

Supporting Information for:

Upconverting Luminescent Nanomaterials: Application to In Vivo Bioimaging

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Experimental

General Considerations. Unless otherwise noted all chemicals and solvents were purchased from Sigma-Aldrich and were used without further purification. The chloro-modified tetrasulfonated carbocyanine precursor (3H-Indolium, 2-[2-[2-chloro-3-[2-[1,3-dihydro-3,3-dimethyl-5-sulfo-1-(4-sulfobutyl)-2H-indol-2-ylidene]ethylidene]-1-cyclohexen-1-yl]ethenyl]-3,3-dimethyl-5-sulfo-1-(4-sulfobutyl)-, inner salt, potassium salt) was prepared according to published procedures.¹ Preparative HPLC was performed on a Varian ProStar 210 instrument equipped with a 335 diode array detector, a model 701 fraction collector, and a Varian RPC18 column (model A6002250X212) at a flow rate of 21 mL/min. Analytical HPLC was performed using a Waters 2695 HPLC system equipped with a 2996 diode array detector, a Micromass ZQ4000 ESI-MS module, and a Grace-Vydac RPC18 column (model 218TP5210) at a flow rate of 0.3 mL/min. HPLC buffer A is 0.1% trifluoroacetic acid (TFA) in water and buffer B is acetonitrile with 10% water and 0.1% TFA. ¹H (400 MHz) spectra were collected on a Bruker Advance-400 NMR spectrometer at ambient temperature. The chemical shifts were measured versus

tetramethylsilane (TMS) as an internal standard. Low-resolution electrospray ionization (ESI) mass spectra were obtained on a Micromass ZQ4000 ESI-MS instrument. Fourier transform infrared (FTIR) spectra were obtained on Perkin-Elmer Model 2000 FT-IR spectrophotometer. Scanning electron microscopy data were collected on a Zeiss Ultra 55 instrument at the Harvard Center for Nanoscale Systems. Dynamic light scattering measurements were obtained on a Nano ZS instrument (Malvern). Data for the absorption spectrum and extinction coefficient of the NIR fluorophore were obtained using a Varian Cary 50-Bio UV-visible spectrophotometer. The extinction coefficient for the NIR fluorophore was measured in phosphate-buffered saline (PBS), pH 7.4 and is the average of three sets of parallel experiments. For each trial, 2-3 mg of the HPLC-purified dye was weighed on a Mettler AT201 analytical balance with an error of ± 0.01 mg and was dissolved in deionized water using a 10 mL volumetric flask to prepare the stock solutions. Quantum yield measurements for the NIR fluorophore were performed in triplicate on a Fluorolog-3 fluorescence spectrophotometer (Horiba Jobin Yvon) with the maximum absorption for each sample less than 0.1, using Cy 7 as a standard ($\Phi = 0.27$).² The standard deviation for both the extinction coefficient and quantum yield measurements is less than 5%. Upconversion fluorescence spectra were obtained on a modified Fluorolog-3 fluorescence spectrophotometer (Horiba Jobin Yvon) equipped with a 300 mW 980 nm diode laser (Roithner Lasertechnik) filtered with a 980 nm bandpass filter. The upconversion emission from the sample was further filtered by a 750 nm short-pass filter to remove any stray excitation light before entering the monochromator.

