Multi-Modal Probes: Super-Resolution and TEM Imaging of Mitochondria, and Oxygen Mapping of Cells, Using Small-Molecule Ir(III) Luminescent Complexes


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SYNTHESIS

All reagents and solvents, unless otherwise stated, were purchased from commercial sources and used as received. $^1$H NMR spectra were recorded using a Bruker AV1-400 MHz spectrometer, chemical shifts are quoted in ppm. All ES mass spectra were recorded with a Micromass LCT ES-TOF mass spectrometer. Photoluminescence spectra were recorded using a Horiba Jobin Yvon Fluoromax-4 Spectrofluorometer. Absorption spectra were measured on Varian Cary 50 Bio UVVisible Spectrophotometer. Time-resolved luminescence measurements in solution at x10$^{-5}$M were performed using Edinburgh Instruments Mini-τ instrument fitted with a 405 nm pulsed laser excitation source.

(Pyridine-2-yl) amidrazone

The synthetic procedure for (pyridine-2-yl) amidrazone was taken from literature.\(^1\) 2-Pyridinecarbonitrile (Sigma Aldrich) (10.3 g, 0.09 mol) was melted and hydrazine monohydrate (5.4 g, 0.09 mol) was added to this and stirred. Ethanol (4 mL) was added to the mixture and then stirred for 16 h at room temperature. The solvent was removed under vacuum and the solid was suspended in petroleum ether (50 mL), cooled in an ice bath, filtered and washed with cold petroleum ether to give a pale yellow solid (8.2 g, 67%).

$^1$H NMR (400 MHz, CDCl$_3$, 298 K): $\delta^H$ 8.53 (ddd, $J$ = 4.9, 1.7, 1.0 Hz, 1H), 8.03 (dt, $J$ = 8.1, 1.1 Hz, 1H), 7.71 (ddd, $J$ = 8.0, 7.5, 1.8 Hz, 1H), 7.30 (t, $J$ = 1.7 Hz, 1H), 7.27 (dd, $J$ = 4.9, 1.2 Hz, 1H), 5.32 (s, 2H). ES-MS: m/z = 137 (MH$^+$).

$L$$_{tol}$

The synthetic procedure for L$_{tol}$ was taken from literature.\(^1\) (Pyridine-2-yl) amidrazone (2.0 g, 0.015 mol) was added to a flame-dried, nitrogen-purged Schlenk tube. To this dry 1,3-Dimethylamylamine (15 mL) and THF (5 mL) were added and the suspension was cooled to 0°C. In a separate flask, p-toluoyl chloride (Sigma Aldrich) (2.3 g, 0.015 mol) was dissolved in dry DMAA (5 mL). This was then cooled and added drop wise to the other mixture and stirred. The mixture was slowly warmed to room temperature and stirred for an addition 5 h. CH$_2$Cl$_2$ (50 mL) was then added resulting in precipitate. This was filtered and the precipitate was heated to 190°C for 25 min in glycerol. Upon cooling H$_2$O (50 mL) was added, resulting in pale yellow precipitate, which was filtered. The product was then obtained by column chromatography on silica gel (200-300 mesh) using CHCl$_3$/methanol (95:5) as the eluent to yield a white solid (2.6 g, 74%).

$^1$H NMR (400 MHz, CDCl$_3$, 298 K): $\delta^H$ 8.84 (d, $J$ = 4.8 Hz, 1H), 8.34 (d, $J$ = 7.9 Hz, 1H), 8.13 (d, $J$ = 8.2 Hz, 2H), 7.93 (td, $J$ = 7.8, 1.7 Hz, 1H), 7.50 (ddd, $J$ = 7.6, 4.8, 1.1 Hz, 1H), 7.36 (d, $J$ = 8.0 Hz, 2H), 2.46 (s, 3H). ES-MS: m/z = 237 (MH$^+$).

