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Biomedical, Inc.

WOLF G2 Cell Sorter Instrument Guide

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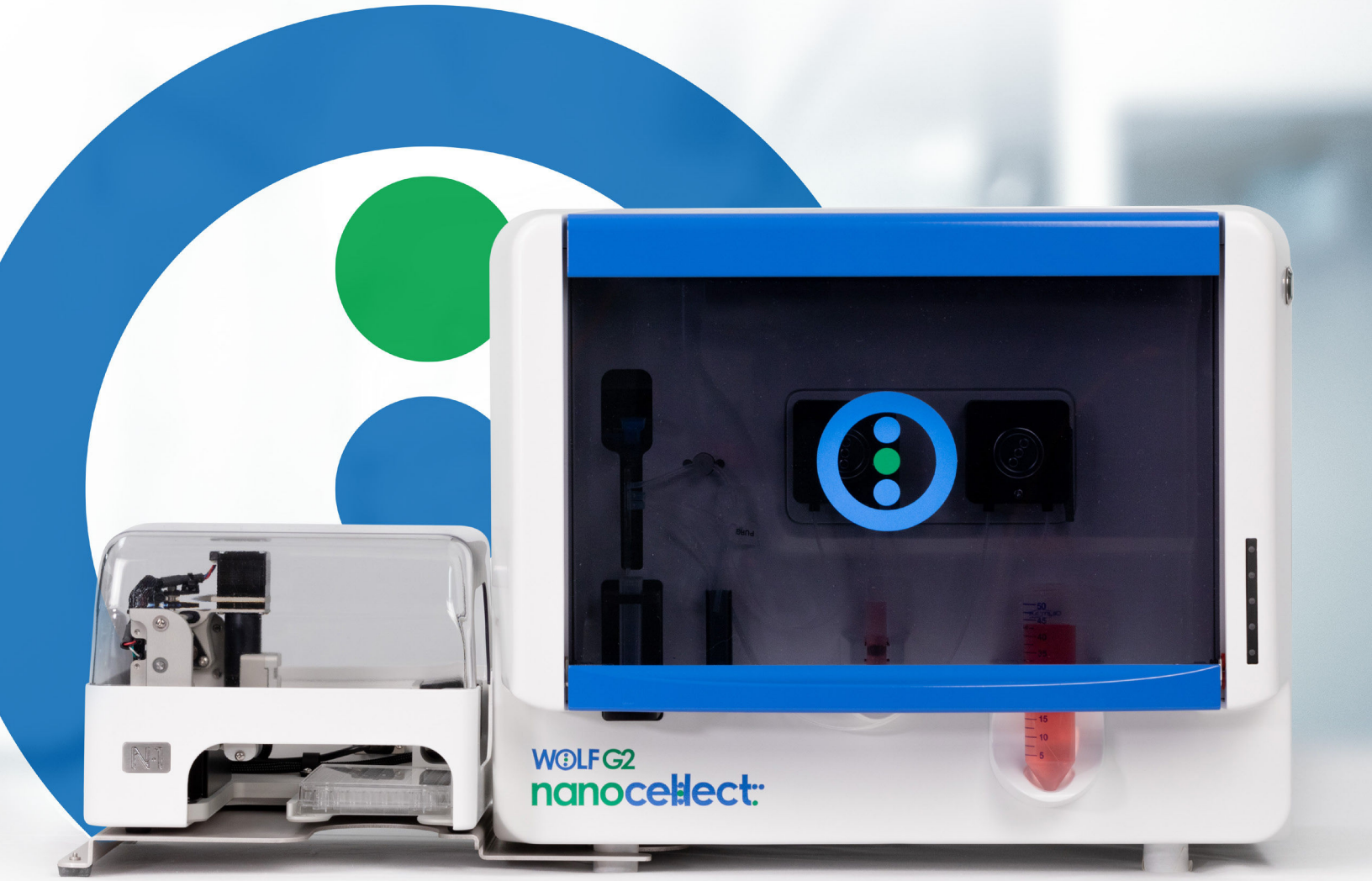

NanoCellect Technical Support

For support, please contact your local **Field Application Scientist** or our **Technical Support Department**.

E-mail: support@NanoCellect.com

Phone: (877) 745-7678 (Extension #3)

Monday–Friday, 6:00 AM–5:00 PM Pacific Standard Time (9:00 AM-8:00 PM EST)

Legal Notices

- The WOLF G2 Cell Sorter is intended for general laboratory **Research Use Only** (RUO).
- The WOLF G2 Cell Sorter is not intended for diagnostic testing or clinical use.
- Read the entire instrument guide 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.

NanoCellect reserves the right to modify its products and services at any time. This instruction guide is subject to change without notice in response to technological advancements and user input.

Although prepared to ensure accuracy, NanoCellect assumes no liability for errors, or for any damages resulting from the application or use of this information.

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488/561 or 488 only Configuration Fluorophore Chart.....80

488/637 Configuration Fluorophore Chart 81

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1. Introduction to the WOLF G2 Cell Sorter

1.1 Overview

The WOLF G2 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 switching 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 Safety

1.2.1 General Safety Warnings

IMPORTANT

- The WOLF G2 Cell Sorter is intended for general laboratory Research Use Only (RUO).
- The WOLF G2 Cell Sorter is NOT intended for diagnostic testing or clinical use.
- Read the entire instrument guide before attempting to operate the instrument.
- Always follow product labeling and manufacturer's recommendations. Please call NanoCellect Biomedical, Inc. if you have questions about how to proceed or if you are uncertain about these instructions, email or call NanoCellect Technical Support team.
- If this instrument is used in a manner not specified by the manufacturer, the protection provided by the instrument may be impaired.

NanoCellect Biomedical, Inc. urges its customers to comply with all national health and safety standards such as the use of barrier protection. This may include, but is not limited to, protective eyewear, gloves, and suitable laboratory attire when operating or maintaining the instrument.

The information in this guide is subject to change without notice. NanoCellect Biomedical, Inc. reserves the right to change its products in response to technological advancements and user input.

Below is a list of terms and their definitions as used within this guide to indicate a potential hazard.

WARNING Can cause injury.

CAUTION Can cause damage to the instrument.

1.2.2 Operator / User Safety

WARNING 

There is a risk of operator injury if:

- The door is not opened and/or closed with care.
- All covers and panels are not closed and secured in place prior to and during instrument operation.
- Contact is made with moving parts.
- Broken parts are mishandled.
- Improper tools are used for troubleshooting.
- The integrity of safety interlocks and/or sensors is compromised.
- Instrument alarms and error messages are not acknowledged and acted upon.

To avoid injury:

- Keep covers and panels closed and secured in place while the instrument is in use.
- Keep away from moving parts.
- Report any broken parts to NanoCellect Technical Support.
- Use the proper tools when troubleshooting.
- Take full advantage of all safety features and do not defeat safety interlocks and/or sensors.
- Acknowledge and act upon instrument alarms and error messages.

IF THIS INSTRUMENT IS USED IN A MANNER NOT SPECIFIED BY THE MANUFACTURER, THE SAFETY PROTECTION PROVIDED BY THE INSTRUMENT MAY BE COMPROMISED LEADING TO OPERATIONAL FAILURE AND/OR OPERATOR INJURY.

To help ensure compliance with intended use:

- Operate the instrument as described in this User Guide.
- Only operate the NanoCellect WOLF G2 Cell Sorter with an original copyrighted version of software authorized by NanoCellect.
- Install, update, and run anti-virus protection software on a regular basis.

If you purchased this product from anyone other than NanoCellect or an authorized NanoCellect distributor, and, if it is not presently under a NanoCellect service contract, NanoCellect cannot guarantee that the product is fitted with the latest mandatory engineering revisions or that you will receive the most current information bulletins concerning the product. For information, call NanoCellect WOLF Technical Support.

1.2.3 Safety Symbols and Labels

Safety symbols and labels alert you to potentially dangerous conditions. These symbols, together with text, apply to specific procedures and appear as needed on the instrument and throughout this guide. Do not remove these labels.

Biohazard / Biological risk
Consider all materials (specimens, reagents, controls, etc.) and areas these materials come into contact with as being potentially infectious and/or life threatening. Wear appropriate laboratory attire, follow universal laboratory safety protocols, and adhere to local regulations.

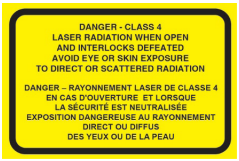
Electrical shock hazard.
There is the possibility of electrical shock when the instrument is plugged into the power source.

Laser radiation / hazard
Consider all laser sources as being potentially hazardous to eyesight. Wear the proper protective eyewear and never look directly into laser light.

Laser aperture hazard
Directed laser light is emitted from the aperture indicated with this label. Follow all laser radiation / hazard warnings.

CLASS 1 LASER PRODUCT.

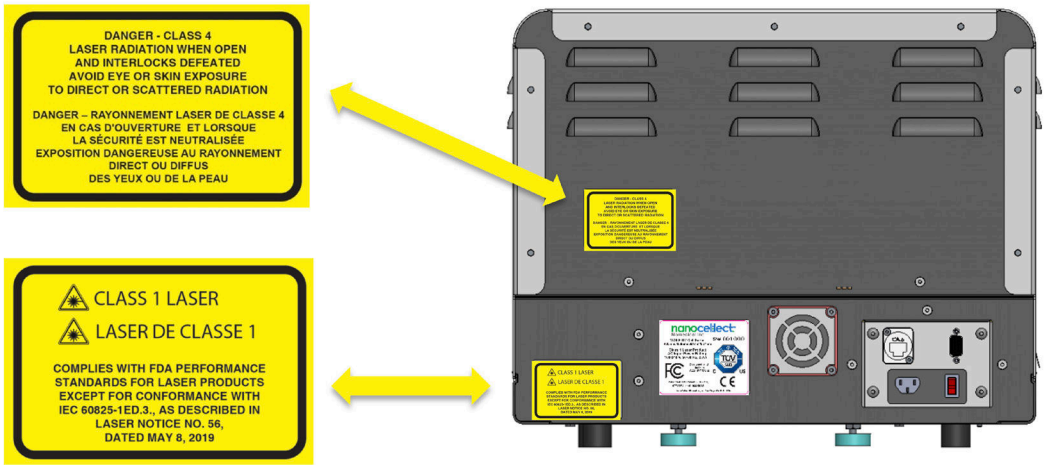
Any laser product which during operation does not permit human access to laser radiation (accessible emission, see IEC 60825-1:2014 3.2) in excess of the AEL of Class 1 for applicable wavelengths and emission duration (see IEC 60825-1:2014 5.3 and 4.3 e).



CLASS 4 LASER RADIATION WHEN OPEN AND INTERLOCKS DEFEATED AVOID EYE OR SKIN EXPOSURE TO DIRECT OR SCATTERED RADIATION

Any laser product which during operation permits human access to laser radiation (accessible emission, see IEC 60825-1:2014 3.2) in excess of the AEL of Class 1 and Class 2, as applicable, but which does not permit human access to laser radiation in excess of the AEL of Classes 3R and 3B (respectively) for any emission duration and wavelength (see IEC 60825-1:2014 5.3 d and 5.3 e).

Warning Labels Location. The laser warning labels are placed on the rear of the instrument. Refer to the figure below for the exact location of the labels.



1.2.4 Biohazard Precautions

Biological samples are potentially dangerous and/or life threatening. Depending on the laboratory environment, there could be a risk of biological, chemical, or radiological contamination.

If you have contact with samples, sample tubes, sample waste, the waste container, and/or associated tubing. Handle all them as if potentially infectious or life threatening. Wear protective clothing, gloves, and eyewear.

The WOLF G2 Cell Sorter produces minimal aerosols during normal operation. Care must be taken when removing the cartridge after completion of a sorting experiment to protect from possible drops of liquid. Dispose of all samples and waste according to proper handling procedures and local regulations.

Never pipette by mouth. Clean up all spills immediately.

Always treat waste with 0.5% NaOCl before disposal. Dispose of waste according to local regulations.

Always empty the waste tank daily or when prompted by software to prevent spillover and possible biohazard risk.

Consult appropriate Material Safety Data Sheets when necessary.

1.2.5 Laser Safety

The WOLF G2 Cell Sorter contains a solid-state 408 nm, 488nm, 561nm and/or 637nm laser under the case, and a secondary optical cover with a safety interlock. The instrument, nevertheless, may pose certain hazards associated with these lasers, if misused.

Wolf G2 Cell Sorter is designed to comply/conform with the IEC/EN 60825-1:2014 and CAN/CSA-E60825-1:15 standards and requirements. Although the laser diode output power is 55 mW, to comply/conform with Class 1 laser product, the laser is securely installed in an optical chamber to limit the Accessible Emission below the Accessible Emission Limit (AEL) of all 408nm, 488nm, 561nm, and 637nm laser. Wolf G2 Cell Sorter is designed to comply/conform with the IEC/EN 60825-1:2014 and CAN/CSA-E60825-1 as well as the Class 1 Laser product standards and requirements. Wolf G2 Cell Sorter is Class 1 laser product.

The following elaborates the conformity and compliance of the significant subclauses selected:

Subclause 6.15.2 Collateral radiation:

The WOLF G2 Cell Sorter, although designed with a 405 nm, 488nm, 561nm and 637nm(55mW) laser, which was properly secured in a protective optical chamber/housing, collateral radiation can still emit. However, under the TUV SUD's laser emission safety check and test, the collateral radiation is less than Accessible Emission Limit (AEL) of all 405nm, 488nm, 561nm and 637nm laser at normal operating conditions and single fault conditions, both of which are significantly lower than the Accessible Emission Limit (AEL) for 405 nm Class 1 laser radiation of 39 µW, or 224 µW for 488 nm laser, 390 µW for 561 nm laser, and 390 µW for 637nm laser.

Subclause 6.16 Power limiting circuit:

The G2 Cell Sorter, although designed with a 405 nm, 488nm, 561nm and 637nm (55mW) laser with a properly regulated power supply and feedback loop circuit to regulate the emission power, a single fault condition might occur during malicious operation. However, under the TUV SUD's LASER emission safety check and test, even at such single fault condition, the LASER module will either shut off/cut off or limit the collateral radiation below the Accessible Emission Limit (AEL) for Class 1 laser radiation, 39 µW for 405nm, 220 µW for 488 nm, 390 µW for 561 nm, and 390 µW for 637 nm laser.

Eye and skin exposure to direct and reflected laser light is hazardous. Never remove or attempt to remove the internal covers. Ensure that all optical filters are securely positioned. Prevent stray reflections from other surfaces. Never place any foreign object in the path of the laser beam. Only NanoCellect personnel can install, remove, or repair the laser. Do not open the laser enclosure for any reason. Always return the instrument to NanoCellect for repair. Never operate the unit in the presence of flammable gases or fumes. Turn off the instrument when not in use.

1.2.6 Laser Interlock

WARNING There is risk of personal injury if the laser safety interlock is bypassed.
Do not tamper with the laser interlock.

The laser interlock deactivates the laser when the top cover of the laser chamber is opened. Never attempt to override the interlock.

1.2.7 Operational Safety

This instrument is designed to be operated at an altitude of up to 2000m.

1.2.8 Ambient Temperature and Humidity

Operating temperature is between 15°C and 30°C (59°F and 86°F) and < 80% relative humidity.

1.2.9 Area of Use

The WOLF G2 Cell Sorter is designed to fit on a laboratory bench top. Provide a safety perimeter of six (6) inches around the instrument and computer to allow for proper ventilation and to protect the instrument and computer from accidental liquid spills.

Special care must be taken while handling fluids around the cytometer. Care must be taken that uncontained fluids do not enter the interior of the instrument or computer. Clean up spills immediately. Never place anything on top of the WOLF, including sample tubes and racks.

Turn off the instrument and unplug the power cord before manual cleaning of the cytometer.

If it becomes necessary to move the instrument, do not cause mechanical shock.

1.2.10 Electrical Precautions

Electrical devices pose the risk of an electric shock. To reduce the risk of an electric shock, do not open or remove the top cover of the cytometer while the instrument is turned on unless specifically indicated in this Instrument Manual. Only authorized NanoCellect personnel should remove any other panels from this device.

Always use the provided power cords, power supplies and cables. Do not abuse the cords. Never use the cords to pull the plug from an outlet. Keep cords away from heat, oil or sharp edges. Damaged cords increase the risk of electric shock.

The instrument is equipped with a fuse rating of 2A and 250V, with fast-acting type and low breaking capacity.

CAUTION This instrument must be plugged into a standard, grounded or earthed mains electrical outlet, conforming to local codes. Non-grounded or non-earthed mains outlet adaptors must not be used.

1.2.11 Reagents

Only fluids approved for use in the WOLF Cell Sorter should be used when operating the WOLF. Most reagents commonly used to prepare cells for sorting are appropriate to be used with the WOLF, however if you plan to use a non-approved reagent is

important to keep in mind the possibility of autofluorescence and degradation of the fluidics system due to chemical reactivity.

The following is a list of approved reagents for use with this system:

Cell culture media, PBS or other typical aqueous based buffers. For 405 nm laser configurations: addition of up to 5% BSA in PBS, and 1% BSA in culture medium is recommended. For all other laser configurations, addition of up to 5% BSA in PBS, and 2% BSA in culture medium is recommended.

The following is a list of non-approved reagents:

Organic solvents, high salt content buffers, bleach, ethanol, oils (mineral oil, etc). Addition BSA in amounts higher than 5%. FBS is not recommended.

Do not use household bleach as a decontamination solution as it contains fluorescent whitening agents that may interfere with fluorescent staining. Bleach may also negatively affect the lifespan of the cartridge components.

1.2.12 Material Safety Data Sheets (MSDS)

To obtain an MSDS:

1. Visit www.NanoCellect.com
2. Email info@NanoCellect.com
3. Call NanoCellect Technical Support at 877-745-7678, extension 3

Or write to: NanoCellect Biomedical, Inc., Attn: Technical Support, 7770 Regents Road #113390; San Diego CA 92122

1.3 Initial System Setup

1.3.1 Installation

This instrument must be installed and operated only in an environment that ensures a pollution degree 2 (or better) according to IEC/EN 61010-1. If used in areas with higher pollution degree, the device needs to be protected accordingly.

1.3.2 Instructions for Lifting and Carrying

To get the best quality of data, to prevent accidents and injury, moving the instrument is not recommended. Please contact the Technical Support Department or your local FAS.

Heavy object. To avoid muscle strain or permanent back injury, use lifting aid or proper lifting techniques when lifting or carrying the instrument. It is recommended to lift or move the instruments with the help of another person or NanoCellect personnel.

1. Make sure that the instrument has been turned off and the power switch on the back of the instrument have been switched off.
2. Unplug the power cable and the ethernet cable from the back of the instrument. If the instrument was paired with an N1 single cell dispenser, the N1 must be uninstalled from the instrument.
3. Slowly lift the instrument with two hands from the bottom of the instrument with one person on each side.
4. Carefully carry the instrument to the desired location. Keep the instrument in horizontal position while carrying.
5. Gently place down the instrument on the desired location.

6. Follow installation procedure on how to install the instrument.

1.4 WOLF G2 Cell Sorter Installation Requirements

Wolf Installation Requirements	
Dimensions	14.8H x 18.0W x 13.6D inch (37.6H x 45.8W x 34.5D cm)
Weight	54 lb (24.5 kg)
Power	AC Input: 100-240V, 50-60Hz, 2A
Fuse	2A, 250V, fast-acting, low breaking capacity
Temperature Range	15-30°C
Humidity Range	<80% relative humidity

N1 Installation Requirements	
Dimensions	8.4W x 6.5H x 8.3D inches (21.4W x 16.5H x 21.1D cm)
Weight	5.5 lb (2.5 kg)
Power	DC input, 24V, 1A
Temperature Range	15-30°C
Humidity Range	<80% relative humidity

1.5 WOLF G2 Cell Sorter Specifications

Software	WOLFViewer
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

Optics	Specifications
Excitation Laser	405 nm; 55 mW DPSS (45,000 hr life) 488 nm; 55 mW DPSS (45,000 hr life) 561 nm; 55 mW DPSS (45,000 hr life) 637 nm; 55 mW DPSS (45,000 hr life)
Beam Size	20 x 90 µm (1/e2)
Scatter Detection	Forward (0 degrees, +/-15) Back (180 degrees, +/-15)

Optics	Configuration	Laser	Optical Filters
Filter configuration (available lasers depend on configuration)	488 only	488 nm (Blue)	• 525/50 (FITC, GFP) • 580/25 (PE) • 620/50 (PI) • 706/95 (PE-Cy5.5®) • 706 LP (PE-Cy7®)
	405/488	405 nm (Violet)	• 450/50 (BFP, DAPI) • 525/50 (BV510) • 525/40 (BV570) • 620/50 (BV605) • 706/95 (BV711)
		488 nm (Blue)	• 525/50 (FITC, GFP) • 575/40 (PE) • 620/50 (PI) • 706/95 (PE-Cy5.5)
	561/488	561 nm (Yellow)	• 580/25 (PE) • 620/50 (mCherry) • 706/95 (7-AAD) • 760 LP (PE-Cy7®)
		488 nm (Blue)	• 525/50 (FITC, GFP) • 580/25 (PE) • 620/50 (PI) • 706/95 (PE-Cy5.5®) • 760 LP (PE-Cy7®)
		637 nm (Red)	• 706/95 (APC) • 760 LP (APC-Cy7)
	637/488	488 nm (Blue)	• 525/50 (FITC, GFP) • 575/40 (PE) • 609/34 (PI) • 706/95 (PE-Cy5.5®) • 760 LP (PE-Cy7®)
Optical alignment	**All**		Fixed

Fluidics	Specifications
Sample Input	1.5, 2, and 5.0 mL tubes
Sheath Input	50 mL conical tubes
Sheath Fluid	1X Phosphate-buffered saline (PBS) or buffer of choice
Sheath Fluid Usage	9.6 mL/hour
Sample Flow Rate	24 µl/minute
Sheath Flow Rate	160 µl/minute
Sample line volume	50 µl
Minimum sample volume	100 µl
Tubing diameter (inner)	200 to 500 µm
Flow Cell	200 x 70 µm
Smaller Channel Dimension	70 µm
Sample pressure	< 2 psi

Fluidics	Specifications
Fluorescence Sensitivity	< 200 MESF FITC (using 488 nm laser) < 250 MESF PE (using 561 nm laser)
Scatter Resolution	<1.5 µm by FSC or BSC
Scatter Resolution	Resolves lymphocytes, monocytes and granulocytes
Fluorescence Resolution	9 peak separation with SPHERO™ Rainbow Calibration Particles
Analysis speed	2,000 events/second
Sorting	1- and 2-way
Sorting speed	200 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/µl), with a target of 10% achieved a 94% purity.

Plate Sorting	Specifications
96-well plate time	9 minutes
384-well plate time	33 minutes

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Operation	Specifications
System power up	3 minutes
System setup and calibration	30 minutes
System shutdown	2 minutes

Computer	Specifications
File Format	FCS 3.1
Computer	Lenovo ThinkCentre TIO24Gen
Memory	16 GB
Storage	256 GB
Screen	23.8”

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1.6 The WOLF G2 Cell Sorter System Components

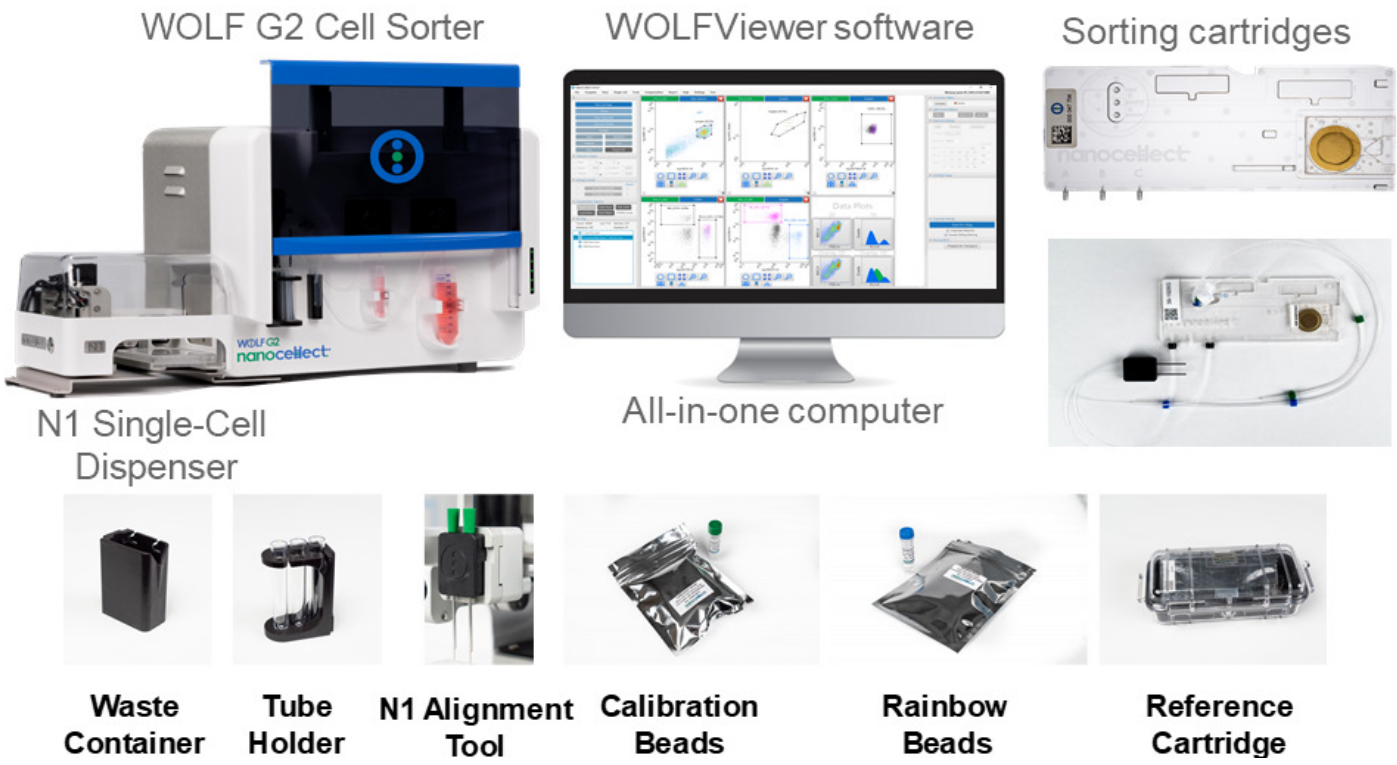


Figure 1. The WOLF G2 Cell Sorter components and accessories.

