Optimization of Seoul-Fluor-based Lipid Droplet Bioprobe and its Application in Microalgae for Bio-fuel Study

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I. General information

$^1$H and $^{13}$C NMR spectra were recorded on a Agilent 400-MR (Agilent Technologies) and Varian Inova-500 (Varian Associates), and chemical shifts were measured in ppm downfield from internal tetramethylsilane (TMS) standard. Multiplicity was indicated as follows: s (singlet); d (doublet); t (triplet); q (quartet); m (multiplet); dd (doublet of doublet); dt (doublet of triplet); br s (broad singlet), etc. Coupling constants were reported in Hz. Routine mass analyses were performed on LC/MS system equipped with a reverse phase column (C-18, 50 x 2.1 mm, 5 μm) and photodiode array detector using electron spray ionization (ESI) or atmospheric pressure chemical ionization (APCI). The identity of desired fluorescence compounds were further confirmed by high-resolution mass spectrometry (HRMS). The HRMS analyses were conducted at the Mass Spectrometry Laboratory of Seoul National University by direct injection on a JEOL JMS 600W spectrometer using electron impact (EI) or chemical ionization (CI), and JEOL JMS AX505WA spectrometer using fast atom bombardment (FAB) method. Excitation maxima and Emission maxima were measured by Cary Eclipse Fluorescence spectrophotometer (Varian Associates) and quantum yield was measured by QE-1000 (OTSUKA Electronics). Triethylamine, diisobutylaluminium hydride, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), sodium borohydride, bromoacetyl bromide, sodium hydride, 4-acetyl pyridine, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ), trifluoroacetic acid, acetic acid, anhydrous dimethyl formamide (DMF) were purchased from Sigma-Aldrich and Tokyo Chemical Industry Co., LTD. The progress of reaction was monitored using thin-layer chromatography (TLC) (silica gel 60, F$_{254}$ 0.25 mm), and components were visualized by observation under UV light (254 and 365 nm) or by treating the TLC plates with anisaldehyde, KMnO$_4$, and ninhydrin followed by heating. Solvents were purchased from commercial venders and used without further purification. Distilled water was polished by ion exchange and filtration. Biochemical reagents were purchased from Sigma-Aldrich. Ez-cytox kit was purchased from Daeil Co. and was used for the cell viability test. Commercial dyes Nile Red were purchased from Invitrogen.

Fluorescence microscope and analysis program for Bio-Imaging experiment.

We carried out fluorescence microscopy studies for mammalian LDs with Olympus Inverted Microscope Model IX71, equipped for epi-illumination using a halogen bulb (Philips No. 7724). Emission signal of each experiments were observed at two spectral setting: green
channel, using a 450–480 band pass exciter filter, a 500 nm center wavelength chromatic beam splitter, a 515 nm-long pass barrier filter (Olympus filter set U-MWB2); and red channel using a 510–550 band pass exciter filter, a 570 nm center wavelength chromatic beam splitter, a 590 nm-long pass barrier filter (Olympus filter set U-MWG2). Emission signal of each experiments were detected with 12.5M pixel recording digital color camera (Olympus, DP71) We carried out algae study using a FluoView FV1000 confocal laser scanning unit with Olympus Inverted Microscope Model IX81 and Confocal PMT detector, equipped for Multi-line Ar Laser (488 nm). Fluorescence images were analyzed and quantified by Image-Pro Plus® 6.2 program, and all graphs were figured by GraphPad Prism 5. Algal figures were processed by IMARIS software (Bitplane). The quantified data are processed via the mean measurement of 40–50 cells from at least three different independent experiments and SEM.

**Cell culture**

HeLa cell line was obtained from American Type Culture Collection. HeLa cells were cultured in RPMI 1640 [GIBCO, Invitrogen] supplemented with heat-inactivated 10% (v/v) fetal bovine serum [GIBCO, Invitrogen] and 1 % (v/v) antibiotic-antimycotic solution [GIBCO, Invitrogen]. Cells were maintained in a humidified atmosphere of 5% CO₂ incubator at 37 °C, and cultured in 100 mm cell culture dish [CORNING]. *Chlamydomonas reinhardtii* cell was obtained from Chlamydomonas Resource Center. Chlamydomonas cell strain number was CC-503 cw 92 mt+. Cell was cultured in 500 mL glass flask with stir incubator at 25°C and normal atmosphere. Algae logarithmic phase was maintained with tris-acetate-phosphate (TAP) medium, and then cell grown under nitrogen privation condition media in 6 days.
II. Experimental procedure

General Synthetic procedure.

