
nanocelllect[®]

Biomedical, Inc.



Instrument Guide

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NanoCellec Technical Support

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Legal Notices

- The WOLF® Cell Sorter is intended for general laboratory **Research Use Only** (RUO).
- The WOLF® Cell Sorter is **not** intended for diagnostic testing or clinical use.
- Read the entire instrument manual before attempting to operate the instrument.
- If this instrument is used in a manner not specified by the manufacturer, the protection provided by the instrument may be impaired and the user put at risk.

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1. Introduction to the WOLF[®] Cell Sorter

1.1 Overview

The WOLF[®] Cell Sorter is designed to provide truly personal sorting flow cytometry for experienced and novice life science researchers.

KEY FEATURES:

Sterile and Disposable Fluid Path: The individually packaged cartridges and tubing sets are sterile, preventing sample contamination. Everything that the sample touches is disposable and can be rapidly exchanged. This avoids sample-to-sample cross contamination.

Low Maintenance: The disposable fluidic path means that the system is dry when not in use and there is no fluidics cart or system to maintain.

High Viability: Low shear stress and low pressure in the microfluidic channels maximize cell viability.

Biohazard Containment: The closed fluid path minimizes biohazard exposure to operators and allows for rapid exchange between hazardous samples with minimal cleanup.

Aerosol-Free: The closed, low-pressure on-cartridge sorting junction does not form droplets or aerosols, reducing the need for containment hoods.

Compact Footprint: At 1.6 square feet, the device can be placed on a benchtop or within a hood.

Simple Software: A robust sorting and analysis platform that is easy-to-use.

1.2 WOLF[®] Cell Sorter Installation Requirements

WOLF INSTALLATION REQUIREMENTS

Dimensions	15.5 x 14.4 x 15 inches (w x l x d) 39.4 x 36.6 x 38.1 cm
Weight	44 lb (20 kg)
Power	AC Input: 100-240V, 50-60Hz, 1.5A
Temperature Range	15-30°C
Humidity Range	<80% relative humidity

N1 INSTALLATION REQUIREMENTS

Dimensions	8.4 x 6.5 x 8.3 inches (w x l x d) 21.4 x 16.5 x 21.1 cm
Weight	5.5 lb (2.5 kg)
Power	24VDC, 2A
Temperature Range	15-30°C
Humidity Range	<80% relative humidity

1.3 WOLF[®] Cell Sorter Specifications

OPTICS

Excitation Laser	488nm; 30mW diode (45,000 hr life)
Beam Size	25 x 75 μm ($1/e^2$)
Scatter Detection	Forward (0 degrees, +/-15) Back (180 degrees, +/-15)
Filter configuration	• 525 $\pm$ 25 nm (FITC, GFP) • 585 $\pm$ 20 nm (PE) • 665 nm Long Pass (PerCP-Cy5.5)
Optical alignment	Fixed

1.3 WOLF[®] Cell Sorter Specifications (Continued)

FLUIDICS	SPECIFICATIONS
Sample Input	1.5 and 5.0 mL tubes
Sheath Input	50 mL conical tubes
Sheath Fluid	1X Phosphate Buffered Saline (PBS) or similar
Sheath Fluid Usage	9.6 mL/hour
Sample Flow Rate	24 µl/minute
Sheath Flow Rate	160 µl/minute
Dead volume	50 µl
Minimum sample volume	250 µl
Tubing diameter (inner)	200 to 500 µm
Flow Cell	200 x 70 µm
Smaller Channel Dimension	50 µm
Sample pressure	< 2 psi

1.3 WOLF® Cell Sorter Specifications (Continued)

PERFORMANCE	SPECIFICATIONS
Fluorescence Sensitivity	TBD
Forward and Backscatter Resolution	1 μ m
Scatter Resolution	Resolves Lymphocytes, Monocytes and Granulocytes
Fluorescence Resolution	TBD
Analysis speed	> 5,000 events/second
Sorting	1 and 2-way
Sorting speed	300 events/second, 1-way sorting
Sorting output	1.5 mL and 5.0 mL, 96- and 384-well plates.
Sorting Purity and Yield	An average of 120 events per second (300 events/ μ l), with a target of 10% achieved a 94% purity.
PLATE SORTING	SPECIFICATIONS
96-well plate time	8 minutes
384-well plate time	32 minutes
OPERATION	SPECIFICATIONS
System power up	3 minutes
System set-up and calibration	30 minutes
System shut-down	2 minutes

1.3 WOLF® Cell Sorter Installation Requirements (Continued)

DATA PROCESSING	SPECIFICATIONS
Software	WOLFViewer
File Format	FCS 3.1
Computer	HP EliteOne 800 G3
Memory	8 GB
Storage	256 GB
Screen	23.8"

SIGNAL PROCESSING	SPECIFICATIONS
Signal Processing	24-bit A/D Conversion
Pulse measurement	Height, Area, and Width
Time	Correlated with any parameter
Channel threshold	Available for all the parameters

1.4 The WOLF® System Components

The WOLF® Cell Sorter and N1 Single Cell Dispenser have different accessories and consumables as well as a computer that are needed to operate the system.

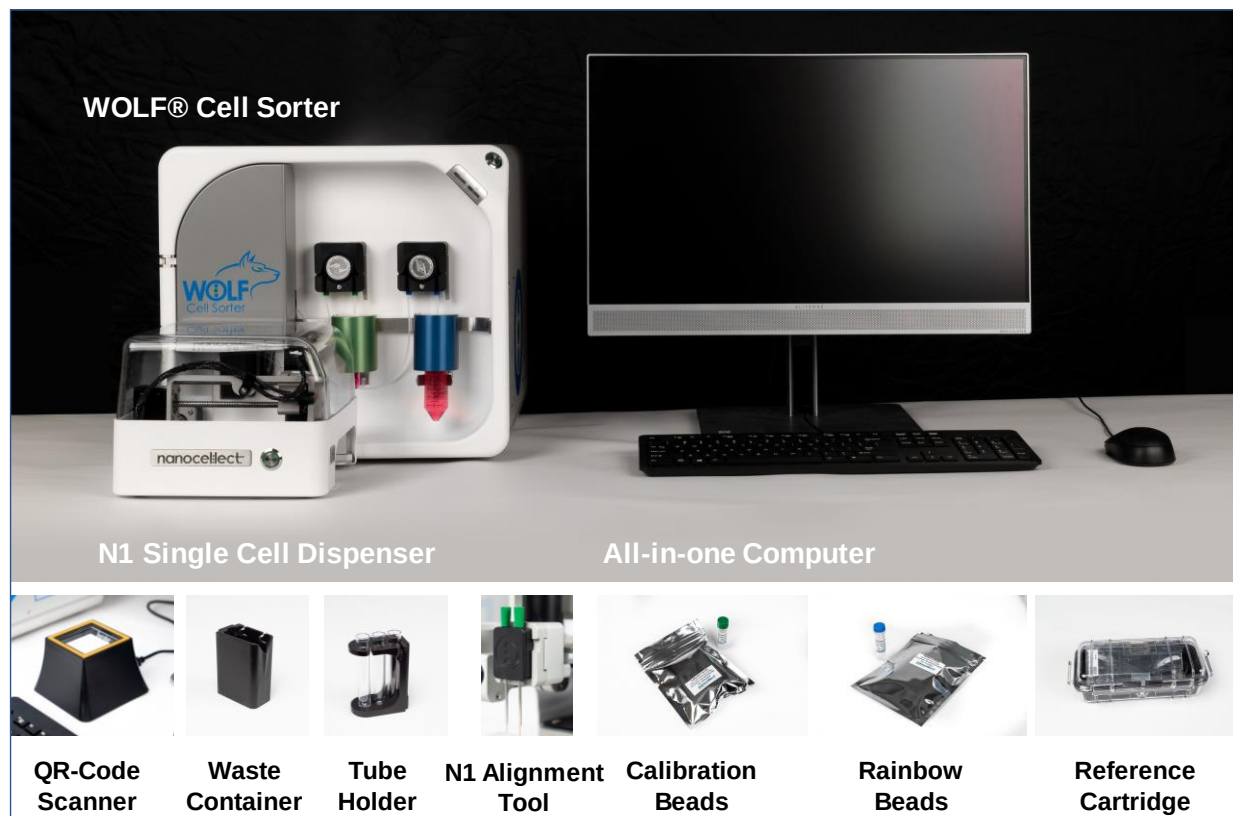


Figure 1. The WOLF® Cell Sorter Components and Accessories.

On installation day you will receive:

- WOLF® Cell Sorter
- N1 Single Cell Module
- All-in-one Computer
- QR-Code Scanner
- Waste Container
- Collection Tube Holder
- Cartridge Starter Pack (10 Sorting or 10 Single Cell Cartridges)
- Beads Starter Pack: 10 x 1 mL vials of Calibration Beads and 5 x 1 mL vial of Rainbow Beads.
- Alignment Cartridge
- Consumables Starter Pack (5 x 5 mL sterile flow tubes, 2 x 0,22 µm syringe filters, 2 x 20 mL syringes, 6 x isopropanol lens-quality wipes, 5 x Precision swabs)

1.5 Front Panel Mechanics and Fluidics

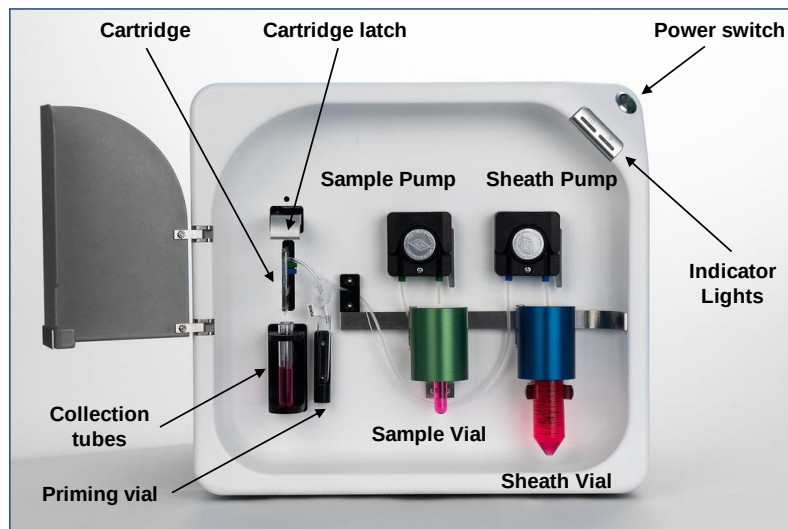


Figure 2. Front panel of the WOLF® Cell Sorter

The front panel of the WOLF® Cell Sorter contains a power switch and Indicator Lights to report on the status of the machine. The cartridge is inserted and latched in the aperture present on the left side of the front panel, behind the door. Cartridge sheath tubing and sample tubing are routed through the peristaltic pumps and into the sheath and sample vials. Sorted cells are collected in the collection tubes situated below the cartridge.

1.6 NanoCelect's Cartridges

The detection and sorting capabilities of the WOLF® rely on a microfluidic chip positioned on the disposable cartridge. The microfluidic chip allows the sample to be **hydrodynamically focused** for detection and sorting based on light scattering and fluorescence emission.

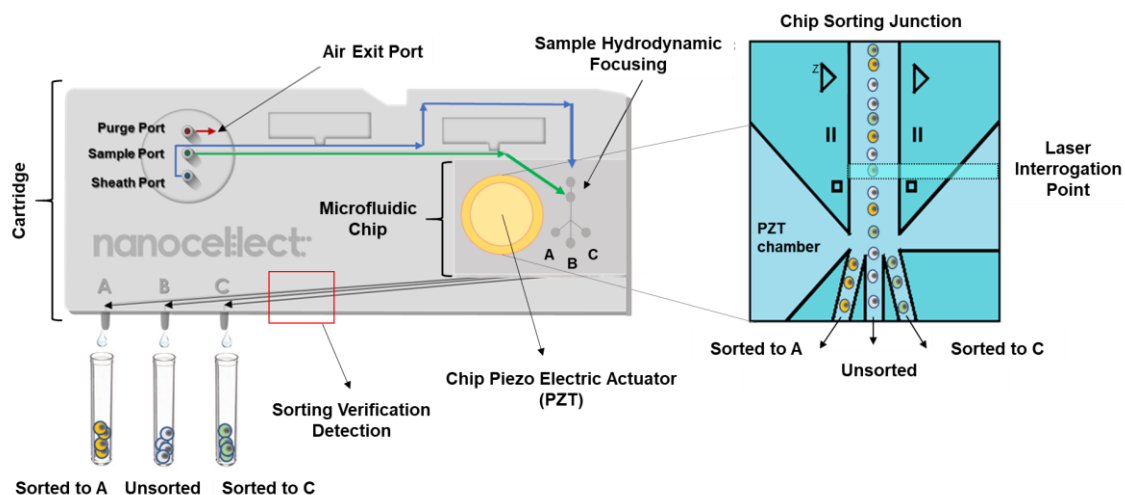


Figure 3. The WOLF® Cell Sorter Cartridge: The cartridge has a purge, sample, and sheath ports on the upper left. The sample is routed into the cartridge microfluidic chip for detection and sorting. Sorted cells exit the cartridge to the collection tubes through three output ports situated on the lower left of the cartridge.

1.6 NanoCelect's Cartridges (Continued)

The WOLF[®] Cell Sorter can be operated with 2 cartridge varieties to sort in bulk or dispense single cells in 96- or 384-well plates.

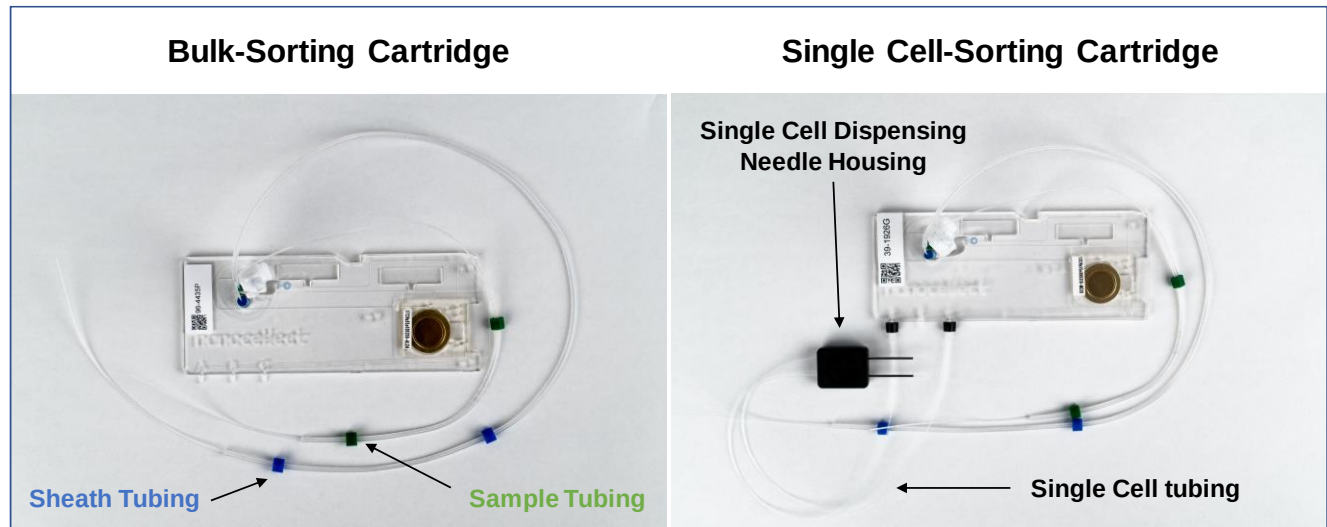


Figure 4. The WOLF[®] Cell Sorter Cartridge Varieties

- **Bulk-Sorting Cartridges** are used to sort cells into collection tubes.
- **Single Cell-Sorting Cartridges** are used to sort individual cells in plates. The difference between these cartridges and the Bulk-Sorting cartridges resides in the tubing attached to the A and C cartridge outputs, and the Dispensing Needle Housing used to deposit cells in the wells.

1.7 WOLF[®] Cell Sorter Computer

In general, the computer supplied with the WOLF[®] Cell Sorter should be treated as a dedicated computer used solely for instrument operation and data management.

The WOLF[®] computer has three accounts:

- **NanoCollect's Account** is used to open WOLFViewer software and operate the instrument.
- **Customer Administrative Account** can be used to change the computer configuration, install software or add the computer to internal networks. Keep the provided account password in a safe place.
- **NanoCollect's Administrative Account** is used for software and firmware updates by NanoCollect's Technical Service personnel.

We recommend considering the following when using the instrument computer:

- Enable automatic Operating System and application security updates.
- Limit computer activities to the minimum needed to operate the system and manage data.
- Connect the computer to an internal network specific to lab equipment. If your organization has a lab specific VLAN, use it.
- Avoid general web browsing, especially social media sites.
- Due to the shared nature of the PC, do not save individual credentials in any apps or browsers.
- Note that corporate requirements around domain membership, group policy objects and anti-malware software may interfere with the normal operation of the system.
- Ensure all device operators are familiar with these best practices.

NOTE: NanoCollect recommends rebooting the WOLF[®] computer once per week to update the Operating System and flush the RAM memory.

1.8 WOLF® Internet Connectivity and Service Support

NanoCelect monitors devices to detect software and firmware errors, issues with operational protocols, and cartridge performance (calibration, alignment, etc). This allows us to provide support or maintenance before it is needed and to improve performance of our hardware, software, and cartridges.

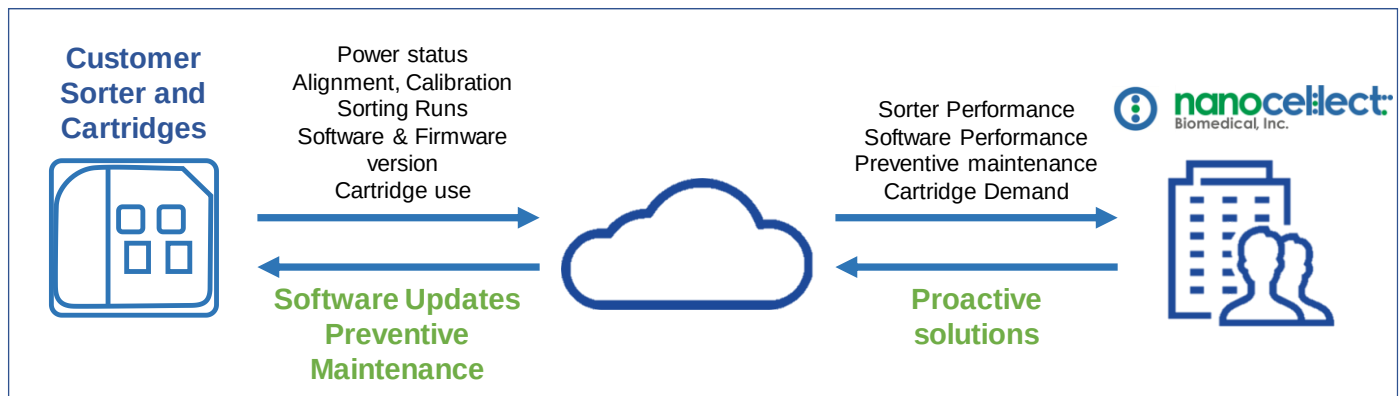


Figure 5. Representation of NanoCelect's Data Monitoring

In addition, we will offer **easy and secure remote control** of the WOLF® Cell Sorter Computer to provide support and training if needed.

We use TeamViewer, a platform that allows NanoCelect's Support personnel to connect with the WOLF® computer without complicated steps. The user needs to provide a secure password, that changes every time a new session is started.

Types of data collected by NanoCelect

- Power status
- Pump performance
- Calibration and alignment performance
- Cartridge serial number
- Operational performance of software and firmware
- Electronic systems performance

1.8. WOLF[®] Internet Connectivity and Service Support (Continued)

Data that is not collected

Sensitive data, experimental data, and user identity is not collected under any circumstances. NanoCollect respects the privacy and intellectual property concerns of our customers. Only the aforementioned “Types of data collected by NanoCollect” are used by NanoCollect. All data collected can be made available for review by Customer upon request including an audit-trail verifying the date, and time data was collected as well as secure storage location, file size and contents.

Data Security features include:

- Encrypted web traffic: All traffic between NanoCollect and the Customer’s browsers use SSL (Secure Socket Layer) protocol to maintain a secure and encrypted session. Unencrypted web traffic (HTTP) is not supported.
- Encrypted data protection: The data is encrypted using AES (Advanced Encryption Standard) 256, a secure symmetric-key encryption standard using 256-bit encryption keys.
- Encrypted database: The database is encrypted using AES and a key size of 256 bits.
- Encrypted file transfer: All data transfers are performed in an encrypted state.
- Access: Data is only accessible by named and authorized personnel who are trained on handling of sensitive data.

1.9 WOLFViewer Software

The WOLFViewer user interface has been designed to provide an intuitive workflow for the user, with all the essential features visible at a glance.

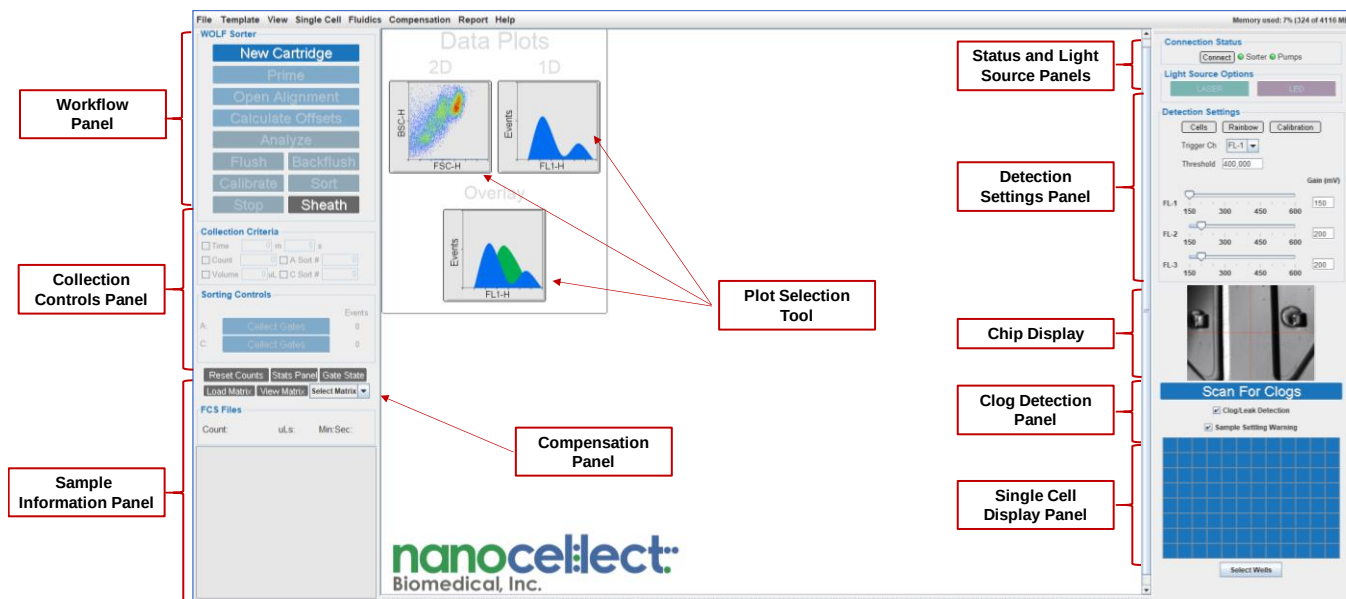


Figure 6. The WOLFViewer Software Graphical User Interface

- **Workflow Panel** has step-by-step processes for running the WOLF®.
- **Collection Controls Panel** allows the user to set sample volume, number of cells, duration and gates for sorting.
- **Sample Information Panel** displays sample names, volumes, collection time and counts. Includes the Statistics Panel and the Gate Status.
- **Plot Selection Tool** allows the user to create histograms or 2D plots for sample analysis.
- **Compensation Panel** guides the user through the process of spillover compensation.
- **Status Panel** reports the connection status of the computer and WOLF®.
- **Light Source Panel** allows the user to turn the laser and the camera LED light on or off.

