Electronic supporting information for:

Time Resolved Confocal Microscopy using Lanthanide Centred near-IR Emission

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Contents

Methods and materials

Lanthanide complexes

The lanthanide used in this work are shown in figure S1, and are ytterbium (Yb.**1**) and neodymium (Nd.**1**) complexes of 1,4,7-Tris(carboxylmethyl)-10-(4'-azido-2-acetophenone)-1,4,7,10-tetraazacyclododecane (H$_3$.**1**), see references 1 and 2.
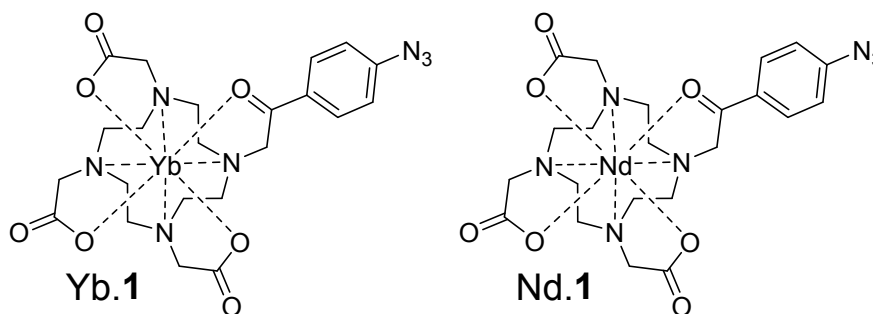

Figure S1. Lanthanide complexes used in this work.

Dyeing of silica particles

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 good mixing. The powders in ether were stirred and left to dry up for further use in microscopy.
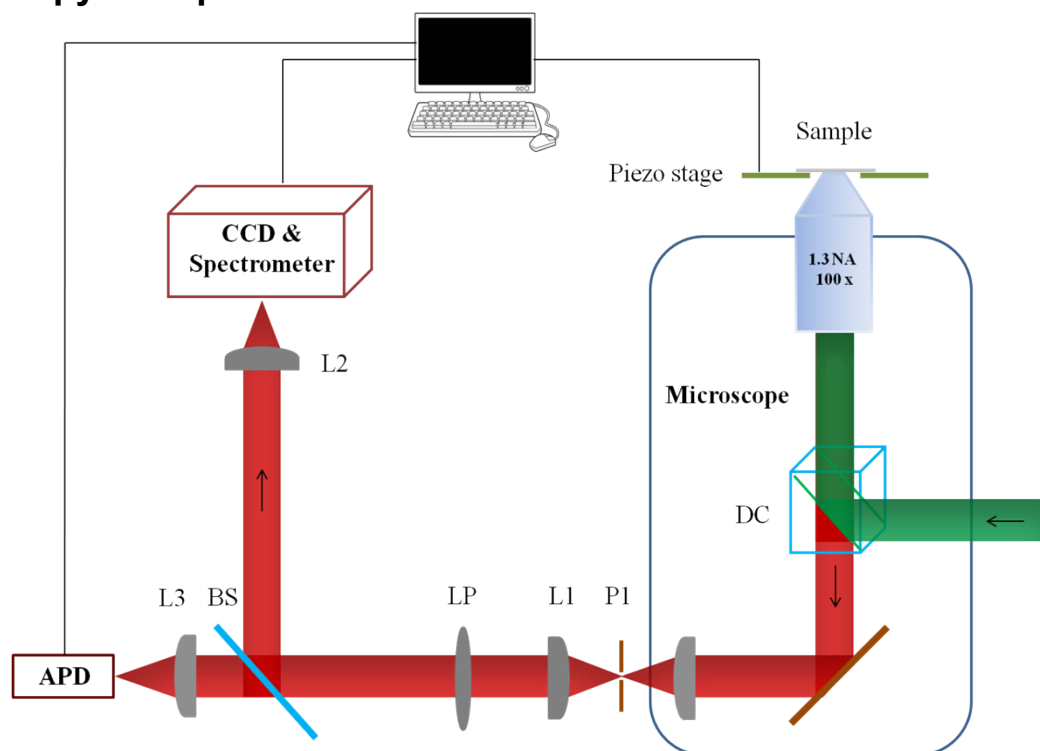
Microscopy set-up

Figure S2. Microscope configuration. Abbreviations: DC, dichroic mirror; P1, pinhole; L1, L2 and L3, lenses; LP, long pass filter; BS, beam splitter; APD, avalanche photo diode; CCD, charge coupled device, adapted from references 1 and 3.

The confocal microscopy set-up depicted in Figure SI2 was used for the imaging. The experiments were carried out on a home-built scanning fluorescence confocal microscopy system based on an Olympus IX71 inverted microscope. A pulsed laser (LDH-P-C-375) from PicoQuant was used as excitation source. The excitation light was separated from the emission by a long-pass filter (Semrock, BLP01-458R-25). Areas with well-separated particles were imaged point by point in a raster scanning fashion. A piezo-driven scanning stage (Physik Intrumente P5173CL) 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 an avalanche photodiode (APD, Perkin-Elmer CD3226) connected to counting electronics (Becker & Hickl SPC-830) for imaging or to a spectrometer for recording of spectra (Princeton Instruments SPEC-10:100B/LN_eXcelon CCD camera, SP 2356 spectrometer with 1-030-500 grating 300 g/mm @ 500 nm).

