# Supporting Information

# Ratiometric Fluorescence Nanoprobes for Subcellular pH Imaging with a Single-Wavelength Excitation in Living Cells

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## Experimental Details:

**Materials.** Tetraethyl orthosilicate (TEOS), hexadecyl trimethylammonium chloride (CTAC), triethanolamine (TEA), hydrogen peroxide ($H_2O_2$, 30%), ammonia solution ($NH_3 \cdot H_2O$), methanol and ethanol were purchased from China National Pharmaceutical Group Corp. (Shanghai, China). 2-(N-Morpholino) ethanesulfonic acid (MES), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 6-aminofluorescein (AF), ethidium bromide (EB) and 3-(4-morpholinyl) propanoic acid hydrochloride (MPP) were obtained from Sigma-Aldrich. Nigericin was obtained from APOLLO Scientific Ltd. (4-carboxybutyl) triphenylphosphonium bromide (TPP), carboxyethylsilanetriol di-sodium salt (CETS), (3-aminopropyl) triethoxysilane (APTES) and 1-ethyl-3-(3-dimethly aminopropyl) carbodiimide (EDC) were obtained from Alfa Aesar Chemical Ltd. (Tianjin, China). Mito-Tracker Green and Lyso-Tracker DND-26 were purchased from Molecular Probes (Invitrogen, USA). The human breast cancer cell line MCF-7 was purchased from KeyGEN

biotechnology Company (Nanjing, China). All the chemicals were analytical grade and used without further purification. Water was purified with a Sartorius Arium 611 VF system (Sartorius AG, Germany) to a resistivity of 18.2 MΩ ·cm.

**Instruments.** High resolution transmission electron microscopy (HRTEM) was carried out on a JEM-2100 electron microscope. Fluorescence spectra were obtained with FLS-920 Edinburgh Fluorescence Spectrometer with a Xenon lamp and 1.0 cm quartz cells at the slits of 2.5 /2.5 nm. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi Device Works, Shanghai, China) with a combined glass-calomel electrode. Absorbance was measured in a microplate reader (Synergy 2, Biotek, USA) in the MTT assay. Confocal fluorescence imaging studies were performed with a TCS SP5 confocal laser scanning microscopy (Leica Co., Ltd. Germany) with an objective lens (×20).

**Preparation of mesoporous silica nanoparticles (MSNs):** MSNs was typically synthesized according to a reported protocol with some modifications. Hexadecyl trimethyl ammonium chloride (CTAC, 0.5 g) and triethanolamine (TEA, 0.08 g) were dissolved in turn in 20 mL water. The mixture was heated at 95 °C under intensive stirring. After 1 h, 1.5 mL of TEOS was added dropwise and the resulting mixture was stirred for another 1 h. The products were collected by centrifugation and washed for several times with ethanol to remove the residual reactants. The collected products were calcined at 450 °C for 10 h to remove the CTAC remaining inside the mesopores absolutely.

**Preparation of carboxyl-functionalized MSNs (MSNs-COOH).** The surface of MSNs was functionalized with carboxyl groups by treatment with CETS. MSNs (15 mg) were first dispersed in 20 mL ethanol. 8 ml of water and 200 μL of $NH_3 \cdot H_2O$ was added to the above solution in order. The mixture was gentlely stirred for 30 min. And then the solution was stirred for overnight, followed by the addition of 50 μL CETS. After centrifugation and washing with water, carboxyl-functionalized MSNs were redispersed in 5 mL of MES buffer (10 mM, pH 6.0).

**Assembly of the nanoprobes.** 2 mL as-prepared MSNs-COOH solution was added to 3 mL EDC solution (10 mM). The mixture was then stirred at room temperature for

30 min to activate the carboxylic group of MSNs-COOH. Subsequently, 3 mM ethidium bromide (EB) and 300 μM 6-aminofluorescein (AF) was added to the mixture and then stirred for 24 h at room temperature. Excess EDC, EB and AF were removed by repeatedly washing the nanoparticles with distilled water several times. The precipitates were dispersed in 10 mL water. 4 mL of ethanol and 200 μL of NaOH (0.1 M) was added to the solution. The mixture was gentlely stirred for 10 min, followed by the addition of 200 μL of 20% TEOS in ethanol along with 10 μL APTES three times under gentle stirring at 30 min intervals. The mixture was reacted for 24 h at 30 °C to form the mesoporous silica shell. The as-synthesized MSNs-EB-AF@$SiO_2$-$NH_2$ was washed with methanol and water for several times. After that, the precipitates were dispersed in 2 mL of MES buffer (10 mM, pH 6.0). The nanoprobes was obtained by coupling the carboxyl group of the TPP or MPP and the amino group on the surface of MSNs-EB-AF@$SiO_2$-$NH_2$ to form the amido bond. EDC solution (1.5 mM) was added to TPP or MPP (0.5 mM) solution, and the solution was mixed and reacted for 30 min at room temperature to activate carboxylate groups. The mixture was then added to 1 mL MSNs-EB-AF@$SiO_2$-$NH_2$ solution with gentle stirring in darkness. The solution was reacted for 24 h with gentle stirring, which resulted in the formation of the amido bond. After that, the resulting precipitates were centrifuged and washed three times with water.

**Quantitation of aminofluorescein and ethidium bromide loaded on the nanoprobes.** The fluorescence intensity of nanoprobes solution (0.2 mg/mL) in 1 mL of PBS buffer (10 mM, pH 7.4) was measured, and the concentrations of aminofluorescein and ethidium bromide were determined according to the standard linear calibration curve of aminofluorescein and ethidium bromide, respectively.

