Electronic Supplementary Information

A bright and responsive europium probe for determination of pH change within the endoplasmic reticulum

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General

Chemicals were purchased from commercial suppliers (Acros, Aldrich, Fluka) and were used without further purification unless otherwise stated. Solvents were dried using an appropriate drying agent when required (CH_3CN over CaH_2, CH_3OH over $Mg(OMe)_2$ and THF over Na/benzophenone). Unless otherwise mentioned, reactions were carried out under an argon atmosphere and the reaction flasks were pre-dried under reduced pressure. Ultra pure de-ionised water (<18 MΩ cm-1) was used throughout. All glassware was washed with acid solution and rinsed with de-ionized, distilled water.

Mass Spectrometry and NMR Spectroscopy

^{1}H, ^{13}C and ^{31}P NMR spectra were recorded in commercially-available deuterated solvents on a Varian Mercury-200 (^{1}H at 199.975 MHz, ^{13}C at 50.289 MHz), Varian Mercury-400 or Bruker Avance-400 (^{1}H at 399.960 MHz, ^{13}C at 100.572 MHz, ^{31}P at 161.943 MHz), Varian Inova-500 (^{1}H at 499.772 MHz, ^{13}C at 125.671 MHz) or Varian VNMRS-700 (^{1}H at 699.731 MHz, ^{31}P at 283.256 MHz) spectrometer. All chemical shifts are given in ppm and coupling constants are in Hz. Electrospray mass spectra were recorded on a Waters Micromass LCT or Thermo-Finnigan LTQ FT instrument operating in positive or negative ion mode as stated, with methanol as the carrier solvent. Accurate mass spectra were recorded using the Thermo-Finnigan LTQ FT mass spectrometer.

Chromatography

Flash column chromatography was performed using flash silica gel 60 (230 - 400 mesh) from Merck. Thin layer chromatography (TLC) was performed on aluminium sheet silica gel plates with 0.2 mm thick silica gel 60 F_{254} (E. Merck) using different mobile phase. The compounds were visualized by UV irradiation (254 nm).

Reverse phase HPLC traces were recorded at 298 K using a Perkin Elmer system equipped with a Perkin Elmer Series 200 Pump, a Perkin Elmer Series 200 Auto-sampler and a Perkin Elmer Series 200 fluorescence detector. A 4.6 x 150 mm 4µm Phenomenex Synergi Fusion RP 80Å analytical column was used. For purification of complex [**EuL^{1}**] a solvent system composed of H_2O + 0.1% HCOOH/methanol + 0.1% HCOOH was used over the stated linear gradient.

Optical Spectroscopy

UV/Vis absorbance spectra were recorded on a Perkin Elmer Lambda 900 UV/Vis/NIR spectrometer using FL Winlab software). Emission spectra were recorded on an ISA Joblin-Yvon Spex Fluorolog-3 luminescent spectrometer using DataMax v2.20 software). Lifetimes were measured on a Perkin Elmer LS55 luminescence spectrometer using FL Winlab Molecular Spectroscopy Version 4.00.02 software. Each sample was contained in quartz cuvettes with a path length of 1cm. Measurements were recorded at 298 K. Generally, an integration time of 0.5 seconds, increment of 0.5 nm and excitation and emission slits of 2.5 and 1.5 nm respectively were used. Quantum yield measurements were calculated by comparison with two standards. For the standards and each of the unknowns, five solutions with absorbance values between 0.05 and 0.1 were used. The quantum yield was calculated according to the equation:

$$\Phi_x = \Phi_r \cdot \frac{A_r}{A_x} \cdot \frac{E_x}{E_r} \cdot \frac{I_r}{I_x} \cdot \frac{\eta_x^2}{\eta_r^2}$$

where *r* and *x* refer to reference and unknown respectively; *A* is the absorbance at λ_{ex} *;* *E* is the corrected integrated emission intensity*;* *I* is the corrected intensity of excitation light*;* *h* is the refractive index of solution.

Confocal Microscopy and Cell Spectral Imaging

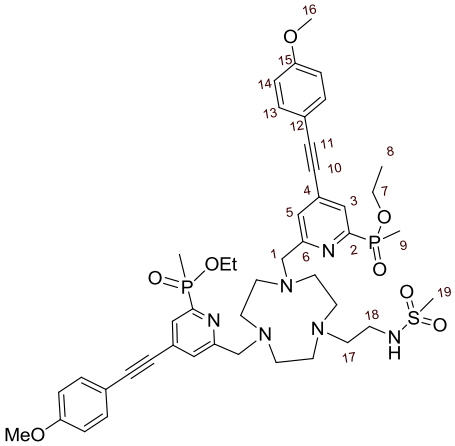
Details of cell culture and assessment of complex toxicity using the MTT assay of mitochondrial redox function have been reported elsewhere. [2,5,9] Cell images and co-localisation experiments were obtained using a Leica SP5 II Laser Scanning Confocal Microscope. In order to achieve excitation with maximal probe emission, the microscope was coupled by an optical fibre to a Coherent 355nm CW (Nd:YAG) laser, operating at 8mW power. A He-Ne or Ar ion laser was used when the commercially available organelle-specific stain ER Tracker Green was used to corroborate cellular compartmentalization. The microscope was equipped with a triple channel imaging detector, comprising two conventional PMT systems and a HyD hybrid avalanche photodiode detector. The latter part of the detection system, when operated in the BrightRed mode, is capable of improving imaging sensitivity above 550 nm by 25%, reducing signal to noise by a factor of 5. The pinhole was always determined by the Airy disc size, calculated from the objective in use (HCX PL APO 63x/1.40 NA LbdBlue), using the lowest excitation wavelength (355 nm). Scanning speed was adjusted to 100 Hz in a unidirectional mode, to ensure both sufficient light exposure and time to collect the emitted light from the lanthanide based optical probes (1024x1024 frame size, a voxel size at x2 digital zoom 120 x 120 x 780 nm). Spectral imaging on this Leica system is possible with the *xyλ*-scan function, using the smallest allowed spectral band-pass (5nm) and step-size (3nm) settings.

