Electronic Supplementary Information for

Azoreductase activatable, endonuclease-gated nanodevice for spatiotemporal amplification imaging microRNA-21 in hypoxic tumor cells

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1. Experimental Section

1.1 Materials and Reagents

Ferric chloride ($FeCl_3$), and sodium dithionite ($Na_2S_2O_4$) were purchased from Energy Chemical Technology Co., Ltd (Shanghai, China). 4,4'-(E)-Diazene-1,2-diyldibenzoic acid (H_2AzDc) was purchased from MREDA (Beijing, China). N, N-Dimethylformamide (DMF) was purchased from MACLIN (Shanghai, China). Apurinic/apyrimidinic endonuclease 1 (APE1) and the 10× NE Buffer 4 were purchased from NEW ENGLAND Biolabs (USA). The rat liver microsomes was purchased from CHI Scientific (Jiangsu China). Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Beyotime (Shanghai, China). All the chemicals were of analytical grade without further purification. Ultrapure water obtained from the Millipore Milli-Q water purification system (18.2 MΩ) was used in all assays. The DNA oligonucleotides were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and purified by high-performance liquid chromatography. The sequences are shown in Table S1.

1.2 Instruments

UV-vis absorption spectra were recorded on a Hitachi U-4100 UV/vis spectrophotometer (Kyoto, Japan). Fluorescence images of cells were obtained by Nikon A1R MP multiphoton and confocal microscope system (Nikon, Tokyo, Japan). Transmission electron microscopy images were obtained on JEM 1200EX microscope (JEOL, Ltd., Japan).

1.3 Synthesis of AMOF

6 mg anhydrous ferric chloride ($FeCl_3$), 10 mg 4,4'- azobenzene dicarboxylic acid (H_2AzDc) and 10 mL N, N-dimethylformamide (DMF) were added in a round bottom flask, then the mixtures were ultrasonicated to dissolve completely. After that, the reaction system was placed at 100℃ for 16 h. Upon reaction, the solution was cooled to room temperature, and centrifuged to take the precipitate. Furthermore, the collected precipitate was washed with DMF until clear and colorless, and then washed with absolute ethanol. Finally, the product was dried in oven at 60℃ and stored at 4℃.

1.4 Extraction of Red Blood Cell Membranes

6 mL fresh sheep blood was centrifuged at 3000 rpm for 10 min at 4℃, then the supernatant was discarded and the precipitates were washed with cold PBS buffer to collect red blood cells. The red blood cells were redispersed with hypotonic cell lysate containing protease inhibitors and cleaved at 4℃ for about 2 h. After cell lysis, the solution was centrifuged at 12000 rpm for 60 min at 4℃ and the supernatant was discarded. Then, the precipitates were washed with cold PBS buffer for several times until clear and no obvious redness. Lastly, the red blood cell membrane was collected and stored at -20℃.

1.5 Construction of Nanodevice

AMOF@AP-CHA:

To obtain AP-CHA probes, AP-LH1 and FH2 were annealed at 95℃ in buffer solution (10 mM PBS, 5 mM $MgCl_2$, pH = 7.4) for 5 min. Next, the product was placed on ice for 5 min immediately and then placed at room temperature for 3 h. After the addition of AP-CHA probes and 1 mg of newly prepared AMOF, the solution was mixed for 30 min. Subsequently, the mixtures were centrifuged, and the precipitates were washed by buffer. Finally, the prepared AMOF@AP-CHA was stored at 4℃.

AMOF@AP-CHA@RBCM:

The red blood cell membrane suspension was added into the prepared AMOF@AP-CHA solution. After vortex mixing, the mixtures were ultrasonicated for 10 min, and then centrifuged at 10000 rpm for 10 min at 4℃. Finally, the products were collected and re-dispersed in the buffer for 4℃ storage.

1.6 Loading Efficiency

The load efficiency (LE) of hairpin FH2 on AMOF was quantitatively determined by fluorimetry. Typically, 0.2 mg/mL AMOF solution was incubated with 100 nM FH2 for 30 min. Then, the solution was centrifuged, and the precipitation and supernatant were collected separately. The fluorescence standard curve of FH2 was constructed by fluorescence spectroscopy to determine the amount of free FH2 in the supernatant. The load efficiency can be calculated as follows: LE (%) = (initial amount of FH2 - free amount of FH2)/initial amount of FH2, where the initial amount of FH2 represents the total amount of FH2 before the addition of AMOF, and the free amount of FH2 is the content of FH2 in the supernatant obtained by centrifugation upon incubated with

AMOF.

1.7 Cell Culture and Cytotoxicity Assay

Cell Culture: Human breast cancer (MCF-7) cells were grown in a fresh complete culture medium (DMEM with 10% fetal bovine serum and 1% double antibody solution of 100 U/mL) in humid air with 5% CO_2 (37℃).

The primary Human breast cancer (MCF-7) cells were donated by Xiangya Hospital (Central South University, China)

Cytotoxicity Assay: 100 μL cell suspension was added into 96-well plate and cultured for 36 h. Upon washed three times, MCF-7 cells were incubated with different concentrations of samples for 24 h. After incubation, the sample solution was washed away and 100 μL MTT solution (0.5 mg/mL) was added to each well and continued to be applied for 4 h. Then, the solution in each well plate was carefully removed. Subsequently, 100 μL DMSO was added to each well, and the purple crystal was completely dissolved by gently oscillating on the oscillator for 5-10 min. Finally, the absorption value at the wavelength of 490 nm in each well was read with RT-6000.

1.8 Imaging Experiment of Cell

MCF-7 cells were seeded in culture dishes and cultured in cell incubator for 48 h before replaced with fresh medium. By adjusting the ratio of nitrogen and oxygen, the cells were cultured under different oxygen concentrations for 4 h, and then washed thoroughly with PBS. Subsequently, MCF-7 cells were incubated with AMOF@AP-CHA@RBCM for 3 h and then washed with PBS. MCF-7 cells were imaged using confocal microscopy. Both the exciting wavelength of TAMRA and Cy5 channel is 561 nm.