**General procedure for ratiometric fluorescence determination:** A series of standard pH buffers were prepared by mixing 10 mM $Na_2HPO_4$ and 10 mM $NaH_2PO_4$ at varied volume ratios. The pH value was measured by a pH-3c digital pH-meter. Then, 1 mL of the standard pH buffer and 20 μL of solution (0.2 mg/mL) were mixed, and the resulting solution was transferred to a quartz cell of 1 cm optical length to measure fluorescence spectrum with $\lambda_{ex}$ = 488 nm. The ratio signal (R = $I_{515}/I_{595}$) was

calculated from the fluorescence intensities at 515 nm and 595 nm. The experiment was repeated three times, and the data are shown as the mean±SD.

**Interference study.** The effects of intracellular species on the fluorescence response of nanoprobes to pH were investigated. The interfering substances, such as metal ions ($Na^+$, $Mg^{2+}$, $K^+$, $Mn^{2+}$, $Ca^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Co^{2+}$), oxidative-stress-associated redox chemicals, including glutathione (GSH) and $H_2O_2$ as well as organism amino acids (Cys, Arg, Try, Lys, L-Cys ) were examined. All data were obtained using an excitation wavelength at 488 nm. The experiment was repeated three times, and the data are shown as the mean±SD.

**Fluorescence reversibility of nanoprobes with pH.** The pH of the nanoprobes (0.2 mg/mL) between pH 4 and pH 9 was adjusted back and forth by 2.0 M HCl or NaOH, and then measured with a pH meter. The fluorescence spectra were recorded with $\lambda_{ex}$ = 488 nm.

**Cell culture.** MCF-7, HeLa cells were cultured in Dulbecco's modified Eagles medium (DMEM). SKOV-3 cells were cultured in RPMI medium 1640. All cell lines were supplemented with 10% fetal bovine serum and 100 U/mL of 1% antibiotics penicillin/streptomycin and maintained at 37 °C in a 100% humidified atmosphere containing 5% $CO_2$.

**Cytotoxicity assay.** The cytotoxicity of nanoprobes was evaluated by the standard MTT assay. Briefly, MCF-7 cells were cultured in 96-well microtiter plates and incubated at 37 °C in a humidified atmosphere with 5% $CO_2$ for 24 h. After the original medium was removed, the cells were incubated with the nanoprobes (0.1, 0.2, 0.4, 1.0, 2.0 mg/mL) for 6, 12 and 24 h, respectively. Next, 150 μL of MTT solution (0.5 mg/mL) was 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. After shaking the plates for 10 min, the absorbance was measured at 490 nm with the RT 6000 microplate reader. The experiment was repeated three times, and the data are shown as the mean±SD.

**Confocal fluorescence imaging.** The cells were plated on chamber slides for 24 h. Then, the nanoprobes (0.2 mg/mL) were delivered into the cells in culture medium for

6 h at 37 °C in 5% $CO_2$. Then, the cells were washed three times with PBS (pH 7.4) to remove the nanoprobes that were not taken up into the cells. The cells were examined by confocal laser scanning microscopy (CLSM) with 488 nm excitation. All experimental parameters (the laser intensity, exposure time, objective lens) were stationary when the different fluorescence images were captured. The fluorescence intensity (FI) was the average fluorescence intensity of the cell area (at least 50 cells) from the confocal fluorescence images, which was quantified by LAS AF software.

**Co-localization fluorescence imaging.** For the mitochondria co-localization study, MCF-7 cells were incubated with 0.2 mg/mL of MSNs-EB@$SiO_2$-$NH_2$ or MSNs-EB@$SiO_2$-TPP for 6 h, washed three times with PBS (pH 7.4), and then incubated with Mito-Tracker Green (MTG, 25 nM) for 15 min. Before fluorescence imaging, the adherent cells were further washed with PBS (pH 7.4) three times to remove the excess MTG. Confocal fluorescence imaging of stained cells were captured using a 488 nm laser, the collection window is 500−550 nm (MTG) and 570−630 nm (EB). For the lysosomes co-localization study, MCF-7 cells were incubated with 0.2 mg/mL of MSNs-EB@$SiO_2$-$NH_2$ or MSNs-EB@$SiO_2$-MPP for 6 h, washed three times with PBS (pH 7.4), and then incubated with Lyso-Tracker DND-26 (LTD, 500 nM) for 15 min. Before fluorescence imaging, the adherent cells were further washed with PBS (pH 7.4) three times to remove the excess LTD. Confocal fluorescence imaging of stained cells were captured using a 488 nm laser, the collection window is 500−550 nm (LTD) and 570−630 nm (EB). The colocalization ratio of the nanoprobes was quantified using Image-Pro Plus Imaging software. More than fifty cells were chosen and circled for measuring the Pearson's correlation coefficient. And the Pearson's correlation coefficient was calculated as the mean of cells.

**Intracellular pH calibration.** The cells were plated on chamber slides for 24 h. Then the MSNs-EB-AF@$SiO_2$-$NH_2$ nanoprobes (0.2 mg/mL) were delivered into the cells in culture medium for 6 h at 37 °C in 5% $CO_2$. The cells were washed three times with PBS (pH 7.4) to remove the nanoprobes that were not taken up into the cells. The nanoprobes-loaded cells were incubated at 37 °C for 15 min in high $K^+$ buffer (30

mM NaCl, 120 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1 mM $NaH_2PO_4$, 5 mM glucose, and 20 mM HEPES) with various pH values in the presence of 10 μM nigericin. After 20 min, cell imaging was carried out after washing cells with PBS (pH 7.4) three times. The cells were examined by confocal laser scanning microscopy (CLSM) with 488 nm excitation. The fluorescence was collected in the ranges of 500−550 nm (AF, green) and 570−630 nm (EB, red), respectively. Pseudo-color ratiometric images were obtained by mediating the green channel image with the red channel at the same pH. The pH calibration was finally obtained based on the average intracellular ratio values shown in the ratiometric images.

