

# Supplementary Information

## Near-Infrared photoacoustic imaging probe responsive to calcium

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## 1. Experimental Procedures

### I. General Procedures

All solvents used were laboratory grade and anhydrous solvents, when required, were freshly distilled over the appropriate drying agent. Water was purified by the 'PuriteSTILLplus' system, with conductivity of  $\leq 4 \mu\text{S cm}^{-1}$ . All reagents and Human Serum were purchased from commercial suppliers (Acros, Sigma Aldrich, and Merck) and were used without further purification unless otherwise stated. Reactions requiring anhydrous conditions were carried out using Schlenk line techniques under an atmosphere of argon.

Thin layer chromatography was performed on neutral aluminium sheet silica gel plates (Merck Art 5554) and visualised under UV irradiation (254 nm), or using specific reagent staining. Preparative column chromatography was performed using silica gel (Merck Silica Gel 60, 230-400 mesh). The compounds were visualized by UV irradiation (254 nm), iodine and Dragendorff staining reagent.

Each synthetic step was characterized by NMR and Mass spectroscopy.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker AV300 and Bruker AV500 spectrometer equipped with a cryoprobe ( $^1\text{H}$ ; internal reference  $\text{CDCl}_3$  at 7.27 ppm or  $\text{D}_2\text{O}$  at 4.75 ppm;  $^{13}\text{C}$ ; internal reference  $\text{CDCl}_3$  at 77.0 ppm). All experiments were performed at  $23^\circ\text{C}$ . Ar = aromatic protons, In = indole protons, InAr = indole aromatic protons,  $\pi$  = Pi-protons.

Electrospray mass spectra (ESI-MS) were recorded on SL 1100 system (Agilent, Germany) with ion-trap detection in positive and negative ion mode.

Preparative Reverse phase HPLC-MS was performed at 298 K on Waters 2767 Autosampler, Waters 600 Multisolvent Delivery System with analytical pump heads (100 $\mu\text{L}$ ); Waters 600 Controller; Waters 2525 Binary Gradient Module with preparative pump heads (500 $\mu\text{L}$ ). At column-dilution, solvent-1, MeCN:H<sub>2</sub>O 70:30 (v/v); flow rate, 5 mL/min. Autosampler 2767 with 10 mL syringe and 10 mL Sample loop. Column 6-position valve Flom 401 with Waters X-Bridge Prep OBD 5  $\mu\text{m}$ , 19x150 mm with X-Bridge RP18 guard cartridge 5  $\mu\text{m}$ , 19x10mm used at flow rate 20 mL/min. Eluent A, H<sub>2</sub>O containing 0.1 % (v/v) HCO<sub>2</sub>H; eluent B, MeCN. Different linear gradients, individually adapted to sample. Injection volume was 9 mL. Make-up solvent, MeOH - MeCN - H<sub>2</sub>O - HCO<sub>2</sub>H [80: 15: 4.95:0.05 (v/v/v/v)]. Make-up pump, Waters Reagent Manager, flow rate 0.5 mL/min. Waters ZQ single

quadrupole mass spectrometer with electrospray source. Positive or negative ion mode scanning  $m/z$  105 - 950 or 300 – 1200 in 1 s; capillary, 3,6 kV; cone voltage, 45 V; multiplier voltage, 700 V; probe and desolvation gas temperature, 120° C and 250° C, respectively. Waters Fraction Collector 2767 with mass or UV-triggered fraction collection. Waters 2487 Dual  $\lambda$  Absorbance Detector, set to 254 nm. Software, Waters MassLynx V 4.0 SP4.

Analytical reverse phase HPLC was carried out on a Perkin Elmer system at 295 K using a 150 x 4.66 mm 4  $\mu$  Phenomenex Synergi Fusion-RP 80i column using method: 95% solvent A (H<sub>2</sub>O, 0.1% HCOOH) and 5% solvent B (MeCN, 0.1% HCOOH) isocratic for 2 min, 5% B to 100% solvent B in 15 min and then running isocratic for 1 min and then back to 5% solvent B in the next 2 min.

All samples for spectrometric characterisation were contained in 96 well-plates with path lengths 0.231 cm and 0.30 cm for volume 75  $\mu$ L and 100  $\mu$ L respectively. Measurements were recorded at 298 K and 310 K. UV/Vis absorbance spectra and Emission spectra were measured on SpectraMax<sup>®</sup> M5 Multi-Mode Microplate Readers (UV/Vis/NIR spectrometer). Samples were measured relative to a reference of pure solvent contained in a matched well and volume. An integration time of 0.5 seconds, increment of 5 nm, excitation and emission slit widths of 2.5 and 1.5 nm, respectively, were used throughout. Free [Ca<sup>2+</sup>] was controlled by a commercial buffer kit (30 mM MOPS, 100 mM KCl, pH 7.2) to generate different ratios of K<sub>2</sub>EGTA/CaEGTA (Calcium Calibration Buffer Kit, Life Technologies). Binding curves were fitted to a variable slope model using Prism 6 (GraphPad Software, La Jolla, California, USA).

