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Transient absorption microscopy of gold nanorods as spectrally orthogonal labels in live cells†

Tao Chen,‡[a] Shouhui Chen,‡[b] Jihan Zhou,[c] Dehai Liang,[c] Xiaoyuan Chen*[d] and Yanyi Huang*[a]

Gold nanorods (AuNRs) have shown great potential as bio-compatible imaging probes in various biological applications. Probing nanomaterials in live cells is essential to reveal the interaction between them. In this study, we used a transient absorption microscope to selectively image AuNRs in live cells. The transient absorption signals were monitored through lock-in amplification. This provides a new way of observing AuNRs with no interference from background autofluorescence.

Metal nanoparticles have shown many promising optical properties, as well as other intrinsic characteristics, which make them great candidates for biomedical research. They have been demonstrated as molecular carriers, diagnostic probes, and therapeutic agents.[1–4] Among the variety of metal nanoparticles, gold nanorods (AuNRs) display particularly unique characteristics. They present a diverse range of optical processes, as their electron energy states can be finely regulated by the size and aspect ratio of the rods.[5] Furthermore, AuNRs can generate surface plasmon and result in a high intensity local electromagnetic field, through which the spontaneous Raman signal can be greatly enhanced.[6] AuNRs are strong light scatterers, which ensure their visualization by dark field microscopy.[7–11] AuNRs can also produce both single-photon and two-photon excitation luminescence (TPL).[12–14] The TPL has shown higher intensity than two-photon excited cellular autofluorescence under the same irradiation power.[15] However, the TPL intensity of AuNRs is not as high as that of the common fluorescent dyes.[15] Moreover, when conjugating AuNRs with intrinsically fluorescent molecules, it can become difficult to distinguish TPL of AuNRs from exgenous fluorescence.

Herein, we report a new imaging modality, transient absorption (TA), to selectively image AuNRs in live cells. TA is a mature method in spectroscopic measurement,[16–19] and it has recently been coupled with scanning microscopy as a contrast source to image single-walled carbon nanotubes and nanodiamonds.[20,21] TA, a multi-photon process, is the secondary absorption happening at the excitation state after the system's primary absorption, which can be characterized by an absorption spectrum (Fig. 1). This nonlinear optical response provides TA with intrinsic three-dimensional sectioning capability. With transient absorption microscopy (TAM), we can selectively

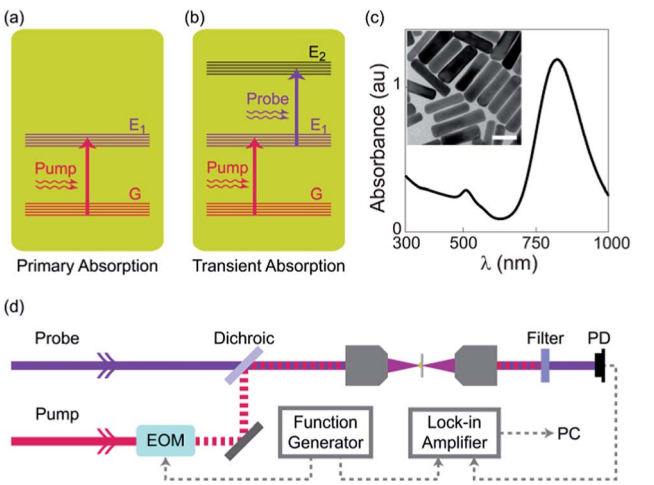

Fig. 1 Transient absorption of gold nanorods. (a) Primary absorption (pump) happened between the ground state (G) and the first excited state (E_1). (b) Transient absorption (probe) is the transition between excited states after the primary absorption. (c) UV-Vis absorption spectrum of gold nanorods. The inset is a TEM image of the same sample. The scale bar is 40 nm. (d) Schematic diagram of the transient absorption microscopy system. PD: photodiode.

[a]*Biodynamic Optical Imaging Center (BIOPIC), College of Engineering, Peking University, Beijing 100871, China. E-mail: yanyi@pku.edu.cn*

[b]*Med-X Research Institutes, Shanghai Jiaotong University, Shanghai 200030, China*

[c]*Beijing National Laboratory for Molecular Sciences and the Key Laboratory of Polymer Chemistry and Physics of Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China*

[d]*Laboratory of Molecular Imaging and Nanomedicine (LOMIN), National Institute of Biomedical Imaging and Bioengineering (NIBIB), National Institutes of Health (NIH), Bethesda, Maryland 20892, USA. E-mail: shawn.chen@nih.gov*

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‡ These authors contributed equally to this work.

highlight AuNRs from any fluorescent environment, providing a superior selectivity and background-free images.

