QC protocol. **Drop Delay Status** – A white icon indicates a good drop delay calculation, and a red icon indicates drop delay must be run or rerun. **Nozzle Size** – Displays the orifice size of the nozzle installed on the system.
9	System Health 	**System Health** – Provides detailed information regarding the Bigfoot Cell Sorter subsystems. Green checkmarks indicate subsystems in a healthy state. Red icons indicate that maintenance is required. Click on the icons for additional information. Available run time is displayed based on the bulk fluids tank with the least capacity. Please note that nozzle size and sheath pressure significantly affect run time. If these are changed, the remaining runtime will update to reflect the current setup. If an OFF status is shown for the run time the system is shutdown.
10	Droplet Camera Image 	**Droplet Camera Image** – Shows the current view of the droplet camera. A strobe, tuned to the same frequency used to make the drops, is used to view any movement in drops. The breakoff is controlled using the droplet maintenance feature. If the breakoff cannot be controlled, the system status shows that droplets are not maintained, if a sort is in progress, it will be stopped. When the **i** symbol is selected a menu appears showing droplet parameters.
11	Shutdown/Startup 	**Shutdown/Startup** – Initiates the automatic shutdown protocol or initiates the automatic startup protocol. Make sure you have maintained system fluids before initiating these actions.
12	Logout 	**Logout** – Logs out the current user and return to the login screen.
13	Template Protocols	**Template Protocols** – Lists universal templates that are available to all users.

Quick Run Workspace

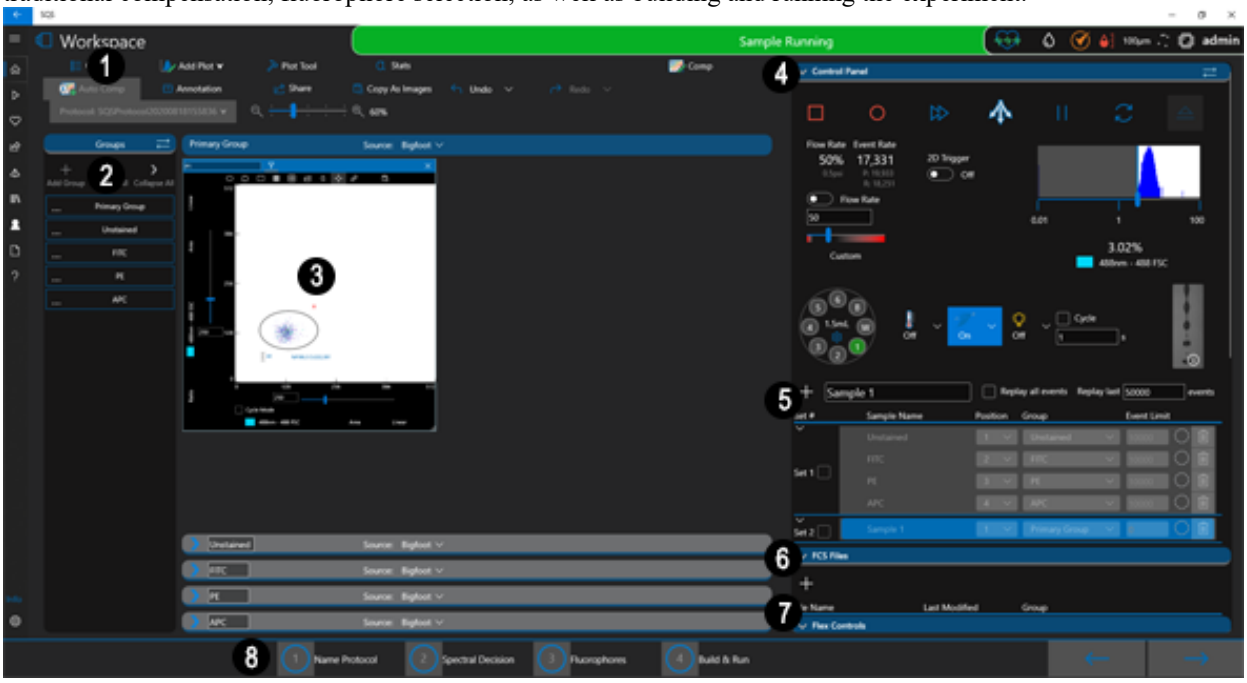
The Quick Run screen bypasses the experiment builder and allows sample to be run from any position on the loader. By default, all lasers and all parameters are active in Quick Run mode. The QC protocol is currently accessed via the Quick Run screen. Note: Spectral sorting is not available in Quick Run mode.



Screen Position	Name	Description
1	Workspace Tools	Workspace Tools are above the plot definition area of the Workspace screen.
2	Plot Definition Area	The Plot Definition Area is location in the middle of the Workspace and is used to create plots and visualize data.
3	Control Panel	The Control Panel is used to start, stop, pause, record and sort the sample.
4	Samples List	The Samples List displays each sample you intend to run including the position on the loader and the Group with which it is associated.
5	FCS Files	FCS files that have been previously exported and saved can be loaded and viewed in any group within the experiment.
6	Flex Controls	The Flex Controls provide QC Setup, Droplet Setup, Streams Setup, Drop Delay Setup, Detection edits, Plot Statistics, Sort Output Setup, Sort Statistics, Keywords and Maintenance.

New Sort Workspace

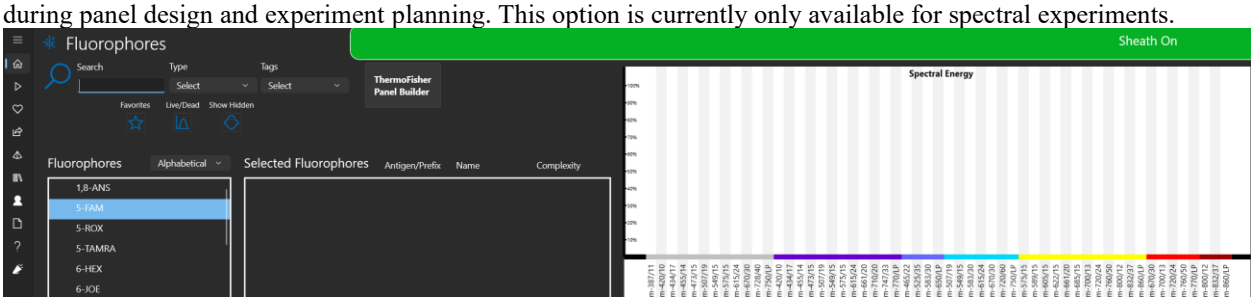
The New Sort Screen is almost identical to the Quick Run screen however, you access this screen by using the experiment builder. The New Sort workflow is an experiment builder that assists with protocol naming, spectral or traditional compensation, fluorophore selection, as well as building and running the experiment.



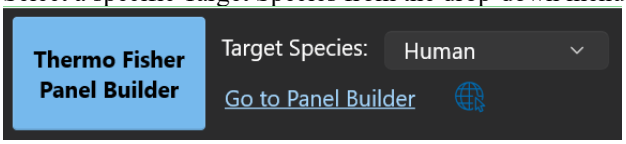
Screen Position	Name	Description
1	Workspace Tools	Workspace Tools are above the plot definition area of the Workspace screen.
2	Groups	Groups automatically appear populated in the New Sort Workspace. Groups are used to partition the workspace into functional sections. Groups can be used to show controls such as a negative control or compensation controls or to display different types of samples with different gating strategies simultaneously. The first group created in every experiment is the Primary Group.
3	Plot Definition Area	The Plot Definition Area is in the middle of the Workspace and is used to create plots and visualize data.
4	Control Panel	The Control Panel is used to start, stop, pause, record, set trigger and threshold and sort the sample.
5	Samples List	The Samples List displays each sample you intend to run including the position on the loader and the Group with which it is associated.
6	FCS Files	FCS files that have been previously exported and saved can be loaded and viewed in any group within the experiment.
7	Flex Controls	The Flex Controls provide QC Setup, Droplet Setup, Streams Setup, Drop Delay Setup, Detection edits, Plot Statistics, Sort Output Setup, Sort Statistics, Keywords and Maintenance.
8	Experiment Builder Progress and Navigation Bar	The Experiment Builder Navigation Bar displays the steps required to build an experiment such as: Name Protocol, Spectral Decision, Fluorophores, Build and Run. The circles around the numbered steps are white until you complete a step at which time the circle turns blue. The Next and Back arrows can be used to navigate backward and forward through the steps.

Thermo Fisher Web Panel Builder

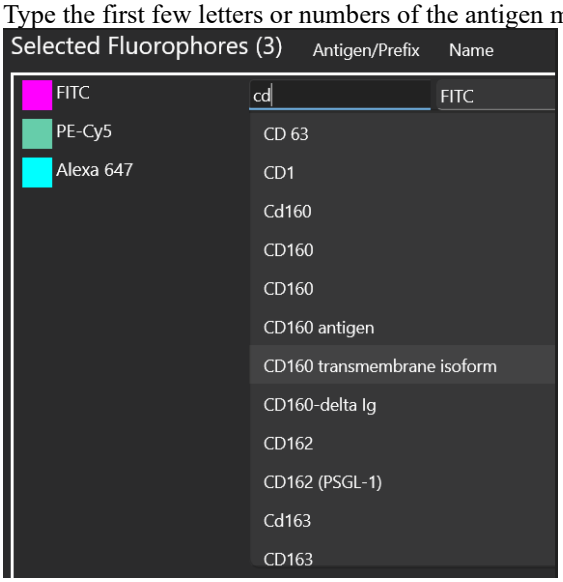
The Thermo Fisher Web Panel Builder allows you to display a list of fluorophores available on the Thermo Fisher website where you can find fluorophore/antigen information and purchase the product. This feature can be helpful during panel design and experiment planning. This option is currently only available for spectral experiments.



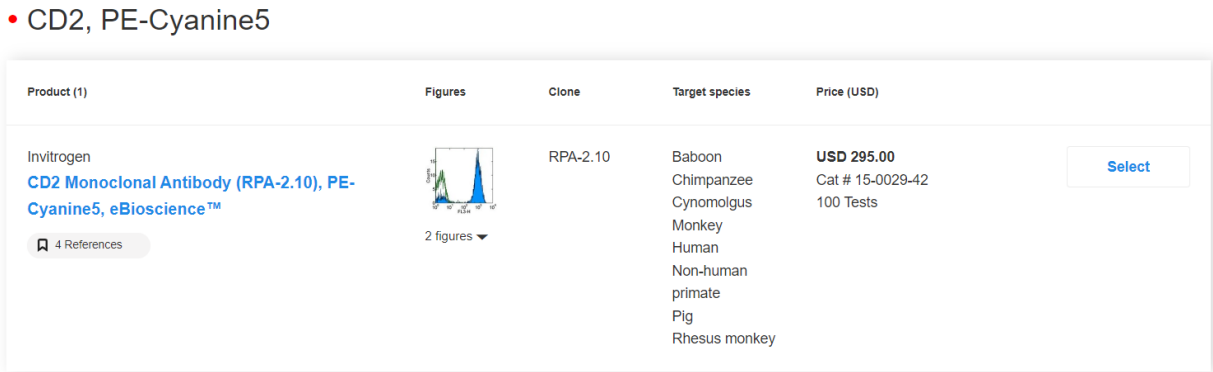
1. From the **Fluorophores** screen click **Thermo Fisher Panel Builder** to display the options available through the Thermo Fisher website.
2. Select a specific Target Species from the drop-down menu.



3. Type the first few letters or numbers of the antigen marker to narrow the options in the list.

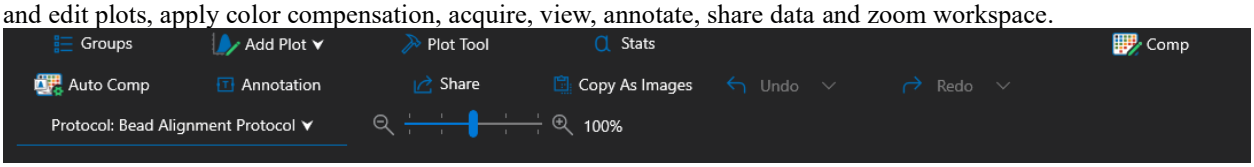


4. When you have selected the fluorophores and antigens click the **Go to Panel Builder** link for more information and the option to purchase the fluorophores.



Workspace Tools

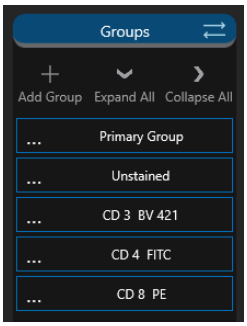
Workspace Tools are above the plot definition area of the Workspace screen. These tools provide the means to build and edit plots, apply color compensation, acquire, view, annotate, share data and zoom workspace.



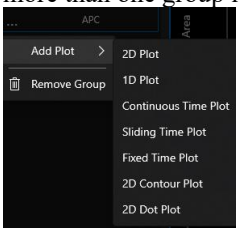
Groups

Groups are used to partition the workspace into functional sections. They can be used to show control samples such as compensation controls or the negative control. Groups can also display different types of samples with different gating strategies simultaneously. The first group in every experiment is labeled Primary Group. Important: Do not remove automatically created Groups.

1. Click **Groups** in the Workspace Tools to expand or collapse the list of groups as shown below.



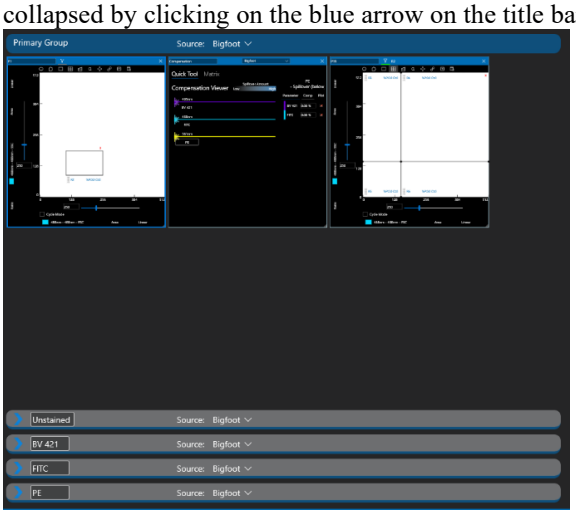
2. If compensation options, such as single-color controls and/or the negative control, were selected in the Fluorophores screen, templates for each sample are automatically created and organized into their own groups. Both the auto compensation wizard and spectral unmixing algorithms interact with these groups. To create an additional group, click **Add Group**. Use the workspace scrollbar to view additional groups if more than one group is present.

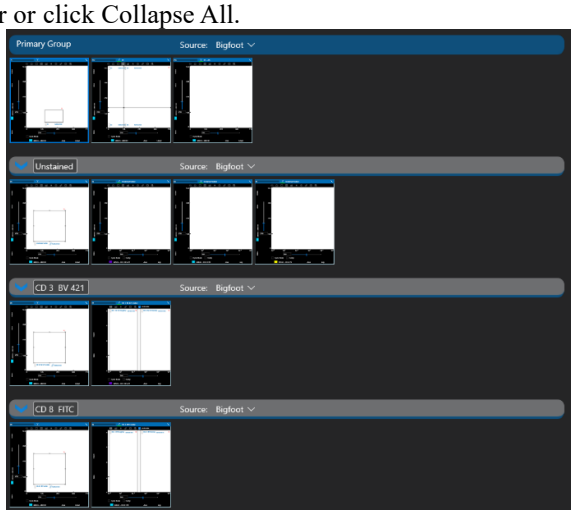


3. Click the **…** button next to the group name to access **Add Plot**, which allows you to add a specific type of plot to the group. The **…** button also allows you to select **Remove Group**, which deletes the group including all its plots and gates from the experiment. Note: The Primary Group cannot be removed.

Group Title Bar

When the workspace first opens, all groups, except the Primary Group are collapsed. Each group can be expanded individually by clicking the blue arrow on group title bar or by clicking Expand All. A group can be individually collapsed by clicking on the blue arrow on the title bar or click Collapse All.

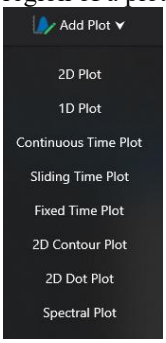




The gray bar above each group shows the name of the group and the data source. Groups are named automatically for selected controls. Names can be changed by directly editing the text in the group name box within the title bar. A data source is applied for each group. This defaults to Bigfoot for acquiring data and sorting on the instrument. If FCS files have been added to the sample list, a group can be set to show the data from an FCS file.

Add Plot

The **Add Plot** tool provides a way to add specific plots within groups. Select a group and then click Add Plot to select a plot type. Another way to add a plot to a group is to double-click in the workspace or double-click in a region of a plot to create a new plot that is gated on that region.

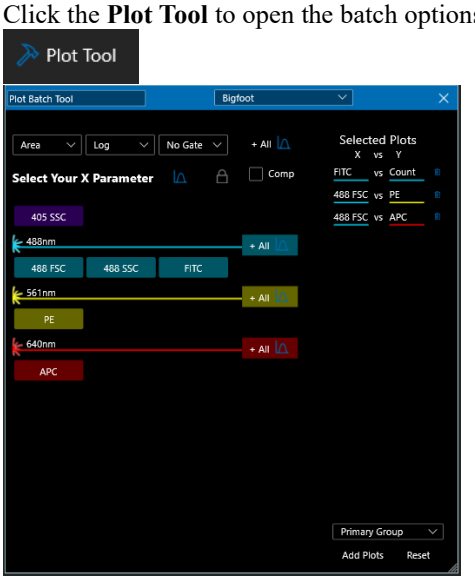


2D Plot – Used to compare two different channels simultaneously, such as FSC vs. SSC.
1D Plot – Used to analyze a specific channel which is useful for looking at the difference of intensity for a specific color in a population.
Continuous Time Plots – Useful for assessing the number of events throughout time, such as Time vs. FSC to determine when the event rate is stable. Continuous plots always show all data with more time being added when the maximum axis count is met.
Sliding Time Plots – Useful for assessing the number of events throughout time, such as Time vs. FSC on a more immediate scale. Sliding time plots keep the same time frame shown at all times and slide the axis values as time increases.
Fixed Time Plots – Fixed time plots are useful for assessing the number of events throughout a fixed time scale. These plots will show only the time frame specified and will not increase as more data is collected.
Spectral Plot – Shows the intensity of a sample in each detector. Note: This option is enabled only for spectral systems in spectral de-mixing experiments.

Plot Batch Tool

The Plot Batch Creation tool provides the means to create one or more plots in a batch with parameters, group and gating already specified.

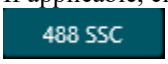
1. Click the **Plot Tool** to open the batch options.



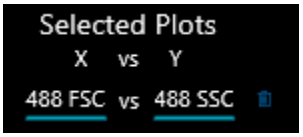
2. Select data handling parameters from the top dropdown lists. These parameters will be applied to all plots created in this batch.

3. Click on the fluorophore to select the X parameter.

4. If applicable, click on the fluorophore to select the Y parameter.



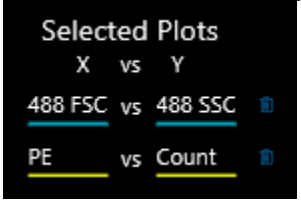
5. You will see the bivariate plot listed in the Selected Plots column on the right. Note: To delete the pair click the **Trash** button.



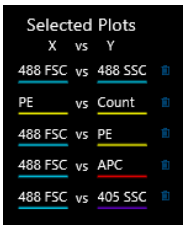
6. Click the **Histogram** button to create a single-parameter plot.

7. Click on the fluorophore to select the X parameter.

8. You will see the univariate plot added to the list.



9. If you intend to add several plots with the same X axis parameter, click **Lock** and then click on the fluorophore for the X axis. Click the fluorophore you want for the Y axis and the plot is added to the list on the right. Continue to click the remaining parameters you want to pair with the locked parameter. See the example below.

