Supporting Information

In-situ Imaging of Cellular Reactive Oxygen Species and Caspase-3 Activity Using A Multifunctional Theranostic Probe for Cancer Diagnosis and Therapy

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cells, respectively.

Figure S11. Dark field images of caspase-3 inhibitor-treated Au-Ag-HM-containing HepG2 cells after laser irradiation and incubation for (A) 0 min and (B) 60 min; Dark field images of ROS inhibitor-treated Au-Ag-HM-containing HepG2 cells after laser irradiation and incubation for (C) 0 min and (D) 60 min.

Figure S12. (A) Cells activity of LO2 cells and HepG2 cells incubated with Au-Ag-HM or AgNPs for different time without laser irradiation. (B) Cells activity of LO2 cells and HepG2 cells incubated with Au-Ag-HM or AgNPs for different time with 10 min of laser irradiation.

Figure S13. Effect of nanoprobe on apoptosis of LO2 cells.

Figure S14. Flow cytometry analysis of HepG2 cells apoptosis treated with (A) control, (B) Au-Ag under dark conditions, (C) Au-Ag-HM under dark conditions, (D) Au-Ag-HM under laser irradiation, (E) NAC and Au-Ag-HM under laser irradiation, (F) Ac-DEVD-CHO and Au-Ag-HM under laser irradiation using Annexin V-FITC/PI staining.

Figure S15. The histogram representing the corresponding concentration of celluar caspase-3 by reading the absorbance of the cell lysis from HepG2 cells treated with (A) control, (B) Au-Ag under dark conditions, (C) Au-Ag-HM under dark conditions, (D) Au-Ag-HM under laser irradiation, (E) NAC and Au-Ag-HM under laser irradiation, (F) Ac-DEVD-CHO and Au-Ag-HM under laser irradiation using caspase-3 kit.

Reagents. Bis(p-sulfonatophenyl)phenylphosphine (BSPP), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), (3-aminopropyl)-triethoxysilane (APTES), hematoporphyrin monomethyl ether (HMME), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT), dopamine·HCl and N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (Shanghai, China). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Life Technologies (California, USA). Caspase-3 protein was purchased from R&D Systems (Minneapolis, USA). Various cell lines，Annexin V-FITC/PI apoptosis detection kit, caspase-3 activity assay kit (colorimetric), caspase-3 inhibitor Ac-DEVD-CHO, caspase-3 specific peptide with a sequence of SH-CGDEVDSG-NH_2 (C-peptide), oligonucleotides for gold nanoflowers (AuNFs) and silver nanoparticles (AgNPs) binding (L-DNA: HS-5′-CCTAACCGGACCATTGGA-3′; S-DNA: HS-5′-TCCAATGGTCCGGTT AGG-3′) were received from KeyGen Biotech (Nanjing, China). Phosphate buffer saline (PBS, pH 7.4) composing of 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4 and 1.41 mM KH_2PO_4, and other reagents were of analytical grade and obtained by Sinopharm Chemical Reagent (Shanghai, China). Ultrapure water (18.2 MΩ cm at 25 °C) was used throughout this study.

Instruments. Transmission electron microscopy (TEM) images were carried out with a JEM-2010 TEM (JEOL, Japan). Fluorescence spectra were carried out on a FluoroMax-4 spectrofluorometer (HORIBA). UV-*vis* spectra were performed on an UV-*visible* spectrometer (Shimadzu UV-2450, Kyoto, Japan). Confocal laser

microscopy images and differential interference contrast (DIC) images were carried out by a confocal laser scanning microscopy (CLSM, FluoViewTM FV1000, Olympus, Japan). MTT assay was performed with a microplate reader (Multiskan GO, Thermo Fisher Scientific, China). Apoptosis experiment was carried out on a flow cytometer (BD FACSCalibur, BD Biosciences). Dark field measurements (DFM) were acquired on an Olympus inverted microscope Eclipse IX71 equipped with a dark-field condenser (0.8<NA<0.92), using 60X objective lens (NA=0.7) and a colour charge coupled device (CCD, DP80, Olympus, Japan) to collect the dark-field images. Scattering spectra were operated with an Acton SP2358 spectrograph mounted on the microscope and a 400BR excelon EMCCD as the detector (Princeton Instruments, USA) by switching the light path into a spectrograph equipped with a grating (grating density: 300 lines per mm; blazed wavelength: 600 nm).