However, spectral imaging in cells was achieved using a custom built microscope (modified Zeiss Axiovert 200M), using a Zeiss APOCHROMAT 63x/1.40 NA objective combined with a low voltage 365 nm pulsed UV LED focused, collimated excitation source (1.2W). For rapid spectral acquisition the microscope was equipped at the X1 port with a Peltier cooled 2D-CCD detector (Ocean Optics) used in an inverse 100 Hz time gated sequence. The spectrum was recorded from 400-800 nm with a

resolution of 0.24 nm and the final spectrum was acquired using an averaged 10,000 scan duty cycle. Probe lifetimes were measured on the same microscope platform using a novel cooled PMT detector (Hamamatsu H7155) interchangeable on the X1 port, with the application of preselected interference filters as detailed in the main text (Fig 6). Both the control and detection algorithm were written in LabView2011 where probe lifetime was determined by using a single exponential fitting algorithm to the monitored signal intensity decay. Time gated imaging was achieved using the same 100 Hz sequence as for spectral acquisition using a gated and cooled high resolution and high sensitivity ThorLabs EOS Monochrome CCD camera.

Spectral imaging and lifetime assessments have been carried out at each pH point using pulsed 365 excitation on a population of 20 cells in the field of view using the above detailed modified Zeiss Axiovert 200M microscope and the slides prepared from LSCM imaging. Spectral acquisition was achieved using a modified 2D CCD with a pre determined pulse sequence comprising 1 ms excitation pulse followed by a 10 μs time gate and a subsequent 3 ms acquisition time, where the final spectrum was recorded as an average of 10000 scans to achieve excellent S/N ratio. Lifetimes were recorded from the same population of cells by monitoring long lived (ms regime) fluorescence as a function of time using a triggerable high sensitivity PMT with a 50 us excitation flash per scan. The recorded spectra were used for ratiometric analysis by plotting the ratio of two well defined spectral region of the long lived Eu(III) emission. This was than compared with the ratios calculated from the LSCM images, using previously established contrast transfer function (CTF) calculation methods. [5a,5b]

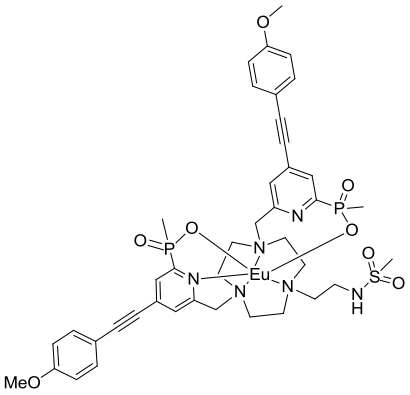
Ligand and Complex synthesis



L[1] The precursor to L[1] has been described in reference 7 (main text)

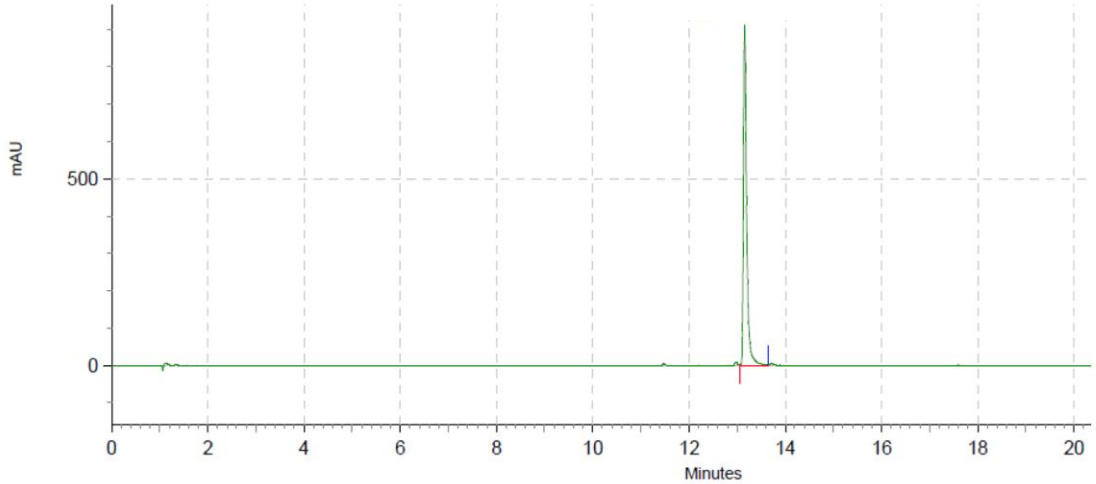
The di-ethyl methylphosphinate ester of **L[1]** (22 mg, 0.03 mmol) and 2-methanesulfonate-*N*-methanesulfonylethylamine, (61 mg, 0.03 mmol) were dissolved in CH_3CN (10 mL) and K_2CO_3 (7.7 mg, 0.06 mmol) was added. The mixture was stirred under argon at 65 °C and monitored by TLC (silica; CH_2Cl_2:5 % CH_3OH, R_f(product) = 0.36). After 12 h, the starting material had been consumed, the reaction was cooled and the solution decanted from excess potassium salts. The solvent was removed under reduced pressure to yield a pale yellow oil, that was used without further purification, (20 mg, 82 %): δ_H (700 MHz, $CDCl_3$) 7.96 (2H, m, H^3), 7.53 (2H, bs, H^5), 7.46 (4H, m, H^{13}), 6.88 (4H,

