Supplementary information for

Design and Synthesis of Deep Tissue Penetrating Near-Infrared Two-Photon Fluorescence Probe for Specific Detection of NQO1

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Experimental section

Materials and reagents

Dimethylacrylic acid, acetonitrile, ethyl acetate, hexanes, acetic acid (AcOH), 1-(2-hydroxyphenyl) ethenone, sulfuric acid, methylene dichloride, toluene, piperidine, triethylamine (TEA), EDC·HCl, 4-imethylaminopyridine (DMAP) and methane sulfonic acid (MsOH) were purchased from Energy Chemical (Shanghai, China). Aqueous *N*-bromosuccinimide (NBS), *N*-hydroxysuccinimidyl, (NHS), nicotinamide adenine dinucleotide disodium salt hydrate (NADH) and bovine serum albumin (BSA) were obtained from J&K Scientific Ltd. (Beijing, China). NAD(P)H:quinone oxidoreductase 1 (NQO1, Human, 100 μg, 1 mg/mL, ab87692) was purchased from Abcam (Boston, USA). Acetylcholinesterase (AChE, bovine liver, #C30, 10000-40000 units/mg protein), carboxylesterase (CE, Porcine liver, #E2884, ≥150 units/mg protein) and Carboxylesterase 2 (CES2, human, #E0412, ≥500 units/mg protein) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Malononitrile, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide and 4-hydroxybenzaldehyde were obtained from Aladdin Industrial Corporation (Shanghai, China). Sodium, Na_2SO_4 and Na_2CO_3 were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Dicoumarol was acquired from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was acquired from Serva Electrophoresis GmbH (Heidelberg, Germany). All siRNA were purchased from GenePharma (Shanghai, China). High-glucose Dulbecco's modified eagle medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), Pierce™ BCA protein assay kit and lipofectamine 3000 were purchased from Thermo Fisher Scientific Co. Ltd (MA, USA). Antibodies used in this study were NQO1 (#3187S), α-Tubulin (DM1A) (#3873S), anti-rabbit/mouse IgG and HRP-linked antibodies (#7074S/7076S), and were obtained from Cell Signaling Technology, Inc (Boston, USA). HeLa (human cervical carcinoma) cells and LoVo (human colorectal cancer) cells used in this paper were purchased from Kaiji Biotechnology Co., Ltd (Jiangsu, China). Female BALB/c nude mice (weight: 16-19 g, age: 4-5 weeks) were acquired from Vital River Laboratory

Animal Technology Co., Ltd (Beijing, China).

Synthesis and Characterization of DCM-NQO1

The structure of the Q_3PA [3-Methyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dienyl) butanoic acid] and DCM-OH were shown in the Scheme S1A and S1B, and the synthesis route of DCM-OH were shown in Scheme S1C following the previous methods.[1,2] **DCM-NQO1** was synthesized by the esterification reaction of Q_3PA and DCM-OH. Briefly, Q_3PA (127 mg, 0.5 mmol) was dissolved in CH_2Cl_2, and was slowly added into a solution of CH_2Cl_2 (40 mL) containing EDC·HCl (210 mg, 1.35 mmol), DCM-OH (213 mg, 0.68 mmol) and DMAP (165 mg, 1.35 mmol) at 0°C for 0.5 h. The mixed liquid was warmed to room temperature and then stirred for 12 h. After washed with 1 M aqueous HCl, anhydrous sodium sulfate was used to dry the organic layer. After the solvent was concentrated *in vacuo*, the residue was purified using silica gel chromatography with CH_2Cl_2/CH_3OH (v/v, 30:1) as the eluent, affording **DCM-NQO1** (164 mg, yield: 60%). ^1H and ^{13}C NMR spectra were performed on a Bruker Avance III HD 400 spectrometer (Bruker, Bremen, Germany). ESI-FTICR MS was recorded on a Bruker SolariX mass spectrometer equipped with a 9.4 T superconducting magnet (SolariX, Bruker, Bremen, Germany).

^1H, ^{13}C NMR and ESI-FTICR mass spectra of **DCM-NQO1** were shown in Fig. S1, S2 and S3 (ESI). ^1H NMR (400 MHz, $CDCl_3$): δ 8.92 (dd, J = 8.4, 1.4 Hz, 1H), 7.75 (m, 1H), 7.62-7.54 (m, 4H), 7.46 (m, 1H), 7.11-7.05 (m, 2H), 6.87 (s, 1H), 6.76 (d, J = 16.0 Hz, 1H), 3.28 (s, 2H), 2.18 (s, 3H), 2.01-1.87 (m, 6H), 1.54 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 190.93, 187.50, 171.18, 157.28, 152.88, 152.45, 152.18, 151.76, 142.91, 139.59, 138.90, 137.75, 134.84, 132.53, 129.17, 126.18, 126.00, 122.51, 119.09, 118.75, 117.97, 116.77, 115.70, 107.17, 63.38, 47.85, 38.61, 29.15, 14.55, 12.78, 12.28. ESI-FTICR MS spectrum: (Calcd. for $C_{34}H_{28}O_5N_2Cl^-$: *m/z* 579.16813 $[M+Cl]^-$; found: *m/z* 579.16951).

General Details for Fluorescence Measurements

Preparation of the stock solutions: 1 mg **DCM-NQO1** was dissolved in DMSO as stock solution (20 μM) for use. The fluorescence measurements were performed by taking appropriate amount of this stock solution.

Absorbance measurements: 1.5 mL of the stock solution of DCM-OH was fully mixed with 1.5 mL PBS buffer (pH = 7.4, 10 mM) and immediately measured at room temperature, 1.5 mL DMSO and 1.5 mL PBS buffer (pH = 7.4, 10 mM) were fully mixed, then measured under the same conditions as a blank solution. (TU-1900 UV-vis double-beam spectrometer (Purkinje General, China).

Fluorescence measurement: All reagents were mixed in 10 mM PBS buffer (pH = 7.4, 800 μM NADH, 0.02% BSA) at room temperature. The stock solution of **DCM-NQO1** (500 μL) was added into 500 μL of buffer solutions at varies concentrations of NQO1 in a quartz cuvette (final concentration = 2.5-80 μg/mL). After incubation at 37°C for 2.5 h, the fluorescence spectra were recorded by F-4600 fluorescence spectrophotometer (Hitachi, Tokyo Japan), using the following parameters: excitation wavelength = 560 nm, excitation/emission slit width = 10 nm， photomultiplier tube voltage = 750 V and scan speed = 1200 nm/min.

Preparation of PBS solutions of various pH values

To obtain the PBS solutions with various pH values, the PBS buffer (10 mM, pH = 7.4, purchased from Sinopharm Chemical Reagent Co., Ltd) were adjusted with 3 M NaOH or 3 M HCl to different pH values.

HPLC Analyses

High performance liquid chromatography (HPLC) analyses were performed to monitor the reaction process. Firstly, 200 μL **DCM-NQO1**(10 μM in PBS containing 50% DMSO), DCM-OH (10 μM in PBS containing 50% DMSO) and reaction system (after 10 μM **DCM-NQO1** reacted with 30 μg/mL NQO1 in PBS containing 50% DMSO) were diluted with 600 μL DMSO, and then analysed on a Shimadzu LC-40 XR preparative HPLC system (Shimadzu, Japan) for detection. The measurement parameters were set as follows. Column: Dikma Bio-Bond column (150 × 2.1 mm), the mobile phase A was H_2O, mobile phase B was acetonitrile. The HPLC separations were 23 min/sample and the gradient were as follows: (1) 0 min, 10% B; (2) 3 min, 10% B; (3) 3.01 min, 40% B; (4) 13 min, 100% B; (5) 18 min, 100% B; (6) 18.01 min, 10% B; (7) 23 min, 10% B. (All the changes were linear, flow rate: 0.3 mL/min, detection wavelength: 450 nm).

