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Attune™ Acoustic Focusing Cytometer

Training Guide

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About This Guide

Purpose

This training guide contains instructions used during the practical training for Attune™ Acoustic Focusing Cytometer. For further details on the Attune™ Acoustic Focusing Cytometer and Attune™ Cytometric software that are not covered in this training guide, refer to the Attune™ Acoustic Focusing Cytometer user guide.

Safety Conventions and Symbols

Brief information on safety conventions and symbols is described below. For details, refer to the Attune™ Acoustic Focusing Cytometer user guide.

Electrical Symbols

The following table describes the electrical symbols that may be displayed on the instrument.

Symbol	Description
	Indicates the **On** position of the main power switch.
	Indicates the **Off** position of the main power switch.
	Indicates a standby switch by which the instrument is switched on to the **Standby** condition. Hazardous voltage may be present if this switch is on standby.
	Indicates the **On/Off** position of a push-push main power switch.
	Indicates a terminal that may be connected to the signal ground reference of another instrument. This is not a protected ground terminal.
	Indicates a protective grounding terminal that must be connected to earth ground before any other electrical connections are made to the instrument.
	Indicates a terminal that can receive or supply alternating current or voltage.
	Indicates a terminal that can receive or supply alternating or direct current or voltage.

Safety Symbols

The following table describes the safety symbols that may be displayed on the instrument. Each symbol may appear by itself or in combination with text that explains the relevant hazard. These safety symbols may also appear next to DANGERS, WARNINGS, and CAUTIONS that occur in the text of this and other product-support documents.

Symbol	Description
	Indicates that you should consult the manual for further information and to proceed with appropriate caution.
	Indicates the presence of an electrical shock hazard and to proceed with appropriate caution.
	Indicates the presence of a hot surface or other high-temperature hazard and to proceed with appropriate caution.
	Indicates the presence of a laser inside the instrument and to proceed with appropriate caution.
	Indicates the presence of moving parts and to proceed with appropriate caution.
	Indicates the presence of a biological hazard and to proceed with appropriate caution.
	Indicates the presence of an ultraviolet light and to proceed with appropriate caution.

Environmental Symbol

The following environmental symbol applies to this product.

Symbol	Description
	**Do not dispose of this product as unsorted municipal waste.** Follow local municipal waste ordinances for proper disposal provisions to reduce the environmental impact of waste electrical and electronic equipment (WEEE). **European Union customers:** Call your local Applied Biosystems® Customer Service office for equipment pick-up and recycling. See www.appliedbiosystems.com for a list of customer service offices in the European Union.

Safety Labels on Instruments

The following CAUTION, WARNING, and DANGER statements may be displayed on the instrument in combination with the safety symbols described in the preceding section.

Hazard Symbol	English	Français
	**CAUTION!** Hazardous chemicals. Read the Safety Data Sheets (SDSs) before handling.	**ATTENTION!** Produits chimiques dangereux. Lire les fiches techniques de sûreté de matériels avant toute manipulation de produits.
	CAUTION! Hazardous waste. Refer to SDS(s) and local regulations for handling and disposal.	**ATTENTION!** Déchets dangereux. Lire les fiches techniques de sûreté de matériels et la régulation locale associées à la manipulation et l'élimination des déchets.
	**DANGER!** High voltage.	**DANGER!** Haute tension.
	WARNING! To reduce the chance of electrical shock, do not remove covers that require tool access. No user-serviceable parts are inside. Refer servicing to Applied Biosystems qualified service personnel.	**AVERTISSEMENT!** Pour éviter les risques d'électrocution, ne pas retirer les capots dont l'ouverture nécessite l'utilisation d'outils. L'instrument ne contient aucune pièce réparable par l'utilisateur. Toute intervention doit être effectuée par le personnel de service qualifié venant de chez Applied Biosystems.
	**DANGER!** Class 3B visible and/or invisible laser radiation present when open. Avoid exposure to beam.	**DANGER!** Rayonnement visible ou invisible d'un faisceau laser de Classe 3Ben cas d'ouverture. Evitez toute exposition au faisceau.
	**CAUTION!** Moving parts. Crush/pinch hazard.	**ATTENTION!** Pièces en mouvement, risque de pincement et/ou d'écrasement.

Safety Alert Word Definitions

The following safety alert words appear in the user documentation where you need to be aware of relevant hazards. Each alert word implies a particular level of observation or action as defined below:

CAUTION! – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING! – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER! – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

IMPORTANT! Provides that is necessary for proper instrument operation, accurate installation, or safe use of a chemical.

Except for IMPORTANT, each safety alert word in the user documentation appears with an open triangle figure that contains a hazard symbol. *These hazard symbols are identical to the hazard icons that are affixed to the instrument* (see **Safety Symbols** above).

Examples of Alert Words

The following examples show the use of safety alert words:

	IMPORTANT! Wear powder-free gloves when you handle the halogen lamp.
	**CAUTION!** The lamp is extremely hot. Do not touch the lamp until it has cooled to room temperature.
	**WARNING! CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
	**DANGER! ELECTRICAL HAZARD.** Failure to ground the instrument properly can lead to an electrical shock. Ground the instrument according to the provided instructions.

General Instrument Safety

 **WARNING! PHYSICAL INJURY HAZARD.** Use this product only as specified in this document. Using this instrument in a manner not specified may result in personal injury or damage to the instrument.

Moving and Lifting the Instrument

 **CAUTION! PHYSICAL INJURY HAZARD.** The instrument is to be moved and positioned only by the personnel or vendor specified in the applicable site preparation guide. If you decide to lift or move the instrument after it has been installed, do not attempt to lift or move the instrument without the assistance of others, the use of appropriate moving equipment, and proper lifting techniques. Improper lifting can cause painful and permanent back injury. Depending on the weight, moving or lifting an instrument may require two or more persons.

Moving and Lifting Stand-alone Computers and Monitors

 **WARNING!** Do not attempt to lift or move the computer or the monitor without the assistance of others. Depending on the weight of the computer and/or the monitor, moving them may require two or more people.

Things to consider before lifting the computer and/or the monitor:

- Make sure that you have a secure, comfortable grip on the computer or the monitor when lifting.
- Make sure that the path from where the object is to where it is being moved is clear of obstructions.
- Do not lift an object and twist your torso at the same time.
- Keep your spine in a good neutral position while lifting with your legs.

- Participants should coordinate lift and move intentions with each other before lifting and carrying.
- Instead of lifting the object from the packing box, carefully tilt the box on its side and hold it stationary while someone slides the contents out of the box

Operating the Instrument

Ensure that anyone who operates the instrument has:

- Received instructions in both general safety practices for laboratories and specific safety practices for the instrument.
- Read and understood all applicable Safety Data Sheets (SDSs).

Cleaning or Decontaminating the Instrument

 **CAUTION!** Using cleaning or decontaminations method other than those recommended by the manufacturer may compromise the safety or quality of the instrument.

Biological Hazard Safety

 **WARNING! BIOHAZARD.** Biological samples such as tissues, body fluids, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective eyewear, clothing, and gloves. Read and follow the guidelines in these publications:

In the U.S:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **www.cdc.gov/OD/ohs/biosfty/bmbl4/bmbl4toc.htm)**
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **www.access.gpo.gov/nara/cfr/waisidx_01/29cfr1910a_01.html)**
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.
- Additional information about biohazard guidelines is available at **http://www.cdc.gov**

In the EU:

- Check your local guidelines and legislation on biohazard and biosafety precaution, and the best practices published in the World Health Organization (WHO) Laboratory Biosafety Manual, third edition **http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/**

Technical Support

For Trouble shooting Guide, refer to the Attune™ Acoustic Focusing Cytometer user guide supplied with the instrument.

For technical support information for all locations, visit www.appliedbiosystems.com/support

Please have the following information available when calling:

Product name: Attune™ Acoustic Focusing Cytometer

Serial number: Located near the back of the instrument. The serial number has a format of letters and numbers.

Error messages:

Chapter 1: Overview of Flow Cytometry

This chapter describes:

- Basic concepts of acoustic cytometry
- Differences between traditional and acoustic focusing cytometry
- Definition of Common Flow Cytometry Terms
- Transit Time
- Exercise 1

Cytometry is the measurement of physical or chemical characteristics of cells or particles. Flow cytometry and cell sorting are important tools to help address many complex biological questions. Flow cytometry measurements are made as cells or particles in suspension pass individually through a flow cytometer instrument. These experiments, typically performed on single cell suspensions, provide discrete measurements from each cell in the sample and provide a distribution of the measured characteristics in the sample. In generic terms, flow cytometry measures cells/particles flowing in a stream. Flow cytometry assays allow researcher's to study many different applications including immunophenotyping of cell populations, functional expression of intracellular proteins, evaluation of apoptosis, cell proliferation, and cell viability.

Traditional flow cytometers are made up of three subsystems:

- Fluidics system which transports the sample to the flow cell and eventually discards as waste
- Optics system that includes lasers, mirrors and lens, and photomultiplier tubes to detect the emitted fluorescent light and scattered light
- Electronics system that converts the data into digital signals that can be visualized in a software program for data acquisition and analysis

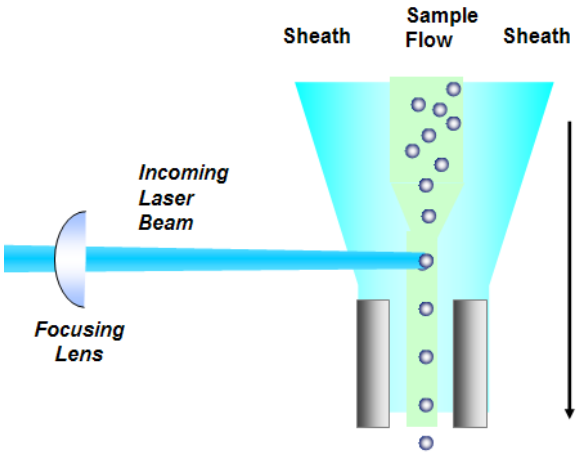
Traditional flow cytometers use a stream-within-a stream method to focus and transport cells throughout the fluidics system, and delivers them for analysis. This process is called hydrodynamic focusing.

Hydrodynamic Focusing

Hydrodynamic focusing for conventional flow cytometry uses relatively high volumetric sheath flow that confines injected sample fluid to a small “core”, which is separate but coaxial within the sheath fluid. The flow of sheath accelerates the particles and restricts them to the center of the sample core. This is the standard method in flow cytometry for positioning particles within the optical interrogation region defined by a tightly focused laser beam.

The technology works best with more concentrated samples as the sheath fluid flow dilutes the sample and the sample is entrained into the flowing sheath fluid. Additionally, as the sample is entrained into the sheath, fluid shear forces are created which can disrupt fragile cells (i.e., apoptotic cells).

As the sample is injected, the sample is caught up in the sheath flow and is accelerated forward (see figure on next page). Because of the high velocity of the sheath flow through a narrow area, mixing of the sample and the sheath is minimal, allowing the sample to remain in its own individual stream. This individual stream is a small percentage of the sheath flow, therefore increasing the rate that the sample is injected has little effect on the speed of the particles in the sample as they pass through the laser. However, increasing the sample injection rate increases the percent fraction of sample in the total fluid flowing, leading to widening of the sample core stream, which can affect the precision of the measurements made by the instrument.

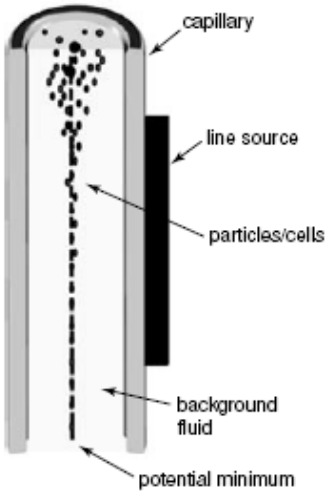


Acoustic Focusing

Acoustic focusing exploits physical differences between cells or particles relative to the background carrier medium to position the particles or cells into a single, focused line along the central axis of a flow channel. The cells or particles move into energy well in the center of the fluid flow. This energy well is created by the acoustic radiation pressure from an ultrasonic piezoelectric transducer.

The ability to focus cells into a tight line without sheath fluid allows many possibilities outside the scope of conventional flow cytometry. Dilute samples can be processed quickly and flow can be controlled in unique ways. Slowing the sample flow through the analysis chamber can be accomplished while maintaining tight focus of the cells. This in turn allows the use of longer laser transit times, enabling higher sensitivity and precision. The acoustically driven capillary is a focusing device that enables the application of acoustic radiation pressure to flow cytometry.

The focusing device is an acoustic resonant device that focuses cells or particles into a single tight line using a capillary coupled to a single piezoelectric transducer. The figure below schematically shows a line-driven capillary depicting tight single line focusing of particles in a flowing liquid using acoustic radiation pressure.



Acoustic focusing cytometry is a powerful technique for the simultaneous measurement and analysis of multiple parameters of individual cells within heterogeneous populations. Acoustic focusing cytometry is useful in applications ranging from immunophenotyping, to ploidy analysis, to cell counting, and GFP expression analysis. The acoustic focusing cytometer performs the analysis by passing thousands of cells per second through a laser beam and capturing the light that emerges from each cell as it passes through. The data gathered is analyzed statistically by software to report cellular characteristics such as size, complexity, phenotype, and health.

Flow Rate

In a conventional cytometer, the diameter of the sample core is varied by the pressure difference between the sample stream and the sheath fluid stream—increasing the sample flow rate enlarges the core diameter, which allows quicker data acquisition but lower resolution.

In contrast, the alignment of cells in the Attune™ Acoustic Focusing Cytometer is independent of the total fluid flow through the instrument. Therefore, large changes in the amount of sample injected or the total fluid flow may alter the diameter of the sample core but not affect the resolution due to the constant focus of the cells and their ability to remain in the 'sweet spot' of the aligned laser. This feature of the instrument allows analysis of dilute samples at a high rate without the loss of resolution. The Attune™ Acoustic Focusing Cytometer has a maximum sample injection rate of 1,000 µL/minute, but is recommended to run from 25 µL/min to 500 µL/min.

If high sensitivity and resolution/precision are more important than throughput, the flow rate can be decreased drastically to maximize the collection of photons, which is dependent upon the laser excitation intensity and cell transit time.

Sample Concentration

All cytometers, including the Attune™ Acoustic Focusing Cytometer, are governed by Poisson statistics, which predict the probability of a given number of cells or particles being intercepted by the interrogating laser beam. While increasing the sample concentration results in a higher sample throughput, it also increases the probability of a coincident event, defined as more than one cell being present in the interrogating laser beam.

The Attune™ Acoustic Focusing Cytometer can maintain its maximum particle analysis rate over a large range of initial sample concentrations without the need to concentrate using centrifugation or filtration. The ability to analyze dilute samples has the added benefits of reduced background fluorescence from free fluorophores in the sample and capability to analyze very small initial samples sizes or to dilute very small samples to larger volumes for more efficient sample use.

Effects of Acoustic Focusing on Cell Viability

Acoustic focusing differs fundamentally from ultrasonic lysis of cells and is generally gentler on cells than the forces occurring in hydrodynamic focusing.

Ultrasonic lysis of cells relies on cavitation and shear forces produced at sub-megahertz frequencies where tiny gas bubbles form and collapse with immense local shear and heating in the solution containing the sample. In contrast, the acoustic focusing capillary of the Attune™ Acoustic Focusing Cytometer operates at a frequency well above 1 MHz, where the possibility of cavitation is greatly reduced. Further, acoustic cytometry is performed with relatively low energy levels at very high sample flow rates and the design of the acoustically driven capillary spreads this energy over the entire length of the capillary. The net effect is that cells experience low shear forces and do not appear to be harmed by the ultrasonic forces.

Advantages of Acoustic Focusing

- **High sensitivity**
  One of the unique capabilities of acoustic cytometry is the ability to select the sample delivery rate. Slowing cell/particle transit times (approximately 20–100 times slower than conventional systems) provides the opportunity to increase the time that a cell is within the laser beam (*transit time*). This maximizes photon collection leading to higher sensitivity optical measurements and photonic events measurements that require longer interrogation times within the laser beam, thus improving sensitivity and producing brighter signals from dim fluorochromes.

- **Dilute sample analysis**
  Dilute samples can be easily analyzed at a high rate using acoustic focusing cytometry. The particle event rate in acoustic or conventional flow cytometry is a function of sample concentration and sample flow rate. Unlike conventional flow cytometry, precise analysis is maintained in acoustic cytometry over a broad range of sample rates by controlling the amount of time the cells spend in the lasers' path allowing you to use a slower flow rate to collect more photons when analyzing dim signals.

  For conventional flow cytometry, increasing the sample flow rate broadens the sample core and results in less precise analysis as a broader sample core allows cells to travel in different streamlines at different velocities where they are illuminated by different parts of the focused laser beam.

  Acoustic cytometry can also be used to maintain maximum particle analysis rate in a wide variety of sample concentrations by diluting the samples and is perfect for analyzing very small sample sizes. Additionally, cells that tend to aggregate can be analyzed as more dilute samples, thus minimizing cellular aggregation.

- **High throughput**

  Acoustic focusing cytometers can tightly focus lymphocytes and larger cells at volumetric flow rates. The high volumetric sample rate enables them to maintain high throughput for even dilute samples without the loss of signal resolution.

- **Improved resolution**

  Acoustically focused sample streams can be slowed without degrading alignment of the particle stream within the flow chamber. The increased residence time within the acoustic field produces a stream of particles whose focus is actually improved.

Attune™ Acoustic Focusing Cytometer

The Attune™ Acoustic Focusing Cytometer is an acoustic cytometer that uses acoustic radiation pressure to confine the cells or particles in a sample solution into a single, focused line along the central axis of the flow channel. The focusing is independent of the sample fluid flow and does not use high fluid velocities or high volumetric sheath fluid, which can damage cells.

The acoustically driven capillary is an acoustic resonant device that focuses the cells or particles into a single tight line using a capillary coupled to a single piezoelectric transducer. The acoustic contrast of

a particle or cell is determined by the density and compressibility differences between the particle/cell and its background medium. The relative magnitudes and signs of the differences determine the size and direction of the acoustic radiation pressure force that is applied to the particles, which allows the particles traveling through the capillary to experience a time averaged force that aligns them along the central line of the capillary independent of fluid flow.

Like a conventional flow cytometer, the Attune™ Acoustic Focusing Cytometer includes three main systems: optics, fluidics, and electronics.