## II. Synthesis

**2-(5-methoxy-2-nitrophenoxy)-1-(morpholin-4-yl)ethan-1-one (1).** A solution of 5-methoxy-2-nitrophenol (0.5 g, 2.96 mmol) and K<sub>2</sub>CO<sub>3</sub> (0.82 g, 5.94 mmol) in anhydrous MeCN (10 mL) was stirred under an atmosphere of argon at room temperature for 15 min. A solution of 2-chloro-1-(morpholin-4-yl)ethan-1-one (0.73, 4.46 mmol) in anhydrous MeCN (2 mL) was added dropwise and the reaction mixture was heated at 80°C for 8 h. The reaction progress was monitored by TLC. Upon consumption of starting materials, the solvent was filtered through G-4 sintered funnel and solvent was removed under reduced pressure. The crude residue was purified by column chromatography (100% Hexane to 80:20 Hexane/EtOAc;  $R_f=0.7$ ) to give **1** (0.59 g, 68%) as a dark yellow gum. <sup>1</sup>H NMR (300 MHz,

CDCl<sub>3</sub>:MeOD)  $\delta$  3.52-3.68 (m, 8H, [-NCH<sub>2</sub>CH<sub>2</sub>O]<sub>2</sub>), 3.82 (s, 3H, -OCH<sub>3</sub>), 4.42 (s, 2H, -OCH<sub>2</sub>CO), 6.56 (s, 1H, -CH<sup>Ar</sup>), 6.62 (d,  $J = 9$ , 1H, -CH<sup>Ar</sup>), 7.92 (d,  $J = 7$ , 1H, -CH<sup>Ar</sup>). <sup>13</sup>C NMR (75 MHz, MeOD)  $\delta$  43.5, 46.9, 48.6, 48.8, 49.4, , 56.7, 61.04, 67.6, , 68.9, 101.9, 107.4, 129.2, 133.7, 154.5, 166.2, 167.8. MS (ES<sup>+</sup>)  $m/z$  C<sub>13</sub>H<sub>16</sub>N<sub>2</sub>O<sub>6</sub> requires 297.2 [M+H]<sup>+</sup>; found 297.2 [M+H]<sup>+</sup>.

**2-(2-amino-5-methoxyphenoxy)-1-(morpholin-4-yl)ethan-1-one (2).** A solution of 2-(5-methoxy-2-nitrophenoxy)-1-(morpholin-4-yl)ethan-1-one (1) (0.3 g, 1.01 mmol) and carbonyl-Fe (0.57 g, 10.17 mmol) in EtOH:H<sub>2</sub>O (9:1 ratio, 5 mL) was stirred at room temperature for 5 min. Concentrated HCl (50  $\mu$ L) as the catalyst was added and the reaction mixture was heated at 100°C for 1 h. The reaction progress was monitored by TLC. Upon consumption of starting materials, the solvent was removed under reduced pressure. The crude residue was purified by column chromatography (100 % Hexane to 60:40 Hexane/EtOAc;  $R_f=0.4$ ) to give **2** (0.15 g, 56%) as a yellow gum. <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  3.58-3.71 (m, 10H, [-NCH<sub>2</sub>CH<sub>2</sub>O]<sub>2</sub>, -NH<sub>2</sub>), 3.82 (s, 3H, -OCH<sub>3</sub>), 4.80 (s, 2H, -OCH<sub>2</sub>CO), 7.09 (d,  $J = 9$ , 1H, -CH<sup>Ar</sup>), 7.15 (s, 1H, -CH<sup>Ar</sup>), 7.37 (d,  $J = 7$ , 1H, -CH<sup>Ar</sup>). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  $\delta$  42.5, 45.9, 56.0, 66.6, 66.8, 69.4, 110.1, 116.6, 120.7, 139.9, 145.0, 153.7, 165.6. MS (ES<sup>+</sup>)  $m/z$  C<sub>13</sub>H<sub>18</sub>N<sub>2</sub>O<sub>4</sub> requires 266.2 [M+H]<sup>+</sup>; found 267.1 [M+H]<sup>+</sup>.