The Calculation of pK_a Value

The formula for pK_a is pK_a = pH + log [(F_{HB}- F) / (F - F_B^-)], where F_{HB}, F and F_B^- represent the fluorescence intensity of the absolute acid form, at a chosen pH, and the absolute base form, respectively. The fluorescence intensity was detected at 680 nm at different pH values.

Enzymatic Assays

Various concentrations of **DCM-NQO1** solution (1-100 μM in PBS containing 50% DMSO, 400 μM NADH and 0.01% BSA) were mixed with 5 μg/mL NQO1. During incubation at 37°C for 2.5 h, the fluorescence intensity with time was monitored. Then relative initial reaction velocity was calculated. By fitting to the Michaelis-Menten equation through linear regression, the kinetic parameters of enzymatic reaction were obtained.

Cell Culture and Cytotoxicity Assay

HeLa and LoVo cells were selected in this paper as NQO1-positive cell lines. In detail, cells were grown in T75 glass culture flask in high-glucose Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin at 37°C in a humidified 5% CO_2 incubator.

Before the cell experiments, we monitored the cytotoxicity of the **DCM-NQO1** by the standard MTT assay on a microplate reader (Molecular Devices SpectraMax i3 BioTek, USA).

siRNA Transfection Experiment and Western Blot Analysis

In this work, siRNA transfection experiments were carried out, and then western blot analysis were performed to determine whether the transfection was successful. Briefly, HeLa and LoVo cells were seeded in 6-well plates and were transfected with 80 nM siNQO1 (antisense-strand: 5'-CCGUACACAGAUACCUUGA-3') or scramble siRNA (a scramble sequence, antisense-strand: 5'-GAACUUCAGGGUCAGCUUG-3') using Lipofectamine 3000 according to the manufacturers' instructions. After cells were treated with siNQO1 and siScramble for 24 h and 36 h, the medium was discarded and cells were washed 3 times using PBS (pH 7.4). Then cells were collected and lysed with 1% NP40 buffer (with 1 mM phenyl methane sulfonyl fluoride) and incubated at 4°C

for 30 min. The lysate was then centrifuged (12,000 g, 20 min, 4°C) to remove insoluble substances. Protein concentrations of whole-cell lysates were quantified using the Pierce™ BCA protein assay kit. Then approximately 20 ug of protein were electrophoresed in SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membrane was incubated with various primary antibodies (NQO1: 1:1000 and tubulin 1:2000 dilution) overnight at 4°C after blocking with 2.5% bovine albumin, and proteins were visualized by enhanced chemiluminescence detection after incubation with the appropriate secondary antibodies.

One or Two-Photon Fluorescence Imaging

One-Photon fluorescence images were recorded on an FV 1200-IX83 confocal laser scanning microscope (PerkinElmer, USA) with 559 nm excitation as well as 650-750 nm emission. Two-photon fluorescence images were detected on an A1R MP confocal laser scanning microscope (Nikon, Japan) with 800 nm femtosecond-pulsed wavelength excitation and 570-620 nm emission. During the confocal experiment, all parameters were remained constant.

Fluorescence Imaging in Nude Mice

All animal care and experimental programs were approved by the National Center for Nanoscience and Technology of China, Chinese Academy of Sciences, and accordance with the Animal Management Rules of the Ministry of Health of the People's Republic of China. Female BALB/c nude mice (age: 4-5 weeks, weight: 16-19 g) were obtained from Vital River Laboratory Animal Technology Co., Ltd (Beijing, China), and raised in SPF animal rooms. HeLa-xenografted tumor-bearing nude mice were used in the study. Detailly, HeLa cells suspension were collected (1×10^7 cells in 100 μL PBS) and orthotopically injected into the armpit of nude mice. *In vivo* imaging was carried out when the tumors grew to approximately 1 cm^3. **DCM-NQO1** was injected *in situ* into the tumor tissue and detected half an hour later by the IVIS *in vivo* imaging system (PerkinElmer, USA) with the excitation wavelength of 560 nm. In the control group, the inhibitor was injected *in situ* first and the **DCM-NQO1** was injected after 1 h, the following operation procedure was consistent with the previous one.

