Supplementary information

Luminescent Molecules towards Precise Cellular Event Regulation

*Ming HU, Qinyu HAN, Linna LYU, Yan TONG, Shuo DONG, Zhiheng LOH, Bengang XING**

M. HU, Q. HAN, L. LYU, S. DONG, Prof Z. LOH, Prof. B. XING
Division of Chemistry and Biological Chemistry, School of Physical & Mathematical Sciences, Nanyang Technological University, 21 Nanyang link, 637371, Singapore
E-mail: Bengang@ntu.edu.sg

Y. TONG
CBIS Confocal Microscopy Laboratory, National University of Singapore, Singapore, 117557, Singapore

Materials and methods

Materials

Boc-glycine (Boc-Gly-OH), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), N,N-Diisopropylethylamine (DIPEA), Trifluoroacetic acid (TFA), Europium(III) Chloride Hexahydrate ($EuCl_3 \bullet 6H_2O$), 4,4,4-Trifluoro-1-(2-naphthyl)-1,3-butanedione (NTA), were obtained from Sigma-Aldrich. 1, 10-Phenanthroline-5-amine, (Phen-NH_2) was purchased from Santa Cruz. Dibenzocyclooctyne-N-hydroxysuccinimidyl ester (DBCO-NHS) and N-azidoacetylmannosamine-tetraacylated (Ac_4ManNAz) were ordered from Click Chemistry Tools. Dulbecco's Modified Eagle Medium (DMEM), Reduced Serum Medium (Opti-MEM), fetal bovine serum (FBS), Antibiotic-Antimycotic, trypsin, Rhod-3 AM calcium imaging kit, Lipofectamine® 3000 transfection kit and PureLink® HiPure plasmid miniprep kit were obtained from Thermo Fisher Scientific. All the reagents indicated above were directly used without further purification.

Instruments.

NMR spectra were acquired on Bruker Avance 500 and Bruker Avance III 400 spectrometers. Mass spectra were obtained on a Thermo Finnigan LCQ Fleet MS. Fluorescence spectra were recorded on a Shimazu RF-5301PC spectrofluorophotometer. The two-photon emission spectrum was recorded at a position perpendicular to the excitation beam (720 nm). The cell viabilities were tested on a microplate reader (BioTek). Fluorescence imaging and two-photon imaging of cells and zebrafish were carried out on a Nikon Eclipse TE2000-E microscope and Leica TCS SP5 X confocal microscope respectively. Light irradiation experiments were performed using either BLAK-RAY B-100AP/R UV lamp (365 nm) or the Coherent Chameleon Ultra II pulsed laser (720 nm).

1. Preparation of Eu(III) complex

1.1) Synthesis of Phen-Gly-Boc

1,10-Phenanthroline-5-amine (80.0 mg, 0.410 mmol), Boc-Glycine-OH (78.9 mg, 0.451 mmol), HATU (311.8 mg, 0.820 mmol) and DIPEA (143 μL, 0.820 mmol) were dissolved in DMF (2 mL) in round bottom flask. The resulting mixture was then allowed to stir overnight at room temperature. After stirring, liquid-liquid extraction was carried out with H_2O-EA (v/v = 1:1) on the solution obtaining the organic layer for 4 times. The organic layer was washed with brine and dried with sodium sulfate. Excess EA was removed using rotary evaporator under reduced pressure. The compound was then dissolved in minimal amounts of EA and purified using silica column using stepwise elution with 150 mL of 1%, 5%, 10%, 15%, 20% 25% MeOH in EA with 1% ammonia as eluents respectively for each round. NMR and MS analysis were carried out to characterize the identity of the compound. 1H NMR (500 MHz, MeOD) of Phen-Gly-Boc: δ 9.12 (d, J = 3.5 Hz, 1H), 9.05 (d, J = 3.4 Hz, 1H), 8.65 (d, J = 8.2 Hz, 1H), 8.48 (d, J = 8.1 Hz, 1H), 8.13 (s, 1H), 7.82 (ddd, J = 10.7, 8.3, 4.4 Hz, 2H), 4.09 (s, 2H), 1.54 (s, 9H). M/Z = 353.30

