Electronic Supplementary Information for

Lysosome-Specific One-Photon Fluorescence Staining and Two-Photon Singlet Oxygen Generation by Molecular Dyad

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Experimental Details.

Materials and Synthesis. Rhodamine B isothiocyanate (RhB–NCS) was purchased from the commercial supplier (Aldrich) and used as received. The syntheses of Irbtp and its precursors, including $[(btp)_2Ir(\mu\text{–}Cl)]_2$, were reported previously by us.[1,2] All glassware and magnetic stirring bars were thoroughly dried in a convection oven. CH_2Cl_2 and THF were purified by filtering over anhydrous alumina columns prior to use. Reactions were monitored using thin-layer chromatography (TLC). Commercial TLC plates (silica gel 60 F_{254}, Merck Co.) were developed and

the spots were visualized under UV illumination at 254 or 365 nm. Silica gel column chromatography was performed using silica gel 60 (particle size 0.063 – 0.200 mm, Merck Co.). ^{1}H and ^{13}C NMR spectra referenced to deuterated solvents were collected with a Bruker Ultrashield 400 plus NMR spectrometer. Mass spectra were recorded using a Thermo Electrons Co. Finnigan LCQ Advantage Max spectrometer (ESI) or a JEOL JMS-600W mass spectrometer (FAB). Elemental analysis was performed using an EA1110 or EA1112 (CE Instrument, Italy) for C, H, N, and S.

[(btp)$_2$Ir(1,10-phenanthroline-5-amine)]PF$_6$. A stirred CH_2Cl_2 solution (30 mL) containing [(btp)$_2$Ir(μ–Cl)]$_2$ (0.329 g, 0.254 mmol) and 1,10-phenanthroline-5-amine (0.0842 g, 0.431 mmol) were refluxed. After 6 h, NH_4PF_6 (15 equiv) was added to the reaction mixture at 0 °C, and the solution was stirred for additional 12 h. The residual NH_4PF_6 was removed by filtration. The solution was concentrated, and subjected to flash column chromatography on silica gel with increasing polarity of the eluent from 100% CH_2Cl_2 to CH_2Cl_2:CH_3OH = 19:1 (v/v). Further purification by using preparative TLC techniques was performed to afford a dark orange solid (0.179 g, 0.191 mmol) in a 75% yield. ^{1}H NMR (MeOD, 400 MHz) δ: 6.14 (t, *J* = 8.7 Hz, 2H), 6.80–6.86 (m, 4H), 7.17 (td, *J* = 8.1, 2.8 Hz, 3H), 7.43 (m, 2H), 7.55 (m, 1H), 7.78−7.87 (m, 8H), 8.26 (m, 2H), 8.90 (dd, *J* = 8.6, 1.3 Hz, 1H). ^{13}C NMR (MeOD, 100 MHz) δ: 119.28, 119.31, 120.78, 122.72, 123.88, 124.50, 124.86, 124.93, 125.28, 125.49, 126.30, 133.78, 135.44, 136.34, 136.50, 139.38, 139.42, 140.51, 142.82, 142.92, 145.46, 145.53, 146.20, 147.45, 147.85, 148.49, 149.41, 149.49, 151.12, 164.86, 164.91. HR MS (FAB, positive, *m*-NBA): Calcd for $C_{38}H_{25}IrN_5S_2$ ([M−PF_6]$^+$), 808.1181; found, 808.1180. Anal. Calcd for $C_{38}H_{25}F_6IrN_5PS_2$: C, 47.89; H, 2.64; N, 7.35; S, 6.73. Found: C, 48.20; H, 2.74; N, 7.18; S, 6.77.

Irbtp–RhB. [(btp)$_2$Ir(1,10-phenanthroline-5-amine)]PF$_6$ (0.100 g, 0.106 mmol) and rhodamine B isothiocyanate (0.114 g, 0.420 mmol) were dissolved in anhydrous THF (20 mL). 2.0 equivalent of dry TEA was delivered slowly to the stirred solution using a micro syringe under an Ar atmosphere, and the solution was stirred for 14 h at room temperature under dark. The reaction mixture was concentrated under vacuum, and the crude product was purified using the preparative TLC technique with CH_2Cl_2:CH_3OH = 9:1 (v/v) to afford a dark pink solid (0.0175 g, 11.7 μmol) in an 11% yield. ^{1}H NMR (MeOD, 400 MHz) δ: 1.29 (t, *J* = 7.3 Hz, 12H), 3.64 (m, 8H), 6.15 (m, 2H), 6.82 (m, 8H), 6.99 (m, 3H), 7.19 (td, *J* = 8.1, 2.9 Hz, 3H), 7.38 (d, *J* = 2.3 Hz, 1H), 7.44 (m, 3H), 7.57 (m, 1H), 7.80-7.87 (m, 7H), 8.27 (m, 2H), 8.93 (dd, *J* = 8.6, 1.2 Hz, 1H). ^{13}C NMR (MeOD, 100 MHz) δ: 9.31, 11.37, 53.37, 95.63, 102.60, 112.02, 113.72, 113.94, 119.29, 120.79, 122.76, 123.86, 124.53, 124.87, 125.51, 126.40, 131.27, 135.50, 136.46, 140.54, 142.84, 145.53, 146.23,

