Supplementary Material (ESI) for Chemical Communications

A Two-Photon Fluorescent Probe for Near-membrane Calcium Ions in Live Cells and Tissues

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Electronic Supplementary Information

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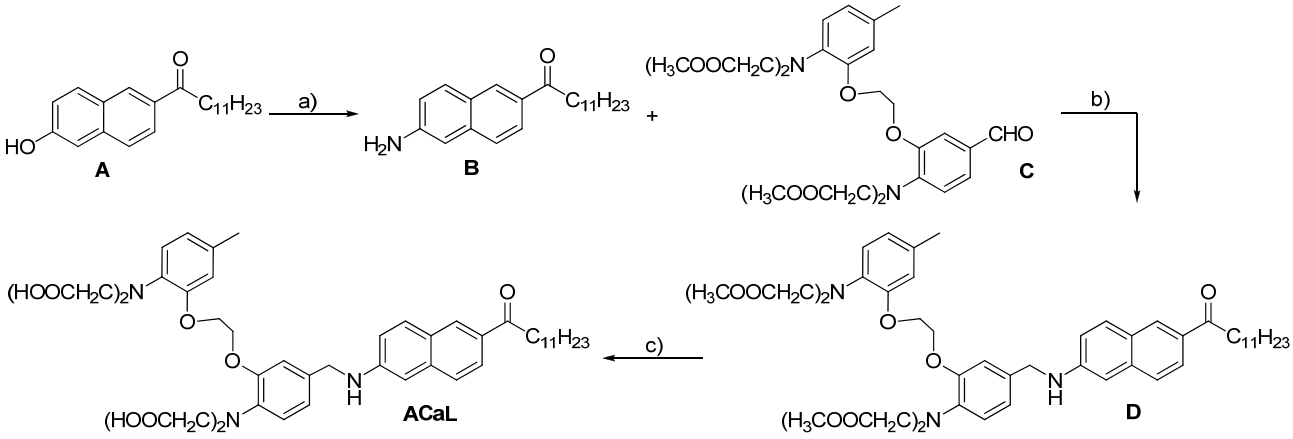
Synthesis of ACaL. 6-Dodecanoyl-2-hydroxynaphthalene (**A**)[1] and 5-methyl-5'-formyl-BAPTA-tetramethyl ester (**C**)[2] were prepared by the literature methods. Synthesis of other compounds is described below.



Scheme. (a) $Na_2S_2O_5$/ NH_4OH (b) i) EA-reflux ii) $NaBH(OAc)_3$/ MC (c) EtOH/ KOH/ HCl.

Compound B. A mixture of **A** (8.0 g, 25 mmol), $Na_2S_2O_5$ (12 g, 61 mmol), and NH_4OH (150 ml) was stirred in a steel-bomb reactor at 140°C for 96 h. The product was collected by filtration, washed with water, and purified by flash-column chromatography by using hexane/ethyl acetate (1:1) as the eluent. It was further purified by recrystallization from MeOH. Yield: 3.9 g (75%); m.p. 102 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.30 (d, 1H, *J* = 2 Hz) 7.92 (dd, 1H, *J* = 2 Hz, *J* = 8 Hz), 7.74 (d, 1H, *J* = 8 Hz), 7.57 (d, 1H, *J* = 8 Hz), 6.96 (dd, 1H *J* = 2 Hz, *J* = 8 Hz), 6.94 (d, 1H, *J* = 2 Hz), 4.04 (s, 2H), 3.00 (t, 2H, *J* = 7 Hz), 1.74 (quin, 2H, *J* = 7 Hz), 1.23 (m, 16H), 0.85 (t, 3Hz, *J* = 7 Hz) ; ^{13}C NMR (100 MHz, $CDCl_3$):

δ = 200.60, 146.84, 137.70, 131.46, 131.40, 130.11, 126.73, 126.15, 124.97, 118.89, 108.10, 38.67, 32.15, 29.88, 29.87, 29.77, 29.72, 29.59, 29.45, 25.00, 22.94, 14.39 ppm. Anal. Calcd for $C_{22}H_{31}NO$: C, 81.18; H, 9.60; N, 4.30. Found: C, 81.55; H, 9.86; N, 4.05.