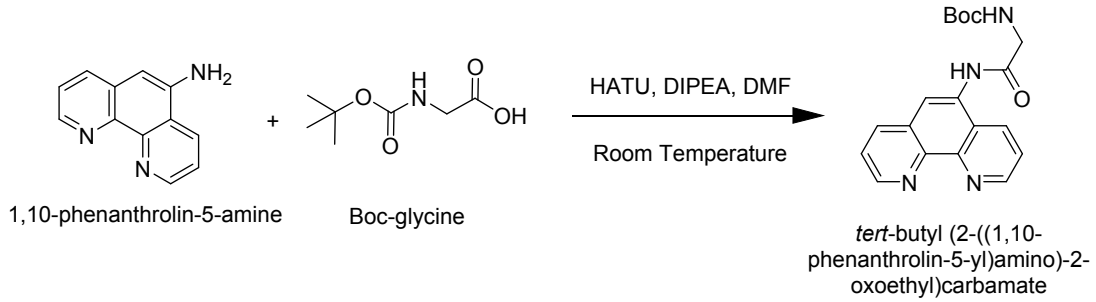

Fig.S1 Synthetic scheme of Phen-Gly-Boc.

1.2) Synthesis of Phen-Gly-DBCO

The removal of the Boc protecting group was achieved using DCM-TFA (v/v = 1:1, 16 mL). Excess TFA was removed by rotary evaporator under reduced pressure. The resulting compound Phen-Gly-NH_2 was used in the following reaction without further purification. Typically, Phen-Gly-NH_2 (6.80 mg, 0.0270 mmol), DBCO-NHS (12.0 mg, 0.0270 mmol) were added into a round bottom flask and dissolved in DMF with a 10-fold excess amount of DIPEA (2 mL). The resulting mixture was then allowed to stir overnight at room temperature. After stirring, liquid-liquid extraction was carried out with H_2O-EA (v/v = 1:1) on the solution obtaining the organic layer for 4 times. The organic layer was washed with brine and dried with sodium sulfate. Excess EA was removed using rotary evaporator under reduced pressure to obtain the crude product. The crude compound was then dissolved in minimal amounts of DCM and purified using silica column using stepwise elution with 100 mL of 0%, 1%, 2%, 3%, 4% 5% and 6% MeOH in DCM with 1% ammonia as eluents respectively for each round. NMR and MS analysis were carried out to characterize the identity of the compound. 1H NMR (400 MHz, $CDCl_3$) of Phen-Gly-DBCO: δ 9.64 (s, 1H), 9.25 (dd, J = 4.3, 1.5 Hz, 1H), 9.20 (dd, J = 4.3, 1.6 Hz, 1H), 8.49 (dd, J = 8.4, 1.5 Hz, 1H), 8.33 (dd, J = 8.1, 1.6 Hz, 1H), 8.18 (s, 1H), 7.80 – 7.62 (m, 2H), 7.57 – 7.46 (m, 1H), 7.46 – 7.34 (m, 3H), 7.19 – 7.06 (m, 2H), 6.82 – 6.67 (m, 1H), 6.67 – 6.55 (m, 1H), 6.45 (d, J = 7.6 Hz, 1H), 4.52 (dd, J = 16.8, 7.5 Hz, 1H), 4.31 (d, J = 13.9 Hz, 1H), 3.82 (dd, J = 16.9, 5.1 Hz, 1H), 3.38 (d, J = 13.8 Hz, 1H), 2.49 – 2.28 (m, 2H), 2.13 – 1.85 (m, 3H). M/Z = 540.52.

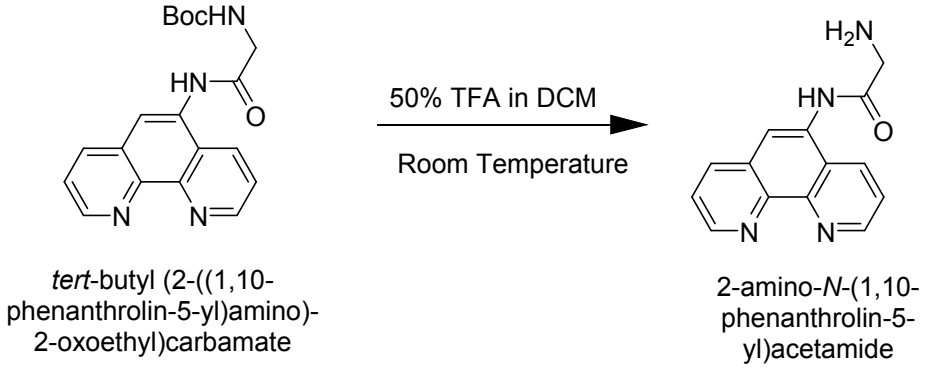

Fig.S2 Synthetic scheme of Phen-Gly.

