Electronic Supplementary Information (ESI)

Reversible two-photon fluorescent probe for imaging of hypochlorous acid in live cells and in vivo

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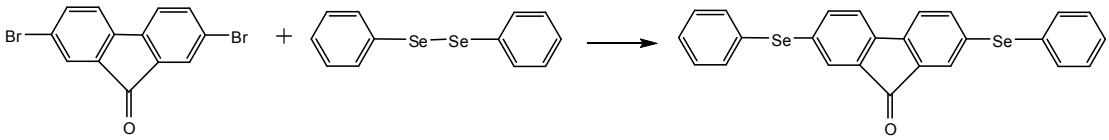
Materials. All chemicals were available commercially and the solvents were purified by conventional methods before use. 2,7-Dibromo-9H-fluoren-9-one, Diphenyl diselenide were purchased from sun Chemical Technology(shanghai) Co., Ltd. DL-Dithiothreitol,1,8-Diazabicyclo[5.4.0]undec-7-ene were purchased from Aladdin Industrial. Reactive oxygen species were as follows. Hypochlorite (NaOCl), H_2O_2, tert-butylhydroperoxide (TBHP), and were delivered from 10%, 30% and 70% aqueous solutions respectively. Hydroxyl radical (•OH) was generated by reaction of 1 mM Fe^{2+} with 200 μM H_2O_2. Nitric oxide (NO) was used from stock solution prepared by sodium nitroprusside. Singlet oxygen ($^1O_2$) was prepared by the ClO^-/H_2O_2 system. Superoxide ($O_2^{\cdot-}$) was delivered from KO_2 in DMSO solution. Peroxynitrite was used from stock solution 10 mM in 0.3 M NaOH.

Instruments. ^{1}HNMR spectra were recorded with Bruker NMR spectrometers at 300 MHz and JOEL JNM-ECA600.The mass spectra were obtained by Bruker maXis ultra high resolution TOF MS system. The one-photon excited fluorescence spectra measurements were performed using FLS-920 Edinburgh fluorescence spectrometer. Two-photon excited fluorescence spectra were measured using a Tsunami 3941-M3-BB: Ti: sapphire femtosecond laser as exciting light source (800 nm) with a pulse width of <150 fs and a repetition rate of 80 MHz, and USB2000 (bought from Ocean Optics Inc.) was employed as the recorder. The one-photon confocal fluorescent images were measured on a Leica TCS SP5, confocal lasers canning microscope with an objective lens (×40). The

excitation wavelength was 405 nm (5 mW). Two-photon confocal fluorescent images were measured on Leica Microsystems. UV/Vis spectra were recorded on TU-1900 UV/Vis spectrometer.

Synthesis of 2,7-bis(phenylselanyl)-9H-fluoren-9-one (FO-PSe).



Scheme S1 Synthesis of probe FO-PSe

Diphenyl diselenide (374 mg, 1.2mmol) was added to a solution of DTT (308mg, 2.0 mmol) in anhydrous DMF (8 mL) under Ar atmosphere. After stirring at 85 °C for 30 min, 2,7-dibromo-9H-fluoren-9-one (135 mg, 0.4 mmol) was added to the reaction mixture and the mixture was stirred for 20 min. Then, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 0.75 mL, 5 mmol) was added and the mixture was stirred for further 36 h. The solvent was removed under reduced pressure and the residue was extracted with CH_2Cl_2 and saturated sodium dithionite aqueous solution. The organic layer was dried over anhydrous $MgSO_4$. The crude product was purified by column chromatography (Petroleum ether: Dichloromethane = 8:1) to give the compound as a orange red solid. Yield: 142 mg (72%). ^{1}H NMR (400 MHz, CDCl3) δ 7.594 (s, 2H; Ar H), 7.528 (d, 2H; Ar H), 7.456(d, 2H; Ar H), 7.180−7.248 (m, 10H; Ar H). ^{13}C NMR (100 MHz, $CDCl_3$) δ

191.54, 141.72, 137.18, 132.77, 132.53, 130.59, 128.60, 127.17, 127.06, 126.69, 119.94. ^{77}Se NMR (150 MHz, $CDCl_3$): δ 431.4. MS data, m/z: 492.9619 (M+H)

MTT Assay. RAW.264.7 cells (10^6 cell mL^{-1}) were dispersed within replicate 96-well microtiter plates to a total volume of 200 μL $well^{-1}$. Plates were maintained at 37 °C in a 5% CO_2/95% air incubator for 24 h. Then RAW.264.7 cells were incubated for 12 h upon different probe concentrations of 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M. MTT solution (5 mg mL^{-1}, HEPES) was then added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in a Triturus microplate reader.