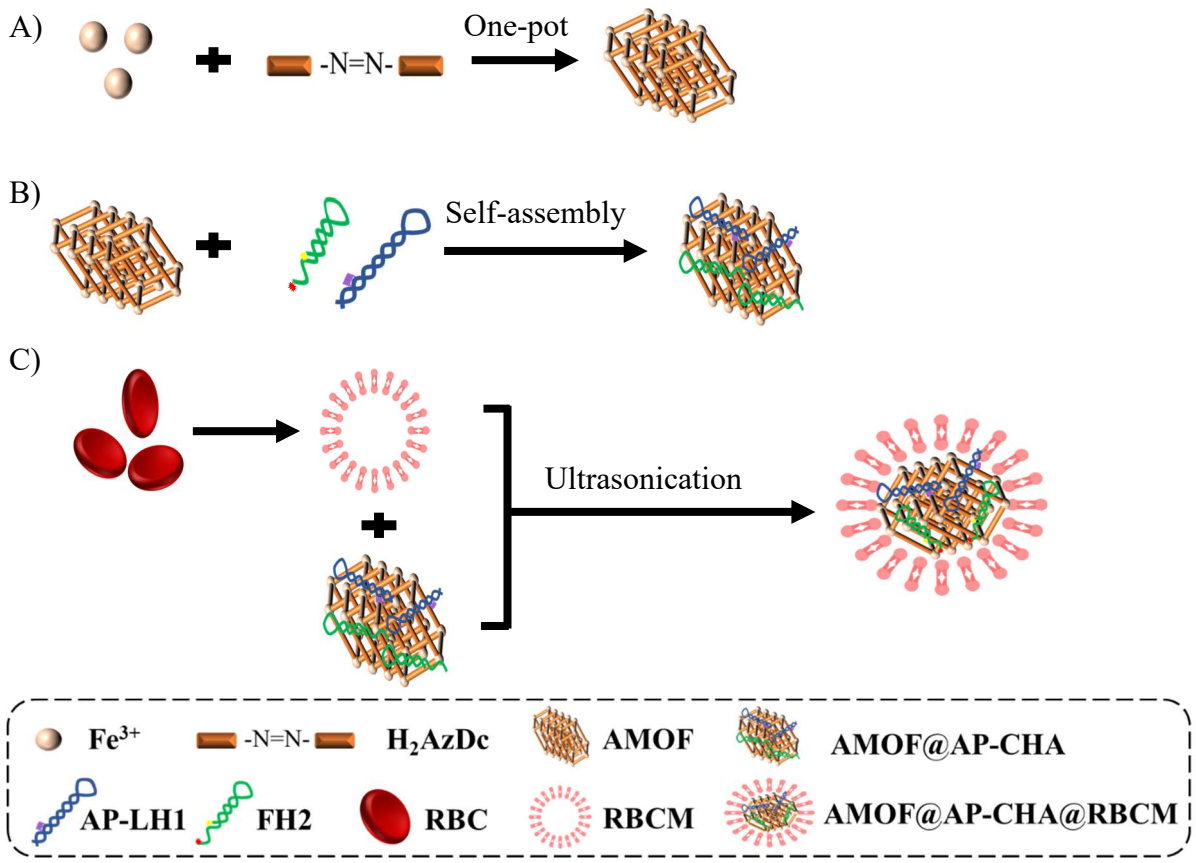
To verify the APE1-activatable performance of AMOF@AP-CHA@RBCM, the MCF-7 cells were pretreated with H_2O_2 (100 μM) for 2 h and washed with PBS. Next, MCF-7 cells were incubated with AMOF@AP-CHA@RBCM at 1% O_2. In order to investigate the magnifying imaging ability of AMOF@AP-CHA@RBCM for miR-21 in cells, MCF-7 cells were up-regulated the expression of miR-21 by introducing DNA mimics of miR-21. Then, MCF-7 cells were incubated with the constructed nanodevices at 1% O_2. Both the exciting wavelength of TAMRA and Cy5 channel is 559 nm.

2. Oligonucleotides Sequences

Table S1

Name	Sequence (5'-3')
miR-21 mimic	TAG CTT ATC AGA CTG ATG TTG A
LH1	CAG CTT ATC AGA CTG ATG TTG AGC AAA GCT CTA TTC AAC ATC AG TCT GAT AAG CTG
AP-LH1	CAG CTT XTC AGA CTG ATG TTG AGC AAA GCT CTA TTC AAC ATC AGT CTG ATA AGC TG
H1	TCA GAC TGA TGT TGA GCA AAG CTC TAT TCA ACA TCA GTC TGA TAA GCT G
H2	GCAAAGCTCTATTCAGACTGATGTTGAATAGAGCTTTGC TCAACATC
FH2	TAMRA- GCA AAG CTC TAT TCA GAC TGA TGT TGA ATA GAG CTT TGC (Cy5) TCA ACA TC
mis-1	TAG CTT ATC AGA CTC ATG TTG A
mis-2	TAG ATT ATC AGA CTC ATG TTG A
Survivin mRNA	ACC ACC GCA TCT CTA CAT TCA A
VEGF mRNA	GTG TGT GCC CAC TGA GGA GTC
Bcl-2 mRNA	ATG GCG CAC GCT GGG AGA

3. Experimental Data



Scheme S1 Schematic synthesis of AMOF (A), AMOF@AP-CHA (B), and AMOF@AP-CHA@RBCM (C).

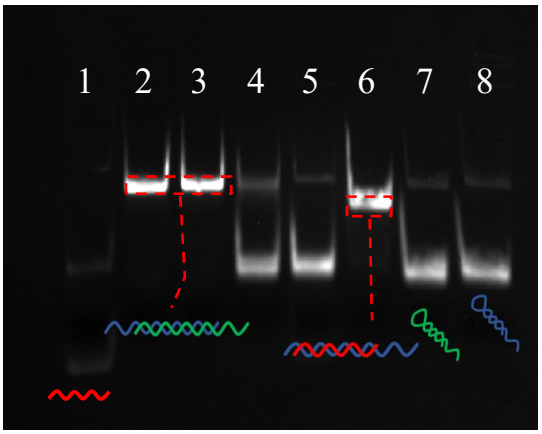


Fig. S1 A) SYBR Gold-stained 12% polyacrylamide gel electrophoresis (PAGE) imaging of H1, H2 with addition of miR-21. Lane 1: miR-21 (1 μM); Lane 2: miR-21 + H1 + H2 (0.5 μM, 1 μM, 1 μM); Lane 3: miR-21 + H1 + H2 (0.2 μM, 1 μM, 1 μM); Lane 4: H1 + H2 (1 μM, 1 μM); Lane 5: H2 + miR-21 (1 μM, 1 μM); Lane 6: H1 + miR-21 (1 μM, 1 μM); Lane 7: H2 (1 μM); Lane 8: H1 (1 μM).

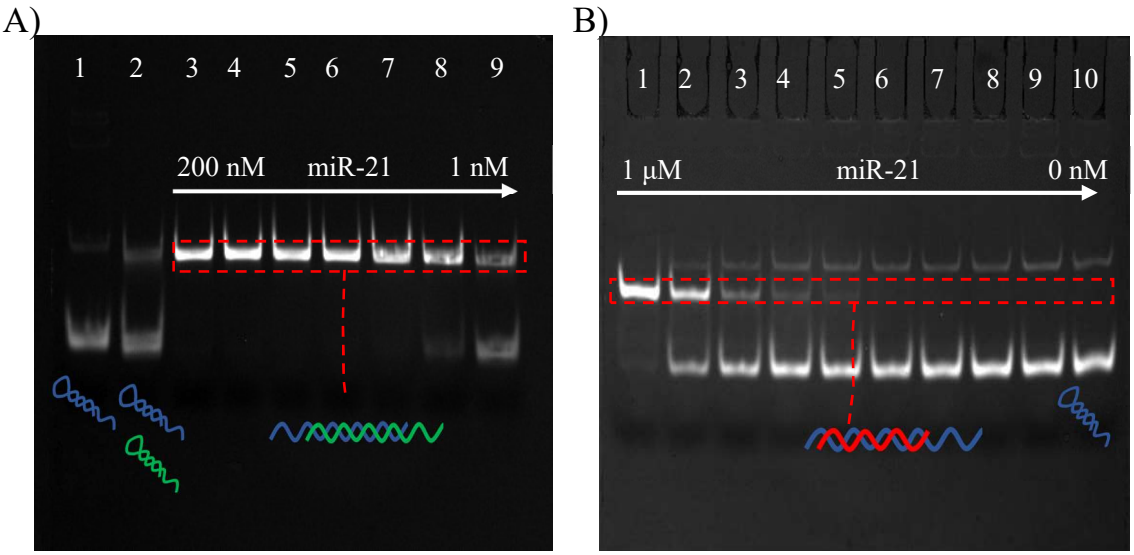


Fig. S2 The analysis of amplification efficiency of CHA. A) SYBR Gold-stained 12% PAGE imaging of CHA responding to different concentrations of miR-21. Lane 1: H1; Lane 2: H2; Lane 3-9: H1 + H2 + miR-21 (200, 100, 50, 20, 10, 5, 1 nM). Both of H1 and H2 was 1 μM. C) SYBR Gold-stained 12% PAGE imaging of H1 responding to different concentrations of miR-21. Lane 1-10: H1 + miR-21 (1 μM, 500, 200, 100, 50, 20, 10, 5,1, 0 nM). H1 was 1 μM.