**Determining the subcellular pH level of nanoprobes.** The nanoprobes-loaded (MSNs-EB-AF@$SiO_2$-$NH_2$, MSNs-EB-AF@$SiO_2$-TPP or MSNs-EB-AF@$SiO_2$-MPP, 0.2 mg/mL) cells in PBS (pH 7.4) as intact cells were directly subjected to fluorescence imaging.

For the monitoring of the mitochondrial pH fluctuation in nanoprobes-loaded (MSNs-EB-AF@$SiO_2$-TPP) MCF-7 cells, a starvation model was employed. For the monitoring of the lysosomal pH fluctuation, the nanoprobes-loaded (MSNs-EB-AF@$SiO_2$-MPP) MCF-7 cells were treated with $NH_4Cl$ at 37 ℃ for 1 min in PBS (pH 7.4). And then the treated cells were subjected to fluorescence imaging.

To explore the intracellular pH fluctuations associated with oxidative stress, the nanoprobes-loaded (MSNs-EB-AF@$SiO_2$-$NH_2$, MSNs-EB-AF@$SiO_2$-TPP or MSNs-EB-AF@$SiO_2$-MPP) MCF-7 cells were first treated with a $H_2O_2$ at 37 °C for 1 h in PBS (pH 7.4), and then the treated cells were subjected to fluorescence imaging.

## Supporting Figures:

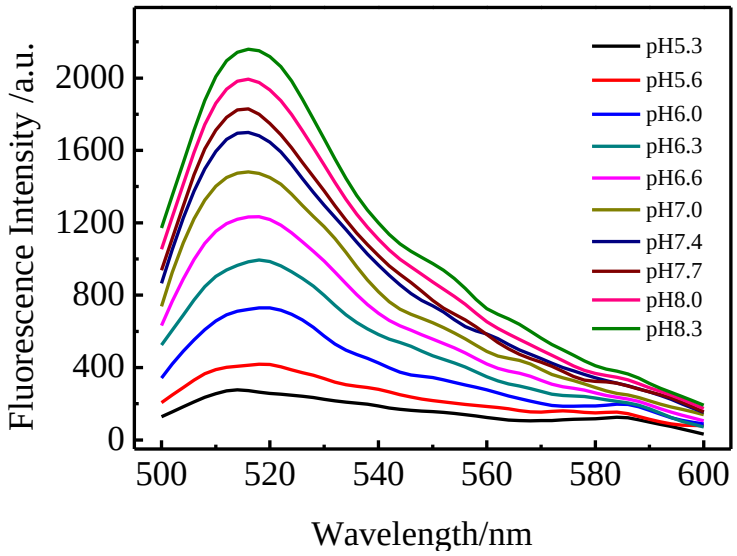


**Figure S1.** Fluorescence emission spectra of the aminofluorescein with various pH values from 5.3 to 8.3 ($\lambda_{ex}$ = 488 nm, 10 mM PBS).

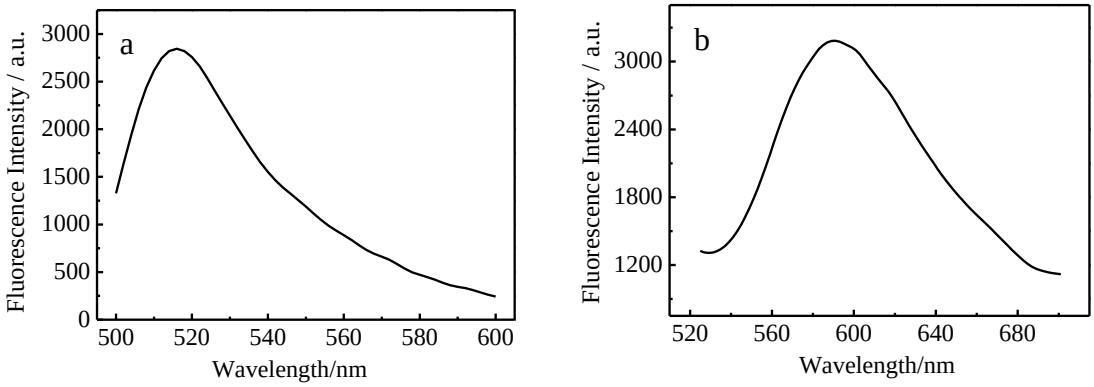


**Figure S2.** Fluorescence emission spectra of the aminofluorescein (a) and the ethidium bromide (b), $\lambda_{ex}$ = 488 nm.

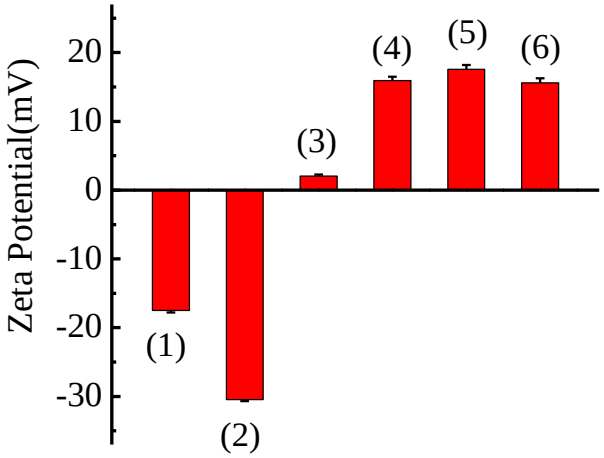


**Figure S3.** Zeta potential of the every step modification: (1)MSNs, (2) MSNs-COOH, (3) MSNs-EB-AF, (4) MSNs-EB-AF@$SiO_2$-$NH_2$, (5) MSNs-EB-AF@$SiO_2$-TPP, (6) MSNs-EB-AF@$SiO_2$-MPP.

