Combined Optical and MR Bioimaging Using Rare Earth Ion Doped NaYF₄ Nanocrystals

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Here, novel nanoprobes for combined optical and magnetic resonance (MR) bioimaging are reported. Fluoride (NaYF₄) nanocrystals (20–30 nm size) co-doped with the rare earth ions Gd³⁺ and Er³⁺/Yb³⁺/Eu³⁺ are synthesized and dispersed in water. An efficient up- and downconverted photoluminescence from the rare-earth ions (Er³⁺ and Yb³⁺ or Eu³⁺) doped into fluoride nanomatrix allows optical imaging modality for the nanoprobes. Upconversion nanophosphors (UCNPs) show nearly quadratic dependence of the photoluminescence intensity on the excitation light power, confirming a two-photon induced process and allowing two-photon imaging with UCNPs with low power continuous wave laser diodes due to the sequential nature of the two-photon process. Furthermore, both UCNPs and downconversion nanophosphors (DCNPs) are modified with biorecognition biomolecules such as anti-claudin-4 and anti-mesothelin, and show in vitro targeted delivery to cancer cells using confocal microscopy. The possibility of using nanoprobes for optical imaging in vivo is also demonstrated. It is also shown that Gd³⁺ co-doped within the nanophosphors imparts strong T₁ (Spin-lattice relaxation time) and T₂ (spin-spin relaxation time) for high contrast MR imaging. Thus, nanoprobes based on fluoride nanophosphors doped with rare earth ions are shown to provide the dual modality of optical and magnetic resonance imaging.

1. Introduction

Multimodal bioimaging is a new frontier in biology and medicine, which combines more than one imaging modality, such as optical, nuclear, ultrasound, and magnetic resonance imaging (MRI). Optical imaging provides the highest sensitivity and spatial resolution for in vitro imaging, is inexpensive, robust, and portable, but still lacks the full capability to obtain anatomical and physiological details in vivo. At the same time, optical imaging is the only technique among all the medical imaging techniques which can provide cellular- or molecular-level information with almost single molecule sensitivity. On the other hand, MRI provides an excellent spatial resolution and depth for in vivo imaging, and provides exceptional anatomic information, but suffers from limited sensitivity and lacks resolution for imaging at the cellular level. Combination of MRI and optical imaging can lead to the development of new approaches to bridge gaps in resolution and depth of imaging between these two modalities. Potential benefits of combined optical and MRI, along with recent advances in nanoscale material engineering, have already stimulated a development of hybrid magnetic fluorescent nanoprobes for imaging in vitro and in vivo. Use of nanoparticles as molecular-specific reporters offers an increased signal over traditional probes as well as simpler conjugation chemistries. Modifying the surface of these multimodal nanoparticles with targeting agents can improve contrast and provide information about specific biomarkers in both modalities.

Highly dispersible nanocrystals of rare-earth compounds, such as oxides, fluorides, phosphates, vanadates, and sulfides, have recently become a new focus of research due to their unique optical properties. The most interesting feature of lanthanide luminescence spectra are their sharp spectral lines much resembling the spectra found in the case of free ions. Lanthanide ions also demonstrate long (sub-millisecond or millisecond range) photoluminescence (PL) lifetimes and exhibit visible emission upon IR excitation when doped with Yb³⁺ and Er³⁺/Tm³⁺ ions. Synthesis of colloidal hexagonal nanocrystals of NaYF₄ doped with various upconversion RE³⁺ ions has been extensively reported. Incorporation of luminescent RE³⁺ ions into NaYF₄ nanocrystals for downconversion luminescence, where the emission wavelength is redshifted relatively to the excitation wavelength, has been also...
shown. Chatterjee et al. have recently demonstrated the use of upconversion nanophosphors (NaYF₄: Er³⁺, Yb³⁺) for in vitro imaging of cancer cells and in vivo imaging in tissues, claiming such advantages of these nanoprobes as absence of photodamage to living organisms, very low autofluorescence, high detection sensitivity, and high light penetration depth in biological tissues.

