Electronic Supplementary Information for

Visualization of O_2/ATP Cross-Talk in Living Cells with Smart Fluorescent Nanoprobe

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Table of Contents

1. Experimental section.....S1

1.1 Instruments.....S1

1.2 Synthesis of AuNPs.....S1

1.3 Synthesis of fluorescent nanoprobe.....S1

1.4 Fluorescence spectroscopy assay.....S1

1.5 Cell culture.....S1

1.6 Confocal fluorescence imaging.....S2

1.7 MTT assay.....S2

1.8 Imaging ATP in MCF-7 cells treat with $CoCl_2$ and glucose.....S2

1.9 Imaging O_2 and ATP in tissues.....S3

1.10 Preparation of AuNP-ATG7-siRNA nanoprobe S3

1.11 Using functionalized nanoprobes to inhibit autophagy.....S4

2. Oligonucleotides sequences.....S4

3. Experimental data..S5

1. Experimental Section:

1.1 Materials and instruments.

Ultrapure water obtained from a Millipore water purification system (18 MΩ) were used in all assays. $HAuCl_4 \cdot 4H_2O$, and N-acetylcysteine were purchased from Sinopharm Chemical Reagent Co. (China). β-cyclodextrin (βCD) was purchased from Zhiyuan Biotechnology Co., Ltd (Shandong China). Azoreductase was purchased from CHI Scientific (Jiangsu China). Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Beyotime (Shanghai, China). Adenosine triphosphate was purchased from Sangon Biotech Co., Ltd. (Shanghai, China). All the chemicals were of analytical grade and used without further purification. Sartorius ultrapure water was used throughout the experiments. DNA oligonucleotides were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) The sequences of the oligonucleotides are described in Part 2. UV-Vis absorption spectra were recorded on a Hitachi U-4100 UV/Vis spectrophotometer (Kyoto, Japan). Fluorescence images of cells were obtained using an Olympus FV1000-MPE multiphoton laser scanning confocal microscope (Japan).

1.2 Synthesis of AuNPs.

The 13 nm AuNPs were prepared according to the classical sodium citrate reduction method. Briefly, 33 mL of ultrapure water was added into 17 mL of 0.1% $HAuCl_4$ solution in a two-neck flask, which was then heated in a hot oil bath pan with stirring. When the solution began to reflux, 5 mL of 38.8 mM sodium citrate was quickly added into the flask, and the solution color began to change into deep red in 1 min. After another 20 min of refluxing, the system was cooled down naturally to room temperature under stirring. The concentration of the prepared colloidal gold solution was measured as about 14 nm by UV-Vis spectrophotometry using the molar extinction coefficient of $2.4 \times 108\ M^{-1}\ cm^{-1}$ at 519 nm for calculation.

1.3 Synthesis of fluorescent nanoprobe

The sequence of S1 (100 μM, 1 μL) and S2 (100 μM, 1 μL) were first incubated in Tris-HCl buffer at 95 ℃ for 5 min, then slowly cooled down for 1 h. The hybridization complex was next added into AuNPs (200 μL, 10 nM) at 200:1 of

molar ratio. Then, the mixture was put in laboratory freezer (-20 ℃) for 5 h, and then thawed at room temperature. The solution was centrifuged (12000 rpm, 15 min) to remove the free *S1S2*. Next, 1 mg of SH-βCD were added into the mixture. After 12 h, the mixture was centrifuged to remove unmodified SH-βCD followed by the addition of S3 at 100:1 of molar ratio. Then, the resulting mixture was subjected to centrifugation at 12000 rpm for 10 min, and the supernatant was removed for the purpose of removing excess S3, while the oil-like precipitate was dissolved in ultrapure water to keep the final volume identical.

1.4 Fluorescence spectroscopy assay

For fluorescence detection of ATP, the constructed nanoprobe (2 nM) was dispersed in Tris-HCl buffer, followed by addition of ATP. For fluorescence detection of oxygen, the nanoprobe was dispersed in Tris-HCl buffer, followed by addition of AzoR and NADPH under hypoxic conditions (1% O_2). Fluorescence emission intensity was recorded from 655 to 730 nm with excitation set at 633 nm for ATP assay, and 560 to 640 nm with excitation set at 540 nm for oxygen assay.

1.5 Cell culture.

MCF-7 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 100 U/mL 1% antibiotic penicillin/streptomycin and maintained at 37 ℃ in a 100% humidified atmosphere containing 5% CO_2.