Cell, zebrafish and mice culture.[S1,S2] RAW.264.7 cells were cultured in DMEM containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37 °C (w/v) in a 5% CO_2/95% air incubator MCO-15AC (Sanyo, Tokyo, Japan). The concentrations of counted cells were adjusted to 1 × 10^6 cells mL^{-1} for confocal imaging in high-glucose DMEM (4.5 g of glucose/L) supplemented with 10% fetal bovine serum (FBS), $NaHCO_3$ (2 ng/L), and 1% antibiotics (penicillin/streptomycin, 100 U/mL). Cultures were maintained at 37 °C under a humidified atmosphere containing 5% CO_2.

Wild type zebrafish was from Shandong Academy of Sciences. Seven days post fertilization zebrafish was incubated in 20 μM probe for 0.5 h and then imaged using confocal fluorescence microscopy. The zebrafish in treatment were placed in confocal

culture dish.

Eight- to ten-week-old wild-type BalB/C mice (male) were used. The mice were anesthetized with 4% chloral hydrate (3 mL/kg) by intraperitoneal injection. The mice were then imaged by using a Leica TCS MP5 in vivo imaging system. The mice were obtained from Shandong University Laboratory Animal Center. The experiments were approved by the institutional committee. All the animal experiments were carried out in accordance with the relevant laws and guidelines issued by the Ethical Committee of Shandong University.

Confocal imaging and two-photon fluorescence imaging. One-photon fluorescent images were acquired on a Leica TCS SP5 confocal laser-scanning microscope with an objective lens (×40). The excitation wavelength was 405 nm (5 mW), and 488 nm (15 mW) respectively. Following incubation, the cells were washed three times with DMEM without FBS and imaged.

The two-photon imaging of cells and zebrafish were obtained with Olympus FV1000MPE with a 60 × water objective and the two-photon imaging of mouse was obtained with Leica TCS MP5 with a 25× water objective. All the Ti: sapphire laser was used to excite the specimen at 770 nm and transmissivity was 6%.

Measurement of two-photon cross section.[S3,S4] The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique. The two-photon induced fluorescence intensity was measured at 800 nm by using

fluorescein ($1\times\ 10^{-5}$ M, pH 11) as the reference, whose two-photon property has been well characterized in the literature. 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).

$$\delta_s = \delta_r \frac{\Phi_r}{\Phi_s}\frac{C_r}{C_s}\frac{n_r}{n_s}\frac{F_s}{F_r} \qquad (1)$$

The subscripts s and r is the sample and the reference material, respectively. δ is the TPA cross sectional value, C is the concentration of the solution, n is there refractive index of the solution, F is two-photon excited fluorescence integral intensity and Φ is the fluorescence quantum yield. Here the fluorescence quantum yield of FO-PSe and FO-PSeO is 0.002 and 0.105, respectively, using fluorescein as the reference. The results showed that the two-photon cross section of FO-PSeO in HEPES at pH 7.4 is 78 GM.

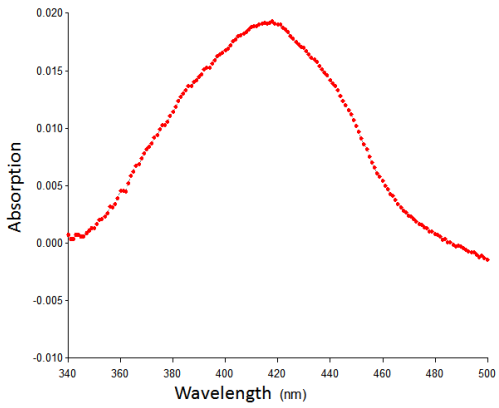


Fig. S1 Absorption spectra of FO-PSe in HEPES (0.015 M) solution (DMSO/water = 1:9 v/v, pH 7.4).

