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Heteronuclear Ir(III)–Ln(III) Luminescent Complexes: Small Molecule Probes for Dual Modal Imaging and Oxygen Sensing


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ABSTRACT
Luminescent, mixed metal $d$-$f$ complexes have the potential to be used for dual (Magnetic Resonance Imaging, MRI and luminescence) in vivo imaging. Here we present di- and tri-nuclear $d$-$f$ complexes, comprising a rigid framework linking a luminescent Ir-center to one ($\text{Ir}^{\bullet}\text{Ln}$) or two ($\text{Ir}^{\bullet}\text{Ln}_2$) lanthanide metal centres (where Ln = Eu(III) and Gd(III) respectively). A range of physical, spectroscopic and imaging-based properties including relaxivity arising from the Gd(III) units and the occurrence of Ir(III)$\rightarrow$Eu(III) photoinduced energy-transfer are presented. The rigidity imposed by the ligand facilitates high relaxivities for the Gd(III) complexes, whilst the luminescence from the Ir(III) and Eu(III) centres provide luminescence imaging capabilities. Dinuclear ($\text{Ir}^{\bullet}\text{Ln}$) complexes performed best in cellular studies, exhibiting good solubility in aqueous solutions, low toxicity after 4 and 18h, and punctate lysosomal staining. We also demonstrate the first example of oxygen sensing in fixed cells using the dyad $\text{Ir}^{\bullet}\text{Gd}$, via two-photon phosphorescence lifetime imaging (PLIM).
**INTRODUCTION**

The use of transition metal luminophores to sensitisce luminescence from lanthanide(III) ions in mixed d-f complexes has attracted recent attention from many researchers.\(^1\) The long-lived excited states of many d-block luminophores with triplet excited states make them excellent energy donors whose excited-state energy can be tuned over a wide range by control of ligand type and substituents; in addition numerous straightforward synthetic methods exist to combine d-block and f-block units such that d-f energy-transfer can occur with the d-block unit acting as the light-harvesting antenna for the lanthanide ion, for which direct f-f excitation is Laporte forbidden.\(^1\) Amongst such d-f hybrids the extensive family of cyclometallated phenyl-pyridine/ Ir(III) complexes, with their high energy and long-lived triplet excited states that result in blue or green luminescence,\(^2\) have proven to be effective energy-donors for generating luminescence from lanthanides such as Eu(III), Tb(III), Yb(III) and Nd(III) in Ir/Ln dyads.\(^3,4\) The first example came from De Cola and co-workers who demonstrated that the combination of blue Ir(III)-based emission and red Eu(III)-based emission from a single molecular edifice could be used to generate white light,\(^4a\) and many other examples have been reported since then.

In addition, the use of luminescent transition metal and lanthanide complexes with long-lived excited states has become very popular in bio-imaging applications over the past decade.\(^5,6\) This, combined with technological developments in electronic shutters and optics, has resulted in time-resolved imaging techniques becoming more commonplace in the biosciences. These techniques include the simple rejection of short-lived background autofluorescence via time gating, and ‘lifetime mapping’ using a combination of single photon counting and scanning confocal microscopy, as demonstrated by Phosphorescence Lifetime Imaging (PLIM) and Time-Resolved Emission-imaging microscopy (TREM).\(^7\)

One area which has benefited from these developments is the real-time detection and quantification of oxygen via phosphorescence quenching.\(^7a,7c-f,8\) Molecular oxygen has a triplet ground state configuration and is an effective quencher of the triplet based phosphorescence displayed by transition metal complexes. Oxygen also plays a key role in many physiological processes, ATP generation, and mitochondrial function.\(^9d-f\) Low or inadequate levels of oxygen, referred to as hypoxia, are important features in solid tumors, inflammatory diseases and Alzheimer's disease.\(^9a-c,9g\) Hence, the ability to monitor oxygen concentration in vivo and in vitro, under real time non-invasive conditions, is extremely desirable for diagnostics and treatment. Optical oxygen monitoring and imaging using
lifetime based techniques have advantages over intensity based ones. Decay of photo-excited molecules typically follows first-order decay kinetics, so lifetime measurements are not affected by the intensity of the excitation light, and lifetime measurements are a property of the individual molecule and are therefore largely concentration independent. Measuring emission lifetime of a probe, rather than the emission intensity, provides a quantitative, non-invasive method by which to monitor intercellular oxygen concentrations. Intensity based measurements can be used for quantitative oxygen sensing with ratiometric probes, where the emissive molecule is both the probe and the internal calibrant. Recent examples of oxygen detection using luminescent transition metal complexes have involved Pt(II) complexes, \textsuperscript{7a,8c-e} Pd(II)–porphyrins, \textsuperscript{8b,11} Ru(II)\textsuperscript{12} and Ir(III)\textsuperscript{8a,13} complexes, including iridium-based up-converting nanoparticles doped with lanthanides.\textsuperscript{13b}

![Figure 1: Structures of the dinuclear and trinuclear complexes discussed in this paper, and a mononuclear Ir(III) complex Ir•L1 used in the DFT calculations.](image)

As part of a study to investigate the use of d/f hybrid complexes as cellular imaging probes, we recently reported the preparation and study of complexes Ir•Eu and Ir•Gd (Fig. 1) in which a strongly luminescent [Ir(F\textsubscript{2}ppy\textsubscript{2})(phen)]\textsuperscript{+} unit is connected to a stable lanthanide(III) polyamino-carboxylate chelate via a fully conjugated alkyne bridge.\textsuperscript{14} The
design of these complexes has three particularly beneficial features. Firstly, the conjugated pathway connecting the two metal centres allows efficient Ir(III) → Eu(III) energy-transfer by the Dexter mechanism which requires electronic coupling between donor and acceptor.\textsuperscript{1c,15} Thus, excitation of the Ir(III) unit under either single-photon or two-photon conditions was followed by partial Ir(III) → Eu(III) energy-transfer to give a mixture of green Ir(III)-based emission (\(\mu\)s timescale) and red Eu(III)-based emission (ms timescale). Secondly, the extended conjugated bridge which is involved in MLCT transitions may afford a significant two-photon absorption cross-section which will assist with imaging under two-photon excitation conditions. Thirdly, the rigidity imparted to the complex by the rigid rod-like structure resulted in a high relaxivity for the mononuclear Gd(III) centre associated with slow tumbling in solution,\textsuperscript{16} and we note that d/f complexes combining a luminescent unit and a Gd(III) unit for dual-mode luminescence imaging / Magnetic Resonance Imaging (MRI) have attracted particular interest recently.\textsuperscript{17} In general, for MRI-based applications, the required high relaxivity for protons of water molecules arises from a combination of factors such as the number of sites available for water to coordinate to the Gd(III) centre and their exchange rate (inner-sphere relaxivity), interactions between the Gd(III) centre and more remote water molecules (outer-sphere relaxivity), the rotational correlation time of the molecules in solution, and the longitudinal and transverse electron spin relaxation times of Gd(III), as encapsulated in Solomon-Bloembergen-Morgan theory.\textsuperscript{18}

In this follow-up paper we report on two related areas. Firstly, we have substantially extended the scope of the cellular imaging studies performed with Ir•Eu and Ir•Gd, demonstrating in particular how we can use the lifetime of the Ir(III)-based luminescence as a probe for the oxygen concentration in cells under in vitro conditions. Secondly, we report the new trinuclear complexes Ir•Eu\(_2\) and Ir•Gd\(_2\) (Fig. 1) in which two Ln(III) units are pendant from the central [Ir(F\(_2\)ppy\(_3\))(phen)]\(^+\) and describe their photophysical properties and use in imaging studies.

