

Information to Accompany “A Two-Photon Fluorescent Probe for Ratiometric Imaging of Hydrogen Peroxide in Live Tissue”

Chul Chung,^{‡,a} Duangkhae Srikun,^{‡,b} Chang Su Lim,^a Christopher J. Chang,^{*,b} and Bong Rae Cho^{*,a}

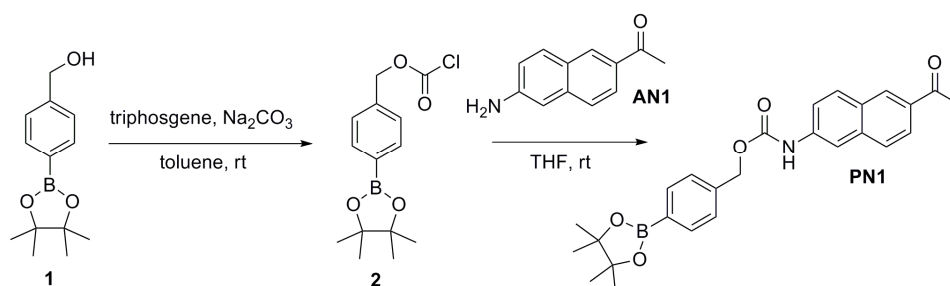
^aDepartment of Chemistry, Korea University, 1-Anamdong, Seoul, 136-701, Korea, and

^bDepartment of Chemistry, and the Howard Hughes Medical Institute, University of California, Berkeley, California 94720, U.S.A.

Table of Contents

	Page
Synthesis of PN1	S2
Spectroscopic measurements	S2
Measurement of two-photon cross section.....	S3
Cell culture	S3
Two-photon fluorescence microscopy.....	S4
Preparation and Staining of fresh rat hippocampal slices	S4
Figure S1. LC/MS traces of PN1, AN1 and the reaction of PN1 with H ₂ O ₂	S5
Figure S2. Solubility of PN1 in HEPES buffer.....	S6
Figure S3. Normalized absorption, excitation, and fluorescence spectra of PN1 and AN1	S7
Figure S4. Excitation spectra of PN1 treated with H ₂ O ₂	S7
Figure S5. Fluorescence spectra PN1 treated with H ₂ O ₂	S8
Figure S6. Two-photon action spectrum of PN1 and AN1	S9
Figure S7. Two-photon fluorescence response of PN1 to H ₂ O ₂	S9
Figure S8. Effect of pH on the $F_{\text{green}}/F_{\text{blue}}$ ratios for PN1 and AN1	S10
Figure S9. Two-photon confocal microscopy of PN1-labeled Raw 264.7 cells in lambda mode scanning using 750 nm excitation.....	S10
Figure S10. Two-photon confocal microscopy of AN1-labeled Raw 264.7 cells in lambda mode scanning using 750 nm excitation.....	S11
Figure S11. Two-photon confocal microscopy of PN1-labeled Raw 264.7 cells after treatment with PMA and H ₂ O ₂	S12
Figure S12. WST-1 viability assays of Raw 264.7 cells treated with AN1 and PN1	S13
Figure S13. TPM images of a fresh rat hippocampal slice stained with PN1.	S14
Figure S14. $F_{\text{green}}/F_{\text{blue}}$ in the CA3 and CA1 regions of PN1-labeled hippocampal slices pretreated with various concentration of H ₂ O ₂	S15
Figure S15. TPM images of a PN1-labeled hippocampal slice after treatment with H ₂ O ₂	S16
Figure S16. TPM images of a fresh rat hippocampal slice stained with AN1.	S17

Synthetic Materials and Methods. Silica gel P60 (SiliCycle) was used for column chromatography. 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzaldehyde was purchased from Oakwood Chemical (West Columbia, SC). The WST-1 cell viability assay kit was purchased from Roche Applied Science (Mannheim, Germany). ProliNONOate was obtained from Cayman Chemical (Ann Arbor, MI, USA). 2-acetyl-6-amino naphthalene (AN1)^[1] and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzylalcohol (**1**)^[2] were prepared by the literature methods. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as received.



Scheme S1. Synthesis of **PN1**.

4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl)benzylchloroformate (2). Na₂CO₃ (3.5 g, 33.0 mmol) was flame dried in a round-bottom flask. The flask was cooled in an ice bath and triphosgene (2.2 g, 7.4 mmol) in toluene (15 mL) was added. After stirring for 0.5 h at 0 °C, **1** (0.87 g, 3.7 mmol) in toluene (5 mL) was added and stirred for 6 h at RT. The crude product after usual work-up was used without further purification; ¹H NMR (CDCl₃, 300 MHz): δ 7.86 (d, *J* = 8.1 Hz, 2H) 7.40 (d, *J* = 8.1 Hz, 2H), 5.31 (s, 2H), 1.35 (s, 12H).

Peroxy Naphthalene 1 (PN1). A solution of **2** (0.50 g, 2.1 mmol) and AN1 (1.1 g, 5.3 mmol) in THF (10 mL) was stirred at RT for 6 h. The solvent was evaporated and the product was purified by silica gel column chromatography using n-hexane/ethyl acetate (3:1) as the eluent. The product was a light yellow solid. Yield, 0.25 g (27 %); M.p. 168.6 °C; ¹H NMR (CDCl₃, 300 MHz): δ 8.39 (d, *J* = 1.8 Hz, 1H), 8.05 (s, 1H), 8.03 (dd, *J* = 8.9 Hz, 1.8 Hz, 1H), 7.92 (d, *J* = 8.9 Hz, 1H), 7.85 (d, *J* = 8.4 Hz, 2H), 7.83 (d, 8.8 Hz, 1H), 7.48 (dd, *J* = 8.9 Hz, 1.8 Hz, 1H), 7.44 (d, *J* = 8.4 Hz, 2H), 6.95 (d, *J* = 1.8 Hz, 1H), 5.27 (s, 2H), 2.70 (s, 3H), 1.35 (s, 12H). ¹³C NMR (CDCl₃, 400 MHz): δ 198.1, 153.3, 138.9, 137.9, 136.7, 135.3, 133.6, 130.9, 130.1, 129.3, 128.1, 127.6, 124.9, 119.9, 114.5, 84.1, 67.4, 26.9, 25.08. HRMS: calculated for [MH⁺] 446.2139, found 446.2136.