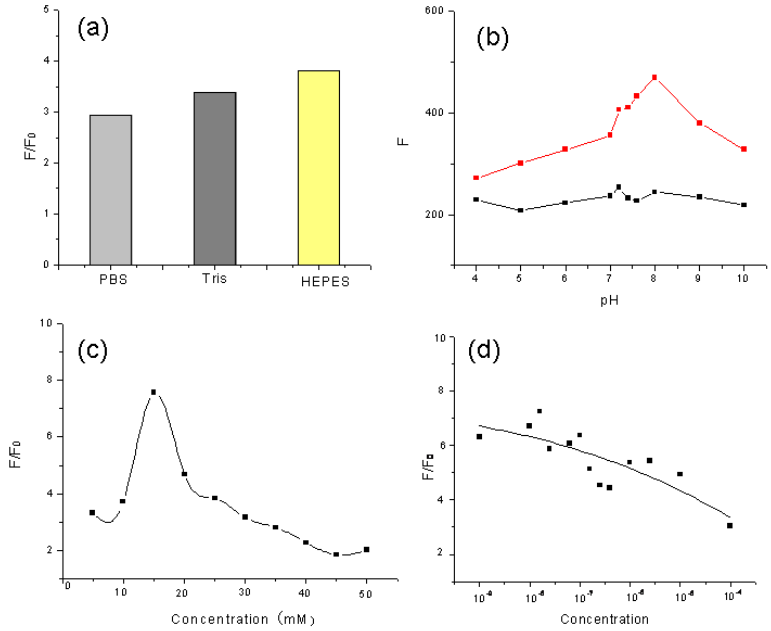


Fig. S2 Conditions optimization experiment. (a) The effect of different kinds of buffer solution. (b) Effect of pH. (c) The effect of buffer solution concentration. (d) The influence of probe concentration on the performance of the FO-PSe.

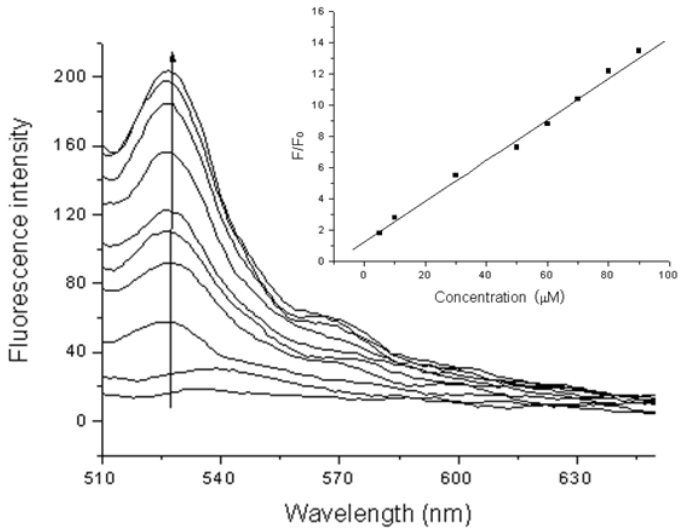


Fig. S3 One-photon fluorescence spectra of 10 μM FO-PSe after adding various concentrations of HOCl (0-100 μM) in HEPES (0.015 M) solution (DMSO/water = 1:9 v/v, pH 7.4). λ_{em} = 415 nm. Inset: A linear correlation between the fluorescence

intensity and HOCl concentrations.

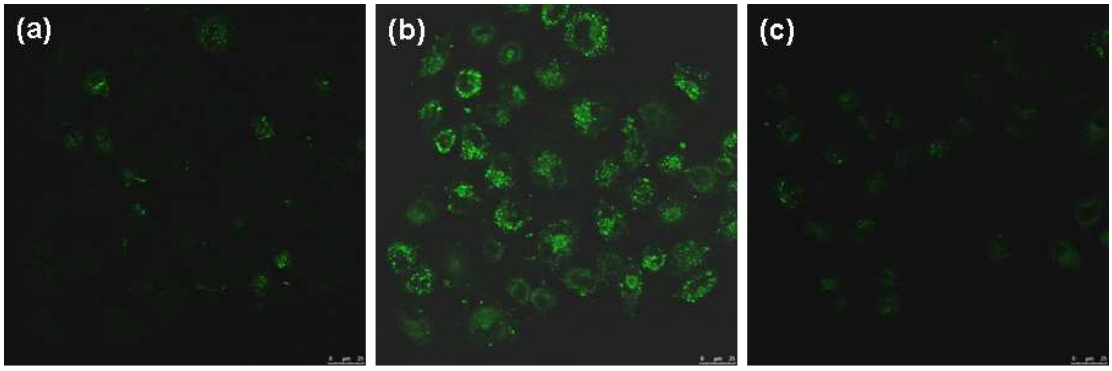


Fig. S4 The reversibility of FO-PSe (10.0 μM). (a) Fluorescent images of probe-loaded cells. (b) Fluorescent images of probe-loaded cells stimulated with PMA. (c) Fluorescent images of probe-loaded stimulated cells treated with 2 equiv of the GSH.