1.6 Confocal fluorescence imaging

For ATP and oxygen responsive imaging in living cells, MCF-7 cells were seeded in confocal dishes and cultured in CO_2 incubator for 24 h. After refreshing the medium, cells were incubated under hypoxic conditions (1% O_2) for 3 h, followed by addition with nanoprobe (4 nM) for another 2 h. Before imaging, the cells were washed with cold PBS for 3 times. The cells were finally subjected to image by Olympus FV1000-MPE multiphoton laser scanning confocal microscope. (TAMRA channel: λex= 559 nm and λem= 560-620 nm; Cy5 channel: λex= 635 nm and λem= 650-720 nm).

1.7 MTT assay.

MCF-7 cells (1×10^6 cells/well) were cultured with 100 μL of fresh culture medium (DMEM) in 96 well microtiter plates in a 5% CO_2/95% air incubator for 24 h. After the supernatant (the medium) was removed and washed three times with DMEM, the nanoprobe or β-lapachone was added to each well with different concentrations for 24 h. Then, 60 μL of MTT solutions (0.5 mg/mL) were added into each well and incubated together for another 4.0 h while the supernatant was following removed. Finally, we added DMSO (150 μL per well) to each well to dissolve the formazan crystals. The absorbance value was read at 490 nm with a RT 6000.

1.8 Imaging ATP in MCF-7 cells treat with $CoCl_2$ and glucose.

For $CoCl_2$ treatment, the MCF-7 cells were cultured with 1 mL fresh culture medium and then incubated with the different concentration $CoCl_2$ for 24 h following by incubation with nanoprobe (4 nM). For glucose treatment, the MCF-7 cells were incubated under hypoxia for 2 h following by culture with glucose (4 nM) for 2h. Then, nanoprobes were added into the cells and incubated for 3 h. Each well was washed twice with cold PBS before imaging and then subjected to Olympus FV1000-MPE multiphoton laser scanning confocal microscope with exiting wavelengths at 559 nm for TAMRA channel and 635 nm for Cy5 channel.

1.9 Imaging O_2 and ATP in tissues.

MCF-7 cells (1×10^6 cells in 100 μL PBS) were injected into the selected positions of the nude mice to establish tumor-bearing nude mice models. When the tumors grew to 200-400 mm^3, we harvested the tumors and liver, and obtained 50 μm-thick tumor/normal tissues slices subsequently. Tumor tissues were incubated under hypoxic conditions for 3 hours. Then, fluorescent nanoprobe (5 nM) were incubated with tumor/normal tissues for another 2 hours. Thereafter, the tissue slices were washed three times with D-PBS to remove free nanoprobe, followed by imaging of the tissue slices using a confocal microscope.

1.10 Preparation of AuNP-ATG7-siRNA nanoprobe.

The sequence of S1 and S2 were treated according to 1.4. The sequence of S3(100 μM,

1 μL), R1 (100 μM, 1 μL) and R2 (100 μM, 1 μL) were first incubated at 95℃ for 5 min, then slowly cooled down for 1 h. The hybridization S3/R1/R2 complex was next added into the solution of S1/S2- and SH-βCD-modified AuNPs. The processing method were consistent with 1.4.

1.11 Using functionalized nanoprobes to inhibit autophagy.

MCF-7 cells were cultured with 1 mL fresh culture medium overnight, and then incubated with the different concentration of $CoCl_2$ for 24 h, following by incubation with AuNP-ATG7-siRNA nanoprobe (2, 4, 8 nM) or nanoprobe (4 nM). Then cell was scrapted and harvested after washing with DPBS. Protein was extracted by RIPA lysates with protease inhibitor cocktail for 30 min at 4℃. After centrifugation of 12000 rpm for 10 min at 4℃, supernatant was collected and protein concentration was measured by BCA assay (Novagen, Germany). 40 μg protein was loaded and electrophoresed in 10% SDS-PAGE gel, and transferred to PVDF membrane (GE healthcare, USA). The samples were immunoblotted with Atg7, LC3-II and β-actin antibody at 4℃ overnight after blocking with skimmed milk, followed by incubation with HRP-Goat anti-Rabbit or anti-Mouse IgG (1:4000) at room temperature for 2 h. Then, the protein expression was detected by Bio-Rad ChemiDoc XRS system. All the western blotting experiments were repeated 3 times.

