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We present a cytometer that measures photosynthetic efficiency and lipid accumulation evaluating diversity within the population and biofuel production potential.
Microfluidic cytometer for high-throughput measurement of photosynthetic characteristics and lipid accumulation in individual algal cells

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Commercially viable algal biofuel production requires discovery of new strains, genetic engineering for higher productivity and optimization of growth conditions. To accelerate research in these areas, we developed a microfluidic cytometer that measures forward light scatter, chlorophyll fluorescence induction and lipophilic stain fluorescence at a rate of 100 cells/sec. The chlorophyll fluorescence data is processed in real-time to measure the fluorescence-based maximum quantum yield, reported as $F_{m}/F_{m}$. We use the cytometer to characterize unstressed (nutrient-replete) and stressed (nutrient-limited) cultures of the marine diatom Phaeodactylum tricornutum and are correlated to values obtained in bulk samples using traditional pulse-amplitude-modulating fluorometry. We then use the cytometer to characterize unstressed and stressed P. tricornutum and show that lipid content (as measured by Nile Red fluorescence) is inversely correlated with $F_{m}/F_{m}$. We believe these findings to be the first time that both photosynthetic efficiency and lipid accumulation have been simultaneously evaluated at the single cell level, and that in doing so, the diversity within these populations was revealed.

Introduction

Algae are a promising source of renewable biofuels because their productivity per given area of land is much higher than that of any terrestrial crop plant, their mass culture requires neither arable land nor freshwater, and their use in generating transportation fuels is projected to have the greatest impact in reducing atmospheric greenhouse gases compared to other biofuels from plants. Significant attention is focused on the potential use of microalgae derived lipids (triacylglycerides) as a renewable source of feedstock for the production of transportation fuels. At this point, research and development is required at all stages of the production process, including characterization of algal strains, genetic engineering for higher productivity, and optimization of growth conditions. These efforts would benefit from new technology for high-throughput screening for algal photochemical energy conversion and lipid accumulation at the single-cell level. Current methods for evaluating algal strains require the growth of algal monocultures that are then subjected to bulk measurements to characterize biofuel production potential. Photosynthetic energy conversion is typically quantified using pulse-amplitude-modulating (PAM) fluorometry techniques that record changes in chlorophyll (Chl) fluorescence upon illumination with actinic light to generate Chl fluorescence induction measurements. These Chl fluorescence induction measurements can then be used to estimate the photochemical quantum yield of photosynthesis. Algal lipid content is often measured using either chromatographic or gravimetric methods. These methods require days per sample because a large number of cells is required to produce sufficient quantities for analysis. In addition, these bulk measurements are averaged over live and dead cells and other material and are incapable of resolving heterogeneity within a culture. These deficiencies generate additional questions that cannot be answered using currently available analytical tools. For example, is there one broadly distributed population or are there multiple modes of productivity? Are the cells performing photosynthesis efficiently, but not producing lipids, or does an unfavorable distribution of light filtration exist within the culture? To investigate these issues, analyses are ideally performed on a cell-by-cell basis.

Chl fluorescence induction in photosynthetic organisms was initially observed and correlated to CO$_2$ assimilation in the 1930’s by Kautsky and Hirsch. Improvements in instrumentation and theory have made Chl induction measurements a laboratory standard to characterize the quantum yield of photosynthesis. The theory of fluorescence induction is based on energy conservation in that the energy of a photon absorbed by a Chl molecule must either be trapped by the reaction centers (RCs) of photosystem II (PSII) to drive electron transfer reactions, or the excitation is dissipated as heat or fluorescence. Under low light conditions, the RCs remain open for excitation trapping and the efficiency of photochemistry remains very high ($>90\%$) with less energy dissipated as heat and fluorescence. If the light flux is sufficiently intense, the RCs will close causing photochemistry to cease and fluorescence and heat dissipation to rapidly rise. Therefore, the observation of Chl fluorescence induction with its inverse relationship to photochemical efficiency provides a rapid and non-destructive method of quantifying the quantum yield of photosynthesis.

