Electronic Supplementary Information for:

Spectrally Resolved Confocal Microscopy using Lanthanide Centred near-IR Emission

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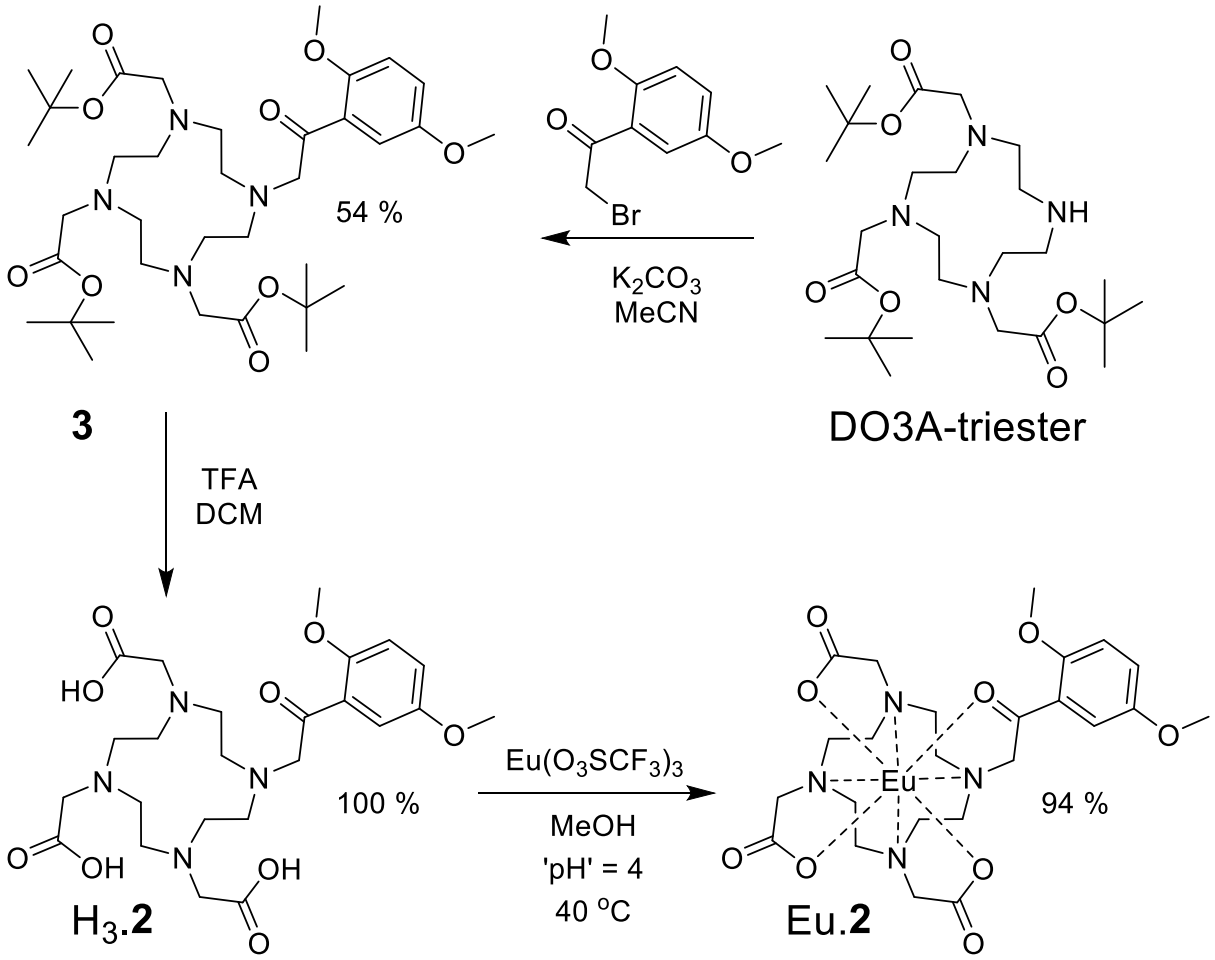
Methods and materials

Compounds

Ln.**1** was synthesised as described in ref. 1. Eu.**2** was synthesised in this work from DO3A-triester, which was prepared as described in ref. 2. All materials were used as received. The synthetic details are given below:

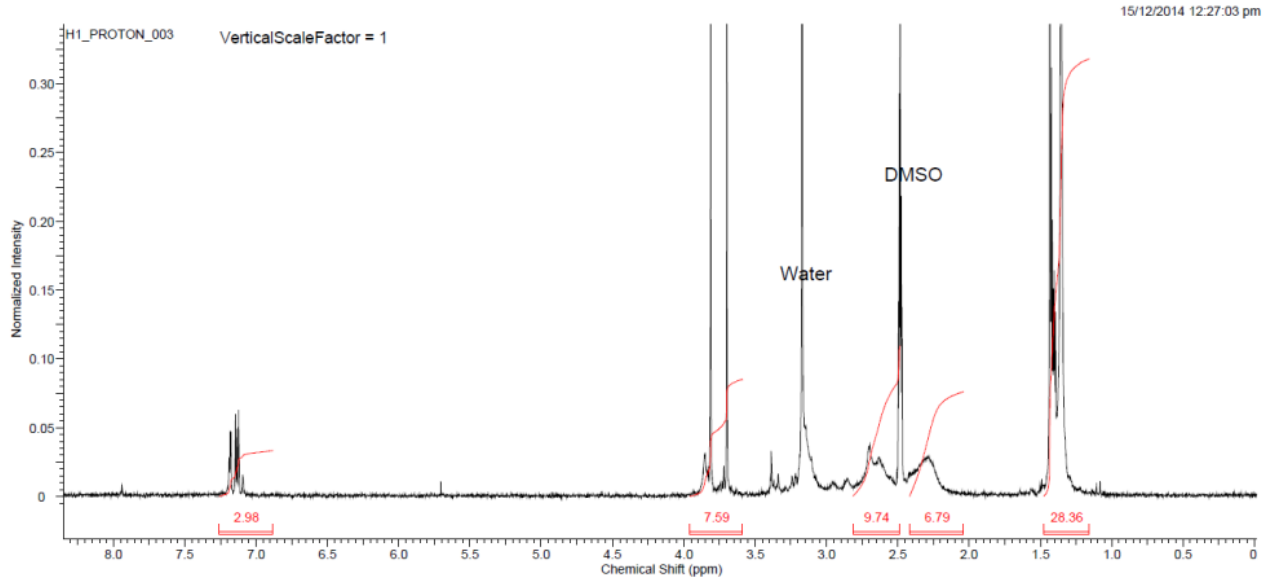


1,4,7-Tris(*tert*-butoxycarbonylmethyl)-10-(2',5'-dimethoxy-2-acetophenone)-1,4,7,10-tetraazacyclododecane (3)

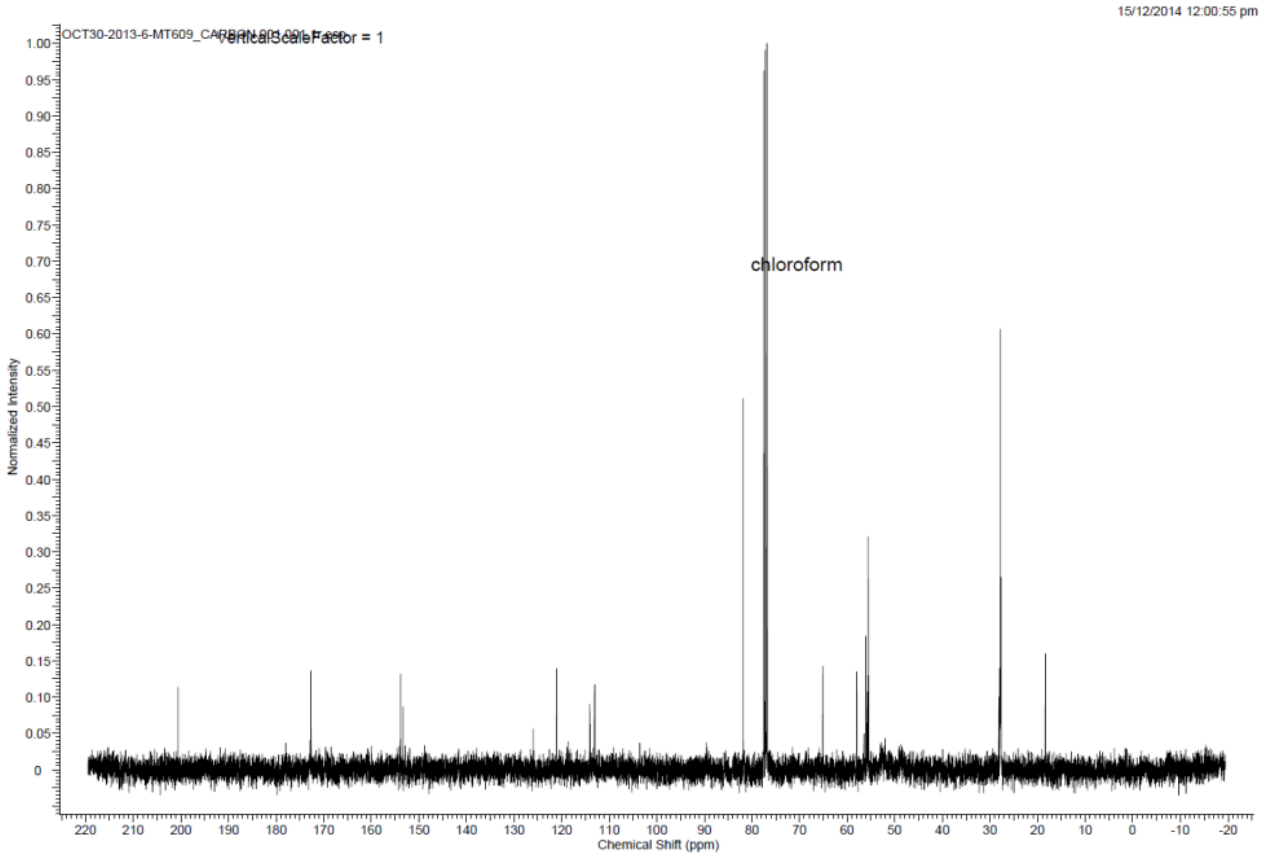
To a solution of DO3A-triester (2.0 g, 3.4 mmol) in 25 ml of dry acetonitrile was added potassium carbonate (0.97 g, 7.0 mmol) and this suspension was left stirring for 20 min. 2-Bromo-2′,5′-dimethoxyacetophenone (0.88 g, 3.4 mmol) was added while keeping the reaction flask in an ice bath. The reaction was left stirring under nitrogen for 16 h at RT and monitored by ES-MS. The inorganic solids were removed by filtration and the solvent evaporated under reduced pressure. The crude product obtained was purified by column chromatography on neutral alumina (DCM/MeOH 95:5 to 80:20) to obtain a yellow solid (1.28g, 54%).

^{1}H NMR (300 MHz; d^6-dmso 353 K): δ 7.20-7.18 (m, 1H), 7.15-7.10 (m, 2H), 3.89 (s, 2H), 3.84 (s, 3H), 3.73 (s, 3H), 3.03 (s, 6H), 2.70 (s, br, 8H), 2.36 (s, br 8H), 1.46 (s, 9H), 1.39 (s, 18H). ^{13}C NMR (76 MHz; d^6-DMSO): δ 28.10, 48.60, 48.98, 50.26, 50.64, 51.08, 54.06, 56.80, 58.68, 115.51, 116.10, 118.11, 119.16, 119.90, 120.89, 155.43, 157.76, 164.23, 165.01, 175.14. LR-ESMS: *m/z* calc. for $[M+Na]^+$ 715.87, found 714.47.

^{1}H NMR DMSO-*d6* VT at 100°C:



^{13}C NMR chloroform-*d1*:

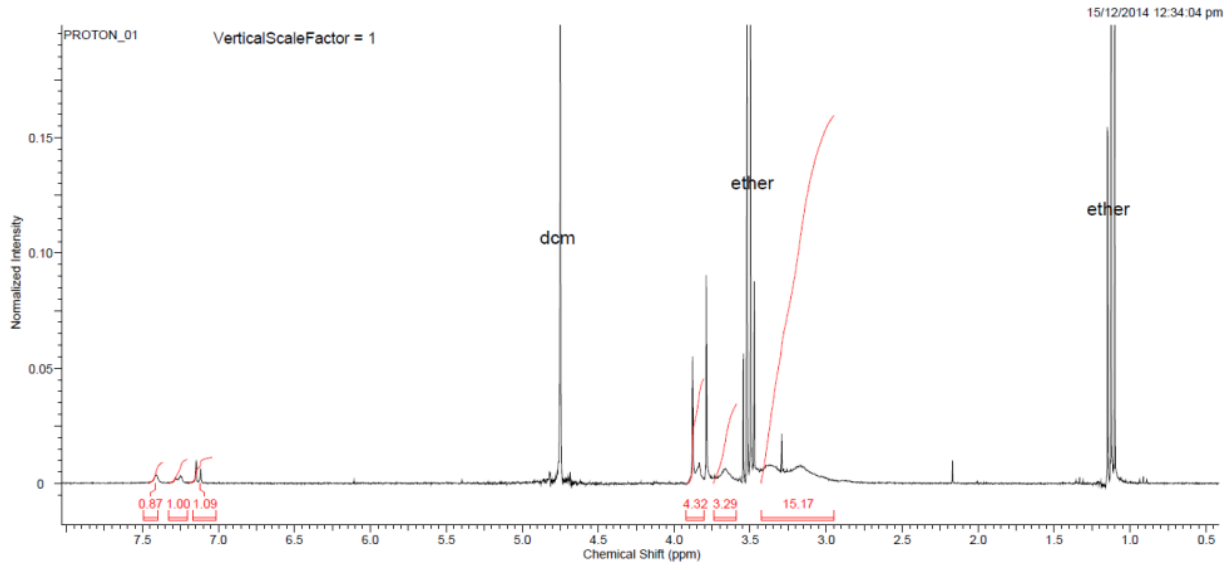


1,4,7-Tris(carboxymethyl)-10-(2',5'-dimethoxy-2-acetophenone)-1,4,7,10-tetraazacyclododecane (H_3.2)

