Supplementary Information

Fluorogenic Nanoreactor Assembly with Boosted Sensing Kinetics for Timely Imaging of Cellular Hydrogen Peroxide

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Materials and Methods. Pentafluorobenzene-1-sulfonyl chloride and 2,4,6-trimethylbenzoic acid were purchased from TCI. Diethyl 2,5-dihydroxylterephthalate and all other chemicals were purchased from Sigma-Aldrich and were used as received. We followed the MSDS guidance for handling 1-phenl yimidazole, to prevent eye/skin exposure, swallowing and inhaling. $^{1}H/^{13}C$ NMR spectra were collected in $CDCl_3$ at 25 ^{o}C on a Bruker AV-300 spectrometer. Chemical shifts are reported in the standard δ notation of parts per million using the peak of residual proton signals of $CDCl_3$ as an internal reference. Elemental analysis was performed on a CHNS-O Analyzer (EA 1180, FISONS Instruments). The mass spectrum of the final compound was obtained from the fast atom bombardment (FAB) ionization mode with m-NBA matrix (JEOL JMS-700). Absorption and fluorescence spectra were acquired using a UV-visible spectrometer (Agilent 8453) and a fluorescence spectrophotometer (Hitachi F-7000, wavelength calibrated for excitation and emission), respectively. The fluorescence quantum yields were determined using ethanol solution of rodamine B ($\Phi f = 0.6$) as a reference. Particle size distribution of nanoparticle dispersions in deionized water was determined at 25 ° C using a particle sizer and a zeta-potential analyzer (90 Plus and Zeta PALS, Brookhaven Instruments Corporation). Transmission electron microscopic (TEM) image of negatively stained particles (with 2 wt% uranyl acetate) was obtained with a CM30 electron microscope (FEI/Philips) operated at 200 kV.

Synthesis of Diethyl 2,5-Bis[(pentafluorophenylsulfonyl)oxy]terephthalate (pFS-DT). To a solution of diethyl 2,5-dihydroxylterephthalate (0.87 mmol) and triethylamine (2.4 mmol) in anhydrous THF (20 mL) was added pentafluorobenzenesulfonyl chloride (2.1 mmol). The reaction mixture was stirred at room temperature overnight under a dry nitrogen atomosphere. The mixture was filtered and concentrated in vacuum. pFS-DT was isolated as a white powder (0.5 g, 90 %) by fresh column chromatography on silica gel ($CHCl_3$:Hexane = 1:10; $R_f = 0.4$). ^{1}H NMR ($CDCl_3$, 300 MHz, ppm): δ 1.42 (t, J = 3.5 Hz, 6H), 4.38 (d, J = 3.6 Hz, 4H), 7.88 (s, phenyl, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz, ppm): 13.88, 62.81,127.29, 129.41, 136.07-136.54, 139.48-139.93, 143.47-143.96, 145.47, 146.96-

147.48, 161.69. Anal. Calcd for $C_{23}H_2F_{10}O_4S_2$: C, 40.35; H, 1.69; S, 8.98. Found: C, 40.3; H, 1.9; S, 9.0. HRMS (FAB$^+$): calculated for $C_{22}H_{20}N_2O_4$ $[M+H]^+$ 714.9790, found 714.9793.

GC/MS Analysis on the Reaction of pFS-DT with Hydrogen Peroxide. Gas chromatography/mass spectrometry analysis was performed on an HP-6890 and HP-5973 instruments equipped with a capillary column (DB-5, 30 m × 0.320 mm × 0.25 μm). H_2O_2 (100 μL) and triethylamine (5 μL) were added to the pFS-DT (10 mg) solution dissolved in $CHCl_3$ (1 mL). After shaking the mixture for 5 min at room temperature, the organic layer was analyzed by GC/MS. The oven temperature was programmed from 80 to 300 °C at 10 °C/min, and final hold 10 min. Main decomposition product was assigned as diethyl 2,5-dihydroxylterephthalate (DT); Rt 15 min, MS (m/z, %): 255 (M+1, 5), 254 (M+, 31), 209 (27), 208 (100), 181 (5), 180 (22), 163 (13), 162 (72), 135 (7), 134 (15), 107 (9), 79 (5), 78 (5), 53 (9).

Preparation of Nanoprobes. Pluronic F-68 (10 mg) and pFS-DT (0.1 mg) were homogeneously mixed with or without catalytic additive (1-phenyl imidazole or 2,4,6-trimethylbenzoic acid at a given weight ratio with pFS-DT) by dissolution in methylene chloride (1.2 mL) and then the solvent was evaporated by air drying. The dried mixture was vortex vigorously with Milli-Q water (1 mL) to give an aqueous dispersion of NPs.

Selectivity Evaluation. H_2O_2, TBHP and OCl^- were delivered from 30 wt%, 70 wt%, and 5 wt% aqueous solutions, respectively. O_2^- and NO• were generated by adding stock solutions of KO_2 and 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5) in DMSO, respectively. •OH and •OtBu were generated by Fenton reaction of 1 mM Fe^{2+} with 100 μM H_2O_2 or 100 μM TBHP, respectively.