For this study, the quantum yield of photosynthesis is
determined from Chl fluorescence induction curves generated by the “pump-and-probe” saturating flash method. In this method a rectangular light pulse is applied to each dark adapted cell with a light intensity that saturates the PSII RCs in tens of microseconds. The initial Chl fluorescence yield (when RCs are open) is designated as \( F_0 \). However, in the light-saturated state created in <100 \( \mu \)sec (when RCs are closed), the fluorescence yield is maximal (\( F_m \)). The difference between \( F_m \) and \( F_0 \) is variable fluorescence (\( F_v \)) that, when normalized to \( F_m \), represents the maximal ceiling for the quantum yield of photosynthesis (\( F_v/F_m = (F_m - F_0)/F_m \)). \( F_v/F_m \) is a well-established, rapidly obtainable and quantitative measure of the quantum yield of photosynthesis.\(^\text{12, 13}\) \( F_v/F_m \) ranges from 0 for dead cells to ~0.65 for healthy algal cells, although this maximal observed value varies somewhat among species and also depends on the excitation and detection conditions.\(^\text{14, 15}\)

Our approach utilizes unique characteristics of microfluidic flow cytometry to combine measurements of algal photosynthetic efficiency and lipid production. Conventional flow cytometers are highly optimized for light scattering and multi-color fluorescence measurements at tens of thousands of cells per sec. However, due to the high flow rate of the hydrodynamic stream in these instruments, the cell/laser interaction time is on the order of one \( \mu \)sec which is too short for measuring photochemical efficiency using time resolved Chl fluorescence induction assays. In contrast, microfluidic chips, with their slower flow rates and stable hydrodynamic sample streams, are ideal for the approximately 100-fold longer cell interrogation times required for these measurements. In addition, the incorporation of microfluidic, diode laser and LED technologies in our design results in an instrument with an inherently small footprint and low power requirements. These characteristics may facilitate future development of a portable device suitable for field studies and environmental monitoring.

Flow cytometers have been successfully developed for use in oceanographic studies and are shown to resolve diverse populations of plankton. This work, though, often focused on increasing the dynamic range of both particle size and fluorescence sensitivity required to examine species typically found in wild type studies.\(^\text{16, 17}\) A highly modified EPIC 753 cytometer has also been used by Olson et al. to acquire Chl fluorescence time-resolved data.\(^\text{18}\) This data was used to generate Chl fluorescence transients for single alga or signals averaged over ~10^4 cells. These graphs were then analyzed post-experimentally to determine \( F_v/F_m \) values. Another approach, developed by Gorbunov et al., measured Chl fluorescence induction using a cell chamber that could generate droplets containing a single cell.\(^\text{19}\) This instrument implemented a fast repetition rate fluorometry technique that necessitated interrogation times ranging from 100 msec to several seconds and also required post-experimental analysis to generate \( F_v/F_m \) values. One of the key innovations in the microfluidic cytometer described here is the ability to calculate \( F_v/F_m \) values on single cells in real-time, thus eliminating the need for post-experimental data analysis.

Quantifying lipid accumulation in bulk algal samples using extraction methods followed by analysis using gas chromatography or high performance liquid chromatography is well developed and capable of accurately identifying lipid classes within the extract.\(^\text{6}\) Extraction methods, though, are complex, equipment intensive and time consuming. More importantly, extraction methods are not useful for single-cell analysis because they require a minimum wet weight sample size of 10 mg.\(^\text{4, 20}\) The use of lipophilic fluorescence dyes, including Nile Red (NR), (9-diethylamino-5H-benzo[alpha]phenoxazin-5-one), BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) and DiO (3',3'-diododecylxocarbocyanine perchlorate), have the advantage that they can be used to measure lipids in single cells, but fluorescence methods are limited in their ability to fully characterize lipid classes.\(^\text{21-24}\) Specifically, BODIPY and DiO preferentially stain neutral and cell membrane structures respectively.\(^\text{25}\) In contrast, NR emission spectra shift depending on the polarity of the environment and can therefore broadly distinguish between neutral lipids that produce emissions of ~580 nm and polar lipids with emissions up to ~640 nm.\(^\text{26}\) NR dye does have the disadvantage of reduced uptake in some algal species which has been attributed to cell wall structure and composition.\(^\text{21, 27}\) The use of NR in many diatoms, though, has been characterized and is therefore well suited for use in this study using Phaeodactylum tricornutum as the model organism.\(^\text{20}\)

For these reasons, we chose to quantify lipid accumulation by detecting NR fluorescence in a second channel both temporally and spectrally separate from the Chl fluorescence channel. However, the second fluorescence channel may be configured for the detection of many other lipophilic dyes or fluorescence markers for diverse analytes.

