Attune™ Acoustic Focusing Cytometer Training

Manik Punj
Attune Training
Attune Training Agenda

Section 1
An Introduction to Flow Cytometry

Section 2
An Introduction to Acoustic Focusing
Hydrodynamic Focusing vs. Acoustic Focusing

Section 3
Instrument Systems
Optics, Fluidics, Electronics

Break

Section 4
Performance Tracking

Section 5
Software Overview
Ribbons and Menus
What is Flow Cytometry?

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

FLOW CYTOMETRY measurements are made as cells or particles in suspension pass individually through a flow cytometer instrument

- Performed on single cell suspensions
- Provides discrete measurements from each cell in the sample
- Provides a distribution of the measured characteristics in the sample
Flow Cytometry: What Can I do?

- Flow cytometric measurement records data from single cells.
- Rapid statistical analysis on large numbers of cells are obtained.
- Identifying subpopulations within a heterogeneous population.
- Ability to identify cell populations on multiple characteristics.
- Ideally suited for blood and other cells in suspension.
- Ability to archive data.
- Data format allows post acquisition analysis.
Flow Cytometry Basics

1. Cells in a single profile pass through the flow cell

2. Laser hits individual cell passing through the narrow tube called flow cell.

3. Deflected light hits a series of detectors (PMTs)

4. The signal from detectors are interpreted by a computer

Flow cell figure taken from http://www.med.umich.edu/flowcytometry/training/lessons/lesson1/index.htm
Particle Delivery: Hydrodynamic Focusing

Hydrodynamic core

Laser Cross Sectional Area

-narrow particle focus = narrow distribution
Particle Delivery: Hydrodynamic Focusing

Low Flow Rate

High Flow Rate

Laser Cross Sectional Area
Particle Delivery: Hydrodynamic Focusing

broad particle focus = broad distribution

- Count
- Intensity

- Increase sample input volume = increase flow rate = decrease in pressure difference = increase core diameter
- Particle distributions broadened
- Instrument resolution decreased
- Volumetric sample rates = 25 ul/min – 150 ul/min
Acoustic Focusing:

How does Attune™ Cytometer Differ from Conventional Flow Cytometers?
What is Acoustic Focusing?

A century old phenomenon

Fundamentals of Acoustic Cytometry

Michael Ward,¹ Patrick Turner,² Marc DeJohn,³ and Gregory Kaduchak¹

¹Life Technologies, Eugene, Oregon
²Los Alamos Technical Services, Los Alamos, New Mexico
³Santa Fe Technical Services, Santa Fe, New Mexico

ABSTRACT

Acoustic cytometry is a new technology that replaces or partly replaces hydrodynamic focusing of cells or particles with focusing derived from acoustic radiation pressure forces. It offers new possibilities for improving current flow cytometry assays and creating new ones. To take full advantage of these possibilities, it is necessary to understand the fundamental benefits and limitations of acoustic focusing as employed in flow cytometry analysis, either as a substitute for hydrodynamic focusing or in combination with it. Curr. Protoc. Cytom. 49:1.22.1-1.22.12. © 2009 by John Wiley & Sons, Inc.
Acoustic Focusing

Low Flow Rate

High Flow Rate

Laser Cross Section

Acoustically focused

Piezoelectric Transducer

sheath

sheath

sheath

sheath
Acoustic Focusing Cytometry: Practical Considerations

- Acoustic forces cause cells to line up in the center of the capillary. No sheath flow is necessary for particle alignment.
- Flow rate past laser can be precisely controlled to very slow rates, allowing more fluorescence and scatter signal per particle (better sensitivity)
- Faster sample flow rates and speedy analysis of dilute samples facilitates rare event analysis
Acoustic Focusing

Acoustic focusing off

Acoustic focusing on

Acoustic focusing on (dilute sample)
Acoustically Focused Sample

-narrow particle focus = narrow distribution

Count

Intensity

Laser Cross Section

Acoustic focusing

-sheath

-sheath
Variable Laser Interrogation Times

High Sensitivity
Transit Time = 40 usec
Total Volume = 600 ul/min
Velocity = ~0.5 m/s

Standard Sensitivity
Transit Time = 10 usec
Total Volume = 2400 ul/min
Velocity = ~2 m/s
Controlling Cell Speed Without Hydrodynamic Focus

High sensitivity
100 ul/min
0.5 meters/sec

Standard sensitivity
100 ul/min
2 meters/sec

Detector
Laser
Piezoelectric ultrasonic device
Focusing Solution Manifold
Cells
Transit Modes and Times

