Supplementary Information

Long-lived metal complexes open up microsecond lifetime imaging microscopy under multiphoton excitation: from FLIM to PLIM and beyond

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Methods and instrumentation

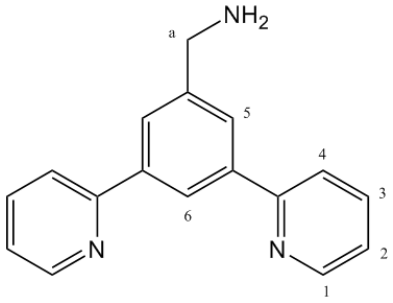
All ^{1}H- and ^{13}C-NMR spectra were recorded on a 400 MHz Bruker Advance 400 spectrometer, except the ^{13}C spectrum of PtL^3Cl which was recorded on a Bruker DRX-500 spectrometer. All NMR chemical shifts are reported in ppm. Positive electrospray ionisation (ES+) spectra were recorded by the mass spectrometry service at the University of Sheffield, on a Waters LCT time of flight instrument. Low-resolution mass spectra are reported to the nearest integer. IR spectra were recorded as solids on a Perkin Elmer Spectrum RX (equipped with DuraSamplIRII SensIR ATR) in attenuated total reflection mode at a resolution of 4 cm^{-1} and are reported in wavenumbers.

The toxicity of the new PtL^3Cl compound towards HDF cells was determined by a standard MTT assay for 100 μm and 50 μm concentration of the complex, the data were averaged over three independent experiments. Cells seeded in a 24 well plate, were grown to approximately 60-70 % confluence, before treatment with PtL^3Cl (at 100 μm and 50 μm) for 10 minutes at 37°C. After removal of PtL^3Cl, cells were washed three times with a phosphate buffered saline solution (3 x PBS at 1ml/well), then incubated with a solution of MTT (0.5 mg/ml in PBS at 1 ml/well) for 40 minutes at 37°C. After careful removal of the MTT solution, acidified isopropanol (400 μl/well) was added to elute the coloured product. After five minutes at room temperature the acidified solution was then transferred in duplicate (2 x 150 μl aliquots) from each well to a 96 well plate and the optical density recorded at 540 nm.

Human dermal fibroblasts, obtained from abdominoplasty or breast reduction operations according to local ethically approved guidelines (Northern General Hospital Trust (NHS), Sheffield, UK), were cultured in a humidified 37°C, 5% CO_2/95% air (v/v) environment in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% (v/v) FCS, 100 units/ml penicillin, 100 μg/mL streptomycin and 0.625 μg/ml amphotericin B. Cells were passaged routinely using 0.02% (w/v) EDTA and used for experimentation between passages 3 and 9 at approximately 60% confluence.

Synthesis of HL^3 and PtL^3Cl.

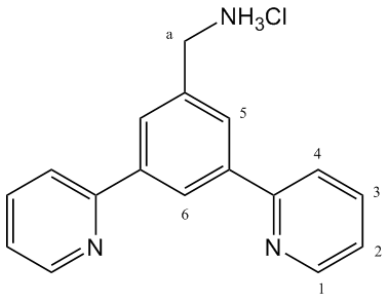
(1) 1-Methylamine(3,5-dipyridyl)benzene.



3,5-dipyridylbenzonitrile (0.340 g, 1.32 mmol) and borane-THF (1.0 M, 13.2 mL, 13.21 mmol) were refluxed under N_2 for 48 hours at 85°C. After cooling, methanol (10 mL) was added and solvent was removed under vacuum. This process was repeated twice. The remaining brown liquid residue was then refluxed in 6M HCl (15 mL) for four hours. Aqueous base (20% KOH, 32 mL) was added until an off-white emulsion was formed (pH 8-9), which was then extracted into CH_2Cl_2 (3 x 15 mL). The combined organic layers were washed with water (3 x 20 mL), dried over anhydrous K_2CO_3 and filtered. The solvent was removed under reduced pressure to give a pale yellow, sticky oil. The crude product was used in the synthetic steps that follow, although purification can be achieved by column chromatography on silica using 10:90, MeOH:CH_2Cl_2 + 1 % Et_3N. Yield: 64%.