Kinetics. The deprotection-induced fluorogenic reaction was monitored using a fluorescence spectrometer. The time courses for the conversion of non-fluorescent to fluorescent states were followed with the fluorescence intensity at 450 nm. In a typical experiment, 100 μM of H_2O_2 was added to the 1 mg/mL aqueous suspension of the nanoprobes. For the highly base-catalyzed nanoprobes (1PI:pFS-DT=10:1 and 5:1), the kinetic constants were determined with nonlinear fitting according to the following steady-state kinetics Eq. (1),

$$I = At + B\left(1 - e^{-kt}\right) \qquad \text{Eq. (1)}$$

where I is increased fluorescence intensity and k is the observed rate constant (k_{obs}). A is steady state velocity defined by Michaelis-Menten equation and B is a multiple kinetic parameter.

For 1PI/pFS-DT NPs at the weight ratio of 1:1, 0.1:1 and 0:1, and TMBA/pFS-DT NPs (10:1), the kinetic constants were determined with nonlinear fitting according to the following Pseudo-first-order kinetics Eq. (2),

$$I = A(1 - e^{-kt}) \qquad \text{Eq. (2)}$$

where I is increased fluorescence intensity and k is the observed rate constant (k_{obs}). A is fluorescence

intensity at equilibrium.

Preparation and Staining of Cell Models. For phagocytosis experiment, RAW264.7 macrophages were cultured in DMEM with 10 % fetal bovine serum (FBS) and stimulated by phorbol 12-myristate 13-acetate (PMA, Sigma, 100 μM stock in Millipore water) for 50 min. The fluorescence images were obtained after uptake of 0.1 mg/mL of TMBA/pFS-DT or 1PI/pFS-DT NPs. In imaging EGF-stimulated H_2O_2 generation, A431 cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10 % FBS and 1 % penicillin-streptomycin in a humidified 5 % CO_2 incubator at 37 °C. The tested cells were seeded onto 35-mm cover glass bottom dishes and allowed to grow until a confluence of 70 %. For all experiments, solution of NPs, epidermal growth factor (EGF, Sigma, 100 μg/mL stock in Millipore water) and inhibitors were added by bath application to the cell culture media while on the microscope stage or incubated at 37 °C for the indicated times and then imaged. All inhibitors were purchased from EMD Bioscience. For the lipotoxicity study, RIN-m cells were cultured in RPMI 1640 medium with 10 % FBS. Palmitic acid (Sigma) was prepared as 1 mg/mL stock solution in ethanol. RIN-m cells were transfected with CellLight® Peroxisome-RFP, BacMam 2.0 (Life Technology Corp.) for 14 h and sequentially incubated with palmitic acid for 12 h. Cells were washed with DPBS and then imaged. For starvation experiment, HeLa cells were incubated in Earle's balanced salt solution (EB SS) or a control medium (DMEM with 10 % of FBS) for 3h. The fluorescence images were observed after treatment with 0.1 mg/mL of NPs for 10 min. To make hypoxic condition, HeLa cells were incubated under an Anaeropack™ (Mitsubishi gas chemical company, Inc.) less than 0.1 % O_2 or 20 % O_2 conditions for 24 h. The fluorescence images of H_2O_2 were observed after treatment with 0.1 mg/mL of NPs for 5 min.

Fluorescence Imaging of Cells. Fluorescence imaging was performed with Nuance FX multispectral imaging system (Cambridge Research & Instrumentation, Inc., USA) set to 350-380 nm excitation and 480-530 nm emission. Real-time imaging was conducted by using iXon Back-illuminated Electron multiplying CCD (EMCCD) Camera with a DAPI/FITC filter set for excitation/emission.

Transfection of Plasmid DNA Expressing Nox1-YFP. A431 cells were plated on 35-mm glass bottom dish (2×10^5) and grown in DMEM for 48 h at 37 °C. Cells were incubated for another 30 min at 37 °C after replacing growth medium with antibiotics- and FBS-free medium. A transfection reagent was prepared by mixing Lipofectamine (10 μL in 240 μL of Optim-MEM incubated for 5 min at room temperature) and Nox1-YFP plasmid DNA (10 μL in 240 μL of Opti-MEM incubated for 5 min at room temperature) and incubating for 20 min at room temperature. The mixture and 1.5 mL of growth medium were then added to the cell dish after suction of existing medium. After 6 h incubation at 37 °C, medium was replaced with medium containing antibiotics and FBS and incubated for 24 h at 37 °C for expression of Nox1-YFP.

