Tuning acoustic forces for flow cytometry

THE ATTUNE™ ACOUSTIC FOCUSING CYTOMETER.

Using acoustic forces to precisely align cells is a key feature of the new Applied Biosystems® Attune™ Acoustic Focusing Cytometer from Life Technologies. Applying acoustic focusing to flow cytometry provides an alternative to conventional flow cytometry, which relies on hydrodynamic forces imparted by sheath fluid. Acoustic focusing offers researchers several unique opportunities, including rapid processing of dilute samples and the ability to control the speed of cells as they pass through the instrument.

Conventional flow cytometry vs. acoustic focusing

Manipulating cells in a conventional flow cytometer is accomplished using hydrodynamic forces. A suspension of cells (the sample stream) is injected into the center of a rapidly flowing sheath fluid, and the forces of the surrounding sheath fluid confine the sample stream to a narrow "core" that carries cells through the path of a laser that excites the associated fluorophores and creates a scatter pattern.

Keeping cells within a confined focal point is important for consistent excitation of the associated fluorophores as they pass through the tightly focused laser beam. However, as the sample rate is increased by raising the pressure behind the sample stream, the pressure differential between the sheath stream and the sample stream decreases, and the sample stream "core" widens. A wider sample core results in a broader distribution of cells as they transit through the laser, and thus fewer cells are accurately aligned with the laser focal point. To obtain optimal data from a conventional flow cytometer, with the lowest variability in signal detection, the instrument must be run at the lowest sample rate, which is typically 10–20 µL/min. Higher sample rates result in greater variability and less precise measurements. Acoustic focusing avoids this compromise in data and sample rates by uncoupling cell alignment from sheath flow.

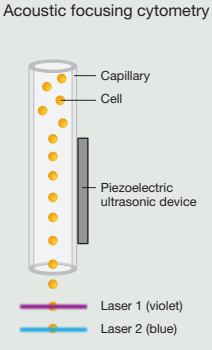

Figure 1. Schematic of an acoustic focusing capillary.

A breakthrough in flow cytometry technology

The effect of acoustic forces on particles was first reported in 1874 by Kundt and Lehmann [1], who observed dust particles levitating in organ pipes. Acoustic forces were more recently used by Curtis and Stephens to separate particles in solution [2–4]. An acoustically driven capillary that applies acoustic radiation pressure to flow cytometry was first described by Kaduchak et al. [5]. This acoustic resonance is driven by a piezoelectric device that precisely aligns cells in the center of a capillary (Figure 1).

The Attune™ Acoustic Focusing Cytometer incorporates many components of a conventional cytometer, including an optical cell for sample interrogation, lasers, and electronics for collecting fluorescence and scatter information. Syringe pumps are used in the Attune™ cytometer to deliver precise volumes of sample, and although delivery of sheath fluid is not required for focusing, the Attune™ cytometer uses a limited amount of fluid to prevent the sample from contacting the optical flow cell.

The resonance frequency required to maintain tight particle alignment in the acoustic focusing capillary is dependent on the capillary diameter and wall thickness. A capillary with an inner diameter of 300 µm, for example, requires 3 MHz for resonance, whereas a 600 µm capillary only requires ~1.5 MHz. Temperature and fluid properties can also affect the resonance of the capillary, so an electronic circuit is required to "tune" the capillary.

Acoustic forces are safe for cells

Unlike the high-energy, lower-frequency, cavitation-inducing forces used to lyse cells in sonication protocols, the acoustic forces used by the Attune™ cytometer are much lower in energy and higher in frequency—similar in frequency to the ultrasound wavelengths commonly used in medical imaging. Thus, the viability of cells is not significantly affected by acoustic focusing.

Does cell size make a difference?

The acoustic force is proportional to the third power of the particle's radius. The Stokes drag force, which resists the acoustic force, is linearly proportional to radius, so the net result is that overall force is proportional to the particle radius squared, and small particles move more slowly than large particles with similar acoustic contrast. Thus, small particles take longer to reach the capillary axis (focus point) than larger particles. This phenomenon suggests that the volumetric sample rate through the capillary should be slowed to focus smaller particles. For subcellular-sized particles, a sample input range similar to that used in conventional cytometers should typically be used (25 µL/min or 100 µL/min on the Attune™ cytometer). For submicron particles, the lowest input rate (25 µL/min) and fluorescence triggers will yield the best data.