The WOLF G2 Cell Sorter and N1 Single-Cell Dispenser require accessories and consumables as well as a computer to operate the system. Please see below for suggested suppliers of typical sorter consumables.

On installation day you will receive:

NanoCellect products:

These can be purchased from NanoCellect if replacements are needed.

- WOLF G2 Cell Sorter
- N1 Single-Cell Dispenser
- All-in-one computer
- Waste container
- Collection tube holder
- Cartridge Starter Pack: 10 sorting or 10 single-cell cartridges
- Beads Starter Pack: 1 mL vial of concentrated NanoCellect Calibration Beads and one vial of rainbow beads
- Alignment cartridge

Consumables Starter Pack:

These are courtesy items that can be purchased from outside suppliers.

- **5 mL sterile flow tubes** (Falcon, #352058** [polystyrene], or #352063** [polypropylene]). Alternatively, similar tubes with a 40 µm cell strainer that simplify sample preparation can be used (for example, Falcon, #352235**).
- **0.22 µm syringe filters** (Genesee Scientific, #25-244**)
- **20 mL syringes** (generic)
- **25 mL N1 waste containers** (VistaLabs, 4054-1002***)
- **isopropanol lens-quality wipes** (any type, ex. VWR, #21910-110**)
- **precision swabs**

** Suggested catalog number

*** Specific catalog number, plese make sure to use this specific part number to order replacements.

1.7 Front Panel Mechanics and Fluidics

The front panel of the WOLF G2 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.

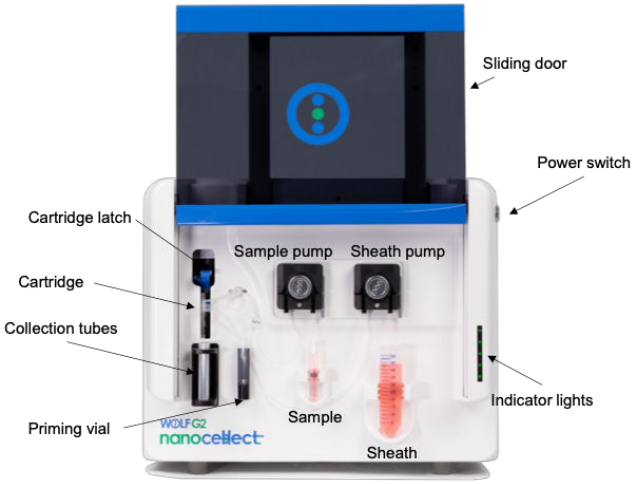


Figure 2. Front panel of the WOLF G2 Cell Sorter.

1.8 NanoCellect Cartridges

The detection and sorting capabilities of the WOLF G2 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**.

The WOLF G2 Cell Sorter can be operated with two cartridge varieties to sort in bulk or dispense single cells in 96- or 384-well plates.

- **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 well by the N1 single-cell dispenser.

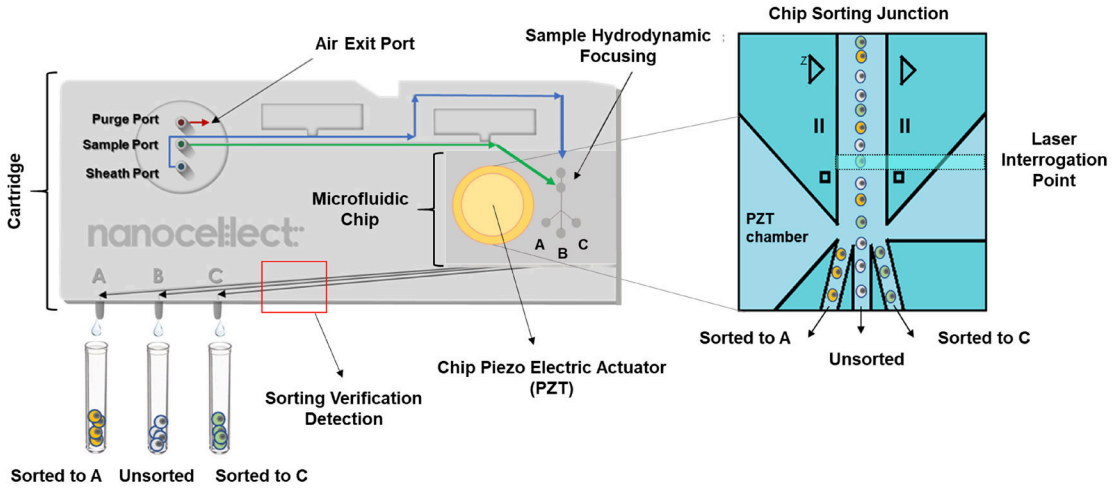


Figure 3. The WOLF G2 Cell Sorter cartridge: The cartridge has a purge, sample, and sheath ports on the upper left. The sample is routed into the cartridge microfluidic chip for detection and sorting. Sorted cells exit the cartridge to the collection tubes through three output ports situated on the lower left of the cartridge.

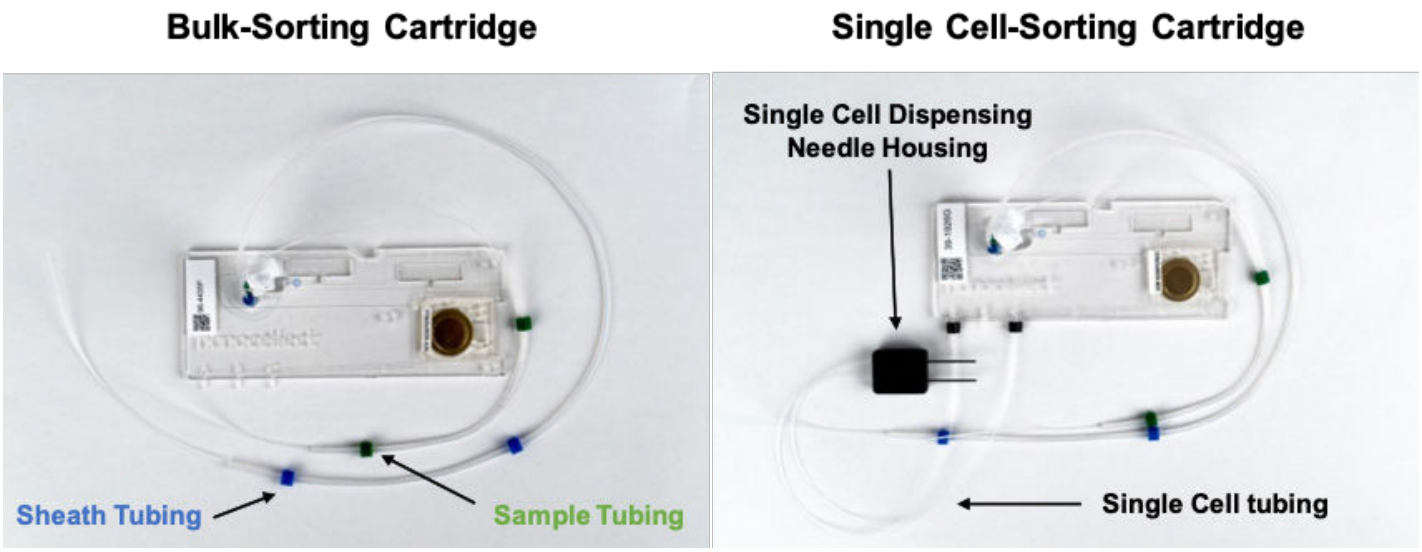


Figure 4. The WOLF G2 Cell Sorter cartridge varieties

1.9 WOLF G2 Cell Sorter Computer

In general, the computer supplied with the WOLF G2 Cell Sorter should be treated as a dedicated computer used solely for instrument operation and data management.

1.9.1 Computer Account Management

The WOLF computer has three accounts:

- **NanoCellect'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.
- **NanoCellect's Administrative Account** is used for software and firmware updates by NanoCellect's Technical Service personnel

1.9.2 Obtaining Administrative Passwords

In the case that the Administrative Account is needed, please contact the support phone line to request it (we cannot provide passwords by email). To obtain it, the user will need the computer serial number. The computer serial number can be found under bottom of the computer monitor.

An alternative way to obtain the computer serial number is through the computer settings. Run the WMIC Command to find serial number for the WOLF computer. This command uses the Windows Management Instrumentation Command-line (WMIC) tool to pull the system's serial number from its BIOS.

- Open a Command Prompt window to get started. On Windows 10 or 8, right-click the Start button and select "Command Prompt". On Windows 7, press Windows + R, type "cmd" into the Run dialog, and then press Enter.
- At the Command Prompt, type the following command and then press Enter:
- wmic bios get serialnumber
- You'll see the computer's serial number displayed beneath the text "SerialNumber".

1.9.3 Computer Maintenance

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: NanoCellect recommends rebooting the WOLF computer once per week to update the Operating System and flush the RAM memory.

NOTE: Excessive external application use may affect resource management and cause buffering issues for the WOLFViewer Software. Please refrain from opening additional software during your sorts.

1.10 WOLF Internet Connectivity and Service Support

As many other equipment manufacturers, NanoCellect 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.

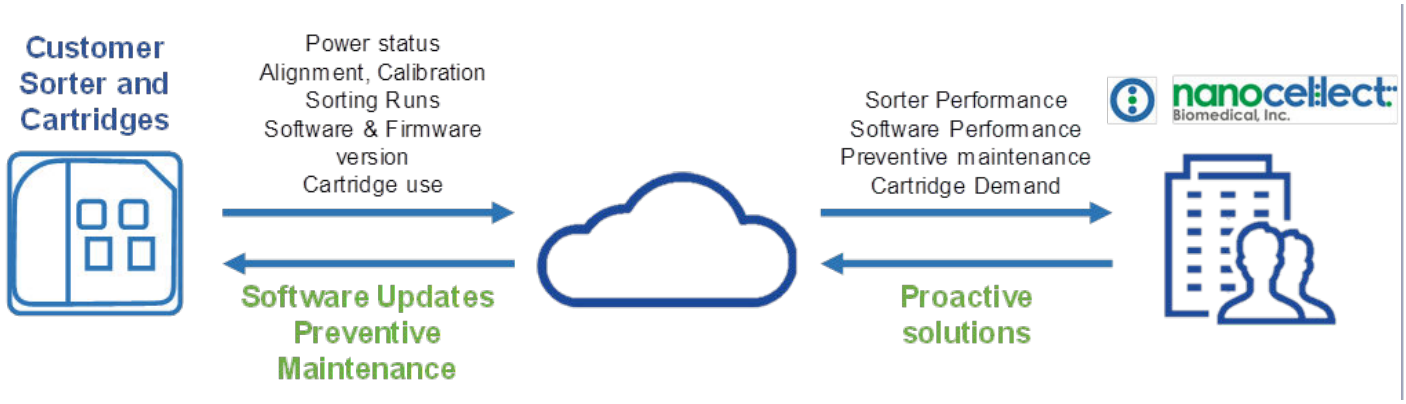


Figure 5. Representation of NanoCellect's data monitoring

In addition, we will offer **easy and secure remote control** of the WOLF G2 Cell Sorter computer to provide support and training if needed.

We use TeamViewer, a platform that allows NanoCellect'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.

1.10.1 Data Privacy

Types of data collected by NanoCellect

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

Data that is not collected

- Sensitive data, experimental data, and user identity is not collected under any circumstances.

NanoCellect respects the privacy and intellectual property concerns of our customers. Only the aforementioned "Types of data collected by NanoCellect" are used by NanoCellect.

All data collected can be made available for review by the 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 NanoCellect 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.

1.10.2 Obtaining Remote Support

Please contact support@NanoCellect.com or the help line to obtain real-time assistance. A TeamViewer shortcut is already on your WOLF computer and can be accessed from the desktop icon.

You can also type in TeamViewer in the Window's search bar to access the TeamViewer. Share Your ID and password with the FAS who is troubleshooting with you. All you need is a WiFi connection on the computer. **We encourage connecting your sorter permanently to take advantage of all the remote support benefits we offer**, including cartridge quality assurance. Please see section above on the type of data we collect. We never collect or store private information.

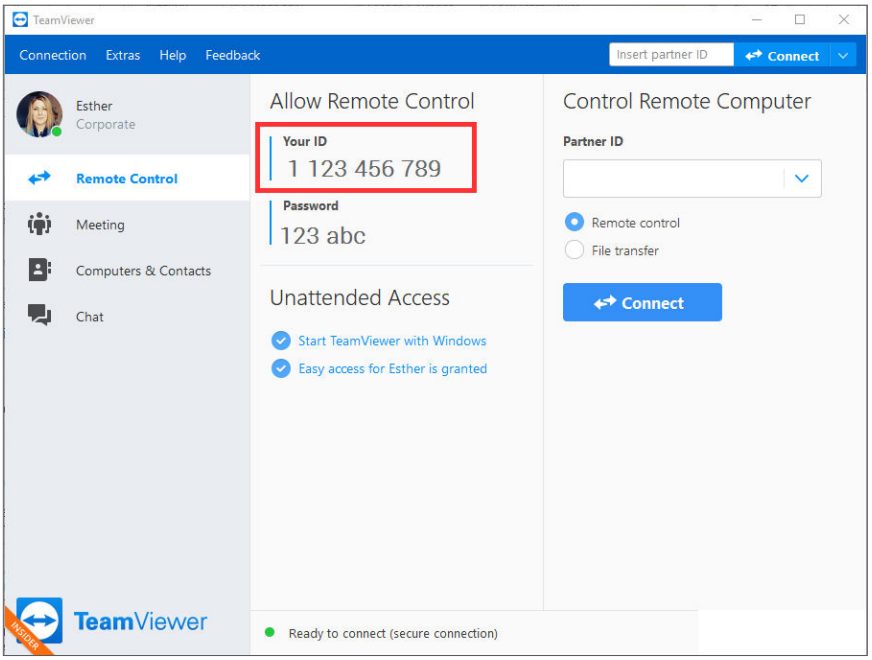


Figure 6. Finding the TeamViewer ID and password. The password changes with each launch

1.11 WOLFViewer Software

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

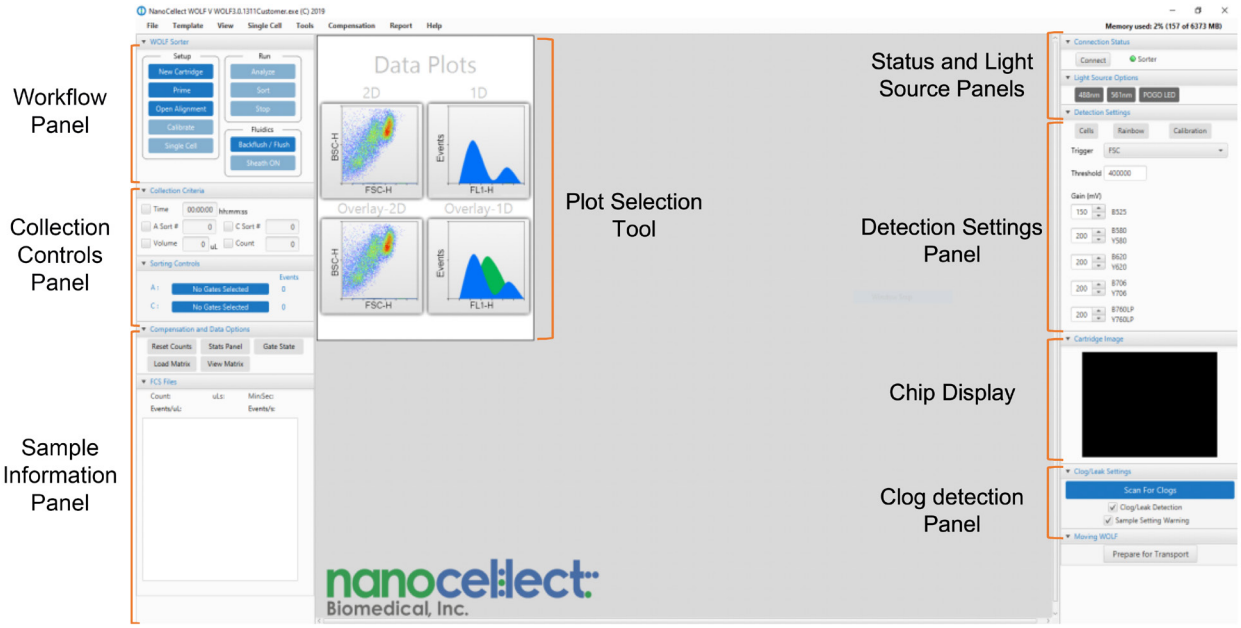


Figure 7. The WOLFViewer software user interface

- **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.
- **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.
- **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.
- **.gates**: 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. Also the format used for export of stats panel.
- **.cmat**: Compensation matrix created for data analysis and sorting.
- **.pdf**: Experimental reports.
- **.jpeg:** Plot and plate analyzer images.

NOTE: NanoCellect recommends saving files to a local location (such as desktop) and then move them to their preferred location such as a server for further analysis.

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.

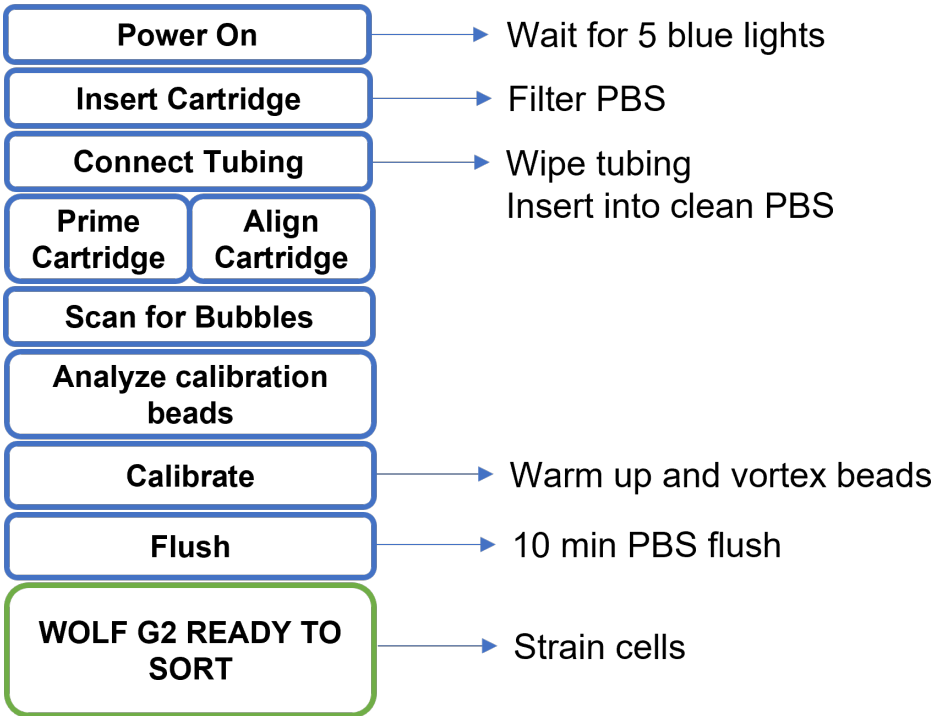


Figure 8. General workflow with the WOLF G2 Cell Sorter

2.1 Materials

- WOLF G2 Cell Sorter cartridge (bulk or single-cell)
- PBS (Phosphate-buffered saline solution)
- 15 µm calibration beads, stock for dilution
- 20 mL syringes
- 0.22 µm syringe filters
- 50 mL conical tubes
- 5 mL FACS tubes
- 37-40 µm cell strainers
- Optical-grade isopropanol wipes
- Reagent reservoirs

NOTE: Please see section 1.6 for suggested catalog numbers.

2.2 Powering On and Cartridge Selection

Powering on:

1. Turn on the WOLF G2 by pressing the button located in the upper right corner of the instrument (Fig. 9 A).
2. Wait until the Instrument Connection Indicator LED shows 5 blue lights. This takes roughly 1 minute.
3. Open WOLFViewer software and check the connection status.
4. If the WOLF G2 is not automatically connected click “Connect” on the Connection Status Panel. The software indicator should turn green (Fig. 9 B).

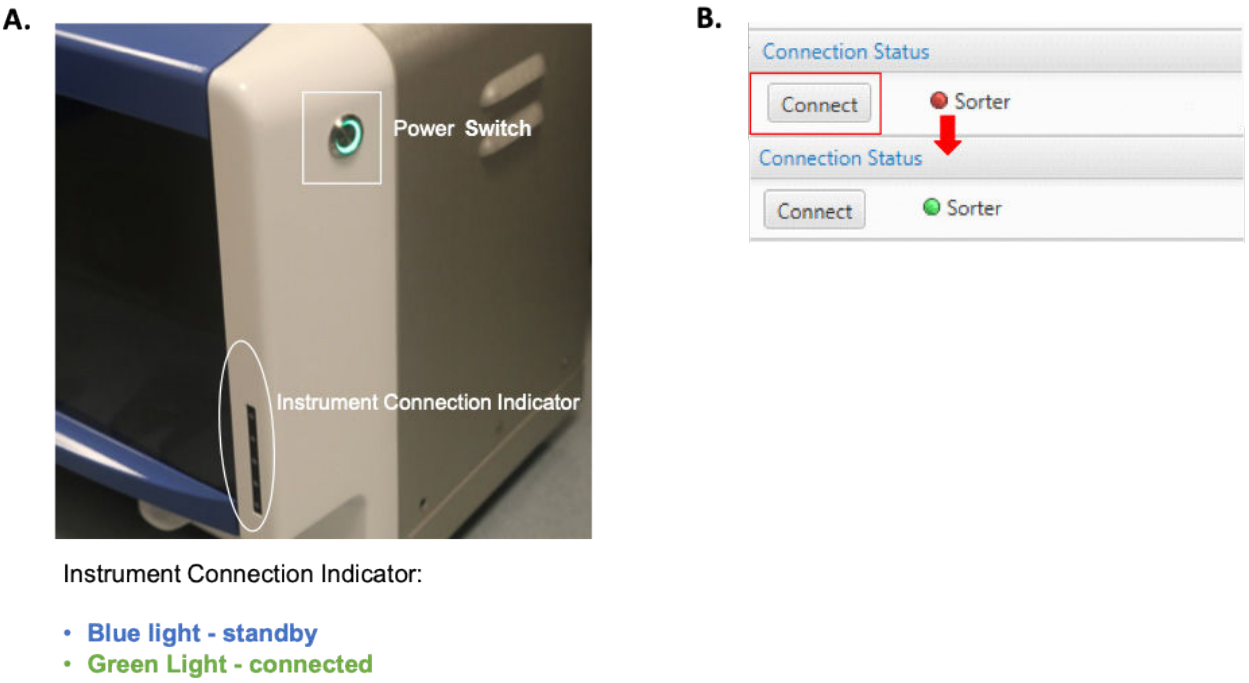


Figure 9. WOLF G2 power on and indicator lights.

The WOLF G2 can be operated with two **types of cartridges** depending on the user experimental design:

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

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

Cartridge selection:

1. Click “New Cartridge” in WOLFViewer. This triggers a pop-up prompting the user to remove a cartridge if present. The motors will move to prepare the system to receive a cartridge (Fig. 10).
2. When the system is ready, a pop-up will prompt to insert a new cartridge. Insert a cartridge and click “OK” (Fig. 11).
3. The cartridge RFID tag will be automatically recognized by the unit once inserted. Alternatively, type in the cartridge ID and click “OK”.
4. Select the type of cartridge that will be used for the experiment (Fig. 12).

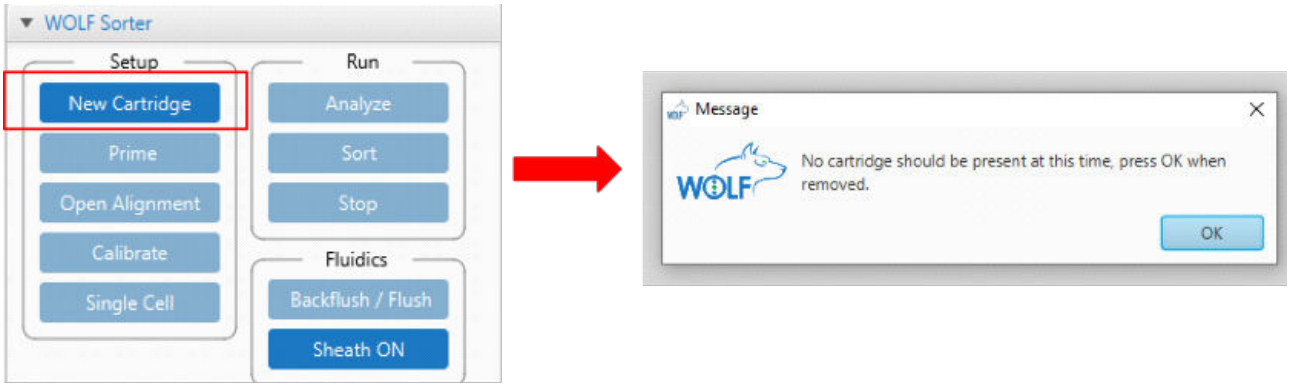


Figure 10. Starting with a new cartridge.

