Electronic Supplementary Information for:

A near-infrared fluorescence off-on probe for sensitive imaging of hydrogen polysulfides in living cells and mice in vivo

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1. Reagents and apparatus

IR 780 iodide, resorcinol, potassium carbonate, benzoyl chloride, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich. Salicylic acid and CH_3CN were obtained from Beijing InnoChem Science & Technology Co. Ltd. 4-Dimethylaminopyridine (DMAP) and cetrimonium bromide (CTAB) were purchased from J&K Scientific Ltd. The phosphate buffer (50 mM, pH 7.4) was prepared by disodium phosphate dodecahydrate and sodium dihydrogen phosphate. Na_2S_2 and Na_2S_4 used in this study were purchased from Dojindo molecular technologies, INC and Alfa Aesar, respectively. Fluorescence quantum yield (Φ) was determined in the phosphate buffer by using indocyanine green (Φ = 0.13 in DMSO) as a standard.

^{1}H-NMR and ^{13}C-NMR spectra were measured with a Bruker DMX-400 spectrometer in $CDCl_3$. Electrospray ionization (ESI) mass spectra were implemented with a Shimadzu LC-MS 2010A instrument (Kyoto, Japan). MTT analysis was made on a microplate reader (Molecular Devices SpectraMax i3). Fluorescence imaging was made on an FV 1200-IX83 confocal laser scanning microscope (Olympus, Japan) with an optical section of 0.5 μm. The incubation was performed in a Shaker incubator (SKY-100C, China). In vivo fluorescence imaging was made on a Kodak In-vivo Imaging System FX Pro.

2. Synthesis of probe 1

The starting material of 2-(benzoylthio)benzoic acid and HXPI were prepared following the reported procedure.[1,2]

To a mixture of HXPI (54 mg, 0.1 mmol), 2-(benzoylthio)benzoic acid (39 mg, 0.15 mmol), EDC (29 mg, 0.15 mmol) and DMAP (6 mg, 0.05 mmol) was added CH_2Cl_2 (10 mL) at room temperature. The mixture was stirred for 30 min, then neutralized with dilute HCl, and finally partitioned between H_2O and CH_2Cl_2. Then the organic phase was dried over anhydrous Na_2SO_4, evaporated under reduced pressure and subjected to silica gel chromatography eluted with CH_2Cl_2/CH_3OH (v/v, 60:1), affording probe **1** as a blue solid (24 mg, yield 31%). ^{1}H-NMR and ^{13}C-NMR spectra of probe **1** are shown below in Figs. S1 and S2, respectively. ^{1}H-NMR (400 MHz, 298 K, $CDCl_3$): δ 8.62 (s, 1H, J=15.2 Hz), 8.50 (m, 1H), 8.03 (s, 1H), 8.01 (s, 1H), 7.75-7.62 (m, 5H), 7.57 (s, 1H), 7.54 (s, 1H), 7.46 (m, 3H), 7.40 (d, 1H, J=8.4 Hz), 7.27 (s, 1H), 7.05 (m, 2H), 6.79 (d, 1H, J=14.8 Hz), 4.67 (t, 2H, J=7.2 Hz), 2.87 (t, 2H, J=5.8 Hz), 2.74 (t, 2H, J=5.8 Hz), 2.06-1.95 (m, 4H), 1.78 (s, 6H), 1.10 (t, 3H, J=7.2 Hz). ^{13}C-NMR (100 MHz, 298 K, $CDCl_3$): δ 189.2, 178.5, 163.9, 159.7, 152.9, 152.5, 146.1, 142.0, 141.4, 137.3, 136.3, 133.8, 133.0, 132.9, 131.7, 130.7, 130.3, 129.7, 129.2, 129.1, 128.7, 128.0, 127.7, 127.7, 122.3, 119.7, 119.0, 115.6, 113.4, 109.4, 106.9, 50.9, 48.4, 29.4, 28.1, 24.9, 21.5, 20.0, 11.5.

