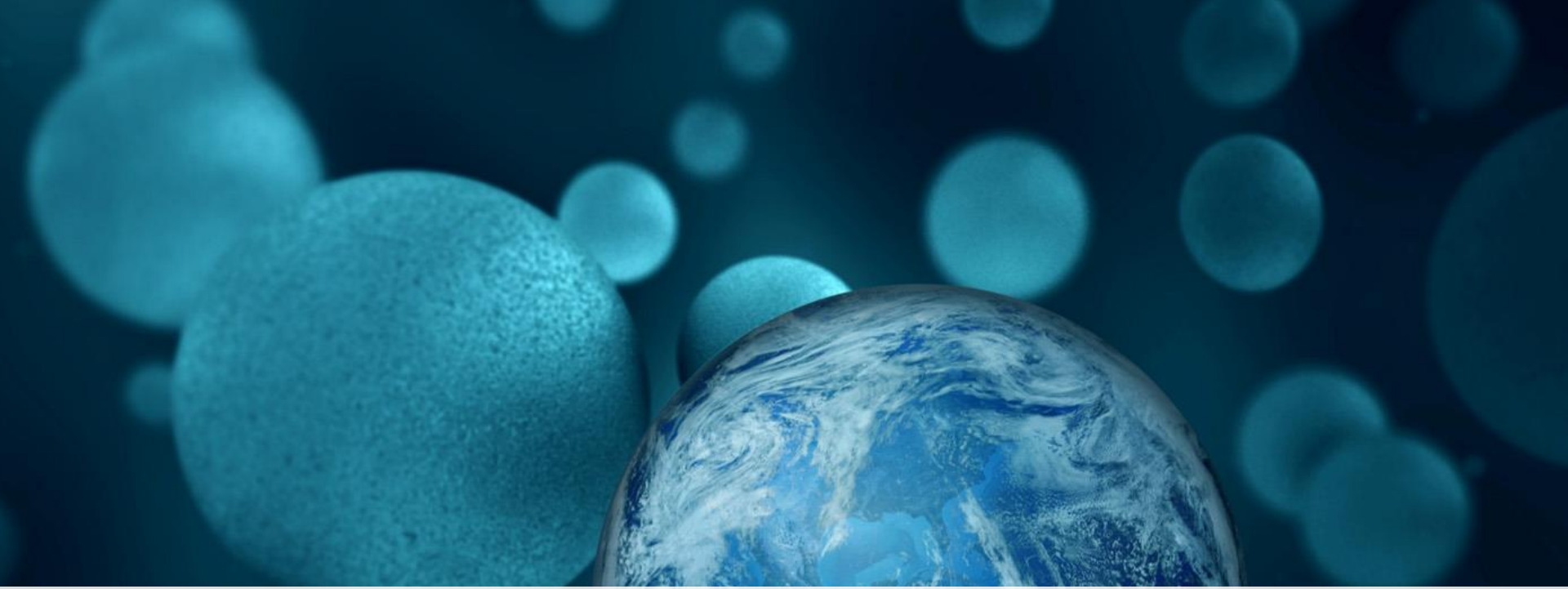

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Attune® NxT™ Acoustic Focusing Cytometer

Two Day Basic Training

Table of Contents – hyperlinked

Agenda – Day 1

9:00 am – 12:00 pm	Welcome and Overview of Training Agenda
	Introduction to Flow Cytometry
	Optional - Activity: Understanding Scatter
	Attune® NxT™ Systems – Fluidics and Optics
	Optional - Activity: Know Your Optics System
	Attune® NxT™ Systems - Electronics
	Software Overview & Experimental Workflow
12:00 pm - 1:00 pm	**LUNCH**
1:00 pm – 4:00 pm	**Startup and Performance Tracking**
	Maintenance Overview
	Voltage Walk

Agenda – Day 2

9:00am – 12:00 pm	Review Day 1
	Attune® NxT™ Maintenance, Data & Account Management
	Optimizing Instrument Settings
	Instrument Startup and Performance testing
12:00 pm – 1:00 pm	**Lunch**
1:00 pm – 4:00 pm	**Customer Samples and Multicolor Compensation (if applicable)**
	Autosampler (if applicable)
	Review of Training – Additional time for practice
	Instrument Shutdown

Hands-on Training Checklist

Attune Training Checklist

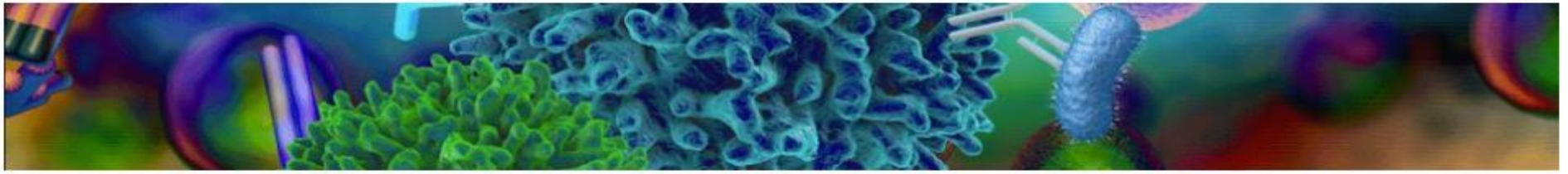
☐	Startup	☐	How to get service
☐	Shutdown	☐	Advance settings - ASF
☐	Performance test/baseline/LevyJennings	☐	How to get follow up support
	Fluidics Functions/Maintenance		Software
☐	Rinse	☐	Create experiments/tubes
☐	SIP Sanitize tube	☐	Create/use/manage templates
☐	SIP Sanitize plate	☐	Parameters
☐	Deep clean	☐	Voltages/voltage walk
☐	Sample Recovery	☐	Threshold
☐	Unclog	☐	Workspace/Edit gates
☐	Debubble	☐	Create experiments/plates
☐	System decontamination	☐	Collection panel/run protocol
☐	Correct way to remove/refill fluid bottles	☐	Customize panel
☐	AAS plate calibration	☐	Compensation
		☐	Options Menu
		☐	Heat Map and HM set up
	Data Analysis		Data Management
☐	Plots/gates	☐	Export (exp/statistics/fcs files)
☐	Statistics	☐	Import (exp, fcs files)
☐	Results tables	☐	Data Base Management (Admin)
☐	Overlaps		

Web Technical Resources

http://www.thermofisher.com/us/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-learning-center.html

Home › Life Sciences › Cell Analysis › Flow Cytometry › Flow Cytometry Learning Center

Flow Cytometry Learning Center



Learn about flow cytometry methods and technologies

The purpose of this learning center is to connect scientists (whether new or experienced) to our many resources for learning about flow cytometry applications, techniques and basic principles by providing a few key points of entry into the vast content.

Flow Cytometry Guided Learning

Find courses for basic applications and techniques used in flow cytometry

Molecular Probes School of Fluorescence

- Flow Cytometry Basics
- Fluorescence Basics

eLearning Courses

- T Cell Stimulation and Proliferation
- Antibody Validation*

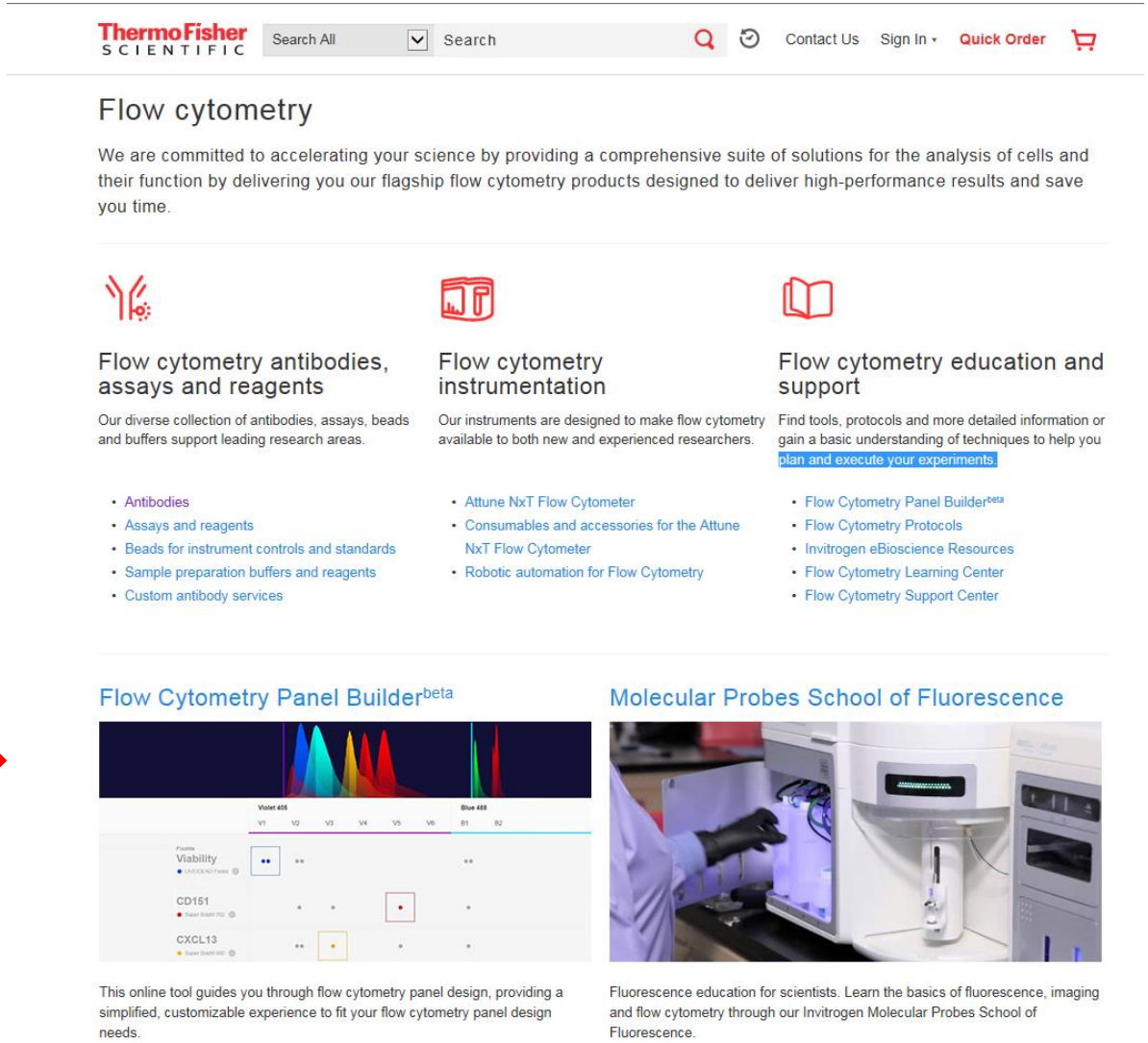
Flow Cytometry Subtopics

Find webinars, application notes, white papers, videos and more for key flow cytometry applications and techniques

- Areas of Biology
- Cell Isolation, Expansion and Differentiation
- Controls, Compensation and Calibration
- Flow Cytometry Assays
- Flow Cytometry Instrumentation
- Immunophenotyping
- Panel Design and Multicolor Flow Cytometry

Web Technical Resources Continued

www.thermofisher.com/flowcytometry



New

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Need additional training?

Application & instrument
training courses

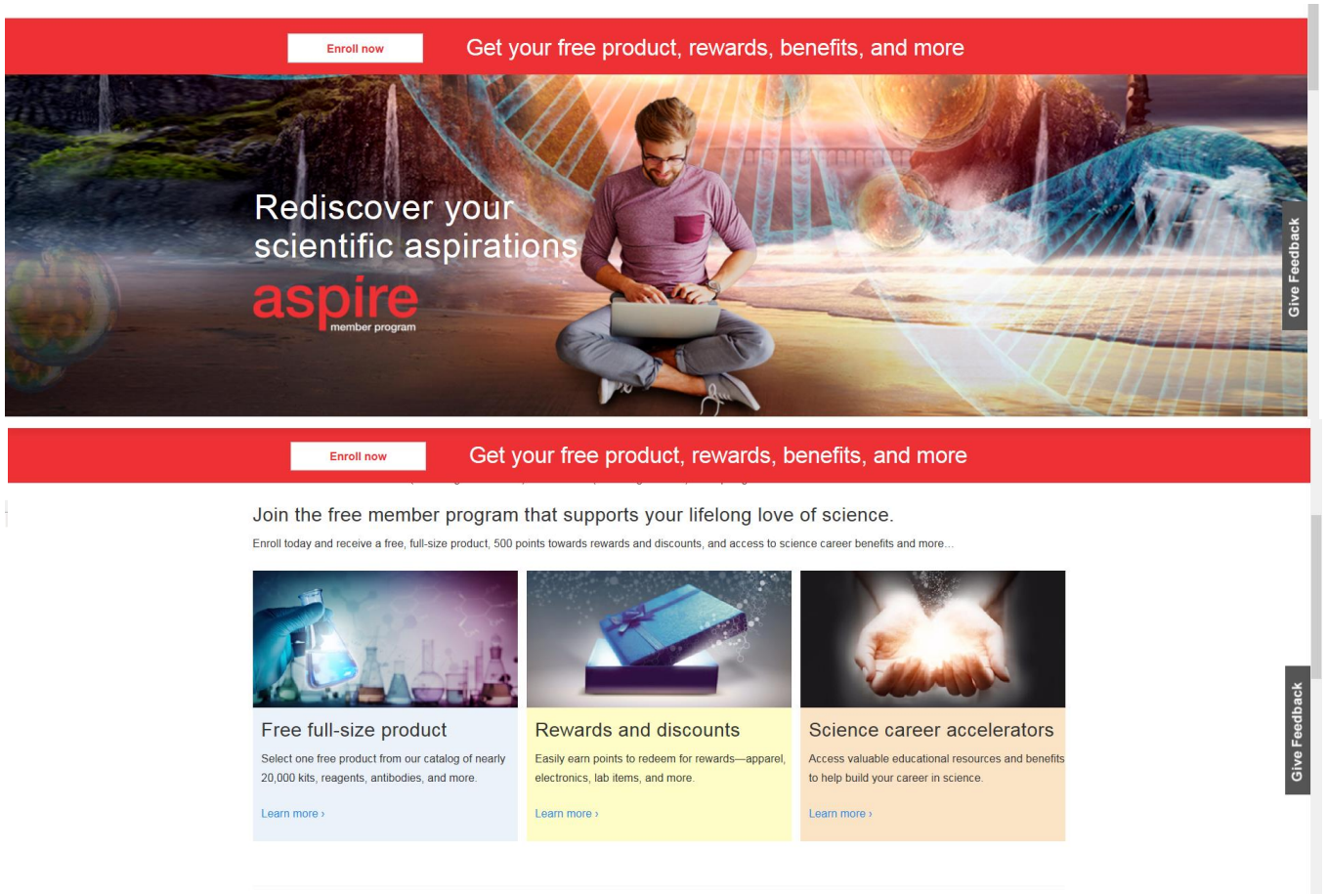
Cat #	Customer site
A25623	2 day NxT training
4484605	1 day NxT extended training
TRN00045	Hourly charge consulting /training

Detailed descriptions: https://learn.thermofisher.com/flowcytometry

For more information, questions or quotes - contact your FAS

Aspire program

www.thermofisher.com/aspire



Flow	**4484604**	**FLO-103: Flow Cytometry FAS On-Site Training (1 Day)**	**Flow Cytometry On-Site Training**	**90,000**	**1**

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Obtaining Support

1. Check the troubleshooting section in the users guide.

   If additional help is required, don't hesitate to contact: **1-800-955-6288**
   (9:00am-8:00pm Eastern, 6:00am-5:00pm Pacific)
   You will need the S/N of your instrument

Choose **Option 3** for Instrument Service and HW support
then **option 1** for a) instrument service or
b) to check status of instrument repair
Option 4, then **option 6** Fluorescent Labeling/ Detection and
Flow Cytometry Reagents and Flow Applications

3. Or Email:
   flowsupport@thermofisher.com
   or

   Instrumenthardwaresupport@thermofisher.com for TAC/Instrument services

Web submission: https://www.thermofisher.com/us/en/home/support/instrument-repair-request.html

Introduction to Flow Cytometry

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

What makes a Flow Cytometer?

Abbreviated : FCM

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

Attune NxT Subsystems

Fluidics

Optics

Electronics

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

Traditional Hydrodynamic Focusing

Particle positioning in laser is important

Low sample flow rate (e.g., 12 µL/min)

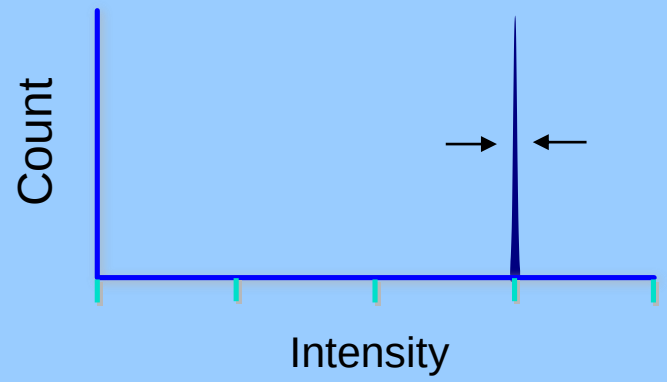
Hydrodynamic core

Focused laser

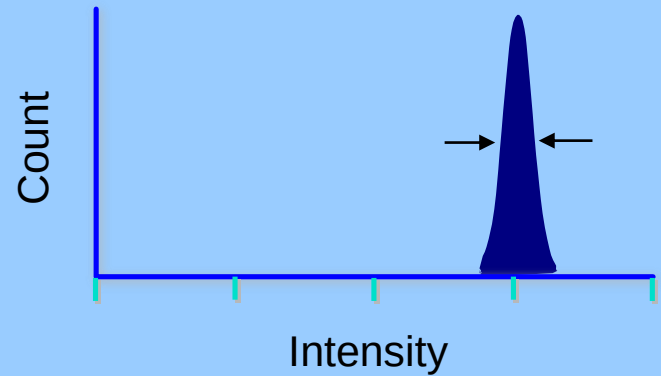
High sample flow rate (e.g., 200 µL/min)

Focused laser

Narrow particle focus = Narrow distribution



Broad particle focus = Broad distribution



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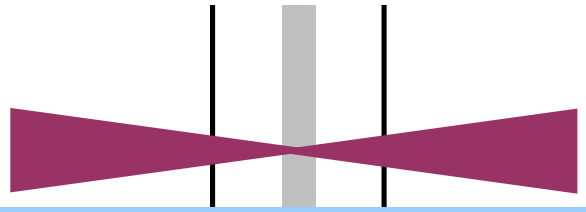
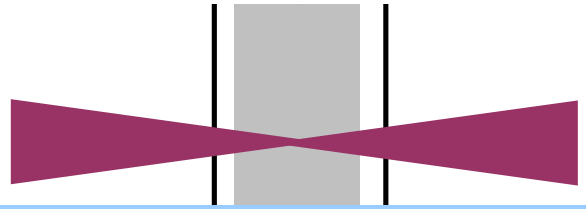
Acoustic Focusing

High sample input flow rates allow for more sample flexibility

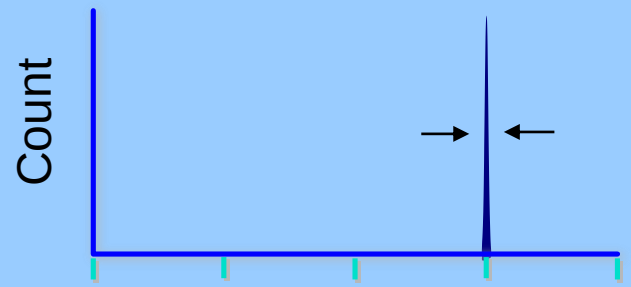
12.5 µL/min

1,000 µL/min

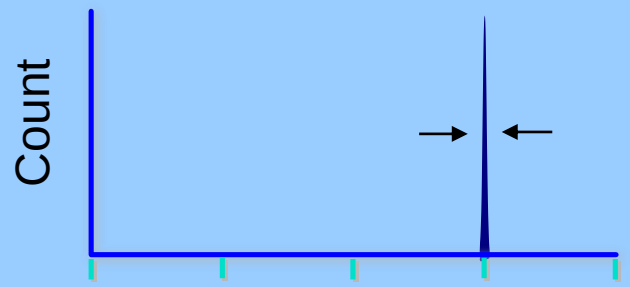




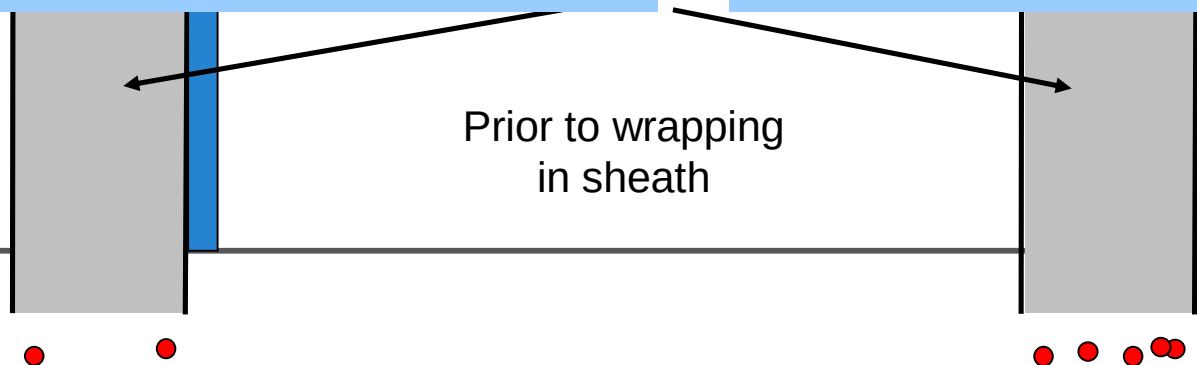
Narrow particle focus = Narrow distribution



Narrow particle focus = Narrow distribution



Prior to wrapping in sheath



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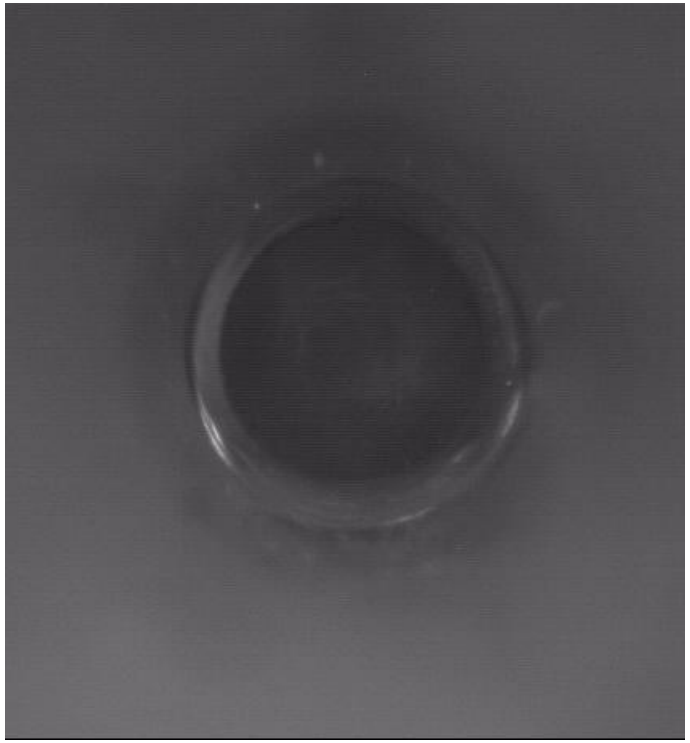
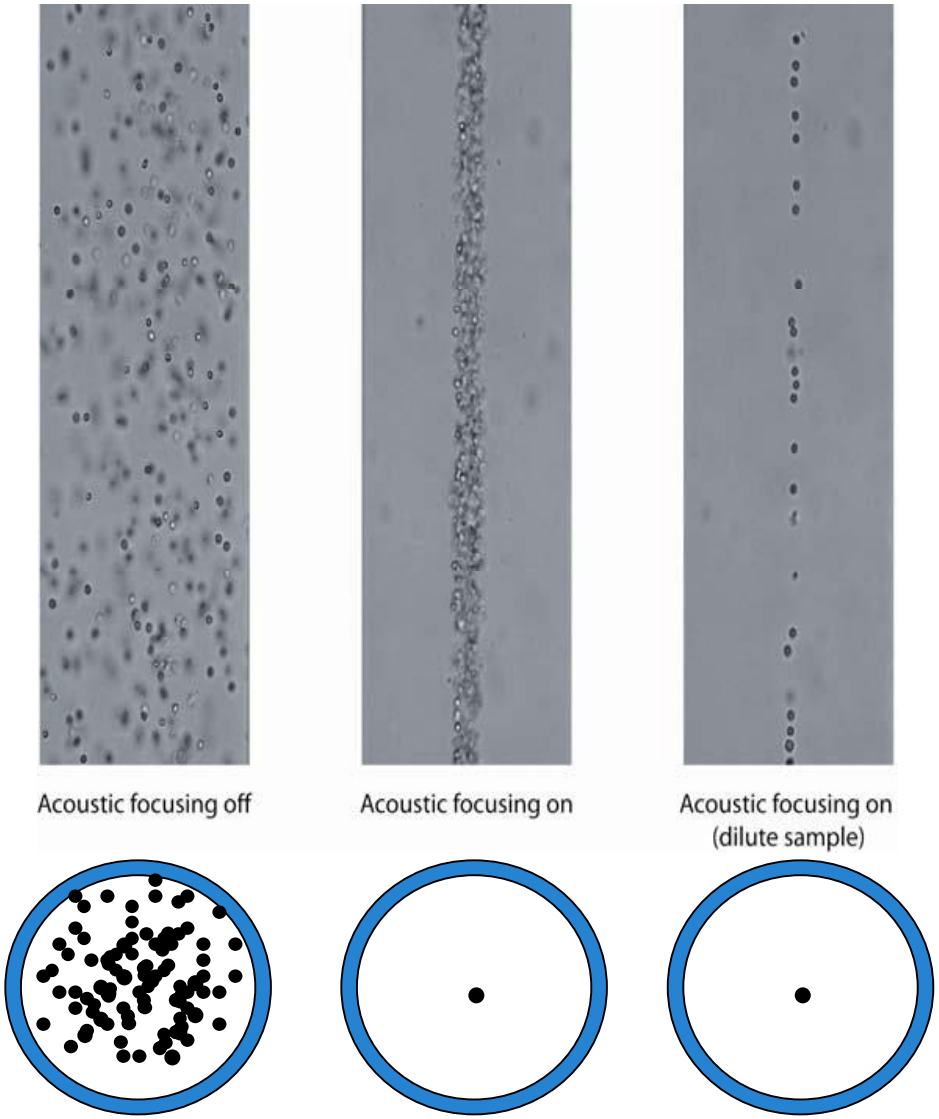
Acoustic Focusing

End-on view of capillary



Acoustic Focusing

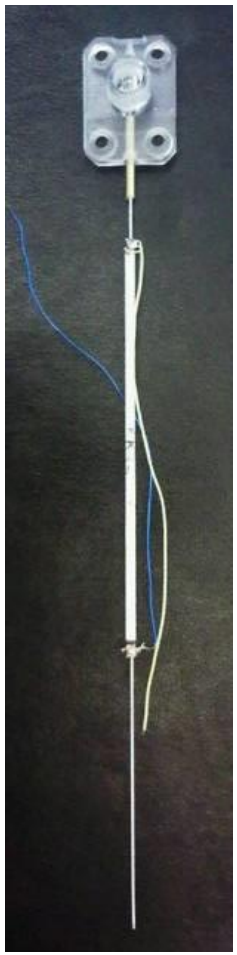


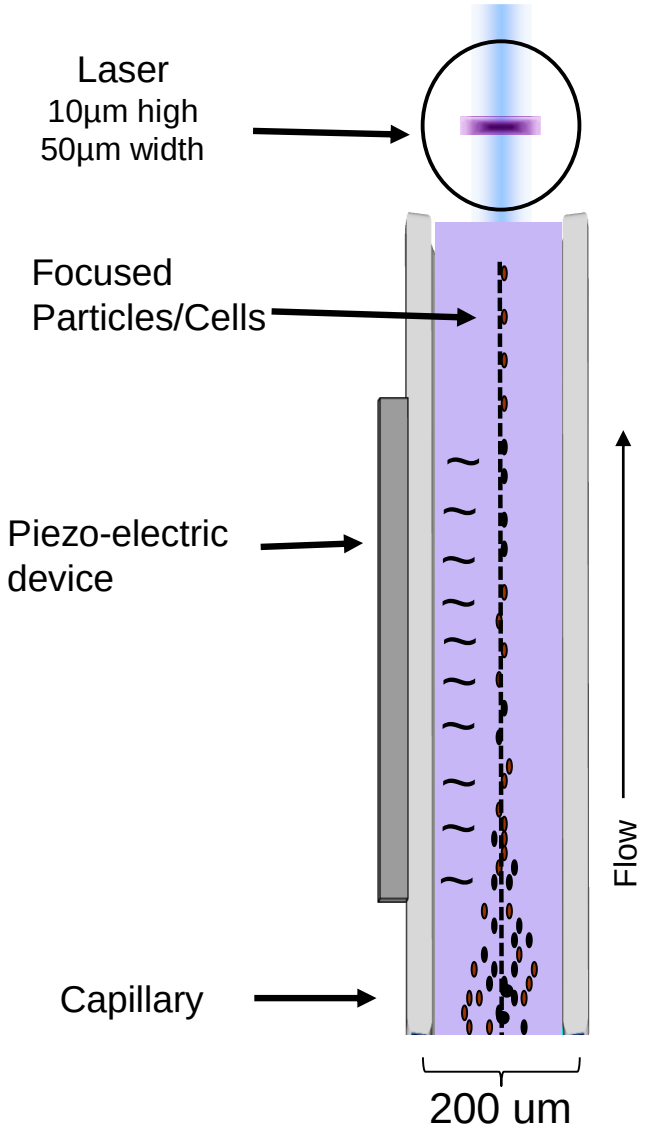
Dilution is the solution!

Acoustic Focusing Capillary

- Acoustic Waves – similar to ultrasound used to visualize a fetus *in utero*
- *2.5 MHz*

~20cm





Flow rate can be increased while maintaining resolution

Concentration and Flow Rates

The event rate will approach maximums stated in the column header when samples of stated concentrations are run at the flow rates below.

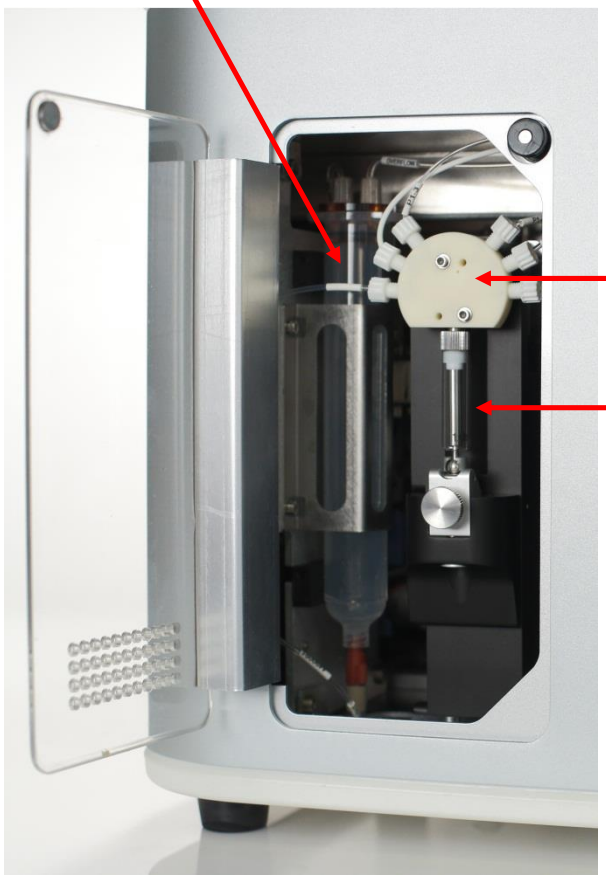
When acquiring large event files (i.e files with > 10^6 events), plot parameters should not be changed while recording.

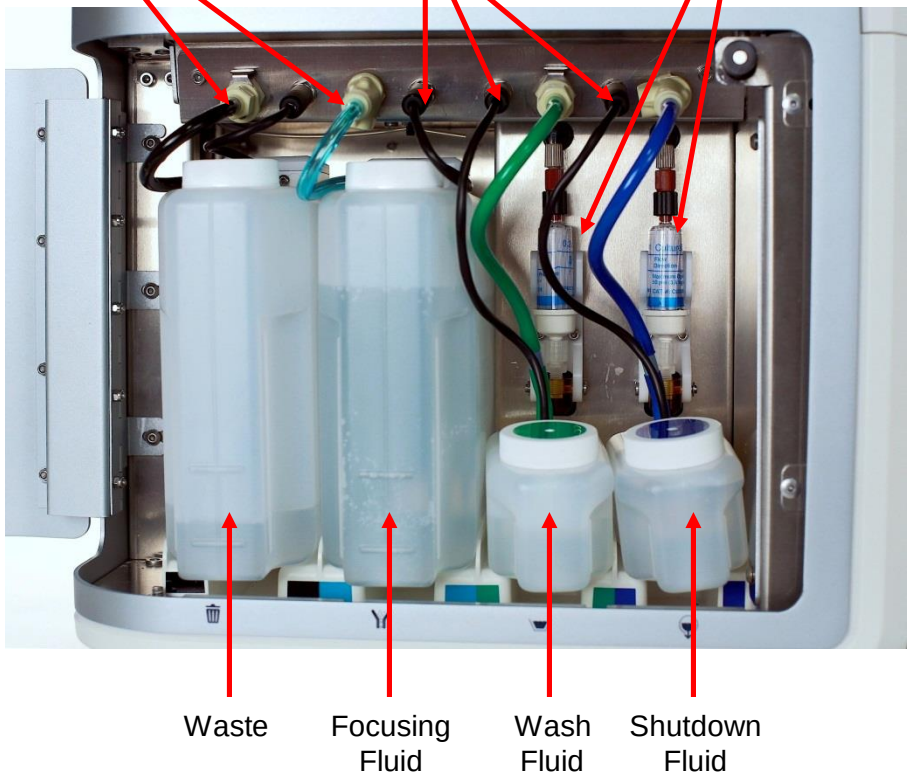
Sample flow rate	***Fastest*** **(35,000 ev/sec)** ***maximum*** **sample concentration**	***Accurate counts*** **(8,000 ev/sec) maximum sample concentration**	**Cell size and flow rate recommendation**
1000 µL/ minute	2.1 x 10^6 cells/mL	0.48 x 10^6 cells/mL	- Particles > 4 µm - Predominantly acoustic focusing
500 µL/ minute	4.2 x 10^6 cells/mL	0.96 x 10^6 cells/mL	- Particles > 2 µm - Predominantly acoustic focusing
200 µL/ minute	6.7 x 10^6 cells/mL	1.5 x 10^6 cells/mL	
100 µL/ minute	1.3 x 10^7 cells/mL	3 x 10^6 cells/mL	
25 µL/ minute	5.4 x 10^7 cells/mL	1.2 x 10^7 cells/mL	- Small particles < 2 µm - Predominantly hydrodynamic focusing - Smallest sample core - Best resolution from background for dimly positives assays
12.5 µL/ minute	1.0 x 10^8 cells/mL	2.4 x 10^7 cells/mL	

Let your biology and data quality be your guide. *If good data is obtained while running at 2-8,000 ev/sec, adjust the sample concentration and flow rate to maintain that.*

Note: Higher flow rates may show some loss of sensitivity

Attune® NxT Fluidics System





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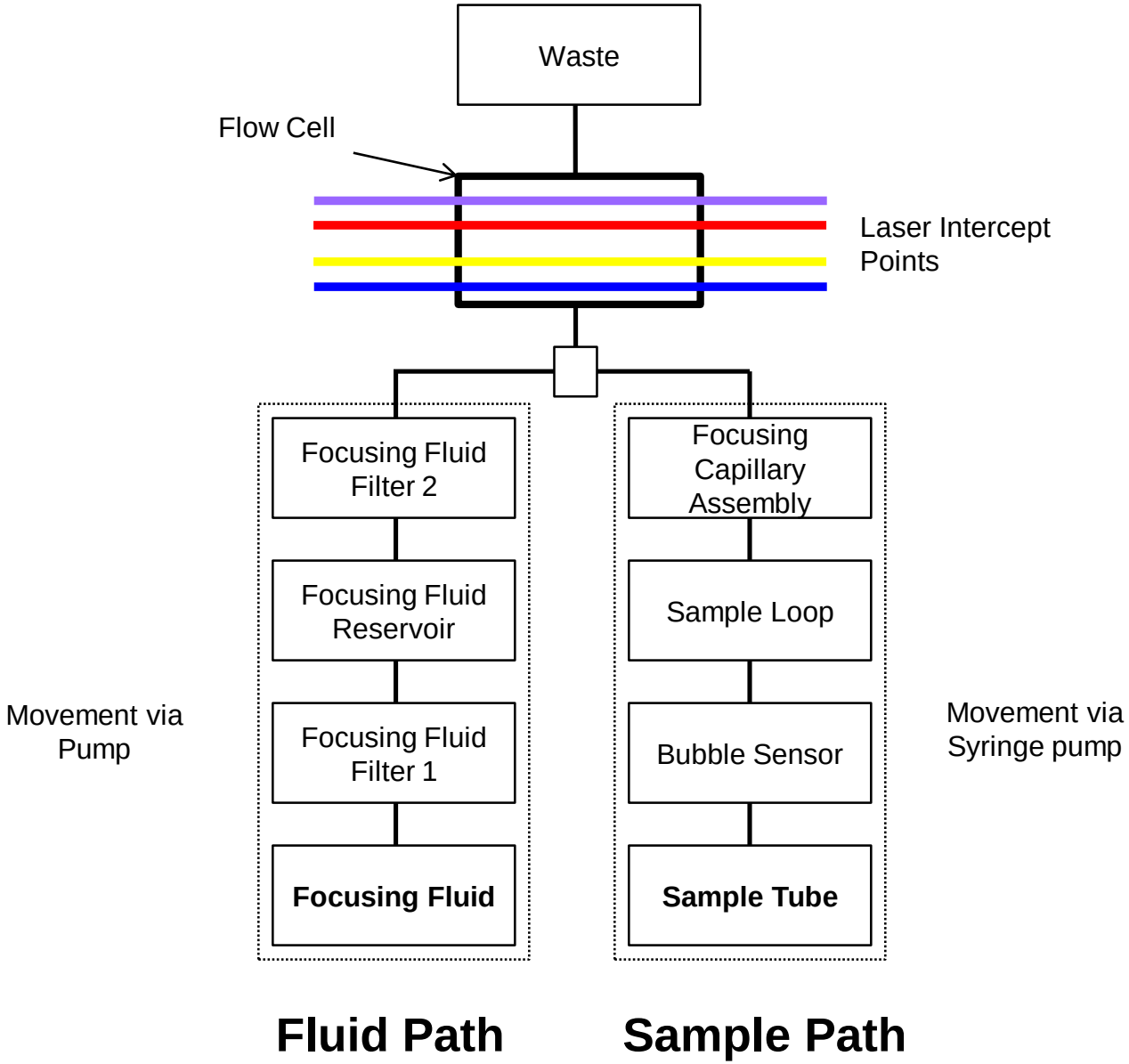
Attune NxT Fluidics System



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Attune NxT Fluidics Solutions

Attune® Focusing Fluid: a buffered, azide-free solution which transports focused particles to the flow cell for laser interrogation. It prevents sample for 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. Fluid must be RT before use.

Attune® Wash Solution: a solution for removing cellular debris and dyes from the fluidic system of the instrument.

Attune® Shutdown Solution: a solution which minimizes bubble formation and crystal deposit in the fluidics system when the instrument is shutdown.

Attune® Debubble solution: a solution formulated to remove bubbles from the fluidics system.

NEW! Attune® Flow Cell Cleaning solution: a solution which when diluted, will minimize contamination buildup that may occur in system lines or the flow cell and system lines.

10% Bleach: Fresh solution used to decontaminate the fluidics lines.

Deionized water: Used for diluting bleach. High quality, filtered and sterile.

All Solutions are 1X and should be at RT when used.

About Bleach

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.

Recently more concentrated formulations (Ultra and Concentrate) have become 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

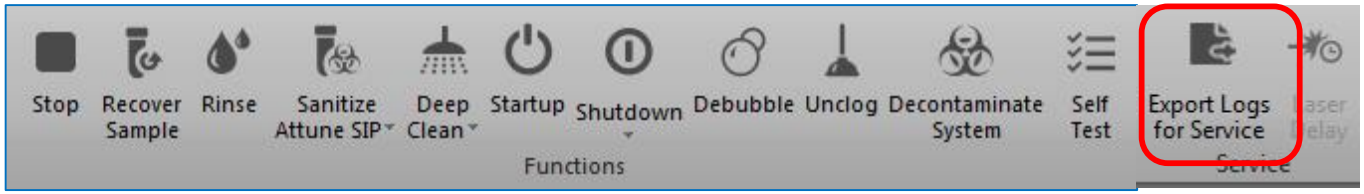
Bleach Solution	Dilution	Chlorine (ppm)
5.25%	None	52,500
	1:10	5,250
Ultra 6.15%	None	61,500
	1:12	5,125
Concentrate 8.25%	None	82,500
	1:16	5,150

http://www.cdc.gov/hicpac/Disinfection_Sterilization/19_00glossary.html

Recommendation:

- Prepare fresh bleach
- Use laboratory-grade bleach
- DO NOT USE bleach with additives (such as perfumes or soap)

Fluidics Functions (on the Instrument tab)



- **Stop** - click to end any running routine.
- **Recover Sample** - returns unused sample volume back to the well or the tube.
- **Rinse -** flushes system between samples to minimize carryover. Rinse runs automatically every time the SIP is lowered, but it can also be user-initiated.
- **Sanitize Attune SIP** - sanitizes the SIP and sample lines between sticky/dirty samples or experiments; requires 10% bleach solution.
- **Deep Clean** - thoroughly washes the system sample lines and flow cell between sticky/dirty samples or experiments; requires 10% bleach solution (can also use debubble solution)
- **Startup -** primes the instrument fluidics with Attune® Focusing Fluid.
- **Shutdown** - automatically cleans, sanitizes and shuts down the instrument.
- **Debubble** - clears bubbles from the fluidics lines of the cytometer; Attune® Debubble solution required.
- **Unclog** - back flush operation to remove clogs from the sample line.
- **Decontaminate System -** semi-automated decontamination of the Cytometer and the Auto Sampler fluidics.
- **New! Export logs for Service –** one button export of log files for field service assistance

All part of fluidics maintenance

New – Attune NxT Flow Cell Cleaning Solution

What is Attune NxT Flow Cell Cleaning Solution?

Alkaline liquid concentrate

Removes contaminants without damaging the quartz flow cell

- Sticky Cells
- Dyes like PI

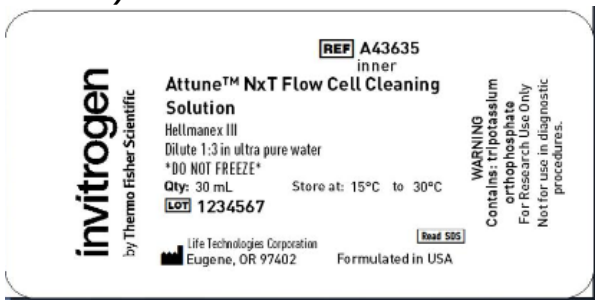
How often should it be used?

Daily - high volume users – running >6 hrs or 8 plates/day

Weekly – lower volume users

Directions

1) Combine 1 mL of Cleaning solution with 2 mL ultra pure water
2) Run Sanitize SIP (substituting the cleaning solution for bleach)
3) Prepare fresh 10% bleach
4) Run Shutdown



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Cleaning Functions

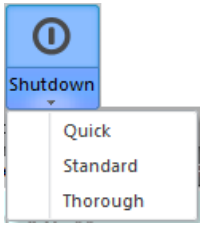
Sanitize SIP

Quick wash/sanitize of sample line.
Duration: 1 min. cycle time.
Requires 3 mL 10% Bleach.
Run between users especially after sticky samples, DNA stains or beads.

Deep Clean

Sanitize system with bleach and wash solutions for selectable period of time.
Three levels: Quick (10 cycles/ 25 min), Standard (20 cycles/50 min, Thorough 30 cycles/75 min.

Shutdown



System clean and flush with bleach, wash, and shutdown.

Quick	10 cycles /30 minutes	Few samples
Standard	20 cycles/60 minutes	Immunophenotyping, apoptosis
Thorough	30 cycles/75 minutes	Samples with sticky dyes (PI), NLNW

Instrument placed in stand-by (dream state) upon completion

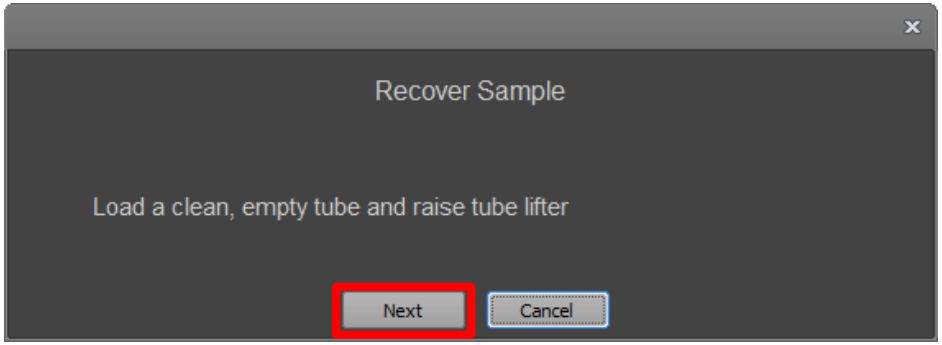
Located on the instrument tab

Instrument Cleaning Guide

Between samples	• **Rinse** – automatically initiated when SIP is lowered (for tubes), or set in *run protocol* for plates • **Sanitize SIP** between sticky samples or cell counts
Between users / experiments **USE:** **1) if there is ≥30 min between users.** **2) If there is <30 min between users.**	**1) Unclog** then **Quick Deep Clean** - 30 minute cleaning routine (click on the arrow below the Deep Clean icon to select ***Quick***) or **2) Unclog** then **2X Sanitize SIP** / **Sanitize Autosampler SIP** (plate experiments) – 1st time with 3 mL 10% Bleach 2nd time with 3 ml Wash or De-bubble solutions
End of day (3 steps)	• ***Unclog*** • *****SIP Sanitize with 1:3 dilution of Attune Flow Cell Cleaning solution*** • ***Thorough* Shutdown** (click on the arrow below the Shutdown icon to select *Thorough*)

Note: Always wipe the outside of the SIP after doing a SIP Sanitize

Sample Recovery - Tubes



When is ***sample recovery*** available?

Anytime sample remains in the sample loop

- Stop option has been reached
- Operator clicks stop

NOTE: Must select **recover sample** before lowering the tube lifter

Sample Recovery - Plates

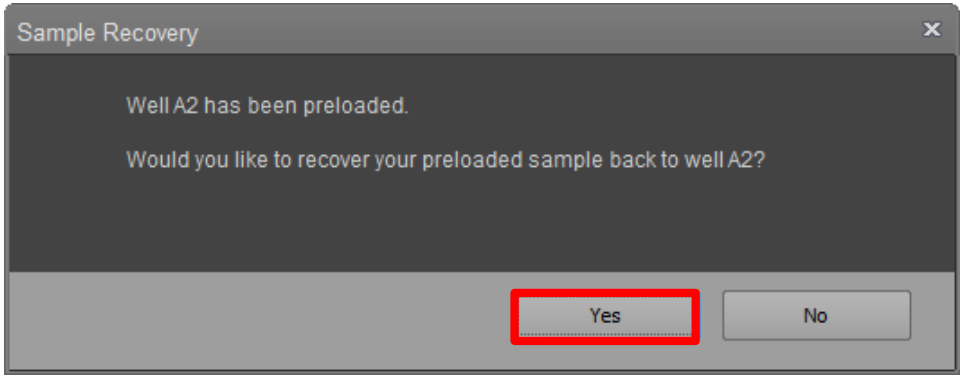
Sample recovery is disabled when moving between wells except when acquisition is stopped.

When Stop is pressed and the next well has been pre-loaded, sample recovery will recover the preloaded sample back into the plate.