Converting the TC-SPC photon stream to images

To create the total intensity and PArTI images from the FIFO data files, a sample with well defined holes was employed as reference for calculating the dwell time of the piezo stage between pixels and lines. The holes are well separated from each other with a distance of 4 μm, and filled with a bright fluorescent organic dye, see the fluorescence intensity image in Figure S3. A 10 x 10 μm area of the sample was scanned, and the photons were recorded using time-correlated single photon counting (TC-SPC) hardware (SPC-830 from Becker & Hickl). The TC-SPC hardware allows us to record two times for every photon. The macrotime, which is related to the time from the start of the experiment and the microtime, which is the arrival time with respect to the next laser pulse. Figure S4 shows an example of the number of photons binned in a 1 second interval from the first 200 seconds. This graph is constructed by using the macrotime of each photon.
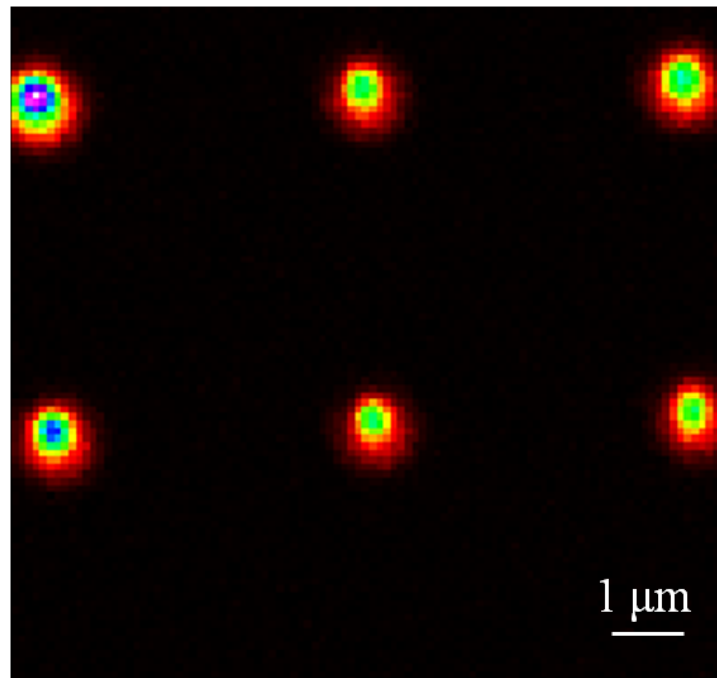

Figure S3. Fluorescence intensity image of the well-defined holes filled with an fluorescent dye.

The TC-SPC hardware was started before starting the scanner. The period before the scanner starts and the time it takes to move to the fist pixel can be easily derived from Figure S4, since the line scans should give a periodic time (T), the starting point of the measurement can hence be found and is indicated as t_0. The period time T consists of two parts, t_1 and t_2, which are the time of scanning from left to right (t_1) and the dwell time (t_2) when the stage moves back to the left starting a new line, respectively. Therefore, there will be more data points recorded than actual pixels. Figure S5 shows the intensity images of a 5 x 5 μm area constructed with the line scan time of T and t_1, respectively. The images presented in the paper used only t_1.
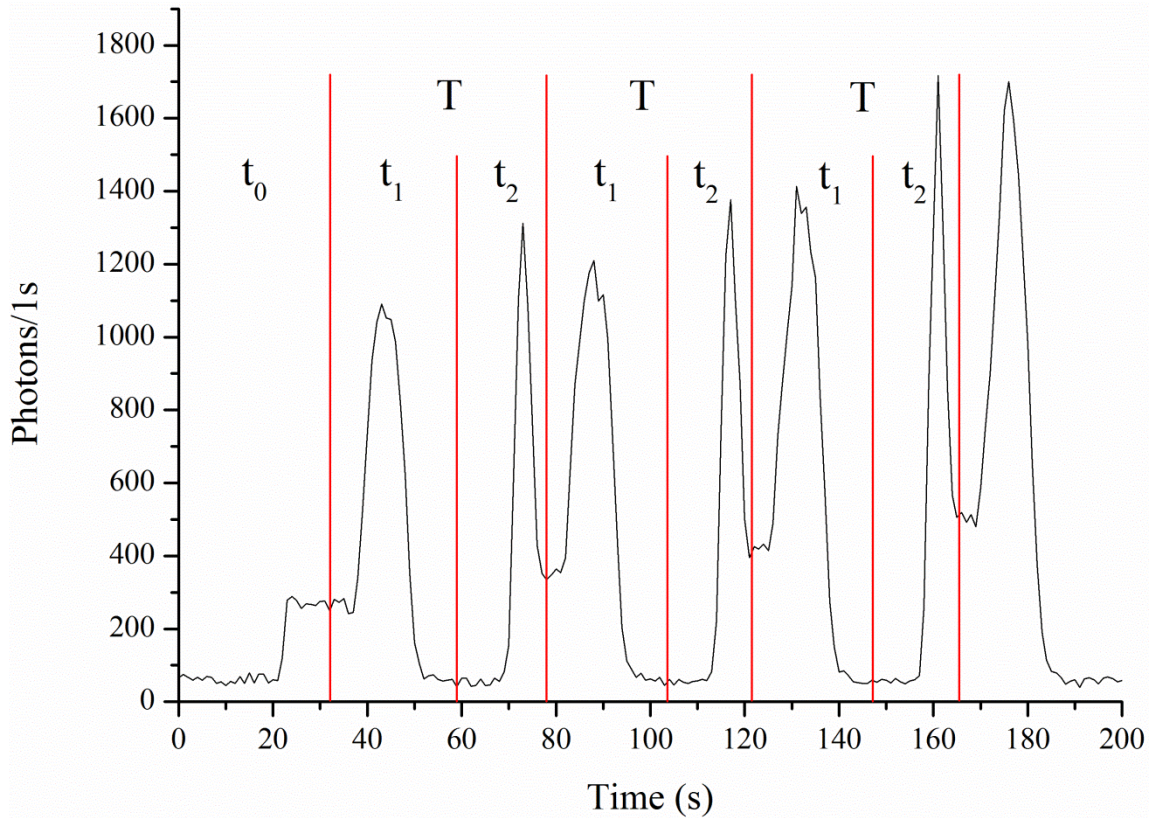
Figure S4. Number of photons detected as a function of time for the first 200 seconds of the experiment (1 second bins).
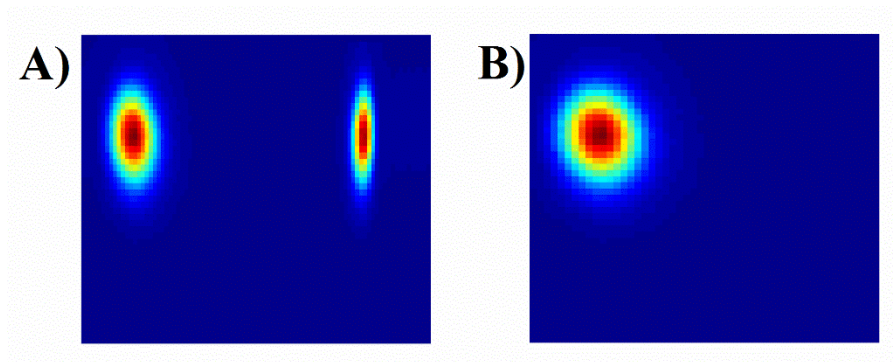

