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Supplementary Information

A Novel Multifunctional Nano-platform with Enhanced Anti-cancer and Photoacoustic Imaging Modalities using Gold-nanorod-filled Silica Nanobeads

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Experimental Section

Materials

Cetyltrimethylammonium bromide (CTAB), ethanol (99.5%), lysine, *n*-octane (99%), 2, 2'-azobis(2-amidinopropane) dihydrochloride (AIBA), hydrogen tetrachloroaurate (III) trihydrate ($HAuCl_4$, 49.5% as Au), sodium borohydride ($NaBH_4$, 99%), silver nitrate ($AgNO_3$, 99%), L-ascorbic acid and sodium hydroxide (NaOH, 97%) were purchased from Sigma-Aldrich. Tetraethylorthosilicate (TEOS, 99%) was purchased from Merck. Styrene monomer (99%) was purchased from Fluka.

Characterization equipments

Nanostructures were investigated using scanning transmission electron (SEM, JSM-6700F, Japan), transmission electron microscopy (TEM, JEM-2010, Japan). PL spectroscopy (PL, Fluorescence Spectrophotometer F-4500, Hitachi, Japan) was used to characterize the fluorescence intensities of AuRNBs. The continuous laser (VD-IIIA Dpss Laser Driver, OPHIR), pulse laser (Surlite II-10, Continuum), ultrasound and photoacouistic imaging system (**Figure S10**) were used to test the photothermal stability and photoacoustic signal of AuRNBs.

Synthesis of porous silica nanobeads (NBs)

300 mg cetyltrimethylammonium bromide (CTAB) was dissolved in a mixture of 45 ml octane and 96 ml dilute water at 70^{o}C. After stirred magnetically for 20 min, 8.5ml styrene monomer, 66 mg lysine, 3000 mg tetraethylorthosilicate (TEOS), and 115 mg AIBA were subsequently added to the system and stirred magnetically for 4 h. After 4 h, the heating was stopped and the suspension was cooled naturally to room temperature. The products were collected by centrifugation at 6000 rpm for 10 min and then washed 3 times with an excess of pure methanol. Finally the template was completely removed by heat treatment at 600^{o}C under atmospheric conditions.[1]

Gold seed filled porous silica nanobeads (AuSNBs)

CTAB solution (5 mL, 0.20 M) was mixed with 5.0 mL of $HAuCl_4$ (0.0005M). To the stirred solution, 0.60 mL of ice-cold 0.010 M $NaBH_4$ was added, which resulted in the formation of a brownish yellow solution. Vigorous stirring of the seed solution was continued for 2 hours. After the solution was stirred, it was kept at 25 °C. Furthermore,

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incorporation of gold seeds was achieved by swelling 1 mM porous silica beads with 1 ml seed solution and stirred magnetically for 2 h. The products were collected by centrifugation at 10000 rpm for 10 min and then were redispersed into dilute water (1 ml).

Templated synthesis of gold nanorods filled porous silica nanobeads (AuRNBs)

CTAB (5 mL, 0.10 M) was added to (0.02, 0.04, 0.06, 0.08, 0.10 mL) of 0.01 M $AgNO_3$ solution at 25 °C. To these solutions, 0.5 mL of 0.01 M $HAuCl_4$ was added, and after gentle mixing of the solution, 55 µL of 0.1 M ascorbic acid was added. Ascorbic acid as a mild reducing agent changes the growth solution from dark yellow to colorless. The final step was the addition of 20 µL of the seed solution (AuSNBs) to the growth solution at 27-30 °C. The color of the solution gradually changed within 10-20 min. For longer NRs, the color change takes place more slowly. The temperature of the growth medium was kept constant at 27-30 °C in all the experiments and stirred magnetically for 24 h. The final products were collected by centrifugation at 10000 rpm for 10 min and then redispersed into dilute water (5 ml).

Selective etching of the gold nanorods filled porous silica nanobeads

The 3 mL gold nanorods filled porous silica nanobeads (AuRNBs) were dispersed in 30 mL of NaOH solution (0.25 M) to obtain gold nanorods. The dispersion was stirred at room temperature for 1 h. Product was collected by centrifugation at 10000 rpm for 10 min and washed with water until neutral pH.