The optical system includes: an optical cell that is integrated with a laser, which acts as the light source for scatter and fluorescence; the detectors, which receive the light to illuminate the particles in the focused stream; and optical filters to direct the resulting light signals to the appropriate detectors.

The fluidics system uses a syringe pump that pushes or pulls the sample through the system. Fluid flow can be controlled at any desired rate using appropriate pumps.

The electronics system converts the detected light signals into electronic signals that can be processed by the computer system, which converts the signals from the detectors into digital data to perform the necessary analyses.

An important difference between the acoustically focused cytometer and a conventional hydrodynamically sheath–focused cytometer is that particles or cells in the acoustic capillary are focused toward a tight central line along the axis of the capillary whether fluid is flowing or not.

Definition of Common Flow Cytometry Terms

Since the Attune™ Acoustic Focusing Cytometer has similar properties to a conventional flow cytometry, some common flow cytometry terms are explained and defined below.

Fluorescence

Fluorescence is the emission of light that occurs when an emitting particle such as a fluorophore-labeled antibody absorbs light from another source such as the intercepting laser beam. When the particle absorbs the intercepting light, it is elevated to an excited electronic state, and as it returns to its ground state, the absorbed energy is radiated where most of it is emitted as light. The emitted light is always a longer wavelength (i.e., less energetic) than the absorbed light. The difference between the excitation and emission wavelengths is known as the Stokes shift.

Flow cytometry uses fluorescence detectors to identify different aspects of cells including functional assays and subset identification. One of the most common ways to study cellular characteristics using flow cytometry involves the use of fluorescent molecules such as fluorophore-labeled antibodies. In these experiments, the labeled antibody is added to the cell sample. The antibody then binds to a specific molecule on the cell surface or inside the cell. When the laser light of the right wavelength strikes the fluorophore, a fluorescent signal is emitted and detected by the flow cytometer.

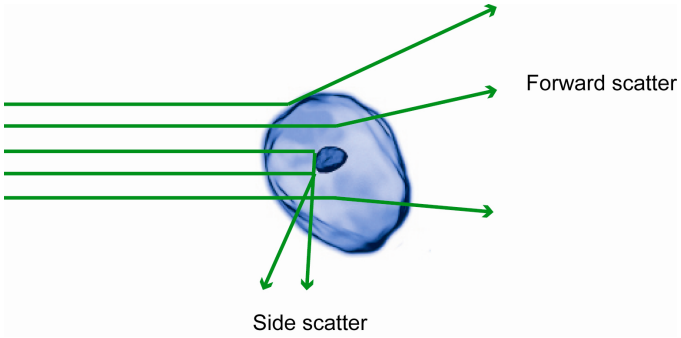
Fluorescence data is collected in generally the same way as forward and side scatter data. In a population of labeled cells, some will be brighter than others. As each cell crosses the path of the laser, a fluorescence signal is generated. The fluorescent light is then directed to the appropriate detector where it is translated into a voltage pulse proportional to the amount of fluorescence emitted. All of the voltage pulses are recorded and can be presented graphically. Multiple colors can be used on a flow cytometer and the number of colors that can be detected depends upon the number

of detectors available in the instrument. The different colors are collected using select optical filters that direct the light to the right detector and capture the peak fluorescent signals.

Light Scatter

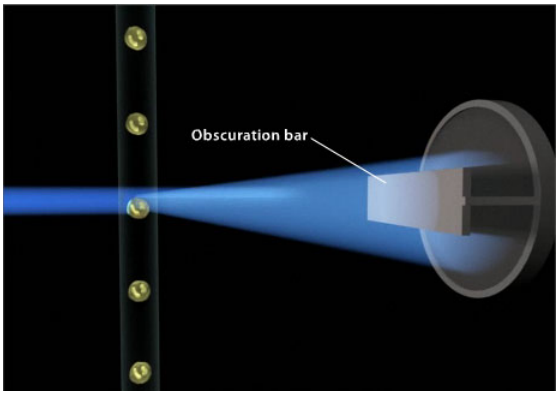
When a cell or particle passes through a focused laser beam, it refracts of scatters light in all directions.

- *Forward scatter*, or low-angle light scatter, is the light that is scattered in the forward direction as laser light strikes the cell. The magnitude of forward scatter is roughly proportional to the size of the cell or particle, and this data can be used to quantify that parameter.
- *Side scatter* is defined as the light that is scattered at larger angles. Side scatter is indicative of the granularity and structural complexity inside the cell or particle.



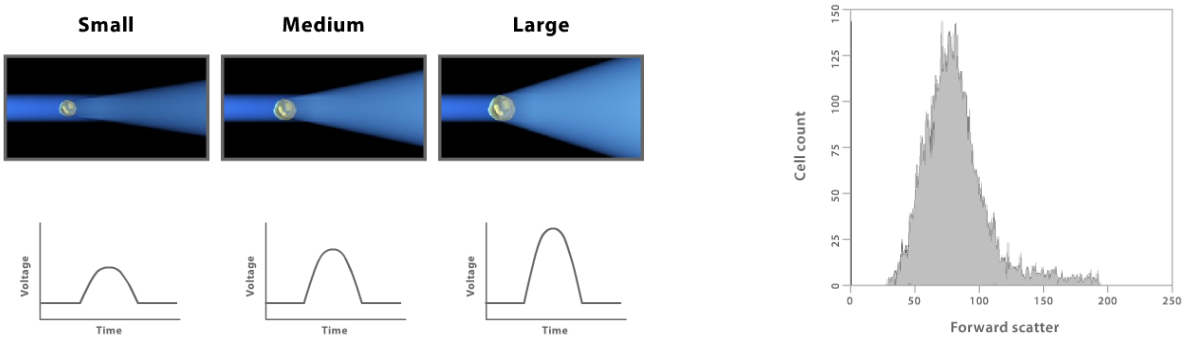
Measuring Light Scatter and Plotting Data

Forward scatter light is quantified by a detector that converts intensity into voltage. In most cytometers, a blocking bar (called an obscuration bar) is placed in front of the forward scatter detector. The obscuration bar prevents any of the intense laser light from reaching the detector. As a cell crosses the laser, light is scattered around the obscuration bar and is collected by the detector.

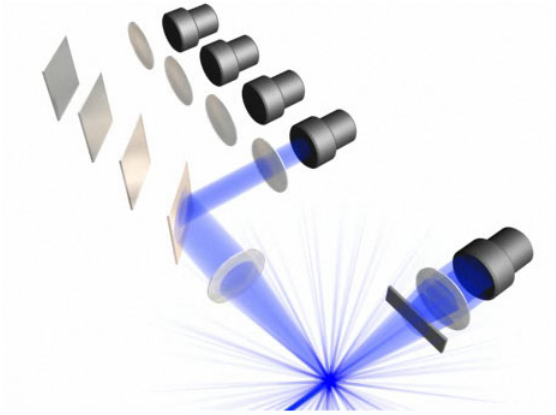


FSC (Forward Scatter) Histogram

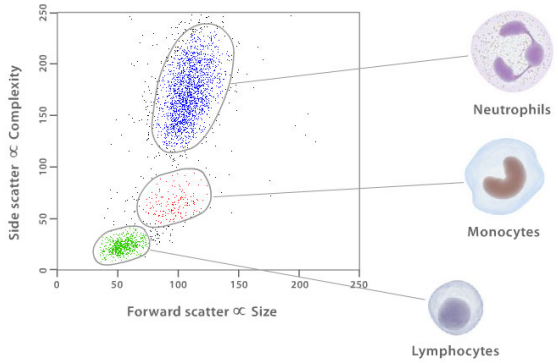
The forward scattered light received by the detector is translated into a voltage pulse. Because small cells produce a small amount of forward scatter and large cells produce a large amount of forward scatter, the magnitude of the voltage pulse recorded for each cell is proportional to the cell size. If we plot a histogram of these data as shown in the figure below, smaller cells appear towards the left and larger cells appear towards the right. A histogram of forward-scatter data is a graphical representation of the size distribution within the population, but such a graph only presents one-dimensional data.



Side-scattered (SSC) light is focused through a lens system and is collected by a separate detector, usually located at 90 degrees from the laser's path. The signals collected by the side-scatter detector can be plotted on one-dimensional histograms similar to forward scatter.



Flow cytometry allows you to visualize objects on two-dimensional dot plots since one-dimensional plots do not always indicate the complexity of samples. By utilizing a dot plot of FSC and SSC, we are able to identify different populations of cells as shown below.



Example: When examining a typical peripheral blood sample, different populations can be identified and gated. The populations that emerge include: lymphocytes which are small cells possessing low internal complexity; monocytes which are medium-sized cells with slightly more internal complexity; and neutrophils and other granulocytes which are large cells with a lot of internal complexity. This multiparametric analysis demonstrates the real power of flow cytometry.

Transit Modes and Times

Hydrodynamic focusing instruments achieve tight focus by adjusting the volumetric flow rate of sheath 100 to 1,000 times greater than the sample solution. Since acoustic radiation is the driving force in an acoustic focusing cytometer to align cells independent of the sheath flow rates, the volumetric input rate of sheath to sample volume can be significantly reduced. This helps alter the interrogation time, which is the amount of time spent by each cell or particle in the path of the laser. Increasing the interrogation time, by reducing the speed of the cells or particles as they travel through flow cell, increases number of photons emitted by the particle while it travels through the path of laser yielding greater sensitivity.

The Attune™ Acoustic Focusing Cytometer allows data acquisition in **Standard** and **High Sensitivity** modes. For each of these two modes, the instrument uses the syringe pump system to control the total flow rate. The total flow volume through the sample chamber of the Attune ™ Cytometer is comprised of two components; part sample and part acoustic focusing solution, which is added to the flow stream prior to entering the sampling cuvette. The two streams remain separated using laminar flow.

The two transit modes are explained below:

Standard Transit Mode

The Standard transit mode is used for most of assays where antigens are abundantly present and/or fluorescent markers used are bright. This is true of most immunophenotyping and cell cycle assays. Standard transit mode transfers 2,400 µL of total volume through the flow cells per minute; particles travel at 2 m/s velocity during this transit mode. The sample input rate in this transit mode can be further adjusted depending on the concentration of the sample and desired data acquisition rate. The sample input rate ranges from 25-1,000 µL per minute and the selectable sample rates are listed below:

Pre-set Sample Input Rate (in µL/minute)*	Focusing Fluid Input Rate (in µL/minute)	Focusing Fluid to Sample Ratio
25	2,375	95:1
100	2,300	23:1
200	2,200	11:1
500	1,900	3.8:1
1,000	1,400	1.4:1

*Note that the particle velocity and interrogation time remains constant regardless of the sample input rate since the total volume (sample and the focusing fluid) is constant at this transit mode.

High Sensitivity Transit Mode

High sensitivity transit mode is ideal for assays where the antigens are not as abundantly expressed and/or the fluorescent makers used are not bright (dim). High sensitivity in this transit mode is achieved by slowing the particle stream down to 0.5 m/s velocity resulting in a greater interrogation time which produces more photons resulting in more robust data. The total volume flowing through flow cell at this transit rate is 600 µL per minute. There are only two selectable sample input rates at this transit mode as listed below:

Pre-set Sample Input Rate (in µL/minute)	Focusing Fluid Input Rate (in µL/minute)	Focusing Fluid to Sample Ratio
25	575	23:1
100	500	5:1

Transit Time Experiment

This section demonstrates a transit time practice experiment using fluorescent Rainbow beads.

Materials Needed

- Spherotech 8 peak Rainbow beads (prepare bead sample by adding 30 µL of Rainbow beads to a 12 × 75 mm tube containing 2 mL PBS)

Experiment

1. Launch the Attune™ software by double-clicking on the shortcut from the desktop.
2. Logon using the admin password and click **Log in**.
3. Go to functions >Start Up to perform **Start up** function of instrument, if not already started.
4. Verify that the Performance Test is complete and it passed the criteria.
5. Open Experiment:
    a. Navigate to the training folder on the experiment explorer.
    b. Right-click on the experiment folder and open the Transit Time Experiment.
    c. Duplicate the experiment.
    d. Rename the experiment by right-clicking on the experiment.
    e. Select **Properties.**
    f. Change experiment name to Sensitivity MMDDYY.
    g. Go to the first sample and double-click to activate the Workspace.
    h. Rename the first sample by right-clicking on sample >Properties >Name standard sensitivity 500 µL per minute.
6. Install bead sample on the tube loader and push up the tube lifter.
7. Press **Run** when bead events appear set at a gate around the main bead population and adjust the PMT voltages such that the lowest peak appears around 10^3 (ensure the axes are on manual scale).

8. Press **Record** after all settings are complete.
9. After completion, unload the tube by pushing the tube lifter down. The **Rinse** function automatically begins.
10. Add additional sample on experiment by right-clicking the sample just collected and select duplicate sample.
11. Rename the sample high sensitivity mode and set speed to 25 µL/min.
12. Double click on the sample to activate and press **Run**.
13. After the events begin and all peaks are visible press **Record**.
14. After completion, perform the rinse function twice.

Exercise 1

1. Define Transit Time:

_____

_____

_____

_____

2. Hydrodynamic focusing requires a _____ to focus the cell sample, where as acoustic focusing is dependent on _____ to focus the cells.

3. Match the following

_____ Interrogation Point	**A.** Detects granularity and structural complexity inside the cell
_____ Forward Scatter	**B.** Prevents any of the intense laser light from reaching the detector
_____ Optical Filter	**C.** Spot where the laser and the sample intersect and the optics collect the resulting scatter and fluorescence
_____ Side Scatter	**D.** Amount of light that is scattered in the forward direction as laser light strikes the cell indicates cell size
_____ Obscuration Bar	**E**. Helps detect laser light to the appropriate fluorescence detector

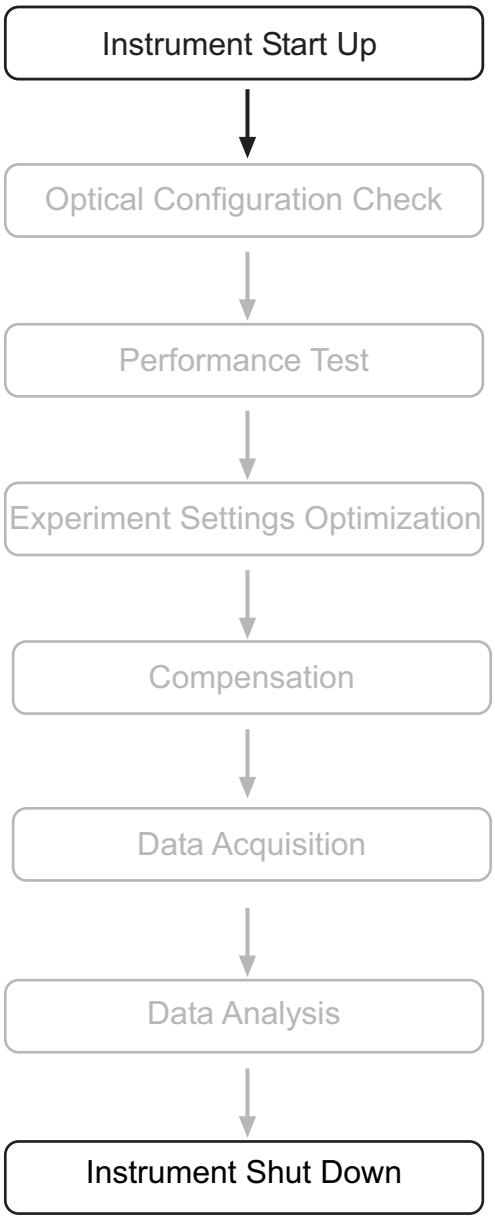
4. _____ transit time should be used to get the most sensitive measurements.

Chapter 2: Fluidics Overview

This chapter covers:

- Fluidics system of the instrument
- Solutions required to operate the Attune™ Acoustic Focusing Cytometer
- Different modes of operation
- Basic instrument start up and shut down procedures
- Maintenance procedures
- Exercise 2 and 3

Workflow


Instrument Start Up
Optical Configuration Check
Performance Test
Experiment Settings Optimization
Compensation
Data Acquisition
Data Analysis
Instrument Shut Down

Fluidics System

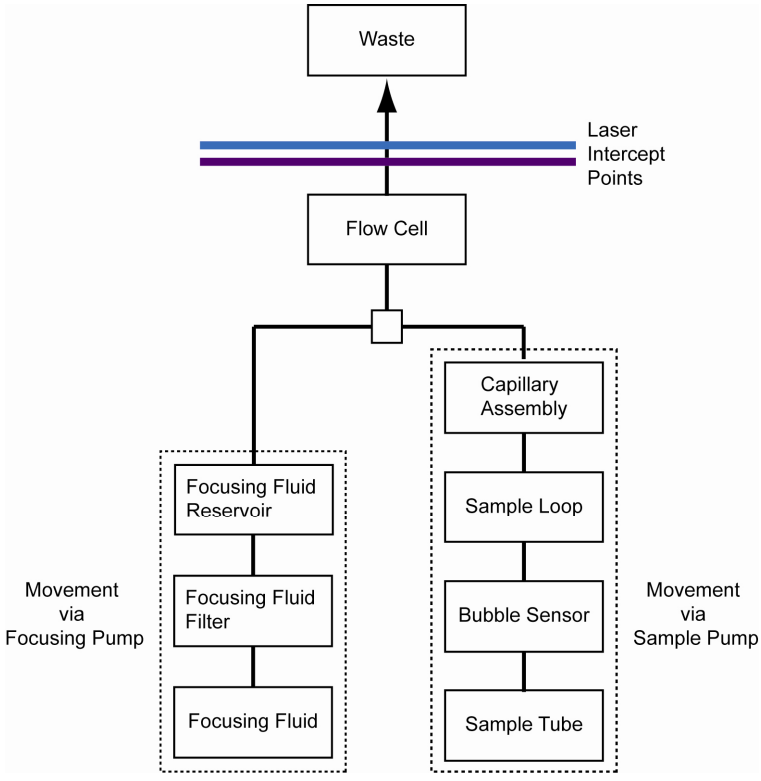
The fluidics system of the Attune™ Acoustic Focusing Cytometer establishes the fluid flows required for acoustic focusing cytometer operation and includes the flows during the Startup, Wash, Rinse, Run, Stop, De-bubble, and Shutdown operations as well as the main data collection operation.

