Application of Acoustic Cytometry in Microbiology

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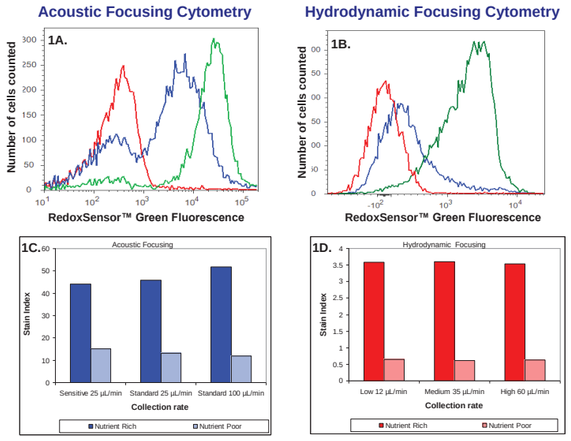
Background: In recent years the application of flow cytometry to the study of various microbiological phenomena has increased, but still represents an under-utilized application in the field. Traditional flow cytometers utilize high velocity, high volumetric sheath fluid to focus particles before laser interrogation. Acoustic focusing concentrates the cells in the center of the fluid with sound energy allowing for flexibility in the sample concentration analyzed. This separates the alignment of cells from the particle flow rate and allows for greater interrogation times and photon collection from inherently low fluorescence particles.

Methods: In the current study we have examined the application of acoustic cytometry to study bacterial physiology to quantitatively determine culture viability and to compare metabolic potential in bacterial populations in nutrient-limiting and nutrient-rich samples. Similarly, acoustic cytometry was used to detect and directly determine cell counts of low abundance picoplankton poulations of *Prochlorococcus* sp. and *Synnechococcus* sp. from marine environmental samples.

Results: Determination of culture viability using traditional hydrodynamic focusing cytometry versus acoustic focusing cytometry yielded comparable results. Metabolic potential, as indicated by changes in staining by a fluorogenic redox indicator dye, was decreased in nutrient-limiting conditions and was detected similarly using the two cytometric methods. Excitation of endogenous fluorescent proteins in *Prochlorococcus* sp. and *Synnechococcus* sp. was used to readily detect and directly enumerate cyanobacterial cells from Surf (5 m) and Deep Chlorophyll Minimum (125 m) depths by acoustic focusing cytometry.

Conclusion: Similar results for culture viability and metabolic potential were found using acoustic focusing cytometry and hydrodynamic focusing cytometry, highlighting the use of this technology in microbiology. Direct enumeration of marine, photosynthetic picoplankton populations was demonstrated. Using 405 nm excitation of the endogenous divinyl chlorophyll *a* combined with the increased interrogation time and photon collection from acoustic focusing cytometry, detailed separation of *Prochlorococcus* sp. in the Surf sample was accomplished.

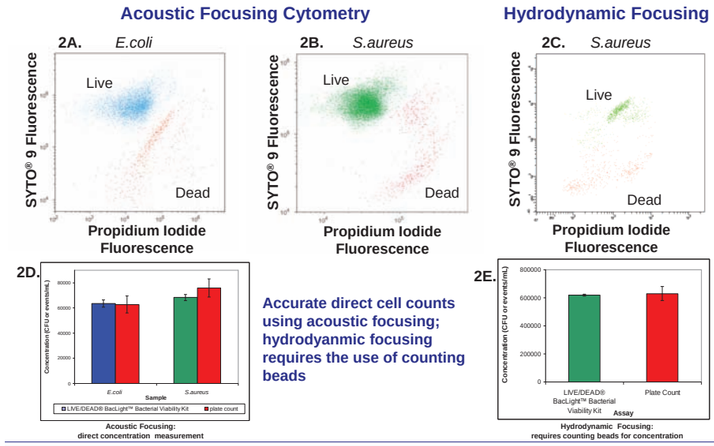
Figure 1. Use of flow cytometry to measure metabolic potential within a bacterial sample



Similar detection of redox potential using acoustic focusing cytometry and hydrodynamic focusing cytometry is observed

