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Abstract

The use of microalgae as a biofuel feedstock is highly desired, but current methods to induce lipid accumulation cause severe stress responses that limit biomass and, thus oil yield. To address these issues, a high throughput screening (HTS) method was devised to identify chemical inducers of growth and lipid accumulation. Optimization was performed to determine the most effective cell density, DMSO and Nile Red (NR) concentrations to monitor growth and lipid accumulation. The method was tested using 1717 compounds from National Cancer Institute (NCI) Diversity Set III and Natural Products Set II in *Chlamydomonas reinhardtii*. Cells were inoculated at low density and 10 μM of the test compound was added. After 72 h, cell density was measured at OD550 and lipid accumulation assessed using NR fluorescence. Primary screening identified 8 compounds with a hit rate of 0.47% and a robust Z′ discrimination factor (0.68 ± 0.1). Of these, Brefeldin A (BFA) was the most successful at inducing lipid accumulation and was used to evaluate secondary screens including measuring levels of fatty acids, photosynthetic pigments, proteins and carbohydrates. The effectiveness of BFA was confirmed in *Chlorella sorokiniana* UTEX 1230. This study demonstrates the power of chemical genomics approaches in biofuel research.

Keywords: Microalgae, Biofuels, Screening, Lipid production

1. Introduction

In recent years, the microalgae, a diverse group of photosynthetic organisms, have been proposed as a feedstock for biofuel production over traditional crops [1]. Algal niche habitats include marine environments, freshwater, desert sands and more extreme environments such as hot springs and cold environments [2]. Algae also demonstrate a physiological plasticity allowing them to adapt to different environmental conditions. This may be attributed, in part, to their ability to synthesize a complex complement of lipid species, as well as several unusual protective compounds [3]. Eukaryotic microalgae accumulate storage lipids (i.e. triglycerides (TAG)) when subjected to different forms of stress such as nutrient starvation (e.g., nitrogen or phosphorous). Nitrogen starvation has been well documented to induce TAG accumulation widely, however, these conditions eventually terminate growth [4–7]. Similar TAG accumulation was observed following phosphorous limitation in *Pavlova lutheri*, *Isochrysis galbana* and *Phaeodacylum tricornutum*. In contrast, phosphorous limitation was correlated with decreased lipid contents in *Nannochloris atomus* [8]. Temperature stress generally effects the fatty acid (FA) composition of microalgae without increasing lipid accumulation [9]. At high temperatures saturated fatty acids (SFAs) are increased relative to unsaturated fatty acids (UFAs) while, at low temperature UFA is increased relative to SFA. Salinity has also been reported to induce lipid accumulation in microalgae as observed in *Dunaliella teritolecta*. In this case TAG content increases when salt concentration is increased up to 4 M coincident with increasing total SFA and monounsaturated FAs (MUFAs) and a reduction in polyunsaturated FAs (PUFAs) [10]. There are also reports that alkaline pH stress leads to TAG accumulation in *Chlorella* CHLOR1 independent of nitrogen or carbon limitation [11]. Additionally, heavy metals including cadmium, iron, zinc and copper induce lipid accumulation in *Euglena gracilis* [12]. Each of these stress conditions commonly terminates growth and reduces photosynthetic complex components coincident with TAG synthesis and storage. In the present work, we sought to develop a screen useful to identify small compounds in a high throughput format that could induce TAG production in algae while maintaining growth states more comparable to non-stressed conditions.

High-throughput screening (HTS) of chemical compounds to identify modifiers of molecular targets and cellular processes has become a central component of the drug discovery process. This approach, referred to as chemical genomics, is a synthetic ligand-driven method that is directed to alter specific cellular metabolic activities [13–15]. This approach has been previously used in a comprehensive portfolio of applications such as the identification of inhibitors of cancer stem cells [16], inhibitors of enzymes [17], identification of modulators of fat storage in *Caenorhabditis elegans* [18], and inhibitors of fatty acid uptake into cells [15,19]. These types of approaches have not been widely adopted in eukaryotic green algae, yet there are several key studies attesting to

the power of this approach. In *Chlamydomonas reinhardtii*, a HTS was used to identify small molecule modulators of growth, motility and photosynthesis [20]. In studies addressing lipid metabolism in four strains of oleaginous microalgae (*Nannochloropsis salina*, *Nannochloropsis oculata*, *Nannochloris* sp., and *Phaeodactylum tricornutum*), a small library of known kinase inhibitors, fatty acid synthetase (FAS) inhibitors and oxidative signaling molecules was used in phenotypic screening to identify classes of compounds that increase growth and lipid production [21]. There had been concerns that the hydroxyproline rich cell wall of *Chlamydomonas* might not amenable for small molecule transport across the cell wall, but recent studies have shown this does not pose a significant barrier [20].

In the present work, we developed a small molecule phenotypic screen to identify compounds that induced lipid accumulation while maintaining growth and minimizing induction of stress response pathways. To evaluate this screening method, we employed two test libraries obtained from the National Cancer Institute (NCI), Diversity Set III and Natural Products Set II. Our results demonstrate that a single assay platform based on a classical live cell drug screening approach can be used to identify small chemical inducers of lipid accumulation. The simultaneous screening for both lipid and biomass accumulation in the presence of compound is ideal and will aid in the elimination of those that induce stress response pathways. Employing a small library design to test these high-throughput screening methods, we were able to demonstrate the feasibility and power of this approach with the eventual goal of screening large chemically diverse libraries to ultimately yield viable candidates that induce lipid accumulation with minimal impact on biomass accumulation.

2. Materials and methods

2.1. Chemicals and materials

All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Nanopure water at 18 Ω was obtained from Milli-Q Millipore (Millipore, Milford, MA). Clear transparent 96- well U-bottom plates were used for growing cells and black-walled flat bottom plates for fluorescent assays were obtained from BD Falcon.

2.2. Organism and growth conditions

C. reinhardtii CC125 wild type strain was obtained from the *Chlamydomonas* stock center (http://www.chlamy.org/). Prior to screening, cells were maintained in Tris Acetate Phosphate (TAP) media at 25 °C with a photon flux density of 54 μmol m^{-2} s^{-1} in 250 mL Erlenmeyer flasks with a rubber stopper adapted for facilitating gas exchange. Ammonium chloride was omitted to yield TAP N− when nitrogen limitation was required to induce lipid accumulation. Flasks were maintained in a horizontal orbital shaking growing chamber (120 rpm; Innova 43, New Brunswick). For screening, cells were dispensed at low cell density to 96-well microtiter plates as detailed below. Similarly, *Chlorella sorokiniana* UTEX 1230 was maintained in TAP media and grown as detailed above.

2.3. Screening library selection

Two compound-screening libraries were obtained from the open chemical repository collection of the National Cancer Institute (NCI). The libraries consisted of aliquots of Diversity Set III and the Natural Products Set II in 96 well microtiter plates. The Diversity Set III consists of 1597 compounds whereas the Natural Products Set II consists of 120 compounds. The compounds in each well were reconstituted using 19 μL of DMSO to obtain a final concentration of 10 mM. Diversity Set III includes a set of compounds that can be used to generate structure-based hypothesis testing for screening methods development as employed herein. These compounds include pharmacores that are relatively rigid, have less than 5 rotatable bonds and have pharmacologically desirable properties. Many of the compounds have well known chemical properties and biological activities.

2.4. Optimization of Nile Red staining

2.4.1. NR concentration optimization

Cells were pre-grown to mid-log phase, centrifuged (5 min; 3000 ×*g*), and resuspended to an OD_{550} of 0.5 in TAP media for negative (Neg) controls. Controls for lipid accumulation were grown in N− TAP (Pos) and were then rinsed three times and then finally resuspended to OD_{550} of 0.5, all in TAP N− media. In both conditions, cells were cultured for 72 h, harvested, centrifuged and plated in a 96-well plate to a final optical density (OD_{550}) of 0.5. To assess optimal lipid staining, Nile Red (NR) (9-(diethyl amino)benzo[a]phenoxazin-5(5H)-one (Sigma-Aldrich)) was prepared as a stock solution at a concentration of 1.0 mg/mL in dimethyl sulfoxide (DMSO) and added to give the desired final concentration as detailed in Section 3. Plates were incubated either at room temperature (22 °C) or at 37 °C in dark for 30 min with or without shaking. NR fluorescence was measured in arbitrary units at 485/20 (Ex) and 590/35 (Em) using a BioTek Synergy Plate Reader.