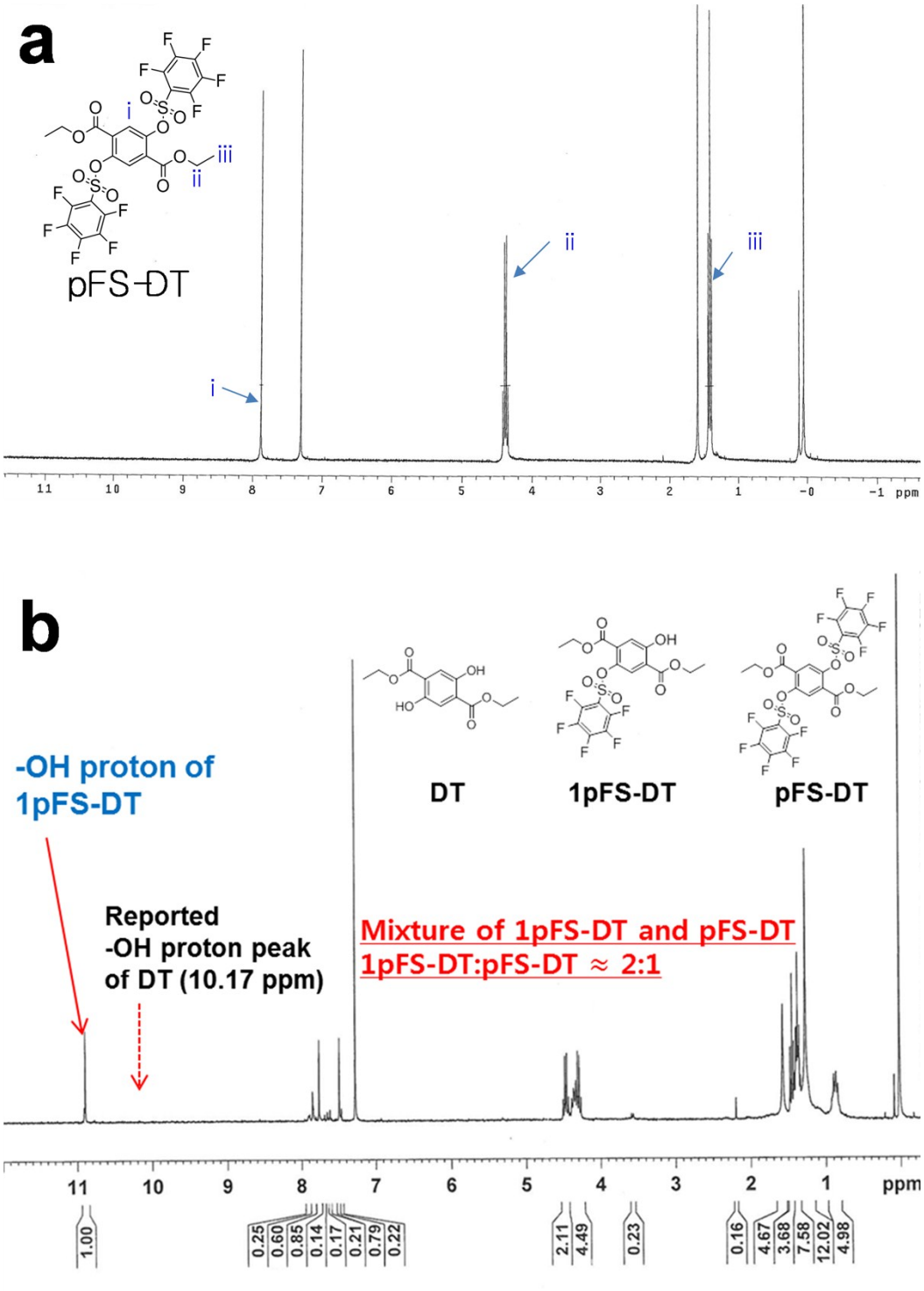

Fig S1. Chemical structures of DT, 1pFS-DT and pFS-DT, and their ^{1}H-NMR spectra: (a) pFS-DT and (b) a mixture of 1pFS-DT and pFS-DT. Even though as-synthesized 1pFS-DT is mixed with a fraction of pFS-DT, this mixture was directly used without further purification for the following optical characterization because pFS-DT has no interference to the optical properties of 1pFS-DT. No appearance of –OH proton peak of DT indicates the absence of DT in the mixture.