Compound D. A mixture of **C** (0.19 g, 0.33 mmol) and **B** (0.11 g, 0.33 m mol) in anhydrous ethyl acetate was refluxed overnight under nitrogen atmosphere. After evaporating the solvent, 1,2-dichloroethane (10 mL) and $NaBH(OAc)_3$ (0.15 g, 0.65 mmol) were added, and stirred overnight. The solvent was removed in vacuo and the product was purified by a column chromatography over silica gel using $CHCl_3$: ethyl acetate : hexane (2:2:1) as eluant. Reddish orange crystals separated out. Yield 0.045g (16%) ; mp 98 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.30 (d, 1H, *J* = 2 Hz) 7.93 (dd, 1H, *J* = 2 Hz, *J* = 9 Hz), 7.73 (d, 1H, *J* = 9 Hz), 7.60 (d, 1H, *J* = 9 Hz), 6.95 (d, 1H, *J* = 2 Hz), 6.92 (dd, 1H, *J* = 2 Hz, *J* = 9 Hz), 6.90 (dd, 1H, *J* = 2 Hz, *J* = 9 Hz), 6.81 (d, 1H, *J* = 9 Hz), 6.75 (d, 1H, *J* = 9 Hz), 6.70 (d, 1H, *J* = 2 Hz), 6.68 (d, 1H, *J* = 2 Hz), 6.67 (dd, 1H, *J* = 2 Hz, *J* = 9 Hz), 4.36 (s, 2H), 4.26 (t, 2H, *J* = 7 Hz), 4.26 (t, 2H, *J* = 7 Hz), 4.16 (s, 4H), 4.12 (s, 4H), 3.59 (s, 6H), 3.53 (s, 6H), 3.04 (t, 2H, *J* = 7 Hz), 2.97 (s, 3H), 1.78 (quin, 2H, *J* = 7 Hz), 1.28 (m, 16H), 0.88 (t, 3Hz, *J* = 7 Hz) ; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 200.50, 172.26, 172.09, 132.66, 132.35, 130.96, 130.20, 129.81, 126.25, 125.00, 122.01, 121.94, 120.63, 119.33, 118.69, 114.13, 113.47, 104.18, 150.73, 150.35, 148.07, 139.48, 138.72, 138.02, 136.88, 67.03, 66.48, 53.57, 53.49, 51.89, 51.79, 47.89, 38.62, 32.17, 29.88, 29.85, 29.77, 29.72, 29.57, 29.25, 25.02, 22.92, 21.31, 21.22, 14.38 ppm. Anal. Calcd for $C_{50}H_{65}N_3O_{11}$: C, 67.93; H, 7.41; N, 4.75. Found: C, 67.95; H, 7.36; N, 4.72.

