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Supporting Information

Two-photon fluorescence imaging of mitochondrial superoxide anion transport mediating liver ischemia-reperfusion injury in mice

Wen Zhang,† Di Su,† Ping Li,* Jiao Zhang, Jihong Liu, Hui Wang, Wei Zhang and Bo Tang*

College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Institutes of Biomedical Sciences, Shandong Normal University, Jinan 250014, People' s Republic of China

E-mail: tangb@sdnu.edu.cn or lip@sdnu.edu.cn

This section includes:

SUPPLEMENTAL METHODS S3

GENERAL MATERIALS AND METHODS S3

SYNTHESIS S3

Synthesis of compound 1 ..... *S3*

Synthesis of probe CST. *S4*

CELLS CULTURE. S4

FLOW CYTOMETRY. S5

STATISTICS. S5

SUPPLEMENTAL SCHEME AND FIGURES S5

Scheme S1. Synthesis of probe CST. *S5*

Figure S1. *The absorption spectra of CST.* *S6*

Figure S2. *Two-photon fluorescence spectra of CST.* *S6*

Figure S3. *The two-photon linear correlation of to $O_2^{\bullet-}$* *S6*

Figure S4*. pH effects to CST.* *S6*

Figure S5*. The one-photon fluorescence time course of CST.* *S7*

Figure S6*. Fluorescent reversibility of CST.* *S7*

Figure S7. *The MTT assay of CST.* *S7*

Figure S8. *Reversible TP fluorescence ratio imaging of $O_2^{\bullet-}$ in hepatocytes.* *S8*

Figure S9*. TP fluorescence ratio imaging of $O_2^{\bullet-}$ in hepatocyte treated with SOD.* *S8*

Figure S10. *The effects of mitochondria membrane potential to CST.* *S9*

Figure S11. *Two-photon imaging of $O_2^{\bullet-}$ within mitochondria and cytoplasm of IR cells.* *S9*

Figure S12. *Two-photon imaging of $O_2^{\bullet-}$ within mitochondria and cytoplasm of IR livers.* *S10*

Figure S13*. Western blot for SOD level and aconitase level.* *S11*

Figure S14*. MTT assay of IR cells.* *S11*

Figure S15. *VDAC channel transports mitochondrial $O_2^{\bullet-}$ to cytoplasm.* *S11*

Figure S16*. Application of CST to explore TOM function for $O_2^{\bullet-}$ transport in IR cells.* *S12*

Figure S17*. MTT assay of cells with different DIDS level.* *S12*

Figure S18*. Flow cytometry analyse.* *S12*

Figure S19*. The ^{1}HNMR data of CST.* *S13*

REFERENCES S13

SUPPLEMENTAL METHODS

General materials and methods

All chemicals for synthesis were purchased from Adamas Reagent, Ltd. (China) or Energy Chemical Ltd. (China), and analytical grade solvents were used without further purification. MTT, annexin V-FITC was purchased from Beyotime. MitoTracker Deep Red was from Invitrogen. Aconitase assay ELISA kit was purchased from Shanghai hi-tech-bioengineering company. ^{1}HNMR spectra were examined at 400 MHz using Bruker NMR spectrometers. The mass spectra were obtained using the Bruker maXis ultra-high resolution-TOF MS system. All one-photon fluorescence measurements were carried out at room temperature on an FLS-980 Edinburgh fluorescence spectrometer. The experiment process of fluorescent reversibility is as follows: First, we prepared 10 mM $O_2^{\bullet-}$ and 2 M ascorbic acid (Vc) as stock solution and 1.0 mL 10 μM CST buffer solution in a quartz cell. Second, after we examined CST fluorescence intensity, we added 1.0 μL $O_2^{\bullet-}$ stock solution into quartz cell and detected fluorescence. Third, we added 1.0 μL Vc stock solution into quartz cell and detected fluorescence. And then, run these steps again and the fluorescent reversibility of CST could be obtained. The two-photon excited fluorescence spectra were measured using a Zeiss LSM 880 NLO with lamda-mode. Fluorescent images were acquired on a Zeiss LSM 880 NLO microscope with an objective lens (20X water objective, N/A 1.0). The Ti: sapphire laser was used to excite the specimen at 800 nm, and the laser power was 80 mW. The laser irradiance is Class IV. The time resolution is 3.72 seconds per frame with 1024*1024 pixels, and we can acquire 18 pictures in one minute. Synthetic procedures and characterization of all compounds were shown in supplementary information. One-way ANOVA tests were applied as required to determine significance. Statistical significance is indicated as follows: *$P \leq 0.05$, **$P \leq 0.01$.

Reactive oxygen species preparation. Reactive oxygen species (ROS) was adapted from previous reports.[1] Hydrogen peroxide (H_2O_2), tert-butylhydroperoxide (TBHP), and hypochlorite (NaOCl) were delivered from 30%, 70%, and 10% aqueous solutions respectively. Hydroxyl radical (•OH) was generated by the reaction of 1 mM $FeCl_2$ with 200 μM H_2O_2. Nitric oxide (NO) was used from stock solution prepared by sodium nitroprusside. Singlet oxygen ($^1O_2$) was prepared by the NaClO-H_2O_2 system. Peroxynitrite ($ONOO^-$) was used from stock solution 10 mM in 0.3 M NaOH. $O_2^{\cdot-}$ was delivered from KO_2 in DMSO solution. Besides, the detection of ROS/RNS concentrations are determined as follows:

1. $O_2^{\bullet-}$: λ_{abs}=250 nm, ε=2682 L mol^{-1} cm^{-1}
2. $ONOO^-$: λ_{abs} =302 nm, ε=1670 L mol^{-1} cm^{-1}
3. ClO^-: λ_{abs} =209 nm, ε=350 L mol^{-1} cm^{-1}
4. H_2O_2: λ_{abs} =240 nm, ε=43.6 L mol^{-1} cm^{-1}
5. NO: Griess method
6. TBHP: Iodometry
7. •OH: Measure reaction with DMSO produces methane sulfinic acid (420 nm) by calorimetric assay.
8. $^1O_2$: Measure reaction with 1,3-diphenylisobenzofuran (410 nm) by calorimetric assay.