Preparation of Gold Nanoflowers Based Nanoprobe (Au-Ag-HM). Firstly, silver nanoparticles were synthesized by sodium citrate reduction method. 5 mL 1% PVP was added into 20 mL ultrapure water in an ice-water bath. 600 μL of freshly prepared 1% $NaBH_4$ solution was quickly added into the above solution under vigorous stirring. Then, the mixture solution of PVP and $NaBH_4$, 5 mL of 10 mM $AgNO_3$ solution were separately injected into a bottle by using two constant-flow pumps at a rate of 30 mL/h. The suspension was heated to 80 ºC and kept for 2 h to remove excess $NaBH_4$ before being stored at 4 °C. The concentration of AgNPs solution was calculated to be 10 nM using the Lambert–Beer law with the extinction coefficient of 1.6×10^9 $M^{-1}cm^{-1}$. Then, 1 μL of thiol-linking S-DNA (100 μM) was

added, followed by rocking gently at room temperature for 24 hours. After centrifugation and washing with ultrapure water for several times, the S-DNA modified AgNPs (S-AgNPs) was thus obtained and stored at 4 °C for further use.

Secondary, AuNFs were synthesized according to our previous reported method. The concentration of AuNFs solution was calculated to be 0.1 nM, the same as gold nanoparticles (used as seed) with the extinction coefficient of 1.5×10^{10} $M^{-1}cm^{-1}$. Then, the BSPP-protected AuNFs were prepared by adding 1mg BSPP into 1 mL 0.1 nM AuNFs solution under stirring, following incubation overnight at room temperature and centrifugation for 20 mins. Then, 1 μL of L-DNA (100 μM) was mixed with above BSPP modified AuNFs and rocked gently at room temperature for 24 hours. After centrifugation and washing with ultrapure water for several times, the L-DNA functionalized gold nanoflowers (L-AuNFs) was collected, which was re-dispersed in PBS and stored at 4 °C for further use.

Thirdly, HMME was assembled on the surface of L-AuNFs by using carbodiimide chemistry. Briefly, 2 mL of EDC (50 mM) and 2 mL of NHS (130 mM) were added into 1 mL of HMME solution (100 μM) and allowed to react for 30 min at room temperature. Then, 1 mL of C-peptide (100 μM) was added to above mixture and stirred for 2 h to obtain HMME-conjugated C-peptide through chemically binding between the carboxyl group in HMME and the amino group in C-peptide. After that, 60 μL of the above conjugates was mixed with 1 mL 0.1 nM L-AuNFs solution and reacted at 4 °C for 12 h. Then, 2.5 μL 0.2 mM thiolated PEG solution was added and incubated for 1 hour to block non-specific binding sites of AuNFs. After

centrifugation and washing, the HMME and L-DNA functionalized gold nanoflowers (L-AuNFs-HM) was thus obtained, which was re-dispersed in PBS to a final volume of 1 mL and stored at 4 °C for further use.

The gold nanoflowers based nanoprobe was prepared by assembling AgNPs onto L-AuNFs-HM by DNA complementary hybridization between S-DNA and L-DNA. The S-AgNPs solution mixed with the L-AuNFs-HM solution at a molar ratio of 1:200. After incubated at room temperature for 24 hours, the mixture was separated by centrifuging at 2500 rpm and washing with water for three times to remove excess S-AgNPs. The resulting gold nanoflowers based nanoprobe (Au-Ag-HM) was re-dispersed in 1 mL of PBS (10 mM) and stored at 4 °C for further use.

Detection of Reactive Oxygen Species and Caspase-3 Activity *in Vitro*. Glass slides were treated with 1% (v/v) APTES for 1 h and washed with ethanol for several times. After dried under N_2 flow and baked at 110 °C for 30 min, the amino-group modified glass slides were thus obtained. After that, 100 μL of diluted Au-Ag-HM (1 pM) was dropped onto the amino-functionalized glass slide and incubated for 4 min. For the in vitro monitoring of ROS, 100 μL of various concentrations H_2O_2 (selected as model ROS) were added onto the above glass slides and incubated for different time for DFM and scattering spectra detection. For the in vitro monitoring of caspase-3, 100 μL of diluted Au-Ag-HM (1 pM) was incubated with various activities of caspase-3 and analyzed by fluorescence spectrum under the excitation wavelength of 392 nm.

