Electronic Supplementary Material (ESI) for New Journal of Chemistry.

Supplementary Information

Two-photon fluorescence imaging of mitochondria viscosity with water-soluble pyridinium inner salts

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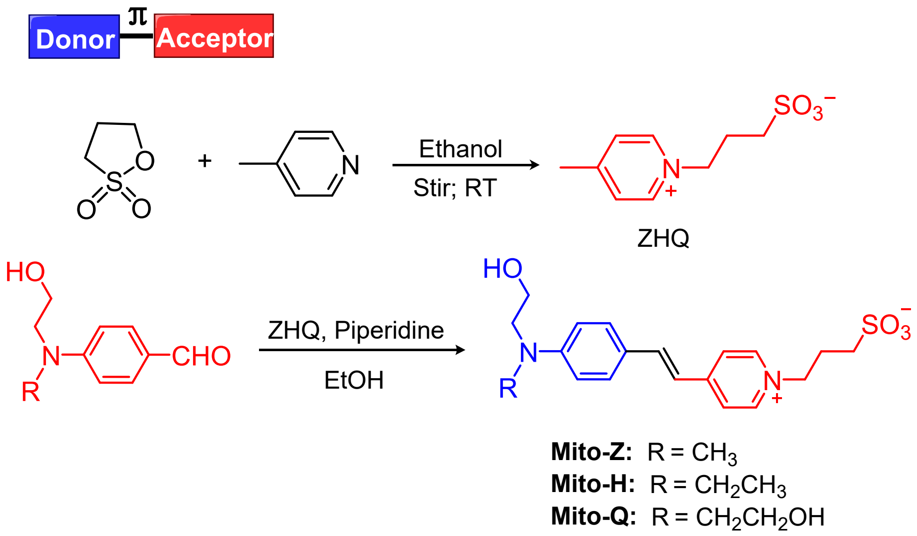
Supplemental Figures and Tables



Scheme S1. The synthesis routes of target compounds.

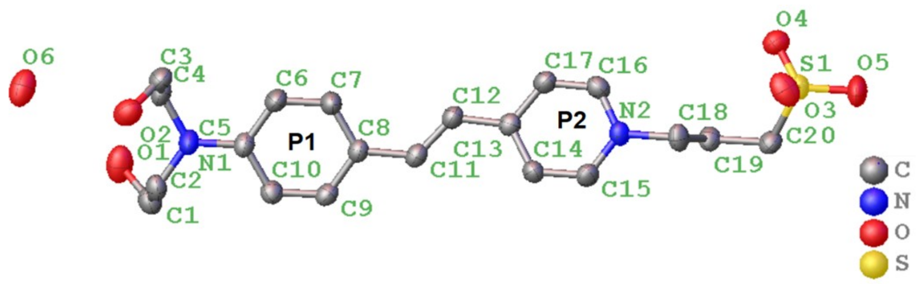


Figure S0. Crystal structures of **Mito-Q**. All H atoms are omitted for clarity.

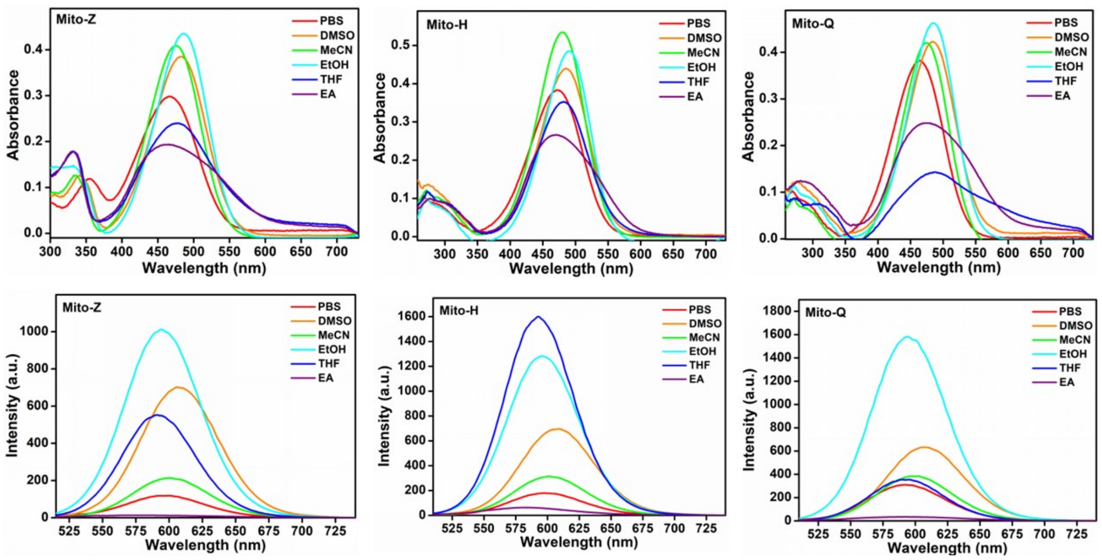


Figure S1. The linear absorption and emission spectra upon excited at 466 nm, 472 nm, 464 nm of **Mito-Z, Mito-H, Mito-Q** in different solvents with different polarities with a concentration of 10 μM.

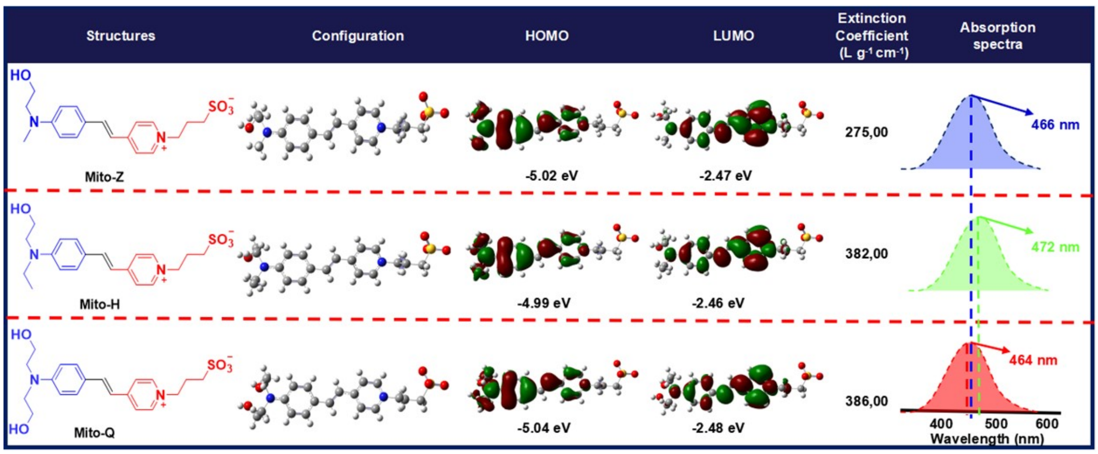


Figure S2. Frontier molecular orbitals of the sensors (HOMO-LUMO energy levels were determined from DFT optimized geometries). The probes were calculated with time-dependent density functional theory (TD-DFT) at the B3LYP/6-31G(d,p) level using Gaussian 09.

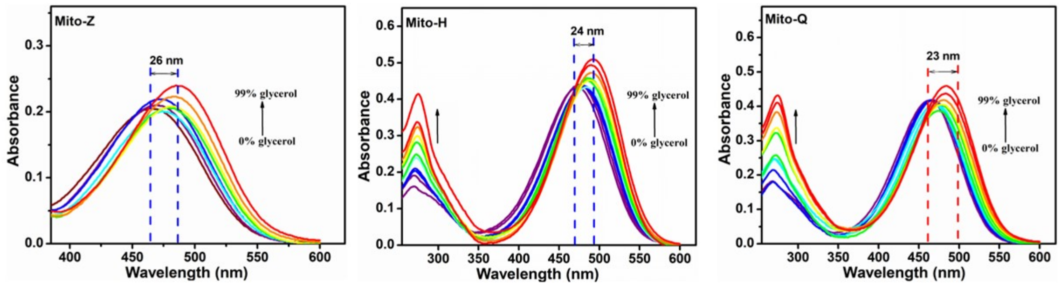


Figure S3. Changes of absorption spectra with variation of solution viscosity in a water-glycerol system. (1) water (2) water/glycerol (9 : 1, v/v), (3) water/glycerol (8 : 2, v/v), (4) water/glycerol (7 : 3, v/v), (5) water/glycerol (6 : 4, v/v) (6) water/glycerol (5 : 5, v/v), (7) water/glycerol (4 : 6, v/v), (8) water/glycerol (3 : 7, v/v), (9) water/glycerol (2 : 8, v/v), (10) water/glycerol (1 : 9, v/v), and (11) 99% glycerol.

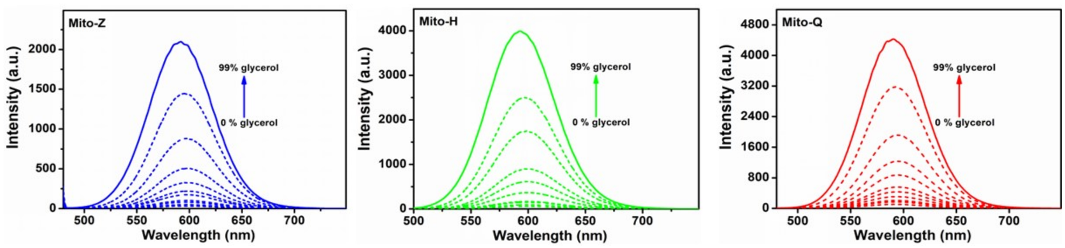


Figure S4. Changes of fluorescence spectra with variation of solution viscosity in water-glycerol media of the sensors with increasing solution viscosity: (1) water (2) water/glycerol (9 : 1, v/v), (3) water/glycerol (8 : 2, v/v), (4) water/glycerol (7 : 3, v/v), (5) water/glycerol (6 : 4, v/v) (6) water/glycerol (5 : 5, v/v), (7) water/glycerol (4 : 6, v/v), (8) water/glycerol (3 : 7, v/v), (9) water/glycerol (2 : 8, v/v), (10) water/glycerol (1 : 9, v/v), and (11) 99% glycerol.