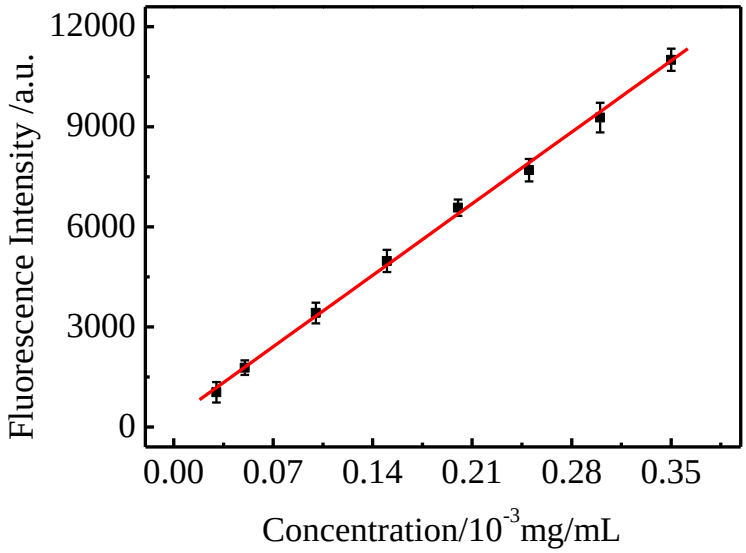


**Figure S4.** Standard linear calibration curve of aminofluorescein (AF).

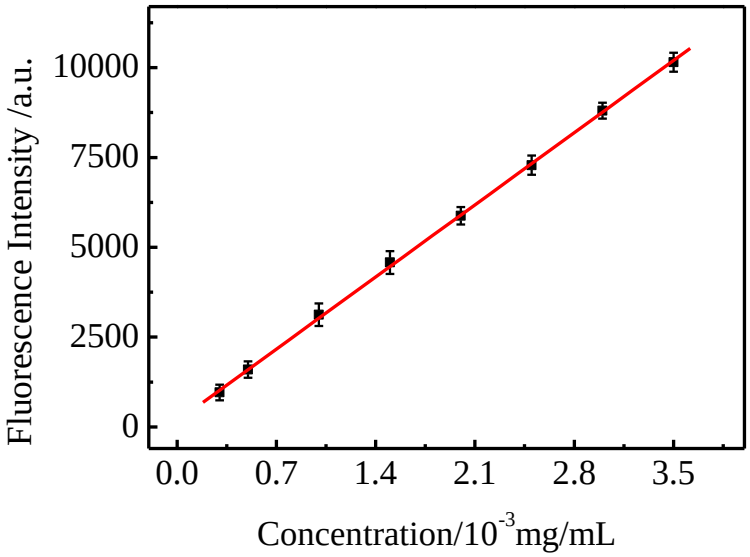


**Figure S5.** Standard linear calibration curve of ethidium bromide (EB).

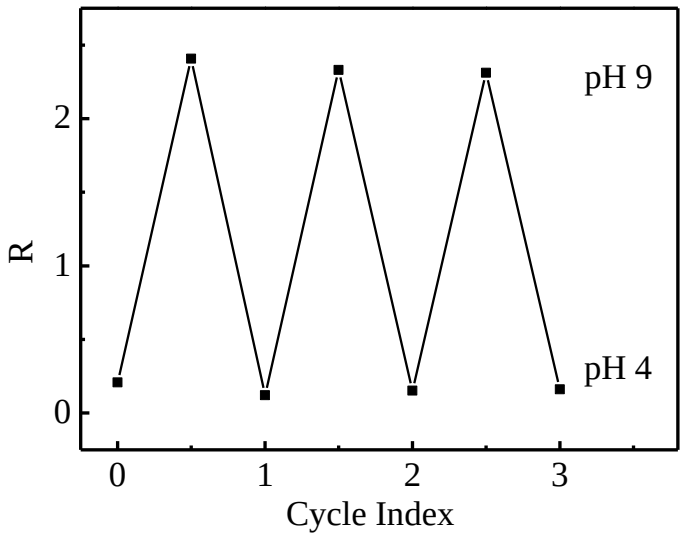


**Figure S6.** pH reversibility study of the nanoprobes between pH 4 and 9 in PBS buffer (10 mM). $\lambda_{ex}$ = 488nm.

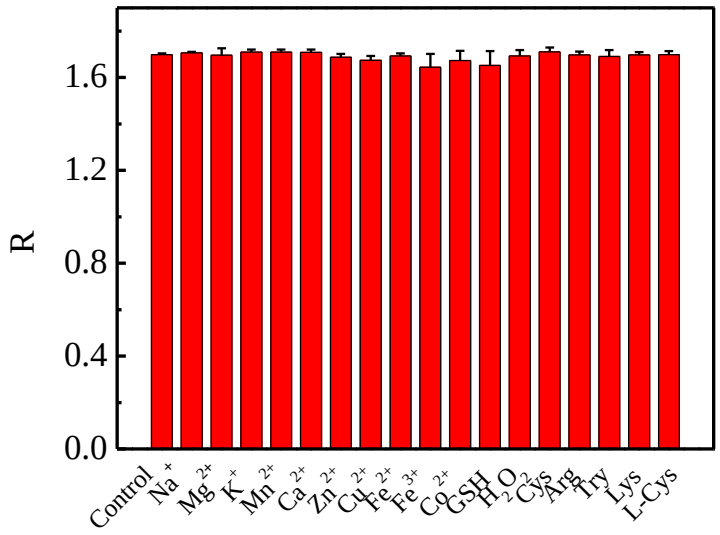


**Figure S7.** Fluorescence responses of nanoprobes to various substances: blank; $Na^{+}$, $Mg^{2+}$, $K^{+}$, $Mn^{2+}$, $Ca^{2+}$, $Zn^{2+}$(1.0 mM); $Cu^{2+}$, $Fe^{2+}$(0.5 mM); $Fe^{3+}$(0.25 mM); $Co^{2+}$(0.2 mM); GSH, $H_2O_2$(1.0 mM); Cys, Arg, Try, Lys, L-Cys (10 μM). All data were obtained using excitation at 488 nm.