m, H^{14}), 4.11 – 4.06 (2H, m, H^{7}), 3.91, (4H, s, H^{1}), 3.90 – 3.84 (2H, m, H^{7}), 3.81 (6H, s, H^{16}), 3.45 – 2.64 (19H, m, ring Hs + $H^{17,18,19}$), 1.75 (6H, m, H^{9}), 1.24 (6H, m, H^{8}); δ_C (176 Hz, $CDCl_3$) 160.8 (d, $^3J_{C-P}$ 20 Hz, C^{6}), 160.5 (s, C^{15}), 153.8 (d, $^1J_{C-P}$ 157 Hz, C^{2}), 133.6 (s, C^{13}), 133.1 (d, $^3J_{C-P}$ 10 Hz, C^{4}), 127.7 (d, $^2J_{C-P}$ 20 Hz, C^{3}), 126.7 (d, $^4J_{C-P}$ 3 Hz, C^{5}), 114.2 (s, C^{14}), 113.7 (s, C^{12}), 95.7 (s, C^{11}), 85.4 (s, C^{10}), 63.2 (s, C^{19}), 61.3 (s, C^{1}), 61.1 (d, $^2J_{C-P}$ 6.1 Hz, C^{7}), 55.3 (s, C^{16}), 55.6 – 39.9 (br m, ring Cs + $C^{17,18}$), 16.4 (d, $^3J_{C-P}$ 6 Hz, C^{8}), 13.3 (d, $^1J_{C-P}$ 104 Hz, C^{9}); δ_P (283 MHz, $CDCl_3$) +39.9; *m/z* (HRMS$^+$) 905.3546 $[M + H]^+$ ($C_{45}H_{59}O_8N_6SP_2$ requires 905.3590).



[EuL^1]

L^1 (28 mg, 31.0 µmol) was dissolved in CD_3OD (3 mL) and NaOH (0.1 M in D_2O, 1 mL) was added. The solution was stirred at 60 °C and monitored by ^{1}H-NMR (loss of C*H*$_3$C*H*$_2$ peaks) and ^{31}P-NMR (reactant 39.9 ppm, product 26.1 ppm) and stopped after 16 h. The pH of the solution was adjusted to 7 by addition of HCl (1 M). $Eu(OAc)_3$ (10.0 mg, 31.8 µmol) in a H_2O: CH_3OH solution (0.5 mL, 1:1 v/v) was added and the solution was stirred at 50 °C for 24 h The solvent was removed under reduced pressure and the crude material purified by preparative-HPLC [gradient: 10 − 100% methanol in water over 20 min; t_R = 13.3 min] to give the *title product* as a cream solid (10.0 mg, 33 %); *m/z* (HRMS$^+$) 999.1969 $[M(^{151}Eu)]^+$ ($C_{41}H_{48}O_8N_6P_2S^{151}Eu$ requires 999.1939); τ_{H2O} = 0.58 ms, τ_{D2O} = 0.75 ms; ε_{MeOH} (332 nm) = 38,500 M^{-1} cm^{-1}.



Analytical RP-HPLC of **[EuL1]**: t_R 13.3 min [gradient: 10 – 100% methanol in water over 20 min]

