Supporting information for

NIR emissive probe for fluorescence turn-on based dead cell sorting and *in-vivo* viscosity mapping in *C. elegans*

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Materials and Methods

All the chemicals were purchased from professional reagent suppliers. Cyclohexanone, 2,3,3 trimethyl indole, 1,4 butyl sulfonate, phosphorus oxychloride, dimethyl foramide, dichloromethane, and 1,2 dichloro benzene were collected from sigma Aldrich Pvt. Ltd and SD fine limited. The column chromatography solvents hexane, methanol, and dichloromethane were collected from Rankem and Finar Pvt. Ltd. All photophysical experiments were carried out in deionized distilled water.

NMR Spectra Measurement

All synthesized intermediate compounds and Cy-Cl probes were characterized using A Jeol ECX-500 (500 MHz for ^{1}H and ^{13}C spectra using different deuterated solvents). After weighing the 10-12 mg sample, the respective deuterated solvents, such as CD_3OD, DMSO-D_6, and CD_3OD, were added to the sample to record the NMR.

High-Resolution Mass Spectrometry (HRMS)

We prepare 1 mg Cy-Cl probe in 1 mL of HPLC grade methanol and by injecting 0.1-0.2 mL diluted samples in a capillary at 4500 V, high-resolution mass spectrum data were obtained using a Bruker Daltonik GmbH (Model - Impact HD, USA) instrument in positive mode. At 2000 V charging voltage, spectra from 100 m/z to 1250 m/z were seen in active mode. The pressure for dry nebulization was set at 0.5 bars.

Fluorescence Lifetime

The fluorescence lifetime and time-resolved anisotropy decays were assessed using the Horiba scientific Delta Flex TCSPC system with Pulsed LED Sources. Ludox has been employed as an instrument response function for spectral value deconvolution. By tri-exponentially fitting the photon decays in different channels with a chi-squared value < 1.2, we measured the fluorescence lifetime of various concentrations of the viscosity-based water-glycerol mixture.

UV-Visible and Steady-State Fluorescence

The absorption and emission spectra of the probe were recorded using Shimadzu UV-2450 and Cary Eclipse spectrophotometer. All photophysical experiments were performed at room temperature. A slit width kept constant, i.e. 10 nm, was used for excitation and emission in the fluorescence spectrophotometer.

Absolute Quantum Yield

We performed the absolute quantum yield measurements using the integrating sphere approach on a Quanta-φ spectrofluorometer (Horiba) instrument. The probe was ensured to have absorbance < 0.1 at all excitation wavelengths to reduce any artifacts. For a particular wavelength, e.g., for 775 nm excitation, an emission spectrum was taken from 765 nm to 850 nm (scattering and emission part combined) first for solvent (water and glycerol) and then with probe. The same settings were employed for both measurements.

Cell Culture

HeLa, HEK 293A, and Hep G2 cell lines were used for the present study. The cells were separately maintained in DMEM (Dulbecco's minimal essential media, Gibco Invitrogen corporation) containing 10% fetal bovine serum (Gibco-invitrogen corporation) and 1% Anti-Anti (penicillin-streptomycin), 1% HEPES, and 1% NEA (Gibco Invitrogen corporation) and incubated in a humidified incubator at 37 °C and 5% CO_2. After growing cells in the T-25 flask, healthy cells were taken from the T-25 flask and seeded on the coverslip into 6-well plates. Each well was supplemented with 2 mL of the growth medium, and cells were allowed to grow overnight for proper adherence. An optical microscope was used to examine the growth and attachment of the cells to the coverslips.

Treatment with the Cy-Cl Probe

Once the cells had adequately adhered to the coverslip, the probe (60 μM) was incubated in HeLa, HEK 293A, and Hep G2 cell lines for 1 hour. Then, the cells were washed three times with PBS buffer to remove any unbound probes and fixed by incubating with 4% paraformaldehyde solution in 1X PBS buffer for 10 minutes. After that, the coverslips were mounted on a glass slide using a drop of glycerol, and the edges were sealed with wax before imaging.

Cell Death Induction

Apoptosis by H_2O_2: HeLa cells were seeded in confocal 35 mm dishes and kept for proper adherence. Once the cells were adhered and adopted proper morphology, cells were incubated with a probe (60 μM) for 1 hour. Further cells were washed with 1X PBS and replenished with fresh media containing 10 mM of H_2O_2. The live cell to dead cell process was monitored for 6-12 hours.

Ferroptosis by Sulfasalazine and Erastin: HeLa cells were seeded in confocal 35 mm dishes and kept for proper adherence. Once the cells were adhered and adopted proper morphology, cells were incubated with a probe (60 μM) for an hour. Further cells were washed with 1X PBS and replenished with fresh media containing 30 μM of sulfasalazine. The cell death process was monitored for 12 hours[1,2]. For erastin-induced ferroptosis, a 10 μM concentration of erastin was used[3,4].

Confocal Microscopy

Nikon Eclipse Ti inverted microscope was used for the confocal microscopy, and images were acquired using Nikon Nis-Element software. The cell samples were excited by a 639 nm laser, and the emission was collected using the appropriate filter sets. The colocalization study was performed using a 60x (1.40 NA) oil immersion objective.

Live to Dead Cell Imaging with Confocal Microscopy

The cells (HeLa, HEK 293A, and Hep G2) were seeded into the 35-mm confocal dishes and kept for proper adherence in the humidified CO_2 incubator. Then, cells were incubated with a Cy-Cl probe (60 μM) for 1 hour, transferred to a humidified benchtop confocal CO_2 incubator, and images were taken. To initiate the apoptosis inside the cells 10 mM of H_2O_2 was added to the confocal dishes, and then time-lapse imaging was started for 6 hours for HeLa and HEK 293A cells and 12 hours for Hep G2 cells.

Cell viability assay

Cell viability of HeLa cells against Cy-Cl probe was performed using an XTT assay. First cells were maintained as per the National Centre for Cell Science (NCCS) Pune, Maharashtra, India guidelines and seeded in 96-well plates with seeding density 2.5 x 10^3 cells at each well. After proper adherence and reaching proper confluency, the cells were exposed to Cy-Cl for 6, 12 and 24 hours respectively. After Cy-Cl probe incubation, an XTT reagent, along with an electron coupler, were added to each well and incubated for 4 hours. The optical density of each well containing cellular media, XTT reagent, and electron coupler was taken using the Tecan Infinite M200 PRO multi-plate reader. The optical density was collected at 450 nm with a reference read at 690 nm. The final absorbance values concerning control and blank samples were analyzed for cell viability measurement.

ROS Estimation

ROS Estimation was performed against the probe and H_2O_2, mainly to check the amount of ROS generated against H_2O_2, which was used to initiate the apoptosis. HeLa cells were seeded into a 96-well plate and allowed to grow properly. After proper adherence, cells were washed with 1X-PBS and stained with 10 µM of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in 1% serum media for 30 minutes. Then, the cells were washed and incubated with a Cy-Cl probe and H_2O_2 for 12 hours. The generated ROS was spectrophotometrically analyzed by exiting DCFH-DA at 485 nm and collecting emissions at 535 nm.

Plant Cell Staining

Tomato seedlings *(Solanum lycopersicum)* were used in this study. The seeds were surface-sterilized with 70% ethanol and thoroughly rinsed with sterile distilled water. The sterilized seeds were germinated on ½ Murashige and Skoog (MS) agar plates. The plates were sealed to maintain sterility, and the seedlings were grown under controlled conditions at 21°C with a photoperiod of 16 hours of light and 8 hours of darkness. Two-week-old tomato seedlings were used for the experiment.

