Electronic Supplementary Material (ESI) for Molecular BioSystems

D, Supporting Information

Synthesis and Applications of LipidGreen2 for lipid and fatty liver imaging

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Experimental Section

General: 1H NMR spectra were recorded on a Bruker 500 MHz and Varian 300 MHz instrument. Chemical shifts (δ) are expressed in ppm downfield from TMS as internal standard. The letters s, d, t, q, and m are used to indicate singlet, doublet, triplet, quadruplet, and multiplet, respectively. All anhydrous solvents (stored over molecular sieves) and chemicals were obtained from standard commercial vendors and were used without any further purification.

All experiments were performed in compliance with the relevant laws and guidelines of the Animal Care Committee at CNU. Animal work was approved by the internal animal ethics committee at Chungnam National University (CNU).

Synthesis of LipidGreen2:

Ethyl 5-methoxyindole-2-carboxylate. A mixture of 5-methoxyindole-2-carboxylic acid (1) (5 g, 26.15 mmol) and sulfuric acid (4 mL) in EtOH (40mL) was refluxed for 10 h. The reaction mixture was evaporated, neutralized to pH 7 with 2N-NaOH and extracted with ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue was purified by silica gel column chromatography using solvent CH_2Cl_2 to give ethyl 5-methoxyindole-2-carboxylate. (5.6 g, 99 %) . ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.80 (bs, 1H), 7.31 (d, *J* = 8.9 Hz, 1H), 7.16-7.05 (m, 2H), 6.99 (dd, J=8.9 Hz, 2.2 Hz), 4.40 (q, J=7.1 Hz, 2H), 3.85 (s, 3H), 1.41 (t, J=7.1 Hz, 3H).

Ethyl 3-formyl-5-methoxy-indole-2-carboxylate. To a solution of ethyl-5-methoxyindole-2-carboxylate (5.6 g, 25.54 mmol) in DMF (47 mL) was added phosphorus oxychloride (5.95 mL, 63.85 mmol) at room temperature. The reaction mixture was stirred for 3 h at room temperature and neutralized to pH 7 by 2N-NaOH at 0°C. The resulting solid was collected and washed with H_2O to give ethyl 3-formyl-5-methoxy-indole-2-carboxylate (6.3 g, 99 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.77 (s, 1H), 10.58 (s, 1H), 7.69 (d, J = 1.9 Hz, 1H), 7.47 (d, J = 8.9 Hz, 1H), 7.03 (dd, J = 8.9 Hz, 2.0 Hz, 1H), 4.43 (q, J = 7.1 Hz, 2H), 3.80 (s, 3H), 1.39 (t, J = 7.1 Hz, 3H).

Ethyl 3-hydroxy-5-methoxyindole-2-carboxylate (2). To a solution of ethyl 3-formyl-5-methoxy-indole-2-carboxylate (9 g, 36.4 mmol) in CH_2Cl_2 (72 mL) were added TSOH.H_2O (6.9 g, 36.4 mmol) and mCPBA (12.6 g, 54.6 mmol) at room temperature. The reaction mixture was stirred for 8 h at room temperature and then evaporated. The residue was purified by silica gel column chromatography using CH_2Cl_2 to give ethyl 3-hydroxy-5-methoxyindole-2-carboxylate (6 g, 70 %) as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.68 (bs, 1H), 7,17 (d, J = 8.9 Hz, 1H), 7.10 (d, J = 1.5 Hz, 1H), 7.01 (dd, J = 8.8 Hz, 2.4 Hz, 1H), 4.43 (q, J = 7.1 Hz, 2H), 3.85 (s, 3H), 1.42 (t, J = 7.1 Hz, 3H).

Ethyl 3-(tert-Butyl-diphenyl-silanyloxy)-5-methoxy-1H-indole-2-carboxylate. A mixture of ethyl 3-hydroxy-5-methoxy-1H-indole-2-carboxylate (2 g, 8.5 mmol), imidazole (1.15 g, 17 mmol) and TBDPSCl (4.4 mL, 17 mmol) in THF (80 mL) was refluex for 5 h. The reaction mixture was evaporated, diluted with H_2O and extracted with ethyl acetate. Organic layer was separated, dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The residue was purified by silica gel column chromatography to give ethyl 3-(tert-butyldiphenylsilyloxy)-5-methoxy-1H-indole-2-carboxylate (3.98 g, 99 %) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.10 (s, 1H), 7.87-7.78 (m, 4H), 7.44-7.30 (m, 6H), 7.07 (d, *J* = 8.9 Hz, 1H), 6.77 (dd, *J* = 9.1 Hz, 1.7 Hz, 1H), 6.34 (s, 1H), 4.30 (q, *J* = 6.9 Hz, 2H), 3.14 (s, 3H), 1.30 (t, *J* = 7.1 Hz, 3H), 1.15 (s, 9H).