Here we describe the design and operational characteristics of a microfluidic cytometer that measures forward light scatter (FS), \( F_v/F_m \) and fluorescence intensity from a lipophilic stain (NR) from each cell, in real-time, at a rate of 100 cells/sec. To demonstrate instrument performance, \( F_v/F_m \) measurements are obtained for unstressed (nutrient-replete) and stressed (nutrient-limited) cultures of the marine diatom P. tricornutum, and we correlate these results to values obtained for bulk samples using traditional PAM fluorometry. We then use the cytometer to measure photosynthetic efficiency and lipid accumulation in unstressed and stressed cells and show that lipid content is inversely correlated with the value of \( F_v/F_m \). By performing these measurements at the single cell level we are able, for the first time, to quantify diversity within these populations. We are optimistic that this cytometer will prove useful in characterizing new algal strains and optimizing productivity of algal biofuels.

### Materials and Methods

The cytometer consists of microfluidic/fluid-handling, optics, electronics and data acquisition/control sub-systems, each of which is described in separation sections below, followed by a description of the operational characteristics and experimental methods.

#### Microfluidics/Fluid-handling

A custom designed, 2D-hydrofocusing microfluidic chip was fabricated (Micronit, The Netherlands) from fused silica (Figure S1). It has a cross-channel configuration with two sheaths, one sample and one waste stream channels that are 90 \( \mu \)m in height and 180 \( \mu \)m in width. The chip has an overall thickness of 1.8
Optics

The microfluidic chip is positioned within an optical path similar to that of an inverted fluorescence microscope, with epi-illumination from a 470 nm LED (Philips Luxeon III Star LXHL-LB3C; luminous flux 23 lm) (Figure 1). This LED provides excitation to Chl and NR channels. Trans-illumination of the sample stream by an 80 mW, 785 nm laser diode (Sanyo DL7140-2015) is used for event triggering by forward light scattering at a wavelength that is unable to excite Chl or NR. This IR/event-trigger laser is coupled with cylindrical optics to a 6.8 μm FWHM line that intersects the entire channel width, thus ensuring that all cells are detected. An obscuration bar blocks the focused beam after transmission through the microfluidic chip and forward-scattered light is detected by an AC-coupled Si photodiode. The low power outputs of the excitation FS diode laser and the 470 nm excitation LED plus the short time period (< 300 μsec) cells are exposed to light ensures that potential temperature fluctuations are negligible. To align and focus sample flow through the microfluidic chip, a removable mirror directs the channel image to a CMOS camera (Marlin F131B, Allied Vision Technologies) using illumination from the laser diode. Additional details of the optical schematic are located in the ESI.

Electronics

Timing and control of the diode laser, LED, and detectors is accomplished with both custom-built circuitry and software. Although Chl excitation intensity is operator controllable from 0.3 to 1.9 W/cm², in these experiments, Chl is excited at 0.8 W/cm². This intensity provides the desirable 20 - 40 μsec rise time in the P. tricornutum used in this study and is similar to the 1.0 W/cm² used by Gorbunov et al. in their Fast Repetition Rate Fluorometer in the examination of Chlorella pyrenoidosa.19

In contrast to the Chl fluorescence channel, the excitation LED was operated in a digital mode to create a higher, but shorter, current pulse that provides an intensity of ~38 W/cm². Though this higher excitation intensity would result in an unacceptably fast rise time if used to measure induced Chl fluorescence, it is desirable in the NR fluorescence channel to improve the signal-to-background ratio (S/B). As in the microfluidic cytometer shown here, Olson et al. also employed dual-intensity excitation beams in a Becton Dickinson FACScan cytometer that was modified to examine natural phytoplankton populations.20 In their cytometer, the low-intensity beam was used to examine Chl fluorescence, and a higher-intensity excitation beam was used in a “classification” channel that examined the natural fluorescence of these mixed populations. These high- and low-intensity excitation beams were generated from a single argon ion laser and spatially separated using an 80:20 beamsplitter and independent optical paths. In contrast, with the microfluidic cytometer described here, we were able to temporally separate the high and low beams by taking advantage of the fast response time of the excitation LED and using software to control the analog/digital LED driver.