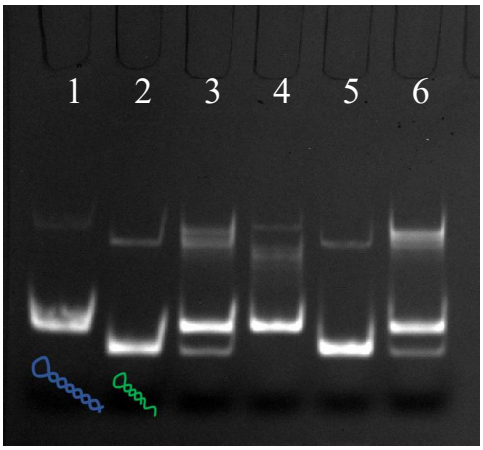


Fig. S3 SYBR Gold-stained 12% PAGE imaging of LH1, H2 with addition of miR-21. Lane 1: LH1; Lane 2: H2; Lane 3: LH1 + H2; Lane 4: LH1 + miR-21; Lane 5: H2 + miR-21; Lane 6: LH1 + H2 + miR-21. The concentration of LH1, H2 and miR-21 was 1 μM.

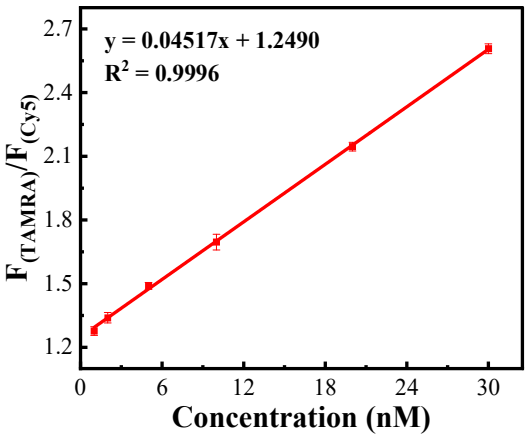


Fig. S4 F_{TAMRA}/F_{Cy5} linear curve of AP-CHA with addition of different concentrations of miR-21 in the presence of APE1 (1 U/mL). F_{TAMRA} is the fluorescence intensity of TAMRA and F_{Cy5} is the fluorescence intensity of Cy5 in each fluorescent spectrum. AP-CHA was 50 nM. λex = 548 nm.

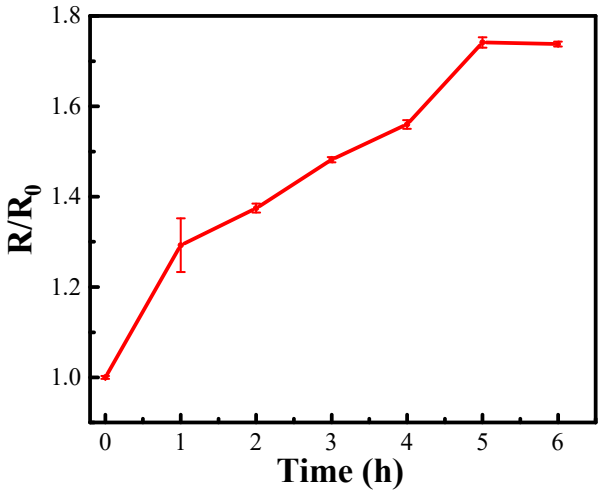


Fig. S5 The analysis of dynamic efficiency of CHA. Pretreated by APE1 (1 U/mL), the mixture of AP-LH1 and FH2 was added with miR-21 (10 nM), then collect the R/R_0 as function of time. R and R_0 is the ratio of fluorescence intensities of TAMRA to Cy5 in fluorescent spectrum before and after the addition of miR-21. AP-LH1 and FH2 was 50 nM. λex = 548 nm.

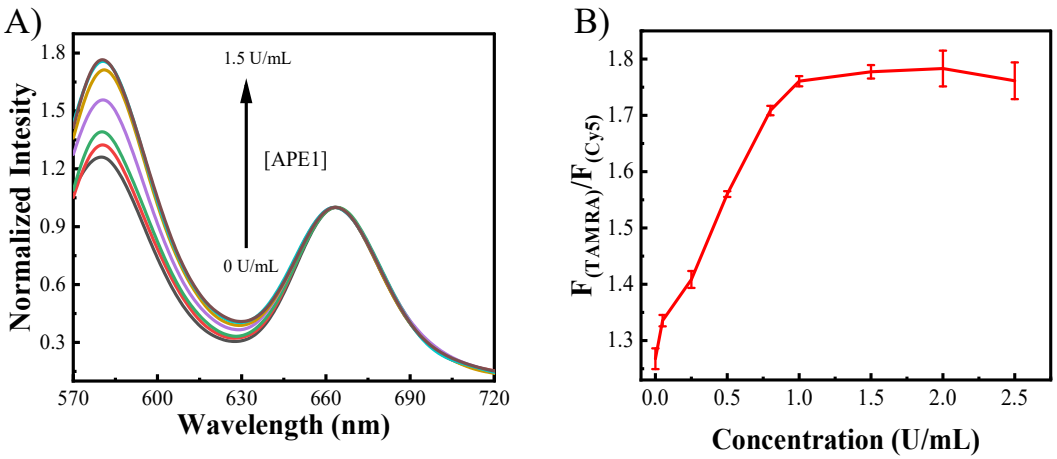


Fig. S6 A) Normalized fluorescence emission spectra of AP-CHA upon addition of different concentrations of APE1 (0, 0.1, 0.25, 0.5, 0.8, 1, 1.5 U/mL). AP-CHA was 50 nM, and miR-21 was 10 nM. The fluorescence intensity of Cy5 in each spectrum was normalized to 1. B) F_{TAMRA}/F_{Cy5} curve of AP-CHA with different concentrations of APE1. F_{TAMRA} is the fluorescence intensity of TAMRA and F_{Cy5} is the fluorescence intensity of Cy5 in each fluorescent spectrum. AP-CHA was 50 nM, and miR-21 was 10 nM. λex = 548 nm.

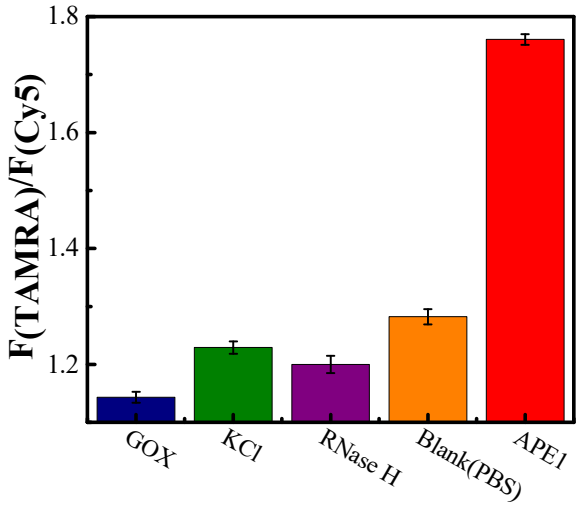


Fig. S7 The F_{TAMRA}/F_{Cy5} of AP-CHA upon treated with GOX (1 μM), KCl (10 mM), RNase H (1 U/mL), blank (PBS, 10 mM) and APE1 (1 U/mL), following by addition of 10 nM miR-21 in PBS. The concentration of AP-CHA was 50 nM. λex = 548 nm.