ACaL. A solution of **D** (0.080 g, 0.11 mmol) in ethanol (5 mL) was dissolved in water (10 mL) containing KOH (1 g) and was stirred overnight. The contents of the flask were partially evaporated to remove ethanol and neutralized with diluted HCl up to a pH range of 3-4. The solid was filtered and crystallized with butanol : acetic acid : water (4:1:1). Yield 0.03g (34 %); mp > 210 °C (dec); ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.31 (d, 1H, *J* = 2 Hz) 7.81 (dd, 1H, *J* = 2 Hz, *J* = 9 Hz), 7.71 (d, 1H, *J* = 8 Hz), 7.51 (d, 1H, *J* = 9 Hz), 7.08 (d, 1H, *J* = 2 Hz), 7.05 (dd, 1H, *J* = 2 Hz, *J* = 8 Hz), 6.94 (dd, 1H, *J* = 2 Hz, *J* = 9 Hz), 6.85 (d, 1H, *J* = 9 Hz), 6.79 (d, 1H, *J* = 9 Hz), 6.75 (d, 2H, *J* = 2 Hz), 6.66 (dd, 1H, *J* = 2 Hz, *J* = 9 Hz), 4.36 (s, 2H), 4.31 (t, 2H, *J* = 7Hz), 4.25 (t, 2H, *J* = 7Hz), 4.06 (s, 4H), 4.00 (s, 4H), 3.02 (t, 2H, *J* = 7Hz), 3.22 (s, 3H), 1.70 (quin, 2H, *J* = 7Hz), 1.27 (m, 16H), 0.87 (t, 3Hz, *J* = 7Hz; ^{13}C NMR (100 MHz, $CDCl_3$): δ = 201.58, 174.80, 174.75, 150.80, 150.66, 138.55, 138.13, 136.46, 133.63, 132.73, 130.51, 130.26, 129.89, 126.10, 125.89, 125.74, 124.04, 121.47, 120.17, 119.43, 118.78, 114.41, 113.02, 104.99, 103.16, 67.27, 67.04, 54.83, 54.52, 49.67, 37.98, 31.90, 29.57, 29.52, 29.49, 29.30, 29.17, 24.96, 22.57, 20.01,19.84, 13.28 ppm. Anal. Calcd for $C_{46}H_{57}N_3O_{11}$: C, 66.73; H, 6.94; N, 5.08. Found: C, 66.52; H, 6.73; N, 5.07.

Spectroscopic measurements. Absorption spectra were recorded on a Hewlett-Packard 8453 diode array spectrophotometer, and fluorescence spectra were obtained with Amico-Bowman series 2 luminescence spectrometer with a 1-cm standard quartz cell. The fluorescence quantum yield was determined by using Coumarin 307 and Rhodamine B as the reference. The spectral data obtained under various conditions are summarized in Figure S1 and Table S1.

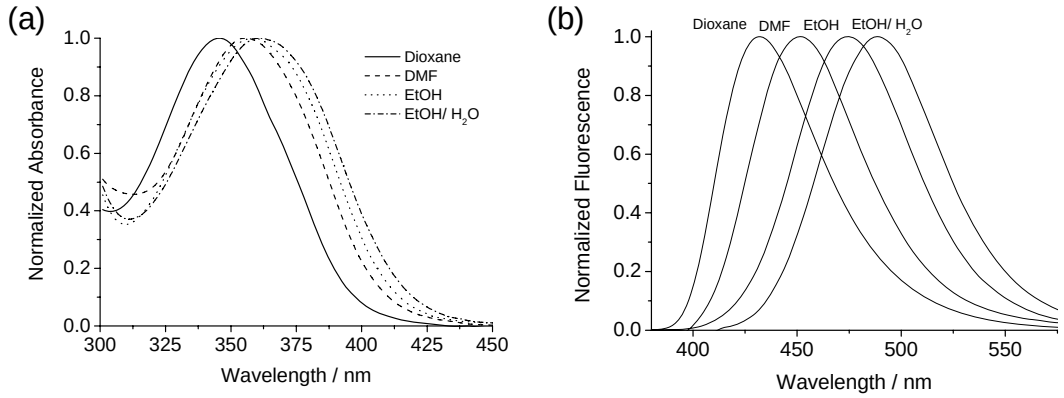


Figure S1. (a) Normalized absorption (b) emission spectra and of ACaL in 1,4-dioxane, DMF, EtOH, and EtOH/ H_2O.

Table S1. Photophysical properties of ACaL in various solvents.

