Video Article

A Simple and Rapid Protocol for Measuring Neutral Lipids in Algal Cells Using Fluorescence

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Abstract

Algae are considered excellent candidates for renewable fuel sources due to their natural lipid storage capabilities. Robust monitoring of algal fermentation processes and screening for new oil-rich strains requires a fast and reliable protocol for determination of intracellular lipid content. Current practices rely largely on gravimetric methods to determine oil content, techniques developed decades ago that are time consuming and require large sample volumes. In this paper, Nile Red, a fluorescent dye that has been used to identify the presence of lipid bodies in numerous types of organisms, is incorporated into a simple, fast, and reliable protocol for measuring the neutral lipid content of *Auxenochlorella protothecoides*, a green alga. The method uses ethanol, a relatively mild solvent, to permeabilize the cell membrane before staining and a 96 well micro-plate to increase sample capacity during fluorescence intensity measurements. It has been designed with the specific application of monitoring bioprocess performance. Previously dried samples or live samples from a growing culture can be used in the assay.

Video Link

The video component of this article can be found at https://www.jove.com/video/51441/

Introduction

Due to their ability to store lipid bodies under certain stress conditions, algae have received a great deal of attention in recent years as a potential renewable fuel source[1,2]. Neutral lipids can account for over 60% of the cell dry weight under appropriate growth conditions[3]. Yet the industry does not have a simple, clean, rapid, and reliable standardized protocol to quantitate lipid content of algal cells in order to properly monitor bioprocess performance, analyze cultures, and screen for new strains.

The Bligh-Dyer gravimetric method developed some 50 years ago remains among the most common techniques used today[4,5]. While this procedure is simple, reliable, and easy to carry out, it is time-consuming, necessitates large sample volumes, and makes use of toxic solvents. It is not practical for analyzing many samples from a fermentation run or screening for new oil-rich strains. Other methods have been developed, but usually require advanced equipment and have not been standardized[6].

An alternative that has garnered a great deal of interest is the Nile Red stain. Nile Red, a dye that fluoresces preferentially in non-polar environments, has been used to identify or quantify lipid bodies in various organisms including nematodes[7], yeast[8], bacteria[9], and algae[10-19]. Initial techniques involving Nile Red were mostly qualitative or semi-quantitative, combining the stain with single-cuvette spectrophotometry or flow cytometry. In addition, some classes of algae such as green algae have thick cell wells that are mostly impermeable to the dye, which limited the range of the technique[10].

Recent improvements to the Nile Red staining method have been reported that bypass the initial shortcomings of the protocol[10,11]. Staining the cells in the presence of a carrier solvent such as DMSO[10] or ethanol[10,11] linearizes the relationship between oil content and absorbance, allowing for reliable quantitative measurements. The solvent helps permeabilize the cell membrane so that the Nile Red molecules can pass through. In addition, incorporating a spectrophotometer with micro-plate reading capabilities enables high throughput protocols suitable for quantitative analysis.

In this article we detail a simple method for measuring oil content of algal cells by staining cultures with Nile Red in the presence of ethanol, a mild solvent. In order to most accurately account for background noise in the measurements, a standard curve correlating fluorescence intensity to oil content is developed using algal cells of known oil composition. The method is adapted from previously published protocols[10,11]. By using a 96-well spectrophotometer, one is able to analyze the same amount of samples in an hour that would take days to monitor by gravimetric methods. Furthermore, by calibrating using representative samples of the desired algal species this method produces relatively precise measurements that are directly interpretable. There exist many protocols outlining methods of staining algae with Nile Red optimized for different strains and applications; the protocol presented here was originally developed by de la Hoz Siegler *et al.*[11] for *Auxenochlorella*

protothecoides, *Chlorella vulgaris*, *Scenedesmus dimorphus*, and *Scenedesmus obliquus*, although it is likely suitable for many more species and classes. It has been designed with the specific application of monitoring bioprocess performance and it works equally well for previously dried samples and wet samples from a growing culture.