Notes: λ_{abs} is the absorption spectrum and ε is molar extinction coefficient.

Synthesis

Synthesis of compound 1. Caffeic acid (1.0 mM) was dissolved in 1,4-dioxane (10 mL), followed by addition of 440 μL thionyl chloride. Under the protection of nitrogen, the mixture was stirred and reflux for 6 hours. After cooling, the solvent was removed under reduced pressure, which can we got the crude

compound **1**.

Synthesis of probe CST. The compound **1** (1.0 mmol) and Safranine T (1.0 mmol) was dissolved in 1,4-dioxane (10 mL). Under the protection of nitrogen, the mixture was stirred and reflux for 6 hours. After the complete reaction, the solvent was removed under reduced pressure affording the crude product, which was purified by thin layer chromatography using dichloromethane/methanol (v/v 10:1) to afford the purple red solid as the probe **CST**. HRMS data, m/z calculated for [$C_{29}H_{25}N_4O_3^+$], 477.1921 found 477.1909. ^{1}HNMR (400 MHz, DMSO): δ 8.13 (s, 2H), 7.80 (d, 2H), 7.65 (s, 1H), 7.50 (s, 1H), 7.46 (s, 1H), 7.31 (d, 1H), 7.19 (m, 1H), 7.07 (m, 2H), 7.00 (s, 1H), 6.93 (d, 1H), 6.88 (d, 1H), 6.73 (d, 1H), 6.49 (s, 1H), 5.38 (s, 2H), 4.72 (s, 1H), 2.07 (s, 3H), 2.03 (s, 3H). ^{13}CNMR (100 MHz, DMSO) δ175.62, 174.85, 168.04, 162.21, 157.85, 156.47, 149.96, 149.63, 148.03, 146.96, 144.86, 142.07, 140.32, 138.01, 135.72, 130.17, 129.98, 128.05, 124.83, 123.69, 123.62, 117.67, 115.51, 111.65, 108.67, 106.66, 97.75, 19.19, 18.92. The product of CST with $O_2^{\bullet-}$ was characterized, HRMS data, m/z calculated for [$C_{29}H_{23}N_4O_3^+$], 475.1764 found 475.1644.

Cells culture.

Human hepatic cells (HL7702) were from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM high-glucose medium supplemented with 10 % fetal bovine serum, 1 % penicillin and 1% streptomycin at 37 °C (w/v) in a 5 % CO_2⁄95 % air incubator MCO-15AC (SANYO, Tokyo, Japan). Hepatic IR model of cells was performed by oxygen and glucose deprivation/reperfusion[3]. Hepatocyte were cultured in DMEM (without glucose) and deoxygenated reagent sodium dithionite (0.5 mmol/L) for 30 min. Afterwards, these cells were incubated with high-glucose DMEM in a 5 % CO_2 and 95% O_2 atmosphere for 30 min. The cells for aconitase assay were treated with RIPA lysis buffer and removed mitochondrial by high-speed centrifuge method (14,000 rmp for 10 minutes at 4 ℃)[4].

Transient transfection. Chemical siRNA for TOM sequences is 5'-CAACUGGUUGGCAACGGUATT-3' and for SOD is 5′-AACCUCACAUCAACGCG-CAtt-3′. All siRNA were purchased from Gene Pharma. A standard Lipofectamine 2000 (Life Technologies) transfection protocol was performed. The ratio of siRNA: Lipfectamine was 2 μL: 4 μL for siRNA knockdown experiments.

Mice culture and maintenance. Eight- to ten-week-old wild-type BalB/C mice (male) were used. The mice were anesthetized with 4% chloral hydrate (3 mL/kg) by intraperitoneal injection and a laparotomy was performed to expose the liver[5]. Hepatic ischemia was induced by clamping the portal vein and artery of the median and the left lateral lobes of the liver with a microvessel clip inducing 70% partial liver ischemia. Thirty minutes later, the ischemic liver was reperfused by opening the vascular clamp. Then CST (100 μM) and DHE (80 μM) were dropped into the left lobe of the liver. As a control, the normal mice were exposed the liver and added CST (100 μM) and DHE (80 μM) in the left lobe of the liver. The permission of animal experiments was obtained from the Shandong normal university authorities.

Fluorescence imaging experiments. HL7702 cells were detached and replanted onto 15-mm glass-bottomed dishes 24 h before imaging. After the addition of CST (10 μM) probe for 10 min, the cell culture media was removed and cells were washed with 1.0 mL PBS for three times. Fluorescence images of CST were obtained with excitation=800 nm, blue channel emission=430-480 nm, green channel emission=550-580 nm and fluorescence ratio channel. Fluorescence images of MitoTracker Deep Red (1.0 nM) were with excitation=633 nm and red channel emission=650-740 nm. Fluorescence images of DHE (8 μM) was with excitation=800 nm and red channel emission=650-700 nm. The anesthetized mice

were fixed on the microscope carrier with 20X water objective (N/A 1.0). Two-photon 3D imaging of mice were acquired with *z*-stack mode of Zeiss 880 NLO microscopy. These 3D images were collected along the z-direction at the depths of approximately 0-170 μm with 24 pictures.