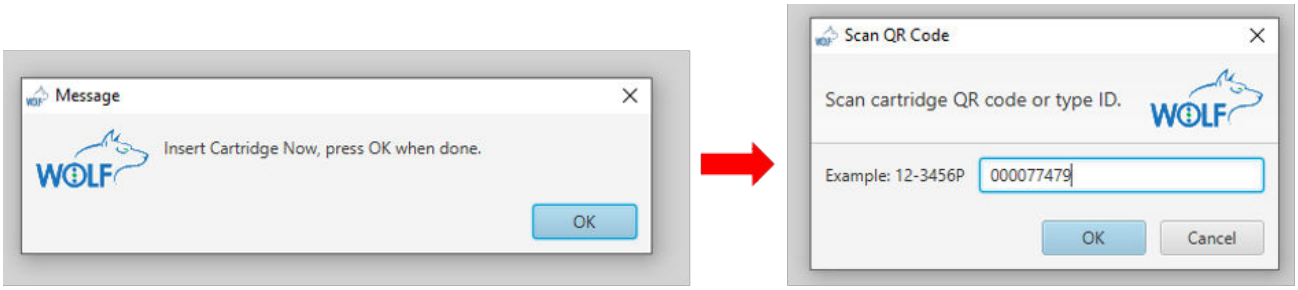


Figure 11. Scanning in a new cartridge.

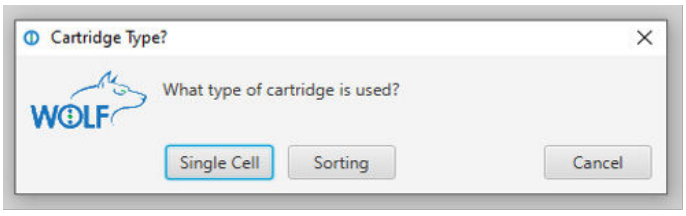


Figure 12. Cartridge selection.

2.3 Cartridge Insertion

Cartridge insertion:

1. Remove the cartridge from the packaging and 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. Hold the cartridge by the QR code sticker to avoid fingerprints. If touched, gently wipe clean with an optical-grade alcohol wipe.
3. Gently insert the cartridge in the slot, avoiding inserting the cartridge at an angle. A minimal amount of pressure is needed to push the cartridge in; if you feel resistance, remove the cartridge and try again. The latch will automatically lift when the cartridge is inserted.
4. When the cartridge reaches its final position, the latch will automatically lower down and secure it in place. You will hear an audible click.
5. If the cartridge gets stuck, lift the latch up with one hand. Use the other hand to pull the cartridge out. Reinsert the cartridge.

Never force the cartridge into the fixture slot as it may damage the cartridge and/or the fixture detectors.

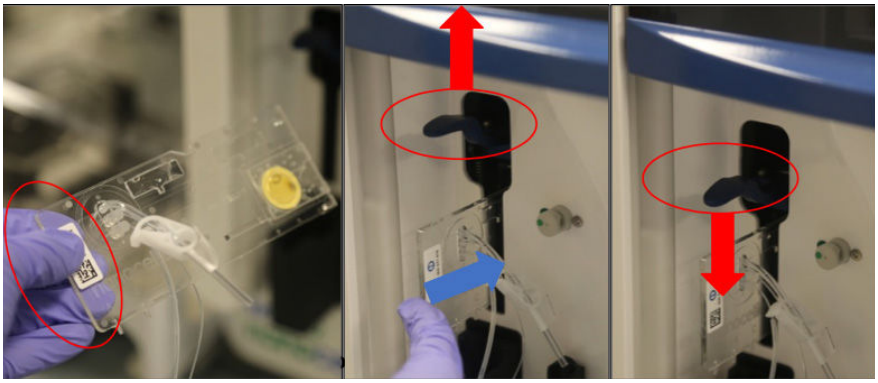


Figure 13. Best way to hold the cartridge for insertion into WOLF G2. Insert gently and release: The blue latch will lower automatically.

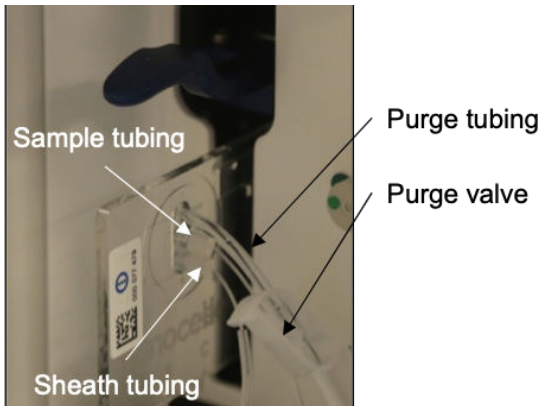


Figure 14. Tubing description.

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 two 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.

Tubing connection:

1. To open the peristaltic pump, release the gray lock tab and gently lift upwards.
2. Thread the sample tubing line over the top of the pump rollers.
3. Gently stretch the tubing and seat the black stoppers below the slots on either side and below the pump rollers.

   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.
5. Ensure that the gray lock tab is securely pushed in.
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.
9. 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 spills.

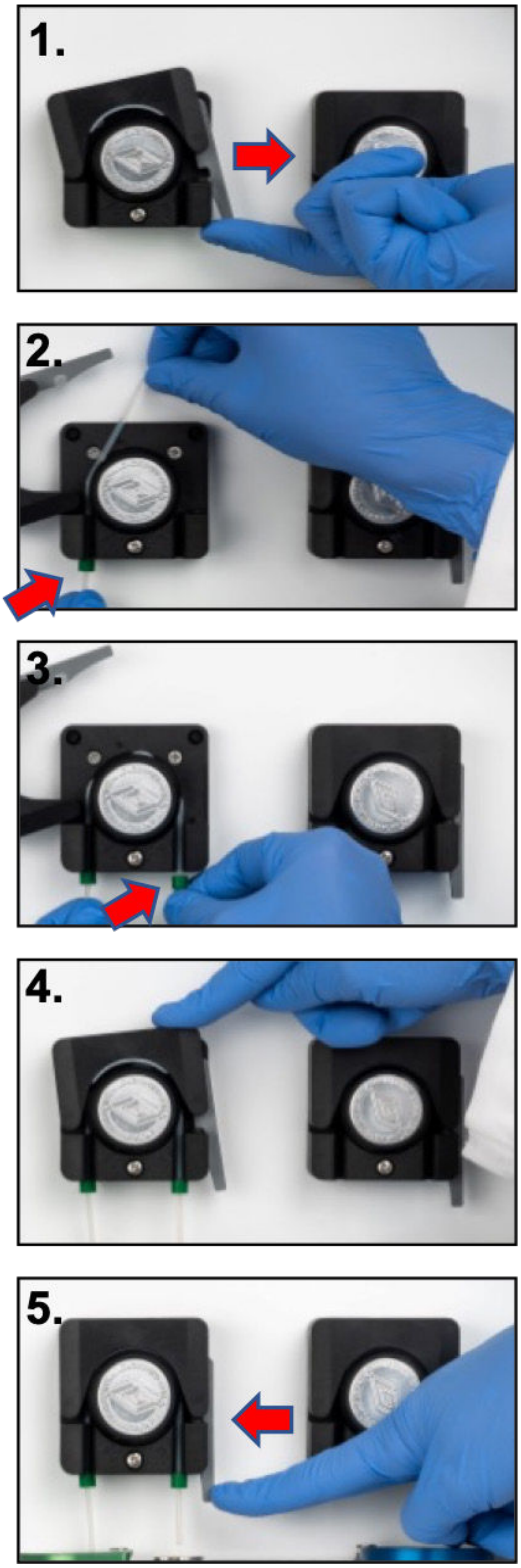


Figure 15. Cartridge tubing setup.

2.5 Cartridge Priming and Chip Alignment

2.5.1 Cartridge Priming

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 5-10-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. To prime, use 1X PBS filtered through a 0.22 µm PES syringe filter for both sheath and sample.

NOTE: Please only use 1X PBS to prime your cartridge. The only exceptions are applications where seawater or insect cell media may be used. These contain high salt concentrations that may react with salt in PBS and result in crystallization. In those cases, prime with your media.

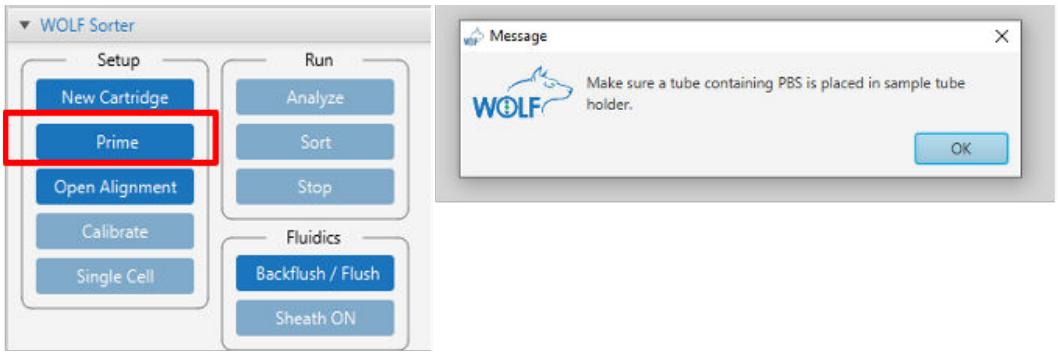


Figure 16. Initiating priming.

Figure 17. Manually closing the purge valve. See the open (left) and closed (right) valve position. The valve sits above the purge sticker.

Cartridge priming:

1. Make sure the purge tube clip is in the open position before starting the priming, enabling air to escape the cartridge. This tube should be placed in a 5 mL waste tube (black holder). Place tubes with filtered PBS on the sheath and sample holders. Wipe the tubing with a lint-free alcohol wipe before immersing in the PBS.
2. In WOLFViewer software, click the “Prime” button.
3. After 5 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. The sample pump will stop turning while the sheath pump continues.
4. Ensure PBS is exiting the purge valve as well as the A, B, and C cartridge outlets before closing the purge tube clip.

2.5.2 Cartridge Alignment

The WOLF G2 Cell Sorter's lasers 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 semi-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 a square XYZ marker found within each cartridge.

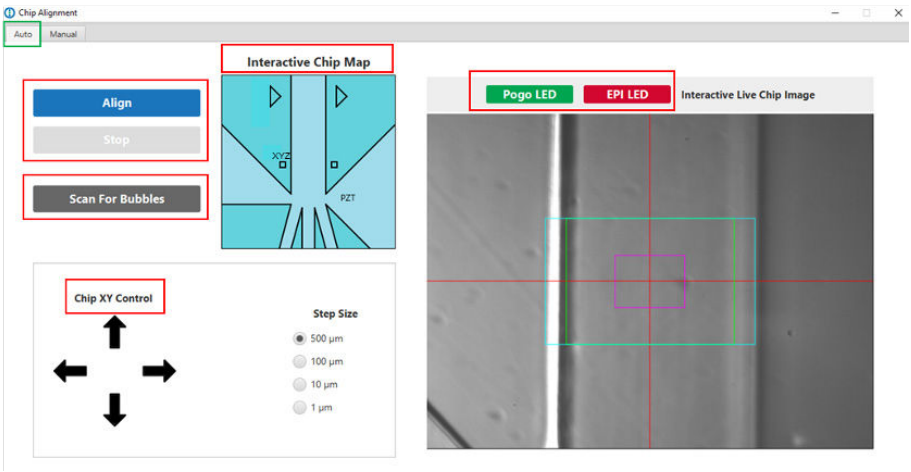


Figure 18. Chip Alignment interface.

The Chip Alignment interface controls the cartridge movement inside the instrument. Upon clicking "Open Alignment" in the workflow, the software opens a new window with the alignment user interface.

The Alignment Window (Fig. 18) 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 XY control arrows: Move the chip in the X-Y plane.
- Step size radio buttons: Control the step size when moving the chip.
- Chip live image: A real-time chip image allows the user to move the chip around when clicking in it.

Make sure cartridge is properly inserted and seated in the fixture slot, and the blue latch automatically closed to ensure the image quality is optimal for alignment before initiating the process. Wait for the purge valve to be closed to initiate alignment, as closing the valve may move the cartridge and could necessitate a new alignment.

To align the chip:

1. Click "Open Alignment" in the workflow menu. The alignment window will pop up.
2. Turn on the POGO camera by clicking on the "POGO LED" button. The button appearing green indicates that the camera is on (Fig. 19).

3. **Using the arrows on the interactive chip map or simply by clicking on the chip camera view, find the left square XYZ marker.**
4. Make sure to focus the marker in Z by using the “Z focus” function and clicking on the “up” or “down” arrows. The features should appear “sharp” and not blurry.
5. Once the XYZ marker is focused, click on the blue “Align” button. The alignment algorithm will center and focus the XYZ marker to determine the chip center.
6. The cartridge will then move to align the chip to the channel center.
7. A pop-up window will notify the user when the alignment has finished.
8. Please verify alignment success by checking the channel is centered and sharp (Figure 19, step 9).

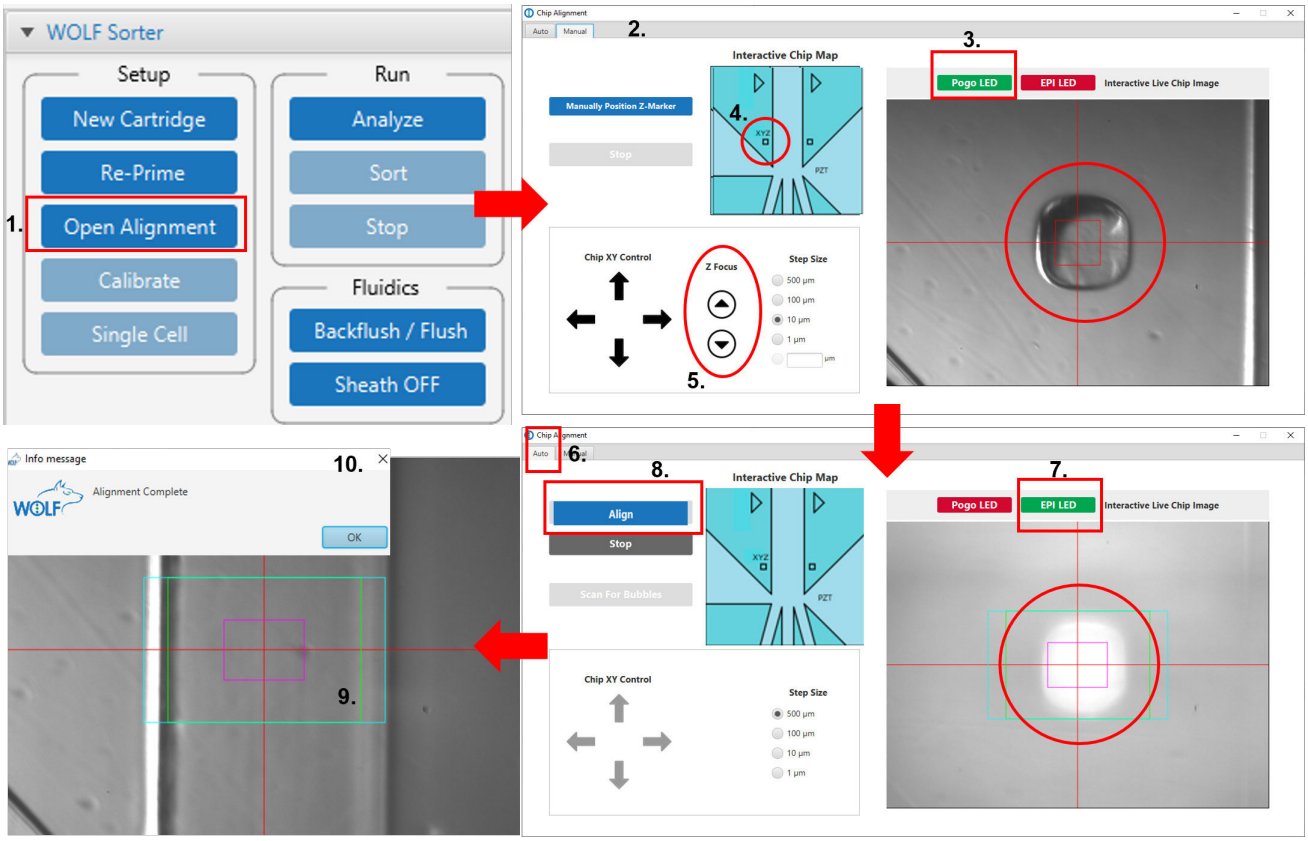


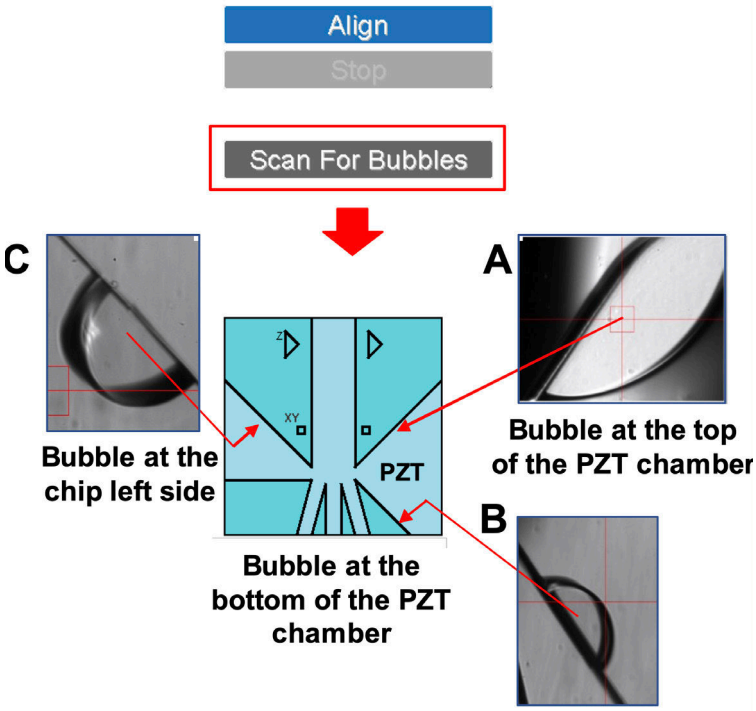
Figure 19. Chip Alignment workflow.

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.

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.

To confirm the absence of air bubbles in the chip:

1. Click the “Scan for Bubbles” button in the chip alignment’s auto window.
2. The chip will be automatically moved for the user to actively monitor the camera image to visualize remaining bubbles.
3. The scan will follow a pattern, viewing first the top chip PZT area, second the lower PZT area, and third the left chip area.
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.



If air remains in the cartridge:

1. Check sample and sheath fluid input to make sure you have sufficient volume and are not taking up air.

2. Check tubing connections to cartridge (Fig 14) for loose connections.

3. Examine purge valve (Fig 17). Ensure the clip is not fully closed and does not sit on top of the white sticker.

4. Re-prime your cartridge, if applicable.

Note: If bubbles remain after 3 re-priming attempts, the software will ask the user to continue to the next step.

Figure 20. The scan for bubbles feature.

2.6 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 µm green fluorescent beads).

Analyze calibration beads:

1. We recommend that you bring the dropper bottle to room temperature 10 minutes prior to preparing a dilution. Follow the instructions included with your calibration beads: vortex vigorously and dilute 1 drop in 650 µl PBS.
2. Vortex calibration beads vigorously before starting the process to prevent aggregation.
3. Position the calibration beads tube in the sample holder.
4. Click the “Analyze” button on the Workflow Panel.
5. Open a 2D log10 FSC/log 10 BSC plot by clicking in the plot selection tool.
6. Analyze ~100 events.
7. Click the “Stop” button to finish analyzing calibration beads.

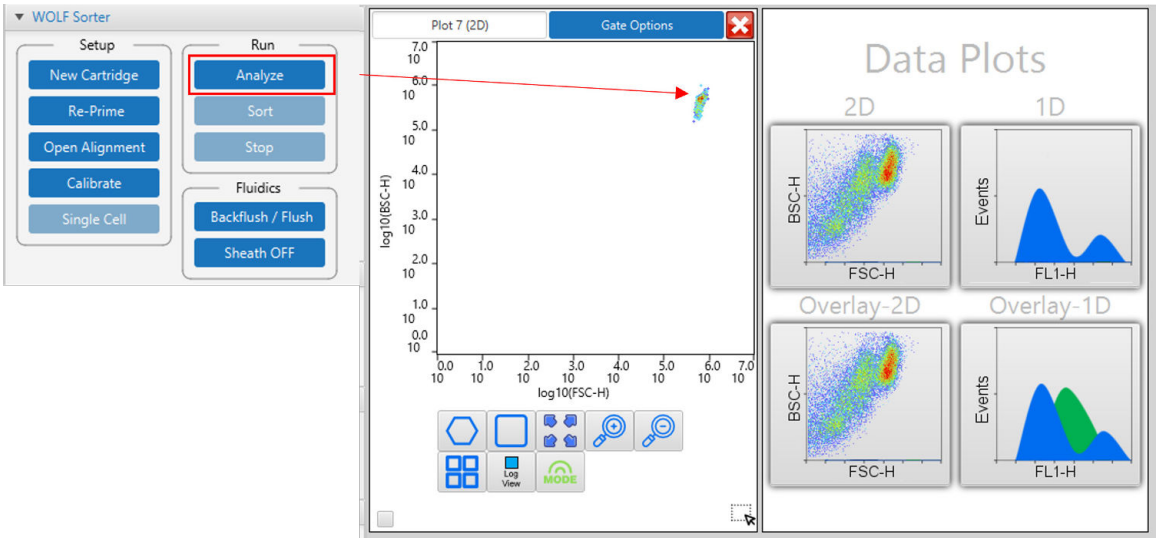


Figure 21. Analyzing calibration beads.

To calibrate the cartridge:

1. It is important to first analyze 100-200 calibration beads. Press “Analyze”, name your sample and click “New Sample”.
2. When the Event Count hits 100, press the “Calibrate” button.
3. The software will calculate the beads concentration and warn the user if it is below 10 beads/µl. The optimal concentration is 50-80 beads/µl.

4. The automated calibration will begin with channel A. A progress bar will inform the user of the calibration status. After channel A, the system will calibrate channel C, for a total of about 4 minutes.
5. 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.
6. Calibration can be paused between channels to mix the beads or replace the tubing if needed.
7. Flush your cartridge once calibration is complete. Place a tube containing 4 mL filtered PBS (or sheath fluid if different) in the sample holder and click the “Flush” button. A 10-minute flush cycle is ideal for removing most beads from the cartridge.

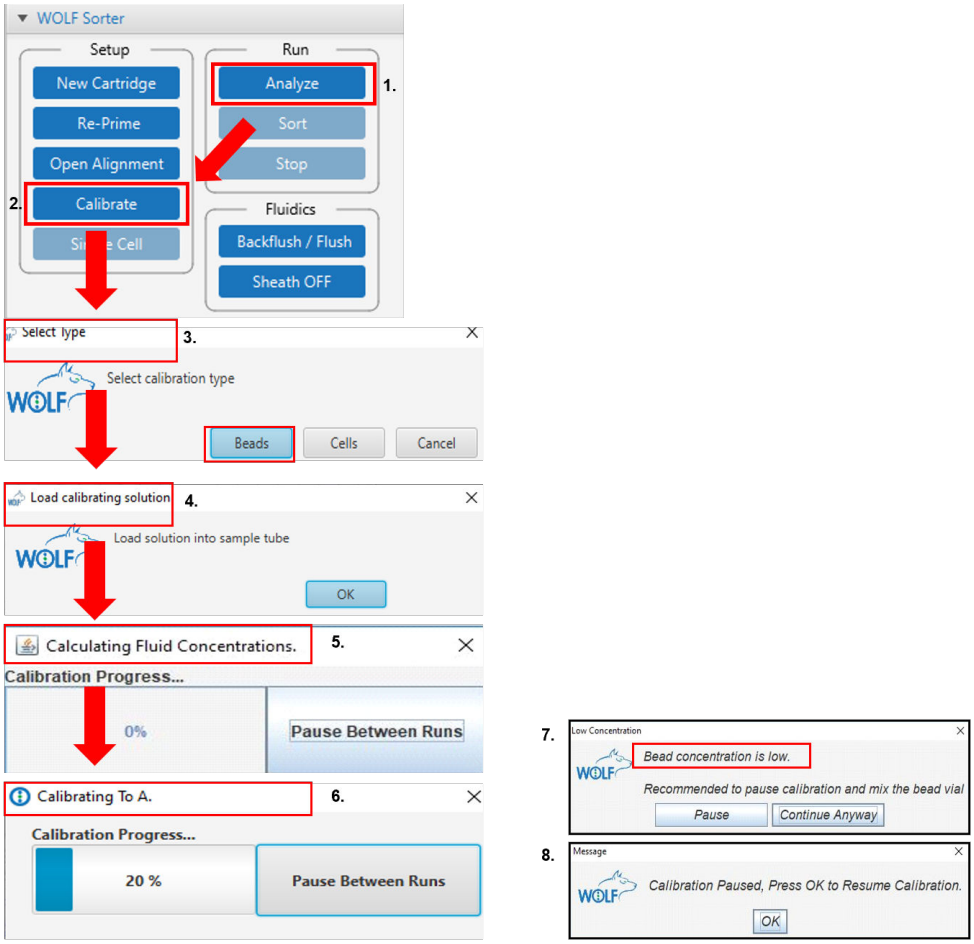


Figure 22. The calibration process. Analyze beads first until they come up on a scatter plot. At that point the calibration button can be pressed to initiate the automatic process.

NOTE: Flushing the beads out after calibration is advised before analyzing and sorting fragile cells that may be particularly sensitive to the bead formulation/additives.

