Supporting Information for

A Dual-Response Fluorescent Probe for N_2H_4 and Viscosity In living Cells and Zebrafish to Evaluate Liver Injury

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Experimental section

Materials and apparatus

All chemicals were commercially available from Energy Chemical and used without further purification. The concentration of PBS solution is 10 mM. ^{1}H NMR and ^{13}C NMR spectra were recorded on Brucker Avance 500 MHz spectrometers. The spectra were reported in ppm (δ) and referenced to a tetramethylsilane (TMS) standard in $CDCl_3$. Thin layer chromatography (TLC) for reaction monitoring was performed on pre-coated silica gel plates (Merck 60 F254 nm) with a UV254 fluorescent indicator and column chromatography was conducted over silica gel (mesh 300-400). The fluorescence and UV–vis spectra were acquired on a SpectraMax M5 (Molecular Devices).

General procedure for viscosity analysis

The parent stock solution was prepared by dissolving probe **FNN** (5 mM) in DMSO. The test solution was prepared by diluting **FNN** to PBS/ Glycerol solutions (5 μM, 1.0 mL). The viscosity solution was prepared by mixing PBS and Glycerol in different proportion. These solutions were ultrasonically treated for 30 minutes. Then, the data of fluorescence spectrum was recorded under the excitation of 500 nm, and the slit width were set at 5 nm. The relationship between the fluorescence emission intensity of the probe and the viscosity of the solvent is well expressed by the Forster Hoffmann equation as follows:

$$\text{Log (If)} = c + x \log \eta$$

Where if is the fluorescence intensity, η is the viscosity of the solution, and X and C are constant.

Determination of the lowest detection limit LOD

The detection limit (LOD) of probe **FNN** for N_2H_4 was determined by fluorescence titration. The equation is as follows:

$$LOD = 3\delta/m$$

Where LOD is the lower detection limit, δ is the standard deviation of the background (obtained by measuring the fluorescence emission intensity of **FNN** and calculating the standard deviation), and M is the sensitivity (slope of the linear curve between the fluorescence intensity and the concentration of N_2H_4).

TD-DFT computing

The density function theory (TD-DFT) method is used to optimize the geometrical space structure of **FNN** and **FNN-1**, and B3-LYP exchange correlation functional and 6-31G basis set are combined. In the calculation, except for the specific instructions, other atom free optimization, the dihedral angle of bond length and bond angle are not fixed, and all optimization is carried out in vacuum environment by default.

Cytotoxicity of probe FNN

The cytotoxicity of probe **FNN** was tested by MTT method. HeLa cells were seeded at a density at 1×10^4 cells per well into 96-well plate, and incubated in 37 °C cell incubator (containing 5% CO_2) for 12 hours. The culture medium was high glucose DMEM with fetal bovine serum and appropriate antibodies (penicillin and streptomycin). Then the probe **FNN** (0-50 μM) was added and incubated for 12 h. 50 μL MTT was added to each pore and the cells were incubated at 37 °C and 5% CO_2 for 4 h. Then, removed the medium and replaced it with DMSO (150μL), and detected the absorption values at 450 nm and 488 nm.

Cellular imaging

To prepare the **FNN** solution, 100 μL of stock solution of **FNN** (DMSO, 10 mM) was added dropwise into 9.9 mL PBS under ultrasonic conditions to obtain the sample solution for cellular imaging.

Cells were cultured in DMEM containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37 °C (w/v) in a 5% CO_2 and 95% air incubator MCO-15AC (Sanyo, Tokyo, Japan). The concentrations of counted cells were adjusted to 1×10^6 cells mL^{-1} for confocal imaging in high-glucose DMEM (4.5 g of glucose/L) supplemented with 10% fetal bovine serum (FBS), $NaHCO_3$ (2.0 ng/L), and 1% antibiotics (penicillin/streptomycin, 100.0 U/mL). Cultures were maintained at 37 °C under a humidified atmosphere containing 5% CO_2.

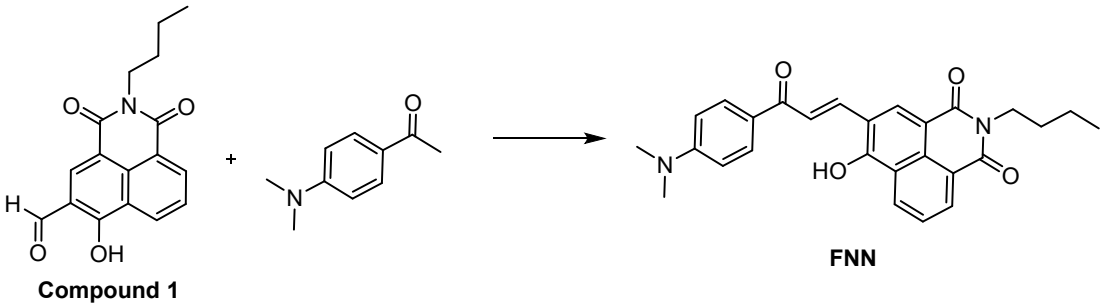
Live subject statement

All experiments were performed in compliance with the relevant laws and institutional guidelines, All zebrafish tested in compliance with the guidelines, which were approved the experiments by institutional and local ethics committee of Zhejiang University of Technology.

Imaging in zebrafish

We incubated 3-day-old zebrafish with **FNN** (10 μM) for 30 min, then washed with PBS buffer and imaged as control group. Another group treated with INH (500 μM/1 mM) for 30 min, and then incubated with **FNN** (10 μM) for 30 min. Thereafter, the

treated zebrafish was washed with PBS buffer three times and imaged using a confocal microscope. Fluorescence images were acquired with Nikon A1R confocal microscope.



Scheme S1. Synthesis route of **FNN**.