^{1}H NMR (400 MHz, $CDCl_3$): δ_H = 4.07 (2H, s, H^a), 7.27 (2H, ddd, *J* 7.6, 4.8, 1.2 Hz, H^2), 7.80 (2H, ddd, *J* 8.0, 7.6, 1.8 Hz, H^3), 7.87 (2H, dt, *J* 8.0, 1.0 Hz, H^4), 8.04 (2H, d, *J* 1.6 Hz, H^5), 8.46 (1H, t, *J* 1.6 Hz, H^6) and 8.74 (2H, ddd, *J* 4.8, 1.8, 0.9 Hz, H^1).
$^{13}C\{^1H\}$ NMR (400 MHz, $CDCl_3$): δ_C = 46.6 (1C, s, CH_2), 120.9 (2C, s, CH-Ar), 122.3 (2C, s, CH-Ar), 124.1 (1C, s, CH-Ar), 126.3 (2C, s, CH-Ar), 136.8 (2C, s, CH-Ar), 140.2 (2C, s, Cquat), 144.5 (1C, s, Cquat), 149.6 (2C, s, CH-Ar) and 157.2 (2C, s, Cquat).
MS (ES^+): m/z = 262 $[MH]^+$, 284 $[M+Na]^+$.

(2) 1-Methylammoniumchloride(3,5-dipyridyl)benzene.



6M HCl (4 mL) was added to solution of 1-methylamine-3,5-dipyridylbenzene (0.29 g, 1.11 mmol) dissolved in CH_2Cl_2 (8 mL). The biphasic solution was stirred vigorously for two hours then transferred into a separating funnel. The organic layer was extracted into water (3 x 5 mL). Aqueous fractions were combined and water removed under reduced pressure to give a pale yellow residue. Yield: quantitative (0.330 g, 1.11 mmol).

^{1}H NMR (400 MHz, D_2O): δ_H = 4.35 (2H, s, CH2 H^a), 7.99 (2H, ddd, *J* 8.0, 5.8, 1.0 Hz, H^2), 8.12 (2H, fd, *J* 1.5 Hz, H^5), 8.27-8.30 (3H, m, H^4, H^6), 8.60 (2H, ddd, *J* 8.3, 8.0, 1.5 Hz, H^3), 8.74 (2H, dd, *J* 5.8, 1.0 Hz, H^1).

$^{13}C\{^1H\}$ NMR (400 MHz, D_2O): δ_C = 42.34 (1C, s, CH_2), 126.48 (2C, s, CH-Ar), 126.90 (2C, s, CH-Ar), 128.77 (1C, s, CH-Ar), 131.83 (2C, s, CH-Ar), 133.32 (2C, s, Cquat), 135.94 (1C, s, Cquat), 141.84 (2C, s, CH-Ar), 147.60 (2C, s, CH-Ar), 150.36 (2C, s, Cquat).

MS (ES^+): m/z = 262 $[M\text{-}Cl]^+$.

IR: ν_{max} (solid) / cm^{-1} = 3354 (broad), 2888 (broad sh), 2620 (broad) 1614, 1536, 1476, 1450, 1398, 1276, 1244, 1147, 1096, 996, 910 (**Fig. S1)**.