**Ethyl 2-[(2-ethoxy-2-oxoethyl){4-methoxy-2-[2-(morpholin-4-yl)-2-oxoethoxy]phenyl}amino]acetate (3).** A solution of 2-(2-amino-5-methoxyphenoxy)-1-(morpholin-4-yl) ethan-1-one (2) (0.12 g, 0.45 mmol) and proton sponge (0.77 g, 3.6 mmol) in anhydrous DMF (5 mL) was stirred under an atmosphere of argon at room temperature for 30 min. A solution of ethyl bromoacetate (0.24, 1.4 mmol) in anhydrous MeCN (0.5 mL) was added dropwise and the reaction mixture was heated at 80°C for 18 h. The reaction progress was monitored by TLC. Upon consumption of starting materials, the reaction mixture was diluted in 100 mL EtOAc and washed with 4X 100 mL H<sub>2</sub>O. The combined organic layer was dried over anhydrous MgSO<sub>4</sub> and solvent was removed under reduced pressure. The crude residue was purified by column chromatography (100% DCM to 95:5 DCM/MeOH;  $R_f=0.4$ ) to give **3** (0.11 g, 53%) as a yellow gum. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  1.24 (t,  $J = 7$ , 6H, [-COOCH<sub>2</sub>CH<sub>3</sub>]<sub>2</sub>), 3.50-3.68 (m, 8H, [-NCH<sub>2</sub>CH<sub>2</sub>O]<sub>2</sub>), 3.72 (s, 3H, -OCH<sub>3</sub>), 4.12 (s, 4H, [-NCH<sub>2</sub>COO]<sub>2</sub>), 4.14 (q,  $J = 7$ , 4H, [-COOCH<sub>2</sub>CH<sub>3</sub>]<sub>2</sub>), 4.78 (s, 2H, -OCH<sub>2</sub>CO), 6.41 (d,  $J = 9$ , 1H, -CH<sup>Ar</sup>), 6.48 (s, 1H, -CH<sup>Ar</sup>), 6.88 (d,  $J = 7$ , 1H, -CH<sup>Ar</sup>). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>: MeOD)  $\delta$  13.9, 20.9, 42.5, 46.0, 55.9, 60.5, 66.5, 66.7, 68.5, 100.4, 106.4, 128.4, 153.3,

164.8, 165.5, 167.9, 171.6. MS (ES<sup>+</sup>) *m/z* C<sub>21</sub>H<sub>30</sub>N<sub>2</sub>O<sub>8</sub> requires 439.4 [M+H]<sup>+</sup>; found 439.1 [M+H]<sup>+</sup>.