$L$$_{pytz}$

The synthetic procedure for L$_{pytz}$ was taken from literature.\(^2\) A 250 mL round bottom flask was charged with 4-amino-3,5-di-2-pyridyl-4H-1,2,4-triazole (Sigma Aldrich) (1.0 g, 4.2 mmol), HNO$_3$ (10 mL, 5 M) and the mixture was stirred at 40°C for 30 min. The
solution was cooled to 0°C and NaNO$_2$ (2.0 g in 10 mL of H$_2$O) was added drop wise. The mixture was maintained at 0°C and stirred for a further 30 min. NH$_3$OH (3 M) was then added drop wise until the mixture was alkaline and a white precipitate formed. The precipitate was filtered and dried to give white crystals of L$_{pytz}$ (0.85 g, 91%).

$^1$H NMR (400 MHz, MeOD, 298 K): δH 8.72 (d, $J = 4.4$ Hz, 2H), 8.27 (d, $J = 7.9$ Hz, 2H), 8.00 (td, $J = 7.8$, 1.7 Hz, 2H), 7.51 (ddd, $J = 7.6$, 4.9, 1.1 Hz, 2H). ES-MS: $m/z = 224$ (MH$^+$).

TosylPEG

The synthetic procedure for TosylPEG was taken from literature. Triethylene glycol monomethyl ether (Sigma Aldrich) (3.01 g 18 mmol), $p$-toluenesulfonyl chloride (Sigma Aldrich) (3.51 g 18 mmol) and 4-dimethylaminopyridine (Sigma Aldrich) (cat.) were added to a two-neck round bottom flask and flushed with N$_2$ (g). To this, CH$_2$Cl$_2$ (80 mL) was added, followed by triethylamine (Sigma Aldrich) (2.22 g, 22 mmol) and the reaction was stirred at room temperature for 18 h. The reaction was quenched by the addition of H$_2$O (75 mL). The organic layer was then washed with H$_2$O (2 × 75 mL). The organic layer was then dried (MgSO$_4$), filtered and the solvent removed under vacuum to give a colourless oil (5.2 g, 90%).

$^1$H NMR (400 MHz, CDCl$_3$, 298 K): δH 7.75 (d, $J = 8.3$ Hz, 2H), 7.31 (d, $J = 8.1$ Hz, 2H), 4.12 (dd, 2H), 3.64 (dd, 2H), 3.59 – 3.53 (m, 6H), 3.49 (dd, $J = 5.7$, 3.3 Hz, 2H), 3.33 (s, 3H), 2.41 (s, 3H). ES-MS: $m/z = 319$ (MH$^+$).

[Ir$_2$(Ald)$_4$Cl$_2$]

The synthetic procedure for [Ir$_2$(Ald)$_4$Cl$_2$] was modified from literature. 4-(2-pyridyl) benzaldehyde (Sigma Aldrich) (0.84 g, 4.6 mmol) was dissolved in a mixture of 2 ethoxyethanol (30 mL) and water (10 mL). To this iridium trichloride hydrate (Alfa Aesar) (0.3 g, 1.0 mmol) was added. The round bottom flask was covered in foil and heated to reflux for 18 h. The solution was cooled to room temperature and the bright red precipitate was collected on a glass filter frit. The precipitate was washed with ethanol (40 mL) and H$_2$O (10 mL) and dried to give [Ir$_2$(Ald)$_4$Cl$_2$] (0.58 g, 48%).

$^1$H NMR (400 MHz, DMSO-d$_6$, 298 K): δH 9.88 (d, $J = 5.7$ Hz, 2H), 9.66 (s, 2H), 9.61 – 9.56 (m, 4H), 8.48 (d, $J = 7.6$ Hz, 2H), 8.40 (d, $J = 7.9$ Hz, 2H), 8.26 (ddd, $J = 7.8$, 1.3 Hz, 1H), 8.17 (ddd, 8.17, $J = 7.8$, 1.3 Hz, 1H), 8.07 (d, $J = 8.1$ Hz, 1H), 8.03 (d, $J = 8.0$, 1H), 7.74 (ddd, $J = 7.6$, 5.7, 1.2 Hz, 1H), 7.65, ddd, $J = 7.6$, 5.7, 1.2 Hz, 1H), 7.44 (dd, $J = 7.8$, 1.4 Hz, 1H), 7.40 (dd, $J = 7.8$, 1.4 Hz, 1H), 6.75 (d, $J = 1.3$ Hz, 2H), 6.14 (d, $J = 1.3$ Hz, 2H). ES-MS: $m/z = 557$ (MH$^{+2}$).