147.93, 149.46, 149.54, 151.17, 157.97, 164.97. MS (ESI, positive): Calcd for $C_{69}H_{58}IrN_9NaO_3S_3$ ($[M+Na+CH_3CN]^{3+}$), 458; found, 458.

Characterization

Determination of the Singlet Oxygen Generation Quantum Yields. The quantum yield for photosensitized singlet oxygen generation ($\Phi(^1O_2)$) was determined according to the method reported previously.[3] Air-equilibrated solutions containing a $^1O_2$ sensitizer and a 100 μM $^1O_2$ substrate, 1,3-diphenylisobenzofuran (DPBF), were photoirradiated with a hand-held UV lamp (10 mW, 365 nm) or a monochromatized beam from high power Xenon lamp. The absorbance of DPBF at 418 nm was recorded at every 10 s during the photoirradiation. The O.D. value at 365 nm of Irbtp−RhB was kept to 0.17. Methylene blue (O.D. = 0.14) was employed as a reference material for singlet oxygen photosensitization ($\Phi_{ref}(^1O_2) = 0.52$). $\Phi(^1O_2)$ values were determined according to the following equation: $\Phi(^1O_2) = \Phi_{ref}(^1O_2)(m/m_{ref})(F_{ref}/F)$, where m and F are the slope of a linear fit to data shown in Fig. S2 and $1-10^{-O.D.}$, respectively.

Spectroscopic Measurements. Spectrophotometric grade acetonitrile (Aldrich) were used to prepare solutions for spectroscopic measurements. Typically, 10 μM solutions were freshly prepared before measurements. A 1 cm × 1 cm fluorimeter cell (Hellma) was used for steady-state optical measurements, unless otherwise noted. UV−vis absorption spectra were collected on a Varian Cary 50 spectrophotometer at room temperature. Photoluminescence spectra were obtained by using a Quanta Master 40 scanning spectrofluorimeter at room temperature. Ar-saturated CH_3CN solutions containing Irbtp-RhB, Irbtp, and RhB were photoexcited by 330–560 nm, 433 nm, and 550 nm, respectively. The photoluminescence quantum yields (Φ_{PL}s) were relatively determined according to the following standard equation: $\Phi_{PL} = \Phi_{PL,ref}(I/I_{ref})(A_{ref}/A)(n/n_{ref})^2$, where A, I, and n are the absorbance at the excitation wavelength, the integrated photoluminescence intensity, and the refractive index of the solvent, respectively. Fluorescein as an aqueous 0.1 N NaOH solution was used as the external reference ($\Phi_{PL,ref} = 0.79$). The refractive index of the 0.1 N NaOH solution was assumed to be identical to the value for pure water. 50 μM solutions (Ar-saturated CH_3CN) were used for the transient photoluminescence measurements. Photoluminescence decay traces were acquired based on TCSPC techniques using a FluoTime 200 instrument (PicoQuant, Germany). A 377 nm diode laser (PicoQuant, Germany) was used as the excitation source. The photoluminescence signals were obtained using an automated motorized monochromator. Photoluminescence decay profiles were analyzed (OriginPro 8.0, OriginLab) using a single- or a biexponential decay models.

Cell Culture. HeLa cells purchased from the Korean Cell Line Bank were cultured in Dulbecco's

modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/mL penicillin at 37 °C in a humidified incubator under 5% CO_2.