2.4.2. DMSO concentration optimization

To determine the optimal DMSO concentration allowable for NR staining of lipid droplets in algal cells, 200 μL of cell suspension with an approximate cell density of 0.5 OD^{500} was used. Dilutions of NR were made to give stock concentrations ranging from 50 μM to 3 mM. The appropriate volume of NR stock was adjusted with DMSO to give the desired final concentrations of NR and DMSO.

2.5. Final screening procedure defined

Cells were grown to mid-log phase (0.5 OD_{550}), harvested by centrifugation, washed twice with TAP media, and then diluted further to give a final optical density OD_{550} of 0.2. Cells were then seeded in a 96-well plate at 200 μL per well and 2 μL of compound solution (stock concentration 1 mM in DMSO) was added to give 10 μM f.c. Separate aliquots of cells and compounds were dispensed using an Eppendorf epMotion 5075 liquid handling robot. The wells in the first column of each plate contained cells alone in TAP media (TAP N+) without compound and served as a positive control for growth and negative control for lipid accumulation (Neg), while the last column of the plate contained cells in nitrogen free TAP media (TAP N−) at a density of 0.5 OD_{550} to serve as the positive control for lipid accumulation (Pos). The latter did not increase significantly in cell density over the course of the experiment due to the lack of nitrogen, thus initial seeding was at a higher cell density. The plates were covered with gas permeable adhesive film (BreathEasy, Diversified Biotech) and incubated in 50–55% humidified atmosphere at 25 °C with a photosynthetic photon flux density of approximately 50–55 μmol m^{-2} s^{-1}. Incubations were continued for 72 h prior to assessment of growth at OD_{550} and assay for lipid accumulation using 10 μM NR and 10% DMSO.

2.6. Data analysis

After removal of the subset of compounds that restricted growth, the raw intensity data for the NR fluorescent signal of each well was background corrected by subtraction of the median intensities across all control wells on the same plate. The background corrected data was used for determining the Z-factor of the plate [22]. The fold-change was calculated by taking the ratio of the NR fluorescence intensity obtained for the treated sample divided by the mean value obtained for the negative control samples (TAP N+). Compounds were identified as hits when the

Z-factor [22] of the plate was greater than 0.5 and the experimental value for NR fluorescence was 3 SD ± mean of the negative control.

2.7. Assessment of Brefeldin A treatment on growth, photosynthetic pigments, carbohydrate and protein

To evaluate the impact of Brefeldin A (BFA) on growth and pigment composition, *Chlamydomonas* cells were treated with a single concentration of BFA, 5 μM, as determined from preliminary experiments and culture density was monitored up to 72 h at OD_{550}. The values for photosynthetic pigments (i.e., chlorophyll a, b and total carotenoids) were measured using the ethanol extraction method [23]. One milliliter of culture equivalent to 1.0×10^6 cells was centrifuged and the pellet was resuspended in 95% ethanol and incubated 4 h in the dark at 4 °C for pigment extraction. The mixture was clarified using centrifugation and chlorophyll a, b and total carotenoids levels in the supernatant were determined by measuring the optical density at 665, 649 and 470 nm, respectively, as previously described [23]. To evaluate the effect of BFA on the total carbohydrate and total protein content, one milliliter of culture equivalent to 1.0×10^6 cells were harvested. The resulting pellet was used for total protein content determination using BioRad DC reagent kit as per the manufacturer's instructions. Total carbohydrate content was determined using a method adapted to a 96-well plate format as described elsewhere [24]. Briefly, approximately 1.0×10^6 cells were harvested in a 1.5mLmicrofuge tube, resuspended in 50 μL of water and transferred to a 96-well plate. Six glucose standards were prepared ranging from 5–50 μg and a standard curve was plotted. To the 50 μL algal cells, 150 μL of anthrone reagent (0.1 g in 100 mL concentrated sulfuric acid (98%)) was added. The plate was first incubated at 4 °C for 10 min and incubated at 100 °C for 20 min. Plates were further cooled at room temperature for 20 min and color development was measured at 620 nm on a Biotek Synergy plate reader.

2.8. Lipid induction by Brefeldin A

The induction of lipid accumulation by BFA treatment was determined by titrating the compound after dilution in DMSO in cultures using the plate assay as described above for screening. Briefly, 200 μL of cells was seeded at an OD_{550} of 0.2 and different concentrations of BFA were added ranging from 0.625 to 50 μM f.c. in triplicate. The final concentration of DMSO was held at 1%. Cells were allowed to grow for 72 h in the presence of compound in 96-well plates as described in the screening assay. At the end of 72 h, the optical density of the cultures was measured at OD_{550} and then the cells were concentrated by centrifugation. NR stain (f.c. 10 μM) in DMSO (10% f.c.) was added and then the samples were incubated in dark at 37 °C for 30 min. Fluorescence intensities were acquired as detailed above.

2.9. HPTLC analysis of lipids

To identify TAGs and other lipid species high performance thin layer chromatography (HPTLC) was performed after compound treatment. For these experiments, approximately 1.0×10^7 cells (BFA treated and control) were harvested and then resuspended and vortexed (10 min) in a mixture containing 2 mL of 50 mM K_2HPO_4, 2 mL of $CHCl_3$ and 4 mL of MeOH to extract lipids. One milliliter of 50 mM K_2HPO_4 and 1 mL of $CHCl_3$ were added to the homogenate, which was then centrifuged at 3000×*g* for phase separation. The organic layer was transferred to a clean glass tube and the solvent evaporated under nitrogen gas flow. The dried lipid extract was resuspended in 25 μL of $CHCl_3$. Triolein obtained from a commercial supplier (Sigma-Aldrich) was dissolved in $CHCl_3$ for use as standards for TAG identification. Ten microliters of standards or samples was sprayed onto a silica gel plate (Whatman) using a Linomat 5 (Camag). The plate had been pre-activated at 10 °C for 1 h. The TLC plate was developed in a chamber filled with a mixture of 220 mL of hexane, 80 mL of ethanol and 1 mL of acetic acid for 30 min. The air-dried plate was dipped in a solution containing 0.63 g of $MnCl_2 \cdot H_2O$, 60 mL of water, 60 mL of methanol and 4 mL of concentrated H_2SO_4 and then charred in an oven at 100 °C for 30 min.

2.10. FAMES analysis of BFA treated cells and imaging of lipid droplets

Total lipid extraction was performed using the liquid–liquid methyl tert-butyl ether (MTBE) method as detailed elsewhere [25]. Briefly, cells were cultured and treated with BFA (f.c., 5 μM) as detailed above. Thirty milliliters of culture were harvested, centrifuged (5000 ×*g* for 5 min at 25 °C) and the biomass was recovered. The cell pellet was plunged into liquid nitrogen to quench metabolic activities and lyophilized overnight at −50 °C. Ten milligrams of the powder was weighed and used for lipid extraction. Ice-cold 75% methanol (400 μL) was added to the dried biomass along with 100 μg of nonadecanoic acid (C19:0) as internal standard. The biomass was homogenized completely and then sonicated on ice for 2 min. One milliliter of MTBE was added and the samples were shaken for 1 h at room temperature. For phase separation, 250 μL of milli-Q water was added and the samples were incubated at room temperature (22 °C) for 10 min to induce phase separation. The upper organic phase was collected and then dried under the stream of nitrogen. Samples were methylesterified using 50 μL of MTBE and 10 μL of trimethyl sulfonium oxide (TMSH) for 30 min at room temperature. GC–MS analysis was carried out on an Agilent 6890 GC–MS system equipped with DB-5 ms (30 m × 0.25 mm × 0.25 μm) column. One microliter was injected in splitless mode onto DB-5 ms column. The injection port was set at 250 °C and the auxiliary temperature was set at 280 °C. The temperature program was started at 70 °C and held for 2 min, then ramped to 140 °C at 20 °C/min and held for 2 min. The second ramp was from 140 °C to 280 °C at 10 °C/min held for 3 min. Helium was used as carrier gas at a constant flow rate of 1.5 mL/min. Spectra were acquired using an Agilent 5973 mass selective detector with a scan range from 50 to 550 amu at 70 eV. Spectra were identified using the NIST (2008) library.

The effect of BFA on lipid accumulation was also visually assessed using confocal laser scanning microscopy as detailed elsewhere [6]. Briefly, the cells in 2 mL culture were collected by centrifugation (2500 ×*g*, 5 min) and then resuspended in 900 μL of fresh TAP media. NR dissolved in DMSO (10 μM f.c.) was added and then the samples were incubated in the dark at 37 °C for 30 min. Microscopic images (100×; oil immersion) were acquired using an Olympus IX-81 inverted confocal laser scanning microscope using FloView v5.0 software. To detect the NR fluorescence, laser excitation was at 543 nm, and the emission/barrier filter was set at 560–610 nm, for chlorophyll fluorescence laser excitation was set at 633 nm and emission was set at 660–700 nm. For consistency, images for BFA treated samples were acquired first and using the same settings images were acquired for TAP N+ cells.