1. Press the “Recover Sample” button

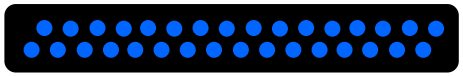
2. If sample has been preloaded, follow instruction to recover sample into plate

3. The remaining sample will be dispensed. This includes the dead volume and sample that has not been acquired



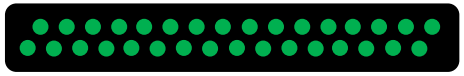
Status Indicator Lights

LED Color	Function/step
Blue (fade)	Warm up
Blue (solid)	Warm up complete
Blue (flashing)	Startup and instrument functions (except rinse)
Green (solid)	Startup complete or instrument idle
Green (flash)	Data/sample acquisition
Multicolor (fade)	Shutdown complete/sleep mode
Amber (blink)	Instrument Errors

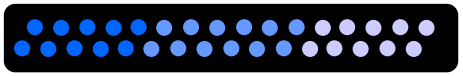


Fade: Warming Up
Flashing: Startup and instrument functions
Solid: Warm up complete

Flashing: Instrument Error



Solid: Start up complete or Idle
Flashing: Data/Sample Acquisition



Fade: Shutdown Complete/Sleep Mode

Attune NxT Systems

Fluidics

Optics

Electronics

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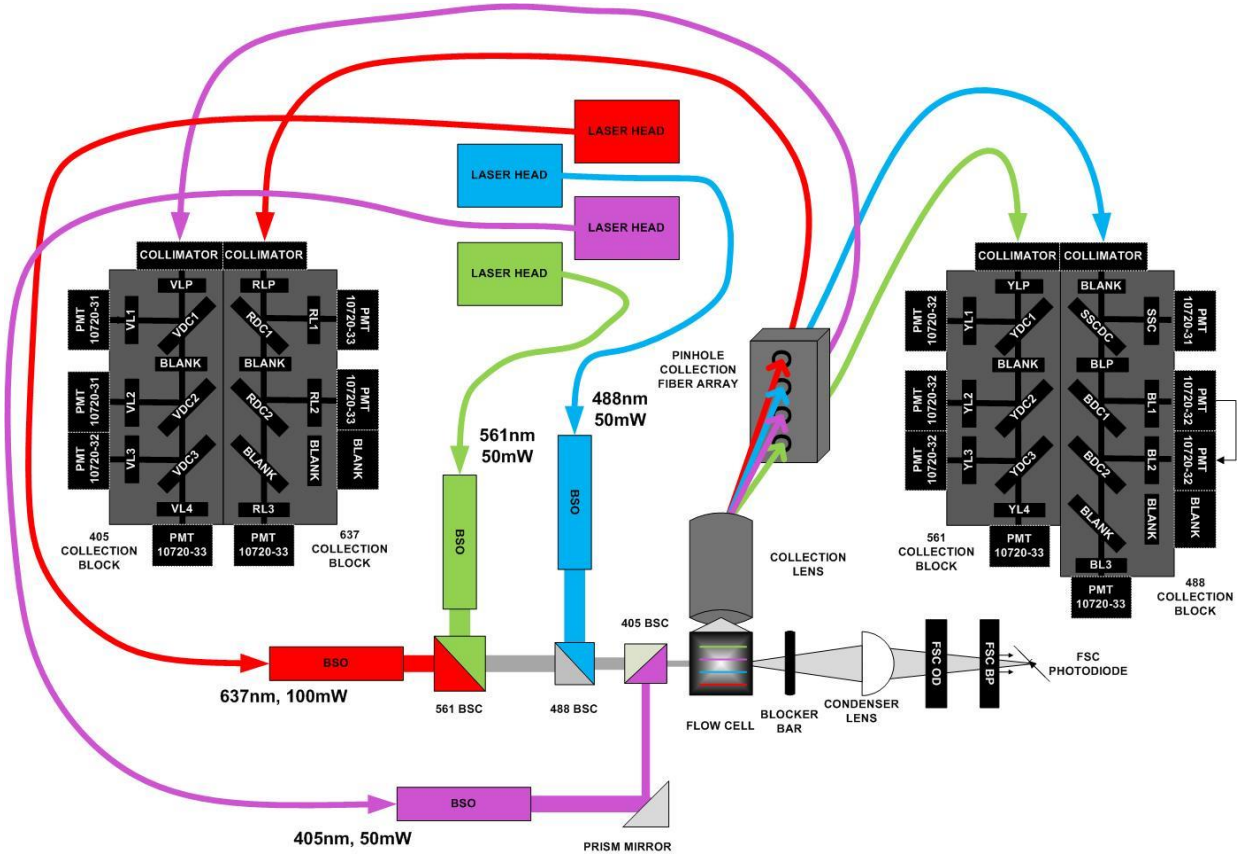
Optical System Anatomy

- Up to 4 lasers
- Up to 14 fluorescence detectors
- 2 scatter detectors
- Different PMTs for different Wavelengths



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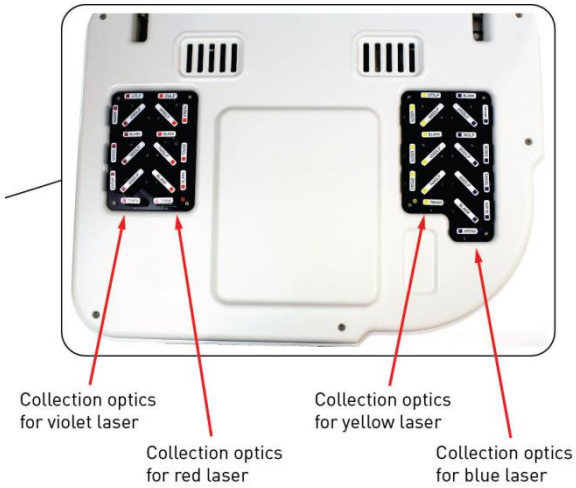
Attune NxT Optical Components

- From 1 to 4 Lasers

Violet	405 nm	50 mW
Blue	488 nm	50 mW
Green	532 nm	100 mW
Yellow	561 nm	50 mW
Red	637 nm	100 mW

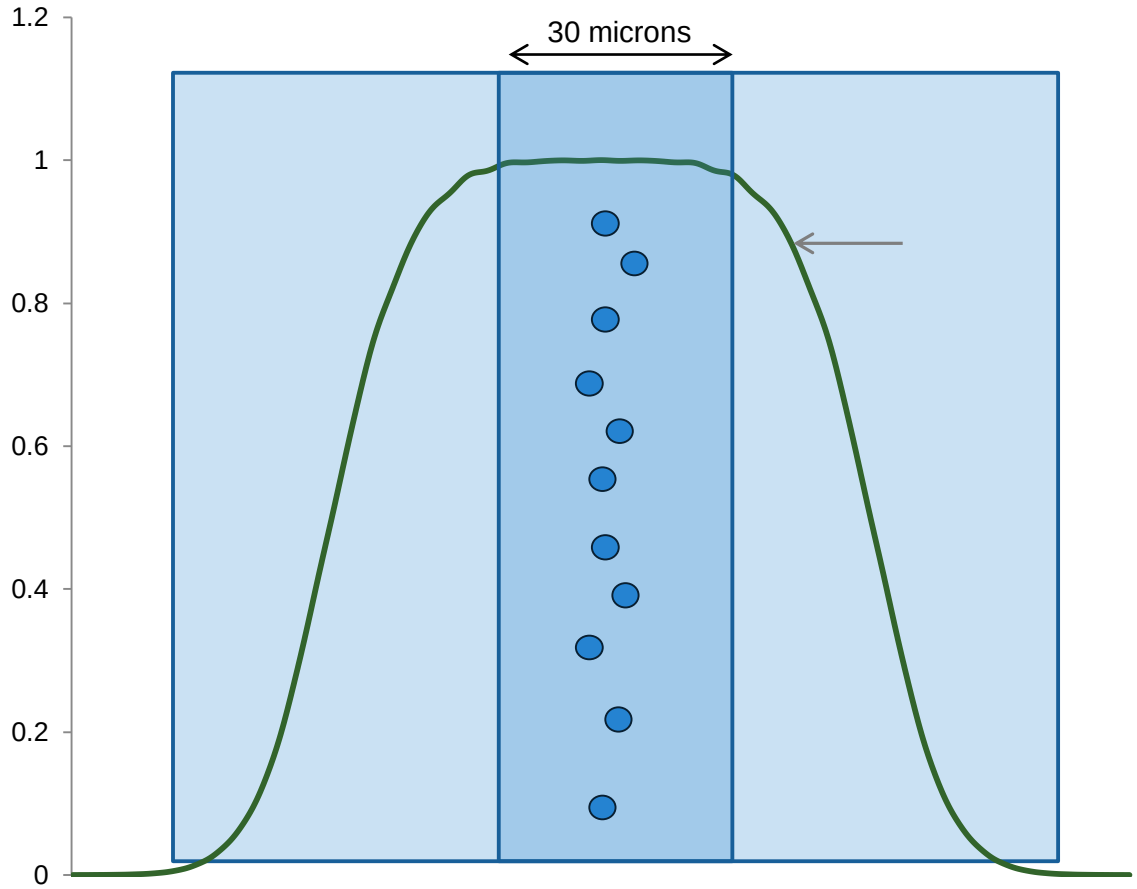


- Option of Green OR Yellow laser

- Filters are user changeable

- 2 Attune® NxT Accessory Filter Configurations
  - Violet Side Scatter kit
  - Fluorescent Protein Optimization kit

Flat Top Laser Profile



- Flattened gaussian profile
- Stable laser alignment
- Minimized down time
- Corrects for fluidic instabilities

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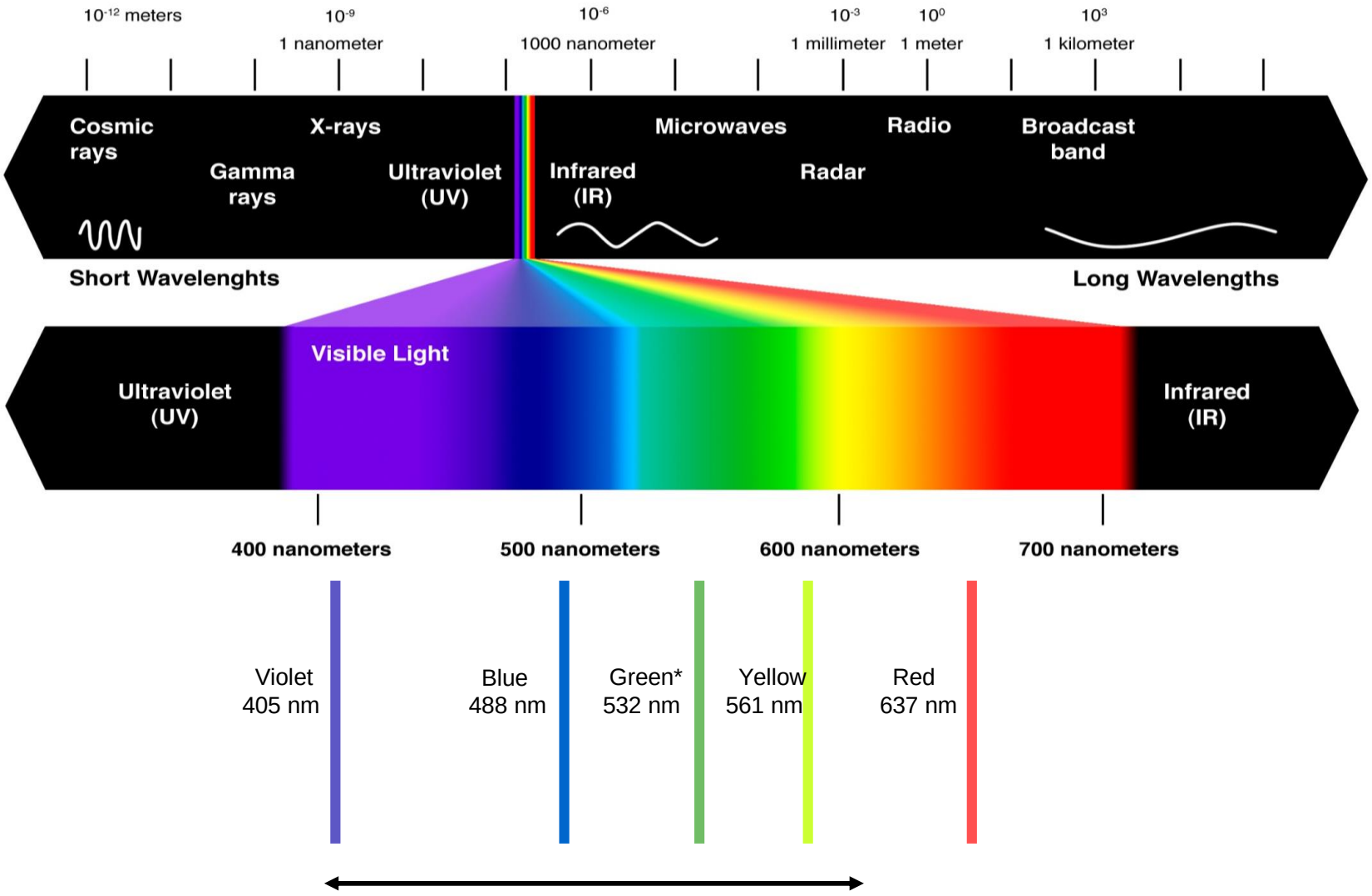
Visible Light

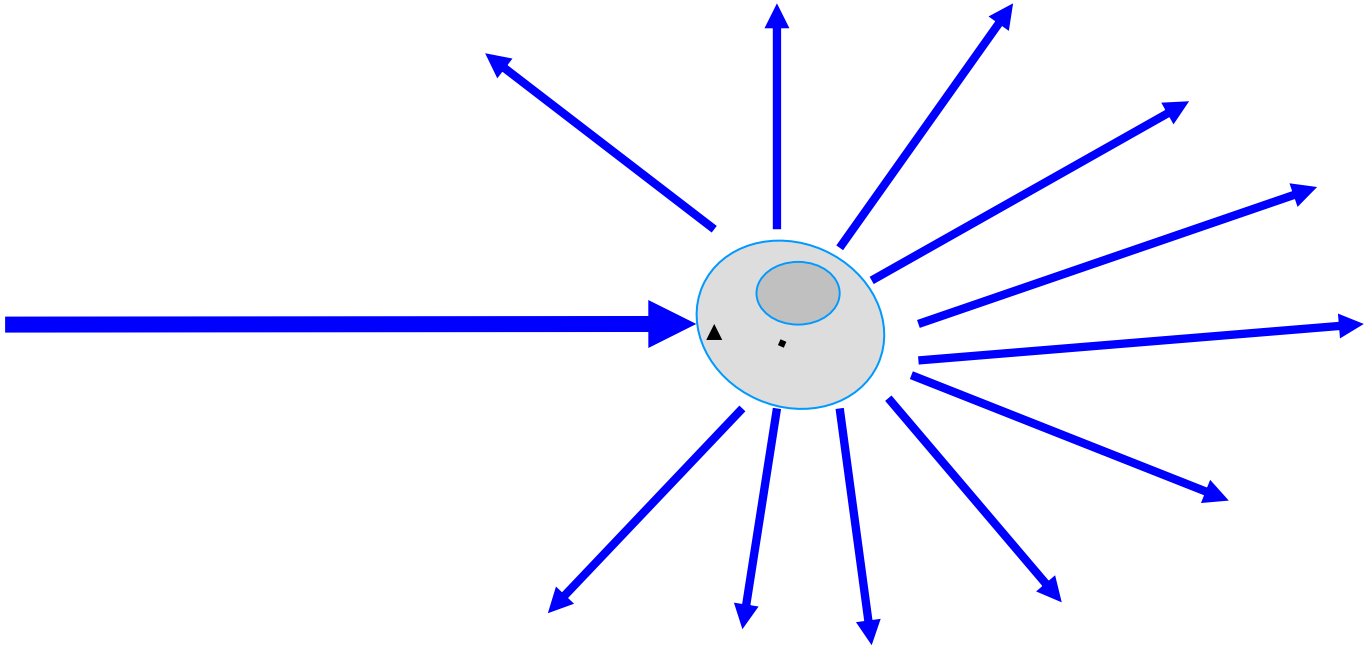


Autofluorescence of mammalian cells can be seen between 400-600nm

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
- We look at Forward Light Scatter and Side Light Scatter

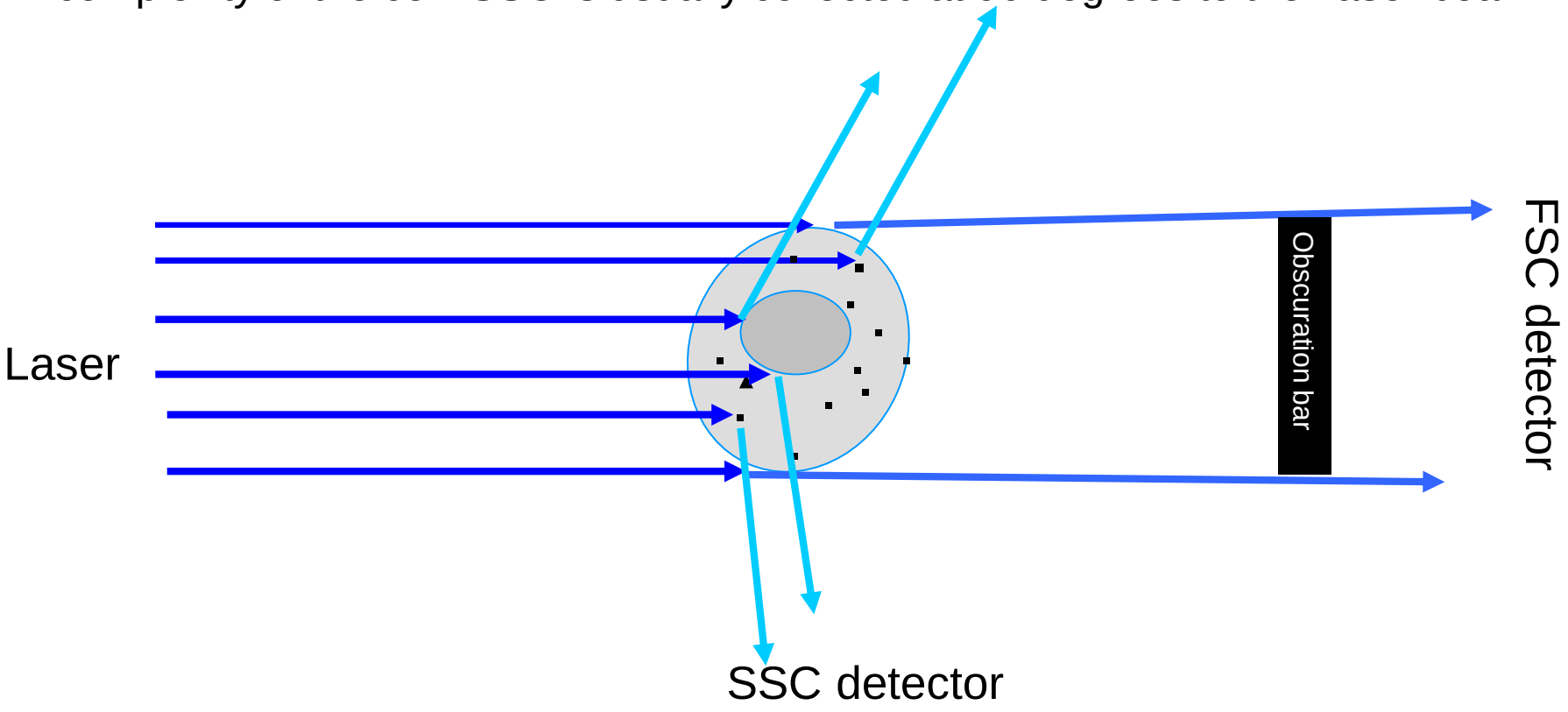


Scatter

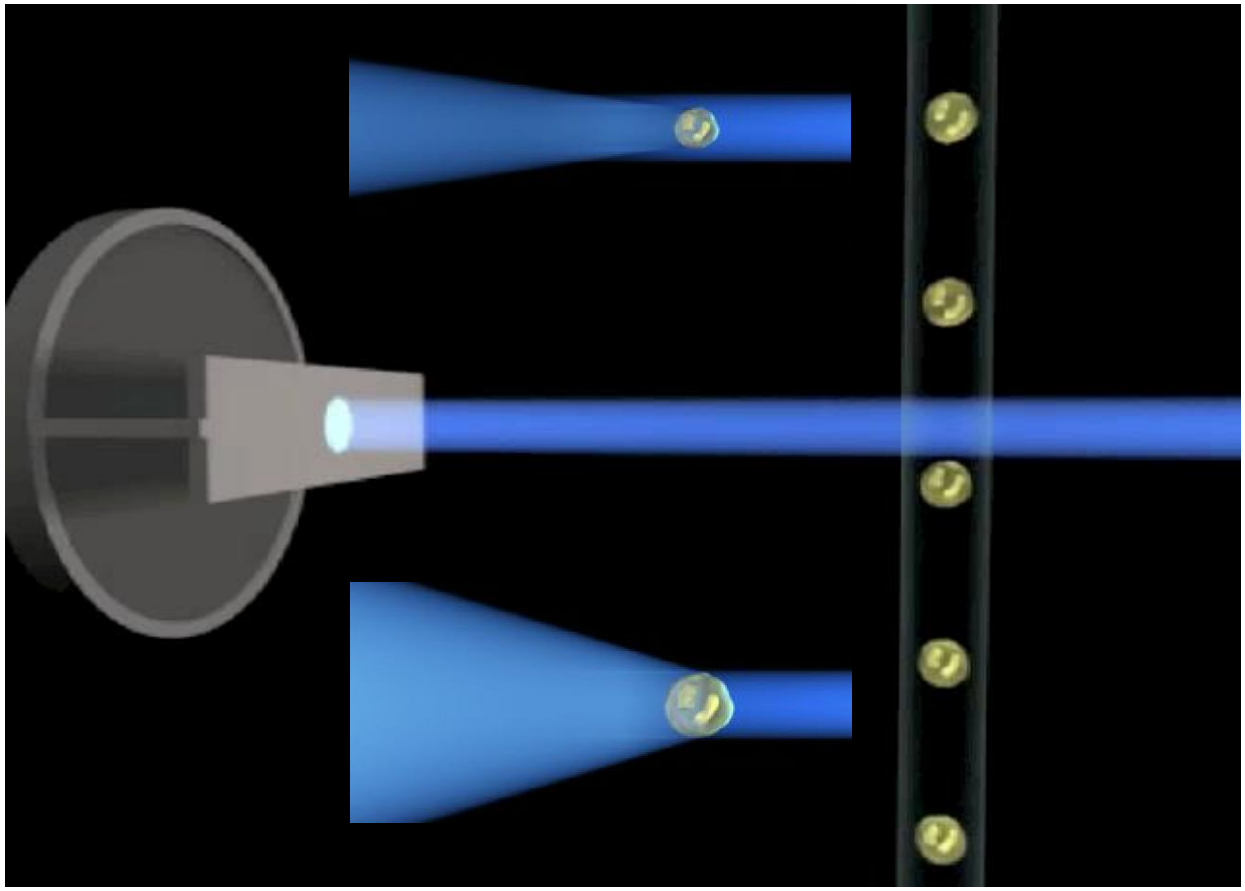
Laser Light Scatter

Forward Scattered light (FSC) is relatively proportional to cell-surface area or size

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



Flow Cytometry – FSC (Size)



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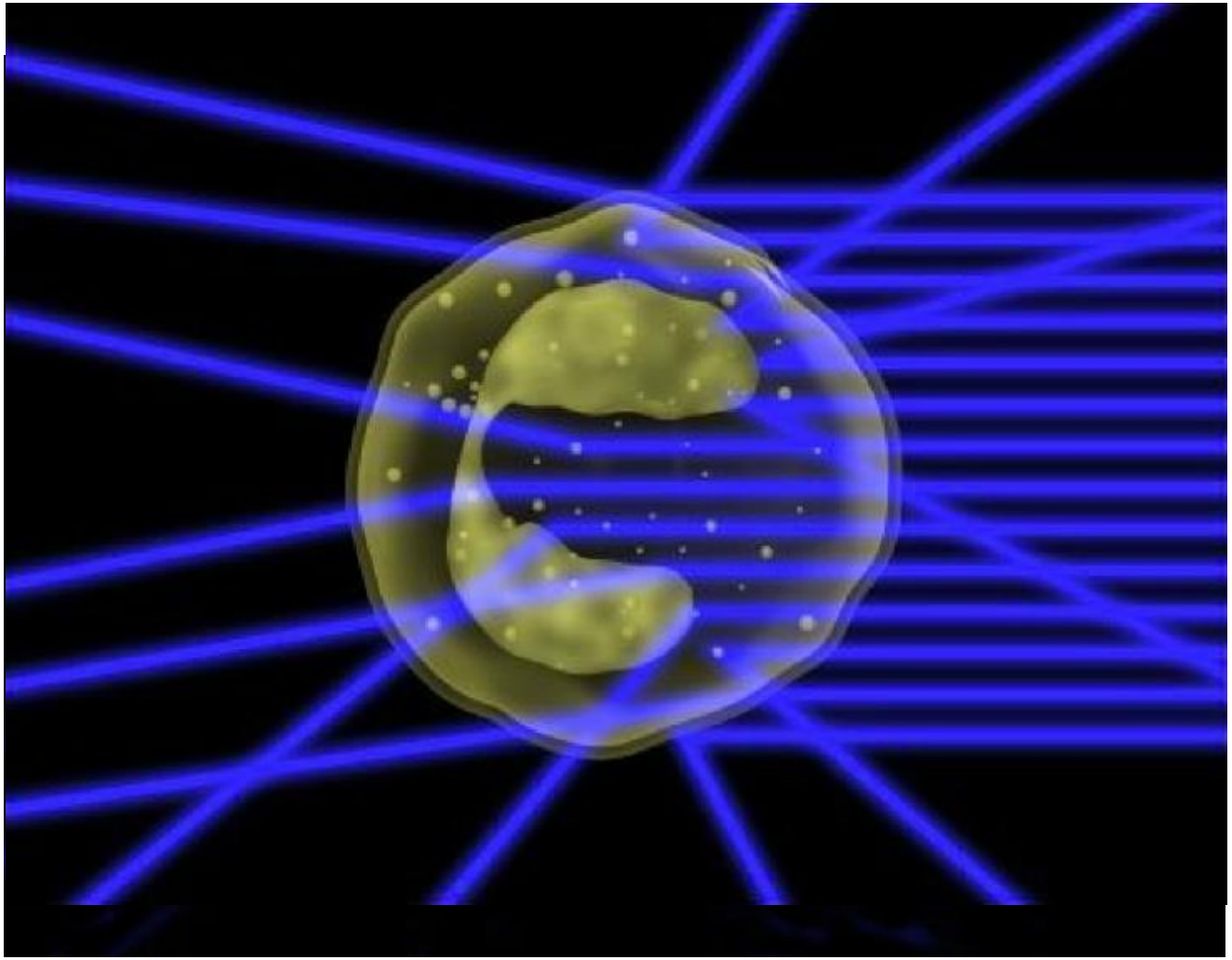
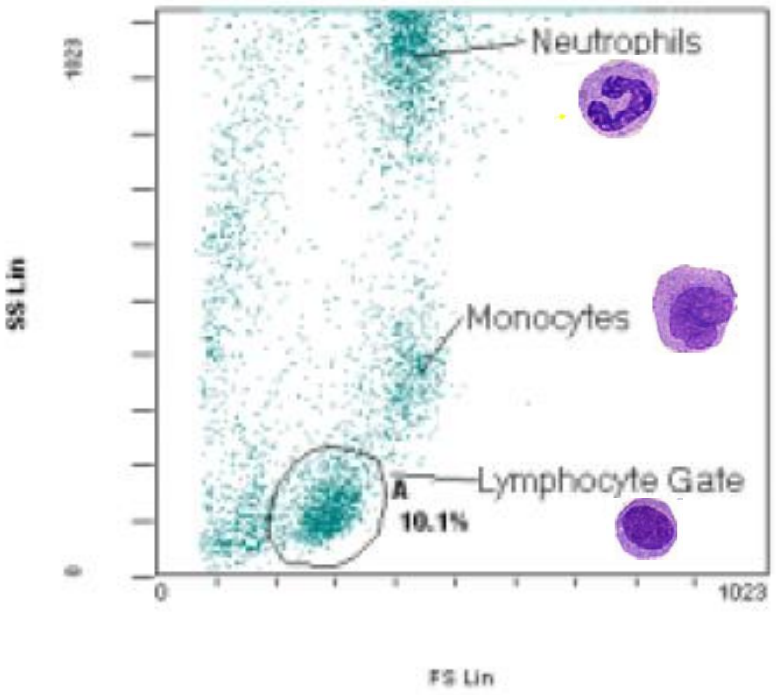
Flow Cytometry – FSC (Size), SSC (Complexity)



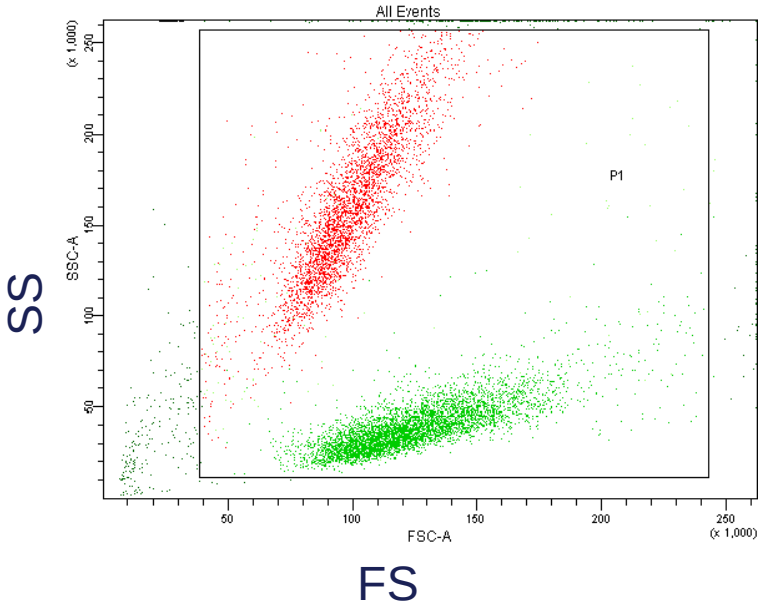
Laser Light Scatter

Measurements of FS and SS

- allow for differentiation of cell types in a heterogeneous cell population
- look for changes in cell health



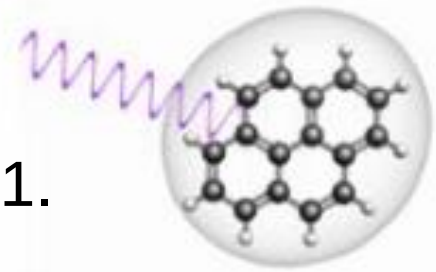
Ammonium chloride lysed whole blood

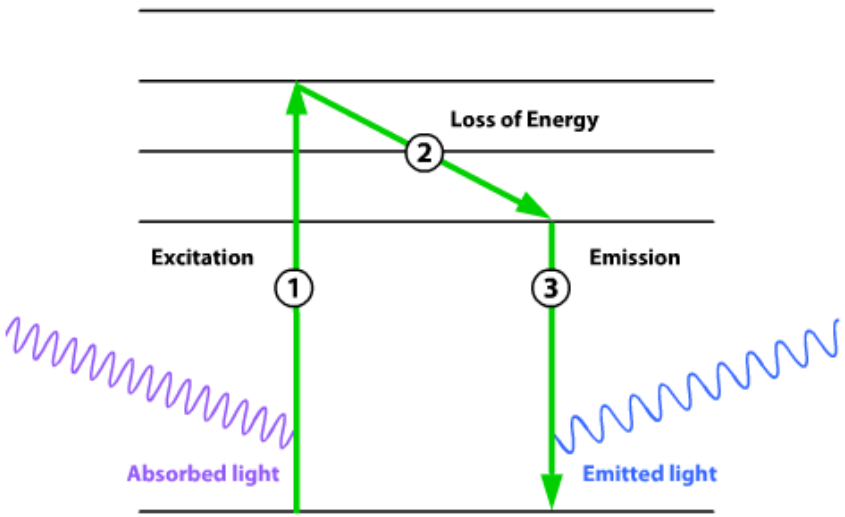


Aged culture of Jurkat T cells: green are live cells & red are dead cells

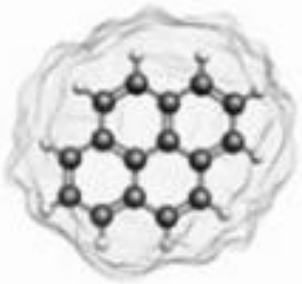
Fluorescence

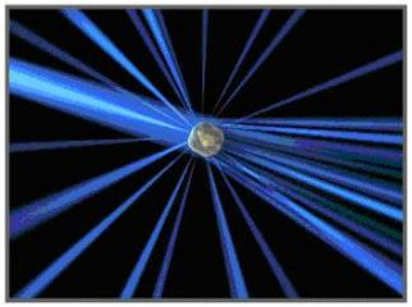
1.





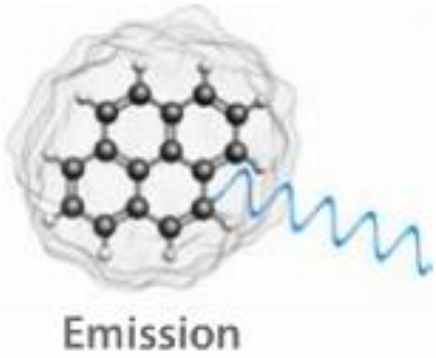
2.



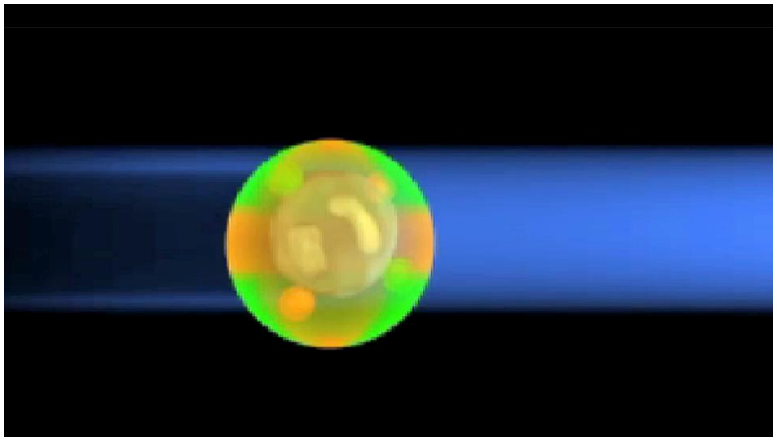


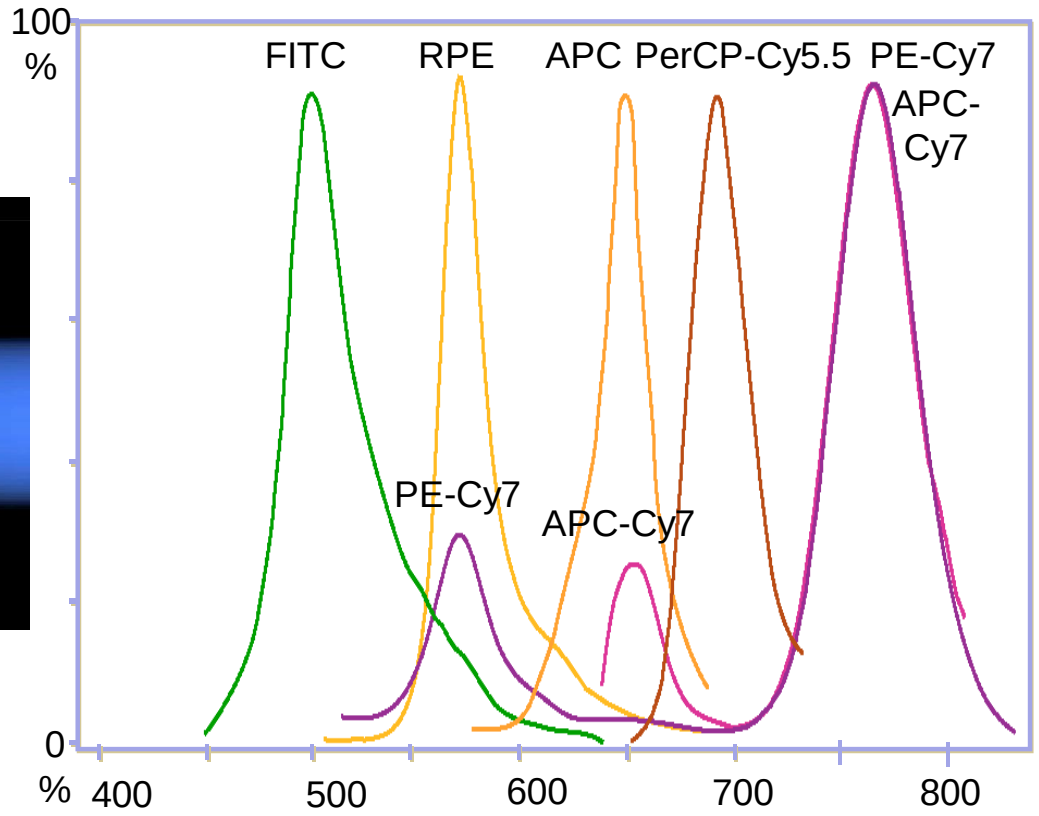
Light measured at 90˚

3.



Flow Cytometry - Fluorescence





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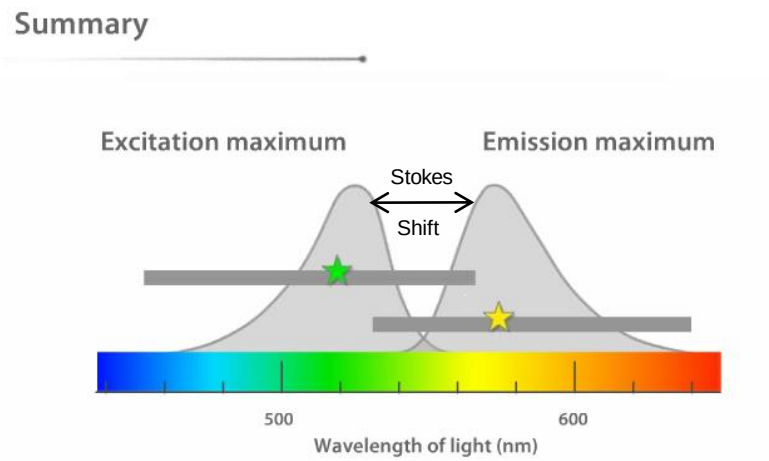
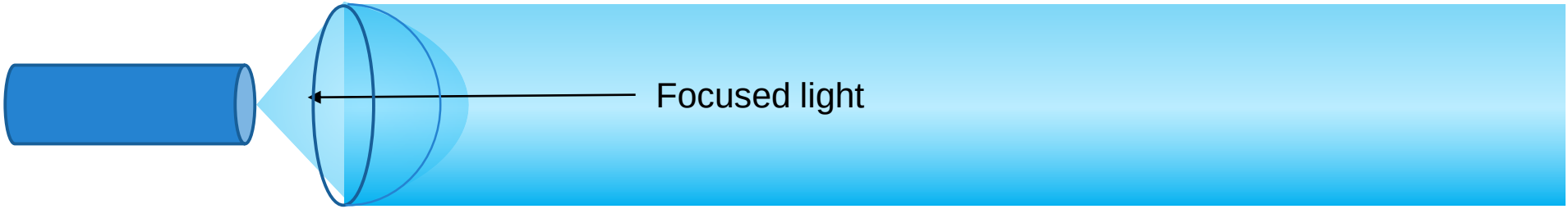
Fluorescent Light – Common Definitions

- **Absorption (Excitation) spectrum**: The wavelength range over which a fluorescent compound can be excited
- **Emission spectrum**: The range of emitted wavelengths of a fluorescent compound, it is a longer wavelength than the absorption wavelength

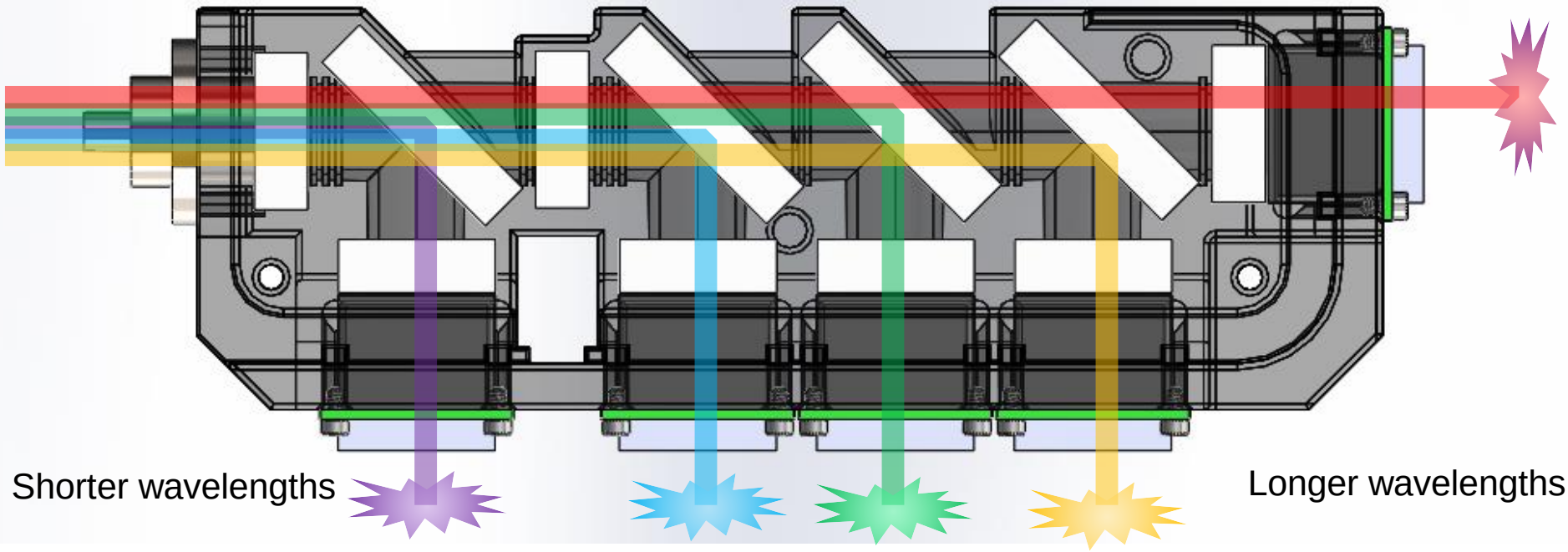


- **Auto-Fluorescence**: fluorescence that originates from endogenous sample constituents which are excited by lasers and detected on PMT usually between 400-600nM.
- **Non-specific fluorescence:** Unbound or nonspecifically bound probes – this may increase 'apparent' autofluorescence.

