High Purity Simultaneous Cell Sorting of Nano and Large Phytoplankton Using the MoFlo Astrios EQ

Introduction

Phytoplankton conversion of light on the upper limits of the ocean consists of half of the photosynthesis on the Earth. Population densities indicate health of phytoplankton, and the entire aquatic ecosystem. The isolation and sorting of aquatic samples using flow cytometry allows for quick and effective population analysis. Forward scatter on the Astrios EQ allows for differentiation of small and large particles from 0.2 to 30 µm on FSC. This design provides researchers greater flexibility to isolate and sort specific phytoplankton of different sizes while utilizing the 7 laser, 42 parameter instrument.

Photosynthetic plankton is one sorting application for the Astrios EQ forward scatter design. Marine Biology has used flow cytometry with phytoplankton previously for: rapid and objective evaluation of water quality;[1] to analyze grazing;[2] cell viability;[3] rapid strain identification;[4] diversity assessment;[5] and to characterize plankton populations through molecular[6,7] and genetic approaches.[8]

Plant Pigments Absorption Spectra

In a general photosystem, as seen in Figure 1, the chlorophyll b transfers energy through the antenna pigment complex to chlorophyll in a reaction center. The accessory pigments—such as Phycocyanin, Allophycocyanin, Allophycocyanin B, and Phycoerythrin—provide a composite absorption spectrum such that a wider range of visible and infrared radiation is absorbed by plants and algae.

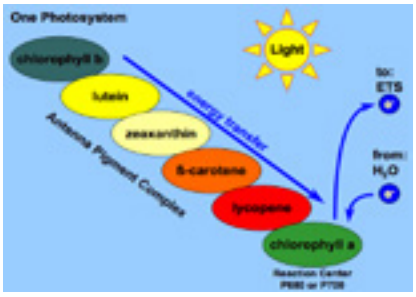

Fig. 1. Plant pigments absorption spectra. Source: Koning Ross E. "Light". *Plant Physiology Information Website*. 1994. Accessed February 9, 2013. http://plantphys.info/plant_physiology/light.shtml.

Quantity of Pigments in Phytoplankton

The quantity and concentration of the plant pigments found in the phytoplankton vary depending on the organism size and oceanic environment (including the habitat depth from ocean surface). Plankton populations may be distinguished by their fluorescence spectra using flow cytometry with multiple laser lines and emission filters, as seen in Figure 2.

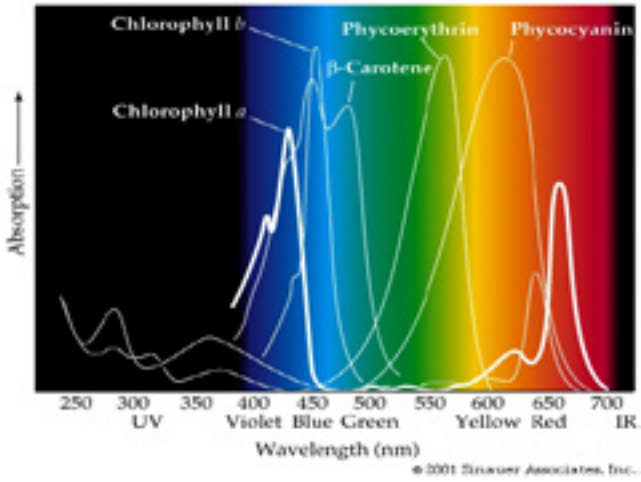

Fig. 2. Plant absorption spectra including chlorophyll a and b, phycoerythrin, phycocyanin and beta-carotene.