The sample to be analyzed is driven by a syringe pump and passes through the bubble sensor and the sample loop before arriving at the capillary assembly. A separate pump forces the focusing fluid through the focusing fluid filter and flow dampener.

The capillary assembly is an acoustic resonant device that focuses cells or particles in the sample fluid into a single tight line (i.e., the sample core) using a capillary coupled to a single piezoelectric transducer. The capillary carries the sample core upward through the center of the optical cell, where the particles to be analyzed are intercepted by a tightly focused laser beam for interrogation.

After passing through the optical cell, the stream arrives at the waste container.

A schematic of the Attune™ Acoustic Focusing Cytometer fluidics system is shown in the figure below.



Types of Fluids and Reagents

The Attune™ Acoustic Focusing Cytometer requires three different fluids to perform the start up and shutdown features and these are supplied with the Attune™ Acoustic Focusing Cytometer. These fluids and reagents are also available separately from Applied Biosystems (see below for ordering information). For details, visit www.appliedbiosystems.com or contact Technical Support.

- **Attune™ Focusing Fluid** to dilute the sample before entering the acoustically driven capillary

  The Attune™ Focusing Fluid is a sterile, buffered, azide-free support/carrier reagent optimized for transporting particles through the Attune™ Acoustic Focusing Cytometer. The focusing fluid provides the balance of total flow solution through the interrogation chamber and aids in maintaining system cleanliness. The solution contains a surfactant to help clean the fluid lines and to minimize bubble formation.

  For best results, store fluid at 2 to 25ºC.

Cat. no.	Quantity:
4449790 (Attune™ Focusing Fluid, 1X)	1 L
4449791 (Attune™ Focusing Fluid, 1X)	6 × 1 L
4449792 (Attune™ Focusing Fluid, 1X)	10 × 1 L

- **Attune™ Wash Solution** is used to clean the sample lines of any build-up of cellular debris or remove any bound dye.

  The Attune™ Wash Solution is a ready-to-use fluid for the shutdown procedure on the Attune™ Acoustic Focusing Cytometer. This wash solution is formulated to remove cellular debris and dyes from the fluidics system of the instrument. The wash solution is stable on the instrument for 30 days after the bottle is opened, and should be stored at 2 to 25ºC.

  Cat. no. 4449755 Quantity: 500 mL

- **Attune™ 10X Shutdown Solution** is used during the shutdown procedure.

  Attune™ Shutdown Solution is a ten-fold concentrated solution that is diluted 1:10 in sterile deionized water. This azide-free solution is formulated to minimize bubble formulation that can occur in the system lines overnight, to help keep the fluid lines clean, and to minimize microbial growth in the fluid lines. The shutdown cycle is used at the end of each day to ensure that all sample fluids and dyes have been removed from the system, and that the fluidic lines and pumps are filled with the Attune™ Shutdown solution to prevent salt deposits from forming in the fluidics lines. Shutdown takes around 20 minutes, but most of the steps are automatic under the control of the Attune™ Cytometric Software. Run the shutdown procedures at least once a day, even if the instrument is intended for continuous use. Proper cleaning of the instrument ensures consistent and accurate operation.

  For best results, store solution at 2 to 25ºC.

  Cat. no. 4454955 Quantity: 250 mL

- **10% bleach** (prepared fresh daily, not supplied with the instrument) installed on to the sample loader during the shutdown procedure.

- **Attune™ Performance Tracking Beads** are designed for use in conjunction with the Attune™ Cytometric Software to automatically characterize, track, and report daily performance measurements of the Attune™ Acoustic Focusing Cytometer.

  Regular calibration of your instrument with the beads ensures the accuracy and sensitivity of the instrument and allows easy identification of potential shifts or trends in the instrument performance. The tracking beads contain distinct sub-populations of beads labeled at different intensities, and are used to establish optimal dynamic range settings for the instrument. The beads are pre-calibrated at the factory and the lot specific bead performance data captured as a downloadable file for use by the Attune™ Cytometric Software. Each vial contains enough beads for 50 measurements.

  Store the beads at 2 to 8ºC.

  Cat. no. 4449754 Quantity: 50 measurements

Modes of Operation

The operation of the Attune™ Acoustic Focusing Cytometer falls under three major modes:

- **Startup Cycle** performed at the beginning of each day and is used to ensure that that all fluidic lines are clean, and the fluidic lines and system's two pumps are filled with fresh sheath fluid. Startup takes approximately 2 minutes.
- **Collect-Run-Rinse Cycle** is used for normal operation with data collection.

  Collect Cycle is used to record all data.

  Run Cycle is used to collect data in two modes—standard- and high-sensitivity modes. Each individual mode can run the sample at different sample rates.

  Rinse Cycle is used to rinse the fluidic lines between samples.
- **Shutdown Cycle** is performed at the end of each day and is used to ensure that all sample fluid and dyes are removed from the system, and the fluidic lines and system's two pumps are filled with shutdown, a low salt solution, to prevent salt crystals from clogging the fluidics system. Shutdown takes around 30 minutes, but most of the steps are automatic and computer-controlled.

Fluidics Functions

The various fluidics functions of the Attune™ Acoustic Focusing Cytometer are defined below:

- **Start Up** is an automated function that starts the fluidics, optics, and electronics of the Attune™ Acoustic Focusing Cytometer. The startup functions include priming the instrument fluidics and allowing laser warm-up time.
- **De-bubble** is a user initiated prime for clearing bubbles in the fluidics lines of the cytometer.
- **Wash** is a user-initiated system cleaning between sticky samples. This function requires user supplied 10% bleach solution.
- **Run** is used to set up the instrument and experiment to collect and run samples through the system but does not collect data.
- **Record** is used to collect and record data.
- **Stop** is used to stop all data collection.
- **Clear** is used to clear the results from the screen. It refreshes the workspace while the instrument is in the Run or Record mode.
- **Rinse** flushes the system between samples to minimize any sample or reagent carryover. This occurs between samples but can also be user-initiated.
- **Unclog**: This is a user initiated back flush operations to remove clogs from the sample probe and flow cell.
- **Shutdown** is an automated function that initiates the cleaning cycle and post-cleaning rinse. This mode requires user-supplied bleach, Attune™ Wash Solution, and Attune™ Shutdown Fluid.

Start Up and Shut Down Procedures

Starting Attune™ Acoustic Focusing Cytometer

1. Turn on the computer if it is not already on by depressing the power button.
2. Check the levels in the fluid tanks.

   Note: The Attune™ Acoustic Focusing Cytometer monitors the fluid levels via floating sensors in all fluidics tanks. When the fluid level is low or the waste bottle is full, the Attune™ Cytometric Software displays the appropriate warning message.

   If the waste tank is full, empty the waste tank.

   If the fluidics tanks are empty, fill the focusing fluid, wash solution, and shutdown solution tanks as follows:

   a. Press the metal release button to free the tubing from the instrument, and remove the tank.

      Note: It is important to use the fittings as pulling on the tubing may cause wires to degrade over time.

   b. Unscrew the lid, and fill the tank with the appropriate solution. The focusing fluid tank has 1 L capacity, and the wash and shutdown solution tanks have 500 mL capacity each. Do **not** overfill the fluid tanks.

   c. Screw the lid back on without over-tightening it.

   d. Replace the tank by sliding it into the appropriate slot and plugging the tubing back into instrument.

3. Turn on the instrument using the power button located on the upper, right hand corner of the fluidics door located on the front of the instrument.

   The instrument lights start blinking until the instrument is ready for use.

4. Launch the Attune™ Cytometric Software as follows:

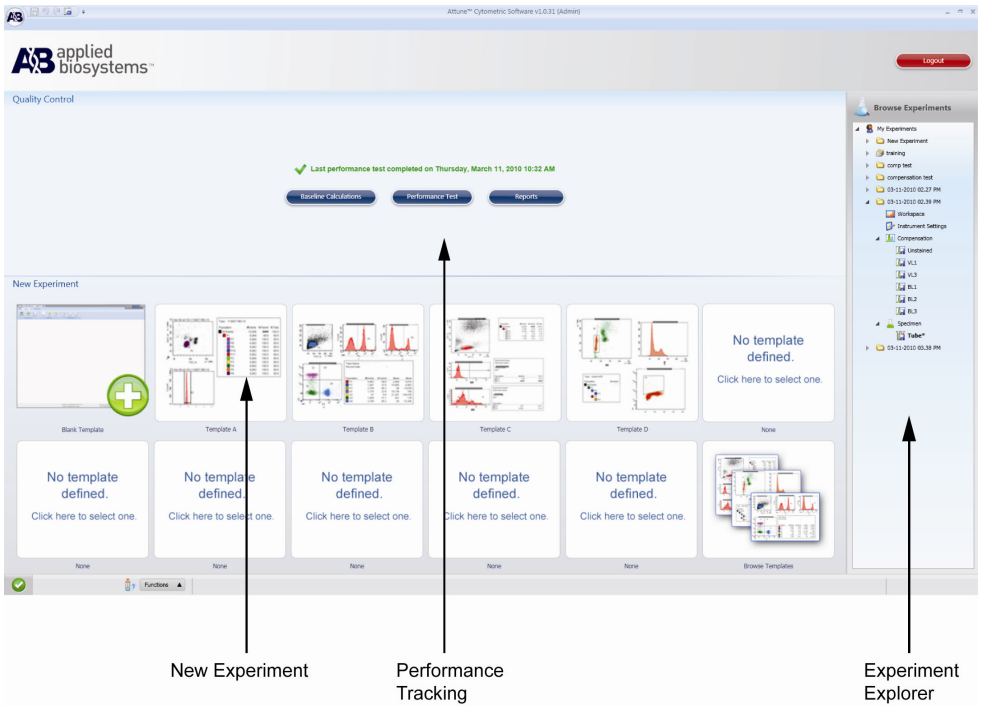
   a Double-click the Attune™ Cytometric Software shortcut icon on the computer desktop or from the Start Menu, **Start ▶All Programs▶Attune™ Cytometric Software**.

   b Enter you user name and password in the user logon screen that is displayed.

      Note: User name is defined by your system administrator. If you have questions, consult your system administrator about your user name assignments. Passwords can be changed by the administrator.

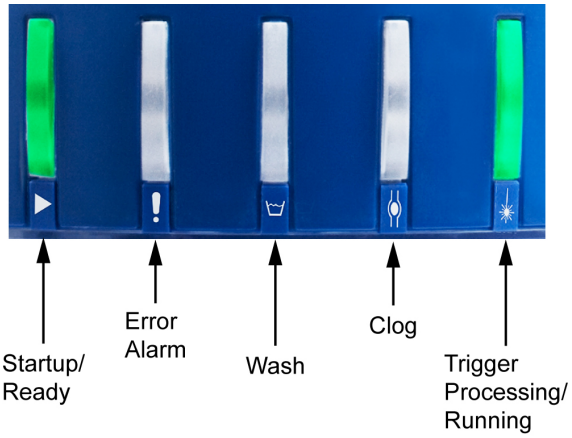
   c Click **log in**. The **Main Menu** screen is displayed (see next page).

Main Menu


Quality Control
Last performance test completed on Thursday, March 11, 2010 10:32 AM
Baseline Calculations
Performance Test
Reports
New Experiment
Browse Experiments
No template defined.
Click here to select one.
New Experiment
Performance Tracking
Experiment Explorer

Instrument Status Indicator Lights

Once the instrument is turned on, a series of five lights located on the right side of the instrument above the tube lifter are turned on that indicate the instrument status.



Instrument Cycle	▶ Green	! Red	Blue	Yellow	✳ Green
Startup	BLINK	ON/OFF*	OFF	OFF	OFF
	*Indicator light blinks for 2 seconds, then turns off.				
Startup complete	ON	OFF	OFF	OFF	OFF
Idle	ON	OFF	OFF	OFF	OFF
Run	ON	OFF	OFF	OFF	BLINK*
	*Indicator light blinks with cycle time proportional to event rate.				
Run complete	ON	OFF	OFF	OFF	OFF
Wash/Declog/De-bubble	OFF	OFF	BLINK	OFF	OFF
Rinse	OFF	BLINK/OFF*	ON	OFF	OFF
	*Indicator light blinks when the rinse cycle is initiated and turns off when it is time to place the sample on the sample injection port.				
Clog detected	OFF	BLINK	OFF	ON	OFF
Focusing fluid tank empty	OFF	BLINK	OFF	OFF	OFF
Waste tank full					
Wash tank empty					
Water tank empty					
Shutdown	OFF	OFF	BLINK	OFF	OFF
Error	OFF	ON	OFF	OFF	OFF

Shutting Down the Attune™ Acoustic Focusing Cytometer

Perform the following shutdown procedures at the end of each day. To ensure consistent and reliable functioning of the instrument, prevent sample tube clogging, and minimize dye carry over in the tubing, perform proper cleaning of the instrument as described below.

Note: Perform a shutdown procedure if viscous samples are analyzed or nucleic acid dyes such as acridine orange, thiozole orange, and propidium iodide are used.

1. Select shutdown procedure on the Attune™ Cytometric software.
2. Ensure that the cleaning solution bottle is at least half full.
3. Install a tube of 10% bleach onto the sample loader.
4. Follow the on-screen instructions for the automatic shutdown procedure.
5. Add two tubes of water when indicated by the shutdown procedure.
6. Leave deionized water on sample tube loader after the shutdown procedure is complete.
7. Turn off the instrument power supply by holding the power button (located on the upper, right hand corner of the fluidics door on the front of the instrument) for 2–3 seconds until the instrument power is turned off.
8. Turn off the computer.

Maintenance Procedures

The Attune™ Acoustic Focusing Cytometer is designed to operate with minimal periodic maintenance. However, to ensure reliable performance of the instrument, we recommend a basic maintenance schedule as described below.

If your instrument is not going to be used for a week or longer, perform a shutdown using 10% bleach, and switch off the instrument and the computer (see **Shutting Down the Attune™ Acoustic Focusing Cytometer)**.

Routine maintenance is performed on the instrument as listed below. For maintenance details, refer to the user guide supplied with the instrument:

Maintenance Type	Performed by	Schedule
Routine		
• Visual examination of fluidics bottles	User	Daily
• Visual examination of syringes	User	Monthly
• Clean Optical filters	User	Monthly
• Change focusing fluid filter	User	Biannually
• Change both syringes	User	Biannually

WARNING: Follow good laboratory practices by wearing Personal Protective Equipment (PPE) when performing maintenance procedures.

Exercise 2

1. Match the following:

_____ Run	**A.** Stops fluid flow
_____ Rinse	**B.** Flushes system between samples to minimize carryover
_____ Stop	**C.** Allows sample to move through system
_____ Record	**D.** Allows sample to move through system and captures data

2. What is the capacity of the following solutions:

   Focusing Fluid is _____ Liter

   Wash Solution is _____Liter

   Shutdown solution is _____Liter

3. In what mode should the instrument be in before refilling the focusing fluid tank?

   _____

4. How often should you perform the following procedures (Daily (D), Weekly (W), Monthly (M), Biannually (B)

   Cleaning of optical filters _____

   Shutdown _____

   Checking of Syringes _____

   Start Up _____

   Replacement of Focusing Fluid Filter _____

Exercise 3

1. Proceed to the Attune™ Acoustic Focusing Cytometer.

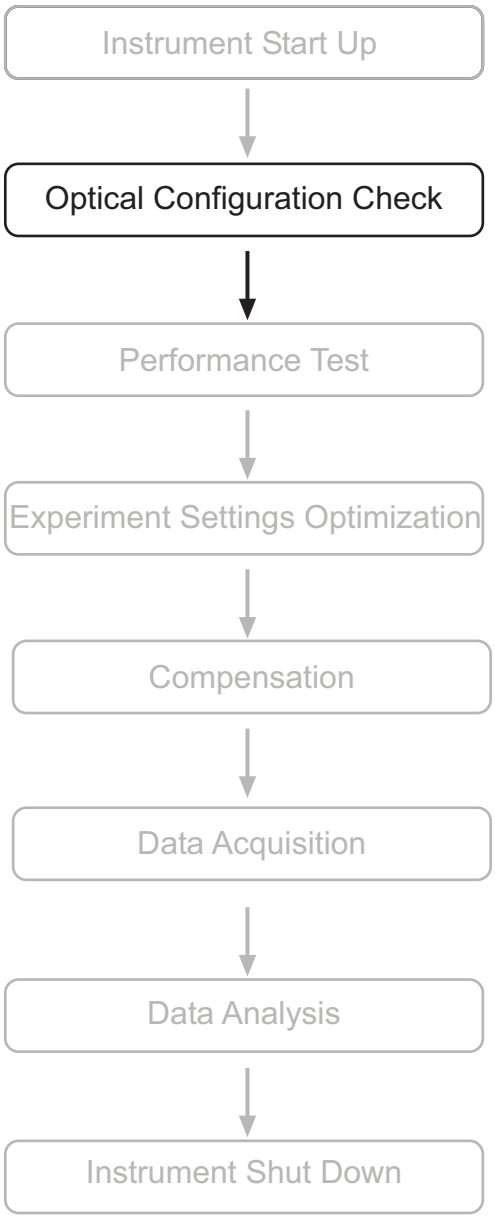
2. Perform startup and shutdown functions.