All amine derivatives (N-Boc-ethylenediamine, N-Boc-1,3-diaminopropane, 2-amino-1-propanol, 3-morpholinopropylamine, N-carbobenzoxy-1,3-diaminopropane hydrochloride) and trans-4-(diethylamino)cinnamaldehyde are commercially available. In case of 2-amino-1-propanol, TBS protection was conducted with a quantitative yield.

Image-based screening of SF44 analogs with HeLa cells.

HeLa cells were treated for 15 min with 5 μM solutions of series of Seoul-Fluor-based bioprobes in RPMI media, supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution. Without media washing, fluorescent images of stained cells were captured using fluorescence microscopy and their fluorescent intensity were determined from the region of interest (ROI) and was quantified by Image-Pro Plus® 6.2 program.

Reagents and conditions: (a) trans-4-(Diethylamino)cinnamaldehyde, AcOH, Na₂SO₄, dichloromethane (DCM), r.t., then NaBH₄, MeOH, 0 °C (70%); (b) Bromoacetyl bromide, triethylamine (TEA), DCM, -78 °C; (c) 4-acetylpyridine; (d) 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), toluene, and DCM (1:1, v/v), then 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (5% for three steps). In case of SF55 and SF56, further de-protection was conducted in quantitative yields.
**Image-based screening of SF44 analogs with *chlamydomonas reinhardtii***.

Nitrogen-starved cells for 6 days (density $2.4 \times 10^6$ cells/ml) were treated with 5 $\mu$M solution of series of Seoul-Fluor-based bioprobe in 2% DMSO solution. Nitrogen starvation facilitates the formation of lipid droplets in microalgae. After 25 min incubation, microalga were observed by Z-depth-controlled image captured in confocal fluorescent microscopy.

**In Vitro Cytotoxicity Test**

Cell viability was measured by the EZ-Cytox assay kit, and the experimental procedure was based on the manufacturer’s manual. HeLa cells were cultured into 96-well plates at a density of $3 \times 10^3$ cells/well for 24 h, followed by the treatment of compounds in various concentrations. After 12 h of incubation with increasing concentration, 10 $\mu$L of WST-1 solution, $(2-(4$-nitrophenyl$)-5-(2$sulfophenyl$)-3-[4-(4$sulfophenylazo$)-2$sulfophenyl$]-2H$-tetrazolium disodium salt, was added to each well, and plates were incubated for an additional 1 h at 37 °C. To measure the viability in microalgae, cells were seeded in 96 well plate and treated with series of Seoul-Fluor-based bioprobes or Nile Red. Final concentration of fluorescent probe was 5 $\mu$M (Seoul-Fluor-based bioprobes) in 2% DMSO solution or 0.1 $\mu$g/mL (Nile Red) in 20% DMSO solution. After 12, 24 and 36 h incubation, cell viability was measured by identical protocol for assay with HeLa cells. The resulting signal was observed after 90 min incubation at 25 °C. Absorbance in 455 nm was measured by microplate reader. The percentage of cell viability was calculated by following formula: % cell viability = (mean absorbance in test wells)/(mean absorbance in control well) × 100. Each experiment was performed in triplicate experiments.
Fig. S1 Normalized excitation wavelengths of each SF-based analog in ethyl ether, ethyl acetate and acetonitrile. Excitations in methanol were excluded because no signals were detected.
Fig. S2 Fluorescent emission shift for SF-based analogs in various solvents. Dye concentration was 1mM.
Fig. S3 Fluorescent(left) and DIC(right) images of SF-based analog for staining of cellular LD in HeLa cell. Final concentration of each analog is 5 μM. Images were taken without washing and fixation step. Scale bar 20 μm.