2.11. Immunoblotting to assess DGAT2A expression

To examine the expression of acyl-CoA: diacylglycerol acyltransferase (DGAT2A), cells were treated with BFA (2.5 or 5 μM) for 72 h and collected by centrifugation (3000 ×*g*, 5 min) and then resuspended in SDT lysis buffer (0.5 mL 4% SDS and 0.1 M DTT in 0.1 M Tris–HCl, pH 7.6) and incubated at 95 °C for 5 min followed by 5 min vortexing (2 cycles). Lysates were further centrifuged at 14,000 ×*g* for 10 min at room temperature and the supernatant was analyzed on a 10% Bis-Tris gel. The antibody against DGAT2A and Histone H3 (Agrisera) used as the loading control were obtained from Agrisera (Sweden).

3. Results and discussion

3.1. Rationale for protocol development

For algae to be useful as a feedstock for biofuel production several hurdles must be overcome. Among these are standardized methods to

maximize lipid production without compromising growth and photosynthetic capacity. Most current methods used to induce lipid storage droplets employ a severe stress such as nutrient limitation, and most commonly nitrogen deprivation [26]. While these methods result in lipid accumulation of 5–50% cell mass, they also result in metabolic shifts commensurate with increased protein and RNA turnover, cessation of cell division, and diminished biomass yield [4–6, 27]. Our goal for devising a high throughput screening system was to identify small synthetic compounds that induce lipid production and storage but that do not inhibit cell growth or induce stress response pathways. The present screening method was tested using small libraries available from the National Cancer Institute selected for their diverse structures and activities. While these libraries are not meant to select a lead hit, they are useful for development of primary and secondary screens necessary when large libraries are to eventually be employed.

Our rationale for screening was relatively simple. It consisted of incubating the compound of interest together with algal cells at low cell density. The mixture was cultured for 1–3 days and the growth was assessed spectrophotometrically at OD_{550} and then lipid accumulation assessed by measuring fluorescence following the addition of the lipophilic dye NR. If the compound prevented growth, the OD_{550} would remain low and the compound would not be selected as a hit whether or not lipid was accumulated. Two controls were included in the assay design: a positive control for lipid accumulation where cells were inoculated at a higher density (generally 0.5 OD_{550}) in media without nitrogen and a negative control for lipid accumulation where cells were inoculated at a low initial density in nitrogen replete media without added compound. This allowed us to distinguish hits and to estimate a Z′ discriminating factor [22]. Only compounds that induced cellular lipid accumulation as evidence by staining with the lipophilic dye NR higher than the untreated control and also allowed cultures to achieve at least 50% of the density of untreated controls were identified as hits. Additionally, all potential hits were evaluated for chlorophyll content, as a proxy for the integrity of the photosynthetic apparatus. This is of value because most methods to induce lipid production in algae by nutrient stress cause loss of photosynthetic capacity and yellowing of the cultures due to reduced chlorophyll concentration.

3.2. Standardization of screening protocol

In order to standardize procedures and maximize sensitivity of cells accumulating lipid, we evaluated both the NR concentration and effects of the concentration of DMSO used as the solvent on the final fluorescence signal. The rationale was that since the DMSO may also act as a cell-permeabilizing agent due to its solvent properties, it was possible it would also affect signal intensity in the treated cell samples. Therefore, we sought to determine the optimal DMSO and NR concentrations for use in screening (Figure 1).We first optimized the staining conditions for lipid accumulation using cells growing in TAP media (N+, negative control) and cells growing in TAP media without nitrogen (N-, positive control). After growth, cells were diluted to 0.5 OD_{550}, 200 µL were dispensed to a 96-well plate, and NR added to give a final concentration of 7.5 or 15 µM. Initial test conditions included with or without shaking and incubation in dark at room temp (22 °C) or 37 °C for 30 min. Figure 1A shows incubating plates at 37 °C resulted in a higher signal when compared to incubation at room temp (22 °C). There were no significant differences in NR staining dependent upon shaking. Once these conditions were optimized, we next addressed the optimal NR and DMSO concentrations required to obtain the maximal fluorescence signal. First NR was varied from 5 to 300 µM at 10% DMSO (Figure 1B); concentrations from 5 to 10 µM were sufficient to obtain optimal lipid staining. Next we tested different concentrations of DMSO, holding NR at 10 µM and found that 7% DMSO gave the highest signal (Figure 1C). On the basis of these two observations we created a matrix of different concentration of NR and DMSO to obtain conditions that resulted in a maximal fluorescence signal. Since relatively low concentrations of NR

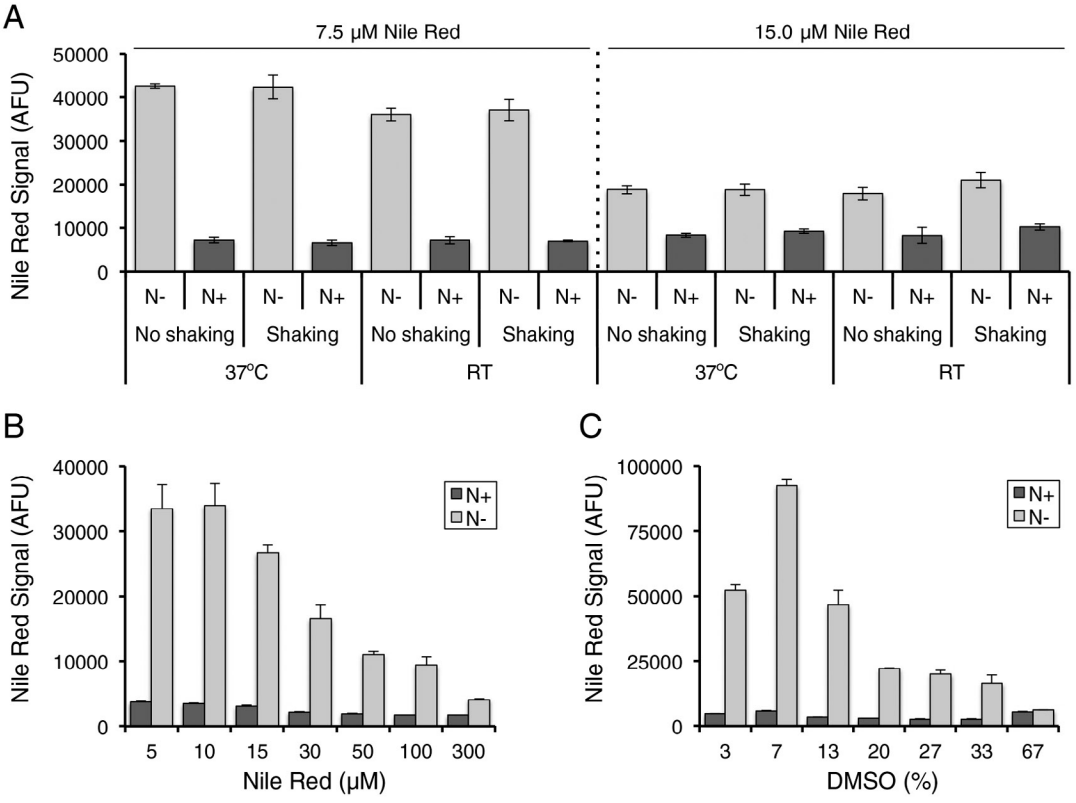


Figure 1. Optimization of staining conditions. Cells growing in TAPN+ or N− were plated in a 96-well plate, and two concentrations of NR were tested either at 22 °C or 37 °C with or without shaking (**A**). Experiments were performed to determine the combined optimal DMSO (**B**) and NR (**C**) concentrations required to attain the highest fluorescent signal from lipid in cells grown in TAP N− or N+ media.

(5–30 μM) resulted in the maximal fluorescent signal over a broad range of DMSO concentrations (6–38%), the final concentrations of NR and DMSO were set at 10 μM and 10% DMSO, respectively, for the test library screening experiments.

3.3. Determination of assay quality

The quality of the primary screening assay was determined using the high throughput Z′-factor parameter [22] employing the positive and negative control samples as detailed above using the NR fluorescence signal obtained to determine lipid accumulation using the test small chemical library.