MTS Assays. HeLa cells were seeded into 96-well plates two days prior to MTS assays. The cells were grown at the confluence of ca. 10^5 cells per well. Irbtp–RhB solutions were prepared by dissolving DMSO stock solutions of Irbtp–RhB in serum-free DMEM to concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 μM. 3 mL of CellTiter 96® Aqueous Solution Reagent (Promega) was diluted in 15 mL serum-free DMEM to prepare the solutions for MTS assays. The HeLa cells were washed with fresh DMEM, and incubated with 120 μL of the Irbtp–RhB solutions for 1 h. After removal of Irbtp–RhB, the treated cells were washed with fresh serum-free DMEM. 120 μL of the MTS solution was delivered to each well, and the cells were incubated in a humidified incubator for additional 1 h. Absorbance at 490 nm of formazan was recorded by using a Molecular Devices, VersaMax 96-well microplate reader. Background absorption due to serum-free DMEM was recorded for correction. To estimate photoinduced cytotoxicity, HeLa cells incubated in the presence and absence of Irbtp–RhB (0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 μM) were photoirradiated for 5 and 15 min before the treatment with the MTS solution. Cell survival (%) was quantitated by calculating the value, $(Abs_{\text{sample}} - Abs_{\text{blank}})/(Abs_{\text{control}} - Abs_{\text{blank}})$, where Abs_{sample}, Abs_{blank}, and Abs_{control} are absorbance for the treated cells, untreated cells, and wells containing serum-free DMEM, respectively.

Confocal Laser-Scanning Microscopy. One day prior to imaging, HeLa cells were plated onto glass-bottom culture dishes. After washing the cells with fresh DMEM, a 10 μM Irbtp-RhB in DMSO (biotech grade, Aldrich) was added to the culture media. The cells were incubated for the indicated periods at 37 °C. The incubated cells were washed twice with fresh DMEM (serum-free), and fluorescence micrographs were taken using a Carl Zeiss LSM 510 META confocal laser scanning microscope using a Newport MaiTai eHP DeepSee multiphoton excitation system. For co-staining experiments, the cells were additionally treated with organelle-specific stains, LysoTracker Red DND–99 (Molecular Probes), MitoTracker Deep Red FM (Molecular Probes), ER–Tracker Blue–White DPX (Molecular Probes), and Hoechst 33258 (Molecular Probes), according to the manufacturer's protocols. An excitation beam was focused onto the dish, and the signals were acquired through the multi-channels by varying the emission ranges. The cellular uptake mechanism of Irbtp–RhB was investigated at various conditions.[4,5] Fluorescence intensities of the HeLa cells incubated with 10 μM Irbtp-RhB at 37 °C and 4 °C for 10 min were compared whether the cellular transport involved energy-dependent uptake mechanisms. To validate this variable-temperature experiments, uptake experiments were performed for HeLa cells that were pretreated

with metabolic inhibitors, 50 mM 2-deoxy-D-glucose (Sigma) + 5 μM oligomycin (Sigma), and endocytic inhibitors, 50 μM chloroquine (Sigma) or 50 mM NH_4Cl (Sigma). Fluorescence micrographs were acquired under identical conditions to estimate the extents of cellular entry of Irbtp–RhB. To demonstrate the two-photon induced cytotoxicity, the HeLa cells incubated with 10 μM Irbtp-RhB (10 min) were photoirradiated under two-photon excitation at 800 nm for 9 min. Irbtp–RhB-treated cells that were photoirradiated under single-photon excitation at 561 nm for the identical period served as controls. Micrograph images were analyzed using the Carl Zeiss ZEN2011 software.

Photoluminescence Lifetime Imaging Microscopy. HeLa cells pretreated with 10 μM Irbtp–RhB for 10 min were washed with PBS three times. The cells were subsequently fixed using 4% formaldehyde, and mounted with Fluoromount–G (Southern Biotech). An inverted time-resolved microscope (PicoQuant, MicroTime 200) was employed for the photoluminescence lifetime imaging microscopic experiments. A 470 nm picosecond pulsed diode laser (<1 μW) operated at a 40 MHz repetition rate was used for photoexcitation. The instrumental response function of the system was ca. 240 ps at fwhm. A dichroic mirror (Z375RDC, AHF), a long-pass filter (HQ405lp, AHF), a 50 μm pinhole, a 550 nm cut-on filter (Thorlabs), and a single photon avalanche diode were used to collect emission from the HeLa cells. The time-resolved emission signals were obtained using a TCSPC technique. Typically, an 80 μm × 80 μm sample area consisting of 250 × 250 pixels was scanned with an acquisition rate of 5 ms/pixel. Photoluminescence lifetime images and their exponential fits were analyzed using the SymPhoTime software provided by the manufacturer.

Cyclic voltammetry. Cyclic voltammograms were obtained at 298 K using a CHI630B instrument (CH Instruments) for deaerated CH_3CN solutions (~ 3 mL) containing 1.0 mM sample and 0.10 M Bu_4NPF_6 supporting electrolyte. A conventional three-electrode cell was used with a platinum disc working electrode and a platinum wire counter electrode. The potentials were recorded with respect to the $Ag/AgNO_3$ pseudo reference electrode, and adjusted to values against SCE by adding 0.29 V. Scan rate was 0.10 V/s.