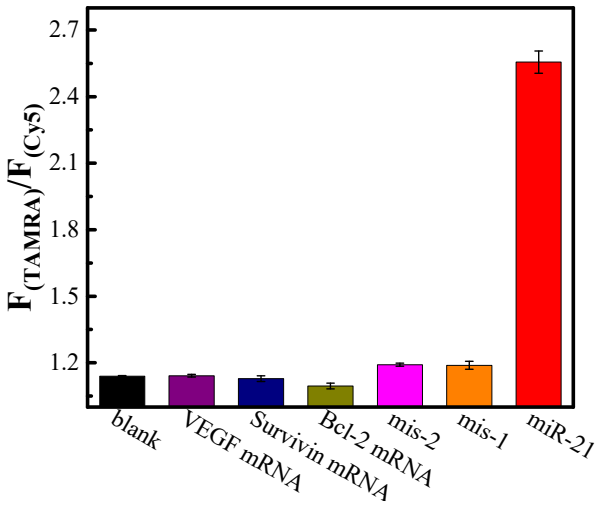


Fig. S8 The F_{TAMRA}/F_{Cy5} of AP-CHA with addition of blank (PBS), VEGF mRNA, survivin mRNA, bcl-2 mRNA, mis-2, mis-1 and miR-21 in the presence of APE1 (1 U/mL). The concentration of all kinds of DNA was 50 nM. λex = 548 nm.

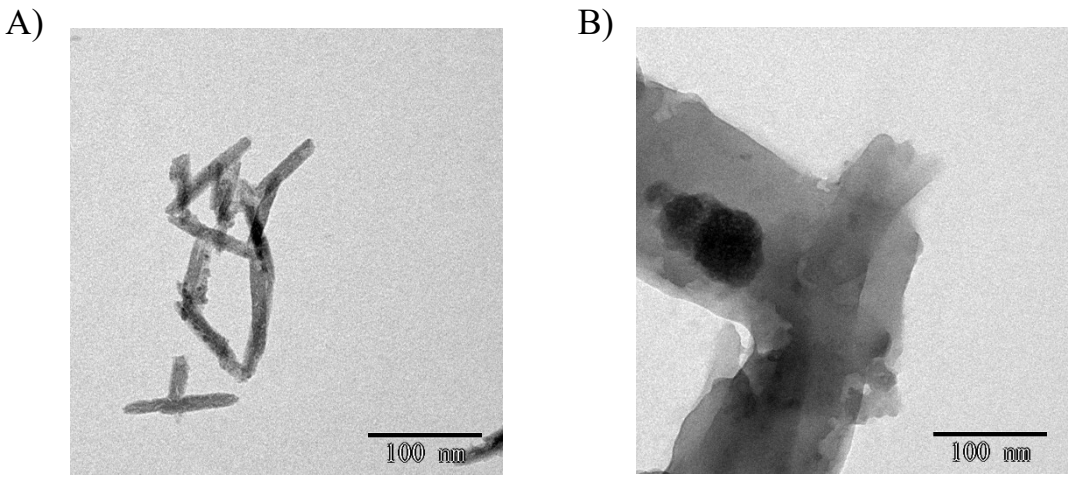


Fig. S9 A) TEM images of AMOF. B) TEM images of AMOF upon treated by $Na_2S_2O_4$ (SDT). Scale bar: 100 nm.

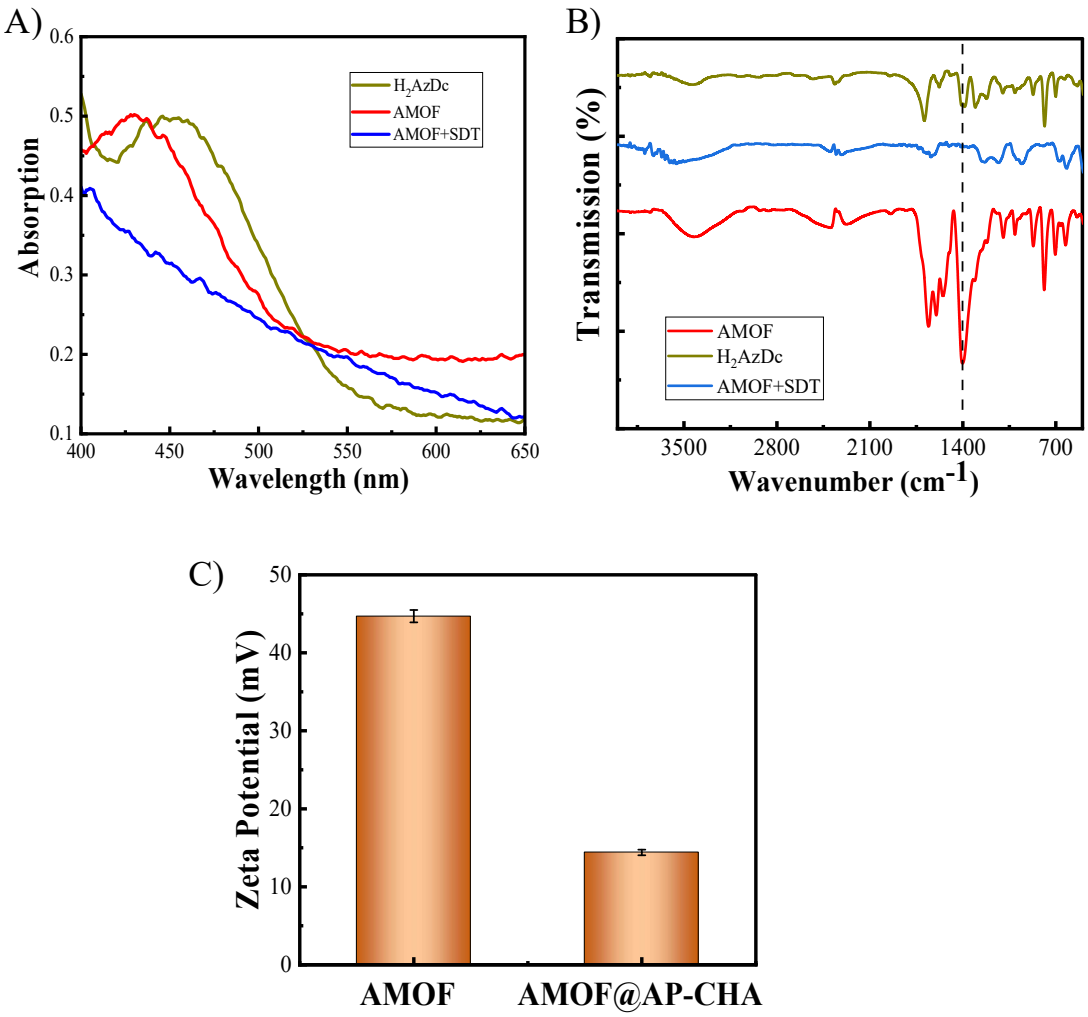


Fig. S10 Characterization of AMOF. A) The UV-vis absorption spectra of H_2AzDc (dark yellow curve), AMOF (red curve), and AMOF upon incubated with $Na_2S_2O_4$ (SDT, blue curve). (B) FTIR spectra of H_2AzDc (dark yellow curve), AMOF (red curve), and AMOF upon incubation with SDT (blue curve). C) ζ-potential of AMOF and AMOF@AP-CHA. Error bars represent the standard deviations from three repeated experiments.