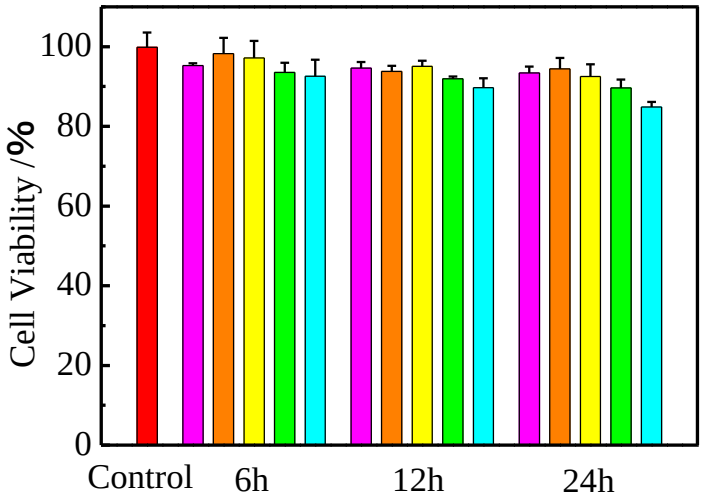


**Figure S8.** Cell viability of MCF-7 cells incubated with different concentrations of nanoprobes for different times (6, 12 and 24 h). Red bar stands for the control, magenta bars, orange bars, yellow bars, green bars and cyan bars stand for the nanoprobe 0.1, 0.2, 0.4, 1.0 and 2.0 mg/mL, respectively.

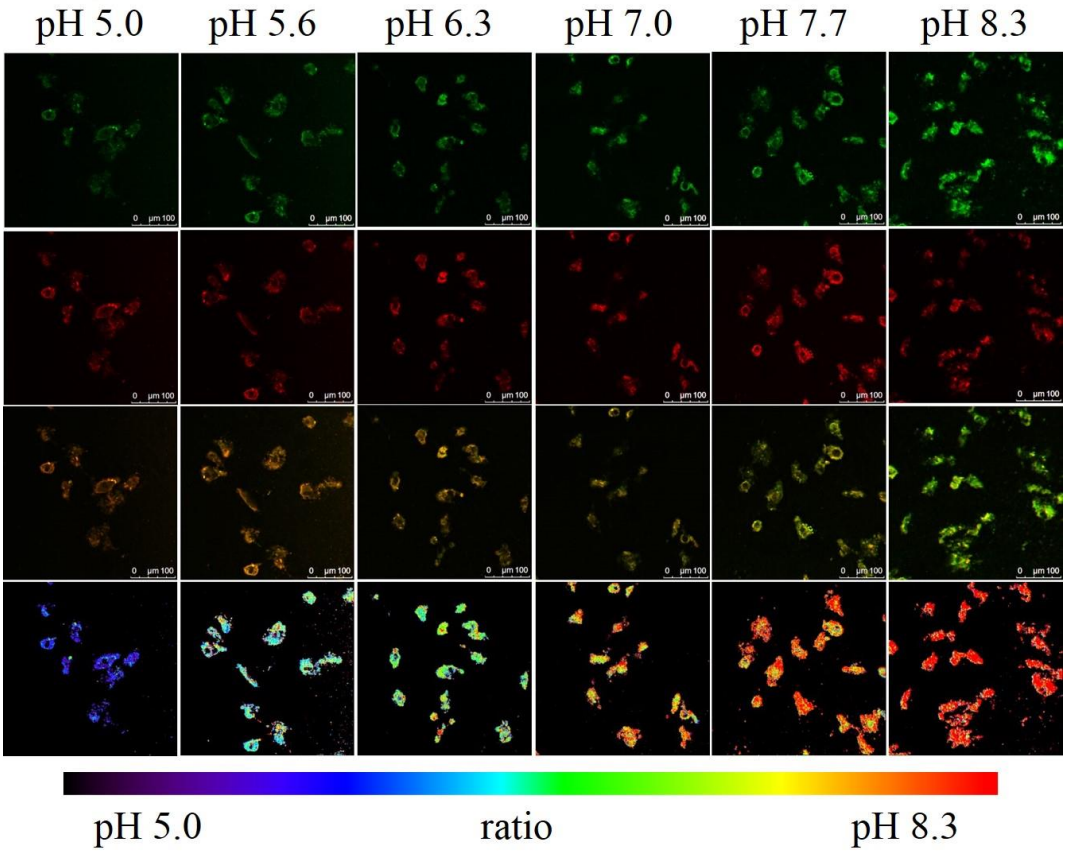


**Figure S9**. Confocal microscopy images of MSNs-EB-AF@$SiO_2$-$NH_2$ nanoprobe-loaded HeLa cells clamped at pH 5.0, 5.6, 6.3, 7.0, 7.7, and 8.3, respectively.