Chapter 3: Optics and Electronics Overview

This chapter describes:

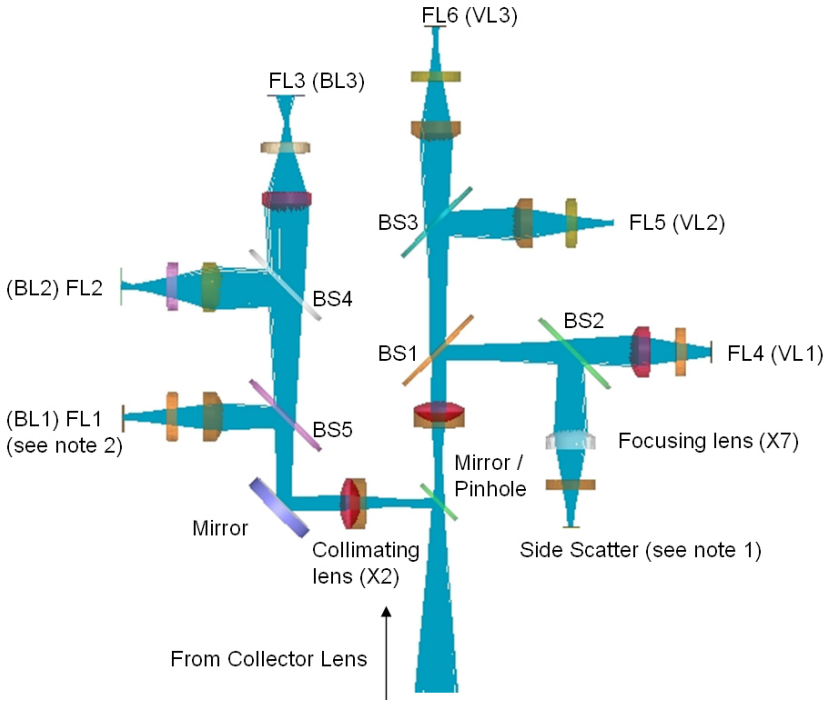
- Optical path of the Attune™ Acoustic Focusing Cytometer
- Types of optical filters
- Excitation lasers and laser power of the instrument
- Compensation
- Electronics
- Exercise 4

Workflow


Instrument Start Up
Optical Configuration Check
Performance Test
Experiment Settings Optimization
Compensation
Data Acquisition
Data Analysis
Instrument Shut Down

Optical Path of the Attune™ Acoustic Focusing Cytometer

The Attune™ Acoustic Focusing Cytometer contains lasers to illuminate cells or particles from the sample, and optical filters and mirrors to direct the resulting light scatter and fluorescence signals to the appropriate detectors. The basic optical layout for the Attune™ Acoustic Focusing Cytometer is shown in the figure below.



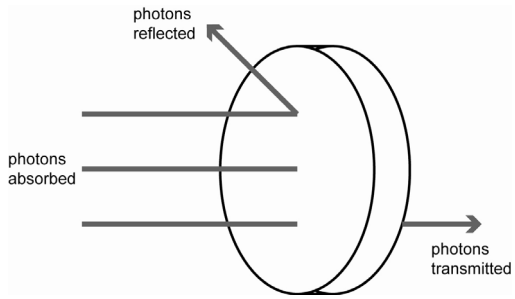
Notes:

1. At Side Scatter location, neutral density (ND) filter is in user-removable filter holder and 405/10 filter is in non-user-removable filter holder tube.
2. At FL1 location, bandpass filter is in user-removable filter holder and long-pass filter is in non-user-removable filter holder tube.

Detector	Laser	Dichroic Mirror	Emission Filter	Designed to Detect
BL1	488	555 nm SP	530/30 nm	FITC, Alexa Fluor® 488, GFP, SYTOX® Green, DyeCycle™ Green Stain, CSFE, LIVE/DEAD® Fixable Green Dead Cell Stain
BL2	488	620 nm LP	575/24 nm	PE, PI, YFP, DyeCycle™ Orange Stains
BL3	488	-	640 nm LP	PerCP-Cy®5.5, PerCP, PE-Cy®5 (TRI-COLOR®), PE-Cy®5.5, PE-Cy®7, DyeCycle™ Ruby, SYTOX® AADvanced, LIVE/DEAD® Fixable Red Dead Cell Stain and Qdot® 655, Qdot® 705, and Qdot® 800 nanocrystal conjugates
VL1	405	410 nm SP	450/40 nm	Pacific Blue™, DyeCycle™ Violet, LIVE/DEAD® Fixable Violet Dead Cell Stain, CellTrace™ Violet, FxCycle™ Violet, SYTOX® Blue, autofluorescence, Alexa Fluor® 405 Dyes
VL2	405	575 nm LP	522/30 nm	LIVE/DEAD® Fixable Aqua Dead Cell Stain, Qdot® 565 nanocrystal conjugates
VL3	405	-	603/48 nm	Qdot® 605 nanocrystal conjugates, Pacific Orange™, LIVE/DEAD® Fixable Yellow Dead Cell Stains

Types of Optical Filters

Optical Filters separate the light scatter and fluorescence directed to detectors by wavelength, which is measured in nanometers (nm). They selectively transmit light having a particular range of wavelengths, while absorbing or reflecting the remainder.



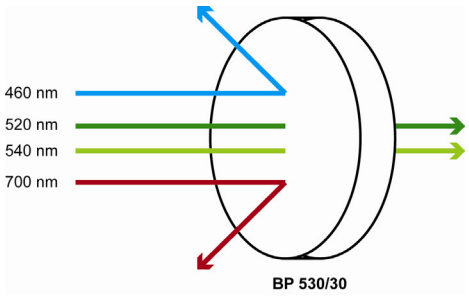
There are five types of optical filters used in flow cytometry:

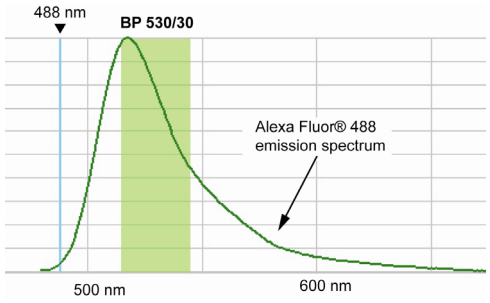
- Bandpass filter (BP)
- Longpass filter (LP)
- Shortpass filter (SP)
- Dichroic mirror (DM)
- Neutral density filter (ND)

Bandpass Filter

A Bandpass (BP) Filter is a device that passes frequencies within a certain range and rejects (attenuates) frequencies outside that range. Combining a longpass filter and a shortpass filter produces a bandpass filter. These filters usually have lower transmittance values than SP and LP filters, and block all wavelengths outside of a selected interval, which can be wide or narrow, depending on the number of layers of the filter. The bandwidth of the filter is the difference between the upper and lower cutoff frequencies. Common bandpass filter nomenclature is the peak emission/bandwidth.

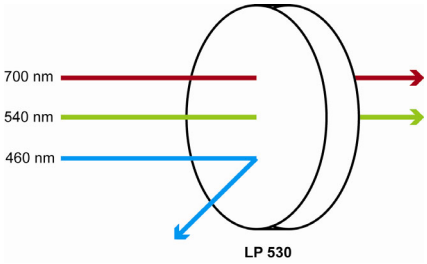
For example, a filter that detects Alexa Fluor® 488 dye would be 530/30 which ranges from 515–545 nm.

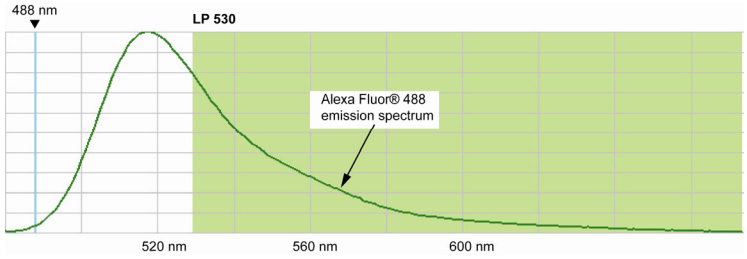




Longpass Filter

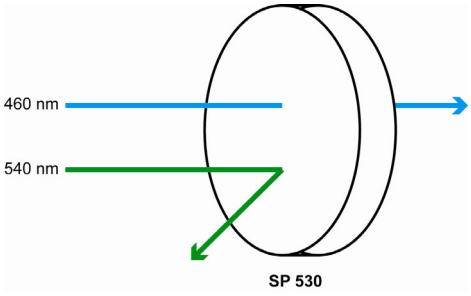
A Longpass (LP) Filter is an optical interference or colored glass filter that attenuates shorter wavelengths and transmits (passes) longer wavelengths over the active range of the target spectrum (ultraviolet, visible, or infrared). Longpass filters, which can have a very sharp slope (referred to as edge filters), are described by the cut-on wavelength at 50 percent of peak transmission.





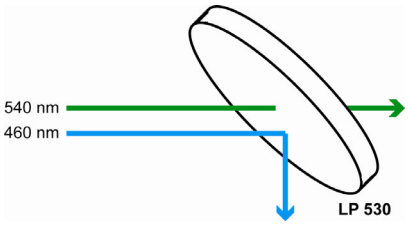
Shortpass Filter

A Shortpass (SP) Filter is an optical interference or colored glass filter that attenuates longer wavelengths and transmits (passes) shorter wavelengths over the active range of the target spectrum (usually the ultraviolet and visible region).



Dichroic Mirror

A Dichroic mirror (also called reflective or thin film or interference filters) can be made by coating a glass substrate with a series of optical coatings. Dichroic filters usually reflect the unwanted portion of the light and transmit the remainder. A dichroic filter is a very accurate color filter used to selectively pass light of a small range of colors while reflecting other colors. By comparison, dichroic mirrors and dichroic reflectors tend to be characterized by the color(s) of light that they reflect, rather than the color(s) they pass. Dichroic mirrors are essential to the optical path of a flow cytometer as they direct the emission light to the photomultiplier tube detector.



Neutral Density Filter

A Neutral Density (ND) filter has a constant attenuation across the range of visible wavelengths, and is used to reduce the intensity of light by reflecting or absorbing a portion of it.

Excitation Lasers and Laser Power of the Instrument

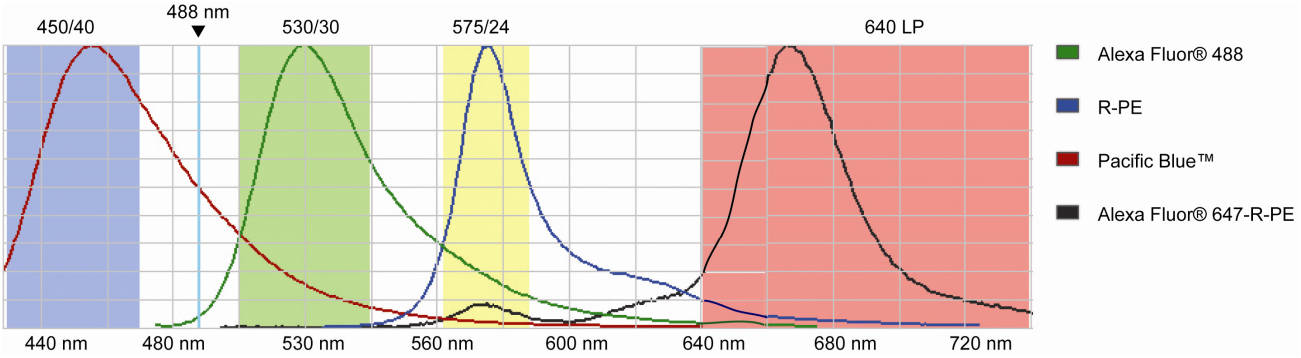
The Attune™ Acoustic Focusing Cytometer has alignment free excitation and emission optics and utilizes two lasers:

Laser Type	Wavelength	Power
Spectra-Physics Cyan	488 nm	20 mW
Power Technology IQ1C50	405 nm	50 mW

Note: Shutting down lasers within 15 minutes of start up time can decrease their lifetime. After performing the shutdown procedure, turn off the lasers for 15 minutes prior to restarting the instrument.

Compensation

Compensation is the process by which the "spillover" is corrected. Every fluorescent molecule emits light with a particular spectrum unique to that molecule. These emission spectra overlap, in some cases very significantly. For instance, see the spectra shown below, for Pacific Blue™, Alexa Fluor® 488, R-phycoerythrin (R-PE), and Alexa Fluor® 647-R-PE dyes.



In this figure, the emission spectra of each dye is highlighted with a shaded area indicating the emission filter where the specific light is captured on the Attune™ Acoustic Focusing Cytometer. The teal line is the laser excitation wavelength of an argon ion laser (488 nm). Each dye emits with a characteristic emission spectrum that is specific for the fluorochrome: Alexa Fluor® 488 dye has a maximum around 520 nm, R-PE at about 575 nm, Alexa Fluor® 647 dye at about 666 nm, and Pacific Blue™ dye at about 454 nm.

To simultaneously measure these emissions, we choose optical filters ("bandpass filters") which only transmit specific wavelengths of light. The filters commonly used in flow cytometry for these dyes are represented by the shaded areas. In general, filters are chosen which collect the emitted light near the emission maximum. For Alexa Fluor® 488 dye, we use a BP 530/30 filter, meaning that the filter has a pass-band centered on 530 nm, and the width of the pass-band is 30 nm.

However, it is impossible to choose filters which measure only one dye. For instance, Alexa Fluor® dye has a significant emission in the region that we measure R-PE (575 nm). Therefore, whenever fluorescein is present, we get signal in the 530 nm band, and also in the 575 nm band. If R-PE is also present, it contributes to the 575 nm band. To differentiate between the fluorescence from R-PE, and Alexa Fluor® 488 dye compensation is used.

Compensation is the mathematical process for correcting for the amount for Alexa Fluor® 488 dye fluorescence in the 575 nm band so that we can measure the R-PE fluorescence.

Performing multicolor analyses complicates compensation further because fluorophores are not usually spectrally separated very well therefore compensation plays a key role in the experimental design. If a fluorescent dye emission is collected through three different filters, then we can always estimate how much emission is in the first filter based on how much spill over or contaminating signal is in the second or the third filters. In the above figure, the Pacific Blue™ conjugate has some spill over in the 530/30 nm filter and very little in the 575/24 nm filter, therefore the amount of compensation required in the 530/30 nm filter will be more than in the 575/24 nm filter.

Fluorescence spillover can be estimated by running single fluorescence controls specific for a certain dye and then subtracting out the fluorescence in the other detection channels, thus leaving the true signal of the other fluorophores.

For a more detailed description of the mechanisms of compensation, refer to the flow cytometry tutorials at:

http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-and-Tissue-Analysis/Flow-Cytometry/FC-Misc/Tools-and-Resources.html

Spectra Viewer

A fluorescence spectra viewer is used to choose the right fluorochromes according to the number of colors you will need in your experiment, the flow cytometer that you are using, and compensation trade-offs.

To use the spectra viewer:

1. Go to www.invitrogen.com and click on the **Support** tab. Click on **Fluorescence SpectraViewer** under Research Tools on the right on the support page.
2. Once the spectra viewer is launched, choose the fluorochromes of interest from the drop down menu, click either excitation, emission, or both spectra boxes.
3. Add laser excitation lines and enter emission filters.

Overlapping spectra are shown for each individual dye to inform the user on the spectral characteristics of each dye. By hovering over each spectra, actual data points may be visualized.

Electronics

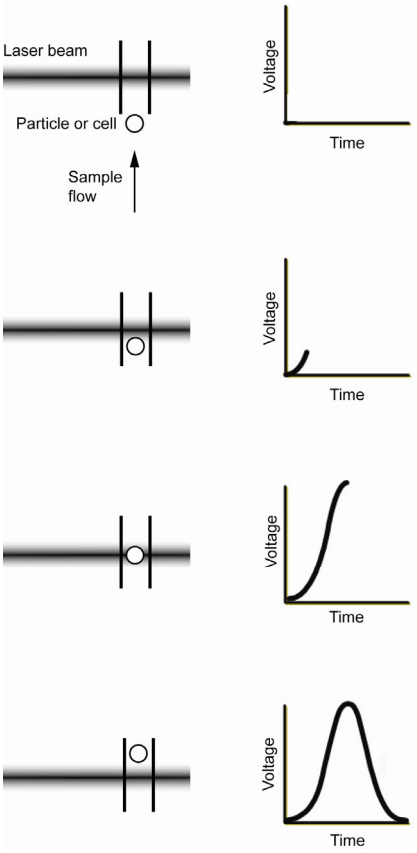
Voltage Pulse

When a cell or particle passes through a focused laser beam, it refracts or scatters light in all directions and can emit fluorescence. The scatter and the fluorescence last only a few microseconds, because the cells or particles are moving very rapidly through the focused laser beam. The detectors convert the momentary flash of light into an electrical signal called a voltage pulse.

When the cell or particle begins to enter the intercepting laser beam, the signal intensity is low, because only a small portion of the particle scatters the light.

The pulse reaches its maximum when the cell or particle is in the middle of the laser beam, and the whole particle scatters the light. Further, the laser beam is brightest in the middle, thus causing more light to scatter off of the particle.

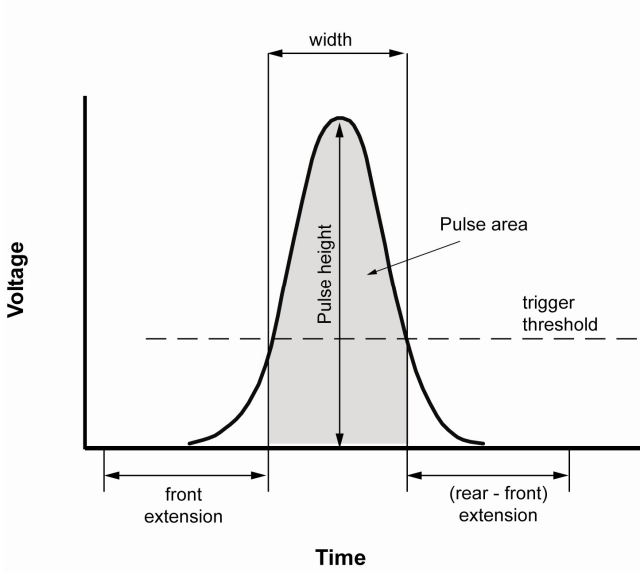
As the cell or particle exits the beam, the signal starts decreasing and eventually trails off below the threshold (see figure on the next page).



Pulse Measurement

The analog signal from the detectors are amplified and relayed to the 24-bit analog-to-digital converter (ADC), which samples the signals at a rate of up to 2.5 MHz, converting the continuous signal into digital data and producing 6 decades resolution for up to 20,000 events per second.

The data is further processed by the field programmable gate array (FPGA), which simultaneously calculates pulse height, area, and width when the pulse exceeds the user-specified threshold value.



Exercise 4

Select True or False for the following questions:

1. A short pass filter allows all light greater than the maximum to the detector (**True** or **False**).

2. The lasers on this instrument require daily alignment (**True** or **False**).

3. The 530/30 nm filter is used to detect light in the VL3 detection channel (**True** or **False**).

4. Compensation is the correction applied to raw data to remove spillover from the signal of interest. (**True** or **False**).