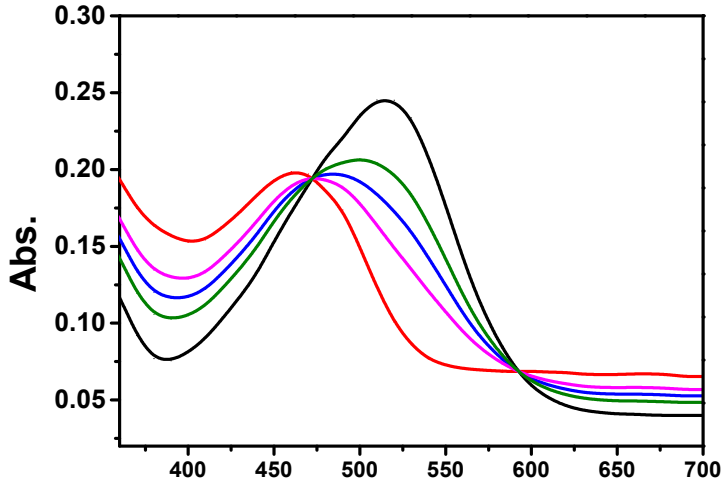


Fig. S1. Absorbance spectra of the probe **FNN** (5 μM) in DMSO/PBS = 1:99 (v/v = 1:1, pH = 7.4) buffer with different concentration of N_2H_4.

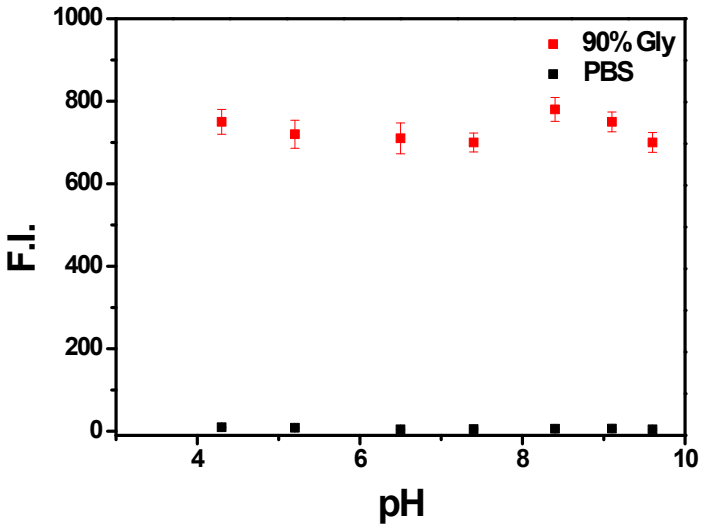


Fig. S2. The effect of pH on the fluorescence intensity changes of **FNN** (5 μM) in 90% glycerol/PBS

solution. Ex = 520 nm/Em = 620 nm.

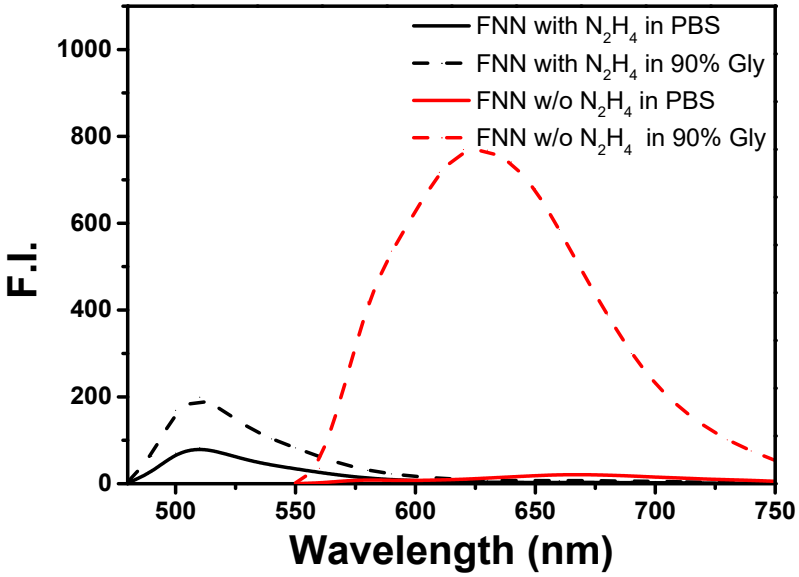


Fig. S3. The fluorescent spectra of **FNN** (5.0 μM) in the absence and presence of N_2H_4 in PBS solution (5 mM, ~0 CP, pH=7.4) and 90% Gly solution (~900 CP). Ex = 460 nm/Ex = 520 nm.

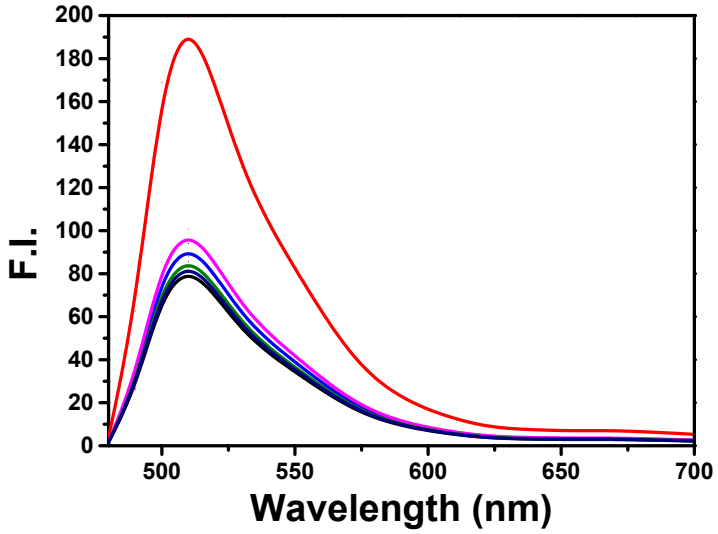


Fig. S4. The fluorescent spectra of **FNN** (5.0 μM) in the presence of N_2H_4 in PBS solution (5 mM, ~0 CP, pH=7.4) 20%, 40%, 60%, 80% and 90% Gly solution (~900 CP). Ex = 460 nm. Slit width: 10 nm/10 nm.

