Deep, high contrast microscopic cell imaging using three-photon luminescence of β-(NaYF₄:Er³⁺/NaYF₄) nanoprobe excited by 1480-nm CW laser of only 1.5-mW

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Abstract: It is challenging to achieve deep microscopic imaging for the strong scattering in biotissue. An efficient three-photon luminescence can effectively increase the penetration depth. Here we report that β-NaYF₄: Er³⁺/NaYF₄ UCNPs were excited by a 1480-nm CW-laser and emitted 543/653-nm light through a three-photon process. With the merit of the hexagonal crystal phase, sub-milliwatt laser power was utilized to excite the UCNP-probed cells to minimize the heating effect. The polymer-coated UCNPs were shown to be harmless to cells. The deep, high contrast in vitro microscopic imaging was implemented through an artificial phantom. Imaging depth of 800 μm was achieved using only 1.5 mW excitation and a 0.7 NA objective. The green/red emission intensities ratio after penetrating the phantom was studied, indicating that longer emission wavelength is preferred for deep multiphoton microscopy. The proposed and demonstrated β-UCNPs would have great potential in three-photon microscopy.

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OCIS codes: (160.5690) Rare-earth-doped materials; (190.4180) Multiphoton process; (180.0180) Microscopy.

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1. Introduction

Multiphoton optical imaging is a powerful, non-invasive tool for life science studies and biological medical applications. It offers a number of important advantages over other imaging technologies, especially for in vivo imaging of brain and vascular structures. In recent years, two-photon microscopy (2PM) has been used for deep tissue imaging. Theer P. et al. achieved a maximum imaging depth of 1 mm with 925-nm excitation in 2PM using a regenerative amplifier (~200 nJ) as the excitation light source [1]. Xu C. et al. achieved the same depth (1 mm) in in vivo imaging of adult mouse brains via a longer wavelength light at 1280 nm with about 93-mW power [2] and later enhanced the excitation power to 120 mW to increase the imaging depth to 1.6 mm [3]. The three photon excitation (3PE) is an optimized choice that provides significant improvement for the longer wavelength excitation with less
scattering in biotissue as well as the better signal background ratio (SBR), and therefore three-photon microscopy (3PM) is used to extend the imaging depth [4, 5]. Recently, Xu C. et al. developed a 1700-nm high-pulse-energy femtosecond (fs) laser light to achieve up to ~1300 μm deep images for an intact mouse brain using 3PM [5]. However, in these multi-photon deep imaging studies, an expensive, complicated and high maintenance fs laser system was involved, and the access to these lasers is not common (e.g. similar high energy 1700-nm fs source is hardly commercially available) [6, 7]. To solve this problem, it is imperative to develop a deep imaging method employing only cost-effective, easily accessible light sources (e.g. CW laser). Another limiting factor is that the dyes or fluorescent proteins, commonly used in the multi-photon microscopy, have intrinsic photo-bleaching issue as contrast agents in bioimaging.

Among the contrast agents, upconversion nanoparticles (UCNPs) have been considered as a good choice of luminescence probe for biological applications because of their high contrast, high resolution, continuous emission, and deep penetration [8–11]. They sequentially absorb two or more low-energy photons (CW laser excitation), which are generally in the near-infrared (NIR) range, and emit anti-Stokes shifted light. UCNPs as an efficient contrast agent has already been used in the 2PM deep imaging in 2009 by F. Li et al. with 980 nm excitation and getting ~0.6 mm depth in tissue [12]. Er³⁺-doped UCNPs have strong optical absorption during the 1450-1580 nm band for the \( ^{4}I_{15/2} \rightarrow ^{4}I_{13/2} \) transition [13]. This band is within the NIR range, which can be used as the excitation light for implementing 3PM. In 2011, Er³⁺-doped LiYF₄ UCNPs were synthesized by Chen G. et al. with a 1490 nm excitation and three-photon UC emission [13]. Later on NaYF₄: Er³⁺ UCNPs were synthesized and excited at 1550 nm and 1523 nm, respectively [14, 15]. J. Qian et al. then used this material in deeper imaging application by loading pure UCNPs solution into glass capillary tubes embedded in phantom [16]. However, the class of ~1500 nm excited UCNPs has not been used to deep imaging in vitro and in vivo. Notably, implementing effective 3PM requires highly efficient nanoparticles because strong absorption of this excitation band allows for low power excitation.