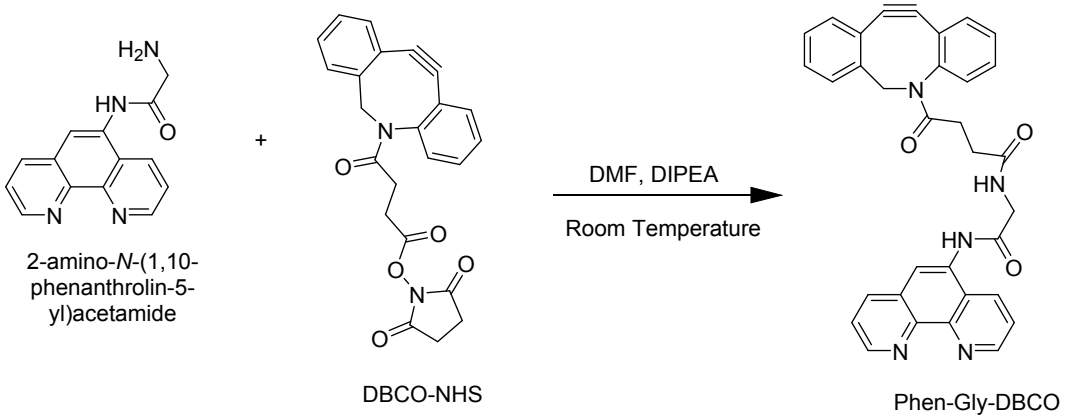

Fig.S3 Synthetic scheme of Phen-Gly-DCBO.

1.3) Synthesis of Eu(III) complex

$EuCl_3 \bullet 6H_2O$ (8.5 mg, 0.0232 mmol) dissolved in MeOH (400 µL) and added into a mixture of Phen-Gly-DBCO (12.6 mg, 0.0234 mmol) or Phenanthroline (4.2 mg, 0.0233 mmol) together with NTA (4,4,4-Trifluoro-1-(2-naphthyl)-1,3-butanedione) (18.6 mg, 0.0699 mmol) that was dissolved in MeOH (600 µL) and DCM (200 µL). The mixture was allowed to be heated with reflux and stirred overnight. After heating with reflux overnight, the leftover MeOH and DCM were removed under reduced pressure using rotary evaporator. Minimum amount of MeOH was added to dissolve the compound. The resulting solution was then added into deionized water to obtain the Europium (III) complex precipitate. MS analysis was done to characterize the compound. M/Z = 1224.13 $[M\text{-}NTA]^+$.