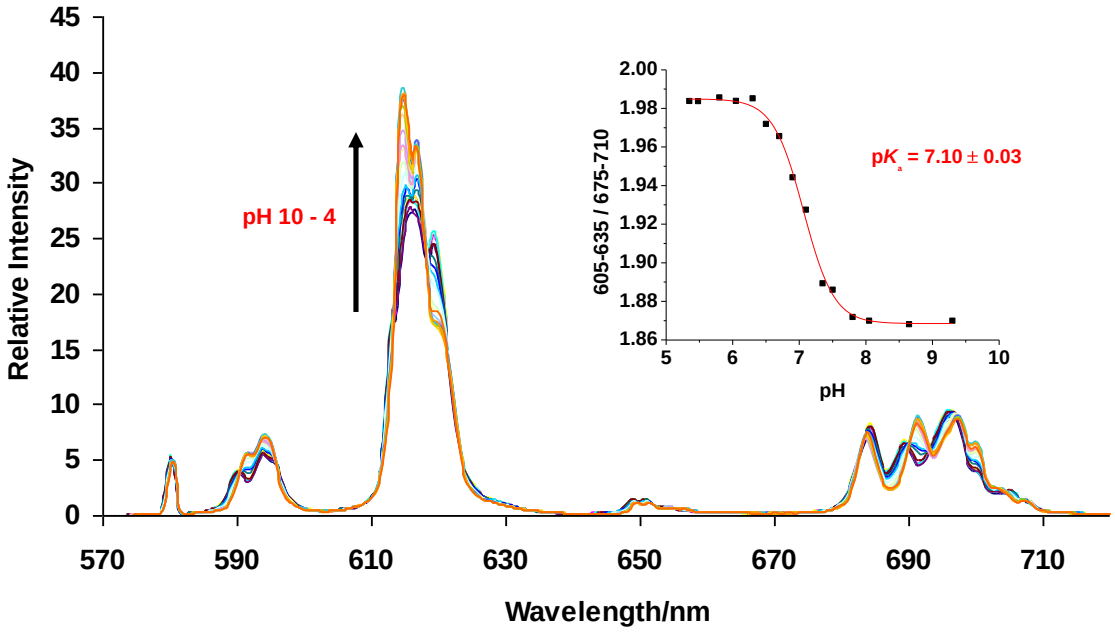


Figure S1: Variation of the Eu^{III} emission of **[EuL¹]** as a function of pH (H_2O, 5 μM complex, 298 K, λ_{exc} = 332 nm) in a 'simulated extracellular' environment (0.4 mM HSA, 0.1 M NaCl, 30 mM bicarbonate, 2.3 mM lactate, 0.9 mM hydrogenphosphate and 0.13 mM citrate) *Inset*: plot of ΔJ = $2/\Delta J = 4$ versus pH, showing the fit to the observed data for an apparent pK_a = 7.10 (±0.03).

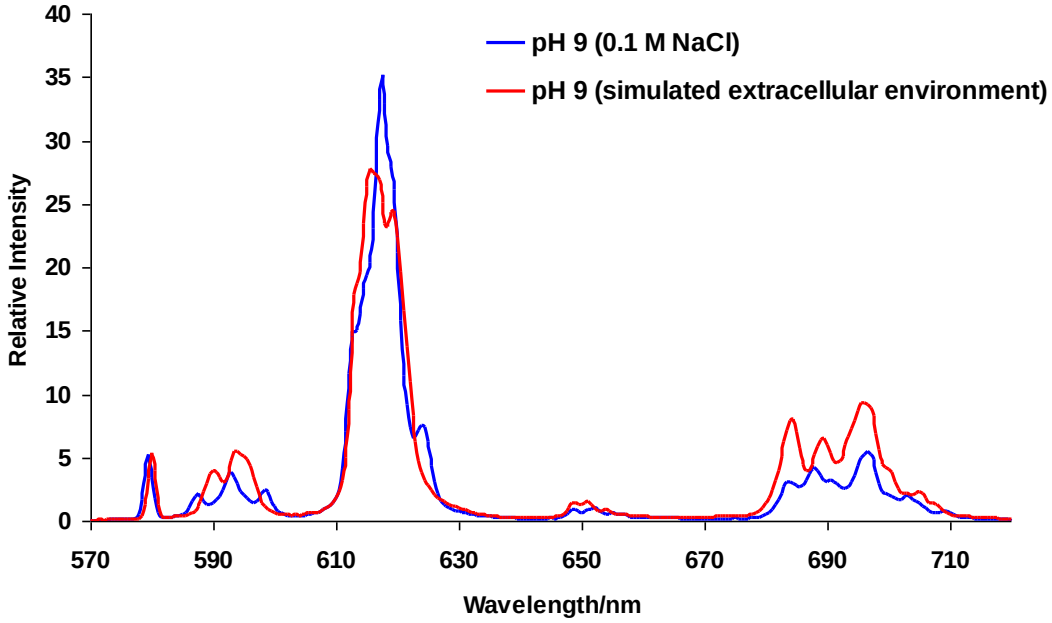


Figure S2: Comparison of the spectral form of **[EuL¹]** at pH 9 in 0.1 M NaCl solution (blue) and in a 'simulated extracellular' environment (0.4 mM HSA, 0.1 M NaCl, 30 mM bicarbonate, 2.3 mM lactate, 0.9 mM hydrogenphosphate and 0.13 mM citrate) (red).

