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Supporting Information

The stepwise photodamage of organelles by two-photon luminescent ruthenium(II) photosensitizers

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Table of Contents

Experimental

Materials

The reagents were purchased from commercial sources and were used without purification unless otherwise specified. Ruthenium chloride hydrate, 5,5',6,6'-tetrachloro-1,1'-3,3'-tretraethyl-benzimidazolylcarbocyanine iodide (JC-1), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), acridine orange (AO), cisplatin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich. 2,2'-bipyridine (bpy), 1,10-phenanthroline (phen), 4,7-dimethyl-1,10-phenanthroline (dmp), 4,7-diphenyl-1,10-phenanthroline (dip), 4,4'-dimethyl-2,2'-bipyridine and morpholine were purchased from Energy Chemical. The commercially available endoplasmic reticulum imaging agent ER-Tracker Green (ERTG), mitochondrial imaging agent MitoTracker Green FM (MTG), MitoTracker Deep Red (MTDR) and lysosomal imaging agent LysoTracker Green DND-26 (LTG) were purchased from Life Technology. The ruthenium precursors (RuX_2Cl_2, X = bpy, phen, dmp, dip) and 4-(bromomethyl)-4'-methyl-2,2'-bipyridine were prepared according to the reported methods.[1,2] Before the experiments, DMSO was used to dissolve the complexes. During the experiments with cells, the concentration of DMSO was less than 1% (v/v).

General instruments

Elemental analyzer (Elementar Vario EL) was used for measuring microanalysis (C, H and N). Liquid chromatography mass spectrometer (Shimadzu, LCMS-2010A) was used for measuring electrospray ionization mass spectrum (ESI-MS). Nuclear magnetic resonance spectrometer (Bruker, AVANCE III, 400 MHz) was used for measuring NMR spectra and tetramethylsilane (TMS) was used as standard. Perkin-Elmer Lambda 850 spectrophotometer was used for measuring the UV-vis spectra. Perkin-Elmer L55 spectrofluorophotometer was used for measuring the emission spectra and $[Ru(bpy)_3]Cl_2$ (φ = 2.8% in air-saturated H_2O) was used as the reference[3] for calculating the quantum yield of luminescence. FLS 920 combined fluorescence-lifetime and steady-state spectrometer (Edinburgh

Instruments ltd.) was used for time-resolved emission measurement and the $^1O_2$ production measurement by the direct method, $[Ru(bpy)_3]Cl_2$ was used as the reference (18% in air-saturated H_2O).[4] Bruker Model A300 spectrometer was used for measuring the electron spin resonance (ESR) spectra and 2,2',6,6'-tetramethylpiperidine (TEMP) was used as the spin trap for $^1O_2$.[5] The two-photon absorption cross sections were obtained by a femtosecond fluorescence spectrum measurement system (tuning range 740-950 nm, SR-500I-D1, Coherent Inc., USA) and calculated according to the reported method.[6,7] The confocal microscope (Zeiss LSM 710 NLO) and the two-photon laser microscopy system consisted of a confocal microscope (Zeiss LSM 880 NLO) and a two-photon laser (Coherent MRU X1) were used for imaging. All measurements were carried out at room temperature. All data was processed by the Origin 8 software package.

Synthesis of 4-((4'-methyl-[2,2'-bipyridin]-4-yl)methyl)morpholine (L)

4-(bromomethyl)-4'-methyl-2,2'-bipyridine (2.62 g, 10 mmol, 1 eq) was mixed with potassium carbonate (2.07 g, 15 mmol, 1.5 eq) and morpholine (1 mL) in CH_3CN (20 mL) and then refluxed under argon for 6 h. After the reaction was cooled to rt and filtered, the filtrate was evaporated under reduced pressure. Finally, the crude product was purified by column chromatography (dichloromethane/ethyl acetate) to obtain **L** (2.09 g, 7.8 mmol, 78%). Anal. Calcd. for $C_{16}H_{19}N_3O$ (%): C, 71.35; H, 7.11; N, 15.60. Found: C, 70.99; H, 7.15; N, 15.52. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (d, *J* = 4.9 Hz, 1H), 8.56 (d, *J* = 4.9 Hz, 1H), 8.35 (s, 1H), 8.25 (s, 1H), 7.39 (d, *J* = 4.5 Hz, 1H), 7.16 (d, *J* = 4.3 Hz, 1H), 3.76 (t, *J* = 4.0 Hz, 4H), 3.61 (s, 2H), 2.52 (s, 4H), 2.46 (s, 3H). ESI-MS: m/z = 270.0 $[M+H]^+$.

Synthesis of the ruthenium(II) complexes

RuX_2**L**Cl_2 (X = bpy, phen, dmp, dip): A mixture of *cis*-$RuX_2Cl_2.2H_2O$[1] (0.200 mmol, 1 eq) and **L** (54 mg, 200 μmol, 1 eq) in the mixture solvent of ethanol and water (10 mL, 9:1, v/v) was refluxed under argon for 8 h to give a clear red solution. The solvent was removed by rotary evaporation. Column chromatography (alumina)

was used to purify the crude products. Acetonitrile and ethanol were used as the eluents. After the solvents were evaporated, brownish red solids were obtained.

$Ru(bpy)_2$**L**Cl_2 (**Ru1**, 128 mg, 170 μmol, 85%): Anal. Calcd. for $C_{36}H_{35}Cl_2N_7ORu$: C, 57.37; H, 4.68; N, 13.01%. Found: C, 57.08; H, 4.70; N, 12.94%. ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.89 (d, *J* = 7.9 Hz, 4H), 8.80 (d, *J* = 13.8 Hz, 2H), 8.17 (t, *J* = 7.5 Hz, 4H), 7.78-7.69 (m, 4H), 7.64 (d, *J* = 5.7 Hz, 1H), 7.58-7.47 (m, 6H), 7.38 (d, *J* = 5.4 Hz, 1H), 3.69 (s, 2H), 3.60 (s, 4H), 2.53 (s, 3H), 2.43 (s, 4H). ESI-MS: m/z = 340.95 $[M\text{-}2Cl^-]^{2+}$.

$Ru(phen)_2$**L**Cl_2 (**Ru2**, 130 mg, 162 μmol, 81%): Anal. Calcd. for $C_{40}H_{35}Cl_2N_7ORu$: C, 59.92; H, 4.40; N, 12.23%. Found: C, 59.62; H, 4.42; N, 12.17%. ^{1}H NMR (400 MHz, D_2O) δ 8.54 (d, *J* = 8.3 Hz, 2H), 8.44 (s, 1H), 8.41 (d, *J* = 5.3 Hz, 2H), 8.33 (s, 1H), 8.22-8.17 (m, 2H), 8.15-8.08 (m, 4H), 7.89-7.83 (m, 2H), 7.71-7.62 (m, 3H), 7.48-7.39 (m, 3H), 7.12 (d, *J* = 5.8 Hz, 1H), 7.02 (d, *J* = 5.7 Hz, 1H), 3.65 (s, 6H), 2.49 (s, 4H), 2.42 (s, 3H). ESI-MS: m/z = 365.40 $[M\text{-}2Cl^-]^{2+}$.

$Ru(dmp)_2$**L**Cl_2 (**Ru3**, 129 mg, 150 μmol, 75%): Anal. Calcd. for $C_{44}H_{43}Cl_2N_7ORu$: C, 61.61; H, 5.05; N, 11.43%. Found: C, 61.30; H, 5.08; N, 11.37%. ^{1}H NMR (400 MHz, D_2O) δ 8.38 (s, 1H), 8.31 (s, 1H), 8.24-8.17 (m, 4H), 7.99 (t, *J* = 5.1 Hz, 2H), 7.67-7.63 (m, 2H), 7.60 (d, *J* = 5.9 Hz, 1H), 7.48 (t, *J* = 4.8 Hz, 2H), 7.43 (d, *J* = 5.8 Hz, 1H), 7.23-7.21 (m, 2H), 7.08 (d, *J* = 5.8 Hz, 1H), 6.98 (d, *J* = 5.7 Hz, 1H), 3.62 (s, 6H), 2.78 (d, *J* = 6.8 Hz, 6H), 2.67 (s, 6H), 2.47 (s, 4H), 2.40 (s, 3H). ESI-MS: m/z = 393.65 $[M\text{-}2Cl^-]^{2+}$.

