Multiparametric Analysis of Microalgae for Biofuels Using Flow Cytometry

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White Paper

Contents

Abstract

Photosynthetic microalgae have received significant attention for their potential as a biomass source for biofuels, leading to an explosion in studies of algal growth and lipid biogenesis. Both research laboratories and commercial facilities need efficient analytical tools to monitor algal cultures in real time. Flow cytometry offers major advantages in screening and analyzing microalgal samples.

This white paper explores the opportunities and challenges involved in applying flow cytometry to microalgal biofuel research and development. The BD Accuri™ C6 personal flow cytometer offers unique advantages for analyzing microalgae of varying sizes, detecting chlorophyll autofluorescence over a wide dynamic range, measuring lipid content using fluorescent stains such as Nile Red and BODIPY 505/515, accurately counting cells, and augmenting throughput via automation. We demonstrate that multiparametric analysis of microalgal cultures using flow cytometry can advance the field of biofuel research and development.

Introduction

Biomass is organic matter derived from common materials such as wood, agriculture crops and their byproducts, and waste streams. Many regard the conversion of biomass to biofuel as a potentially renewable solution to the world's energy needs. For example, the US Energy Independence and Security Act of 2007 (EISA) called for increased production of biofuels to help meet the country's energy requirements and alleviate its petroleum dependence.[1] Similarly, in 2009, the European Parliament called for research on sustainable advanced biofuels as part of its renewable energy initiative.[2]

Recently, the use of algae as a biomass source has received significant attention.[3] In fact, EISA required the US Secretary of Energy to initiate studies on the use of algae as a feedstock for biofuel production. Algal biofuels were one of seven value chains proposed in the European Bioenergy Industrial Initiative (EIBI), and the Asia Pacific Economic Cooperation (APEC) Biofuels Task Force initiated an algal biodiesel project in 2009.[4]

Photosynthetic microalgae exhibit a high theoretical rate of conversion of solar energy to chemical energy.[5,6,7] Early studies conducted under laboratory conditions projected that oleaginous microalgae could produce oil at a much higher rate than conventional oil crops such as jatropha, soy, or palm.[8] Some authorities argue that algal biofuel is the best renewable candidate to fully replace fossil petroleum.[9]

Current status of research

Fulfilling the promise of microalgae will require significant research and development. The US Department of Energy's Aquatic Species Program, initiated in 1978, demonstrated that the production of energy from algae was feasible but required technological advances to achieve economic viability.[10] This qualification still holds true today.[11] Current research efforts are directed at screening large numbers of new algal isolates, some obtained from nature or commercial algal culture collections, and others artificially engineered in the laboratory. Targeted characteristics that are important for algal crop production include rapid biomass generation, high oil content, tolerance for environmental perturbations, resilience against contamination, and biosynthesis of desirable secondary products.

Research laboratories need efficient analytical tools to monitor algal cultures in real time, but there are significant technical challenges to overcome. The methodology must be able to characterize microalgae that vary widely in size, chlorophyll autofluorescence, and lipid content. Sample analysis must attain high throughput without sacrificing data quality.

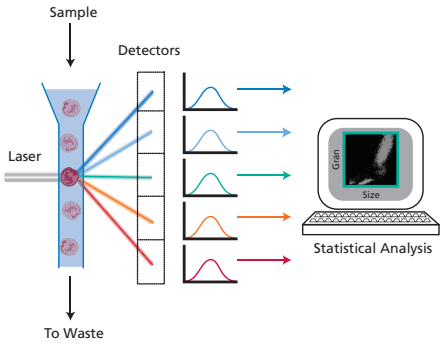

Figure 1. How flow cytometry works.

Impact of flow cytometry

Flow cytometry offers a powerful and effective method for screening microalgal cultures. In flow cytometry, particles or cells suspended in a hydrodynamically focused liquid stream pass through a pulsed beam of laser light. Optical detectors collect scattered laser light and fluorescent emissions, and electronics digitize these signals for computational analysis. The light-scatter data provides basic information about the cells, such as relative size, shape, and surface features. The fluorescence data allows researchers to measure the cells' intrinsic fluorescence (from chlorophyll, for example). Or, by labeling the cells with fluorescent tags such as dye-conjugated antibodies or lipophilic stains, researchers can assess numerous other cellular properties.