In this work, for the first time, we synthesized uniformed \( \beta \)-(NaYF₄: Er³⁺/ NaYF₄) UCNPs. Thanks to the highly efficient hexagonal (β)-phase and the most efficient host material NaYF₄, we can use low power lasers as the excitation that can decrease the damage to the cells. In this study, the as-prepared \( \beta \)-(NaYF₄: Er³⁺/ NaYF₄) was efficiently excited by a low power 1480-nm CW diode laser to implement 3PM. The UCNPs were coated with a biocompatible polymer through electrostatic adsorption and then cultured with HeLa cells to be probed. The deep microscopic imaging was implemented with an artificial tissue phantom as the spacer. The 800 μm depth imaging were obtained through this method using less than 1.5 mW only. The red/green emission ratio, resolution and signal-to-noise ratio (SNR) in these images were analyzed. This result was limited by the low NA (0.7) objective as well as several refraction index-mismatched interfaces involved in the imaging sample [17].

2. Materials and method

2.1 Materials

Lanthanide chlorides (YCl₃·6H₂O and ErCl₃·6H₂O ≥99.9%), 1-octadecene (ODE) (≥90%), polydactylous hydrochloride (PAH) and polychromatic acid (PAA) were purchased from Sigma-Aldrich. NH₄F was purchased from Aladdin Co., Oleic acid (AR), ethyl alcohol (AR), chloroform (AR) and cetyltrimethyl ammonium bromide (CTAB) were purchased from Sinopharm Chemical Reagent Co., China. TiO₂ (AR) was purchased from Kermel, China. Regular agarose G-10 was purchased from BIORWEST, Spain. Deionized (DI) water was used in all the experimental procedures.
2.2 Synthesis of $\beta$-(NaYF$_4$: 25% Er$^{3+}$/NaYF$_4$)

The NaYF$_4$: 25% Er$^{3+}$ was synthesized through a modified method [18]. Briefly, YCl$_3$·6H$_2$O (0.75 mmol) and YCl$_3$·6H$_2$O (0.25 mmol) were heated to 120 °C in oleic acid and ODE for 40 min. The solution was cooled down to room temperature, after which NH$_4$F (4 mmol) and sodium oleate (2.5 mmol) were added. The solution was then heated to 310 °C for 60 min under the protection of argon air, then cooled rapidly, and 10 mL ethanol was added when the solution reached <75 °C. Nanoparticles were precipitated by ethanol and collected by centrifugation at 7500 rpm for 4 min. Then the UCNPs were cleaned by ethanol several times and stored in the chloroform solution.

The NaYF$_4$ shell was synthesized by a similar process. Briefly, YCl$_3$·6H$_2$O (1 mmol) was heated to 120 °C in oleic acid and ODE for 40 min. The solution was cooled down to 70 °C, and then the previous core solution in chloroform was added. The chloroform was removed by vacuum and then the solution was cooled to room temperature after which NH$_4$F (4 mmol) and sodium oleate (2.5 mmol) were added. The solution was then heated to 280 °C for 40 min under the protection of argon air, then cooled rapidly, and 10 mL ethanol was added when the solution reached < 75 °C. Nanoparticles were precipitated by ethanol and collected by centrifugation at 7500 rpm for 4 min. Then the UCNPs were cleaned and stored in 4 mL chloroform.

2.3 Hydrophilic process of the UCNPs

1 mL of the as-prepared UCNPs chloroform solution was added into 10 mL 0.1 mM violently revolving CTAB solution with a temperature of 70 °C. Then the solution was cooled down to 40 °C gradually and kept for 2 hours. The UCNPs were collected by centrifugation at 7500 rpm for 4 min. Then the UCNPs were cleaned by DI water several times to remove the residual CTAB. The UCNPs were stored in 1 mL DI water.

