Electronic supplementary information (ESI)

Matrix targeted fluorescent probe to monitor mitochondrial dynamics

Madhu Ramesh,[a] Kolla Rajasekhar,[a] Kavya Gupta,[c] Vardhaman Babagond,[a] Deepak Kumar Saini,[c] and Thimmaiah Govindaraju*[ab]

[a.] *New Chemistry Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur P.O., Bengaluru, 560064 Karnataka, India.*

[b.] *VNIR Biotechnologies Pvt. Ltd., Bangalore Bioinnovation Center, Helix Biotech Park, Electronic City Phase I, Bengaluru 560100, Karnataka, India.*

[c.] *Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bengaluru 560012, India.*

**Corresponding author – Email: tgraju@jncasr.ac.in*

Table of contents

Experimental Procedures

1. Reagents and apparatus

All the chemicals and reagents were purchased from Merk or Spectrochem and used without further purification unless mentioned. The commercial fluorescent probes (Mitotracker Green FM, Lysotracker Blue DND, Mitotracker orange and Rho 123) from Invitrogen (Thermo Fisher Scientific). All the media and reagents for cell culture were purchased from Gibco (Thermo Fisher Scientific) or HiMedia and plastic wares from NEST, SPL life sciences or Eppendorf. The absorbance and fluorescence spectral measurements were performed in Agilent Cary series UV−Vis-NIR absorption and Agilent Cary eclipse fluorescence spectrophotometers, respectively. NMR spectra were recorded in Bruker AV−400 spectrometer and HRMS spectra by Agilent 6538 UHD HRMS/Q-TOF high-resolution spectrometer. The microscopy imaging and measurements were carried out using Leica DMi8 fluorescent microscope with stage top incubator for live cell imaging and IX83 microscope (Olympus, Japan). The incubator is maintained at 37 °C and 5% CO_2 with humidification for all culture and experiments. Huygens essential software is used for the deconvolution of images and ImageJ for all image quantification analysis. Olympus FV3000 confocal microscope was used for photostability experiments. Origin 8.5 or Prism 6 software was used to process and analyze the raw data.

2. Synthesis of Mito-TG

Synthesis of cyno-2-(2-methyl-4H-chromen-4-ylidene)malononitrile (3). 1-(2-Hydroxyphenyl)ethanone (10.0 g, 73.45 mmol) was suspended in ethyl acetate (200 mL). The solution was purged with nitrogen for 10 min and stirring was continued under nitrogen atmosphere. Sodium (8.0 g, 0.34 mmol) was added and the reaction mixture was stirred for 4 h at room temperature (RT). The grayish-green solid was filtered and resuspended in 200 mL deionized water followed by adjusting the solution pH to neutral. The aqueous solution was extracted with ethyl acetate (200 mL) and the collected organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated to yield the crude product **1** as a brown solid (Yield: 7.2 g, 55%). The crude product **1** was used for the next step without further purification. The compound 1 (7.2 g) was suspended in acetic acid (10 vol) and sulfuric acid was slowly added. The reaction mixture was refluxed at 120 °C for about 30 min and poured into ice cold water (800 mL), followed by adjusting the solution pH to neutral using Na_2CO_3 solution. The reaction mixture was extracted with DCM twice and the organic layers were dried over Na_2SO_4, filtered, and concentrated to yield the final crude product (**2**) as gray solid (4.8 g, 78.8%). **2** and malononitrile (2.40 g, 36.2 mmol) were suspended in 25 mL acetic anhydride, and refluxed at 140 °C for 14 h. The reaction mixture was dried under reduced pressure, resuspended in water (80 mL) and the mixture was refluxed for another 0.5 h. The reaction mixture was extracted with dichloromethane (3 x 100 mL) and the organic layer was dried, and concentrated. The obtained crude product was purified by flash chromatography silica column chromatography (30% ethyl acetate and hexane) to yield compound **3** as an orange solid (2.3 g, 35.93 %).[1]