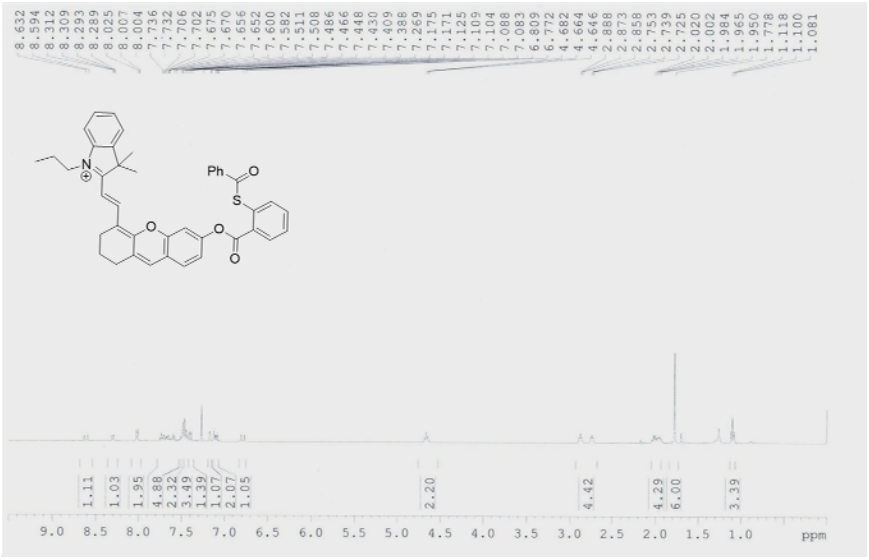

Fig. S1 ^{1}H-NMR spectrum of probe **1** (400 MHz, 298 K, $CDCl_3$).

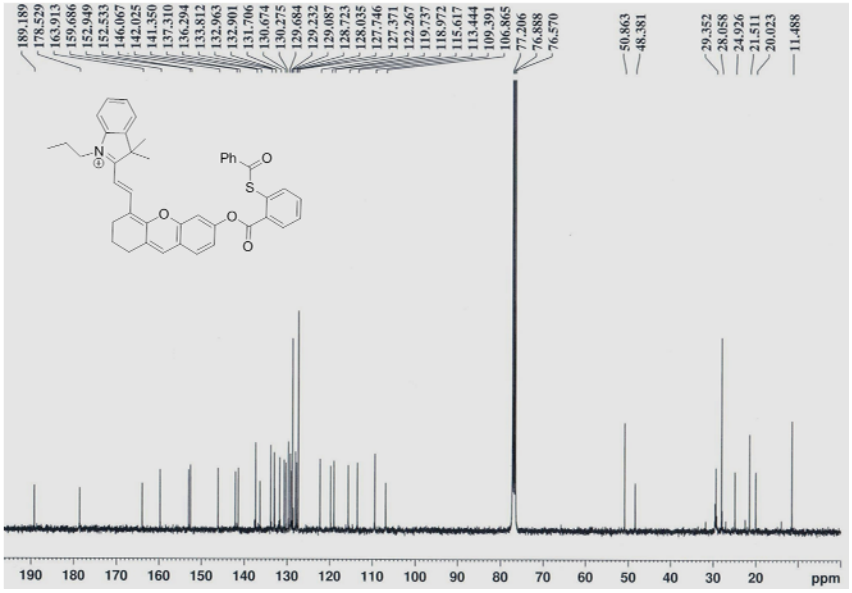

Fig. S2 ^{13}C-NMR spectrum of probe **1** (100 MHz, 298K, $CDCl_3$).

3. Solution preparation and general procedure for spectroscopic analysis

The stock solution (1 mM) of probe **1** was prepared in DMSO. Ultrapure water (over 18 MΩ·cm) was used throughout. The solution of hydrogen polysulfides was prepared from Na_2S_2, or Na_2S_4 in 50 mM phosphate buffer. The stock solution of CTAB (5 mM) was prepared in EtOH. The stock solution of S_8 (200 mM) was prepared in CH_2Cl_2, and then diluted to 10 mM with EtOH. Superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), H_2O_2 and $ONOO^-$ were prepared according to the reported literature.[3] All of the test solutions were prepared freshly.