This white paper explores opportunities and challenges of applying flow cytometry to microalgal biofuel research, and discusses key capabilities and techniques for successful analysis. All research examples in this paper were analyzed on the BD Accuri C6, the first personal flow cytometer, which is especially well suited for the rapid and efficient assessment of microalgal cultures.

Figure 2. The BD Accuri C6 flow cytometry system.

Exterior dimensions of the flow cytometer unit (H x W x D) are 27.9 x 54.6 x 41.9 cm (11 x 21.5 x 16.5 in) with fluid tanks in place.

Technical considerations of the BD Accuri C6

Operating environment

The BD Accuri C6 flow cytometer offers performance, simplicity, and affordability. Small, light (30 lb, 13.6 kg), and rugged, the system connects to a standard electrical outlet, making it readily portable and suitable for any space. Fixed optics and capillary sheath-flow fluidics enable continuous operation even during motion and vibration, including aboard ship.[12]

BD Accuri™ C6 software makes operation menu-driven and intuitive for novice and proficient users alike. Most new users become fluent with the software in less than 30 minutes, assisted only by a 3-page pictorial *Quick Start Guide*.

Routine instrument decontamination, cleaning, and priming are automated. Maintenance is easy, even for novice users, and requires no tools. The recommended sheath fluid is 0.22 µm-filtered, deionized water. These attributes effectively remove flow cytometry from the core facility and place it on the laboratory bench.

Optical and analytical modalities

The BD Accuri C6 features two lasers, two scatter detectors, and four fluorescence detectors. A state-of-the-art digital signal processing (DSP) system gives the BD Accuri C6 a dynamic range of six full decades. This means that it can finely resolve both faint and bright signals at once and analyze a wide span of variation in intrinsic microalgal fluorescence in a single run. The instrument detects this broad dynamic range using standard factory detector settings, without the need for optimization or tuning.

If additional flexibility is needed, the optical configuration is flexible as shown in Table 1. The Selectable Lasers Module (Cat. No. 653126) allows reassignment of the standard laser-detector associations, and optional filters can modify the effective detector characteristics.

Table 1. Standard and optional optical configuration of the BD Accuri Cytometer system.

Laser/Detector Associations	"3 blue 1 red" (standard)	488 nm (emissions read in: FL1, FL2, FL3) 640 nm (emissions read in: FL4)
	"2 blue, 2 red" (653126 Module)	488 nm (emissions read in: FL1, FL2) 640 nm (emissions read in: FL3, FL4)
	"4 blue" (653126 Module)	488 nm (emissions read in: FL1, FL2, FL3, FL4)
Scatter Detection	Forward (0° ±13°) Side (90° ±13°)	
Emission Detection	Four colors, user-swappable optical filters	
	Standard set: FL1 533 ±15 FL2 585 ±20 FL3 670 LP FL4 675 ±12.5	**Optional:** 510 ±7.5 (Cat. No. 653184) 540 ±10 (Cat. No. 653528) 565 ±10 (Cat. No. 653185) 610 ±10 (Cat. No. 653186) 780 ±30 (Cat. No. 653187)

High-performance fluidics

Microalgae can vary greatly in size and can live as single cells or in colonies, which may make them difficult to process through a flow cytometer's fluidics system. The BD Accuri C6's "push-pull" peristaltic pump enables independent regulation of both the sheath and sample flow rates. Users can quickly adjust the sample core diameter from 5 to 40 µm to match the anticipated size range of sample microorganisms.

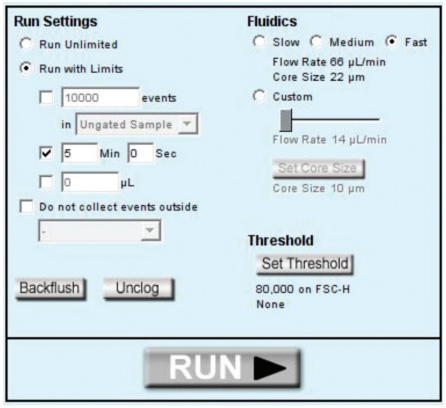

Figure 3. BD Accuri C6 software sample collection navigator.

The BD Accuri C6's preset sample flow speeds (Fast, Medium, or Slow) automatically adjust the core diameter to 22 µm, 16 µm, or 10 µm respectively. Alternatively, to optimize detection of very small or large microorganisms, the software provides custom user-variable flow rate and core size control from 5 to 40 µm in diameter. The Unclog and Backflush buttons help to remove clogs.