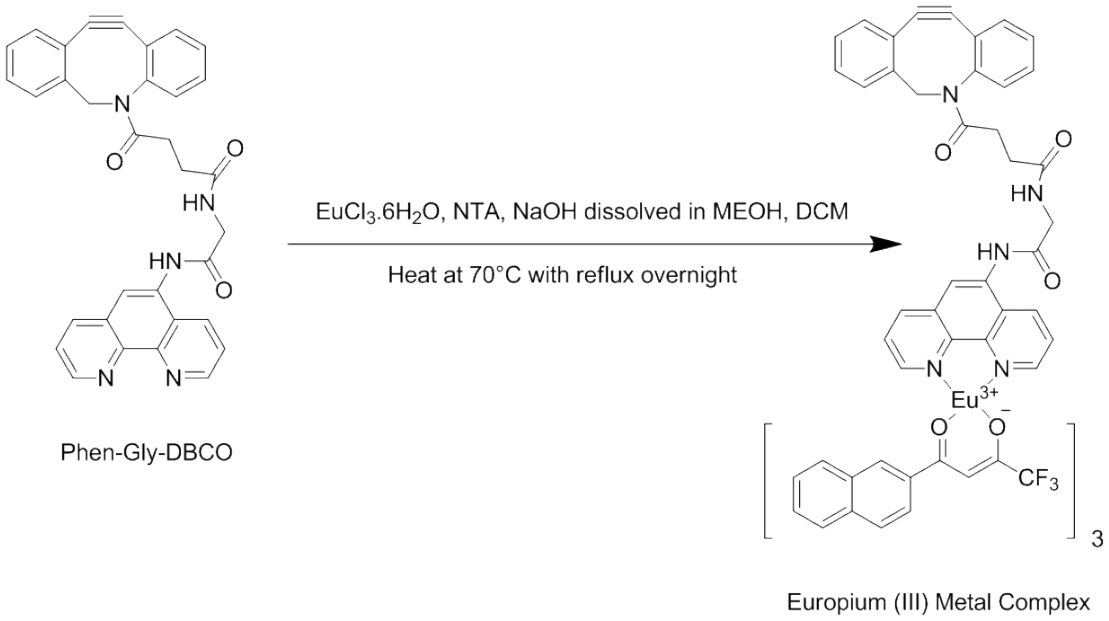

Fig.S4 Synthetic scheme of Eu(III) complex **Eu-DBCO**.

2. Cell culture and metabolic labeling on cell membrane

The human embryonic kidney 293 (HEK293) cell lines were purchased from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units mL^{-1} penicillin and 100 µg mL^{-1}

streptomycin and maintained in a humidified incubator with 5% CO^2 at 37 °C. The cells were seeded at a density of 1×10^5 in 12-well plate and incubated with 50 μM of Ac_4ManNAz for 2 days. The cells were trypsinized and cultured in an ibidi dish (35 mm, plastic bottom) at 1×10^5 cells/well in 1 mL DMEM medium overnight. The azido-labeled cell membrane was stained with CellMask DeepRed (5 μM, 10 min) separately and imaged using confocal microscope. For cell viability test, HEK293 cells were seeded in 96-well plate (1×10^4 HEK293 cells per well) and incubated for 24 h. Then, cells were incubated with Ac_4ManNAz or Eu(III) complex of different concentrations for another 24 h and the cell viabilities were evaluated using TOX8 assay kit (resazurin based) by following standard manufacturer's protocol.

3. Membrane channel expression and covalent labeling of Eu-DBCO on HEK293 cell membrane

Generally, ChrimsonR-GFP plasmid was purchased from Addgene[6c]. The plasmid DNA was replicated and purified with commercial available kit by following manufacturer's procedure. For ion channel expression, HEK293 cells were seeded with the density of 1×10^5 and incubated for 24 h. The commercial plasmid transfection reagent, Lipofectamine® 3000, was used according to the manufacturer's protocol in Opti-MEM medium.[12] After 4 h incubation, the medium was replaced with fresh DMEM medium containing Ac_4ManNAz (50 μM). The cells were cultured in incubator for 48 h and re-plated in ibidi dish (35 mm, 1×10^5 cells) for another 24 h. For confocal imaging experiments, cell samples were washed twice with DMEM medium and incubated with Eu-DBCO at a concentration of 60 μM for 3h at 37 °C. The cells were washed 3 times with DMEM medium prior to imaging (λ_{ex}=350±25 nm).

4. Intracellular calcium analysis by Rhod-3 AM

Calcium imaging kit (Rhod-3AM) was dissolved in 100 μL DMSO to prepare a stork solution (10 mM). After Eu-DBCO labeling on transfected HEK293 cells, the samples in dishes (1×10^5 cells per well) were incubated with Rhod-3 AM (10 μM) for 30 min in the dark by following manufacturer's protocol. Then the cells were washed 3 times with serum-free DMEM medium and irradiated with 720 nm NIR light. [7a]The intracellular calcium imaging was recorded on Leica TCS SP5 X confocal microscope (λ_{ex}= 560 nm).