NOTE: At this point, please check that you have not introduced any fibers into your cartridge by using the Scan For Clogs funcionality. Consult section 7.9. If there is an issue, refer to the Troubleshooting Microfluidic Cartridges note on the Users’ Knowledge Base.

2.7 Fluidic Controls

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

To flush and backflush the cartridge:

1. Click “Flush” to remove beads after calibration or clean carryover between samples. The user can flush the cartridge for either 3 or 10 minutes.
2. **In the event of running out of sample, to remove any air from the cartridge inlet lines (sample only) 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 at that point for optimal sorting efficiency.
3. If repriming after a major quantity of air was detected in the cartridge, it is advised to run the bubble scan and calibration steps again to confirm that the air was removed and the cartridge is sorting properly.

NOTE: To avoid air intake into sample or sheath tubing, monitor sample and sheath usage or set volume uptake limits in the Collection Criteria Panel.

To change the PBS sheath buffer for other cell media:

1. Stop the sheath pump by clicking the “Sheath Off” button to prevent air from entering the chip. The button name will change to “Sheath On”.
2. Exchange the sheath buffer tube as quickly as possible to avoid the cartridge drying out.
3. Click the “Sheath On” button to start the pump again.
4. Check the Sample Preparation section for tips on sheath buffer composition.

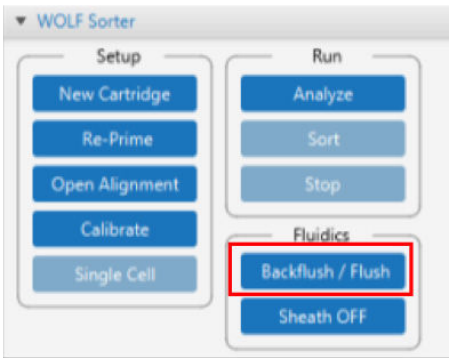


Figure 23. The flush and backflush feature.

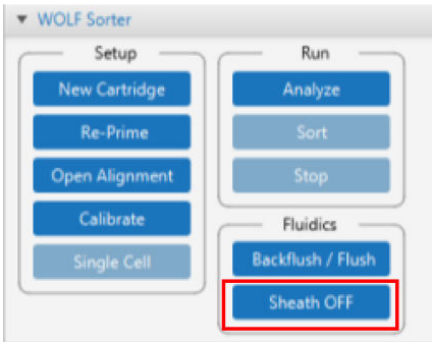


Figure 24. Changing the Sheath Fluid.

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 G2 Cell Sorter Best Practices of this manual for suggestions on how to avoid problems during sorting.

3.1 Sample Analysis

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.

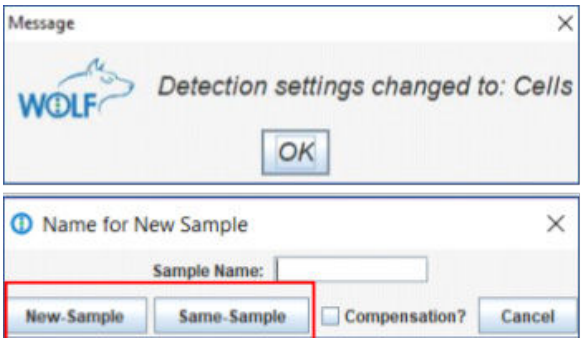


Figure 25. Analyzing a new sample.

When clicking “New Sample”, 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).

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.

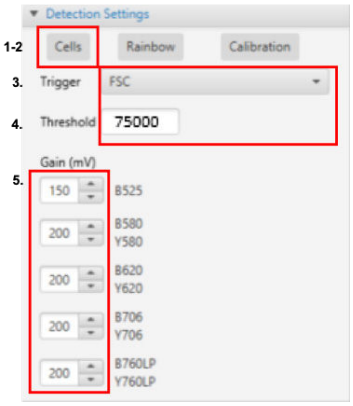


Figure 26. Detection settings. Predetermined "Cells" setting should be optimal for most cells. The trigger and threshold can be customized for small cell detection. The gain on each detector may be applicable to 1-2 channels sharing the same filter.

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

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 103-104 in the logarithmic scale. Lower intensity values are considered background noise.

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.

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.
2. Click the "Cells", "Rainbow", or "Calibration" button depending on the type of sample to analyze.
3. Select the triggering channel from the drop-down menu.
4. Type the threshold value for that channel in the blank box.
5. Adjust the PMT gains by clicking on the up/down arrows or inputting values in the appropriate boxes to optimize your sample detection.

4. 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 two 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

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.
3. To change one of the axes to a different parameter, simply right-click the label and choose the desired channel.

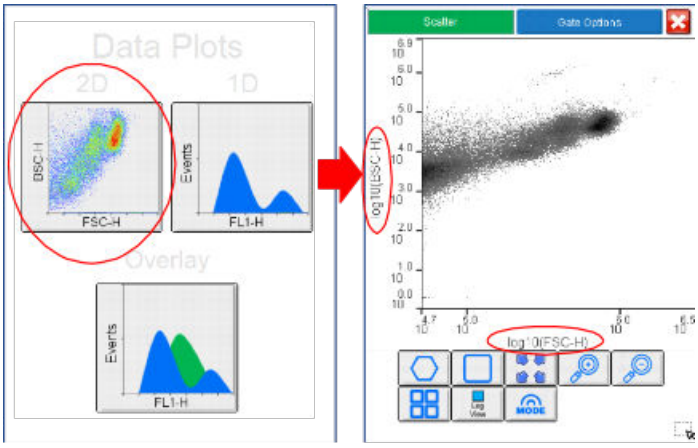


Figure 27. Creating bivariate plots.

The “Mode” option in the plot tool allows the user to change the plot display between dot, density, and contour (Fig. 28).

- **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, with blue representing low, green as medium, and red as high-density.
- **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.

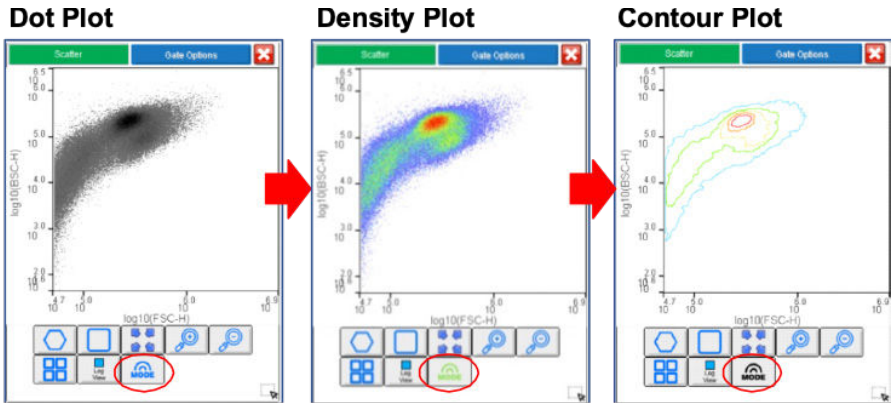


Figure 28. Dot, density, and contour bivariate plots.

4.1.2 Histogram Plots

The user can analyze the frequency (number of 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.

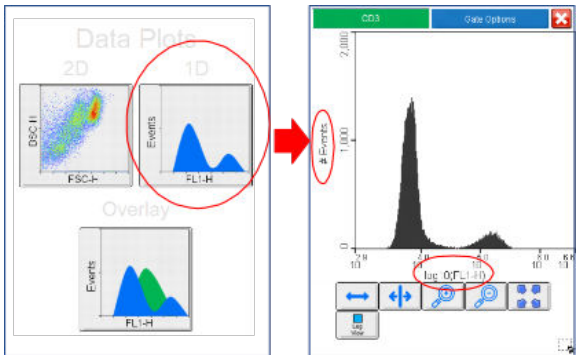


Figure 29. Creating univariate plots.

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 two or more data sets in the same axis for analysis of several samples in one graph to compare controls with diverse experimental outcomes.

To create an overlay plot:

1. On the Data Plots display, click the “Overlay Plot” button (Fig 30 A).
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 (Fig 30 B).
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.

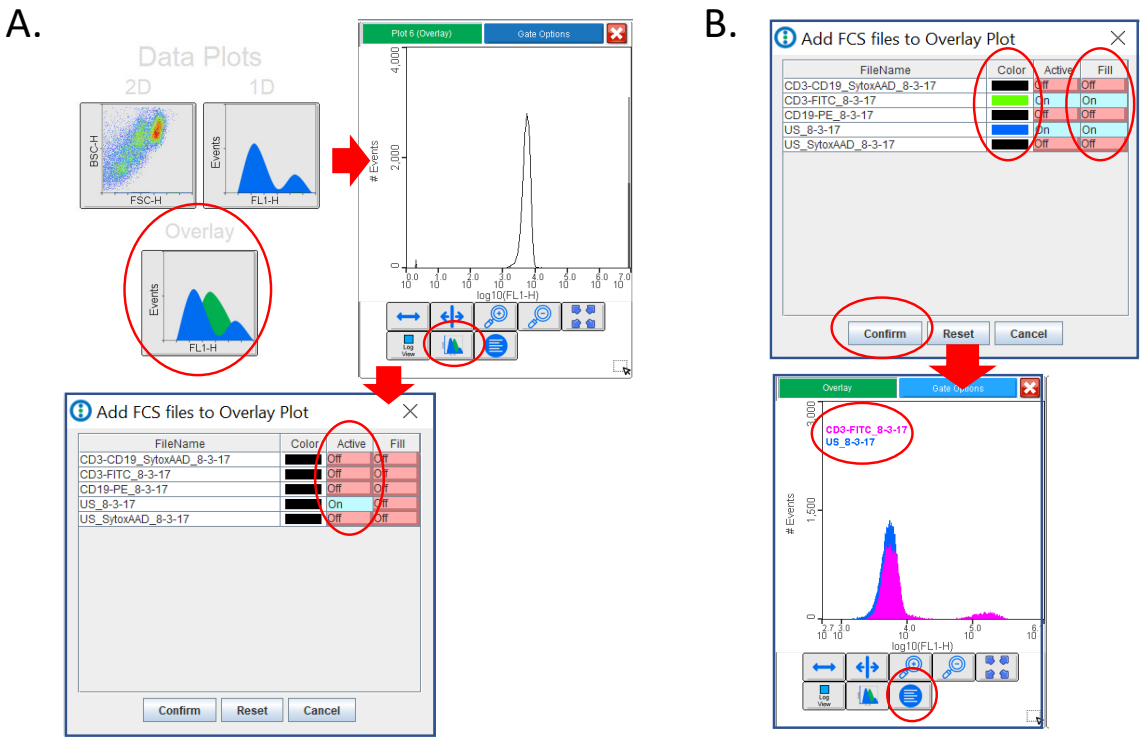


Figure 30. Creating univariate, overlay plots.

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 resolution.

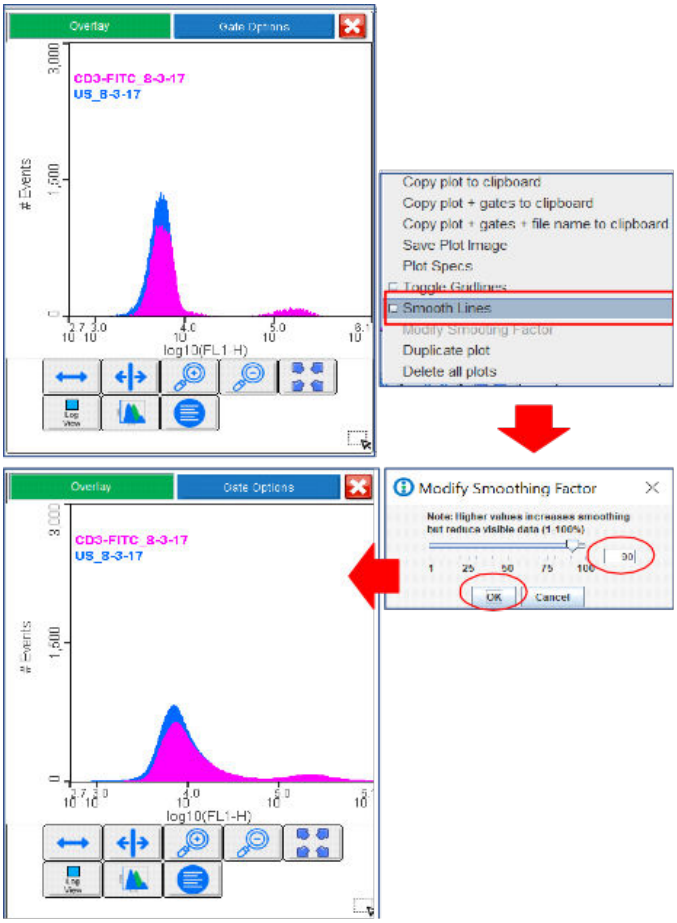


Figure 31. Smoothing lines in overlay plots.

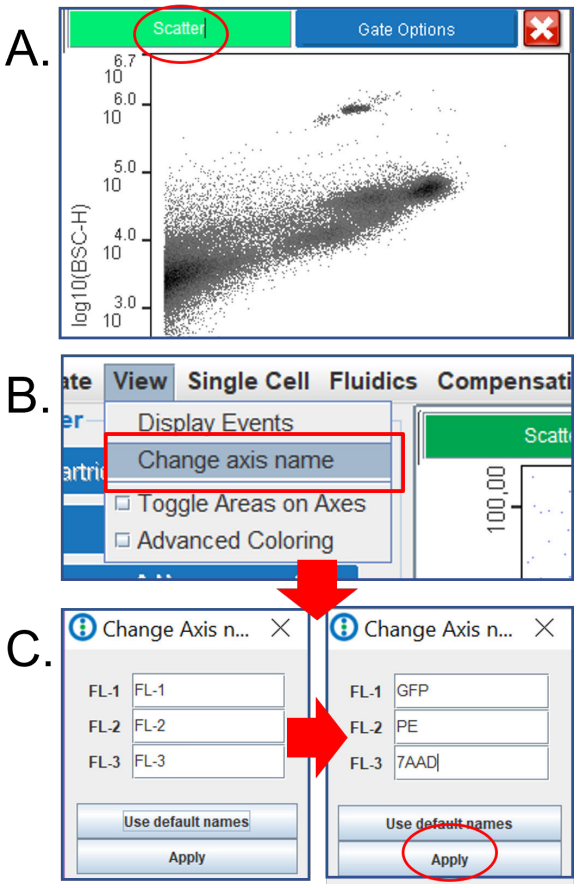


Figure 32. Changing plot and axis name.

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.

To change plot name:

1. Type the new plot name in the green box situated in the top left corner of the plot (Fig. 32 A).

To change axis name:

1. In the software main menu, select the “View” tab to access the “Change axis name” window (Fig. 32 B).
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. 32 C).

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 axes:

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

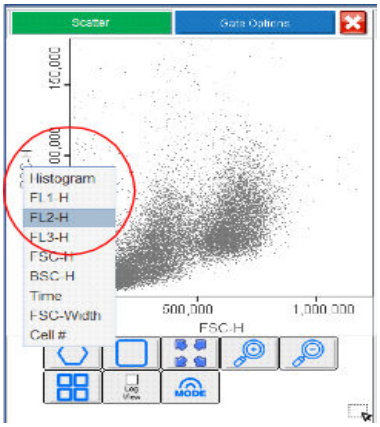


Figure 33. Changing plot axis.

4.2.3 Customizing Plot Axis Values

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

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.

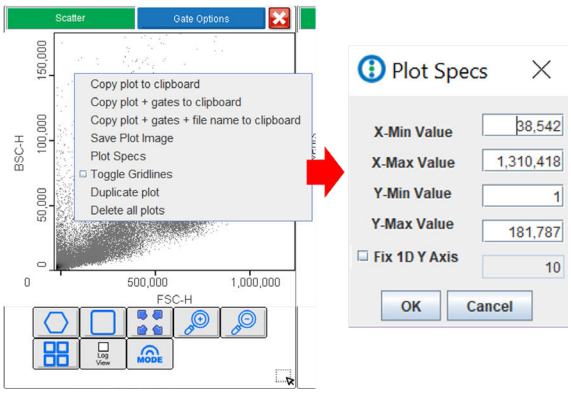


Figure 34. Setting new plot axis values.

4.2.4 Adding Gridlines to Plots

To add gridlines to plots:

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

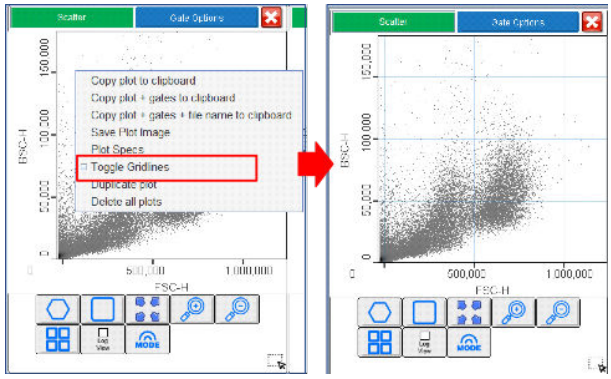


Figure 35. Adding gridlines to plots.

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. 36 A).
- **Rectangular gates** in bivariate plots are generally used to select big areas of data and in scatter versus fluorescence plots to distinguish positive cells from negative cells (Fig. 36 B).
- **Quadrant gates** are used to divide the plot into four 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. 36 C).
- **Vertical gates** divide the univariate plot into two main regions. They are also useful for discriminating positive cells from negative cells (Fig. 36 D).
- **Horizontal gates** can be applied to histograms for analysis of several populations, like positive versus negative and higher versus lower expressor cells (Fig. 36 D).

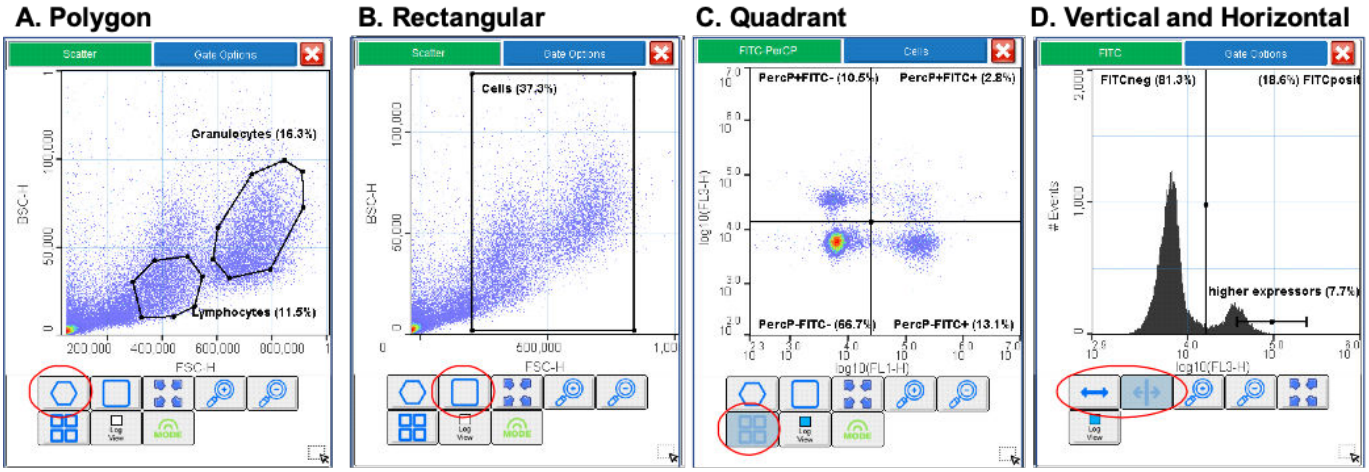


Figure 36. Different types of gates in WOLFViewer.

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. 37 A).
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. 37 B).

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.

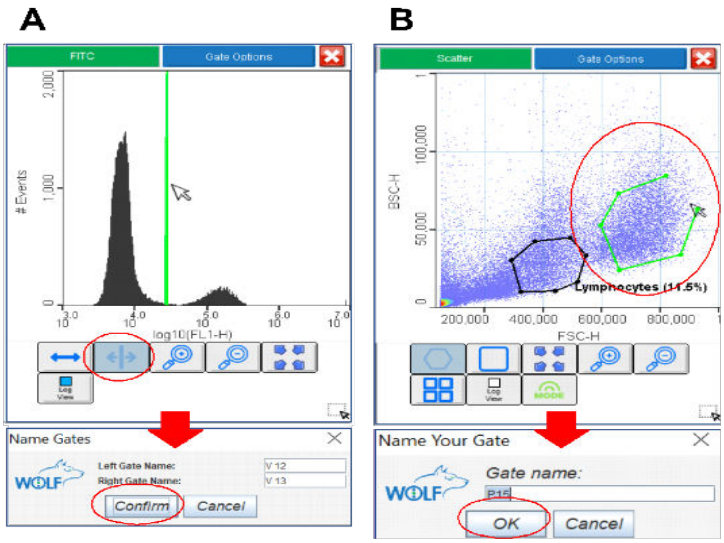


Figure 37. Creating plot gates.

4.3.2 Gate Tools

To delete and rename gates:

1. Position the mouse on the gate limit.
2. Right-click and select the desired option (Fig. 38).

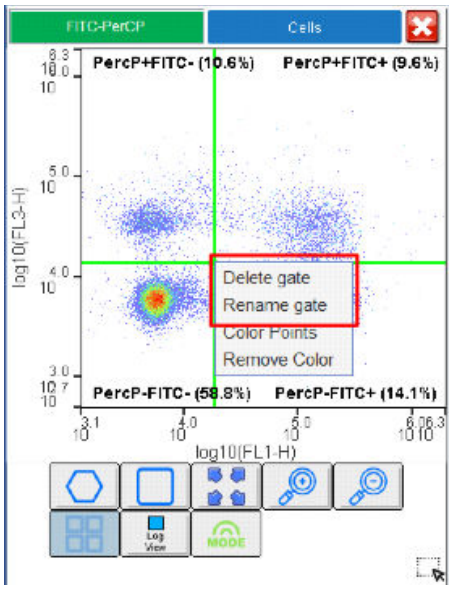


Figure 38. Deleting or renaming gates.

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”.

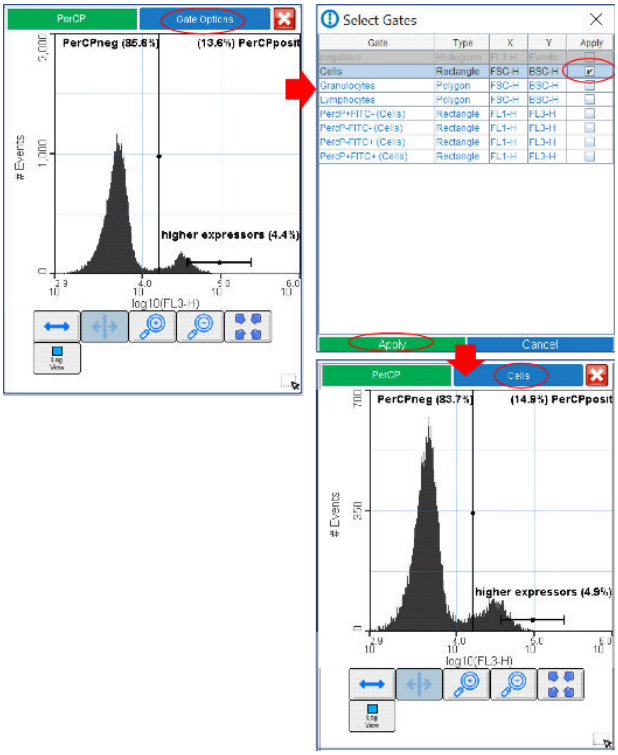


Figure 39. Applying gates to plots.

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.

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. 40).
3. Up to ten gates can be colored in a plot. Gates in different plots can also be colored. The color pattern will appear superposed (Fig. 41).
4. To remove the gate color, right-click on the gate and select “Remove Color”.

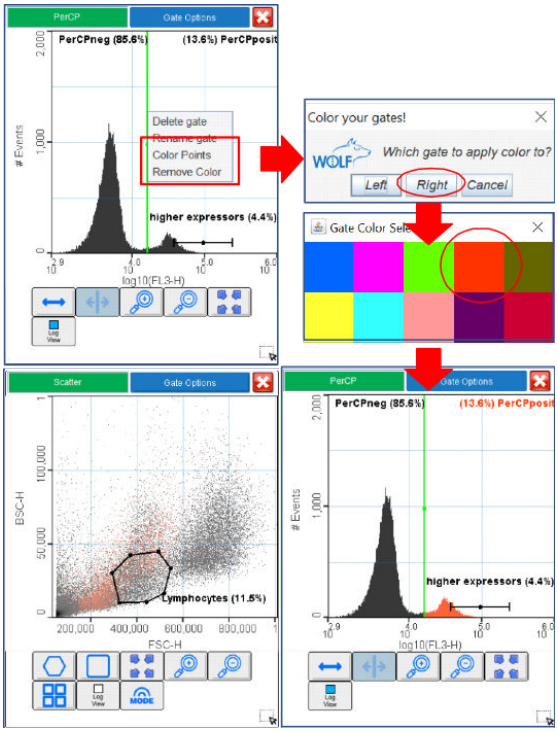


Figure 40. Applying color to gates.

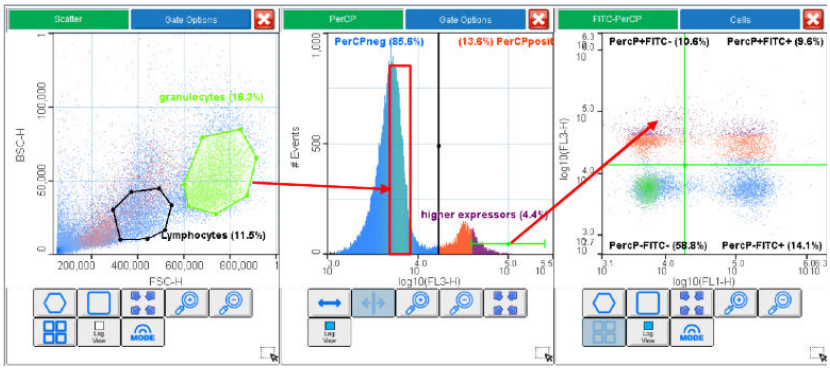


Figure 41. Applying color to gates in different plots.