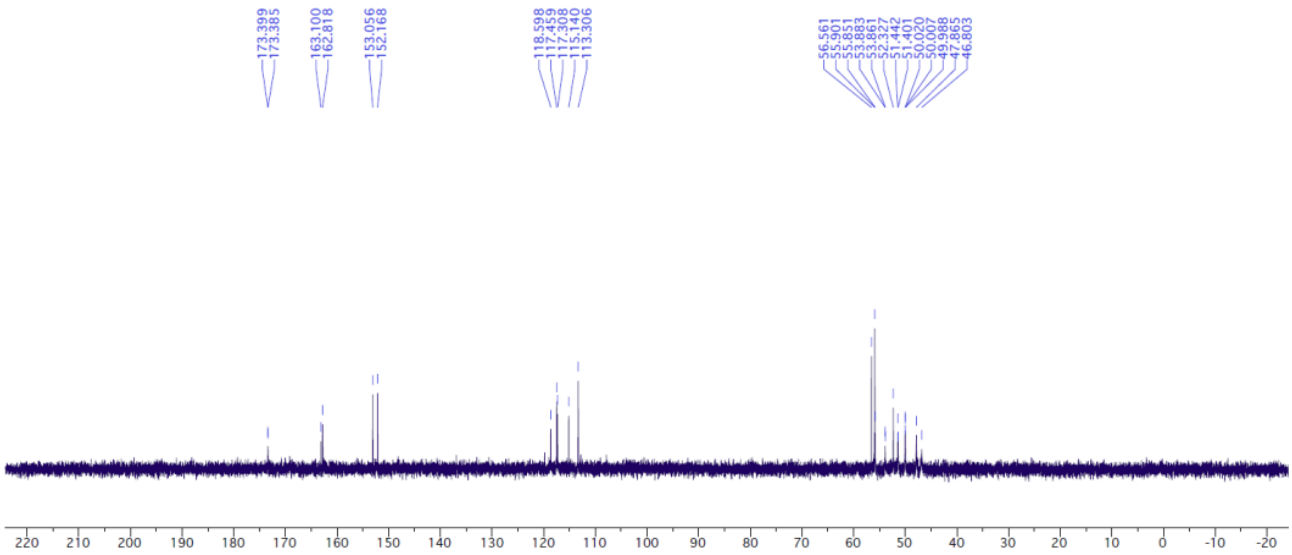
Compound **3** (1.0 g, 1.90 mmol) was dissolved in DCM (8 mL) and TFA (8 mL) was added dropwise. The solution was stirred at RT for 24 h. The solvent was removed under reduced pressure and the residue dissolved in the minimum amount of methanol and precipitated with diethyl ether. Trituration with diethyl ether yielded the TFA salt of the title compound as a white solid in quantitative yield.

^{1}H NMR (500 MHz; D_2O): δ 7.14 (dd, 1H), 7.11-7.10 (m, 2H), 4.52 (s, 2H), 4.08 (s, 2H), 3.85 (s, 3H), 3.82 (s, 3H), 3.61-3.03 (m, br, 24H). ^{13}C NMR (126 MHz; D_2O): δ 46.80, 47.86, 50.09, 51.44, 52.33, 53.88, 55.90, 56.56, 113.31, 115.14, 117.31, 117.46, 118.60, 119.78, 152.17, 153.06, 162.82, 163.10, 173.40. HR-ESMS: *m/z* calc. for $[M+H]^+$ 525.256, found 525.254.

^{1}H NMR D_2O:



^{13}C NMR D_2O:

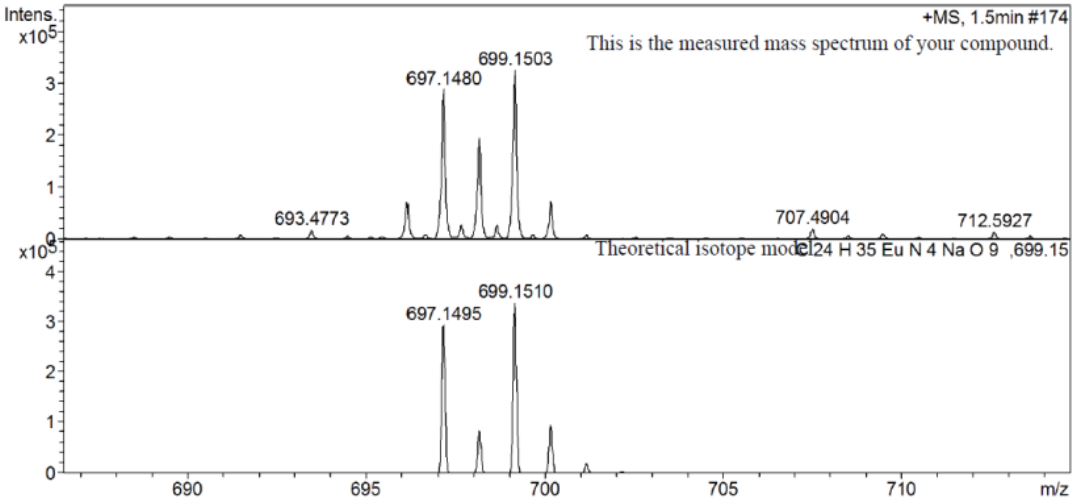


General method for the synthesis of the lanthanide complexes from H_3.2

To a solution of the ligand (**H_3.2**) in methanol the appropriate lanthanide triflate salt was added (1.05 eq.) and the reaction mixture was stirred at 40º C for 30 min. The pH was adjusted to 4 by dropwise addition of an aqueous NaOH solution. The reaction was left stirring at 40º C for 48 h. The methanol was removed under reduced pressure leaving an oil that was dissolved in water. The pH of this solution was adjusted to 10 by addition of aqueous NaOH to remove excess of lanthanide as its insoluble hydroxide. The resulting precipitate was centrifuged and the supernatant filtered. The solvent was removed under reduced pressure to yield the product as a yellow solid.

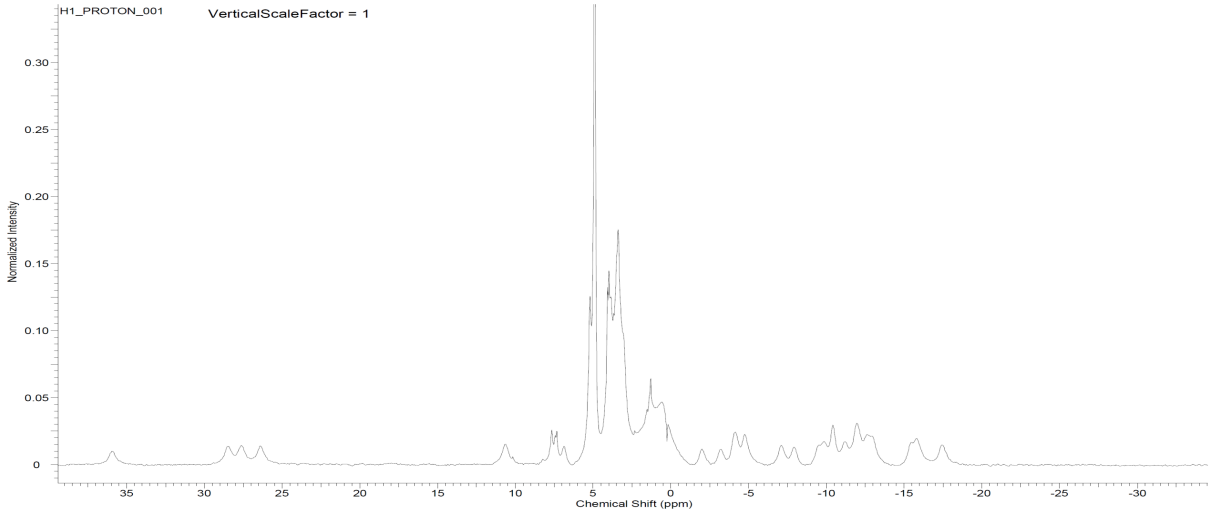
Eu.**2** (Ln: Eu). Yield: 94 %. ^{1}H NMR (300 MHz; D_2O): δ 35.85, 28.34, 27.52, 26.33, 10.52, -2.10, -3.33, -4.24, -4.84, -7.21, -8.01, -9.99, -15.91, -17.55. Only resolved peaks outside 0 to 10 ppm region are reported. HR-ESMS: *m/z* calc. for $[M+Na]^+$ 697.135, found 697.137. UV-Vis (H_2O) λ_{max}: 268, 373 nm. Lifetimes: 0.27 ms (H_2O); 0.49 ms (D_2O); q=1.7. Elemental analysis: Calcd. for $C_{24}H_{35}EuN_4O_{10}\cdot 3NaCl$: C, 33.25; H, 4.07; N, 6.46. Found: C, 33.04; H, 4.14; N, 6.54.

HR-ESMS:



Meas. m/z	#	Formula	m/z	err [ppm]	Mean err [ppm]	rdb	e⁻ Conf	mSigma
699.1503								
	1	C 24 H 35 Eu N 4 Na O 9	699.1509	0.8	2.8	9.0	odd	146.58

^{1}H NMR:



Solutions

The lanthanide complexes were dissolved in D_2O and the concentration monitored by recording absorption spectra. Note that the emission spectra are given in total intensity, which is not corrected for absorbance at the point of excitation or dye concentration. That is, the intensity reported cannot be used to judge the brightness of the probes.

Silica coating and mixing

Firstly the dyes were dissolved in deuterated water. The deuterated solutions were then separately mixed with silica particles. The amount of silica particles was chosen in a manner that there was 3 to 4 times more solution than particles. The solutions of each lanthanide dye were then left to dry. Upon drying two different powders with dye-coated silica particles were mixed together in a separate vial. Diethyl ether was added to the powder mixture to ensure the good mixture degree. The powders in ether were stirred and left to dry up for further use in microscopy.

Microscopy set-up

The confocal microscopy setup depicted in Figure SI1 was used for the imaging. A pulsed laser (LDH-P-C-375) from PicoQuant was used as excitation source, and the power used for all the imaging and recording of spectra is ~ 2.4 kW/cm^2 in the focal spot. The excitation light is separated from the emission by a long-pass filter (Semrock, BLP01-458R-25 for Eu and Nd and an additional filter Semrock, LP02-633RU-25for Yb). Areas with well-separated particles were imaged point by point in a raster scanning fashion. N_2 was flown over sample during the scan, which helped to reduce bleaching (see figure SI 22).

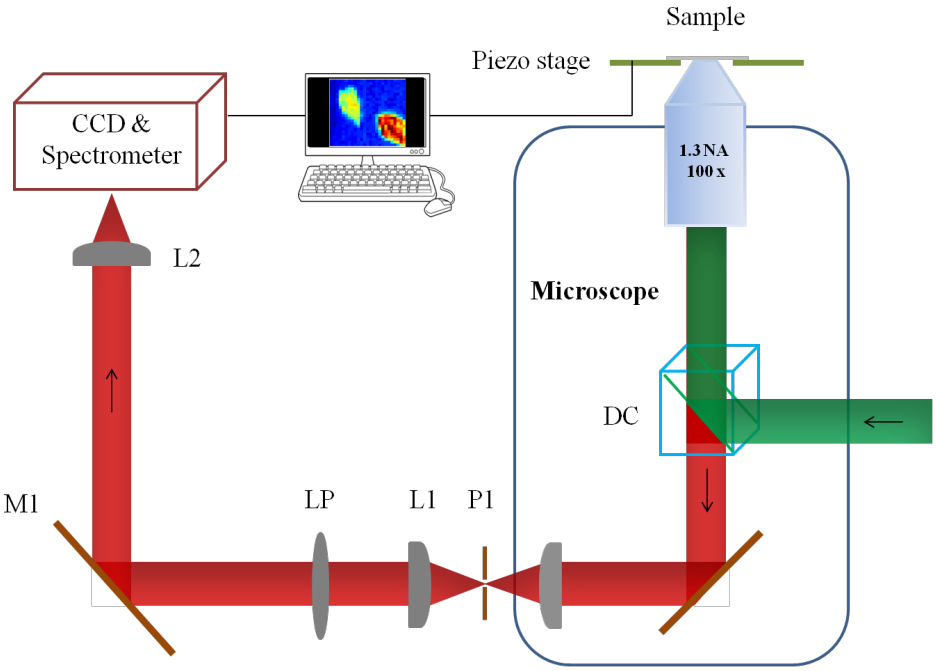


Figure SI1. Microscope configuration. Abbreviations: DC, dichroic mirror; P1, pinhole; L1 and L2, lenses; LP, long-pass filter; M1, mirror; CCD, charge coupled device.

The experiments were carried out on a home-built scanning fluorescence confocal microscopy system based on an Olympus IX71 inverted microscope. A piezo-driven scanning stage (Physik Intrumente P5173CL), which is controlled by a home-written software program (LabView, National Instruments), allows for

imaging the sample point by point in a raster scanning fashion in a range up to 100 μm × 100 μm. Upon laser illumination, the fluorescence emission signal from the sample was collected by a 100× oil immersion objective (Olympus UPLFLN 100×), and directed into a spectrometer for the measurements (Princeton Instruments SPEC-10:100B/LN_eXcelon CCD camera (back-illuminated CCD), SP 2356 spectrometer with 1-030-500 grating 300 g/mm @ 500 nm, all controlled by the same LabView program that controls the scanner). The X axis of the emission spectra was calibrated using emission lines of a Neon lamp. The Y axis (Intensity) was not corrected for differences in optical transmission and detection efficiency in order to provide a better impression of the raw, uncorrected signal on can detect using the previously mentioned equipment.

Software and data analysis

A home-written software program (Labview, National Instruments) was used for the imaging, and a full fluorescence emission spectrum was recorded at each pixel of the image. The acquisition time at each pixel of each spectrum is 5 s for all the imaging data shown in this paper.

The reconstruction of the images is based on the fluorescence intensity in the spectral region of interest (800-830 nm for Eu.**2** and 860-890 nm for Nd.**1**). To minimize the influence from background, for instance the continuous decreasing slope in the spectrum, the spectral region of interest is background subtracted by the average intensity of spectral region on both sides (*vide infra*) .