In-situ Detection of ROS Level and Caspase-3 Activity in Cells. To in-situ

detection of intracellular ROS level and caspase-3 activity related to Au-Ag-HM treatment, the cells were first incubated in confocal culture dishes at a density of approximately 5×10^2 cells per dish for 24 h. Then, ROS inhibitor (NAC), caspase-3 inhibitor (Ac-DEVD-CHO) or blank control group were added and incubated for 2 h, followed by the addition of Au-Ag-HM (50μL, 1 pM) for 2 h. The confocal culture dishes were reacted under dark conditions or 638 nm laser irradiation at 100 mW cm^{-2} for 10 min and further incubated for 60 min. The dishes were washed and immersed in PBS for dark field scattering imaging of cellular ROS and scattering spectra scanning. Corresponding fluorescent confocal imaging of cellular ROS (in the green fluorescence channel) and caspase-3 activity (in the red fluorescence channel) was taken after the Au-Ag-HM-treated cells were further stained with DCFH-DA for 15 min. Image J software was used for fluorescence statistical analysis. To investigate the intracellular ROS after treated with inhibitor, the cells were incubated with different concentrations of NAC in confocal dish for 2 h. Then dark field scattering imaging and scattering spectra scanning were collected.

Cellular Viability and Light-mediated Cancer Therapy Effect. HepG2 cells and LO2 cells were selected for cellular experiments. Cells were cultured in DMEM supplemented with 15% fetal calf serum (FCS, Sigma), 100 μg mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin in 5% CO_2 and 37 °C incubator. The cell number was determined with a cell-counting board. MTT assay and cellular apoptosis assay were carried out to investigate the cytotoxicity and light-mediated cancer therapy effect of nanoprobe. Cells were first seeded into cell culture well plates at a density of

approximately 5×10^4 cells per well, and incubated at 37 °C for 24 h. After rinsing with PBS, 50 μL of nanoprobe (0.1 nM) was added to each well and incubated for 2 h. These wells were irradiated by a 638 nm laser at power density of 100 mW cm^{-2} for 10 min or maintained in the dark for 10 min as control. After incubation for different time, 50 μL MTT solution (1 mg mL^{-1}) was added and incubated at 37 °C for 4 h, and then 100 μL of dimethyl sulphoxide was added and vibrated for 15 min. The absorbance at a wavelength of 490 nm was measured using a microplate reader for cell viability assay. Meanwhile, 50 μL of nanoprobe (0.1 nM) was incubated with cells in well plates, irradiated by a 638 nm laser at power density of 100 mW cm^{-2} for 10 min or maintained in the dark for 10 min, then continue to incubate for 24 h. Each fraction of cells was digested, centrifuged, re-suspended, stained with Annexin V-FITC/PI and collected by flow cytometry for cell apoptosis measurement.

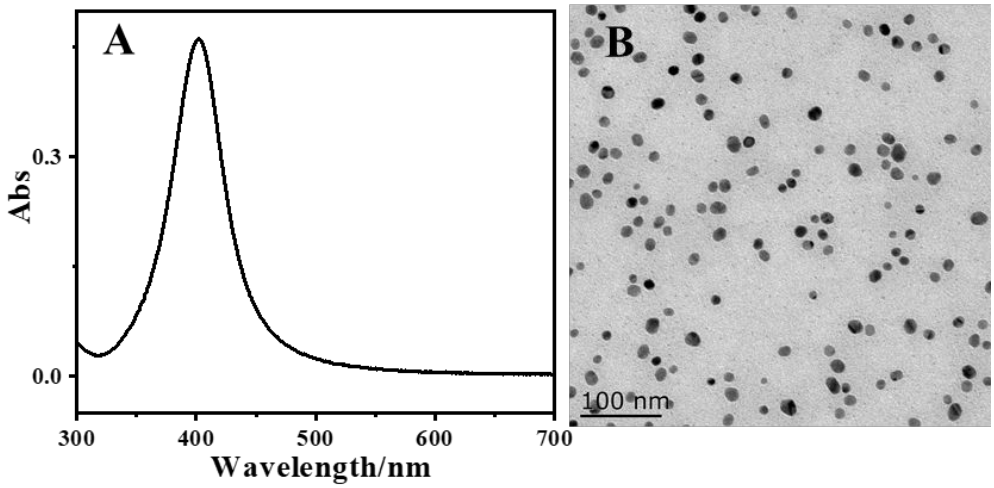

Fig.S1 (A) UV spectrum of AgNPs. (B) TEM spectrum of AgNPs.

