

Supporting Information

Nonlinear optical properties of Au/Ag alloyed nanoboxes and their applications in both *in vitro* and *in vivo* bioimaging under long-wavelength femtosecond laser excitation

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Experimental

Materials: Synthesis of Au/Ag alloyed nanoboxes (ANBs): aqueous AgNO₃, aqueous trisodium citrate, aqueous poly (sodium styrenesulphonate) (PSSS), aqueous NaBH₄, ascorbic acid (AA), gold chloride (HAuCl₄•3H₂O). They were all obtained from Sigma-Aldrich Co.

Surface modification of Au/Ag alloyed nanoboxes (ANBs): hexadecyltrimethylammonium bromide (CTAB), poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) (PSS), aqueous sodium chloride (NaCl), poly(allylamine hydrochloride) (PAH), methoxy-poly(ethylene glycol)-thiol (mPEG-SH). They were purchased from Sigma-Aldrich Co. Deionized (DI) water was applied in all the procedures of experiments.

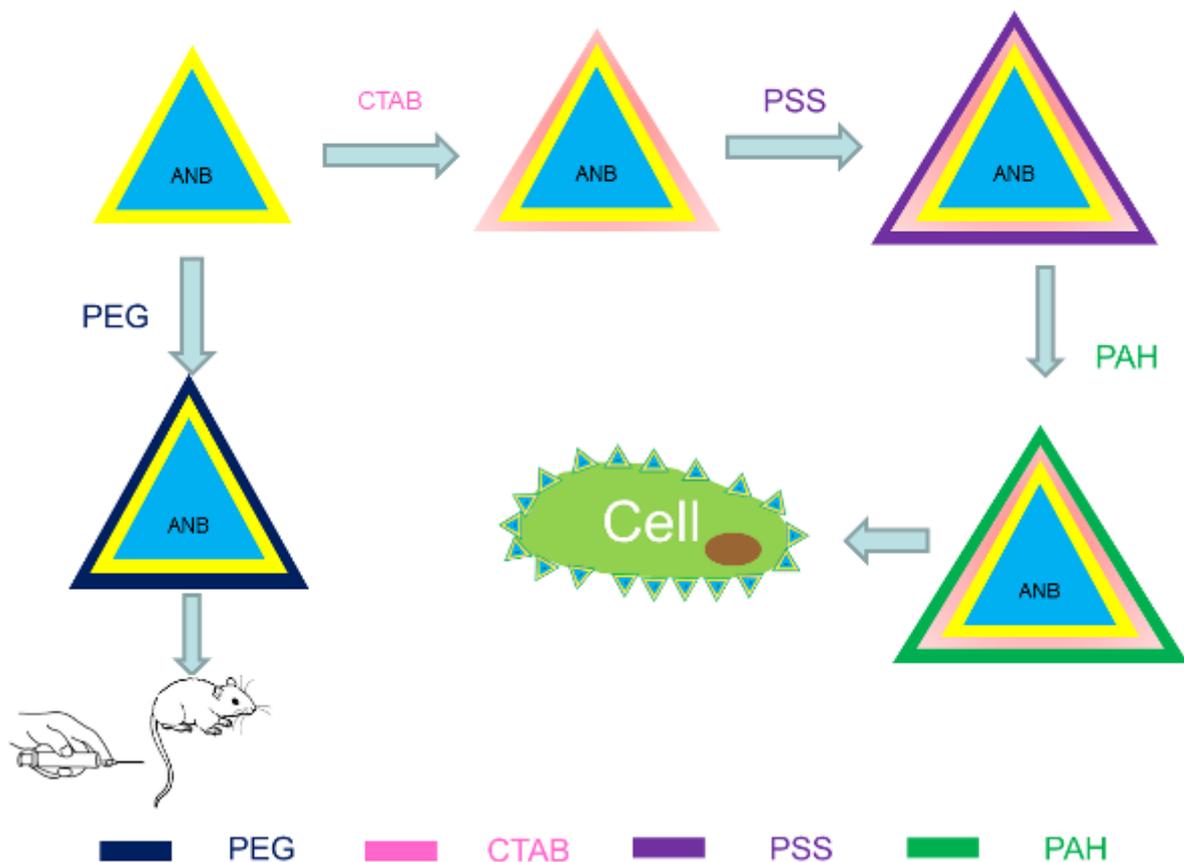
Synthesis of Au/Ag ANBs: In the first step, silver seeds were produced. 0.25 ml of 500 mg L⁻¹ poly(sodium styrenesulphonate) (PSSS) was added into 5 ml of 2.5 mM trisodium citrate, followed by addition of freshly prepared 0.3 ml of 10 mM NaBH₄. Then 5 ml of 0.5 mM AgNO₃ was added into the solution by a syringe pump at a rate of 2 ml min⁻¹. The whole process was under vigorous stirring. Secondly, Ag nanoprisms were prepared by combining 20 ml DI water, 300 µl of 10 mM ascorbic acid and certain amount of seeds, followed by addition of 12 ml of 0.5

