

Three-Photon Luminescence of Gold Nanorods Excited by 1040 nm Femtosecond Laser for High Contrast Tissue and *In Vivo* Imaging

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Abstract. Gold Nanorods (GNRs) with tunable aspect ratios can strongly absorb and scatter light in the NIR region due to their localized surface plasmon resonance (LSPR) property, and have been demonstrated to exhibit strong plasmon enhanced multi-photon luminescence (MPL) with brightness many times stronger than the conventional organic chromophores. In this study, we synthesized GNRs with longitudinal LSPR peak at 1036 nm to match our home-built light source 1040 nm femtosecond laser, which locates in the “optical window” where the tissue absorbs relatively little light. PEGylated GNRs with great biocompatibility were intravenously injected through the tail vein into mice. Excited by 1040 nm laser, the GNRs exhibit bright three-photon luminescence (3PL) signals while circulating in the blood vessels. The use of GNRs as bright contrast agents for 3PL imaging of mouse ear blood vessels *in vivo* was demonstrated. And GNRs targeted in tissues can be excited by 1040 nm laser and could be clearly visualized with no autofluorescence background. These results indicated that 3PL of GNRs is very promising for deep *in vivo* bio-imaging and assessing the distribution of GNRs in tissues with high contrast.

1. Introduction

Gold nanoparticles display many unique optical properties including localized surface plasmon resonance (LSPR), which makes them ideal enhancement agents for imaging, sensing and theranostics in biological systems [1]. Gold Nanorods (GNRs) with well-defined shapes and sizes are very attractive for their plasmon resonant absorption and scattering in the NIR region, making them promising probes for *in vitro* and *in vivo* imaging [2]. GNRs have been demonstrated to exhibit strong plasmon enhanced multi-photon luminescence (MPL) with brightness many times stronger than the conventional organic chromophores [3,4].

In this paper, three-photon luminescence of high aspect ratio gold nanorods with longitudinal LSPR peak at 1036 nm excited by 1040 nm femtosecond laser was investigated and its applications for high



contrast tissue and *in vivo* imaging were also demonstrated. Due to the bright three-photon luminescence (3PL) of PEGylated GNRs excited by 1040 nm laser, GNRs targeted in tissues could be clearly visualized with negligible autofluorescence background. The results demonstrated that 3PL imaging could be used to assess the distribution of GNRs in tissues with high contrast. After intravenously injected through the tail vein into mice, 3PL of GNRs can be excited and visualized in mice ear blood vessels, suggesting that GNRs are promising in *in vivo* imaging.

2. Materials and Methods

2.1 Synthesis of GNRs

GNRs with the longitudinal LSPR peak at 1036 nm were synthesized using an improved seed-mediated method proposed by Xingchen Ye [5]. Briefly, 0.2 mL of 25 mM HAuCl₄ and 5 mL of 0.2 M CTAB were mixed in 5 mL water solution and 0.6 mL of 0.01 M ice-cold sodium borohydride was quickly injected. The solution was stirred for 2 min and kept for 2 h before use. 250 mL of GNRs growth solution containing 3.5 g CTAB and 0.617 g NaOL and 0.5 mM HAuCl₄ was prepared in warm water (30 °C). 12 mL of 4 mM AgNO₃ solution was added and the mixture solution was stirred for 60 min. 2.6 mL of HCl (37 wt. %) and 0.625 mL of 64 mM ascorbic acid were added. Afterwards, 0.4 mL of seed solution was injected into the growth solution and left undisturbed at 37 °C for 12 h to let GNRs grow.

2.2 Surface modification of GNRs

50 mL of the as-prepared GNRs were centrifuged twice at 6000 rpm for 10 min, and then the precipitate was dispersed in 25 mL of 2 mg/mL PEG (SH-PEG-CH₃, MW = 5000) aqueous solution. The solution was stirred magnetically for 12 h. The final PEGylated GNRs solution was washed twice and dispersed in 1 × PBS solution for use.

