Supplementary Information

Near-Infrared Fluorescence Imaging of Vimentin Dynamic Enables Evaluation of Stroke Therapy in Live Mice

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1. Materials and Methods

BCA Protein Concentration Assay Kit was obtained from Boster Biological Technology Co., Ltd. 4-HNE and Withaferin A were purchased from MedChemExpress. 2% TTC was purchased from Beijing Solarbio Science & Technology Co., Ltd. Amoxicillin, Ranitidine, Hydrochloride, Omeprazole, Ginkgolide, Citicoline, Hydroxocobalamin, Mecobalamin and Cyanocobalamin were purchased from Shanghai Macklin Biochemical Co., Ltd. PC12 cells were purchased from Center of Excellence in Molecular Cell Science. Hela cells were purchased from Wuhan Pricella Biotechnology Co., Ltd. MCAO spigot line was purchased from Beijing Cinontech Co., Ltd. Fluorescence spectra were obtained with a Hitachi F-4700 fluorescence spectrophotometer. CCK-8 assay was performed using a Triturus microplate reader. Absorption spectra were recorded on a UV-Visible spectrophotometer (Evolution 220, Thermo Scientific). Confocal imaging was performed on LEICA STELLARIS. *In vivo* imaging was performed on PerkinElmer IVIS Lumina II.

2. Experimental Section

2.1 Peptide synthesis

The synthesis of peptide was done by Shanghai Apeptide Co., Ltd.

N, N-Dimethylformamide (DMF) and methanol were soaked overnight with G3 molecular sieves to remove impurities and water. A total of 2.0 g of blank Wang resin was placed in a clean, dry reaction tube. Then, 15 mL of DMF was added, and the mixture was activated at room temperature (rt) for approximately 30 min. At rt, the solvent was removed by vacuum filtration. Subsequently, 1 mmol of cysteine, along with 5-fold molar excess of 4-dimethylaminopyridine (DMAP) and N, N-Diisopropylcarbodiimide (DIC), was added to the tube, with DMF as the solvent. The reaction was conducted at rt for 3 hours. After the reaction, the resin was washed with DMF 4-6 times (5-6 mL each time). Thereafter, pyridine and acetic anhydride (1:1 volume ratio) were added, and the reaction was allowed to proceed for 30 min. Finally, the resin was washed again with DMF 4-6 times (5-6 mL each time), which served to block the unreacted active sites on the resin. The solvent was removed by vacuum filtration, and 10 mL of 20% piperidine in DMF solution was added to the resin. The mixture was stirred under N_2 for 10 min, followed by filtration to remove the solution. Another 10 mL of 20% piperidine in DMF solution was added, and the mixture was stirred under N_2 for 5 min before filtration. This operation was repeated twice. Afterward, the resin was washed with DMF 4 times and methanol 2 times (5-6 mL each time). The resin was washed with DMF 4 times and methanol 2 times (5-6 mL each time). A ninhydrin test showing a blue color indicated that the next step could be conducted. Repeat the procedure until the synthesis of Threonine is completed. After removing the Fmoc protecting group, dry the mixture by suction. The resin was cleaved with a trifluoroacetic acid (TFA) cleavage solution (95% TFA: 2% Triisopropylsilane (TIS): 2% 1,2-ethanedithiol (EDT): 1% H2O) for 2 hours. The reaction solution was subjected to vacuum filtration to obtain a TFA solution of the peptide. The cleavage solution was dried as much as possible under a stream of N2, followed by precipitation with diethyl ether and centrifugation. The resulting precipitate was washed with diethyl ether 3-5 times to obtain a white solid. The solid was dissolved in pure water and subjected to high-performance liquid chromatography (HPLC) for desalting and purification. Crystals were obtained

after lyophilization, and a small amount of the product was analyzed by mass spectrometry (MS).

2.2 Synthesis of Vim-NIG

The synthesis of the probe Vim-NIG was done by Shanghai Apeptide Co., Ltd.

80.85 mg (0.1 mmol) of the peptide was dissolved in DMF, followed by the addition of 84.95 mg (0.1 mmol) of IR820. Subsequently, an appropriate amount of triethylamine (TEA) was added to afford Vim-NIG.

2.3 Preparation of stock solution of probe Vim-NIG

When using probe Vim-NIG to detect other analytes, the preparation protocol for the probe stock solution is as follows: dissolve 1 mg of probe Vim-NIG in 62.6 μL of DMSO to prepare a 10 mM probe stock solution.