Protocol

1. Isolation of Dry Algal Biomass to be Used as Standards for Fluorescence Readings

1. Remove a sample volume from the growing algal culture that will provide at least 200 mg of dry biomass, 400-600 mg is preferable.
2. Centrifuge sample at 4 °C for 10 min at 10,000 x g. Discard the supernatant and wash the pellet with an equal volume of phosphate buffer formulated to the same pH as the growth media.
3. Repeat step 1.2 for a total of 3 washing steps.
4. Re-suspend the pellet in de-ionized water and transfer to a pre-weighed weigh dish. Let dry at 50 °C for 48 hr. NOTE: Drying under vacuum will decrease drying time and drying the culture at temperatures above 50 °C may make resuspension difficult.
5. Store dried algal cultures at room temperature for future use.

2. Gravimetric Quantification of Neutral Lipids by Hexane Extraction (Adapted from Bligh and Dyer[4])

1. Measure approximately 50 mg of dry algal biomass in a weigh dish. NOTE: Masses ranging from 40-80 mg can be used without loss of reproducibility.
2. Transfer the biomass to a mortar pre-washed with hexane. If necessary, wash the weigh dish with a small amount (1 ml) of hexane using a Pasteur pipette in order to completely transfer the biomass to the mortar. NOTE: Hexane is a highly volatile and toxic substance. It must be handled in the fume hood with proper protective clothing.
3. Grind the algal biomass for 5 min using a pestle. Begin with gentle grinding and gradually increase intensity. Grind the biomass into a fine and smooth paste in the 5 min period. If excess hexane is used when transferring the biomass to the mortar, it is best to wait for the hexane to evaporate before grinding.
4. Add a few ml of hexane to the mortar and mix the resulting slurry with the pestle until it is homogenized. Ensure that all the cell debris adhered to the walls of the mortar are knocked free and suspended in the liquid.
5. Transfer the hexane-cell mass mixture to a centrifuge tube. NOTE: The centrifuge tube must be either glass or a suitable polymer compatible with hexane such as Teflon.
6. Repeat steps 2.4 and 2.5 until all the biomass has been transferred to the centrifuge tube (3-5x).
7. Centrifuge the sample at 4 °C for 20 min at 10,000 x g.
8. Carefully pipette the supernatant into a pre-weighed metal weigh dish. Store in the fume hood.
9. Perform a 2nd hexane extraction by adding 3 ml of hexane to the pellet and vortexing vigorously for 1 min.
10. Repeat steps 2.7 and 2.8. If necessary, run the samples for 30 min in the centrifuge during the second extraction to ensure all cell debris fully settles. Determine mass of oil extracted gravimetrically after hexane has completely evaporated.

3. Fluorometric Quantification of Neutral Lipids Using Nile Red (as Reported by de la Hoz Siegler *et al.*[11])

NOTE: Only 10 µl of an algal suspension at 5 g/L is needed for the fluorescence reading. Generally, isolation of dry algal biomass from 1.5 ml of culture broth is more than sufficient. Also, the light intensity of the lamp in the spectrophotometer can degrade over time. It is recommended to include standards in every experiment to ensure that variations in the instrument do not add unnecessary error to the measurements.