1.9 WOLFViewer Software (Continued)

- **Detection Settings Panel** provides preset gain and threshold values. The user can select the triggering channel and manually adjust the threshold and gain settings of the PMT detectors.
- **Chip Display Panel** allows the user to monitor a live image of the chip, scan for clogs, and directly access the chip alignment interface.
- **Clog Detection Panel** allows the user to turn the clog detection and sample settling detection warnings on or off.
- **Single Cell Display Panel** shows the sorting progress in a 96- or 384-well plate display.

File Types Generated by WOLFViewer

.fcs: Data file standard for the reading and writing of data from flow cytometry experiments. WOLFViewer .fcs files can be opened with third party software.

.temp: Saves plot, gate and collection settings templates that can be applied in future experiments.

.csv: Spreadsheet format that stores the XY coordinates of the cells sorted in the plate in a Single Cell Sorting experiment.

.cmat: Compensation matrix created for data analysis and sorting.

.pdf: Experimental reports.

.jpeg: Plot and plate analyzer images.

2. System Setup

The initial steps to start analyzing samples with the WOLF[®] can be simultaneously performed to save time. A total of 20 to 30 minutes is needed to set up the system.

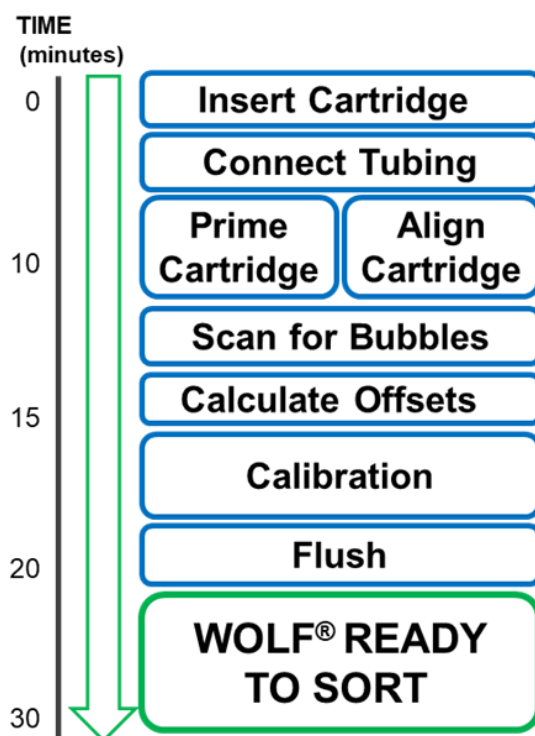


Figure 7. General workflow with the WOLF[®] Cell Sorter

2.1 Materials

- WOLF[®] Cell Sorter Cartridge
- PBS (Phosphate Buffered Saline Solution)
- 15 μ m Calibration Beads
- 20 mL Syringes
- 0.22 μ m Syringe Filters
- 50 mL Conical Tubes
- 5 mL FACS Tubes
- 37-40 μ m Cell Strainers
- Optical-Grade Isopropanol Wipes
- Reagent Reservoirs

2.2 Powering-On and Cartridge Selection

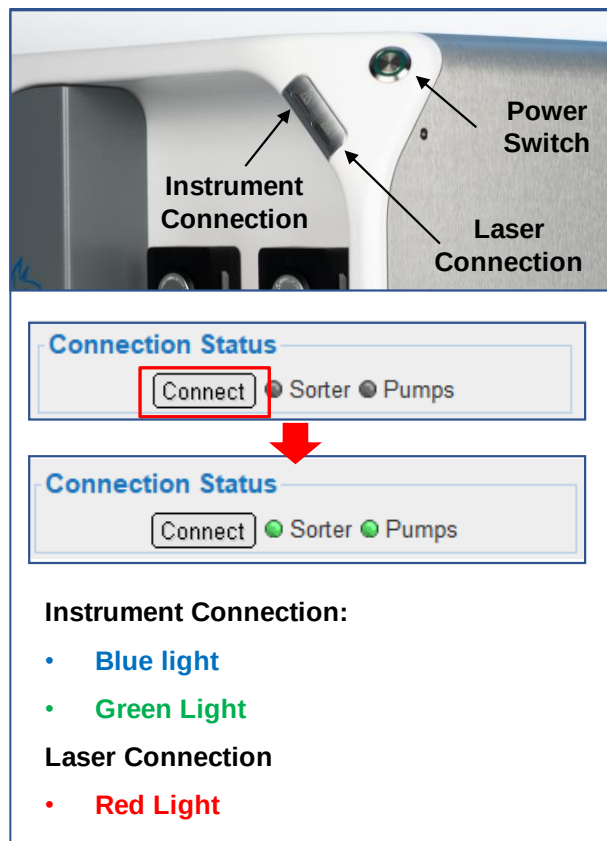


Figure 8. WOLF® Power on and indicator lights.

Powering On:

1. Turn on the WOLF® by pressing the button located in the upper right corner of the instrument.
2. Wait until the upper indicator (Instrument Connection) LED turns blue.
3. Open WOLFViewer Software, and if WOLF® is not automatically connected click “Connect” on the Connection Status Panel.
4. The upper (Instrument Connection) LED turns green when the instrument is connected.
5. The lower (Laser Connection) LED will turn red when the laser is on.

The WOLF® can be operated with two **types of cartridges** depending on the user experimental design:

Sorting cartridges are used for 1- or 2-way sorting into collection tubes.

Single Cell cartridges are used for 1-way sorting into 96- or 384-well plates using the N1 Single Cell module. The cartridge sorting outlets are connected to an extra length of tubing that attaches to the N1 module for cell dispensing into plates.

2.2 Powering-On and Cartridge Selection (Continued)

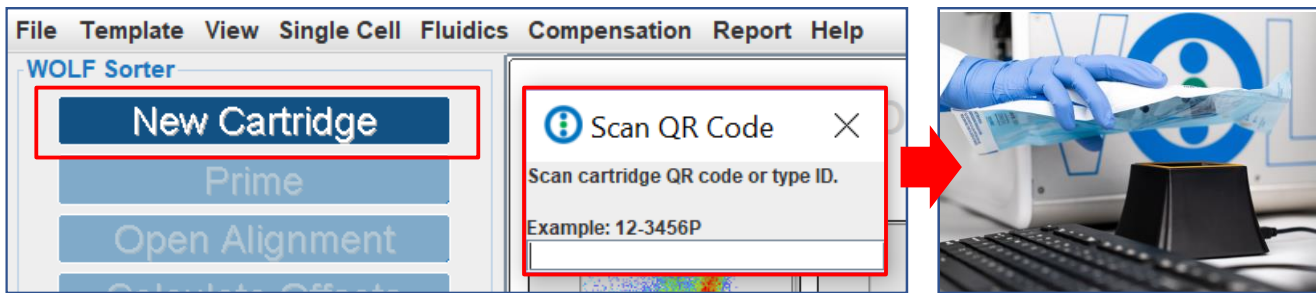


Figure 9. Cartridge QR Code Scan

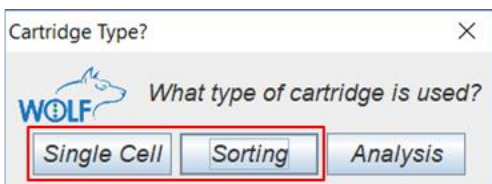


Figure 10. Cartridge Selection

Cartridge Selection:

1. Click "New Cartridge" in WOLFViewer. This triggers a "System Check" to ensure WOLF® is ready.
2. Scan the cartridge code using the QR code reader, or type the cartridge ID.
3. Select the type of cartridge that will be used for the experiment.

2.3 Cartridge Insertion



Figure 11. Cartridge Packing and Latch Opening Display.

Cartridge Insertion:

1. Remove the cartridge from the packaging, inspect it for any signs of damage that may compromise the performance of the cartridge.
2. Do not touch the glass portion of cartridge/chip as fingerprints can harm the optical signal. If touched, gently wipe clean with an optical-grade alcohol wipe.
3. Open the latch at the top of the cartridge slot.

2.3 Cartridge Insertion (Continued)

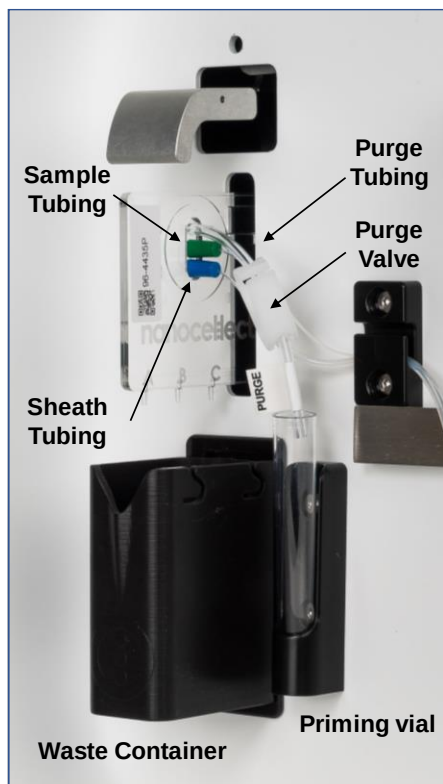


Figure 12. View of tubing, waste container and priming vial.

Cartridge Insertion (Continued):

4. Rest the bottom of the cartridge on the fixture floor to ensure it is not inserted at an angle.
5. Gently and smoothly insert the cartridge into the slot while avoiding jiggling, until a click is felt, and the cartridge feels securely seated.
6. Close the cartridge latch.
7. **Never force the cartridge into the fixture slot, it may damage the cartridge and/or the fixture detectors.**
8. If the cartridge gets stuck, lift the latch and hold it up with one hand. Use the other hand to pull the cartridge out while gently pushing it towards the left side of the fixture (see arrow in picture).
9. Reinsert the cartridge.

2.4 Tubing Connection

There are three tubing lines connected to the inlet ports of each cartridge. Single Cell Dispensing cartridge also possess tubing lines from 2 outlet ports.

- **Purge tubing:** shortest length and largest internal diameter
- **Sample tubing:** middle length tubing
- **Sheath tubing:** longest length tubing
- **Needle housing and tubing (for Single Cell Cartridges only):** connected to the outlets A and C. The magnetic needle housing attaches to the N1 module for single cell deposition.

2.4 Tubing Connection (Continued)

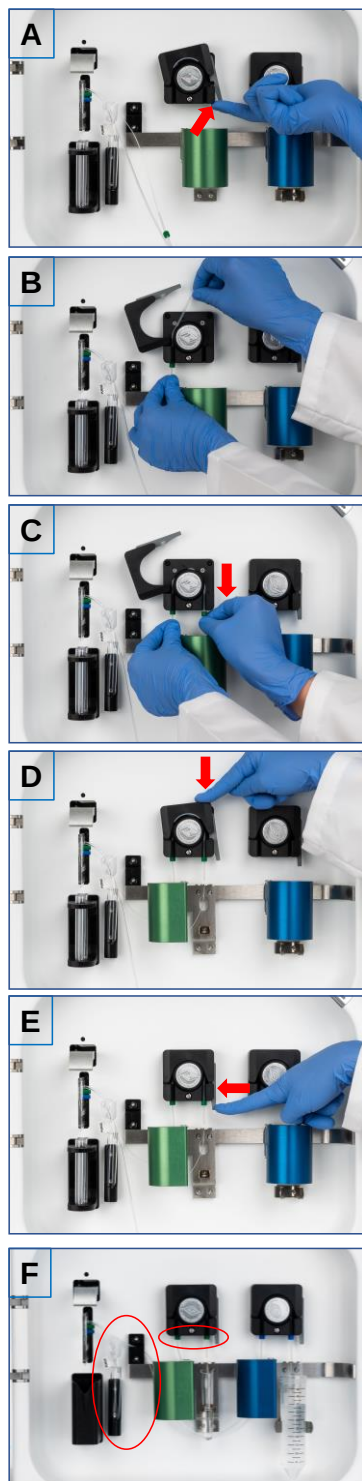


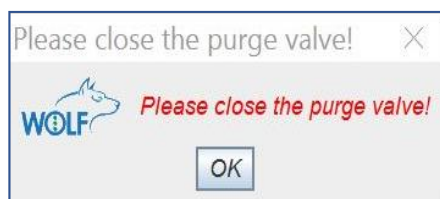
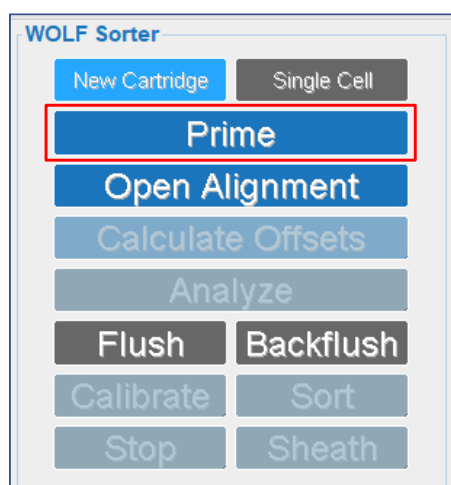
Figure 13. Cartridge tubing installation.

Tubing Connection:

1. To open the peristaltic pump, release the gray lock tab and gently lift upwards (Fig. 13A).
2. Thread the sample tubing line over the top of the pump rollers. (Fig. 13B).
3. Gently stretch the tubing and seat the black stoppers below the slots on either side and below the pump rollers (Fig. 13C). **Do not move the stoppers or overstretch the tubing as that may affect performance.**
4. Close the pump lock by gently pushing down until it clicks (Fig. 13D).
5. Ensure that the gray lock tab is securely pushed in (Fig. 13E).
6. Repeat the process with the sheath tubing.
7. Clean the tubing with an optical-grade alcohol wipe and place the free end into the respective sample or sheath tube.
8. Ensure both stoppers are seated directly below the bottom edge of the pump (Fig. 13F).
9. Hold Sample and Sheath tubing using the front panel, black holder. (Fig. 13F).
10. Make sure the purge tubing is in front of the sample and sheath tubing for easy access to the purge valve. Purge tubing should be positioned in the Priming Vial to prevent spillage (Fig. 13F).

2.5 Cartridge Priming and Chip Alignment

Priming assures that sheath fluid displaces all air in the cartridge fluidics to ensure good performance. Any excess fluid passes through the purge tubing into the priming vial. The priming protocol is a 10-minute automated process that has two phases. Phase 1: PBS is brought into the cartridge to displace air through the open purge valve; Phase 2: The purge valve is closed, generating an increase in pressure to dissolve any remaining air bubbles.



Cartridge Priming:

1. Make sure the purge tube clip is in the open position before starting the priming, enabling air to escape the cartridge.
2. In WOLFViewer Software, click the “Prime” button.
3. After 4 minutes, a pop-up window will appear prompting the user to close the purge valve. First, close the valve, then click “OK” on the pop-up window (Fig. 14). The user will notice that the pumps change speed and direction.
4. Ensure PBS is exiting the purge valve as well as the A, B, and C cartridge outlets before closing the purge tube clip.
5. We recommend opening the latch and reseating the cartridge. Once reseated, close the latch and align the cartridge by clicking the “Open Alignment” button.

Figure 14. Purge Valve Closing

2.5 Cartridge Priming and Chip Alignment (Continued)

The WOLF[®] Cell Sorter's laser and mirrors are stationary; however, the cartridge can be moved to optimize the light path, ensuring the best fluorescence and scatter measurements.

The Alignment protocol is a completely automated process that will move the cartridge in the X, Y and Z planes to situate the channel center at the optimal position for laser interrogation. The algorithm for cartridge alignment relies on focusing on various markings found within each cartridge

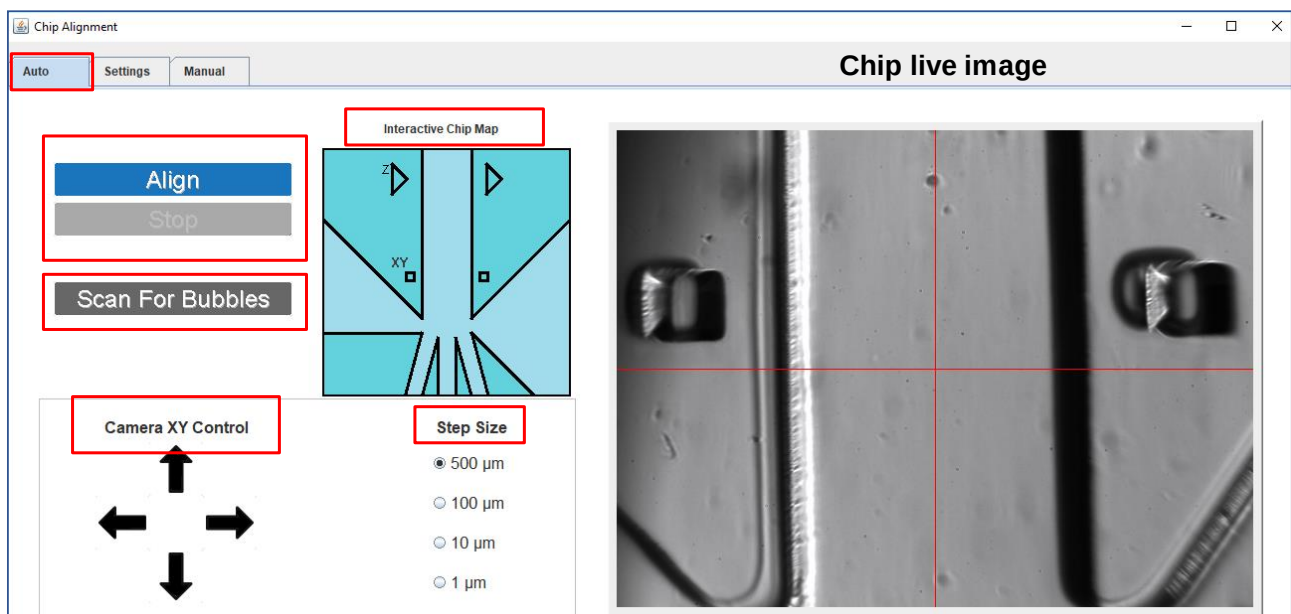


Figure 15. Chip Alignment User Interface - Auto Tab

The Chip Alignment Interface has 3 tabs to control the cartridge movement inside the instrument.

The **Auto Tab** displays from left to right

- “Align” and “Stop” buttons: Initiate and halt the automatic alignment process.
- “Scan For Bubbles” button: Automatically scans the chip after priming for air pockets.
- Interactive Chip Map: Moves the chip to specific areas without using the arrows.
- Chip live image: A real-time chip image allows the user to move the chip around when clicking in it.
- Camera XY Control arrow: Move the chip in the X-Y plane.
- Step Size radio buttons: Control the step size when moving the chip.

2.5 Cartridge Priming and Chip Alignment (Continued)

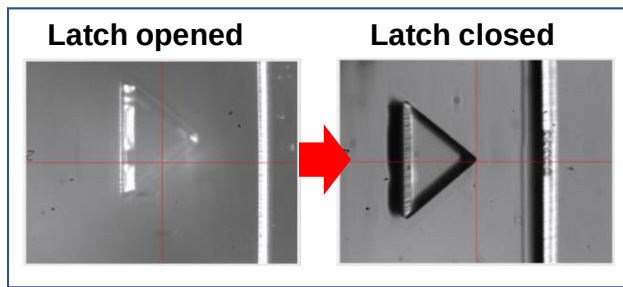


Figure 16. Chip Image quality displays with latch opened and closed

Make sure cartridge is properly inserted and seated in the fixture slot, and the latch is closed to ensure the image quality is optimal for alignment before initiating the process.

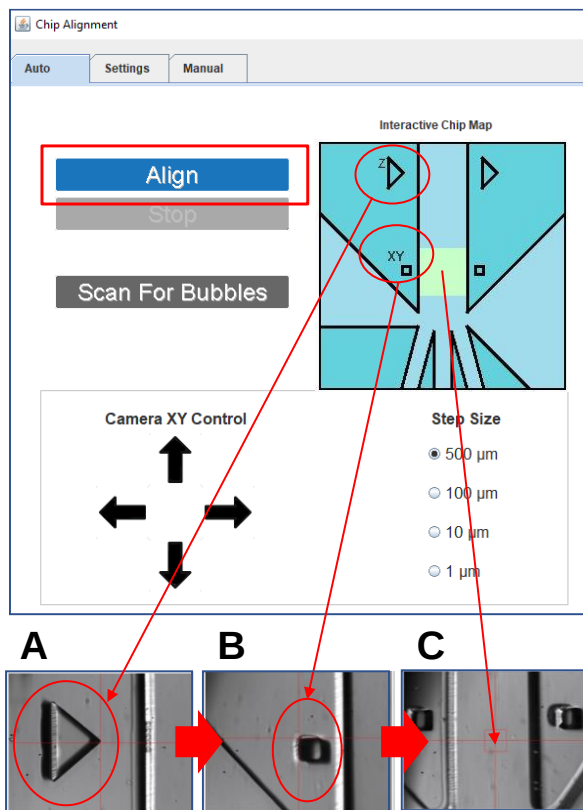


Figure 17. Chip Alignment Process

To Start Chip Alignment:

1. Click “Align” to initiate the automated process.
2. The cartridge will move to find the chip Z-marker (Fig. 17A) to focus in the Z-plane.
3. The cartridge will then move to the XY-marker (Fig. 17B) to estimate the distance to the chip channel center.
4. Alignment will be completed after this step, when the cartridge is moved to align the chip to the channel center (Fig. 17C).
5. A pop-up window will notify the user when the alignment has finished.