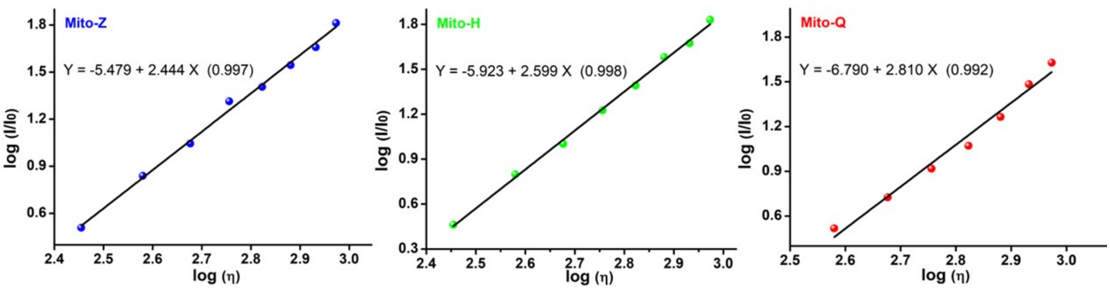


Figure S5. (A) The linear relationship of fluorescence intensity of compound **Mito-Q** with different viscosity. I and I_0 represent the fluorescence intensities of **Mito-Q** in glycerol/water mixture and water, respectively (water/glycerol = 80:20, 70:30, 60:40, 50:50, 20:80, 10:90, 1:99).

Fluo. intensities at 595 nm (Mito-Z)		σ (Fluo.)	Fluo. intensities at 595 nm (Mito-H)		σ (Fluo.)	Fluo. intensities at 595 nm (Mito-Q)		σ (Fluo.)
31.46	31.56	0.764	58.85	58.95	0.782	104.1	102.3	0.732
31.23	34.01		58.26	58.01		105.2	104.6	
33.11	33.16		58.97	59.16		104.8	105.1	
32.45	32.58		60.12	60.32		105.3	105.1	
33.56	33.46		59.85	60.06		105.5	104.8	
32.98	32.12		60.22	59.65		104.9	105.2	
32.45	33.25		58.34	59.39		104.6	104.9	
32.68	32.96		58.19	60.23		104.9	105.1	

Figure S6. Multi-recorded fluorescence spectra of blank measurement. Insert: the data of standard deviation (σ) of blank measurement from fluorescence spectra.

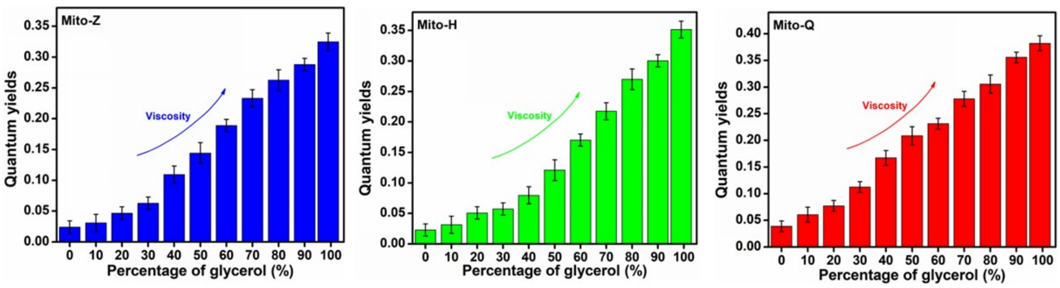


Figure S7. The percentage of fluorescence quantum yields of the sensors with increasing solution viscosity: (1) water (2) water/glycerol (9 : 1, v/v), (3) water/glycerol (8 : 2, v/v), (4) water/glycerol (7 : 3, v/v), (5) water/glycerol (6 : 4, v/v) (6) water/glycerol (5 : 5, v/v), (7) water/glycerol (4 : 6, v/v), (8) water/glycerol (3 : 7, v/v), (9) water/glycerol (2 : 8, v/v), (10) water/glycerol (1 : 9, v/v), and (11) 99% glycerol.

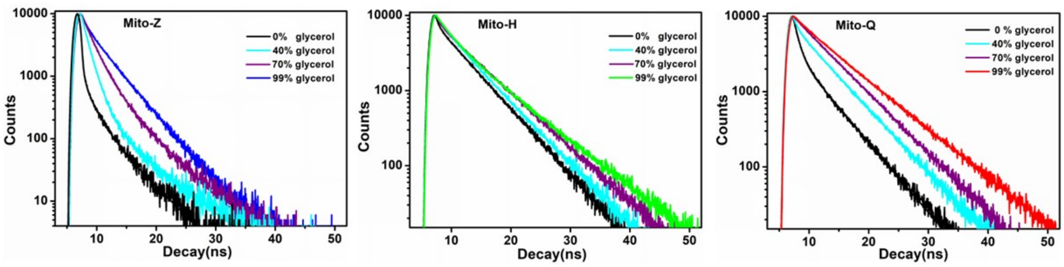


Figure S8. Fluorescence lifetimes of the sensors in different viscosity media. (1) water, (2) water/glycerol (6 : 4, v/v), (3) water/glycerol (3 : 7, v/v), (4) 99% glycerol.

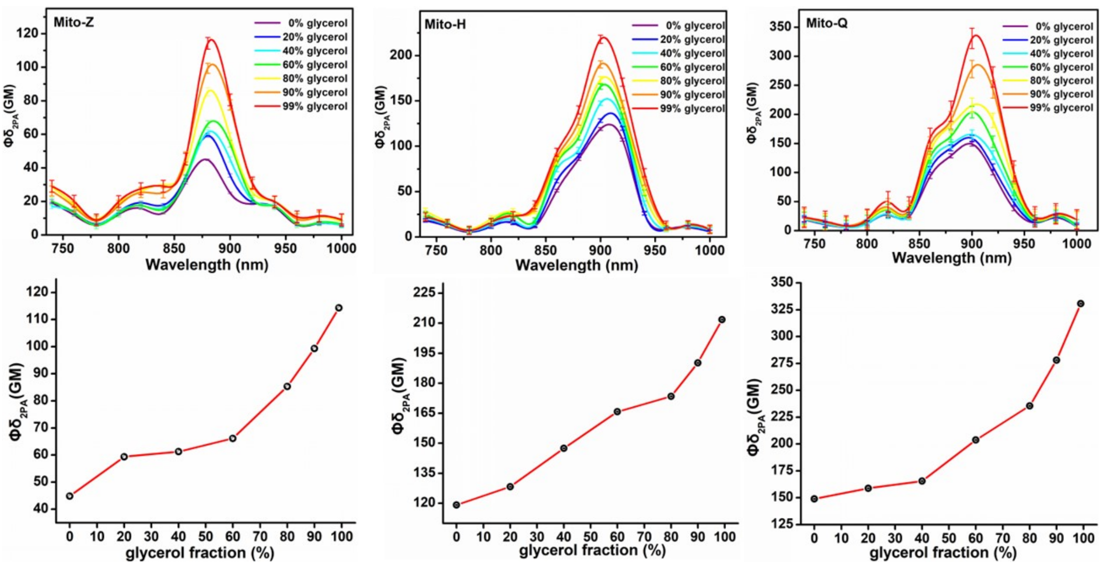


Figure S9. Two-photon (TP) action cross-section spectra of the sensors with variation of solution viscosity in the wavelength region of 740-1000 nm. And TP action cross-section spectra of the sensors with variation of solution viscosity at 880nm, 900 nm, 900 nm.

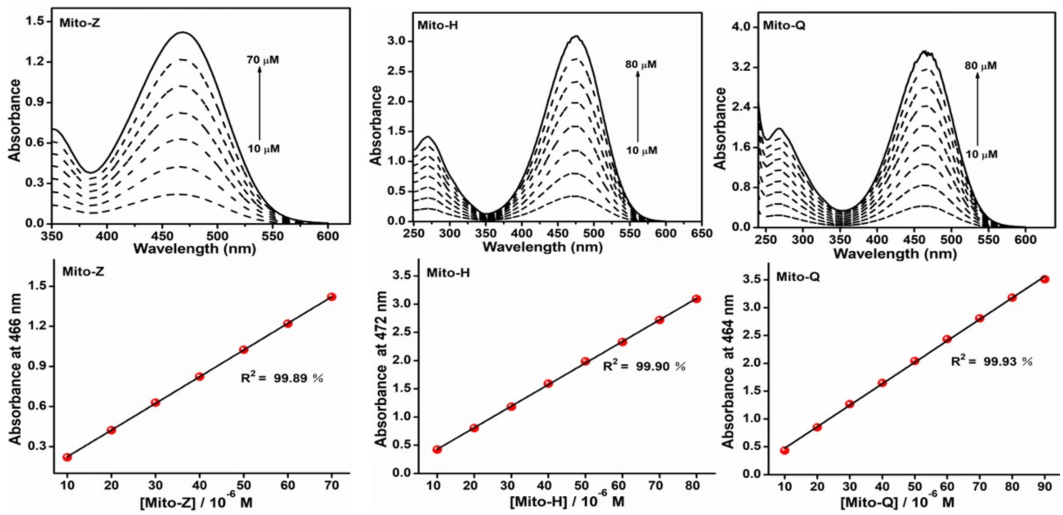


Figure S10. Absorption spectra and plot of intensity against the concentration of the sensors in pure PBS buffer (pH = 7.4), respectively.

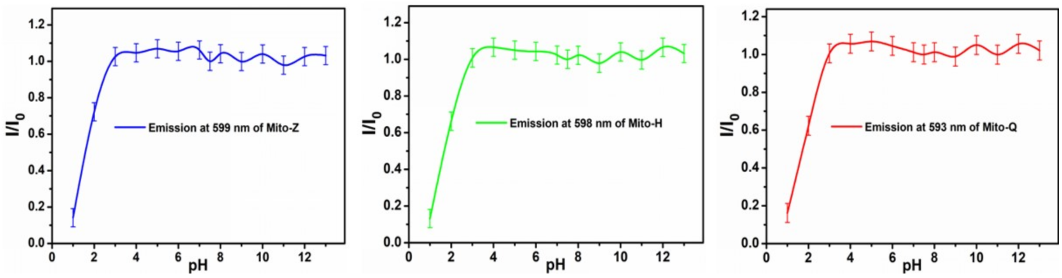


Figure S11. The changes of emission intensity of the sensors (10 μM) in PBS-buffer (10 mM) at various pH. (I_0 is the emission intensity with pH at 7.40 and I represent the emission intensity at other pH values).