5. A dichroic mirror is used to direct light to the appropriate photomultiplier tube (**True** or **False**).

6. Bandpass filters transmit specific wavelengths of light (**True** or **False**).

7. Using a fluorescence spectra viewer at http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html

   What detection channels would you detect the following fluorochromes:

   A. Pacific™ Orange antibody conjugate _____

   B. Alexa Fluor® 488 antibody conjugate _____

   C. Live/Dead® Fixable Aqua stain _____

   D. SYTOX® Blue dye _____

   E. R-PE antibody conjugate _____

   F. Qdot® 605 antibody conjugate _____

   G. Vybrant® DyeCycle™ Violet dye _____

Chapter 4: Software Overview

This chapter describes:

- Launching the Software
- Log in Screen
- Main Menu
- Workspace Screen
- Software Administration
- Perform Basic Software Functions
- Exercise 5

The Attune™ Acoustic Focusing Cytometer is controlled by the Attune™ Cytometric Software. This chapter provides a basic overview of the software and its features.

Launching the Software

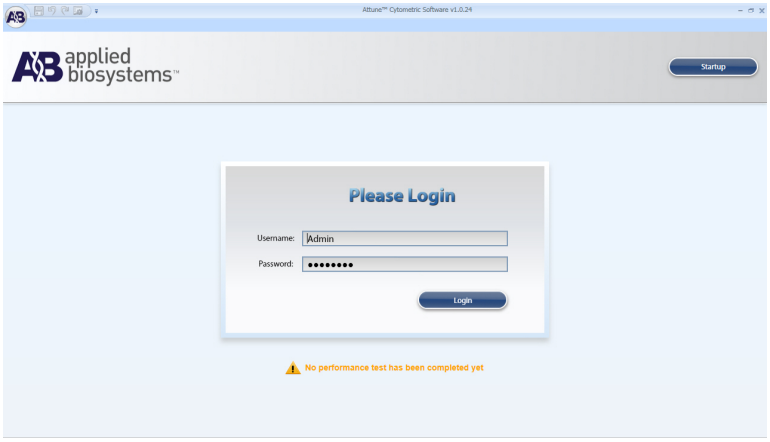
Upon installation of the Attune™ Acoustic Focusing Cytometer, the Attune™ Cytometric Software is installed on your instrument under programs, a folder for Applied Biosystems, and the Attune™ software icon or a shortcut is placed on the computer desktop and under programs from your Start menu.

To launch the Attune™ Cytometric Software, turn on the computer and double-click on the Attune™ software icon on the desktop. The Log in screen is displayed.

The software consists of the following screens: Log in Screen, Main Menu screen, and Workspace Screen.

Log in Screen

The Log in screen allows users to log into the software.



There are three types of user accounts—Administrator, Operator, and Service.

- Administrator users perform a variety of tasks that are described at the end of this chapter
- Operators are users who can access and modify their own workspace and experiments, but can not add or delete users.
- Service is used for Applied Biosystems' service personnel to trouble shoot and maintain the performance of the instrument.

After a successful log in, the software directs you to the **Main Menu** Screen.

Main Menu Screen

The Main Menu screen consists of three main sections: Performance Test Panel, New Experiment Panel, and Experiment Explorer.

Performance Test Panel

This screen provides information of when the last performance test was complete and the status of that run. It also allows you to set up a baseline for your daily performance monitoring, runs the Performance Tracking Program, and review the Performance Tracking Report. It also allows you to download new lot information for Attune™ Performance Tracking Beads.

New Experiment Panel

In this panel, you can create an experiment using a blank template or use a pre-populated template that can be user-defined or preset template. This panel displays up to 10 individual experiment templates.

Experiment Explorer

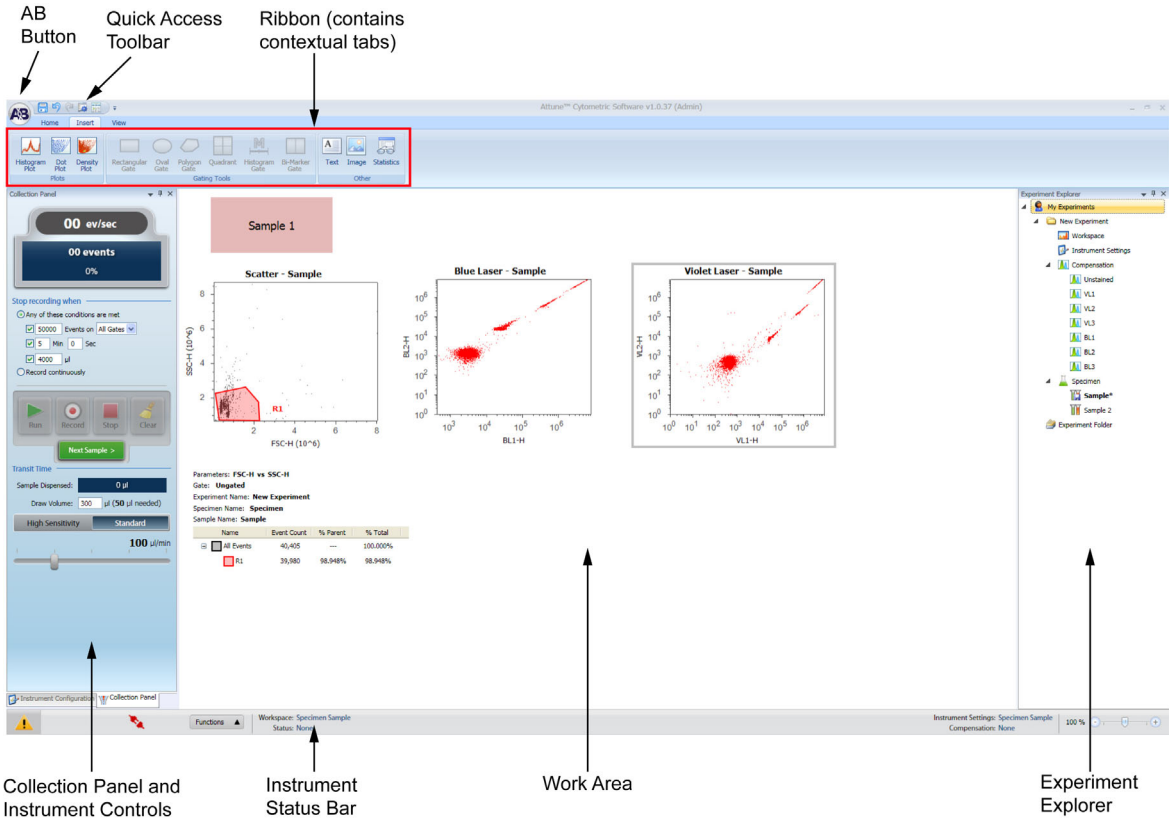
This panel shows the experiments of the operator that is logged on to the instrument. Each experiment is stored in individual folders or can be organized in larger folders. Double-clicking on any sample within the experiment opens the Workspace screen.

Workspace Screen

The Workspace is used for controlling the Attune™ Acoustic Focusing Cytometer to run samples, generate data, and analyze the results.

- Collection Panel
- Instrument Controls

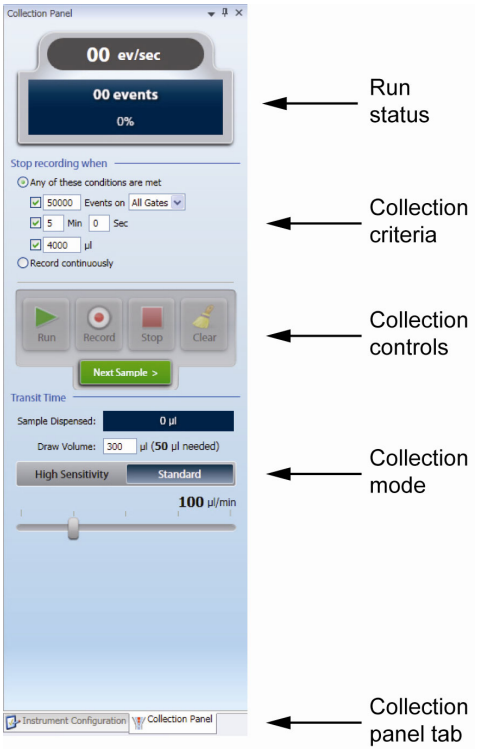
- Experiment Explorer
- Work Area.
- Contextual Tabs
- Instrument Status Bar
- Ribbon Bars
- Quick Access Toolbar
- AB Button



Note: You can move the experiment explorer, collection panel, and instrument control panel throughout the Workspace to make more room on the work area. These panels also can be hidden or minimized.

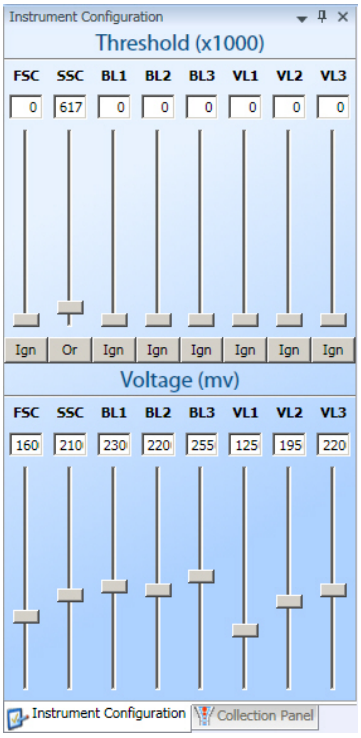
Collection Panel

The collection panel displays a status bar as well as number of events collected. The collection panel allows you to define the run limits, select the mode of sample collection, set up samples for a run, and record data.



Instrument Configuration Panel

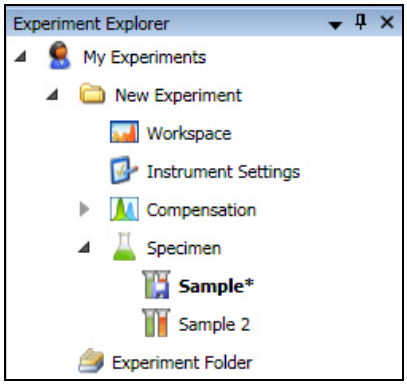
The Instrument Configuration Panel allows you to specify the *Threshold* and *Voltage* parameters for each of the Scatter and Fluorescence detectors on the Attune™ Acoustic Focusing Cytometer. To set threshold and voltage values, adjust the slider bars below each detector or enter the desired number directly in the appropriate window.



Experiment Explorer

The Experiment Explorer lists experiments in a hierarchal view and functions as an interface for creating new Experiments and recording data. Experiment Explorer is also an interface for creating Experiments and recording data. Only one experiment can be opened at a time. You can organize your experiments in higher level folders, which can contain multiple experiments.

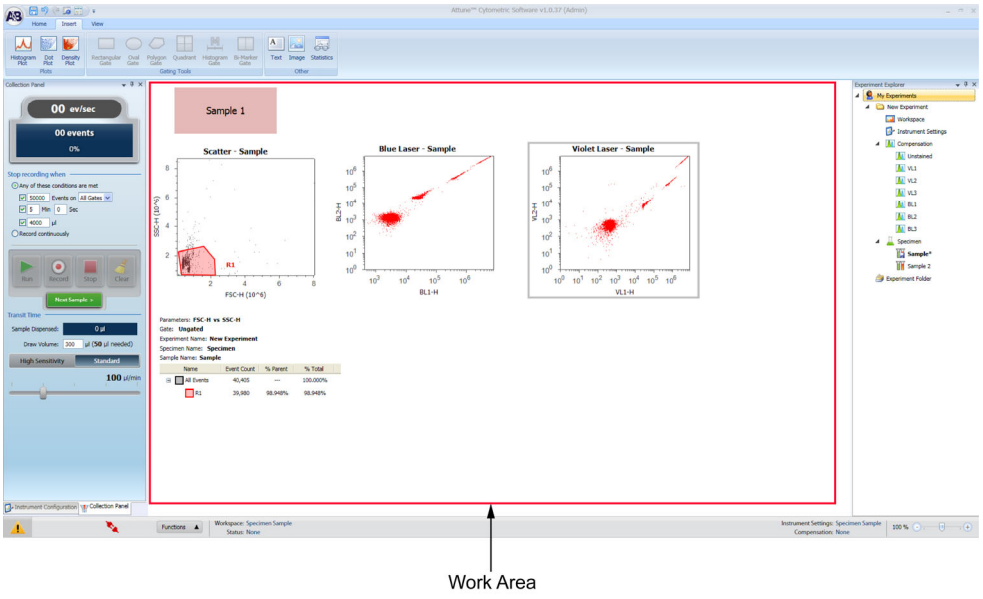
Double-click on any sample to open an experiment to display the work area. An active sample is the one with a tube icon. A sample that has data recorded has a tube icon overlayed with a disk.



Work Area

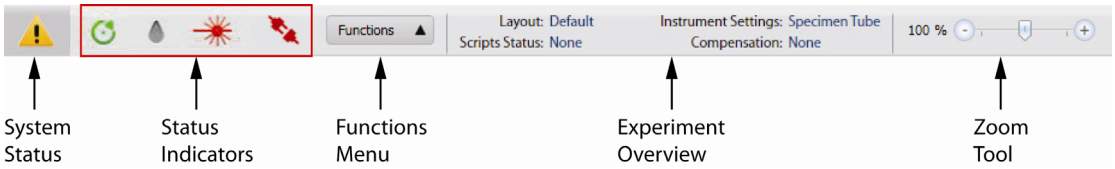
Work Area displays Analysis objects (plots, gates, and statistics views). The work area is empty for new experiments and pre-populated for experiments from a template or a saved experiment.

Double-click any Sample (or) in the Experiment Explorer to open and display it in the Work Area.



Instrument Status Bar

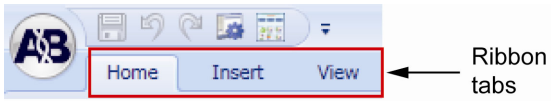
The Instrument status bar indicates the status of the instrument. This bar indicates On/Off, Fluidics, laser power status, USB connection, and functions such as unclog.



Ribbon Tabs

The Attune™ Cytometric Software utilizes the Microsoft Ribbon interface. The Ribbon consolidates related functionality by organizing control elements in logical groups under contextual tabs. Each tab relates to a type of activity.

The main *contextual (Ribbon) tabs* are the *Home*, *Insert*, and *View* tabs, and they contain common commands that apply only to the selected item. Objects that are not available as a function in a particular context are grayed out in the tab.



Home Tab

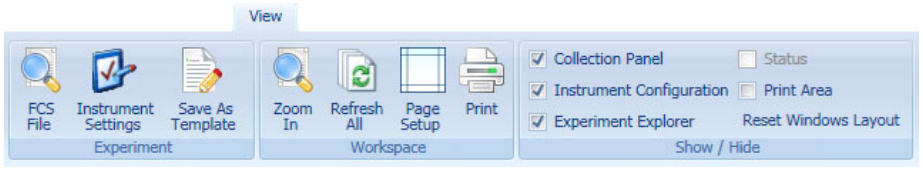
The Home tab provides editing and formatting functions for your workspace.

Insert Tab

The Insert tab includes ribbons that facilitate the insertion of different types of charts onto the work area, identify gating parameters, and allow addition of text, statistics, and images. Objects are grayed out if they are not available as a function.

View Tab

The View tab facilitates the configuration of an experiment and application. You can view the contents of the currently open FCS file; modify instrument settings, and customize the workspace.



Instrument Settings are the individual parameters that are measured such as the PMT voltages, threshold settings for an experiment, specimen, and sample. The Instrument Settings are saved as part of the experiment and can be viewed on the experiment explorer by double-clicking on the

Instrument Settings icon. To adjust the Instrument Settings, double-click on the Instrument Settings icon and then type a PMT value or use the slider bars to increase or decrease the voltage.

Instrument Controls is a summary of the entire instrument functions) i.e., unclog, run, rinse, wash, and prime).

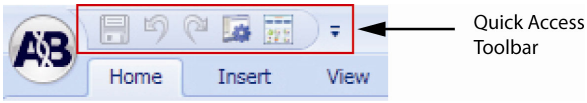
Options allows you to define storage paths, format workspace, and objects.

Compensation ribbon allows you to access the compensation set up guide, and view the compensation matrix.

Instrument Set Up allows you to choose the parameters that you want to collect data including mode, area, height and width selection, change threshold settings, and customize parameters such as defining a ratio parameter and adjusting PMT voltages.

Quick Access Toolbar

The Quick Access Toolbar contains the Save Workspace, Undo, Redo, and Options buttons.



Save workspace button allows you to save workspace to the disk.

Undo function undoes the last action.

Redo function redoes the last action.

Options which allows you to perform administrator and user management functions, (if authorized), determine file and folder pathways, change general formatting, and define start up options.

AB Button

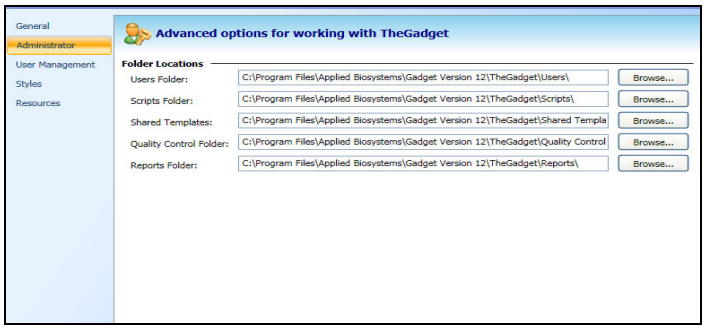
The AB Button allows you to save FCS Files, Workspace, and Instrument Settings, and to print Workspace. It also lets you open the Options window, access the Main Menu, and logout of the software. Double clicking the AB Button closes the Attune™ Cytometric Software.

Software Administration

Several functions are only available to the software administrator or a super user. These include:

- Adding Users:
  a. Click on the show options icon () in the upper, left of the screen.
  b. Click the **User Management** option.
  c. Select **New User**.
  d. Enter appropriate information and permissions.
  e. Assign password by selecting change password.
  f. Enter new password and confirm.

- Assigning Data Storage paths for user's folders, scripts, shared templates, QC folders, and reports folders:



  a. Click on the show options icon ( ) in the upper, left of the screen.
  b. Click on **Administrator** option.
  c. Assign the appropriate path using the **Browse** function.

- Changing Passwords:

  a. Click on the show options icon ( ) in the upper, left of the screen.
  b. Click the **User Management** option.
  c. Select **User**.
  d. Assign password by selecting **Change Password**.
  e. Enter a new password and confirm.

- Resetting Password:

  a. Click on the show options icon ( ) in the upper left of the screen.
  b. Click the **User Management** option.
  c. Select **User**.
  d. Assign password by selecting **Change Password**.
  e. Enter a new password and confirm.