Amine modified NIR fluorophore synthesis. To a solution of tyramine (2 mmol, 274 mg) in 0.5 mL of dimethylformamide (DMF) and triethylamine (3 mmol, 418 μ L) was added di-*tert*-butyl dicarbonate (3 mmol, 665 mg). After stirring for 1 h, the reaction was subjected to rotary evaporation, giving a viscous oil. To this oil, was added DMF (0.5 mL) and NaH (2 mmol, 80 mg) as a 60% w/w dispersion in mineral oil. The reaction was allowed to stir for 30 min, until no more gas evolution was observed. To this solution was added the chloro-modified tetrasulfonate dye (0.1 mmol, 89 mg) giving a yellow solution. After stirring for 3 h, 10 mL of a 1:1 water/trifluoroacetic acid solution was added and stirring was continued for 2 h. Following solvent removal by rotary evaporation, the crude reaction was purified by preparative HPLC using a gradient from 0 to 100% buffer B giving the pure product (63 mg, 64%) as a dark green solid. Purity was verified by analytical HPLC (**Figure S3**). UV-vis (PBS): $\lambda_{\text{max}} (\epsilon) = 773 \text{ nm} (215,000 \text{ M}^{-1} \text{cm}^{-1})$. Fluorescence (PBS): $\lambda_{\text{max}} (\phi_f) = 792 \text{ nm} (7 \%)$. $^1\text{H NMR}$ (400 MHz, DMSO- d_6 , δ): 7.79 (2H, d, $J = 14.1 \text{ Hz}$), 7.62 (2H, s), 7.59 (2H, dd, $J = 8.1, 1.5 \text{ Hz}$), 7.33 (2H, d, $J = 8.3 \text{ Hz}$), 7.29 (2H, d, $J = 8.6 \text{ Hz}$), 7.14 (2H, d, $J = 8.6 \text{ Hz}$), 6.23 (2H, d, $J = 14.2 \text{ Hz}$), 4.13 (4H, br s), 2.89-2.76 (8H, m), ~ 2.55 (4H, broad singlet overlapping the residual DMSO resonance), 1.92 (2H, br s), 1.76-1.67 (8H, m), 1.25 (12H, s). LRMS (ESI), m/z : $[\text{M}]^+$ calcd for $[\text{C}_{46}\text{H}_{58}\text{N}_3\text{O}_{13}\text{S}_4]^+$, 988.3; found 988.4.

Nanoparticle preparation. Following sonication of the red emitting $\text{Y}_2\text{O}_3\text{:Er:Yb}$ nanoparticles (500 mg, lot # 1LZL062A1 from Nanocerox Inc.) in ethylene glycol (15 mL) for 8 h, 500 mg of 15,000 MW polyacrylic acid (PAA) as the free acid (prepared by cation exchange from its sodium salt) was added and sonication was continued for 20 h. Following sonication, methanol (20 mL) was added and the nanoparticles were recovered

by centrifugation (30 min at 10,000 G). The pellet washed by re-suspension in 25 mL 0.1 M phosphate buffer, pH 7.4 followed by centrifugation (30 min at 10,000 G). The washing procedure was repeated 3 times to ensure removal of all non-nanoparticle bound PAA. After the final wash, the PAA coated particles were re-suspended in 2.5 mL of deionized water. The particle concentration was determined to be 160 mg/mL by weighing a lyophilized 50 μ L aliquot of the stock. The PAA coated nanoparticles (400 mg, 2.5 mL) were added to 55 mL of dimethylsulfoxide (DMSO) and 0.1 M bicarbonate buffer, pH 7.4 (1.5 mL). To this suspension, was added the amine modified carbocyanine fluorophore (0.032 mmol, 31.6 mg) in 5 mL of DMSO, 2000 MW mPEG-NH₂ (320 mg in 16 mL of 0.1 M bicarbonate buffer, pH 7.4) from Creative PEGWorks and the mixture was sonicated for 1 h to break up any nanoparticle aggregates. Subsequently, 4.00 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was added and sonication was continued for 30 min. At this time, an additional 4.00 g portion of EDC was added followed by further sonication for 30 min. The labeled nanoparticles were then isolated by centrifugation (20 min at 10,000 G) and the supernatant was decanted. The particles were washed by re-suspension in 50 mL 0.1 M bicarbonate buffer, pH 7.4 followed by centrifugation (30 min at 10,000 G). This washing procedure was repeated 3 times. To remove trace amounts of larger nanoparticle aggregates, the particles were then suspended in an additional 50 mL of bicarbonate buffer, pH 7.4 and centrifuged at 2000 G for 10 min. The small pellet, containing large nanoparticle aggregates was discarded and the opaque supernatant was centrifuged again at 2000 G for 10 min and the resulting supernatant was collected. The final coated nanoparticles were recovered by centrifugation of the cloudy nanoparticle dispersion at 15,000 G for 30 min. The