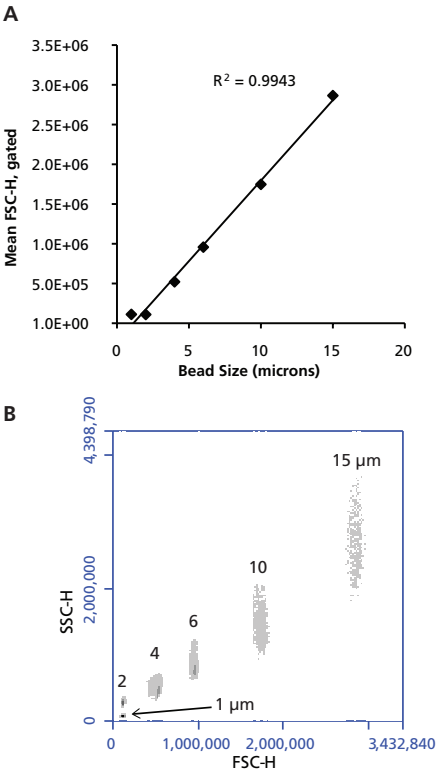

Figure 4. Light-scatter characteristics of beads on the BD Accuri C6.

***A.** Mean FSC-A signal is highly correlated with bead size. **B.** Forward and side scatter of a single sample of beads varying from 1 to 15 µm in diameter. Each sample was run for 60 seconds using Medium fluidics speed and a core size of 16 µm.*

Algal cultures frequently contain debris and other contaminating organisms (especially when grown in open ponds) that can clog fluidics lines. The direct-drive fluidics design of the BD Accuri C6 allows easy removal of clogs that might form during sampling. By design, most clogs will occur in the sample injection probe, not in the interior fluidics lines. The operator can push clogs out of the flow cell with a brief burst of sheath fluid followed by a high-volume flush, simply by clicking the Unclog and Backflush buttons in BD Accuri C6 software (Figure 3).

Applications in the analysis of microalgae

Size distribution of sample organisms

Because microalgae vary greatly in size, it is important to accurately measure forward-angle light scatter (FSC) signals for a broad range of particle sizes. Figure 4 shows a high linear correlation between the mean FSC-A values obtained from samples containing single bead populations of known diameter (ranging from 1 to 15 µm) after analysis and gating on the BD Accuri C6 (Barker J, Cattolico RA, unpub). Light-scatter signal for beads up to 30 µm can be displayed on scale. The 200-µm diameter of the flow cell allows particles and cells up to 100 µm to pass, although the light-scatter signals may be off scale.

As Figure 4 demonstrates, the BD Accuri C6 is capable of processing particles across a range of sizes, as well as any particulate material likely to be present in environmental or bioreactor samples. Table 2 shows 11 strains of microalgae ranging in size from 2 to 15 µm, analyzed on a single instrument under identical conditions. Figure 5 shows the forward-scatter profiles of all 11 strains plotted as histogram overlays in BD Accuri C6 software, allowing simultaneous comparison of all strains on a single plot.

Table 2. Forward scatter characteristics of 11 microalgal strains.

Strain	Length (µm)	Width (µm)	Mean FSC-A
A	2–5	2–5	61,037
B	6–10	6–8	324,817
C	4–6	4–8	406,640
D	4–6	4–8	213,550
E	3–6	3–6	278,934
F	10–15	10–14	701,813
G	3–6	3–6	343,692
H	5–8	5–8	305,581
J	2–4	2–4	162,400
K	3–10	3–8	345,186
L	6–15	4–10	488,418