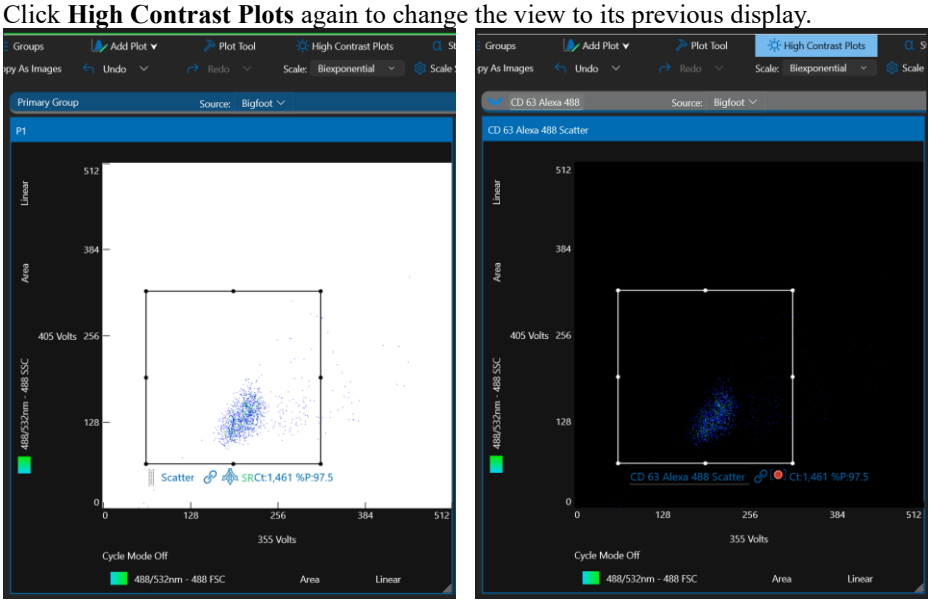


10. Select the **Group** with which the plots will be associated.

11. Click **Add Plots** and the software will create the plots and put them in the selected group.
12. **Reset** clears the Selected Plots list.

High Contrast Plot View

1. Click **High Contrast Plots** to change the plot background color from white to black.
2. Click **High Contrast Plots** again to change the view to its previous display.



Stats

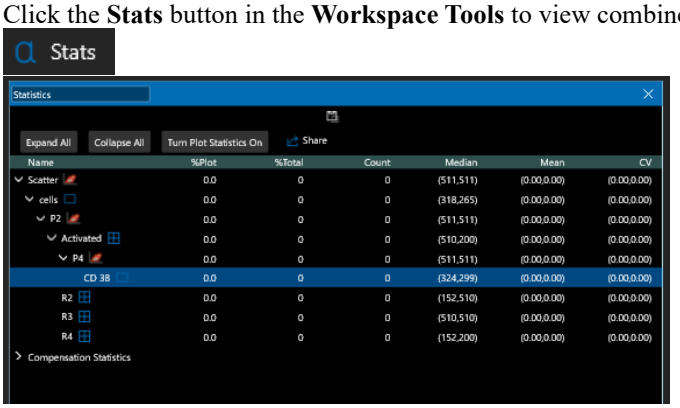
The **Stats** button shows the combined statistics from all the plots and regions in the workspace. The upper section of the table contains statistics from the primary group. The lower section shows any statistics from groups automatically created in the single color and negative control templates, which are minimized by default. Within each statistics section, the plot and gate hierarchy are shown. Options such as expand all, collapse all, turn plot statistics on, share and copy image are available in the statistics window.

The statistics column headings are those selected in the User Settings or Global settings screens. The available statistics are:

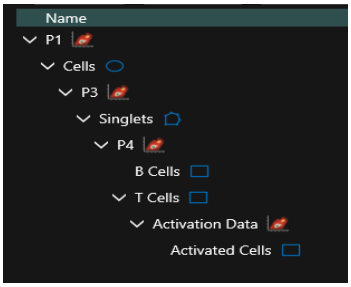
- %Plot – Number of events as a percentage of all the events in the plot.
- %Total – Number of events as a percentage of all triggered data.
- Count – Total number of events.
- Median – The channel number of a plot at which there are 50 percent of events either side.
- Mean – The average channel number in a distribution.
- StdDev – A measurement of data heterogeneity defined as the average distance of each point from the mean
- %CV – Coefficient of Variation, a measurement of data heterogeneity independent of the position of the data on the plot, is given by Standard Deviation / Mean * 100.
- Max – The maximum value in that plot or region.
- Min – The minimum value in that plot or region.
- Mode – The channel that has the most events.

- Variance – The variation within the plot or region.

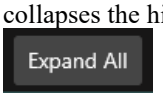
1. Click the **Stats** button in the **Workspace Tools** to view combined statistics.



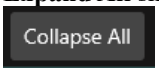
2. Pn is the name of the plot. Plots can be renamed on the plot itself. Any new names entered will be reflected in the statistics table. If no name has been entered, the plot will have the default name “P1, P2,” and so forth.



3. The indented view shows the hierarchy of the plots and regions.
4. The V arrow shows the hierarchy of all the plots and regions below the selected plot or region. The > arrow collapses the hierarchy of all the plots and regions below the selected plot or region.



5. **Expand All** shows the hierarchy of all the plots and regions.



6. **Collapse All** hides the hierarchy of all the plots and regions.

7. **Turn Plot Statistics On** shows plot statistics on all plots in the experiment. Default statistics can be selected in Settings, User Setting/Global settings statistics, plot stats.

8. **Share** opens a windows file save menu allowing a CSV file of the statistics to be Exported to any drive available.

9. **Region** is a sort region. When a region is selected for sorting, a sort icon is displayed next to the region name.

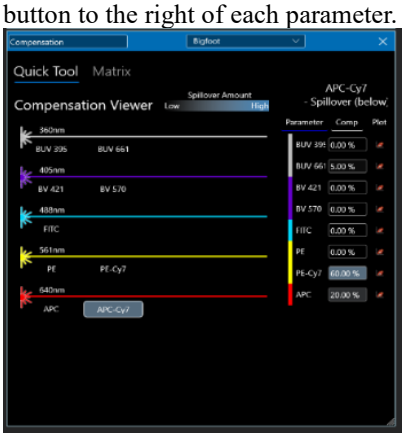
Comp

The Comp tool allows verification of Automatic Compensation as well as Manual Color Compensation. This tool is not used for spectral experiments.

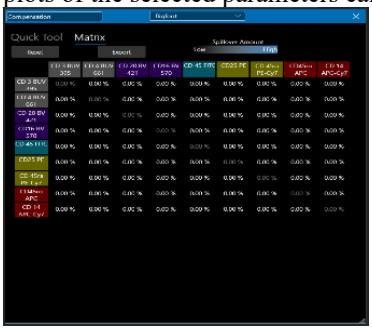
There are two modes: **Quick Tool** and **Matrix**.

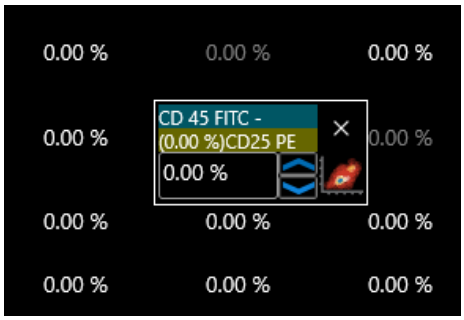
Click **Comp** and the **Quick Tool** opens first. Note: To learn how this feature is used in the context of setting up an experiment see the Build and Run – Manually section of this document.

Quick Tool –This tool shows parameters on the left in laser order and the possible combinations of each parameter on the right. The amount of compensation is shown in the Comp column. Each compensation value can be adjusted in real time to see its effect. Additionally, new plots can be created from each combination by clicking the Plot button to the right of each parameter.



Matrix Tool – The matrix shows a table of each parameter compared against the other with the compensation value at their intersection point. Clicking on the value opens a box where the compensation can be changed, and density plots of the selected parameters can be created.





Auto Comp

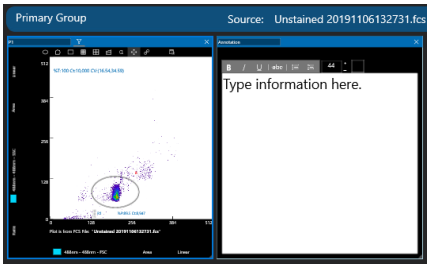
The Auto Comp tool is used in context of the experiment builder when you have set up an experiment using single-color controls. See Build and Run using Automated Color Compensation for more details.

Annotation Plot

The Annotation Plot tool provides a rich text plot to add notes within the Primary Group. This will be stored with the protocol and can be printed using the **Share** button. This plot can be used to make notes about the cell preparation or any experimental factors and only one annotation plot is allowed per experiment. However, individual, plots can be annotated using the annotation button on the specific plot.

1. Click the **Annotation** button in the **Workspace Tools**.

2. A text box opens within the Primary Group.



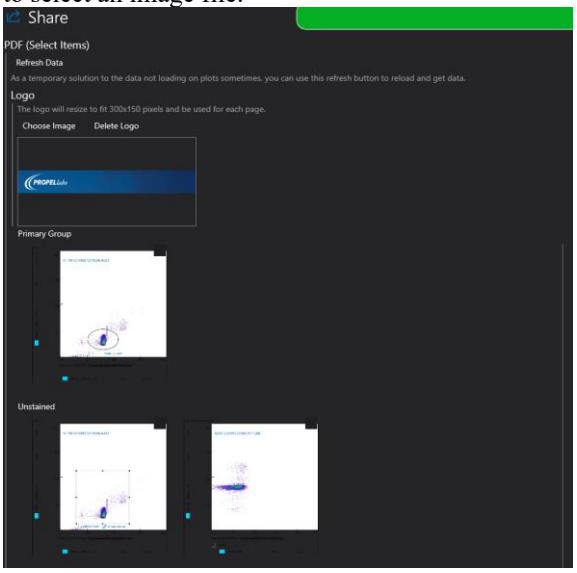
3. Click inside the empty text box and type the desired information.

Share

The Share tool allows you to select Groups one at a time and print them or save them to a PDF file.

1. Click **Share** in the **Workspace Tools**.

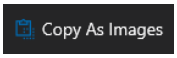
2. Click on the **Group** you would like to print.
3. If you would like to add a logo to your printout, click **Choose Image**. A file manager opens and allows you to select an image file.



4. Scroll down and click **Print**.

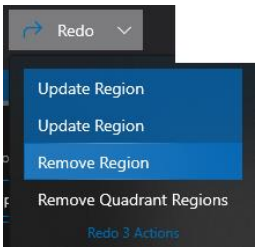
Copy as Images

The **Copy as Images** tool copies the plots from all Groups to the clipboard. You can then paste the images into another document. Click **Copy** in the plot header to copy individual plots.



Undo/Redo

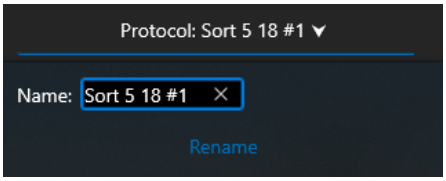
The workspace toolbar includes Undo and Redo buttons. Clicking once on either option executes a single action of undo or redo. Clicking on the down arrow presents a list of recent actions that can be undone or redone at once. Moving down this list adds to the total number of actions to be either undone or redone. The text in blue at the bottom of the box indicates the selected number of undo or redo actions that will be carried out.



Protocol

The current protocol name is displayed at the top of the Workspace.

Use the down arrow to rename the protocol if desired. Highlight the text you want to change, type in the new text and select **Rename**.

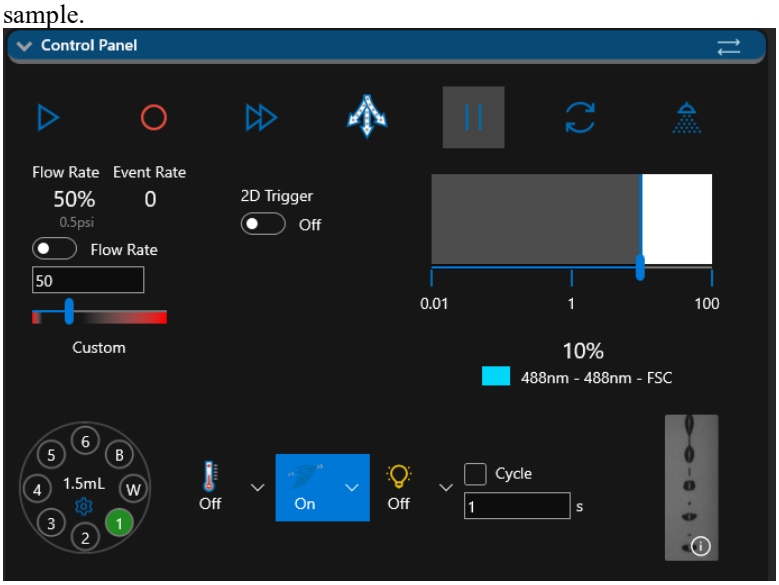


Zoom Workspace

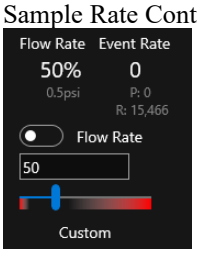
The workspace can be zoomed in or out to allow more or fewer plots to be visualized at once. The default value is 100%. Click – or + to decrease or increase the value by 10%. Click the scale to adjust the zoom percentage to within 10% of the position selected.

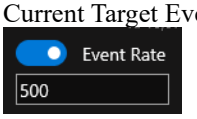
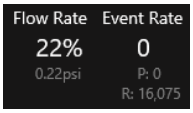
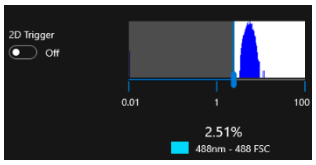
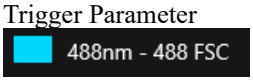
Control Panel

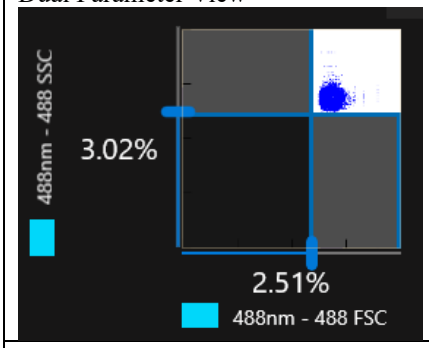
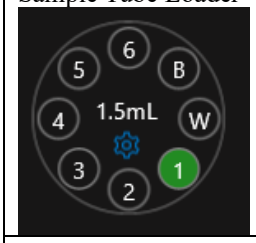
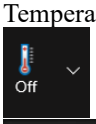
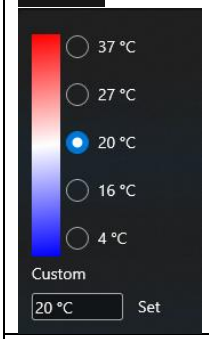
The Control Panel is used to control the instrument including start, stop and pause sample, record sample data, set trigger and threshold, adjust flow and event rate, set sample temperature and agitation, view droplets and sort the sample.

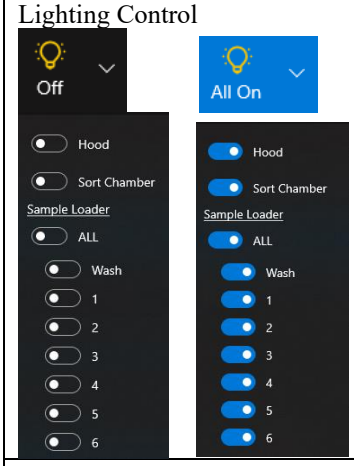
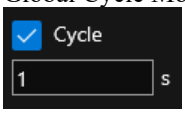
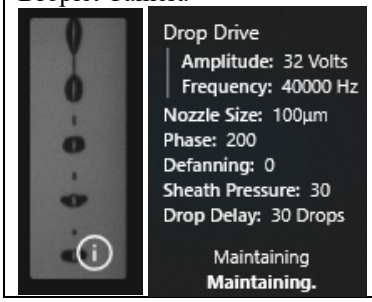


Name	Description
Start Sample 	Starts the sample acquisition process. The loader rotates to the selected position and lowers the probe. Once sealed, a boost pressure is applied to the sample. Once boosted, the user-defined sample pressure is used to continuously deliver sample to the nozzle. When running, data is streamed to SQ Software. The system is in setup mode allowing adjustments, such as PMT voltage and threshold changes Note: This data is not stored.
Stop Sample 	Stop Sample appears when the run sample button is activated. This button stops the acquisition, turns off sample pressure, lifts the probe out of the sample tube and initiates probe wash.

Record Data 	Starts recording data. This button can be used in setup mode or to directly start and record sample. The system will record data to an FCS file until the stop button is clicked, a preset limit is reached, or an error condition is detected.
Stop Recording Data 	Stops recording data.
Skip to Next Sample 	Stops the current sample and skips to the next one in the queue. This can be activated during data acquisition, which ends data acquisition for that sample and proceeds to the next.
Start Sort 	Opens the sort preview screen which indicates if everything is set up properly to start sorting. Sort logic, limits and modes can be reviewed as well as the output media camera. You can click Start Sort while acquiring to switch to sorting or to start sample acquisition and sort simultaneously.
Pause Sample 	Pauses sampling while maintaining the sample pressure and the sample in the line. During pause, sample is stopped and data monitoring / data acquisition continues so a gap will appear in the data file that corresponds to time.
Resume Sample 	Resume sample flow. Click to resume the flow of sample and continue acquisition/sorting.
Refresh Data 	Refreshes the data displayed on plots in the workspace, which can be helpful during setup. Note that this function **does not** erase data being recorded during an acquisition or sort.
Wash Sample Line 	Wash Sample Line moves the loader to the wash position, backflushes the sample line and rinses the exterior of the probe.
Sample Rate Control 	The sample flow rate can be set to a given pressure or to a target event rate. Generally, during setup it is best to use a set pressure and then switch to a target event rate for sorting based on cell concentration and sort efficiency.
Current Percentage Sample Pressure Set Point 	The default mode for sample control is percent sample pressure. The percent sample pressure can be adjusted to consume sample at a slower or faster rate. Increasing the percent sample pressure increases the amount of sample delivered to the nozzle. When sample concentration is 1 million/ml particles per ml, the following settings will produce the approximate events per second. Low = 100 eps Medium = 500 eps High = 1000 eps Note: Actual sheath and sample pressures differ based on the size of the nozzle tip selected.

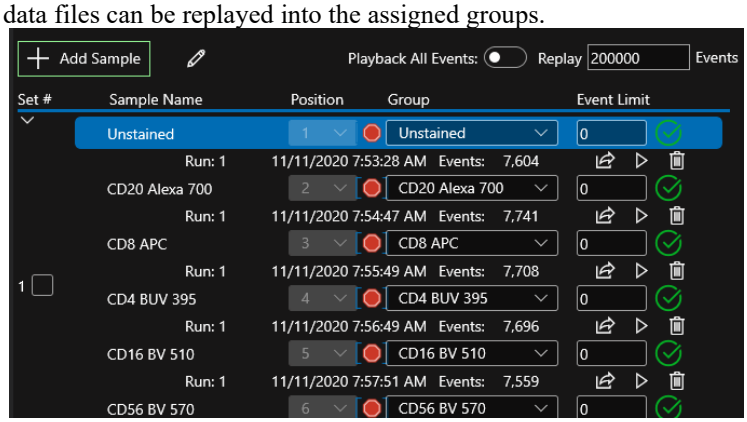
Current Target Event Rate Set Point 	Instead of setting a sample pressure, target event rate can be used to regulate sample flow. The SQ software will automatically adjust the sample pressure to maintain the set event rate. Note: The system will only adjust the sample pressure within a given range. When using event rate control and switching between samples with varying concentrations, the system may take a few seconds to adjust the sample pressure to the correct level. This will affect the amount of sample consumed. Dilute samples will be consumed more rapidly than concentrated ones. Therefore, when starting a sample prior to adjusting trigger and threshold, use a set pressure rather than event rate control.
Actual Flow Rate and Event Rate 	Displays the current sample pressure as well as the event rate expressed as the number of events per second.
Trigger and Threshold 	The trigger parameter alerts the system to the presence of an event over a user-defined threshold. When an event is detected the system acquires all selected parameters for that event. Users can exclude or include data from the acquisition by adjusting the threshold. Data below the threshold will not be saved as part of the FCS file. Only events above the threshold are saved. All live data registered by the Bigfoot data acquisition electronics for any given parameter is displayed in the trigger plot. With the 2D Trigger switch set to off this plot is a 1D Histogram. The X axis is in Log format. The current threshold position is shown at the solid line. The data considered to be below the threshold is to the left of the solid line and in the shaded gray area. The data considered to be above the threshold (and therefore collected in the FCS file) is to the right of the solid line in the white area. Although the Threshold Plot shows every event measured in the trigger parameter, events below the threshold will not be saved in the data file.
Trigger Parameter 	The default trigger parameter is forward scatter (488 nm laser). SQ Software enables data triggering by up to two unique parameters, either scatter or/and fluorescence. These are selected from the list of enabled parameters by clicking on the parameter name. The threshold is set using the trigger parameter selected.
Threshold Setting 	Threshold is a percentage of the signal in the trigger detector. The range that can be entered is 0.01 to 99.99%. The default value is 10.00%. Tip: When utilizing a trigger parameter in log display, set a threshold value of less than 1% to allow display of data in the lower log decades.