Solvent (E_T^N)[a]	$\lambda_{max}^{(1)}$ [b]	λ_{max}^{fl} [b]	Φ[c]
Dioxane(0.164)	346	433	0.36
DMF (0.386)	356	453	0.059
EtOH (0.654)	359	475	0.11
EtOH/ H_2O (1.00)	356	487	0.096

[a] The numbers in the parenthesis are normalized empirical parameter of solvent polarity.[3] [b] λ_{max} of the one-photon absorption and emission spectra in nm. [c] Fluorescence quantum yield. The uncertainty is ± 15 %.

Determination of apparent dissociation constants. A series of calibration solutions containing various [Ca^{2+}] was prepared by mixing two solutions (*solution A* containing 10 mM K_2EGTA and *solution B* containing 10 mM CaEGTA) in various ratios.[4,5] Both solutions contained 1 μM ACa1, 100 mM KCl, 30 mM MOPS, and they were adjusted to pH 7.2.

To determine the K_d for Ca^{2+}–ACa1, the fluorescence spectrum was recorded with 2.0 mL of *solution A* (0 μM free Ca^{2+}) at 20 °C. Then 203 μL of this solution was discarded and replaced by 203 μL of *solution B* (39 μM free Ca^{2+}), and the spectrum was recorded. This brings the CaEGTA concentration to 1.00 mM and the $[Ca^{2+}]_{free}$ to about 0.017 μM with no change in the concentration of the probe or of the total EGTA. The $[Ca^{2+}]_{free}$ is calculated from the K_d of EGTA for Ca^{2+} (150.5 nM) using Eq (1).[5,6]

$$[Ca^{2+}]_{free} = K_d^{EGTA} \times \frac{[CaEGTA]}{[K_2EGTA]} \qquad (1)$$

Further iterations attained 0.038, 0.065, 0.101, 0.150, 0.230, 0.350, 0.601, 0.800, 1.00, 1.30, 2.50, 5.30, 10.0, and 20.0 μM free Ca^{2+} by successively discarding 223, 251, 285, 327, 421, 479, 667, 420, 350, 412, 905, 1028, 926, and 992 μL of *solution A* and replacing each with an equal volume of *solution B*.

When a 1:1 metal-ligand complex is formed between probe and Ca^{2+}, one can describe the equilibrium as follows, where L and M represent probe and Ca^{2+}, respectively.

The total probe and metal ion concentration are defined as $[L]_0 = [L] + [LM]$ and $[M]_0 = [M] + [LM]$, respectively. With $[L]_0$ and $[M]_0$, the value of K_d is given by:

$$[LM]^2 - \left([L]_0 + [M]_0 + K_d\right)[LM] + [L]_0[M]_0 = 0,$$

$$[LM] = \frac{\left([L]_0 + [M]_0 + K_d\right) - \sqrt{\left([L]_0 + [M]_0 + K_d\right)^2 - 4[L]_0[M]_0}}{2} \qquad (2)$$

or $$\left(F - F_{min}\right) = \left(\frac{\left([L]_0 + [M]_0 + K_d\right) - \sqrt{\left([L]_0 + [M]_0 + K_d\right)^2 - 4[L]_0[M]_0}}{2[L]_0} \right)(F_{max} - F_{min}) \qquad (3)$$

where F is the observed fluorescence intensity, F_{min} is the minimum fluorescence intensity, and F_{max} is the maximum fluorescence intensity. The K_d value that best fits the titration curve (Figure S2c) with Eq 3 was calculated by using the Excel program as reported.[6]

In order to determine the K_d^{TP} for the two-photon process, the TPEF spectra were obtained with a DM IRE2 Microscope (Leica) excited by a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 780 nm and output power 1230 mW, which corresponded to approximately 10 mW average power in the focal plane. The TPEF titration curves (Figure S2c) were obtained and fitted to Eq 3.