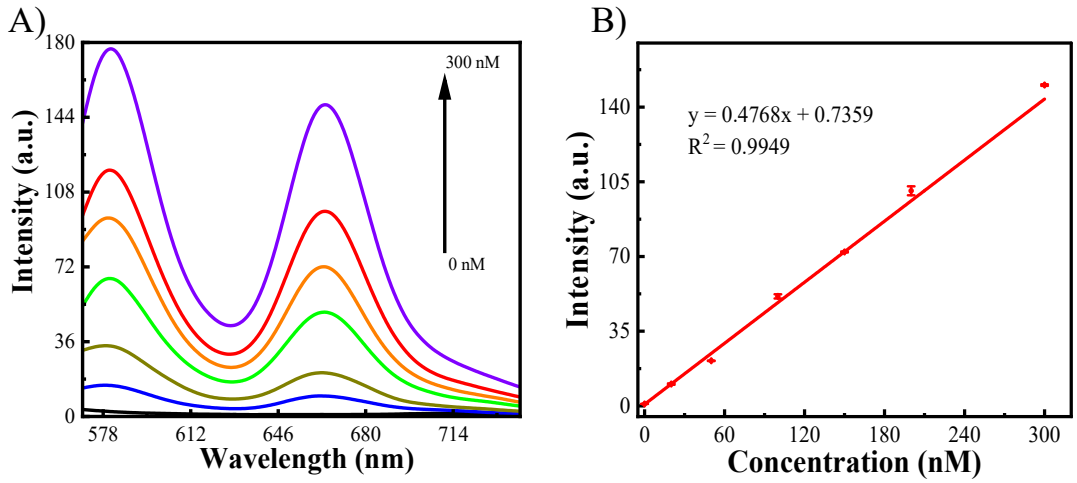


Fig. S11 A) The fluorescence spectra of different concentrations of FH2 (0, 20, 50, 100, 150, 200, 300 nM). B) Standard curve based on fluorescence intensity of Cy5 collected from FH2. Error bars represent the standard deviations from three repeated experiments. λex = 548 nm.

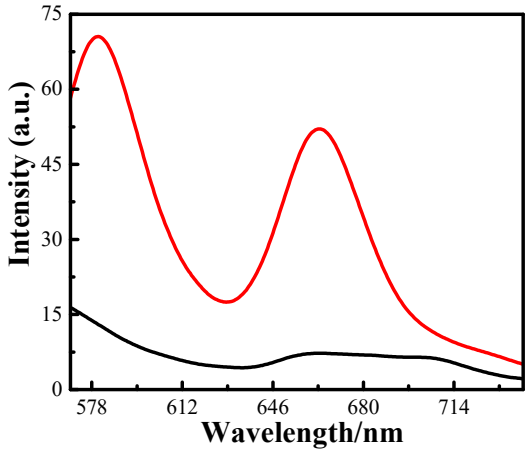


Fig. S12 The fluorescence spectra of 100 nM free FH2 (red line) and the supernatant collected from AMOF@FH2 following by centrifugation (black line, AMOF: 200 μg/mL, FH2: 100 nM). λex = 548 nm.

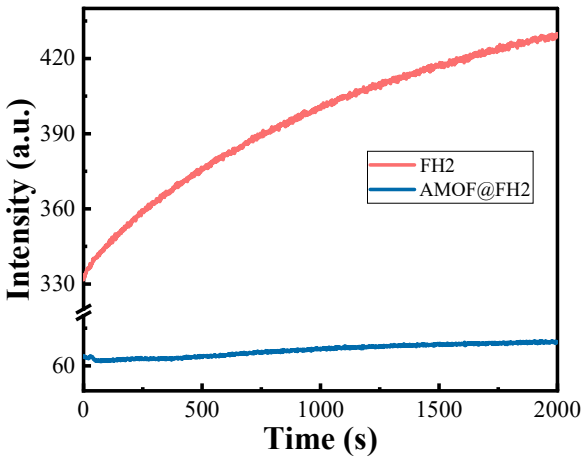


Fig. S13 Fluorescence changes with time of 100 nM FH2 (red curve) and AMOF@FH2 (blue curve) upon the addition of DNase I (3 U/mL). λex = 548 nm, λem = 580 nm.

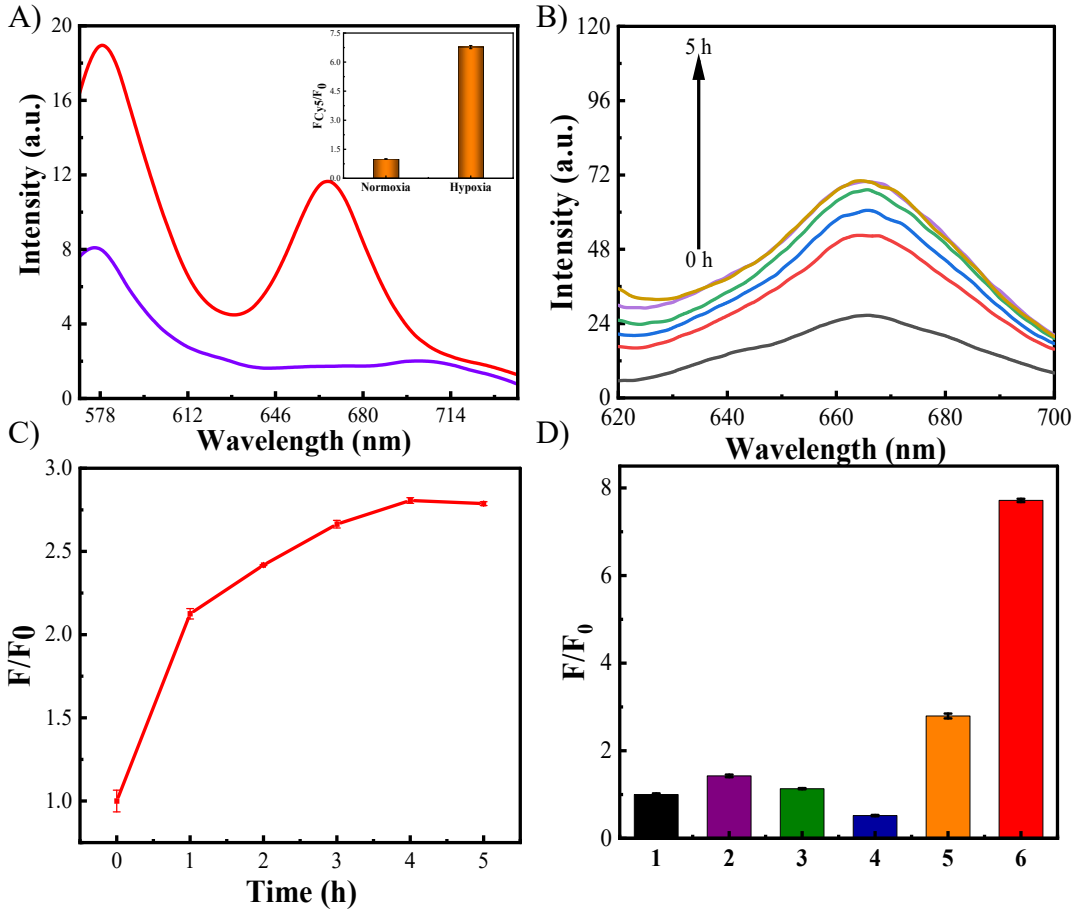


Fig. S14 A) Fluorescence emission spectra of AMOF@FH2 in the presence (red curve: rat liver microsomes: 100 μg/mL, NADPH: 50 μM) and absence (purple curve) of rat liver microsomes and NADPH. Inset: F_{Cy5}/F_0 of AMOF@FH2 under normoxia and hypoxia conditions. F_{Cy5} and F_0 represent the fluorescence intensity of Cy5 under hypoxia conditions (rat liver microsomes: 100 μg/mL, NADPH: 50 μM)) and normoxia conditions respectively. λex = 548 nm. B) Fluorescence emission spectra of AMOF@FH2 in the presence of rat liver microsomes (100 μg/mL) and NADPH (50 μM) with different time (0, 1, 2, 3, 4, 5 h). λex = 548 nm. C) F/F_0 of AMOF@FH2 upon treated with rat liver microsomes (100 μg/mL) and NADPH (50 μM) after different time. F and F_0 represent the fluorescence intensity of Cy5 before and after treatments, λex = 548 nm. D) F/F_0 of AMOF@FH2 upon treated by different biospecies. 1: PBS; 2: APE1 (1 U/mL); 3: ATP (1 μM);4: GSH (10 mM); 5: NADPH (50 μM); 6: rat liver microsomes (100 μg/mL), NADPH (50 μM). F and F_0 represent the fluorescence intensity of TAMRA before and after treatments, λex = 548 nm.