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 503 as the reference by the literature method.^[3] Reactive oxygen species (200 μM unless otherwise stated) were administered to PN1 in 20 mM HEPES (pH 7.1, 25 °C) as follows. H₂O₂, *tert*-butylhydroperoxide (TBHP), and hypochlorite (NaOCl) were delivered from 30%,

70%, and 10% aqueous solutions respectively. Hydroxyl radical ($\bullet\text{OH}$), and *tert*-butoxy radical ($\bullet\text{O}^t\text{Bu}$) were generated by reaction of 1 mM Fe^{2+} with 200 μM H_2O_2 or TBHP, respectively. Nitric oxide (NO) was generated from proliNNOate (stock solution 70 mM in 0.1M NaOH, the concentration of intact compound was determined from its characteristic UV absorption at 254 nm ($\epsilon = 8,400 \text{ M}^{-1} \text{ cm}^{-1}$). Superoxide (O_2^-) was delivered from the enzymatic reaction of xanthine oxidase (0.02 unit/mL) and hypoxanthine (1 mM in phosphate buffer pH 7.8) in the presence of 5 units catalase as a scavenger for any H_2O_2 . The rate of O_2^- production observed from the reduction of cytochrome c was 1.5 $\mu\text{M}/\text{min}$. Singlet oxygen was generated from the thermodissociable endoperoxide of disodium 3,3'-(1,4-naphthalene)bispropionate (400 μM in phosphate buffer pH 7.4 at 37 °C).^[4]

Measurement of Two-Photon Cross Sections. The two-photon cross section (δ) of dyes were determined by using a femto second (fs) fluorescence measurement technique as described.^[5] PN1 and AN1 were dissolved in 20 mM HEPES buffer (pH 7.1) at concentrations of $2.0 \times 10^{-6} \text{ M}$ (PN1) and $2.0 \times 10^{-6} \text{ M}$ (AN1) and then the two-photon induced fluorescence intensity was measured at 720–940 nm by using Rhodamine 6G in MeOH as the reference, whose two-photon properties have been well characterized in the literature.^[6] 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 by using $\delta = \delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$, 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.

Cell Culture and Imaging. Raw 264.7 cells were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and glutamine (2 mM). One day before imaging, cells were passaged and plated on 24 well plates with coverslips coated with poly(lysine) at a density of $2 \times 10^5/\text{well}$. For labeling, the growth medium was removed and replaced with DPBS containing calcium and magnesium and 5 μM PN1 or AN1. Cells were incubated with the staining solution for 30 min. The coverslips of cells loaded with dye were transferred into a fresh DPBS solution. Hydrogen peroxide was delivered from a 100 mM stock solution in MilliQ water. PMA was added from stock solution of 1 mg/mL in DMSO. For proliferation assays, one day before experiments, Raw 264.7 cells were passaged and plated on 96 well plates at a density of $3 \times 10^4/\text{well}$. Cells were incubated with WST-1 solution in DMEM containing DMSO, AN1, or PN1 at indicated concentrations for 2 h at 37 °C with 5% CO_2 . The formation of the formazan product was measured by absorption at 440 nm using SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). AN1 and PN1 in DMEM have no background absorption at 440 nm.

Two-photon fluorescence microscopy. Confocal fluorescence imaging of Raw 264.7 cells was performed with a Zeiss 510 Axioimager laser scanning microscope and 40x Acroplan water-immersion objective lens. Excitations of AN1 and PN1 were carried out with a MaiTai two-photon laser (mode-locked Ti:sapphire laser, Spectra-Physics, 80 MHz, 80 fs) using 750 nm pulses and output power 2400 mW. Lambda mode scanning was acquired with META spectral detector with 10.7 nm steps. Data were analyzed using ImageJ software (NIH) and Ratio Plus plugin (programmer: Paulo Magalhes).

Two-photon (TP) fluorescence microscopy images of probe-labeled tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP2) with 100x (NA = 1.30 OIL) and 20x (NA = 0.30 DRY) objective lens. The TP fluorescence microscopy images were obtained using 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 750 nm and output power 1230 mW, which corresponded to approximately 10 mW average power in the focal plane. To obtain images at 390–465 nm and 500–550 nm range, internal PMTs were used to collect the signals in an 8 bit unsigned 512 × 512 pixels at 400 Hz scan speed.

Preparation and staining of fresh rat Hippocampal slices. Rat Hippocampal slices were prepared from the hippocampi of 2-days-old rat (SD) according to an approved institutional review board protocol. Coronal slices were cut into 400 µm-thick using a vibrating-blade microtome in artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 3 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 10 mM D-glucose, 2.4 mM CaCl₂, and 1.3 mM MgSO₄). Slices were incubated with 20 µM PN1 in ACSF bubbled with 95% O₂ and 5% CO₂ for 2 hr at 37 °C. Slices were then washed three times with ACSF and transferred to glass-bottomed dishes (MatTek) and observed in a spectral confocal multiphoton microscope.

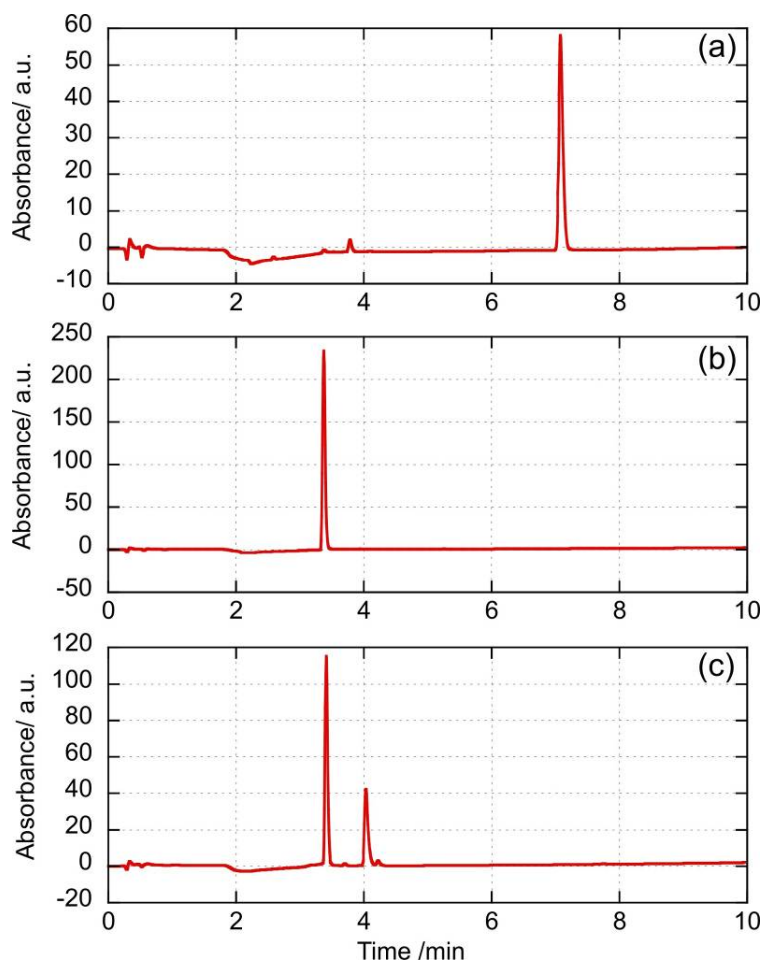


Figure S1. LC/MS traces monitored by UV-Vis absorbance at 350 nm of (a) PN1, the 7.08 min peak corresponds to the positive ion $[MH^+] = 446$. (b) AN1, the 3.37 min peak corresponds to the positive ion $[MH^+] = 186$. (c) The reaction between PN1 (50 μ M in PBS pH 7.4) and H_2O_2 (20 mM) after 60 min at room temperature: the 3.41 min peak corresponds to the positive ion $[MH^+] = 186$, confirming that AN1 is the major product of the reaction. The additional 4.03 min peak corresponds to the 4-hydroxy benzyl carbamate intermediate.