Synthesis of (4-(5-(diethylamino)-2-formylphenoxy)butyl)triphenylphosphonium (4). To the solution of 4-(diethylamino)salicylaldehyde (500 mg, 2.59 mmol) dissolved in acetonitrile (20 mL) triethylamine (1.08 mL, 7.77 mmol) was added and stirred for 15 min at RT. (4-Bromobutyl)triphenylphosphonium bromide (1.48 g, 3.1 mmol) was added, and

refluxed at 75 °C for 6 h under argon atmosphere. The reaction mixture was dried under reduced pressure, resuspended in water (50 mL) and stirred for 90 min. The reaction mixture was extracted with dichloromethane (3 x 80 mL) and the organic layer was dried, and concentrated. Deep yellow coloured sticky product (**4**) was obtained, the crude product was used without any further purification.

Synthesis of Mito-TG. To the solution of **3** (100 mg, 0.48 mmol) and (4-(5-(diethylamino)-2-formylphenoxy)butyl)triphenylphosphonium (266 mg, 0.57 mmol) in toluene (15 mL), piperidine (0.23 mL) and acetic acid (0.23 mL) were added, and refluxed at 115 °C for 3 h under argon atmosphere. The reaction mixture was concentrated and the crude product was purified by column chromatography (chloroform/methanol: 98/2) to obtain Mito-TG as a crystalline green solid (Yield 23%).

^{1}H NMR (DMSO-*d*6, 400 MHz) δ 8.70-8.68 (m, 1H), 7.76-7.71 (m, 12H), 7.64-7.62 (m, 6H), 7.00 (s, 2H), 6.96 (s, 1H), 6.56 (s, 1H), 6.40-6.38 (m, 1H), 6.19 (s, 1H), 4.24-4.22 (t, J = 40 Hz, 2H), 3.74-3.71 (m, 2H), 3.47-3.71 (m, 4H), 2.08-2.07 (m, 2H), 1.91-1.85 (m, 2H), 1.15-1.12 (m, 6H); ^{13}C NMR (DMSO-*d*6, 100 MHz) δ 160.6, 159.9, 152.6, 152.2, 151.8, 135.2, 133.9, 133.8, 130.6, 124.9, 119.2, 119.1, 118.3, 117.7, 117.1, 116.1, 111.4, 105.5, 104.6, 94.9, 66.3, 56.8, 44.5, 29.3, 29.2, 20.5, 20, 19.2, 13.1; HRMS (ESI-MS): found. 700.3043 [M^+], calcd. 700.3087 for $C_{46}H_{43}N_3O_2P^+$.

3. Photophysical measurements

The compound stock of 10 mM was made in DMSO and aliquots (µL) in buffer were used for further spectral measurements. The absorbance and fluorescence measurements were performed in phosphate buffered saline (10 mM, pH 7.4) and with 0.01% detergents. For all the studies spectroscopy grade solvents were used and all the spectra were acquired at room temperature.

Quantum yield calculation[2]

Fluorescence emission spectra of Mito-TG were measured in PBS as solvent and PBS with isolated mitochondria. Rhodamine B in water (ϕ = 0.31) was used as the standard for quantum yield calculation using absorption of the test sample. The area under the emission spectra was obtained from 580-800 nm. Diluted solutions of probe (submicromolar) were used to minimize reabsorption effects. Fluorescence measurement were made three times for each dye and averaged. Quantum yields were determined using the following formula

$$\phi_{\text{Mito-TG}} = \phi_{\text{Stand}} (F_{\text{Mito-TG}}/F_{\text{Stand}}) \times (A_{\text{Stand}}/A_{\text{Mito-TG}}) \times (n_{\text{Mito-TG2}}/n_{\text{Stand2}})$$

ϕ = Quantum yield, F = Area under fluorescence spectra, A = Absorbance, n = Refractive index

Mitochondria isolation

For photophysical measurements, mitochondria were isolated from cultured cells using reported protocols.[3] Briefly, the cells were cultured in T75 culture flask, harvested by trypsinization and made the cell pellet. The pellet was resuspended in 11 mL hypotonic buffer and incubated for 10 min and monitored for swelling of cells. The swollen cells were homogenized using 15 mL Dounce homogenizer and immediately added 2.5x

homogenization buffer. Cell lysate was centrifuged at 1300 g for 5 min at 4 °C to remove cell membrane debris and nucleus. The supernatant was centrifuged further twice to remove trace of cellular debris. The mitochondria were pelleted by centrifuge at 10500 g for 15 min. The pellet was suspended in buffer of choice (PBS) and used immediately for the experiments.