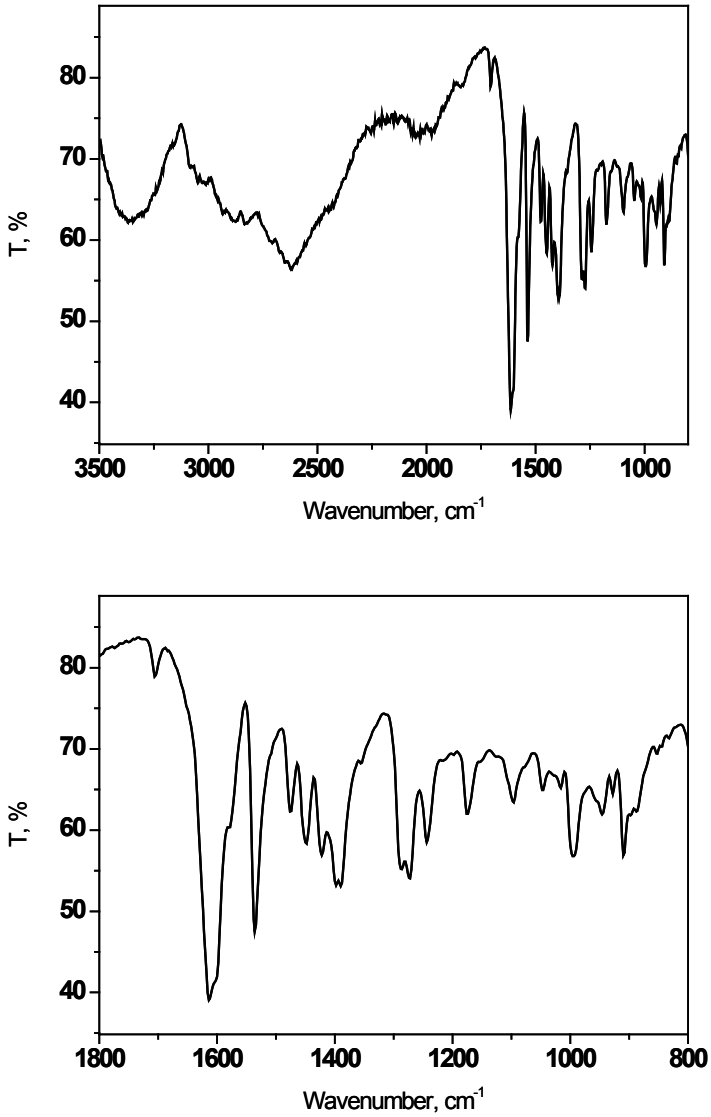
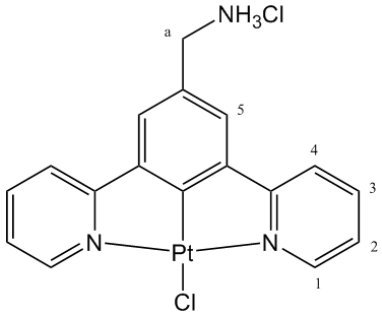


Fig. S1. Infrared spectrum of ligand **2**, 1-Methylammoniumchloride(3,5-dipyridyl)benzene (recorded as a solid). Top panel – full spectrum, bottom panel – expanded fingerprint region.

Synthesis of PtL^3Cl, 1-Methylammoniumchloride-3,5-dipyridylbenzene platinum chloride.



Acetic acid (10 mL) was added to a solution of 1-methylammoniumchloride-3,5-dipyridylbenzene (0.113 g, 0.382 mmol) and K_2PtCl_4 (0.159 g, 0.382 mmol) in water (3 mL). The peach coloured, opaque mixture was degassed via three freeze-pump-thaw cycles before being heated to reflux under N_2. After 72 hours the yellow heterogenous mixture was cooled to room temperature and filtered, the solid residue was washed with a large volume of water until the filtrate was clear. Water was removed from the filtrate under vacuum to give a bright yellow solid. Yield: 48 % (0.070 g, 0.132 mmol).

^{1}H NMR (400 MHz, d_6-DMSO): δ_H 4.05 (2H, s, H^a), 7.62 (2H, ddd, *J* 7.7, 5.7, 1.3 Hz, H^2), 7.91 (2H, s, H^5), 8.02 (2H, d, *J* 8.0 Hz, H^4), 8.26-8.32 (5H, ddd, *J* 8.0, 7.8, 1.5 Hz, H^3 and bs, NH_3), 9.15 (2H, dd, *J* (^{195}Pt) 35 Hz, *J* 5.8, 1.2 Hz, H^1), **Fig. S2**.

^{13}C{^{1}H} NMR (400 MHz, d_6-DMSO): δC = 43.15 (1C, s, CH_2), 120.21 (s, Ar), 124.54 (s, Ar), 125.85 (s, Ar), 128.94 (s, Ar), 140.63 (s, Ar), 140.77 (s, Ar), 150.54 (s, Ar), 151.53 (s, Ar), 161.02 (s, Ar), 166.00 (s, Ar), **Fig. S3.**

MS (ES^+): m/z = 455 $[M-(2xCl)-H]^+$, 496 $[M-(2xCl)-H]^+$ + MeCN.
Note: The use of ASAP (solids probe) or MALDI does also lead to detection of the m/z = 491 $[M-(HCl)]^+$ peak (containing one Cl ligand), **Fig. S4.**

IR: ν_{max} (solid) / cm^{-1} = 3378 (broad), 2950 (broad), 2624, 1698, 1606, 1480, 1468, 1428, 1380, 1286, 1206, 1124, 970, 876, 832, **Fig. S5.**