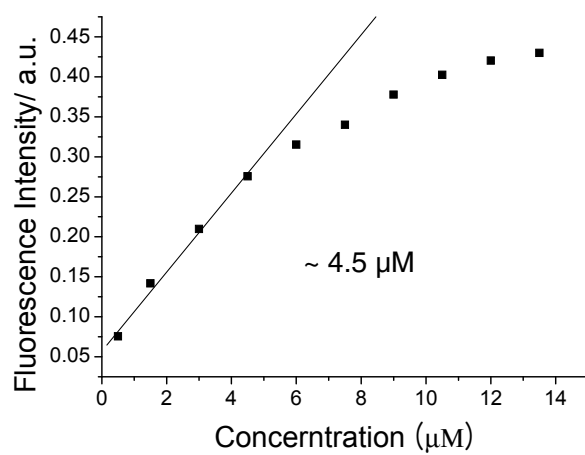


Figure S2. Plot of fluorescence intensity against PN1 concentration in HEPES buffer (20 mM HEPES, pH 7.1).

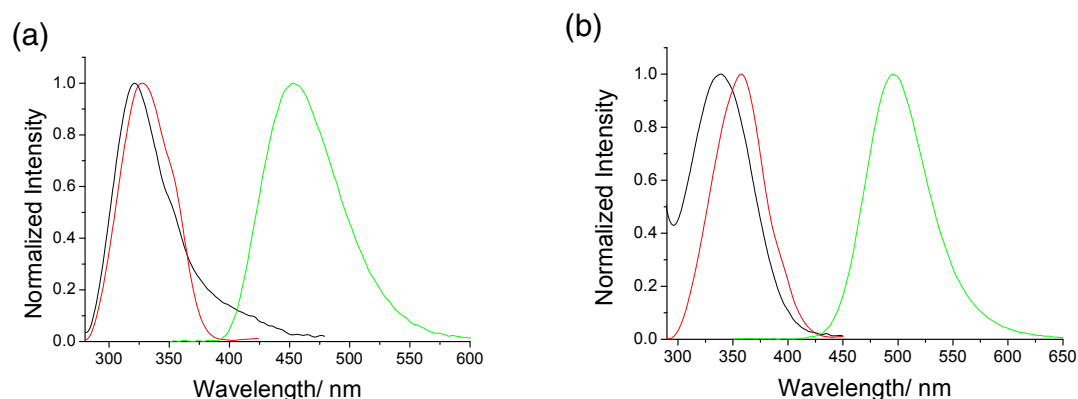


Figure S3. Normalized absorption (black), excitation (red), and fluorescence (green) spectra for (a) PN1 and (b) AN1 in HEPES buffer ([HEPES] = 20 mM, pH 7.1). The absorption (λ_{abs}), excitation (λ_{exc}), and fluorescence maxima (λ_{fl}) of PN1 and AN1 are as follows: PN1, $\lambda_{\text{abs}} = 321$ nm ($\epsilon = 12,200$ M⁻¹ s⁻¹), $\lambda_{\text{exc}} = 328$ nm, $\lambda_{\text{fl}} = 453$ ($\Phi = 0.70$); AN1, $\lambda_{\text{abs}} = 338$ nm ($\epsilon = 13,200$ M⁻¹ s⁻¹), $\lambda_{\text{exc}} = 358$ nm, $\lambda_{\text{fl}} = 495$ nm ($\Phi = 0.40$).

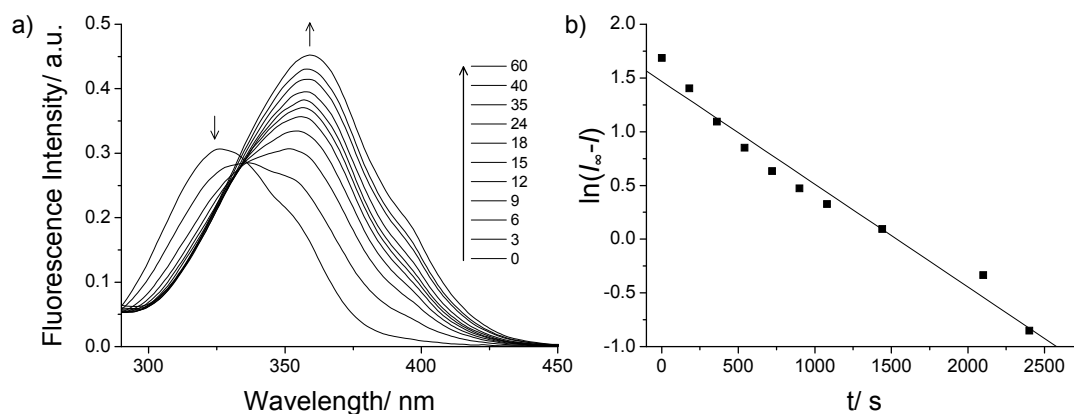


Figure S4. (a) The change in the excitation spectra of PN1 (3 μM) with time after addition of 1 mM H₂O₂ in HEPES buffer (20 mM HEPES, pH 7.1) at 25 °C. (b) Plot of $\ln(I_{\infty} - I)$ vs time at 358 nm, where I_{∞} and I are the fluorescence intensities at 358 ± 10 nm at t_{∞} (= 60 min) and t , respectively. The k_{obs} calculated from the slope of this plot is 9.5×10^{-4} s⁻¹.