Because of the higher excitation intensity of the digital pulse, Chl fluorescence can potentially exceed the safe operating level of the Chl photomultiplier tube (PMT) since it is operated at high gain to measure the weak fluorescence signal. To prevent damage to the Chl PMT during NR fluorescence excitation, a custom-built gating circuit was implemented to disable the PMT during the digital pulse. To eliminate a background contribution from the 785 nm diode laser in the Chl channel, the current source driving the trigger laser is also provided with a modulation circuit for turning it off during the Chl and NR fluorescence induction measurements. The diode laser is returned to an active state after data acquisition and processing is completed for each event.

The photocurrent from each PMT is processed by custom-built AC-coupled trans-impedance operational amplifiers having a 10 μA to 1 VDC current to voltage conversion. Output signals are conditioned with a 0.16 Hz high-pass filter that improves the signal to noise ratio and a 500 kHz low-pass filter providing signal smoothing.

Data acquisition/control software

The signal voltages are digitized at 500 kHz with a data acquisition board (NI 6251, National Instruments) and LabView 8.6 (National Instruments) was used to develop the control software. The software design incorporates an operator interface to adjust (i) FS, Chl and NR data acquisition periods, (ii) event triggering levels, (iii) timing delays after event detection, (iv) Chl excitation flash intensity and (v) state of the Chl PMT.

Data output includes real time display of FS, Chl and NR fluorescence, Fm and F/Fm histograms (Figure S2). Also, a real-time graph is updated for each event showing FS, Chl and NR signals versus time. Output files are exported at the end of each run that contain complete datasets for FS, F/Fm, Chl and NR values that can be used, if desired, in post-run data analyses.
Operational characteristics/timing

The event sequence and timing for each data collection event are shown in Figure 2. In the initial state, before a cell arrives, the IR laser is on, and the LED is off. When a cell enters the interrogation region, the FS signal is detected by the photodiode, the IR laser is switched off, the Chl PMT is switched on, and the LED is switched on to the appropriate intensity to measure Chl fluorescence induction. The rise time of the LED, as measured by a Si photodiode, is < 2 µsec. After a sufficient interval to ensure the Chl fluorescence signal is saturated (typically 20 - 100 µsec), the Chl PMT is switched off, and the LED current is stepped up to higher power. After another variable interval (typically 10 - 50 µsec) during which the average fluorescence signal for the NR channel is determined, the instrument is reset for the next measurement event: the IR laser is switched on, and the LED is switched off. This measurement sequence on each cell can occur in less than 200 µsec, thus setting a maximum theoretical throughput for this instrument of 5000 cells/sec, but the current configuration is limited to 100 cells/sec to allow sufficient time for the control software to perform data analysis and update the display.

Growth conditions and sample preparation

Cultures of *P. tricornutum* (UTEX 646) were grown in 150 ml borosilicate flasks in f/2 medium at 25° C. Illumination using 12 hr alternating light/dark cycle was provided by a 15 W cool-white fluorescent bulb (GE F16T12CW) at a distance of 25 – 30 cm. Unstressed cultures were passaged 1:20 approximately 7 days before each experiment. Physiologically stressed cultures were prepared by a 1:10 passage and grown to a nutrient depleted condition in approximately 21 days. Samples of both unstressed and stressed cultures were prepared by passing 1 ml through a 40 µm cell strainer. To quantitatively determine lipid content the samples were stained with 30 µM NR solution in acetone at a 1:100 dilution. Samples were dark adapted for a minimum of 5 min prior to analysis.