The Z′ factor was calculated using the equation:

$$Z' = 1 - 3 \times \frac{SSD}{R}$$

where, $SSD = \sigma p + \sigma n$ and $R = \mu p - \mu n$; σp represents the standard deviation of the NR signal of the positive control samples (given in arbitrary fluorescent units); σn is the standard deviation of the NR signal of the negative control samples; μp is the mean value of the NR signal of the positive control samples, and μn is the mean value of the NR signal of the negative control samples.

The calculated mean Z′ factor for 22 plates in the primary screen based on NR fluorescent signal was 0.68 ± 0.1 (SD) and thus could be described as an "excellent assay" capable of distinguishing differences between the positive and negative control and thus hits with high degree of confidence [22]. The Z′-factor for the individual screened plates is given in Figure 2 and the growth analysis of the cells after compound treatment for 72 h is given in Figure 3.

3.4. Pilot screen for inducers of lipid accumulation in algae

For the screening experiments, cells were grown to mid-log phase in TAP N+ media and harvested by centrifugation (5 min; 3000 ×*g*). The cell pellets were washed three times using either TAP N+ or TAP N− media and then resuspended to a final cell density of 0.2 OD_{550} for cells with nitrogen and 0.5 OD_{550} for cells without nitrogen. Cells (200 μL aliquots) were dispensed robotically to wells of a 96-well plate containing the compound of interest, or DMSO alone for controls, giving a final concentration of 10 μM for each test compound. The samples were incubated at room temperature under lights for 72 h and plates were shaken once every day to disperse cells that had settled to the bottom of the well. After 72 h, the final cell density was measured and recorded. NR in DMSO (see above)was added to each well, samples were incubated in the dark at 37 °C for 30 min without shaking, and fluorescence as a measure of lipid accumulation was measured at 485/20 (Ex) and 590/35 (Em) using a Biotek Synergy Plate reader.

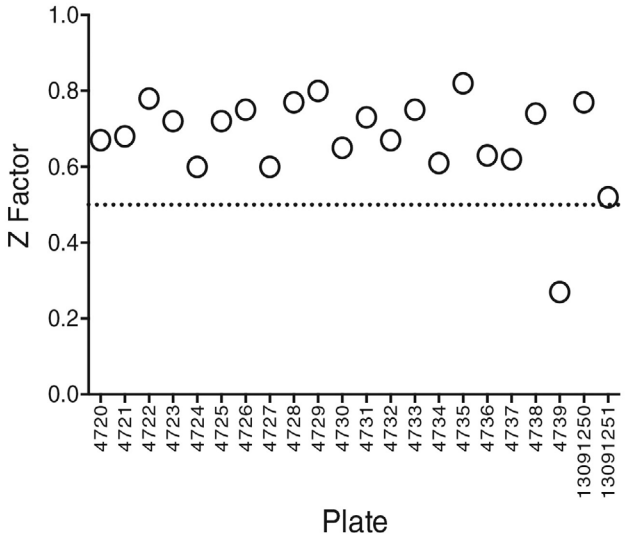


Figure 2. Z-factor analysis of the primary screen. See text for details. The circles indicate the calculated Z′ calculated for each of the 22 plates employed. The plate code is indicated on the X-axis.

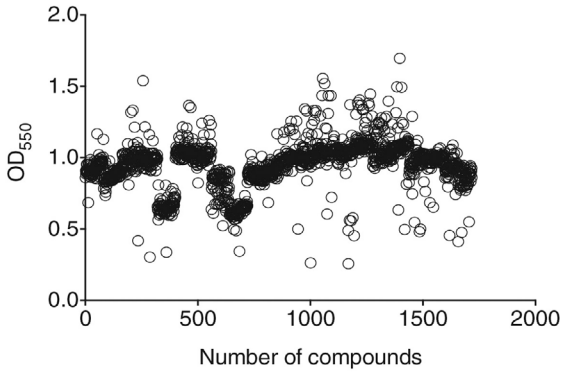


Figure 3. Growth analysis of *Chlamydomonas* cells after screening. Scatterplot of end-point readings of cell density for each well treated with a unique compound.

To test our screening method, we carried out an HTS screening experiment using 1717 compounds from the NCI Diversity Set III and the Natural Products Set II. This required 22 96-well plates each containing 8wells of the positive control for lipid accumulation (TAP N–), 8wells of the negative control (TAP N+), and 80 wells with aliquots of one compound each at a final concentration of 10 μM. The performance of the optimized screening assay was evaluated by calculating the Z′-factor for each plate from the data acquired for the positive and negative controls as detailed above. As expected, most compounds (1344 or 78%) had no effect on growth (Figure 3). However, 18 compounds (1.05%) caused severe growth retardation. This demonstrated that the devised screening assay displayed a robust performance with a mean Z′-factor of 0.68 ± 0.1 and could also be used to assess cell growth (Figs. 2 and 3).

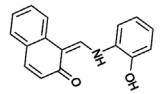
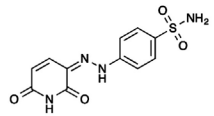
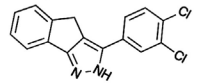
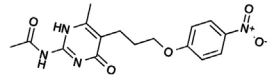
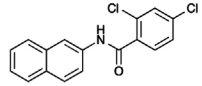
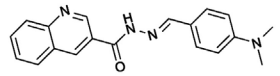
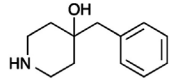
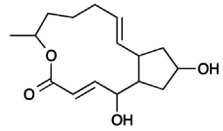
A summary of the HTS screening data is presented in Table 1. Values for growth and lipid accumulation as well as structural information for primary hit compounds from the initial screening are shown in Table 2. Additional data including the complete primary screening results and physicochemical properties of the compound libraries can be found in Additional File 1. For the primary screen, compounds that induced at least a 5-fold increase in total fluorescence intensity compared to the negative control and did not eliminate growth were considered candidate lipid activators (Figure 4a and Table 2). Using this approach we identified 8 compounds at a hit rate of 0.47%.

To validate the 8 hits, each compound was retested at 3 concentrations in triplicate using 10 mL cultures applying the same criteria described above for the primary screen (i.e., measurement of lipid accumulation by NR staining and growth by measuring cell density at OD_{550}) (Figure 4b). Of these, one compound (4736-A05; see Table 2) failed to show lipid accumulation above control levels in the untreated control samples and was eliminated. Six of the 7 gave modest increases in lipid yield ranging from 1.6- to 2.4-fold above untreated controls. One compound, Brefeldin A (BFA), had the best performance in terms of lipid

Table 1. Summary of data from the *Chlamydomonas* HTS screen for compounds that induce lipid accumulation. Details of the HTS screen are in Section 3.4. Experimental data from the primary screen are in Figs. 3, 4 and Appendix 1.

	Value
Number of compounds screened	1717
Total number of primary hits	8
Overall primary hit rate	0.47%
Number of compounds repeated (n = 3)	8
Z-factor, mean ± SD	0.68 ± 0.11

Table 2. Summary of results for compounds selected in the primary screen and confirmation of activity.

Comp ID	NSC code	IUPAC name	OD_{500}[a]	1st screen[b]	Confirmatory screen[c]			Structure
				10 μM	10μM	5μM	2.5 μM	
4722-C07	111847	(1Z)-1-[(2-hydroxyanilino) methylidene] naphthalen-2-one	0.64	6.36	1.58 ± 0.15	1.59 ± 0.25	1.22 ± 0.30	
4722-C08	134199	4-[(2Z)-2-(2,6-dioxopyridin-3-ylidene)hydrazinyl]benzenesulfonamide	0.7	5.6	2.39 ± 0.35	1.89 ± 0.11	1.34 ± 0.26	
4726-E05	331968	3-(3,4-dichlorophenyl)-1,4-dihydroindeno[1,2-c]pyrazole	0.8	5.2	2.42 ± 0.22	1.59 ± 0.14	1.81 ± 0.01	
4726-E07	211356	N-[6-methyl-5-[3-(4-nitrophenoxy)propyl]-4-oxo-1H-pyrimidin-2-yl]acetamide	0.86	6.49	2.43 ± 0.28	1.71 ± 0.35	1.41 ± 0.29	
4726-E08	158959	2,4-dichloro-N-naphthalen-2-ylbenzamide	0.78	5.37	2.30 ± 0.52	2.02 ± 0.75	1.38 ± 0.14	
4726-E09	201868	N-[(E)-[4-(dimethylamino)phenyl] methylideneamino]quinoline-3-carboxamide	1.04	5.18	2.37 ± 0.43	2.04 ± 0.18	1.42 ± 0.29	
4736-A05	83237	4-benzylpiperidin-4-ol	0.82	5.34	0.93 ± 0.19	0.92 ± 0.14	0.96 ± 0.12	
13091250-F09 Brefeldin A	89671	(1S,2E,7S,10E,12R,13R,15S)-12,15-dihydroxy-7-methyl-8-oxabicyclo[11.3.0]hexadeca-2, 10-dien-9-one	0.649	5.41	9.89 ± 2.64	4.06 ± 0.75	1.78 ± 0.53	

a. Final OD_{550} after 72 h growth with compound.

b. Fold-change in NR fluorescence intensity for one well treated with the indicated compound at 10 μM final concentration.

c. Average of 3 experiments ± standard error given as the fold change in NR fluorescence intensity compared with untreated controls, each assayed in triplicate at the indicated concentration.

