Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Synthesis of a new fluorescent small molecule probe and its use for *in vivo* lipid imaging

Jae Hong Lee [‡a], Ju-Hoon So [‡a,b], Jong Hyun Jeon[a], Eun Bok Choi[a], Yu-Ri Lee [b], Young-Tae Chang [c], Cheol-Hee Kim* [b], Myung Ae Bae*[a], Jin Hee Ahn*[a]

General. All reported yields are isolated yields after column chromatography or crystallization. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Bruker AVANCE-300 using tetramethylsilane (TMS) as an internal reference. High-resolution mass spectra were obtained on the Autospec magnetic sector mass spectrometer (Micromass, Manchester, UK).

Abbreviations. dpf, day post fertilization; an, anus; ap, anterior periorbital; pp, posterior periorbital; sk, skull; op, opercular; pf, pectoral fin; cl, clavicle; lj, low jaw; ve, vertebra; dcp, dorsal caudal peduncle; vcp, ventral caudal peduncle; hp, hypural; go, gonad; L, liver; I, intestine. an, anus; ap, anterior periorbital; pa, pancreas; sb, swim bladder; pf, pectoral fin; mi, mid intestine; ci, caudal intestine; pe, pericardial; lj, low jaw; pp, posterior periorbital; anf, anal fin; pvf, pelvic fin; ve, vertebra; dcp, dorsal caudal peduncle; vcp, ventral caudal peduncle; op. opercular; hp, hypural; df, dorsal fin; sk, skull; cl, clavicle; vl, ventral midline.

Synthetic procedures

Ethyl 3-formyl-5-methoxy-indole-2-carboxylate (2)

A mixture of 5-methoxyindole-2-carboxylic acid (5 g, 26 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 to give ethyl 5-methoxyindole-2-carboxylate. (5.5 g, 96 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.84 (bs, 1H), 7.30 (d, J = 9.0 Hz, 1H), 7.14 (dd, J =2.4 Hz, J =1.0 Hz, 1H), 7.07 (d, J =2.4 Hz, 1H), 6.99 (dd, J =9.0 Hz, 1H), 4.40 (q, J =7.0 Hz, 2H), 3.85 (s, 3H), 1.41(t, J =7.0Hz, 3H).

To a solution of ethyl-5-methoxyindole-2-carboxylate (4.8 g, 21.9 mmol) in DMF (40mL) was added phosphorus oxychloride (5.1 mL, 54.75mmol) at room temperature. The reaction mixture was stirred for 3 h at room temperature and neutralized to pH 7 by 2N-NaOH at 0 ^{o}C. The resulting solid was collected and washed with H_2O to give ethyl 3-formyl-5-methoxy-indole-2-carboxylate (5 g, 92 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.73 (s, 1H), 9.25 (bs, 1H), 7.90 (d, *J* =2.4 Hz, 1H), 7.60 (d, *J* =9.0 Hz, 1H), 7.07 (dd, *J* =9.0 Hz, *J* =2.4 Hz, 1H), 4.48 (q, *J* =7.0 Hz, 2H), 3.90 (s, 3H), 1.28 (t, *J* =7.0 Hz, 3H). HRMS ($C_{13}H_{13}NO_4$): calcd, 247.0845 found, 247.0841.

Ethyl 3-hydroxy-5-methoxyindole-2-carboxylate (3)

To a solution of ethyl 3-formyl-5-methoxy-indole-2-carboxylate (5 g, 0.02 mol) in CH_2Cl_2 (40mL) were added pTSA (3.8 g, 0.02 mol) and mCPBA (7 g, 0.03 mol) at room temperature. The reaction mixture was stirred for 8 h at room temperature and then evaporated. The resulting mixture was diluted with H_2O and extracted with ethyl acetate. The 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-methoxyindole-2-carboxylate (4.6 g, 88 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70 (bs, 1H), 7.18 (d, *J* =9.0 Hz, 1H), 7.10 (d, *J* =2.4 Hz, 1H), 7.00 (dd, *J* =9.0 Hz, *J* =2.4Hz, 1H), 4.44 (q, *J* =7.0 Hz, 2H), 3.85 (s, 3H), 1.41 (t, *J* =7.0 Hz, 3H). LC-MS (m/z): 236 (MH^+). HRMS ($C_{12}H_{13}NO_4$): calcd, 235.0845 found, 235.0843.