resulting light green pellet was re-suspended in deionized water (2.0 mL). The concentration of the final upconverting nanoparticle stock solution was determined to be 106 mg/mL by weighing a lyophilized 100 μ L aliquot of the stock. The molarity of this stock nanoparticle solution was then determined by calculation of the number of nanoparticles in the 100 μ L aliquot of the stock NP solution. Briefly, the mass of a single particle first was approximated using the radius of the NP cores (38 nm), as determined by SEM, and the density of Y_2O_3 (5.01 g/cm³). The nanoparticle concentration in the aliquot was then determined by dividing the total mass of the NPs in the aliquot by the mass of the individual particles. This number, together with the volume of the aliquot (100 μ L), was then used to calculate the molarity of the stock solution (153 nM).

Nanoparticle characterization. Particle coating. Presence of the polyethylene glycol coating was verified by comparing FTIR spectra (KBr pellets) of the uncoated particles, the free mPEG-NH₂ polymer, and the final coated nanoparticles. The diameter of the Y_2O_3 nanoparticle cores were determined by SEM (average of 125 particles) and the hydrodynamic diameter of the nanoparticles were measured by dynamic light scattering. Fluorophore loading on the nanoparticle surface was approximated by measuring the fluorophore absorbance at 773 nm from a 200-fold dilution of the 106 mg/mL stock nanoparticle solution with a final concentration of 0.76 nM.

Cell viability. RAW 264.7 Leukemic monocyte/macrophage (RAW) cells were maintained in a 5% CO₂, water-saturated atmosphere and grown in DEMI media supplemented with 10% FBS, 1% penstrep, 1% L-glutamine and 2% Na₂CO₃. RAW cells were seeded in a 96-well plate at a density of ~17,500 cells per well and were allowed to grow for 24 hours before addition of the upconverting nanoparticles. After addition of

the nanoparticles, the cells were incubated at 37 °C for 4 h and cell viability was assessed with the MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)) assay. The assay solution was prepared by diluting the CellTiter 96 AQueous one solution reagent (Promega) 6-fold with cell media. After removing the cell media, cells were gently washed twice by fresh media. Then 120 μ L of the assay solution was added to each well and the plate was incubated for 30 minutes at 37 °C in a humidified, 5% CO₂ atmosphere. The absorbance of each well at 490 nm was recorded by an XFluor4 96-well plate reader (TECAN) to determine cell viability. All of the data were averaged over three trials.