mM AgNO₃ at a rate of 2 ml min⁻¹. Finally, 3 ml of 10 mM ascorbic acid was added into the Ag nanoprisms solution, followed by addition of certain amount of 0.5 mM HAuCl₄ at a rate of 2 ml min⁻¹ until the LSPR wavelength of ANBs was satisfactory.

Characterization of Au/Ag ANBs: A JEOL JEM-1200EX microscope operating at 160kV was used to get TEM images of Au/Ag ANBs. The absorption spectra of ANBs in water were measured by a Shimadzu 3600 UV–vis scanning spectrophotometer. Since water absorption is intense above 1350 nm, it was difficult to measure the absorption spectrum of ANB1450 in water. Instead, it was dispersed in dimethylformamide (DMF) for measurement.

Surface modification of Au/Ag ANBs: To make the ANBs more biocompatible, we did some surface-modification on them (Scheme S1). PSS/PAH-coating is a commonly used surface modification method for metal nanoparticles because they can be easily connected to nanoparticles through electrostatic force. Since the membrane of cancer cells is usually negatively charged and PAH is positively charged, nanoparticles coated with PAH can easily stain *cell membranes* due to electrostatic attraction. After adhesion to membranes, some of ANBs may also be endocytosed by the cell and enter into cytoplasm. For *in vitro* cellular imaging, in the first step, 5ml of 0.2M CTAB was added to 15 ml ANBs solution, 3 hours later the mixed solution was centrifuged twice (12000 rpm, 15 mins) to remove excess CTAB and redispersed in 1 ml DI water. 200 µl of 210 mgml⁻¹ PSS and 100 µl of 30 mM NaCl in the solution were added into 1 ml aqueous dispersion of CTAB-ANBs and vortexed in the tube for 3 mins. Three hours later it was centrifuged twice (12000rpm, 15mins) again to remove excess PSS. The collected PSS-CTAB-ANB was redispersed in 1ml DI water followed by adding 200 µl of 10 mg ml⁻¹ PAH and 100 µl of 10 mM NaCl in the solution, and it was vortexed in the tube for 3mins. Three hours later it was

centrifuged twice (12000 rpm, 15 mins) to remove excess PAH and the remaining PAH-PSS-CTAB-ANB was redispersed in 1 ml of DI water. To confirm the validity of each coating step mentioned above, the Zeta potential of remaining nanoparticles in each step was tested. It was found that the typical Zeta potential of freshly prepared ANB, CTAB-ANB, PSS-CTAB-ANB and PAH-PSS-CTAB-ANB were -8.6mV, +49.9mV, -26mV and +38.9mV respectively. The PAH-PSS-CTAB-ANB sample was then used to treat A549 cells (human lung adenocarcinoma epithelial cell line). For *in vivo* vasculature imaging, 60 mg of mPEG-SH was added into 120 ml of freshly prepared ANBs solution followed by vortex for 5 mins, and then the solution was left undisturbed for 3 hours. In this way, ANBs could be encapsulated by mPEG-SH. After concentration and redispersed in 1× PBS (phosphate-buffered saline), the mPEG-ANB sample was then injected into mice via tail vein. As for their final distribution, they may accumulate in liver or kidney and cleared out from the mouse body through metabolism process.



Scheme S1. The two surface modification procedures of Au/Ag ANBs for cellular and vasculature bioimaging, respectively.

Cells culture and ANBs treatment: A549 cells were cultured with RPMI 1640 with 10% fetal bovine serum at 37°C and 5% CO₂. A549 cells were seeded in 6-well plates (BD, 353046) at a density of 1×10⁵ cells/well in 2 ml of culture medium. After 24 hours of incubation, cells were treated with 40 μl of 200 μg ml⁻¹ PAH-PSS-CTAB-ANB for 2 hours. Then the cells were washed with PBS three times for cellular imaging.

In vivo experiments: All *in vivo* experiments were performed in compliance with Zhejiang University Animal Study Committee's requirements for the care and use of laboratory animals in research. The animal housing area (located in Animal

Experimentation Center of Zhejiang University) was maintained at 24 °C with a 12 h light/dark cycle, and animals were fed with water and standard laboratory chow.