***5. In vivo* animal studies and preparation:**

All the animal involved experiments were carried out by following the approved guidelines of Nanyang Technological University Institutional Animal Care and Use Committee (IACUC). Typically, zebra fish embryos were kept in E3 media which contains 0.1% Methylene Blue, 0.33 mM $MgSO_4$, 0.33 mM $CaCl_2$, 0.17 mM KCl and 5 mM NaCl, pH 7.0−7.2. The fish embryos were raised at 28.5 °C. Prior to experiments, the fish larvae were anesthetized at 48hours post fertilization by using 0.003 % tricaine. Cells expressing ChrimsonR-GFP were incubated with Ca^{2+} indicator and harvested (5×10^6 cells/mL). The harvested cells were injected into the yolk sac of zebra fish larvae through pressurized microinjector (MPPI-3 Applied Scientific). After the implantation of cells, the larvae were washed with E3 media. The Eu(III) complex was injected into the yolk sac of these larvae which have been already implanted with cells. In Ca^{2+} imaging experiment, the anesthetized larvae were imaged in ibidi dish (35 mm). The larvae were irradiated with NIR light (720 nm) for different time durations. Fluorescence images of zebra fish larvae were captured on a Leica TCS SP5 X confocal microscope before and after NIR light stimulation.

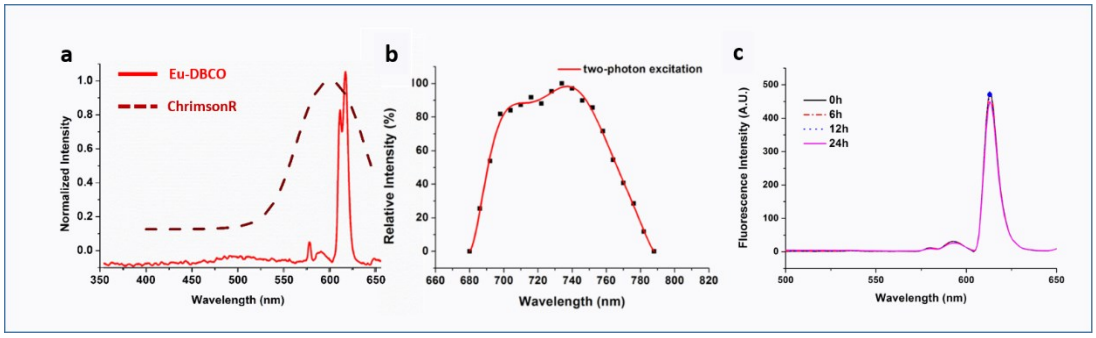

Fig.S5 Fluorescence spectra of Eu-DBCO under two-photon excitation. a) Action spectra of ChrimsonR and emission spectra of Eu-DBCO λ_{ex}=720 nm, b) Excitation spectra of Eu-DBCO λ_{em}=615 nm, c) Stability of Eu-DBCO under physiological condition, emission spectra of Eu-DBCO (50μM) in HEPS buffer pH 7.4, λ_{ex}=350 nm.

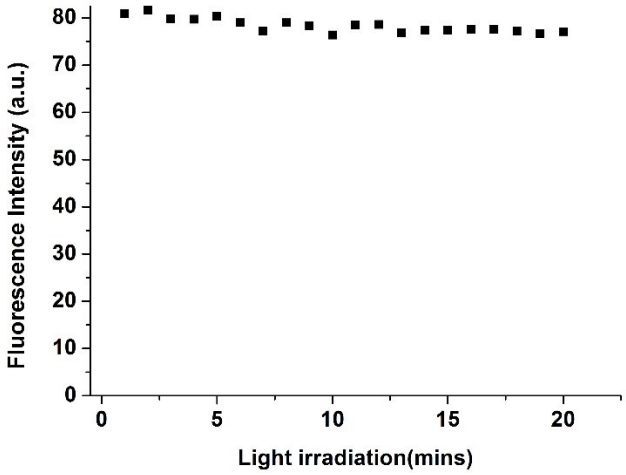

Fig.S6 Luminescence stability of Eu complex (60μM) under light treatment (λ_{ex}=350 nm, λ_{em}= 615 nm).