Unless otherwise specified, the spectroscopic analyses were made according to the

following procedure. In a tube, 5 mL of the phosphate buffer (50 mM, pH 7.4) and 100 μL of the stock solution of CTAB were mixed, followed by the addition of an appropriate volume of sample solution and 100 μL of the stock solution of probe **1**. The final volume was adjusted to 10 mL with the phosphate buffer and the reaction solution was mixed well. After incubation at 37 ^{o}C for 10 min, a 3-mL portion of the reaction solution was transferred to a quartz cell of 1-cm optical length to measure absorbance or fluorescence with $\lambda_{ex/em}$ = 680/708 nm (both excitation and emission slit widths were set to 10 nm). Under the same conditions, a blank solution containing no testing species (control) was prepared and measured for comparison. The results are expressed as the mean ± standard deviation of three measurements.

4. Cell incubation and fluorescence imaging

HeLa cells were grown on glass-bottom culture dishes (MatTek Co.) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin at 37 °C in a humidified 5% CO_2 incubator. RAW264.7 cells (mouse leukemic monocyte macrophage) were grown in Roswell Park Memorial Institute-1640 medium (RPMI-1640) supplemented with 10% (v/v) heat-inactivated new-born calf serum (NBS) and 1% (v/v) penicillin-streptomycin at 37 °C in a humidified 5% CO_2 incubator. Before use, the adherent cells were washed three times with FBS-free DMEM or NBS-free RPMI-1640. For fluorescence imaging of HeLa cells, the cells were incubated with 10 μM probe **1** in FBS-free DMEM at 37 °C for 20 min. After removal of excess probe and washing with FBS-free DMEM three times, the cells were incubated with Na_2S_2, Na_2S_4 or Na_2S for 20 min in the phosphate buffer (pH 7.4, containing 50 μM CTAB). For fluorescence imaging of RAW264.7 cells, endogenous H_2S_n was produced under the stimulation with lipopolysaccharides (LPS, bacterial endotoxin). In brief, 1 μg/mL LPS was added to the dishes containing RAW264.7 cells in RPMI 1640 supplemented with 10% (v/v) NBS and 1% (v/v) penicillin-streptomycin at 37 °C in a humidified 5% CO_2 incubator, and the cells were incubated for different periods of time. Then the cells were incubated with 10 μM probe **1** in the phosphate buffer containing 50 μM CTAB for 20 min, and washed three times with NBS-free RPMI 1640 for imaging use. Cell imaging was performed with 635 nm excitation and 650-750 nm emission through a 100 × 1.4 NA objective. The pixel intensity of the cells in the fluorescence image was determined by using ImageJ software (version 1.45s, NIH). The cells were taken as a Region of Interest (ROI) based on their periphery. For comparison, the pixel intensity at least from three cells in each fluorescence image was measured and averaged in this work.

5. In vivo imaging of mice

Five-week-old male BALB/c nude mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd., and were divided into two groups (A, control; B,

test) for imaging. All animal experiments were approved by the Beijing Association on Laboratory Animal Care and the Association for Assessment and Accreditation of Laboratory Animal Care, and performed according to their guidelines. Na_2S_2 was dissolved in warm saline (2 mM); the saline contains 50 μM CTAB for both group A and group B.

6. Spectral properties of probe 1

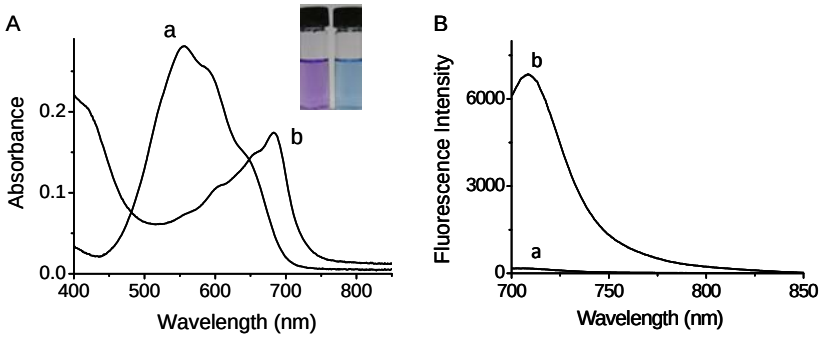

Fig. S3 (A) Absorption and (B) fluorescence emission (λ_{ex} = 680 nm) spectra of probe **1** (10 μM) before (a) and after (b) reaction with Na_2S_2 (20 μM) at 37 °C for 10 min. The color change of the reaction solution is shown in the inset of panel A.