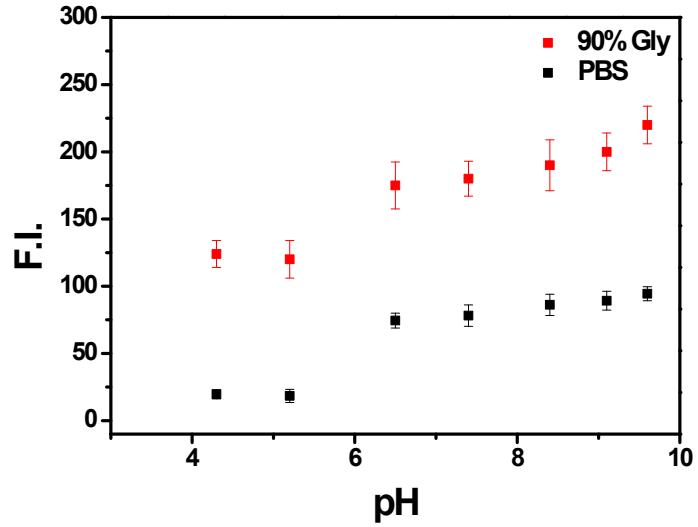


Fig. S5. The effect of pH on the fluorescence intensity changes of **FNN** (5 μM) in the presence of N_2H_4 in PBS solution and 90% glycerol/PBS solution. Ex = 460 nm/Em = 520 nm.

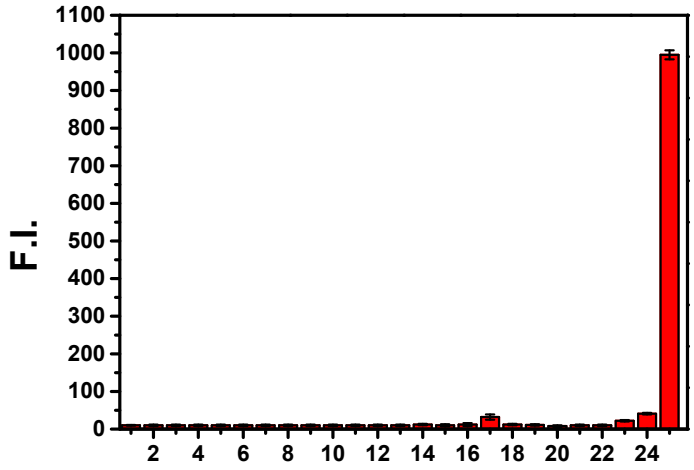


Fig. S6. The fluorescent responses of probe **FNN** (5.0 μM) to N_2H_4 (20.0 μM) in the presence of various relevant species at 520 nm (1, Serine; 2, Asparticacid; 3, Arginine; 4, Alanine; 5, Lysine; 6, Glutamicacid; 7, TBHP; 8, H_2O_2; 9, HClO; 10, NO; 11, Glutamicacid; 12, Tyrosine; 13, Histidine; 14, Tryptophan; 15, Valine, 16, GSH; 17, GSH (10 mM); 18, Homoncysteine; 19, Cysteine, 20, SO_3^{2-}; 21,$S_2O_3^{2-}$, 22, aqueous ammonia; 23, methylamine; 24, H_2S; 25, N_2H_4; 100 μM, incubated 30min) in DMSO/PBS buffer (1:99, v/v, pH 7.4) at 37 °C.

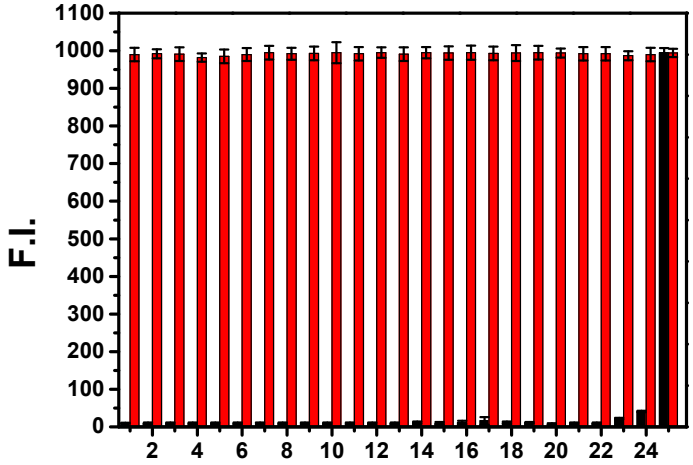


Fig. S7. The fluorescent responses of probe **FNN** (5.0 μM) to N_2H_4 (20.0 μM) in the presence of various relevant species at 520 nm (1, Serine; 2, Asparticacid; 3, Arginine; 4, Alanine; 5, Lysine; 6, Glutamicacid; 7, TBHP; 8, H_2O_2; 9, HClO; 10, NO; 11, Glutamicacid; 12, Tyrosine; 13, Histidine; 14, Tryptophan; 15, Valine, 16, GSH; 17, GSH (10 mM); 18, Homoncysteine; 19, Cysteine, 20, SO_3^{2-}; 21,$S_2O_3^{2-}$, 22, aqueous ammonia; 23, methylamine; 24, H_2S; 25, N_2H_4; 100 μM, incubated 30min) in DMSO/PBS buffer (1:99, v/v, pH 7.4) at 37 °C.