Absorption spectra in D_2O solution

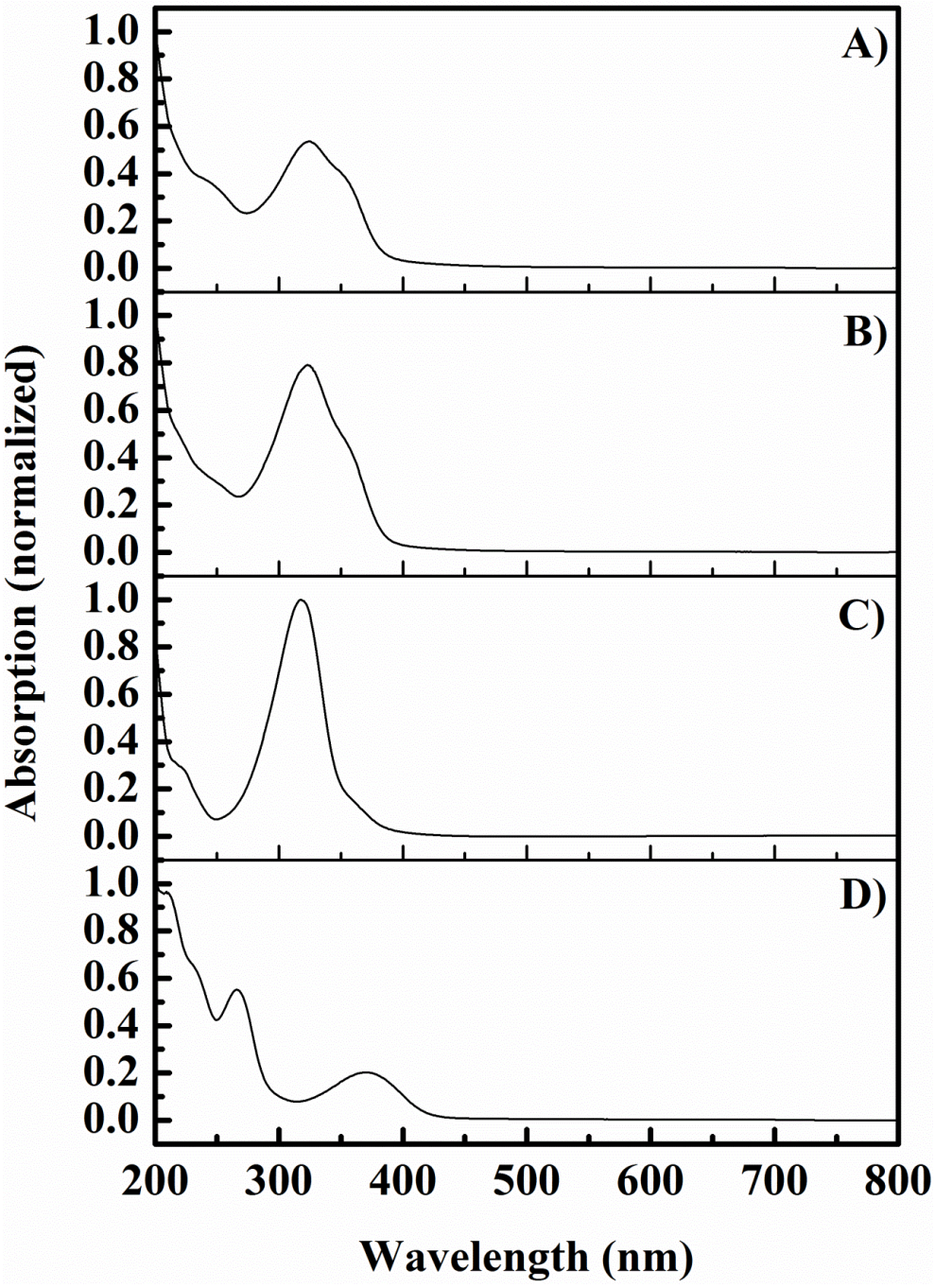


Figure SI2A. Normalised absorption spectra of A) Yb.**1**, B) Nd.**1**, C) Eu.**1** and D) Eu.**2** dissolved in D_2O, recorded prior to determining the emission spectra.

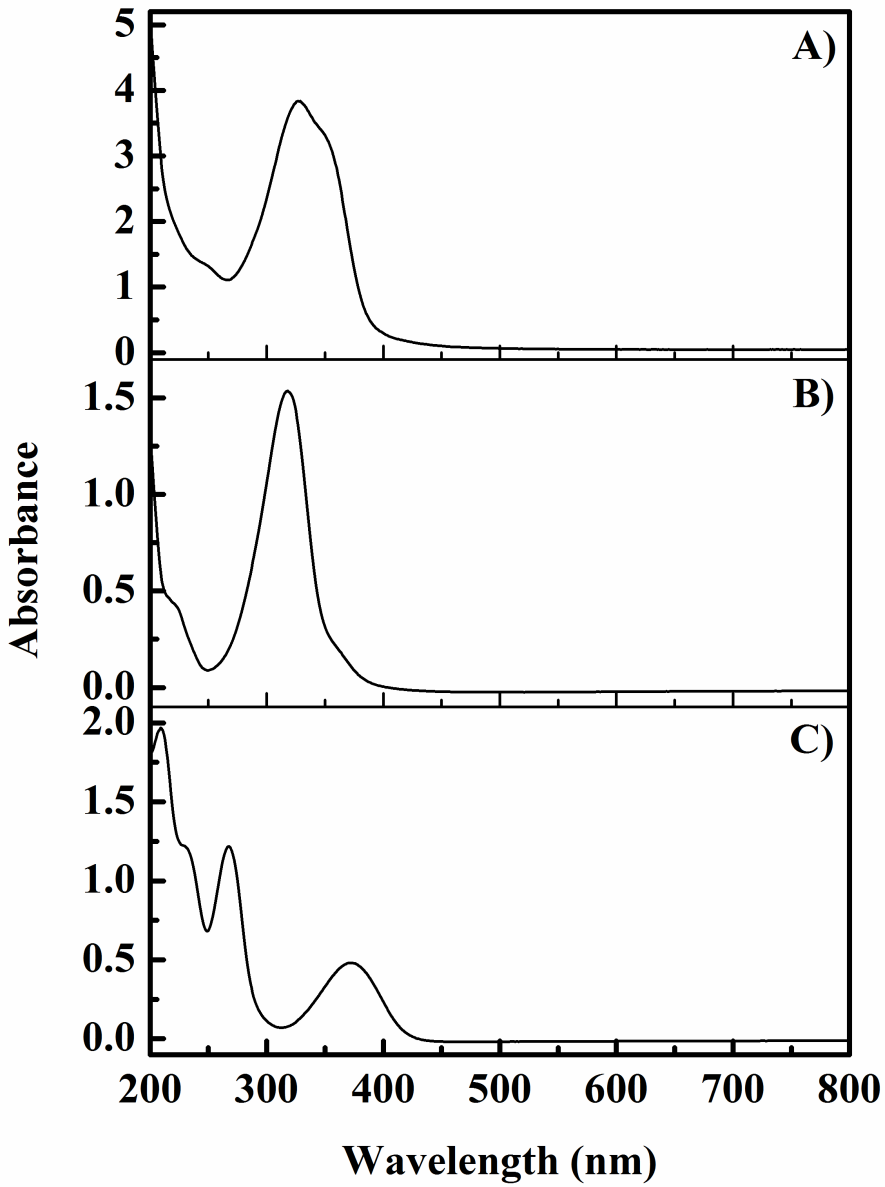


Figure SI2B. Absorption spectra of A) Nd.**1**, B) Eu.**1**, C) Eu.**2** dissolved in D_2O, recorded after determining the emission spectra.

Emission spectra in D_2O solution

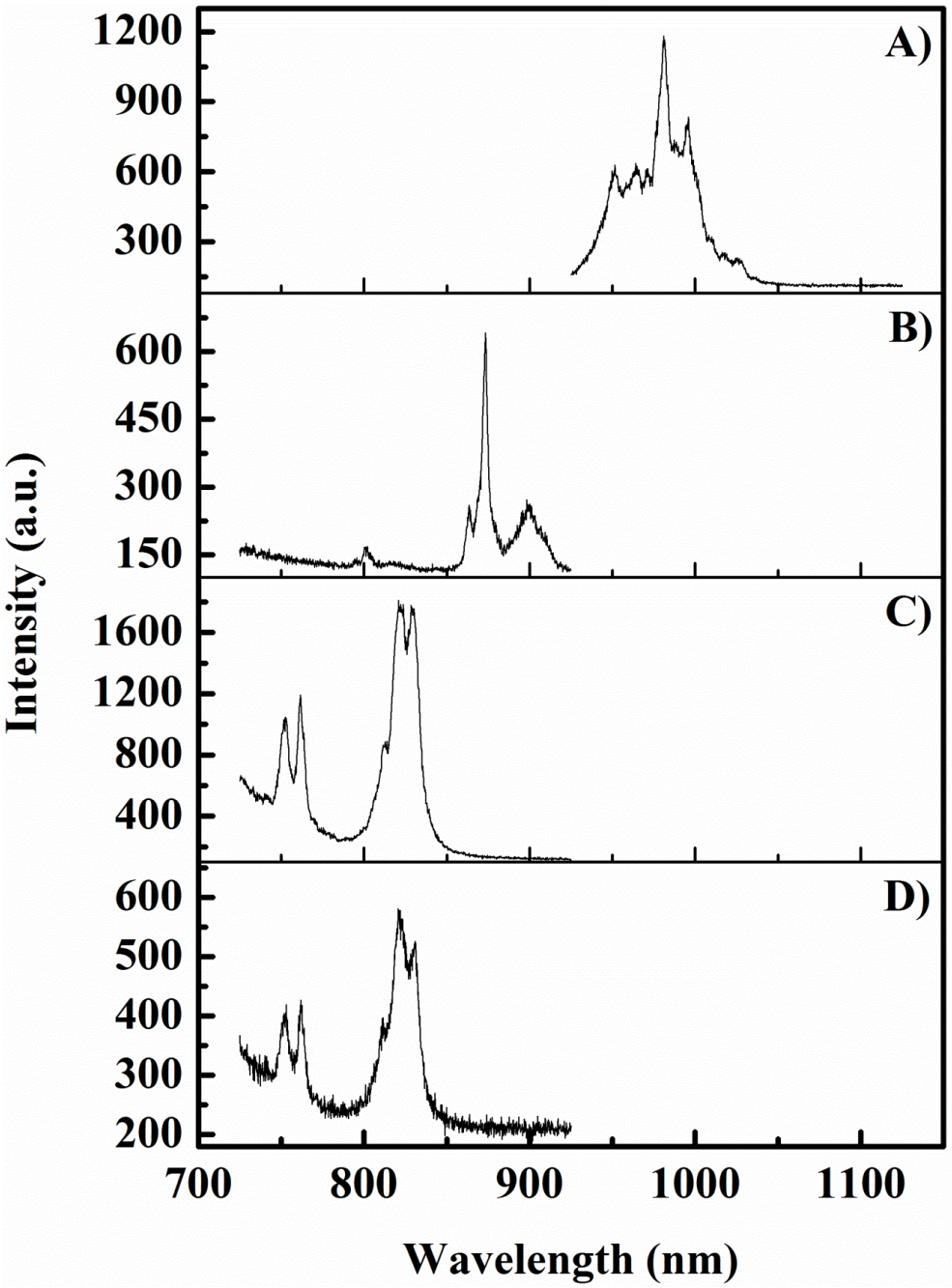


Figure SI3. Emission spectra of Yb.**1** Nd.**1**, Eu.**2** and Eu.**1** measured on the microscope using the CCD camera. A) Yb.**1** in D_2O, integration time, 600 s. B) Nd.**1** in D_2O, integration time, 200 s. C) Eu.**2** in D_2O, integration time, 200 s. D) Eu.**1** in D_2O, integration time, 600 s.

Spectra of lanthanide dyes on silica particles and background spectrum

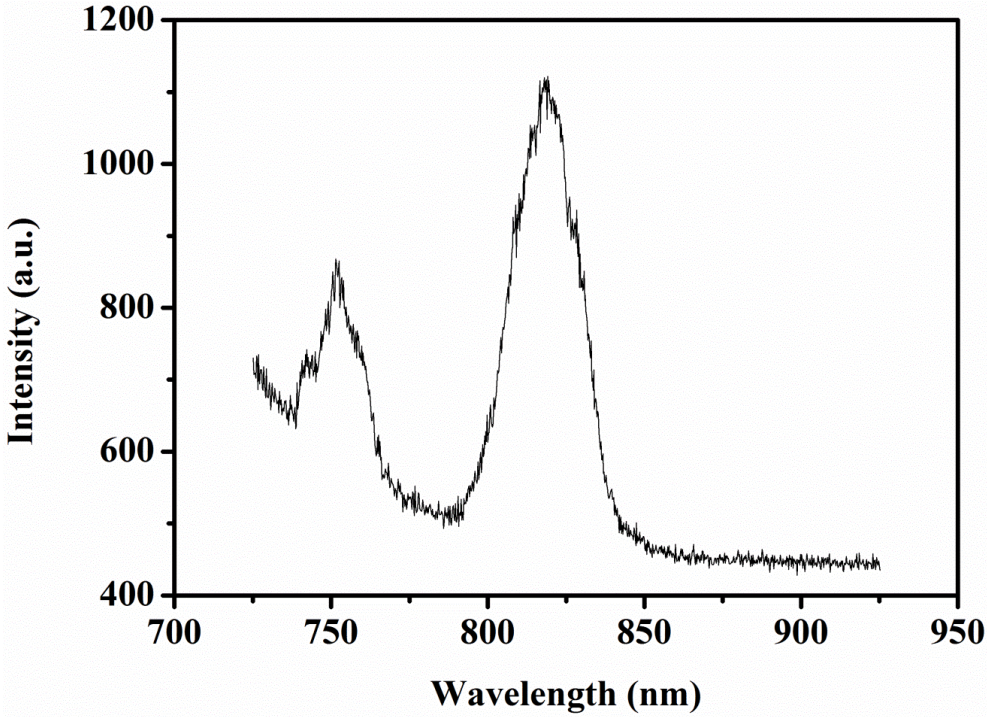


Figure SI4. Emission spectrum of Eu.**2** absorbed on silica particle, integration time, 200 s.