2. Oligonucleotides Sequences

Name	Sequence（5'-3'）
S1	acc tgg ggg agt att gcg gag gaa ggt ggg tta ggg ata-Cy5
S2	tat ccc taa ccc taa ccc ata-SH
S3	ttt(ABZ) ttt tgt tag tgt tag tgt tag-TAMRA
R1	cag ccu ggc auu uga uaa att-cta aca cta a
R2	uuu auc aaa ugc cag gcu gtt

3. Experimental data

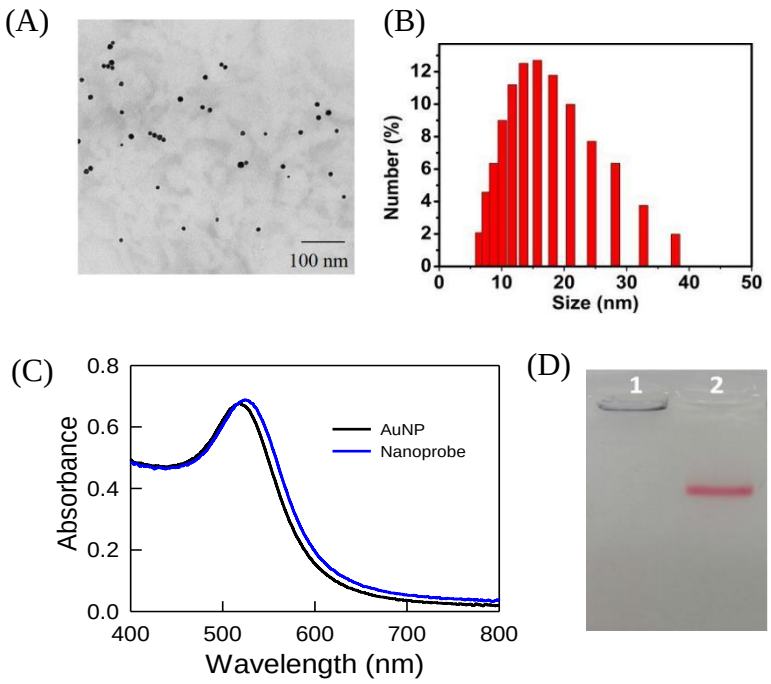


Fig. S1 . (A) TEM characterization of AuNPs. (B) DLS analysis of AuNPs. (C) Absorption spectra of AuNPs (black curve), and the constructed nanoprobe (blue curve). (D) 2% agarose gel electrophoresis image of AuNPs (lane 1), and the constructed nanoprobe (lane 2)

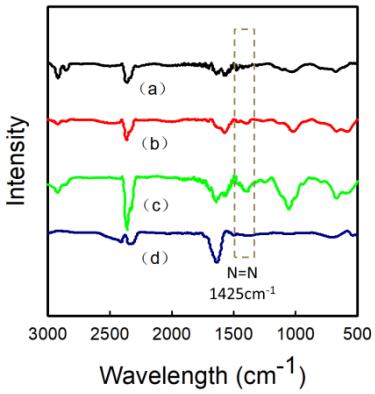


Fig. S2 FTIR spectra of (a) AuNPS, (b) βCD-decorated AuNPs, (c) fluorescent nanoprobe, and (d) fluorescent nanoprobe upon with hypoxic treatment. (The wavenumber located at 1425 cm^{-1} was assigned to the N=N stretching in the

compounds of azobenzene).

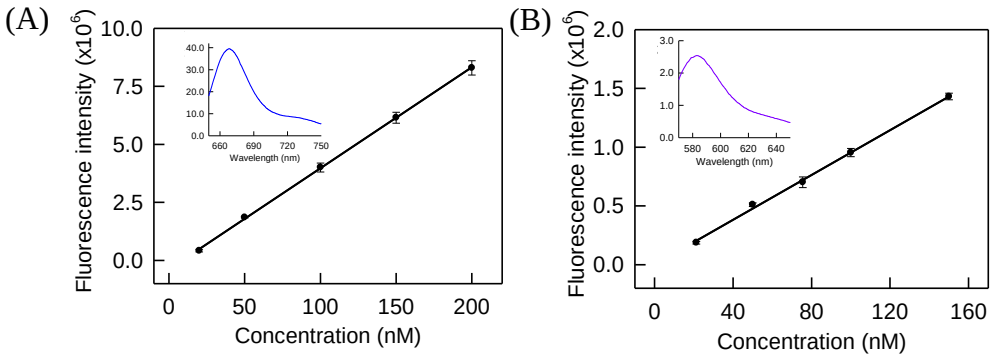


Fig. S3 Evaluation of amounts of substrate strands on each AuNP. Standard linear calibration curve of fluorescence signal against the concentration of Cy5 (A) and TAMRA (B) labeled strands. The error bars represent the standard deviations.