1. Prepare a Nile Red solution at a concentration of 10 µg/ml dissolved in alcohol reagent grade ethanol. Store this solution in the dark at 4 °C.
2. Prepare a 30% (v/v) ethanol solution in deionized water and store at 4 °C.
3. Prepare all algal samples at the same biomass concentration (5 g/L is recommended) and in the same manner as the standards used in the measurement. Do this by either suspending pre-dried samples in the appropriate amount of phosphate buffer (0.6 g/L potassium phosphate dibasic, 1.4 g/L potassium phosphate monobasic), or adjusting the concentration of a growing algal culture to 5 g/L with phosphate buffer after measuring the turbidity. NOTE: measurements performed on live algal cultures will often have larger error associated with them depending on the precision of the turbidity calibration curve. Resuspending dried samples may require the use of a homogenizer to fully disperse the biomass.
4. For each sample, mix 80 µl of the 30% ethanol solution, 10 µl of the Nile Red solution, and 10 µl of algal suspension in a single well of a 96-well plate. In order to properly account for the variability of the fluorescence measurement, perform 5 replicates of each sample.
5. Run a two point calibration curve with standards prepared previously in order to account for day-to-day variations in the instrument and preparation. Prepare the standards for fluorescence measurement using the same procedure as the samples. NOTE: Generally two points is sufficient for recalibration of the instrument, three points can be run to verify linearity.
6. Perform the fluorescence measurements in a multi-well plate reader spectrophotometer. The following conditions were found to yield the most consistent results[11]:
    1. Shake at 1,200 rpm, orbit 3 mm, for 30 sec.
    2. Incubate at 40 °C for 10 min.
    3. Shake at 1,200 rpm, orbit 3 mm, for 30 sec.
    4. Record fluorescence, excitation at 530 nm, emission at 604 nm.

7. Convert the fluorescence measurements to oil content using the results from the internal standards.

4. Fluorescence Microscopy Technique

NOTE: The staining protocol described in section 3 is designed for quantitative analysis, but it can also be useful to provide visual representations of stain-based techniques for educational and illustrative purposes. To produce images of sample fluorescence one requires an optical microscope with traditional transmission and additional epifluorescence illumination sources. Excitation and emission light filters in the 530 nm (green) and 604 nm (red) range, respectively, are needed for the Nile Red stain as well as a microscope mounted camera with associated software. The images shown in this study (**Figure 1**) were acquired using a bright field microscope equipped with a camera and Monochrome to RGB converter unit. The procedure for producing Nile Red fluorescence images using these tools is outlined below:

1. Prepare a culture sample with the Nile Red stain according to section 3 of the protocol. NOTE: a sample in the 5 g/L concentration range produces slides of adequate cell density without overcrowding.
2. After completing step 3.6 of the staining protocol, prepare a microscope slide of the processed sample according to standard laboratory procedures.
3. Starting with the microscope in transmission mode, load the prepared slide into the microscope, and locate the cells at the desired magnification (images shown in this article were acquired with the 100X objective).
4. Once focused, switch the microscope from transmission to epifluorescence illumination mode. The light source should now be coming directly from the objective lens (this can be confirmed visually by observing the space between the slide and the objective lens).
5. Insert a green excitation filter into the light source and a red emission filter into the observation light path; the fluorescence of the stained cells should now be directly visible through the eyepiece.
6. Switch the microscope observation mode from the eyepiece to the mounted camera and use viewing software to capture an image of the fluorescing cells. Depending on the sensitivity of the camera, the sample may not initially appear in the preview window (*i.e.* the screen will be black); to remedy this, adjust the exposure time and gain of the camera to a level where the cells are visible. Specific settings will vary with instruments, equipment, and cell types.

Representative Results

Representative algal cells stained with Nile Red dye are depicted in **Figure 1**. Parts **A** and **B** of **Figure 1** display images of *A. protothecoides* grown in excess nitrogen, leading to very low intracellular lipid accumulation. In parts **C** and **D**, samples of *A. protothecoides* grown under nitrogen limitation are shown. Under transmission illumination, the lipid bodies of the cell can be visualized with careful inspection of **Figure 1C**, where they appear as shiny circular structures and constitute the majority of the cell volume. The cells shown in **Figure 1A**, which were grown in excess nitrogen, are only 5% oil by dry weight and do not contain significant levels of lipid bodies.

