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Supporting Information

Monitoring glutathione dynamics in the DNA replication (S-phase) using a two-photon reversible ratiometric fluorescent probe

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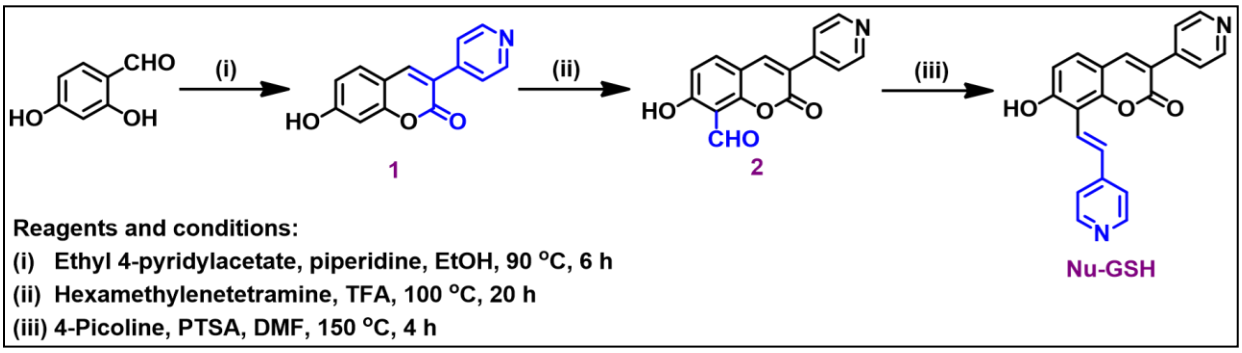
Materials, methods, and instrumentations.

2,4-Dihydroxybenzaldehyde (BLD Pharm, China), ethyl 4-pyridylacetate (TCI, Japan), piperidine (Avra, India), hexamethylenetetramine (SRL, India), p-toluenesulfonic acid (Avra, India), 4-picoline (Avra, India), TFA (Alfa Aesar, USA), ethanol (Honyon International, Inc., China), DMF (Merck, India), $FeCl_2$ (SRL, India), $Zn(OAc)_2.2H_2O$ (SRL, India), $CaCl_2$ (SRL, India), $MgCl_2$ (Merck, India), NaCl (SRL, India), H_2O_2 (Avra, India), diethylamine NONOate diethylammonium salt (Sigma-Aldrich, USA), reduced glutathione (TCI, Japan), alanine (SRL, India), phenylalanine (Spectrochem, India), glycine (SRL, India), leucine (SRL, India), histidine (SRL, India), methionine (SRL, India), cysteine (SRL, India), homocysteine (Sigma-Aldrich, USA), glutathione (GSH) (Sigma-Aldrich, USA), pepsin (SRL, India), trypsin (SRL, India), tyrosinase (SRL, India), sodium sulfide (Na_2S) (Qualigens, India), DL-DTT (SRL, India), thiophenol (SRL, India), heparin (SRL, India), albumin (SRL, India) were purchased commercially and used without further purification. 5-chloromethylfluorescein diacetate (CMFDA) and DMEM medium powder were procured from Sigma-Aldrich. Antibiotics solution (100X) was purchased from Gibco and fetal bovine serum (FBS) and trypsin solution (0.25%) from Himedia. Flash column chromatography was performed using silica gel (100–200 mesh) and analytical thin-layer chromatography was performed using silica gel 60 (per coated sheet with 0.25 mm thickness). Mass spectra were recorded on an anion SpecHiResESI mass spectrometer. NMR spectra were recorded on 400 MHz spectrometer (Bruker, Germany).

Reaction scheme.



Scheme S1. Synthetic scheme for **Nu-GSH**.

Synthesis of compound 1.

To a stirred solution of 2,4-dihydroxybenzaldehyde (2 g, 14.48 mmol) and ethyl 4-pyridylacetate (2.43 mL, 15.92 mmol) in ethanol (40 mL), piperidine (2.86 mL, 28.96 mmol) was added and the resulting mixture was refluxed for 6 h. After completion (checked by TLC), the reaction mixture was cooled and the precipitate was collected by filtration. The crude product was recrystallized in ethanol to afford compound **1 (**1.7 g, yield 49%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.77 (s, 1H); 8.63 (bs, 2H); 8.42 (s, 1H); 7.76–7.75 (d, *J* = 3.88 Hz, 2H); 7.66–7.63 (d, *J* = 8.44 Hz, 2H); 6.87–6.85 (d, *J* = 8.36 Hz, 1H); 6.78 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.64, 160.12, 156.21, 149.99, 143.53, 143.17, 131.05, 122.84, 118.15, 114.98, 111.33, 102.34. ESI-HRMS m/z [M+H$^+$]: calcd, 240.0661; found, 240.0664.

Synthesis of compound 2.

A solution of compound **1** (600 mg, 2.51 mmol) and hexamine (387.12 g, 2.76 mmol) in TFA (7 mL) was heated under reflux for 20 h. After completion of the reaction (checked by TLC), TFA was evaporated. The crude reaction mass was washed with ether and azeotroped with toluene in order to remove the excess TFA. Finally, we afforded 1.39 g of compound **2** as a dark brown liquid having an LCMS abundance of 61% and was taken for the next step without further purification. LCMS (m/z) [M+H$^+$]: 268.0.

Synthesis of probe Nu-GSH.

To a stirred solution of compound **2** (290 mg, 1.09 mmol) in DMF (10 mL), p-toluenesulfonic acid (523.22 mg, 3.04 mmol) and 4-picoline (0.13 mL, 1.30 mmol) were added in a round-bottomed flask at room temperature. Then, the reaction mixture was heated to reflux under an argon atmosphere for 4 h. The cooled reaction mixture was diluted with water (50 mL) and extracted with dichloromethane (3 x 50 mL). The combined organic layer was washed with chilled water, and brine, dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and concentrated under reduced pressure to afford the crude material. The resulting crude material was purified by column chromatography (using SiO_2; eluent: 5–10% MeOH-DCM) to afford **Nu-GSH** (75 mg, yield 20%) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.63–8.57 (m, 3H); 8.44 (d, *J* = 9.1 Hz, 1H); 8.37 (bs, 1H); 7.81–7.80 (m, 1.5H); 7.72–7.70 (m, 2H); 7.64–7.58 (m, 1.5H); 7.54–7.52 (m, 1H); 7.08–7.06

(m, 1H); 6.97–6.92 (m, 1H); 6.85 (d, *J* = 12.0 Hz, 0.5H), 6.70 (d, *J* = 12.2 Hz, 0.5H). ^{13}C NMR (125 MHz, DMSO-d_6): 161.73, 159.85, 159.62, 159.44, 153.54, 152.70, 150.63, 150.14, 149.94, 145.54, 145.35, 143.83, 143.65, 142.72, 131.69, 130.88, 130.43, 130.30, 123.84, 122.86, 122.67, 119.22, 119.06, 114.26, 113.75, 112.18, 112.10, 111.49, 110.47. ESI-HRMS m/z ($M+H^+$): calcd, 343.1083; found, ($M+H^+$): 343.1083.

Preparation of GSH adduct with Nu-GSH.

To a solution of compound **Nu-GSH** (5 mg, 0.014 mmol) in PBS (5 mL) was added GSH (10 mM) and the mixture was stirred for 2 h at room temperature. After 2 h, the aliquot was subjected to HRMS analysis. ESI-HRMS m/z ($M+H^+$): calcd, 650.1921; found, 650.1227.

UV-Vis. and fluorescence spectroscopy.

The UV-absorption and fluorescence spectra were collected from Multiskan SkyHigh Microplate Spectrophotometer (A51119500C) and F4700 spectrofluorometer (Hitachi, Japan) using a 1 cm standard quartz cell. The emission spectra were recorded at an excitation wavelength of 350 nm and emission was monitored over the wavelength range of 460 and 510 nm. The stock solutions of probe **Nu-GSH** (1 mM) and GSH (1 M) were prepared in DMSO. In UV/fluorescence titration, the probe concentration was maintained at 10 μM in phosphate-buffered saline (PBS, DMSO: 0.05%). The stock solutions of various biological analytes, Na(I), K(I), Ca(II), Mg(II), Fe(II), Fe(III), Zn(II), Cu(I), Cu(II), H_2O_2, NO, $^{\cdot}OH$, $O_2^{-\cdot}$, $TBO^{\cdot}$, ClO^-, alanine, phenylalanine, glycine, leucine, histidine, methionine, trypsin, pepsin, tyrosinase, heparin, albumin, DTT, cysteine, homocysteine, H_2S, and PhSH were prepared in PBS and used as per the requirement (100 μM). All spectroscopic data were acquired up to 30 min after the addition of various biological analytes and reduced glutathione in PBS buffer under physiological conditions (pH = 7.4 and temperature 37 °C). The excitation and emission slit width is set at 5 nm.