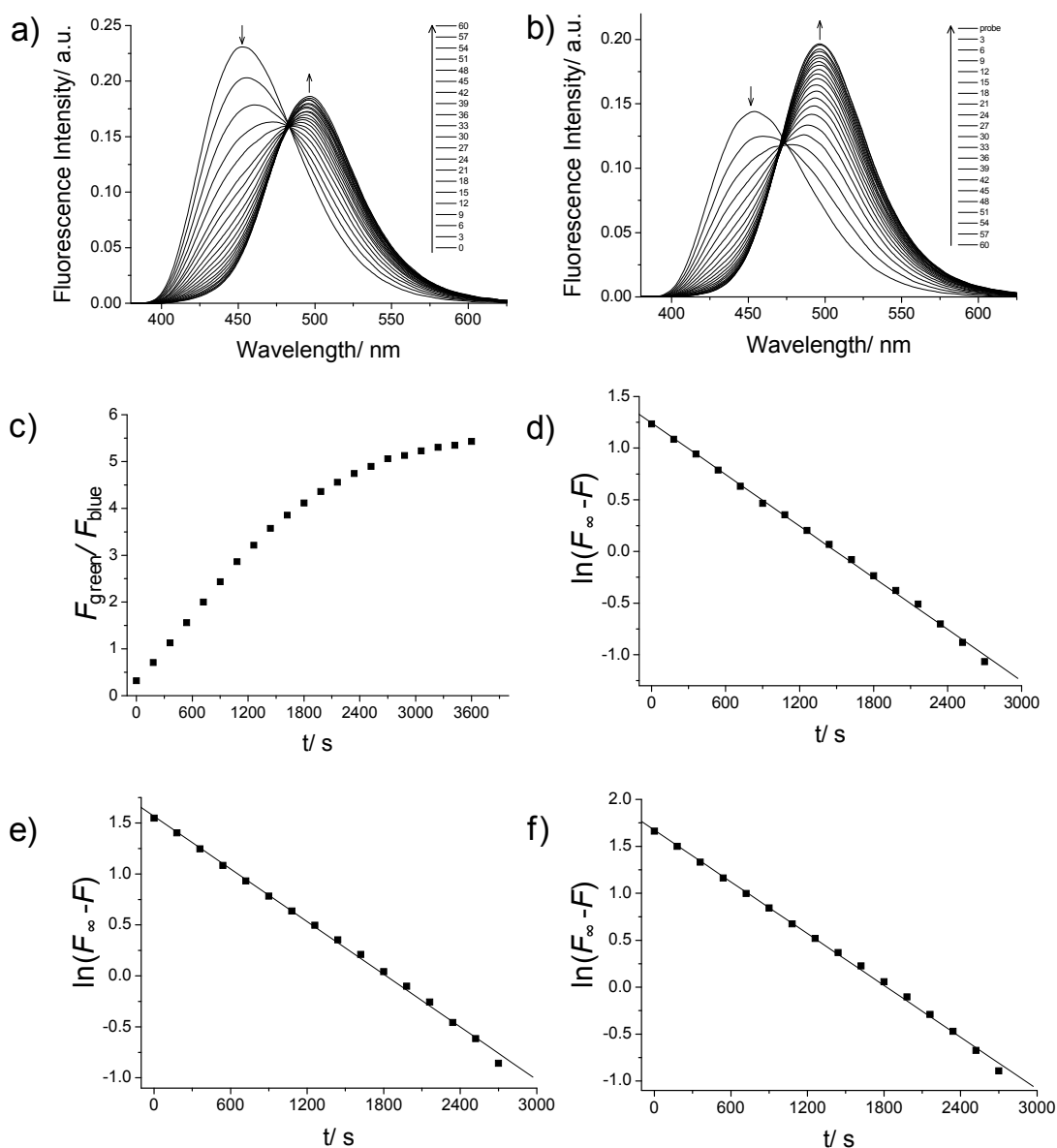


Figure S5. Fluorescence responses of 3 μM PN1 to 1 mM H_2O_2 with excitation at (a) 350 and (b) 360, respectively. (c) The changes in the emission intensity ratios ($F_{\text{green}}/F_{\text{blue}}$, green = 500–550 nm, blue = 390–465 nm) for the reaction of 3 μM PN1 with 1 mM H_2O_2 upon excitation at 370 nm. The $F_{\text{green}}/F_{\text{blue}}$ ratio increases by 17-fold after complete conversion to AN1. The $k_{\text{obs}} = 8.3 \times 10^{-4}$, 8.6×10^{-4} , and $9.2 \times 10^{-4} \text{ s}^{-1}$ were obtained from plot of $\ln(F_{\infty} - F)$ vs time using excitation wavelength (d) 350 nm, (e) 360 nm, and (f) 370 nm. F_{∞} and F are $F_{\text{green}}/F_{\text{blue}}$ at time t_{∞} (= 60 min) and t , respectively.

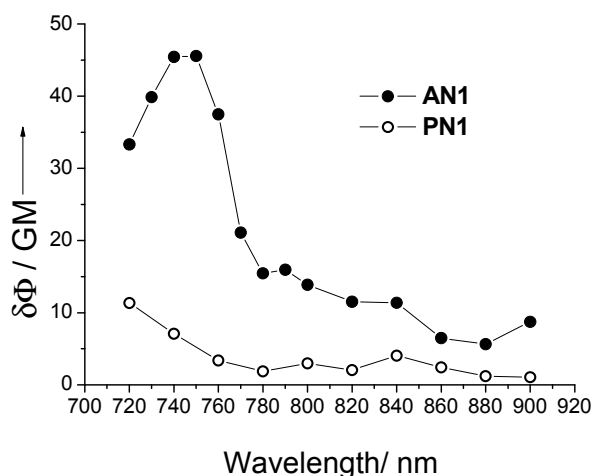


Figure S6. Two-photon action spectrum of 2 μM PN1 and 2 μM AN1 in HEPES buffer ([HEPES] = 20 mM, pH 7.1). The estimated uncertainties for the two-photon action cross section values ($\delta\Phi$) are $\pm 15\%$.

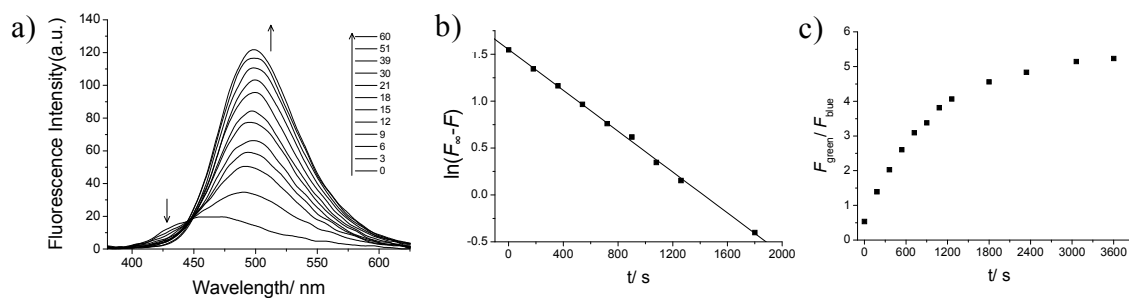


Figure S7. (a) Two-photon fluorescence response of 3 μM PN1 to 1 mM H_2O_2 with excitation at 750 nm. (b) Plot of $\ln(F_\infty - F)$ vs time at 750 nm, where F_∞ and F are $F_{\text{green}}/F_{\text{blue}}$ at time t_∞ (= 60 min) and t , respectively. The k_{obs} calculated from the slope of this plot is $1.0 \times 10^{-3} \text{ s}^{-1}$. (c) The changes in the emission intensity ratios ($F_{\text{green}}/F_{\text{blue}}$, green = 500–550 nm, blue = 390–465 nm) for the reaction of PN1 (3 μM) with H_2O_2 (1 mM) upon excitation at 750 nm. The $F_{\text{green}}/F_{\text{blue}}$ increased by 10-fold after complete conversion to AN1.

