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Label-free measurement of algal triacylglyceride production using fluorescence hyperspectral imaging ☆

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ABSTRACT

Microalgae have been identified as a promising renewable feedstock for production of lipids for feeds and fuels. Current methods for identifying algae strains and growth conditions that support high lipid production require a variety of fluorescent chemical indicators, such as Nile Red and more recently, Bodipy. Despite notable successes using these approaches, chemical indicators exhibit several drawbacks, including non-uniform staining, low lipid specificity, cellular toxicity, and variable permeability based on cell-type, limiting their applicability for high-throughput bioprospecting. In this work, we used in vivo hyperspectral confocal fluorescence microscopy of a variety of potential microalgae production strains (*Nannochloropsis* sp., *Dunaliella salina*, *Neochloris oleoabundans*, and *Chlamydomonas reinhardtii*) to identify a label-free method for localizing lipid bodies and quantifying the lipid yield on a single-cell basis. By analyzing endogenous fluorescence from chlorophyll and resonance Raman emission from lipid-solubilized carotenoids we deconvolved pure component emission spectra and generated diffraction limited projections of the lipid bodies and chloroplast organelles, respectively. Applying this imaging method to nutrient depletion time-courses from lab-scale and outdoor cultivation systems revealed an additional autofluorescence spectral component that became more prominent over time, and varied inversely with the chlorophyll intensity, indicative of physiological compromise of the algal cell. This signal could result in false-positives for conventional measurements of lipid accumulation (via spectral overlap with Nile Red), however, the additional spectral feature was found to be useful for classification of lipid enrichment and culture crash conditions in the outdoor cultivation system. Under nutrient deprivation, increases in the lipid fraction of the cellular volume of ~500% were observed, as well as a correlated decrease in the chloroplast fraction of the total cellular volume. The results suggest that a membrane recycling mechanism dominates for nutrient deprivation-based lipid accumulation in the microalgae tested.

1. Introduction

Biochemical conversion of lipids into high energy density forms, especially triacylglycerol (TAG), by algae is among the most highly sought-after traits for commodity-scale algae culture. Numerous species of algae show considerable promise as biofuel producing candidates and have been shown to accumulate quantities of lipids between 20 and 50% of dry weight [1,2], as well as other noteworthy properties, such as high biomass productivity, tolerance to high salinity and temperature variation, ease of harvest, and predation resistance (for reviews see [2–4]). Extensive efforts have been applied in characterizing the lipid profile of algae of interest for biofuels and other applications [5,6]. With the exception of a few recent studies employing minimally invasive and single-cell spectroscopic methods [7–9], extraction-based studies have primarily been used for assessing the lipid profile of algae [10,11]. However, the results of such studies suffer from high variability based on the solvents employed for lipid extraction [12] and the inability to be used in high-throughput screening.

The fluorescence dye Nile Red (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one) has frequently been used as a stain of neutral lipids [13,14]. Nile Red staining has been used successfully in a number of fluorescence spectroscopic investigations of algae by showing a linear relationship between fluorescence intensity and lipid quantity [14–17]. Nile Red staining protocols for lipid quantity have been developed using bulk, plate reader, fluorescence microscopy, and flow cytometry formats [14,18]. Nevertheless, the use of Nile Red can be problematic for several reasons. First, Nile Red staining of lipids is highly variable among different species of alga [16]. For example, members of the

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genus *Nannochloropsis* are strong candidates for biofuel development, however intracellular uptake of Nile Red is highly variable [19,20]. Other species that are readily stained by Nile Red have dramatically different uptake kinetics and useful concentration windows [21], requiring that the staining protocol be optimized for each strain under investigation [16,19,22]. Second, Nile Red is often solubilized in acetone and can be toxic to algal cells in low concentrations [23] greatly limiting the possibilities for using this probe in in situ studies. On the other hand, protocols using dimethyl sulfoxide (DMSO) as a solvent have been established with reported greater reliability of the stain and viability of the organism [5,16]. Nile Red has also been observed to preferentially stain cells with compromised cellular envelopes, and alter osmotic responses leading to greater uncertainty in using this stain in assessing lipid content.

A more favorable means to assess lipid quantity would be with the use of an endogenous reporter of lipid and to measure the oil content directly. Efforts have been made to address this point by means of Raman microscopy where the molecular vibrational modes of lipid bodies in individual algal cell were detected and quantified [9,24,25]. However, while these reports have shown the utility of detecting lipid in a label-free manner, the lipid volume content on a per cell basis has not been demonstrated and would be useful in determining the lipid quantity.

In this work we sought to use a different endogenous marker for algal lipid quantification; resonance enhanced Raman and fluorescence signatures from carotenoids. Similar to TAG and fatty acids, carotenoid synthesis can be triggered by stress conditions [26]. Indeed, the biosynthesis for TAG is usually interdependent with secondary carotenogenesis in algae [27–29] as secondary carotenoids are esterified with TAG and stored in cytosolic lipid vesicles outside of the chloroplast [30]. Thus, quantification of carotenoid, outside of the chloroplast, provides a highly sensitive, label-free and specific method for tracking lipid production in algal cultures.