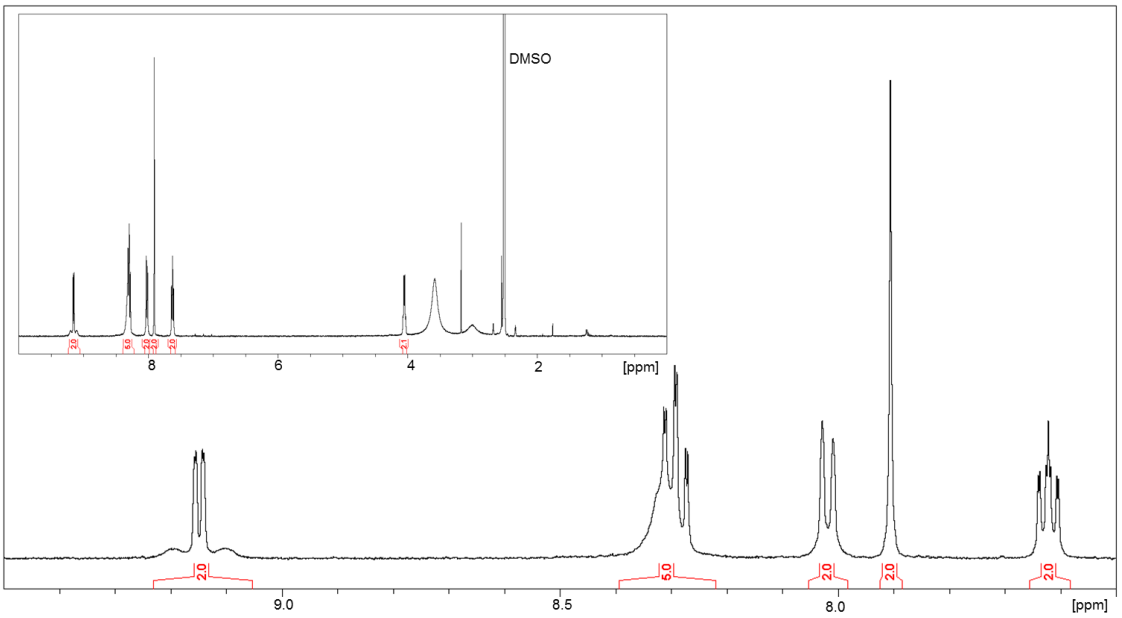


Fig. S2. ^{1}H NMR (400 MHz) spectra of complex PtL^{3}Cl in d_6-DMSO, in the aromatic region (7.5-9.5 ppm) to show Pt satellites and peak splitting. The inset shows the full spectrum from 0 to 10 ppm. Peak integration shown in red.

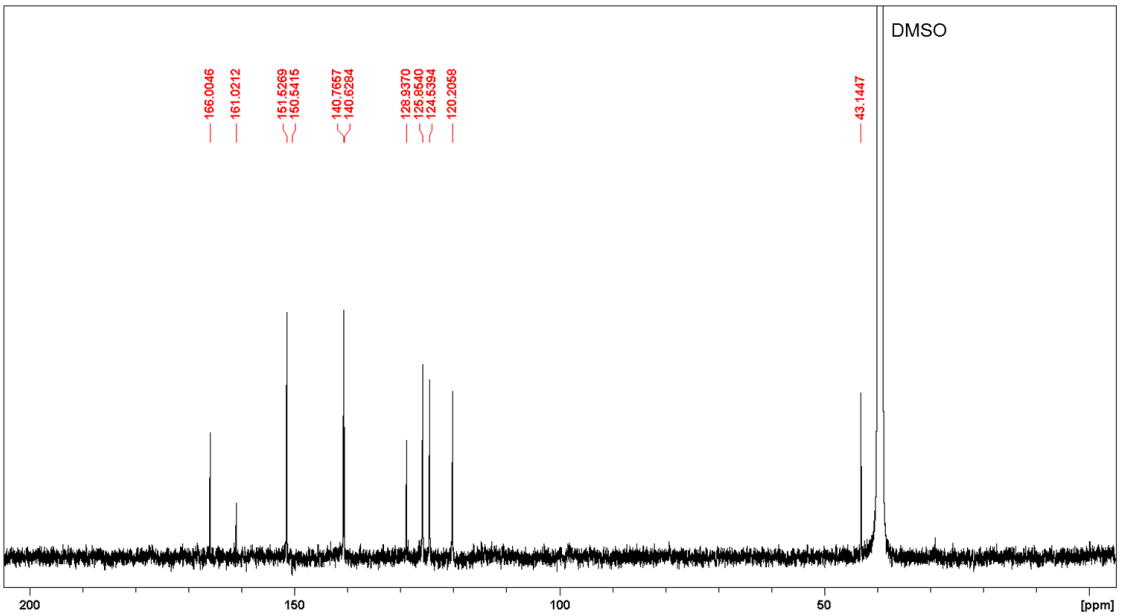


Fig. S3. ^{13}C NMR (500 MHz) spectrum of complex PtL^{3}Cl in d_6-DMSO.