$Ru(dip)_2$**L**Cl_2 (**Ru4**, 170 mg, 154 μmol, 77%): Anal. Calcd. for $C_{64}H_{51}Cl_2N_7ORu$: C, 69.49; H, 4.65; N, 8.86%. Found: C, 69.14; H, 4.67; N, 8.82%. ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.86 (d, *J* = 13.2 Hz, 2H), 8.37 (d, *J* = 5.5 Hz, 1H), 8.32-8.21 (m, 7H), 7.96 (dd, *J* = 5.3, 2.3 Hz, 2H), 7.77 (d, *J* = 5.4 Hz, 3H), 7.73-7.60 (m, 21H), 7.49 (d, *J* = 5.7 Hz, 1H), 7.37 (d, *J* = 5.7 Hz, 1H), 3.73 (s, 2H), 3.61 (s, 4H), 2.57 (s, 3H), 2.45 (d, *J* = 3.9 Hz, 4H). ESI-MS: m/z = 517.50 $[M\text{-}2Cl^-]^{2+}$.

Lipophilicity

A shake-flask ultraviolet absorptive spectrophotometry method[8] was used to

determine the *n*-octanol/water (log $P_{o/w}$) partition coefficients of **Ru1-4**. First, the mixed solution of 50 mL of *n*-octanol and 50 mL of water was left shaking at 37 °C for 48 h. The **Ru1-4** standard solutions were prepared using the water phase or organic phase. Second, the **Ru1-4** detection solutions (C = 10 μM) were prepared with mixed solvent containing 25 mL of *n*-octanol and 25 mL of water and shaking for 48 h. Third, the concentrations of **Ru1-4** in the water phase (C_w) and organic phase (C_o) of the detecting solution were determined separately using ultraviolet spectrophotometry. The *n*-octanol/water partition coefficient, namely, log P, is calculated by the following equation (1):

$$\log P = \lg (C_o/C_w) \quad (1)$$

Cell culture conditions

In a humidified incubator with an atmosphere of 95% air and 5% CO_2 at a constant temperature of 37 °C, Dulbecco's modified Eagle medium (DMEM) with fetal bovine serum (10%, v/v) were used to culture A549 cells, which were obtained from the Experimental Animal Center, Sun Yat-sen University (Guangzhou, China).

Cellular uptake

The cells were trypsinized, counted, and adjusted to 1×10^4 cells/mL and 1 mL was added to 6-well plates. After 24 h, the cells were treated with 10 μM of the Ru(II) complexes in the dark for different time (2 h, 6 h, 12 h, 18 h, 24 h). For flow cytometry, after being washed with PBS three times, the cells were trypsinized and centrifugated in PBS buffer. Cells were harvested, and single cell suspensions in 0.5 mL PBS buffer were prepared and subjected to flow cytometric analysis. A flow cytometer (Coulter Co. USA) was used to measure the luminescence intensity of the complexes.

(Photo)cytotoxicity test

The IC_{50} values were determined using A549 cells. In 96-well plates, the exponentially grown A549 cells (7×10^3) were seeded per well. After 24 h, increasing

concentrations of the tested complexes were treated with the cells in the dark for 24 h. The media was removed and replaced with fresh DMEM containing 10% FBS. For phototoxicity studies, cells were irradiated for 600 s (flat light with LED system, 450 nm, 7.32 J/cm^2). Both cells from the dark and light groups were incubated for an additional 48 h. 10 μL/well of MTT (5 mg/mL) was added to stain the viable cells for 4 h. Removed the media, 200 μL/well of DMSO was added. At 595 nm, the optical density of each well was measured by the Tecan Infinite M200 monochromator-based multifunction microplate reader. The cell survival rate was considered 100% cell survival for the control wells without complexes solutions.

Confocal imaging

A549 cells were plated onto 35 mm glass bottom dishes (Corning) and allowed to adhere for 24 h.

For lysosomal co-localization, after treatment with 10 μM of **Ru4** in the dark for 24 h, LTG was added and the cells further incubated for 0.5 h. Cell imaging was performed after the media was replaced.

For mitochondrial co-localization, after treatment with 10 μM of **Ru4** in the dark for 24 h, MTG was added and the cells further incubated for 0.5 h. Cell imaging was performed after the media was replaced.

For endoplasmic reticular co-localization, after treatment with 10 μM of **Ru4** in the dark for 24 h, ERTG was added and the cells further incubated for 0.5 h. Cell imaging was performed after the media was replaced.

For analysis of the uptake mechanism, A549 cells were treated with 10 μM complex **Ru4** at 37 °C for 4 h in the dark for normalized incubation. For low temperature inhibition, the cells were incubated with the complex **Ru4** (10 μM) for 4 h at 4 °C in the dark. Cell imaging was performed after the media was replaced.

For singlet oxygen detection, after treatment with 10 μM of **Ru4** in the dark for 24 h, the cells were washed with PBS three times and incubated with DCFH-DA for 0.5 h. Cell imaging was performed after the media was replaced.

For the analysis of lysosomal integrity, after the treatment with 10 μM of **Ru4** in

the dark for 24 h, acridine orange (AO) was added and further incubated for 15 min. Cell imaging was performed after the media was replaced.

For analysis of the mitochondrial membrane potential, after treatment with 10 μM of **Ru4** in the dark for 24 h, the irradiated cells (450 nm, 7.32 J/cm^2) and the unirradiated cells were stained with JC-1 for 0.5 h. Cell imaging was performed after the media was replaced.

For tracking the dynamic process during PDT, the cells were washed with PBS, incubated with 10 μM of **Ru4** in the dark for 24 h, then incubated with MTDR (50 nM) for 0.5 h. Cell imaging was performed during light irradiation.

Zeiss LSM 710 NLO confocal microscope and Zeiss LSM 880 NLO confocal microscope (63×/NA 1.4 oil immersion objective) were used for imaging the cells. At 458 nm (for **Ru4**), 488 nm (for LTG, MTG, ERTG, DCF, AO and J-monomer), 543 nm (for J-aggregates) or 633 nm (for MTDR), the luminescence (fluorescence) was excited. For two-photon imaging/irradiation, the laser of 810 nm was used. At 630 ± 20 nm (for **Ru4**), 610 ± 20 nm (for AO-red and J-aggregates), 510 ± 10 nm (for LTG, MTG, ERTG, DCF, AO-green and J-monomer) or 650 ± 10 nm (for MTDR), the emission signal was collected.

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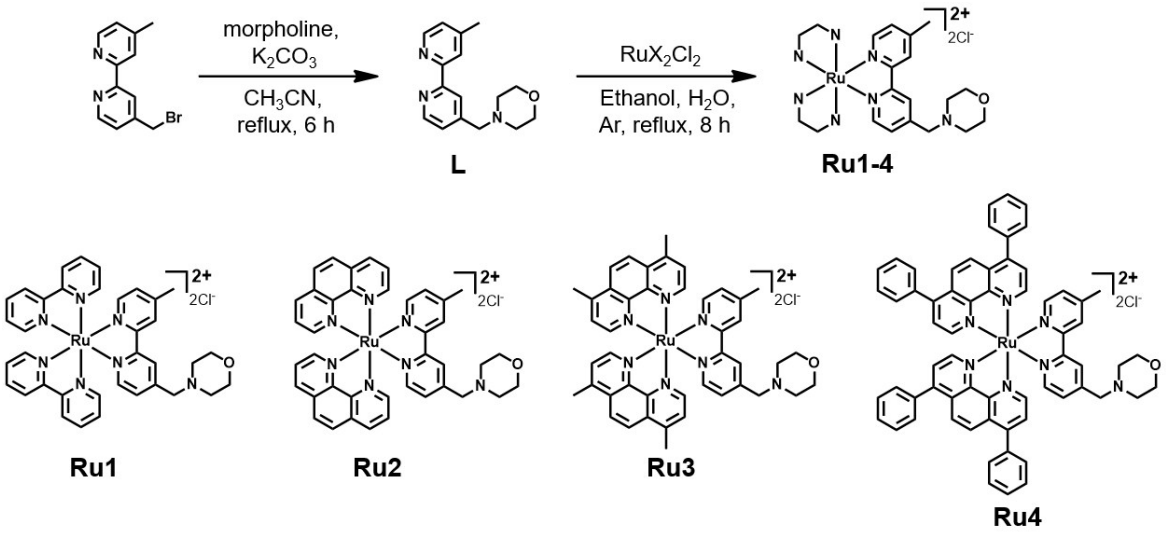

Scheme S1. Synthetic route to **Ru1-4**.