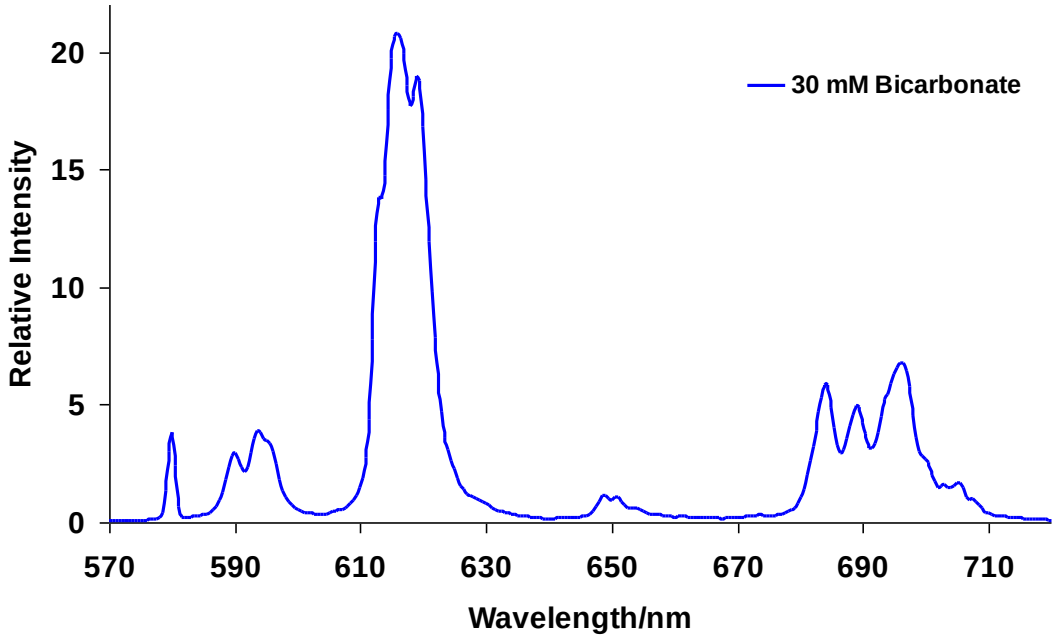


Figure S3: Europium emission spectra for **[Eu.L¹]** in the presence of 30 mM sodium bicarbonate.

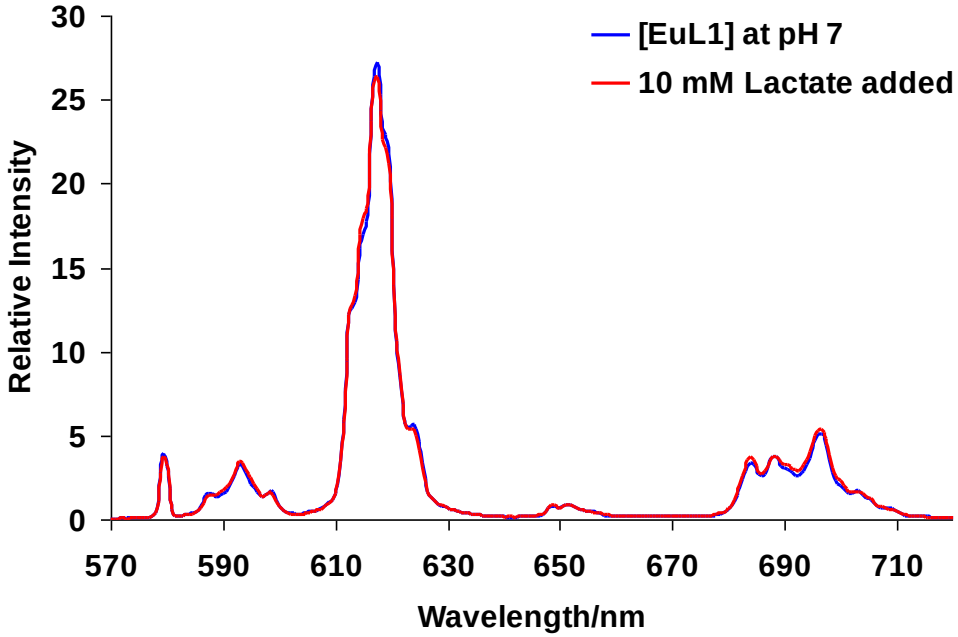


Figure S4: Europium emission spectra for **[Eu.L¹]** in the presence of 10 mM sodium lactate.

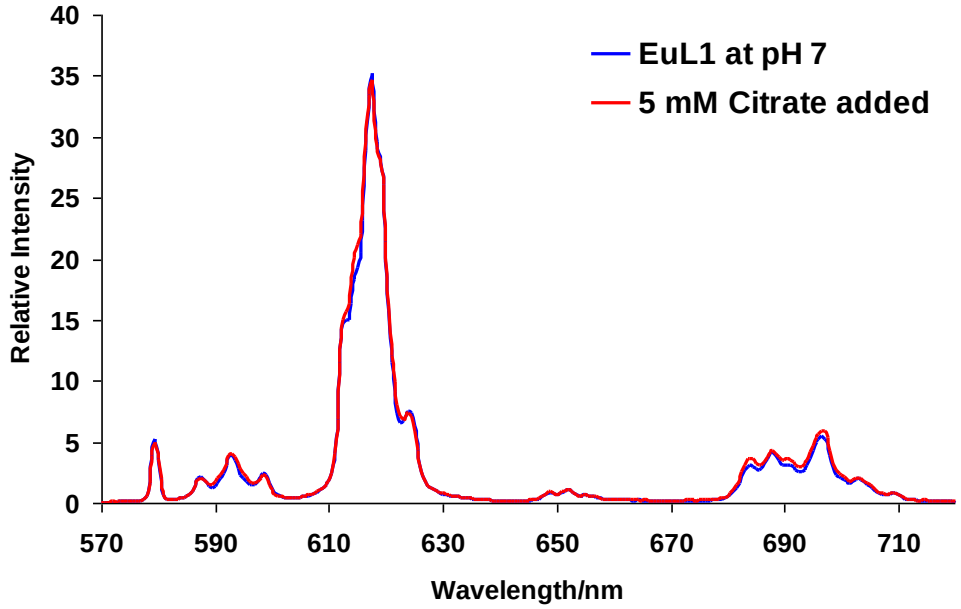


Figure S5: Europium emission spectra for **[Eu.L^1]** in the presence of 5 mM trisodium citrate.