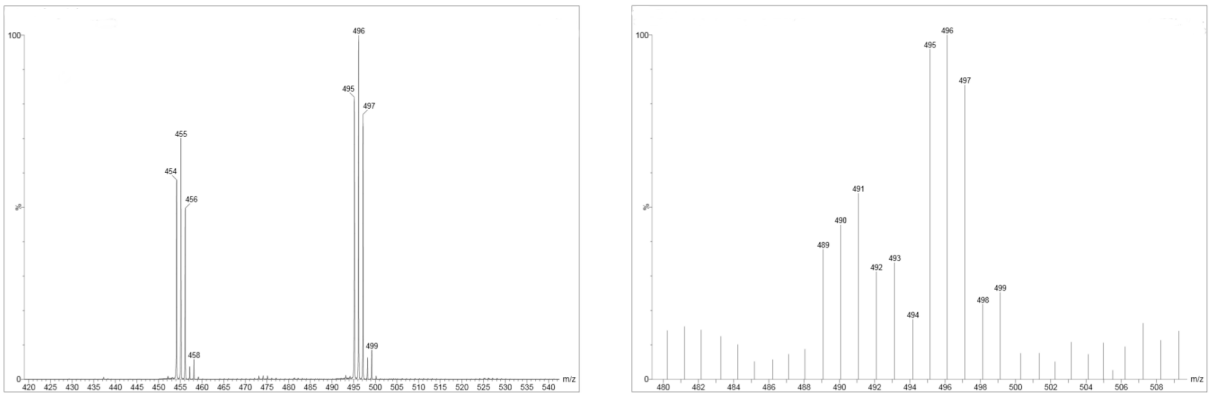


Fig. S4. Left: Electrospray mass spectrum of complex PtL^3Cl, showing $[M-(HCl)-Cl]^+$ ion at m/z = 455 and the corresponding acetonitrile adduct at m/z = 496 (both splitting patterns consistent with presence of one Pt and no chlorides). **Right**: ASAP (solids probe) mass spectrum of PtL^3Cl, showing the $[M-(HCl)]^+$ ion at m/z = 491 (splitting pattern consistent with the presence of one Pt and one chloride), and the $[M-(HCl)-Cl]^+$ acetonitrile adduct.