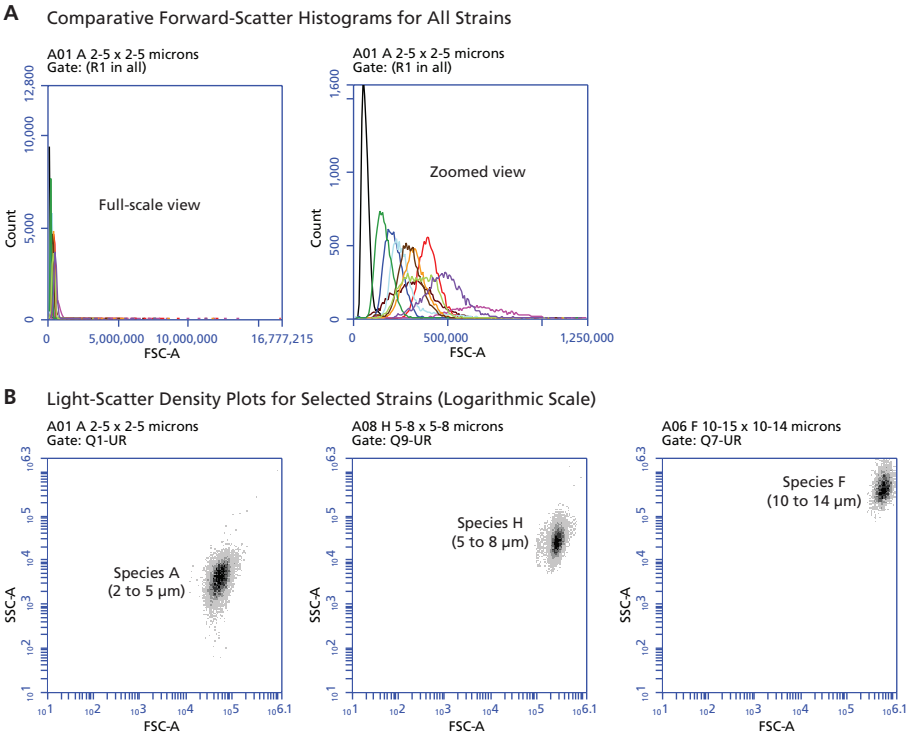

Figure 5. Light-scatter profiles for microalgae of various sizes on the BD Accuri C6.

A. FSC-A histograms for all 11 algal species are overlaid across the instrument's entire dynamic range (left) and zoomed for easier comparison (right). B. FSC-A vs SSC-A plots of the smallest, largest, and an intermediate algal species (A, F and H) using logarithmic axis scaling. The maximum channel values of the axes are identical for each plot (FSC-A=1.25 x 10^6, SSC-A=2.0 x 10^6). Growth conditions for each strain were 12 h light:12 h dark photoperiod, 100 µE/m^2/s, and 20°C. Cultures were sampled and analyzed using Medium fluidics speed (core size 16 µm) at the transition from logarithmic to stationary growth phase. Ten thousand events were measured for each sample and gated to exclude debris (Barker J, Cattolico RA, unpub).

A unique feature of BD Accuri C6 software is the ability to "zoom" in on the channel range where the data of interest occurs. Plotting the FSC data across all 1.6 x 10^7 channels (Figure 5A, left) does not allow visual differentiation of the strains' individual FSC-A profiles. However, a zoomed view of the x-axis showing only the first 1.25 x 10^6 channels (Figure 5A, right) reveals their different profiles. The Zoom tool is essential for optimal data analysis across a wide channel range.

Two-dimensional density plots of forward- vs side-angle scatter can further define the varying morphologic characteristics of each algal strain. When using BD Accuri C6 software, any parameter can be plotted on either linear or logarithmic scale. When the species to be analyzed vary greatly in size, as in the strains used here, the log scale is the best choice. Figure 5B shows light-scatter density plots for three representative strains, plotted in zoomed view. For these data, the logarithmic axes allow easier comparison among species by bringing all populations into clear view on the same scale.

Zooming, scaling, and other transformations do not overwrite the base data, which is always preserved at high resolution. Researchers can try various analytical approaches without putting the data set at risk.

Table 3. Mean channel values of chlorophyll fluorescence intensities for 11 microalgal strains.

Strain	Mean Chlorophyll-A (FL3-A)
A	139,339
B	1,081,883
C	1,813,107
D	1,044,973
E	974,225
F	7,035,768
G	759,615
H	1,514,209
J	385,538
K	338,308
L	1,523,488

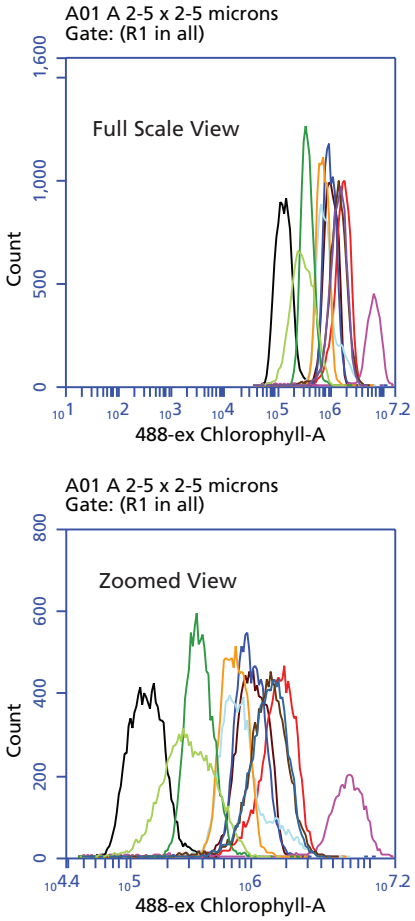

Figure 6. Chlorophyll fluorescence profiles for 11 microalgal species on the BD Accuri C6.