Volume fraction of gold nanorods in nanobeads

The atomic concentration fraction of gold (Au) to silicon (Si) was analyzed to be 0.186 from inductively coupled plasma (ICP). The volume fraction of Au nanorods is determined using the equation (1) :

$$\frac{\text{Au (ppm)}}{\text{Si (ppm)}} = \frac{\text{Au (mmol)}}{\text{Si (mmol)}} = \frac{\text{Au (mol)}}{\text{Si (mol)}} = 0.186$$

$$\frac{\text{Au (v\%)}}{\text{SiO}_2\text{(v\%)}} = \frac{\text{Au } (\frac{\text{gcm}^3}{\text{g}})}{\text{SiO}_2\ (\frac{\text{gcm}^3}{\text{g}})} = \frac{\frac{\text{Au (mol)}}{\text{D}_{\text{Au}}} \text{ x } \text{m}_{\text{Au}}}{\frac{\text{SiO}_2\text{ (mol)}}{\text{D}_{\text{SiO2}}} \text{ x } \text{m}_{\text{SiO2}}} = \frac{\text{Au (mol) x } \frac{\text{m}_{\text{Au}}}{\text{D}_{\text{Au}}}}{\text{Si (mol) x } \frac{\text{m}_{\text{SiO2}}}{\text{m}_{\text{Si}}} \text{ x } \frac{\text{m}_{\text{SiO2}}}{\text{D}_{\text{SiO2}}}}$$

$$= 0.0837 = 8.37\%$$

, where m_{Au} (196.97), m_{Si} (28.08) and m_{SiO2} (60.08) are the molecular weight of Au, Si and SiO_2, respectively. D_{Au} (19.3 g/cm^3) and D_{SiO2} (2.648 g/cm^3) are the density of Au and SiO_2, respectively.

Cell culture

MDA-MB-231, human breast cancer cells were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100µg ml^{-1} streptomycin. Cells were cultured with

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complete medium at 37°C in a humidified atmosphere of 5% CO_2 in air. For all experiments, cells were harvested from sub-confluent cultures by use of trypsin and were resuspended in fresh complete medium before plating. A comparison of *in vitro* cytotoxicity of NBs, AuSNBs and AuRNBs with different concentrations was performed on MDA-MB-231 cells with an *in vitro* proliferation method using MTT. Briefly, 10^4 cells were plated in 96-well plates to allow the cells to attach and then exposed to the serial concentrations of NBs, AuSNBs and AuRNBs at 37°C. After the NBs, AuSNBs and AuRNBs incubated with cells for 24 h, 20 μL of MTT solution was added and incubated for another 2 h. Then, the medium was replaced with 200 μL of DMSO, and the absorbance was monitored using a Sunrise absorbance microplate reader at dual wavelengths of 570 and 650 nm. To estimate the cellular uptake of the nanocarriers, the nanocarriers were labeled FITC (green emitting) for the study. The AuR (gold nanorods) or AuRNBs were incubated with the MDA-MB-231 cell for 4 h, 12h and then studied by confocal laser scanning microscopy (CLSM) and flow cytometry.

In vivo experiments

Subcutaneous tumors were induced in either the left hind flank of nude mice after injection of about ten million cells (MDA-MB-231) in 0.1 mL media. Tumors were allowed to grow to ~100 mm^3 before experimentation. The MDA-MB-231 xenograft nude mice were given single injection of the AuRNBs via intracortical injection. The concentration of particles (AuRNBs) administered were 0.5 μM given in 50 μL injection. Then it was anesthetized with isoflurane and irradiated the tumor region with NIR light (4 W cm^{-2} for 2 min) at 12 h post-injection and use infrared thermal mapping apparatus to monitor the temperature raise. The experimental protocol used for animals was evaluated and approved by the Institutional Animal Care and Use Committee of National Chiao Tung University and National Tsing Hua University, Taiwan. Care of the animals and surgical procedures were performed according to the standards of National Chiao Tung University and National Tsing Hua University Protocol on Laboratory Animals.

