Supplementary Information *for*

Construction of fluorescent probes for ratiometric visualization of mitochondrial depolarization based on FRET mechanism

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Contents

Materials

All chemicals used are of analytical grade. 1,3,3-Trimethyl-2-(formylmethylene) indoline, iodomethane, etc. were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). 4-Methylpyridine, pyrrolidine etc. were purchased from J&K Chemical (Beijing, China). The solvents used in the spectral measurement are of chromatographic grade.

Spectroscopic measurements

The UV-visible-near-IR absorption spectra of dilute solutions were recorded on a U2910 spectrophotometer using a quartz cuvette having 1 cm path length. One-photon fluorescence spectra of dilute solutions were obtained on a HITACH F-2700 spectrofluorimeter equipped with a 450-W Xe lamp. PBS buffer solution: 10 mM, NaCl, $NaHPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot 2H_2O$, pH = 7.40.

Cell culture and staining methods

HeLa cells were bought from Procell Life Science&Technology Co,.Ltd., and the cells were grown in H-DMEM (Dulbecco's Modified Eagle's Medium, High Glucose) supplemented with 10% FBS (Fetal Bovine Serum) in a 5% CO_2 incubator at 37 $^{\circ}$C. For living cells imaging experiment of the probes, 1 mM (M1, R2) stock solutions of the probe in DMSO were prepared. After that, 2 μL of stock solutions were mixed evenly with 1 mL culture medium in a tube. The culture medium surrounding the cells were firstly removed. The cells were incubated with the above mixed solutions at 37 $^{\circ}$C. After incubation, the cells were imaged immediately without further washing procedure.

Fluorescence imaging methods

Confocal fluorescence images were obtained with a Nikon A1R confocal laser

scanning microscope. The fluorescent images of M1 and R2 were acquired with the excitation of 405 nm and 561 nm, respectively. The fluorescence was collected in blue and red channel in the wavelength range of 425-475 nm and 570-620 nm, respectively. For co-localization experiments, the commercialized probe (MTDR) were excited by 647 nm, and the emission were collected in NIR channel (665-735 nm). For FRET experiments, 405 nm was used for excitation and the emission was collected in blue and red channel in the wavelength range of 425-475 nm and 570-620 nm, respectively.

Co-stain with commercialized mitochondial probe

The HeLa cells cultured in glass-bottom culture dish were stained by 2 μM M1, or 2 μM R2 together with 50 nM MTDR (a deep-red emissive commercial probe for mitochondria) for 20 min. The cells were then imaged under confocal microscope without additional washing procedure. The colocalization coefficients were calculated with the Nikon software.

RNA digestion experiments

Live HeLacells were initially fixed with 4% paraformaldehyde, and then penetrated with 0.3% triton X-100 for 15 min. Then 20 μg/mL of RNase in PBS buffer solution were used to treat the cells for 2 hours to hydrolyze the intracellular RNA. After washing the cells three times with PBS buffer, 2 μM R2 was used to incubate the cells for 20 min. Finally, the cells were visualized under confocal microscope in red channels with the excitation at 561 nm.

Visualization of the $\Delta\Psi_m$ change

The change in $\Delta\Psi_m$ was induced by the addition and removal of carbonyl cyanide 3-chlorophenyl-hydrazone (CCCP). To realize the in-situ monitoring of $\Delta\Psi_m$ change of the same cells, living cells were initially found under microscope. Half of the

culture medium (0.5 mL) was carefully removed, and 0.5 mL of culture medium containing 20 μM CCCP was added in. Then a series of cell images with gradually decreased $\Delta\Psi_m$ was obtained. Afterwards, the culture medium with CCCP was carefully removed, and fresh culture medium was added in. The $\Delta\Psi_m$ could be recovered and the cell images were acquired.

