Electronic Supporting information for

A new ligand skeleton for imaging applications with d/f complexes: combined lifetime imaging and high relaxivity in an Ir/Gd dyad

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Contents

A. General Experimental Section

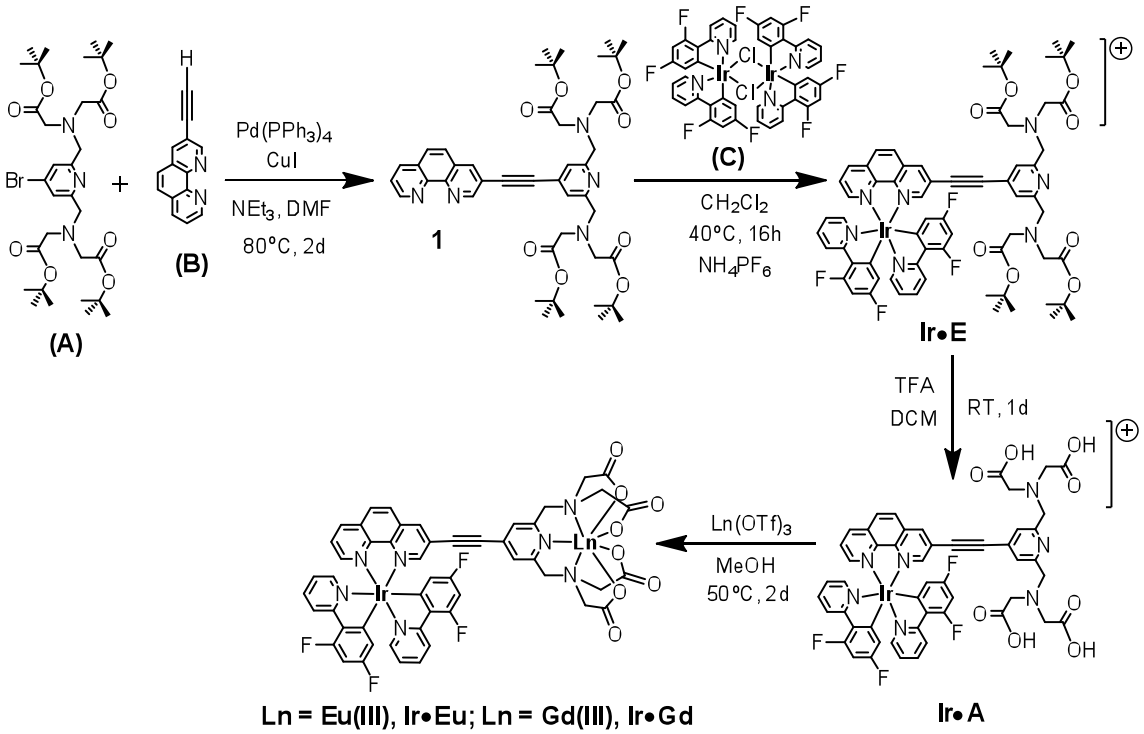
Materials. 1,10-phenanthroline, trimethylsilylacetylene, triethylamine, 2,4-difluoro(2-phenyl pyridine), bromine, phosphorus tribromide, chelidamic acid, di-*tert*-butyl iminodiacetate, sodium borohydride, $Pd(PPh_3)_2Cl_2$, $Pd(PPh_3)_4$, CuI, $IrCl_3.nH_2O$, $Eu(OTf)_3$ and $Gd(OTf)_3$ were purchased from Aldrich Chemical Co. and used as received without further purification. Precursors **A**, **B** and **C** were synthesized using the literature procedure[S1]. All anhydrous solvents were acquired from Aldrich and used as obtained. All reactions were carried out under a dry argon atmosphere unless noted otherwise. Chromatographic separations were performed using column chromatography, with 100–200 mesh silica gel obtained from Merck.

Instrumentation.

^1H NMR spectra (400 MHz and 500 MHz) and ^{13}C NMR spectra (125 MHz) were recorded on Brucker DPX-400 or Brucker DPX-500 spectrometers in $CDCl_3$, CD_3OD and DMSO-d_6 at 298 K using either residual solvent signals or tetramethylsilane as internal standards. Chemical shifts are reported as δ in ppm. ESI mass spectra were recorded using a Micromass LCT instrument. UV/Vis-NIR spectra were recorded on a Varian Cary 50 spectrophotometer in deionised H_2O at 298 K. Luminescence spectra were measured on a Jobin Yvon Fluoromax 4 fluorimeter, at 298K and 77K respectively. Ir(III)-based luminescence lifetimes were measured using the time correlated single photon counting (TCSPC) technique with an Edinburgh Instruments "Mini τ" luminescence lifetime spectrometer, equipped with a 410 nm pulsed diode laser as an excitation source and a Hamamatsu H577303 photo-multiplier tube (PMT) detector. The lifetimes were calculated from the measured data using the supplied software. Preparative HPLC measurements were performed on a Waters 2695 chromatograph furnished with a Waters 2487 UV detector by using 1% TFA in 5 to 95% CH_3CN in water as eluent.

B. Synthesis and Characterisation

The receptors and their metal complexes were prepared by using the standard procedures. All the reactions were carried out using anhydrous solvents using standard Schlenk line under argon atmosphere. Purification of the compounds was done by column chromatography using Al_2O_3, Brockmann-III (often multiple rounds were required to obtain pure material). The compound **1** and **Ir•E** were purified by using a long Shephadex LH-20 column. The products were recrystallized from suitable solvent combinations and used for characterization, photophysical analyses and biological studies. The synthetic route for the ligand and complexes is summarised in Scheme S1.



Scheme S1 Synthetic scheme for **Ir•Ln** [Ln = Gd(III) and Eu(III), respectively] dyads.

1: At first a solution containing precursor **A** (0.673 g, 1 mmol) and **B** (0.163 g, 0.8 mmol) in anhydrous DMF (15 mL) was prepared which was purged by argon for 30 min. $Pd(PPh_3)_4$ (69 mg, 0.06 mmol) and CuI (38 mg, 0.20 mmol) were placed in a 100 mL Schlenk tube which was evacuated and back-filled with argon several times. The previously prepared DMF solution of **A** and **B** was transferred into the reaction flask *via* a clean and dry cannula. Then anhydrous triethylamine (5 mL) was added to the reaction mixture and the resulting mixture was kept at 80°C for two days with constant stirring. After removal of the solvent, the deep brown residue was dissolved in dichloromethane (150 mL), washed with aqueous potassium cyanide (2%, 50 mL) and with water (2 × 200 mL). The organic layer was dried over $MgSO_4$ and purified by column chromatography on Al_2O_3, eluting initially with DCM and then with DCM/MeOH, 99:1 [R_f (DCM) = 0.50] to furnish 550 mg of **1** as an orange yellow semi solid. Yield ~86%; ^{1}H NMR (500 MHz, $CDCl_3$, 298 K, TMS): δ = 1.44 (s, 36H), 3.50 (s, 8H), 4.05 (s, 4H), 7.20-7.29 (m, 4H), 7.42-7.45 (m, 2H), 7.69 (s, 1H), 8.26 (bs, 1H), 8.90 (bs, 1H); ^{13}C NMR spectra (125 MHz, $CDCl_3$, 298 K, TMS): δ = 28.0, 55.8, 59.4, 80.9, 88.5, 92.7, 120.0, 122.7, 124.5, 126.1, 127.4, 127.8, 128.2, 129.2, 129.4, 133.4, 136.1, 137.9, 149.8, 151.2, 159.3, 170.3; ESI-MS: *m/z* calcd for $C_{45}H_{57}N_5O_8$, 795.9; found, 797.0 $(M + H)^+$ (100%) and 819.0 $(M + Na)^+$(10%).

Ir•E: A mixture of **1** (0.398 g, 0.5 mmol) and fluorinated Ir(III)-dimer, **C**, (0.304 g, 0.25 mmol) was placed in a 100 mL three necked flask, which was evacuated and back-filled with argon several times. Anhydrous DCM (40 mL) and MeOH (10 mL) were added to this and the resulting mixture was heated to reflux for overnight. When all the starting materials were

consumed (confirmed by TLC), the reaction mixture was cooled to RT and ~15 equivalents of NH_4PF_6 (1.22 gm, 7.5 mmol) added slowly to the reaction mixture with continued stirring at RT. After 6 h the reaction mixture was evaporated near to dryness and 5 mL of DCM was added to it to completely dissolve the organic substances. Residual NH_4PF_6 was filtered off and the dark organic solution was concentrated *in vacuo*. The crude reaction mixture was then subjected to column chromatography on Al_2O_3 [Eluent: (1) neat CH_2Cl_2, (2) 2% MeOH in CH_2Cl_2] to furnish 0.567 g of **Ir•E** as a deep yellow crystalline material**.** Yield ~75%; ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ = 1.48 (s, 36H), 3.50 (s, 8H), 4.04 (s, 4H), 5.77 (dd, J = 4.0 Hz, J = 4.0 Hz, 1H), 5.81 (dd, J = 4.0 Hz, J = 4.0 Hz, 1H), 6.62-6.67 (m, 2H), 7.02-7.06 (m, 2H), 7.37-7.40 (m, 2H), 7.71 (s, 2H), 7.79-7.83 (m, 2H), 7.94-7.97 (m, 2H), 8.21 (d, J = 12.0 Hz, 2H), 8.29-8.39 (m, 4H), 8.74 (s, 1H), 8.82 (d, J = 8.0 Hz, 1H); ^{13}C NMR spectra (125 MHz, $CDCl_3$, 298 K, TMS): δ = 28.1, 55.8, 59.5, 81.2, 86.8, 94.9, 114.1, 122.6, 122.9, 123.78 124.1, 127.5, 127.7, 128.1, 130.4, 130.9, 132.5, 139.2, 140.1, 141.6, 145.4, 145.9, 148.6, 149.1, 151.0, 151.7, 152.3, 159.6, 160.4, 162.57, 164.1, 164.7, 170.4; ESI-MS: *m/z* calcd for $C_{67}H_{69}F_4N_7O_8Ir$, 1368.5; found, 1368.0 $(M)^+$ (100%).