Ethyl 1,2-diallyl-5-methoxy-3-oxoindoline-2-carboxylate (5) and ethyl 1-allyl-3-(allyloxy)-5-methoxy-1H-indole-2-carboxylate (6)

A mixture of ethyl 3-hydroxy-5-methoxyindole-2-carboxylate (100 mg, 0.37 mmol), K_2CO_3 (235 mg, 1.85 mmol) and allyl iodide (0.2 mL, 1.85 mmol) in acetone (5 mL) was refluxed for 5 h. The reaction mixture was evaporated, diluted with H_2O and extracted with ethyl acetate. The 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 1,2-diallyl-5-methoxy-3-oxoindoline-2-carboxylate (5a, 15 mg, 12 %) and ethyl 1-allyl-3-(allyloxy)-5-methoxy-1H-indole-2-carboxylate (6a, 20 mg, 17 %).

(5a) ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.14 (dd, *J* =9.0 Hz, *J* =2.4 Hz, 1H), 7.02 (d, *J* =2.4 Hz, 1H), 6.79 (d, *J* =9.0 Hz, 1H), 5.96~5.83 (m, 1H), 5.53~5.39 (m, 1H), 5.33~5.26 (m, 1H), 5.24~5.12 (m, 1H), 5.00~4.97 (m, 1H), 4.20~4.08 (m, 2H), 4.05~3.82 (m, 2H), 3.76 (s, 3H), 3.04~2.88 (m, 2H), 1.41 (t, *J* =7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$); δ 195.5, 167.7, 157.8, 152.7, 134.0, 131.0, 128.3, 119.8, 119.5, 117.3, 110.9, 105.1, 78.0, 62.1, 55.8, 47.6, 37.1, 14.1; HRMS ($C_{18}H_{21}NO_4$): calcd, 315.1471 found, 315.1464.

(6a) ^{1}H NMR (300 MHz, $CDCl_3$) δ , 7.21 (d, *J* =9.0 Hz, 1H), 7.05 (d, *J* =2.4 Hz, 1H), 6.98 (dd, *J* =9.0 Hz, *J* =2.4 Hz, 1H) 6.23~6.10 (m, 1H), 6.01~5.89 (m, 1H), 5.44~5.37 (m, 1H), 4.29~5.23 (m, 1H) 5.09~5.04 (m, 3H), 4.88~4.82 (m, 1H), 4.67~4.64 (m, 2H), 4.39 (q, J =7.0 Hz, 2H) 3.85 (s, 3H), 1.41 (t, *J* =7.0 Hz, 3H). HRMS ($C_{18}H_{21}NO_4$): calcd, 315.1471 found, 315.1470.

Synthesis of compound 5 (route b)

A mixture of ethyl 3-hydroxy-5-methoxy-1H-indole-2-carboxylate (500 mg, 2.12 mmol), imidazole (288 mg, 4.24 mmol) and TBDPSCl (1,1 mL, 4.24 mmol) in THF (20 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 (795 mg, 79 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.11 (s, 1H), 7.84-7.81 (m, 4H), 7.42-7.25 (m, 6H), 7.06 (d, *J* = 8.9 Hz, 1H), 6.76 (dd, *J* = 8.9 Hz, 2.2 Hz, 1H), 6.34 (d, *J* = 2.2 Hz, 1H), 4.30 (q, *J* = 7.1 Hz, 2H), 3.15 (s, 3H), 1.32 (t, *J* = 7.1 Hz, 3H), 1.15 (s, 9H). HRMS ($C_{282}H_{31}NO_4Si$): calcd, 473.2022 found, 473.2016.

To a solution of ethyl 3-(tert-butyldiphenylsilyloxy)-5-methoxy-1H-indole-2-carboxylate (500 mg, 1.05 mmol) in DMF (10 mL) was added NaH (50 mg, 2.10 mmol) and allyl iodide (0.23 mL, 2.10 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 1-allyl-3-(tert-butyldiphenylsilyloxy)-5-methoxy-1H-indole-2-carboxylate (360 mg, 66 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.83-7.80 (m, 4H), 7.42-7.31 (m, 5H), 7.26 (d, *J* = 2.2 Hz, 1H), 7.07 (dd, *J* = 8.9, 2.2 Hz, 1H), 6.90-6.77 (m, 1H), 6.47-6.45 (m, 1H), 5.95-5.87 (m, 1H), 5.05-5.02 (m, 3H), 4.79 (d, *J*= 17 Hz, 1H), 4.22-4.10 (m, 2H), 3.18 (s, 3H), 1.28-1.20 (m, 3H), 1.07 (s, 9H). HRMS ($C_{31}H_{35}NO_4Si$): calcd, 513.2335 found, 513.2329.