Perform Basic Software Functions

1. Launch the Software by double-clicking the shortcut from the desktop.
2. Logon to the log in screen using admin. Enter the password (use default) and click log in.
3. Perform Start up Function of instrument (Go to functions; Start Up), if not already started.
4. Verify Performance Test is complete and it passed criteria.
5. Create an Experiment:
   a. Navigate to the training folder on the experiment explorer.
   b. Select a Workspace exercise.

c. Right-click on the workspace exercise folder.

d. Duplicate an experiment.

e. Find a newly created experiment (it may be at the bottom of the experiment explorer).

f. Rename the experiment by right-clicking on the experiment and select **Properties**.

g. Change the experiment name to Workspace Experiment-Your name.

h. Go to the Sample and double-click to activate the Workspace.

i. Setup a FSC histogram, a SSC histogram, and an FSC vs. SSC dot plot (manual axes).

6. Install a bead sample on tube loader.
7. Click **Run** on the Collection panel. Place a region around the bead population of interest by clicking on the gate and then moving to the location of population.
8. View Instrument Control Panel to Adjust Voltages:

   a. Adjust FSC parameter by using the slider bars and increase the voltage by 1,000. Note: record Median values prior to adjusting.

   b. Adjust the SSC parameter by using the slider bars and increase the voltage by 400. Note: record Median value prior to adjusting.

   c. Record data for FSC and SSC using different samples as a reference.

   d. After recording, change scale from linear to log. Right-click on plot and choose scale **Logarithmic**.

   e. Adjust the voltage by typing numbers into the box.

   f. Increase the FITC voltage up and down using slider bars.

9. Use Instrument Control Panel to change the threshold:

   a. Choose a threshold on SSC.

   b. Adjust the threshold up and down.

   c. Apply multiple thresholds.

Exercise 5

1. What happens if you change FSC and SSC to logarithmic mode?

_____

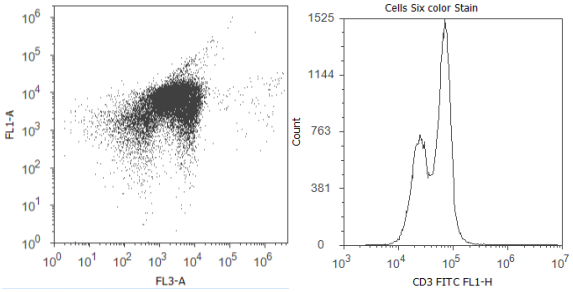
2. Increase the FSC PMT voltage by 200 volts. Which direction does the population move?

_____

3. Decrease the SSC PMT voltage by 100 volts. Which direction does the population move?

_____

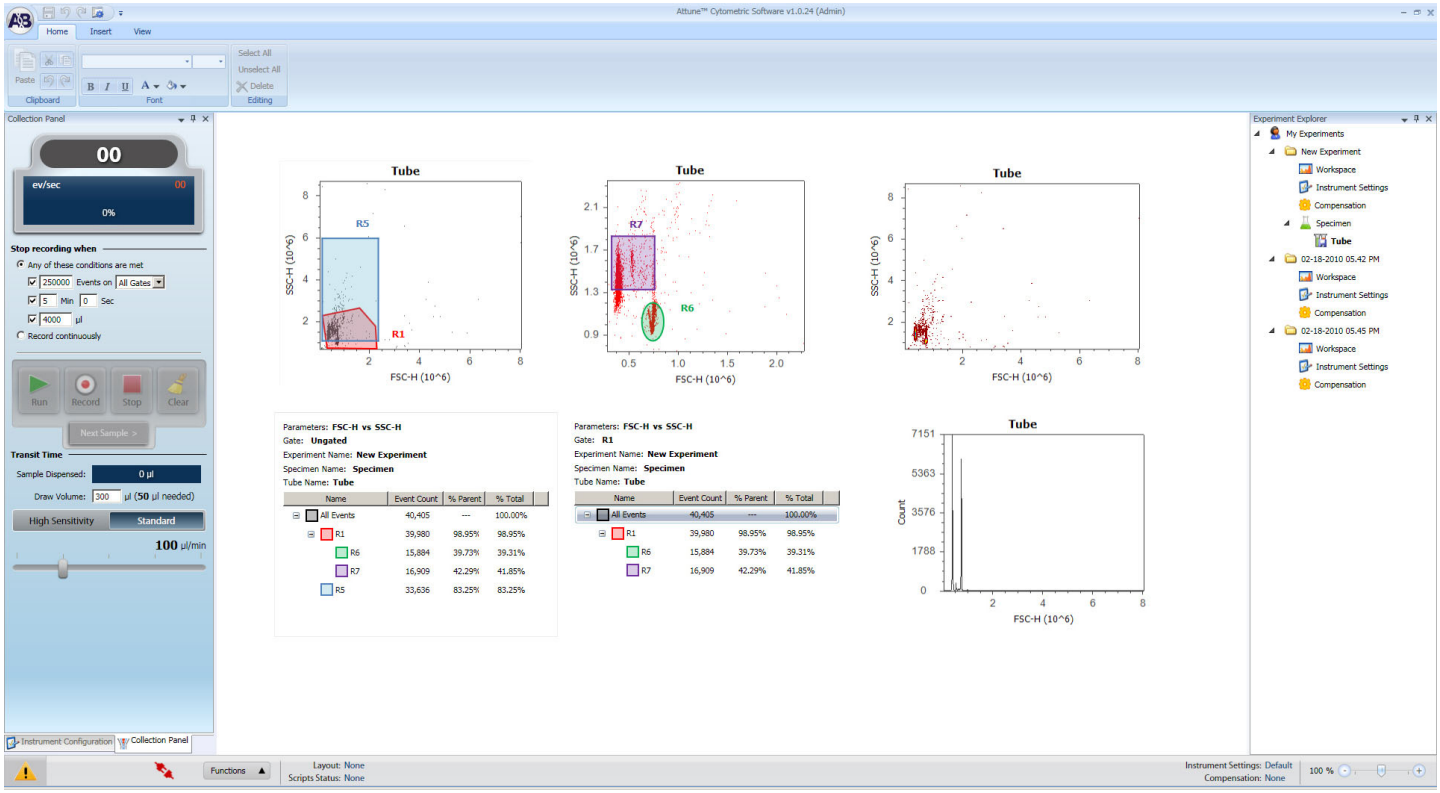
4. Identify the following plot types.



_____

_____

5. Identify the different parts of the workspace and define their functions.



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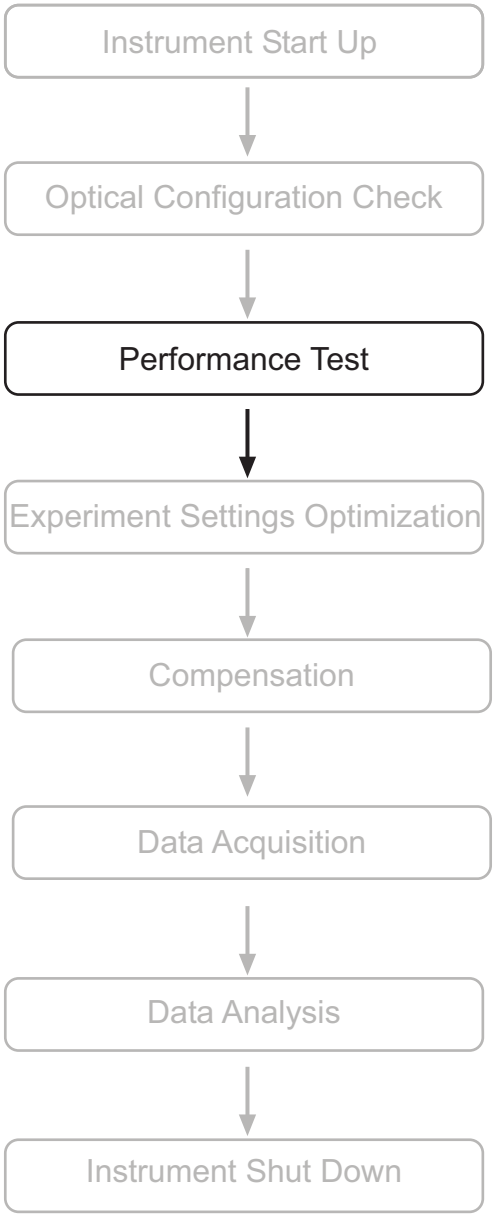
Chapter 5: Instrument Performance Tracking

This chapter describes:

- Performance Tracking of Attune™ Acoustic Focusing Cytometer
- Attune™ Performance Tracking Beads
- Baseline Calculations
- Interpretation Daily Performance Reports
- Performance Tracking Procedure using Attune™ Performance Tracking Beads
- Exercise 6

Workflow


Instrument Start Up
Optical Configuration Check
Performance Test
Experiment Settings Optimization
Compensation
Data Acquisition
Data Analysis
Instrument Shut Down

Performance Tracking of Attune™ Acoustic Focusing Cytometer

Instrument performance tracking is the process in which you can monitor the daily performance of the instrument. Performance tracking is very critical to ensure the accuracy and sensitivity of the instrument. The performance check report provides you with information on all of the lasers and detection channels available on the Attune™ Acoustic Focusing Cytometer.

The performance tracking process includes:

- Running the Attune™ Performance Tracking bead particle set at the same instrument settings on a daily basis.
- Monitoring the change in the coefficient of variation and changes in PMT voltages.
- Tracking the linearity of the instrument.
- Evaluating the detector quantum efficiency (Q) and optical background (B).

Once baseline values are defined, the beads are used to run daily performance measurements to track the cytometer performance daily. We recommend running the performance test at least once per day that the instrument is used. The voltage that is required to place the bright bead in the target channel is recorded, as well as the day-to-day delta PMT voltage. The half-peak coefficient of variation (%HPCV) of the bright bead is recorded. Using assigned MESF values for each fluorescent bead, the relative quantum efficiency (rQ) and relative Background (rB) is calculated for each channel, and the linear regression is calculated and recorded. The laser delay setting is also automatically calculated. Levey-Jennings charts provide a visual to track the %HPCV and PMT voltage to check for shifts and trends.

Attune™ Performance Tracking Beads

The Attune™ Performance Tracking Beads are supplied with the instrument and also available separately (Cat. no. 4449754). Each vial of the Attune™ Performance Tracking Beads contains a mixture of equal concentrations of beads of four fluorescence emission intensities: intensity levels 1, 2, 3, and 4. The beads are used to define a baseline and conduct daily measurements of the cytometer. Each 3-mL vial contains beads sufficient for approximately 50 daily measurements or 50 baseline definitions.

The intensity level 1 beads in Attune™ Performance Tracking Beads are 2.4 µm in nominal diameter. The intensity level 2, 3, and 4 beads are 3.2 µm in nominal diameter and stained with a combination of multiple fluorophores that can be excited by the lasers used in the Attune™ Acoustic Focusing Cytometers and emit fluorescence signals at designed levels to all the channels in the instrument.

Baseline Calculations

The Attune™ Performance Tracking Beads are used to define a cytometer baseline which is performed anytime a new lot of beads are used, or after any major maintenance is performed on the instrument. Diluted beads are run on the Attune™ Acoustic Focusing Cytometer using the Attune™ Cytometric software. The percent half-peak coefficient of variation (%HPCV) of the intensity level 4 bead is recorded. Using assigned MESF values for each fluorescent bead, the relative quantum efficiency (rQ)

and relative background (rB) is calculated for each channel and the linear regression is calculated and recorded. The laser delay setting is also automatically calculated

Interpretation of Daily Performance Reports

Several reports need to be reviewed to determine the performance of the instrument. These reports include a summary of the baseline check, review of the linearity of the PMTs, track laser performance, and alignment. These reports also include Levey-Jennings plots that graph out daily voltage differences of the instrument over time. Using these reports, you should review changes in delta PMT voltage, CV for all channels including scatter parameters.

Example reports are attached as appendices.

Performance Tracking Procedure Using Attune™ Performance Tracking Beads

A brief procedure for using the Attune™ Performance Tracking Beads (Cat. no. 4449754) is described below. For detailed instructions and troubleshooting information, refer to the user's manual for the Attune™ Acoustic Focusing Cytometer.

Note: Prepare the Performance Tracking Bead suspension immediately before use. Optimization of cytometer settings for applications using stained biological samples may be required following cytometer setup.

Materials Required but Not Provided

- Disposable 12 × 75-mm tubes or flow tubes or 1.5 mL microcentrifuge tubes
- Vortex mixer, optional
- Attune™ Acoustic Focusing Solution (Cat. no. 4449790) or phosphate buffered saline (PBS) for use as a diluent

Running a New Lot of Beads

Before running a new lot of beads, go to www.appliedbiosystems.com, navigate to the Attune™ Acoustic Focusing Cytometer's product page, and import the lot-specific data file. Refer to the user guide for instructions on how to import the lot-specific information into the Attune™ Cytometric Software. To verify the lot number of the Attune™ Performance Tracking Beads, look at the **first six digits** (L/T) printed on the label.

Preparing Attune® Performance Tracking Beads in Tubes

1. Label a flow tube, polystyrene tube or a 12 × 75 mm tube, or 1.5 mL microcentrifuge tube.
2. Mix the Attune™ Performance Tracking Bead vial by gentle inversion or gentle vortexing.
3. For defining baseline or running daily measurements, prepare the beads suspension by adding the following to the labeled tube:

   1 mL diluent (Attune™ Acoustic Focusing Solution or PBS)

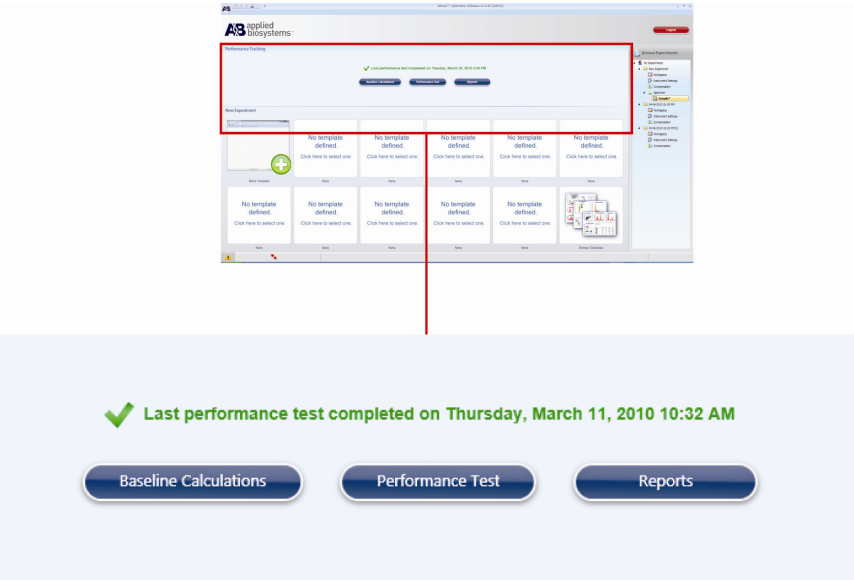
   1 drop of Attune™ Performance Tracking Beads

4. Mix the bead suspension by gentle inversion or vortexing.

   Note: If the beads are not used immediately, store the diluted beads suspension at 2°C to 25°C, **protected from light,** for no more than 4 hours.

5. To access the performance tracking program, log on to the Attune™ Cytometric software

6. After log-in, navigate to the **Performance Tracking** panel located on the upper, half of the workspace.



   Note: After installing the instrument for the first time, a baseline calculation will be performed followed by a performance test.

7. To perform a baseline calculation, the Attune™ Cytometric Software prompts you through the following steps:

   a. Prepare Attune™ Performance Tracking Bead sample for baseline as indicated above.

   b. Check bead lot number on the tube label.

   c. Select the bead lot number from the drop-down menu.

   d. Load the bead sample.

   e. Run the baseline calculation.

8. Once the baseline calculation is complete, the Attune™ Cytometric Software prompts you to complete the performance tracking test (recommended to perform daily to ensure proper performance of the instrument) using the following steps:

   a. Prepare Attune™ Performance Tracking Bead sample for baseline as indicated above.

   b. Check bead lot number on the tube label. If beads from new lot are used, download the correct lot information and perform a new base line calculation as described in Step 7.

   c. Load the bead sample.

   d. Run the performance test.

Exercise 6

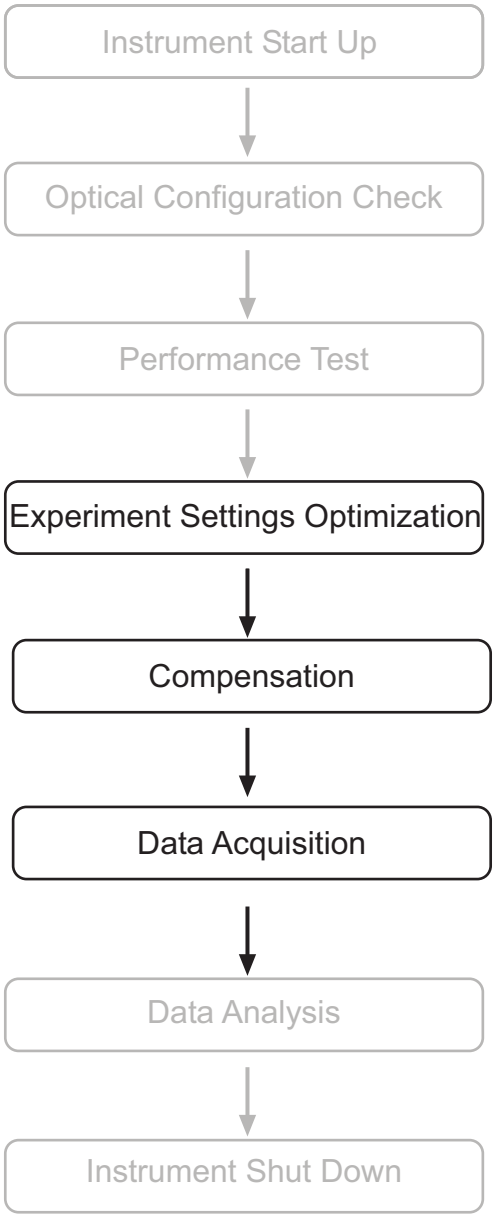
Perform a performance tracking run on the cytometer.

