Article

Measurement of Lipid Droplet Accumulation Kinetics in *Chlamydomonas reinhardtii* Using Seoul-Fluor

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Abstract: Alternative energy resources have become an important issue due to the limited stocks of petroleum-based fuel. Microalgae, a source of renewable biodiesel, use solar light to convert CO_2 into lipid droplets (LDs). Quantification of LDs in microalgae is required for developing and optimizing algal bioprocess engineering. However, conventional quantification methods are both time and labor-intensive and difficult to apply in high-throughput screening systems. LDs in plant and mammalian cells can be visualized by staining with various fluorescence probes such as the Nile Red, BODIPY, and Seoul-Fluor (SF) series. This report describes the optimization of LD staining in *Chlamydomonas reinhardtii* with SF probes via systematic variations of dye concentration, staining time, temperature, and pH. A protocol for quantitative measurement of accumulation kinetics of LDs in *C. reinhardtii* was developed using a spectrofluorimeter and the accuracy of LD size measurement was confirmed by transmission electron microscopy (TEM). Our results indicate that our spectrofluorimeter-based measurement approach can monitor kinetics of intracellular LDs (in control and nitrogen-source-starved *Chlamydomonas reinhardtii*)

accumulation that has not been possible in the case of conventional imaging-based methods. Our results presented here confirmed that an SF44 can be a powerful tool for *in situ* monitoring and tracking of intracellular LDs formation.

Keywords: microalgae; *Chlamydomonas reinhardtii*; SF44; lipid droplets; biodiesel

1. Introduction

Petroleum-based fuels have been the main energy source of modern society since the Industrial Revolution. Due to increasing energy demands, we are facing the risk of an energy shortage due to decreasing reserves of fossil fuels. In addition, accumulation of CO_2 as a by-product of fossil fuel consumption has emerged as a source of global warming. Numerous governmental and industrial efforts are therefore focused on exploring alternative energy sources that are renewable and carbon neutral [1]. One of these alternative energy sources is biodiesel derived from crops including corn, soybean, canola, coconut, and palm oils. However, these materials cannot entirely replace petroleum as transport fuels due to their relatively low oil yield (L/ha) [1]. Microalgae has been discussed as a source of biodiesel for over 50 years [2]. Microalgae convert CO_2 and solar energy into biofuel [3–7], food [8], feed [9] and high-value molecules [10–16]. Compared with traditional crops, oil yield from microalgae can be up to 10^3 to 10^4 time higher [1]. In addition, microalgae can grow rapidly and require only 1 to 10% of the cultivation area needed for most crops [1]. Generally, the oil content of microalgae is 20%–50% by weight of dry biomass [1]. Biodiesel consists of mono-alkyl esters that are formed from triglycerides via transesterification [17]. In microalgae, triglycerides form lipid droplets (LDs), an intracellular organelle containing neutral lipids surrounded by a phospholipid monolayer that are used for storing food energy [18].

Conventional methods for lipid quantitation are solvent extraction and gravimetric determination [19]. Other methods including thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC), and gas chromatography (GC), require dedicated analytical instrumentation [20]. These methods are both time- and labor-intensive and difficult to apply in high-throughput screening systems. Therefore, there is a strong need for the development of *in situ* lipid quantification methods [21].

Nile Red, one of the most frequently used lipid-soluble fluorescence dyes, has been used to measure lipid content of animal cells [22] and microorganisms [21,23–25]. Since the first applications of Nile Red to microalgae, [26] Nile Red has continued to be used to measure lipid contents as an industrial standard. Recently, combined with flow cytometry, Nile Red has been more powerful tool for high-throughput screening systems and single cellular level dynamics of lipid accumulations [27]. Moreover, Nile Red has continuously been reported to enhance applications of high-throughput screening systems. Due to the dense and complex network of cell wall of microalgae, some groups optimized staining conditions through considered physico-chemical factors, including dye concentration, staining time, temperature and pH in diverse classes of microalgae [25].