Here, we report the synthesis of a new generation of functionalized fluoride nanophosphors of size <50 nm, which form stable aqueous dispersion that can be used for bimodal imaging, both optical and MRI. Down- and upconverted luminescence from the rare-earth ions (Er³⁺, Eu³⁺, and Yb³⁺) doped into nanometer-sized fluoride matrices allows optical imaging modality for the nanoprobes and co-doping with Gd³⁺ provides high contrast MRI modality. These nanoprobes are devoid of the drawbacks inherent to other optical imaging probes, namely broad emission and lack of photostability. Furthermore, upconversion nanophosphors demonstrate nearly quadratic dependence of the PL intensity on the excitation power, which provides the benefits of conventional two-photon induced imaging, such as intrinsic 3D localization of two-photon excitation and low photobleaching and photodamage of the sample outside the excitation volume.

Use of UCNPs does not require high photon intensity, which is necessary for two-photon excitation of organic dyes or quantum dots used for two-photon microscopy. Because of the existence of a metastable intermediate energy level in nanophosphors, an upconversion process involves the sequential absorption of two photons, as opposed to simultaneous absorption in the case of two-photon absorption, where the intermediate level is virtual. This makes the upconversion process much more efficient in comparison to two-photon absorption and allows excitation and imaging of upconversion nanophosphors using low-power and continuous wave (CW) laser diodes, which are inexpensive and ready available, in contrast to the femtosecond pulsed lasers needed for simultaneous two-photon excitation.

Keeping in mind that gadolinium (Gd³⁺) is a paramagnetic relaxation agent extensively used in MRI, we have also co-doped the fluoride nanophosphors with Gd³⁺ ions, thus introducing MRI modality. We have shown that relaxivity, which is the measure of an MRI agent’s ability to shorten the inherent water-proton relaxation times and is the quantitative measure of MRI contrast agent efficacy, is higher for the Gd³⁺ doped nanophosphors than for the gadolinium chelate compounds currently used in conventional MRI. We believe that use of such contrast agents can boost MRI signal and improve contrast at a lower dosage. It is also worth noting that most Gd³⁺-based contrast agents currently used in clinical medicine are fluid agents that are distributed extracellularly and excreted exclusively via the kidneys. Because of its toxicity, the hydrated Gd³⁺ ion is sequestered by chelation to reduce potential toxic effects. However, even for chelated Gd³⁺-complexes, the release of the metal ion in vivo during metabolic processes and the subsequent toxicity are pertinent issues. Gd³⁺ ions doped into rigid matrices of fluoride nanocrystal, as fabricated here, can avoid this release issue. In addition, the dual imaging modality of the nanoprobes, based on fluoride nanophosphors doped with rare earth ions for optical and magnetic resonance imaging, can provide complementary anatomic, functional, and molecular information. We have also demonstrated active targeting of pancreatic cancer cells by using anti-claudin 4 and anti-mesothelin as targeting biomolecules conjugated to the nanophosphors. To the best of our knowledge, the synthesis and biomedical application of water-soluble hybrid NaYF₄:RE³⁺, Gd³⁺ nanoparticles, combining both optical and MR imaging modalities, has not been reported before.

2. Results and Discussion

NaYF₄ nanophosphors co-doped with various rare earth ions were synthesized with a size distribution in the range of 25–30 nm, as shown by the transmission electron microscopy (TEM) image in Figure 1A. The size of the water-dispersible nanophosphors remains practically the same after the ligand exchange process, as can be seen in Figure 1B. Dispersions of these nanophosphors demonstrate good colloidal stability, optical transparency and produce intense photoluminescence (Fig. 2). To investigate how different ratios of rare-earth co-dopants influence the optical/MRI properties of the nanophosphors, we synthesized upconversion NaYF₄ nanophosphors co-doped with three ratios of the lanthanides: 18% of Yb³⁺ and 2% of Gd³⁺; 15% of Yb³⁺ and 5% of Gd³⁺; and 10% of Yb³⁺ and 10% of Gd³⁺. The percentage of Er³⁺ remained the same for all samples (2%). The results for the steady-state and time-resolved absorption and