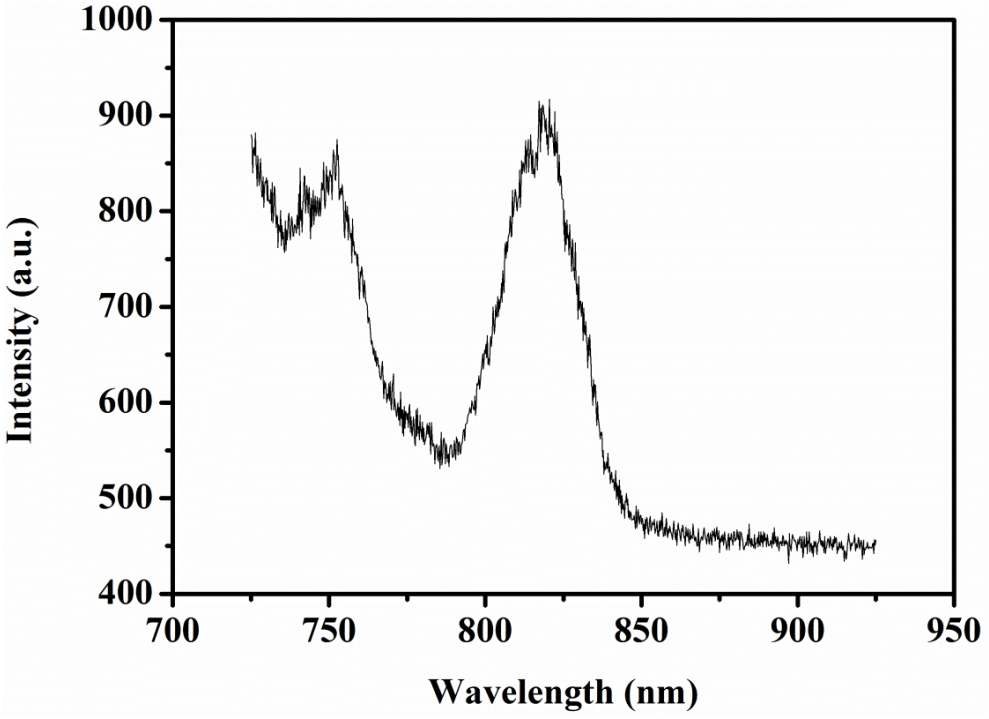


Figure SI5. Emission spectrum of Eu.**1** absorbed on silica particle, integration time, 200 s.

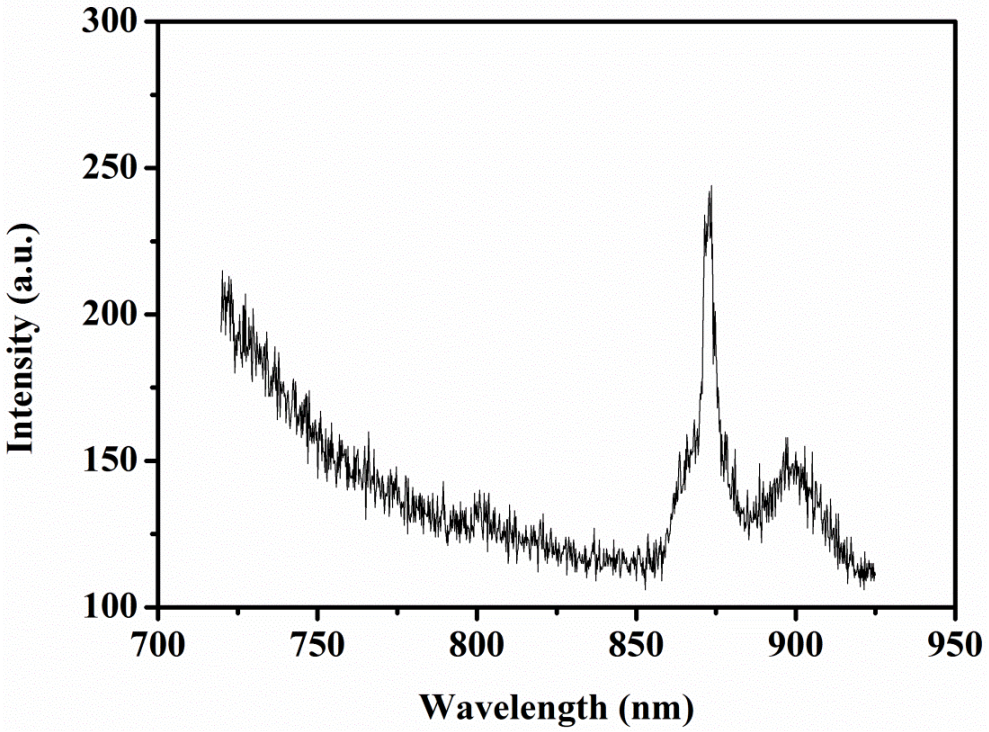


Figure SI6. Emission spectrum of Nd.**1** absorbed on silica particle, integration time, 200 s.

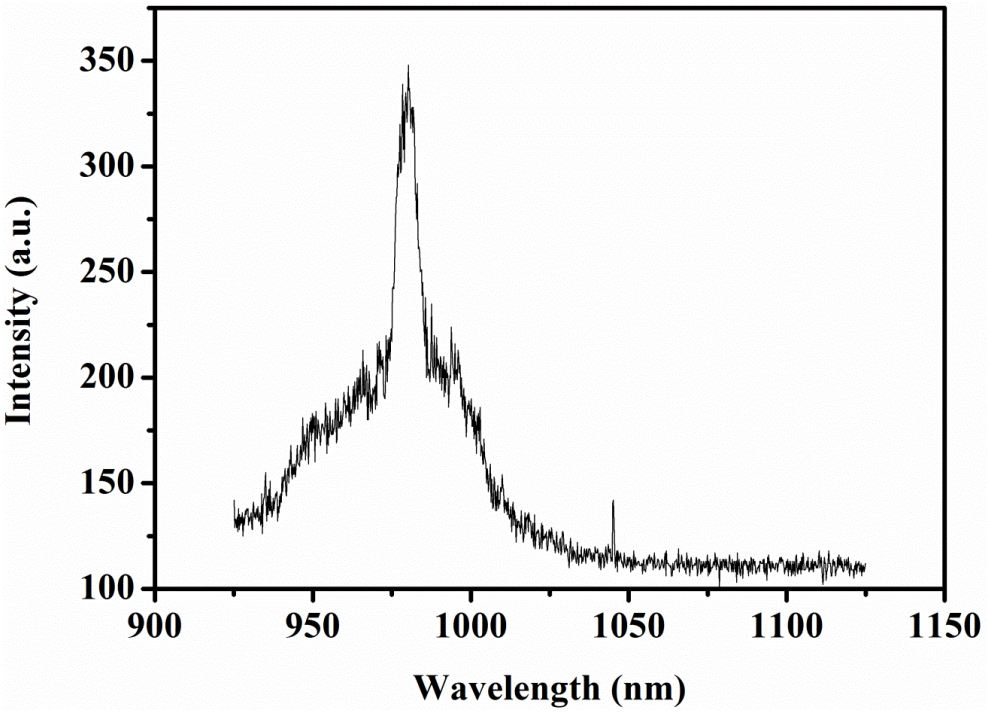


Figure SI7. Emission spectrum of **Yb.1** absorbed on silica particle, integration time, 900 s.

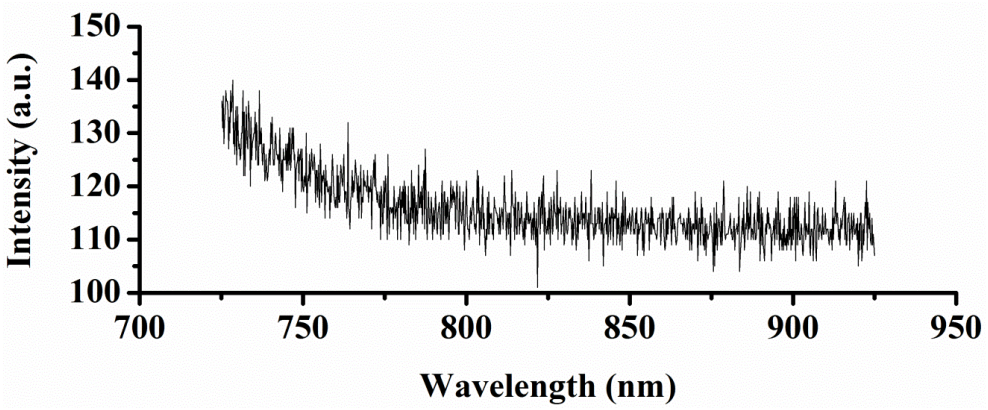


Figure SI8. Spectrum from silica beads with the same excitation power as imaging, integration time: 200 s.

Raw images of silica particles and spectrally resolved images without background subtraction

Figure SI9 shows the comparison between long-pass filter based imaging and spectrally resolved imaging. Figure SI9A shows the fluorescence image of silica particles based on the intensity in the spectral region of 725 – 925 nm, as a 725 nm long-pass filter was used for the imaging. Figure SI9B is the spectrally resolved imaging based on the intensity in the spectral region of 800 – 890 nm. As a reference, an example of image based on the intensity in the spectral region of 720 -740 nm, where there is no emission from the dyes (see figure SI9B), is given in figure SI9C. The comparison between these images indicates that the background may give false signals as shown in figure SI9C, even though there is no emission from the dyes.

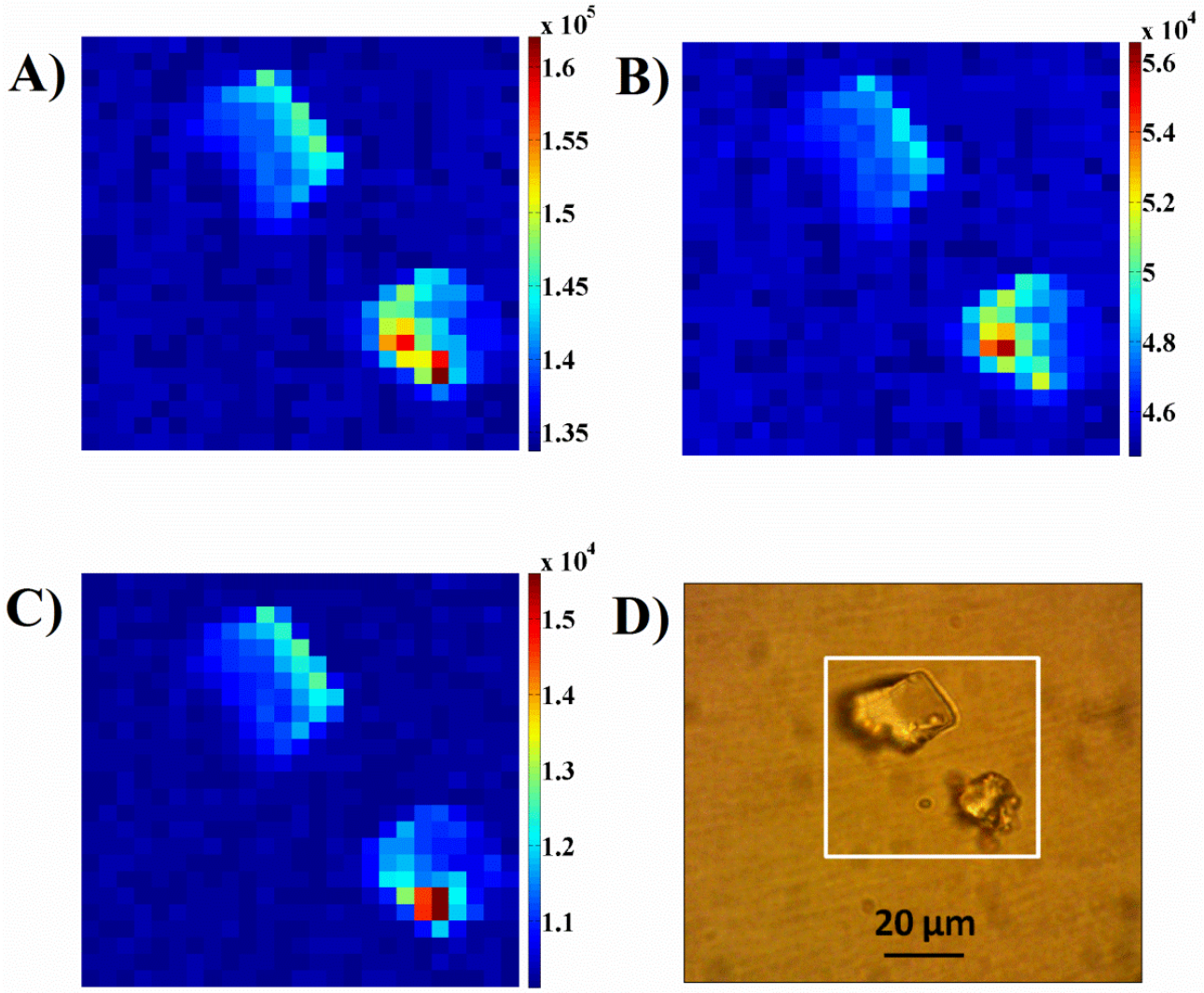


Figure SI9. 50 x 50 µm (25 x 25 pixels) fluorescence images recorded using a CCD based detector system of silica beads dyed with Eu.**2** and Nd.**1**. A) Fluorescence image of silica particles based on the intensity in the spectral region of 725 – 925 nm, as a 700 nm long-pass filter was used. B) Image based on the intensity in the spectral region of 800 – 890 nm. C) Image based on the intensity in the spectral region of 720 – 740 nm (out of the emission peaks). D) The corresponding optical image.