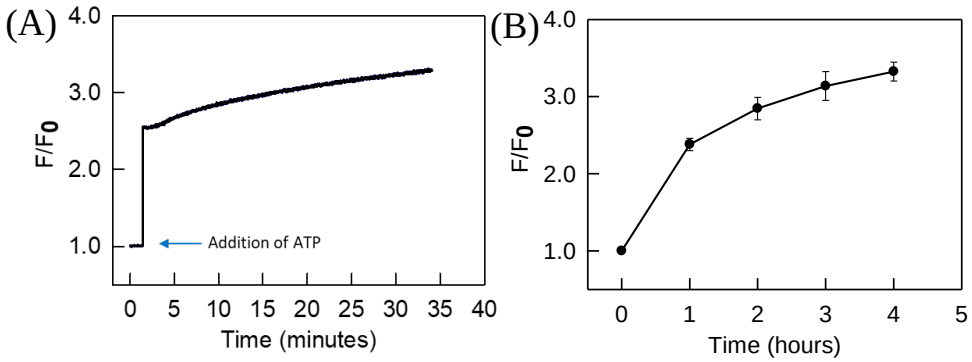


Fig. S4 (A) Time-dependent fluorescence spectra of nanoprobe (2 nM) upon ATP (20 mM) addition. λex=635 nm. (B) Time-dependent fluorescence spectra of nanoprobe (2 nM) upon azoretuctase (75 μg/mL) addition under hypoxic conditions. Hypoxia conditions in solution were mimicked by addition of 75 μg/mL azoreductase and 50 μM NADPH under 1% O_2 at 37 ℃ for 3 h. λex=540 nm.

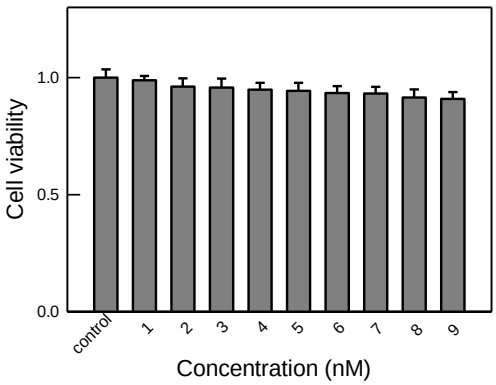


Fig. S5 Cell relative viability of MCF-7 cells upon treated with the different concentration of nanoprobe (from left to right: 0, 1, 2, 3, 4, 5, 6, 7, 8 and 9 nM, respectively). The concentration of nanoprobe was defined by AuNPs. Error bars represent variations between three measurements.

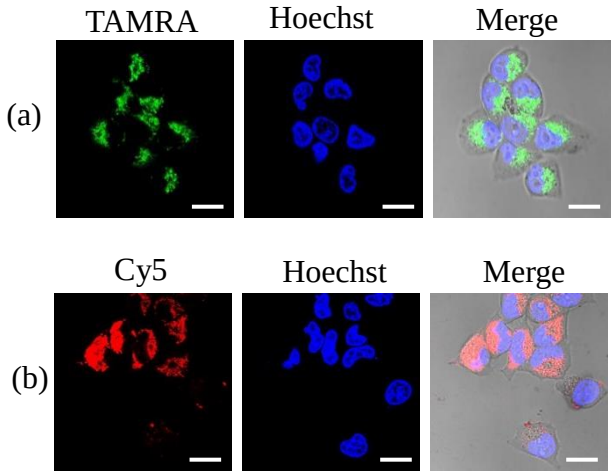


Fig. S6 Confocal fluorescence images of MCF-7 cells incubated under (a) hypoxic, (b) normoxic conditions, and followed by the incubation with nanoprobe (4 nM) and Hoechst 33342. (Blue color: nuclei stained by Hoechst 33342, green color: TAMRA, red color: Cy5). Scale bar: 15 μm.