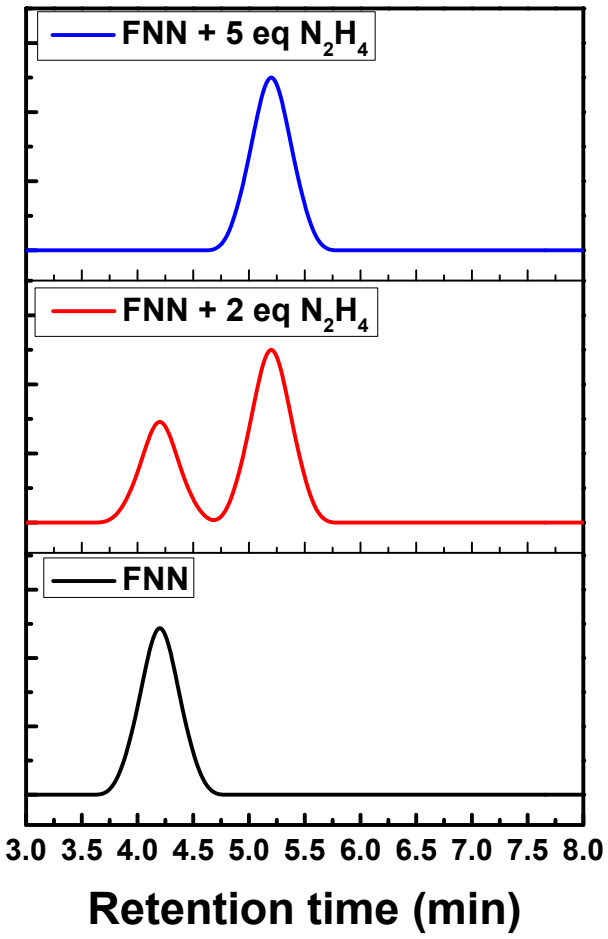


Fig. S8. HPLC results for probe **FNN** (5.0 μM, black line), probe **FNN** (5.0 μM, red line) incubated with N_2H_4 (2 eq., 10.0 μM) for 30 min, probe **FNN** (5.0 μM, blue line) incubated with N_2H_4 (4 eq., 20.0 μM) for 30 min. The HPLC method for the determination of the above-mentioned analytes with isocratic elution was established, and the mobile phase is 80% methanol and 20% water.

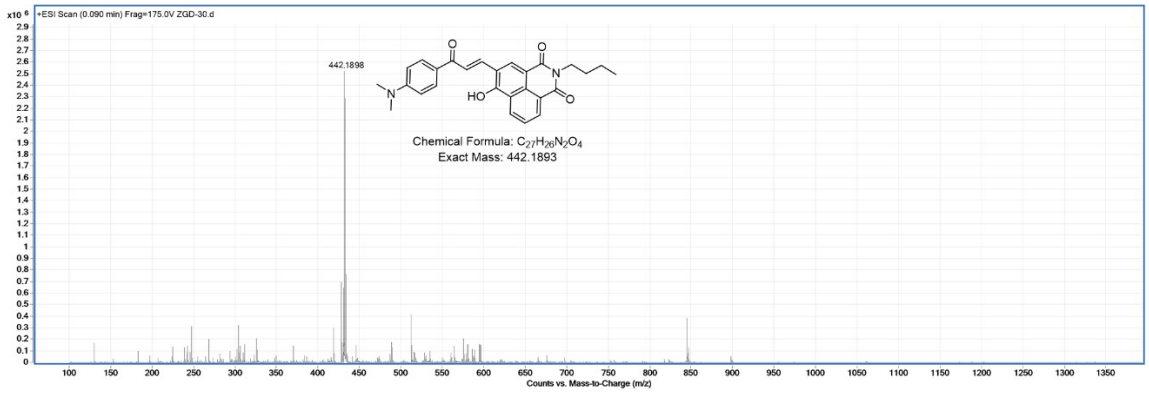


Fig. S9 HRMS spectrum of **FNN**.

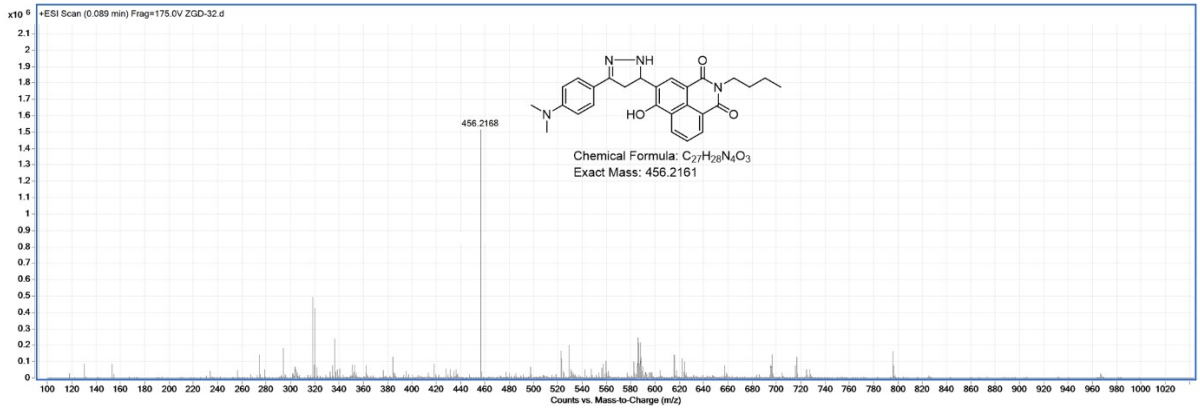


Fig. S10 HRMS spectrum of **FNN** after adding N_2H_4.

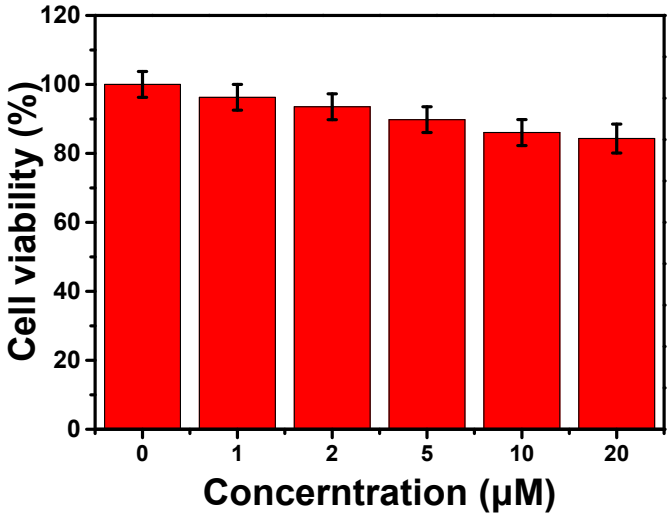


Fig. S11. MTT results of Huh7 viabilities after incubation with **FNN** for 24 h. Data are expressed as mean ± SD (experiment times n = 3).