2.4 Viscosity experiment

Solutions were prepared by mixing glycerol and water at ratios of 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10. After homogenization by vortexing, the viscosity of each solution was measured using a viscometer, followed by subsequent experiments.

2.5 Cell culture

PC12 cells were cultured in RPMI 1640 supplemented with 10 % fetal bovine serum, 1% penicillin and 1% streptomycin at 37°C (w/v) in an MCO-15AC incubator in 5% CO_2 / 95% air. One day before imaging, the cells were detached and placed in glass-bottomed dishes.

Hela cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin at 37°C (w/v) in an MCO-15AC incubator in 5% CO_2 / 95% air. One day before imaging, the cells were detached and placed in glass-bottomed dishes.

2.6 BCA protein assays

The BCA protein assays were carried out by BCA Protein Assay Kit (Boster). All of the samples were lysed in the lysis buffer. Then 25 μL of reference sample and the sample to be tested were added in microplate, respectively. 200 μL of BCA working solution was added in each well and vibrate to mix sufficiently. The microplate was covered and incubated for 30 min at 37°C. After cooling to room temperature, the absorbance at 562 nm was measured using a Triturus microplate reader. The protein concentrations of samples were determined according to the standard curve of reference sample and the dilution ratio of samples.

2.7 Total protein extraction of Hela cells

Take the culture dishes out of the cell culture box, place them on ice, remove the culture medium, then wash them twice with pre-cooled PBS, add the lysis solution, scrape off the cells with a cell scraper, collect them in a centrifuge tube, centrifuge at 4℃, 12000 rpm for 5 min, and then take the supernatant. Use the BCA kit to determine the protein concentration and store it in a

-80℃ refrigerator.

2.8 Experimental procedure for fluorescence measurement

Different concentrations of whole proteins extracted from Hela cells were mixed with the probe Vim-NIG and diluted to a final volume of 1 mL using PBS buffer (10 mM, pH 7.4). The final concentration of Vim-NIG in the PBS solution was 10 μM/L. $\lambda_{ex}/_{em}$ = 773 /817 nm.

2.9 Cytotoxicity assays

Cell Counting Kit-8 (CCK-8) assays were carried out to evaluate the toxicity of Vim-NIG. PC12 cells (10^6 cells mL^{-1}) were seeded into 96-well microtiter plates with total volumes of 200 μL $well^{-1}$. After 24 h of incubation, various concentrations of Vim-NIG (0 M, 1×10^{-6} M, 2×10^{-6} M, 3×10^{-6} M, 5×10^{-6} M, 1×10^{-5} M, 2×10^{-5} M, 3×10^{-5} M and 5×10^{-5} M) were added, and the PC12 cells were cultured for another 24 h. Afterwards, 10 μL of CCK-8 solution was added to each well. After 4 h of incubation, the absorbance at 450 nm was measured using a Bio-Tek multimode reader.

2.10 Oxygen-glucose deprivation/reperfusion (OGD/R) model.

PC12 cells were incubated in RPMI 1640 without glucose with the deoxygenation reagent 0.5 mmol/L sodium dithionite for 30 min to simulate the oxygen-glucose deprivation process, and then incubated in RPMI 1640 containing glucose with 5% CO_2 and 95% O_2 for 30 min to simulate the reperfusion process.

2.11 Fluorescence Imaging of living cells

PC12 or HeLa cells were trypsinized, seeded into glass-bottomed dishes, and cultured for 24 hours. Before imaging, the cells were washed three times with 1 mL of phosphate-buffered saline (PBS). Imaging was performed using a Leica STELLARIS laser confocal microscope. Analysis was performed using Leica software. Each set of experiments was repeated at least 3 times. λ_{ex} = 638 nm, λ_{em} = 700 - 839 nm.