**RESULTS AND DISCUSSION**

**Synthesis of new trinuclear complexes.**

Synthesis of the trinuclear Ir•Ln\(_2\) complexes follows the same general methodology as used for the dinuclear Ir•Ln complexes that were reported earlier (Scheme 1).\textsuperscript{12} The ester-protected pyridine-2,6-bis(imino-diacetate) unit which ends up as the Ln(III) binding site contains a central pyridyl group which can be functionalized with a Br atom at the C\(^4\) position.
(precursor A). This permits its connection to the \([\text{Ir(F}_2\text{ppy)}_2\text{(phen)}]^+\) sensitiser via alkynyl linkages introduced in a Sonogashira coupling reaction. Whereas use of 3-ethynyl-1,10-phenanthroline afforded the dinuclear Ir•Ln complexes using this methodology,\(^{14}\) in this new work use of 3,8-di(ethynyl)-1,10-phenanthroline (precursor B) allowed connection of the Br-functionalised bis(imino-diacetate) unit to both alkynyl sites to give compound C. After assembling the component binding sites in this way, coordination of the phenanthroline unit of C to an \(\{\text{Ir(F}_2\text{ppy)}_2\}_2\)\(^+\) unit, and unmasking of the amino/carboxylate binding site by removal of the esters, followed by incorporation of Eu(III) or Gd(III), all used standard methods. Incorporation of the pyridyl group into the lanthanide binding site – in contrast to use of the cyclen-tricarboxylate chelate that we used in our earlier work\(^3f\) – allows the Sonogashira coupling to provide a fully conjugated pathway between the Ir(III) and lanthanide centres.
Accordingly these trinuclear complexes \( \text{Ir} \cdot \text{Ln}_2 \) should be expected to have all of the same benefits arising from their structure as do the dinuclear ones, \( \text{viz.} \) efficient \( \text{Ir(III)} \rightarrow \text{Eu(III)} \) energy-transfer; a two-photon absorption cross-section sufficient for imaging purposes; and structural rigidity to impart high relaxivity to the \( \text{Gd(III)} \) units.

### Steady-state and time-resolved luminescence properties of the trinuclear complexes.

UV/Vis absorption spectral data of the complexes in water are summarised in Table 1. Intense absorptions in the UV region are due to ligand-centred \( \pi \rightarrow \pi^* \) electronic transitions; the weak shoulder and long tail between 400 nm and 550 nm are ascribed to \( \text{Ir(III)} \rightarrow \text{phen} \) \( ^3\text{MLCT} \) transitions (Fig. S13). It was observed that there is approximately a 10 to 15 nm bathochromic (red) shift, as well as an increase in intensity, of the absorption bands in the case of \( \text{Ir} \cdot \text{Ln}_2 \) compared to the \( \text{Ir} \cdot \text{Ln} \) complexes which we ascribe to (i) the greater extent of the conjugated network on the substituted phenanthroline ligand in the latter case, and (ii) the presence of two \( \text{Ir(III)} \) units and their associated aromatic ligands instead of just one.

Luminescence spectra (see Fig. 2) of \( \text{Ir} \cdot \text{Ln} \) and \( \text{Ir} \cdot \text{Ln}_2 \) were recorded in aerated aqueous solution upon excitation at 400 nm. In \( \text{Ir} \cdot \text{Gd}_2 \) the \( \text{Ir(III)} \)-based luminescence maximum is at 595 nm: this is significantly red-shifted compared to \( \text{Ir} \cdot \text{Gd} \) (560 nm), a consequence of the more extended \( \pi \)-network around the phen ligand involved in the MLCT transition which will stabilise its LUMO. At 77K (1:4 \text{MeOH/EtOH} frozen glass) the rigidochromism expected from a charge-transfer state is clear (Fig. 3), with the highest-energy emission feature now occurring at 529 nm, indicating a \( ^3\text{MLCT} \) energy of 18,900 cm\(^{-1}\) (compared to 20,200 cm\(^{-1}\) for \( \text{Ir} \cdot \text{Gd} \)). Significantly this energy value for the \( \text{Ir(III)} \rightarrow \text{phen} \) \( ^3\text{MLCT} \) state is now marginal for efficient sensitisation of the emissive \( ^5\text{D}_0 \) level of Eu(III) which lies at 17,500 cm\(^{-1}\) – the gradient for energy-transfer is only 1400 cm\(^{-1}\) – so on this basis we might not see efficient sensitisation of Eu(III)-based luminescence in \( \text{Ir} \cdot \text{Eu}_2 \), as a gradient of ca. 2000 cm\(^{-1}\) for energy-transfer at RT is normally considered necessary to prevent thermally-activated back energy-transfer.\(^{3e,19}\) Quantum yield values of dyad \( \text{Ir} \cdot \text{Gd} \) (\( \Phi = 0.048 \)) and triad \( \text{Ir} \cdot \text{Gd}_2 \) (\( \Phi = 0.026 \)) were measured in DMSO, showing that the lower-energy luminescence of \( \text{Ir} \cdot \text{Gd}_2 \) is also slightly lower in intensity.
Comparison of the luminescence spectra of dyad Ir•Eu and triad Ir•Eu₂ demonstrate nicely the differing ability of their Ir(III)-centred ³MLCT states to sensitise Eu(III) emission (Fig. 2). The luminescence spectrum of Ir•Eu in water (reported previously¹⁴) shows how significant Ir(III) → Eu(III) EnT (energy transfer) has occurred, with the Ir(III)-based luminescence reduced in intensity compared to what was observed for Ir•Gd and the characteristic sharp emission lines from Eu(III) superimposed on the low-energy tail of the broad Ir(III)-based luminescence. In contrast, in the luminescence spectrum of Ir•Eu₂ (Fig. 2, green trace) the sharp Eu(III)-based emission lines are barely visible. They are clearly present to some extent as they distort the envelope of the Ir(III)-based luminescence: a small just-visible feature at 615 nm may be ascribed to the tip of the most intense Eu(III)-emission component, but the sensitised Eu(III)-based luminescence is clearly very much weaker in Ir•Eu₂ than in Ir•Eu. Quantum yield determinations for dyad Ir•Eu and triad Ir•Eu₂ were
not performed due to substantial overlap of the Ir(III)-based Eu(III)-based emission components; and in any case a single quantum yield value encapsulating the combined emission from two quite different luminophores is not a useful parameter.