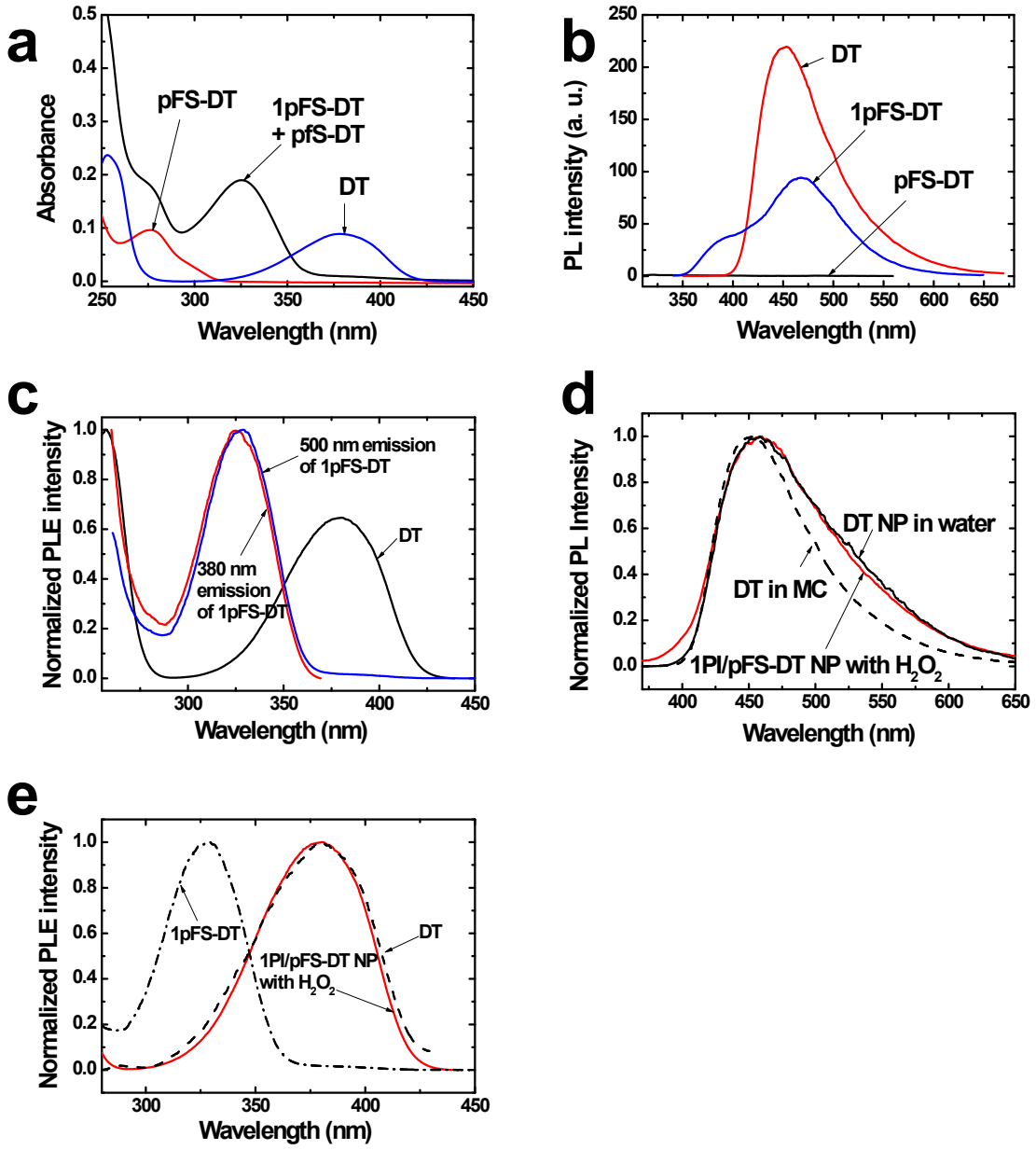

Fig S2. (a) Absorption spectra of pFS-DT, 1pFS-DT (mixture) and DT. (b) Fluorescence spectra of pFS-DT, 1pFS-DT (mixture) and DT. (c) Excitation spectra for the fluorescence of DT and 1pFS-DT (mixture). (d) Fluorescence spectra of DT (in dichloromethane, MC), DT NPs (in water) and 1PI/pFS-DT NPs (in water) reacted with H_2O_2. (e) Excitation spectra for the fluorescence of DT, 1pFS-DT (mixture) and 1PI/pFS-DT NPs reacted with H_2O_2.

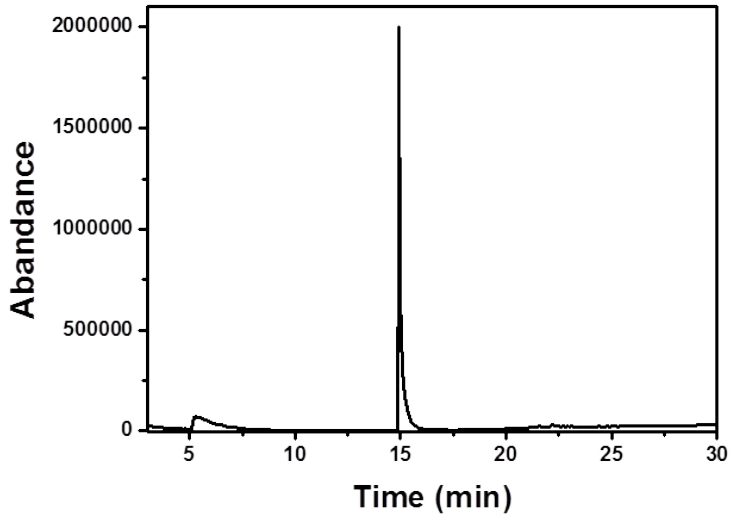

Fig S3. Gas chromatography analysis of pFS-DT after treatment with H_2O_2. Main decomposition product at Rt 15 min was assigned as diethyl 2,5-dihydroxylterephthalate (DT); MS (m/z, %): 255 (M+1, 5), 254 (M+, 31), 209 (27), 208 (100), 181 (5), 180 (22), 163 (13), 162 (72), 135 (7), 134 (15), 107 (9), 79 (5), 78 (5), 53 (9).