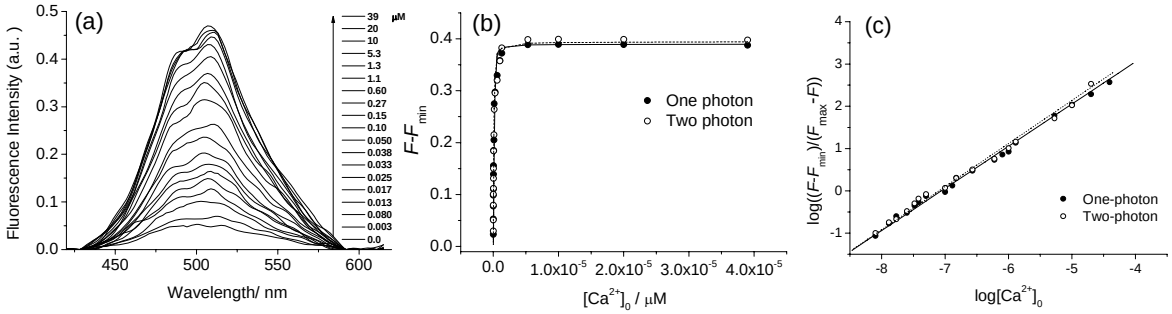


Figure S2. (a) Two-photon emission spectra of ACaL (30 mM MOPS, 100 mM KCl, 10 mM EGTA, pH 7.2) in the presence of free Ca^{2+} (0–39 μM). (b) One- (●) and two-photon (○) fluorescence titration curve for the complexation of ACaL with free Ca^{2+} (0-39 μM). (c) Hill plots for the complexation of ACaL with free Ca^{2+} (0–39 μM).The excitation wavelengths for one- and two-photon processes were 365 and 780 nm, respectively.

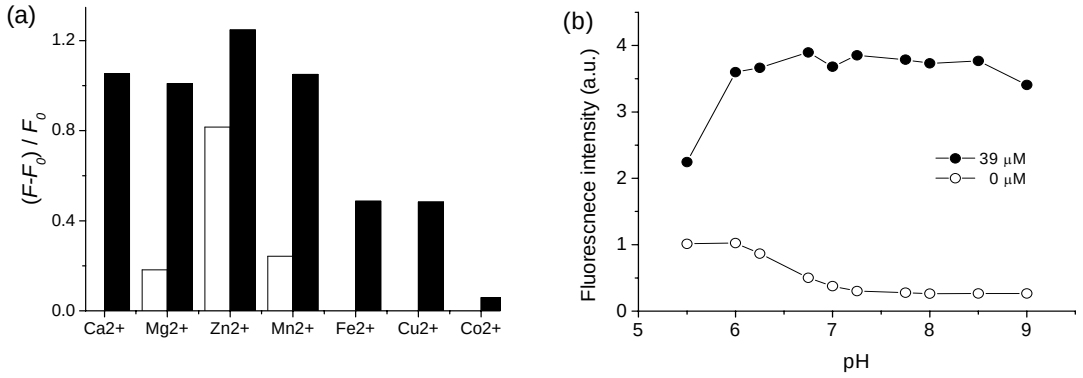


Figure S3. (a) The relative fluorescence intensity of 1 μM ACaL in the presence of 2 mM for Mg^{2+}; 100 μM for Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+} (empty bars) followed by addition of 100 μM of Ca^{2+} (filled bars). (b) Effect of the pH on the one-photon fluorescence intensity of 1 μM ACaL in the presence of 0.0 (○) and 39 μM (●) of free Ca^{2+} in 30 mM MOPS and 100 mM KCl. The data at $[Ca^{2+}]$ = 0 μM were determined by adding 10 mM EGTA. These data were measured in 30 mM MOPS buffer (100 mM KCl, 10 mM EGTA, pH 7.2). The excitation wavelength was 365 nm.

Vesicle preparation. Vesicles for the measurements of one photon fluorescence spectra were prepared by the solvent evaporation method.[7] The dissociation constants in the vesicles were determined by the same method as described above except that the lipid/probe ratio was maintained to be 300/1.