Filtered Light Emission Path – Generalized Configuration

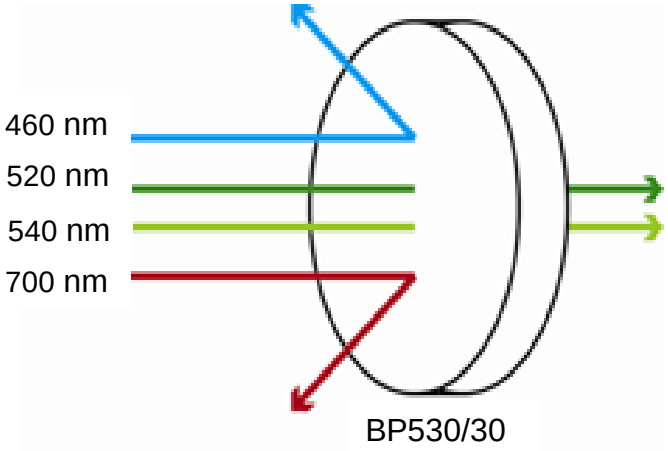


Optical filters and mirrors to direct and split light

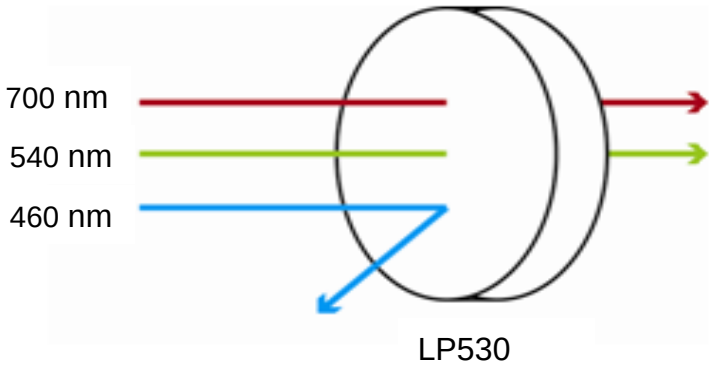


Filters

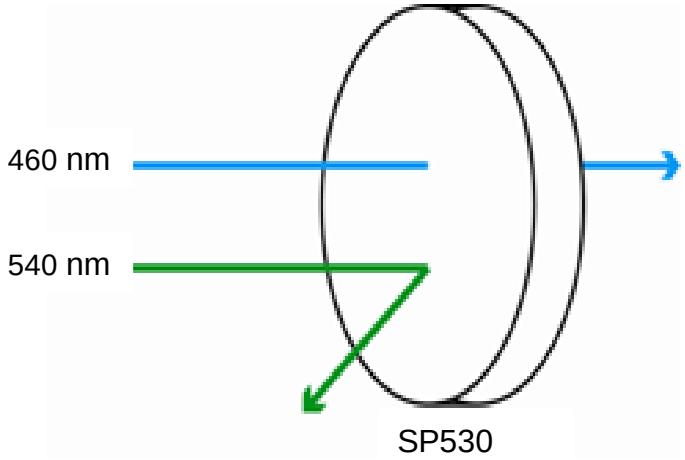
Bandpass Filter



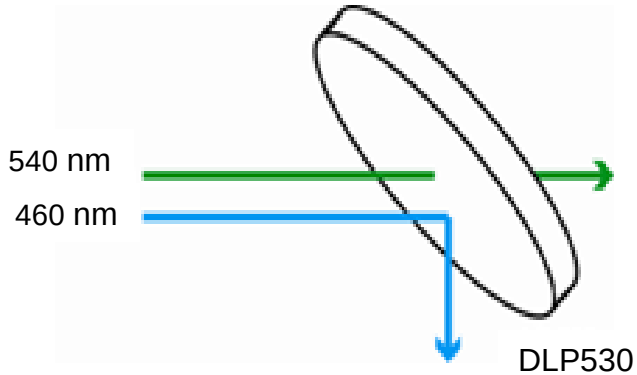
Longpass Filter



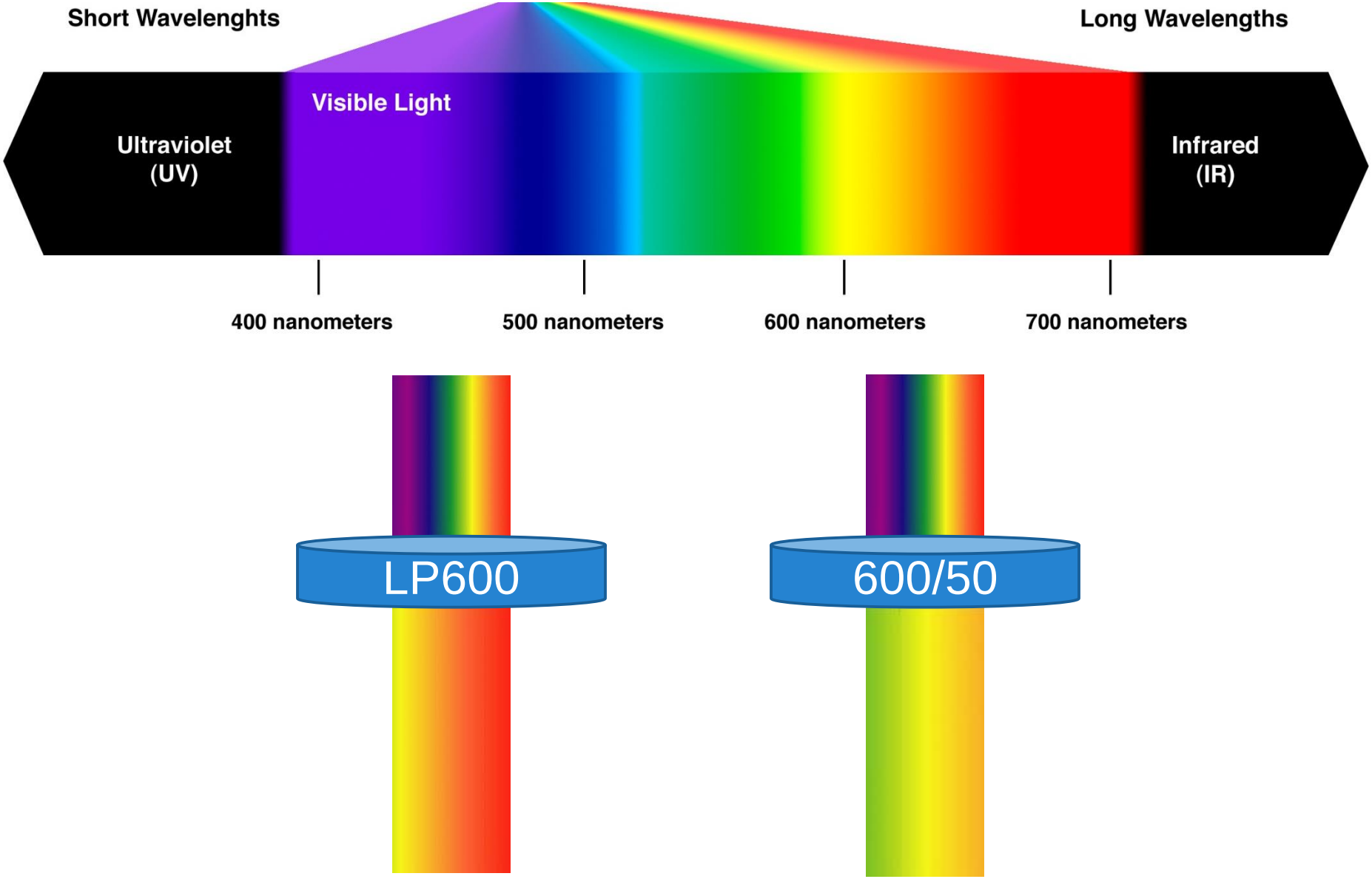
Shortpass Filter



Dichroic Mirror



Filtering Emitted Light



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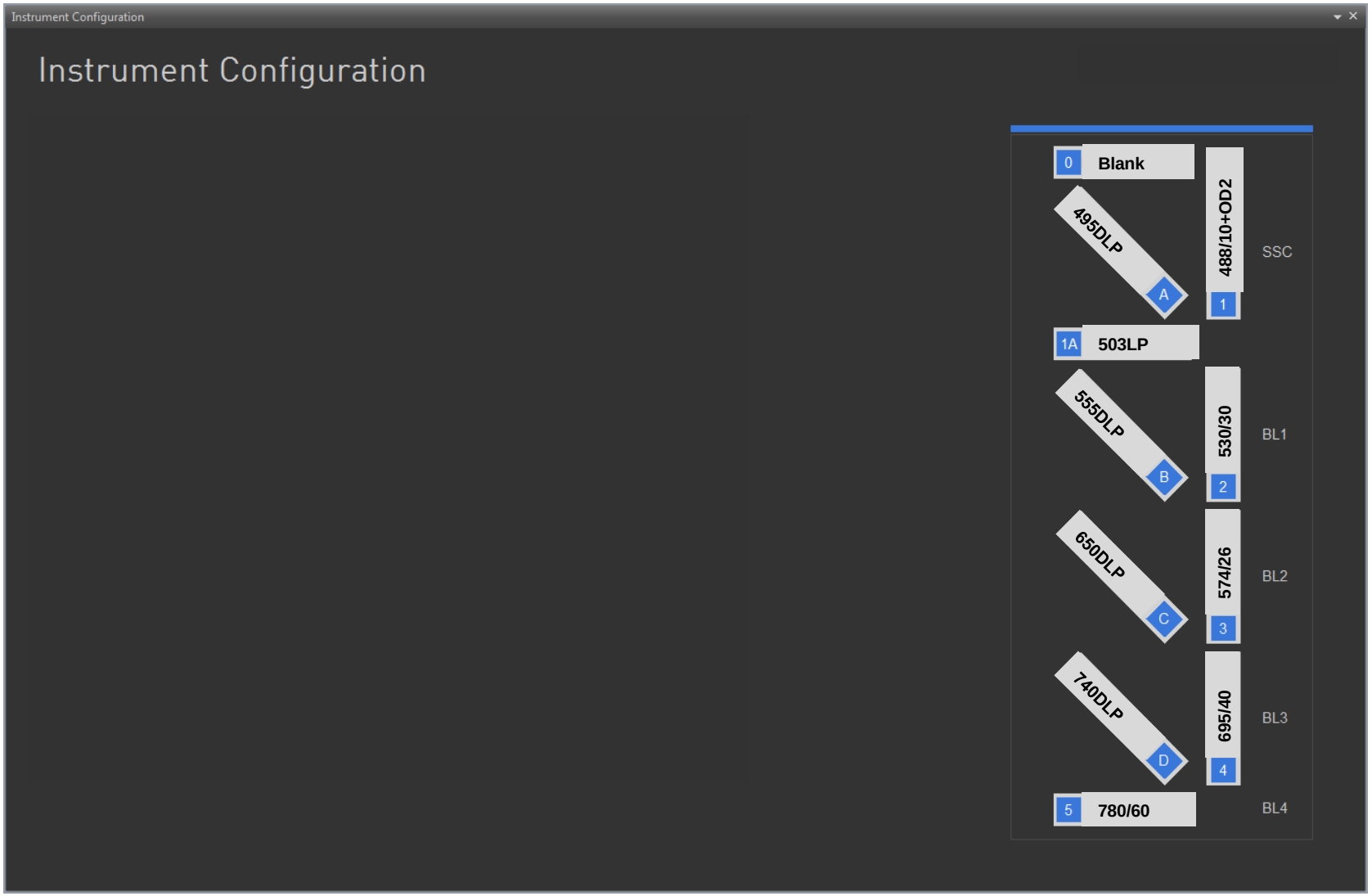
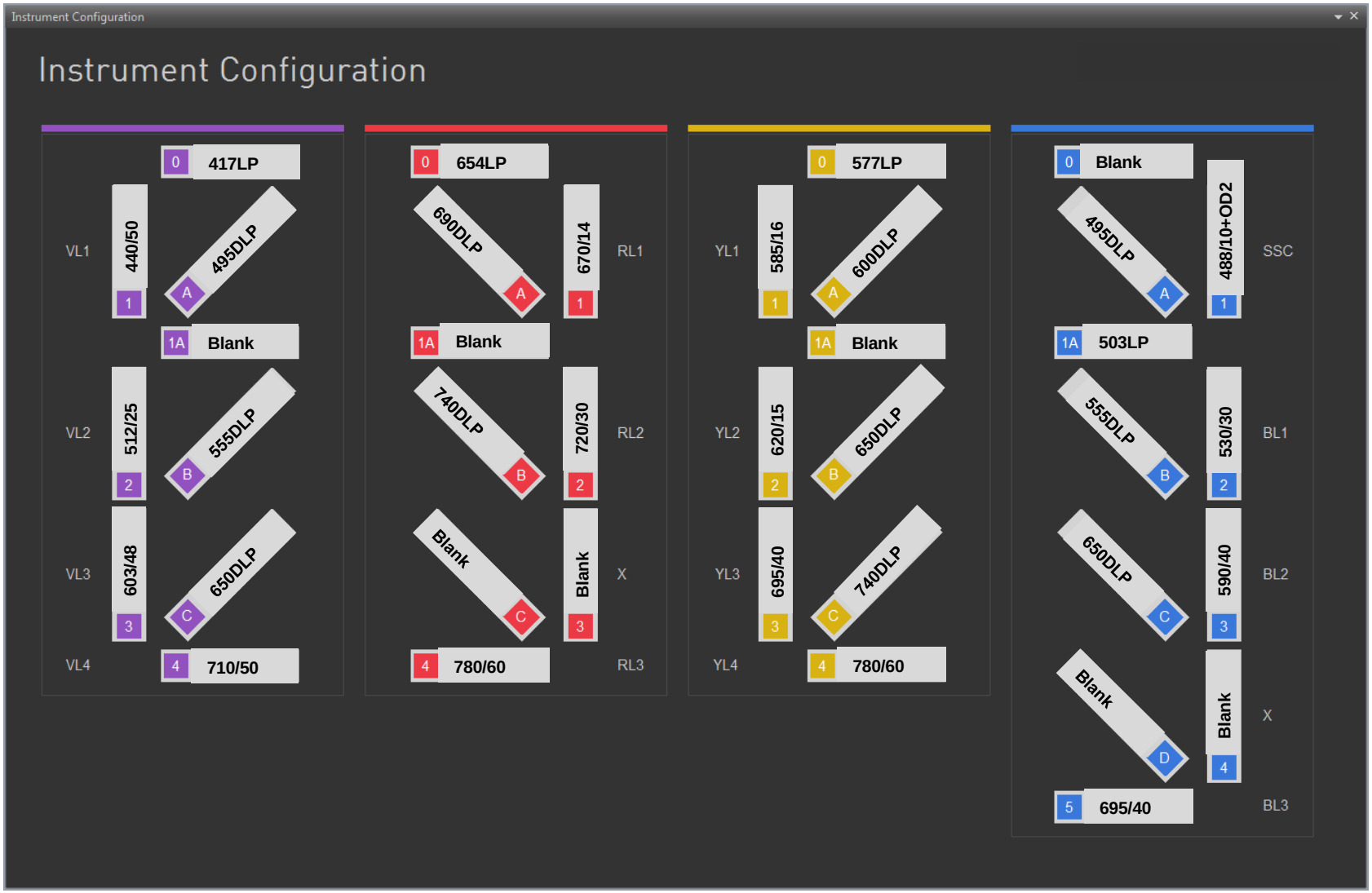
Blue - Standard Configuration



ThermoFisher SCIENTIFIC

Violet Blue Yellow Red - Standard Configuration



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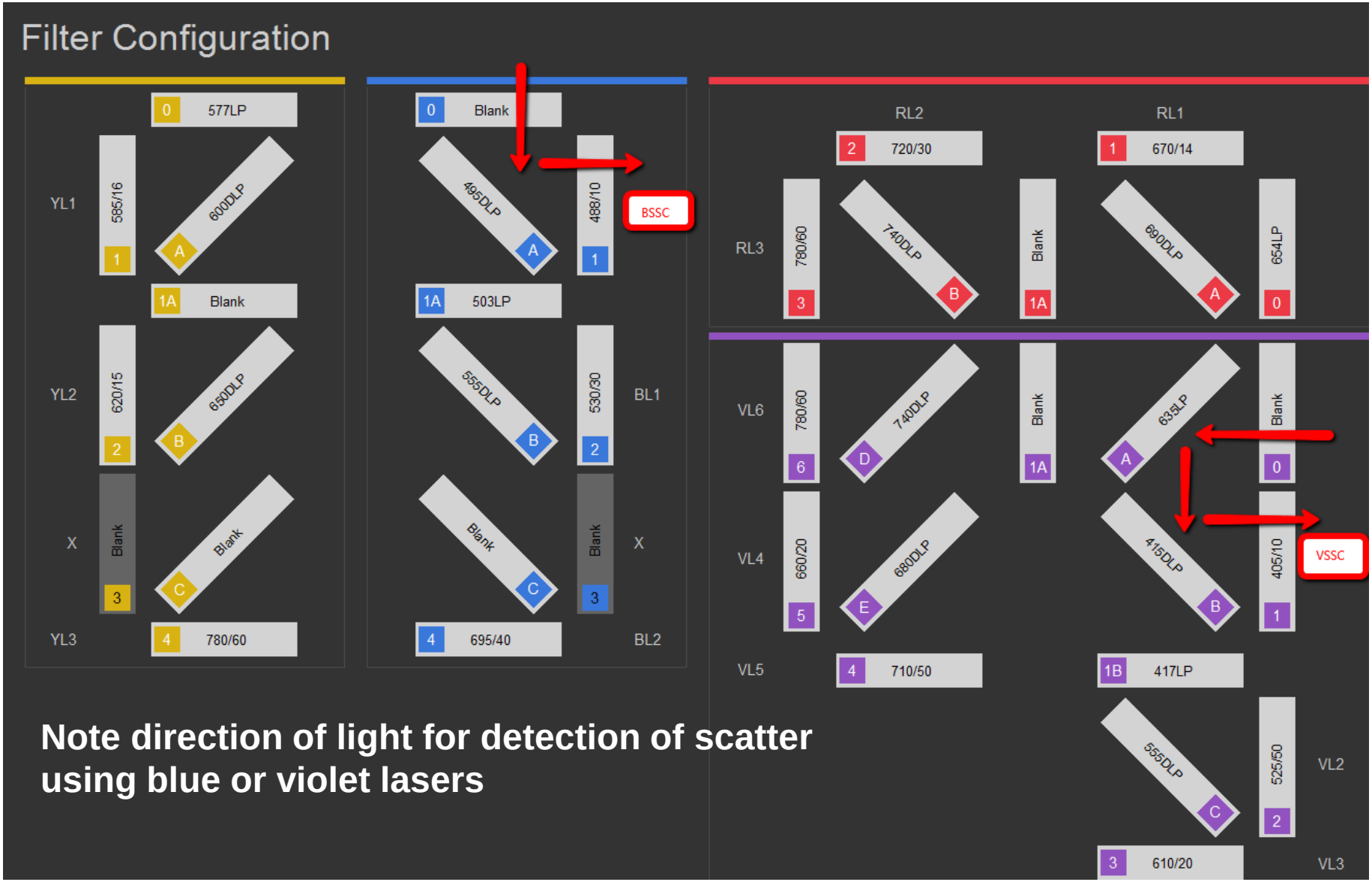
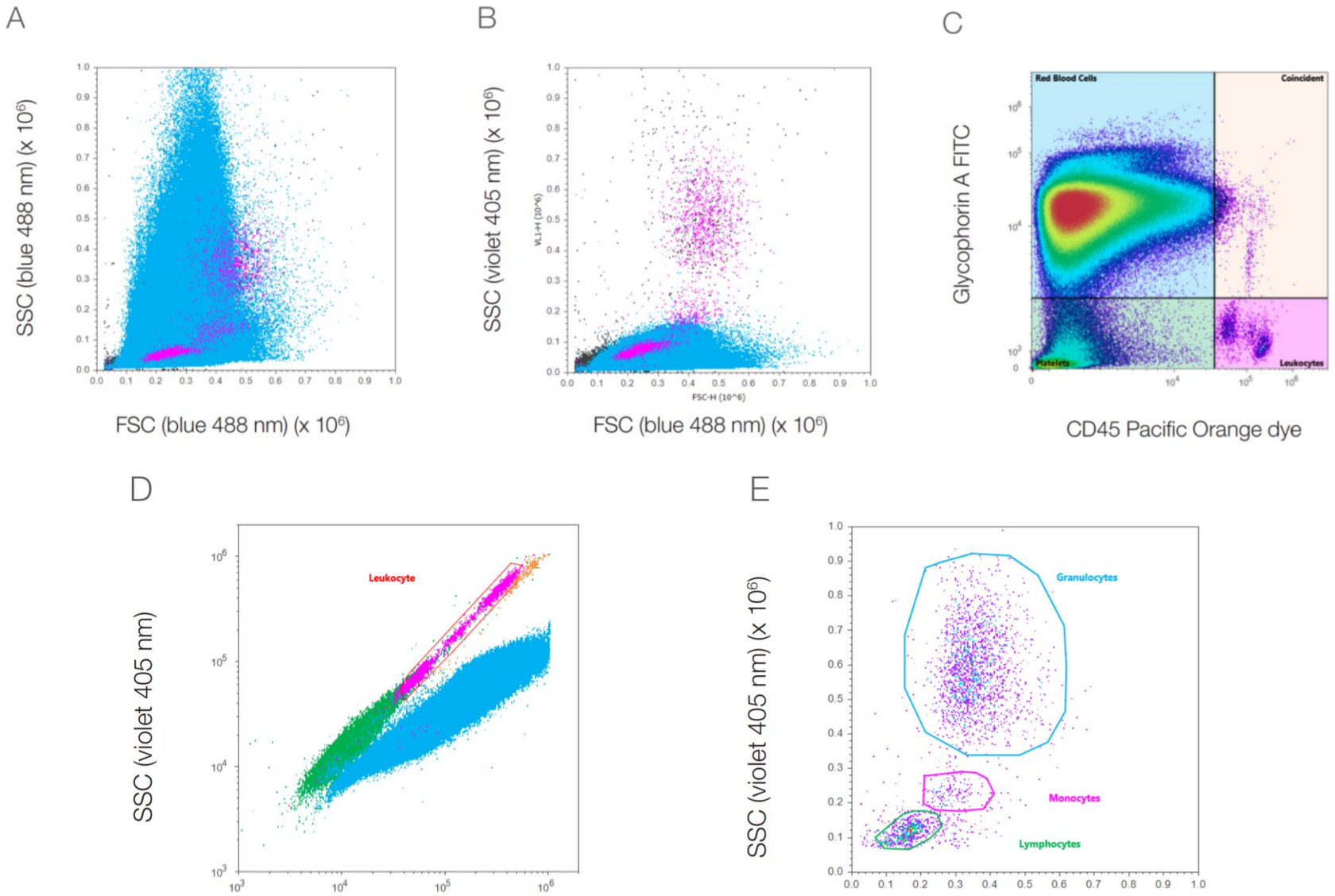
Blue and Violet Side Scatter (with NLNW filter kit installed)



Note direction of light for detection of scatter using blue or violet lasers

No-Wash, No-Lyse Detection of Leukocytes



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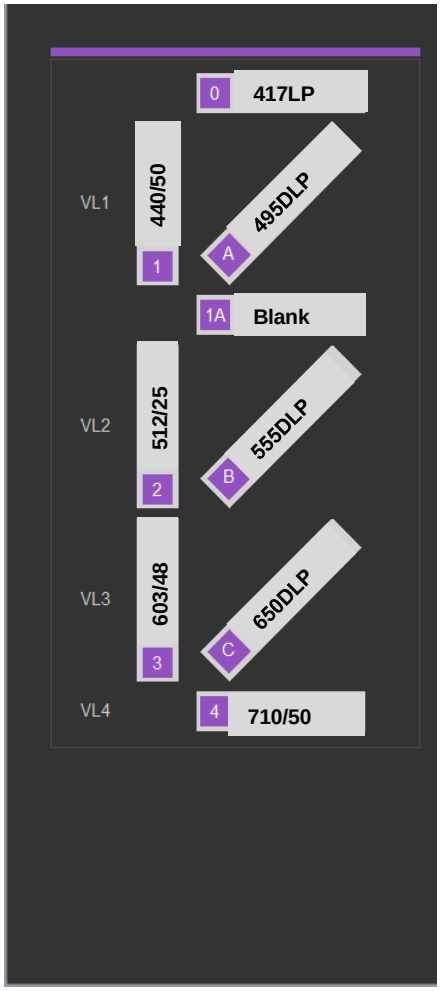
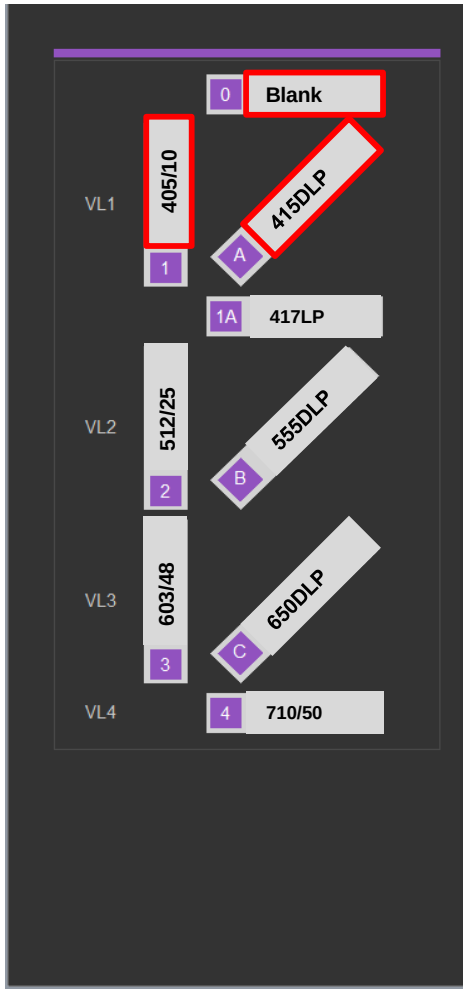
Violet SSC Configuration



Violet Side Scatter Kit

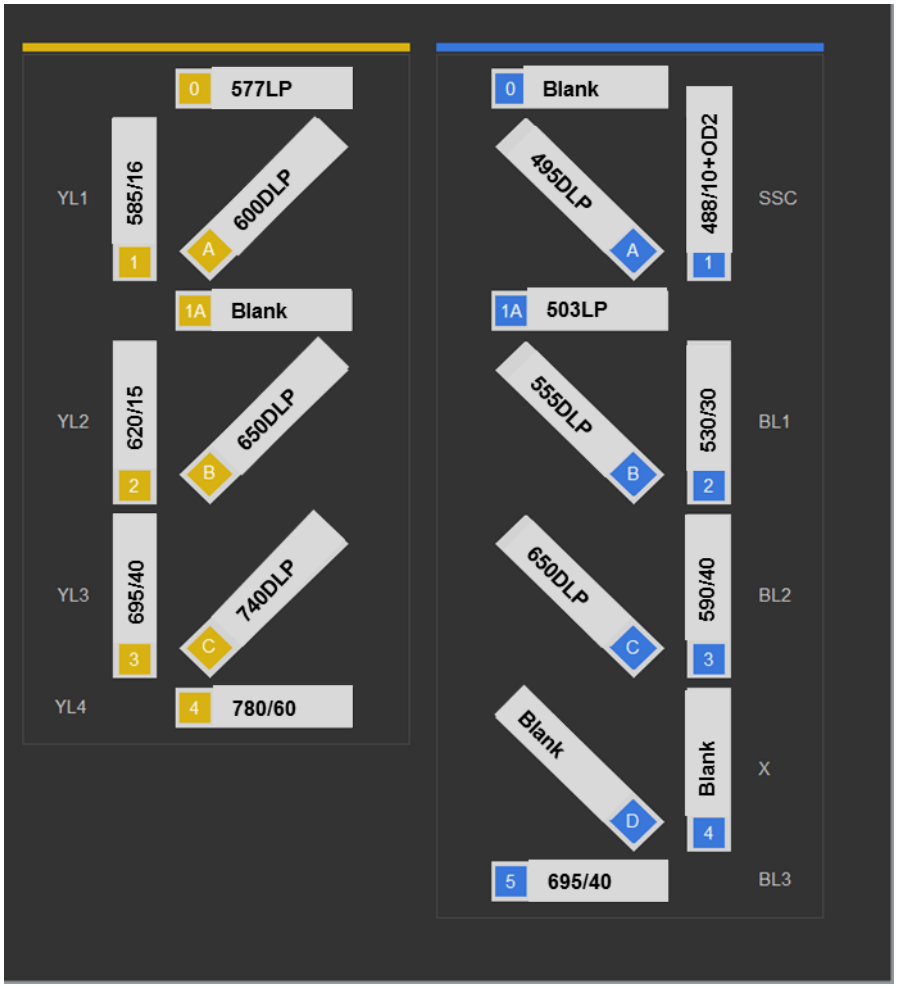
415 dichroic LP
405/10 BP filter
Blank 25 mm holder

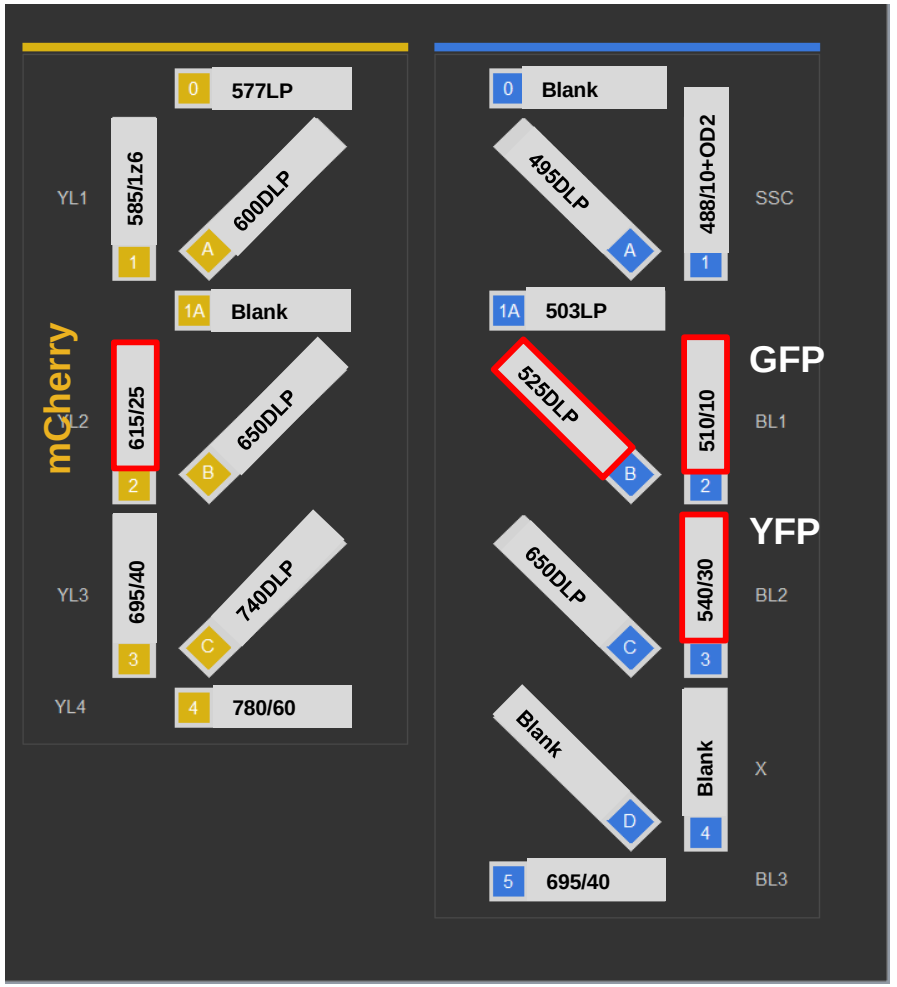


Fluorescent Protein Configuration

mCherry 615/25 BP filter

GFP 510/10 BP filter
YFP 540/30 BP filter
525 dichroic LP





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Attune NxT Systems

Fluidics

Optics

Electronics

Electronics

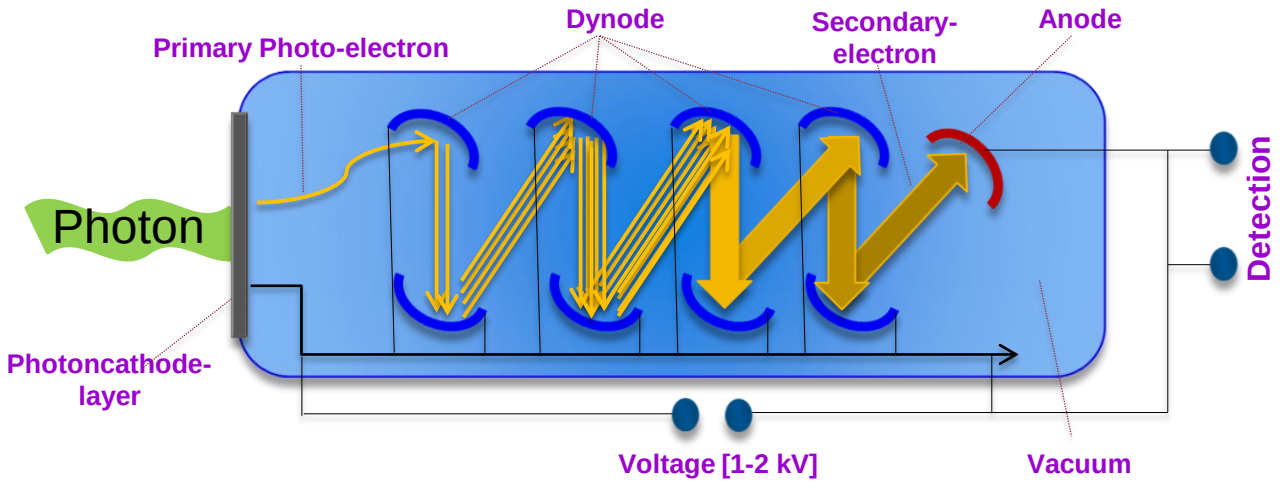
Functions of Electronics:

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

Flow Cytometry Detectors

Photomultiplier tube (PMT):

- Often referred as the “detector”
- PMT convert photons into electrons and amplify them to create a voltage pulse.



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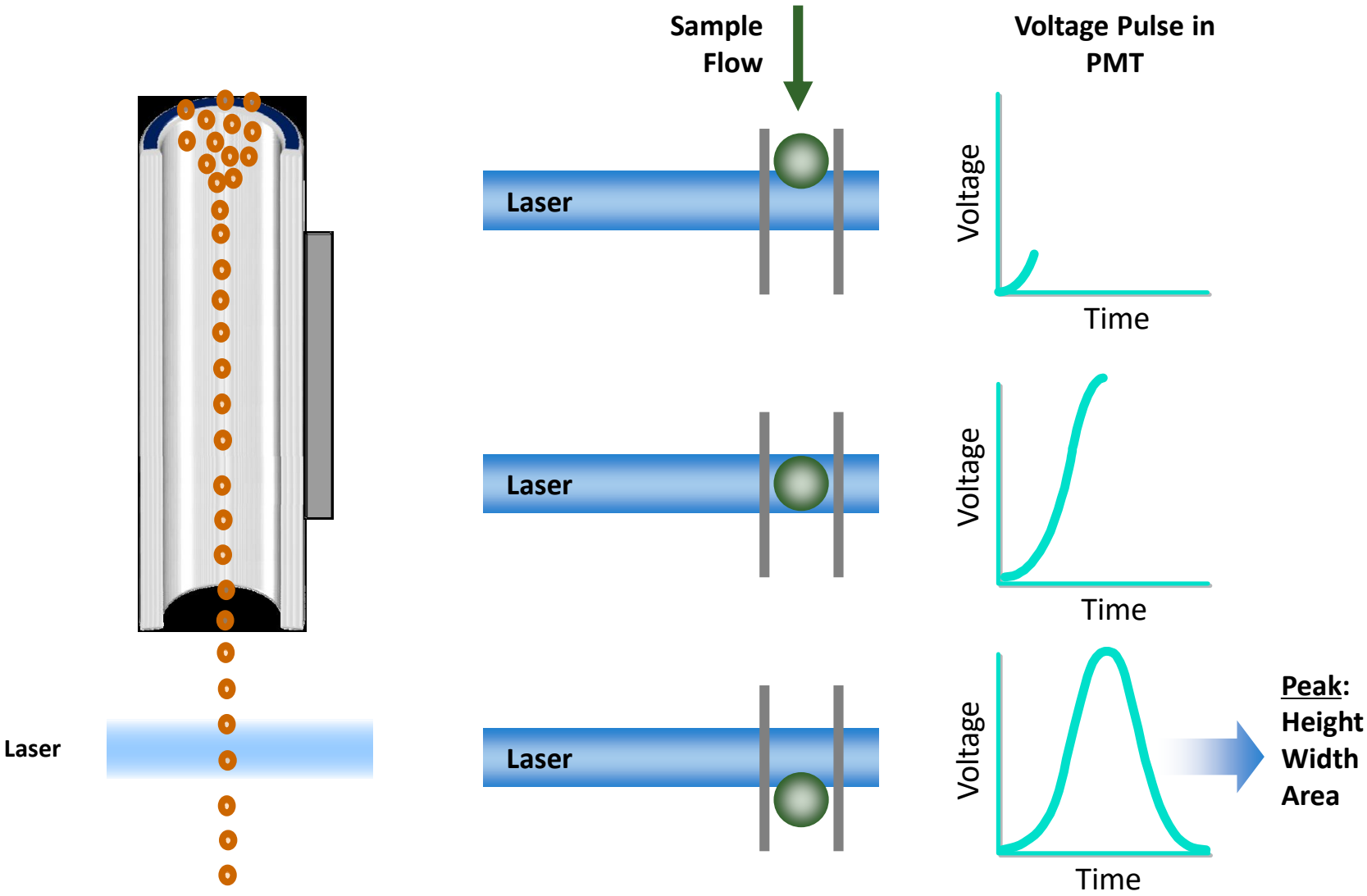
Sample Presentation: Voltage Pulse



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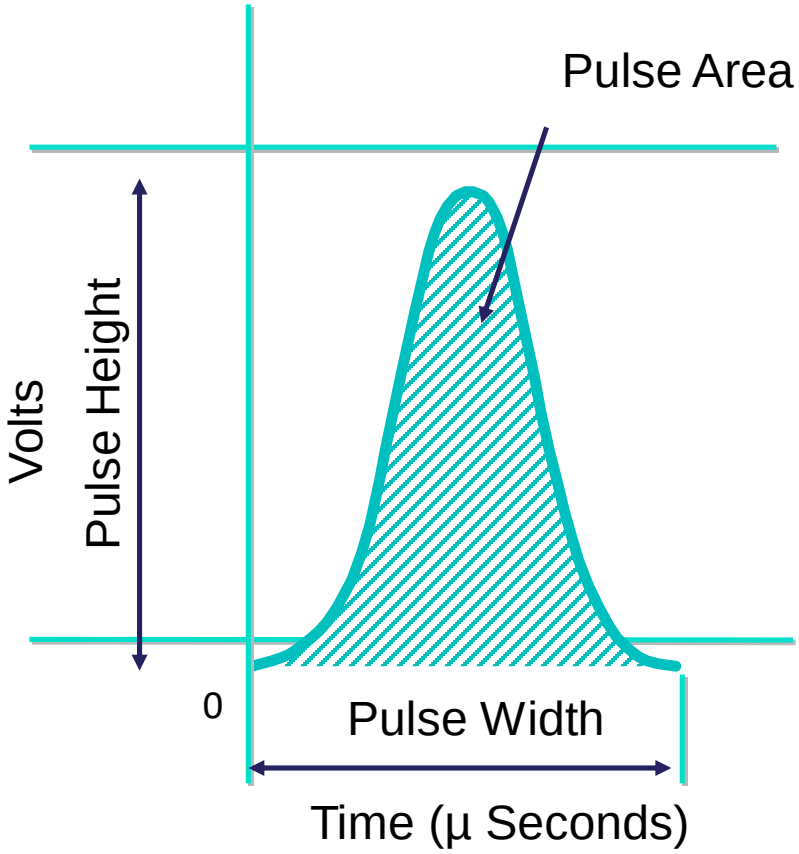
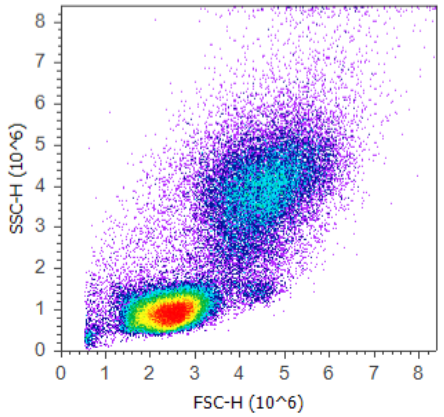
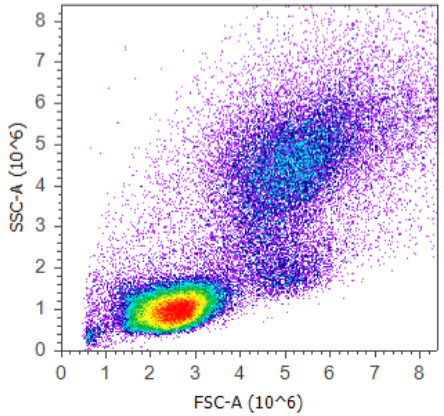
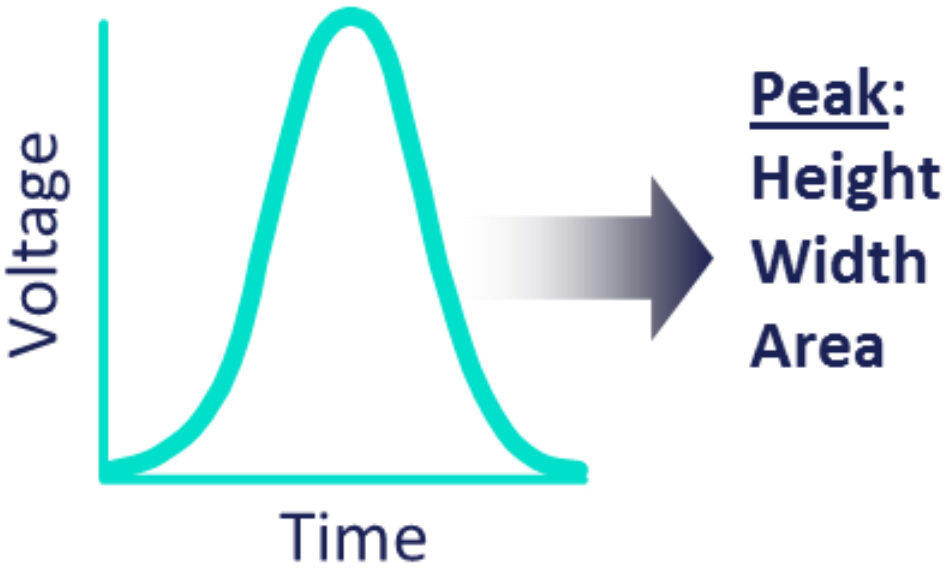
Sample Presentation: Voltage Pulse







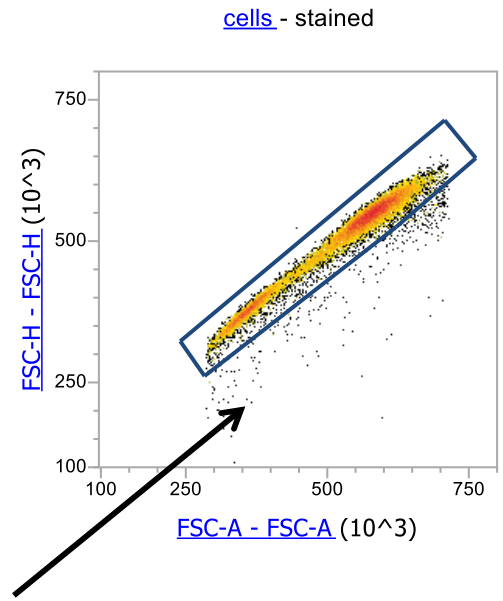
Pulse to Parameters



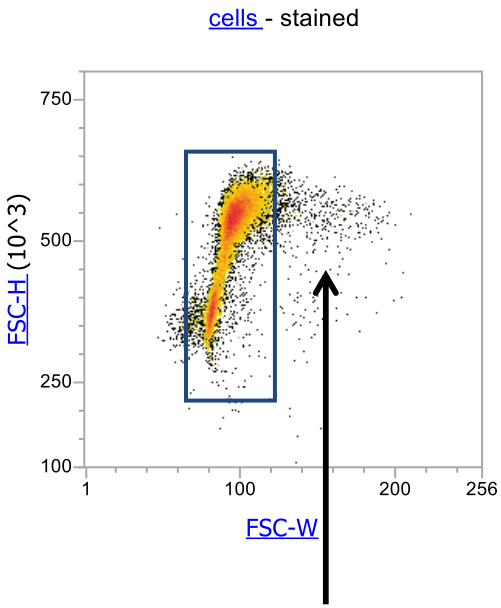
Attune default: All parameters (A-H-W) on all channels

Doublet Discrimination – 3 Ways to Display (blood)

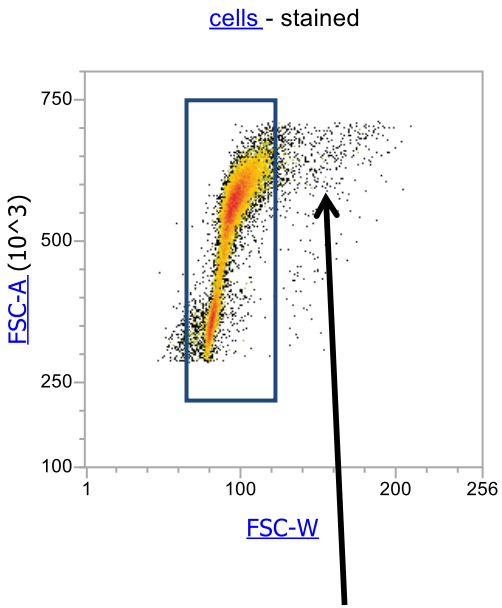
In most cases, data analysis should include gating on single cells.



Doublets



Doublets



Doublets

Pulse height = pulse area

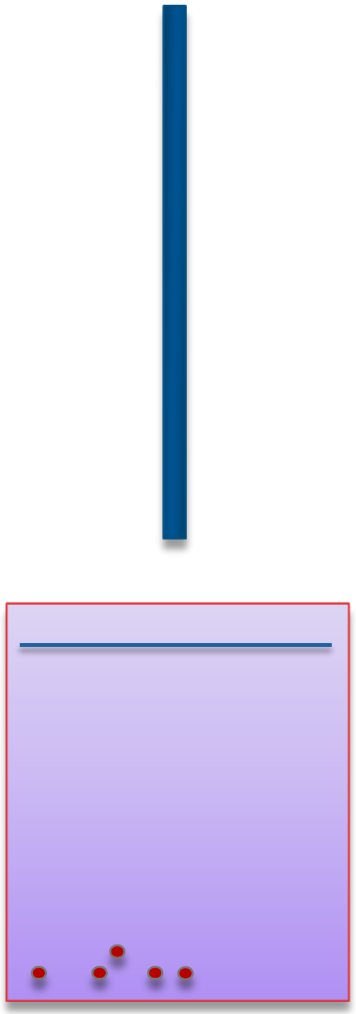
Attune NxT Systems

Attune® NxT AutoSampler

Attune® Autosampler

- Compatible with many different standard plate formats, including 96-well, flat, round and V-bottom.
- Round Bottom plates are recommended for optimal mixing
- 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

Attune® Autosampler Mixing procedure



The user sets:

- The plate type
- The total sample volume
- The number of mixes

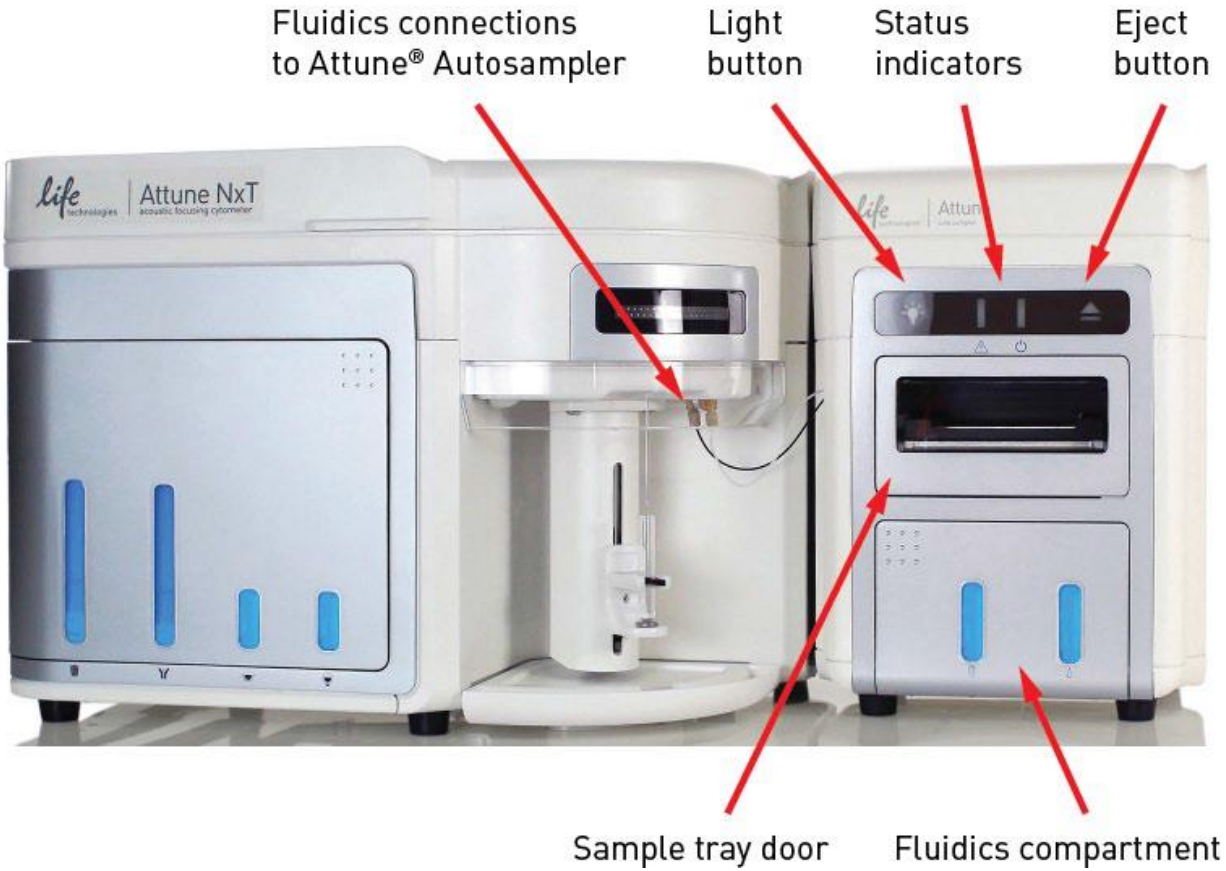
(Max. 3 mixes, recommended at this time)

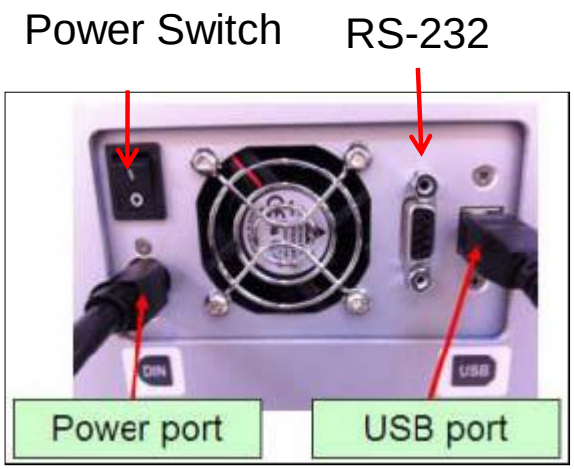
The system defines:

- The liquid level in well
- The probe position
- The mixing method

Mixing sample by aspiration instead of shakings ensures homogeneity of the sample and maintains cell viability

Exterior Components





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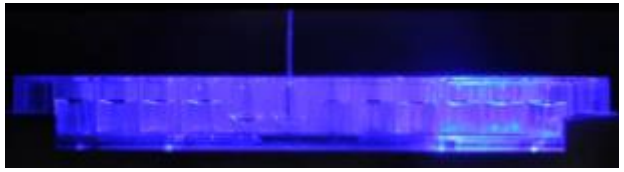
Interior Components

Sample Probe



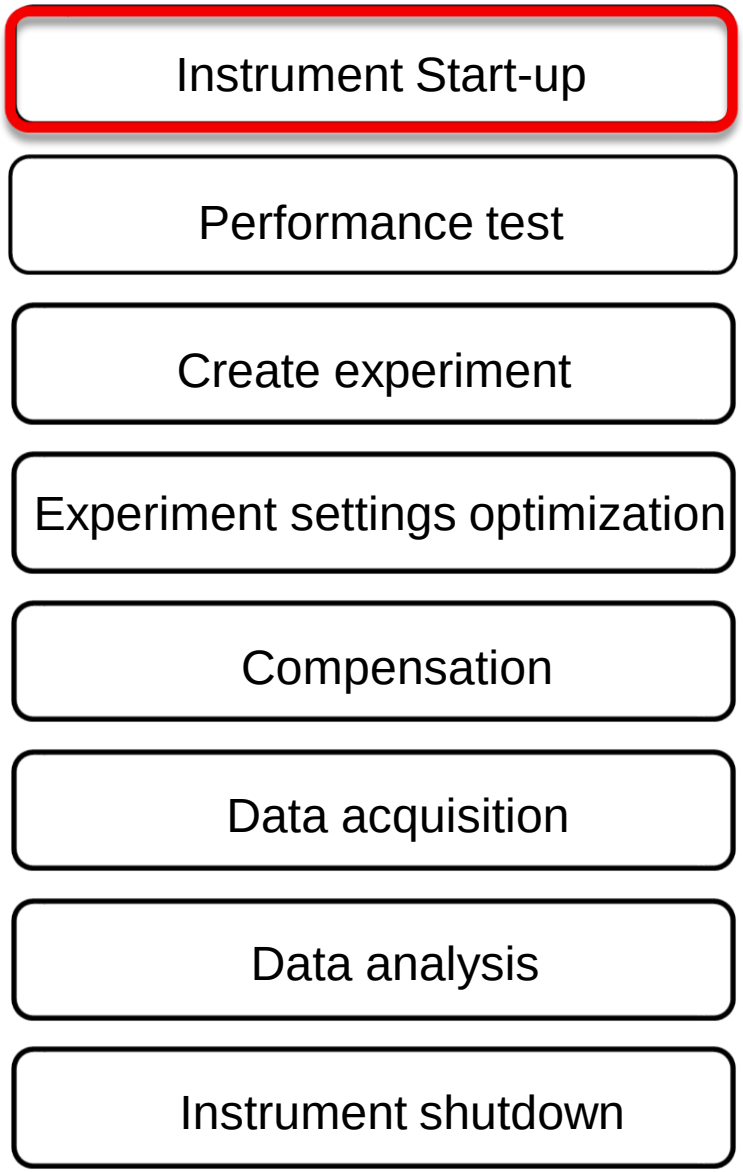
Leak Sensor

1ml Syringe

Waste

Focusing Fluid

Workflow



Instrument Startup

Startup function:

- Warms up the lasers
- Initializes the pumps
- Primes the instrument fluidics
- Flushes out the shutdown solution

Ensures that:

- All fluidic lines are clean
- The fluidic lines and the syringe pump are filled with fresh focusing fluid
- The lasers are warmed to operating temperature

Startup

Takes 3.5 minutes

Uses ~25 ml of focusing fluid

Instrument Startup

From power off

1. Turn on autosampler remove cleaning plate and close tray door
2. Turn on the cytometer and wait until status light remain a solid, bright blue
3. Open the Attune NxT Software
4. Open the instrument tab and click startup icon