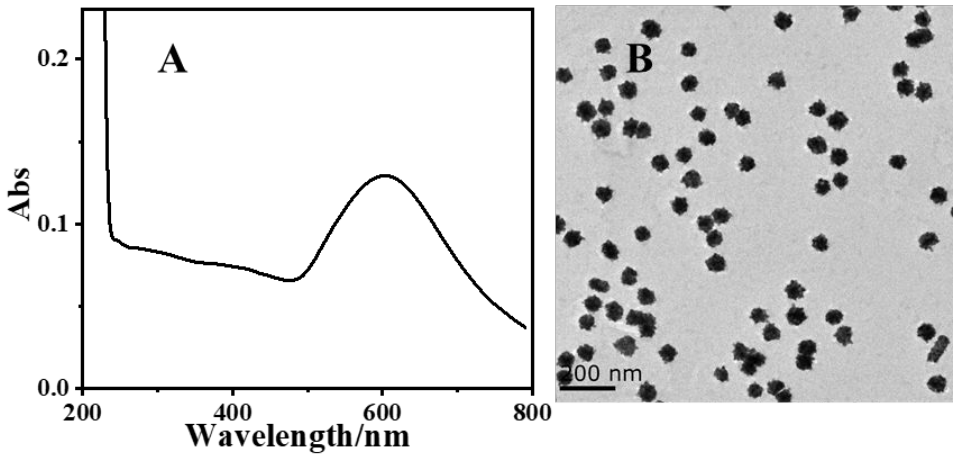

Fig.S2 (A) UV spectrum of AuNFs. (B) TEM spectrum of AuNFs.

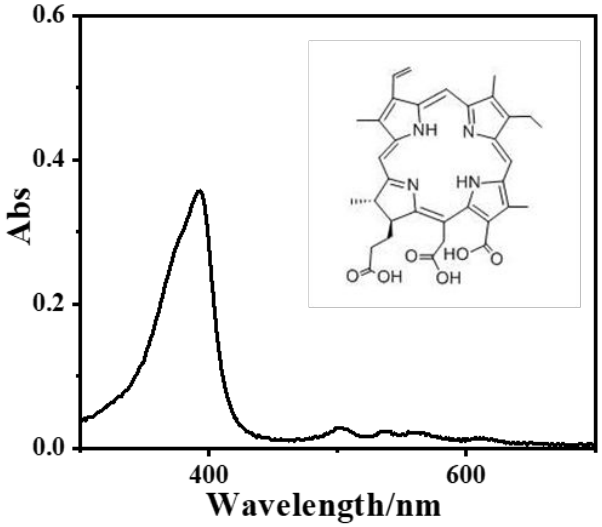

Fig.S3 UV spectrum of HMME.

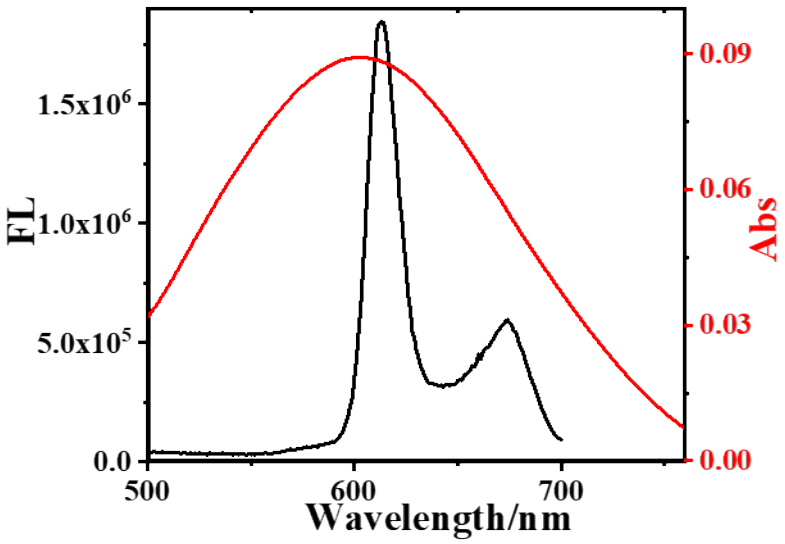

Fig.S4 Fluorescence spectrum of HMME and UV spectrum of AuNFs.