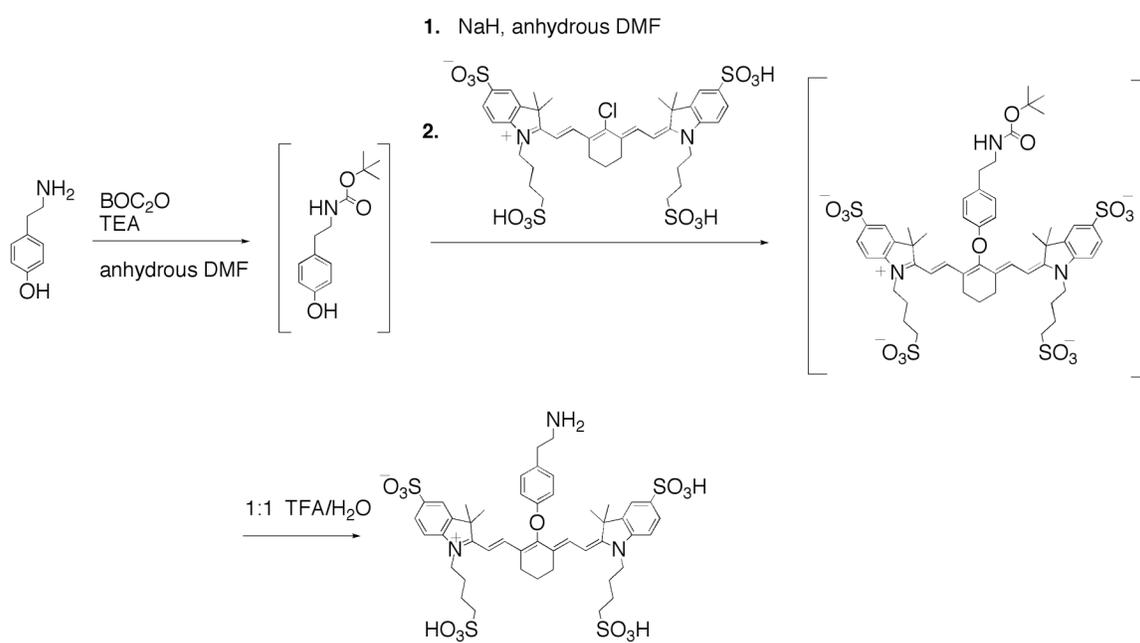
Imaging System. A whole-field-illumination upconverting microscope was constructed and used for the in vivo imaging (**Figure S5**). Interchangeable illumination sources shone from below the glass sample stage. The imaging stage was positioned above the sample. Light was gathered using a 25x microscope objective. Interference filters housed in a filter ring (Edmund Optics) then selected the imaging channel: 430nm-460nm for blood absorption imaging, 765nm-800nm for NIR fluorescence, and 750nm short-pass for up-conversion imaging. An additional 950 short-pass filter in the imaging path further rejected 980 nm excitation light. A 40 mm lens (Edmund Optics) created an image on the PixelFly QE camera sensor. Separate illumination sources were used for each imaging channel. Blue illumination was provided by a blue LED. A 737 nm fiber coupled laser (Ceramoptec) was coupled to a collimator and diffuser for NIR fluorescence imaging. Upconversion illumination was provided by a 2 W 980 nm diode laser (Roithner Lasertechnik). The 980nm laser light was collimated by an aspherical

lens (Edmund Optics), filtered by a 950 nm long-pass filter, and diffused by a square pattern diffuser (Thorlabs).

In vivo imaging. All animal experiments presented in this paper were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care under protocol 2003N000209. The mice were anesthetized by intraperitoneal injection of a mixture of 100 mg/kg Ketamine and 5 mg/kg Xylazine. After the anesthesia took effect, the probe was injected intravenously and the mice were placed in the imaging system. Images were then taken in each of the three channels: blue illumination, NIR fluorescence, and upconversion. After each image, an additional black image was taken without illumination. These black images were subtracted from the illuminated images to reduce the effects of camera noise. For the upconversion imaging movie, each frame was captured with a 200 ms exposure time. After capturing the frames, the frames were post processed with curve adjustments and despeckle to remove hot pixels, Gaussian blur to remove artifacts, and gradient luminance adjustments to balance illumination.

References:

- 1 S. A. Hilderbrand, K. A. Kelly, R. Weissleder, C.-H. Tung, *Bioconjugate Chem.* **2005**, *16*, 1275.
- 2 R. B. Mujumdar, L. A. Ernst, S. R. Mujumdar, C. J. Lewis, A. S. Waggoner, *Bioconjugate Chem.* **1993**, *4*, 105.