2.12 Middle Cerebral Artery Occlusion Model

The mice were allowed to acclimate for 1 week before the experiments. Animals were kept in a controlled environment with a stable temperature (22 ±2 °C), and had free access to food and water. All animal care and experimental protocols complied with the Animal Management Rules of the Ministry of Health of the People's Republic of China and were approved by the Animal Care Committee of Shandong Normal University (AEECSDNU2025160). The use of animals for the experiments followed the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) Model, as described previously. Mice were anesthetized with isoflurane (4% induction; 2% maintenance) throughout the operation. The left external carotid artery was completely exposed and ligated

using a 6-0 suture. A nylon monofilament was introduced through the external carotid stump into the left internal carotid artery to occlude the origin of the MCA and block distal blood flow. After 2 h of occlusion, the nylon monofilament was carefully removed to restore blood flow. Body temperature was maintained at 37 ± 0.5°C throughout the procedure. The sham group underwent an operation without the insertion of a nylon monofilament.[1]

2.13 *In vivo* Fluorescence imaging

In vivo imaging of Vim-NIG was performed on tribromoethanol-anesthetized mice. We chose intraperitoneal injection of Vim-NIG for the mice. Intraperitoneal injection has the advantages of large absorption areas by rich utilizing vessels of peritoneal membrane vessels and omentum. Through intraperitoneal injection, the drugs could enter the blood circulation and acts on the whole body. Importantly, intraperitoneal injection is easier to administer, which is widely used for the research of the blood-brain barrier-permeable drugs or imaging agents for the central nervous system.[2-4] Following intraperitoneal administration of 150 μL of 100 μM Vim-NIG, the mice were subjected to imaging for 60 min in a PerkinElmer IVIS Lumina II system. The imaging parameters were set at λ_{ex} = 780 nm and λ_{em} = 845 nm.

2.14 3, 5-Triphenyltetrazolium Chloride (TTC) Staining

Mice were euthanized and perfused intracardially with saline. After perfusion, the brains were immediately removed and cut into 1-mm sections in a mold. The sections were incubated in 2% TTC staining solution for 15 min at room temperature before fixation with 4% PFA.

2.15 Neurological function assessment

As shown in previous researches,[5,6] neurological function assessment of mice was recorded after I/R based on the modified Neurological Severity Score (mNSS), including raising the mouse by the tail, walking on the floor, beam balance tests and reflexes absence. The score ranges from 0 to 14, and it was positively correlated with the severity of ischemia-reperfusion injury. Detailed scores can be found in Table below:

Modified neurologic severity scores (mNSS)

Motor tests	**Points**
Raising the mouse by the tail	**3**
1 Flexion of forelimb	
1 Flexion of hindlimb	
1 Head moved more than 10° to the vertical axis within 30 seconds	
Walking on the floor (normal=0; maximum=3)	**3**
0 Normal walk	
1 Inability to walk straight	
2 Circling toward the paretic side	
3 Falling down to the paretic side	
Beam balance tests (normal=0; maximum=6)	**6**
0 Balances with steady posture	
1 Grasps side of beam	

2 Hugs the beam and one limb falls down from the beam

3 Hugs the beam and two limbs falls down from the beam, or spins on beam (>30 seconds)

4 Attempts to balance on the beam but falls off (>20 seconds)

5 Attempts to balance on the beam but falls off (>10 seconds) S-8

6 Falls off: No attempt to balance or hang on to the beam (<10 seconds)

Reflexes absence **2**

1 Pinna reflex (a head shake when touching the auditory meatus)

1 Corneal reflex (an eye blink when lightly touching the cornea with cotton)

Maximum points **14**

2.16 Immunofluorescence imaging

After the various treatments, cells were fixed with 4% paraformaldehyde for 25 min at rt Cells were permeabilized with 0.1% Triton X-100 in PBS, blocked by incubation in 1% bovine serum albumin in PBS (blocking solution) and incubated primary antibodies Rabbit-Vimentin at 1:700 dilution in the same solution. When necessary, secondary antibodies were used at 1:700 dilution in blocking solution.

2.17 Western blot assay

All the pre-treated cells were lysed in cell lysis buffer containing the protease inhibitor PMSF. The cell lysates were centrifuged at 12,000 rpm for 10 min at 4°C. The hippocampus was sonicated in RIPA lysis buffer containing protease and phosphatase inhibitors. The samples were then centrifuged at 12,000 rpm for 20 min at 4 °C. Protein concentrations were measured using the BCA Protein Assay Reagent with BSA to establish a standard curve. Equal proteins were lysed in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 6% glycerol, 0.005% bromophenol blue, and 2.5% 2-mercaptoethanol) and then boiled for 5 min at 95°C. Proteins were separated using SDS-polyacrylamide gel electrophoresis and were then transferred to polyvinylidene difluoride membranes at 100 V for 1 h. Membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T) (20 mM Tris pH 7.5, 137 mM NaCl, and 0.1% Tween 20) for 60 min and then incubated overnight at 4°C with the antibody. Then the membranes were washed with TBS-T, they were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (Abcam) at room temperature for 1 h. Then the samples were washed with TBS-T, protein bands were detected by using Immobilon Western Chemiluminescent HRP Substrate with the luminescent image analyzer (ChemiDoc MP, BIO RAD).