Subcutaneous tumor xenografts: The xenografted mouse models were generated by subcutaneously injecting 5×10^6 Hela cells/mouse in the right scapular region of 18-21 g male nude mice. Tumor growth was monitored every 2 days until a tumor size of approximately 5 mm in diameter was observed.

3PL brain imaging of mice: 8-week-aged male BALB/c mice were used for 3PL brain imaging. After anesthetized with pentobarbital, the skulls of the mice were opened up through microsurgery. A metal ring (Thickness: 1.2 mm, inner diameter: 8 mm) with a handle was mounted onto the mouse brain. A round thin cover glass slide (Thickness: 0.15 mm, Diameter: 6 mm) was then embedded in the metal ring and directly adhered to the mouse brain through dental cement. The brain was flattened by the cover glass slide to improve the 3PL imaging quality. The metal ring was then connected with a metal plate, which also has a handle, via handle-handle fix. The metal plate was heavy and firm enough to keep the mouse's head immobilized during the whole imaging process.

The nonlinear optical signals of Au/Ag ANBs under the fs excitation of 1600 -1800 nm: When excited by a fs laser with wavelengths in the optical tissue window of 1600 -1800 nm, Au/Ag ANBs could also response and emit strong nonlinear optical signals. Au/Ag ANBs with an LSPR peak at 544 nm (abbreviated as ANB544) were tested in our measurement system. The output of the home-made fs laser (In Tianjin University, China) can be tuned from 1400 nm to 1800 nm. Figure S1 (a) illustrates the emission spectrum of ANB544 when the excitation wavelength was 1650 nm. As can be seen, strong THG signal (centered at 550 nm) can still be observed. Figure S1

(b) shows the spectra of ANB544 under the excitation wavelengths ranging from 1650 nm to 1800 nm. The split of THG peak under 1750 nm excitation was due to the instability of fs laser source.

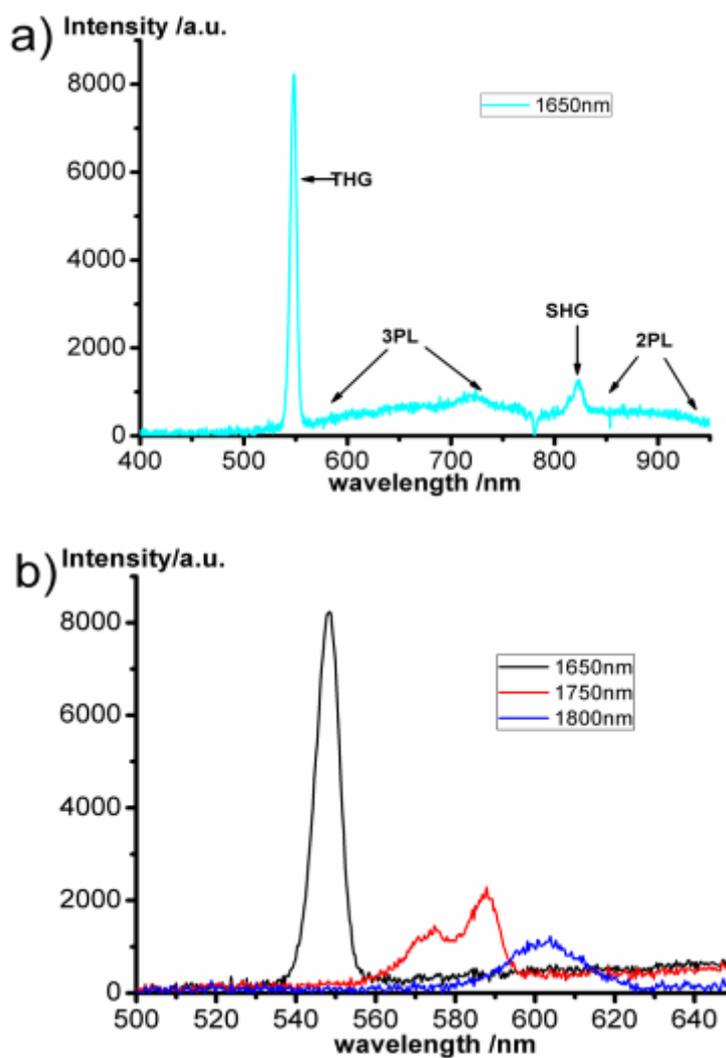


Figure S1. a) Emission spectrum of ANB544 when the excitation wavelength was 1650nm. b) Emission spectra of ANB544 under the excitation wavelengths ranging from 1650-1800nm. The split of THG peak under 1750 nm excitation was due to the instability of the fs laser.