2.3 Tissue slices preparation

PEGylated GNRs (in 200 μL 1 × PBS, 30 nM) were intravenously injected into mice and the control group were treated with the same amount of saline. The liver was excised at 0.5 h post-injection and fixed in 4% paraformaldehyde solution. The samples were embedded in paraffin and sectioned at 50 μm thickness and mounted on slides.

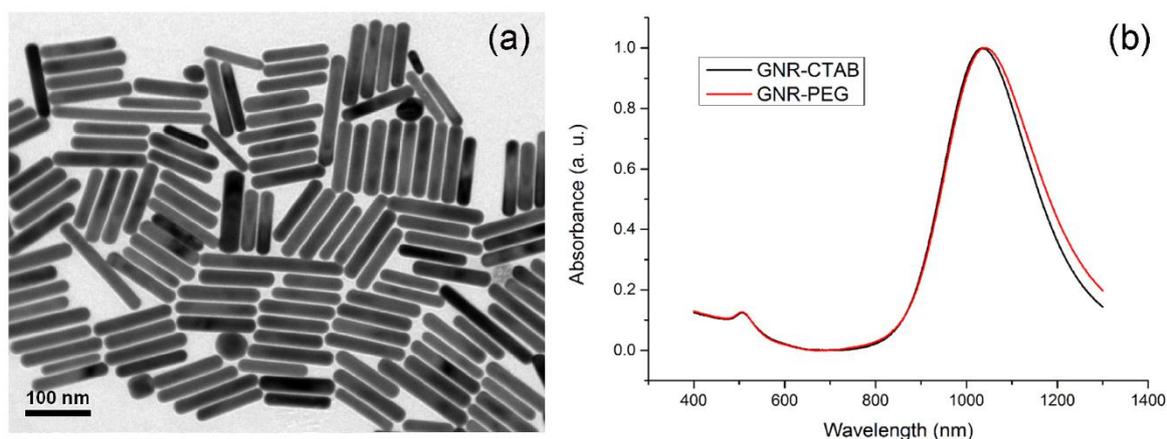


Figure 1. (a) Representative TEM image of GNRs; (b) Normalized UV-vis-NIR absorption spectra of GNRs (GNR-CTAB) and PEGylated GNRs (GNR-PEG).

3. Results and Discussion

3.1 Characterizations of GNRs

Transmission electron microscope (TEM) image of GNRs were captured by a JEOL JEM-1200EX microscope at 160 kV (Figure 1a). The GNRs are $\sim 15 \times 100$ nm, with aspect ratio = ~ 6.5 . As shown in Figure 1b, the absorption spectra of GNRs and PEGylated GNRs were measured using a Shimadzu UV3600 UV-vis-NIR scanning spectrophotometer. The longitudinal LSPR peak of PEGylated GNRs showed a little red shift compared with the as-prepared GNRs, indicating a successful coating of PEG polymer on GNRs. In addition, the absorption spectrum did not broaden after the PEG modification and the optical properties of GNRs were maintained for the imaging.

3.2 3PL imaging of GNRs in liver tissue

To access the biodistribution of GNRs in liver tissue, 1040 nm fs laser beam was focused onto the sample by a $20 \times$ objective lens (NA = 1.00) with a power of 10 mW. A short pass filter (492 nm) and a long pass filter (590 nm) were used to collect 3PL and 2PL signals from GNRs and liver tissue, respectively. As shown in Figure 2a and 2b, PEG-GNRs accumulated in the mouse liver due to the reticuloendothelial system (RES) and appeared as bright dots in the sliced tissue images (white circle in Figure 2a). When excited by 1040 nm laser, GNRs exhibit plasmon-enhanced multi-photon luminescence, including three-photon luminescence (3PL) and two-photon luminescence (2PL) [6,7]. The autofluorescence from liver was clearly observed in the 2PL channel (> 590 nm) and disappeared in the 3PL channel (< 492 nm), whereas the 3PL from GNRs can be visualized with super high contrast and little autofluorescence background. As a control, no bright dots of GNRs were observed in images of the tissue explanted from mice liver injected with saline (Figure 2c and 2d). These results demonstrated that bright 3PL of GNRs could be excited by 1040 nm femtosecond laser and this feature enables us to access the biodistribution of GNRs in tissues with high contrast.