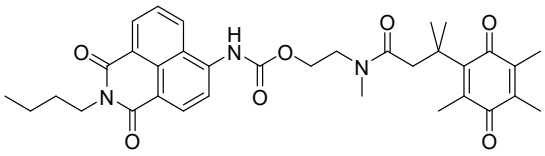
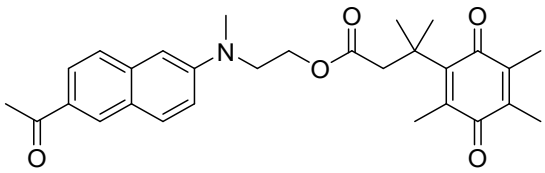
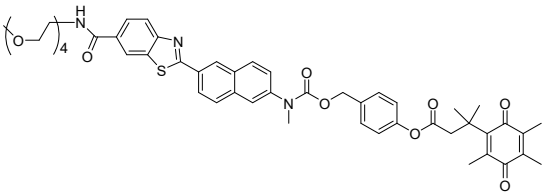
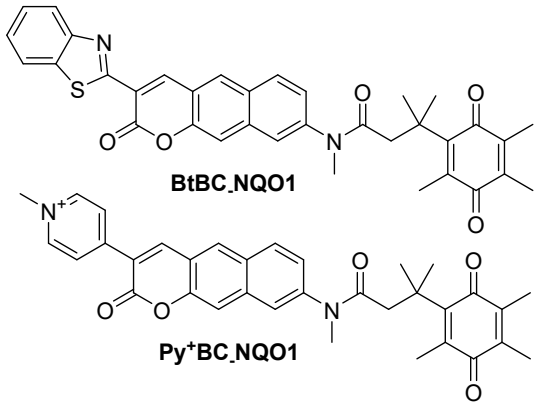
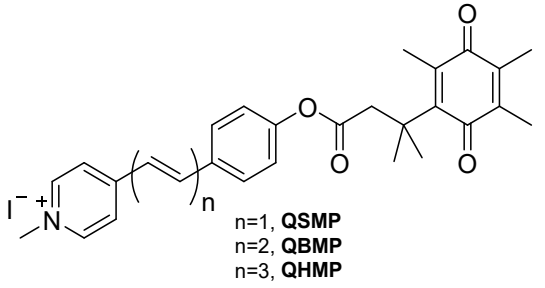
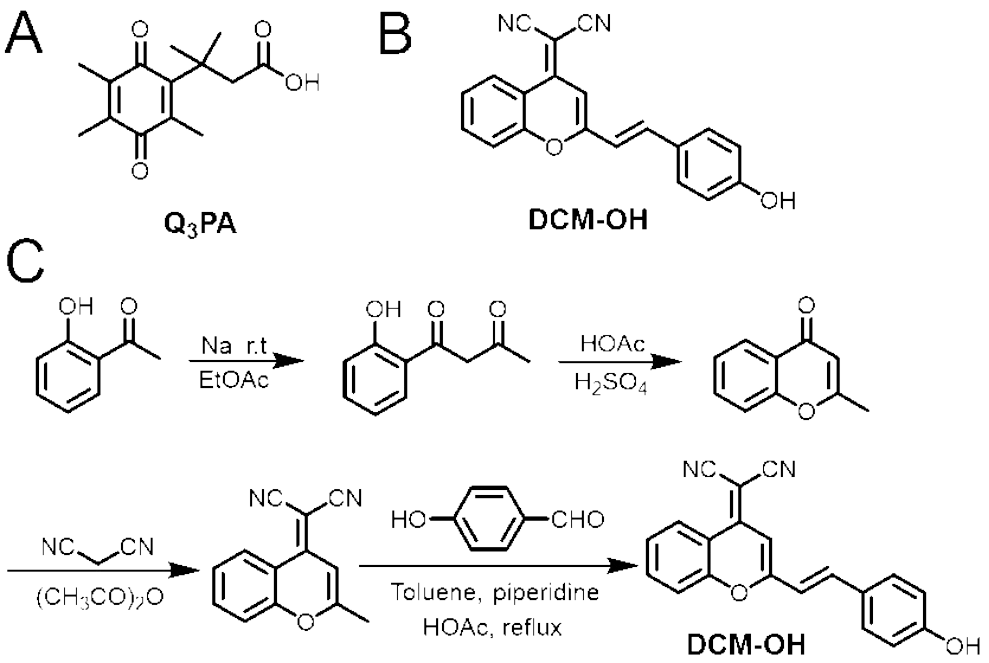
	Turn-on 1st Two-photon $\lambda_{ex/em}$ = 370/470 nm	R. L. McCarley and co-workers. *J. Am. Chem. Soc.,* **2013**, *135*, 309-314.
	Turn-on 2nd Two-photon $\lambda_{ex/em}$ = 400/520 nm	L. Cui and co-workers. *Chem. Commun.,* **2017**, *53*, 525-528.
	3rd Ratiometric, Two-photon $\lambda_{ex/em}$ = 365/461&540 nm	H.W. Kim and co-workers. *Asian J. Org. Chem.,* **2019**, *8*,1707-1712.
 **BtBC_NQO1** **Py$^+$BC_NQO1**	4th **BtBC_NQO1**: Turn-on, Confocal & Two-photon $\lambda_{ex/em}$ = 470/535 nm; **Py+BC_NQO1**: Ratiometric, Confocal & Two-photon $\lambda_{ex/em}$ = 405/555 & 650 nm	K. H. Ahn and co-workers. *Sens. Actuators. B.,* **2021**, *330*,129277-129286.
 n=1, **QSMP** n=2, **QBMP** n=3, **QHMP**	5th Ratiometric, Confocal & Two-photon $\lambda_{ex/em}$ = 378/464, 402/564 nm	S. Zhang and co-workers. *Anal. Chem.,* **2021**, *93*, 2385-2393.

Table S1. Reported Two-photon NQO1 fluorescence probes.



Scheme S1. Structures of trimethyl-locked quinone propionate (Q_3PA) (A) and DCM-OH (B). (C) Synthetic route of and DCM-OH.

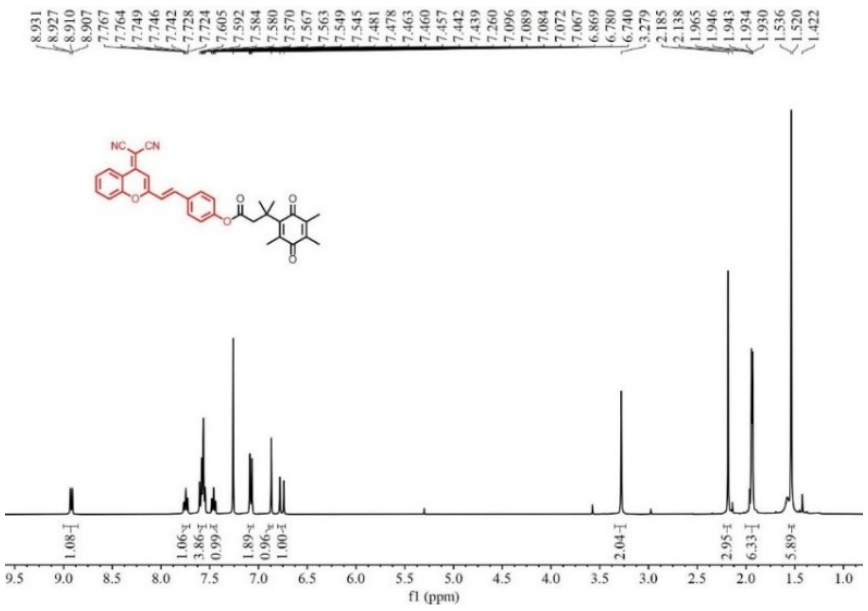


Fig. S1 ^1H NMR spectrum of **DCM-NQO1**. (400 MHz, $CDCl_3$, 298 K).

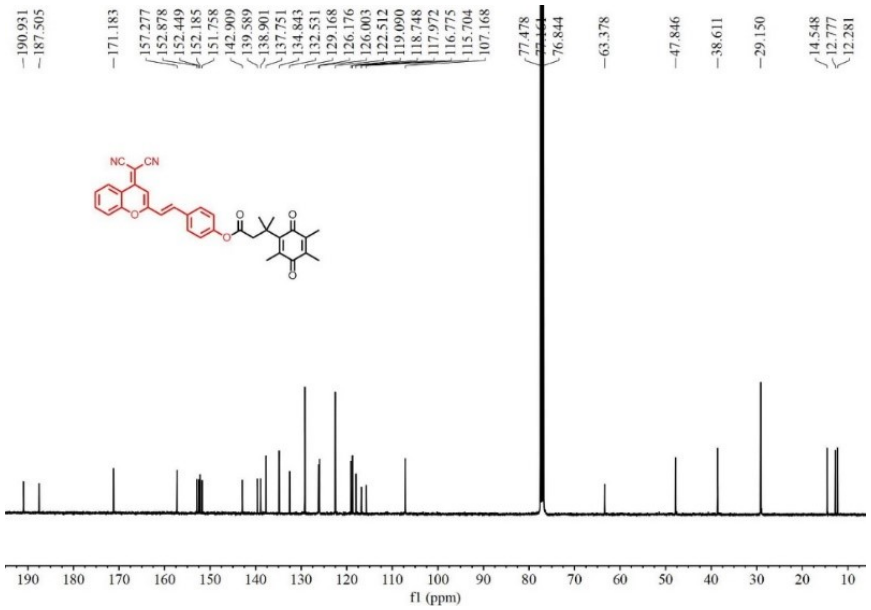


Fig. S2 ^{13}C NMR spectrum of **DCM-NQO1**. (100 MHz, $CDCl_3$, 298 K).