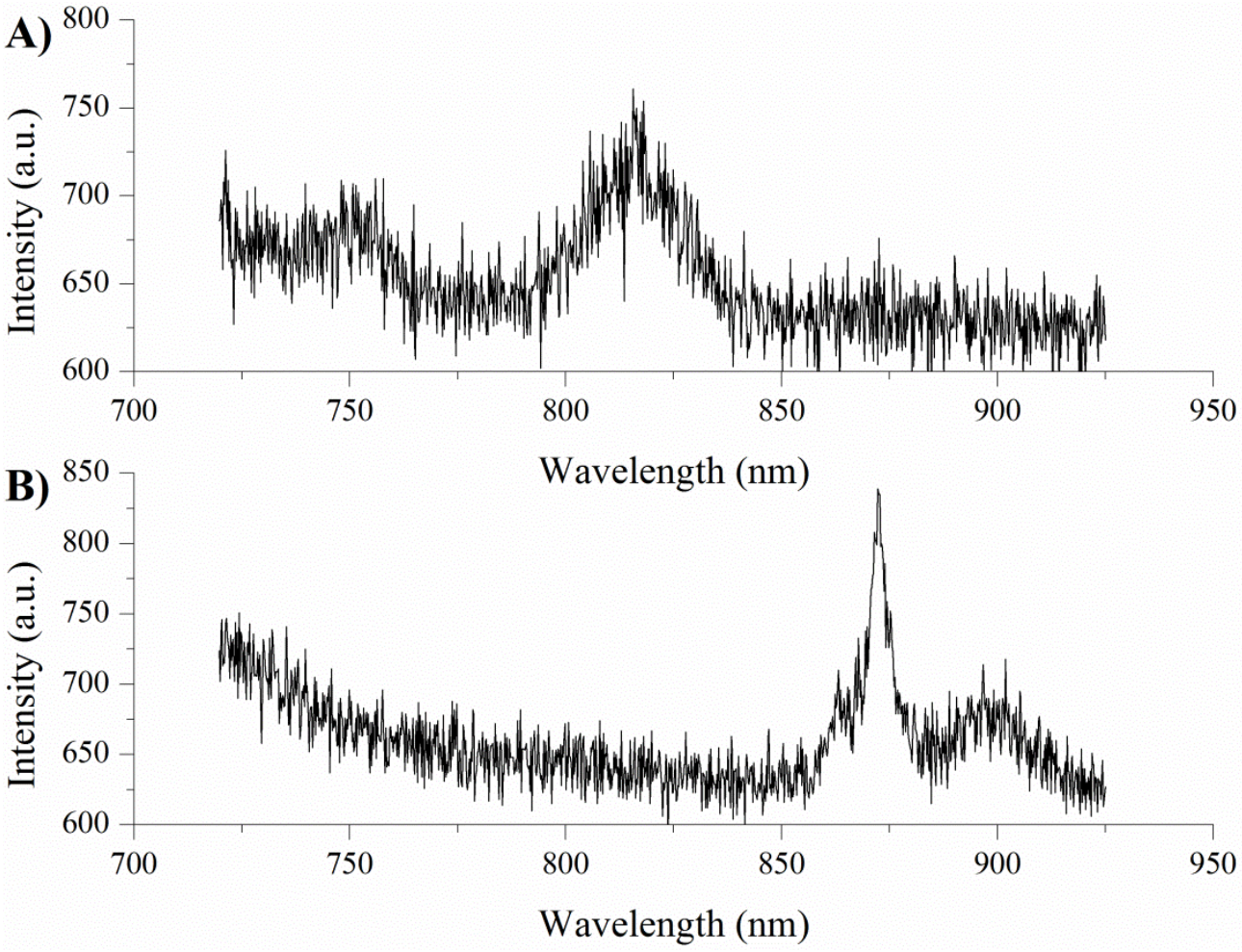


Figure SI10. Emission spectra extracted from image shown in Figure SI9. A) Spectrum from silica particle absorbed with Eu.**2**, sum of 6 spectra (5 s/spectrum). B) Spectrum from silica particle absorbed with Nd.**1**, sum of 6 spectra (5 s/spectrum).

Images simulated to correspond to results from filter based experiments

To minimize the influence from the background (as shown in figure SI9C), all the images in this paper were reconstructed with a MATLAB program. Figure 11 shows the basic processing of the data. Figure SI11A presents the image using all photons above 725 nm. Two silica particles can be seen from the image, but it's impossible to tell which one is stained with Eu.**2** or Nd.**1**. Figure SI11B is the spectrally resolved image, based on the intensity in the spectral region of 800 - 830 nm. From this image, the emission from the particle in the lower right corner can be easily assigned to Eu.**2**. However, the other particle stained with Nd.**1** can also be seen in the image. This is due to the background (see the continuous decreasing slope in the spectrum shown in figure SI1 B). To solve this problem, the spectral region of interest (800 – 830 nm) is background subtracted by the average intensity in the spectral regions on both sides (790 – 795 nm and 845 – 850 nm), where the intensity is from the background. In this way, the influence from the background can be minimized, see figure SI11C.

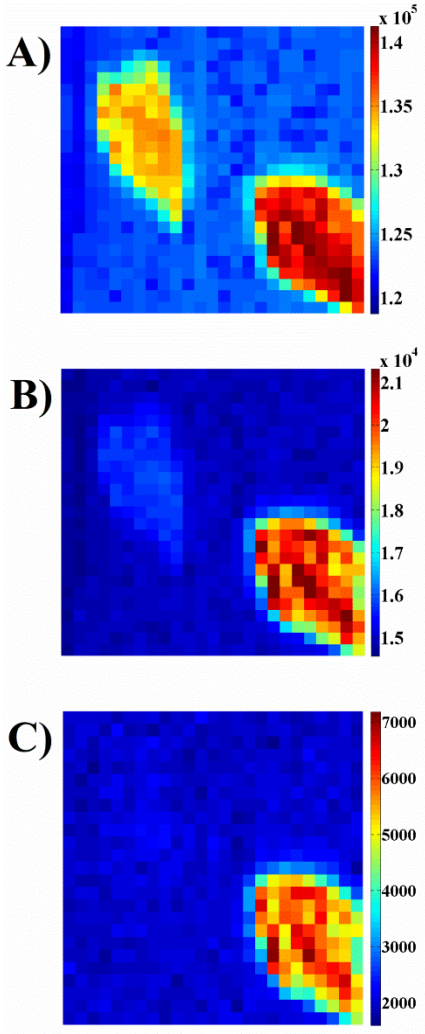


Figure SI11. Comparison of images constructed with different methods. A) Image of silica beads dyed with Eu.**2** and Nd.**1** as a 700 nm long-pass filter was used. B) Image of the beads integrated in the spectral region of 800 – 830 nm, without background subtraction. C) Image of the beads integrated in the spectral region of 800 – 830 nm, the background is removed by subtracting the average intensity of spectral region of 790 – 795 nm and 845 – 850 nm.

Images of silica particles, background corrected

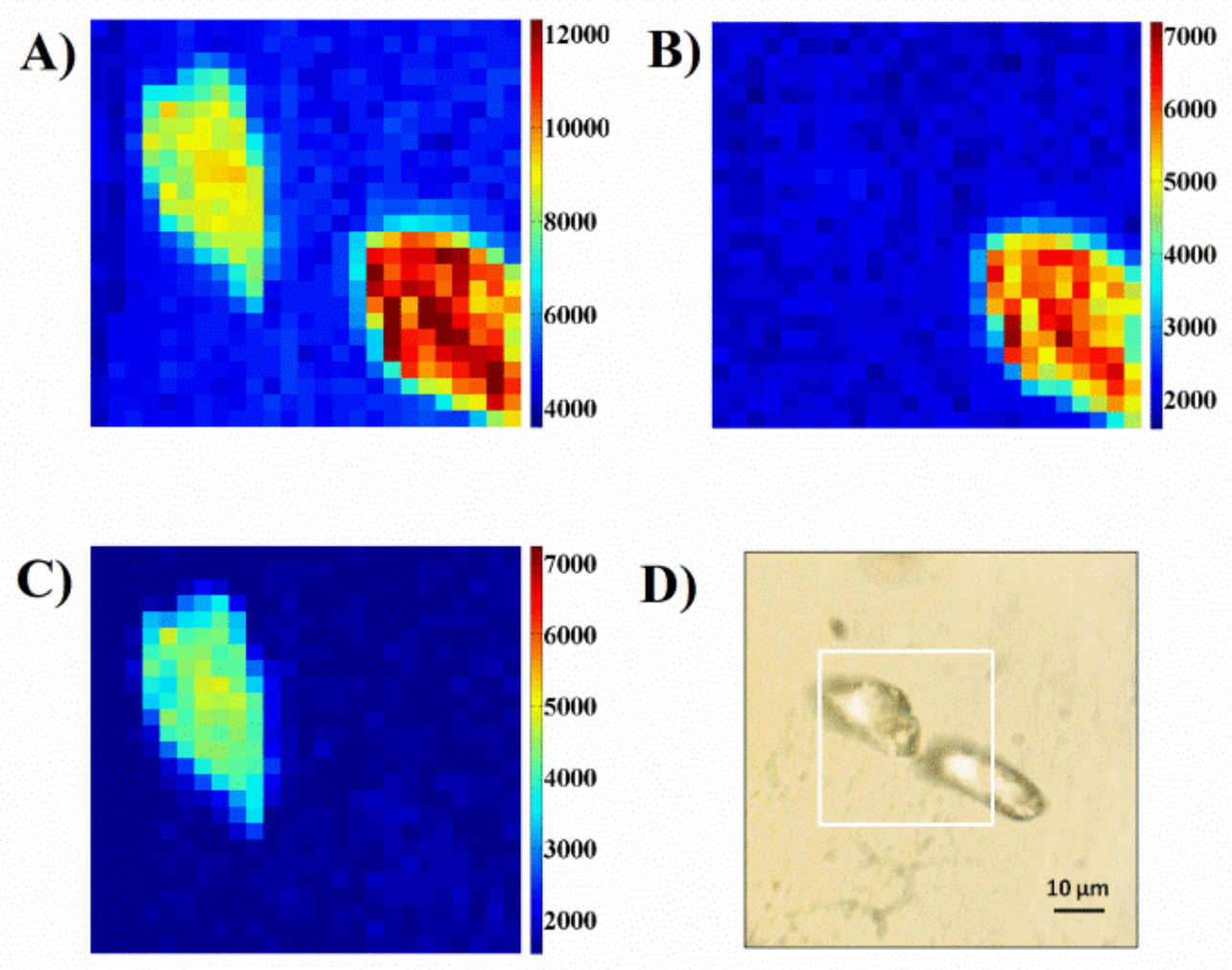


Figure **SI12**. 50 x 50 µm (25 x 25 pixels) fluorescence images recorded using a CCD based detector system of silica beads dyed with Eu.**2** and Nd.**1**. A) Image constructed by integrating the intensity in the spectral region R1 (800-830 nm) and R2 (860-890 nm), see figure SI13. B) Image constructed by integrating the intensity in the spectral region R1. C) Image constructed by integrating the intensity in the spectral region R2. D) Optical image of the scan area, marked by the white square.