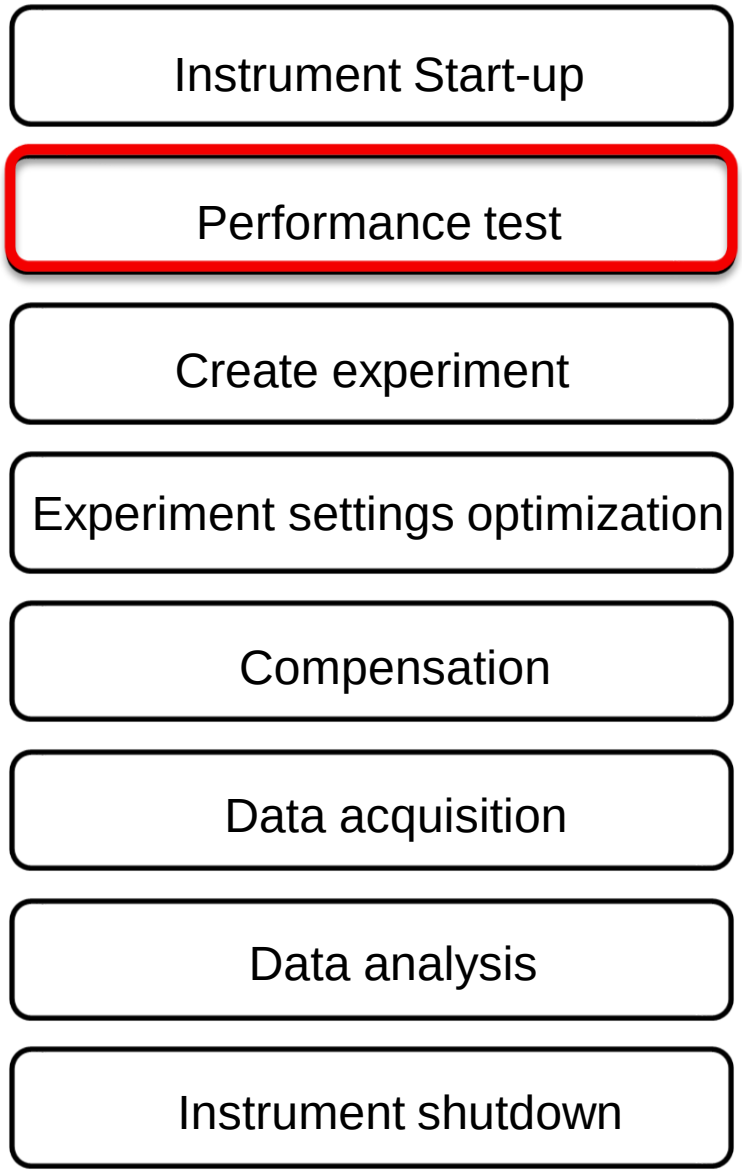
From Sleep mode

After shutdown completes

1. Close/re-open the software
2. Login
3. Open the instrument tab
4. Open the instrument tab and click startup icon

Once Startup is complete, the status lights are solid green and the status bar displays the *Ready* icon

Workflow



Performance Test

- Allows you to monitor performance of the instrument
- Critical to ensure accuracy and sensitivity of instrument
- Provides information about the lasers and detection channels
- Run immediately after launching the software application and running **Startup**

<u>There are 2 parts to Instrument Performance Tracking:</u>

1. Baseline Calculation (BL)
2. Daily Performance test (PT)

Performance Test ‘when to run’

Daily performance Test

Run daily - everyday samples are run/recorded

Baseline Calculation (advanced user/admin/sysadmin only)

Performed at time of installation by Field Service Engineer (FSE)

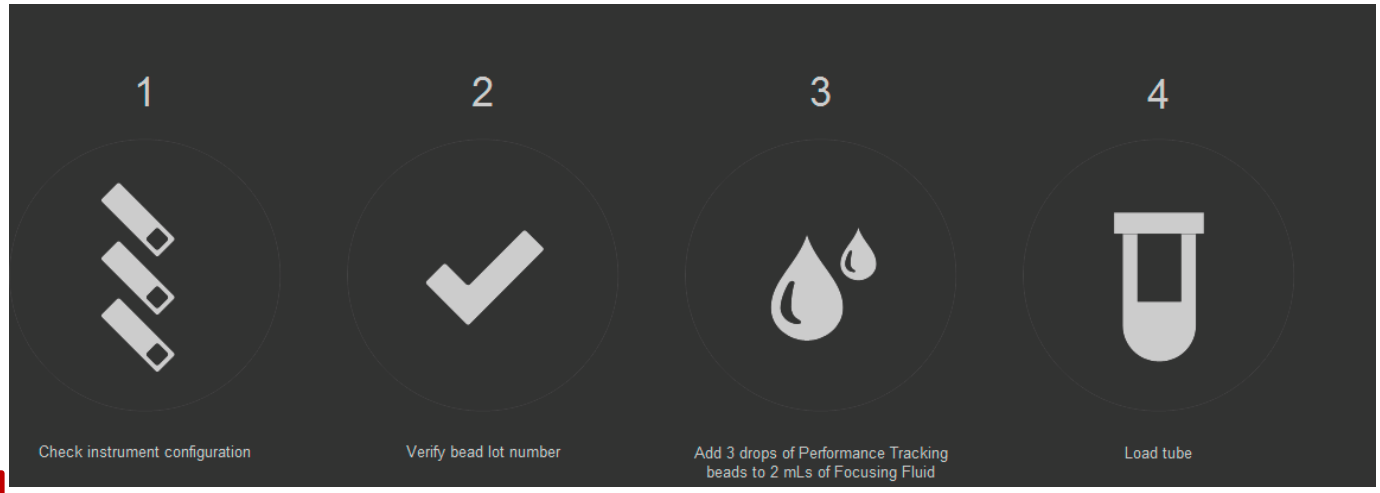
After any major service (FSE)

Every time the bead lot changes (User)

When recommended by FSE or FAS

Attune® Performance Tracking Beads

- A mixture of beads of four fluorescence emission intensities in equal concentration
  - Blank
  - Dim
  - Medium
  - Bright



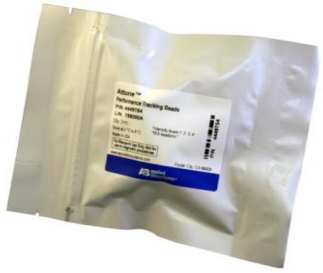
- 3mL vial

  PN: 4449754

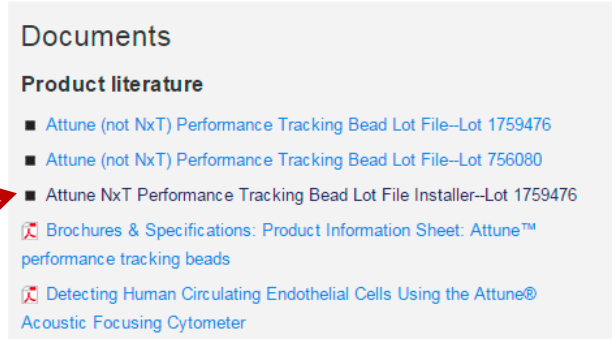
Lot # 2029773xx

Lot # batch #

3 drop of beads per 2 ml of Focusing Fluid or PBS



Notes: Data for a new lot of beads can be downloaded from the Performance Tracking beads webpage on the ThermoFisher website



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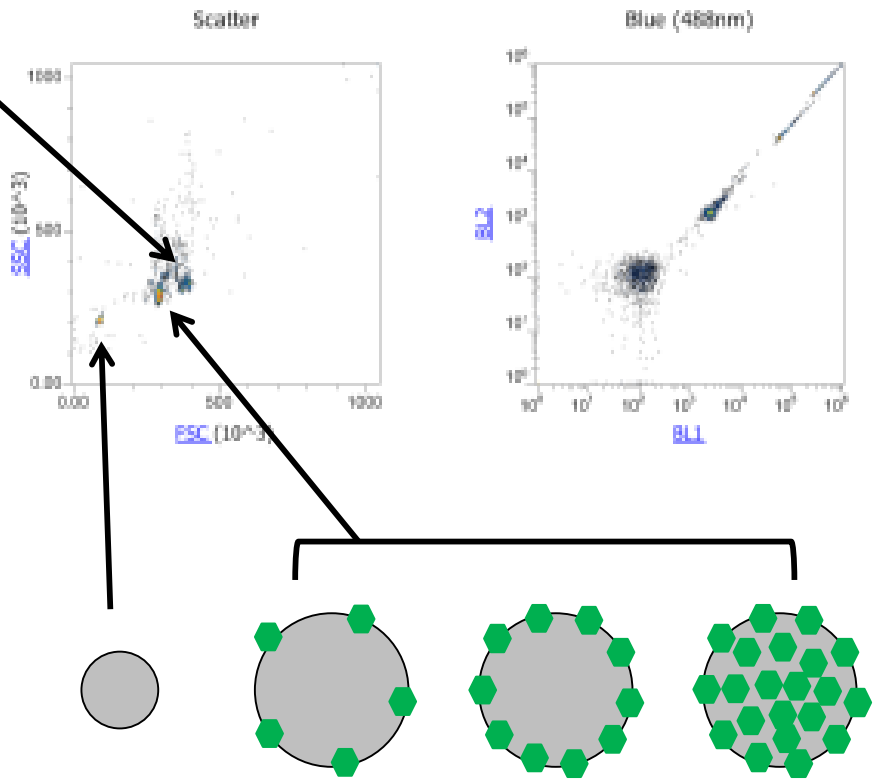
Performance tracking beads

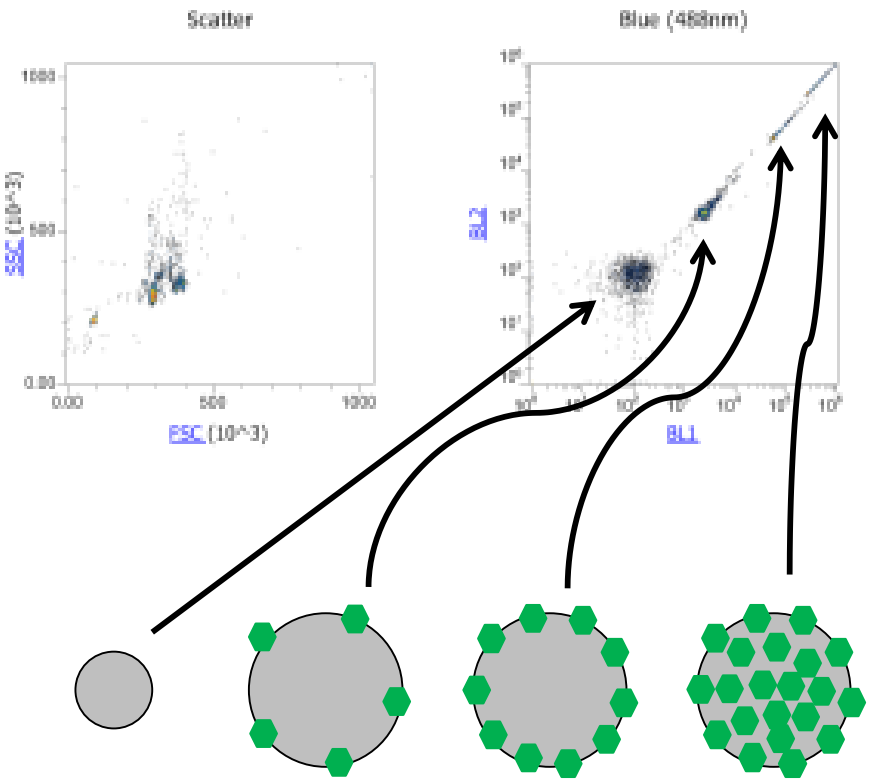
Bead doublets and multiples



Size:	small	large	large	large
Dye level:	none	low	med	high

ThermoFisher SCIENTIFIC

Performance tracking beads



Size:	small	large	large	large
Dye level:	none	low	med	high

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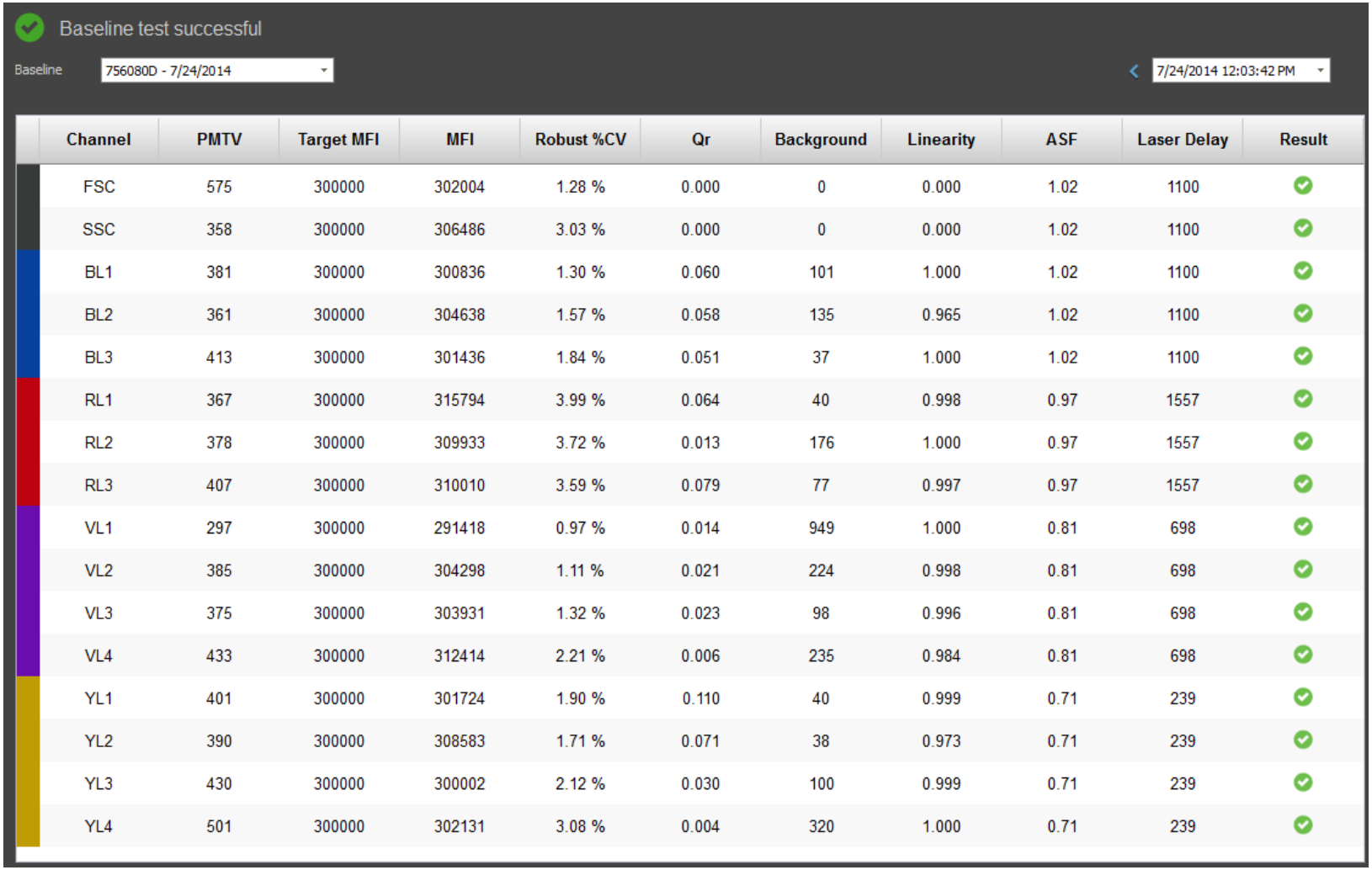
Baseline Calculation and Performance Test

- **Baseline** uses Performance Tracking beads 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 reported
  - The robust % coefficient of variation (Robust %CV) of the brightest bead is recorded
  - Relative quantum efficiency (Qr) of each detector is determined
  - Relative Background level (Background) of each detector is determined
  - Linear regression (Linearity) is calculated and recorded
  - Area scaling factor (ASF) is calculated and reported for every laser and automatically updated in "Advanced Settings"
  - Laser delay setting is automatically calculated

- Performance test uses Performance Tracking beads to monitor changes over time
  - Same process/measurements as Baseline - reports all
  - The change of the PMT voltage **(Δ PMT)** from baseline is also reported

Baseline Report



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	✓

Pass 

Fail 

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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 

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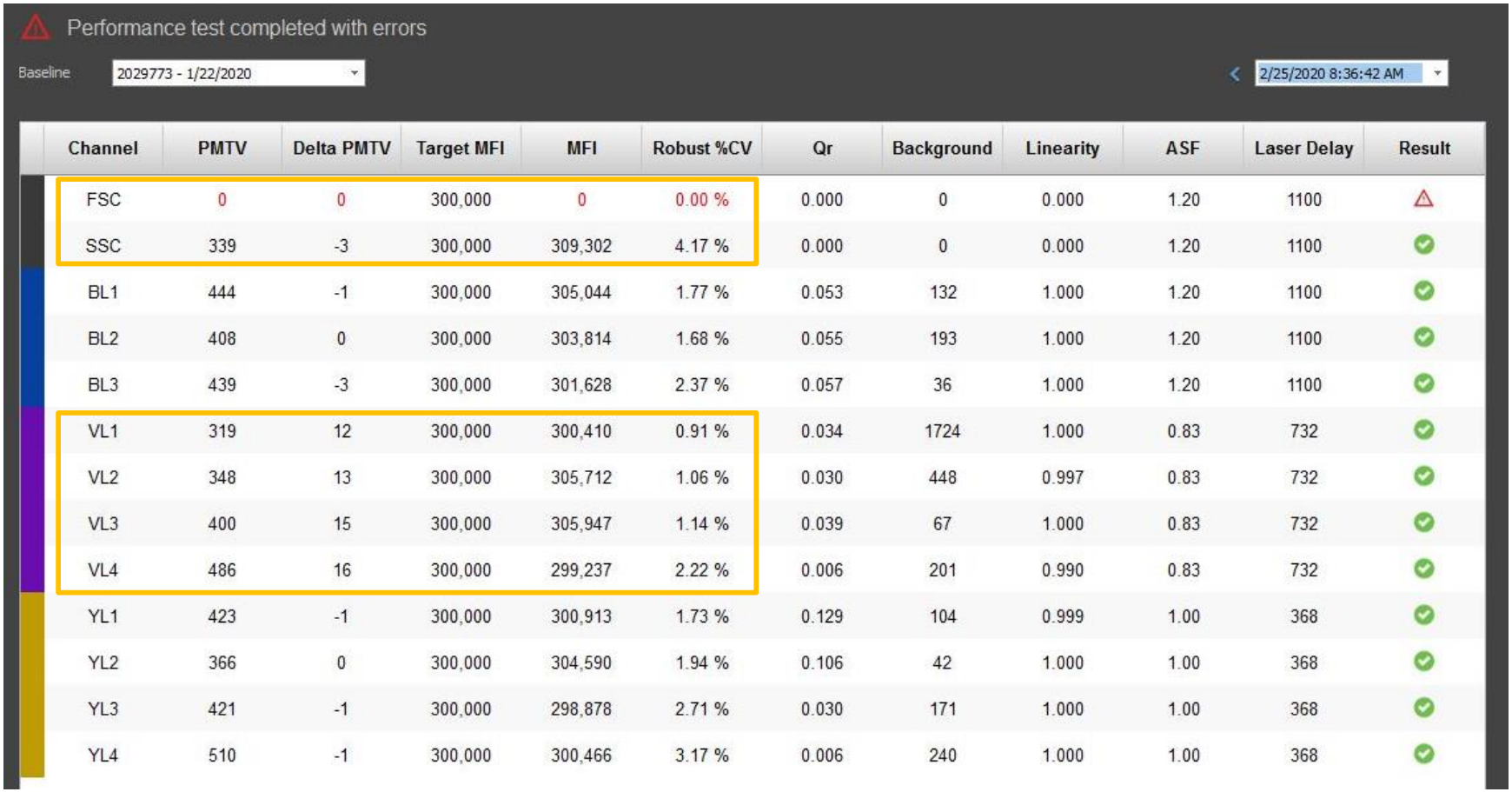
Performance Test Territory



Channel	PMTV	Delta PMTV	Target MFI	MFI	Robust %CV	Qr	Background	Linearity	ASF	Laser Delay	Result
FSC	0	0	300,000	0	0.00 %	0.000	0	0.000	1.20	1100	⚠
SSC	339	-3	300,000	309,302	4.17 %	0.000	0	0.000	1.20	1100	✓
BL1	444	-1	300,000	305,044	1.77 %	0.053	132	1.000	1.20	1100	✓
BL2	408	0	300,000	303,814	1.68 %	0.055	193	1.000	1.20	1100	✓
BL3	439	-3	300,000	301,628	2.37 %	0.057	36	1.000	1.20	1100	✓
VL1	319	12	300,000	300,410	0.91 %	0.034	1724	1.000	0.83	732	✓
VL2	348	13	300,000	305,712	1.06 %	0.030	448	0.997	0.83	732	✓
VL3	400	15	300,000	305,947	1.14 %	0.039	67	1.000	0.83	732	✓
VL4	486	16	300,000	299,237	2.22 %	0.006	201	0.990	0.83	732	✓
YL1	423	-1	300,000	300,913	1.73 %	0.129	104	0.999	1.00	368	✓
YL2	366	0	300,000	304,590	1.94 %	0.106	42	1.000	1.00	368	✓
YL3	421	-1	300,000	298,878	2.71 %	0.030	171	1.000	1.00	368	✓
YL4	510	-1	300,000	300,466	3.17 %	0.006	240	1.000	1.00	368	✓

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.

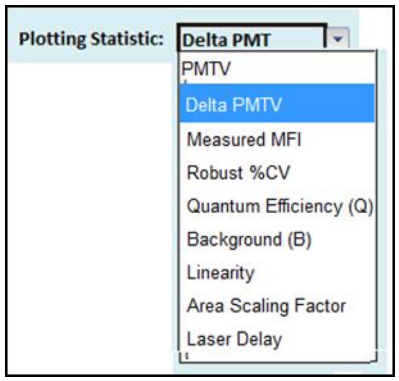
e.g. Δ PMT exceeds 100 mV
%rCV – detector specific but ranges from 3-5%

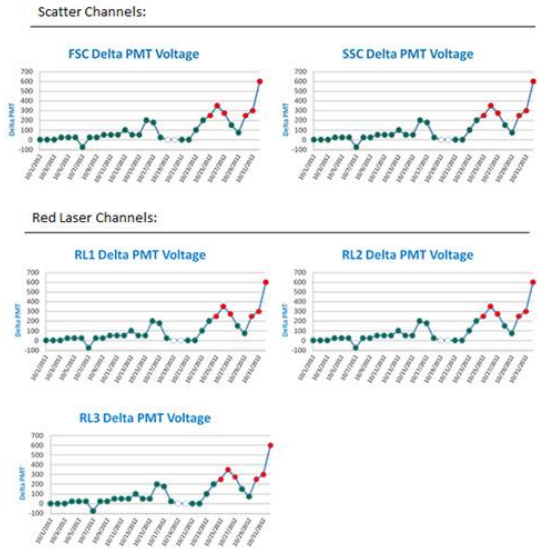
Levey-Jennings Report

- Tracks “Performance Test” data
- Provides a visual indication of the cytometer performance over time.
- Monitor shifts and trends in cytometer performance and

Select laser to view

Select: statistic





View/Print individual reports

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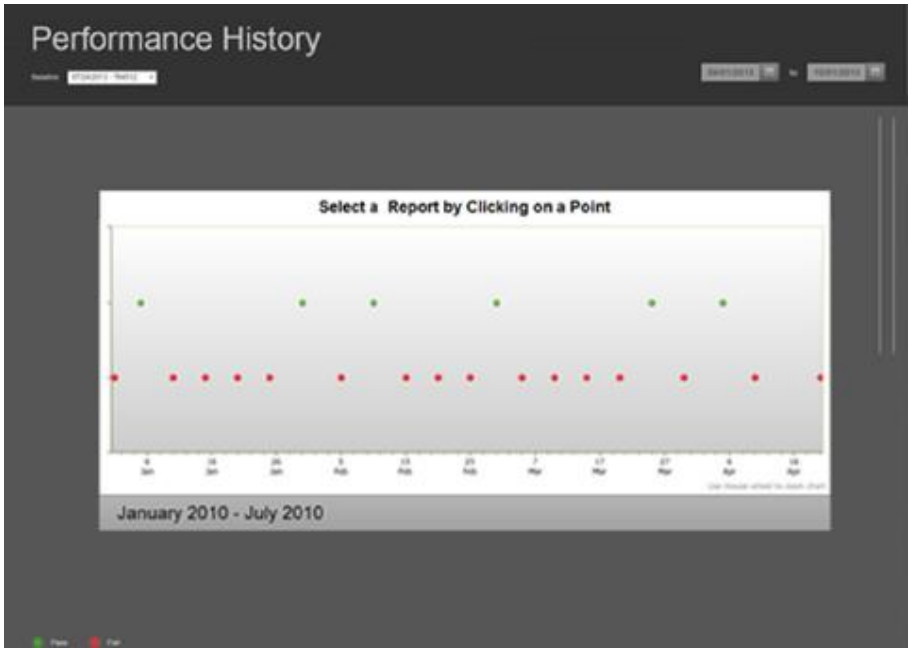
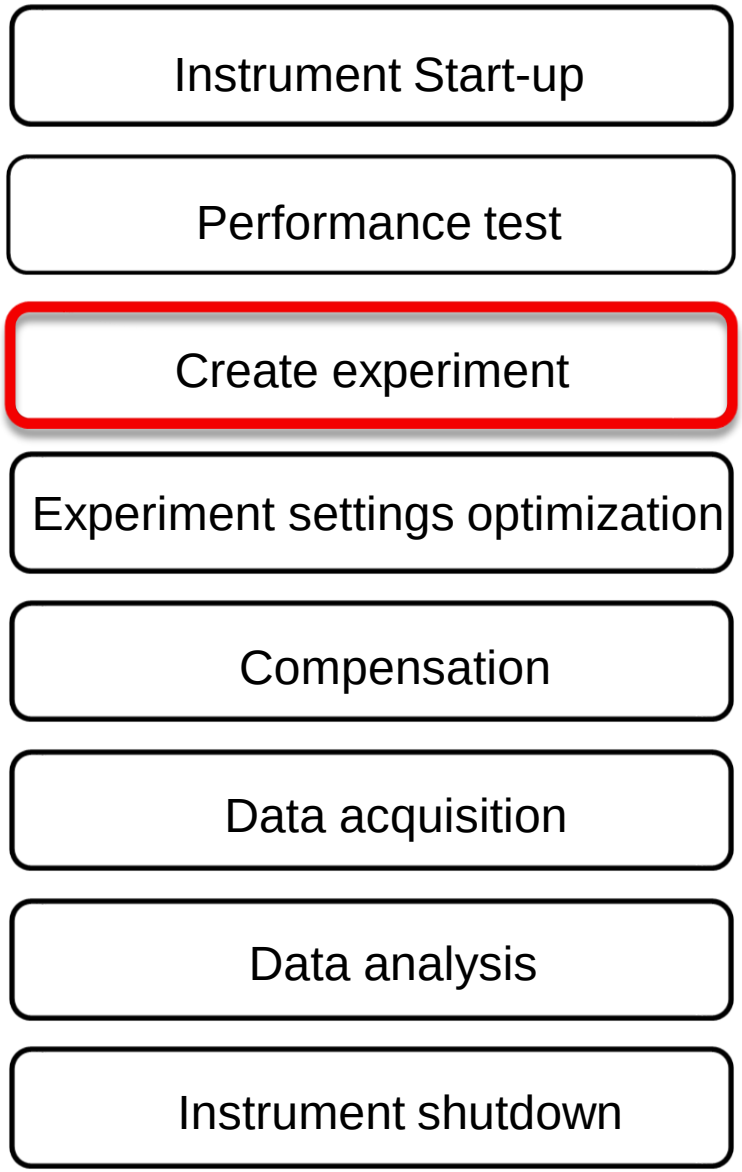
Performance History Report

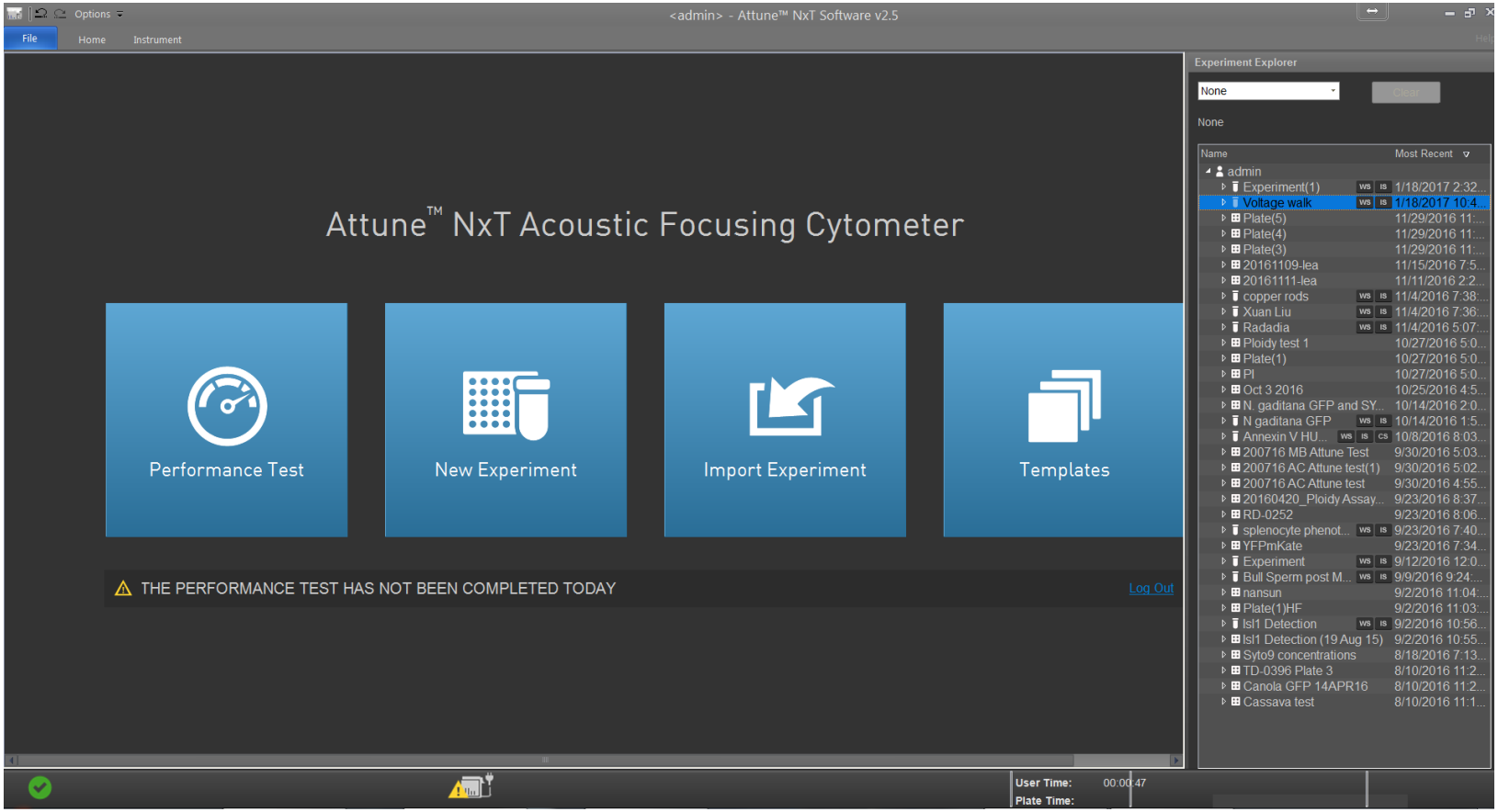
- Pass/fail status of all PT runs
- Filter date range
- Quick visualization of ‘health’ of instrument
- Click on result to open associated PT test
- Max 180 days for current baseline
- Not available if no baseline exists



Workflow

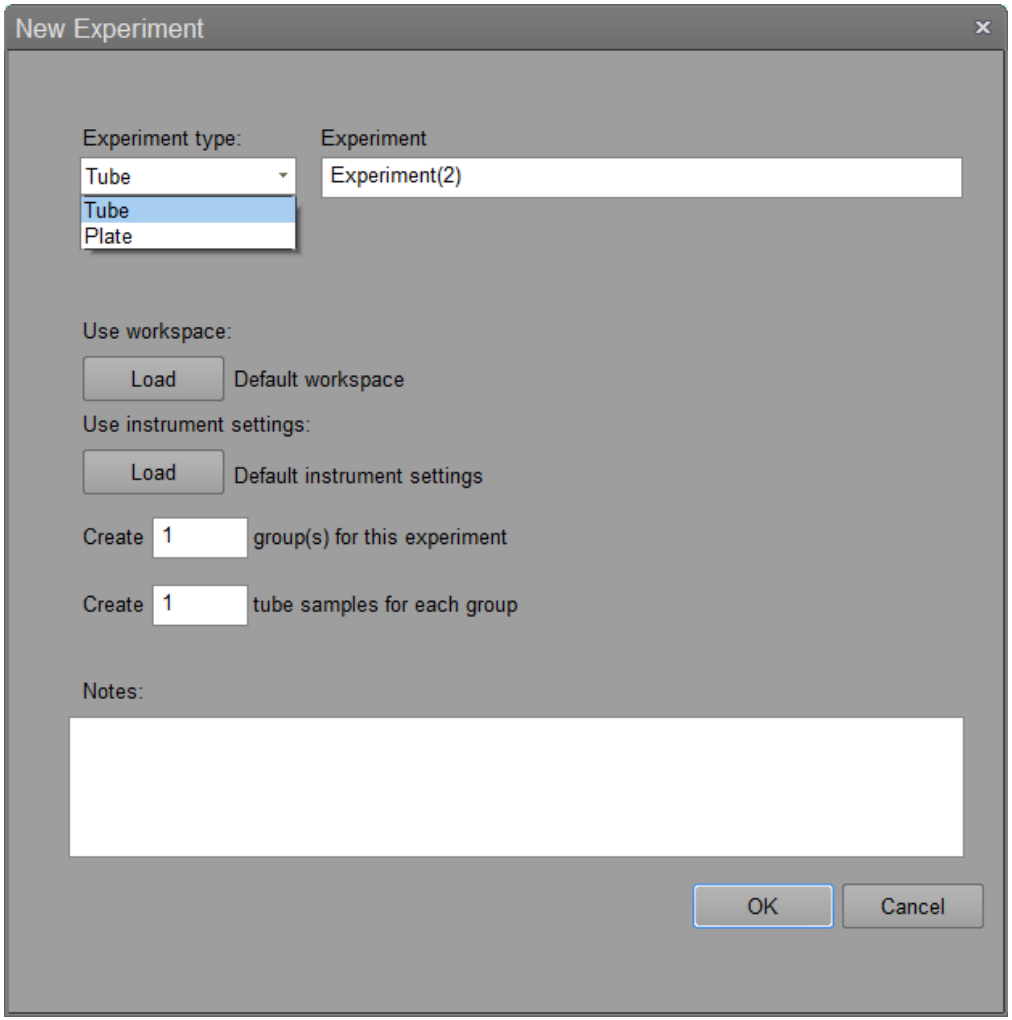


Workflow



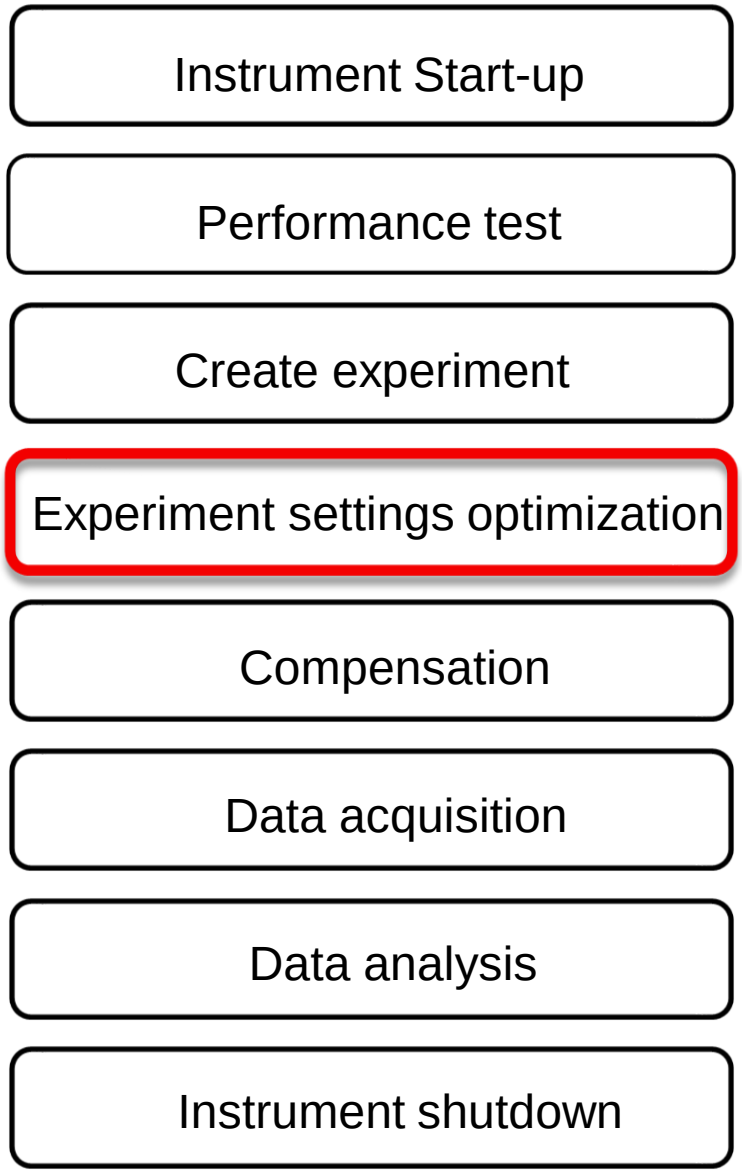
Create a new experiment by selecting New Experiment

Workflow



Select Experiment type and click OK

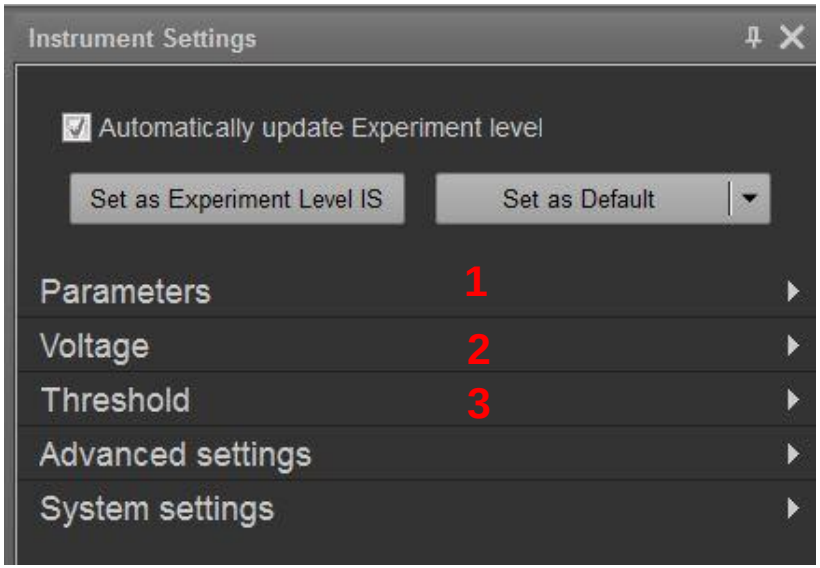
Workflow



Workflow

Experiment settings optimization

Instrument settings panel



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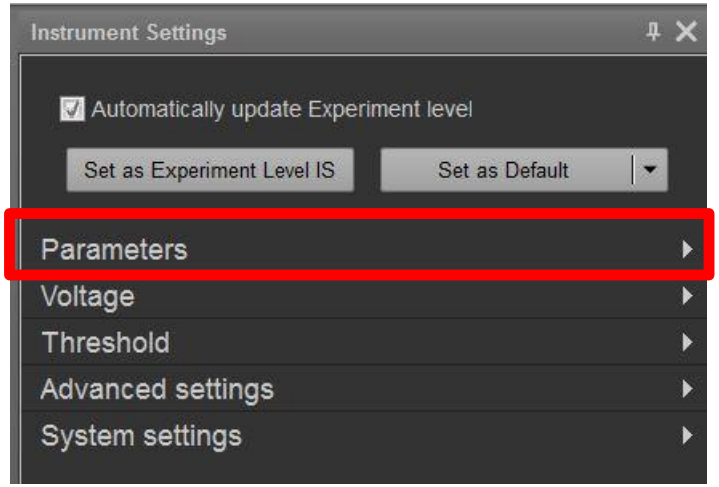
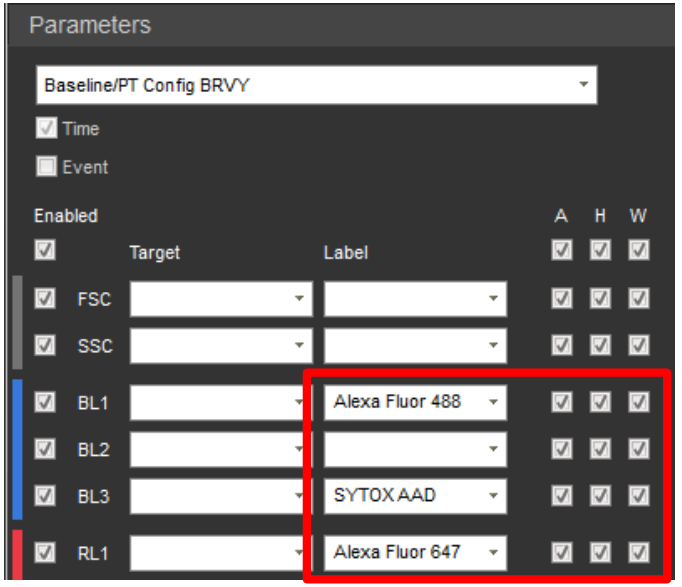
1. Parameters

- Expand parameters section
- Optional: Select customized configuration
- Deselect detectors/channels not required
- Select A – H – W as needed
- Select ***label*** from drop down menu
- Enter target name
- After information has been entered, collapse the section





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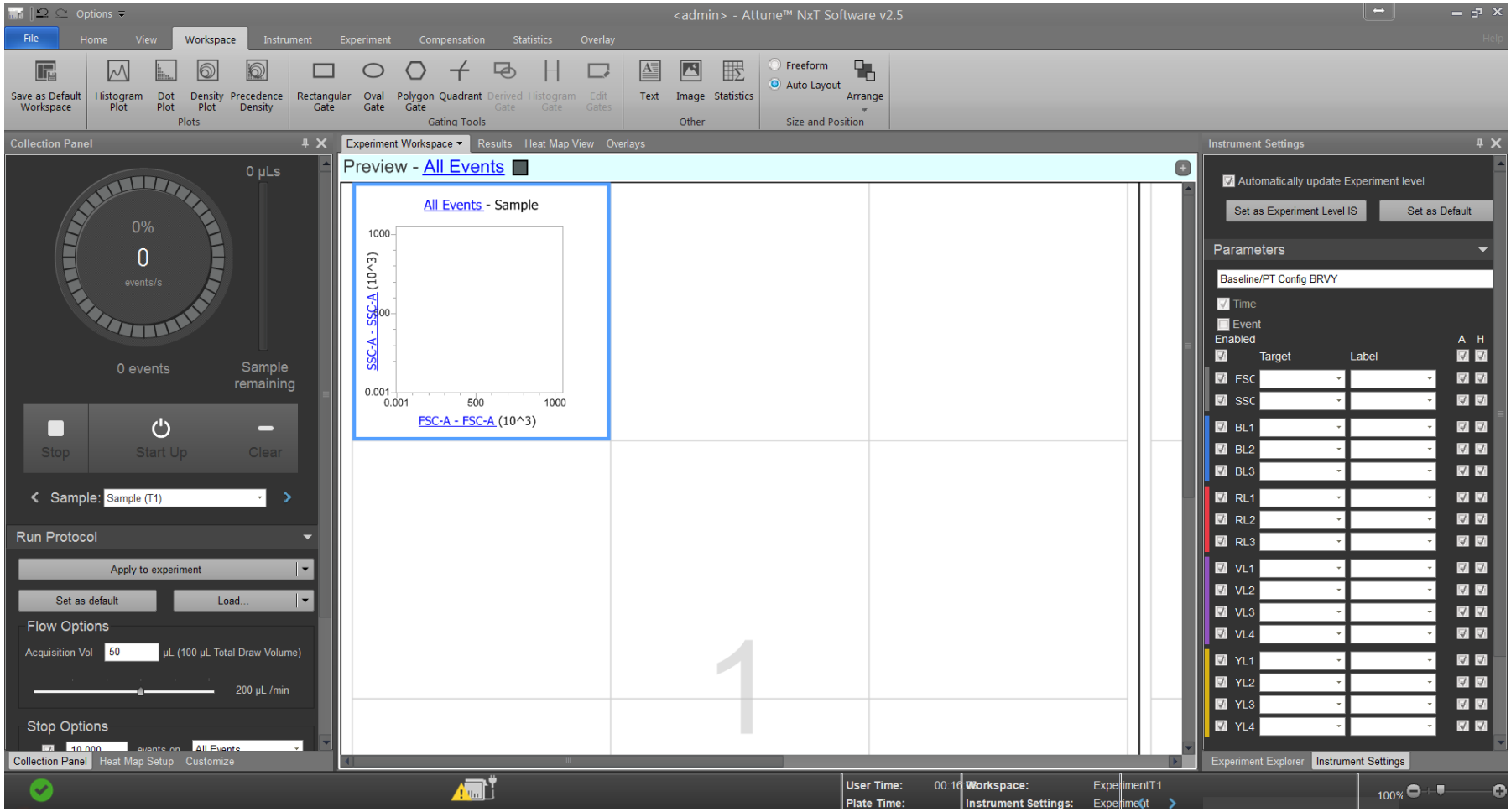
Create Workspace



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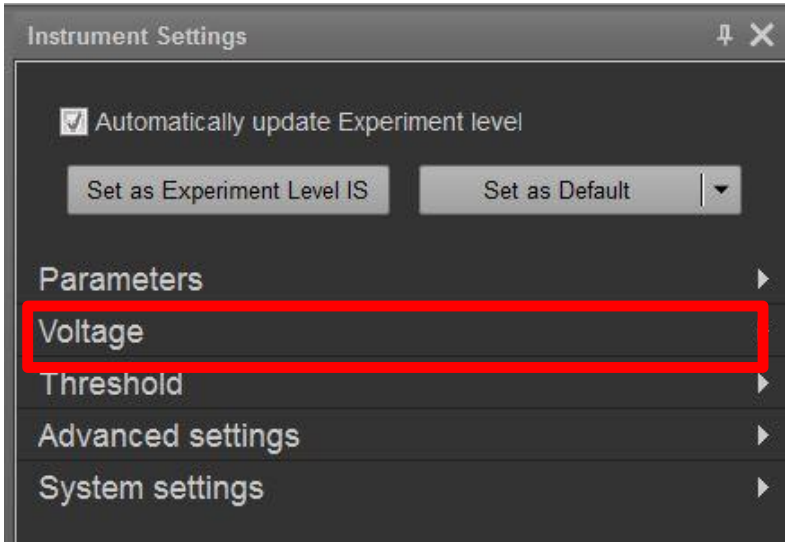
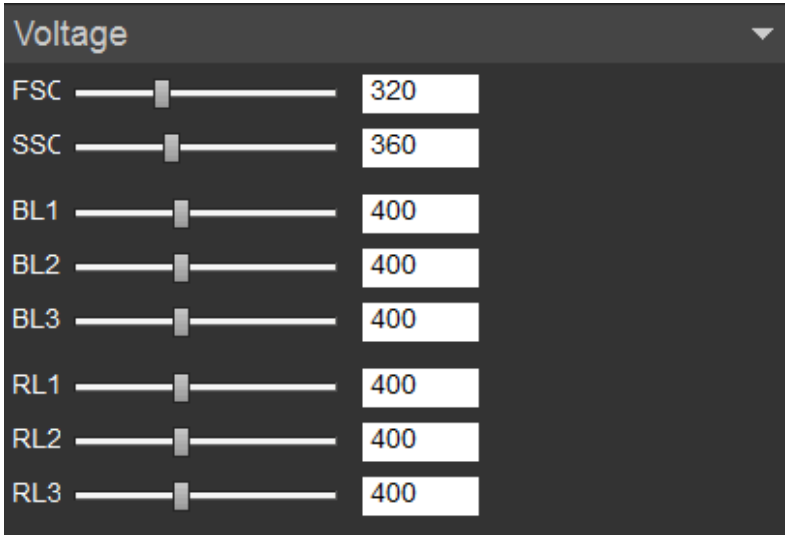
2. Adjust PMT Voltages

- Expand the ***Voltage*** section
- Adjust FSC & SSC voltages to position cell population on the scatter plot
- Adjust Fluorescence Channels voltages based upon the positive control
- If you do not have a positive control, set the voltage for the negative population so that the events are displayed between 100-1000 relative fluorescent units

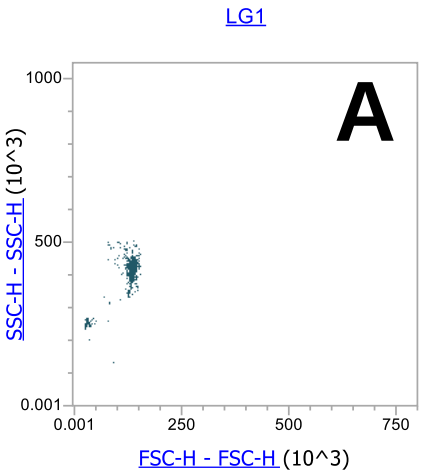


Note:

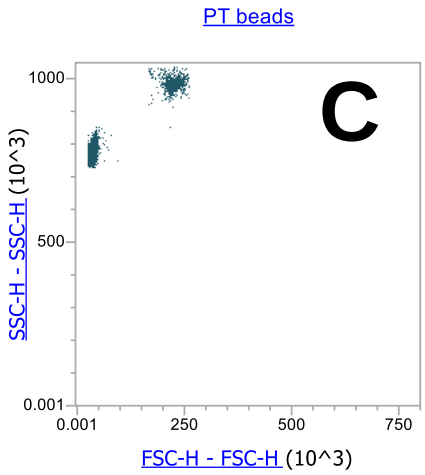
- It is recommended to perform a voltage walk and calculate the staining index for each fluorophore
- If compensation controls are recorded, all the fluorescence channels voltages are disabled (i.e. grayed out)



