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Attune™ NxT

Flow Cytometer

Basic Training

Revision 2.5
Revision Date: Aug2019

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Agenda – Day 1

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10.45 – 12.00	Attune™ NxT Cytometer Systems
12.00 – 12.30	Instrument Setup and Daily Maintenance
12.30 – 13.30	LUNCH
13.30 – 14.15	Overview of the Attune™ NxT Software
14.15 – 16.30	Experiment Setup & Data Acquisition Single Color Lab Exercise
16.30 – 17.00	Post-Acquisition Analysis Tools

Agenda – Day 2

09.00 – 09.30	Review of Day 1
09.30 – 11.00	Compensation – Theory and Activity
11.15 – 12.30	Compensation – Multicolor Lab Exercise
12.30 – 13.30	LUNCH
13.30 – 14.30	Advanced Software Tools & Data Management
14.30 – 15.00	Attune™ NxT Cytometer Maintenance
15.15 – 16.45	Basic Troubleshooting scenarios and Review of Training
16.45 – 17.00	Resources – Q&A

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Introduction to Flow Cytometry

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TRUE or FALSE

A flow Cytometer can provide the distribution of cellular characteristics within a sample

True

False

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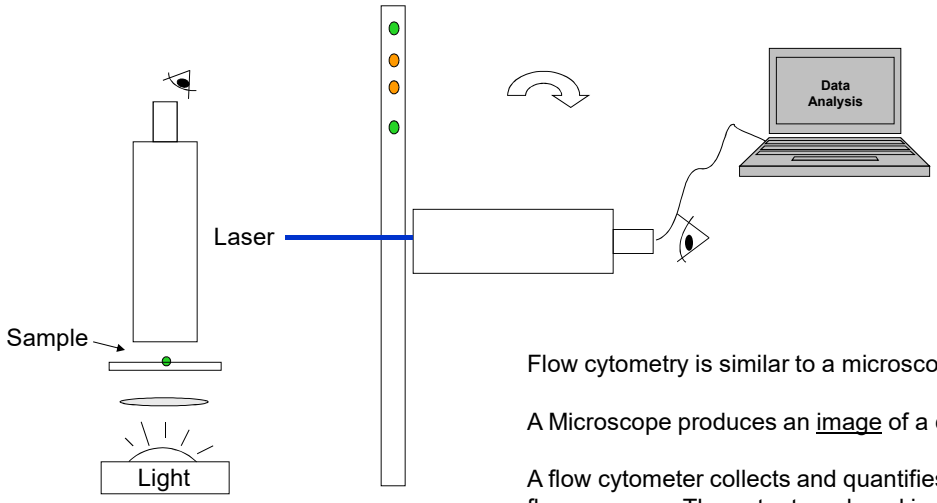
What is Flow Cytometry?

CYTOMETRY is the measurement of physical or chemical characteristics of cells or particles

FLOW CYTOMETRY measurements are made as individual cells or particles in flowing stream pass through a flow cytometry instrument

- Performed on single cell suspensions
- Provides discrete measurements from each cell in the sample
- Provides a distribution of the measured characteristics in the sample

The Flow Cytometer – "A Different kind of Microscope"



Flow cytometry is similar to a microscope.

A Microscope produces an image of a cell

A flow cytometer collects and quantifies scattered light and fluorescence. The output produced is numbers.

What makes a Flow Cytometer?

Flow Cytometer is made up of 3 subsystems:

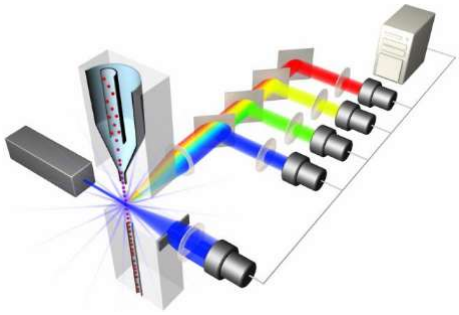
- Fluidics
  To introduce and focus the cells for interrogation
- Optics
  To generate and collect the light signals
- Electronics
  To convert the optical signals to proportional electronic signals for computer analysis

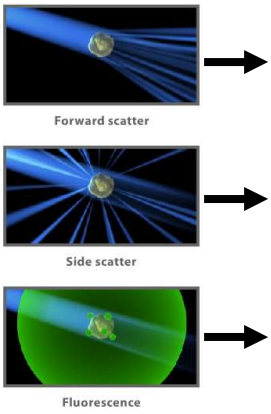
TRUE or FALSE

A Flow Cytometer only collects and quantifies fluorescence

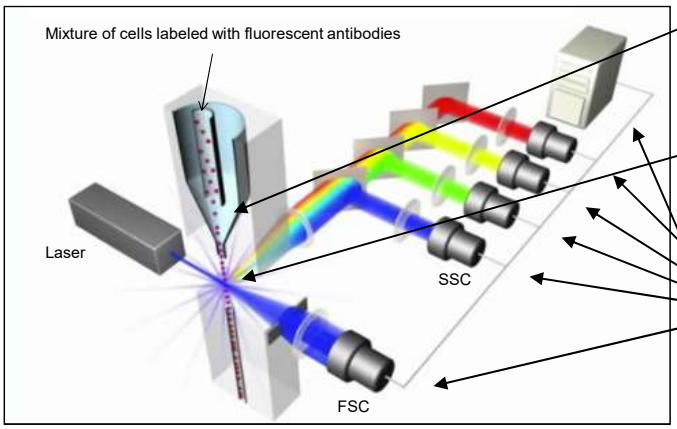
True False

Principle of Flow Cytometry





Principle of Flow Cytometry

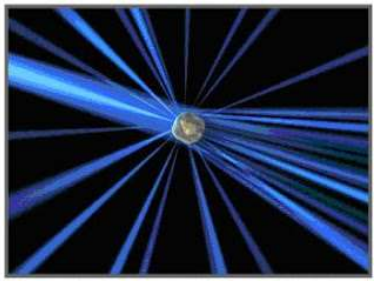


1. Cells in a single profile pass through the flow cell
   - **Cells Focusing**
2. Laser hits individual cell passing through the narrow tube called flow cell
   - **Interrogation Point**
3. Deflected light hits a series of detectors
4. The signal from detectors are interpreted by a computer

What happens to light when it hits a cell?

Laser Light Scatter

- When laser light interacts with a cell, light is scattered in all directions
- The light scatter depends on size and internal complexity of the cell
- We look at Forward Light Scatter and Side Light Scatter.

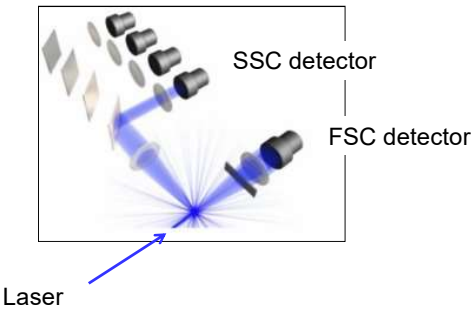


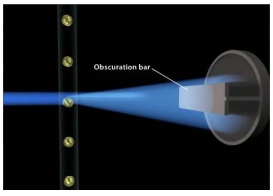
What happens to light when it hits a cell?

Laser Light Scatter

Forward Scattered light (FSC) is proportional to cell-surface area or size. Obscuration bar (also known as Blocker bar) stops direct laser light .

Side-scattered light (SSC) is proportional to cell granularity/internal complexity of the cell. SSC is usually collected at 90 degrees to the laser beam





Obscuration bar protects the FSC detector from being hit by the direct laser light

Practical Session

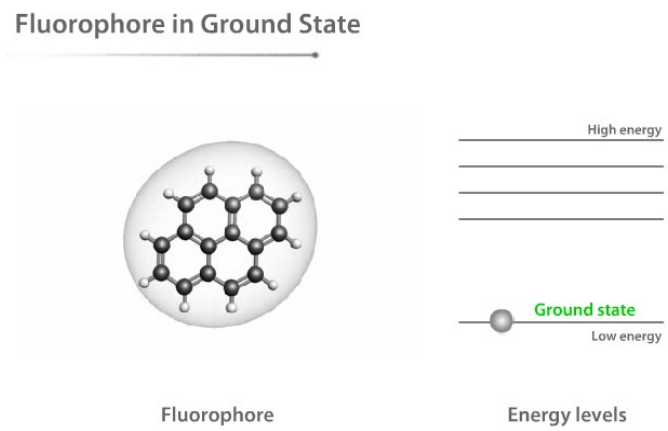
- Activity 1 – Fruit Scatters

What happens to light when it hits a cell?

Fluorescent Light – Common Definitions

- **Fluorescence** is the result of a three stage process in molecules called fluorophores, or fluorescent dyes.
- **Absorption spectrum**: The wavelength range over which a fluorescent compound can be excited. Also known as Excitation range.
- **Emission spectrum**: The range of emitted wavelengths of a fluorescent compound, it is a longer wavelength than the absorption wavelength.
- **Auto-Fluorescence**: is a natural fluorescence that occurs in cells and originates from endogenous constituents such as cyclic ring compounds like NAD(P)H, collagen, riboflavin and aromatic amino acids including phenylalanine, tyrosine and tryptophan.

Fluorescence 3-steps process



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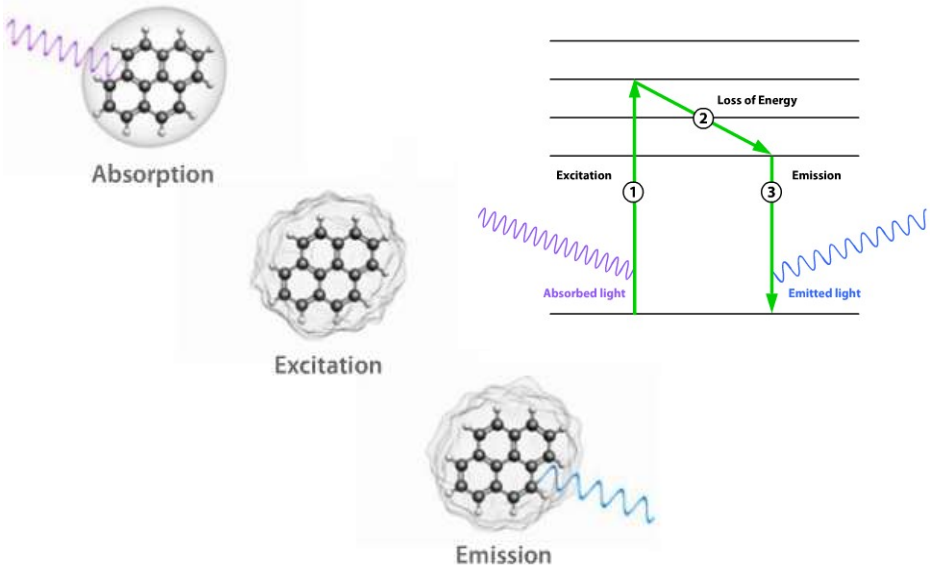
Fluorescence 3-steps process



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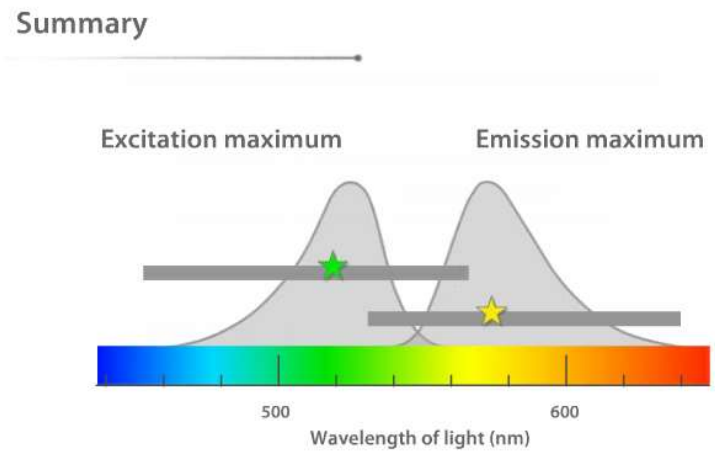
Fluorescence Spectrum



Practical Example

Three Colors Experiment

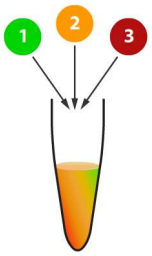
Sample:

Lysed Blood from Human

Measure:

% T-lymphocytes CD4+

% T-lymphocytes CD8+

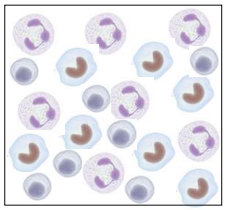


	Antibody	Fluorescent Probe
1	Anti-CD3	Alexa Fluor™ 488
2	Anti-CD4	R-PE
3	Anti-CD8	R-PE Alexa Fluor 700 tandem dye

→ T-lymphocytes specific

Three colors Experiment – Data representation

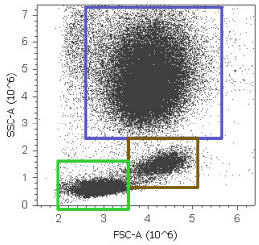
Lysed human whole blood



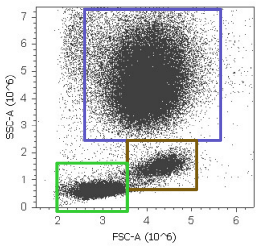
Data representation →

Dual parameters representation

Morphology ***Dot*** plot (size vs complexity)



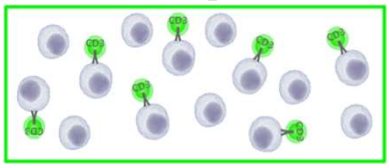
Three colors Experiment – Data representation



3 different identifiable regions (i.e. populations)

1 region of interest (i.e. lymphoid populations)

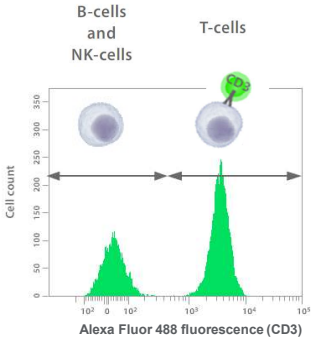
GATING



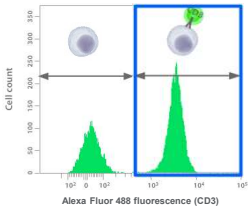
Data representation

One parameter representation

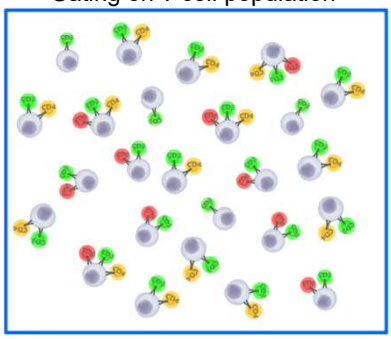
Fluorescence ***Histogram*** plot (Fluorescence intensity vs cell count)



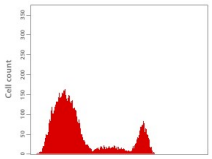
Three colors Experiment – Data Representation

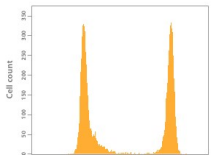


Gating on T-cell population



Data representation

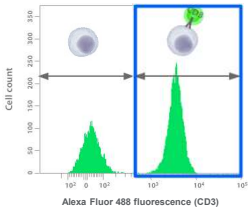




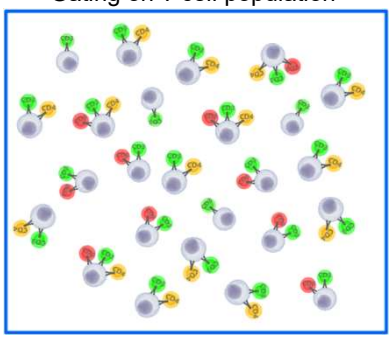
Two histogram plots, one for each fluorescence

Loss of information!

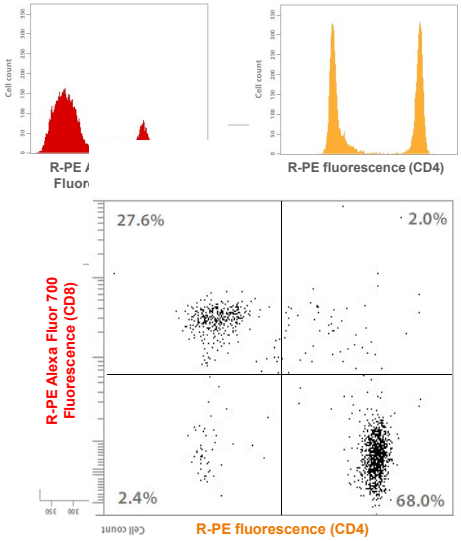
Three colors Experiment – Data Representation



Gating on T-cell population



Data representation



Dual fluorescences ***Dot*** plot

Applications in Flow Cytometry

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-
-
-
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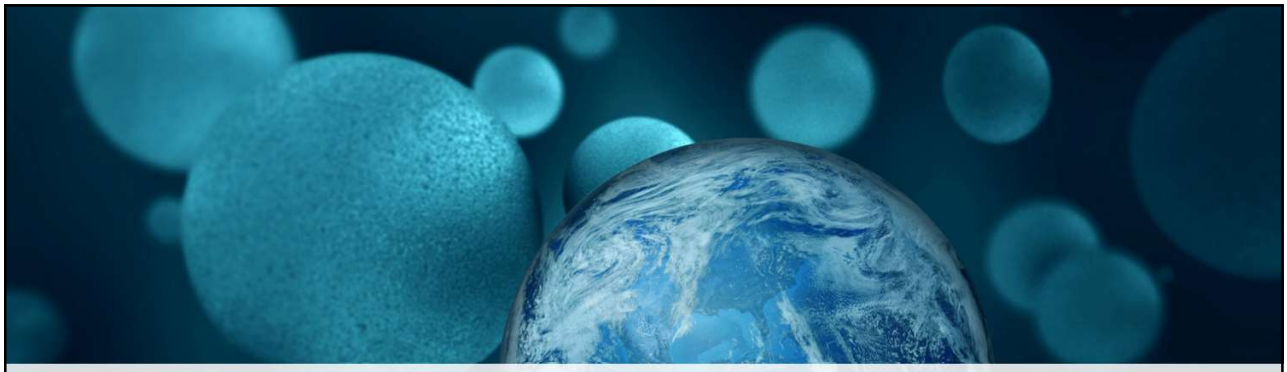
Key Learning Points

- Every Flow Cytometer consists of three subsystems (Fluidic, Optic, Electronic)
- The readout is forward scatter, side scatter and fluorescence
- Every fluorophore has a unique absorption- and emission- spectra
- Data can be represented using histograms or dual parameter plots including a reasonable gating strategy



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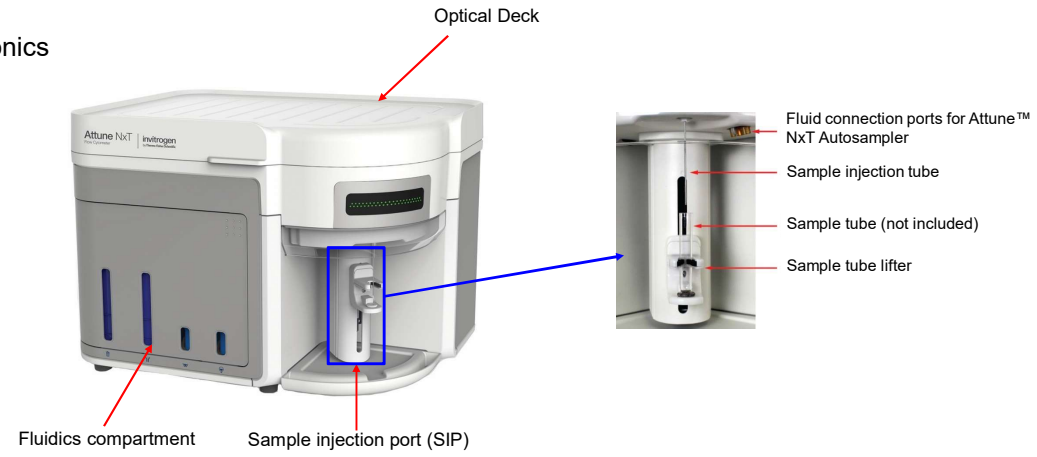
Attune™ NxT Cytometer Systems

Attune™ NxT Acoustic Focusing Cytometer

Three main Systems:

- Fluidics
- Optics
- Electronics



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Attune™ NxT Fluidics System

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Fluidics System

The purpose of a fluidics system is to transport particles in a fluid stream to the laser beam for interrogation

For optimal illumination, the stream transporting the particles should be in the center of the laser beam.

Only one particle should move through the laser beam at a time.

Fluidics system needs to be free of air bubbles & debris.

TRUE or FALSE

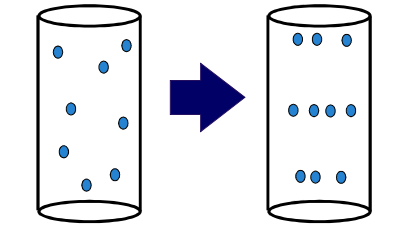
The Attune™ NxT Cytometer uses fluids to align cells into a single stream

True

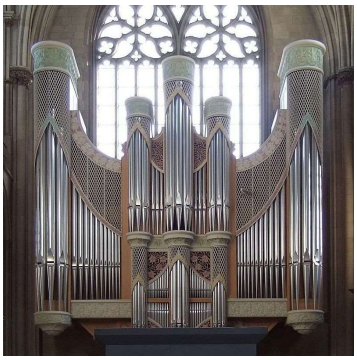
False

The Acoustic Idea

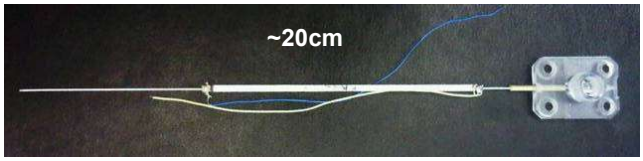
A century old phenomenon



Cells not damaged by acoustic waves – similar to ultrasound used to visualize a fetus *in utero*.

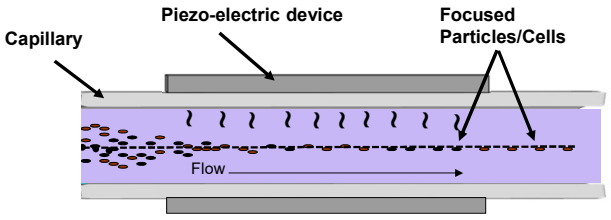


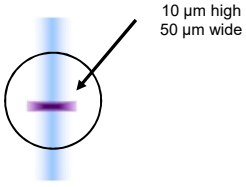
Acoustic Focusing Capillary



Acoustic Waves:

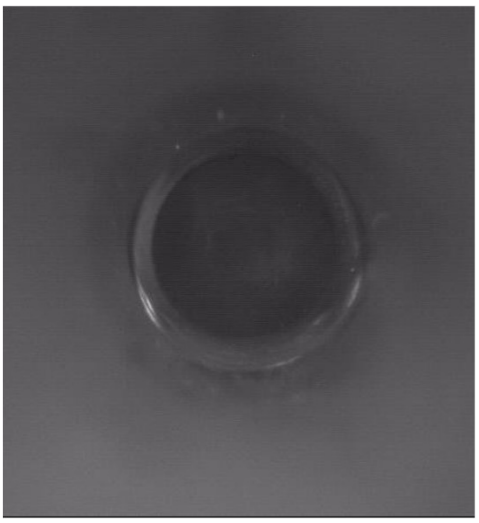
Similar to ultrasound used to visualize a fetus *in utero*.





- Sheath fluid is not required to focus cells.
- Flow rate can be increased while maintaining resolution.

Acoustic Focusing in Action



10 µm fluorescently-labeled beads

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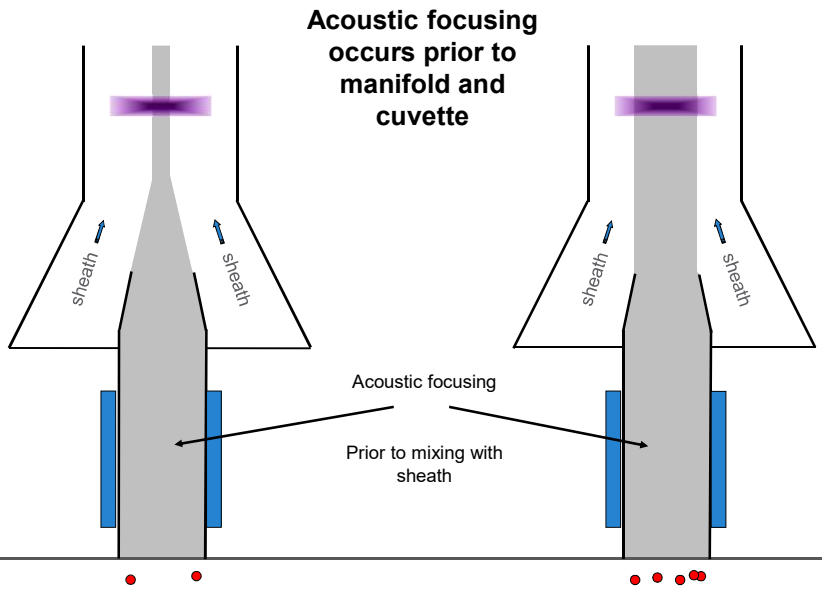
Acoustic Focusing



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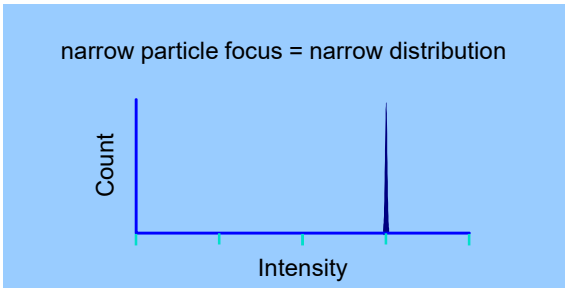
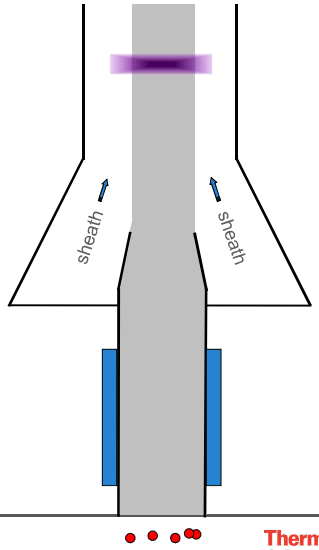
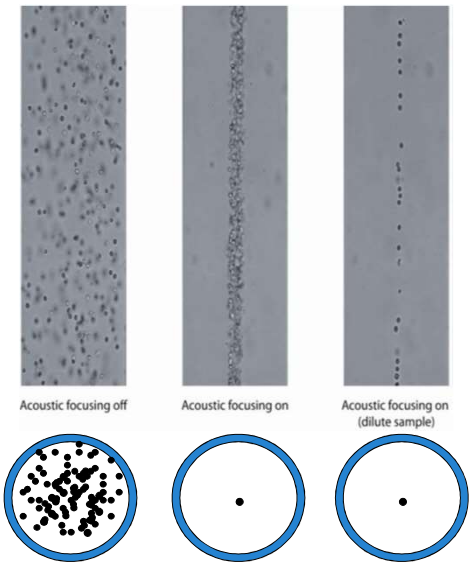
Acoustic Focusing – High Flow Rate



- Increase sample input volume = increase flow rate = NO pressure difference
- SAME Particle distributions
- SAME Instrument resolution
- Volumetric sample rates = 12.5 µl/min – 1000 µl/min

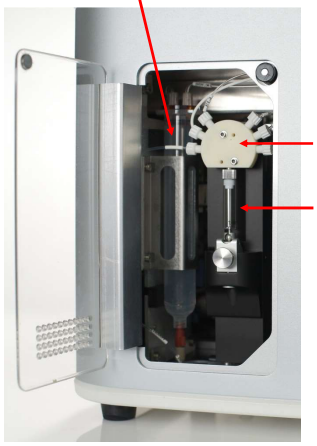


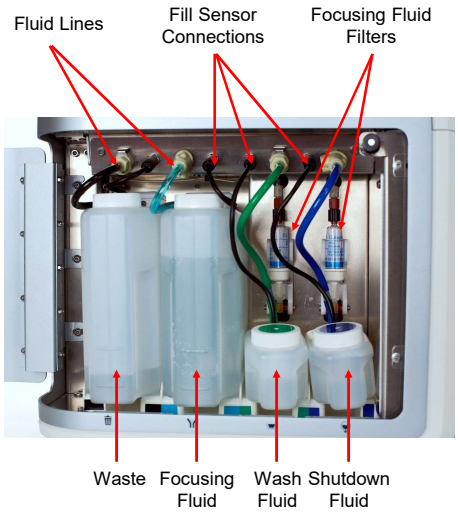
Acoustic Focusing Benefits



Dilution is the solution!

Attune™ NxT Fluidics System





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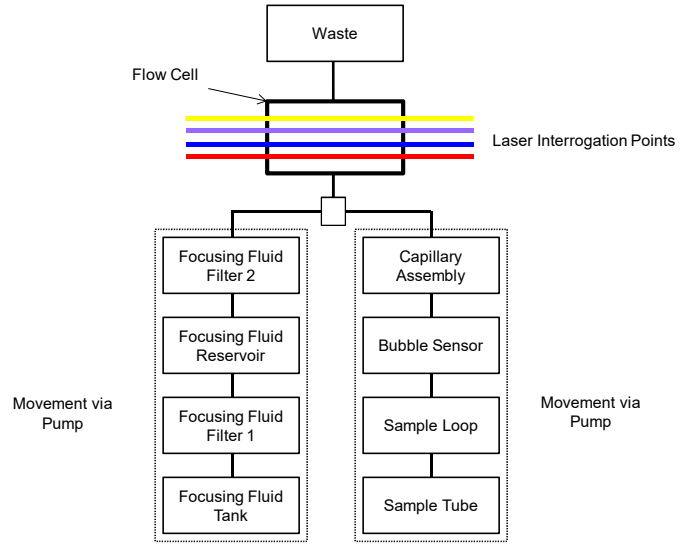
Attune™ NxT Fluidics System



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Attune™ NxT Fluidics Solutions

Attune™ Focusing Fluid: 1X buffered, azide-free solution which transports focused particles to the flow cell for laser interrogation. It prevents sample from coming into contact with the walls of the flow cell. It contains an anti-microbial agent, a preservative and a detergent designed to minimize bubble formation. (Cat.No. 4488621, available in different sizes)

Attune™ Wash Solution: 1X solution to minimize background by removing cellular debris and dyes from the fluidic system of the instrument. (Cat.No. A24974)

Attune™ Shutdown Solution: 1X solution to minimize bubble formation and crystal deposit in the fluidic system when the instrument is shutdown. It contains an anti-microbial agent. (Cat.No. A24975)

Attune™ Debubble solution: 1X solution to remove bubbles and sticky particles from the fluidics system (Cat.No. A10496).

Attune™ NxT Flow Cell Cleaning Solution: 3X Alkalyne liquid concentrate to clean out the flow cell (Cat.No. A43635).

10% Bleach: To decontaminate the fluidics lines. To be prepared fresh daily.

Deionized water: Used for diluting bleach. To be highly filtered and sterile.

Notes: All solutions must be Room Temperature (RT) before use.

"10% Bleach" = **0.5% - 1%** NaOCl

The final concentration of sodium hypochlorite should be **0.5% to 1%.**

Some facts about "bleach":

- Sodium Hypochlorite is **not stable** >>> tends to decompose over time releasing chlorine gas.
- Factors that promote this decomposition: **heat, light, metal ions** (including water hardness), low pH (less than 11), and more.
- Decomposition rate is **exponential** >>> occurs in the first week after production
- Best storage conditions are low temperature (fridge) in a closed opaque container.

10% Bleach = 0.5% to 1% sodium hypochlorite

The final concentration of sodium hypochlorite to be used in the instrument should be **0.5% to 1%.**

Example: 10% bleach is defined as a 1 in 10 dilution (1 part bleach to 9 parts water) of 5.25% sodium hypochlorite in water. This gives a final concentration of **~0.5% sodium hypochlorite** equivalent to ~5000 ppm of available chlorine.

More concentrated formulations (e.g. Ultra and Concentrate) are also available:

- ✓ Ultra is 6.15% Sodium Hypochlorite and should be diluted 1 part bleach to 11 parts water.
- ✓ Concentrate is 8.25% Sodium Hypochlorite and should be diluted 1 part bleach to 15 parts water

Bleach Solution	Dilution	Chlorine (ppm)
5.25%	None	52,500
	1:10	5,250
Ultra 6.15%	None	61,500
	1:12	5,125
Concentrate 8.25%	None	82,500
	1:16	5,150

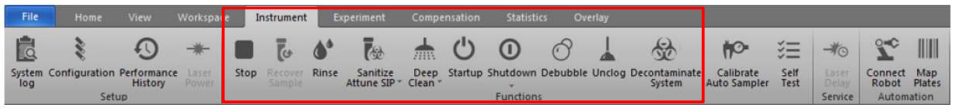
https://www.cdc.gov/infectioncontrol/pdf/guidelines/disinfection-guidelines.pdf

Recommendation:
Prepare fresh bleach
Use laboratory-grade bleach
Avoid bleach with additives (such as perfumes)

Attune™ NxT External Fluid Supply

- Optional accessory for the Attune™ NxT Cytometer
- Provides extended Focusing Fluid and Waste capacity
  - 10 L for Focusing Fluid
  - Up to 18 L for the Waste (including 2L of bleach for biosafety requirements)
- Fully controlled and powered by the Attune™ NxT Cytometer
- Automatically monitors the empty/full states of the Cytometer containers for Focusing Fluid and Waste

Fluidics Functions



Stop is used to end any running script.

Recover Sample is a user-initiated function allowing the return of unused sample volume back to the well or the tube

Rinse flushes system between samples to minimize carryover. This function is run automatically every time the SIP is lowered, but it can also be user-initiated.

Sanitize Attune SIP is a user-initiated function for sanitizing the SIP and sample lines between sticky/dirty samples or experiments. This function requires 10% bleach solution.

Deep Clean is a user-initiated function that thoroughly washes the system sample lines and flow cell between sticky/dirty samples or experiments. This function requires 10% bleach solution.

Startup script primes the instrument fluidics with Attune™ Focusing Fluid.

Shutdown script allows the user to sanitize and shut down the instrument.

Debubble is a user-initiated function for clearing bubbles from the fluidics lines of the cytometer. This function uses Attune™ Debubble solution.

Unclog is a user-initiated back flush operation to remove clogs from the sample line.

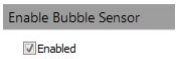
Decontaminate System is a user-initiated function for automated decontamination of the Cytometer and the Auto Sampler fluidics.

Bubble Sensor

The Attune™ NxT Cytometer is equipped with a Bubble Sensor designed to detect "bubbles" (air in the system) with a volume greater than 40 µL.

What is the Bubble Sensor?

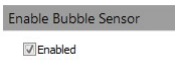
The Bubble Sensor is a hardware component located along the fluid path between the sample loop and the flow cell. It is always "ON" BUT the user will only be notified of air in the system if the "Enable Bubble Sensor" option is enabled in the Attune™ NxT software.



How does the Bubble Sensor Work?

After a sample is aspirated it passes through the bubble sensor and is directed towards the flow cell. If bubbles (air) are present in the sample, or in this section of the fluidics line, a system notification will be triggered.

Bubble Detection

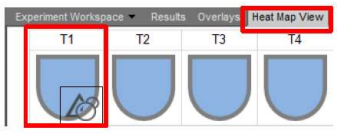


- By default, the bubble sensor option is enabled
  - This setting is applied to all users
  - Only an Administrator, System Administrator or Service account may access the Enable Bubble Sensor option, from the *Options* menu under *Configuration* section
- Bubbles may be detected
  - During sample aspiration or during acquisition
  - In tube or in plate

Bubble Detection

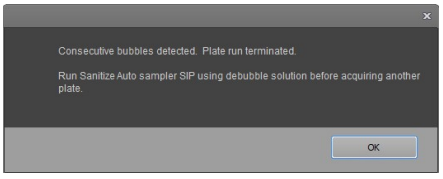
In Tube mode

- If recoverable volume of sample is within the system, the user will be given the option to recover the remaining sample
  - Click “Yes” to recover the sample
  - Click “No” to discard the sample
- The Heat Map view will label the tube sample with the bubble detected icon

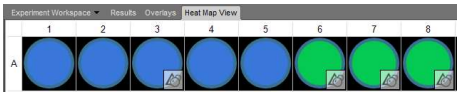


In Plate mode

- After a bubble is detected in 3 consecutive wells, the bubble detected dialogue box will appear and the plate run is terminated



- The Heat Map view will label wells with the bubble detected icon



Leak Detection

The leak sensor is located on bottom-front of the Attune™ NxT Cytometer centered under the focus fluid bottle. There is also a sensor located under the Attune™ Autosampler.

Leak Detection

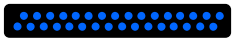
Instrument status bar shows instrument status and alerts indicator icons:

Plate Leak Detected: The Plate Leak Detected icon is displayed when a leak in the Auto Sampler has been detected. Tooltip displays “Auto Sampler leak detected”.

Instrument Leak Detected: The Instrument Leak Detected icon is displayed when a leak in the instrument has been detected. Tooltip displays “Instrument leak detected”.

If a leak is also detected in the Auto Sample, the Tooltip displays “Instrument leak and Auto Sampler leak detected”

Status Indicator Lights



Flashing: Booting up
Fluidic functions

Solid: Idle (after Startup)
Flashing: Running sample

Flashing: Instrument error

Fade: Sleep mode

Attune™ NxT Optical System

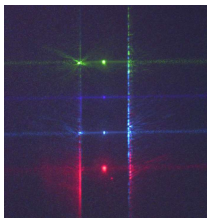
TRUE or FALSE

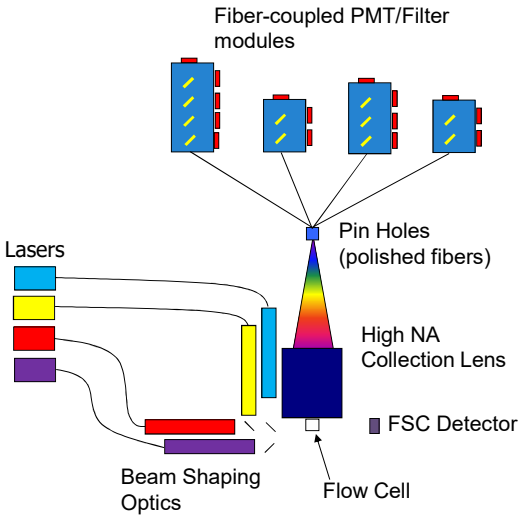
The Attune™ NxT Cytometer has a flexible optical configuration

True False

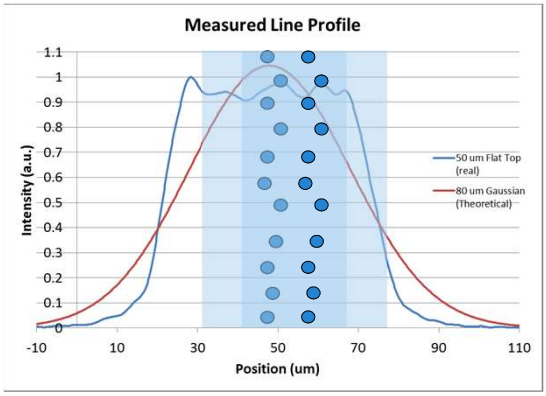
Modularity – Single Platform Optics

- Up to 4 Lasers
- Up to 14 Fluorescence Detectors
- 2 Scatter Detectors
- Different PMTs for different Wavelengths





Minimized Down Time - Stabilize Alignment



- Flatten gaussian profile
- Provide safety net/margin for alignment drift
- Alignment ease in field/manufacture
- Corrects for fluidic instabilities

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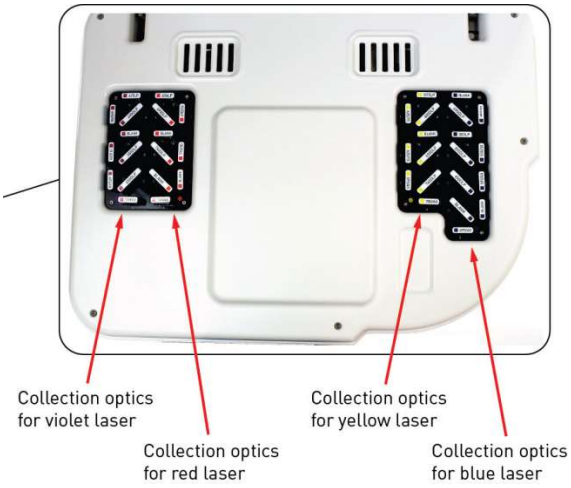
Attune™ NxT Optical Components

- From 1 to 4 Lasers:
  - Violet 405 nm 50 mW
  - Blue 488 nm 50 mW
  - Yellow 561 nm 50 mW
  - Green 532 nm 100 mW
  - Red 637 nm 100 mW
- Filters are User-Changeable
- 4 Attune™ NxT Accessory Filter Configurations
  - Small Particle Side-Scatter Filter
  - No-Wash No-Lyse Filter Kit
  - Fluorescent Protein Filter kit
  - Custom Filter Holder Kit



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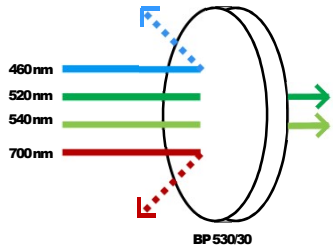
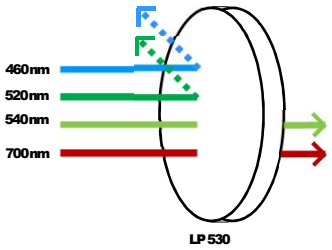
Filters

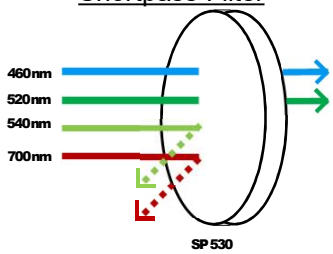
Bandpass Filter



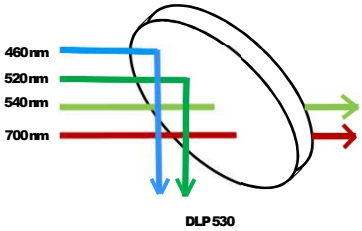
Longpass Filter



Shortpass Filter



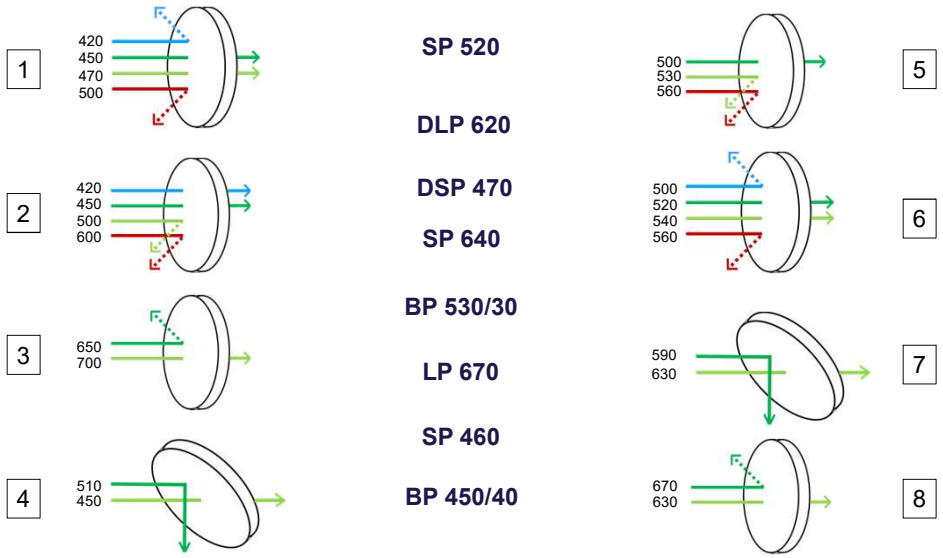
Dichroic Mirror



Practical Session

- Activity 2 – Optical Filters

Select the filter type and wavelength that matches best



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Attune™ NxT Optical Configurations

4 violet channels configurations and 561nm (yellow) laser

Configuration	# colors	Pac Blue	Pac Green	Pac Orange	Qdot 705	FITC	PE	PI	PerCP-Cy5.5	PE-Cy7	PE	PE-Texas Red	PE-Cy5.5	PE-Cy7	APC	AF700	APC-AF750
filters		440/50	512/25	603/48	710/50	530/30	574/26	590/40	695/40	780/60	585/16	620/15	695/40	780/60	670/14	720/30	780/60
Blue	4					■	■		■	■							
Blue Red	7					■	■		■	■					■	■	■
Blue Violet	8	■	■	■	■	■	■		■	■							
Blue Yellow	7					■		■	■		■	■	■	■			
Blue Red Violet	11	■	■	■	■	■	■		■	■					■	■	■
Blue Violet Yellow	11	■	■	■	■	■		■	■		■	■	■	■			
Blue Red Yellow	10					■		■	■		■	■	■	■	■	■	■
Blue Red Violet Yellow	14	■	■	■	■	■		■	■		■	■	■	■	■	■	■

Note: The Attune™ NxT Cytometer laser configuration can be upgraded by our Field Service Engineers.