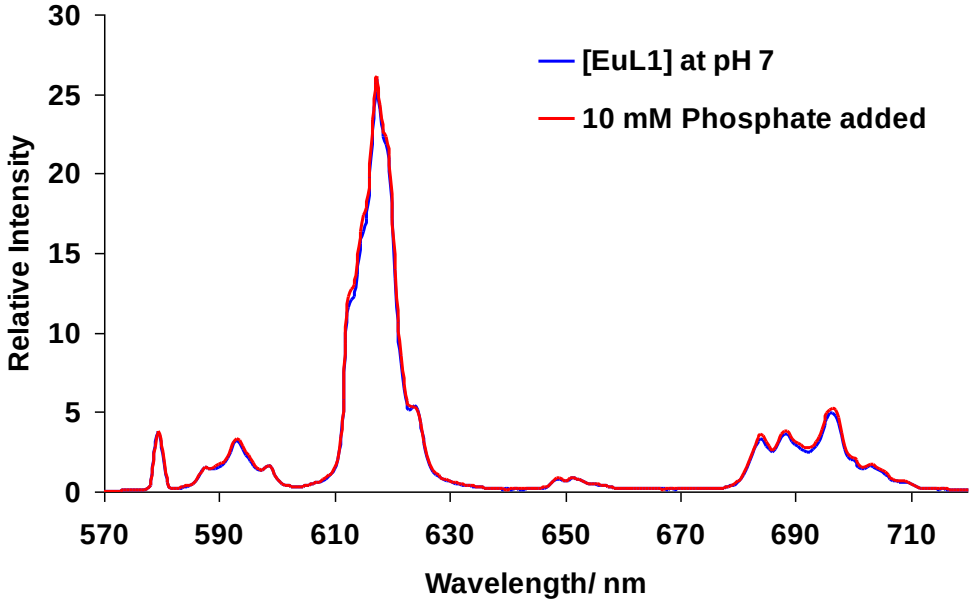


Figure S6: Europium emission spectra for **[Eu.L^1]** in the presence of 10 mM sodium hydrogen phosphate.

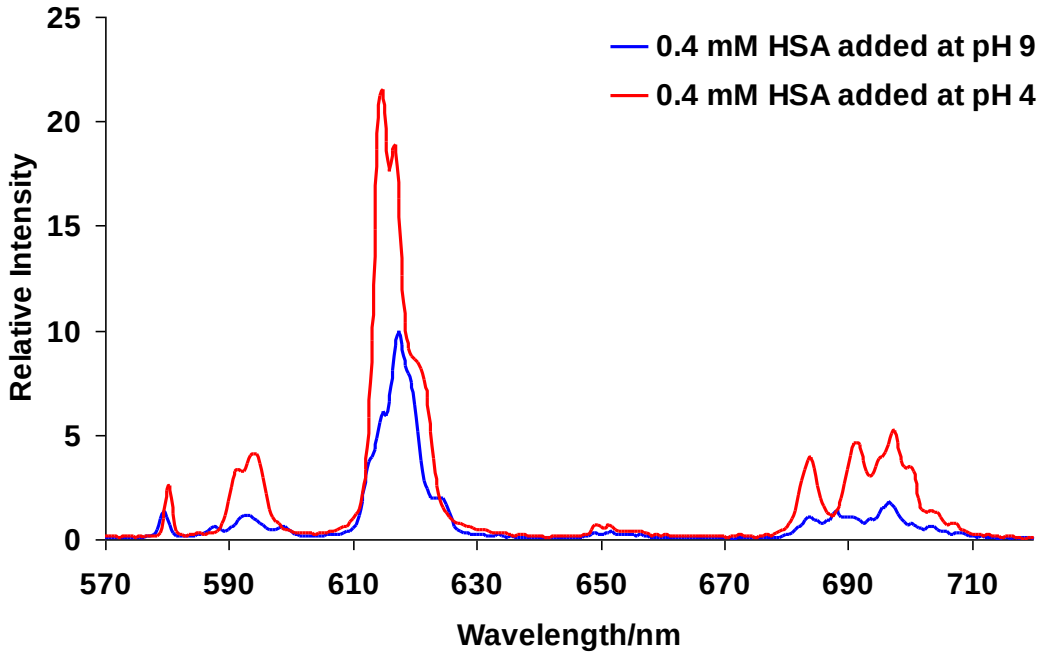


Figure S7: Europium emission spectra for **[Eu.L¹]** in the presence of 0.4mM HSA at pH 4 and pH 9.