In this study, we introduced a new LD-specific fluorescence probe; SF44. 9-Aryl-1,2-dihydropyrrolo[3,4-*b*]indolizin-3-one, named Seoul-Fluor (SF), is a tunable and predictable fluorescent core skeleton. (Scheme 1) SF44 and various analogues containing electron-donating amine moieties

have been introduced as a LD-specific fluorescence bioprobes in mammalian cells [28] and microorganisms [29]. SF44 has been successfully applied to the specific measurement of LD organelles using its fluorogenic properties under unique hydrophobic environment of LDs in cytosols [28]. SF44-based LD staining does not require any washing and fixation steps to acquire fluorescence signals as same with conventional Nile Red method. Therefore, SF44 can be considered a powerful tool for developing algal bioprocesses and for optimizing quantitative, high-throughput screening.

This study describes the demonstration of staining conditions using SF44 with *Chlamydomonas reinhardtii* (a species of microalgae widely used as a model organism). The optimized protocol is a sensitive and quantitative method for high-throughput screening of intracellular lipid accumulations of microalgae for biodiesel research.

Scheme 1. A schematic diagram showing the chemical structure of SF44. LDs and liposome specific fluorescence intensity was monitored at 540 nm with excitation at 450 nm.

λ_{ex} = 450 nm / λ_{em} = 540 nm

$C_{29}H_{36}N_4O_4$

MW: 504.621

(9-Aryl-1,2-dihydropyrrolo[3,4-*b*]indolizin-3-one)

2. Results and Discussion

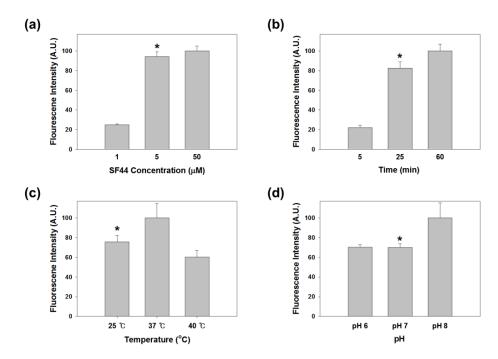### 2.1. Optimization of SF44 Staining for Microalgae

SF44 has been successfully used to stain LDs in mammalian cells such as 3T3-L1 murine adipose and HeLa cells [28]. Unlike mammalian cells, microalgae are surrounded by a dense, complex network of cell walls [30], therefore, additional physical and chemical factors need to be considered for staining *C. reinhardtii*. Moreover, we wanted to validate the possibility of *in situ* tracking of accumulations of LDs in *C. reinhardtii*, so we chose the optimal conditions for applicable live cell determination.

Considered physico-chemical factors were dye concentration, staining time, temperature, pH, and solvent concentration. (Figure 1) The fluorescence intensity of the *C. reinhardtii* stained with SF44 was affected by dye concentration (Figure 1a). When SF44 concentration was increased from 1 μM to 5 μM, fluorescence intensity changed up to 5 μM. However, compared to 5 μM, no significant difference was observed at 50 μM of SF44. Similar to the optimal concentration used for LDs staining in mammalian cells [27], we identified 5 μM as the optimal concentration.

Staining time is one of the critical factors for high-throughput screening determination. Figure 1b shows that staining efficiency of LDs in *C. reinhardtii* increased up to 60 min. Compared to 5 min, there was significant increase in fluorescence intensity at 25 min and 60 min. We considered the correlation between staining time and intensity as an optimization for *in situ* LD content determination. Although

intensity of 25 min was 17% less than intensity of 60 min, we settled on 25 min as an optimal time by trading off small gain in fluorescence intensity for reducing staining time more than 30 min.