Chlorophyll fluorescence intensities in FL3 are overlaid across the instrument's entire dynamic range (top) and zoomed for easier comparison (bottom).

Detection of chlorophyll autofluorescence over a wide dynamic range

Microalgal species vary not only in size but also in chlorophyll content. Table 3 shows mean channel values of chlorophyll fluorescence intensity for the 11 microalgal strains in Table 2, which vary in volume from about 8 to >300 µm^3. With a 24-bit analog-to-digital converter and standardized detectors calibrated to operate in a linear range, the BD Accuri C6 flow cytometer accurately resolves fluorescence intensity signals over 6 orders of magnitude without voltage adjustments. This wide dynamic range enables simultaneous collection of bright and dim fluorescence emissions during the same sample run, simplifying direct comparison of autofluorescence across different species and ensuring that no data is lost due to incorrect settings.

Chlorophyll fluorescence intensities for these 11 microalgal species are plotted as histogram overlays in Figure 6. The fluorescence of chlorophyll-containing algae is dominated by signal in the FL3 detector of the BD Accuri C6 (488-nm laser excitation, 670 LP filter emission). All 11 species display a high chlorophyll fluorescence signal, ranging from a mean channel value of 1.4 x 10^5 (strain A) to 7.0 x 10^6 (strain F) on a scale with a maximum channel value of 16.7 x 10^6 (Barker J, Cattolico RA, unpub). For reference, non-fluorescent sizing beads have FL3 autofluorescence values between 500 and 5,000, depending on bead size (data not shown).

If off-scale fluorescence signals are encountered, 90% and 99% attenuated versions of all standard BD Accuri C6 optical filters will bring them back on scale while maintaining detector operation within the optimal linear range.

Multiparametric lipid analysis using fluorescent stains

Light-scatter signals on the BD Accuri C6 can profile microalgae based on size and morphological complexity (Figure 5), while fluorescence detectors (channels FL3 and FL4 in the standard configuration) can measure and characterize chlorophyll autofluorescence (Figure 6). The addition of fluorescing dyes and probes can measure more specific features such as cell viability, DNA content, and protein abundance. Dyes for multiple purposes can be multiplexed without interference, permitting simultaneous measurement of several characteristics, such as algal size, chlorophyll content, and lipid content.

Of particular interest to algae-based biofuel research is the ability to assess lipid concentration in living algal cells. Various physiological factors impact the synthesis of fatty acids in algae. To obtain optimal growth and lipid production, especially in large-scale operations, growers need to assess the productivity of their crop frequently, rapidly, and with precision. The monitoring of fatty acid content by conventional means requires the time-consuming extraction, conversion, and measurement of algal lipid. Flow cytometry with lipophilic dyes can rapidly estimate neutral lipid storage in live algal cells while requiring only a small sample (<0.5 mL), usually without needing to concentrate or remove the alga from its growth medium.

Example: Nile Red

Nile Red is intensely fluorescent in organic solvents and hydrophobic environments. The dye is excited by the 488-nm laser, and its emission maximum shifts from red to yellow wavelengths in a non-polar environment.[13] Nile Red staining allows neutral lipids to be detected in the FL2 (585 ±20 nm) channel of the BD Accuri C6.

Figure 7 shows the labeling of neutral lipid in *Isochrysis* sp. using Nile Red (Sigma). Staining resulted in a 200-fold increase in FL2 signal, observable along the y-axis of the density plot in Figure 7B (vs unstained sample in Figure 7A), and on the single-parameter histogram in Figure 7C. The density plot allows simultaneous assessment of chlorophyll *a* autofluorescence as an internal control. In this case, chlorophyll *a* fluorescence was minimally (2.7-fold) changed by Nile Red staining.

