Supporting Information for
A TEMPO-Conjugated Fluorescent Probe for Monitoring Mitochondrial Redox Reactions

Shota Hirosawa, a Satoshi Arai b and Shinji Takeoka* a
aDepartment of Life Science and Medical Bioscience, Graduate School of Advanced Science and Engineering, Waseda University (TWIns), Tokyo 162-8480, Japan.
bConsolidated Research Institute for Advanced Science and Medical Care, Waseda University, Tokyo 162-0041, Japan.

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1. **General experimental information**

All reagents and solvents were purchased from Kanto Chemical Industry Co. (Japan), unless otherwise mentioned. The NMR spectra were obtained using samples prepared in a deuterated solvent and were recorded using an OXFORD NMR AS400 (400 MHz) spectrometer. The electrospray ionization mass spectra (ESI-MS) were recorded using a LCQ Fleet (Thermo Fisher Scientific) mass spectrometer. The FT/IR spectra were recorded using a JASCO FT/IR-4100 Fourier transform infrared spectrometer. Plastic sheets coated with 0.2 mm silica gel 60 (Merck Co.) were used for thin-layer chromatography (TLC). Fluorescent spectrum was obtained by using a RF-5300PC spectrofluorophotometer (Shimadzu Co., Japan). ESR spectrum was obtained by using JES-PX1060 (JOEL Ltd., Japan). Confocal laser scanning microscopic (CLSM) images were obtained by using FV1000 (Olympus Co., Japan).

2. **Synthesis of MitoRP**

**Scheme 1. Synthesis of MitoRP**

Reagents and conditions; 4-amino TEMPO, DMT-MM, methanol, RT, 12h.

Coumarin 343 (28.5 mg, 0.100 mmol, Sigma-Aldrich, USA) was added to a solution of 4-amino TEMPO (51.2 mg, 0.300 mmol, Tokyo Chemical Industry, Co., Japan) in methanol (20 mL) and the mixture was kept stirring for 15 min. DMT-MM 15.7 % hydrate (98.5 mg, 0.300 mmol, Kokusan Chemical Co., Japan) was subsequently added and the reaction mixture was stirred for 12 h at room temperature, protecting from light. The volatiles were then evaporated *in vacuo*. Water (30 mL) was added to the slurry and precipitation was collected by filtration after washing with water (2×10 mL). The crude mixture was chromatographed over ODS silica gel with methanol as a moving phase. The obtained yellow solution was lyophilized from tert-BuOH to yield MitoRP as a yellow powder (20.0 mg, 0.045 mmol, 45 %) having RF 0.4 (Methanol, RP-TLC).$^1$H-NMR (δ ppm in DMSO-δ6, chemically reduced by phenylhydrazine) 8.41(1H, s), 8.38(1H, d, 7.6 Hz), 7.20(1H, s), 4.08(1H, m), 2.64(4H, m), 1.80(4H, m), 1.74(2H, m), 1.30(2H, m), 1.03(12H, m). ESI-MS *m/z* : 461.50 [M + Na]$^+$, 899.25[2M + Na]$^+$ (*exact mass* = 438.24). IR (KBr, ν / cm$^{-1}$) 3446, 2939, 1689, 1618, 1585, 1560, 1522, 1442, 1367, 1309, 1286, 1240, 1213, 1176, 1076, 793, 623.
Fig. S1 NMR spectrum in CDCl₃. The nitroxide radical was chemically reduced with phenylhydrazine. *peaks from phenylhydrazine.

Fig. S2 COSY NMR spectrum in CDCl₃. The nitroxide radical was chemically reduced with phenylhydrazine.
Fig. S3 MS spectrum.

Fig. S4 IR spectrum.
3. **Excitation and emission spectra of MitoRP**

Excitation and emission spectra of MitoRP were measured in Hepes buffer containing 1% DMF as a co-solvent.