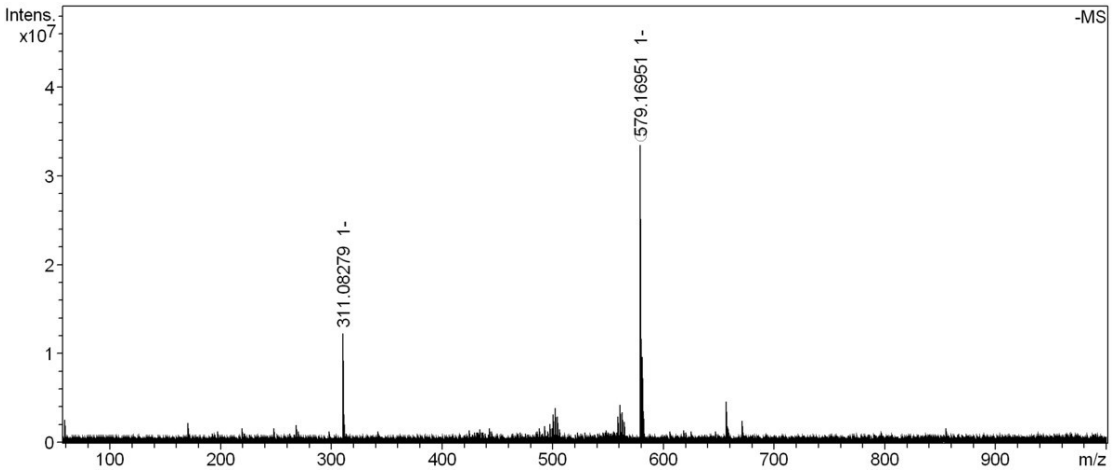


Fig. S3 ESI-FTICR MS spectrum of **DCM-NQO1**.

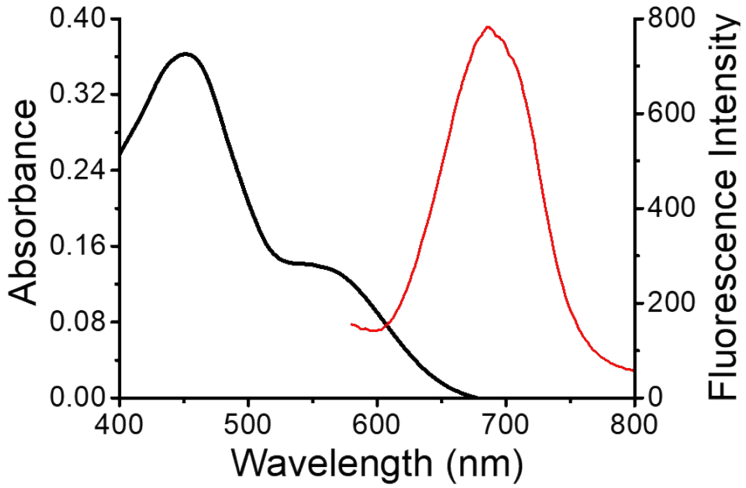


Fig. S4 UV-vis absorption spectrum (black line) and fluorescence spectrum (red line) of DCM-OH (10 μM).

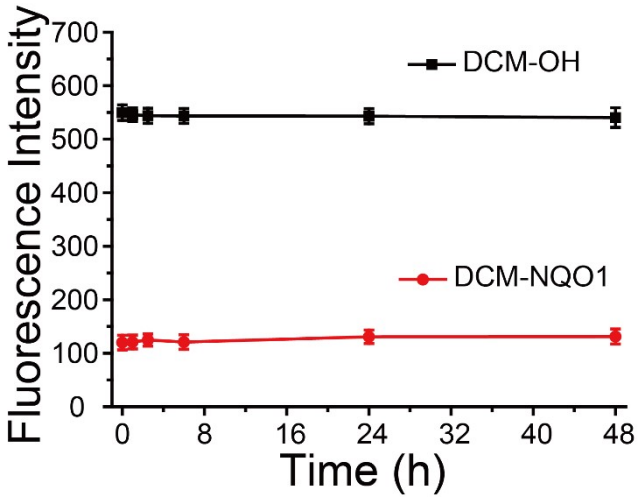


Fig. S5 Stability analyses of **DCM-NQO1** and DCM-OH by monitoring its fluorescence changed for different time (10 μM, 0-48 h). The results were expressed as the mean ± SD (n = 3), $\lambda_{ex}/_{em}$ = 560/680 nm.

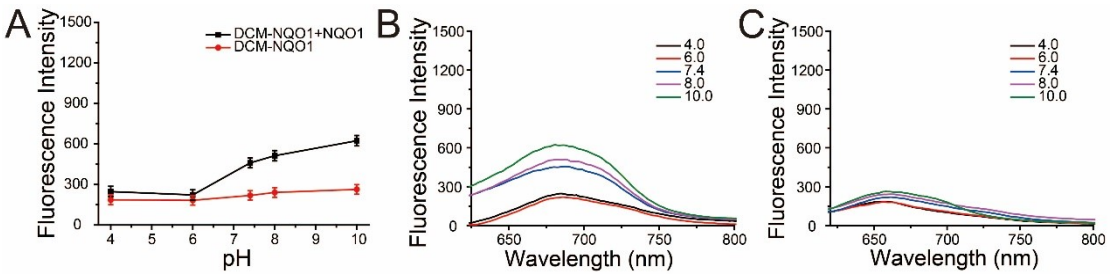


Fig. S6 Effects of pH on the fluorescence intensity of reaction solution (**DCM-NQO1**+NQO1) and **DCM-NQO1**. The measurements were performed for 2.5 h in PBS buffer containing 50% DMSO. (10 mM, pH = 7.4). (B) Fluorescence intensity of **DCM-NQO1**+NQO1 in pH range from 4.0-10.0. (C) Fluorescence intensity of **DCM-NQO1** in pH range from 4.0-10.0. λ_{ex} = 560 nm.

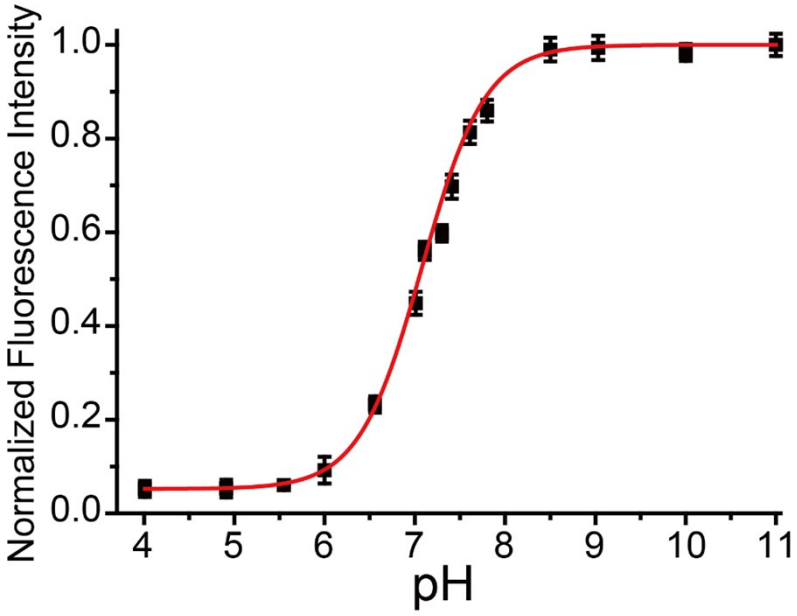


Fig. S7 Plot between normalized fluorescence intensity of DCM-OH (10 μM) in the PBS solution and different pH values. The results were expressed as the mean ± SD (n = 3), $\lambda_{ex}/_{em}$ = 560/680 nm.