MOFLO ASTRIOS EQ
FLOW CYTOMETER SYSTEM

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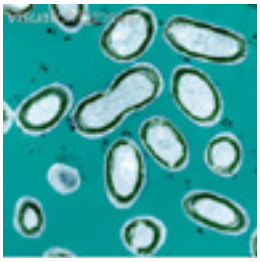
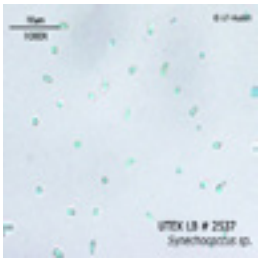
Materials

Plankton species—*Chlorella, Phormidium inundatum, Phormidium persicinum, Cryptomonas, Rhodosorus, Synechococcus, Skeletonema, Fremyella*—were acquired from UTEX "The Culture Collection of Algae", grown in photobioreactors and cultured in specialized salt and fresh water media (Figures 3a–3h). Plankton species *Prochlorococcus marinus* and *Emiliana huxlei* were grown in sterile 2 L containers supplied with 0.2 µm filtered air in salt water with fertilizer. Instant Ocean, 1/2-cup per gallon of deionized water, was added to the culture with Microalgae Grow Mass Pack with Silicate.

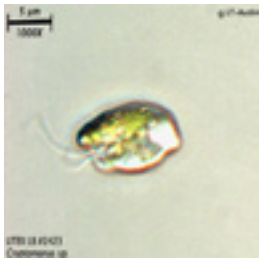
Fig. 3. The Culture Collection of Algae, grown in photobioreactors and cultured in specialized salt and fresh water media.



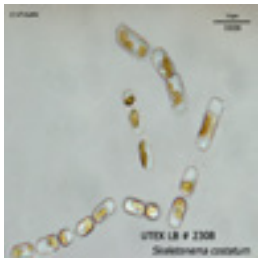
a. Prochlorococcus, 0.8 µm



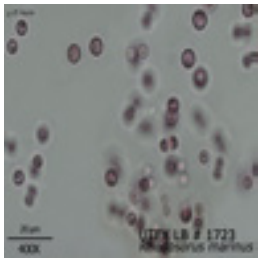
b. Synechococcus, 0.8–1.6 µm



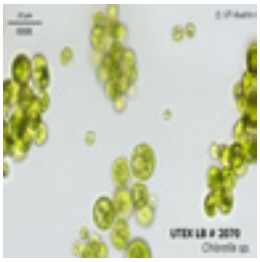
c. Cryptomonas, 10–50 µm



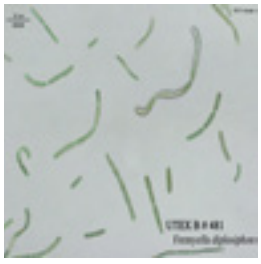
d. Skeletonema, 10 µm



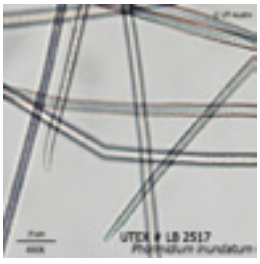
e. Rhodosorus, 7.6 µm



f. Chlorella, 8–50 µm



g. Fremyella, 7–10 µm



h. Phormidium

Methods

Viability was tested using the Beckman Coulter Vi-CELL for the plankton and then less than $5x10^6$ cells were stained with Sytox Green (Invitrogen, S7020) 2 µl, per 1 mL of culture, incubated for 20 minutes in the dark. Three mL were stained for each culture as single controls. The FSC 1 and FSC 2 detectors were designated for large and small plankton with an ND absorbing filter in front of the FSC 2 detector. A mixture of YG beads size 0.2 to 6 µm (Polysciences, Fluorobrite kit 1 and 2, 21636-I and 21637-I) were tested prior to plankton to measure the instrument noise/detection threshold.

FSC 1 had the P1 mask and ND 1 absorbing filter and FSC 2 had the M2 mask. The FSC was aligned to the 488 nm laser with the trigger on 640 SSC to visualize the FSC parameters clearly.

Sorting out the plankton populations required a 100 µm tip at 10 K EPS sort speed. Samples were sorted into Enriched Seawater media and reanalyzed after cleaning the fluidics with bleach and DI.