For photoacoustic imaging experiment, the mouse (Balb/c) were induced tumors subcutaneously in the head after injection of about million cells (CT26, mouse colon cancer) in 0.1 mL media. The mouse was given single injection of the pure AuR (0.5 μM, 50 μL) and AuRNBs (0.5 μM, 50 μL) via intracortical injection. Then the mouse was restriction of movement in the fixer. A frequency-doubled pulsed laser with 10-ns pulse width pumped an optical parametric oscillator to illuminate the tumor site at 808 nm wavelength and 10 mJ/cm^2 fluence. PA signals were received by the central 32 elements of an ultrasound linear array and recorded by an ultrasound scanner.

Table S1 Different aspect ratio of the AuRNBs.

LSPR (nm)	$AgNO_3$ (μL)	Length (nm)	Width (nm)	Aspect ratio
636	20	28.7	12.5	2.3
688	40	35.2	12.1	2.9
753	60	40.7	11.3	3.6
793	80	47.7	11.1	4.3
821	100	49.1	10.9	4.5

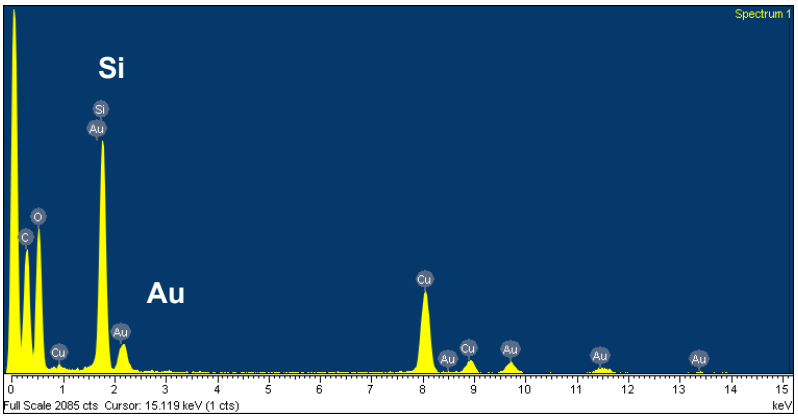
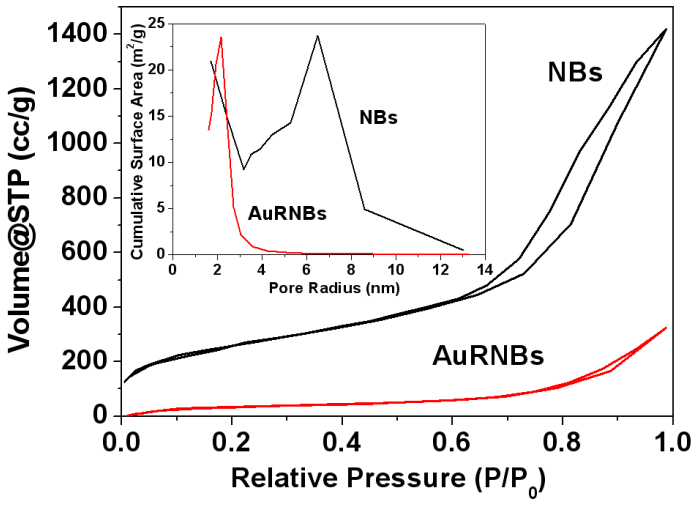


Figure S1 The energy-dispersive X-ray spectroscopy of the mesoporous silica nanobeads with gold nanorods pore-filled structure by TEM.

A Brunauer-Emmett-Teller (BET) analysis also showed a considerable reduction of surface area and average pore size from 862.25 m^2g^{-1} and 6.5 nm (radius) to 128.96 m^2g^{-1} and 2.1 nm (Figure S2), which supported that the nanoporosity of the silica nanobeads was extensively filled up by the gold nanorods.



	Surface area (m^2/g)	Pore volume (cc/g)	Pore radius (nm)
NBs	862.25	1.908	6.5
AuRNBs	128.96	0.378	2.1

Figure S2 The N_2 adsorption/desorption isotherms of the nanobeads with Brunauer-Emmett-Teller (BET). The inset showed that the Barret-Joiner-Halenda (BJH) analysis of the nanobeads and AuRNBs.