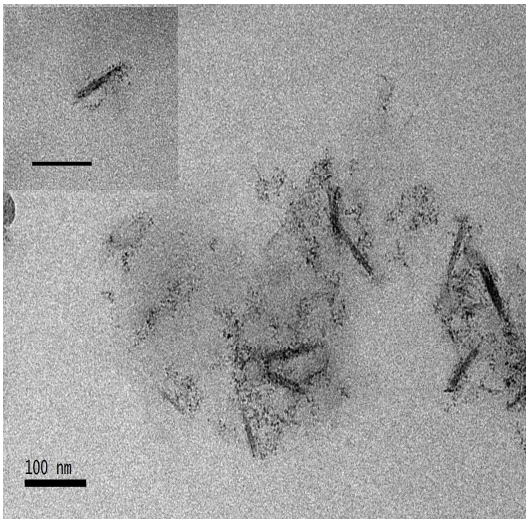


Fig. S15 TEM image of AMOF@AP-CHA@RBCM. Inset: partial enlargement of TEM image. Scale bar: 100 nm.

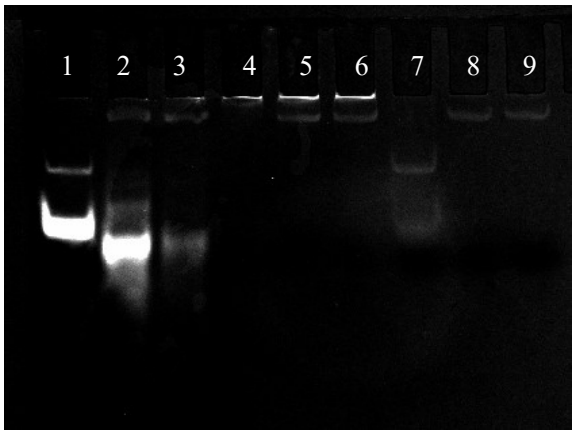


Fig. S16 SYBR Gold-stained 12% PAGE imaging. Lane 1: H2 in FBS for 0 day; Lane 2: H2 in FBS for 1 day; Lane 3: H2 in FBS for 2 days; Lane 4: AMOF@H2@RBCM in FBS for 0 day; Lane 5: AMOF@H2@RBCM in FBS for 1 day; Lane 6: AMOF@H2@RBCM in FBS for 2 days; Lane 7: AMOF@H2 in FBS for 0 day; Lane 8: AMOF@H2 in FBS for 1 day; Lane 6: AMOF@H2 in FBS for 2 days. The concentration of FBS was 10%.

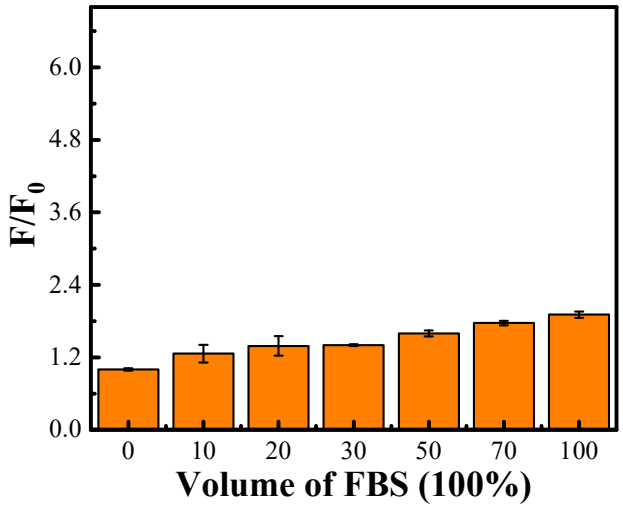


Fig. S17 F/F_0 of AMOF@FH2@RBCM upon incubated with different concentraions of FBS for 6 h. F is the fluorescence intensity of TAMRA and F_0 is the fluorescence intensity of TAMRA in PBS for 6 h. λex = 548 nm. λem = 580 nm.

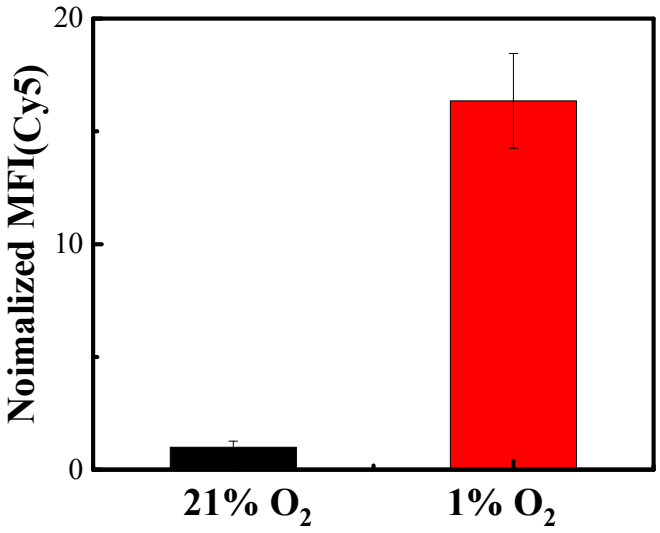


Fig. S18 The normalized MFI of Cy5 collected from MCF-7 cells upon AMOF@AP-CHA@RBCM pre-treatment under 21% O_2 and 1% O_2 for 3 h. The MFI of Cy5 collected from MCF-7 cells under 21% O_2 was normalized to 1. The concentration of AMOF@AP-CHA@RBCM was 0.15 mg/mL. λex = 561 nm.