Figure 1. Representative TEM images of NaYF₄ nanophosphors doped with RE³⁺ ions. Samples for TEM were prepared from A) chloroform and B) water dispersions.
photoluminescence spectroscopy for upconversion nanophosphors (NaYF₄, co-doped with Gd³⁺, Er³⁺, and Yb³⁺) are shown in Figure 3. The absorption spectra of the dispersions show the presence of Yb³⁺ ions doped into a nanocrystal matrix by a peak in the area of ~970 nm. The presence of Er³⁺ ions is indicated by the absorption peaks at around 520 and 650 nm.⁴² As one can see, the water dispersions of the nanophosphors demonstrate a higher level of light scattering, apparently caused by the attachment of the ligands and resulting in the appearance of significant “background” in the absorption spectra. It is worth noting that we have not observed any colloidal instability for the nanophosphors in water over a period of at least one month. Dynamic light scattering experiments on the aqueous dispersions of nanophosphors have also indicated the absence of nanoparticle aggregation (see the Supporting information). The PL emission spectra of the nanophosphors show multiple sharp spectral lines, characteristic of luminescent nanophosphors. Yb³⁺ excited at 975 nm transfers energy to Er³⁺, which results in the characteristic Er³⁺ emission with peaks at ~520, 538, and 550 nm (“green” bands) and ~649, 653, and 667 nm (“red” bands).⁴³ The “green” PL is a radiative deactivation of the ²H₁₁/₂ and ⁴S₃/₂ energy levels of the Er³⁺, whereas “red” PL corresponds to ⁴F₅/₂ → ⁴I₇/₂ transition.³²,⁴³ Both levels are populated through the ⁴F₅/₂ level of Yb³⁺, thus upconverting energy (see Fig. S1, Supporting information).

It is worth noting that the ratio of the intensity of the red band to that of the green band is noticeably different for aqueous and chloroform dispersions of the nanophosphors; red emission is more intense in water dispersion (Fig. S2A in the Supporting information). This variable green-to-red ratio (GRR) has been investigated and reported by several groups.¹⁹,⁴³ Its value is considered to depend on the excitation density, crystal phase, and any additional impurities which can increase the rate of multiphonon relaxation between the green-emitting ²H₁₁/₂ and ⁴S₃/₂ levels and the red-emitting ⁴F₅/₂ level.¹⁹,⁴³ The presence of Gd³⁺ ions in our case can be an additional factor, decreasing the value of GRR for UCNPs. At the same time, in nanophosphors a larger number of Er³⁺ ions are located closer to the surface when compared to bulk material. This is why surface ligands used to disperse nanocrystals in water can significantly increase the rate of the ²H₁₁/₂ → ⁴S₃/₂ transition. On the other hand, higher light scattering in water nanophosphor dispersion results in a lower excitation density for the exciting light and causes a decrease in the rate of the ⁴F₅/₂ → ⁴I₇/₂ process in comparison to that of ⁴F₅/₂ → ⁴F₇/₂. A similar change in GRR has been also seen in the PL spectra of the aqueous dispersion of the UCNPs for different excitation powers (Fig. S2B in the Supporting information).

The photoluminescence lifetime for nanophosphors is very different from the fluorescence lifetime characteristic for conventional fluorescence imaging probes, which is typically in the nanosecond range. For UCNPs, it is in the sub-millisecond range (Fig. 3D). The
lifetime does not noticeably depend on the percentage of \( \text{Er}^{3+} \) and \( \text{Yb}^{3+} \), but is significantly different for the red and the green bands, indicating a higher rate for the deactivation of the \( ^{5}S_{3/2} \) level (Fig. S1 in the Supporting Information). Different \( \text{Gd}^{3+} \) percentage does not noticeably influence the rate of the PL decays.

The power dependence for both the red and the green PL bands is close to quadratic (Fig. 3C), thus unambiguously confirming a two-photon-induced process. It is important to note that two-photon excitation in two-photon fluorescence laser scanning microscopy and other fluorescence imaging techniques is an essential tool, providing a number of advantages over single photon excitation. The use of near-IR light for excitation allows a larger penetration depth in biological tissues and results in lower phototoxicity and reduced background due to the relatively low two-photon absorption cross-section of most biomolecules responsible for autofluorescence in biological systems. The quadratic dependence of the excitation efficiency on the power of excitation leads to an intrinsic 3D localization of two-photon excitation and also results in low photobleaching and photodamage of the sample outside the focal region.[3,36,37] Relatively low two-photon excitation cross-sections of the nanoprobes mean that the use of a high-repetition femtosecond-pulsed laser is necessary for two-photon induced imaging, as is the use of objective lenses with high numerical apertures to focus the laser beam.[37] UCNP use does not require high photon flux and allows excitation and imaging of upconverting nanophosphors using low-power and CW laser diodes, which are inexpensive and readily available when compared to femtosecond pulsed lasers.