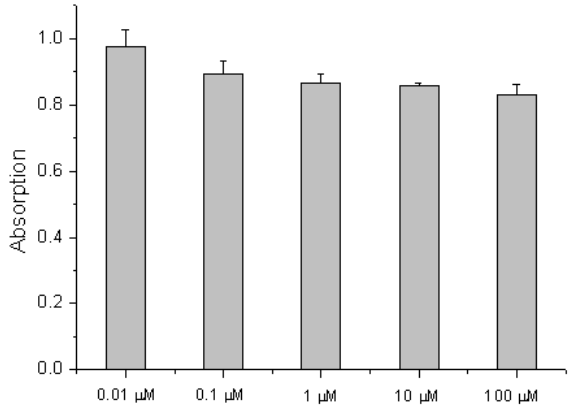


Fig. S5 Viability of RAW.264.7 cells in the presence of probe as measured by using MTT assay. The cells were incubated with probe for 12 h.

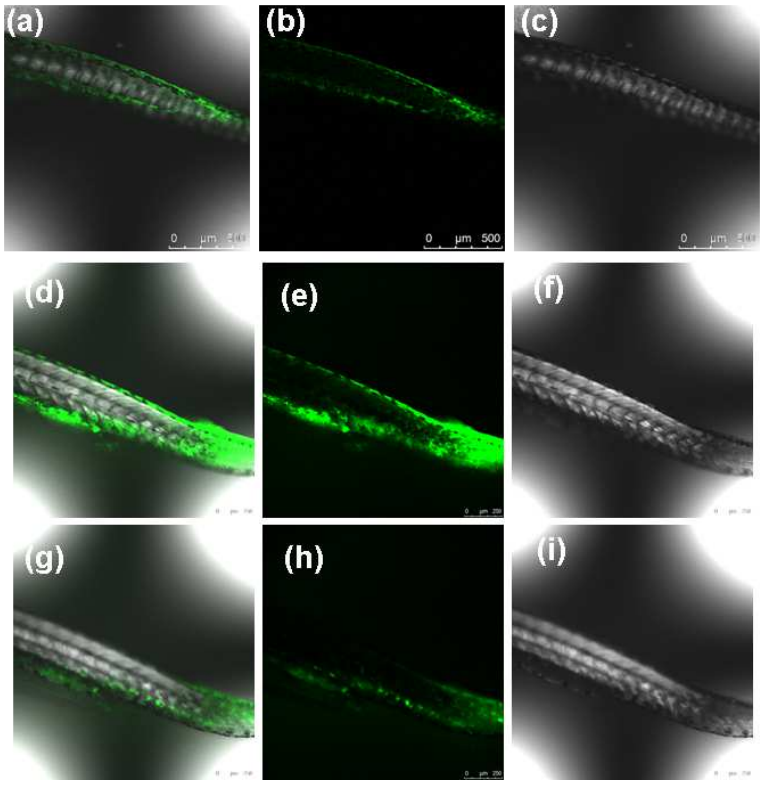


Fig. S6 Fluorescent images of HOCl levels in zebrafish. (a) OP fluorescent images of zebrafish and the overlay of darkfield and brightfield. (b) darkfield of zebrafish fluorescent images. (c) brightfield of zebrafish fluorescent images; (d) Fluorescent images of zebrafish by adding HOCl and the overlay of darkfield and brightfield. (e) darkfield. (f) brightfield; (g) Fluorescent images of the stimulated zebrafish treated with Vc the overlay of darkfield and brightfield. (h) darkfield. (i) brightfield.