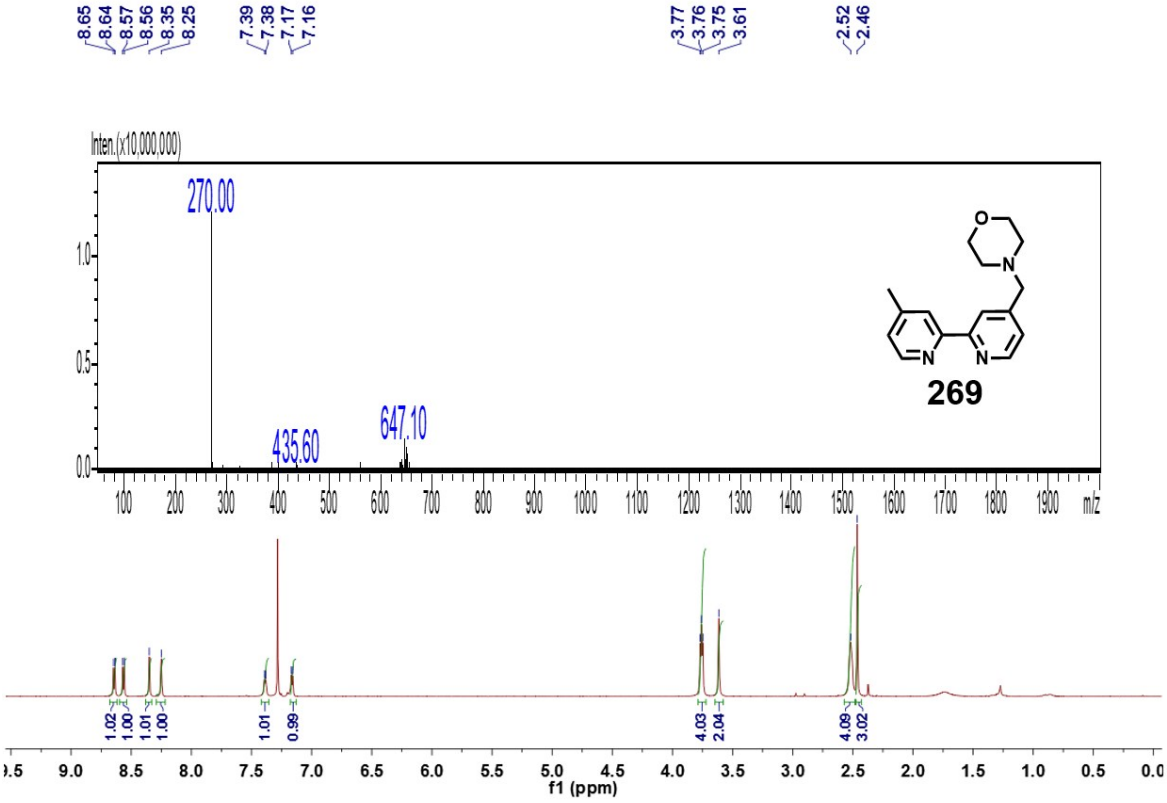

Fig. S1 ESI-MS spectrum and ^{1}H NMR spectrum of ligand **L**.

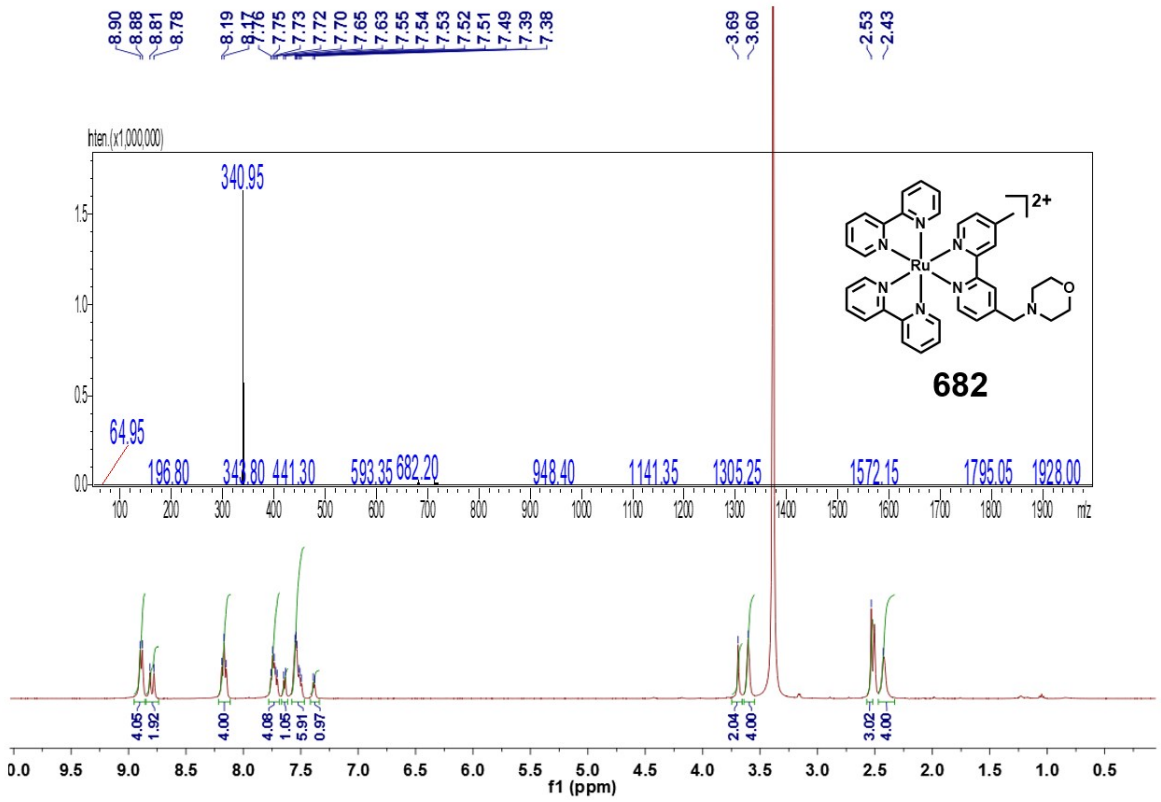

Fig. S2 ESI-MS spectrum and ^{1}H NMR spectrum of **Ru1**.