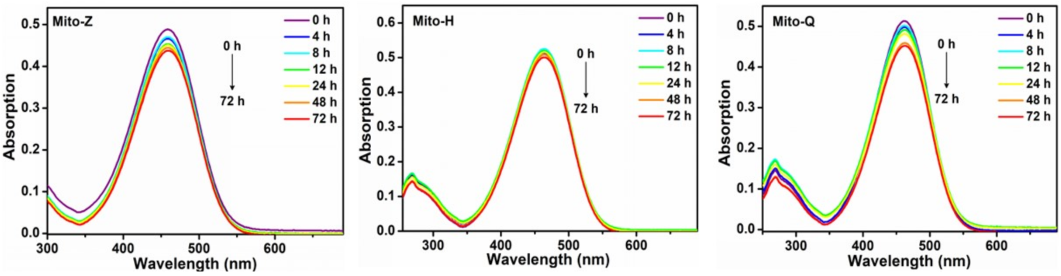


Figure S12. Time evolution of UV-*vis* absorption spectra of the targeting compounds in PBS buffers (pH = 7.40).

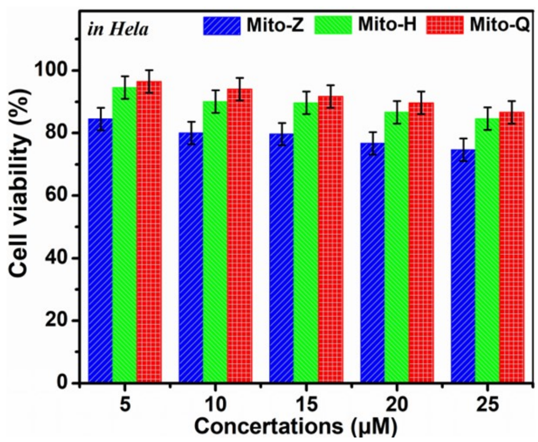


Figure S13. MTT assay of HepG2 cells treated with the sensors at different concentrations for 24 h.

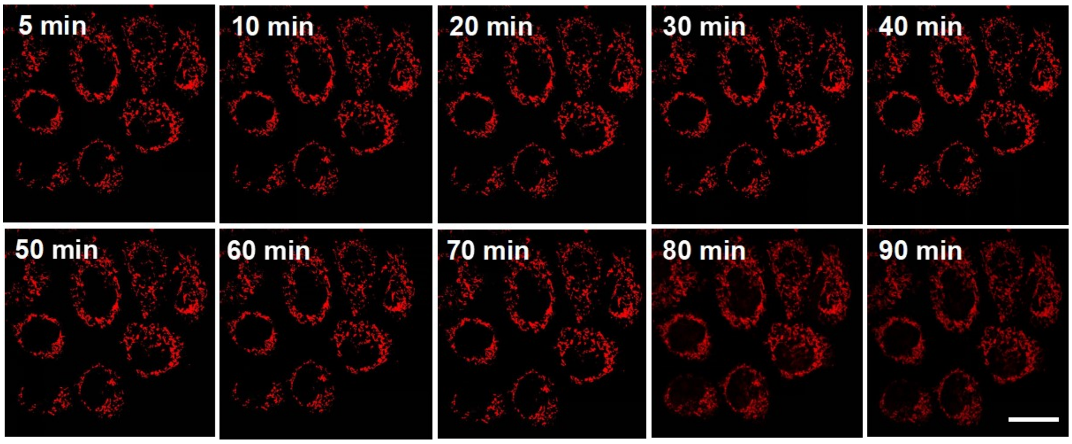


Figure S14. Real time monitoring images under OP fluorescence microscopy using **Mito-Q** (1 uM). Scale bar =20 μm

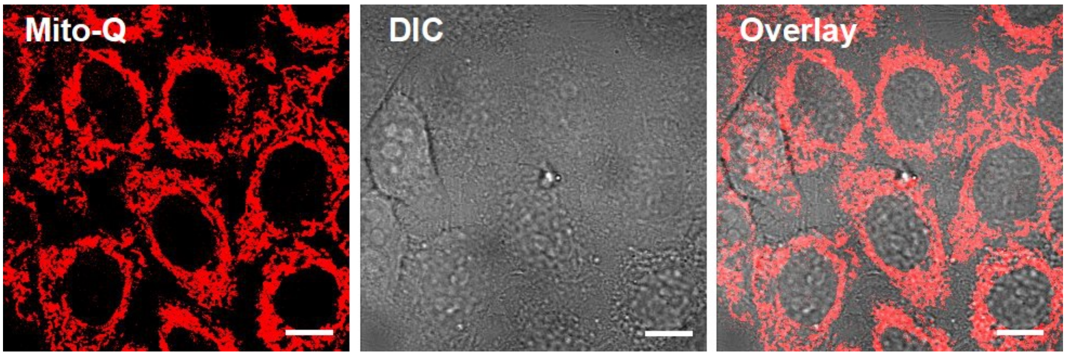


Figure S15. One-photon confocal laser fluorescence microscopy images of HepG2 cells treated with **Mito-Q** (10 μM). Scale bar =20 μm

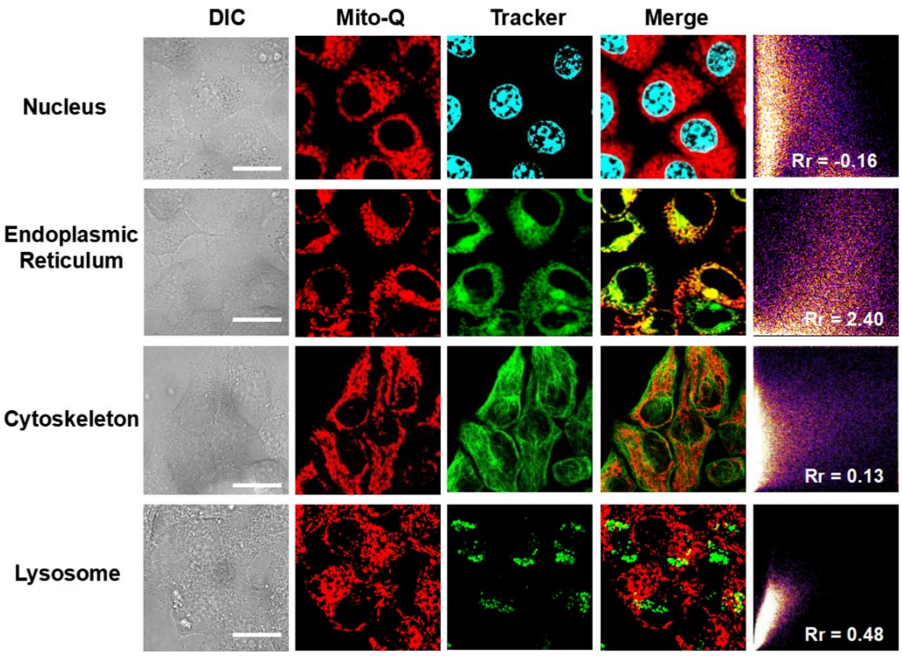


Figure S16. Determination of intercellular localization of Mito-Q by confocal microscopy. Hela cells were incubated with Mito-Q for 30 min, and then co-incubated with Nuc-red for 15 min, ER tracker for 30 min, Sir-tubulin for 30 min and lysotracker for 30 min, respectively. The scale bars represent 20 μm.

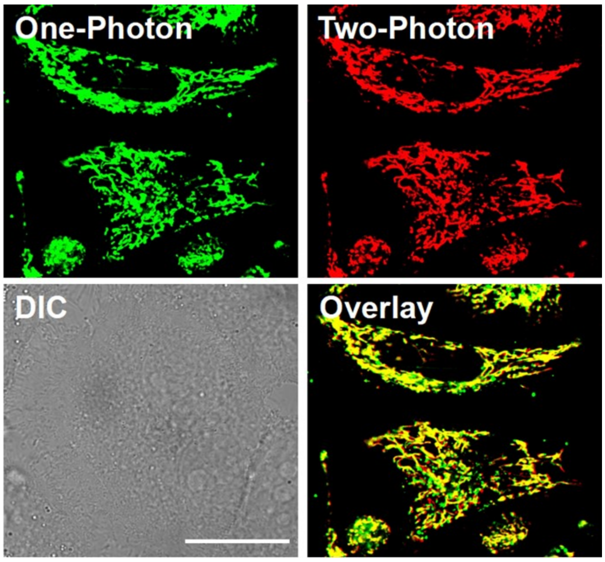


Figure S17. One- and two-photon fluorescence images of living Hela cells incubated with Mito-Q (10 μM) for 30 min. Scale bar =20 μm.