Scheme S1. Synthesis of the amine modified NIR carbocyanine fluorophore

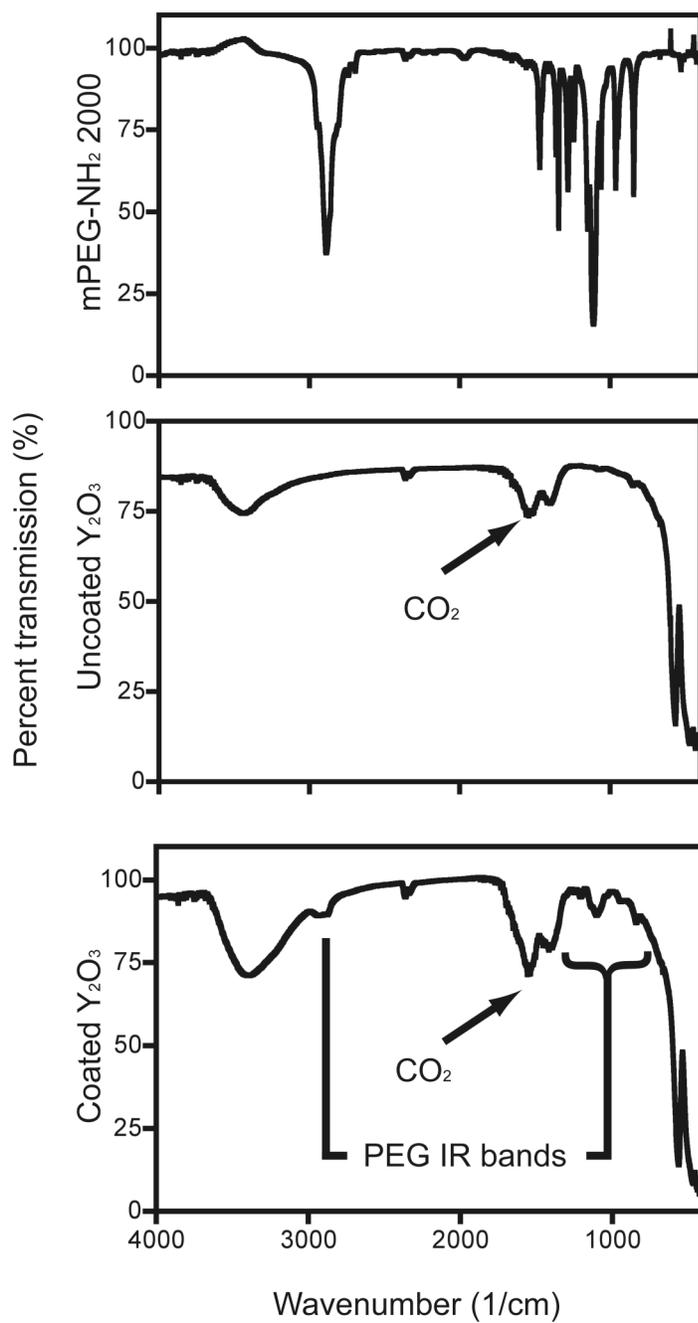


Figure S1. FTIR spectra of the mPEG-NH₂ 2000, uncoated Y₂O₃ and coated Y₂O₃ nanoparticles from top to bottom, respectively. IR bands corresponding to the PEG coating can clearly be seen in the spectrum of the final particles.

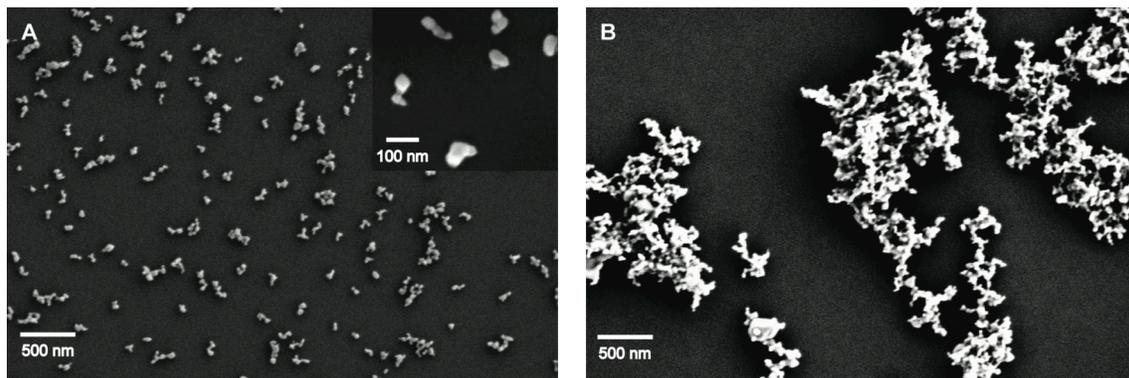


Figure S2. SEM images of the Y_2O_3 upconverting nanoparticle cores after (a) and before (b) coating the particles with polyacrylic acid, polyethylene glycol, and NIR fluorophores. Strong clumping of the particles can be seen with the uncoated particles. The average diameter of the Y_2O_3 cores of the coated particles is 76 nm, as determined from the average of 125 individual particles. The magnification for a and b is 60,000X and the inset magnification is 175,000X.