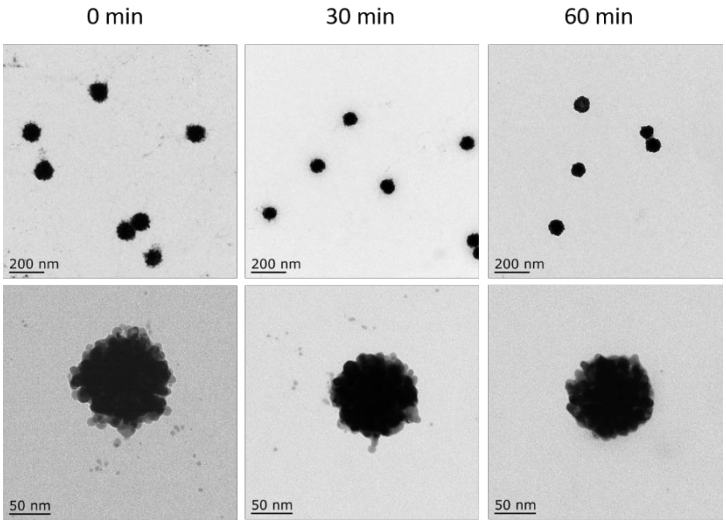

Fig.S5 TEM images of Au-Ag-HM nanoprobe incubated with 10 μM of H_2O_2 for different time.

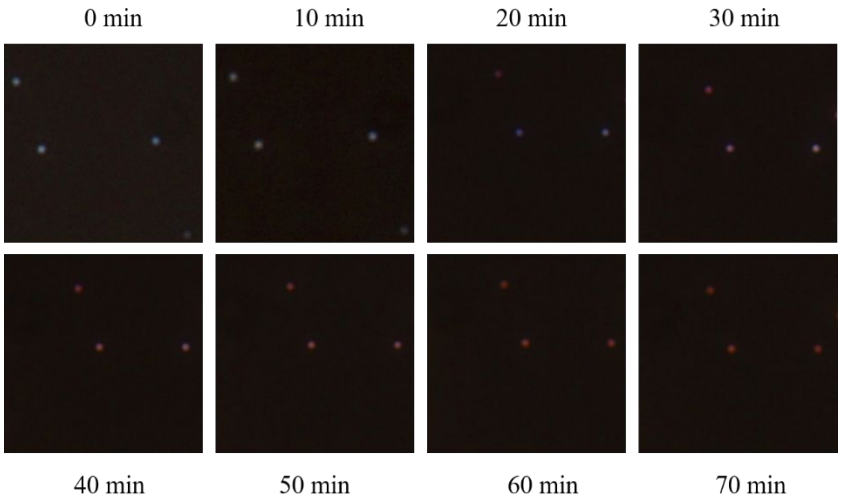

Fig.S6 Dark-field images of Au-Ag-HM nanoprobe incubated with 10 μM of H_2O_2 for different time.

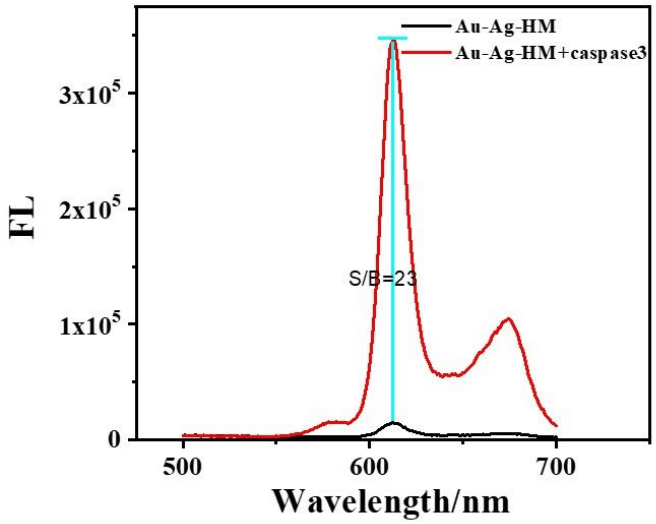

Fig.S7 Fluorescence spectra of Au-Ag-HM solution before and after adding 20 nM of caspase-3.