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Attune™ NxT Optical Configurations

4 violet channels configurations and 532nm (green) laser

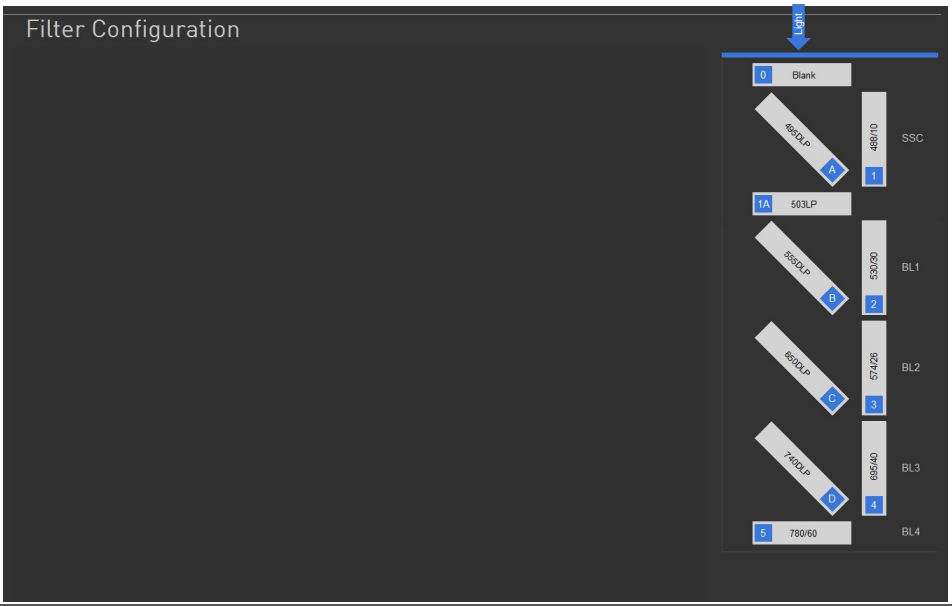
Configuration	# colors	Pac Blue	Pac Green	Pac Orange	Qdot 705	FITC	PE	PI	PerCP-Cy5.5	PE-Cy7	PE	PE-Texas Red	PE-Cy5.5	PE-Cy7	APC	AF700	APC-AF750
filters		440/50	512/25	603/48	710/50	525/50	574/26	590/40	695/40	780/60	575/36	620/15	695/40	780/60	670/14	720/30	780/60
Blue Green	7					■		■	■		■	■	■	■			
Blue Violet Green	11	■	■	■	■	■		■	■		■	■	■	■			
Blue Red Green	10					■		■	■		■	■	■	■	■	■	■
Blue Red Violet Green	14	■	■	■	■	■		■	■		■	■	■	■	■	■	■

6 violet channels configurations

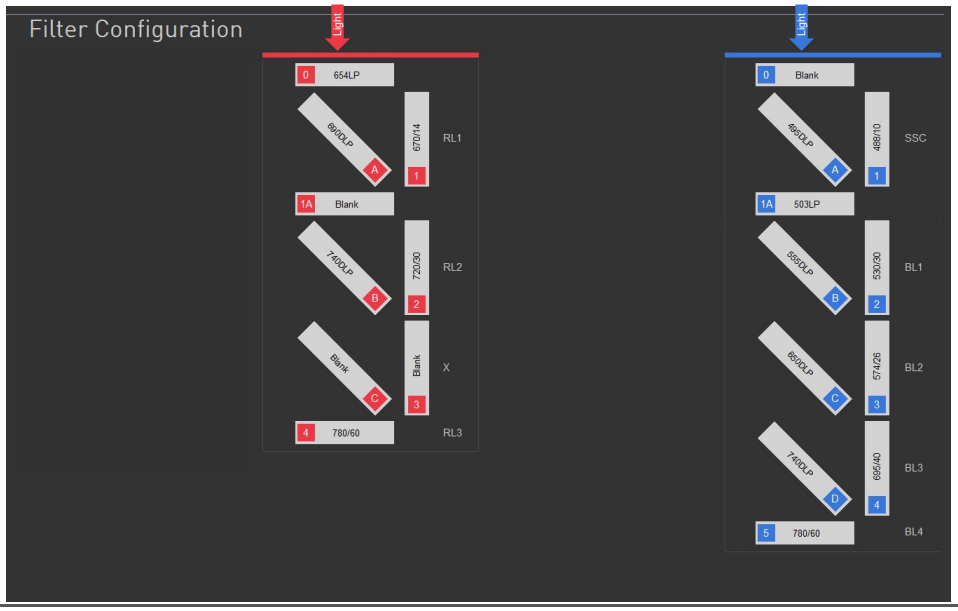
Configuration	# colors	Pac Blue SB436	Pac Green	Pac Orange SB600	SB645 BV650	Qdot 705 SB702	BV786	FITC	PE	PerCP-Cy5.5	PE	PE-Texas Red	PE-Cy7	APC	AF700	APC-AF750
filters		450/40	525/50	610/20	660/20	710/50	780/60	530/30	574/26	695/40	585/16	620/15	780/60	670/14	720/30	780/60
Blu Violet6	9	■	■	■	■	■	■	■	■	■						
Blu Red Violet6	12	■	■	■	■	■	■	■	■	■				■	■	■
Blue Red Violet6 Yellow	14	■	■	■	■	■	■	■		■	■	■	■	■	■	■

Note: The Attune™ NxT Flow Cytometer laser configuration can be upgraded by our Field Service Engineers.

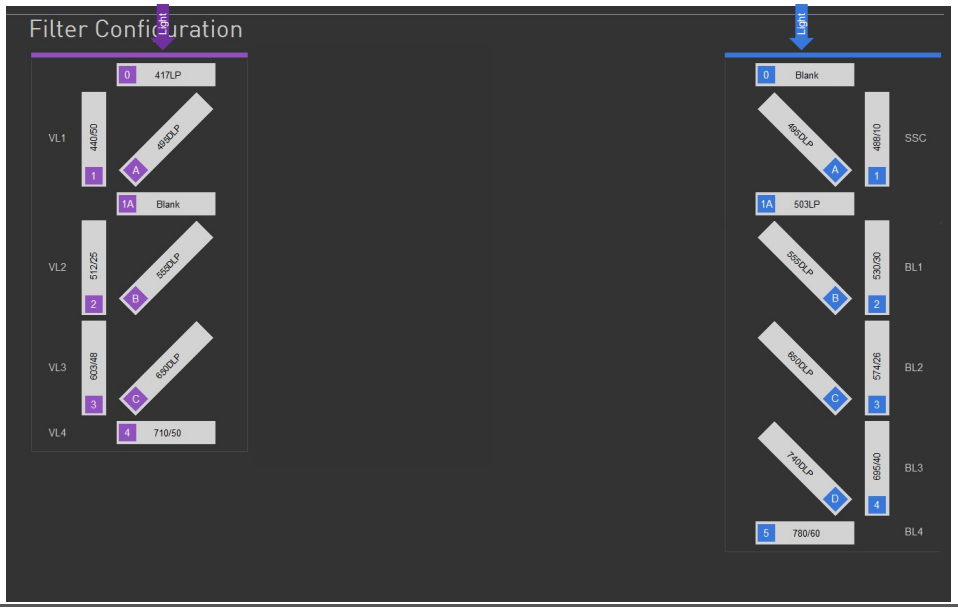
Blue (P/N A24864) – default configuration



Blue Red (P/N A24863) – default configuration



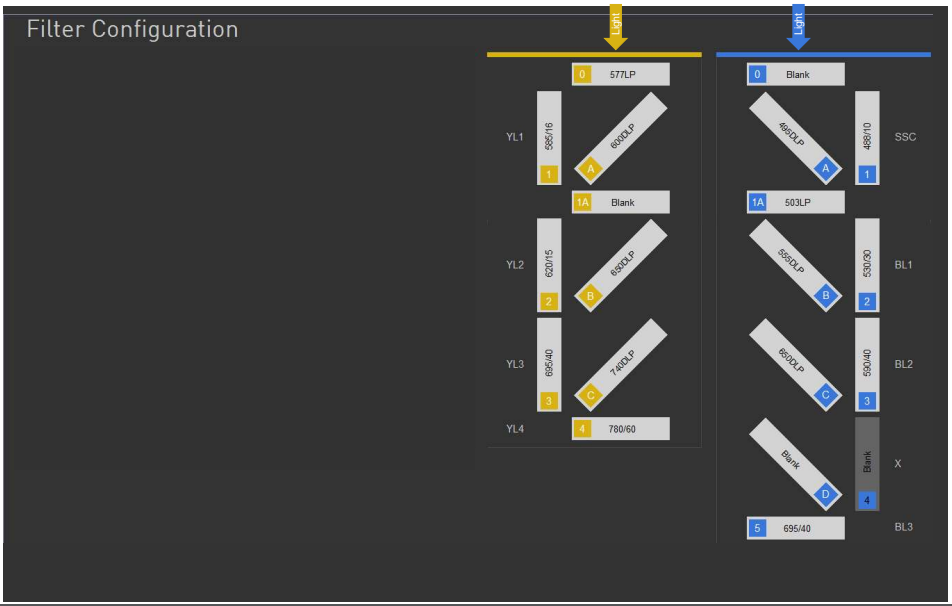
Blue Violet (P/N A24862) – default configuration



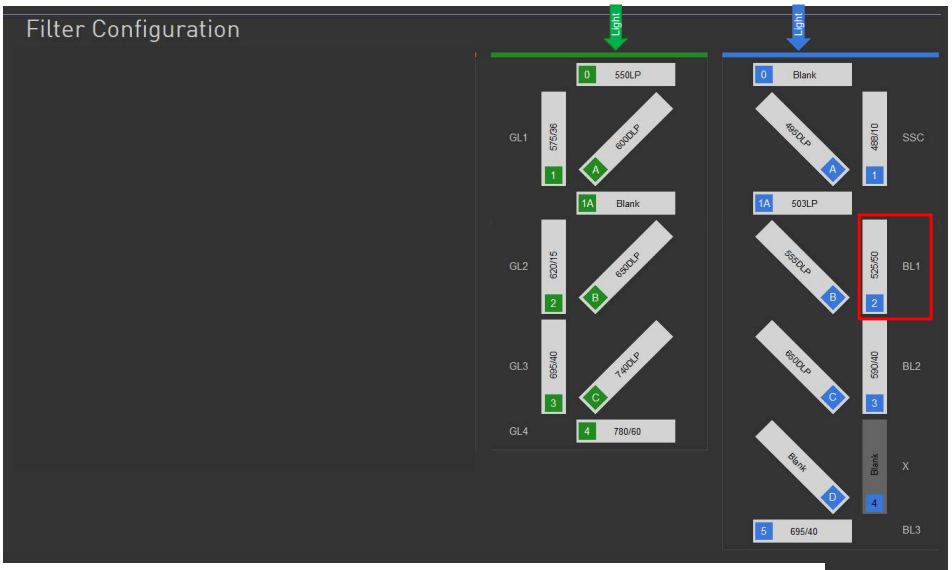
Violet6 Blue (P/N A29002) – default configuration

NOTE: Different filter in VL1 position (450/40) and optical deck orientation for implementing 6 violet channels. Only 3 channels out of blue laser

Blue Yellow (P/N A24869) – default configuration

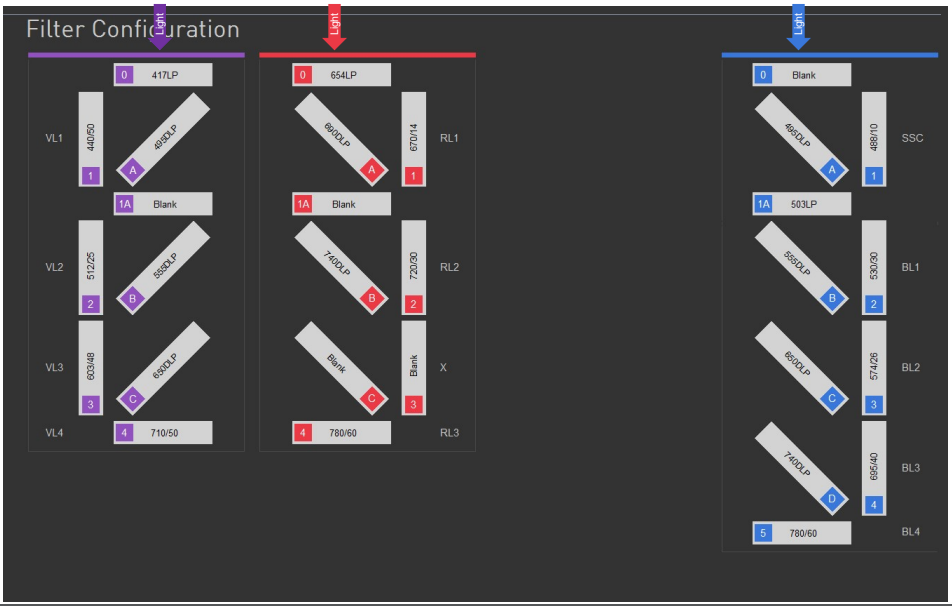


Blue Green (P/N A28995) – default configuration

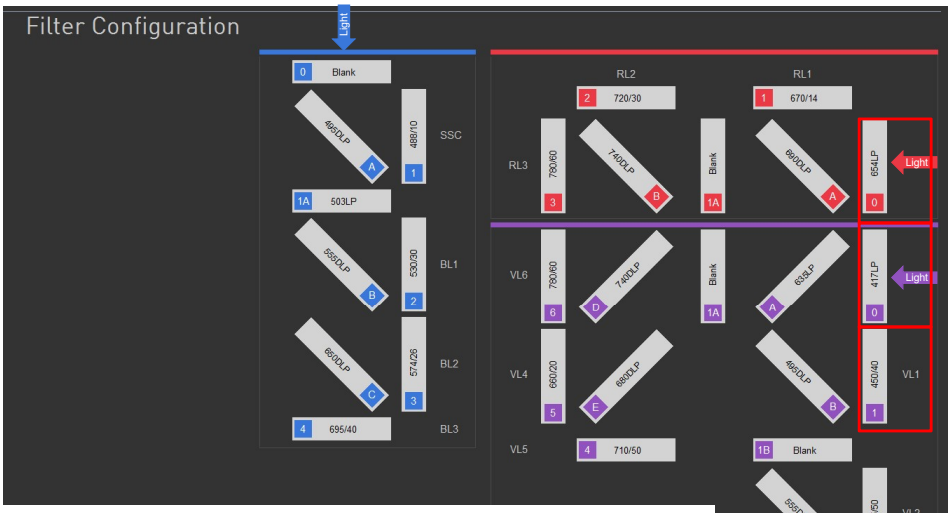


NOTE: Different filter in BL1 position (525/50) for Green laser system

Violet Blue Red (P/N A24860) – default configuration

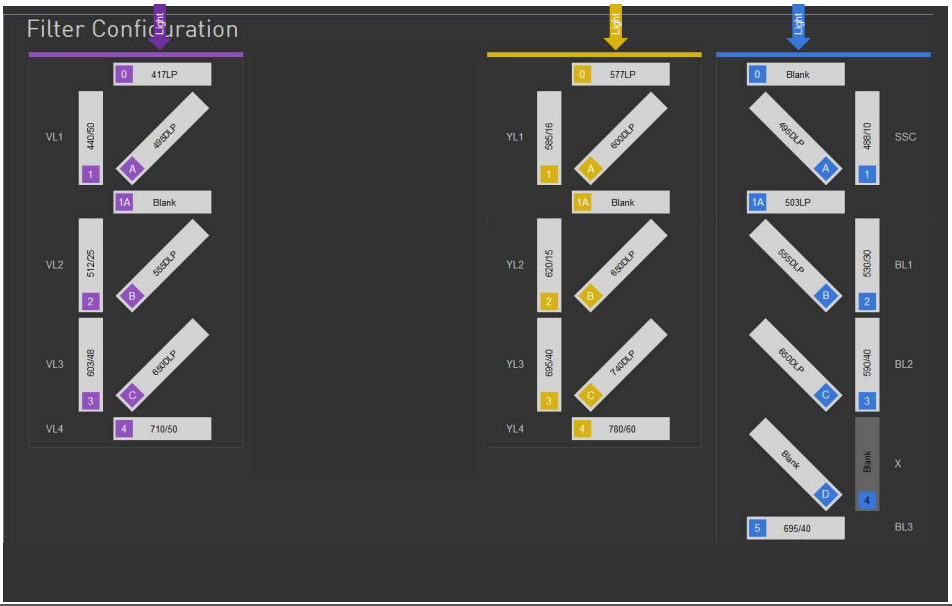


Violet6 Blue Red (P/N A24860) – default configuration

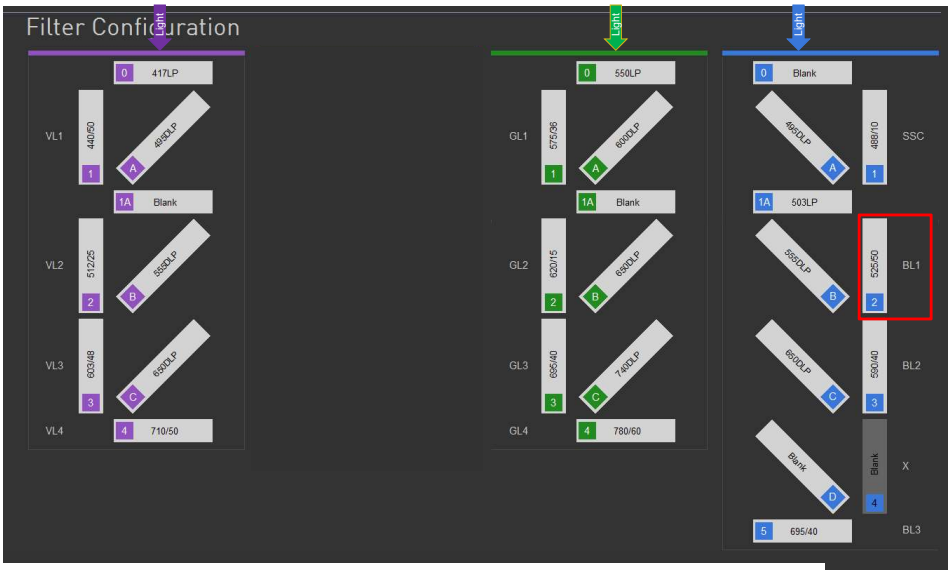


NOTE: Different filter in VL1 position (450/40) and optical deck orientation for implementing 6 violet channels. Different number of channels on blue (3 channels)

Violet Blue Yellow (P/N A24859) – default configuration

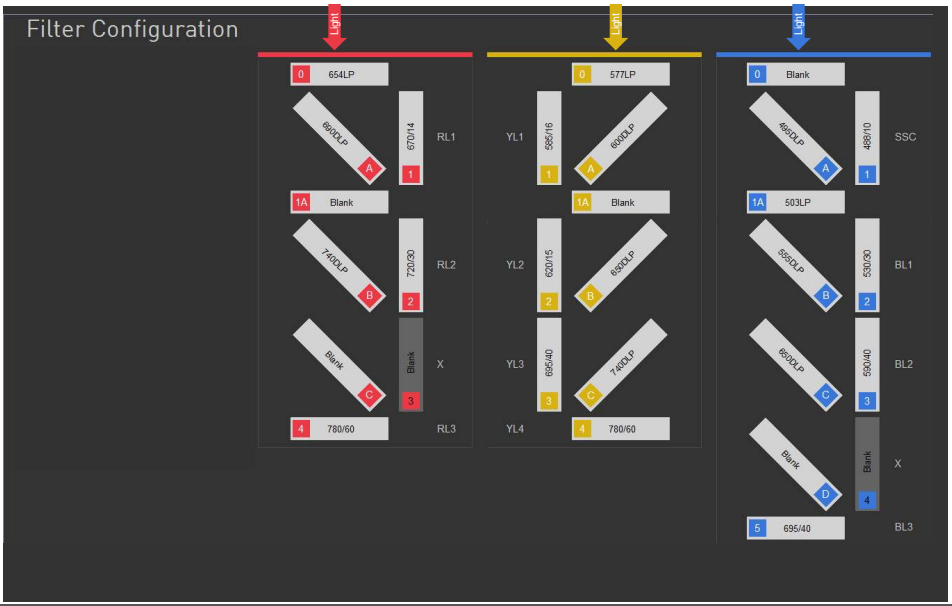


Violet Blue Green (P/N A28999) – default configuration

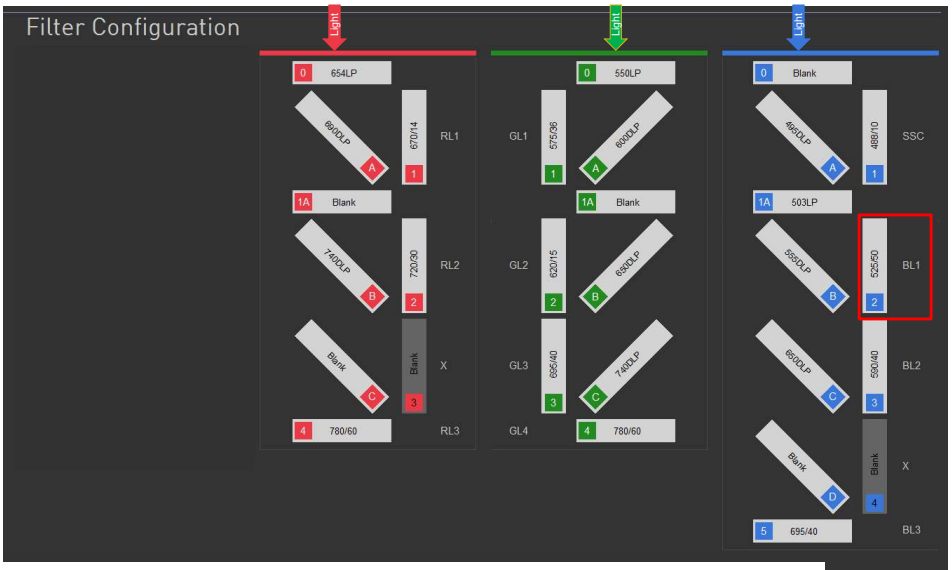


NOTE: Different filter in BL1 position (525/50) for Green laser system

Blue Red Yellow (P/N A28993) – default configuration

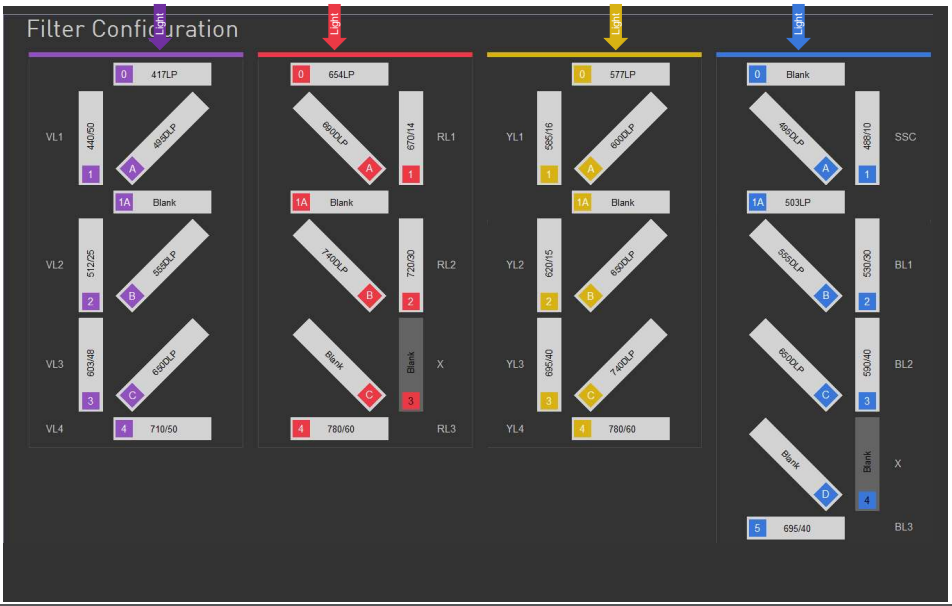


Blue Green Red (P/N A28997) – default configuration

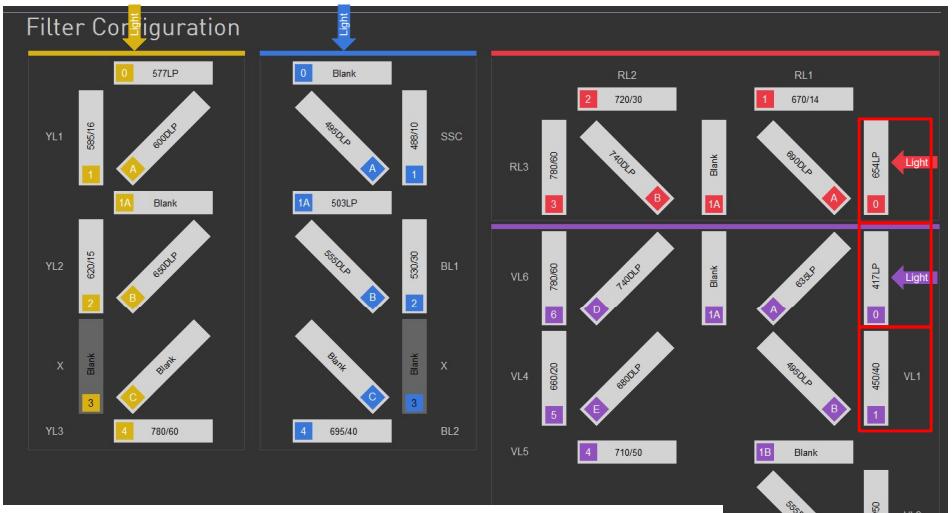


NOTE: Different filter in BL1 position (525/50) for Green laser system

Violet Blue Yellow Red (P/N A24858) – default configuration

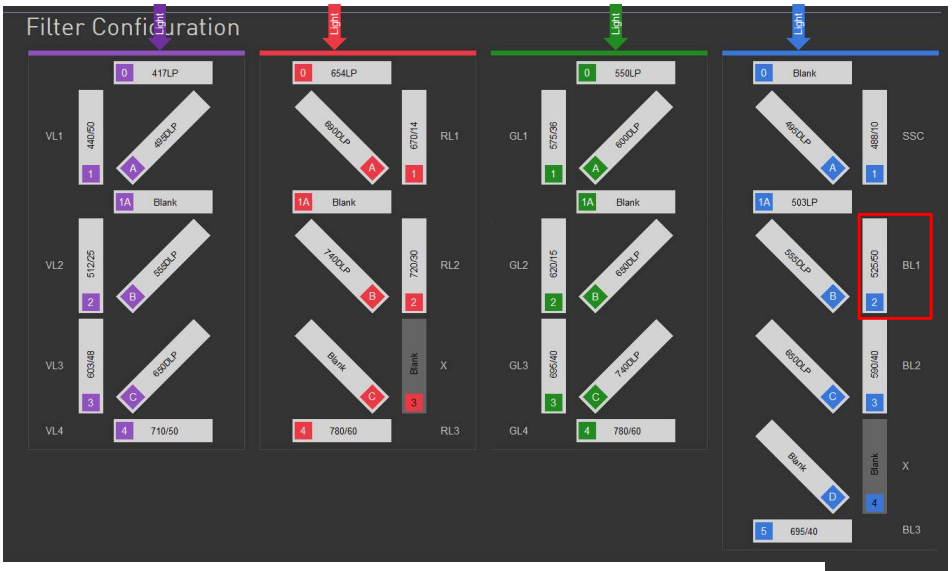


Violet6 Blue Yellow Red (P/N A29004) – default configuration



NOTE: Different filter in VL1 position (450/40) and optical deck orientation for implementing 6 violet channels. Different number of channels on blue (2 channels) and yellow (3 channels) lasers

Violet Blue Green Red (P/N A29001) – default configuration



NOTE: Different filter in BL1 position (525/50) for Green laser system

Attune™ NxT Accessory Filter Configuration

- vSSC configuration: Attune™ NxT No-Wash No-Lyse Filter Kit (Cat.No. 100022776)
  For additional side scatter channel off of the violet laser:
  - 405/10 BP filter
  - 415 Dichroïc LP

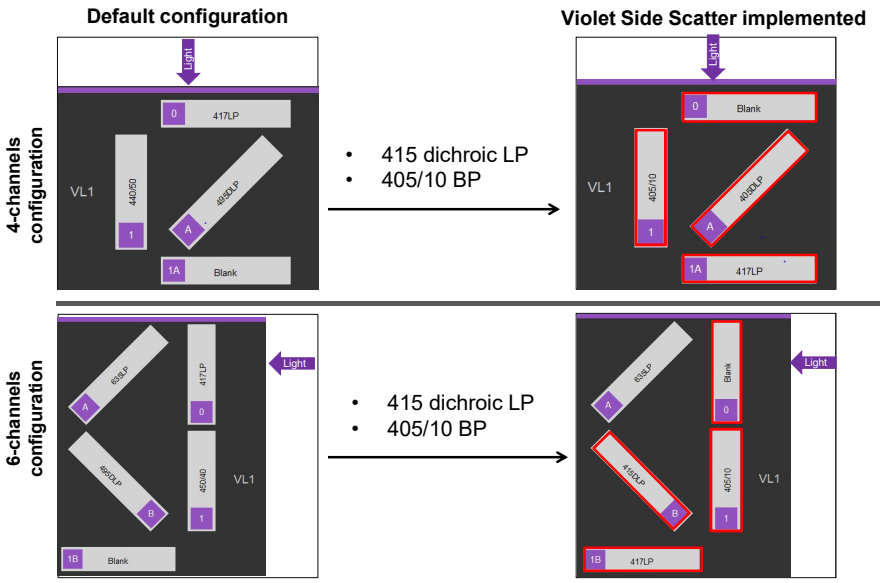
- Attune™ NxT Small Particle Side-Scatter Filter (Cat.No. 100083194):
  To increase the dynamic range of the side-scatter detection:
  - 488/10 BP filter

Attune™ NxT Accessory Filter Configuration

- Fluorescent protein configuration: Attune™ NxT Fluorescent Protein Filter Kit (Cat.No. 100022775)
  For multiplex detection of GFP, YFP, and mCherry fluorescent proteins:
  - 510/10 BP filter (GFP)
  - 540/30 BP filter (YFP)
  - 615/25 BP filter (mCherry)
  - 525 dichroic LP

- Attune™ NxT Custom Filter Holder Kit (Cat.No. A27784):
  To make custom emission or dichroic filters:
  - 2 dichroic filter blades
  - 2 emission filter blades

Violet SSC (vSSC) Configuration



NOTES: vSSC configuration will cause the loss of the first channel out of the violet deck to detect fluorescence → Reordering the remaining filters will allow you to use different fluorochromes combinations

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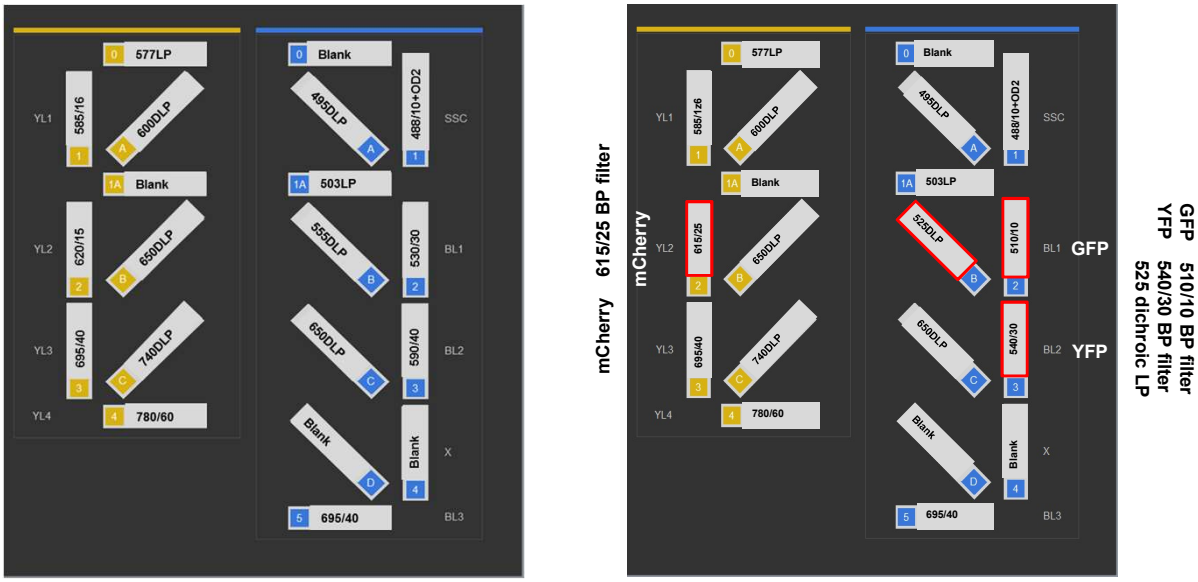
Fluorescent Protein Configuration



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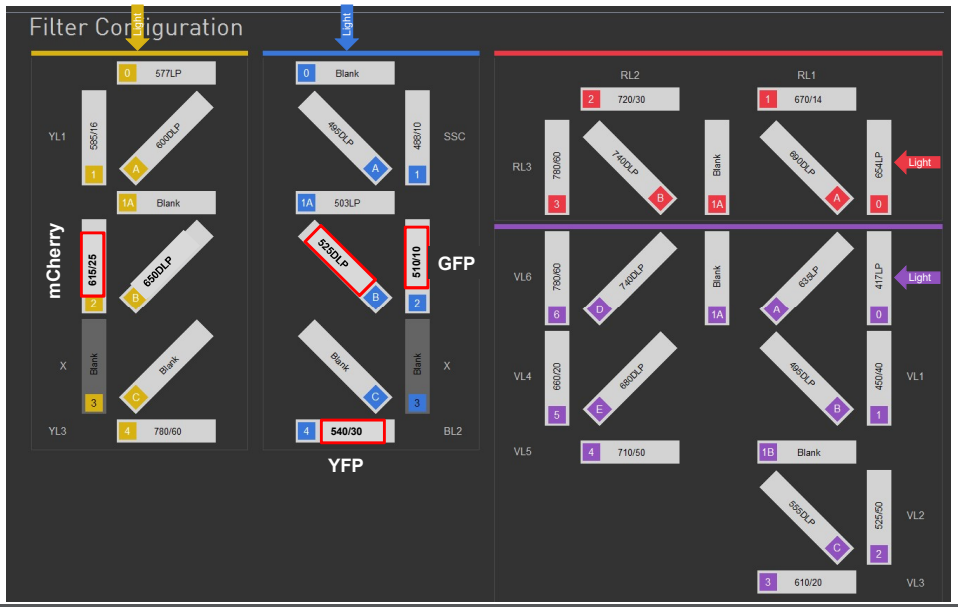
Fluorescent Protein Configuration on Violet6 Blue Yellow Red



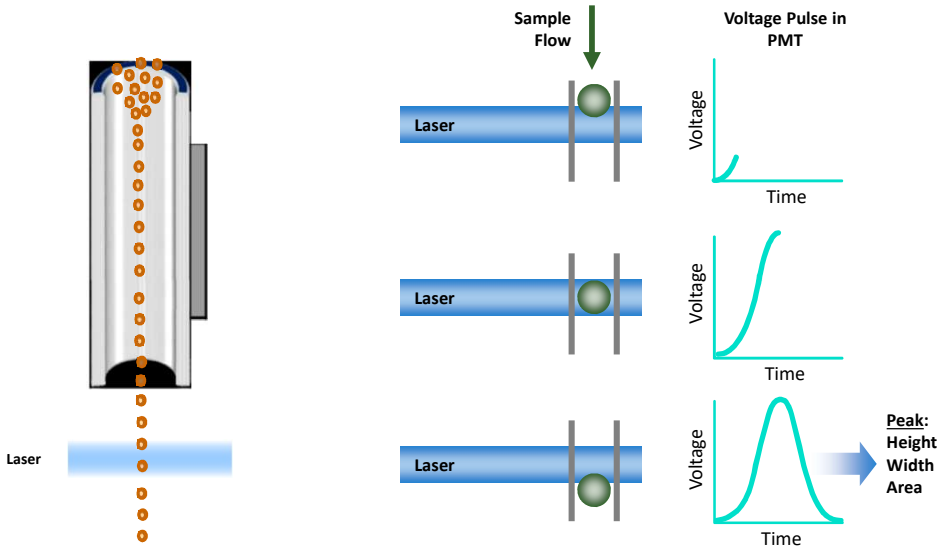
Attune™ NxT Electronics System

Electronics

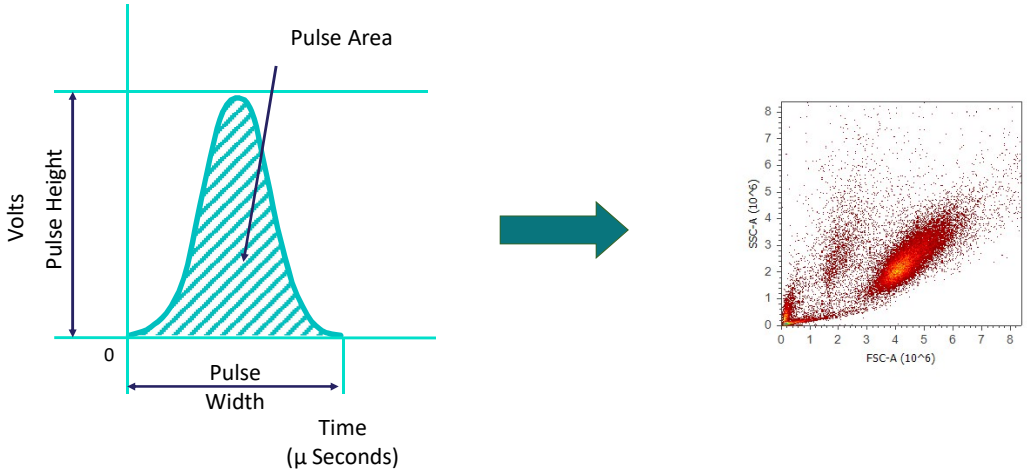
Functions of Electronics:

- Convert detected light signals into proportional electronic signals (voltage pulses)
- Electronic signals are processed by the onboard processor
- Convert electronic signals from the detectors into digital data used for analysis
- Interface with the computer for data transfer

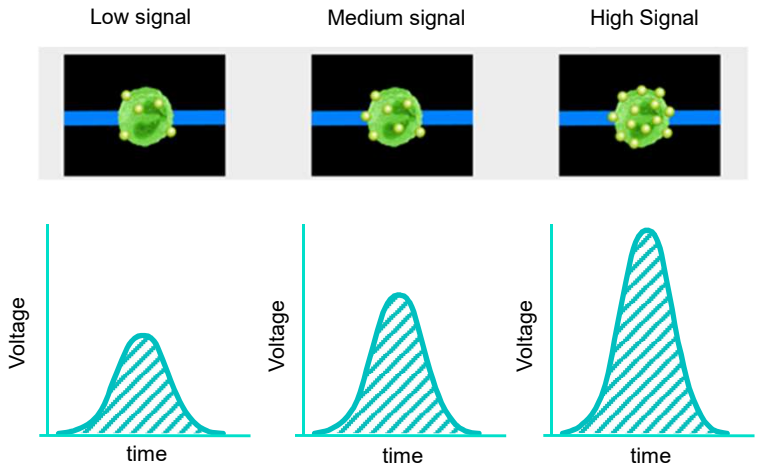
Sample Presentation: Voltage Pulse



Sample Presentation: Voltage Pulse



Interpreting the voltage pulse of a parameter



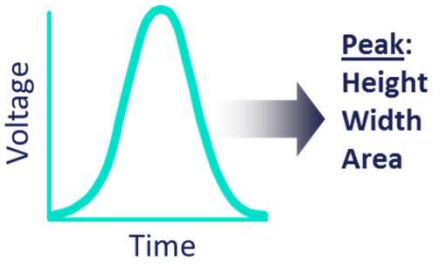
https://www.thermofisher.com/de/de/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/flow-cytometry-basics/flow-cytometry-fundamentals/electronics-flow-cytometer.html

TRUE or FALSE

With the Attune™ NxT Cytometer you only collect the Area of the Voltage Pulse

True False

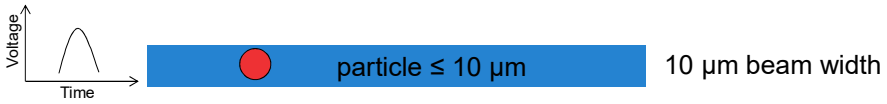
Pulse to parameters



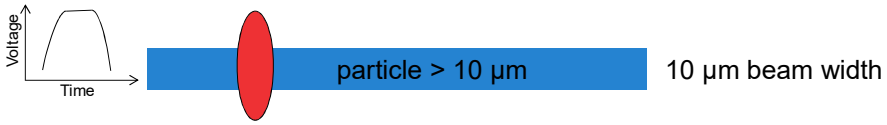
Attune™ NxT Software default: All parameters (A-H-W) on all channels

Area or Height - What should I use?

- Particle size < Laser width: Height = Area



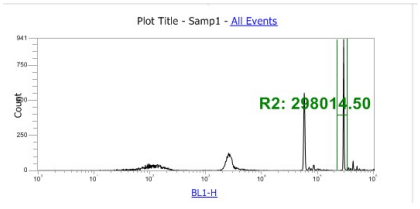
- Particle size > Laser width: Height ≠ Area

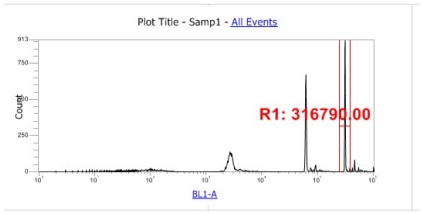


➢ Two single particles of very different size might generate a voltage pulse with the same height measurement (same amplitude) but with substantially different area.

Area Scaling Factors - What do they do?

- The Area Scaling Factor (ASF) is a correction parameter that sets the Area and Height measurements at parity:



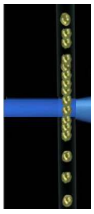


- ASF are calculated for each laser as part of the Baseline and Performance tests and are stored as a System Setting that is applied globally to all users

Channel	PMTV	Target MFI	MFI	Robust %CV	Qr	Background	Linearity	ASF	Laser Delay	Result
FSC	555	300000	302000	1.80 %	0.000	0	0.000	1.01	1100	✓
SSC	318	300000	304325	3.44 %	0.000	0	0.000	1.01	1100	✓
BL1	403	300000	301618	1.34 %	0.051	86	1.000	1.01	1100	✓
BL2	348	300000	311511	1.34 %	0.068	138	0.966	1.01	1100	✓
BL3	385	300000	305088	1.72 %	0.047	18	1.000	1.01	1100	✓
RL1	480	300000	304847	7.33 %	0.016	59	0.994	0.78	1512	✓
RL2	493	300000	307636	7.70 %	0.002	448	0.999	0.78	1512	✓
RL3	510	300000	307720	7.87 %	0.007	217	1.000	0.78	1512	✓

Coincident events – What are they?

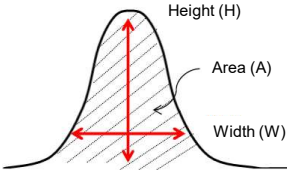
"**Coincident events**" occur when two particles pass through the laser interrogation point so quickly that their respective voltage pulse cannot be separated.