4. Cell culture

HeLa, A459, MDA-MB-231 and L929 cells were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% Penicilin-streptomycin (PS) in humidified incubator with 5% CO_2 at 37 °C. SH-SY5Y cells were cultured in DMEM F12 (without phenol red) media supplemented with 10% FBS and 1% PS in standard conditions. For imaging experiments, cells were seeded onto 35 mm glass bottom confocal dishes (SPL Life Sciences), cultured for 24 h in standard conditions and used for further experiments.

5. Cytotoxicity assay

The cytotoxicity of the probe Mito-TG was assessed in HeLa and SH-SY5Y cells by MTT assay. Briefly cells were seeded in 96 well plates with cell density of 25,000 cells per well and maintained at 37 °C in 5% CO_2 for 24 h. Cells were treated with different concentrations of Mito-TG (0.1, 0.25, 0.5 and 1 µM) and incubated for 24 h. Followed by treatment with thiazolyl blue tetrazolium bromide (MTT) dye (5 mg/mL) and incubated for 4 h. Media was removed and formazan crystals were dissolved in 100 µL DMSO/methanol (1:1). Absorbance at 570 nm was recorded in a microplate reader and the cell viability was calculated.

6. Transfection protocols

For transfection experiments, $1x10^5$ HeLa cells were seeded in a 12 well plate and allowed to adhere overnight at 37 °C with 5% CO_2. Next day, a cocktail of 100 µL DMEM, 500 ng of mito-PAGFP/Rab14 plasmid (mito-PAGFP was a gift from Richard Youle; Addgene plasmid # 23348; http://n2t.net/addgene:23348; RRID:Addgene_23348)[4,5] along with turbofect (ThermoFischer) was made and incubated at room temperature for 30 min. Cells were washed twice with 1X PBS and fresh DMEM media containing 2% FBS was added. The transfection cocktail was added to the cells and incubated for 48 h at 37 °C with 5% CO_2. Cells were further seeded onto confocal dishes for imaging experiments.

7. Fluorescence microscopy experiments

7.1. Concentration and time dependent staining. HeLa cells were seeded onto glass bottom confocal dishes cultured for 24 h at 37 °C with 5% CO_2 in humidified condition. Cells were incubated with different concentrations of Mito-TG (100 nM, 250 nM and 500 nM) for 15 min in DMEM media. The cells were washed with dPBS (Dulbecco's phosphate buffered saline) to remove unbound probe and immediately imaged in live cell imaging set up (Leica DMi8 system). Further to optimize the time required for staining, the cells were stained with 250 nM of Mito-TG for 15 min, washed with dPBS and immediately imaged in time dependent manner.

7.2. Photobleaching experiment. The HeLa cells were cultured in confocal dishes for 24 h and incubated with Mito-TG (250 nM) for 15 min, followed by dPBS wash (twice) to remove unbound probe. Cells were imaged in media under confocal microscope with continuous

scanning of the same area with line scan mode in single Z-plane. The fluorescence intensity profile of different scans was analyzed to check photostability of probe. Same protocol was followed for the commercially available Mitotracker for comparison.

7.3. Colocalization experiments. HeLa cells expressing mito-PAGFP and Rab14eGFP were seeded in 35 mm glass bottom dishes (NEST) and allowed to adhere overnight at 37 °C with 5% CO_2. After 24 h, cells were washed twice with dPBS and only DMEM containing 250 nM of Mito-TG was added. Cells were incubated for 30 min at 37 °C followed by washing with dPBS. The cells were imaged in dPBS acquired in both GFP (λ_{ex}: 488 nm and λ_{em}: 507 nm) and RFP (λ_{ex}: 554 nm and λ_{em}: 581 nm) channel using IX83 microscope (Olympus, Japan).