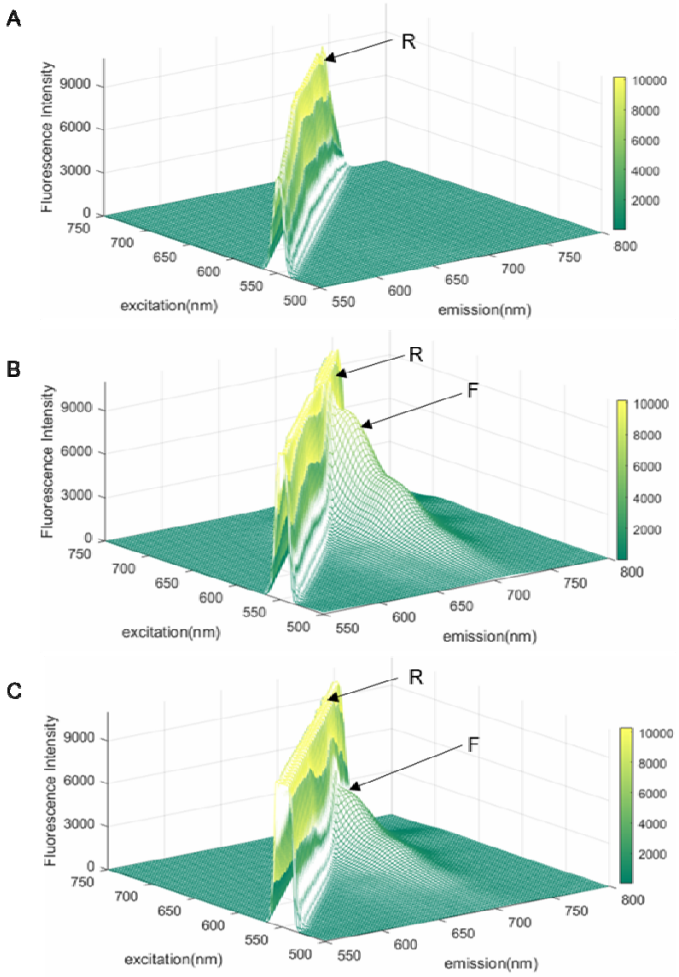

Fig. S4 Fluorescence excitation-emission matrix scans of (A) probe **1** (10 μM), (B) HXPI

(10 μM) and (C) probe **1** (10 μM) with Na_2S_2 (10 μM) in the presence of 50 μM CTAB. The Reyleigh (R) and fluorescence (F, $\lambda_{ex/em}$ =680/708 nm) peaks were indicated by arrows. Matrix scans were performed at 3 nm resolution and 10 nm band pass.