- Standard (2400 ul/min total volume)

<table>
<thead>
<tr>
<th>Pre-set Sample Input Rate (in ul/min)*</th>
<th>Focusing Fluid Input Rate (in ul/min)</th>
<th>Focusing Fluid to Sample Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>2375</td>
<td>95:1</td>
</tr>
<tr>
<td>100</td>
<td>2300</td>
<td>23:1</td>
</tr>
<tr>
<td>200</td>
<td>2200</td>
<td>11:1</td>
</tr>
<tr>
<td>500</td>
<td>1900</td>
<td>3.8:1</td>
</tr>
<tr>
<td>1000</td>
<td>1400</td>
<td>1.4:1</td>
</tr>
</tbody>
</table>

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

- High Sensitivity (600 ul/min total volume)

<table>
<thead>
<tr>
<th>Pre-set Sample Input Rate (in ul/min)</th>
<th>Focusing Fluid Input Rate (in ul/min)</th>
<th>Focusing Fluid to Sample Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>575</td>
<td>23:1</td>
</tr>
<tr>
<td>100</td>
<td>500</td>
<td>5:1</td>
</tr>
</tbody>
</table>
Questions ?
Section 2 - Instrument Systems

- **Flow Cytometer** is comprised of 3 subsystems:
  - **Fluidics** - To introduce and focus the cells for interrogation
  - **Optics** - To generate and collect the light signals
  - **Electronics** - To convert the optical signals to proportional electronic signals for computer analysis
Fluidics

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

Three conditions needed for optimal interrogation:

• The stream transporting the particles should pass through the focal point of the laser beam.

• Optimally one particle should move through the laser beam at one time.

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

**Attune Focusing Fluid**—is an isotonic, buffered, azide-free support/carrier reagent for transporting particles through the capillary assembly to the flow cell for laser interrogation. It contains a preservative and detergent designed to minimize bubble formation. Prevents sample from coming into contact with the walls of the flow cell (optical cuvette).

**Attune™ Wash Solution**—is a ready-to-use solution for removing cellular debris and dyes from the fluidics system of the instrument.

**Attune™ Shutdown Solution**—is a 10X solution that prevents bubble formation in the fluidics system of the instrument. Prepare a 1:10 dilution of the shutdown solution in deionized water.

**10% Household Bleach Solution in deionized water (0.5% Sodium hypochlorite)**—decontaminates the fluidics lines. Prepare this solution fresh daily and use during the shutdown procedure.

**Deionized water**—used for diluting Attune™ Shutdown Solution and bleach, as well as for long-term storage of the instrument.
**Fluidics Functions**

- **De-bubble** is a user-initiated function 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.

- **Unclog** function is a user-initiated back flush operation to remove clogs from the sample probe and flow cell.

- **Rinse** flushes system between samples to minimize carryover. This function is run automatically between samples, but it can also be user initiated.

- **Shutdown** is an automated function that decontaminates, cleans, rinses and powers off the cytometer. This mode requires user supplied bleach, Attune™ Wash Solution, and Attune™ Shutdown Fluid.

- **Startup** primes the instrument fluidics with Attune™ Focusing Fluid.

- **Stop** is used to end any running script.

- **Clear Error** is used to erase any error prompts.
Status Indicator Lights

The *Status Indicator Lights* above the sample injection port identify the status of the instrument.

![Status Indicator Lights Diagram]

<table>
<thead>
<tr>
<th>Instrument Cycle</th>
<th>Green</th>
<th>Red</th>
<th>Blue</th>
<th>Yellow</th>
<th>Green</th>
</tr>
</thead>
<tbody>
<tr>
<td>Startup</td>
<td>BLINK</td>
<td>ON/OFF*</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Indicator light blinks for 2 seconds, and then turns off.</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Startup complete</td>
<td>ON</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
</tr>
<tr>
<td>Idle</td>
<td>ON</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
</tr>
<tr>
<td>Run</td>
<td>ON</td>
<td>OFF</td>
<td>OFF</td>
<td>OFF</td>
<td>BLINK*</td>
</tr>
<tr>
<td><em>Indicator light blinks with cycle time proportional to event rate.</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Routine Maintenance