The ratio images were the ratio calculation of blue/green channel by Zen 2.1 (Zeiss 880 NLO microscopy software) according to the following equation (Eq 1). Subsequently, depending on the each pixel ratio, the pseudo-color map was described by microscopy software. The ratio color bar on the right corner of the figures indicated the gray scale (0-255) of pseudo-color. For data analysis, the average fluorescence intensity per image in each experimental condition was obtained by selecting regions of interest. Each experiment was repeated at least three separate times with identical results.

$$Ratio = \frac{I_{blue-channel} - I_{backgroud}}{I_{green-channel} - I_{backgroud}} \qquad \text{Eq 1}$$

Flow cytometry.

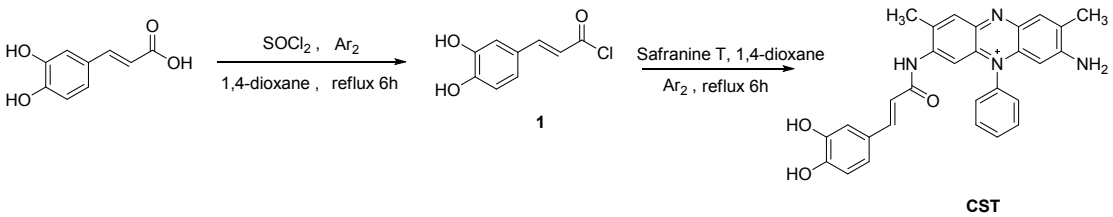
Cells were plated on six well plates with 2 mL of DMEM/High Glucose in each plate 24-36 hours prior to the day of experiment and maintained at 37 °C, 5 % CO_2 to obtain 80-90 % confluency. Before the flow cytometry, cells were treated with none, IR, DIDS or interleukin 6, then cells were washed with PBS and trypsinized with 1 mL 0.05 % Trypsin in each plate for 5 min. Trypsin was discarded and 5 mL of DMEM/High Glucose was added in each plate and cells were spun down at 1000 rpm for 5 min. Media was discarded and cells were resuspended in 5 mL of PBS and again spun down at 1000 rpm for 5 min. Next, each pellet was incubated with 195 μL binding buffer of Annexin V-FITC, 5 μL Annexin V-FITC and 10 μL Propidium Iodide (PI) at room temperature away from the light for 20 min, cells were spun down at 1000 rpm for 5 min and resuspended in 100 μL of PBS. After these procedures, cells were used for the flow cytometry. Fluorescence signals in FITC channel and PI channel were measured for 5000 cells on a flow cytometer of ImageStream^X Mark II (Merck Millipore, Seattle, WA). Data was analyzed by IDEAS version 6.2.

Statistics.

In vitro fluorescence experiments of CST for $O_2^{\bullet-}$ sensing were done in three independent replicates and data is presented as mean and error bars represent s.e.m. for indicated conditions. For in cellular and *in vivo* imaging of $O_2^{\bullet-}$, three such experiments were performed for each experimental condition. The regions of interests mean and the s.e.m. of these three different experiments were then calculated and plotted. Differences were analyzed by one-way ANOVA followed by Tukey's post-hoc test.

SUPPLEMENTAL SCHEME AND FIGURES

Scheme S1. Synthesis of probe CST.



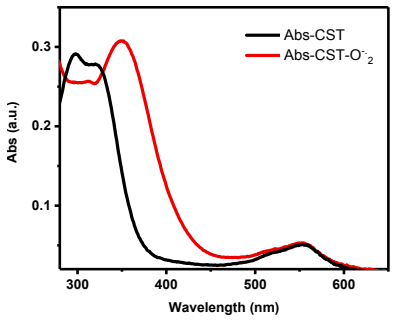


Figure S1. The absorption spectra of 10 μM CST before and after addition of $O_2^{\bullet-}$ with 30 mM PBS buffer (pH 7.4).

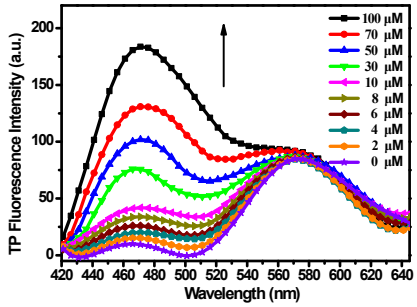


Figure S2. Two-photon fluorescence spectra of 10 μM CST after adding various concentrations of $O_2^{\bullet-}$ (0-100 μM) with 30 mM PBS buffer (pH 7.4) at λ_{ex} = 800 nm.

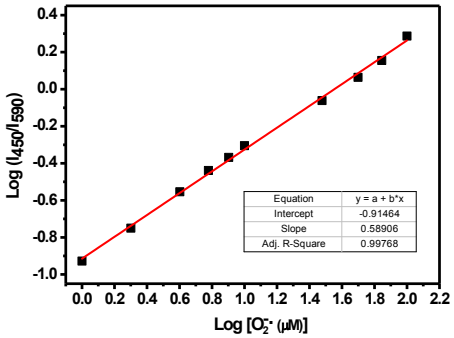


Figure S3. A linear correlation between the ratio fluorescence intensity and $O_2^{\bullet-}$ concentrations with 30 mM PBS buffer (pH 7.4) at λ_{ex} = 800 nm.