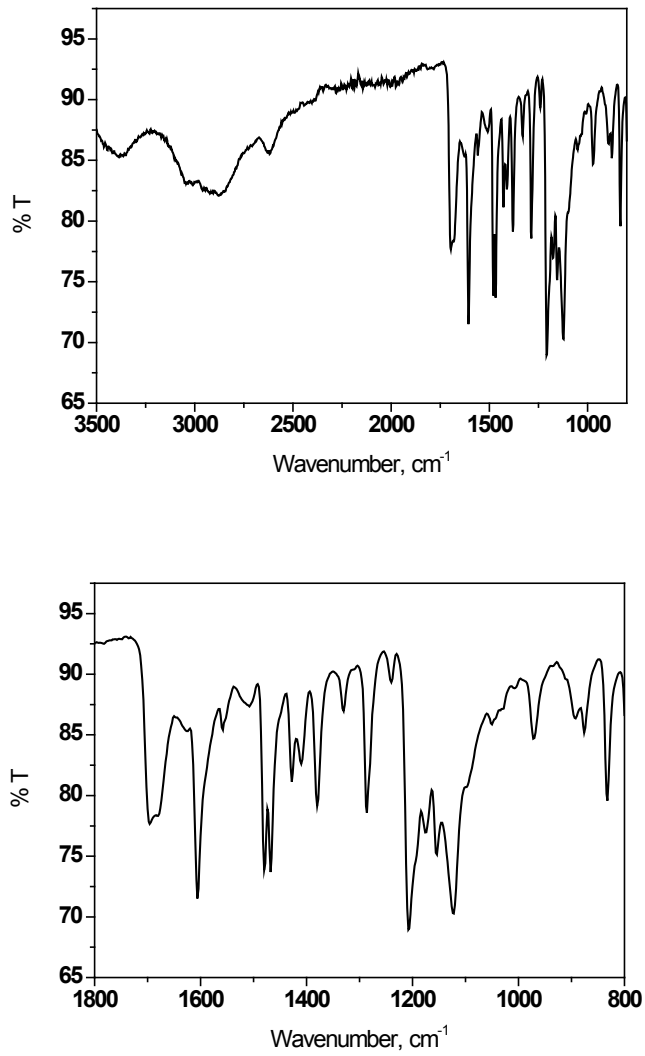


Fig. S5. Infrared spectrum of complex PtL^3Cl (recorded as a solid). Top panel – full spectrum, bottom panel – expanded fingerprint region.

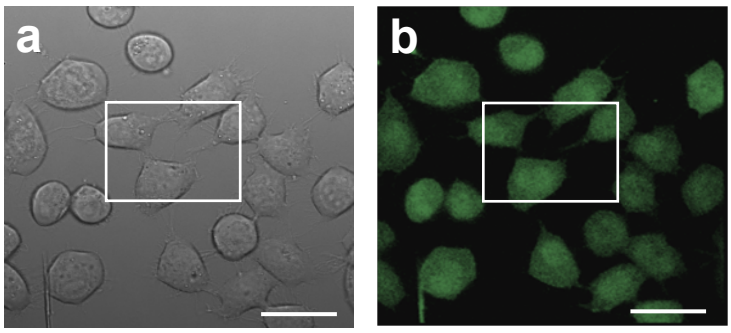


Fig. S6. CHO-K1 cells labelled with PtL^1Cl: (a) Steady-state transmission image; (b) confocal emission image, 488 nm excitation / 515 nm detection. The highlighted area has been investigated by TP-TREM (Fig. 2 in the main text). Scale bar 20 μm.

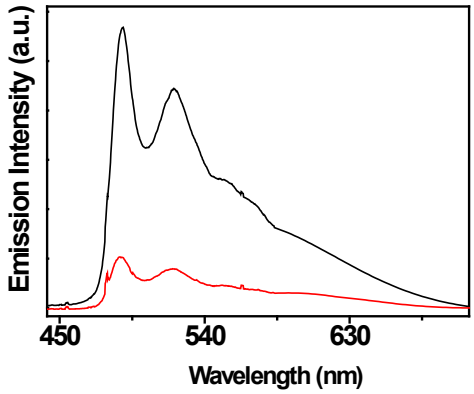


Fig. S7. Emission spectra from live CHO cells labelled with 100 μM PtL^3Cl in PBS, 5 min incubation time: black, nucleus; red, cytoplasm.