We first studied the optical properties of AuNRs with TAM. The experimental setup has been reported previously.[21] Briefly, two spatially and temporally overlapped picosecond pulse lasers were introduced into a scanning microscope, serving as pump beam (1064 nm) and probe beam (tunable between 780–990 nm) in TAM. The TA signal strength is dependent on the incident power of laser beams. The power dependences on both pump and probe beams were verified by fixing the power of one beam, and the altering power of the other beam. TA signal intensity was recorded against both increasing and decreasing laser power (Fig. 2(a) and (b)). The probe wavelength was then scanned from 790 nm to 910 nm (Fig. 2(c)), and the signal intensity was almost maintained constant, indicating a broadband secondary absorption of AuNRs around the near-infrared region. We also measured the dependence of signal intensity against the delay time between two pulses (Fig. 2(d)). There was no signal when the probe pulse was ahead of the pump pulse. As the probe pulse was delayed with reference to the pump pulse, the TA signal first increased and then decreased. The asymmetric temporal behavior is a result of the transient nature in this process. The TA signal of AuNRs has a much longer lifetime than the pulse width.

We then applied TAM to image the cell uptake of AuNRs. HeLa cells were incubated in the culture medium with AuNRs for 8 h, and then imaged with TAM using the wavelength combination of 1064 nm (pump) and 816.7 nm (probe). The imaging system is also capable of performing stimulated Raman scattering (SRS) microscopy.[22] SRS can directly probe Raman-active vibrations, without labeling, from specific chemical bonds or structures, such as CH_2, which is abundant in cellular lipids. However, SRS can only be carried out when the two laser pulse trains are perfectly overlapped in time-domain. Through these wavelengths we used the SRS signal of CH_2 stretching (2845 cm^{-1}), when the time delay was adjusted to 0, to provide the lipid distribution inside live cells for comparison. When the SRS reveals the lipid components of live cells, we also observed bright dots scattered in cells, mostly in the cytoplasm (Fig. 3(a)). The SRS signal of lipid was relatively weak and evenly distributed cross the whole cell body except the nuclei, and it clearly sketched the outline of cells.

The unusually bright dots were actually the TA signal from AuNRs inside the cells. We adjusted the delay time between pump and probe pulse trains to differentiate the TA signal from SRS. When we tuned the probe pulses ahead of the pump pulses

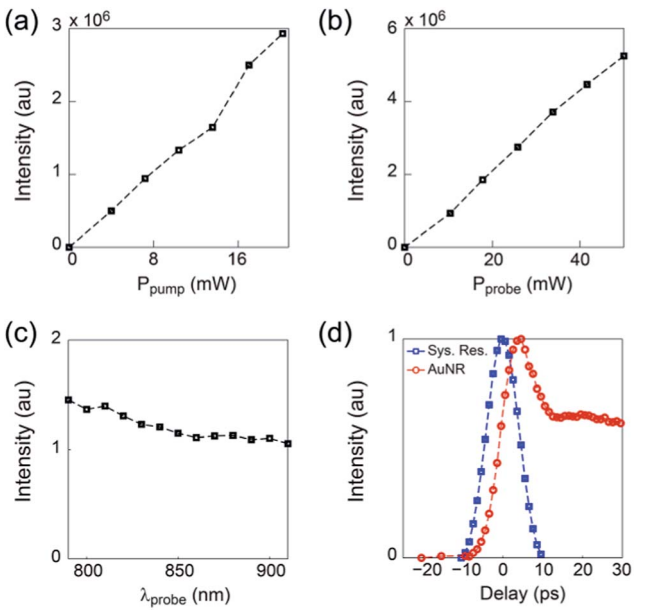

Fig. 2 Characterization of the transient absorption property of AuNRs. (a) The dependence of the TA intensity on the excitation power of the pump beam (1064 nm). (b) The dependence of the TA intensity on the excitation power of the probe beam (850 nm). (c) The dependence of the TA intensity on the probe wavelength. (d) The time-resolved TA intensity (red), with the stimulated Raman scattering signal of dodecane to present the system response (blue).