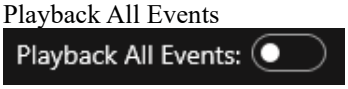
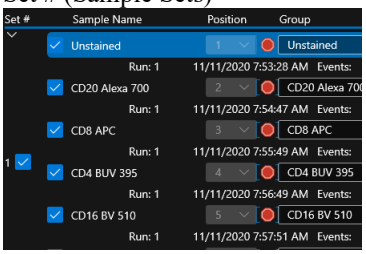
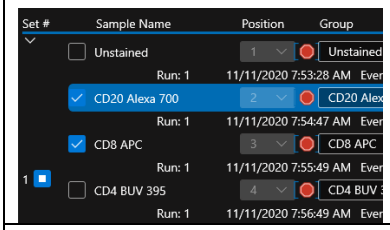
2D Trigger 	By default, a single parameter trigger value is required for data acquisition. The user can define a second trigger parameter if desired. The dual trigger can be set using And or Or logic.
Threshold position, Dual Parameter View 	When activated, the trigger plot changes from a single parameter histogram to a density plot. The parameter and trigger value for the Y axis can be set in addition to and independent of the X axis value. Events must meet both threshold criteria to be included in the data file.
Sample Tube Loader 	This image of the sample loader shows the current position (highlighted in green) and the tube type as 1.5mL. Right-click to define the tube type that will go in each location. Valid tube types are 1.5ml, 5ml and 15ml.
Sample Loader and Sort Output Temperature Control  	Allows the user to set temperature in degrees Celsius for the sample loader and the sort collection stage. The default is off. If desired, click the down arrow to select a temperature. Click the button to enable a temperature control. You can also enter a value in the **Custom** field. Click **Set** to confirm the choice. Note: Allow up to 30 minutes for temperature changes to the sample loader and sort output area to stabilize.
Sample Tube Agitation  	This control enables sample tube agitation. The default is On. The agitation level adjusts depending on tube type. If desired, enable agitation each time an experiment is loaded. Click **All On** to enable all positions. Select the arrow on the right side of the control to select specific loader positions for agitation, to change the speed, or add an intermittent delay.

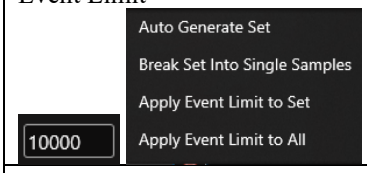
Lighting Control 	This control adjusts lighting for the biosafety containment hood, the sample loader and the sort chamber. The default is Off. Click **All** to enable/disable all lights. Selecting the arrow on the right side of the control allows individual control of the lights as indicated.
Global Cycle Mode 	When activated, all plots in the workspace will display only a certain number of events, based on the time that is entered into this field. This is useful during setup and voltage adjustment. Note: If data recording/sorting is active, Cycle Mode will refresh the plots, however, all events will still be stored. Individual plots can be cycled when the checkbox on the axis is selected.
Droplet Camera 	This is the view from the droplet camera. A stroboscopic light tuned to the same frequency used to create the drops is used to view movement. Droplet formation is controlled and kept constant using the droplet maintenance mechanism. If it cannot be controlled, the system shows maintenance is lost and, if a sort is in progress, it will be stopped. Click the **i** symbol to view details that affect droplet maintenance.

Sample List

The sample definition area of the SQ Software allows programming of the sample loader. Samples can be defined individually and run singly or sequentially as a set, such as in a compensation panel of controls. Once collected, the data files can be replayed into the assigned groups.

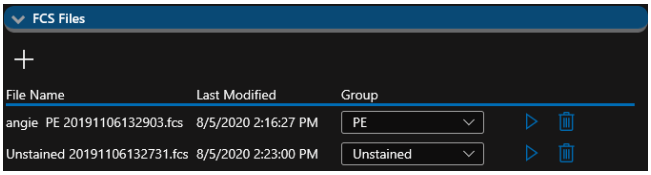


Name	Description
Add Sample 	Selecting the +Add Sample button adds one sample to the sample list. If single-color or negative controls are selected in the fluorophores screen, they are automatically added to the Sample List.
Name Sample 	Samples are added to the list using a chronological default name such as Sample 1, Sample 2 and Sample 3. To change the name, double-click the sample in the list and edit the name. This name will be used to label the FCS file created during acquisition or sorting.
Playback All Events 	Turn this button On to replay every event in the FCS file. This can be time consuming for large FCS files, but necessary for viewing samples that may contain rare events.
Replay last (n) events 	To save time while replaying FCS files, enter a maximum number of events to replay from the file.
Set # (Sample Sets)  	Samples can be acquired or sorted in a batch of up to six tubes if a different sample loader location is designated for each tube. These batches are called sets. When the event limit is reached for a sample in a set, the system washes the probe and proceeds to the next sample. This continues until all samples in a set have been processed. Click **Stop Sample** at any time to stop the batch run. Deselect the checkbox next to an individual sample to prevent it from being acquired as part of the set.
Sample Name Column	The name of the sample to be run. For user defined samples, double-click to edit the name. SQ Software automatically creates control samples using the names that were defined in the Fluorophores screen during setup.

Position	Identifies the loader location for the sample. The dropdown list is used to designate a different location for the sample.
Group	Each Sample can be run using a group of plots within the experiment. By default, control samples are associated with their own, automatically created groups. These should not be changed because they are used for auto compensation or spectral unmixing. By default, samples (other than controls) are associated with the Primary Group. The Primary Group should be used for sorting.
Event Limit 	An event limit can be entered for a given sample. Right-click in the event limit field to auto generate the set, break the set into single samples, apply the value to all samples in the set or all samples in the sample list.
Export FCS 	Opens the Export FCS Files screen where this and other samples can be selected for exporting to another location.
Play FCS 	Loads the saved FCS file into the assigned sample group in the workspace.
Delete FCS 	Removes the sample from the sample list. If a data file has been acquired for that sample, it will also be deleted. SQ Software will issue a warning and require a confirmation to proceed. Once a data file has been deleted it cannot be retrieved.
FCS File Recorded 	Indicates that at least one FCS file has been recorded for the sample.
FCS File Not Recorded 	Indicates that an FCS file has not been recorded for the sample.

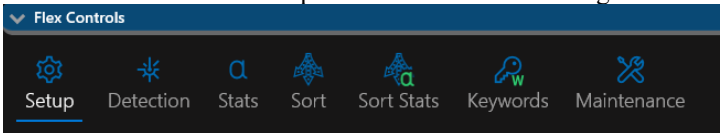
FCS Files

FCS files can be selected and viewed in any group within the experiment. Load the file, select the Group from the dropdown list and click **Play** to load the data. The **Trash** button removes the FCS file from the list but does not delete it from its saved location.



Flex Controls

Flex Controls allow direct control of many of the instrument hardware components and processes. They are located at the bottom of the control panel and have the following main headings.



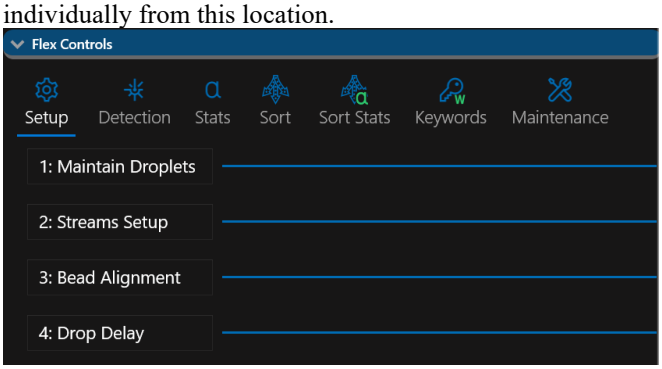
- Setup – Automated functions normally associated with getting the system ready for analysis and or sorting can be run individually from this location.
- Detection – Shows the available lasers and fluorophores, allows settings to be changed individually or imported from the experiment metadata library.
- Stats – Summarizes all plot and region stats available in the experiment. The current plot/gate hierarchy is displayed, which can be modified by the user. Statistics can be shared or exported.
- Sort – Guides the user through the sort process enabling the selection of sort media type, gates, sort modes

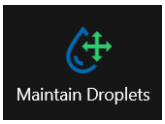
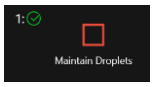
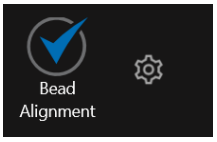
and numbers of cells for sorting.

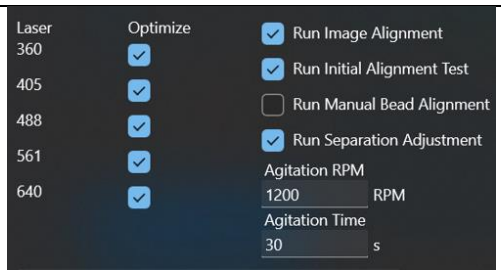
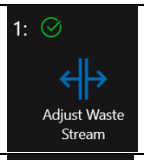
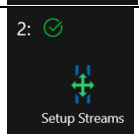
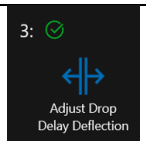
- Sort Stats – Real-time reporting of the current sort statistics. The report can be printed or stored as a record of the sort.
- Maintenance – Tools to aid in cleaning and user maintenance of the system including a wizard to swap the nozzle tip.

Setup – System Calibration

Automated functions normally associated with getting the system ready for analysis and or sorting can be run individually from this location.

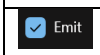
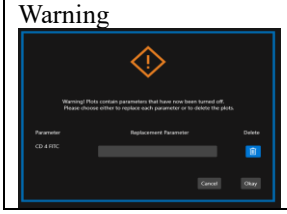


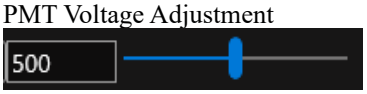
Maintain Droplets	  	Click the blue Maintain Droplets button to automatically set up the Droplet Camera. This button is present when the system is not maintaining droplets and the red droplet icon is also present in the status bar. When the system is maintaining droplets, the **Stop Maintaining Droplets** icon will be visible and the droplet icon in the status bar will be white.
Streams Setup		The stream image view enables visualization of the stream during the calibration processes. The center waste stream and deflected side streams are displayed in the main camera image shown in yellow. On the right is a small section showing a side view of the streams shown in red. This allows automated calibration of the stream positions. The streams camera is located behind the sort area and the streams viewer background adaptor.
Bead Alignment		This button runs the bead alignment process using the on-board QC beads. This includes automated alignment of the stream to the lasers with a single peak bead and separation factor calculation with two beads. FCS files are stored for each of these processes and can be accessed in the Reports menu.

		**Optimize** – When all laser wavelength checkboxes are selected, the Automatic QC process optimizes all lasers equally. Deselecting a laser checkbox does not turn the laser off, but it deprioritizes it for the bead alignment process. **Run Image Alignment** – Select for automated QC to move the nozzle and try to match the saved nozzle position. If you have manually saved a good nozzle position, deselect this box. **Run Image Initial Alignment Test** – Select for automated QC to run 1000 events to quickly check alignment. **Run Manual Bead Alignment** – Select to allow manual bead alignment. **Run Separation Adjustment** – Select for automated QC to adjust voltages to move the blank bead population at 101. These will be the starting voltages for new protocols. **Agitation RPM** – Set the RPM at which the QC bead bottle will be agitated before the test is run. **Agitation Time** – Set the seconds that the QC bead bottle will be agitated before the test is run.
		Automatically adjusts the waste stream to the center of the waste catcher to the left and right as well as front to back, using the nozzle up and down and side to side motorized positioners.
		Enables a four-way test pattern (two left streams and two right streams). Droplet Charge phase and Droplet Charge de-fanning are automatically adjusted to give the best resolution of the four side streams and center stream. These values are used for all combinations of sort directions.
		The drop delay deflection process utilizes a port to the left of the waste catcher to collect sorted droplets. As droplets are deflected into this port, they are interrogated for the presence of drop delay beads. During this process, the waste stream is deflected to the right. and the position of the sorted stream is automatically adjusted to the correct position.
		Enables a stream that is directed straight down. Droplet Charge phase and Droplet Charge de-fanning are automatically adjusted to provide the best resolution. These values are used for straight down sorting.
		Utilizes the on-board QC beads to run the drop delay process and measure the delay between the interrogation point and the droplet breakoff point.

Detection

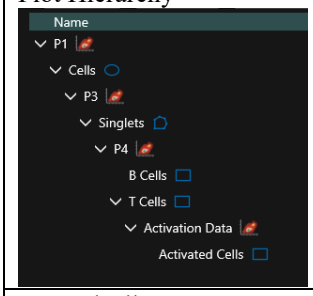
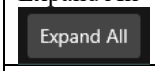
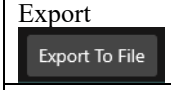
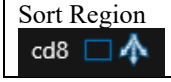
Shows the available lasers and fluorophores, allows settings to be changed individually or imported from the experiment metadata library.

Select Laser 	Click a laser label to view and edit laser and detector settings.
Laser On/Off  	Toggles the laser On or Off (standby mode). When in standby mode, no light from this laser is delivered to the stream and all parameters on the laser path are inactivated. The PMT voltage is 0. If the laser is switched off, plots from these parameters are present in the workspace a warning message will be shown. The toggle button must be in the **On** position for parameters to be selected. The lasers are automatically placed in standby mode if no parameters for the laser have been selected in the experiment.
	The **Emit** checkbox on the right side of the Detection panel allows you to turn a laser on and off while preserving the associated plots and channels/PMTs. This may be helpful if an investigator wants to verify possible spillover from unrelated lasers.
Edit Parameter Names Off  Edit Parameter Names On 	When selected (on), the parameter name and prefixes are shown and editable. Any changes will be saved with the acquired FCS data. When changed, this name appears in the plot axes, compensation screens and filter configuration.
Parameter Disabled  Parameter Enabled 	Parameters can be enabled or disabled from the detection panel. If a parameter is disabled, the SQ Software will warn if plots exist in the experiment with that parameter. Only enabled parameters will be saved in the FCS file.
Warning 	If you attempt to delete a parameter and it has active plots available, this message is displayed. You can replace it with another parameter to preserve any plot and gating strategy. **Delete** will delete the parameter. **Cancel** will leave this menu without making any changes **Okay** will enable any changes you have made.

Filter Information 	Displays the filter information for each detector.
Laser Required Power, Actual Power and Status 	The maximum laser power for each laser is 100 mW. This is the default setting for power. Laser power can be reduced to 10mW if needed for an experiment. The actual laser power output is displayed to the right. When in use, the laser status shows emitting. When the laser is switched to standby mode the laser power is grayed out and the status changes to Ready.
Import Settings from the Metadata Library 	Settings such as PMT voltages, laser powers, etc. can be imported from the experiment metadata library.
PMT Voltage Adjustment 	The PMT voltage for each detector can be adjusted individually. Photomultiplier Tubes (PMTs) are electron cascade devices and varying the voltage changes the sensitivity. Data with a low level of heterogeneity should be displayed in linear and the voltage changed in 1- or 10-volt steps. Data with a high level of heterogeneity should be displayed on a log scale and voltage changed in steps of 10 or 20 to optimize population display and separation. Changing the voltage through a range of voltages will produce a linear response. This range can be determined by the user. The typical voltage range is 300-900.
ND Filter Retracted  ND Filter In 	Each scatter detector has a retractable Neutral Density (ND) filter. The filter is an ND 1 (i.e. 10% light transmission). It is retracted by default. If signal reduction is desired, the filter can be moved into the light path of the detector by clicking on the button.
Polarization Filters 	This button appears when polarization filters are present on the instrument for the corresponding scatter parameter. When you click a Polarization Filter, a filter moves into place on the detection path.
Laser Sync Mode for Spectral Setup	Adjusting the voltages for a given laser in sync gives the ability to move up or down all the corresponding signals. This can be helpful if the location of the negatives is too low across a given laser detection path, if the positive population is off-scale, or if the laser power was adjusted. The software also allows sync mode across all parameters.

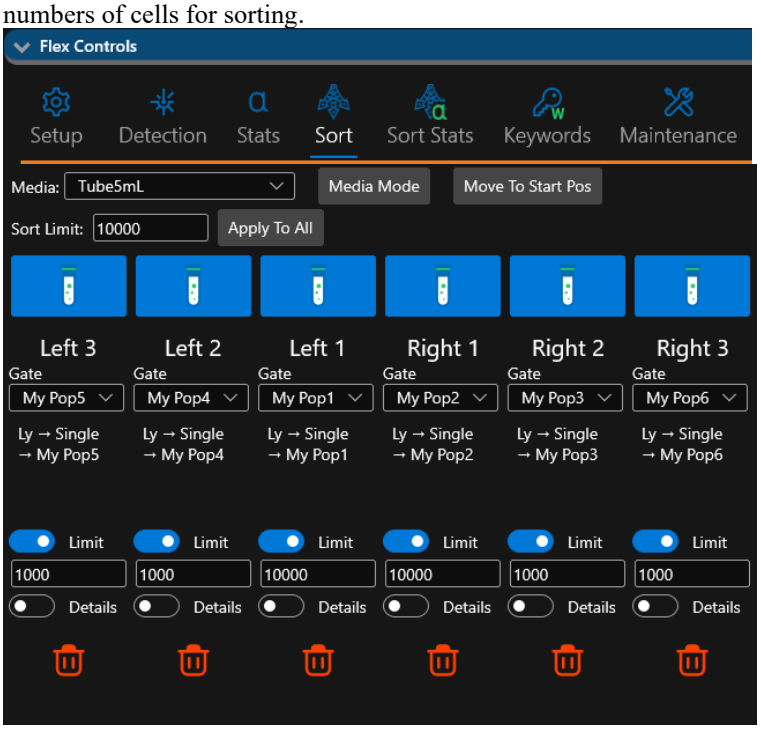
Stats

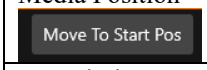
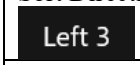
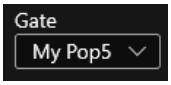
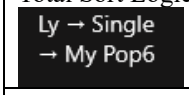
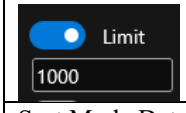
Summarizes all plot and region stats available in the experiment. Current plot/gate hierarchy is displayed which can be modified by the user. Statistics can be shared or exported. The statistical information found in the Flex Tools is the same as in the Workspace tools.