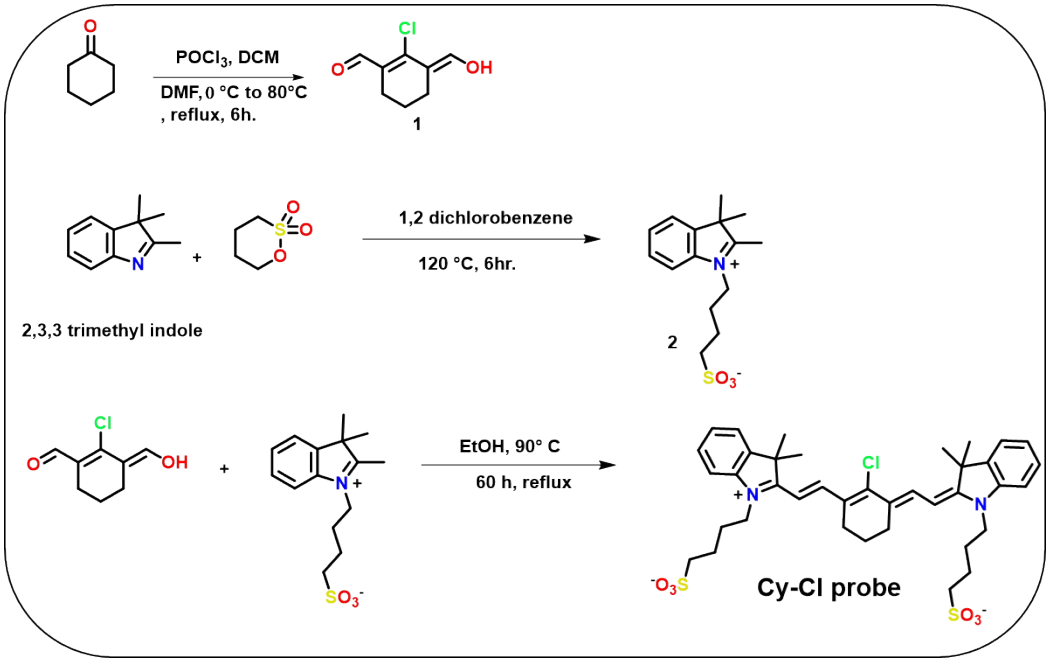
***C. elegans* Staining**

To perform basic imaging using staining*, C. elegans* was extracted from the NGM plate using M9 solution. The sample was then centrifuged at a speed of 2000 RPM for 2 minutes, during which the supernatant was discarded and replaced with a new M9 buffer. The operation was repeated to eliminate any remaining bacteria, which were transferred to a tube containing 2 mL of the buffer solution. A 60 µM dye concentration was introduced into the buffer solution containing the worms. The mixture was then incubated at 20 °C for 6 hours. Following this, the worms were subjected to two additional rounds of centrifugation with M9, as outlined in the methodology above, to eliminate any artifacts. After this, a volume of 10 µL was extracted from the resulting pellet using a pipette and then deposited onto a 2% agar pad to prevent desiccation. In order to render the worms immobile for imaging purposes, a concentration of 10 µM sodium azide (MERCK-769320) was introduced into the agar before creating the pad. A cover slip was placed on the pad to prepare the worms for imaging[5].

For staining of the intestinal lumen (60 µM of the dye was added), ~~to~~ 1 ml bacteria OP50 and plated onto the 60 mm NGM plates. Age-synchronized worms are added to this plate and allowed to grow for 24 hours. After this, the animals were washed from the plate with the help of M9 buffer and centrifuged twice at 2000 RPM for 2 minutes to remove artifacts. 10 µL was extracted from the pellet and placed on an agar pad containing sodium azide as per the protocol

mentioned above to immobilize the worms. A coverslip was then added on top before proceeding with imaging[6,7].

Synthesis route of Cy-Cl probe:



Scheme Sc1: Synthesis protocol of Cy-Cl probe.

Synthetic Procedure of Cy-Cl probe:

Synthesis of compound 1:

DMF (10 mL) and dichloromethane (10 mL) were mixed in the ice bath condition, and then $POCl_3$ (10 mL) was added drop by drop with the help of a dropping funnel. After stirring for 30 min, 4.0 g cyclohexanone was added and refluxed at 80 °C. After 6 hours, the mixture was poured into the ice cold water to get the yellow solid product after that, the product was filtered by a Buchner funnel and dried on rota vapor. Further, the product formation was confirmed by ^{1}H-NMR spectroscopy (Yield: 2.8 gm; 39.82%).

Synthesis of compound 2

1 equivalent of 2.3.3-trimethyindolec (5.0 gm, 31.40 mmol) and 1, 4-butylsulfonate (3.3 mL, 31.40 mmol) were mixed in 20 mL of 1, 2-dichlorobenzene. The mixture was refluxed at 120 °C for 6 hours. After the completion of the reaction, it was poured into iced acetone for extraction and washed several times with acetone to obtain the product as a light pink solid in

color. The product formation was further confirmed by the ^{1}H-NMR spectroscopy (Yield: 7.0 gm; 64.40%).

Synthesis of probe Cy-Cl:

Compound 1 (300 mg, 1.7 mmol) and Compound 2 (1.014 gm, 3.43 mmol) were dissolved in anhydrous ethanol and refluxed at 90 °C for 60 hours to produce dark green solids. The reaction mixture evaporated on rota vapor to remove all solvents. The compound was purified by column chromatography with a 20% DCM-MeOH solvent system to get the final product as Cy-Cl probe[8,9] (Yield: 500 mg; 39.68%).

^{1}H NMR of Compound 1 ((E)-2-chloro-3-(hydroxymethylene)cyclohex-1-ene-1-carbaldehyde) (500 MHz, DMSO-D_6): δ 10.81 (s, 1H), 10.02 (s, 1H), 7.47 (s, 1H), 2.41 – 2.21 (t, 4H), 1.52 (p, 2 H J = 5.7 Hz)

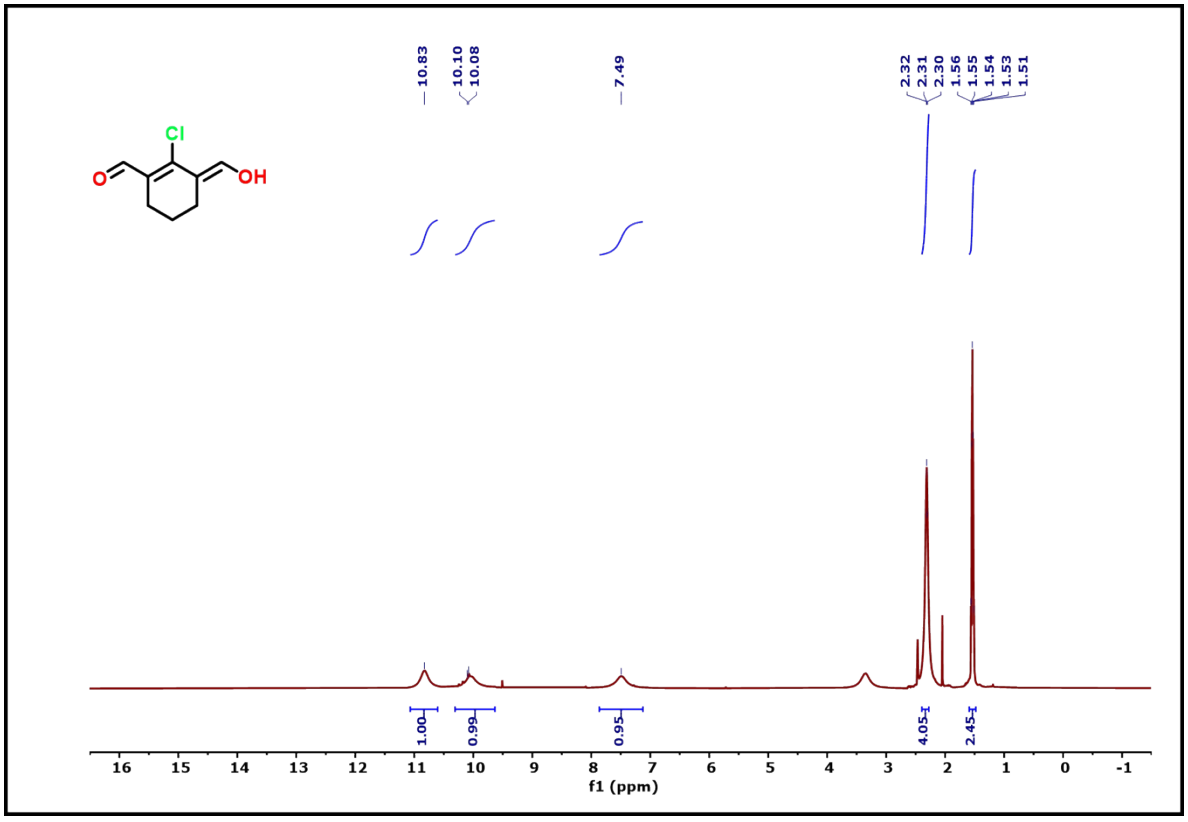


Figure S1: ^{1}H NMR (E)-2-chloro-3-(hydroxymethylene)cyclohex-1-ene-1-carbaldehyde (500 MHz, DMSO-d_6)

^{1}H NMR of Compound 2 (4-(2,3,3-trimethyl-3H-indol-1-ium-1-yl)butane-1-sulfonate) (500 MHz, $CDCl_3$) δ 7.83 (d, J = 7.3 Hz, 1H), 7.57 – 7.48 (m, 3H), 4.83 – 4.75 (t, 2H), 3.04 (s, 3H), 2.91 (t, J = 6.8 Hz, 2H), 2.20 – 2.11 (m, 2H), 2.05 – 1.99 (m, 2H), 1.58 (s, 6H).