Cell viability assay.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed using HeLa (human cervical cancer) and WI-38 (human lung fibroblast) cells to prove the suitability of the probe for biomolecule sensing purposes. For this, both the cells were grown on the flat bottom 96 well plates at a seeding density of 1 x 10^5 cells/well. After 24 h, different concentrations of **Nu-GSH** in serum-free DMEM were added to the cells and again incubated for 6 and 24 h respectively. MTT (0.5 mg/mL, Sigma-Aldrich, USA) in HBSS was added to each well and kept at 37 °C, under dark conditions for 4 h to form the formazan crystals. Finally, the crystals were dissolved in DMSO and shaken for 30 min and the OD was measured at 570 nm (BioTek Instruments, USA). Percentage cell viability was calculated by the formula {[treated OD/ control OD] x 100}.

Concentration and time-dependent internalization of Nu-GSH.

HeLa cells were seeded on 96 well plates at a seeding density of 7 x 10^3 cells/well and incubated for 24 h. Then the cells were treated with varying concentrations of GSH probe viz., 5, 10, and 20 μM in serum-free DMEM for 60 min, and images were acquired in both the blue and green channels (Olympus fluorescence microscope IX83).

Similarly, for time-dependent studies, the GSH probe (10 μM) was treated for 15 min, 30 min and 60 min respectively to acquire the images. The fluorescence intensity profile was semi-quantitatively measured using ImageJ software to plot the histogram.

BSO, GSE, H_2O_2 and NEM dependent studies.

In order to study the effect of GSH inhibitor BSO (L-buthionine-sulfoximine), HeLa cells were pre-treated with 5- and 10 mM concentrations of BSO for 4 h and then with **Nu-GSH** (10 μM) for 1 h. At the same time, **Nu-GSH** (10 μM) treated cells were subjected to H_2O_2 (200 μM) and NEM (100 μM) treatment and images were captured by Olympus fluorescence microscope IX83 at different time points and further GSH ester (100 μM) was added to the same wells.

Colocalization studies.

Organelle-specific localization of **Nu-GSH** was observed in HeLa cells. **Nu-GSH** pre-treated cells were incubated with mitotracker red (100 nM for 30 min, Invitrogen), lysotracker red (75 nM for 15 min, Invitrogen), and nuclear tracker red (2 drops/mL PBS for 15 min, Invitrogen) and then colocalization images were collected using the microscope. For time-resolved nuclear colocalization studies, HeLa cells were seeded on the flat bottom 96 well plates (7×10^3 cells/well) and cells were incubated with the probe **Nu-GSH** for 24, 48, and 72 h, succeeded by nuclear tracker red dye treatment. A replica of the experiment was carried out with 2 μM CMFDA probe (15 min) treatment followed by nucleus tracker incubation. The images were acquired using Olympus fluorescence microscope IX83.

Nuclear colocalization in fixed cells.

HeLa cells were treated with probe **Nu-GSH** initially and washed thrice with PBS. Cells were then fixed with 70% methanol for 15 min. After rinsing with PBS cells were treated with 2 μg/mL propidium iodide in PBS. After repeated PBS rinsing cells were imaged under the Olympus fluorescence microscope IX83.

Cell cycle and flow cytometric analysis.

HeLa cells were grown on 6 well culture plates for both cell cycle analysis and flow cytometry of probe **Nu-GSH** treated cells. Cell cycle analysis was performed with the untreated cells at 24 h, 48 h and 72 h post-seeding stages according to the manufacturer's instruction (BD Cycletest™ plus DNA reagent kit). The kit is provided with propidium iodide as a nucleus staining agent. Percentage cells in the S-phase was quantified according to the nuclear DNA content that will get doubled during the S-phase from 2N to 4N (diploid to tetraploid) by analyzing the mean fluorescence intensity using BD FACS DIVA 8.0.3 software. Similarly, in another experiment cells at the same time points were treated with the **Nu-GSH** (10 μM) and subjected to flow cytometric analysis under CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) lasers.

Two-photon imaging.

Two-photon images were recorded using Leica TCS SP5 II Advanced System and a 25× objective lens (obj. HCX PL APO 25× / 1.10 W CORR CS, Leica, Germany). The two-photon excitation wavelength was tuned to 800 nm. Emission light was collected in 400–660 nm.