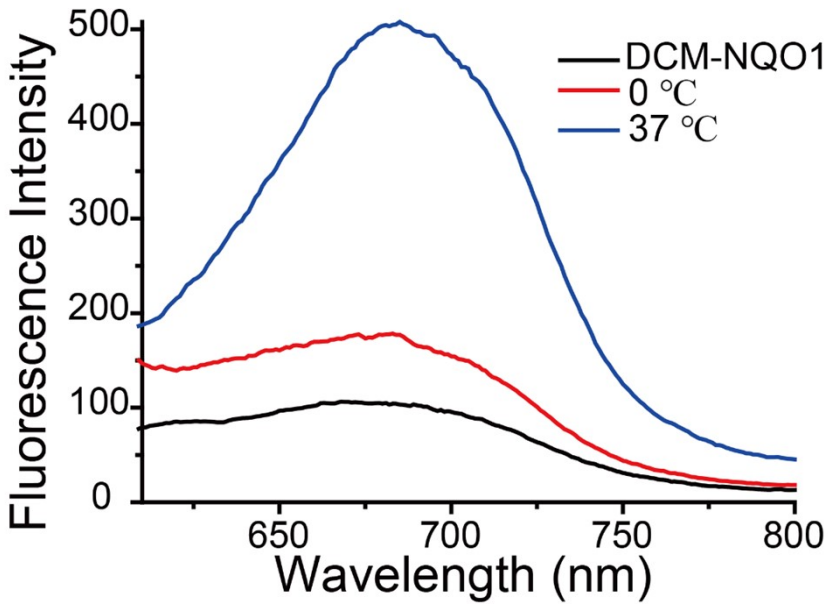


Fig. S8 Effects of temperature on the fluorescence intensity of **DCM-NQO1** and reaction solution. The measurements were performed for 2.5 h in PBS buffer (10 mM, pH = 7.4), λ_{ex} = 560 nm.

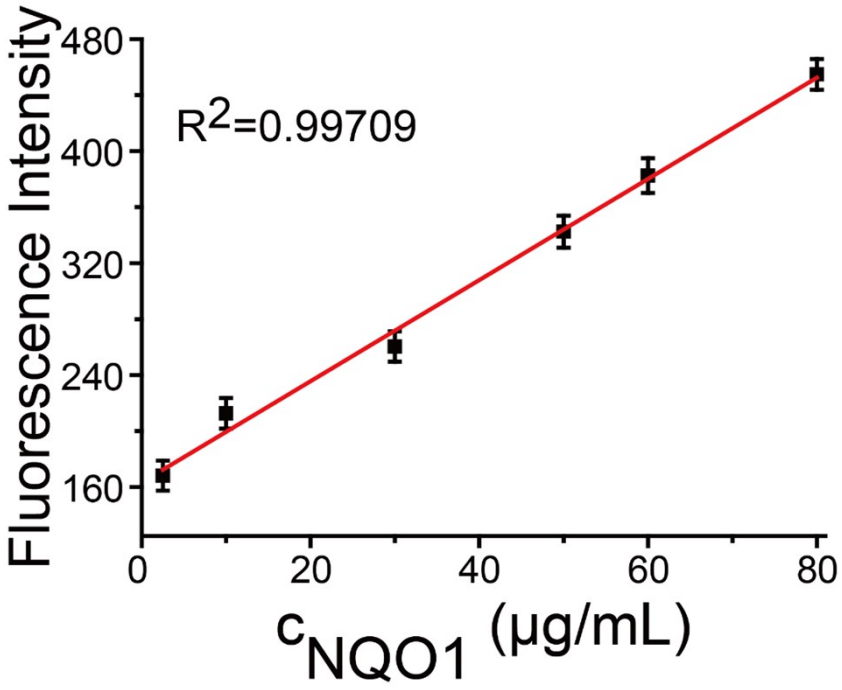


Fig. S9 The linear curve of fluorescence intensity of **DCM-NQO1** against various NQO1 concentrations. ($\lambda_{ex}/_{em}$ = 560/680 nm).

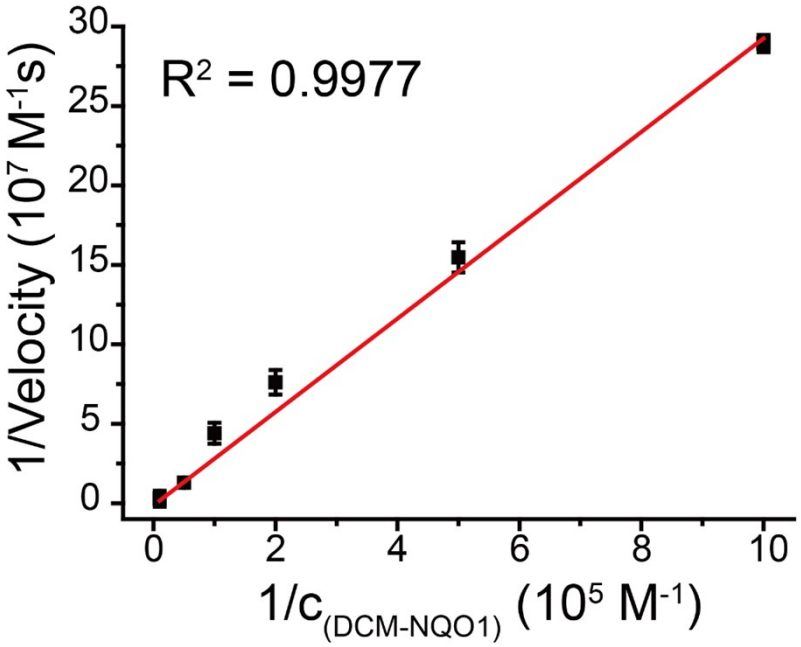


Fig. S10 The Lineweaver-Burk plot for the enzyme-catalyzed reaction of NQO1 (10 μg/mL) with **DCM-NQO1** (1-100 μM) in PBS (pH = 7.4, 10 mM) containing 50% DMSO at 37°C. ($1/V = (K_m/V_{max})/[S] + 1/V_{max}$). where V is initial velocity, Vmax is final velocity, [S] is **DCM-NQO1** concentration. The results were expressed as the mean ± SD (n = 3), $\lambda_{ex}/_{em}$ = 560/680 nm.