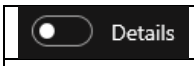
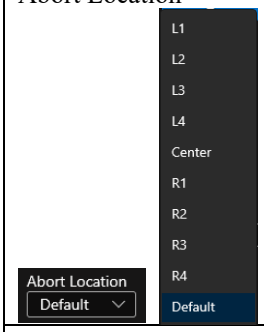
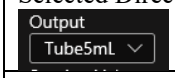
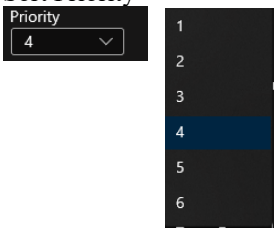
Stats Table Headings 	The selected statistics for the created plots and regions are shown in this table. Columns can be added or removed in User or Global Settings, Statistics or Combined Statistics.
Plot Statistics 	Next to the plot name is an icon representing the plot type. Plots can be renamed on the plot itself. New names entered will be reflected in the stats table.
Region Statistics 	Next to the region name is an icon representing the region type. Regions can be renamed on the plot itself. New names entered will be reflected in the stats table.
Plot Hierarchy 	The indented view shows the hierarchy of the plots and regions. Click the > arrow to show or collapse the hierarchy beneath a plot or region.
Expand All 	Expands the hierarchy for all the plots and regions.
Collapse All 	Collapses the hierarchy for all plots and regions.
Show Plot Statistics 	Display plot statistics on all plots in the experiment. Note: The statistics to be displayed are selected in Settings, User Settings/Global Settings.
Export 	Opens a windows file save menu allowing the CSV file to be saved to any available drive.
Sort Region 	When the region is selected for sorting, the sort icon is displayed next to the region name.

Sort

Sort Setup guides the user through the sort process enabling the selection of sort media type, gates, sort modes and numbers of cells for sorting.



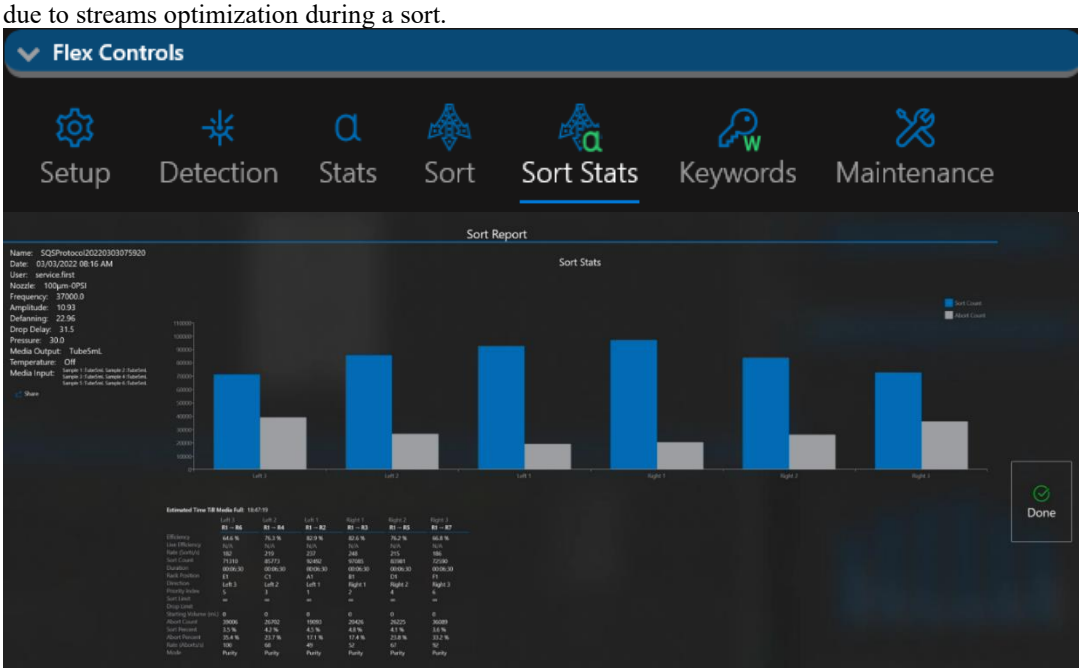
Output Media Selection 	Select the desired output media type for sorting.
Media Position 	The move to start position will move the output stage to the starting position for the sort.
Sort Limit 	A global sort limit can be set for all sort directions. Note: The default sort limit is set to 1000. Individual sort limits may be set under the specific sort direction.
Sort Media Type 	This icon represents the sort media type in the selected direction.
Sort Direction 	Depending on the output media selection, different numbers of sort directions will be available.
Sort Gate Name 	The final gate in a sort logic string for a specific direction is selected and displayed. When the user defines the sort gates the software automatically assigns the sort directions. When the user moves the cursor over a sort gate field, the corresponding sort region on the plot in the workspace highlights in solid green. The parent gates for the sort gate flash green.
Total Sort Logic String 	Shows the full gating scheme for the sort direction.
Enable/Disable Sort Limit 	When selected, the sort direction will have a limit enabled (set to 1000 in this case). When disabled, no limit is set in this sort direction and sorting will continue until the sort media is full or the sample is consumed.
Sort Mode Details Off	Details are not shown and default sort mode is set.

	
Sort Mode Details On 	Sort Mode Details On provides the details of a sort direction and allows the user to edit different sort modes and limits.
Abort Location 	Events that are aborted due to sort mode or envelope can be captured in another location for further sorting or reanalysis. The location for the aborts can be assigned here. Note: Multiple aborts can be assigned to the same location.
Sort Collection Tube Type for Selected Direction 	Individual sort output media can be selected from the dropdown list per sort direction.
Starting Volume for Sort Output 	Enter an initial volume for the output media. This most commonly would represent the volume of buffer added to the tube to prepare it to receive sorted cells. This, combined with the volume of sorted drops will be used to determine when the sort media is full and the sort will halt.
Sort Priority 	Sort Priority is automatically assigned in the order sort gates are selected. This can be changed if desired. If one population for sorting is a subset of another, prioritize the smaller subset higher to sort those first.
Sort Mode 	**Enrich** This mode will sort all positive events even if negative events are nearby. Purity will be reduced to maintain high recovery. Enrich should be used for sorts when recovery is critical, and purity is not as important. This mode minimizes aborts and provides the highest efficiency. **Purity** This mode will sort if no negative events are present in the droplet envelope. If multiple positive events are present, sorting will still occur. Purity should be used for general sorts where both purity and recovery are important. This mode provides the second-best efficiency. **Single** This mode requires that one and only one positive event be in the center of

	the droplet envelope without any negative events nearby. Recovery will be reduced due to these strict sort restrictions. Single should be used for single cell deposition, or where an accurate number of cells is required because other modes will still sort when multiple target cells are present. This mode provides approximately 50 percent efficiency.
	**Recovery Enhance** – When the target event is close to the upper or lower edge of the target drop, the adjacent drop will be sorted if it meets the sort mode criteria.
Delete Sort Direction 	Delete the sort direction from the experiment.

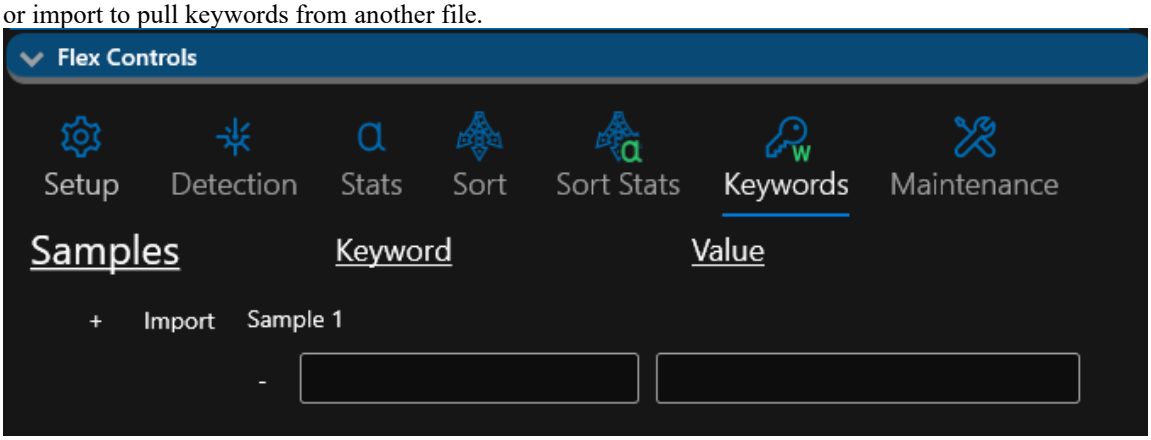
Sort Stats

Sort Statistics are available in the Flex Controls area of SQ Software during and immediately after a sort. Information includes the sort status, system software settings during the sort and a graph that displays the sort count and abort count per sort direction. Live efficiency is a recent addition that allows you to view changes to efficiency due to streams optimization during a sort.



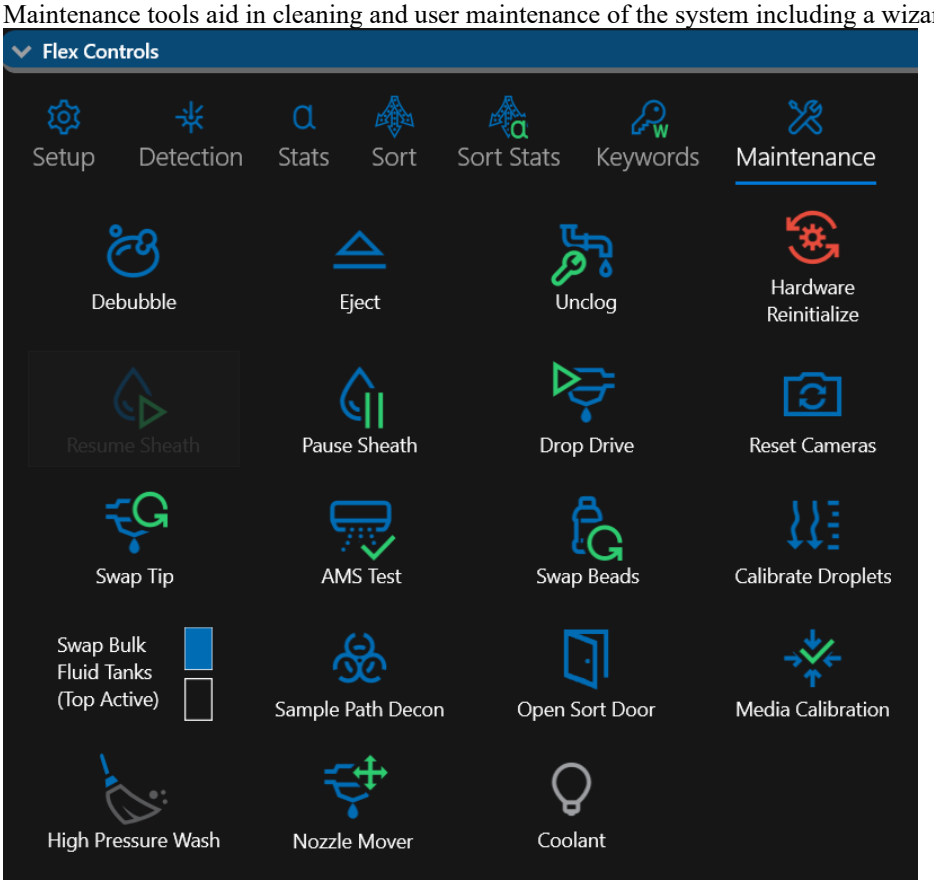
Keywords

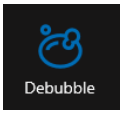
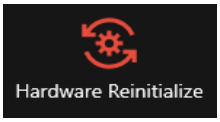
Keywords are helpful when you intend to use different software packages to analyze Bigfoot FCS files. Keywords such as cell type and treatment time can be added to a sample using the keyword section of the flex space. These keywords will be saved with the file and can be accessed through the metadata display. Click + to add new keywords or import to pull keywords from another file.

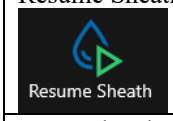
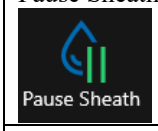
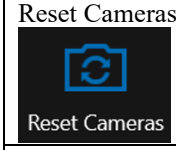
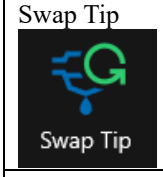
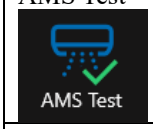
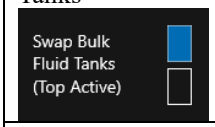
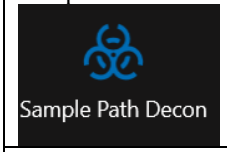


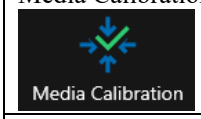
Maintenance

Maintenance tools aid in cleaning and user maintenance of the system including a wizard to swap the nozzle tip.



Nozzle Debubble 	The debubble process uses the sheath valves to eliminate bubbles from the nozzle body and tip. If the droplet image is shifting or the droplets are not symmetrical, it is likely that a bubble has formed in the nozzle. The debubble process automatically runs as part of startup and nozzle swap. Nozzle debubble may cause droplet maintenance to be lost. If this is the case, re-establish sort maintenance, stream setup and drop delay before commencing your sort.
Eject 	Eject stops the acquisition, turns off sample pressure, lifts the probe out of the sample tube and initiates probe wash. This is only used in the rare case where software has crashed with sample running and you have reopened the software.
Unclog 	Moves the nozzle to the docking location for unclogging. This process applies high pressure to force fluid back through the nozzle tip and away to waste, removing cellular debris. This process is done automatically during nozzle swap and shutdown. If you have utilized the unclog process droplet maintenance will be lost. Re-establish QC, sort maintenance, stream setup and drop delay before commencing your sort.
Hardware Reinitialize 	Occasionally, the need may arise to reinitialize the hardware. For example, if an adapter or tube is not placed correctly and the loader crashes or if something is in the way of the sort door closing, an error occurs and the hardware may need to be reset. This process will rehome the loader and doors to ensure the obstruction has been cleared.

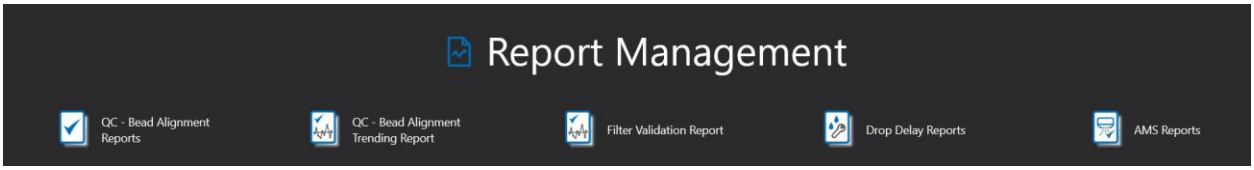
Resume Sheath 	This button resumes the flow of sheath if it has been paused.
Pause Sheath 	The sheath stream may need to be paused while the system is running to clean the sort plates. This button allows you to pause the stream. Pause Stream will cause droplet maintenance to be lost. Re-establish sort maintenance, stream setup and drop delay before commencing your sort.
Enable Drop Drive 	This button can be used to enable and disable drop drive voltage to the nozzle. If clicked while the system is maintaining drops, the maintenance will stop.
Reset Cameras 	There are several cameras used in the Bigfoot system. If a camera error occurs, this button can be used to reset them.
Swap Tip 	Opens the swap tip wizard and walks the user through tip removal and replacement. This can be used to remove a stubborn clog (if sonication is required) or to change between nozzle tip sizes. After the tip change process, it is necessary to run QC and drop delay again. Swap Tip is disabled while running QC, acquiring, or sorting.
AMS Test 	Opens a wizard to test the aerosol management system according to the ISAC guidelines using Dragon Green beads and Cyclex-D cartridges. This wizard will walk the user through the process and create a report at the end to confirm the results. AMS Test is disabled while running QC, acquiring, or sorting.
Swap Beads 	Rotates the loader to the bead bottle position and opens the cap to allow you to remove and replace the bead bottle. After installing a new bead bottle, click **Wash** to replace the cap and reset the loader. **Swap Beads** is disabled while running QC, acquiring, vortexing or sorting.
Calibrate Droplets 	When changing between nozzle tip sizes, droplet calibration may be necessary to optimize the breakoff position. This process sets the amplitude and adjusts the droplet frequency to optimize the last attached drop for stability.
Swap Bulk Fluids Tanks 	Changes the system from using the top to bottom (or bottom to top) bulk fluid bottles. If the bottles in use are low and you need to refill or empty them, switch to the other set of bottles. Important: Do not remove the illuminated, active bottles from the system.
Sample Path Decon 	Starts a decontamination process for the sample line and sheath path back to the in-line filters. This process fills the fluidic lines with AHP/DI solution, allows soaking time and then thoroughly rinses all the lines with DI water. This process takes approximately an hour. This is disabled while running QC, acquiring, or sorting.
Open Sort Door 	Opens and closes the sort chamber door. When running QC, acquiring, or sorting the door cannot be opened for safety reasons.

Media Calibration 	Opens the media calibration screen to calibrate or check sort output media positions. Here you can adjust side stream positions for both plates and tubes to fine tune or check positions for sorting.
High Pressure Wash 	Starts a high-pressure wash.
Nozzle Mover 	Opens the Nozzle Mover panel that shows live images of the sample stream, provides controls to manually or automatically move the nozzle and a control to save the new nozzle position.
Coolant 	Turns on/off the lighting to the coolant tank to aid in viewing fluid levels.

Report Management

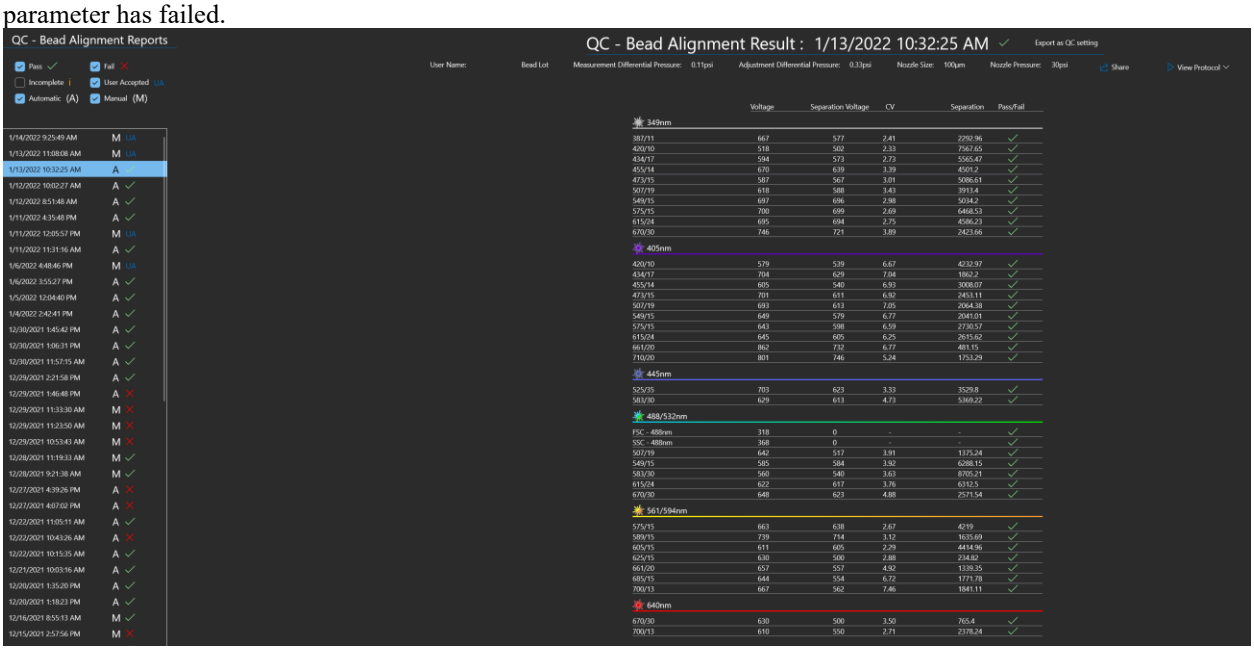
Click **Report** in the **Quick Menu** to access the **Report Management** screen.

Click the report you would like to view.



QC Report

QC Report allows you to select a specific QC session, view the data, view the protocol used to run QC and share the results. You can filter results based on nozzle size, automated, manual, user accepted and incomplete QC sessions. The report also includes sheath pressure and variation from max CV per filter/PMT that is displayed when a parameter has failed.



Automated QC Filters

Automated QC results can be filtered by sessions that passed, failed or were incomplete.