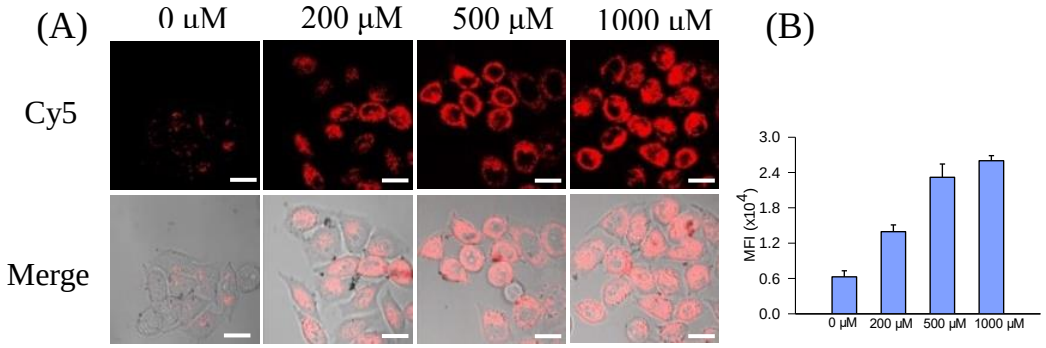


Fig. S7 (A) Confocal fluorescence images of MCF-7 cells incubated with different concentration of ATP, followed by the addition of nanoprobe (4 nM) at 37 ℃ for 1 h. (B) Relative pixel intensity of the Cy5 fluorescence images from panel A. λex = 635 nm. Scale bar: 15 μm.

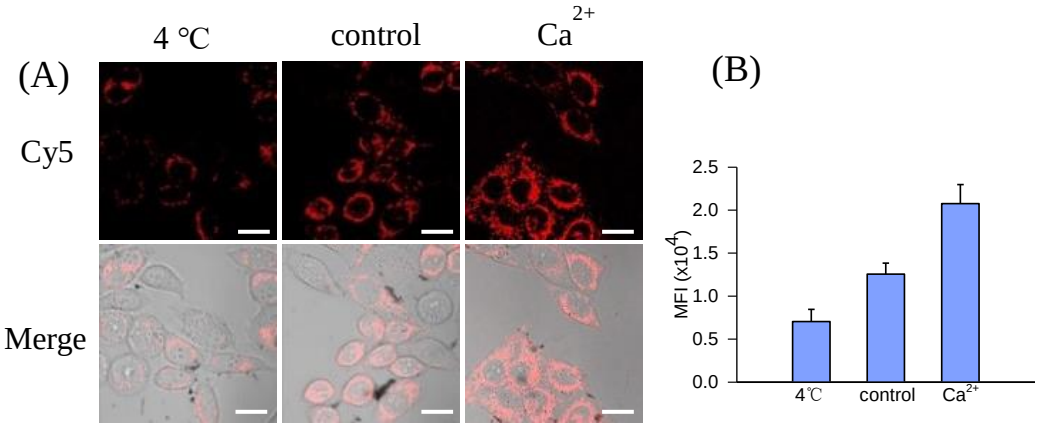


Figure S8. (A) Confocal fluorescence images of MCF-7 cells cultured under different treatments, followed by incubation with 4 nM nanoprobe for 4 h at 37 ℃. From left to right: 4 ℃, control, and 5 mM Ca^{2+}. (B) Relative pixel intensity of the Cy5 fluorescence images from panel A. λex = 635 nm. Scale bars: 15 μm.

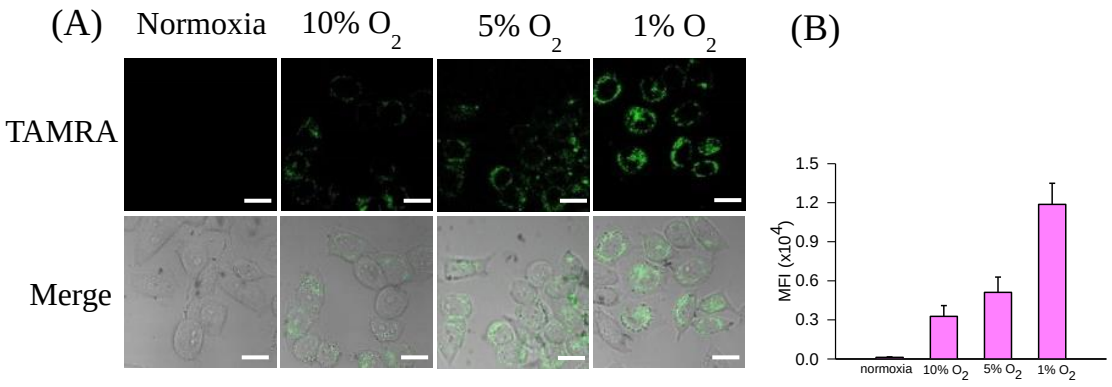


Fig. S9 (A) Confocal fluorescence images of MCF-7 cells incubated with different concentration of O_2, followed by the addition of nanoprobe (4 nM) at 37 ℃ for 1 h. (B) Relative pixel intensity of the TAMRA fluorescence images from panel A. λex = 559 nm. Scale bar: 15 μm.