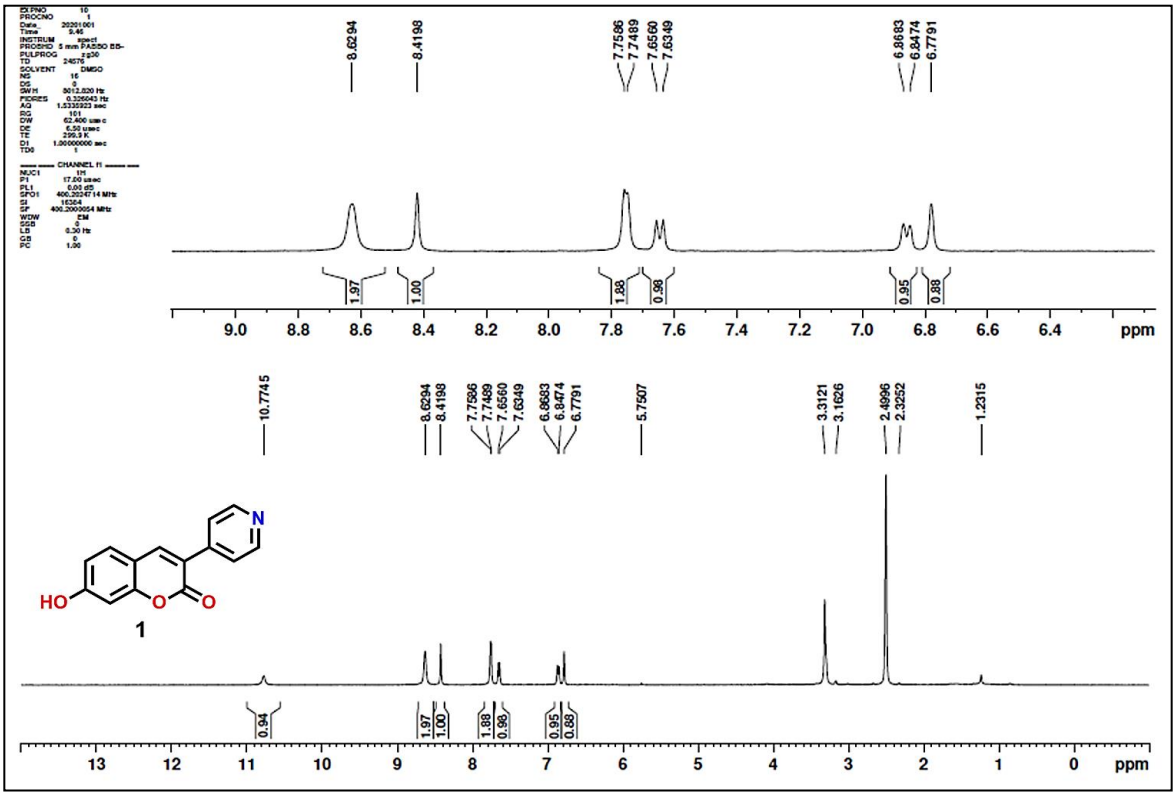


Fig. S1 ^{1}H NMR of compound **1** in DMSO-d_6

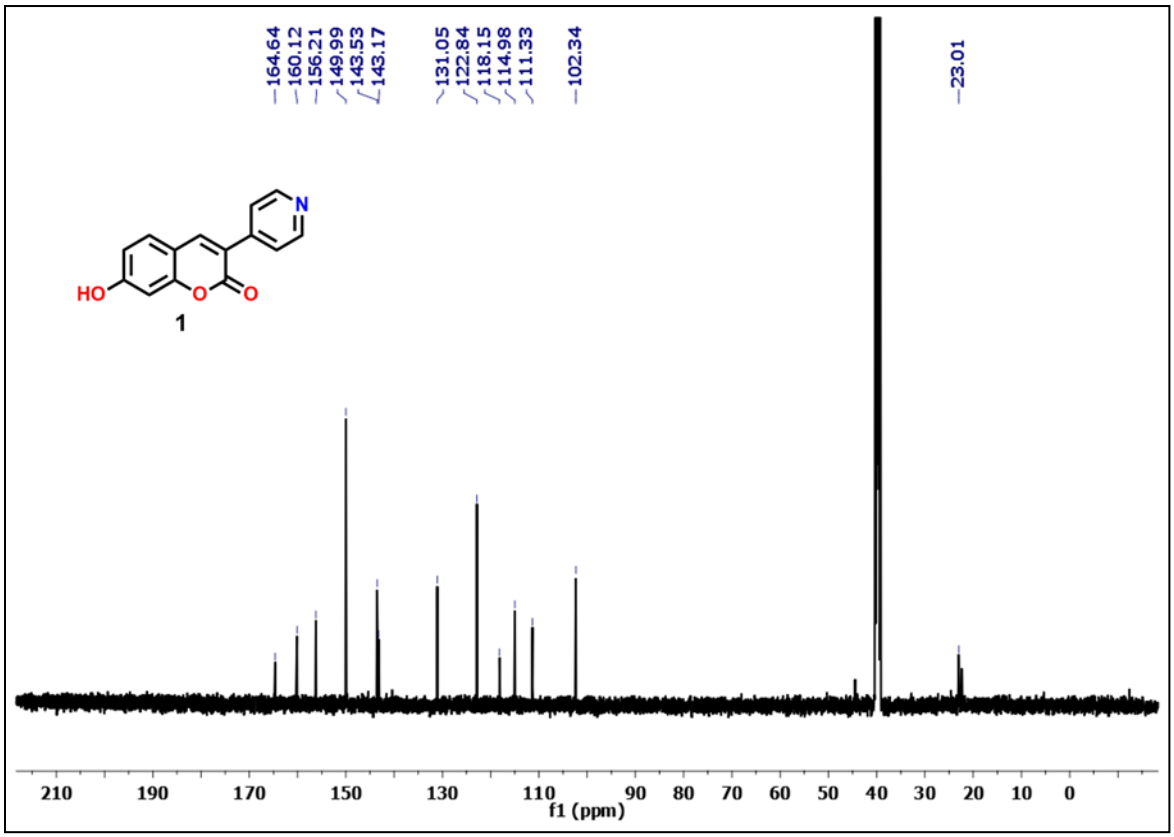


Fig. S2 ^{13}C NMR of compound **1** in DMSO-d_6

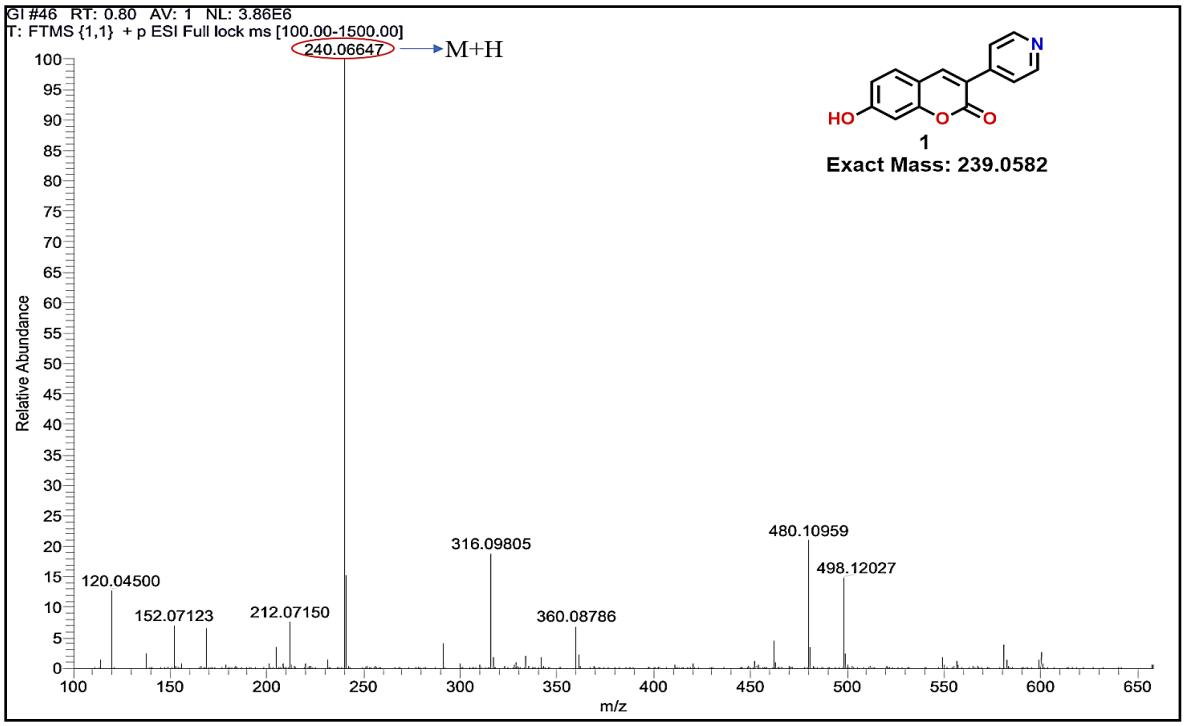


Fig. S3 HRMS of compound **1**

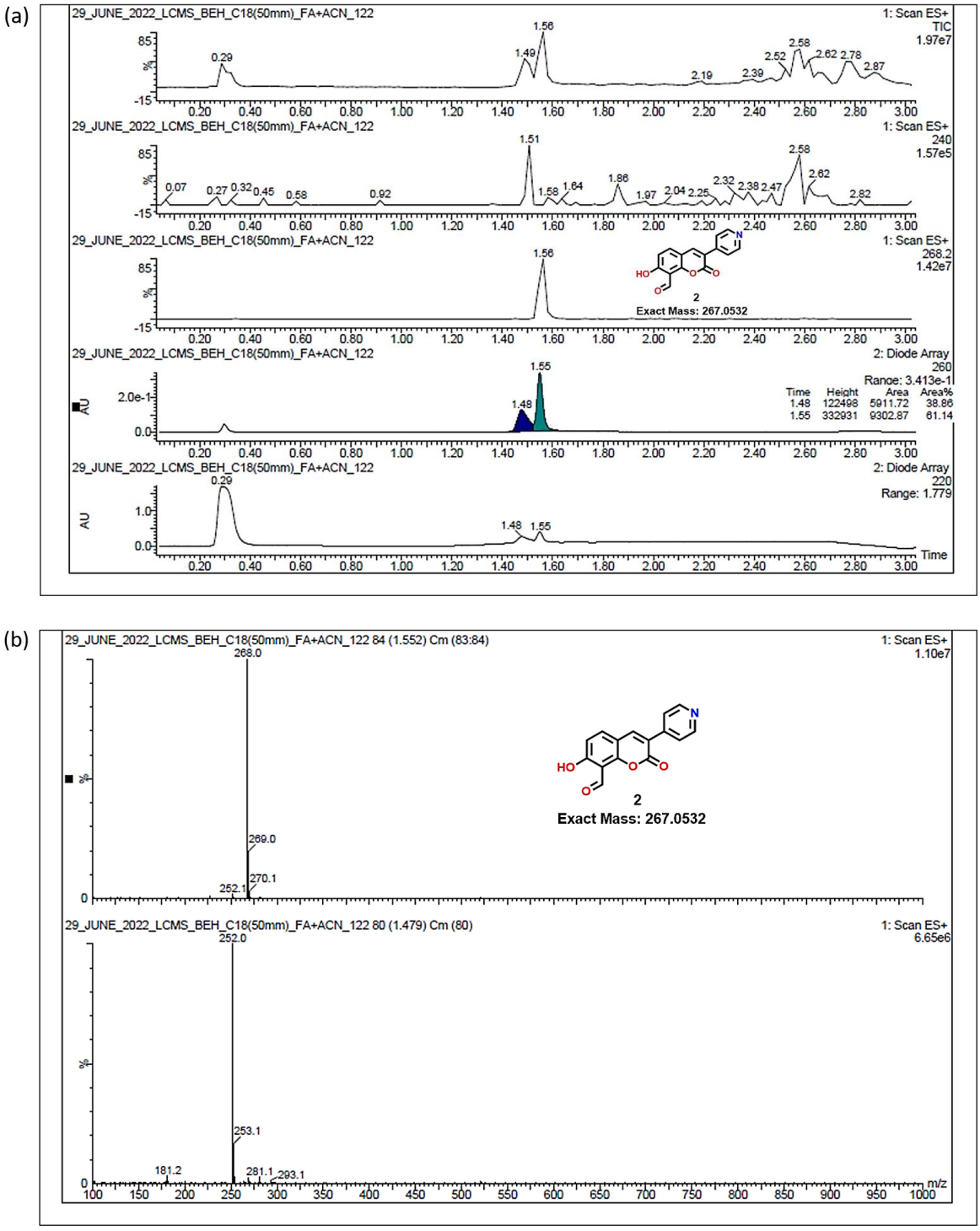


Fig. S4 LCMS of compound **2**. (a) Chromatogram. (b) MS spectrum

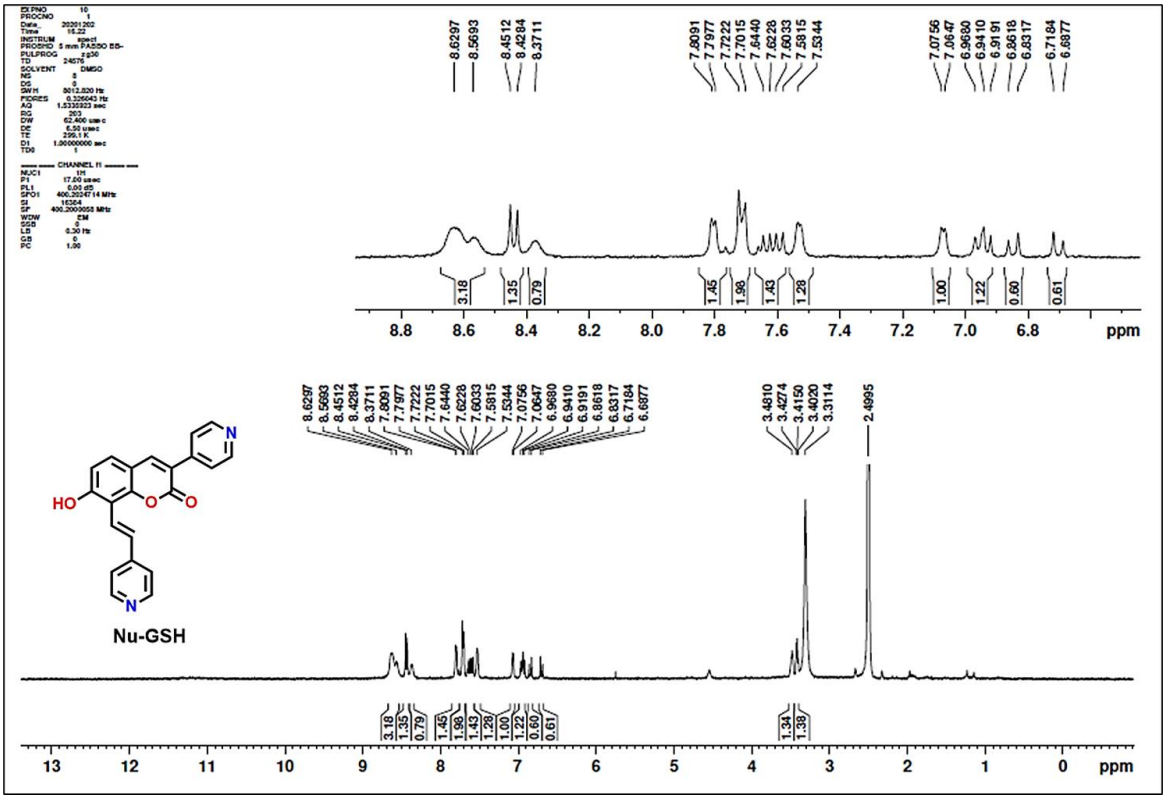