Ethyl 3-(tert-butyl-diphenyl-silanyloxy)-5-methoxy-1-(3,3 dimethylallyl)-1H-indole-2-carboxylate. To a solution of ethyl 3-(tert-butyldiphenylsilyloxy)-5-methoxy-1H-indole-2-carboxylate (3.94 g, 8.32 mmol) in DMF (80 mL) was added NaH (0.396 g, 9.98 mmol) and 3,3-dimethylallylbromide (1.48 g, 9.98 mmol). The reaction mixture was stirred for 3 h at room temperature. The resulting mixture was diluted with H_2O and extracted with ethyl acetate. Organic layer was separated, dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The residue was purified by silica gel column chromatography to give ethyl 3-(tert-butyl-diphenyl-silanyloxy)-5-methoxy-1-(3,3 dimethylallyl)-1H-indole-2-carboxylate (3.3 g, 74 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.84-7.77 (m, 4H), 7.43-7.29 (m, 6H), 7.08 (d, J = 9.0 Hz, 1H), 6.78(dd, J = 9.1 Hz, 2.2 Hz, 1H), 6.44 (d, J = 2.0 Hz, 1H), 5.14-5.06 (m, 1H), 5.05-4.97 (m, 2H), 4.17 (q, J = 7.1 Hz, 2H), 3.17 (s, 3H), 1.78 (s, 3H), 1.65 (s, 3H), 1.23 (t, J = 7.1 Hz, 3H), 1.12 (s, 9H).

Ethyl 3-hydroxy-5-methoxy-1-(3,3dimethylallyl)-1H-indole-2-carboxylate (3). To a solution of ethyl 3-(tert-butyl-diphenyl-silanyloxy)-5-methoxy-1-(3,3 dimethylallyl)-1H-indole-2-carboxylate (3.32 g, 6.13 mmol) in THF (100 mL) was added TBAF (13.4 mL, 13.4 mmol). The reaction mixture was stirred for 2 h at room temperature. The reaction mixture was evaporated, diluted with H_2O and extracted with ethyl acetate. Organic layer was separated, dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The residue was purified by silica gel column chromatography to give ethyl 3-hydroxy-5-methoxy-1-(3,3dimethylallyl)-1H-indole-2-carboxylate (1.7 g, 91 %) as a yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.68 (bs, 1H), 7.17-7.08(m, 2H), 7.02(dd, J = 9.1 Hz, 2.0 Hz, 1H),5.14-5.06 (m, 1H), 4.96-4.89 (m, 2H), 3.84 (s, 3H), 1.80 (s,3H), 1.65 (s, 3H), 1.43 (t, J=7.1 Hz, 3H).

5-Methoxy-1,2-bis-(3-methyl-but-2-enyl)-3-oxo-2,3-dihydro-1H-indole-2-carboxylic acid ethyl ester (LipidGreen2). A mixture of ethyl 3-hydroxy-5-methoxy-1-(3,3dimethylallyl)-1H-indole-2-carboxylate (1.58 g, 5.21mmol), K_2CO_3 (483 mg, 20.8 mmol), potassium iodide (1.73 g, 10.4 mmol) and 3,3-dimethylallyl bromide (1.55 g, 10.4 mmol) in acetone (80 mL) was refluxed for 12 h. The reaction mixture was evaporated, diluted with H_2O and extracted with ethyl acetate. Organic layer was separated, dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The residue was purified by silica gel column chromatography to give 5-methoxy-1,2-bis-(3-methyl-but-2-enyl)-3-oxo-2,3-dihydro-1H-

indole-2-carboxylic acid ethyl ester (727 mg, 38 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.13 (dd, J = 8.8, 2.5 Hz, 1H), 7.01 (d, J = 2.5 Hz, 1H), 6.73 (d, J = 8.9 Hz, 1H), 5.24-5.16 (m, 1H), 4.77-4.68 (m,1H), 4.13 (q, J = 7.2 Hz, 2H), 4.04-3.82 (m,, 2H), 3.76 (s, 3H), 3.05-2.74 (m, 2H), 1.73 (s, 6H), 1.63 (s, 3H), 1.55(s, 3H), 1.19 (t, J = 7.1 Hz, 3H). HRMS ($C_{22}H_{29}NO_4$):): calcd, 371.2097 found, 371.2094.