Bulk F$_{v}$/F$_{m}$ measurements

Bulk measurements of F$_{v}$/F$_{m}$ were measured with a Walz PAM fluorometer with excitation at 455 nm and detection with a 710 nm longpass filter. For these measurements, 6 ml of algal culture was centrifuged and the remaining pellet was transferred to filter paper (Wattman GF/F porosity 0.45 microns). Cells were dark-adapted for a minimum of 2.5 minutes before measurements, which were performed on three separate sample preparations. In these measurements, low intensity excitation is used to obtain the F$_{v}$ fluorescence value, and a high intensity flash saturates the photosystems thus providing the F$_{m}$ fluorescence value. The measured fluorescence signals are corrected for background fluorescence from the filter paper and medium at both intensities.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare differences among groups of three or more followed by Tukey’s *post hoc* test to assess differences within groups. Significance between two groups was demonstrated by one-tailed t-test. P-value defined as p.

Results and Discussion

Data acquisition and analysis

The major instrument design challenge is a result of conflicting requirements for the Chl fluorescence induction measurement and the NR emission measurement. To address this issue, the cytometer incorporates an LED excitation source with an intensity that is controlled during the data acquisition on each cell. The NR measurement, that takes place after Chl measurements, uses a relatively short, but high intensity, excitation pulse. In contrast, the excitation pulse to generate the Chl fluorescence signal from each cell must be much lower, falling within a narrow regime. As discussed by Olson et al., the intensity and duration of the Chl excitation is set to ensure that the PSII RCs are closed in tens of µsec, so that accurate values of F$_{v}$ and F$_{m}$ are obtained. If the excitation intensity is insufficient, PSII does not saturate, and the value of F$_{m}$ is not attained. If excitation intensities are too high, Chl radical formation causes a decrease in the fluorescence transient, so F$_{m}$ is again underestimated. We observe single Chl fluorescence transients, and adjust the intensity and duration of the low-power excitation pulse to ensure the appropriate fluorescence saturation kinetics are observed. This procedure allows the operator to optimize the excitation configuration for different growth conditions and for species having different photosynthetic properties.

Analysis of each event is performed and displayed before resetting the FS event trigger in preparation for the next event. Though complete datasets for each event are stored in memory for export, the data for each event are also analyzed for real time display. The FS dataset is analyzed to find the peak value. This value is used to update a FS histogram displayed on the monitor. For the NR channel, the digitized signal is averaged for the time period of high excitation intensity, and this value is used to update the NR histogram. The last half of the Chl dataset is averaged and then used to update the F$_{m}$ histogram.

Determining real-time values of F$_{v}$ requires more extensive data analysis. In previous investigations, F$_{v}$ was obtained from fluorescence induction by using manual curve fitting techniques.
during post-experimental analysis assuming an exponential rise in Chl fluorescence.\(^3\) In contrast, our goal was to perform real-time calculations to determine the \(F_{m}/F_{m}'\) value of each cell during the experimental run. A key step in this approach is associating accurate time points to the Chl signal data. The time points consider delays inherent in the system that are comprised of multiplexing delays during data acquisition, the response time of the LED driver and the signal response of the transimpedance amplifier. These corrected time points are then correlated to the first four data points of the Chl dataset and used in a least-squares, linear fit algorithm that generates an equation relating Chl fluorescence to time. Though the linearization algorithm is an approximation of the exponential induction of Chl fluorescence, it is computationally efficient, and \(F_{0}\) is easily determined by extrapolation to the \(t = 0\) time point. Figure 3 demonstrates the implementation of this approach to calculate \(F_{m}/F_{m}'\) using data for a single cell from an experimental run.

Although the \(F_{m}/F_{m}'\) results from the cytometer for unstressed cells correlate well to the values obtained from a commercial Chl fluorometer, the cytometer exhibits slightly higher \(F_{m}/F_{m}'\) values in stressed cells. There are several possible explanations for this inconsistency. A simple explanation may be that the linearization method, used to determine \(F_{0}\), slightly underestimates this value (causing an overestimation of \(F_{m}/F_{m}'\)) in cells that have a lower fluorescence induction (e.g., under stressed conditions). Thus, \(F_{m}/F_{m}'\) values will be slightly inflated. Another explanation may be that there is a difference in the in the range of fluorescence wavelengths detected by the two instruments. The Chl channel in the cytometer employs a 775/46 nm bandpass filter, that selectively detects fluorescence only from PSII, whereas the fluorometer measurements employed a 710 longpass filter, which may detect emissions from both PSI and PSII. Since the former has a lower quantum efficiency\(^3\), it is to be expected that selective measurements of PSII will observe higher values of \(F_{m}/F_{m}'\). Note that it is generally not possible to selectively measure PSII in bulk (e.g., in leaves) because the fluorescence is reabsorbed by the longer-wavelength pigments in these optically-dense conditions. In contrast, the cytometer operates on single cells, which are optically thin, so fluorescence is not re-absorbed by other photosynthetic components of each cell.