For localization experiments of different cells lines, cells were seeded onto 35 mm glass bottom confocal dishes and incubated in standard conditions. After 24 h the cells were incubated with Hoechst 33342 (1 μM) for 1 h to stain nucleus and washed thrice with dPBS followed by MitoTracker Green FM (500 nM) and Mito-TG (250 nM) for 15 min and washed with dPBS to remove unbound probe. The images were acquired in DAPI channel (λ_{ex}: 359 nm and λ_{em}: 461 nm) for nuclear staining, FITC channel (λ_{ex}: 495 nm and λ_{em}: 519 nm) for Mitotracker Green FM and Rho channel (λ_{ex}: 553 nm and λ_{em}: 627 nm) for Mito-TG in Leica DMi8 microscope. The images were deconvoluted by using Huygens essential software. The colocalization analysis was performed using ImageJ software.

7.4. Mitophagy experiments

Absorbance and fluorescence spectra of Mito-TG (10 μM) was recorded in PBS with 0.01% Triton X 100 adjusted to various pH. The ability of Mito-TG to stain the mitochondria in acidic pH was verified in HeLa cells. Cells were seeded onto confocal dishes and maintained under standard conditions. After 24 h cells were washed with dPBS and stained with Mito-TG (250 nM) for 15 min in PBS (10 mM, pH 7.4) and followed by wash. Cells were incubated with PBS (10 mM, pH 7.4) for 30 min and imaged. For acidic pH, cells were stained with Mito-TG (250 nM) in PBS of pH 4.5, incubated cells for 30 min and imaged.

The effect of CCCP on the localisation of Mito-TG was analysed by assessment of colocalisation of with Mitotracker green which is unaffected. The HeLa cells were stained with MitoTG (250 nM) for 20 min followed by dPBS wash twice. Cells were treated with CCCP (20 μM) and incubated for 2 h, without treatment as control and then imaged for colocalisation analysis.

In mitophagy experiments HeLa cells were seeded onto confocal dishes and maintained in standard conditions with DMEM media supplemented with 10% FBS and 1% PS. After 24 h cells were incubated with lysotracker Blue DND (500 nM) for 1 h in complete media and washed with dPBS, stained with Mito-TG (250 nM) for 15 min and washed with dPBS to remove unbound probe. Cells were incubated with serum free media, which results in serum starvation and time lapse imaging was carried out to monitor the mitophagy process.

7.5. Aβ42 mediated mitochondrial changes

Imaging of mitochondria in presence of ROS. To test the effect of ROS on Mito-TG (10 μM), fluorescence was measured in presence of different ROS (1 mM). Different ROS were generated in vitro as reported earlier.[6] To generate ROS *in cellulo*, HeLa cells were treated with varying concentrations of H_2O_2 to arrive at optimum concentration at which cells are

viable. HeLa cells were treated with different concentration of H_2O_2 for 4 h and cell viability was assessed by MTT assay. Up to 500 µM of H_2O_2 and incubation period of 4 h, cells were viable and potentially generate ROS. This concentration was further used to generate ROS *in cellulo*, cells were stained with Mito-TG (250 nM) and imaged for 3 h.

Preparation of Aβ42. Peptide was dissolved in 1 mL of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and incubated at room temperature with intermittent vortex at moderate speed. Solution was sonicated for 20 min in bath sonicator, followed by drying the HFIP/peptide solution under nitrogen gas. Resuspend the peptide in 100% DMSO at higher concentration and vortex it at moderate speed, and immediately used for cell treatment studies by diluting in low serum media.

Aβ42 treatment and mitochondrial imaging. SH-SY5Y cells were cultured in DMEM F12 (Phenol red free) media with 10% FBS and 1% PS in standard conditions and seeded onto glass bottom dishes. After 24 h the cells were incubated with Mito-TG (250 nM), washed with dPBS and treated with Aβ42 (20 µM) peptide. Cells were imaged at different time points to monitor the mitochondrial dynamics. Similarly, SH-SY5Y cells without Aβ42 treatment were imaged as control.