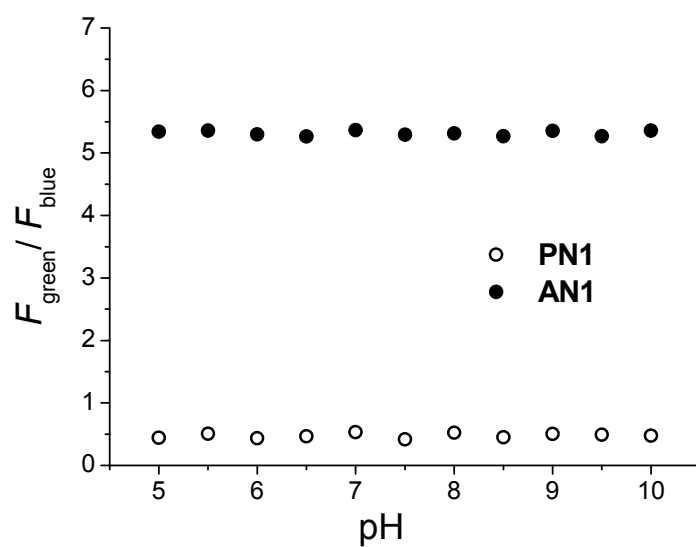


Figure S8. Effect of pH on the $F_{\text{green}}/F_{\text{blue}}$ ratios for PN1 (○) and AN1 (●) in HEPES buffer (20 mM HEPES, pH 7.1). The excitation wavelength was 370 nm.

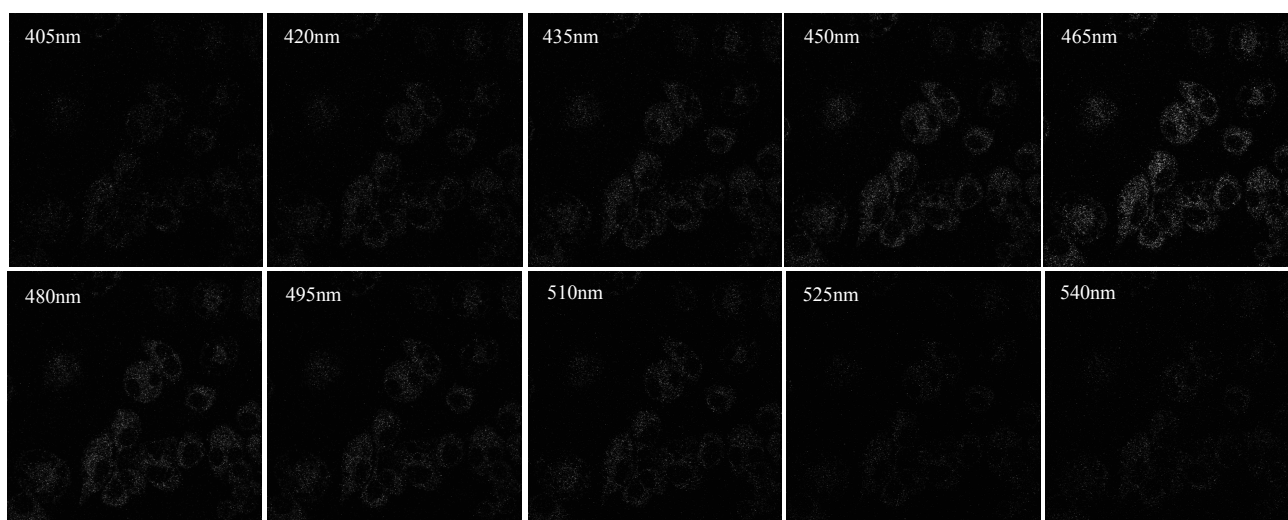


Figure S9. Two-photon confocal microscopy of PN1-labeled Raw 264.7 cells in lambda mode scanning using 750 nm excitation.

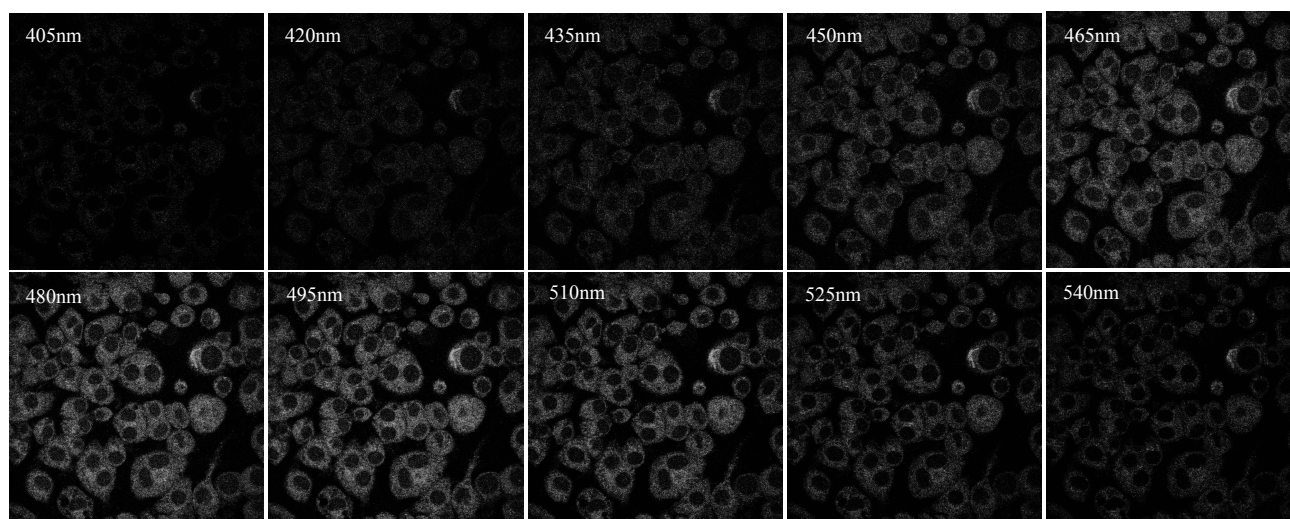


Figure S10. Two-photon confocal microscopy of AN1-labeled Raw 264.7 cells in lambda mode scanning using 750 nm excitation.