ES-MS: $m/z = 319$ (MH$^+$).
[Ir(Ald)_2L_{pytz}]

The synthetic procedure for [Ir(Ald)_2L_{pytz}] was modified from literature. A two-neck 250 mL round bottom flask was flushed with N₂ and charged with L_{pytz} (0.28 g, 1.3 mmol), MeOH (90 mL) and CH₂Cl₂ (45 mL). To this [Ir(Ald)_2Cl₂] (0.51 g, 0.42 mmol) was added. The flask was covered in foil and heated to reflux for 18 h under N₂. The mixture was cooled to room temperature and the solvent was removed. The crude was columned twice on alumina gel (Brockmann Grade III), first using CH₂Cl₂/MeOH (99:1) and then using CH₃CN/H₂O (99:1) as the eluents, to give an orange precipitate (0.25 g, 78%).

1H NMR (400 MHz, CDCl₃, 298 K): δH 9.75 (s, 1H), 9.70 (s, 1H), 8.68 (d, J = 4.6 Hz, 1H), 8.49 (d, J = 7.9 Hz, 1H), 8.13 (d, J = 7.9 Hz, 1H), 8.03 - 7.96 (m, 3H), 7.89, (ddd, J = 7.8, 7.8, 1.5 Hz, 4H) 7.71 (d, J = 5.7 Hz, 1H), 7.69 – 7.62 (m, 2H), 7.52 (ddd, J = 8.0, 1.5 Hz, 1H), 7.47 (dd, J = 8.0, 1.5 Hz, 1H), 7.17 (ddd, J = 7.6, 4.9, 1.0 Hz, 1H), 7.14 (ddd, J = 7.6, 5.6, 1.2 Hz), 7.09 (dd, J = 7.6, 5.7, 1.2 Hz), 7.03 ((dd, J = 7.6, 5.7, 1.2 Hz), 6.81 (d, J = 1.4 Hz, 1H), 6.79 (d, J = 1.4 Hz, 1H). ES-MS: m/z = 780 (MH⁺).
The synthetic procedure of reduction of the aldehyde groups was modified from literature. [Ir(ppy-Ald)$_2$L$_2$pytz] (0.25 g, 0.33 mmol) was dissolved in CH$_2$Cl$_2$ (15 mL) this was then added to a solution of ethanol (50 mL) followed by sodium borohydride (0.04 g, 1.1 mol). The reaction was stirred at room temperature for 30 min. The solvent was removed under vacuum and the mixture was dissolved in CH$_2$Cl$_2$ /H$_2$O (30 mL/ 100 mL). Sodium carbonate (0.17 g, 1.6 mol) was then added and the mixture was stirred at room temperature for 30 min. The organic layer was extracted into CH$_2$Cl$_2$ (5 × 30 mL), combined, dried over MgSO$_4$ and the solvent was removed under vacuum. The crude product was columned on alumina gel (Brockmann Grade III) using CH$_2$Cl$_2$/MeOH (95:5) to give a yellow precipitate (0.24 g, 92%).

$^1$H NMR (400 MHz, CDCl$_3$, 298 K): δH 8.67 (ddd, J = 4.9, 1.8, 0.8 Hz, 1H), 8.53 (bs, 1H), 8.19 (bd, J = 7.8 Hz), 7.91 – 7.82 (m, 4H), 7.76 (d, J = 5.3 Hz, 2H), 7.70 – 7.64 (m, 4H), 7.55 (d, J = 5.6 Hz, 2H), 7.21 (dd, J = 7.5, 5.3 Hz, 2H), 7.15 (ddd, J = 7.5, 5.6, 1.2 Hz, 2H), 7.03 (td, J = 8.7, 1.7 Hz, 2H), 6.94 (ddd, J = 7.3, 5.7, 1.2 Hz, 2H), 6.89 (ddd, J = 7.3, 5.7, 1.2 Hz, 2H), 6.40 (d, J = 1.3 Hz, 1H), 6.34 (d, J = 1.3 Hz, 1H), 4.48 (s, 2H), 4.43 (s, 2H). ES-MS: m/z = 784 (MH$^+$).
Figure S3: $^1$H NMR (400 MHz, CDCl$_3$) spectrum of [Ir(OH)$_2$L$_{pytz}$]