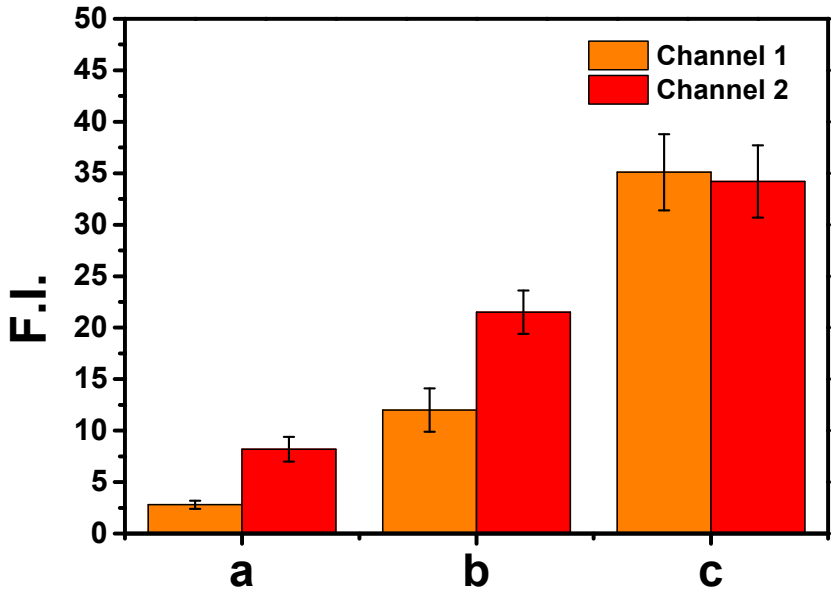


Fig. S12. The quantitative analysis of Huh7 cells in channel 1 and channel 2. (a) The cells only stained with **FNN** (10 μM) as control group. (b) Preincubate the cells with INH (100 μM) for 4 h then treat them with **FNN** (10 μM) for 30 min. (c) Preincubate the cells with INH (500 μM) for 4 h then treat them with **FNN** (10 μM) for 30 min. Data are expressed as mean ± SD.

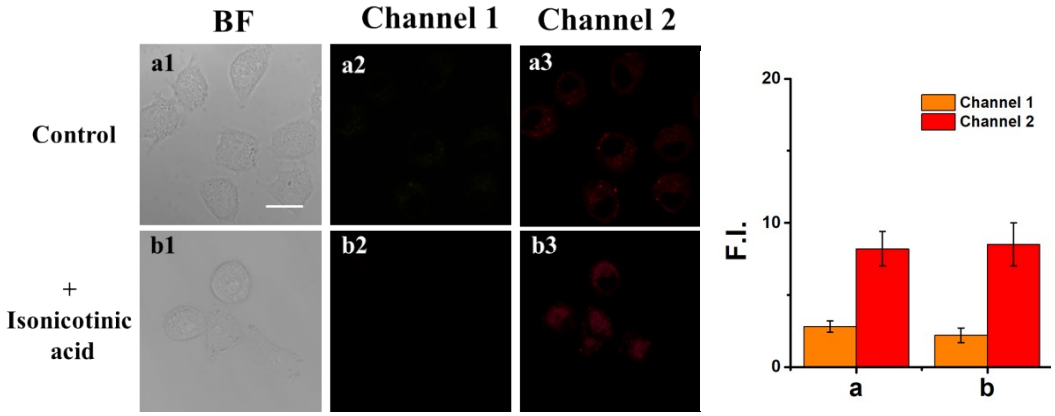


Fig.S13. Fluorescence images of Huh7 cells incubated with **FNN** (10 μM) for 30 min before (a a) and after (b) treatment of isonicotinic acid (10 μM) for 30 min. Scale bar: 20 μm. (1) Bright field. (2) channel 1 (Ex = 460 nm, Em = 500–550 nm) and (3) channel 2 (Ex = 520 nm, Em = 600–650 nm).