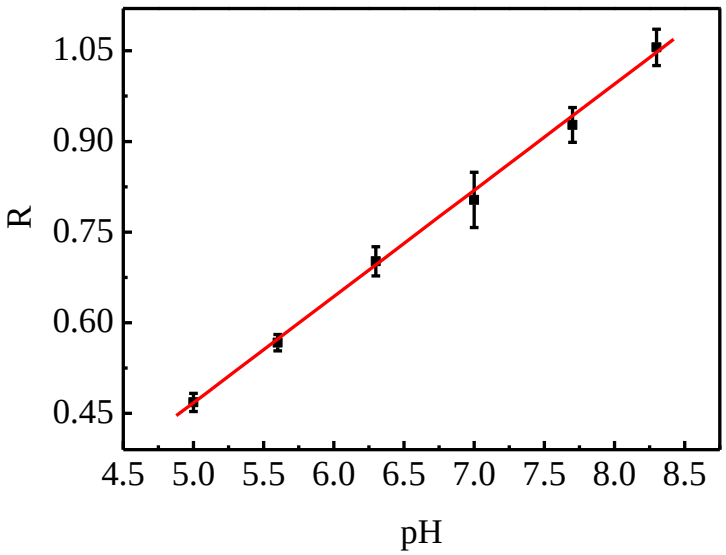


**Figure S10**. Intracellular pH calibration curve constructed by plotting $I_{green}/I_{red}$ vs pH in HeLa cells.

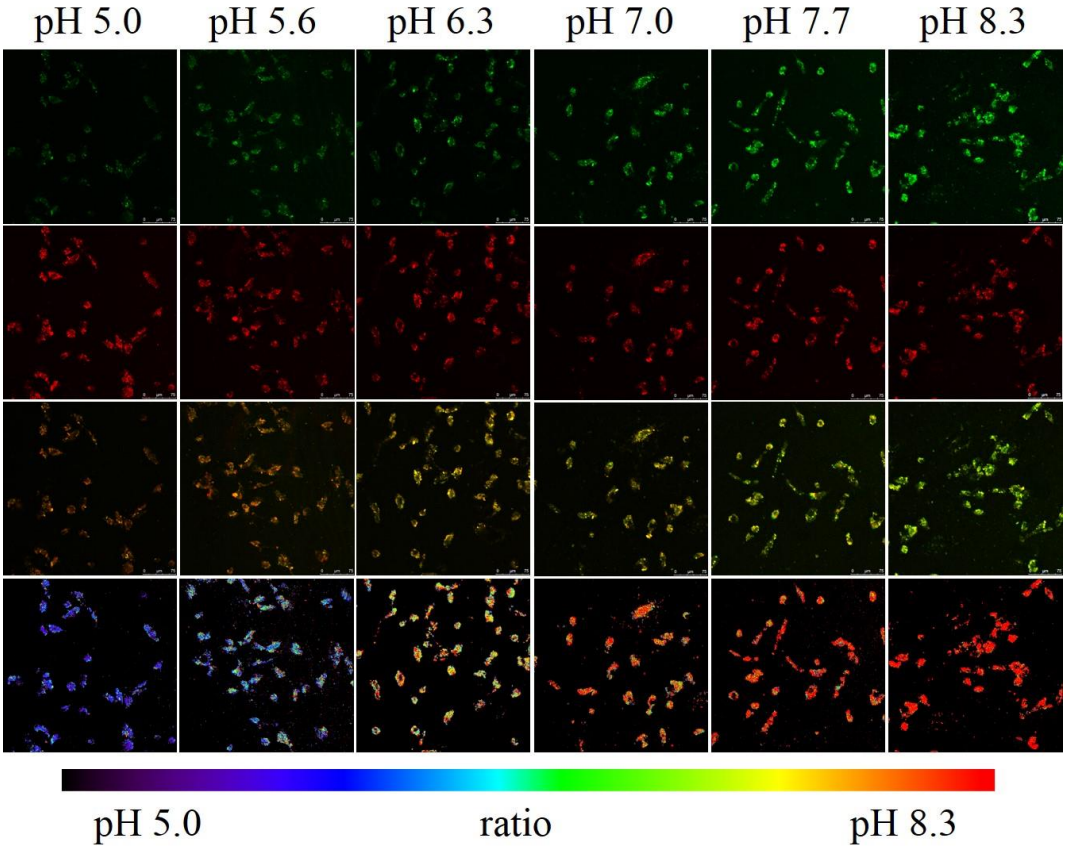


**Figure S11**. Confocal microscopy images of MSNs-EB-AF@$SiO_2$-$NH_2$ nanoprobe-loaded SKOV-3 cells clamped at pH 5.0, 5.6, 6.3, 7.0, 7.7, and 8.3, respectively.