To a solution of ethyl ethyl 1-allyl-3-(tert-butyldiphenylsilyloxy)-5-methoxy-1H-indole-2-carboxylate (500 mg, 0.97 mmol) in THF (15 mL) was added TBAF (50 mg, 2.10 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 1-allyl-3-hydroxy-5-methoxy-1H-indole-2-carboxylate (222 mg, 83 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.64 (s, 1H), 7.16 (d, *J* = 8.9 Hz, 1H), 7.11 (d, *J* = 2.2 Hz, 1H), 7.03 (dd, *J* = 8.9, 2.2 Hz, 1H), 5.94-5.82 (m, 1H), 5.08-5.03 (m, 1H), 4.94-4.84 (m, 3H), 4.44 (q, *J* = 7.1 Hz, 2H), 3.84 (s, 3H), 1.25 (t, *J* = 7.1 Hz, 3H). HRMS ($C_{15}H_{17}NO_4$): calcd, 275.1158 found, 275.1157.

A mixture of 1-allyl-3-hydroxy-5-methoxy-1H-indole-2-carboxylate (210 mg, 0.76 mmol), K_2CO_3 (483 mg, 3.80 mmol) and allyl iodide (0.43ml, 3.80mmol) in acetone (10 mL) was refluxed for 7 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 1,2-diallyl-5-methoxy-3-oxoindoline-2-carboxylate (5a, 120 mg, 50 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.14 (dd, *J* =9.0 Hz, *J* =2.4 Hz, 1H), 7.02 (d, *J* =2.4 Hz, 1H), 6.79 (d, *J* =9.0 Hz, 1H), 5.96~5.83 (m, 1H), 5.53~5.39 (m, 1H), 5.33~5.26 (m, 1H), 5.24~5.12 (m, 1H), 5.00~4.97 (m, 1H), 4.20~4.08 (m, 2H), 4.05~3.82 (m, 2H), 3.76 (s, 3H), 3.04~2.88 (m, 2H), 1.41 (t, *J* =7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$); δ 195.5, 167.7, 157.8, 152.7, 134.0, 131.0, 128.3, 119.8, 119.5, 117.3, 110.9, 105.1, 78.0, 62.1, 55.8, 47.6, 37.1, 14.1; HRMS ($C_{18}H_{21}NO_4$): calcd, 315.1471 found, 315.1464.

Cell culture and treatments

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), NIH3T3 (mouse embryonic fibroblast cell line) and HEK293 (human embryonic kidney cell line) cells were propagated in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS, GIBCO-BRL). These cells were incubated with LipidGreen at 10 μM for 24 h in DMEM containing 10% FBS at 37 □ and then observed under a fluorescence microscope. 3T3-L1 cells were seeded onto 0.1% gelatin-coated coverslips and allowed to grow to confluence. Differentiated adipocytes were induced at confluence in DMEM containing 10% FBS, 0.25 μM dexamethasone (Sigma aldrich), 0.5 mM isobutylmethylxanthine (IBMX, Sigma aldrich) and 166 nM insulin (Sigma aldrich) for 2 days (from day 0 to day 2). This medium was replaced with DMEM containing 10% FBS and 166 nM of 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. Then we treated LipidGreen at 10 μM for 24 h with DMEM containing 10% FBS. HepG2 cell was treated with LipidGreen in 24 h, and then was diluted in Dimethyl sulfoxide (DMSO).

Maintenance of fish

Zebrafish were raised and kept under standard laboratory condition at 28.5°C. Zebrafish embryos were obtained from spontaneous spawning. Zebrafish are staged and fixed at specific days post fertilization (dpf). A high-cholesterol diet for zebrafish was made by artificial artemia in a diethyl ether solution of cholesterol (Sigma) to achieve a content of 4% (wt/wt) cholesterol in the food after ether evaporation. For starvation and refeeding experiments, zebrafish were maintained in individual cages.