Cell culture and Fluorescence Image. 3T3-L1 (Mouse embryonic fibroblast cell line), PC3 (Human prostate cancer cell line), MCF7 (Human breast adenocarcinoma cell line), HT29 (Human colon cancer cell line), HepG2 (Human liver carcinoma cell line), CHL-1 (Human melanoma cell line), and NIH3T3 (Mouse embryonic fibroblast cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37℃ and 5% CO_2. LLC-PK1 (Porcine kidney cell line) was cultured in Medium 199 supplemented with 15% fetal bovine serum. Differentiated adipocytes were induced at confluence in DMEM containing 10% FBS, dexamethasone (0.25 μM, Sigma Aldrich, St. Louis, MO), isobutylmethylxanthine (0.5 mM, IBMX, Sigma Aldrich) and insulin (166 nM, Sigma Aldrich) for 2 days (from day 0 to day 2). This medium was replaced with DMEM containing 10% FBS and insulin for a further 2 days (from day 2 to day 4). From day 4 onwards, cells were fed with DMEM containing 10% FBS and this medium was replaced every 2 days. Various cell lines were incubated with LipidGreen2 at 10 μM for 4 h in DMEM containing 10% FBS at 37℃. To visualize lipid droplets, we stained LipidGreen2 or Nile Red at 5 μM for 30 min, and then was diluted in Dimethyl sulfoxide (DMSO). After compound staining, cells were washed three times with PBS and were observed by fluorescence microscope.

LipidTOX™ Dyes staining. Cells were incubated with LipidTOX™ (phospholipid) Red phosholipidosis detection reagent in propranolol (30 μM) (50 μL/well). Cyclosporin A (30 μM) was used as positive control (50 μL/well). Cells were incubated under normal culture conditions for a period of time that allowed assessment of the effects of LipidGreen2, after which the medium was removed. A formaldehyde/Hoechst 33342 fixative solution (100 μL) was added to each well and incubated for 30 min at room temperature. After removing the fixative, cells were observed under a fluorescence microscope. For neutral lipid staining, 100 μL of LipidTOX™ (neutral lipid) Red detection reagent was added to each well. After 30 min incubation at room temperature, cells were imaged.

Induction of Lipid droplets in HepG2 cells. Cells were incubated with 2% alcohol, 10 μM amiodarone, 50 μM tetracycline and 5 μM tamoxifen for 72 h. After compound treat, then treated for 30 min with 10 μM LipidGreen2 and 40 μg/ml BODIPY 493/503. The stock compounds were diluted in the culture medium to obtain the desired final concentrations. The final DMSO concentration in the culture medium never exceeded 1%, and vehicle sample were treated with the same amount of DMSO.

Maintenance of zebrafish. Zebrafish were raised and kept under standard laboratory condition at 28.5°C. Zebrafish embryos were staged and fixed at specific hours post fertilization (hpf) and days post fertilization (dpf).

Staining and bioimaging of live zebrafish with LipidGreen2. Zebrafish embryos were incubated in 10 μM LipidGreen2 or 10 μM LipidGreen for 30 min at 28 hpf. After staining with LipidGreen2 or LipidGreen, zebrafish were washed with egg water for 30 min. After washing, zebrafish were anesthetized with 0.016% Tricaine and mounted in 3% methyl cellulose. The photographs were performed on a fluorescence stereomicroscope.

Oil Red O staining Whole larvae were fixed with 4% paraformaldehyde, washed with PBS, infiltrated with a graded series of propylene glycol, and stained with 0.5% Oil Red O in 100% propylene glycol overnight. Stained larvae were washed with decreasing concentrations of propylene glycol followed by several rinses with PBS and transferred to 75% glycerol. Images were captured on a Leica MZ10F.

Tunicamycin treatment Tunicamycin (Sigma) was dissolved in dimethyl sulfoxide (DMSO) as 1 mg/ml concentration and diluted with egg water at a final concentration of 0.5 μg/ml. Tunicamycin treatment started at 3 dpf until 6 dpf.

Imaging and Quantitative analysis Zebrafish images were captured by using a fluorescence stereomicroscope (MZ10F, Leica Microsystems, Wetzlar, Germany) with a coupled device camera (DFC425C, Leica Microsystems, Wetzlar, Germany). The fluorescence stereomicroscope equipped with the filter set GFP Plant (470/40 nm excitation, 525/50 nm emission) and DsRed (545/30 nm excitation, 620/60 nm emission). The Fluorescence intensity of whole individual embryos was calculated within the yolk fluorescence region of one focal plane because the size of embryos are relatively small, 2 mm total body length, as shown in Figure by use of LAS v3.8 Leica Microsystems software. Cell lines images were acquired using a fluorescence microscope (Leica DMI3000B) equipped with the filtercubes set I3 (BP 450-490, LP 515), N2.1 (BP 515-560, LP 590) and D (BP 355-425, LP 470).

