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Detection of phosphoproteins using the Attune® Acoustic Focusing Cytometer

The study of mitogen-activated protein kinase (MAPK) is important for investigations of human diseases such as cancer. MAPK signaling cascades play important roles in the critical decision processes within the cell, including cellular responses to environmental stimuli and disease progression [1]. MAPKs regulate diverse cellular programs including embryogenesis, proliferation, differentiation, and apoptosis based on cues derived from the cell surface and on the metabolic and environmental state of the cell [2]. Multiparameter flow cytometry provides an important tool for dissecting signaling pathways in cell populations using intracellular staining with fluorescent antibodies against phosphorylation site–specific proteins. While reagents and techniques aimed at phosphoprotein-specific detection have progressed, the signals that result from these experiments are usually dim and difficult to distinguish. Advancements in instrumentation using acoustic focusing allow improved detection of dim signals. The Attune® Acoustic Focusing Cytometer employs high-frequency sound waves to maintain a tightly focused sample stream, allowing greater precision at the laser interrogation point. By using the High Sensitive transit time setting to slow the sample stream, longer laser interrogation time is permitted, which increases the sensitivity of detection.

Here we chose three phospho-proteins, Akt, Erk1/2, and p38, to demonstrate an ideal research application for the Attune® Acoustic Focusing Cytometer, which enables the detection of dim signals through highly sensitive and precise data-gathering capabilities.

Basic protocol

Materials

- Jurkat T cell leukemia cells (ATCC # TIB-152)
- Gibco® 1640 RPMI medium + 10% FBS (Invitrogen Cat. No.16000)
- LY294002 (Cell Signaling Technology)
- Phorbol 12-myristate 13-acetate (PMA) (Sigma)
- Ionomycin, calcium salt (Invitrogen Cat. No. I24222)
- Anisomycin, from *Streptomyces griseolus* (Sigma)
- Phosphate-buffered saline with 1% bovine serum albumin (PBS + 1% BSA)
- Methanol-free formaldehyde (Polysciences, Inc.)
- Methanol (Baker)
- 1% saponin (Sigma) in PBS
- Akt Alexa Fluor® 488 (Cell Signaling Technology)
- Erk1/2 Alexa Fluor® 488 (Cell Signaling Technology)
- p38 Alexa Fluor® 488 (Cell Signaling Technology)
- Attune® Acoustic Focusing Cytometer (Applied Biosystems Cat. No. 4445315)
- BD™ LSR II flow cytometer (BD Biosciences)
- BD FACSCalibur™ flow cytometer (BD Biosciences)
- FlowJo software (Treestar, Inc.)

Staining and analyzing cells

Jurkat T cell leukemia cells, adjusted to 1 x 10^6 cells/mL in 1640 RPMI medium + 10% FBS, were either inhibited with LY294002 (a selective inhibitor of phosphatidylinositol 3-kinase (PI3K)) or stimulated with PMA/ionomycin or anisomycin for 30 min at 37°C, 5% CO_2. Cells were washed twice with ice-cold PBS + 1% BSA and fixed for 10 min at 37°C with methanol-free formaldehyde at a final concentration of 4%. After fixation, the cells were washed once using ice-cold PBS + 1% BSA, then permeabilized on ice for 30 min using ice-cold methanol at a final concentration of 90%. Following permeabilization, the cells were washed and resuspended in 1% saponin/PBS and stained with the respective phosphospecific antibody for 30 min at room temperature, then washed and resuspended to 1 mL in 1% saponin/PBS. Samples were acquired on an Attune® Acoustic Focusing Cytometer and on a BD™ LSR II and/or BD FACSCalibur™ flow cytometer, using low (12 µL/min) and high (60 µL/min) flow rates, each using a 488 nm laser with a 530/30 nm bandpass filter. The main population of cells, excluding debris, was gated, and 10,000 events were acquired at each of seven collection rates ranging from 25 µL/min to 1,000 µL/min, using Standard and High Sensitive modes on the Attune® Acoustic Cytometer. Single-color histograms were generated, and sample analysis with overlay plots was performed using the Attune® Cytometric Software. The samples collected on the BD™ LSR II and BD FACSCalibur™ flow cytometers were analyzed and plot overlays of the BD™ LSR II data were derived using FlowJo software. The stain index (SI) was calculated for each phosphoprotein using the untreated/stained sample and the treated/stained sample. The SI is defined as the difference between the positive and negative peaks, divided by 2 times the standard deviation of the negative peak.