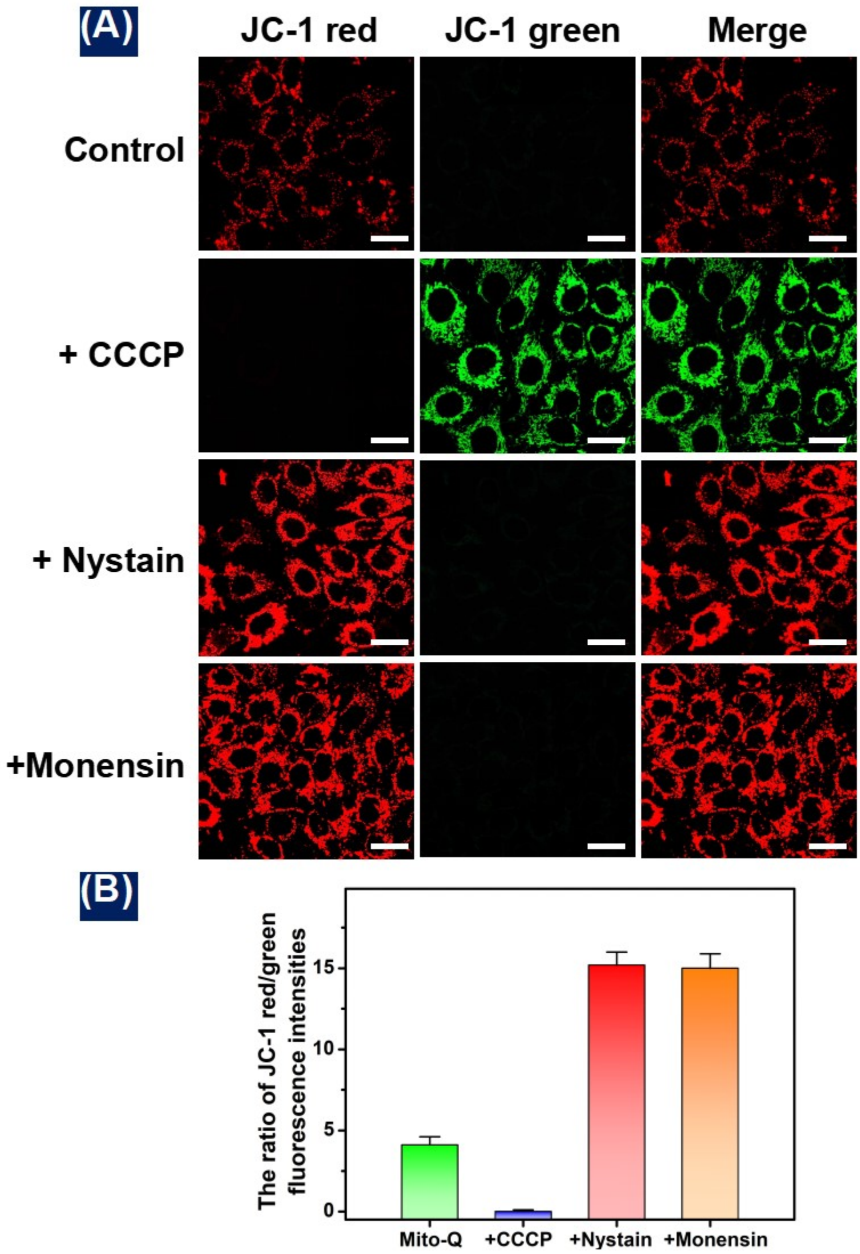


Figure 18. Mitochondrial membrane potential (MMP) changes in Hela cells with treatment by (-), CCCP (+), nystatin (+) and monensin (+), respectively. Scale bar = 20 μm. (B) Histogram of the changes in the mitochondrial membrane potential in Hela cell treated with (-), CCCP (+), nystatin (+) and monensin (+), respectively. Data reference is the mean of three replicate ± SD.

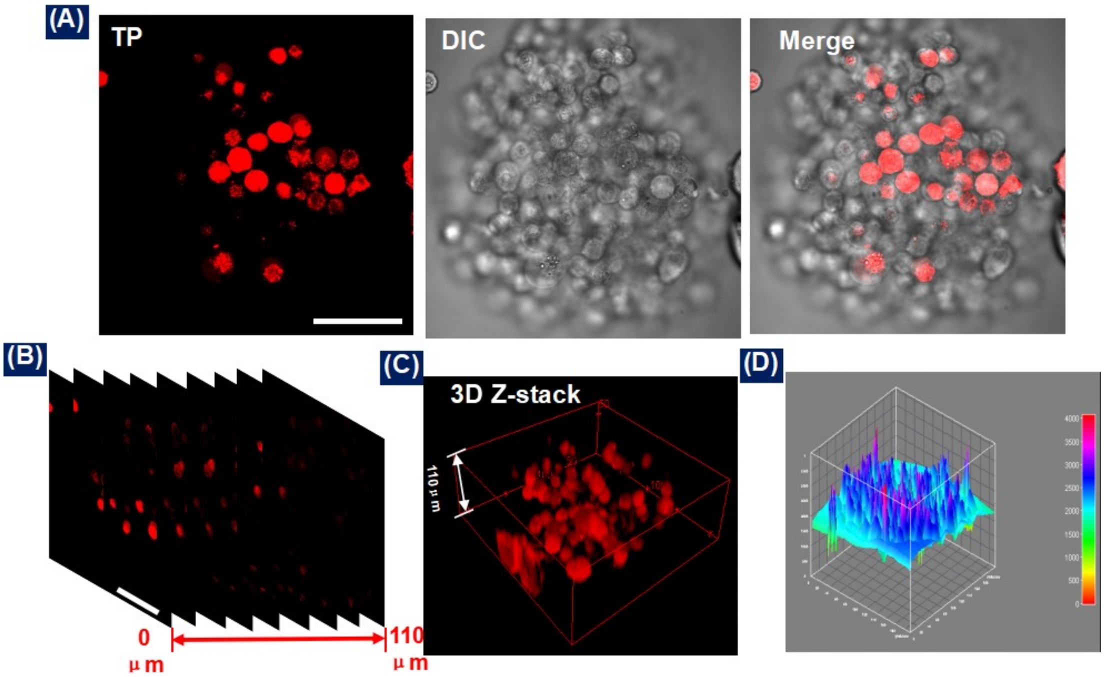


Figure S19. (A) TP fluorescence images of the 3D multicellular spheroids of HepG2 cells incubated with 10 μM **Mito-Q**, 30 min. (B) The TP Z-stack images were taken for 0-110 μm. (C) The TP 3D Z-stack images of an intact spheroid; (bottom), λ_{em} = 550-630 nm and λ_{ex} = 900 nm. (D) 3D fluorescent intensity with **Mito-Q**.

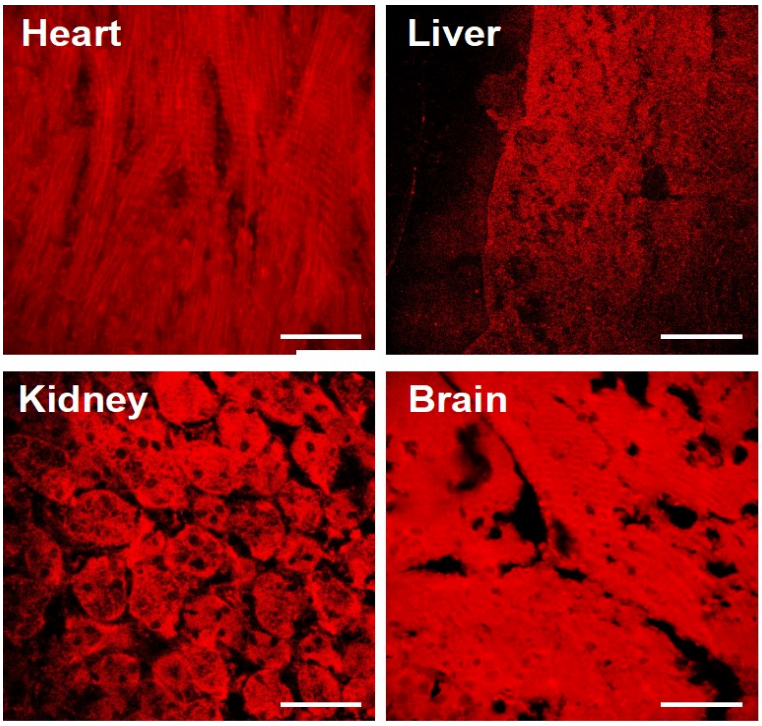


Figure S20. Fresh Tissue sections from brain, liver, spleen and heart (10 μm) incubated with probe **Mito-Q** for 30 min, Scale bar = 100 μm.

Characterization spectra

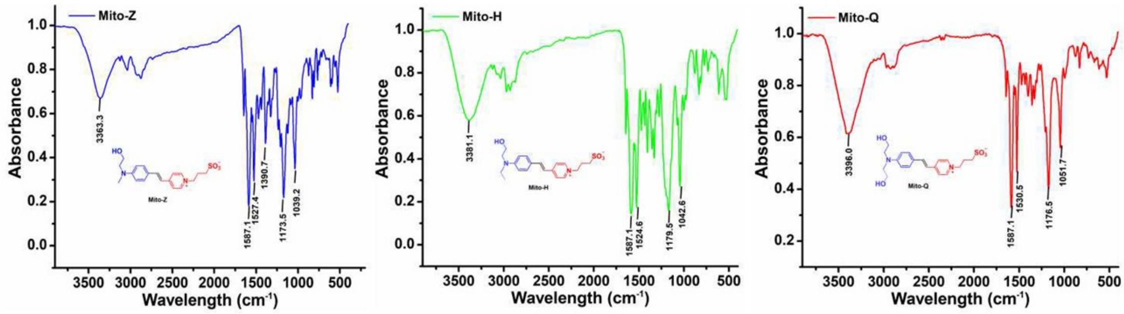


Figure S21. IR spectra of the probes.