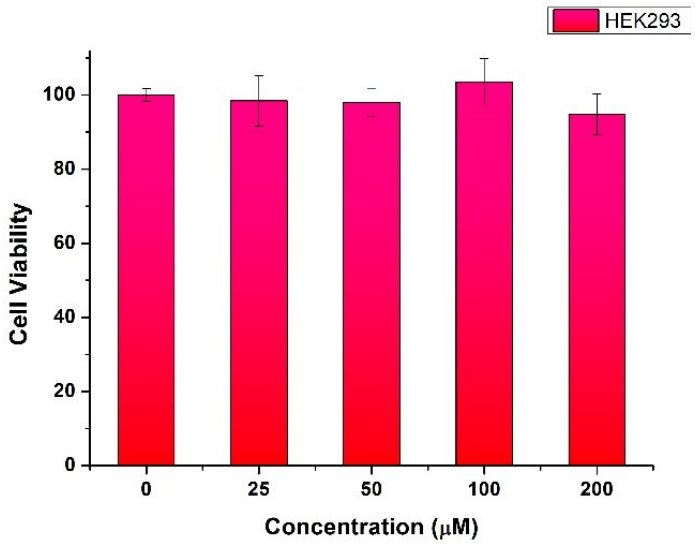

Fig.S7 Cell viability of HEK293 cells treated with Ac_4ManNAz of different concentrations.

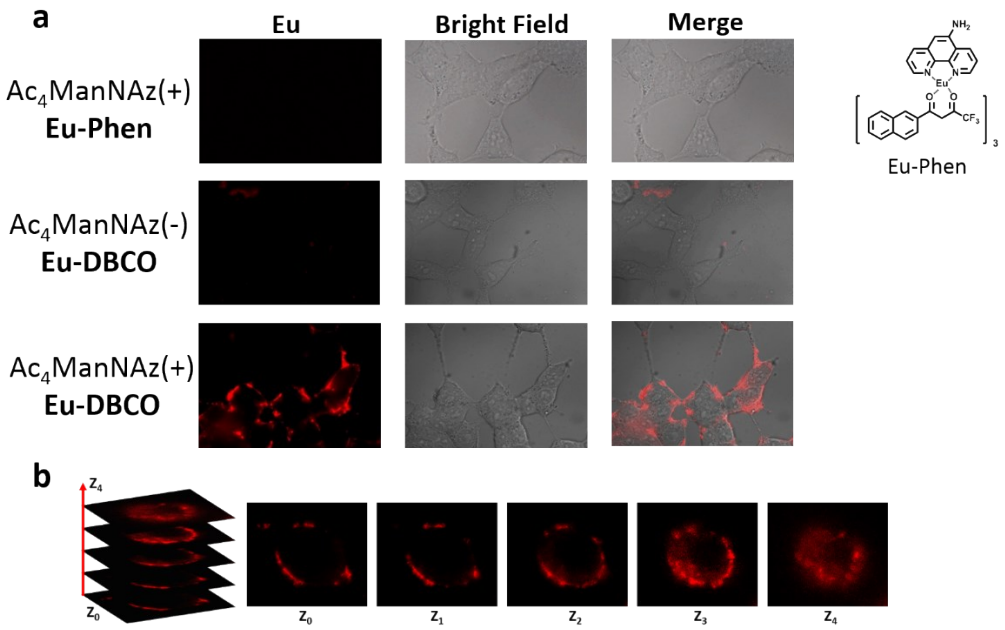

Fig.S8 a)Fluorescence images of HEK293 cells labelled with Eu(III) complex (60μM, λ_{ex} =350nm, λ_{em} =610/50nm); b) Fluorescence images at different axial position of HEK293 cells labelled with Eu-DBCO.

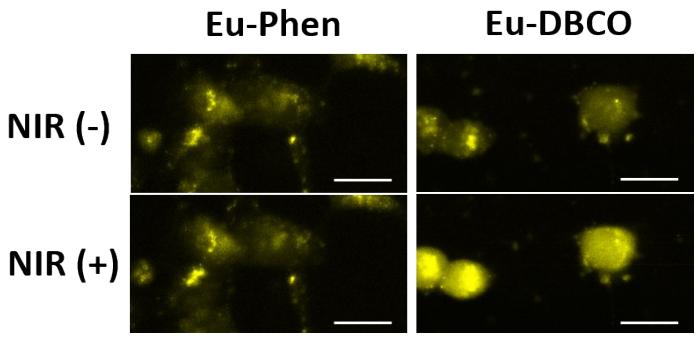

Fig.S9 Membrane channel activity manipulated through NIR illumination: fluorescence images of Ca^{2+} indicator (Rhod-3 AM, 10 μM, λ_{ex}=560 nm, λ_{em}=610/75nm) in cells treated with Eu(III) complexes before and after NIR light stimulation (scale bar = 15μm).