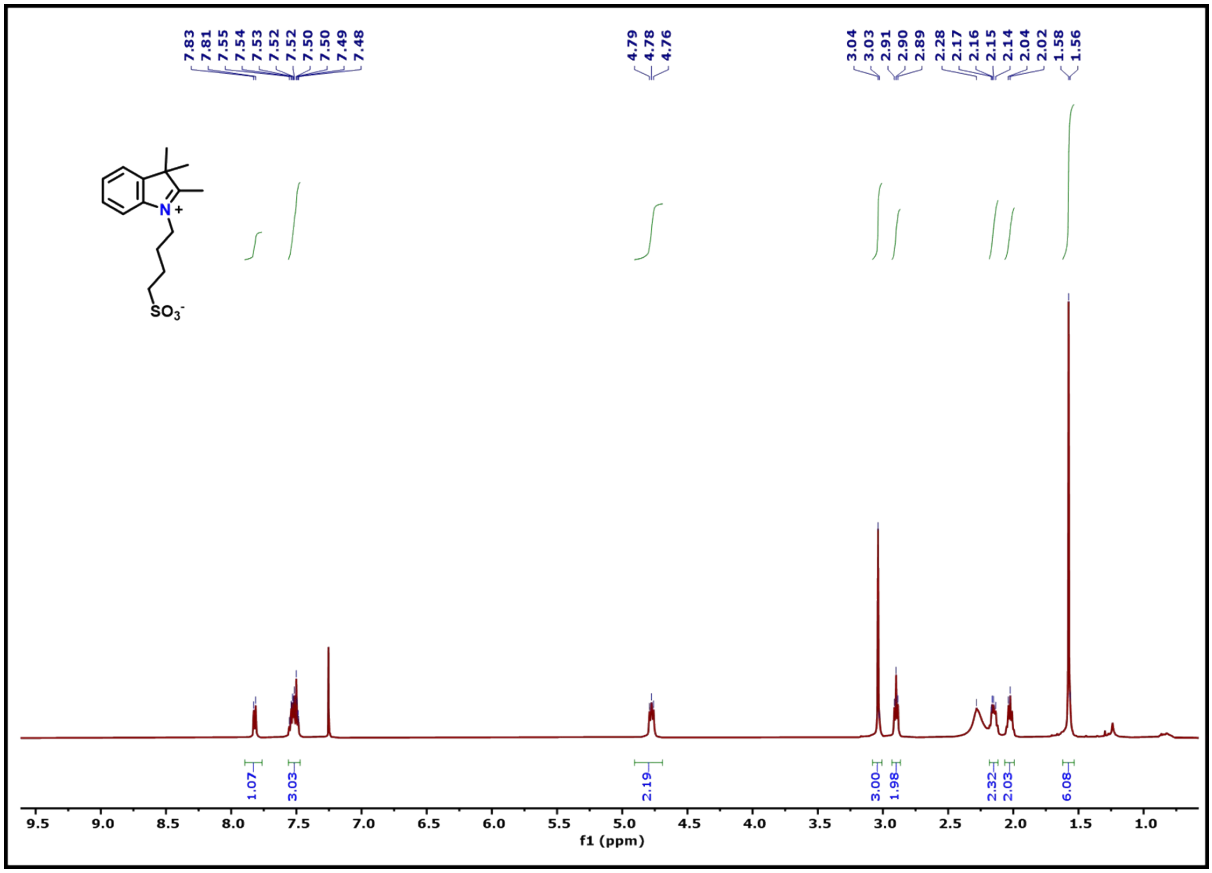


Figure S2: ^{1}H NMR of 4-(2,3,3-trimethyl-3H-indol-1-ium-1-yl)butane-1-sulfonate ($CDCl_3$, 500 MHz)

^{1}H NMR of Cy-Cl probe (500 MHz,CD_3OD) δ 8.22 (dd, *J* = 13.9 Hz, 2H), 7.59 (d, *J* = 7.4 Hz, 2H), 7.45 (d, *J* = 7.9 Hz, 2H), 7.38 (dd, *J* = 7.7 Hz, 2H), 7.24 (dd, *J* = 7.3 Hz, 2H), 6.34 (dd, *J* = 14.3 Hz, 2H), 4.18 (t, *J* = 7.5 Hz, 4H), 2.69 (t, *J* = 6.2 Hz, 4H), 2.46 (m, 4H), 1.79 (tq, *J* = 22.7, 7.4, 6.6 Hz, 6H), 1.70 (m, *J* = 7.3 Hz, 4H), 1.63 (s, 12H).

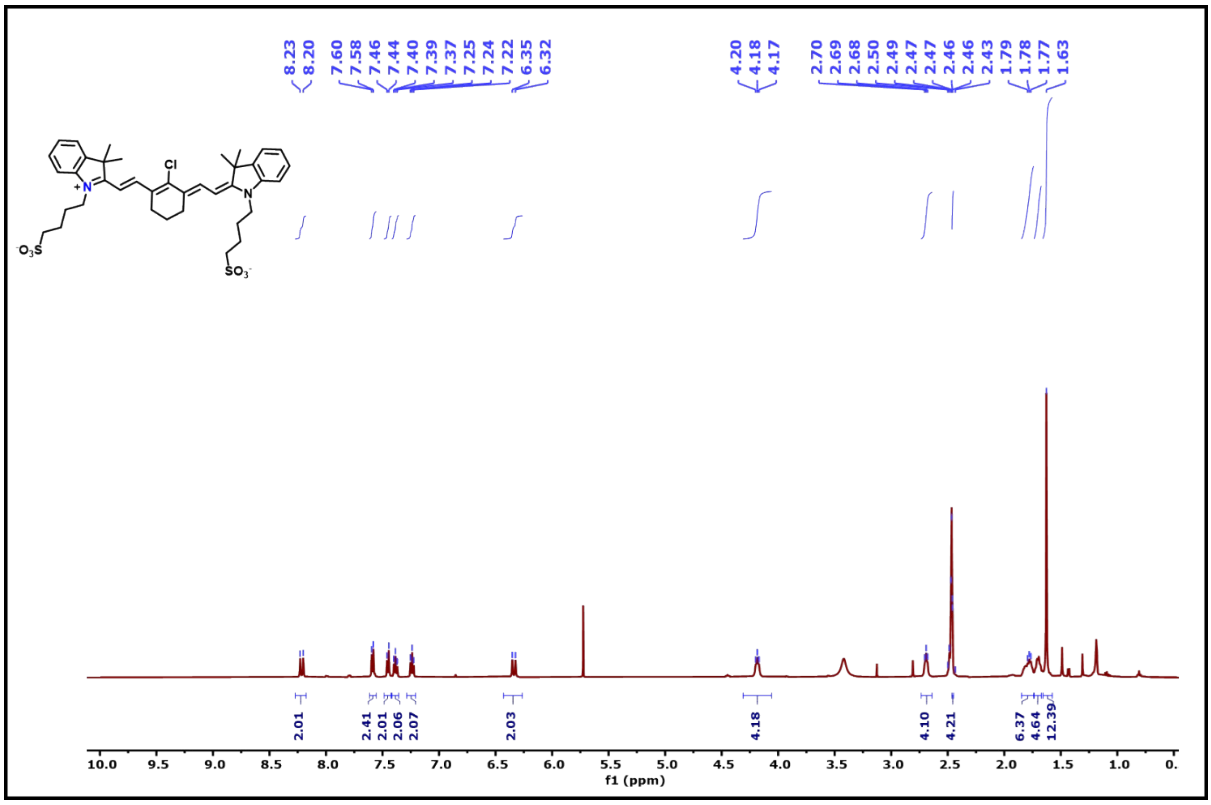


Figure S3: ^{1}H NMR of Cy-Cl probe (CD_3OD, 500 MHz)