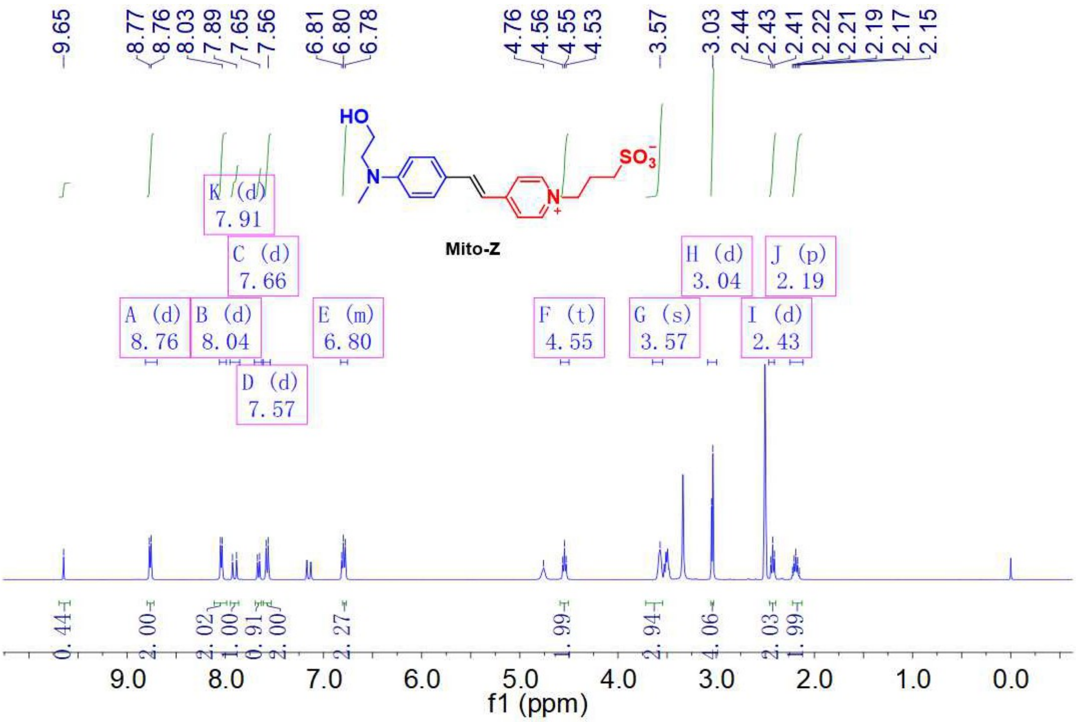


Figure S22. ^{1}H NMR spectrum of **Mito-Z** (400 MHz, 298 K, d_6-DMSO).

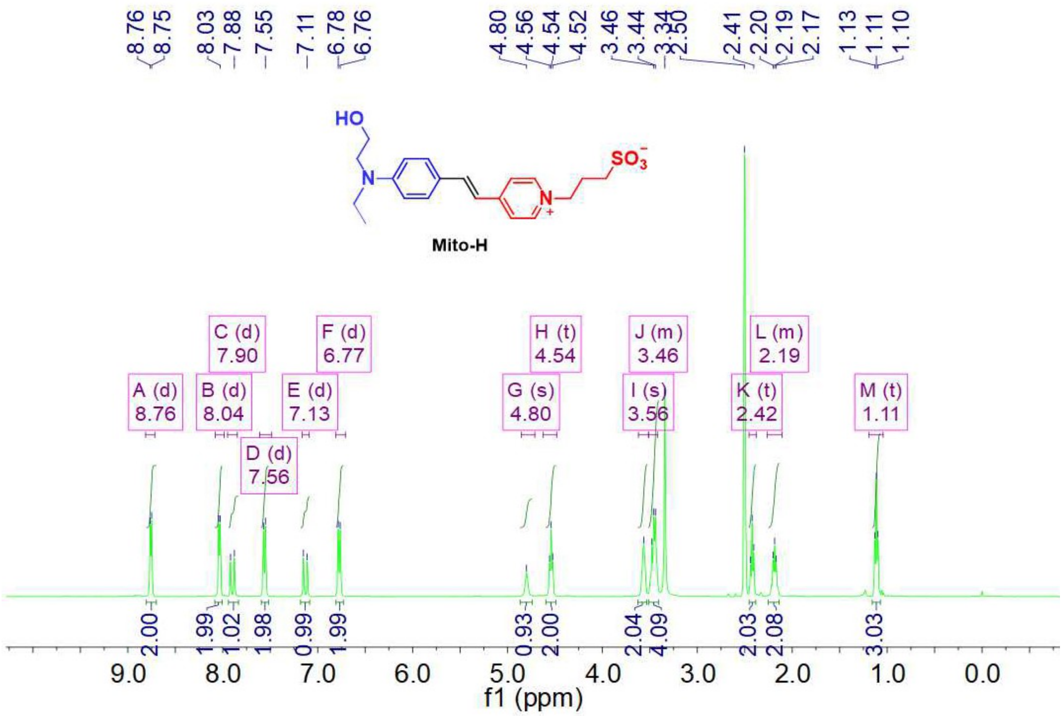


Figure S23. ^{1}H NMR spectrum of **Mito-H** (400 MHz, 298 K, d_6-DMSO).

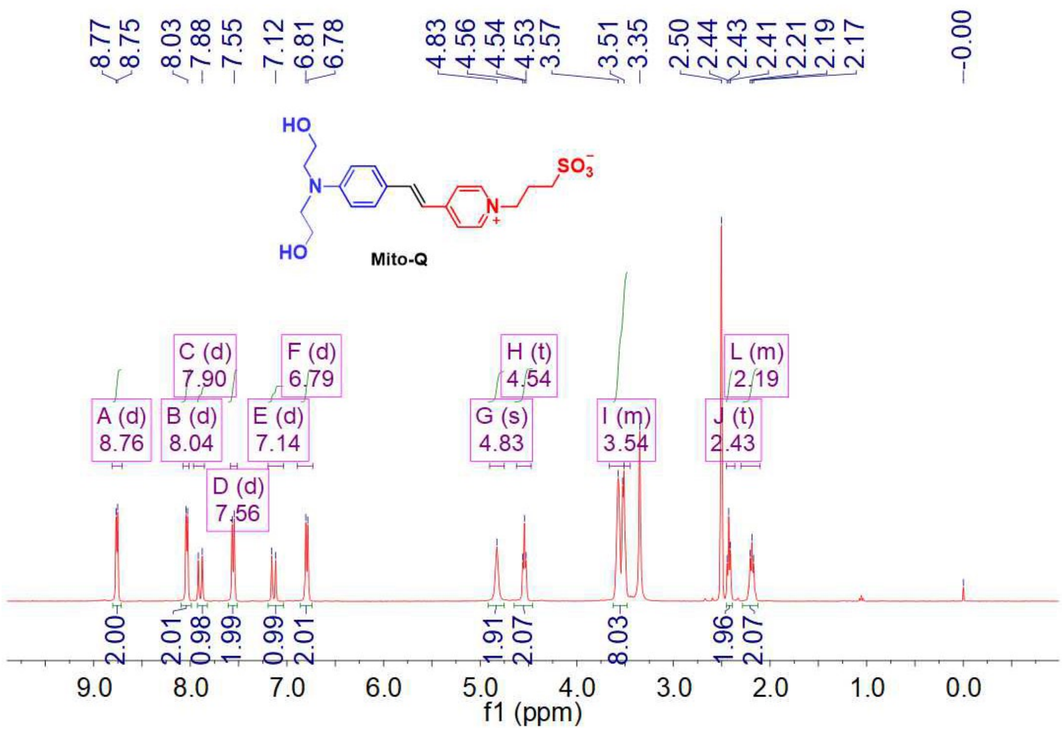


Figure S24. ^{1}H NMR spectrum of **Mito-Q** (400 MHz, 298 K, d_6-DMSO).

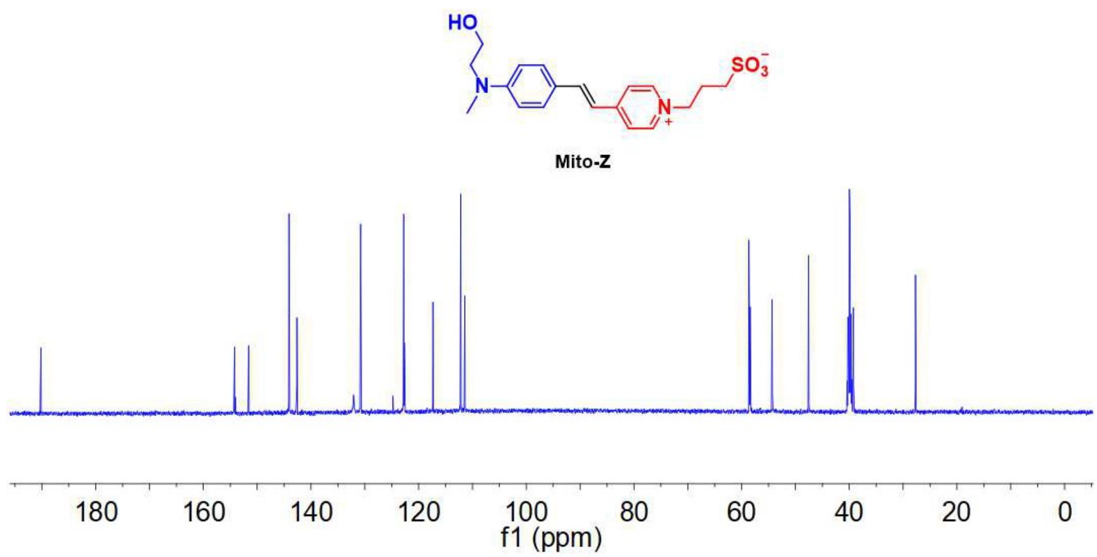


Figure S25. [13]C NMR spectrum of **Mito-Z** (100 MHz, 298 K, d_6-DMSO).

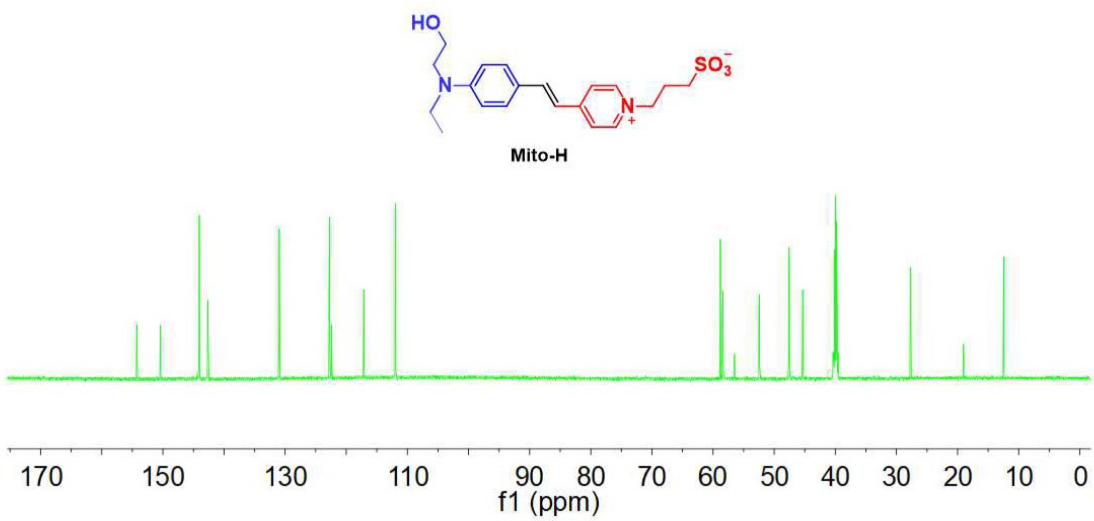


Figure S26. [13]C NMR spectrum of **Mito-H** (100 MHz, 298 K, d_6-DMSO).