**2-[(carboxymethyl)(4-hydroxy-2-[2-(morpholin-4-yl)-2-oxoethoxy]phenyl)amino]**

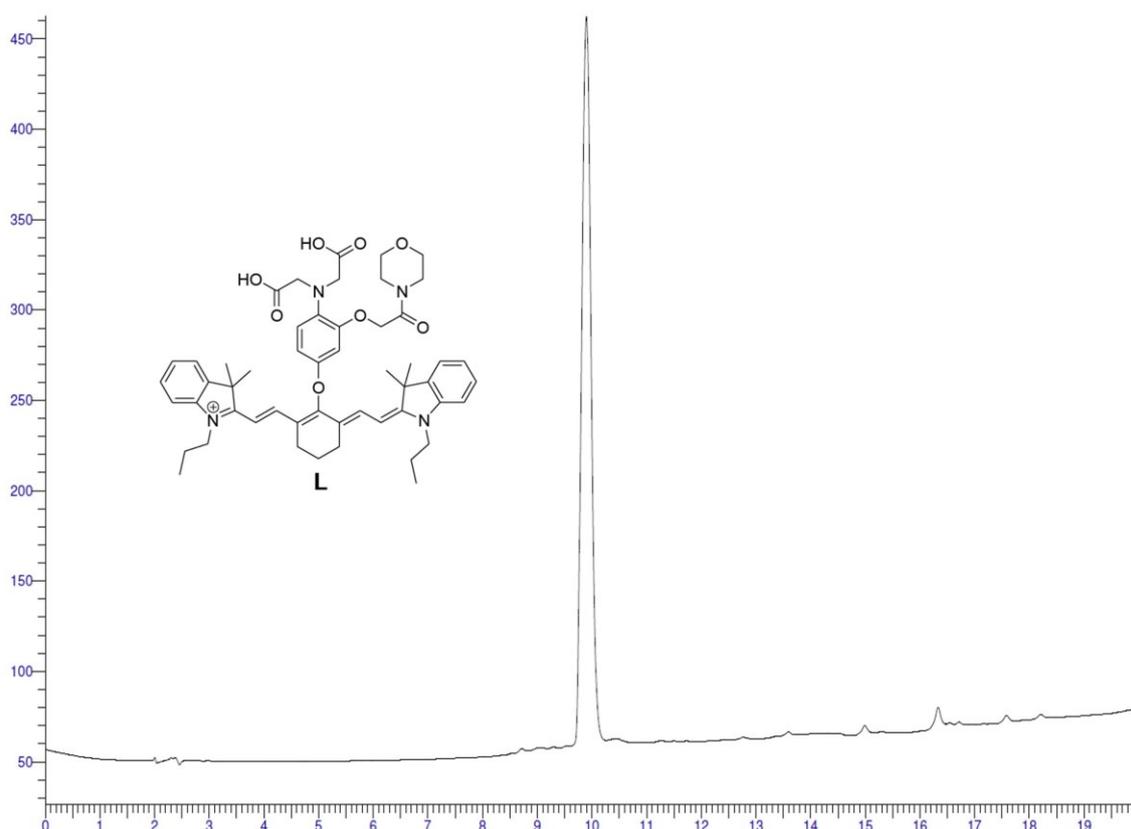
**acetic acid (4).** 2-[(carboxymethyl)(4-hydroxy-2-[2-(morpholin-4-yl)-2-oxoethoxy]phenyl)amino]acetic acid (4) (0.10 g, 0.23 mmol) was taken in anhydrous DCM (0.5 mL) and stirred at 0°C for 10 minutes. 1 mL of 1M BBr<sub>3</sub> in DCM was added in reaction mixture at 0°C and stirred further at room temperature for 2 h. The reaction progress was monitored by LC-MS. Upon consumption of starting materials, the solvent was removed under reduced pressure. A crude dried product was re-dissolved in THF:H<sub>2</sub>O (1:1, 2 mL) and KOH (0.04 g, 0.69 mmol) was added in reaction mixture. The reaction mixture was stirred at room temperature for 6 h, pH was maintained to 7 and solvent was dried under reduced pressure. The product, **4** (0.08 g, 100% w/w, as a yellow gum) was used in next step without any purification. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 3.38-3.84 (br. m, 8H, [-NCH<sub>2</sub>CH<sub>2</sub>O]<sub>2</sub>), 4.15-4.30 (m, 4H [-NCH<sub>2</sub>COO]<sub>2</sub>), 4.84 (s, 2H, -OCH<sub>2</sub>CO), 6.50 (s, 1H, -CH<sup>Ar</sup>), 6.57 (d, *J* = 3, 1H, -CH<sup>Ar</sup>), 7.00 (d, *J* = 7, 1H, -CH<sup>Ar</sup>). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 42.4, 43.8, 54.0, 61.3, 66.8, 68.6, 104.1, 109.8, 119.6, 133.0, 151.6, 155.9, 169.8, 174.7. MS (ES<sup>+</sup>) *m/z* C<sub>16</sub>H<sub>20</sub>N<sub>2</sub>O<sub>8</sub> requires 369.6 [M+H]<sup>+</sup>; found 369.1 [M+H]<sup>+</sup>.

**2-[(E)-2-[(3E)-2-{4-[bis(carboxymethyl)amino]-3-[2-(morpholin-4-yl)-2-oxoethoxy]phenoxy}-3-{2-[(2E)-3,3-dimethyl-1-propyl-2,3-dihydro-1H-indol-2-ylidene]ethylidene}cyclohex-1-en-1-yl]ethenyl]-3,3-dimethyl-1-propyl-3H-indol-1-ium**

**(L).** 2-[(carboxymethyl)(4-hydroxy-2-[2-(morpholin-4-yl)-2-oxoethoxy] phenyl)amino]acetic acid (4) (20 mg, 0.054 mmol) and NaH (5.2 mg, 0.33 mmol) were taken in anhydrous DMF:DMSO (9:1, 1 mL) and stirred at 0 °C for 30 mins. 2-((E)-2-((E)-2-chloro-3-((E)-2-(3,3-dimethyl-1-propylindolin-2-ylidene)ethylidene)cyclohex-1-enyl)vinyl)-3,3-dimethyl-1-propyl-3H-indolium iodide (54 mg, 0.081 mmol) was added dropwise as a solution in anhydrous DMF (0.5 mL) and the resulting mixture heated to 80 °C for 6 h. Upon completion of the reaction, the reaction mixture was cooled down to 4°C and added dropwise on stirred diethylether (200 mL at 0°C). The crude compound was crashed down in diethylether, the solvent was decanted and the product was dried under reduced pressure. The crude product was purified by preparative RP-HPLC to give **L** as dark green solid (2.2 mg, 5%).