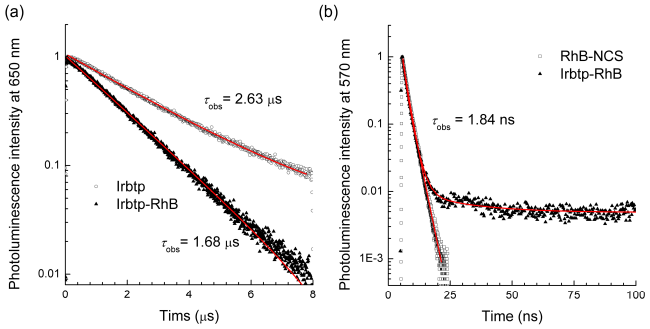

Fig. S1 Comparisons of the fluorescence decay traces after nanosecond (a) and picosecond (b) pulsed photoexcitation at 377 nm. (a) Photoluminescence decay traces of 50 μM Irbtp (empty circles) and 50 μM Irbtp–RhB (filled triangles) observed at 650 nm. Red lines are nonlinear least-squares fits to a monoexponential decay model. The k_{TTET} value was calculated using the relationship, $k_{TTET} = 1/\tau - 1/\tau_0$, where τ and τ_0 are the photoluminescence lifetimes of Irbtp–RhB and Irbtp, respectively. (b) Fluorescence decay traces of 50 μM RhB–NCS (empty squares) and 50 μM Irbtp–RhB (filled triangles) observed at 570 nm. Red curves are nonlinear least-squares fits to a biexponential decay model. τ_{obs} in the Fig. S1b is the lifetime of the fast component. Argon-saturated acetonitrile solutions were used for the measurements.

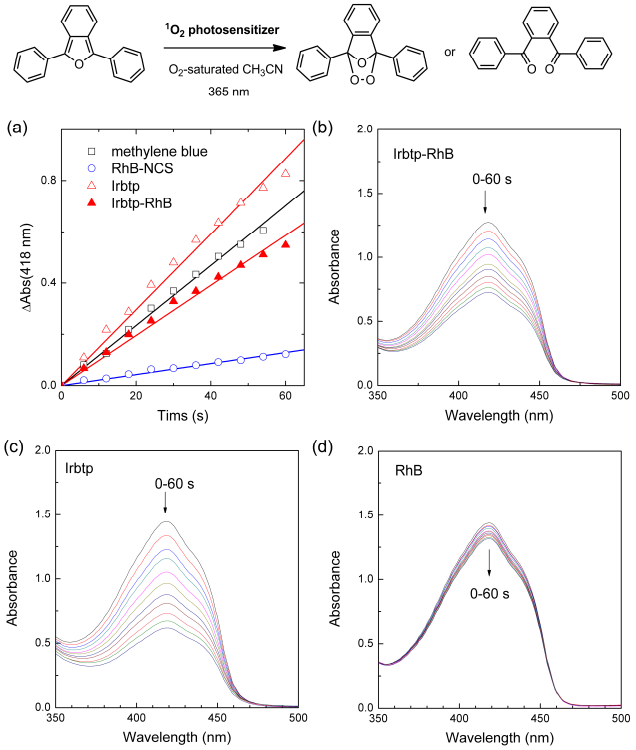

Fig. S2 Determination of the singlet oxygen generation quantum yields ($\Phi(^1O_2)$). Air-saturated CH_3CN solutions (2 mL) containing a singlet oxygen photosensitizer (O.D. = 0.17 at 365 nm) and a 100 μM $^1O_2$ substrate, DPBF, were photoirradiated under a UV light of 365 nm. Decreases in the DPBF absorption (λ_{abs} = 418 nm) were monitored during the photoirradiation. (a) Temporal changes of the DPBF absorption (ΔAbs(418 nm)) for Irbtp (empty red triangles), methylene blue (empty black squares), Irbtp–RhB (filled red triangles), and RhB–NCS (empty blue circles). UV–vis spectral changes of DPBF upon photoirradiation of (b) Irbtp–RhB, (c) Irbtp, and (d) RhB–NCS.

