Novel detection and direct cell counting of marine, photosynthetic picoplankton by acoustic focusing cytometry

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Background

Flow cytometry is a powerful tool for studying the biology, ecology and biogeochemistry of marine, photosynthetic picoplankton. Populations of picoplankton are intrinsically fluorescent due to their photopigment content and differences in these photopigment compositions allows for discriminating populations of species. *Prochlorococcus* spp. and *Synechococcus* spp. are the two major groups of prokaryotes that comprise the photosynthetic picoplankton and have been extensively studied for their principal role in primary production. *Prochlorococcus* spp. are the smallest and most abundant photosynthetic organisms known, and along with *Synechococcus* spp. have a large impact on the global carbon cycle. *Prochlorococcus* spp. are approximately 0.6 μm in size and contain the red fluorescent molecules divinyl-chlorophylls *a* and *b*. Cells of *Synechococcus* spp. are larger at 1 μm and contain the orange fluorescent phycoerythrin in addition to red fluorescent chlorophyll. These differences allow for the discrimination of natural populations of *Prochlorococcus* spp. and *Synechococcus* spp. from environmental samples (1-5).

Typical picophytoplankton population analysis

Analysis of marine, photosynthetic picoplankton is routinely done using flow cytometry, 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. Instruments that utilize high velocity or high volumetric sheath fluid to focus cells (hydrodynamic focusing) before laser interrogation have been used. Most flow cytometers are pressure driven and direct cell counting of discrete populations is not possible without weighing of the sample pre and post analysis or the addition of counting beads to the sample. In addition, the inclusion of 488 nm excitable nucleic acid binding dyes for determining cell counts of the heterotrophic population (*Bacteria* and *Archaea*) obscures the intrinsic fluorescence of the picoplankton populations, requiring multiple samples to assess the entire microbial population.

Simplifying picophytoplankton population detection with the Attune® Acoustic Focusing Cytometer

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, low background 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 (Figure 1). 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 2 demonstrates the utility of combining a slow particle flow rate and excitation of divinyl-chlorophylls with the 405 nm laser.

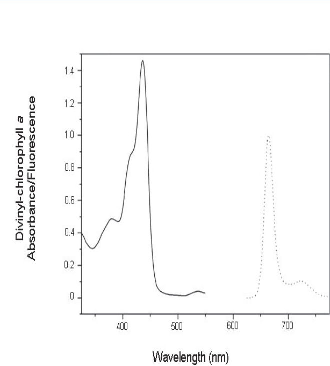


Figure 1. Absorption (solid line) and fluorescence emission (dotted line) spectra of divinyl-chlorophyll *a*.

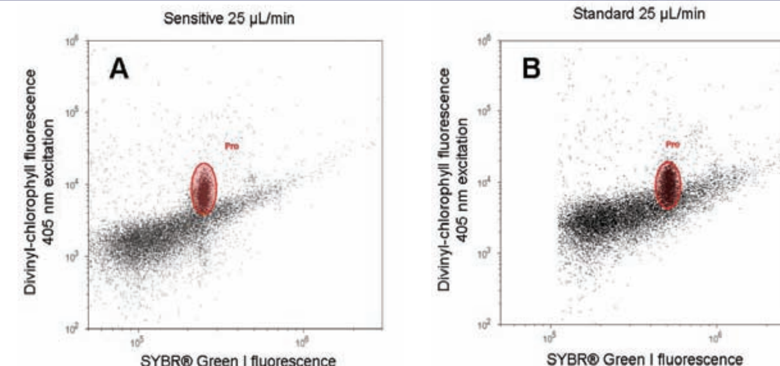
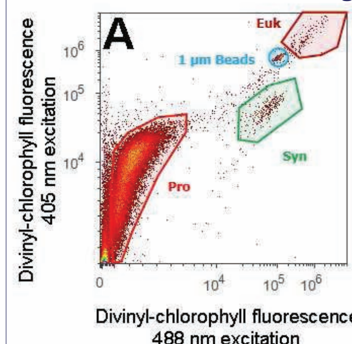


Figure 2. Oligotrophic Station ALOHA surface water sample analyzed with Sensitive and Standard transit times (particle flow rates). Sensitive 25 μL /min (A) allows for better separation of the inherently dim red fluorescent (y-axis) *Prochlorococcus* spp. (Pro) populations from the remaining SYBR® Green I stained cells as compared Standard 25 μL /min (B). Slowing the particle flow rate can increase the laser interrogation and photon collection times from dim, low background populations.

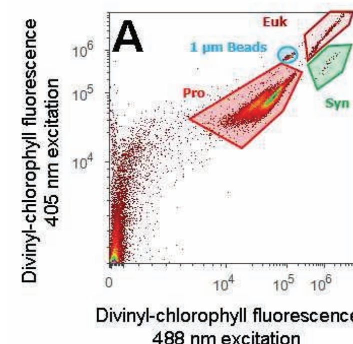
Direct Cell Counts of Picophytoplankton Based on Intrinsic Fluorescence with the Attune® Acoustic Focusing Cytometer



B

Name	Event Count	Concentration
All Events	35,264	282.02
Pro	27,100	216.73
Syn	261	2.09
Euk	138	1.10
1 µm Beads	401	3.21

Figure 3. Divinyl-chlorophyll fluorescence from 488 nm excitation (x-axis) versus divinyl-chlorophyll fluorescence from 405 nm excitation (y-axis) showing separation of populations (A) and direct cell counts (B) 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* spp. (Pro) (27000 events) observed from the oligotrophic Station ALOHA surface water sample was 216,000 cells/mL. The *Synechococcus* spp. (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.