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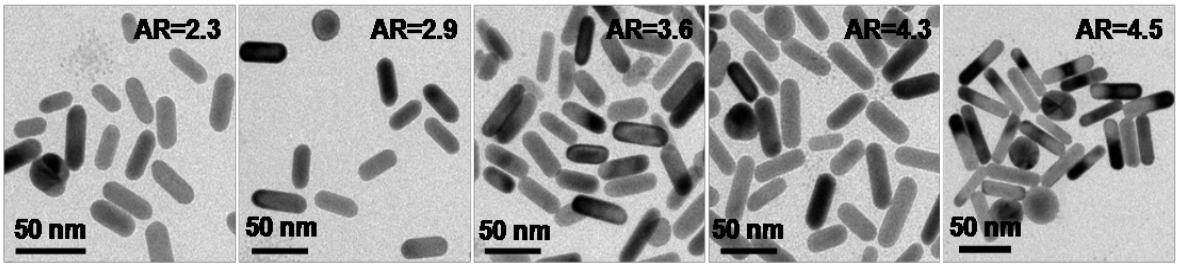

Figure S3 TEM images of AuRNBs after removal of silica matrix.

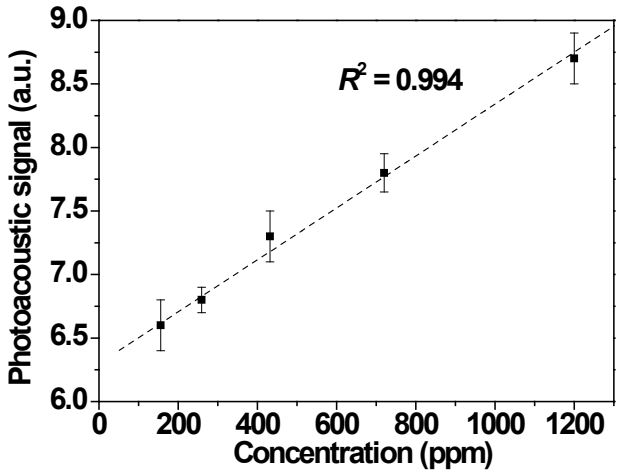

Figure S4 The photoacoustic signal produced by AuRNBs was observed to be linearly dependent on the concentration. (R^2= 0.994).

The amplitude of the photoacoustic signal at nanosecond time scales is related to the temporal temperature gradient generated in the surrounding medium by heat dissipation from the nanorod.[2] Figure S5 shows a mechanism for the photoacoustic signal enhancement from gold nanorod-filled nanobeads, emphasizing the importance of the heat transfer to the water. Pure gold nanorods have a slower heat transfer resulting in a broader heat peak due to their interfacial properties, but the gold nanorods filling in silica nanobeads can reduce the interfacial resistance and lead to a sharper peak. Furthermore, the gold nanorods filled nanobeads shows three-fold higher photoacoustic signal amplitude than pure gold nanorods. We therefore expected that the photoacoustic signal enhancement must come from an increased thermal transfer through the gold interface. Thus, the porous silica nanobeads with AuR-filled structure could amplify the photoacoustic response by increasing the interfacial heat conduction from gold to water due to the presence of silica matrix.