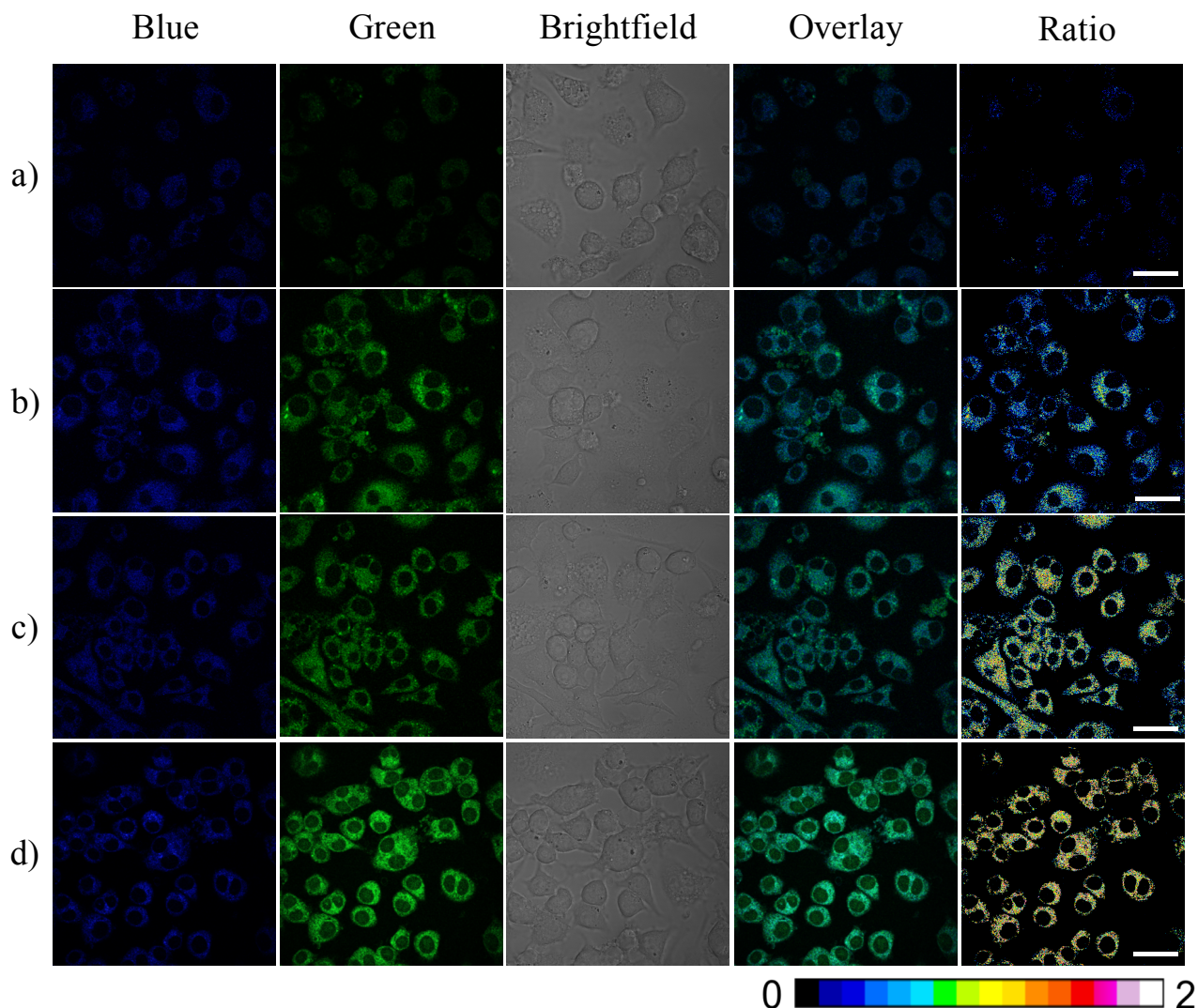


Figure S11. Two-photon confocal microscopy of Raw 264.7 cells after 30 min incubation with (a) PN1 and d) AN1. (b,c) Cells were pretreated with 1 μ L PMA (1 μ g/mL) (b) and 100 μ M H_2O_2 (d) for 30 min before labeling with PN1. The ratio of green to blue ($F_{\text{green}}/F_{\text{blue}}$) images are shown in pseudocolor ratiometric image mode with 16 color look-up tables, which represent the ratio from 0 to 2. Images were acquired using 750 nm excitation and fluorescent emission windows: Blue = 390-465 nm, Green = 500-550 nm. Scale bar = 30 μ m.

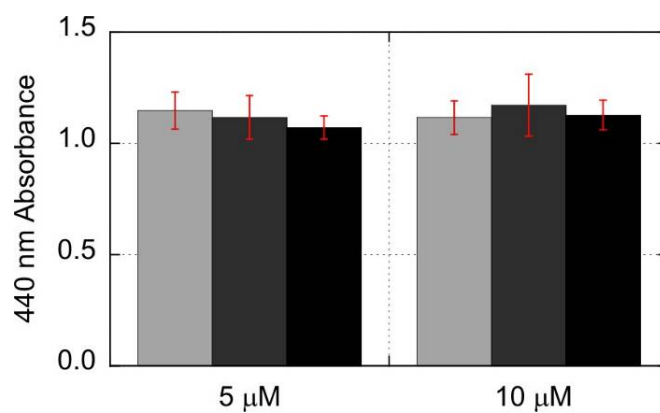


Figure S12. WST-1 viability assays of Raw 264.7 cells after incubation with 5-10 μM AN1 (dark grey) and PN1 (black) in DMEM for 2 h. An equal volume of DMSO was added to DMEM as a control (light grey). Error bars represent standard deviation of four measurements.