Figure 1. Effects of (**a**) dye concentration; (**b**) staining time; (**c**) temperature; and (**d**) pH on the staining of *Chlamydomonas reinhardtii* cells are shown. The optical density of the cell suspensions was 1.0 (OD$_{680\ nm}$). All data are expressed as the mean of 30 replicates with a single standard deviation. Asterisk indicates the optimal condition for staining SF44.
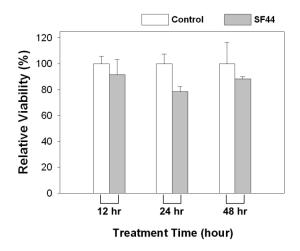

For the measurement of LDs accumulation kinetics, real-time observation without additional processing is ideal. Moreover, conditions compatible with live-cell imaging considered as an important factors such as temperature, pH, and solvent concentration. As indicated in Figure 1c,d, fluorescence intensity was influenced by the temperature and pH used during the staining process. In the case of temperature, intensity was increased with the staining temperature up to 37 °C, but decreased significantly at 40 °C. In case of pH, there was no significant difference between 6.0 and 7.0, but there was increase of fluorescence intensity at pH 8.0. Although fluorescence intensity at 25 °C and pH 7.0 were less than 37 °C and pH 8.0, we chose 25 °C and pH 7.0 as an optimal temperature and pH by considering the conventional freshwater *C. reinhardtii* culture system. In case of DMSO concentration, similar results were observed for different concentration from 1% to 5% (data not shown). We identified 2% of DMSO as an optimal concentration to minimize the amount of DMSO exposure to microalgal cells.

The optimal condition for staining LDs in *C. reinhardtii* therefore involve using SF44 is 5 μM of SF44 dissolved in 2% (*v/v*) DMSO (pH 7.0) and incubated at 25 °C for 25 min. That condition leads to capability for *in situ* tracking of LDs in *C. reinhardtii*.

Based on the above optimized condition, we performed cytotoxic assays for testing feasibility of live-cell continuous monitoring of LDs accumulation in microalgae. To determine cell viability, we used EZ-Cytox that enhanced sensitivity of MTT assays and has been widely used in various organisms [29,31,32]. SF44 staining condition was evaluated with EZ-Cytox assays over 2 days (Figure 2). Compared to control, SF44 showed no significant cytotoxic effect. It seemed that condition

for staining with SF44 is similar to conventional *C. reinhardtii* culture system, so these result shows that the optimal staining conditions determined herein for SF44 are sufficiently non-cytotoxic and can be used to monitor LDs accumulation kinetics while keeping the cells alive.

Figure 2. The viability of *C. reinhardtii* treated with SF44 (gray bar) and control (white bar) is shown with treatment of 12, 24, and 48 h.

Throughout the optimization and cell viability test, we concluded that SF44 is suitable for *in situ* determination and real time live-cell monitoring for accumulations of LDs. To verify that SF44 based measurement of accumulation kinetics of LDs, we evaluated the possibility for quantification of lipid contents in *C. reinhardtii*.
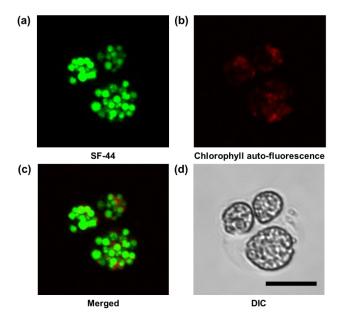
2.2. Quantification of Lipid Contents Using SF44

SF44 has been successfully used to visualize LDs in mammalian cells and microorganism [28,29]. The validity of using SF44 as a LDs specific molecular probe was assessed by analyzing fluorescence micrographs. Figure 3 shows fluorescence micrographs of N-source-starved *C. reinhardtii* during 2 weeks. SF44 exhibited LDs specific staining properties with no overlapping fluorescence signals from chlorophyll, due to the different emission wavelength between SF44 (λ_{em} = 540 nm) and chlorophyll (λ_{em} = 600 nm). This result demonstrates that SF44 may be useful as a LDs specific probe.

However, visualization of LDs stained with SF44 was not validated on initial stage of nitrogen starvation. (Initial stage from 1 day to 2 day, Data was not shown.) After 3 days of nitrogen starvation, LDs formed visible droplets when stained with SF44. It seems that resolution of conventional fluorescence microscope is not capable of resolving smaller size LDs before day 3.