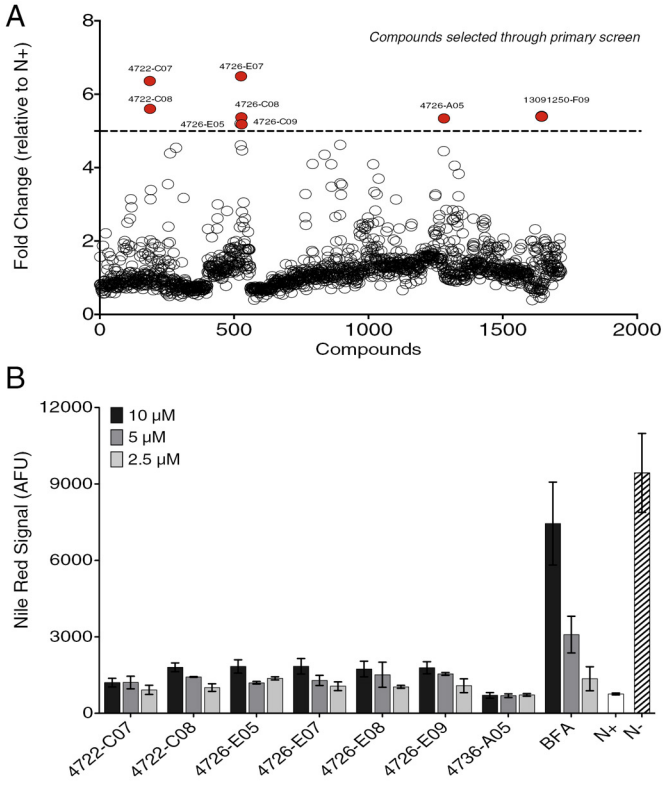


Figure 4. Results of the screening showing lipid accumulation after compound treatment. Scatterplot of lipid accumulation during primary screen assayed using NR (**A**). The red filled circles indicate primary hits. Verification of the primary hits by treatment of cells at 3 different concentrations of each compound (**B**). The black bar indicates the relative fluorescence units (RFU) obtained at 10 µM, the dark gray bar at 5 µM, and the light gray bar 2.5 µM compound. The white bar is the mean of the data for the negative control (cells growing in TAP N+ media) and the bar with the black hatches indicates the positive control (cells growing in TAP N− media). The bar height indicates the mean of three experiments (±SD).

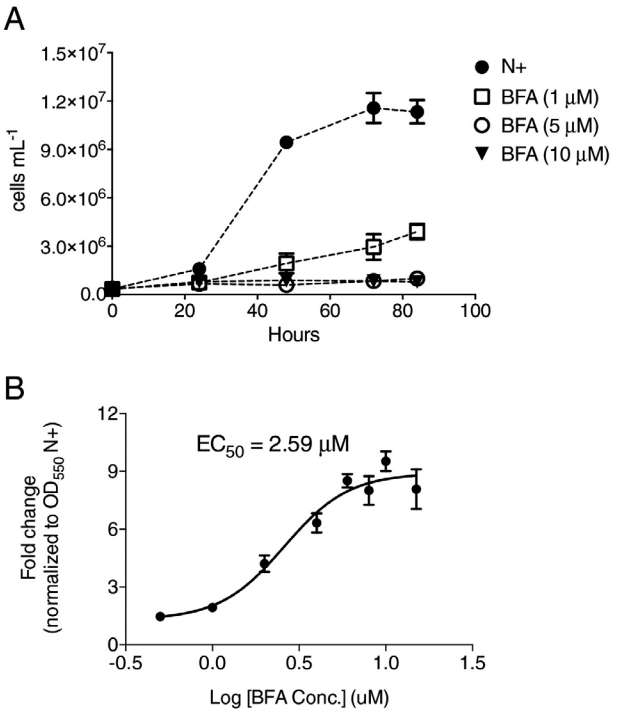


Figure 5. Growth and dose response of *Chlamydomonas* with BFA treatment. Time course of cell growth in the presence of BFA at 3 dosages (**A**). Dose-dependent increases in lipid accumulation as assessed using NR and normalized to cell density (**B**). The error bars indicate the standard deviation for 3 experiments assayed in triplicate.

accumulation and yielded a reasonable dose response curve giving an apparent EC_{50} of 2.59 µM (Figure 5a and b). We noted that all the selected compounds resulted in reduction in total cell density by about 30% at 10 µM f.c. indicating each had some depressive effect on cell growth. Despite this limitation, BFA, which gave a higher lipid yield, was employed as a test compound to develop secondary assays useful to investigate potential lipid activators in a future screen employing larger compound libraries.

3.5. Effect of Brefeldin A on cellular growth and levels of photosynthetic pigments

To study the impacts of BFA on cell growth and structure, as well as on the photosynthetic apparatus, *Chlamydomonas* cells were grown in the presence of 1, 5, or 10 µM BFA in 10 mL liquid cultures (Figure 5a and b). Without added compound, the number of cells in TAP N+ media increased rapidly and reached a plateau at approximately 72 h. By comparison, *Chlamydomonas* cells ceased growing when transferred to TAP N−media almost immediately [28]. The lipid activators we seek would have a limited affect on growth to maximize both biomass and lipid accumulation. With BFA treatment cell growth continued to a limited extent while lipid accumulation was stimulated to high levels. This growth inhibitory effect was more severe than we had observed in the primary screen and during validation (Table 2 and Additional File 1). This may be due to significant differences in the experimental design including higher culture volume (10 mL versus 200 µL), continuous shaking and aeration. Implications of these results with regard to scaling up HTS experiments are discussed below.

During nitrogen starvation the chloroplasts are remodeled and photosynthetic pigments and proteins are decreased in abundance [29–31]. To determine the effect of BFA on the pigments associated with the photosynthetic complexes, cells were treated with 5 µM BFA dissolved in DMSO or DMSO alone (vehicle control) and pigment levels quantified. Treatment with BFA at 5 µM decreased the total chlorophyll a levels approximately 2-fold (log2 fold ratio −2.6 ± 0.02) when compared with untreated control cells. A similar profile was observed for chlorophyll b and total carotenoids (Figure 6a). BFA treatment also decreased total protein as compared to the untreated TAP N+ cells (Figure 6b). Surprisingly, treatment with BFA did not alter carbohydrate levels significantly up to 72 h (Figure 6c). The correlation between increased lipid droplet accumulation and decreased pigment and protein levels is consistent with a stress response. This response is similar to metabolic changes that occur during nitrogen deprivation where degradation of the photosynthetic apparatus and elevated protein turnover are common features [29–31].

To visually assess the effect of BFA treatment on lipid accumulation and cellular architecture, cells were stained with NR and imaged using both bright field and fluorescent confocal microscopy (Figure 7a). The cell morphology of BFA treated cells was altered when compared with untreated controls. Of particular note was the loss of flagella, increased cell size, and increased starch granules and lipid droplets. These observations were consistent with what has been observed previously with BFA treatment [32].When the cells were imaged using the green channel there appeared to be a reduction in the size of the chloroplast. This was consistent with the measured reduction in the chlorophyll a and b pigments levels as summarized above (Figure 7a). Triglyceride accumulation induced by BFA was dose-dependent as assessed using HPTLC (Figure 7b).

