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Toward the Use of Two-Color Emission Control in Upconverting NaYF₄:Er³⁺, Yb³⁺ Nanoparticles for Biomedical Imaging

Christian F. Gainer^a, Gihan S. Joshua^a, and Marek Romanowski^a

^aDepartment of Biomedical Engineering, University of Arizona, 1657 E Helen St, Tucson, AZ USA 85721

Abstract

In the interest of generating new biomedical sensing techniques as well as improving those that currently exist, a great deal of attention has been given to upconverting lanthanide nanoparticles in recent years. In order to develop these nanoparticles for use in multiplexed and ratiometric sensing techniques, many recent studies have focused on experimental control of their emission wavelengths. Here we describe a new method for controlling the relative intensity of green and red emission bands in NaYF₄:Yb³⁺, Er³⁺ nanoparticles via control of the excitation pulse repetition rate. Using this method, particles of the same composition may be tuned to produce red and green light in user-defined ratios. We discuss the mechanism behind this control as well as potential applications that could make use of this property, specifically in super resolution imaging techniques.

Keywords

Upconversion; nanoparticle; lanthanide; erbium

1. INTRODUCTION

Over the course of the last decade, many advances have been made in the development of upconverting lanthanide nanoparticles (UNPs) for use in biomedical imaging.¹⁻³ These nanoparticles are typically composed of a crystalline host matrix doped with a mixture of trivalent lanthanide ions. Yb³⁺, Er³⁺, and Tm³⁺ are of particular interest due to the fortuitous matching of several electronic transitions between metastable states. These states provide a convenient route for two photon excitation processes, resulting in the conversion of NIR excitation to anti-Stokes shifted emission by energy transfer upconversion (ETU), which occurs with much greater efficiency than excited state absorption (ESA) or direct excitation without intermediate states, commonly referred to as two-photon excitation (2p).⁴

UNPs have numerous advantages over fluorescent dyes and quantum dots for biomedical imaging applications. NIR excitation is scattered less than UV and visible wavelengths, improving depth of penetration in tissue. Anti-Stokes shifted emission effectively negates interference from endogenous fluorescence in most confocal and wide-field microscopy

techniques. Additionally, the relatively high efficiency of ETU allows these particles to be used in 2p microscopy with minimal interference from endogenous fluorophores. While the long luminescence lifetime of these particles renders their use in scanning microscopy techniques difficult, this can be mitigated through image processing techniques. UNPs are also exceptionally photostable; photobleaching effects are practically nonexistent.⁵

NaYF₄ nanoparticles codoped with Yb³⁺ and either Er³⁺ or Tm³⁺ are commonly regarded as being among the most efficient UNPs, due in part to the low phonon energy of the host matrix.⁶ Excitation of these particles occurs through ytterbium's $^2F_{7/2} \rightarrow ^2F_{5/2}$ transition at $\sim 10,200 \text{ cm}^{-1}$. Once excited, these ions act as sensitizers for a nearby Er³⁺/Tm³⁺ ion, exciting it multiple times in order to produce higher energy emission. For Er³⁺ doped UNPs, this emission can occur at a number of wavelengths, including 417, 488, 525, 546, 668, and 847 nm. Among these, the emission peaks at 546 and 668 nm are typically the strongest.

Numerous methods for controlling the relative intensities of these emission bands have been investigated in recent years. A wide variety of colors can be produced by modifying parameters such as the lanthanide doping ratio or reaction temperature during synthesis.⁷⁻⁹ Alternatively, NaYF₄:Yb³⁺, Er³⁺(Tm³⁺) emission bands may be selectively quenched through surface modification.^{10,11} We have recently reported an additional means of controlling the ratio of green to red emission (GRR) in cubic phase NaYF₄:Yb³⁺, Er³⁺ through excitation repetition rate and pulse duration.¹² Herein, we discuss control of the GRR in hexagonal phase UNPs, which exhibit as much as 10-fold increased green luminescence efficiency over their cubic phase counterparts³ and explore the use of this phenomenon in super resolution microscopy.