Figure S5. A) Intensity image constructed with line scan time of T, 88 x 50 pixels. B) Intensity image constructed with line scan time of t_1, 50 x 50 pixels.

Data analysis

Once the period t_1 is established, this part of the photon trace can be divided in the right number of pixels. As a result of this, we know now from the macro time which photons belong to which pixel in our final image. Now that each photon has an X and Y pixel label, we can use the microtimes of these specific photons to create the PArTI images, which is done by using a home-written MATLAB routine. The PArTI images are built using the RGB color model (see Figure S6), and the colors of red, green and blue represent the photons arriving in different micro time ranges, as indicated in Figure S10B for example. For each pixel in the image of Figure S10 A, if the photons collected in a pixel are dominant by those with arrival time in the red range, the color will be fairly red for this pixel. Similarly the color will be fairly green if most of the photons collected in this pixel arrived in the time frame of the green range. Since only two dyes with different fluorescence decay times presented, the third color 'blue' is defined as the background (dark counts). The dark counts in these three selected ranges are equal, so the color of the background will be 'white' or 'black', depending on the total counts of photons, which determines the brightness of the pixels. The more counts of photons, the brighter the color. To make the brightness of green and red color even, the red range is defined broader than the green, so that the total counts of photons will be similar. To sum up, the arrival time of photons defines the color of pixels in the time-gated image, and the photon number in the selected range in each pixel determines the brightness.
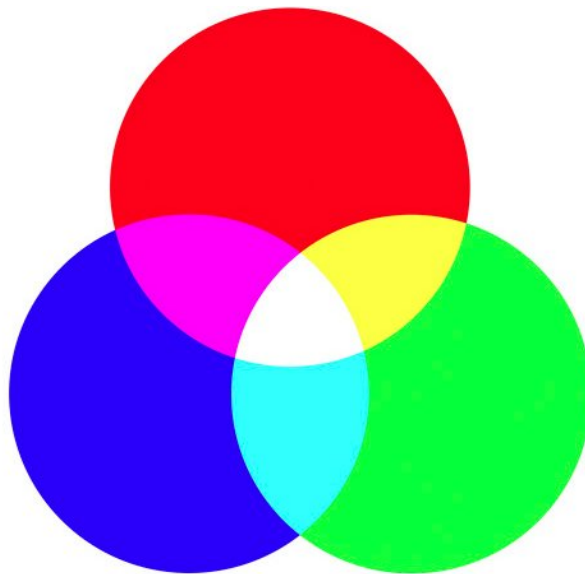