The resolution of conventional optical microscopes are limited to 180 nm in focal plane and 500 nm in optic axis [33]. The size of LDs at beginning microalgae is only a few tens of nanometers, therefore, microscopic imaging-based validation for LD accumulation, especially the early state, is not ideal for quantification of lipid content. There is a need for a quantitative measurement technique for early stage of LD formation.
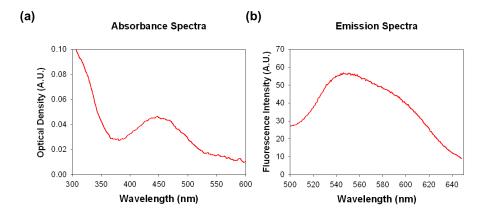
Figure 3. Micrographs showing (**a**) the fluorescence of LDs stained by SF44 (in green); (**b**) chlorophyll auto-fluorescence (in red); (**c**) the merged fluorescence SF44 and chlorophyll auto-fluorescence; and (**d**) a DIC image. All images were acquired through a confocal microscope (FV1000). The scale bar represents 10 μm.
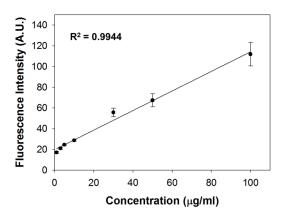

As an alternative, we used a spectrofluorimeter to monitor SF44 stained LD quantitatively at 540 nm with excitation at 450 nm (Figure 4). We prepared liposomes as model LDs using mechanical dispersion methods. Phosphatidylcholine (PC) liposomes were prepared at various concentrations ranging from 1 μg/mL to 100 μg/mL by diluting a stock solution. Fluorescence intensities were measured at each concentration. Figure 5 shows a linear ($R^2 = 0.9944$) standard curve acquired by plotting fluorescence intensity as a function of liposome concentration. This approach shows the possibility of indirect measurement for lipid content using a spectrofluorimeter.

Throughout the quantification of liposome, we concluded that SF44 is suitable for *in situ* determination for LDs content. So, we want to evaluate that SF44 based measurement of accumulation kinetics of LDs.

Figure 4. Fluorescence characteristics of SF44. (**a**) Absorbance and (**b**) emission spectra are shown for SF44 with 1 mg/mL of phosphatidylcholine (PC) liposome. Absorbance and emission wavelength were 450 nm and 540 nm, respectively.
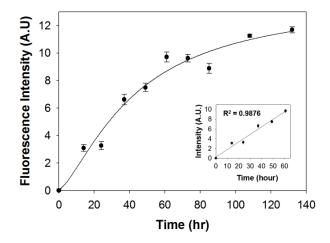
Figure 5. SF44 fluorescence intensity is shown with different concentrations of phosphatidylcholine (PC) liposome. All data are expressed as the mean of five replicates and a single standard deviation ($R^2 = 0.9944$).

2.3. Measurement of Intracellular LDs Accumulation through SF44

Based on correlation between concentration of PC liposome and fluorescence intensity after SF44 staining, we confirmed that fluorescence intensity can be used to track the kinetics of intracellular lipid accumulation with N-source-starved *C. reinhardtii*. Figure 6 shows the fluorescence intensity associated with LD accumulation as a function of time. Fluorescence intensity was monitored at 540 nm with excitation at 450 nm. From the onset of the experiment to 60 h, the LDs concentration increased linearly as a function of time ($R^2 = 0.9876$). No further increase in LDs concentration was observed after 3 days of incubation.
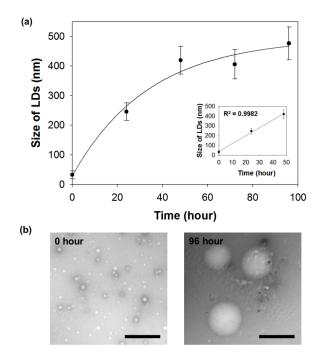
Figure 6. Accumulation kinetics of intracellular LDs. The fluorescence intensity of SF44 was monitored with a spectrofluorimeter. Regression result from initial to 60 h incubation is presented in insert. ($R^2 = 0.9876$) The sample contained *C. reinhardtii* in N-free TAP medium (pH 7.5) incubated at 25 °C, and 5% CO_2 with continuous shaking at 125 rpm. All data are expressed as the mean of 5 replicates and a single standard deviation.