The picture of optical setup of 3PL scanning microscope:

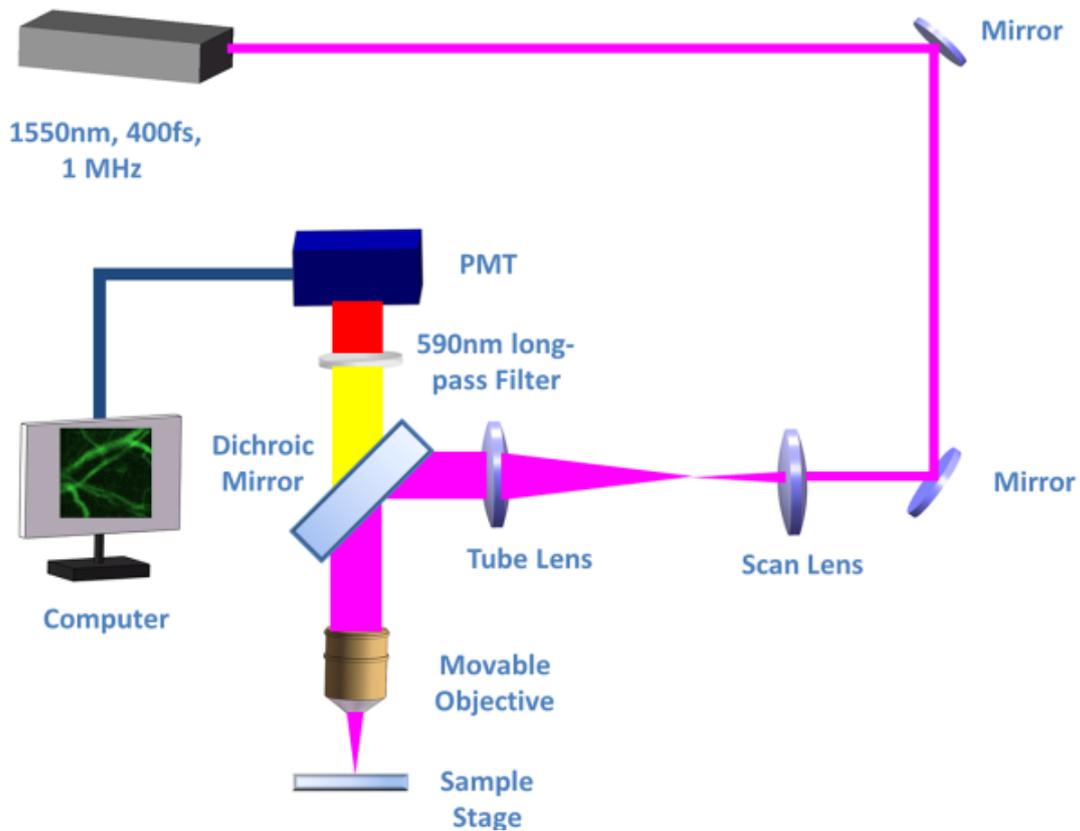


Figure S2 The optical setup of 3PL scanning microscope.

The contrast test results of A549 cells without any Au/Ag alloyed nanoboxes:
 living A549 cells without Au/Ag alloyed nanoboxes were imaged by our 3PL scanning system, and no 3PL signals were obtained, as described in figure S3.