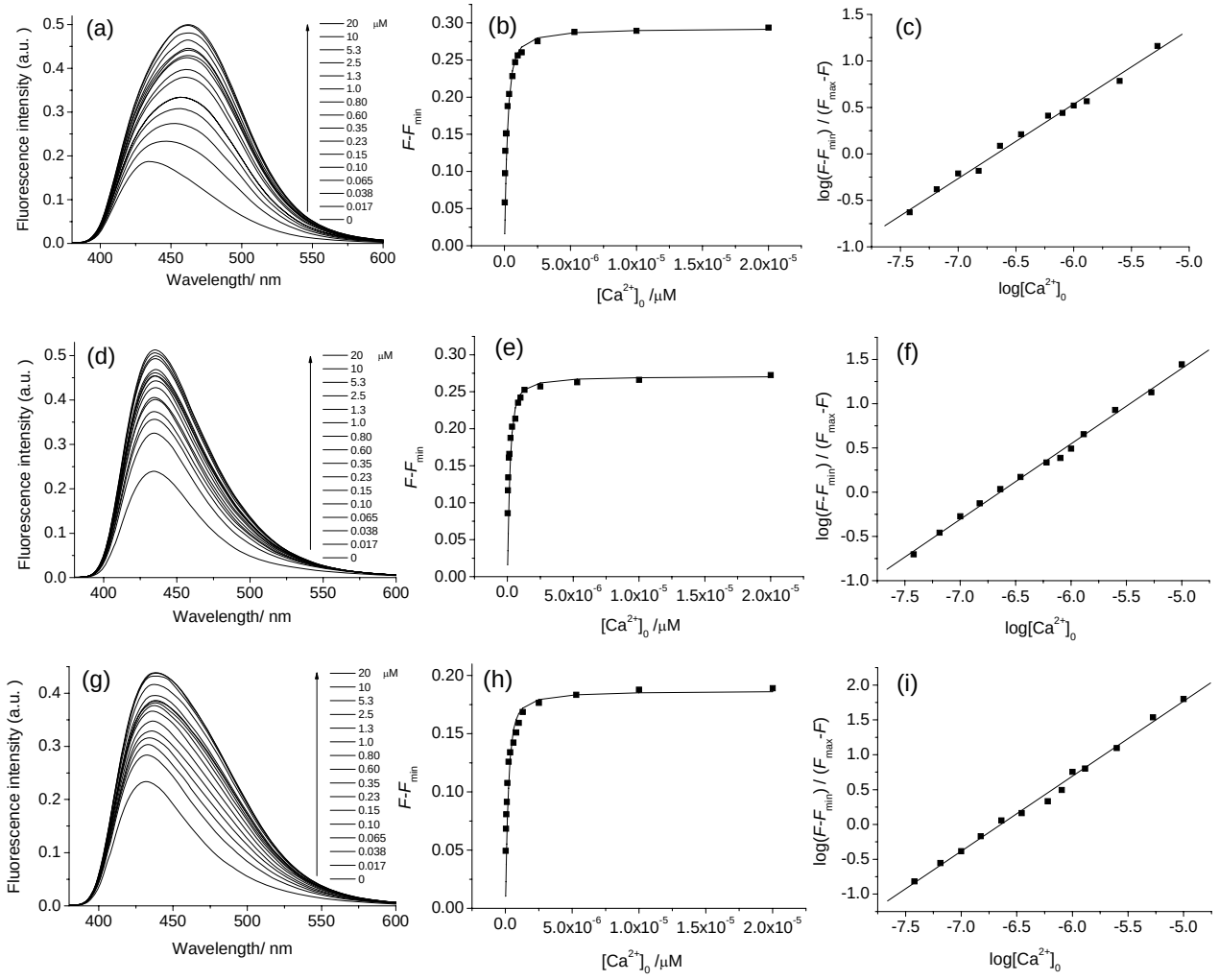


Figure S4. (a,d,g) One-photon emission spectra, (b,e,h) one-photon fluorescence titration curve, and (c,f,i) Hill plots for the complexation of ACaL with free Ca^{2+} (0-39 μM) in (a,b,c) DOPC, (d,e,f) DPPC and (g,h,i) DOPC/sphingomyelin/cholesterol (1:1:1, raft mixture). In all cases, lipid/ACaL was 300/1 and the excitation wavelength was 365 nm.