The differences in Ir(III) → Eu(III) EnT between Ir•Eu and Ir•Eu₂ are also apparent from time-resolved measurements. Whereas Ir•Eu showed substantial quenching of the Ir(III)-based emission lifetime compared to Ir•Gd, associated with an Ir(III) → Eu(III) EnT rate of ca. 6 × 10⁶ s⁻¹, there is much less difference between the unquenched Ir(III)-based emission lifetimes of Ir•Gd₂ (τ = 1260 and 230 ns) and Ir•Eu₂ (τ = 1240 and 168 ns). Based on the shorter-lived emission component we can estimate an Ir(III) → Eu(III) EnT rate of ca. 1.6 × 10⁶ s⁻¹ in Ir•Eu₂; this comes from equation 1, in which τ_u is the ‘unquenched’ lifetime (i.e. in the Ir/Gd complex) and τ_q is the ‘quenched’ lifetime (i.e. in the Ir/Eu complex).

\[ k_{\text{EnT}} = \frac{1}{\tau_q} - \frac{1}{\tau_u} \]  

(1)

The energy-transfer rate constant for Ir•Eu₂ is clearly considerably smaller than for Ir•Eu due to the smaller thermodynamic gradient, and this is also in agreement with the appearance of the steady-state spectra.

Table 1: UV/Vis absorption and luminescence spectral data for the complexes

| Compound | \(\lambda_{\text{max}}/\text{nm} \)  
\( (10^3 \varepsilon / \text{M} \cdot \text{cm}^{-1}) \) | \(\lambda_{\text{em}}/\text{nm} \) | \(\tau_1^0, \tau_2^0/\text{ns}^b \) | \(\tau_1, \tau_2/\text{ns}^b \) | \(\phi^d \) | Ref. |
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<tr>
<td>Ir•Gd</td>
<td>242 (46), 285 (42), 338 (22)</td>
<td>560</td>
<td>1100, 450</td>
<td>640, 220</td>
<td>0.048</td>
<td>14</td>
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<tr>
<td>Ir•Eu</td>
<td>242 (50), 283 (44), 343 (23)</td>
<td>578, 590, 615, 684, 697</td>
<td>780, 116</td>
<td>510, 95</td>
<td>–</td>
<td>14</td>
</tr>
<tr>
<td>D</td>
<td>241 (81), 285 (79), 355 (52)</td>
<td>552</td>
<td>1470, 275</td>
<td>–</td>
<td>–</td>
<td>This work</td>
</tr>
<tr>
<td>E</td>
<td>241 (93), 290 (96), 356 (73)</td>
<td>590</td>
<td>890, 150</td>
<td>–</td>
<td>–</td>
<td>This work</td>
</tr>
<tr>
<td>Ir•Gd₂</td>
<td>242 (112), 292 (119), 358 (104)</td>
<td>595</td>
<td>1260, 233</td>
<td>–</td>
<td>0.026</td>
<td>This work</td>
</tr>
<tr>
<td>Ir•Eu₂</td>
<td>241 (118), 292 (127), 357 (111)</td>
<td>616</td>
<td>1240, 168</td>
<td>–</td>
<td>–</td>
<td>This work</td>
</tr>
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*All measurements were carried out in air-equilibrated water at room temperature. The excitation wavelength for luminescence studies was 400 nm in every case.*
Ir(III)-based luminescence lifetimes: all complexes showed dual-exponential luminescence decay (see main text). \( \tau \): air equilibrated solution. \( \tau^0 \): Argon equilibrated solution.

Eu(III)-based emission maxima which partly obscure the Ir(III)-based emission, see main text.

Quantum yield values in aerated solvent recorded in DMSO against 1,3-di(2-pyridyl)benzene Platinum (II) Chloride in CH\(_2\)Cl\(_2\) as a reference (\( \phi = 0.039 \), see ref. 20)

**Theoretical calculations.**

Quantum mechanical calculations were carried out in an effort to understand the optical properties and charge transfer behavior of the two major Ir(III)-containing rigid skeletons. Calculations were performed on mononuclear Ir(III) complexes \( \text{Ir} \bullet \text{L}^1 \) (Fig. 1) and \( \text{E} \) (Scheme 2), i.e. the fully functionalized and deprotected Ir(III) complexes, with the pendant pyridyl/amine/acid binding sites but without the attached Ln(III) ions. All calculations were performed using the Gaussian 09 program suite\(^{21} \) with Becke’s three-parameter hybrid exchange functional (B3LYP);\(^{22} \) a basis set of 6-31G* was employed for H, C, N and O atoms and for Ir(III) we used the LanL2DZ\(^{23} \) basis set. The calculations revealed that the conjugated phenanthroline-based ligands with alkyne substituents in both \( \text{Ir} \bullet \text{L}^1 \) and \( \text{E} \) are planar. Moreover, there is a prominent (0.149 eV) reduction of energy gap (\( \Delta E \)) between the HOMO and LUMO on moving from \( \text{Ir} \bullet \text{L}^1 \) (\( \Delta E = 2.630 \) eV) to \( \text{E} \) (\( \Delta E = 2.481 \) eV; cf. Fig. S24 and S25 respectively) which is attributed to the stabilization of the LUMO that results from an effective expansion of the \( \pi \)-electron cloud throughout the rigid rod ligand, with the second alkyne/pyridine substituent. This finding is fully in agreement with the observed red shift of the absorption bands in \( \text{E} \) which are in the UV region, characteristic of \( \pi \rightarrow \pi^* \) electronic transition (Fig. S13).

The systems were then carefully analyzed in accord with a number of molecular orbital digrams from HOMO–20 to LUMO+20 to explain other electronic features. In the case of \( \text{Ir} \bullet \text{L}^1 \), the HOMO–2 is mainly concentrated on Ir(III)-based antenna group and the LUMO is located throughout the conjugated fragments (Fig. S24). On this basis, we assign the \( {}^3 \)MLCT band observed in the visible region for \( \text{Ir} \bullet \text{L}^1 \) to be due to the HOMO–2 \( \rightarrow \) LUMO electronic transition (Fig. S26). In the case of the larger complex \( \text{E} \), the HOMO–4 and HOMO–16 have substantial Ir(III)-based character and the LUMO is centered mainly on the extended phen/alkyne ligand, extending to the include the pyridyl rings (Fig. S25). Thus the \( {}^3 \)MLCT transition could be assigned due to the HOMO–4 \( \rightarrow \) LUMO and HOMO–16 \( \rightarrow \) LUMO electronic transitions (Fig. S27).