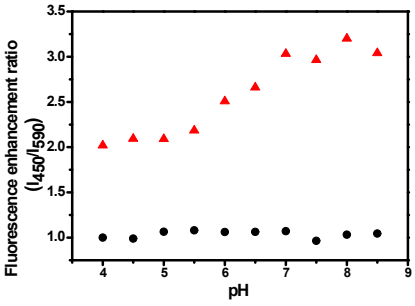


Figure S4. The one-photon fluorescence intensity of CST (10 μM, ●) and reaction system (10 μM $O_2^{\bullet-}$, ▲) with various pH.

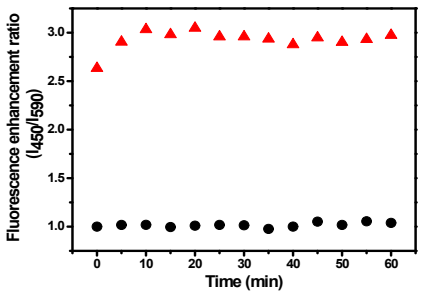


Figure S5. The time course of one-photon fluorescence for 10 μM CST (●) and the product of CST with 10 μM $O_2^{\bullet-}$ (▲). The one-photon spectra were acquired in 30 mM PBS buffer (pH 7.4, 37 ℃) at λex = 400 nm and λem = 450/590 nm. The ratio value was calculated with $I_{0(450/590)}$ as 1.0.

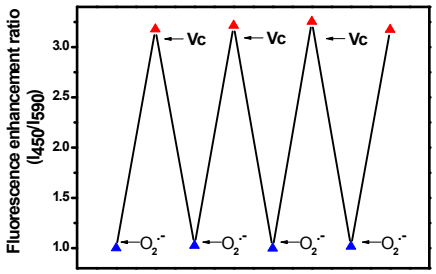


Figure S6. Fluorescent reversibility of CST with 10 μM $O_2^{\bullet-}$ and 2.0 mM ascorbic acid (Vc) alternately. The one-photon spectra were acquired in 30 mM PBS buffer (pH 7.4, 37 ℃) at λex = 400 nm and λem = 450/590 nm. The ratio value was calculated with $I_{0(450/590)}$ as 1.0.

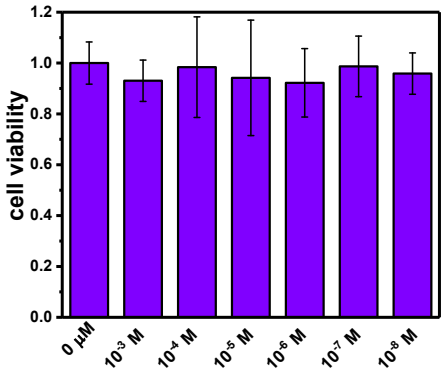


Figure S7. The MTT assay of CST.