3. Supplemental Figures

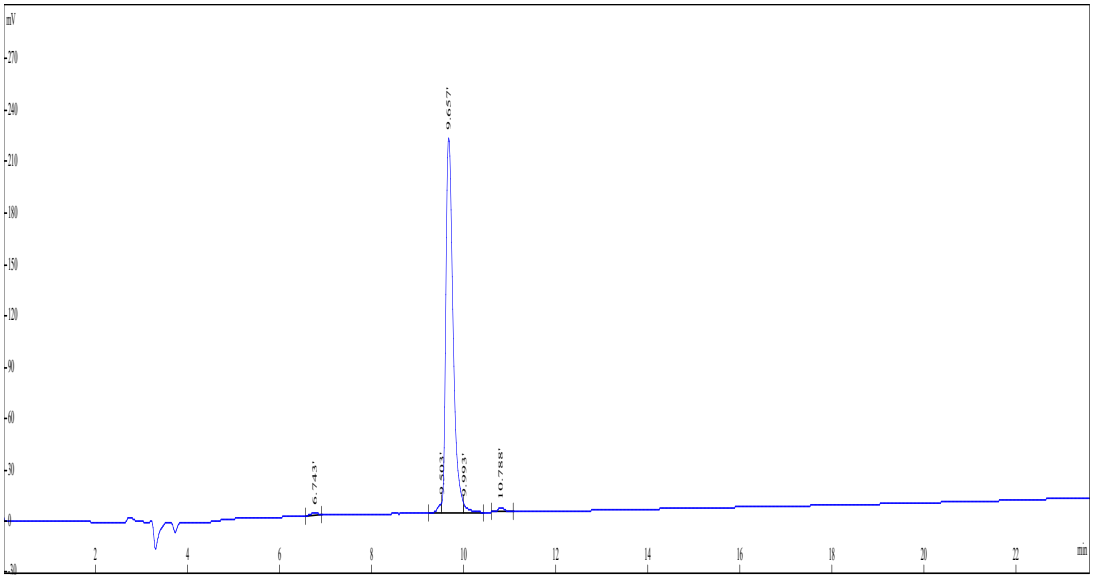

Figure S1. HPLC analysis of Vim-NIG. Vim-NIG was passed through a 0.22 μm filter, and 10 μL of sample was loaded onto Gemini-NX 10μ C18 100A, reversedphase column (4.6×250 mm). The column was eluted with acetonitrile/water (7:3, v/v). The flow rate was set at 1.0 mL/min. A UV/vis detector was used to monitor the desiring product at wavelength from 220 nm.