Fig. S4 Cell viability result of SF58 against HeLa cells. Various concentration of SF58 was treated to HeLa cells for 12 h. Experiment was performed in triplicate and the results were normalized by DMSO control as 100 %.
**Fig. S5** Optimization of SF44 for monitoring LDs in *chlamydomonas reinhardtii*. Various concentration (A) and staining time (B) of SF44 was screened under DMSO 2% at 25°C in triplicate.
**Fig. S6** Fluorescent staining patterns by SF44 in a single cell of *chlamydomonas reinhardtii* were demonstrated in different angles. The continuous monitoring of cellular LD staining was documented in 3D-movie which is available in ESI †

**Fig. S7** Fluorescent staining by each analogs in *chlamydomonas reinhardtii*. a), d), g), j) and m) are fluorescent images, b), e), h), k) and n) are DIC images, and c), f), i), l) and o) are merge images. Final concentration of each analog was 5 μM under 2% DMSO at room temperature. Scale bar represents 5 μm.
IV. Compound Characterization data and NMR spectrum

7-Acetyl-9-(4-(dimethylamino)phenyl)-2-(1-hydroxypropan-2-yl)-1H-pyrrolo[3,4-β]indolizin-3(2H)-one (SF55)

\[ 7\text{-Acetyl-9-(4-(dimethylamino)phenyl)-2-(1-hydroxypropan-2-yl)-1H-pyrrolo[3,4-β]indolizin-3(2H)-one (SF55)} \]

\[^1\text{H NMR (500 MHz, CDCl}_3\text{)} \delta 8.46 \text{ (dd, } J = 1.0, 7.5 \text{ Hz, 1H), 8.33 (s, 1H), 7.42 (d, } J = 8.5 \text{ Hz, 2H), 7.19 (dd, } J = 1.5, 7.0 \text{ Hz, 1H), 6.86 (dd, } J = 2.0, 7.0 \text{ Hz, 2H), 4.52 (q, } J = 16.5 \text{ Hz, 2H), 4.41 (m, 1H), 3.92–3.75 (m, 2H), 3.03 (s, 6H), 2.57 (s, 3H), 1.35 (d, } J = 7.0 \text{ Hz, 3H)}; 13\text{C NMR (125 MHz, CDCl}_3\text{)} \delta 195.7, 162.4, 134.5, 134.1, 128.4, 124.5, 122.9, 121.8, 121.5, 114.8, 113.3, 113.2, 109.2, 70.7, 65.6, 50.9, 43.8, 40.7, 26.2, 15.5; HRMS (FAB): m/z calcd for C\text{23}H\text{25}N\text{3}O\text{3} [\text{M}]+ : 391.1896; found : 391.1893.\]

7-Acetyl-2-(3-aminopropyl)-9-(4-(diethylamino)phenyl)-1H-pyrrolo[3,4-β]indolizin-3(2H)-one (SF56)

\[ 7\text{-Acetyl-2-(3-aminopropyl)-9-(4-(diethylamino)phenyl)-1H-pyrrolo[3,4-β]indolizin-3(2H)-one (SF56)} \]

\[^1\text{H NMR (400 MHz, CDCl}_3\text{)} \delta 8.51 \text{ (dd, } J = 0.8, 7.2 \text{ Hz, 1H), 8.39 (s, 1H), 7.41 (d, } J = 9.2 \text{ Hz, 2H), 7.23 (dd, } J = 1.8, 7.4 \text{ Hz, 1H), 6.81 (d, } J = 9.2 \text{ Hz, 2H), 4.48 (s, 2H), 3.71 (t, } J = 6.8 \text{ Hz, 2H), 3.43 (q, } J = 7.1 \text{ Hz, 4H), 2.77 (t, } J = 6.6 \text{ Hz, 2H), 2.60 (s, 3H), 1.82 (pentet, } J = 6.7 \text{ Hz, 2H), 1.23 (t, } J = 7.0 \text{ Hz, 6H); 13\text{C NMR (100 MHz, CDCl}_3\text{)} \delta 195.6, 161.8, 146.8, 134.0, 133.9, 128.7, 128.3, 124.4, 122.8, 122.0, 120.2, 115.0, 112.2, 109.1, 46.5, 44.4, 40.1, 39.0, 32.3, 26.0, 12.7; HRMS (FAB): m/z calcd for C\text{25}H\text{31}N\text{4}O\text{2} [\text{M}+\text{H}]^+ 419.2447, found 419.2445.\]
7-Acetyl-9-(4-(diethylamino)phenyl)-2-(3-morpholinopropyl)-1H-pyrrolo[3,4-β]indolizin-3(2H)-one (SF57)