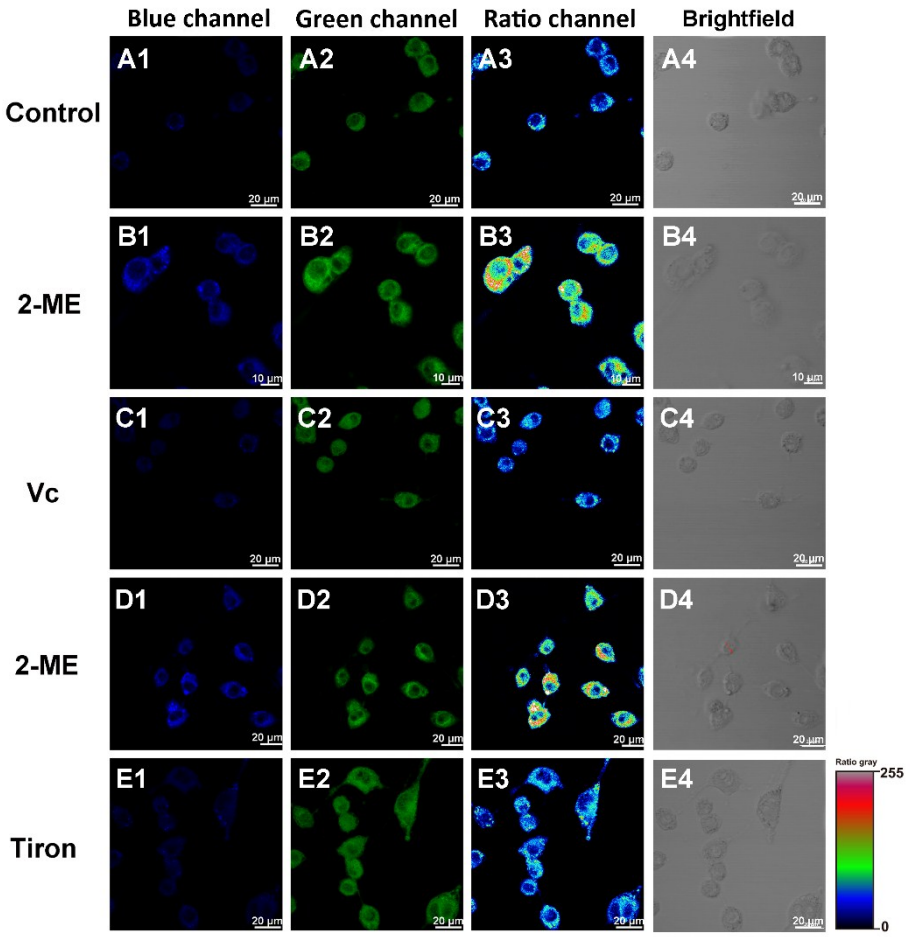


Figure S8. Reversible TP fluorescence ratio imaging of $O_2^{\bullet-}$ in hepatocytes. (A) images were loaded with 10 μM CST for 10 min. (B) images were A cells with 1.0 μg/mL 2-methoxyestradiol (2-ME). (C) images were B cells with 1.0 mM Vc. (D) images were C cells with 1.0 μg/mL 2-ME again. (E) images were cells with 10 μM Tiron 30 min before CST addition. Images were acquired by using excitation of 800 nm and emission windows of blue channel=430-480 nm, green channel=550-630 nm and fluorescence ratio was blue channel/green channel. Scale bar= 20 μm.

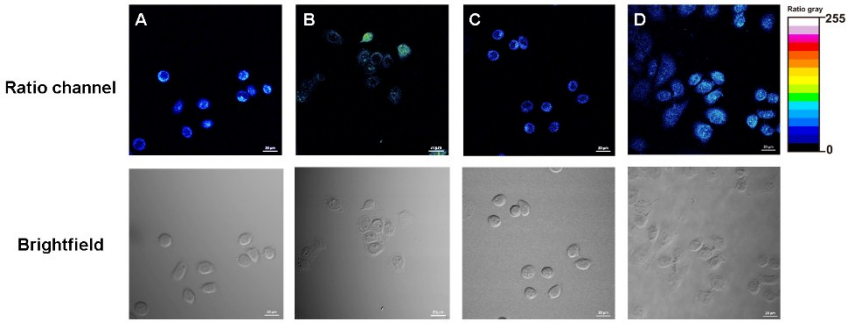


Figure S9. TP fluorescence ratio imaging of $O_2^{\bullet-}$ in hepatocyte treated with SOD. The hepatic cells (A) and IR hepatic cells treated with none (B), with 10 μM interleukin 6 (C) and with Mn-SOD siRNA treatment (D) were loaded with 10 μM CST for ratio imaging. Images were acquired by using excitation of 800 nm and emission windows of blue channel =430-480 nm, green channel =550-630 nm and fluorescence ratio was blue channel/green channel.

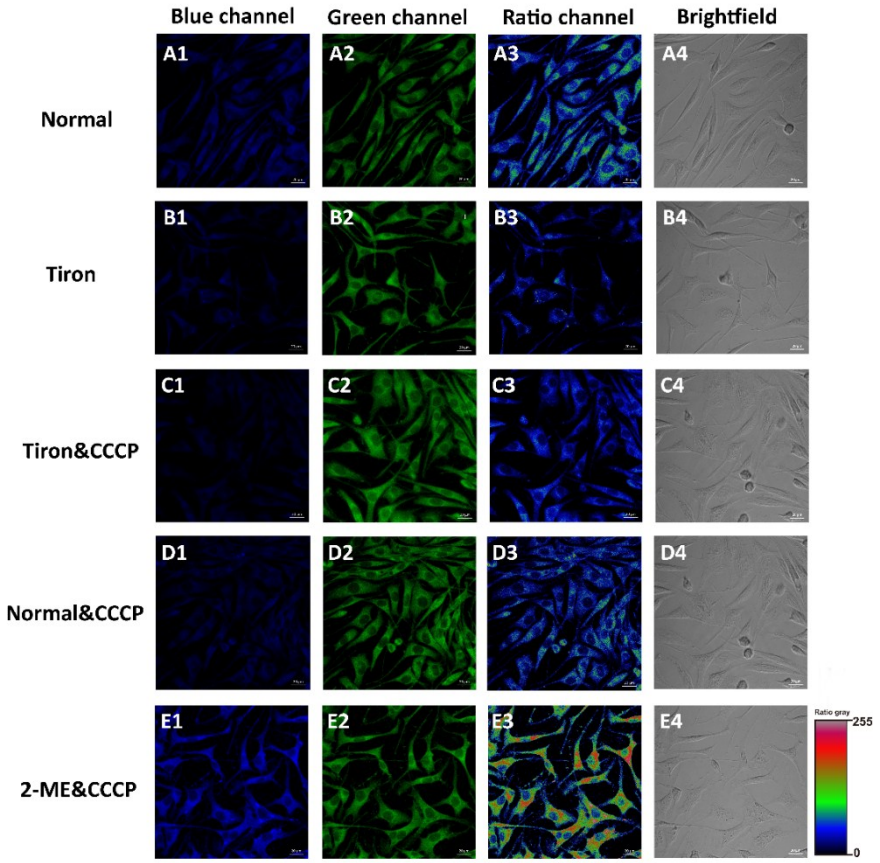


Figure S10. The effects of mitochondria membrane potential to CST. (A) Normal cells were loaded with 10 μM CST for 10 min. (B) Cells were loaded with 10 μM Tiron for 30 min before 10 μM CST addition. (C) Cells were loaded with 10 μM Tiron for 30 min and 20 μM CCCP for 20 min before 10 μM CST addition. (D) Cells were loaded with 20 μM CCCP for 10 min before 10 μM CST addition. (E) Cells were loaded with 20 μM CCCP for 20 min and 1.0 μg/mL 2-ME for 30 min before 10 μM CST addition.