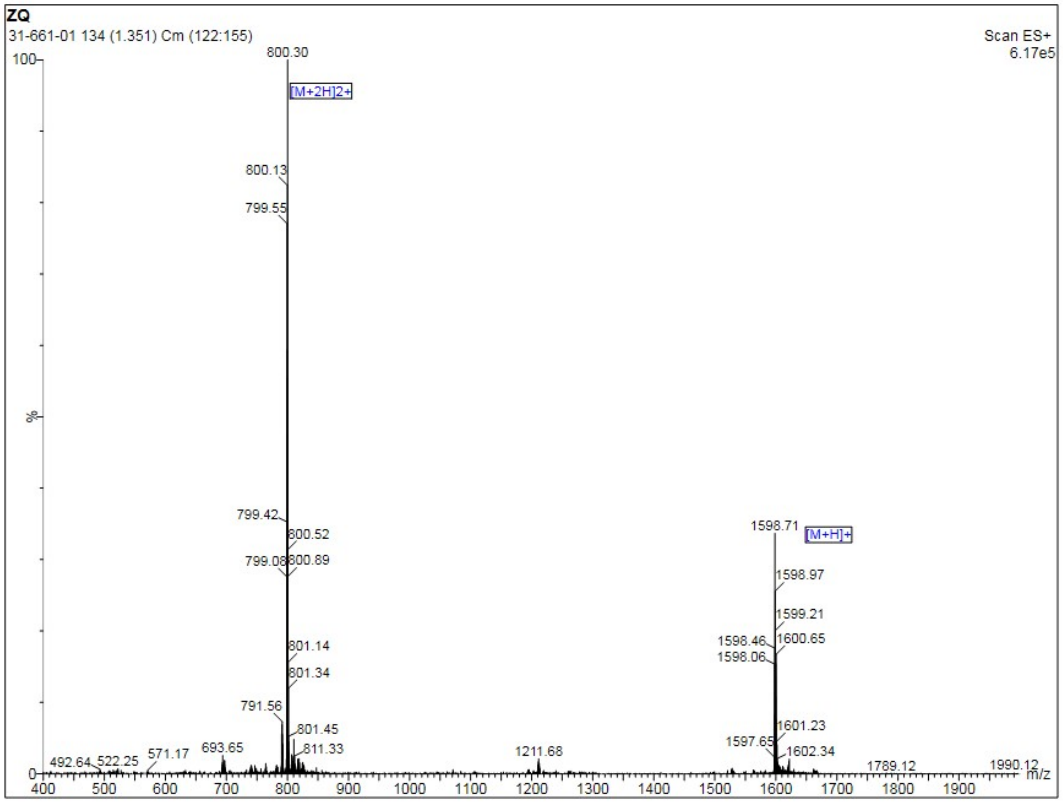

Figure S2. LC-MS of Vim-NIG.

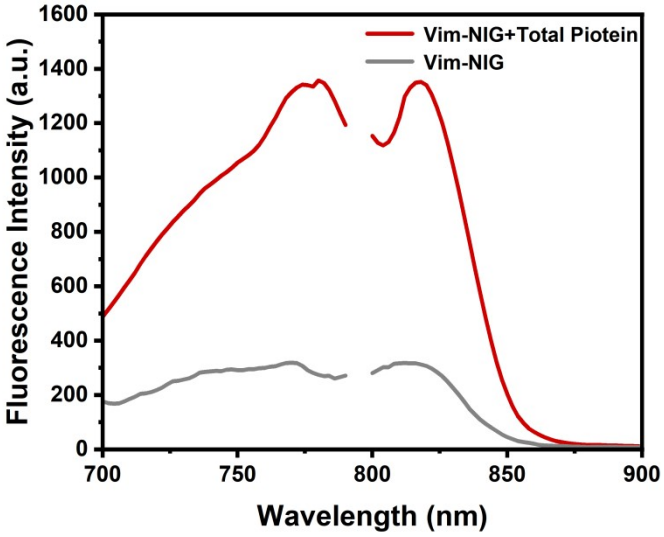

Figure S3. Fluorescence spectra of Vim-NIG (25 μM) before (black) and after (red) incubation with total protein for 30 min.

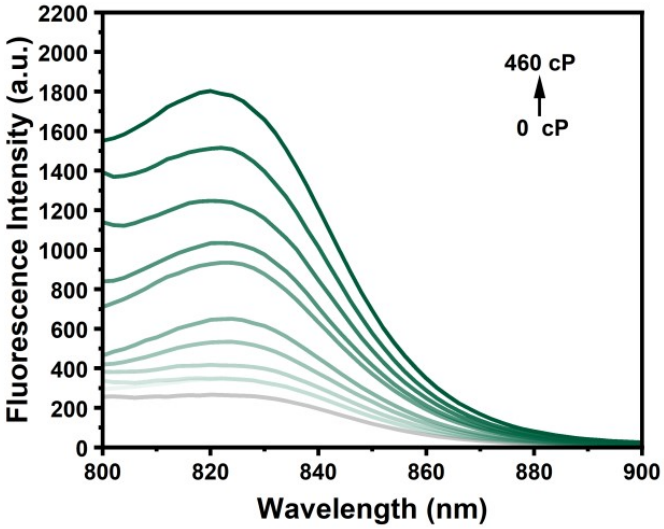

Figure S4. Fluorescence response of Vim-NIG (25 μM) in media with varying viscosities. λ_{ex} = 773 nm, λ_{em} = 817 nm.