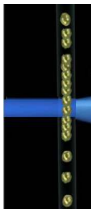
High quality flow cytometry data is obtained from **single** cell analysis, so removal/exclusion of this phenomenon is highly recommended

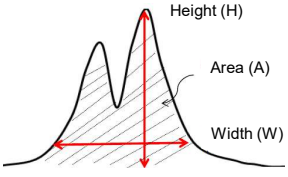
Coincident events and Voltage Pulse parameters

Voltage pulse from coincident events will have same height (H) but area (A) and width (W) measurements larger than the corresponding pulse of a single event



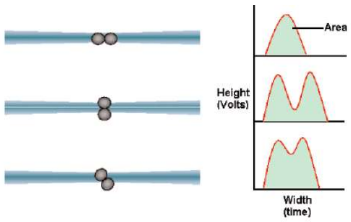
Voltage pulse from a single event





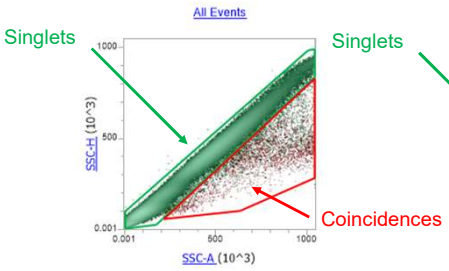
Voltage pulse from a coincident event
(2 particles)

Coincident events removal using Pulse Parameters (manual)

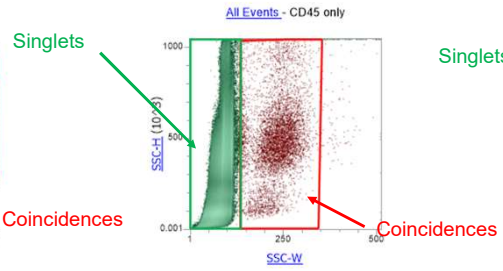


All events are RECORDED AND SAVED
but ONLY single cells must be used for analysis

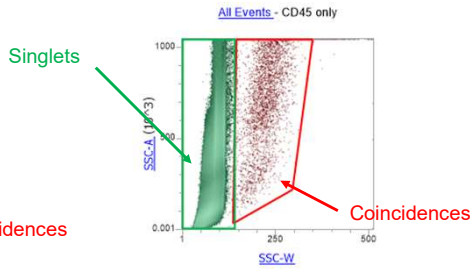
Three Methods of Determination



Linear Relationship of Pulse *Height* (-H) to Pulse *Area* (-A)



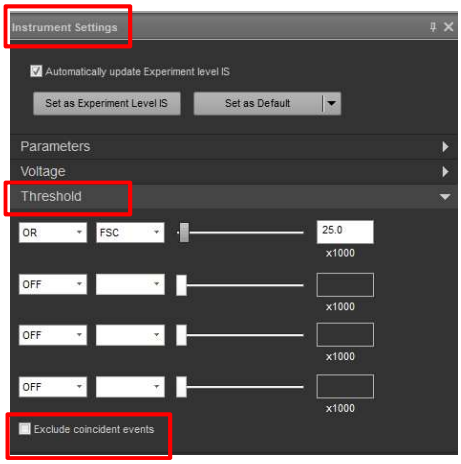
Relationship of Pulse *Height* (-H) to Pulse *Width* (-W)



Relationship of Pulse *Width* (-W) to Pulse *Area* (-A)

"Exclude coincident events" option

To remove automatically coincident events from collection by selecting "Exclude coincident events" option within the Threshold section of the instrument settings menu

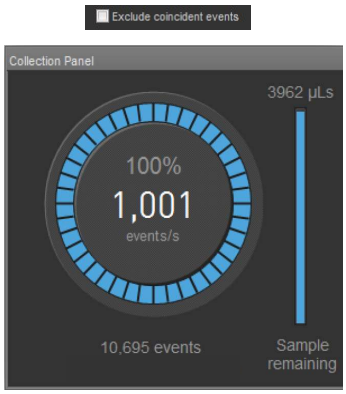


IMPORTANT NOTE: By default this option is **NOT SELECTED**

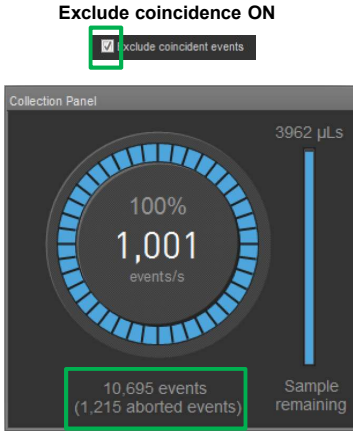
Number of aborted events

When “Exclude Coincident Events” is enabled the **number** of events that excluded from the data stream (due to coincidence) is **displayed** in the collection panel and **recorded** within the FCS file

Exclude coincidence OFF



Exclude coincidence ON



Aborted events are NEITHER PROCESSED or DISPLAYED in plots

Attune™ NxT Electronic Specifications

- **Maximum Single-Event File:** 20 million with option to append
- **Data Acquisition rate:** Up to 35.000 events/sec
  - Up to 34 parameters
  - Based on 10% coincidence rate
  - No Abort, No Lost
- **Maximum Electronic Speed:** 65.000 events/sec with all parameters

Attune™ NxT Autosampler

Attune™ NxT Autosampler

- Compatible with many different standard plate formats including 96-well, 384-well, and deep-well plates; flat, round and V-bottom.
- Intelligent probe design minimizes clogging and carryover (<0.5%) and prevents damage to the instrument.
- Performs automated cleaning between wells (from 1 to 10 rinses) and when the instrument is shutting down.
- Minimal variation regardless of sampling method (tube vs. plate) and collection rates.
- Easy to plug and unplug on the Attune™ NxT Cytometer.

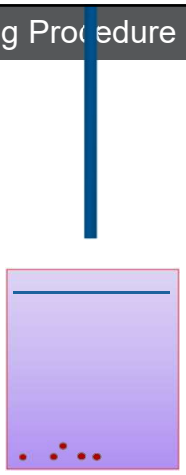
TRUE or FALSE

The Attune™ NxT Autosampler is mixing samples by vortexing.

True

False

Attune™ Autosampler Mixing Procedure

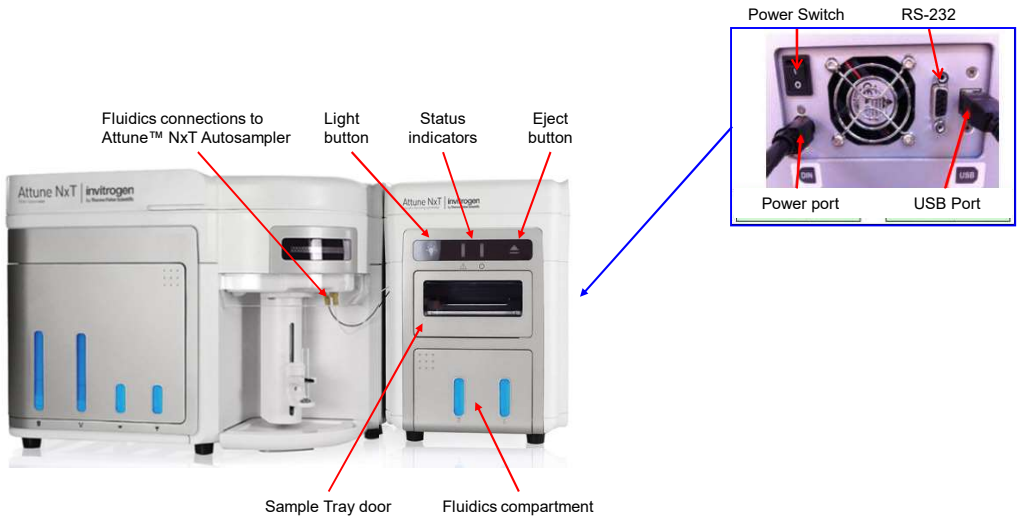


The user sets:
- The plate type
- The total sample volume
- The number of mixes (up to 10)

The system defines:
- The liquid level in well
- The probe position
- The mixing method

Mixing samples by aspiration instead of shaking ensures homogeneity of the sample and maintains cell viability.

Exterior Components



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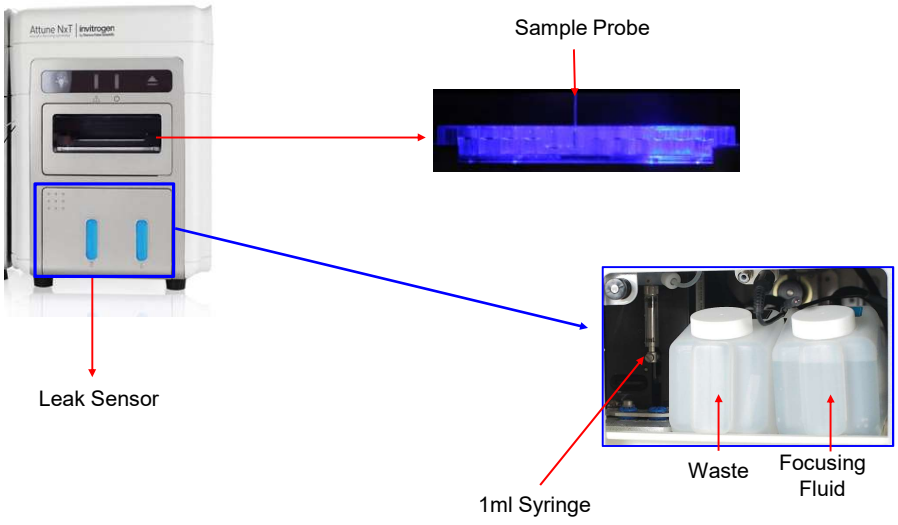
Interior Components



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Thermo Scientific™ Orbitor™ RS Microplate Mover

Revision 2.5
Revision Date: Aug2019

ThermoFisher SCIENTIFIC

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Thermo Scientific™ Orbitor™ RS Microplate Mover

- A high-speed robotic mover designed for integrated lab automation
- Automatic retrieval of a plurality of microplates in association with a variety of applications, including flow cytometry

- Allow to scale up to the volume of samples needed for statistical significance and rich data from which to validate research and discovery.

Revision 2.5
Revision Date: Aug2019

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Key Learning Points

- The Attune™ NxT Flow Cytometer uses acoustic and hydrodynamic focusing
- Samples are delivered by a positive-displacement syringe pump
- The optical system is flexible (up to four lasers, can be upgraded in the field, filters are interchangeable and can be customized)
- The optical part consists of flat-top lasers, fiber optic cables, collection lense, optical filters, PMTs
- The electronical system converts the light-signal into an electronical signal (Voltage pulse, A-H-W)
- The Attune™ NxT Autosampler is an optional accessory for the Attune™ NxT Flow Cytometer and enables rapid processing of multiple samples from 96- or 384-well plates

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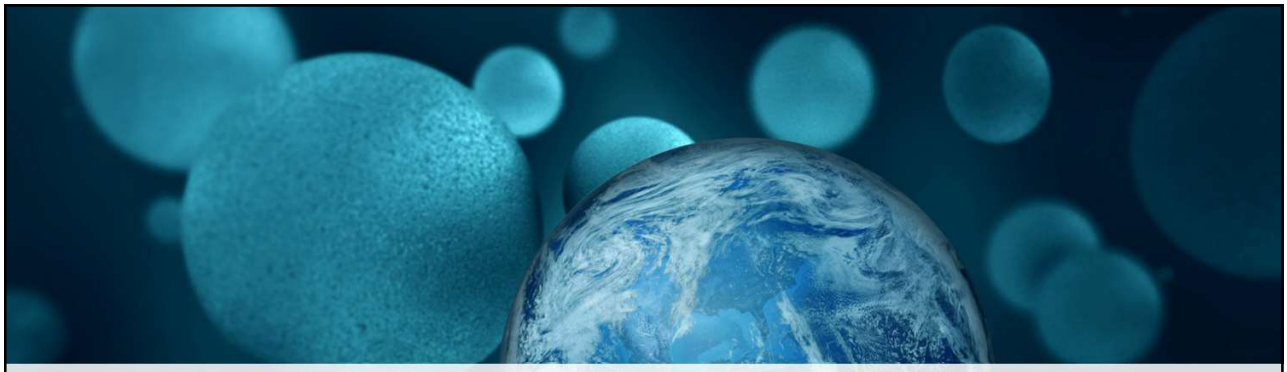

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Experiment Setup

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Before to start

Preliminary operation
with
Attune™ NxT Flow Cytometer

Workflow

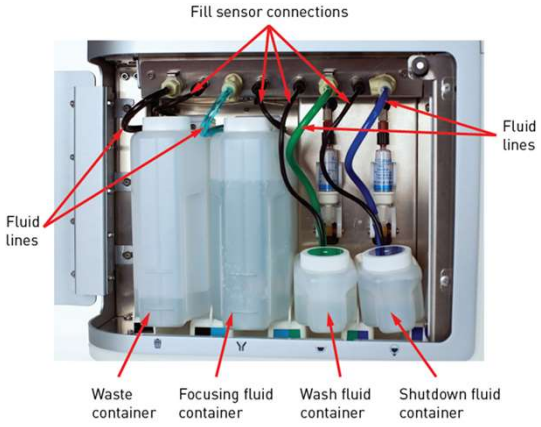
- Visual Inspection
- Instrument Startup
- Optical Configuration Check
- Performance Test
- Experiment Settings Optimization
- Compensation
- Data acquisition
- Data Analysis
- Instrument Shutdown

Instrument visual inspection

- Fluidics compartment: Make sure there are no fluids or salt residues on the floor of the compartment, around the connectors, or on tube junctions.

- Check the fluids level. Fill/empty as needed:
  - Focusing fluid
  - Wash solution
  - Shutdown solution
  - Waste

- Visually inspect the SIP.



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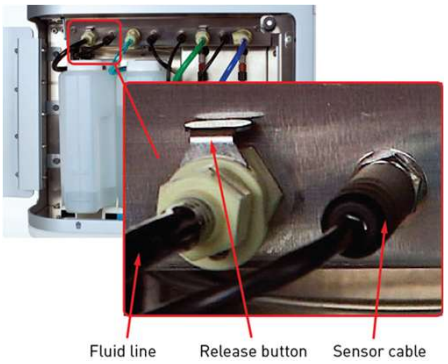
Filling (or emptying) Fluid Tanks



1. Remove the sensor cable from the instrument.
2. Press the metal release buttons to free the tubing.
3. Fill or empty as needed with RT solutions:
   1. Large tanks – 1.9 L
   2. Small tanks – 175 mL
4. Return tanks to cytometer and reconnect the fluid line, then the sensor line.

IMPORTANT

1) Connecting the sensor cable while leaving the fluid line disconnected may result in increased back pressure and introduction of air into the system.

2) The Attune™ NxT Flow Cytometer must be idle before refilling the fluidics containers.

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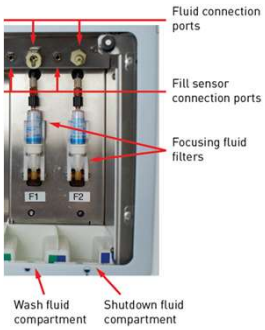
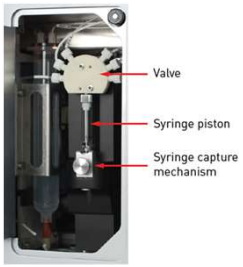
Daily - Visual inspection

- **Focusing Fluid Filters** – Located behind the wash and shutdown fluidics bottles. Change if there are any signs of debris/dirt, or if the sample pump stays on too long.



- **Syringe Compartment** – Make sure there is no fluid or salt residue on the floor of the compartment.
- **Syringe** – Finger-tighten the syringe; change if there is a leak or if salt residue builds up.



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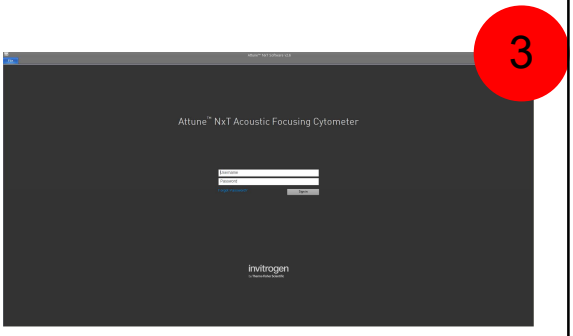
Switching ON the Attune™ NxT Flow Cytometer



Important Notes:

- If Attune™ NxT Flow Cytometer and Attune™ NxT Autosampler are on sleep mode, close the software and relaunch.
- Power Cycle Attune™ NxT Flow Cytometer and Attune™ NxT Autosampler and Computer at least once a week

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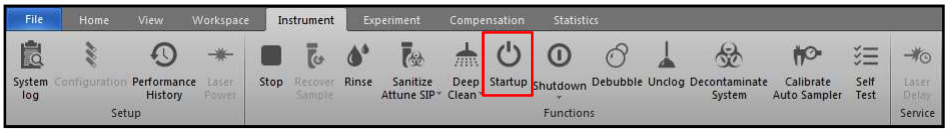
Workflow

- Visual Inspection
- Instrument Startup
- Optical Configuration Check
- Performance Test
- Experiment Settings Optimization
- Compensation
- Data acquisition
- Data Analysis
- Instrument Shutdown

Instrument Startup

- If Instrument is in Sleep Mode, the Startup script will wake it up



- During the Startup, the Attune™ NxT Cytometer software:
  - Automatically turns on instrument systems
  - Initializes the pump in the cytometer
  - Initializes the motors and pumps in the Autosampler when it is connected and powered on
  - Primes the fluidic lines

- Once Startup function is complete, the Status Lights are solid **Green**

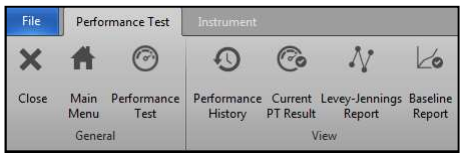
Workflow

- Visual Inspection
- Instrument Startup
- Optical Configuration Check
- Performance Test
- Experiment Settings Optimization
- Compensation
- Data acquisition
- Data Analysis
- Instrument Shutdown

Instrument Performance

- Allows you to monitor performance of the instrument.
- Critical to ensure accuracy and sensitivity of instrument.
- Provides information about the lasers and detection channels.

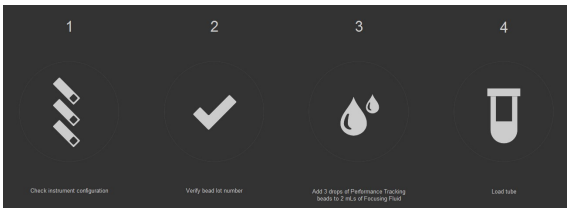
- There are 2 parts to Instrument Performance Tracking:
  - Baseline Calculation (BL)
  - Daily Performance test (PT)



Attune™ Performance Tracking Beads

- A mixture of beads of four fluorescence emission intensities in equal concentration
  - Blank
  - Dim
  - Medium
  - Bright
- 3 ml vial: use 3 drops of beads per 2 ml of Focusing Fluid (vortex beads!)
- Run a SIP Sanitize following Performance Test

Part No. 4449754



Instrument Performance

- Baseline Calculation
  - Access to this function can/should be restricted
  - Performed at time of installation by Field Service Engineer (FSE)
  - Performed after any major service (FSE)
  - Performed every time the bead lot changes (User)
  - Performed whenever recommended by FSE
- Daily Performance Test
  - Run daily - everyday samples are run/recorded

Baseline Calculations

- To be performed as recommended earlier.

Note: Data for a new lot of beads can be downloaded from the ThermoFisher Scientific website

- Uses Performance Tracking bead specifications (MESF values) to define initial status of the Attune™ NxT Cytometer.
  - PMT voltages are adjusted to place the brightest bead at target MFI values; voltage value for each channel is recorded.
  - The robust % coefficient of variation (%rCV) of the brightest bead is recorded.
  - Relative quantum efficiency (rQ) of each detector is determined.
  - Relative Background level (rB) of each detector is determined.
  - Linear regression is calculated and recorded.
  - Laser delay setting is automatically calculated.
  - Area scaling factor calculated and reported for every channel.

Baseline Calculations

Baseline test successful

Baseline: 756080D - 7/24/2014 | 7/24/2014 12:03:42 PM

Channel	PMTV	Target MFI	MFI	Robust %CV	Qr	Background	Linearity	ASF	Laser Delay	Result
FSC	575	300000	302004	1.28 %	0.000	0	0.000	1.02	1100	✓
SSC	358	300000	306486	3.03 %	0.000	0	0.000	1.02	1100	✓
BL1	381	300000	300836	1.30 %	0.060	101	1.000	1.02	1100	✓
BL2	361	300000	304638	1.57 %	0.058	135	0.965	1.02	1100	✓
BL3	413	300000	301436	1.84 %	0.051	37	1.000	1.02	1100	✓
RL1	367	300000	315794	3.99 %	0.064	40	0.998	0.97	1557	✓
RL2	378	300000	309933	3.72 %	0.013	176	1.000	0.97	1557	✓
RL3	407	300000	310010	3.59 %	0.079	77	0.997	0.97	1557	✓
VL1	297	300000	291418	0.97 %	0.014	949	1.000	0.81	698	✓
VL2	385	300000	304298	1.11 %	0.021	224	0.998	0.81	698	✓
VL3	375	300000	303931	1.32 %	0.023	98	0.996	0.81	698	✓
VL4	433	300000	312414	2.21 %	0.006	235	0.984	0.81	698	✓
YL1	401	300000	301724	1.90 %	0.110	40	0.999	0.71	239	✓
YL2	390	300000	308583	1.71 %	0.071	38	0.973	0.71	239	✓
YL3	430	300000	300002	2.12 %	0.030	100	0.999	0.71	239	✓
YL4	501	300000	302131	3.08 %	0.004	320	1.000	0.71	239	✓

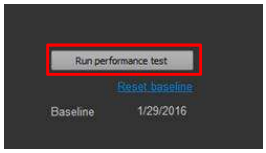
Instrument Performance

- Baseline Calculation
  - Access to this function can/should be restricted
  - Performed at time of installation by Field Service Engineer (FSE)
  - Performed after any major service (FSE)
  - Performed every time the bead lot changes (User)
  - Performed whenever recommended by FSE

- Daily Performance Test
  - Run daily - everyday samples are run/recorded

Performance test

- Recommended to be performed every day instrument is going to be used



- Uses Performance Tracking bead and data generated during baseline calculations
  - PMT voltages are adjusted to place the brightest bead at target MFI values.
  - Reports the voltage change (Δ PMT) from Baseline voltage.
  - The robust % coefficient of variation (%rCV) of the brightest bead is recorded.
  - Relative quantum efficiency (rQ) of each detector is determined.
  - Relative Background level (rB) of each detector is determined.
  - Linear regression is calculated and recorded.
  - Laser delay setting is automatically calculated.
  - Area scaling factor calculated and reported for every channel.

Performance Test Report

Baseline 756080D - 7/9/2014 7/23/2014 3:27:05 PM

Channel	PMTV	Delta PMTV	Target MFI	MFI	Robust %CV	Qr	Background	Linearity	ASF	Laser Delay	Result
FSC	568	-9	300000	299756	2.06 %	0.000	0	0.000	1.02	1100	✓
SSC	350	-10	300000	288385	3.89 %	0.000	0	0.000	1.02	1100	✓
BL1	379	-1	300000	300519	1.24 %	0.060	115	1.000	1.02	1100	✓
BL2	359	1	300000	306803	1.34 %	0.057	118	0.965	1.02	1100	✓
BL3	410	-3	300000	297859	1.99 %	0.052	45	1.000	1.02	1100	✓
RL1	366	-2	300000	319156	3.83 %	0.070	42	0.998	0.96	1563	✓
RL2	374	-6	300000	292375	3.76 %	0.012	159	1.000	0.96	1563	✓
RL3	407	-3	300000	303972	3.82 %	0.059	72	0.997	0.96	1563	✓
VL1	301	4	300000	297676	1.58 %	0.008	579	1.000	0.82	694	✓
VL2	383	-3	300000	297463	1.15 %	0.020	282	0.998	0.82	694	✓
VL3	374	-6	300000	300917	1.40 %	0.023	93	0.995	0.82	694	✓
VL4	429	-8	300000	289260	2.17 %	0.005	221	0.984	0.82	694	✓
YL1	400	-3	300000	292582	1.44 %	0.092	34	0.999	0.68	229	✓
YL2	390	-3	300000	304246	1.31 %	0.067	36	0.973	0.68	229	✓
YL3	430	-3	300000	294263	2.16 %	0.028	94	0.999	0.68	229	✓
YL4	500	-3	300000	288991	3.16 %	0.005	309	1.000	0.68	229	✓

Pass ✓ Fail ⚠

Attune™ NxT Performance Specifications

✓ Pass - All statistics and calculations for the channel meet the criteria set by the Baseline calculation.

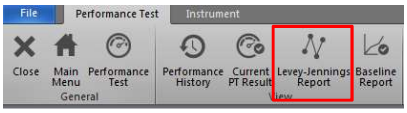
⚠ Fail - One or more of the statistics or calculations for the channel deviate significantly from the target set by the Baseline calculation.

e.g. Delta PMT exceeds 100 mV
%rCV – detector specific but ranges from 6-8%

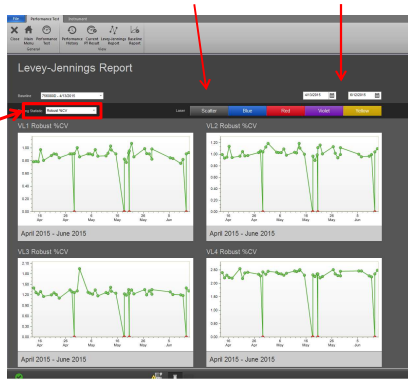
Levey-Jennings Report

The **Levey-Jennings Report** tracks the parameters listed in “Performance Test Report” for all channels.

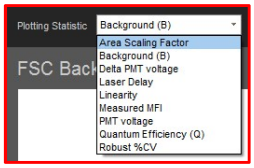
- Checks for shifts and trends in cytometer performance, providing visual indication of the cytometer performance over time.
- Use this report to look for individual channel trends and shifts.



Select laser and period to view

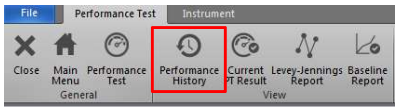


Select Statistic



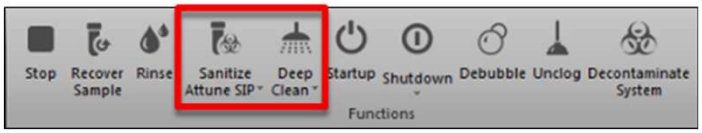
Performance History Report

Displays the **Pass/Fail status** of all Performance Tests run against a selected Baseline test and during a selected time period.



- Useful for gauging the overall “health” of the instrument over the selected time period.
- Data is displayed from the last 180 days for the current baseline.
- Report will only be active if a current baseline exists.
- Can be filtered using a date range.
- A result from any given day takes into account **all** Pass/Fail criteria for the instrument (e.g. Fail for one parameter, the entire test fails).

Clean between Experiments



- **After non-sticky cells, Run 2 Sanitize SIP**

Quick wash/sanitize of sample line and sample probe
Duration: ~3-4 min
- 1st iteration: Use 3 mL of 10% Bleach
- 2nd iteration: Use 3mL of Wash solution instead of 10% Bleach

- **After Sticky cells, Run Deep Clean**

Sanitize system with bleach and wash solutions for selectable period of time.
Three levels:
- Quick 5 cycles/~25min
- Standard 15 cycles/~45min
- Thorough 25 cycles/~75min

10% Bleach = 0.5% to 1% sodium hypochlorite

The final concentration of sodium hypochlorite to be used in the instrument should be **0.5% to 1%.**

Example: 10% bleach is defined as a 1 in 10 dilution (1 part bleach to 9 parts water) of 5.25% sodium hypochlorite in water. This gives a final concentration of **~0.5% sodium hypochlorite** equivalent to ~5000 ppm of available chlorine.

More concentrated formulations (e.g. Ultra and Concentrate) are also available:
- ✓ Ultra is 6.15% Sodium Hypochlorite and should be diluted 1 part bleach to 11 parts water.
- ✓ Concentrate is 8.25% Sodium Hypochlorite and should be diluted 1 part bleach to 15 parts water

Bleach Solution	Dilution	Chlorine (ppm)
5.25%	None	52,500
	1:10	5,250
Ultra 6.15%	None	61,500
	1:12	5,125
Concentrate 8.25%	None	82,500
	1:16	5,150

https://www.cdc.gov/infectioncontrol/pdf/guidelines/disinfection-guidelines.pdf

Recommendation:
Prepare fresh bleach
Use laboratory-grade bleach
Avoid bleach with additives (such as perfumes)

Workflow

- Visual Inspection
- Instrument Startup
- Optical Configuration Check
- Performance Test
- Experiment Settings Optimization
- Compensation
- Data acquisition
- Data Analysis
- Instrument Shutdown

Important sample guidelines

Sample Flow Rate	Max. Sample Concentration	Particle Size	Particle Velocity
1000 µL/ min	2.1 x 10^6 cells/mL	- Particles > 4 µm - Predominantly acoustic focusing	8 m/sec*
500 µL/ min	4.2 x 10^6 cells/mL	- Particles > 2 µm - Predominantly acoustic focusing	8 m/sec*
200 µL/ min	6.7 x 10^6 cells/mL		4 m/sec
100 µL/ min	1.3 x 10^7 cells/mL		
25 µL/ min	2.0 x 10^7 cells/mL	- Small particles < 2 µm - Best resolution from background for dimly positives assays - Smallest sample core - Predominantly hydrodynamic focusing	4 m/sec
12.5 µL/ min	2.0 x 10^7 cells/mL		

*Higher flow rates may show some loss of sensitivity

Important Guidelines for Absolute Counting

- Sample Preparation: accurate measurements are obtained for samples between 500 cells/mL-1x10^6 cells/mL
  - Dilute sample if a higher starting concentration is expected
- Maintain the event rate at <8,000 events/second to keep coincidence <10%.
- Cells must be kept in single cell suspension – minimize clumping by use of EDTA (from 2 to 5 mM) or protein in buffer (FBS, BSA… – from 0.5 to 5%)
- Ensure to account for all dilutions after collecting concentration statistic or calculating concentration manually

Important Guidelines for Absolute Counting

Recommended conditions for accurate counting based on particle/cell size

Particle or cell size range			
	0.5 – 3 µm	3 – 15 µm	> 15 µm
Sample type	Bacteria Microspheres	Jurkat cells Ramos cells Leukocytes Microspheres	Cardiomyocytes Microspheres
Flow rates	12.5 – 1000* µl/min	100 - 1000 µl/min	
Sample concentration range	500 - 10^6 particles/ml		
Event Rate	< 8 000 events/sec		
Sample volume	50 – 4000 µl (in tube) or 20 – 2000 ul (in plate)		

*Flow rates 100ul/min and above should only be used if measuring off a single laser

Important Guidelines for Absolute Counting

- Collect enough events to achieve statistically significant detection (>400 events for cells or particles of interest for 5% CV).
- Threshold: exclude debris
  - No-lyse-no-wash assays: use fluorescence threshold to identify WBC or exclude RBC (pan-leukocyte marker or DNA binding dye)
- Start with a clean instrument: ensure regular maintenance is completed
- Clean between each measurement: SIP sanitize
- Only use round-bottom plates when measuring concentration from 96- or 384-well plates

Important Guidelines for Absolute Counting

- Proper sample preparation and pipetting technique are critical:
  - Ensure samples are thoroughly mixed during each stage of sample preparation and before acquisition
  - Minimize transfer steps where possible; pipetting and mixing errors compound with the number of steps
  - Use calibrated pipettes and rigorous pipetting techniques
- Validate concentration measurement accuracy
  - Use bead produce (CountBright™ Absolute Counting Beads)
  - Expect 10% variation from bead specification
  - If greater variation observed, consider sample preparation

Buffer Density

Sample buffers **with very high salt concentration** create a large density difference between sample buffer and focusing fluid, causing artifacts in the data including:

- Delay in events at beginning of data streaming
- Absence of events at low sample flow rates
- Pulsing of data at medium to high sample flow rates
- Low event counts

Sample buffer should **not exceed** 5X salt concentration of focus solution, or **4.5% weight by volume**, or **1.03 g/ml specific gravity**.

If working in a very cold environment focus fluid should be brought to room temperature before running.

Experiment Settings Optimization

- **Experiment Setup** - Create a new experiment and using *Instrument Settings* Panel and *Workspace* Tab:
  - Select Parameters, Target & Label
  - Set-up the workspace
- **Run Protocol Setup** - In *Collection Panel*, set:
  - Acquisition Volume
  - Sample Flow Rate
  - Stop Options
- **Adjust PMT Voltages** – In *Instrument Settings* Panel, adjust Scatters and fluorescent detectors voltages
- **Set Threshold** – In *Instrument Settings* Panel, adjust threshold if necessary

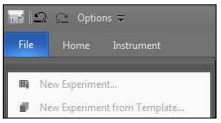
New Experiment Setup

4 ways to open the *New Experiment* dialogue box:

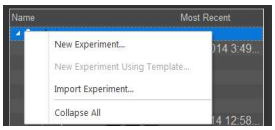
- From the *Main Menu* Screen

- From the *Home* tab, click the New Experiment icon

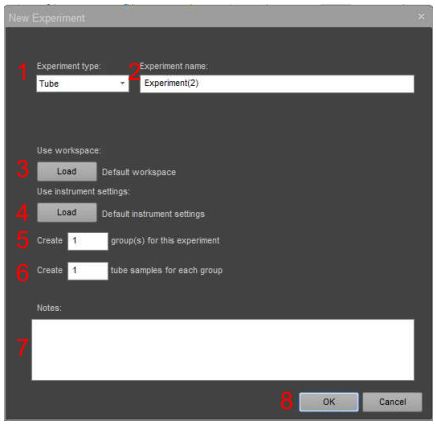
- From the *File* tab, select New Experiment



- From the Experiment Browser, right click on User Name, select New Experiment

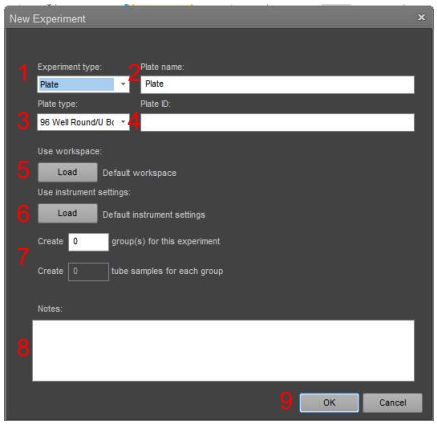


New Experiment Dialog Box - Tube Experiment



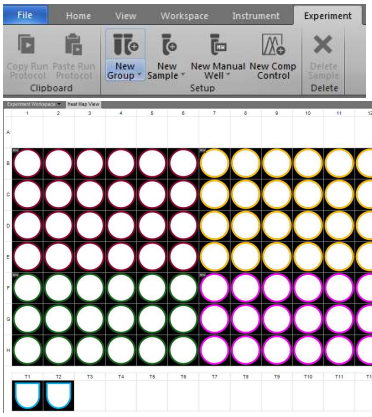
1. Select Experiment Type: Tube
2. Name Experiment
3. Load Workspace or use Default
4. Load Instrument Settings or use Default
5. Enter number of sample groups
6. Enter number of samples per group
7. Add notes (Optional)
8. Click OK

New Experiment Dialog Box – Plate Experiment



1. Select Experiment Type: Plate
2. Name Experiment
3. Select Plate type
4. Enter plate ID (Optional)
5. Load Workspace or use Default
6. Load Instrument Settings or use Default
7. Add number of groups and sample per group
8. Add notes (Optional)
9. Click OK

Plate Experiment Setup



1. Use the Heat Map to define mapping of samples for plate
2. Heat Map will show blank wells until they are assigned to an experiment
3. Select wells for Groups within the experiment
4. Click New Group or choose existing within the *Experiment tab*
5. Customize the Group and Sample names or titles (Note: the default sample name used is the well location)

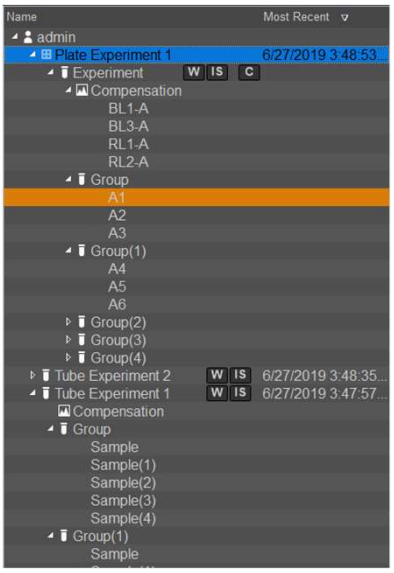
Notes:

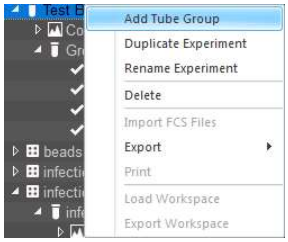
- One experiment can have multiple groups
- One plate can have a single experiment
- One Experiment can have a single plate

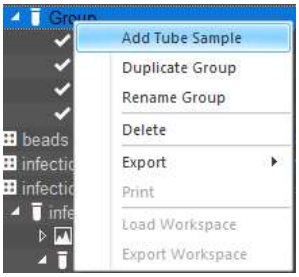
Group and Sample Setup

To add a New Tube Group or a New Tube Sample:

1. Right-click on the Experiment Name or the Group Name
2. Select Add Tube Group or Add Tube Sample
3. Right-click on Group Name or Sample Name and select Rename (or alternatively, click on F2 to rename).







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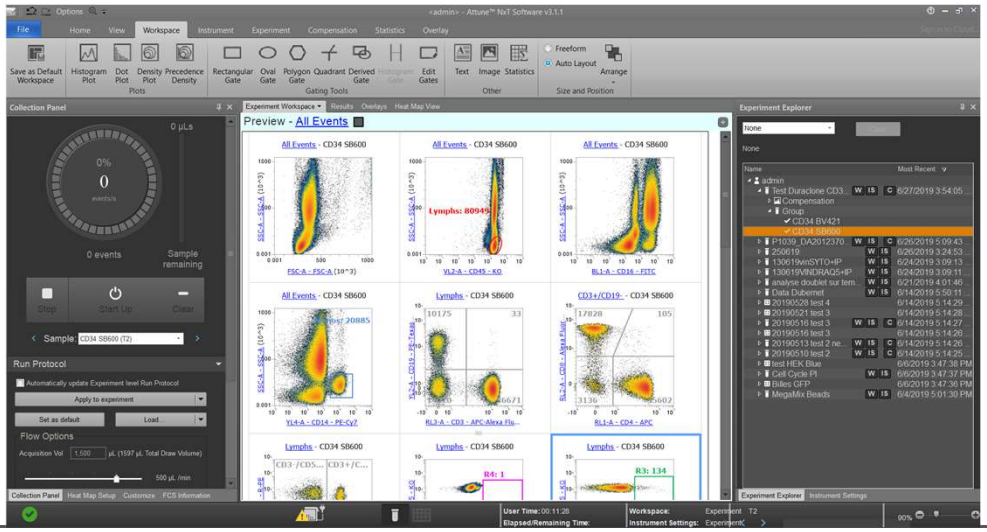
Main application workspace

The Main application workspace accommodates the Application area, a number of Application panels, the Experiment Explorer, and the Status notification display.



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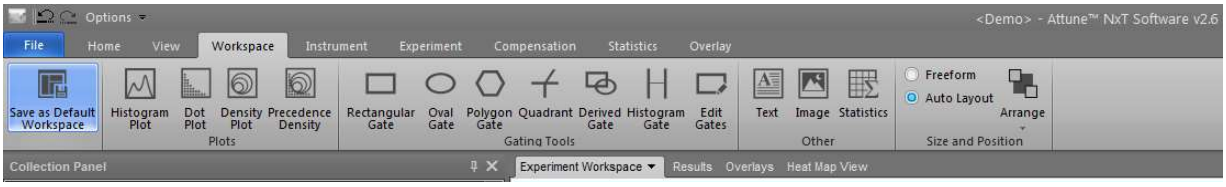
Ribbon tabs

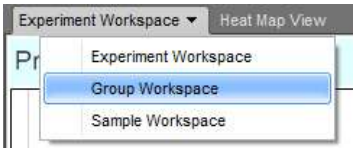


Depending on the context of the application, the Ribbon bar contains one or more of the following tabs :

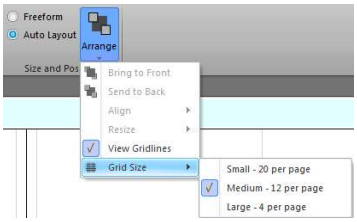
- File tab
- Home tab
- View tab
- Workspace tab
- Instrument tab
- Compensation tab
- Statistics tab

Workspace – Views and Modes

- **3 Workspace Views**:
  - Experiment workspace – experiment specific (i.e. global)
  - Group workspace – group specific
  - Sample – sample specific (i.e. local)



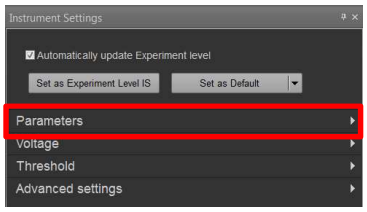
- **2 Workspace Modes**:
  - Freeform
    Add plots, text boxes, images, or statistics of any size anywhere on the Workspace. Objects can be freely resized and moved on the Workspace
  - Auto Layout (default)
    Workspace split into a set number (4 – 20) of *grid slots*. All objects of fixed size and placement. The default size is 12 grids per page and might be changed in *grid size* menu.



Instrument Settings – Parameter Selection and Naming

Expand the **Parameters** section:

- Default setting includes all channels and all parameters (A – H – W)
- De-select the fluorescent channels that are not needed in the experiment to reduce file size
- De-select the parameters (A-H-W) not needed
- Add names to Target and Label for each channel needed (e.g.: CD4-FITC)
- Select/deselect event count and/or time



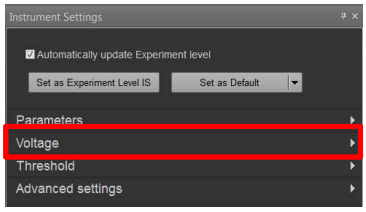
Notes:
No data will be collected for the deselected channels and parameters.

Large files: $20x10^6$ events are limited to 34 parameters IF collected at highest event rates (~30-35K/second). For slower runs, can use all.

Instrument Settings - Adjust PMT Voltages

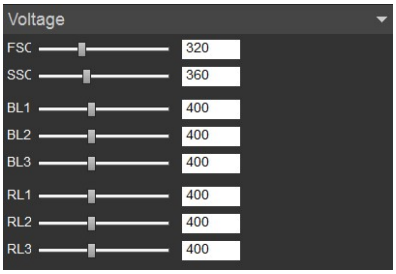
Expand the **Voltage** Section:

- Adjust FSC & SSC voltages to position cell population on the scatter plot
- Adjust Fluorescence Channels voltages to position the auto fluorescence signal (unstained population) to ~10^3

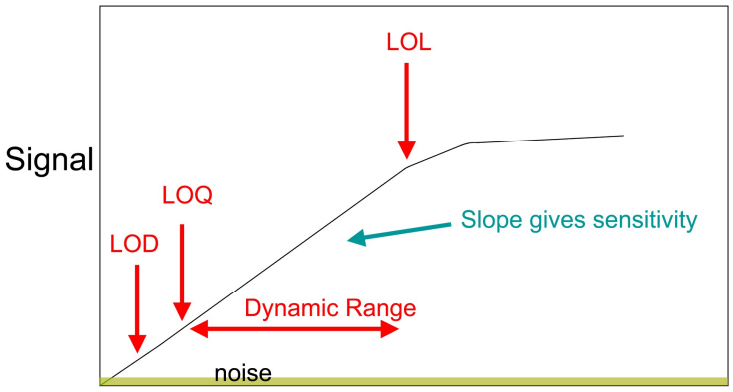


Notes:

Following acquisition of compensation controls, you will be unable to adjust voltage in fluorescence channels (voltages are disabled i.e. grayed out).



About PMTs

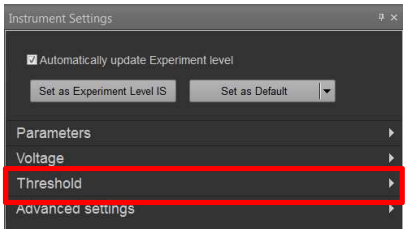


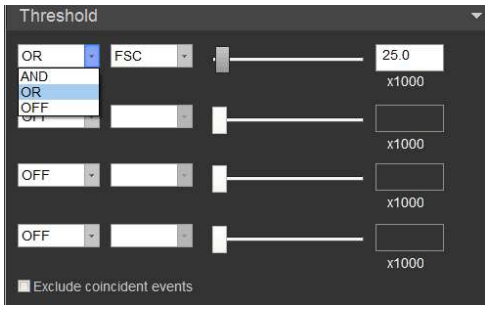
A calibration curve plot showing limit of detection (LOD), limit of quantification (LOQ), dynamic range, and limit of linearity (LOL).

Instrument Settings - Adjust Threshold

Expand the Threshold section:

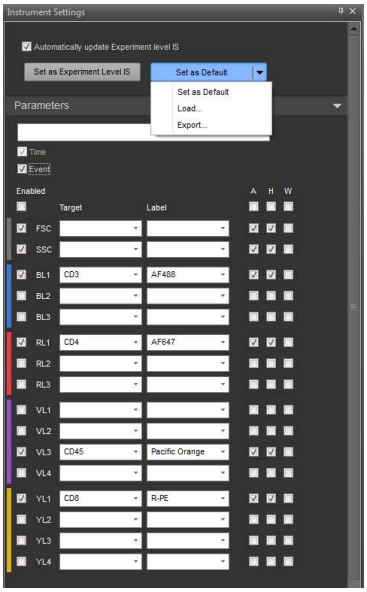
- Use threshold to remove unwanted events (i.e. noise or debris) before sample has been recorded.
- Can be set for 1-4 channels (scatter and/or fluorescence).
- Data not meeting threshold criteria is permanently lost.





Instrument Settings – Default, Export/Import, Level

- Instrument Settings can be exported, imported, or set to a Default setting that will be applied to all future experiments.
- Once there is a new Default IS, subsequent experiments will include the parameters selected and target and label titles set as default; these may be customized further if needed



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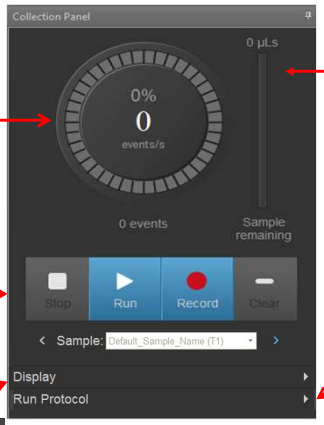
Collection Panel - Tube Mode Setup

Acquisition Status

- Progress dial
- Event rate
- Total # events collected

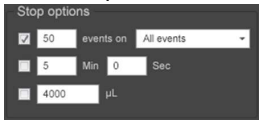


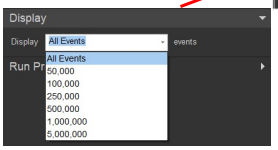
Sample remaining indicator

Collection Commands

Select Flow Rate options

Select Stop Recording options





Select # of events to be displayed during acquisition.