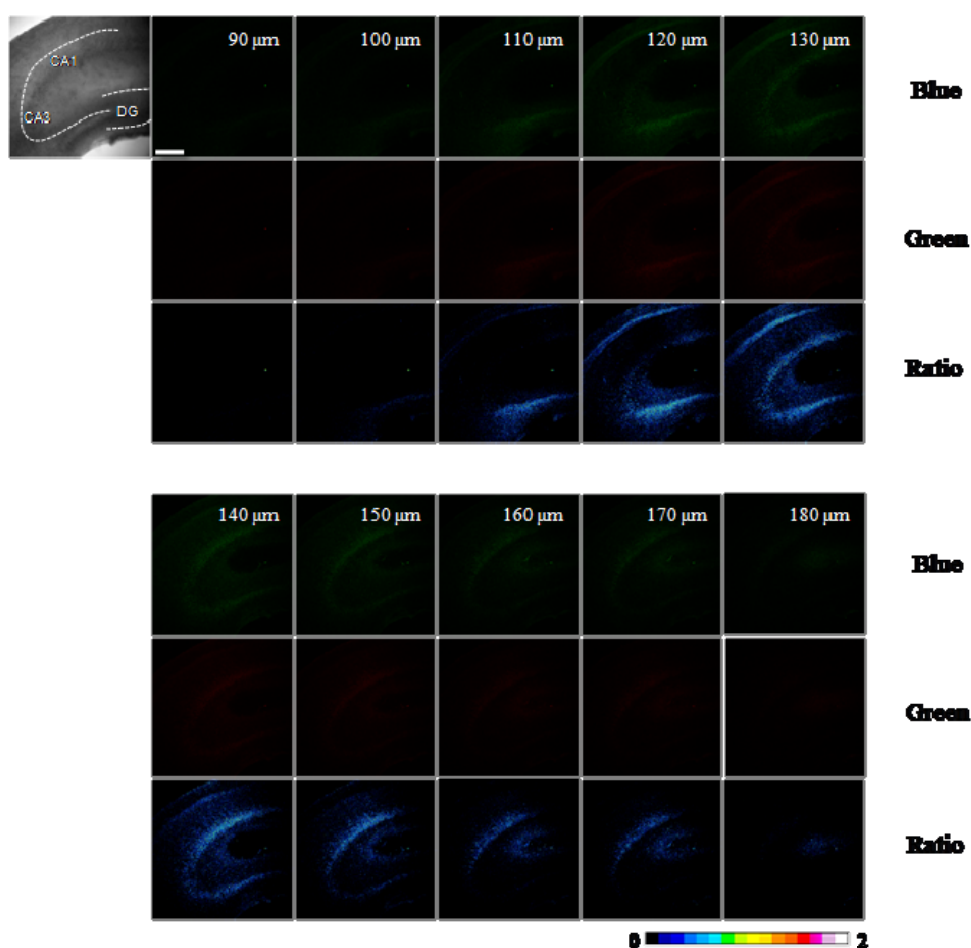


Figure S13. TPM images of a fresh rat hippocampal slice stained with 20 μM PN1. The TPEF were collected at 390-465 (blue) and 500-550 nm (green) upon excitation at 750 nm with fs pulses. The images were taken at depths of 90-180 μm with magnification at 10 $\times$. The ratio represents the ratiometric images constructed from the images in the blue and green rows. Scale bar = 300 μm .

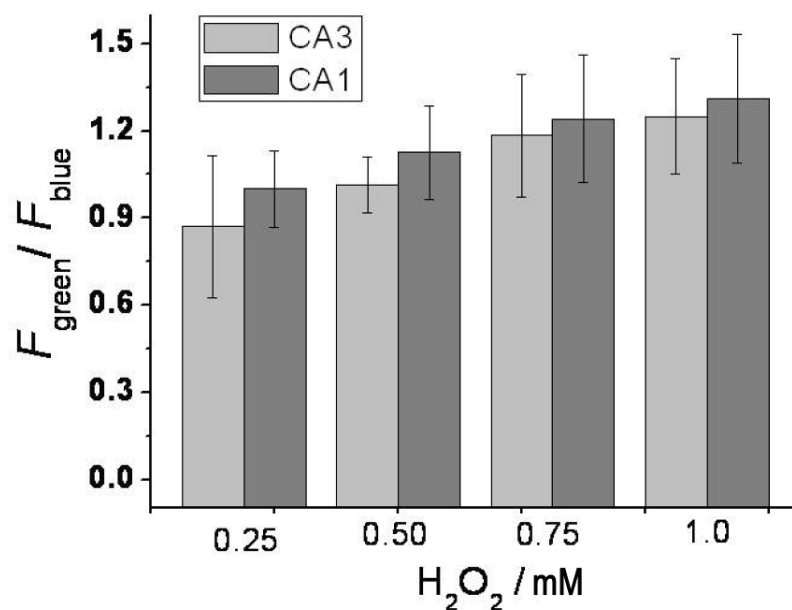


Figure S14. Average $F_{\text{green}}/F_{\text{blue}}$ in the CA3 and CA1 regions of a fresh rat hippocampal slice pretreated with 0.25, 0.50, 0.75, and 1.0 mM H_2O_2 for 30 min before labeling with 20 μM PN1. The TPEF were collected at two channels (Blue = 390-465 nm, Green = 500-550 nm) upon excitation at 750 nm with fs pulse.