Fig. S5 ^{1}H NMR of probe **Nu-GSH** in DMSO-d_6

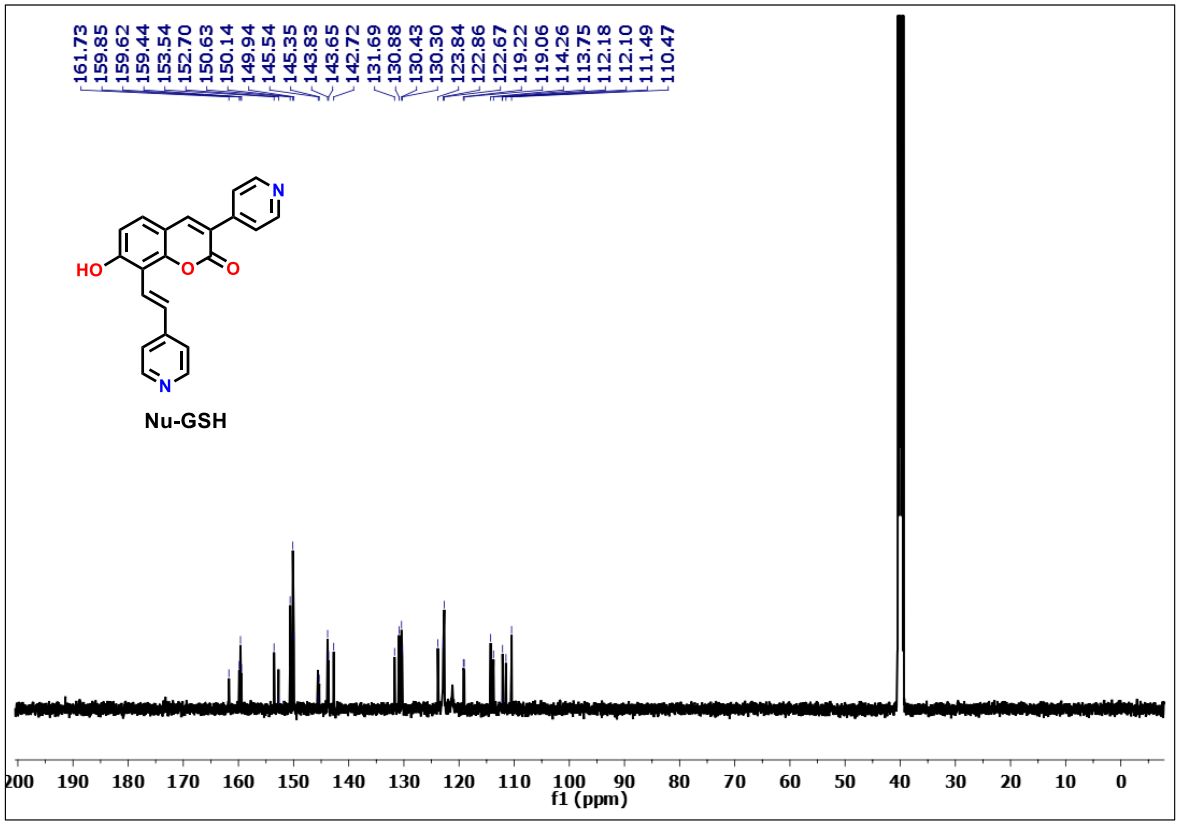


Fig. S6 ^{13}C NMR of probe **Nu-GSH** in DMSO-d_6

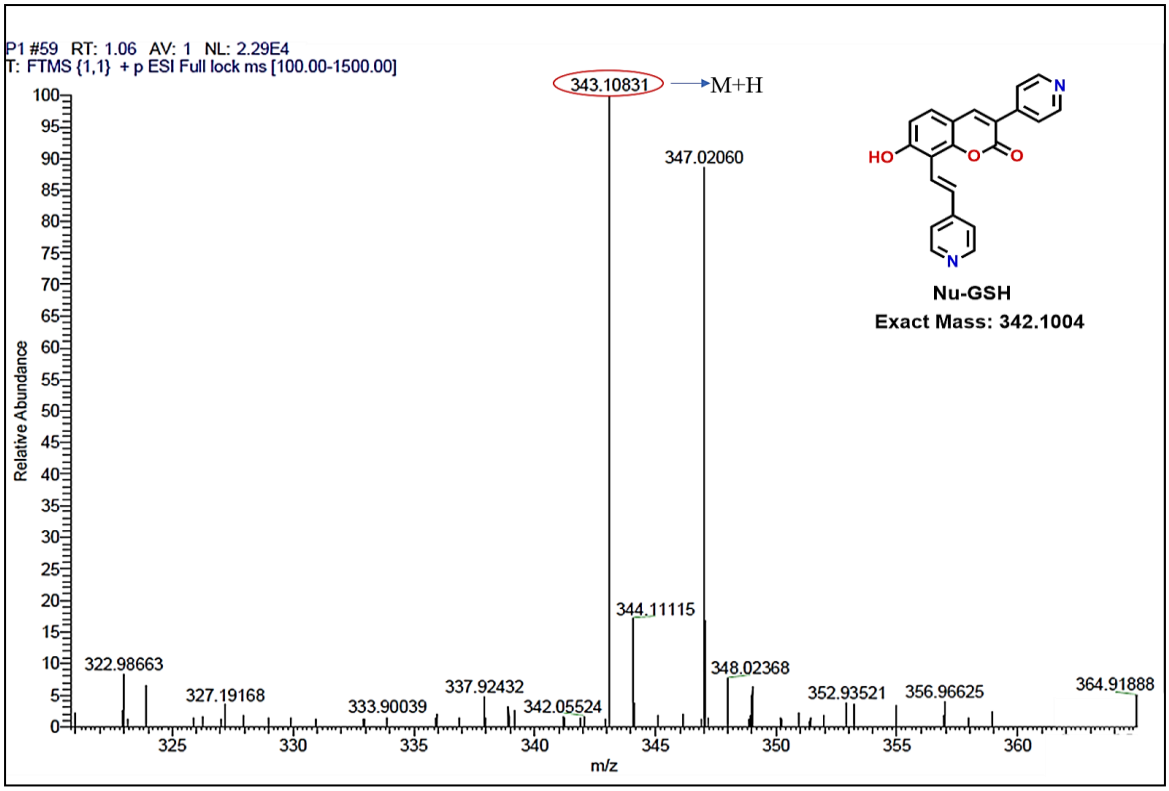


Fig. S7 HRMS of the probe **Nu-GSH**

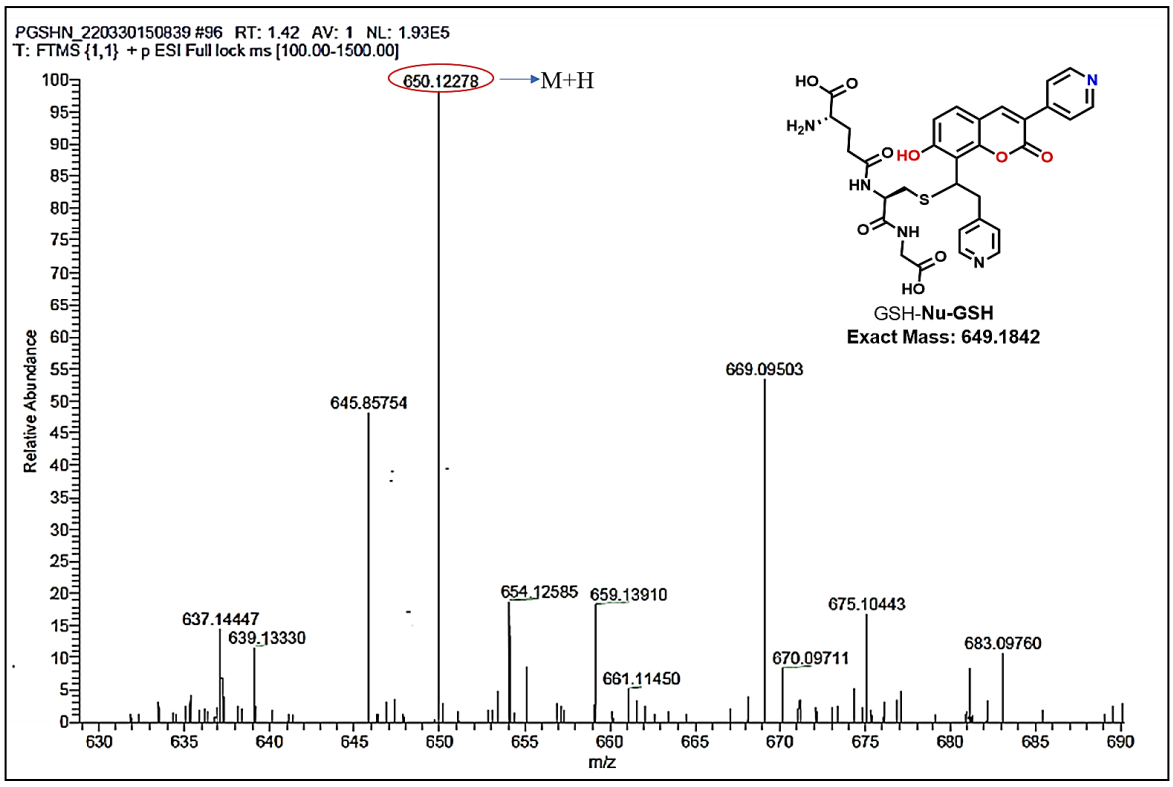


Fig. S8 HRMS of the GSH adduct of probe **Nu-GSH**

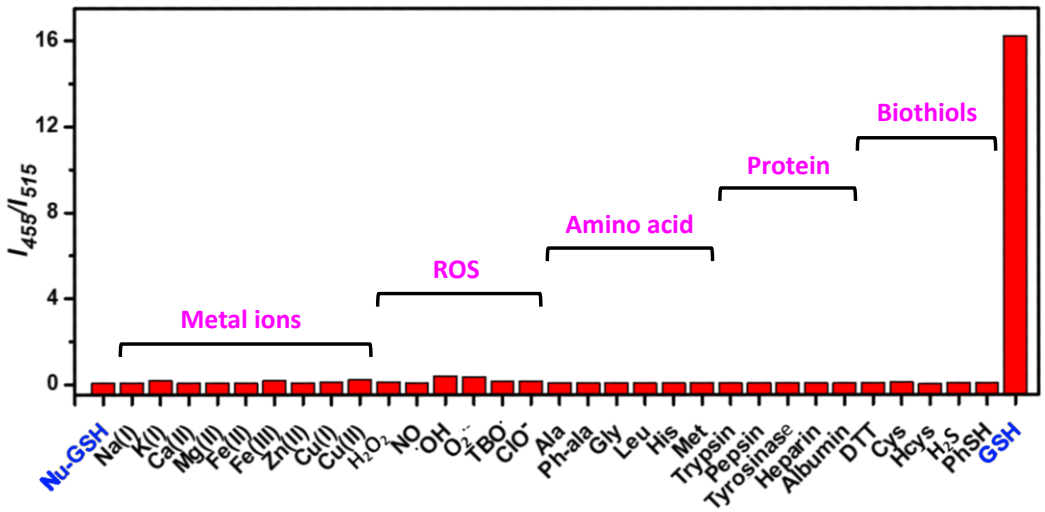


Fig. S9 Fluorescence change of probe **Nu-GSH** in the presence of various analytes. Emission is collected at 455 nm upon excitation at 380 nm.

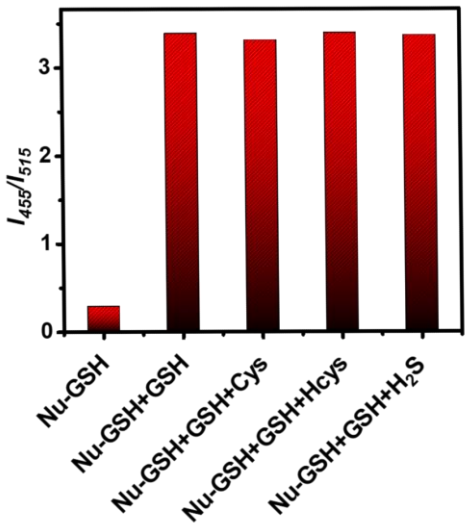


Fig. S10 Fluorescence behavior of GSH adduct of **Nu-GSH** in the presence of other biothiols (Cys, Hcys, and H_2S). The fluorescence data of the GSH adduct were recorded upon incubation with other biothiols (100 μM) for 30 min.