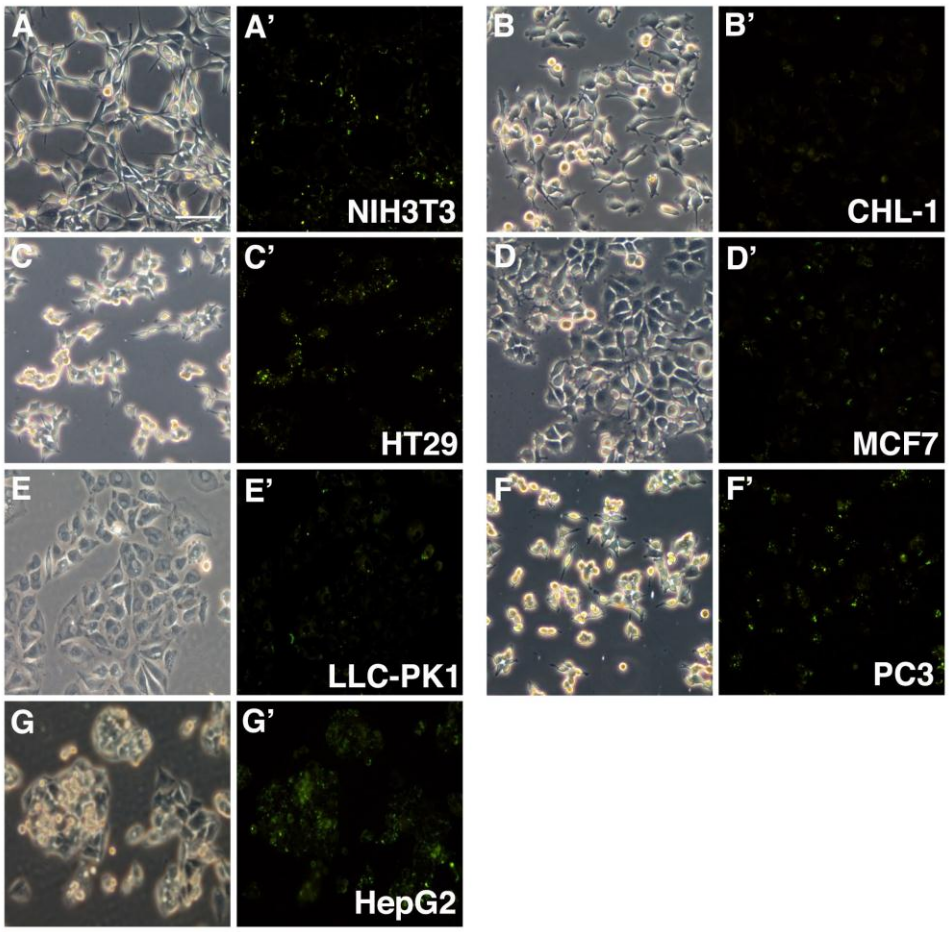


Figure S1. **Various cell lines were stained with the LipidGreen2.** Subcellular localization of LipidGreen2 in NIH3T3 cells (Mouse embryonic fibroblast cell line) (A and A‘), CHL-1 cells (Human melanoma cell line) (B and B’), HT29 cells (Human colon cancer cell line) (C and C’), MCF7 cells (Human breast adenocarcinoma cell line) (D and D’), LLC-PK1 cells (Porcine kidney cell line) (E and E’), PC3 cells (Human prostate cancer cell line) (F and F‘), HepG2 cells (Human liver carcinoma cell line) (G and G’). Scale bars represent 100 μm (A-G‘).