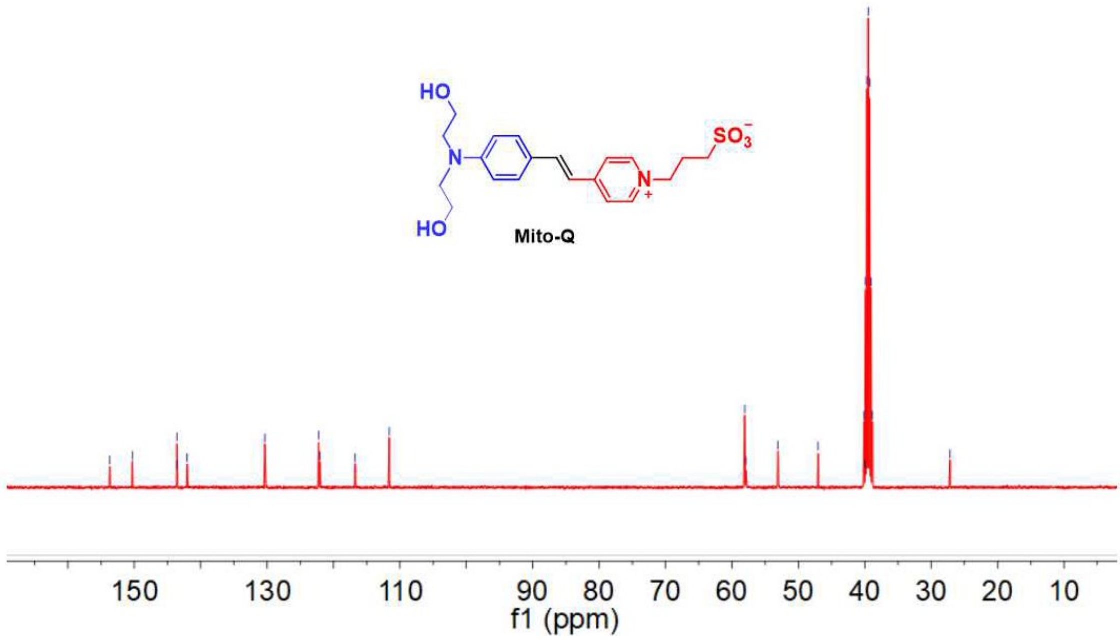


Figure S27. ^{13}C NMR spectrum of **Mito-Q** (100 MHz, 298 K, d_6-DMSO).

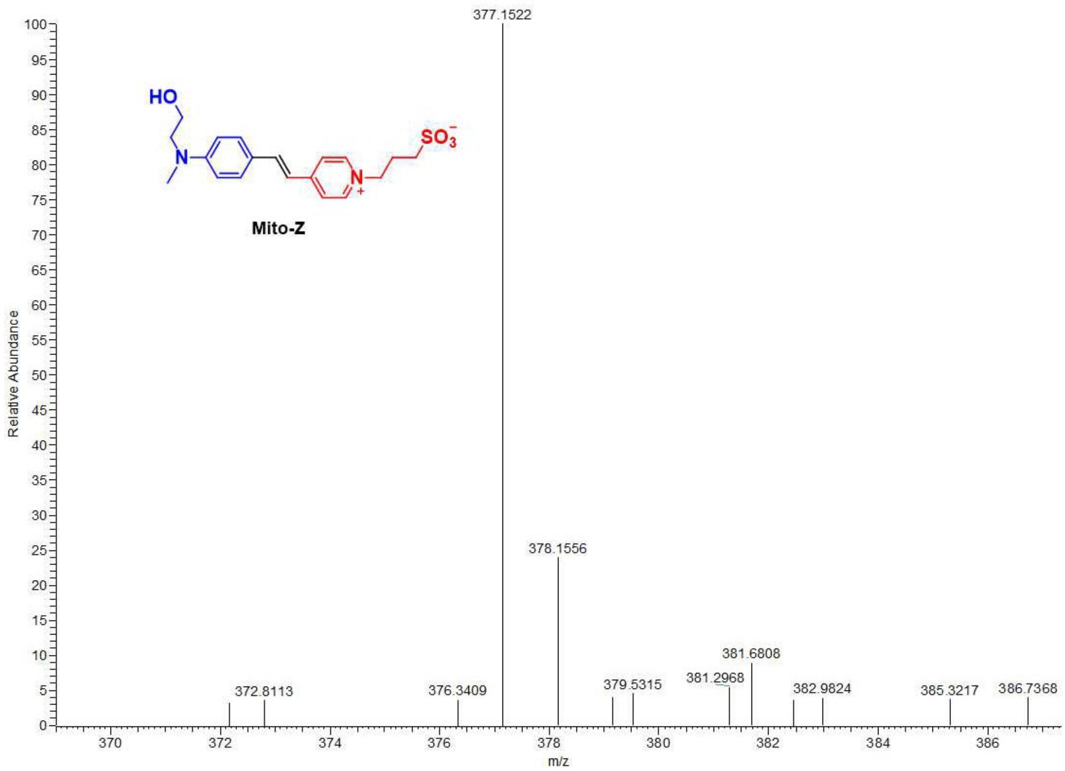


Figure S28. HR-MS spectrum of **Mito-Z**.

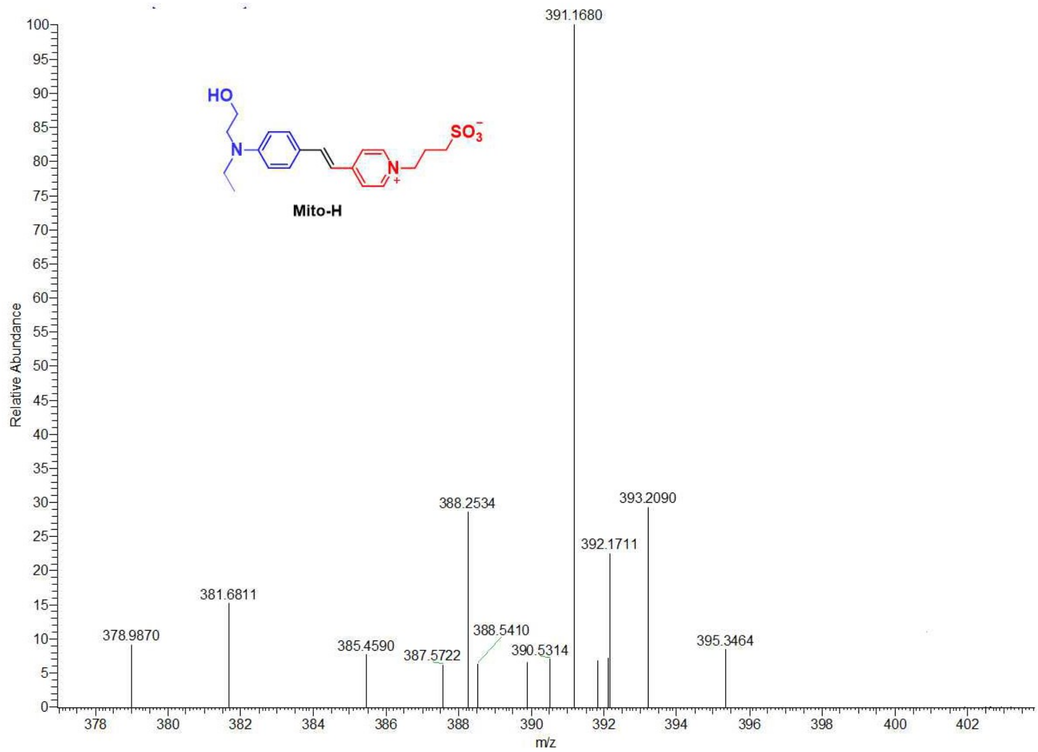


Figure S29. HR-MS spectrum of **Mito-H**.

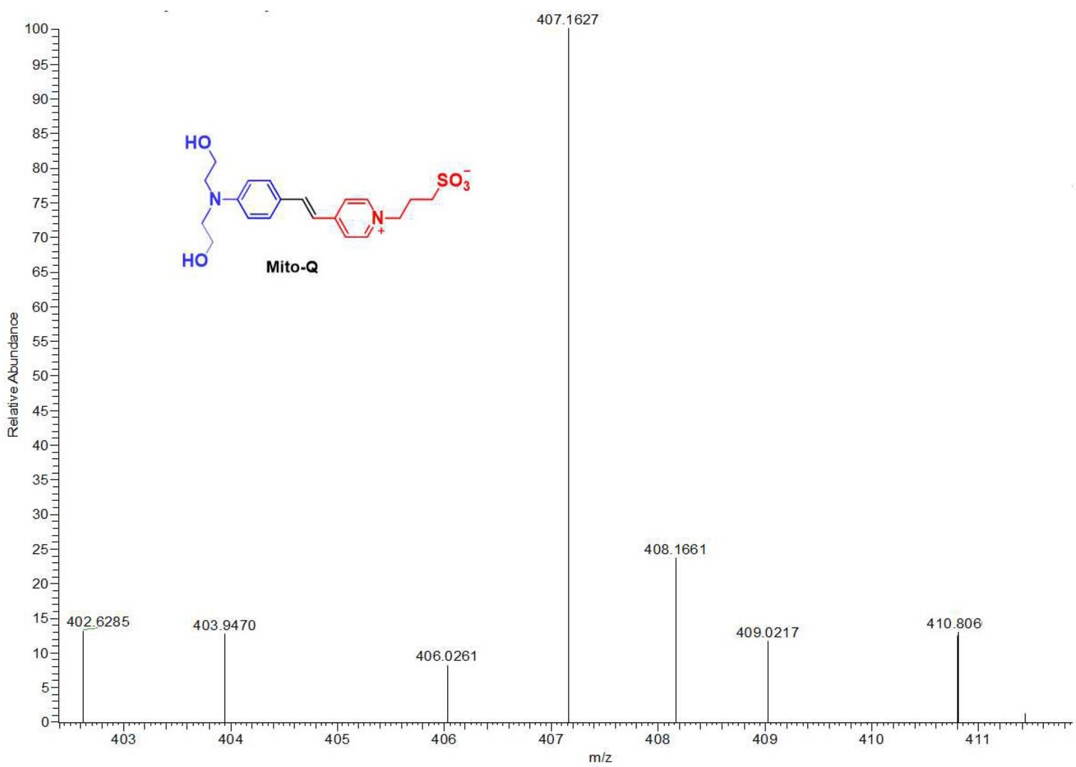


Figure S30. HR-MS spectrum of **Mito-Q**.