Once the priming process has been completed, the software will prompt the user to scan the chip for bubbles that could affect the sorting performance.

2.5 Cartridge Priming and Chip Alignment (Continued)

The Scan for Bubbles automated process is designed for an easy-to-use experience where the instrument operator must monitor the chip scanning to visualize the bubbles.

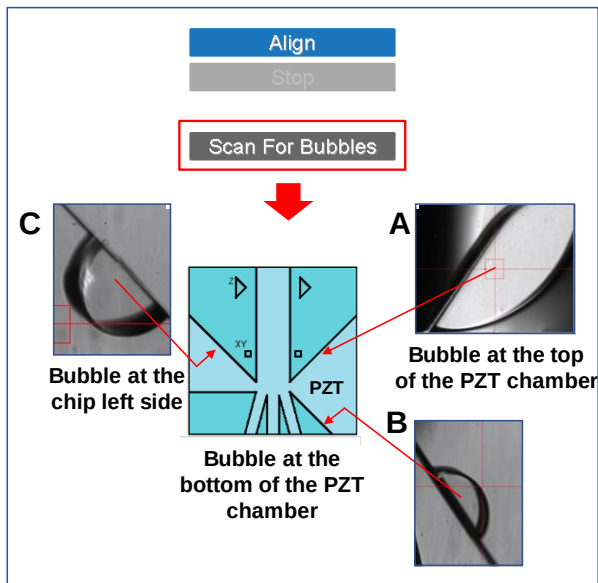


Figure 18. The Scan For Bubbles Feature

To Confirm the Absence of Air Bubbles in the Chip:

1. Click the “Scan for bubbles” button in the Chip Alignment window (Fig. 18).
2. The chip will be automatically moved for the user to actively monitor the camera image to visualize remaining bubbles.
3. The top chip PZT area will be scanned first (Fig. 18A), lower PZT area will be scanned second (Fig. 18B), and the left chip area will be scanned last (Fig. 18 C).
4. If bubbles are present, Pause the Bubble Scan and click the “Re-Prime” button in the Workflow Panel.
5. Once all bubbles are dissolved into the buffer, resume the bubble scan to confirm that the priming is complete. Re-Prime if needed until chip is air-free.

Note: If bubbles remain after 3 re-priming attempts, the software will force the user to continue to the next step, “Calculate Offsets”.

2.5 Cartridge Priming and Chip Alignment (Continued)

The Settings and Manual tabs are designed for NanoCollect's personnel to control the process and evaluate potential failures.



Figure 19. Chip Alignment User Interface - Settings Tab

The **Settings Tab** displays from top to bottom and left to right

- Current Stage Position: Chip location values within the cartridge fixture.
- Options Panel: User can set a new chip center if needed and reset the servos (motors that move the cartridge) or the camera.
- Analysis – 1 Panel: A graphical representation of the alignment algorithm progress.
- Camera Image Panel: A real-time chip image and buttons to change the camera exposure time.

Note: We recommend that you do not change any of the values in the Setting Tab without guidance of NanoCollect's support personnel.

2.5 Cartridge Priming and Chip Alignment (Continued)

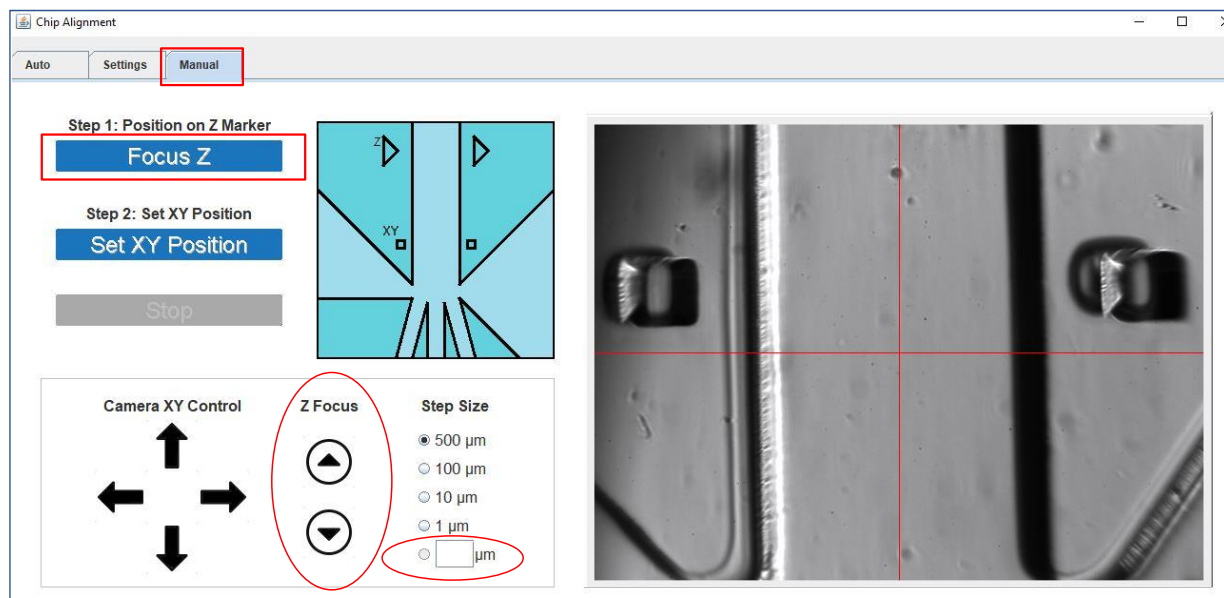


Figure 20. Chip Alignment User Interface - Manual Tab

The Manual Tab has a similar interface to the Alignment Tab. The user can manually find the Z-marker and initiate the focusing by clicking the button “Focus Z”.

The user can focus in the Z-plane and manually input a preferred step value for the process.

2.6 Chip Offsets Calculation

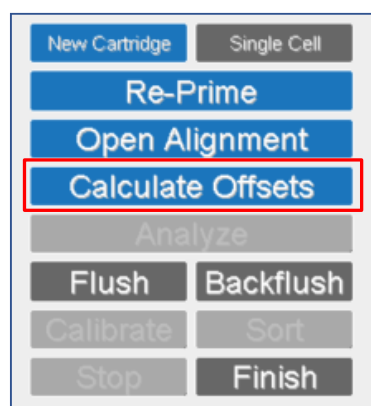


Figure 21. Workflow Panel and Offset Calculation

During this step, the software will automatically calculate the chip background signal to subtract it from the signals detected during the experiment.

Chip Alignment:

1. Make sure the WOLF® front door is closed. Vibrations, like centrifuges and vortexers, may affect the process.
2. Click the “Calculate Offsets” button on Workflow Panel to begin the automatic process.

2.7 Chip Calibration

The calibration step is an automated process to determine the optimal sort delay (time between cell detection and cell sorting) for a given cartridge. The calibration protocol is a multi-step process that evaluates the efficiency of sorting 100 beads into a particular channel for ten different delay times. This protocol is completed for Channel A and Channel C. The automatic Calibration step has been optimized for use with our Calibration Beads (15 μ m green fluorescent beads).

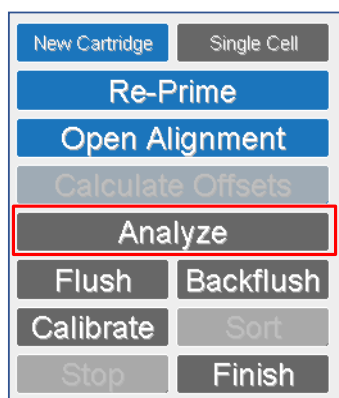


Figure 22. Workflow Panel, Analysis and Plot Selection

Analyze Calibration Beads:

1. Vortex and load the calibration beads vial in the sample tube holder.
2. Click the “Analyze” button on the Workflow Panel.
3. Open a 2D and a histogram plot by clicking in the plot selection tool. Analyze 300-500 events.
4. Click the “Stop” button to finish analyzing calibration beads

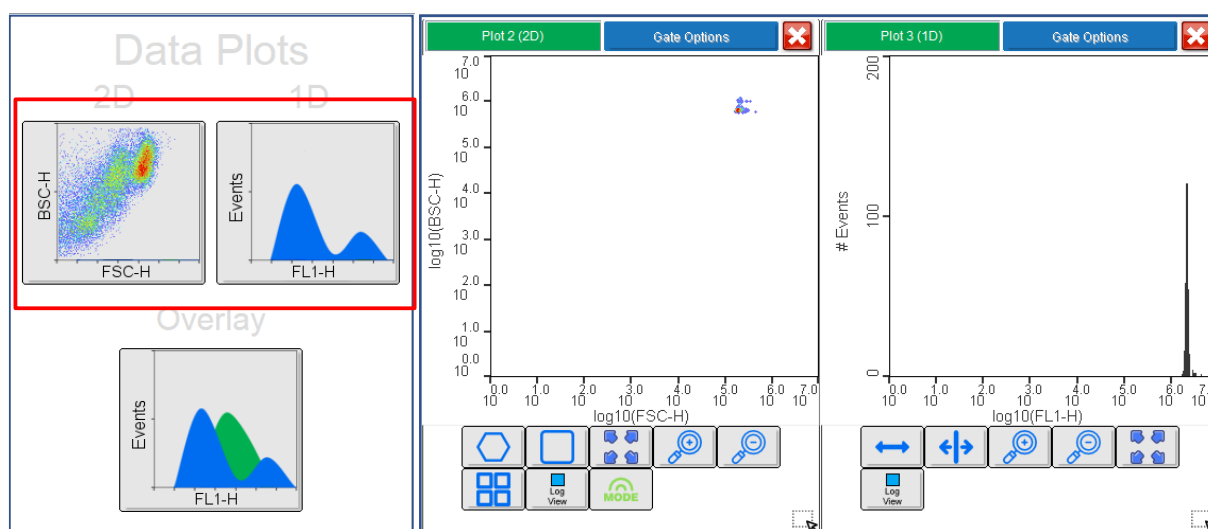


Figure 23. Plot Selection Tool and Scatter and Fluorescent Plots displaying the scatter and fluorescence intensity of the Calibration Beads. With proper cartridge alignment, the beads will appear as a tight cluster in the Forward and Back Scatter plot (2D) and display small CVs (Coefficient of Variation) in the FL1 channel plot.

2.7 Chip Calibration (Continued)



Figure 24. Calibration Beads vortexing.

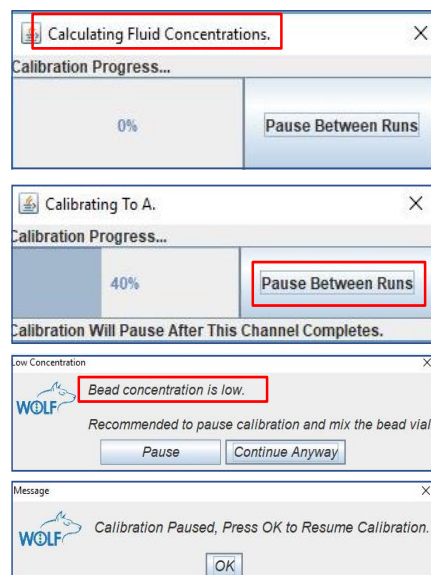


Figure 25. Calibration Process

To Calibrate the Cartridge:

1. Vortex Calibration Beads vigorously before starting the process to prevent aggregation.
2. Position the Calibration Beads tube in the sample holder. Make sure the cartridge tubing does not reach the very bottom of the vial, as beads can be filtered out.
3. Press the “Calibrate” button on the Workflow Panel.
4. The software will calculate the beads concentration and warn the user if it is below 10 beads/ μ l.
5. The automated calibration will begin with channel A. A progress bar will inform the user of the calibration status.
6. The beads can settle at the bottom of the vial, decreasing the concentration available to calibrate the chip. A pop-up will inform the user of the number of beads detected by the laser.
7. Calibration can be paused between channels to mix the beads or replace the tubing if needed.
8. Flush your cartridge once calibration is complete. Place a tube containing 4 mL filtered PBS in the sample holder and click the “Flush” button. A 10-minute flush cycle is ideal for removing most beads from the cartridge.

2.8 Fluidic Controls

The user can control the instrument fluidics after priming for flushing and backflushing the chip, as well as stopping the sheath pump.

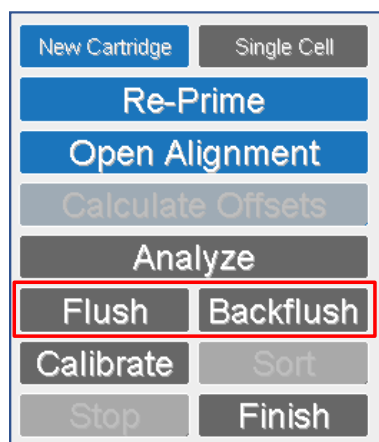


Figure 26. Flush, Backflush Tools.

To Flush and Backflush the Cartridge:

1. Click "Flush" to remove beads after calibration, or clean carry over between samples. The user can flush the cartridge for either 3 or 10 minutes.
2. To remove air bubbles from the cartridge inlet lines (sample and sheath) before they reach the chip, the user can click "Backflush" to change the sample pump direction for a minute. Big bubbles in the chip area will not be removed by backflush. The user needs to re-prime for optimal sorting efficiency.

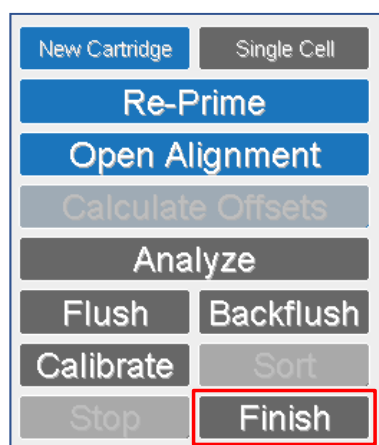


Figure 27. Sheath Pump Control

To Change the PBS Sheath Buffer for Other Cell Media:

1. Stop the sheath pump by clicking the "Finish" button to prevent air from entering the chip.
2. Exchange the sheath buffer tube as quickly as possible to avoid the cartridge drying out.
3. Click the "Sheath" button to start the pump again.

3. Sample Analysis

Prior to sorting, one must analyze basic parameters, such as scatter and level of fluorescence. Analysis of the particles allows one to identify populations and establish the desired gates for sorting.

Note: Refer to [Section 8. WOLF® Cell Sorter Best Practices](#) of this manual for suggestions on how to avoid problems during sorting.

3.1 Sample Analysis

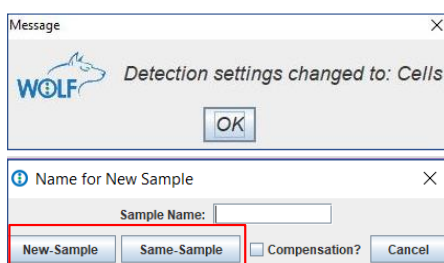


Figure 28. Sample Analysis Process

To Begin a New Sample Analysis:

1. Detection settings change automatically to “Cells” when the chip calibration has been completed to apply the appropriate threshold and PMT gains. PMT gains and threshold can be manually adjusted to suit sample intensity if needed.
2. Click the “Analyze” button in the Workflow Panel.
3. A pop-up window will request naming the sample.

When clicking “New Sample” (Fig. 28), the instrument will temporarily accelerate the sample pump, reducing the time for the sample to reach the detection point. “Same Sample” does not accelerate the pump for faster detection and saves 30 µl of volume.

3.2 Setting Detector Gains

For the best sample detection, the user should select a triggering channel and the optimal detector gains. WOLFViewer provides standard settings for cell detection that will be automatically loaded once calibration is completed. However, the user should optimize the settings according to the sample needs.

3.2.1 Threshold and Triggering Channel

During sample analysis, not all the particles that pass the laser interrogation point are of interest to the user. To discriminate unwanted signals the user should set a triggering channel (detector) and a signal intensity (threshold) (Fig. 29).

When choosing a triggering channel, the user should consider that cells will be registered as events if they have an intensity greater than the threshold in the selected trigger channel. We recommend using FSC-H as triggering channel for cell detection with a threshold between 25,000-75,000 to remove most of the cell debris.

Note: The minimum threshold value that can be applied in WOLFViewer is 1,024.

3.2.2 PMT Gains

Adjusting the PMT gains increases or decreases the intensity of the fluorescent signal to determine the minimal voltage required for each detector to sense signals above the electronic noise. When adjusting the voltages, the user should make sure the negative population is between 10^3 - 10^4 in the logarithmic scale. Lower intensity values are considered background noise.

3.2.2 PMT Gains (Continued)

WOLFViewer provides preset values for Cells, Calibration, and Rainbow Beads detection. However, the user should optimize the values to achieve the best detection for their samples.

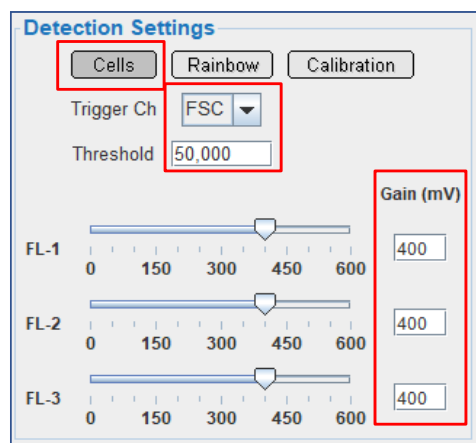


Figure 29. Detection Settings

To Adjust the Detection Settings:

1. Detection settings change automatically to “Cells” when the chip calibration has been completed to apply the appropriate threshold and PMT gains.
4. Click the “Cells”, “Rainbow”, or “Calibration” button depending on the type of sample to analyze.
5. Select the triggering channel from the drop-down menu.
6. Type the threshold value for that channel in the blank box.
7. Adjust the PMT gains by moving the sliders to optimize your sample detection.

4. The WOLFViewer Analysis Tools

The WOLFViewer Software has all the tools needed for real-time as well as post-collection **sample** analysis.

4.1 Analyzing Sample Information with Plots

To initiate the analysis, the user needs to create plots to display sample information. WOLFViewer offers the option to create 1D (histograms), overlay and 2D plots for the analysis.

4.1.1 Dot, Density, and Contour Plots

Bivariate (2D) dot-plots are widely used to show 2 parameters of any individual cell at the same time. There are three types of dot-plots:

- Scatter Plots (FSC vs. BSC)
- Fluorescence vs. Scatter (FSC/BSC vs. FL)
- Fluorescence vs. Fluorescence

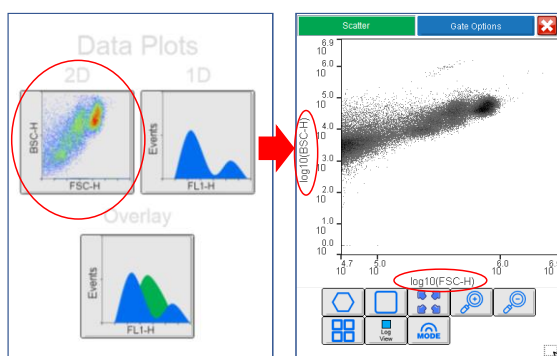


Figure 30. Creating bivariate plots

To Create a Dot Plot:

1. On the Data Plots display, click the 2D Plot button.
2. A dot-plot displaying the forward versus back scatter channels will automatically open.

The “Mode” option in the plot tool allows the user to change the plot display between dot, density and contour (Fig. 31).

- Dot plots are helpful when studying populations with moderate or low frequency populations.
- Density plots show all the events collected represented in a color scale based on density, blue being low, green medium, and red high intensity.

4.1.1 Dot, Density and, Contour Plots (Continued)

- **Contour plots** display event densities with lines (gradients). They are useful when the plot has concentrated high density regions or when there is a build-up of events along axes, to estimate the events that are off-scale.

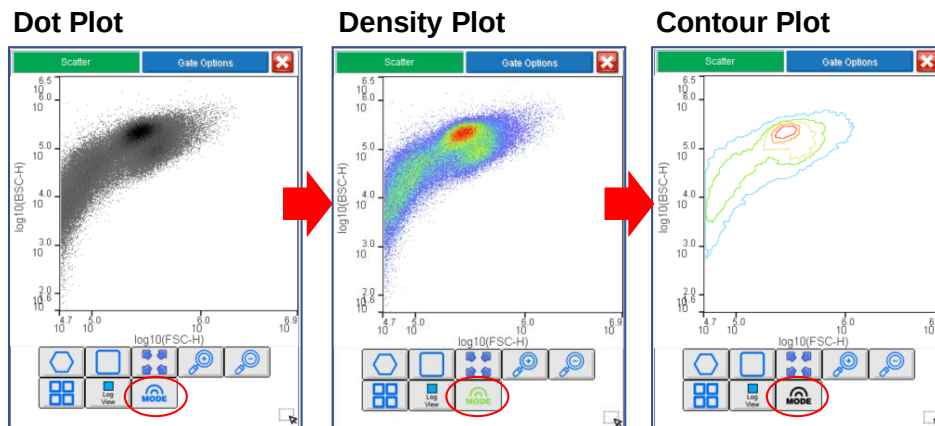


Figure 31. Dot, density and contour bivariate plots

4.1.2 Histogram Plots

The user can analyze the frequency (# Events) and distribution of the data (Intensity) from a single parameter using histogram plots. 1D plots are useful for analyzing the total number of cells in a sample that possess the properties or express the marker of interest.

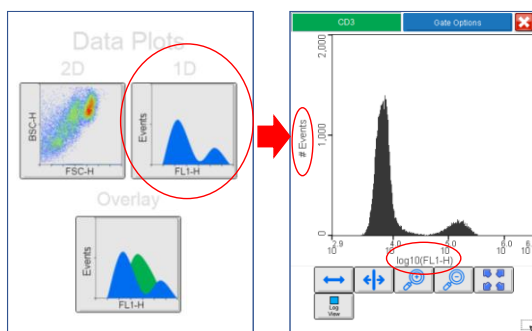


Figure 32. Creating univariate plots

To Create a Histogram Plot:

1. On the Data Plots display, click the 1D Plot button.
2. A histogram displaying the FL1 channel and number of events will automatically open.

4.1.3 Overlay Plots

Overlay plots are univariate and display 2 or more data sets in the same axis. The user can analyze several samples in one graph, comparing controls with diverse experimental outcomes.