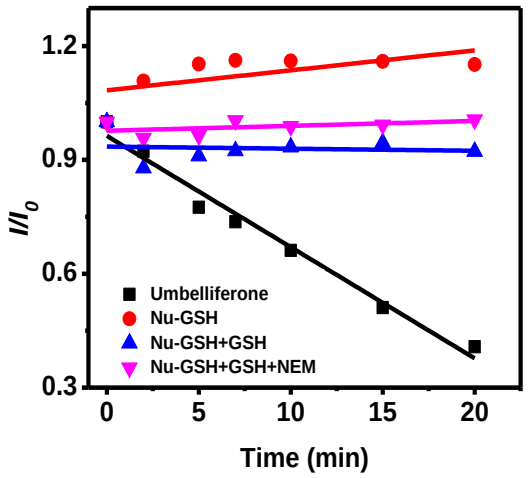


Fig. S11 Photostability of **Nu-GSH** and its GSH adduct in the presence/absence of NEM (1 mM) in comparison to the blue emitting dye Umbelliferone. 10 μM dye was dissolved in PBS 7.4 and irradiated with a 4-Watt 365 nm UV lamp.

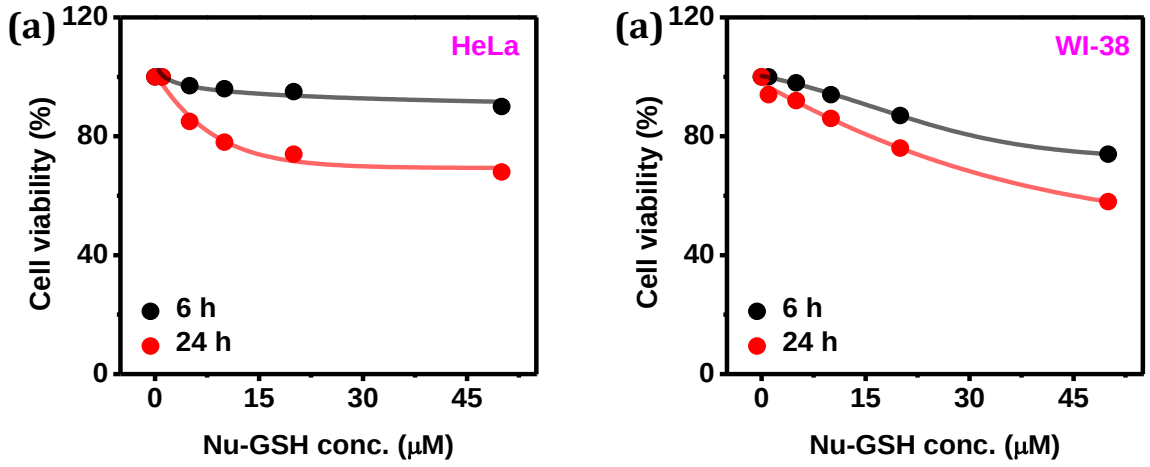


Fig. S12 MTT assay of the probe **Nu-GSH** in HeLa and WI-38 cells: Cells were treated with **Nu-GSH** for 6 h and 24 h to check the effect on cell proliferation.

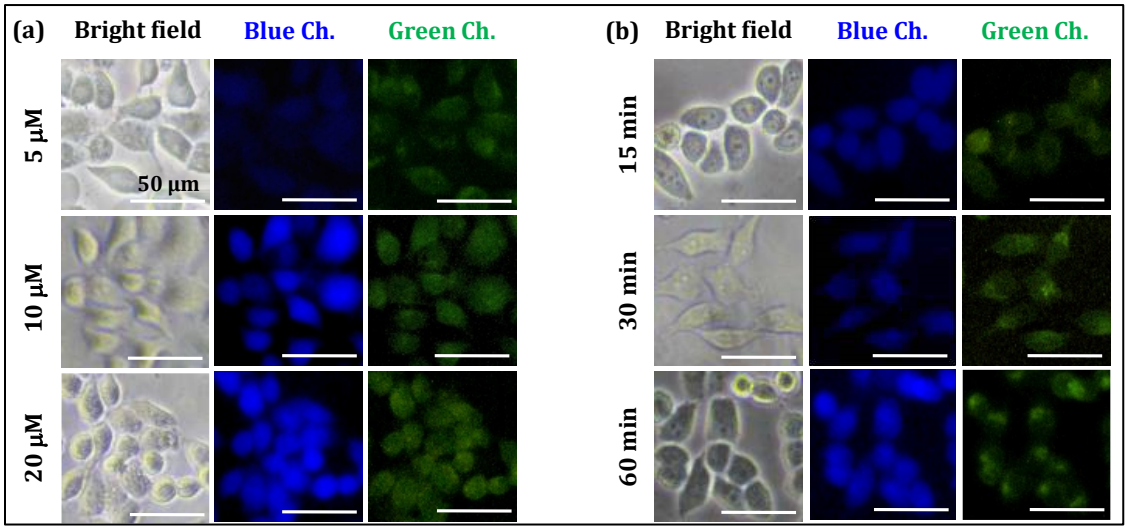


Fig. S13 (a) HeLa cells were incubated with varying concentrations of probe **Nu-GSH** (5–20 µM) for 60 min and images were acquired. (b) Cells were incubated for 15 min to 60 min with **Nu-GSH (**10 µM). Cells were imaged under blue and green channels. Blue channel (Blue Ch.) excitation range, 340–390 nm and emission collection range, 430–490 nm. Green channel (Green Ch.) excitation range, 460–495 nm, emission collection range, 510–550 nm. Scale bar: 50 µm.

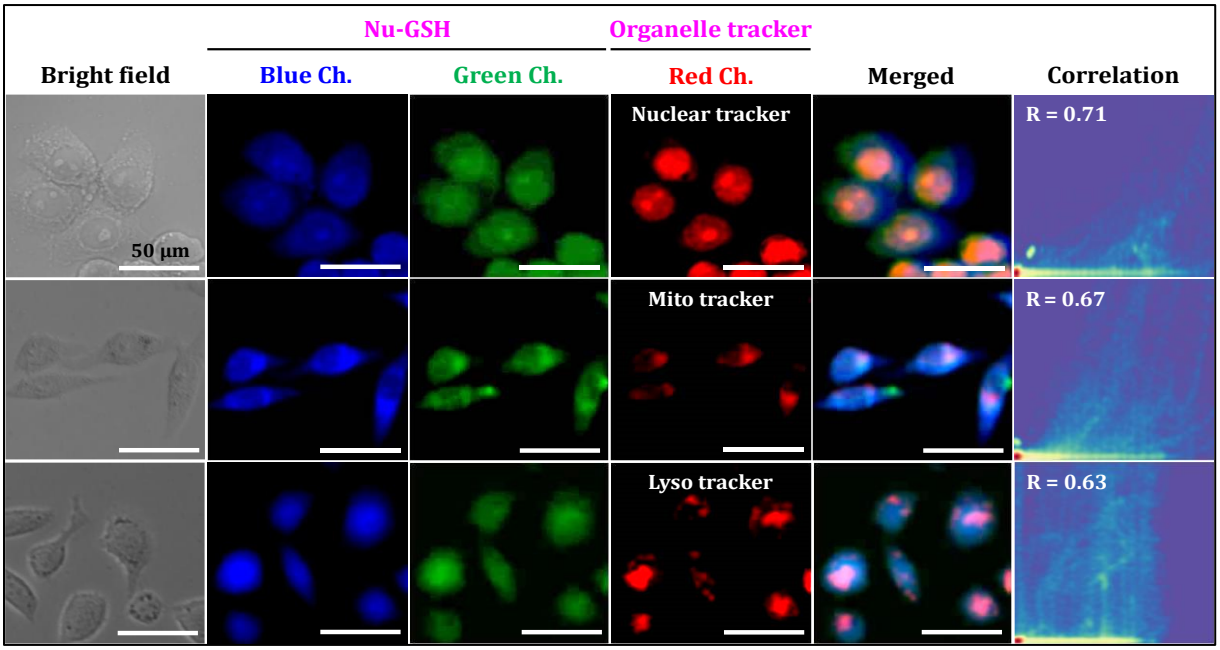


Fig. S14 Nu-GSH treated HeLa cells were again incubated with lysotracker red, mitotracker red and nuclear tracker red. Cells were visualized under the fluorescent microscope and were imaged under blue, green and red channels. Blue channel (Blue Ch.) excitation range, 340–390 nm and emission collection range, 430–490 nm. Green channel (Green Ch.) excitation range, 460–495 nm, emission collection range, 510–550 nm and red channel (Red Ch.) excitation range, 530–550 nm, emission range 575–650 nm. Scale bar: 50 µm.