Time-dependent (TD) DFT (B3LYP/6-31G*) simulations of the electronic spectra of \( \text{Ir} \bullet \text{L}^1 \) and \( \text{E} \) were also carried out. The TD-DFT stick spectra obtained from this analysis are
in good agreement with the experimental spectra (cf. Fig. S26–S27, Tables S2–S4 in ESI). In fact, the $^3$MLCT-based transitions involving the Ir(III)-dominated MOs are seen to give rise to very weak bands observed in the visible region of the experimentally determined steady-state absorption spectra (cf. Tables S2–S3).

**Relaxivity of Ir•Gd and Ir•Gd$_2$.**

We reported previously that dinuclear Ir•Gd has a relaxivity of 11.9 mM$^{-1}$s$^{-1}$ in aqueous solution at 37°C and 20 MHz, a value considerably higher than normally observed for mononuclear Gd(III) chelates of this type, which was ascribed to the rigidity of the rod-shaped complex.\(^\text{14}\) Trinuclear Ir•Gd$_2$ is not sufficiently soluble for relaxivity measurements in pure water as solubilities of the order of mM are required. Instead the relaxivity of Ir•Gd$_2$ was measured in a DMSO/water (5:95, v/v) mixture, and the relaxivity of Ir•Gd was re-measured under the same conditions for comparison as the presence of DMSO increases solvent viscosity. Note that the relaxivity values for the analogous compounds Ir•Eu and Ir•Eu$_2$ were not measured as the non-symmetrical electron configuration of Eu(III) is well known to result in a spin relaxation time which is too short to alter the relaxivity of protons.\(^\text{24}\)

Complex Ir•Gd, in this solvent mixture, has a relaxivity slightly higher than in aqueous solution (14.0 mM$^{-1}$s$^{-1}$, cf. 11.9 mM$^{-1}$s$^{-1}$ in water, both at 20 MHz, 37°C). The relaxivity of Ir•Gd$_4$ is slightly lower than that of Ir•Gd (12.6 mM$^{-1}$s$^{-1}$) at this frequency despite the presence of a second Gd(III) centre. The relaxivity values of these species are comparable to those of complexes with very similar Gd(III) co-ordination environments in aqueous solution,\(^\text{25}\) but significantly higher (in the case of Ir•Gd) than those observed with DTPA (diethylenetriamine-pentaacetic acid) and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid)-like molecules which are typically 4–5 mM$^{-1}$s$^{-1}$. This is mostly due to the rigidity of the local ligand framework about the metal centre leading to fast water exchange rates.\(^\text{25a}\) The lower relaxivity of Ir•Gd$_4$ than Ir•Gd at 20 MHz may arise from the complex relationship between rotational correlation time, relaxivity and measurement frequency:\(^\text{26}\) but also, in part, it may arise from the poor solubility of Ir•Gd$_2$ which would cause aggregation of the complex and change the effective hydration state of the Gd(III) centre.

*Imaging studies using Ir•Eu and Ir•Gd.*
During the initial imaging studies with dyads $\text{Ir}\cdot\text{Eu}$ and $\text{Ir}\cdot\text{Gd}$ we observed that $\text{Ir}\cdot\text{Gd}$ was taken up by MCF7 cells considerably more effectively than $\text{Ir}\cdot\text{Eu}$, which was a surprise given their structural similarity: this might be related to a change in coordination number between Eu(III) and Gd(III). Two-photon PLIM measurements confirmed that the differences in Ir(III)-based emission lifetimes, arising from partial quenching by $\text{Ir(III) → Eu(III) EnT}$ in $\text{Ir}\cdot\text{Eu}$ but not in $\text{Ir}\cdot\text{Gd}$, could also be detected in cells.

Here we report additional imaging studies using these complexes in HeLa cells. As with MCF7 cells, we noticed that cellular uptake of the two dyads was markedly different. The luminescence intensity observed after 18 hours incubation with $\text{Ir}\cdot\text{Gd}$ was much higher than that observed with $\text{Ir}\cdot\text{Eu}$, implying a more efficient uptake of $\text{Ir}\cdot\text{Gd}$. This is apparent in the distance vs. luminescence intensity line profiles for the two complexes following incubation at 100 µM (Fig. 4): across individual cells $\text{Ir}\cdot\text{Gd}$ displays intensity values that are approximately double that of $\text{Ir}\cdot\text{Eu}$, with the brightest pixels for the punctate emission showing almost triple the luminescence intensity that was obtained with $\text{Ir}\cdot\text{Eu}$. If there were no difference in uptake between the two complexes we would expect to see only a ca. 20% enhancement in emission intensity from $\text{Ir}\cdot\text{Eu}$ to $\text{Ir}\cdot\text{Gd}$ [based on the differences in Ir(III)-based emission intensity between the two complexes arising from partial quenching in $\text{Ir}\cdot\text{Eu}$]. We tentatively attribute this to difference in cellular uptake with a subtle difference in co-ordination sphere around the lanthanide ion, as other attributes of the dyads, overall size, charge and lipophilicity are virtually the same. Fig. S15 shows the comparative uptake of $\text{Ir}\cdot\text{Eu}$ and $\text{Ir}\cdot\text{Gd}$ in HeLa cells across a range of concentrations (100–25 µM), with images being recorded using the same instrument parameters (initially optimised for $\text{Ir}\cdot\text{Eu}$ at 100 µM) to allow direct comparison. Optimising imaging parameters per sample (Fig. S16) allowed the staining pattern of the dyads in HeLa cells to be clearly observed. The staining of $\text{Ir}\cdot\text{Eu}$ and $\text{Ir}\cdot\text{Gd}$ in HeLa cells is very similar to that observed in MCF7 cells, in that emission from the dyads is observed in the cell cytosol with some additional punctate staining. The complexes were not observed to cross the nuclear membrane.
Co-localisation studies with lysotracker red (Fig. 5) show a very good correlation with the punctate staining observed for the dyads. Qualitative overlay images (Fig. 5, right panel, grey scale), generated using co-localisation threshold in ImageJ, show a good correlation between the punctate Ir(III)-based emission (green) and lysotracker emission (red). Control images (lysotracker only, Fig. S17) confirm there is no crosstalk of lysotracker emission under 405 nm excitation.