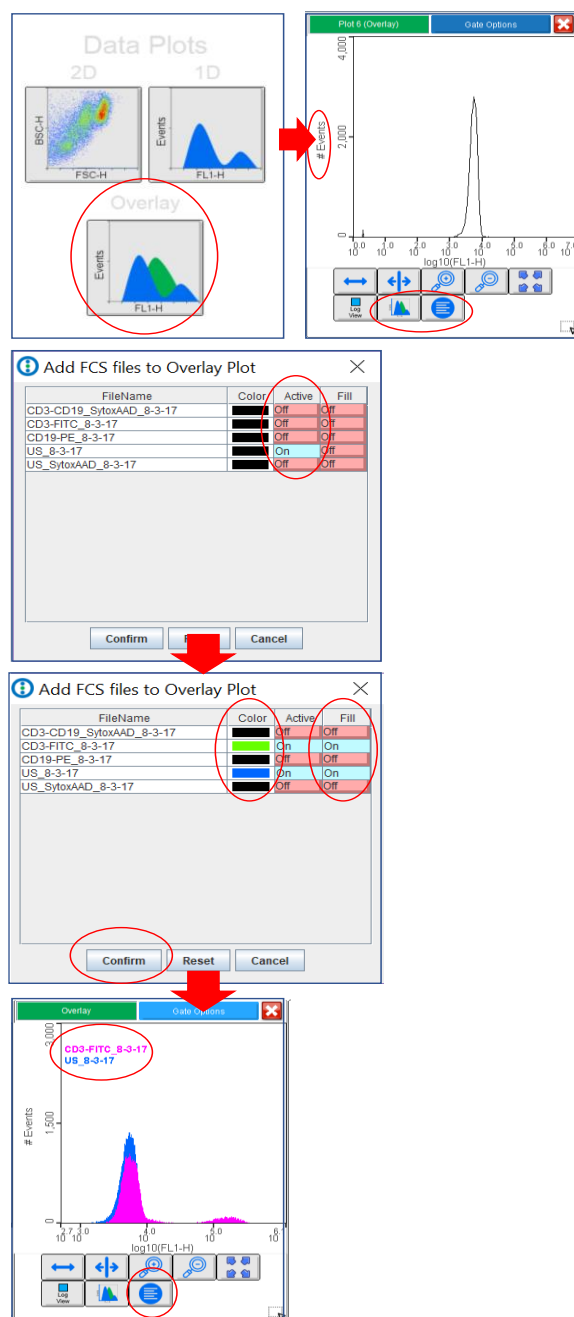


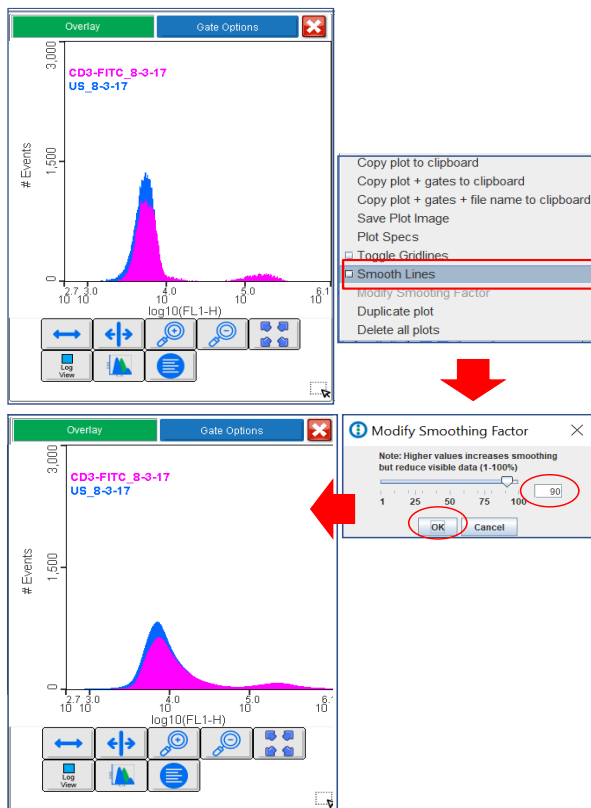
Figure 33. Creating univariate, overlay plots

To Create an Overlay Plot:

1. On the Data Plots display, click the Overlay Plot button.
2. A FL1 histogram of the sample file selected for analysis, and a window to work on the plot settings will automatically open.
3. The “Add fcs files” window will display all the fcs files currently loaded in WOLFViewer. The user should make sure that all files needed for analysis are loaded in the software before creating the overlay plot.
4. To add more files to the plot, click the “Off” button to “On” in the “Activate” column.
5. Select a color to label the file display by clicking in the “Color” button by the file name.
6. The curves can be filled with the color selected “Off” button by the file name in the “Color” column.
7. Click the display button below the graph to display the file names in the plot.
8. Click “Confirm” to finalize the process or “Reset” to start over.

4.1.3 Overlay plots (Continued)

The lines of overlay plots can be smoothed for graphic display.



To Smooth Plot Lines:

1. Right click on the plot.
2. Select “Modify Smoothing Factor”
3. Move sliders to increase and decrease curves’ smoothness.
4. Click “OK”.

NOTE: Overlay plots are used for sample analysis but cannot be used for sorting purposes. Also, be aware that applying high smoothing factors will reduce data display.

Figure 34. Smoothing lines in overlay plots

4.2 Customizing plots

The user can customize the plots created for sample analysis and sorting using the different features present in the software.

4.2.1 Changing Plot and Axis Names

Plots and axes names can be customized to make the sample analysis more intuitive.

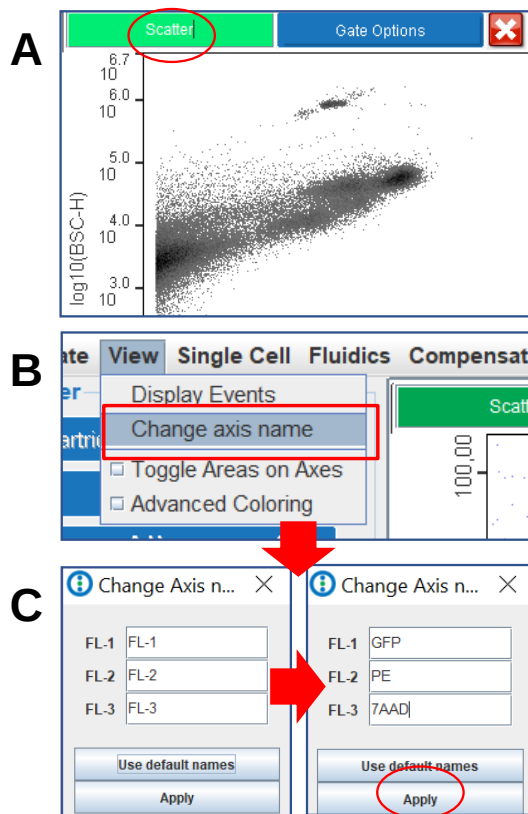


Figure 35. Changing plot and axis name

To Change Plot Name:

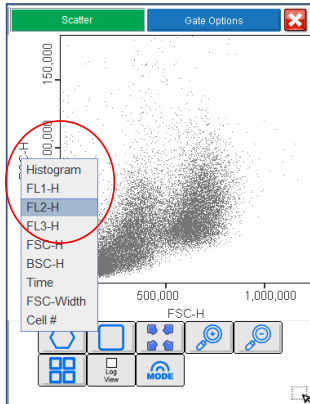
1. Type the new plot name in the green box situated in the top left corner of the plot (Fig. 35A)

To Change Axis Name:

1. In the software main menu, select the "View" tab to access the "Change axis name" window (Fig. 35B).
2. Type the new name in the channel boxes.
3. Click "Apply". The new axis names will automatically load in all the existing and new plots (Fig. 35C).

4.2.2 Changing Plot Axes

The user can customize pre-existing plots, changing from bivariate to univariate or selecting a different channel to analyze the sample.



To Change Plot Axis:

1. Right-click on the plot name.
2. Select the channel of interest.

Figure 36. Changing plot axis

4.2.3 Customizing Plot Axis Values

The user can set specific plot axis values to always display the same representation when analyzing data.

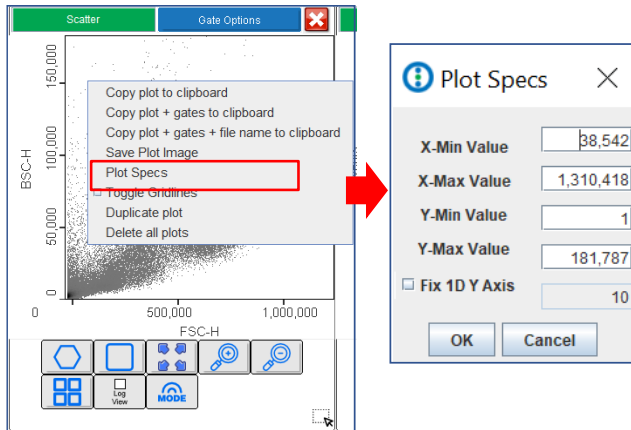


Figure 37. Setting new plot axis values

To Set Plot Values:

1. Right-click on the plot.
2. Select "Plot Specs".
3. Type the desired axis values in the boxes.
4. The user can fix the number of events displayed in the Y-axis for histogram plots by checking the "Fix 1D Y Axis" box and typing the desired value.

4.2.4 Adding Gridlines to Plots

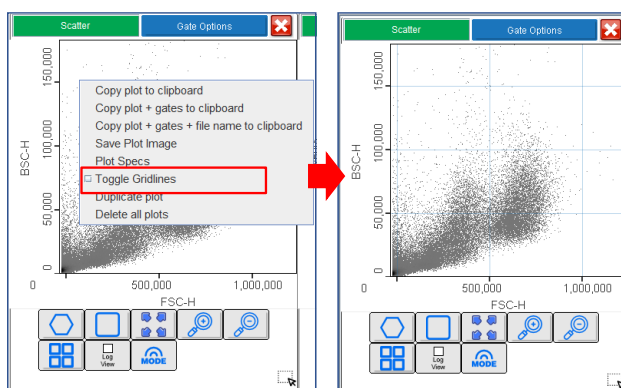


Figure 38. Adding gridlines to plots

To Add Gridlines to Plots:

1. Right-click on the plot.
2. Check "Toggle Gridlines"

4.3 Creating Gates

The gating process is essential for flow cytometry data analysis and sorting. During this process, the user applies gates to discriminate unwanted cells from the cells of interest. Forward and backscatter, as well as markers of interest, are used to identify the target cells for sorting.

4.3.1 Types of gates

The user can create different types of gates for analysis in bivariate and univariate plots.

- **Polygon gates** allow the user to select specific populations within regions with diverse data. They are widely used to select cells based on size (FSC) and complexity (BSC) in bivariate plots (Fig. 39A).
- **Rectangular gates** in bivariate plots are generally used to select big areas of data and in scatter vs fluorescence plots to distinguish positive cells from negative cells (Fig. 39B).
- **Quadrant gates** are used to divide the plot into 4 areas in bivariate plots. They allow the user to determine the single positive cells for each marker as well as both double negative and double positive cells (Fig. 39C).
- **Vertical gates** divide the univariate plot into 2 main regions. They are also useful for discriminating positive cells from negative cells (Fig. 39D).
- **Horizontal gates** can be applied to histograms for analysis of several populations, like positive vs negative and higher vs lower expressor cells (Fig. 39D).

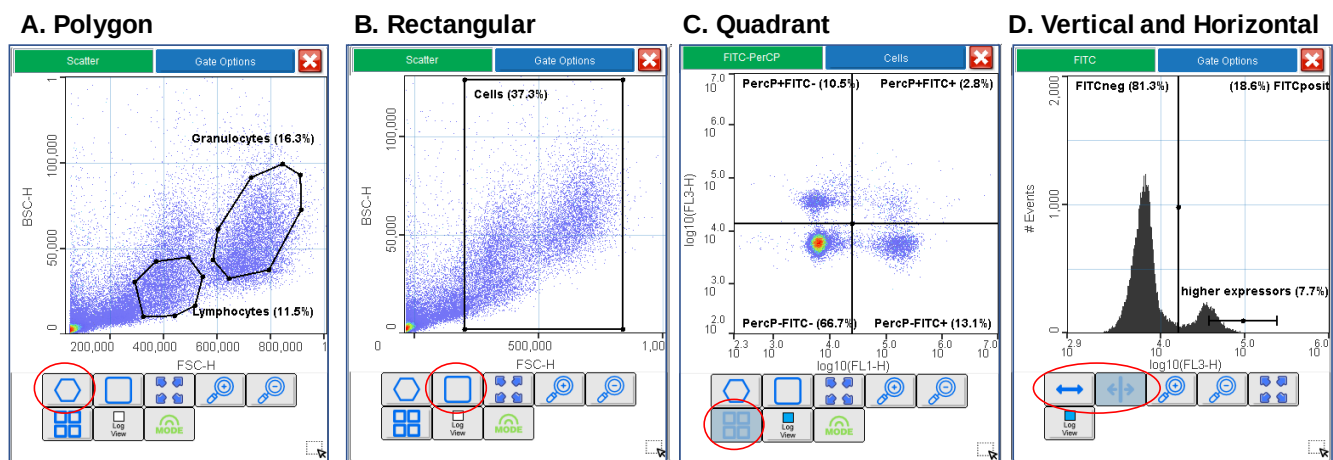


Figure 39. Different types of gates in WOLFViewer

4.3.1 Types of gates (Continued)

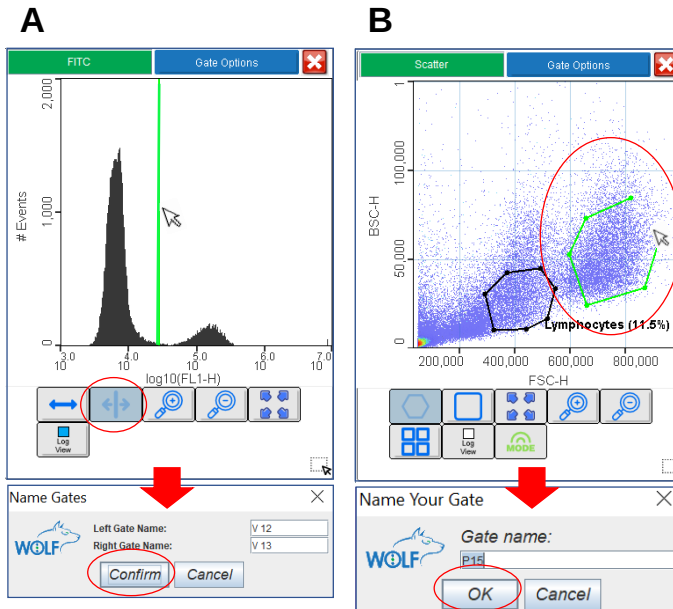


Figure 40. Creating plot gates

4.3.2 Gate Tools

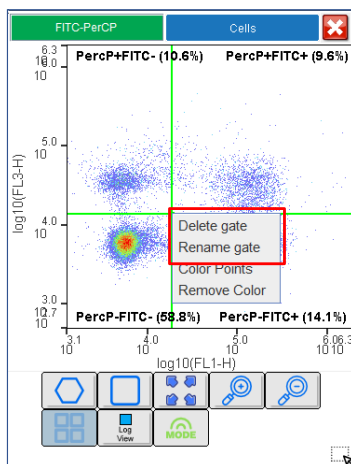


Figure 41. Deleting or renaming gates

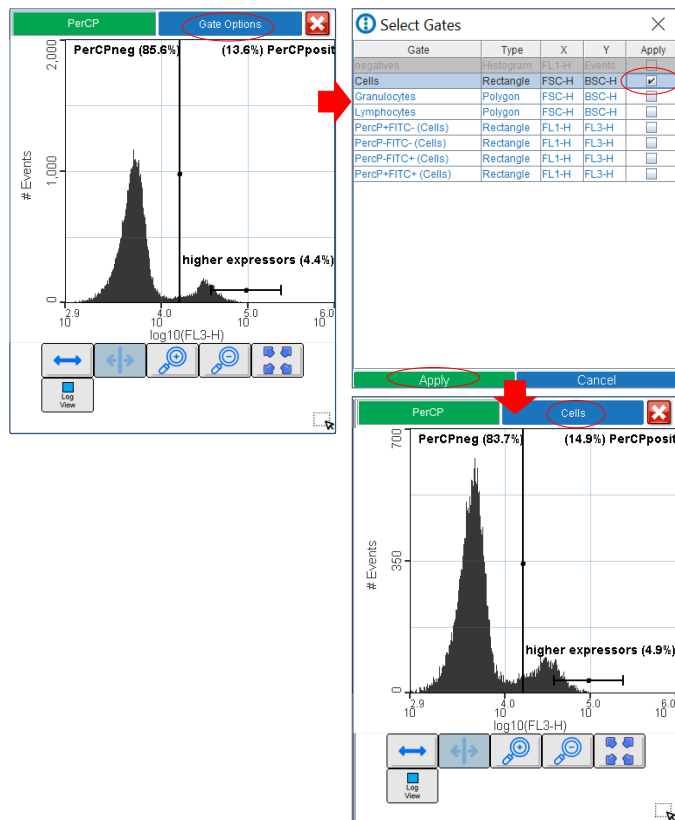
To Create a Gate:

1. Select the gate tool under the plot.
2. For **vertical and quadrant gates**, click on the plot to apply it (Fig. 40).
3. For **horizontal and rectangular gates**, drag the gate after the initial click.
4. For **polygon gates** click several times on the plot to draw the desired shape. To close the polygon, connect the last point with the first one or double-click for automatic closing (Fig. 40B).
5. Name the gate in the pop-up window.
6. To move a gate, click on one of the walls and drag it.
7. To resize a gate, click on one of the vertices and drag it.

To Delete and Rename Gates:

1. Position the mouse on the gate limit.
2. Right-click and select the desired option (Fig. 41).

4.3.3 Applying Gates to Other Plots



To Apply a Gate to a Plot:

1. Click on "Gate Options"
2. Check the boxes to apply the gates of interest. Gates already present in the plot will be greyed-out in the Selection window.
3. The user can apply more than 1 gate per plot to display events that are only present in both gates.
4. Click "Apply".

Figure 42. Applying gates to plots

4.3.4 Coloring Populations or Back-Gating

Coloring the populations of interest, or back-gating, is used to analyze cells identified in a gate in different plots with other parameters. The goal is to confirm a staining pattern or a gating strategy, non-specific binding, or dead cells.

4.3.4 Coloring Populations or Back-Gating (Continued)

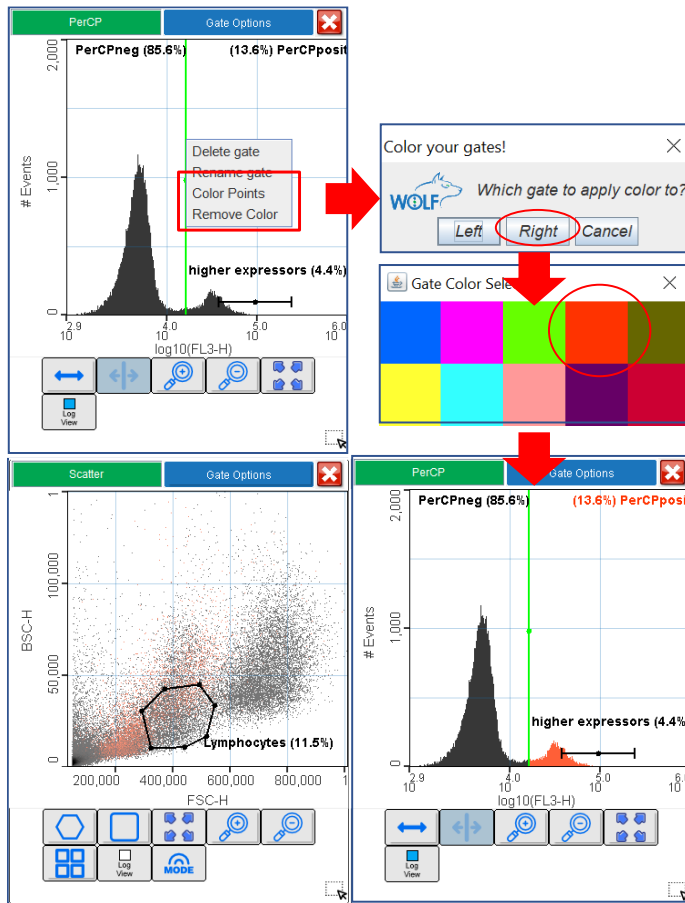


Figure 43. Applying color to gates

To Apply Color to a Gate:

1. Right-click on the gate and select "Color Points".
2. Click on the desired color to apply. Gate name and % will be colored too. Set the plot in Regular Mode (black dots) for better display (Fig. 43).
3. Up to 10 gates can be colored in a plot. Gates in different plots can also be colored. The color pattern will appear superposed (Fig. 44).
4. To remove the gate color, right-click on the gate and select "Remove Color".

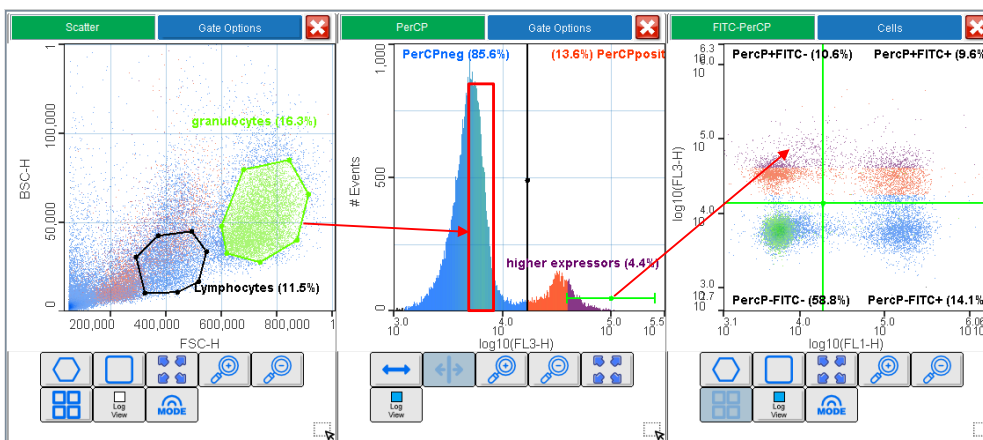


Figure 44. Applying color to gates in different plots

4.3.5 Other Plot View Options

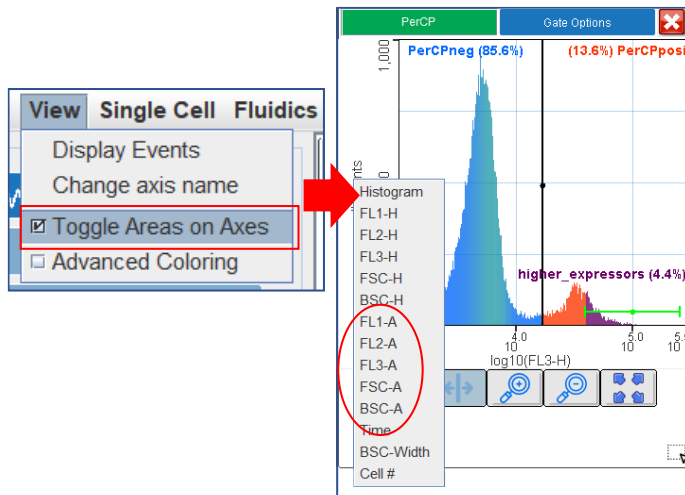


Figure 45. Adding Area to data plots

To Add Area to Plots:

1. Click on the “View” in the Main Menu.
2. Check “Toggle Area on Axes”.
3. The option Area will automatically load in the plot Axes.