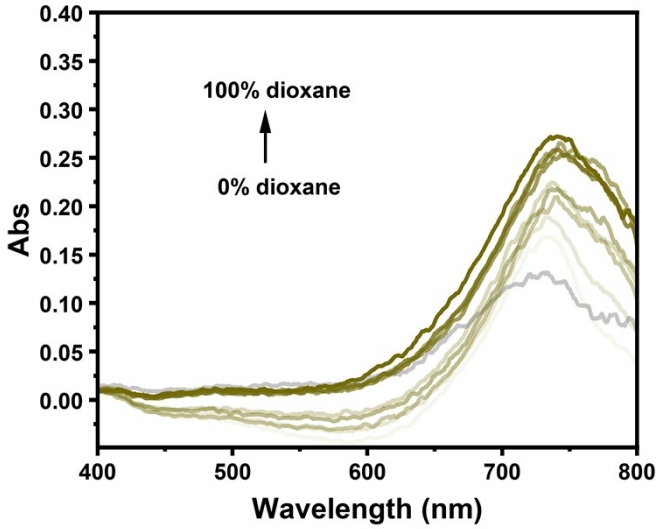

Figure S5. The absorption spectra of Vim-NIG in solvents with different polarities.

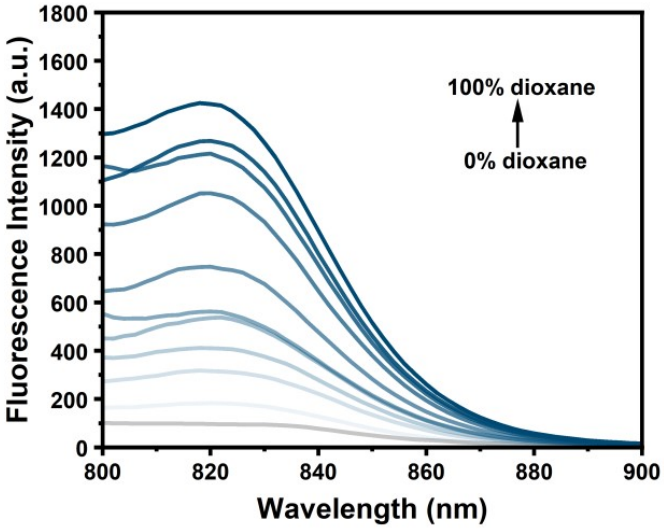

Figure S6. The fluorescence emission spectra of Vim-NIG in solvents with different polarities. λ_{ex} = 773 nm, λ_{em} = 817 nm.

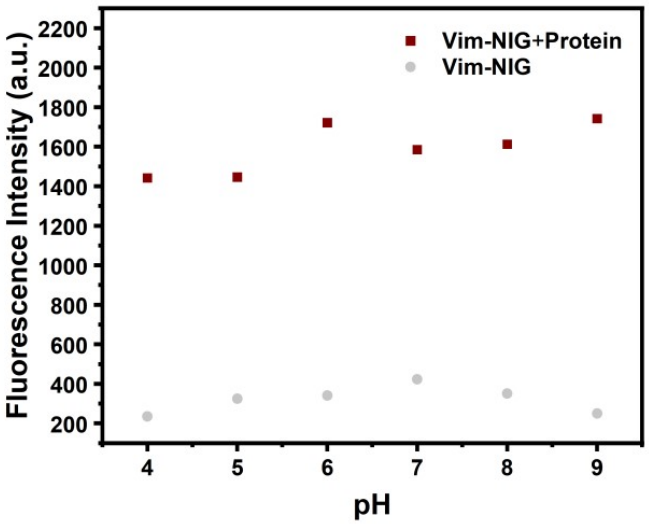

Figure S7. Fluorescence intensity of Vim-NIG (25 μM) before and after reacted with vimentin under different pH values.

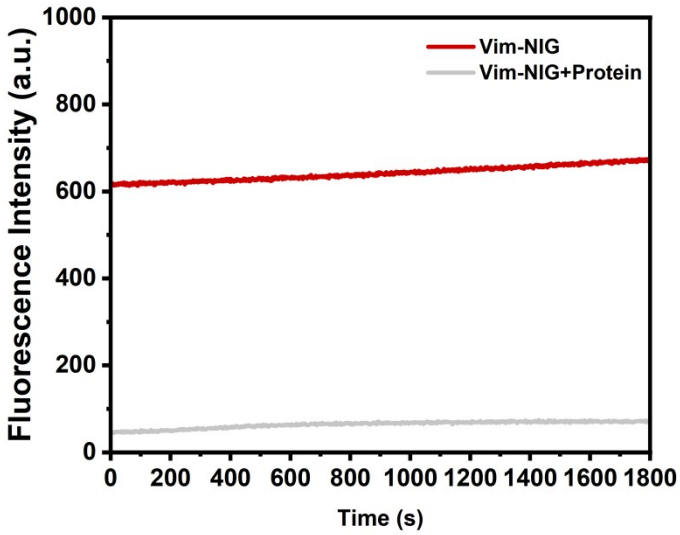

Figure S8. Fluorescence intensity of Vim-NIG (25 μM) within 30 min after reacting with 10 μg/mL total protein for 30 min. λ_{ex} = 773 nm, λ_{em} = 817 nm.