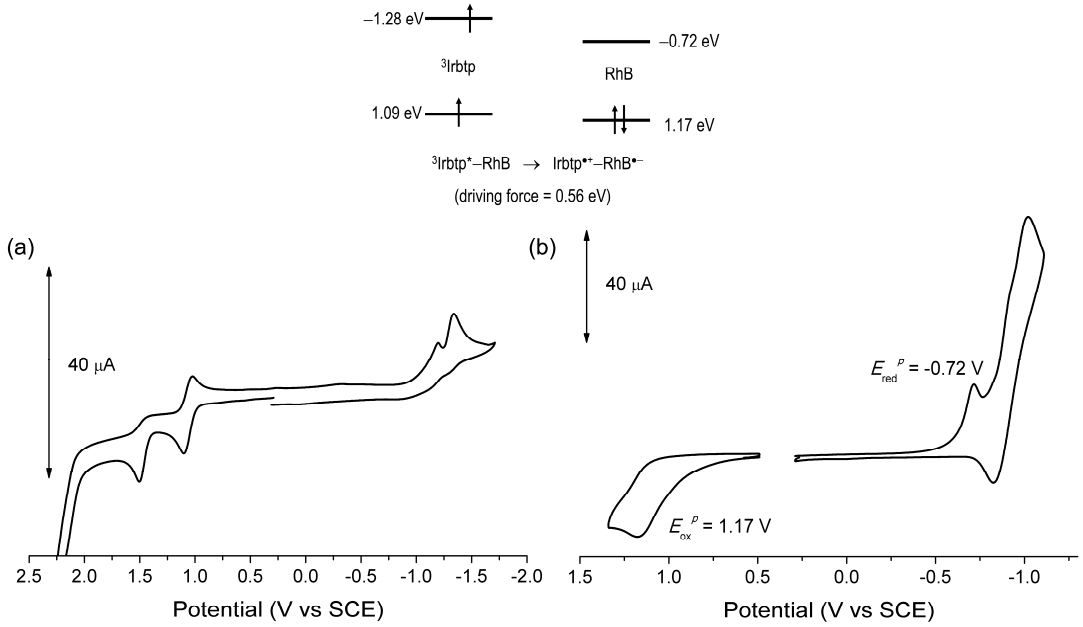

Fig. S3 Cyclic voltammogram (V vs SCE) of (a) 1.0 mM Irbtp and (b) 1.0 mM RhB–NCS in Ar-saturated CH_3CN solutions containing 0.10 M Bu_4NPF_6 supporting electrolyte. A Pt wire and a Pt microdisc were used as a counter electrode and a working electrode, respectively. $Ag/AgNO_3$ was employed for a pseudo reference electrode. The electrochemical potentials suggest the occurrence of photoinduced electron transfer between the Irbtp and RhB entities (see the top diagram).

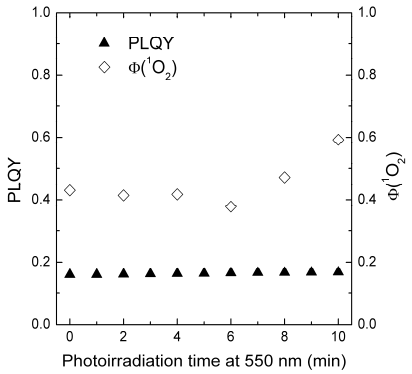

Fig. S4 Photostability of the photoluminescence quantum yields (PLQY, filled triangles) and $\Phi(^1O_2)$ (empty diamonds) values of Irbtp–RhB upon the continuous exposure to 550 nm photoirradiation.