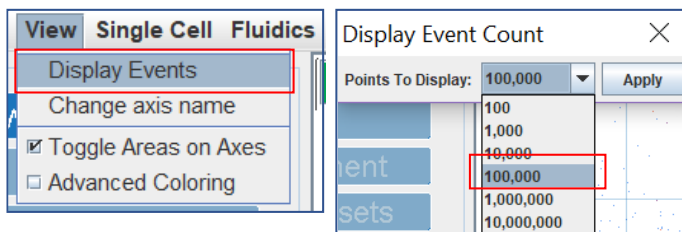


Figure 46. Changing event display in plots

To Change Event Display in Plots:

1. Click on the “View” in the Main Menu.
2. Select “Display Events”.
3. Change to the desired number of events to display in all existing and newly created plots.

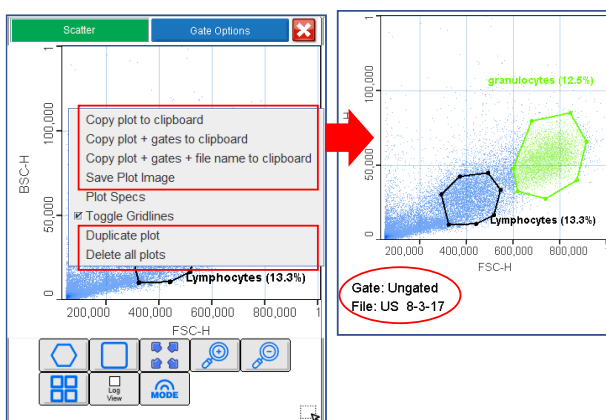


Figure 47. Copying data plots

To Copy Plots:

1. Right-click on the plot.
2. Select the desired “Copy” option and paste it in another document.
3. Plot images can also be saved in JPEG format.

4.4 Sample Statistical Analysis

The Stats and Gate State Panels provide additional information on the populations gated for sample analysis and sorting.

To Open the Stats Panel:

1. Click on the “Stats Panel” button.
2. Information from all the events displayed in plots and gates is present in the panel (% of Parent, Mean, Coefficient of Variance (CV), Median and Mean) for the parameters plotted in the axis.
3. The user should keep in mind that the plots display the last 100,000 events collected and not the entire data set. However, the Stats Panel shows the analysis of all events collected.
4. The panel needs to be refreshed to display the latest numbers if left open.
5. To copy an image or save the information in a .csv file, click on the grey background by the data table.
6. To close the panel, click again on the “Stats Panel” button.

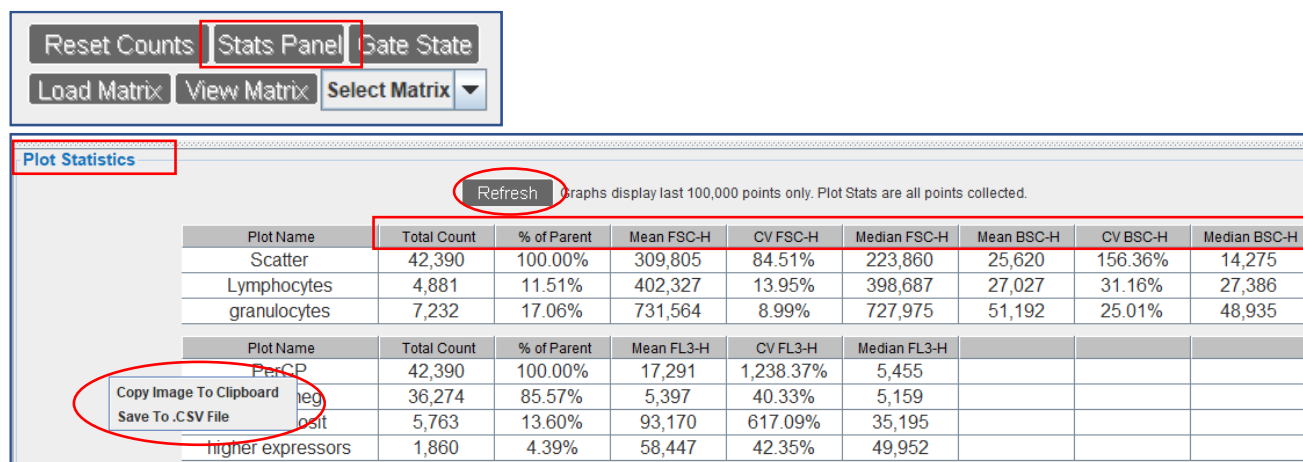


Figure 48. WOLFViewer Stats Panel

4.4 Sample Statistical Analysis (Continued)

To Open the Gate State Panel:

1. Click on the “Gate State” button.
2. A Boolean display will show all the gates created in the plots currently opened in WOLFViewer.
3. The statistical information displays the number of events collected per gate, the percentage when compared to the total events in the plot (% of Data (Total)), and compared to the events present in the parent gate (% of Data (Parent)).
4. The panel needs to be refreshed to display the latest numbers if left open.
5. To copy an image or save the information in a .csv file, click on the grey background by the data table.
6. To close the panel, click again on the “Gate State” button.

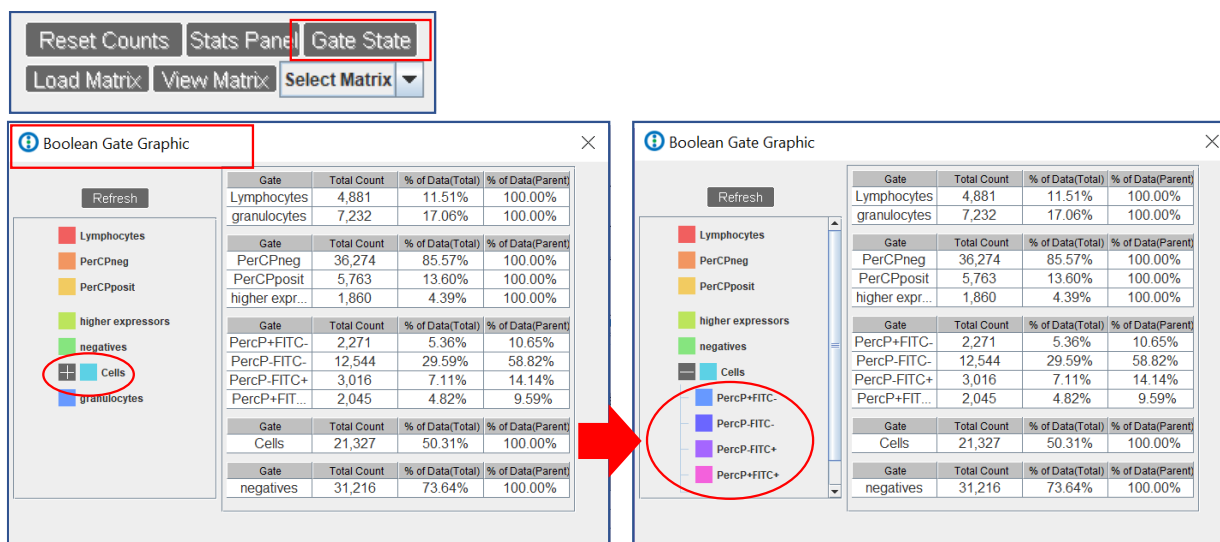


Figure 49. WOLFViewer Gate State Panel

4.5 Compensation

“Spillover” or “cross talk” occurs when the emission spectrum of a fluorochrome is sensed by a detector that is assigned to another fluorochrome (Fig. 50A). Compensation can be used to correct this fluorochrome spillover by using a compensation matrix (Fig. 50B).

The matrix is created by analyzing single-color controls in all the detection channels and determining the spectral overlap for each detector.

The values are computed to generate a Spillover matrix that will show the % of fluorochrome bleeding into other channels.

A Compensation matrix, generated based on the Spillover matrix, will be applied to the sample fluorescence intensity values in the different channels to correct the spillover.

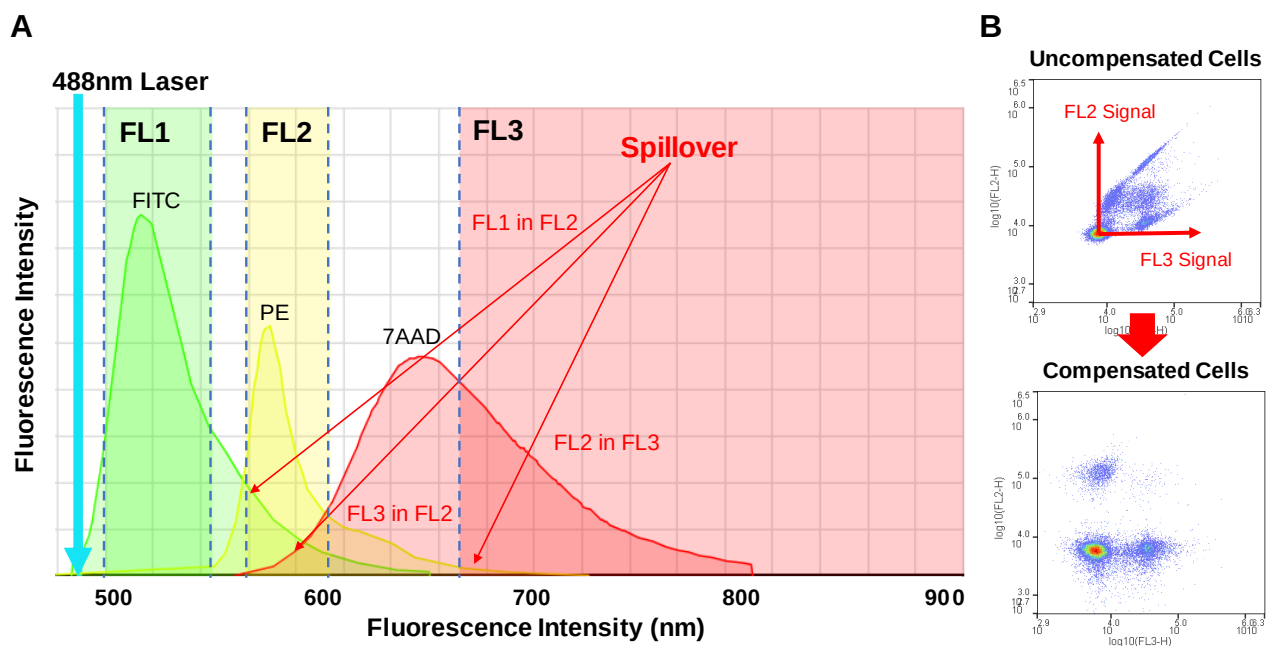


Figure 50. Representation of fluorescence spillover in the different WOLF® detectors.

4.5.1 Compensation Experimental Design

The WOLFViewer compensation feature collects the data of every control and automatically calculates the appropriate compensation for each fluorochrome combination. However, optimal controls should be analyzed for an accurate compensation process.

The software offers the possibility of Automatic and Manual Compensation, as well as editing existing matrices.

To Prepare Compensation Controls

1. Prepare your single-stained controls by labeling your cells with each individual fluorochrome. Use the same fluorochrome that is used in the sample to compensate (do not use Alexa488 to compensate FITC).
2. Make sure your compensation controls are as bright or brighter than the sample.
3. Prepare an unstained control. A 3-color experiment requires 4 samples (unstained control + 3 single-stained controls). Do not rely on a universal negative control. Autofluorescence, which is related to cell size, should be the same in positive and negative controls.
4. Establish optimal Detector gains to avoid saturating signals (events against the axes walls). Use the same Detector gains for all the compensation controls and the sample of interest, as the degree of compensation applied is gain-dependent.
5. Analyze your controls to have a minimum of 10,000 events in the gates used for compensation to obtain accurate median values.
6. Once a compensation matrix is created and saved, it can be applied to other samples with the same experimental conditions.

4.5.2 Automatic Compensation

We recommend using automatic compensation algorithms to correct for spillover. If results after applying a matrix are not the expected, the user should redesign the controls. The automatic compensation feature will analyze the single stained and unstained controls to generate the values of the compensation matrix.

To Create a Compensation Matrix with Automatic Compensation

1. Select “Automatic Compensation” from the Compensation tab in the main menu.
2. Click the “Load Files” button to open the .fcs files that were generated while analyzing the single-stained and unstained controls.
3. Choose the files to load. A minimum of 3 files (2-color compensation) need to be loaded to initiate the process.
4. Click the “Select” button.
5. Assign the control files to each detector channel by checking the boxes. The unstained control should be checked as “Blank”.

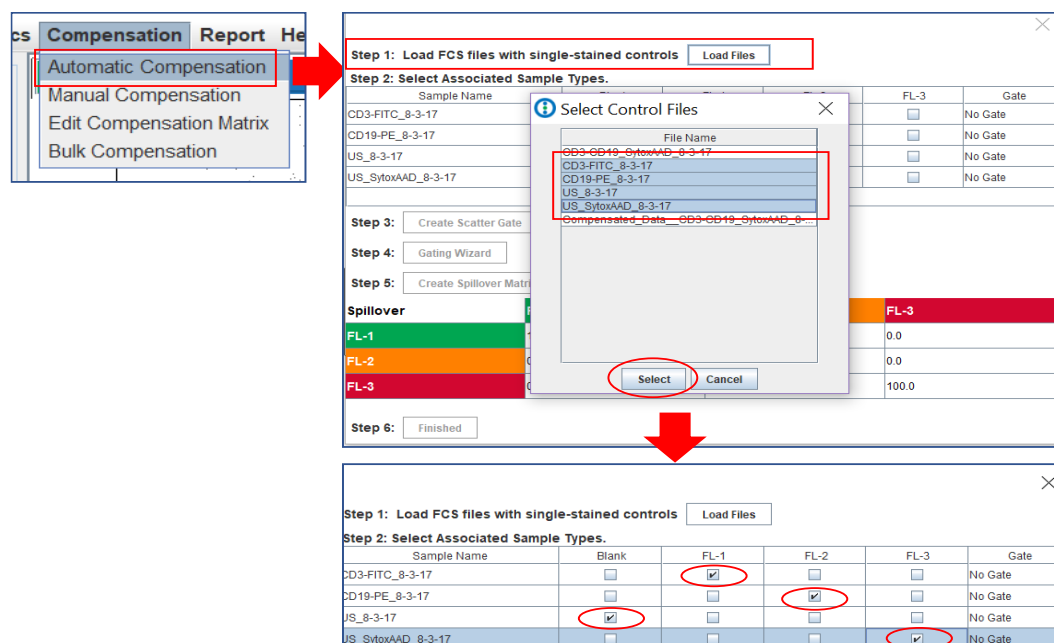


Figure 51. Selecting control files for Automatic Compensation.

4.5.2 Automatic Compensation (Continued)

The user has the option to create a scatter gate to discriminate fluorescence signals coming from debris.

6. Click “Create Scatter Gate” and select “Draw New Gate”. The software will automatically open an FSC-BSC scatter plot from the Blank (unstained) control. The user will draw a gate using all the available plot tools, name it, and click “Apply”.
7. To use an existing gate, select the option “Use Existing Gate” and pick one from the “Select Gates” window.

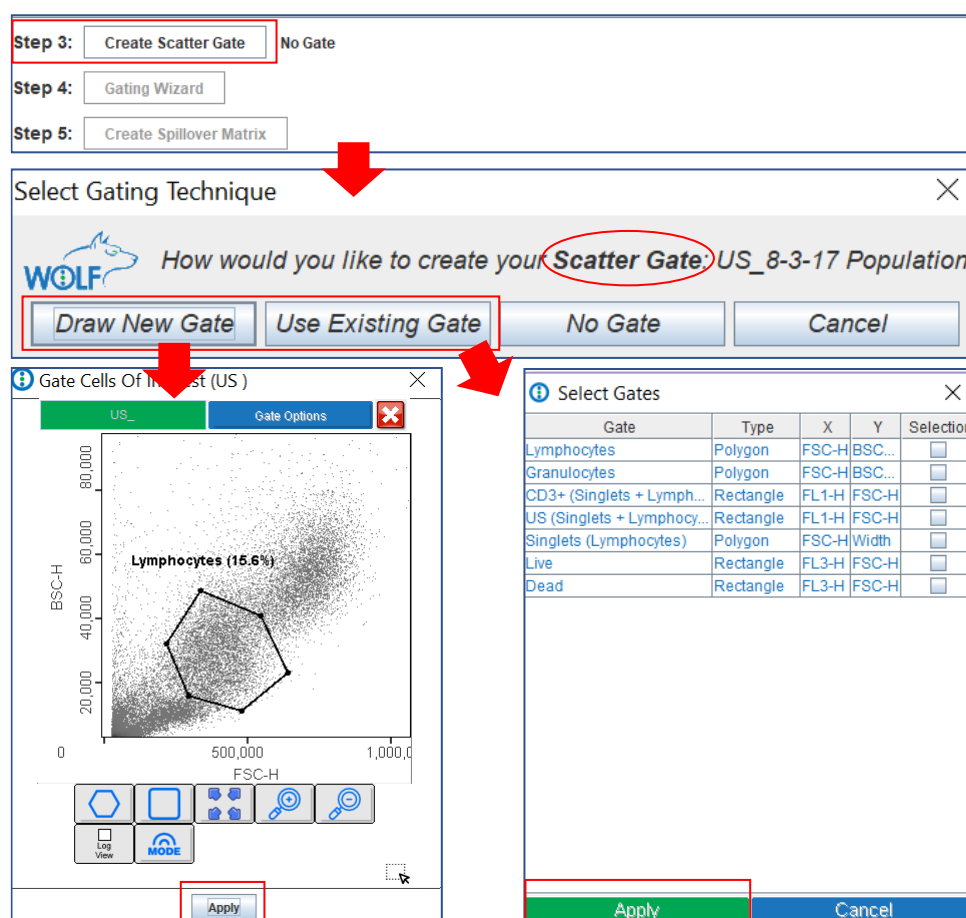


Figure 52. Applying a scatter gate to the Compensation Process.

4.5.2 Automatic Compensation (Continued)

The user can proceed to create gates in the fluorescence channels, taking into consideration that events gated should be within the dynamic range (do not gate events that are too close to the axes). If there is not a clear separation between positive and negative cells, the user should draw gates that are as far from each other as possible.

8. Click on “Gating Wizard”. The software will automatically open a plot with FSC vs FL1 on the axes and the scatter gate applied for the Unstained sample. The user can change plot representation and use all the available gate tools.
9. Once the Unstained control gate has been applied, the software will open plots to create gates for FL1, FL2, and FL3 channels consecutively.
10. New plots containing the gates generated with the wizard will automatically open when the process is completed.

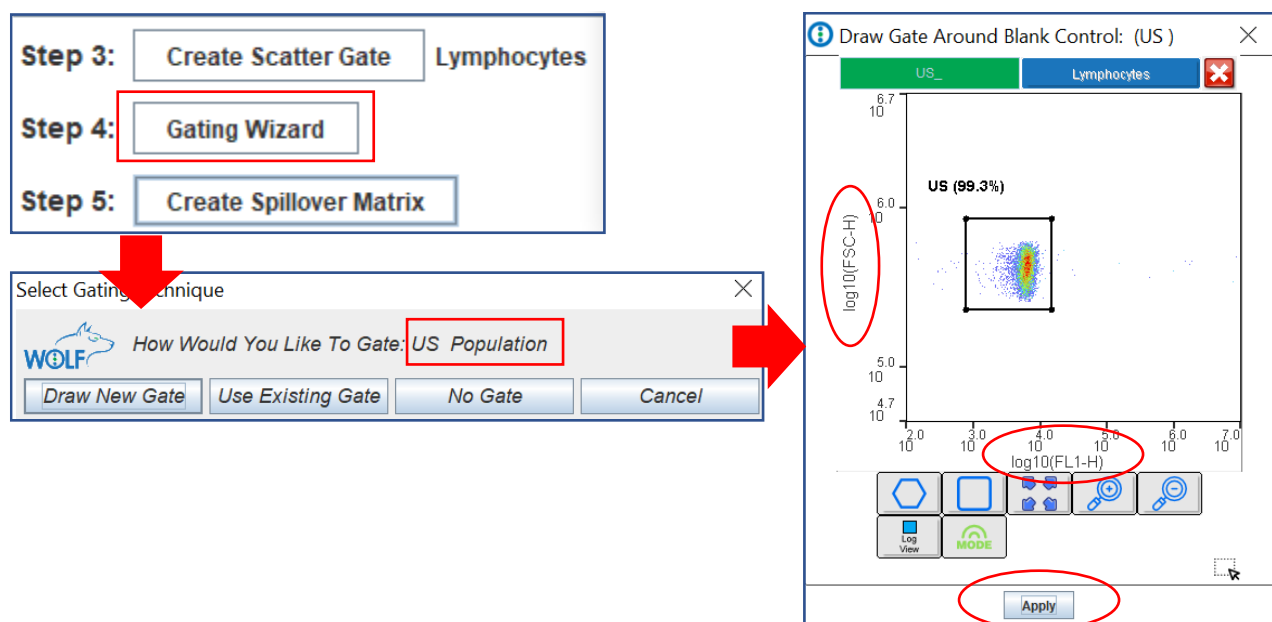


Figure 53. Gating fluorescence on Compensation Controls.

4.5.2 Automatic Compensation (Continued)

11. Click the “Create Spillover Matrix” button (Fig. 54A)
12. Save the Spillover Matrix (.cmat format) for later use.
13. The software will automatically display the Spillover Matrix (not a Compensation Matrix) with updated values for the Fluorescence Detectors (columns) versus the fluorochrome signal (rows) (Fig. 54B).
14. Click “Finished” to close the wizard.