![Excitation and emission spectra](image)

**Fig. S5** Excitation spectrum (gray) and emission spectrum (black).

4. **Influence of pH, ionic strength, temperature, H$_2$O$_2$ and O$_2^·$ on MitoRP**

**pH, ionic strength and temperature**

The buffer pH ranging from 5.8 to 7.8 was controlled by mixing of NaH$_2$PO$_4$ and Na$_2$HPO$_3$, changing the ratio of their amount. The ionic strength was controlled by KCl, ranging from 0 to 200 mM as a final concentration. MitoRP was dissolved in these buffers with 1% DMF as a cosolvent and incubated for 30 min (final concentrations; MitoRP: 10 µM, phosphate: 100 mM, DMF: 1%) at RT or 37°C on black 96-well plate. Then the fluorescence intensity was measured with microplate reader using a $\lambda_{ex}/\lambda_{em}=485\pm20$ nm/528±20 nm filter set. The influences of pH, ionic strength and temperature on the fluorescent intensity of MitoRP are summarized in Fig. S6. These results show the MitoRP is not affected by these parameters.
**Fig. S6** Stability of MitoRP to the physiological conditions. Normalized fluorescent intensity vs. pH (a) and ionic strength (b) were shown. Temperature was also controlled to 25 °C (black) and 37 °C (red). Data = mean ± s.d. (n = 3)

H₂O₂ and O₂⁻

To study the influence of H₂O₂ and O₂⁻ on reduced MitoRP, the time course of fluorescent intensity was performed in presence of 1 mM ascorbic acid and these reactive oxygen species. H₂O₂ was added to the solution directly. O₂⁻ was generated by xanthine in presence of 100 mU/mL xanthine oxidase (Sigma-Aldrich, USA). The result is shown in Fig. S7. We found the detectable but small decreases in fluorescent intensity of MitoRP. Therefore, these oxygen species also do not affect the fluorophore scaffold.

**Fig. S7** The influence of H₂O₂ and O₂⁻ on reduced MitoRP. Concentrated H₂O₂ solution or xanthine solution were added to 100 μM MitoRP in presence of ascorbic acid. Arrows indicate the addition of H₂O₂ or xanthine with their final concentrations.
5. **UV-visible spectrum and fluorescence excitation spectra during the reduction of MitoRP**

Time course of the UV-visible absorption and fluorescence excitation spectra was measured during the reduction of the TEMPO moiety.

![Graph showing UV-visible spectra](image)

**Fig. S8** Time courses of UV-visible and fluorescence excitation spectra during the reduction of 100 µM MitoRP. Reduction was conducted with 1 mM ascorbic acid in 20 mM Hepes buffer (pH 7.4).

6. **Cell culture**

For all *in vitro* experiments, human carcinoma cell line HeLa was cultivated in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO, USA) supplemented with 10 % fetal bovine serum (Nichirei Bioscience Inc., Japan), 500 units/ml penicillin and 500 µg/ml streptomycin (used penicillin-streptomycin cocktail, GIBCO, USA). The cells were maintained in a humidified incubator at 37 °C under 5 % CO₂ atmosphere.

7. **Cytotoxicity of MitoRP to the cultured cell**

To study the cytotoxicity, we used a Cell Counting Kit-8 solution (Dojindo Laboratories, Japan) which works similarly to conventional MTT assay. HeLa cells were seeded on the 96-well plate with a density of 5 x 10⁴ cells/mL (100 µL/well) and cultivate for 12 hr in DMEM at 37 °C under 5 % CO₂ atmosphere. The 30 µL of concentrated MitoRP was added to each well and incubated for 2 h. Then the 30 µL of 3 fold diluted Cell Counting Kit-8 solution was added and incubated for additional 1 h. The absorbance value at 450 nm of the each well was measured by using a microplate reader. We investigated the cell viability at the concentration of MitoRP ranging from 1.6 nM to 10 µM (Fig. S9). We found that there was no remarkable cytotoxicity under these concentrations, but relative high cytotoxicity was observed at 10 µM MitoRP. Therefore, we determined the concentration for cell staining as 5 µM at which no cytotoxicity was found.
Fig. S9 Cytotoxicity of MitoRP to the human carcinoma cell line HeLa. Cell viability was measured by WST-8.