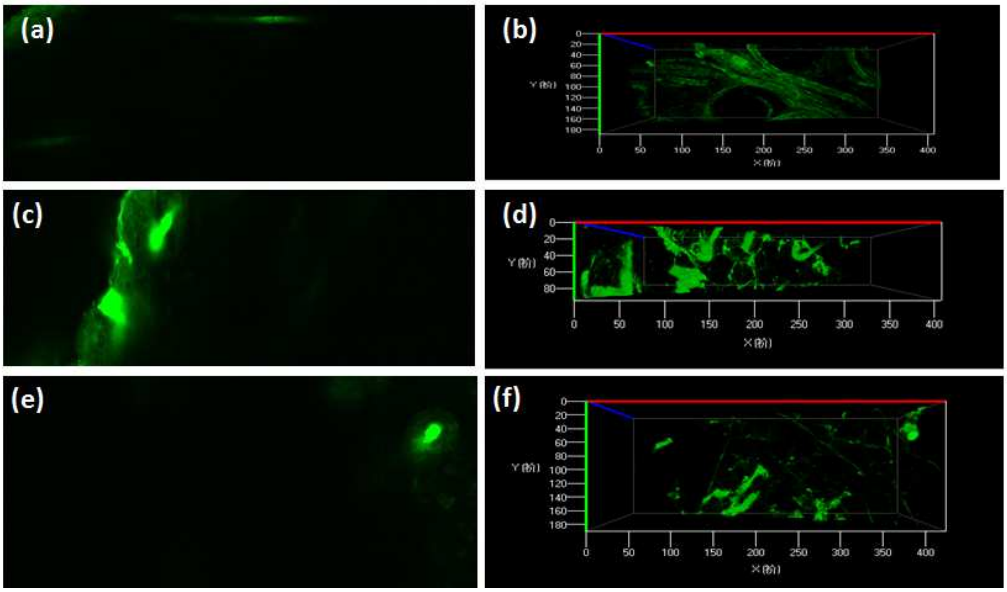


Fig. S7 TP fluorescent images of HOCl levels in the abdomen of mice. (a) Fluorescent images of probe-loaded mice. (b) Fluorescent images of the 3D distribution for HOCl in mice. (c) Fluorescent images of probe-loaded mice stimulated with zymosan. (d) Fluorescent images of the 3D distribution for HOCl in mice stimulated with zymosan. (e) Fluorescent images probe-loaded stimulated mice treated with Vc. (f) Fluorescent images of the 3D distribution for HOCl in stimulated mice treated with Vc. Images were acquired using 800 nm TP excitation. TP fluorescent emission windows: 500-550 nm.

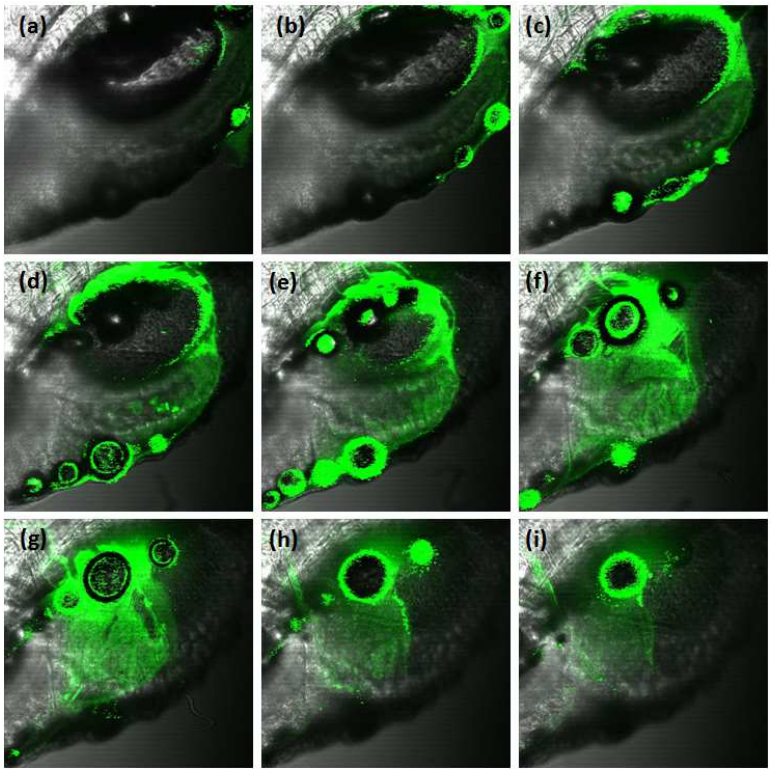


Fig. S8 The changes of HOCl levels at different depth in zebrafish (0-288 μm). TP fluorescence images of zebrafish with FO-PSe (20 μM). Images were acquired using 800 nm TP excitation. TP fluorescent emission windows: 500-550 nm.

Reference

(S1) R. Liu, L. Zhang, X. Lan, L. Li, T. T. Zhang, J. H. Sun and G. H.Du, *Neuroscience.,* 2011, **10**, 408.

(S2) S. Thiberge, S. Blazquez, P. Baldacci, O. Renaud, S. Shorte, R. Ménard and R. Amino, *Nature protocols*., 2007, **2**, 1811.

(S3) C. Xu and W. W.Webb, *J. Opt. Soc. Am. B*., 1996, **13**, 481.

(S4) J. R. Lakowicz, Principles of Fluorescence Spectroscopy, 2nd Ed. Kluwer Academic/Plenum Publishers, New York, London, Moscow, Dordrecht, **1999**.