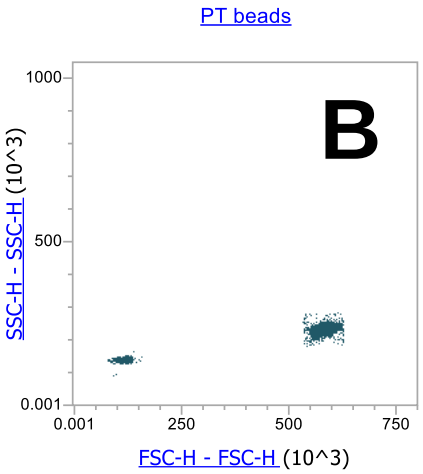
Match the FSC/SSC voltage settings with the plots



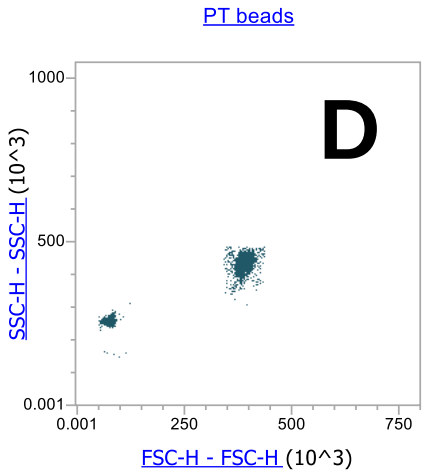
FSC 400
SSC 400



FSC 450
SSC 350



FSC 350
SSC 350

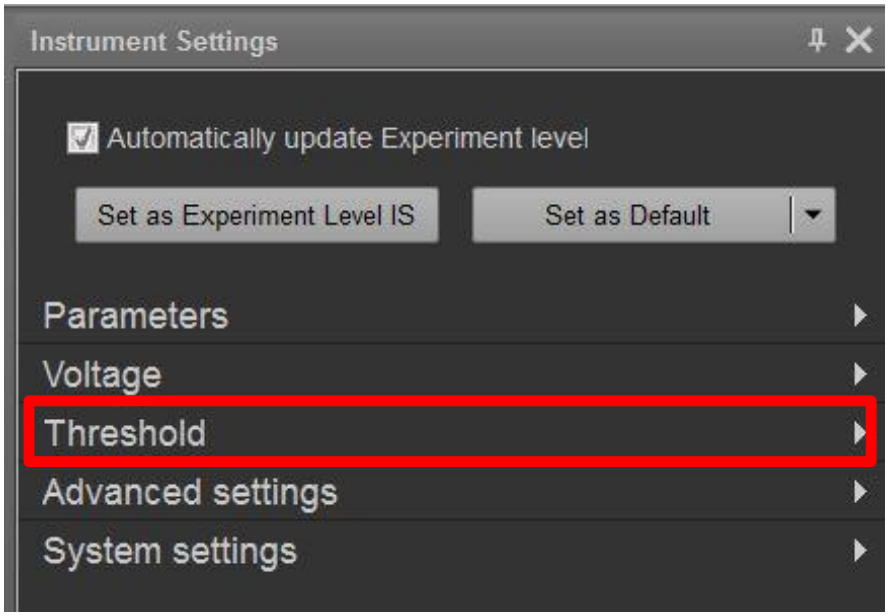


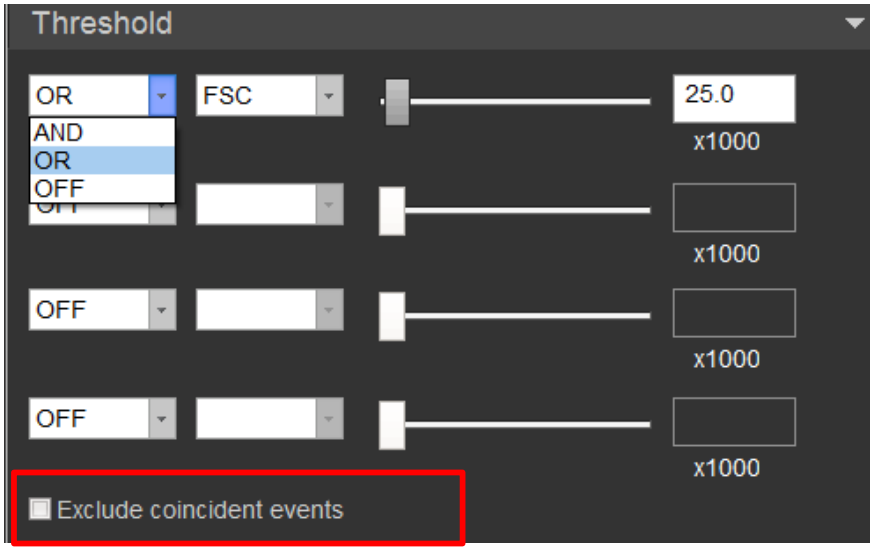
FSC 500
SSC 325

3. Adjust Threshold (optional)

- Expand the ***Threshold*** section
- Way to get rid of unwanted events (i.e. noise) before sample has been recorded
- Default setting: OR FSC 25 x 1000.
- Can be set on a single or up to 4 scatter and/or fluorescence channels
- Data not meeting threshold criteria is <u>permanently lost</u>

- Exclude coincident events option:
  - checkbox
  - # of aborted events in FCS file header



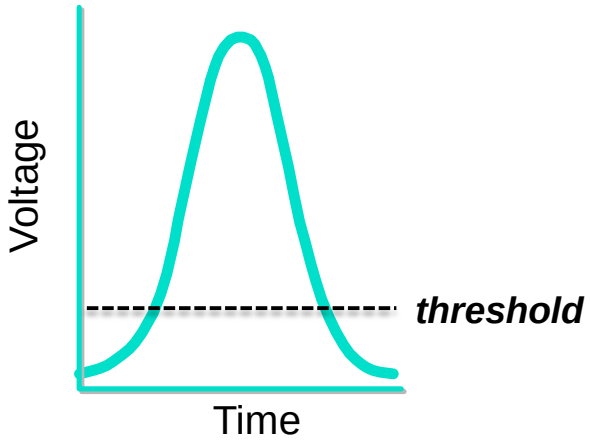


At least 1 threshold must be set . If all are set to OFF, no data is displayed on the workspace

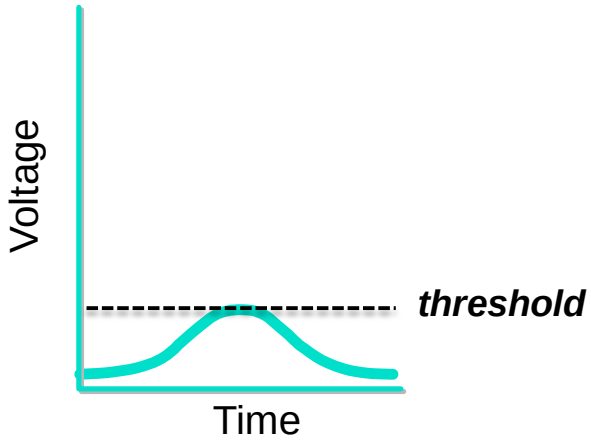
3. Adjust Threshold (optional)

A threshold: is a way to get rid of unwanted events (e.g. debris) before a sample is recorded

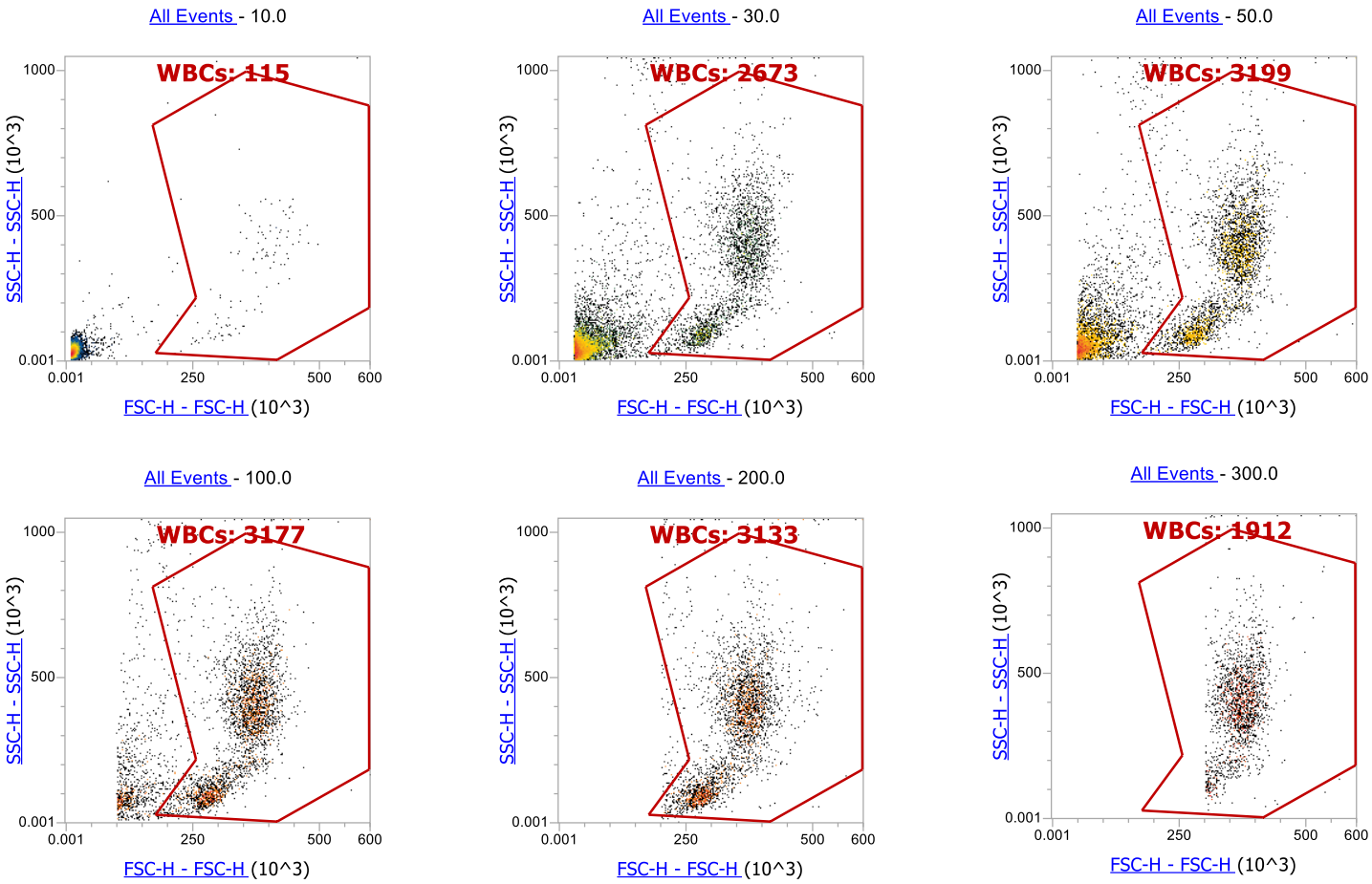
Above threshold: an "event"



Below threshold: not an "event"



Examples of Threshold Adjustment



Stop Option – 10,000 events on All Events

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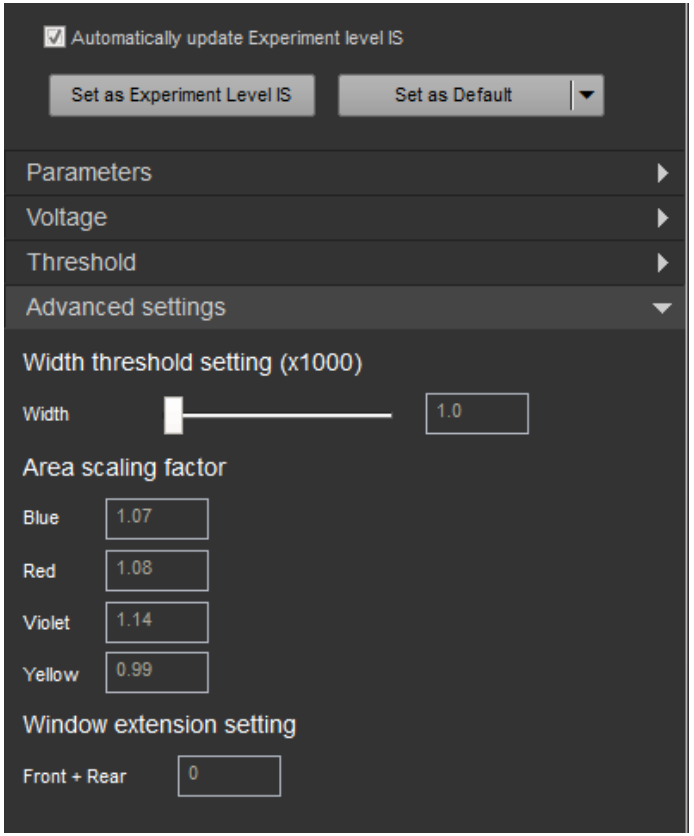
Advanced instrument settings

(*Administrator* and *Advanced User* permission required)

Advanced Settings

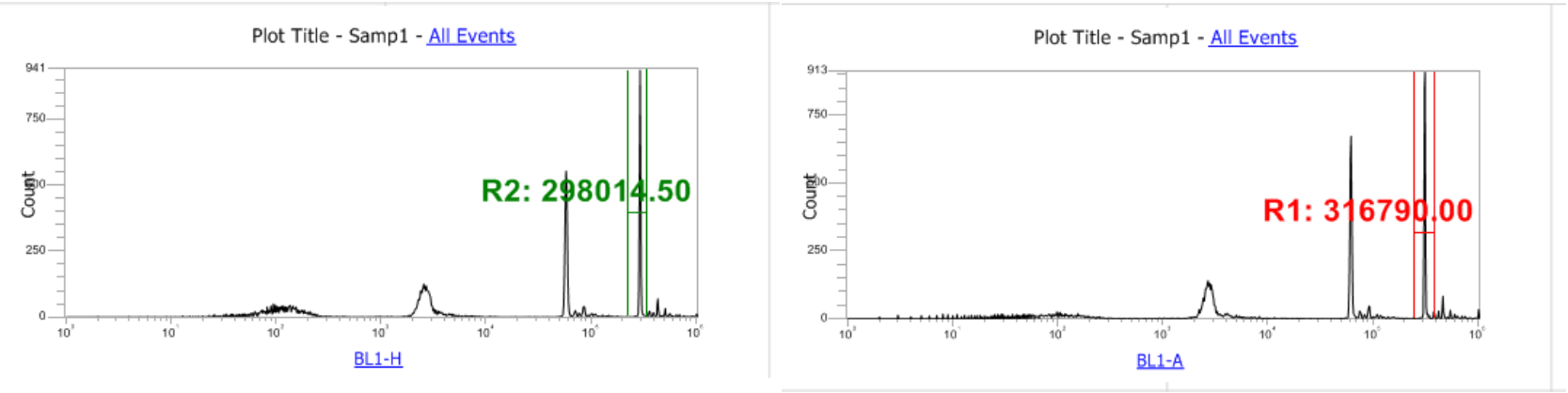
- **Width threshold setting**
- **Area scaling factor (ASF)**
- **Front + Rear window extensions**



More information can be found in the
SW User Guide Rev C (loaded on the desktop)

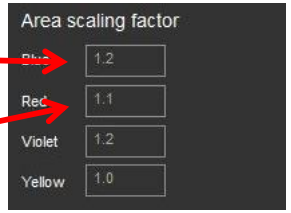
Area Scaling Factors

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



- Area Scaling Factors are Calculated and Applied from the results of the performance test (V2.4 – not V2.2)

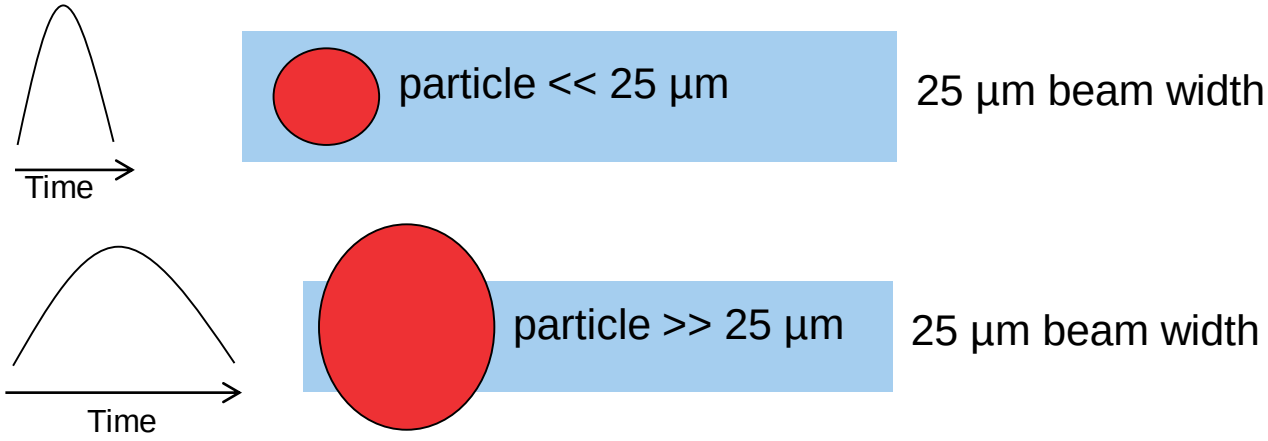
Channel	PMTV	Target MFI	MFI	Robust %CV	Qr	Background	Linearity	ASF	Laser Delay	Result
FSC	555	300000	302000	1.80 %	0.000	0	0.000	1.01	1100	
SSC	318	300000	304325	3.44 %	0.000	0	0.000	1.01	1100	
BL1	403	300000	301618	1.34 %	0.051	86	1.000	1.01	1100	
BL2	348	300000	311511	1.34 %	0.068	138	0.966	1.01	1100	
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	
VL1	306	300000	304797	1.07 %	0.015	2479	1.000	1.09	676	



ThermoFisher SCIENTIFIC

Area Scaling Factors - Why would I change them?

ASF changes as a function of particle size

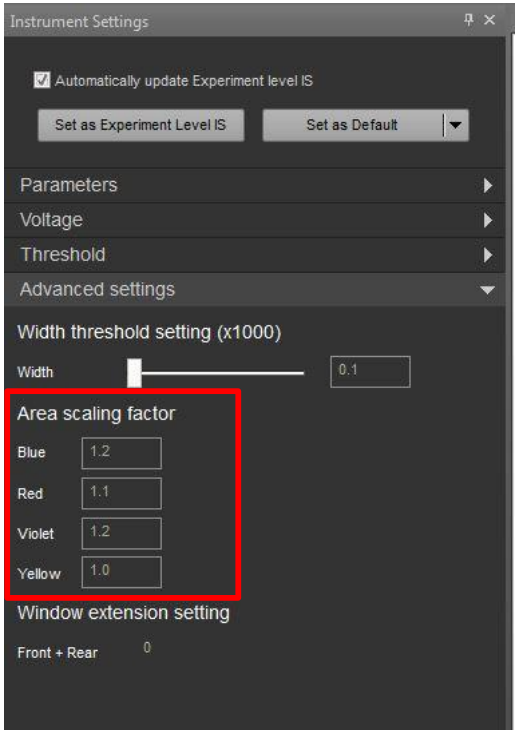


Adjusting ASF

If Area (MFI) is greater than Height (MFI); enter a value <1

If Area (MFI) is less than Height (MFI); enter a value >1

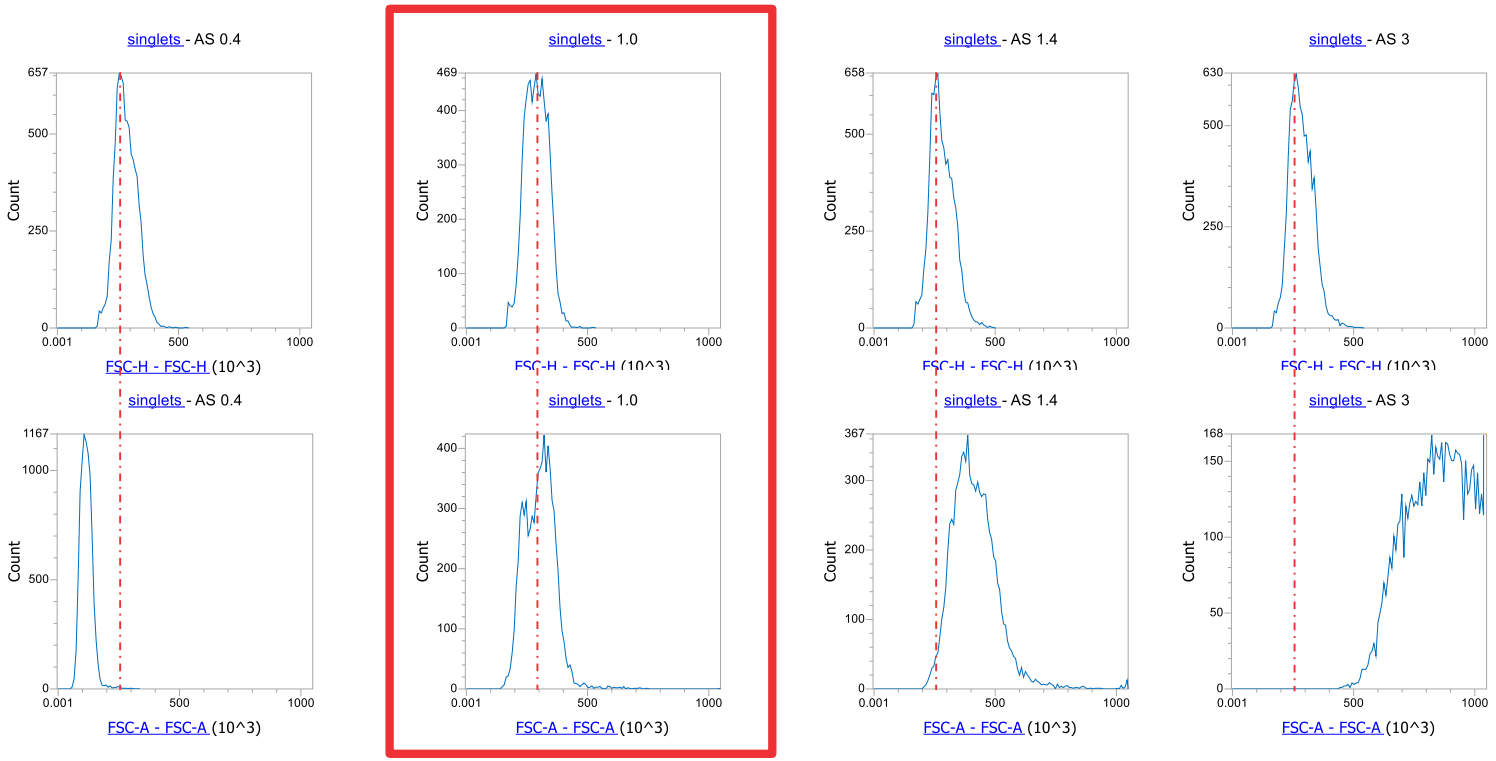
*Note: Adjustment must be made **prior** to recording data*



ThermoFisher SCIENTIFIC

Examples of Adjusting Area Scaling – 1st Method

Compare FSC-H vs FSC-A in histograms



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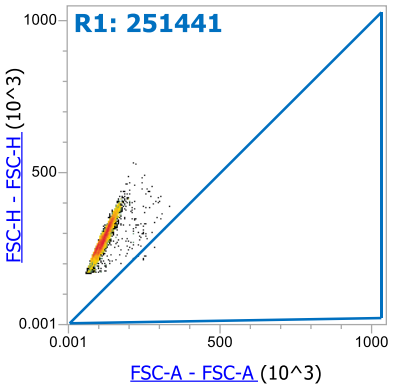
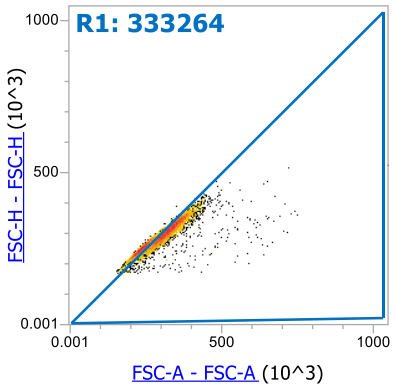
Examples of Adjusting Area Scaling – 1st Method

Compare FSC-H vs FSC-A a bivariate plot

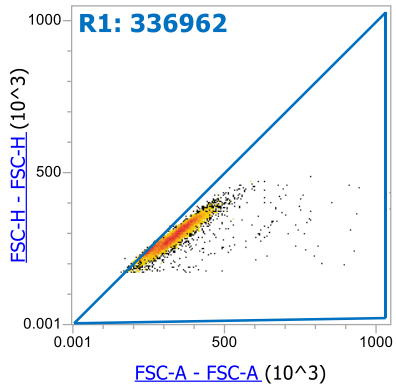
singlets - AS 0.4



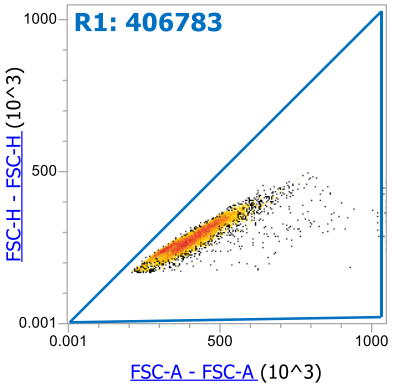
singlets - AS 1.0



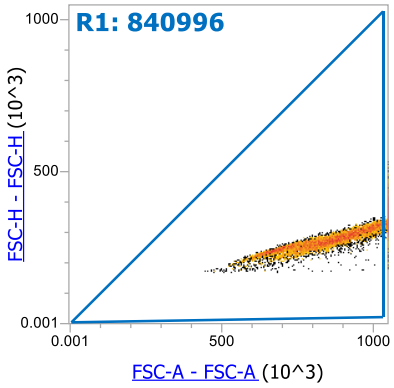
singlets - AS 1.1



singlets - AS 1.4

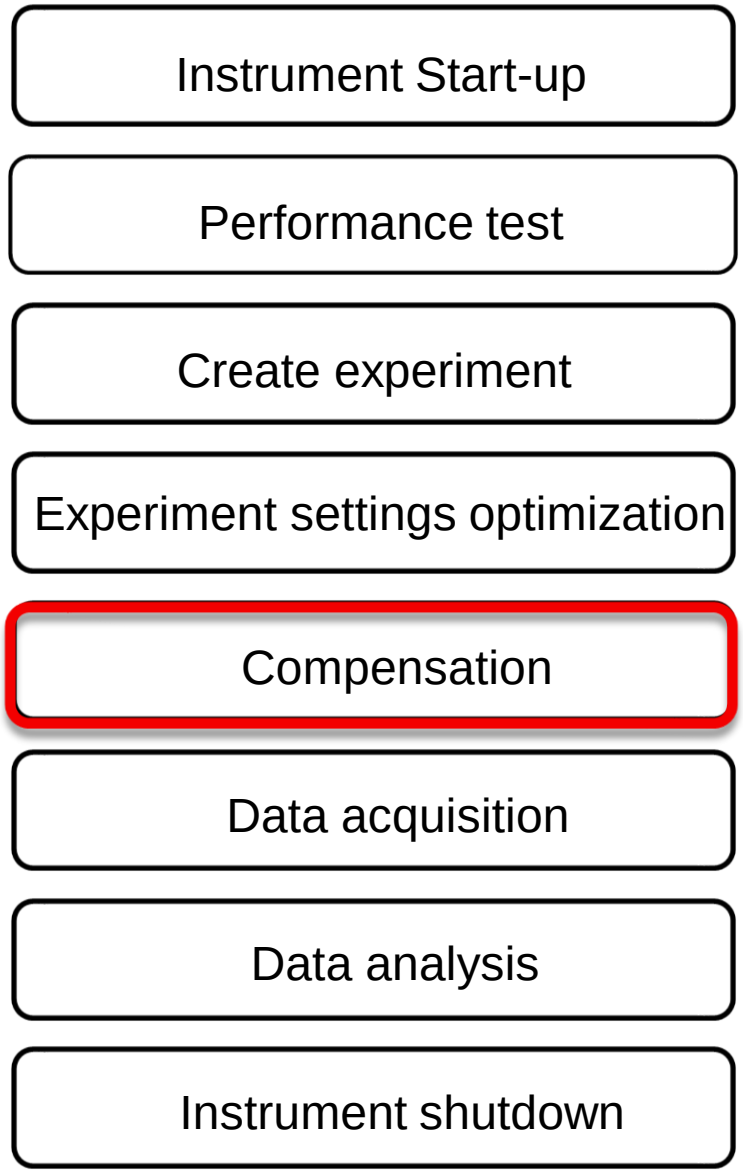


singlets - AS 3



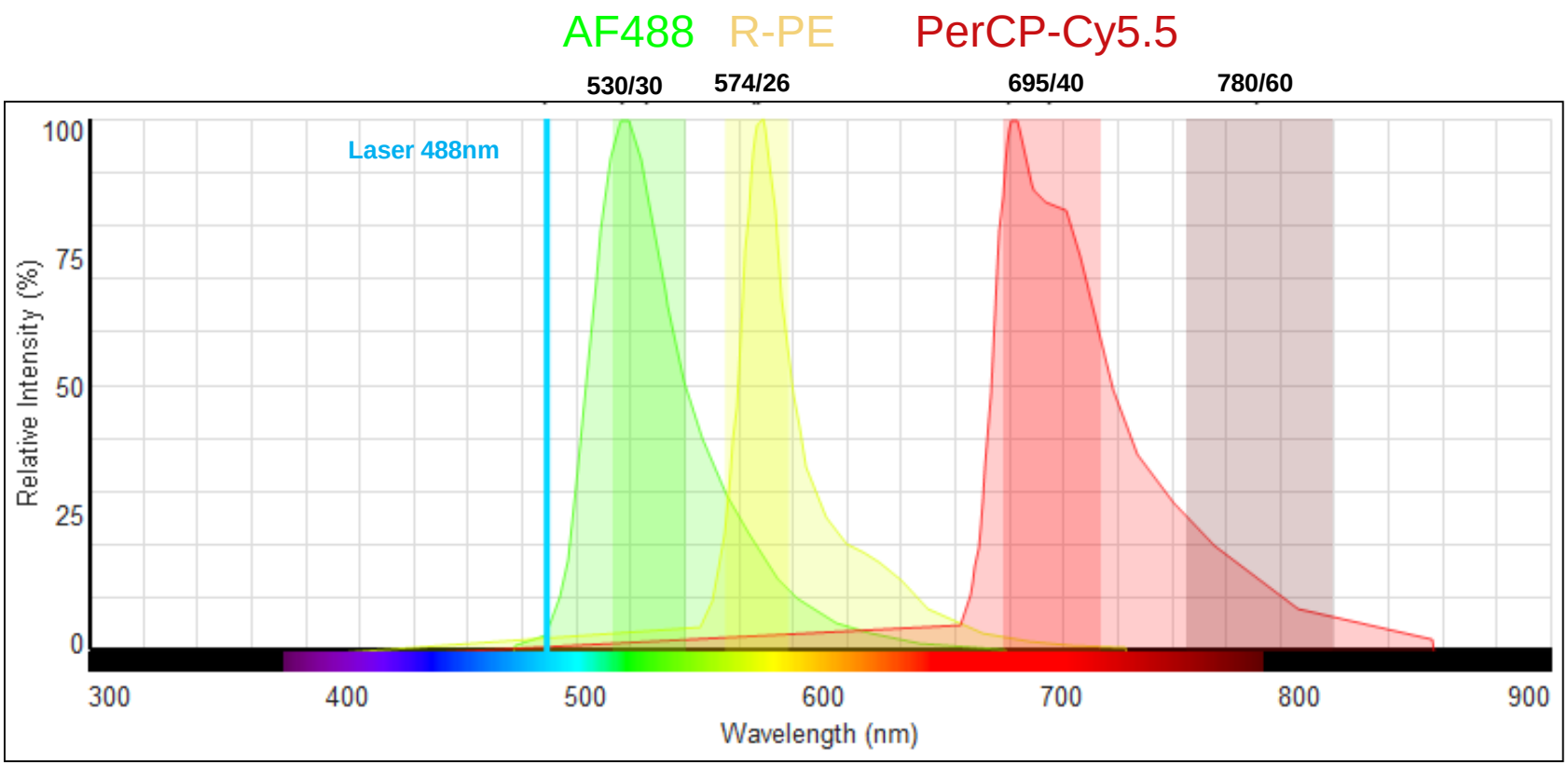
1. Draw a diagonal line
2. Cells should fall as close to the line as possible
3. But towards the right of the line
4. AS 1.0 and 1.1 are optimal for this sample
5. Cell lines usually need to be adjusted

Workflow



Why do we need to compensate?

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

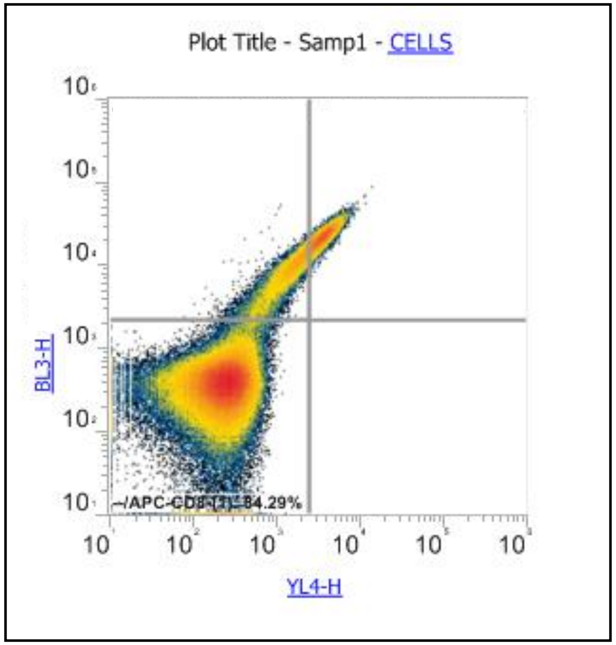


https://www.thermofisher.com/order/spectra-viewer

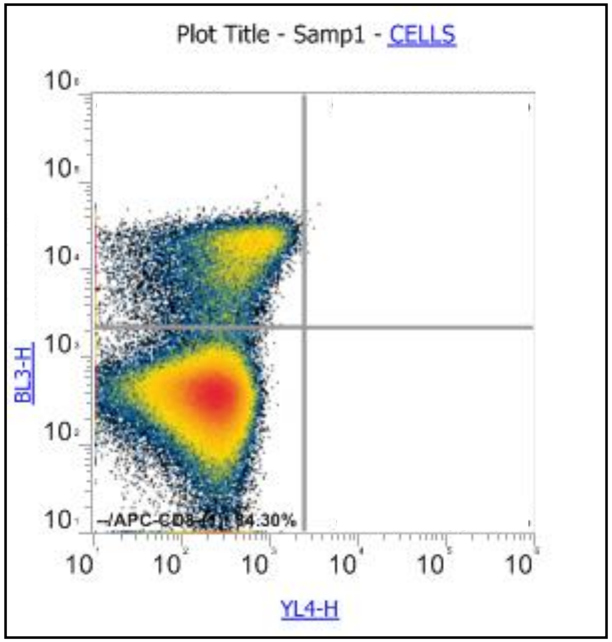
How does fluorescence 'spillover' look in data plots?

Single stained sample: PE-Cy5.5

Uncompensated



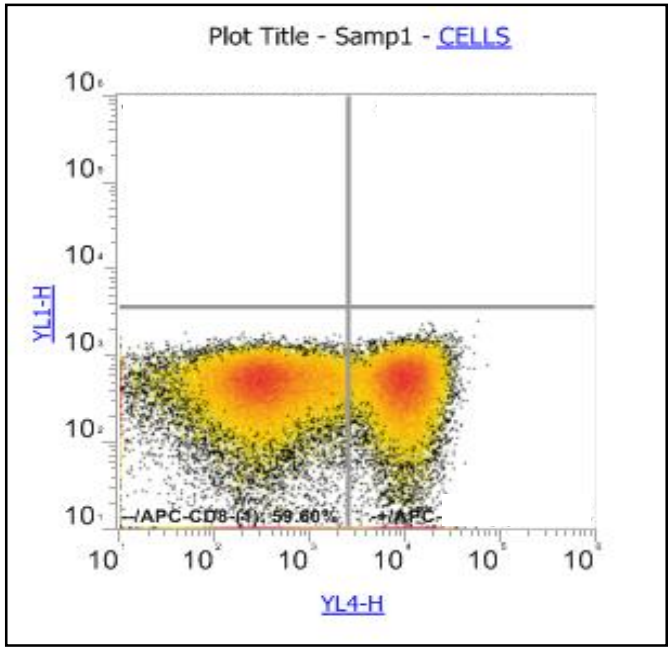
Compensated



Compensation is the process of correcting the **spillover** from our signal (e.g. PE-Cy5.5) into YL4 and each secondary channel into which it is detected/measured.

How? We match median fluorescence of positive and negative populations

Compensation Confirmation



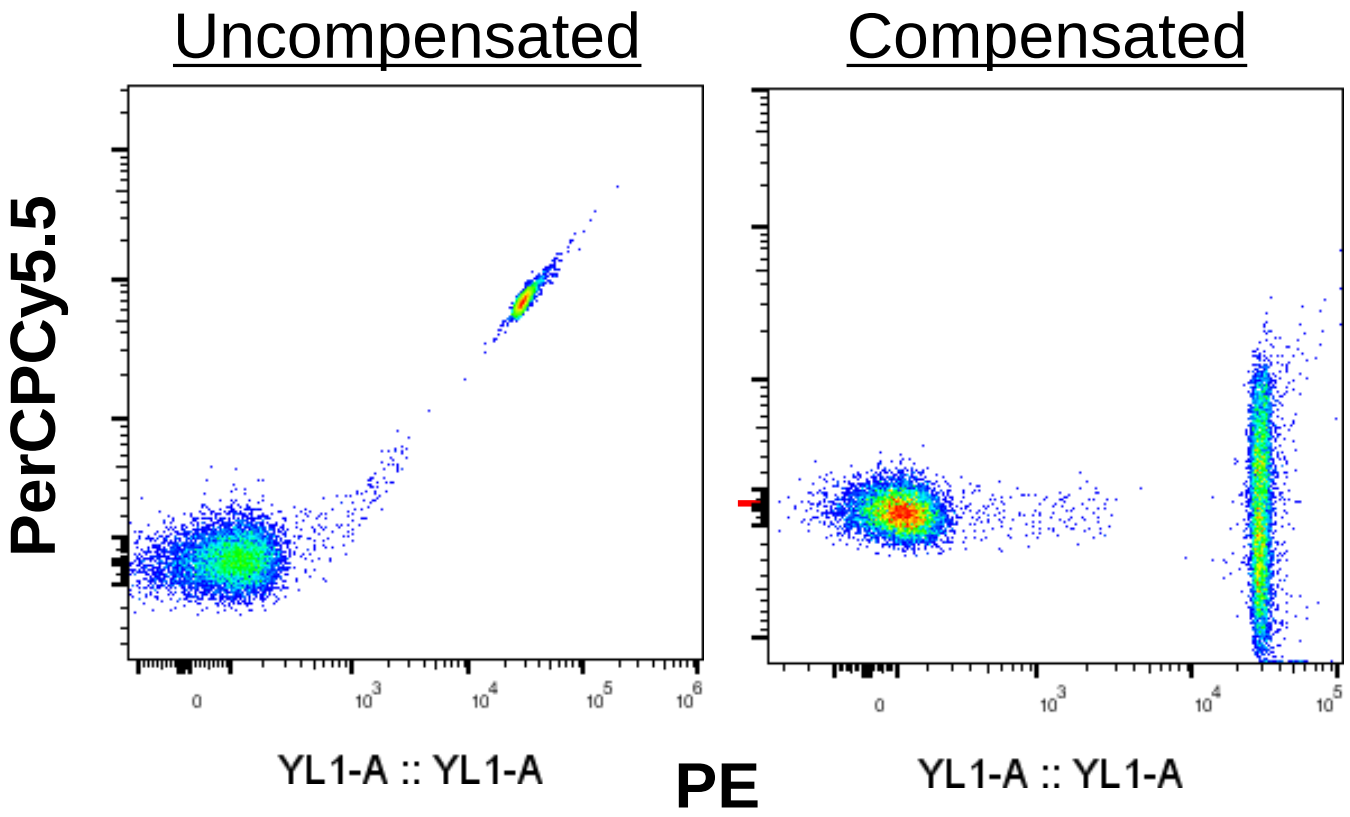
Parameters: **YL4-H vs YL1-H**
Gate: **CELLS**
Experiment: **14H13_INKT**
Group: **INKT MATRIX**
Sample: **NOT-IN_STATS**

Name	Count	%Gated	%Total	Y Median
All Events	1106537	100.00	100.00	413.88
--/APC-CD8+(1)	1	0.00	0.00	4104.51
-+/APC-CD8+(1)	2	0.00	0.00	3967.83
--/APC-CD8-(1)	515380	59.60	46.58	419.63
-+/APC-CD8-(1)	349313	40.40	31.57	410.26

- Check Median of fluorescence is equivalent in non-targeted channel
- Check for all dual parameters plot combination

Compensation and Spreading Error

PE into PerCPCy5.5



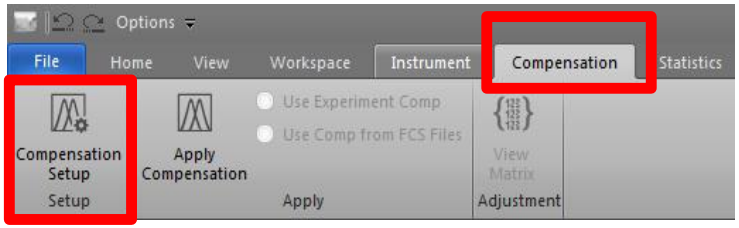
Basic Rules of Compensation

1. Unstained cells
   - Only required if sample does not have an unstained population
   - Background fluorescence should be the same for the positive and negative populations
2. Single color controls for each fluorochrome
   - Compensation color must be matched to your experimental color (FITC cannot substitute for GFP)
   - The actual tandem dye being used in the sample staining must be used in the single-color control. Lot numbers must match.
3. Controls need to be at least as bright as the brightest positive sample
   - Titrate antibody on compensation beads. Beads are efficient at antibody binding and maybe too bright and off scale compared to cell staining
   - Log separation between the negative and positive peaks
   - Brightest in the primary detector (its own cannel) or spilling over into other channels less than 100%
4. **Collect enough events.**

Compensation Set up

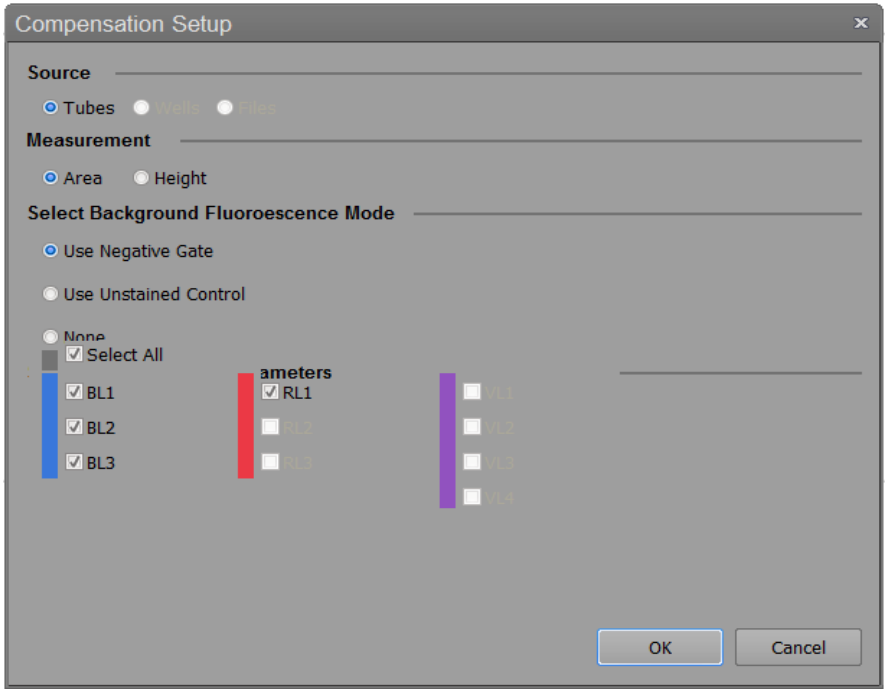
In *Compensation* tab, click on **Compensation Setup**. Or

Double-click on Compensation in Experiment Explorer



Compensation setup options:

- Source: Tube
- Parameter: Area or Height
- Autofluorescence:
  - Negative gate,
  - Unstained control
  - None
- Fluorescent channels



Compensation: Background Fluorescence Modes

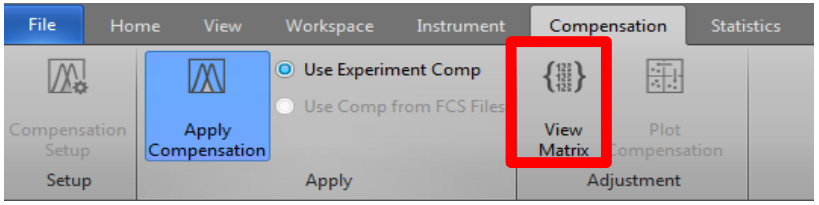
Auto Fluorescence Correction Choices:

- Negative Gate
- Unstained Control
- None

Background Mode	When to Use?
Negative Gate	With different 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 background is negligible or cannot be ascertained.

Spillover Matrix

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



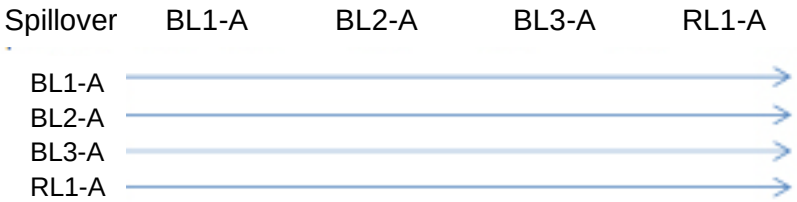
Spillover

	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

Spillover Matrix

The spillover matrix will assign a numeric value of percent spillover of a fluorophore into other detectors once all controls have been recorded. To read the matrix, consider the column on the left the fluorophore (ie FITC) and the top row the detector.

If FITC is the fluorophore assigned to BL-1 detector, then the table below shows that FITC spills into the BL-1 detector 100%, the BL-2 detector 38.10% and the BL-3 detector 5.86%

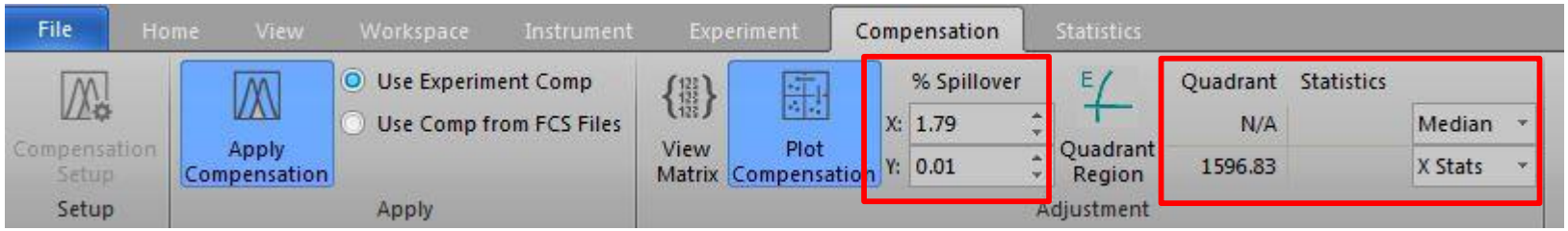


	BL1-A	BL2-A	BL3-A	RL1-A
BL1-A	100.00	38.10	5.86	0.01
BL2-A	0.51	100.00	24.18	0.00
BL3-A	0.22	25.40	100.00	13.92
RL1-A	0.06	0.04	0.01	100.00

On Plot Compensation Adjustment Tools

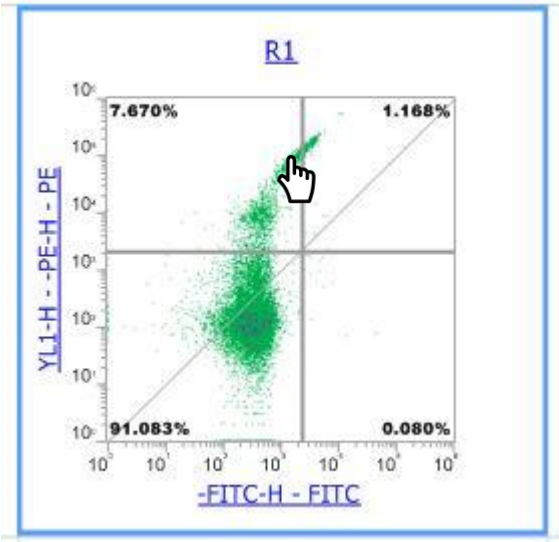
NOTE: Be very careful when using manual compensation as it may poorly effect your results.