2. MATERIALS AND METHODS

2.1 Cubic Phase Nanoparticle Preparation

Cubic phase (α -) NaYF₄:Er³⁺, Yb³⁺ UNPs were synthesized by a method similar to that described by Boyer et al in 2006.¹³ First, lanthanide precursors were prepared by dissolving lanthanide oxides at a molar ratio of 2% Er³⁺, 20% Yb³⁺, and 78% Y³⁺ in 50% aqueous trifluoroacetic acid at 80 °C. Ln-TFA precursors were then added to a solution of 1-octadecene and oleic acid and heated to 100 °C for 30 minutes. The resulting solution was heated to 300 °C for 1.5 hours under Ar. Following synthesis, the sample was precipitated with acetone, separated from solution via centrifugation, and washed twice with ethanol before being vacuum dried and dispersed in chloroform or dichloromethane for analysis.

2.2 Hexagonal Phase Nanoparticle Preparation

Hexagonal phase (β -) NaYF₄:Er³⁺, Yb³⁺ UNPs were synthesized using a method similar to that described by Li et al in 2008.¹⁴ In a typical synthesis, 2 mmol of lanthanide chlorides at a molar ratio of 2% Er³⁺, 20% Yb³⁺, and 78% Y³⁺ were added to 10–12 mL oleic acid and 30 mL 1-octadecene. This solution was then heated to 160 °C while cycling between Ar flow and vacuum. The solution was maintained at 160 °C under vacuum for 30 minutes, and then cooled to 50 °C. 8 mmol NH₄F and 5 mmol NaOH were dissolved in 20 mL methanol and added to the reaction vessel containing the lanthanide precursors. The resulting solution was stirred under Ar flow for 30 minutes, then heated to 80 °C and placed under vacuum to

remove any remaining methanol. The solution was then heated to 300 °C and maintained at this temperature for 1.5 hours under Ar atmosphere. The resulting nanoparticles were precipitated from solution with ethanol and separated via centrifugation. The resulting pellet was dispersed in minimal dichloromethane, and then washed with ethanol 4 times.

2.3 Characterization of UNPs

Emission spectra were collected using a QE65000 back-thinned CCD array spectrometer (Ocean Optics, Dunedin, Florida, USA). Excitation for emission spectra was provided by a 980 nm laser diode. Luminescence lifetime measurements were made using single photon counting avalanche photodiode (PerkinElmer, Vaudreuil, Québec, Canada) and multichannel scaler (Picoquant, Berlin, Germany) with excitation by a tunable optical parametric oscillator pumped by an Nd:YAG laser Q-switched at 20 Hz with a 3 ns pulse width. Nanoparticles in suspension were sized by quasi elastic light scattering using a ZS-90 Zetasizer (Malvern, Worcestershire, United Kingdom). The green to red ratio of nanoparticle emission was determined by dividing the integrated spectral peaks at 525 and 546 nm by the integrated peak at 668 nm.

2.4 Super Resolution Microscopy

Proof of principle for super resolution microscopy via control of the GRR was investigated using the system diagrammed in Figure 3. A Ti:Sapphire laser provided excitation light at 980 nm. The laser was modelocked at 80 MHz and had a pulse width ~100 fs. The output of the Ti:Sapphire was expanded to match the back aperture of a 100x/1.4 NA objective lens. The beam was then split into two arms. Both arms contained Glan-Laser polarizers and half-wave plates to independently control excitation power of the two beams used. One beam was mechanically chopped to a frequency below 1 kHz while the other passed through a vortex phase plate (RPC Photonics, Rochester, New York, USA). This phase plate has a transmission function $t=e^{i\theta}$, resulting in a donut shaped excitation profile when focused into the sample, much like that used for stimulated emission depletion (STED) microscopy (Fig. 4).¹⁵ The two beams were then recombined and focused into the sample. The resulting emission profile was recorded on a CCD camera after being passed through either a red or green bandpass filter.