Mitochondria quantification. Mitochondrial counts and perimeter was quantified by using ImageJ software with Mito-Morphology macro developed by Ruben K. Dagda at the University of Pittsburgh and currently maintained and supported by NIH/NINDS R01NS105783-01 and NIH/NIGMS R25 1R25-OD023795-01 grants.[7]

8. Results and Discussion

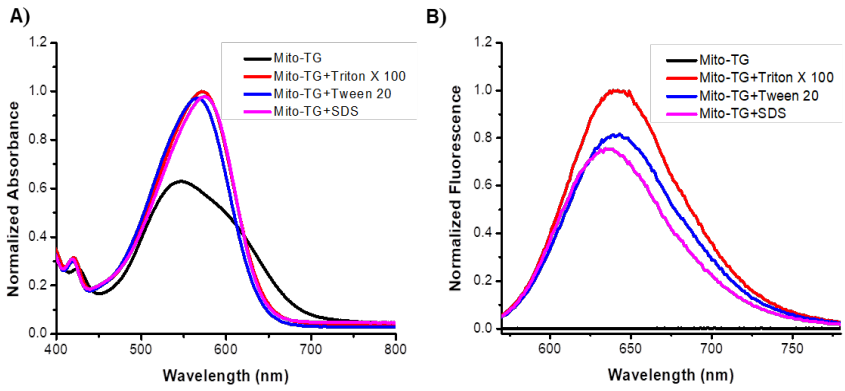


Fig. S1 (A) Absorbance and (B) fluorescence spectra of Mito-TG (5 µM) in PBS and with detergent molecules (0.1%).

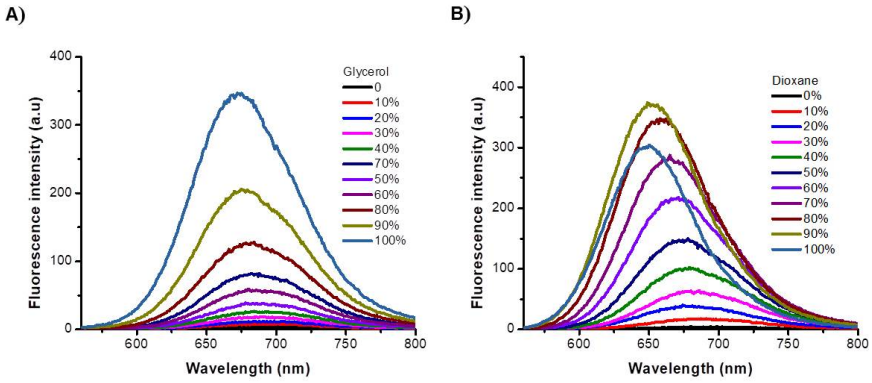


Fig. S2 Fluorescence spectra of Mito-TG (5 µM) with increasing ratios of (A) glycerol in water/glycerol mixtures and B) dioxane in water/dioxane mixtures.

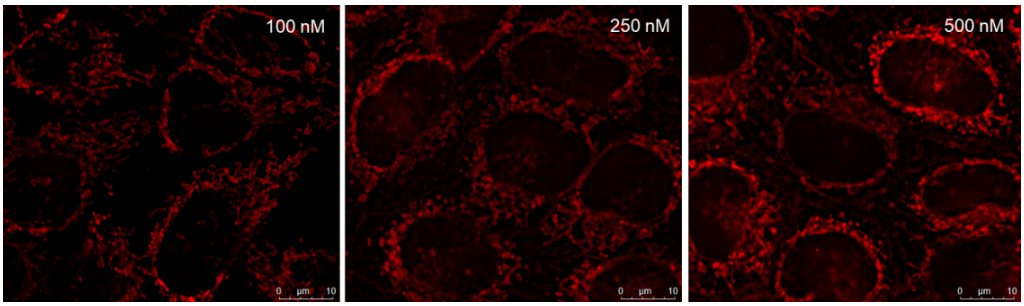


Fig. S3 Fluorescence images of live HeLa cells incubated with different concentrations of Mito-TG for 15 min (Scale bar 10 µm).