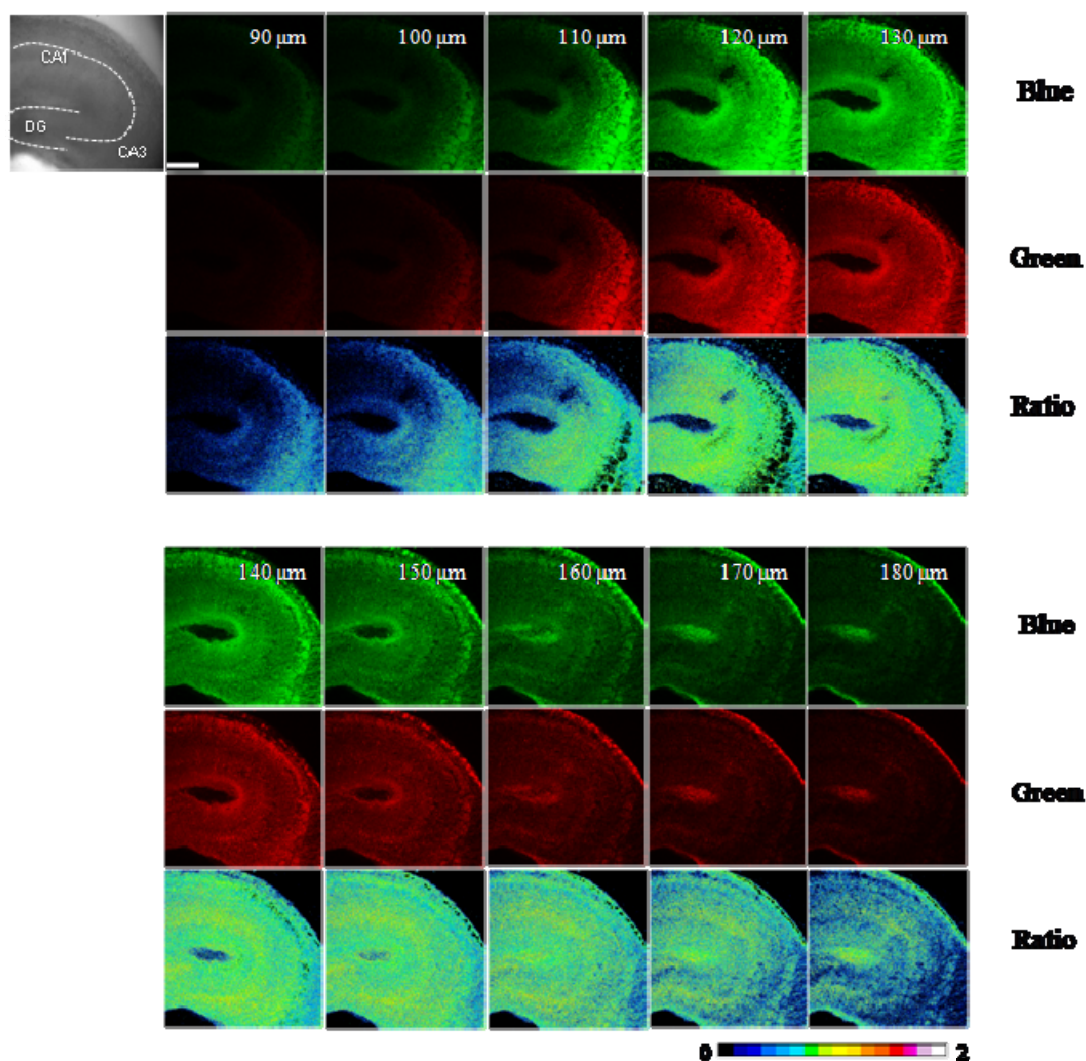


Figure S15. TPM images of a fresh rat hippocampal slice stained with 20 μM PN1 after treatment with 1 mM H_2O_2 for 30 min. The TPEF were collected at 390-465 (blue) and 500-550 nm (green) upon excitation at 750 nm with fs pulses. The images were taken at depths of 90-180 μm with magnification at 10 $\times$. The ratio represents the ratiometric images constructed from the images in the blue and green rows. Scale bar = 300 μm .

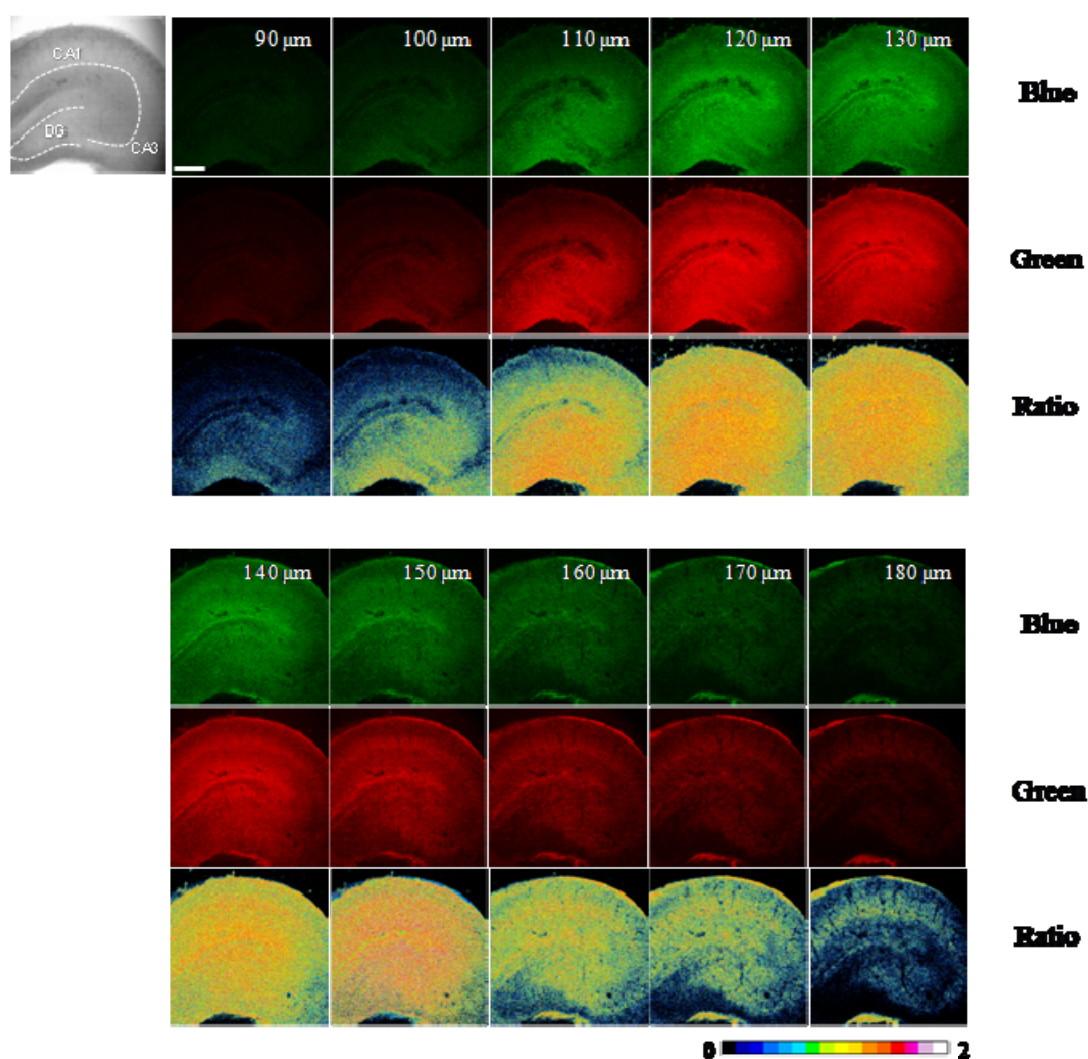


Figure S16. TPM images of a fresh rat hippocampal slice stained with 20 μM AN1. The TPEF were collected at 390-465 (blue) and 500-550 nm (green) upon excitation at 750 nm with fs pulses. The images were taken at depths of 90-180 μm with magnification at 10 $\times$. The bottom row represents the ratiometric images constructed from the images in the blue and green rows. Scale bar = 300 μm .

References

- 1 H. M. Kim, B. R. Kim, M. J. An, J. H. Hong, K. J. Lee, B. R. Cho, *Chem. Eur. J.* 2008, **14**, 2075-2083.
- 2 A. de Filippis, C. Marin, C. Thimon, *Syn Commun.* 2002, **32**, 2669-2676.
- 3 J. N. Demas, G. A. Crosby, *J. Phys. Chem.* 1971, **75**, 991-1024
- 4 D. Costa, E. Fernandes, J. L. M. Santos, D. C. G. A. Pinto, A. M. S. Silva, J. L. F. C. Lima, *Anal. Bioanal. Chem.* 2007, **387**, 2071-2081.
- 5 K. Lee, W. J. Tang, J. J. Choi, C. H. Kim, S. J. Jeon, B. R. Cho, *Org. Lett.* 2005, **7**, 323-326

- 6 Xu, W. W. Webb, *J. Opt. Soc. Am. B.* 1996, **13**, 481-491