Flow Cytometry

The Astrios EQ was configured with 7 lasers, and set up with a 100 µm tip to accommodate the larger phytoplankton (*Cryptomonas*). Cells were selected on their "live" status by being highly fluorescent in the red channels (chlorophyll) and low in the green channels (Sytox -). For small particle analysis, *Prochlorococcus* and *Synechococcus* were simultaneously analyzed on FSC-Log parameters. Populations were sorted based on fluorescence and size as a 6-way sort into 5 mL tubes. The plankton were sorted at 25 K EPS to collect at least 100,000 events per each population using sort mode Purify 1–2.

Fluorescence

The Fisher Distance was used to determine the optimum excitation laser and emission bandpass using Kaluza software. Fisher Distance quantifies the separation between a stained and unstained population in terms of the population widths. The Kaluza software was used to find the initial Fisher Distance formula optimum median channel and then used to calculate the Fisher Distance in the analysis package for quick data analysis. You can see an example of the fluorescence of the *Cryptomonas* plankton (Figure 4) and the resulting Fisher Distance of all of the plankton analyzed in Kaluza (Figure 5) according to the indicated fluorescence parameters. The red indicates parameters of poor resolution and blue indicates parameters of high separation.

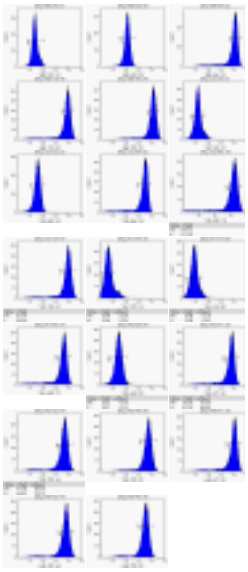


Fig. 4. Fluorescence of the *Cryptomonas* plankton example.

Fluorescence Parameter	Chlorella	Cryptomonas	E. Huxley	Fremyella	Phormidium fragile	Phormidium inundatum	Phormidium pericinium	Prochlorococcus	Rhodosaurus	Skeletonema	Synechococcus
488-576/21			0.89	0.01	1.18	0.01	0.01	0.01	2.64		
488-620/29		0.01	1.96	-0.01	1.18	0.02			2.89		0.09
488-664/22	2.53	2.07	3.23	1.32	1.83			0.08	3.11	2.97	2.01
488-710/45	2.12	1.80	3.18	0.68	1.69	0.40	1.01	0.09	2.66	2.64	1.69
488-795/70	1.84	1.77	3.07	0.97	1.71		0.13		2.60	2.42	
532-576/21				0.01	1.73	0.02	-0.05		3.24		1.79
532-622/22			1.56	0.01	1.63	0.01	0.03		2.96		
532-664/22	2.90	1.98	3.03	1.04	2.02	0.51	0.69	1.56	3.07	3.14	2.99
532-692/18	2.29	1.41	3.11	1.07	1.95	1.00	1.06	1.34	2.99	2.72	1.50
532-736/47	1.87	1.35	2.88	1.00	1.94	0.56	0.89	1.37	2.71	2.55	
561-579/16					1.75		0.01	0.03	2.98		1.82
561-614/20		0.09	0.05	0.58	1.66	0.13	0.39	1.23	2.83		3.15
561-692/75	3.25	1.53	2.90	1.07	1.98	0.54	0.64	1.92	3.10	2.89	2.36
592-620/29		0.22	2.02	3.35	1.63	1.72	3.18	1.71	2.74		0.01
592-671/30	4.42	1.72	2.95	1.17	1.94	0.54	0.74	2.36	3.14	2.99	
592-722/44	4.42	1.59	2.81	1.20	2.06	1.11	1.18	2.20	2.84	2.63	0.15
592-795/70	2.58	1.88	3.13	1.46	2.01	0.45	0.72	2.26	3.17	2.96	0.26
640-671/30	5.21	1.88	3.03	0.99	1.89	0.25	0.59	2.26	3.51	3.10	2.61
640-722/44	4.42	1.73	2.86	1.10	1.84	0.47	0.95	1.99	3.13	2.77	0.15
640-795/70	2.76	1.95	2.98	1.18	1.81	0.93	1.30	2.17	2.97	2.59	0.26

Fig. 5. Fisher Distance of all plankton analyzed.