Manual QC Filters

Manual QC results can be filtered by sessions that passed, failed, or were incomplete and whether the results were accepted by the user.

Separation Voltage and Separation Index Display

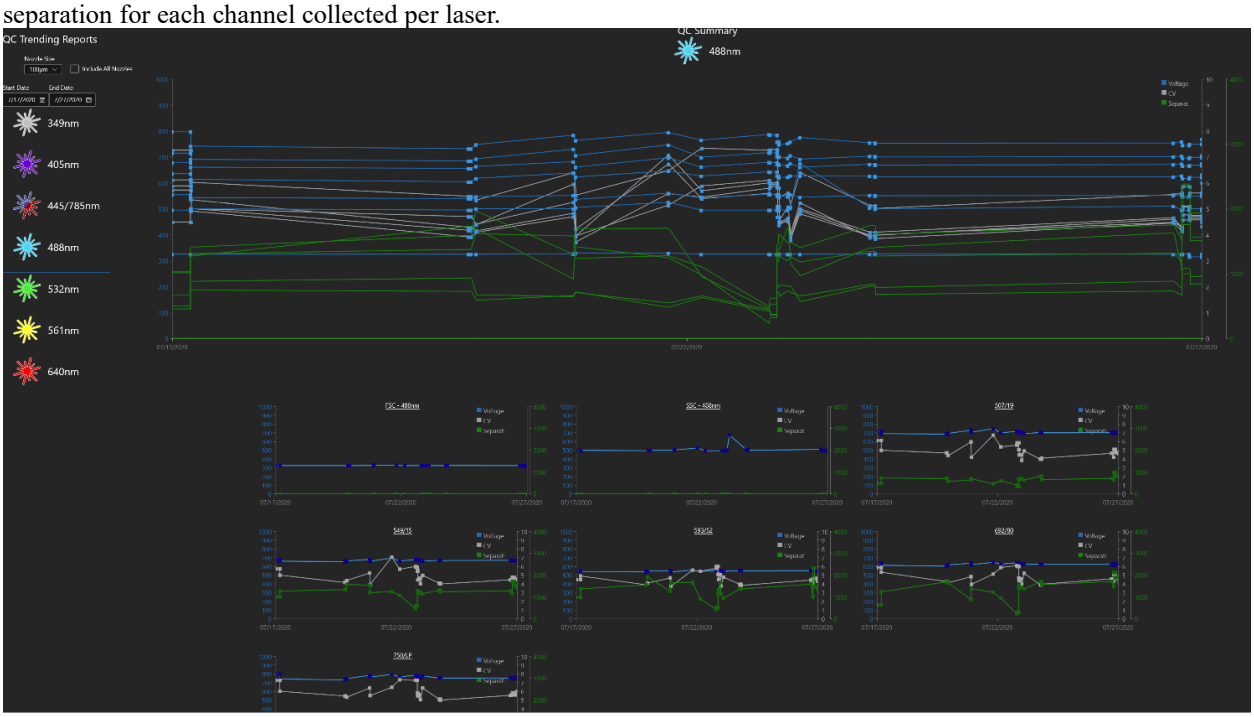
The QC Report displays the per-filter separation voltage, which is the voltage that was required to move the blank bead population to the first log decade and is used to calculate the separation index. The per-filter separation index for the Bigfoot QC Bead Mix is also displayed.

Nozzle Pressure Display

Sheath pressure at the nozzle is displayed as well as measurement differential pressure and adjustment differential pressure.

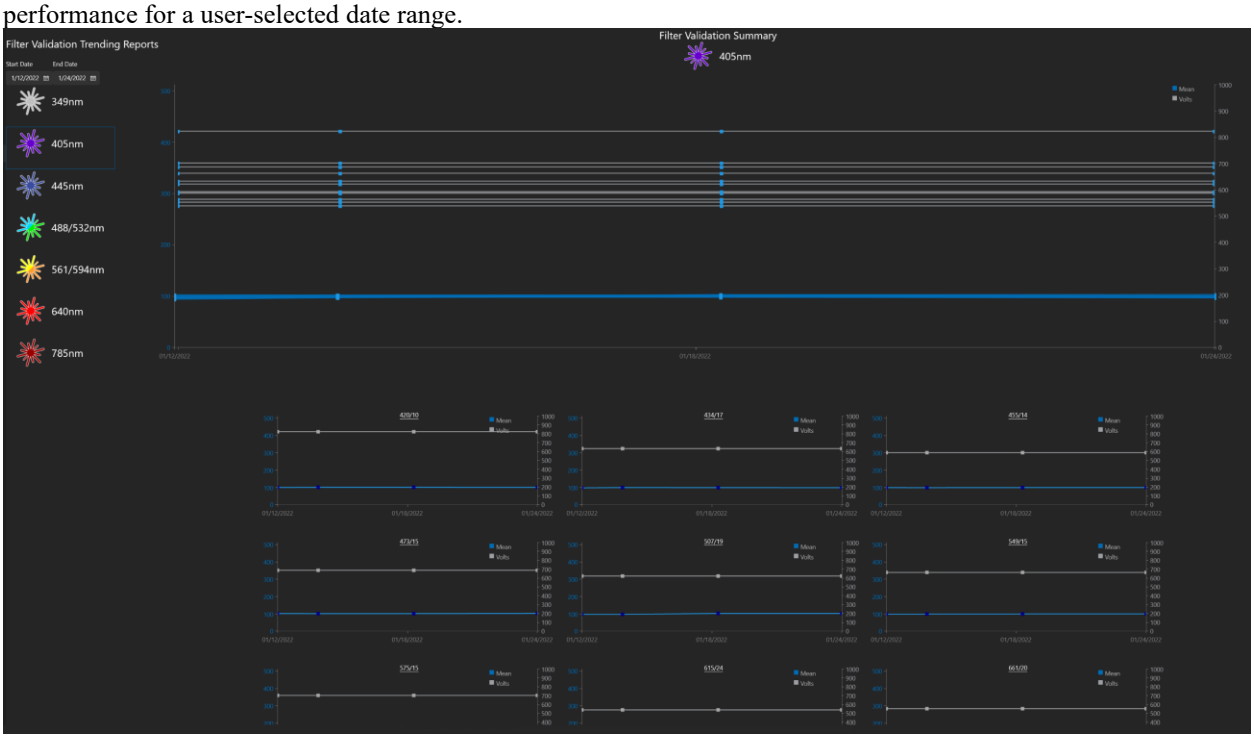
QC Bead Alignment Trending Report

The QC Bead Alignment Trending Report allows you to select a specific nozzle size, or include all nozzle sizes and specify a date range for which you would like to view a report of instrument performance. View voltage, CV and separation for each channel collected per laser.



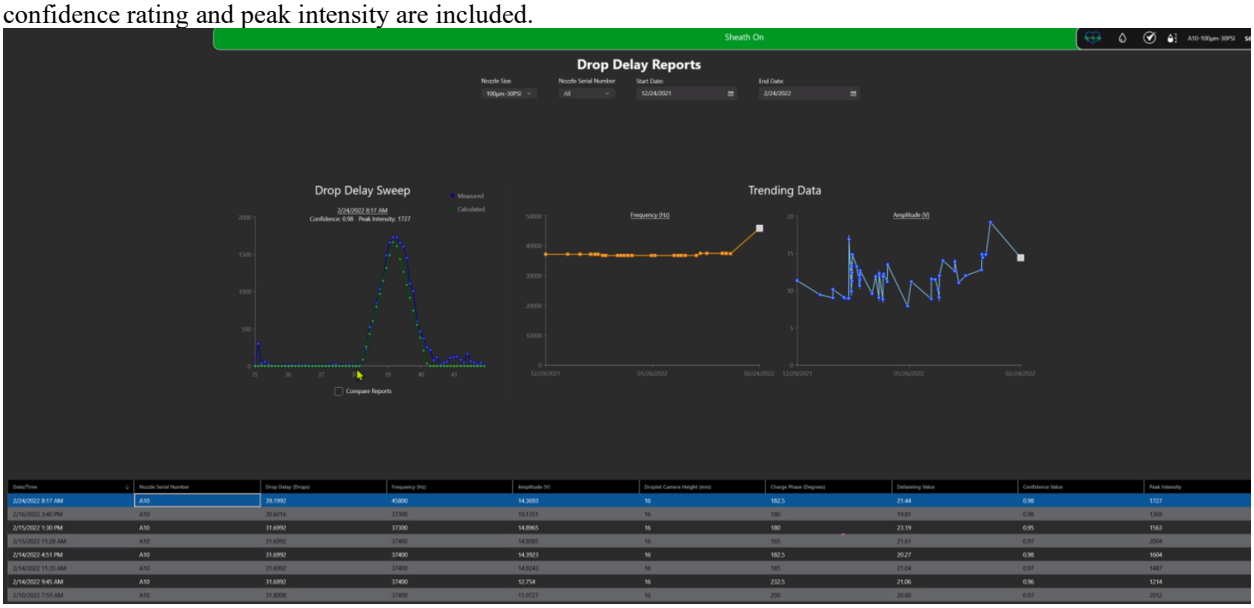
Filter Validation Report

The Filter Validation Report displays the results of the automatic comparison of filters that are installed on the system with filters that are listed in the software. Data from this report can reveal variations in filter or PMT performance for a user-selected date range.



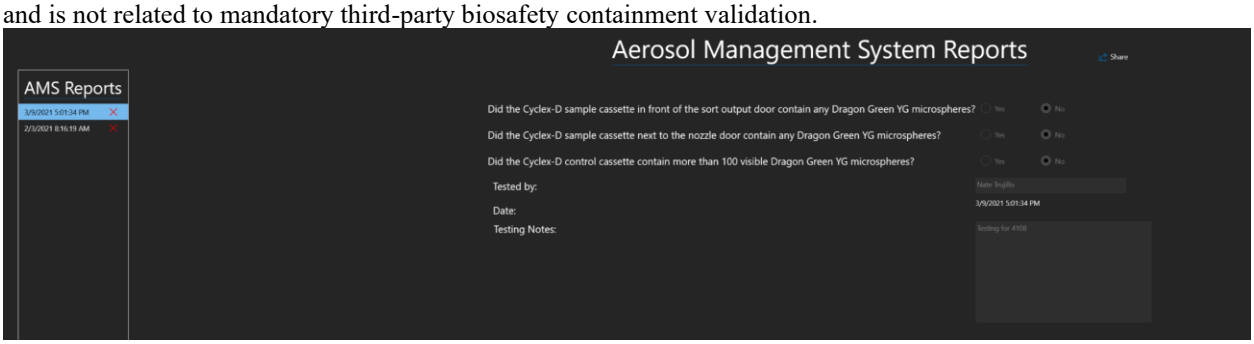
Drop Delay Reports

Drop Delay Reports allow you to select a specific nozzle size, nozzle serial number, or include all nozzle sizes and view a trending report of drop delay sweep, frequency and amplitude for every time drop delay was run on the system. Additional information such as nozzle serial number, droplet camera height, charge phase, defanning value, confidence rating and peak intensity are included.



AMS Report

The AMS Report displays the results of aerosol management tests that were performed using the AMS Wizard. The AMS Wizard guides the user through AMS validation as outlined by the International Society for the Advancement of Cytometry (ISAC). The frequency with which the AMS validation test is performed is determined by each facility and is not related to mandatory third-party biosafety containment validation.



Settings

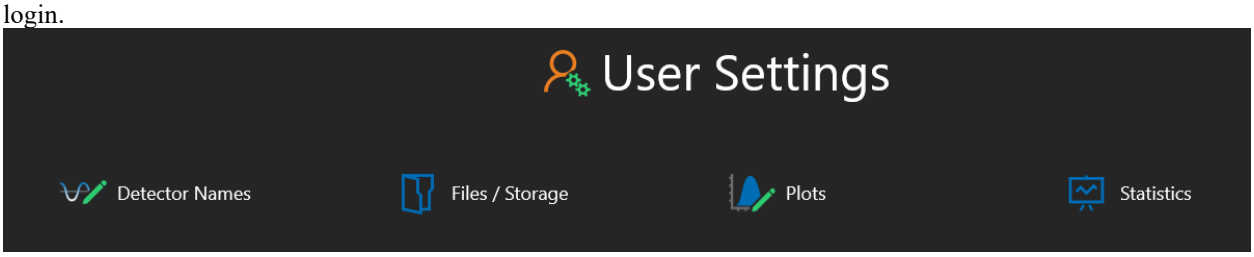
SQ software can be customized in Settings via Global and User settings. User settings inherit their values from Global Settings, but some features can be overridden in the User Settings section or by the person with an individual User Login.

Click the **Gear** button at the bottom of the **Quick Menu** to access **User Settings** and **Global Settings**.

Administrators can set **Global** and **User Settings**. Individual users can customize settings that are specific to their own login.

User Settings

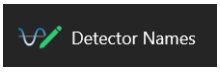
User Settings inherit their initial values from Global Settings. People with User Logins can customize detector names, file storage locations, plot building features and the statistics that will be displayed specific to their own login.



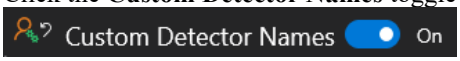
Detector Names

Custom detector names entered from the User Settings screen will appear throughout the software whenever this user is logged in. Detector names can also be changed in the New Sort workflow or in Flex Controls when running samples.

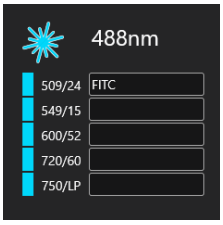
1. Click **Detector Names** to enter or edit custom detector names.



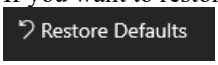
2. Click the **Custom Detector Names** toggle button to **On**.



3. Type the custom name in the field next to the corresponding filter number. Changes are automatically saved.



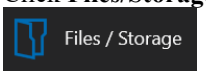
4. If you want to restore all filter names to the defaults that are set in Global Settings, click **Restore Defaults**.



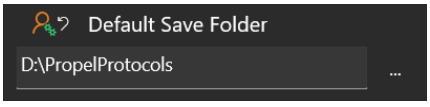
Files/Storage

The file storage location, FCS export location and default parameter types entered from the User Settings screen will apply for this user's session every time they are logged in.

1. Click **Files/Storage**

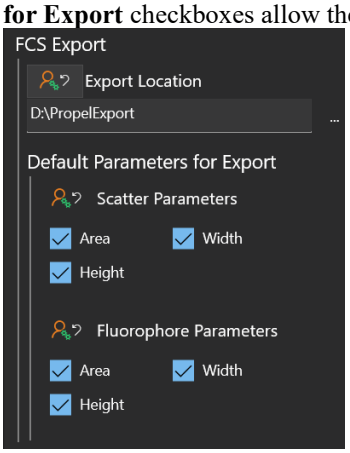


2. **Default Save Folder** is the save location for protocols created during this user's session.



3. Enter a new folder name, or click the **Ellipses** to navigate to a fixed drive local to the Bigfoot PC.
   Note: A subfolder with the current username will automatically be created in this folder.

4. **FCS Export** is the save location for FCS files exported during this user's session. The **Default Parameters for Export** checkboxes allow the user to include or exclude parameters in exported FCS files.

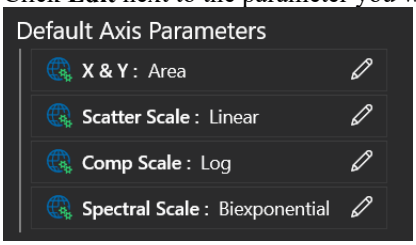


Plots

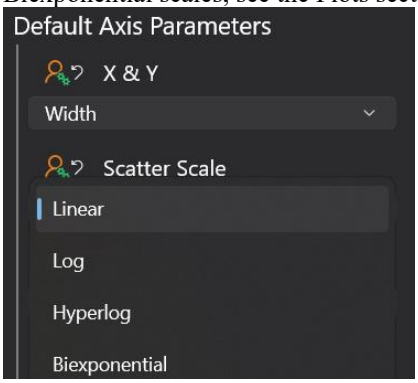
In SQ Software the user can create custom defaults for parameter type and parameter scaling that will be used when plots are created.

1. To select default parameters and scaling, click **Plots**.

2. Click **Edit** next to the parameter you want to change.



3. Select the parameter type and scaling. Note: For in-depth information regarding the Hyperlog and Biexponential scales, see the Plots section in Global Settings.

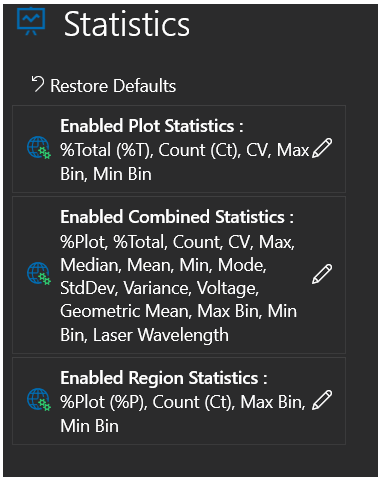


4. To restore the single selection to its default value, click the following button.

5. To restore all selections to the global default values, click **Restore Defaults**.

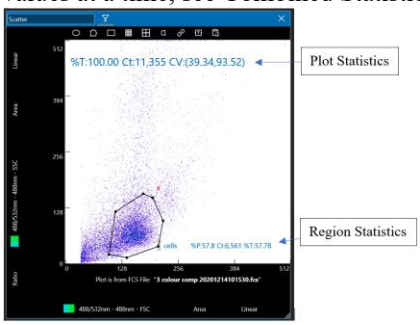
Statistics

Experiment Statistics can be displayed in a variety of locations within the SQ Software workspace Plot, Region and Combined Statistics are shown below. The user can customize the statistics that appear in each context.



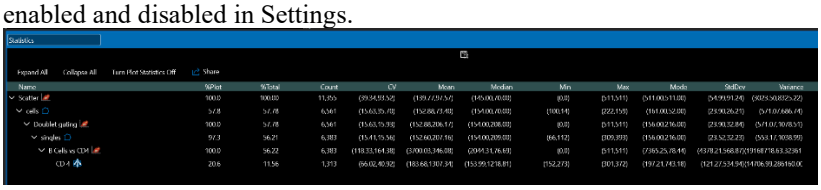
Plot and Region Statistics

Due to limited space on plots and regions, up to three values should be displayed at a time. To view more than three values at a time, see Combined Statistics.



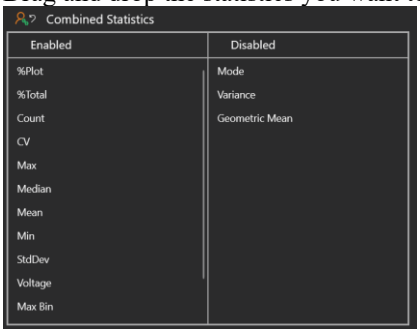
Combined Statistics

Combined Statistics can be viewed from the Workspace tools and in the Flex Controls. The available options are enabled and disabled in Settings.



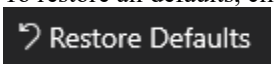
Enable and disable Combined Statistics

1. Click the edit button next to **Combined Statistics**.
2. Drag and drop the statistics you want to enable or disable.



3. To restore defaults for a specific context, such as Plot Statistics, click **Enable Individual Defaults**.

4. To restore all defaults, click **Restore Defaults**.



Global Settings

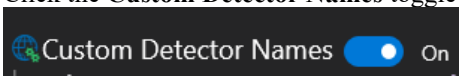
Administrators have access to Global Settings as well as User Settings. Global Settings include the options allowed in User Settings and include additional system settings such as diagnostics, emergency contacts, fluorophores, QC, drop delay and startup/shutdown settings. User Settings inherit their values from Global Settings unless they are overridden in the User Settings section or by a person with a User Login.

Detector Names

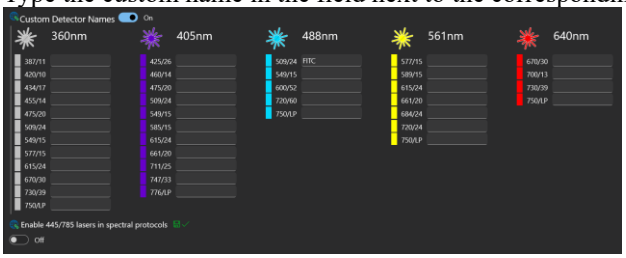
Custom detector names entered from the Global Settings screen will appear throughout the software as the system default. Detector names set in this screen can be individually customized in User Settings. They can also be changed in the New Sort workflow or in Flex Controls when running samples.