7. Study on reaction mechanism of probe 1 with H_2S_n

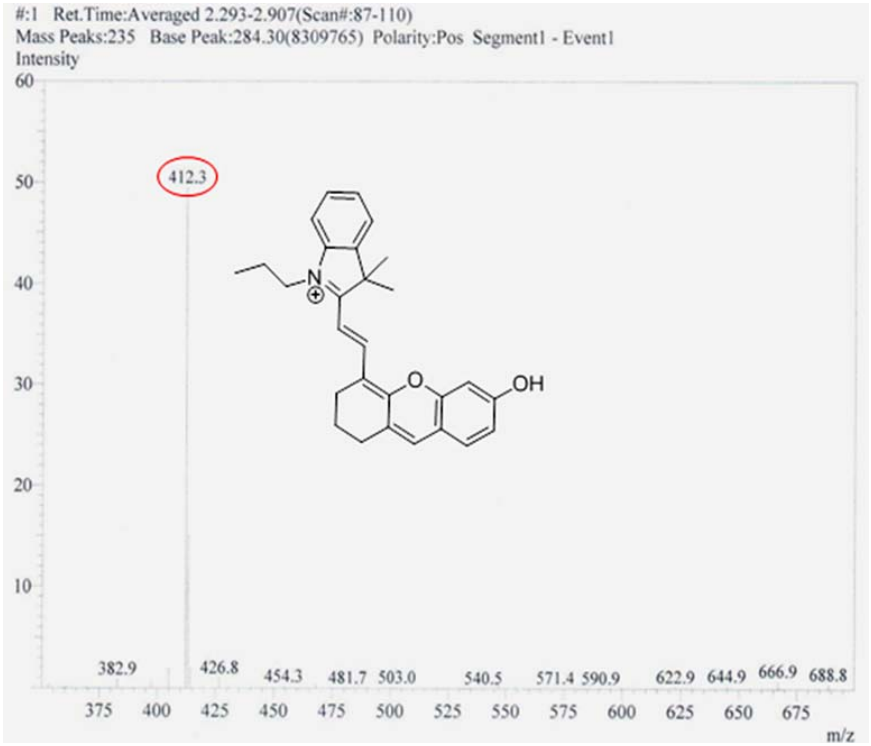

Fig. S5 ESI mass spectrum of the reaction solution of probe **1** (10 μM) with Na_2S_2 (20 μM). The peak at *m/z* = 412.3 indicates the generation of HXPI (the probe was completely converted to HXPI in the presence of Na_2S_2).

8. Optimization of experimental conditions

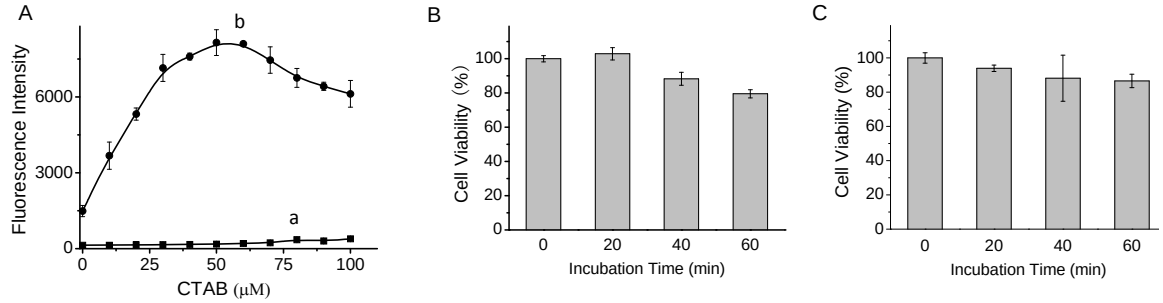

Fig. S6 (A) The effect of CTAB concentration on the fluorescence intensity of probe **1** (10 μM) in the absence (a) and presence (b) of Na_2S_2 (20 μM). The measurements were performed at 37 ^oC in the phosphate buffer (50 mM, pH 7.4) with $\lambda_{ex/em}$ = 680/708 nm. The optimum concentration of CTAB is 50 μM, at which the viability of (B) HeLa or (C) RAW264.7 cells is still over 90% within 40 min.

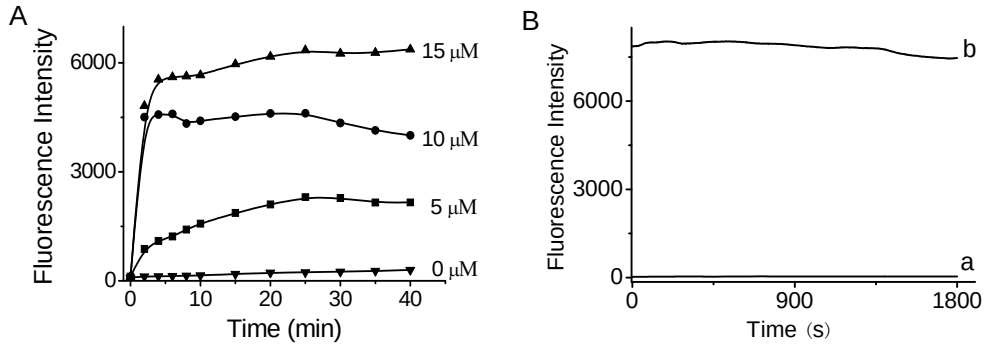

Fig. S7 (A) Plots of fluorescence intensity vs. the reaction time of probe **1** (10 μM) with varied concentrations of Na_2S_2 (0, 5, 10, 15 μM) in the presence of 50 μM CTAB. (B) Photostability of probe **1** (a) and HXPI (b) under the continous irradiation of xenon lamp (150 W).