Supporting Tables

Table S1. Crystal data and structure refinement for **Mito-Q**.

Compound	**Mito-Q**
Empirical formula	$C_{20}H_{26}N_2O_5S$, H_2O
Formula weight	424.50
Temperature	296(2) K
Wavelength	0.71073 Å
space group	$P2_1/n$
Crystal system	monoclinic
a/Å	17.617(5)
b/Å	13.441(5)
c/Å	8.825(3)
β/(°)	100.752(4)
V/Å^3	2053.0(12)
Z	4
D_c/Mg m^{-3}	1.373
μ/mm^{-1}	0.198
F(000)	904
Final R indices [I>2*σ*(I)]	R_1 = 0.0488, wR_2 = 0.1563
Goodness-of-fit on F^2	1.091

Table S2. Photophysical data of the target compounds in different solvents.

Mito-Z

Solvents	λex[a]	λem[b]	ΔV [c]	Logε$_{max}$	Φ[d]	Brightness (εΦ)[e]
PBS	466	600	134	2.13	0.012	0.026
DMSO	485	606	121	2.08	0.018	0.037
ACN	476	600	124	2.09	0.014	0.029
EtOH	485	594	109	2.04	0.026	0.053
THF	480	595	115	2.06	0.015	0.031
EA	465	589	124	2.09	0.010	0.021

Mito-H

Solvents	λex[a]	λem[b]	ΔV [c]	$Log\varepsilon^{max}$	Φ[d]	Brightness (εΦ)[e]
PBS	472	597	125	3.88	0.016	0.062
DMSO	485	609	124	3.94	0.023	0.091
ACN	479	601	122	4.03	0.018	0.073
EtOH	490	595	105	3.99	0.034	0.136
THF	482	593	111	3.85	0.019	0.073
EA	468	580	112	3.72	0.012	0.045

Mito-Q

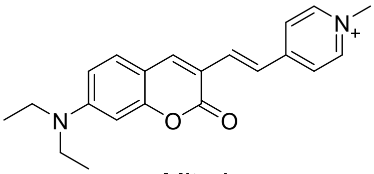
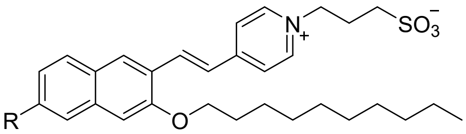
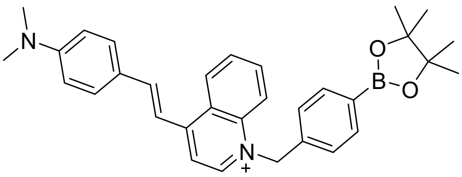
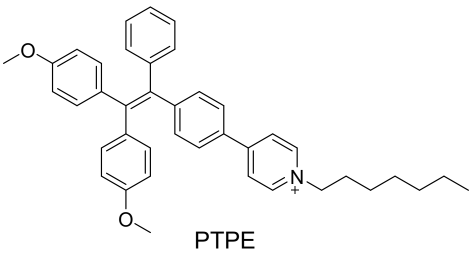
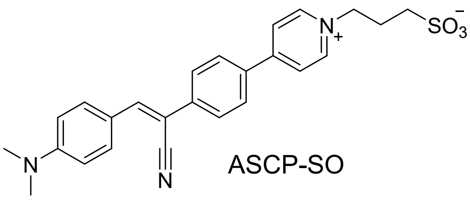
Solvents	λex[a]	λem[b]	ΔV [c]	$Log\varepsilon^{max}$	Φ[d]	Brightness (εΦ)[e]
PBS	464	593	129	3.88	0.019	0.074
DMSO	485	606	121	3.93	0.032	0.126
ACN	475	598	123	3.92	0.022	0.086
EtOH	484	595	111	3.97	0.042	0.167
THF	486	595	109	3.46	0.021	0.073
EA	472	595	123	3.70	0.013	0.048

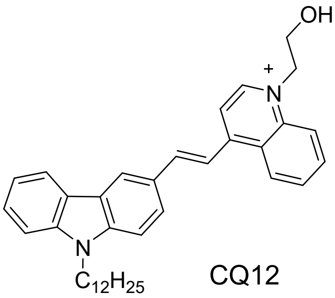
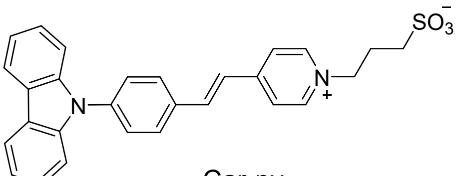
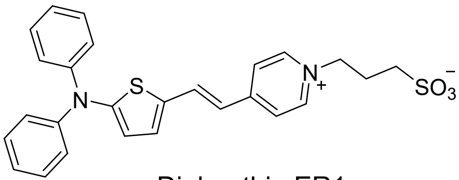
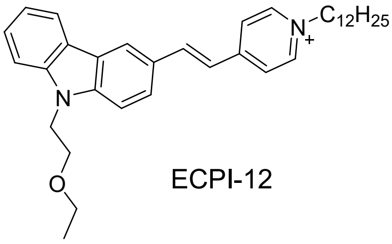
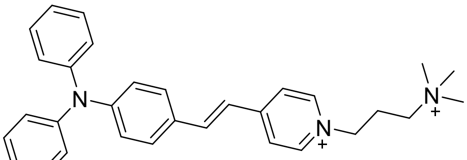
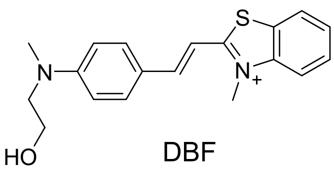
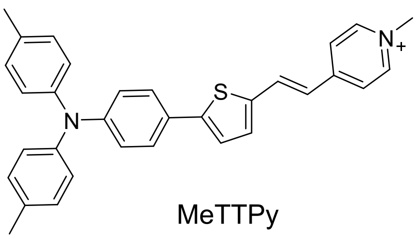
[a]Peak position of the longest absorption band in nm. [b]Peak position of SPEF, excited at the absorption maximum in nm.[c]Stokes' shift in nm. [d]Quantum yields determined by using rhodamine (RhB) as standard. [d]Brightness(εΦ) is proportional to the product of the extinction coefficient (ε, at the relevant excitation wavelength, 10^4) and the fluorescence quantum yield (Φ).

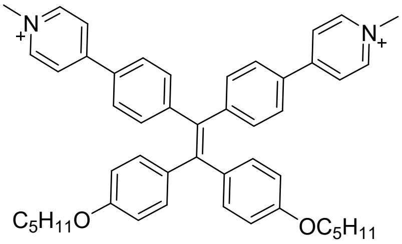
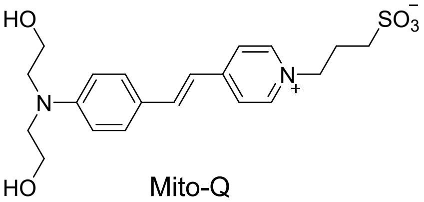
Table S3. Calculated linear absorption properties (nm), excitation energy (eV), oscillator strengths and major contribution for the target compounds .

Cmpds	λ(nm)	E(ev)	*f*	Composition	Character
Mito-Z	486	2.55	0.01455	100→101 (H→L) (0.64)	ICT
Mito-H	490	2.53	0.01575	104→105 (H→L) (0.64)	ICT
Mito-Q	484	2.56	0.01836	108→109 (H→L) (0.64)	ICT

Table S4. The reported structurally similar probes and their sensing properties

Structures	Targeting	Comments	Ref.
 Mito-Lyso	Mitochondria	Indicator of mitochondrial membrane potential (MPP)	2018 Anal. Chem.
 mNVP-B: R=-OAc; mNVP-G: R=-OMe; mNVP-R: R=-$N(CH_2)_4$.	Cell Membrane	Fluorescence trackers for the outer ccell membrane	2020 ACS Appl.Bio Mater.
 QCD-B	Mitochondria	Reveal Mitochondrial Nucleoprotein Dynamics with Reactive Oxygen Species Regulation	2020 Angew. Chem. Int. Ed.
 PTPE	Mitochondria	Autophagy Modulators for Cancer Therapy	2020 Angew. Chem. Int. Ed.
 ASCP-SO	Mitochondria	Visually for mitochondria uptake and retention	2020 Chem.Commun.

 CQ12	Cell Membrane	Visually Discriminating Near-Zeroand Normal Situations of Cell Membrane Potential	2021 Anal. Chem.
 Car-py	Cell Membrane	Visually plasma membranes	2021 Sens. Actuators B Chem.
 Diphenthio ER1	Endoplasmic Reticulum(ER)	inducingcellularstressand mitochondria-mediatedapoptosisincancercells	2018 J.Mater.Chem.B
 ECPI-12	Mitochondria	long-term mitochondria visualization and tracking	2018 Chem.Sci.
 TVP	Cell Membrane	Visually plasma membranes	2019 Mater.Horiz.
 DBF	Nucleic acid	Fluescence imaging of the viscosity of live cells and tissues	2016 Chem.Sci.
 MeTTPy	Mitochondria	Fluorescence-imaging-guided photodynamic therapy	2018 Adv. Mater.