Optimizing FSC

Masks

Material (M) is designed to pull apart particles of different material types. It is for relation to particle size over large ranges of particles. Separation (S) is better for pulling out small differences in similar particles. It reduces the sensitivity to different particle types. All-Purpose (P) is best when matched against "M" or "S" masks to pull out details. It is for overall general performance. Reference Figure 6 below.

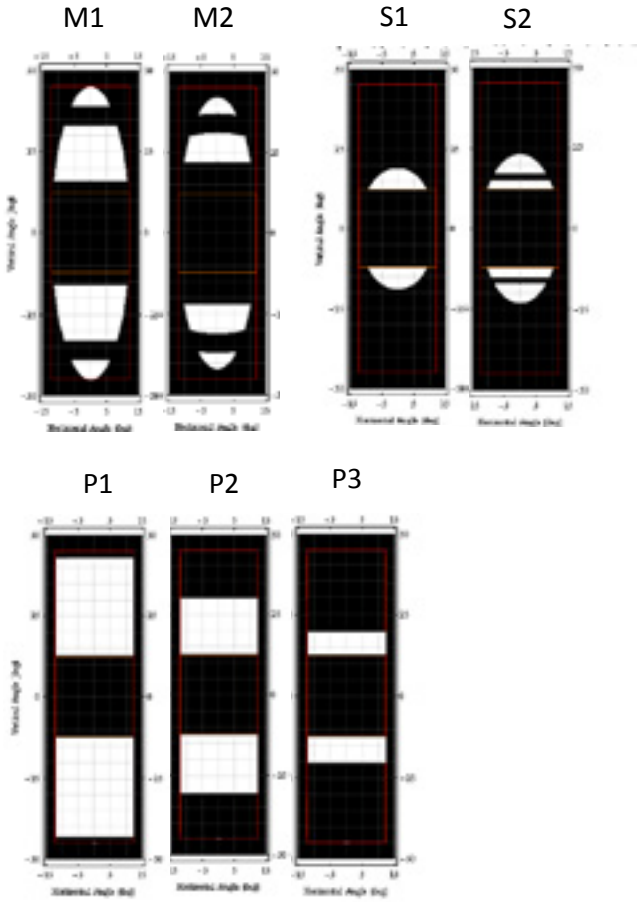


Fig. 6. Masks.

Dual FSC Used to Find Unique Populations

The Astrios EQ forward scatter provides 2 scatter parameters to analyze populations. Within the conventional FSC vs. SSC populations, the Synechococcus population appears as a "blob" with the FSC 1 with a material (M) mask (Figures 7a and 7b).

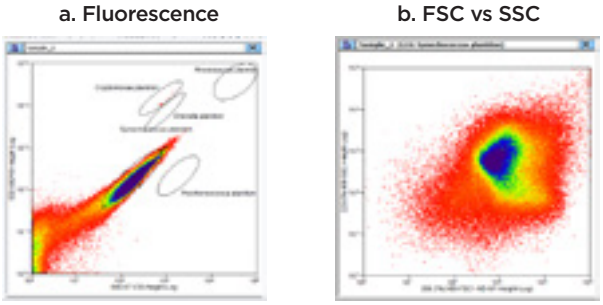
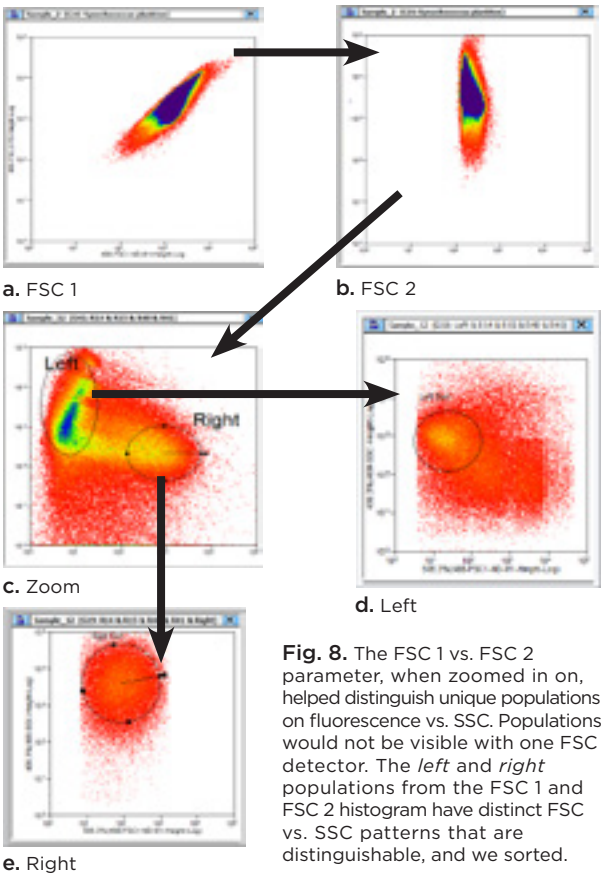