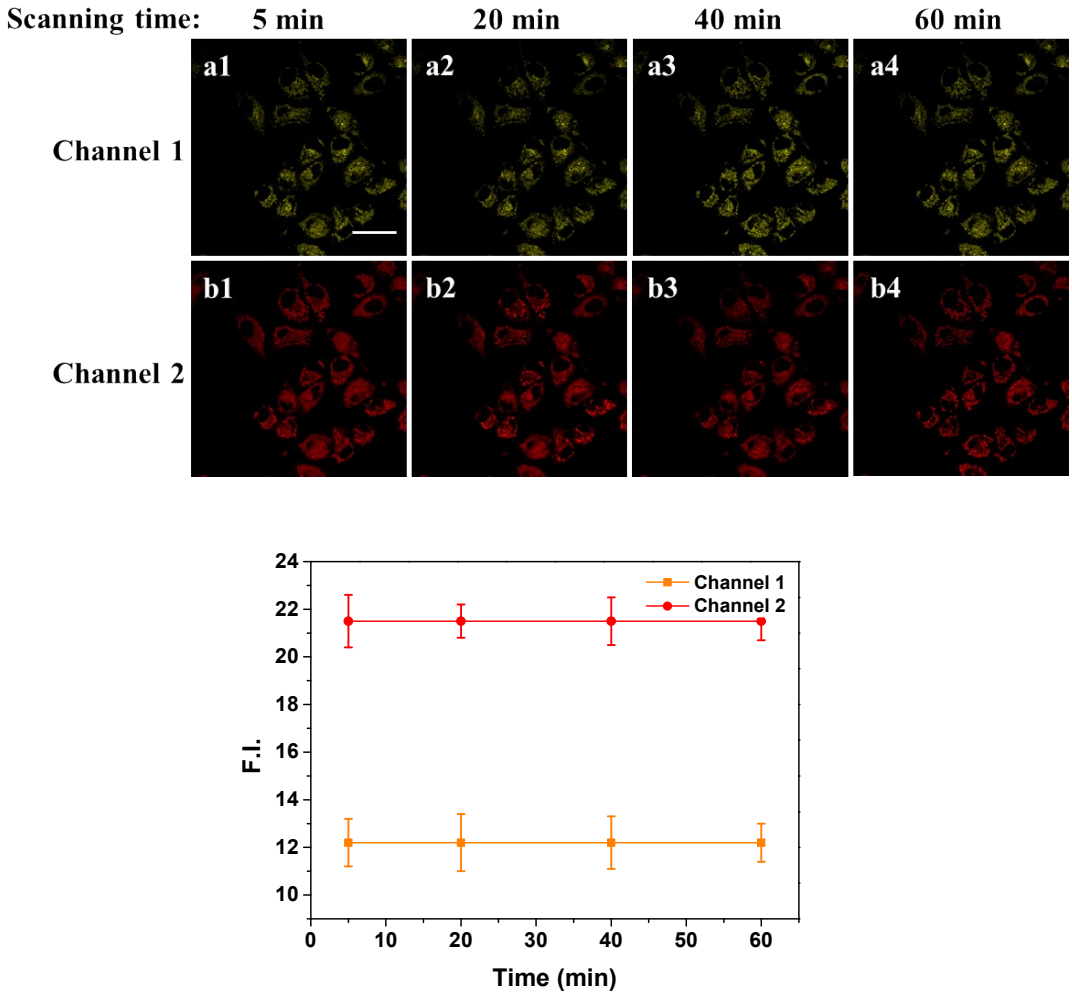


Fig. 14. Fluorescence images of Huh7 cells after different continuous scanning time of 5 min, 20 min, 40 min, 60 min. Preincubate the cells with INH (500 μM) for 4 h and then treat them with **FNN** (10 μM) for 30 min. The cells were washed briefly with 1.0 mL of PBS twice before recorded by fluorescence confocal microscopic.

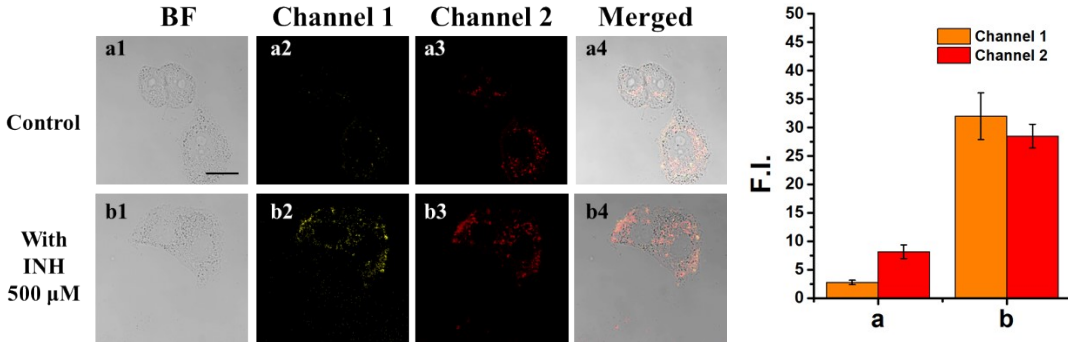


Fig. S15. Fluorescence images of HeLa cells labeled with **FNN** (10 μM) for 30 min (a) before and after treatment of (b) INH (500 μM) for 4 h. (1) bright field (BF); (2) channel 1 (Ex=460 nm, Em=500–600 nm); (3) channel 2 (Ex=520 nm, Em=600–700 nm); (4) merged images of (1), (2) and (3). Scale bar = 20 μm.

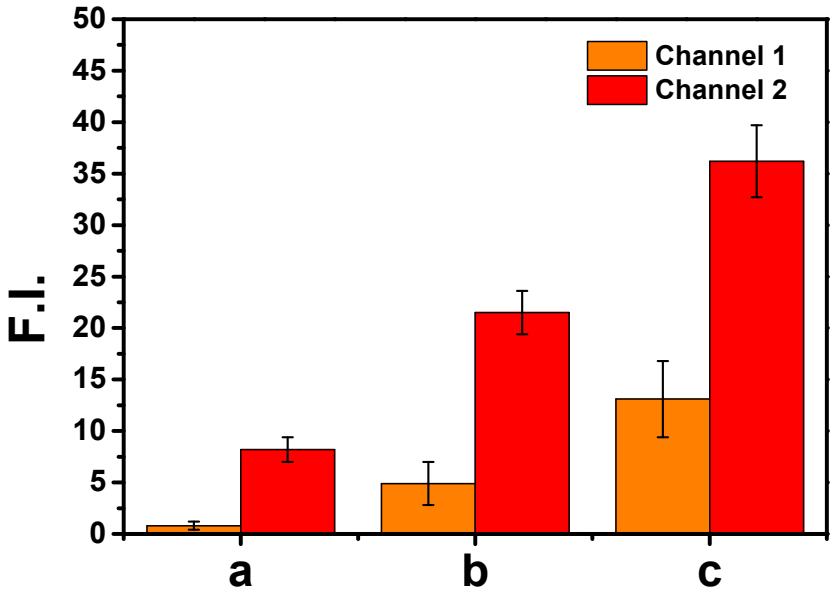


Fig. S16. The quantitative analysis of N_2H_4 and viscosity in living 3-day-old zebrafish. (a) Incubate zebrafishes with probe **FNN** (10 μM) for 2h; (b) Preincubated zebrafishes with INH (500 μM) firstly for 4 h and then with **FNN** for 2 h. (c) Preincubated zebrafishes with INH (1 mM) firstly for 4 h and then with **FNN** for 2h. Data are expressed as mean ± SD.