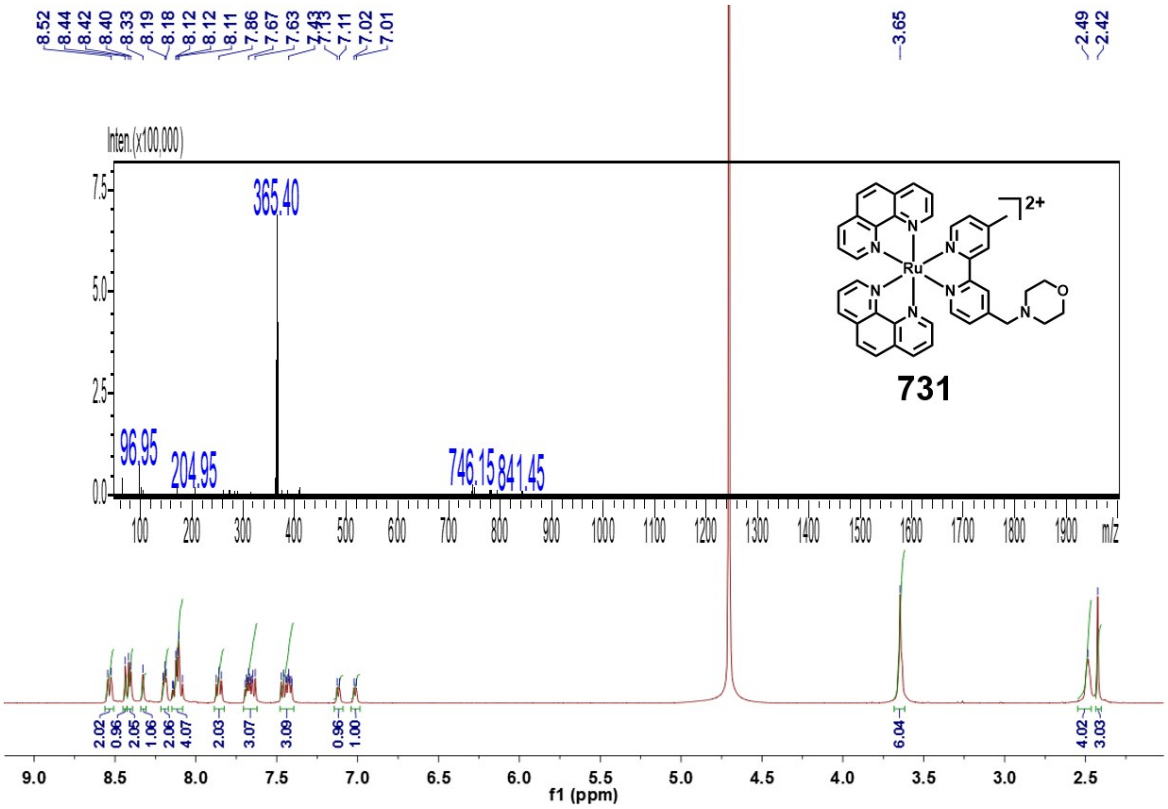

Fig. S3 ESI-MS spectrum and ^{1}H NMR spectrum of **Ru2**.

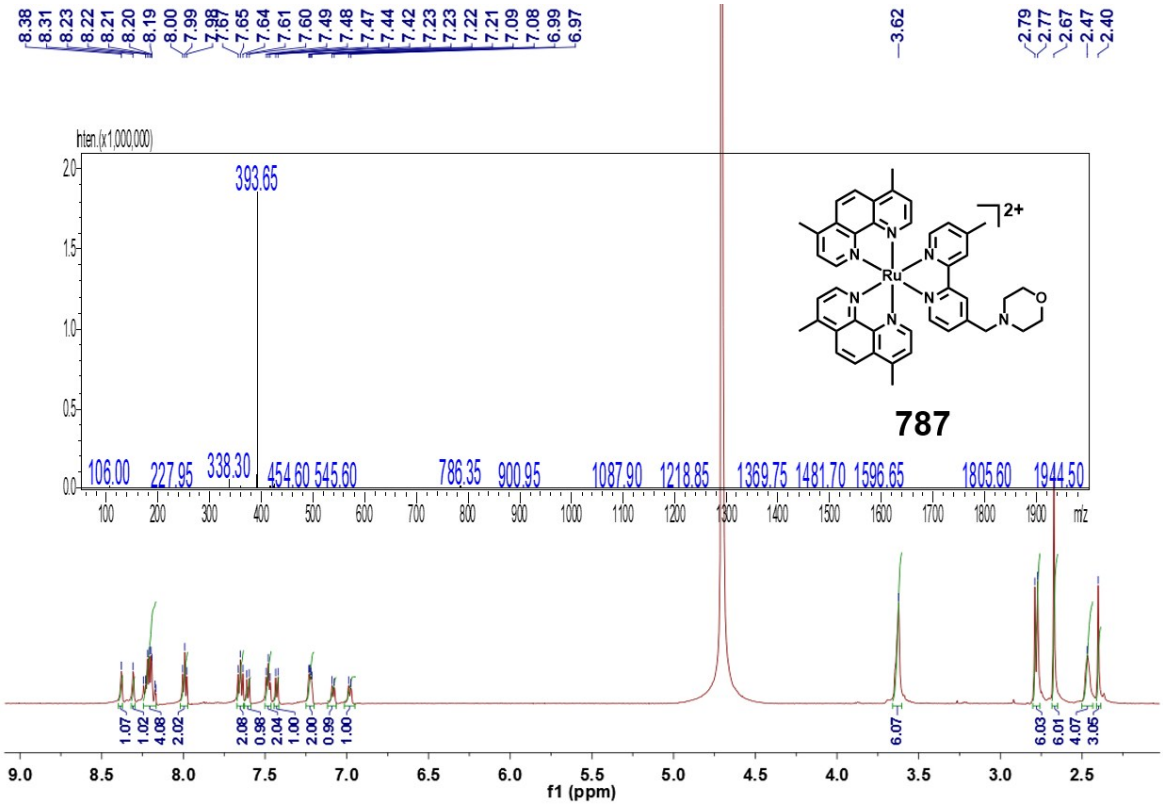

Fig. S4 ESI-MS spectrum and ^{1}H NMR spectrum of **Ru3**.

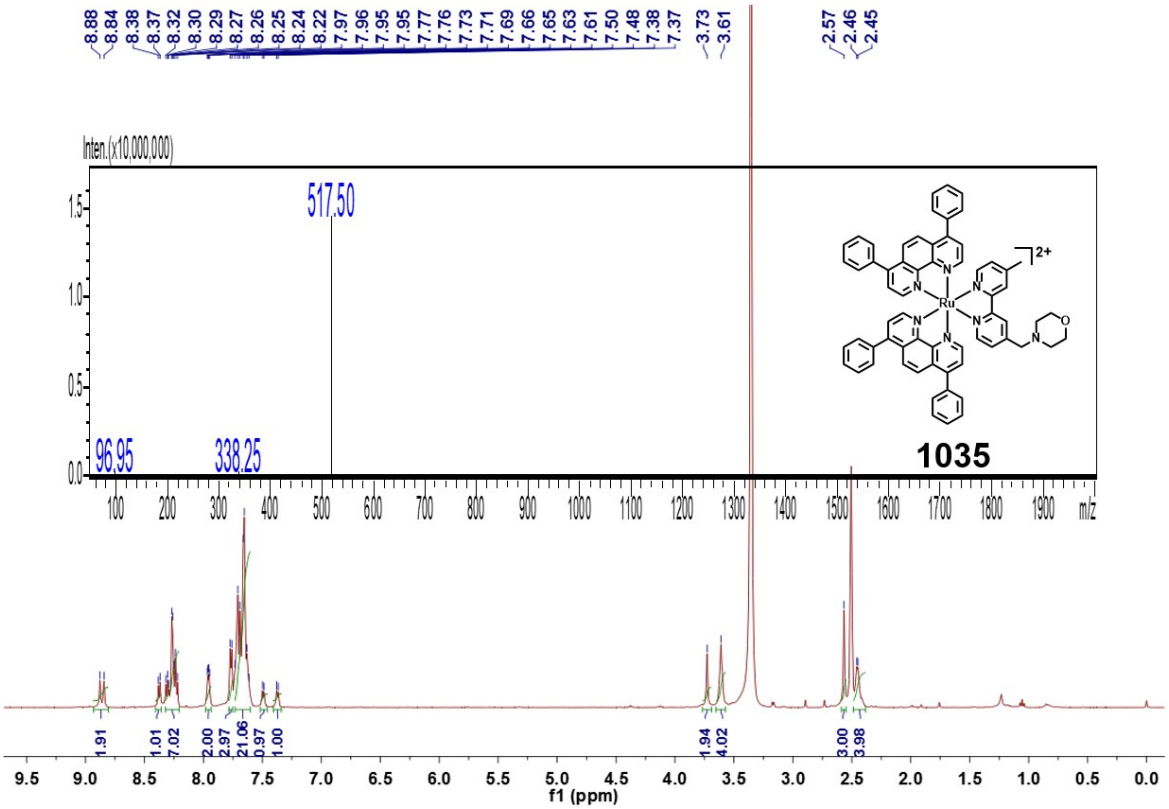

Fig. S5 ESI-MS spectrum and ^{1}H NMR spectrum of **Ru4**.