Chapter 6: Data Collection

This chapter describes:

- Experiment Optimization Overview
- Experiment Set up
- Creating Experiment Templates
- Optimizing Instrument Settings
- Adjusting Thresholds
- Performing Compensation
- Acquiring Data
- Recording Data
- Saving Experiment
- Exercise 7

Workflow


Instrument Start Up
Optical Configuration Check
Performance Test
Experiment Settings Optimization
Compensation
Data Acquisition
Data Analysis
Instrument Shut Down

Data collection occurs in several steps:

1. Instrument start up
2. Performance tracking
3. Experiment optimization
4. Compensation
5. Recording Data
6. Data Analysis
7. Instrument shutdown

Experiment Optimization Overview

Before recording data for a sample, perform experiment optimization, which includes PMT voltage optimization, compensation, and threshold settings for each dyes and samples that are used in the experiment. Instrument settings optimization needs to be done by adjusting the position of the populations of interest on scale for scatter and fluorescence parameters.

Each individual experiment needs to have experiment optimization performed or verified prior to data collection.

This chapter demonstrates an experiment optimization practice experiment using fluorescently labeled beads, PeakFlow™ beads. The beads are used to demonstrate the process of performing experiment optimization, but are not to be used as an absolute standard for your experimental conditions.

Experiment Set up

Materials Needed to complete the optimization experiment

- FACS Tubes
- Attune™ Focusing Fluid
- Attune™ Performance Tracking Beads
- PeakFlow™ Blue Beads
- PeakFlow™ Green Beads
- AbC™ anti-mouse Negative beads

Instrument Set Up

1. Start Up the Attune™ Acoustic Focusing Cytometer as described in Chapter 2.
2. Double-click on the Attune™ Cytometric software shortcut from the desktop.
3. Log in as the administrator and enter the password. Click on log in.

4. Ensure the performance test is complete and it passed criteria. If needed, perform Instrument Performance Tracking as described in Chapter 5.
5. Check the optical layout of the instrument to verify that the filters are appropriate for the use of Alexa Fluor® 488, R- PE, Tricolor, APC, Pacific Blue™, Pacific Orange™, and Qdot® 605 dyes.

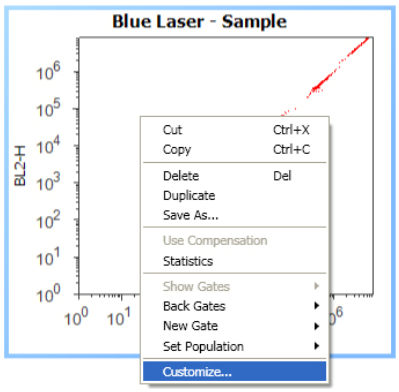
Creating Experiment Templates

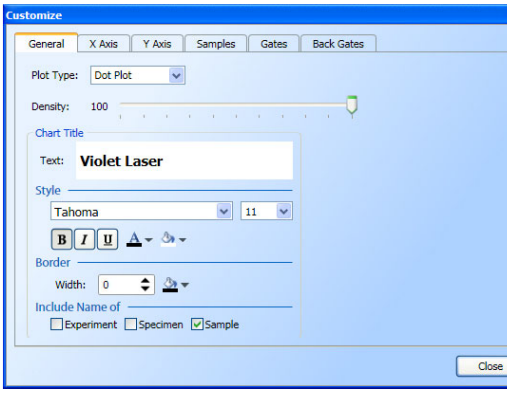
1. Open the training experiment explorer by going to the quick access tool bar and AB symbol.
2. Browse and select **Training Folder**.
3. Right-click on **Data Acquisition Folder**.
4. Select **Duplicate Experiment**. Right-click on Duplicate Experiment and select **Properties**.
5. Rename the Data Acquisition with the desired name.
6. Confirm the following setup:
    - Select **Specimen** by right-clicking and choose **Properties** option.
    - Rename Beads.
    - Select **Sample** by right-clicking on sample.
    - Duplicate sample 3X creating a total of 4 samples.
    - Select each sample and rename it with unstained, blue, green, and mixed by right-clicking on sample and select **Properties**.
    - Open **Experiment** by double-clicking on sample.
    - Create FSC vs. SSC dot plot.
    - Create a BL1 vs. VL1 dot plot.
    - Create a BL1 histogram.
    - Create a VL1 histogram.

Creating Plots

1. To insert a Plot to your Work Area, select the **Specimen** or the **Sample** in the Experiment Explorer, then choose the type of plot from the Insert tab.

2. To change the Parameter and/or the Scale, right-click directly on the plot axis. Alternatively, right-click on the plot and select **Customize** to open the Customize dialog box.





Optimizing Instrument Settings

1. Double-click on the unstained sample to activate the workspace.
2. Install unstained bead sample on the tube lifter.
3. Select standard transit time.
4. Adjust the flow rate to 100 µL/min by adjusting slider bar.
5. Define stop criteria by setting auto-stop to 10,000 events on all events.
6. Click the **Run** button. Events appear in the FSC vs. SSC plot. You can obtain real-time data without saving the data to a file.
7. Adjust the FSC and SSC voltage to place the bead population on scale by sliding the FSC slider bar up or down.
8. Adjust threshold on instrument control panel to remove debris.
9. Adjust the fluorescence channels to place your unlabeled beads in the round to the far, left of all of the dot plots (negative should run around 10^3).
10. Record data from unstained beads.
11. Remove the unstained beads from the tube loader.

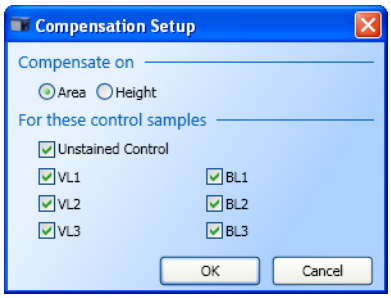
Adjusting Thresholds

A threshold is a way to get rid of unwanted events (i.e., debris before a sample is recorded). Thresholds can be set on a single or dual parameter either on scatter or fluorescence channels.

1. Select **Insert** tab to display the Gating Tools.
2. Select the **FSC vs. SSC** dot plot on the Work Area.
3. Select the appropriate **Gate** from Gating Tools. Gate appears automatically on the selected plot.
4. Drag the **Gate** over the population of interest and adjust its boundaries to include the population.
5. Check settings are fine for all samples. Place mixed bead sample on to the instrument.

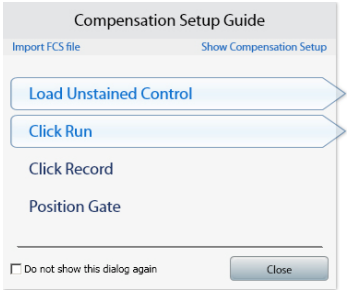
Performing Compensation

1. Click **Compensation** in the Home tab to open up the Compensation Setup dialog box. Alternatively, right-click and select **Compensation Setup** under the same folder that contains your optimized Experiment in the Experiment Explorer.

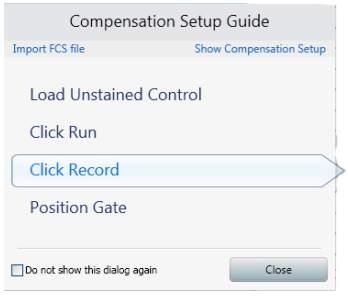


2. Select the type of compensation you want to perform and the channels, and click **OK.**
3. **Compensation Setup Guide** opens and the Workspace is automatically populated with the plots necessary to calculate compensation.

   The Compensation Setup Guide directs you through the steps necessary for calculating compensation for your samples.



4. Install the tube containing the unstained control beads on the sample injection port as prompted by the software.
5. Push up the tube loader to the active position in the sample injection port and click **Run** on the Collection Panel.
6. Wait until the sample equilibrates, and click **Record**.



7. Repeat the process for each of the single-stained controls.
8. After you have calculated the compensation settings, you are ready to run your samples to acquire and record data.

Acquiring Data

1. Select the Sample of interest from the Experiment Explorer panel. Double-click **Sample** to activate it. Workspace displays the plots setup for the Experiment.
2. Enter the collection criteria in the Collection Panel. You can specify the collection criteria as unlimited, or set limits to collection by the number of events for specified gates, total sample volume interrogated, or by elapsed time.
3. Install the tube containing the sample on the sample injection port and lift up the tube loader to the active position.
4. Click **Run**. The events are displayed on the plots as the graphs are being populated. Wait a short time for the sample to equilibrate.

Customizing Plots

1. On the work area, select each individual dot plot one at a time.
2. Right-click on Dot plot and select **Properties**.
3. Rename Axis labels as follows:
   - FSC. Vs. SSC
   - Blue vs. Green
4. Select each individual histogram plot.
5. Right-click on the Histogram plot and select **Customize**.
6. Rename **Axis** labels as follows:
   - Blue 450/40—VL1 axis
   - Green 530/30—BL1 axis

Recording Data

1. Select Blue sample and double-click to activate sample.
2. Install Blue bead sample and press run.
3. Adjust VL1 PMT to make sure beads are on scale and not off scale too high (around 10^5).
4. When on scale, press **Record**.
5. After completion, unload the tube by pushing the tube lifter down. The **Rinse** function automatically begins.
6. Select **Green** Sample and double-click to activate the sample.
7. Install the Green Bead sample and press **Run**.
8. Adjust BL1 PMT to make sure beads are on scale and not off scale too high (around 10^5).
9. When on scale, press **Record**.
10. Unload tube and perform **Rinse**.

11. Double-click and select the sample on experiment explorer.
12. Place mixed sample on the tube loader.
13. Push up the tube loader to the active position.
14. Select **Run** on collection panel; wait for the sample to equilibrate.
15. Select **Record** and acquire data.
16. Click **Stop** to stop the data collection and save the data in a unique FCS file.
17. After recording data, unload the tube.
18. Place the tube of 1 mL deionized water on the tube loader in the active position.

Exercise 7

1. List the Instrument Optimization Steps in order:

_____Define Threshold

_____Calculate Compensation

_____Define Gating Parameters

_____Performance Tracking

_____Adjusting FSC and SSC parameters

_____Create Experiment objects on Workspace

_____Adjust PMT settings

_____Instrument Start- Up

_____Check Optics

_____Record Data

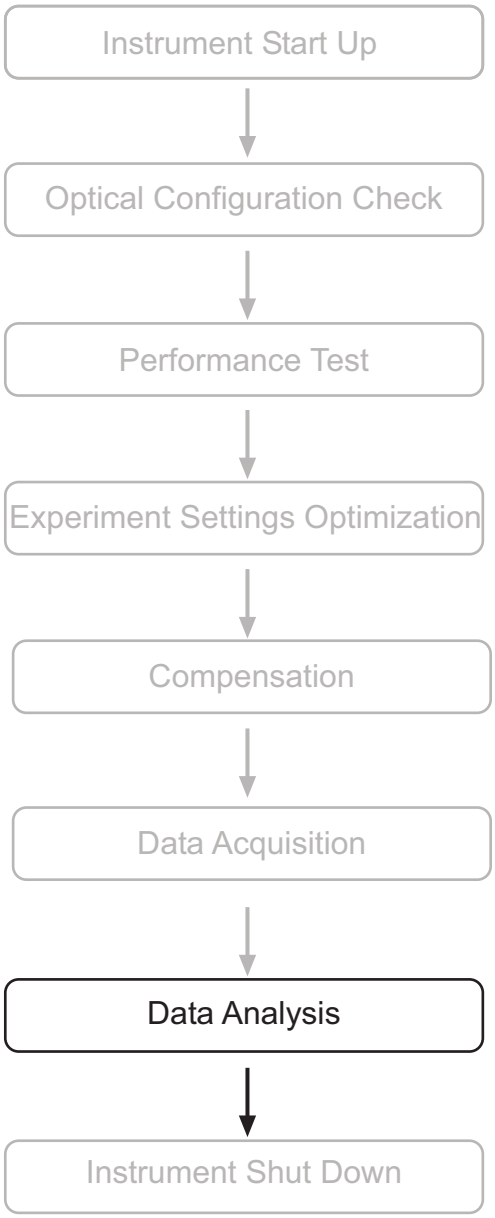
2. Compensation can only be set using automated program (**True** or **False**).
3. It is not an important part of data acquisition to perform instrument performance tracking (**True** or **False**).

Chapter 7: Data Analysis

This chapter describes:

- Types of Data Plots
- Gates and Gating Tools
- Statistical Analysis
- Data Analysis Exercise
- Exercise 8

Workflow

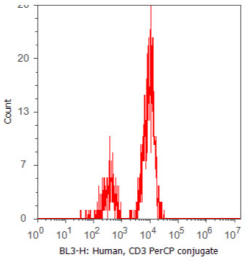

Instrument Start Up
Optical Configuration Check
Performance Test
Experiment Settings Optimization
Compensation
Data Acquisition
Data Analysis
Instrument Shut Down

Analyzing Data is a multi-step process. In the previous chapter we learned about the different plots that we use in flow to acquire data and perform instrument optimization. In this chapter you will learn how to use the software features in which to analyze your data including setting gates, defining gate parameters, and introduce various statistical analysis tools.

Types of Data Plots

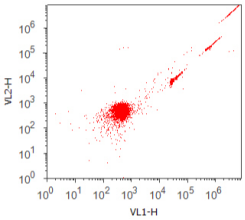
The Attune™ Cytometric Software displays data in *histogram*, *dot*, or *density* plots.

Histogram Plot



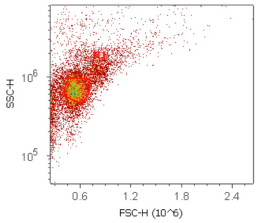
Histogram is a graphical representation of single-parameter data and shows the relative number and distribution of events. In a histogram, the horizontal axis corresponds to the signal intensity of the selected parameter while the vertical axis represents the number of events (count) per channel number.

Dot Plot



A dot plot is a graphical representation of two-parameter data where each axis represents the signal intensity of one parameter. One parameter is displayed on the x-axis and the other parameter is displayed on the y-axis. Each dot in the plot corresponds to one or more events detected above the threshold.

Density Plot



A density plot is a graphical representation of two-parameter data where the colors represent the collection of events with the same intensity and each axis represents the signal intensity of one parameter.

Gates and Gating Tools

Regions and gates are commonly used in data analysis to identify different subsets of populations in multi-color experiments.

Regions are shapes or objects that are drawn around a population of interest on one and two parameter plots.

A **gate** is defined when regions are used to isolate a specific group of cytometric events from a large set of data. Gates can be customized by using Boolean logic (OR, AND, NOT) to link multiple gates together. However, the most popular application is to use gates in sequential order to limit data display and define the parameters for statistical data display.

Creating Gates

1. The Gate ribbon is grayed out until a plot on the work area is selected by clicking on the plot. The plot is highlighted with a blue border to indicate the active plot.
2. Once a plot is activated then the gate ribbon is available.

3. After selecting the appropriate region by clicking the tool, a gate appears on the plot. This region will have a default color as well as a default name, color, and border which can be customized.
4. To create multiple regions, click the tool multiple times to display multiple gates. The types of objects that are available to create regions on two parameter plots are: rectangle gate, oval gate, polygon gates, and quadrant gates.

   Quadrant gates are used to divide a dot or contour plot into four individual populations. Quadrant gates are typically used for sub-setting populations. The objects available for use on a single parameter histogram plots are histogram markers, and Bi-marker gate. Histogram markers are used to select a single range of events in a plot, whereas a Bi-marker gate is used to identify two separate ranges in a histogram plot (i.e., negative vs. positive events).
5. After a population is assigned, it displays in hierarchy under the statistics tab.
6. After the gating parameters are defined, you can customize the gates to create joined, intersected, or inverted gates by setting a customized gate.

Editing Gates

Objects are automatically created using default settings. These objects can then be customized as per the user's preference.

Left-click on the object (object handles appear on the outside of the object) to customize the objects.

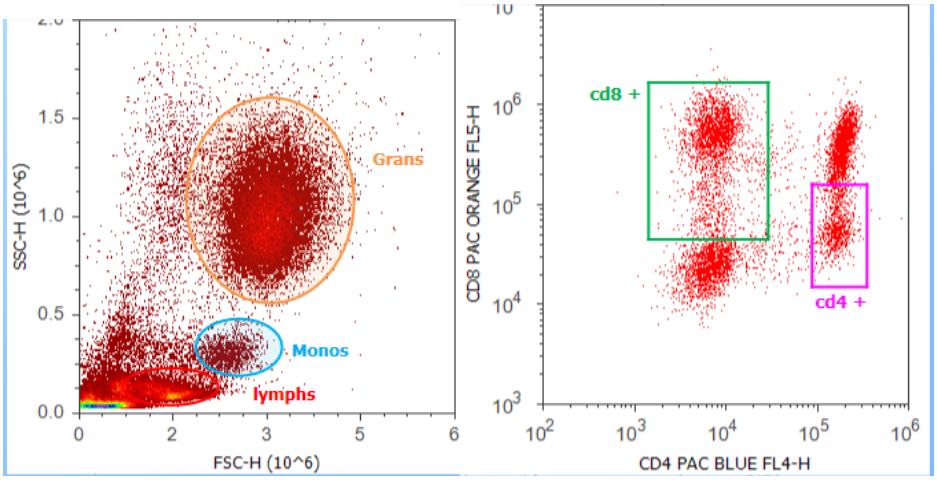
- To move the region, click on the center of the region, then hold the left mouse key down and move the cursor to where you want it on the plot. Release the left mouse.
- To resize the gate, click on one of the handles around the region. Hold the left mouse key down, drag handle in or out, and then release the left mouse key.
- To change formatting of the region, select the region, then right-click on center of region and select **Customize Function**. Under the Customize Function, you can change the name of the region from the default name. The default names for the plots are generated using the region label of R followed by the number order that the object was created.

  Note: If you change the name of the region, the next region created will be the lowest number in order that is not assigned a number. Regions created automatically come highlighted with the default color. The highlight color as well as the opacity can be adjusted using the population option.
- To change the look of the object, right-click on the different options under the Customize Function to change shading and population color.
- To delete an object, select the object, and right-click on the object. Select **Delete**.
- To create a daughter plot of a gate, select **Object**, right-click on object and select create daughter plot. Select the type of plot. The plot created only displays data from the region that was selected.
- To hide gates, select the object, right-click on center of object, and then select **Hide**. The gate and the boundary are now hidden.
- To show the gate, click on the center of the plot, right-click on the plot, and select **Show Gate**. Select the gate to be displayed and gate is automatically displayed.

Hierarchy or Gating Tree

All defined gates are found on a **Gating Tree** under the **Statistics** box. Use this view to show defined populations and to show the relationships between populations for a particular sample tube.