The degree of co-localisation between lysotracker and the \textit{Ir\cdotLn} complexes is reflected in the Manders coefficients\textsuperscript{27} (obtained using Coloc2 plugin, ImageJ): These are values between 0 and 1, which measure the extent of co-occurrence between red and green pixels (where 0 is no co-occurrence and 1 is perfect co-occurrence). The coefficient M1 takes into account the red channel first, asking the question ‘if there is a red pixel is there also a co-localised green one’? The coefficient M2 is calculated using the green channel first (\textit{i.e.} if there is a green pixel, is there also a co-localised red one)?
Figure 5: Co-staining of HeLa cells with Ir•Ln and lysotracker red. Top: Ir•Eu, Bottom: Ir•Gd. Live HeLa cells were incubated with Ir•Eu at 100 µM and Ir•Gd at 50 µM in full media for 18 hours before the addition of lysotracker red. Live cells were washed with PBS and imaged in full (phenol-red free) media. Left: Ir•Ln = $\lambda_{\text{ex}}$: 405 nm, $\lambda_{\text{em}}$: 500–550 nm; Middle: Lysotracker red = $\lambda_{\text{ex}}$: 561 nm, $\lambda_{\text{em}}$: 590–700 nm; right: overlay, with co-localisation depicted by grey. Scale bar = 10 µm.

The M1 / M2 values for lysotracker with either Ir•Eu or Ir•Gd are similar at 0.99 / 0.73 (lysotracker + Ir•Eu) and 0.91 / 0.87 (lysotracker + Ir•Gd). The fact that M1 is close to 1 and M2 is not 0 in both cases indicates that there is a good co-localisation between lysotracker and Ir•Ln, and that the Ir•Ln complexes do not exclusively stain the lysosome, which supports what we see in the steady state confocal images.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) toxicity assays show a decrease of ca. 20% in cell survival, particularly at higher incubation concentrations (100 µM) and incubation times (up to 18h), associated with uptake of the complexes into the cells. However, the reduction in cell viability with the Ir•Ln complexes is much less significant with an incubation time of only 4 hours: under these conditions, cell survival fractions here are similar to the control experiments. Overall, the dinuclear Ir•Ln complexes appear to be only slightly toxic to HeLa cells even at high concentrations and long incubation
times, and under conditions used for imaging and oxygen sensing (see below) these dinuclear complexes do not appear to have any significant effect on cell viability.

**Imaging studies using $\text{Ir}^{\text{Eu}}_2$ and $\text{Ir}^{\text{Gd}}_2$.**

The trinuclear complexes $\text{Ir}^{\text{Eu}}_2$ and $\text{Ir}^{\text{Gd}}_2$ display a very similar staining pattern in HeLa cells to that of the dinuclear complexes (Fig. 6). However, their uptake into HeLa cells was observed to be generally poorer than $\text{Ir}^{\text{Ln}}$ complexes. As size and charge have transpired to be two key factors in cellular uptake of metal based imaging agents, where small cationic complexes tend to perform the best, it is therefore logical that the larger, anionic triads ($\text{Ir}^{\text{Ln}}_3$) are less efficiently taken up in to mammalian cells than the smaller, neutral dyads ($\text{Ir}^{\text{Ln}}_2$). The uptake trend between the two triad complexes appeared to exhibited the opposite behavior to that of the dinuclear complexes, in that $\text{Ir}^{\text{Eu}}_2$ was taken up more effectively than $\text{Ir}^{\text{Gd}}_2$. Again such a difference is unexpected given the structural similarity of the triads, and may arise from a change in coordination number between Eu(III) and Gd(III). However, the mediocre solubility of the triads in aqueous solution, when diluted from a DMSO stock, means that this difference between uptake of $\text{Ir}^{\text{Eu}}_2$ and $\text{Ir}^{\text{Gd}}_2$ may actually be related to solubility. Confocal images of HeLa cells stained with $\text{Ir}^{\text{Ln}}_2$ (Fig. 6, left and middle), obtained using an identical set of parameters (initially optimized for $\text{Ir}^{\text{Eu}}_2$ at 100 µM) depict the strong difference between uptake of $\text{Ir}^{\text{Eu}}_2$ and $\text{Ir}^{\text{Gd}}_2$: the emission intensity arising from the cells stained with $\text{Ir}^{\text{Eu}}_2$ is clearly the brighter of the two despite the inherently weaker emission of the Ir-unit [arising from slight quenching by the Eu(III) ions].

![Figure 6](image_url)

**Figure 6**: Left and Middle: Comparison of $\text{Ir}^{\text{Eu}}_2$ (left) and $\text{Ir}^{\text{Gd}}_2$ (middle) uptake in live HeLa cells. Cells were incubated with $\text{Ir}^{\text{Ln}}_2$ at 100 µM in full media for 18 hours, followed by washing and fixation. Imaging parameters (optimised for $\text{Ir}^{\text{Eu}}_2$ at 100 µM) remained constant for both compounds. Right: $\text{Ir}^{\text{Gd}}_2$ imaged with optimised parameters for this sample. $\lambda_{\text{ex}}$: 405 nm, $\lambda_{\text{em}}$: 500–530 nm. Scale bar = 20 µm.
Poor cellular uptake of the trinuclear complexes is reflected in the MTT toxicity data (Fig. S19). The survival fractions of HeLa cells after incubation with 100 µM of either of the trinuclear complexes for 18 hours, are very similar to those obtained for 0.5% DMSO and 1% DMSO control experiments, implying that uptake into the cells is poor. The relatively poor solubility of the Ir•Ln\textsubscript{2} trinuclear complexes (in comparison to the dinuclear Ir•Ln analogues) may contribute to this. Nonetheless, luminescence spectra recorded directly from live HeLa cells (Fig. 7) do show that the complexes remain intact during transport across the cell membrane (compare the spectra in Fig. 7 with the spectra shown in Fig. 2).

**Figure 7:** Emission spectra recorded from live HeLa cells after incubation with dinuclear Ir•Ln complexes (left) and trinuclear Ir•Ln\textsubscript{2} complexes (right).

**O\textsubscript{2} Sensing using Ir•Gd.**

The Ir•Gd dyad is an ideal candidate to investigate further in time-resolved imaging applications. It is highly soluble in aqueous solution; taken up efficiently into both MCF7 and HeLa cells; exhibits low toxicity; shows appreciable excitation under two-photon excitation; and it has a luminescence lifetime of the order of hundreds of ns which makes lifetime variations easy to measure. We therefore wished to evaluate its suitability for luminescence-based O\textsubscript{2} sensing in cells. To do this we investigated the emission lifetime of Ir•Gd under increasing concentrations of O\textsubscript{2} (using O\textsubscript{2}:N\textsubscript{2} mixtures) in aqueous solution, in full cell media using single-photon PLIM, and in fixed HeLa cells using two-photon PLIM.

Solution measurements (in water and cell media) were recorded by scanning a small area of the bulk solution (inside a MatTek 35 mm glass bottomed dish), using a 256 × 256 pixel array on an inverted Nikon Ti-E microscope with single photon PLIM imaging capabilities. The entire (homogenous) 256 × 256 pixel array was analysed as a single region
of interest (ROI) for each oxygen concentration. Superimposed decay traces (Fig. S20) clearly show the change in \textbf{Ir•Gd} emission lifetime with changing concentration of \textit{O}_2. For both solutions, decay curves were best fit to a double exponential model; the major lifetime component ($\tau_1$) matches those recorded in a conventional spectroscopy cuvette using a lifetime spectrometer.