 TPE-OM	Mitochondria	Photodynamic therapy	2020 J.Am.Chem.Soc.
 Mito-Q	Mitochondria	Two-photon fluorescence imaging of mitochondria viscosity	This Work

Commands for theoretical calculation of target molecules

Mito-Z:

```
%nproc=6
%chk=1.chk
%mem=6000MB
# opt b3lyp/6-31g scf=xqc
Mito-Z
0 1
 C     6.99749400    1.03942200   -0.24319600
 H     7.14286300    1.58666100    0.54415300
 H     7.38224600    1.50740800   -1.00064500
 C     5.49930900    0.90221700   -0.46891200
 H     5.13268000    1.77529300   -0.67817800
 H     5.34952000    0.32809500   -1.23541800
 C     4.76089500    0.33220900    0.72250100
 H     5.10358300   -0.55262500    0.92096700
 H     4.91490100    0.89524900    1.49678500
 C     2.45245900    1.13465700    1.00759800
 H     2.78118800    1.79651600    1.57318900
 C     1.11293900    1.07247400    0.74968400
 H     0.54337500    1.69892600    1.13371200
 C     0.58345900    0.07878700   -0.08709500
 C     1.50565500   -0.80735100   -0.64984300
 H     1.20792100   -1.47403800   -1.22587100
 C     2.83194800   -0.70818000   -0.36958800
 H     3.42502900   -1.31342000   -0.75269700
 C    -0.83329400   -0.08266900   -0.35370200
```

H	-1.10118600	-0.86307900	-0.78462600
C	-1.78127500	0.79488500	-0.03191000
H	-1.49232000	1.59339800	0.34647600
C	-3.21553900	0.64297400	-0.20477400
C	-4.06413800	1.72444000	0.00929400
H	-3.69101600	2.54743800	0.23291000
C	-5.43093100	1.62601100	-0.09635900
H	-5.95479200	2.38406200	0.02732200
C	-6.04886300	0.40084200	-0.38699600
C	-5.19179100	-0.69475300	-0.61149800
H	-5.55954000	-1.52172900	-0.82199900
C	-3.83137800	-0.57173400	-0.52812700
H	-3.30056700	-1.31726500	-0.69106000
C	-8.29660500	1.41302600	-0.28363900
H	-7.94268000	1.99930200	0.40383000
H	-9.16986800	1.10711000	0.00822400
C	-8.03721400	-1.04306500	-0.66131500
H	-7.56824900	-1.48543600	-1.38540500
H	-8.95422600	-0.90624900	-0.94367600
C	-8.02887100	-1.94638500	0.54789400
H	-8.32659400	-2.82968000	0.27810600
H	-7.11655300	-2.02859300	0.86756600
N	3.30511800	0.25005300	0.45240300
N	-7.41574900	0.26264700	-0.42314400
O	7.36555000	-1.37622300	-1.07394200
O	7.50541100	-0.98895400	1.28862000
O	9.26232900	-0.20335300	-0.16976700
O	-8.84532100	-1.50013700	1.61775300
H	-9.61158700	-1.76867600	1.50647800

```
 S                      7.86122600   -0.50089800   -0.03097400
 H                     -8.40862700    1.96928900   -1.19079000

--link1--
%nproc=6
%chk=1.chk
%mem=6000MB
# td=(singlets,nstates=50,root=1) b3lyp/6-31g(d) scf=xqc guess=read geom=allcheck
```

Mioto-H:

```
%nproc=6
%chk=1.chk
%mem=6000MB
# opt b3lyp/6-31g scf=xqc
Mioto-H
0 1
 C                     -7.29981100   -1.12809100   -0.09328900
 H                     -7.44572800   -1.53696000    0.77392200
 H                     -7.66424100   -1.72605000   -0.76441500
 C                     -5.80151700   -0.99834100   -0.32266100
 H                     -5.41472700   -1.88600900   -0.37660500
 H                     -5.65201400   -0.55935400   -1.17385000
 C                     -5.09182900   -0.21852900    0.76263400
 H                     -5.45476100    0.67941800    0.80420000
 H                     -5.24587900   -0.64536900    1.61956100
 C                     -2.77236600   -0.91022500    1.20504400
 H                     -3.09611400   -1.47364500    1.87119600
```

C	-1.43074200	-0.86332900	0.95495300
H	-0.85453700	-1.40295900	1.44598300
C	-0.90893000	-0.01454700	-0.03259200
C	-1.84020200	0.74291400	-0.74768400
H	-1.54738400	1.30864100	-1.42527900
C	-3.16819600	0.66365100	-0.46915200
H	-3.76750500	1.18202300	-0.95593000
C	0.50807000	0.13038000	-0.30726400
H	0.76670800	0.83210500	-0.86151100
C	1.46843700	-0.65891500	0.16921000
H	1.18986000	-1.38784600	0.67452900
C	2.90177500	-0.50711400	-0.01127600
C	3.76837700	-1.51776000	0.39259100
H	3.40838100	-2.29890300	0.74863600
C	5.13434500	-1.40873600	0.28668300
H	5.67122900	-2.12317300	0.54301200
C	5.73210500	-0.23734100	-0.20103200
C	4.85690500	0.78527200	-0.61767200
H	5.21126000	1.57244000	-0.96152800
C	3.49812200	0.64838200	-0.52947400
H	2.95510600	1.34367500	-0.82243300
C	7.99766500	-1.16783100	0.09724400
H	7.64547200	-1.63666800	0.87038600
H	8.86040000	-0.79779200	0.34243100
C	8.18912500	-2.15171000	-1.02561600
H	7.33355700	-2.41494500	-1.36983000
H	8.65397000	-2.92564300	-0.69685000
H	8.70394400	-1.74352500	-1.72495900

C	7.69531800	1.18237600	-0.69485500
H	7.22824000	1.48518000	-1.48862700
H	8.61884400	1.01979700	-0.93985300
C	7.65163200	2.27724100	0.34322200
H	7.93572900	3.10831300	-0.06938500
H	6.73333700	2.39244500	0.63431200
N	-3.63420000	-0.15147000	0.49841400
N	7.09637600	-0.07735000	-0.24520200
O	-7.70343400	1.10306200	-1.32616700
O	-7.86980800	1.11901700	1.06617500
O	-9.58957200	0.05919500	-0.25670400
O	8.46116200	2.03686100	1.48217100
H	9.22350600	2.29937400	1.33527900
S	-8.19677400	0.40660200	-0.15517200

--link1--

%nproc=6

%chk=1.chk

%mem=6000MB

td=(singlets,nstates=50,root=1) b3lyp/6-31g(d) scf=xqc guess=read geom=allcheck

Mito-Q:

%nproc=8

%chk=1.chk

%mem=8000MB

opt td(singlets,nstates=6,root=1) b3lyp/6-31g

Mito-Q

0 1

C	7.93355600	-2.08929900	0.44455400
H	7.08766900	-2.48349600	0.70844700
H	8.42566700	-2.75758700	-0.05849700
C	7.66249400	-0.91046400	-0.45150500
H	8.50420000	-0.47561400	-0.65555600
H	7.28967700	-1.23451700	-1.28640900
C	7.22001300	2.50996700	-0.17986300
H	6.29790700	2.61898000	-0.45719500
H	7.46894100	3.29354200	0.33462600
C	7.32586700	1.29638800	0.69781200
H	8.26154300	1.13598000	0.89702900
H	6.87390700	1.47817500	1.53652900
C	5.39407900	-0.10378700	0.05894400
C	4.51101000	0.81479100	0.65600100
H	4.85827700	1.53442300	1.13350100
C	3.15394800	0.67381400	0.55049300
H	2.60074000	1.30082700	0.95568400
C	2.57560000	-0.39465900	-0.15369100
C	3.44914100	-1.32351900	-0.71491600
H	3.09780400	-2.04870500	-1.17852200
C	4.81316200	-1.20184000	-0.60721300
H	5.36156900	-1.85435500	-0.97865400
C	1.13952000	-0.53451000	-0.34113700
H	0.86646900	-1.19462600	-0.93606100
C	0.17013400	0.16922300	0.23861100
H	0.42205700	0.79747700	0.87586700
C	-1.24257200	0.03865400	-0.04026900

C	-1.75870200	-0.70697400	-1.11040400
H	-1.17763300	-1.17432100	-1.66608600
C	-3.10813100	-0.75576700	-1.34872300
H	-3.43033900	-1.24867600	-2.06831700
C	-3.50747300	0.62281500	0.49020400
H	-4.11050000	1.07635800	1.03461100
C	-2.18255300	0.69842500	0.75756300
H	-1.89130700	1.19834800	1.48560400
C	-5.43768400	-0.15885400	-0.80486200
H	-5.59886500	-0.46048400	-1.71215900
H	-5.81735800	0.72888300	-0.71080100
C	-6.11667800	-1.10228400	0.16797800
H	-5.93831400	-0.80366000	1.07292200
H	-5.73192300	-1.98692300	0.06786100
C	-7.62133200	-1.19264600	-0.03415000
H	-7.97068300	-1.88726000	0.54566500
H	-7.79620400	-1.45899700	-0.95030200
N	6.75448900	0.07616900	0.10906200
N	-3.96979700	-0.09912800	-0.55347200
O	-8.21672700	1.21049000	-0.80827100
O	-7.96062000	0.83137900	1.54135900
O	-9.89817600	-0.02936800	0.40053800
S	-8.49554000	0.31674900	0.29722900
O	8.04287400	2.43497700	-1.33376100
H	7.77091100	2.96460200	-1.89828800
O	8.66406800	-1.76461800	1.60267000
H	9.41984600	-1.52536100	1.39085900

References:

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2. A. D. Becke, Density-functional thermochemistry. III. The role of exactexchange, *J. Chem. Phys.*, 1993, **98**, 5648-5652.
3. T. G. Gray, C. M Rudzinski, E. E. Meyer, R. H. Holm, D. G. Nocera, Spectroscopic and photophysical properties of hexanuclear rhenium(III) chalcogenide clusters. *J Am. Chem. Soc.*, 2003, **125**, 4755-4770.