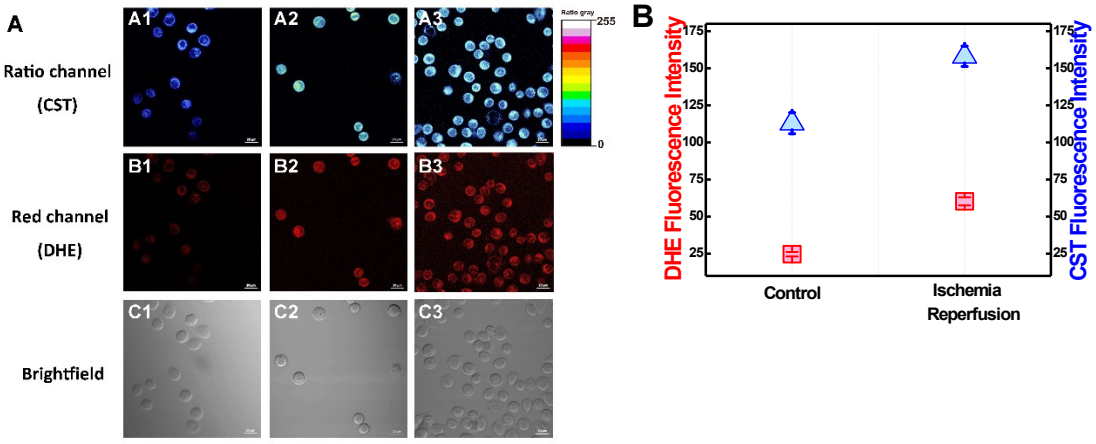


Figure S11. Two-photon imaging of $O_2^{\bullet-}$ within mitochondria and cytoplasm of IR cells. (A) The normal (A1and B1), IR hepatic cells (A2 and B2) and IR hepatic cells with 25 μM ebselen (A3 and B3) were loaded with 10 μM CST for ratio imaging and 8 μM DHE for the red channel. (B) The average fluorescence intensity output of A1, B1, A2 and B2. Images were acquired by using 800 nm excitation and red channel=650-700 nm and fluorescence ratio was blue channel/green channel. Scale bar= 20 μm.