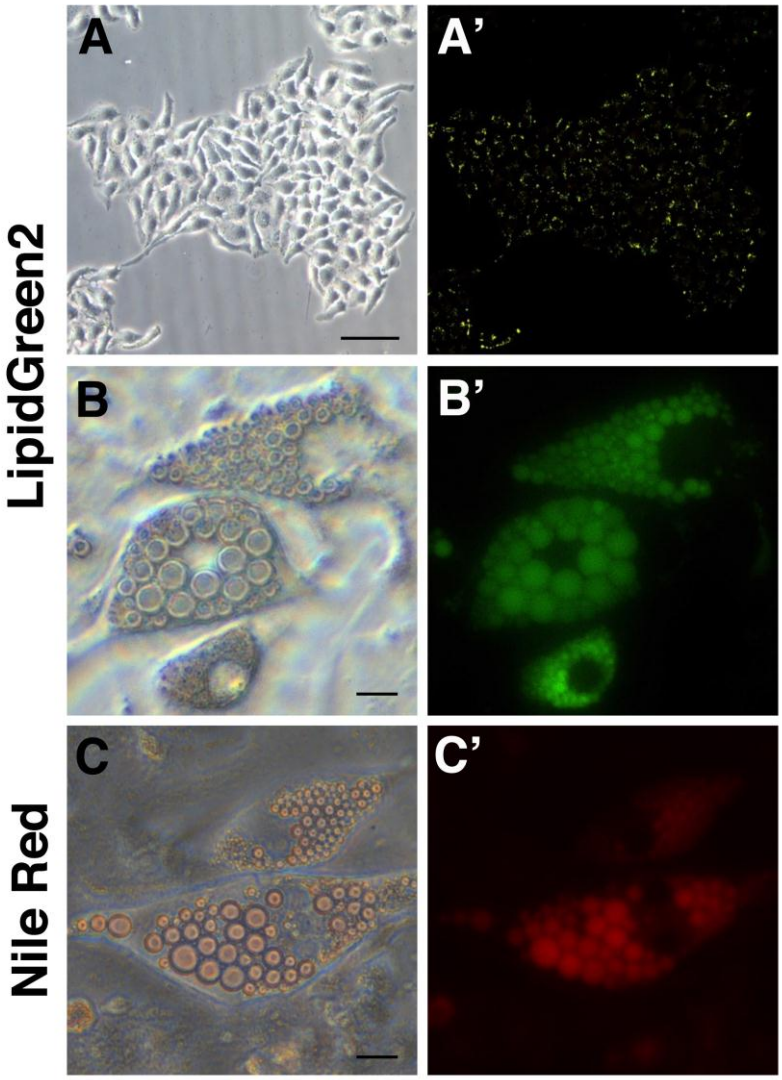


Figure S2. 3T3-L1 cell lines stained by LipidGreen2. Subcellular localization of LipidGreen2 in undifferentiated 3T3-L1 (mouse embryonic fibroblast cell line) (A and A'). Differentiated 3T3-L1 cell lines were stained with the LipidGreen2 (B and B') and Nile Red (C and C'). Scale bars, 100 μm (A and A') and 20 μm (B, B', C and C')

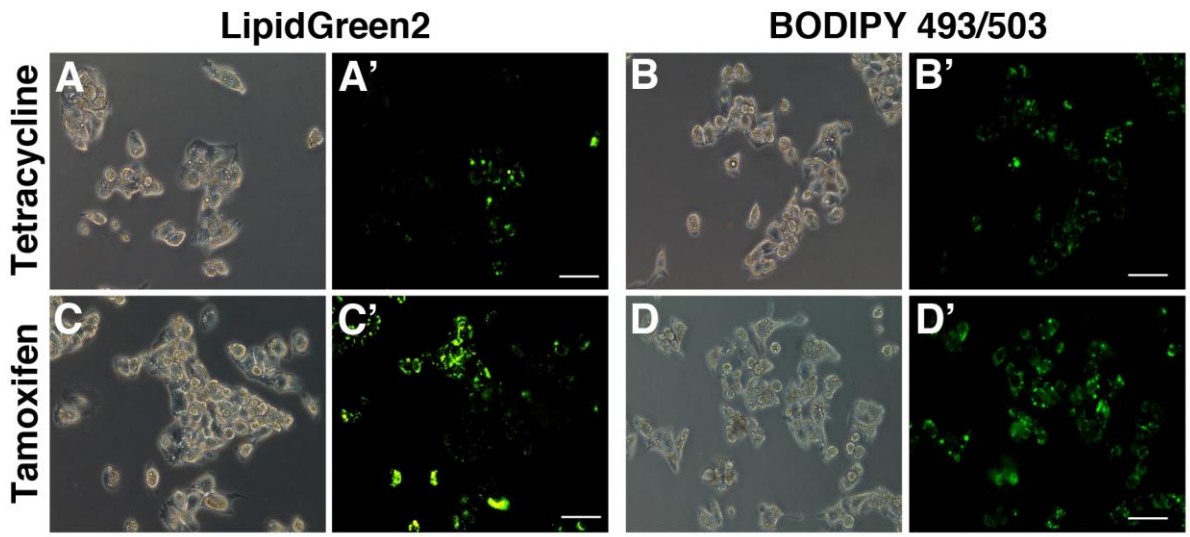


Figure S3. Validation of steatosis assay using the LipidGreen2 in HepG2 cell line. Cultured cells were treated tetracycline (A-B') or tamoxifen (C-D') for 72 h. Fat accumulation assessed by LipidGreen2 (A', C') or BODIPY® 493/503 (B', D') fluorescence for 30 min. Scale bars, all figures 100 µm.