Treatment and imaging of LipidGreen and Nile red with zebrafish

Individual zebrafish was treated with LipidGreen that was diluted in DMSO. Zebrafish were incubated with 100 μM LipidGreen in the dark for 15mins. For staining with Nile red, zebrafish were incubated 100 μM of Nile red in the dark for 15mins. After incubation of LipidGreen or Nile red, zebrafish were washed with egg water for 30 min. After washing, photography was performed on a Leica MZ10F.

Immunostaining

Zebrafish fat deposits were fixed for 4 h at room temperature in 4% paraformaldehyde. Fixed fat droplets were transferred to methanol at -20 □ overnight. Embryos were rehydrated in graded methanol: PBST series (3:1, 1:1, 1:3) for 5 min. After fat deposits were washed 3 times by PBST for 5 min, those were blocked for 1 h in 5% horse serum. Next, adipose tissues were incubated with anti-perilipin antibody (1:200) in for 4 h at room temperature. After incubation, wash 6 times at 10 min interval with PBST. Then fat deposits were incubated for overnight at 4 □ with Rhodamine-conjugated anti-mouse secondary antibody (1:500). Embryos were washed with PBST until the signals is clear. Photography was performed on a Zeiss LSM5 (Confocal Laser Scan Microscope)

Hoechst 33342 DNA staining

Zebrafish adipose tissues were stained with Hoechst 33342 (1μg/ml) (SIGMA) at room temperature in darkness for 5 min. After staining, adipose tissues were washed several times for 1hours in PBST.

Histological Analysis

After LipidGreen staining, the stained-visceral organs were dehydrated with a graded ethanol series. Xylene-soaked specimens were embedded in paraffin, cut at 12 μm thickness, and then subjected to histological evaluations.

Imaging and Quantitative analysis

LipidGreen or Nile red stained-fishes were imaged by using a LeicaMZ10F microscope. After compound staining and washing, fishes were anaesthetized with tricaine and embedded in 3% methylcellulose for imaging. Laser at 470/40nm or 545/30nm filter was used for excitation. Images were acquired with 525/50nm 620/60nm emission filter. Quantitative analysis was performed by fluorescent microscopy and LAS v3.6 Leica Microsystems software.