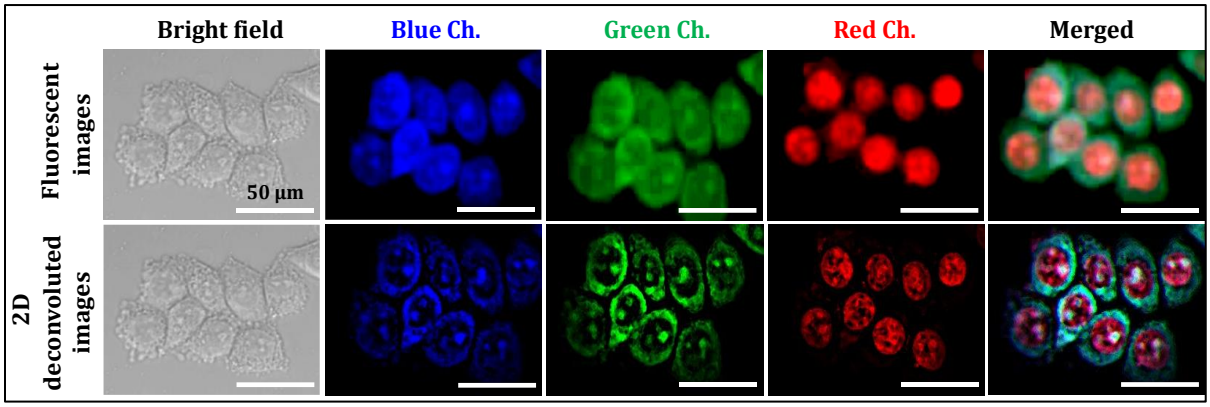


Fig. S15 HeLa cells were pretreated with the probe for 1 h and fixed with 70% methanol followed by propidium iodide (PI) treatment for 10 min. Cells were visualized in the blue, green and red channel under a fluorescence microscope. Blue channel (Blue Ch.) excitation range, 340–390 nm and emission collection range, 430–490 nm. Green channel (Green Ch.) excitation range, 460–495 nm, emission collection range, 510–550 nm and red channel (Red Ch.) excitation range, 530–550 nm, emission range 575–650 nm. Scale bar: 50 µm.

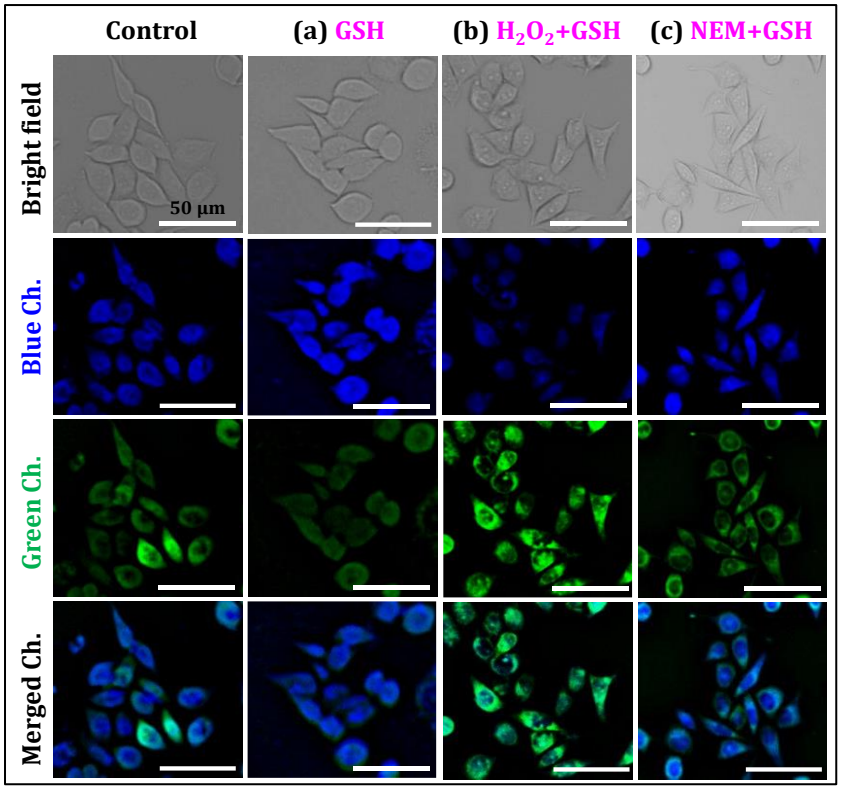


Fig. S16 Cellular GSH dynamics in the presence of GSH scavenger H_2O_2 and NEM. HeLa cells were treated with **Nu-GSH** (10 μM) for 1 h (control). (a) **Nu-GSH** (10 μM) pre-treated HeLa cells were incubated with GSH (100 μM) for 15 min and imaged under the blue and green channels. (b) **Nu-GSH** treated cells were incubated with H_2O_2 (200 μM) and GSH (100 μM) for 15 min to perform the fluorescence imaging. (c) **Nu-GSH** pre-treated cells were incubated with NEM (100 μM) followed by GSH (100 μM). Blue channel (Blue Ch.) excitation range, 340–390 nm and emission collection range, 430–490 nm. Green channel (Green Ch.) excitation range, 460–495 nm, emission collection range, 510–550 nm. Scale bar: 50 μm. The fluorescence intensity profile was calculated using ImageJ software. Data are average ± SD of three analyses.

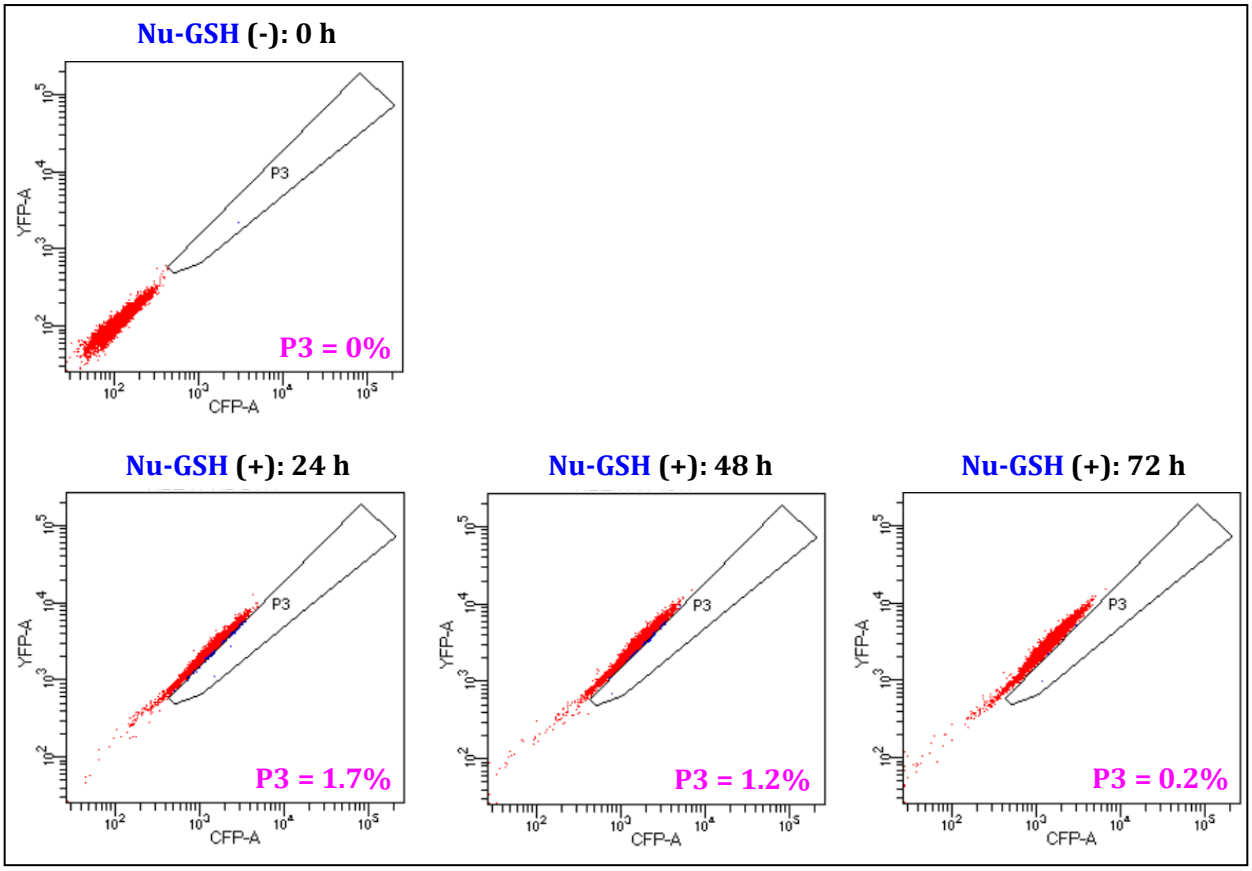
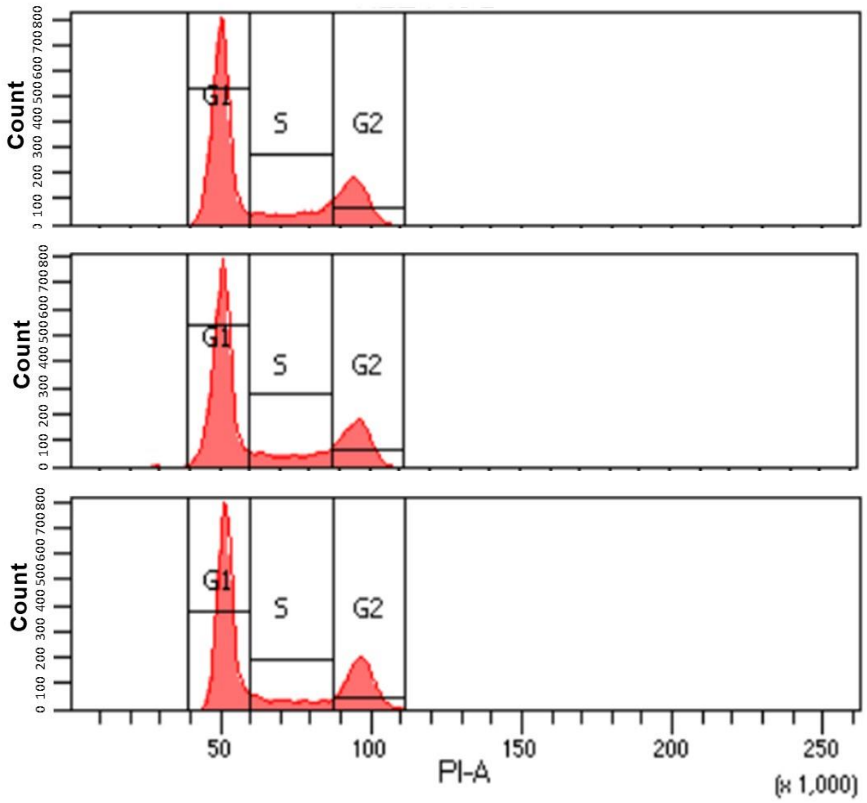