Fig. 7. a. Synechoccocus fluorescence (APC vs. Chlorophyll) and **b.** FSC vs. SSC populations.

Within the dual FSC parameters, the Synechococcus population demonstrates 2 specific and unique populations only visible on FSC vs. FSC on a zoomed population. Those 2 populations have distinct SSC signatures that are not visible on the fluorescence population pictured (Figures 8a through 8e).



a. FSC 1
b. FSC 2
c. Zoom
d. Left
e. Right

Fig. 8. The FSC 1 vs. FSC 2 parameter, when zoomed in on, helped distinguish unique populations on fluorescence vs. SSC. Populations would not be visible with one FSC detector. The *left* and *right* populations from the FSC 1 and FSC 2 histogram have distinct FSC vs. SSC patterns that are distinguishable, and we sorted.

Results

Plankton populations were distinguishable using fluorescence and scatter patterns on both log and linear scales simultaneously. The plankton fluorescence spectra were optimized for signal to noise using the optical flexibility of the Astrios EQ. Isolation of the *Prochlorococcus*, *Synechococcus* and other plankton species using cell sorting achieved 99% purity for all populations.

The *Prochlorococcus* and *Synechococcus* cyanobacteria have distinct fluorescence characteristics and were used to "Color" gate to find unique populations of each plankton (Figure 9). Note the "Noise" gate was very far from the submicron plankton populations.