For example, for a normal whole blood sample there are several different cell types that can be identified using immunophenotyping protocols. In a normal whole blood sample, there are at least 3 clearly identifiable populations of cells; lymphocytes, monocytes, and granulocytes based on scatter properties alone (left plot in the figure below). These populations can be further subdivided by viewing the antigen expression on the cells. Lymphocytes are commonly subgated on T cells, B cells, and NK cells. These cells can then be further divided into T-helper cells, T-cytotoxic cells by CD4 and CD8 expression. (right plot in the figure below).



Statistical Tools

There are many different statistics used in the analysis of flow cytometry data. The statistics that are available or can be calculated using the Attune™ Cytometric Software are:

- **Percent Total**: The percentage of a population of the percent of the total events collected.
- **Percent Parent:** The percentage of a population based on the number of events collected in the parent gate.
- **Median (50th percentile):** This is the value that corresponds to the middle item in a ranked list (i.e., sorted by magnitude) of all measurements.
- **Mean:** This is calculated by summing the values of all measurements and then dividing by the number (n) of measurements.
- **Standard Deviation:** The standard deviation is a measure of how spread out your data are. The steps for computing the standard deviation are:

  1. Compute the mean for the data set.
  2. Compute the deviation by subtracting the mean from each value.
  3. Square each individual deviation.

4. Add up the squared deviations.
5. Divide by one less than the sample size.
6. Take the square root.

- **Coefficient of Variation:** Percent coefficient of variation is a measure of peak distribution. The percent coefficient of variation is the standard deviation of the peak divided by the mean channel number of the peak, multiplied by 100.
- **Stain Index:** Mean of positive-Mean of Negative/(2 * SD of the Negative).

Note: The median is robust in that it doesn't necessarily move in response to small numbers of outliers, or to skewing of the tails of a distribution, whereas the mean is tugged by both. One situation where the median is probably the only valid measure is where data pile up at one extreme of measurement, as long as more than 50% of the cells are clear of the sides you get a valid median, but either type of mean will be way off. So if you've got normally distributed data, and want to be able to reflect small changes, use the arithmetic mean, if you've got slightly skewed data use the geometric mean, and if you want a robust indicator use the median. The geometric mean is most useful when data is not normally distributed.

Data Analysis Exercise

1. Start Up the Attune™ Acoustic Focusing Cytometer as described in Chapter 2.
2. Double-click on the Attune™ Cytometric software shortcut from the desktop.
3. Log in as the administrator and enter the password. Click on log in.
4. Ensure performance test is complete and it passed criteria. If needed, perform Instrument Performance Tracking as described in Chapter 5.
5. Check the optical layout of the instrument to verify that the filters are appropriate for the use of Alexa Fluor® 488, R- PE, Tricolor, APC, Pacific Blue™, Pacific Orange™, and Qdot® 605 dyes.
6. Open the Experiment and navigate to the training folder on the experiment explorer.
   a. Select the workspace exercise.
   b. Right-click on **Data analysis exercise folder**.
   c. Duplicate an experiment.
   d. Find the newly created experiment (it may be at the bottom of the experiment explorer).
   e. Rename the experiment by right-clicking on the experiment.
   f. Select **Properties.**
   g. Change the experiment name to Data analysis Experiment-Your name.
7. Go to the Sample and double-click the sample to activate the Workspace.
8. On **FSC vs. SSC dot plot**, create three regions around the lymphocytes, monocytes, and granulocytes. Adjust the gate sizes around the population by clicking on the gate and then using the dots to size the gate tightly around the population of interest.

9. Right-click the region name box, hold the right mouse down and drag the R1, 2, and 3 symbols off of the region so they are easier to see. Rename them by clicking on the Region box and rename lymphs, monos, and grans.
10. Create a histogram plot for the VL1 channel that shows only data from your lymphocyte gate.
    - Right-click in the center of your desired gate, select **Create Daughter Plot**, and then select **Histogram**. Define the parameter of interest to VL1-H and set the scale to log if necessary

    OR

    - From workspace, select **Histogram** plot tool on the insert tab and a plot automatically appears on the workspace. Define the parameter of interest- VL1-H and set the scale to log, if necessary.
11. Using the bi-marker tool, select the negative and positive population.

    Note: Make sure the plot is active by clicking in middle of plot and the outline in light blue. Adjust the bi-marker right-click and hold on the handle in the middle of the plot and slide to capture the population of interest or right-click and hold on the colored portion of the gate and then slide to capture the population.
12. Create a **Statistics** box by clicking on the histogram plot and then selecting the statistics tool on the other ribbon under the insert tab.

    Note: The gate tree is now visible and you can only see the gates under the lymphocyte gate because all other cell populations are eliminated by creating a daughter gate or setting your population to lymphs.
13. Select the VL1 vs. BL1, VL1 vs. BL3, and BL3 vs. BL1 dot plots.
14. Plot to display only data from the lymphocyte gate by doing the following:
    a. Right-click on the center of the plot.
    b. Select **Set Population**- Lymphs.
15. Create a histogram plot for BL1 and BL3 to display only data from the lymphocyte gate.
    - Right-click in the center of your desired gate, select **Create Daughter Plot**, and then select **Histogram**. Define the parameter of interest to BL1H and BL3 H and set the scale to log, if necessary.

    OR

    - From the workspace, select **Histogram** plot tool on the insert tab and a plot automatically appears on the workspace. Define the parameter of interest—BL1 H and BL3 H, and set the scale to log, if necessary.
16. Add histogram markers to BL1 positive cells and right-click on center of BL1 + marker.
17. Create a daughter dot plot VL1 vs. BL3 H.
18. Create rectangle gates around CD8 and CD4 positive populations.
19. Rename regions T cytotoxic for CD8 positive and T helper for CD4 positive regions.
20. Select BL1 histogram and rename CD3 positive cells.
21. Select **Statistic** boxes for both dot plots for newly created VL1 vs. BL3 plot:

a. Right-click on the center of the desired plot and select **Statistics**.

b. Select **Plot of Interest** and use the statistics tool under the other ribbon on the insert contextual tab.

22. Customize the statistics box to show % Total, % of Parent, Event Count, Median, Standard Deviation, and CV.

23. Save workspace by selecting the **Save Disk** icon on the quick access tool bar located at the top left of the screen.

    Note: Once you have saved, the analysis strategy is defined for your experiment and this strategy can be applied to all sequential samples within your experiment.

Exercise 8

1. When creating a daughter plot, why are some of the data points missing?

   _____

   _____

   _____

2. Explain the difference between % Total vs. % Parent.

   _____

   _____

   _____

3. The Hierarchy or Gating Tree is found on the instrument explorer (**True** or **False**).

4. A gate is defined when regions are used to isolate a specific group events from a large set of data (**True** or **False**).

5. Names of regions cannot be changed (**True** or **False**).

6. A bi-marker tool is a good way to identify positive events from negative events (**True** or **False**).

7. Rectangle gates can be used as a gate on histogram plots (**True** or **False**).

8. What statistic is recommended when data is collected in logarithmic mode—Mean or Median?

Chapter 8: Data Management

This chapter describes:

- Options for Maintaining Computer Efficiency
- Importing Experiment
- Exporting Experiment
- Maintain Data Quality Using Routine Maintenance Procedures

Data management is very important but often forgotten in maintaining a properly functioning instrument system. There are many tools to help manage experimental data including: ability to build folders on the experiment explorer to categorize or file day-to-day experimental results, and exporting experiments to a back-up storage device such as an external hard drive, CD, or network drive.

The Attune™ Cytometric software does not generate separate files for instrument settings, experiments, and data. All experimental data is stored within an experiment and is stored in the C drive (hard drive) on your computer. To maximize the efficiency of the computer system, routinely export the FCS files and Experiments to a network drive, external hard drive, or the D drive of your desktop system.

Some of the options for maintaining your computer efficiency are described below.

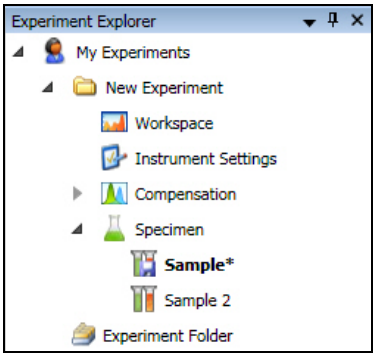
However, there are some maintenance procedures that can help maintain the quality of your data and ensure proper function of the computer:

- Back up your experiments on a regular basis to a secondary storage device.
- Defragment the hard drive of the computer weekly.
- Minimize memory usage by deleting parameters that you do not need (such as only collect parameters in either area or height, not both, unless you need both parameters for a certain application such as cell cycle) when planning the experiments. Also, if the experiment has several samples, consider collecting some of the samples under one experiment and then collecting the rest under a second experiment.
- Save the experiment data after every experimental run.

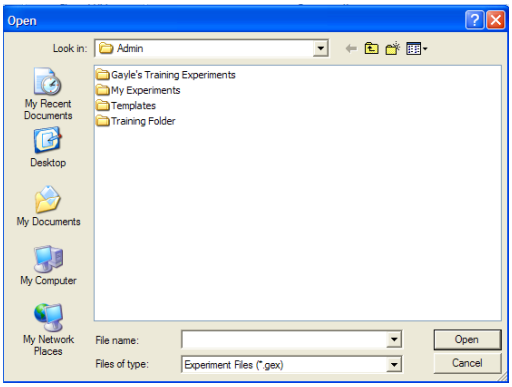
Importing Experiments

It is very easy to import an experiment using the Attune™ Cytometric Software.

1. In the experiment explorer highlight, select **My Experiments** on the experiment hierarchy in the experiment explorer by left-clicking on title.



2. Right-click on **My Experiments**, then choose import experiment to bring up a navigation panel.



3. Navigate to the folder where you have the experiment stored that you want to import.
4. Select the file by left-clicking on it, then select **Open**. Experiment, instrument settings, compensation settings, workspace, and specimens and samples automatically appear at the bottom of the experiment explorer.

Exporting Experiments

You may want to export experiments for long-term data storage, use the experiment template for other experiments, and perform data analysis using other tools.

1. Right-click on the experiment folder on the experiment explorer.
2. Select **Export Experiment**.
3. Navigate to the storage location and select **Save**.

Chapter 9: Advanced Applications—Immunophenotyping

This chapter describes:

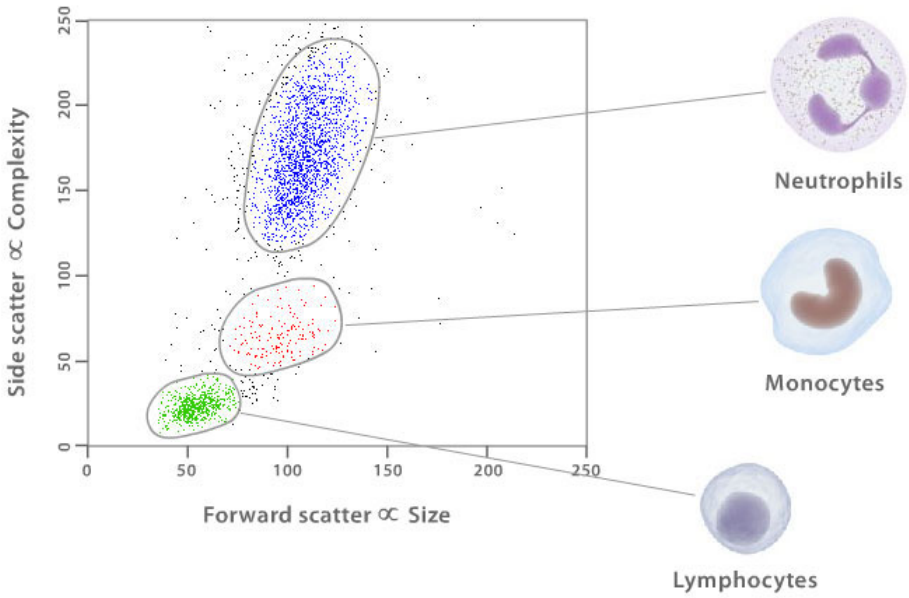
- Immunophenotyping
- List of fluorescent markers for use with the Attune™ Acoustic Focusing Cytometer
- Immunophenotyping Experiment

Immunophenotyping

Immunophenotyping is a technique to detect or study proteins expressed by cells; this technique is used to identify cell type in clinical settings to diagnose various disease states and in laboratory setting to study protein expression and function. Immunophenotyping is one of the major applications of flow cytometry.

Immunophenotyping allows the identification of cells types that cannot be delineated based on morphology alone. Immunophenotyping assays are performed on different types of cells, including but not limited to peripheral blood cells, bone marrow cells, splenocytes, and various cells lines.

An example of human peripheral leukocytes scatter profile (red blood cells were lysed in this assay) is shown below. Although major cell types such as lymphocytes, monocytes, and neutrophils were easily distinguishable, further identification of T cells, B cells, NK cells, and dendritic cells is not clear; these cell populations are found within the lymphocyte gate.



Antigen specific antibodies are used to study different cell types and protein expression/function. The antibodies used for this application are tagged with fluorescent markers that help to identify the protein or antigen of interest. A variety of fluorescent-tagged antibodies for flow cytometry applications are commercially available. In addition to conjugated antibodies, flow cytometry also utilizes various cells stains such as propidium iodide, DAPI, and various SYTOX® stains that distinguish healthy cells from dead or dying cells.

List of Fluorescent Markers

A list of fluorescent markers that can be used in the standard configuration of Attune™ Acoustic Focusing Cytometer are listed below. For details on the labeled antibodies and dead cell stains, visit www.invitrogen.com. Visit the Fluorescence Spectra Viewer on www.invitrogen.com (under support tab) to find the optimal excitation and emission of fluorescent proteins and the optimal filter configuration required.

Note: The instrument can be optimized for other fluorescent markers since the filters can be easily replaced.

Detector	Laser	Dichroic Mirror	Emission Filter	Designed to Detect
BL1	488	555 nm SP	530/30 nm	FITC, Alexa Fluor® 488, GFP, SYTOX® Green, DyeCycle™ Green Stain, CSFE, LIVE/DEAD® Fixable Green Dead Cell Stain
BL2	488	620 nm LP	575/24 nm	PE, PI, YFP, DyeCycle™ Orange Stain
BL3	488	-	640 nm LP	PerCP-Cy®5.5, PerCP, PE-Cy®5 (TRICOLOR®), PE-Cy®5.5, PE-Cy®7, DyeCycle™ Ruby, SYTOX® AADvanced, LIVE/DEAD® Fixable Red Dead Cell Stain and Qdot® 655, Qdot® 705, and Qdot® 800 nanocrystal conjugates
VL1	405	410 nm SP	450/40 nm	Pacific Blue™, DyeCycle™ Violet, LIVE/DEAD® Fixable Violet Dead Cell Stain, CellTrace™ Violet dye, FxCycle™ Violet dye, SYTOX® Blue dye, autofluorescence, Alexa Fluor® 405 dye
VL2	405	575 nm LP	522/30 nm	LIVE/DEAD® Fixable Aqua Dead Cell Stain, Qdot® 565 nanocrystal conjugates
VL3	405	-	603/48 nm	Qdot® 605 nanocrystal conjugates, Pacific Orange™ dye, LIVE/DEAD® Fixable Yellow Dead Cell Stain

Immunophenotyping Experiment

In this experiment, you will perform a multicolor staining experiment using a variety of fluorescent dyes, perform compensation to compensate the signal, and acquire data using Attune™ Acoustic Focusing Cytometer.

Materials Required

- CYTO-TROL™ Control Kit from Beckman Coulter Cat. no. 6604248 (contains lyophilized cells and Reconstitution Buffer)
- Mouse anti-Human CD45, Pacific Orange™ (Cat. no. MHCD4530)
- Mouse anti-Human CD3, PE-Cy®5.5 (Cat. no. MHCD0318)
- Mouse anti-Human CD8, Pacific Blue™ (Cat. no. MHCD0828)
- Mouse anti-Human CD19, R-PE (Cat. no. MHCD1904)
- Mouse anti-Human CD4, Alexa Fluor® 488 (Cat. no. MHCD0420)

Prepare Sample

1. Add 1 mL of Reconstitution Buffer into the vial containing lyophilized cells to reconstitute cells.
2. Mix gently by inverting the vial or placing on a rocker (if available).
3. Allow the cells to stabilize for 10 minutes before use.

Stain Cell Suspension

1. Label tubes as described in the table below:

Label	Description	Assigned Tube No.
Cells Alone	Negative Control (Auto-fluorescence)	
VL1 Comp.	VL1 Channel Compensation Control (Pacific Blue™)	
VL3 Comp.	VL3 Channel Compensation Control (Pacific Orange™)	
BL1 Comp.	BL1 Channel Compensation Control (Alexa Fluor® 488)	
BL2 Comp.	BL2 Channel Compensation Control (R-PE)	
BL3 Comp.	BL3 Channel Compensation Control (PE-Cy®5.5)	
Sample	All conjugates added together	

2. Add 100 µL of cell suspension into the individual tubes.
3. Stain individual tubes with appropriate samples and/or controls. Add 5 µL of antibody conjugates.
4. Incubate the cells for 15 minutes in the dark.
5. After the incubation period, add 1 mL of PBS solution to all tubes and acquire the data on the Attune™ Cytometer as described below.

Data Acquisition:

1. Create a new experiment under the **Experiment Explorer**.
2. Rename the new experiment as "Immunophenotyping Experiment" followed by your initials.
3. Create new samples and required templates.
4. Under **Workspace**, create following plots: FSC versus SSC (Dot Plot); BL1 versus BL2 (Dot Plot); BL2 versus BL3 (Dot Plot); BL3 versus VL1 (Dot Plot); VL3 versus SSC (Dot plot).
5. Use the **Compensation setup** guide to compensate the signal in all channels with the exception of VL2 (use the table in staining section of procedure to find appropriate controls).
6. Create appropriate statistics window(s) for all plots and display the mean values and standard deviations in the statistics window(s).
7. Acquire data.
8. Calculate the Stain Index for following fluorochromes/channels using the formula:
   Stain Index = Mean of positive – Mean of Negative/(2 * SD of the Negative)

Fluorochrome Conjugate	Stain Index Value
R-PE	
Pacific Blue™	
Pacific Orange™	
Alexa Fluor® 488	
PE-Cy®5.5	

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Technical Resources and Support:

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