[Ir-L$_{pytz}$]

A round bottom flask was flushed with N$_2$ and to this NaH (0.3 g, 0.013 mol) was added. 100 mL of hexane was added and the mixture was stirred for 5 min. The hexane was then removed and dry THF was added (50 mL). DMF (50 mL) and [Ir(OH)$_2$L$_{pytz}$] (0.24 g, 0.3 mmol) was then added and the mixture was stirred under N$_2$ for 30 min. TosylPEG (1.5 g, 4.7 mmol) and Bu$_4$NI (Sigma Aldrich) (cat.) were then added and the mixture was heated to reflux for 18 h. The reaction was allowed to cool and unreacted NaH was quenched with MeOH (10 mL). The solvent was removed under vacuum and the crude mixture was dissolved in CH$_2$Cl$_2$ (50 mL). The organic layer was washed with H$_2$O (5 × 40 mL), dried over MgSO$_4$ and the solvent was removed under vacuum. The crude was columned on alumina gel (Brockmann Grade III) using CH$_2$Cl$_2$/MeOH (99:1). The product was obtained through further purification by analytical HPLC in 60% H$_2$O (0.1% TFA) and 40% CH$_3$CN (retention time = 6 min) to give a yellow precipitate (0.078 g, 24%).

$^1$H NMR (400 MHz, CDCl$_3$, 298 K): $\delta$H 8.76 (d, $J = 4.8$ Hz, 1H), 8.70 (d, $J = 7.9$ Hz, 1H), 8.23 (d, $J = 7.8$ Hz, 1H), 8.04 (ddd, $J = 7.8$, 7.8, 1.3 Hz, 1H). 7.96 (ddd, $J = 7.8$, 7.8, 1.3 Hz,
1H), 7.94 – 7.87 (m, 2H), 7.84, (d, J = 5.3 Hz, 1H), 7.79 – 7.70 (m, 2H), 7.34, (ddd, J = 8.0, 5.7, 0.8 Hz, 1H), 7.09-6.99 (m, 3H), 6.95 (ddd, J= 7.5, 5.7, 1.2 Hz, 1H), 6.2s (bs, 2H), 4.44 – 4.27 (m, 4H), 3.69 – 3.46 (m, 24H), 3.40 – 3.31 (m, 6H). **ES-MS:** m/z = 1076 (MH+).

**Elemental analysis** (IrC_{49}H_{36}N_{7}O_{8}): found (%): C: 53.7; H: 5.9; N: 8.7; calculated with two coordinated H2O molecules (%): C: 54.0; H 5.5; N: 8.8.

**Figure S4:** $^1$H NMR (400 MHz, CDCl$_3$) spectrum of Ir-L$_{pytz}$

**[Ir(Ald)$_2$L$_{tol}$]**

The synthetic procedure for [Ir(Ald)$_2$L$_{tol}$] was modified from literature. A two-neck 250 mL round bottom flask was flushed with N$_2$ and charged with L$_{tol}$, MeOH (90 mL) and CH$_2$Cl$_2$ (45 mL). To this [Ir$_2$(Ald)$_4$Cl$_2$] (0.51 g, 0.42 mmol) was added. The flask was covered in foil and heated to reflux for 18 h under N$_2$. The mixture was cooled to room temperature and the solvent was removed. The crude was columned twice, first on silica gel (200-300 gel mesh) using CH$_2$Cl$_2$/MeOH (98:2) as the eluent, and then on alumina gel (Brockmann Grade III), using CH$_2$Cl$_2$ as the eluent to give an orange precipitate (0.2 g, 60%).
$^1$H NMR (400 MHz, CDCl$_3$, 298 K): $\delta$H 9.76 (s, 1H), 9.69 (s, 1H), 8.35 (d, $J = 7.8$ Hz, 1H), 8.04 – 7.98 (m, 5H), 7.87 (td, $J = 7.5$, 1.5 Hz, 1H), 7.84 – 7.74 (m, 4H), 7.70 (d, $J = 4.7$ Hz, 1H), 7.64 (d, $J = 5.7$ Hz, 1H), 7.53 (dd, $J = 8.0$, 1.7 Hz, 1H), 7.46 (dd, $J = 8.0$, 1.7 Hz, 1H) 7.14, (d, $J = 8.0$ Hz, 2H), 7.13–7.08 (m, 4H), 7.03 (ddd, $J = 7.5$, 5.7, 1.4 Hz, 1H) 6.86 (d, $J = 1.5$ Hz, 1H) 6.80 (d, $J = 1.5$ Hz, 1H), 2.33 (s, 3H). ES-MS: $m/z = 792$ (MH$^+$).