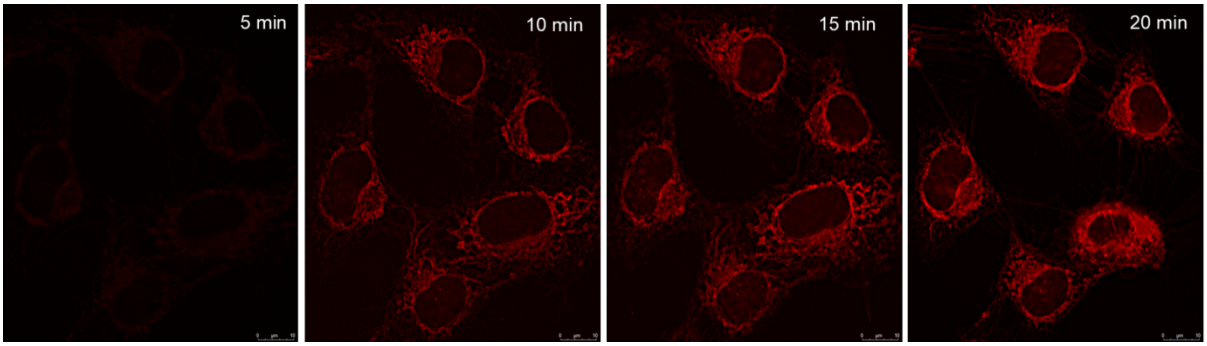


Fig. S4 Time dependent live cell imaging of HeLa cells treated with 250 nM Mito-TG (Scale bar 10 µm).

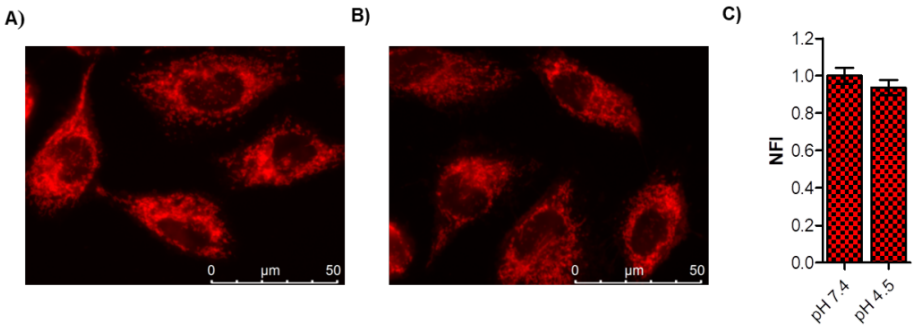


Fig. S5 (A) Fluorescence images of HeLa cells stained with Mito-TG (250 nM) at physiological (pH 7.4) and (B) acidic pH (4.5). (C) Normalized fluorescence intensity (NFI) of Mito-TG stained cells from (A) and (B) shows that fluorescence of Mito-TG is unaffected in acidic pH.

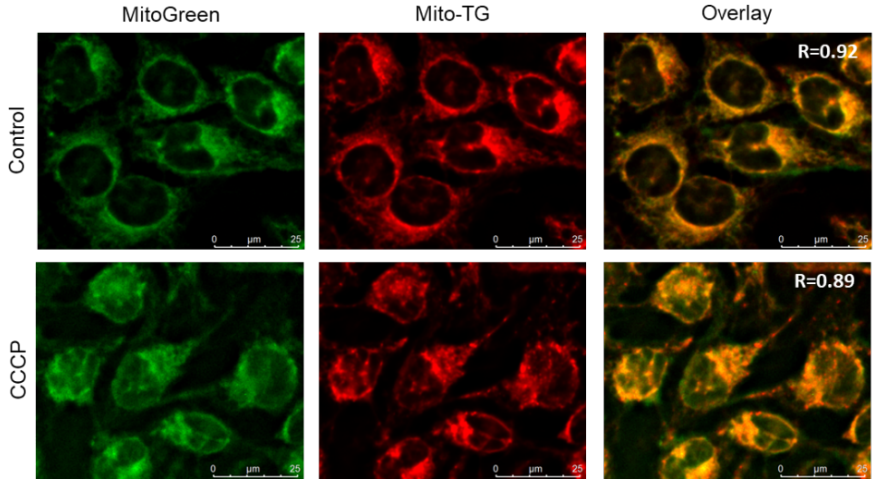


Fig. S6 Colocalisation of Mito-TG and Mitotracker green after treatment of CCCP (20 µM).