2.4 Synthesis of polymer-functionalized UCNPs

First a PAA layer was modified onto the UCNPs, following a PAH layer. The methods of the two layers are the same. Briefly, 200 μL of the core/shell UCNPs solution were diluted to 2 mL, and then 400 μL PAA (10 mg/mL) that were dissolved in a 10 mM NaCl solution and 200 μL 10 mM NaCl solution were added. Then this mixture was revolving gently for 30 minutes, after which it was centrifuged at high speed for 5 minutes. The precipitated UCNPs were redispersed in 2 mL deionized water, and an identical process was conducted to modify a PAH layer. The UCNPs were stored in 1 mL DI water.

2.5 Phantom preparation

The tissue phantom consisted of 0.5% agarose, which was typically used as phantom matrix material. 0.7 grams per liter (g L$^{-1}$) of TiO$_2$ were added as scatterers, while 25 × 10$^{-6}$ liters of ink per liter of tissue phantom were added as absorbers. The TiO$_2$ concentration was chosen according to the data in [19], which suggests a reduced scattering coefficient of 0.34 mm$^{-1}$. The absorption coefficient was controlled within a range between 0.006 to 0.009 mm$^{-1}$ [20]. The optical properties of the phantom are in the range of human epidermis and dermis tissues, suggesting that this phantom mimics human skin tissues well [21].

2.6 Cell culture and treatment

HeLa cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 IU/mL penicillin–streptomycin at 37 °C under 5% humidified CO$_2$. Upon reaching confluence, the cells were trypsinized and replanted at a density of 1 × 10$^6$ cells/mL onto a confocal petri dish (35 mm) and grown at 37 °C under 5% humidified CO$_2$ for 48 hrs. For UCNPs labeling, the cells were rinsed gently with pre-warmed PBS and incubated with 400 μL 10 mM PAA-
PAH-UCNPs added in a diameter of 6-cm cell-dish with 4 mL DMEM for 4 hours at 37°C. After that, the cells were gently rinsed twice with PBS to remove unbound UCNPs.

2.7 Cell viability evaluation of UCNPs, 1480 nm laser treatments using CCK-8 assay

HeLa cells ($6 \times 10^3$) in 100 μl of the DMEM medium were seeded onto 96-well plates and incubated with a 10 μL PAA-PAH-UCNP solution. After 12 hours of incubation, the medium was replaced by PBS and the cells were exposed to a 1.5 mW 1480-nm CW laser for 10 min. And then the PBS was replaced with fresh medium and the cells were incubated again at 37 °C with 5% CO₂ for 24h. After treatment, 10 μL of the CCK-8 solution were added to each well of the plate, followed by incubation for another 1 hour. Then, cell viability was determined by measuring absorbance at the wavelength of 450 nm with a microplate reader. All the measurements were conducted in triplicate.

2.8 Characterization methods

The size and shape of the nanoparticles were characterized by transmission electron microscopy (TEM) using a JEM-2100HR microscope at an acceleration voltage of 100~200 kV. X-ray diffraction (XRD) patterns were obtained on a Bruker D8 ADVANCE X-ray diffractometer system with Cu Kα1 radiation from 2θ of 25 to 70°. The Fourier Transform Infrared spectroscopy (FTIR) was measured by using a Thermo Nicolet 6700 device. Zeta potential measurements were performed by a NanoPlus instrument (Micromeritics, USA) at room temperature.

2.9 Photoluminescence spectroscopy and deep 3PM

The emission spectra of UCNPs in Fig. 2 were recorded by a fiber spectrograph (QE65000, Ocean Optics) in the microscope system. A CW diode laser (LEO Photonics, China) working at a 1480 nm wavelength was utilized in this experiment. The pump-power dependence was measured with an optically thin sample on the coverslip through a microscope. The beam profile after the objective was estimated to be a circle with the diameter of 4 μm. This in vitro cell UC imaging experiment was implemented in the multiphoton laser scanning microscopy system (FV10MPE-S, Olympus). Green and red UC photoluminescence from UCNPs were obtained using two channels with proper filters. The setup is illustrated in Fig. 6(a). The NA of the objective (×20, 1.6 mm WD, Olympus) is 0.7.