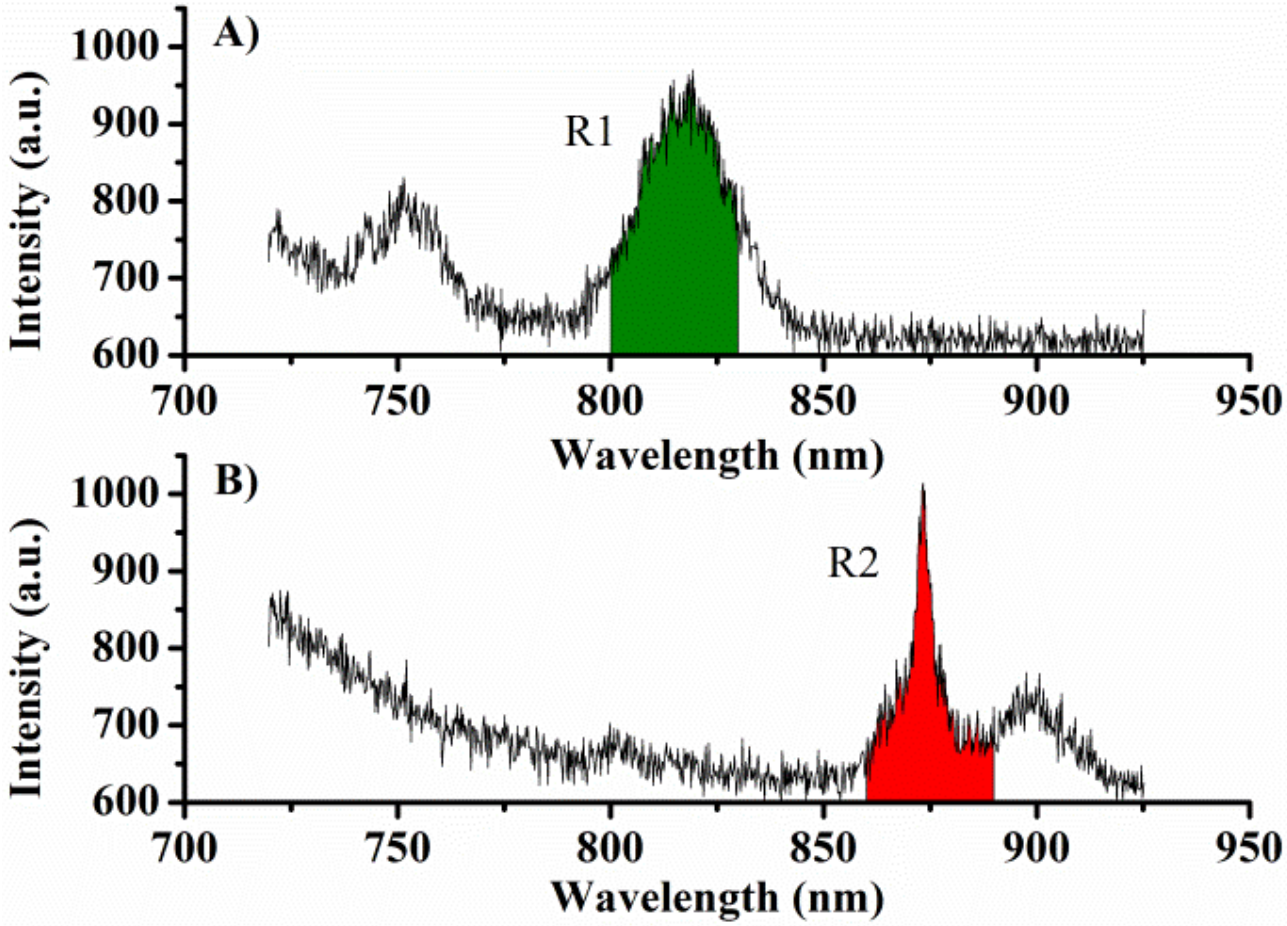
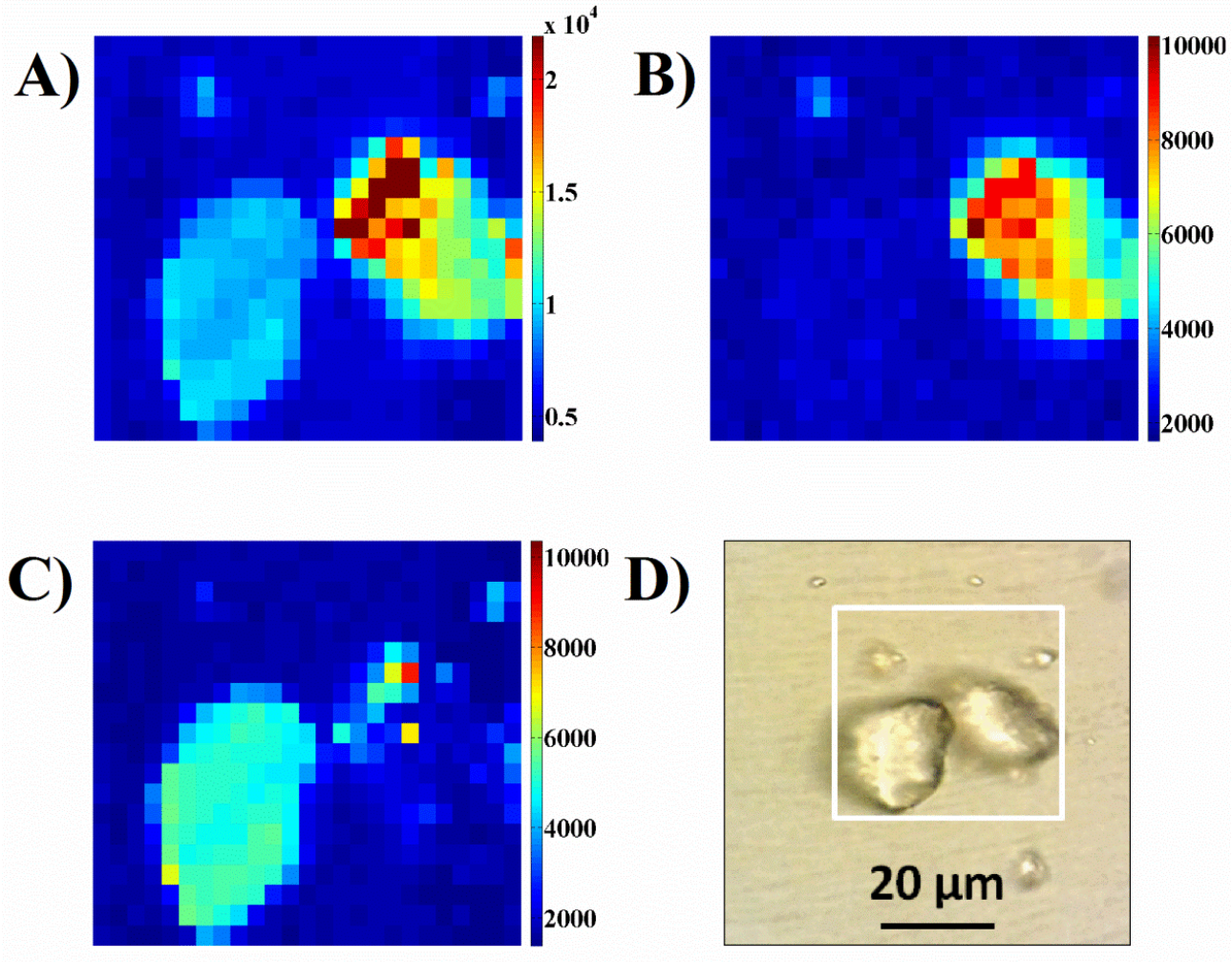


Figure SI13. Representative of spectra from the images in Figure SI12. A) Sum of 6 spectra (5 s/spectrum) from the particle shown in Figure SI12B. B) Sum of 6 spectra (5 s/spectrum) from the particle shown in Figure SI12C.

Additional examples of spectral based imaging of two different Lanthanides.

The examples shown below were taken under identical conditions (5 s integration time per pixel, N_2 was flown over sample during the scan etc), and the treatment to the images are all the same, as described above (figure SI 11). The resolution of the images is specified in each figure description.

Example 1



FigureSI 14. 40 x 50 µm (20 x 25 pixels) fluorescence images of silica beads dyed with Eu.**2** and Nd.**1**. A) Image constructed by integrating the intensity in the spectral region R1 (800-830 nm) and R2 (860-890 nm), see figure SI13. B) Image constructed by integrating the intensity in the spectral region R1. C) Image constructed by integrating the intensity in the spectral region R2. D) Optical image of the scan area, marked by the white square.

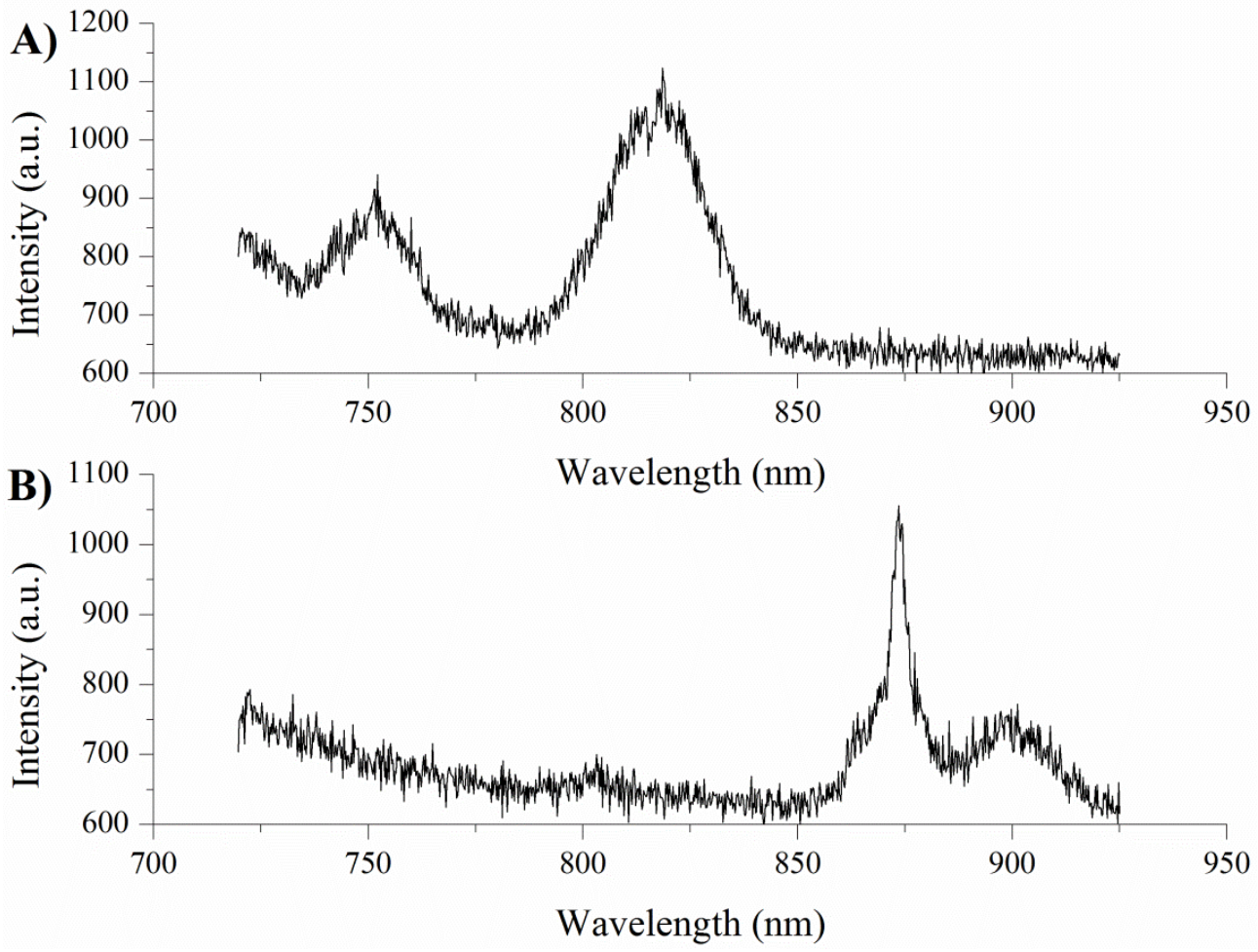


Figure SI15. Representative of spectra from the images in Figure SI14. A) Sum of 6 spectra (5 s/spectrum) from the particle shown in Figure SI14B. B) Sum of 6 spectra (5 s/spectrum) from the particle shown in Figure SI14C.

Example 2

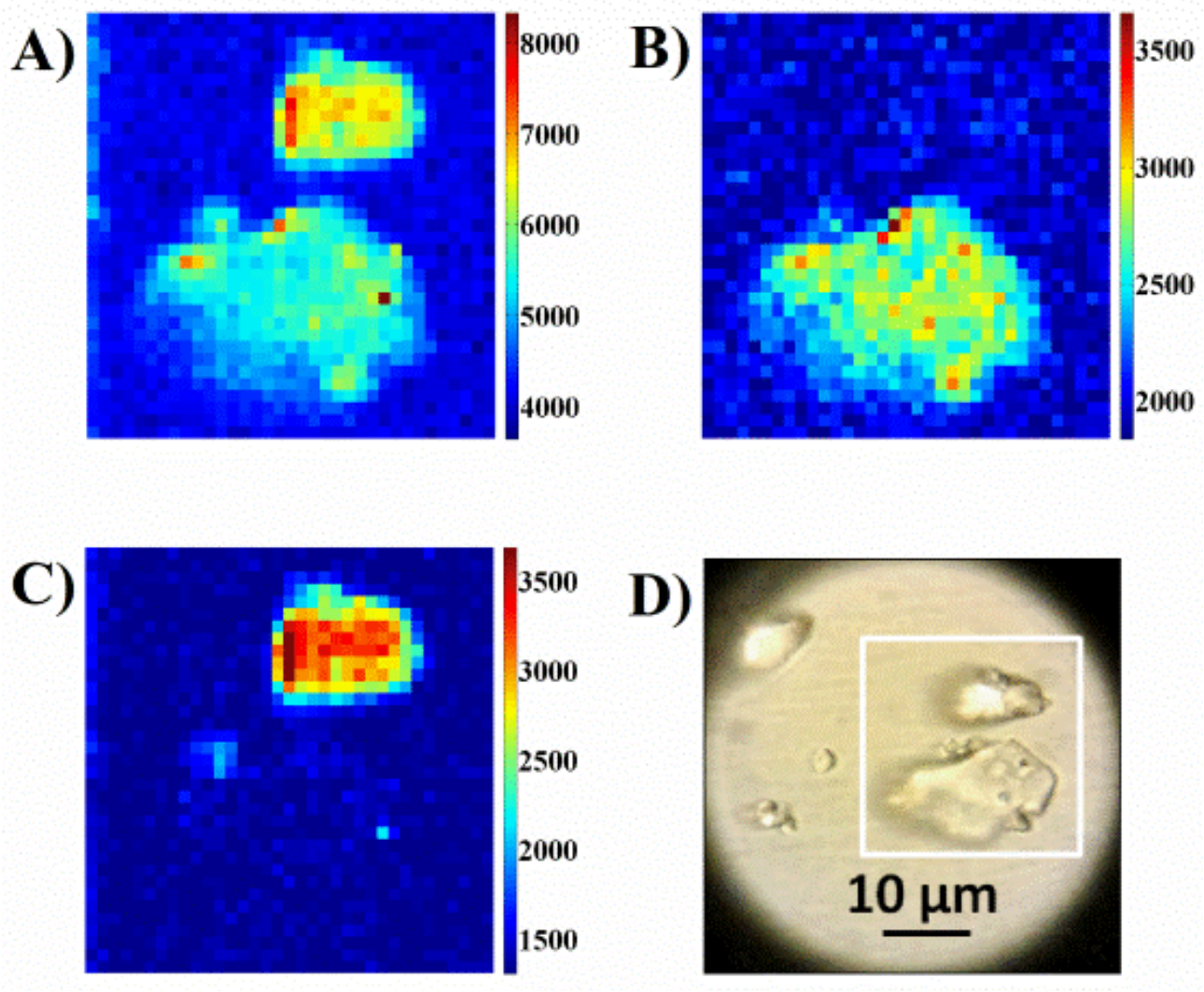


Figure SI16. 35 x 35 µm (35 x 35 pixels) fluorescence of silica beads dyed with Eu.**2** and Nd.**1**. A) Image constructed by integrating the intensity in the spectral region R1 (800-830 nm) and R2 (860-890 nm), see figure SI13. B) Image constructed by integrating the intensity in the spectral region R1. C) Image constructed by integrating the intensity in the spectral region R2. D) Optical image of the scan area, marked by the white square.