Visualization of mitochondrial depolarization by H_2O_2

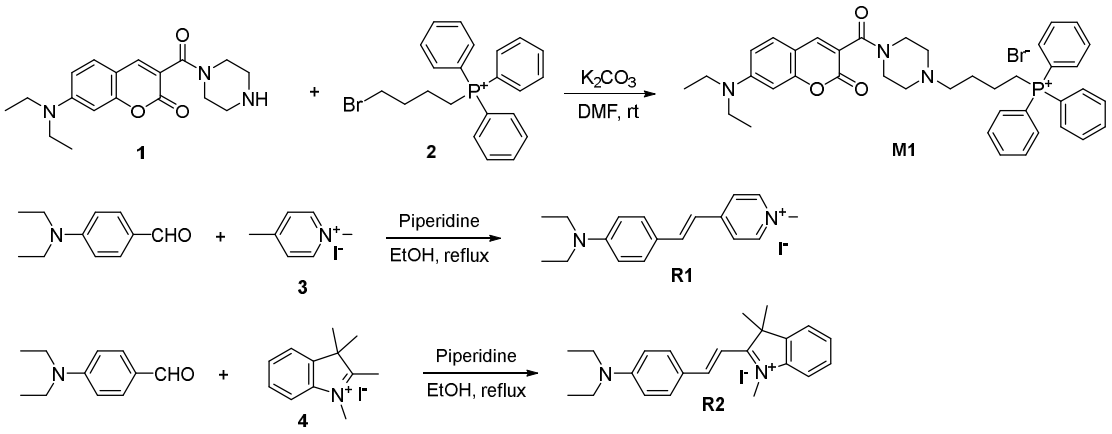
Living cells cultured in the glass-bottom culture dish were initially stained by the probes. Then the cells for experiments were found under confocal microscope, and half of the culture medium (0.5 mL) was removed. 0.5 mL of culture medium containing 16 mM H_2O_2 was added in to obtain solution with 8 mM H_2O_2, and a series of cell images were acquired to record the response to oxidative damage.

Molecular simulations

The geometrical structures of M1, R1, and R2 were obtained with Gaussian 09 software, by means of the sequential optimization with the basic sets of PM3, B3lYP/3-21G, and B3LYP/6-31G, respectively.[1]

The binding modes of M1, R1, and R2 to RNA were obtained with AutoDock 4.2 software.[2] The initial structure of RNA was obtained from the crystal structure (crystal structure of RNA was downloaded from the protein data bank with a PDB ID of 6ND4).[3] For AutoDock calculations, number of GA runs was set as 50, Maximum number of evals was set as 25000000, and the other parameters were set as the default ones without changes.

Synthesis of M1, R1, and R2



Scheme S1. The synthetic routes of probes M1, R1, and R2.

Synthesis of (4-(4-(7-(diethylamino)-2-oxo-2H-chromene-3-carbonyl)piperazin-1-yl)butyl)triphenylphosphonium bromide (M1). The compounds **1** and **2** were synthesized according to the previously reported procedures.[4-5] To synthesize probe M1, compounds 1 (0.5 mmol) and 2 (0.5 mmol) were added into a round-bottom flask. 20 mL DMF was poured into the flask to dissolve the two compounds, and K_2CO_3 (1 mmol) were then added in. The mixture was stirred at room temperature for 24 h to accomplish the reaction, which was then filtrated. The DMF in the liquid phase was then removed by distillation under reduced pressure. The residues were purified via column chromatography with dichloromethane and methanol (v/v = 20:1) as the fluent. The product was further purified by recrystallization in ethanol to obtain green-yellow powder (yield, 37.2%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.96 (s, 1H), 7.94 – 7.88 (m, 3H), 7.88 – 7.71 (m, 12H), 7.50 (d, J = 8.9 Hz, 1H), 6.75 (dd, J = 9.0, 2.5 Hz, 1H), 6.56 (d, J = 2.4 Hz, 1H), 3.57 – 3.39 (m, 8H), 3.28 (s, 2H), 2.34 (t, J = 12.1 Hz, 6H), 1.63-1.61 (m, 4H), 1.13 (t, J = 7.0 Hz, 6H). HRMS (ESI): m/z, for $C_{40}H_{45}N_3O_3P^+$, Calc., 646.3193, found, 646.3207.

Synthesis of (E)-4-(4-(diethylamino)styryl)-1-methylpyridin-1-ium iodide (R1). To a round-bottom flask containing 5 mL ethanol, compound **3** (1.0 mmol) and 4-(diethylamino)benzaldehyde (1.0 mmol) were added. The system was vigorously

stirred for 10 min to mix evenly, and drops of piperidine was added in. The mixture was stirred and refluxed for 24 h to accomplish the reaction, and solid product was precipitated after cooling down to room temperature. The product was isolated by filtration and further purified via recrystallization in ethanol. The pure product was presented in red powder (yield 71.8%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.67 (d, J = 6.6 Hz, 2H), 8.13 – 7.97 (m, 2H), 7.90 (d, J = 16.1 Hz, 1H), 7.68 – 7.47 (m, 2H), 7.13 (d, J = 16.1 Hz, 1H), 6.84 – 6.60 (m, 2H), 4.17 (s, 3H), 3.43 (q, J = 7.0 Hz, 4H), 1.13 (t, J = 7.0 Hz, 6H). HRMS (ESI): m/z, for $C_{18}H_{23}N_2^+$, Calc., 267.1856, found, 267.1857.