3. Results and discussion

3.1 Characterization of core and core-shell UCNPs

The NaYF₄: 25% Er³⁺ core and NaYF₄ shell were synthesized through a solvothermal procedure which is stated in the methods. The size and morphology were characterized by TEM, where the core UCNPs clearly exhibited a uniform, spherical shape with an average diameter of 21.92 nm (Fig. 1(a)-1(b)). After being coated with a ~12 nm NaYF₄ shell, the nanoparticles are nearly 45.3 nm (Fig. 1(d)) and present a regular hexagon as seen in Fig. 1(c). The UCNPs are still very uniform with the NaYF₄ shell. As shown in Fig. 1(f), XRD patterns of the core-shell UCNPs have well-defined peaks, and this measured data matched well with the hexagon crystal structure (JCPDS 16-0334) to be found with a β phase. This shell is very helpful in enhancing the emission for both inhibiting the quenching of surface activator ions and formation of the β phase structure [22]. With the high-resolution TEM analysis (Fig. 1(e)), one can easily observe a d-spacing of 0.51 nm, and this result demonstrated the high crystallization of UCNPs.

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3.2 Emission spectra of core-shell UCNPs

The emission spectrum of core-shell UCNPs was measured under 1480 nm CW laser excitation and the results are shown in Fig. 2(a). Four upconversion emission bands are clearly resolved and are maximal at 524, 543, 653, 809, and 990 nm, respectively. These emissions are assigned to the transitions \( ^{2}H_{11/2} \rightarrow ^{4}I_{15/2} \) (524 nm), \( ^{4}S_{3/2} \rightarrow ^{4}I_{15/2} \) (543 nm), \( ^{4}F_{9/2} \rightarrow ^{4}I_{15/2} \) (653 nm), \( ^{4}I_{9/2} \rightarrow ^{4}I_{15/2} \) (809 nm), and \( ^{4}I_{11/2} \rightarrow ^{4}I_{15/2} \) (990 nm) of Er\(^{3+}\) (Fig. 2b) [23]. The 990 nm is the strongest emission band that submerges the emissions at 543 and 653 nm, which were magnified in Fig. 2(a). To investigate the upconversion mechanism at 543 and 653 nm for the \( \beta-\text{(NaYF}_4: 25\% \text{Er}^{3+}/\text{NaYF}_4) \) nanoparticles, the dependence of the emission intensities on different pump laser powers was measured. The number of photons transferred was calculated by the formula \( I \propto P^n \), where \( I \) is the emission intensity, \( P \) is the pump laser output power, and \( n \) is the number of photons involved in the excitation process [24]. The \( n \) was calculated by measuring the emission images in the microscope system and its corresponding pump laser power, and the results were used to draw a double-logarithmic plot in which the slope was \( n \). The laser intensity range was from 0.07 mW to 1.2 mW, during which the \( n \) had three different values from low power intensity (Slope L), middle power intensity (Slope M) to high power intensity (Slope H). This is because of the saturation of the energy levels at high power intensity and this saturation power differs in different luminescent materials [25]. Figure 2(c) shows the dependence of the two different luminescent emission bands versus the incident excitation power density, which fits well with the linear relation. The slopes are 2.92, 1.95 and 1.02 for the detected red emission, 3.21 and 2.67 for green emission, respectively. This demonstrated the three photon process of red emission especially for a low power intensity excitation. The slope of green emission in low power situation was larger than 3 because four-photon process would be partially involved in the process of green emission, which could be attributed to the transition GSA\( \rightarrow \)ETU1\( \rightarrow \)ETU2\( \rightarrow \)ETU3 as shown in Fig. 2(b). In this way, even the excitation above or below the focal plane will still be 3PE or more than a 3PE.
Fig. 2. (a) The emission spectrum of β-(NaYF₄: 25% Er³⁺/NaYF₄). (b) Mechanism diagram of upconversion via ETU processes between two Er³⁺ ions. It concludes ground state absorption (GSA), excited state absorption (ESA) and also energy transfer upconversion (ETU). (c) Pump-power dependence for green and red UC emission intensities of β-(NaYF₄: 25% Er³⁺/NaYF₄) with 1480 nm CW excitation.