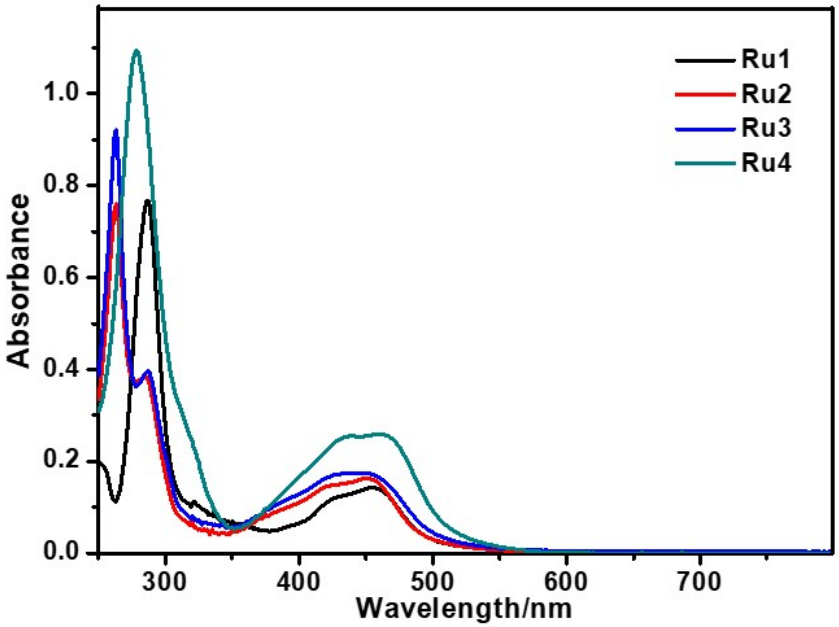

Fig. S6 Absorption spectra of **Ru1-4** (10 μM) in a Britton-Robison buffer solution at pH 7.0.

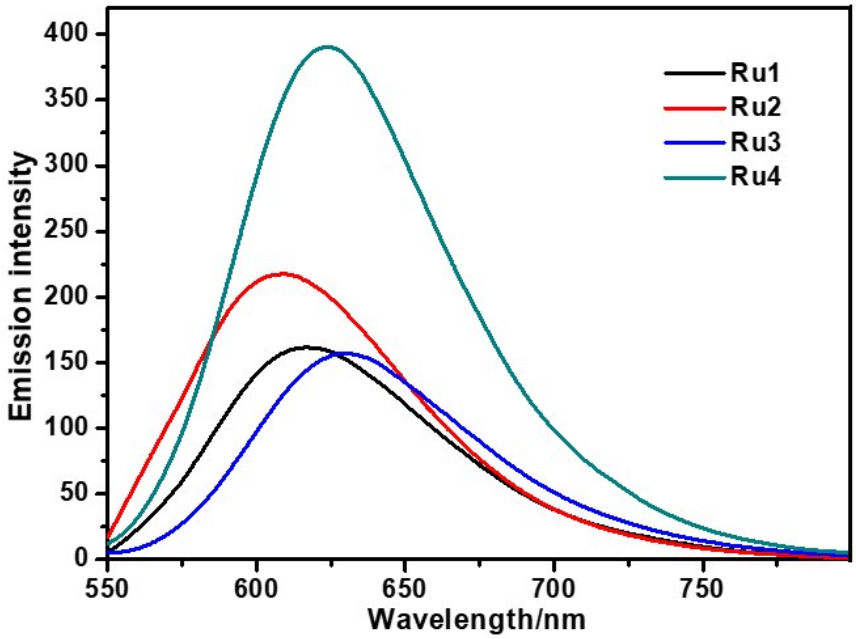

Fig. S7 Emission spectra of **Ru1-4** (10 μM) in a Britton-Robison buffer solution at pH 7.0.

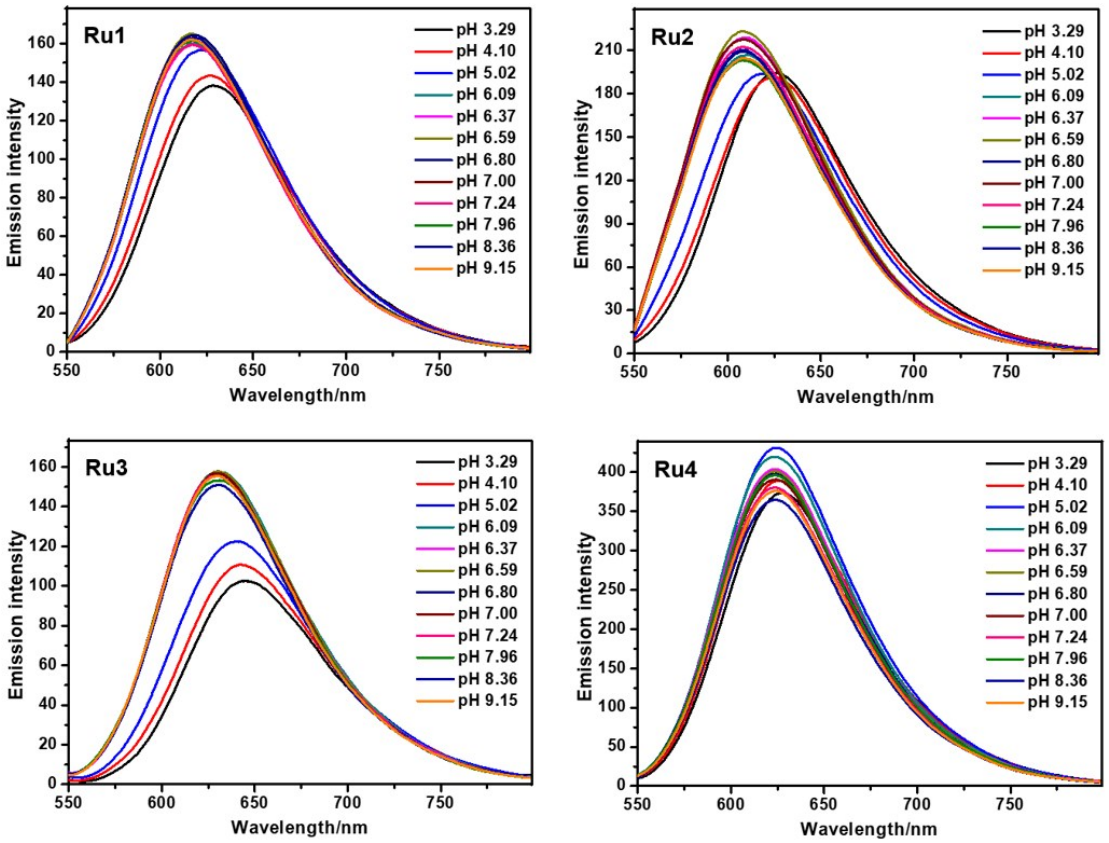

Fig. S8 The emission spectra of **Ru1-4** (10 μM) in different pH of Britton-Robison buffer solutions.