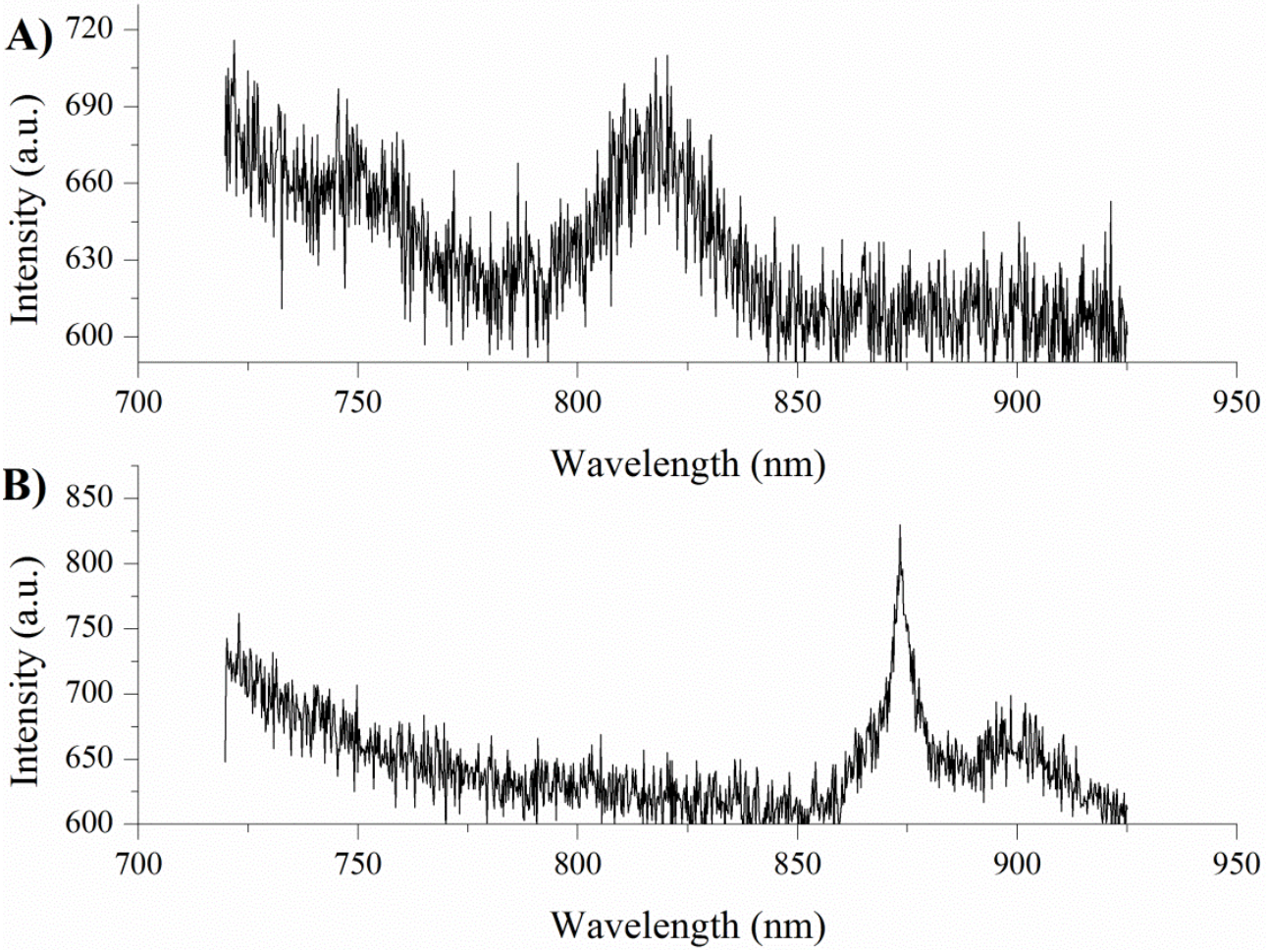


Figure SI17. Representative of spectra from the images in Figure SI16. A) Sum of 6 spectra (5 s/spectrum) from the particle shown in Figure SI16B. B) Sum of 6 spectra (5 s/spectrum) from the particle shown in Figure SI16C.

Example 3

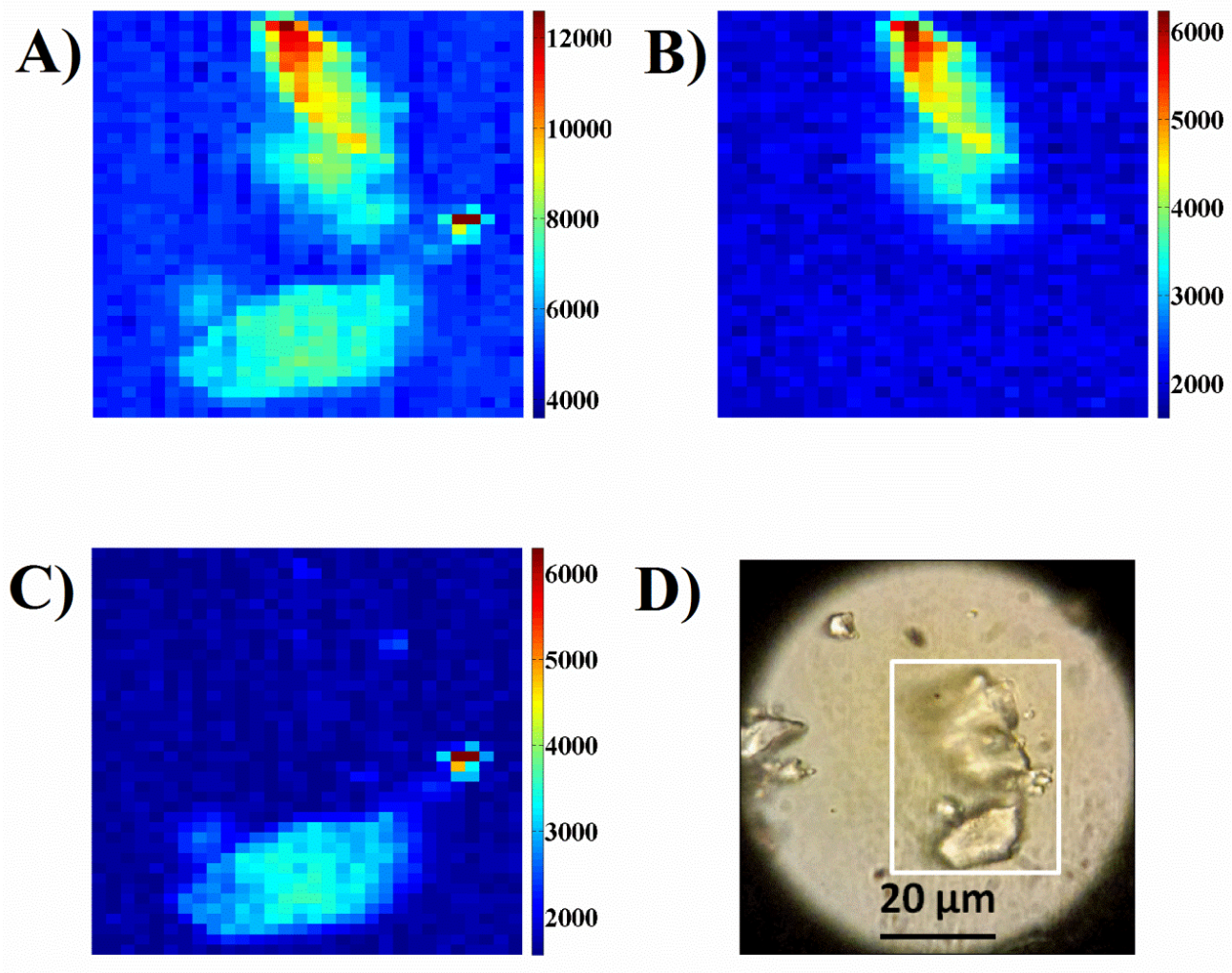


Figure SI18. 30 x 40 µm (30 x 40 pixels) fluorescence images of silica beads dyed with Eu.**2** and Nd.**1**. A) Image constructed by integrating the intensity in the spectral region R1 (800-830 nm) and R2 (860-890 nm), see figure SI13. B) Image constructed by integrating the intensity in the spectral region R1. C) Image constructed by integrating the intensity in the spectral region R2. D) Optical image of the scan area, marked by the white square.

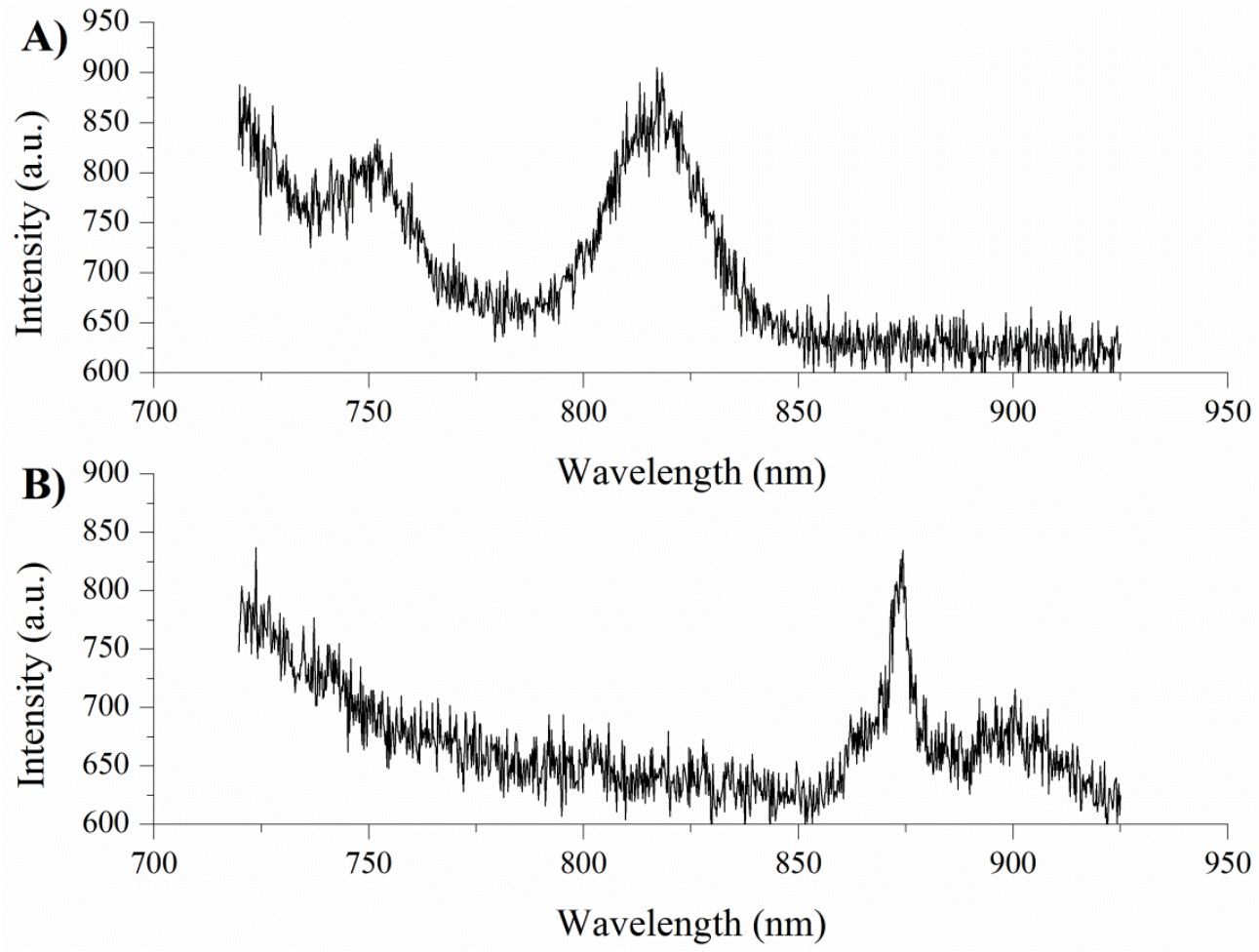


Figure SI19. Representative of spectra from the images in Figure SI18. A) Sum of 6 spectra (5 s/spectrum) from the particle shown in Figure SI18B. B) Sum of 6 spectra (5 s/spectrum) from the particle shown in Figure SI18C.

Example 4

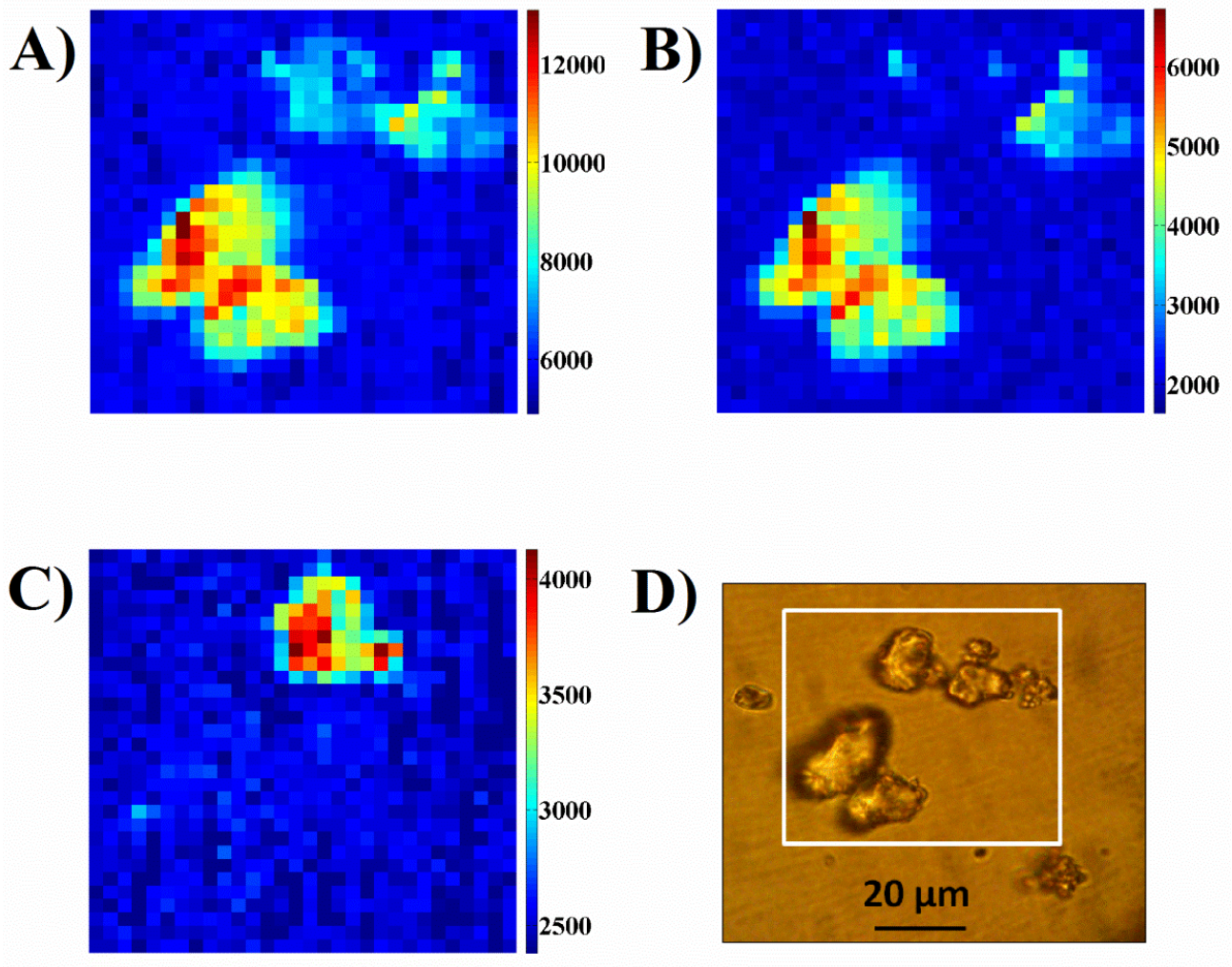


Figure SI20. 30 x 40 µm (30 x 40 pixels) fluorescence images of silica beads dyed with Eu.**2** and Nd.**1**. A) Image constructed by integrating the intensity in the spectral region R1 (800-830 nm) and R2 (860-890 nm), see figure SI13. B) Image constructed by integrating the intensity in the spectral region R1. C) Image constructed by integrating the intensity in the spectral region R2. D) Optical image of the scan area, marked by the white square.