To evaluate the fatty acid composition of the cells following BFA treatment, total lipids were extracted from treated and untreated cell

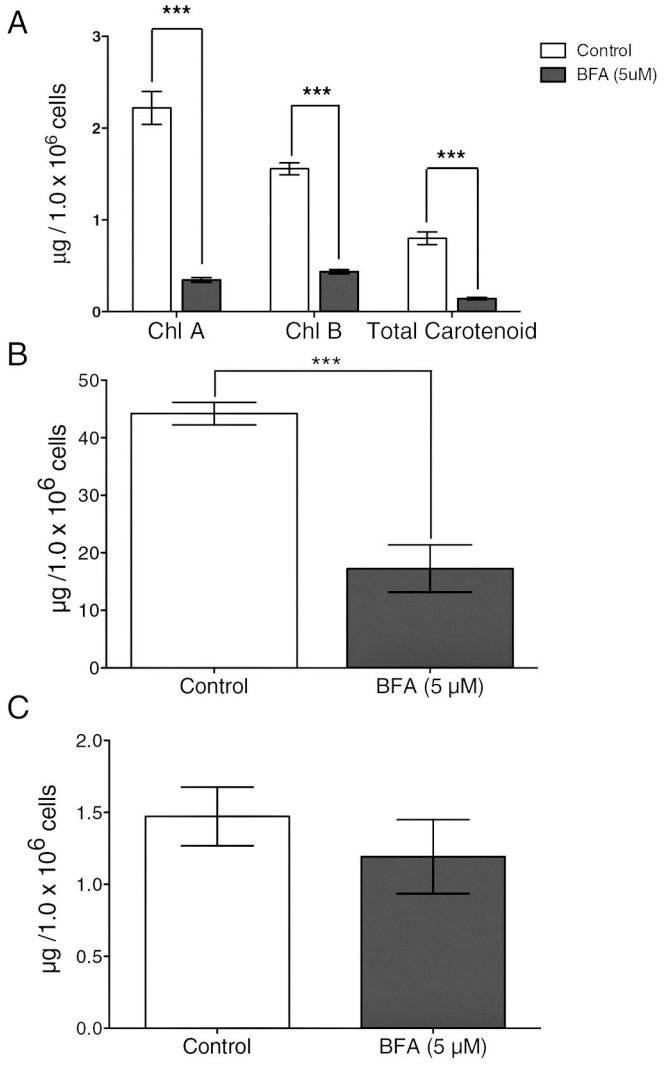


Figure 6. Cellular metabolites assessed after treatment with 5 µM BFA. Chlorophyll (Chl) a and b and total carotenoids (**A**); total protein (**B**); and total carbohydrate (**C**). The bar height indicates the mean of 3 experiments assayed in triplicate; the error bars indicate the standard deviation.

cultures, fatty acid methyl esters (FAMEs) prepared, and analyzed using GC–MS. BFA-treated samples had higher levels of C16:0, C16:1 (Δ9), C16:3 (Δ7,10,13), C18:0, C18:3 (Δ5,9,12), and C20:0 (Figure 7c). In contrast, the two major components of plastidic lipids C16:4 (Δ4,7,10,13) and C18:3 (Δ9,12,15) were reduced in BFA treated cells compared to controls. These data suggests that the storage lipids were derived from membrane lipids outside the plastid.

3.6. Effect of BFA on the expression of DGAT2A

Diacylglycerol acyl-transferase (DGAT2A) activity is required for TAG accumulation in algae. This enzyme has also been reported to contribute to the incorporation of hydroxylated fatty acids into TAG [33]. There are 6 genes encoding DGAT in *Chlamydomonas* and previous studies have shown that the expression of DGAT2A (DGTT1) is increased in nitrogen starved cells [29]. Since BFA treatment induced lipid accumulation, we addressed whether DGAT2A protein expression was also increased using Western blotting. As shown in Figure 8, BFA increased the expression of DGAT2A. However, at 5 µM BFA, DGAT expression was lower than what we observed in the nitrogen starved cells.

3.7. Effect of BFA treatment on C. sorokiniana UTEX 1230

To determine whether BFA treatment increased lipid accumulation in other industrially useful strains, we tested the effect of treatment on *C. sorokiniana* UTEX 1230, which represents a fast growing, temperature tolerant, algal strain. BFA treatment of these cells for 72 h, showed the expected lipid accumulation, but there was amore limited accumulation of lipid across the range of BFA concentrations tested compared with *Chlamydomonas* (Figure 9). We speculate that one reason for this difference may be due to the unique cell wall composition of each. The *Chlamydomonas* cell wall is made up of hydroxyproline rich glycoproteins [34], while the *C. sorokiniana* cell wall is composed of 83% of carbohydrate and 17% protein with very small amounts of hydroxyproline [35]. The major sugar moieties present are rhamnose, glucuronic acid, galactose, xylitol, and mannose. By comparison with *Chlamydomonas*, this makes the cell wall of *C. sorokiniana* a formidable barrier for the passage of exogenous molecules, which may account, at least in part, for the decreased efficacy of BFA [35–37].

4. Conclusions

The present study was undertaken to devise and evaluate methods to screen large compound libraries for those that increase lipid accumulation in microalgae without inducing a stress response such as occurs in nutrient deprivation. We expect this high throughput screening method will facilitate the identification of small molecule inducers of lipid production and storage without severely compromising growth. The hit compounds are expected to obviate the large metabolic shifts during nutrient stress induction that compromise growth and limit photosynthetic capacity as occurs with nutrient starvation methods commonly used to induce lipid accumulation in algae. Such compounds may impact lipid production to: a) target a single regulatory or metabolic protein; b) allow investigators to unravel the metabolic pathways and regulatory factors of lipid synthesis and storage; and c) to help advance algal biofuels to commercial viability.

The platform we present in this work is well suited for exploration of chemical space and use of small molecules to uncover biochemical stimulus that produce a desired phenotype. We have designed and tested this screen to identify small molecules that increase growth and lipids in the freshwater algae *Chlamydomonas*. However, the methods are easily adapted to other single celled photosynthetic organisms suited to biofuels production. While our method employed 96-well microplates to screen a pilot collection of diverse compound library obtained from National Cancer Institute this method can be easily be scaled up to 384- or 1536-well high content screening formats to accommodate larger compound libraries. The microplate assay we have developed monitors growth by measuring the optical density of the culture and following the addition of lipophilic dye NR, the intracellular neutral lipid levels at the end of the experiment. The secondary screens include: imaging to assess cell structure and lipid droplet formation; and the measurement of photosynthetic pigments, protein and carbohydrate levels. Each of these secondary screens is required to assess impact of compound treatment on cellular physiology and metabolism.

BFA identified in the primary screen conducted here using small test libraries cannot be pursued as a lead compound because, while it did satisfy the criterion of high lipid accumulator, secondary screening determined it limited photosynthetic pigments and growth as compared with the control. This points to the necessity to verify hits early in the follow-up validation protocols using larger culture volumes inoculated a range of cell densities and compound concentrations. Despite the aforementioned limitations, BFA was characterized further as a test case to discern other analytical and biological methods that will be useful to characterize lead compounds in future large platform screenings. Since high lipid accumulation was achieved in nitrogen replete media, we suggest BFA may increase TAG synthesis via a pathway that is different from that which occurs during nitrogen starvation.