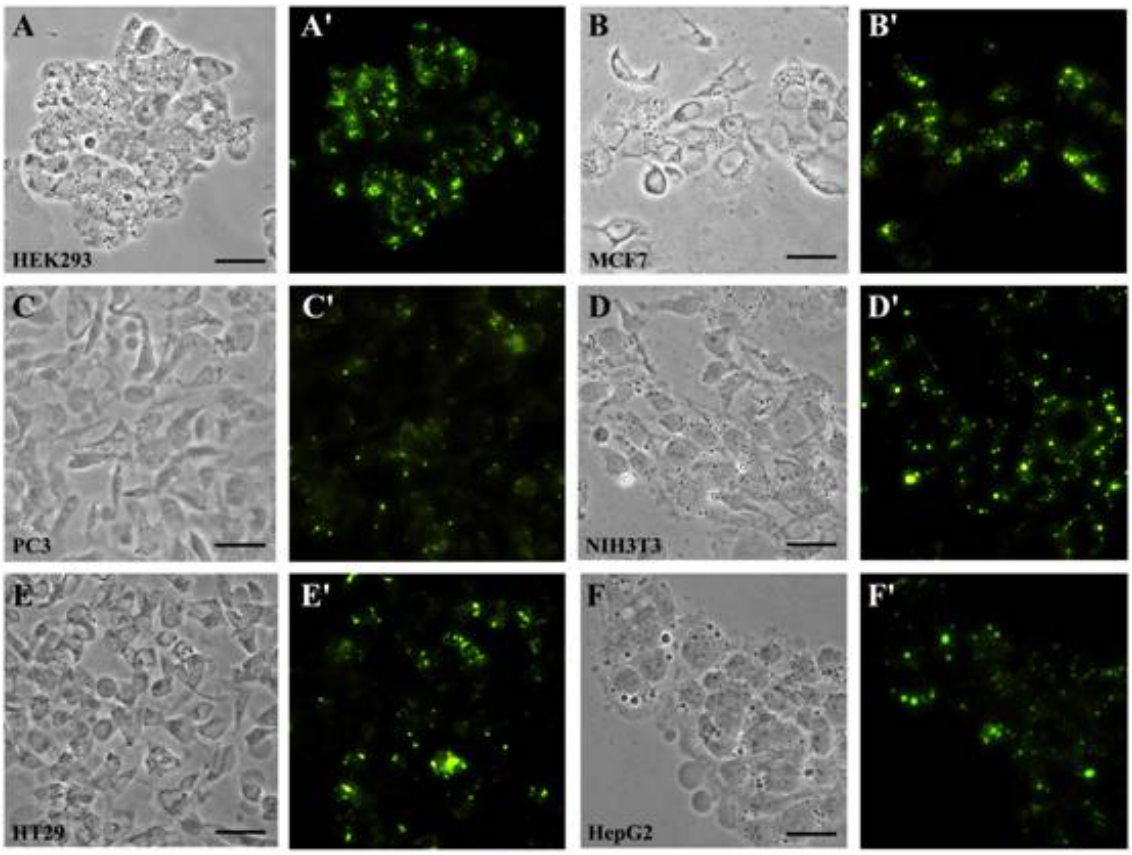

Figure S1. Various cell lines were stained with the compound 5. Subcellular localization of LipidGreen in HEK293 cells (Human embryonic kidney cell line) (A and A′), MCF7 cells (Human breast adenocarcinoma cell line) (B and B′), PC3 cells (Human prostate cancer cell line) (C and C′), NIH3T3 cells (Mouse embryonic fibroblast cell line) (D and D′), HT29 cells (Human colon cancer cell line) (E and E′), HepG2 cells (Human liver carcinoma cell line) (F and F′) Scale bars represent 100μm (A-F′).