1. To enter or edit custom detector names, click **Detector Names**.

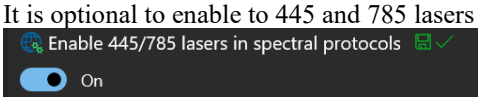
2. Click the **Custom Detector Names** toggle button to **On**.



3. Type the custom name in the field next to the corresponding filter number.



4. It is optional to enable to 445 and 785 lasers for spectral protocols.



5. If you want to restore all filter names to the blank default, click **Restore Defaults**.

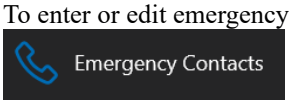
Diagnostics

Do not make changes on this screen unless specifically directed to do so by support representative.

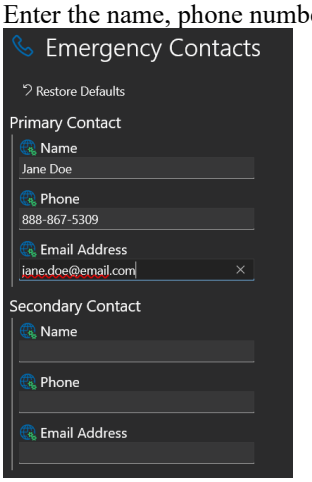
Emergency Contacts

Emergency Contact information is displayed when you click **Info** in the lower-left corner of the screen at the bottom of the Quick Menu.

1. To enter or edit emergency contacts, click **Emergency Contacts**.

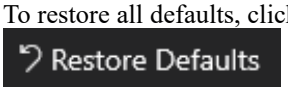


2. Enter the name, phone number and email of the Primary and Secondary contact.



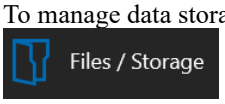
3. Click the **Info** button in the lower-left corner of the screen to verify the contact information.

4. To restore all defaults, click **Restore Defaults**.



Flies/Storage

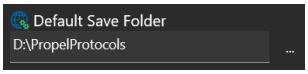
The Files/Storage settings allow an administrator to set the default folders for saving, backing up and archiving data files. Administrators can also set the default folder for FCS exports and the parameters that will be included.
To manage data storage options, click **Files/Storage**.



Default Save Folder

SQ Software stores FCS files that are created during setup and sorting to the Propel/Protocols folder. If you change the location of this folder, it should still be on a fixed drive that is local to the Bigfoot PC.

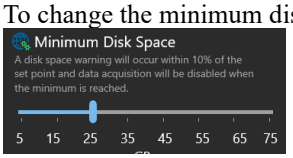
1. To change the **Default Save Folder**, click the ellipses next to Propel/Protocols and navigate to a new folder.



Minimum Disk Space

Experiments run on the Bigfoot system can create FCS files that are 10's of GB in size. Therefore, SQ Software warns the user when the remaining storage space is within 10% of the set point and disables data acquisition when the minimum is reached.

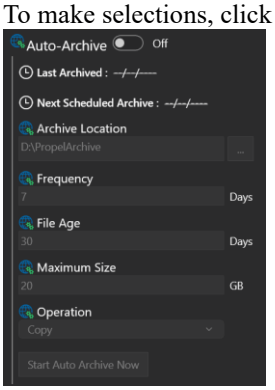
1. To change the minimum disk space value, click and drag the slider. The new value is automatically saved.



Auto Archive

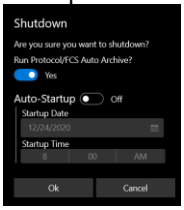
SQ Software can automatically archive data stored in the Bigfoot PC Default Save Folder.
During Acquisition and Sorting the whole experiment is stored, including the protocol plot definition, instrument settings, sort settings and sort statistics together with all FCS files associated with the experiment.
The auto archive is designed to move or copy this information to another location, to preserve the data safe location or to free up space on the local storage device.
By default, auto archive is set to Off and no selections are active.

1. To make selections, click the **Auto-Archive** toggle button to On.



Last Archived – The date of the last archive event.
Next Scheduled Archive – The date when the next auto archive event is scheduled.
Archive Location – Location of files after the archive operation. Do not use the same drive as the original save location because archive is intended to free up space on the default data storage device.
Frequency – The interval between auto archive events.
File Age – If the experimental protocol is older than the number of days set here then it will be auto archived.
Maximum Size – The maximum file size that will be archived, files bigger than this will not be automatically archived.
Operation – Designates whether files are copied or moved during auto archive. Note on successive archive runs, this utility will create only one copy of the file on the target drive.
Start Auto Archive Now – Starts the archiving process.

2. When shutting down the system, ensure that auto archive is enabled. This will log out of SQ Software workspace but allow the software to process the auto archive request.

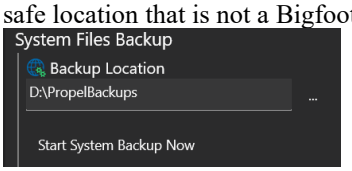


System Files Backup

SQ Software builds of a library of files to store information such as sort media definitions, fluorophore information, nozzle size default settings, QC settings and more. It is useful to back up these files to quickly recover from data loss

such as a PC hardware failure. System Files Backup is automated and happens throughout operation of the system. The system will automatically delete old files when new ones are created.

1. To change the System Files Backup location, click the ellipses and navigate to the storage location. Select a safe location that is not a Bigfoot PC local drive.

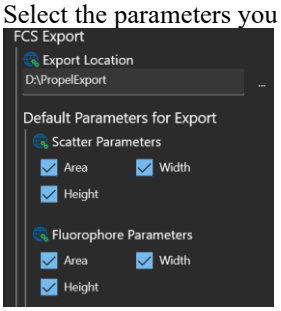


2. Click **Start System Backup Now** to activate the process.

FCS Export

This sets the default location and parameters that will be included when the user exports FCS files.

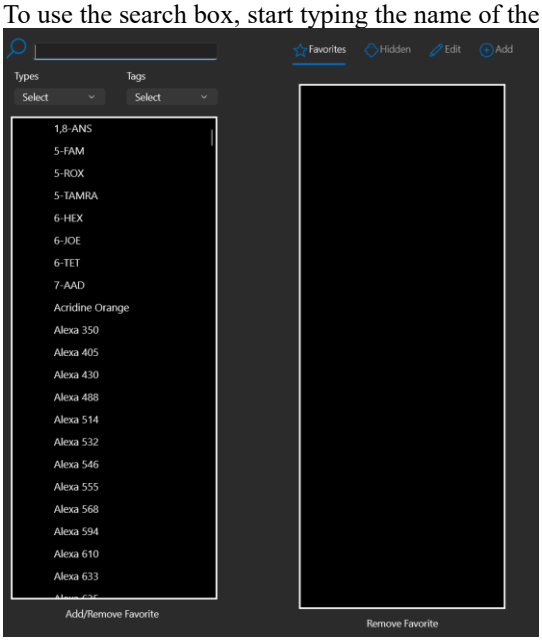
1. To set the FCS Export folder, click the ellipses and select a location accessible to the Bigfoot PC.
2. Select the parameters you want to export by default.



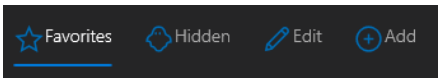
Fluorophores

SQ Software utilizes the excitation and emission spectra in two locations. First, in non-spectral sorts to select which available laser is most suitable to excite a given dye, to display the theoretical spectra of each dye for each available laser and select which available optical filter can be used to collect fluorescence emission. Second, its used in the spectral sort definition to show the emission of a given dye at each of the detection points for each laser as well as to calculate the complexity and simplicity of parameters. If a fluorochrome has a defined type or tag the list of displayed fluorochromes will be filtered to reflect this. The search box can be used to find fluorochromes already available in SQ Software and fit any type or tag terms.

1. To use the search box, start typing the name of the required fluorochrome and the list is filtered as you type.



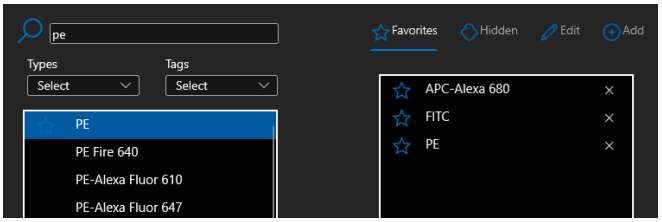
2. Click **Favorites**, **Hidden**, **Edit**, or **Add** to customize the **Fluorophores** list users will see in SQ Software.



Favorites

Fluorophores designated as Favorites appear in the users' fluorophores list with a star next to them. Favorites can simplify protocol creation by allowing users to find the most used fluorophores easily during protocol creation.

1. To mark a fluorophore as a favorite, click **Favorites** to make the list active.
2. Find the fluorophore in the fluorophores list and double-click on it. The fluorophore appears in the Favorites list with a star next to it.

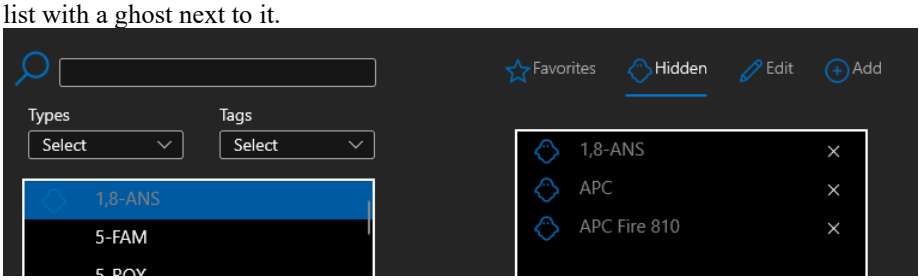


3. To remove a fluorophore from the list, click the X next to the fluorophore name.

Hidden

Fluorophores designated as Hidden do not appear in the users' fluorophores list. Administrators can hide fluorophores that are not available in the lab to help simplify protocol creation.

1. To hide a fluorophore, click **Hidden** to make the list active.
2. Find the fluorophore in the fluorophores list and double-click on it. The fluorophore appears in the Hidden list with a ghost next to it.

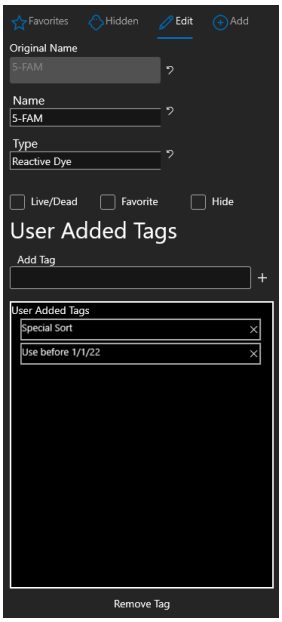


3. To remove a fluorophore from the list, click the X next to the fluorophore name.

Edit

Administrators can edit information for specific fluorophores, mark as favorite or hidden, add tags and more. If a fluorochrome was not a default fluorochrome delivered with the software it can also be deleted

1. Select the fluorophore you want to edit in the Fluorophores list.
2. Click **Edit** and make the desired changes. Information is automatically saved.



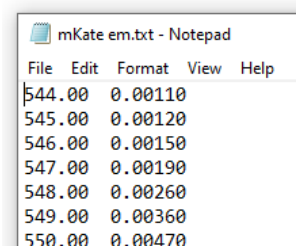
3. To remove User Added Tags, click the X next to the Tag name.

Add

Administrators can add custom fluorophores to the Fluorophores list if they have the emission and excitation information files from the manufacturer.

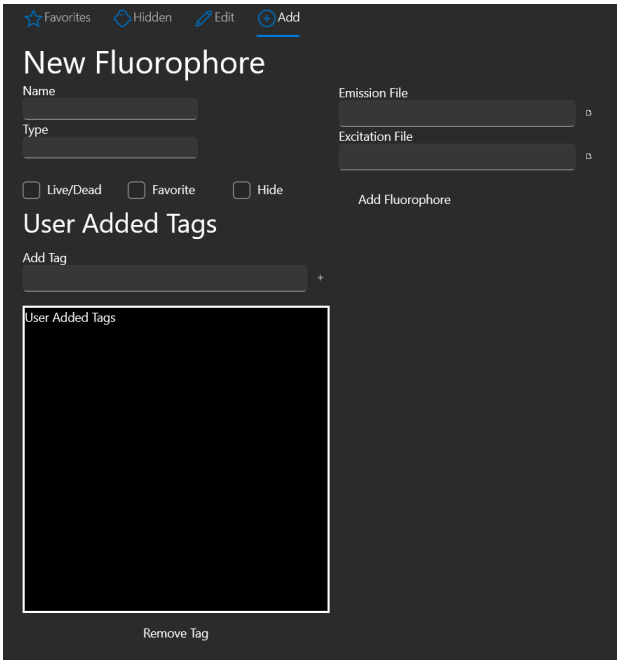
Emission and Excitation File Format

Obtain the excitation and emission information from the dye manufacturer. Make sure the information is in two CSV files- one CSV file format named dyename em .txt and in another CSV file named dyename ex.txt. Save the files to a folder the Bigfoot can access.



The left column is the wavelength low-to-high in increments of 1.
The right column the relative ex/em 0 to 1.

1. To add a new fluorophore, click **Add**.
2. Enter the name for the fluorophore.
3. Click the button next to the **Emission File** field and navigate to the CSV file named “dyename em.txt”
4. Click the button next to **Excitation File** fields and navigate to the CSV file named “dyename ex.txt”
5. It is optional to include the remaining details or to add tags.

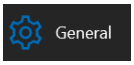


6. When at least a name and the CSV files are entered, click Add Fluorophore.

General Global Settings

Administrators can include a custom logo to the workspace and set the system to wash the probe twice between samples.

1. Click **General**.



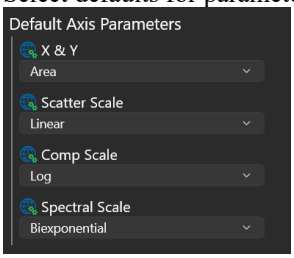
2. Click **Choose Image** and navigate to the image file.

3. Set **Double Wash Between Samples** to the desired state.
4. To restore defaults, click **Restore Defaults**.

Plots

In SQ Software the administrator sets defaults for parameter type and parameter scaling that will be used when plots are created.

Select defaults for parameter type and parameter scaling from the dropdown lists.



Understanding Biexponetional and Hyperlog Scaling

On Bigfoot the parameter data streamed from the instrument during acquisition and sorting consists of positive values only. Therefore, all events have values of 0 or higher. It is for this reason that when setting PMT voltages most events should be set to have values above 0.

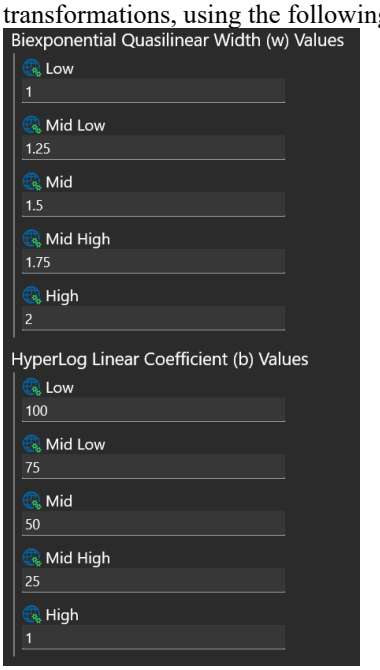
As is customary with the manipulation of flow cytometry data, negative values for parameters can be generated during parameter calculation in both Color Compensation and Spectral Unmixing. Using Biexponential and Hyperlog axes scaling it is possible to view this data. Additionally, as a general effect of manipulating log data, small changes in signal intensity are spread further in the lower decades as compared to the higher decades. To mitigate this, Biexponetional and Hyperlog utilize log compression and compound log/linear displays to limit the spread of this mainly negative data to create a more visually proportional distribution compared to positive events.

Biexponential scaling in SQ Software allows a log compression of the data to view a more appropriate spread of the data above and below 0. See reference: Herzenberg LA, Tung J, Moore WA, Herzenberg LA, Parks DR. Interpreting flow cytometry data: a guide for the perplexed. Nat Immunol. 2006 Jul;7(7):681-5.

Hyperlog in SQ Software is based on the "Logicle" method utilizing a linear transformation around 0. See reference: David R. Parks, Mario Roederer, Wayne A. Moore. A new "Logicle" display method avoids deceptive effects of logarithmic scaling for low signals and compensated data. Cytometry Part A, Volume 69A, Issue 6, pages 541-551, June 2006.

Biexponential or Hyperlog scales can be used during acquisition, sorting and FCS file replay. The user can adjust the transformation to compress or expand the scale around 0 by selecting the left and right arrows on each of the plot axes.

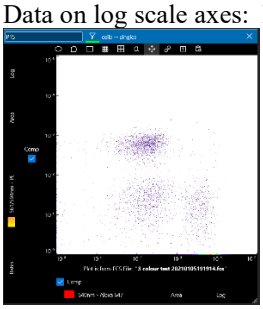
The administrator can set the Biexponential and Hyperlog scale transformation variables which are used for the transformations, using the following menus in Global Settings.



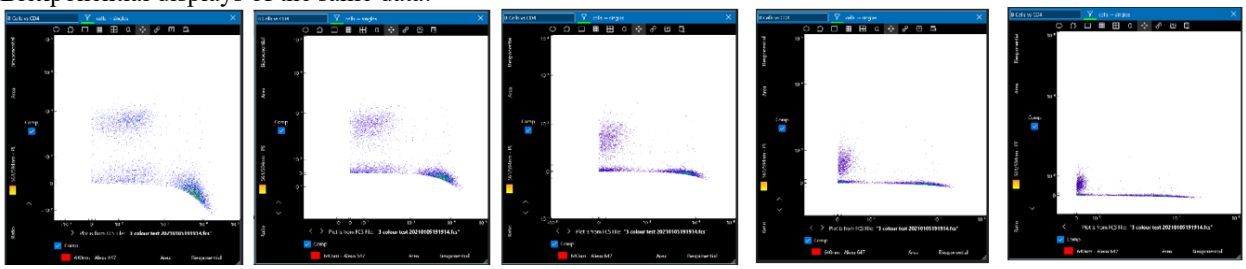
Biexponential Scaling

Selecting the right arrow, steps from the low to high Quasilinear width values or from the low to high Hyperlog Linear Coefficient (b) Values. For Biexponential, this function compresses the data around 0 and expands the data in higher decades. Proper use of control samples at the same plot settings is advised when setting sort gates. Please note for illustration purposes color compensation has been incorrectly set to increase the number of negative events

on the PE axis.
Data on log scale axes: Please note the events on the x axis below.



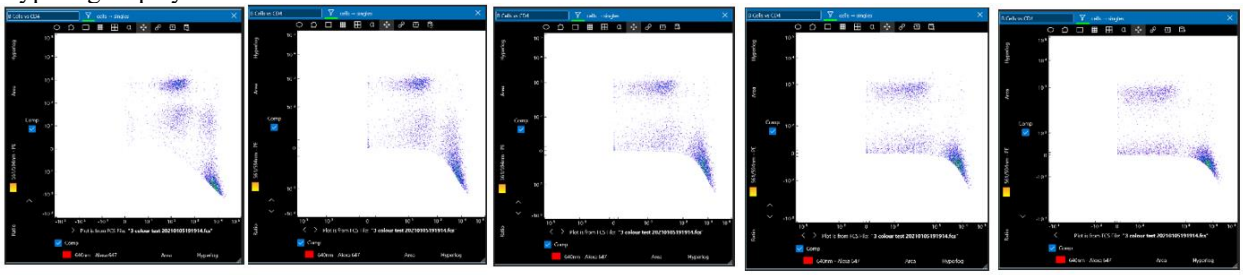
Biexponential displays of the same data:



Data displayed on both axes in Low (1), Mid Low (1.25), Mid (1.5), Mid High (1.75) and High (2) Biexponential Levels with Quasilinear Width Values displayed in brackets.