Lifetime mapping of fixed HeLa cells labeled with \textbf{Ir•Gd}, using two-photon PLIM, reveals a distribution of lifetimes across a single cell under air equilibrated conditions. The higher intensity emission emanating from the punctate staining of the cell lysosomes exhibits a shorter emission lifetime compared to that of the more diffuse cytoplasmic staining (Figs. 8 & 9). Fig. 8 highlights the two different staining patterns as ROI-1 (cytoplasm) and ROI-2 (lysosomes) on a typical two-photon PLIM intensity based image (where all emitted photons are binned into one channel). Overlaid decay traces [Fig. 8(c)] and distribution histograms [Fig. 8(a), depicting the distribution of the major component $\tau_1$, after fitting all pixels to a double exponential model] from each ROI [see Fig. 8(b)] shows the difference in emission lifetime between cellular locations; ROI 1: $\tau_1 = ca. 620$ ns, ROI 2: $\tau_1 = ca. 500$ ns. This variation in lifetime is tentatively attributed to the different local environments experienced by the complex, \textit{i.e.} acidic cell lysosomes in comparison to diffuse cytoplasm, rather than concentration of \textbf{Ir•Gd} within the cell. In solution, the emission lifetimes of \textbf{Ir•Gd} remain constant with concentration across \textit{ca.} two orders of magnitude (1.0 x$10^{-6}$ M to 1.0 x$10^{-4}$ M, Fig. S22). Lifetimes recorded in aqueous solution have a larger percentage contribution from the shorter lifetime component ($\tau_2$ \textit{ca.} 60\%) in comparison to CH$_2$Cl$_2$ solutions ($\tau_2 > 10\%$), however, this contribution decreases to \textit{ca.} 40\% with increasing concentration. The cellular environment is more complex than that of a homogenous solution and the local concentration of \textbf{Ir•Gd} within the lysosomes may be higher than that measured in solution. Therefore, it is possible that aggregation and self-quenching (as is observed in solution for these types of complex)$^{3b,3d,14}$ also plays a part in the shorter emission lifetime observed at the lysosomes.
Having established the range of luminescence lifetimes within a cell under air, we then repeated the experiment under different concentrations of O$_2$. Two-photon PLIM imaging of Ir$\cdot$Gd in fixed HeLa cells across a range of O$_2$ concentrations (Fig. 9) shows that (i) the Ir(III) based emission lifetime is sensitive to changing levels of O$_2$ even when fixed inside a complex cell environment, and (ii) the distribution of lifetimes across a single cell is maintained. Four lifetime maps are shown in Fig. 9, plotted on the same colour scale with the variation from red to blue as the lifetime varies across the range 0–1000 ns. Within Fig. 9 the $\tau_1$ distribution plots change visually from principally orange to principally blue, showing how the luminescence lifetime increases as the O$_2$ concentration decreases. The range of $\tau_1$ values within each image are also plotted as histograms (below) on the same scale, depicting the change in average luminescence lifetime in a cell with decreasing levels of O$_2$: average lifetime values of 435, 520, 586 and 644 ns are obtained with relative O$_2$ concentrations of 100%, 50%, 21% and 0% respectively. The response of specific lysosomal and cytoplasmic ROI’s with changing O$_2$ levels are shown in Fig. 10.
The sensitivity of Ir•Gd luminescence lifetime to changing levels of oxygen across the three conditions (fixed cells, water and full cell media) was quantified using the Stern-Volmer equation (2). Quenching constants \( K_{SV} \) determined from a straight line plot of \([O_2]\) (µM) against \( \tau_0/\tau \), Fig. 10 (where \( \tau_0 \) is the emission lifetime at 100% N\(_2\)), were very similar for fixed HeLa cells and water (3.54 × 10\(^{-4}\) µM\(^{-1}\) and 3.80 × 10\(^{-4}\) µM\(^{-1}\), respectively) but slightly lower for full cell media (2.02 × 10\(^{-4}\) µM\(^{-1}\)), which indicates some environment-dependent sensitivity of the luminescence towards O\(_2\).

Luminescence lifetime values in full media were observed to be slightly longer than those obtained from aqueous solution or in fixed cells. We believe the longer observed lifetime – and lower sensitivity towards quenching by O\(_2\) – in full Modified Eagles Medium (MEM) cell media is due to the dyad being in a more protected environment when a large protein such as fetal calf serum is present in the bulk solution. Binding to large protein molecules is known to protect small molecules from collisional quenching with dissolved oxygen; protein titrations with a luminescent Ru(II) complex have shown that the emission intensity and lifetime increase as the concentration of protein increases. Although proteins and sub-cellular structures also exist in fixed cells the region of interest (in this case the whole cell) is non-homogenous; meaning the lifetime observed from a single cell is a summative average of the emission properties of Ir•Gd from multiple cellular environments (potentially at different concentrations). The more homogeneous nature of MEM in cell media solution provides a more uniformly protected environment for Ir•Gd, which when compared to a whole cell exhibits a longer lifetime.
Figure 9: Two-photon PLIM ($\lambda_{ex} = 760$ nm) imaging of Ir•Gd stained HeLa cells (50 µm, 18 h, fixed) under varying concentrations of O$_2$. Scale bar = 10 µm.

For a complex to be a suitable biological O$_2$ sensor, a significant difference in emission lifetime is required across physiological concentrations of O$_2$, typically 1-11%. Platinum and palladium porphyrin based systems, which have received a lot of attention as oxygen sensors in recent years, typically show a 2-fold reduction in emission lifetime ($\tau_{air} = 60–70$ µs) as the proportion of O$_2$ increases from 0 to 21%. Although, the O$_2$ sensitivity of Ir•Gd is lower than such porphyrin systems, this small molecule design does have some advantages; synthetically they are less time consuming (fewer synthetic steps than large porphyrins), the Gd centre potentially enables this single molecule to act as an MRI probe in addition to a luminescence-based probe, and the luminescence lifetime (> 10 µs) allows for faster data collection using point scanning, time resolved techniques. The fact that a clear difference in luminescence lifetime is observed in fixed HeLa cells with Ir•Gd and changing
[O\textsubscript{2}] is promising for a dynamic live cell model. Furthermore, modification of the Ir(III) chromophore to elongate the $\tau^0$Ir(III) lifetime (closer to ca. 5 $\mu$s rather than ca. 1 $\mu$s) would contribute towards improved O\textsubscript{2} sensitivity, as is observed with other Ir(III) containing oxygen sensors.\textsuperscript{13b,13d}

Figure 10: Top: Stern-Volmer plots of Ir•Gd under different oxygen concentrations in fixed HeLa cells (red, K\textsubscript{sv} = 3.54 $\times$ 10\textsuperscript{-4} $\mu$M\textsuperscript{-1}, $\tau_1$ cell average); full cell media at (green, K\textsubscript{sv} = 2.02 $\times$ 10\textsuperscript{-4} $\mu$M\textsuperscript{-1}); and water (black, K\textsubscript{sv} = 3.80 $\times$ 10\textsuperscript{-4} $\mu$M\textsuperscript{-1}). Right: Histogram showing Ir•Gd lifetime variation across fixed cell. Diffuse cytoplasmic lifetimes (grey) are consistently longer than punctate staining (blue) under varying concentrations of oxygen.