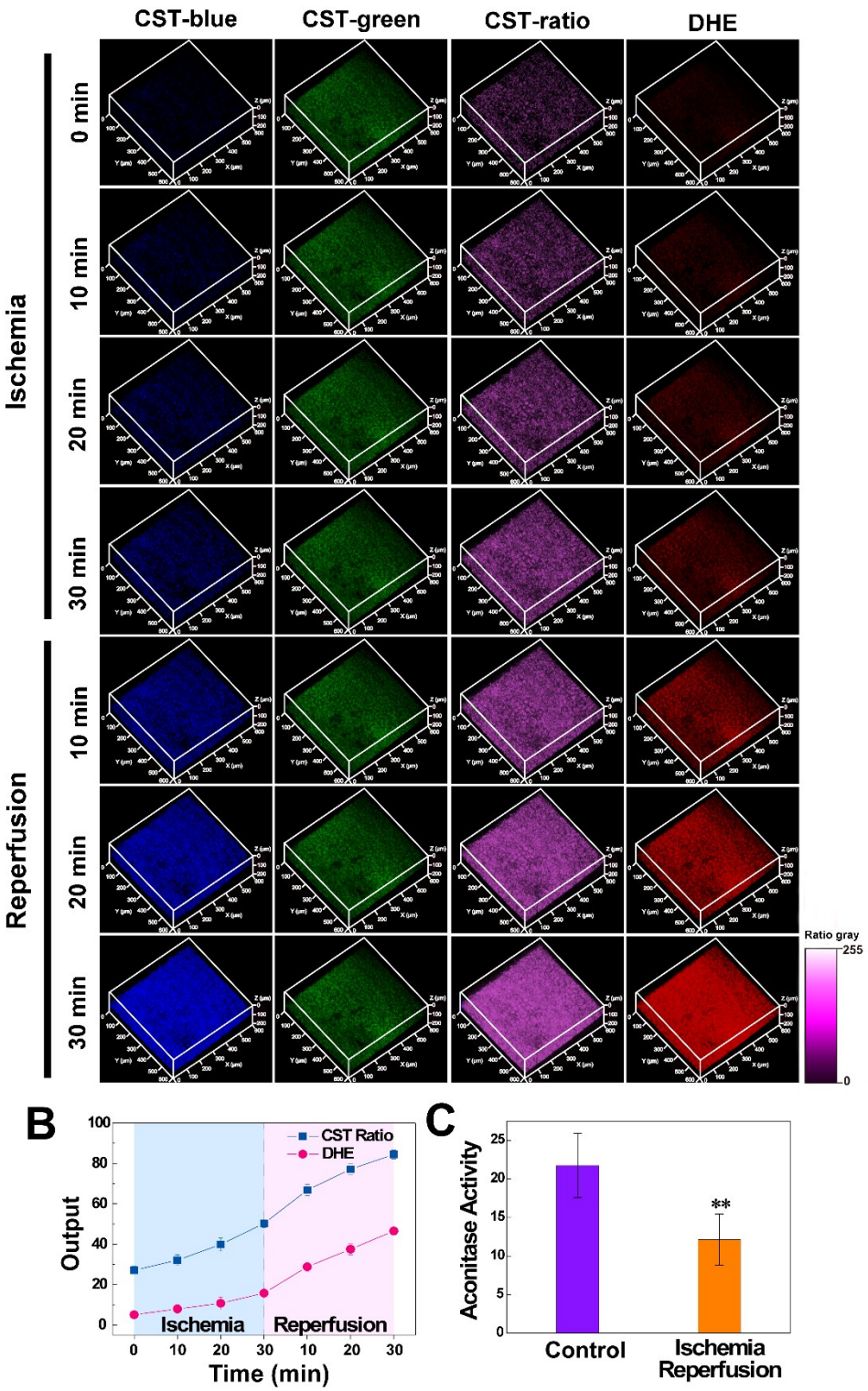


Figure S12. Two-photon imaging of $O_2^{\bullet-}$ within mitochondria and cytoplasm of mouse IR livers. (A) The time-dependent 3D images of $O_2^{\bullet-}$-mediate redox state during IR procedure in mice loaded with 100 μM CST and 80 μM DHE. (B) Average gray output of the CST-ratio channel and the DHE channel in A. (C) Results from aconitase activity assay kit of normal and IR cells (n=3). Images were acquired by using 800 nm excitation, blue channel emission=430-480 nm, green channel emission=550-580 nm and red channel emission=650-700 nm. The fluorescence ratio was blue channel/green channel.

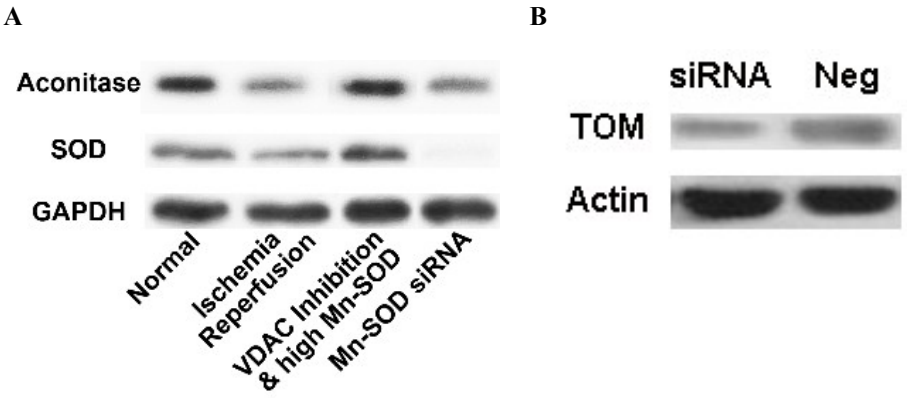


Figure S13. **(A)** Western blot for SOD level (Abcam, ab137037) and aconitase level (Abcam, ab126595) in normal cells, IR hepatic cells, VDAC inhibition & Mn-SOD high level cells (1 mM DIDS and10 μM interleukin 6 and Mn-SOD siRNA knockdown. (B) Western blot for TOM (Abcam: ab185543) showing decreased expression in hepatic cells with siRNA treatment.

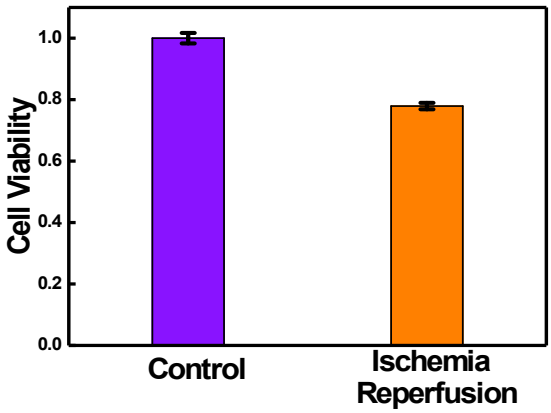


Figure S14. MTT assay of normal and IR cells (n=3).

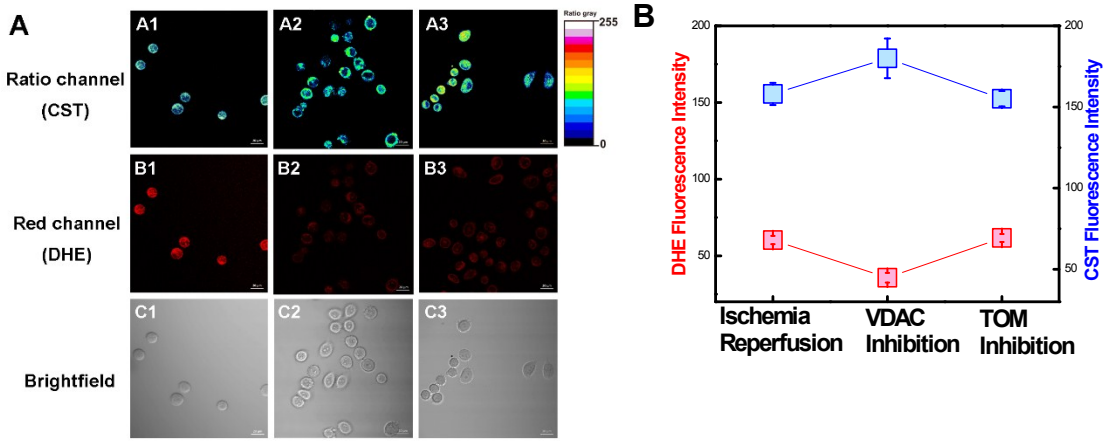


Figure S15. VDAC channel transports mitochondrial $O_2^{\bullet-}$ to cytoplasm. (A) The IR hepatic cells treated with none (A1 and B1), 1 mM DIDS (A2 and B2), dextran sulfate (A3 and B3) were loaded with 10 μM CST for ratio imaging and 8 μM DHE for the red channel. (B) The average fluorescence intensity output of A1, B1, A2, B2 and S15. Images were acquired by using excitation of 800 nm and emission windows of red channel =650-700 nm and fluorescence ratio was blue channel/green channel. Scale bar= 20 μm.