Ir•A: The Ir(III)-complex, **Ir•E**, 0.378 gm (0.25 mmol) was dissolved in 10 mL of DCM and the flask placed in an ice bath. An excess of cold TFA (5 mL) was added to this cold solution dropwise. The resulting mixture was stirred for 16 h at 273 K under an argon atmosphere. The solvents and other volatile substances were removed under vacuum without heating. The residue was then triturated with ether (5 × 25 mL) and finally filtered to afford the tetracarboxylic acid **Ir•A** as a yellow mass. The compound was dried under vacuum pump. Yield: 0.24 g (~74%); ^{1}H NMR (500 MHz, $CDCl_3$, 298 K, TMS): δ = 3.75 (s, 8H), 4.32 (s, 4H), 5.77 (dd, J = 5.0 Hz, J = 5.0 Hz, 1H), 5.83 (dd, J = 5.0 Hz, J = 5.0 Hz, 1H), 6.71-6.77 (m, 2H), 6.98-7.01 (m, 2H), 7.49 (d, J = 5.0 Hz, 1H), 7.61 (d, J = 5.0 Hz, 1H), 7.77 (s, 1H), 7.89-7.92 (m, 2H), 7.99-8.01 (m, 2H), 8.33-8.41 (m, 4H), 8.44 (s, 1H), 8.85 (d, J = 10.0 Hz, 1H), 9.10 (s, 1H), 12.01 (bs, 8H); ^{13}C NMR spectra (125 MHz, $CDCl_3$, 298 K, TMS): δ = 56.4, 58.6, 91.6, 92.9, 115.1, 122.9, 124.9, 125.1, 126.3, 128.8, 129.2, 129.5, 130.9, 132.5, 133.9, 136.0, 140.8, 143.8, 147.4, 147.2, 150.8, 152.9, 153.5, 154.1, 154.5, 157.2, 162.1, 163.9, 165.3, 166.2, 173.6; ESI-MS: *m/z* calcd for $C_{51}H_{37}F_4N_7O_8Ir$, 1144.1; found, 1144.0 $(M)^+$ (10%), 573 $(M + H)^{2+}$ (100%).

Synthesis of Ln(III) Complexes: Complexations with lanthanides was achieved by reacting **Ir•A** with $Ln(OTf)_3$ in MeOH at 50°C for 2 days under argon atmosphere. After evaporating the reaction mixture, analytically pure products were isolated in almost quantitative yield. There are single traces in analytical HPLC for each final compounds which confirm its purity.

Ir•Eu: Typically, this was prepared by mixing 0.052 gm (0.04 mmol) of the acid **Ir•A** and $Eu(OTf)_3$ (0.027 g, 0.045 mmol) in a 50 mL two necked flask. Then 15 mL of MeOH was added to dissolve the starting materials. The transparent solution was stirred for another 30 min and 1M aqueous NaOH solution was added slowly to obtain a pH of *ca*. 5. The reaction mixture was heated to 50°C with constant stirring for 2 days. After cooling the reaction

mixture the solvent was evaporated under reduced pressure. The residue was dissolved in minimum amount of MeOH and re-precipitated by the gradual addition of ether. This was done several times and the light yellow solid mass was collected by filtration. HPLC analysis shows a single peak with the retention time of 20.54 min which confirms purity. Yield: 0.035 g (~67%); ESI-MS: *m/z* calcd for $C_{51}H_{33}F_4N_7O_8IrEu$, 1292.0; found, 1292.0 $(M)^+$ (10%), 669 $(M + 2Na)^{2+}$ (100%).

Ir•Gd: By following the same experimental conditions the corresponding Gd(III)-complex was prepared. Starting from **Ir•A** (0.052 g, 0.04 mmol) and $Gd(OTf)_3$ (0.027 g, 0.045 mmol) the isolated yield of **Ir•Gd** was 0.043 g (~82%). ESI-MS: *m/z* calcd for $C_{51}H_{33}F_4N_7O_8IrGd$, 1297.3; found, 1297.0 $(M)^+$ (12%), 672 $(M + 2Na)^{2+}$ (100%).

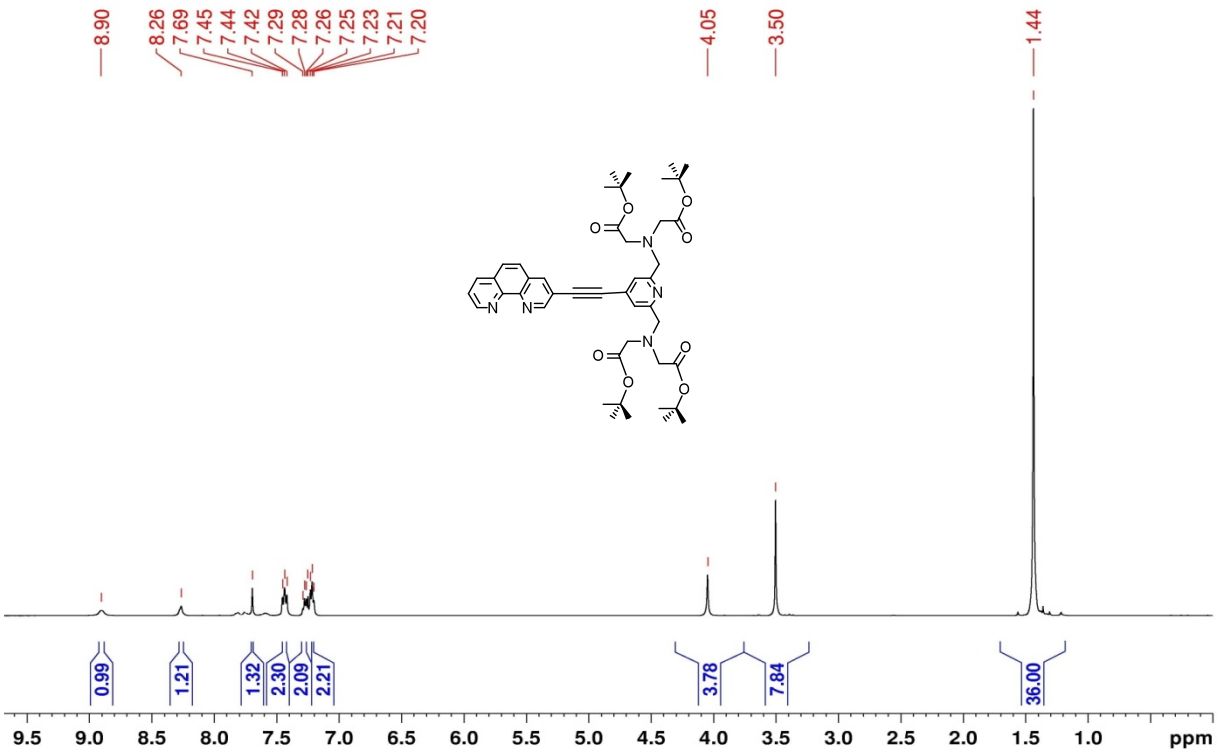


Fig. S1 500 MHz ^{1}H NMR spectrum of **1** in $CDCl_3$ at RT.

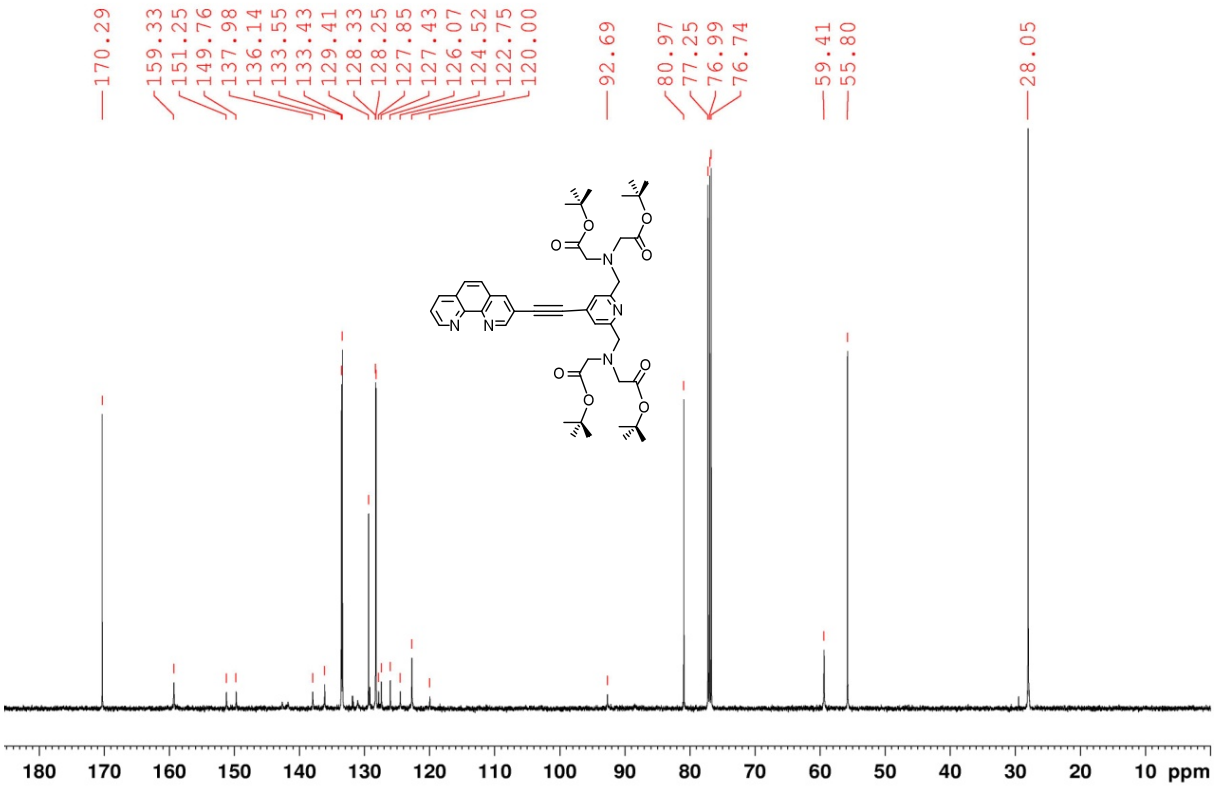


Fig. S2 125 MHz ^{13}C NMR spectrum of **1** in $CDCl_3$ at RT.

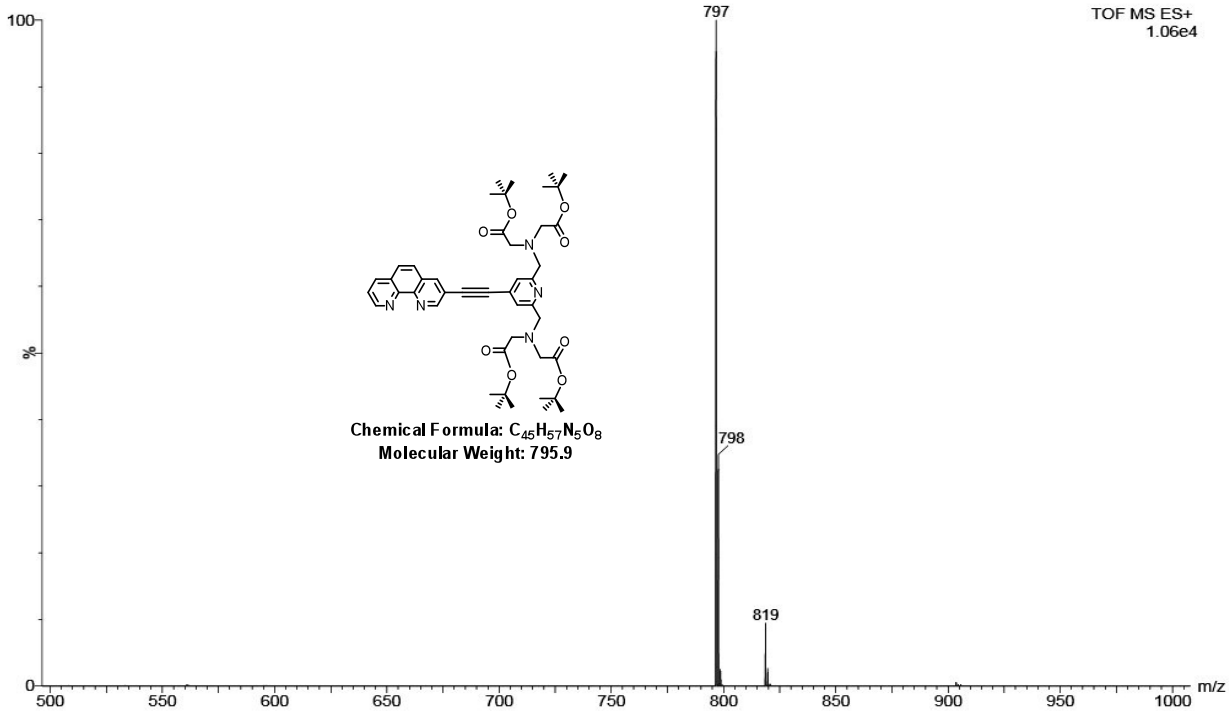


Fig. S3 ESI MS of **1** receptor.