Synthesis of (E)-2-(4-(diethylamino)styryl)-1,3,3-trimethyl-3H-indol-1-ium (R2). To a round-bottom flask containing 5 mL ethanol, compound **4** (1.0 mmol) and 4-(diethylamino)benzaldehyde (1.0 mmol) were added. The system was vigorously stirred for 10 min to mix evenly, and drops of piperidine was added in. The mixture was stirred and refluxed for 24 h to accomplish the reaction, and solid product was precipitated after cooling down to room temperature. The product was isolated by filtration and further purified via recrystallization in ethanol. The pure product was presented in red powder (yield 62.3%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.29 (d, J = 15.6 Hz, 1H), 8.06 (d, J = 8.7 Hz, 2H), 7.77 (d, J = 7.4 Hz, 1H), 7.69 (d, J = 8.0 Hz, 1H), 7.55 (t, J = 7.8 Hz, 1H), 7.47 (t, J = 7.4 Hz, 1H), 7.22 (d, J = 15.6 Hz, 1H), 6.88 (d, J = 8.9 Hz, 2H), 3.96 (s, 3H), 3.56 (q, J = 7.0 Hz, 4H), 1.75 (s, 6H), 1.18 (t, J = 7.0 Hz, 6H). HRMS (ESI): m/z, for $C_{23}H_{29}N_2^+$, Calc., 333.2325, found, 333.2325.

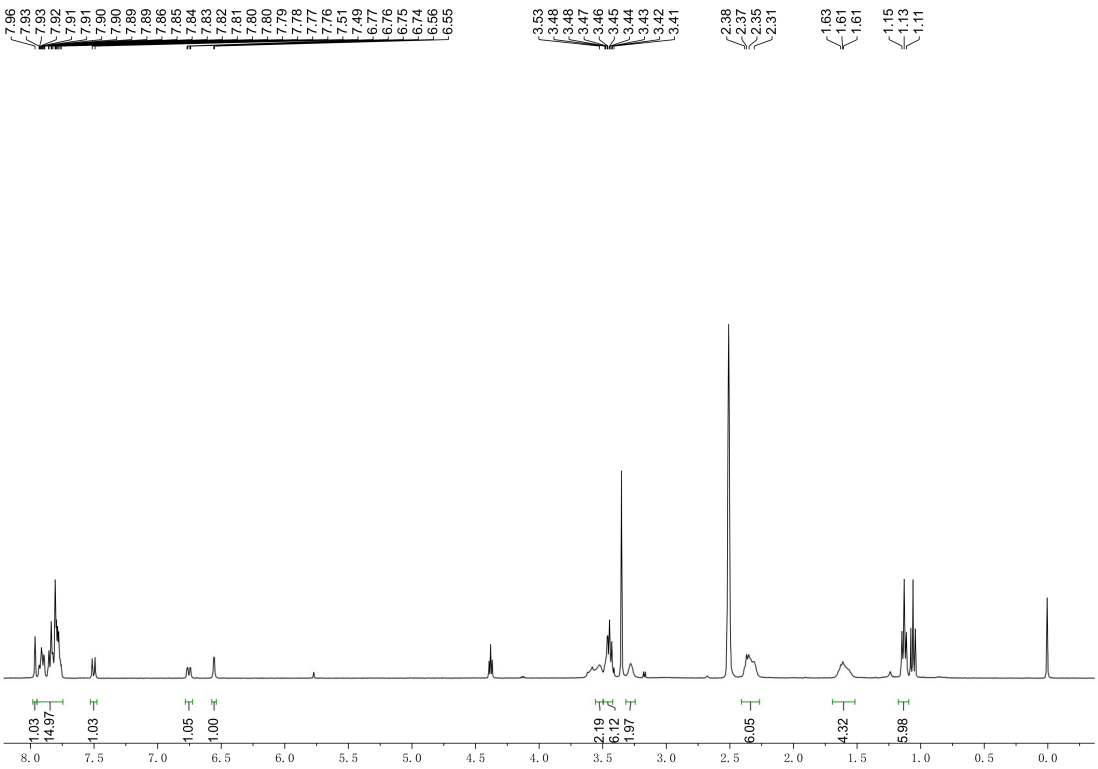


Figure S1. The ^{1}H NMR spectrum of probe M1 in DMSO-d_6.