**Precision of FS and fluorescence intensities**

Coefficient of variation (CV), a measure of signal dispersion using the standard deviation normalized by the mean, was used to determine the precision of the FS and fluorescence channel signals. Datasets for each channel were generated using commercially available, fluorescent calibration beads (10 \(\mu\)m diameter) (6605359, Beckman Coulter). Chauvenet’s criterion was used to eliminate the calibration bead doublets from the datasets.\(^3\) FS measurements, using the peak signal for each event, result in an 11.6% CV. This level of performance is similar to reports of CVs that range from 8% to 30% in microfluidic devices with similarly sized channels and 10 \(\mu\)m calibration beads.\(^3\) For the NR fluorescence channel, measurements demonstrate an 11.2% CV when the signal is averaged over 10 \(\mu\)sec and excitation is 38 W/cm\(^2\). Chl fluorescence channel measurements demonstrate a 9.0% CV using an excitation intensity of 0.8 W/cm\(^2\) with the signal is averaged over 20 \(\mu\)sec. These values are also comparable to previously reported CVs that range from 6% to 16% in microfluidic devices with channels of similar dimensions using 6 to 12 \(\mu\)m calibration beads and either a microscope objective having a high numerical aperture or an optical waveguide detection system.\(^3\) In summary, the precision of FS scatter and fluorescence intensity measurements in this instrument is comparable to other microfluidic cytometers of similar configuration.

**Bulk versus single cell \(F_{m}/F_{m}'\) measurements**

Nutrient limitation has been linked to decreases in photosynthetic energy conversion efficiencies in multiple algal species.\(^5\) We utilize this phenomenon to characterize the accuracy of the cytometer photosynthetic efficiency measurements by comparing averaged \(F_{m}/F_{m}'\) values for cultures of \(P.\) tricornutum in unstressed (nutrient-replete) and stressed (nutrient-limited) cells to bulk
Fig. 5 Combined cytometric data for unstressed and stressed cultures of *P. tricornutum* showing differences in the photosynthetic quantum yield ($F_v/F_m$) and lipid content (NR fluorescence) of cells. Stressed cells show decreased $F_v/F_m$ values and increased NR fluorescence compared to unstressed cells. (A, B, C) Histograms for FS, NR and $F_v/F_m$ (respectively), (D) $F_v/F_m$ vs. NR scatter plot with each data point representing a single cell.

$F_v/F_m$ measurements obtained from a commercial PAM fluorometer (Figure 4). Statistically significant decreases in $F_v/F_m$ values for the stressed samples are observed in both the cytometer (-41.2%) and fluorometer (-53.4%) measurements (bar 1 vs. 2 and bar 3 vs. 4). Importantly, in the unstressed sample, no difference is observed between measurement methods as determined with Tukey’s post hoc test ($p \leq 0.05$). However, for the stressed sample, a significant increase (4.29%) is observed between the two measurement methods ($p > 0.05$). Thus, the data show that (i) differences in $F_v/F_m$ values between unstressed and stressed samples are detected by both the cytometer and the fluorometer, (ii) in unstressed samples, the cytometer accurately replicates the $F_v/F_m$ values of the commercial fluorometer and (iii) in stressed samples, averaged $F_v/F_m$ measurements observed in the cytometer are slightly higher than values obtained from the commercial PAM fluorometer.