Figure S6. The RGB (red-green-blue) colour scale used in the images. This colour scheme is limited to imaging three co-localising probes in a single image. Note the colours occurring upon co-localisation.
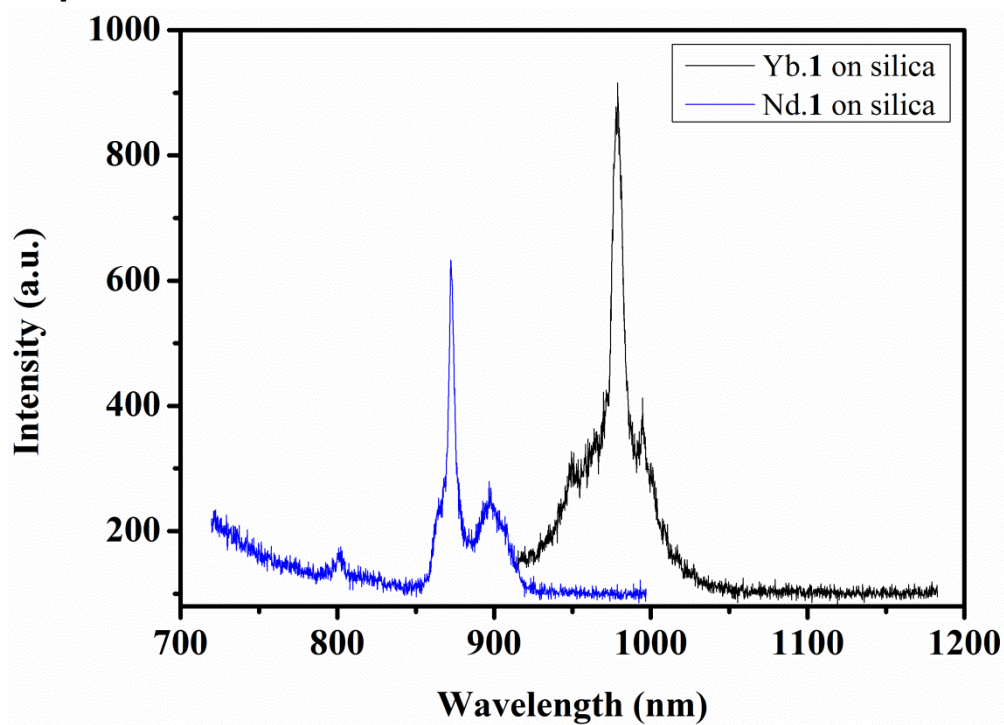
Emission spectra

Figure S7. Emission spectra from silica particles dyed with Nd.**1** (blue, 10s) and Yb.**1** (black, 50s).