Acoustic focusing with a hydrodynamic element

Since cell alignment with the Attune™ cytometer is accomplished by acoustic forces, sheath fluid can be introduced in varying ratios to the sample stream. Unlike traditional flow cytometers, where the volume of sheath fluid is typically up to 1,000 times greater than the sample flow, sheath fluid is used in the Attune™ cytometer at a much lower ratio—between 1:1 and 100:1, approximately. The flow rate of Attune™ Focusing Fluid (effectively a "sheath" fluid) automatically adjusts with the sample input rate to maintain a constant cell velocity for the wide range of sample throughput volumes (25 µL/min to 1,000 µL/min). For dim, low-background samples such as low-expression fluorescent proteins, researchers can reduce cell velocity and increase the number of photons collected by switching to high-sensitivity mode. This reduces the ratio of Attune™ Focusing Fluid to sample for the 25 µL/min and 100 µL/min sample input rates, decreasing the overall fluid velocity through the optical flow cell.

In addition, Attune™ Focusing Fluid performs the critical role of keeping the walls of the optical chamber clean by preventing sticky proteins, biomolecules, and reagents from making contact. →

The physics behind the design

Acoustic forces on a particle in medium are described in the following equation, where a = particle radius; β_0 = compressibility of the surrounding fluid in the absence of particles; ρ_0 = density of the surrounding fluid; p = pressure; and v = velocity of the acoustic field.

$$U = \frac{4}{3}\pi a^3 \left[\left(\beta_0 \frac{\langle p^2 \rangle}{2} \right) f_1 - \frac{3}{2} \left(\frac{\rho_0 \langle v^2 \rangle}{2} \right) f_2 \right]$$

The time-averaged quantity is bracketed. Terms f_1 and f_2 determine how the mechanical properties of the particle differ from the medium, where:

$$f_1 = 1 - \frac{\beta_p}{\beta_0}$$

$$f_2 = \frac{2(\rho_p - \rho_0)}{2(\rho_p + \rho_0)}$$

The force acting on the particle is related to the gradient of the force potential (U) as follows: $F = -\nabla U$. Under acousatic forces, particles localize at a stable equilibrium where the potential U is at a minimum. The acoustic contrast of a particle is determined by the density and compressibility differences between it and the surrounding medium. The relative magnitudes of f_1 and f_2 determine the magnitude and the direction of the radiation force. For example, if a particle and the surrounding medium share the same density, then f_2 is zero and the acoustic contrast is due only to compressibility differences (f_1). If both f_1 and f_2 equal zero, then no acoustic contrast exists.

Cells inside the acoustic focusing capillary of the Attune™ cytometer experience a "standing wave" force that transports them to the minimum force potential at the axial center of the capillary. Nearly all particles and cells of biological origin have a positive acoustic contrast in aqueous buffer. A few materials, such as fat globules and air bubbles, carry a negative acoustic contrast with surrounding aqueous medium and migrate toward the walls of the capillary.

Advantages of the Attune™ cytometer

A fundamental difference between the Attune™ Acoustic Focusing Cytometer and conventional cytometers is that focusing cells is largely independent of the sample input rate, enabling cells to be tightly focused regardless of the sample-to-sheath ratio. This, in turn, allows cell velocity to be slowed to collect more photons for high-precision analysis at unprecedented volumetric sample throughput.

By uncoupling cell alignment from hydrodynamic forces and sheath flow, the Attune™ cytometer allows rapid detection of rare events. The instrument is designed to collect up to 20,000,000 events per run, enabling detection of those rarest of rare events. Because there is minimal variation in results regardless of sample throughput rate, the Attune™ cytometer is ideal for detecting cell proliferation, where it is critical to precisely detect differences in fluorescence intensity between multiple cell populations (Figure 2). The Attune™ cytometer also provides clear resolution of difficult-to-detect signals—for example, from dim antigens such as ZAP-70—to help facilitate cell signaling studies.