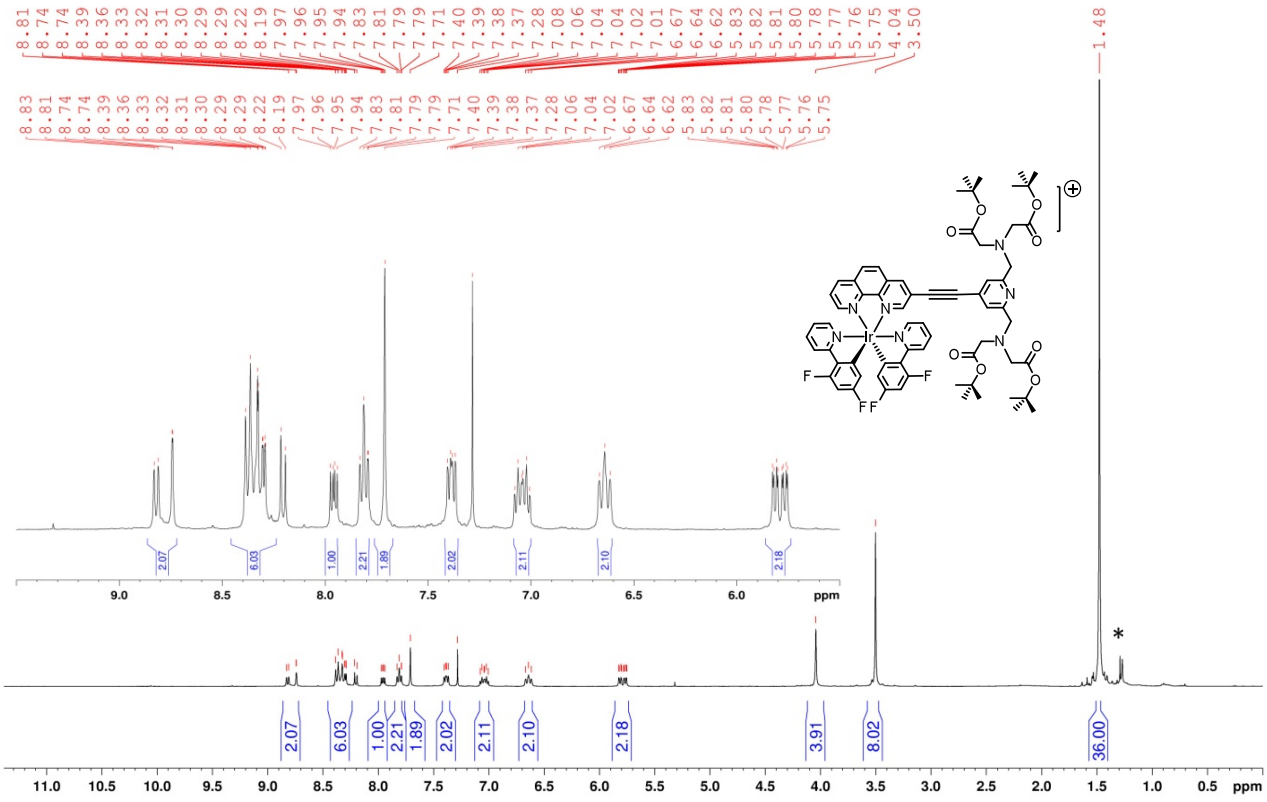


Fig. S4 400 MHz ^{1}H NMR spectrum of **Ir•E** in $CDCl_3$ at RT.

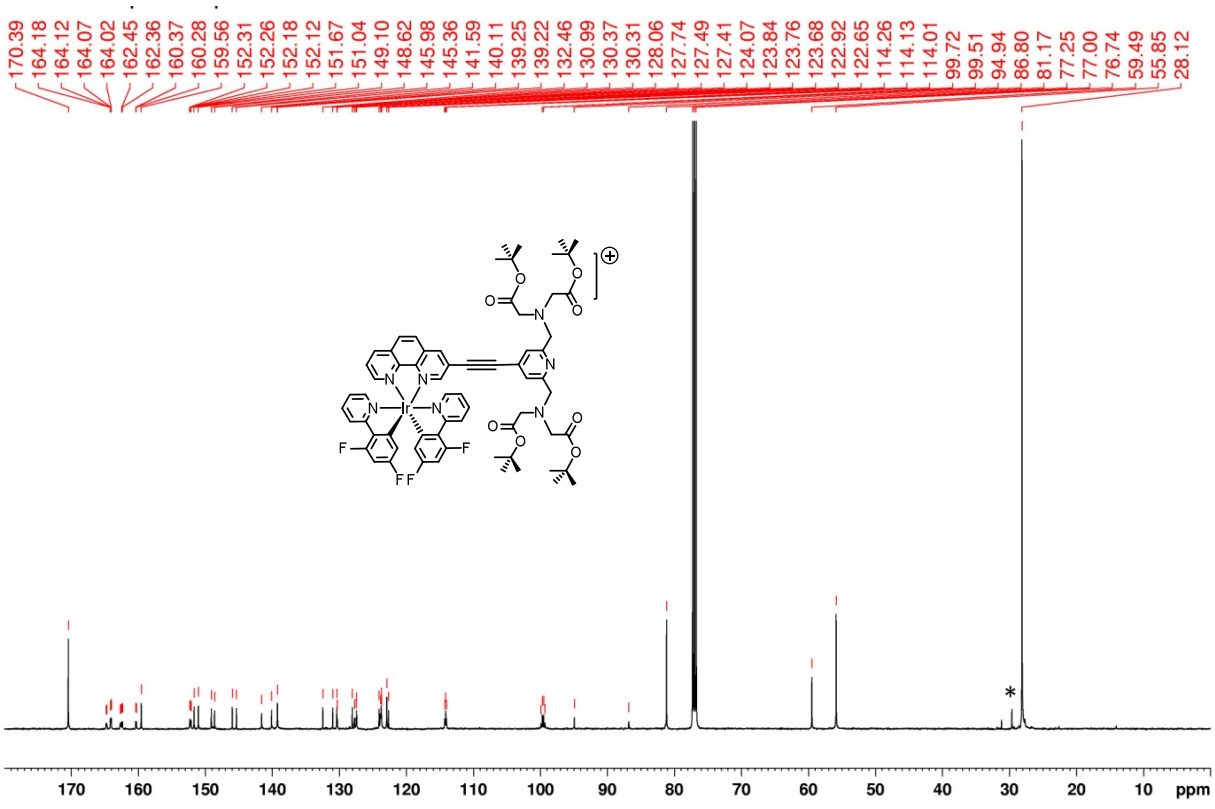


Fig. S5 125 MHz ^{13}C NMR spectrum of **Ir•E** in $CDCl_3$ at RT.

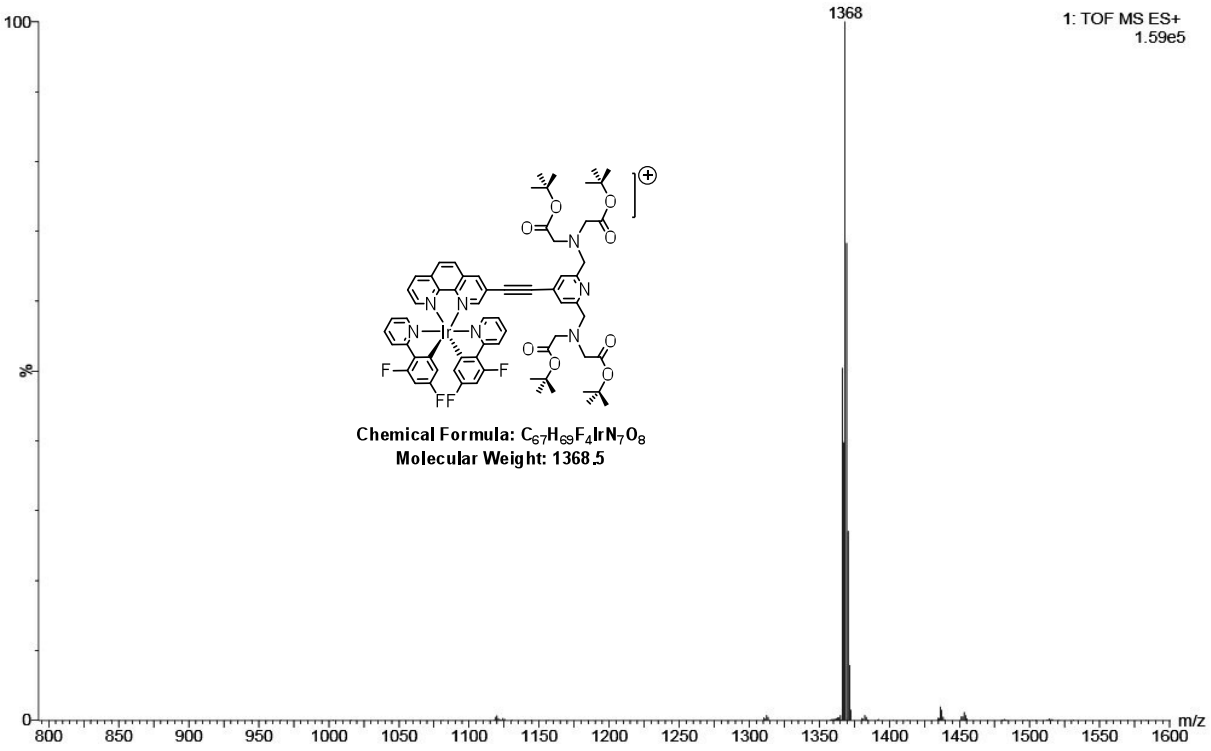


Fig. S6 ESI MS of **Ir•E**.