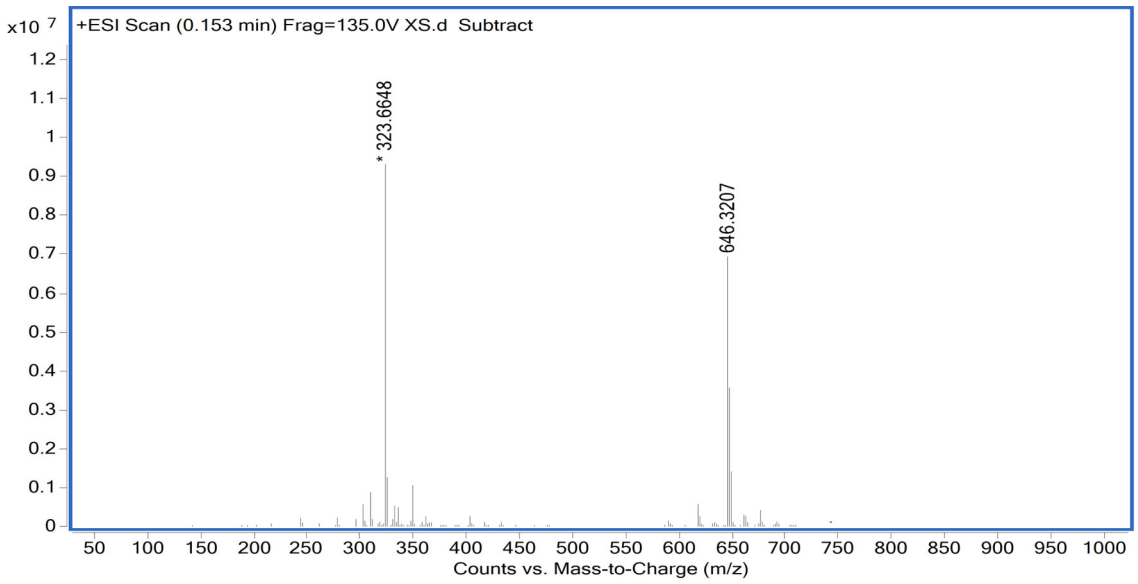


Figure S2. The HRMS spectrum of probe M1.