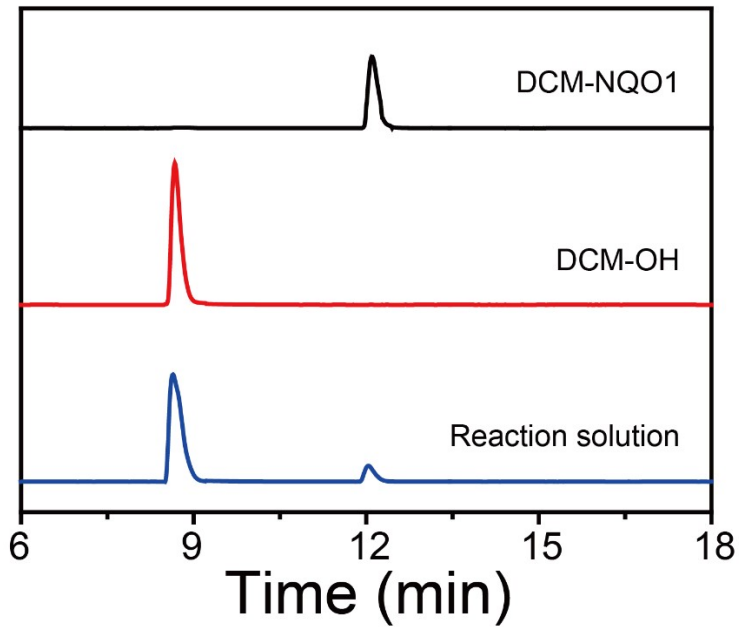


Fig. S11 Chromatograms of **DCM-NQO1** (2.5 μM, black line), DCM-OH (2.5 μM, red line) and reaction system (blue line). gradient: (1) 0 min, 10% B; (2) 3 min, 10% B; (3) 3.01 min, 40% B; (4) 13 min, 100% B; (5) 18 min, 100% B; (6) 18.01 min, 10% B; (7) 23 min, 10% B. (A: H_2O, B:

acetonitrile, flow rate: 0.3 mL min^{-1}, detection wavelength: 450 nm).

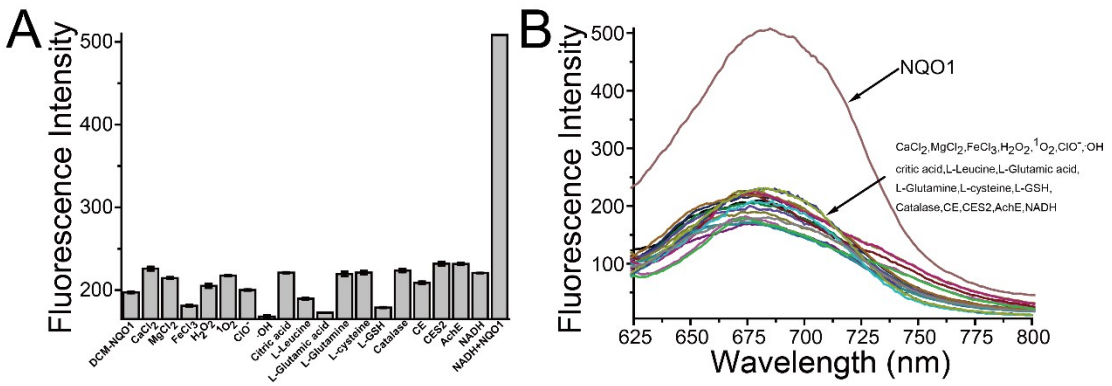


Fig. S12 Fluorescence responses (A) and fluorescence spectra (B) of **DCM-NQO1** (10 μM) to various potential interfering species. $CaCl_2$ (100 μM), $MgCl_2$ (100 μM), $FeCl_3$ (100 μM), H_2O_2 (100 μM), $^1O_2$, (100 μM), ClO^- (100 μM), ·OH (100 μM), citric acid (100 μM), L-Leucine (100 μM), L-Glutamic acid (100 μM), L-Glutamine (100 μM), L-cysteine (100 μM), L-GSH (100 μM), Catalase (100 mU/mL), CE (100 mU/mL), CES2 (100 mU/mL), AchE (100 mU/mL), NADH (400 μM) and NQO1 (30 μg/mL). The measurements were performed at 37°C for 2.5 h in PBS buffer (pH = 7.4) with $\lambda_{ex}/_{em}$ = 560/680 nm.

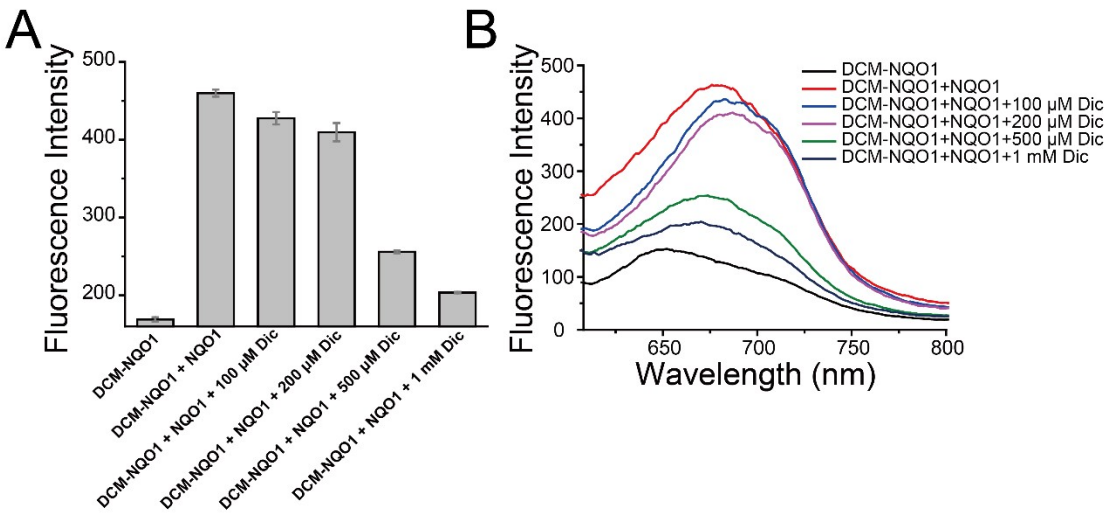


Fig. S13 The effect of Dic concentration on florescence intensity (A) and spectra (B) of **DCM-NQO1**. **DCM-NQO1**: 10 μM, NQO1: 30 μg/mL, Dic (100 μM, 200 μM, 500 μM, 1 mM). Reaction solution were incubated with Dic for 15 min, then treated with DCM-NQO1.The measurements were performed at 37°C for 2.5 h in PBS buffer (pH = 7.4) with $\lambda_{ex}/_{em}$ = 560/680 nm. The results were the mean ± standard deviation of three separate measurements.

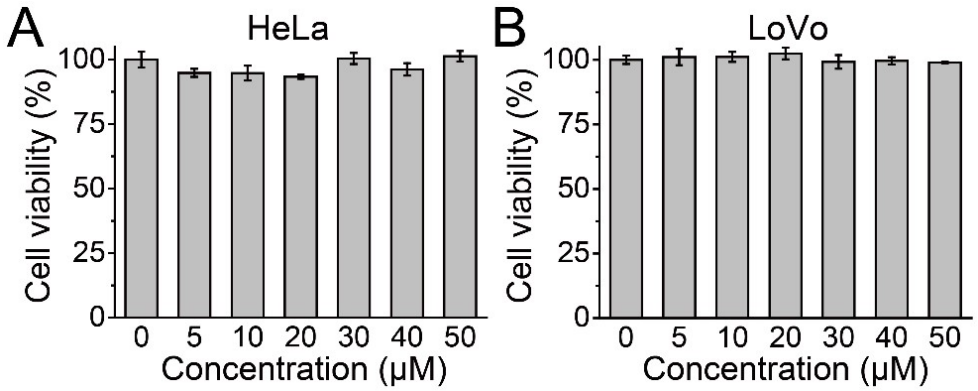


Fig. S14 The cell cytotoxicity of **DCM-NQO1**. Viability changes of HeLa cells (A) and LoVo cells (B) after 6 h of incubation with **DCM-NQO1** (5, 10, 20, 30, 40, 50 μM). The viability of cells without treatment was defined as 100%. The results were the mean ± standard deviation of three separate measurements.