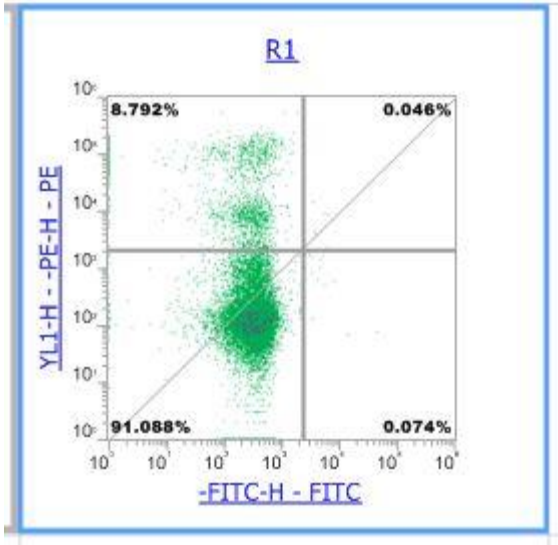
Tool allows manual adjustment of a selected plot



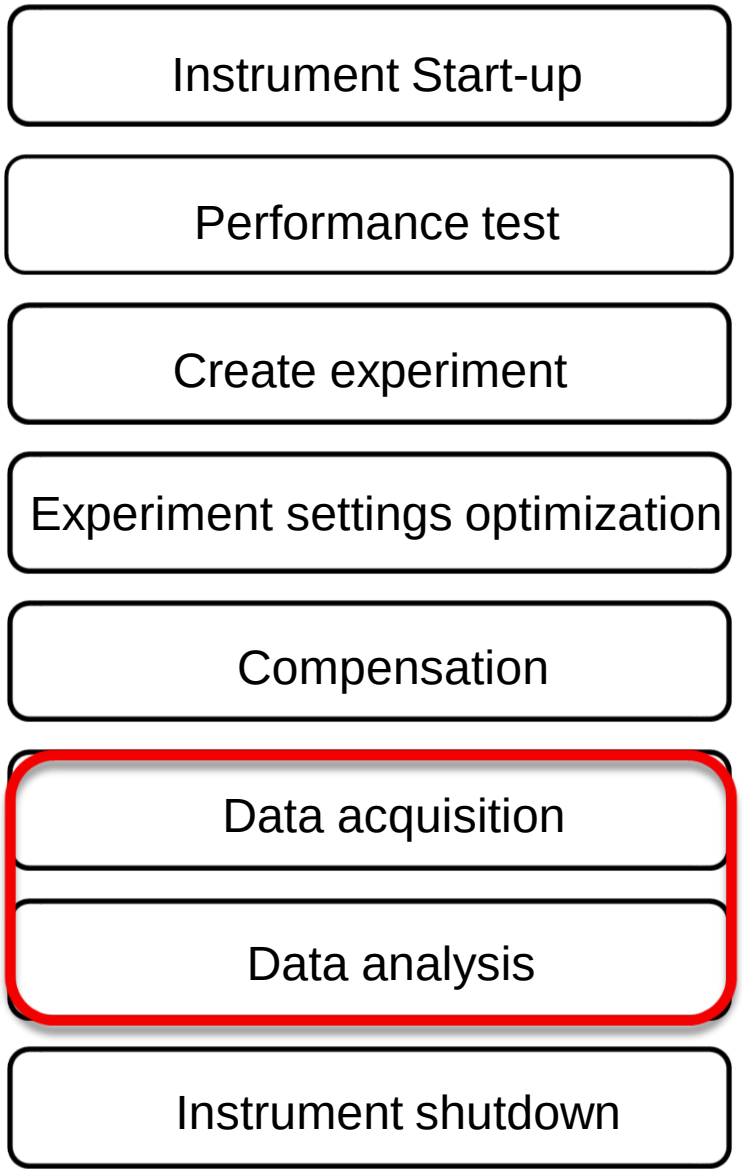
% Spillover Quadrant statistics

Dragging populations





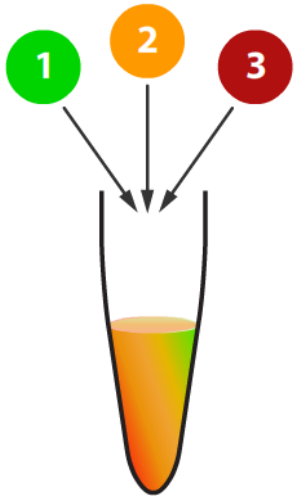
Workflow



Immunophenotyping Example: Three Color Experiment

Sample: Leukocytes from Human Blood

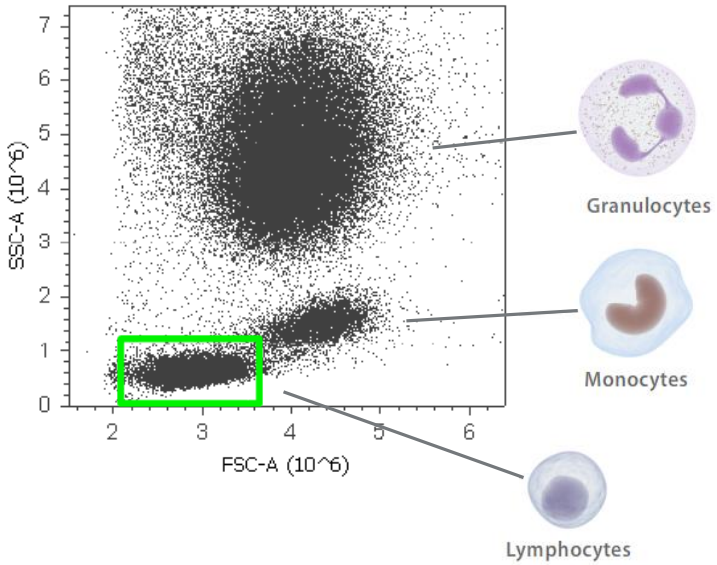
Measure: % T-lymphocytes CD4+
% T-lymphocytes CD8+

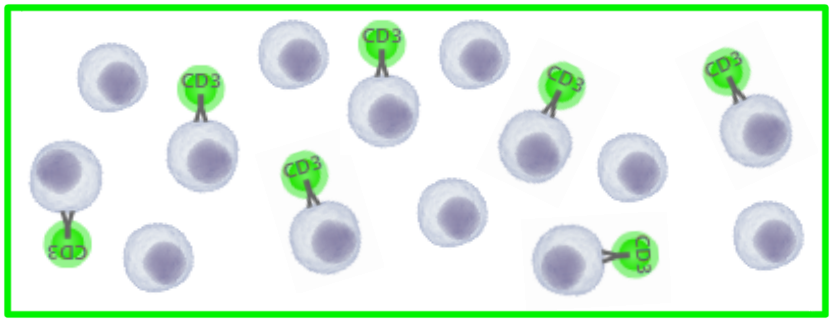


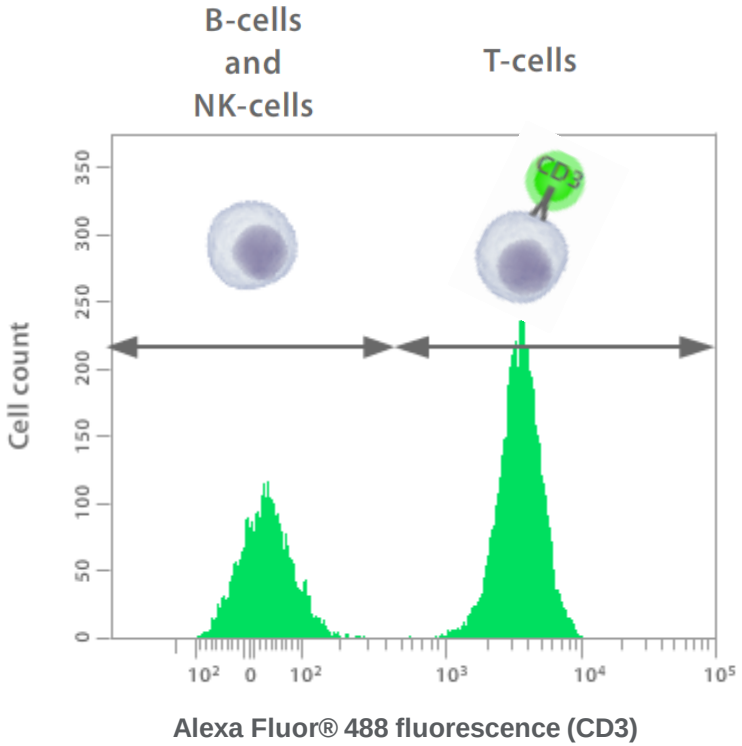
	Antibody	Fluorescent Probe
1	Anti-CD3	Alexa Fluor® 488
2	Anti-CD4	R-PE
3	Anti-CD8	R-PE Alexa Fluor® 700 dye tandem

→ T-lymphocytes specific

Three Color Experiment – Data presentation







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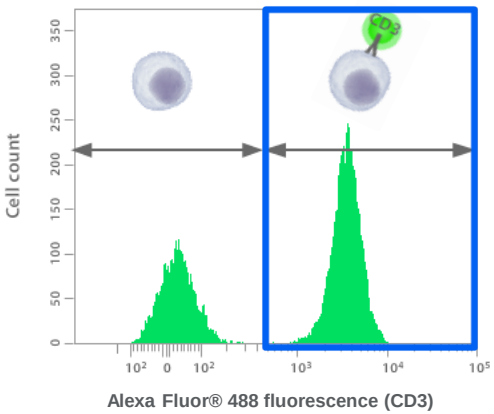
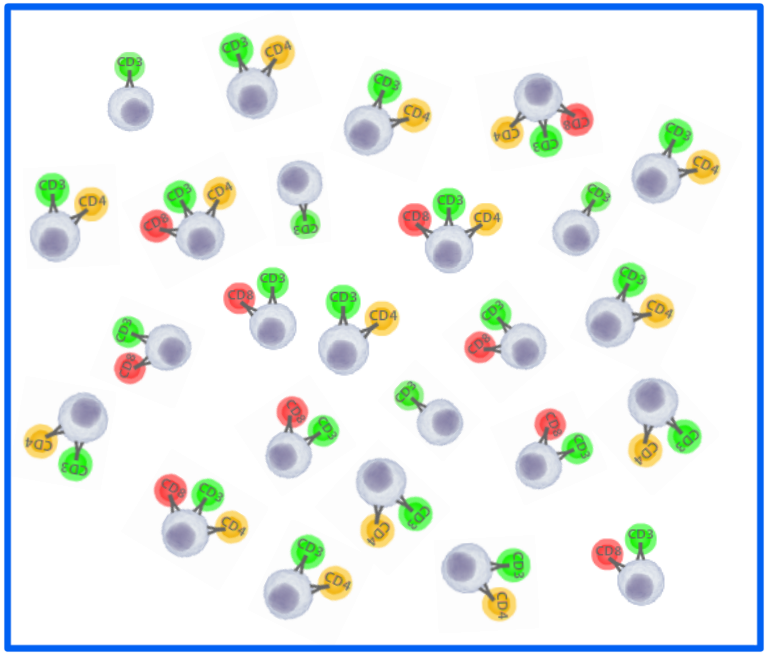
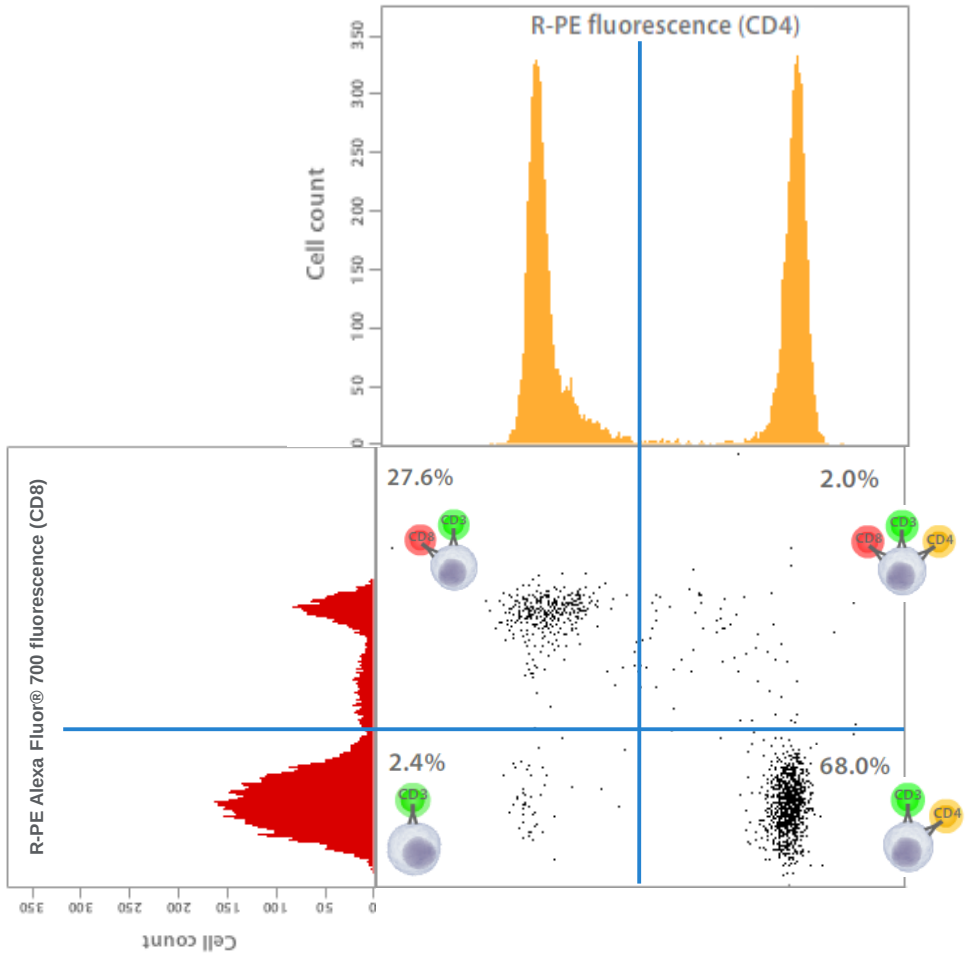
Three Color Experiment – Data Representation







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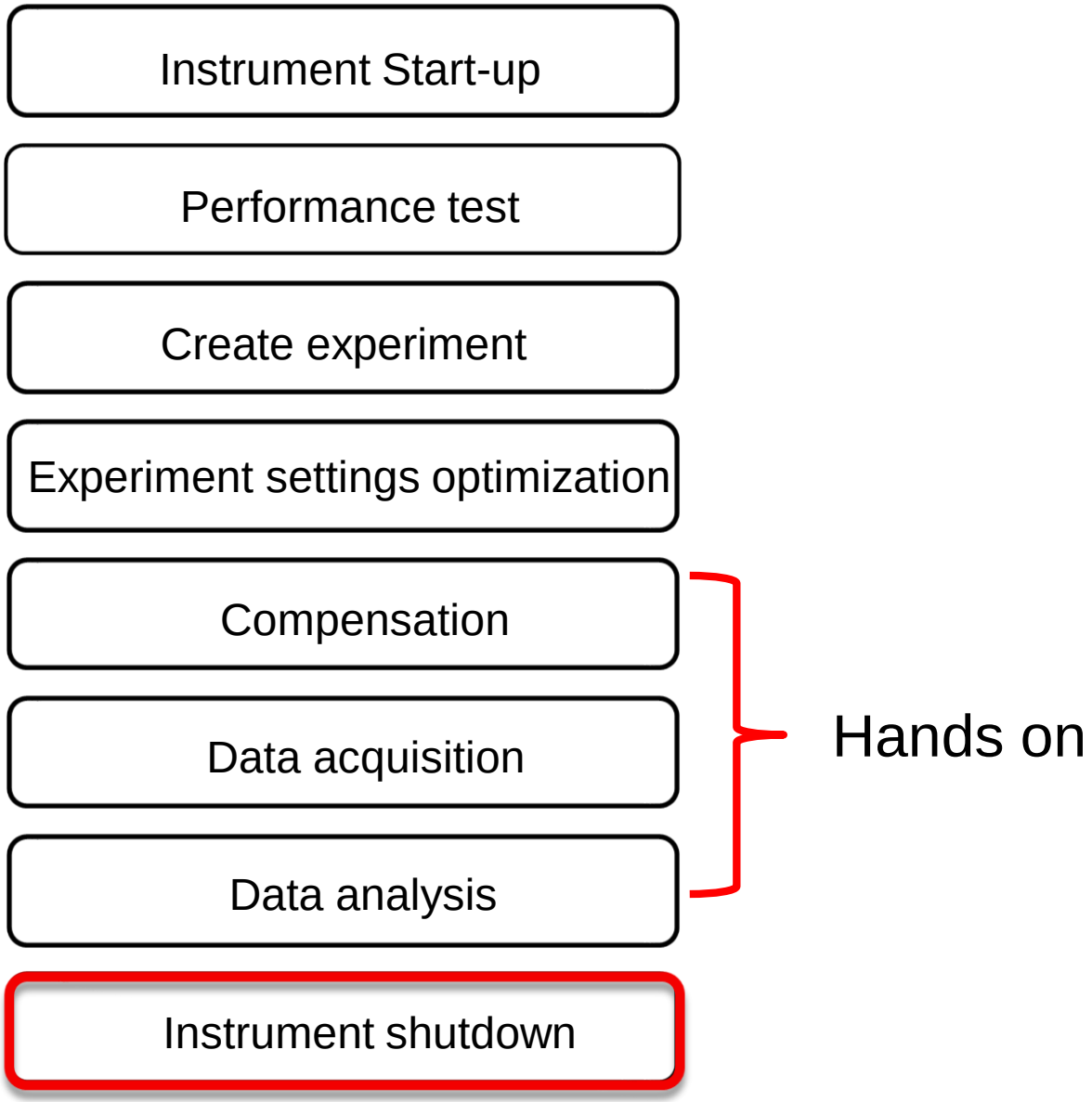
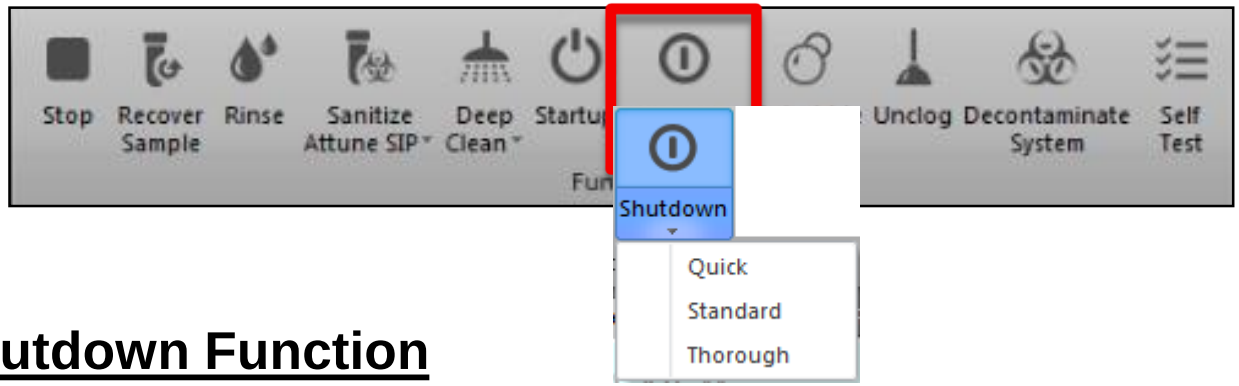
Workflow



Instrument Shutdown



Shutdown Function

- Sanitizes the instrument
- Cleans and rinses the fluid lines
- Refills fluid lines with shutdown solution
- Requires 10% Bleach freshly prepared

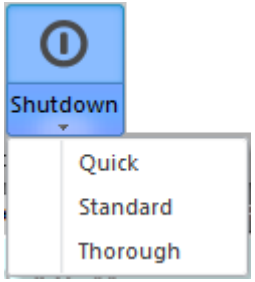
Ensures that:

- Fluid lines refilled with a solution that prevents crystal formation and bubbles.

Instrument Shutdown

- In *Instrument* tab, select **Shutdown** function
- Select:

Quick	15 cycles / 30 minutes
Standard	25 cycles / 45 minutes
Thorough	35 cycles / 60 minutes



- Follow the on screen instructions
- Once *Shutdown starts, log out of the software if necessary*

- Use 10% bleach - freshly prepared
- At the end of the Shutdown script:
  - the Cytometer will be in 'dream state'
  - the Autosampler will be in standby.
  - The fluid in the tube on the SIP will be shut down solution, not bleach solution

Attune® NxT Cytometer System Maintenance

Instrument cleaning guide

Between samples	• **Rinse** – automatically initiated when SIP is lowered (for tubes), or set in *run protocol* for plates • **Sanitize SIP** between sticky samples or cell counts
Between users / experiments **Use:** **1) if there is ≥30 min between users.** **2) If there is <30 min between users.**	**1) Unclog** then **Quick Deep Clean** - 30 minute cleaning routine (click on the arrow below the Deep Clean icon to select ***Quick***) or **2) Unclog** then **2X Sanitize SIP** / **Sanitize Autosampler SIP** (plate experiments) – 1st time with 3 mL 10% Bleach 2nd time with 3 ml Wash or De-bubble solutions
End of day (3 steps)	• ***Unclog*** • *****SIP Sanitize with 1:3 dilution of Attune Cleaning solution*** • ***Thorough* Shutdown** (click on the arrow below the Shutdown icon to select *Thorough*)

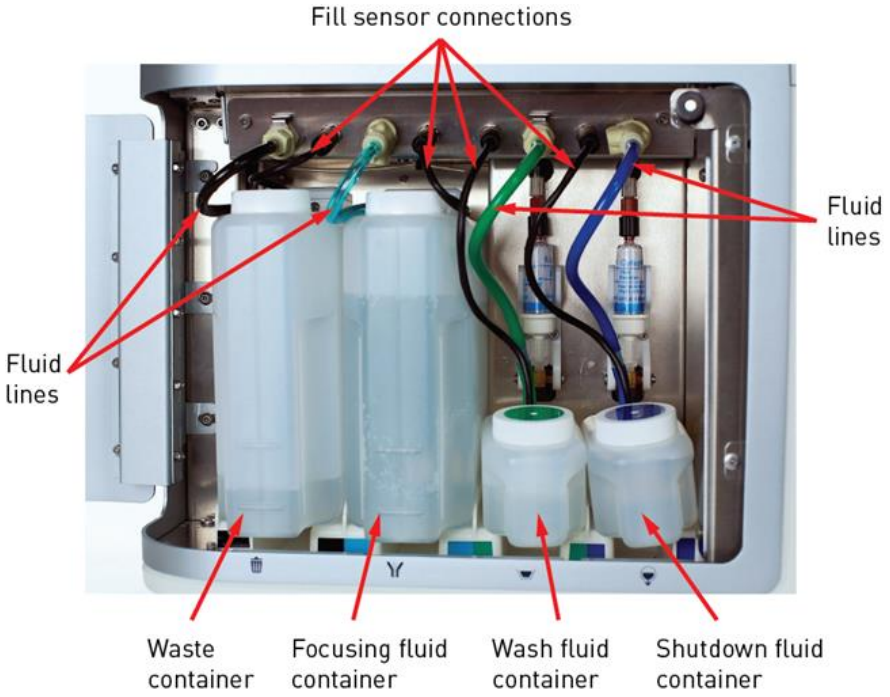
Note: Always wipe the outside of the SIP after doing a SIP Sanitize

Daily - Visual inspection

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

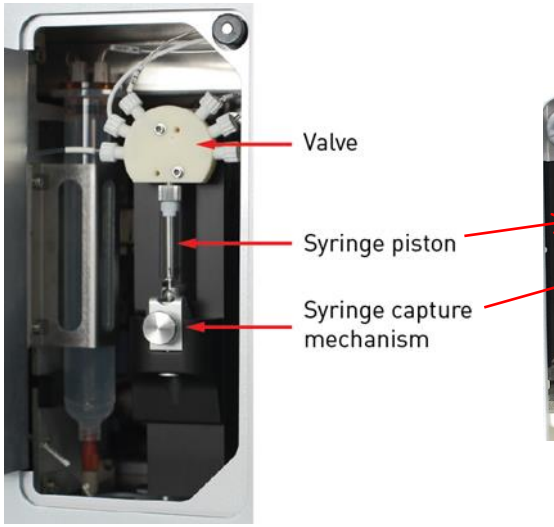
- Check the fluids level. Fill/empty as needed:
  - Focusing fluid
  - Wash solution
  - Shutdown solution
  - Waste

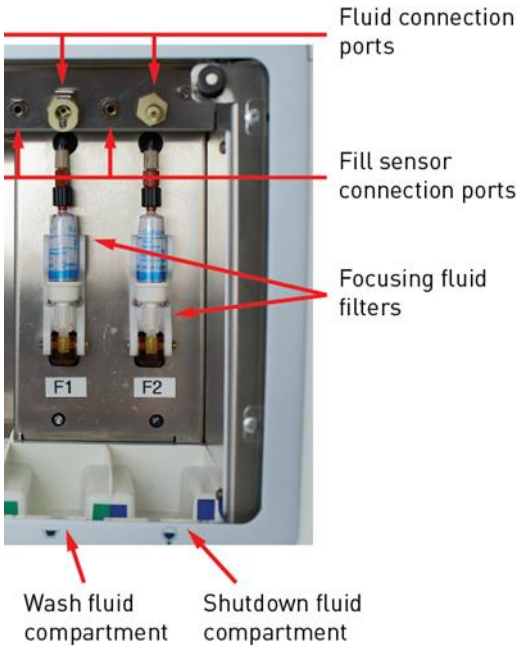
- Visually inspect the SIP



Daily - Visual inspection

- **Syringe compartment** – confirm 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 salt residue builds-up
- **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





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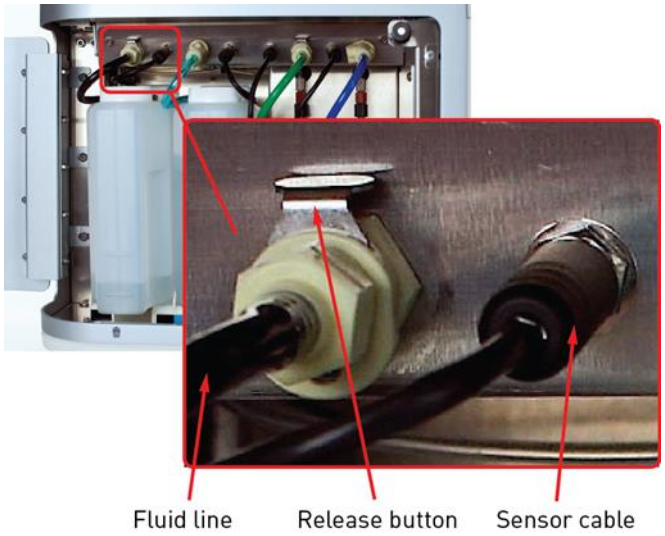
Filling (or emptying) Fluid Tanks



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
   Large tanks – 1.8 L
   Small tanks – 175 mL
4. Return to cytometer and reconnect the fluid line then the sensor line

	Fluid line	Sensor cable
Disconnect:	2	1
Reconnect:	1	2

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.

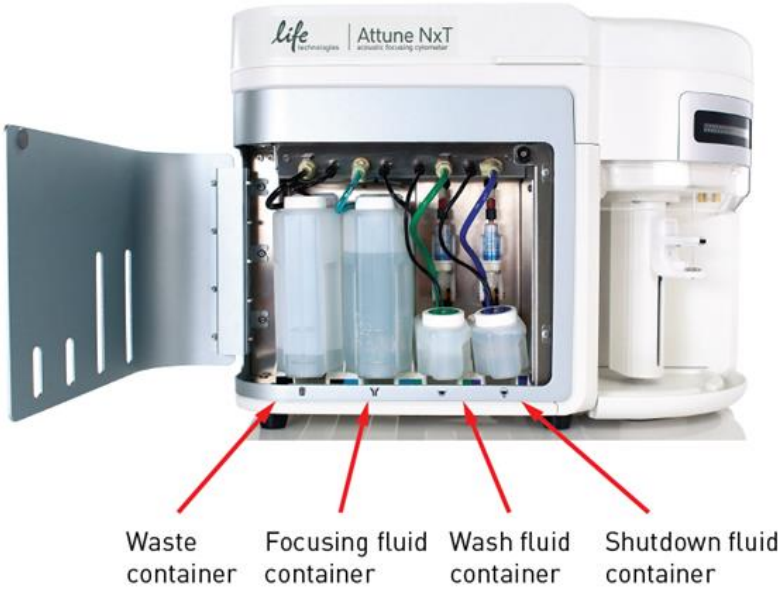
2) The Attune® NxT Acoustic Focusing Cytometer must be idle before refilling the fluidics containers.

3) Do not pull on the lines.

Contamination

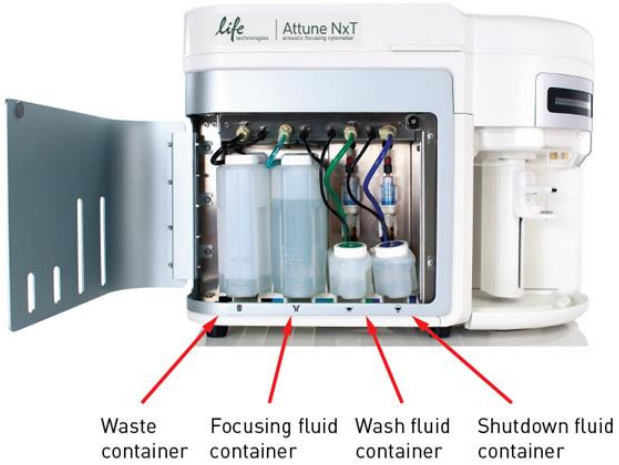
Potential problem – Contamination

- Check tanks for cloudiness or debris in the solutions or brown marks on the sensor
- Fill the emptied waste container with a **full strength bleach up to the bleach fill mark (bottom line) on the bottle



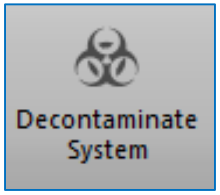
**
112 mL of conc bleach (8.25%)
120 mL of ultra bleach (6.15%)
180 mL standard bleach (5.25%)

Fluidics Replacement Parts



- Replacement part's:
  - 1.9 L waste tank # 100022156
  - 1.9L focusing fluid solution tank # 100022155
  - 175 ml wash solution tank # 100022151
  - 175 ml shutdown solution tank # 100022154
  - AAS focusing fluid solution tank # 4477847
  - AAS waste tank # 4477850

System Decontamination



- Function which facilitates the decontamination of the Attune® NxT™ Acoustic Focusing Cytometer and the Attune® Autosampler fluidics.
- Sanitizes the system and fluidics bottles with Bleach and Wash solutions.
- Mostly automatic - 60 minutes, 3 phase operation with on screen instructions
- REPLACE FOCUSING FLUID FILTERS

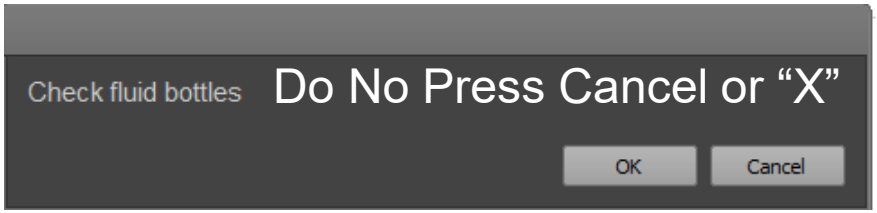
Note: this function is only available to advanced users and administrators

When?

- As a **quarterly** 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
- Anytime contamination in the fluid lines is suspected – i.e. event rate is too high
- Prior to any service work or shipment for service

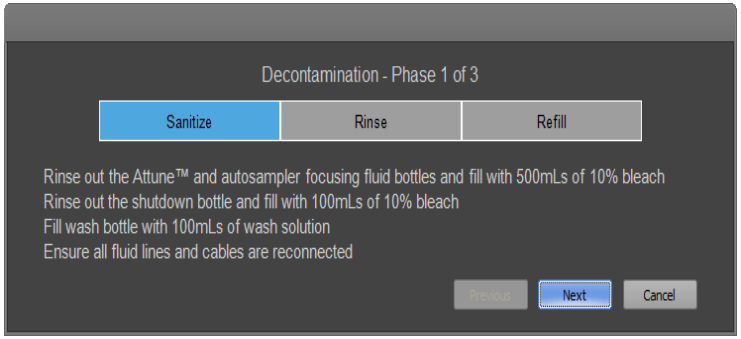
NEW: Improved instructions for System Decontamination

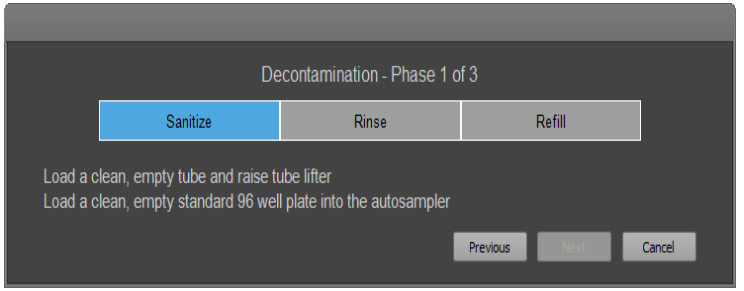
- Decontaminate System is divided in to three phases: **Sanitize, Rinse and Refill**



This screen is displayed when bottles are disconnected

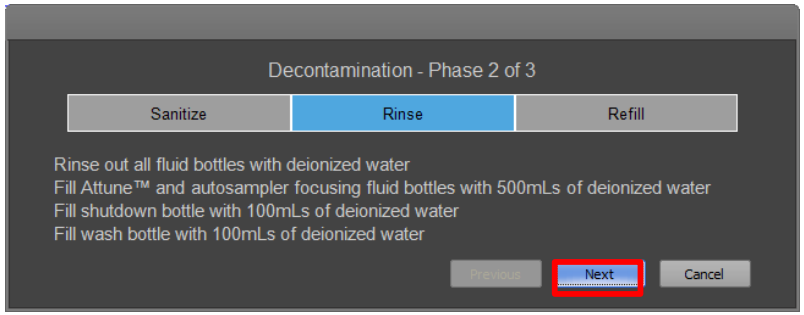
Phase 1

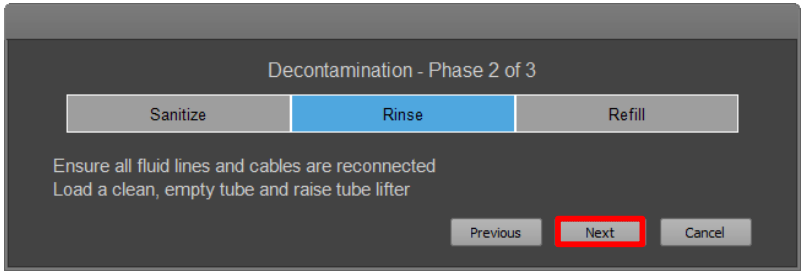




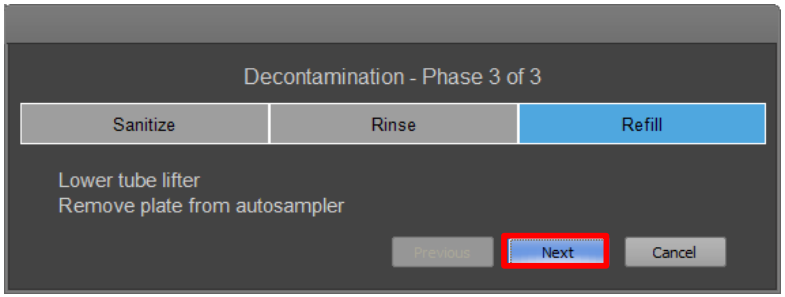
Decontaminate System

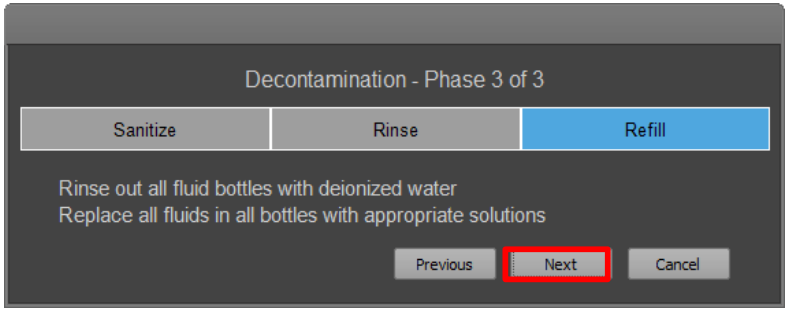
Phase 2

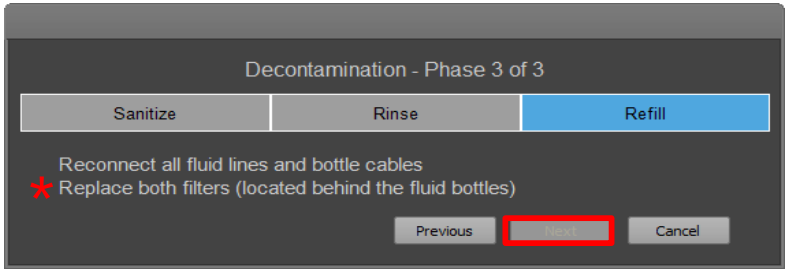




Phase 3







Phase 3 - *Replace filters before reconnecting fluid lines

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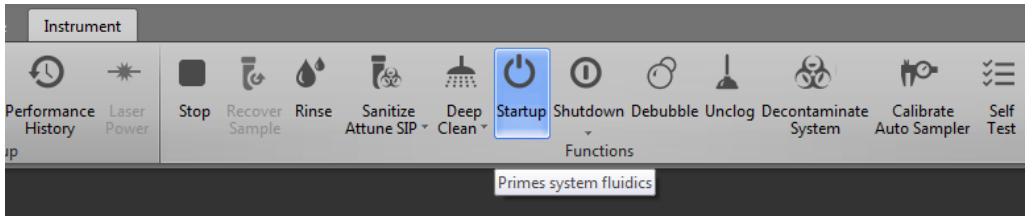
After System Decontamination: Prime Fluidics

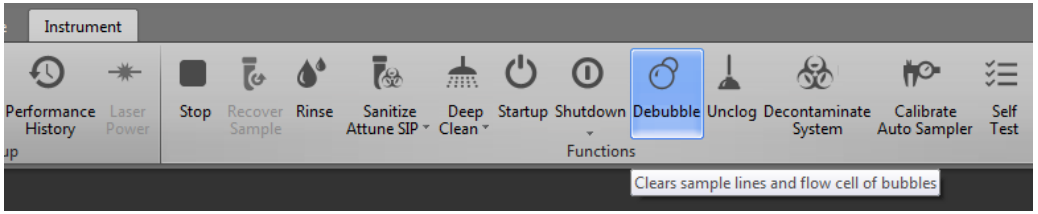
After System Decontamination and replacement of focusing fluid filters, the Attune NxT must be primed to remove air from the new filters

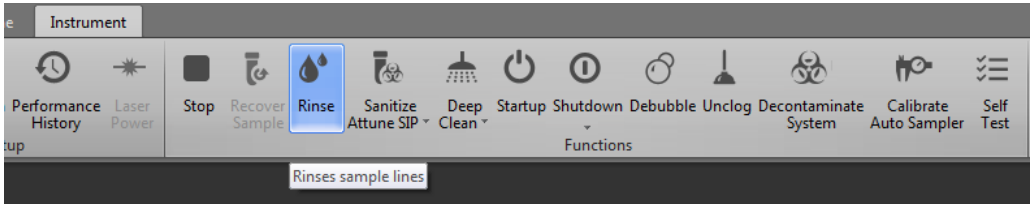
To Prime the Fluidics System:

1. Run 3 Startup cycles
2. Run 2 Debubble cycles using Debubble solution or wash solution
3. Run 1 Rinse cycle
4. Run Performance Test. If PT fails, run two debubble cycles and repeat Performance Test.

Your system is now ready to use







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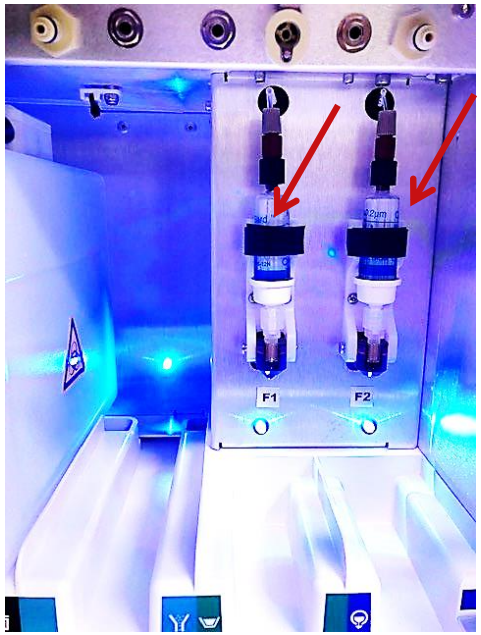
Maintenance – Focusing Fluid Filters

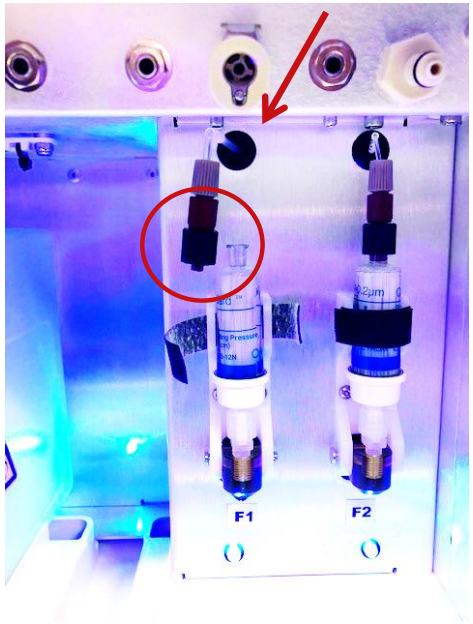
Focusing fluid filter
PN 100022587

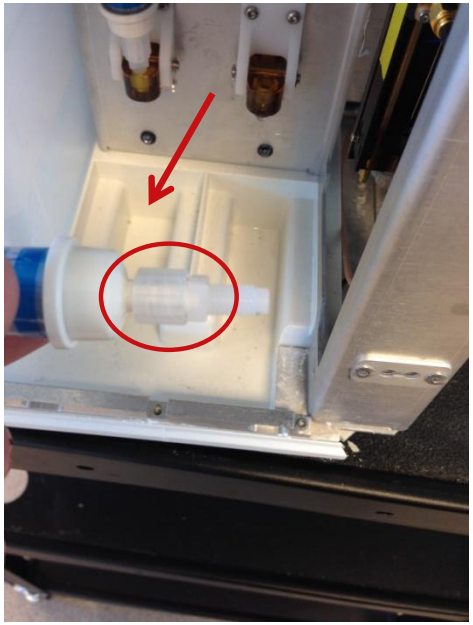
- Two filters located behind the Wash and Shutdown bottles
- The filters may grow some contamination over time. If discoloration is evident, replace the filters.
- Replacing focusing fluid filters **quarterly** reduces the risk of any potential contamination in the lines. ALWAYS REPLACE AFTER SYSTEM DECONTAMINATION.

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)
- **Prime the filters by running 3X Startups and 2X Debubble**







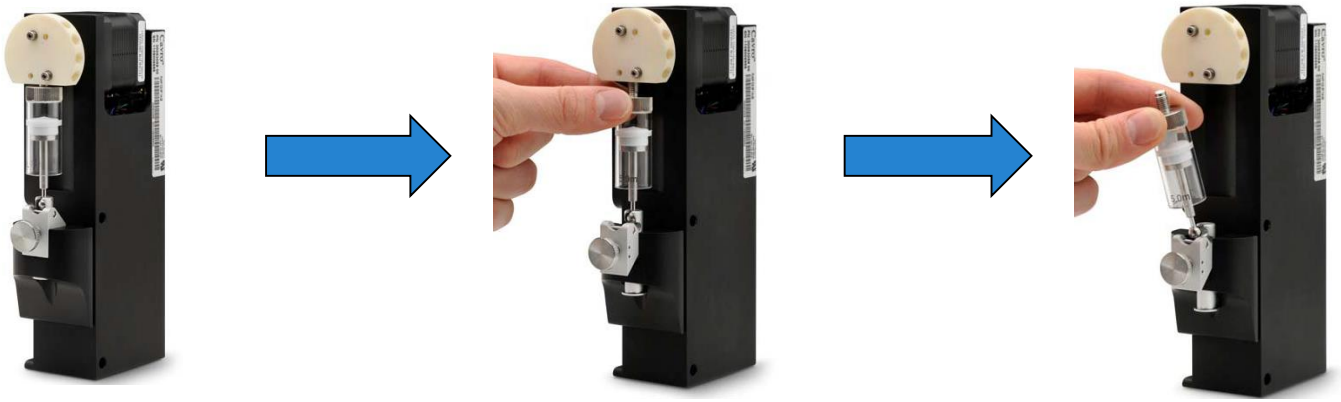
Maintenance – Sample Syringe

- Potential problems:
  - 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 # 100022591
- Replacing Frequency: as needed, at least bi-annually

How to change the sample syringe?

- Run 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 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.

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



Maintenance - Optics

A27784 Filter Holder Kit
2 dichroic filter holder
2 bandpass filter holder

- Check for dust or scratches on the filters
- Gently remove any dust from the surface with a blower (bulb or compressed gas).
- **If there is any other issue with the filter, please call Technical Support.**

Attune NxT Database Utility

What does the Attune NxT Database utility do?

1. **Backup User Data:** backups the entire database, files plus database data to a folder a single time (duplicates everything upon initiation)

2. **Restore User Data:** restores the contents of the folder from a backup so that the backup becomes the current version of the database (eg, the “live” database)

3. **Automated backup:** schedules automatic backup of user data and the database

4. **Re-Install Database:** used to reset the database to the **new, no data added state**. This should rarely need to be used
   - When would this be suitable? If recommended by FSE
   - **Please reference detailed slide deck for more information**

Maintaining computer efficiency & data quality

Administrator

Set up user accounts with operator privileges
User, Advanced User, Administrator

Back up experiments to secondary storage

Virus protection – scan thumb drives before connecting to Attune computer

Defragment the hard drive *weekly*

Network connection (optional)

Maintaining computer efficiency & data quality

- **Operator/user**

  De-select those parameters not needed - minimize file size

  ***Do not clutter the Experiment Explorer ***

  Close experiments not currently active (collapse all)

  Export then delete experiments from the browser

  Export experiments - .atx or .apx

  FCS files – 3.0 or 3.1 format

If you need to scroll through the list of experiments in the experiment explorer – it is time to backup and delete

Virus protection – scan thumb drives before connecting to Attune computer

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

Maintenance Summary

Procedure		Frequency
Startup and Shutdown		Daily (2-3 x per week)
Visual inspection of	sample injection port (SIP), fluidics tanks and connections syringe pumps	Daily
Fluidics maintenance – cleaning routines on Instrument tab *see the posted daily cleaning guidelines*	Rinse	Daily – between samples
	Sanitize SIP	Daily – between samples, experiments, or users
	Deep Clean	Daily – between experiments
	Debubble	Daily – as needed
	Unclog	Daily – as needed
Sanitize SIP with Attune Cleaning solution	Usage - heavy: ≥6 hr per day, ≥6 plates per day, multi-lab shared system	Daily before shutdown
	Usage - light to moderate <6 hr per day	Once a week before shutdown
Fluid bottles	Empty, clean, rinse, refill	Monthly
System Decontamination		Every 3-6 months
Replace focusing fluid filters		Every 3-6 months
Syringe replacement		6 months or as needed
Optical filter and mirror inspection		Monthly or less
Secondary backup of experiments	Export experiments (.atx or .apx) or FCS files	Daily, weekly
Attune Database	Schedule auto backup	Daily, weekly or monthly

Service Preventive Maintenance (PM)

- Replacement of:
  - Focusing Fluid Filters
  - 1 mL Syringe
  - Sample probe (SIP)

- Cleaning of:
  - Interior and Exterior of unit
  - Optical filters

- Check for leaks
- Check fittings and valves
- Run a system diagnostics test
- Check pinhole and laser alignment
- Computer Maintenance

Ensures maximal performance

Attune® NxT Service Plan

√ 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	Typical 2 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

Changing the location of the Attune® NxT?

Bench space needed: W x H x D

Width: 50 inches
- 22.9 in. for Attune® NxT
- 3.75 in. for access to side syringe compartment
- 22.5 in. for Computer system

Height: 29 inches to allow the hinged lid to fully open

Depth: 23.1 inches
- 17.1 in. for Attune® NxT cytometer
- 2.5 in. for adequate ventillation behind the instrument
- 4 in. for the fluidics bottles in front of the unit

Logins - all case sensitive

Instrument is powered on in a set order:

1) NxT Autosampler
2) NxT Cytometer
3) Open Attune NxT software

Instrument:

User name: INSTR-ADMIN

Password: INSTR-ADMIN

Attune NxT Software:

User name: admin

Password:

Individual users (account can be setup by someone with administrator privileges):

User name: avoid ALL CAPS

Password: same as user name. Temporary until first login

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Thank you!