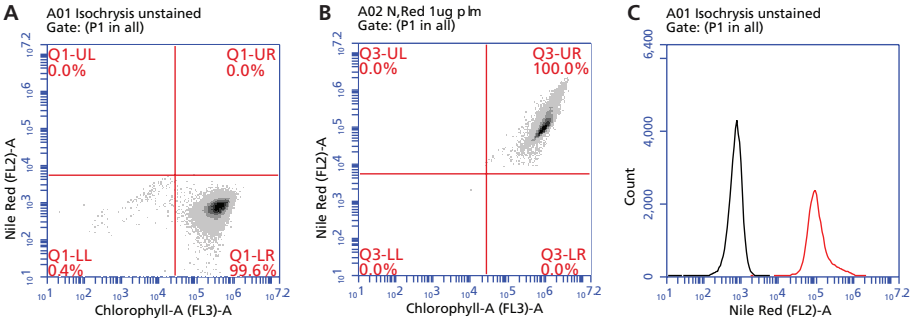

Figure 7. Assessment of neutral lipid content in *Isochrysis* sp. using Nile Red.

Isochrysis *cells, either* ***(A)*** *unstained or* ***(B)*** *stained with 1 µg/mL of Nile Red, were analyzed on a BD Accuri C6. Data are plotted on 2D density plots of chlorophyll fluorescence (x-axis: FL3; ex: 488 nm, em: 670 nm LP) vs Nile Red fluorescence (y-axis: FL2; ex: 488 nm, em: 585 ±20 nm).* ***C.*** *Data from unstained (black) and stained (red) cells are plotted on a single-parameter (Nile Red; FL2) histogram. Debris was excluded from analysis by gating (Wolfe G, unpub).*

Example: BODIPY

A new dye, BODIPY® 505/515 (BODIPY FL; 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene; www.probes.com), has recently been developed to monitor in vivo algal lipid storage.[14] BODIPY 505/515 is a green lipophilic fluorescent dye with a high oil/water partition coefficient. It readily penetrates cellular and organelle membranes and uses a diffusion trap to accumulate in intracellular lipid bodies (Figure 8).

The green emission spectrum of BODIPY 505/515 is spectrally separate from algal chloroplast autofluorescence. The 488-nm laser excites the dye enough to detect a significant fluorescence signal in the FL1 channel (533 ±15 nm) of the BD Accuri C6 (Figure 9). BODIPY 505/515 has several advantages over Nile Red: it does not bind to proteins, it fluoresces at wavelengths more green-shifted from chlorophyll than Nile Red, it may be more specific for neutral lipids, and it can assess neutral lipid levels across a broad taxa of algae, including those that have thick cell walls, scales, or thecae (Barker J, Cattolico RA, unpub; see Cooper et al, 2010[14] for discussion).

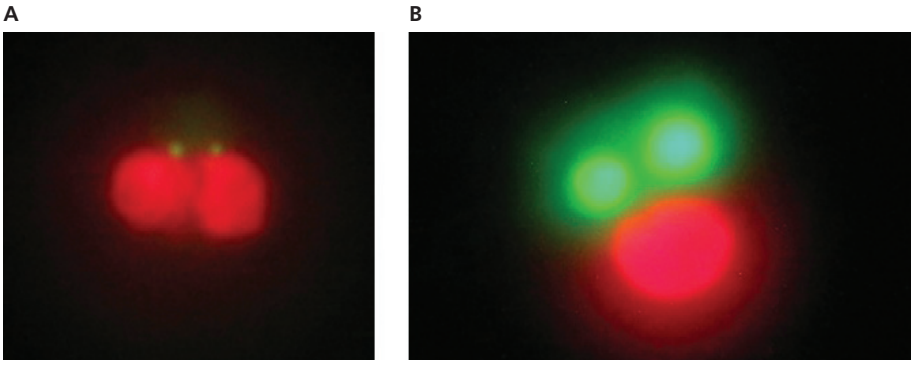

Figure 8. Lipid body staining with BODIPY 505/15 in *Chrysochromulina* sp.