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197 **^{13}C NMR of Cy-Cl probe (500 MHz, CD_3OD):** δ 181.6, 157.4, 152.5, 151.6, 150.5, 138.1,
198 135.8, 134.6, 131.9, 121.1, 111.2, 64.4, 60.2, 58.4, 53.3, 39.9, 38.5, 37.0, 35.6, 35.4, 32.0,
199 31.55, 31.5, 29.9

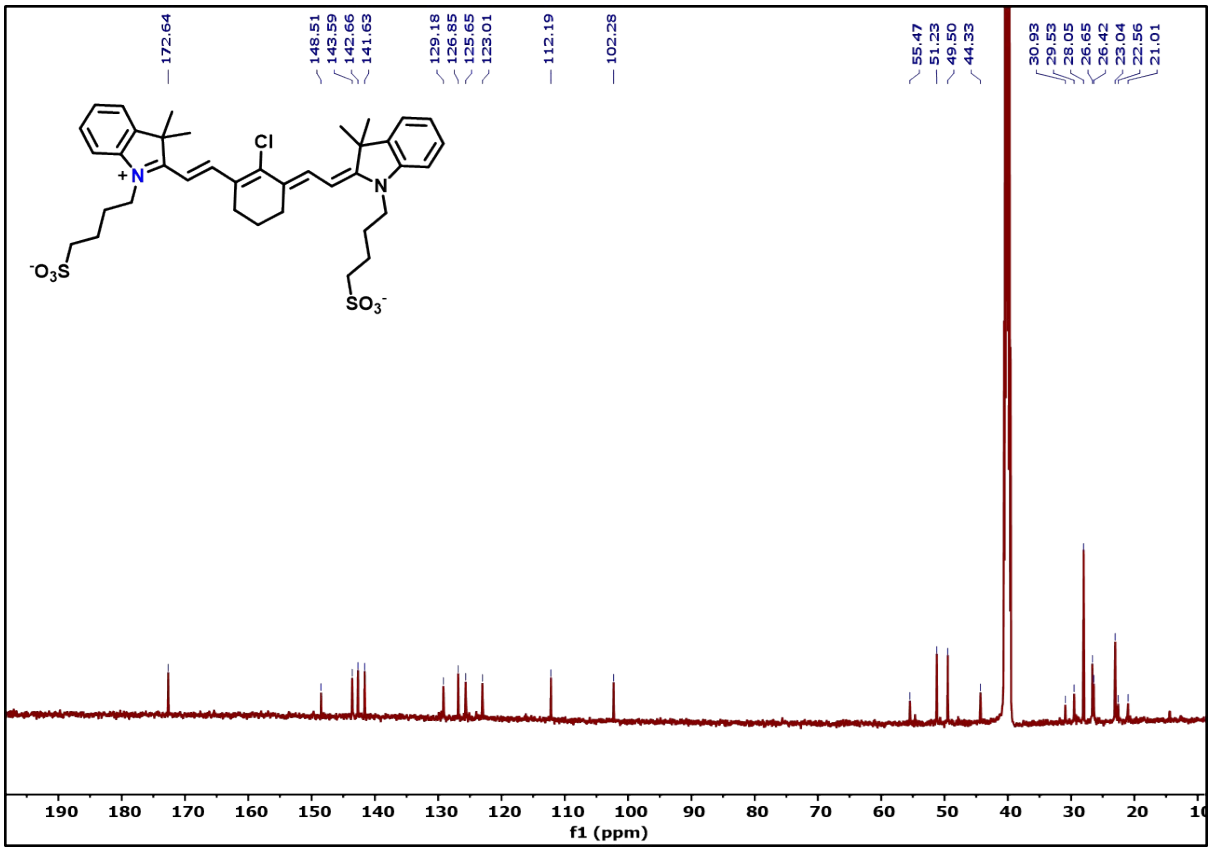

Figure S4: ^{13}C NMR of Cy-Cl Probe ($CDCl_3$, 500 MHz)

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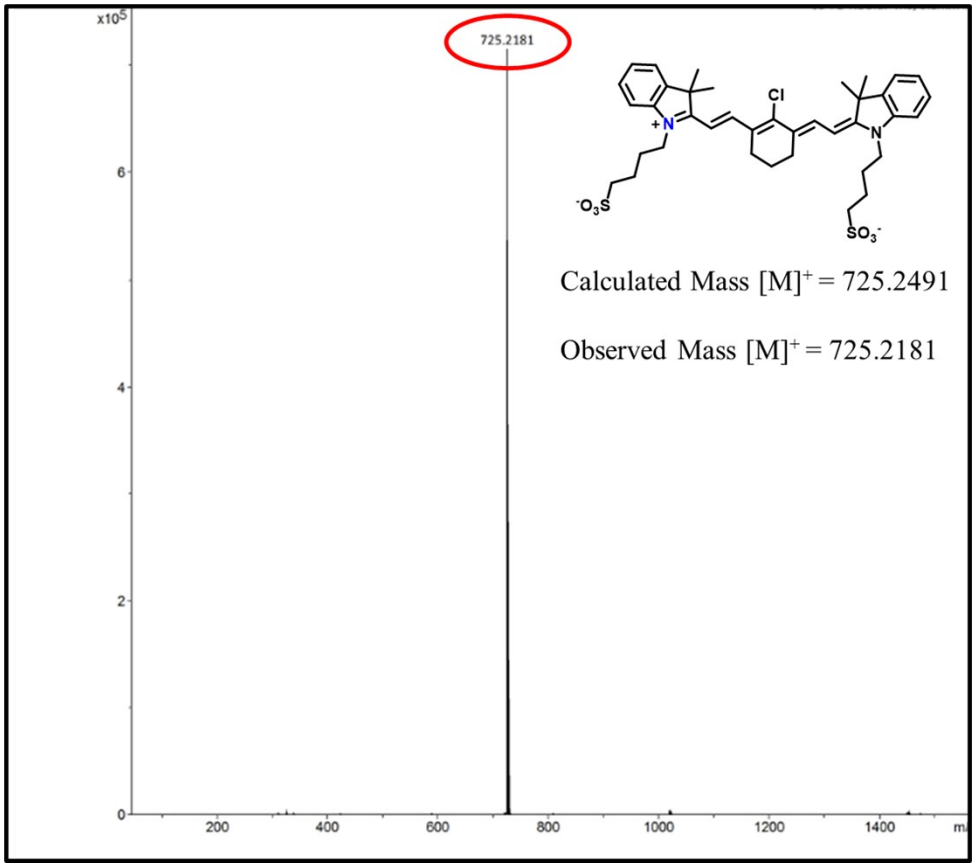

Figure S5: Mass spectra of the Cy-Cl probe (Calculated Mass = 725.2491, Observed mass = 725.2181)

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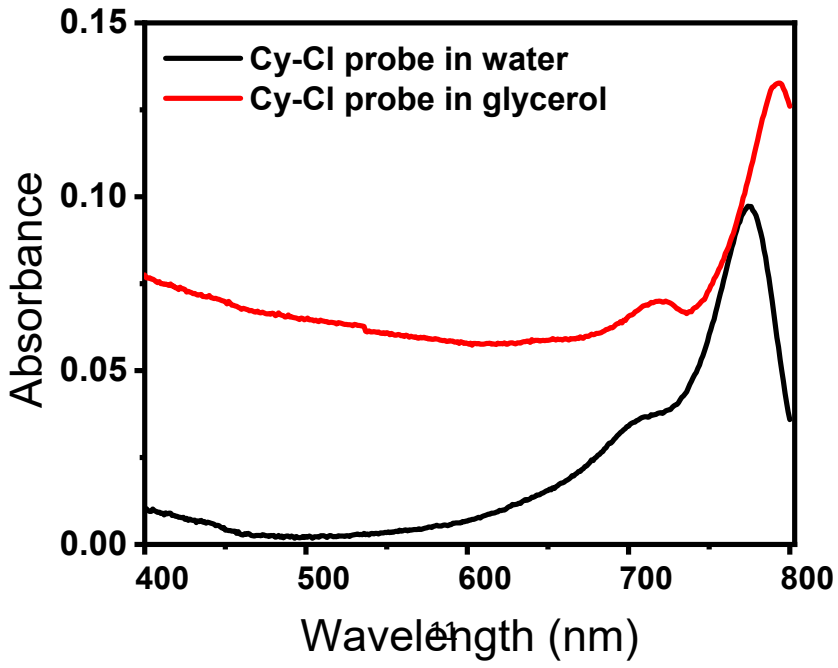

Figure S6: Absorption spectra of Cy-Cl probe in water and glycerol medium. A 10 nm red shift in glycerol showed the possibility of J-type aggregation.