Results and conclusion

The Attune® Acoustic Focusing Cytometer was able to maintain highly sensitive detection of Akt in LY294002-treated cells at flow rates much higher than those used with traditional hydrodynamic focusing instruments (SI = 2.5 at 1,000 µL/min, compared to SI = 1.9 for 12 µL/min). SI values for all flow rates on the Attune® cytometer were superior to the SI values for the slow flow rates on both of the conventional instruments (Figure 1). Similar results were obtained for Erk1/2 detection in PMA/ionomycin-treated cells (Figure 2) and p38 detection in anisomycin-treated cells (Figure 3).

The Attune® Acoustic Focusing Cytometer allows consistent phosphoprotein detection at all collection rates. We illustrate the strength of the Attune® Acoustic Focusing Cytometer to detect and separate the significant differences in phosphorylation states of target proteins in stimulated vs. unstimulated cell populations. While the High Sensitive rate delivers the best separation between positive and negative populations, the higher collection rates confer excellent results as well. Acoustic focusing enables better detection of dim signals than does conventional hydrodynamic focusing.

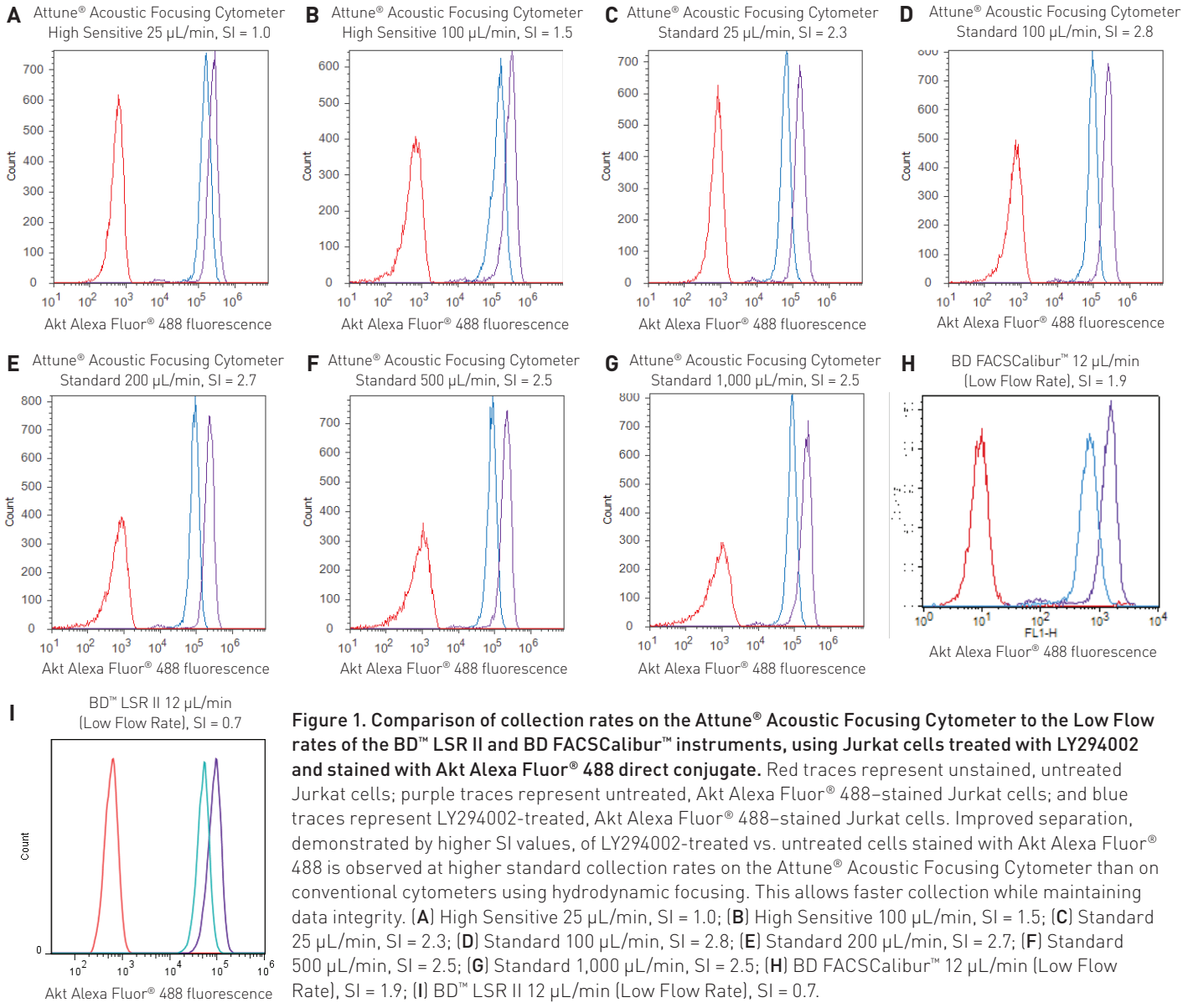
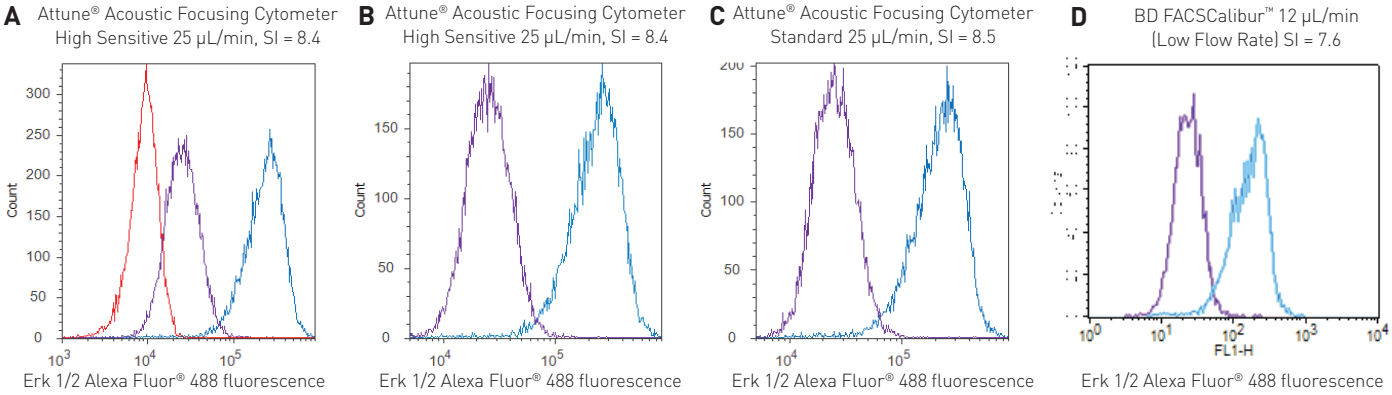