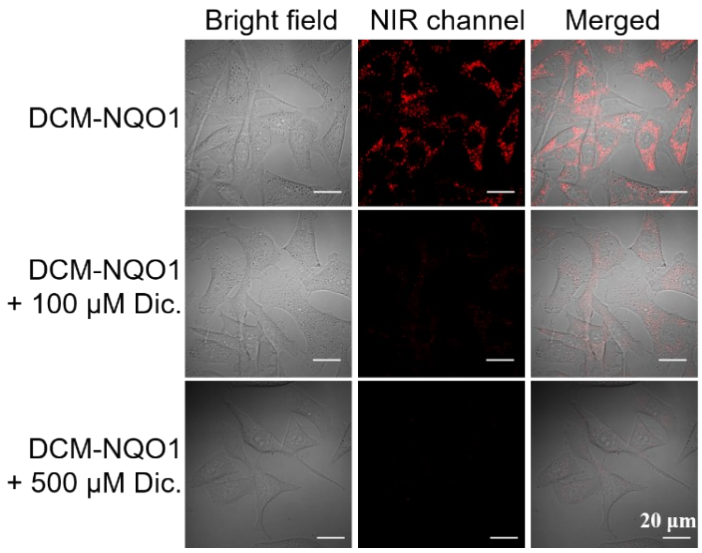


Fig. S15 One-photon confocal fluorescence images of **DCM-NQO1** in LoVo cells. The 1st row: LoVo cells incubated with **DCM-NQO1** (10 μM) for 30 min. The 2nd row: LoVo cells incubated with Dic (100 μM) for 4 h, then treated with **DCM-NQO1** (10 μM) for 30 min. The 3rd row: LoVo cells incubated with Dic (500 μM) for 4 h, then treated with **DCM-NQO1** (10 μM) for 30 min. (excited at 559 nm, collected from 650 to 750 nm; Scale bar: 20 μm).

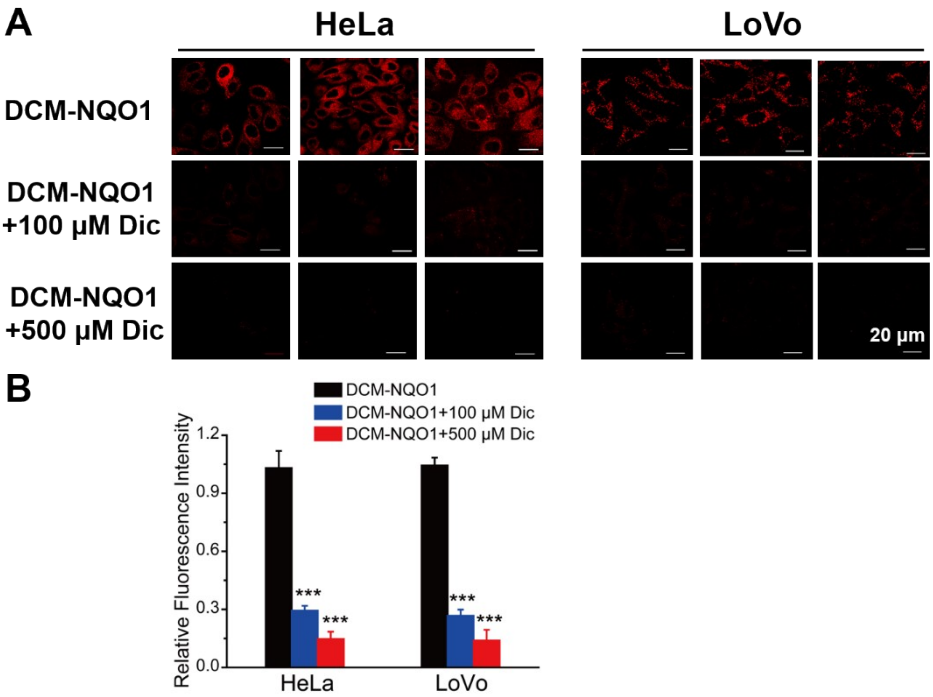


Fig. S16 (A) One-photon confocal fluorescence images of **DCM-NQO1** in HeLa and LoVo cells. The 1st row: Cells incubated with **DCM-NQO1** (10 μM) for 30 min. The 2nd row: Cells incubated

with Dic (100 μM) for 4 h, then treated with **DCM-NQO1** (10 μM) for 30 min. The 3rd row: Cells incubated with Dic (500 μM) for 4 h, then treated with **DCM-NQO1** (10 μM) for 30 min. (excited at 559 nm, collected from 650 to 750 nm; Scale bar: 20 μm). (B) Relative fluorescence intensity in pane (A). All data present mean ± SD. n = 3 repeats. *P* values were calculated using Student's t-test relative to **DCM-NQO1** and results were represented as ***$P < 0.001$.

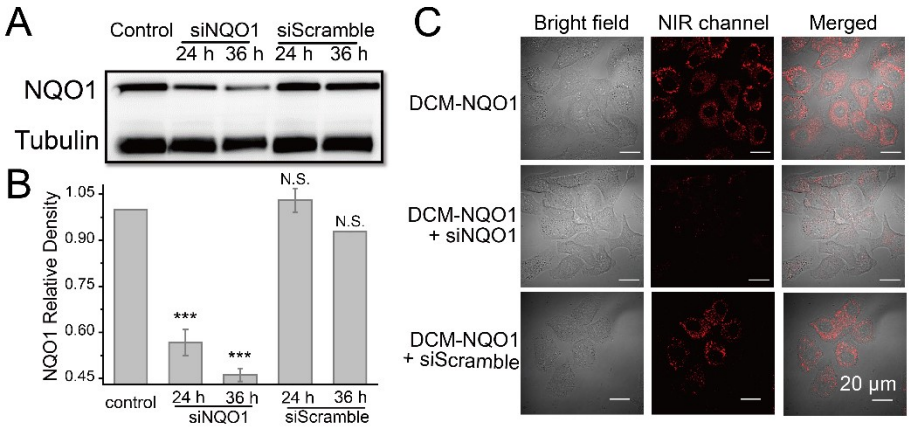


Fig. S17 (A) Western blot analysis of LoVo cells which were transfected with siNQO1 (80 nM) and siScramble (80 nM) using Lipofectamine 3000 (2.7 μg/mL). The above treated cells were harvested and lysed for western blot analysis after 24 or 36 h incubation. (B). NQO1 relative density of the corresponding images in (A) (the NQO1 relative density of control is defined to be 1.0). All data present mean ± SD. n = 3 repeats. *P* values were calculated using Student's *t*-test relative to the control and results were represented as N.S. non-significant and ***$P < 0.001$. (C) One-photon confocal fluorescence images of LoVo cells with 10 μM **DCM-NQO1**. The 1st row: LoVo cells stained with **DCM-NQO1** (10 μM) for 30 min. The 2nd row: LoVo cells incubated with siNQO1 (80 nM) for 24 h, then treated with **DCM-NQO1** (10 μM) for 30 min. The 3rd row: LoVo cells incubated with siScramble (80 nM) for 24 h, then treated with **DCM-NQO1** (10 μM) for 30 min. (excited at 559 nm, collected from 650 to 750 nm; Scale bar: 20 μm).