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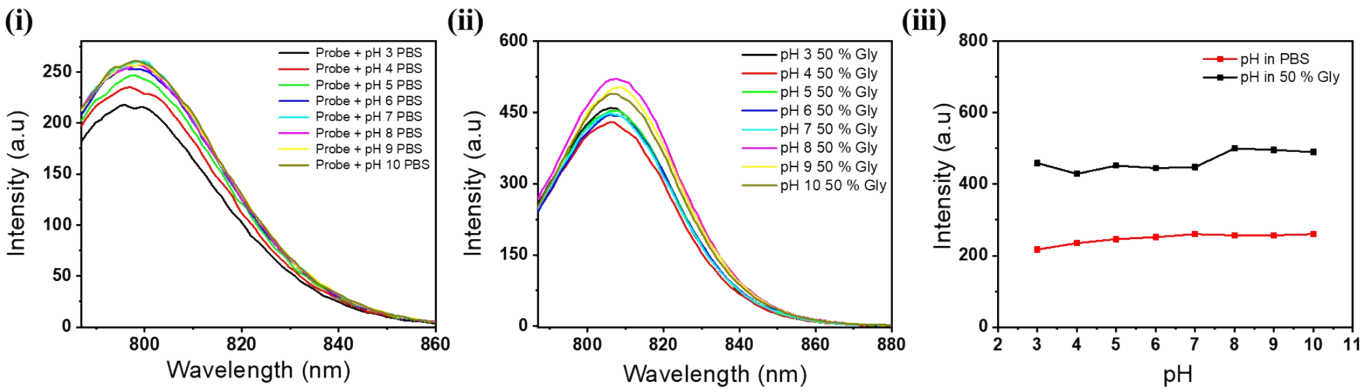

Figure S7: Fluorescence response of Cy-Cl probe in different viscosity at various pH (i) in 0% Glycerol-PBS (ii) 50 % Glycerol PBS mixture (iii) is the maximum fluorescence intensity at various pH.

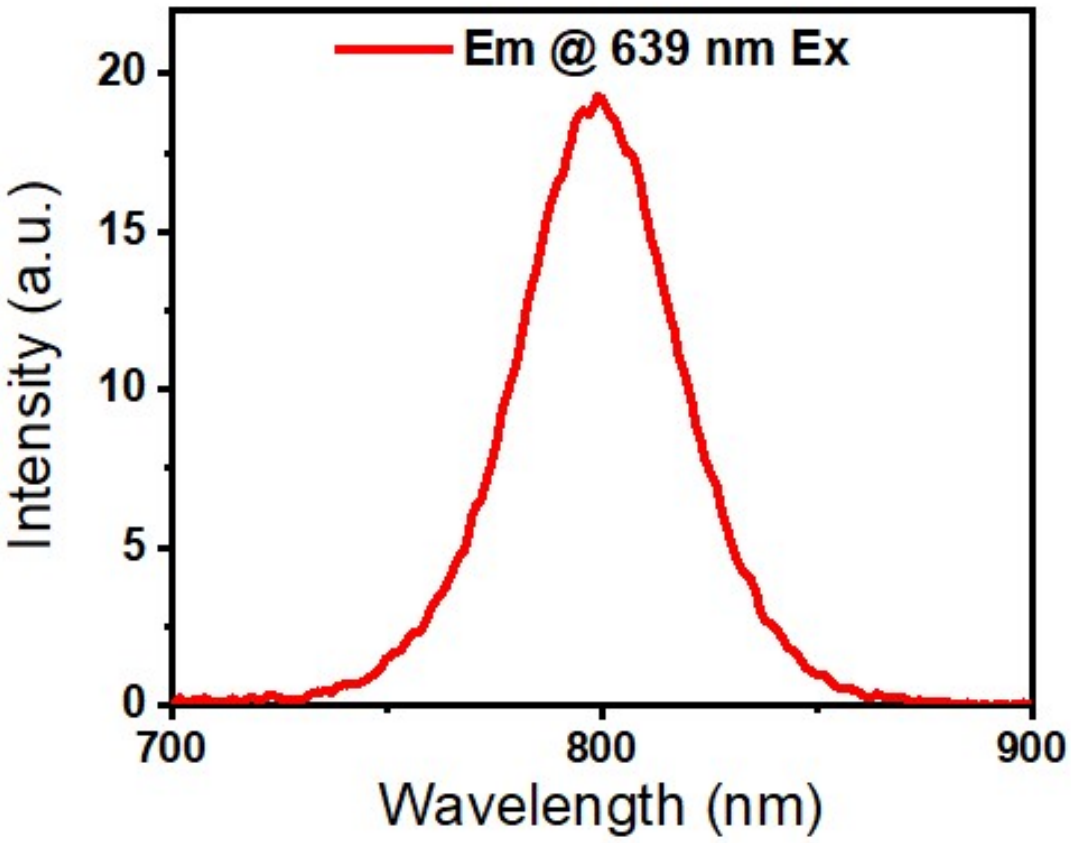

Figure S8: The Cy-Cl probe exhibits significant emission when excited at 639 nm, indicating that a 639 nm laser source is suitable for bioimaging.

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Cy-Cl (Viscosity)	τ_1 (ns)	τ_2(ns)	τ_3 (ns)	A_1(%)	A_2 (%)	Average Lifetime (ns)
Water only	0.02173	0.1772	0.5303	99.97	0.03	0.02173
10% gly+water	0.01389	0.1977	0.5969	100	0.00	0.01389
20% glyc+water	0.2311	0.4954	-	90.21	9.79	0.2438
30% gly+water	0.2454	0.4373	-	81.68	18.32	0.2669
40% gly+water	0.2950	0.4728	-	75.77	24.23	0.3246
50% gly+water	0.3469	0.5084	-	65.02	34.98	0.3903

Table S1: (a) The fluorescence lifetime of the Cy-Cl probe in a water-glycerol mixture increases, indicating the probe response towards viscosity changes.

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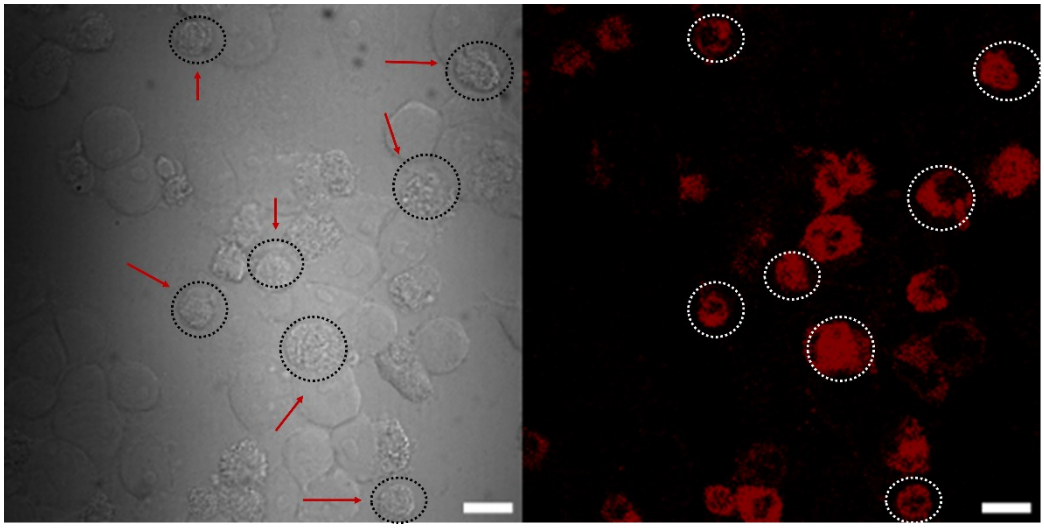