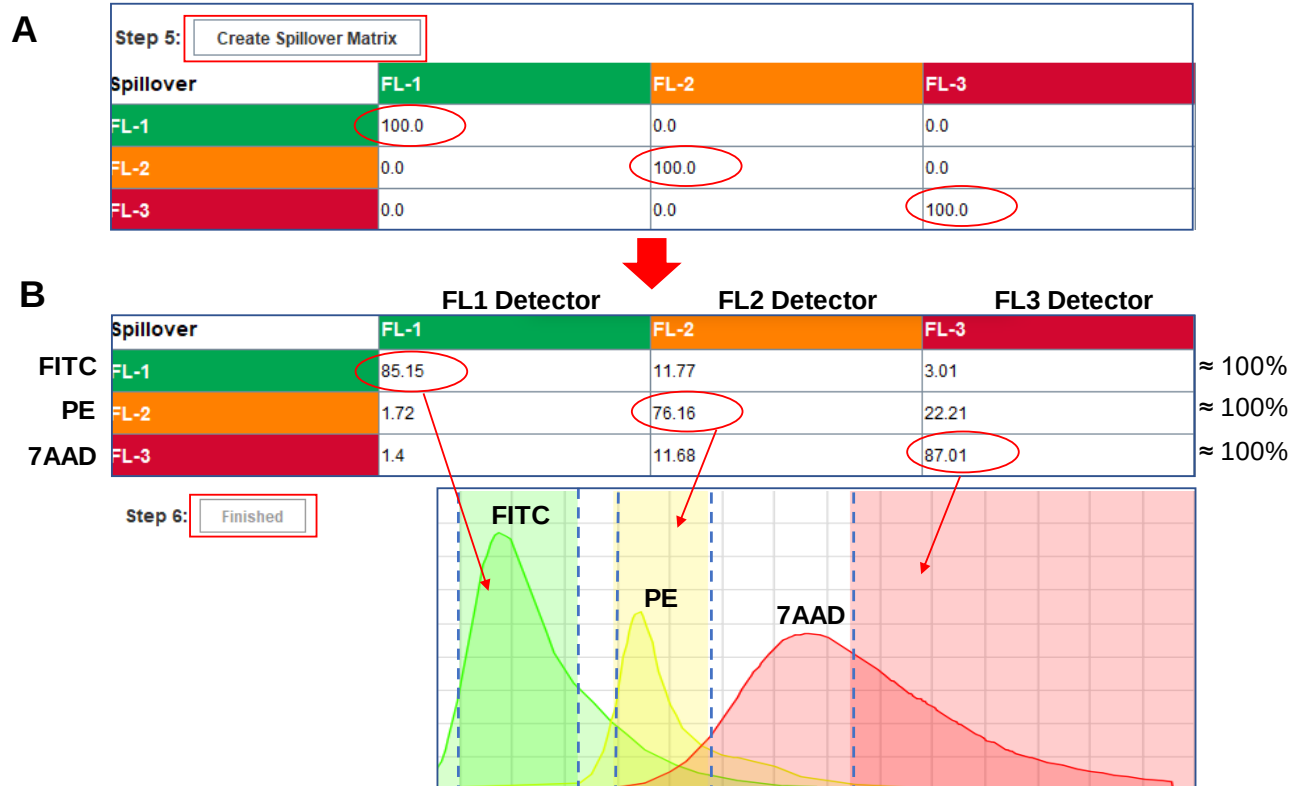


Figure 54. Creating a Spillover Matrix in WOLFViewer. A representation of the fluorescence spillover into the different detectors is shown in Panel B.

4.5.3 Manual Compensation

Manual compensation is the process of adjusting compensation based on how the data visually looks in the plots. WOLFViewer offers the option to adjust the uncompensated data by moving the sliders present in the 3 plots represented (FL1/FL2, FL1/FL3, FL2/FL3) in the wizard.

To Create a Compensation Matrix with Manual Compensation

1. Select the file to compensate by clicking in the Fcs Files Panel.
2. Select “Manual Compensation” from the Compensation tab in the main menu.
3. Adjust compensation values in the plots by moving the sliders or typing values in the boxes and clicking enter.
4. The user can reset the values and disable the plot preview by selecting the “Off” option.
5. Save the new compensation matrix by clicking “Save Compensation Matrix”.

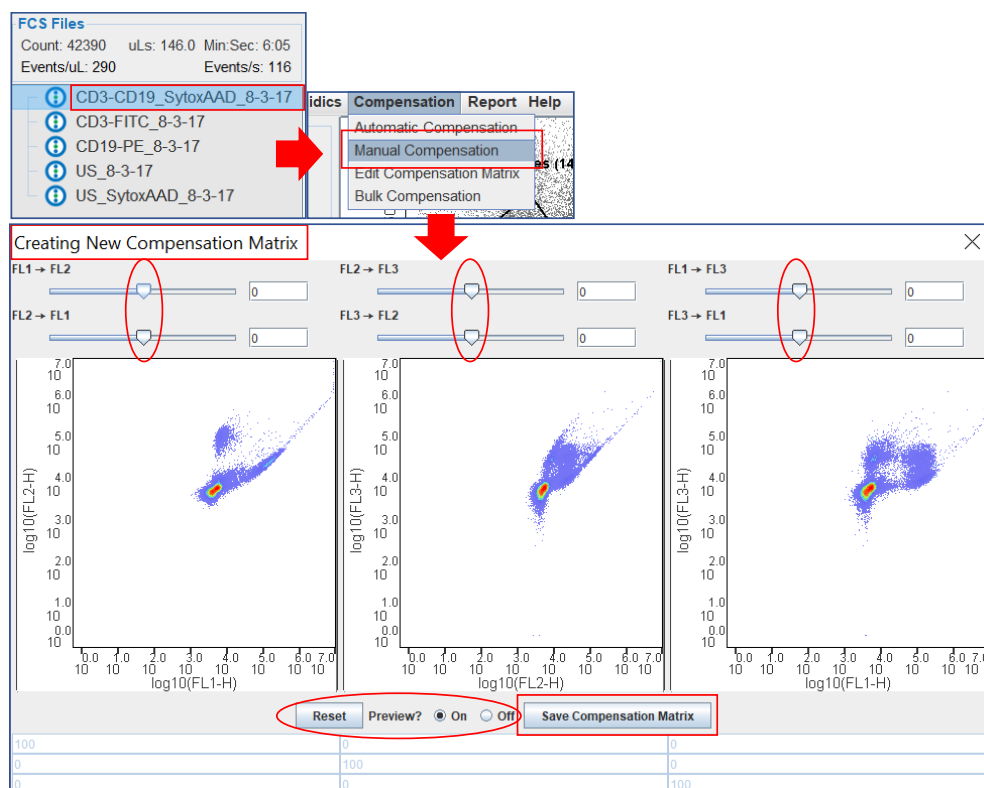


Figure 55. Manual Compensation Workflow

4.5.4 Editing an Existing Compensation Matrix

The user can modify an existing matrix to fit new experimental needs.

To Edit a Compensation Matrix

1. Load a .cmat file in the software by clicking the “Load Matrix” button.
2. Select the file to compensate by clicking on it within the Fcs Files Panel.
3. Select “Manual Compensation” from the Compensation tab in the main menu.
4. Adjust compensation values in the plots by moving the sliders or typing values in the boxes and clicking enter.
5. Save the new compensation matrix by clicking “Save Compensation Matrix”.

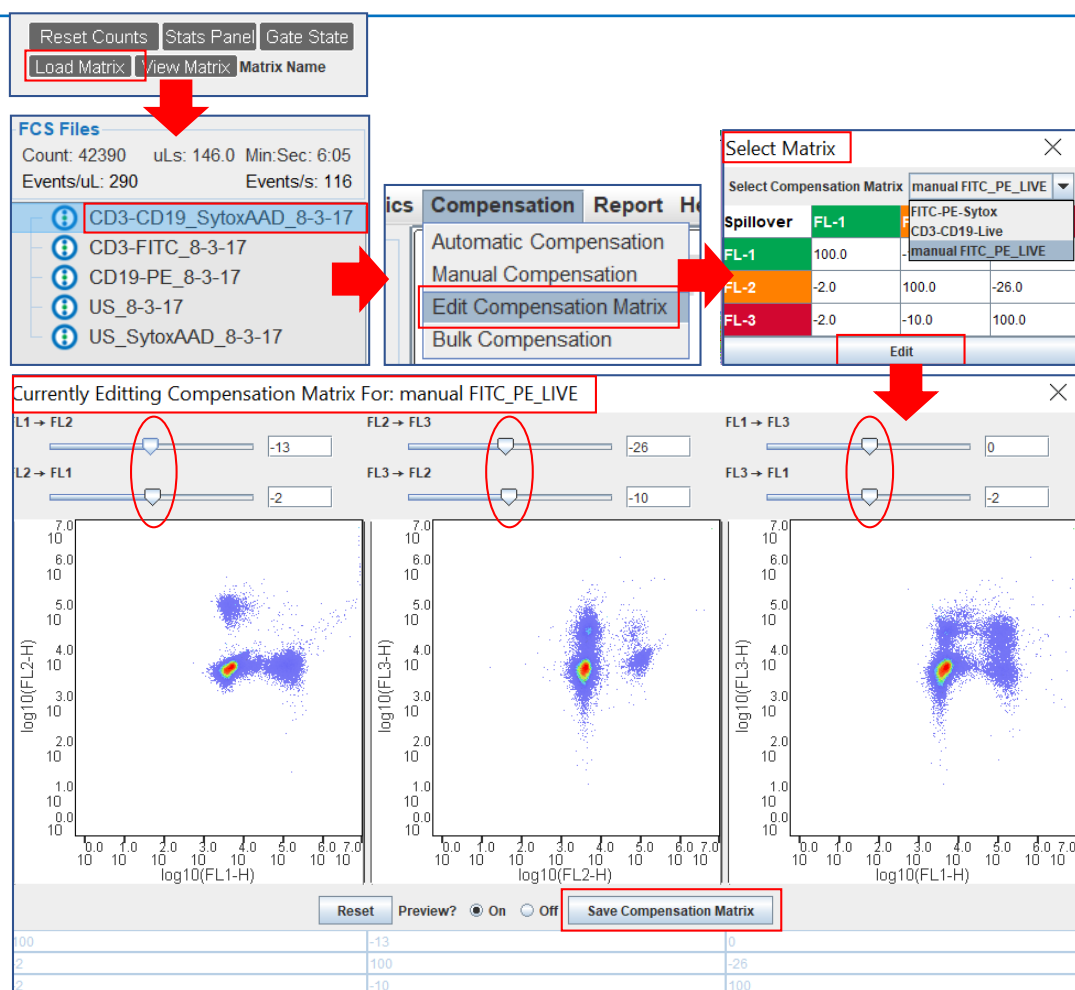


Figure 56. Editing a compensation Matrix

4.5.5 Applying a Compensation Matrix

Compensation matrices can be used to analyze existing sample files and to sort based on compensation.

To Apply a Compensation Matrix to Existing Files

1. Newly created matrices are automatically loaded in the software to be applied to samples.
2. When using a previously created matrix, load it by clicking “Load Matrix” (Fig. 57A).
3. Select the file of interest in the Fcs Files Panel.
4. Right-click and select “Apply Compensation”.
5. The Matrix Selection Tool will open for the user to pick the matrix of interest from the dropdown menu.
6. Click “Matrix For Compensation”.
7. The software will automatically create a compensated file in the Fcs Files panel. See Fig. 57B for a look at the same file before and after compensation has been applied.

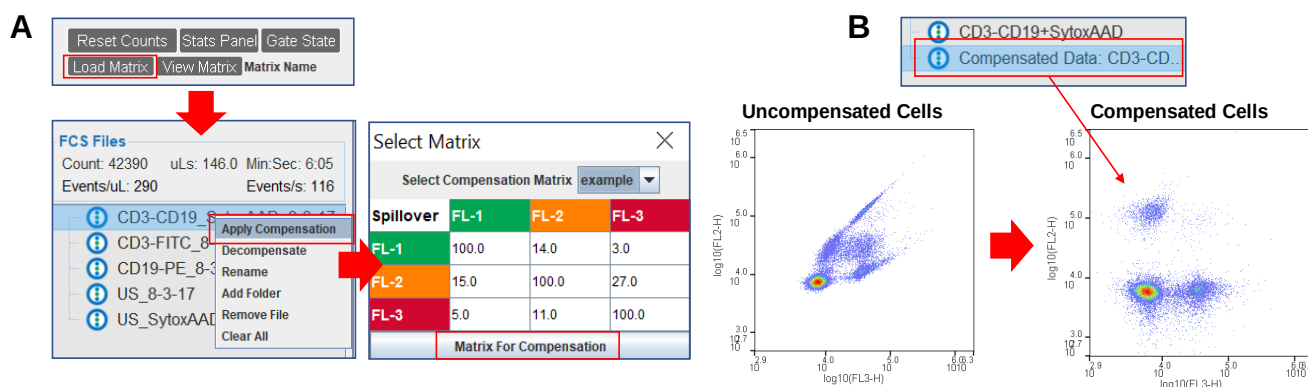


Figure 57. Applying a compensation matrix

4.5.5 Applying a Compensation Matrix (Continued)

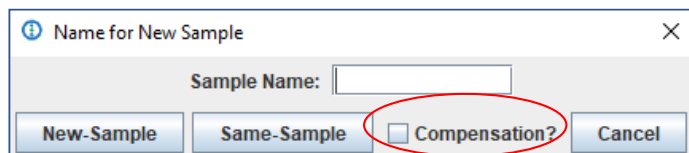


Figure 58. Applying a compensation matrix

To Analyze and Sort with Compensation

1. When starting sample analysis or sort, check the “Compensation?” box in the “Name for New Sample” window.

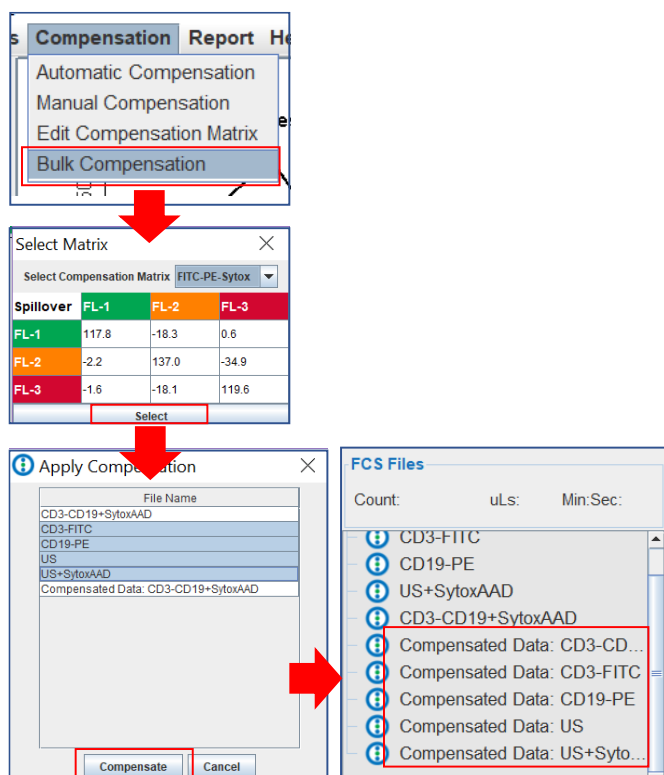


Figure 59. Compensating files in bulk

To Compensate Existing Files in Bulk

1. In the Software main menu, select “Bulk Compensation” from the “Compensation” tab.
2. Choose a matrix from the dropdown menu and click “Select”.
3. Select the files of interest and click “Compensate”.
4. The software will automatically generate compensated files in the Fcs File Panel.

5. Bulk Cell Sorting

The user can perform 1- and 2-way sorting when using “Sorting” cartridges.

To Initiate Bulk Sorting:

1. If desired, select time, volume, or trigger counts in the Collection Criteria Panel to automatically stop the sort (Fig. 60).
2. Select 1 or more gates to each sorting channel in the Controls Panel. Cells can be sorted to channel A or C. There are no efficiency differences between channels; the user can pick either of them when performing a 1-way sorting experiment.
3. When applying more than 1 gate for sorting in 1 channel, the user should keep in mind that only events present in both gates will be sorted.
4. Click the “Sort” button.
5. Exchange the waste container for the tube holder. It will take approximately 1 minute for the system dead volume to flow through the tubing and cartridge. Collection tubes may be added at this time (Fig. 60).
6. Click the “Pause” button to modify or change the gates used for sorting. Click “Resume” to continue sorting.
7. Click the “Stop” button to end the sorting process.
8. The number of events (cells) that have been triggered during sorting is displayed in the Sorting Controls Panel next to the gate selected for each channel.
9. Before removing the collection tubes from the holder, pay attention into which tube each population is being sorted. Cells sorted to the A-channel are collected in the tube that is Away of the instrument. Cells sorted to the C-channel are sorted in the tube that is Closer to the instrument. Unsorted cells are collected in the central tube (B) and can be used for other experimental needs (Fig. 61).

Bulk Cell Sorting (Continued)

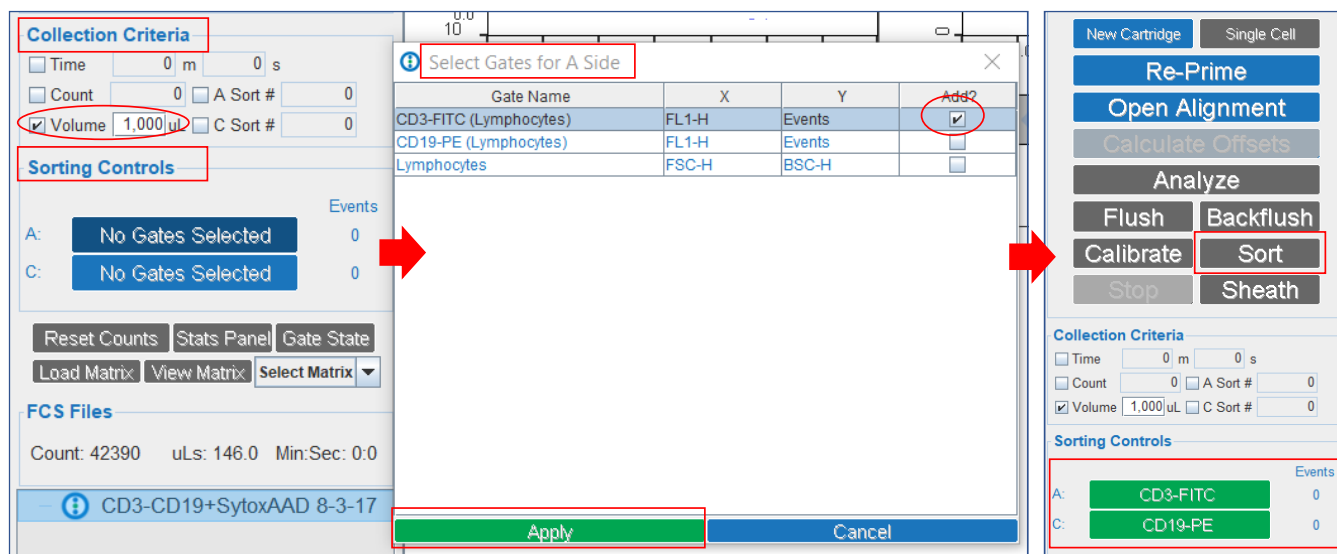


Figure 60. Bulk Sorting Process



Figure 61. Collection Tube Holder

6. Single Cell Sorting

The WOLF[®] Cell Sorter, in combination with the N1 Single Cell Module, has the capability to dispense single cells into 96- and 384-well plates for clone generation and single cell analysis.

6.1. Single Cell Module Set-Up

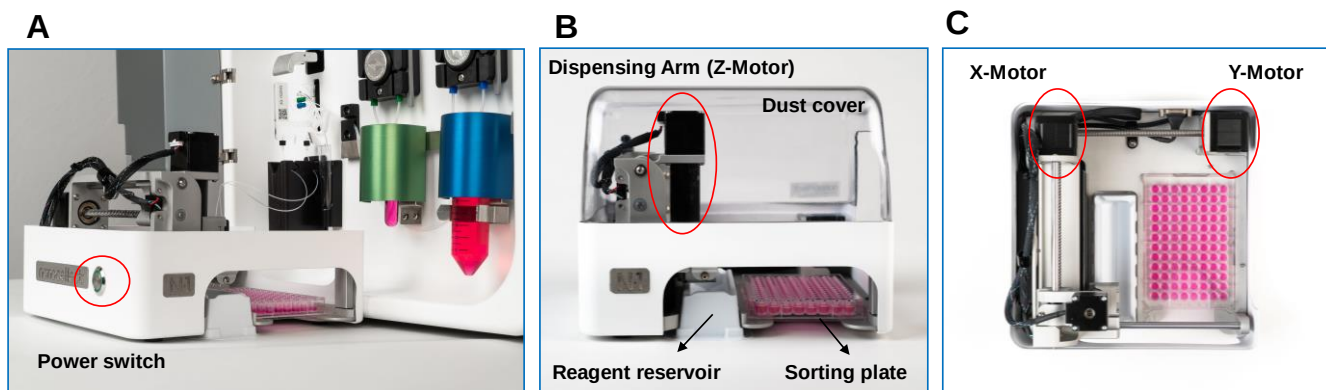


Figure 62. The N1 Single Cell Module Set-Up

To Set Up the Single Cell Sorting Module:

1. Connect the N1 to the back of the WOLF[®] and power it on. The module power-button should be facing away from the WOLF[®], as shown in the image above (Fig. 62A)
2. Insert the waste tray (reagent reservoir) in the module (Fig. 62B).
3. Attach the waste container to the magnetic tray receptacle on the WOLF[®] front panel (Fig. 63B).

6.2. Single Cell Cartridge Tubing Assembly

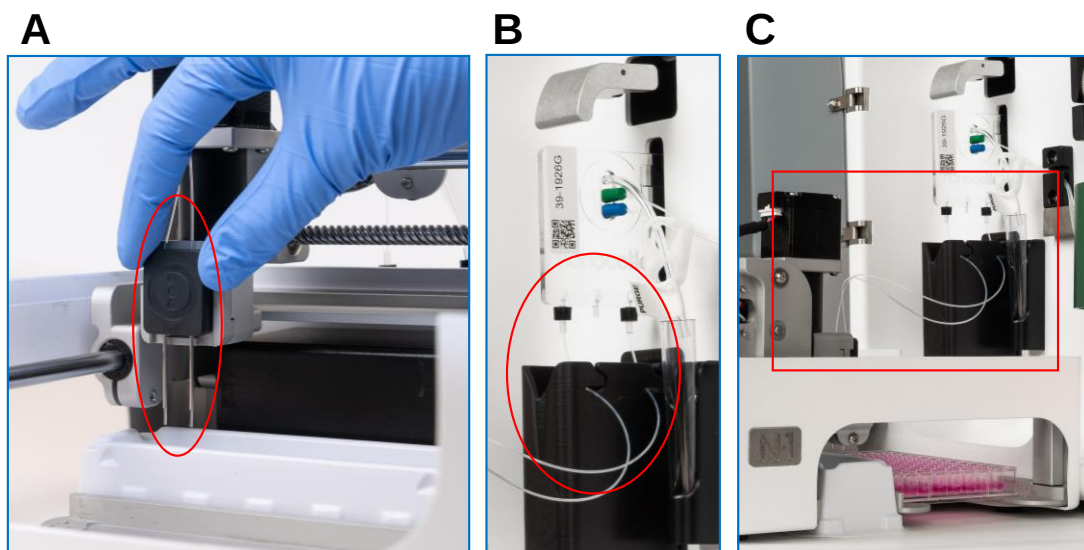


Figure 63. Cartridge Assembly in the N1 Single Cell Module

To Set Up the Single Cell Sorting Cartridge:

1. Open the cartridge pouch and unwrap the tubing.
2. Attach the dispensing needles to the magnetic receptacle on the N1 dispensing arm (Fig. 63A).
3. Insert the cartridge in the instrument cartridge fixture. Route the output tubing through the grooves in the waste container (Fig. 63B).
4. Connect the sample and sheath tubing to the peristaltic pumps and position the purge tubing into the priming vial (Fig. 63C).
5. After closing the purge valve in during the Priming step, position the N1 Dust Cover in the module to prevent plate contamination (Fig. 62B).