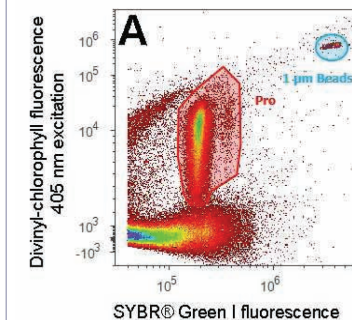


B

Name	Event Count	Concentration
All Events	24,056	192.32
Pro	14,107	112.78
Syn	37	0.30
Euk	299	2.39
1 µm Beads	346	2.77

Figure 4. Divinyl-chlorophyll fluorescence from 488 nm excitation (x-axis) versus divinyl-chlorophyll fluorescence from 405 nm excitation (y-axis) showing separation of populations (A) and direct cell counts (B) of picophytoplankton from unstained Station ALOHA site Deep Chlorophyll Maximum (DCM) water sample. The direct cell count for *Prochlorococcus* spp. (Pro) observed for the Station ALOHA site DCM sample was 113,000 cells/mL. The *Synechococcus* spp. (Syn) cell count calculated from the DCM sample was 300 cells/mL. Picoeukaryote (Euk) cell count for this sample was 2400 cells/mL.

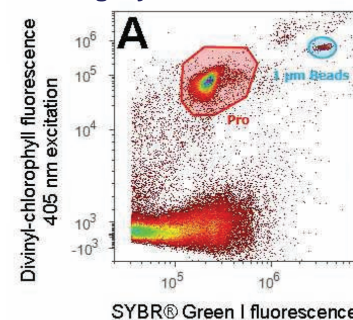
Direct Cell Counts of Bacterioplankton and *Prochlorococcus* spp. Based on SYBR® Green I DNA Staining and Intrinsic Fluorescence with the Attune® Acoustic Focusing Cytometer



B

Name	Event Count	Concentration
All Events	108,167	864.47
SYBR® Green I	93,284	745.53
Pro	27,100	216.58
1 µm Beads	388	3.10

Figure 5. SYBR® Green I fluorescence (x-axis) versus divinyl-chlorophyll fluorescence from 405 nm excitation (y-axis) showing separation (A) 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 strongly agrees with the cell count from the unstained sample. 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.



B

Name	Event Count	Concentration
All Events	72,135	576.70
SYBR® Green I	58,446	467.26
Pro	13,688	109.43
1 µm Beads	358	2.86

Figure 6. SYBR® Green I fluorescence (x-axis) versus divinyl-chlorophyll fluorescence from 405 nm excitation (y-axis) showing separation (A) and direct cell counts of SYBR® Green I stained bacterioplankton and *Prochlorococcus* spp. (Pro) from Station ALOHA site DCM sample with detection from 405 nm laser excitation. *Prochlorococcus* spp. population cell count of 109,000 cells/mL strongly agrees with the cell count from the unstained sample. The heterotrophic population cell count calculated from this analysis was 358,000 cells/mL. FluoSpheres® 1.0 μm yellow-green fluorescent microspheres were added as an internal reference.

Materials and Methods

Attune® Acoustic Focusing Cytometer and standard associated fluids equipped with 640 LP filter in VL3 channel (standard filter = 603/48). Station ALOHA Surf (5 m) and DCM (125 m) marine water samples.

Sample Preparation for Unstained Samples

Marine water samples can be analyzed with or without fixation (final concentration of 1% paraformaldehyde or 0.1% gluteraldehyde, 20 min).

FluoSpheres® 1.0 μm yellow-green fluorescent microspheres diluted to 105 beads/mL in distilled water. Add 10 μL of the diluted bead suspension to 1 mL of sample. This is an optional internal reference that may be used.

Sample Preparation for SYBR® Green I Stained Samples

Marine water samples may be analyzed with fixation (final concentration of 1% paraformaldehyde or 0.1% gluteraldehyde, 20 min). The fixation step ensures that all cells within the sample are stained with SYBR® Green I.For DNA staining, prepare a 1:10 dilution of SYBR® Green I (supplied as a 10,000X stock solution) in distilled water. Add 1 μL of 1:10 dilution of SYBR® Green I per mL of sample and incubate in the dark for 30 min. The sample should not be washed before analysis.

Conclusions

Prochlorococcus spp., *Synechococcus* spp., picoeukaryote and heterotrophic populations are readily detectable and directly enumerated by acoustic focusing cytometry, using the Attune® Acoustic Focusing Cytometer. In addition, *Prochlorococcus* spp. are detected and directly counted from the oligotrophic surface waters from Station ALOHA, even when the sample is stained with the DNA stain SYBR® Green I for calculation of the heterotrophic population. The direct cell counts determined from the samples reported in this brief communication concur with published data from Station ALOHA near Hawaii.

References

1. **Campbell, L., Nolla, H. A. & Vaulot, D. (1994).** The importance of *Prochlorococcus* to community structure in the North Pacific Ocean. *Limnol Oceanogr* **39**, 954-961.
2. **Marie, D., Partensky, F., Jacquet, S. & Vaulot, D. (1997).** Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Appl Environ Microbiol* **63**, 186-193.
3. **Marie, D., Partensky, F., Vaulot, D. & Brussaard, C. (1999).** Enumeration of phytoplankton, bacteria, and viruses in marine samples. *Curr Protocol Cytom* **47**, 11.11.1-11.11.15.
4. **Partensky, F., Hess, W. R., & Vaulot, D. (1999).** *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. *Microbiol Mol Biol Rev* **63**, 106-127.
5. **Veldhuis, M. J. W., & Kraay, G. W. (2000).** Application of flow cytometry in marine phytoplankton research: current applications and future perspectives. *Sci Mar* **64**, 121-134.

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