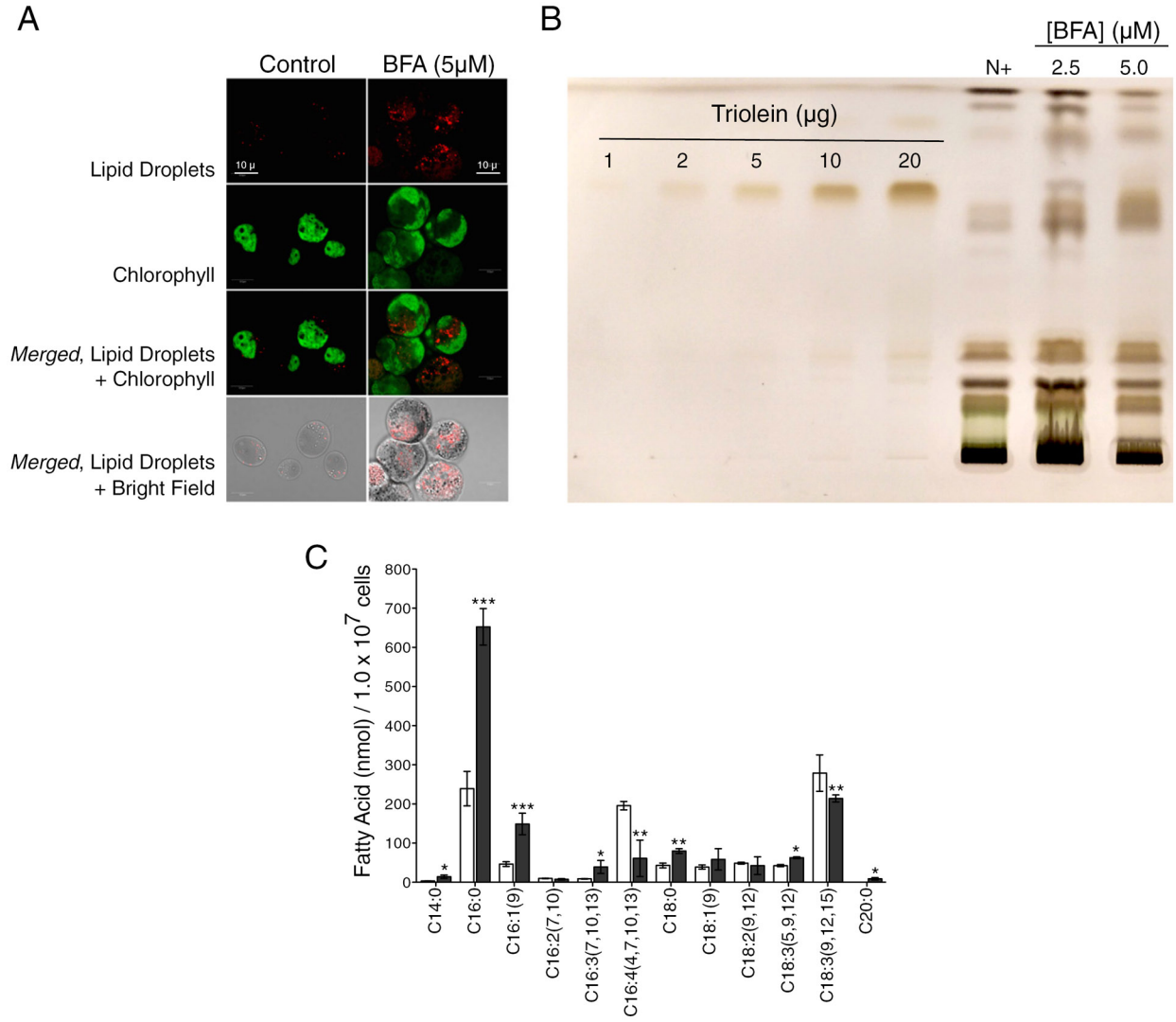


Figure 7. Lipid analysis of BFA treated cells. Representative images of BFA-treated *Chlamydomonas* cells to show lipid droplets after NR staining (red) and chloroplasts imaged using chlorophyll autofluorescence (green) (**A**). HPTLC analysis showing dose-dependent TAGs accumulation in *Chlamydomonas* when treated with BFA (**B**). Cells were cultured in 250 mL flasks with 100 mL of culture with various concentrations of BFA for 72 h. Lipids were extracted from 1.0×10^7 cells. Extracted lipids were resolved on HPTLC plates and TAGs were visualized by charring the plates. Comparison of the fatty acid composition of BFA treated cells and untreated controls (**C**). For the fatty acid species listed, the number in parentheses indicates the position of a double bond. The bar height is the mean of 3 experiments assayed in triplicate; the error bars indicate the standard deviation. The significance of the differences between untreated (white bars) and BFA treated cells (black bars) was determined using the student's *t*-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

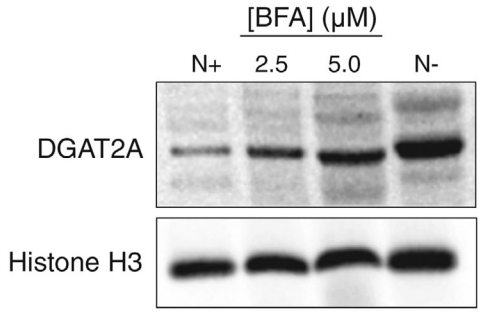


Figure 8. Western blot analysis of DGAT2A (Acyl-CoA: Diacylglycerol acyltransferase) after BFA treatment. After growth for 72 h in the presence of BFA at 2.5 and 5 μM, protein was extracted and resolved on 10% Bis-Tris Gel in denaturing condition. Expression of DGAT2A was found increased in BFA treated and positive control (TAP N−) cells as compared to untreated cells. Histone H3 was used as a loading control.

The use of the chemical genetic methods detailed in this work promises to advance our understanding of algal lipid production necessary to transition algal oils into economically feasible and sustainable feedstock for biofuel production. The current study highlights the power of chemical genetics to identify in vivo pathways through which a drug-like chemical compound exerts its effect. The advantage of live algal screening with diverse compound libraries is that hit selection does not depend on any preconceived notions as to which pathway should be targeted to generate the desired phenotype, thus favoring the possibility of identifying novel pathway shifts. Since biochemical pathways and the chemical structures of cofactors and signaling intermediates are often conserved from plants to insects and animals, the knowledge obtained by evaluating drug activity in algae may direct applications to mammalian studies or vice versa. Further, studies employing such hit compounds will inform on algal biology and biochemistry to catalyze future research.

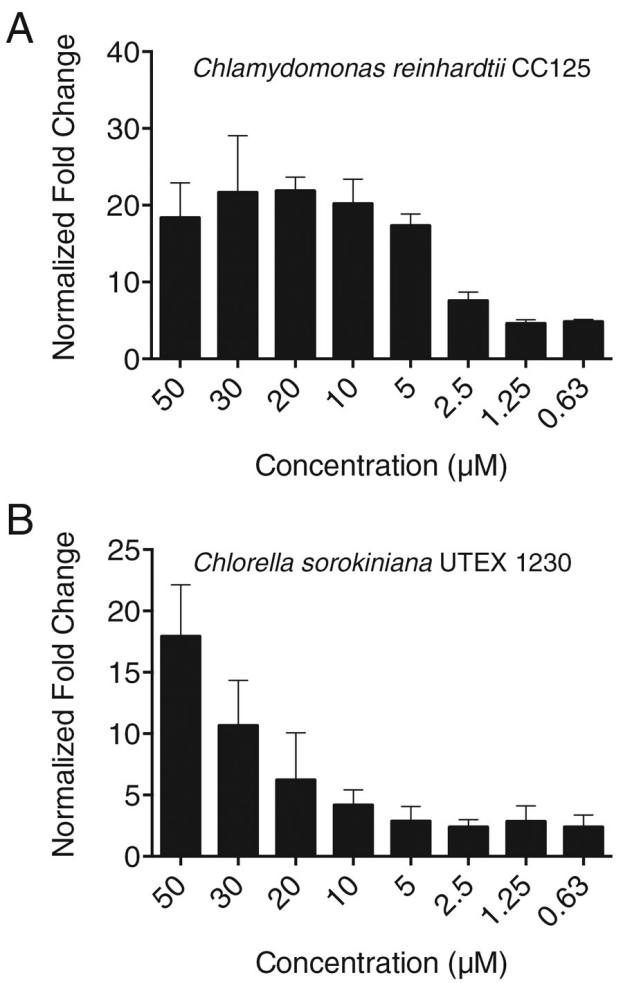


Figure 9. Comparison of the effect of BFA on *Chlamydomonas reinhardtii* CC125 (**A**) and *Chlorella sorokiniana* UTEX 1230 (**B**). After growth for 72 h in the presence of the compound, cells were stained with NR and lipid accumulation was quantified in a plate reader. Data was reported as fold change relative to the control, untreated cells, as indicated. The bar height indicates the mean of 3 experiments assayed in triplicate; the error bars indicate the standard deviation.

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Appendix A. Supplementary data — Supplemental information including the primary screening data, verification of the primary hits and details of the libraries used can be found in the MS Excel file attached to the cover page of this article in the UNL DigitalCommons repository, or online (for subscribers) at http://dx.doi.org/10. 1016/j.algal.2015.06.002.

References

[1] Y. Chisti, Biodiesel from microalgae, Biotechnol. Adv. 25 (2007) 294–306.

[2] C. Hoek, Algae: An Introduction to Phycology, Cambridge University Press, 1995.

[3] G. A. Thompson Jr., Lipids and membrane function in green algae, Biochim. Biophys. Acta (BBA)-Lipids and Lipid, Metabolism 1302 (1996) 17–45.

[4] A. Converti, A. A. Casazza, E. Y. Ortiz, P. Perego, M. Del Borghi, Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production, Chem. Eng. Process. Process Intensif. 48 (2009) 1146–1151.

[5] J. Msanne, D. Xu, A. R. Konda, J. A. Casas-Mollano, T. Awada, E. B. Cahoon, H. Cerutti, Metabolic and gene expression changes triggered by nitrogen deprivation in the photoautotrophically grown microalgae *Chlamydomonas reinhardtii* and Coccomyxa sp. C-169, Phytochemistry 75 (2012) 50–59.

[6] N. Wase, P. N. Black, B. A. Stanley, C. C. DiRusso, Integrated quantitative analysis of nitrogen stress response in *Chlamydomonas reinhardtii* using metabolite and protein profiling, J. Proteome Res. 13 (2014) 1373–1396.