To validate the fluorescence-based indirect measurement for LD accumulation, we measured the size of purified LDs and verified the kinetics. Figure 7 shows the size measurement (from TEM imaging of LD) associated with LDs accumulation. From the onset of the experiment to 48 h, the size
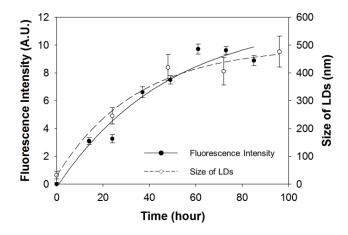
of LDs increased linearly as a function of time (R^2 = 0.9982). Like with fluorescence-based indirect measurements, no further increase in size of LDs was observed after 3 days of incubation.

Figure 7. (**a**) Kinetics of size for intracellular LDs. The size of purified LDs was determined using TEM. Regression result from initial to 48 h incubation is presented in the insert (R^2 = 0.9982); (**b**) TEM micrographs at 0 h and 96 h incubation. The scale bar represents 500 nm. The sample contained *C. reinhardtii* in N-free TAP medium (pH 7.5) incubated at 25 °C, and 5% CO_2 with continuous shaking at 125 rpm. All data are expressed as the mean of 100 replicates and a single standard deviation.

Both indirect and direct measurement methods show an increase until 3 days, then a steady state at the highest level (Figure 8). This result indicates that SF44 can be used as a LD-specific probe for quantitatively tracking intracellular LD accumulation.

Figure 8. Accumulation kinetics of intracellular LDs. The fluorescence intensity of SF44 was monitored with a spectrofluorimeter (closed dot and line). The size of purified LDs was determined using TEM micrograph (open dot and dashed line).

3. Experimental Section

3.1. Material

Chlamydomonas reinhardtii (CC-503; cw92 mt+) was purchased from the *Chlamydomonas* Resource Center at the University of Minnesota (St. Paul, MN, USA). SF44 was synthesized as described in a previous report [28] and provided by Prof. Park's group. To obtain the SF series and more information, readers should contact the Park group. EZ-Cytox kits were purchased from DoGen (Seoul, Korea). Other chemicals and reagents used in this study were purchased by Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise noted.

3.2. Culture Conditions

Chlamydomonas reinhardtii (CC-503) in tris acetate phosphate (TAP) medium were cultured at 23 °C and 5% CO_2 while shaking on an orbital shaker at 125 rpm. The cultures were exposed to 12 h cycles of light (40 μmol/s/m^2) and dark. When the optical density (OD) of the culture (measured at 680 nm) was approximately 1.0 (early stationary phase), the cells were precipitated by centrifugation (1500 rpm, 3 min) in a test tube. After removal of the supernatant by vacuum aspiration, the pellet was re-suspended with TAP without a nitrogen source to induce accumulation of the LDs.

3.3. Visualization of LDs in C. reinhardtii *with SF44*

C. reinhardtii cells were washed with phosphate buffered saline (PBS) twice by centrifugation (1500 rpm, 3 min) and treated with SF44. Cells were observed with an Olympus FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with a multiline Ar laser (488 nm). The incubation chamber set was to 23 °C, 5% CO_2. Micrographs were acquired with a ×60 oil objective and an appropriate excitation and emission filter set. Acquired fluorescence micrographs were processed with the Image software (NIH, Bethesda, MD, USA).