CONCLUSIONS

In conclusion, dinuclear and trinuclear Ir•Ln and Ir•Ln\textsubscript{2} complexes, in which a phosphorescent Ir(III)-based unit is connected to one or two water-stable lanthanide/aminocarboxylate units via a rigid, conjugated bridging connection, have been prepared. The dinuclear complexes performed better than their trinuclear counterparts for luminescence cell imaging and also in terms of relaxivity (for Gd complexes). For dual (luminescence + MRI)
imaging applications the complex **Ir•Gd** is a promising candidate. With excellent luminescence imaging capabilities and low toxicity, it also displays unusually high relaxivity for a small molecule containing just one Gd(III) center. Variations in luminescence lifetime allow it to be used as a sensor towards molecular oxygen, an important biological analyte, in solution and *in vitro*. However, to be considered an ideal *in vivo* sensor for O₂ a more significant change in the phosphorescence lifetime across physiological O₂ concentrations would be required: achieving this in future generations is well within the scope of well-understood synthetic modifications to the Ir(III)-complex core. Thus, this complex provides an excellent platform to optimize a dual imaging, oxygen sensitive probe.

**EXPERIMENTAL SECTION**

**Syntheses**

Ligand C (see Scheme 1). Precursors A (1.48 g, 2.2 mmol) and B (0.228 g, 1.0 mmol) (see Scheme 1) were dissolved in anhydrous DMF (20 cm³) under argon. Pd(PPh₃)₄ (92 mg, 0.08 mmol) and CuI (67 mg, 0.35 mmol) were placed in a separate 100 cm³ Schlenk tube which was evacuated and back-filled with argon several times. The solution of A and B was transferred into this reaction vessel *via* a clean and dry cannula. Finally, anhydrous triethylamine (5 cm³) was added to the reaction mixture which was kept at 80°C for three days with constant stirring. After removal of the solvent under reduced pressure, the deep brown residue was dissolved in dichloromethane (150 cm³), washed with aqueous potassium cyanide (2%, 70 cm³) and with water (2 × 200 cm³). The organic layer was dried over MgSO₄ and purified by column chromatography on alumina, eluting initially with CH₂Cl₂ and then with 1% MeOH in CH₂Cl₂, to furnish C as an orange semisolid (1.16 g, 82%). **Note:** potassium cyanide (KCN) is highly toxic, it can prevent cellular respiration and should be handled with care. Extra training may be required by the user. ¹H NMR (500 MHz, CDCl₃, 298 K, TMS): δ 1.49 (s, 72H), 3.55 (s, 16H), 4.10 (s, 8H), 7.53, (bs, 4H), 7.76 (bs, 2H), 7.92 (bs, 2H), 8.45 (bs, 2H). ¹³C NMR (125 MHz, CDCl₃, 298 K, TMS): δ = 28.2, 56.1, 59.5, 81.3, 92.0, 122.9, 127.2, 128.5, 129.7, 131.9, 132.2, 133.0, 133.4, 133.8, 138.5, 152.2, 160.1, 170.3. ESI-MS: m/z 1411.8 [20%, (M + H)⁺]; 706.4 [100%, (M + 2H)²⁺].

**[Ir(dfppy)]₂(C)[PF₆] (Complex D).** A suspension of C (0.706 g, 0.5 mmol) and [{Ir(dfppy)₂Cl}₂] (0.304 g, 0.25 mmol) in dry CH₂Cl₂/MeOH (50 cm³, 4:1 v/v) was kept at reflux for 16 hours under an inert atmosphere. After cooling the reaction mixture to RT, *ca.*
15 equivalents of NH$_4$PF$_6$ (1.22 g, 7.5 mmol) was added to the reaction mixture with stirring. After 6 h the reaction mixture was evaporated near to dryness and 5 cm$^3$ of CH$_2$Cl$_2$ was added to the residue to completely dissolve the organic substances. Residual NH$_4$PF$_6$ was filtered off and the dark solution was concentrated. The crude product was purified by column chromatography on alumina, eluting initially with CH$_2$Cl$_2$ and then with 2% MeOH in CH$_2$Cl$_2$, to furnish complex D as a deep yellow crystalline material (0.724 g, 70%). $^1$H NMR (400 MHz, CDCl$_3$, 298 K, TMS): $\delta$ 1.47 (s, 7H), 3.50 (s, 16H), 4.05 (s, 8H), 5.76 (dd, 1H), 5.78 (dd, 1H), 6.62-6.67 (m, 2H), 7.08-7.10 (m, 2H), 7.25 (s, 1H), 7.41-7.43 (m, 2H), 7.63 (s, 1H), 7.70 (s, 2H), 7.81-7.85 (m, 2H), 8.25-8.33 (m, 4H), 8.37-8.39 (m, 2H), 8.76 (d, 1H), 8.83 (d, 1H). $^{13}$C NMR (125 MHz, CDCl$_3$, 298 K, TMS): $\delta$ 28.1, 55.9, 59.4, 81.1, 86.8, 95.2, 114.2, 121.7, 122.9, 124.1, 127.6, 129.5, 130.3, 131.6, 139.3, 141.9, 142.3, 145.0, 149.0, 151.6, 152.0, 157.9, 159.4, 160.3, 162.4, 163.9, 164.0, 170.4. ESI-MS: m/z 1984 [3%, (M – PF$_6$)$^+$], 992 [20%, (M – PF$_6$ + H)$^{2+}$], 662 [100%, (M – PF$_6$ + 2H)$^{3+}$] all with correct isotope patterns and spacings.