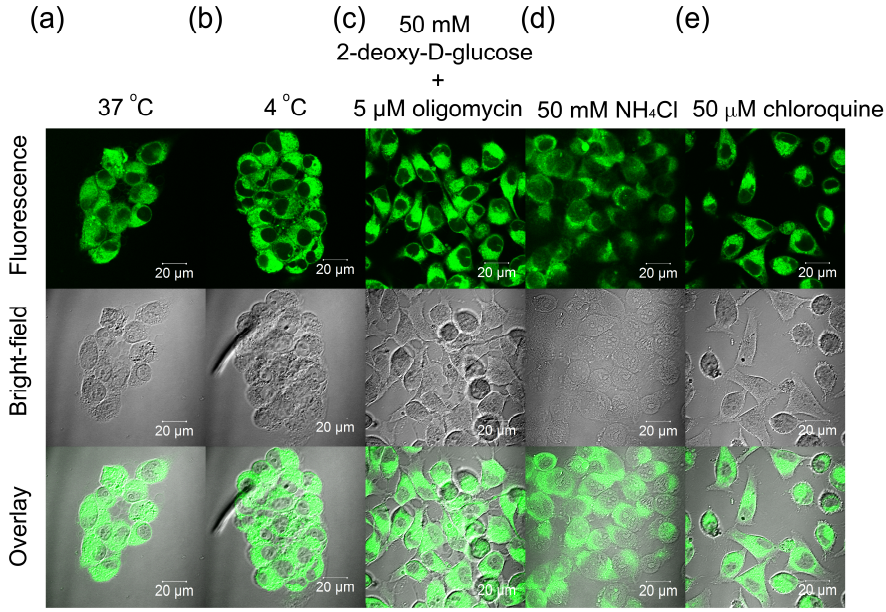

Fig. S5 Intracellular uptake of 10 μM Irbtp–RhB in HeLa cells at various conditions: (a) Incubation at 37 °C. (b) Incubated at 4 °C. (c) HeLa cells were pretreated with 50 mM 2-deoxy-D-glucose + 5 μM oligomycin for 30 min at 37 °C. (d) HeLa cells were pretreated with 50 mM NH_4Cl for 30 min at 37 °C. (e) HeLa cells were pretreated with 50 μM chloroquine for 30 min at 37 °C. Top panels, fluorescence images; middle panels, bright-field images; bottom panels, overlay images.

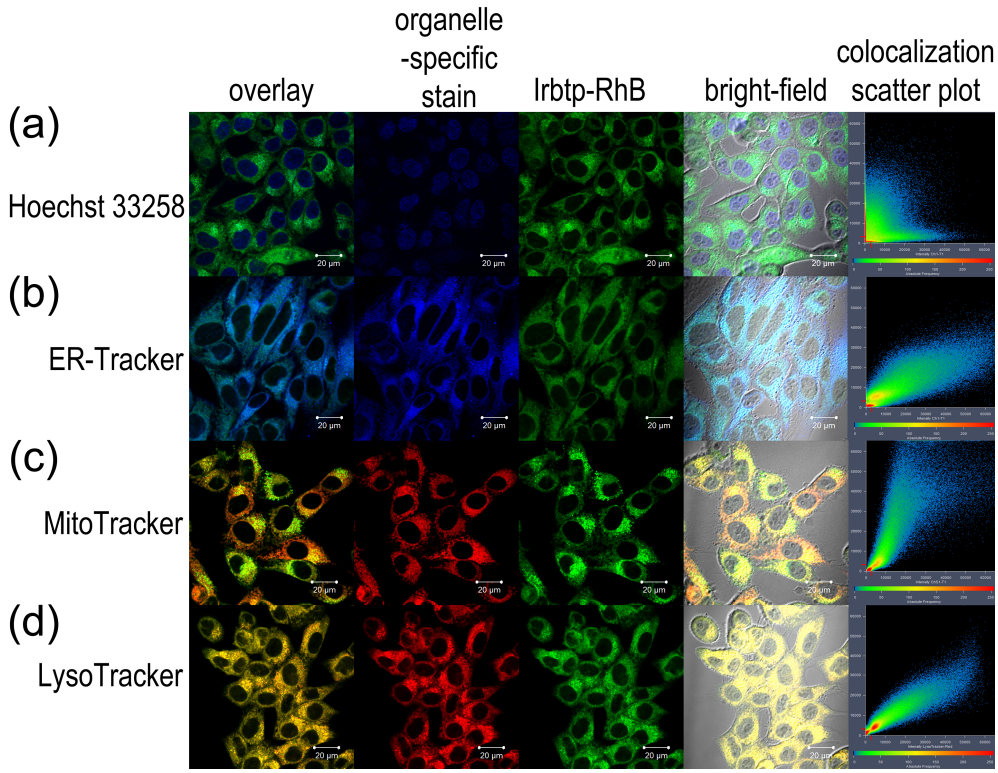

Fig. S6 Organelle-specific subcellular localization of Irbtp–RhB in HeLa cells. (a) 10 μM Irbtp–RhB (10 min incubation at 37 °C; λ_{ex} = 488 nm; λ_{em} = 551–691 nm) and 16 μM Hoechst 33258 (10 min incubation at 37 °C; λ_{ex} = 405 nm; λ_{em} = 415–481 nm). (b) 10 μM Irbtp–RhB (10 min incubation at 37 °C; λ_{ex} = 488 nm; λ_{em} = 551–691nm) and 1 μM ER–Tracker Blue–White DPX (20 min incubation at 37 °C; λ_{ex} = 374 nm; λ_{em} = 430–540 nm). (c) 10 μM Irbtp–RhB (10 min incubation at 37 °C; λ_{ex} = 488 nm; λ_{em} = 551–621 nm) and 500 nM MitoTracker Deep Red FM (45 min incubation at 37 °C; λ_{ex} = 633 nm; λ_{em} = 641–758 nm). (d) 10 μM Irbtp–RhB (10 min incubation at 37 °C; λ_{ex} = 488 nm; λ_{em} = 551–691 nm) and 500 nM LysoTracker Red (2 h incubation at 37 °C; λ_{ex} = 577 nm; λ_{em} = 590–747 nm). Overlap coefficient = 0.52 (Hoechst 33258), 1.20 (ER–Tracker Blue–White DPX), 1.24 (MitoTracker Deep Red FM), and 0.99 (LysoTracker Red).