4.3.5 Other Plot View Options

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.

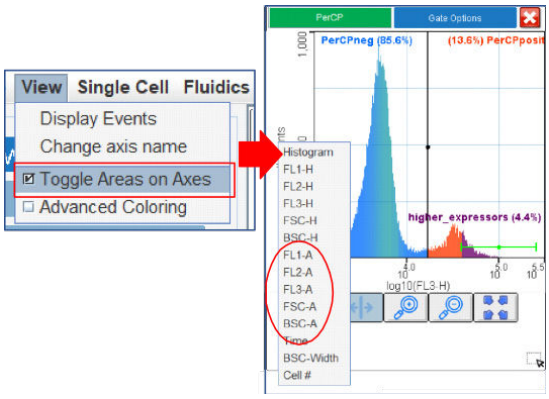


Figure 42. Adding Area to data plots.

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.

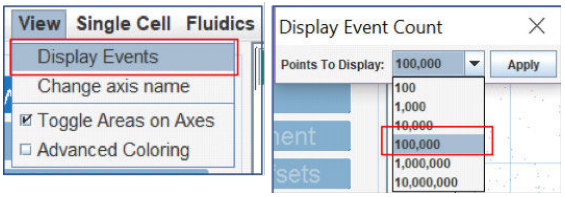


Figure 43. Changing event display in plots.

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.

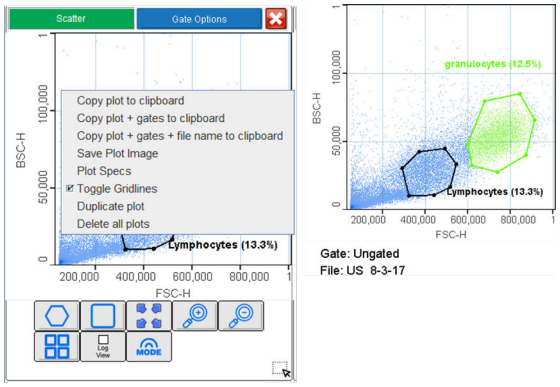


Figure 44. Copying data plots.

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, 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.

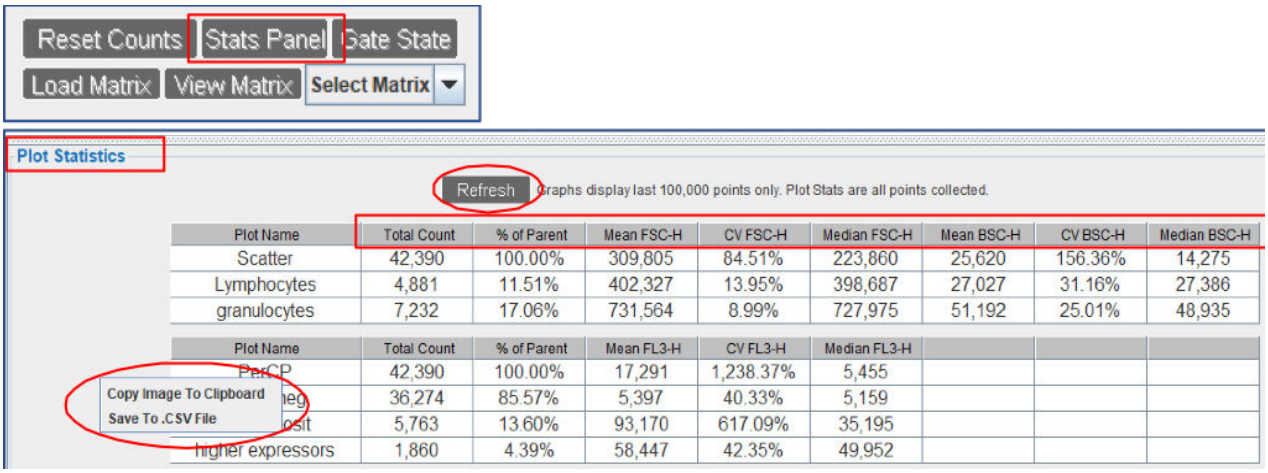


Figure 45. WOLFViewer Stats Panel.

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.

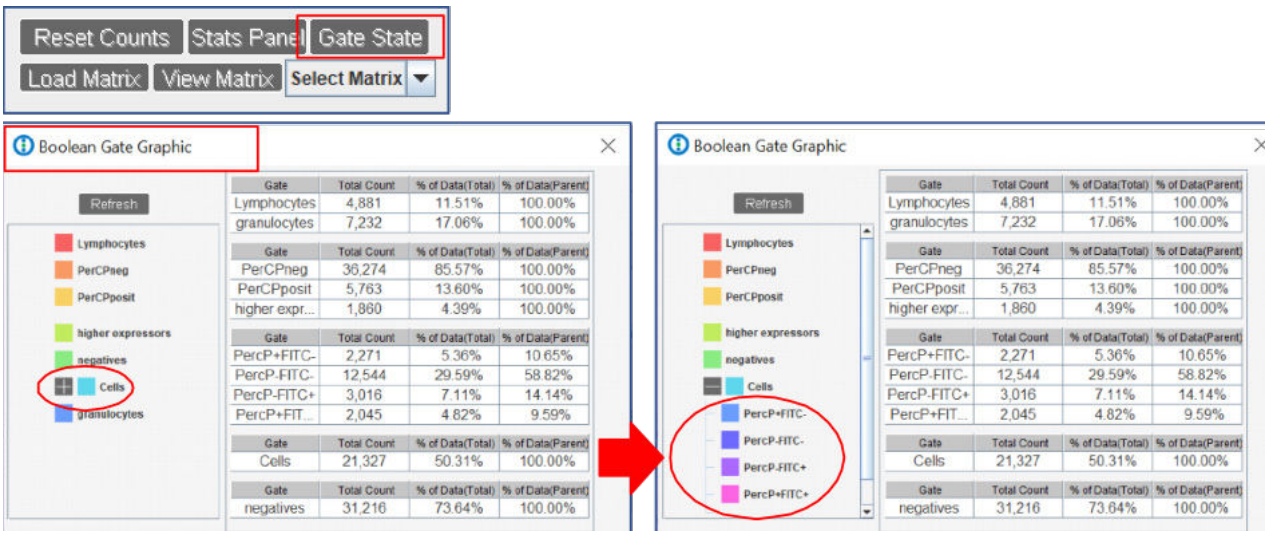


Figure 46. Expanding sub-populations within the Gate State tool can be done by clicking the “+” icon by the parent gate name.

5. Compensation

5.1 Why Compensation?

In multicolor flow cytometry assays, compensation is needed to prevent spectral overlap of fluorochrome emissions into neighboring filters. Whenever more than one color is involved, compensation is necessary and should be planned for.

5.2 What is Compensation?

Fluorescent compensation is a mathematical method to correct for fluorochrome spillover by creating a spillover matrix. “Spillover” or spectral “crosstalk” occurs when the emission spectrum of a fluorochrome is sensed by a detector that is assigned to another fluorochrome (Fig.46 A-C). The reason this happens is that emission spectra exhibit a maximum value at well-defined wavelengths of light (this is typically called the emission wavelength of a fluorophore), but they actually emit light within a range of wavelengths. Part of this emission peak may fall into the neighboring light detectors meant to capture the maxima of other colors. This spectral crosstalk is measured by analyzing individual fluorochromes (single-stained controls). **For the following examples, FL1 through FL3 refer to arbitrary detectors.**

These controls allow the software to assess the emission wavelengths falling within non-specific detector channels (for example, FITC signal in the FL2 and FL3 detectors), and then to correct for this signal by subtracting these values from the non-specific detectors (Fig.46 A-C). The values are computed to generate a spillover matrix that will show the fraction or percent of each fluorochrome bleeding into other channels. This is essentially a visual representation of the spectral overlap within your panel of fluorophores (Fig.46 A-D). A compensation matrix, generated by inverting the spillover matrix, is then applied to the sample fluorescence intensity values in each channel to correct for spillover mathematically.

Compensation should be employed whenever an experiment contains 2 or more fluorochromes. The workflow involves first analyzing an unstained or parent population, followed by single-color controls for each active detection channel to allow automatic calculation of the spectral overlap for each detector.

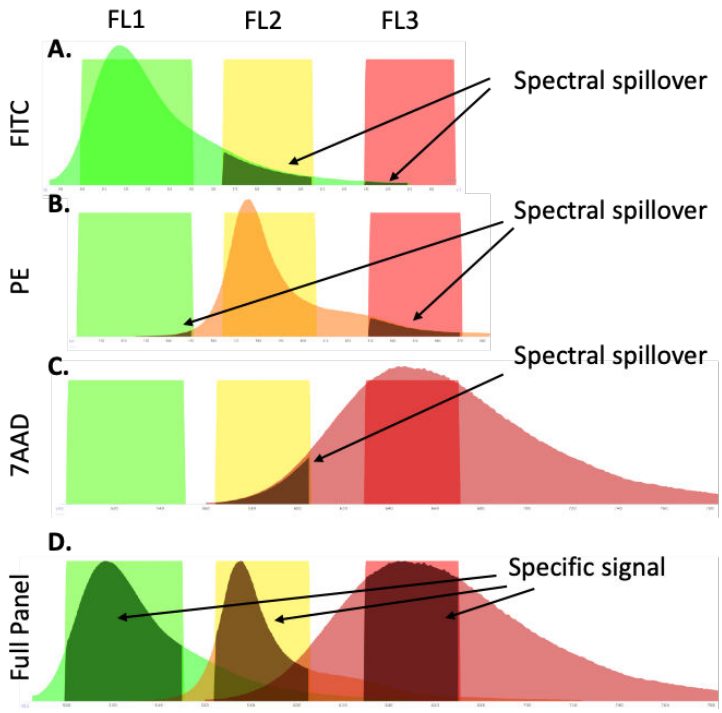


Figure 47. Visualization of spectral spillover. Y axis indicates fluorescence intensity (peak height), and X axis represents visible light wavelengths (~499 to 780 nm). The solid color blocks are bandwidth filters that divert fluorescent signal to FL detectors. **A.** FITC emission spectra are detected specifically in FL1 detector (green). However, a small percentage of it will “spill over” into FL2 and FL3. **B.** PE emits in FL2, however, neighboring detectors FL1 and FL3 may capture part of the spectrum. **C.** Same is true for 7AAD DNA stain that emits in FL3 but can also be detected to a lesser degree in FL2. **D.** All three fluorophores can still be combined into a 3-color panel, since compensation will subtract the spillover to only display specific signal, shaded here.

Figure 46. WOLFViewer Gate State Panel.

5.3 WOLF G2 Configurations

Fluorescent panel design for the WOLF G2 2-laser system offers many possibilities. The system operates using up to 9 fluorescence detectors per experiment. Each configuration is uniquely tailored to different research needs. Please review Figure 48 for an overview of each optical configuration.

Figure 48. WOLF G2 optical configurations. Each configuration contains a blue (488 nm) laser, plus an additional custom laser (either 405, 561, or 637 nm). This changes the available color number (9 total for 405 and 561 nm configurations, and 7 total for 637 nm configuration). **A.** 405/488 nm configuration offers 9 fluorescent channels and is best for researchers working with green and blue fluorescent proteins and antibodies. **B.** 488/561 nm configuration offers 9 fluorescent channels and is best for researchers working with green and red fluorescent proteins and antibodies. **C.** 488/637 nm configuration offers 7 fluorescent channels and is best for researchers requiring flexibility for work with APC-based antibodies or far-red fluorescent proteins.

When planning work around Brilliant Violet (BV) antibodies, DAPI and blue fluorescent proteins, the researcher may consider the 405 nm laser. For research involving red fluorescent proteins and antibodies, the 561 nm laser line may be ideal. If flexibility to work with APC conjugates is required, one may consider the 637 nm laser configuration.

5.4 Fluorescent Panel Design

5.4.1 Principles of Choosing Conjugates for Labeling Antibodies

The traditional wisdom of panel design holds that, if given the option, the user can customize their fluorescent label (whether a fluorescent protein or a covalent fluorescent antibody conjugate) so that all the labels are compatible with each other, as well as the cytometer. The following are a few simple rules to help you design your experiment. Please consult with a cytometrist if in doubt.

1. **The most highly expressed markers should be paired with the dimmest fluorophores, and the rarest markers with the brightest fluorophores.** Following this advice can improve detection of weakly expressed markers and avoid having to readjust detectors because of overly bright labeling of common molecules. Generally, more molecules of the fluorophore, and the higher its brightness, the better the detection of that labeled antigen For context, there are approximately 10^5 T cell receptors (and accompanying CD3 molecules) expressed on the surface of a cytotoxic T lymphocyte.
2. **Minimize spectral overlap between the fluorochromes in your system as much as possible.** Use a spectral analyzer to visualize predicted emission overlaps in your panel. A number of free websites provide this service. Your WOLF G2 filter configuration can be found in Figure 48.
3. **Place bright markers together**, i.e. avoid grouping common and rare proteins within one detector. This will allow you to set a common voltage (sensitivity) without potential risks to the resolution of your marked populations. See more below.
4. **Be mindful of tandem dyes and their potential to degrade**. This may release the donor molecule signal and contribute to compensation issues.

5.4.2 Combining Colors on a Detector

From a technical point of view, the lasers are modulated (alternated) within the system, and most detectors will be assigned two colors at a time (one from each laser). While the detectors will be able to detect and discriminate signals from the two lasers based on the timing of the signal, the gain of the individual detector may be shared between the two lasers. You will see this in the software when adjusting the detector voltage.

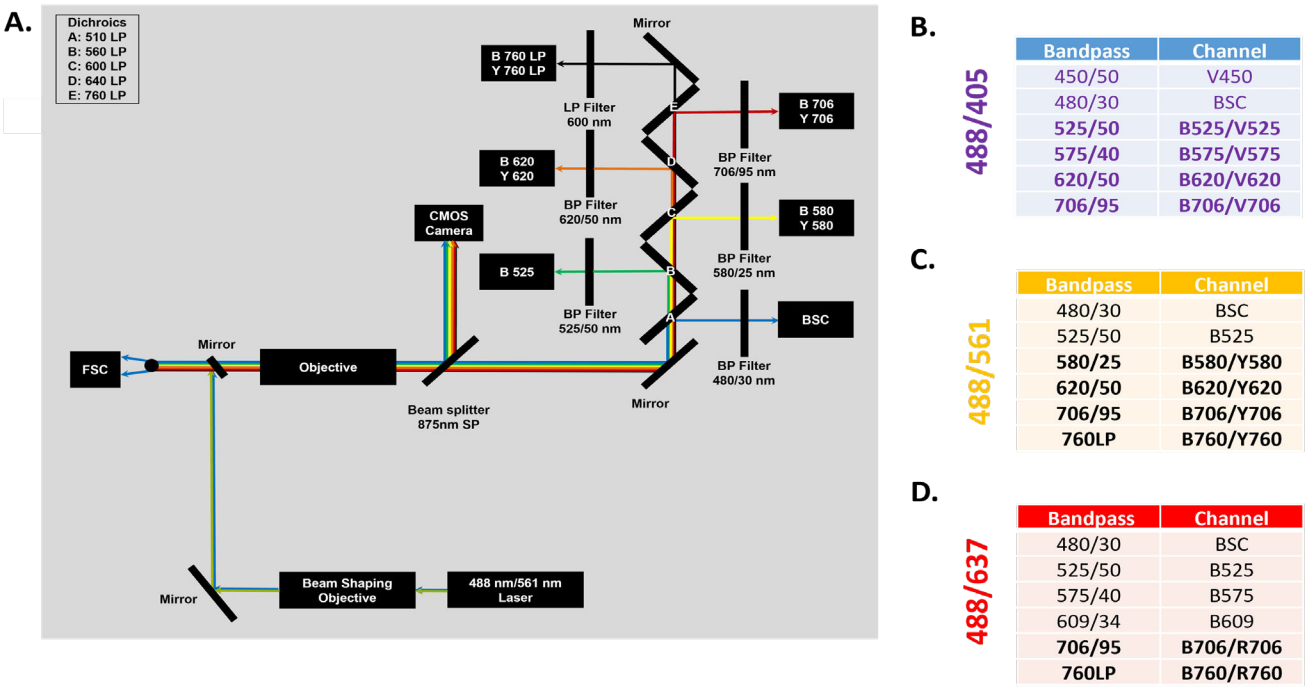
It is essential to pair fluorochromes of similar intensities to be detected by the same detector to make sure that the gain will work for both fluorochromes. If two fluorochromes of very different intensities are paired on the same detector, adjusting the gain to work for both may be challenging. Please see below for a list of paired colors on each detector (Fig. 49). To check fluorophore brightness, refer to manufacturer's informational materials. Typically, brightness is indicated as a relative measure to other fluorophores on offer, and is published by the reagent manufacturer.

5.4.3 Building a Panel for your Experiment

Please see the fluorophore charts on the following pages to review suggested fluorophores for each channel.

To use the charts, first, identify your WOLF G2 configuration. Find the chart with the secondary laser for your machine (405 nm, 561 nm, or 637 nm). Second, find the category of the marker that you are looking for in the right-hand columns (fluorescent protein or an antibody conjugate), and look for the name of your favorite marker to identify the best detector for it.

Please keep in mind that some markers will be excited by more than one laser (ex. PerCP or PE) in certain configurations. This may impact your fluorophore choices. See the notes at the bottom of the chart.



B.

488/405

Bandpass	Channel
450/50	V450
480/30	BSC
525/50	B525/V525
575/40	B575/V575
620/50	B620/V620
706/95	B706/V706

C.

488/561

Bandpass	Channel
480/30	BSC
525/50	B525
580/25	B580/Y580
620/50	B620/Y620
706/95	B706/Y706
760LP	B760/Y760

D.

488/637

Bandpass	Channel
480/30	BSC
525/50	B525
575/40	B575
609/34	B609
706/95	B706/R706
760LP	B760/R760

Fig 49. Fluorescent detector set for each available WOLF G2 configuration: A. Detector setup: the fluorescence detectors may be shared between two fluorophores if the two are excited by different lasers. A-E indicates long-pass pre-filters (cut off light below wavelength noted in Dichroics table). Diagram shown for 488/561 nm configuration. **B**. 488/405 nm WOLF G2 configuration will combine detection of 8 channels on four shared detectors. **C.** Analogous representation of 488/561 nm configuration. **D**. Chart for the 488/637 nm configuration.

5.5 Best Compensation Practices and Controls

To successfully compensate a sample, proper negative and single-color fluorescent controls are needed. One control is needed for each fluorochrome; the more complex your experiment, the more single-color controls will be needed for automatic compensation.

To prepare compensation controls:

1. Prepare your fluorescent compensation controls (one for each color) by labeling your cells with each individual fluorochrome.
2. It is recommended that the brightness of your fluorescent controls be at least as bright as, but ideally brighter than, your sample. This can be achieved by using compensation beads or cells known to express high amounts of the target marker. **The fluorescent compensation controls must be uniform throughout the whole experiment – either cells or beads, but not both.**
3. Label your cells or beads with each individual fluorochrome. Use the same fluorochrome that is used in the sample you wish to compensate (do not use AF488 to compensate FITC). **If you are working with a new antibody, a titration may be recommended to determine the best concentration for your sample.**
4. Prepare an unstained control.

5. Your WOLF G2 uses a universal negative control. This means beads and cells cannot be used together to calculate compensation. **Autofluorescence, which is much different between beads and cells. This can affect compensation. That is the reason to use only beads or only cells across all the compensation controls.**
6. Analyze the negative control to establish the negative and positive gates in all channels. Acquire at least 10,000 events in the gates used for compensation to obtain accurate median values.
7. Analyze the positive controls. Acquire at least 10,000 events in the gates used for compensation to obtain accurate median values. Establish optimal detector gains for each fluorochrome to avoid saturating signals (events against the axes walls).
8. **The values of the gains for each detector must stay constant across all the compensation controls**. If a gain needs to be modified, all the compensation controls will need to be re-acquired for the compensation to work properly. Use the same detector gains for your sample of interest as for your controls.
9. **We recommend acquiring your fully stained control and adjusting detector gains to appropriate levels before moving on to acquire all your single-stained controls. This will save you time.**
10. Once all controls are acquired, you can calculate the compensation matrix and apply to your sample of interest. The experimental conditions of your sample of interest (fluorochromes, gains) must be the same as those used to acquire the compensation

5.6 Automatic Compensation

The WOLFViewer auto-compensation algorithm processes the data of every control and automatically calculates the appropriate compensation for each fluorochrome combination.

However, appropriate controls should be analyzed for accurate compensation. See notes in the previous section. Currently, WOLFViewer facilitates generating automatic compensation matrices as well as manual editing of existing matrices.

We recommend using automatic compensation algorithms to correct for spillover with high accuracy. The automatic compensation feature will analyze the single stained and unstained controls to generate the values for the compensation matrix without further user input. If results after applying a matrix are not as expected, the user should look at redesigning the controls, or adjusting the matrix using the Edit Compensation Matrix functionality.

NOTE: Auto compensation in WOLFViewer may require the application of "automatic offsets" if compensated values appear as zeros on the axes.

To create a compensation matrix with automatic compensation:

1. Select "Automatic Compensation" from the Compensation tab in the main menu (Fig. 50 A).
2. Click the "Load Files" button to open the .fcs files that were generated for the single-stained and unstained controls (Fig. 50 B).
3. Choose the files to load. A minimum of 3 files (2-color compensation with unstained control) must be loaded to initiate the process. Every fluorochrome or dye represented in the experimental sample must have its corresponding single-color control to build a matrix (Figure 50 B).
4. Click the "Select" button.

5. Assign the control files to the correct detector channel by checking the boxes (Fig. 50 C). The unstained control should be checked as “Blank”.

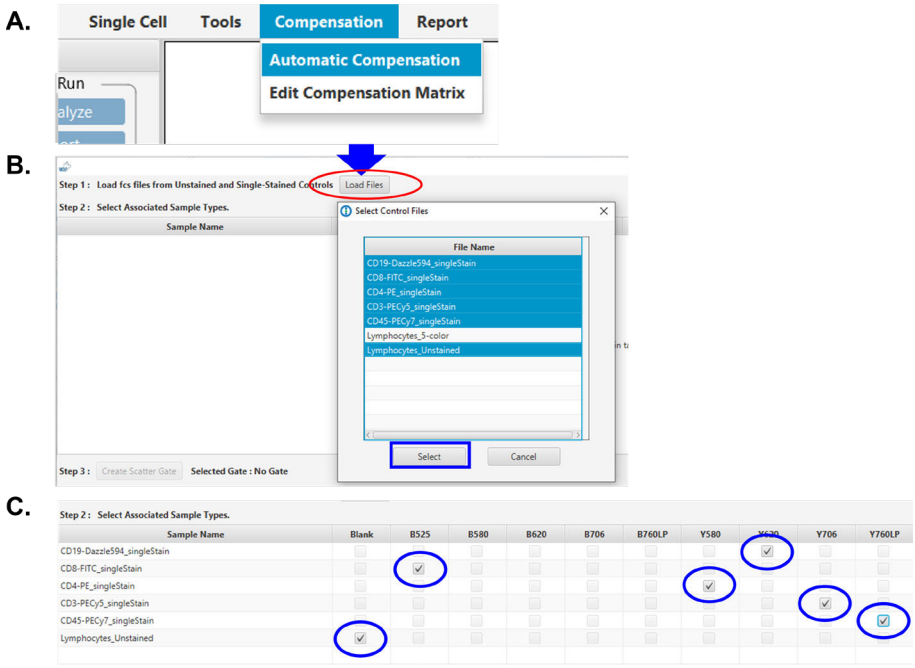


Figure 50. Automatic Compensation Workflow, Part 1: Selecting Controls. A. Open the auto-compensation menu **B.** Load controls by using the “Load Files” button and select the correct files from the list. **C.** Assign the files to the corresponding channels.

After the control files are selected, the user will create a general light scatter-based gate to discriminate cellular fluorescence signals from those coming from debris or other particles.

6. Click “Create Scatter Gate” under Step 3, and select “Draw New Gate” (Fig. 51 A). The software will automatically open the appropriate FSC-FL1 scatter plot from the blank (unstained) control. The user will draw a gate using all the available plot tools, name it and click “Apply”.

NOTE: if the user already drew gates on the plots during analysis and wishes to use these gates instead of drawing new ones, instead of clicking on “Draw New Gate,” the user will select “Use Exisiting Gate” and pick the appropriate gate from the “Select Gates” window (Fig. 51 B-C).

7. Click “Gating Wizard” (Fig. 52 A), select “Draw New Gate” (Fig. 52 B) and name your control gate (Fig. 52 C). WOLFViewer will automatically open a scatter plot with the X-axis matching your control sample fluorescence channel vs backscatter on the Y-axis. This will be FL1 for the unstained control, FL1 for FITC, FL2 for PE, etc.
8. Once the first gate has been applied, WOLFViewer will open new plots, one-at-a-time, to create gates for all single-channel controls (Fig. 52 D).
9. Click “Create Spillover Matrix” (Fig. 53 A) and then save (.cmat format). Note default <Enable auto-offsets> object.

This function helps to display negative and null data.

NOTE: One should save a matrix with and without auto-offsets to be able to compare the impact on your experiment.

10. WOLFViewer will automatically display the spillover matrix with updated values in the fluorescence detectors (columns) versus the fluorochrome signal (rows) (Fig. 53 B).