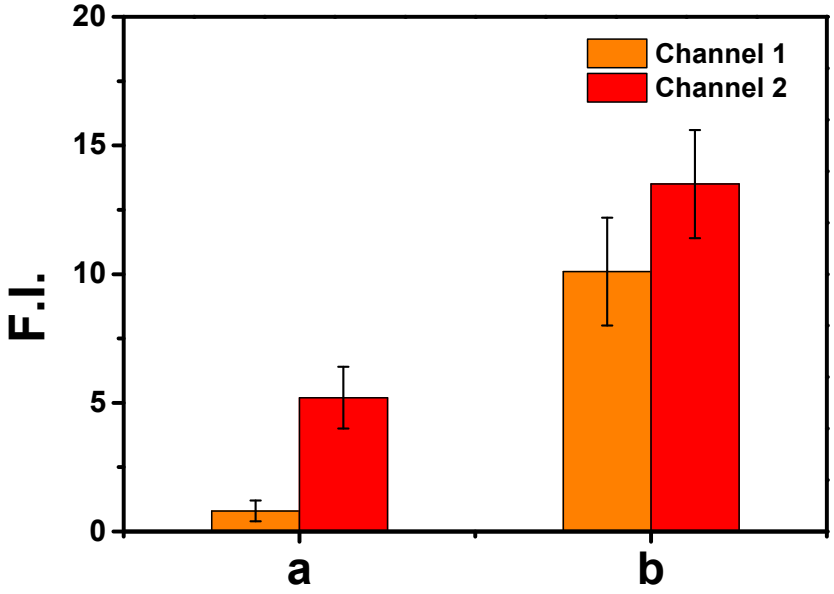


Fig. S17. The quantitative analysis living zebrafish embryo. (a) The cells only stained with **FNN** (10 μM) as control group. (b) Preincubate the cells with INH (500 μM) for 4 h then treat them with **FNN** (10 μM) for 2 h. Data are expressed as mean ± SD.