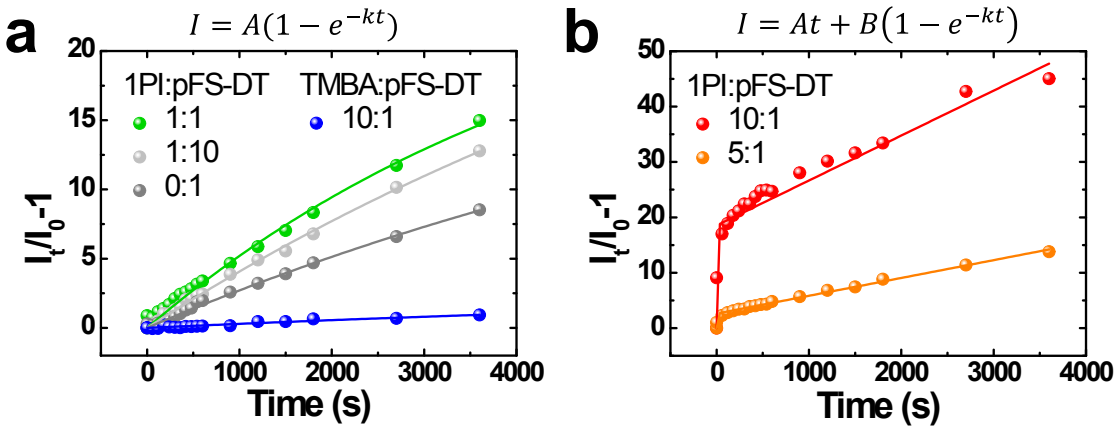

Fig S4. Kinetic profiles for H_2O_2 sensing of NPs with different types and amounts of the catalyst. I_t is fluorescent intensity at a given time t, and I_0 is initial fluorescent intensity before H_2O_2 addition. Solid lines are fitted with equations for pseudo-first-order kinetics in (a) and pre-steady-state burst kinetics in (b).

Table S1. Observed rate constants (k_{obs}) for H_2O_2 sensing of NPs with different types and ratios of the catalytic additives.

Catalytic additives	Additive-to-probe ratio (w/w)	k_{obs} (s^{-1})[a]	Model Kinetics[b]
1PI	10:1	5.6×10^{6}	Burst
	5:1	4.9×10^{-1}	Burst
	1:1	2.0×10^{-4}	1st order
	0.1:1	1.2×10^{-4}	1st order
None	0:1	1.1×10^{-4}	1st order
TMBA	10:1	8.8×10^{-5}	1st order

[a] Observed rate constants (k_{obs}) obtained from nonlinear fitting to kinetic models provided in experimental section. [b] Burst: pre-steady-state burst and 1st order: pseudo-first-order kinetics.

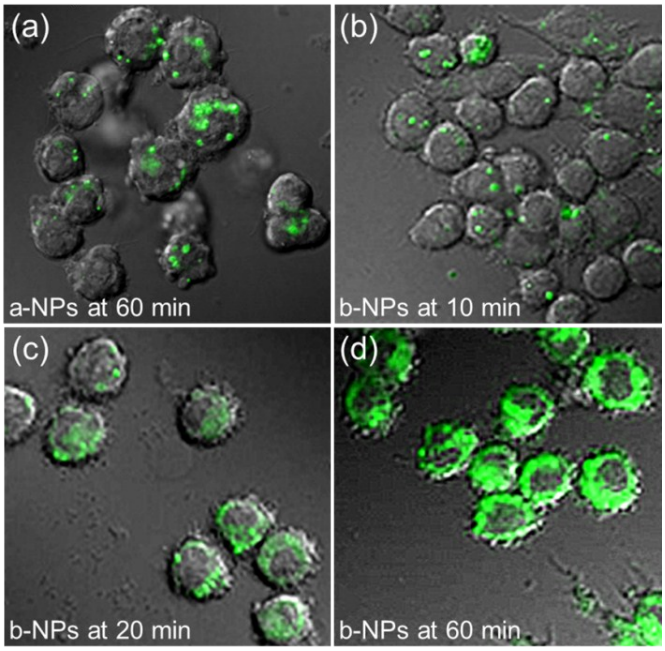

Fig S5. Fluorescence imaging of PMA-stimulated RAW264.7 macrophages with (a) acidic NPs (a-NPs; TMBA:pFS-DT=10:1) or (b-d) basic NPs (b-NPs; 1PI:pFS-DT=10:1). Imaging time points after the NPs treatment are indicated.