When shown under the appropriate light conditions, the differences in these samples are magnified. The oil-lean cells appear as fluorescent rings with dark bodies (**Figure 1B**) while the oil-rich cells display a bright orange-red glow where they have accumulated lipid bodies (**Figure 1D**). In **Figure 1D**, it is more difficult to see the faint fluorescence of the cell membrane and other cell structures. Nile Red will fluoresce in the presence of non-polar proteins as well as lipids, which is why the cell membrane and other cellular structures provide a background level of fluorescence during the spectrophotometer measurements.

Under the optimized conditions of the assay, calibration curves with R^2 values greater than 0.980 are readily achievable (**Figure 2A**). The relationship between cell oil content and fluorescence becomes non-linear if the cells are stained in a solution lacking a carrier solvent such as ethanol. The data presented in **Figure 2B**, with an R^2 of 0.395, were obtained by carrying out the protocol in a 0% ethanol solution (pure deionized water).

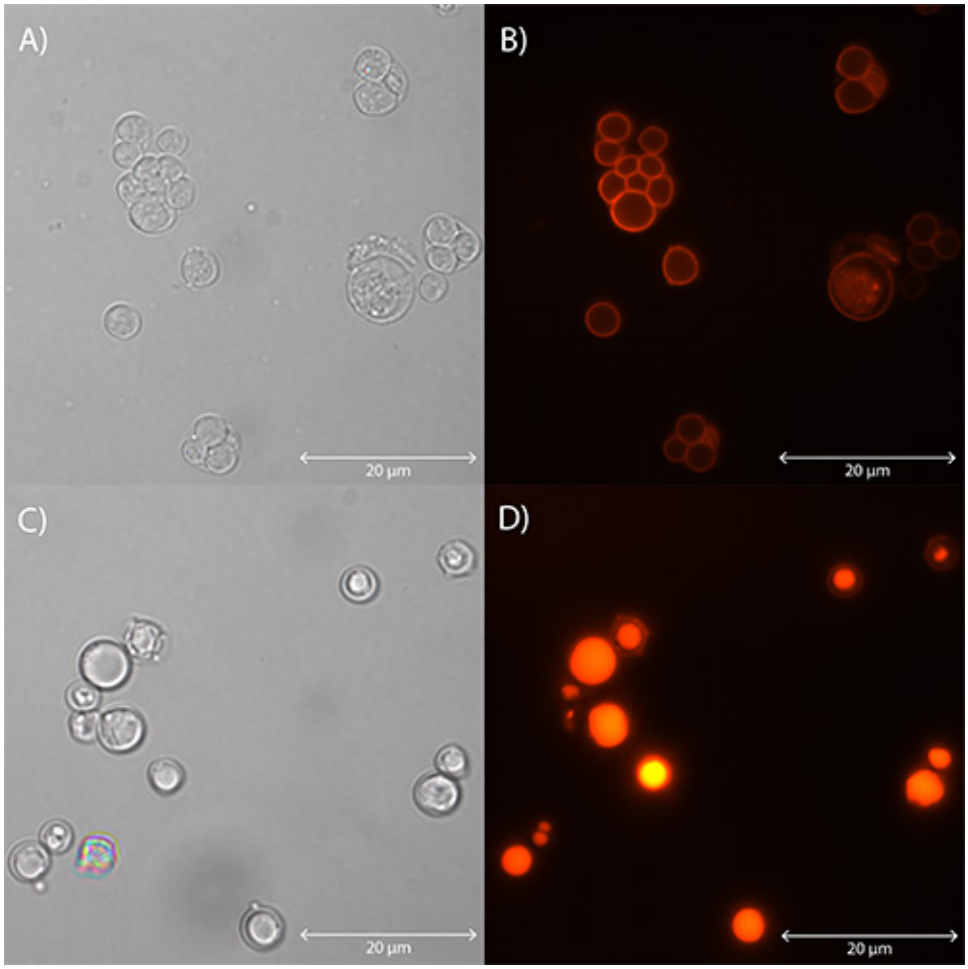

Figure 1. Images of *A. protothecoides* stained with Nile Red. An oil-lean sample (4.4% oil content by dry weight) is shown under **A)** transmitted light microscopy and **B)** epifluorescence illumination with green excitation filter (510 nm). The lipid bodies stained with Nile Red fluoresce at 604 nm. An oil-rich sample (54.7% oil content by dry weight) is shown in **C)** and **D)** under the same transmitted and epifluorescence light conditions as **A)** and **B)**, respectively. Click here to view larger image.