Figure S9: The Cy-Cl probe specifically stains dead cells, which are marked as black circles in the TD image, while white circles indicate emissions from the dead cells in the red region of the right image. (Scale bar: 25 μm)

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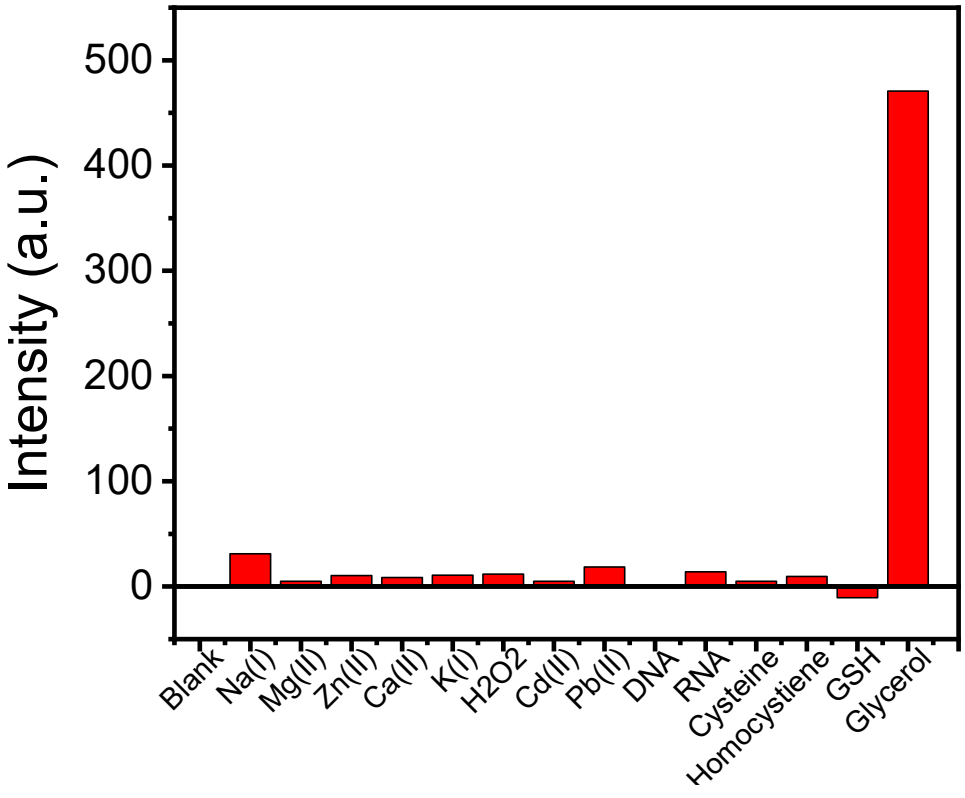

Figure S10: The fluorescence bar plot illustrates the Cy-Cl probe's response to metal ions and biomolecules, indicating that fluorescence is primarily influenced by the viscosity of the medium.

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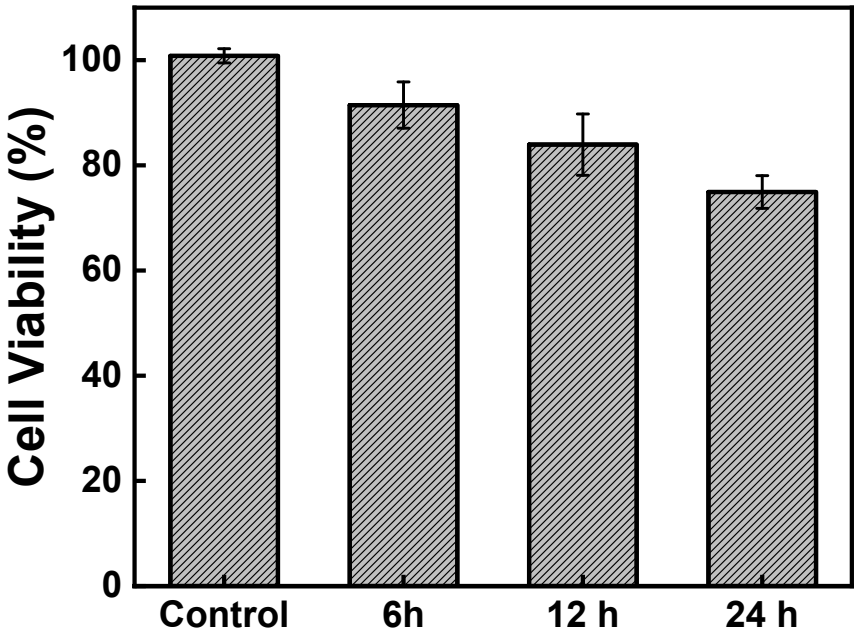

Figure S11: Cellular cytotoxicity experiment of Cy-Cl probe at 6 hours, 12 hours, and 24 hours respectively in HeLa cells. The result shows that ~80% cell viability even after 24 hours of Cy-Cl treatment.

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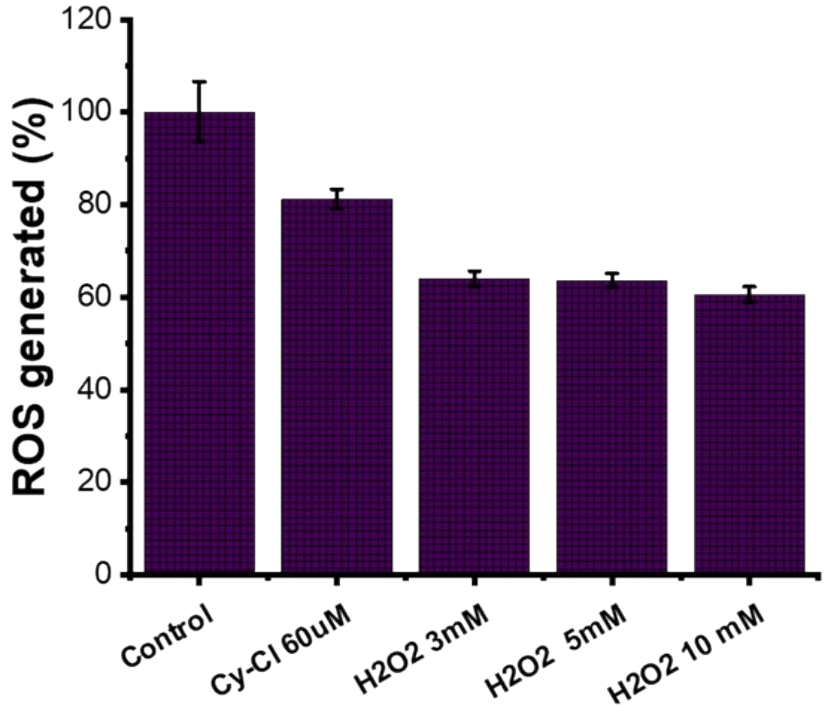

Figure S12: Spectrophotometric estimation of ROS generation against H_2O_2 confirms the initiation of the apoptosis process.