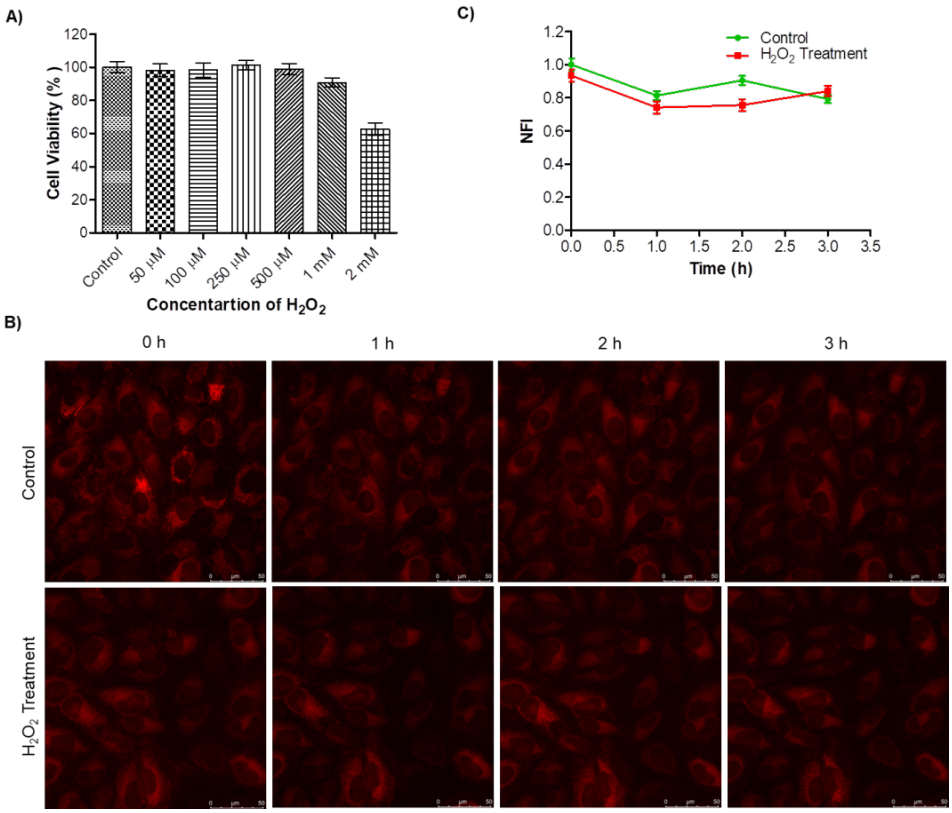
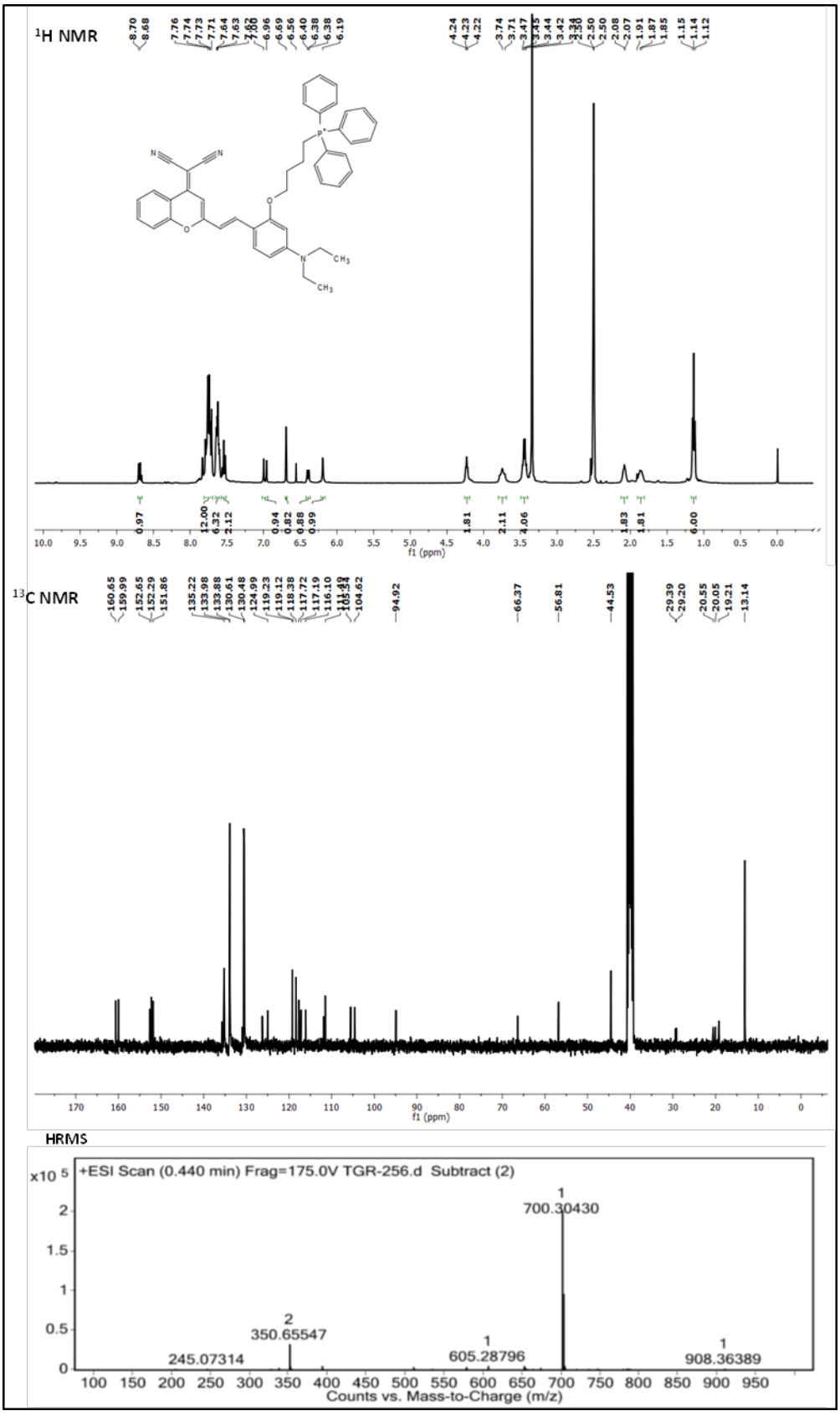