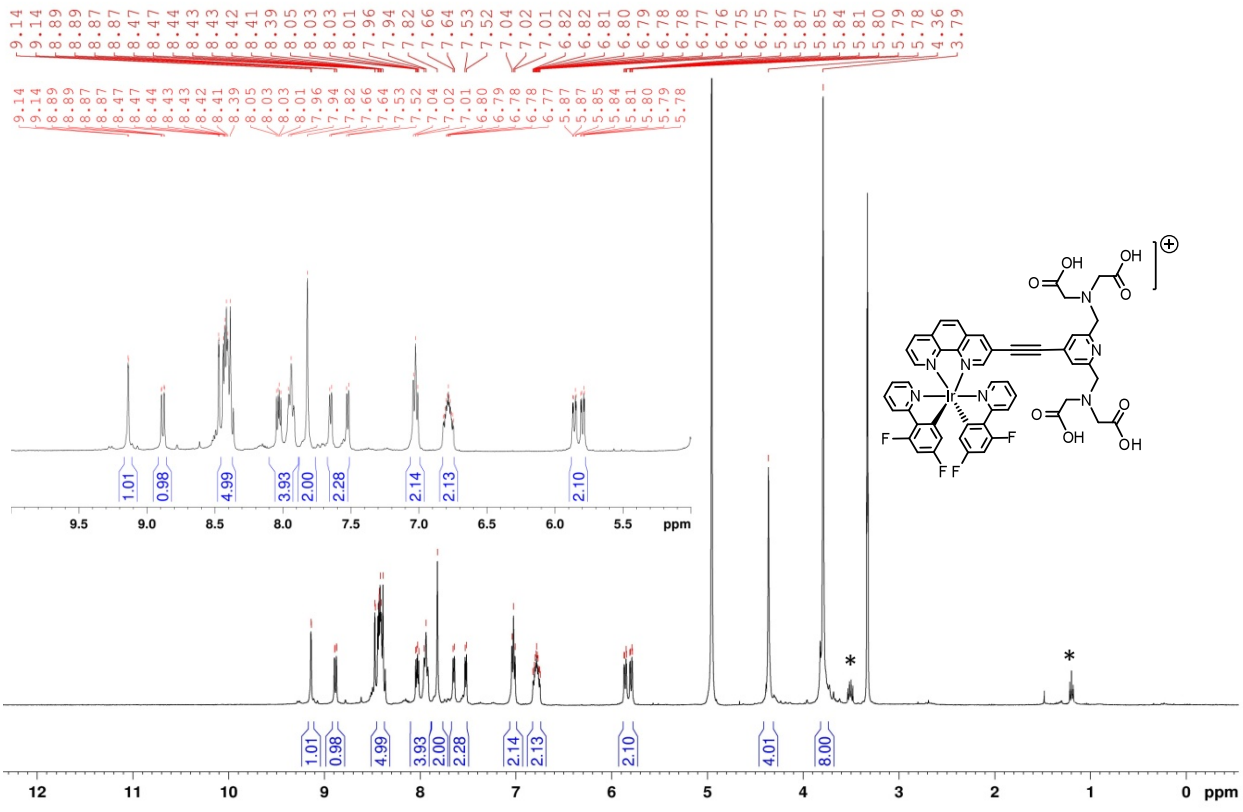


Fig. S7 500 MHz ^{1}H NMR spectrum of **Ir•A** in CD_3OD at RT.

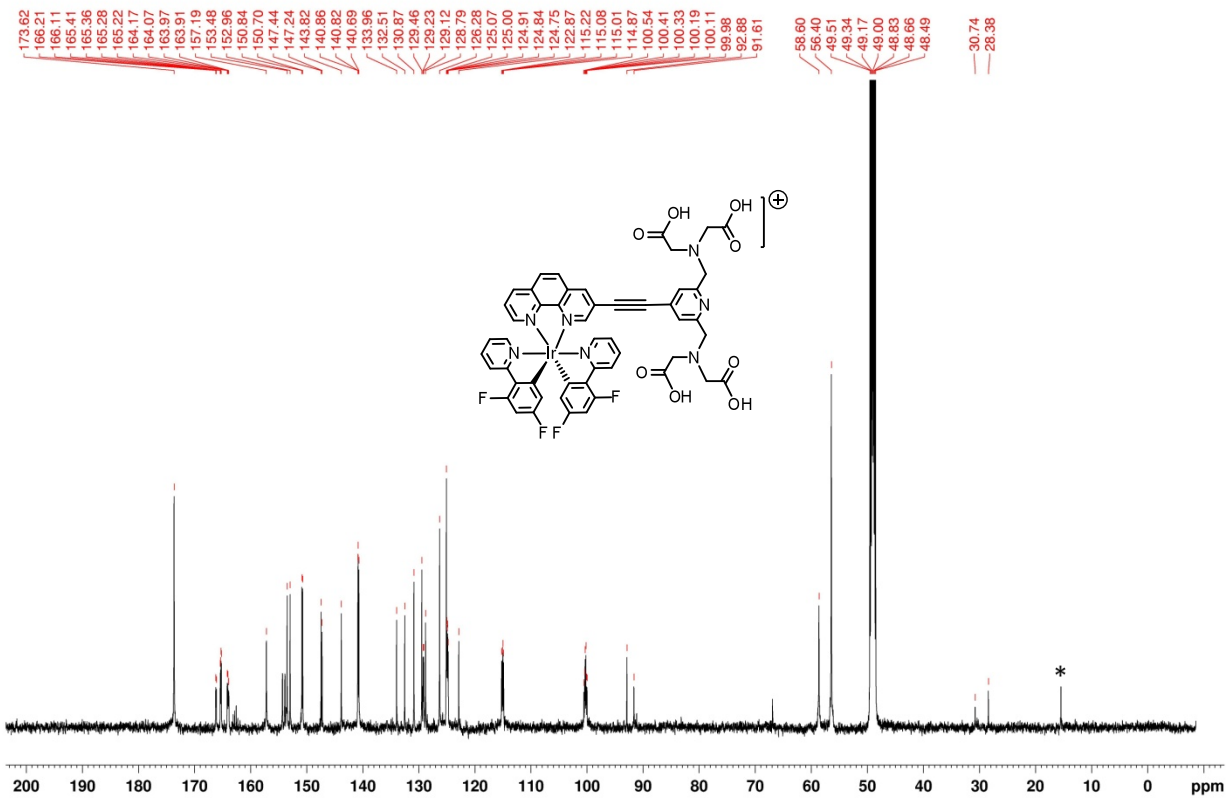


Fig. S8 125 MHz ^{13}C NMR spectrum of **Ir•A** in CD_3OD at RT.

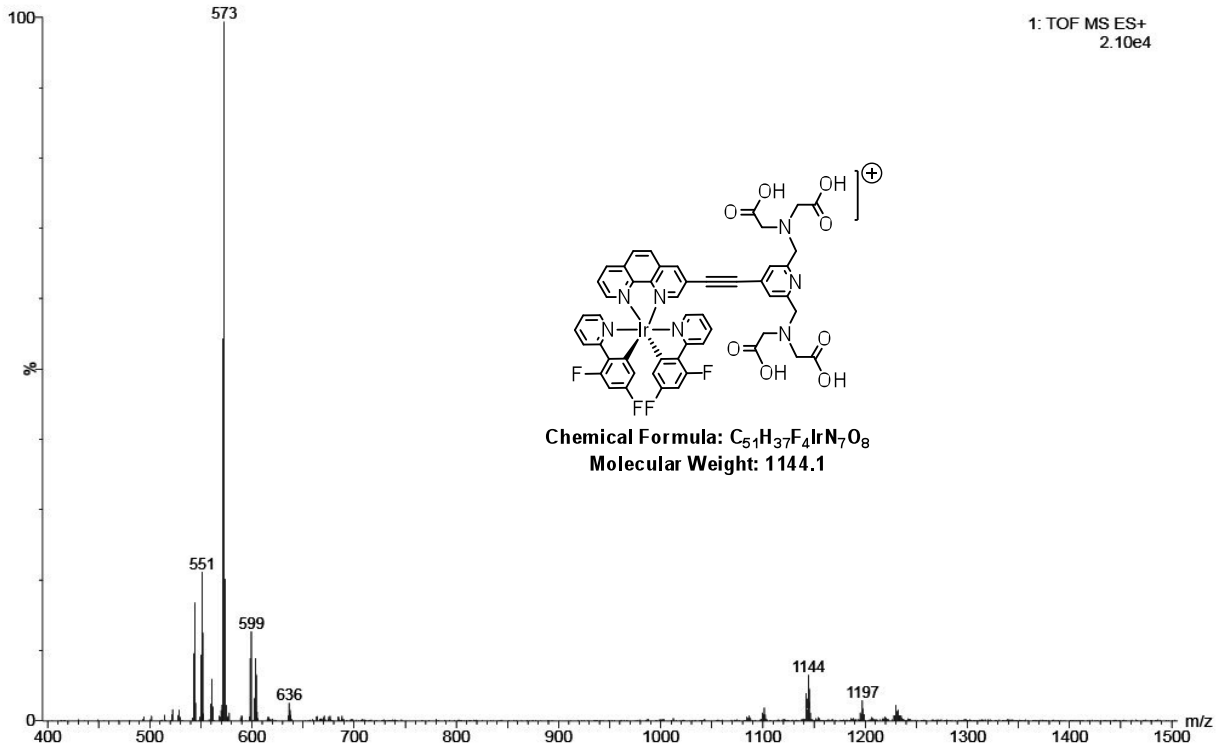


Fig. S9 ESI MS of **Ir•A** receptor.