3.4. Optimization of SF44 Staining

Dye concentration, staining time, temperature, and pH were optimized. *C. reinhardtii* samples (98 μL, 2.4×10^6 cells/mL) were introduced into a test tube and treated with 2 μL of SF44 stock solution. Final concentrations of SF44 were 1 μM, 5 μM, and 50 μM. Test tubes were vortexed and incubated at 25 °C for 25 min. Algal cells were stained with 5 μM of SF44 in solutions of 2% (*v/v*) DMSO. Staining times of 5, 25, and 60 min were evaluated. Staining temperatures was evaluated at 25 °C, 37 °C, and 40 °C and were optimized as described previously. Solution pH was evaluated at pH 6.0, 7.0, and 8.0.

3.5. Viability Assay

Cells were plated on 96-well plates at 2.4×10^6 cells/mL. After the early stationary phase ($OD_{680} = 1.0$), cells were treated with the fluorescence probe using the optimized methods. After treatment, 10 μL of EZ-Cytox solution was added to each well and the cells were incubated for 30 min at room

temperature. Cell viability was monitored by measuring the solution absorbance at 450 nm. The assay solution containing the control was used as a blank.

3. 6. Liposome Concentration Measurement Using SF44

Phosphatidylcholine (PC; 1 mg/mL) from soybean was dissolved in hexane in a test tube. Hexane was evaporated away by gently blowing N_2 gas into the tube, which resulted in the formation of a thin lipid film on the surface of the tube. The tube was stored at −80 °C for 1 h and then moved to water bath of 60 °C to peel off lipid layer from wall. 1 ml of PBS with three glass bead was added to the tube and the mixture was vortexed vigorously. After removing the glass beads, the mixture was sonicated for 10 min in a bath sonicator. The suspension was extruded using Avanti® mini-extruder with 100 nm membrane (Mini-extruder, Avanti® Polar Lipids INC., Burnaby, Canada). Several concentrations of liposomes were prepared. Mixtures (196 μL) were stained with 250 μM SF44 in DMSO (4 μL) to a final volume of 200 μL. Fluorescence intensity was measured at 540 nm with excitation at 450 nm using a spectrofluorimeter (LS-55, Perkin-Elmer, Waltham, MA, USA).

3.7. Determination of LDs Contents via Luminescence Spectrometry

Accumulation of intracellular LDs was tracked by monitoring SF44 fluorescence at 540 nm with excitation at 450 nm. During incubation, cells (196 μL) were combined with 250 μM SF44 in DMSO (4 μL) to a final volume of 200 μL. Cells were collected by centrifugation (1500 rpm, 3 min) in a test tube. After removing the supernatant by vacuum aspiration, the pellet was re-suspended in PBS buffer. Fluorescence was measured with a spectrofluorimeter.

3.8. LDs Purification and Size Measurement

LDs were purified from N-source-starved *C. reinhardtii* with slight modifications of a previously reported procedure [34]. Cell samples were harvested by centrifugation (2000 rcf, 4 min) at 4 °C and re-suspended in a solution of ice-cold digitonin (5 mM potassium phosphate buffer, pH 6.5, containing 6% (*w/w*) polyethylene glycol 6000, and 0.004% (*w/v*) digitonin). Cells were rapidly incubated at 30 °C for 7 min. After incubation, the solution was transferred to an ice bucket and harvested by centrifugation (2000 rcf, 4 min) at 4 °C. Cell pellets were re-suspended in cold osmotic lysis buffer (150 mM Tric-HCl, pH 7.5, containing 10 mM KCl, 1.5 mM EDTA, 0.1 mM $MgCl_2$, 15% (*w/v*) sucrose). Samples were immediately spun at 20,000 rcf for 45 min. LDs were collected with a pipette after floating to the surface of the solution. For size measurement, micrographs of collected LDs were acquired with a transmission electron microscope (TEM, JEME 1010, JEOL, Tokyo, Japan). Aliquots (10 μL) of each sample were placed onto a carbon-coated copper grid (200 mesh) and incubated for 5 min to adsorb on carbon film. After the incubation, unadsorbed LDs and excessed solution were removed by wetting with a filter paper (Whatman™ Grade 1, GE Healthcare, Piscataway, NJ, USA). The samples were negative stained with 2% uranyl acetate for 30 s and visualized with a TEM. All of the acquired micrographs were processed using Image J (NIH).