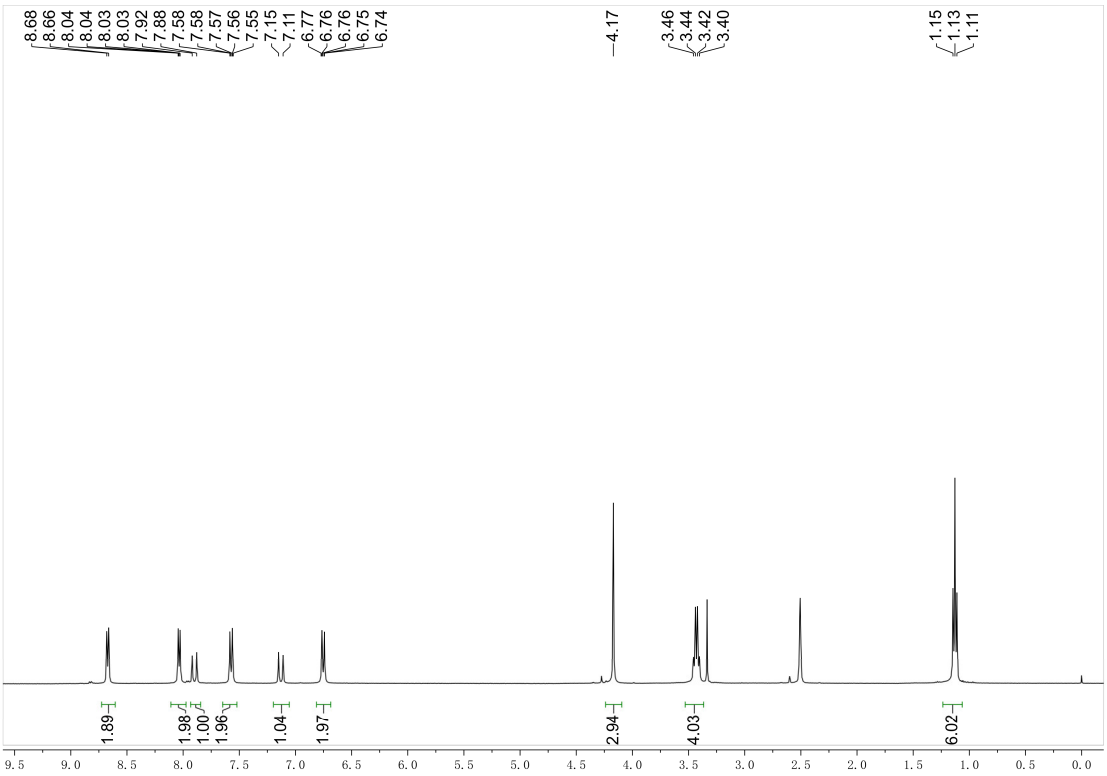


Figure S3. The ^{1}H NMR spectrum of probe R1 in DMSO-d_6.

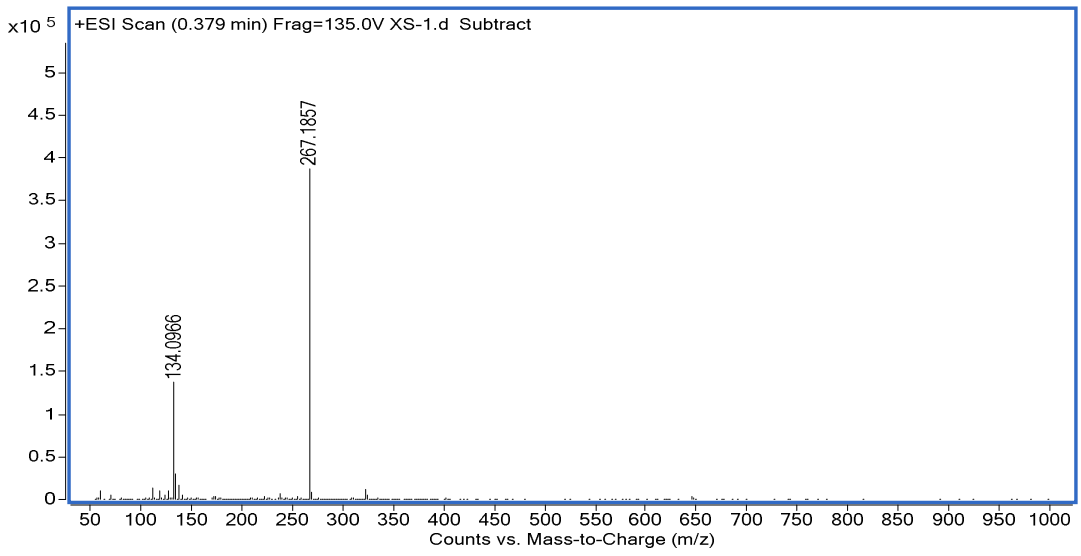


Figure S4. The HRMS spectrum of probe R1.