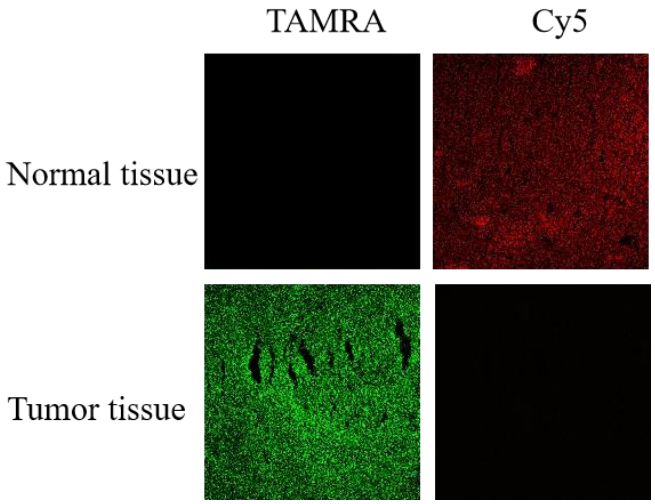
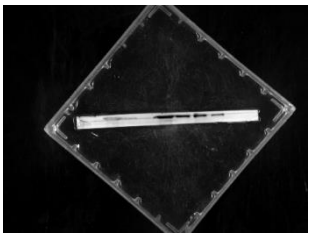
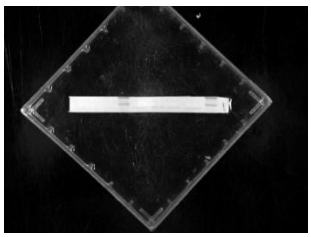
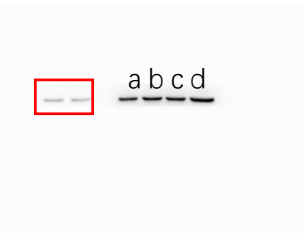
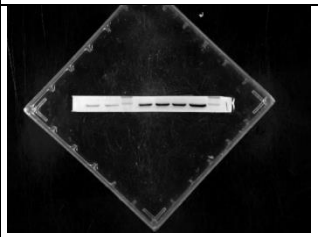
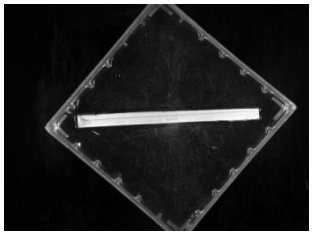
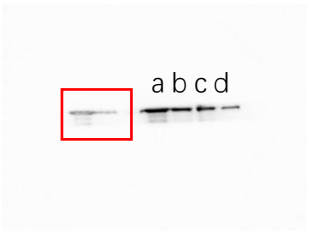
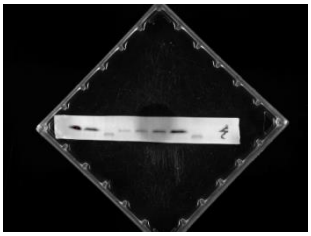
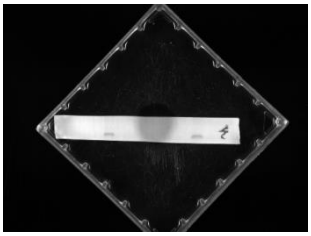
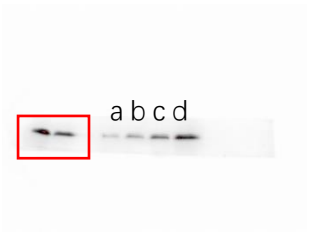


Fig. S10 Fluorescence imaging of tumor tissue and normal tissue upon treated with fluorescent nanoprobe (5 nM).

Protein	Merged	White light	Blotting
β-actin			
ATG-7			
LC-3			

Note: in red box: irrelevant experiment

Fig. S11. Western blot analysis of Atg7, LC3 II and β-actin protein levels in MCF-7 cells upon treated with (a)AuNPs, (b)2 nM AuNP-Atg7 siRNA, (c) 4 nM AuNP-Atg7 siRNA and(d) 8 nM AuNP-Atg7 siRNA under hypoxic conditions.