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.57 (d, $J = 7.5$ Hz, 1H), 8.38 (s, 1H), 7.73 (d, $J = 8.5$ Hz, 2H), 7.68 (d, $J = 8.5$ Hz, 2H), 7.36 (d, $J = 7.0$ Hz, 1H), 4.58 (s, 2H), 4.00–3.90 (m, 4H), 3.75 (t, $J = 6$ Hz, 2H), 3.62 (q, $J = 6.5$ Hz, 4H), 3.52 (d, $J = 11$ Hz, 2H), 3.13 (t, $J = 8$ Hz, 2H), 2.88 (br s, 2H), 2.65 (s, 3H), 2.24 (pentet, $J = 7.1$ Hz, 2H), 1.23 (t, $J = 7.0$ Hz, 6H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 195.4, 161.9, 136.0, 135.7, 135.3, 135.2, 130.4, 129.2, 125.0, 123.5, 122.3, 120.4, 112.1, 110.3, 63.8, 55.1, 53.4, 52.3, 46.1, 39.6, 26.2, 23.1, 10.4; HRMS (FAB): $m/z$ calcd for C$_{29}$H$_{37}$N$_4$O$_3$ [M+H]$^+$ 489.2866, found 489.2864.

tert-Butyl (2-(7-acetyl-9-(4-(diethylamino)phenyl)-3-oxo-1H-pyrrolo[3,4-β]indolizin-2(3H)-yl)ethyl)carbamate (SF44)

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.50 (dd, $J = 1.0$, 7.5 Hz, 1H), 8.39 (s, 1H), 7.41 (d, $J = 9.0$ Hz, 2H), 7.23 (dd, $J = 1.7$, 7.2 Hz, 1H), 6.81 (d, $J = 9.0$ Hz, 2H), 4.97 (br s, 1H), 4.56 (s, 2H), 3.73 (t, $J = 5.7$ Hz, 2H), 3.45–3.41 (m, 6H), 2.60 (s, 3H), 1.35 (s, 9H), 1.23 (t, $J = 7.0$ Hz, 6H); $^{13}$C NMR (125 MHz, CDCl$_3$) 195.6, 162.2, 156.3, 146.8, 134.3, 133.9, 128.6, 128.2, 124.3, 122.4, 122.0, 120.1, 115.0, 112.3, 108.9, 79.5, 47.3, 44.5, 43.1, 39.8, 28.4, 26.0, 12.7; HRMS (FAB): $m/z$ calcd for C$_{29}$H$_{36}$N$_4$O$_4$ [M]$^+$ 504.2737, found 504.2743.
Benzyl (3-(7-acetyl-9-(4-(diethylamino)phenyl)-3-oxo-1H-pyrrolo[3,4-β]indolizin-2(3H)-yl)propyl)carbamate (SF58)

\(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.47 (dd, \(J = 0.75, 7.3\) Hz, 1H), 8.38 (s, 1H), 7.40 (d, \(J = 9\) Hz, 2H), 7.37–7.26(m, 5H), 7.22 (dd, \(J = 1.5, 7.0\) Hz, 1H), 6.81 (d, \(J = 9\) Hz, 2H), 5.82 (br s, \(J = 5.75\) 1H), 5.10 (s, 2H), 4.47 (s, 2H), 3.68 (t, 6 Hz, 2H), 3.43 (q, \(J = 7.2\) Hz, 4H), 3.23 (t, \(J = 5.8\) Hz, 2H), 2.60 (s, 3H), 1.84 (pentet, \(J = 5.75\) Hz, 2H), 1.23 (t, \(J = 7.0\) Hz, 6H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 195.9, 162.5, 156.9, 147.1, 137.1, 134.5, 134.3, 129.0, 128.77, 128.75, 128.3, 128.0, 124.8, 122.7, 122.3, 120.3, 115.4, 112.6, 109.5, 66.9, 47.0, 44.8, 40.3, 37.9, 28.9, 26.3, 13.0; HRMS (FAB) \(m/z\) calcd for C\(_{33}H_{36}N_4O_4\) [M]+ 552.2737, found 552.2744.
SF56
SF58
SF55