Hyperlog Scaling

Hyperlog displays of the same data:

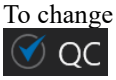


Data displayed on both axes in Low (1), Mid Low (25), Mid (50), Mid High (75) and High (100) Hyperlog Linear Coefficient (b) Values displayed in brackets.

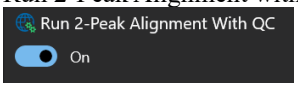
Quality Control Settings

The QC setting allows an administrator to define settings for the QC material and definitions of QC limits that will affect the Pass or Fail result on all parameters.

1. To change QC parameters, click **QC**.



2. Run 2-Peak Alignment with QC.

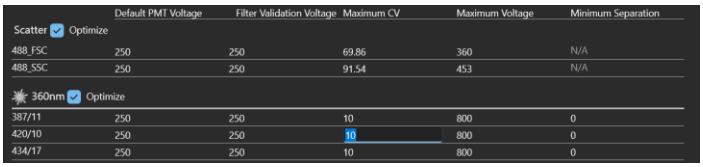


3. Normally, QC beads are kept in the bead station on the loader and the system is set to use the bead station for QC. If for any reason the bead station is not available, or you would like to use your own QC reagent, click the **Use Bead Station** toggle button to Off and put the QC reagent in loader station 1.

Changing QC Limits and Settings

For each detector, an administrator can set several defaults and limits.

1. Click on the value you would like to change and type a new value.



Default PMT Voltage – The voltage required to center the alignment bead population on a linear, area axis. It is loaded as the first phase of QC is initiated and stored when a QC protocol is passed. The administrator can type in a value to overwrite the current setting.

Filter Validation Voltage – (See ORB process.) The running default PMT voltage SQ Software uses to assess if changes have occurred for each fluorescence detector. Changes in this voltage can occur daily and can be tracked in the report section of the software.

Maximum CV – If the Coefficient of Variation (CV) of the alignment bead population exceeds this value the parameter will fail QC. The administrator can type in a value to overwrite the current setting.

Maximum Voltage – If the voltage required to center the alignment bead population on a linear, area axis is higher than this value the parameter will fail QC. The administrator can enter a value to overwrite the current setting.

Minimum Separation – During the second phase of the QC process, the blank and alignment beads within the QC Reagent are plotted on a log fluorescence axis. The separation between the negative and positive peaks is given by dividing the median intensity of the alignment beads by the median intensity of the negative beads.

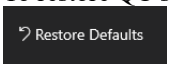
Import Settings and Export Settings

QC limits and settings can be imported as well as exported as CSV files. Below is an example of the file format.

	A	B	C	D	E	F
1	Laser488_Filter488_FSC	356	250	10	600	0
2	Laser488_Filter488_SSC	448	250	10	600	0
3	Laser360_Filter387/11	649	250	6	800	2345
4	Laser360_Filter420/10	692	250	6	800	3456

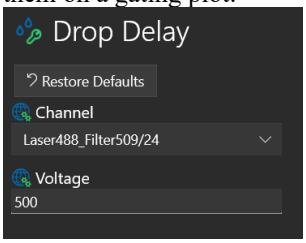
Restore Defaults

To restore QC settings to factory defaults, click **Restore Defaults**.



Drop Delay

When the drop delay process is initiated, the software selects the bright drop delay bead population withing the QC reagent. This is done by setting the trigger and a gating plot to a parameter on the 488nm laser such as the 583/30 detector. The default voltage is the setting required to trigger acquisition of the drop delay beads as well as to display them on a gating plot.

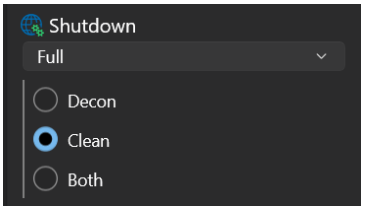


Startup/Shutdown

An administrator can configure startup and shutdown details.

Quick or Full Shutdown

1. To set the type of shutdown, click the dropdown list and select **Full** or **Quick**.

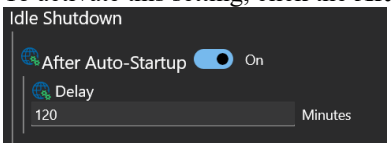


Quick Shutdown – Sets the system to rinse with DI water before shutting down.
Full Shutdown – Sets the system to either run cleaner, decontamination solution, or both before shutting down.

Idle Shutdown

The system can be set to automatically shut down after a period of idleness.

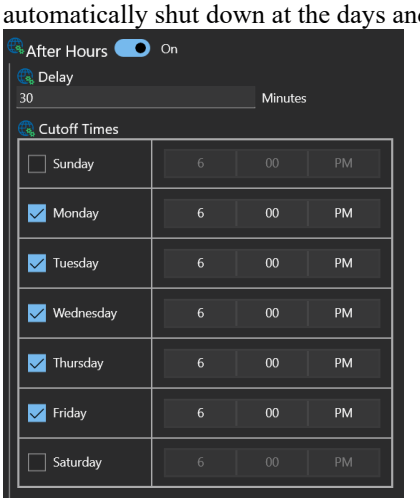
1. To activate this setting, click the **After Auto-Startup** toggle button to On.



2. Enter the minutes you want the system to be idle before it is automatically shut down.

After Hours Shutdown

The system can be set to automatically shut down after the facility is scheduled to close. When activated it will initiate an auto shutdown after a set number of idle minutes. When activated after a set idle time the system will automatically shut down at the days and times selected in the table.

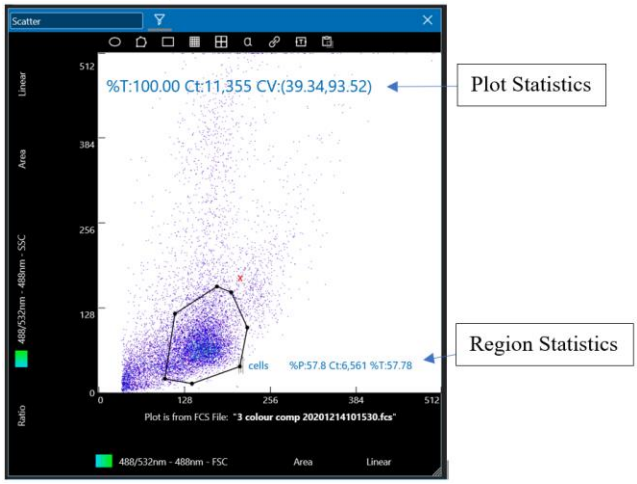


Global Statistics

Experiment Statistics can be displayed in a variety of locations within the SQ Software workspace Plot, Region and Combined Statistics are shown below. In Global Settings the administrator can set the statistics that appear by default in each context.

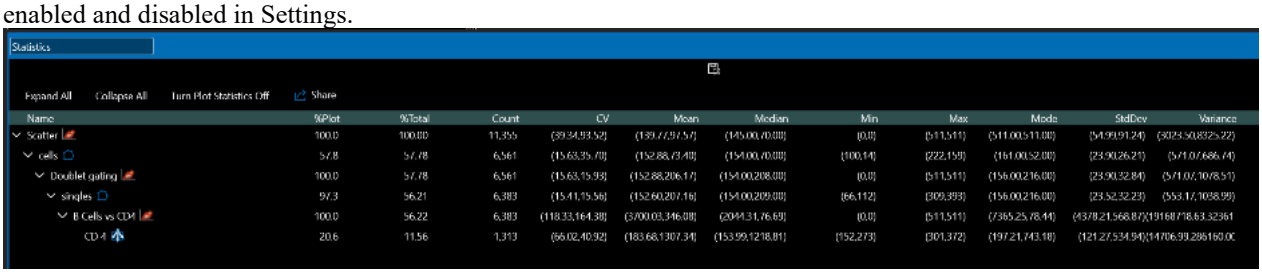
Plot and Region Statistics

Due to limited space on plots and regions, up to three values should be displayed at a time. To view more than three values at a time, see Combined Statistics.



Combined Statistics

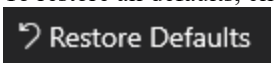
Combined Statistics can be viewed from the Workspace tools and in the Flex Controls. The available options are enabled and disabled in Settings.



Enable and disable global statistics

1. Drag and drop the statistics you want to enable or disable into the appropriate list.

2. To restore all defaults, click **Restore Defaults**.



User Management

SQ software allows administrators to designate users to perform several functions by person, group or set of applications. User logins can be used to:

- Segregate groups of experiment protocols and data.
- Account for system usage time.
- Customize file storage, plot and statistics display and names of fluorochromes.
- Allow access to SQ software for a limited (standard) or expanded (admin) set of privileges.

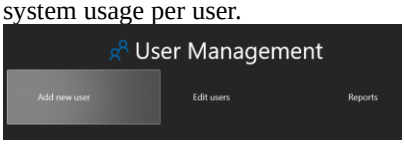
User Login

A standard user login only allows the user to reset their password by entering a new password and confirming it.

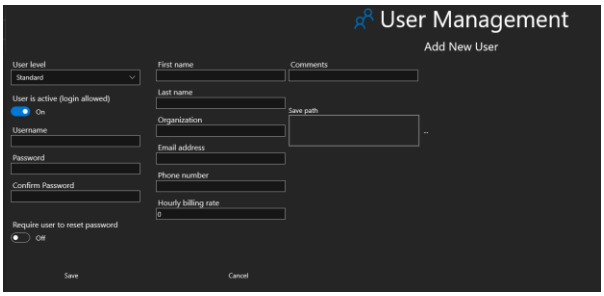
Click **Save** to save the password. Click **Cancel** to abandon any changes and exit the **User Management** screen.

Administrator Login

An administrator login allows you to create a new user, edit users, or create a CSV-formatted report of the total system usage per user.



Add New User



User level

Select Standard user level to allow a limited set of custom system settings. Users with Standard privileges cannot add or edit users. Select Admin to allow a more advanced set of custom system settings. See the Settings section for more information.

User is Active (login allowed)

This switch allows an administrator to enable or disable a User login and is set to On by default.

Username

Enter the name the user wants to use to enter SQ software.

Password/Confirm Password

Enter and confirm the password required for the user to login.

Require user to reset password

This switch is set to Off by default. When this switch is set to On, a new user will be required to reset their password the first time they log on.

First Name, Last name Organization Phone Number

These fields allow the user to enter their registration details.

Email Address

Will enable the software to contact the user with notifications from SQ software.

Hourly billing rate

Administrators can create a user report CSV file detailing the time the user was logged in. Entering the hourly billing rate allows calculation of the final bill for a particular user.

Comments

A free text space to enter any required text about this user.

Save path

Sets the save path for experiment protocols and data for the user. Note it is recommended this path is a fixed drive local to the Bigfoot computer.

Save

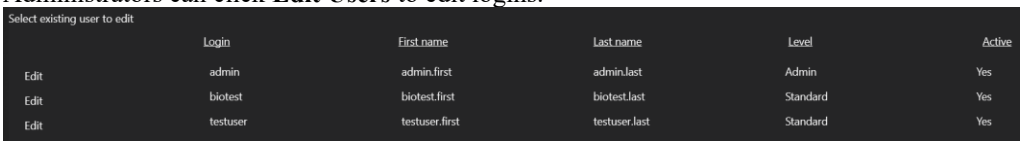
Saves the information for this User login.

Cancel

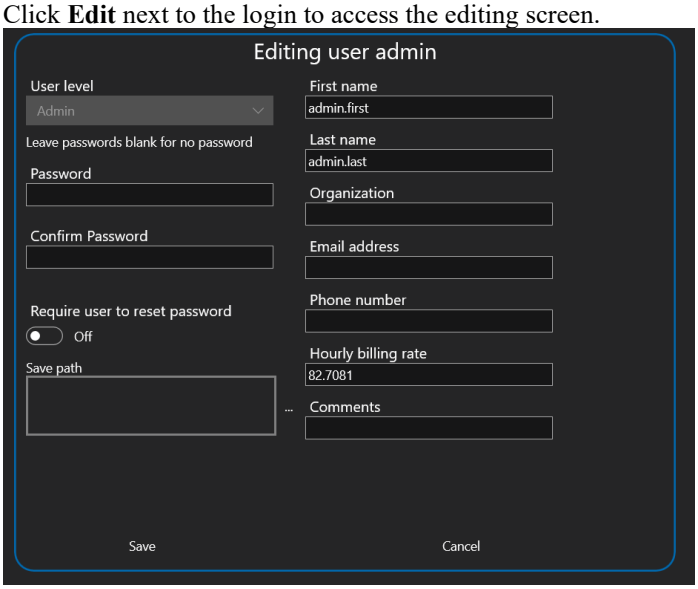
Abandons this user creation.

Edit Users

Administrators can click **Edit Users** to edit logins.



Click **Edit** next to the login to access the editing screen.



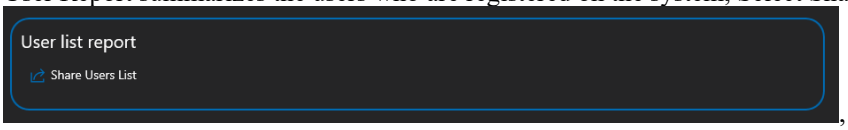
Make the required changes and click **Save** to save changes or **Cancel** to abandon changes.

Reports

SQ Software can provide a user report and a usage report associated with user logins.

Users List

User Report summarizes the users who are registered on the system, Select Share Users List.



,

Click **Export**.

Designate a location in which to save the report and click **Save**. Here is an example of the report.

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	User Active	Username	Created Date	Last Login Date	User Leve	User Right	First Name	Last Name	Organization	Email	Phone	Billing Rate	Comment
2	Active	admin	3/13/2020 11:43	12/4/2020 16:19	Admin		admin.first	admin.last				82.71	
3	Active	biotest	7/1/2020 8:29	11/24/2020 12:55	Standard		biotest.first	biotest.last				51.7	
4	Active	testuser	3/13/2020 11:43	12/23/2020 10:41	Standard		testuser.first	testuser.last				94.93	

Usage Report

The Usage Report summarizes the usage of the system for billing purposes. Select Share Usage Report.

Click **Export**.

Designate a location in which to save the file. Here is an example of the exported file.

	A	B	C	D	E	F	G	H	I	J
1	Login Date	Logout Date	Forced	Hours	Rate	Session Cost	Username	First Name	Second Name	Organization
2	12/3/2020 11:12	12/3/2020 11:12		0.01	$82.71	$0.48	admin	admin.first	admin.last	
3	12/3/2020 11:25	12/3/2020 11:43		0.3	$82.71	$24.86	admin	admin.first	admin.last	
4	12/3/2020 11:45	12/3/2020 12:23		0.62	$82.71	$51.40	admin	admin.first	admin.last	
5	12/4/2020 16:19	12/4/2020 17:05		0.76	$82.71	$62.98	admin	admin.first	admin.last	
6		Total	admin	1.69		$139.72				
37	12/14/2020 9:10	12/14/2020 13:48		4.64	$79.72	$369.55	service	service.first	service.last	
38	12/14/2020 13:50	12/14/2020 17:12		3.38	$79.72	$269.34	service	service.first	service.last	
39	12/14/2020 17:19	12/14/2020 17:55		0.6	$79.72	$47.94	service	service.first	service.last	
40	12/14/2020 22:04	12/14/2020 22:10		0.1	$79.72	$8.19	service	service.first	service.last	
41	12/15/2020 8:35	12/15/2020 10:28		1.89	$79.72	$150.55	service	service.first	service.last	
42	12/15/2020 10:30	12/15/2020 14:04	F	3.57	$79.72	$284.69	service	service.first	service.last	
43	12/15/2020 14:05	12/15/2020 16:41	F	2.61	$79.72	$207.80	service	service.first	service.last	
44	12/15/2020 16:42	12/15/2020 17:07	F	0.41	$79.72	$32.57	service	service.first	service.last	
45	12/15/2020 17:09	12/15/2020 17:11	F	0.04	$79.72	$3.20	service	service.first	service.last	
46	12/15/2020 17:13	12/15/2020 18:18		1.09	$79.72	$86.69	service	service.first	service.last	
47	12/15/2020 22:34	12/15/2020 22:35		0.01	$79.72	$0.41	service	service.first	service.last	
48	12/16/2020 14:16	12/16/2020 17:21		3.08	$79.72	$245.44	service	service.first	service.last	
49	12/17/2020 14:35	12/17/2020 14:35	F	0	$79.72	$0.33	service	service.first	service.last	
50	12/17/2020 14:37	12/21/2020 15:59		97.37	$79.72	$7,762.09	service	service.first	service.last	
51	12/21/2020 16:48	12/21/2020 16:48		0.01	$79.72	$1.01	service	service.first	service.last	
52	12/21/2020 16:48	12/21/2020 16:50		0.03	$79.72	$2.32	service	service.first	service.last	
53	12/21/2020 17:20	12/22/2020 20:05	F	26.76	$79.72	$2,133.03	service	service.first	service.last	
54	12/23/2020 9:41	12/23/2020 10:41		1	$79.72	$79.60	service	service.first	service.last	
55		Total	service	273.83		$21,829.23				

System Health

The Bigfoot Cell Sorter continuously monitors the status of the instrument utilizing sensors that detect if the system is in a normal state or if an error condition has occurred. The user is notified if an error occurs and if necessary, the affected functions of the system are stopped as a precaution.

Viewing System Health

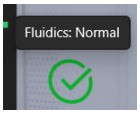
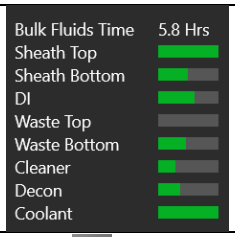
System Health can be viewed in the following areas within the software.

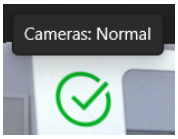
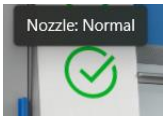
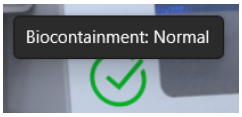
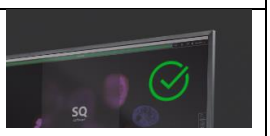
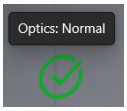
- Home Screen
- Bigfoot Health Status Icons
- System Health Banner
- Quick Menu

Viewing System Health on the Home Screen

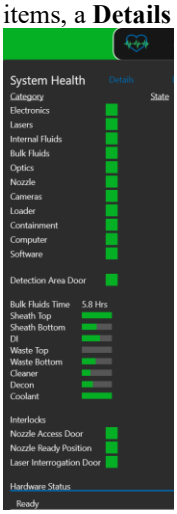
After logging in the **Home screen** appears. System health is represented by a picture of the system with green checkmarks that indicate subsystems are in a good state or red triangles that indicate maintenance is required. Hover the mouse over the status icons for detailed subsystem information.

Available run time is displayed based on the bulk fluids tank with the least capacity. Nozzle size and sheath pressure significantly affect run time. If these are changed, the remaining runtime will update to reflect the current nozzle and sheath pressure setup. If the run time shows the word “Off” the system is shut down.

Subsystem	Context	Status Details
Fluidics	System fluidics are responsible for maintaining sheath flow and pressure, sample flow and pressure and the removal of waste.	
Bulk Fluidics	Seven bottles of bulk fluids are weighed and monitored throughout system operation.	
Bulk Fluidic Level Details	DI water, waste and 1x PBS sheath fluid are stored in 4 L bottles and cleaner and decontamination fluids are stored in 1 L bottles. Green indicates healthy status and red indicates attention is required.	
Overall Fluidic Level	For quick reference, the total amount of fluids available is shown. Green indicates fluid levels are adequate. Red indicates that attention is required.	