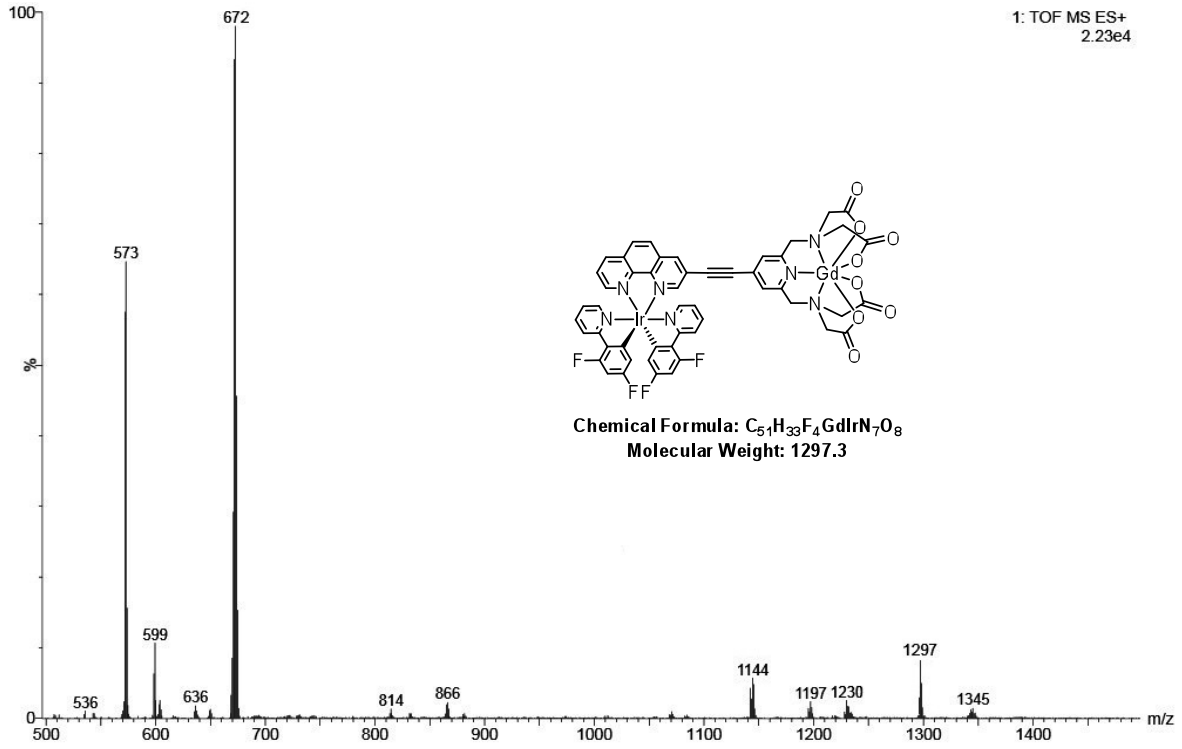


Fig. S10 ESI MS of **Ir•Gd** dyad.

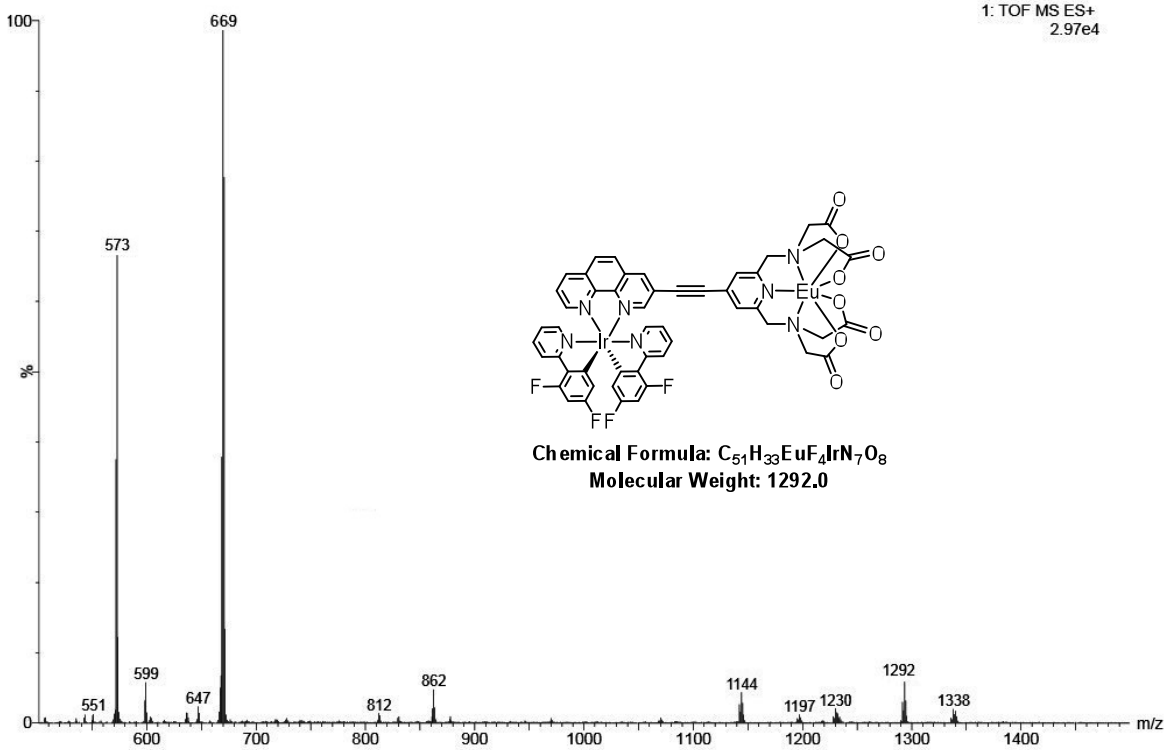


Fig. S11 ESI MS of **Ir•Eu** dyad.

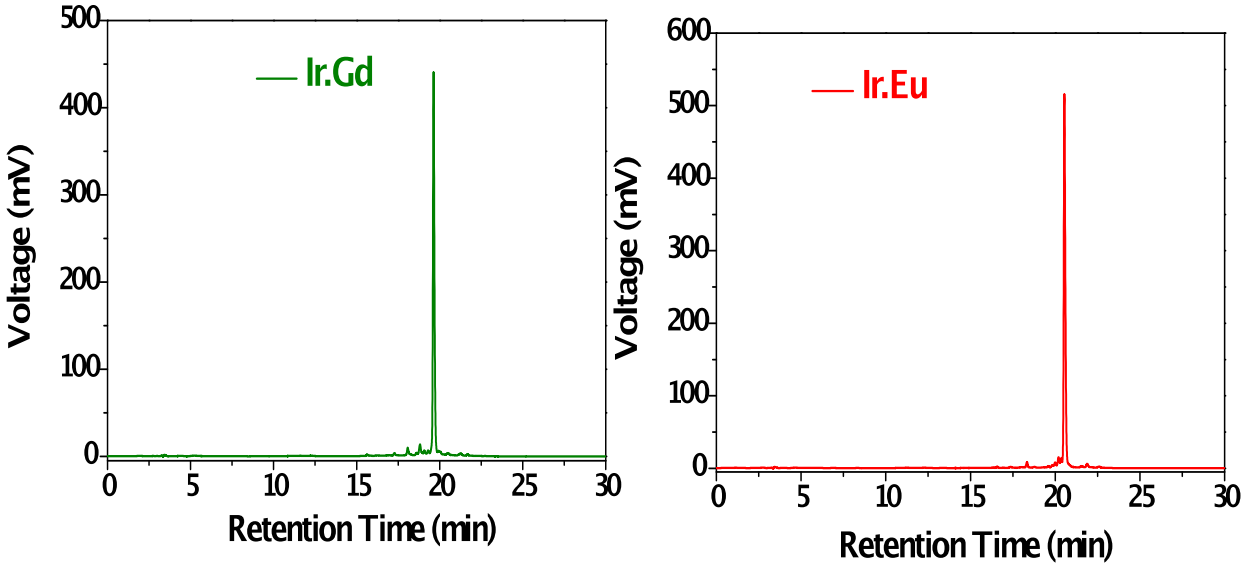


Fig. S12 HPLC data for the dyads **Ir•Gd** and **Ir•Eu** respectively.

C. Photophysical Experiments

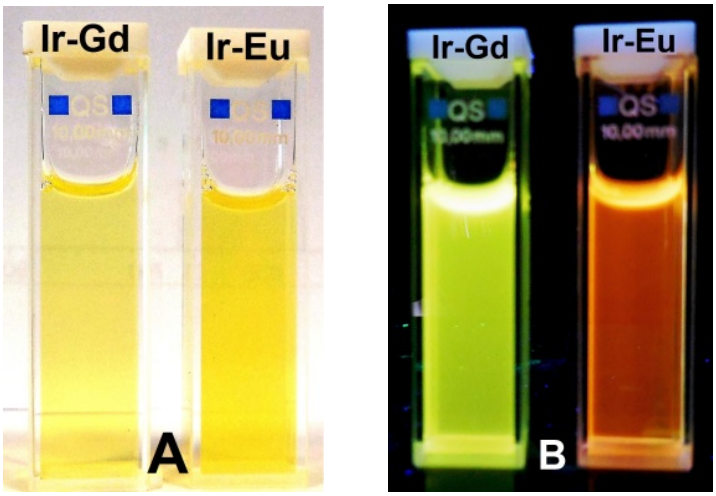


Fig. S13 Photographs of the **Ir•Ln** dyads in normal light (A); and under UV lamp (365 nm) respectively (B). The concentrations are in the order of 10^{-5} M in water.

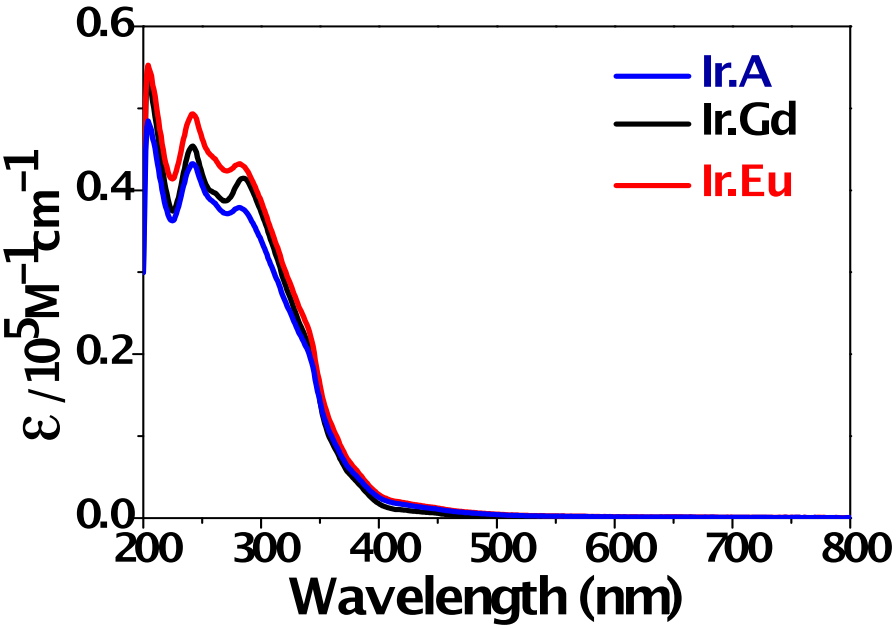


Fig. S14 The UV-vis spectra of the compounds **Ir•A, Ir•Gd** and **Ir•Eu** respectively in aqueous solution.