Figure S5: $^1$H NMR (400 MHz CDCl$_3$) spectrum of [Ir(Ald)$_2$L$_{tol}$]

[Ir(OH)$_2$L$_{tol}$]

The synthetic procedure of reduction of the aldehyde groups was modified from literature.$^{6}$ [Ir(Ald)$_2$L$_{tol}$] (0.2 g, 0.25 mmol) was dissolved in CH$_2$Cl$_2$ (15 mL) this was then added to a solution of ethanol (50 mL) followed by sodium borohydride (0.038 g, 1.0 mmol). The reaction was stirred at room temperature for 30 min. The solvent was removed under vacuum and the mixture was dissolved in CH$_2$Cl$_2$/H$_2$O (30 mL/ 100 mL). Sodium carbonate (0.13 g, 1.3 mmol) was then added and the mixture was stirred at room temperature for 30 min. The organic layer was extracted into CH$_2$Cl$_2$ (5 × 30 mL), combined, dried over MgSO$_4$ and the solvent was removed under vacuum. The crude product was columned on alumina gel (Brockmann Grade III) using CH$_2$Cl$_2$/MeOH (98:2) to give a yellow precipitate (0.22 g, 62%).
**1H NMR (400 MHz, CDCl₃, 298 K):** δH 8.26 (d, J = 7.9 Hz, 1H), 8.01 (d, J = 7.9 Hz, 2H), 7.91 (d, J = 5.4 Hz, 1H), 7.82 (m, 4H), 7.64 – 7.48 (m, 5H), 7.13 (d, J = 7.6 Hz, 2H), 7.06 (dd, J = 7.6, 6.3 Hz, 1H), 6.97, (d, J = 7.6 Hz, 1H), 6.92 – 6.81 (m, 3H), 6.35 (d, J = 20.7 Hz, 2H), 4.36 (d, J = 4.0 Hz, 2H), 4.33 (d, J = 4.0 Hz, 2H), 2.33 (s, 3H).

**ES-MS:** m/z = 796 (MH⁻).

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**Figure S6:** ¹H NMR (400 MHz, CDCl₃) spectrum of [Ir(OH)₂L₉tol]

**Ir-L₉tol**

A round bottom flask was flushed with N₂ and to this NaH (0.3 g, 0.013 mol) was added. 100 mL of hexane was added and the mixture was stirred for 5 min. The hexane was then removed and dry THF was added (50 mL). DMF (50 mL) and [Ir(OH)₂L₉tol] (0.22 g, 0.28 mmol) was then added and the mixture was stirred under N₂ for 30 min. TosylPEG (1.3 g, 4.2 mmol) and Bu₄NI (Sigma Aldrich) (cat.) were then added and the mixture was heated to reflux for 18 h. The reaction was allowed to cool and unreacted NaH was quenched with MeOH (10 mL). The solvent was removed under vacuum and the crude mixture was dissolved in CH₂Cl₂ (50 mL). The organic layer was washed with H₂O (5 × 40 mL), dried.
over MgSO₄ and the solvent was removed under vacuum. The crude was columned on silica gel (200-300 mesh) using CH₂Cl₂/MeOH (98:2). The product was obtained through further purification by analytical HPLC in 60% H₂O and 40% CH₃CN (retention time = 6 min) to give a yellow precipitate (0.062 g, 21%).