3. RESULTS AND DISCUSSION

3.1 Control of Green to Red Emission Ratio

$\text{NaYF}_4:\text{Er}^{3+}, \text{Yb}^{3+}$ nanoparticles used in excitation frequency dependence experiments had a mean diameter of ~14 nm for cubic phase and ~70 nm for hexagonal phase. Emission peaks were observed at 409, 525, 541, 667, 800, and 847 nm. The relative intensities of emission peaks in the cubic phase UNPs were not observed to vary for excitation powers at or below ~16 kW/cm²; however, the GRR of $\beta\text{-NaYF}_4:\text{Er}^{3+}, \text{Yb}^{3+}$ particles was dependent on peak power. Increasing peak power to 8 kW/cm² while maintaining a repetition rate of 100 Hz resulted in an increase of the GRR from 1.73 to 6.68. Therefore, peak excitation power was held constant at 800 W/cm² for the measurements presented in Figure 5. As can be seen in Figure 5, the GRR of both cubic and hexagonal phase nanoparticles exhibit a dependence on excitation repetition rate. As the excitation frequency is increased from 20 Hz to 10 kHz, the

GRR decreases from 1.40 to 0.31 in cubic phase particles and 1.75 to 0.68 in hexagonal phase particles. While the difference between maximum and minimum GRR for the two sets is roughly equivalent, it is interesting to note that the GRR reaches a local minimum between 4 and 6 kHz in α -NaYF₄Er³⁺, Yb³⁺ particles while the β -NaYF₄Er³⁺, Yb³⁺ particles do not reach a local minimum. This suggests that hexagonal phase UNPs are sensitive to excitation repetition rate over a greater range than their cubic phase counterparts, possibly due to a difference in the lifetime of the ⁴I_{13/2} state.

As can be seen in Figure 6, population of each of the excited states leading to emission from Er³⁺ can occur through numerous pathways. Of interest to this work are those routes which populate ⁴S_{3/2} and ⁴F_{9/2}, from which radiative relaxation produces emission at approximately 546 and 668 nm respectively. Initial population of ⁴I_{11/2} from ⁴I_{15/2} can occur by energy transfer from a nearby Yb³⁺ ion in the ²F_{5/2} state or by direct excitation at 980 nm. From there, an additional excitation event results in population of ⁴F_{7/2} which may undergo nonradiative relaxation to ⁴S_{3/2}. This is the dominant pathway by which green emission is generated, regardless of excitation repetition rate. The dependence of the GRR on this repetition is instead linked to the population of ⁴F_{9/2}. This state is predominantly populated by either nonradiative relaxation from ⁴S_{3/2} or by excitation of ⁴I_{13/2} through ETU or ESA. Population of ⁴I_{13/2} may occur through either nonradiative relaxation from ⁴I_{11/2} or radiative relaxation from ⁴S_{3/2} at ~847 nm.

At short pulse widths and low frequencies, ⁴I_{13/2} decays completely between each excitation pulse and the dominant pathway for the generation of red emission is ⁴I_{15/2}→⁴I_{11/2}→⁴F_{7/2}→⁴S_{3/2}→⁴F_{9/2}→⁴I_{15/2}. Since ⁴S_{3/2}→⁴I_{15/2} is more probable than ⁴S_{3/2}→⁴F_{9/2}→⁴I_{15/2}, the GRR is greater than 1. As we increase the repetition rate, ⁴I_{13/2} has less time to relax between excitation pulses and the dominant pathway for red emission shifts to ⁴I_{15/2}→⁴I_{11/2}→⁴I_{13/2}→⁴F_{9/2}→⁴I_{15/2}, causing the GRR to decrease, reaching a minimum when the UNPs are excited at peak ⁴I_{13/2} population.

Taking advantage of this phenomenon, we have begun work on a two-photon super resolution microscopy technique for use in measuring subdiffraction distances between particles that are beyond the range of fluorescence resonance energy transfer (FRET). By using a donut shaped illumination pattern running at a high repetition rate to excite the periphery of a diffraction limited spot at low repetition rate, particles located outside the center of this spot are forced to emit light at a lower GRR. In this way, it may be possible to distinguish between multiple particles located within a single diffraction limited spot (Fig. 7).