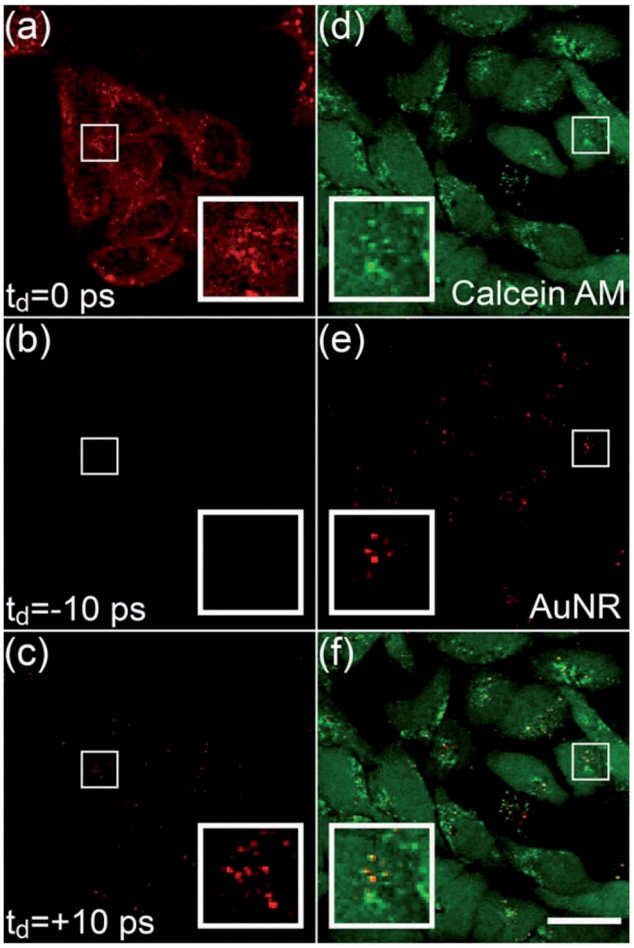

Fig. 3 Imaging AuNRs in live cells. (a)–(c) Imaging AuNRs in live HeLa cells at different delay time between the pulse trains of pump and probe beams. (d) Two-photo luminescence (TPL) image of AuNRs-containing live HeLa cells stained with calcein AM. (e) TA image of AuNRs demodulated by a lock-in amplifier. (f) Merged image of (d) and (e), showing the distribution of AuNRs in a highly fluorescent background. Insets in (a)-(f) show the zoomed regions for clarity. Specifically, yellow in (f) inset indicates colocalized TPL and TA signal from AuNRs. Scale bar: 25 μm.

with the separation of 10 ps (we defined this as −10 ps), we found that both weak and strong signals have disappeared (Fig. 3(b)). However, when we tuned the delay to be +10 ps, the weak signal disappeared while the strong dots were still visible (Fig. 3(c)). With this experimental configuration, we can clearly monitor the AuNRs through TA and completely eliminate the influence from SRS background signal generated by any other compounds. Furthermore, by adjusting the proper delay between pulse trains, we can achieve the multi-modal imaging with the TAM setup, combining the chemical specificities from both SRS and TA. Both SRS and TA are multiphoton processes, which provide comparable 3D sectioning capabilities.[21]

TA, like SRS, can probe the AuNRs in high spatial resolution at the diffraction limit. To verify whether the AuNRs form aggregates in aqueous solutions, we measured the hydrodynamic radius, R_h, of AuNRs that dispersed in the cell culture medium by dynamic light scattering. The apparent $R_{h,app}$ value determined at 30° was 18 nm. The diameter was thus comparable to the length of AuNRs (40 nm) measured by TEM, suggesting that AuNRs stayed mainly as single particles in the culture medium.

TA signal was acquired by a large-area photodiode that collected all the signals generated by laser excitation within the focal volume, including the fluorescence. However, the TA signal was encoded with the same modulation frequency as the pump beam while the fluorescence signal would not carry this frequency if it could not be excited by the pump laser. With a lock-in amplifier, which only demodulated the signal with the encoded pumping frequency, we should be able to easily decouple the weak TA signal from strong fluorescence background. We then tested the imaging capability of AuNRs using TAM with the existence of calcein AM, a fluorescent dye to indicate the cell viability.[23,24] After the HeLa cells were cultured with AuNRs-containing culture medium for 8 h, calcein AM was introduced to stain the live cells. Subsequently, all the live cells exhibited strong two-photon fluorescence under the excitation of probe laser (816.7 nm). This high-intensity green fluorescence spectrally well overlapped with the AuNR's two-photon luminescence emission and makes the differentiation of them impossible through the wavelength selection under the two-photon imaging channel (Fig. 3(d)). This disadvantage has created various difficulties of applying AuNRs as labels in biological research. However, the TA image (Fig. 3(e) and (f)) shows only AuNRs with extremely clean background. This spectral orthogonal imaging modality of AuNRs allows us to use them as a new category of labeling nanomaterials for bioimaging.