Chrysochromulina *cells, maintained on a 12-h light:12-h dark photoperiod, were harvested* ***(A)*** *11.5 h into the dark period or* ***(B)*** *11.5 h into the light cycle. Harvested cells were excited at 450 to 490 nm, and emission wavelengths were imaged through a 515-nm LP filter using high-contrast microscopy. Green represents lipid bodies stained with 0.5 µM of BODIPY 505/515 dye, while red represents chloroplast autofluorescence. This data was previously published in Bigelow et al (2011).*[15]

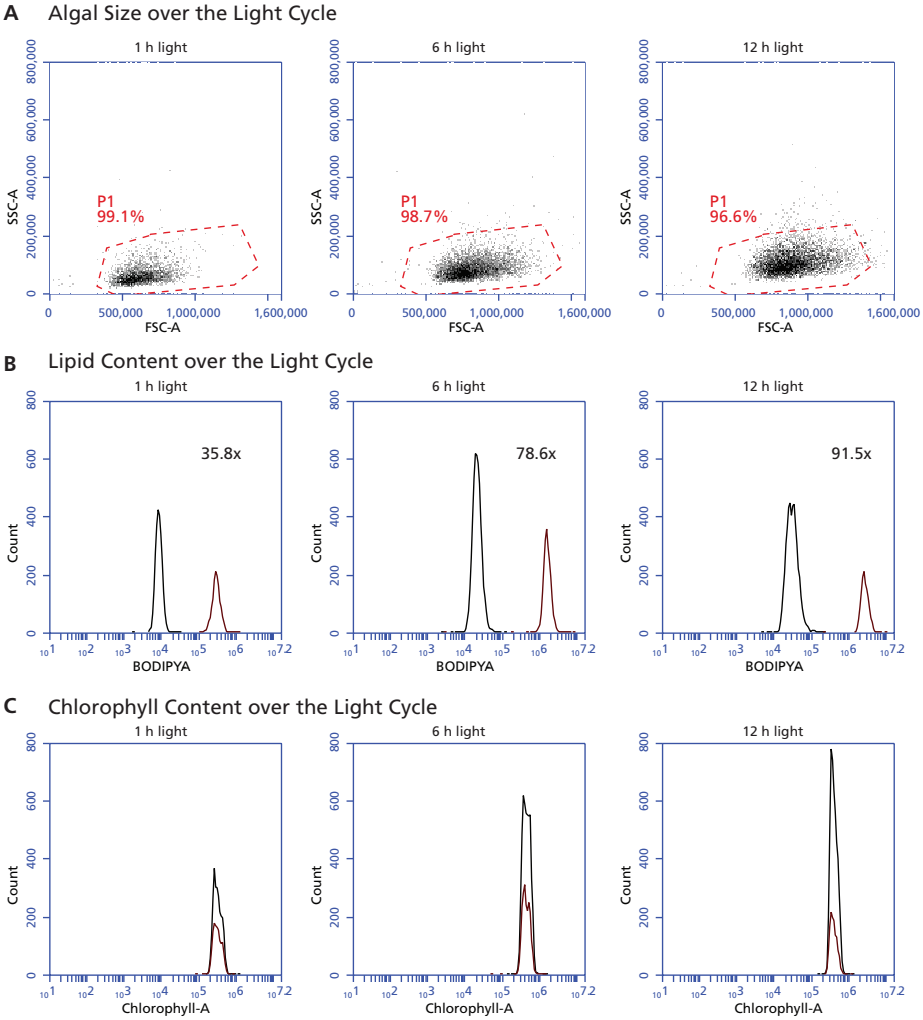

Figure 9. Analysis of size, lipid content, and chlorophyll content of *Chrysochromulina* sp. on the BD Accuri C6.

Cells were cultured as in Figure 8 and stained after harvest with 12.5 µM of BODIPY 505/515 dye. ***A.*** *Representative FSC-A vs SSC-A plots from samples harvested 1 h, 6 h, and 12 h into the light portion of the 24-h photoperiod. The increase in FSC-A signal reflects the increase in algal size over the light cycle. Gate P1 was drawn to exclude debris.* ***B.*** *Histogram overlays of BODIPY 505/515-stained samples (red lines) vs unstained controls (black line) from the same cultures. Fold increases (indicated) in BODIPY 505/515 median fluorescence values over control show that that neutral lipid content also increased over the light cycle.* ***C.*** *Overlays of chlorophyll fluorescence for the same samples show that chlorophyll content remained relatively unchanged over the light cycle and was not affected by BODIPY 505/515 staining (Barker J, Cattolico RA, unpub).*

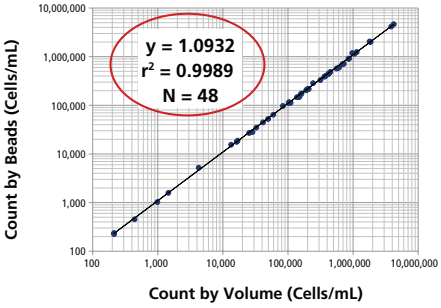

Figure 10. Absolute cell counts measured by two methods on the BD Accuri C6.