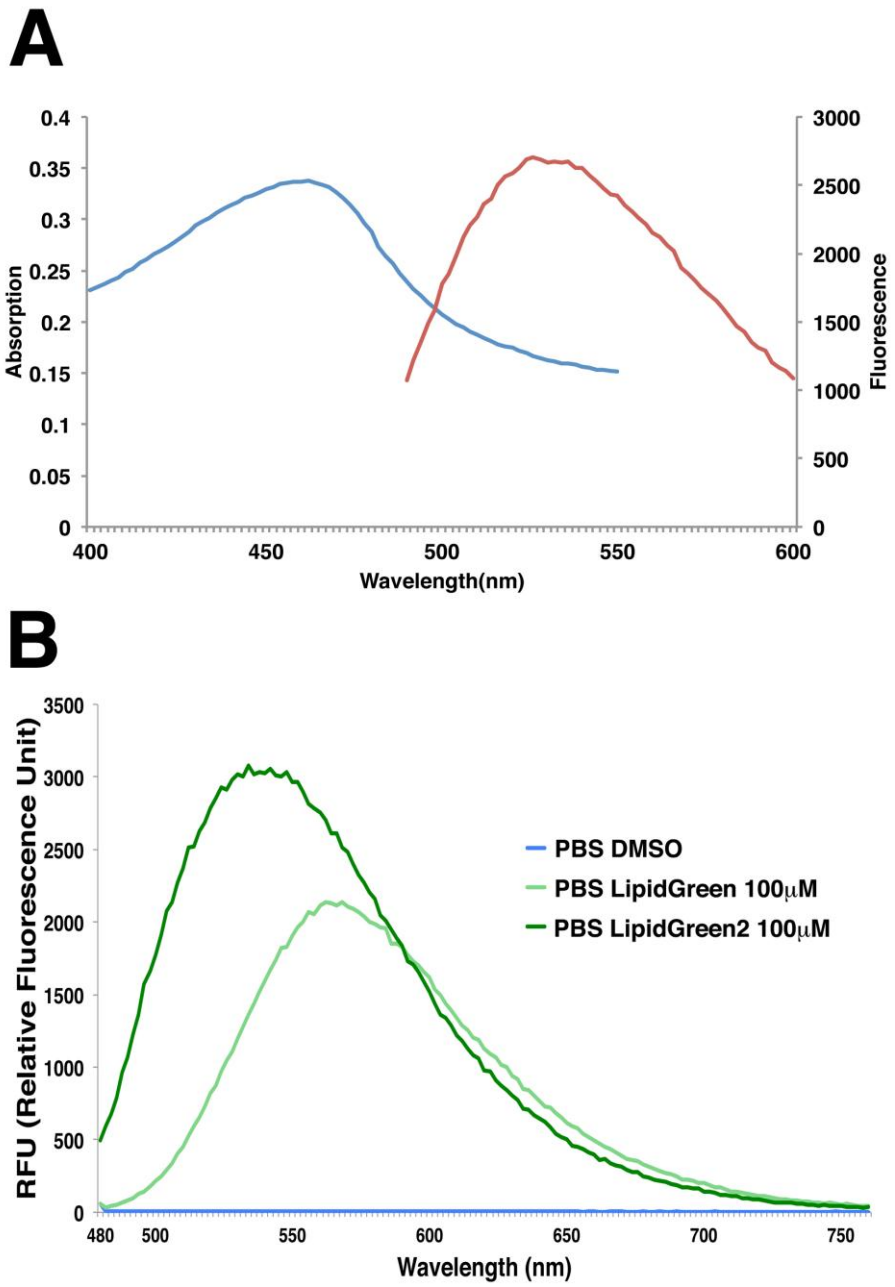


Figure S4. A) Spectrum of excitation (456nm) and emission (534nm) in LipidGreen2. B) Fluorescence intensity of LipidGreen or LipidGreen2 in PBS solution.