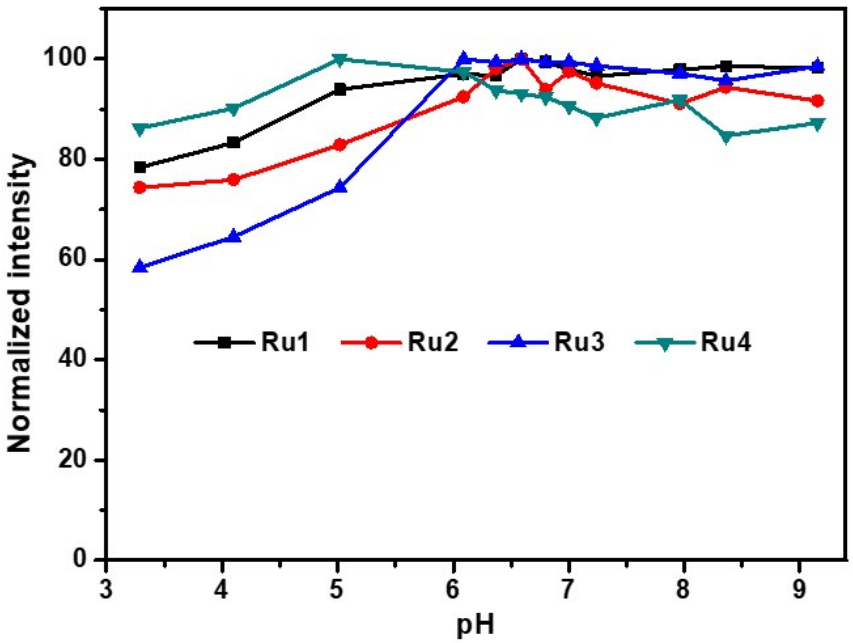

Fig. S9 Plots of the emission intensities of the complexes (**Ru1** at 617 nm, **Ru2** at 608 nm, **Ru3** at 630 nm, and **Ru4** at 624 nm) *vs* pH.

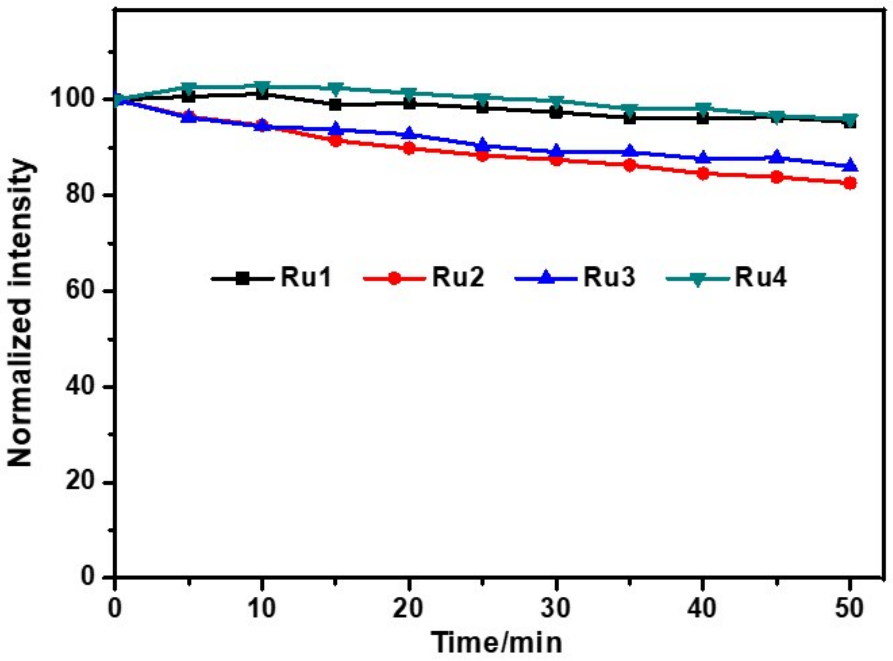

Fig. S10 Stability of the luminescence intensity of **Ru1-4** (10 μM) in a Britton-Robison buffer solution at pH 7.0 under 450 nm irradiation by a surface light source of 24.4 mW/cm^2.

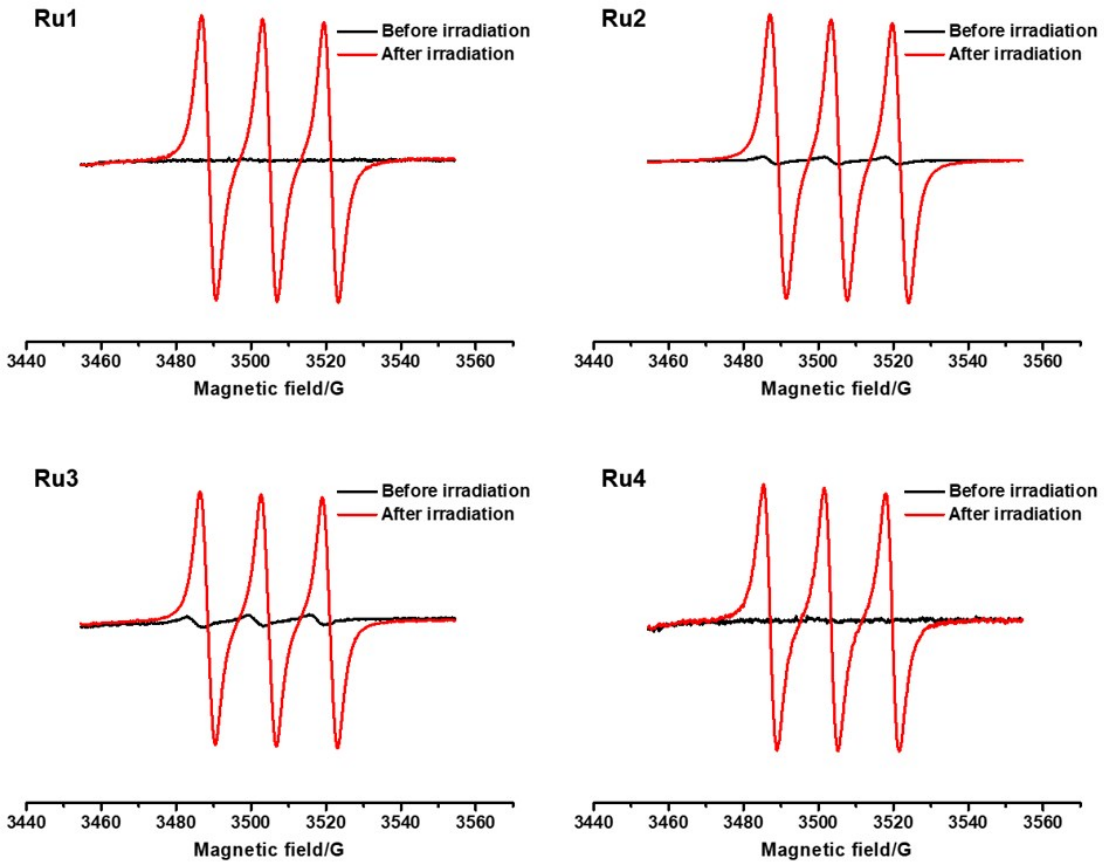

Fig. S11 ESR signals of **Ru1-4** (10 μM) trapped by TEMP in CH_3OH before and after irradiation.

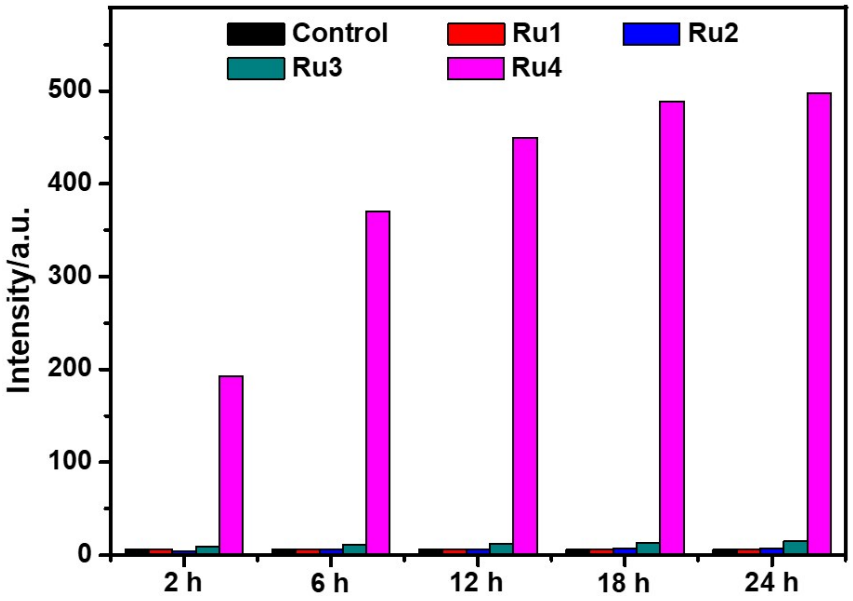

Fig. S12 Cellular uptake of **Ru1-4** (10 μM) in A549 cells measured by luminesence intensity measured by flow cytometry at increasing treatment time within 24 h.