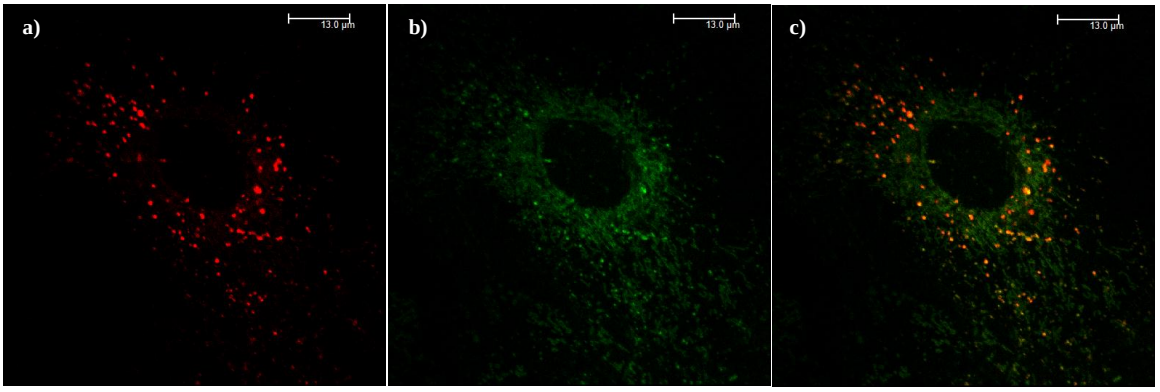


Figure S8: a) LSCM microscopy images of NIH 3T3 cells (mouse skin fibroblasts) loaded with **[EuL¹]** (4h incubation, 30 μM complex concentration in the growth medium); λ_{exc} = 355 nm, λ_{em} = 605-720 nm b) corresponding MitoTracker Green emission (λ_{exc} = 488 nm, λ_{em} = 500-540 nm) c) RGB merge image, showing rather poor correspondence (see Fig. 2 in the main text for ER 90% correspondence)

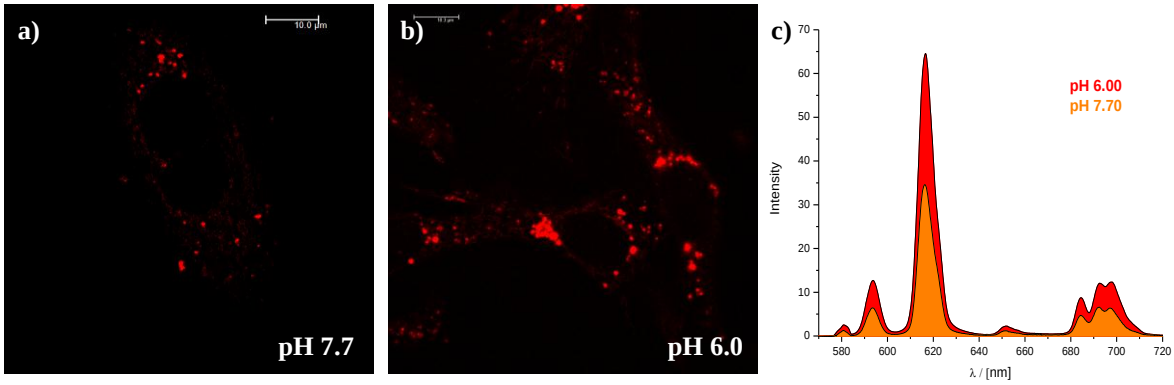


Figure S9: LSCM microscopy images of NIH 3T3 cells (mouse skin fibroblasts) loaded with **[EuL¹]** (4h incubation, 30 μM complex concentration in the growth medium) at a) pH 7.7 and b) pH 6.0; λ_{exc} = 355 nm, λ_{em} = 605-720 nm c) time-gated spectral image of a cell at pH 7.7 and pH 6.0 (λ_{exc} 365 nm, t_{int} 2 ms, t_{gate} 10 μs, 10 000 scans averaged duty cycle using a 2D CCD detector, 100 s acquisition time). (Two fold increase in brightness observed at pH 6 in comparison to pH 7.7)