In summary, transient absorption can serve as a novel modality to image gold nanorods. The selective imaging capability can distinguish AuNRs from high backgrounds of Raman scattering or wavelength-overlapped fluorescence, avoiding the difficult identification of AuNRs with other optically active species. TAM, excited with near-infrared lasers, also provides the intrinsic 3D imaging capability with high spatial resolution, making it a suitable for live cell imaging using AuNRs as spectrally orthogonal labels. With the long-wavelength excitation provided by near-infrared lasers, TAM is a promising modality in tissue imaging, which would be hard for TPL or dark-field microscopy. By tuning the pump/probe wavelengths, hopefully, multiplex imaging can also be achieved. In addition, the absorption nature in TA process creates the possibility to quantitatively analyze single nanoparticles.

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Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Transient absorption microscopy of gold nanorods as spectrally orthogonal labels in live cells

Tao Chen,‡[a] Shouhui Chen,‡[b] Jihan Zhou,[c] Dehai Liang,[c] Xiaoyuan Chen,*[d] and Yanyi Huang*[a]

[a] *Biodynamic Optical Imaging Center (BIOPIC), and College of Engineering, Peking University, Beijing 100871, China*

[b] *Med-X Research Institutes, Shanghai Jiaotong University, Shanghai 200030, China. E-mail: yanyi@pku.edu.cn*

[c] *Beijing National Laboratory for Molecular Sciences and the Key Laboratory of Polymer Chemistry and Physics of Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China*

[d] *Laboratory of Molecular Imaging and Nanomedicine (LOMIN), National Institute of Biomedical Imaging and Bioengineering (NIBIB), National Institutes of Health (NIH), Bethesda, Maryland 20892, USA. E-mail: shawn.chen@nih.gov*

‡ These authors contributed equally to this work.

1. Methods and Materials

Synthesis of AuNRs

$HAuCl_4 \cdot 3H_2O$, $AgNO_3$, ascorbic acid, sodium borohydride and other chemicals were purchased from major suppliers such as Sigma-Aldrich and Alfa Aesar, and were used as received. The UV-Vis spectra were recorded with a Genesys 10s UV-Vis spectrophotometer.

AuNRs were synthesized using the seed-mediated growth method developed by Murphy et al [1]. Briefly, a seed solution of gold nanoparticles with an average diameter of around 4 nm was produced by the reduction of $HAuCl_4 \cdot 3H_2O$ (5 mL, 5 mM) with $NaBH_4$ (0.6 mL, 10 mM) in the presence of a cationic surfactant cetyltrimethylammonium bromide (CTAB, 5 mL, 200 mM). The resulting seed nanoparticles were used in the synthesis of AuNRs. In a typical procedure, to 8 mL of 0.1 M ascorbic acid solution was added 100 mL of 0.10 M CTAB, 2 mL of 25 mM $HAuCl_4$, 1.25 mL of 10 mM $AgNO_3$, and 2 mL of 0.5 M H_2SO_4 under gentle stirring at ambient temperature. Finally, 240 μL of the seed solution was added to the mixture, and the resulting solution was stirred for 1 min and then left undisturbed overnight at room temperature. According to TEM images, the average length, diameter, and aspect ratio of AuNRs were calculated to be 67 ± 8 nm, 16 ± 3 nm, and 4.2 ± 0.5, respectively. The concentration of AuNRs can be calculated by means of their UV-Vis spectra and the extinction coefficient at the longitudinal plasmon peak.

Transient absorption microscopy (TAM)

The TAM was constructed on a modified two-photon scanning microscope system. Light source for TAM is a pump source integrated optical parametric oscillator, also known as one-box OPO (picoEmerald, APE, Berlin, Germany). This one-box OPO can simultaneously provide two spatially and temporally overlapped pulse trains, with a repetition rate of 80 MHz. The wavelength of one beam is 1064 nm (the pump beam in TAM), and the other is tunable between 780-990 nm (the probe beam in TAM). Pulse duration is 8 ps for the pump beam and 6 ps for the probe beam. The two beams are sent into an inverted multiphoton microscope (IX81/FV1000, Olympus, Japan). Intensity of the pump beam is modulated with an electro-optic modulator (EO-AM-NR-C2, Thorlabs, USA) at 9.825 MHz. The two beams are collinearly aligned and focused by a 60X water immersion objective (UPLSAPO 60XW, Olympus, Japan), where they scan over the sample through galvano mirrors in the scanning unit. After transmitting through the sample, the probe beam is collected by a water immersion condenser (N.A. 0.9, Olympus, Japan), then focused to a large area photodiode (FDS1010, Thorlabs, USA). The photo-electric signal was sent into a home-made lock-in amplifier. After demodulation, the amplified signal was sent into an A/D converter, and then used to reconstruct images. Stimulated Raman scattering signal of paraffin's CH_2 stretching mode (2845 cm^{-1}) was acquired to characterize the system response and to evaluate the correlation between the two pulse trains.