6.3 Single Cell Sorting

To Initiate Single Cell Sorting:

1. Scan the cartridge QR code and select “Single Cell” in the cartridge pop-up.
2. A window with a plate map will automatically open for plate type selection.
3. Select the plate type from the dropdown menu. 96- and 384-flat bottom plates are default for sorting (Fig 64A).
4. Type the plate pre-fill volume.
5. Enter the number of cells/well. Keep in mind that the system is optimized to sort 1 cell/well. The accuracy for dispensing numbers above 1 cell/well may be lower.
6. Cells can be deposited in the last 2 wells for focusing purposes if a plate imager is used downstream. Select the “Deposit cells in focusing wells” checkbox to use this function. The cells will be deposited in wells H1 and H2 will in a 96-well plate, and P1 and P3 in a 384-well plate (Fig. 64B).
7. Select an even number of columns for sorting. For the entire plate, click A1 and H12 (P24 for 384 plates). It is possible to select different sections of a plate in 2-well (for 96-well) or 4-well (384-well increments).
8. **Prepare the sample at a 100,000 events/ μ l concentration to ensure high single cell dispensing efficiency.**
9. Pass your cells through a 37-40 μ m cell strainer and proceed to analyze your controls and sample.
10. Select the gate for sorting in the Sorting Controls Panel. Remember that the N1 performs only 1-way sorting (Fig. 64C).
11. Click the “Sort” button.
12. Load plate in the module positioning the A1 well by the A1 symbol engraved on the module. Push the plate all the way down until it feels seated and secured (Fig. 64D).

6.3 Single Cell Sorting (Continued)

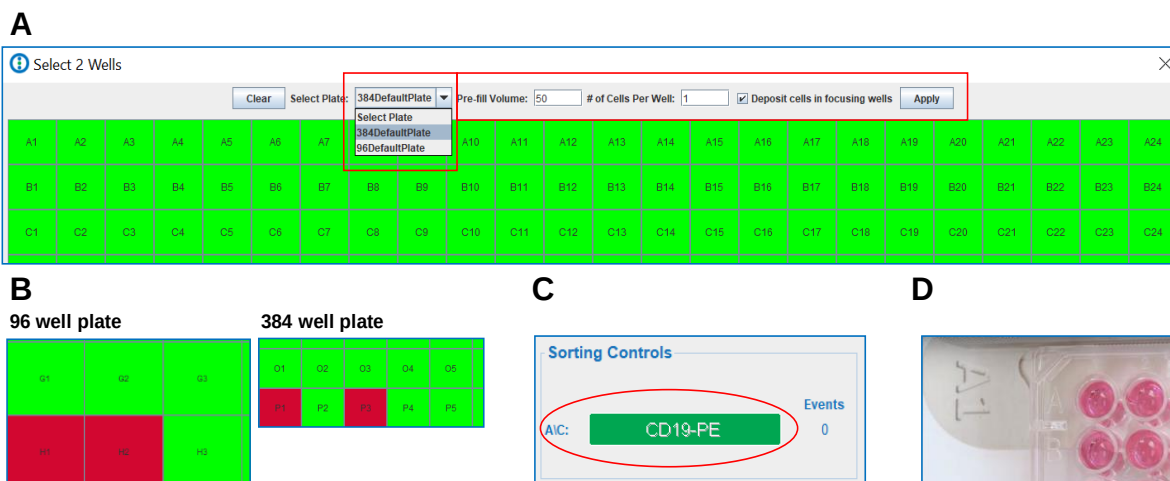


Figure 64. Steps in the Single Cell Sorting Process

6.4 Single Cell Features

6.4.1 Single Cell Plate Time Calculator

The Plate Time Calculator allows the user to estimate the plating time for a given sample.

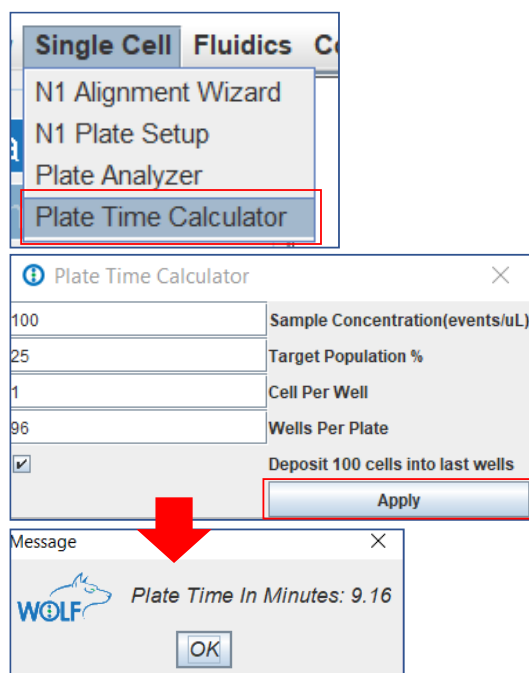


Figure 65. Applying a compensation matrix

To Calculate Plating Time

1. Select "Plate Time Calculator" in the main menu View tab.
2. Input the information on cell concentration, % of target population, cells per well and type of plate.
3. The software will automatically calculate the time if a gate for sorting has already been selected.

6.4.2 Single Cell Data Indexing

The Index Sorting file (.csv) stores the XY coordinates of the cells sorted in the plate. The information can be of help to relate markers of a specific cell with its phenotype or gene expression profile.

The information stored in the file includes:

- Well ID
- Cell Fluorescence Intensity in the 3 channels
- Forward and Backscatter values
- Forward Scatter-width values
- Channel used for sorting
- Drop volume
- Time the dispensing needles spent in the well.
- Cell number

Once the plate sorting has finalized, a pop-up window will prompt the user to save the Index Sorting File.

6.5 N1 Module Alignment

The Alignment Wizard is used the first time a N1 Single Cell module is connected to a WOLF® Cell Sorter, and after extensive usage or transportation. The wizard will allow the user to set the module baseline specifications to sort into 96- and 384-well plates, which will be used to create other plate templates.

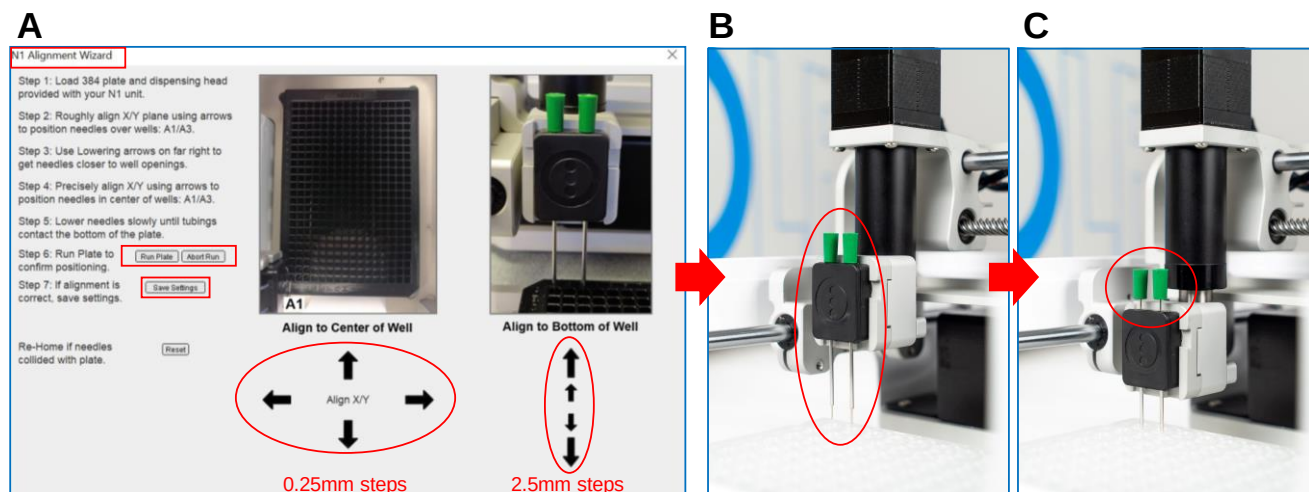


Figure 66. Steps in the Single Cell Sorting Process

To Align the N1 Single Cell Module:

1. Select “N1 Alignment Wizard” from the Single Cell main menu tab.
2. The dispensing arm will home and move to the last A1/A3 well position where the module was aligned.
3. Load the 384-well, flat bottom plate provided in the installation kit. This plate serves as a universal alignment tool for most commonly used 96- and 384-well flat bottom plates.
4. Place the N1 Alignment Tool provided in the installation kit on the N1 dispensing arm (Fig. 66B).
5. Align the needles in the X/Y plane to the A1/A3 wells on the plate using the arrows under “Align to Center of Well” (Fig. 66C).

6.5 N1 Module Alignment (Continued)

To Align the N1 Single Cell Module (Continued):

6. Use arrows under “Align to bottom of wells” to get needles closer to the well for easier alignment (Fig. 66A).
7. Once the needles are precisely positioned at the center of the wells, begin the z-plane alignment (Fig. 66B).
8. Move the needles down clicking the big arrows (2.5 mm steps). Once the tubing that comes out of the needles is close to the bottom, click the small arrows (0.25 mm steps) until it contacts the bottom of the plate **and the bumpers move up slightly** (Fig. 66C).
9. Click “Run Plate” to confirm proper positioning.
10. Click “Abort Run” if the needles collide with the plate. Re-start alignment by clicking “Re-Home.”

6.5 N1 Plate Set Up

The N1 Plate Setup allows the user to create templates based on different plates specifications (dimensions).

To Create a New Plate Template:

1. Select “N1 Plate Setup” from the Single Cell main menu tab (Fig. 67).
2. The dispensing arm will automatically move to the center of wells A1/A2 (96-well) or A1/A3 (384-well).
3. Load the plate of interest for the alignment.
4. Place the magnetic N1 Dispensing Head provided in the installation kit on the N1 dispensing arm.
5. Click “New Plate” from the dropdown menu (Fig. 67).

6.5 N1 Plate Set Up (Continued)

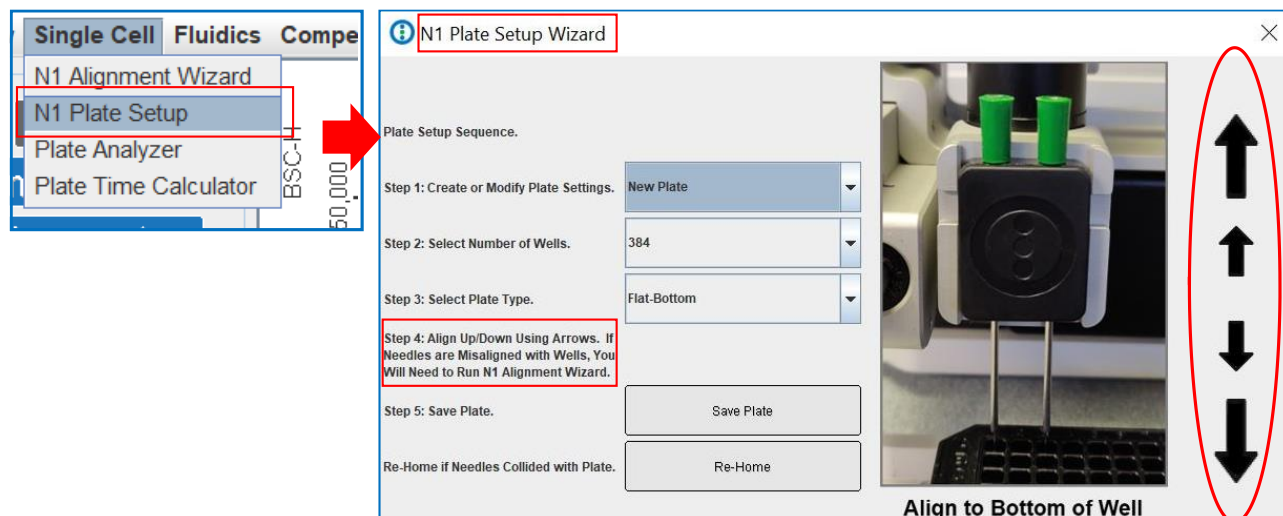


Figure 67. The N1 Plate Set Up Wizard

6. Select the number of wells from the dropdown menu.
Select "Flat Bottom" from the plate type dropdown menu.
7. Move the needles down by clicking the big arrows (2.5 mm steps). Once the tubing that comes out of the needles is close to the bottom, click the small arrows (0.25 mm steps) until it contacts the bottom of the plate and the bumpers slightly move up.
8. Save the plate specifications by clicking the "Save Plate" button. We recommend naming the files with well and catalog numbers for easier future reference (i.e. #96-PCR #CLS6551). The new template will appear in the Single Cell Selection main menu.
9. To change an existing plate type specification, pick the plate type to change from the dropdown menu in Step 1 of the Wizard. It will query all available plate specifications that have been created.

NOTE: We recommend using skirted plates to achieve reliable alignments.

6.6 Single Cell Plate Analyzer (Post-Sort Analysis)

WOLFViewer's Plate Analyzer tool loads the entire plate allowing the user to visualize the data that is captured for each individual cell sorted into the plate.

The Heat Map View can be used to identify the brightest cells sorted into the plate. The fluorescence intensity from each cell is mapped to a color, and a scale bar shows the specific intensity values that correspond to the brightest and dimmest cells in the plate.

NOTE: Cells sorted based only on scatter values cannot be analyzed with this tool.

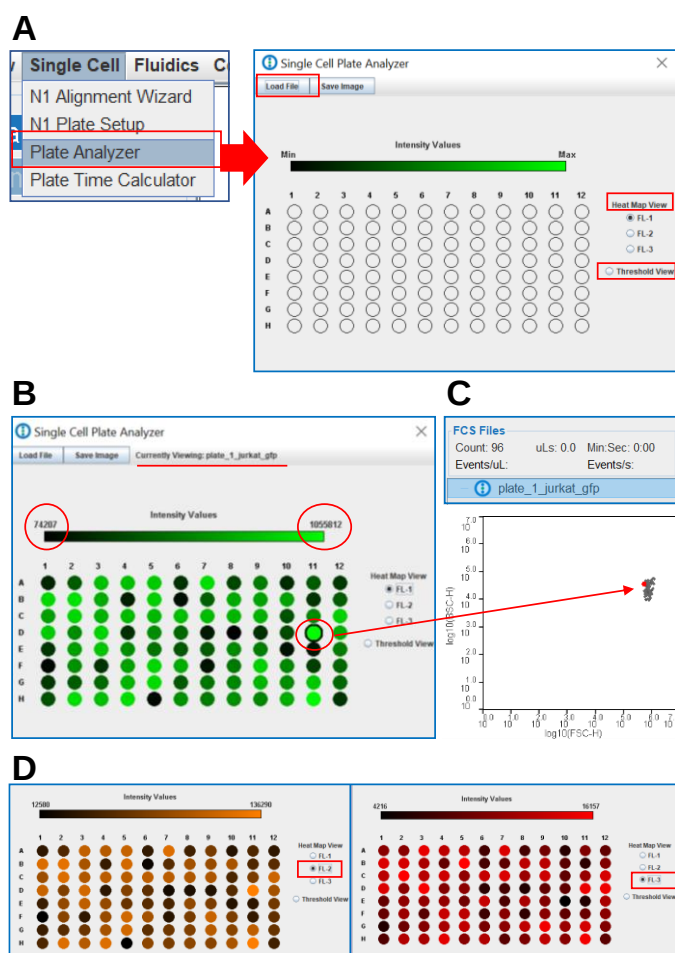


Figure 68. The Plate Analyzer Heat Map View

To Use the Heat Map View

1. Click the "Single Cell" tab from the main menu to select "Plate Analyzer".
2. Click "Load File" and select a csv file from a single cell sort (Fig. 68A).
3. The minimum and maximum intensity values in the heat map correspond to the ones in the gate selected for sorting.
4. An .fcs file containing the information from the events sorted into the plate will be automatically created.
5. Click on any of the wells to see the event displayed in red on any of the plots opened for analysis (Fig. 68B and 68C).
6. The user can see the map in the FL2 and FL3 channels (Fig. 68D).

6.6 Single Cell Plate Analyzer (Post-Sort Analysis) (Continued)

The Threshold View can be used to characterize the profile of the cells at the time of sorting. This data can be useful for determining correlation with another data set, for example genomic analysis or expression and viability data obtained at a later time point.

In this view, a well in the grid will only be displayed as a solid circle if it contains a cell with fluorescence and scatter intensities all exceeding the “threshold” values in the text boxes.

The threshold boundaries in the sliders are set based on the gate used for sorting.

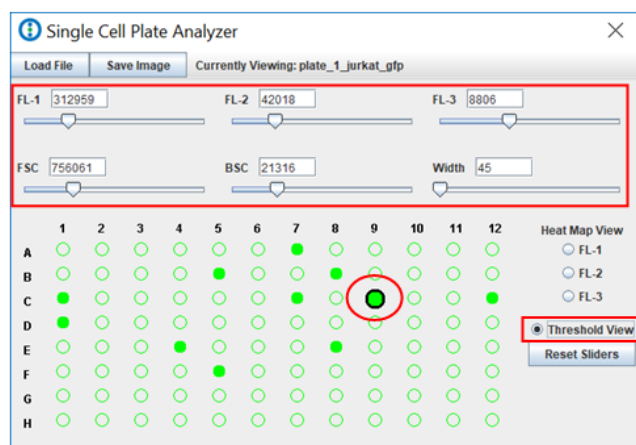


Figure 69. The Plate Analyzer Threshold View

To Use the Heat Map View

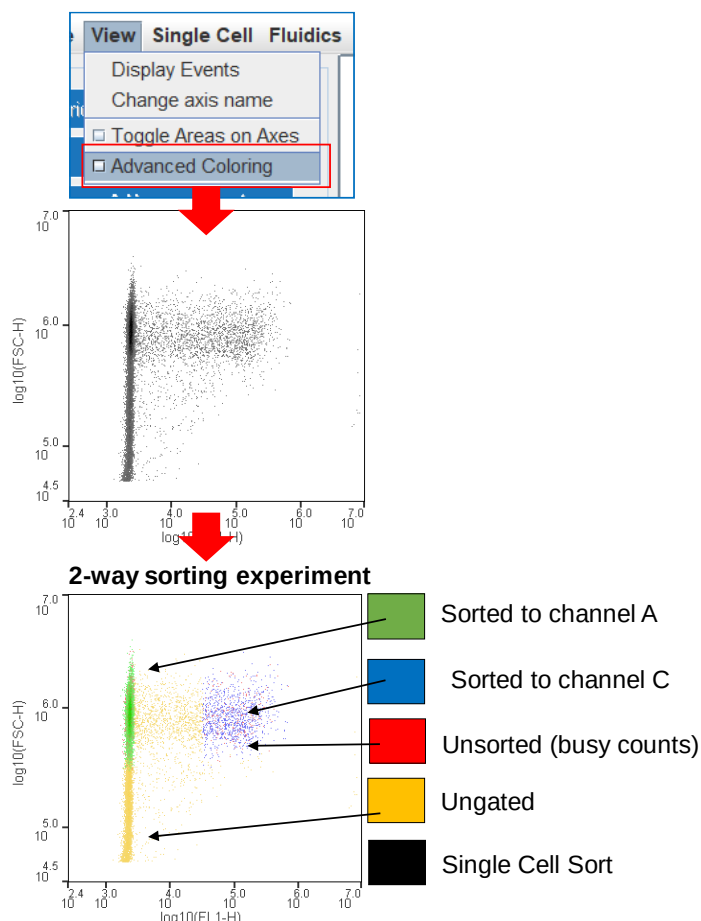
1. Check the Threshold View.
2. Adjust thresholds individually moving the sliders or by manually inputting a number (Fig. 69).
3. Reset Values and select a well to see an event on the plots (Fig. 69).

6.7 The Advanced Coloring Feature (Post-Sort Analysis)

The WOLF® Cell Sorter has a sorting speed of 300 cells/second for 1-way sorting and 150 cells/second for 2-way sorting. Some cells may not be sorted when processing very concentrated samples with high target populations.

The Advanced Coloring Feature color-codes the populations in a plot from a file generated during a sorting experiment. It is designed to monitor trigger counts and highlight the cells that are not sorted due to a high event rate (Fig. 70). It indicates which cells are sorted to A or C-channel in a 2-way experiment. It can also be used as a permanent record of the gates used for sorting if a template was not saved.

6.7 The Advanced Coloring Feature (Post-Sort Analysis) (Continued)



To Use the Advanced Coloring Tool:

1. Go to the "View" tab in the main menu and select the "Advanced Coloring" check box.
2. Different colors display events gated and sorted for a given experiment (Fig. 70).
3. For Single Cell Experiments where the same population is sorted to A and C-Channels, the sorted events are all labeled black.

Figure 70. Post-sorting analysis with the Advanced Coloring Feature

NOTE: To minimize the number of unsorted cells that are lost due to busy counts, the user should dilute the sample to decrease cell concentration .

6.8 The Clog/Leak and Sample Settling Detection Features

The user can actively check the sorting junction for clogs anytime using the “Scan for Clogs” software option. In addition, WOLFViewer has the capability to warn the user of a potential clog or leak in the chip, or cells settling down in the tube while sorting.