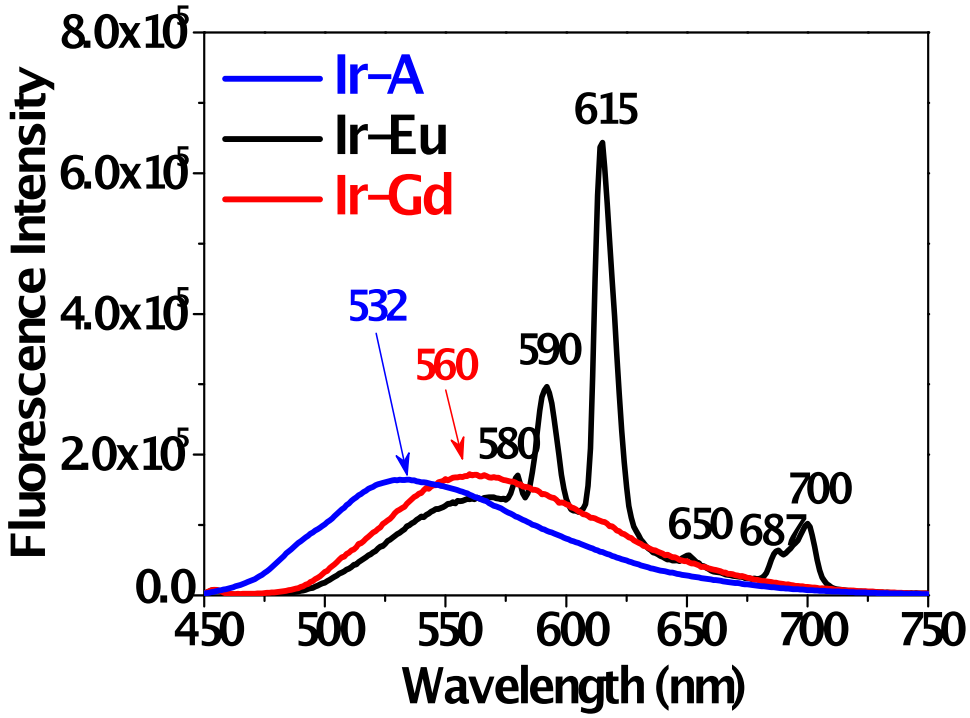


Fig. S15 A comparative fluorescence spectra of the **Ir•A** and **Ir•Ln** dyads respectively, in aqueous medium (λ_{ex} = 400 nm).

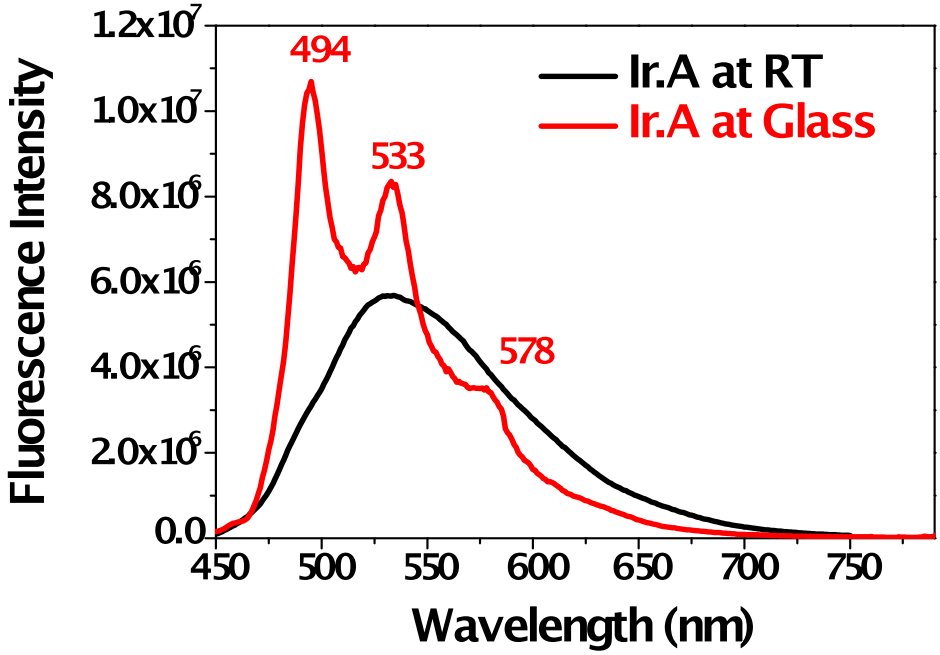


Fig. S16 Fluorescence spectra of **Ir•A** in room temperature and glass temperature respectively in MeOH and EtOH (1:4) mixture. λ_{ex} = 400 nm.

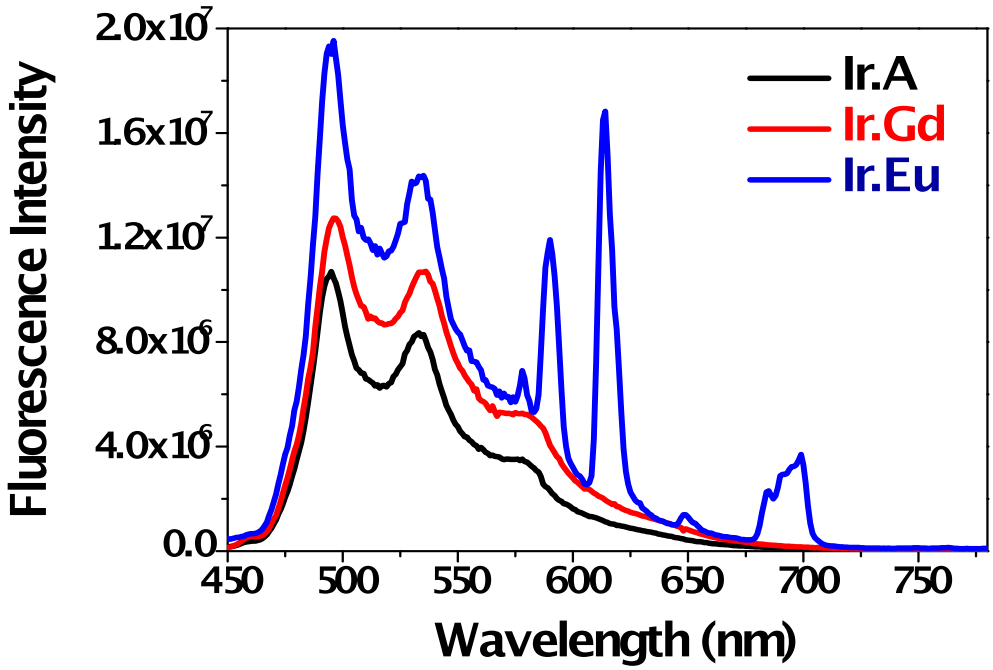


Fig. S17 Fluorescence spectra of **Ir•A**, **Ir•Gd** and **Ir•Eu** in glass temperature respectively in MeOH and EtOH (1:4) mixture. λ_{ex} = 400 nm.

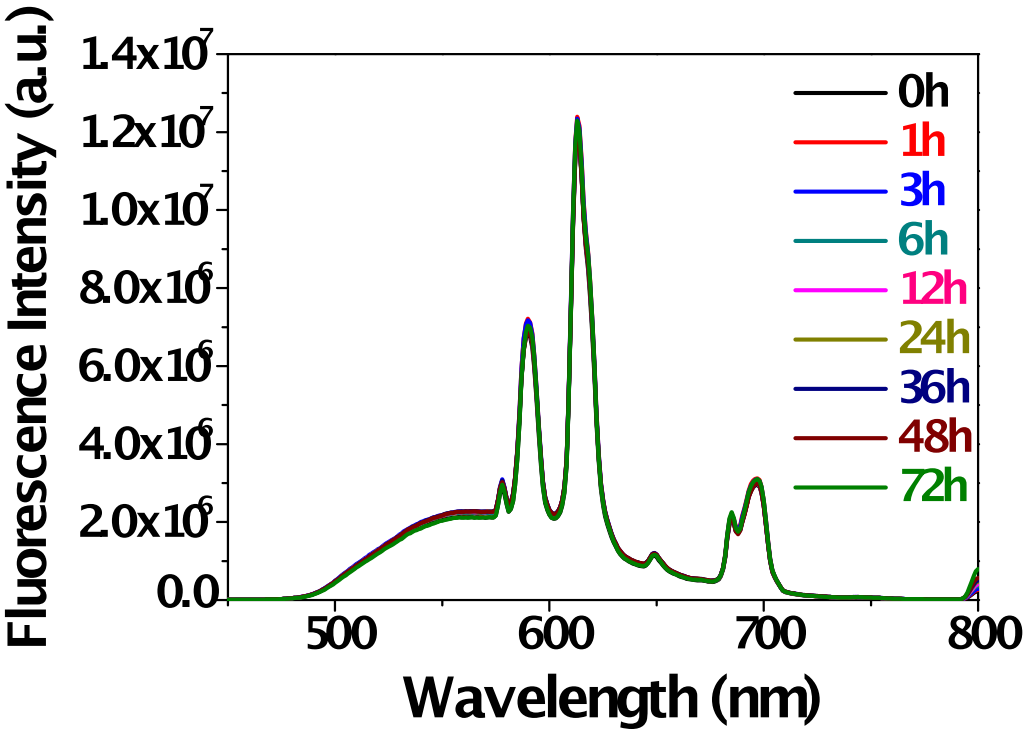


Fig. S18 Stability test of 5×10^{-4} M solution of the fluorophore **Ir•Eu** in H_2O.

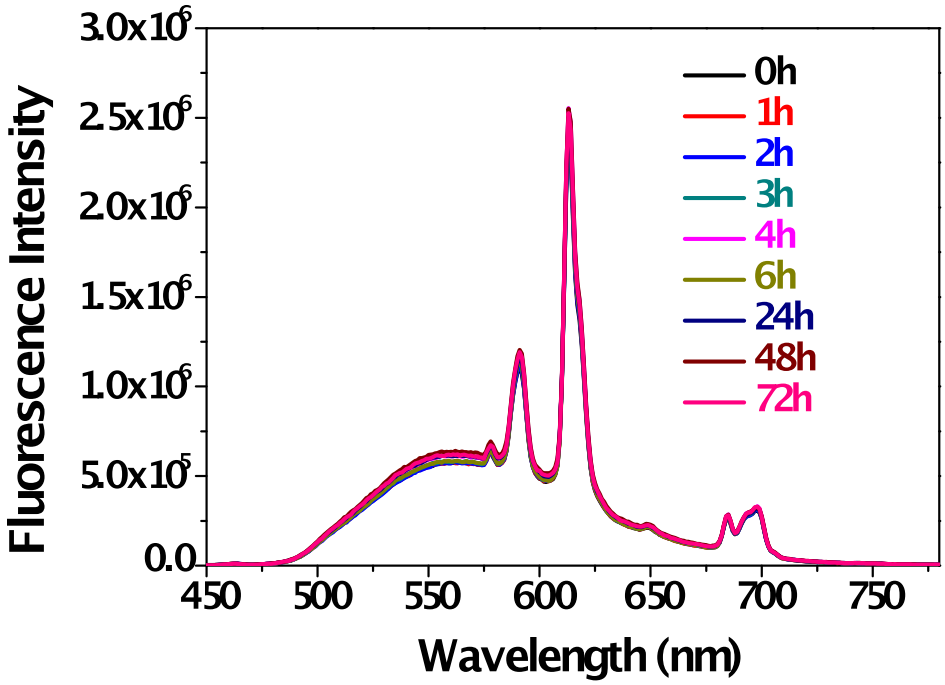


Fig. S19 Kinetic stability experiment of 10^{-4} M solution of the fluorophore **Ir•Eu** in presence of DOTA (1:1.2) in aqueous medium.