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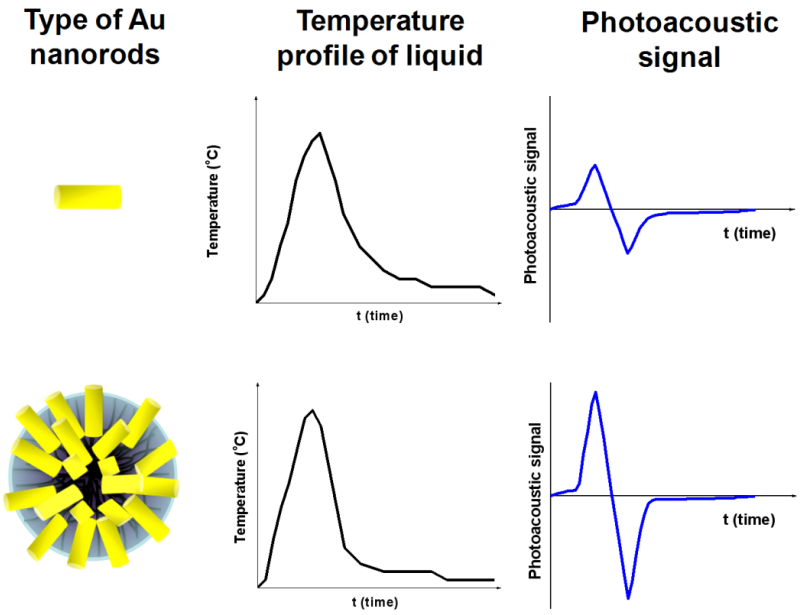

Figure S5 The proposed thermal transport processes from the nanoparticle to the environment and resulting temporal profiles of the temperature and the amplitude of the photoacoustic signal.

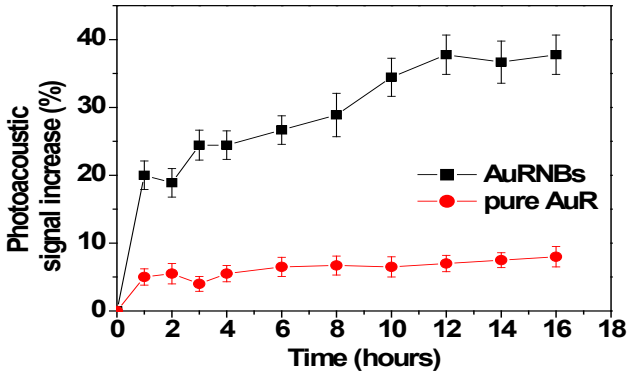

Figure S6 Mice injected with AuRNBs showed a significantly higher photoacoustic signal than mice injected with pure AuR.

Cellular uptake of the FITC-labeled AuRNBs (green color emitted) was monitored by confocal laser scanning microscopy (CLSM). Figures S7a and S7b show that the AuRNBs are gradually internalized after incubating with the MDA-MB-231 cell for 4-h and 12-h durations. The green fluorescent dye was clearly observed in the region of cytoplasm over short time periods of incubation. For 4-h incubation, some of the nanoobjects appeared to attach rapidly to the cell membranes. However, numerous regions of the cytoplasm displayed strong green fluorescent emission at 12 hours. This observation implies that the nanoprobes can be internalized rapidly upon cellular uptake. A further investigation using flow cytometry toward the AuR and AuRNBs with MDA-MB-231 cells is given in Figure S7c. The AuR taken by MDA-MB-231 (blue color) displayed low signal intensity but for the AuRNBs (brown color), the signal intensity was 30 times higher than that of AuR in 4 h incubation. There are high density gold nanorods in one AuRNBs.

That is to say, when the cancer cells uptake one AuRNBs nanoparticles, it is equal to uptake several pure AuR. Thus, AuRNBs showed higher fluorescence intensity than pure AuR. Furthermore, for a time period from 4 h to 12 h, the fluorescence intensity determined by flow cytometry rapidly increased, indicating a highly efficient cellular internalization.