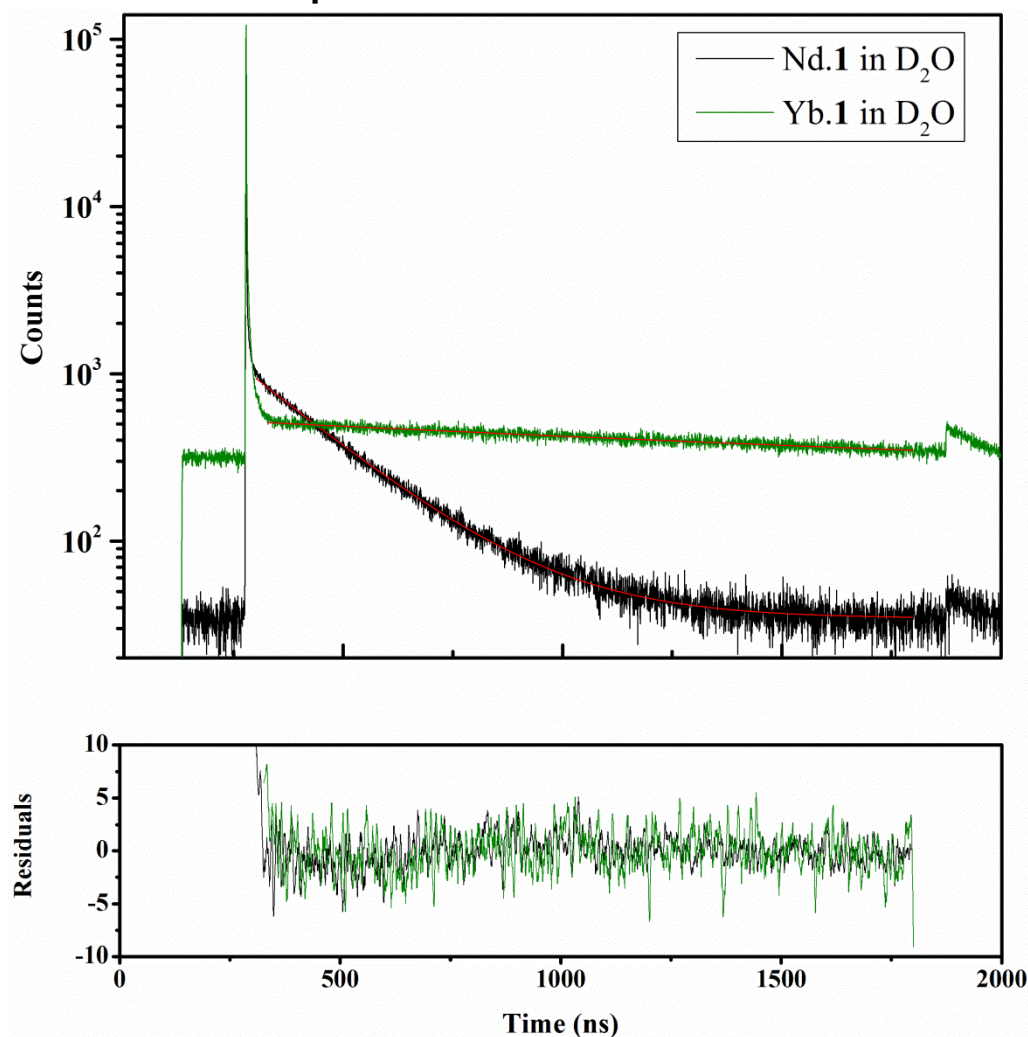## Time-resolved emission profiles

Figure S8. Time-correlated single photon counting (TC-SPC) measurements for compound Nd.**1** (black, 660 s integration time) and compound Yb.**1** (green, 1400 s integration time) in D_2O. The excitation source was a 375 nm pulsed laser from PicoQuant, operated at 5×10^5 Hz. Nd.**1** and Yb.**1** decayed bi-exponentially in D_2O, with a time constant of 0.203 μs and 2.24 μs for the long component, respectively. Both the criterion values (χ^2) for the tail fitting are below 1.1.
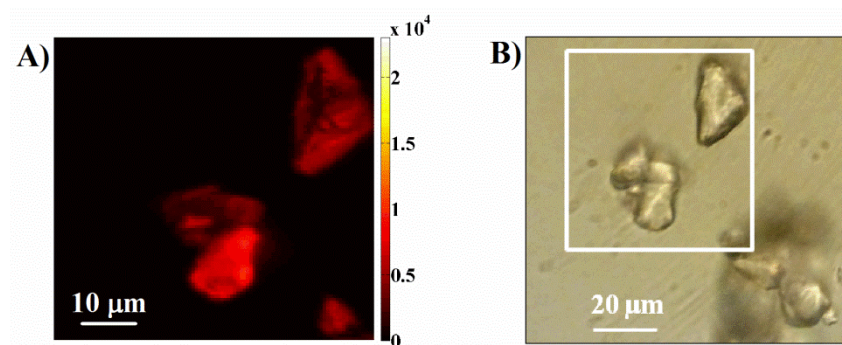
Additional examples

Example 1:

Figure S9. (A) Intensity image (100 x 100 pixels) and (B) optical transmission image of silica particle mixture dyed with lanthanide complexes Nd.**1** and Yb.**1**. The construction of intensity image is based on the photon stream recorded by the TCSPC during the scan over the sample. The white square in Figure S9B marks the scanning area, which is 60 x 60 μm.
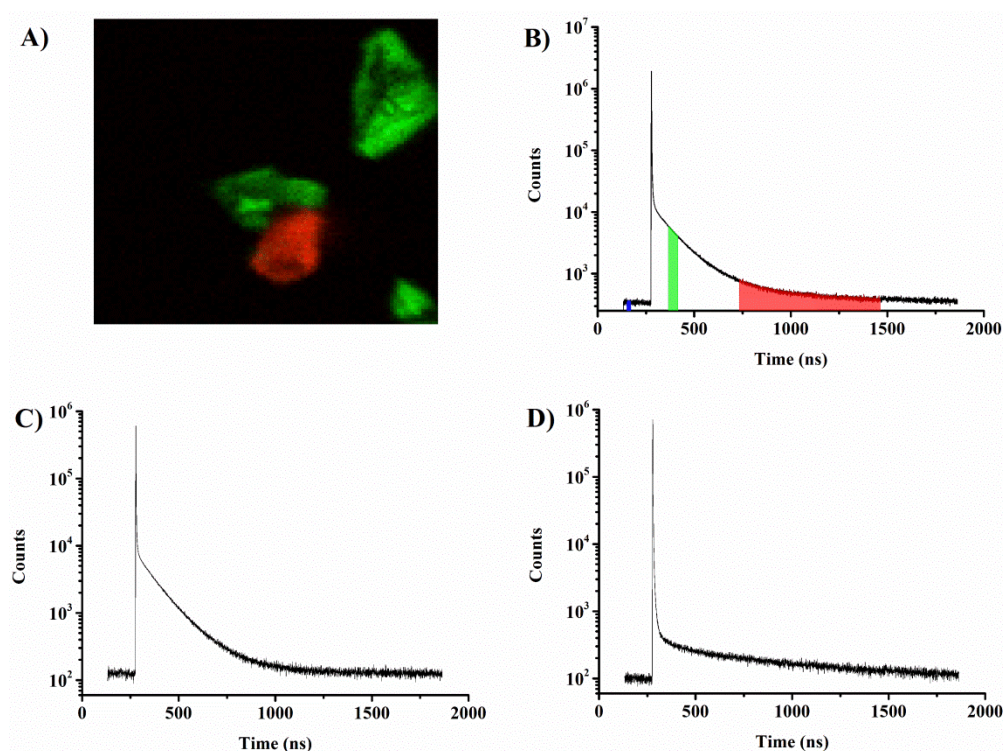