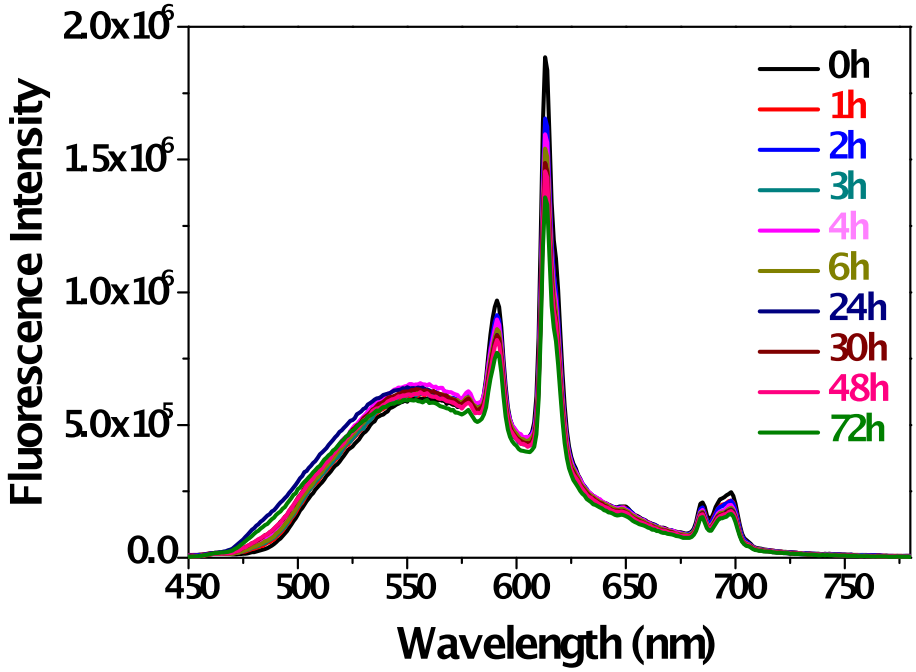


Fig. S20 Kinetic stability experiment of 10^{-4} M solution of the fluorophore **Ir•Eu** in presence of DOTA (1:1.2) in PBS buffer.

Table S1 Photophysical data for all the compounds under present studies in aqueous solution. The excitation wavelength (λ_{ex}) is 400 nm in each case.

Entity	λ_{max}[nm]	ε[$M^{-1}cm^{-1}$]	λ_{fl}[nm]	τ_1[ns]	τ_2[ns]
1	279,315	44300,29200	-	-	-
Ir•E	140,280,342	49330,45290,21850	520	1470(73%)	406(27%)
Ir•A	242,282,341	43450,38200,20680	532	750(83%)	258(17%)
Ir•Gd	242,285,338	45890,41800,21700	560	1100(56%)	450(44%)
Ir•Eu	242,283,343	49640,43500,22600	564,580,590,615,650,687,700	780(63%)	116(37%)
Ir•Gd[a]	-	-	496,535,580	-	-
Ir•Eu[a]	-	-	495,532,580,590,615,650,687,700	-	-

[a] The measurements were carried out in frozen glass at 77K .

D. Imaging Experiments

Cell Culture: MCF7 cells were cultured in a humidified 37°C, 5% CO_2/95% air (v/v) environment in Roswell Park Memorial Institute, RPMI1640 (Sigma Aldrich) supplemented with 10% (v/v) FCS (fetal calf serum), L-glutamine (200 mM, 5 mL), penicillin/streptomycin (5 mL), and fungizone (1.25 ml). Cells were cultured as monolayers in T-75 flasks and passaged using trypsin-EDTA. For PLIM and confocal imaging experiments, cells were seeded in to sterile 6 well plates and cultured until 60% confluent.

Staining: After removal of growth media, cells were washed with PBS (phosphate buffered saline, 1 ml/well) before treating with a solution of the appropriate **Ir•Ln** complex: 10-100 μM in full RPMI (0.04–0.4% DMSO, 4h at 37°C, 1 ml/well). All incubation solutions were diluted from a 20 mM stock solution of **Ir•Ln** in DMSO. After incubation, cells were washed with PBS (3 × 1 ml/well) to remove excess complex, and were then imaged in PBS.

MTT Assay for Cell Viability

MCF7 cells were cultured in sterile 6 well 12 well plates until 60–70% confluent then incubated with **Ir•Ln** for the desired incubation time and concentration. Conditions investigated: 25 μM, 50 μM and 100 μM for 4 and 24 hours. 0.5% DMSO in RPMI and RPMI only were also investigated as controls. After **Ir•Ln** incubation, MCF7 cells were washed with PBS and treated with a solution of MTT (0.5 mg/ml in PBS, 1ml/well) for 40 minutes at 37°C. After careful removal of the MTT solution, acidified isopropanol was added (400 μl/well, 10 minutes at RT). 150 μl aliquots of the acidified isopropanol solution were then transferred to a 96 well plate (two from each 24 well) and the absorbance at 540 nm was recorded using a plate reader. Each incubation condition was assessed in triplicate.

Confocal and Time-Resolved PLIM Imaging

MCF7 cells were imaged in PBS using a × 40 water-dipping objective (in 6 well plates). Time-resolved, phosphorescence lifetime imaging (PLIM) was carried out using a Ti:Sa pulsed laser (λ_{ex}: 780 nm) and a Becker and Hickl combined FLIM/PLIM system, which comprises a SPC-150 TPSPC module and a DDG-210 pulse generator module,[S2] connected to a Zeiss LSM 510 upright confocal microscope. PLIM imaging of the iridium unit was carried out using the 12 μs predefined set-up on the SPCM software (laser on time: 2 μs, PLIM decay window: 12 μs) and data processed using SPCImage software, with a pixel bin of 3 unless stated otherwise. In all cases decay traces were best fit to a double exponential decay model, with longest component being the more significant Ir lifetime. Confocal images were recorded using standard settings on the LSM 510, λ_{ex}: 780 nm / λ_{em}: 500–550 nm for **Ir•Ln**.

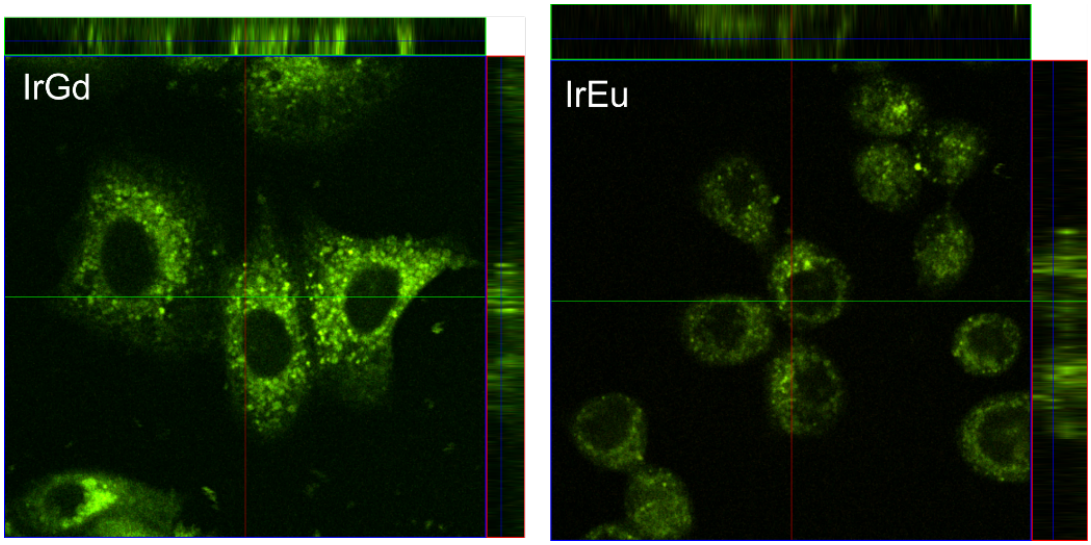


Fig. S21 Steady-state, Z-stack images of **Ir•Gd** and **Ir•Eu** in live MCFZ cells, showing complex internalization. λ_{ex} = 780 nm, λ_{em} = 500–550 nm.

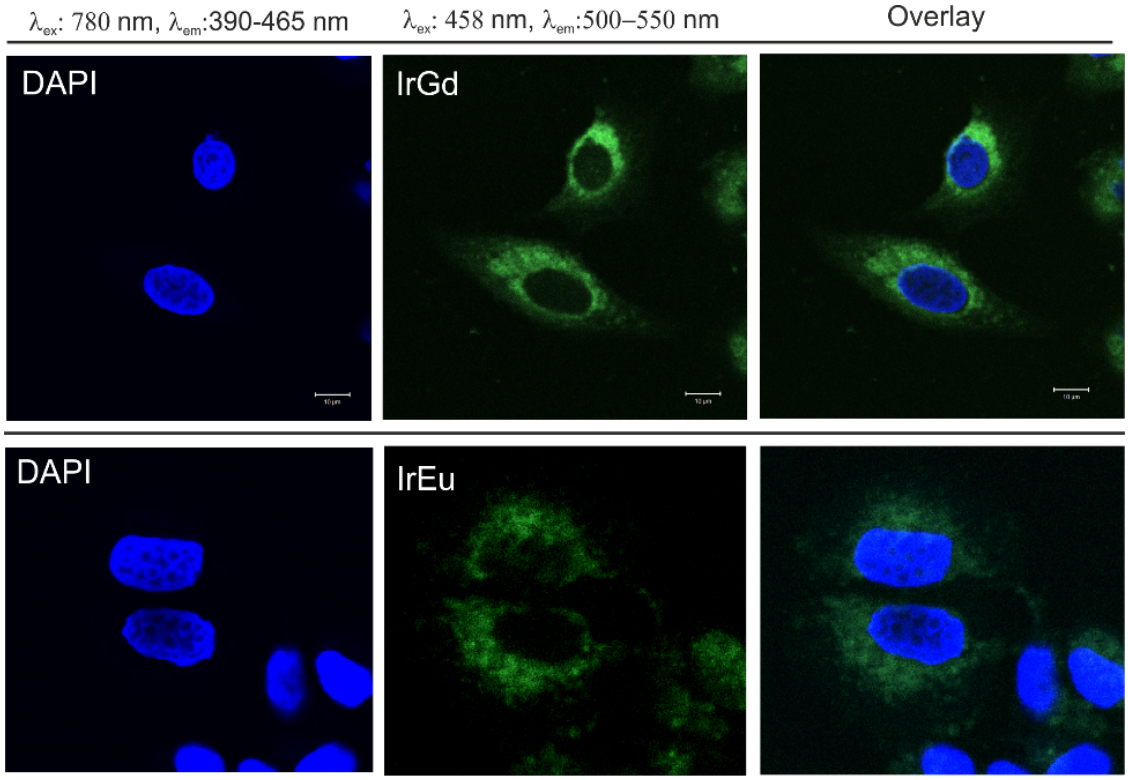


Fig. S22 Steady-state confocal images of MCF7 cells stained with DAPI and **Ir•Ln** dyads, **Ir•Gd** (50 μM, top) and **Ir•Eu** (100 μM, bottom). For **Ir•Ln**: λ_{ex} = 458 nm, λ_{em} = 500–550 nm; for DAPI: λ_{ex} = 780 nm, λ_{em} = 390–465nm. Scale bars: 10 μm.