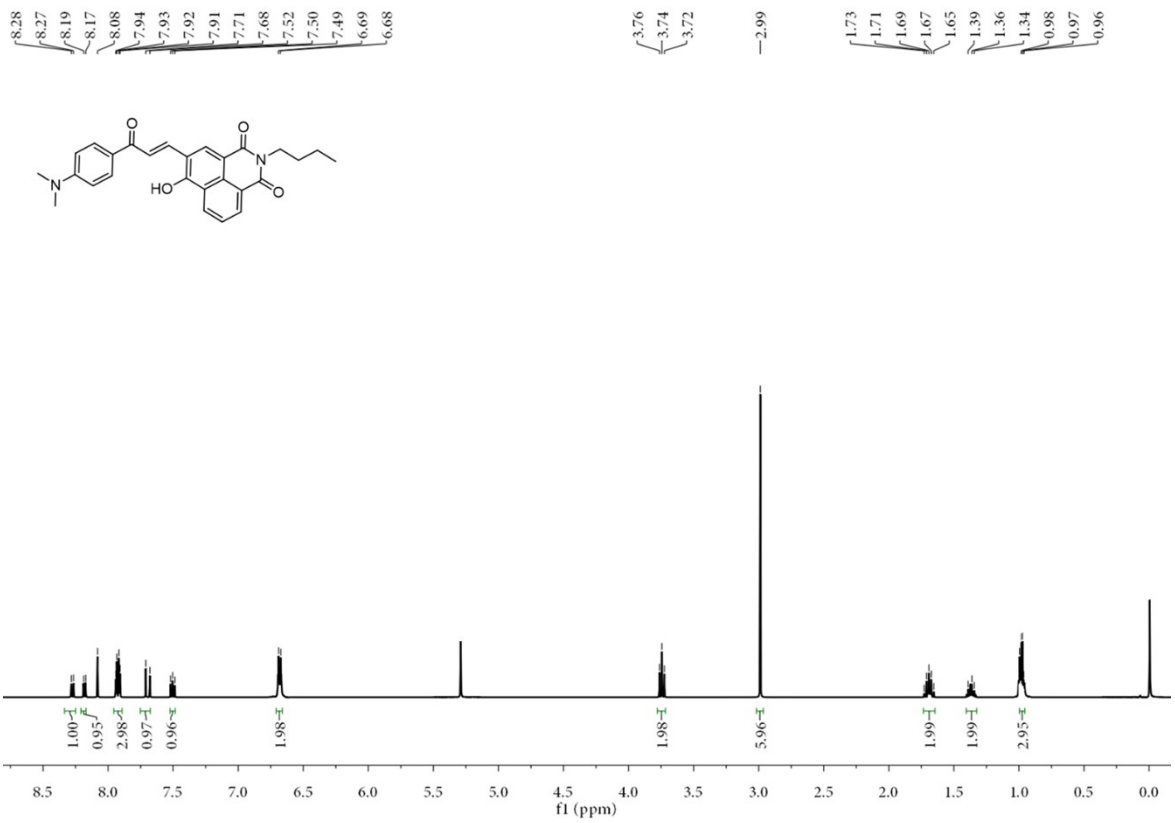


Fig. S18 ^{1}H NMR spectrum of **FNN** in $CDCl_3$

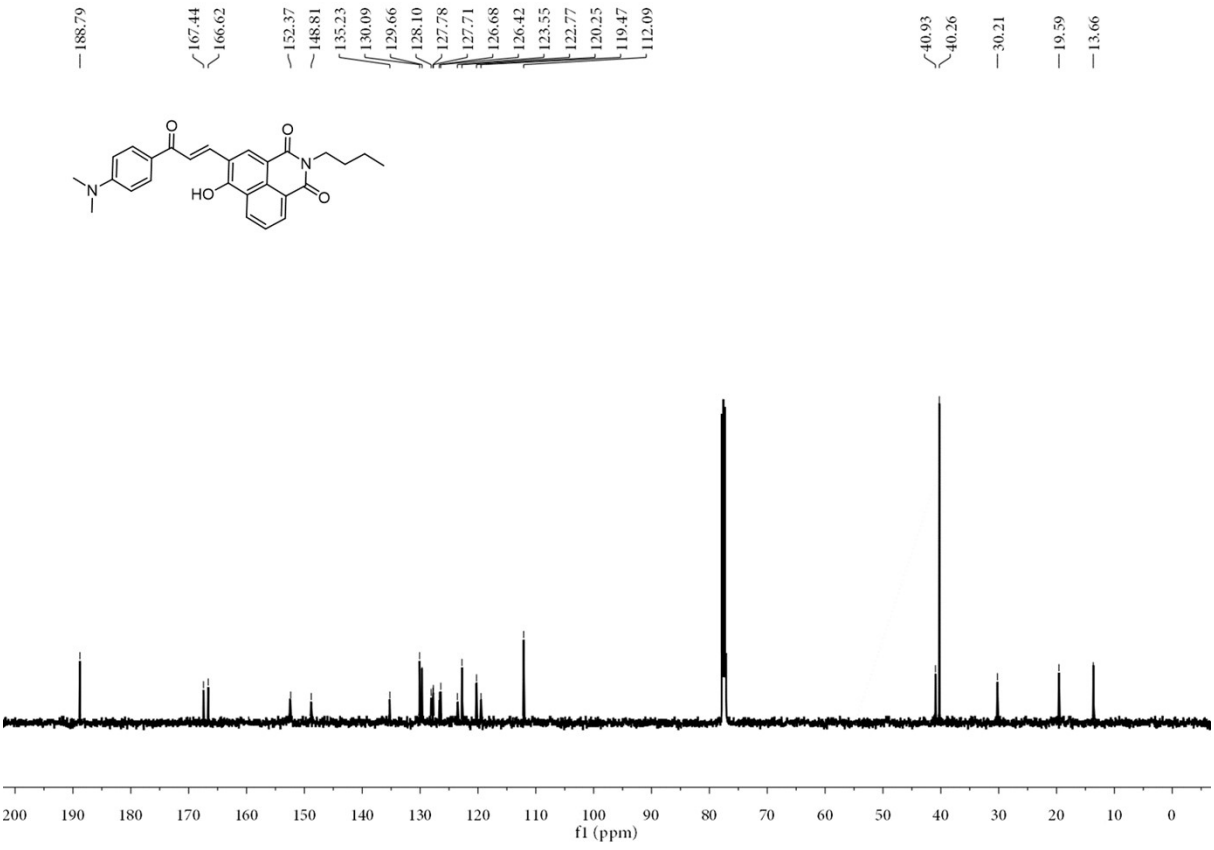


Fig. S19 ^{13}C NMR spectrum of **FNN** in $CDCl_3$

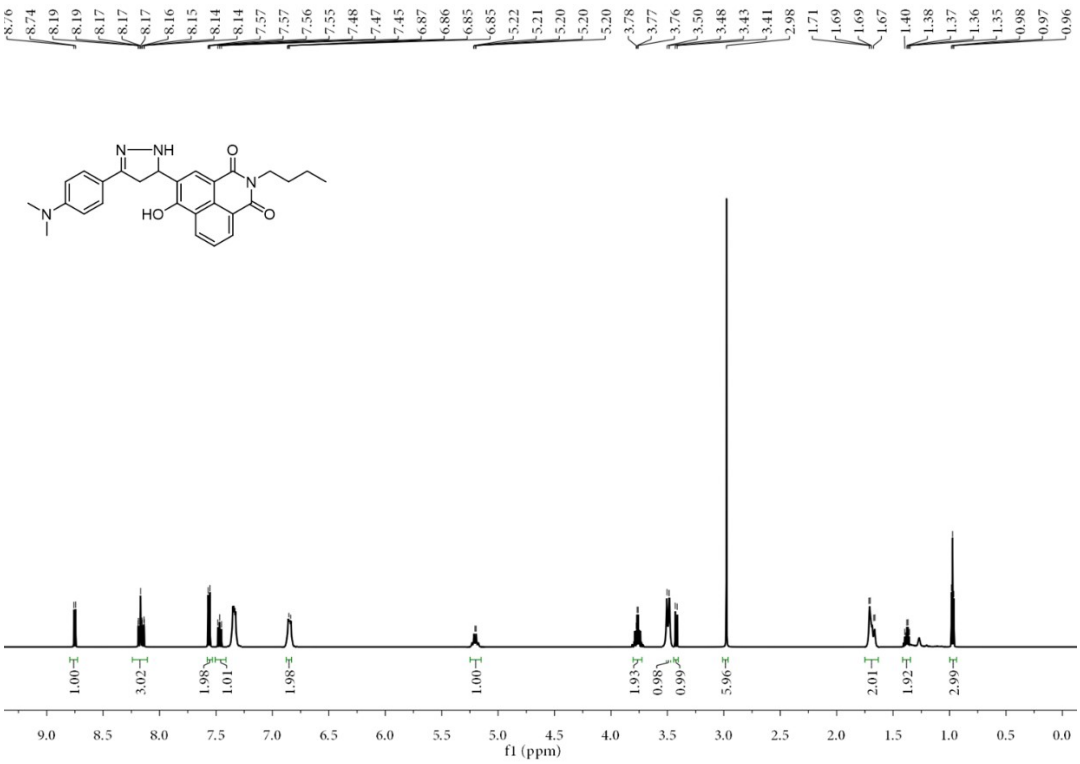


Fig. S20 ^{1}H NMR spectrum of **FNN-1** in $CDCl_3$