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Shutdown</td>
<td>Daily</td>
</tr>
<tr>
<td>• Visual inspection of sample injection port</td>
<td>Daily</td>
</tr>
<tr>
<td>• Visual inspection of fluidics tanks and connections</td>
<td>Daily</td>
</tr>
<tr>
<td>• Visual inspection of syringe pumps</td>
<td>Daily</td>
</tr>
<tr>
<td>• Computer maintenance</td>
<td>Weekly</td>
</tr>
<tr>
<td>• Optical filter cleaning</td>
<td>Monthly</td>
</tr>
<tr>
<td>• Fluidics maintenance</td>
<td>As needed*</td>
</tr>
<tr>
<td>• Replacing syringes</td>
<td>As needed*</td>
</tr>
<tr>
<td>• Changing focusing fluid filter</td>
<td>As needed*</td>
</tr>
</tbody>
</table>

* The frequency of maintenance depends on how often you run the cytometer. If the Attune is to be unused for a period of time exceeding two weeks, perform the shutdown function using distilled water.
Regular Computer Maintenance Procedures

• Back up your experiments on a regular basis to a secondary storage device

• Defragment the hard drive of the computer weekly

• Minimize memory usage:
  - When planning the experiments remember to delete parameters that you do not need i.e. only collect the parameters needed
Questions?
The image below shows the optics compartment of the Attune™ Acoustic Focusing Cytometer.

- **Dichroic Mirrors**
- **Band Pass Filters**
- **Excitation lasers**
  - 50mW 405nm Violet Laser
  - 20mW 488 Blue Laser
- **Beam shaping optics**
- **Storage for spare collection optics**
- **Collection optics**
- **Collection lens**
- **Recessed PMTs and amplifiers**
Laser Light Scatter

Forward Scattered light (FS) is proportional to cell-surface area or size.

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

**Fluorescence** - is the result of a three stage process in certain molecules called fluorophores, or fluorescent dyes.

**Absorption spectrum** - The wavelength range over which a fluorescent compound can be excited.

**Emission spectrum** - The range of emitted wavelengths of a fluorescent compound, it is a longer wavelength than the absorption wavelength.

**Auto-Fluorescence** - Otherwise know as background fluorescence that originates from endogenous sample constituents or from unbound or nonspecifically bound probes.
### Blue (488 nm laser)

<table>
<thead>
<tr>
<th>Channel</th>
<th>Possible Fluorochromes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL1</td>
<td>FITC, AF488, GFP, CSFE</td>
</tr>
<tr>
<td>BL2</td>
<td>PE, PI, YFP</td>
</tr>
<tr>
<td>BL3</td>
<td>PerCP, PE Tandems, QDot 705 &amp; 800</td>
</tr>
</tbody>
</table>

### Violet (405 nm laser)

<table>
<thead>
<tr>
<th>Channel</th>
<th>Possible Fluorochromes</th>
</tr>
</thead>
<tbody>
<tr>
<td>VL1</td>
<td>Pac Blue, DyeCycle Violet, AF405</td>
</tr>
<tr>
<td>VL2</td>
<td>Fixable Aqua, QDot 525</td>
</tr>
<tr>
<td>VL3</td>
<td>Pacific Orange, Qdot 605</td>
</tr>
</tbody>
</table>
Optical Filters

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)
Band Pass Filters

Long Pass

Short Pass

Dichroic Mirror
Compensation

• Compensation is the process by which we correct for "spillover"

• Every fluorescent molecule emits light with a particular spectrum unique to that molecule

• These emission spectra overlap, in some cases very significantly
Questions?
Electronics

Functions of Electronics:

• Converts detected light signals into electronic signals
• Electronic signals are process by the computer system
• Converts signals from detectors into digital data used for analysis
Electronic Pulse

Volts

Pulse Height

Pulse Area

Pulse Width

Time

(μ Seconds)
Doublet Discrimination

One G0/G1 cell

Two G0/G1 cells (doublet)

One G2/M cell

Laser beam  

Pulse area (integral)  

Pulse width

Doublets

Singlets
Questions?
Instrument Performance Tracking

• Allows you to monitor performance of the instrument

• Critical to ensure accuracy and sensitivity of instrument

• Includes:
  
  • Running the same bead particle set (Attune™ Performance Beads)
  • Monitoring changes in the CV
  • Monitoring changes in mean fluorescent intensity
  • Tracking linearity of instrument
  • Evaluating quantum efficiency and background
  • Sets laser delay
  • Sets the instrument’s performance baseline

• Provides information about all the lasers and detection channels
Attune Performance Tracking Beads

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

• 3mL vial
PN: 4449754
Running Baseline

- Uses performance tracking beads
- CSV file obtained from Applied Biosystems® website
- Performed any time a new lot of Attune™ Performance beads are used
- Performed after any major maintenance on the instrument
- The percent half-peak coefficient of variation (%HPCV) of the brightest bead is recorded
- PMT is adjusted to place the brightest bead at target MFI values, and voltage values for each channel are recorded
- Relative quantum efficiency (rQ) and relative Background (rB) is calculated for each channel
- Linear regression is calculated and recorded
- Laser delay setting is also automatically calculated
### Baseline Calculations Report