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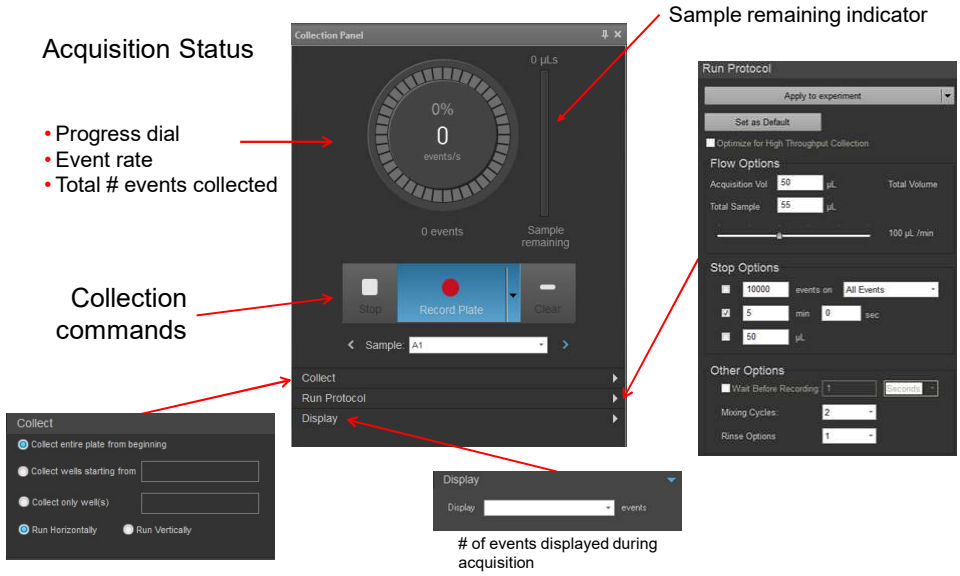
Collection Panel - Plate Mode Setup



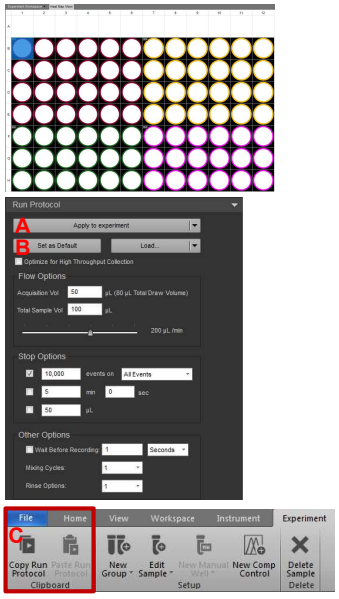
Collection Panel – Plate Mode Setup

Establish a Run Protocol

1. In the Heat Map View, double click on the well.

2. Set a run protocol
   1. Flow Options
   2. Sample Flow Rate
   3. Stop Option
   4. Mixing Cycles
   5. Rinse Options

3. ***Apply to experiment/group*** (A) OR ***set as Default*** (B) OR Click ***Copy Run Protocol,*** select wells on the plate and Click ***Paste Run Protocol*** (C)



Automatic update of Run Protocol

The **Run Protocol** of an experiment are **instructions on how the sample will be acquired**, including flow rate, acquisition volume, stop conditions, storage gate conditions, and for plate based experiments wait before recording, mixing and rinse instructions.

Tube sample

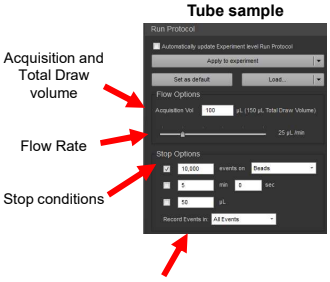
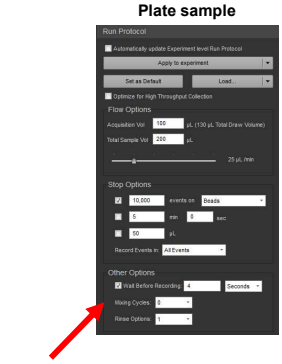
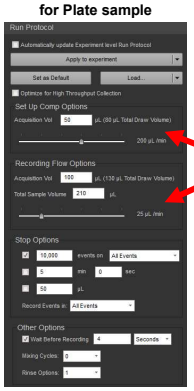


Plate sample



Compensation control for Plate sample



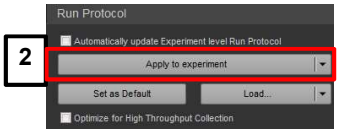
Automatic update of Run Protocol

The **Run Protocol** may be applied to individual samples, to a group, or to all samples in the experiment

To Apply the Run Protocol to all samples in the experiment:

Option 1: Automatic update
Select "Automatically update Experiment level Run Protocol" to update Protocol for ALL samples
Notes: For compensation samples from a plate, the run protocol will apply ONLY to the 'Recording Flow Option'. **TIP:** set run protocol for 'Set up Comp Options' manually or use default settings

Option 2: Apply to Experiment
When automatic update of experiment level Run Protocol is NOT enabled, press "Apply to Experiment" button to update Protocol for ALL samples within the experiment



Option 3: Apply to Group
When automatic update of experiment level Run Protocol is NOT enabled, click arrow and press "Apply to Group" button to update Protocol for ALL samples within the group

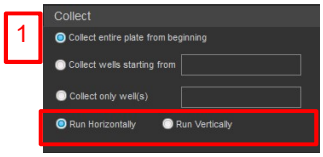
Option 4: Apply to Sample
When automatic update of experiment level Run Protocol is NOT enabled, set run protocol for each sample individually

Collection Options for Plate-based Experiments

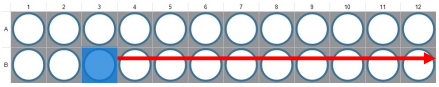
The order that wells are collected in is defined by the User

Three options are available for acquisition of plate samples:

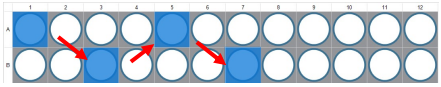
1. **Record entire plate from beginning**: samples will be acquired for the entire plate either **HORIZONTALLY**, left to right across each row or **VERTICALLY** from top to bottom, down columns

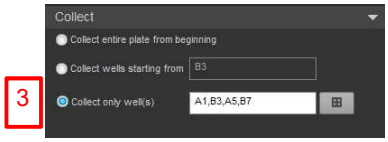


2. **Collect wells starting from a defined location "….."** : samples will be acquired for the entire plate starting with the well indicated in the text field (example, well B3) and proceeding through the last well (for example, well B12) for this example collected horizontally.



3. **Collect only well(s):** only samples listed in the text field are acquired from the plate (for example, wells A1, B3, A5, B7)





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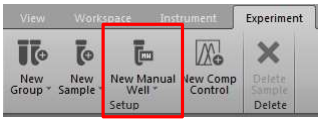
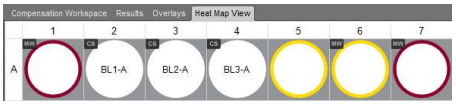
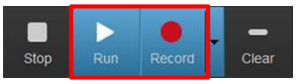
Manual Well set up

Adding Manual well to a plate allows to acquire sample in well in the same manner as it was a tube sample





When a Manual well is selected a user has an option to Run or Record the Manual well(s)



- **Run:** instrument setting adjustment (voltage, threshold) may be modified during acquisition. **No FCS file is saved**

- **Record**: instrument settings are locked and may not be modified during acquisition. **A FCS file is saved**

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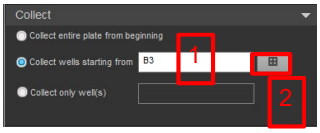
How to Select a Starting Well or a Subset of Wells for Collection

When collecting a subset of samples from a plate, the user may set the collection options using 2 methods:

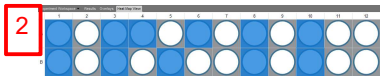
1. Typing directly in the text field
2. Selection of well(s) from the heat map after activation of the "Plate" indicator icon



To select wells directly from the heat map:

1. Click the Plate indicator icon to activate it

2. Select wells from the Heat Map View



3. Click the Plate indicator icon to deactivate it

The text field will be updated to indicate the selected wells. Wells are recorded in the order indicated in the text field and NOT across rows

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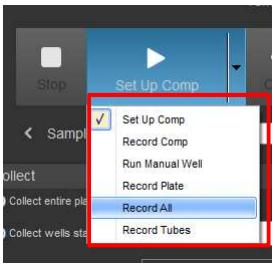
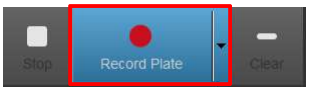
Collection Options for Plate-based Experiments

Plate samples are collected in "phases" if multiple well types are included in the plate experiment

Well Type	Phase collected
Compensation well	Run in "Set Up Comp" phase and recorded in "Record Comp" phase
Manual well	Run Manual Well
Sample well	Record Plate
Tube sample	Record Tubes



Users must progress through phases by selection of the phase



NOTE: IF the **"Record All"** option is selected, plate acquisition of all compensation wells and sample wells from the plate will proceed automatically without the requirement of the user to switch between compensation and sample well phases

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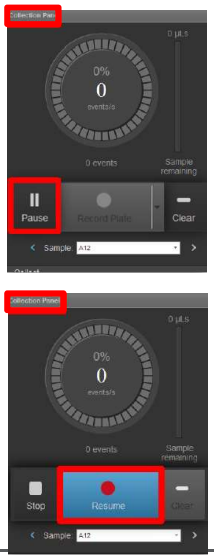
How to Pause and Resume Plate-based Acquisition

Sample acquisition may be paused during a plate run using the "Pause" button in the collection panel

To pause acquisition from a plate experiment:



1. Click the "Pause" button in the collection panel

2. Acquisition will temporarily stop **AFTER** the current well **has reached the stop condition (time, volume or number of events)**
   - If the next sample was pre-loaded **BEFORE** «Pause» was pressed, the system will **automatically recover** the sample into a plate.
   - Once paused, the autosampler door unlocks and the «Record Plate» button will change to the "Resume" button

3. **To resume plate acquisition**, press the «Resume» button
   - Acquisition will re-initiate with the next well

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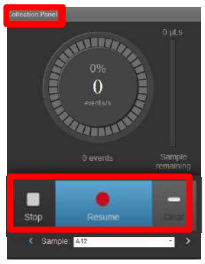
How to Stop Plate-based Acquisition

Sample acquisition may be stopped during a plate run using the "Stop" button in the Collection Panel

Options to Stop Acquisition from a Plate Experiment:



1. Press the stop button
2. IF the plate run has been **paused**, the user may press "**Stop**" or "**Resume**" in the collection panel. Notes: no sample recovery options are available because "Pause" will initiate sample recovery automatically

Pressing Stop will revert the plate acquisition to a pre-run state where the user may

- **Change** order of well collection
- **Add/delete/duplicate** samples and groups
- **Remove, import, export FCS** files from samples
- **Modify compensation** controls (add/delete)
- **Proceed through different phases** of plate collection (Set Up Comp, Record Comp, Run Manual Well, Record Plate, Record Tubes)
- **Execute Instrument functions** (Rinse, Sanitize SIP, Deep Clean, Startup, Shutdown, Debubble, Unclog, etc)
- **Create, duplicate, or delete an experiment**

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Collection Panel - Append Data Options

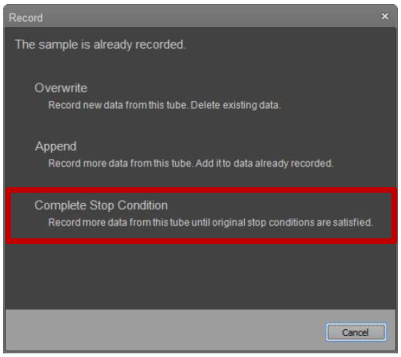
If the *Record* button is selected before proceeding to the next sample AFTER a FSC file is recorded:

Two dialog boxes with **3 options**:

1) **Overwrite:** New data replaces existing data

2) **Append:** New data added to existing data

3) Depends on whether stop condition has been met:
   - **Complete Stop Condition:** Add to existing data until stop condition met
   - **New Sample:** Creates a new sample and records data



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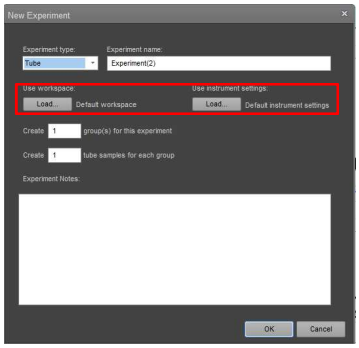
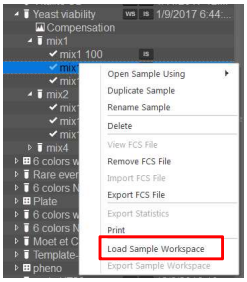
Load Previous Settings

Software is enabled to load:
- Compensation
- Workspace
- Instrument Settings

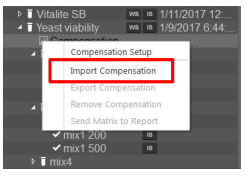
New Experiment Window



Sample Menu



Compensation Menu



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Experiment Explorer - Shortcuts

Drag & drop within the Experiment Explorer:

- WS Workspace from experiments, groups or samples can be applied to another experiment, group or sample
- IS Instrument settings from experiments, groups or samples can be applied to experiments, groups or samples not containing recorded data. Samples with existing data remain unchanged and are updated to display the Sample level
- CS Compensation Matrix from an experiment can be applied to another experiment. Compensation controls will be shown as UC and compensation workspace will be disabled

Sample Recovery

The Sample Recovery Option allows recovery of sample remaining in sample loop tube during the following conditions:

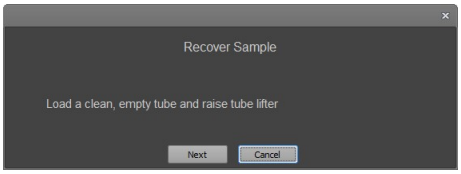
- Stop is pressed during acquisition operation
- Stop Criteria are met and significant volume remains in the sample loop
- Fluidic errors detected in instrument such as a bubble

The Sample Recovery button is located on the *Instrument* Tab

As well as in *Collection Panel*

Sample Recovery

To use sample recovery in **tube mode**:



1. Press the “Recover Sample” button
2. When instructed, place a new tube on the tube lifter
3. Raise the tube lifter and press Next
4. The remaining sample will be dispensed. This includes the dead volume and sample that has not been acquired
5. Lower the tube lifter. A Rinse cycle will initiate after sample recovery is complete

Practical Session

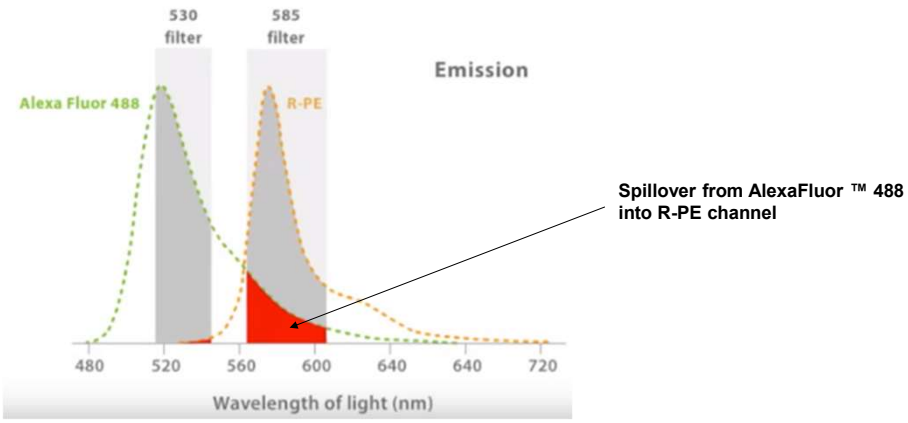
- Exercise 1 – Performance Test
- Exercise 2 – Experiment Setup and Data Acquisition
  Single Color Experiment

Workflow

- Visual Inspection
- Instrument Startup
- Optical Configuration Check
- Performance Test
- Experiment Settings Optimization
- **Compensation**
- Data acquisition
- Data Analysis
- Instrument Shutdown

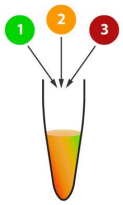
Why do we need to Compensate?

- Every fluorescent molecule emits light with a particular spectrum, unique to that molecule.
- These emission spectra overlap - in some cases, very significantly.
- *Compensation* is the process by which we correct for "spillover".



Three Color Experiment

Which Controls do you need to Compensate?



	Antibody	Fluorescent Probe
1	Anti-CD3	Alexa Fluor 488
2	Anti-CD4	R-PE
3	Anti-CD8	PE-Cy7 tandem dye

Compensation Controls

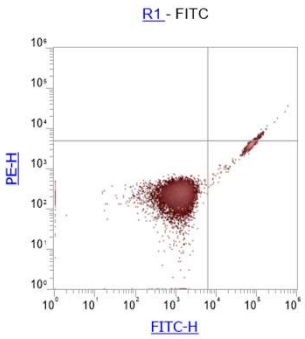
1. Cells or beads only (optional)
2. Cells or beads + Anti-CD3 only
3. Cells or beads + Anti-CD4 only
4. Cells or beads + Anti-CD8 only

AbC™ Total Antibody Compensation Bead Kit

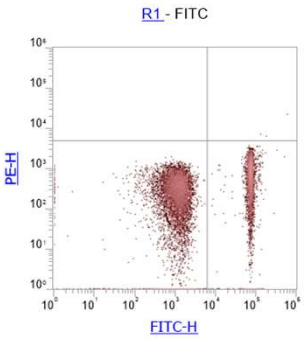
Why do we need to Compensate?

Single stained control for FITC

Uncompensated



Compensated

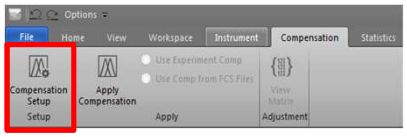


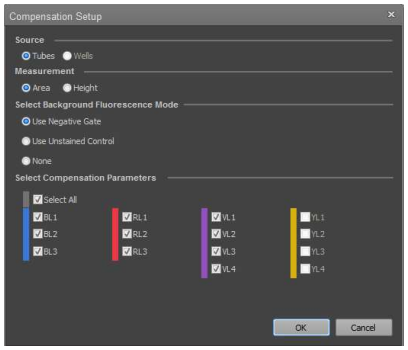
Options for Compensation Controls

- There are many different options for compensation within tube and plate experiments
- Compensation Controls may be recorded with each experiment
  - Tube based samples – for tube experiments
  - Tube samples – for plate experiments
  - Well samples – for plate experiments
- Previously recorded Compensation Controls may be applied to a new experiment
  - Manual export (save) of compensation files as a .acs file and load (import) into a new experiment
  - Drag-n-drop of compensation settings from one experiment to another

Automatic Compensation

- Prepare Compensation Controls using experimental cells/particles or Compensation beads.
- In *Compensation* tab, click on Compensation Setup. Alternatively, you may double-click on Compensation in Experiment Explorer.
- Select Compensation options:
  - Source: Tube, Well or File
  - Parameter: Area or Height
  - Autofluorescence: Negative gate, Unstained control or none
  - Fluorescent channels
- Run all controls and adjust gates accordingly.

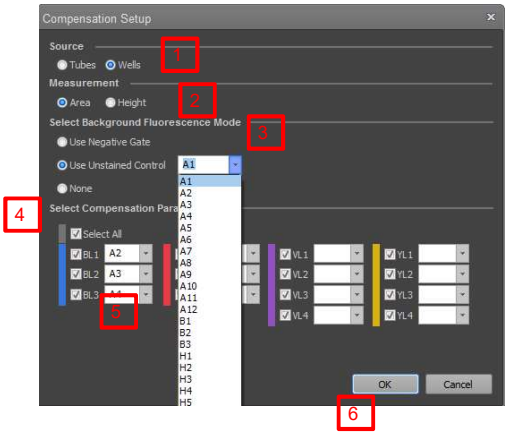




Compensation Controls in Plate

To Create Compensation Controls for plate-based acquisition:

1. Select “Wells” as a compensation source
2. Select the desired measurement
3. Select background fluorescence mode and well location
4. Select the parameters requiring compensation
5. Set the well locations for each compensation control
6. Press “OK”



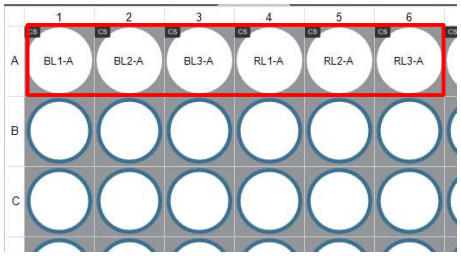
Compensation Controls in Plate

Each compensation control is labeled with the **well location** and **parameter**
E.g.: well A1 = BL1 control (Alexa Fluor 488)

Controls will also be labeled with the **stain name** if fluorophore in the “Label” field in the Instrument Settings Menu

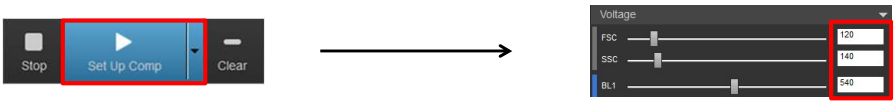
In the *Heat Map View* Compensation Wells are identified by the “CS” indicator icon in the top right corner of the well.

The parameter used for the compensation control is listed by name in the center of the well

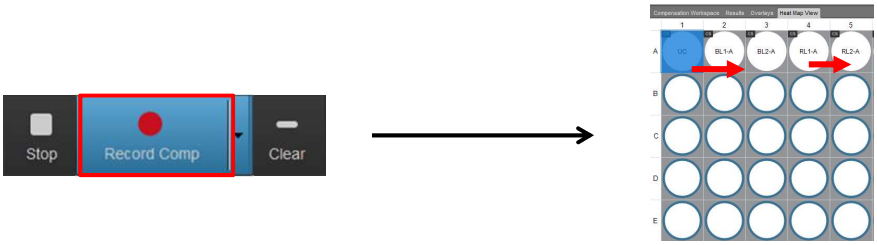


Compensation Controls in Plate

- Set Up Comp phase is used to adjust instrument settings



- Record Comp phase is used once instrument settings are established and compensation controls are ready to be recorded



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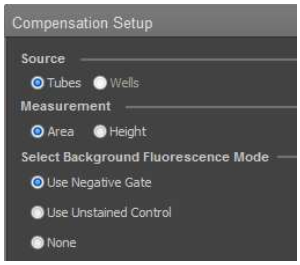
Compensation Modes

Three options to correct for background fluorescence:

1. Negative Gate
2. Unstained Control
3. None



Background Mode	When to Use?
Negative Gate	With a “mixed bag” of controls such as cells and beads; or using different cell populations (lymphs and monos).
Unstained Control	When all controls are of the same type (beads, all lymphs)
None	Rarely used, but in cases where autofluorescence is negligible or cannot be ascertained, compensation is calculated without correcting for background auto-fluorescence.

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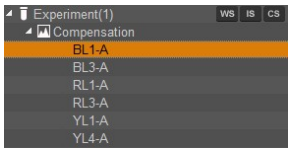
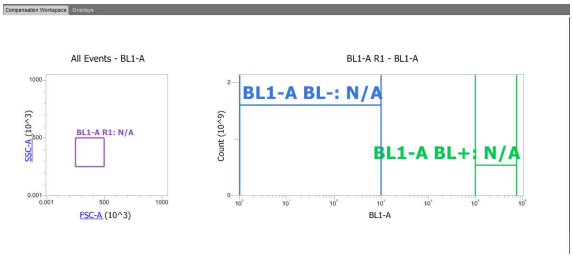
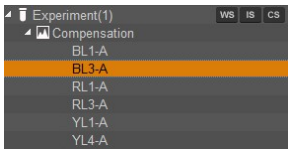
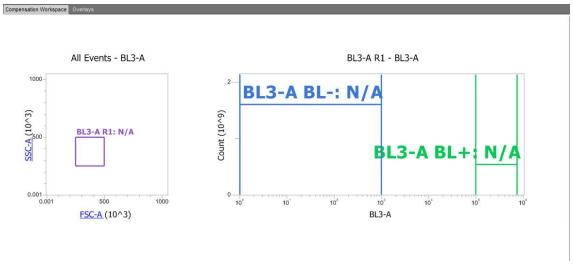
"Negative gate" compensation workspace









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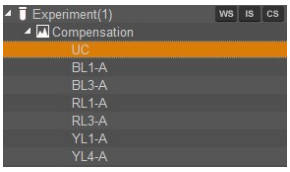
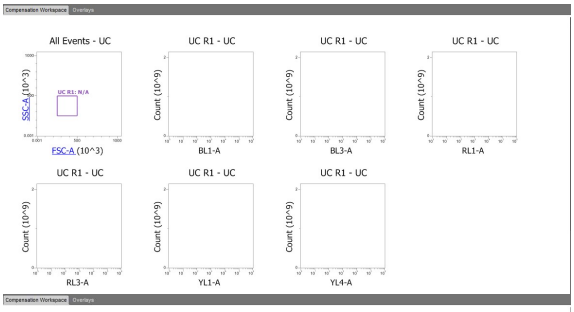
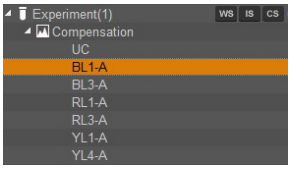
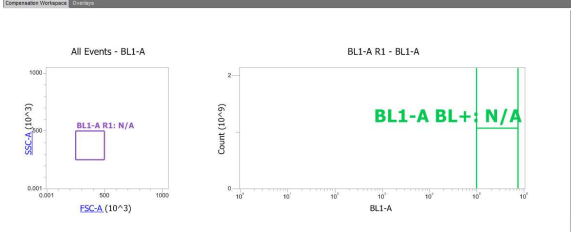
"Unstained control" compensation workspace









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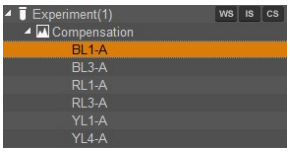
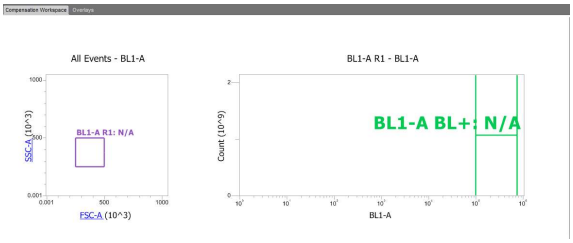
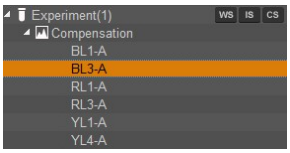
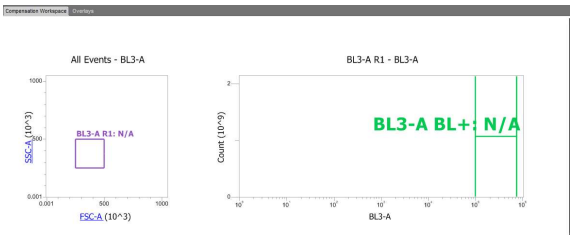
"None" compensation workspace









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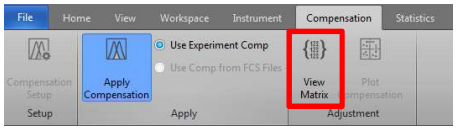
Spillover Matrix

- At the end of Auto-compensation:
  - Spillover Matrix is automatically calculated
  - Compensation is applied on all samples



Spillover

	BL1-H	BL3-H	RL1-H	RL3-H	YL1-H	YL4-H
BL1-H	100.00	0.31	0.12	0.14	0.04	0.04
BL3-H	1.76	100.00	1.65	0.67	81.71	14.70
RL1-H	0.10	0.29	100.00	22.10	0.00	1.08
RL3-H	0.17	0.00	0.39	100.00	0.01	3.64
YL1-H	1.29	3.68	0.09	0.02	100.00	0.50
YL4-H	3.24	0.22	0.11	34.20	2.44	100.00

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Spillover Matrix

- Read across rows
- Should be <100%

- In the matrix below, the spillover from BL1 into BL3 is 0.31%

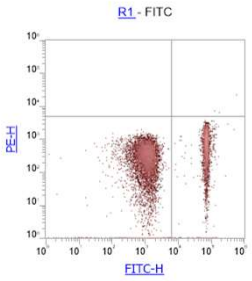
Spillover

	BL1-A	BL3-A	RL1-A	RL3-A	YL1-A	YL4-A
BL1-A	100.00	0.31	0.12	0.14	0.04	0.04
BL3-A	1.76	100.00	1.65	0.67	37.40	14.70
RL1-A	0.10	0.29	100.00	22.10	0.00	1.08
RL3-A	0.17	0.00	0.39	100.00	0.01	3.64
YL1-A	1.29	3.68	0.09	0.02	100.00	0.50
YL4-A	3.24	0.22	0.11	34.20	2.44	100.00

  - What is the spillover of BL3 into BL1?
  - What is the spillover of RL3 into YL4?

Manual Confirmation

Single stained control for FITC



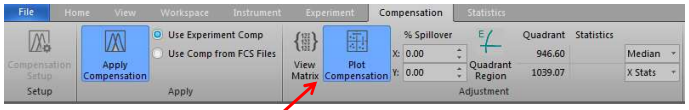
Group: **Group**
Sample: **FITC**
Time Recorded: **14:34:14**

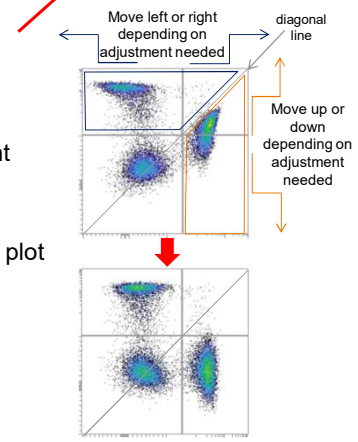
Name	Count	%Gated	X Median	Y Median
All Events	30,000	100.000	N/A	N/A
R1	23,081	76.937	359,104	515,975
FITC-/PE+	0	0.000	N/A	N/A
FITC+/PE+	3	0.013	182,626	7,262
FITC-/PE-	13,259	57.446	1,227	92
FITC+/PE-	9,819	42.541	73,867	98

- Check that Median of fluorescence is similar in non-targeted channel
- Check for all dual parameters plot combinations

On Plot Compensation Tools

Allow for manual adjustments or tweaks to a selected plot.

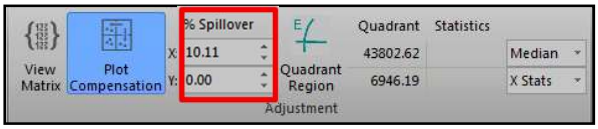




- Allow to drag populations to new positions and change the associated compensation matrix values.
- For selected plot, a diagonal line distinguishes the two coefficient sections.
- The sensitivity of the dragging action is dependent on where the plot is clicked. This is correlated to the scale of plot and the range in which a population is moved.

Spillover Adjustment

Consist of two spin box controls that allow adjustment of the compensation or the spillover matrix.

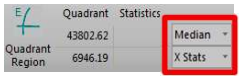


- Visible only when On Plot Compensation Tool is active and a dual-parameter plot is selected
- Increments of 1 when using up/down arrows or the up/down keys on the keyboard
- Increments of 0.1 if pressing the **Shift** while using up/down arrows or the up/down keys on the keyboard
- The textbox accepts numbers from 0 to 100 with 2 decimal places displayed at all times.

Quadrant Gate

Quadrant Region:
Allows the insertion of a Quadrant gate on any dual parameter plot.

Quadrant Statistics:
Displays the mean or the median values for selected Y-axis or X-axes statistics.



- Only displayed for a selected dual-fluorescence parameter plot for recorded samples
  - X-axis statistics reflect statistics for the lower left and lower right quadrants.
  - Y-axis statistics reflect statistics for the lower left and upper left quadrants.

- If a statistics value cannot be calculated, the respective quadrant will display “N/A” (i.e., not applicable).

Practical Session

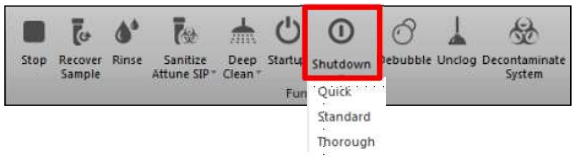
- Activity 3 – Compensation and Spectra Viewer
- Exercise 3 – Multicolor Acquisition and Compensation
  3 to 4 Colors Experiment

Workflow

- Visual Inspection
- Instrument Startup
- Optical Configuration Check
- Performance Test
- Experiment Settings Optimization
- Compensation
- Data acquisition
- Data Analysis
- **Instrument Shutdown**

Instrument Shutdown

- Prior to Shutdown, run a Sanitize SIP function using Attune™ NxT Flow Cell Cleaning Solution
  - Daily for high volume users (>6 hours or >8 plates per day)
  - Weekly for less frequent users
- Then, select Shutdown function in *Instrument* tab, and select the number of cycles



Shutdown option	Nb of Cycles Duration	When?
Quick	5cycles/~25min	Few samples
Standard	15cycles/~45min	Standard Applications
Thorough	25cycles/~75min	Sticky samples or dyes, NLNW…

  - Follow the instructions on the screen
  - Use 10% bleach, freshly prepared

Note: At the end of the Shutdown script, the Attune™ NxT Flow Cytometer and Attune™ NxT Autosampler will be in Sleep mode.

ThermoFisher SCIENTIFIC

Basic Software Analysis Features

Revision 2.5
Revision Date: Aug2019

The world leader in serving science

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List of Features

- Plot types
- Previews panel
- Gate types and gate modifications
- Customize panel
- Statistics options
- Zoom in options

Revision 2.5
Revision Date: Aug2019

ThermoFisher SCIENTIFIC

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List of Features

- Plot types
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Plot types – Workspace Tab

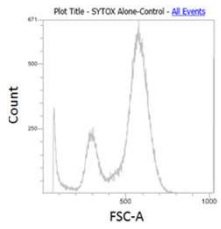
Use the *Customize* Panel to change plot titles, axis labels, axis scaling, plot type etc.

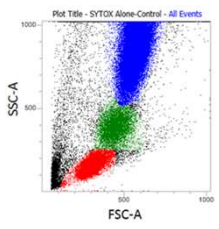
Histogram Plot – Single parameter plot showing number and distribution of events

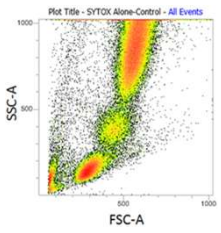
Dot Plot – Two parameters plot where each axis represents the signal intensity of one parameter

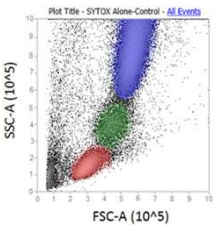
Density Plots – Two parameters plot where colors represent the density of a population of events with the same intensity

Precedence Density – A combination of Dot and Density display. A gradient is used to indicate the number of events within each of the plot bins and color is used to display the parent gate of events present.







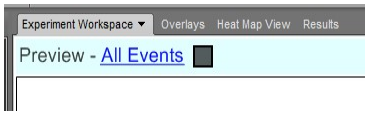


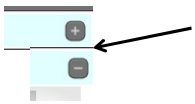
List of Features

- Plot types
- Previews panel
- Gate types and gate modifications
- Customize panel
- Statistics options
- Zoom in options

Previews panel

- The **Previews panel** of the Workspace displays all permutations of Histogram and Precedence Density plots based on the parameters selected. It provides an easy way of adding plots to the current Workspace.
- The Previews panel is located at the top portion of the Workspace view. By default, the panel is displayed as a minimized bar.





- The Previews panel cannot be opened during acquisition.

Previews panel

- When opened, the Previews panel occupies 50% of the vertical space of the Workspace view. The size of the panel cannot be changed.

List of Features

- Plot types
- Previews panel
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Regions and Gates – Workspace Tab

- Regions and Gates are commonly used in data analysis to identify population subsets.

- **Gate** is a shape or object that is drawn around a population of interest on one or two parameters plots.
- **Region** is defined when gates are used to isolate a specific group of cytometric events from a large set of data.
- Gates are displayed in a hierarchy or family tree.

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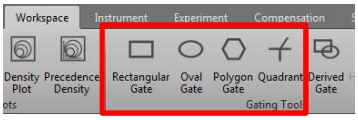
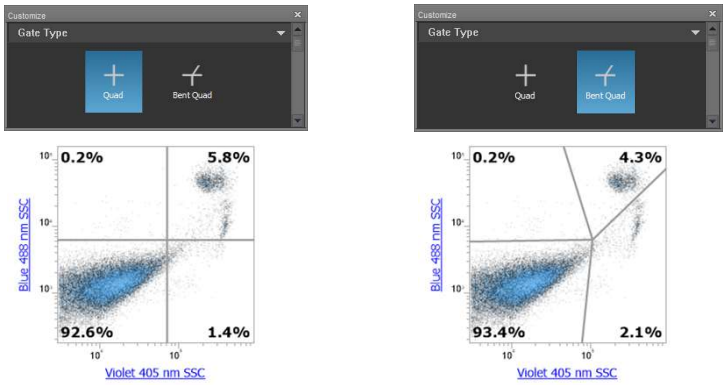
Bendable Quadrant Gates

Data presented in dual parameter plots can be identified for subsequent analysis using different types of gates including rectangle, oval, polygon and quadrant gates



Quadrant gates may be square or hinged and 'bendable'



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Using Bendable Quadrant Gates

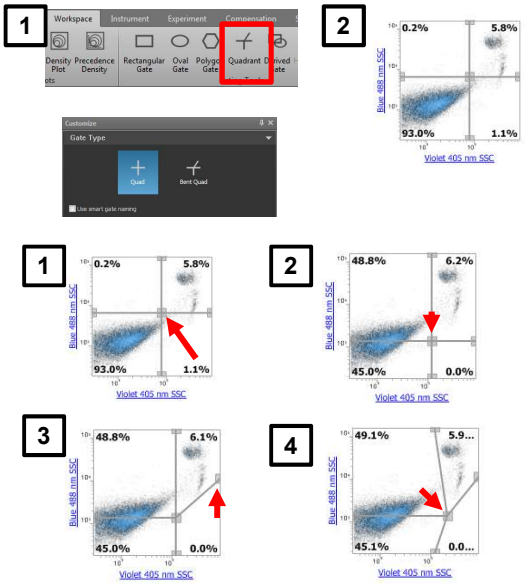
To add a Quadrant gate to a plot:

1. Click the “Quadrant” button in the Workspace tab
2. Click the plot where the gate will be placed.
   - Inserted quadrant gate is orthogonal unless changed by user.

To reposition a quadrant gate:

1. Click on the center point intercept of the quadrant gate to activate the gate
2. Move the center point to reposition orthogonal gates
3. Click ‘Bent Quad’ button in the Customize menu OR move an endpoint to change an orthogonal gate to a bent quadrant gate.

4. Moving the center point of a bent quadrant gate will keep endpoints on each axis in same position and move center only



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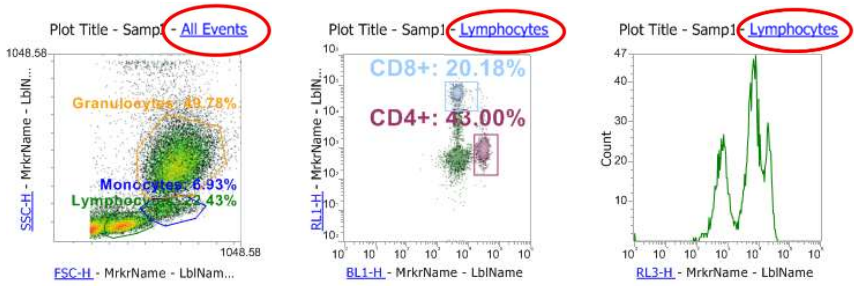
Daughter Plot

- Displays data only from the region selected.



- To create:
  - Right click on the gate and select Daughter plot and the type of plot, OR
  - Right click on the plot and select Set population, OR
  - Left click on the hyperlink in the plot title and select gate name from the drop down list.

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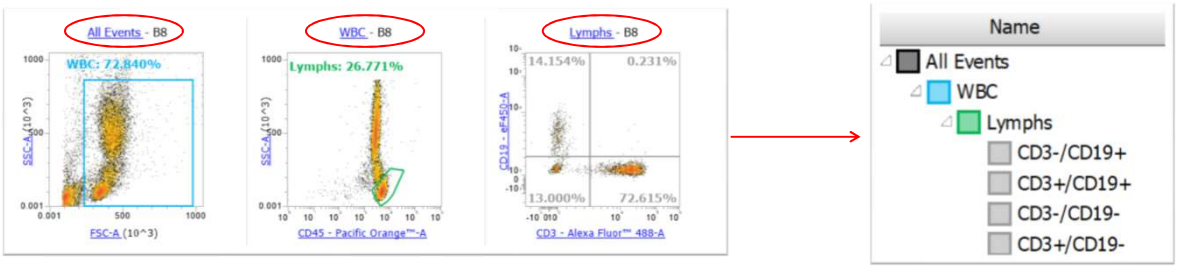
Hierarchy/Gating Tree



- Used to demonstrate how Gates and Regions are interrelated to one another.
- Used commonly for identifying subsets of cells population.

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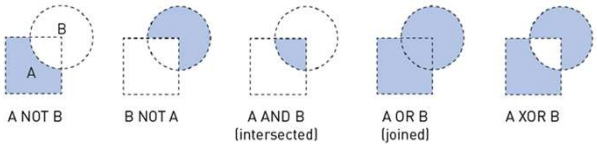
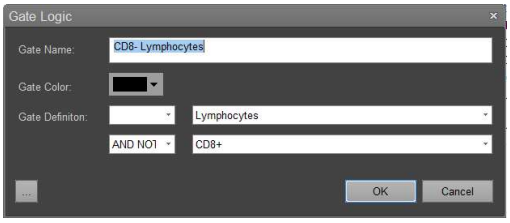
Derived Gates

- Gates can be customized by using Boolean logic (OR, AND, NOT, XOR) to link multiple gates together.





AND gates = All events that are shared.

OR gates = All events found within 2 or more individual gates.

NOT gates = All events found outside the gate.

XOR gates = Unique events found within an individual gate.

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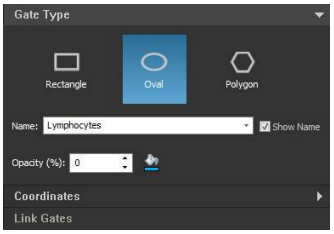
Gate Editing

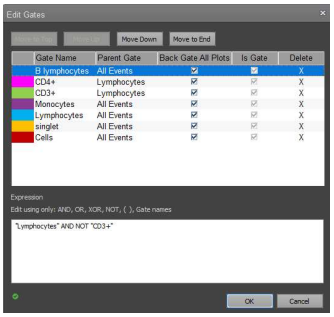
A single selected gate can be edited from the *Customize* Window.

- Change Gate type, Name, color and opacity, Coordinates or Link Gates.



The **Edit Gates** button located on the *Workspace* Ribbon lists all available gates on the active workspace. Here you can edit:

- Gate color
- Parent gate
- Gate math expression
- Z-order in which gates are painted,
- Toggle on/off for Backgate All Plots and Deletion



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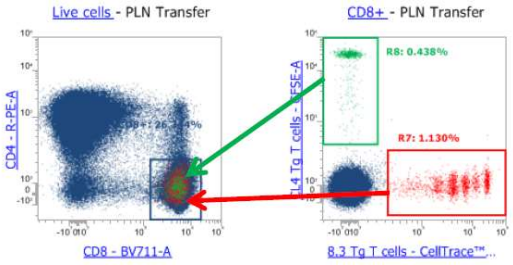
What is "Backgating"?

"Backgating" refers to coloring all events within a gate to match the gate color.

- Populations may be identified on parent plots (i.e. Dot Plot and Precedence Density plot) by color of population



- By default, automatic backgating is set to "ON" within the Attune™ Nxt Software

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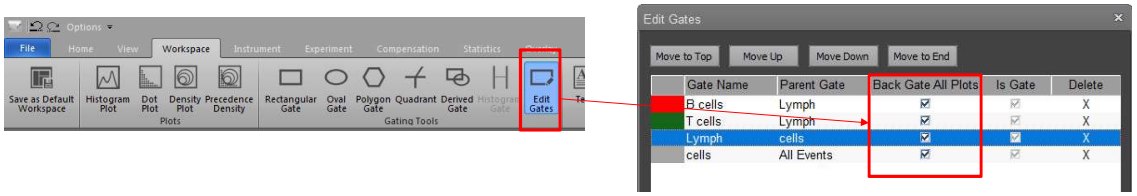
How to control Automatic backgating?