Figure 1. Comparison of collection rates on the Attune® Acoustic Focusing Cytometer to the Low Flow rates of the BD™ LSR II and BD FACSCalibur™ instruments, using Jurkat cells treated with LY294002 and stained with Akt Alexa Fluor® 488 direct conjugate. Red traces represent unstained, untreated Jurkat cells; purple traces represent untreated, Akt Alexa Fluor® 488–stained Jurkat cells; and blue traces represent LY294002-treated, Akt Alexa Fluor® 488–stained Jurkat cells. Improved separation, demonstrated by higher SI values, of LY294002-treated vs. untreated cells stained with Akt Alexa Fluor® 488 is observed at higher standard collection rates on the Attune® Acoustic Focusing Cytometer than on conventional cytometers using hydrodynamic focusing. This allows faster collection while maintaining data integrity. (**A**) High Sensitive 25 µL/min, SI = 1.0; (**B**) High Sensitive 100 µL/min, SI = 1.5; (**C**) Standard 25 µL/min, SI = 2.3; (**D**) Standard 100 µL/min, SI = 2.8; (**E**) Standard 200 µL/min, SI = 2.7; (**F**) Standard 500 µL/min, SI = 2.5; (**G**) Standard 1,000 µL/min, SI = 2.5; (**H**) BD FACSCalibur™ 12 µL/min (Low Flow Rate), SI = 1.9; (**I**) BD™ LSR II 12 µL/min (Low Flow Rate), SI = 0.7.