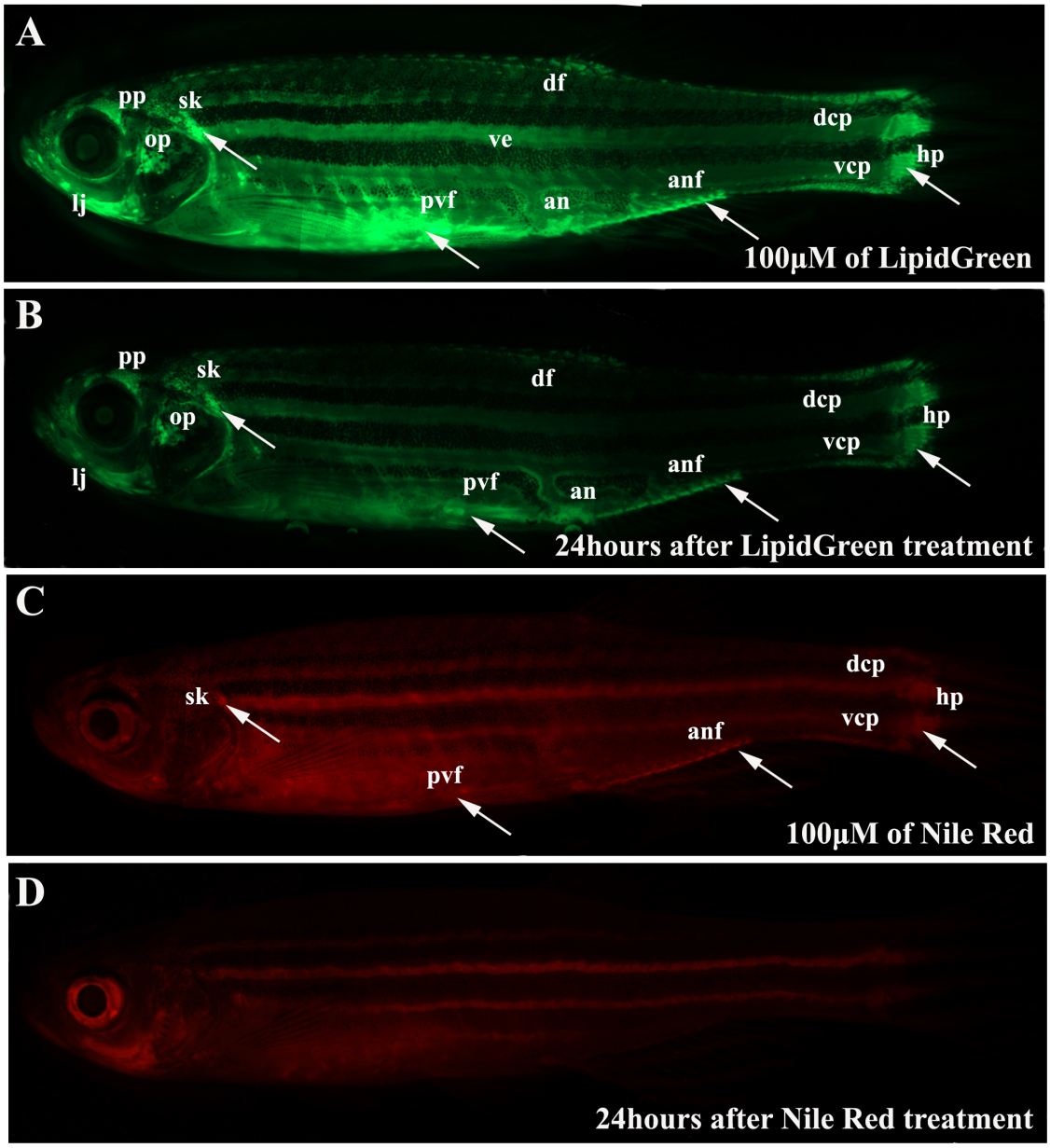

Figure S2. Staining comparison with LipidGreen and Nile red in zebrafish. (A) Zebrafish stained with 100μM of LipidGreen. (B) Image of zebrafish 24 h after LipidGreen-staining; LipidGreen-stained fat deposits still remained. (C) In contrast, 100μM of Nile red-stained fat deposits are less bright than that of LipidGreen -stained fat depots. (D) Image of Nile red-stained fat deposits determined 24 h later. Arrows indicate LipidGreen or Nile red-stained fat deposits.

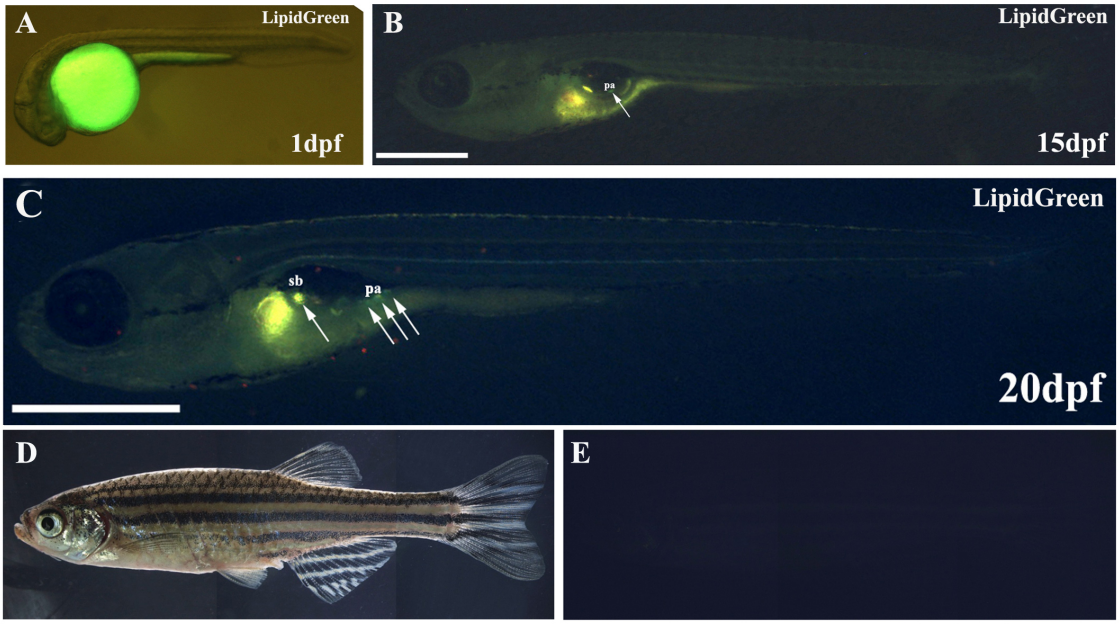

Figure S3. Fat tissues imaging with LipidGreen in embryo and juvenile zebrafish. (A) LipidGreen stained the yolk of developing zebrafish embryos. (B) LipidGreen-stained lipids accumulated around the pancreas at 15dpf and 20dpf. Arrows indicate lipids. (D and E) Auto-fluorescence is not detectable in wild-type zebrafish. Scale bars represent 2 mm (B and C)