Figure S10. (A) Time-gated image (100 x 100 pixels) of the sample depicted in Figure S9, the silica particles dyed with Nd.**1** and Yb.**1** can be distinguished by the color. (B) Fluorescence decay recorded by TCSPC during the scan. Filled areas under the curve show the ranges of photons arrival time that selected for construction of Figure S10 A. (C) Fluorescence decay extracted from the total photon stream, containing only photons from areas in the image that were identified to contain compound Nd.**1**. (D) Fluorescence decay extracted from the total photon stream, containing only photons from areas in the image that were identified to contain compound Yb.**1**.
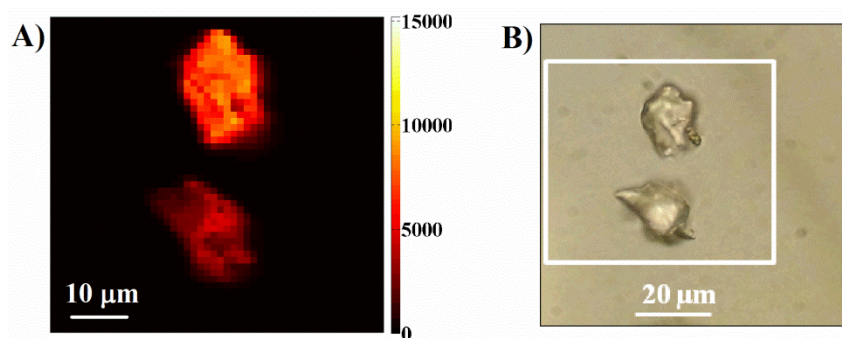
Example 2:

Figure S11. (A) Intensity image (50 x 50 pixels) and (B) optical transmission image of silica particle mixture dyed with lanthanide complexes Nd.**1** and Yb.**1**. The construction of intensity image is based on the photon stream recorded by the TCSPC during the scan over the sample. The white square in Figure S11 B marks the scanning area, which is 60 x 60 μm.
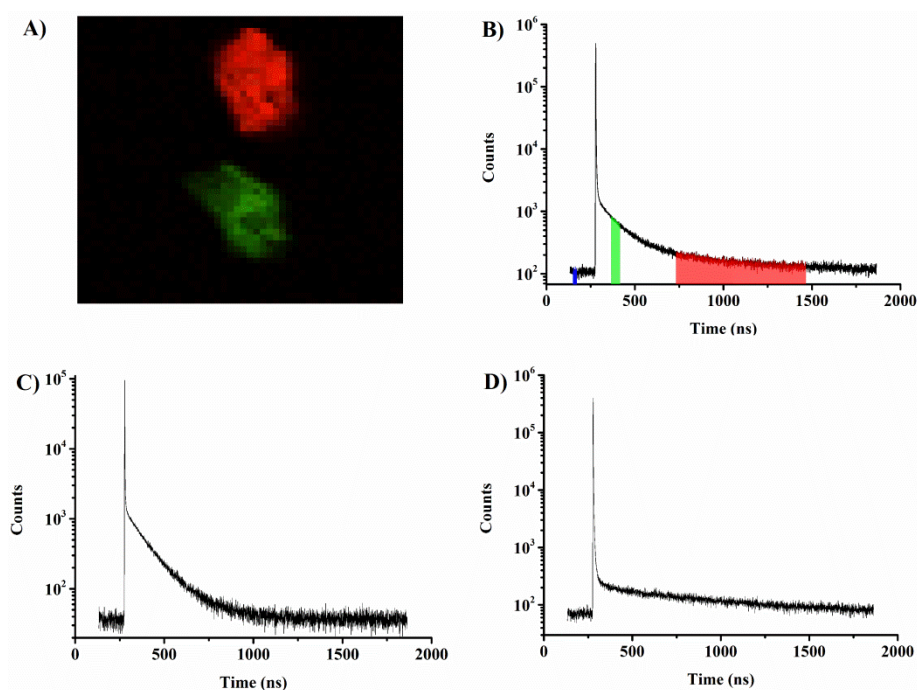

Figure S12. (A) Time-gated image (50 x 50 pixels) of the sample shown in Figure S11, and silica particles dyed with Nd.**1** and Yb.**1** can be distinguished by the color. (B) Fluorescence decay recorded by TCSPC during the scan. Filled areas under the curve show the ranges of photons arrival time that selected for construction of Figure S12 A. (C) Fluorescence decay extracted from the total photon stream, containing only photons from areas in the image that were identified to contain compound Nd.**1**. (D) Fluorescence decay extracted from the total photon stream, containing only photons from areas in the image that were identified to contain compound Yb.**1**.
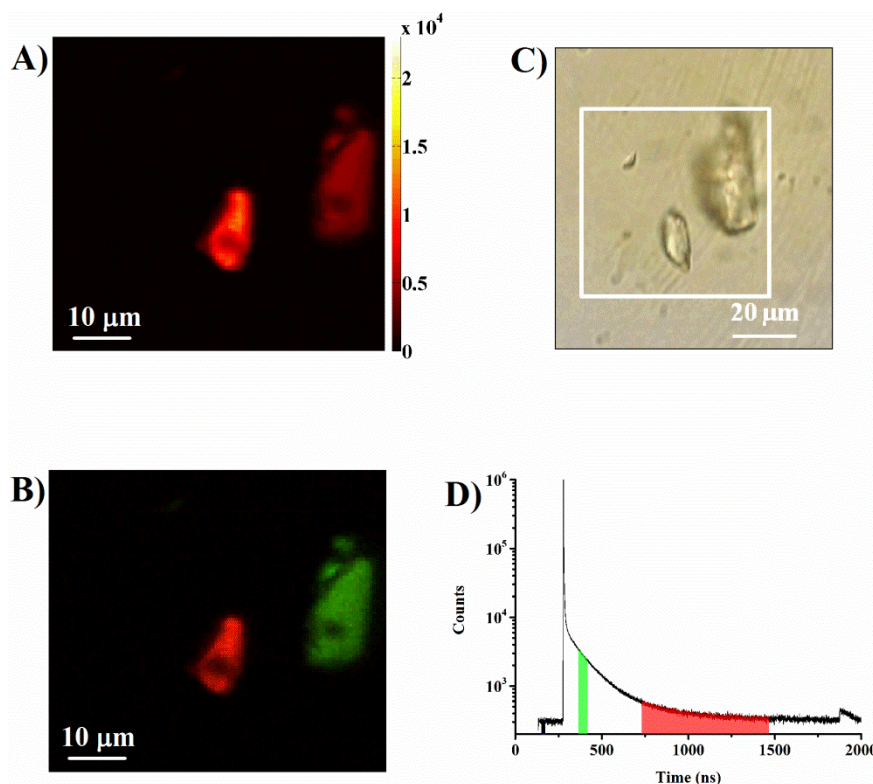
Example 3:

Figure S13. A) Intensity image (100 x 100 pixels), B) Time-gated image (100 x 100 pixels) and C) optical transmission image of silica particles dyed with Nd.**1** and Yb.**1**. The white square in image C marks the scanning area, which is 60 x 60 μm. D) Fluorescence decay curve recorded during the scanning by TCSPC. Filled areas under the curve show the arrival time ranges of photons that selected for construction of image B.
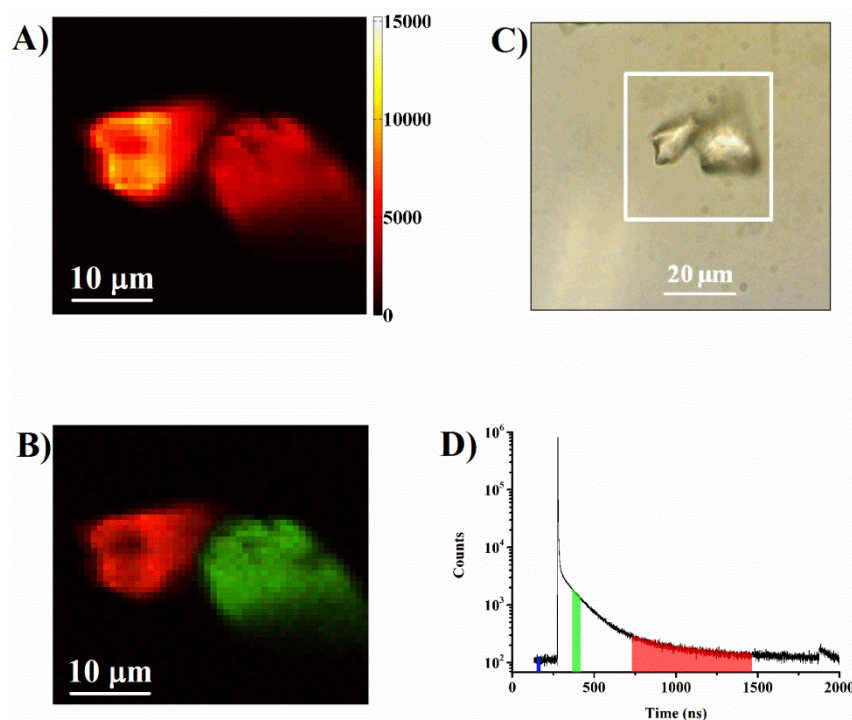
Example 4:

Figure S14. A) Intensity image (50 x 50 pixels), B) Time-gated image (50 x 50 pixels) and C) optical transmission image of silica particles dyed with Nd.**1** and Yb.**1**. The white square in image C marks the scanning area, which is 40 x 40 μm. D) Fluorescence decay curve recorded during the scanning by TCSPC. Filled areas under the curve show the arrival time ranges of photons that selected for construction of image B.