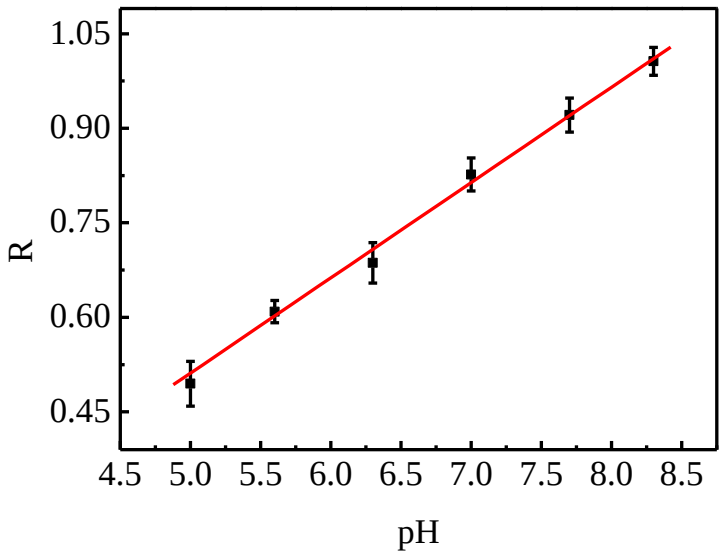


**Figure S12**. Intracellular pH calibration curve constructed by plotting $I_{green}/I_{red}$ vs pH in SKOV-3 cells.

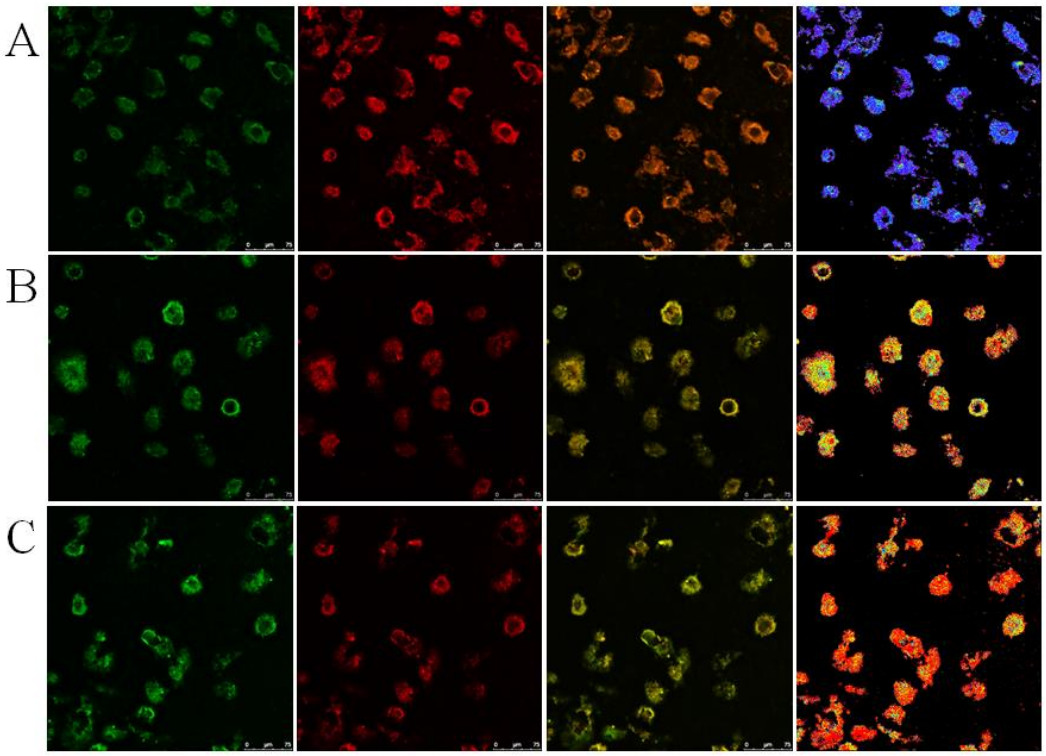


**Figure S13**. Confocal fluorescence images of HeLa cells treated with MSNs-EB-AF@$SiO_2$-MPP (A), MSNs-EB-AF@$SiO_2$-$NH_2$ (B), and MSNs-EB-AF@$SiO_2$-TPP (C).

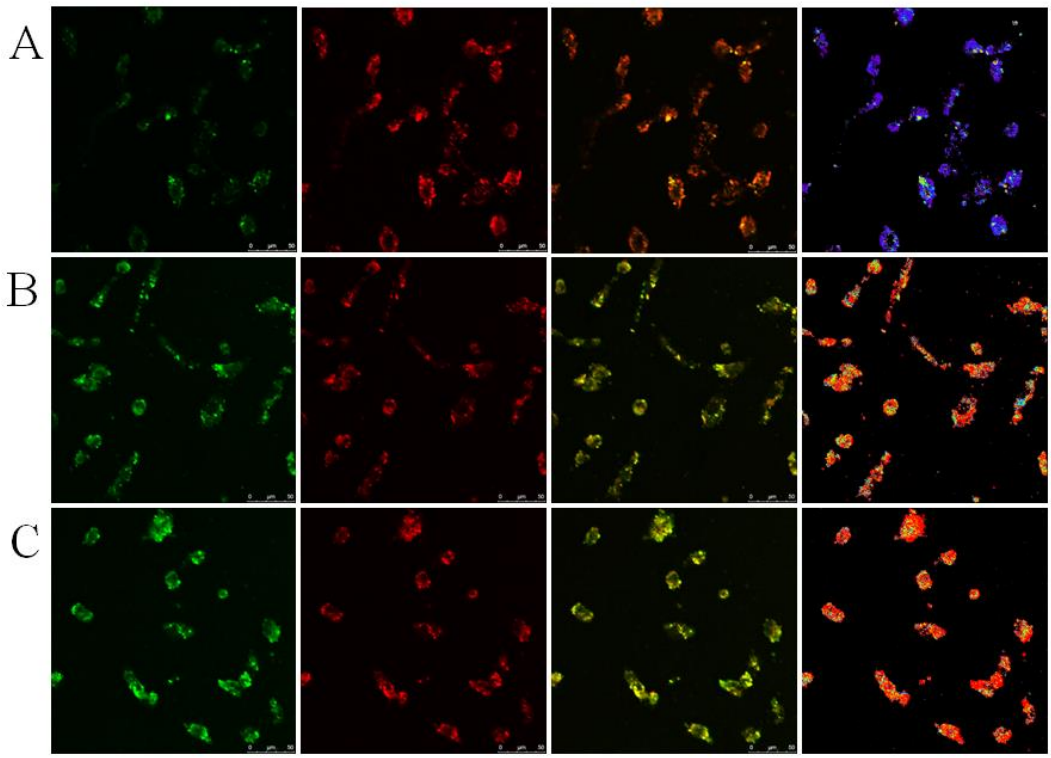


**Figure S14**. Confocal fluorescence images of SKOV-3 cells treated with MSNs-EB-AF@$SiO_2$-MPP (A), MSNs-EB-AF@$SiO_2$-$NH_2$ (B), and MSNs-EB-AF@$SiO_2$-TPP (C).