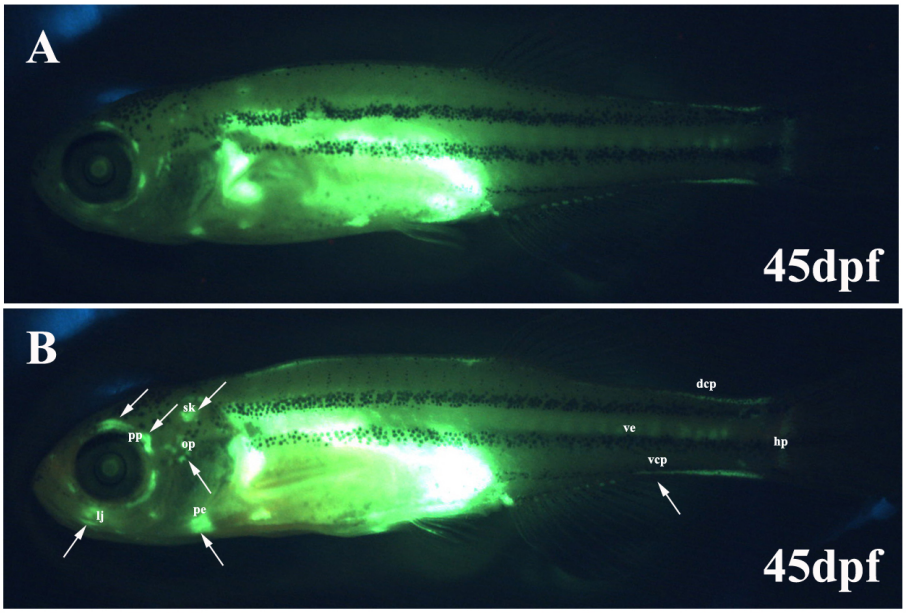

Figure S4. Effect of a high-fat diet on fat accumulation in zebrafish. Zebrafish were fed with 4% cholesterol-rich high fat diet for 15 days beginning at 30 dpf. After feeding with 4% cholesterol-rich high fat diet for 15 days of 4% cholesterol, elevated fat deposits were detected in the lj, pp, pe, op, sk, and vcp. Abbreviations; pe, pericardial; lj, low jaw; pp, posterior periorbital; ve, vertebra; dcp, dorsal caual peduncle; vcp, ventral caudal peduncle; op. opercular; hp, hypural; sk, skull.

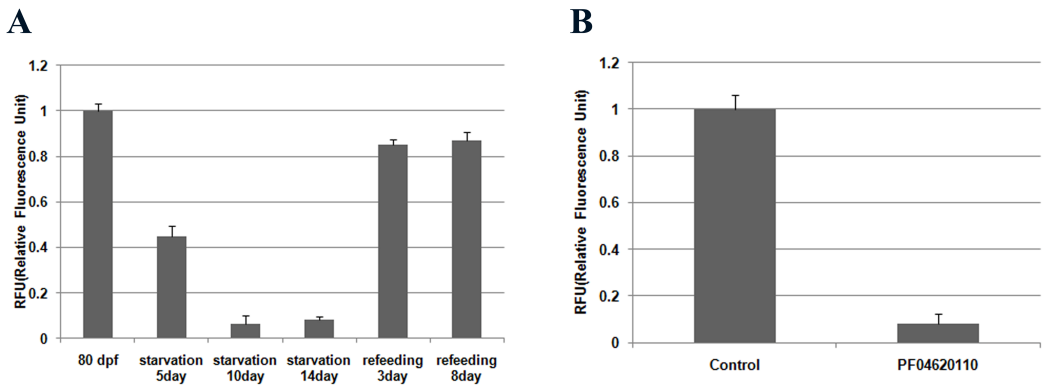

Figure S5. Quantitative anaylsis of the changes in **LipidGreen** fluorescence. Monitoring of fat deposits by **LipidGreen** after starvation and refeeding in Figure 3 (A). Detection of fat accumulation in DGAT1 inhibitor-treated zebrafish larvae in Figure 4 (B).