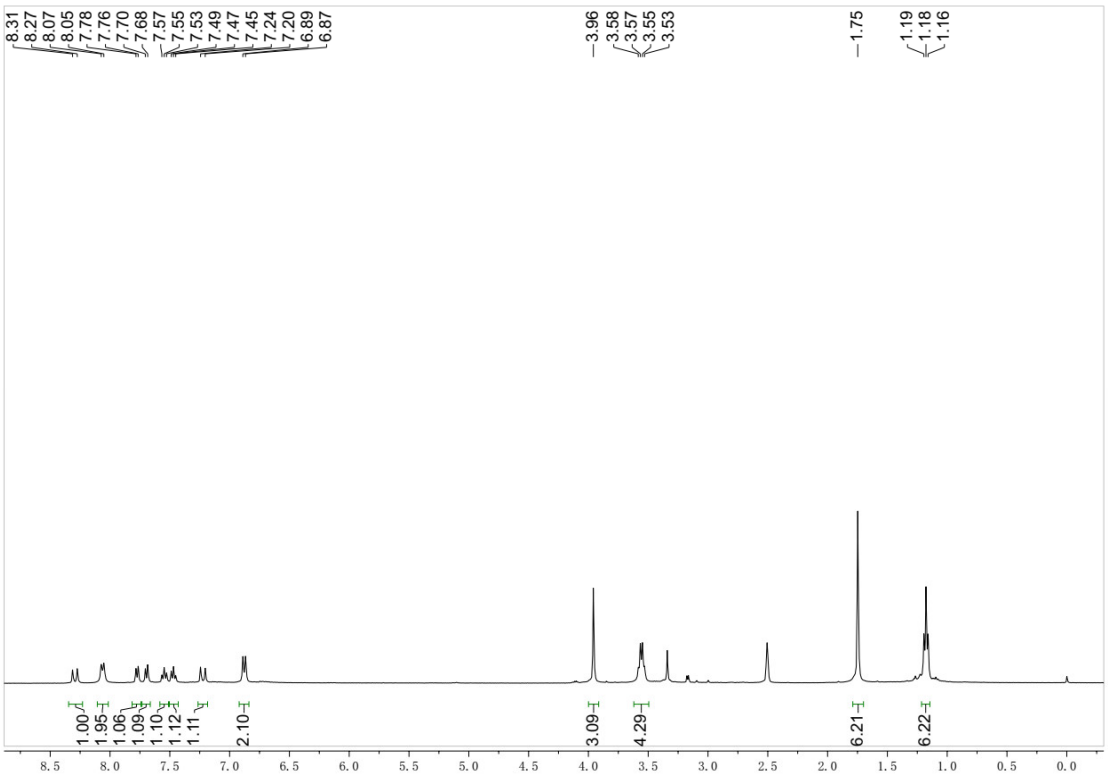


Figure S5. The ^{1}H NMR spectrum of probe R2 in DMSO-d_6.

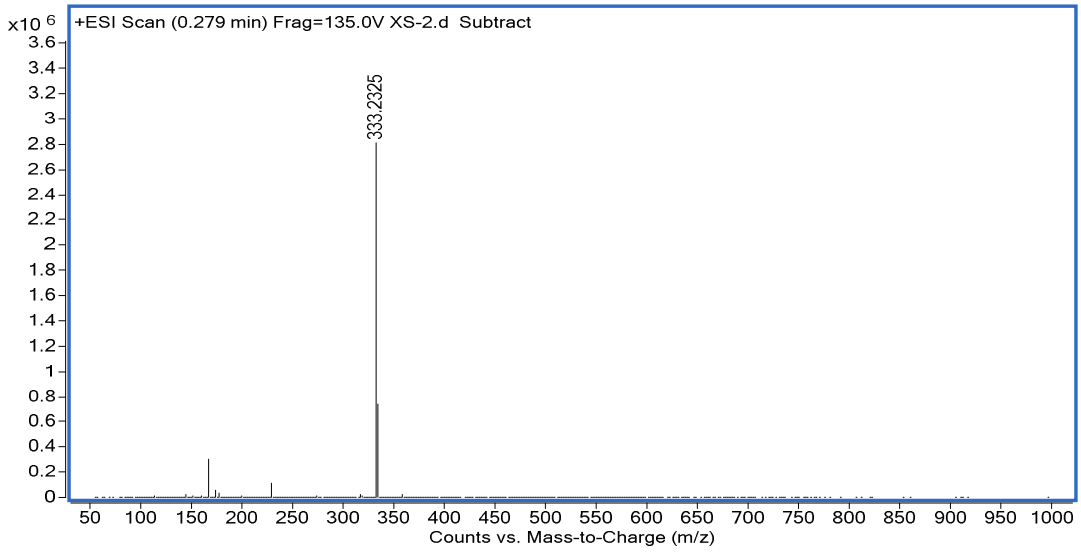


Figure S6. The HRMS spectrum of probe R2.