Volumetric throughput—achieving incredible sample rates

The Attune™ cytometer achieves sample throughput at rates nearly 10 times faster than other cytometers—up to 1,000 µL per minute. However, like all cytometers designed to analyze a single cell at a time, avoiding coincidence (the entry of two or more cells at once into the event window) limits the sample rate at any concentration. As sample input increases with concentration, the probability of coincidence increases, and sample concentration may need to be adjusted. However, the ability to run very dilute samples at high sample rates provides some clear advantages. This is particularly useful for samples that are inherently dilute or that must be diluted to avoid aggregation.

This feature also allows researchers to intentionally dilute samples to avoid washing by centrifugation. In the case of very low amounts of available sample, dilution to 1 mL or more can help preserve the sample while not significantly affecting data acquisition time. For example, a sample volume of 5 µL diluted 800-fold to a final volume of 4 mL can be processed in a few minutes at a run rate of 1 mL/min. Diluting a sample to this level prior to analysis with the Attune™ cytometer reduces background more than does a single round of centrifugation and can minimize or eliminate altogether the need for washing (and subsequent sample loss). For whole blood samples, this technique can be combined with a fluorescence trigger such as CD45 to perform sensitive, no-lyse, no-wash immunophenotyping without the variability and cell loss or degradation common to lysis and centrifugation sample prep.

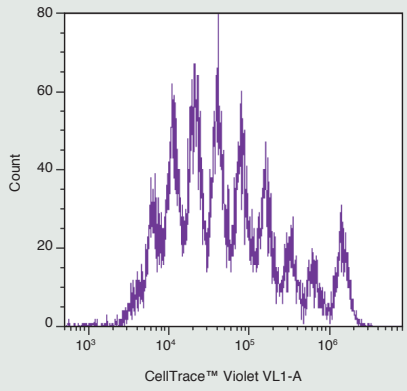

Figure 2. Ten cell divisions identified with the Attune™ Acoustic Focusing Cytometer and the Molecular Probes® CellTrace™ Violet Cell Proliferation Kit. Human peripheral blood mononuclear cells were isolated from whole blood, stained with the CellTrace™ Violet Cell Proliferation Kit, and stimulated to proliferate in culture. Cells were then analyzed on the Attune™ cytometer at a flow rate of 25 µL/min. The histogram shows fluorescence intensity, with each peak representing one subsequent generation of proliferating cells.

The future of acoustic focusing cytometry

Using acoustic forces to precisely align cells provides less signal variability and better data clarity. Acoustic focusing cytometry has the potential to redefine the performance standards for flow cytometry and open new applications for this technology. Life Technologies is committed to advancing acoustic focusing technology for cellular analysis and realizing its full potential through continued innovation. Learn more, and view the new acoustic focusing tutorial, at **www.invitrogen.com/bp63**. ■

References

1. Kundt A, Lehmann O (1874) *Annalen der Physik und Chemie (Poggendorff's Annalen)* 153:1–11.
2. Curtis HW, Stephans EJ (1982) *IBM Technical Disclosure Bulletin* 25(1).
3. Yasuda K, Haupt SS, Unemura S (1997) *J Acoust Soc Am* 102:642–645.
4. Jonsson H, Nilsson A, Petersson F et al. (2005) *Perfusion* 20:39–43.
5. Kaduchak G, Goddard G, Salzman G et al. (2008) US Patent 7,340,957.

Product	Quantity	P/N*
Attune™ Acoustic Focusing Cytometer	1 each	4445315
Attune™ Focusing Fluid, 1X solution	1 L	4449790
Attune™ Focusing Fluid, 10X solution	1 L	4449792
Attune™ Focusing Fluid, 1X solution	6 x 1 L	4449791
Attune™ Wash Solution	1 each	4449755
Attune™ 10X Shutdown Solution	1 each	4454955
Attune™ Performance Tracking Beads (5×10^6 beads/mL)	1 each	4449754

* These products can be ordered from **www.appliedbiosystems.com**.