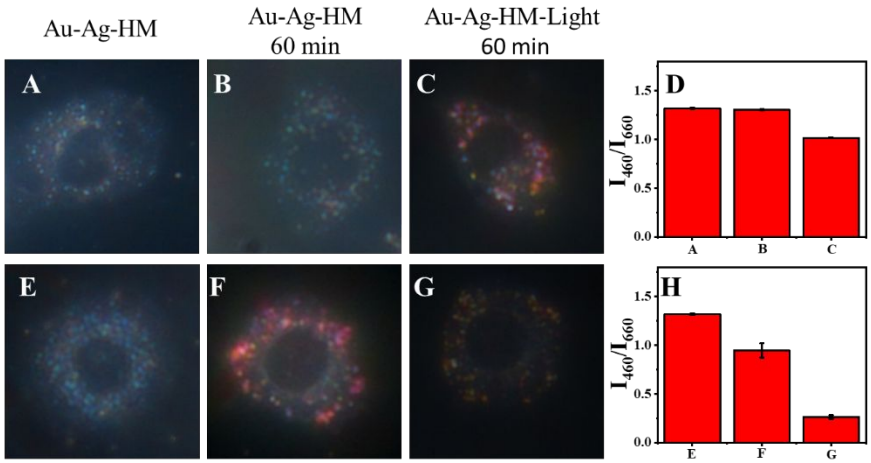

Fig. S8 Dark field images of Au-Ag-HM-treated LO2 cells under dark conditions and further incubation for (A) 0 min and (B) 60 min. Dark field images of Au-Ag-HM-containing HepG2 cells under dark conditions and further incubation for (E) 0 min and (F) 60 min. Dark field images of (C) Au-Ag-HM-containing LO2 cells (G) Au-Ag-HM-containing HepG2 cells after laser irradiation for 10 min and further incubation for 60 min. Relative LSPR scattering peak intensity ratio of Au-Ag- HM in (D) Au-Ag-HM-containing LO2 cells (H) Au-Ag-HM-containing HepG2 cells.

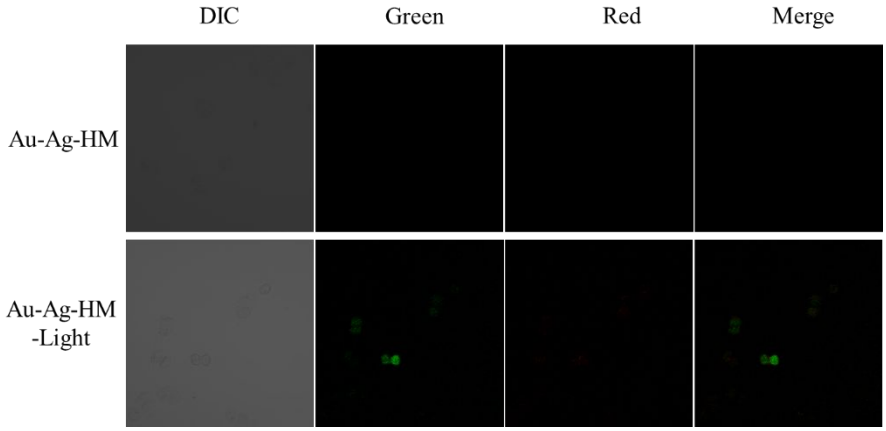

Fig.S9 Effect of laser irradiation on confocal imaging of LO2 cells incubated with Au-Ag-HM.