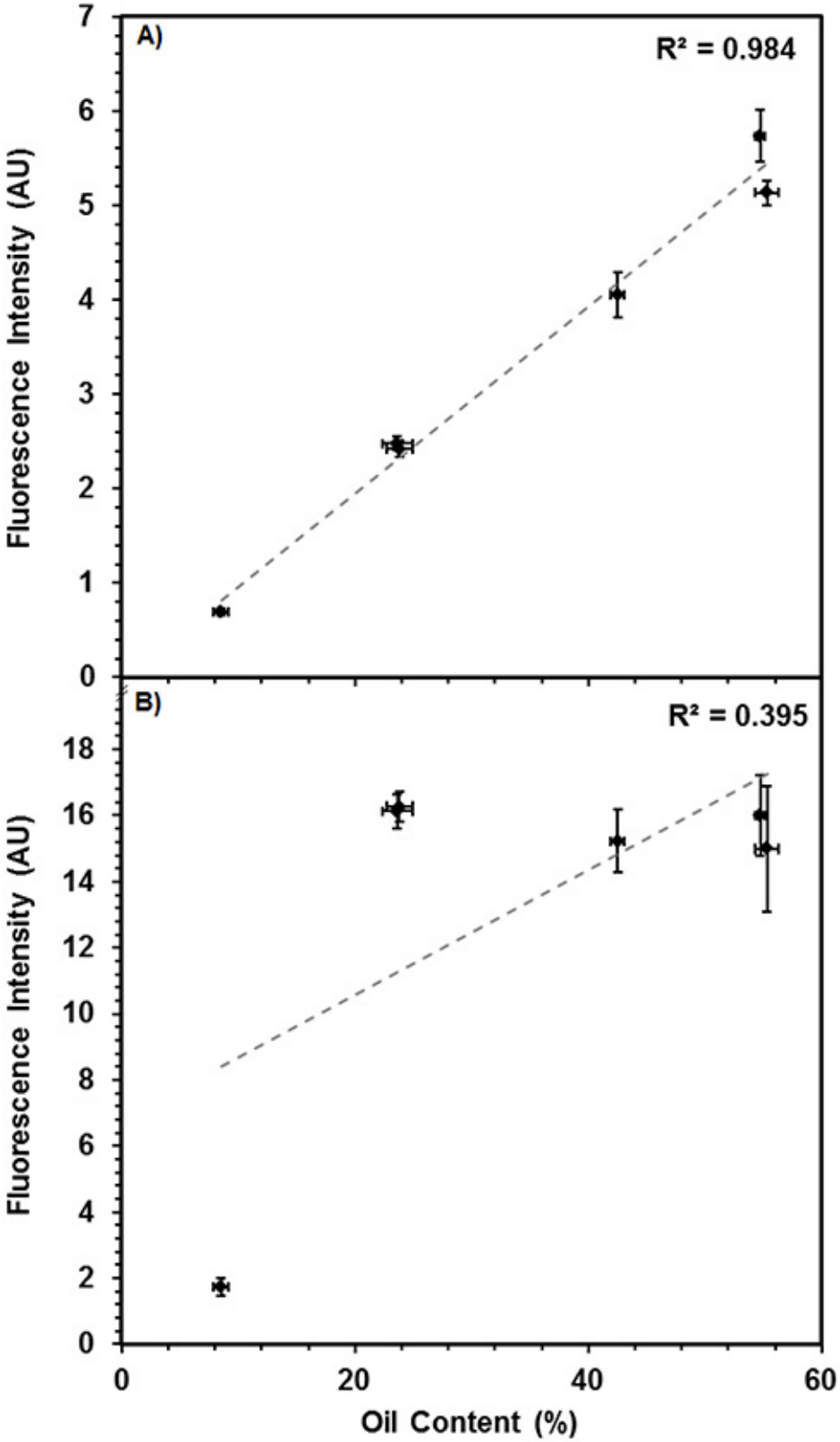

Figure 2. Calibration curves correlating the oil content of *A. protothecoides* to fluorescence intensity. In **A)**, samples were prepared according to the described protocol. In **B)**, samples were prepared in a 0% ethanol solution (deionized water). Vertical error bars represent the standard deviation of five replicates in the spectrophotometer. Horizontal error bars report the standard deviation of at least three replicates from the gravimetric quantification of neutral lipids. Click here to view larger image.

Chemical	Company	Concentration
KH_2PO_4	Fisher Scientific	2.8 $g \cdot L^{-1}$
K_2HPO_4	Fisher Scientific	1.2 $g \cdot L^{-1}$
$MgSO_4 \cdot 7H_2O$	Fisher Scientific	1.2 $g \cdot L^{-1}$
$FeSO_4 \cdot 7H_2O$	Fisher Scientific	48 $mg \cdot L^{-1}$
H_3BO_3	Fisher Scientific	11.6 $mg \cdot L^{-1}$
$CaCl_2 \cdot 2H_2O$	Fisher Scientific	10 $mg \cdot L^{-1}$
$MnCl_2 \cdot 4H_2O$	Fisher Scientific	7.2 $g \cdot L^{-1}$
$ZnSO_4 \cdot 7H_2O$	Fisher Scientific	0.88 $mg \cdot L^{-1}$
$CuSO_4 \cdot 5H_2O$	Fisher Scientific	0.32 $mg \cdot L^{-1}$
$Na_2MoO_4 \cdot 4H_2O$	Fisher Scientific	0.12 $\mu g \cdot L^{-1}$
thiamine hydrochloride	Fisher Scientific	40 $\mu g \cdot L^{-1}$
glucose	Fisher Scientific	30 $g \cdot L^{-1}$
glycine	Fisher Scientific	0.2-2.0 $g \cdot L^{-1}$

Table 1. Culture media recipe. All reagents were of analytical grade. Solutions were prepared using deionized water. The glycine concentration was used to control the carbon to nitrogen ratio in the culture and consequently, the final oil content of the algae. Cells ranging from 8.5% to 55% neutral lipid content by mass (dry basis) were used to make the standard curve.

Discussion