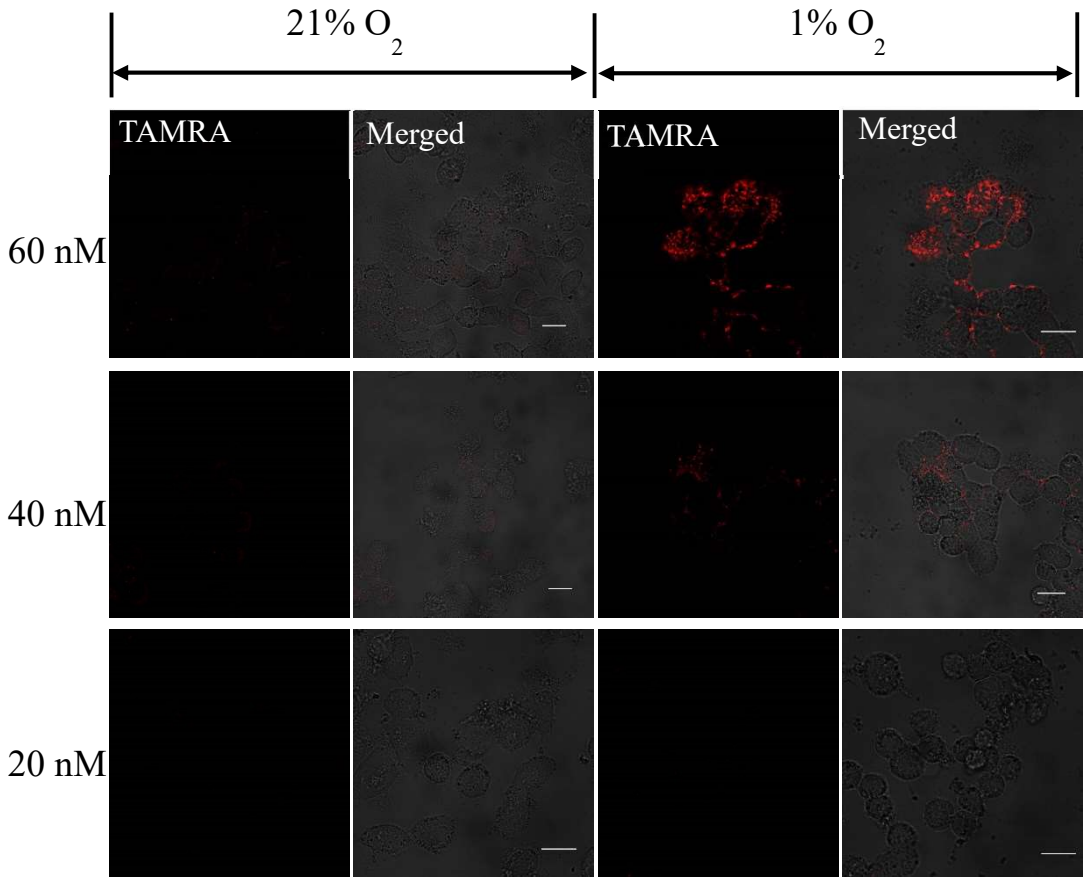


Fig. S19 Confocal fluorescence images of MCF-7 cells upon treated with AMOF@FH2 (AMOF: 0.15 mg/mL, FH2: 20, 40, 60 nM) for 3 h under 21% O_2 and 1% O_2. λex = 559 nm, Scale bar: 20 μm.

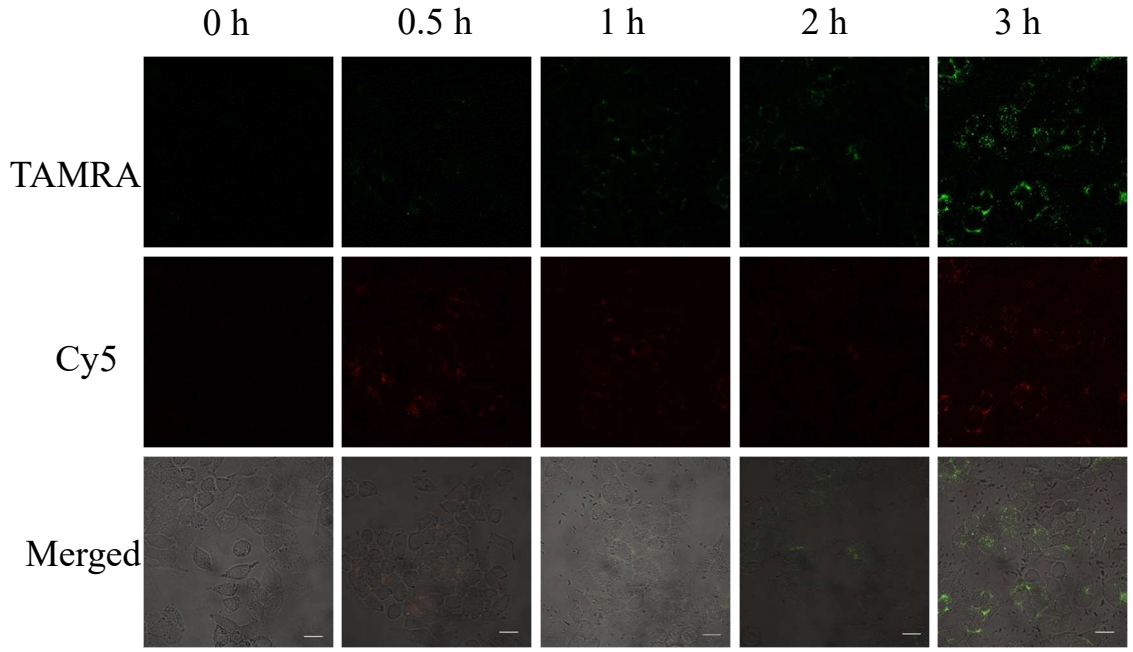


Fig. S20 Confocal fluorescence images of MCF-7 cells upon pre-treated with AMOF@AP-CHA@RBCM (0.15 mg/mL) for different time (0, 0.5, 1, 2, 3 h) under 1% O_2. Scale bar: 20 μm. λex = 559 nm.

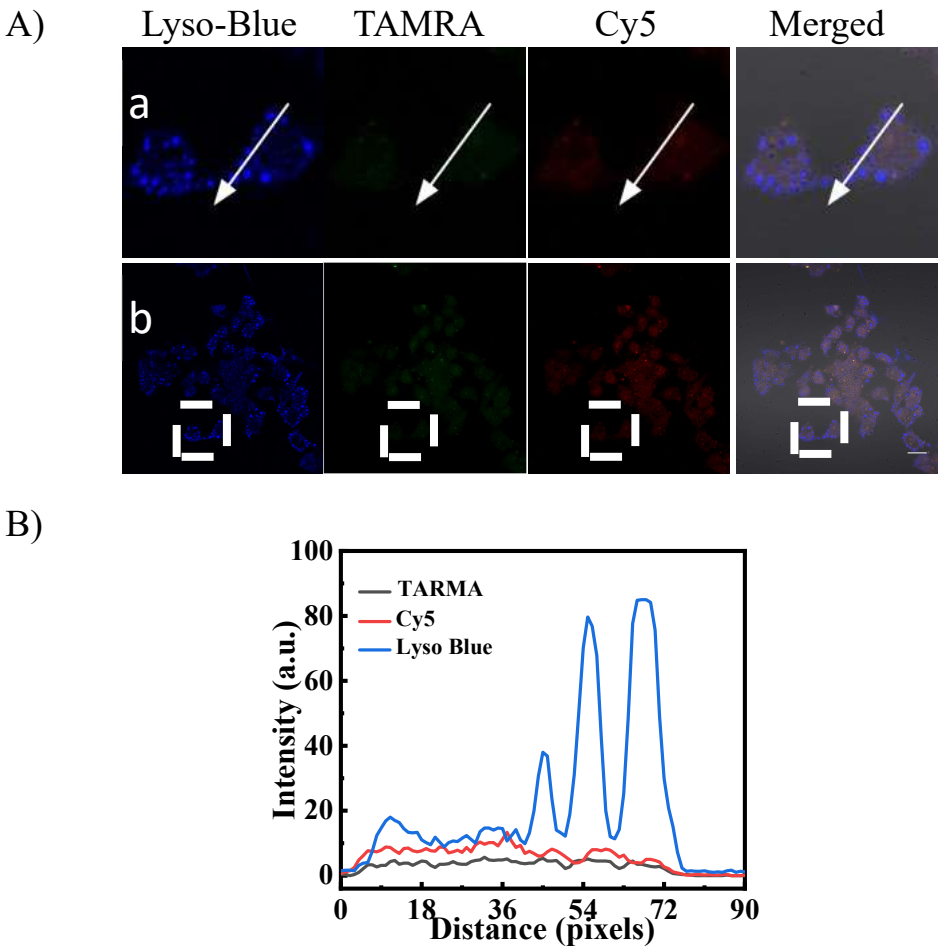


Fig. S21 A) Confocal fluorescence imaging of the MCF-7 cells which were pre-treated with AMOF@AP-CHA-RBCM (0.15 mg/mL) for 3 h under 1% O_2. a is a partial enlargement of b. Scale bar: 20 μm. Lyso-Blue channel: λex = 405 nm, TAMRA and Cy5 channel: λex = 561 nm. B) Fluorescence intensity distribution of white arrow areas in Image A).