**1H NMR (400 MHz, CDCl₃, 298 K):** δH 8.29 (d, J = 7.5 Hz, 1H), 8.02 (d, J = 8.0 Hz, 2H), 7.97 - 7.90 (m, 4H), 7.73 (d, J = 5.4 Hz, 1H), 7.71 - 7.60 (m, 4H), 7.54 (d, J = 5.5 Hz, 1H), 7.16 (ddd, J = 7.8, 5.5, 1.2 Hz, 1H), 7.00 (dd, J = 7.3, 1.5 Hz, 2H, 6.92 (ddd, J = 7.3, 5.6, 1.0 Hz, 1H, 6.58 (ddd, J = 7.3, 5.6, 1.0 Hz, 1H), 4.40-4.30 (m, 4H) 3.78 - 3.46 (m, J = 21.7, 15.3, 9.5, 4.8 Hz, 24H), 3.39 (t, J = 4.3 Hz, 6H), 2.34 (s, 3H). **ES-MS:** m/z = 1090 (MH⁺). **HRMS ES⁺:** Calculated for IrC₅₂H₆₀N₆O₈: 1089.4102; Found: 1089.4102.

*Figure S7: 1H NMR (400 MHz, CDCl₃) spectrum of Ir-L*
EMISSION SPECTRA

**Figure S8:** Emission spectra of Ir-L$^{pytr}$ at 298 K in H$_2$O (blue line, excitation at 436 nm), CH$_2$Cl$_2$ (black line, excitation at 446 nm), toluene (green line, excitation at 448 nm) and CH$_3$CN (red line, excitation at 446 nm). The optical density was 0.1 in every case so intensities are directly comparable.

**Figure S9:** pH response curve (left) and emission spectra (right) and for Ir-L$^{pytr}$ from pH 2 to 14.

CELL CULTURE AND IMAGING

**Cell culture**

HeLa cells were cultured at 37°C in a humidified 5% CO$_2$ / 95% air (v/v) environment in Dulbecco’s modified Eagle’s medium (DMEM, Aldrich), supplemented with 5 mL L-glutamine (200 mM solution) and 50 mL fetal calf serum (10%). Cells were cultured as monolayers in T-75 flasks and passaged using trypsin-EDTA.

For steady-state confocal and PLIM imaging, cells were seeded on 35 mm glass bottomed dishes (Mat Tek Corporation) until approximately 60% confluent. After the removal of growth media, the cells were washed with PBS (1 mL / well) and then stained with 50 μM Ir-L$^{pytr}$ or Ir-L$^{tol}$ in full DMEM (<1% DMSO) at 37 °C for 4 h, washed with PBS (1 mL), and immersed in phenol red-free media for imaging.

For 3D SIM, cells were seeded in 6-well plates on high precision coverslips and grown to ~60 % confluency. After the growth media was removed, the cells were washed with PBS (1
x 1 mL) and then treated with 50 µM Ir-L^{pyr} or Ir-L^{tol} in full DMEM (<1% DMSO) at 37 °C for 2 h. The media was then removed, the cells washed with PBS (1 x 1 mL) and then fixed using 4% paraformaldehyde in PBS for 10 minutes. Finally, the cover slips were dipped in deionised water to remove any salts on the cover slip, mounted on microscope slides using Slowfade® gold antifade reagent and then imaged.

**Oxygen Sensing**

Gas mixtures of O₂ and N₂ (flow rate: 100 ml/min) were controlled using Mass View flow meters (MV-302, Bronkhorst). Samples were equilibrated for 20 minutes (at RT) with each gas mixture, using a bespoke lid (with inlet and outlet), before PLIM imaging.

*Two-Photon PLIM imaging.* A multi-photon (760 nm) phosphorescence lifetime imaging unit comprised an adapted Becker & Hickl DCS120 confocal scanning system and Coherent Mira 900F laser pumped by a Verdi V10, connected to a Nikon Ti-E inverted microscope, was used to live HeLa cells stained with Ir-L^{pyr} under varying levels of O₂. A water immersion 60 × (NA 1.2) objective was used for all samples. Emission light was collected from 485-650 nm using appropriate filters.

*PLIM Data collection and processing.* A PLIM imaging window of 24 µs was used for all samples. Regions of interest (whole field of view for solutions, individual cells for *in-vitro* studies) were analysed in SPCImage (Becker & Hickl software, version 5.0) and in Origin (version 6.0). In all cases the data was most appropriately fitted to a double exponential decay model. Reported lifetimes and lifetime maps depict the major emission component τ₁. A thresholding function within the analysis software ensured that non-correlating photons leading to background noise arriving at the detector were not included in the analysis. Pixel binning was set to 3 for all samples. Lifetime maps represent τ₁ values and are presented as a rainbow colour continuum without further image processing.