The algae used in the standard curve must be the same species cultivated under the same experimental conditions as those being measured. Significant changes in media composition, cultivation technique, and staining protocol can affect the intensity of the fluorescence reading. Hexane extraction (described in sections 1 and 2) was used to determine the neutral lipid content of samples used in the standard curve. For accurate fluorescence intensity measurements, all samples must be analyzed at the same biomass concentration (5 g/L was used in this study). Oil content can then be easily determined by comparison to the standard curve. For this protocol, algae were cultivated in shake flasks under heterotrophic conditions (28 °C, 100 rpm) with glucose as the carbon source (30 g/L) and glycine as the nitrogen source (0.2-2.0 g/L). For a detailed media recipe, see **Table 1**.

An important consideration in carrying out the protocol is the conditions for drying the wet algal biomass when preparing the standard curve. A vacuum oven at 45 °C is recommended. A conventional oven at 45-50 °C will also suffice so long as additional time is allowed to completely dry the samples (24-48 hr). Temperatures above 50 °C may cause the biomass to fuse together or form an impenetrable crust that does not readily resuspend in phosphate buffer. Resuspension may be aided with the use of a homogenizer. Chemical additives such as surfactants or a strong base should be avoided as these usually influence the fluorescence reading. Dried cultures can be stored for up to 6 months in a sealed container without experiencing significant lipid degradation. Note that measurements performed on live algal cultures will often have larger error associated with them depending on the precision of the turbidity calibration curve.