Figures used in the manuscript

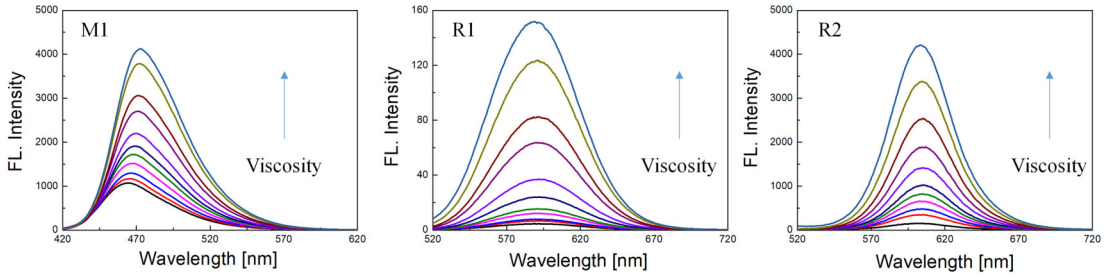


Figure S7. The fluorescence spectra of M1, R1, and R2 in mixed solvents of methanol and glycerol with different glycerol content (0%-100%).

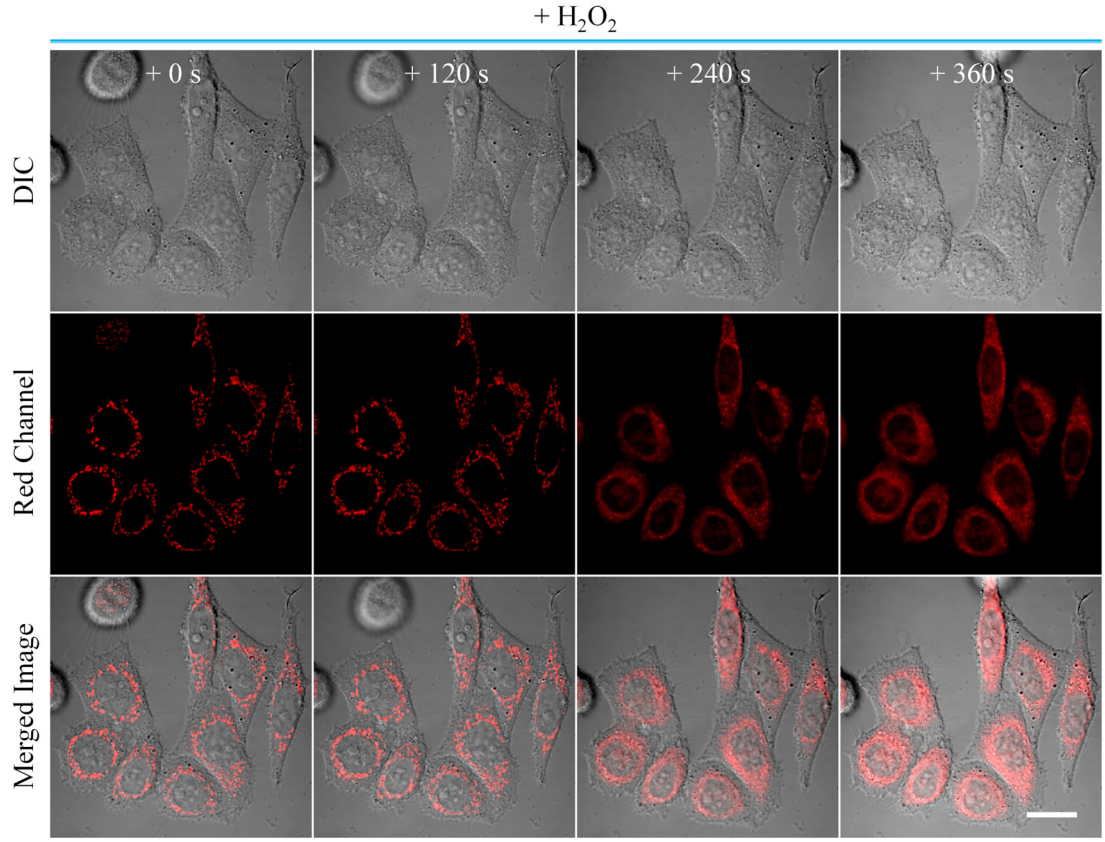


Figure S8. The fluorescence images of living HeLa cells pre-loaded by R2, and then treated by H_2O_2 for 0-360 s. Scale bar = 20 μm.