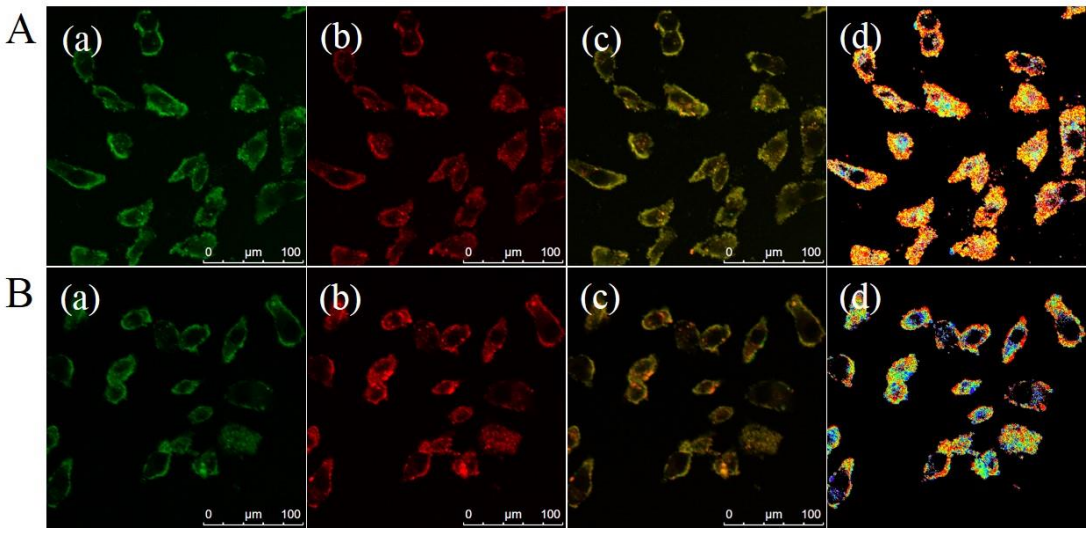


**Figure S15.** Confocal fluorescence images of (A) intact and (B) nutrient-deprived MCF-7 cells treated with MSNs-EB-AF@$SiO_2$-TPP.

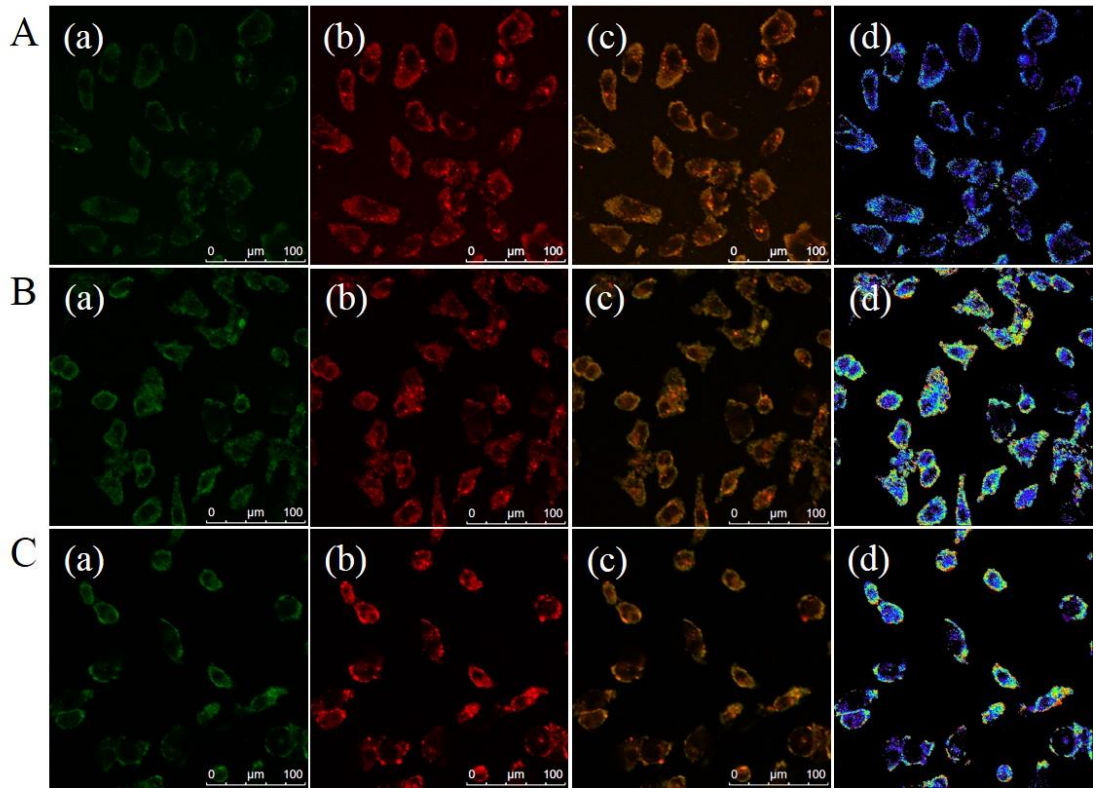


**Figure S16.** Fluorescent images of MCF-7 cells treated with MSNs-EB-AF@$SiO_2$-MPP. (A) intact cells, (B) the treated cells with 10 mM $NH_4Cl$, (C) the treated cells with 100 μM $H_2O_2$.