As a part of establishing proof of principle for this technique, we illuminated a sample of α -NaYF₄:Er³⁺, Yb³⁺ nanoparticles on a glass slide using the diffraction limited spot, donut pattern, and a combination of the two. Images of the diffraction limited spot were visually inspected and grouped into one of two categories based on the location of the emission PSF, “on center” or “off center.” Figure 8 presents data from preliminary experiments to establish proof of principle for this technique as well as example images of upconversion luminescence from the sample considered in the center of illumination and off center. As can be seen, it is possible to delineate between these particles based on their GRR alone.

4. CONCLUSIONS

In conclusion, we have demonstrated control of the relative intensities of the two strongest emission bands in $\text{NaYF}_4\text{:Er}^{3+}$, Yb^{3+} nanoparticles via excitation repetition rate in both cubic and hexagonal phase nanoparticles. We have applied this control and presented preliminary data demonstrating the ability to identify nanoparticles in different locations within a diffraction limited illumination spot. Future experiments will include the use of a ratiometric point spread function and a scanning system with the goal of generating images with a spatial resolution beyond the diffraction limit. It should be noted that the use of this technique will likely be limited to small areas due to the long luminescence lifetime of these nanoparticles. Hexagonal phase UNPs may allow for similar results to be obtained at higher repetition rates of the center excitation spot. This super resolution microscopy system is currently contemplated for use in particle tracking and distance measurements in biological systems.

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Figure 1.

Upconversion luminescence from $\beta\text{-NaYF}_4\text{:Er}^{3+}, \text{Yb}^{3+}$ nanoparticles 80 nm in diameter in suspension following excitation at 980 nm by a Ti:Sapphire laser modelocked at 80 MHz with ~ 100 fs pulse width. Left: Luminescence resulting from excitation focused through a 10X objective at 80 mW average power. Right: Collimated excitation at 400 mW average power.

Figure 2. Luminescence from α -NaYF₄:Er³⁺, Yb³⁺ nanoparticles 52 nm in diameter following pulsed excitation by 980 nm laser diode. From left to right, excitation was at 1 MHz, 3.33 kHz, 1.11 kHz, 555 Hz, and 167 Hz (0.1, 30, 90, 180, 300 μ s pulse width).

Figure 3.

Diagram of the imaging system used for proof of principle of subdiffraction imaging. The final imaging system will include an X/Y scanner between the polarizing beamsplitter and microscope as well as two PMTs to detect luminescence at different wavelengths.

Figure 4.

A. Image of center beam (980 nm) focused onto a mirror with a 100x/1.4 NA objective. B. Image of donut beam (980 nm) focused onto a mirror with a 100x/1.4 NA objective. C. Horizontal profiles of A and B taken from the same region.

Figure 5. Plot of GRR vs. excitation frequency. Excitation was by 980 nm laser diode with pulse width of 10 μ s.

Figure 6.

Energy level diagram of energy transfer system between Yb^{3+} and Er^{3+} in $\text{NaYF}_4:\text{Yb}^{3+}$, Er^{3+} nanoparticles. Grey arrows represent routes for nonradiative decay, dashed arrows represent excitation and decay by energy transfer from Yb^{3+} to Er^{3+} . The diagram was constructed based on prior work by Carnall et al.¹⁶ as well as experimental data.

Figure 7.

Conceptual diagram of super resolution system. Maximum GRR is obtained when a particle is located in the center of the illumination pattern. When multiple particles are present, some mixture of GRRs is measured by PMT detectors. By setting a threshold that is only surpassed when a particle is in the center of this pattern, it may be possible to delineate between particles within a diffraction limited distance of each other.

Figure 8.

Top. Example images of a particle considered “on center” (left) and one considered “off center” (right). A white circle has been included in the same position on both images for reference. Bottom. Comparison of the GRR of $\alpha\text{-NaYF}_4\text{:Er}^{3+}, \text{Yb}^{3+}$ emission for diffraction limited spots located in the center of the illumination focus and slightly off center. The center excitation beam was pulsed at 140 Hz with a 10% duty cycle and had an average power of 16 μW . The donut shaped beam had a repetition rate of 80 MHz, simulating CW excitation of these particles, and had an average power of 24 μW . Excitation was at 980 nm and was focused onto the sample through a 100x/1.4 NA objective lens. Particles used had an average diameter of 40 nm. The combined illumination used center and donut patterns at the same time.