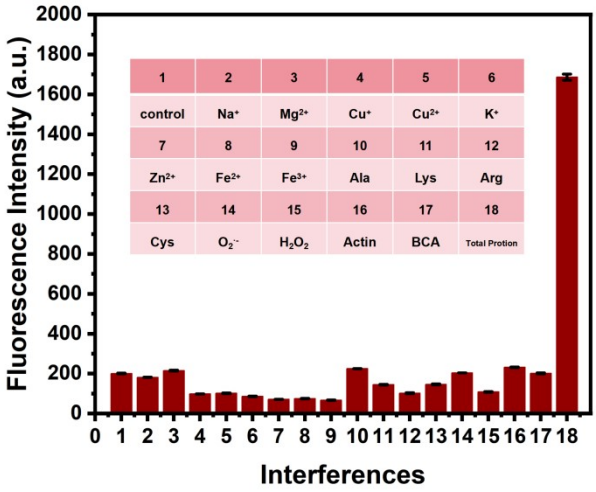

Figure S9. Spectral response of Vim-NIG. Fluorescence response of Vim-NIG to metal ions (1 mM), Ala (100 μM), Lys (100 μM), Arg (100 μM), Cys (100 μM), ROS (50 μM $O_2^{\bullet-}$, 1 mM H_2O_2), Actin (50 μg/mL), BCA (100 μg/mL) and Total Protein (100 μg/mL).

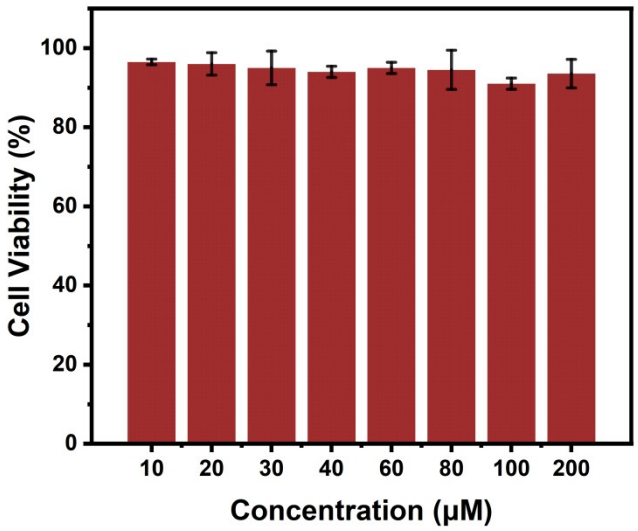

Figure S10. Effect of different concentrations of probe Vim-NIG after pretreated for 24 hours on cell viability was measured using CCK-8.

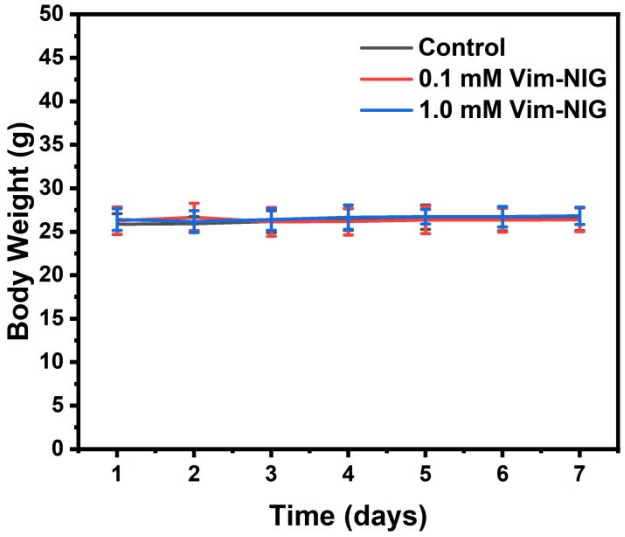

Figure S11. In vivo toxicity assay of the probe Vim-NIG. Mice were given a daily intraperitoneal injection of 0 mM (grey), 0.1 mM (red) and 1.0 mM (blue) of Vim-NIG.