To show the possibility of single-photon-induced luminescence optical imaging with \( \text{Gd}^{3+} \)-doped nanophosphors, we have also synthesized downconversion nanophosphors co-doped with \( \text{Eu}^{3+} \) as described above. DCNPs co-doped with \( \text{Eu}^{3+} \) and \( \text{Gd}^{3+} \) show specific and distinct spectral lines of \( \text{Eu}^{3+} \) in their PL excitation and emission spectra (Fig. 4A). Similarly to the UCNPs, DCNPs also demonstrate substantially delayed PL emission; its lifetime is in millisecond range (\(~10\) ms), as seen in Figure 4B. This characteristic feature of \( \text{Eu}^{3+} \) photoluminescence provides one more option which can be potentially used for time-resolved photoluminescence imaging.[20,45]

Confocal microscopy was used to study the selective uptake of the up- and downconversion nanophosphors by pancreatic cancer cells in vitro, using pre-equated numbers of nanoparticles. As shown in Figure 5, there is almost no uptake of the nanophosphors capped with mercaptopropionic acid only (A, C), whereas when the same nanophosphors were conjugated with targeting biomolecules such as anti-claudin 4 (B) and anti-mesothelin (D), the uptake of the nanoparticles was considerably enhanced. As can be seen in Figure 5, nanophosphors mostly label the cell membrane. This enhanced labeling efficiency is a result of receptor-mediated uptake of these bioconjugated nanoparticles with their corresponding receptors, which are known to be overexpressed on the surface of these cells.[19] The cell-viability assay of the nanoparticles showed no considerable cellular toxicity over a period of 48h (see Fig. S3, Supporting Information).

We have also checked whether nanophosphors can be imaged using a Maestro GNIR FLEX system, an instrument used typically for whole body optical imaging in small animals. Figure 6 presents images of nanoparticles in aqueous dispersions acquired with this system. As can been seen, spectrally unmixed images clearly demonstrate the potential to image and spectrally distinguish the characteristically emitting nanophosphors (shown as orange and red) using the Maestro imaging software. This experiment underlines the possibility of extending the application of these nanophosphors for in vivo bioimaging.

We have also examined the paramagnetic behavior of the \( \text{Gd}^{3+} \)-doped fluoride nanocrystals and their ability to be used as contrast agents in MRI. Figure 7 shows the effect of different concentration of \( \text{Gd}^{3+} \)-doped UCNPs on the relaxivity \((R_1 = 1/T_1 \text{ and } R_2 = 1/T_2)\) of the
Studies performed on aqueous solutions of different Gd\textsuperscript{3+}-doped nanophosphors confirmed that T1 is inversely proportional to the concentration of the co-doped paramagnetic agent, as shown previously for commercial MRI contrast agents.

In order to determine whether these gadolinium-doped nanophosphors could be used as contrast agents in MRI, their contrast effect in solution was tested. Different concentrations of suspensions of nanophosphors, containing 0–10 mM of Gd\textsuperscript{3+}, as well as pure water for the background signal, were placed in the centrifuge tubes. Representative T1-weighted (TR/TE = 500 ms, 10 ms) and T2-weighted (TR/TE = 10 000 ms, 70 ms) MRI images are shown in Figure 8. The MR images of the nanophosphor suspension clearly showed the negative enhancement effect on T2-weighted sequences, a result very similar to that obtained for the superparamagnetic iron oxide nanocrystals used as contrast agents in MRI. As a result, the tubes containing the Gd\textsuperscript{3+}-doped nanophosphors appeared dark and invisible in the T2-weighted MR image (Fig. 8), while the tubes that contained water and void nanophosphors remained brighter and visible.