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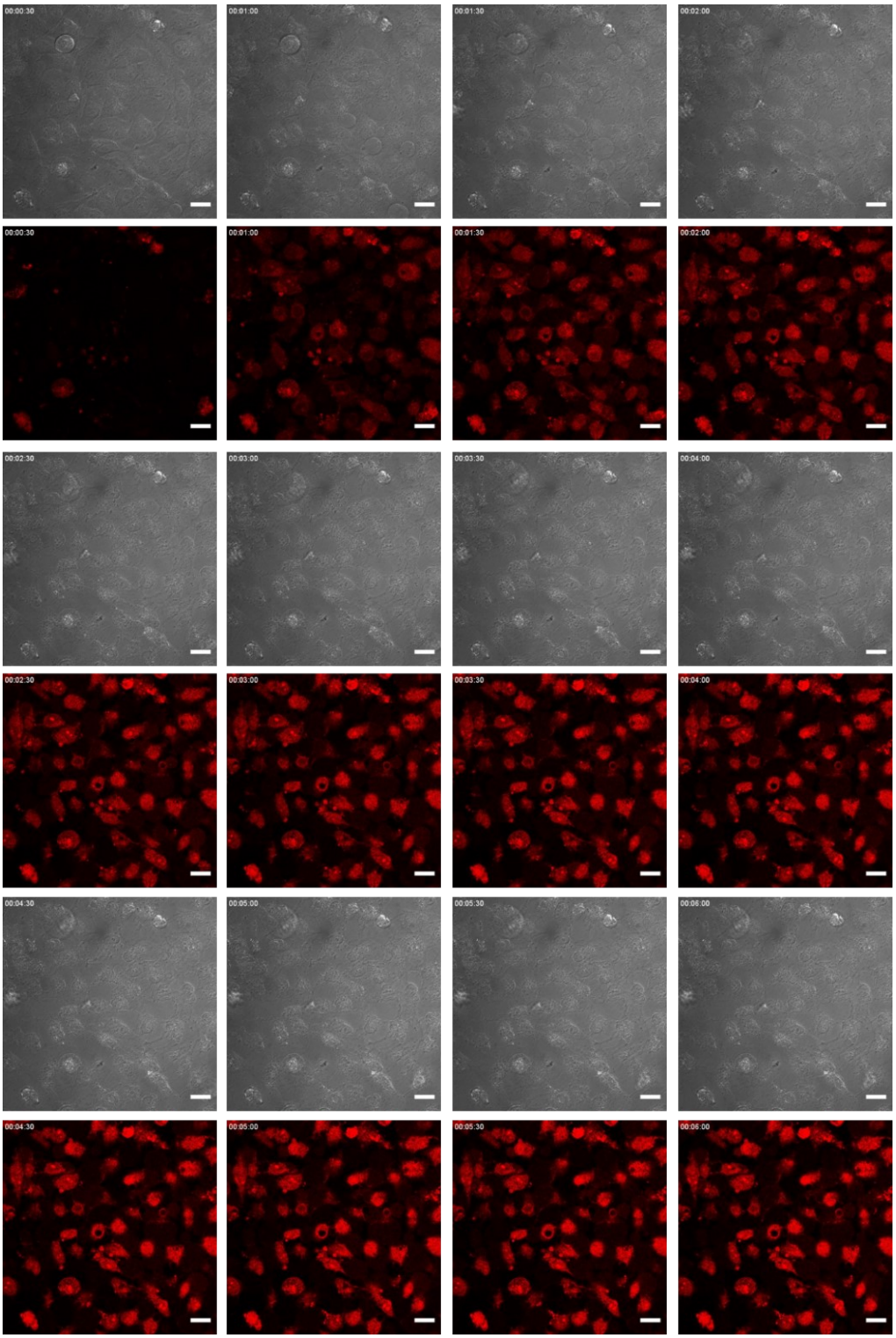

Figure S13: The live imaging of dead and live HeLa cells using the Cy-Cl probe (60 μM), followed by the addition of 10 mM H_2O_2 for 6 hours, demonstrates the probe's capability for long-term tracking of dead cells. (Scale bar: 25 μm)

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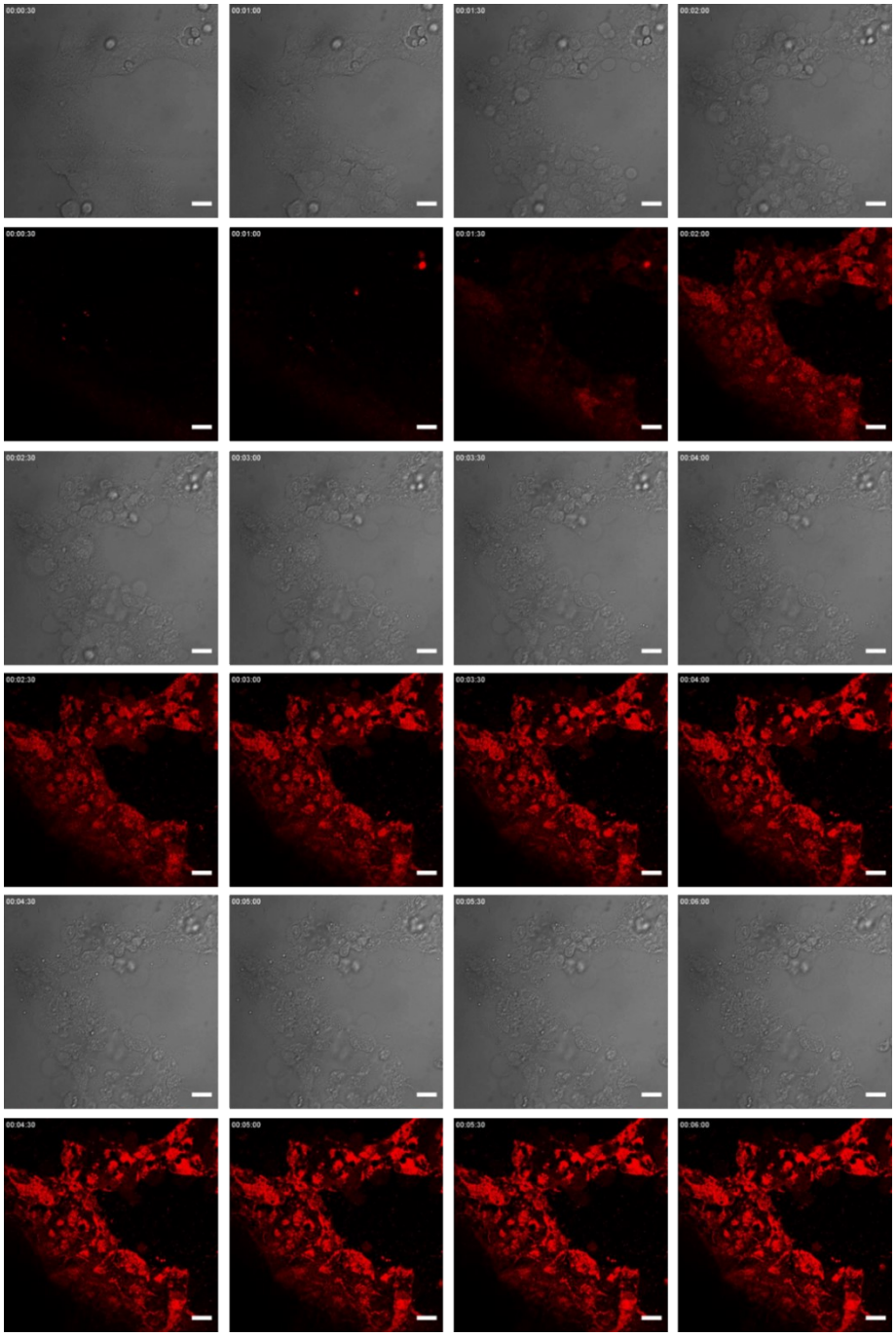

Figure S14: The live imaging of dead and live HEK 293A cells using the Cy-Cl probe (60 μM), followed by the addition of 10 mM H_2O_2 for 6 hours, demonstrates the probe's capability for long-term tracking of dead cells. (Scale bar: 25 μm)