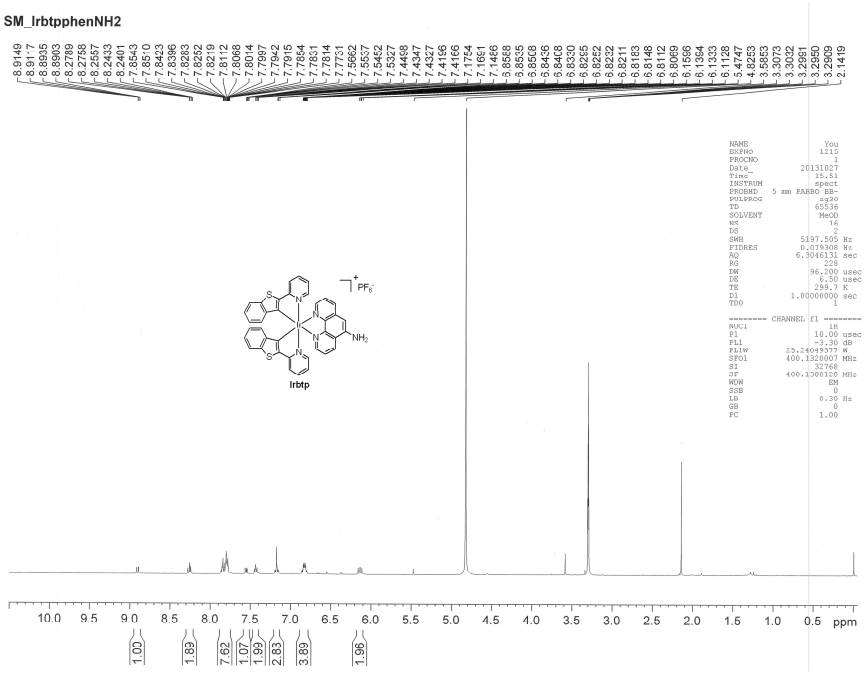

Fig. S7 ^{1}H NMR spectrum (400 MHz, MeOD) of $[(btp)_2Ir(1,10\text{-phenanthroline-5-amine})]PF_6$.

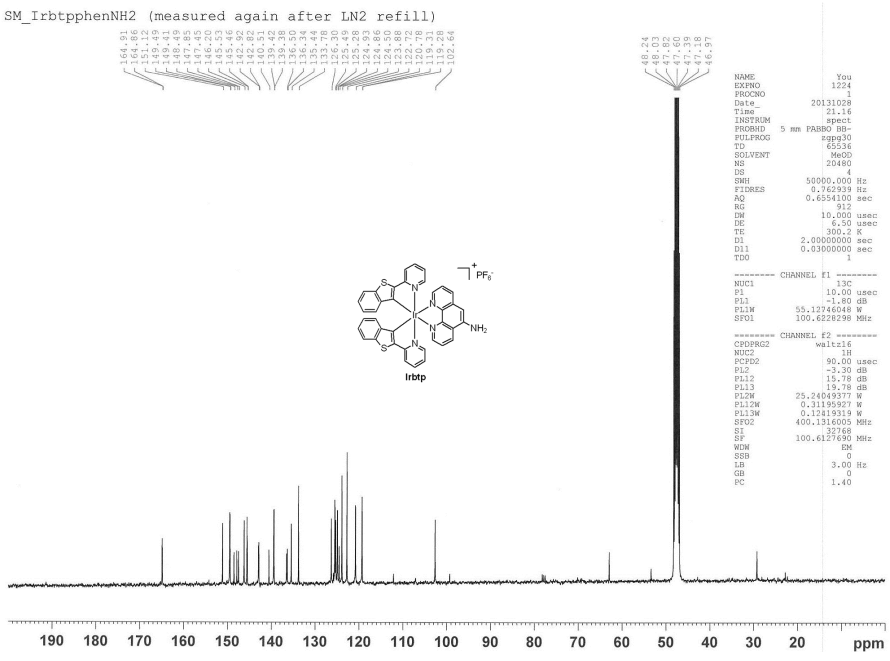

Fig. S8 ^{13}C NMR spectrum (100 MHz, MeOD) of $[(btp)_2Ir(1,10\text{-phenanthroline-5-amine})]PF_6$.