<sup>1</sup>H NMR (500 MHz, MeOD:CDCl<sub>3</sub>) δ 1.10-1.31 (m, 6H, [-N<sup>ln</sup>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>]<sub>2</sub>), 1.34-1.73 (br. m, 13H, [-N<sup>ln</sup>CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>]<sub>2</sub>, [-C<sup>ln</sup>CH<sub>3</sub>]<sub>3</sub>), 1.80-1.96 (m, 5H, -C<sup>ln</sup>CH<sub>3</sub>, -CH<sub>2</sub><sup>CH</sup>), 2.42-2.53 (m,

2H,  $-CH_2^{CH}$ ), 2.63-2.74 (m, 2H,  $-CH_2^{CH}$ ), 3.41-3.77 (br. m, 10H,  $-[NCH_2CH_2O]_2$ ,  $-N^{In}CH_2CH_2CH_3$ ), 4.06 (s, 4H,  $[-NCH_2]_2$ ), 4.75 (s, 2H,  $-OCH_2$ ), 5.24-5.36 (m, 2H,  $-N^{In}CH_2CH_2CH_3$ ), 6.01-6.10 (m, 1H,  $-CH^{\pi}$ ), 6.35-6.43 (m, 1H,  $-CH^{Ar}$ ), 6.51-6.79 (m, 4H,  $-CH^{Ar}$ ,  $[-CH^{\pi}]_2$ ,  $-CH^{InAr}$ ), 6.90-7.12 (m, 5H,  $-CH^{Ar}$ ,  $[-CH^{InAr}]_4$ ), 7.19 (t,  $J = 7$ , 1H,  $-CH^{InAr}$ ), 7.30-7.42 (m, 2H,  $-CH^{InAr}$ ,  $-CH^{\pi}$ ), 7.87 (d,  $J = 9$ , 1H,  $-CH^{InAr}$ ). MS (ES<sup>+</sup>)  $m/z$   $C_{52}H_{62}N_4NaO_8$  requires 894.05 [M+Na]<sup>+</sup>; found 894.49 [M+Na]<sup>+</sup>. Analytical HPLC;  $t_R = 9.95$  min.  $\lambda_{max}/\lambda_{em}$  (H<sub>2</sub>O) 765/792 nm. Molar extinction coefficient = 194,000 M<sup>-1</sup>cm<sup>-1</sup>.



**Figure S1.** Analytical HPLC chromatogram of **L**.

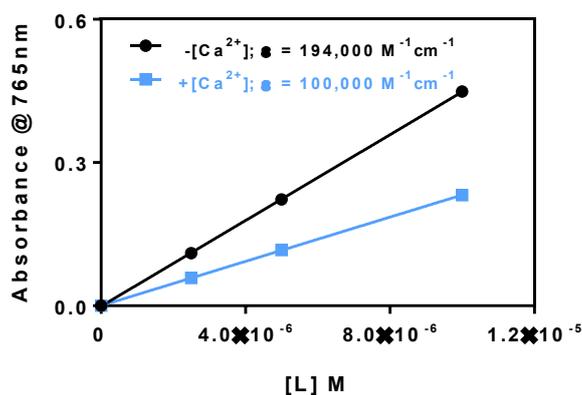
### III. Photoacoustic imaging.

Photoacoustic imaging was performed on a small animal system (inVision 256-TF, iThera Medical GmbH, Munich, Germany) containing a tunable optical parametric oscillator pumped by an Nd:YAG laser (InnoLas Laser GmbH, Krailling, Germany) that allows for excitation from 680 nm to 900 nm. Laser pulse duration is below 9 ns and a pulse repetition frequency of 10 Hz was used. The beam was coupled into a custom fiber bundle that is divided into 10 output arms, allowing even illumination around the circumference of the agar-phantom with a ring-shaped pattern. A custom-made piezocomposite ultrasonic

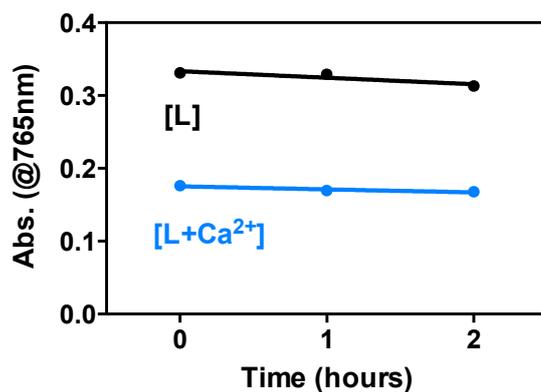
cylindrically focused transducer array (Imasonic SAS, Voray, France) with 256 elements and a central frequency of 5 MHz was used for detection. The array covers an angle of 270 degrees around the sample to create cross-sectional image. Transducer array, fibre bundle outputs and sample holder were submerged in a water bath maintained at 37°C.