Another consideration is the ethanol concentration used to run the samples in the micro-plate reader. Ethanol acts as a carrier solvent, facilitating the transport of Nile Red molecules into the cell. At low concentrations (< 30%) the relationship between oil content and fluorescence becomes non-linear due to poor diffusion across the cell membrane (**Figure 2B**). At higher ethanol concentrations, improved linearity is obtained at the expense of decreased fluorescence intensity (**Figure 2A).** For *Auxenochlorella protothecoides*, *Chlorella vulgaris*, *Scenedesmus dimorphus*, and *Scenedesmus obliquus*, an ethanol concentration of 30% was found to give the optimal trade-off between improved linearity and decreased fluorescence intensity[11]. It may be necessary to adjust the ethanol concentration to optimize results for other organisms.

Choosing the proper excitation and emission wavelengths for reading the fluorescence in the algal samples is crucial to ensure only neutral lipids are included in the measurement. Nile Red fluorescence intensity will vary at different wavelengths dependent on the polarity of the environment[11,13]. For *A. protothecoides*, the emission peak for neutral lipids is observed at 590 nm; a secondary peak representing more polar lipids can be observed at 645 nm[11]. Consequently, fluorescence intensity measurements outlined in this procedure were performed using a 530 nm excitation filter, with half band-width = 10 nm, and a 604 nm emission filter, with half bandwidth = 10 nm. Choice of excitation and emission wavelength should be verified for every algal strain tested.

Since Nile Red will fluoresce in any non-polar environment, cell structures in addition to lipid bodies will be detected by the spectrophotometer (see **Figure 1B**). This fact, in combination with the inherent heterogeneity of cell biomass, leads to a non-linear relationship between cell biomass and fluorescence[11]. This problem is solved by ensuring all samples are prepared at a constant dry weight (5 g/L recommended). At dilute concentrations the signal becomes faint and its trends are difficult to distinguish. Additionally, the fluorescence signal can be noisy due to cell-to-cell variations and particulate suspensions in the media. Consequently, 5 replicates of each sample are recommended for reliable results[11].

Some protocols use an oil standard such as triolein to calibrate the measurement[10,20,21]. While this technique has been reported with success for certain species of algae such as *Bacillariophyceae* and *Dinophyceae*, it is less suitable for thick-walled algae such as *Chlorella* due to diffusion limitations across the cell membrane. Furthermore, the addition of triolein may disturb the distribution of Nile Red molecules between the standard and the lipid bodies, leading to inaccurate estimations of oil content. Using algal samples of known oil content as standards avoids these potential shortfalls and accounts for any background fluorescence caused by the biomass and growth conditions.