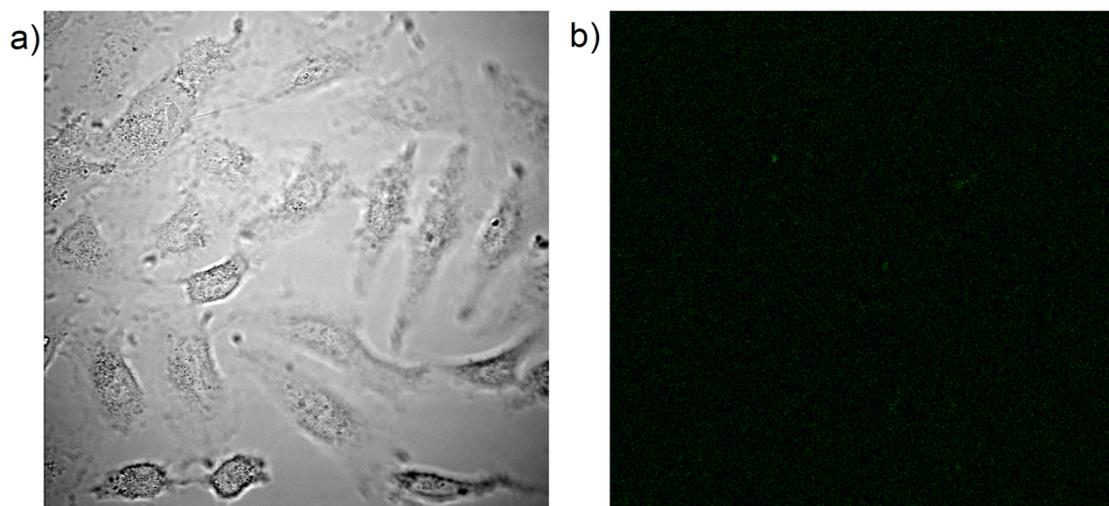


Figure S3 living A549 cells without Au/Ag alloyed nanoboxes were imaged. a) is the transmission images of cells, b) is the 3PL imaging results.

More evidence about nanoboxes: To confirm that the nanoparticles we synthesized are nanoboxes instead of solid nanoprisms, we conducted TEM analysis of nanoparticles which are “standing” on their edges. Figure S4 a) shows some edge-oriented nanoparticles marked by green circles. As can be seen, the edges of the sides of nanoparticles are darker than their interior part. The sides of nanoparticles are composed of Au, and one would not expect a contrast between the edge and the interior part of the sides unless there is a hollow in each nanoparticle (which makes the edge of sides of nanoparticle darker than its interior part).

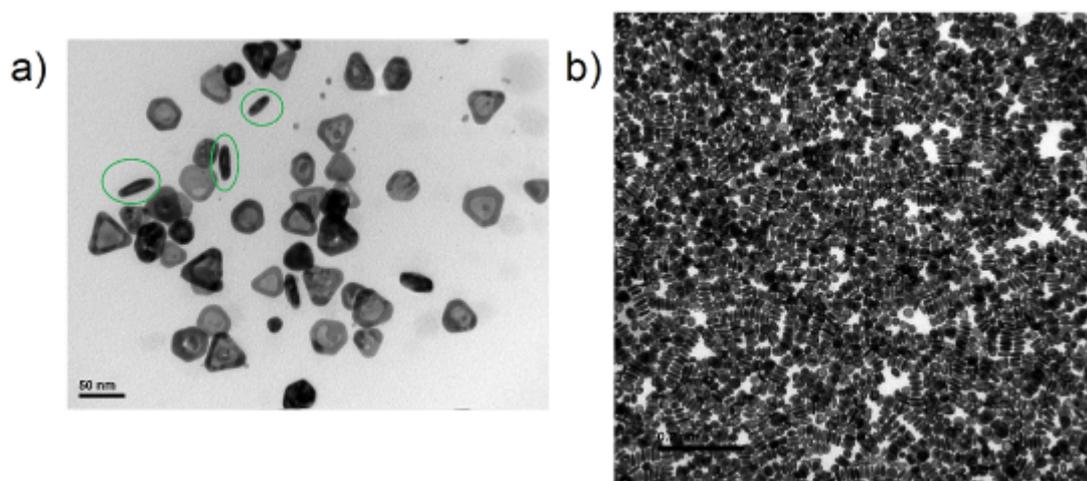


Figure S4 TEM images of edge-oriented nanoparticles.

The cytotoxicity of surface-modified ANBs: When we carried out *in vitro* 3PL bioimaging of A549 cells stained with surface-modified ANBs, ANBs were added into cells. Two hours later, the nanoparticle-treated cells were imaged. From the transmission images, we can tell that surface-modified Au/Ag alloyed nanoboxes had little cytotoxicity to cells because the morphology of cells still kept very well after 2-hour-treatment, as shown in the following picture:

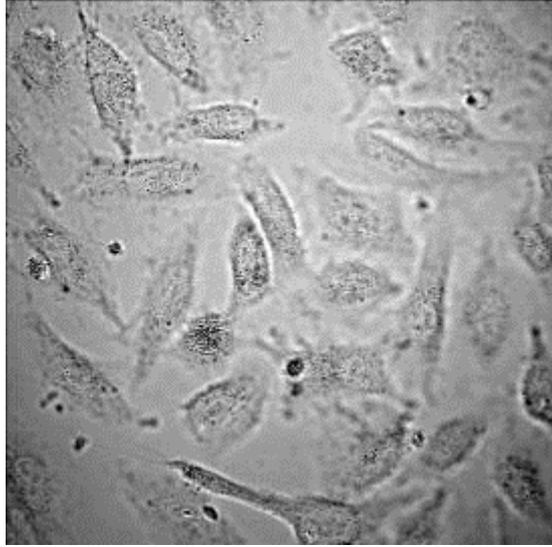


Figure S5 Transmission images of A549 cells stained with ANBs.