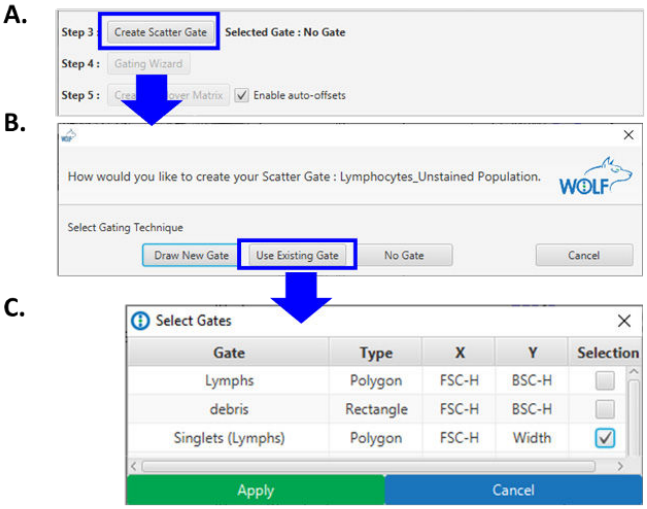


Figure 51. Automatic Compensation Workflow, Part 2: Assigning Channels to Controls and Gating. A. Select or create gates. **B.** User decides between drawing new gate or using a pre-existing gate. **C.** All pre-existing gates are listed and the choice can be approved via the “Apply” button.

The user will now proceed to create gates in the fluorescence channels, taking into consideration that gated cells should be within the dynamic range. (Do not gate events that are too close to the axes. If this happens, analyze your controls again with adjusted detector gains). 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, relying on the negative or FMO control to discriminate positive and negative signal.

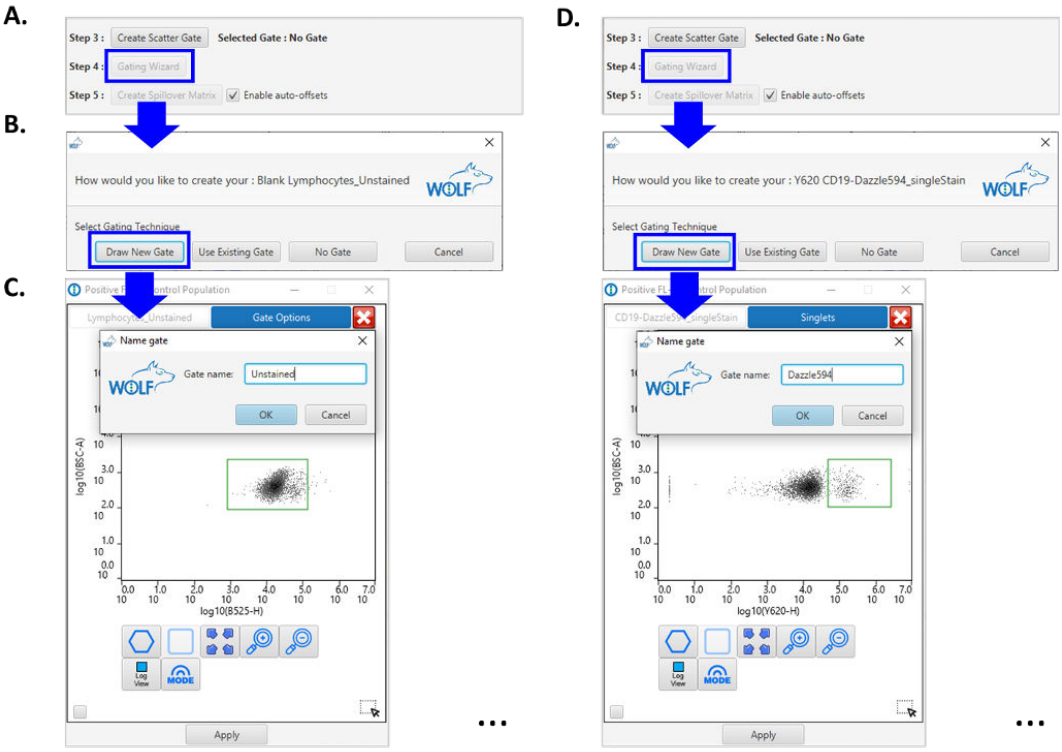


Figure 52. Automatic compensation workflow, Part 3: Selecting the fluorescent populations. **A.** Enter the gating wizard. **B.** Choose new or pre-existing gate. **C.** The correct plot is automatically preloaded. Select and name the unstained population and click “Apply”. Values between 10^3 and 10^4 are typically negative populations. **D.** The user is guided to select the positive (fluorescent) cells from the control files.

A.

B.

Source	Target								
Spillover	B525	B580	B620	B706	B760LP	YS80	Y620	Y706	Y760LP
B525	100.28	0.0	0.0	0.0	0.0	-0.14	-0.09	-0.04	-0.01
B580	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
B620	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0
B706	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
B760LP	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
YS80	2.88	0.0	0.0	0.0	0.0	19.43	77.07	0.61	0.01
Y620	-1.21	0.0	0.0	0.0	0.0	1.96	97.94	1.27	0.04
Y706	-2.09	0.0	0.0	0.0	0.0	1.75	40.35	56.5	3.48
Y760LP	6.9	0.0	0.0	0.0	0.0	5.82	23.83	5.31	58.13

Step 6 : Finished

Figure 53. Automatic Compensation Workflow, Part 4: Creating Spillover Matrix. A. After the Gating Wizard is complete, create the matrix in Step 5. Please note that you should save a matrix with and without auto-offsets to be able to compare the impact on your experiment. **B.** Your spillover values will display below. Save the matrix and click “Finished.”

5.7 Applying Compensation

To compensate prior to sorting and real-time flow analysis:

1. Load a .cmat file in the software by creating compensation via the wizard, or by clicking the “Load Matrix” button (Fig. 55 A).
2. When starting sample analysis or sort, check the “Compensation?” box in the “Name for New Sample” window (Fig. 54).
3. Select the desired compensation matrix from the dropdown and proceed with analysis or sort.

NOTE: Compensation matrices can be used to analyze existing sample files, but also applied to new samples. Once loaded, the most recent compensation matrix will be applied to the next analyzed sample by default. To disable this, deselect the “Compensation?” box in the “Analyze” window (Fig. 54).

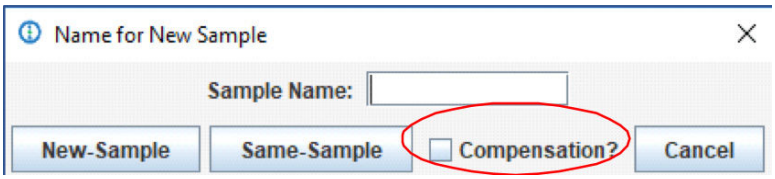


Figure 54. Applying compensation to new samples.

To retroactively apply compensation to an existing FCS file

1. Load a .cmat file into WOLFViewer by clicking the “Load Matrix” button (Fig. 55 A). If the matrix was created within the current WOLFViewer session, proceed to step 2 directly.
2. Right-click the active FCS file, select “Apply Compensation” (Fig. 55 B).
3. WOLFViewer will generate a new FCS file with the loaded matrix applied.

NOTE: Displayed below are two compensated FCS files with the same compensation matrix applied generated with (Fig. 55 C) or without (Fig. 55 D) auto-offsets enabled.

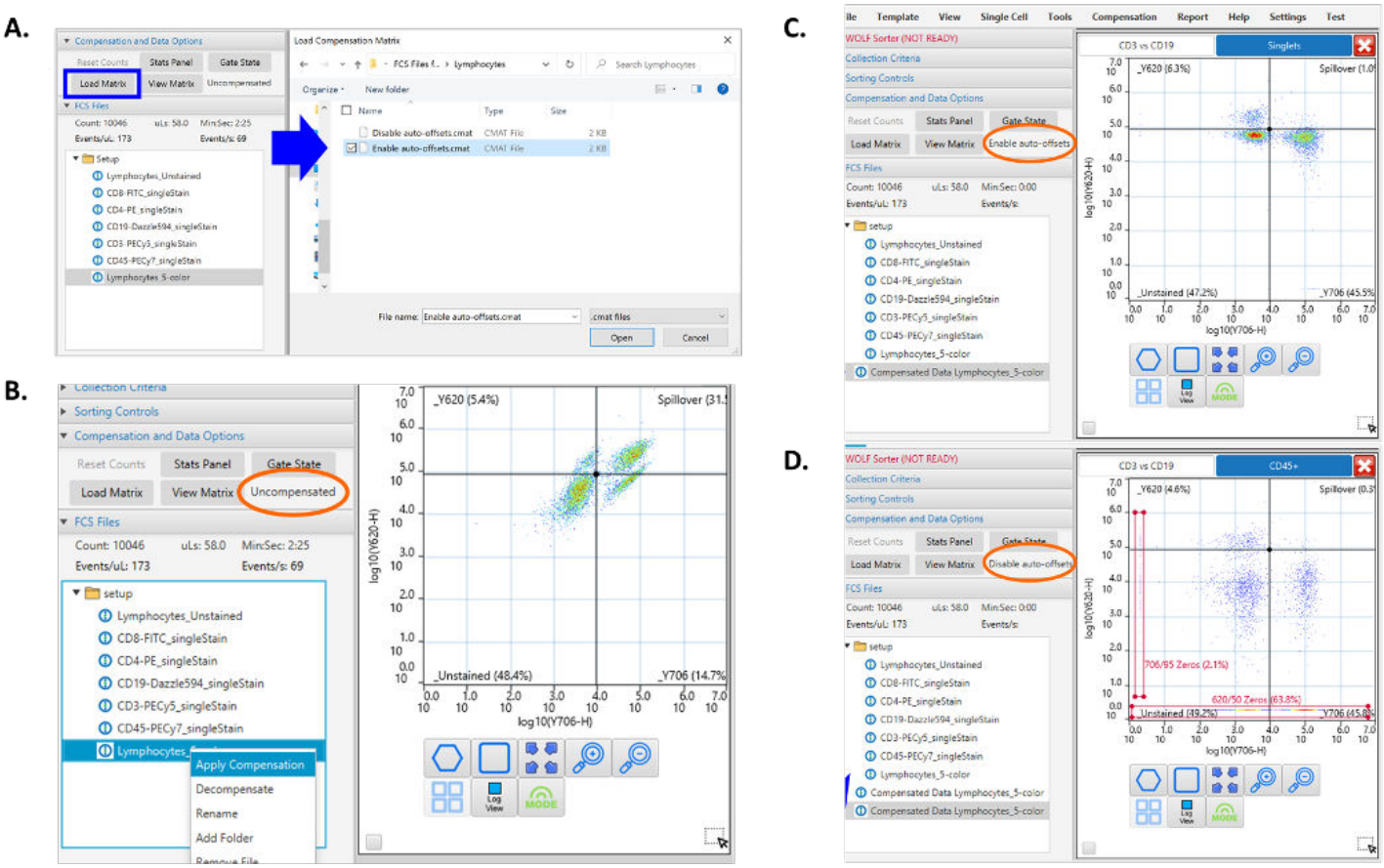


Figure 55. Applying compensation matrices to existing files. A. Load a pre-existing compensation matrix by choosing its location via the "Load Matrix" button. **B.** Select an uncompensated FCS file from the list. Right-click the file and choose "Apply Compensation". Select your pre-loaded matrix name. **C.** Compensation matrices are calculated with "auto-offsets" by default. **D.** When auto-offsets are disabled, negative or null data points may be displayed close to the plot axes.

5.8 Editing an Existing Compensation Matrix Manually

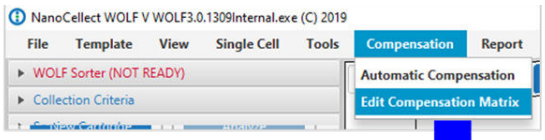
Manual compensation is the process of adjusting compensation based on how the data visually looks in the plots. WOLFViewer offers the option to adjust a compensation matrix by editing the associated spillover matrix in the wizard. The user may choose to do this to correct a mathematical error resulting from mismatched controls or another unexpected experimental error. To adjust individual fields in a matrix, the user will need to know how to read it.

HINT: Look for the "Source" designation in Fig. 10 B. That column can be mentally relabeled as "My Fluorophores". For example, B525 will become FITC. Then look for the "Target" designation (Fig. 10 B). Those columns can mentally be labeled as "My Detectors". And so, the first field in the first column, FITC, should be 100% detected in the B525 detector. And so on with the other fluorophores and detectors.

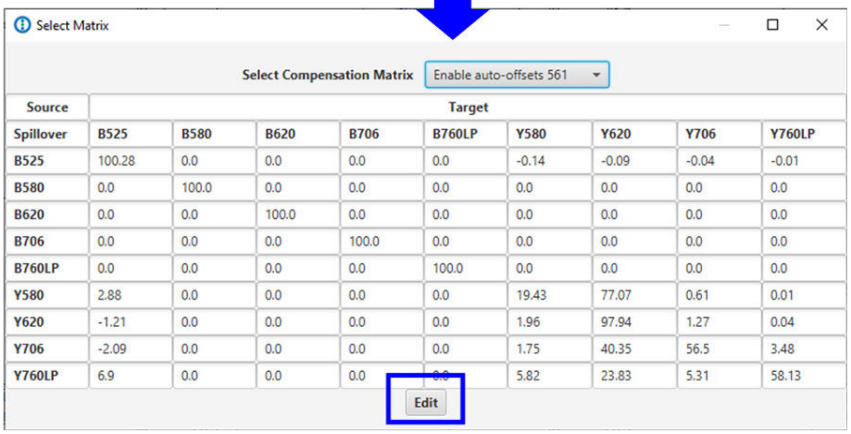
To edit a compensation matrix:

1. Load a .cmat file in the software by clicking the “Load Matrix” button (Fig. 55 A).
2. Select the file to compensate by clicking on it within the FCS Files Panel.
3. From the Compensation tab in the main menu, select “Edit Compensation Matrix” (Fig. 56 A).
4. With the desired spillover matrix displayed, click “Edit” to proceed (Fig. 56 B).

(A)



(B)



Source	Target								
Spillover	B525	B580	B620	B706	B760LP	Y580	Y620	Y706	Y760LP
B525	100.28	0.0	0.0	0.0	0.0	-0.14	-0.09	-0.04	-0.01
B580	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
B620	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0
B706	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
B760LP	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0
Y580	2.88	0.0	0.0	0.0	0.0	19.43	77.07	0.61	0.01
Y620	-1.21	0.0	0.0	0.0	0.0	1.96	97.94	1.27	0.04
Y706	-2.09	0.0	0.0	0.0	0.0	1.75	40.35	56.5	3.48
Y760LP	6.9	0.0	0.0	0.0	0.0	5.82	23.83	5.31	58.13

Figure 56. Edit Compensation Matrix functionality. A. How to find the Edit Matrix menu. **B.** Once the file is selected, click “Enter” to change a particular matrix.

To edit a compensation matrix (continued):

5. Adjust apparent spillover displayed in the plots by engaging the arrows or typing values in the boxes and clicking enter (Fig. 57 A).
6. Save the new .cmat by clicking “Save Compensation Matrix” (Fig. 57 B) and type a new name for the manually edited compensation matrix (Fig. 57 C).

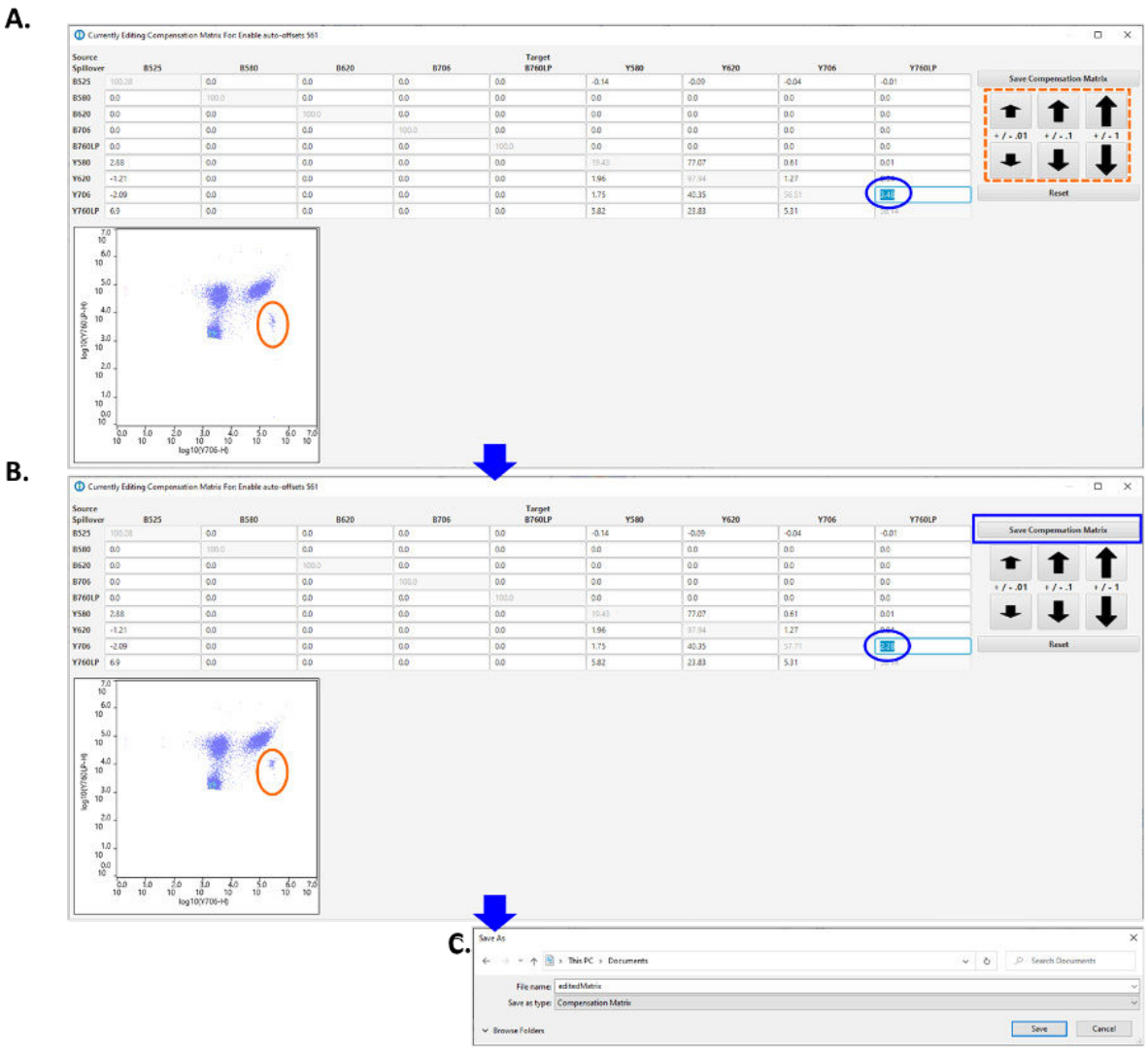


Figure 57. Manual edits to the compensation matrix: **A.** Identify the field to be adjusted based on data display. Here, the algorithm subtracted excessive signal from the Y760 detector (Y axis), causing the red-circled population to drop down. The user finds the Source (Y706) in the first column and lowers the Y760 detector compensation value. **B.** The population is now totally visible on the plot. **C.** The user saves the modified compensation matrix.

6. Bulk Cell Sorting

The user can perform 1- and 2-way sorting when using bulk-sorting cartridges.

To initiate bulk sorting:

1. If desired, select time, volume, total event count, or triggered counts in the Collection Criteria Panel to automatically stop the sort once that number has been obtained (Fig. 58 A).
2. Select one gate to each sorting channel in the Sorting 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. Click the “Sort” button.
4. Exchange the waste container for the collection tube holder. It will take approximately 1 minute for the sample volume to flow through the tubing and cartridge. Collection tubes may be added at this time (Fig. 58 B).
5. Click the “Pause” button to modify or change the gates used for sorting. Click “Resume” to continue sorting.
6. Click the “Stop” button to end the sorting process.
7. 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.
8. Before removing the collection tubes from the holder, pay attention into which tube each population is being sorted. Cells sorted to channel A are collected in the tube that is Away of the instrument. Cells sorted to the channel C 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. 58 B).

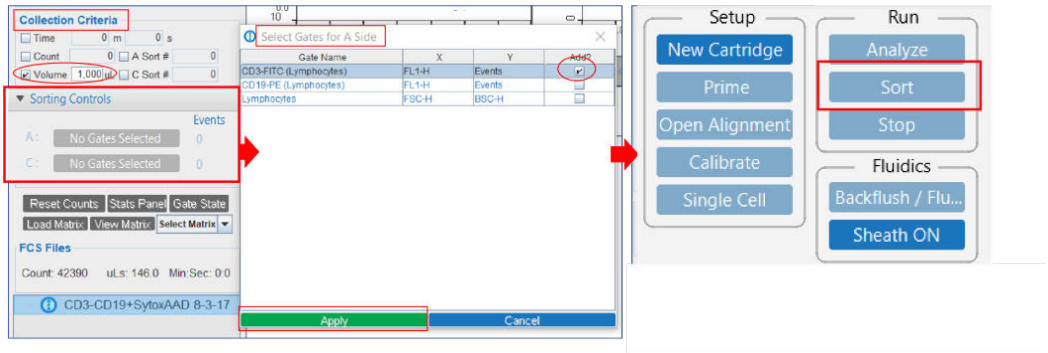


Figure 58 A. Bulk sorting process.

Figure 58 B. Collection tube holder.

7. Single-Cell Sorting

The WOLF G2 Cell Sorter, in combination with the N1 Single-Cell Dispenser, has the capability to simultaneously sort and dispense single cells into 96- and 384-well plates for clone generation and single-cell analysis.

7.1 N1 Single-Cell Module Setup

To set up the N1 Single-Cell Sorting Dispenser:

1. Connect the N1 adaptor stand to the WOLF G2 (Fig. 59). The stand should be positioned adjacent to the left edge of the WOLF G2. Secure the stand in place by pressing it firmly under the WOLF G2 and hitching the stand around the feet (Fig. 59 A-C). The notches of the stand fit the front and back left feet of the WOLF G2.
2. Place the N1 Single-Cell Dispenser on top of the stand. The N1 should be positioned so that the slot for the reagent reservoir and sorting plate are facing forward, and the two back feet of the WOLF G2 are inserted in the corresponding round areas on the stand (Fig. 59 D).
3. Connect the N1 to the WOLF G2 through the use of a DB9 cable.
4. Power on the N1 by pressing the module power button. The power button should be facing away from the WOLF G2, as shown in the image above (Fig. 60 A).
5. Insert the waste tray (reagent reservoir) into its respective slot within the N1 Single-Cell Dispenser (Fig. 60 B).
6. Attach the waste container to the magnetic tray receptacle on the WOLF G2 front panel.

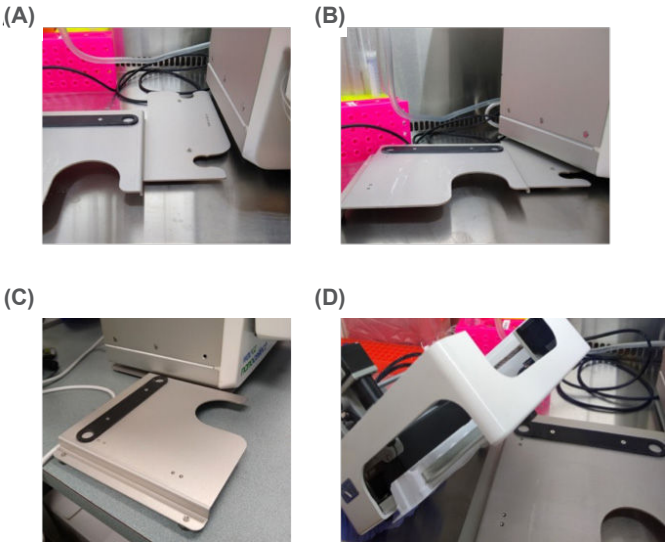


Figure 59. The N1 Single-Cell Dispenser setup.

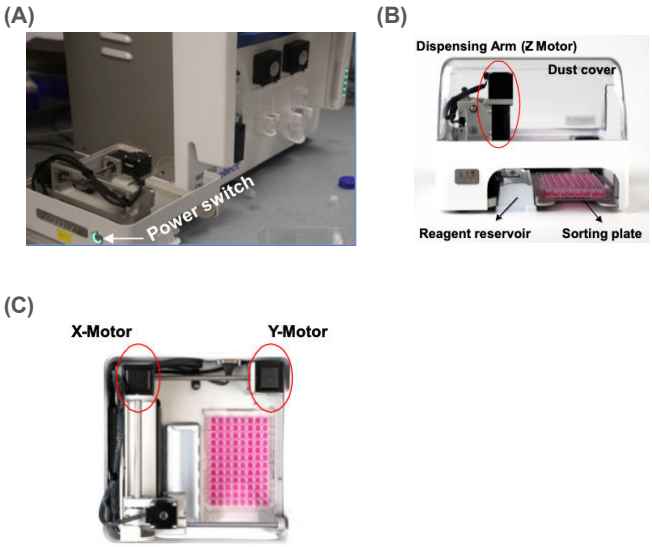


Figure 60. The N1 Single-Cell Dispenser anatomy.

7.2 N1 Single-Cell Dispenser Alignment

The Alignment Wizard is used the first time the N1 Single-Cell Dispenser is connected to a WOLF G2 Cell Sorter or 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.