Hyperspectral confocal microscopy provides a sensitive analytical tool for simultaneously quantifying total algal biomass, lipid content, and the concentration of other high-value algal products, including carotenoids [31]. In this study, we employed hyperspectral fluorescence microscopy with multivariate analysis to track the morphological dynamics and local concentrations of chlorophyll, carotenoids, and lipids in multiple microalgal species, including *Nannochloropsis* sp., *Neochloris oleoabundans*, *Dunaliella salina*, and *Chlamydomonas reinhardtii*. By quantifying the volume of carotenoid-containing globules outside the chloroplast we have determined the absolute amount of stored lipid on a per cell basis and compared these results to analysis using traditional Nile Red staining. This imaging strategy should be generally applicable to novel and recently isolated strains of interest for algal biofuel development, and amenable to grab-sampling assays from large-scale algae biomass production systems.

2. Materials and methods

2.1. Algae strains and culture conditions

Nannochloropsis sp. (Eustigmatophyceae) cultures were grown in NC-media, an enriched artificial seawater medium [32] supplemented with additional sodium nitrate and sodium bicarbonate to generate a broad range of initial nutrient conditions. Specifically, condition 1 contained 1.55 mM nitrate and 2.1 mM and bicarbonate, condition 2 contained 1.55 mM nitrate and 7.1 mM bicarbonate, condition 3 contained 21.55 mM nitrate and 2.1 mM bicarbonate, and condition 4 contained 21.55 mM nitrate and 7.1 mM bicarbonate. *C. reinhardtii* (Chlorophyceae) cell wall-deficient mutant cw92 and wild type strains cc124 & 125 carrying the nit1/nit2 mutation conferring inability to grow on nitrate as the sole nitrogen source were grown in Sueoka's High Salt medium [33]. *D. salina* (Chlorophyceae, UTEX 20/21) cultures were grown in 2X Erdschreiber [34], a mildly enriched carbon (0.5 mM) and nitrogen (2.3 mM) medium. *N. oleoabundans* (Chlorophyceae, UTEX 1185) was cultured in fresh water Bristol media supplemented with 0.05 mM $CaCO_3$ and sterile filtered soil water. Culture tubes were positioned in the outer slots of a Roller drum and placed next to a fluorescent tube illuminator to simulate a continuous 12 h diurnal cycle, corresponding to photosynthetically active radiation (PAR) levels up to 100 μE. Initial culture densities were standardized to an optical density at 700 nm of 0.3 (OD_{700}), measured with a Beckman Coulter DU800 spectrophotometer, and four replicates of each culture were maintained. Cultures maintenance was performed by sub-culturing aliquots of each strain at mid- to late-log phase growth as required.

A laboratory culture of *Nannochloropsis* sp. was used to inoculate two ponds (west and east) at a greenhouse located in southern Albuquerque, NM. Experiments consisted of imaging sample aliquots from the two ponds during a two-week period of time in November 2011. The two ponds were operated at different CO_2 levels. For the first week, the west pond was bubbled at 3 scfh CO_2, while the east pond only had ambient CO_2 levels. For the second week the CO_2 levels were swapped for the two ponds. Following this two week experiment, the ponds were subject to a simulated culture crash by addition of ~0.005% (v/v) sodium hypochlorite.

2.2. Sample preparation and Nile Red staining

For data collection, 250 μL aliquots were removed from each culture, with alternating sampling among the 4 replicates on a daily basis. Nile Red staining was performed by adding a 5 μL bolus of a 5 mg/mL acetone solution of NR to 250 μL of algae sample. The NR exposed algae samples were briefly vortexed and incubated for 10 min prior to imaging. Samples were then gently centrifuged to concentrate the cells, which were subsequently transferred to an imaging slide, and sealed under a #1.5 coverslip. Following a brief (5 min) settling period, images were collected for ~20 min before a new sample was prepared. A total of 10 imaging experiments were conducted over a 16 day time period.

2.3. Hyperspectral confocal fluorescence microscopy

High spectral resolution imaging and confocal sectioning was performed using a custom 3D hyperspectral confocal fluorescence microscope [35]. In this system, a 488 nm continuous wave laser is used for raster illumination covering a 12.5 × 12.5 μm field of view using a 60×, 1.4 NA Plan Apo objective (Nikon, Inc.), corresponding to a maximum excitation power of up to ~15 kW/cm^2 at the sample. At each imaging voxel, a spectrum is collected with resolution of 1–3 nm over a 500–800 nm wavelength region at a rate of 4160 spectra/s. This spectral imaging rate is achieved using a high performance galvanometer (Cambridge Technology, Inc.) triggered via hardware synchronization with an EMCCD camera (Andor, Inc.). Spatial resolution under these conditions is 250 nm in the XY plane (2–3 pixels), and ~500 nm in the Z direction. Emission wavelength calibration was performed using a hollow-cathode Kr+ lamp, and dark images were collected at regular intervals during data collection to correct for instrument offset and background signals [36].

Due to excessively large fluorescence observed from the photosynthetic pigments (>600 nm) compared to other components, a dual image data collection approach was employed to provide data appropriate for multivariate analysis. For the first stage of data collection, laser excitation was performed at low laser power (50 W/cm^2 at the sample) which allows discrimination of the photosynthetic pigments without detector saturation or significant photobleaching of the pigments. For the second stage of data collection the maximum laser power (~15 kW/cm^2) was employed in coordination with an absorptive filter (Newport BG-40) placed in front of the spectrometer to attenuate the signal from the photosynthetic pigments, while minimally perturbing the bluer emission components, especially the resonance Raman signal originating from carotenoids.