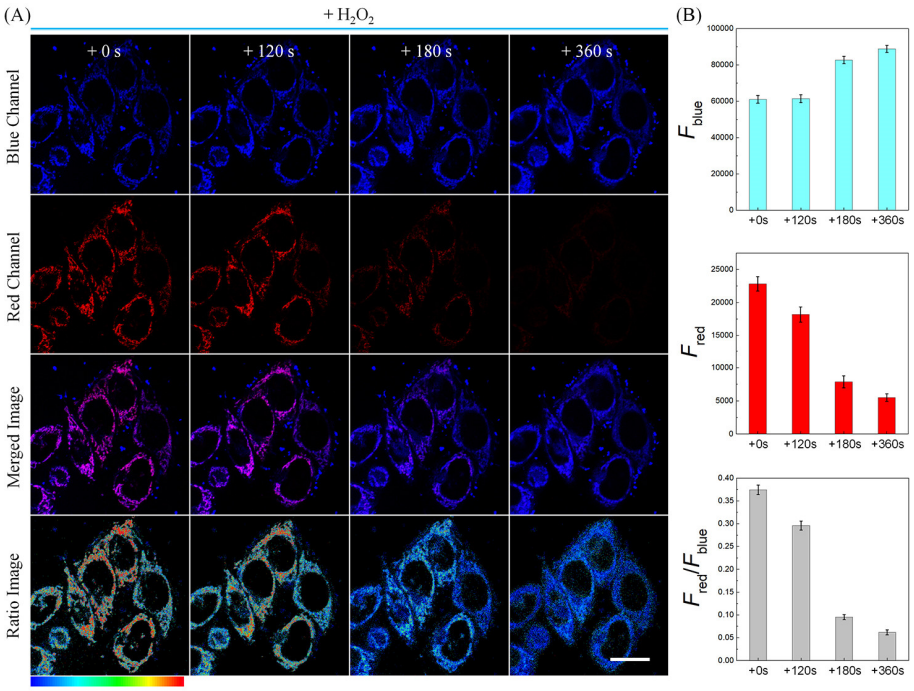


Figure S9. (A) The fluorescence and ratiometric images of HeLa cells initially co-stained by M1 and R2, treated by CCCP for 0-90 s, and then incubated in fresh culture medium for 0-120 s. (B) The relative fluorescence intensity in blue and red channels in cells of (A), and the ratio values in (A). Scale bar = 20 μm.

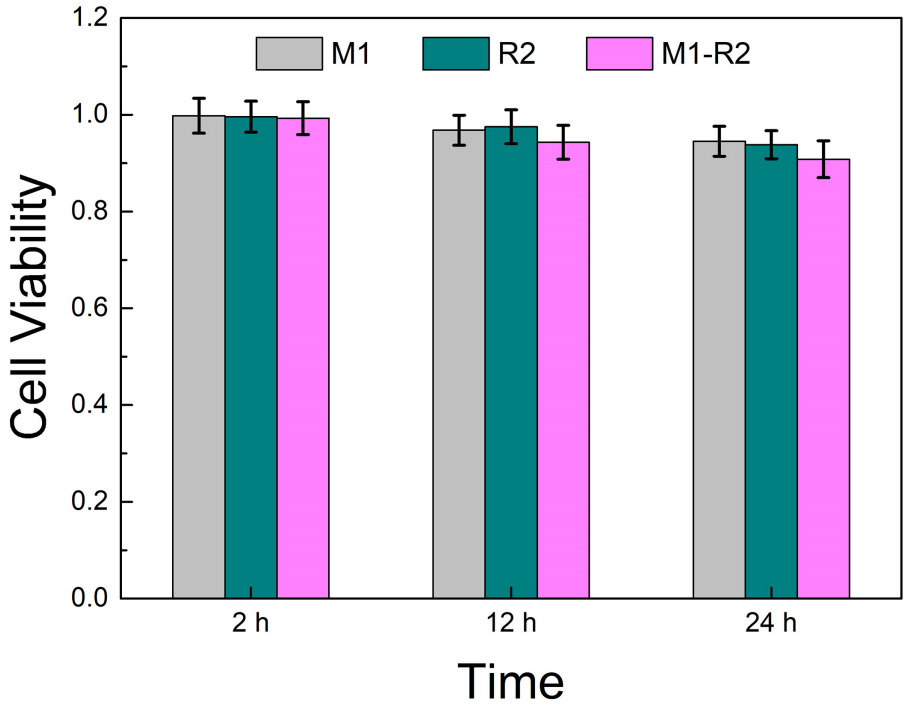


Figure S10. The cell viability of living HeLa cells after incubation by 2 μM M1, 2 μM R2, or 2 μM M1 and 2 μM R2 for 2 h, 12 h, and 24 h.

References

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