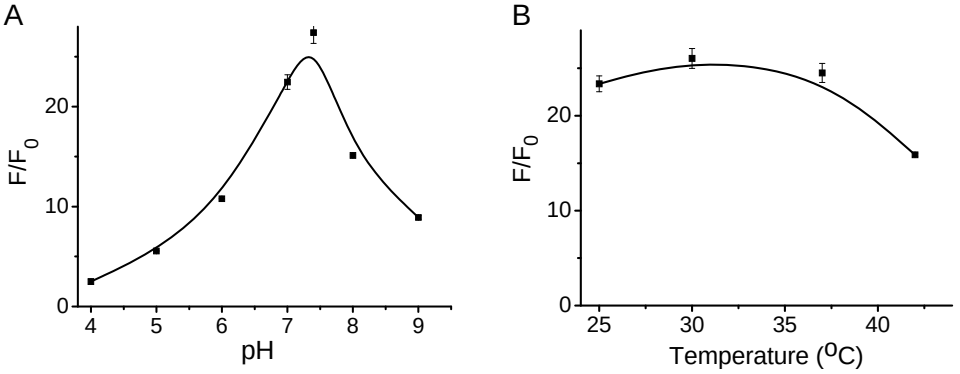

Fig. S8 Effects of (A) pH and (B) temperature on the fluorescence intensity of probe **1** (10 μM) reacting with Na_2S_2 (10 μM). $\lambda_{ex/em}$ = 680/708 nm. F and F_0 represent the fluorescence intensity from the corresponding reaction solution and the reagent blank, respectively.

9. Cytotoxicity of probe 1 to cells

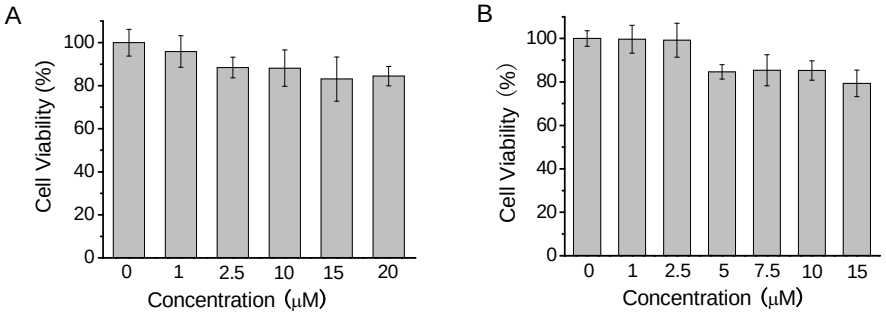

Fig. S9 Effects of probe **1** at varied concentrations on the viability of (A) HeLa and (B) RAW264.7 cells. The cytotoxicity of probe **1** to HeLa and RAW264.7 cells was evaluated by MTT assay following the reported procedure.[4] The viability of the cells without probe **1** is defined as 100%. The results are expressed as the mean ± standard deviation of five separate measurements.

10. Comparison of probe 1 with other fluorescent probes for H_2S_n

Table S1. Comparison of probe 1 with other small molecular probes for H_2S_n

Recognition unit of probe	$\lambda_{ex/em}$ (nm)	Detection range (μM)	Detection limit (nM)	Application	Depletion by thiols	Ref.
2-Fluoro-5-nitro-benzoic ester	490/515	0.5-15	71	Cell	Yes	5a
	707/737	1-20	50	Cell, mice		5b
	675/730	1-10	25	Cell, mice, organ		5c
	368/534	1-20	500	Cell, zebrafish		5d
	405/460, 518	1-8	100	Cell, tissue		5e
	370/448, 541	0.5-15	700	Cell, tissue, organ		5f
	730/780	5-50	80	Cell, mice		5g
	680/720	0.5-10	22	Cell, mice		5h
Ring-opening of aziridines	350/530	1-20	300	Bovine plasma	No	6
Phenyl 2-(benzoyl-thio)benzoate	490/515	0.25-20	3	Cell	No	7
	680/708	0.5-14	35	Cell, mice		This work

11. References

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