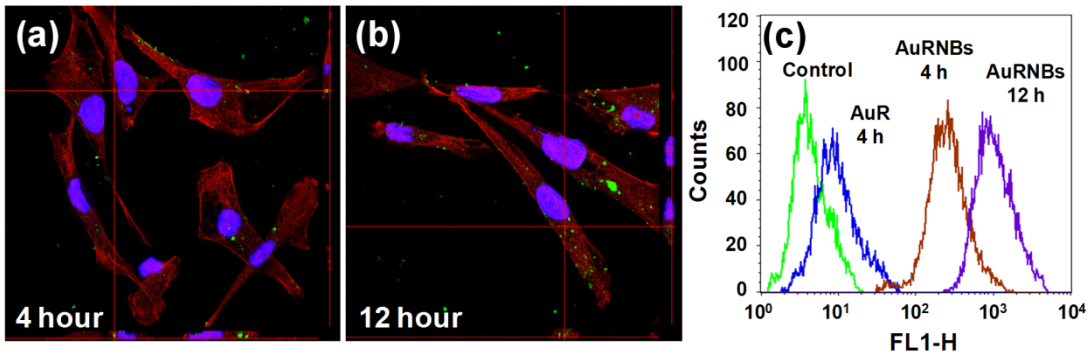

Figure S7 Time-course PL microscopy images of MDA-MB-231 cells labeled with FITC-AuRNBs nanoprobes and incubated for (a) 4 hour and (b) 12 hours. The cell skeleton was stained with rodamin phalloidin (red), and the cell nucleus with DAPI (blue). (c) Quantitative flow cytometric data shows that the fluorescence brightness and uniformity levels of pure AuR and AuRNBs uptake by MDA-MB-231 for incubation 4 h and 12h.

The result of the MTT (3-(4,5-dimethyl diazol-2-yl)-2,5 diphenyl tetrazolium bromide) assay as a measure of metabolic competence of the cell with different concentrations of the NBs (silica nanobeads), AuSNBs (AuS-filled silica nanobeads) and AuRNBs (AuR-filled silica nanobeads) were shown in Figure S8a. The nanoparticles were incubated with MDA-MB-231 cancer cells for 24 hours. The AuSNBs and AuRNBs demonstrated equivalent cell viability to the NBs, indicating a relatively low or little cytotoxicity of these newly-designed AuRNBs to the MDA-MB-231 cells. To evaluate photothermal therapy, we irradiated MDA-MB-231 cells administrated with AuR and AuRNBs (the same dose of gold, incubated for 24 hr) at a NIR wavelength of 808 nm with 2 W/cm^2 for different irradiated time and monitored cell viability as shown in Figure S8b. With increasing NIR laser treatment time, the cell viability of MDA-MB-231 obviously reduced. However, after irradiation, the AuRNBs showed the lower cell viability than pure AuR. From flow cytometric data (Figure S7c), the cell uptake efficiency of AuRNBs are higher than pure AuR under the same concentration and incubation time, which accordingly the AuRNBs should expect to have higher hyperthermia efficiency than that from pure AuR upon NIR laser treatment.

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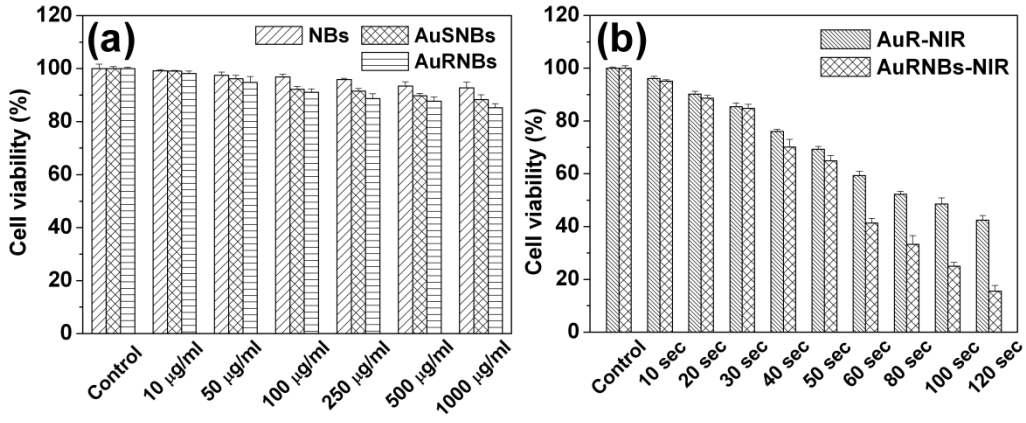

Figure S8 (a) Cell viability of MDA-MB-231 cells after 24 h of incubation with nanobeads, AuSNBs, and AuRNBs. (b) Cell viability of MDA-MB-231 cells after 24 h of incubation with pure AuR and AuRNBs versus duration of irradiation.