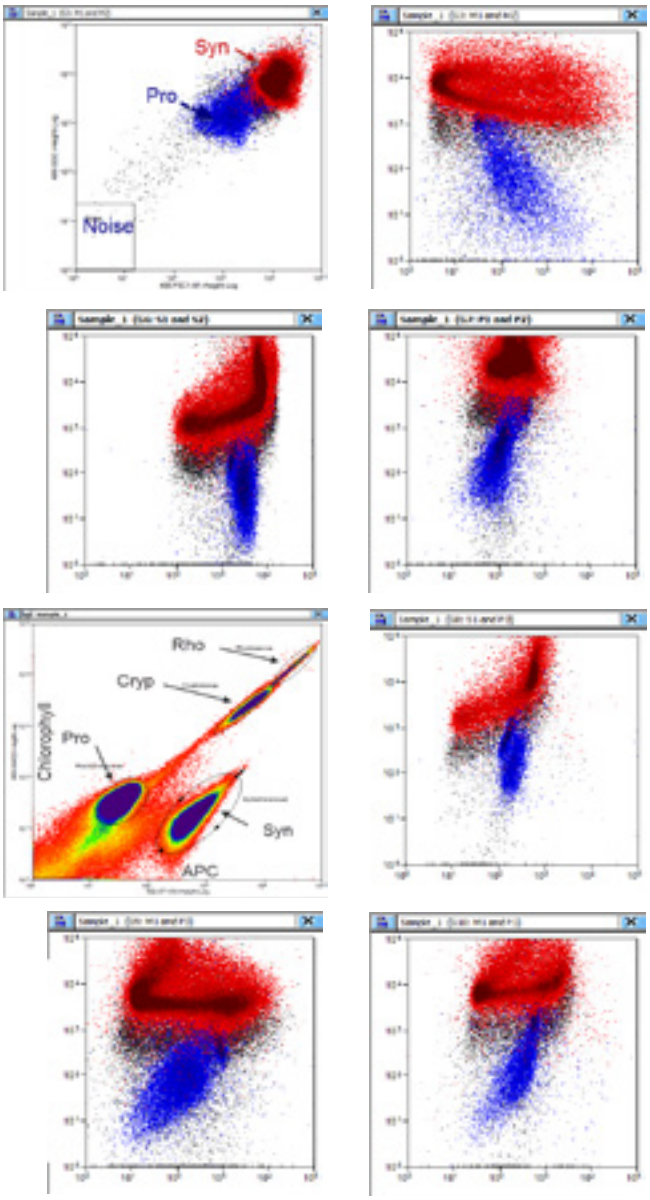
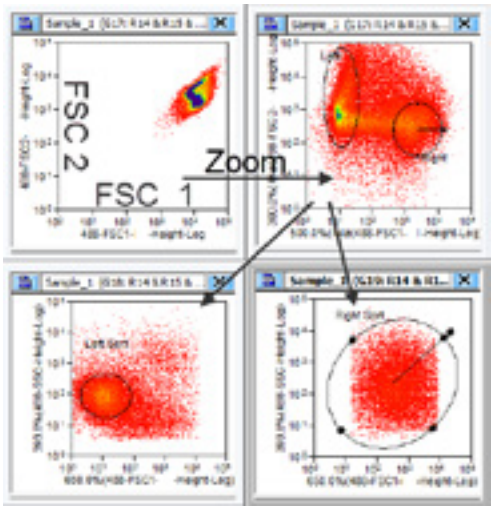


Fig. 9. Mask selection of *Prochlorococcus* and *Synechococcus*.

The sort contained populations of *Rhodosaurus*, *Cryptomonas*, *Prochlorococcus* and the 2 populations found in the *Synechococcus* (Figure 10). The sort was run at 50 K EPS on Purify 1–2 into 5 mL tubes. The purity was: Syn Right–94.33%; Syn Left–98.70%; *Prochlorococcus*–98.56%; *Rhodosaurus*–98.59%; and *Cryptomonas*–99.54%.

Synechococcus Left and Right Pre-Sort



Synechococcus Post-Sort

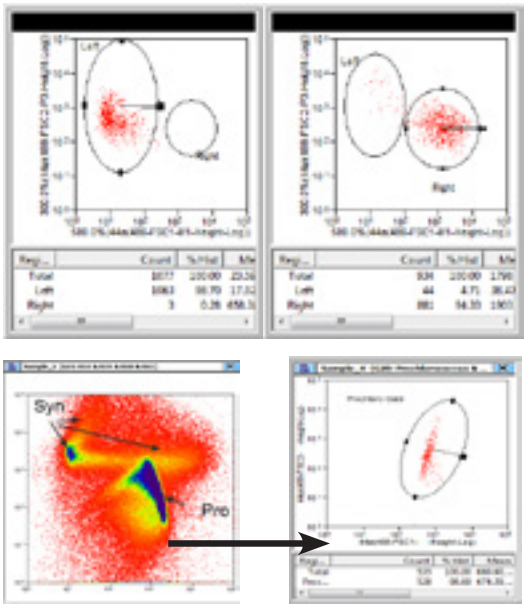


Fig. 10. 5-way high-speed sort.

Conclusion

The Astrios EQ new FSC module provided the ability to sort unique FSC populations for the *Synechococcus* population with high purity and high speed. The FSC module was able to distinguish two submicron populations (*Prochlorococcus* and *Synechococcus*) from noise. The plankton populations were sorted with high purity and speed, indicating the robust nature of the Astrios EQ sorting system.

Authors

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