"Automatic backgating" is controlled at 2 separate levels:

1. Globally within the "Gate Option window" from "Options" Menu

NOTE: Change ONLY applies to **newly** created workspaces **AFTER** the change is made

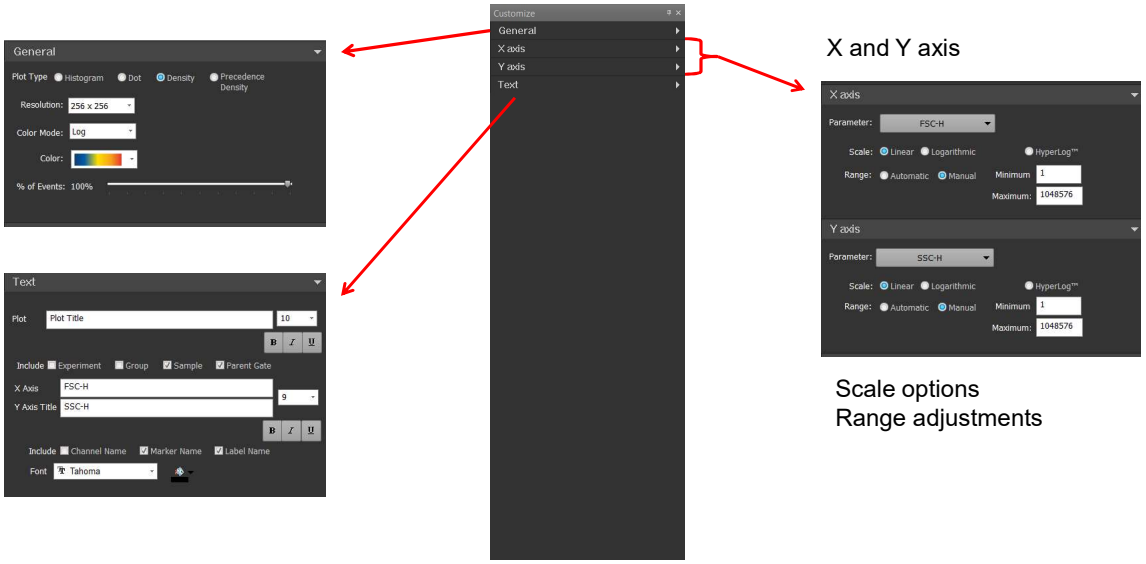
2. Experimentally within the "Edit Gates" menu by **ticking/unticking** the relative option



List of Features

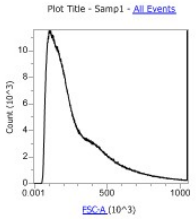
- Plot types
- Previews panel
- Gate types and gate modifications
- Customize panel
- Statistics options
- Zoom in options

Customize Plots

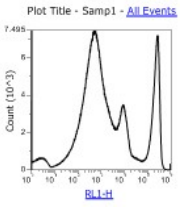


Axis Scale Options

- Impact the way data is visualized
- **Linear** – data spread over a single order of magnitude
  - FSC
  - SSC
  - DNA content

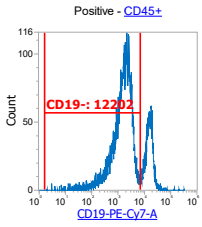


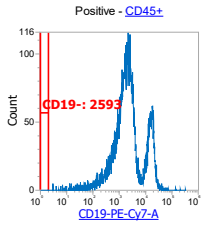
- **Logarithmic** – data spread over a wide range (>1 order of magnitude)
  - Fluorescent channels
  - FSC +/or SSC – bacteria, small particles and blood samples



Axis Scale Options - Logarithmic Scale

- Log scale: do not represent cells whose fluorescence values are below 0 (10^0 =1)



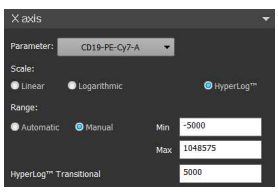


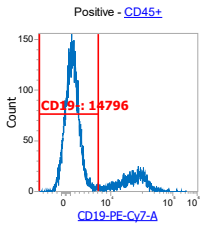
- Values below 0 result from:
  - Background subtraction
  - Compensation

Axis Scale Options - HyperLog™ Scale

HyperLog™ Scale uses log-linear hybrid transformations to represent cells whose fluorescence values are below 0

- Logarithmic scale at the high end
- Transitions to linear scale in the region around zero (Transition value is approximately 2 time the autofluorescence value)
- The HyperLog™ Transitional value is used to determine the width of the linear region of a HyperLog display
- Values are adjusted for each axis on a plot and persist across all plots that display that parameter in HyperLog.

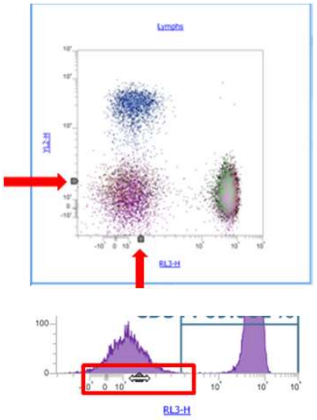




Hyperlog - a flexible log-like transform for negative, zero, and positive valued data. *Cytometry A.* 2005 Mar;64(1):34-42
Modern Flow Cytometry: A Practical Approach *Clin Lab Med.* 2007 September ; 27(3): 453–v.

Axis Scale Options - HyperLog™ Scale

On-plot slider bars available to make Transition Value adjustment



- When a plot axis is on HyperLog™scale and the plot is selected the slider bars will display.
- The transitional value can be adjusted by dragging the slider along the axis.
- When the mouse cursor is over the transition slide bar it will change to a double headed arrow indicating the direction the slider can be moved (to the left or right).

➢ Use to determine if compensation is correct

- Correct – double negative population will distribute symmetrically around autofluorescence level
- Overcompensation – double negative population center below autofluorescence value
- Undercompensation – double negative population centers the distribution above autofluorescence value

List of Features

- Plot types
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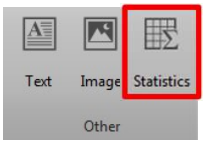
Statistics Table

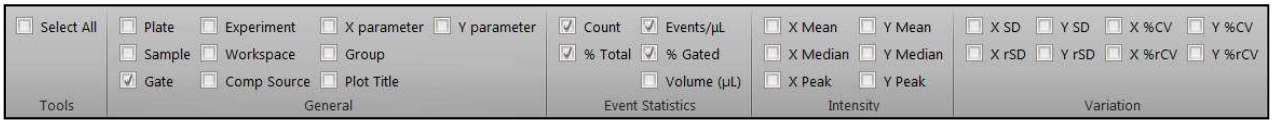


Experiment: **6 color immuno**
Group: **Default_Group_Name**
Sample: **NOT-IN_STATS**

Name	Count	%Gated	%Total
All Events	30000	100.00	100.00
Lymphocytes	6728	22.43	22.43
CD4+	2893	43.00	9.64
CD8+	1358	20.18	4.53
Monocytes	2080	6.93	6.93
Granulocytes	14933	49.78	49.78

- To display **Workspace Statistics Table**, click Statistics without selecting a plot. Workspace statistics contains data of all the gates in the Workspace.
- To display **Plot Statistics Table**, select a plot in the Workspace and then click Statistics. Local statistics only displays data pertaining to the selected plot.
- Alternatively, you can insert a statistics table by right-clicking on a plot or on the workspace, and select Insert Statistics

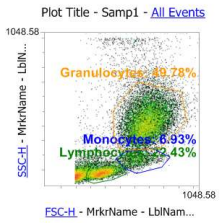
Customize Statistics



Experiment: **6 color immuno**
Group: **Default_Group_Name**
Sample: **NOT-IN_STATS**

Name	Count	%Gated	%Total
All Events	30000	100.00	100.00
Lymphocytes	6728	22.43	22.43
CD4+	2893	43.00	9.64
CD8+	1358	20.18	4.53
Monocytes	2080	6.93	6.93
Granulocytes	14933	49.78	49.78

- To customize a statistics table, select the Table and check statistics to display in the *Statistics* Tab



- To customize statistics value displayed on a plot, select the plot and choose the statistic

Statistical Values

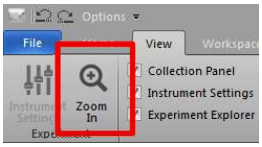
- **Count**: Number of events collected
- **Events/µl**: Concentration of events/µl in the sample tube
- **% Total**: Percentage of total events collected
- **% Parent**: Percentage of a population based on the number of events collected in the parent gate
- **Mean**: Sum of the signal intensities of a gate divided by the number of values
- **Median** (50th percentile): signal intensity of a gate separating the higher half of a data population
- **Mode**: signal intensity that appears most often in a set of data
- **SD**: Standard Deviation, amount of dispersion of signal intensity around the Mean
- **rSD**: Robust Standard Deviation, amount of dispersion of signal intensity around the Median
- **%CV**: Percent coefficient of variation, Standard Deviation of the peak divided by the Mean of the peak, times 100
- **%rCV**: Percent Robust coefficient of variation, Standard Deviation of the peak divided by the Median of the peak, times 100

List of Features

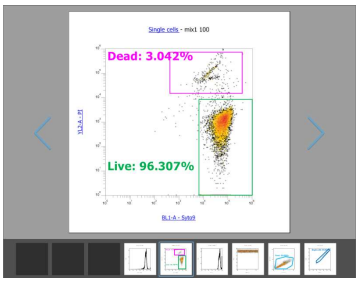
- Plot types
- Previews panel
- Gate types and gate modifications
- Customize panel
- Statistics options
- Zoom in options

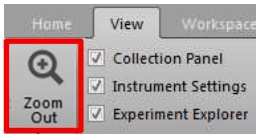
Zoom in and out options – plot zoom in and out

- **Zoom In and Out option** allows to zoom in (maximize) and out (minimize) each plot displayed in the area of the Workspace view. Zoom in icon is present in *View* tab and is enabled when the Workspace view is active and contains plots.

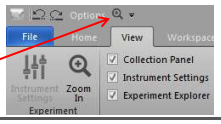


Zoomed in plots





Zoom In icon can be added to tool bar



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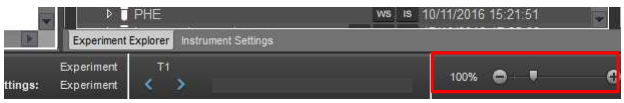
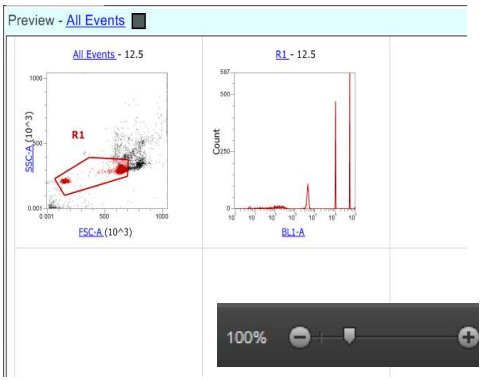
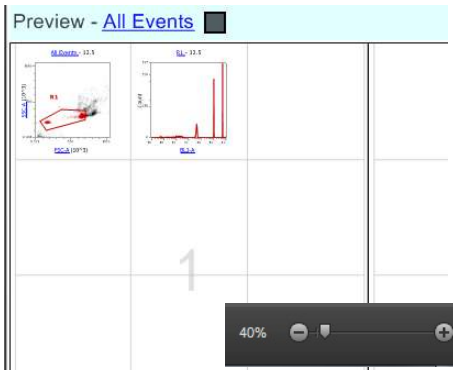
Zoom in and out options – workspace zoom in and out

- **Zoom In and Out option** for workspace allows to zoom in and zoom out the whole workspace view. This zoom in/out option is displayed in the right lower corner of the software:







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Advanced Software Features

Revision 2.5
Revision Date: Aug2019

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Advanced Software Features

- Template management
- Filter Configuration Manager
- Heat Map Analysis Tools
- Results Table
- Overlay Builder
- Sample List
- Keyword Manager
- Storage Gate / Gate Export to fcs file
- User Options

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Advanced Software Features

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Templates: what are they?

Templates provide a **convenient method to quickly collect flow cytometry data in the same manner for multiple experiments**

Experiment Templates include all information needed to run an experiment: Groups and samples, workspaces, instrument & compensation settings, run protocols, heat map settings and plate layout

Templates can be:

1. Created (saved)
2. Exported
3. Imported
4. Modified
5. Deleted

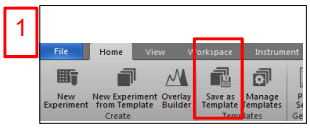
Notes: Templates are unique to each user profile (account restricted) and are stored in the Attune™ NxT Database

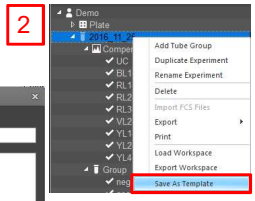
How to create a template

After an experiment is created, it may be saved as a template

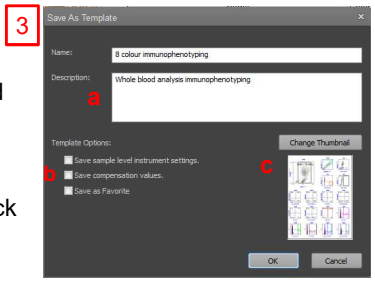
To create a template:

1. Select the **"Save as Template"** button from the **Home Tab** of the Ribbon bar
   OR
2. **Right click** the **Experiment name** and **select** "Save as template"



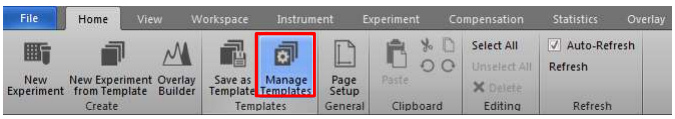


3. In the "Save as template" dialogue box:
   a. Enter Name (required) and description (*optional*) for new template
   b. Provide instructions on what will be included in the template
      - ✓ sample level instrument settings
      - ✓ compensation values
      - ✓ Save as favorite (*optional*): used for quick and easy identification for automation
   c. Change thumbnail (*optional*)

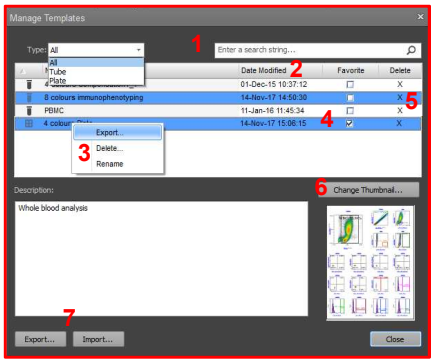


Managing Experiment templates

Templates can be sorted, listed as favorites, deleted, imported and exported from the "**Manage templates**" menu



1. **Sort** through templates (by "search string" or by display only Tube or Plate experiment templates)
2. **View** the date of last template modification
3. **Rename** a template by right click on template name
4. Designate a template as a "**Favorite**"
5. **Delete** a template by pressing the "X" next to the template name
6. **Change the thumbnail**
7. **Export** or **Import** a template to/from another folder location



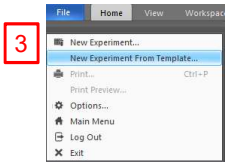
Using Experiment templates

There are **3 ways** a new experiment can be quickly created from a saved experiment template:

1. "New experiment from Template" button on the **Main menu**

2. "New experiment from Template" button from the **Home tab**

3. "New experiment from Template" button from the **File tab**



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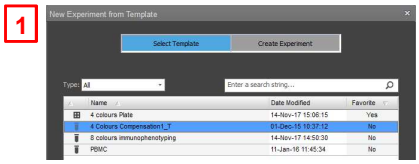
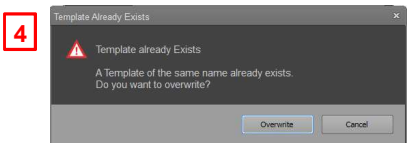
How to modify a template

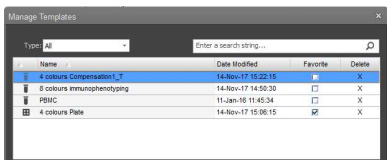
After a template is created, it may be modified

To make changes to an existing template:

1. **Create** a new experiment **using** an existing template
2. **Modify** the template as needed and **save as template**
3. **Rename** the template using the exact same name as original
4. Select "**Overwrite**" when warned that "A template of the same name already exists"
5. The manage Templates menu will list the date of last modification of the template







Notes: Design a template as a "Favorite" will change the date of last modification

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Advanced Software Features

- Template management
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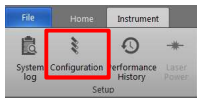
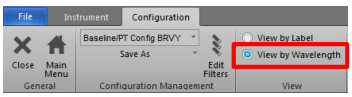
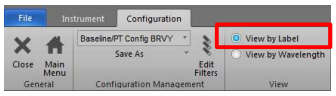
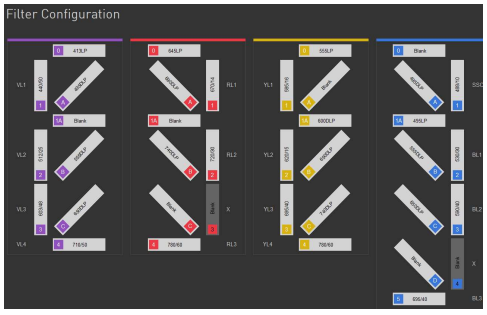
Filter Configuration Manager

The configuration manager is used to create virtual configuration settings



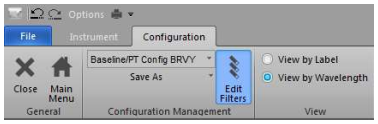




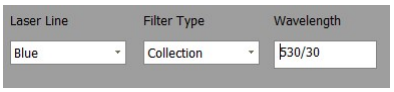


Filter Configuration Manager – Add or Edit Filters

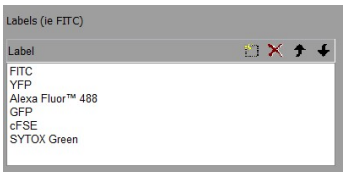
1. Open the Edit Filters menu



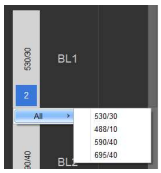
2. Input information about the new filter: Laser Line, Filter Type (Collecting or Directing), Wavelength



3. Optional: List associated labels



4. Select the correct filter in the Configuration Manager



Advanced Software Features

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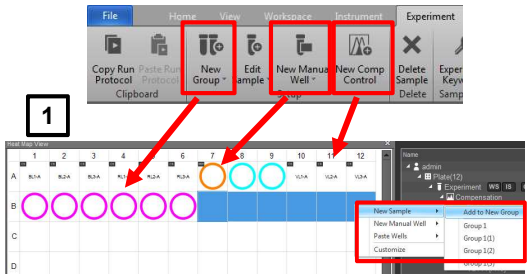
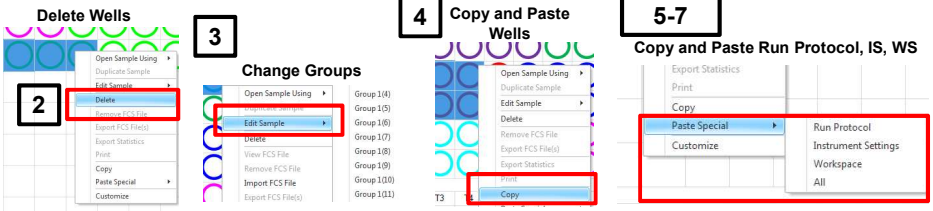
Using the Heat Map View to Define Samples and Groups

Samples and Groups for tube and plate experiments may be quickly defined using the Heat Map View

Use the Heat Map View, Experiment tab of the Ribbon bar, and right-click context menus to quickly:

1. Define wells in a plate
2. Delete wells
3. Edit groups (move wells to different groups)
4. Copy/paste sample wells
5. Copy/paste Run Protocols
6. Copy/paste Instrument Settings
7. Copy/paste Workspaces





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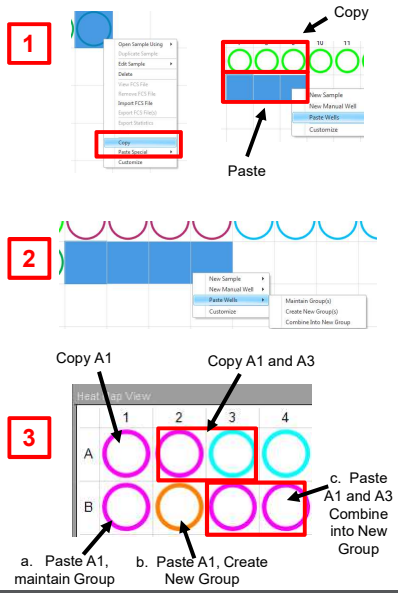
Copy/Paste of Samples using the Heat Map

To copy Sample wells or Groups using the Heat Map:

1. Right click on the well(s) to copy and select “Copy”
2. Select location to Paste wells to.
   Ensure that the size of the **paste location** includes enough wells to match the **size of the copy location** (i.e., copy 6 wells, ensure select 6 wells are available **across a row** or selected from wells in different rows to paste into). Number of samples pasted = number of samples copied
3. Right click and select “Paste Wells”. Select option to “Maintain Group”, “Create a New Group”, or “Combine into a New Group”
   a. Choose “**Maintain Group**” if the new samples are to be part of the copied group
   b. Choose “**Create New Group**” if the new samples are to be part of a new group
   c. Choose “**Combine into a New Group**” if new samples are copied from multiple groups but will be combined into a new group.

   Notes: Run protocol of new group will map to run protocol of copied wells



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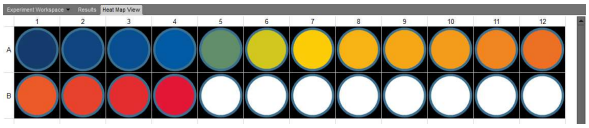
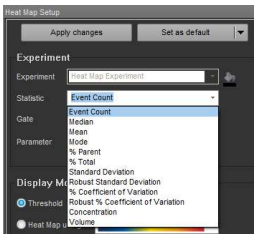
Heat Map Analysis Tools

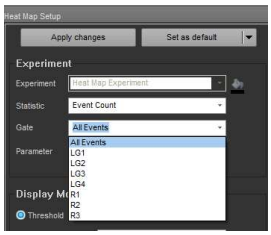
The *Heat Map view* provides a graphical method (i.e., *Heat Map*) for setting up and analyzing plate- and tube-based experiments.

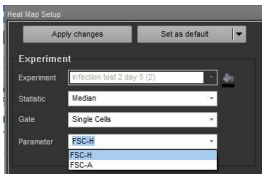
- Each sample with a saved FCS file will be colored to reflect a user-specified statistic for a specific gate and parameter



- Select statistic, gate, and parameter for heat map display from drop down menus on the Heat Map Setup Menu



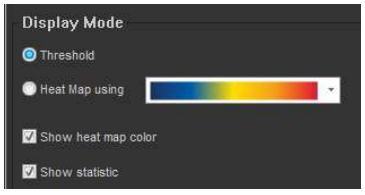


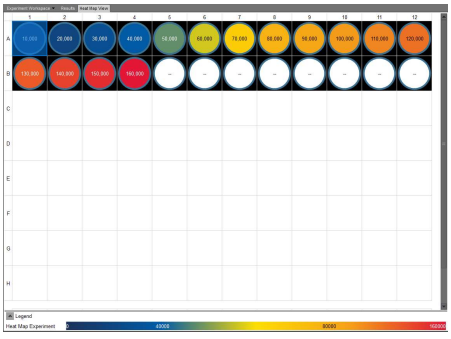


**only gate specific parameters are available*

Heat Map Analysis Tools

- Statistical data is overlaid on heat map by selection of the “Show statistic” box under Display mode





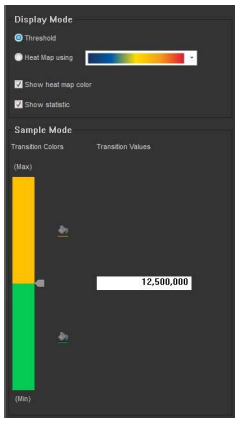
- A legend is displayed on the bottom of the Heat Map Display. Indicates experiment name, color scheme and transition points

Heat Map Analysis Tools

Two display modes are available: Threshold and Heat Map Mode

Threshold mode:

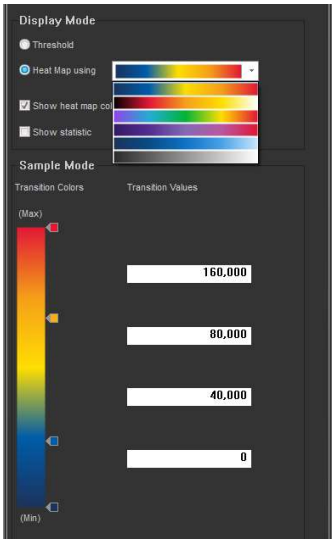
- Data displayed using discreet colors to indicate user-specified transition points in data set
- Once level is exceeded, color will change
- Color scheme may be changed by selection of pain colors



Heat Map Analysis Tools

Heat Map Mode:

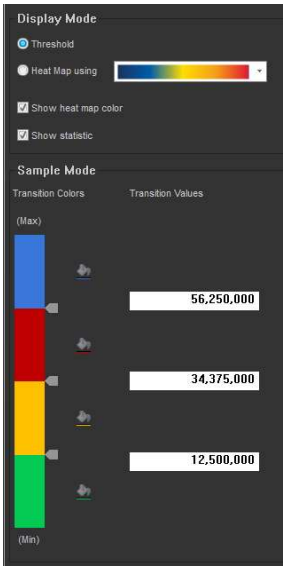
- Data displayed using color gradient to indicate user-specified transition points in data set
- Multiple color choices available from drop down menu



Heat Map Analysis Tools

In both modes:

- Define min/max range and transition values by typing value in text boxes
- Add transition points by clicking on colored bar
- Transition points can be repositioned by selecting and dragging arrow to new position
- Delete transition points by clicking on arrow and dragging it away from the colored bar

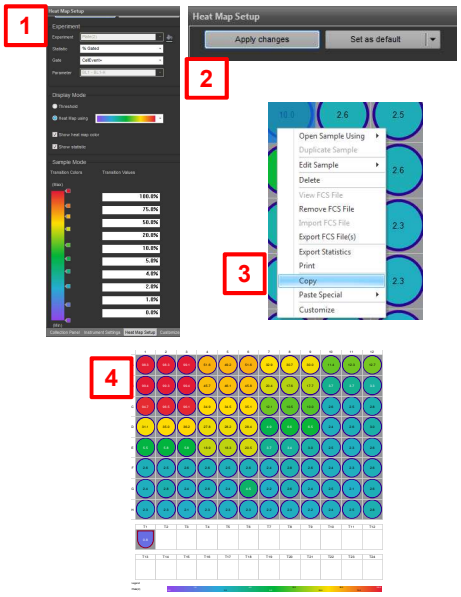


How to Export a Heat Map Image

To export an image of a Heat Map:

1. Adjust heat map settings within the “Heat Map Setup” menu
2. Press “Apply changes”
3. Right click on a defined well (not empty) on the HeatMap image; select “Copy”
4. Paste the image to another program such as Microsoft Word™, Excel™, PowerPoint™, FlowJo®, De Novo™ FCS Express

Notes: The complete Heat Map image is copied, including tube samples and the heatmap legend



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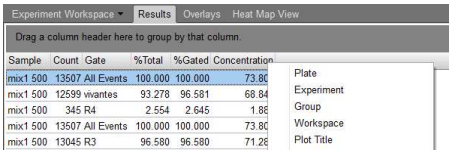
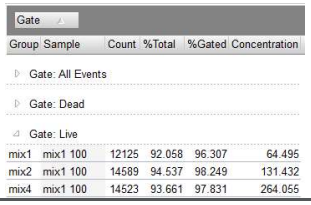
Results table

The *Results view* displays results from all samples consolidated into a single table

- Statistics can be added by selection of statistics from *Statistics* Ribbon (including "Select all" option), or from the pop-up list generated from right-click of any column header of the table

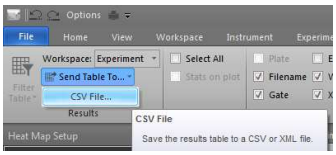


- Dragging a header onto the "Drag a column header …" group control removes the header from the table and places it in the group control. If you hold down the **Ctrl** key while dragging a header, the header is copied rather than moved.



Results table

- Columns can be re-ordered by dragging and dropping their headers.
- The table can be sorted by any column in an ascending or descending order (case insensitive) by clicking the column title.
- The Results Table can be exported by selection of “Send Table To”



- Specific rows can be selected, copied and paste into an Excel sheet by holding down the **Ctrl** or **Shift** keys to select, then **Ctrl+C** to copy, and **Ctrl+V** to paste

Advanced Software Features

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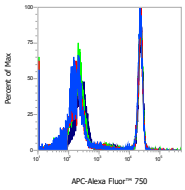
Overlay Builder

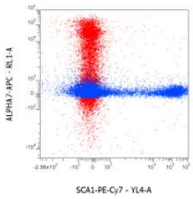
Overlays provide a graphical method for comparing data from samples within a single experiment by superimposing selected plots.

- Overlays may be created using either Histograms or Dot Plots:

Histogram overlay

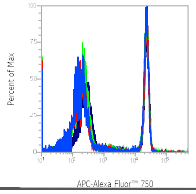


Dot-plot overlay

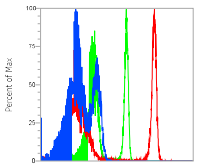


- Data may be superimposed in different ways:

Different samples, same plot overlay (example: antibody titration)



Same sample, different parameter or population overlay (example: spillover into other channels, intensity of signal into different populations)

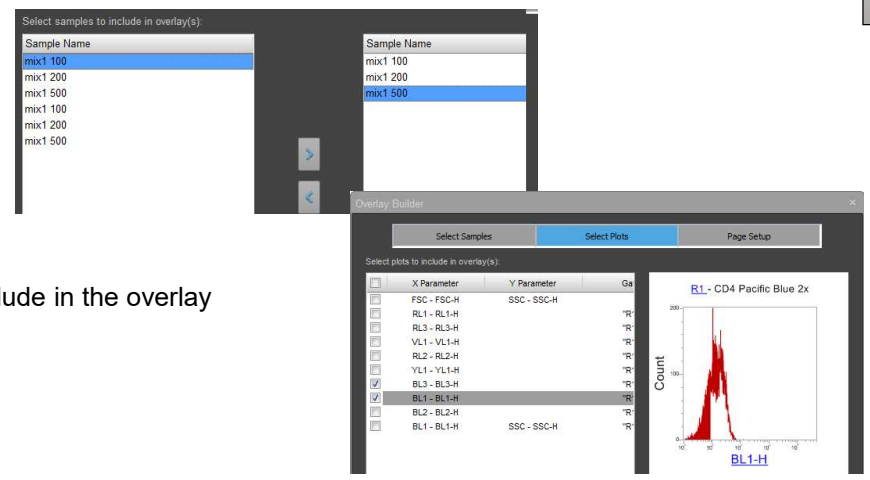


Alexa Fluor™ 488 fluorescence viewed in BL1, BL2, and BL3 channels

Overlay Builder

The Overlay Builder is a wizard that guides creation of new Overlay plots. It is accessible from the *Home* or the *Overlay* tabs

1. Select samples

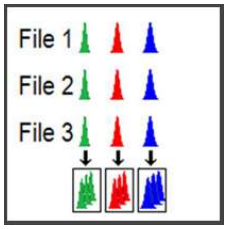


2. Select plots to include in the overlay

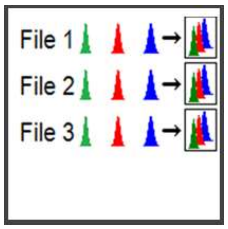
Overlay Builder

3. In the “Page Setup”, select Plot Layout:

   a. To create an overlay of different samples shown on the same plot, select “Display the same plots from all files together”

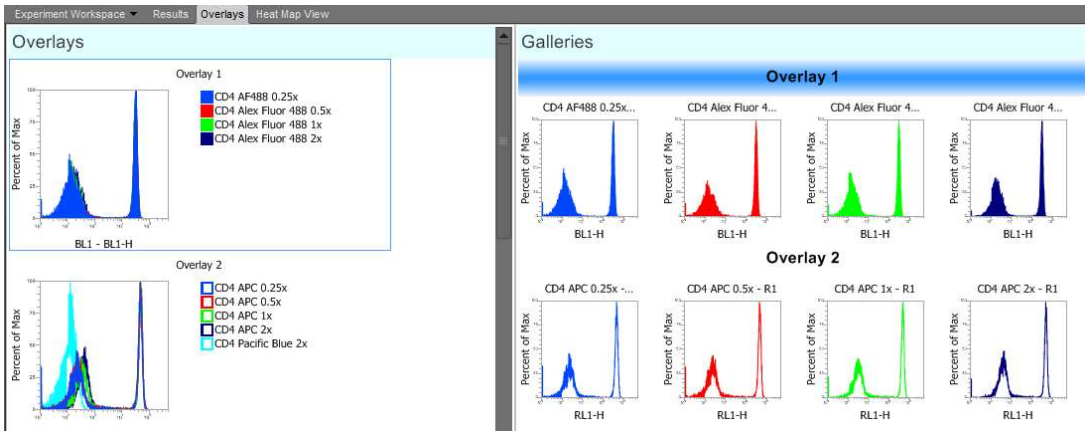


   b. To create an overlay that displays various parameters or populations of the same sample on the same plot, select “Display plots from the same file together”



Overlays

The *Overlay view* is divided vertically into two windows by a splitter bar.

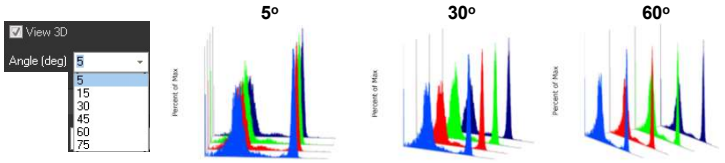


Overlays area displays the overlay plots
Notes: multiple overlay plots will be created if >10 samples are selected for overlay and default number of plots is not changed

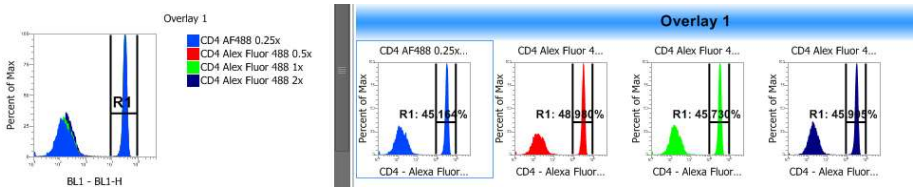
Galleries area displays source plots used in the overlays

Overlay Analysis

- There are many options for customization of overlays available from the *Customize* Panel, as the 3D View:



- Gates may be added directly to Overlay plots and source plots in the *Overlays* View



Notes: Gates added to the overlay plot will not be added to the source plot.
Gates may not be added to plots displayed in 3D.
Displayed statistic can be changed from *Statistics* Ribbon

Advanced Software Features

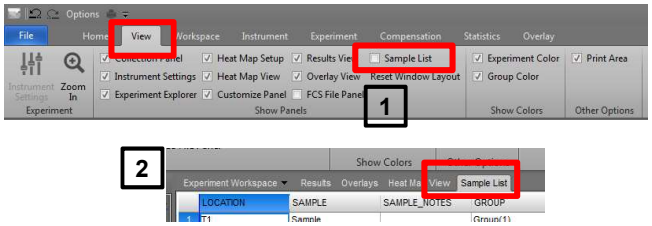
- Template management
- Filter Configuration Manager
- Heat Map Analysis Tools
- Results Table
- Overlay Builder
- Sample List
- Keyword Manager
- Storage Gate / Gate Export to fcs file
- User Options

Sample List View Enables Quick Annotation of Samples

The Sample List View is a tabular view of all samples in the current experiment.

To View the Sample List:

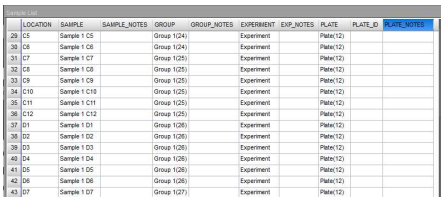
1. Select “Sample List” within the View Tab of the Ribbon Bar
2. The Sample List will open within a tab behind the Experiment WS



Sample List – Tube Experiment

	LOCATION	SAMPLE	SAMPLE_NOTES	GROUP	GROUP_NOTES	EXPERIMENT	EXP_NOTES
1	T1	Sample 1 T1		Group 1		Default Group and...	
2	T2	Sample 1 T2		Group 1		Default Group and...	
3	T3	Sample 1 T3		Group 1		Default Group and...	
4	T4	Sample 1 T4		Group 1		Default Group and...	
5	T5	Sample 1 T5		Group 1		Default Group and...	
6	T6	Sample 1 T6		Group 1		Default Group and...	
7	T7	Sample 1 T7		Group 1		Default Group and...	
8	T8	Sample 1 T8		Group 1		Default Group and...	
9	T9	Sample 1 T9		Group 1		Default Group and...	
10	T10	Sample 1 T10		Group 1		Default Group and...	

Sample List – Plate Experiment



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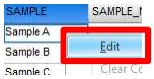
Sample List View enables quick annotation of Samples

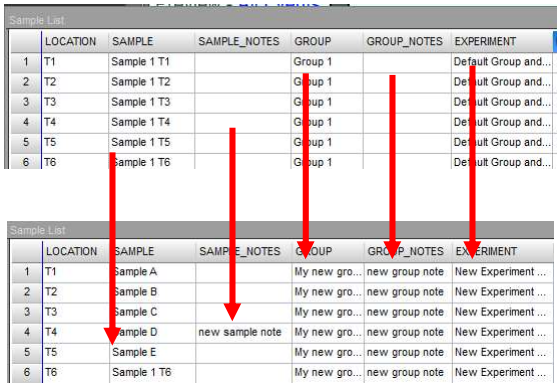
The Sample List View includes information on the location, sample name, sample notes, group name, group notes, experiment name, experiment notes, plate name, plate notes, plate ID, AND any custom keywords created by the user

Users can edit the sample, group, experiment, and plate information by typing directly into the table or selecting “edit” from the right click context menu



Notes:

- Changes to samples are sample-specific
- Changes to group names or properties apply to the group
- Changes to the experiment or the plate apply to the experiment or plate (all samples)



Changes can be made pre or post acquisition

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Import a sample list file

The Sample List can be updated quickly by import of a sample list file OR copy/paste from a .csv file

To Import a Sample List File:

1. Create a new experiment (plate or tube) or locate an experiment without any FCS files recorded to import the sample list into. It is not necessary to define samples or groups if importing a sample list.
2. Create a Sample list with information in the following order:
   - Location
   - Sample
   - Sample Notes
   - Group
   - Group Notes
   - Experiment
   - Experiment Notes
   - Plate*
   - Plate ID*
   - Plate Notes*
   - Keywords**

*for plate experiments
**Keywords must be defined and added to experiment before importing Sample List

1

2

	A	B	C	D	E	F	G	H	I	J
1	LOCATION	SAMPLE	SAMPLE_NOTES	GROUP	GROUP_NOTES	EXPERIMENT	EXP_NOTES	PLATE	PLATE_ID	PLATE_NOTES
2	A1	1001	test sample 1	Group A	control treatment	plate 1	plate 1	plate 1	plate 1	This is a test pla
3	A2	1002	test sample 2	Group A	control treatment	plate 1	plate 1	plate 1	plate 1	This is a test pla

IMPORTANT NOTES:

- The "Location" column in the Sample List must be listed in the format A1, A2, …etc. for plates and T1, T2, … etc. for tube samples
- The sample list in the .csv file MUST match the properties of the sample list in the current experiment (ie, the csv file should not include new keywords or have more samples than possible for the current experiment)

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Import a sample list file (cont.)

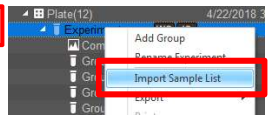
3. Right click on the experiment or plate name in the Experiment Explorer.

4. Select the correct **.csv file** and click "Open"
   Notes: .xlsx or .xls files may not be imported as Sample Lists

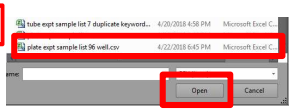
5. In the "Map Sample List Data" Menu, ensure that the columns in the .csv file are mapped to the correct location for the sample list table in the Attune NxT Software. Adjust as needed using the dropdown menus.
   Notes: If the experiment already has a sample list the software will display an "Update Sample Information" dialogue.

6. The Imported Sample List will be applied to the experiment

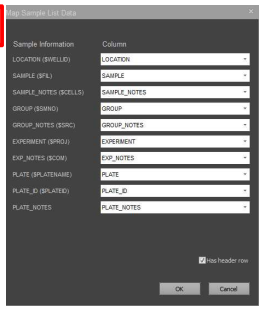
3

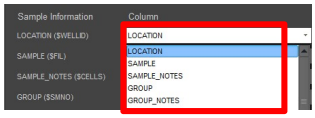


4

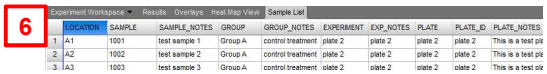


5





6



IMPORTANT NOTES:

If the .csv file has extra columns or wells/tube locations are not in the standard order the import may fail or have an error message

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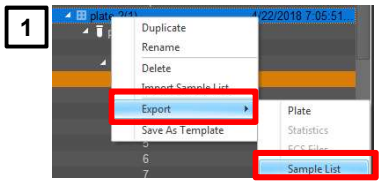
Export Sample List to .csv file

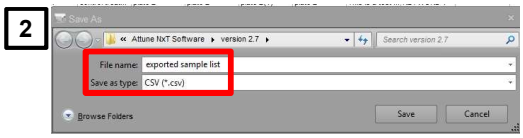
The Sample List can be exported to a .csv file.

To Export a Sample List:

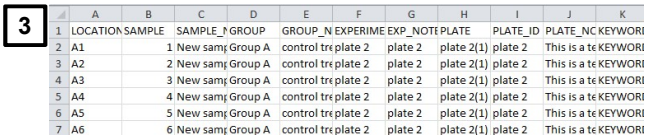
1. Right-click on the plate or experiment that includes the sample list to be exported and select “Sample List” from the drop down menu



2. In the “Save As” dialogue box, name the sample list and select “Save”. The file is saved as a .csv file



3. Open the saved file and use



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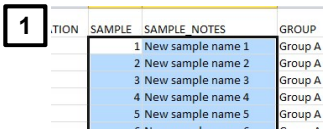
Copy/Paste of Data into Sample List for Quick Annotation

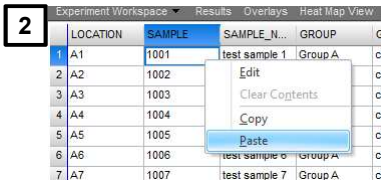
The Sample List can be updated quickly by copy/paste from a .csv file

To Copy/Paste data from a .csv file

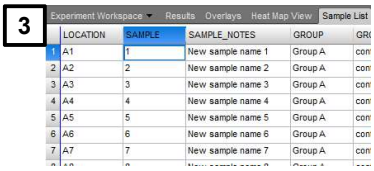
1. Open .csv file, select range of data and copy (right click/copy or use Ctrl+C)



2. Open Sample List in Attune NxT Software, select range to paste information to, select paste (or use Ctrl+V) OR select first well of range to copy information to and paste



3. Data will be copied into Sample List



IMPORTANT NOTES:

- If range selected does not match range of cells available in Sample List, will get error message
- It is not possible to copy a different location name (e.g.: 1, 2, 3, 4 instead of T1, T2, T3.. etc)
- Names cannot end with a "period" character and cannot be any of the following words: "CON", "PRN", "AUX", "CLOCK$", "NUL", "COM1", "COM2", "COM3", "COM4", "COM5", "COM6", "COM7", "COM8", "COM9", "LPT1", "LPT2", "LPT3", "LPT4", "LPT5", "LPT6", "LPT7", "LPT8", "LPT9".

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Advanced Software Features

- Template management
- Filter Configuration Manager
- Heat Map Analysis Tools
- Results Table
- Overlay Builder
- Sample List
- Keyword Manager
- Storage Gate / Gate Export to fcs file
- User Options

What are “Keywords”?

Keywords describe a characteristic of the flow cytometry data set and paired with a value. Keywords are unique in data sets, i.e., there are no multiple instances of the same keyword in the data set.

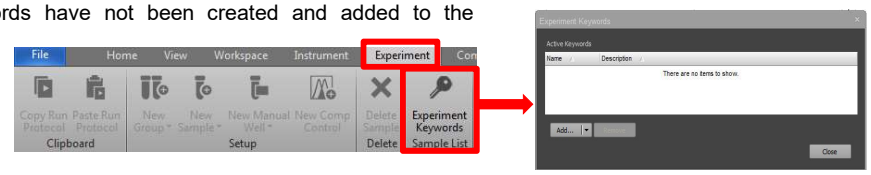
- There are **required and optional FCS keyword-value** pairs. The **required** keyword-value pairs represent the **minimum set needed to successfully read and write an FCS data set.** Conformant FCS file reading programs must recognize required FCS keywords. **Example: $TOT** is the keyword that describes the **total number of events** in the data set
- **Optional keywords** include keywords (characteristics) defined in the file standard that are **helpful for data annotation** but not required to be written to the FCS file by the manufacturer at the time of acquisition. **Example: $VOL** is the keyword that describes the **volume of sample** run during data acquisition
- **Custom keywords** include keywords (characteristics) **defined by the instrument manufacturer** that are written to the file at the time of acquisition. Custom keywords are identified by a “#”. **Example: #FLOWRATE** is the keyword that describes the **flow rate of sample during sample acquisition**

Customer Created, User Defined Keywords

Attune™ NxT software allows users to create custom keywords that can be included in the FCS file when recording.