Cell culture

The Hela cells were maintained in Dulbecco's Modified Eagle Medium(DMEM) complete media containing 10% Fetal Bovine Serum(FBS), and 1% penicillin-streptomycin mix. Cells were seeded into 35mm petri-dish at a concentration of $10^5/cm^2$. Cells were cultured in a humidified incubator at 37 °C and 5% CO_2 atmosphere.

Cell uptake of AuNRs

Hela cells were passaged into petri-dish with glass bottom. Cultured as mentioned above. Before cells grew to confluence, changed medium with AuNRs. Stocked AuNRs were added to culture medium with ratio of 1:1000. Then, changed the AuNRs-contained medium. Cultures the cells in incubator for 8h before imaging.

3-Dimensional time-lapse imaging

Hela cells were prepared as described above. AuNRs were added to Hela cells 2 hours before imaging. Wash the cells with PBS to remove remaining AuNRs in culture medium. Then an automatic 3-D time-lapse imaging was performed for about 20 mins.

Particle size measurement

AuNRs were suspended in culture medium 1X(1μg/mL) and 10X concentrations. Suspensions are then used to measure particle size distribution with light scattering analyzer (ZetaPALS, Brookhaven Instruments Co., NY, USA).

2. Supplementary Figures

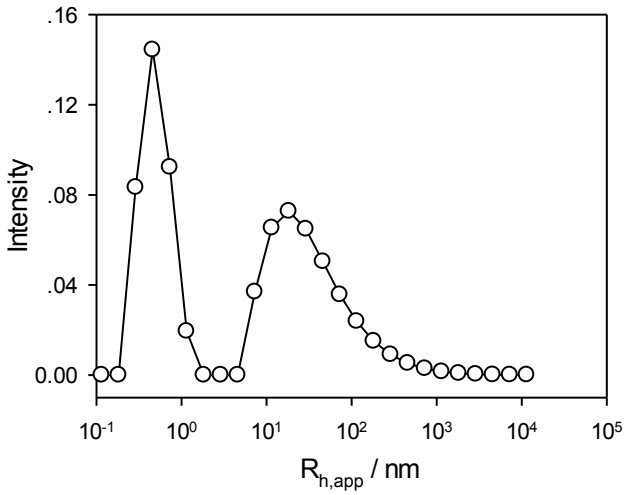

Fig S1. The appearance hydrodynamic radius ($R_{h,app}$) of AuNRs in cell-culture medium measured by dynamic laser light scattering at 30°. The distribution with the average $R_{h,app}$ about 0.5 nm is attributed to the diffusion caused by the culture medium, while the distribution with the average $R_{h,app}$ about 18 nm is caused by the diffusion of AuNRs.

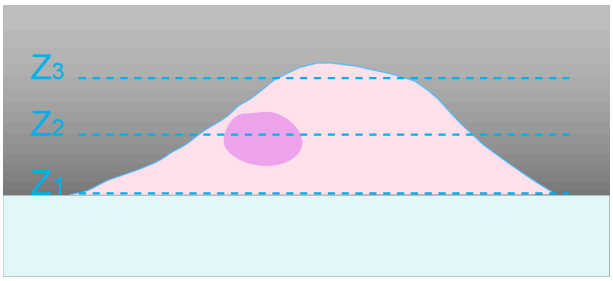

Fig S2. Illustration of focal plane selected in 3-D time-lapse. z_1, z_2, and z_3 corresponds to movies S1 - S3.

3. Supplementary Movies

Movie S1-S3: Motion of AuNRs in live HeLa cells lasting for ~ 20 min at different focal planes, as shown in Fig S2.

Movie S4: 3D distribution of AuNRs in live Hela cells. Faint blue showed the cell profiles. Bright green/white showed the AuNRs. Color code remains the same in the movies.

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