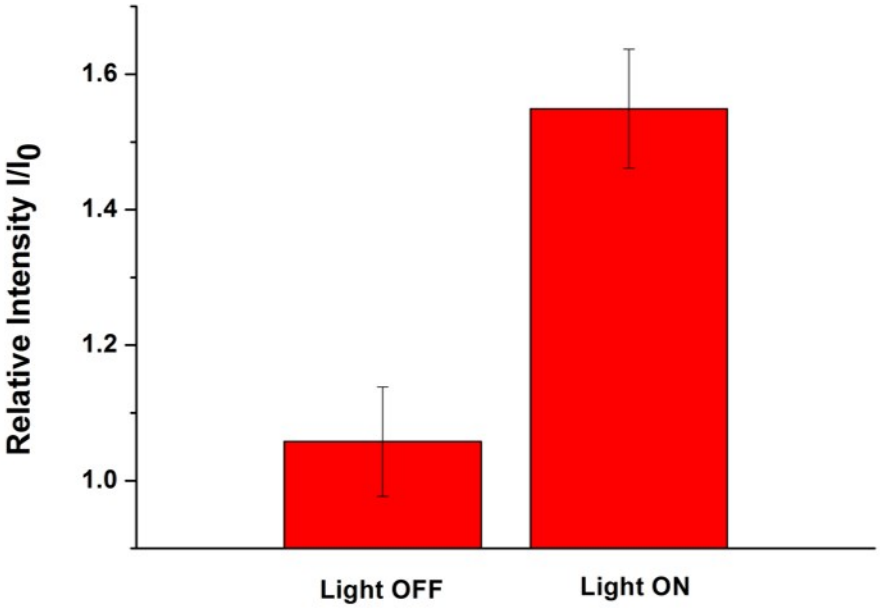

Fig.S10 Relative fluorescence intensity of membrane potential dye (di-3-ANEPPDHQ) on Eu-labelled HEK293 cells in the absence/presence of light stimulation.

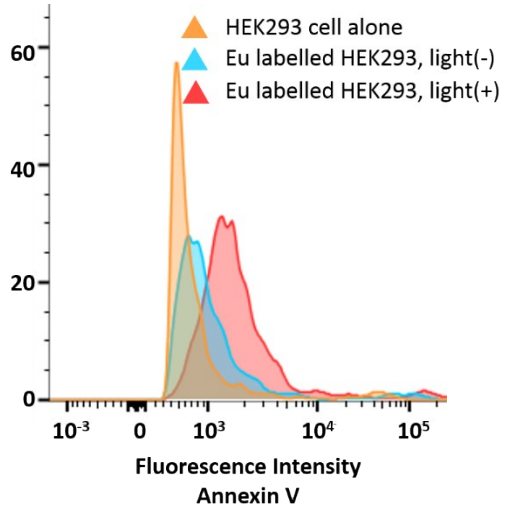

Fig.S11 Apoptotic event revealed by flow-cytometry analysis of ChrimsonR-expressed HEK293 cells stained by Annexin V-Cy3.

[1] N. C. Klapoetke, Y. Murata, S. S. Kim, S. R. Pulver, A. Birdsey-Benson, Y. K. Cho, T. K. Morimoto, A. S. Chuong, E. J. Carpenter, Z. Tian, *Nat. Methods* **2014**, *11*, 338.

[2] https://tools.thermofisher.com/content/sfs/manuals/lipofectamine3000_protocol.pdf.

[3] S. K. Mohanty, R. K. Reinscheid, X. Liu, N. Okamura, T. B. Krasieva, M. W. Berns, *Biophys. J.* **2008**, *95*, 3916-3926.