6.8.1 Scan For Clogs Feature

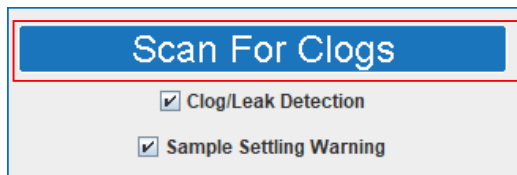


Figure 71. The Clog Detection Panel

To Scan the Sorting Junction for Clogs:

1. Click “Scan for Clogs” in the Clog Detection Panel.
2. Actively scan the chip sorting junction area while the LED light is on.

6.8.2 Sample Settling Warning Feature

This feature detects if there is a decrease in the overall event rate detected by the laser over time. It advises the customer to check if there is still enough volume in the sample tube and resuspend the cells by mixing or pipetting.

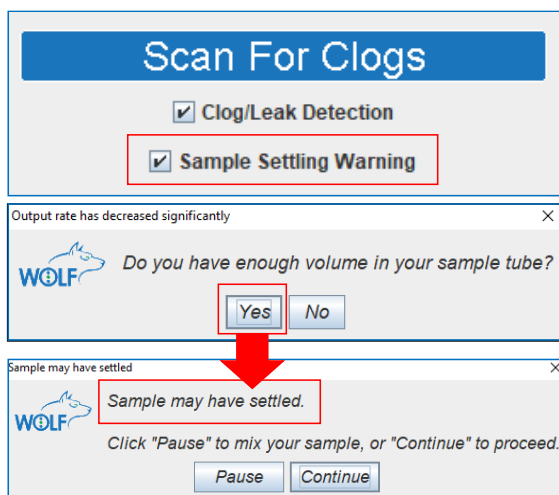


Figure 72. Sample Settling warnings

To Detect Sample Settling:

1. The software will automatically warn the user if the event rate at detection has decreased over time.
2. The user may check first if there is still enough volume in the sample tube and proceed to mix the sample if needed.
3. To deactivate the function, unselect the checkbox in the Clog Detection Panel.

6.8.3 Chip Clog and Leak Detection

The main challenge of using microfluidic systems is clogging of channels, which leads to chip leaking. The Clog and Leak Detection Feature uses the optical sorting verification system to detect a decrease in the event rate in the channels used for sorting.

NOTE: False positives may arise if the cells sorted are smaller than 8 μ m, as detection of small particles by the sorting verification system is not accurate.

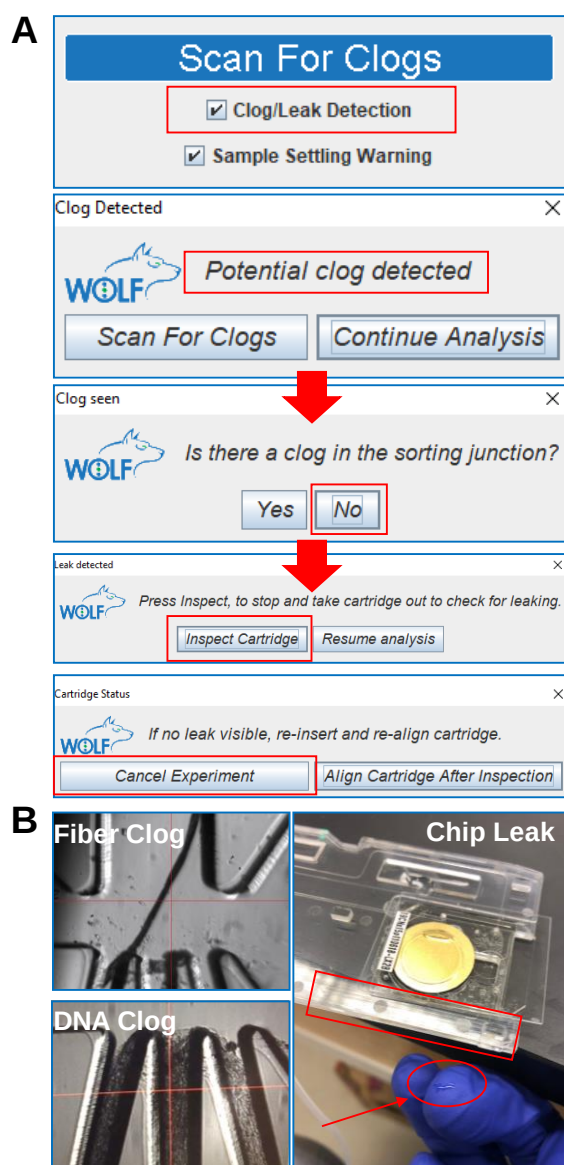


Figure 73. The Clog and Leak Detection System

To Check for Clogs and Leaks:

1. A pop-up window will warn the user of the presence of a clog. The user must scan the sorting junction at this time (Fig. 73A).
2. If a clog is seen in the junction, the user should replace the cartridge.
3. Fiber clogs can be prevented by filtering all buffers used for priming and sorting and wiping the front panel. DNA clogs are due to cell viability. Sample preparation should be revisited.
4. If clogs are not visible, the user must check the chip for potential leaks.
5. Gently take the cartridge out. Slide your finger over the cartridge edge to check for wetness (Fig. 73B).
6. If a leak is present, the user must remove the cartridge and clean the cartridge fixture following the protocol in section 6.8.4.

6.8.4 Cartridge Fixture Cleaning Protocol

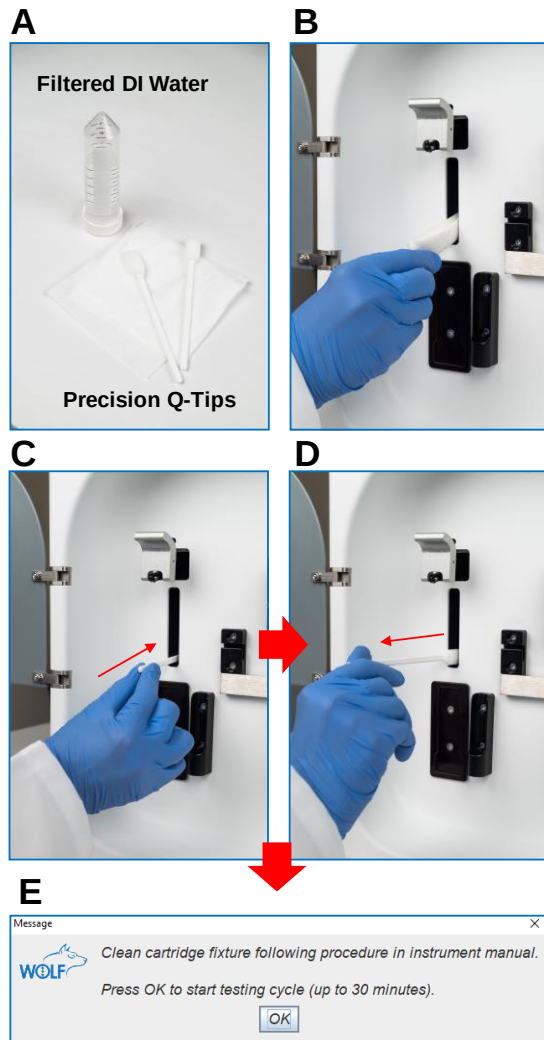


Figure 74. The Clog and Leak Detection System

To Clean the Instrument After a Leak:

1. Insert a lint-free wipe in the cartridge fixture to clean for buffer residues.
2. Clean the calibration detectors using a precision swab wetted with filtered, distilled water (Fig. 74A).
3. Insert the tip and rub gently the bottom left and right fixture walls. It is critical that all the sorting buffer is cleaned from the detectors to avoid salt precipitates that could lead to errors in detection (Fig. 74B-D).
4. Rinse the swab with water and dry it, damping on lint-free wipes.
5. Dry the detectors by rubbing gently with the swab.
6. Once the cartridge fixture is clean, the user can start a Testing Cycle. A system check will run every 3 minutes and advise the user when the system is ready (Fig. 74E).
7. If the cartridge fixture is not ready after 30 minutes, contact NanoCelect's Technical Support Department.

7. Shutting Down the WOLF[®] Cell Sorter

Once the sorting experiment has been completed, the user can save the collection tubes or plates sorted and dispose of the cartridge.

7.1 Saving Files and Templates

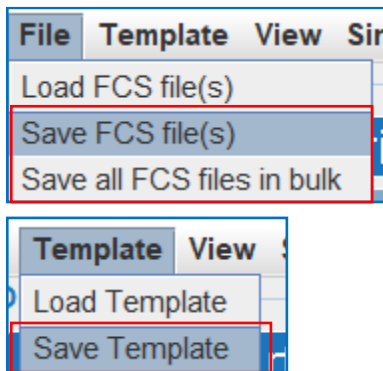


Figure 75. Saving Files and Templates

To Save Files and Templates

1. Go the “File” tab in the main menu to save the analysis and sorting files. Files can be saved in bulk if needed.
2. Go to the “Template” tab to save the experiment template. Templates contain the gate, zooming and plot coloring information used.

7.1.1 Saving Sample Experimental Reports

The following information is saved in a PDF Report:

- Graphs with the gates created during the analysis.
- Sorting Parameters.
- Statistic Panel.
- Gate State.
- Plate Analyzer displays.
- Compensation Matrix used.

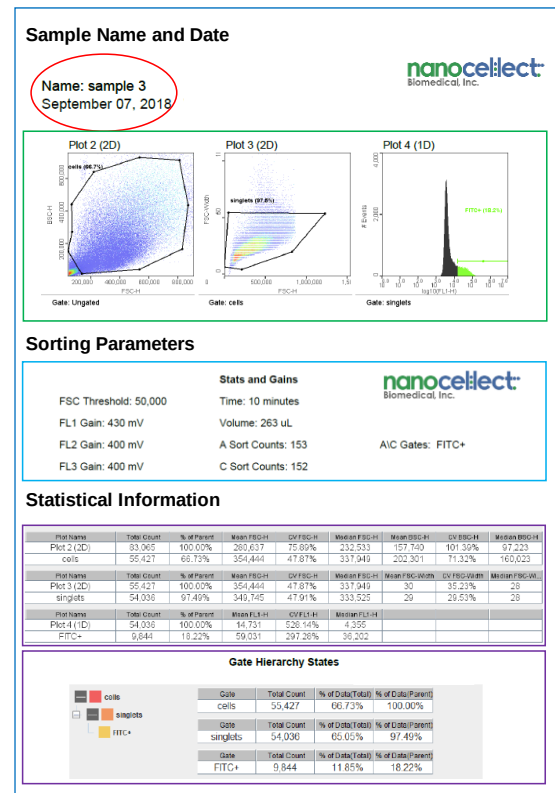


Figure 76. Example of a WOLFViewer PDF Report

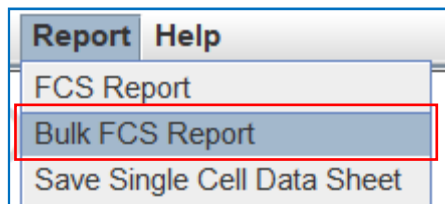


Figure 77. Saving Sample PDF Reports

To Save Experiment PDF Reports:

1. Select the file of interest in the FCS Panel.
2. Go to the "Report" tab in the main menu and save the report. Reports can be saved in bulk if needed.

7.2 Removing a Cartridge

To Remove the Cartridge:

1. Stop the Sheath fluid pump by clicking the “Finish” button. Remove your sample and sheath vials from the WOLF® front panel.
2. Remove the waste container or the collection tube holder from the WOLF® front panel.
3. Open both peristaltic pump lock tabs and remove the tubing from the sheath fluid and sample tubes.
4. For Single Cell cartridges, detach the needle head from the dispensing arm and set it aside on a paper towel.
5. Remove the output tubing from the waste container grooves.
6. Lift the cartridge latch and gently push the cartridge slightly to the left (Fig. 78A).
7. Pull the cartridge to remove it from the instrument while holding the latch up (Fig. 78B and 78C).
8. Dispose of the cartridge set in accordance with biohazard disposal best practices.
9. Treat the waste container with 0.5% bleach for decontamination. Do not autoclave it.
10. Remove the N1 Reagent Reservoir and dispose of it in accordance with biohazard disposal best practices

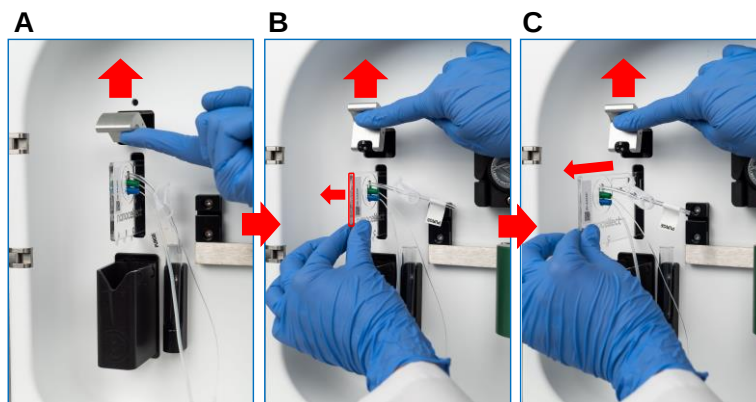


Figure 78. Cartridge Removal

7.3 Turning the WOLF® Off



Figure 79. Turning the WOLF® Off

To Turn the WOLF® and N1 Module Off:

1. Turn WOLF® Cell Sorter off by depressing the power button.
2. Turn the N1 Single Cell Module off by depressing the power button (Fig. 79).
3. Close WOLFViewer Software.
4. Restart computer.

7.4 Moving the WOLF®

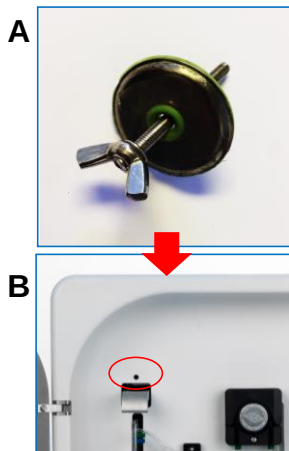


Figure 80. Using the Moving Lock

To Move the WOLF®:

1. Open WOLFViewer and click “Align” to allow the motors to home the cartridge fixture.
2. Using the latch, gently move the fixture to align the front panel with the fixture holes.
3. Insert the moving lock (Fig. 80A) in the front panel hole above the cartridge latch (Fig. 80B).
4. Turn the screw until the cartridge fixture feels secured to prevent damage of internal optics components during movement.

8. WOLF® Cell Sorter Best Practices

8.1. Selecting Fluorochromes for the Experiment

Selecting the right staining for your cells is critical for optical detection and sorting. Apart from back (BSC, granularity) and forward (FSC, size) scatter, the WOLF® has 3 fluorescence detection channels, FL1, FL2 and FL3 (Fig. 81).

nanocelllect Biomedical, Inc.		Conjugated Fluorophores	Viability Dyes	Fluorescent Proteins	Tracking Dyes
Excitation: 488nm Laser	Channel 1 (500-550)	- FITC - Alexa Fluor 488 - DyeLight 488 - Qdot 525 nanocrystal	- Calcein - Sytox Green - LIVE/DEAD® Green stain	- GFP - YFP	CellTracker™ Green
	Channel 2 (565-605)	- PE - Alexa Fluor 546 - Qdot 565 and 585 nanocrystals	PI	CyOFP1	
	Channel 3 (665+)	- PE-Cy5 - PerCP/Cy5.5 - PE-Cy7	- PI 7AAD - Sytox AADvanced - LIVE/DEAD® Red stain	RFP (10% excitation)	CellTracker™ Red

Figure 81. Recommended Fluorophores for the WOLF® Cell Sorter Configuration

To Optimize Fluorochrome Choice:

1. Select the brightest fluorochromes for your experiments to ensure positive signal detection.
2. Choose fluorochromes with minimal spectral overlap.
3. Compensation may be needed to correct overlapping emission spectra. Refer to [Section 4.5 Compensation](#) of this manual for more information.
4. Use the brightest fluorochromes for the dimmest populations and vice versa.

For example, if staining white blood cells for lymphocyte sorting, use CD3-FITC (high antigen expression) and CD19-PE (medium antigen expression).

8.2. Sample Preparation

Good sample preparation is essential to the success of a sorting experiment. We encourage optimizing sample preparation before sorting to determine if it is necessary to improve the conditions for:

- Obtaining a single cell suspension
- Preserving viability
- Cell staining

To Reduce Cell Aggregation and Increase Cell Viability:

1. Use a gentle cell dissociation reagent for cell detachment to achieve single cell suspensions. Trypsin can be used for more adherent cells but may remove membrane receptors. Accutase™, TrypLE and EDTA are all used as gentle dissociation reagents for sensitive cells.
 - a. Use EDTA if cells tend to clump.
 - b. Use DNase I if cells are clumping due to cell death. Note that EDTA should not be used with DNase I as DNase I is Mg²⁺ dependent.
2. Consider keeping cells and reagents at 4°C, or on wet-ice, throughout the preparation and sorting process.
3. Avoid excessive centrifugation and washing to minimize cell loss.
4. Use the minimum incubation times to shorten the process.
5. Use of Ca²⁺ and Mg²⁺ free PBS with BSA or FBS to suspend cells as it may reduce clumping.
6. Use culture medium as sheath fluid to preserve viability.
7. Consider using HEPES in sample buffer or sheath fluid to prevent pH changes and preserve cell viability.

8.2. Sample Preparation (Continued)

To Reduce Cell Aggregation and Increase Cell Viability (Continued):

10. Always filter samples with a 40 µm mesh immediately before sorting to minimize cartridge clogging.
11. Consider restraining cell samples multiple times over the course of a long sorting day.
12. Use round-bottom 1.5 mL or 5 mL polypropylene tubes to prepare and collect samples. Polypropylene is less adherent than polystyrene and reduces cell loss.

8.2.1 Saving Sample Experimental Reports

Suggested sample and sheath fluid buffers:

- HBSS sorting buffer: HBSS
 1-5% FBS (heat inactivated)
 1mM EDTA
- PBS sorting buffer: PBS (Ca²⁺ and Mg²⁺ Free)
 1-5% FBS (heat inactivated)
 1mM EDTA
 25mM HEPES pH 7.0
- Sorting media: Cell culture media
 1-5% FBS (heat inactivated)
 1mM EDTA

8.2.2 Number of Cells to Prepare

The **number of cells** to be prepared for a sorting experiment depends upon how many cells are needed for a given application. Because sorting is not a perfectly efficient process, we recommend determining the number of cells required and then doubling that number, to be conservative, until optimization is performed.

For example, if the target GFP-positive population is 10%, and 5×10^4 cells are needed, a minimum of 1×10^5 cells would be required. Preparing 1×10^6 will provide enough cells for analysis, setting of gates, and accommodate various inefficiencies in the cell sorting process.

8.2.3 Optimizing Sample Staining

To Optimize Sample Staining:

1. Titrate your antibodies to make accurate measurements of fluorescence. An optimal antibody concentration will increase the signal, while reducing the noise due to non-specific binding of the antibodies to low affinity targets.
2. Use a viability dye to prevent sorting dead cells. Propidium Iodide, 7-AAD, or SYTOX® Green are good options. Unspecific antibody binding to the dead cell membranes can mask the real signal.
3. Consider trying multiple clones for your antigen of interest to determine best binding.

8.3 Sorting Controls

Controls are required for all sorting experiments that involve more than 1 color. Always include an unstained and stained positive control, even for 1 color experiments.

Fluorescence Minus One (FMO) controls mimic the sample except for one fluorescent reagent that is absent. These controls allow an accurate gating in the presence of fluorescence spillover between detection channels and will be useful to create the compensation matrix.

8.3 Sorting Controls (Continued)

For example: In a 3-color experiment using FITC, PE, and 7AAD stained cells, the following may be required:

- 1) Unstained control cells
- 2) FITC-only stained cells
- 3) PE-only stained cells
- 4) 7AAD-only stained cells
- 5) FITC + PE stained cells
- 6) FITC + 7AAD stained cells
- 7) PE + 7AAD stained cells
- 8) FITC + PE + 7AAD stained cells

8.4 Sample Concentration, Sorting Purity, and Sorting Time

The pre-sorted sample has a varying distribution of cells that depends on the total cell concentration. When sorting a desired cell, there is a Poisson statistical likelihood of sorting a second unwanted cell as a “tailgater”. Therefore, increasing the sample concentration will decrease the purity of the sort. In addition, highly concentrated samples may clog the cartridge.

The graph below represents the performance of the WOLF sorting different target populations at increasing sample concentrations.

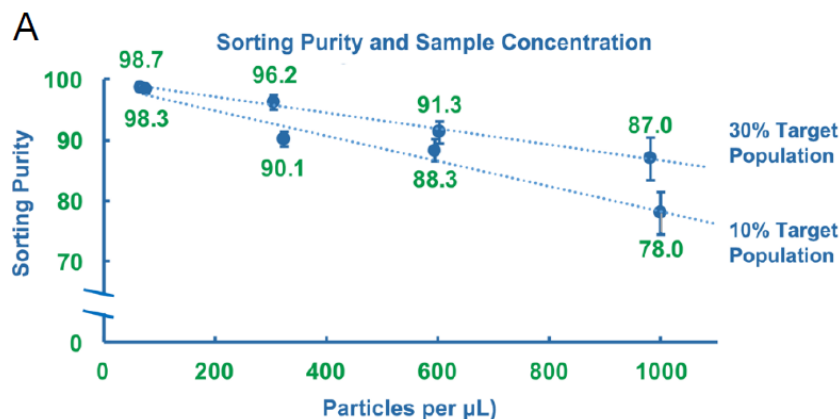


Figure 82. Representation of Sorting Purity versus Sample Concentration

8.4 Sample Concentration, Sorting Purity, and Sorting Time (Continued)

The WOLF[®] has a fixed flow rate of 24 µl per minute to process 1 mL of sample in 42 minutes. The instrument can analyze more than 5,000 events per second and has a sorting speed of 300 cells per second in a 1-way sorting experiment.

Please note that sorting rare target populations may significantly extend sorting times.

8.5 Maintaining Sterility

The WOLF[®] Cell Sorter is small enough to be placed inside a tissue culture hood. Sterile cartridges and tubing sets assure sterility.

To Maintain Sterility:

1. Wipe the instrument front panel with an alcohol wipe to reduce tubing contamination. Cartridge tubing may come in contact with the panel during installation.
2. Use sterile technique and open the cartridge envelope inside a tissue culture hood.
3. Clean the cartridge tubing with optical-grade alcohol wipes to remove particles and fibers that could clog the cartridge. This also helps to clean the tubing if it has come in contact with non-sterile surfaces.
4. Filter PBS and all media with a 0.22 µm syringe filter inside the hood.
5. Use sterile sample and collection tubes.



The WOLF[®] Cell Sorter is
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by
NanoCollect Biomedical, Inc.
www.nanocollect.com

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A 488 nm 30 mW
Class 1 Laser Product

US and International Patents Pending

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U.S. Patents: 20150268244 20130083315.
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