Fig. S7 (A) Viability of HeLa cells treated with different concentrations of H_2O_2 for 4 h. (B) Time dependent live cell images of HeLa cells treated with H_2O_2 (500 µM) for 4 h and then stained with Mito-TG (250 nM) and without H_2O_2 treatment used as control. (C) Normalized fluorescence intensity of cells at different time points from (B)

.

9. Mito-TG characterization data



References

1. K. Rajasekhar, C. J. Achar, T. Govindaraju, *Org. Biomol. Chem*, 2017, **15**, 1584-1588.
2. K. Rajasekhar, N. Narayanaswamy, N. A. Murugan, K. Viccaro, H.-G. Lee, K. Shah, T. Govindaraju, *Biosens. Bioelectron*, 2017, **98**, 54-61.
3. D. A. Clayton, G. S. Shadel, *Cold Spring Harb. Protoc*. 2014, **2014**, pdb.prot080002.
4. M. Karbowski , D. Arnoult , H. Chen , D. C. Chan , C. L. Smith , R. J. Youle, *J. Cell Biol*. 2004, **164**, 493-499;
5. S. Seto, K. Tsujimura, Y. Koide, *Traffic,* 2011, **12**, 407-420.
6. S. Samanta, T. Govindaraju, *ACS Chem. Neurosci,* 2019, **10**, 4847-4853.
7. R. K. Dagda, S. J. Cherra, S. M. Kulich, A. Tandon, D. Park, C. T. Chu, *J. Biol. Chem*, 2009, **284**, 13843-13855.