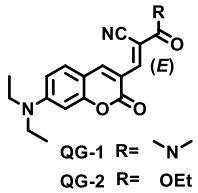
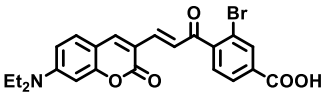
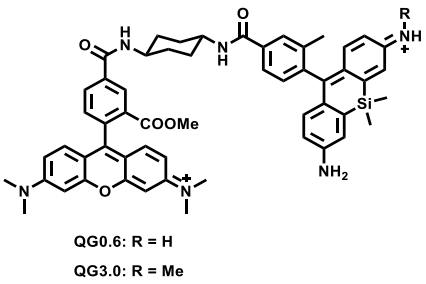
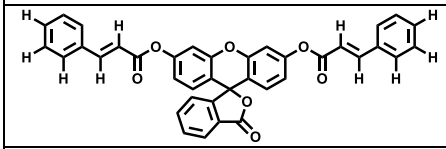
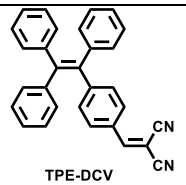
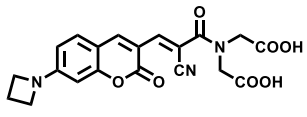
Fig. S17 HeLa cells were seeded and after different time points like 24 h, 48 h and 72 h cells were treated with probe **Nu-GSH** (10 μM) and subjected to flow cytometry analysis with single excitation (405 nm) and dual emission mode (CFP- 485 nm YFP- 515 nm ranges). The ratio plot for CFP (Cyan fluorescent protein, indicating the emission range) vs. YFP (Yellow fluorescent protein, indicating the emission range) was plotted to find out the time point where more blue emissions can be observed from the cells (Area P3, is the subpopulation of cells with more blue fluorescence).

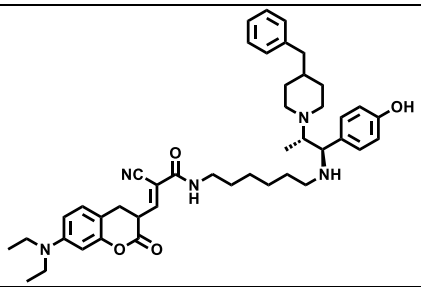
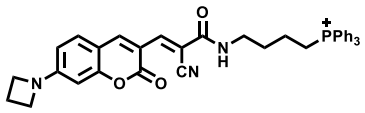
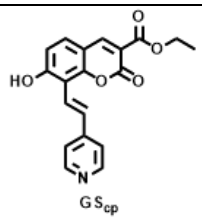
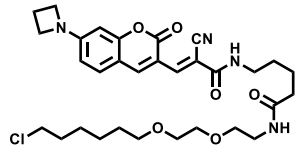
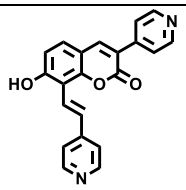


Time point (h)	% Cells in S-phase
24	17.6
48	15.0
72	14.7

Fig. S18. Cell Cycle analysis. HeLa cells were seeded at different time points and cell cycle analysis was performed using BD cycletest™ plus DNA reagent kit to find out the % of cells in the S phase.

Table S1. Comparison between various reported reversible fluorescent probes for GSH and present study

Probe structure	Absorption/Emission ($\lambda_{abs}/\lambda_{em}$)		Two-photon	Target organelle	Bio-relevant application	Reference
	Probe	Probe-GSH				
 QG-1 R= QG-2 R= OEt	474 nm/560 nm	409 nm/488 nm	⊗	Cytosol	Quantitative information on cellular GSH dynamics	*Angew. Chem. Int. Ed.*, **2017**, 56, 5812 (Ref. 17)
 ThiolQuant Green (TQ Green)	479 nm/590 nm	406 nm/463 nm	⊗	Cytosol	Quantification of cellular GSH	*ACS Chem. Biol.*, **2015**, 10, 864 (Ref. 18)
 QG0.6: R = H QG3.0: R = Me	**QG0.6**: 595 nm/617 nm	557 nm/594 nm	⊗	Cytosol	Quantification of cellular GSH	*Nat. Chem.*, **2017**, 9, 279 (Ref. 19)
	QG3.0: 609 nm/632 nm	558 nm/593 nm				
	-	470 nm/530 nm	⊗	Cytosol	Cellular GSH quantification	*Chem. Sci.*, **2018**, 9, 8065 (Ref. 20)
 TPE-DCV	-	415 nm/520 nm	⊗	Cytosol	Application in enzymatic activity assay	*Sci. Rep.*, **2014**, 4, 4272 (Ref. 21)
 RealThiol(RT)	488 nm/562 nm	405 nm/487 nm	⊗	Cytosol	Realtime imaging of cellular GSH	*Nat. Commun.*, **2017**, 8, 16087 (Ref. 23)

	-	400 nm/475 nm	✓	Cytoplasm	Quantifying GSH level near to the N-methyl-D-aspartate receptors	*Chem. Commun.*, **2022**, 58, 3633 (Ref. 24)
 Mito-RealThiol (MitoRT)	488 nm/567 nm	405 nm/488 nm	✗	Mitochondria	Quantitative information on GSH dynamics in mitochondria	*ACS Sens.*, **2017**, 2, 1257 (Ref. 25)
 GS_{cp}	410 nm/510 nm	350 nm/488 nm	✗	Nucleus	Quantitative information on GSH in static nuclei	*Anal. Chem.*, **2020**, 92, 10989 (Ref. 26)
 HaloRT	488 nm/565 nm	405 nm/480 nm	✗	Nucleus	GSH dynamics in nuclei at steady state	*Antioxid. Redox Signal.*, **2019**, 30, 1900 (Ref. 27)
 Nu-GSH	410 nm/515 nm	365 nm/455 nm	✓	Nucleus	Monitoring GSH dynamics in nucleus during DNA replication stage	*This work*