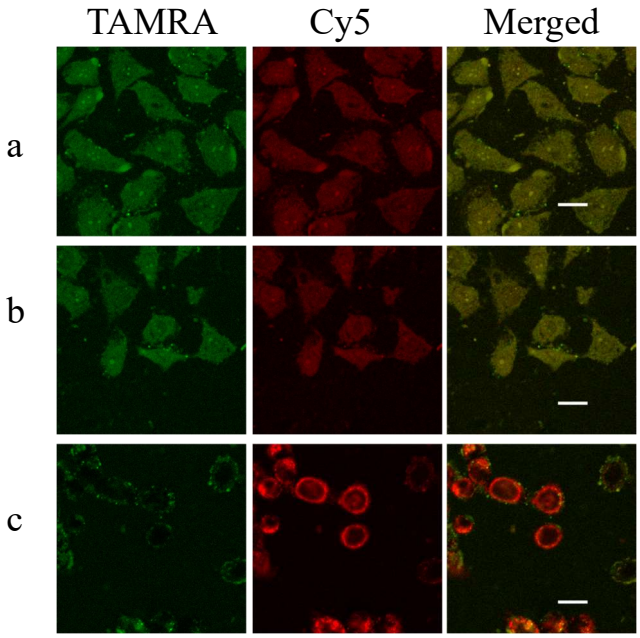


Fig. S22 Partial enlargement of Fig. 4A, scale bar: 20 μm.

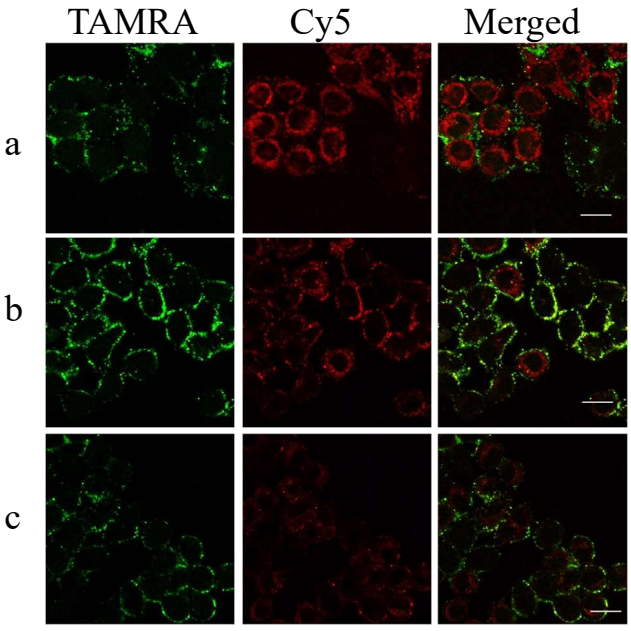


Fig. S23 Partial enlargement of Fig. 4B, scale bar: 20 μm.

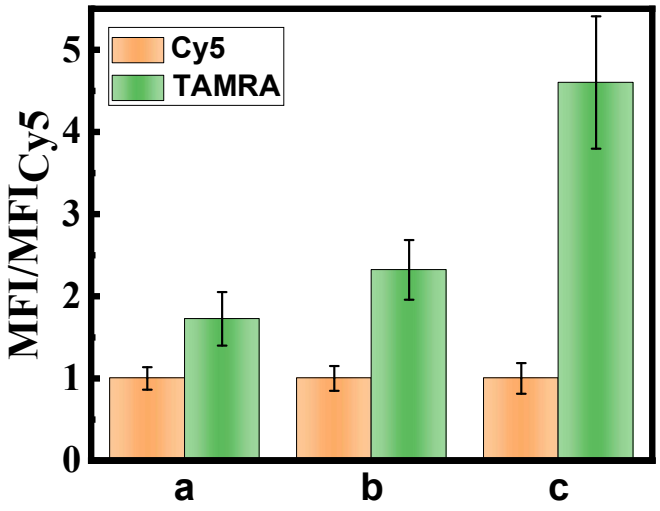


Fig. S24 The ratio of MFI between TAMRA channel and Cy5 channel in group a, b, c of Fig 4B. Error bars represent the standard deviations from three repeated experiments.

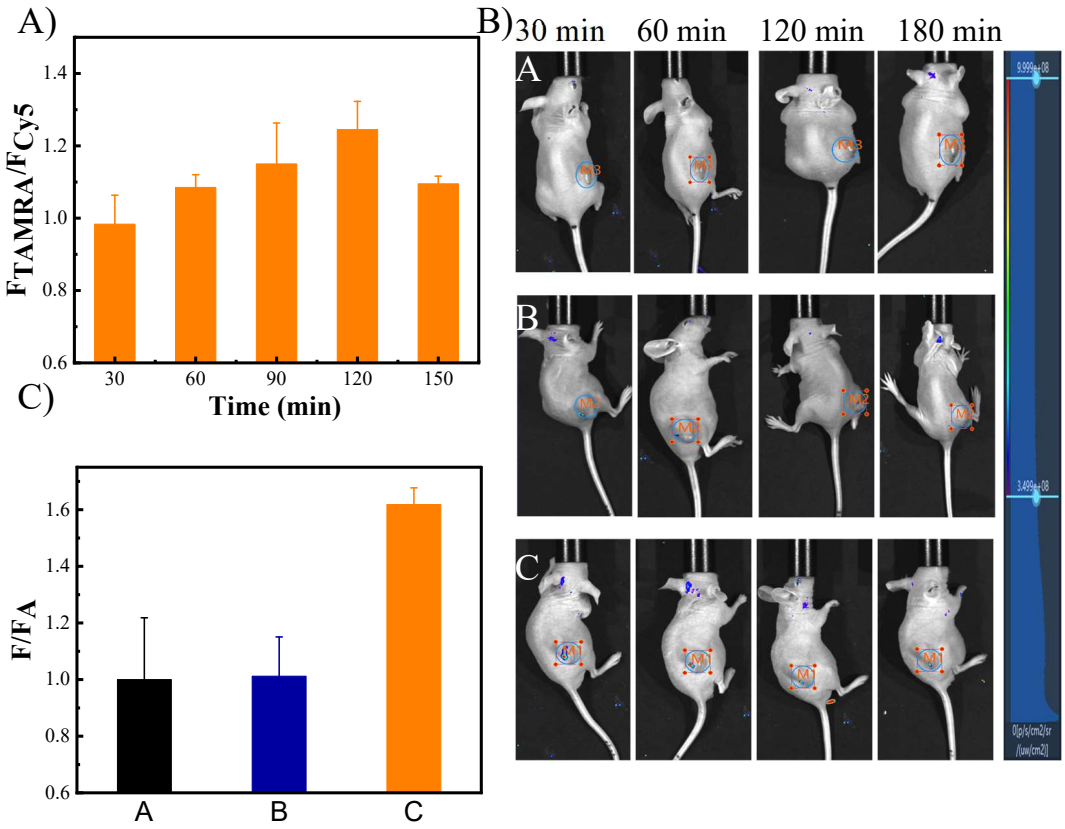


Fig. S25 The imaging investigation of AMOF@AP-CHA@RBCM *in vivo*. A) F_{TAMRA}/F_{Cy5} of tumor-bearing mice at different time points upon injected with AMOF@AP-CHA@RBCM. F_{TAMRA} and F_{Cy5} represent fluorescence of TAMRA and Cy5 respectively, λex = 535 nm. B) Representative fluorescence images of the tumor-bearing mice at the different time points upon injected with: (A) PBS, (B) AMOF@AP-CHA, (C) AMOF@AP-CHA@RBCM. The circles represent the tumor sites. C) Fluorescence quantification analysis of the tumor sites upon injected for 180 min of A-C in Fig. S23 B).