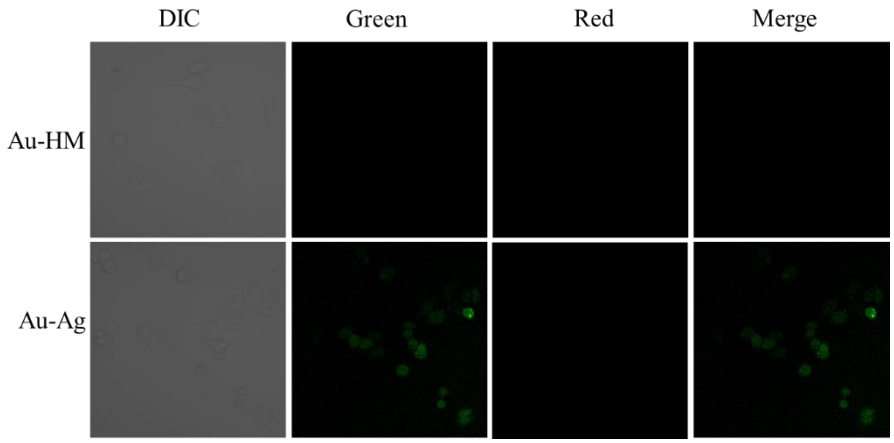

Fig.S10 Confocal images of Ag-HM and Au-Ag after incubation with HepG2 cells, respectively.

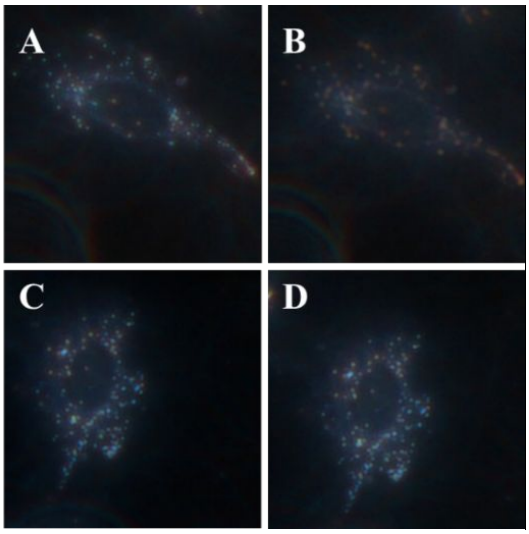

Fig.S11 Dark field images of caspase-3 inhibitor-treated Au-Ag-HM-containing HepG2 cells after laser irradiation and incubation for (A) 0 min and (B) 60 min; Dark field images of ROS inhibitor-treated Au-Ag-HM-containing HepG2 cells after laser irradiation and incubation for (C) 0 min and (D) 60 min.

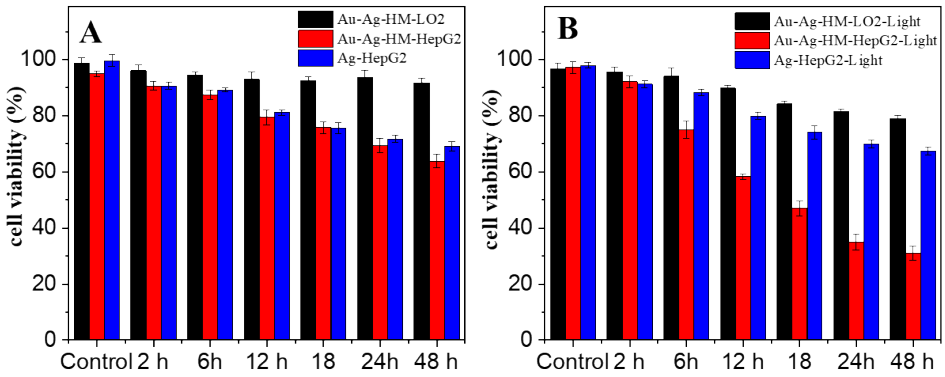

Fig.S12 (A) Cells activity of LO2 cells and HepG2 cells incubated with Au-Ag-HM or AgNPs for different time without laser irradiation. (B) Cells activity of LO2 cells and HepG2 cells incubated with Au-Ag-HM or AgNPs for different time with 10 min of laser irradiation.

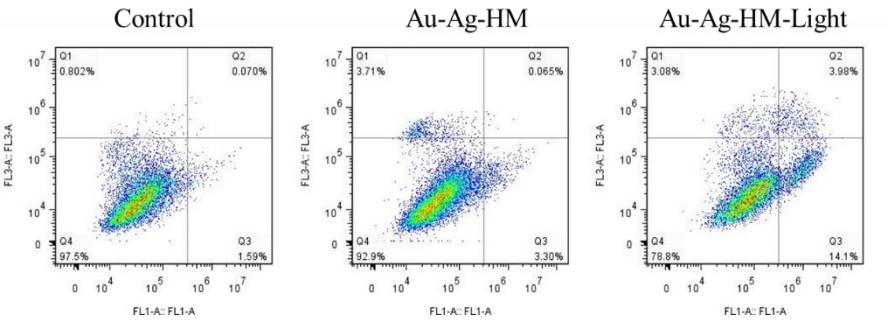

Fig.S13 Effect of nanoprobe on apoptosis of LO2 cells.