Camera	High resolution video cameras are used throughout the system to automatically position the nozzle, maintain droplet location, place side streams on predesignated markers and show the sort media.	
Nozzle		
Biocontainment	The Bigfoot has two independent biocontainment zones: the front area sample handling and sample loading, the second, placed inside the first zone, is the AMS (Aerosol Management System) that contains the nozzle, stream deflection and sort collection areas.	
PC	The Bigfoot acquires data, controls the system, and performs sorting via a system interface and FPGA arrays. This system utilizes firmware to perform these tasks. The PC which stores data communicates user commands to the firmware is mounted at the back of the system. Alongside the PC is a UPS to maintain the system for a short time in the event of a power failure.	
Optics	Lasers are used to illuminate cells and filters are used to direct emitted light to the appropriate detectors. The Bigfoot system automatically verifies the configuration of the optical filter setup by pulsing eleven different wavelengths of light from eleven LEDs into the optical fibers leading to the detector banks. This is called Optical Response Baselining (ORB). The ORB filter confirmation process is part of automated QC and startup. The system tracks the median of the signals over time.	

Click the heart-shaped **System Health** button to access the summary table that includes a list of System Health items, a **Details** tab and a **Last System Health Issue** tab to view the most recent error condition.

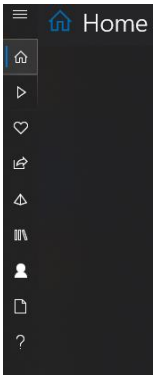


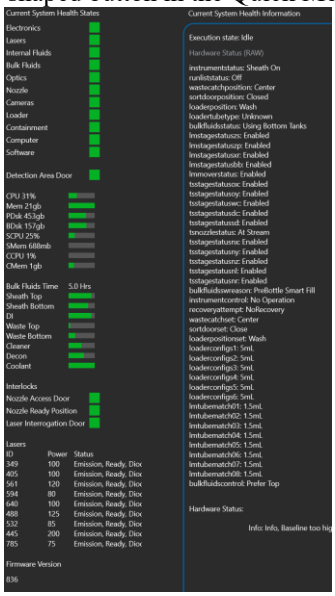
System Health Banner

Overall system health is indicated by the color of the workspace banner at the top of the screen. Green indicates the system is okay. Orange indicates an issue has been found and maintenance may be required. Red indicates the system has stopped due to an error condition. Within the banner is the heart-shaped System Health button that can be selected to show system status details.

System Health Quick Menu

Click the heart-shaped button in the Quick Menu on the far left of the screen to a view system health detail.





Chapter 6: Troubleshooting

Problem	Possible Cause	Solution
No events are present during QC process.	Probe position is not sufficient to aspirate low fluid levels in QC bead bottle.	Recalibrate the probe position for the bead station.
	Bead bottle is empty.	Replace bead bottle.
	The bead bottle may have been diluted (drips, etc.) so it cannot reach event rate, or no events are detected.	Replace bead bottle.
	Sample line is blocked.	Clean or replace sample line.
	Sample line was not replaced correctly.	Reinstall sample line.
	Nozzle is blocked.	Clean or replace nozzle.
	Nozzle alignment is incorrectly set.	Check image alignment of the nozzle.
High voltage present in certain channels during QC.	Incorrect beads on bead station.	Ensure Bigfoot QC Beads are used.
	Beads have gone bad or are expired.	Install a new bottle of Bigfoot QC Beads on the instrument.
No events during acquisition	Probe is too high and not in sample fluid.	Recalibrate the probe with Service assistance.
	Laser(s) off	Turn on the relevant lasers in SQ Software. Modify the protocol so that required fluorophores are activated to ensure that laser is on when the run is initiated.
	Laser(s) not functioning properly	Service support is necessary.
	Events are below the threshold/ threshold is set too high.	Adjust the trigger signal PMT voltage or decrease the threshold percentage.
	Threshold is not correctly set.	Change the threshold value.
	PMTs are set too high or too low to see data.	Edit PMT voltages or change trigger and threshold to visualize data.
	There is a clog in the system.	Run the cleaning panel or unclog feature.
	Sample is not aspirated.	Ensure there are no leaks in the sample line connection. Ensure that the probe is moving correctly.
	Incorrect optical filter.	Ensure that all filters are correct and in the right locations.
	Sample is too dilute.	Recreate the experiment with a more concentrated sample.
	Sample has run out.	Stop the acquisition and check the tube/plate to see if sample has in fact run

Problem	Possible Cause	Solution
		out.
	Air is in the sample line.	Perform backflush from wash station.
	Plots created in the workspace do not match the enabled parameters.	Create new plots that match the enabled parameters or enable parameters that match the plots.
The system suddenly shuts down.	Bulk fluidics bottles are empty.	Empty the waste and refill DI bottles, as necessary.
Data suddenly disappears from the plots in the workspace and threshold plot and the event rate drops to 0 during acquisition.	Trigger channel voltage is too high.	Decrease the trigger channel voltage until data starts to appear in the threshold plot. Then, adjust the trigger voltage and threshold value until data appears as expected in the trigger channel plot in the workspace.
Event rate decreases unexpectedly.	Sample has run out.	Stop sample acquisition and check the tube/plate to see if sample has in fact run out.
	Sample has settled.	Turn on agitation in the Control Panel to suspend sample. Note that settled samples may require a longer agitation than usual.
	Possible clog.	Perform backflush. Vortex sample/filter sample.
There is a lower event rate than expected based on the density of samples.	Air bubbles in the sample line.	Stop sample acquisition and run a tube filled with at least 500 µL DI water.
Noisy threshold plot.	Debris in the sample line.	Run the decontamination process.
	Debris in the sheath or DI bottles.	Clean the bulk fluids bottles.
	Dead cells in sample.	Adjust gates or repeat experiment with fresh cells.
Unable to press the play button.	Sample is back flushing, or the previous run is not complete.	Wait for the process to complete.
Unexpected fluorescence signal.	Free dye accumulated in the sample line.	Run the cleaning process.
High CV in data plots.	Poor sample preparation.	Prepare a new sample.

Problem	Possible Cause	Solution
	Air in system.	Stop sample acquisition and run a tube filled with at least 500 µL DI water.
	Dirty optical filters.	Inspect and clean filters.
	Improper laser delay.	Run QC again.
Dramatic change in PMT for one single channel.	Improper bandpass filter.	Run the ORB process and inspect filters.
	Improper dichroic mirror placement.	Run the ORB process and inspect filters.
	PMT malfunction.	Service support is necessary.
	Scratched mirror or filter.	Inspect and replace if necessary.
Dramatic change in PMTs for all channels.	Incorrect beads.	Ensure Bigfoot QC Beads are used. Proline Beads or Universal Proline beads will not fluoresce in certain channels, especially for the UV and violet lasers.
	Clogged nozzle or partially clogged nozzle.	Run unclogging process.
	Air in system.	Stop sample acquisition and backflush from wash station.
Weak or no fluorescence.	Not enough antibody was used during sample preparation.	Prepare a new sample.
	Intracellular target.	Ensure correct techniques are used to fix cells.
	Incorrect fluorophore selection.	Check sample preparation and experiment set up.
	Poor compensation.	Run the compensation process.
	Reagent is old or degraded.	Antibody may not have been stored in the proper conditions (refrigerated and kept in the dark).
	Antibodies are not compatible.	Verify that the secondary antibody used has been grown against the species in which the primary antibody has been grown.
	Lasers are turned off.	Turn on lasers in software.
	Lasers are misaligned.	Service support is necessary.
High fluorescence.	Antibody concentration in sample is too high.	Prepare a new sample.
	Inadequate cell preparation or washing.	Prepare a new sample.
	Cells have naturally high auto fluorescence.	Adjust PMT voltages to place cells on scale.

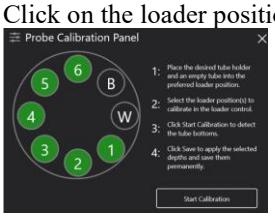
Problem	Possible Cause	Solution
	Poor compensation.	Run the compensation process.
	Bacterial contamination that can cause auto fluorescence.	Run the decontamination process.
	A secondary antibody may cross react with cells.	Evaluate sample preparation.
Two or more populations are present when one is expected.	Gating is inaccurate.	Adjust gating.
	Target protein is expressed on multiple cells.	Evaluate sample preparation or experiment setup.
	Inadequate cell preparation.	Ensure adequate cell separation and preparation because multiple cell types or debris could be present in a sample.
	Cells are detected as doublets.	Adjust the flow rate down.
	Nonspecific staining due to dead cells in the sample.	Prepare a new sample.
Salt buildup is present on the quick-connect fluidic connectors.	Cleaning is necessary.	Thoroughly clean the connectors and their port with 70% alcohol. Reattach the connectors and pull on them to make sure they are fully sealed.
Sample probe is not sealing consistently.	Sample probe O-ring is dirty, not lubricated or damaged.	Check the orange O-ring located under the sample probe. Thoroughly clean the O-ring to remove any debris that could cause an insufficient seal. Lubricate the O-ring.
Waste stream and sort streams exhibit excessive fanning.	Static charge has accumulated in the nozzle chamber.	Pause the stream and slightly open the nozzle door but do not click it into place. Open the chamber door and use an anti-static wipe to wipe the chamber floor. Do not touch the nozzle tip or the optical windows on the walls of the chamber. Let the floor air dry.
	Static charge has accumulated in the sort output area.	Remove the streams handle and clean it with DI water and a Kim wipe. Clean the streams window with DI water and a Kim wipe. Use an anti-static wipe to wipe the streams window. Use another anti-static wipe to wipe the streams handle including the mirrors.

Sample Probe Calibration

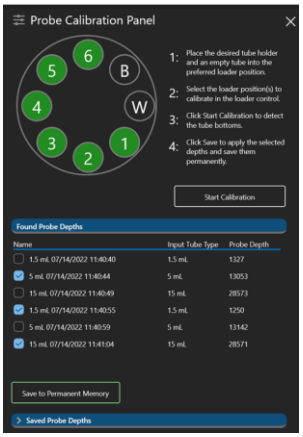
Sample Probe Calibration is the process by which the probe is set to the ideal sampling depth for each input tube type of set of tubes within a particular tube type.

1. From the **Flex Tools**, **Maintenance** tab, select **Probe Calibration**.

2. To calibrate more than one tube type at a time, set up the sample loader with the tube holders for the various tube types you would like to calibrate. Or to calibrate one tube type using several data points, set up the sample loader with the same tube holder in each position.
3. Put an empty tube in each tube holder. Make sure that the tube type matches the tube holder type.
4. Click on the loader positions in which you have loaded the tubes. The active positions will appear green.



5. Click **Start Calibration**. The instrument automatically detects the bottom of each tube and displays the ideal depths with corresponding checkboxes selected. You can change the selection if needed, but only one depth calculation per tube type can be saved.
6. Click **Save to Permanent Memory**.

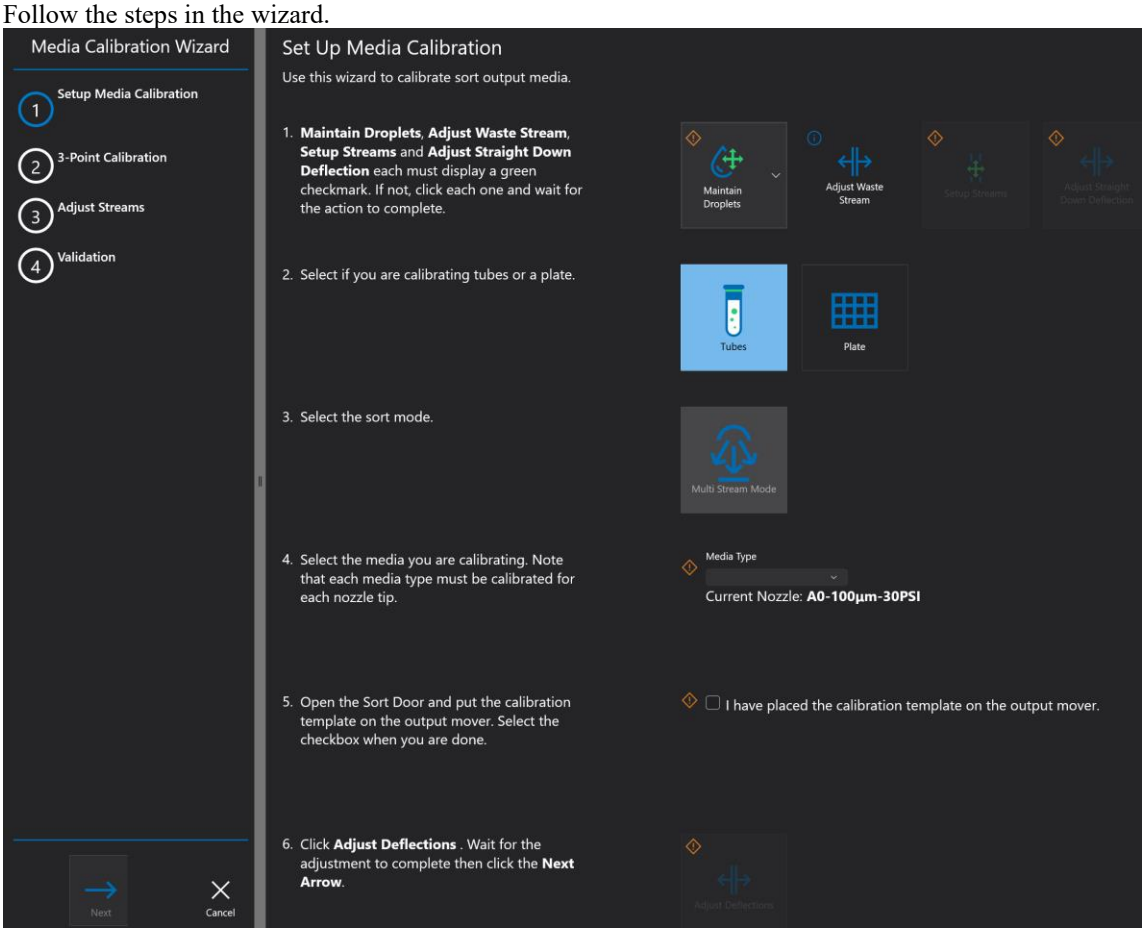


3-Point Media Calibration

To mediate the effect of additive tolerance of mechanical parts, we have added the ability to calibrate sort media on three different points.

1. From the Flex Controls select the Maintenance tab.
2. Click Media Calibration Wizard.

3. Follow the steps in the wizard.



Spectral Warning and Error Messages

Tooltip Message	Cause	Possible Solutions
Autofluorescence Matrix is null or did not have the required number of events	Less than 1500 events acquired for the autofluorescence creation algorithm or autofluorescence matrix is null.	Check gating to ensure at least 1500 events are acquired. Rerun the control if necessary. If more than 1500 events are collected and this message still appears, contact customer support.
Autofluorescence Matrix has one or more dimensions <= 0.	There is an issue with the data for creating autofluorescence. Either there are no rows or no columns.	Rerun the unstained control. If this does not resolve the issue, contact customer support.
Autofluorescence (Matrix, Covariance, Signal) is null. Please run Negative Controls prior to Single Controls.	There is no autofluorescence object available for creating single-color control signals.	Run the unstained sample if it has not been run. Otherwise, contact customer support.
All events appear to be Autofluorescence/Negative based on global distance comparisons.	There are no events that have been run for that single-color control that are outside the distribution of the unstained control.	Make sure the unstained control was not run unintentionally. If the control is dim, consider using a bead for that control and implementing the multiple unstained function. If the software version allows, let the user choose positives. This can also be a true "Error", which means the experiment is stopped.
All events appear to be Autofluorescence/Negative based on statistical comparisons.	Although some events were far enough from the autofluorescence in high dimensions to pass the global distance test, no filters had a mean value that was different from the autofluorescence in that same filter at a statistically significant level.	Make sure the unstained control was not run unintentionally. If the control is dim, consider using a bead for that control and implementing the multiple unstained function. If the software version allows, let the user choose positives. This can also be a true "Error", which means the experiment is stopped.
No autofluorescence channel and control channel are significantly different with a 99% level of confidence.	No filters had a mean value that was different from the autofluorescence in that same filter at a statistically significant level.	Make sure the unstained control was not run unintentionally. If the control is dim, consider using a bead for that control and implementing the multiple unstained function. If the software version allows, let the user choose positives. * This is only a warning, so the experiment will be able to continue. Advise the researcher to inspect their control.

Tooltip Message	Cause	Possible Solutions
Significant overlap detected in controls run to this point. Please check for possible signal duplication.	The actual computed complexity index for the single color controls is much higher than the predicted complexity index on the fluorophore selection screen for the panel up to this color.	Make sure there is not duplication of signals. In the protocol folder, inspect the diagonal of the spectral demix_(fluorophore name here).csv file. If two diagonal values are very large, visually inspect those two colors in SQ software. These are the most likely to have been duplicated. Make sure that tandem dyes have not broken down. We have seen many instances of BV 786 becoming BV 421 due to bonds breaking down. If nothing is duplicated, this is generally a warning, so the investigator will be able to continue.
The current signal for this fluorophore has less fluorescence than predicted in its expected primary filter.	The max channel for the computed signal for this control has 50 percent or less of the fluorescence expected for this fluorophore in its predicted primary channel.	Note that software scales the data, so we do not expect a given value for fluorescence. Inspect the spectral graph for this control. In some cases, the fluorophore may fluoresce more brightly for other lasers than expected. For example, the BV dyes that have primary channels >= 600 often fluoresce heavily on the yellow and red lasers. Look for evidence of carryover. Look for evidence of the incorrect control having been run. * This is only a warning, so the experiment will be able to continue. Advise the researcher to inspect their control.
Total fluorescence in positive channels does not exceed lower bounds.	Although some events were far enough from the autofluorescence in high dimensions to pass the global distance test, no filters had a mean value that was different from the autofluorescence in that same filter at a statistically significant level.	Make sure the unstained control was not run unintentionally. If the control is dim, consider using a bead for that control and implementing the multiple unstained function. If the software version allows, let the user choose positives. * This will most likely be a warning, so experiment can continue if researcher believes the signal is okay.
Number of outliers is higher than expected. Please inspect the spectral graph.	The number of events outside of outlier threshold precludes finding sufficient positive events.	Check the spectral graph for saturation. This will generally occur when all or most events are against the axes.

Tooltip Message	Cause	Possible Solutions
Only (a number) of events were identified as positives. Please select a positive region for creation of the spectral signature.	Using only the global distance, fewer than necessary positive events were found. The algorithm will create a signature using all events, but the user should move the region to their desired positives region.	Check the spectral signature and positive/negative graph. Ensure the user selects a better positive region or moves to beads or another suitable control media.
No positives found for calculating positive event stat distances.	The number of positives was incorrectly zero.	Contract support.
Only (a number) of non-outlier events were identified as positives.	A possible mode was found, but during the calculation of the bounds, the number of positives slipped below the cutoff.	The user must select a positive region.
One or more values are too large in the demixing matrix. Please try rerunning the control for the fluorophore.	There is enough overlap that software cannot send the matrix to firmware because it contains values that are too large to be represented in the firmware data formats.	This is common if there is a duplication of signals. Check for duplication and fix accordingly. If not, this is an error, and the panel/controls must be reviewed. The experiment will have to be stopped.

Chapter 7: Consumables

The following consumable items for the Bigfoot Cell Sorter are available to order. Please contact your sales associate.

Part Number	Description
PL00286	10X Sterile Sheath Solution, six 1 gal. bottles
PL00287	Bigfoot QC Beads, three 5ml bottles
PL00305	Sample Line and Probe Assembly
PL00330	Seismic Lockdown
PL00312	70um Nozzle
PL00313	100um Nozzle
PL00315	120um Nozzle
PL00314	150um Nozzle
PL00306	Sort Output Loader Box
PS00242	15mL adapter for sample input with built in agitation
PS00236	5mL adapter for sample input with built in agitation
PS00241	1.5mL adapter for sample input with built in agitation
PL00338	50mL sort output tube adapter (can be used for right or left direction)
PL00337	15mL sort output tube adapter (can be used for right or left direction)
PL00336	5mL sort output tube adapter (can be used for right or left direction)
PL00335	1.5mL sort output tube adapter (can be used for right or left direction)
PL00317	Waste bottle with cap and connector
PL00318	DI bottle with cap and connector
PL00319	Sheath bottle with cap and connector
PL00320	Cleaner bottle with cap and connector
PL00321	Decon bottle with cap and connector
PL00340	10X chip holder adapter