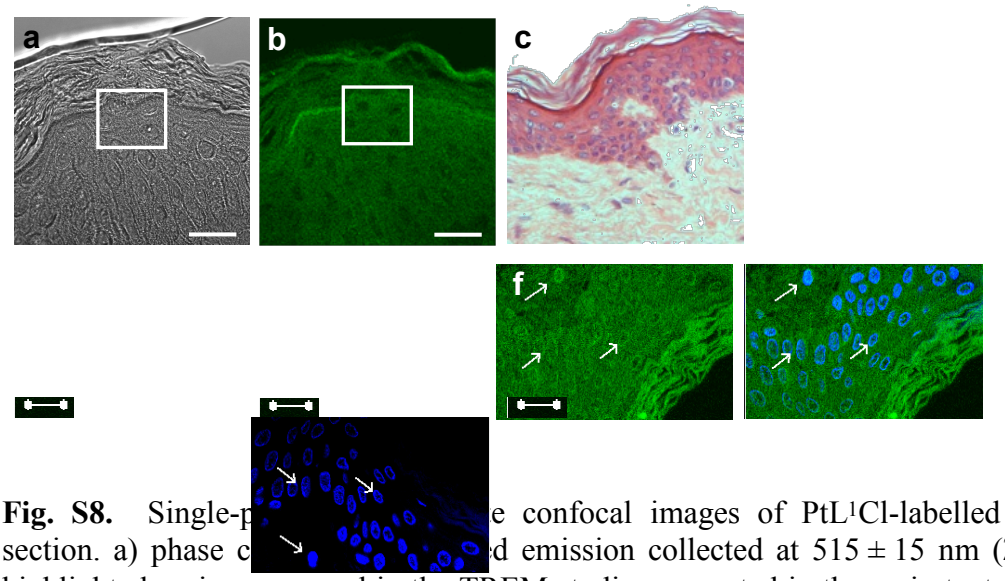


Fig. S8. Single-p e confocal images of PtL^{1}Cl-labelled rat skin tissue section. a) phase c d emission collected at 515 ± 15 nm (λ_{ex} 488nm); the highlighted region was used in the TREM studies presented in the main text. c) H&E stained skin section (×40 magnification), showing distribution of cell nuclei. d-f) Steady-state confocal images of PtL^{1}Cl and DAPI co-stained skin section, d) DAPI emission (λ_{ex} 800 nm, λ_{em} 436-485 nm), e) PtL^{1}Cl emission (λ_{ex} 488 nm, λ_{em} 530 ± 20 nm), f) overlay of (d) and (e). Arrows highlight cell nuclei. Scale bar 20 μm.

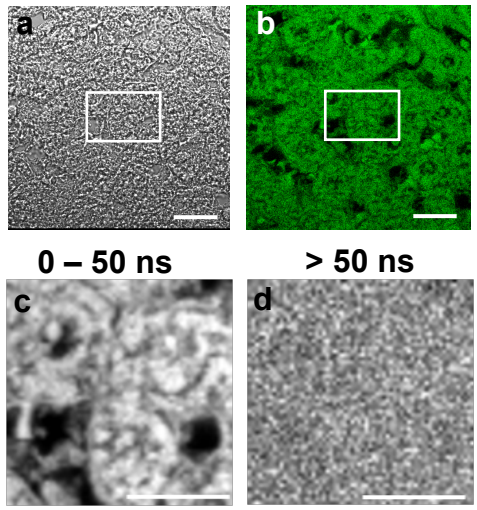


Fig. S9. Steady-state confocal images of unlabelled rat liver tissue section obtained under one-photon excitation. a) Phase contrast, b) auto-fluorescence λ_{em} 515±15 nm (λ_{ex} 488nm); the highlighted region was used in TREM studies (c and d). Time-gated intensity images obtained under two-photon excitation (λ_{ex} 760 nm) of unlabelled liver tissue section, recorded over the time intervals of c) 0-50 ns and d) > 50 ns.

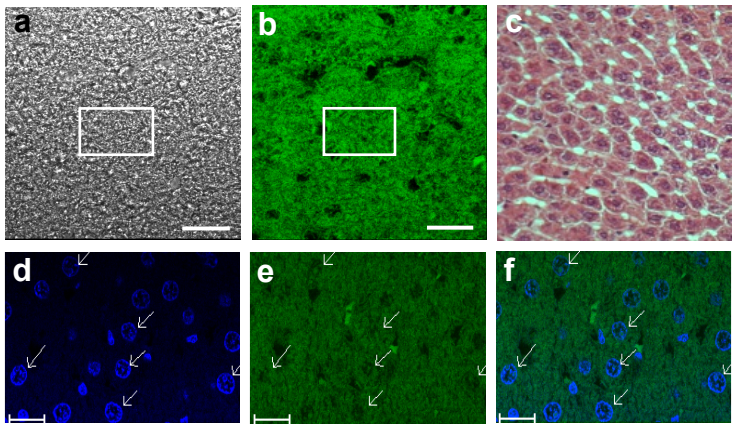


Fig. S10. Steady-state confocal images of PtL^{1}Cl-labelled liver tissue section obtained under one-photon excitation. a) Phase contrast, b) Pt(II) emission registered in the region 515±15 nm (λ_{ex} 488nm); the highlighted region was used in TREM studies presented in the main text. c) H&E stained liver section (×40 magnification), showing distribution of cell nuclei (purple). d-f) Steady-state confocal images of PtL^{1}Cl and DAPI co-stained liver section, d) DAPI emission (λ_{ex} 800 nm, λ_{em} 436-485 nm), e) PtL^{1}Cl emission (λ_{ex} 488 nm, λ_{em} 530±20 nm), f) overlay of d) and e). Arrows highlight cell nuclei. Scale bar 20 μm.

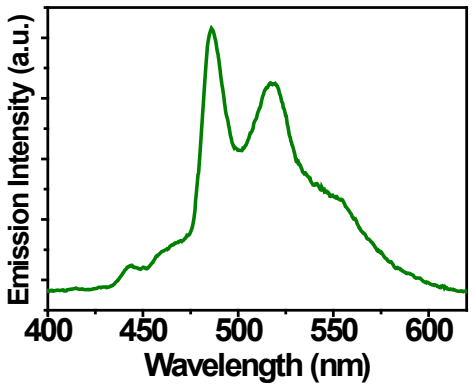


Fig. S11. Liver tissue section labelled with PtL^{1}Cl. Emission spectrum from a cellular region (position 1 in Fig. 4c, main text). The features in the 400 – 450 nm region are due to tissue autofluorescence.