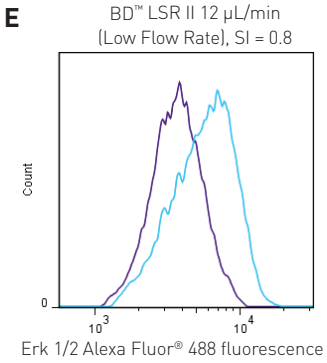
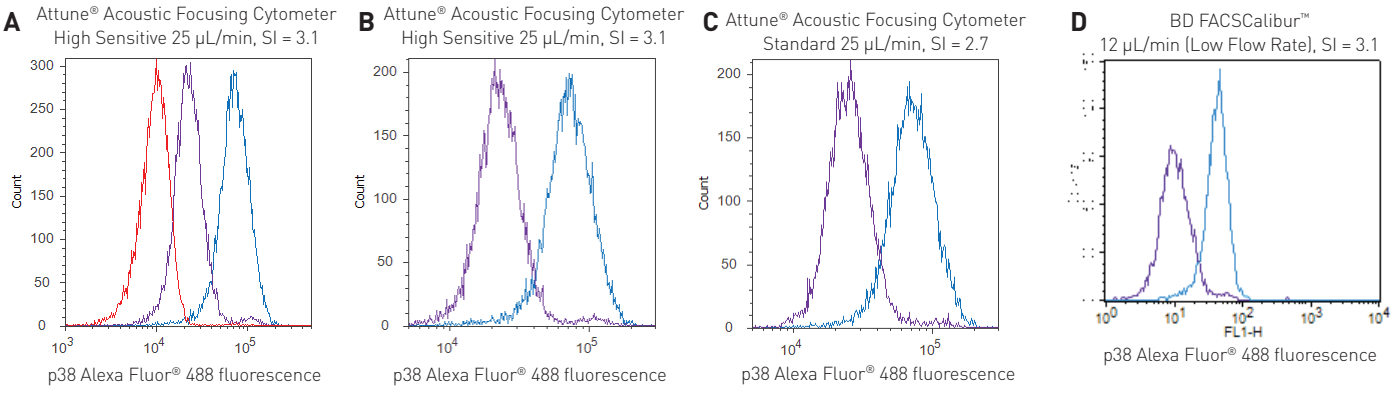


Figure 2. Comparison of High Sensitive and Standard transit times (using 25 µL/min sample injection rate) on the Attune® Acoustic Focusing Cytometer to the Low Flow rates (12 µL/min) of the BD™ LSR II and BD FACSCalibur™ instruments, using Jurkat cells treated with PMA/ionomycin and stained with Erk1/2 Alexa Fluor® 488 direct conjugate. Purple traces represent untreated, Erk1/2 Alexa Fluor® 488–stained Jurkat cells; blue traces represent PMA/ionomycin-treated, Erk1/2 Alexa Fluor® 488–stained Jurkat cells. The Attune® Acoustic Focusing Cytometer demonstrates improved separation of low-expressed proteins using the High Sensitive mode, compared to the conventional instruments using hydrodynamic focusing. (**A**) High Sensitive 25 µL/min, showing unstained, untreated Jurkat cells for reference (red trace), SI = 8.4; (**B**) High Sensitive 25 µL/min (unstained cells not shown), SI = 8.4; (**C**) Standard 25 µL/min (unstained cells not shown), SI = 8.5; (**D**) BD FACSCalibur™ 12 µL/min (Low Flow Rate) (unstained cells not shown), SI = 7.6; (**E**) BD™ LSR II 12 µL/min (Low Flow Rate) (unstained cells not shown), SI = 0.8.



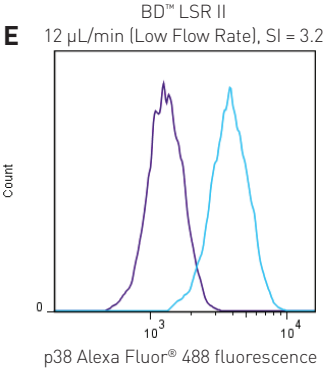


Figure 3. Comparison of High Sensitive and Standard transit times (using 25 µL/min sample injection rate) on the Attune® Acoustic Focusing Cytometer to the Low Flow rates (12 µL/min) of the BD™ LSR II and BD FACSCalibur™ instruments, using Jurkat cells treated with anisomycin and stained with p38 Alexa Fluor® 488 direct conjugate. Purple traces represent untreated, p38 Alexa Fluor® 488–stained Jurkat cells; blue traces represent anisomycin-treated, p38 Alexa Fluor® 488–stained Jurkat cells. The Attune® Acoustic Focusing Cytometer demonstrates improved separation of low-expressed proteins using the High Sensitive mode, compared to the conventional instruments using hydrodynamic focusing. (**A**) High Sensitive 25 µL/min, showing unstained, untreated Jurkat cells for reference (red trace), SI = 3.1; (**B**) High Sensitive 25 µL/min (unstained cells not shown), SI = 3.1; (**C**) Standard 25 µL/min (unstained cells not shown), SI = 2.7; (**D**) BD FACSCalibur™ 12 µL/min (Low Flow Rate) (unstained cells not shown), SI = 3.1; (**E**) BD™ LSR II 12 µL/min (Low Flow Rate) (unstained cells not shown), SI = 3.2.

References

1. Junttila MR, Li SP, Westermarck J (2008) Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. *FASEB J* 22(4):954–965.
2. Raman M, Chen W, Cobb MH (2007) Differential regulation and properties of MAPKs. *Oncogene* 26(22):3100–3112.

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