Please provide feedback if/when surveyed

*The following are reference slides for Attune NxT software

Updated Version of Manuals

- Hardware User Guide

  https://assets.thermofisher.com/TFS-Assets/LSG/manuals/100024235_AttuneNxT_HW_UG.pdf

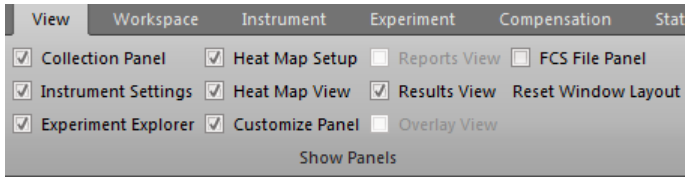
- Software User Guide

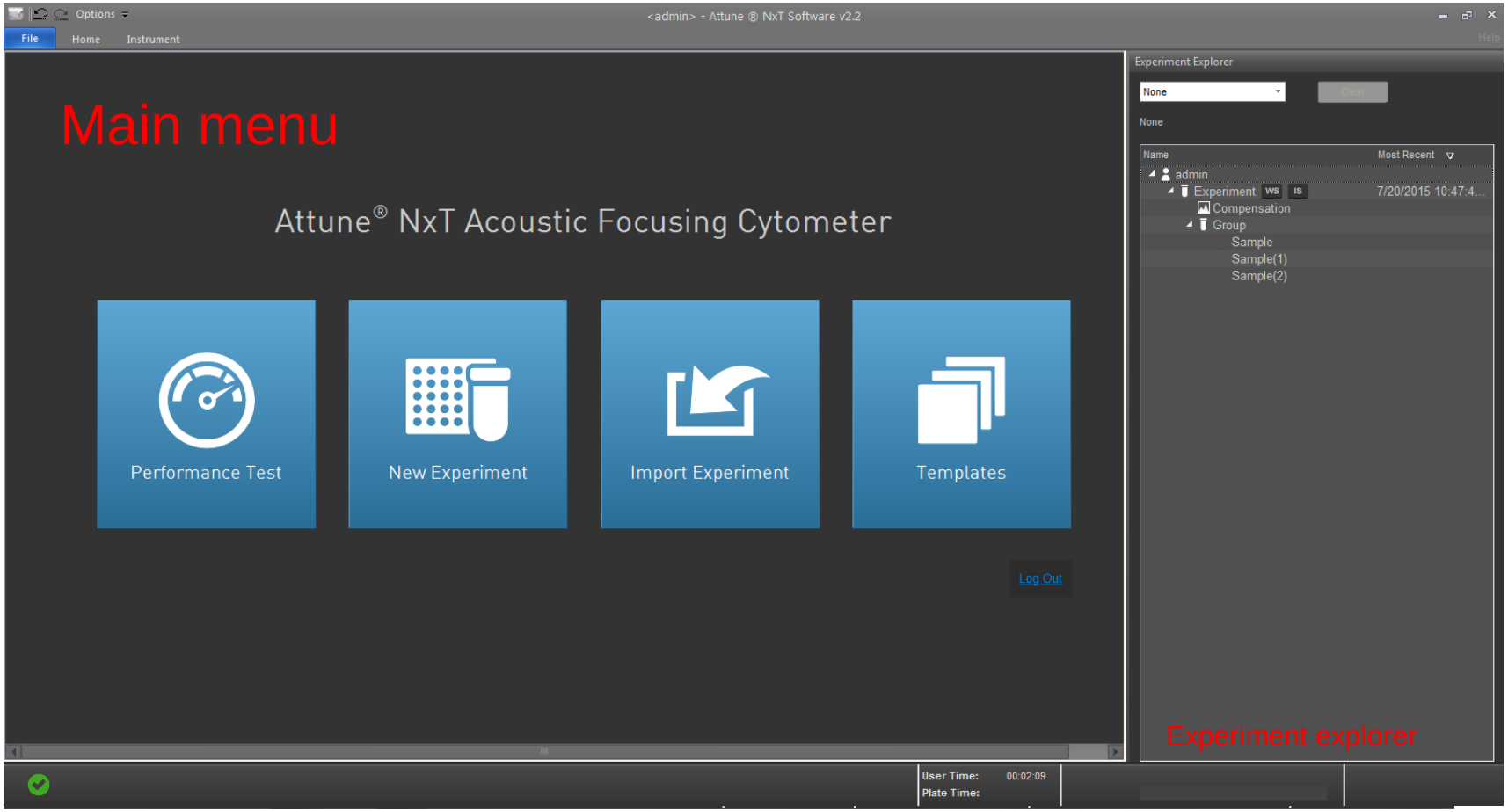
  https://assets.thermofisher.com/TFS-Assets/LSG/manuals/100024236_Attune_NxT_SW_UG.pdf

- Maintenance and Troubleshooting Guide

  https://assets.thermofisher.com/TFS-Assets/BID/Product-Guides/attune-nxt-maintenance-troubleshooting-guide-quick-reference.pdf

Software





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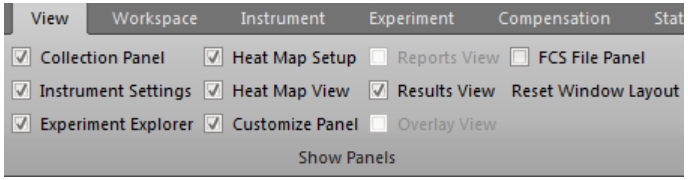
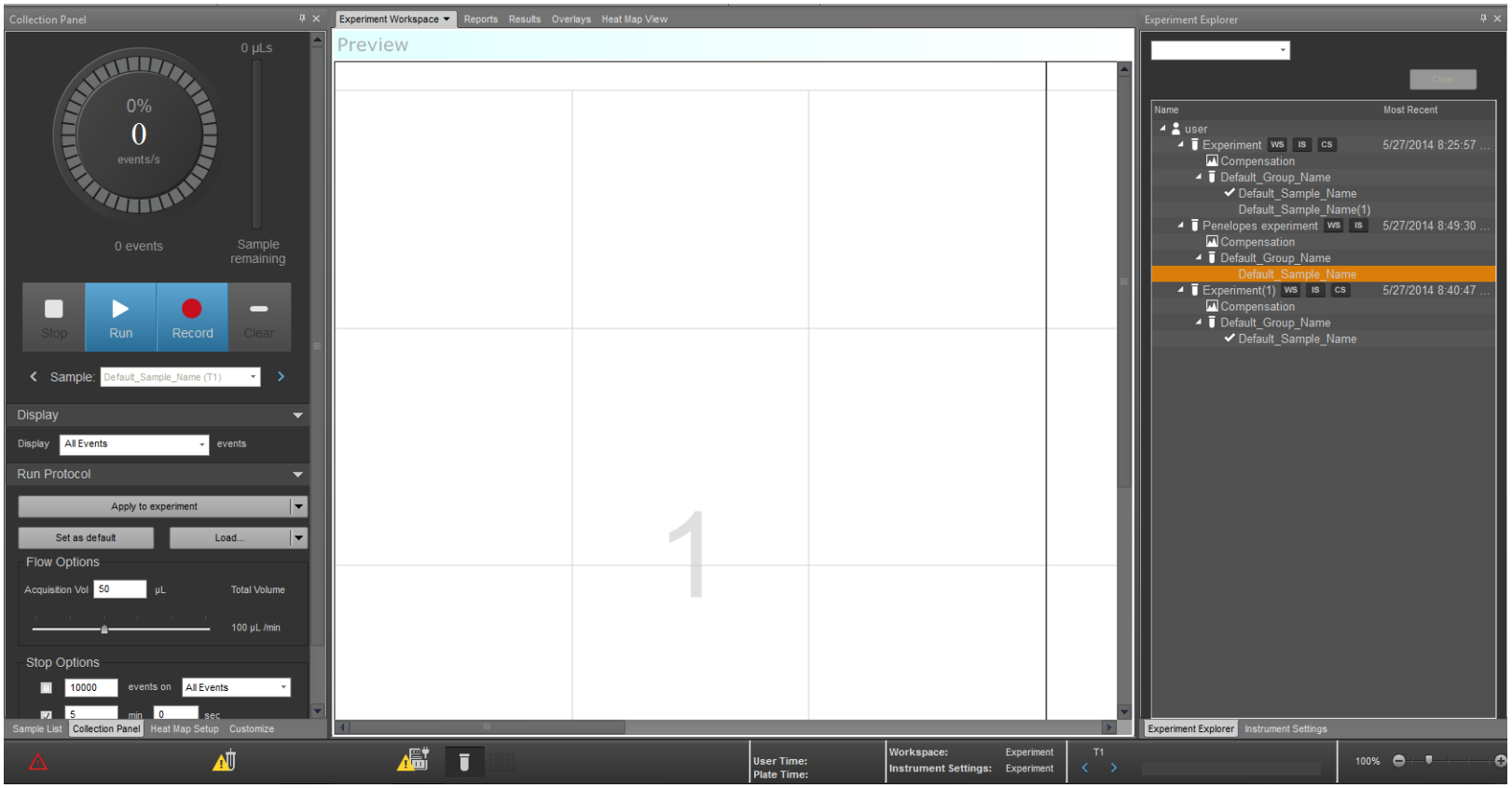
Panels





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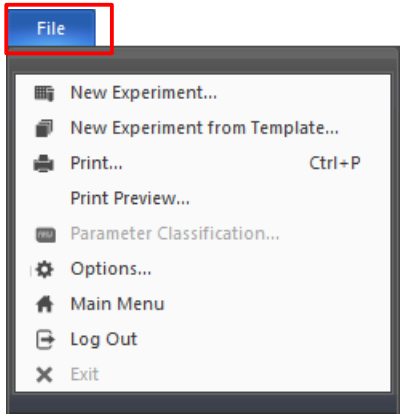
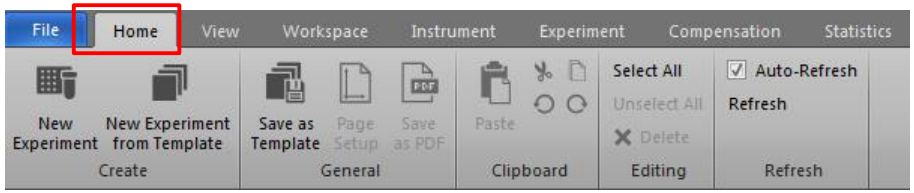
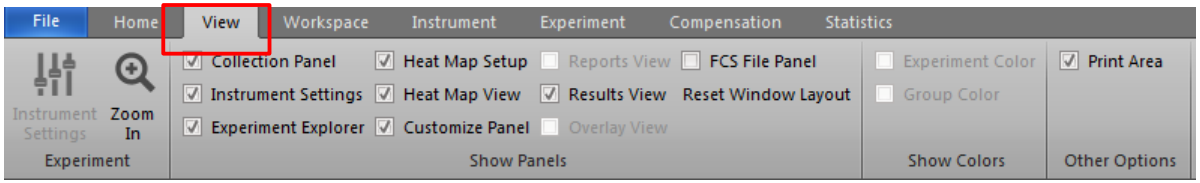
Ribbons and Tab (#1)

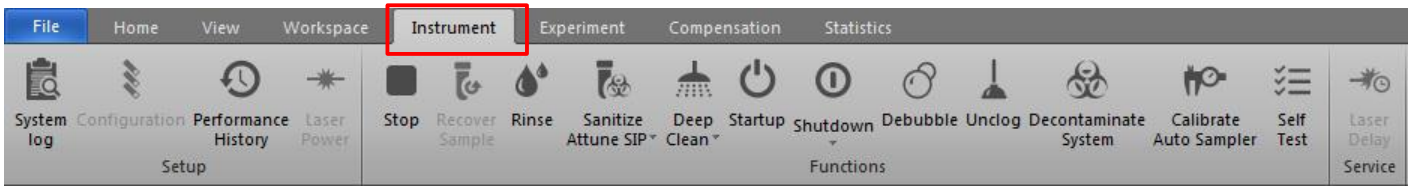




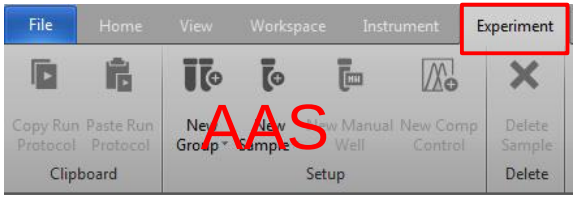
Home



View



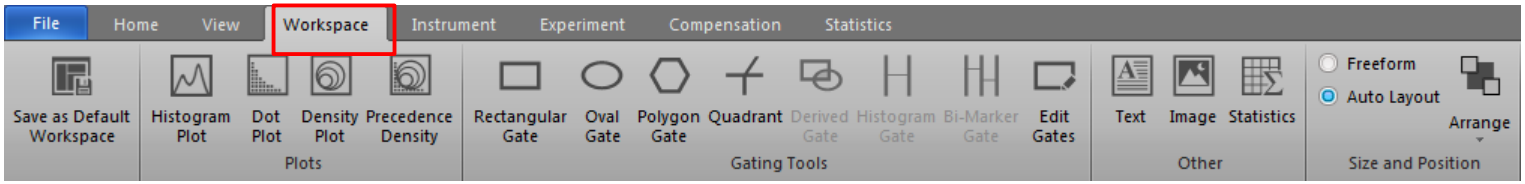
Instrument



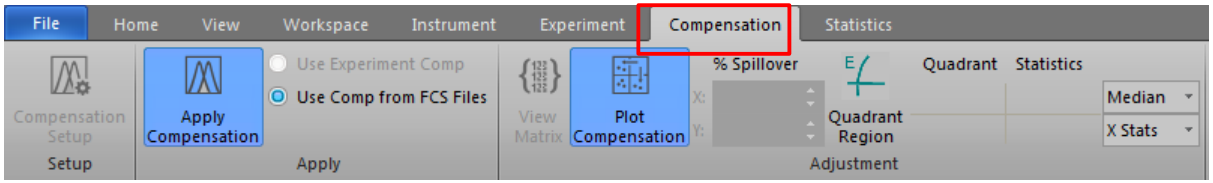
Autosampler

More information in the SW user guide

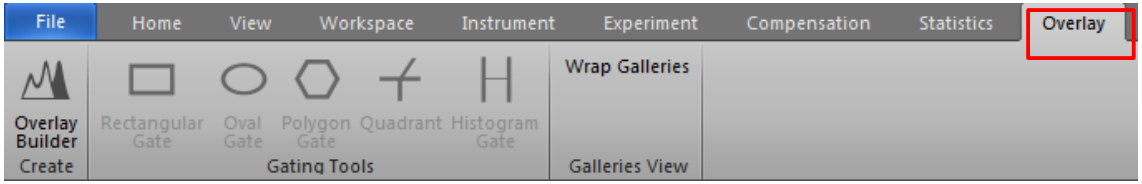
Ribbons and Tab (#2)



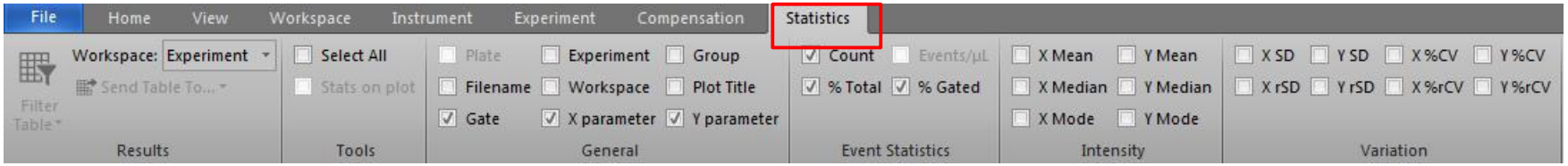
Workspace



Compensation



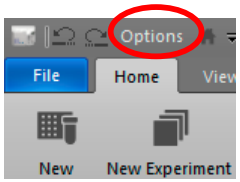
Overlay builder

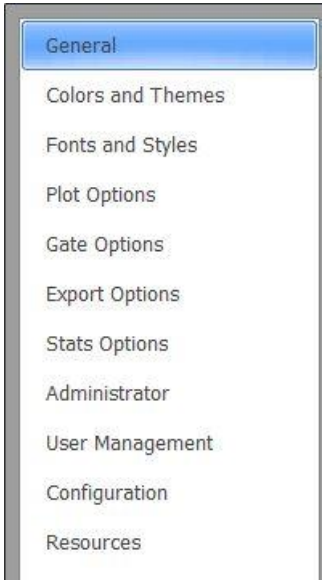


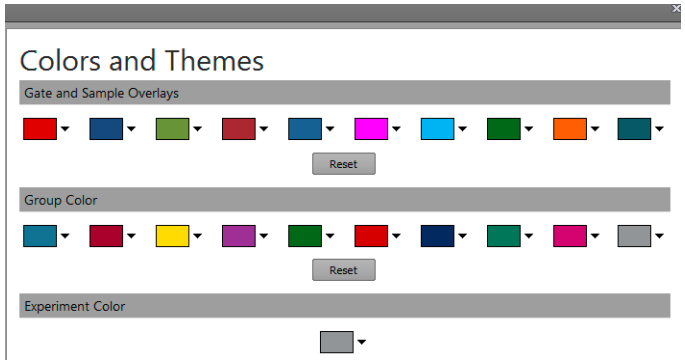
Statistics

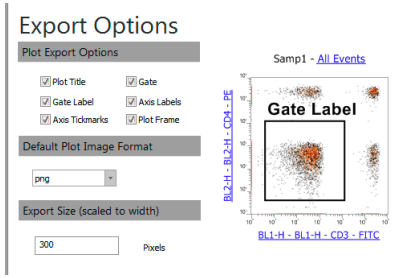
More information in the SW user guide

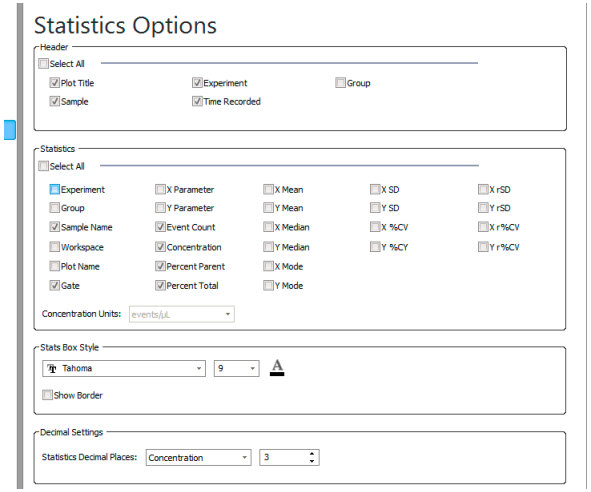
Setting User Preferences

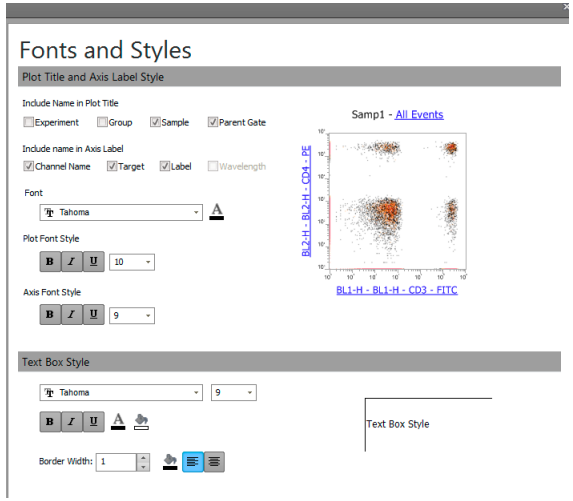


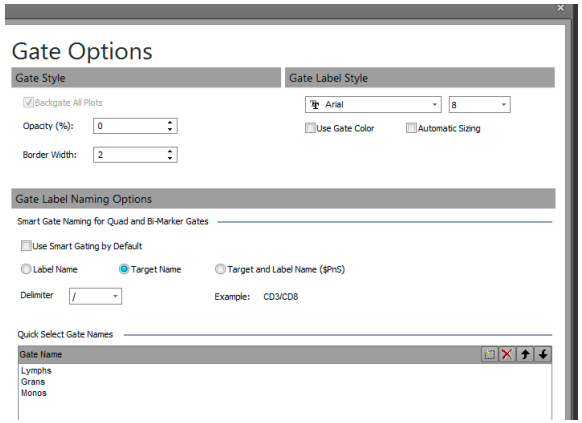












Software Overview

Workspace
- plots
- gate
- scales - linear, log and hyperlog
- statistics box and results table

Customize

Heat Map View and Setup

Filter Configuration Manager

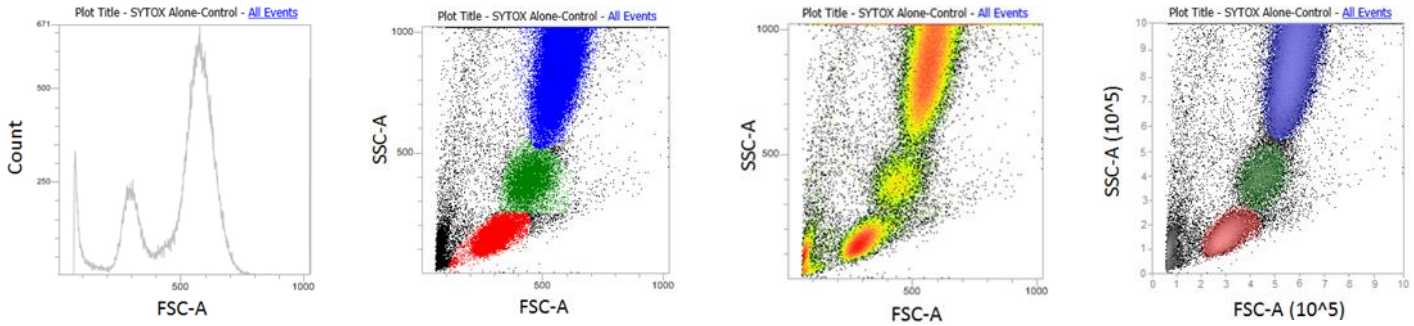
Workspace - Plots

Histogram – 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

Density precedence – 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.



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

Note: *Defining the workspace and gating strategy prior to data acquisition is highly recommended. Final gate adjustment should be made after a file is recorded.*

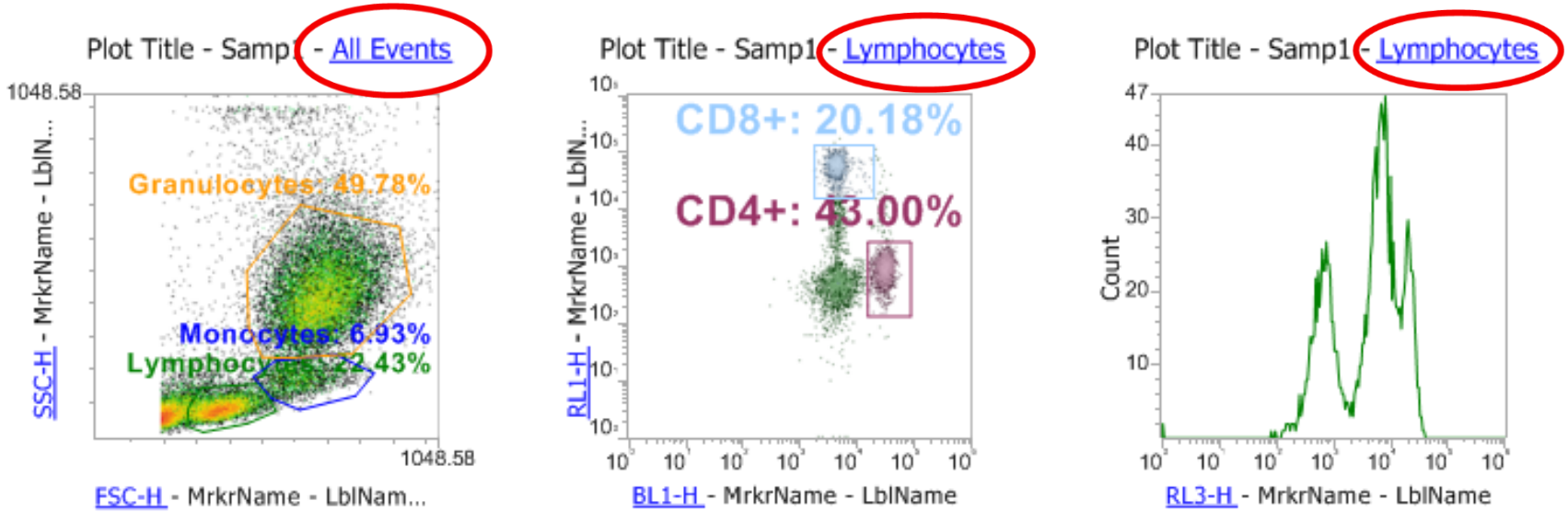
Workspace – Gating Tools

- 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 parameter 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
- Region may be exported as an fcs file by right-clicking on the gate and choose Export to fcs file

Daughter Plots

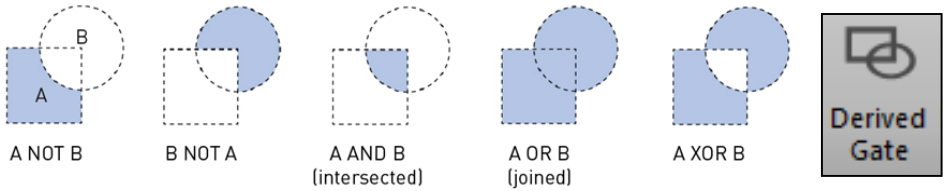
- Displays data only from the region selected

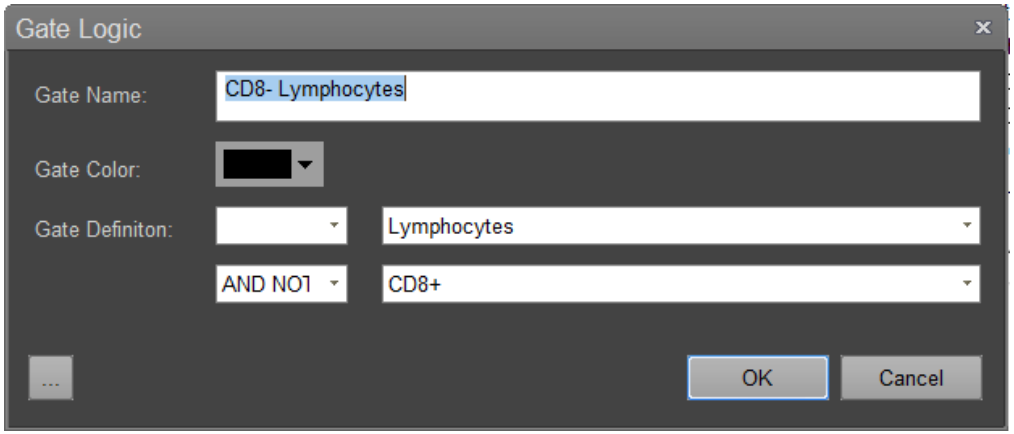


- To create
  - Right click on the gate and select **Daughter plot** and the type of plot
    OR
  - Right click on the plot and select **Set population**
    OR
  - Left click on the Hyperlink in the plot title and select gate name from the drop down list
  - Note: do not use quadrant gate as a parent gate

Workspace - 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 gates

Note:	*When naming a derived gate with two words, use parentheses to enclose both words.*
	Derived gates can only be created using regions.

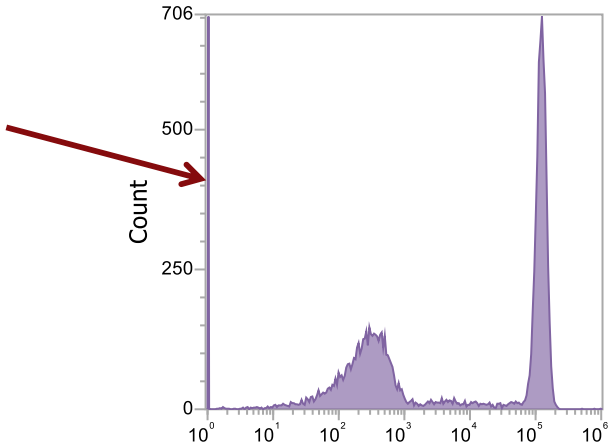
Scale Options

Impact the way data is visualized

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

HyperLog™ Scale

- Log scale – cannot correctly represent values for cells whose fluorescence values fall at/or below zero - compensated data piles up on the axes

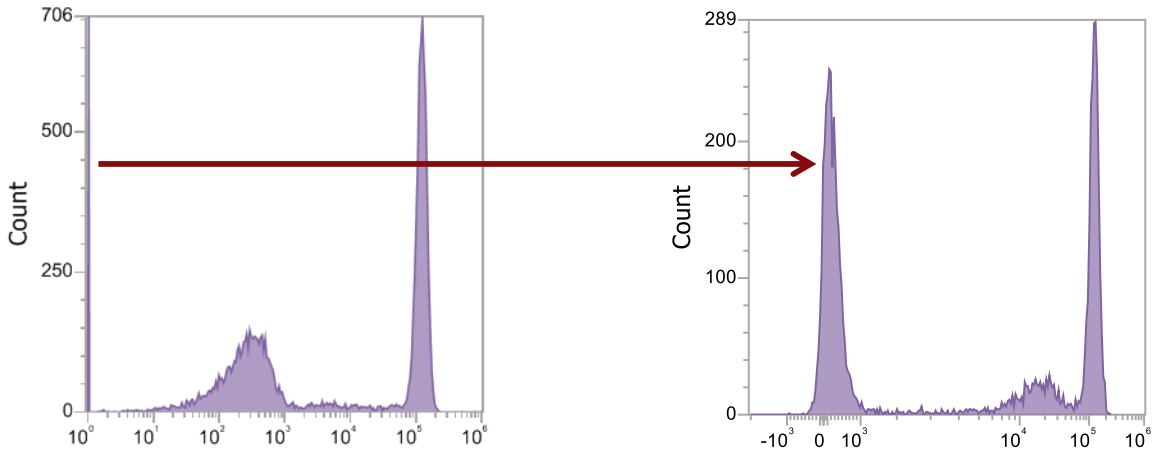


Negative fluorescence results from

- Background subtraction
- Compensation

HyperLog™ Scale

- HyperLog™ – similar to LinLog scale used in classic Attune®
  - Logarithmic scale at the high end
  - Transitions to linear scale in the region around zero



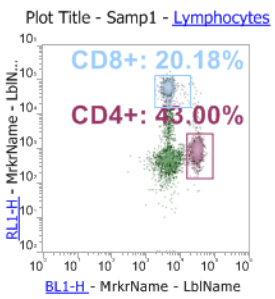
- Use to determine if compensation is correct
  - Correct – double neg population will distribute symetrically around autofluorescence level
  - Overcompensation – double negative population center below autofluorescence value
  - Undercompensation – double negative population centers the distribution above autofluorescence value

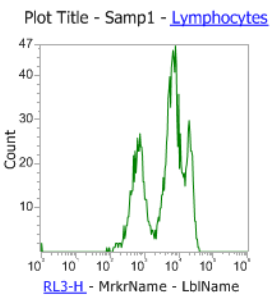
Modern Flow Cytometry: A Practical Approach *Clin Lab Med*. 2007 September ; 27(3): 453–v.

Results displays

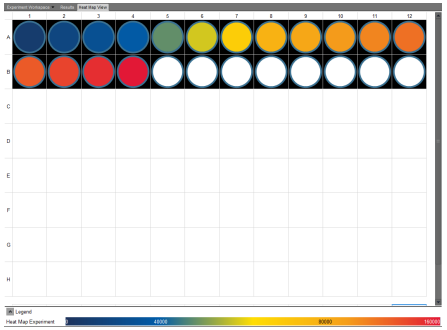
Visual

Workspace plots





Heat Map

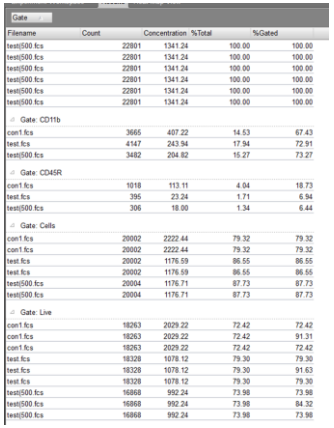


Statistics

Statistics box (on plot)

Name	Count	%Total	%Gated	Concentration
All Events	25,218	100.000	100.000	2,802.000
Cells	20,002	79.316	79.316	2,222.444
Live	18,263	72.420	91.306	2,029.222
RBC	12,163	48.231	66.599	1,351.444
live wbc	5,435	21.552	29.760	603.889
CD45R	1,018	4.037	18.730	113.111
CD11b	3,665	14.533	67.433	407.222

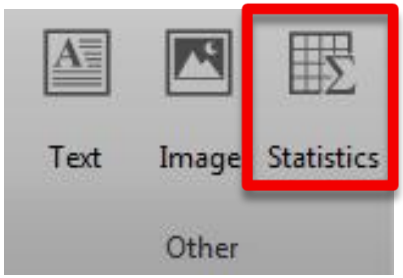
Results table



Statistics export

- CSV spreadsheet

Statistics Table

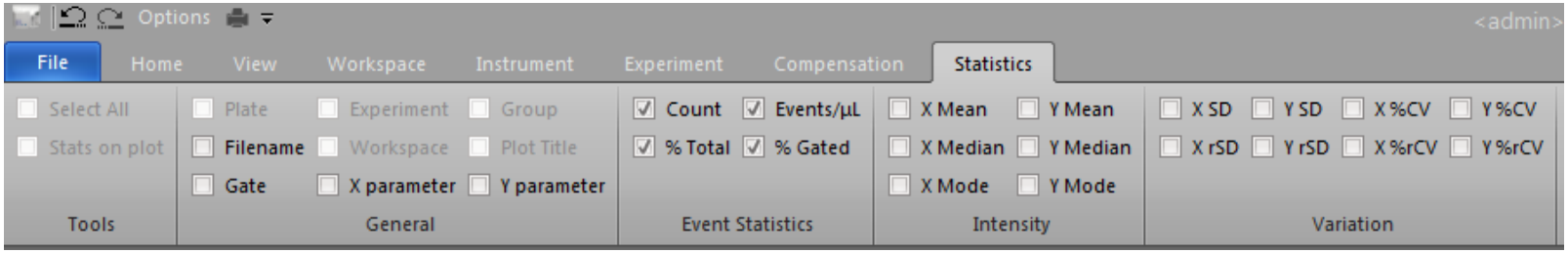


Experiment: **6 color immuno**
Group: **Default_Group_Name**
Sample: **NOT-IN_STATS**

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 Statistics table by right-clicking on a plot or on the workspace, and select Insert Statistics
- Prior to adding a statistics box, make sure the workspace has at least one plot.

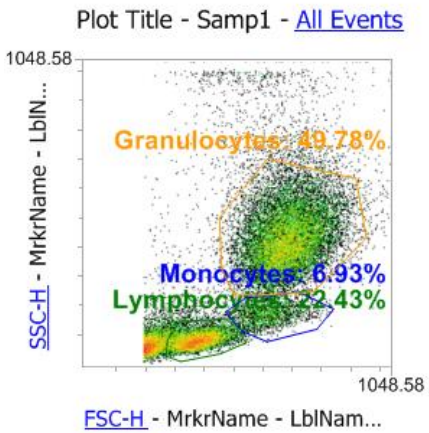
Customize Statistics



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 Statistics table, select the Table and check statistics to display in the *Statistics* Tab



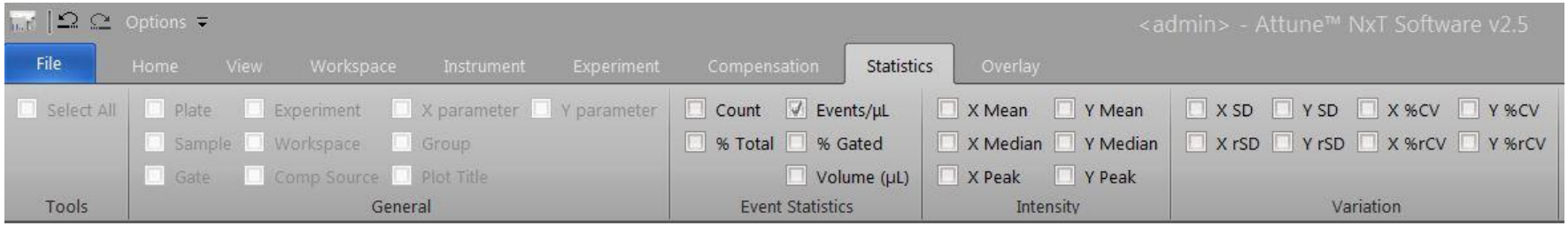
- To customize Statistics value displayed on a plot, select the plot and choose the statistic

Statistical Values

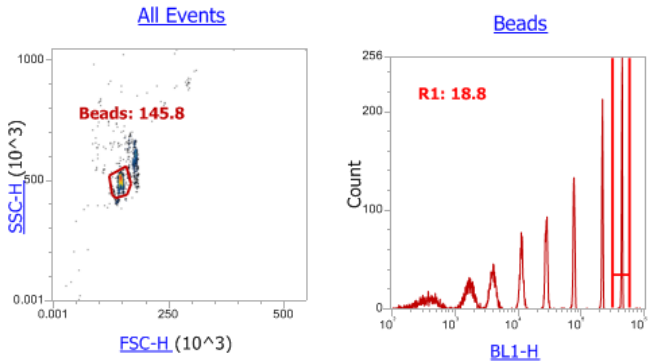
- **Count**: Number of events collected
- **Events/µl**: Concentration of events/µl in the gated region
- **% Total**: Percentage of total events collected
- **% Parent**: Percentage of a population based on the number of events collected in the parent gate
- **Mean**: Sum of the signal intensities of 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

Sample Concentration

The **Concentration Statistic** can be selected from the Statistics Ribbon



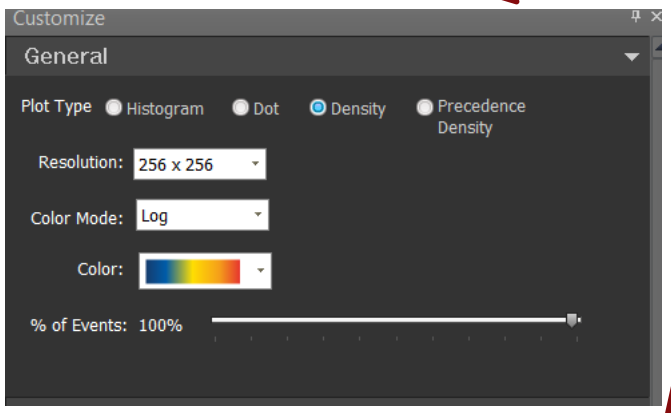
- Values are displayed as Events/µL

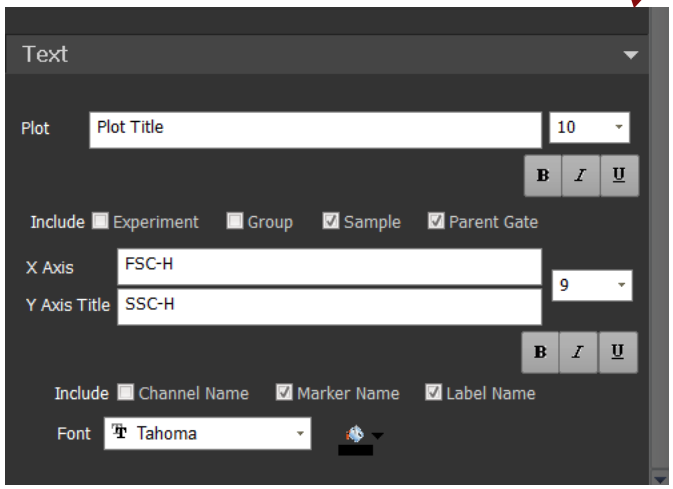


Parameters: **BL1-H**
Gate: **All Events**
Experiment: **8 peak beads**
Group: **Group**
Time Recorded: **10:45:51**

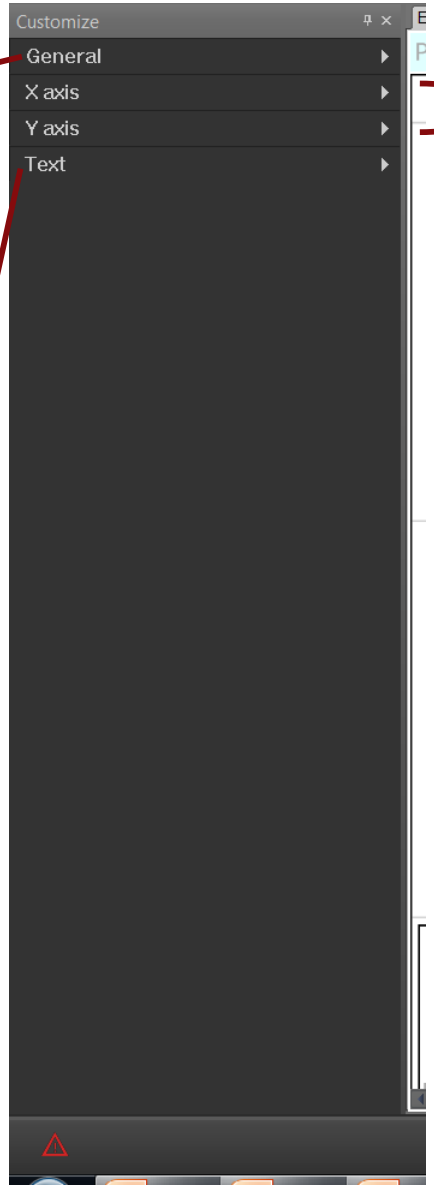
Name	Gate	Count	%Total	%Gated	X Median	Concentration
All Events	All Events	10,000	100.000	100.000	26,705	161.3
Beads	Beads	9,038	90.380	90.380	27,084	145.8
R1	R1	1,166	11.660	12.901	467,501	18.8

Customize Panel

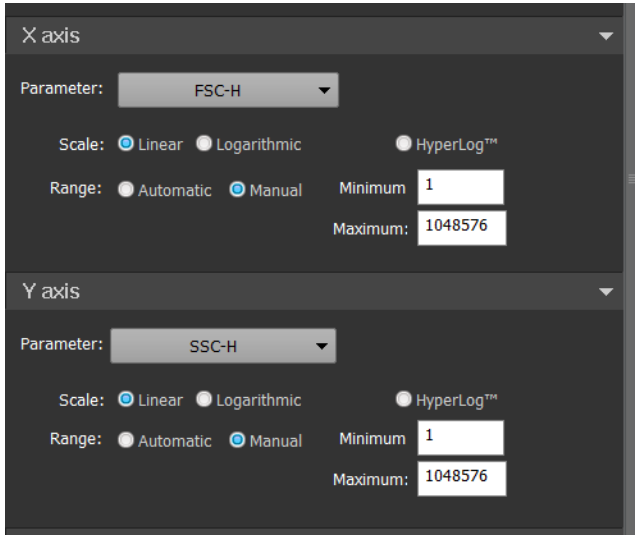




Note: Use < 50 characters when naming gates.
Quad gate names > 31 characters will be truncated.