40  $\mu\text{M}$  **L** was mixed with varying  $[\text{Ca}^{2+}]$  (0  $\rightarrow$  60  $\mu\text{M}$ , at 20  $\mu\text{M}$  incremental points) and inserted in 3 mm diameter tubes that were placed within a 20 mm diameter phantom made of 1.3% agar resulting in an optically diffusive medium with acoustic properties similar those of tissue. Samples were placed in a horizontal position inside a thin polyethylene membrane without direct contact with water. This arrangement allowed a convenient acoustic coupling between the sample and the transducer array. PAI was done in one cross-sectional imaging plane ( $\sim$ 200  $\mu\text{m}$  in plane resolution). The phantom images were reconstructed using the interpolated model-matrix inversion.<sup>[1]</sup>

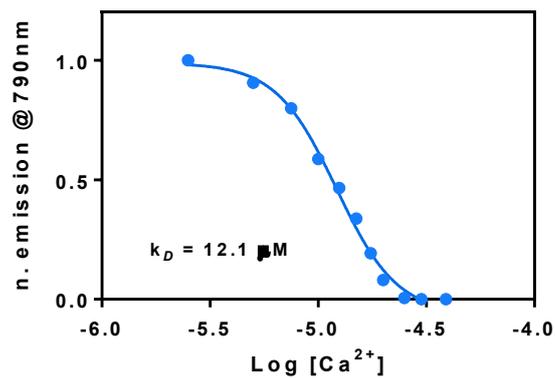
[1] A. Rosenthal, V. Ntziachristos and D. Razansky, *Med. Phys.*, 2011, **38**, 4285.



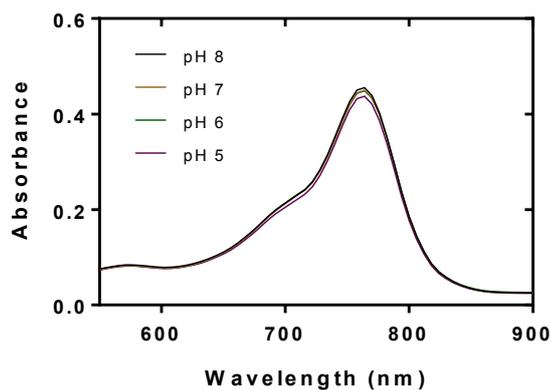
**Figure S2.** Molar extinction coefficient of **L** without and with  $\text{Ca}^{2+}$ . **Black line:** Absorbance obtained at 765 nm with a dilution series of **L** (10  $\mu\text{M}$ , 5  $\mu\text{M}$ , 2.5  $\mu\text{M}$ , 0  $\mu\text{M}$ , pH 7.2) measured without  $\text{Ca}^{2+}$  at 294K. **Blue line:** Absorbance of **L** obtained at 765 nm with dilution series (10  $\mu\text{M}$ , 5  $\mu\text{M}$ , 2.5  $\mu\text{M}$ , 0  $\mu\text{M}$  pH 7.2) measured with  $\text{Ca}^{2+}$  (20  $\mu\text{M}$ , 10  $\mu\text{M}$ , 5  $\mu\text{M}$ , 0  $\mu\text{M}$  respectively) at 294K. In all series, volume and path length were 75  $\mu\text{L}$  and 0.231 cm respectively.



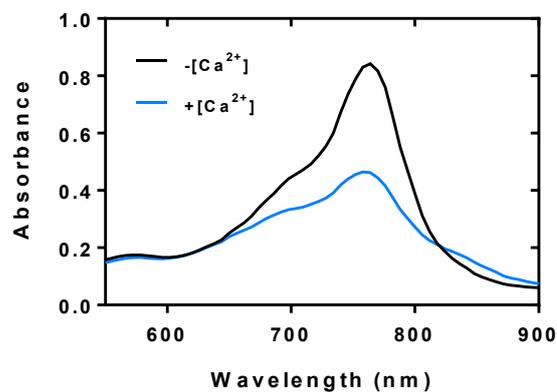
**Figure S3.** Stability studies of **L** and **L+Ca<sup>2+</sup>** in buffer with time. Absorbance at 765 nm of **L** and **L+Ca<sup>2+</sup>** (1 equivalent  $\text{Ca}^{2+}$ ) were obtained over time in 100 mM KCl, 30 mM MOPS (20  $\mu\text{M}$  **L**, pH 7.2, 310 K). The signal change over 2 hours was ~-5%.