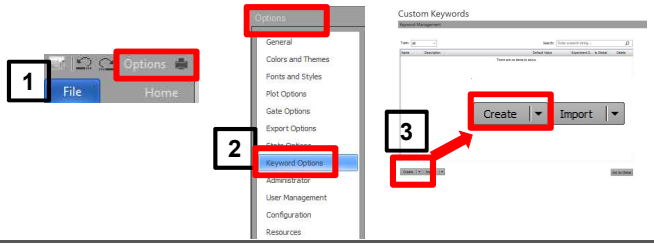
Why create a custom keyword? Creating custom keywords are helpful for data annotation because they provide additional information about the sample that is useful for sorting data and analyzing data using offline analysis programs.

Experiment Keywords are listed in the "Experiment Keywords" Menu. No Keywords will be listed if Keywords have not been created and added to the experiment.



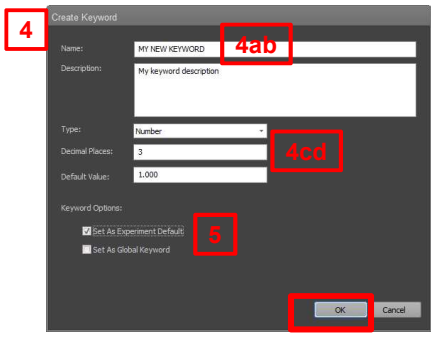
To create a new custom keyword:

1. Open the Options Menu
2. In the Options Menu, open Keyword Options
3. Click "Create" to add a new keyword



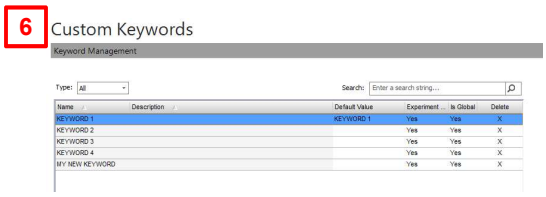
Customer Created, User Defined Keywords

4. Enter properties for the new keyword:
   a. **Name (required)**
   b. **Description**
   c. **Type (required; number or characters/string)**
   d. **Default value**



5. Set the keyword as an **Experiment Default Setting** or as a **Global keyword**
   - Setting a Keyword as an "**Experiment Default"** will cause the keyword to be included in **all new experiments for all users**
   - Setting a Keyword as a "**Global Keyword**" will cause the Keyword to be available for other user accounts

6. Click OK. New Keywords will be listed in the Custom Keywords Menu



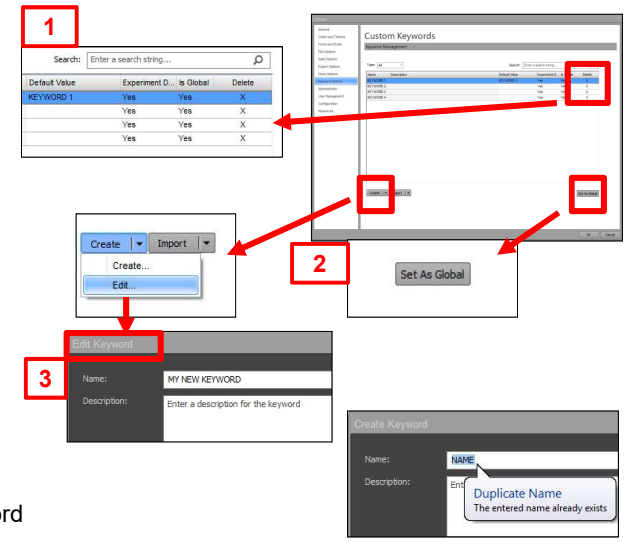
Default Keywords will be automatically added and used in new experiments

Global Keywords are available in all user profiles

Keyword Management

Keywords are managed globally in the Administrator account and locally by User accounts

1. Administrators can Delete Global Keywords
2. Administrators can change setting of Keyword as Global after the Keyword is created
3. Keyword Properties may be edited after creation, including setting the name, type, default value, and setting the Keyword as a Default



IMPORTANT NOTES : Keywords can not be named using a name that is already used in another user's account, EVEN if the keyword is NOT a global keyword

Share Keyword Lists through Export/Import of Keywords

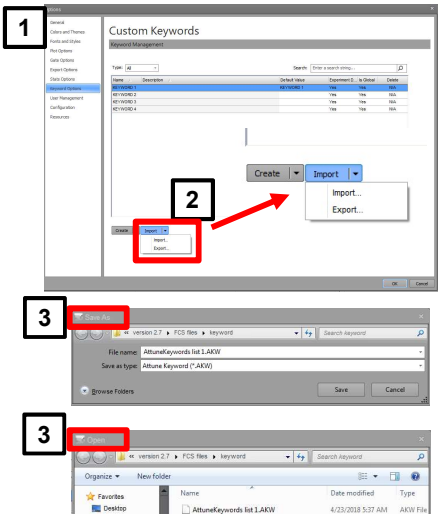
Keyword lists can be shared between user profiles or instruments by Export/Import of Keyword lists

To Export a Keyword List:

1. Open the Options Menu and navigate to "Keyword Options"
2. Click the arrow next to the "Export" button and select "Export" from the drop down menu
3. In the "Save As" dialogue, name the Keyword list and click Save

To Import a Keyword List:

1. Open the Options Menu and navigate to "Keyword Options"
2. Click the "Import" button and select "Import" from the drop down menu
3. In the "Open" dialogue, locate the Keyword list and click Open.



Adding keywords to an Experiment

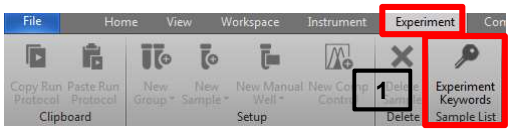
Keywords must be added to an experiment before they can be used.

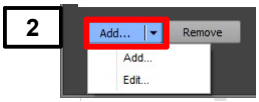
Experiment Keywords are listed in the "Experiment Keywords" Menu. No Keywords will be listed if Keywords have not been created and added to the experiment by the user or added by default.

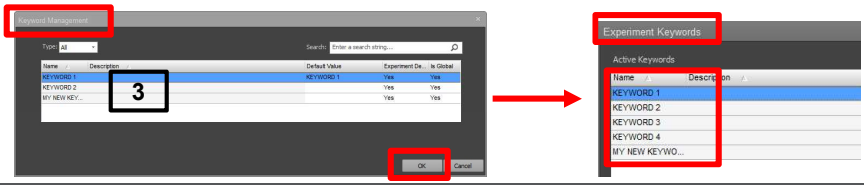
Keywords are generally added to an experiment during experiment setup.

To add a Keyword to the experiment:

1. Open Experiment Keywords Menu
2. Select "Add"
3. Select the Keywords from the Keyword Management Dialogue
4. Click OK. The new keywords have been added to the active experiment.







Advanced Software Features

- Template management
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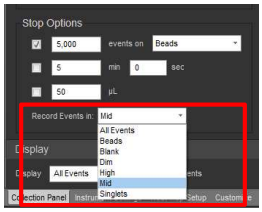
Limit file size by Recording Data within a Specific Gate

The Attune™ NxT Software enables recording of sample data within a specified storage gate.

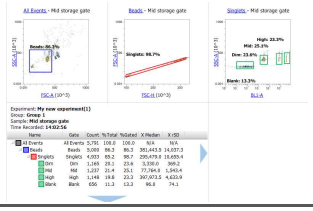
When is this feature useful? Experiments that require collection of a large number of events that are NOT of interest to the study (examples: stem cell studies involving collection of lineage negative populations, No Lyse No Wash assays, etc.).



How does this feature work?

- User designates a storage gate **before recording data**
- As the sample is acquired **all events are displayed** but only the events within the **specified gate are saved**.
- Hierarchical gating strategy preserved for data within the storage gate is preserved, however data NOT falling within the storage gate are excluded from the final data file

At the end of recording



Reviewing file (after moving to another sample)

Revision 2.5
Revision Date: Aug2019

ThermoFisher SCIENTIFIC

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How to record data within a specific gate

To limit the data recorded to an FCS file:

1. Create workspace to identify storage gate
2. Set Run protocol (acquisition volume, flow rate, stop conditions, and for plate experiments total sample volume, rinse and mix cycles)
3. Pre-run sample, adjust instrument settings, and adjust gate positions as necessary
4. Set storage gate by selecting gate from list next to “Record Events in” menu

   Notes: Compensation controls will only list available gates for compensation samples
5. Record sample

   Notes: All data will be displayed while recording and when acquisition is complete, however only data within the storage gate is actually saved to the FCS file.

IMPORTANT NOTES for using post acquisition “Save” option: If the storage gate is repositioned after acquiring sample and then the save button pressed, data included in the repositioned/resized gate will not be captured. Only data within the original storage gate position and size is saved to the FCS file.

Tube sample

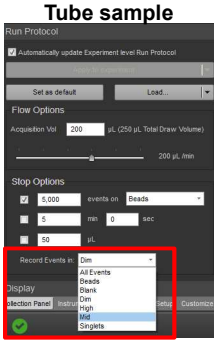
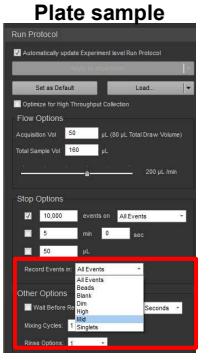
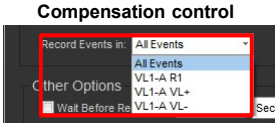


Plate sample



Compensation control



Revision 2.5
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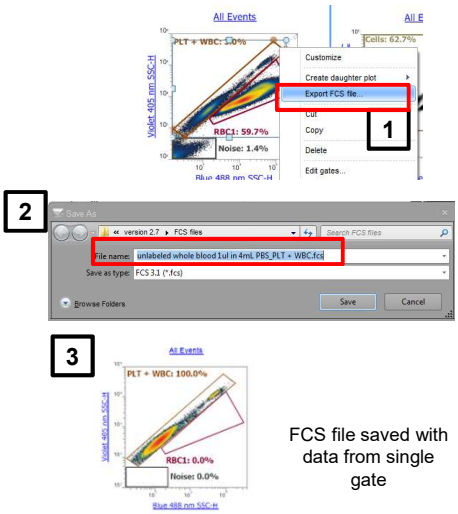
Export of a FCS file from a gated population

The Attune™ NxT Software enables export of an FCS file from a sample OR a gated population

To export an FCS fie from a specific gate:

1. Select the gate
2. In the right click context menu, select “Export FCS file”
3. In the Save As dialogue box the file will be named by default using “sample name_gate name”
4. Click “Save”.
   Notes: A FCS file will be created that includes data ONLY from the gated population (data shown is the .fcs file imported into another sample)

IMPORTANT NOTES: The originality of the FCS file, date and account responsible for modification is preserved in the header of the exported FCS file to indicate that the data was exported from another data set ($Originality, $LAST_MODIFIER, and $LAST_MODIFIED keywords



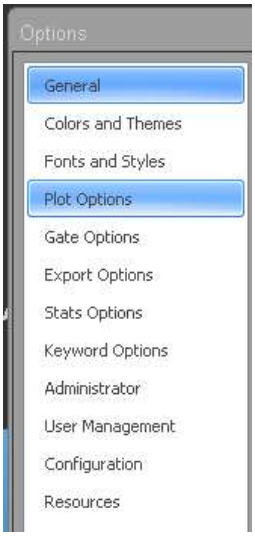
FCS file saved with data from single gate

Advanced Software Features

- Template management
- Filter Configuration Manager
- Heat Map Analysis Tools
- Results Table
- Overlay Builder
- Sample List
- Keyword Manager
- Storage Gate / Gate Export to fcs file
- User Options

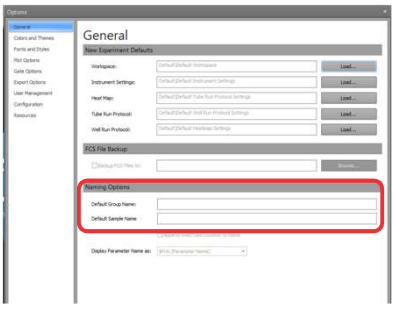
Options Dialog

- The *Options* Dialog allows to customize the Attune™ NxT software by configuring personal settings and changing the default options.
- Some options are user-specific, while others are application-specific (i.e. global to all users) and customizable only by an authorized user.



User Options

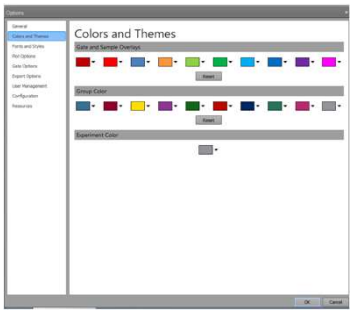
General



- Define the default ***Group*** and ***Sample*** Names
- Select the mode to display ***Parameter Name***

Notes: Workspace, Heat Map, Tube and Well Run Protocol default customization are not available at this time.

Colors & Themes

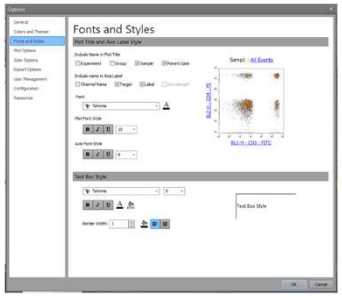


Change color rotation of:

- Gates on Workspace
- Samples on Overlays
- Group coloring on Plate View

User Options

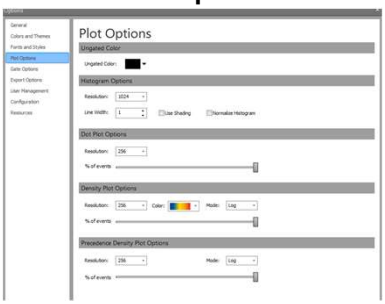
Fonts and Styles



Define default Display options:

- Plot title
- Axis labels
- Tick mark labels
- Text box

Plot Options

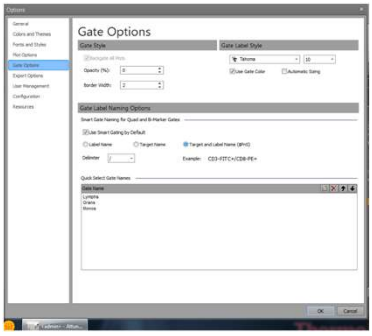


Set preferences for plot types:

- All plot types: Resolution
- Histograms: Shading, line width, normalization.
- Density plots: Color scheme and % events to display
- Dot and Precedence Density plots: % of events to display on plots.

User Options

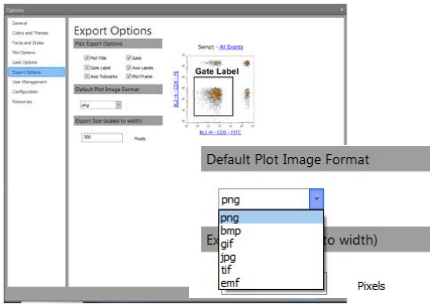
Gate Options



Set preferences for Gate Options:

- Gate style: Opacity, Border Width
- Gate Label Style: Font type and size
- Quad Gate: Smart Label options
- Quick select Gate Names

Export Options

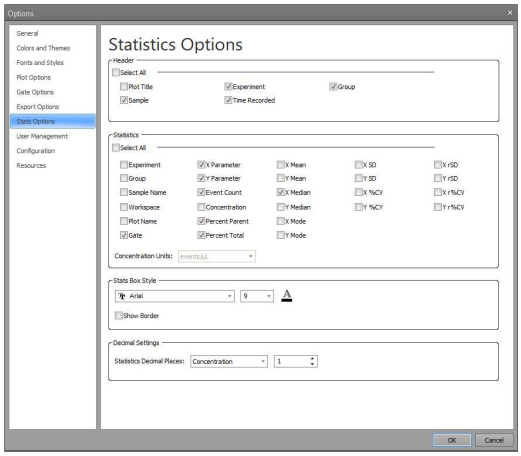


Set Preferences for Export Options:

- Plot Options: Title, Axis Titles, Gates, Gate titles, Tick marks
- Format: png, jpg, bmp…
- Export Size (300 pixels default)

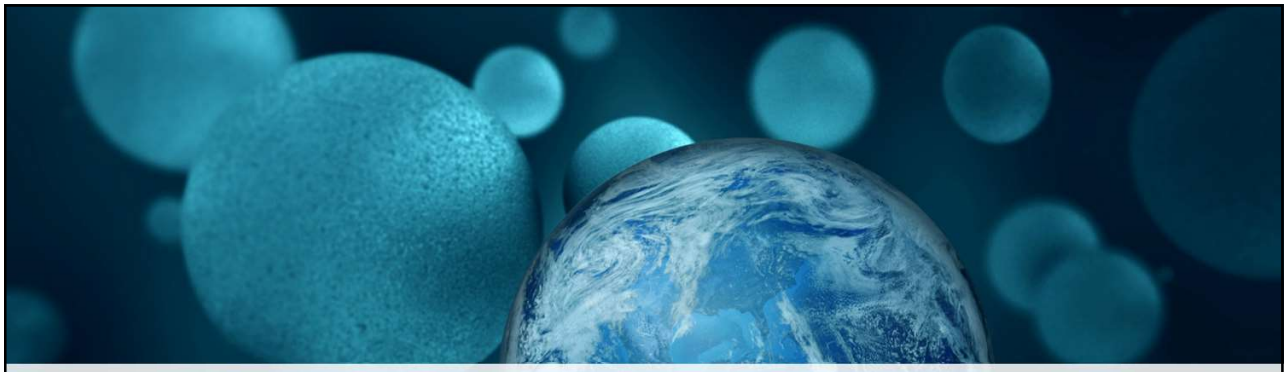
User Options

Statistics Options



Set preferences for Stats options

- Header
- Statistic values
- Style: Font type and size
- Decimal settings



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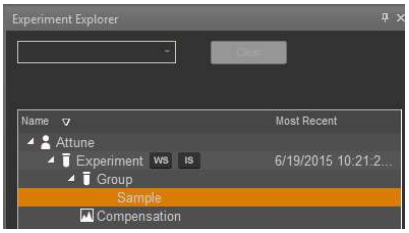
Data and User Management

Experiment Explorer

- Interface for creating, viewing and managing experiments.



- Icons indicate various elements and conditions

Icon	Indication	Icon	Indication
	User		Compensation
	Plate Experiment node		Read-only compensation
	Tube Experiment node	WS	Workspace
	Group node	IS	Instrument Settings
	Sample with recorded data	CS	Compensation Settings

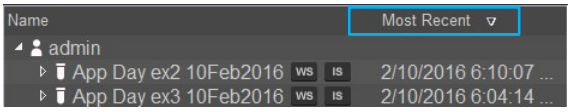
Experiment Explorer

Search

- Filters the files and reports listed
- Reduces the number of experiments displayed
- By Date or by Keywords

Sort

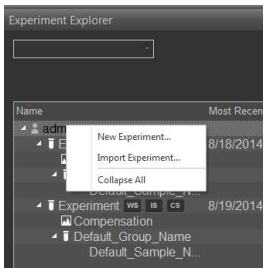
- Sort by Name or Date using column headings.



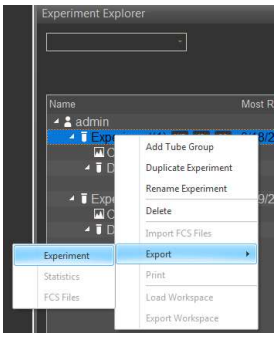
Experiment Explorer - Context Menus

- Right-click will display menus associated with different levels of the explorer hierarchy:

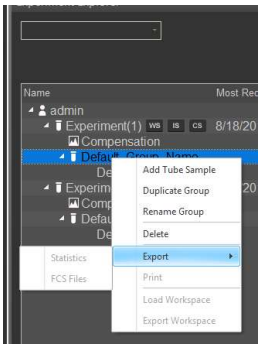
User



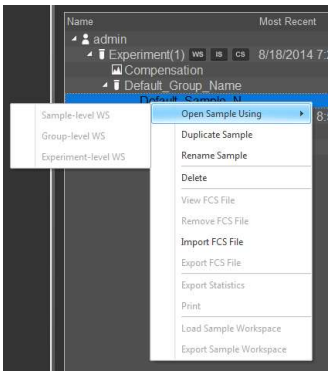
Experiment



Group



Sample



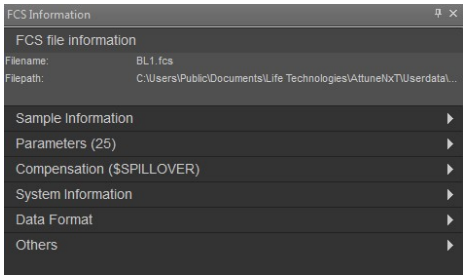
File Management - Types and Extensions

- Data format: FCS 3.1 or FCS 3.0
- Storage Location: The directory selected by users.
- File extensions: Automatically added to each file.

.fcs – Data file
.ahm – Heat map file
.arp – Run protocol
.aws – Workspace file
.ais – Instrument settings file
.acs – Compensation settings
.aic – Instrument configuration
.apt – Plate template
.att – Tube template
.afs – System log in, system local format

FCS File

- FCS file info
  - File name and path
- Sample information
  - Start/end time
  - Flow rate
  - Volume
  - Total events
  - Lost events
  - Aborted events
- Parameters
  - Channel
  - Target & label
  - Voltage
- Compensation
  - Spillover values
- System information
  - Configuration
  - Laser, ASF, laser delay



Options for Data Backup with Attune™ NxT Software

- **Option 1:** Manual Export of Experiments and Data files
  - To be done by all users
  - Recommended after each experiment
- **Option 2:** Use the Attune™ NxT Database Utility
  - Separate program but supplied with Attune™ NxT software
  - Backs up the whole database as a snapshot at that time
  - Not incremental, whole thing every time – may result in large files
  - Can be scheduled to backup at defined times

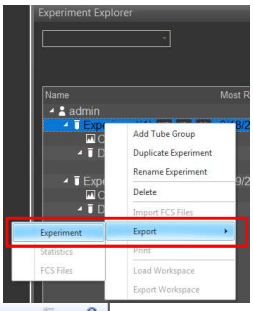
Notes: DO NOT rename or move files or folders within the AttuneNxT\Userdata folder

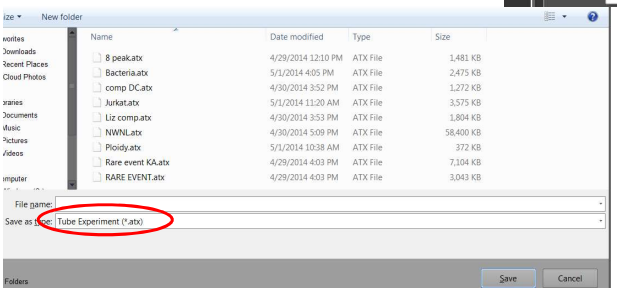
Experiment Export

- Right-click on the Experiment Name, open context menu.
- Select Export Experiment.
- Windows browser opens to last ‘saved’ directory.
- Batch Export enabled

- Creates files:
  - .atx Tube experiment
  - .apx Plate experiment

Note: Convert .atx to .zip. Unzip to show all the .fcs files





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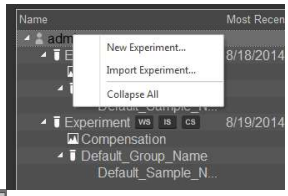
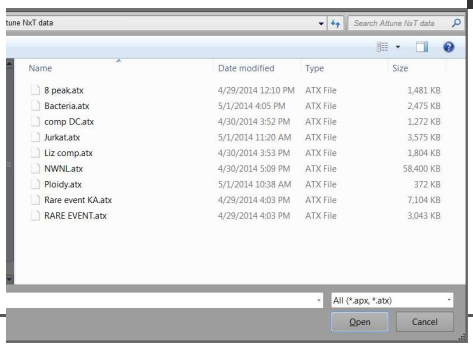
Experiment Import

- Click on Import Experiment from the Main Menu
  OR
- Right-click on the User name, open context menu.
- Select Import Experiment.

- Windows browser opens to last ‘saved’ directory.

- Select:
  - .atx Tube experiment
  - .apx Plate experiment





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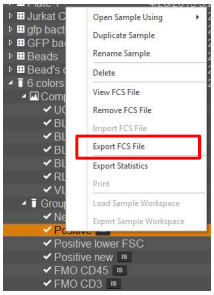
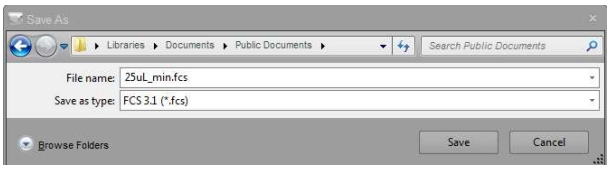
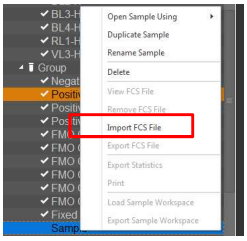
FCS File Management – Export/Import

Right-click on the *sample* name, open context menu



1. Export Raw data to FCS file
   - Multi-select enabled
   - Batch export enabled
   - File extension added
   - Type: FCS 3.1 or FCS 3.0

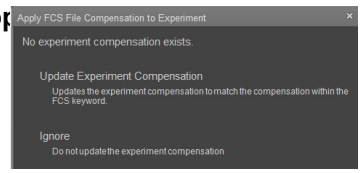




2. Import Raw data from FCS files

Import of FCS Files by drag-n-drop

FCS files can be imported into Attune™ NxT Software by drag-n-drop



To import files by drag-n-drop

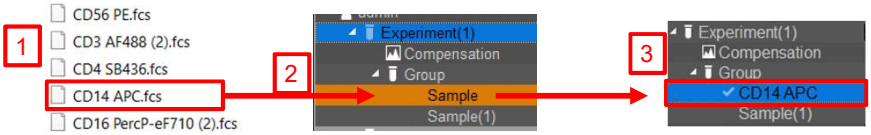
1. Select individual file for import

2. Click and "drag" file onto a sample tube or well

   → Select "ignore" or "updated" when asked to update experiment compensation (exported files may have null or empty compensation matrix

   - "Ignore": the file will be imported without creation of an experiment level compensation (if none exists)
   - "Update Experiment Compensation": all Attune™ NxT FCS files have a compensation matrix embedded in the file, even if no compensation was recorded. When no compensation was recorded, the compensation matrix is called a "null" or "empty " matrix because no data exists within it.

3. The file will be imported into the selected sample tube or well, and the sample name will be updated



Attune™ NxT Database Utility

The Attune™ NxT Database Utility Program has 4 functions:

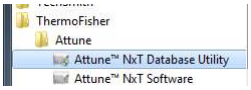
- **Backup User Data**: used to backup the whole database, files plus database data to a folder a single time
- **Restore User Data**: used to restore the contents of the folder from a backup so that the backup becomes the current version of the database.
- **Schedule an automated backup**: used to automatically backup user data and the database
- **Re-Install Database**: used to reset the database to the new, no data added state. Only available for Administrators and System Administrators and should be used only if recommended by Service or Support from ThermoFisher Scientific

MUST NOT occur while the Attune™ NxT Cytometer is acquiring samples

Attune™ NxT Database Utility

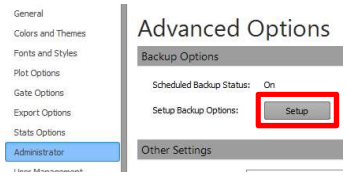
Open the Attune™ NxT Database Utility Program

- From the Windows Start Menu



OR

- From the Options Menu, under Administrator



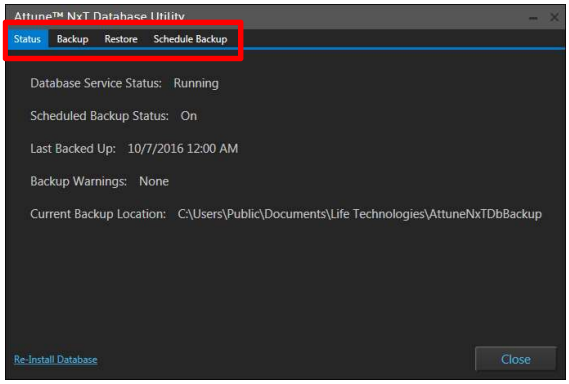
Notes: The Administrator page is only available to administrator, system administrator, and service accounts. **All** user accounts may launch the Database Utility **BUT** features are restricted for certain account types

Attune™ NxT Database Utility

After login, up to 4 pages are visible from the Main Database Utility Menu:

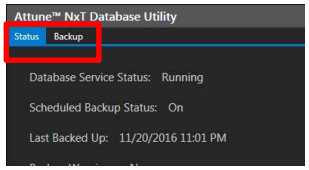
1. Status
2. Backup
3. Restore
4. Scheduled Backup

IMPORTANT NOTE: Restoration of User Data from a saved Database will overwrite all Data in the Active, instance of Attune™ NxT Software.



All options are available for **System Administrator** and Administrator accounts

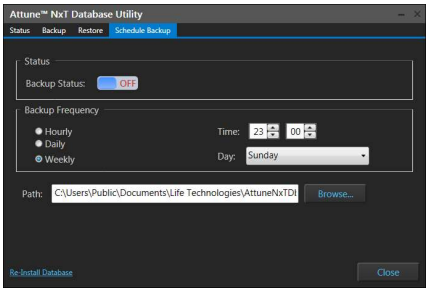
Service, **Advanced User**, and **User** accounts may only view the **status** of the database service and **initiate a database backup**



Attune™ NxT Database Utility – Schedule Backup

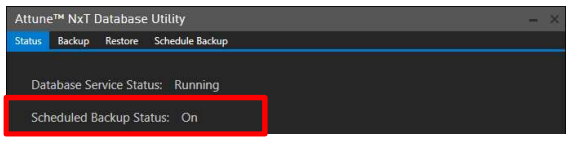
The *Schedule Backup* tab allows an authorized user to set the backup policy and schedule

1. From the *Schedule Backup* Tab, set the Status to "ON"



2. Set the Backup Frequency and Path
   Notes: It is possible to set automatic backup of the Attune™ NxT Database to a network location.

3. In the Status Tab, Scheduled Backup Status should be ON



Attune™ NxT Database Utility – Important Notes

The Attune™ NxT Database Utility saves a **snapshot of the entire database**

Single Time Backup: all files and the database structure will be saved as they are in that moment in time.

Regular Automatic Backup: the system will check for files that have changed between the current backup and previous backup and will only back up those that have changed. When the backup utility runs the next scheduled backup it will replace the existing backup with the new one.

EXAMPLE:

1. User records *Experiment 1.* The Database Utility runs and saves *Backup 1* to the predefined location
2. User records *Experiment 2* and deletes *Experiment 1* from the experiment explorer
3. The Database Utility performs an automated backup and overwrites *Backup 1* with *Backup 2*.

>>> Database *Backup 2* will contain *Experiment 2* and **NOT** *Experiment 1*.

Notes: If the user wants to keep each individual Database Backup file, they should move each file to a new location prior to the next scheduled Backup to prevent it being overwritten.

Connectivity to the Thermo Fisher Cloud

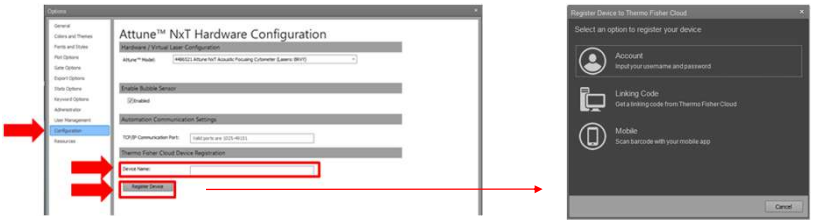
- Thermo Fisher Cloud offers up to 1 TB storage – free – easily share files without any cost.
- Available to :
  - Back up data files (not currently possible for Database)
  - Share data or other files easily with users at other locations
  - Upload experiments, instrument settings, workspace... then share with another user or download to another computer for analysis
  - View Baseline and Performance Test data.

NOTES: Thermo Fisher Cloud functionality is dependent on good internet speeds. Poor speed may result in connection errors.

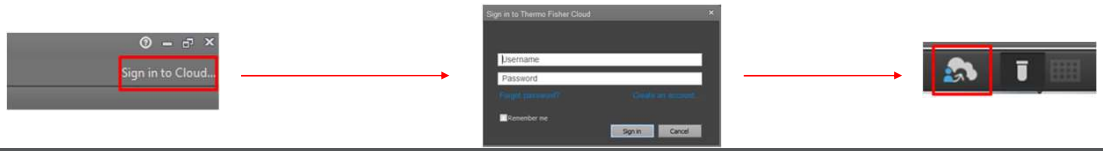
Connect your device to the Thermo Fisher Cloud

1. Create a ThermoFisher Cloud account

2. Register your device



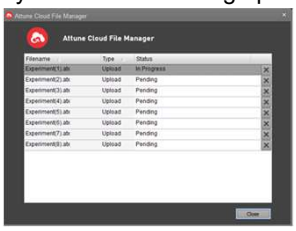
3. Sign in to Thermo Fisher Cloud in the Attune™ NxT Software



Data management with Thermo Fisher Cloud

- Users can export or import data files to and from the Thermo Fisher Cloud using any of the export/import options within the software:

- The **Attune Cloud File Manager** displays a list of files being uploaded or downloaded:



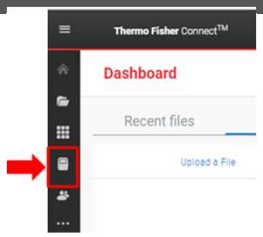
NOTES: The Attune Cloud File Manager system tray icon is only available when a cloud user is logged into their cloud account in the Attune™ NxT Software.

IMPORTANT RULES:

- The Attune Cloud Service allows for concurrent upload and/or download of data queues from up to 8 users.
- If more than 8 users attempt to add data to the upload/download queue then additional users must wait for the data transfer of one of those users to complete before they can add data to the queue.
- In each User account only 1 file will be uploaded or downloaded at a time.
- Once the total size of all uploading files exceeds 4GB then no more uploads will be processed until the current ones have completed.

View your device within the Thermo Fisher Cloud

- Once connected users can view their instrument/device within their Thermo Fisher Cloud account by clicking on the instrument connect button



- Connected devices will display under the Instruments tab

- Users can view Baseline and Performance Test data, Manage Users, Schedule Instrument

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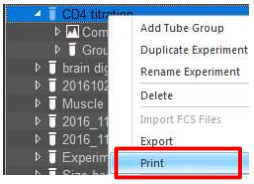
Print option

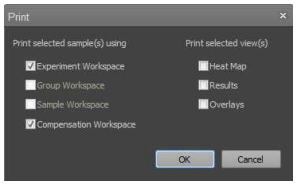
Workspaces, the Results Table, Heatmaps and Overlays may be printed

1. From the experiment explorer.
   - 1A: Right click on experiment or group name **to batch print all** samples within the selected experiment or group:
   - 1B: Right click on sample name to print currect active only select sample

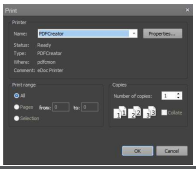


2. Select "Print" from the dropdown menu

3. In the Print Dialogue box, select items to print



4. Select destination printer



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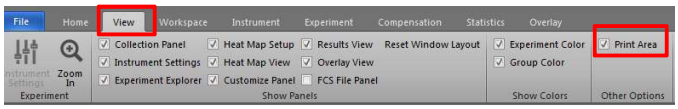
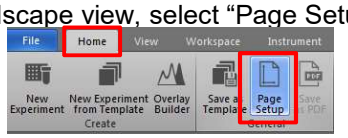
Changing the Page Setup

The Page Setup and Zoom will determine how a workspace is printed.

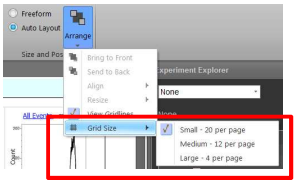
- To view the print area, select “Print Area” in the *View* Tab.



- To change from portrait to landscape view, select “Page Setup” in the *Home* Tab



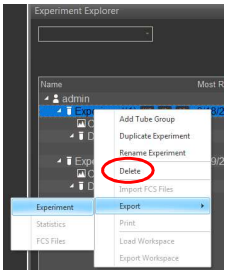
- To change number of plots per page In Auto Layout Mode, select “Grid Size” in the *Workspace* Tab

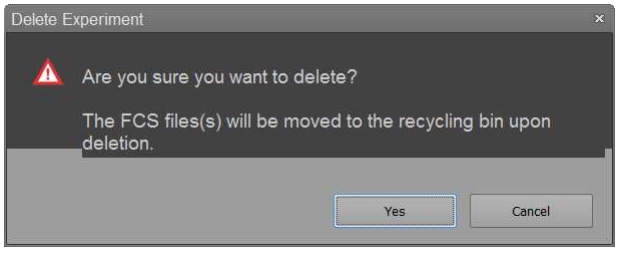


File Management - Delete

- Right click on Experiment, Group or Sample
- Select *Delete* from the context menu
- Files moved to the Recycling Bin

- Multi-select enabled with **Ctrl** button

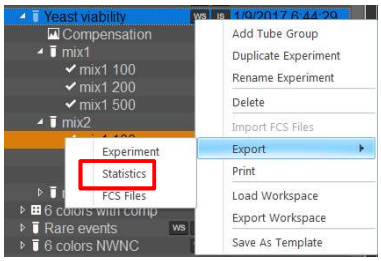


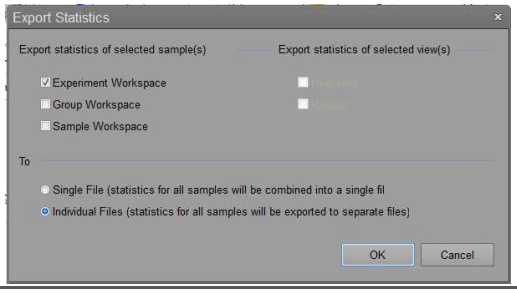


Data Management - Export Statistics

Select the Experiment, Group, or Sample data to Export:

- Right-click and select *Export, Statistics*
- Select statistics level and view of export
  Multi-selection enabled with the **Ctrl** button
- Select *Single File* or *Individual Files* for exported data
- Export to .csv file





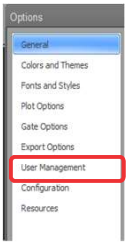
User Management

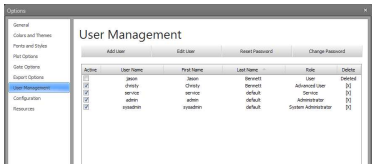
The *User Management* tab allows authorized users to add, edit, and view User accounts and manage User account passwords.

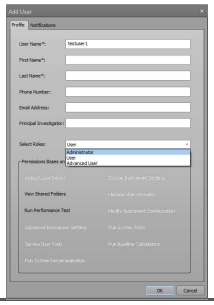
Add User:

Allows an authorized user to create a new User Account.

- Enabled only for Administrators.
- Clicking **Add User** opens the *Add User* dialog.
- There are different types of User accounts that provide different levels of system permissions.







User Management

Edit User:

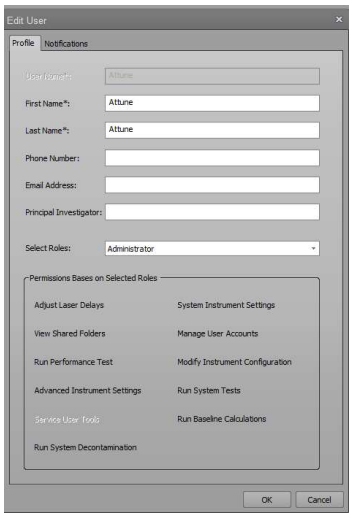
- Open by clicking ***Edit User***
- Modify a User account profile
- Administrator level can modify lower permission Users or self.
- Users can modify their own profile.

Reset Password:

- Authorized User to reset the password for any User account.

Change Password:

- Open by clicking ***Change Password.***
- Enables users to change their own passwords.
- Enabled for all Users.



Account Permissions

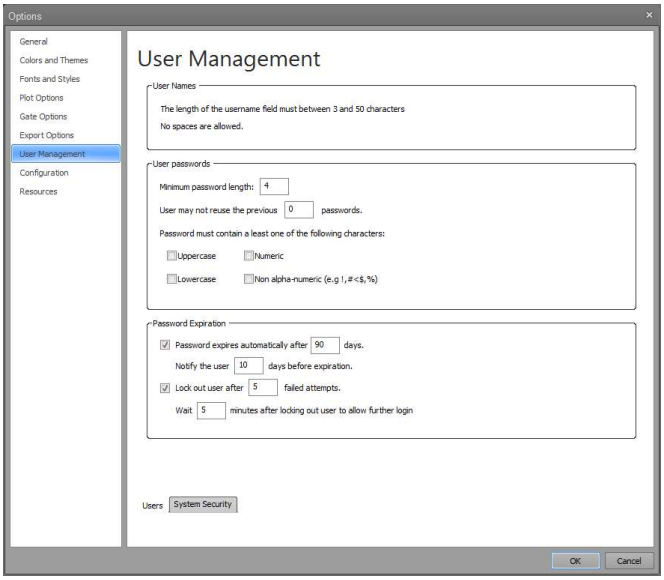
Permission	Description	User	Advanced User	Administrator	System Administrator
Run Performance Test	Allows a user to run the Performance Test and view the Performance Test reports	X	X	X	X
Run Baseline calculations	Allows a user to run and set new Performance Test Baseline calculations		X	X	
Run system decontamination	Allows a user to run the Decontaminate System function		X	X	
Run System Tests	Allows a user to run the system tests		X	X	
Manage User accounts	Allows a user to create user accounts, edit user accounts, reset passwords, change passwords, view login/logout times for all users, and view the length of all user sessions			X	X
Set security policy	Allows a user to set system security settings for username length, password length, password expiration and lock-out, and the auto lock out time due to system inactivity				X

System Administrator Account

- Highest level account
- Created upon Software Installation
- Allowed to define System Security Policy

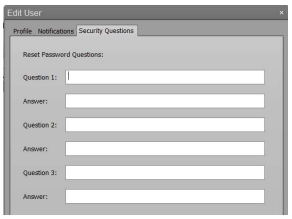
1) Set password rules
   - Length
   - Re-use rules
   - Password format

2) Password expiration rules



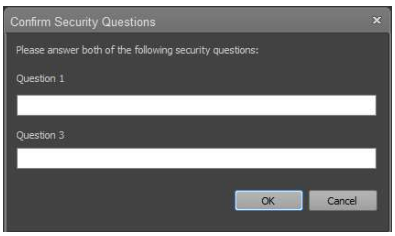
Forgotten Password

- The Administrator must reset a forgotten Password.



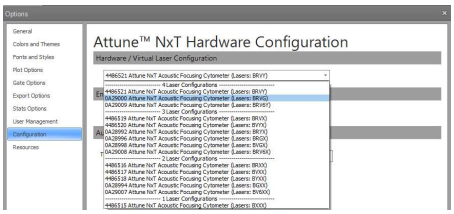
- *Forgot Password*? dialogue available at login.

- Linked to Security Questions answered during account creation.



Options

Configuration

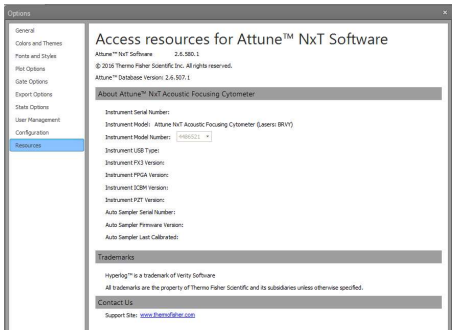


The instrument configuration (# of Lasers) is set by your FSE at installation.

Note: If using the software on a non-instrument computer, the default setting will be set to a 4 laser instrument.

If files are imported from another instrument configuration, (e.g., 1-3 laser systems), errors will be occur. Change the default setting to match your instrument configuration.

Resources



- Software, firmware, and database versions
- Link to website

Note: Autosampler information, and acoustic device calibration information are not available at this time.