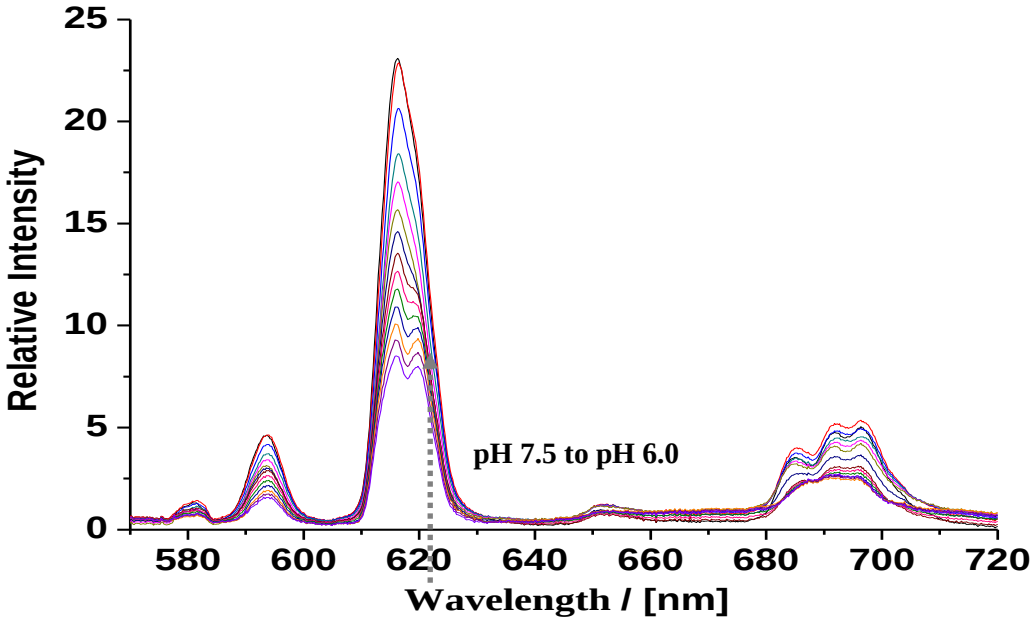


Figure S10: Time gated spectral images of NIH 3T3 cells (mouse skin fibroblast) loaded with **[EuL¹]** (4h incubation, 30 μM) recorded at several pH values between pH 7.5 and pH 6.0 (λ_{exc} 365 nm, t_{int} 2 ms, t_{gate} 10 μs, 10 000 scans averaged duty cycle using a 2D CCD detector, 100 s acquisition time)

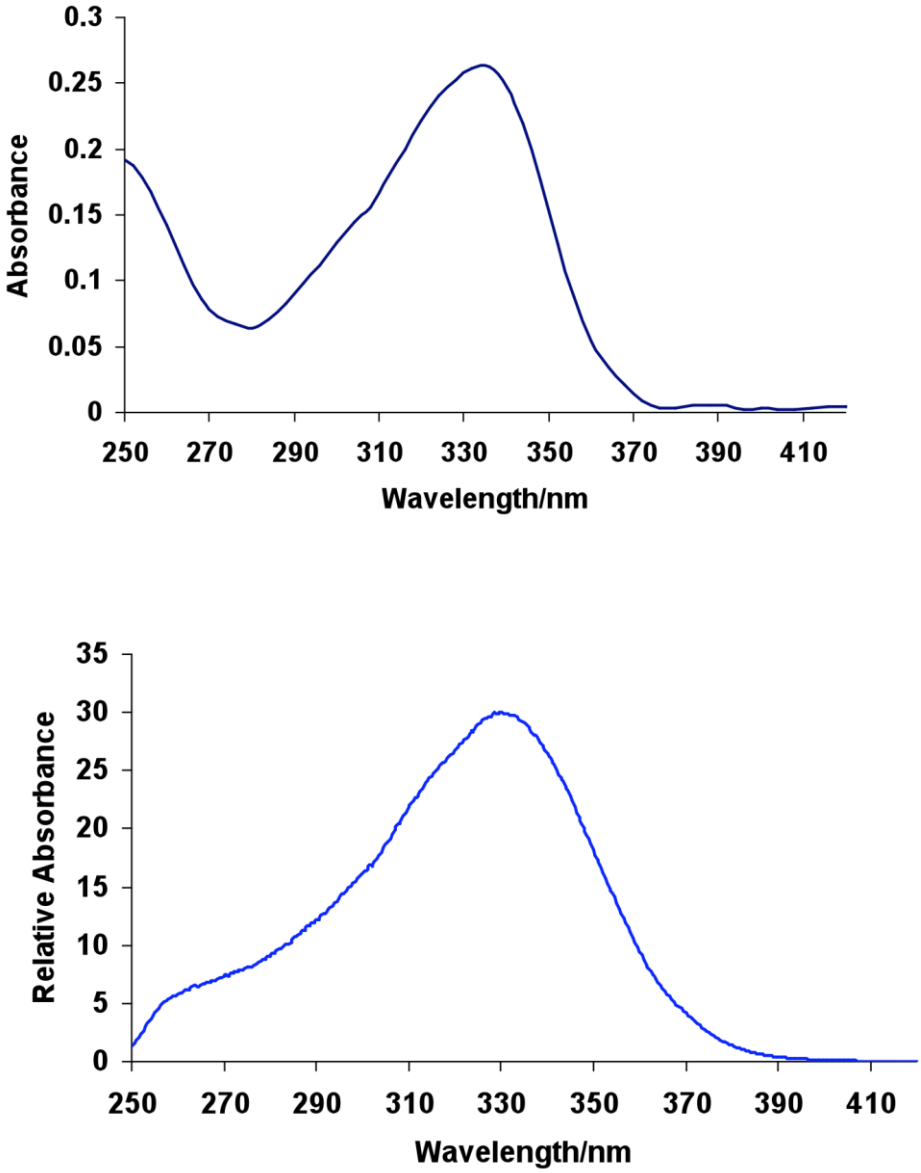


Figure S 11 upper: absorption spectrum of [**Eu.L**^{1}] in water (5μM complex, 295K); lower: excitation spetrum for (water, 295K 5 mM complex, λ_{exc} 355 nm, λ_{obs} 615 nm)