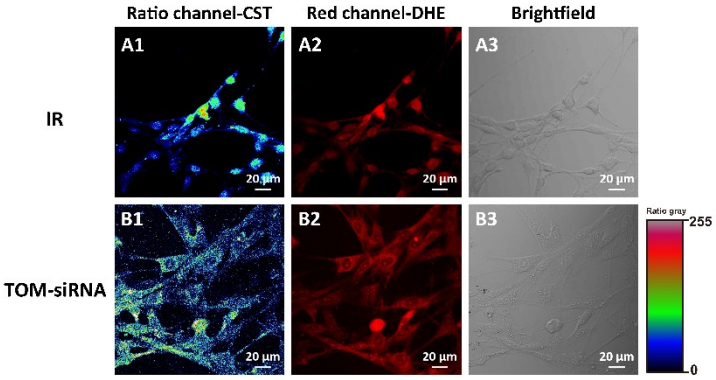


Figure S16. Application of CST to explore TOM function for $O_2^{\bullet-}$ transport in IR cells. (A) The IR hepatic cells (A1 and A2) and IR hepatic cells with TOM siRNA (B1 and B2) were loaded with 10 μM CST for ratio imaging and 8 μM DHE for the red channel. Images were acquired by using excitation of 800 nm and red channel =650-700 nm and fluorescence ratio was blue channel/green channel.

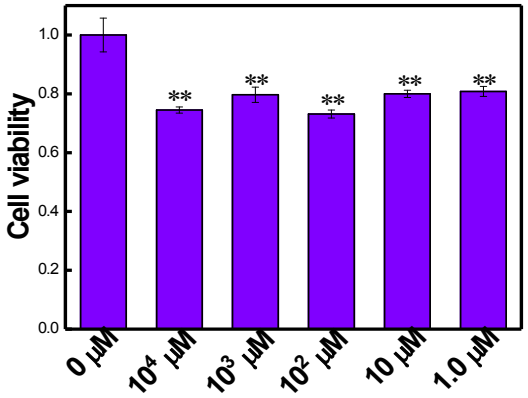


Figure S17. MTT assay of cells with different DIDS level (0-10 mM, n=3).

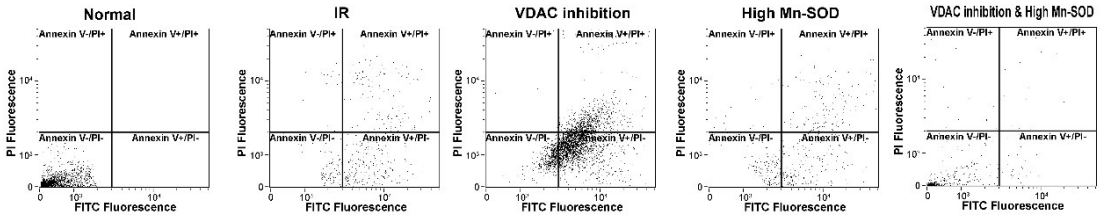


Figure S18. Flow cytometry analysed normal cells, IR hepatic cells, VDAC inhibitied group (1 mM DIDS), Mn-SOD high level group (10 μM interleukin 6) and VDAC inhibition & Mn-SOD high level (1 mM DIDS and 10 μM interleukin 6).

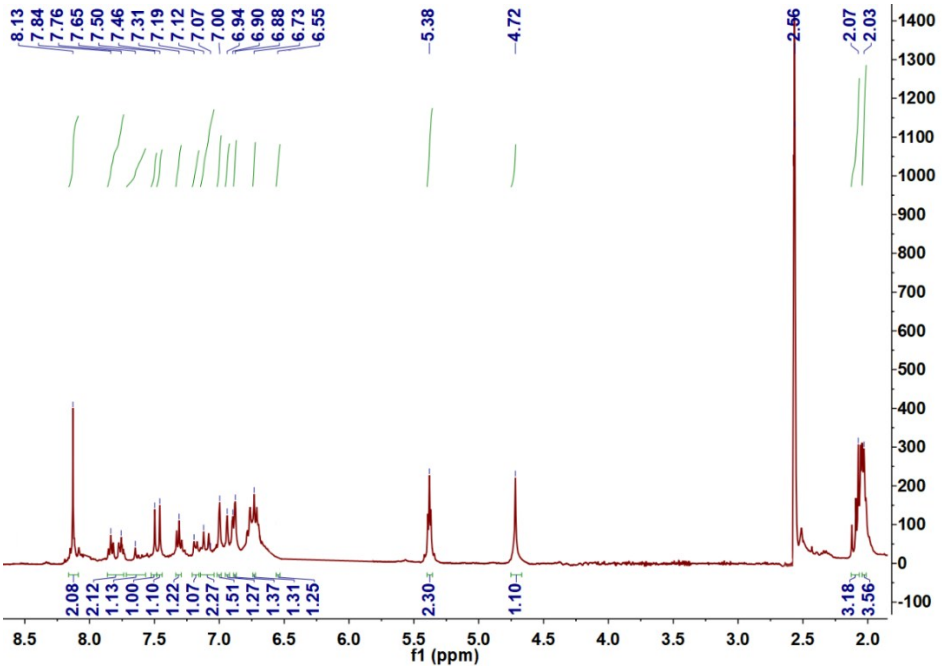


Figure S19. The [1]HNMR data of CST.

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