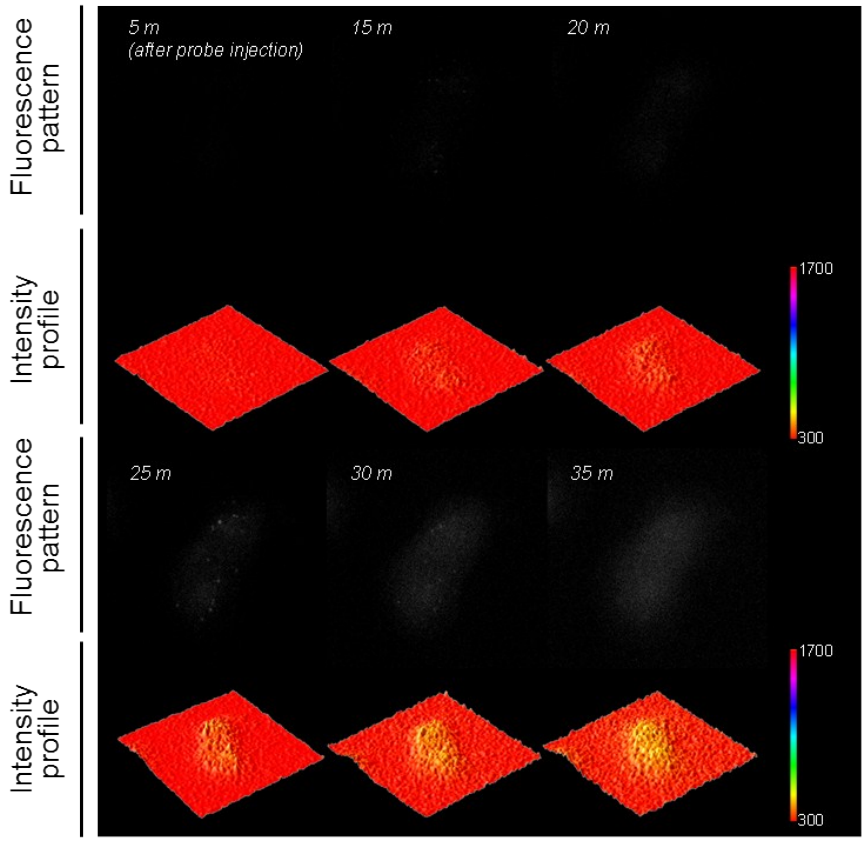

Fig S6. Temporal evolution of fluorescence patterns and corresponding intensity profiles after uptake of 0.1 mg/mL b-NPs. Exposure time was 300 s and a filter set of DAPI/FITC was used for excitation/emission.

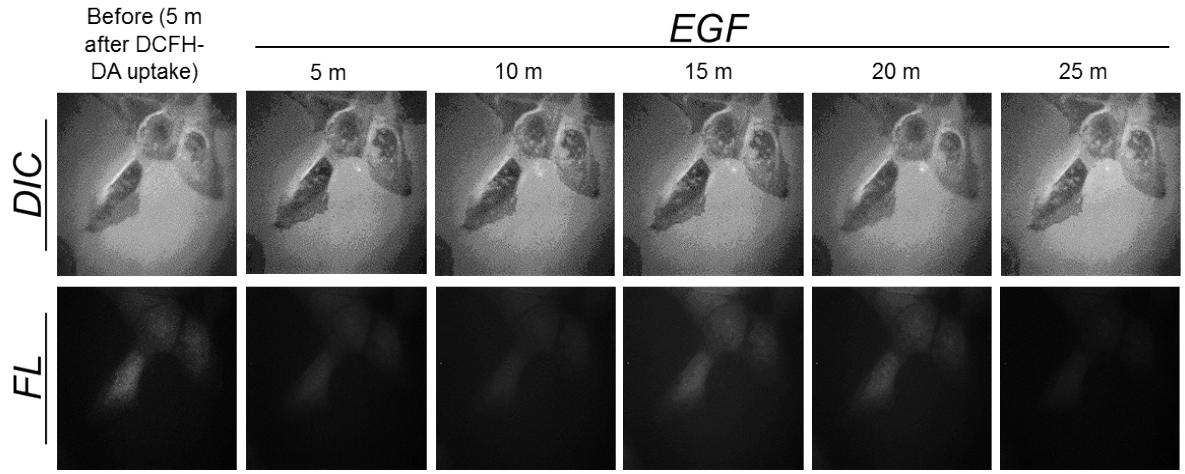

Fig S7. Differential interference contrast (DIC) and fluorescence (FL) images of DCFH-DA treated A431 cells at the elapsed time after EGF treatment. Imaging was conducted under the same experimental setup and condition with Fig 2.

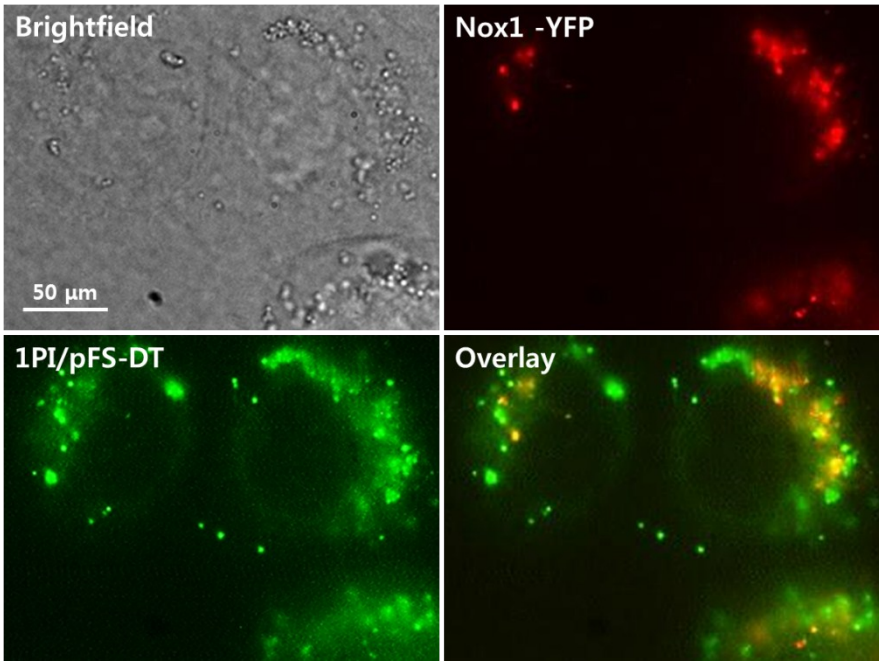

Fig S8. Fluorescence imaging of EGF-triggered production of H_2O_2 in A431 cells (transfected with Nox1-YFP plasmid DNA) with b-NPs. Red and green color corresponds to the fluorescence signals of Nox1-YFP and b-NPs, respectively, and yellow signals (Overlay) indicate the co-localization of the two signals. Cells were incubated with b-NPs (0.1 mg/mL) for 10 min and subsequently treated with EGF (500 ng/mL). Images were taken at 10 min after the EGF treatment.