**Instrument Name:** Life Technologies Proprietary & Confidential  
**Serial Number:** NA  
**Institution:**  
**Software Version:** 1.0.46  
**Laser Delay:** 338

**Operator:** Administrator  
**Run Date:** 4/27/2010 10:23:58AM  
**Product Name:** 4 Peak Beads  
**Lot Number:** 756080  
**Expiration Date:** 02/11/2013

### Detector Settings

<table>
<thead>
<tr>
<th>Laser</th>
<th>Parameter</th>
<th>Bright Bead Target MFI</th>
<th>Bright Bead Actual MFI</th>
<th>PMT Voltage</th>
<th>Bright Bead %HPCV</th>
<th>Quantum Efficiency (Q)</th>
<th>Background (B)</th>
<th>Lin.Reg.</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Violet</td>
<td>FSC</td>
<td>700000</td>
<td>682198.56</td>
<td>2875</td>
<td>2.01 %</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Criteria Met</td>
</tr>
<tr>
<td>Violet</td>
<td>SSC</td>
<td>700000</td>
<td>698779.94</td>
<td>2600</td>
<td>5.22 %</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Criteria Met</td>
</tr>
<tr>
<td>Blue</td>
<td>BL1</td>
<td>2000000</td>
<td>2039192.38</td>
<td>2250</td>
<td>1.53 %</td>
<td>0.097</td>
<td>185.831</td>
<td>1.000</td>
<td>Criteria Met</td>
</tr>
<tr>
<td>Blue</td>
<td>BL2</td>
<td>2000000</td>
<td>1922056.75</td>
<td>2300</td>
<td>1.28 %</td>
<td>0.314</td>
<td>195.302</td>
<td>1.000</td>
<td>Criteria Met</td>
</tr>
<tr>
<td>Blue</td>
<td>BL3</td>
<td>2000000</td>
<td>1986913.25</td>
<td>2700</td>
<td>1.56 %</td>
<td>0.098</td>
<td>59.31</td>
<td>1.000</td>
<td>Criteria Met</td>
</tr>
<tr>
<td>Violet</td>
<td>VL1</td>
<td>2000000</td>
<td>1884634.50</td>
<td>1400</td>
<td>1.37 %</td>
<td>0.051</td>
<td>4013.693</td>
<td>1.000</td>
<td>Criteria Met</td>
</tr>
<tr>
<td>Violet</td>
<td>VL2</td>
<td>2000000</td>
<td>1929080.88</td>
<td>2275</td>
<td>1.39 %</td>
<td>0.016</td>
<td>2171.233</td>
<td>1.000</td>
<td>Criteria Met</td>
</tr>
<tr>
<td>Violet</td>
<td>VL3</td>
<td>2000000</td>
<td>1917445.13</td>
<td>2350</td>
<td>1.55 %</td>
<td>1.493</td>
<td>38.295</td>
<td>.996</td>
<td>Criteria Met</td>
</tr>
</tbody>
</table>
Running Daily Performance Check

- Performed after baseline values have been defined
- Attune™ Performance Beads are used to run daily performance measurements to track the performance of the cytometer
- Run the performance test at least once per day that the instrument is used
- Determines the voltage required to place the brightest bead in the target MFI, and calculates the delta PMT voltages as compared to the baseline.
- %HPCV of the bright bead is recorded
- Relative quantum efficiency (rQ) and relative Background (rB) is calculated for each channel
- Linear regression is calculated and recorded
- Laser delay setting is also automatically calculated
- Levy-Jennings charts provides a record of %HPCV and PMT voltage to check for shifts and trends
Daily Performance Check
Levey-Jennings Reports

Instrument Name: NA
Serial Number: NA
Institution: NA
Software Version: 1.0.32

Operator: Odyssey Admin
Run Date: 3/16/2010 7:56 AM
Product Name: 4 Peak Beads
Lot Number: 1212
Expiration Date: 1/19/2011

FSC Detector Voltage

FSC Bright Bead %HPCV

SSC Detector Voltage

SSC Bright Bead %HPCV
A calibration curve plot showing limit of detection (LOD), limit of quantification (LOQ), dynamic range, and limit of linearity (LOL).
Software Overview

Main Features:

Tracking and Performance

Experiment Explorer

Collection Panel

Ribbons

Menus
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