Figure 1. Analysis of relative cell vitality within a bacterial culture using flow cytometry. Measurement of oxidation-reduction activity within a bacterial cell is a good indicator of cell vitality because bacterial oxidases and reductases play key functions in the electron transport chain, catabolic and anabolic pathways, and xenobiotic compound catabolism. *Bac*Light™ RedoxSensor™ Green will produce a bright fluorescent green signal in actively respiring cells. *E. coli* cells were grown in nutrient-rich conditions (rich media, Luria Bertani broth, LB) or in nutrient-poor conditions (minimal media) before staining with the *Bac*Light™ RedoxSensor™ Green Vitality Kit (Invitrogen). Stained cells were analyzed using both an acoustic focusing cytometer and a hydrodynamic focusing cytometer. Both cytometers were equipped with 488 nm laser for *Bac*Light™ RedoxSensor™ Green excitation and a 530/30 bandpass filter for fluorescence emission detection. **1A, 1B**. Fluorescence histogram overlay indicating greater redox potential (as indicated by greater RedoxSensor™ Green fluorescence) in cells grown in rich media (green) compared to cells grown in nutrient-poor media (blue); unstained cells are shown in red. Samples were analyzed at a collection rate of Sensitive 25 µL/min on the Attune® instrument (**1A**) or analyzed on the "low" setting of a hydrodynamic focusing cytometer (~12µL/min, **1B**). **1C, 1D**. Relative culture vitality, as indicated by increased stain index, was calculated for cells grown in nutrient-rich or nutrient-poor conditions. Stain index was calculated by dividing the difference between the median fluorescence signal of the positive and unstained populations by twice the standard deviation of the unstained population. Stain index is reported for samples run at multiple collection rates on both cytometers. Cell vitality is greater in cells grown in rich media as compared to cells grown in nutrient poor media and is detected similarly using either acoustic focusing cytometry (**3C**),or hydrodynamic focusing cytometry (**1D**).

Figure 2. Bacterial culture concentration determined using flow cytometry



Accurate direct cell counts using acoustic focusing; hydrodyanmic focusing requires the use of counting beads

Figure 2. Analysis of culture concentration of a gram-negative or gram-positive bacterial culture using flow cytometry. *E.coli* (gram-negative) and *S.aureus* (gram-positive) cultures were grown and cells were stained and analyzed according to protocols listed in the LIVE/DEAD® *Bac*Light™ Viability Kit (Invitrogen) for (**2A**) *E.coli* and (**2B, 2C**) *S.aureus*. Culture concentration was determined for both organisms analyzed using either cytometry method and compared to viable cell concentration as determined using traditional plate count methods. **2D**. Using the Attune® Acoustic Focusing Cytometer software *E.coli* and *S.aureus* concentration were determined to be 6.37 +/- 0.29 x 10^4 events/mL and 6.83 +/- 0.25 x 10^4 events/mL, respectively. Similarly, concentration determined by plating on Luria Bertani (LB) agar was 6.28 +/- 0.67 x 10^4 CFU/mL for *E.coli* and 7.60 +/- 0.72 x 10^4 CFU/mL for *S.aureus*. No significant difference was detected between culture concentration determined by cytometry or by plate count methods. **2E**. Using a hydrodynamic focusing cytometer, counting beads of known concentration were included in the sample and used to determine culture concentration. Culture concentration determined using hydrodynamic focusing cytometry was determined to be 6.18 +/- 0.06 x 10^5 cells/mL as compared to 6.30 +/- 0.5 x 10^5 CFU/mL using the plate count method.

Figure 3. Detection of low abundance picoplankton ppopulations

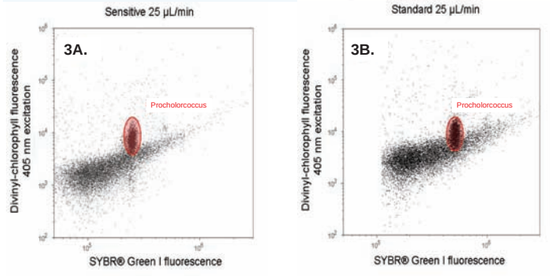


Figure 3. Oligotrophic Station ALOHA surface water sample analyzed with Sensitive and Standard transit times (particle flow rates). Sensitive 25 µL /min (A) allows for better separation of the inherently dim red fluorescent (y-axis) *Prochlorococcus* spp. populations from the remaining SYBR® Green I stained cells as compared Standard 25 µL /min (B). Slowing the particle flow rate can increase the laser interrogation and photon collection times, enabling the detection of very dim populations.