8. Membrane permeability of MitoRP

We studied the transport pathway of MitoRP through the plasma membrane. Generally, passive diffusion is an energy-independent process and is observed even at low temperatures, but active transportation like endocytosis is drastically inhibited at low temperatures.\textsuperscript{S2, S3} We therefore investigated the cellular uptake of MitoRP to the cultured cell at 4 \textdegree C and 37 \textdegree C.

HeLa cells were seeded to the 35 mm glass bottom dish (5 \times 10^4 cells/mL, 2 mL/dish) and cultivated for 1 day at 37 \textdegree C under 5 \% CO\textsubscript{2} atmosphere. For the interaction at 4 \textdegree C, the cells were washed by room-temperature DMEM and subsequently washed by cold DMEM. For the interaction at 37 \textdegree C, cells were washed by pre-warmed DMEM and then stained with 5 \mu M MitoRP for 1 hr at respective temperature. Nucleolus was also stained by 1 \mu M DAPI to observe the nuclear morphology. After washing by room-temperature DMEM, cells were imaged by CLSM in the 1 mL DMEM without phenol red. MitoRP was excited with 440 nm laser and light ranging from 460 nm to 560 nm was collected. DAPI was excited with 405 nm laser and light ranging from 415 nm to 455 nm was collected. The CLSM images with different depth were averaged (Fig. S10a) and assessed semi-quantitatively by Image J software. To measure the fluorescent intensity, the region of interest (ROI) was selected by drawing the cell morphology and the averaged fluorescent intensity was calculated after background correction by using the region in the absence of the cell (Fig. S10b). This result shows that the nearly same fluorescence intensities were appeared at both temperatures. Therefore, MitoRP permeated into the cell membrane by the passive diffusion process. The bright field images and nuclear morphology indicated that cells were viable.
Fig. S10 Cellular uptake of MitoRP at 4 °C and 37 °C. (a) The CLSM images were acquired after incubation in DMEM containing 5 µM MitoRP for 1h. Data of over five z-images (bottom to top of the cell) were averaged. (b) Semi-quantitative analysis of the fluorescent intensity calculated by Image J software. data = mean ± standard deviation. Scale bar = 100 µm.

9. Influence of NADH in the experiment of the interaction between MitoRP and mitochondria

NADH is a fluorescent compound with maximum excitation/emission wavelength at 405 nm/480 nm. Additionally, NADH is capable of working as a reductant. Therefore we studied the influence of background fluorescence emitted by NADH itself and the reduction of MitoRP by NADH as a reductant. In order to clarify the influence of NADH as background fluorescence, fluorescent intensity was measured as the same manner described in main text but not the MitoRP and mitochondria (condition: 1 mM NADH in the 80 µL of 20 mM Hepes buffer containing 120 mM KCl and 1 mM EDTA, λ<sub>ex</sub>/λ<sub>em</sub>=485±20 nm/528±20 nm). Reduction of MitoRP by NADH itself was investigated by associating MitoRP with only NADH (condition: 10 µM MitoRP, 1 mM NADH in the 80 µL of 20 mM Hepes buffer containing 120 mM KCl and 1 mM EDTA, λ<sub>ex</sub>/λ<sub>em</sub>=485±20 nm/528±20 nm). The fluorescent intensity was normalized to the solution containing only MitoRP. As shown in Fig.S11, we demonstrated that the fluorescence of NADH is negligible under these conditions. In the interaction between MitoRP and NADH, we observed the slight increase in the fluorescent intensity of MitoRP due to the reduction by NADH. However the changes are comparatively lower than that in presence of mitochondria, suggesting that MitoRP is effectively reduced by the complex I-mediated electron pathway.
**Fig. S11** Control experiment for the interaction between MitoRP and mitochondria. Background fluorescence emitted by NADH (left column) and the fluorescent increase in MitoRP due to the reduction by NADH (middle column) are shown. The fluorescent intensity in the interaction among the MitoRP, mitochondria and NADH (right column) is also shown. Data = mean ± s.d.

9. **References**