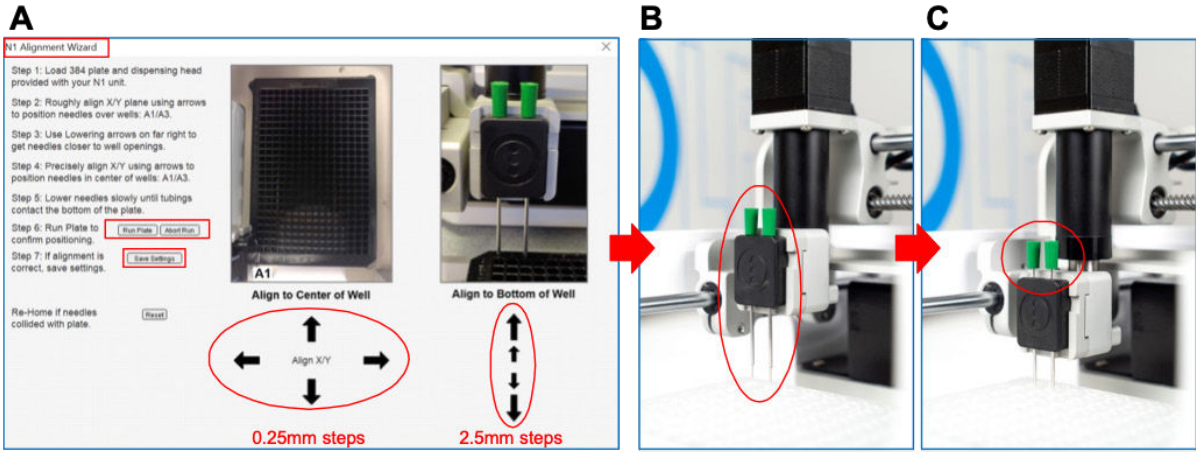


Figure 64. Steps in the single-cell sorting process.

To align the N1 Single-Cell Dispenser:

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. 64 B).
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. 64 C).
6. Use arrows under “Align to Bottom of Wells” to get needles closer to the well for easier alignment (Fig. 64 A).
7. Once the needles are precisely positioned at the center of the wells, begin the Z-plane alignment (Fig. 64 B).
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 the it contacts the bottom of the plate **and the bumpers move up slightly** (Fig. 64 C).
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.”

7.3 N1 Plate Setup

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

To create a new plate template:

1. Select “N1 Plate Setup” from the Single Cell main menu tab (Fig. 65).
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. 65).
6. Select the number of wells from the dropdown menu.
7. Select the appropriate plate type from the plate type dropdown menu. Compatible plate types include: flat bottom, round bottom, V-bottom, half-area, and PCR.
8. 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 the it contacts the bottom of the plate and the bumpers slightly move up.
9. Save the plate specifications by clicking the “Save Plate” button. We recommend naming the files with well and catalog numbers for easier future reference (e.g., #96-PCR #CLS6551). The new template will appear in the Single Cell Selection main menu.
10. 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.

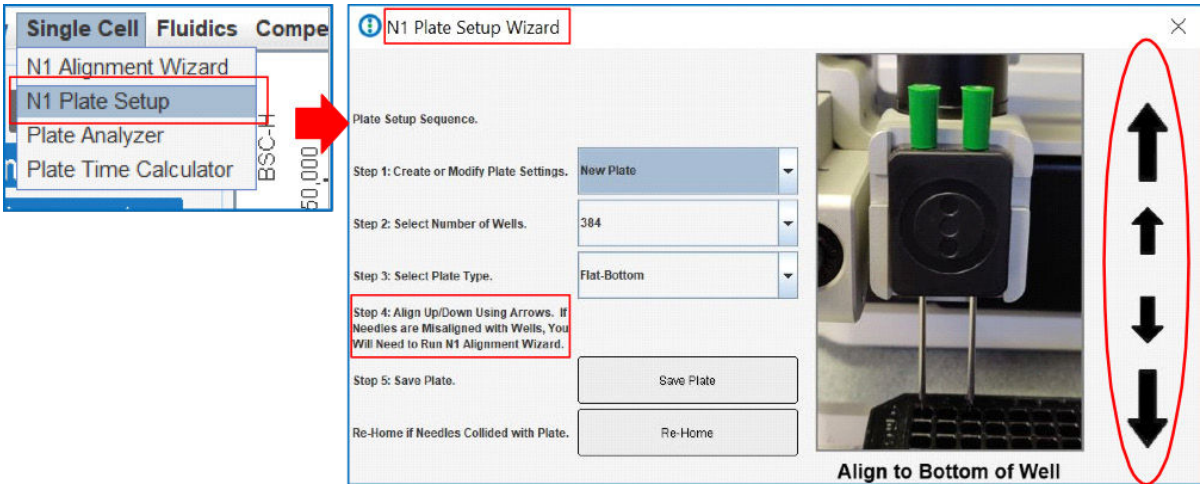


Figure 65. The N1 Plate Setup Wizard.

7.4 Single-Cell Cartridge Tubing Assembly

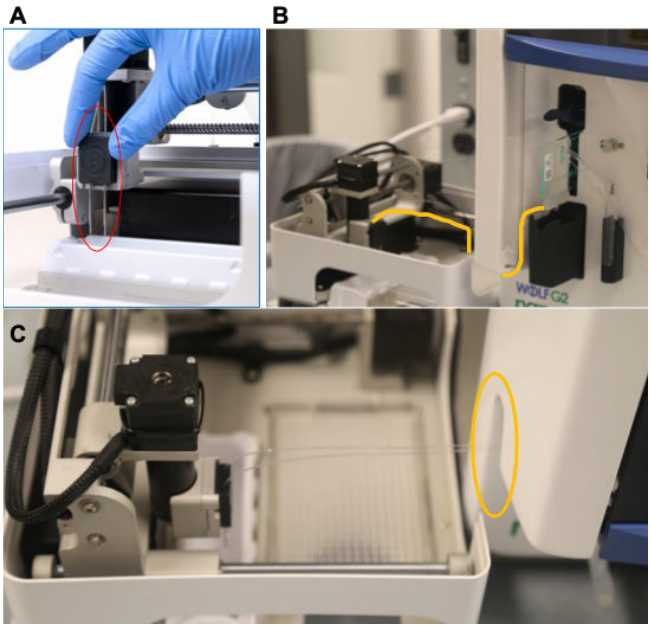


Figure 61. Cartridge assembly in the N1 Single-Cell Dispenser.

To set up the cartridge for single-cell sorting:

1. Open the cartridge pouch and unwrap the tubing.
2. Attach the dispensing needles to the magnetic receptacle on the N1 dispensing arm (Fig. 61 A).
3. Thread the outlet tubing attached to the dispensing needle through the plastic flap within the WOLF G2 (Fig. 61 B).
4. Insert the cartridge in the instrument cartridge fixture. Route the output tubing through the grooves in the waste container (Fig. 61 B).
5. Connect the sample and sheath tubing to the peristaltic pumps and position the purge tubing into the priming vial (Fig. 61 C).
6. Position the N1 Dust Cover in the module to prevent plate contamination.

7.5 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. 62 A).
4. Type the plate pre-fill volume.
5. Enter the number of cells per well. Keep in mind that the system is optimized to sort 1 cell per well. The accuracy for dispensing >1 cell per well may be lower.
6. Cells can be deposited in the last two 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. 62 B).
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 events/µl (about 10^5 cells/mL) concentration to ensure high single-cell dispensing efficiency.
9. Pass your cells through a 37-40 µm 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. 62 C).
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. 62 D).

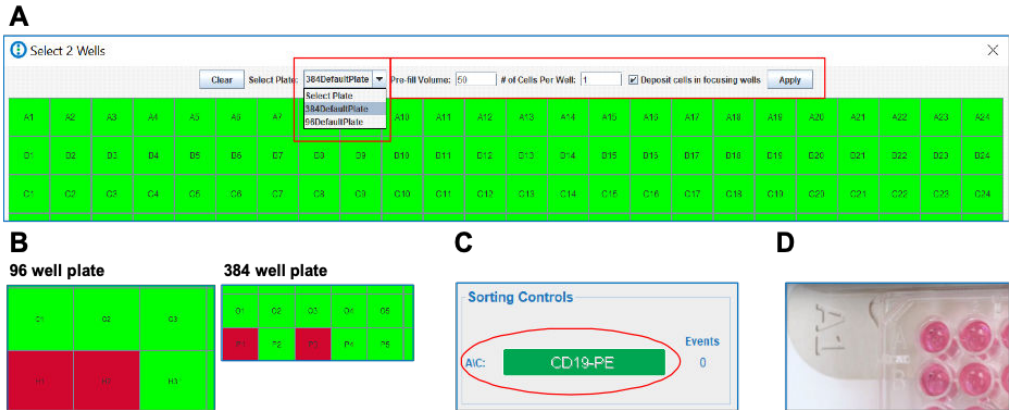


Figure 62. Steps in the single-cell sorting process.

7.6 Single-Cell Features

7.6.1 Single-Cell Plate Time Calculator

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

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.

NOTE: Bent tubing decreases single cell sorting efficiency. Please do not pinch or bend the tubing. Please take a picture if you notice any bent tubing during shipping and send the picture back to support@NanoCellect.com. We will work to amend the issue.

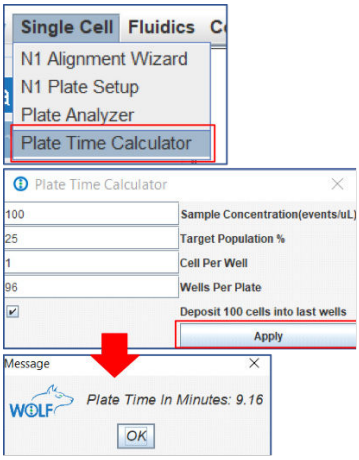


Figure 63. Applying a compensation matrix.

7.6.2 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 channels
- Forward and backscatter values
- Forward scatter width values
- Channel used for sorting
- Droplet 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.

NOTE: A CSV file is not available for plates with multiple cells per well.

7.7 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.

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. 66 A).
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. 66 B-C).
6. The user can see the map in the FL2 and FL3 channels (Fig. 66 D).

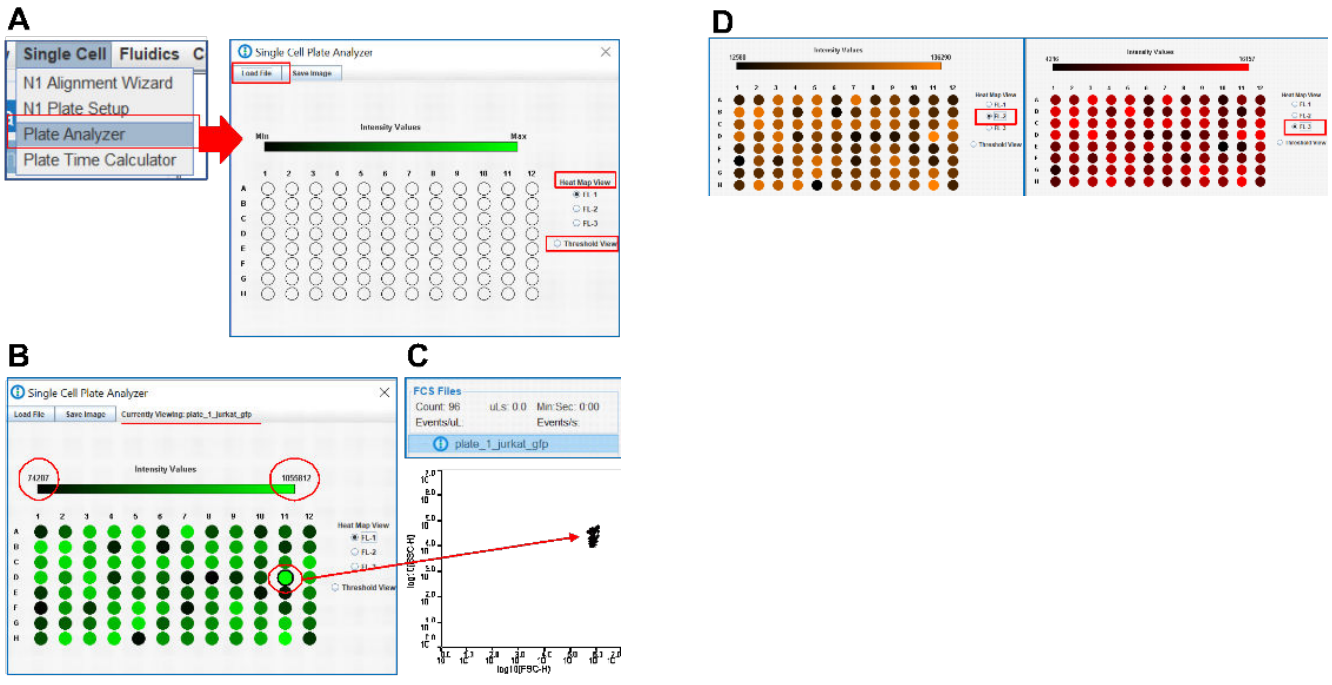


Figure 66. The Plate Analyzer Heat Map View.

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.

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. 67).
3. Reset values and select a well to see an event on the plots (Fig. 67).

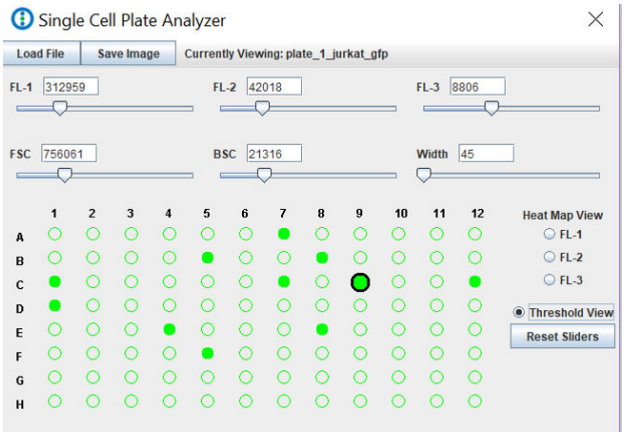


Figure 67. The Plate Analyzer Threshold View.

7.8 The Advanced Coloring Feature (post-sort analysis)

The WOLF G2 Cell Sorter has a sorting speed of 200 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. 68). It indicates which cells are sorted to channel A or C 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.

To use the Advanced Coloring Tool:

1. Go to the “View” tab in the main menu and select the “Advanced Coloring” checkbox.
2. Different colors display events gated and sorted for a given experiment (Fig. 68).
3. For single-cell experiments where the same population is sorted to channels A and C, the sorted events are all

labeled black.

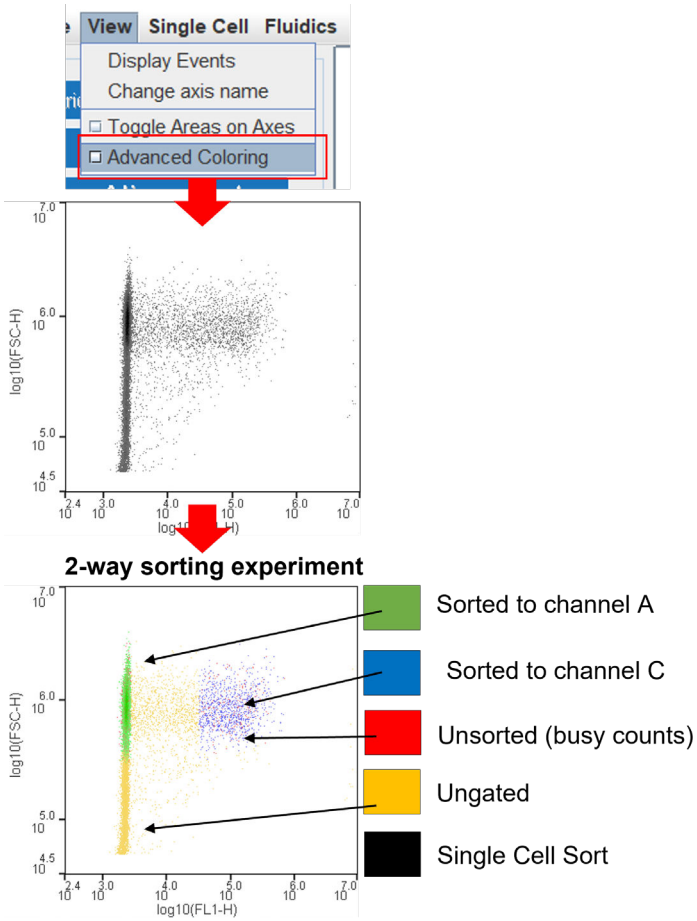


Figure 68. Post-sorting analysis with the Advanced Coloring Feature.

NOTE: If red counts are present, to minimize the number of unsorted cells that are lost due to busy counts, the user should dilute the sample. This will increase the percentage of target cells captured in the sorting channel.

8. Troubleshooting

8.1 The Clog and 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.

8.1.1 Scan for Clogs Feature

To scan the sorting junction for clogs:

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

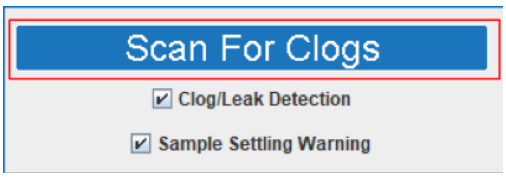
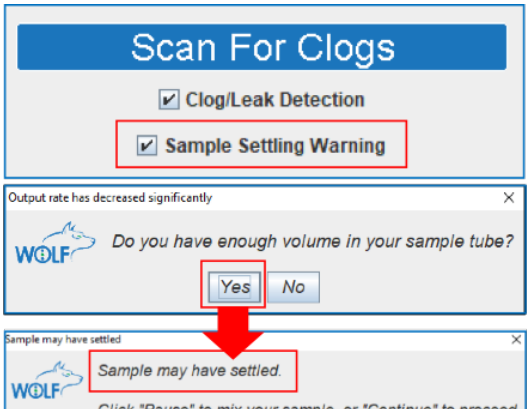


Figure 69. The Clog Detection Panel.

8.1.2 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 if they need to resuspend the cells by mixing or pipetting.



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.

Figure 70. Sample settling warnings.

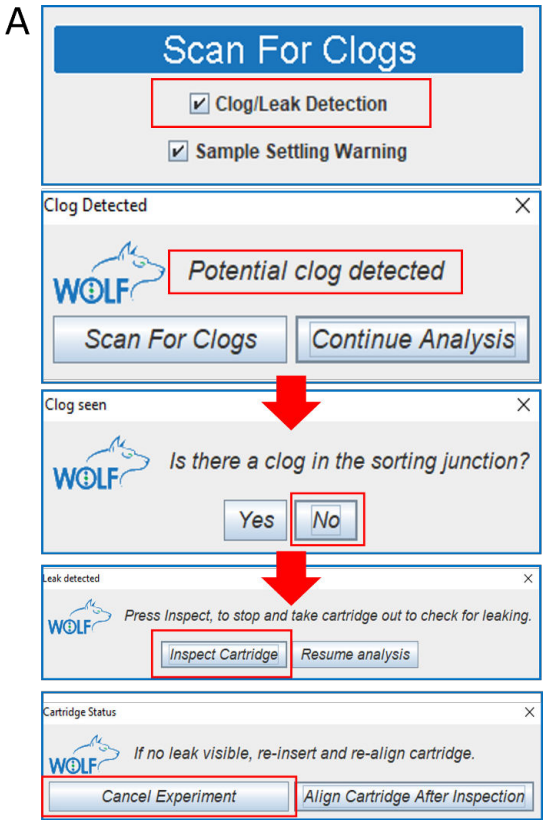
8.1.3 Chip Clog and Leak Detection

While much more tolerant to large cells than a typical sorter, the occasional challenge of using microfluidic systems is clogging of channels, which can lead 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 µm, as detection of small particles by the sorting verification system is not as accurate. In that case, this functionality can be disabled (see below).

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. 71 A).
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. **Be careful not to pull any tubing out of the cartridge, and to keep it perpendicular to the ground.** Slide a paper towel over the cartridge edge to check for wetness (Fig. 71 B).
6. If a leak is present, the user must remove the cartridge and clean the cartridge fixture following the protocol in section 8.2.2. If it is OK, reinsert, **reprime**, **realign and recalibrate** the cartridge.



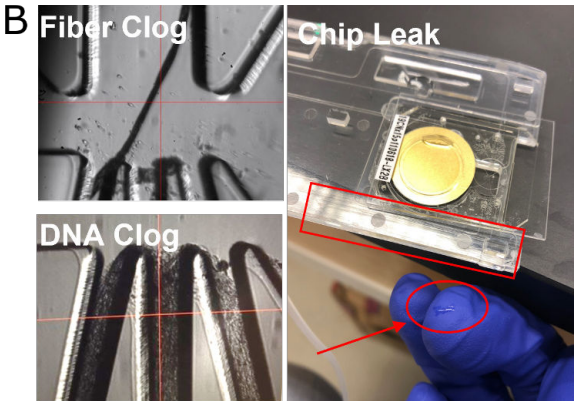


Figure 71. The Clog and Leak Detection Feature. **A.** The user can disable these features if needed. If a potential clog is detected, this means that cells seen by the laser do not seem to be passing through the cartridge. First, please scan the sorting junction for obstructions. Some of these are shown in **B.** as a fiber and DNA clogs. If none is visible, ensure there is not a leak in the system.

8.2 Removing Minor Fibers

Microfluidics-based sorting affords many advantages over traditional sorting but can be plagued by its inability to easily modulate pressure to blast away a potential fiber. Environmental contaminants can occasionally get lodged in the channel, obstructing sorting and becoming a significant annoyance at best, or, at worst, a hindrance to completing important experiments. These can happen due to a wide variety of reasons, including sample or environmental contaminants, such as DNA or foreign fibers. In the past, clogs typically required discarding the blocked cartridge and restarting your experiment with a new one. Many clogs, however, can be resolved with a set of simple steps. To allow for an easier fix that can save you from discarding the cartridge, our team at NanoCellect is excited to provide some tips and tricks to decrease cartridge replacements.

In short, fibers happen, especially to beginners. If you see a simple fiber in your sorting junction at priming, check the following:

- There is no direct airflow onto the WOLF from facility air vents
- Your sheath and media were filtered using a recommended syringe filter

You may attempt to remove a simple fiber at priming, or before a sample accumulation at the sorting junction has occurred. If in doubt, you may attempt the following procedure and change the cartridge if unable to remove the foreign object.

8.2.1 Procedure

1. **Reprime**: This is always the first step after noticing a clogged cartridge.
   Make sure the sample is loaded with filtered PBS and the purge valve is closed. Press the “reprime” button. If, after 2 minutes, the clog does not disappear, stop the reprime step and proceed to step 2.
2. **Purge Valve:** Opening the purge valve alters the pressure within the cartridge, which may dislodge a clog that was not previously cleared by repriming. Open the “Chip Alignment” window and move the live chip image to the sorting junction. Gently open the purge valve (Fig. 72). If the clog remains, proceed to close and open the purge valve two additional times.
3. **Flush:** If the clog is still present after opening and closing the purge valve 3 times, perform a flush step. This change in fluidic flow rates can often clear a clog.
   Make sure the purge valve is open and sterile, filtered PBS is loaded into the sample holder. Press the “flush” button and select a 3-minute flush. If flushing still does not remove the clog, proceed to step 4, carefully following instructions.
4. **Remove and tap**: If all of the above fails, follow the below steps to manually tap the cartridge. The physical tapping will dislodge the clog from the sorting junction.

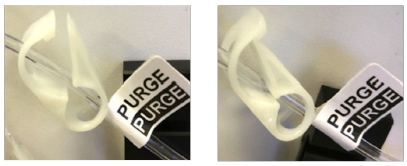


Figure 72. During step 2, the purge valve should be opened (left) and then close (right).

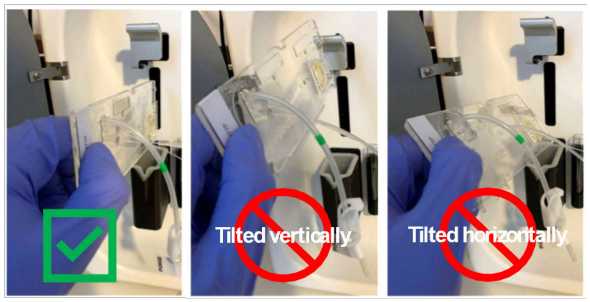


Figure 73. Be sure to hold the cartridge straight and vertical when removing or reinserting and do not tilt to the side. This could cause air to enter the cartridge.

First, unlatch the cartridge and gently remove the cartridge from the WOLF, holding it by the edge of the cartridge with the QR code and label. Make sure not to detach tubing, which should remain threaded in the pumps (Fig. 73).

Ensure the purge valve is open and the sheath pump is on. Then, using a Sharpie or similar plastic pen, GENTLY tap the side of the cartridge (Fig. 74).

Figure 74. Cartridge diagram with tap point marked (step 4).

8.2.2 Cartridge Fixture Cleaning Protocol

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. 75 A).
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. 75 B-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. 75 E).
7. If the cartridge fixture is not ready after 30 minutes, contact NanoCellect's Technical Support Department.

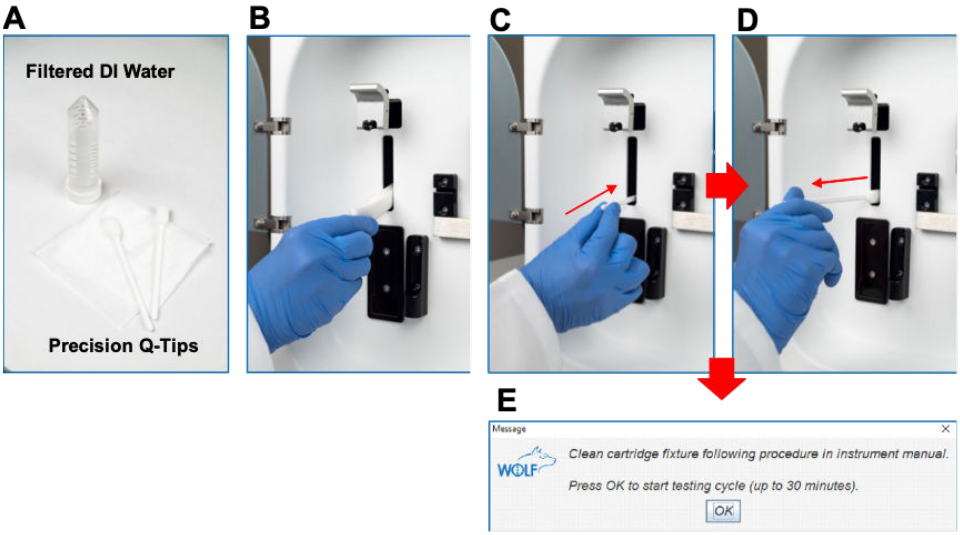


Figure 75. Cartridge fixture cleaning process.