Software Licensing Options

Part Number	License	Includes
A25554	Attune™ NxT Software - Single Individual Copy License	1. One license dongle 2. One software USB card 3. One document kit CD
A24856	Attune™ NxT Software - Five Individual Copy License	1. Five license dongle 2. Five software USB card 3. Five document kit CD
A24855	Attune™ NxT Software - Ten Individual Copy License	1. Ten license dongle 2. Ten software USB card 3. Ten document kit CD
A25555	Attune™ NxT Software - Multiple User Copy License - Five	1. One enterprise license dongle for server 2. One software USB card 3. One document kit CD
A25556	Attune™ NxT Software - Multiple User Copy License - Ten	1. One enterprise license dongle for server 2. One software USB card 3. One document kit CD

System Requirements: Windows® 7; 64-bit; 16 GB RAM; 500 GB Hard Drive

Document: “Data Management in the Attune™ NxT Software”

Data Management in the Attune NxT™ Software

Thermo Fisher Scientific

Attune™ NxT Software v2.7 or higher

- Saving User data
- Types of Data included in the Attune™ NxT Software
- Choosing one or more data archiving methods
- Database backup
- How to Save and Export Plate and Tube Experiment Files
- How to Save and Export FCS Files
- How to Use the Attune™ NxT Database Utility Program
- How to Restore Data using a Saved Database
- How to Copy a Database to a Different Computer
- Deletion of Experiment and Plate Files
- Re-installation of a New Database
- Deleting a User Account to Remove Data
- Permanent Removal of old Baseline and Performance Test Data

Revision 2.5
Revision Date: Aug2019

ThermoFisher SCIENTIFIC

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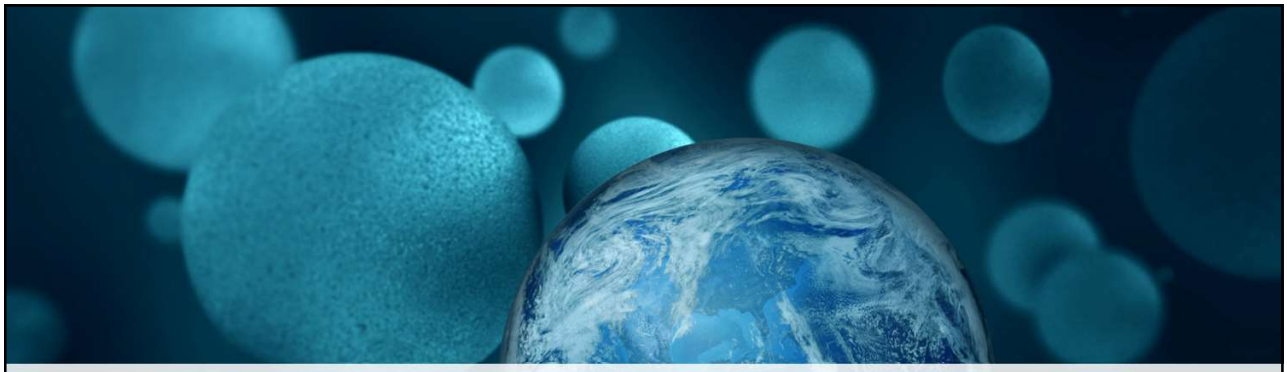

ThermoFisher SCIENTIFIC

Attune™ NxT Cytometer System Maintenance

Revision 2.5
Revision Date: Aug2019

The world leader in serving science

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System Maintenance

- Daily Maintenance
  - Visual Inspection
  - Start-up / Shutdown
  - Performance Test
  - Clean between Experiments
- General Maintenance
  - Optics
  - Fluidics
    - System Decontamination
    - Replacing Focusing Fluid Filters
    - Replacing Syringes
  - Attune™ NxT AAS calibration
  - Informatics

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    - Replacing Syringes
  - Attune™ NxT AAS calibration
  - Informatics

General Maintenance

Maintenance - Optics

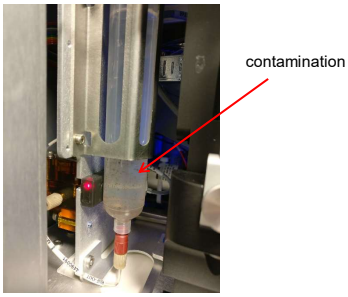
100022651 - dichroic filter holder
100022652 - bandpass filter holder

- If there is dust, use compressed air or a bulb blower to gently blow away the dust.
- If there is grease (e.g. fingerprints), gently wash, rinse with deionized water, and air-dry; do not wipe dry.
- **Frequency: as needed**

Maintenance – Fluidics

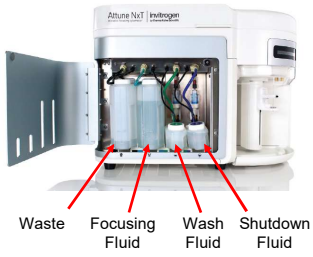
Potential problem – Contamination

- Check tanks for cloudiness or debris in the solutions, or brown marks on the sensor.
- Fill the emptied waste container with a 10% bleach solution up to the bleach fill mark (bottom line) on the bottle.



Maintenance – Fluidics

- Cleaning option:
  - System Decontamination Script
- Frequency: **Every 3-6 months**
- Replacement parts:
  - 1.9 L Attune™ NxT waste bottle - Cat. No. 100022156
  - 1.9 L Attune™ NxT focusing fluid bottle - Cat. No. 100022155
  - 175 ml Attune™ NxT Wash Bottle - Cat. No. 100022151
  - 175 ml Attune™ NxT Shutdown Bottle - Cat. No. 100022154
  - Attune™ NxT Auto Sampler Focusing Fluid Bottle - Cat. No. 4477847
  - Attune™ NxT Auto Sampler waste Bottle - Cat. No. 4477850



System Decontamination

- Function which facilitates the decontamination of the Attune™ NxT Flow Cytometer and the Attune™ NxT Autosampler fluidics.
- Sanitizes the system and fluidics bottles with Bleach and Wash solutions.
- Mostly automatic - 45 minutes, 3 phase operation – **Sanitize, Rinse, Refill**

Notes: This function is only available to Administrators and Advanced Users

WHEN?

- **Every 3-6 months** as a maintenance routine to prevent and reduce microbial growth within the instrument and fluidic bottles.
- If the system is likely to be **idle for more than two weeks** (run it in place of the Shutdown function).
- If the instrument has been **idle for more than two months**.
- Any time **contamination** in the fluid lines is suspected – i.e. event rate is too high.
- Prior to any service work or shipment for service.

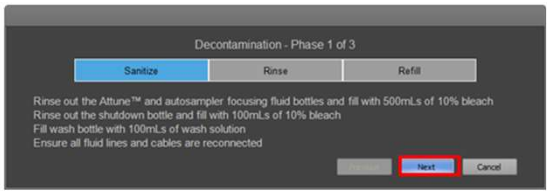
IMPORTANT NOTE: Make sure 2 replacement Attune™ NxT Focusing Fluid Filters are available before starting a System Decontamination.

System Decontamination – Phase 1 - Sanitize

During this the phase the Attune™ NxT and Attune™ NxT Autosampler (if connected) are sanitized with 10% bleach

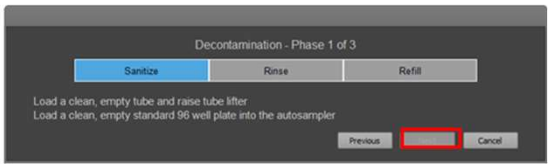
- **Step 1**: Follow the on-screen instructions and click *Next*

1



- **Step 2**: Once the tube lifter raised and a 96 well plate is in the autosampler (if connected) the *Next* button will be available.

2



Once *Next* is pressed the dialogue will close until Phase 1 is complete.

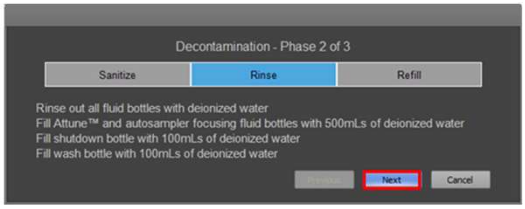
System Decontamination – Phase 2 - Rinse

During this the phase the Attune™ NxT and Attune™ NxT Autosampler (if connected) are rinsed with deionized water

Once Phase 1 is finished the instructions for Phase 2 will display

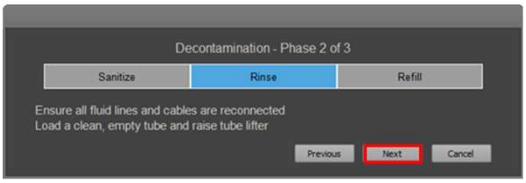
- **Step 1**: Follow the on-screen instructions and click *Next*

1



- **Step 2**: Once the tube lifter raised the *Next* button will be available.

2



Once *Next* is pressed the dialogue will close until Phase 2 is complete

System Decontamination – Phase 2 - Refill

During this phase the Attune™ NxT and Attune™ NxT Autosampler (if connected) are purged with Attune Fluids

Once Phase 2 is finished the instructions for Phase 3 will display

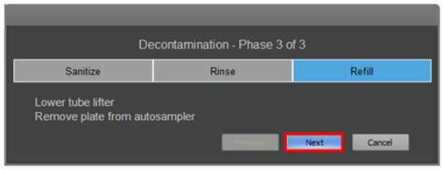
- **Step 1**: Follow the on-screen instructions. Once the tube lifter is lowered and the plate is removed from the autosampler the *Next* button will become available

- **Step 2**: Follow the instructions prior to clicking *Next*

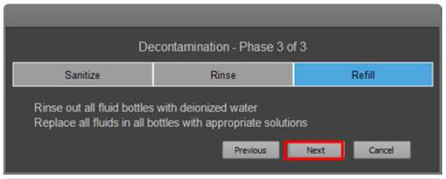
- **Step 3**: Once the fluidics lines and bottle cables are reconnected the Next button will become available.
  IMPORTANT NOTE: Change fluidics filters **before** clicking *Next*

Once Phase 3 is complete, the pump will activate and Focusing Fluid will fill the back reservoir. Users should then proceed to prime the fluidics.

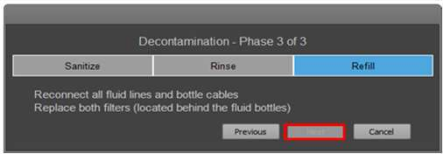
1



2



3



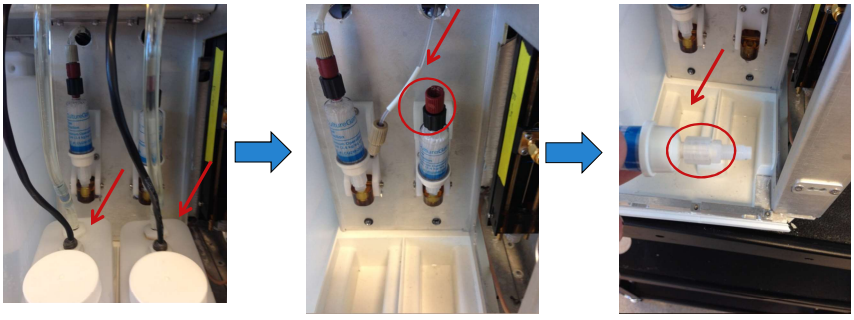
Focusing Fluid Filters

Focusing Fluid Filter
Part No. 100022587

- Two filters located behind the Wash and Shutdown bottles
- The filters may grow some contamination over time. If discoloration is evident, replace the filters.
- Replacing focusing fluid filters **every 3-6 months** (always after Decontamination Script has been run) reduces the risk of any potential contamination in the lines.

How to change Focusing Fluid Filters?

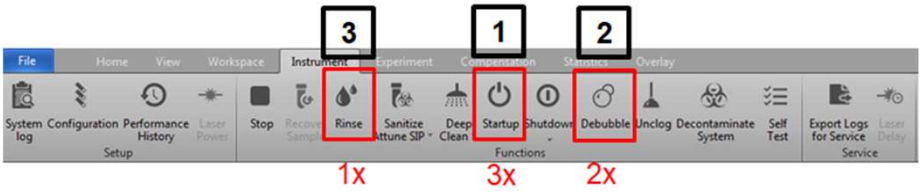
- Remove shutdown and wash bottles from bottle bay
- Unscrew the top luer fitting
- Unscrew bottom luer fitting and remove filter
- Put fittings on new replacement filter and re-attach to unit
- Ensure arrow on filter points in the direction of fluid flow (down).



Priming the Fluidics after changing the Fluidics Filters

Once System Decontamination is complete and Fluidics Filters are changed it is **very important users ensure the fluidics are primed**, this is to ensure any air is purged from the new fluidics filters.

Follow the 3-2-1 rule!
Run 3 Startup scripts, 2 Debubble scripts, 1 Rinse script



Notes: Failure to prime the fluidics can lead to air being trapped in the system. This can cause increased CV's and instability of the fluidics flow.

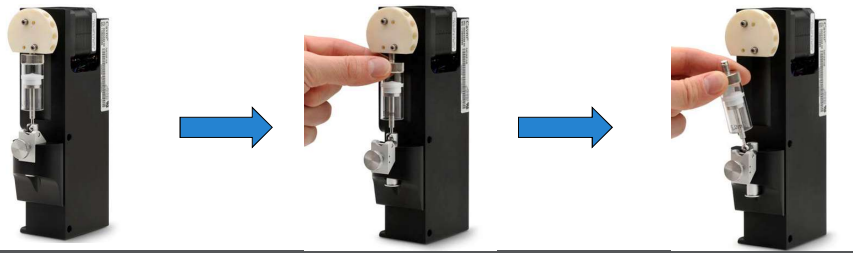
Maintenance – Sample Syringe in Attune™ NxT Cytometer

- Potential problem:
  - Check for leaks
  - Erratic or no fluid draws up from SIP
  - Erratic or no fluid draws up from fluidics tanks
- Part numbers:
  - 1 ml syringe Cat. No. 100022591
- Replacing Frequency: As needed, but **at least bi-annually**

How to change the sample syringe?

- After running Shutdown, open the Syringe Pump door, located on the left side of the cytometer.
- Loosen the knurled thumbscrew below ball end of syringe.
- Unscrew top portion of syringe from valve head.
- Remove syringe from ball end, pull out and replace with new syringe.
- Tighten the syringe 1/4 turn past initial contact of the Teflon insert into the valve for a liquid seal.
- Properly seat the ball end of the syringe. Tighten the knurled thumbscrew below the ball end.
- Prime the syringe by running 3X Startups, 2X Debubble and 2X Rinse

Notes: No tools should be used to tighten the syringe to the valve.



Attune ™ NxT Maintenance Kit

This convenient kit includes sufficient supplies to perform all recommended preventative maintenance procedures for 12 months:

- 1 qty Waste Tank for Attune™ NxT Cytometer
- 1 qty Focusing Fluid Tank for Attune™ NxT Cytometer
- 12 qty Focusing Fluid Filters
- 1 qty SIP Tube
- 2 qty Attune™ NxT Sample Syringe
- 1 qty Debubble Solution

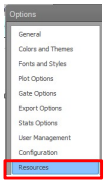
Cat. No.: A43038

Attune™ NxT Autosampler calibration

The Auto Sampler Calibration function sets the plate tray position to ensure that the probe consistently measures from the same spot in each well.

- The Attune™ NxT Auto Sampler is pre-calibrated before the unit is shipped and the instrument auto re-calibrates every 30 days.
- After running Startup, on the Instrument ribbon, click "Calibrate Auto Sampler"

- The Attune™ NxT Auto Sampler calibration operation takes approximately 1 minute to complete
- Last calibration date might be visualized from "Resources" window of option menu



Maintenance - Informatics

- **Administrator**
  - Set up user accounts with Operator privileges
  - Check space on D drive (at least 50 GB free)
  - Back up Experiments and/or Database to secondary storage
  - Virus protection – scan thumb drives before connecting to Attune™ NxT computer
  - Network connection (optional)

- **Operator/User**
  - Set up Security Questions in case the User password is forgotten.
  - Minimize file size, de-select the parameters not needed
  - Do not clutter the Experiment browser:
    - Collapse all experiments not currently active
    - Export & delete experiments from the browser
  - Virus protection – scan thumb drives before connecting to Attune™ NxT computer

Notes: Keep Default Windows access rights

Compatibility with antivirus software

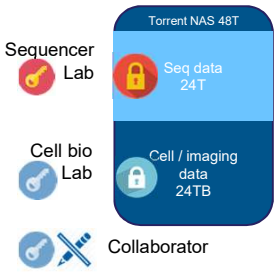
- Symantec Endpoint Protection antivirus software v12 and above is validated to be used on the computer monitoring the Attune™ NxT Cytometer
- Other antivirus software may be used at the discretion of the user. However these have not been tested with Attune™ NxT Software to ensure compatibility.
- **Do not** run the antivirus software while the Attune™ NxT Cytometer is acquiring data
- Set virus scans to run at a time when the instrument will not be in use

DataSafe

Storing data from any instrument

Replication and disaster recovery (DR)

Collaboration and Privacy



Recommended for labs who have **multiple data types**, **data backup needs**, and **collaboration** needs

Networking and Smart Monitor

- **Networking:** Connection of the Attune™ NxT system to your local network is recommended for easy data transfer, FCS file backup to a network folder, and connection to our Remote Monitoring and Diagnostics (included in Service Contract).

- **Smart Monitor:** A real-time remote instrument monitoring service that provides feedback on instrument parts (pumps, PZT, valves, USB and system logs).
  - Proactive problem detection for decreased instrument downtime
  - Decreased time for troubleshooting and repair
  - Remote control and desktop collaborative problem resolution (*only with customer permission at time of troubleshooting)
  - Manage software/firmware version control
  - Confirm the problem remotely
  - File transfers to/from instrument

Attune™ NxT Maintenance Summary

For Cytometer and Autosampler

Procedure	Frequency
Startup and Shutdown	Daily
Visual inspection	Daily
Performance test	Daily
Optical filter and mirror inspection	As needed
Fluidics decontamination	Every 3-6 months
Change focusing fluid filters	Every 3-6 months
Syringe replacement	At least Bi-annually
Sanitize SIP	Between each experiment
Deep Clean	As needed
Power-cycle instrument	Weekly
Calibrate Attune™ NxT Autosampler	As needed
Computer maintenance	Monthly

Notes: The frequency of maintenance depends on how often you run (or do not run) the instrument

Miscellaneous

Uninterrupted Power Supplies

- We recommend the use of a 1.5-kVA uninterruptible power supply (UPS), especially in areas prone to Power failure.

Anti-Virus software

- Disable or deactivate antivirus software and antispyware during use of the Attune™ NxT Acoustic Focusing Cytometer.
- Antivirus and antispyware monitoring can interfere with data collection, resulting in data loss.

Changing the location of the Attune™ NxT (EMEA)

Bench space needed: W x H x D

- Width: 127 cm
  - 58.2 cm for Attune™ NxT cytometer
  - 9.5 cm for access to side syringe compartment
  - 57.2 cm for Computer system
- Height: 74 cm to allow the hinged lid to fully open
- Depth: 58.5 cm
  - 23.1 cm for Attune™ NxT cytometer
  - 6.5 cm for adequate ventilation behind the instrument
  - 10.2 cm for the fluidics bottles in front of the unit

IMPORTANT NOTE: Unplug cables before you move the instrument. Please feel free to get in contact with your local FSE or FAS for further instructions.

Service Maintenance

- Check for leaks
- Check fittings and valves
- Confirm functionality of vents
- Run System Decontamination
- Run System Test
- Check pinhole, laser and blocker bar alignment
- Run baseline and performance test
- Software Upgrade
- Computer Maintenance

- Replacement of:
  - Focusing Fluid Filters
  - Syringes (Attune™ NxT cytometer + AAS)
  - Sample probe (SIP)

- Cleaning of:
  - Interior and Exterior of unit
  - Inspection of optical filters (cleaning only if necessary)
  - Tube lifter

Helps ensure maximal performance

Attune™ NxT Service Plans

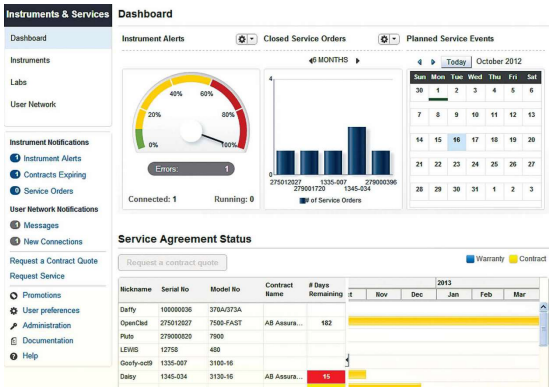
√ Included O Option	AB™ Complete	AB™ Assurance	AB™ Maintenance
Planned maintenance	√	√*	√
On-site service--Labor	√	√	
On-site service--Parts	√	√	
On-site service--Travel	√	√	
Remote instrument monitoring diagnostics	√	√	
Telephone Support (within 3 hours)	√	√	√
Application technical support	√	√	√
On-site application consulting	√		
Qualification service	√	O	O
Computer System Validation	O	O	O
On-site response time	Guaranteed next business day	Guaranteed 3 business days	Guaranteed 3 business days**

* Available with 1 or 2 pm/year
** After purchase order has been received by ThermoFisher Scientific

A service plan from ThermoFisher Scientific can help you:

- Maximize productivity
- Optimize your laboratory's efficiency
- Lower the cost of ownership
- Obtain unmatched availability of critical laboratory systems
- Increase quality
- Lower costs by minimizing lost data, samples, or reagents

Instrument Management



Online tool to manage the use and care of all your instruments.

- Get instant access to complete service histories
- Track service contract and warranty expiration dates
- Check availability and schedule time on your instruments
- See all serial numbers as wells as software version and computer details

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Best Practices and Troubleshooting

Known Issues related to Sample Acquisition

- Running startup after running a shutdown doesn't always reset the startup icon in the collection panel to run/record
  - Run a Rinse by pressing “Rinse” from Instrument tab or Repeat Startup
- When creating experiments, the software doesn't check to see if it there is enough disk space to create the necessary files.
  - Check space available on D drive
  - Export experiment data from the experiment explorer and then remove experiments to free up disk space
- Adding more than 400 samples to an experiment can cause software instability.
  - If an experiment requires more than 400 samples, duplicate the experiment for additional samples beyond 400.

Known Issues related to Sample Acquisition

- A plate experiment isn’t automatically active after it is created.
  - Double click on the new experiment to activate it, create samples or groups of samples on heat map tab.
- Syringe Pump Error – Step Loss Plunger error observed when starting a plate experiment immediately after Performance Test.
  - After Performance Test perform a SIP sanitize. If error is observed, follow instructions in dialog.

Known Issues related to Data Analysis

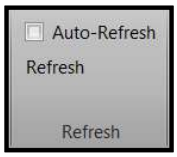
- Quadrant gate names can't be moved
- When both X and Y axis are set to HyperLog™ and the X axis scale is set to 'manual' scale, any changes to 'manual' or 'automatic' setting of the Y axis will revert the X axis range to default settings (Min: 1, Max: 1048575).
  - If a manual range for the X axis is needed for hyperlog, set Y axis first (manual or automatic setting, adjusting range as needed for manual setting), then adjust manual setting for X axis range.
- The width parameter scale range will default to 1,048,576.
  - Set the maximum scale range to 1024 for ease of viewing

Known Issues related to Data Analysis

- No warning is given if attempting to overlay FCS files that were acquired using different instrument settings
- Gallery plots cannot be printed at this time
- When printing overlay plots, ensure the Overlay view's zoom setting is less than 400% otherwise the plots may be too big to print on a page
- Avoid Automatic scale for overlay plots

Best Practices for Sample Acquisition

- If running samples at very high event rates or collecting very large amount of data:
  - Set all plots on the workspace to manual scaling
  - Deselect “Auto-Refresh” option from the Home Tab
  - Limit plots and workspace complexity
  - Select only the channels that are needed for experiment
  - Wait to make adjustments on the workspace until after the file has completed.



- At the end of sample recording, lower the tube lifter to initiate a Rinse and to avoid sample staying in the loop
- When Stop Criteria are based on *Time* or *Volume*, make sure sample volume drawn in the system is enough if you press Run before starting the recording, as the “complete stop condition” option will not work.

Best Practices for using Autosampler

- Keep the tube lifter in the DOWN position when using the Auto-sampler
- Setup *Total volume* lower than actual volume expected to be in the well to minimize the introduction of air bubbles in the plate due to pipetting/dispensing errors
- Depending on sample viscosity, it might be suggested to limit the number of mixes to 2 or less to prevent bubbles being introduced into the sample

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Attune™ NxT Cytometer
"Tune-up your knowledge"

Revision 2.5
Revision Date: Aug2019

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1. What should you do if you see no events displayed on the plots?

A. Adjust the Voltages and Thresholds accordingly
B. Verify that you are using correct filters configuration
C. Confirm cell concentration is sufficient
D. Run Performance Tracking Beads as a sample using PMT voltages from the last Performance Test

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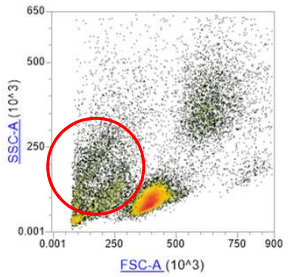
2. What should you do if you see a large number of events with low FSC and Low SSC together with your cells of interest?



A. Increase FSC and SSC voltages
B. Increase FSC and/or SSC thresholds
C. Decontaminate your system
D. Run performance test

3. What should you do if no positive fluorescence signal is detected?

A. Repeat the whole experiment with fresh solutions
B. Confirm correct parameters are selected
C. Adjust voltages
D. Check Optical Configuration is properly setup in the software

4. Dead cells have different scatters properties than live cells

❑ TRUE

❑ FALSE

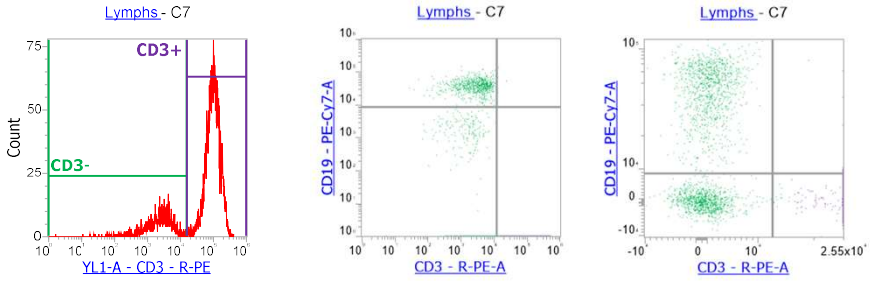
5. What to do if aggregates or coincident events are detected?

A. Run Unclog script
B. Dilute your sample and run with higher flow rate
C. Decontaminate your system and filter Attune solutions
D. Filter your sample and rerun

6. How to diagnose a clog?

A. High event rate
B. Low event rate
C. Sample is automatically backflushed into the tube
D. The software reports an error
E. Look at Time vs Fluorescence or Scatter Density Plot

7. You do not see your positive population on a dual parameter plot while it's present on an histogram plot. What do you do?



A. You analyze all parameters on histograms only
B. You change the scale on your dual plot parametric
C. You check compensation is properly calculated
D. You repeat the experiment with fresh solutions

8. You noticed carry over between samples while using the AAS. How can you prevent that?

A. You change the flow rate
B. You record more events
C. You wait 2 seconds before recording
D. You increase the number of rinse cycles between 2 wells
E. You increase the number of mixing cycles

9. You have a low number of event using the AAS. The sample runs correctly in a tube. What do you do?

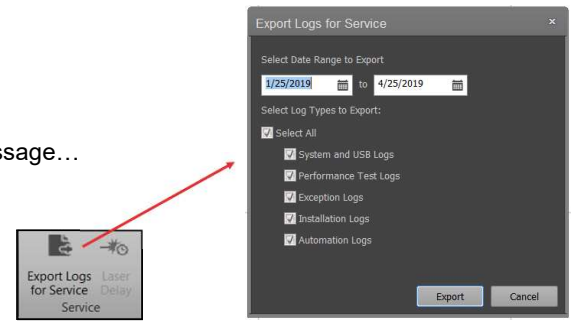
A. You check the acquisition volume is properly setup
B. You check the total sample volume is properly setup
C. You check your plate type is validated and properly setup
D. You increase the number of rinse cycles between 2 wells
E. You increase the number of mixing cycles

10. What should you do if the Performance Test fails?

A. Switch OFF the instrument, and call it a day
B. Repeat the Performance test with freshly prepared beads
C. Follow the instruction provided by software interface
D. Run a Deep Clean/Debubble script and repeat the performance test with freshly prepared beads
E. Contact Technical Support or Service Admin

Information to provide when contacting Thermo Fisher Scientific Technical Support

- Your Contact Information
  - Name, Institute, Email address, Phone number
- Instrument Information
  - Optical configuration, w/wo Auto sampler
  - Serial Number
- Issue Information
  - What is the issue?
  - When does it occur?
  - Experiment Files (.atx or .apx), Screen shots of error message…
  - Log Files from *Instrument* Tab selecting Date Range at which the issue occurs (a .zip file will be saved to the selected location)



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Resources

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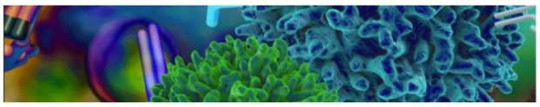
Web Technical Resources

Flow Cytometry Learning Center



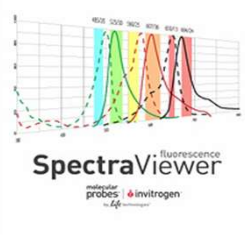
Learn about flow cytometry methods and technologies
www.thermofisher.com/flowlearning

www.thermofisher.com/antibodies

Molecular Probes™ Handbook
www.thermofisher.com/handbook

Flow Cytometry Panel Builder

https://www.thermofisher.com/order/panel-builder



www.thermofisher.com/spectraviewer

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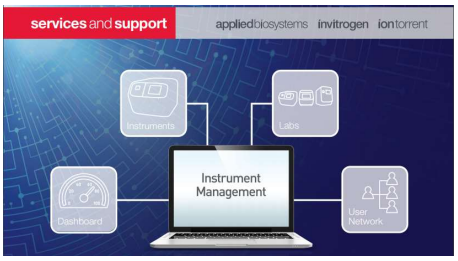
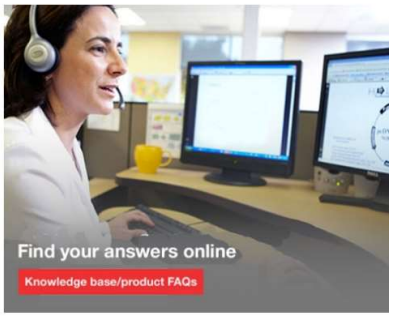
Service and Support Web Resources

Instrument Management



www.thermofisher.com/easiertomanage

Services & Support



www.thermofisher.com/support

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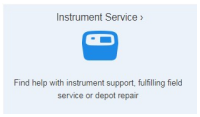
Contact Us

- Technical Support
  By phone: 00 800 5345 5345
  By email: eurotech@thermofisher.com
  Web site: www.thermofisher.com/contactus

- Instrument Service
  By phone: 00 800 5345 5345
  Check for your specific country details
  Web site: www.thermofisher.com/contactus



- Customer Service
  By phone: Check for your specific country details
  Web site: www.thermofisher.com/contactus

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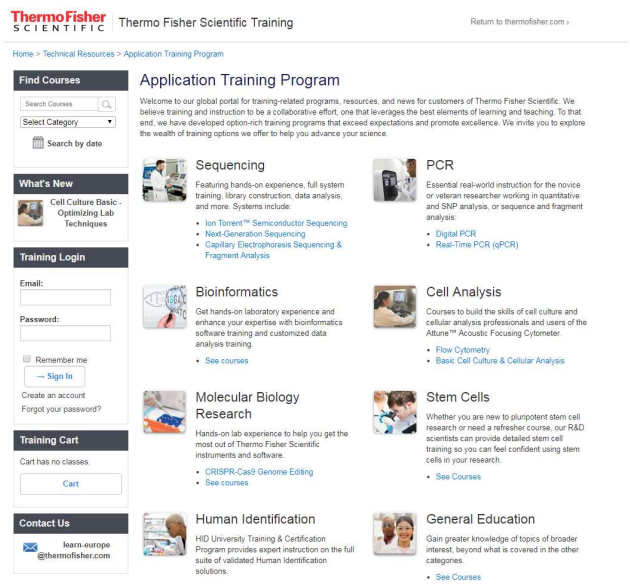
Applications training courses and workshops



Increase your working knowledge of applications and instrument operation, including hands-on lab experience

https://learn.thermofisher.com/europe/

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Flow Cytometry Application and Instrument Training Courses

Flow Cytometry

Our interactive and laboratory instruction courses deliver flexible, hands-on training, from refresher to in-depth, to help meet the needs of cellular biology professionals and users of the Attune™ Acoustic Focusing Cytometer.

Available in our Customer Experience Centers or in Your Laboratory

- **Attune™ NxT Operational Training** (2 days) - Interactive course focusing on basic operation of the Attune NxT Acoustic Focusing Cytometer.
- **Microbiology and Small Particle Analysis** (1 day) – Interactive course covering the analysis of small particles and microbiological samples.
- **Multiparametric Analysis** (1 or 2 days) – Interactive course covering multiparametric analysis such as immunophenotyping.

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Legal and Regulatory Statements

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

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Attune™ NxT

Flow Cytometer

Basic Training

Exercises and Appendix

Exercise 1 - Performance Test

This preliminary exercise is part of the daily maintenance of the Attune™ NxT Cytometer. When conducted daily, this test monitors the performance and helps ensure the instrument's accuracy and sensitivity.

Objective: Conduct performance tracking and review reports.

Materials:
- Diluent: Attune™ Focusing Fluid
- Attune Performance Tracking Beads (Cat. No. 4449754)

Prepare beads:
1. Beads are sticky. Thoroughly vortex the vial of performance beads.
2. Prepare a flow tube with 2 ml of Attune™ Focusing Fluid and add 3 drops of performance tracking beads. Vortex thoroughly.
3. Use immediately or protect from light and use within 4 hrs.

Procedure:

1. Power on instrument, then launch the software.

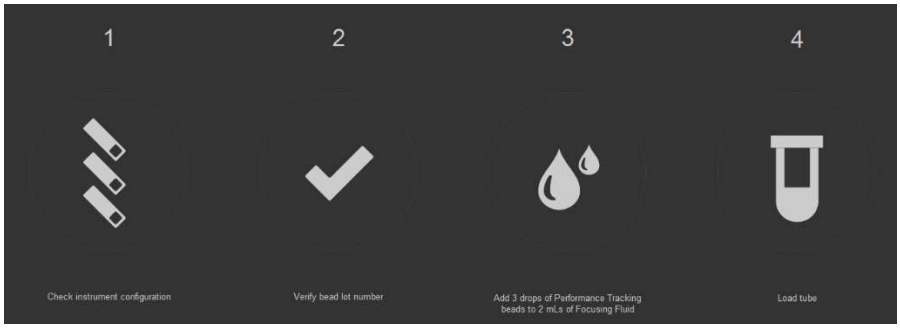
2. Log in with your username and password

   Note: If you did not yet create your own account, ask your administrator for setting up

3. The Main Menu page opens:

4. Click on: 

The Performance Test Setup opens.



5. Startup the Instrument by clicking on:

   Alternatively, you may open the Instrument Tab and choose:

6. Setup the Performance Test by following the instructions on the screen.

   Note: The Lot Number should be checked every time a new tube of beads is ordered. **The Alpha character is not part of the Lot number**. It's an indication of when the product has been packaged and will have a different expiration date.

7. Click **Run Performance Test.** It will take few minutes.

8. Once complete, the Daily Performance Report is displayed. Review the report.

9. Clean the Attune NxT by using the function available in the *Instrument* Tab, followed by a Rinse

Exercise 2 – Experiment Setup and Data Acquisition
Single Color Experiment

Objective:

- Create a new Tube experiment.
- Set up the *Workspace* for the new experiment.
- Use the *Collection Panel* to run a sample.
- Use the *Instrument Settings* panel to adjust thresholds and PMT voltages.
- Set a gate on a population of interest.

EXERCISE A: Use green fluorescent beads to perform basic software functions.

Materials provided:

- Cell Sorting Set-up Beads for Blue Lasers (Cat. No. C16508)
- Negative Beads from AbC™ Total bead kit (Cat. No. A10513)
- PBS

Sample Preparation:

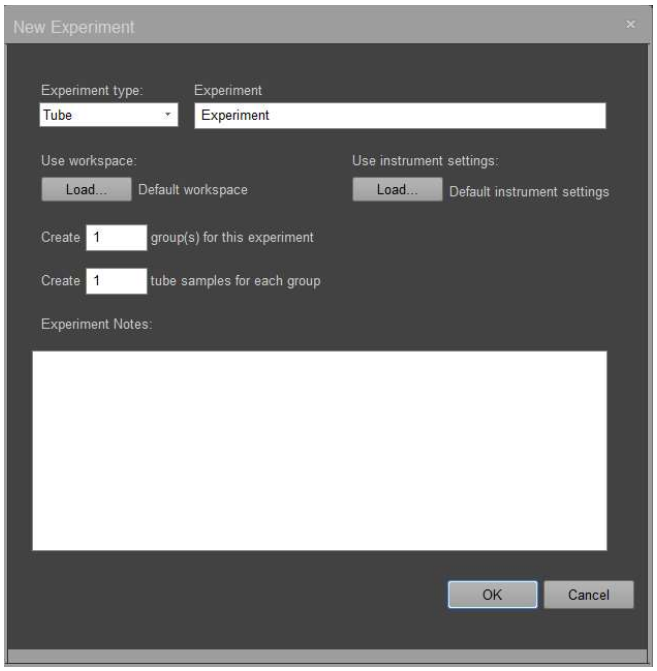
1. Add 1 ml of PBS to each of 2 tubes.
2. Add 1 drop of Negative beads in tube #1.
3. Add 1 drop of Negative beads + 1 drop of Cell Sorting beads to tube #2.
4. Vortex.

Procedure:

Create a New Experiment

1. On the *Main Menu,* click on 

and the *New Experiment* window opens.



2. Create the experiment as follows:
    a. Experiment Type: Tube
    b. Experiment name: **date - your name – 1 color bead**
    c. Use Default Workspace and Default Instrument Settings.
    d. Include 1 tube group with 2 samples and click **OK.**

3. Rename the samples “negative beads” and “green beads” respectively.

Create a Workspace

1. In the *Instrument Settings/Parameters* Panel, deselect parameters and channels not needed.

2. Use the *Workspace* tab to populate the *Experiment Workspace* as follows:

- Dot Plot FSC-A (linear) vs. SSC-A (linear)
- Histogram BL1-A (log)
- Default Statistic table - Global

Experiment optimization – Adjusting PMT Voltages, Setting Threshold, Setting Gates

1. In *Collection Panel*, set the Run Protocol as follows:
   - Acquisition volume : 200 µl
   - Sample Flow Rate: 25 µl/min
2. Load tube 1 (negative beads only) onto the SIP, click: 

3. In the *Instrument Settings/Voltages* panel, adjust FSC and SSC voltages to properly discriminate beads from background.
4. In the *Instrument Settings/Threshold* panel, adjust Threshold to remove debris from FSC vs. SSC plot.
5. Adjust BL1 PMT voltage to position the negative bead population peak ~10^3 MFI.

   Click 

6. Remove tube 1 and load tube 2 onto the SIP. Click Run.
   Two peaks should be displayed on the BL1 histogram. If not, adjust the BL1 voltage. Click *Stop.*
   Notes: Instrument settings are now optimized. Remove tube 2
7. Optional: clean the Attune by using the function 

available in the *Instrument* Tab, followed by a Rinse
8. Record data for both samples using the following Run Protocol:
   - Acquisition volume : 300 µL
   - Sample Flow Rate: 200 µL/min
   - Stop Options: 20,000 on All events

Data Analysis

1. In the “Green beads” sample, use the *Workspace* tab to create a gate around the bead population on FSC vs. SSC dot plot, and rename “beads”.

2. Apply beads gate to BL1 Histogram.

3. Create a gate around green beads and rename “green beads”.

4. Identify % of green beads.

5. Export the Experiment to the hardrive, then transfer to a USB driver.

EXERCISE B – Tube mode: Use single-stained cells to perform basic software functions.

Materials provided:

- Coulter™ IMMUNO-TROL™ Control Cells (Fisher Beckman Coulter Cat. No. CO6607077) – BSL2 sample
- Mouse Anti-Human CD45 Alexa Fluor™ 488 Antibody
- High-Yield Lyse solution (Cat. No. HYL250)

Sample Preparation:

1. In tubes 1 and 2, add 100 µl of IMMUNO-TROL cells to each.
2. Add 5 µl of antibody (Ab) to tube 2.
3. Incubate 15 minutes in the dark at room temperature (RT).
4. After incubation, add 2 mL of High Yield Lyse solution and mix.
5. Incubate for 10 min in the dark at RT, mix after 5 minutes.

Procedure:

Create a New Experiment

1. Create the experiment as follows:
    a. Experiment Type: Tube
    b. Experiment name: **date - your name – 1 color cells**
    c. Use Default Workspace and Default Instrument Settings.
    d. Include 1 tube group with 2 samples.

2. Rename the samples “unstained cells” and “stained cells” respectively.

Create a Workspace

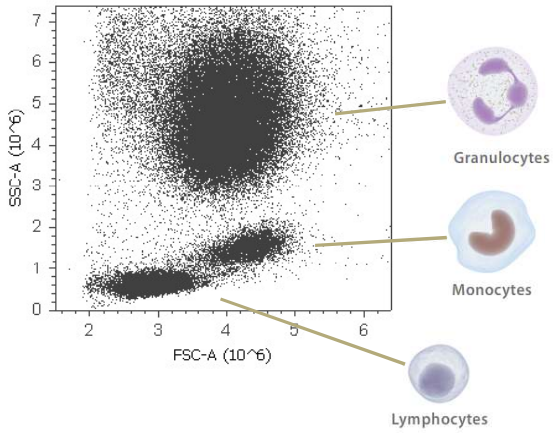
1. In *Instrument Settings* Panel, deselect parameters and channels not needed and enter Target and Label name for selected channel.

2. The *Experiment Workspace* should contain:
    - Precedence Density Plot: FSC-A (lin) vs. SSC-A (lin)
    - Density Plot: CD45 AF448-A (log) vs. SSC-A (lin)
    - Histogram: CD45 AF488-A (log)
    - Default Statistic table - Global

Experiment Optimization – Adjusting PMT Voltages, Setting Threshold, Setting Gates

1. In *Collection Panel*, set the Run protocol as follows:
    a. Acquisition volume : 300 µl
    b. Sample Flow Rate: 25 µl/min

2. Using unstained cells, adjust Scatters voltages to properly position populations on the Scatters plot as follows:



Tip: you can use a quadrant gate, display % of event in each square, to facilitate scatters voltages adjustments

3. Adjust Threshold to remove debris from Scatters plot.

4. Adjust BL1 PMT voltage to position the unstained cells around 10^3 MFI.

5. Create a gate around cells and rename “Cells”.

Alternative Experiment Optimization: If adjusting the FSC and SSC voltages proves difficult using unstained cells, single stained cells may alternatively be used.

1. Adjust BL1 PMT voltage to discriminate stained from unstained cells.

2. Create a histogram gate around the positive population and rename “stained cells”.

3. Back gate the “stained cells” population on the Scatters plot.

4. Adjust FSC and SSC PMT voltages to get all “back gated” events on the plot.

Sample Recording:

1. Optional: Once Instrument Settings are properly adjusted, Sanitize the Attune SIP.

2. Record the samples using the following Run Protocol:
    a. Acquisition volume: 300 µl
    b. Sample Flow Rate: 200 µl/min
    c. Stop Options: 30.000 on Cells

Data Analysis

1. Update the workspace to identify % of lymphocytes based on CD45 expression level (CD45High/SSCLow).

2. Explore differences in Plot types (DotPlot, DensityPlot, PrecedenceDensity).

3. Play with *Statistics* tab to change values on Plots and Statistic table.

4. Export the Experiment to a USB driver.

EXERCISE B – Plate mode: Use single-stained cells to perform basic software functions.