Both DCNPs and UCNPs also showed a pronounced contrast on the T1 weighted image sequences. It can be seen in Figure 8 that samples with 10 mM of Gd\textsuperscript{3+} (for both DCNPs and UCNPs) showed the maximum contrast. The samples with 2 and 5 mM of Gd\textsuperscript{3+} showed a slightly smaller contrast, which was still very high in comparison to plain water and void nanophosphors. The direct proportionality of the Gd\textsuperscript{3+} concentration on the contrast enhancement can be readily seen by the linearity of the plots of R1 (1/T1) and R2 (1/T2) as a function of gadolinium concentration. We have determined the specific relaxivities r1, r2 from these plots, the values are: r1 = 0.14 mM\textsuperscript{-1}s\textsuperscript{-1}, r2 = 8.7 mM\textsuperscript{-1}s\textsuperscript{-1}. As has been reported previously, the most commonly used commercial contrast agent, Gd-DTPA, has r1 = 3.7 mM\textsuperscript{-1}s\textsuperscript{-1} and r2 = 5.8 mM\textsuperscript{-1}s\textsuperscript{-1}. It is worth also noting that these values are reported per Gd\textsuperscript{3+} ion. A single nanophosphor probe can contain many Gd\textsuperscript{3+} ions, thus providing much higher signal per single probe, in comparison with single-molecule-based probes such as Gd-DTPA.

The T1/T2 relaxation plots for all of the imaging agents as well as a deionized water sample as control are shown in Figure 9. Estimated T1/ T2 values from the least squared error regression of the curves are shown in Table 1. Gd\textsuperscript{3+} (10 mM) doped in DCNPs had little effect on either T1 or T2 in comparison with the same concentration of Gd\textsuperscript{3+} doped in UCNPs. However, this difference may be due to a variation in the sample preparation or differences in the relaxivity provided by the different lanthanides (Eu vs Er); this needs to be determined. In any case, the pronounced contrast enhancement for both T1- and T2-weighted image sequences as well as high relaxivities for both DCNPs and UCNPs suggests the potential use of these nanoparticles as excellent contrast agents in MRI.
Experimental tumors are underway. In vivo experiments using small animals bearing these non-toxic nanoprobes can be considered as ideal candidates for a new generation of diagnostic probes for routine clinical application. In vivo experiments using small animals bearing experimental tumors are underway.

3. Conclusions

In conclusions, we have synthesized organic phase NaYF4 nanocrystals of size 20–30 nm, co-doped with various rare-earths ions (Er3+, Er3+, Yb3+, Gd3+), and subsequently dispersed them in aqueous phase. These nanoparticles are colloidally stable and easily detectable by both MR and optical imaging methods. Their excellent photoluminescence properties make them suitable for in vitro and in vivo optical bioimaging. We have functionalized these nanoparticles by conjugation with tumor-specific antibodies and shown targeted imaging of living cancer cells in vitro, without any sign of cellular toxicity. The superior magnetic contrast of these Gd3+-doped nanophosphors also underscores their potential as robust MRI probes. In summary, owing to the combined presence of efficient optical and MR imaging probes, along with the facility of site-specific delivery, these non-toxic nanoprobes can be considered as ideal candidates for a new generation of diagnostic probes for routine clinical application. In vivo experiments using small animals bearing experimental tumors are underway.

4. Experimental

Synthesis of NaYF4 Nanocrystals Doped with Rare Earth Elements: The synthetic strategy followed a similar protocol as has been described previously [31,50] with modifications. Fixed amounts of commercial Gd2O3, Er2O3, Yb2O3 and Y2O3 were mixed and solubilized in hot ~50% concentrated trifluoroacetic acid and slowly evaporated to dryness in a vacuum oven at 70 °C. The different molar compositions of the feed external solution were [Er/(Gd + Er + Yb + Y)] = 0.18; 0.15; 0.10, and [Gd/(Gd + Er + Yb + Y)] = 0.02; 0.05; 0.10. Next, the obtained trifluoroacetate precursor salt was added to a three-necked flask with octadecane (30 mL), oleic acid (30 mL), and 4.5 mmol of sodium trifluoroacetate. The molar ratio of Na(ClF3·COO) to Er(ClF3·COO) was kept at 1.8 to form a pure nanocrystalline matrix of α-NaREF4. The solution was heated to 110 °C under vacuum with stirring for ~30 min to remove water and oxygen, during which the flask was purged with dry nitrogen every 10 min. The resultant yellow solution was then heated to 300 °C under nitrogen and kept under vigorous stirring for 1 h. After synthesis, the mixture was cooled to room temperature and precipitated by acetone using an ultrasonic bath, collected by centrifugation at 11 000 rpm for 30 min, and then washed with ethanol. Finally, the entire quantity of nanocrystals was directly dispersed in chloroform (4 mL). For down-conversion luminescence, NaYF4: Er3+,Gd3+ nanophosphors were synthesized using the same procedure as that used for the synthesis of the NaYF4:Er3+, Yb3+, Gd3+ co-doped sample, except that Er2O3 was replaced with EuCl3. The different molar compositions of the feed external solution were [Eu/(Gd + Eu + Y)] = 0.1, and [Gd/(Gd + Eu + Y)] = 0.1.