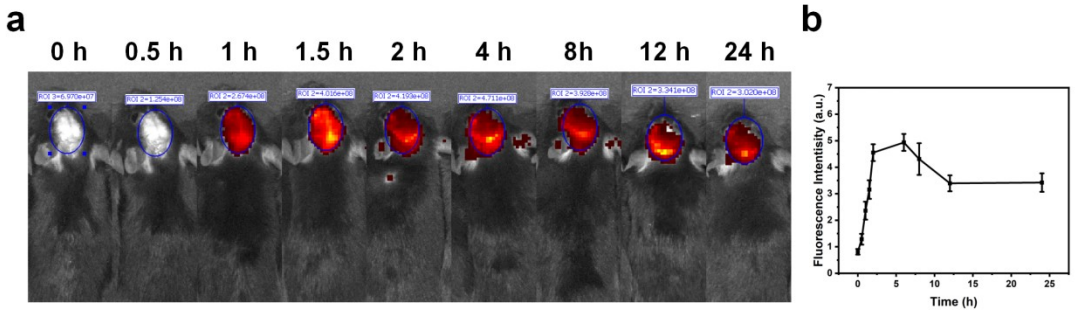

Figure S12. Metabolism of Vim-NIG in mice. (a) Fluorescence imaging of mice after intraperitoneal administration of 0.96 mg/kg of Vim-NIG. (b) Data output of relative fluorescence intensity in a.

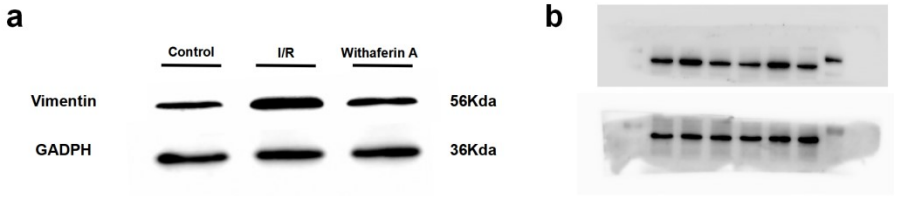

Figure S13. (a) Withaferin A inhibits vimentin content in the brain of mice with ischemia-reperfusion injury. (b) Uncropped immunoblot gels for Figure a.

Reference

(1) Tuo, Q.; Lei, P.; Jackman, K. A.; Li, X.; Xiong, H.; Li, X.; Liuyang, Z.; Roisman, L.; Zhang, S.; Ayton, S.; Wang, Q.; Crouch, P. J.; Ganio, K.; Wang, X.; Pei, L.; Adlard, P. A.; Lu, Y.; Cappai, R.; Wang, J.; Liu, R.; Bush, A. I. Tau-Mediated Iron Export Prevents Ferroptotic Damage after Ischemic Stroke. *Mol. Psychiatry* **2017**, *22* (11), 1520-530.

(2) Schiffer, W. K.; Mirrione, M. M.; Dewey, S. L. Optimizing Experimental Protocols for Quantitative Behavioral Imaging with18F-FDG in Rodents. *J. Nucl. Med.* **2007**, *48* (2), 277-287.

(3) Kimura, R.; Murata, T. Influence of Alkylamides of Glutamic Acid and Related Compounds on the Central Nervous System. I. Central Depressant Effect of Theanine. *Chem. Pharm. Bull. (Tokyo)* **1971**, *19* (6), 1257-1261.

(4) Yokoyama, H.; Itoh, O.; Ogata, T.; Obara, H.; Ohya-Nishiguchi, H.; Kamada, H. Temporal Brain Imaging by a Rapid Scan ESR-CT System in Rats Receiving Intraperitoneal Injection of a Methyl Ester Nitroxide Radical. *Magn. Reson. Imaging* **1997**, *15* (9), 1079-1084.

(5) Chen, J.; Zhang, C.; Jiang, H.; Li, Y.; Zhang, L.; Robin, A.; Katakowski, M.; Lu, M.; Chopp, M. Atorvastatin Induction of VEGF and BDNF Promotes Brain Plasticity after Stroke in Mice. *J. Cereb. Blood Flow Metab.* **2005**, *25* (2), 281-290.

(6) Li, Y.; Chopp, M.; Chen, J.; Wang, L.; Gautam, S. C.; Xu, Y. X.; Zhang, Z. Intrastriatal Transplantation of Bone Marrow Nonhematopoietic Cells Improves Functional Recovery after Stroke in Adult Mice. *J. Cereb. Blood Flow Metab.* **2000**, *20* (9), 1311-1319.