Serial dilutions of Jurkat, 3T3, and U937 cells, and T cells, B cells, and platelet samples from four human peripheral blood donors, were counted on the BD Accuri C6 by two methods. X-axis values represent direct-volume measurements, while y-axis values were obtained from counting beads. The high correlation demonstrates the reliability of direct-volume counting.

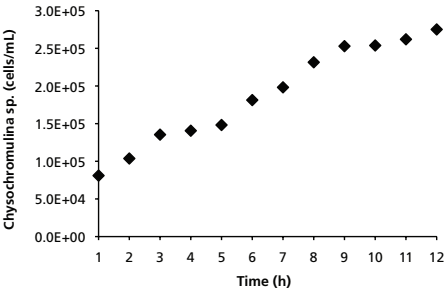

Figure 11. Absolute counts of *Chrysochromulina* sp. cells on the BD Accuri C6.

Cells were cultured as in Figures 8 and 9 and counted by the direct-volume method on the BD Accuri C6. Cell concentration (in cells/mL) increased over a 12-h light exposure (Barker J, Cattolico RA, unpub).

Table 4. Estimated time to count 30 samples using various methods.

Method	Time
BD Accuri C6 (with BD CSampler)	22.5–37.5 min
BD Accuri C6 (manual run)	60 min
Particle Counter	150–262.5 min
Hemocytometer	390 min

Estimates for single samples courtesy of James Barker and Rose Ann Cattolico, PhD, University of Washington, Seattle, WA.

Absolute counting

In addition to assessing the size, chlorophyll, and lipid content of microalgal cells (Figure 9), biofuel researchers and developers also need to assess the cell density of their cultures (in cells/mL). The BD Accuri C6 flow cytometer can provide absolute cell counts directly, eliminating the need to use a hemocytometer or counting beads. Its microprocessor-controlled, peristaltic pump system accurately meters volume (in µL) and reports it in the BD Accuri C6 software statistics tables. Densities of total and gated populations are automatically calculated and can be selectively displayed in tabular format. As shown in Figure 10, the BD Accuri C6 can accurately calculate cell density in samples with concentrations ranging from 1.0 x 10^3 to 5.0 x 10^6 cells/mL (>3-log concentration range), without the need to concentrate or dilute the samples.

Figure 11 shows progressive growth of absolute counts of *Chrysochromulina* sp. cells obtained at hourly intervals during a 12-hour light cycle.

The capacity to count cells while simultaneously collecting data is important in processing large sample sets. Although cell counting is a basic function served by alternative technologies, its integration into the BD Accuri C6 saves valuable time. Table 4 shows estimates of the time required to count 30 samples on the BD Accuri C6 with and without automation, compared to a particle counter or hemocytometer. The BD CSampler™ automation accessory is described in the following section.

Automated flow cytometry

Production and commercialization of algal biofuels will require close monitoring of growth rate, maximum cell density, tolerance to environmental factors, nutrient requirements, strain robustness, and other variables. Frequent data collection or large experimental matrices can produce many samples, requiring rapid population analysis and enumeration.

The BD CSampler adds simple, unattended, reliable, and easy-to-use automation to the BD Accuri C6 flow cytometer system. Automation can significantly increase throughput (Table 4), allowing algaculture researchers to run large experiments that would otherwise be infeasible. The BD CSampler is compatible with both 48- and 96-well plates and deep-well plates, and includes a 24-tube rack for standard 12 x 75- mm tubes. The option adds minimal footprint to the system—about 3 ft^2 (0.28 m^2) for the pair—keeping the benchtop free for other uses.

Figure 12. The BD Accuri C6 with BD CSampler option.

The optional BD CSampler provides automated sample processing from a 24-tube rack or from 48- and 96-well plates.

Summary

Flow cytometry represents a powerful methodology for microalgal biofuel researchers and developers. The BD Accuri C6 personal flow cytometer offers unique advantages, including analysis of organism size and autofluorescence over a wide range, multiparametric lipid analysis using fluorescent stains, direct cell counting capability, and high throughput via automation. Its combination of power, simplicity, and durability make the BD Accuri C6 a valuable tool to help researchers bring the promise of biofuels to fruition.

Acknowledgments

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