As shown in Figure S9, the tumor injected with AuRNBs via a single dose of irradiation with NIR laser completely regressed, whereas tumors in the control group were under developing after 14 days. On this base, it can be conceivable that the AuRNBs currently developed in this study are proved to be a potential photothermal therapeutic vehicle with exceptionally higher heating efficiency than conventional pure Au nanoobjects for anti-cancer therapy.

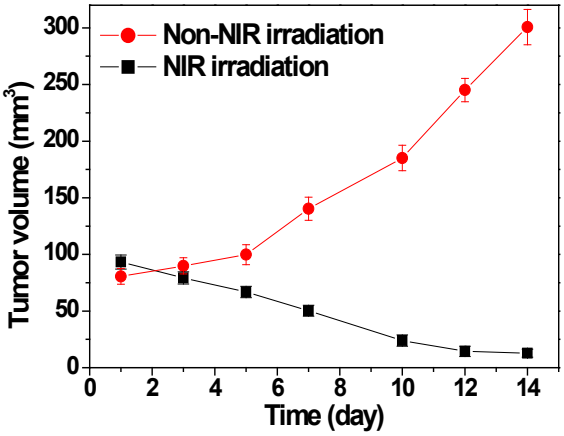

Figure S9 The volume of tumor variation by treated with NIR light.

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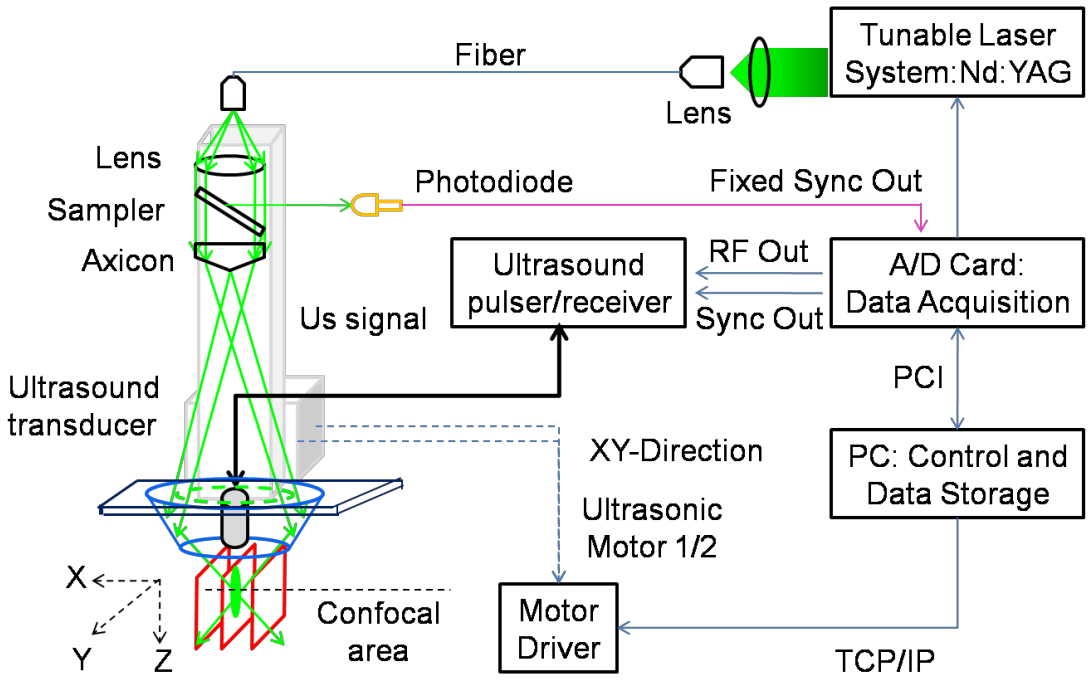

Figure S10 The photograph of the ultrasound and photoacoustic imaging system.

[1] A. B. D. Nandiyanto, S. G. Kim, F. Iskandar and K. Okuyama, *Micropor. Mesopor. Mater.,* 2009, **120**, 447.

[2] C. Frez and G. J. Diebold, *Eur. Phys. J. Spec. Top.*, 2008, **153**, 307.