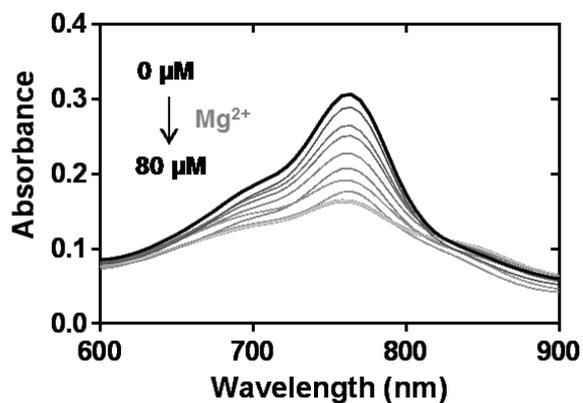
**Figure S4.**  $IC_{50}$  values for  $Ca^{2+}$  were determined from the peak emission value at 790 nm in 100 mM KCl, 30 mM MOPS (20  $\mu$ M L, pH 7.2, 310 K) (Figure 1B). The  $Ca^{2+}$  binding curve obtained from the peak emission amplitude is shown in blue curve.



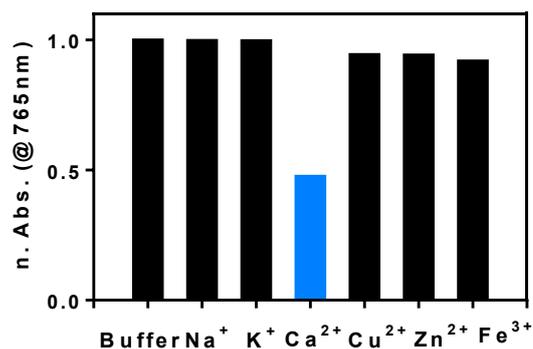
**Figure S5.** Absorbance spectra of L (10  $\mu$ M) measured at various pH (5, 6, 7, 8) in water at 294K.



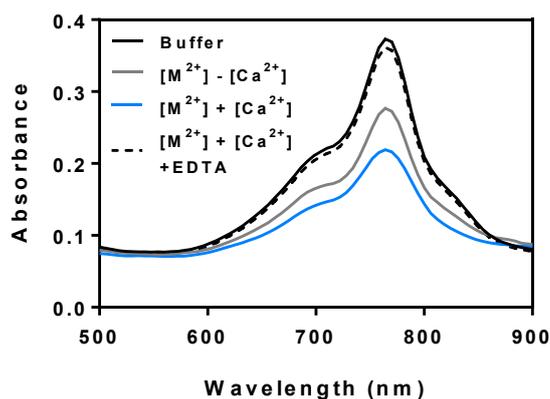
**Figure S6.** Absorbance spectra of **L** (10  $\mu\text{M}$  in 5 mM BSA, pH 7.2) measured without and with  $\text{Ca}^{2+}$  (20  $\mu\text{M}$ ) at 294K.



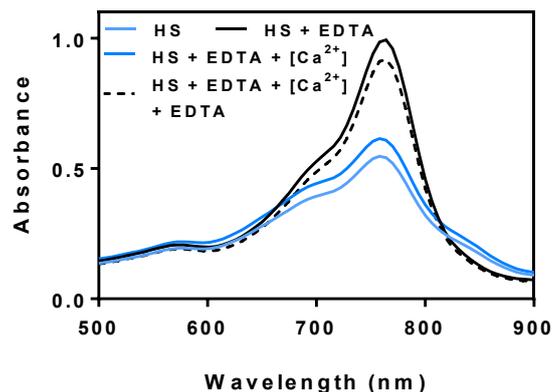
**Figure S7.** Spectroscopic characterisation of **L** to determine  $\text{Mg}^{2+}$  sensitivity. Changes in absorbance induced by various concentrations of free  $[\text{Mg}^{2+}]$  (0, 7.5, 15, 22.5, 30, 37.5, 45, 52.5, 60, 67.5 and 80  $\mu\text{M}$ ) in 30 mM MOPS buffer containing 100 mM KCl (pH 7.2, 20  $\mu\text{M}$  **L**) at 310K.