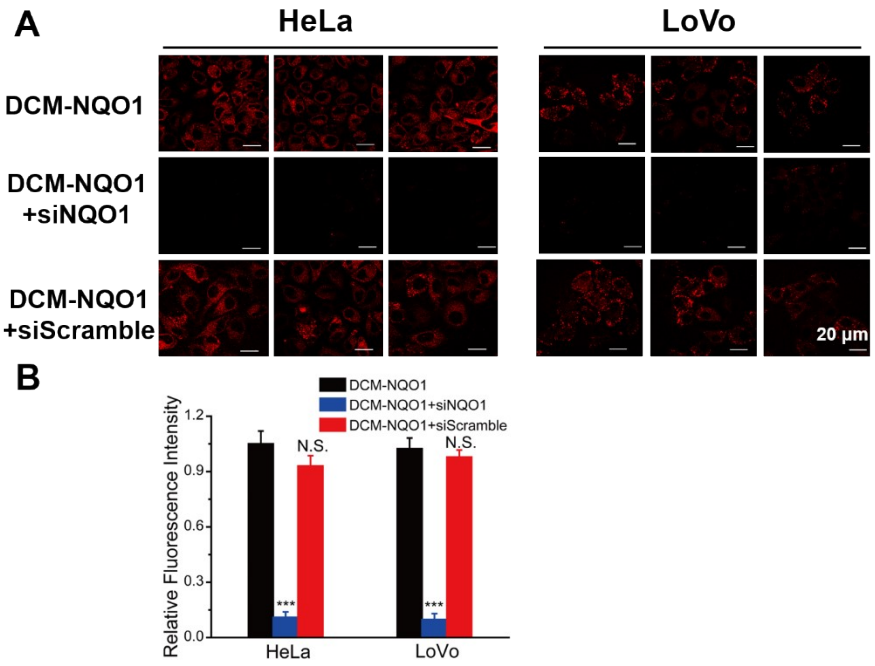


Fig. S18 (A) One-photon confocal fluorescence images of HeLa and LoVo cells with 10 μM **DCM-NQO1**. The 1st row: Cells stained with **DCM-NQO1** (10 μM) for 30 min. The 2nd row: Cells incubated with siNQO1 (80 nM) for 24 h, then treated with **DCM-NQO1** (10 μM) for 30 min. The 3rd row: Cells incubated with siScramble (80 nM) for 24 h, then treated with **DCM-NQO1** (10 μM) for 30 min. (excited at 559 nm, collected from 650 to 750 nm; Scale bar: 20 μm). (B) Relative fluorescence intensity in pane (A). All data present mean ± SD. n = 3 repeats. *P* values were calculated using Student's t-test relative to **DCM-NQO1** and results were represented as N.S. non-significant and ***$P < 0.001$.

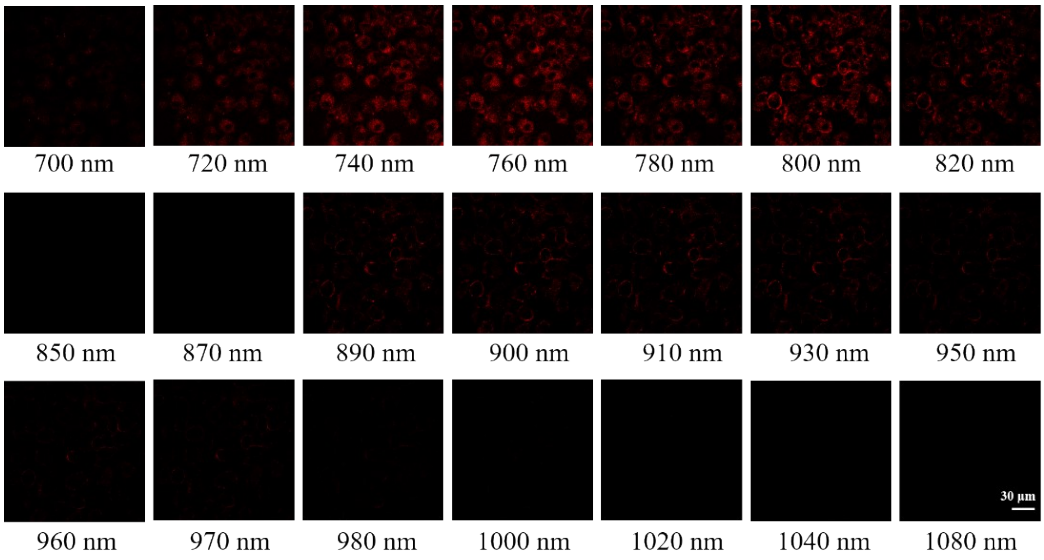


Fig. S19 Fluorescence response of **DCM-NQO1** (10 μM) towards various two-photon excitation wavelengths (from 700 nm to 1080 nm) excited by near-infrared pulsed laser after incubated in LoVo cells for 30 min. (collected from 570 to 620 nm; Scale bar: 30 μm).

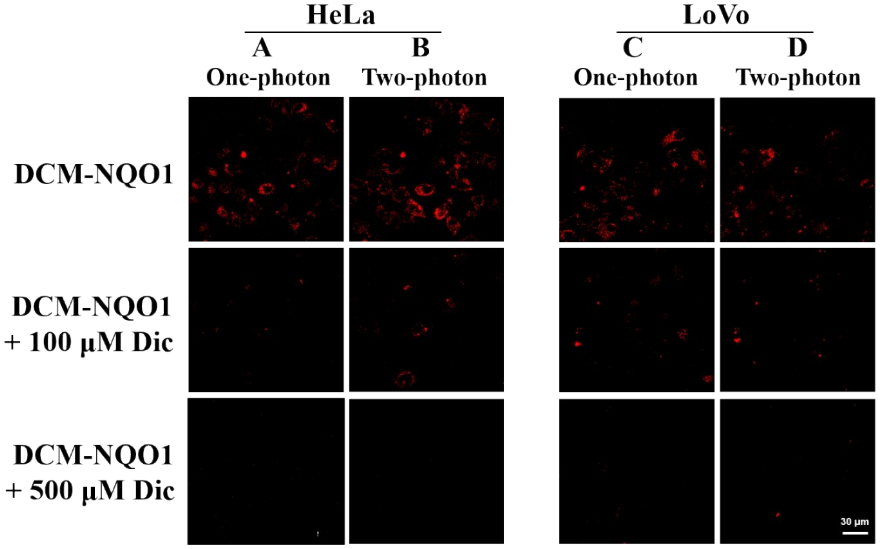


Fig. S20 One-photon (A, C) and two-photon (B, D) confocal fluorescence images of **DCM-NQO1** in HeLa and LoVo cells. The 1st row: the cells incubated with **DCM-NQO1** (10 μM) for 30 min. The 2nd row: the cells incubated with Dic (100 μM) for 4 h, then treated with **DCM-NQO1** (10 μM) for 30 min. The 3rd row: the cells incubated with Dic (500 μM) for 4 h, then treated with **DCM-NQO1** (10 μM) for 30 min. (excited at 561 nm, collected from 570 to 620 nm for one-photon confocal fluorescence images; excited at 800 nm, collected from 570 to 620 nm for two-photon confocal fluorescence images. Scale bar: 30 μm).

Reference

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