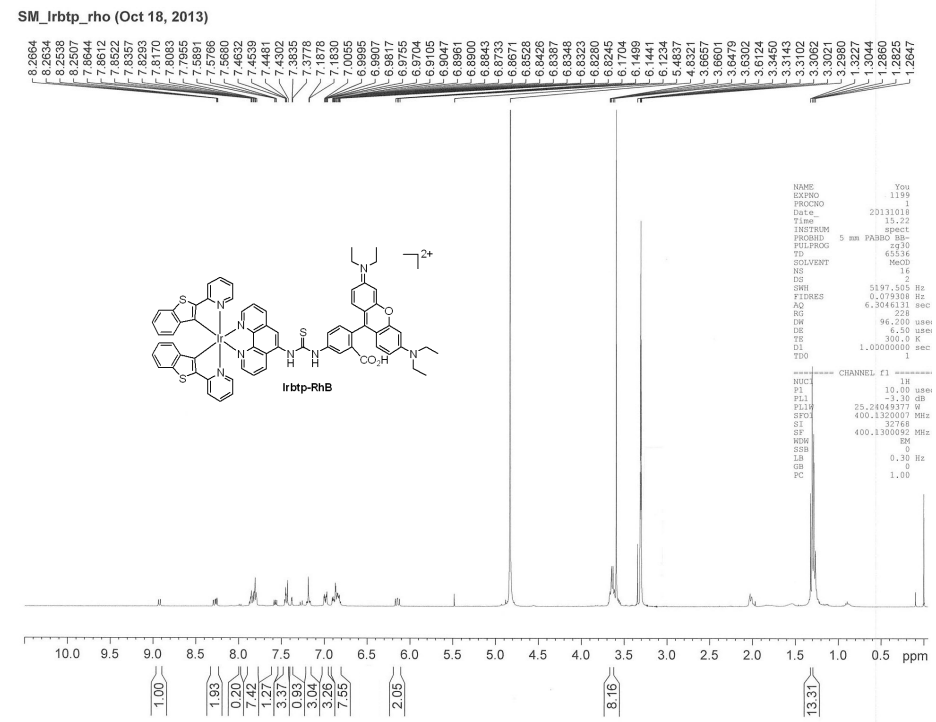

Fig. S9 ^{1}H NMR spectrum (400 MHz, MeOD) of Irbtp–RhB.

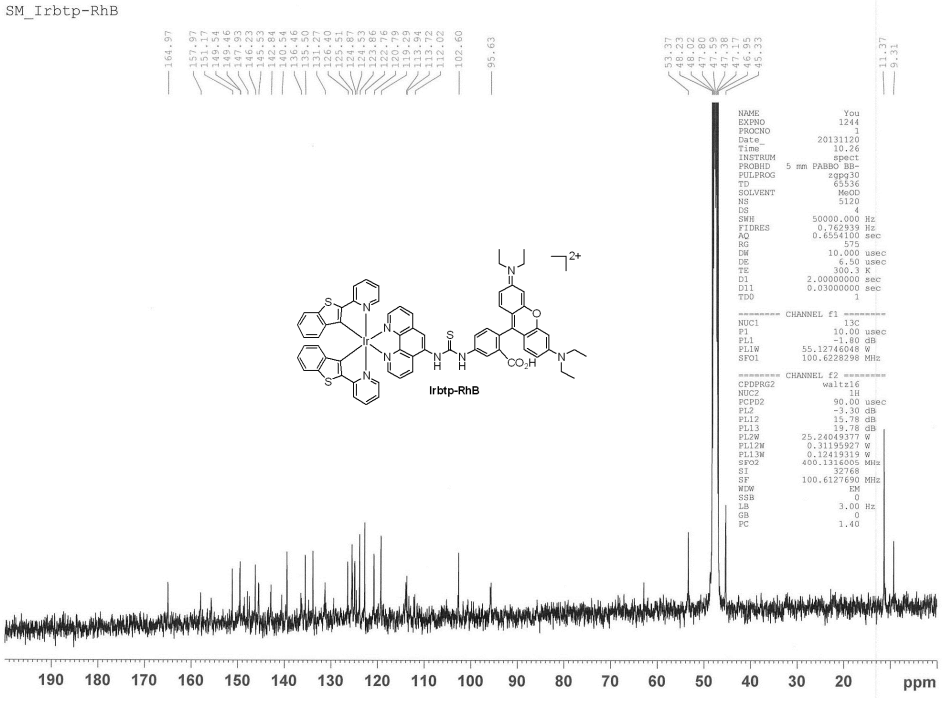

Fig. S10 ^{13}C NMR spectrum (100 MHz, MeOD) of Irbtp–RhB.

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