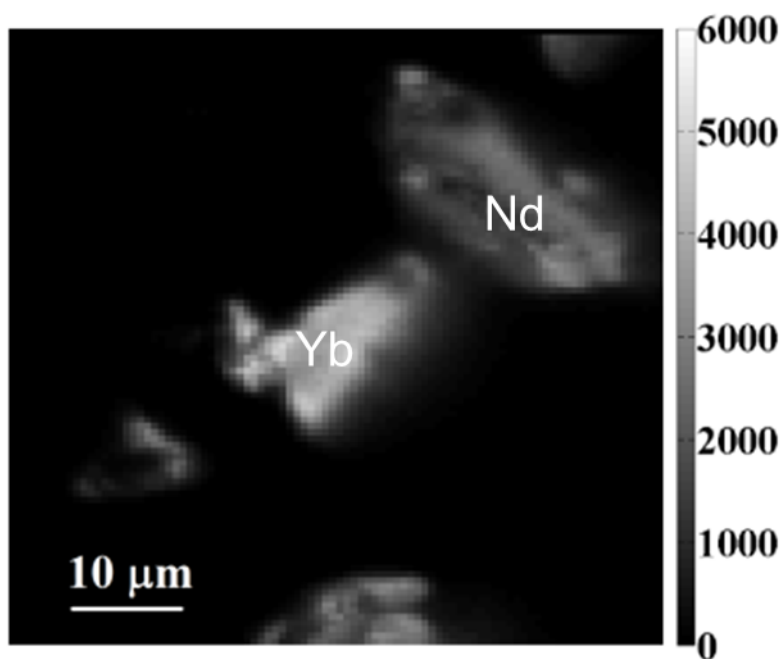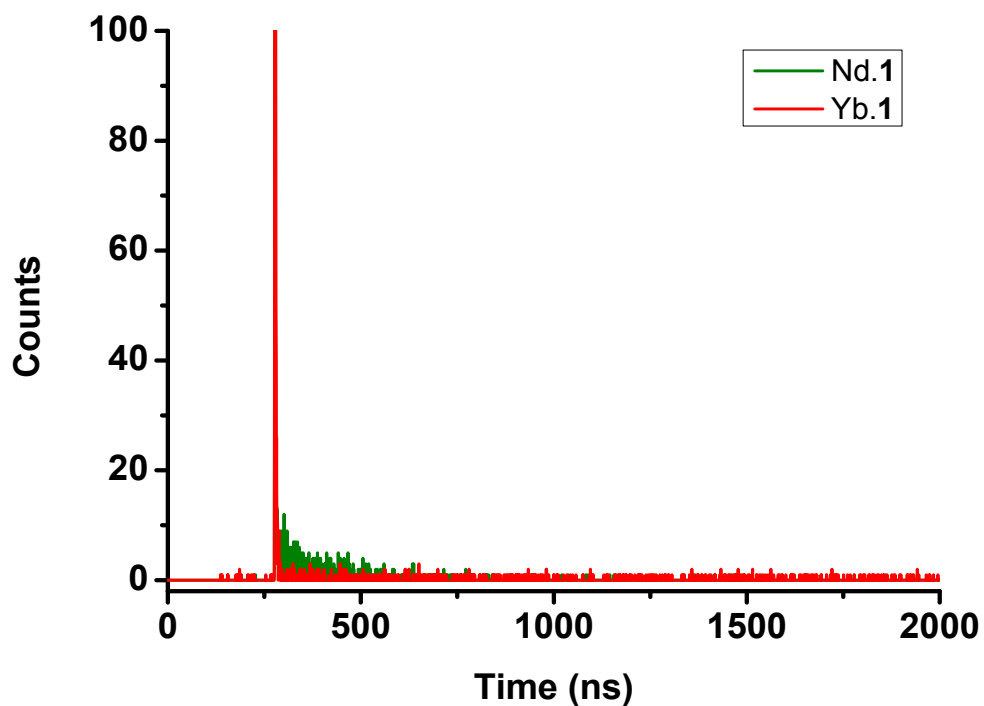## Time-resolved emission decay profile from a single pixel

Figure S15. Fluorescence decay curves (top) based on the photons collected in one pixel. The pixels are taken from the particles labelled with Nd and Yb (bottom) in the image that correspond to Figure 2 in the main document.
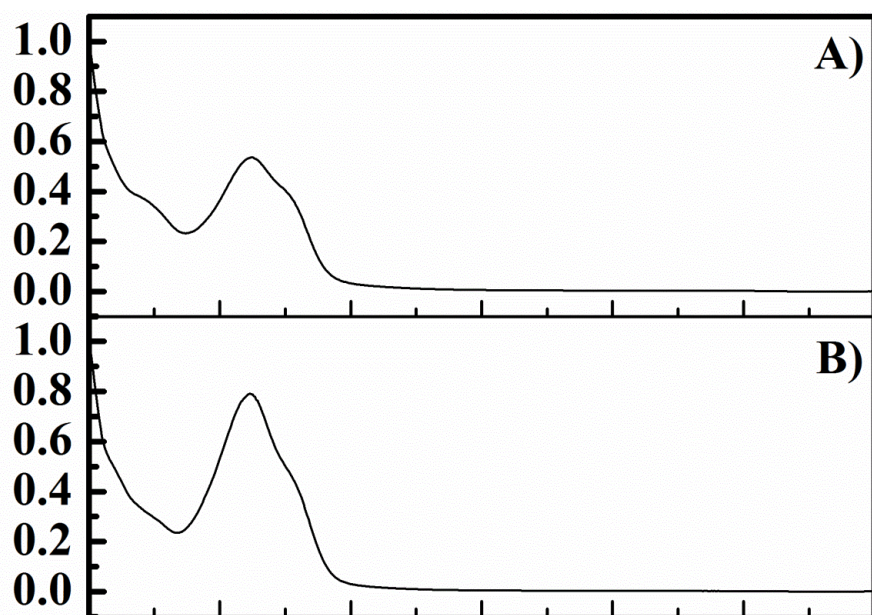
Absorption spectra in D$_2$O solution

Figure S16. Normalised absorption spectra of A) Yb.1, B) Nd.1 dissolved in D$_2$O.

References

1.   Z. Liao, M. Tropiano, K. Mantulnikovs, S. Faulkner, T. Vosch and T. Just Sørensen, *Chemical Communications*, 2015, **51**, 2372-2375.

2.   M. Tropiano and S. Faulkner, *Chem Commun (Camb)*, 2014, **50**, 4696-4698.

3.   Z. Liao, E. N. Hooley, L. Chen, S. Stappert, K. Müllen and T. Vosch, *Journal of the American Chemical Society*, 2013, **135**, 19180-19185.