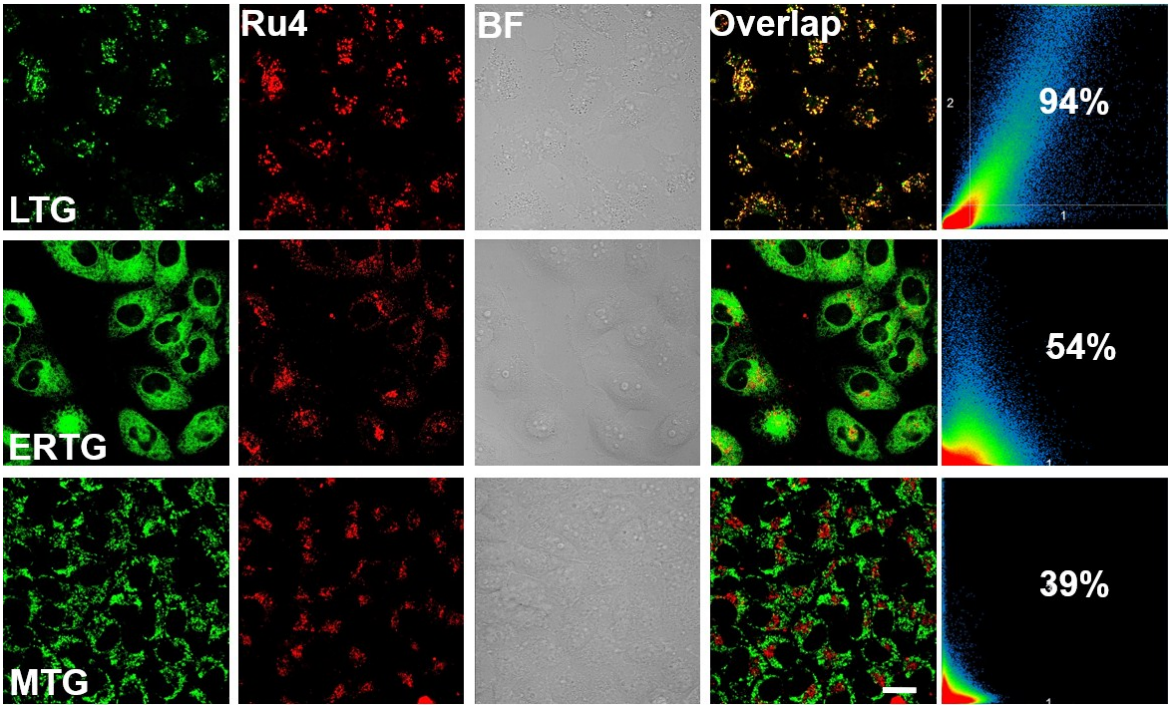

Fig. S13 Confocal images of A549 cells co-labeled with the complex **Ru4** (10 μM, 24 h) and the commercial organelle imaging agents LTG, ERTG and MTG (0.5 h). The complex was excited at 458 nm. The organelle imaging agents were excited at 488 nm. The fluorescence/luminescence was collected at 510 ± 10 nm and 630 ± 20 nm for the organelle imaging agents and **Ru4**, respectively. LTG: LysoTracker Green DND-26, 50 nM; ERTG: Endoplasmic Reticulum Tracker Green, 1 μM; MTG: MitoTracker Green FM, 50 nM. BF: bright field. The 5th column is the Pearson's correlation coefficient. Scale bar: 20 μm.

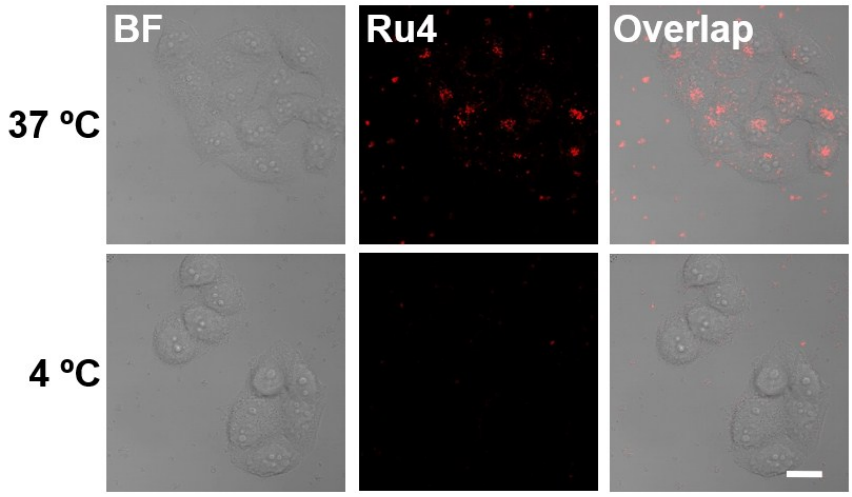

Fig. S14 Confocal images of A549 cells incubated with **Ru4** (10 μM, 4 h, λ_{ex} = 458 nm, λ_{em} = 630 ± 20 nm) under the conditions of 37 ºC and 4 ºC. BF: bright field. Scale bar: 20 μm. Under the low temperature, the metabolism of cells could be inhibited.

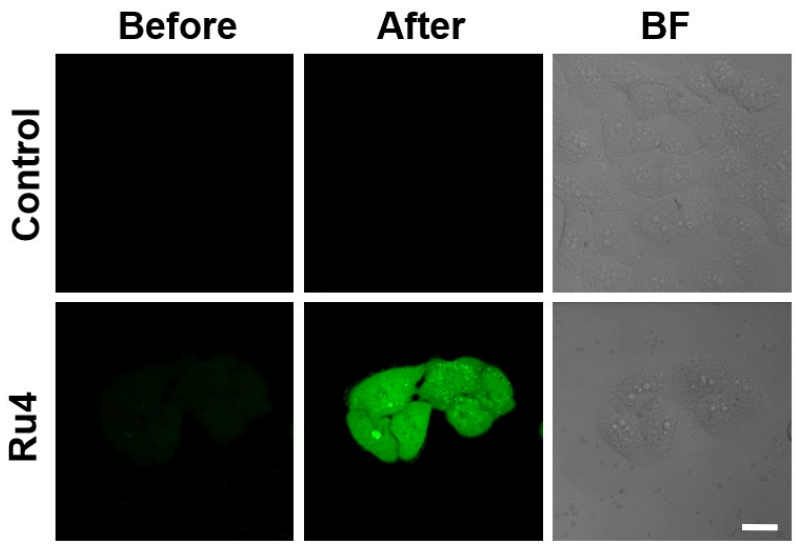

Fig. S15 Images of $^1O_2$ generation measured by DCF (10 μM, 0.5 h, λ_{ex} = 488 nm, λ_{em} = 510 ± 10 nm) in A549 cells incubated with/without **Ru4** (10 μM, 24 h) before and after the light of 458 nm irradiation. BF: bright field. Scale bar: 20 μm.

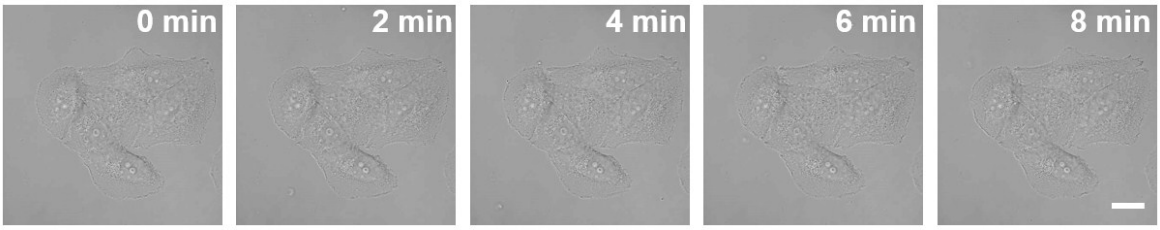

Fig. S16 The dynamic bright field images of A549 cells without **Ru4** during the 458 nm laser irradiation as a control group. No damage was found under light irradiation. Scale bar: 20 μm.