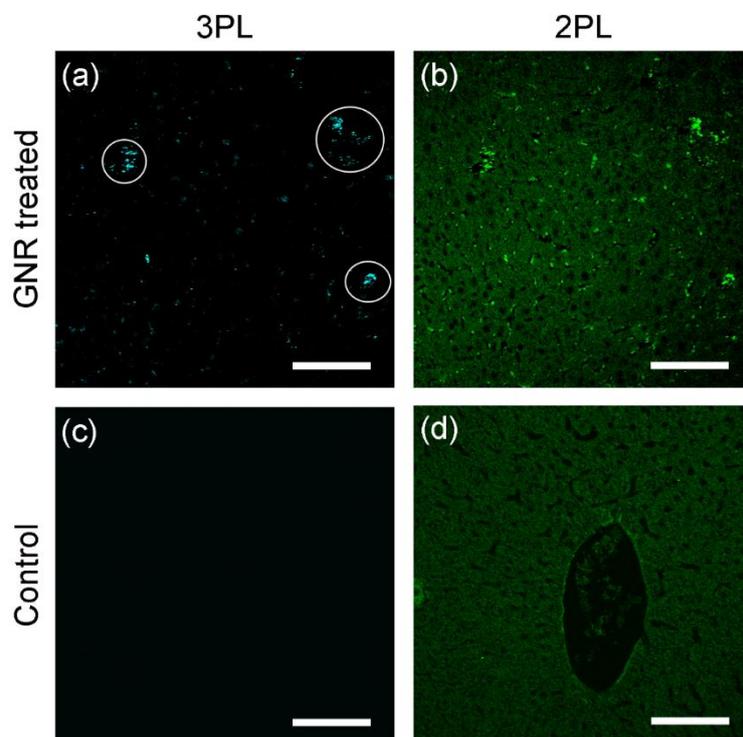


Figure 2. Multi-photon luminescence (3PL and 2PL) images of tissue slices harvested from mice liver injected with PEGylated GNRs (2a and 2b) and saline (2c and 2d). $\lambda_{\text{ex}} = 1040$ nm, emission signals collected within < 492 nm for 3PL channel and > 590 nm for 2PL channel. The white circles indicate the aggregates of PEG-GNRs in liver tissue. Scale bars: 50 μm .

3.3 *In vivo* 3PL imaging of GNRs in mouse ear blood vessels

PEGylated GNRs (in 200 μL $1 \times$ PBS, 30 nM) were intravenously injected into mice and the mice were anesthetized and placed on a petri dish with one ear attached to the coverslip [3]. The 1040 nm fs laser beam was focused by a water-immersion objective lens ($20 \times$, NA = 1.00) onto the earlobe immersed by water and 3PL signals were collected using a filter within < 492 nm. An 80 μm -deep stacks for 3PL imaging was taken with 5 μm step depth. As shown in Figure 3, 3PL of PEG-GNRs flowing in the blood vessels excited by 1040 nm laser at various depths could be clearly observed. Aside from major veins and arteries, the small capillaries could also be visualized with the GNRs. This result demonstrated that GNRs hold great promise to serve as a three-photon agent for intravital vasculature imaging.