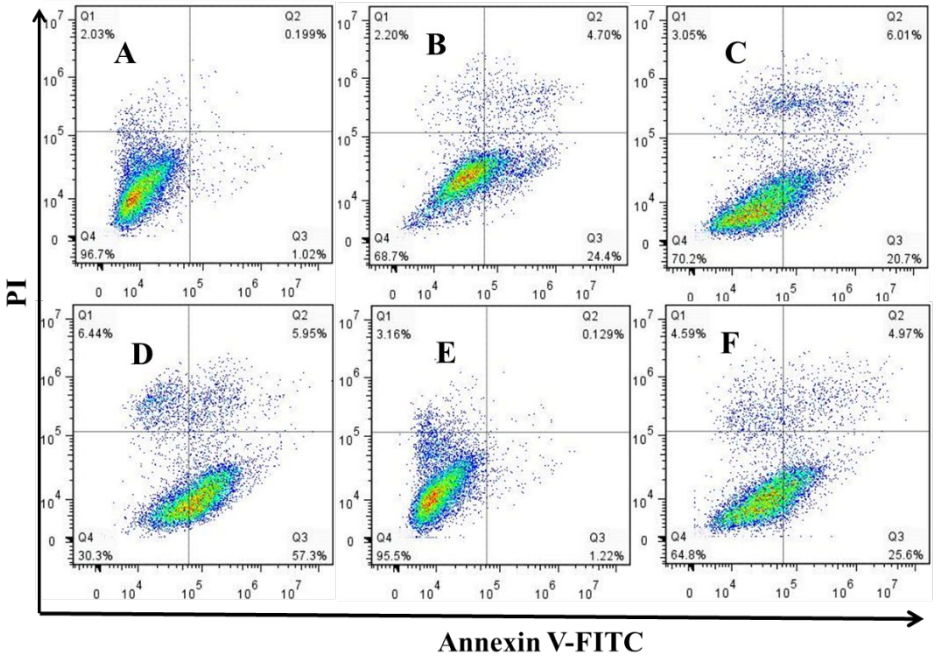

Fig.S14 Flow cytometry analysis of HepG2 cells apoptosis treated with (A) control, (B) Au-Ag under dark conditions, (C) Au-Ag-HM under dark conditions, (D) Au-Ag-HM under laser irradiation, (E) NAC and Au-Ag-HM under laser irradiation, (F) Ac-DEVD-CHO and Au-Ag-HM under laser irradiation using Annexin V-FITC/PI staining.

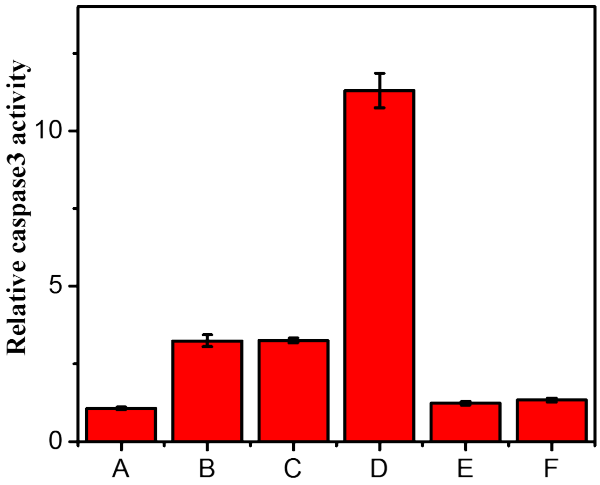

Fig.S15 The histogram representing the corresponding concentration of celluar caspase-3 by reading the absorbance of the cell lysis from HepG2 cells treated with (A) control, (B) Au-Ag under dark conditions, (C) Au-Ag-HM under dark conditions, (D) Au-Ag-HM under laser irradiation, (E) NAC and Au-Ag-HM under laser irradiation, (F) Ac-DEVD-CHO and Au-Ag-HM under laser irradiation using caspase-3 kit.