Simplifying photosynthetic picoplankton population detection with the Attune® Acoustic Focusing Cytometer

Analysis of marine, photosynthetic picoplankton is routinely done using flow cytometery, although this testing has presented some challenges. The excitation of the intrinsically fluorescent photosynthetic picoplankton has conventionally been performed using a 488 nm laser, although this excitation is not optimal for the divinyl-chlorophyll containing *Prochlorococcus* spp. and violet 405 nm provides more efficient excitation. Conventional cytometers utilize large sheath to sample flow rates to hydrodynamically focus particles. In contrast, the Attune® Acoustic Focusing Cytometer uses standing sound waves to focus particles and requires significantly lower sheath fluid flow rates. The Sensitive mode on the Attune® further reduces the instrument sheath flow rate, thereby slowing the particle velocity. By slowing the particle velocity, the researcher can increase the laser interrogation and photon collection times for dim populations (e.g. the inherently low fluorescent *Prochlorococcus* spp. from oligotrophic surface water samples). The 405 nm laser enables better excitation of divinyl-chlorophylls from *Prochlorococcus* spp. and enhances separation of distinct picoplankton populations from background signal. Syringe driven sample fluidics permits direct counting of cells in a given population. Combining syringe driven sample handling with excitation of divinyl-chlorophylls with the 405 nm laser allows for direct enumeration of *Prochlorococcus* spp. in SYBR® Green I stained samples. Figure 3A and 3B demonstrates the utility of combining a slow particle flow rate and excitation of divinyl-chlorophylls with the 405 nm laser.

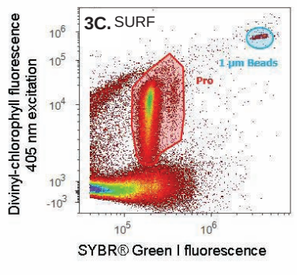


Figure 3C. SYBR® Green I fluorescence (x-axis) versus divinyl-chlorophyll fluorescence from 405 nm excitation (y-axis) showing separation and direct cell counts of SYBR ® Green I stained bacterioplankton and *Prochlorococcus* spp. (Pro) from Station ALOHA surface water sample. *Prochlorococcus* spp. population cell count of 216,000 cells/mL. The heterotrophic population cell count calculated from this analysis was 530,000 cells/mL. Fluospheres® 1.0 µm yellow-green fluorescent microspheres were added as an internal reference.

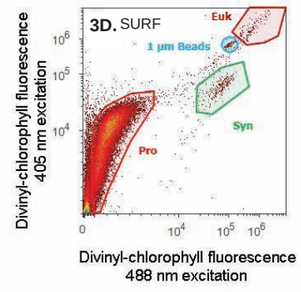


Figure 3D. Divinyl-chlorophyll fluorescence from 488 nm excitation (x-axis) versus divinyl-chlorophyll fluorescence from 405 nm excitation (y-axis) showing separation of populations of picophytoplankton from unstained Station ALOHA site surface water sample. The analyzed sample is from an oligotrophic area of the ocean. The Attune® Acoustic Focusing Cytometer is capable of resolving the *Prochlorococcus* spp. population from this oligotrophic part of the ocean and provide a direct cell count. The concentration for *Prochlorococcus* (Pro) observed from the oligotrophic Station ALOHA surface water sample was 216,000 cells/mL. The *Synechococcus* (Syn) cell count from this sample was 2100 cells/mL. Picoeukaryote (Euk) cell count for this sample was 1100 cells/mL. Fluospheres® 1.0 µm yellow-green fluorescent microspheres were added as an internal reference.