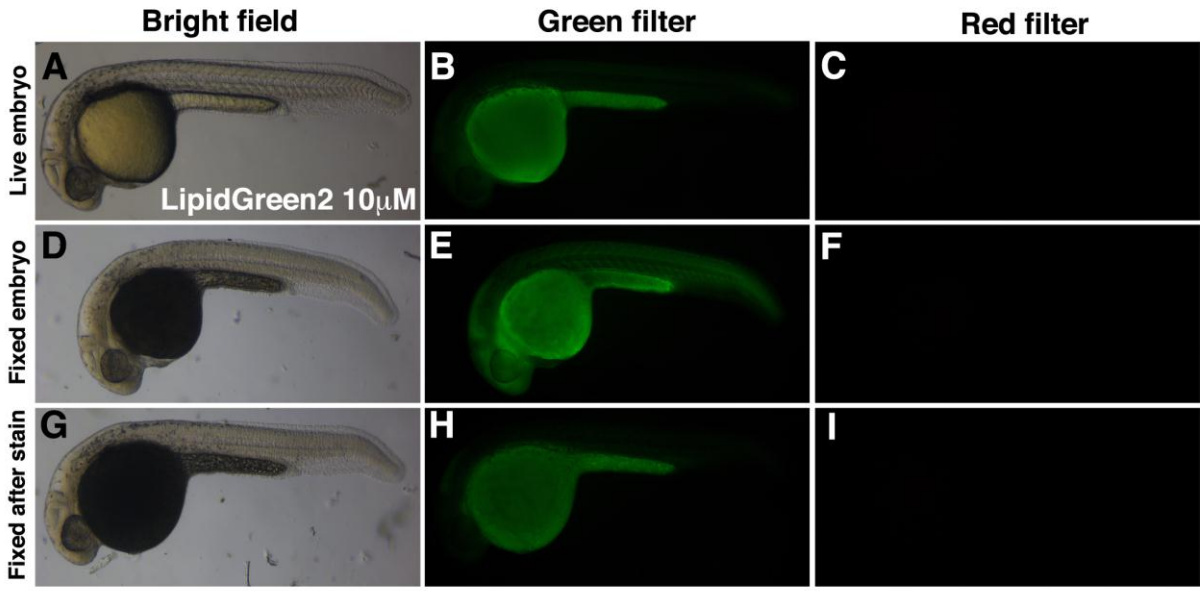


Figure S5. **Characteristics of LipidGreen2.** Zebrafish live and fixed embryos were incubated with the 10μM LipidGreen2 for 30min. Embryos were fixated for 2 h in 4% paraformaldehyde in PBS. Fixed embryos were stained with the 10μM LipidGreen2 in PBS. LipidGreen2 wasn't detected fluorescence signal in red channel. Non-specific green fluorescence signals were showed in fixed embryos.