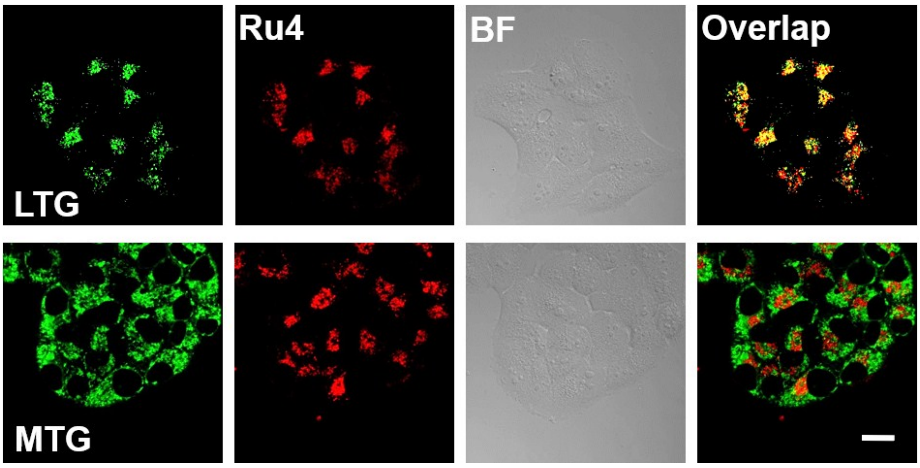

Fig. S17 Two-photon confocal images of A549 cells co-labeled with the complex **Ru4** (10 μM, 24 h) and the commercial organelle imaging agents LTG and MTG (0.5 h). The complex was excited at 810 nm. The organelle imaging agents were excited at 488 nm. The fluorescence/luminescence was collected at 510 ± 10 nm and 630 ± 20 nm for the organelle imaging agents and **Ru4**, respectively. LTG: LysoTracker Green DND-26, 50 nM; MTG: MitoTracker Green FM, 50 nM. BF: bright field. Scale bar: 20 μm.

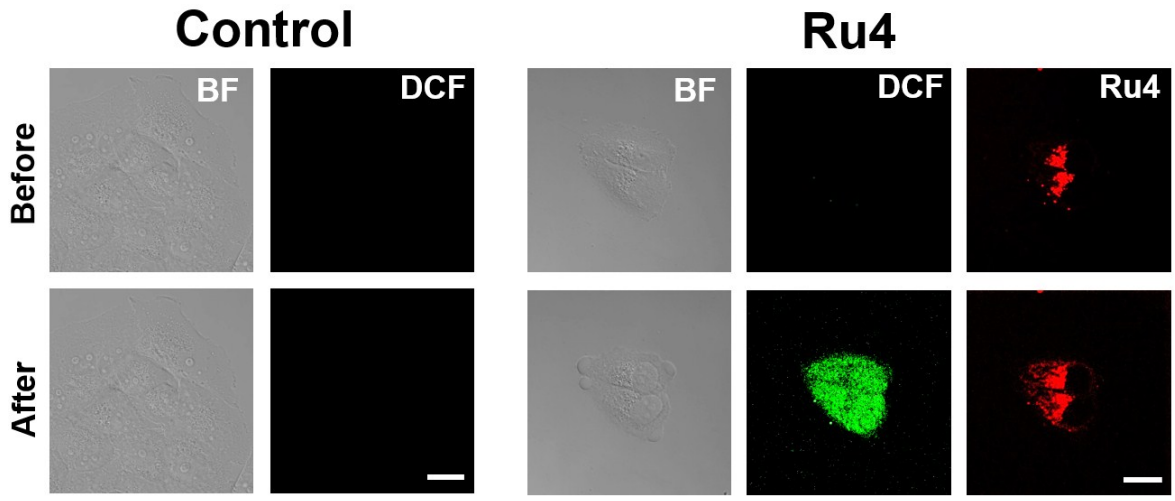

Fig. S18 Images of $^1O_2$ generation measured by DCF (10 μM, 0.5 h, λ_{ex} = 488 nm, λ_{em} = 510 ± 10 nm) in A549 cells incubated with/without **Ru4** (10 μM, 24 h, λ_{ex} = 810 nm, λ_{em} = 630 ± 20 nm) before and after the laser of 810 nm irradiation. BF: bright field. Scale bar: 20 μm.

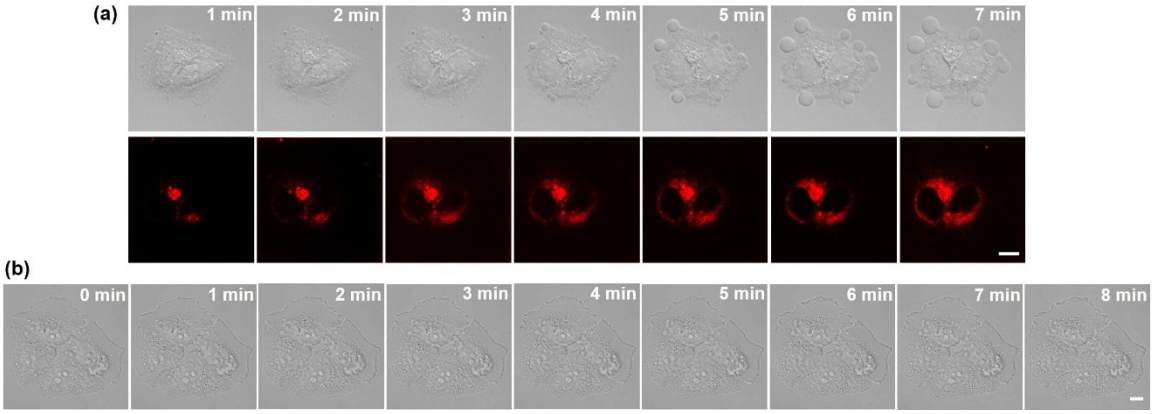

Fig. S19 (a) The dynamic confocal images of A549 cells incubated with **Ru4** (10 μM, 24 h, λ_{ex} = 810 nm, λ_{em} = 630 ± 20 nm) during the laser of 810 nm irradiation between 1 min to 7 min. (b) The dynamic bright field images of A549 cells without **Ru4** during 810 nm laser irradiation as a control group. No damage was found under two-photon irradiation. The shared scale bar: 10 μm.

Table S1 Photophysical data for the complexes at 298 K.

Complexes	λ_{ab}[a]	ε[b]	λ_{em}[c]	φ[d]	t[e]
Ru1	456	1.42	618	3.2	361
Ru2	450	1.62	617	3.6	436
Ru3	443	1.75	630	2.3	442
Ru4	460	2.59	628	4.0	722

[a] λ_{ab} maximum values of the absorption spectra (nm). [b] Extinction coefficient in (1×10^4 M^{-1} cm^{-1}). [c] λ_{em} maximum values of the emission spectra (nm). [d] Luminescence quantum yield (%). [e] Luminescent life time (ns).

Table S2 (Photo)cytotoxicity (IC_{50}, μM) of the complexes toward A549 cells.

Complexes	Dark[a]	Light[b]	PI[c]
Ru1	>500	98.8 ± 8.3	>5.1
Ru2	>500	92.4 ± 7.7	>5.4
Ru3	>500	31.6 ± 1.9	>15.8
Ru4	>100	0.4 ± 0.1	>250
cis-platin	31.4 ± 2.8	27.9 ± 2.2	1.1

[a] The IC_{50} values in the dark. [b] The IC_{50} values upon light irradiation. [c] PI is the phototoxicity index, which is the ratio between the IC_{50} values in the dark and upon light irradiation.