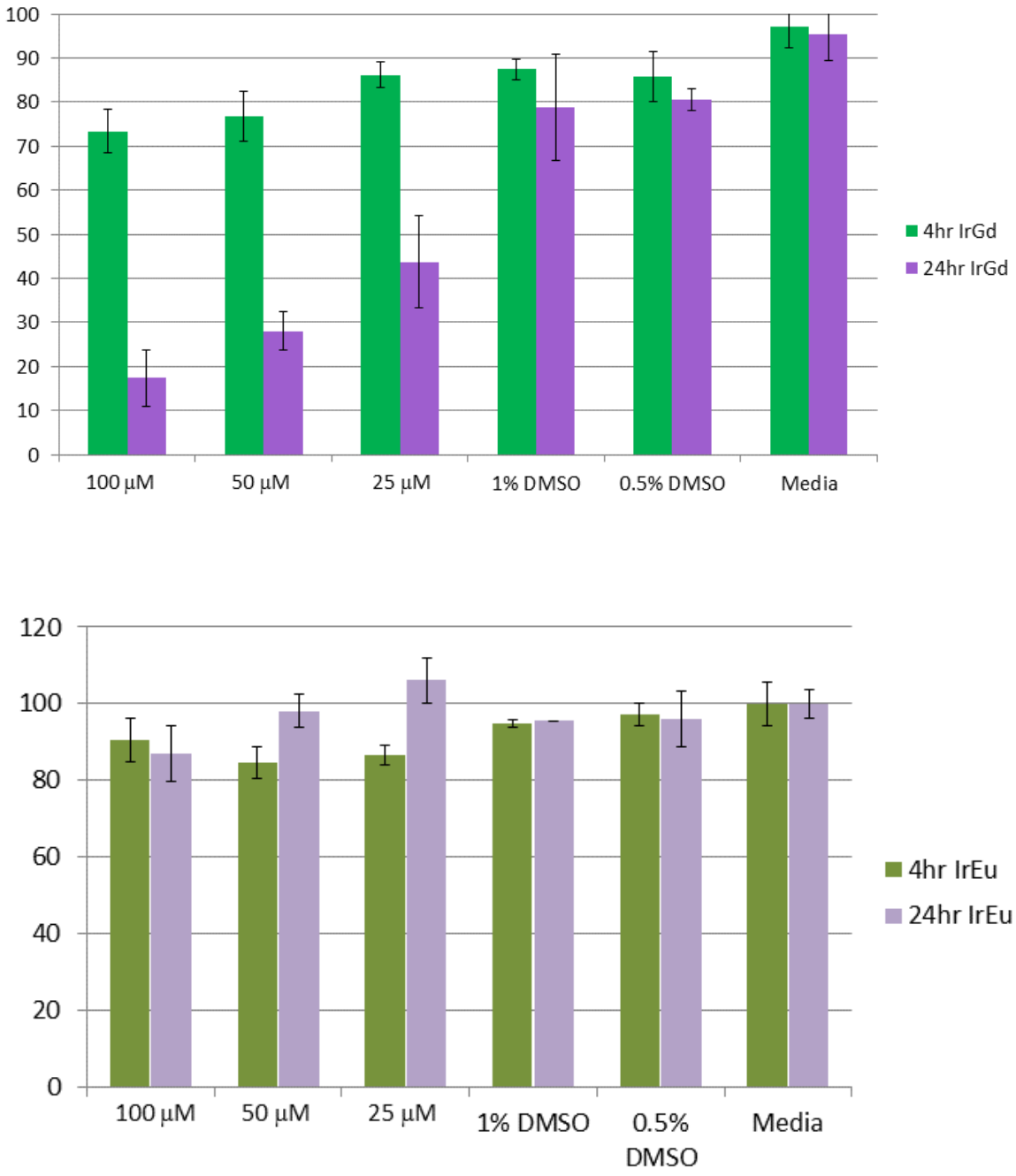


Fig. S23 MTT toxicity assay on MCF7 cells incubated with **Ir•Gd** (top) and **Ir•Eu** (bottom). **Ir•Ln** incubations were done in full RPMI media at 37°C, for 4 and 24 hours. Error bars represent the standard deviation of six data points (two absorbance readings from each well of the triplicate data set).

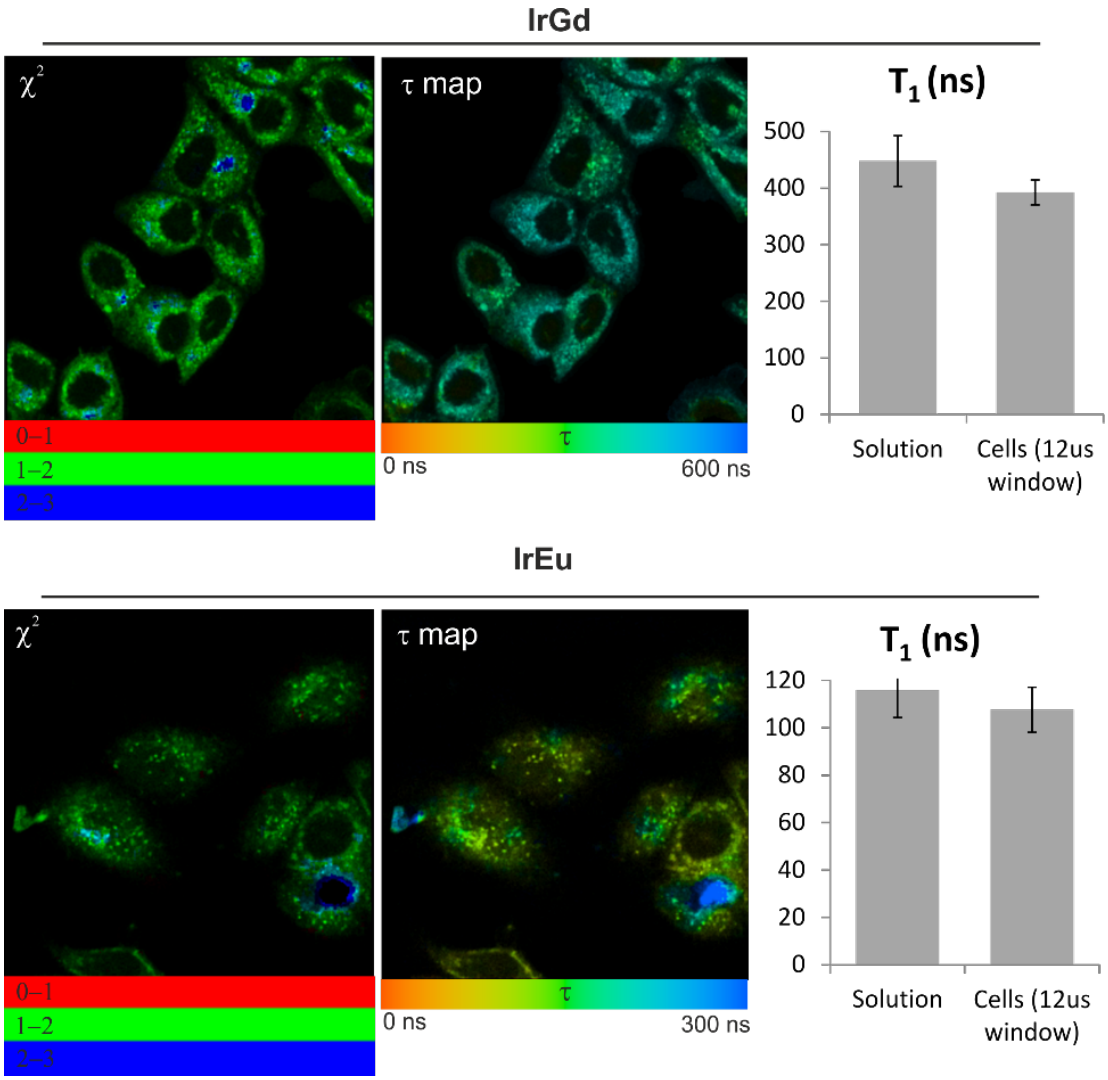


Fig. S24 TP-PLIM (λ_{ex}: 780 nm) imaging of **Ir•Gd** (50 μM, top, 4 hr) and **Ir•Eu** (100 μM, bottom, 20 hr) in live MCF7 cells. **Centre**: Lifetime map of τ_1, with rainbow colour legend (**Ir•Gd**: 0–600 ns, **Ir•Eu**: 0–300 ns); **Left**: χ^2 of double exponential fit for each pixel, with discrete tri-colour legend; **Right**: τ_1 lifetime values from ten pixels from across the 256 × 256 array compared with aerated solution lifetimes. Error bars represent the standard deviation of the ten pixel data set.

E. Relaxivity Measurements

The T_1 relaxation time of **Ir•Eu** at a frequency of 20MHz was measured on a Stelar Spinmaster FFC-2000 relaxometer, using a non-polarising (NP) magnetic field sequence for standard inversion recovery. A Spinmaster Variable Temperature Controller allowed the setting and monitoring of the sample temperature at 37(±0.1) °C. The temperature in the probe head was measured with a Fluke 52 k/j digital thermometer. Each sample was allowed to equilibrate at the desired temperature for 10 minutes prior to data collection. The reproducibility in T_1 measurements was within < 1% variance. The sample was measured at three different concentrations and a plot of the observed $1/T_1$ *vs.* concentration (in mM) gave a gradient of 11.9 mM^{-1} s^{-1}, after correcting for the inherent relaxivity of the solvent.

F. References

S1: a) H. Takalo, J. Kankare, *Acta Chem. Scand*., *Ser.* **1987**, *B41*, 219; b) H. Takalo, P. Pasanen, J. Kankare, *Acta Chem. Scand*., *Ser.* **1988**, *B42*, 373; c) J. Kupai, P. Huszthy, K. Székely, T. Tóth, L. Párkányi, *ARKIVOV*, **2011**, *ix*, 77; d) D. Tzalis, Y. Tor, S. Failla, J. S. Siegel, *Tetrahedron Lett*. **1995**, *36 (20)*, 3489; e) C. Michel, D. Habibi, M. Schmittel, *Molecules*, **2001**, *6*, M224; f) C. Michel, D. Habibi, M. Schmittel, *Molecules*, **2001**, *6*, M225; g) Q. Shu, L. Birlenbach, M. Schmittel, *Inorg. Chem*. **2012**, *51*, 13123.

S2: Becker & Hickl GmbH, Combined Fluorescence and Phosphorescence Lifetime Imaging (FLIM / PLIM) with the Zeiss LSM 710 NLO Microscopes, Application note. Available on www.becker-hickl.com.