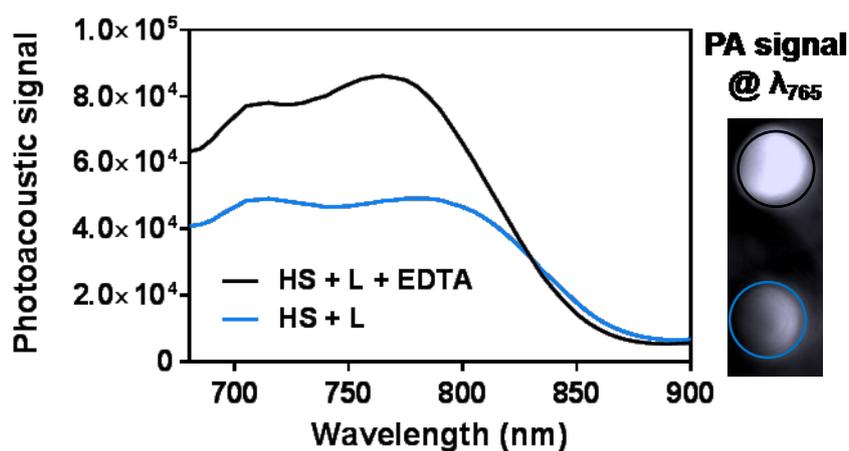
**Figure S8.** Spectroscopic characterisation of **L** to determine selectivity for  $\text{Ca}^{2+}$  over other biologically relevant mono-, di- and tri-valent cations. Peak absorbance maxima (at 765 nm) measured from **L** (20  $\mu\text{M}$ ) upon addition of 50  $\mu\text{M}$  [ $\text{Na}^+$ ], [ $\text{K}^+$ ], [ $\text{Ca}^{2+}$ ], [ $\text{Cu}^{2+}$ ], [ $\text{Zn}^{2+}$ ] and [ $\text{Fe}^{3+}$ ] in 30 mM MOPS, 100 mM KCl (pH 7.2, 310 K).



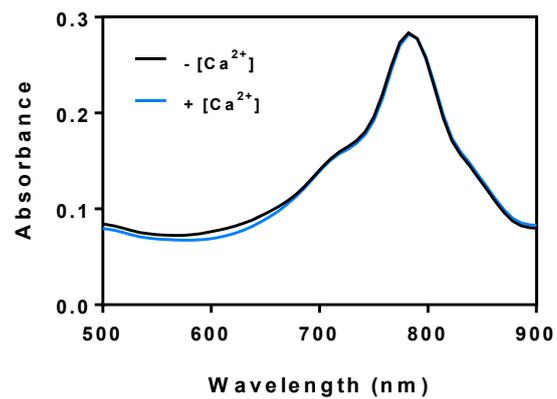
**Figure S9.** Absorbance at 765 nm obtained from **L** (20  $\mu\text{M}$ ) in 30 mM MOPS, 100 mM KCl (pH 7.2, 310 K), mixing with 20  $\mu\text{M}$  (1 equi.) each of [ $\text{Mg}^{2+}$ ]/[ $\text{Zn}^{2+}$ ]/[ $\text{Cu}^{2+}$ ], and subsequent addition of 30  $\mu\text{M}$  [ $\text{Ca}^{2+}$ ] and 60  $\mu\text{M}$  EDTA. [ $\text{M}^{2+}$ ] denotes the mixture of several cations { [ $\text{Mg}^{2+}$ ]/[ $\text{Zn}^{2+}$ ]/[ $\text{Cu}^{2+}$ ] }.



**Figure S10.** L (20  $\mu\text{M}$ ) was added to human serum (pH 7.2, 310 K) and the absorbance was sequentially measured before and after mixing with 4 mM EDTA, and after subsequent addition of 1 mM  $[\text{Ca}^{2+}]$  and 1 mM EDTA.



**Figure S11.** Reversible photoacoustic signal change of L in human serum. To 20  $\mu\text{M}$  L in human serum [pH 7.2, 310 K] 0 or 1 mM of EDTA were added. Both samples were filled into plastic tubes (3 mm) that were inserted in the centre of a 20 mm diameter phantom made of 1.3% agar. Photoacoustic images were acquired for different wavelengths (680 to 900 nm in 5 nm steps, 9 ns laser pulses at a repetition rate of 10Hz, 50 averages per image). Photoacoustic spectra are plotted extracted from the average pixel intensities of the color-coded circular region of interests (ROIs) covering the samples on the photoacoustic images.



**Figure S12.** Absorbance spectra of **IR-780 only** (10  $\mu\text{M}$ , pH 7.2) measured without and with  $\text{Ca}^{2+}$  (20  $\mu\text{M}$ ) at 294K.