Materials provided:

- Coulter IMMUNO-TROL Control Cells (Fisher Beckman Coulter Cat. No CO6607077) – BSL2 sample
- Mouse Anti-Human CD45 Alexa Fluor 488 Antibody
- High-Yield Lyse solution (Cat. No. HYL-250)

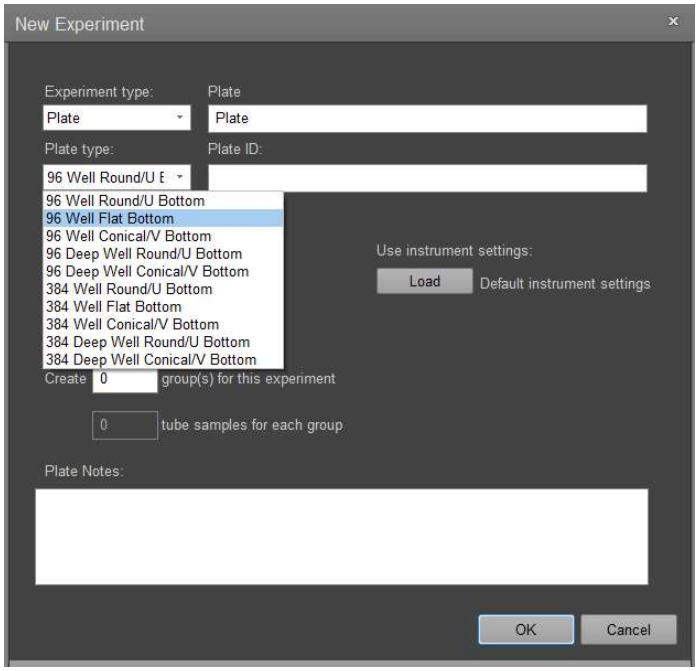
Sample Preparation:

1. In tubes 1 and 2, add 100 µl of IMMUNO-TROL cells to each
2. Add 5 µl of antibody (Ab) to tube 2
3. Incubate 15 minutes in the dark at room temperature (RT)
4. After incubation, add 2 mL of High Yield Lyse solution and mix
5. Incubate for 10 min in the dark, mix after 5 minutes
6. In a 96-well plate, load:
    a. 250µL of sample 1 in well A1 to A4
    b. 250µL of sample 2 in well A5 to A7

Procedure:

Create a New Experiment

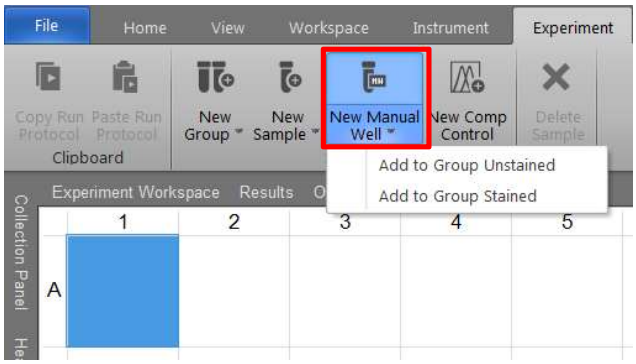
1. Create the experiment as follows:
    a. Experiment Type: Plate
    b. Select the correct plate type in the drop-down list
    c. Experiment name: **date - your name – 1 color cells**
    d. Use Default Workspace and Default Instrument Settings
    e. Include 2 groups



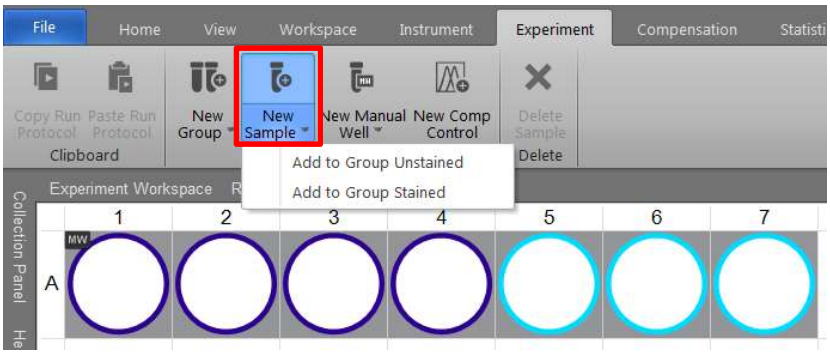
2. Open the Plate experiment

3. Rename groups as “Unstained” and “Stained”

4. In the *Experiment* Tab, set A1 as a Manual Well in “Unstained” group. This well will be used to adjust PMT voltages



5. Set A2 to A4 as new samples within “Unstained” group and A5 to A7 as new samples within “Stained” group



<u>Create a Workspace</u>

1. In *Instrument Settings* Panel, deselect parameters and channels not needed and enter Target and Label name for selected channel.

2. Open a sample well and update the Experiment Workspace as follows:
    a. Precedence Density Plot: FSC-A (lin) vs. SSC-A (lin)
    b. Density Plot: CD45 AF448-A (log) vs. SSC-A (lin)
    c. Histogram: CD45 AF488-A (log)
    d. Default Statistic table - Global

Alternatively, you can drag and drop the workspace (WS) created for the previous experiment.

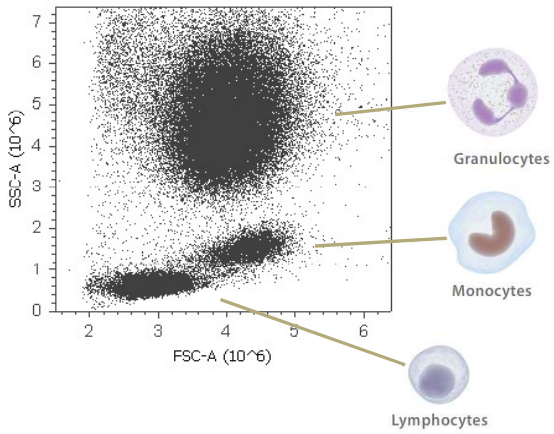
<u>Experiment Optimization</u> **–** Adjusting PMT Voltages, Setting Threshold, Setting Gates

Experiment Optimization can be done in tube or in plate. This procedure will show how to adjust PMT voltages and thresholds in plate mode.

1. Open the Manual Well (A1)

2. In *Collection Panel*, set the Run protocol as follows:
    a. Acquisition volume : 200 µl
    b. Total Sample volume : 230µl
    c. Sample Flow Rate: 25 µl/min
    d. 1 mix and 1 rinse

3. Click Run and adjust Scatters voltages to properly position populations on the Scatters plot as follows:



Tip: you can use a quadrant gate, display % of event in each square, to facilitate scatters voltages adjustments

4. Adjust Threshold to remove debris from Scatters plot.

5. Adjust BL1 PMT voltage to position the unstained cells around 10^3 MFI.

6. Create a gate around cells and rename “Cells”.

Alternative Experiment Optimization: If adjusting the FSC and SSC voltages proves difficult using unstained cells, single stained cells may alternatively be used.

1. Adjust BL1 PMT voltage to discriminate stained from unstained cells.

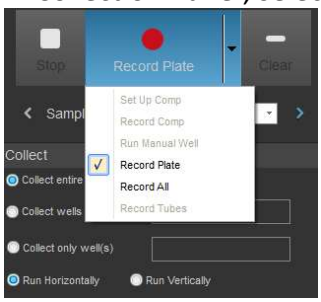
2. Create a histogram gate around the positive population and rename “stained cells”.

3. Back gate the “stained cells” population on the Scatters plot.

4. Adjust FSC and SSC PMT voltages to get all “back gated” events on the plot.

Sample Recording:

1. In *Collection Panel*, select Record Plate



2. Setup Run Protocol as follows:
    a. Collect entire plate from the beginning
    b. Acquisition volume : 200µL
    c. Total Sample: 230µL
    d. Flow rate: 200µL/min
    e. Stop after 30.000 Cells
    f. 1 mix and 1 rinse
    g. Apply to experiment

3. Record the plate

Data Analysis

1. Update the workspace to identify % of lymphocytes based on CD45 expression level (CD45High/SSCLow)

2. Explore differences in Plot types (DotPlot, DensityPlot, PrecedenceDensity)

3. Play with *Statistics* tab to change values on Plots and Statistic table

4. Explore *HeatMap Analysis* using Threshold on % Lymphocytes

5. Export the Experiment (export "plate") to the hardrive, then transfer to a USB driver

Exercise 3 – Multicolor Acquisition and Compensation
4 Colors Experiment

Multicolor experiments often require compensation to remove the spectral overlap of fluorochrome emissions into non-targeted detectors. This exercise is designed to demonstrate a 4 colors experiment using fluorescently labeled beads for compensation.

Objective:

- Create a new experiment with compensation.
- Set up the Compensation.
- Validate Compensation.
- Setup a plate experiment using the auto-sampler (optional)

Background information:

All normal cells express a variety of cell surface markers, dependent on the specific cell type and degree of maturation. However, when cells are growing abnormally, the natural expression of these antigens may be either overly expressed or under-expressed. In this experiment, normal human blood will be analyzed for its immunophenotype to identify white blood cells (WBC), monocytes and T and B lymphocyte subsets. The antigens that will be detected in this stain are CD3, CD19, CD45 and CD14:

- CD3 is a marker that labels the T cell lymphocyte subset
- CD19 is a marker that labels the B cell lymphocyte subset
- CD45 is a pan WBC marker and labels all normal white blood cells
- CD14 is a marker that labels monocytes

Materials provided:

- Coulter™ IMMUNO-TROL™ Control Cells (Fisher Beckman Coulter Cat. No CO6607077) – BSL2 sample
- AbC™ Total bead kit (Cat. No. A10513)
- Mouse Anti-Human CD45 Alexa Fluor™ (AF) 488 Antibody
- Mouse Anti-Human CD3 R-PE Antibody
- Mouse Anti-Human CD19 PE-Cy®7 Antibody
- Mouse Anti-Human CD14 APC Antibody
- High-Yield Lyse solution (Cat. No. HYL-250)
- PBS
- 12 x 75 mm tubes
- Optional: 96-well plate

EXERCISE A - Tube mode

Sample Preparation:

1. Prepare Samples according to the staining chart below; ensure you mix the bead and Coulter IMMUNO-TROL samples prior to pipetting.

Sample		Sample volume	CD45 AF488	CD3 PE	CD19 PE-Cy7	CD14 APC	Post incubation add	AbC Bead negative **Add immediately prior to running**
Detector used if Yellow laser present								
Detector used if Yellow laser absent								
1	AbC Bead capture						3 mL PBS	2 drops
2	AbC Bead capture	2 drops	1 µL				3 mL PBS	2 drops
3	AbC Bead capture	2 drops		1 µL			3 mL PBS	2 drops
4	AbC Bead capture	2 drops			1 µL		3 mL PBS	2 drops
5	AbC Bead capture	2 drops				1 µL	3 mL PBS	2 drops
6	IMMUNO-TROL Cells	100 µL					2 mL HYL	
7	IMMUNO-TROL Cells	100 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	2 mL HYL	

Notes: Compensation auto fluorescence setup will use Unstained Beads.
If no red laser available, do not include CD14-APC.

2. Incubate samples with Antibody conjugates for 20 minutes in the dark at room temperature (RT) for all samples.

3. After incubation, add 2 mL of High Yield Lyse to IMMUNO-TROL Cells and mix (tubes 6 and 7). Incubate for 10 min protected from light at RT, mix after 5 minutes.

4. Add 3 mL PBS to the AbC Bead capture mix (tubes 1 to 5).

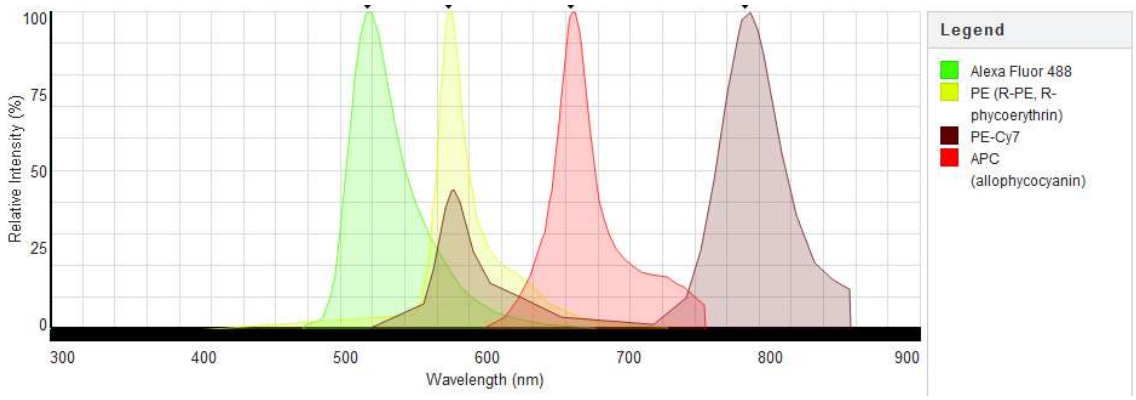
5. Add the AbC Bead negative prior to instrument set up (tubes 1 to 5).

Procedure:

Create a New Experiment

1. In the staining chart above, indicate the channel used to detect each antibody conjugate.

SpectraViewer

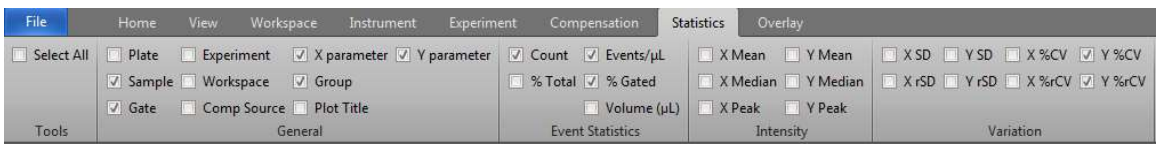


2. Create the experiment as follows:
   - Experiment type: tube
   - File name: **Date – Name – 4 colors Compensation**
   - Default Workspace and Default Instrument Settings
   - 1 group – 2 samples
   - Rename group- "Lysed Whole Blood"
   - Rename samples - "Unstained cells" and "Stained cells"

3. Deselect parameters and channels not needed and enter Target and Label name.

4. The Experiment Workspace should contain:
   - Scatters Precedence Density Plot
   - CD45 vs. SSC Density Plot
   - CD3 vs. CD19 Dot Plot
   - CD14 vs. CD3 Dot Plot
   - CD14 vs. CD19 Dot Plot
   - Global Statistics including Median X and Y

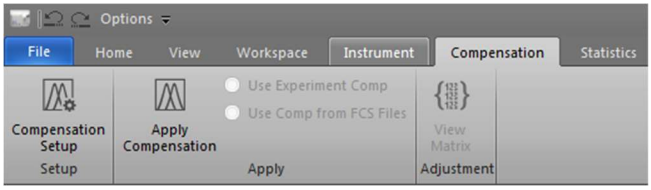
     Open the *Statistics* tab and select parameters needed.



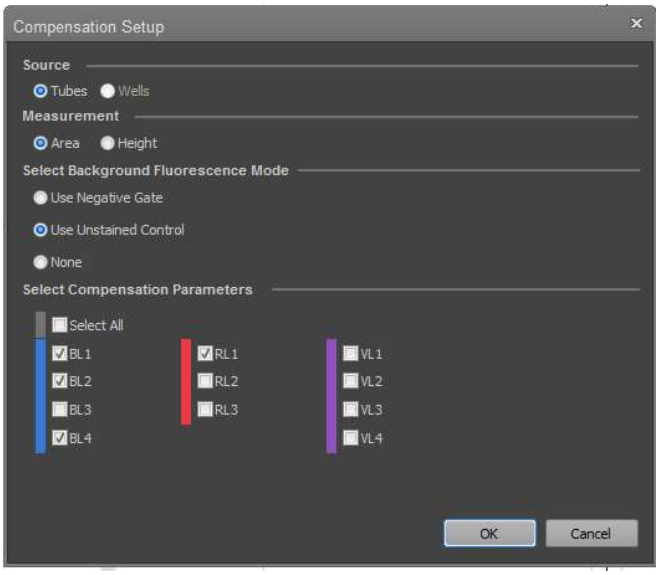
Experiment Optimization and Compensation Set Up

1. Set up compensation criteria

    a) In the *Compensation* tab, choose *Compensation Setup*.



    b) Set compensation measurement to Area, set Background Fluorescence mode to Unstained Control, and verify all parameters that are needed for the experiment are selected (as indicated below).



    Notes: the UC compensation control tube will automatically open

2. Optimize Instrument Settings

    a) Using unstained cells (Tube 6), adjust PMTs voltages for FSC, SSC, and all fluorescent parameters, and threshold using the following Run Protocol:
        - Acquisition volume : 200 µl
        - Sample Flow Rate: 25 µl/min

    b) Document the optimal FSC and SSC Settings:
        Forward Scatter Voltage Setting for cells _____
        Side Scatter Voltage Setting for cells _____
        FSC Threshold _____

c) Verify fluorescent compensation controls are on scale (Tubes 2 to 5) and adjust detector voltages if needed to ensure all positive beads are on scale. If not on scale, make slight voltage adjustments to bring the population on scale.
Tip: use a 3 steps process for voltages settings
- Adjust positive population on plot
- Adjust negative population outside of background level
- Optimize distance between negative and positive peaks

Note: you will need to adjust FSC and SSC voltages to get the beads in the R1 gate. Once done, "Apply gate to all controls".

d) Sanitize the Attune SIP before recording Compensation controls.
Note: At this step you have not yet recorded any data.

3. Record compensation controls
Note: Be sure you have added the negative bead to the bead sample tubes 1 to 5

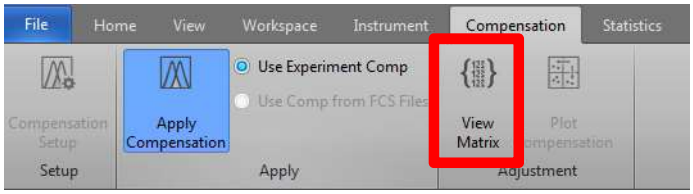
a) Using tubes 1 to 5 (Bead controls) record compensation using the following Run Protocol:
- Acquisition volume = 300µL
- Flow rate = 200µL/min
- Stop criteria: 5000 total events

Note: Check that the correct control is being measured.

b) After recording of each control tube, adjust the R2 gate around the positive bead population.

c) (*optional*) Sanitize the SIP before running cell samples.

5. Check compensation is properly calculated by viewing the Matrix in the *Compensation* tab.



<u>Sample acquisition</u>

1. Open the "Unstained cells" tube sample and Run the unstained cells (Tube 6).

2. Adjust the FSC and SSC voltages and Threshold as necessary to visualize cells on Scatter Plot (see values noted in step 2b under compensation set up).

3. Record the "Unstained cells" (tube 6) and "Stained cells" (tube 7) samples according to the following Run Protocol:
    - Acquisition volume: 300 µl
    - Sample Flow Rate: 200 µl/min
    - Stop criteria: 50.000 events total

4. Check that compensation has been properly calculated and adjust if needed.
   Note: To verify matrix values are correct, compare Median values between positive population and negative population using On-Plot Compensation tool in the *Compensation* Tab. Values should be in the same range.

Data Analysis

1. Open one of the "Stained cells" sample.

2. On the CD45 vs. SSC plot, create a gate around the lymphocytes and rename lymphs.

3. On the CD45 vs. SSC plot, create a gate around the monocytes and rename monos.

4. Set population on the CD3 vs. CD19 plot to lymphs.

5. Create and adjust quadrant gate on CD3 vs CD19 dot plot.

6. Create a new derived gate using lymphs OR monos and rename "L and M".

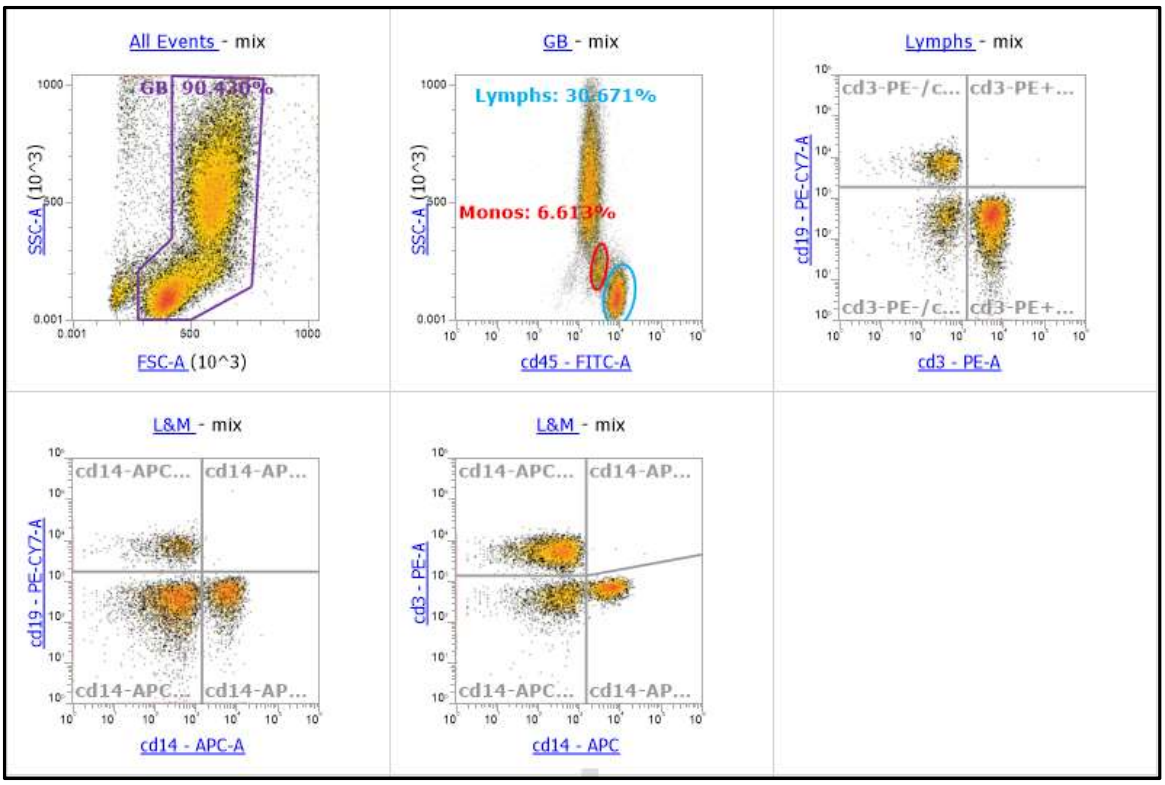
7. Open the Edit Gates dialog box. Select and move the "L and M" gate to the top of the list.

8. On the CD19 vs. CD14, and CD3 vs. CD14 dot plots set population to "L and M".

9. Create a rectangle gate or a quadrant gate so that you can identify the monocyte CD14 positive population.

10. Identify % of
    a. T lymphocytes in L and M population
    b. B lymphocytes in L and M population
    c. Monocytes in L and M population

11. Export the Experiment and transfer to a USB drive.



Post-acquisition Questions:

1. Which samples were used as the controls?
2. Were there any adjustments to the instrument settings when using beads and cells?
3. Why are the voltage settings locked after recording compensation?

EXERCISE B – Plate mode

Sample Preparation:

1. Prepare Samples according to the staining chart below; ensure you mix the bead and Coulter IMMUNO-TROL samples prior to pipetting.

Sample		Sample volume	CD45 AF488	CD3 PE	CD19 PE-Cy7	CD14 APC	Post incubation add	AbC Bead negative **Add immediately prior to running**
Detector used if Yellow laser present								
Detector used if Yellow laser absent								
1	AbC Bead capture						3 mL PBS	2 drops
2	AbC Bead capture	2 drops	1 µL				3 mL PBS	2 drops
3	AbC Bead capture	2 drops		1 µL			3 mL PBS	2 drops
4	AbC Bead capture	2 drops			1 µL		3 mL PBS	2 drops
5	AbC Bead capture	2 drops				1 µL	3 mL PBS	2 drops
6	IMMUNO-TROL Cells	100 µL					2 mL HYL	
7	IMMUNO-TROL Cells	100 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	2 mL HYL	

Notes: Compensation auto fluorescence setup will use Unstained Beads.
If no red laser available, do not include CD14-APC.

2. Incubate samples with Antibody conjugates for 20 minutes in the dark at room temperature (RT) for all samples

3. After incubation, add 2 mL of High Yield Lyse to IMMUNO-TROL Cells and mix (tubes 6 and 7). Incubate for 10 min protected from light, mix after 5 minutes

4. Add 1mL of PBS in Stained sample (tube 7)

5. Add 3 mL of PBS to the AbC Bead capture mix (tubes 1 to 5)

6. Add the AbC Bead negative prior to instrument set up (tubes 1 to 5)

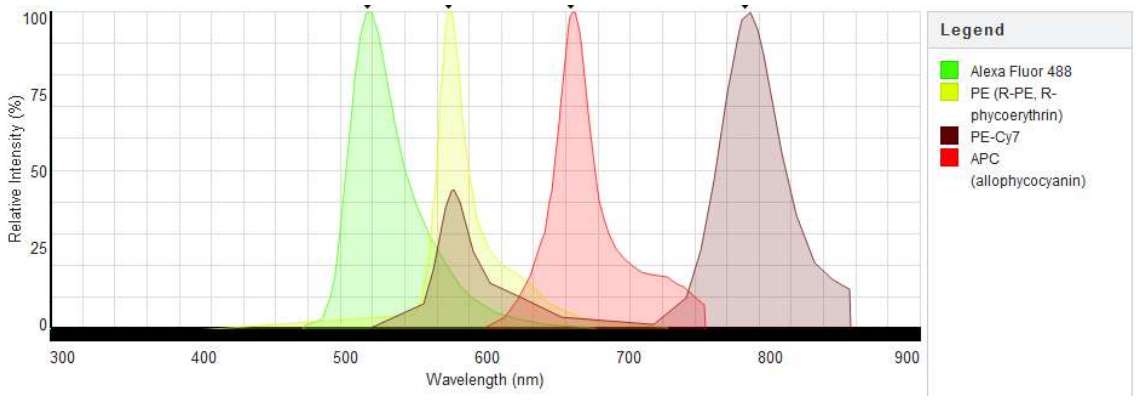
7. In a 96-well plate, load:
    a. 50µL of sample 7 + 150µl of PBS in wells B1 to B3 and C1 to C3
    b. 100µL of sample 7 + 100µl of PBS in wells B4 to B6 and C4 to C6
    c. 200µL of sample 7 in wells B7 to B9 and C7 to C9
    d. 200µl of PBS in wells B10 to B12 and C10 to C12

Procedure

Create a New Experiment

1. In the staining chart above, indicate the channel used to detect each antibody conjugate.

SpectraViewer



2. Create the experiment as follows:
   - Choose "Plate" in experiment type and choose the correct plate type in the drop-down list.
   - File name: **Date – Name – 4 colors Compensation**
   - Default Workspace and Default Instrument Settings
   - 2 groups

3. Open the Plate and rename the 2 groups "1 mix 1 rinse" and "2 mix 2 rinses" respectively

3. Assign wells Raw B to "1 mix 1 rinse" group and Raw C to "2 mix 2 rinses" group

4. Deselect parameters and channels not needed and enter Target and Label name. Alternatively, you may use Instrument Settings from Exercise 3A.

5. The Experiment Workspace should contain:
   a. CD45 vs. SSC Density Plot
   b. Scatters Precedence Density Plot
   c. CD3 vs. CD19 Dot Plot
   d. CD14 vs. CD3 Dot Plot
   e. CD14 vs. CD19 Dot Plot
   f. Global Statistics including Median X and Y
      Open the *Statistics* tab and select parameters needed

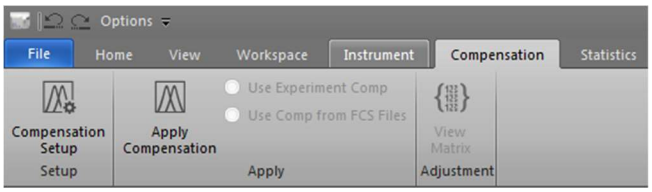
Alternatively, you may drag and drop the Experiment Workspace (WS) from Exercise 3A.

Experiment Optimization and Compensation Set Up

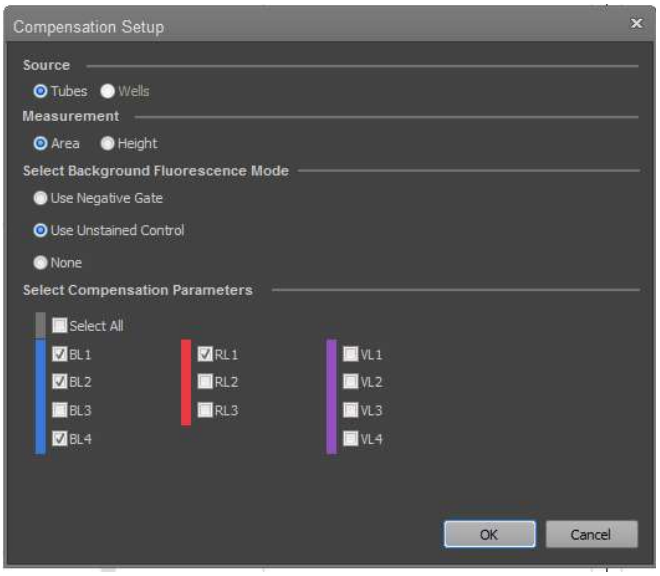
Experiment Optimization and Compensation can be done in tube or in plate. This procedure will show how to adjust PMT voltages and thresholds, and Setup Compensations in tube mode.
Alternatively, you may use Instrument Settings and Compensation Matrix from Exercise 3A.

1. Set up compensation criteria

    a. In the *Compensation* tab, choose *Compensation Setup*.



    b. Set compensation measurement to Area, set Background Fluorescence mode to Unstained Control, and verify all parameters that are needed for the experiment are selected (as indicated below).



Notes: the UC compensation control tube will automatically open

2. Optimize Instrument Settings

a. Using unstained cells (Tube 6), adjust PMTs voltages for FSC, SSC, and all fluorescent parameters, and threshold using the following Run Protocol:
   - Acquisition volume : 200 µl
   - Sample Flow Rate: 25 µl/min

b. Document the optimal FSC and SSC Settings:
   Forward Scatter Voltage Setting for cells _____
   Side Scatter Voltage Setting for cells _____
   FSC Threshold _____

c. Verify fluorescent compensation controls are on scale (Tubes 2 to 5) and adjust detector voltages if needed to ensure all positive beads are on scale. If not on scale, make slight voltage adjustments to bring the population on scale.
   Note: you will need to adjust FSC and SSC voltages to get the beads in the R1 gate.

d. Sanitize the Attune SIP before recording Compensation controls.
   Note: At this step, you have not yet recorded any data.

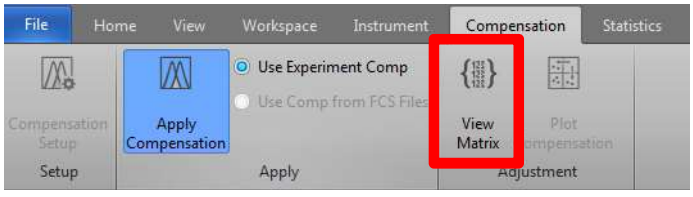
3. Record compensation controls
   Note: Be sure you have added the negative bead to the bead sample tubes 1 to 5

a. Using tubes 1 to 5 (Bead controls) record compensation using the following Run Protocol:
   - Acquisition volume = 300µL
   - Flow rate = 200µL/min
   - Stop criteria: 5000 total events
   Note: Check that the correct control is being measured.

b. After recording of each control tube, adjust the R2 gate around the positive bead population.

c. (*optional*) Sanitize the SIP before running cell samples.

6. Check compensation is properly calculated by viewing the Matrix in the *Compensation* tab.



Sample acquisition

1. Adjust the FSC and SSC voltages and Threshold as necessary to visualize cells on Scatter Plot (see values noted in step2b under compensation set up).

2. Setup the Run Protocol as follows:
    a. Collect entire plate from the beginning
    b. Acquisition volume: 150µL
    c. Total sample Volume: 180µL
    d. Flow rate: 200µL/min
    e. Stop after 50.000 events total
    f. 1 mix and 1 rinse in Raw B
       2 mix and 2 rinses in Raw C

3. Collect the plate

Data Analysis

1. Open one of the "Stained cells" sample.

2. On the CD45 vs. SSC plot, create a gate around the lymphocytes and rename lymphs.

3. On the CD45 vs. SSC plot, create a gate around the monocytes and rename monos.

4. Set population on the CD3 vs. CD19 plot to lymphs.

5. Create and adjust quadrant gate on CD3 vs CD19 dot plot.

6. Create a new derived gate using lymphs OR monos and rename "L and M".

7. Open the Edit Gates dialog box. Select and move the "L and M" gate to the top of the list.

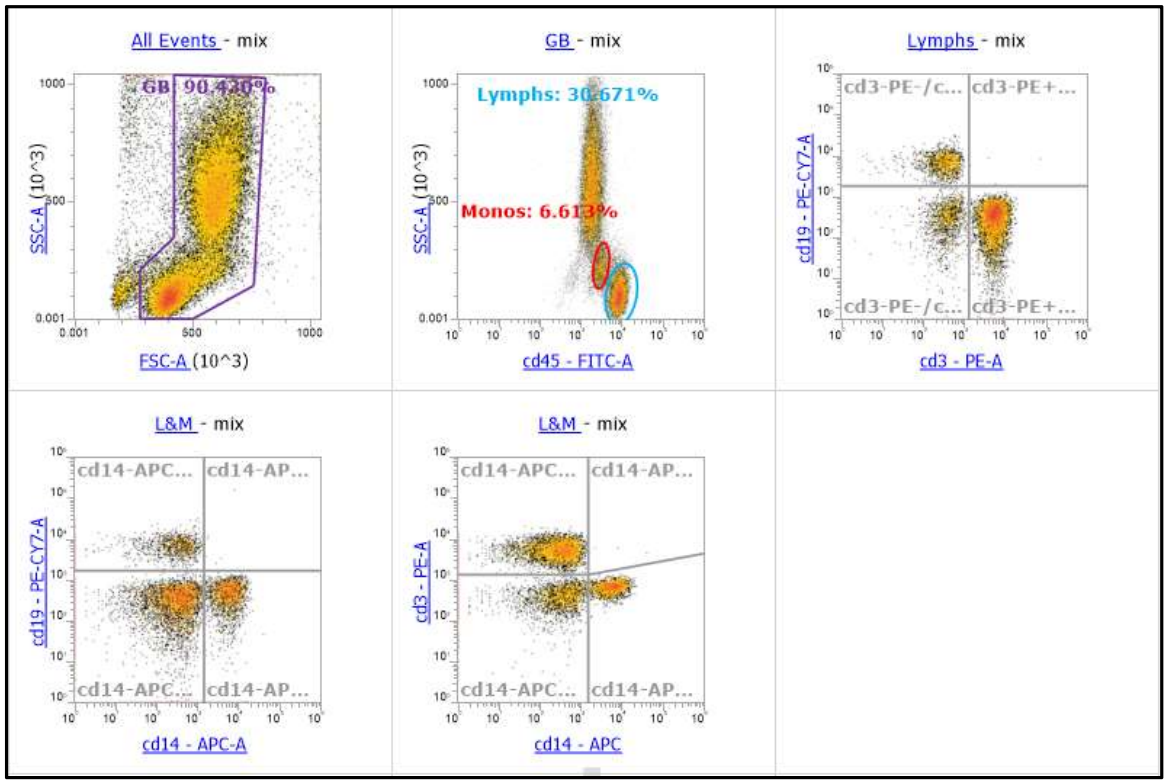
8. On the CD19 vs. CD14, and CD3 vs. CD14 dot plots set population to "L and M".

9. Create a rectangle gate or a quadrant gate so that you can identify the monocyte CD14 positive population.

10. Identify % of
    a. T lymphocytes in L and M population
    b. B lymphocytes in L and M population
    c. Monocytes in L and M population

11. Explore *HeatMap* Analysis Tools

12. Export the Experiment and transfer to a USB drive



Post-acquisition Questions

1. Which samples were used as the controls?

2. Were there any adjustments to the instrument settings when using beads and cells?

3. Why are the voltage settings locked after recording compensation?

4. Which Run protocol conditions are the best for:
    a. Accurate counting?
    b. Low carryover?

Appendix: "Tune-up your knowledge"

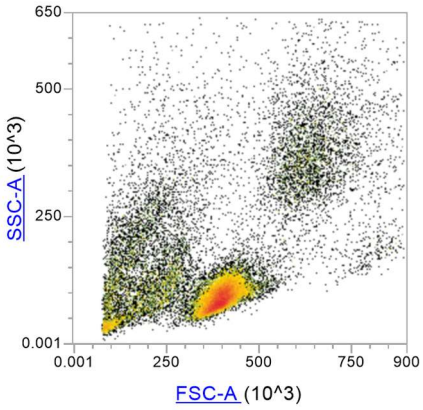
1. What should you do if you see no events displayed on the plots?

All answers can be correct, following the order below:

A. Adjust the voltages and thresholds accordingly
B. Verify that you are using correct filter configuration
C. Confirm cell concentration is sufficient
D. Run Performance Tracking Beads as a sample using PMT voltages from the last Performance Test

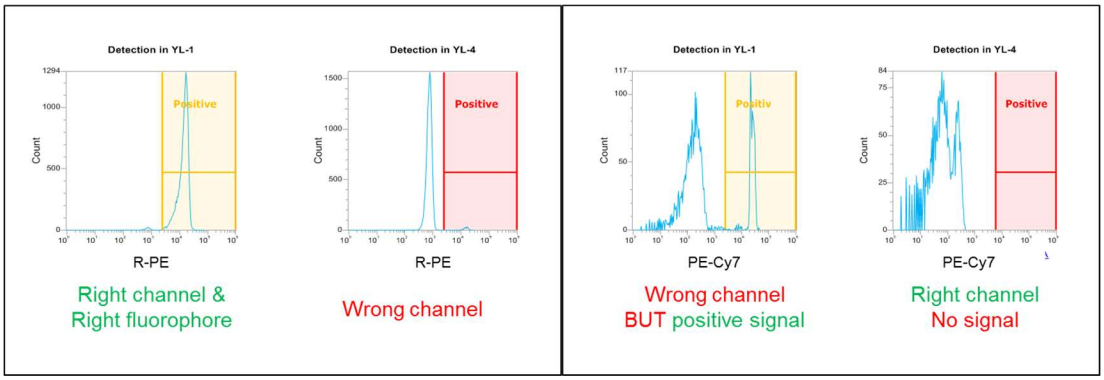
2. What should you do if you see a large number of events with low FSC and Low SSC together with your cells of interest?

B. Increase FSC and/or SSC thresholds
C. Decontaminate your system



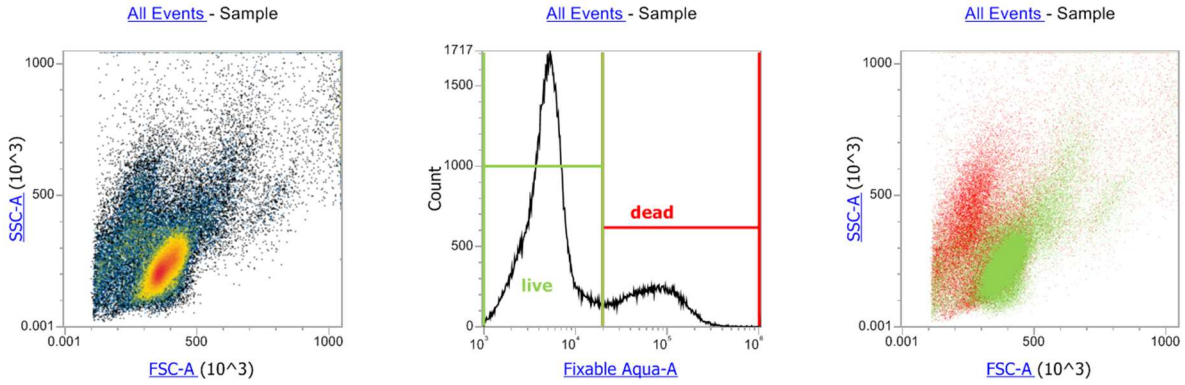
3. What should you do if no positive fluorescence signal is detected?

A. Repeat the whole experiment with fresh solutions
B. Confirm correct parameters are selected and check fluorochrome is present
C. Adjust voltages
D. Check Optical Configuration is properly setup in the software



4. Dead cells have different scatters properties than live cells

✓ TRUE



Live/Dead™ Fixable Dead Cell Stain Kits

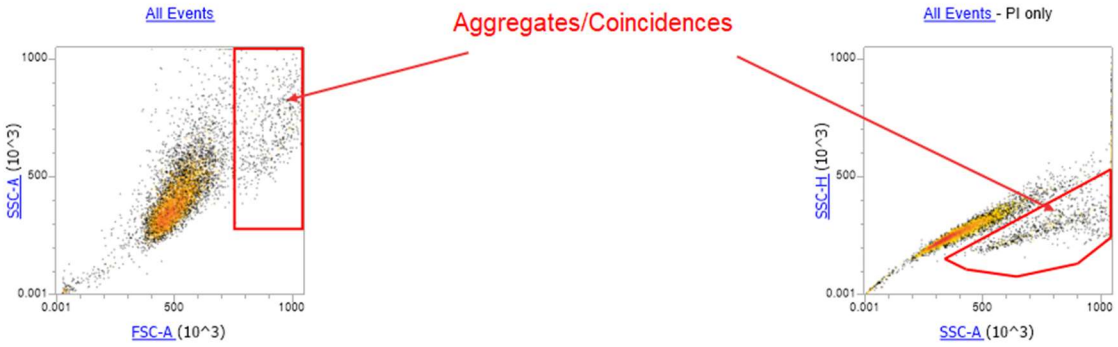
SYTOX™ Dead Cell Stain Sampler Kit

5. What to do if aggregates/coincident events are detected?

B. Dilute your sample and run with higher flow rate (if coincidences)

C. Decontaminate your system and filter Attune solutions (if aggregates are in the instrument, no need for filtering solutions)

D. Filter your sample and rerun (if aggregates are in the sample)

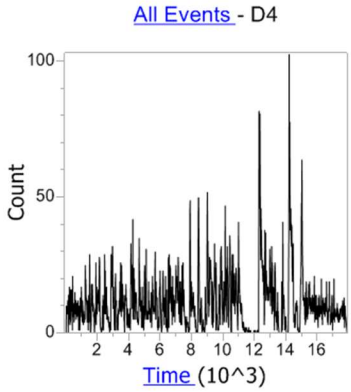


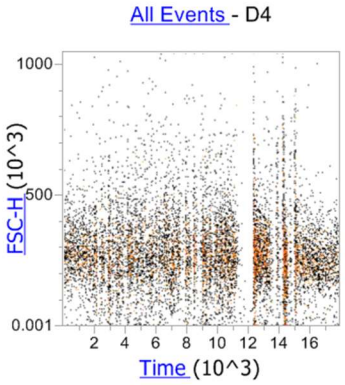
6. How to diagnose a clog?

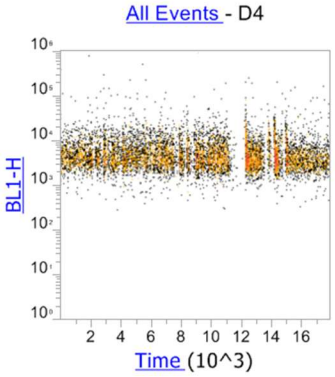
B. Low event rate

D. The software reports an error (only if it is a full clog)

E. Look at Time vs Fluorescence or Scatter Density Plot





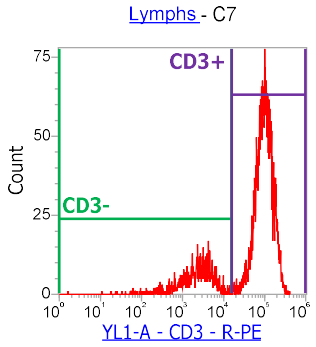


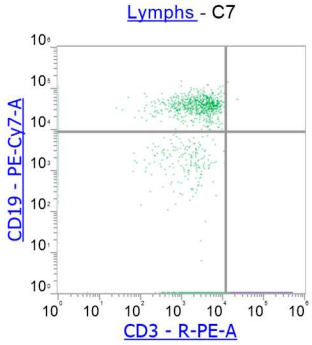
7. You do not see your positive population on a dual parameter plot while it's present on an histogram plot. What do you do?

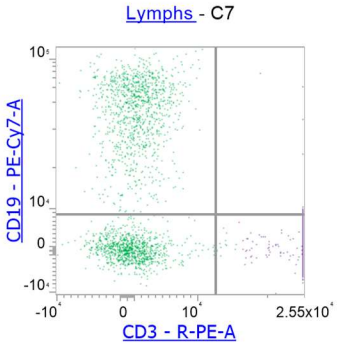
B. You change the scale on your dual plot parametric

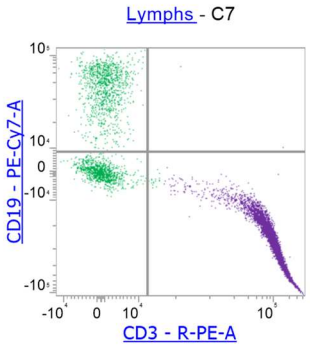
C. You check compensation is properly calculated

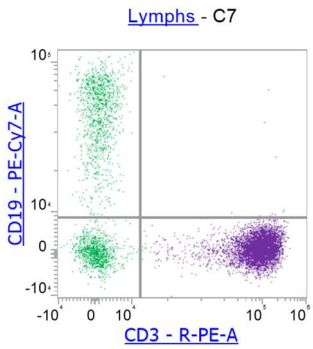
.





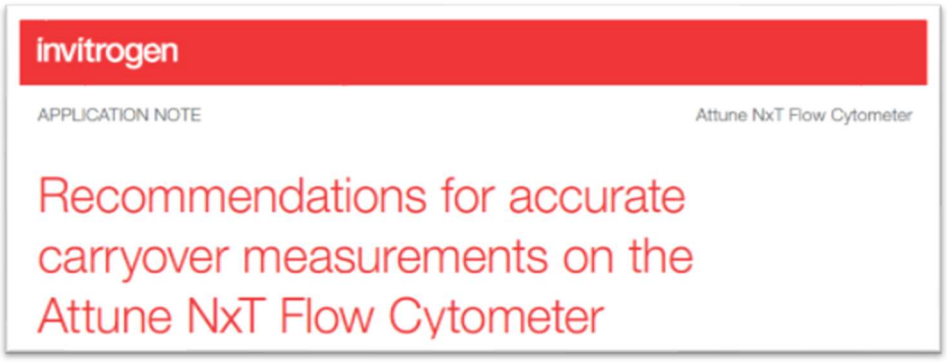






8. You noticed carry over between samples while using the AAS. How can you prevent that?

D. You increase the number of rinse cycles between 2 wells



9. You have a low number of events using the AAS. The sample runs correctly in a tube. What do you do?

B. You check the total sample volume is properly setup
C. You check your plate type is validated and porperly setup
E. You increase the number of mixing cycles

Notes: If there are no events using the AAS, check the sample probe is properly entering into the well. If this is not the case, run the Autosampler. Calibration script from the *Instrument* Tab

10. What should you do if the Performance Test fails?

Actions should be taken as follows:

C. Follow the instruction provided by software interface
B. Repeat the Performance test with beads freshly prepared
D. Run a Deep Clean/Debubble script and repeat the performance test with beads freshly prepared
E. Contact Technical Support or Service Admin