3.3 Hydrophilization of core-shell UCNPs and cell imaging

Before deep microscopy, the UCNPs probed cancer cells in *in vitro* microscopy were performed. However, the β-(NaYF₄: 25% Er³⁺/NaYF₄) nanoparticles are capped with oleic acid (OA), which is not biocompatible. In this work, a strategy using CTAB was adopted to turn the UCNPs hydrophilic. Then the hydrophilic UCNPs were dissolved in deionized water, and the surface of the UCNPs exhibited a strong positive potential of 167.72 mV. Afterwards, the nanoparticles were successively capped with water-soluble PAA and PAH to enhance the biocompatibility as well as to reduce toxicity [26, 27]. When the PAA was capped onto the surface, the UCNPs were negatively charged, and this allowed for positively-charged PAH to be coated on next. All the surface zeta potentials were measured by a Zeta-Plus and the results are shown in Fig. 3(b)-3(c). The schematic diagram showed in Fig. 3(a) illustrates the surface modification strategy and process for the as-synthesized UCNPs. In the FTIR spectrum of OA-UCNPs, the peak at 3008 cm⁻¹ was attributed to the C-H stretching vibration, and peaks at approximately 2926 cm⁻¹ and 2855 cm⁻¹ were attributed to the asymmetric and symmetric stretching vibrations of methylene (CH₂) in the long alkyl chain, indicating that OA was coated on the surface of the nanoparticles. The peak at 1680 cm⁻¹ as well as the inconspicuous peak located around 745 cm⁻¹ indicates the existence of an additional N-H bond, which manifests the existence of PAH.

Then, the polymer-coated UCNPs were cultured with HeLa cells for 4 hours before the microscopy imaging. The power level at the sample surface was 0.8 mW, which was a really low power and cannot damage cells. Considering the detection wavelength range of PMT, we couldn’t detect the transmission channel image. However, it didn’t affect detection of the luminescence signals as shown in Fig. 4. The signals of green and red emission through a 3PE were strong and the images show a high contrast. It also indicated that the PAA-PAH-coated UCNPs were easily endocytosed by cells and did little harm to them [26].
As 1480nm laser can be strongly absorbed by water, which could result in damage to biological tissues due to overheating effect, the theoretical cell-in-celldish model is used to simulate laser-induced spatiotemporal temperature distributions [28, 29]. Considering the low power (<1.5 mW) and high water absorption coefficient near 1480 nm, the $Q_{1480\text{nm}}$ is calculated to be 3553 W/m$^3$. The radiation time was set to be 20 minutes. And the temperature increased less than 0.3°C after 20-minute irradiation from 37°C (initial temperature) to 37.25 °C. The simulation result showed in Fig. 5(a) clearly indicates that such a low power of 1480-nm excitation won’t induce overheating effect in biological tissues. Since the cell-in-celldish model are based on PBS solution, this water-based sample will have more absorbance than animal tissue, which means heating effect would be ever less in the actual tissue. The inviability in Fig. 5(b) also proved this after 12 hours cultivation with PAA-PAH-UCNPs. When the PAA-PAH-UCNPs and 1480 nm laser were added to the cells together, there was still little harm to them. It could be found in Fig. 5(b) that the cell still had a high viability after 10 minutes of irradiation by a ~1.5 mW 1480 nm CW laser using the CCK8 method.
3.4 Deep microscopy by 3PE

To implement the deep microscopy, a layer of artificial phantom made of agarose was used. The TiO$_2$ was added and dispersed into agarose as scatterers. We used two pieces of coverslip to shape the phantom as well as to conveniently control the thickness. To minimize the refraction effect of the bottom of the culture disk, the cancer cells were cultured on a coverslip as shown in Fig. 6(a). When the thickness of the phantom was 500 $\mu$m, the high contrast UCNP-probed fluorescence imaging of cancer cells with a size of 320 $\times$ 320 pixels acquired as showed in Fig. 6(b). When the thickness of the phantom increased to 800 $\mu$m, the UCNP-probed cells can also be observed with the clear cell profile as an example in the white dots area (Fig. 6(d)). This imaging depth was affected by several factors including the low NA and more Fresnel refraction of reflection due to several air-glass interfaces between the sample and the objective. Also a low power (1.5 mW) output of the objective degraded the SNR of the images and this excitation intensity would be much attenuated after penetrating the phantom. The scale relating the pixel values to the color level in the red channel image was set aside the pictures, which could quantitatively show the emission intensity of the difference in 500 $\mu$m and 800 $\mu$m (Fig. 6(b), 6(d)).