9. Shutting Down the WOLF G2 Cell Sorter

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

9.1 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.

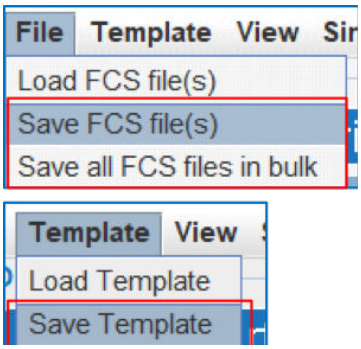


Figure 76. Saving files and templates.

NOTE: It is important to remember to save files at the end of the experiment. However, if the user accidentally forgets to save files, they may have been automatically backed-up. Files are automatically saved every 30 minutes into the “Recovery Files” folder within the WOLFViewer folder. Go to C:\Program Files\NanoCellect\WolfViewer\Recovery Files to view these files. However, these files will be automatically deleted after 72 hours.

9.2 Saving Experimental Reports as a PDF

The following information is saved in a PDF Report:

- Graphs with the gates created during the analysis
- Soting parameters
- Statistic Panel
- Gate state
- Plate Analyzer displays
- Compensation matrix used

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.

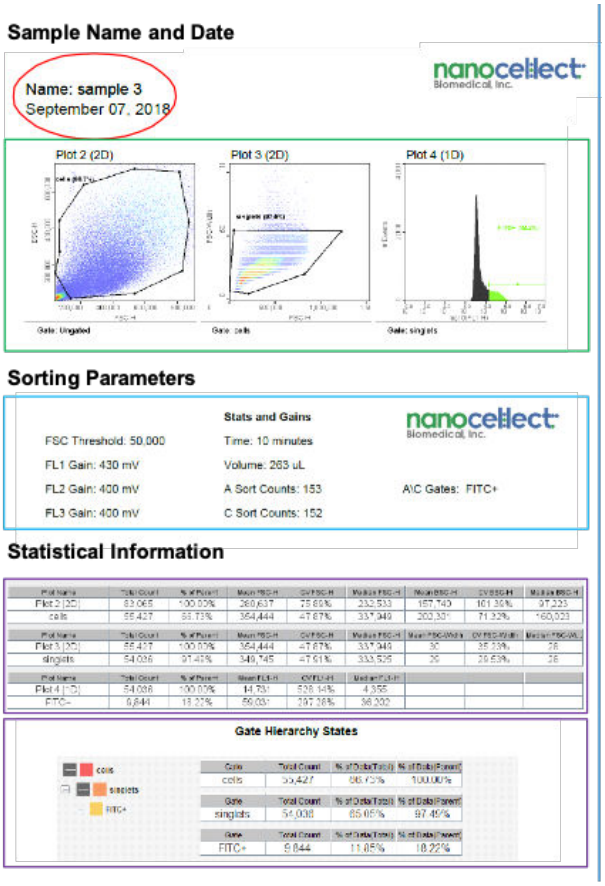


Figure 77. Example of a WOLFViewer PDF report.

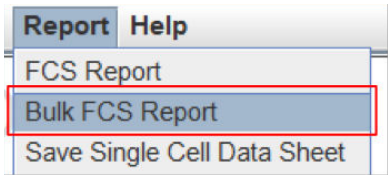


Figure 78. Saving sample PDF reports.

9.3 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 G2’s front panel.
2. Remove the waste container or the collection tube holder from the WOLF G2’s 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 pull the cartridge to remove it from the instrument.
7. Dispose of the cartridge and tubing in accordance with biohazard disposal best practices.

8. Treat the waste container with 10% bleach for decontamination. Do not autoclave it. The white waste inserts can be autoclaved.
9. Remove the N1 Reagent Reservoir and dispose of it in accordance with biohazard disposal best practices.

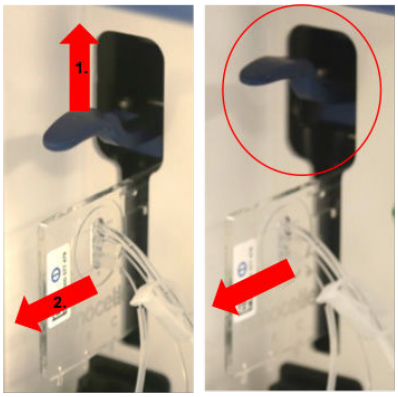


Figure 79. Cartridge removal process.

9.4 Powering Off the WOLF G2 Cell Sorter

To turn the WOLF G2 and N1 Single-Cell Dispenser off:

1. Turn WOLF G2 Cell Sorter off by depressing the power button (Fig. 80).
2. Turn the N1 Single-Cell Dispenser off by depressing the power button.
3. Close WOLFViewer software.

Figure 80. Turning off the WOLF G2 Cell Sorter.

10. WOLF G2 Cell Sorter Best Practices

10.1 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 G2 has 5 fluorescence detection channels, which depend on the particular 2-laser configuration of the WOLF G2 (Fig. 48).

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 the compensation section of this guide 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).

10.2 Sample Preparation

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

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

Please keep in mind that the exact protocol will be cell type-specific. We strive to provide good general starting points for most cell types below. It is always a good idea to check for cell preparation protocols with researchers and publications that use your specific type of cell for flow cytometry.

Good Sample Preparation for Optimal Flow Cytometry Success

10.2.1 Cell Number Requirements

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 a conservative approach by determining the number of cells required and then doubling that number until optimization is performed.

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

10.2.2 Staining the Sample

Staining the sample with dyes or antibodies typically requires some optimization via titration. No matter the experiment, and especially for single-cell sorting, we recommend using viability dyes. These are an important part of selecting live, healthy cells. Please follow manufacturer's recommendations for staining protocols. NanoCellect suggests the dyes in our fluorophore table based solely on optical compatibility with the WOLF G2 instrument (Fig. 48).

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. In addition, 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 optimal binding.

10.2.3 Sample and Sheath Buffers

Generally, sample buffer and sheath composition are highly specific to the type of sample. We make only general recommendations for simple buffer solutions below based on most cell types' preference for protein content and stable pH levels. Please consult your field for the best sorting condition for your sample. NanoCellect additionally provides experimental conditions used in our application notes, which are available online.

Suggested sample buffers:

• HBSS sorting buffer:	HBSS
	1-2% BSA
	1 mM EDTA
• PBS sorting buffer:	PBS (Ca^{2}+ and Mg^{2}+ Free)
	1-5% BSA
	1 mM EDTA
	25 mM HEPES pH 7.0
• Sorting media:	Cell culture media (DMEM)
	1% BSA (for 405 nm laser configurations)
	2% BSA (for all other laser configurations)

NOTE: Please limit the BSA additive on the high-powered 405 nm laser configuration to 1%. Higher BSA content can cause laser focusing at the sample core and temperature damage to the cells.

10.2.4 Sample Condition Optimization

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.
2. Consider keeping cells and reagents at 4°C, or on 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 Ca^{2+}- and Mg^{2+}-free PBS with BSA (up to 5% v/v) to suspend cells as it may reduce clumping.
6. Use EDTA if cells tend to clump.
7. Use DNase I (10 units/mL or 2 to 5 µg/mL) if cells are clumping or releasing DNA due to cell death. Note that EDTA should not be used with DNase I as DNase I is Mg^{2+} dependent.
8. Use **CO_2-independent** cell medium as sheath fluid to preserve viability.
    - Consider using HEPES in sample buffer or sheath fluid to prevent pH changes and preserve cell viability.
9. Always strain samples through a 40 µm mesh immediately before sorting to minimize cartridge clogging. This may be done periodically throughout the experiment. Please contact NanoCellect support before using a larger mesh, up to 60 µm. Consider re-straining cell samples multiple times over the course of a long sorting day.
10. 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.
11. Never use cell culture media for long sorting experiments. The rapid pH change at room conditions can result in significant cell death. Use CO2-independent media instead that is buffered with Tris or HEPES. Check your supplier for the appropriate media for your cell type.
12. Consider aliquoting your cells to break up long sorts into manageable segments. This can decrease the time your cells spend outside of the incubator.
13. Always use a viability dye for your sample or stain a portion of your cells to assess viability before the sort.

10.2.5 WOLFie's Tips for Sample Preparation for Flow Cytometry

The WOLF G2 system provides unprecedented opportunities to customize sheath and sample fluid composition. To improve sample compatibility with the microfluidic system, we recommend following the simple guidelines below.

- Consider PBS with BSA (up to 5% v/v and 1% v/v for the 405 nm laser) for your sheath fluid. We do not recommend using FBS for flow cytometry experiments due to its optical properties. If you must use serum in your experiment, please use the least amount possible. The addition of serum can decrease cartridge lifetime.
- Always use the same buffer for sample preparation and sheath fluid to minimize scatter distortion due to different refraction indexes.

10.3 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. These are needed for compensation.

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 adjusting the compensation matrix if needed. These are optional, and will not be needed to compensate via the automatic wizard.

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. 7-AAD-only stained cells

And, optionally:

5. FITC + PE stained cells
6. FITC + 7-AAD stained cells
7. PE + 7-AAD stained cells
8. FITC + PE + 7-AAD stained cells

10.4 Sample Concentration, Sorting Purity, and Sorting Time

10.4.1 Considerations for Cell Concentration in Sorting

The pre-sorted sample contains a target population as a percentage of total cell count. 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 G2 sorting different target populations at increasing sample concentrations. In general, avoid preparing the cells at high concentrations. Prepare single-cell suspensions at concentrations between **250,000 – 500,000 events/mL for bulk sorting** and **100,000 cells/mL for single-cell sorting.**

The suggested sample concentration intervals correspond to the intended experimental outcome. For ultra-rare target populations (less than 0.5%), we recommend considering a 2-step sort by pre-enriching at higher concentrations and sorting the resulting fraction again to high purity. For typical bulk sorting, concentrations up to about half a million cells per mL are appropriate. For single-cell dispensing, the recommended cell concentration is about 100,000 cells per mL.

10.4.2 Fluidics Speed and Cell Processing

The WOLF G2 has a fixed flow rate of 24 µl per minute to process 1 mL of sample in ~42 minutes. The instrument can analyze 2,000 cells per second with high accuracy and has a sorting speed of 200 cells per second.

Please note that sorting rare target populations may significantly extend sorting times. Consider pre-enriching highly rare target populations by pre-sorting at higher concentrations, or by another method.

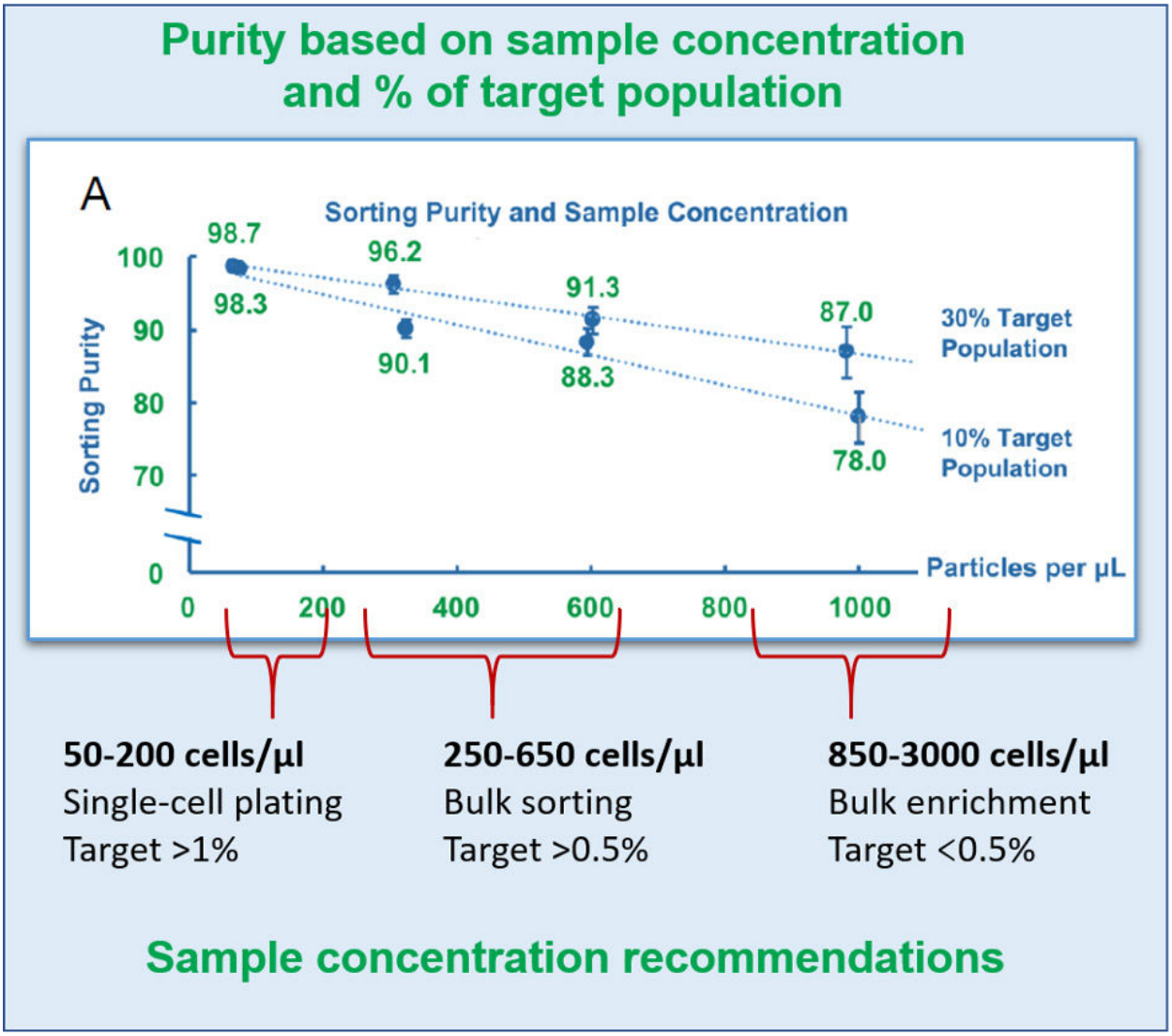


Figure 81. Representation of sorting purity versus sample concentration. For single-cell dispensing, a target of 1-5% is advised at concentrations around 100,000 cells per mL. For bulk sorting, the concentration can be increased to 500,000 cells per mL approximately, and further if quick pre-enrichment of rare targets is required.

10.5 Maintaining Sterility

The WOLF G2 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.

NOTE: Avoid any vacuum-driven filters. They may release fibers that can clog microfluidics.

11. Customer Resources

11.1 WOLF User's Knowledge Base

Registered customers can access our scientific content library called WOLF User's Knowledge Base. The page contains:

- WOLF/N1 training videos
- Quick-start guide for processes like sample prep or sorting small cells
- WOLFViewer software information, release notes, and resources
- MSDS and instructions for consumables like calibration and rainbow beads
- Cartridge information
- Frequently asked questions (FAQ)
- Consumables order form

Register to use the UKB at https://NanoCellect.com/registration/, and use the appropriate form: customer (left), or distributor (right). Your registration will generally be approved within 24 hours. Upon registration, access the database at https://NanoCellect.com/wolf-users-knowledge-base-login/.

11.2 Additional Online Resources

The NanoCellect website contains a constantly updated list of scientific resources (application and technical notes) that showcase sorting workflows for a variety of applications. To access these resources, visit https://NanoCellect.com/scientific-content/.

11.3 Suggested Fluorophore Tables

Please see the following pages for your particular WOLF G2 configuration. Feel free to place this next to your sorter for quick reference!

11.3.1 Disclaimer

Brilliant Violet is a trademark of Becton, Dickinson and Company or its affiliates. CellTrace and CellTracker are a trademark of Thermo Fisher Scientific. SYTOX is a registered trademark of Thermo Fisher Scientific. Cy is a registered trademark of GE Healthcare UK Ltd. DRAQ5 is a trademark of BioStatus Ltd. PE/Dazzle is a trademark of BioLegend Inc. Vio, VioBlue, VioGreen, PE-Vio, and PerCP-Vio are trademarks of Miltenyi Biotec GmbH. Accutase is a trademark of Innovative Cell Technologies. APO-BrdU is a trademark of Phoenix Flow Systems Inc. SPHERO is a trademark of Spherotech, Inc.

405/488 Configuration Fluorophore Chart

WOLF G2 Cell Sorter		Conjugated Fluorophores	Viability Dyes	Fluorescent Proteins	Tracking or Lipid Dyes
Excitation: 405 nm (V)iolet Laser	**V450** (450/50)	BV421 Pacific Blue eFluor 450 BD V Horizon 450 Alexa Fluor 405	DAPI ☼ Sytox Blue LIVE/DEAD Violet Zombie Violet	BFP CFP ☼ Cerulean ☼	CellTrace Violet
	V525 (525/50)	BV480	LIVE/DEAD Aqua ☼ DAPI	T-Sapphire Cerulean CFP	CellTracker Violet ☼
	V575 (575/40)	BV570	LIVE/DEAD Aqua		CellTracker Violet
	V620 (620/50)	BV605 Qdot 605			
	V706 (706/95)	BV711 Qdot 705 PerCP ▲ PerCP-Cy5.5 ▲			

WOLF G2 Cell Sorter		Conjugated Fluorophores	Viability Dyes	Fluorescent Proteins	Tracking or Lipid Dyes
Excitation: 488 nm (B)lue Laser	**B525** (525/50)	FITC Alexa Fluor 488 DyeLight 488	Calcein AM Sytox Green LIVE/DEAD Green SYTO 9	GFP YFP mVenus	Cell Mask Green CellTracker Green
	B575 (575/40)	PE		dsRed tdTomato	
	B620 (620/50)	PE/Dazzle 594 PE-Texas Red	Propidium iodide ☼	dsRed ∞ tdTomato ∞	
	B706 (706/95)	PerCP ▼ PerCP-Cy5.5 ▼ PE-Cy5 PE-Cy5.5	Sytox AADvanced DRAQ5 DRAQ7 Propidium iodide		

▼ This fluorophore is also excited well by the 405 nm laser; expect spillover into the corresponding (V)iolet channel.

▲ This fluorophore is also excited well by the 488 nm laser; expect spillover into the corresponding (B)lue channel.

☼ This fluorophore also emits in the neighboring channel; consider leaving the **next** channel open. Expected spillover channel indicated in blue. Proper compensation will likely be needed.

∞ This fluorophore also emits in the neighboring channel; consider leaving the **previous** channel open. Expected spillover channel indicated in blue. Proper compensation will likely be needed.

For more information, visit nanocellect.com
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488 Single Laser Configuration Fluorophore Chart

WOLF G2 Cell Sorter

		Conjugated Fluorophores	Viability Dyes	Fluorescent Proteins	Tracking or Lipid Dyes
Excitation: 488 nm (B)lue Laser	**B525** (525/50)	FITC Alexa Fluor 488 DyeLight 488 Qdot 525	Calcein AM Sytox Green LIVE/DEAD Green SYTO 9	GFP YFP mVenus	Cell Mask Green CellTracker Green
	B580 (580/25)	PE		dsRed tdTomato	
	B620 (620/50)	Qdot 605 PE/Dazzle 594	Propidium iodide ☼	dsRed ∞ tdTomato	
	B706 (706/95)	PerCP PerCP-Cy5.5 PE-Cy5 PE-Cy5.5	Sytox AADvanced 7-AAD Propidium iodide DRAQ5 DRAQ7		
	B760 (760LP)	PE-Cy7			

☼ This fluorophore also emits in the neighboring channel; consider leaving the **next** channel open. Expected spillover channel indicated in blue. Proper compensation will likely be needed.

∞ This fluorophore also emits in the neighboring channel; consider leaving the **previous** channel open. Expected spillover channel indicated in blue. Proper compensation will likely be needed.

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488/561 Configuration Fluorophore Chart

WOLF G2 Cell Sorter

		Conjugated Fluorophores	Viability Dyes	Fluorescent Proteins	Tracking or Lipid Dyes
Excitation: 488 nm (B)lue Laser	**B525** (525/50)	FITC Alexa Fluor 488 DyeLight 488 Qdot 525	Calcein AM Sytox Green LIVE/DEAD Green SYTO 9	GFP YFP mVenus	Cell Mask Green CellTracker Green
	B580 (580/25)	PE ▲		dsRed tdTomato ▲	
	B620 (620/50)	Qdot 605 PE/Dazzle 594 ▲	Propidium iodide ▲☼	dsRed ∞ tdTomato ▲	
	B706 (706/95)	PerCP ▲ PerCP-Cy5.5 ▲ PE-Cy5 ▲ PE-Cy5.5 ▲	Sytox AADvanced ▲ 7-AAD ▲ Propidium iodide ▲ DRAQ5 ▲ DRAQ7 ▲		
	B760 (760LP)	PE-Cy7 ▲			

WOLF G2 Cell Sorter

		Conjugated Fluorophores	Viability Dyes	Fluorescent Proteins	Tracking or Lipid Dyes
Excitation: 561 nm (Y)ellow Laser	**Y580** (580/25)	PE ▼☼ Alexa Fluor 546 ☼ Alexa Fluor 555 ☼		RFP tdTomato	BODIPY 558/568 CellTrace Yellow ☼
	Y620 (620/50)	PE/Dazzle 594 ▼ Alexa Fluor 568 Alexa Fluor 594 ☼ Alexa Fluor 610 ☼ Alexa Fluor 546 Alexa Fluor 555 Alexa Fluor 633 PE	Propidium iodide ▼☼ Sytox AADvanced 7-AAD	mCherry ☼ RFP ∞ tdTomato ▼∞ mKate ☼ mRuby	CellTracker™ Red ☼ Nile Red ☼ CellTrace Yellow
	Y706 (706/95)	Alexa Fluor 633 ∞ PE-Cy5 ▼ PE-Cy5.5 ▼ PerCP-Cy5.5 ▼ Alexa Fluor 594	Sytox AADvanced▼∞ 7-AAD ▼∞ Vybrant DyeCycle Ruby DRAQ5 ▼ DRAQ7 ▼ Propidium iodide	mCherry mKate	CellTracker™ Red Nile Red
	Y760 (760LP)	PE-Cy7 ▼			

▼ This fluorophore is also excited well by the 488 nm laser; expect spillover into the corresponding (B)lue channel.

▲ This fluorophore is also excited well by the 561 nm laser; expect spillover into the corresponding (Y)ellow channel.

☼ This fluorophore also emits in the neighboring channel; consider leaving the **next** channel open. Expected spillover channel indicated in blue. Proper compensation will likely be needed.

∞ This fluorophore also emits in the neighboring channel; consider leaving the **previous** channel open. Expected spillover channel indicated in blue. Proper compensation will likely be needed.

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488/637 Configuration Fluorophore Chart

WOLF G2 Cell Sorter		Conjugated Fluorophores	Viability Dyes	Fluorescent Proteins	Tracking or Lipid Dyes
Excitation: 488 nm (B)lue Laser	**B525** (525/50)	FITC Alexa Fluor 488 DyeLight 488	Calcein AM Sytox Green LIVE/DEAD Green SYTO 9	GFP YFP mVenus	Cell Mask Green CellTracker Green
	B575 (575/40)	PE		dsRed tdTomato	
	B609 (609/34)	PE/Dazzle 594	Propidium iodide ☼	dsRed ∞ tdTomato ∞	
	B706 (706/95)	PerCP PerCP-Cy5.5 ▲ PE-Cy5 ▲	Sytox AADvanced 7-AAD Propidium iodide ▲ DRAQ5 ▲ DRAQ7 ▲		
	B760 (760LP)	PE-Cy7			

WOLF G2 Cell Sorter		Conjugated Fluorophores	Viability Dyes	Fluorescent Proteins	Tracking or Lipid Dyes
Excitation: 637 nm (R)ed Laser	**R706** (706/95)	APC Alexa Fluor 633 Alexa Fluor 647 PE-Cy5 ▼ PerCP-Cy5.5 ▼	DRAQ5 ▼ DRAQ7 ▼	E2-Crimson	CellTracker Deep Red
	R760 (760LP)	APC-Cy7 Alexa Fluor 750	Zombie NIR		

▼ This fluorophore is also excited well by the 488 nm laser; expect spillover into the corresponding (B)lue channel.

▲ This fluorophore is also excited well by the 637 nm laser; expect spillover into the corresponding (R)ed channel.

☼ This fluorophore also emits in the neighboring channel; consider leaving the **next** channel open. Expected spillover channel indicated in blue. Proper compensation will likely be needed.

∞ This fluorophore also emits in the neighboring channel; consider leaving the **previous** channel open. Expected spillover channel indicated in blue. Proper compensation will likely be needed.

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WOLF G2 Cell Sorter

The WOLF G2® Cell Sorter is designed and built in California by NanoCellect Biomedical, Inc.
www.NanoCellect.com

488 nm 55 mW
405 nm 55 mW
561 nm 55 mW
637 nm 55 mW
Class 1 Laser Product

US and International Patents Pending

Covered by one or more of the following
U.S. Patents: 20150268244 20130083315.
US and International Patents Pending