Example 5

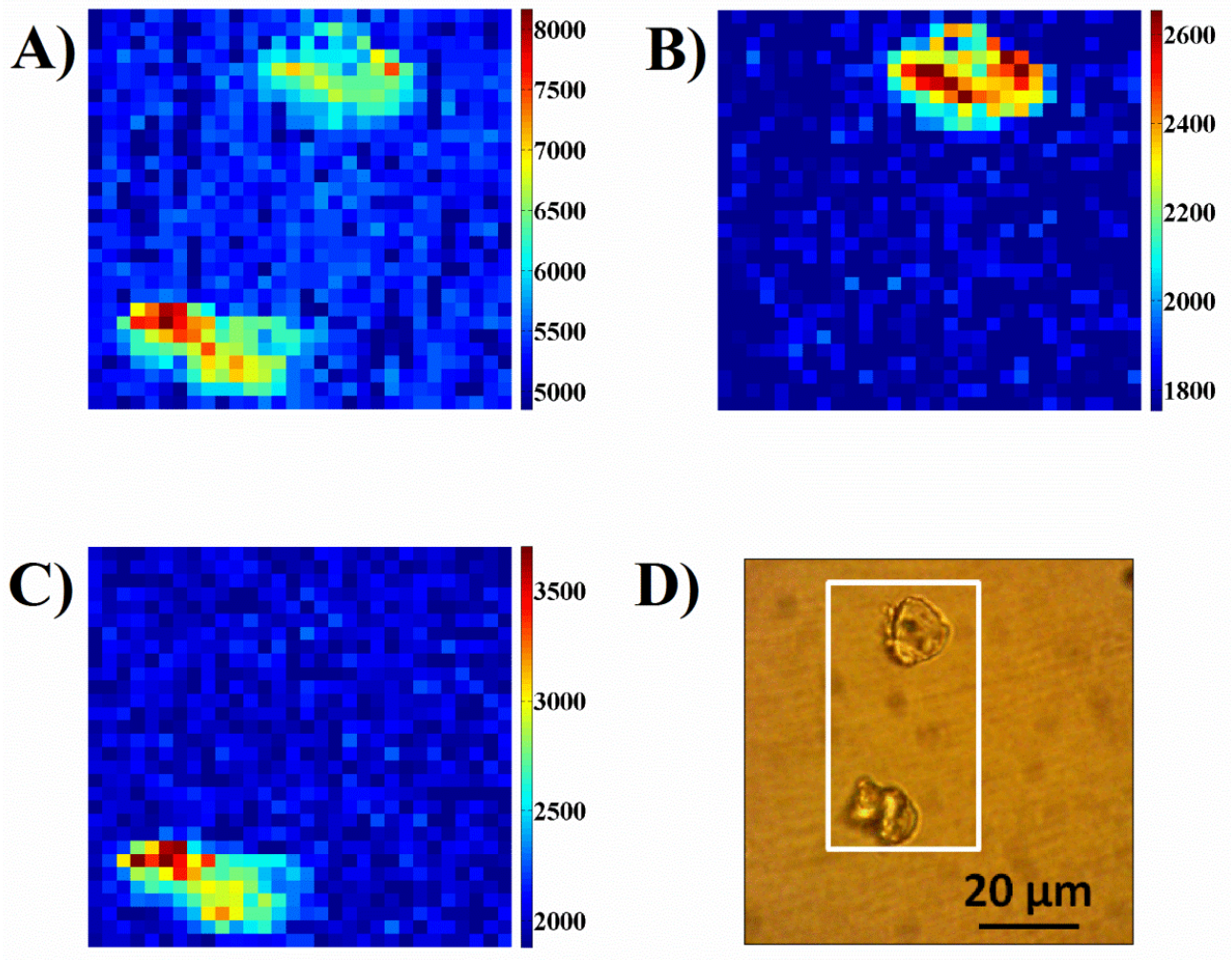


Figure SI21. 30 x 40 µm (30 x 20 pixels) fluorescence images of silica beads dyed with Eu.**2** and Nd.**1**. A) Image constructed by integrating the intensity in the spectral region R1 (800-830 nm) and R2 (860-890 nm), see figure SI13. B) Image constructed by integrating the intensity in the spectral region R1. C) Image constructed by integrating the intensity in the spectral region R2. D) Optical image of the scan area, marked by the white square.

Bleaching curves in air and under nitrogen

Bleaching experiments were carried out with Eu.**2** and Eu.**1** in air under identical conditions (excitation power etc). The dyes were dissolved in deuterated water and mixed with silica particles seperately. After evaporation of deuterated water, the dye absorbed on the partilces, and the bleaching experiments were done with the silica particles on the mircoscope. Each decay curve shown in Figure SI23 represents the average extracted from 5 measurements.

The bleaching experiment was performed using experimental conditions identical to those used for imaging, see above.

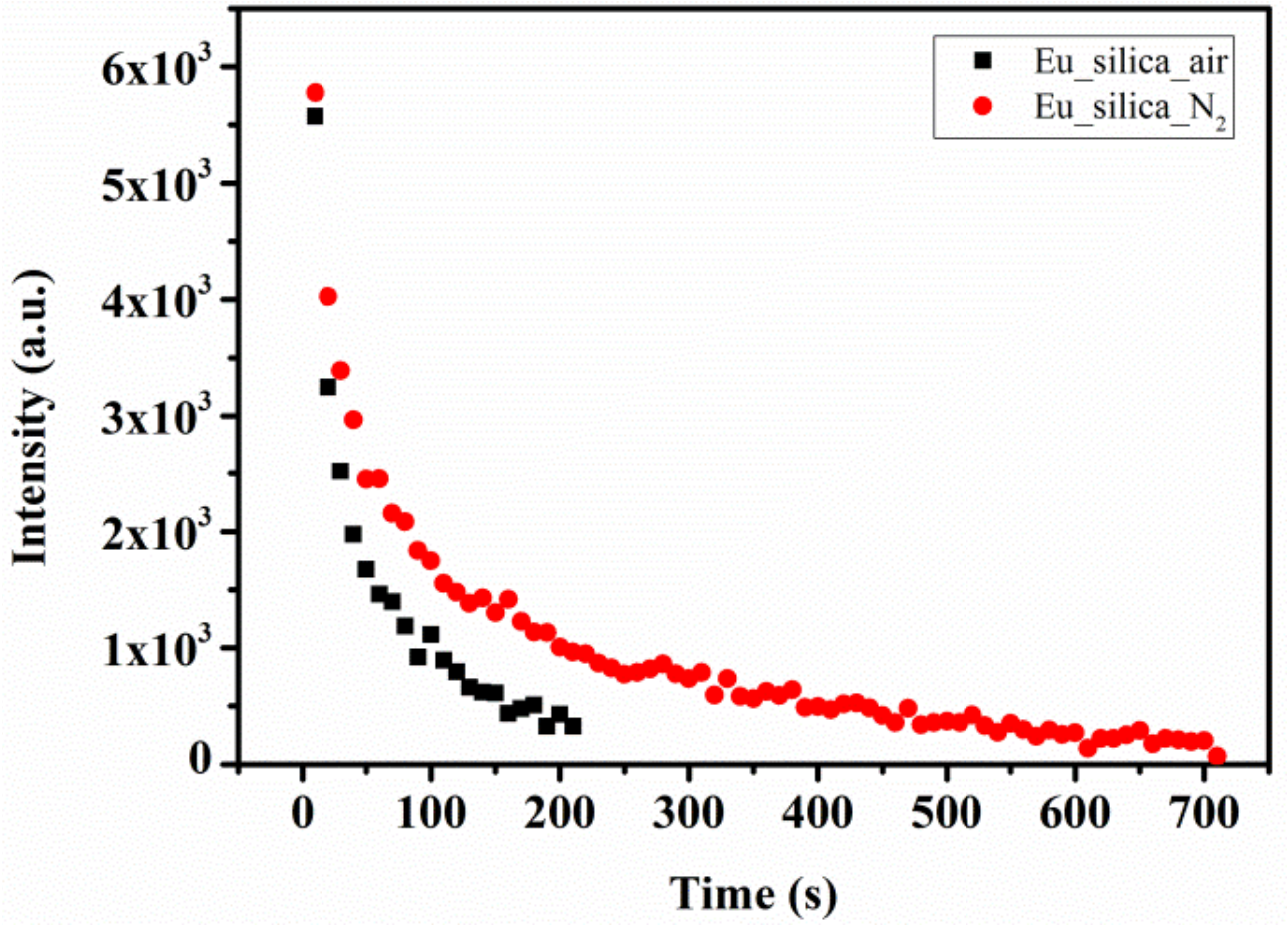


Figure SI22. Bleaching curves of Eu.**2** on silica beads in air (black) and with N_2 flow (red). Each point in the curves represents the intensity integrated in the spectral region R1. The time interval between each point is 10 s.

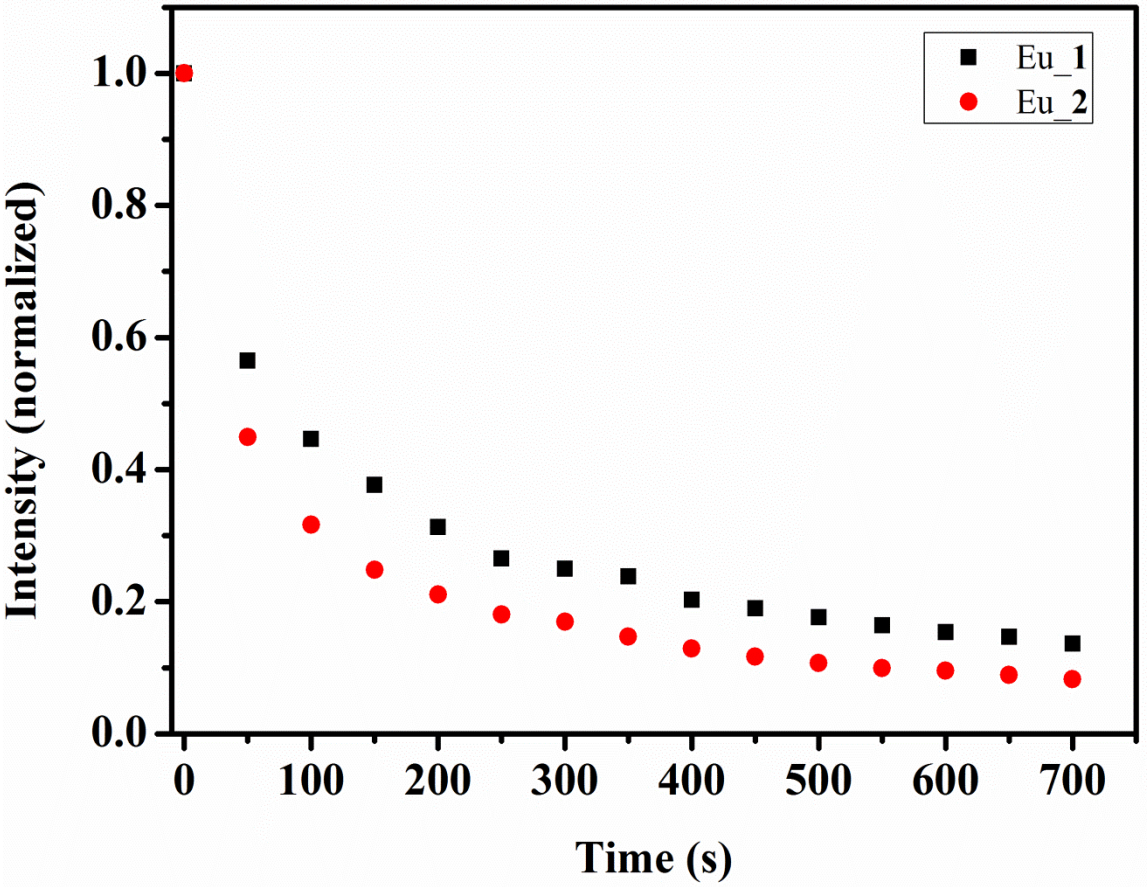


Figure SI23. Normalized bleaching curves of Eu.**1** (black) and Eu.**2** (red) on silica beads in air. Each point in the curves represents the intensity integrated in the spectral region R1. The time interval between each point is 50 s.

References

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2. Dadabhoy, A.; Faulkner, S.; Sammes, P. G., Long wavelength sensitizers for europium(iii) luminescence based on acridone derivatives. *Journal of the Chemical Society, Perkin Transactions 2* **2002,** (2), 348-357.