[7] Z. T. Wang, N. Ullrich, S. Joo, S. Waffenschmidt, U. Goodenough, Algal lipid bodies: Stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*, Eukaryot. Cell 8 (2009) 1856–1868.

[8] K. I. Reitan, J. R. Rainuzzo, Y. Olsen, Effect of nutrient limitation on fatty acid and lipid content of marine microalgae1, J. Phycol. 30 (1994) 972–979.

[9] I. A. Guschina, J. L. Harwood, Lipids and lipid metabolism in eukaryotic algae, Prog. Lipid Res. 45 (2006) 160–186.

[10] X.-Q. Xu, J. Beardall, Effect of salinity on fatty acid composition of a green microalga from an Antarctic hypersaline lake, Phytochemistry 45 (1997) 655–658.

[11] J. B. Guckert, K. E. Cooksey, Triglyceride accumulation and fatty acid profile changes in *Chlorella* (Chlorophyta) during high pH-induced cell cycle inhibition, J. Phycol. 26 (1990) 72–79.

[12] M. Einicker-Lamas, G. Antunes Mezian, T. Benevides Fernandes, F.L.S. Silva, F. Guerra, K. Miranda, M. Attias, M. M. Oliveira, *Euglena gracilis* as a model for the study of Cu^{2+} and Zn^{2+} toxicity and accumulation in eukaryotic cells, Environ. Pollut. 120 (2002) 779–786.

[13] J. Inglese, D. S. Auld, A. Jadhav, R. L. Johnson, A. Simeonov, A. Yasgar, W. Zheng, C. P. Austin, Quantitative high-throughput screening: A titration-based approach that efficiently identifies biological activities in large chemical libraries, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 11473–11478.

[14] J. Inglese, R. L. Johnson, A. Simeonov, M. Xia, W. Zheng, C. P. Austin, D. S. Auld, High-throughput screening assays for the identification of chemical probes, Nat. Chem. Biol. 3 (2007) 466–479.

[15] H. Li, P. N. Black, C. C. DiRusso, A live-cell high-throughput screening assay for identification of fatty acid uptake inhibitors, Anal. Biochem. 336 (2005) 11–19.

[16] P. B. Gupta, T. T. Onder, G. Jiang, K. Tao, C. Kuperwasser, R. A. Weinberg, E. S. Lander, Identification of selective inhibitors of cancer stem cells by high-throughput screening, Cell 138 (2009) 645–659.

[17] D. A. Bachovchin, S. J. Brown, H. Rosen, B. F. Cravatt, Identification of selective inhibitors of uncharacterized enzymes by high-throughput screening with fluorescent activity-based probes, Nat. Biotechnol. 27 (2009) 387–394.

[18] G. A. Lemieux, J. Liu, N. Mayer, R. J. Bainton, K. Ashrafi, Z. Werb, A whole-organism screen identifies new regulators of fat storage, Nat. Chem. Biol. 7 (2011) 206–213.

[19] A. Sandoval, A. Chokshi, E. D. Jesch, P. N. Black, C. C. DiRusso, Identification and characterization of small compound inhibitors of human FATP2, Biochem. Pharmacol. 79 (2010) 990–999.

[20] S. E. Alfred, A. Surendra, C. Le, K. Lin, A. Mok, I. M. Wallace, M. Proctor, M. L. Urbanus, G. Giaever, C. Nislow, A phenotypic screening platform to identify small molecule modulators of *Chlamydomonas reinhardtii* growth, motility and photosynthesis, Genome Biol. 13 (2012) R105.

[21] A. K. Franz, M. A. Danielewicz, D. M. Wong, L. A. Anderson, J. R. Boothe, Phenotypic screening with oleaginous microalgae reveals modulators of lipid productivity, ACS Chem. Biol. 8 (2013) 1053–1062.

[22] J.-H. Zhang, T. D. Chung, K. R. Oldenburg, A simple statistical parameter for use in evaluation and validation of high throughput screening assays, J. Biomol. Screen. 4 (1999) 67–73.

[23] A. R. Wellburn, The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution, J. Plant Physiol. 144 (1994) 307–313.

[24] A. Leyva, A. Quintana, M. Sanchez, E. N. Rodriguez, J. Cremata, J. C. Sanchez, Rapid and sensitive anthrone–sulfuric acid assay in microplate format to quantify carbohydrate in biopharmaceutical products: method development and validation, Biologicals 36 (2008) 134–141.

[25] V. Matyash, G. Liebisch, T. V. Kurzchalia, A. Shevchenko, D. Schwudke, Lipid extraction by methyl–tert–butyl ether for high-throughput lipidomics, J. Lipid Res. 49 (2008) 1137–1146.

[26] M. Y. Roleda, S. P. Slocombe, R. J. Leakey, J. G. Day, E. M. Bell, M. S. Stanley, Effects of temperature and nutrient regimes on biomass and lipid production by six oleaginous microalgae in batch culture employing a two-phase cultivation strategy, Bioresour. Technol. 129 (2013) 439–449.

[27] S. Kim, H. Kim, D. Ko, Y. Yamaoka, M. Otsuru, M. Kawai-Yamada, T. Ishikawa, H. M. Oh, I. Nishida, Y. Li-Beisson, Y. Lee, Rapid induction of lipid droplets in *Chlamydomonas reinhardtii* and *Chlorella vulgaris* by Brefeldin A, PLoS One 8 (2013) e81978.

[28] V. H. Work, R. Radakovits, R. E. Jinkerson, J. E. Meuser, L. G. Elliott, D. J. Vinyard, L. M. Laurens, G. C. Dismukes, M. C. Posewitz, Increased lipid accumulation in the *Chlamydomonas reinhardtii* sta7–10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains, Eukaryot. Cell 9 (2010) 1251–1261.

[29] R. Miller, G. Wu, R. R. Deshpande, A. Vieler, K. Gartner, X. Li, E. R. Moellering, S. Zauner, A. J. Cornish, B. Liu, B. Bullard, B. B. Sears, M. H. Kuo, E. L. Hegg, Y. Shachar- Hill, S. H. Shiu, C. Benning, Changes in transcript abundance in *Chlamydomonas reinhardtii* following nitrogen deprivation predict diversion of metabolism, Plant Physiol. 154 (2010) 1737–1752.

[30] S. Schmollinger, T. Muhlhaus, N. R. Boyle, I. K. Blaby, D. Casero, T. Mettler, J. L. Moseley, J. Kropat, F. Sommer, D. Strenkert, D. Hemme, M. Pellegrini, A. R. Grossman, M. Stitt, M. Schroda, S. S. Merchant, Nitrogen-sparing mechanisms in *Chlamydomonas* affect the transcriptome, the proteome, and photosynthetic metabolism, Plant Cell 26 (2014) 1410–1435.

[31] I. K. Blaby, A. G. Glaesener, T. Mettler, S. T. Fitz-Gibbon, S. D. Gallaher, B. Liu, N. R. Boyle, J. Kropat, M. Stitt, S. Johnson, C. Benning, M. Pellegrini, D. Casero, S. S. Merchant, Systems-level analysis of nitrogen starvation-induced modifications of carbon metabolism in a *Chlamydomonas reinhardtii* starchless mutant, Plant Cell 25 (2013) 4305–4323.

[32] N. Kato, T. Dong, M. Bailey, T. Lum, D. Ingram, Triacylglycerol mobilization is suppressed by brefeldin A in *Chlamydomonas reinhardtii*, Plant Cell Physiol. 54 (2013) 1585–1599.

[33] M. Zhang, J. Fan, D. C. Taylor, J. B. Ohlrogge, DGAT1 and PDAT1 acyltransferases have overlapping functions in *Arabidopsis* triacylglycerol biosynthesis and are essential for normal pollen and seed development, Plant Cell Online 21 (2009) 3885–3901.

[34] D. H. Miller, I. S. Mellman, D. T. Lamport, M. Miller, The chemical composition of the cell wall of *Chlamydomonas gymnogama* and the concept of a plant cell wall protein, J. Cell Biol. 63 (1974) 420–429.

[35] B. L. Russel, Determination of Factors Limiting Enzymatic Hydrolysis of the *Chlorella sorokiniana* Cell Wall, University of Florida, Florida, 1995. 153.

[36] H. Takeda, Sugar composition of the cell wall and the taxonomy of *Chlorella* (*Chlorophyceae*), J. Phycol. 27 (1991) 224–232.

[37] H. Takeda, T. Hirokawa, Studies on the cell wall of Chlorella I. Quantitative changes in cell wall polysaccharides during the cell cycle of *Chlorella ellipsoidea*, Plant Cell Physiol. 19 (1978) 591–598.