**Complex E.** The Ir(III)-complex D (0.532 g, 0.25 mmol) was dissolved in DCM (20 cm$^3$) and cooled to 0 °C. An excess of cold trifluoroacetic acid (5 cm$^3$) was added to this cold solution dropwise whilst stirring under an argon atmosphere. The resulting mixture was stirred for 16 h at 0 °C after which solvents and other volatile substances were removed in vacuo. The residue was triturated with ether ($5 \times 25$ cm$^3$) and finally filtered to afford the product as a yellow mass which was dried in vacuo overnight. The isolated yield was 0.260 g (62%). $^1$H NMR (500 MHz, DMSO-d$^6$, 298 K, TMS): $\delta$ 3.48 (s, 16H), 3.95 (s, 8H), 5.68 (d, 1H), 5.72 (d, 1H), 6.98-7.09 (m, 4H), 7.28 (s, 1H), 7.51 (s, 1H), 7.59 (s, 2H), 7.70-7.73 (m, 2H), 7.97-8.00 (m, 2H), 8.28-8.31 (m, 4H), 8.43 (s, 2H), 9.29 (d, 2H), 12.42 (br s, 8H). $^{13}$C NMR (125 MHz, CDCl$_3$, 298 K, TMS): $\delta$ 54.4, 58.9, 88.3, 93.3, 113.5, 121.1, 121.5, 122.4, 122.9, 123.5, 124.4, 128.0, 128.9, 129.6, 131.1, 139.9, 142.5, 144.6, 150.6, 152.8, 157.9, 159.6, 161.6, 162.6, 163.6, 172.3. ESI-MS: m/z: 1535 [25%, (M – CF$_3$CO$_2$)$_2$], 1490 [90%, (M – CF$_3$CO$_2$ – HCOOH)]$^+$

**Complexes Ir$^*$Ln$_2$ (Ln = Eu(III), Gd(III); isolated as Na$^+$ salts).** To a solution of complex E (0.059 g, 0.035 mmol) in MeOH (5 cm$^3$) was added the appropriate Ln(OTf)$_3$ (0.045 g, 0.075 mmol). The mixture was stirred for 30 min after which 1M aqueous NaOH was added slowly to afford a pH of 5. The reaction mixture was then stirred at 50°C for 2 days. Solvents were then evaporated, and the residue was dissolved in the minimum amount of MeOH and re-precipitated by the gradual addition of ether; this reprecipitation was
repeated several times after which the product was dried *in vacuo*. Isolated yield: *ca.* 0.05 g (75%); the products were pure by HPLC (see ESI). Negative-ion ESI-MS for \textit{Ir•Eu}_2: \textit{m/z} 1831 (100%, [\textit{M} – \textit{Na}]^{-}). ESI-MS for \textit{Ir•Gd}_2: \textit{m/z} 1842 (100%, [\textit{M} – \textit{Na}]^{-}). These molecular ions in ESI-MS showed the correct isotope patterns and spacings.

**Cell culture and staining**

*Culture.* HeLa cells were cultured in a humidified 37°C, 5% CO\textsubscript{2}/95% air (v/v) environment in Modified Eagles Medium (MEM) supplemented with 10% (v/v) foetal calf serum (FCS), 1% penicillin-streptomycin and 2mM L-Glutamine. Cells were cultured as monolayers in T-75 flasks and passaged using trypsin-EDTA. For confocal and PLIM imaging experiments, cells were seeded in to 35 mm MatTek glass-bottomed dishes 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: 25–100 µM in full MEM (0.04–0.4% DMSO, 16 h at 37°C, 1ml/well). All incubation solutions were diluted from a 20 mM stock solution of \textit{Ir•Ln} in DMSO, with the exception of \textit{Ir•Eu}_2 which was diluted from a 10mM stock. After incubation, cells were washed with PBS (3 × 1 ml/well) to remove excess complex, then imaged in 2–3 ml of phenol-red free MEM media.

*Co-staining with lysotracker red.* After incubating with \textit{Ir•Ln}, cells were washed with PBS (3 × 1 ml/well) before the addition of lysotracker red (*Life Technologies*), 50nM in full MEM media for 30 minutes at 37°C, after which cells were washed with PBS and covered with fresh media before imaging.

**Confocal imaging.**

Confocal images were recorded using an inverted Nikon Eclipse C2 attached to a Nikon Ti-U, with 405, 488 and 561 nm coupled lasers, as a combined laser-hub unit from Omicron, UK. For imaging, a Nikon 100 × Apo oil immersion objective (NA 1.49) was used along with 405nm excitation for \textit{Ir•Ln} and \textit{Ir•Ln}_2, and 561nm excitation for lysotracker red. Emission filters used were 515/30 for \textit{Ir•Ln} and 590 nm lp for lysotracker red.

**O\textsubscript{2} dependence on Ir•Gd and lifetime imaging**
Sample preparation. Solutions of \textbf{Ir•Gd} in distilled water and full MEM media (1 × 10^{-4} M), diluted from a 20mM DMSO stock, were placed in Mat Tek 35mm glass bottomed dishes, and the excitation light focused into the bulk of the solution. For fixed cell samples, HeLa cells were cultured to 60% confluency in Mat Tek 35mm glass bottomed dishes before incubation with \textbf{Ir•Gd} (50 µM, 16 hours at 37°C). After washing and fixation, cells were covered with PBS for imaging.

Gas mixtures of O\textsubscript{2} and N\textsubscript{2} (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.

Single Photon PLIM imaging. A single-photon (405nm) phosphorescence lifetime imaging unit was used to record emission lifetimes of \textbf{Ir•Gd} solutions at varying levels of O\textsubscript{2}. This system comprised a Becker & Hickl BDL405 SM laser and DCS120 attached to a Nikon Ti-E inverted microscope.

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 image fixed cells stained with \textbf{Ir•Gd} under varying levels of O\textsubscript{2}.

A water immersion 60 × (NA 1.2) objective was used for all samples. Emission light was collected from 485-650nm 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 fixed cell samples) 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 \(\tau_1\).

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The Authors declare no competing financial interest.

Supporting information

Characterisation of $\text{Ir}\cdot\text{Ln}_2$ and precursor complexes (NMR, mass spectra, Uv-vis and emission spectra, and calculated MO diagrams). MTT procedure and toxicity data for dyads ($\text{Ir}\cdot\text{Ln}$) and triads ($\text{Ir}\cdot\text{Ln}_2$). Luminescence decay traces of $\text{Ir}\cdot\text{Gd}$ at varying concentration, and from 0-100% $O_2$ in cell media and aqueous solution. Confocal imaging of $\text{Ir}\cdot\text{Ln}_2$ in HeLa cells, two-photon absorption cross section (calculation and data processing), calculation showing conversion of % $O_2$ to [$\mu$M] $O_2$. Tabulated DFT data.

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Summary:

Dinuclear (Ir•Ln) and trinuclear (Ir•Ln₂) d-f complexes are investigated for cellular imaging. A range of photophysical, spectroscopic and imaging-based properties are reported, including relaxivity arising from Gd(III) units and the occurrence of Ir(III)→Eu(III) photoinduced energy-transfer. Non-invasive O₂ sensing with Ir•Gd (a potential dual probe: MRI + luminescence) is demonstrated in aqueous solution, full cell media and in vitro, via lifetime mapping and two-photon, phosphorescence lifetime imaging microscopy (PLIM).