**Correlation of $F_v/F_m$ with lipid content**

Previous studies have revealed correlations between quantum yield and intercellular carbon-to-nitrogen ratios in some algal species. In one study, Berges *et al.* investigated the correlation between nitrogen stress (N-stress) and $F_v/F_m$ values the marine phytoplankton *Thalassiosira weissflogii* and *Dunaliella tertiolecta*. This group reported up to 3.5-fold increases in carbon to nitrogen ratios (as measured by CHN analysis) and up to 3.5-fold decreases in $F_v/F_m$ over an 18-day period of N-stress in *D. tertiolecta*. In another study, Stehfest *et al.* utilized Fourier transform infrared (FTIR) spectroscopy to examine multiple phytoplankton species that included *P. tricornutum*. A portion of this study examined the ratio of FTIR absorption band indentifying lipids (1780-1708 cm$^{-1}$) to the amide II band identifying the N-H bond of amides in proteins (1575-1480 cm$^{-1}$).
Nitrogen depleted *P. tricornutum* cultures were shown to have increasing lipid/amide II ratios and decreasing quantum efficiencies over the initial 28 days of the experiment. Neither of these studies attempted to quantify lipid accumulation. Other research has quantified lipid accumulation in several algal species when subjected to nutrient limitations, but this work did not report photosynthetic energy conversion efficiencies.\(^2\),\(^3\)

For this study, we directly investigated the relationship between lipid accumulation and photosynthetic energy conversion efficiencies by collecting data on unstressed and stressed cultures of *P. tricornutum*. Separate measurements were performed on 1765 unstressed and 2001 stressed individual cells using 20 μL of each culture that were additionally diluted with 1 mL of culture medium to achieve sample concentrations of ~20,000 cells/mL. As anticipated, evaluation of peak FS signals shows no significant difference for the unstressed (0.107 a.u. (s.d. 0.024)) and stressed (0.104 a.u. (s.d. 0.028)) samples (p ≤ 0.05) suggesting algal cell size was not affected by the short time period required to stress the culture (Figure 5A). In contrast, the average Fc/Fl for unstressed cells was 0.532 (s.d. 0.088) compared to 0.382 (s.d. 0.083) for stressed cells demonstrating a 28.2% decrease (Figure 5B). The average NR fluorescence signal for the unstressed sample was 0.285 a.u. (s.d. 0.021) and 0.897 a.u. (s.d. 0.171) for the stressed sample demonstrating a 3.2-fold increase (Figure 5C). The datasets for unstressed and stressed cells were also combined to create a scatter plot in which each data point represents the Fc/Fl and NR fluorescence of a single algal cell (Figure 5D). With the scatter plot, it is easily discerned that stressed cells do have significantly lower photosynthetic efficiency, yet higher lipid content than the unstressed cells, and thus show that nutrient-limited *P. tricornutum* exhibit an inverse correlation between lipid content and quantum efficiency. Though this correlation is suggested from the previously cited research, we do not believe this phenomenon has been directly reported. Additionally, we note that previous studies investigating aspects of lipid content, C:N ratios and photosynthetic efficiencies, were performed in bulk samples, and by using the microfluidic cytometer the diversity within these populations is now revealed.

**Conclusions and future work**

We have developed a microfluidic cytometer that measures photosynthetic efficiency (as Fc/Fl) and lipid accumulation (as NR fluorescence) on single algal cells in real time. Previous methods for evaluating these parameters typically required large samples and time-consuming analytical techniques. Additionally, when measurements have been performed on single cells, evaluation of Fc/Fl was determined by time-consuming, post-experimental analysis. We used the cytometer to evaluate *P. tricornutum* in nutrient stressed cells, and demonstrated both a decrease in Fc/Fl values and an increase in NR fluorescence when compared to unstressed cells. Interestingly, within the observed distribution of the stressed population, a number of algal cells retain photosynthetic efficiencies comparable to that of unstressed cells, yet exhibit substantial lipid accumulation. This has significant implications for biofuels productivity, and it would clearly be of interest to isolate these cells using high-throughput screening techniques to examine the genetic basis of these physiological differences. We believe these findings to be the first time that both photosynthetic efficiency and lipid accumulation have been simultaneously evaluated at the single cell level, and that in doing so, the diversity within these populations was revealed.

With the ability to characterize the diversity of both quantum yield and lipid accumulation within a single population we anticipate the cytometer will have immediate applications in algal research. We believe this technology will accelerate identification of promising wild type and mutant organisms directly leading to improved strains. We foresee potential applications in areas that include characterization of mixed cultures in bioprospecting expeditions, genetic/functional analysis, strain improvement and optimization of commercial-scale photobioreactor/raceway pond operations.

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**Notes and references**

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