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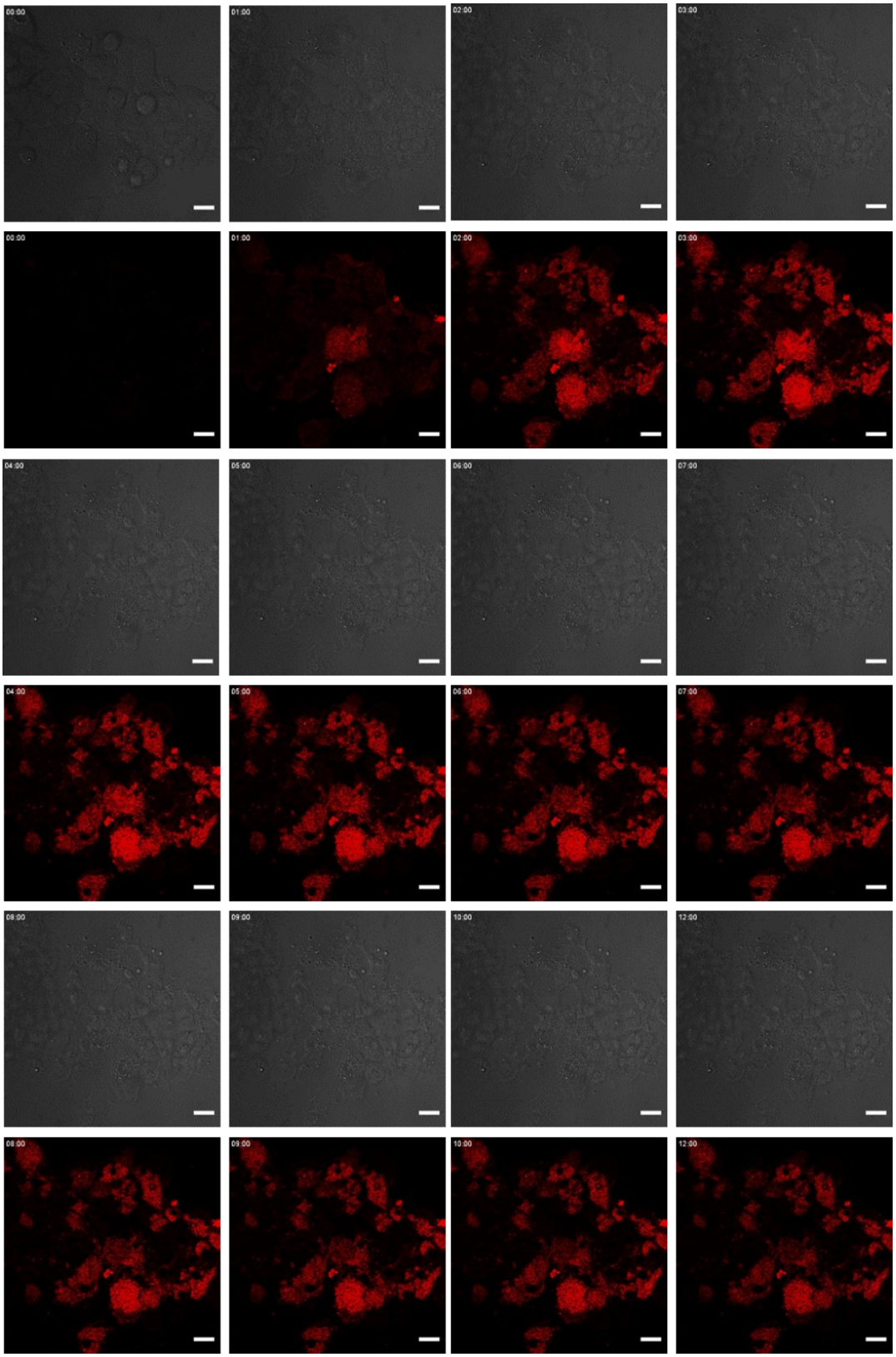

Figure S15: The live imaging of dead and live HepG 2 cells using the Cy-Cl probe (60 μM), followed by the addition of 10 mM H_2O_2 for 12 hours, demonstrates the probe's capability for long-term tracking of dead cells. (Scale bar: 25 μm)

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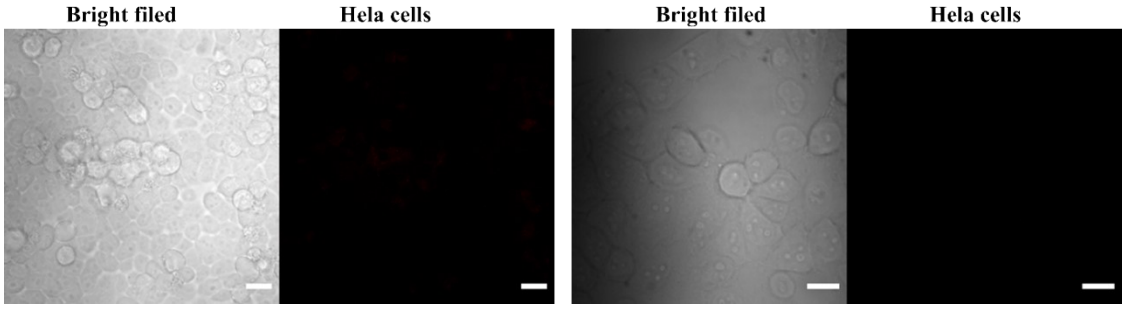

Figure S16: Fixed Hela cells treated with 0.1% Triton X-100 for pore formation incubated with Cy-Cl probe, Images do not show any emission after fixed cell confocal imaging (Scale bar: 25 μm).

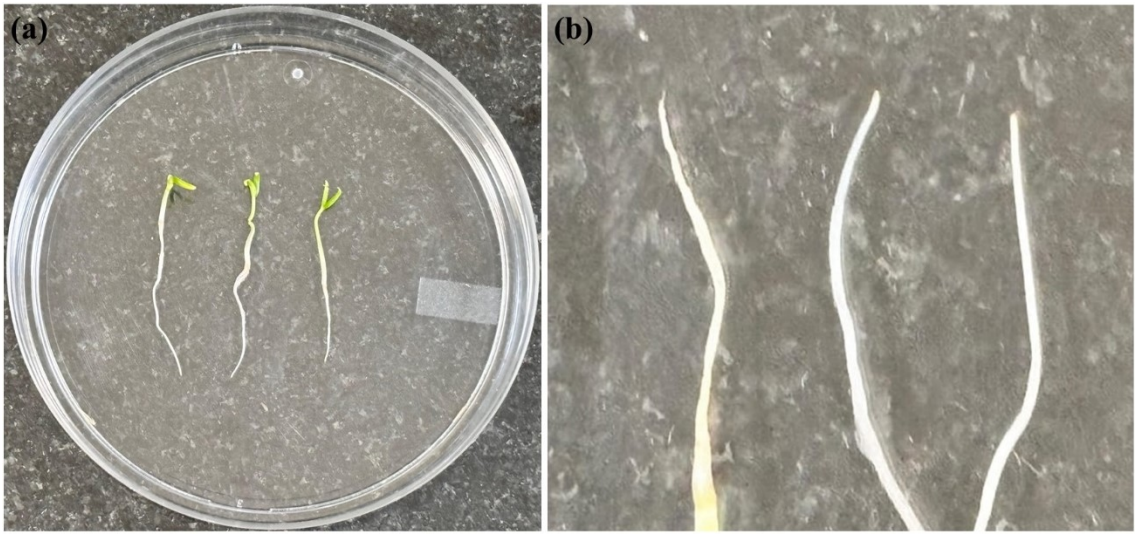

Figure S17: (a) The root image of tomato seedlings used for dead cell study. **(b)** The zoomed image of **(a)** tomato plant root

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References:

1 J. Lim, J. Lee, Y. Boo and W. J. Kim, *Nanoscale*, 2023, **16**, 742–751.

2 J. Liu, C. Lou, C. Zhen, Y. Wang, P. Shang and H. Lv, *Metallomics*, , DOI:10.1093/mtomcs/mfac027.

3 H. Li, W. Shi, X. Li, Y. Hu, Y. Fang and H. Ma, *J. Am. Chem. Soc.*, 2019, **141**, 18301–18307.

4 M. Adamiec-Organisciok, M. Wegrzyn, L. Cienciala, D. Sojka, J. Nackiewicz and M. Skonieczna, *Pharmaceuticals*, 2023, **16**, 1–14.

5 M. Rahimi, S. Sohrabi and C. T. Murphy, *Biophys. J.*, 2022, **121**, 515–524.

6 A. Bidaud-Meynard, F. Demouchy, O. Nicolle, A. Pacquelet, S. K. Suman, C. N. Plancke, F. B. Robin and G. Michaux, *Dev.*, 2021, **148**, 1–8.

7 T. Saito, K. Kikuchi and T. Ishikawa, *Biochem. Biophys. Res. Commun.*, 2024, **706**, 149762.

8 N. Zhou, F. Huo, Y. Yue and C. Yin, *J. Am. Chem. Soc.*, 2020, **142**, 17751–17755.

9 A. Samanta, M. Vendrell, R. Das and Y. T. Chang, *Chem. Commun.*, 2010, **46**, 7406–7408.