4. Conclusions

Intracellular LDs are a primary source of triglycerides, a starting material for biodiesel, in algal cells. Therefore, having a method for measuring LDs concentration and accumulation is important for algal bioprocess development and optimization. Conventional methods for measuring LDs in microalgae are based on solvent extraction and gravimetry [19]. Although several chromatographic methods have emerged [20], these methods are cumbersome and require multiple pretreatment steps thus there are many requirements on *in situ* determination of LDs through fluorescence probe.

Nile Red, one of the most representative molecular probes for detecting intracellular LDs, has been used to evaluate the lipid contents of various mammalian cells and microoganisms such as animal cells, bacteria, yeast and fungi. Several researchers have recently published results with Nile Red as an industrial standard for detecting and measuring LDs in microalgae [25,26,34–36]. Nile Red can be used not only to visualize, but also to quantify intracellular LDs using an optical microscope and spectrofluorimeter, respectively. Moreover, combined with flow cytometer and microfluidic system, Nile Red successfully measured single cellular level dynamics of lipid accumulations [27].

In this work, SF44, a tunable and hydrophobic organelle specific fluorescence probe, was used to monitor LD accumulation in live *C. reinhardtii* cells. In previous reports, SF44 had been successfully used to visualize intracellular LDs in mammalian cells and other microorganisms [28,29]. Optimal staining conditions for SF44 in *C. reinhardtii* were identified with regard to dye concentration, staining time, temperature, and pH. The optimal conditions for visualizing LDs through a fluorescence microscope were 5 μM SF44 for 25 min at 25 °C in TAP medium (pH 7.0). However, as Nile Red has emerged as an industrial standard through application on numerous microalgal species of various classes, optimized staining process for SF44 must also be verified in other species. Moreover, microalgal lipid productivity is commonly affected by their surrounding environmental stresses and culture conditions such as nutrient, light, gas concentration, temperature and pH. Especially, pH (about 10.0)-induced LD accumulation is the best conditions in green algae for application in industrial systems. For the advanced applications of SF44 in industrial systems, methodology should be considered as a "tailor-made" system and reconstructed with their optimized conditions. In this report, our optimization was only for conventional culture system, so it needs to be re-optimized with specific experimental conditions.

The feasibility of measuring the kinetics of intracellular LDs accumulation *via* SF44 staining was also demonstrated. SF44 emission at 540 nm was monitored in live cells using an excitation wavelength of 450 nm. Kinetic parameters were also determined by measuring the size of purified LDs using TEM. Both of these data sets indicated that LD accumulation was linear for 3 days before reaching a steady state. At the end point of linear growth (3 day), fluorescence intensity and size showed 88% and 84% of maximum level, respectively. Moreover, the kinetics were well fitted by the same nonlinear regression equation and showed similar R^2 values. (0.9955 and 0.9884, respectively) This result indicated that similar kinetic trends were observed between two methods. Therefore, SF44 provides a simple means of tracking intracellular LDs. This approach allows not only indirect measurement but also tracking for initial stage of LDs accumulation which is impossible to validate their fluorescence intensity through microscopic image analysis with their low resolution.

The tracking of intracellular LDs in microalgae is essential for high-throughput screening of samples for determining their biodiesel potential. Conventional chromatographic methods involve

several sample preparation steps and are not always suitable for *in situ* measurements. To overcome the limitations of chromatographic methods, fluorescence probes have emerged as a rapid and reliable means of acquiring *in situ* data on LDs accumulation and LDs content of a sample [25]. This study shows that SF44 is a powerful, LDs specific fluorescence probe, that is suitable for use in multi well plate based read-out systems and high-throughput screening of intracellular LDs accumulation.

Acknowledgments

Conflicts of Interest

The authors declare no conflict of interest.

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