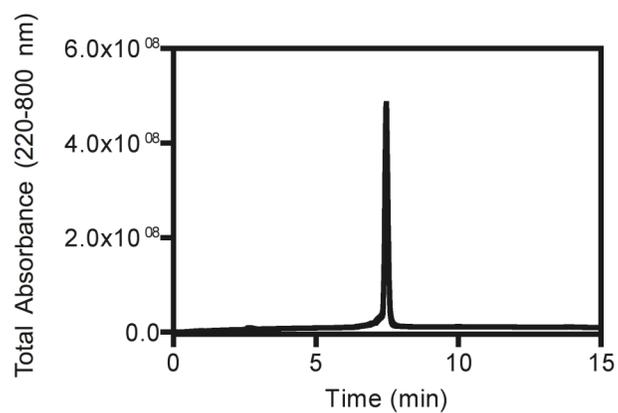


Figure S3. HPLC trace of the amine modified NIR carbocyanine fluorophore verifying purity of the compound.

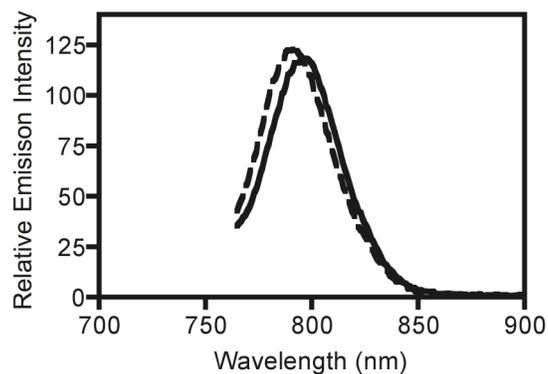


Figure S4. Fluorescence emission traces for absorbance matched solutions of the free amine modified carbocyanine dye (dashed line) and the same fluorophore conjugated to the upconverting Y₂O₃ nanoparticles (solid line). The relative fluorophore concentration in both samples is 350 nM, the nanoparticle concentration is 760 pM. Less than 5% fluorescence quenching is observed when the nanoparticle bound fluorophore solution is compared to the free fluorophore solution. A slight 5 nm red shift in the fluorescence emission from 792 (free fluorophore) to 797 nm (nanoparticle bound fluorophore) is observed. Both samples were excited under identical conditions at 750 nm.

Transillumination Upconverting Imager

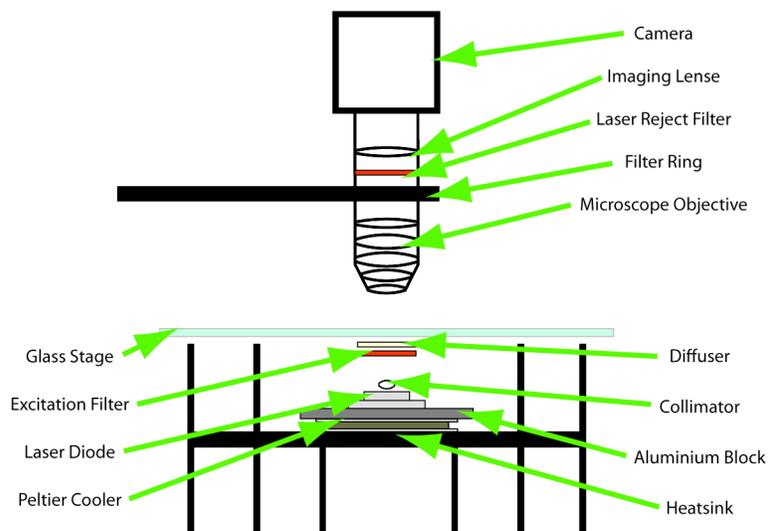


Figure S5. Schematic of the transillumination upconverting imager used for the in vivo imaging experiments.