Besides, what also interested us was that the intensity ratio of the green and red emission varied with the phantom thickness. When there was no phantom, the same area of green and red was chosen. The intensity ratio in Fig. 4(d) indicates that the intensity of green was twice as high as that of the red. These results were somewhat affected by the PMT, which was more sensitive to green light. However, after penetrating the 500-$\mu$m phantom, the green emission was much lower than the red, with a ratio of 0.32:1 shown in Fig. 6(c). The ratio was aggravated by the 800-$\mu$m phantom, for the green emission was only 17% of the red (Fig. 6(e)). All these results demonstrated that the green light had a stronger attenuation than the red in the biological tissue. In the microscopy imaging the red emission was mainly used and analyzed. This study, for the first time, indicates longer emission wavelength would be preferred for deep microscopic imaging as for the same intensity dependence of excitation.

![Fig. 6. (a) Schematic diagram for the setup of the objective, phantom and the cells on the coverslip. (b) In vitro cancer cell 3PM imaging based on a layer of 500 $\mu$m phantom. (d) In vitro cancer cell 3PM imaging based on a layer of 800 $\mu$m phantom. They are green channel, red channel and overlay images, respectively. (c) and (e) are the relative value of emission intensity of green and red in the dotted box area. The excitation laser intensity was 1.5 mW output of the objective. The yellow dashed box area was shown in Fig. 7. The scale bar is 30 $\mu$m.](image)

In order to quantify the SNR in the images, the intensity line profiles across the cell border shown in Fig. 7 were used in the red channel images. We calculated the average noise and signal intensity by using a small area in the images like the method in the literature [12]. The SNR was calculated to get the results that SNR$_{500\mu m}$ was 46.75 and SNR$_{800\mu m}$ was 15.87. This finding quantitatively shows the SNR decrease with the depth of artificial tissue in vitro for both the attenuation of excitation and emission light. The value of the full width half...
maximum (FWHM) of the line profiles across the cell border was obtained to show a fine resolution (Fig. 7).

![Intensity line profiles across the cell border](image)

**Fig. 7.** Intensity line profiles across the cell border used to characterize the SNR and resolution. The intensity profile was along the yellow line in the corresponding image.

### 4. Conclusion

We have synthesized efficient $\beta$-(NaYF$_4$: Er$^{3+}$/ NaYF$_4$) UCNPs which, excited by a 1480-nm CW laser, could efficiently emit 543 and 653 nm light bands through a three-photon excitation process. After being coated with PAA and PAH, it can be taken up by cancer cells and exhibited very low cytotoxicity. The UCNP-stained cells can be excited by a low, safe power laser and achieved clear, high contrast cancer cell imaging. Furthermore, these two emissions were used in 3PM to perform the deep, high contrast *in vitro* microscopic imaging. An artificial phantom with mimic properties was used and an imaging depth of 500 μm and 800 μm was demonstrated, though limited by the specific experimental conditions including low NA (0.7) and several refraction index-mismatched interfaces involved in the imaging sample. One can easily follow our proposal and increase the imaging depth with better experimental conditions and alternative excitation wavelengths. The proposed CW laser excited 3PM imaging using UCNPs would completely eliminate the femtosecond laser induced autofluorescence from tissue itself, facilitating better SBR. This study has showed that the Er$^{3+}$-doped $\beta$-UCNP is a powerful contrast agent and has great potential in 3PE deep microscopic imaging. In addition, the influence of emission light scattering on the microscopic imaging depth has been discussed and may provide good guidance for other related studies.

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