The protocol presented in this study provides a simple, cheap and rapid method of quantifying neutral lipids in algal samples. By using a multi-well plate, high sample throughputs are achieved with significant time savings over other staining protocols. In addition, the conditions of the assay have been optimized to yield reproducible linear trends.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Chisti, Y. Biodiesel from microalgae. *Biotechnol. Adv.* **25**, 294-306, doi:10.1016/j.biotechadv.2007.02.001 (2007).
2. U.S. Department of Energy. National Algal Biofuels Technology Roadmap. *Energy Efficiency & Renewable Energy*. U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, Biomass Program. http://www1.eere.energy.gov/bioenergy/pdfs/algal_biofuels_roadmap.pdf (2010).
3. de la Hoz Siegler, H. *et al.* Optimization of microalgal productivity using an adaptive, non-linear model based strategy. *Bioresour. Technol.* **104**, 537-546, doi:10.1016/j.biortech.2011.10.029 (2012).
4. Bligh, E. G., & Dyer, W. J. A Rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911-917 (1959).
5. Hara, A., & Radin, N. S. Lipid extraction of tissues with a low-toxicity solvent. *Anal. Biochem.* **90**, 420-426, doi:10.1016/0003-2697(78)90046-5 (1978).
6. Han, Y. *et al.* Review of methods used for microalgal lipid-content analysis. *Energ. Procedia.* **12**, 944-950 (2011).
7. Pino, E. C. *et al.* Biochemical and high throughput microscopic assessment of fat mass in *Caenorhabditis elegans*. *J. Vis. Exp.* **73**, e50180, doi:10.3791/50180 (2013).
8. Sitepu, I. R. *et al.* An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. *J. Microbiol. Meth.* **91**, 321-328, doi:http://dx.doi.org/10.1016/j.mimet.2012.09.001 (2012).
9. Izard, J., & Limberger, R. J. Rapid screening method for quantitation of bacterial cell lipids from whole cells. *J. Microbiol. Meth.* **55**, 411-418 (2003).
10. Chen, W. *et al.* A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *J. Microbiol. Meth.* **77**, 41-47, doi:10.1016/j.mimet.2009.01.001 (2009).
11. de la Hoz Siegler, H. *et al.* Improving the reliability of fluorescence-based neutral lipid content measurements in microalgal cultures. *Algal Res.* **1**, 176-184, doi:10.1016/j.algal.2012.07.004 (2012).
12. de la Jara, A. *et al.* Flow cytometric determination of lipid content in a marine dinoflagellate, *Crypthecodinium cohnii*. *J. Appl. Phycol.* **15**, 433-438, doi:10.1023/a:1026007902078 (2003).
13. Elsey, D. *et al.* Fluorescent measurement of microalgal neutral lipids. *J. Microbiol. Meth.* **68**, 639-642, doi:10.1016/j.mimet.2006.11.008 (2007).
14. Feng, G.-D. *et al.* Evaluation of FT-IR and Nile Red methods for microalgal lipid characterization and biomass composition determination. *Bioresour. Technol.* **128**, 107-112, doi:10.1016/j.biortech.2012.09.123 (2013).
15. Guzmán, H. *et al.* Estimate by means of flow cytometry of variation in composition of fatty acids from *Tetraselmis suecica* in response to culture conditions. *Aquacult. Int.* **18**, 189-199, doi:10.1007/s10499-008-9235-1 (2010).
16. Huang, G.-H. *et al.* Rapid screening method for lipid production in alga based on Nile red fluorescence. *Biomass Bioenerg.* **33**, 1386-1392, doi:10.1016/j.biombioe.2009.05.022 (2009).
17. Lee, S. *et al.* Rapid method for the determination of lipid from the green alga *Botryococcus braunii*. *Biotechnol. Tech.* **12**, 553-556, doi:10.1023/a:1008811716448 (1998).
18. Montero, M. *et al.* Isolation of high-lipid content strains of the marine microalga *Tetraselmis suecica* for biodiesel production by flow cytometry and single-cell sorting. *J. Appl. Phycol.* **23**, 1053-1057, doi:10.1007/s10811-010-9623-6 (2011).
19. Vigeolas, H. *et al.* Isolation and partial characterization of mutants with elevated lipid content in *Chlorella sorokiniana* and *Scenedesmus obliquus*. *J. Biotechnol.* **162**, 3-12, doi:10.1016/j.jbiotec.2012.03.017 (2012).
20. Bertozzini, E. *et al.* Application of the standard addition method for the absolute quantification of neutral lipids in microalgae using Nile red. *J. Microbiol. Meth.* **87**, 17-23, doi:10.1016/j.mimet.2011.06.018 (2011).
21. Kou, Z. *et al.* Fluorescent measurement of lipid content in the model organism *Chlamydomonas reinhardtii*. *J. Appl. Phycol.*, 1-9, doi:10.1007/s10811-013-0011-x (2013).