For transferring the nanocrystals from chloroform solution to aqueous phase, we used the ligand exchange method using 3-mercaptopropionic acid, which results in water-dispersible carboxyl-terminated nanoparticles. In a typical protocol, 2 mL of nanocrystal chloroform dispersion was further diluted with 3 mL of chloroform, incubated with 3-mercaptopropionic acid (5 mL). After the solution was stirred overnight, HPLC grade water (5 mL) was added to it and it was further stirred for 30 min. The turbid solution obtained was centrifuged and the pellet was redispersed in HPLC grade water (5 mL) by sonication. Finally, the aqueous dispersion of the nanocrystals was filtered with a 0.45 μm syringe filter and stored at 4 °C for future use.

Conjugation of –COOH-Terminated Nanocrystals with Anti-Claudin 4: For in vitro cellular targeting studies, the carboxyl terminated aqueous dispersion of nanocrystals was conjugated to different biomolecules such as anti-claudin 4 and transferrin by using a typical EDC chemistry. 1 mL stock solution of –COOH-terminated nanocrystals was mixed with 0.1 M EDC solution (25 μL) and gently stirred for 30 minutes. Next, anti-claudin 4 (5 μL of 0.5 mg mL−1) was added into this mixture and incubated at room temperature for 2 h to allow the free amino groups of the antibody to covalently bond to the carboxyl groups on the nanoparticles. Human pancreatic cancer cell line Panc 1 was cultured in Dulbecco minimum essential media (MEM-α) with 10% fetal bovine serum (FBS), 1% penicillin, and 1% amphotericin B. The day before nanoparticle treatment, cells were seeded in 35-mm culture dishes. On the treatment day, the cells, at a confluence of 70–80% in serum-supplemented media, were treated with the nanophosphors at a specific concentration (1 mg mL−1) media) for two hours at 37 °C.

Table 1. T1 and T2 values as estimated from the MRI images.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Control</th>
<th>UCNPs</th>
<th>DCNPs</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0 mM Gd3+</td>
<td>Water</td>
<td>Gd3+</td>
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<tr>
<td>T1 (ms)</td>
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<td>3140</td>
<td>1417</td>
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<tr>
<td>T2 (ms)</td>
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<td>1330</td>
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Figure 9. Plots of R1 (1/T1) and R2 (1/T2) as a function of gadolinium concentration for Gd3+ doped nanophosphors as measured from 9.4T MRI.
Optical spectroscopy: Optical absorption, photoluminescence steady-state and time-resolved spectroscopy were used to characterize the spectral properties of the luminescent nanomaterials. UV–vis absorption spectra were acquired using a Shimadzu UV-3600 spectrophotometer. PL excitation/emission spectra were recorded on a Fluorolog-3 spectrophotometer (Jobin Yvon, Longjumeau, France). A fiber-coupled laser diode (Qphotonics) emitting at 975 nm was used as the excitation source in case of the UCNPs. A second harmonic 532-nm beam from a nanosecond-pulsed Nd:YAG laser (Lotis TII, Belarus) operating at 20 Hz was used to excite the DCNPs. A Qphotonics laser diode operating in pulsed mode (pulse width of 200 µs) at 200 Hz was used as the PL excitation source in case of the UCNPs.

In Vitro Confocal Microscopy and Optical Imaging: Confocal fluorescence laser scanning microscopy was performed using a confocal microscope (MRC-1024, Bio-Rad, Richmond, CA), which was attached to an upright microscope (Nikon model Eclipse E800). A water immersion objective lens (Nikon, Fluor-60X, NA 1.0) was used for cell imaging. We used a diode-pumped solid state laser (Millenia, Spectra-Physics) as a source of excitation, providing a 532-nm line for excitation of DCNPs, or a single-mode laser diode (Qphotonics) operating at 975 nm to image UCNPs. Excitation light was coupled to the upper port of the microscope through a single-mode optical fiber.

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