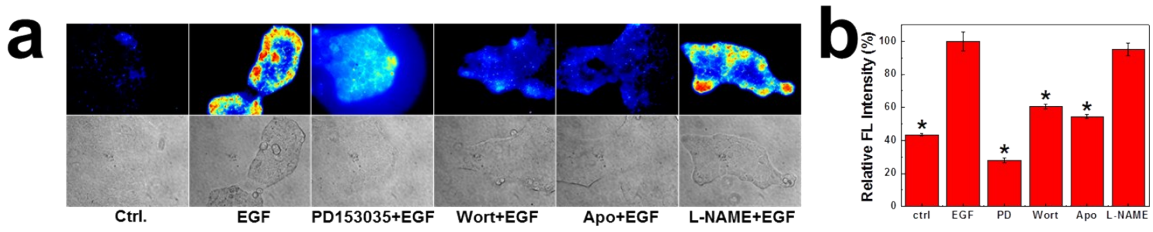

Figure S9. (a) Effects of various inhibitors on fluorescence responses in EGF signaling: fluorescence (top row) and bright field (bottom row) images of A431 cells sequentially incubated with 100 μM each inhibitor for 25 min, EGF (500 ng/ml) and then 0.1 mg/mL b-NPs for 15 min. Control experiments were conducted without EGF/inhibitor treatment. (b) Relative fluorescence intensities in (a). * indicates that $p < 0.05$ when compared to EGF treated cells. Error bars are ± s.e.m.