*Live Cell Emission spectra* under two-photon excitation were recorded by sending the emission signal from a specific pixel position through a spectrometer to a CCD detector setup (Andor iDUS) at another port of the microscope.
Figure S10: MTT toxicity data for \textbf{Ir-L}^{Pyrz} and \textbf{Ir-L}^{tol} in HeLa cells at 10 – 75 \, \mu M after 4 hour incubations. Control cells: 0.1\% DMSO in DMEM.

Figure S11: Steady-state confocal images of HeLa cells stained with \textbf{Ir-L}^{Pyrz} (left) and \textbf{Ir-L}^{tol} (right), showing typical staining pattern after 4h at 50 \, \mu M.
Figure S12: Steady-state confocal images of HeLa cells treated with Ir-L^Pytz (top) and Ir-L^tol (bottom) (50 µM, 4 h) and cellLight ER-RFP tracker (30 p/c). Scale bar = 10 µm.

Figure S13: Confocal microscopy images of MitoTracker Orange in HeLa cells at 400 nM (35 minutes), clear nucleoli staining is observed at this concentration. Left: λ<sub>ex</sub> = 561 nm, λ<sub>em</sub> = <590; Right: λ<sub>ex</sub> = 405 nm, λ<sub>em</sub> = 475 - 575 nm; no cross talk observed when using Ir(III) excitation and detection parameters.
Figure S14: Confocal images of HeLa cells treated with Ir-L_{pytz} (50 µM, 4 h) and LysoTracker red (50 nM, 35 mins). (A) Ir-L_{pytz} only, (B) LysoTracker® red (C) overlay. Scale bar = 10 µm.

Figure S15: 3D SIM images of HeLa cells treated with Ir-L_{pytz} (50 µM, 4 h, green). (A) Zoomed single Z-slice displaying emission from Ir-L_{pytz}, (B) 2D slice of whole cell displaying emission from Ir-L_{pytz}, (C) Emission line profile (yellow line on image A), showing relative intensities of Ir-L_{pytz} emission at the edge and centre of two mitochondria. Emission intensities are slightly higher at the mitochondrial membrane (edge) in comparison to the centre of the mitochondria. The relative difference between centre and edge is very similar to that observed in the presence of MitoTracker® Orange.
**Figure S16**: Single Z slice from a 3D rendered SIM image of fixed HeLa cells stained with Ir-L\textsuperscript{tol} (50 µM, 4h). Processing defect due to complex precipitate (white arrow). Mitochondrial staining (yellow box). Scale bar = 5 µm.

**Figure S17**: 3D rendered SIM images of fixed HeLa cells treated with Ir-L\textsuperscript{pytz} (50 µM, 4 h, green) and MitoTracker® Orange (100 nm, 20 mins, magenta). Full cell (left), zoomed view (right). White arrows: co-staining, blue arrows: nuclear membrane staining from Ir-L\textsuperscript{pytz}, red boxes: regions of Ir-L\textsuperscript{pytz} emission only.
**Figure S18**: 3D-SIM Z-stack: Mean intensity graph of all raw slices form HeLa cells treated with **Ir-L\textsuperscript{pytz}** (50 µM, 4 h, green) and MitoTracker® Orange (100 nM, 20 mins, red). Red line shows significant decrease in emission intensity per-image slice. Green line remains at the same intensity per-image slice.

**Figure S19**: TEM images of HeLa cells stained with **Ir-L\textsuperscript{pytz}** (top) and **Ir-L\textsuperscript{tol}** (bottom), without contrast agents (left), with UC and LC only (right). Damage to cell (blue arrow) is due to the lack of OsO\textsubscript{4}. Imaging without OsO\textsubscript{4} was necessary to confirm the contrast enhancement brought about by addition of Ir complexes only.
Figure S20: PLIM imaging of Live HeLa cell stained with $\text{Ir-L}^{\text{pyz}}$ under atmospheric conditions. a) Black and white intensity image (all photons binned into one time-channel). b) Rainbow-coloured lifetime map showed major component ($\tau_1$) across whole cell. c) $\tau_1$ lifetime distribution of cellular mitochondria, average $\tau_1 = 830$ ns.

References