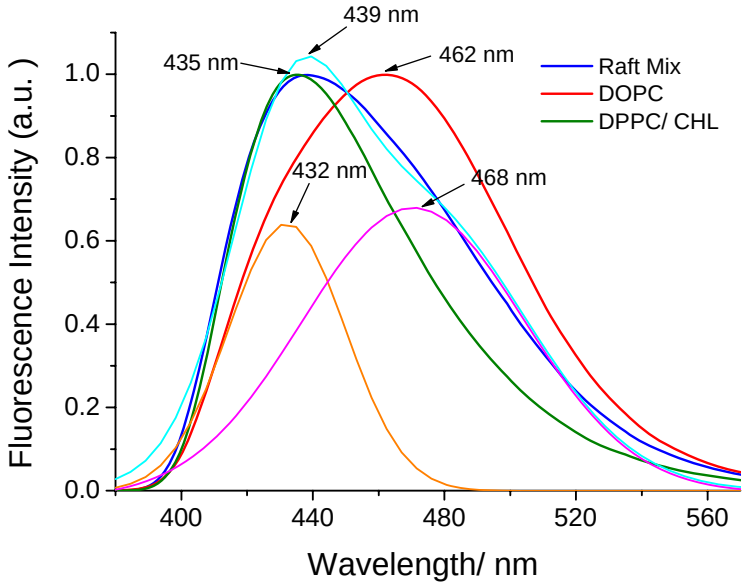


Figure S5. The normalized fluorescence spectra of ACaL in the presence of 39 μM for Ca^{2+} in vesicles composed of DPPC/cholesterol (green curve), DOPC (red curve) and DOPC/sphingomyelin/cholesterol (1:1:1, raft mixture, blue curve). The spectrum from the raft mixture could be fitted to two Gaussian functions centered at 432 (orange curve) and 468 nm (pink curve). The excitation wavelength was 365 nm.

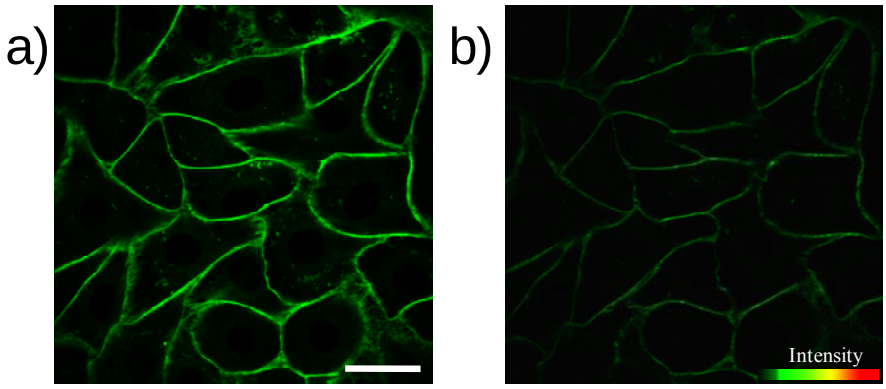


Figure S6. (a) Pseudo colored TPM images of ROS cells labeled with ACaL (5 μM). (b) TPM image of the same cells after treatment with EGTA (50 μM). Images were collected at 360-620 nm upon excitation at 780 nm. Cells shown are representative images from replicate experiments (n = 5). Scale bar, 30 μm.