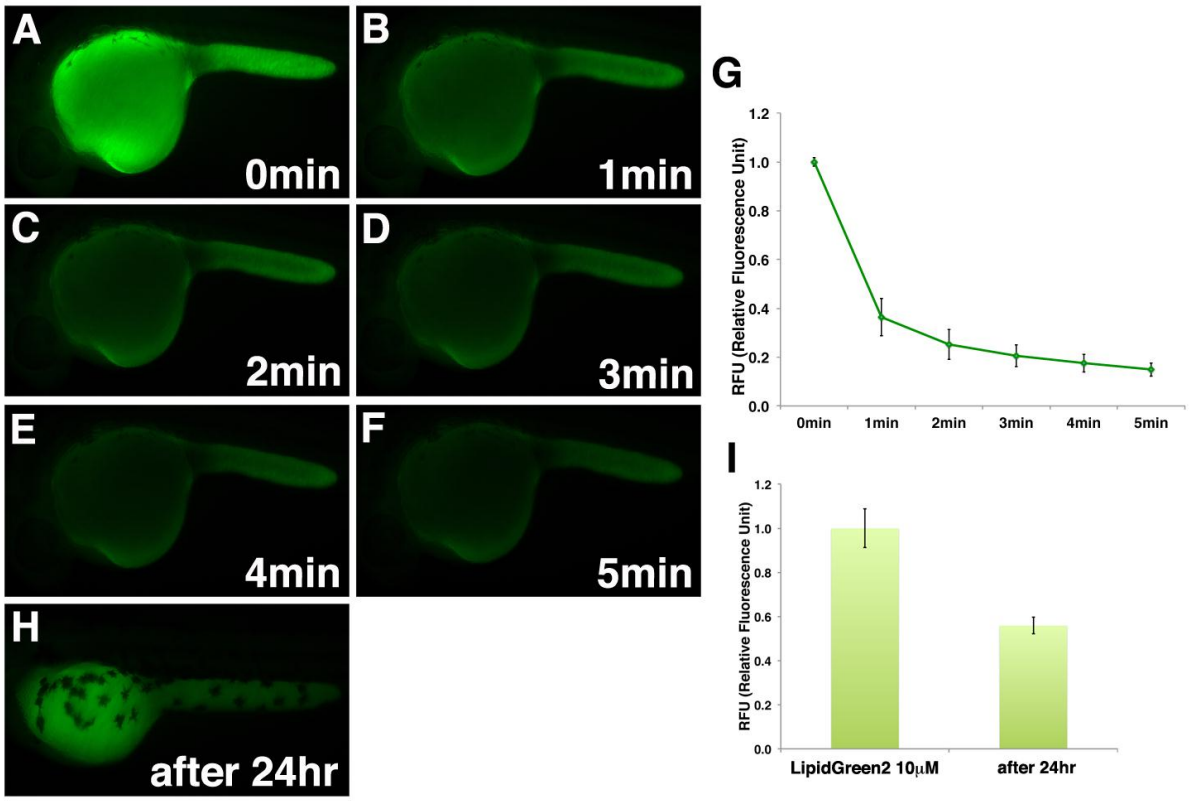
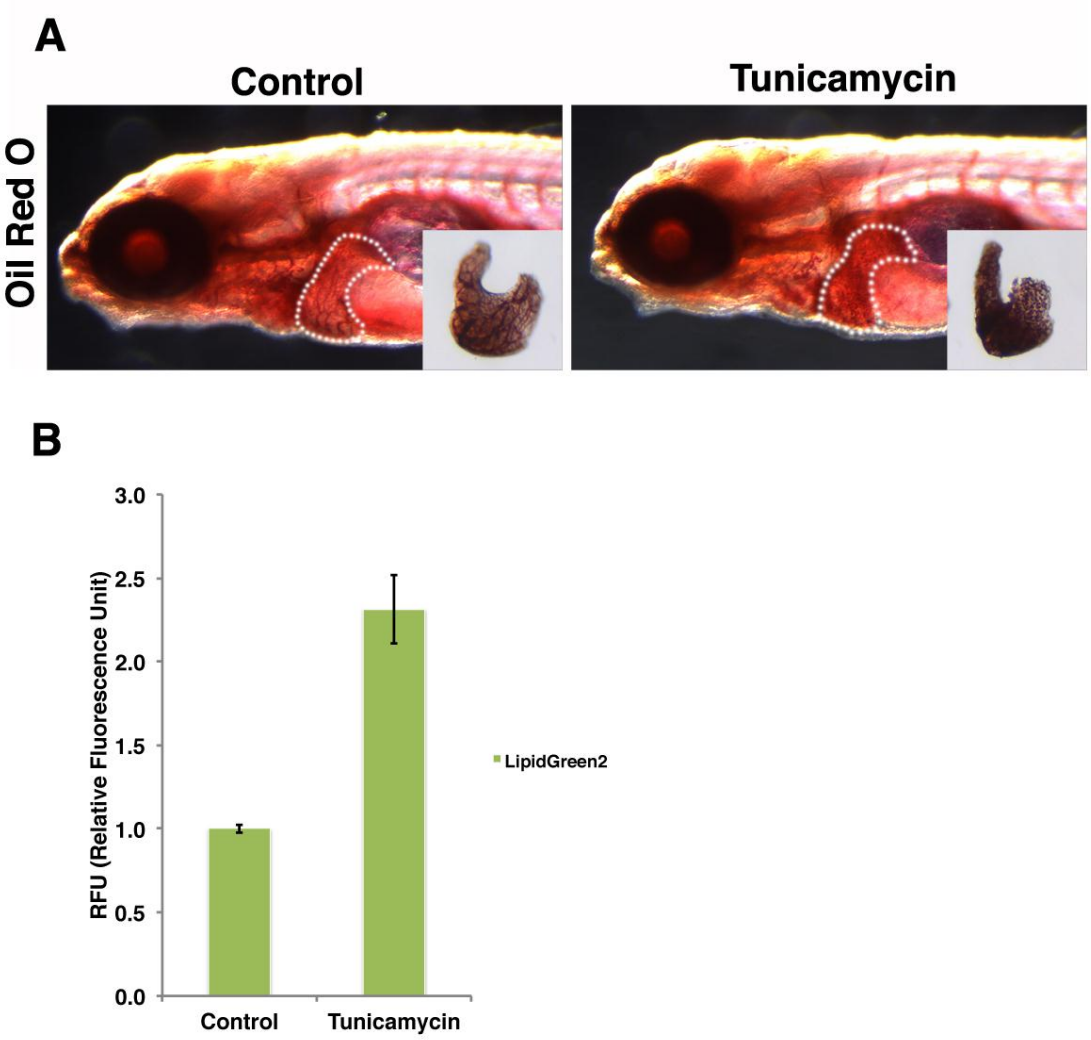


Figure S6. **Photostability of LipidGreen2.** Zebrafish embryos were incubated with the 10μM LipidGreen2 for 30min. Then, embryos were continuously exposed to using GFP filter in UV light sources for 5 min to investigate photostability of the LipidGreen2 (A-G). To demonstrate long-term stability of LipidGreen2, we observed stained embryos, which washed for 24h in egg water. Fluorescence signal was decrease to half after 24h(H,I). Images were taken same condition (expose and gain) of microscope. The each bar represents the mean ± SD of five embryos.



Figrure S7. **Stain of fatty liver using Oil Red O and quantitative analysis of the changes in LipidGreen2 Fluorescence.** (A) Whole-mount staining with Oil Red O on the Figure 5 same larvae. (B) Quantitative analysis (dotted line) of LipidGreen2 fluorescence on tunicamycin-induced zebrafish larvae in Figure 5 (dotted line). The each bar represents the mean ± SD of five embryos.