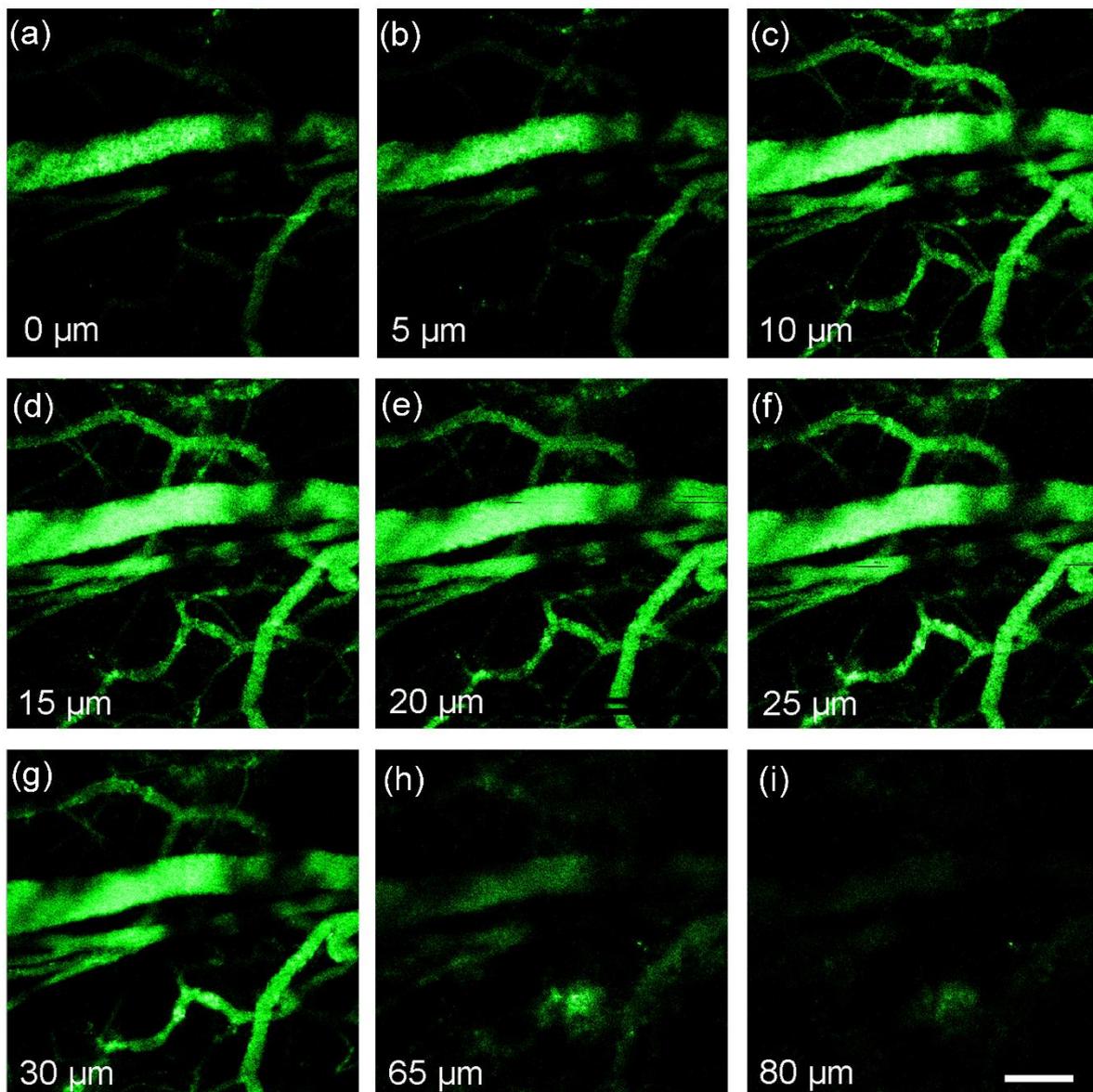


Figure 3. Intravital 3PL images of PEG-1036GNR-stained mouse ear blood vessels. (a)-(i) Images at various vertical depths of mouse ear skin. $\lambda_{\text{ex}} = 1040$ nm. Signal collected within < 490 nm. Scale bars: 100 μm .

4. Conclusions

In summary, high aspect ratio gold nanorods with the longitudinal LSPR peak at 1036 nm were synthesized and their three-photon luminescence excited by 1040 nm laser were observed in *in vitro* and *in vivo* imaging. PEGylated GNRs were intravenously injected through the tail vein into mice and flowed *via* blood circulation. Bright 3PL imaging of GNRs accumulated in liver tissue clearly illustrated the distribution of GNRs in major organs with high contrast. In addition, 3PL of GNRs in *in vivo* imaging of mouse ear blood vessels was demonstrated. Excited by longer wavelength laser, which penetrates deeply into tissues, 3PL imaging of GNRs holds great potential in high contrast and deep *in vivo* imaging.

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