Measurement of two-photon cross section. The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique as described.[8] ACaL, Calcium Green C_{18}, and C_{18}-Fura-2 were dissolved in 30 mM MOPS buffer (30 mM MOPS, 100 mM KCl, 10 mM EGTA, pH 7.2) at concentrations of 5.0×10^{-6} M and then the two-photon induced fluorescence intensity was measured at 740–940 nm by using fluorescein (8.0×10^{-5} M, pH = 11) as the reference, whose two-photon property has been well characterized in the literature.[9] The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated according to Eq 1,

$$\delta = \frac{S_s \Phi_r \phi_r c_r}{S_r \Phi_s \phi_s c_s} \delta_r \qquad (1)$$

where the subscripts *s* and *r* stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as *S*. Φ is the fluorescence quantum yield. ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as *c*. δ_r is the TPA cross section of the reference molecule.

Two-photon fluorescence microscopy. Two-photon fluorescence microscopy images of probe-labeled cells and tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP2) with a ×100 (NA = 1.30 OIL) and ×20 (NA = 0.30 DRY) objective lens, respectively. The two-photon fluorescence microscopy images were obtained with a DM IRE2 Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength 780 nm and output power 1230 mW, which corresponded to approximately 10 mW average power in the focal plane. To obtain images at 360–460 nm and 520–620 nm range, internal PMTs were used to collect the signals in an 8 bit unsigned 512 × 512 pixels at 400 Hz scan speed.

Cell culture and imaging. ROS17/2.8 rat clonal osteoblastic cell line, were cultured in ascorbic acid-free α-MEM (WelGene Inc, Seoul, Korea) supplemented with heat-inactivated 10% FBS (v/v, WelGene Inc, Seoul, Korea), 100 units/mL penicillin, and 100 ug/mL streptomysin. Cells were grown at 37°C in a humidified atmosphere with air/CO_2 ratio of 95:5. Two days before imaging, the cells were detached with trypsin-EDTA solution and ~10,000 cells/mm^2 were replated on glass-bottomed dishes (MatTek). For labeling, the growth medium was removed and changed with a reduced calcium balanced salt solution (RCBSS) (127 mM NaCl, 3.8 mM KCl, 1.2 mM KH_2PO_4, 0.8 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES buffer). The cells were exposed to 5 μM probe at room temperature. After 5 min, the cells were washed three times with RCBSS and then imaged. For characterization of Ca^{2+} efflux by ROS17/2.8 cells, ACaL-labeled cells were stimulated with 100 nM of hPTH (parathyroid hormone 1-34, human, Calbiochem, Merck, Germany).[10] To test the effects of the inhibitor, cells were pre-incubated with thapsigargin (5 μM) for 10 min at 37°C in the incubator.

Preparation and staining of fresh rat Hippocampal slices. All experiments were performed in accordance with the guidelines established by the Committee of Animal Research Policy of Korea University College of Medicine. The 400 μm thick of hippocampal coronal slices were prepared from 2 day-old Sprague Dawley rat with a vibrating-blade microtome in artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 26 mM $NaHCO_3$, 10 mM D-glucose, 1.3 mM $MgCl_2$, 3 mM KCl, 1.25 mM NaH_2PO_4, 2.4 mM $CaCl_2$). Slices were incubated with 10 μM ACaL in ACSF solution bubbled with 95/5 (v/v) of O_2/CO_2 for 40 min at 37°C. After washing three times with ACSF, the slices were transferred to glass-bottomed dishes (MatTek) and observed under a spectral confocal multiphoton microscope. To observe the effect of EDTA, a solution of EDTA in MOPS buffer (200 μM) was added to this sample and TPM image was obtained. In cases where a study involves the use of live animals or human subjects, the author should include in the Methods/Experimental section of the manuscript a statement that all experiments were performed in compliance with the relevant laws and institutional guidelines, and also state the institutional committee(s) that have approved the experiments.

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