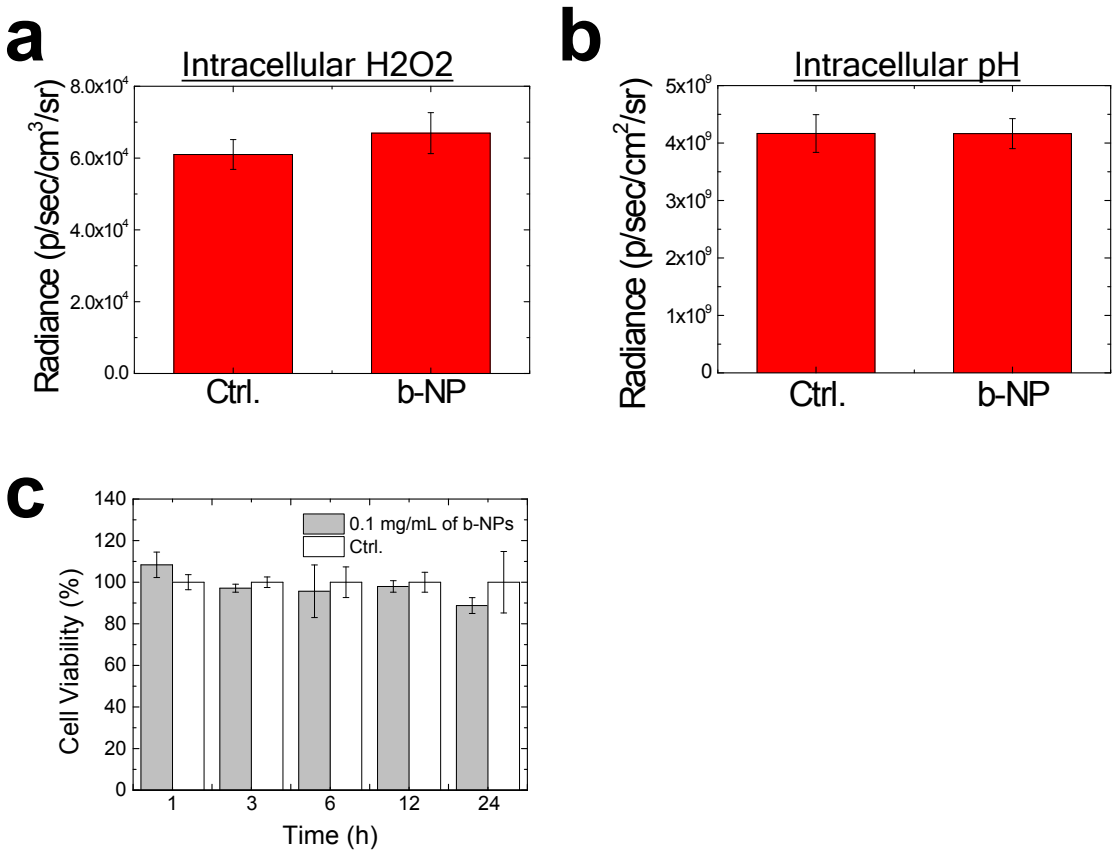

Fig S10. (a) Chemiluminescence (CL) intensities in response to intracellular H_2O_2. HeLa cells were incubated with or without (Ctrl.) 0.1 mg/mL of b-NPs for 1h, and H_2O_2-responsive CL nanoparticles (FPOA NPs)[1] (0.4 mg/mL) were added to both the incubation media. After 10 min, the CL imaging was done with IVIS Lumina Series III (Caliper, USA). The graph shows the average CL intensities. (b) Fluorescence (FL) intensities in response to intracellular pH. HeLa cells were incubated with or without (Ctrl.) 0.1 mg/mL of b-NPs for 1h, and 0.1 mL of pHrodo® RED AM intracellular pH indicator (Molecular Probes™) was added to both the media. After 30 min, the FL imaging was done with IVIS Lumina Series III (Caliper, USA) with ex/em=560/600 filter. The graph shows the average FL intensities. (c) Cytotoxicity of b-NPs against HeLa cells, evaluated by the colorimetric MTT assay. HeLa cells were treated with or without (Ctrl.) 0.1 mg/mL of b-NPs.

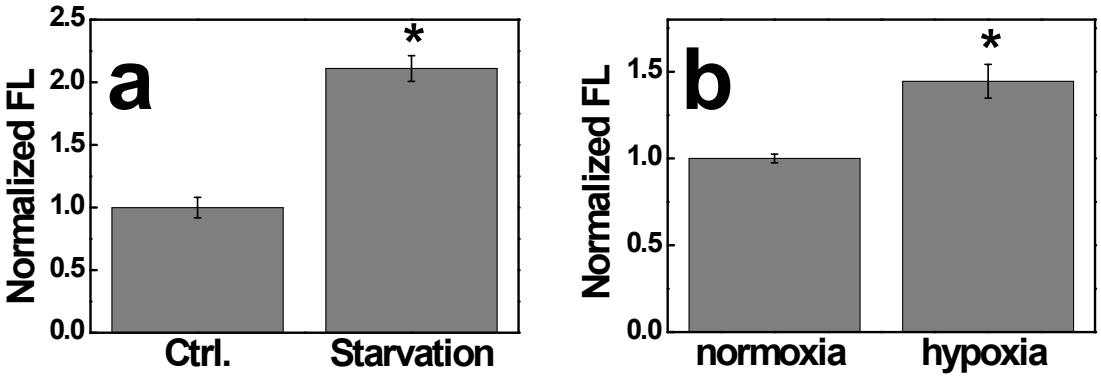

Fig S11. Relative fluorescence intensities from (a) starvation and (b) hypoxia studies in Fig 3c-d. The intensities were normalized to signals from control (Ctrl. and normoxia) experiments. * indicates that $p < 0.05$ and the error bars are ± s.e.m in each graph.

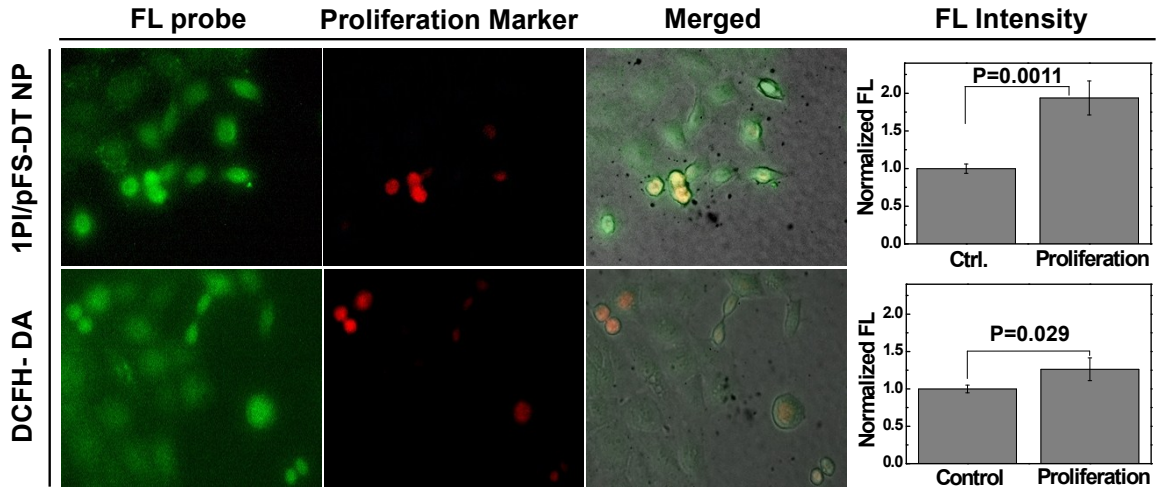

Fig S12. Fluorescence imaging of H_2O_2 produced in cell proliferation of HeLa cells incubated with 0.1 mg/mL of NPs (top row) or DCFH-DA (bottom row) for 15 min at 25 °C. In both experiments, cells were pre-treated with a proliferation marker kit (Click-iT EdU imaging kits, Invitrogen) for 15 min and images were taken after addition of Alexa Fluor 647 picolyl azide. Graphs show the relative fluorescence intensities normalized to signals from the cells before proliferation.

References

[1] Y.-D. Lee, C.-K. Lim, A. Singh, J. Koh, J. Kim, I. C. Kwon, S. Kim, *ACS Nano* 2012, **6**, 6759-6766.