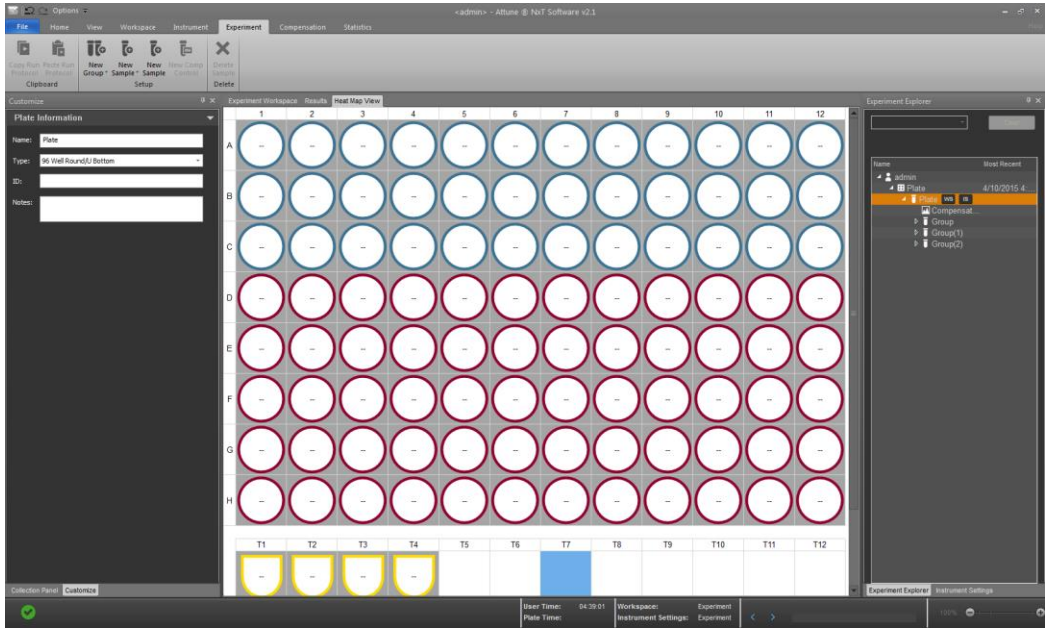
X and Y axis



Scale options
Range adjustments

Heat Map View

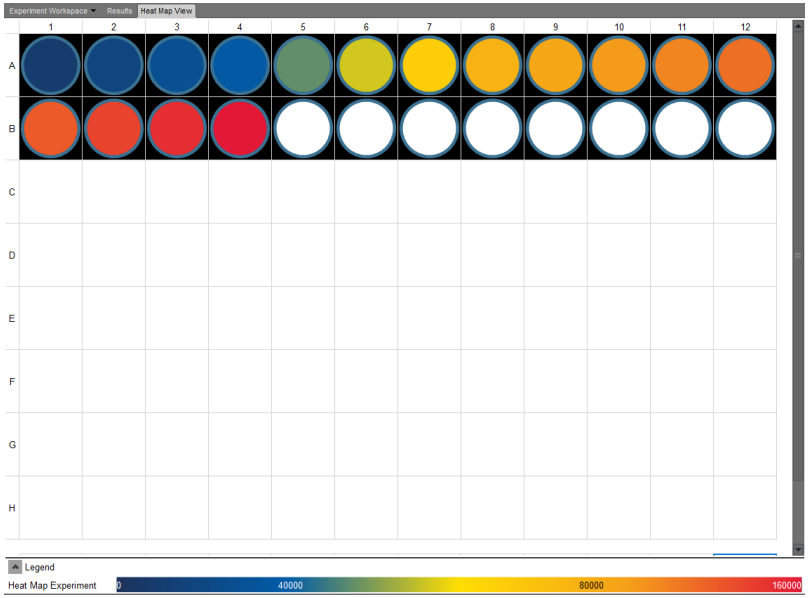
- The *Heat Map view* provides a graphical method (i.e., *Heat Map*) for setting up and analyzing plate- and tube-based experiments
  - Access from the “Heat Map” tab of the workspace desktop
  - Displays plate and tube samples

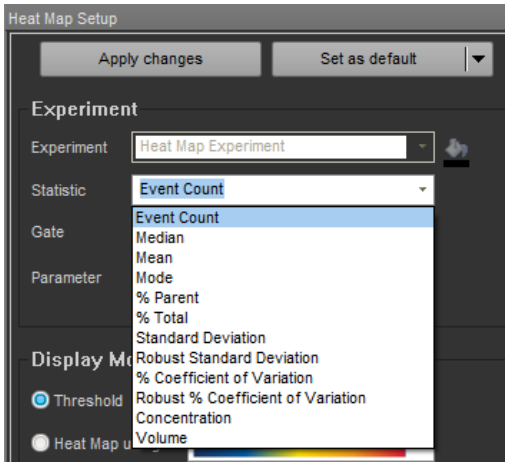


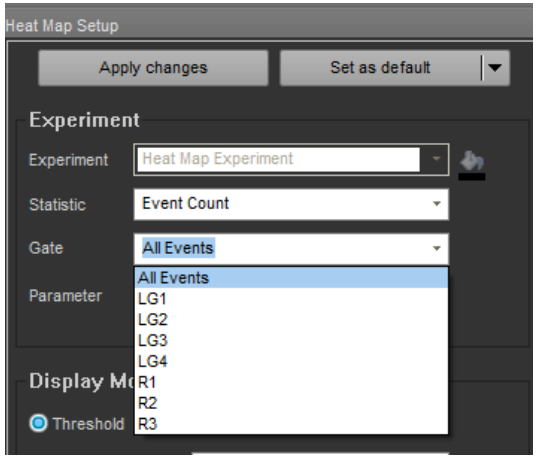
For tubes and plates

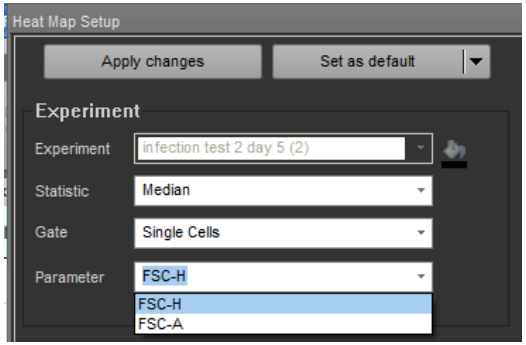
Heat Map Setup

- 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*

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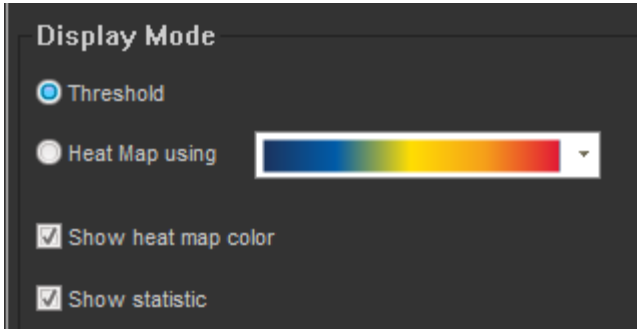
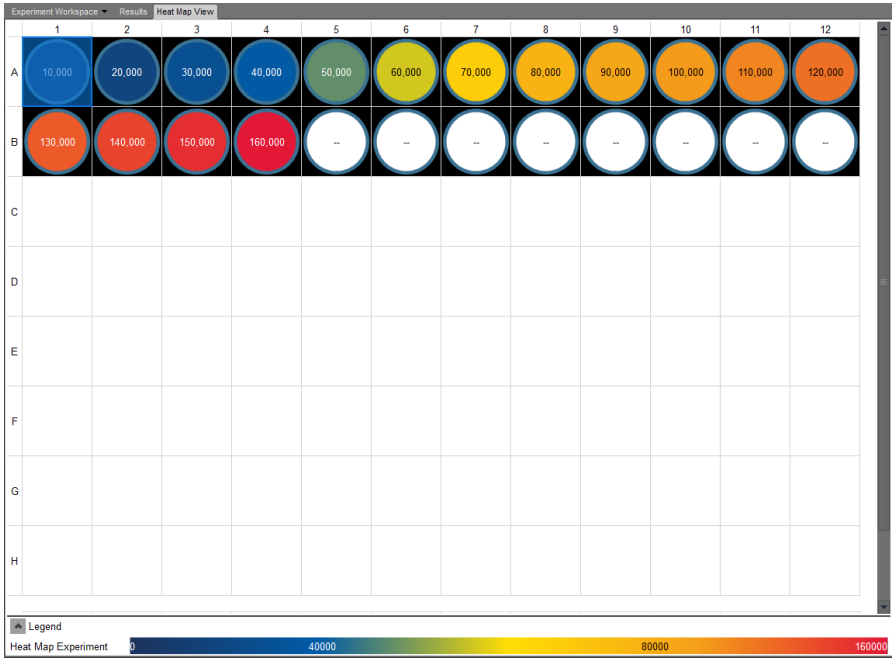
Heat Map Setup

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





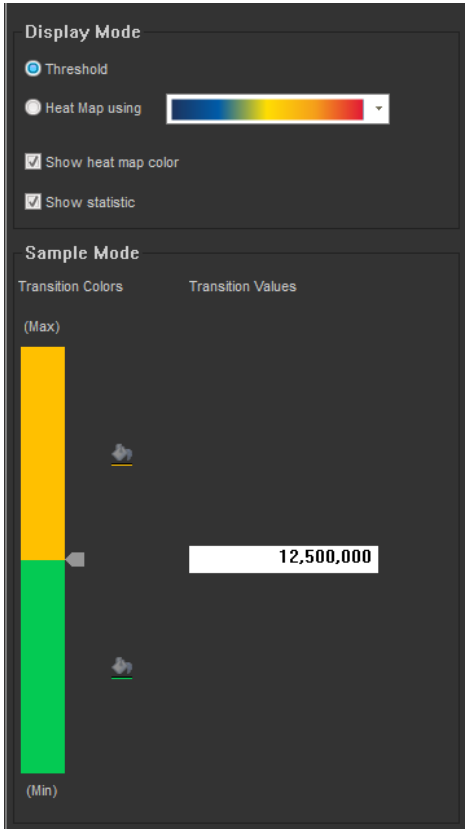
- A legend is displayed on the bottom of the Heat Map Display
  - Indicates experiment name, color scheme and transition points

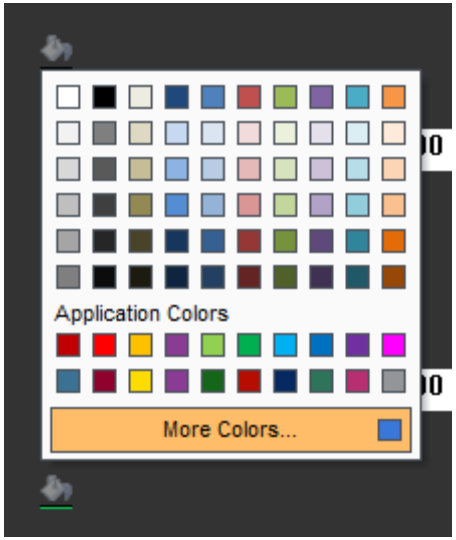
Heat Map Setup

Two display modes are available: Threshold and Heat Map Mode

1. 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 panel of colors

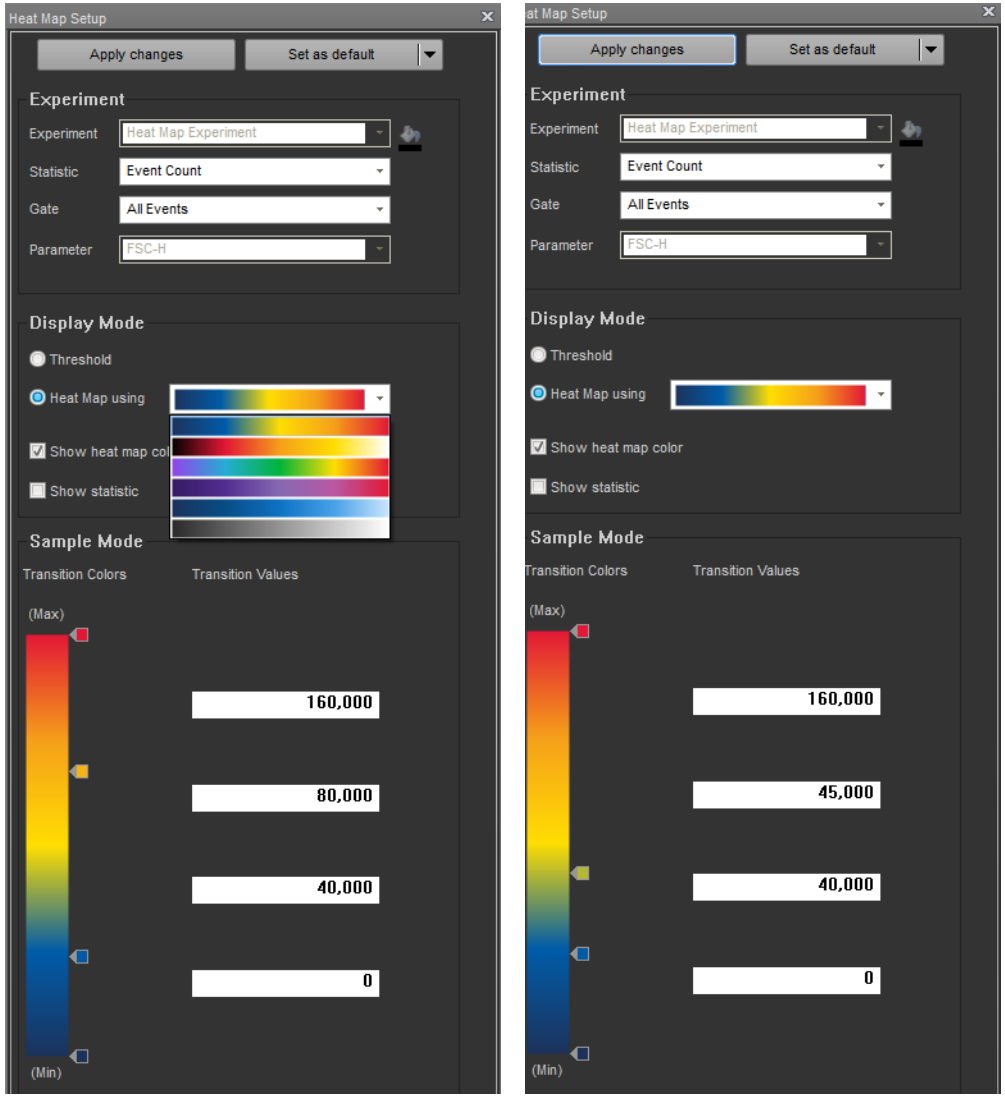




Heat Map Setup

2. Heat Map Mode:

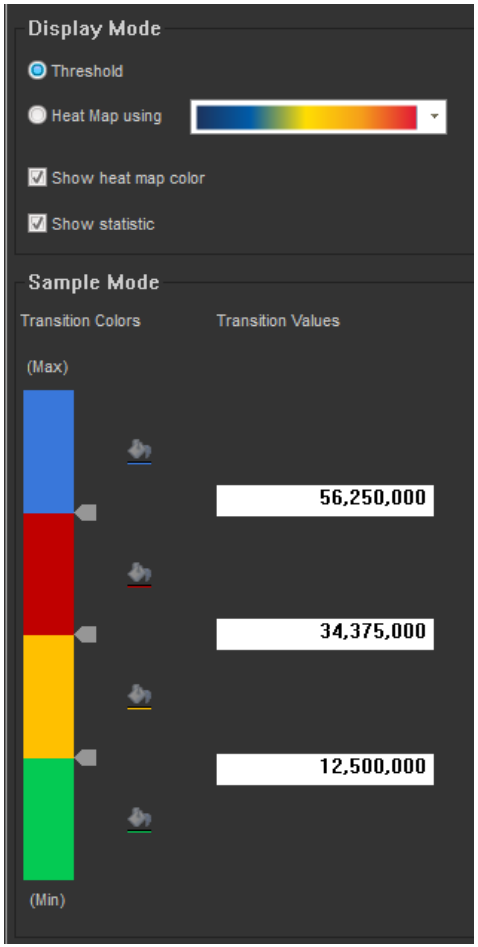
- Data displayed using color gradient to indicate user-specified transition points in data set
- Multiple color choices available from drop down menu

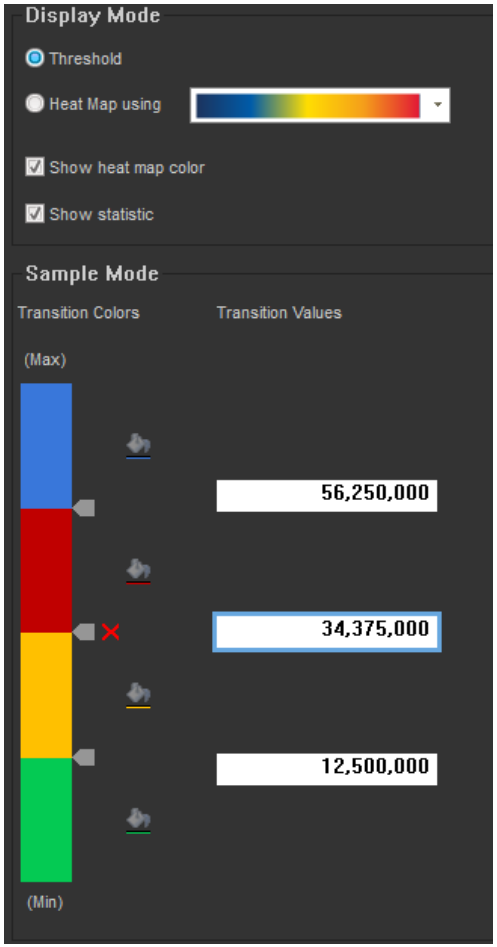


Heat Map Setup

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





Filter Configuration Manager

Set up user-specific filter configurations

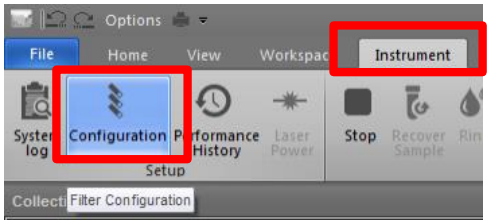
Add new filters to the configuration

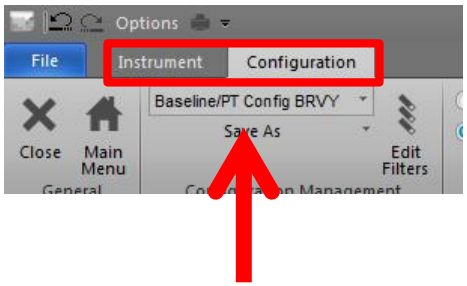
Add new labels to the detectors/channels

Filter Configuration Manager – Instrument Manager

Allows creation of experiments while not connected to an Attune NxT instrument by maintaining correct instrument settings and channel mapping

Configurations are user-specific – but configurations can be exported and shared .aic files





TIP: the name of the configuration shown is located under the configuration tab

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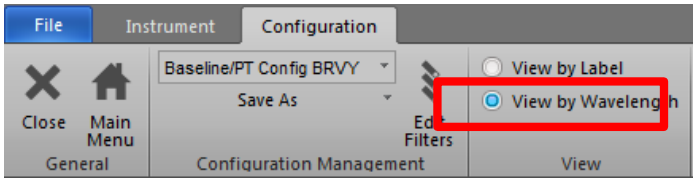
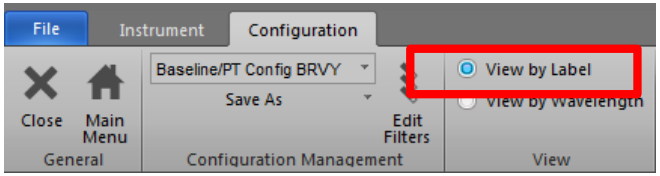
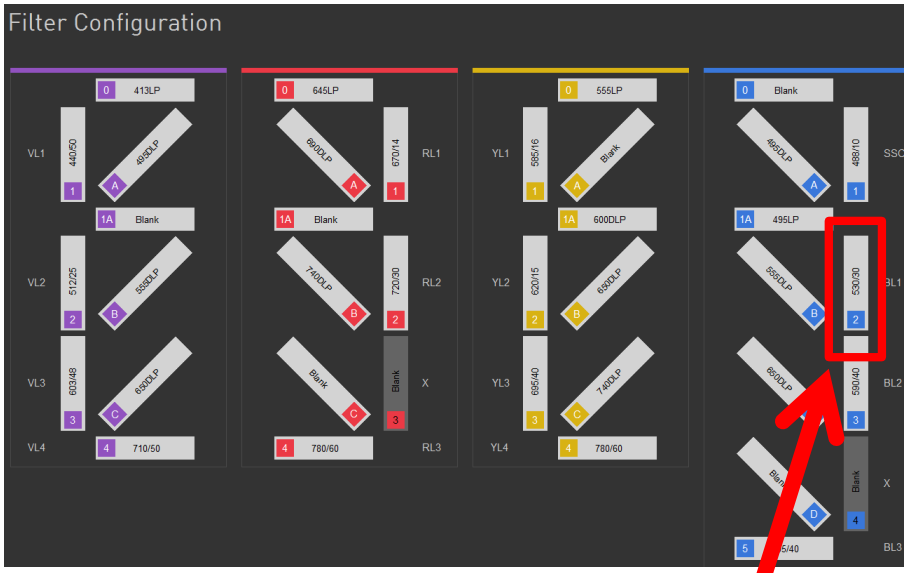
Filter Configuration Manager

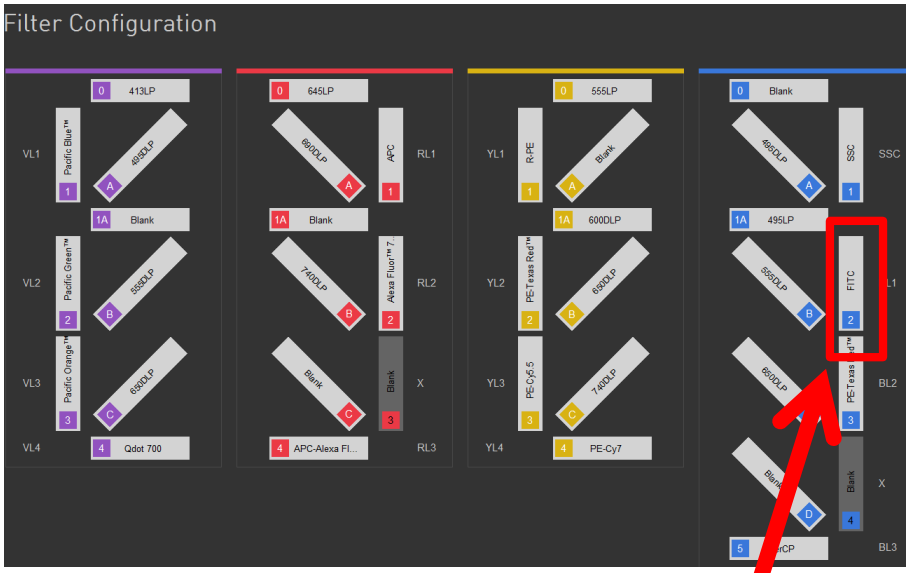
The filter labels shown in the configuration display can be viewed by “Label” or by “Wavelength”







530/30 as defined in current configuration



FITC as defined in current configuration

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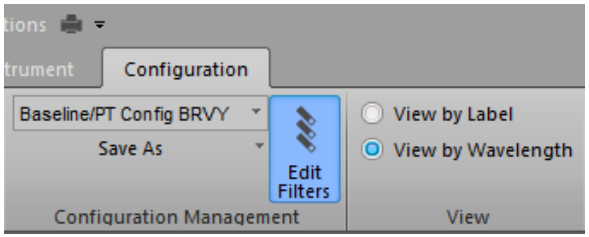
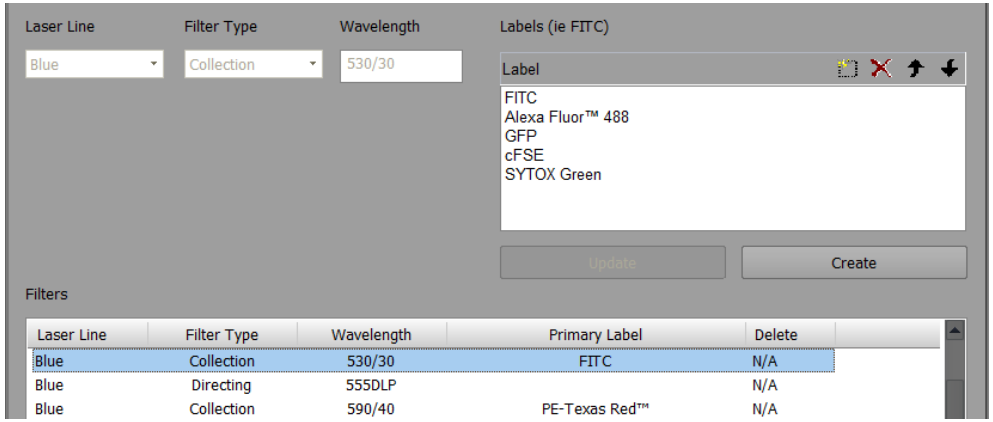
ADD a new filter to the drop down label menu

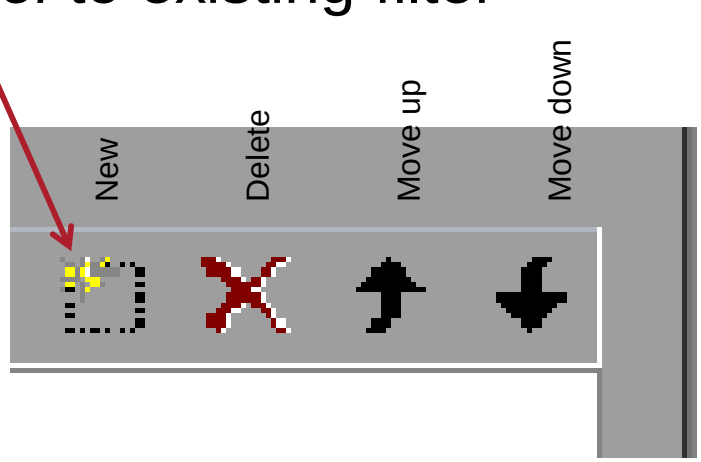
1. Instrument > Configuration > Edit Filters
2. Input information about the new filter:
   - Laser Line
   - Filter Type:

     Collecion: bandpass (BP)
     Directing: Longpass (LP) or Dichroic LP filter
   - Wavelength – specific wavelengths collected or directed by filter



Create new filter or add *New label* to existing filter





Update to save

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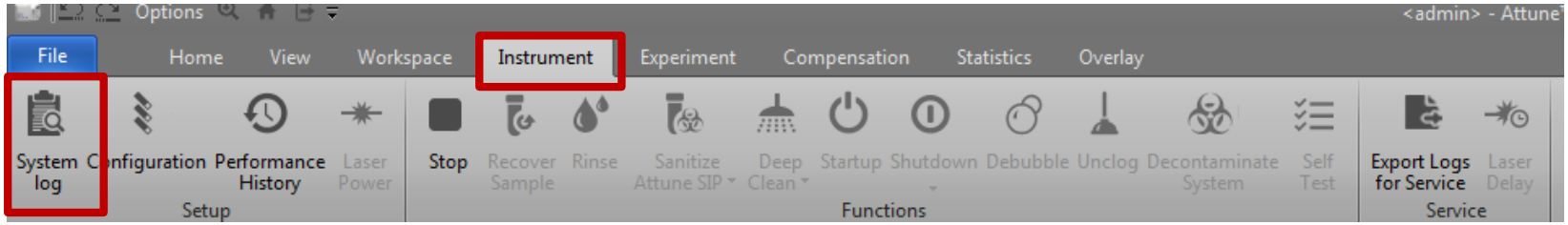
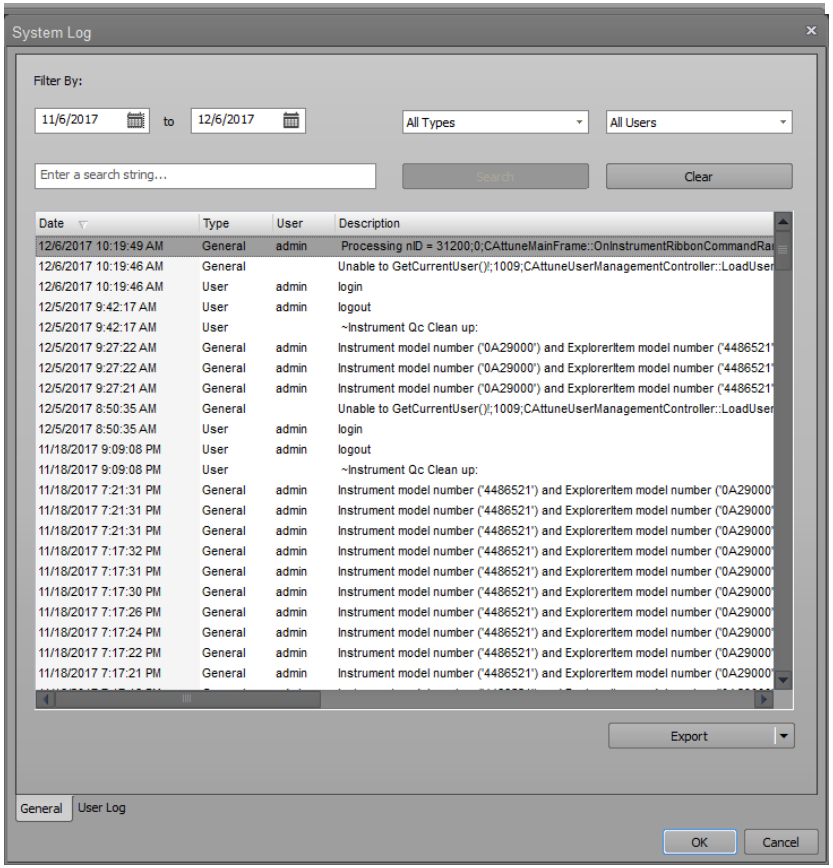
Account and Data Management

Attune NxT v 2.5 SW 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	
Advanced instrument settings	Allows a user to adjust the width threshold, window extensions, and area scaling factor within an experiment		X	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 (Button on instrument ribbon is labeled “Self Test”)		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 Logs / User Logs





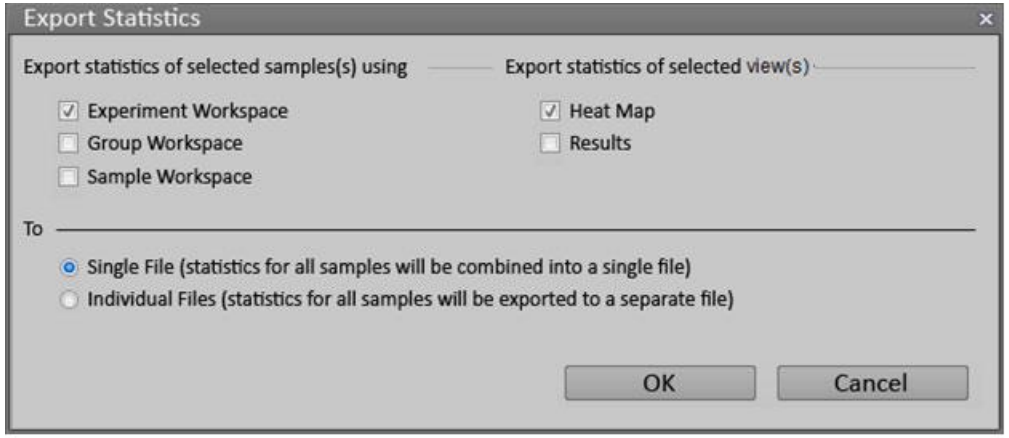
- **General Log**
- Log of system transactions.
- The information displayed for administrator
- Important for troubleshooting/support

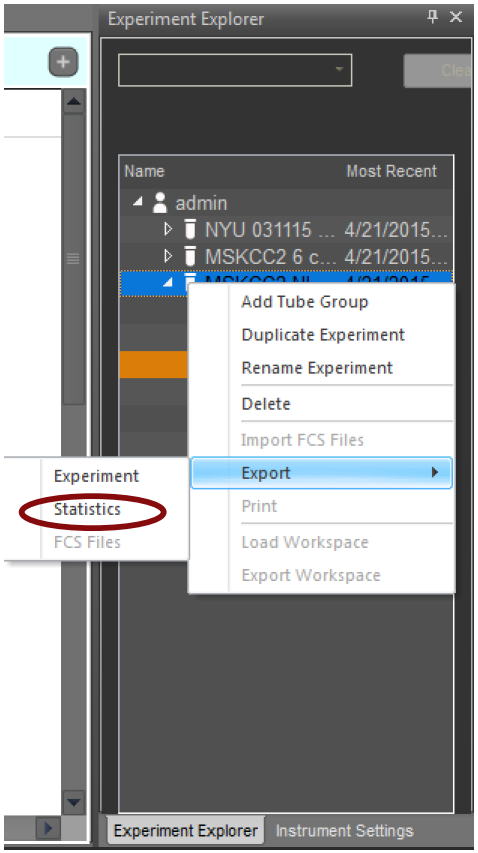
  - **User Log**
  - Filter by:

    Date
    User
    Elapsed time or Sample Count
  - Shared instrument? – helpful for tracking usage

Export Statistics to CSV

- Select the Experiment, Group, or Sample files to export
- Multi-selection enabled with the Ctrl button
- Right-click and select Export
- Select statistics level and views
- Select single or separate files





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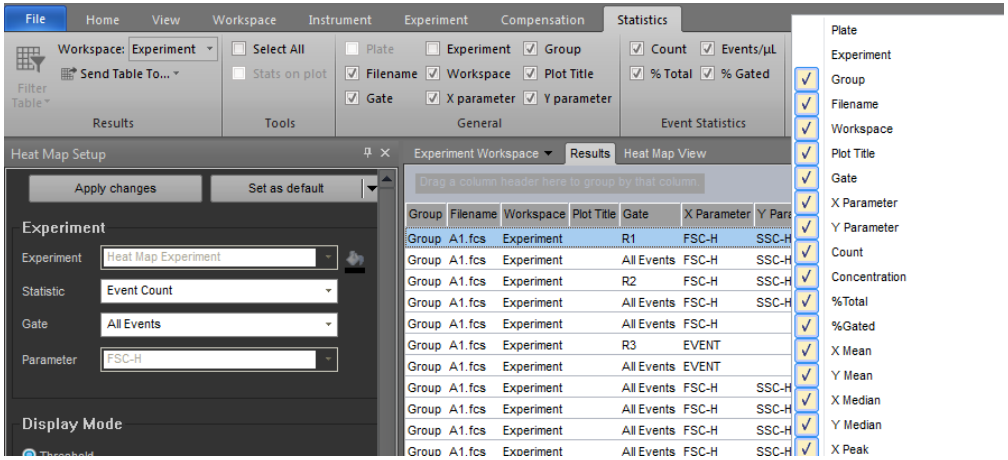
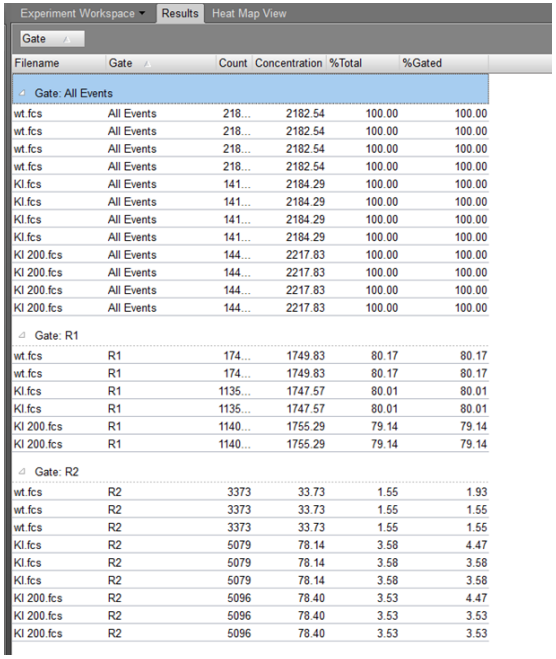
Results table

- View the statistics associated in a tabulated format

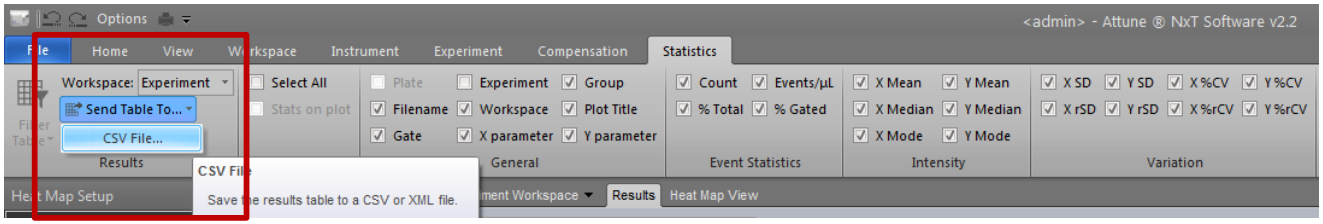
Select statistics



Group results



Export



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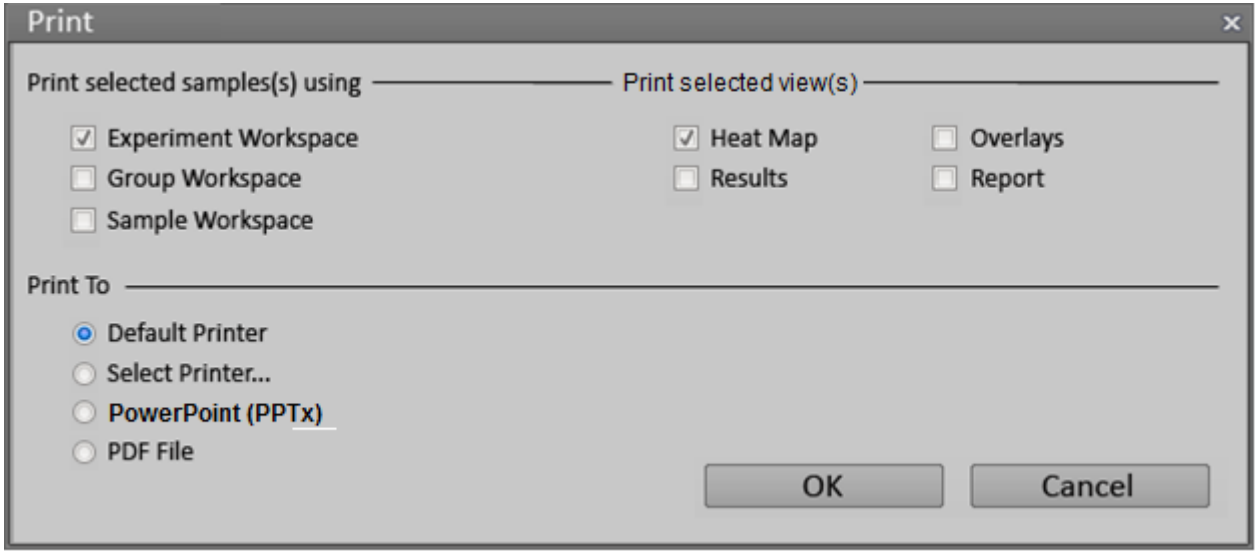
Print Results

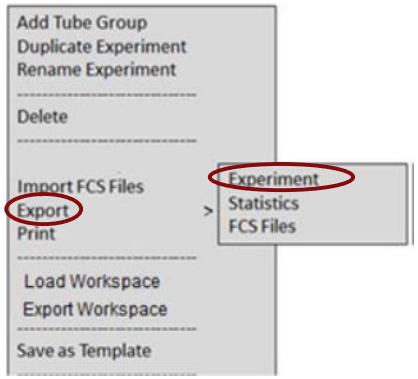
- Select the Experiment, Group, or Sample files
- Multi-selection enabled with the Ctrl or shift button
- Select workspace level and views
- Select print destination

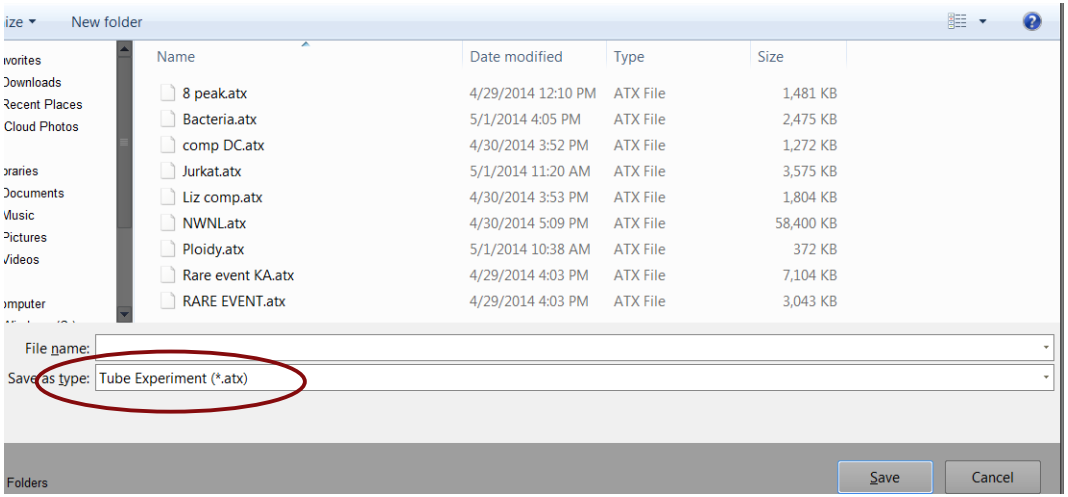


Experiment Export

- Right click on the experiment name, open context menu
- Select *Export Experiment*
- Windows browser opens to last ‘saved’ directory
- Creates files:

  .atx tube experiment
  .apx plate experiment





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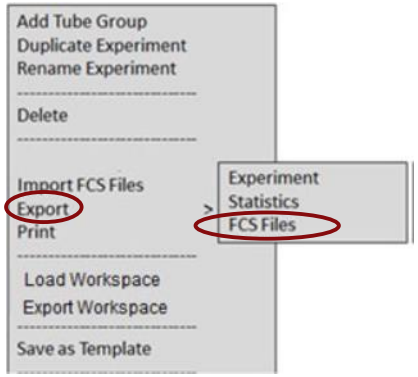
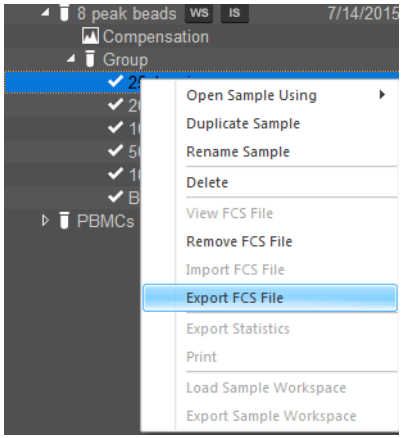
Export/Save of files as FCS 3.0 and FCS 3.1

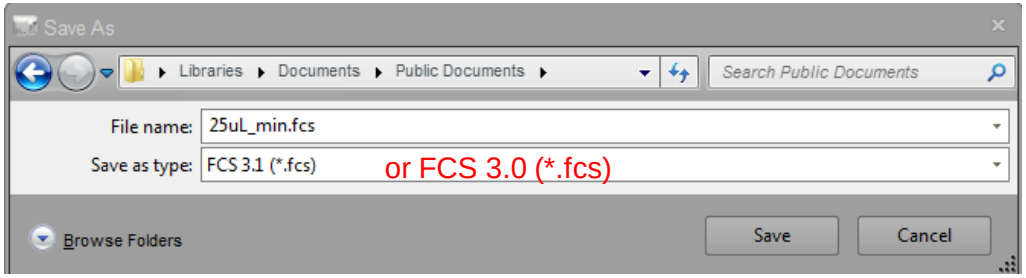
All experiment samples



Selected experiment samples



Right click on the experiment name or selected sample(s) and select “Export FCS file” from the drop down menu

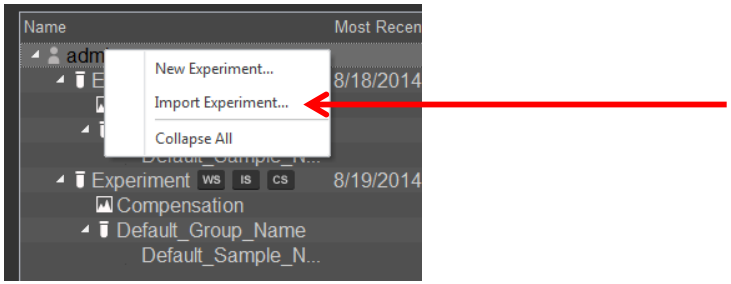


FCS 3.1 files are compatible with: FlowJo V10+, Kaluza, FCS Express V4+, and VenturiOne 3rd

FCS 3.0 files are compatible with: FlowJo V7+, FCS Express V3+, VenturiOne, and Kaluza

Experiment Import

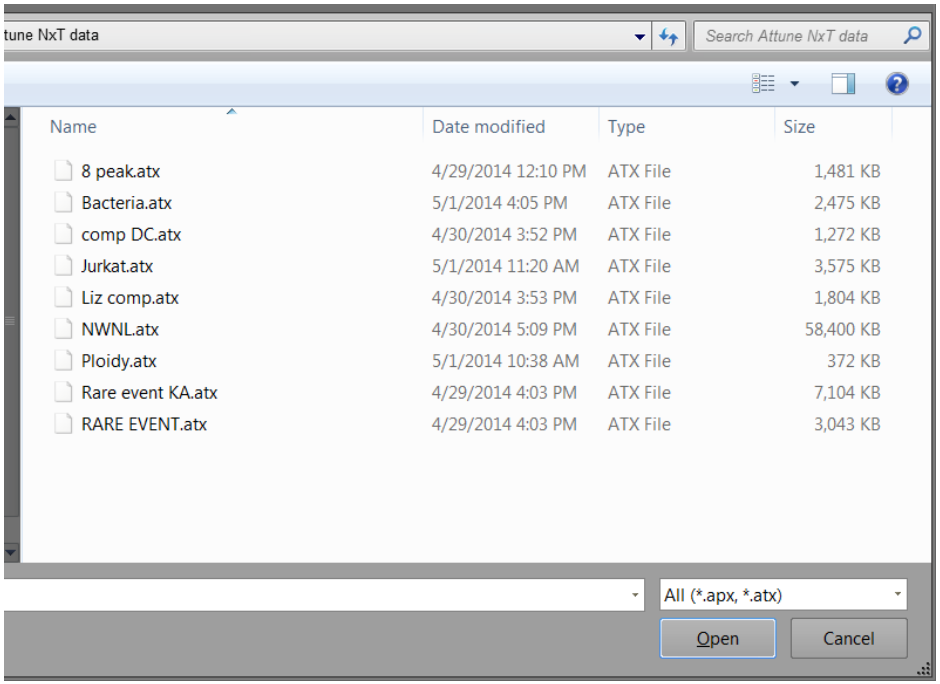
Right click on the *User* name, open context menu select *Import Experiment*



Windows browser opens to last ‘saved’ directory

Select:

.atx	tube experiment
.apx	plate experiment



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File Types and Extensions

Data format: FCS 3.1 (default)
Storage location: the directory where last file was saved

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*
.afs – system log in system local format

* These files can be saved (exported) and used (loaded) in other experiments

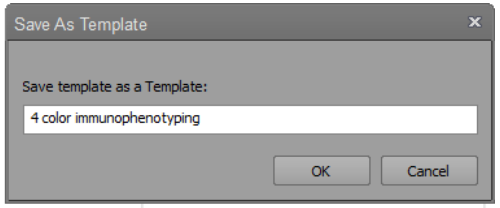
Sample Naming Limitations

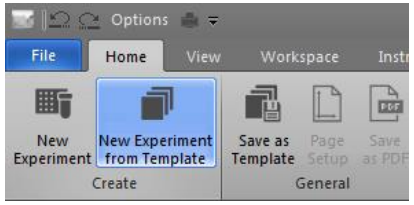
If samples are named using any of the following words, the FCS file will not be recorded:

CON, PRN, AUX, CLOCK$, NUL, COM1, COM2, COM3, COM4, COM5, COM6, COM7, COM8, COM9,LPT1, LPT2, LPT3, LPT4, LPT5, LPT6, LPT7, LPT8, LPT9

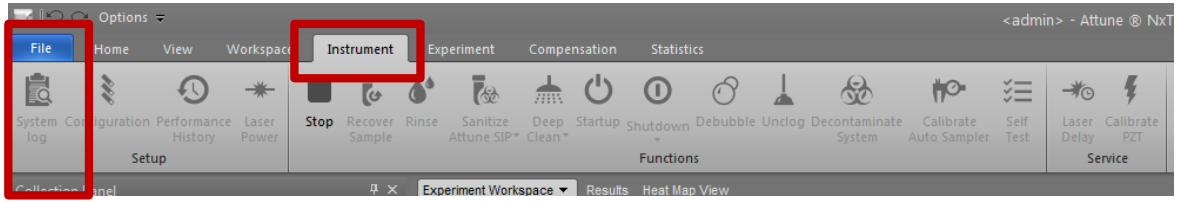
Templates

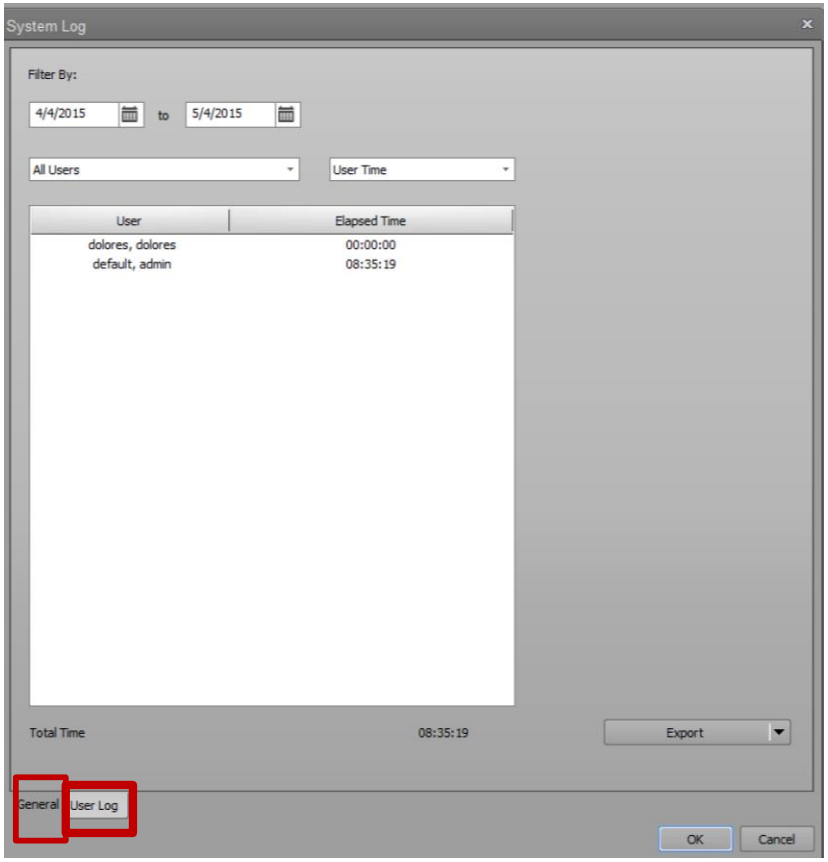
- Experiment Templates are directions for a new experiment that include workspace objects, run protocol, and instrument settings as defined by the user
- Templates can be accessed from a single user profile
- To create a template from a recorded experiment, select “Save as Template” from Home Tab or by right-clicking on user name in Experiment Explorer
- To create a new experiment from template, select the “Templates” button located on the Main Menu desktop or from the Home Tab
- Once a Template is created, it cannot be deleted





System Log





User Log

Filter by:

- Date
- User
- Elapsed time or Sample Count

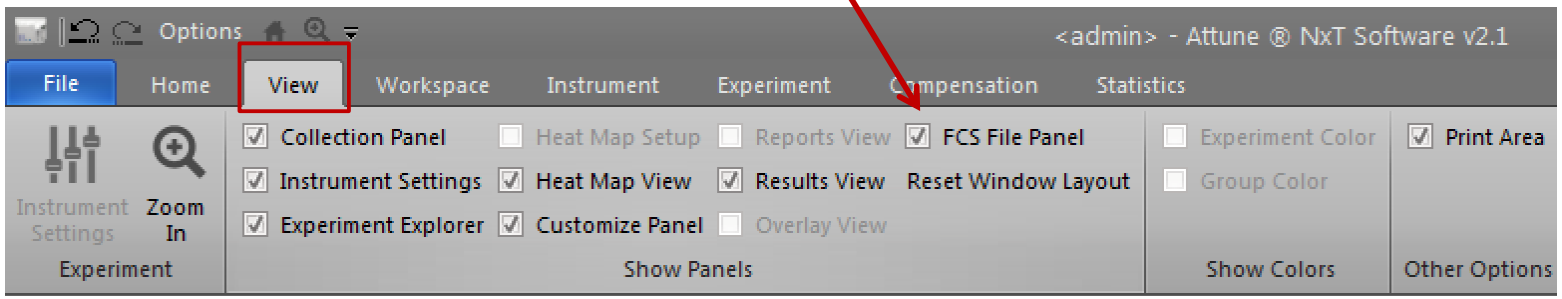
General Log

- Log of system transactions.
- The information displayed for administrator

FCS File Panel

Open the *View* tab

Check the FCS file panel



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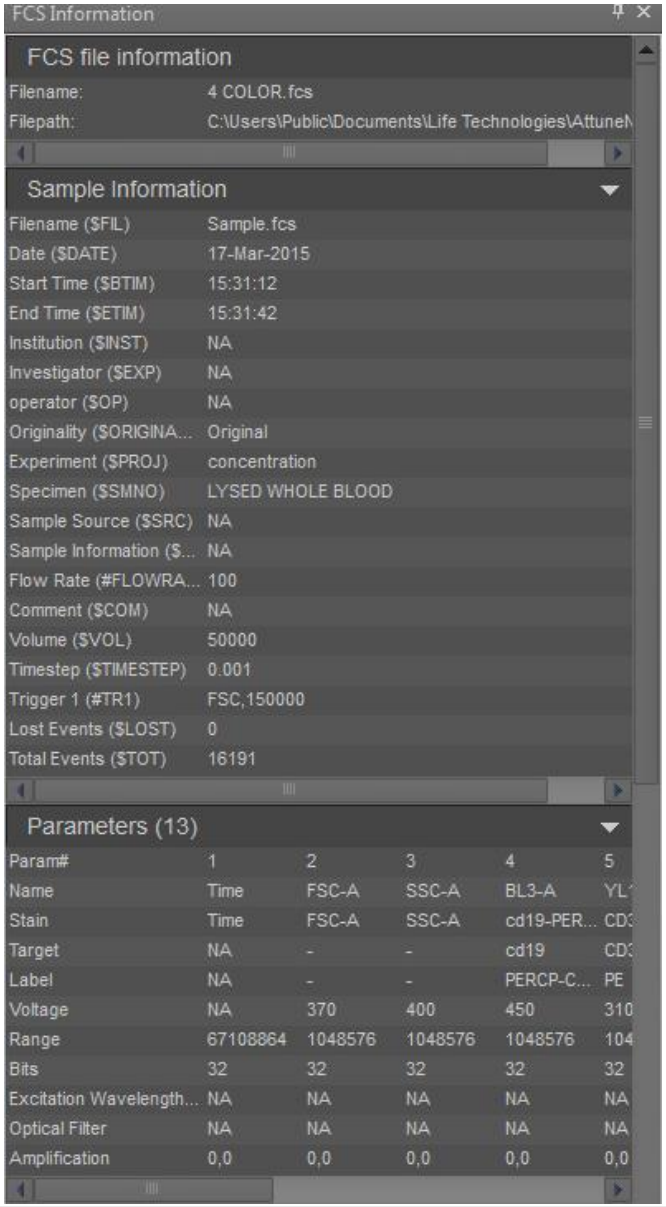
FCS File

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



Legal Statements

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