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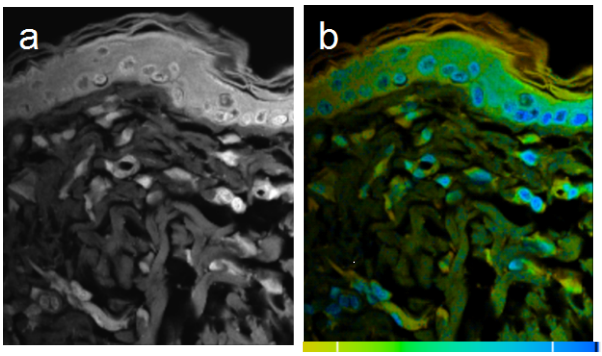

Fig. S12. Skin tissue sections incubated with 100 μM solution of water-soluble PtL^3Cl in PBS, 5 min at r.t. **a** – intensity image obtained by integration of the kinetic data of the long-lived (Pt-based) component; **b** – corresponding emission lifetime map, 2 – 10 μs.

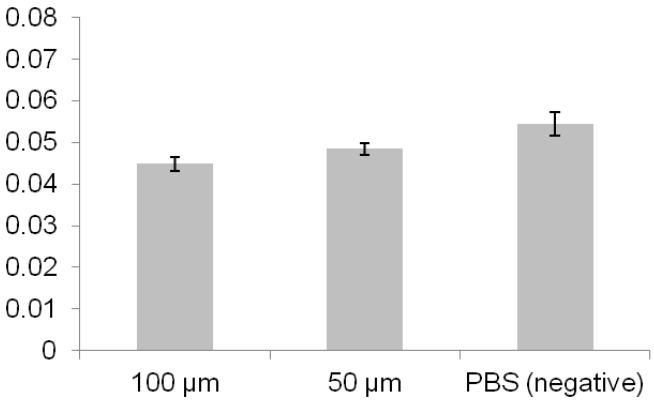

Fig. S13. HDF cell viability determined by MTT, immediately after incubation with PtL^3Cl. Cells were incubated for 10 min with PtL^3Cl (in PBS) at the two different concentrations, covering the range normally used for imaging. Cells were washed with PBS (x3) then treated with MTT directly. No significant difference between test and control conditions was observed (mean±SEM, n=3).

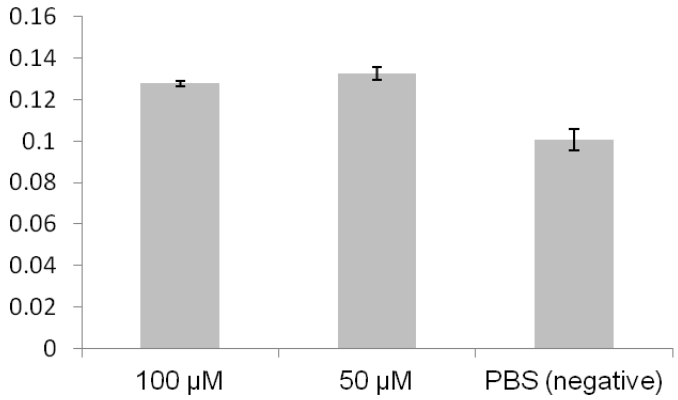

Fig. S14. HDF cell viability determined by MTT, 1h after incubation with PtL^3Cl. Cells were incubated for 10 min with PtL^3Cl (in PBS) at the two different concentrations indicated, covering the range normally used for imaging. Cells were washed with PBS (x 3), and then incubated with fresh culture medium for 1 h. No significant difference between test and control conditions was observed (mean±SEM, *n*=3).

Supplementary Video:
Z-stack of the images of CHO cells incubated with PtL^1Cl (1% DMSO/PBS) at room temperature for 5 min, and then washed with PBS, recorded under 780 nm excitation, 250 KHz repetition rate of ~120 fs pulses from the cavity dumped Ti:Sapph laser (see Experimental section for details). The total intensity shown for each slice is obtained by binning all emission decay photons per pixel as collected between successive laser pulses.