Electronic Supplementary Information

In situ detection of hydroxyl radicals in mitochondrial oxidative stress with nanopipette electrode

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Contents:

1. EXPERIMENTAL SECTION

Chemicals and Reagents

Borosilicate glass capillaries (BF100-78-10; i.d. = 0.78 mm; o.d. = 1 mm, with filament) were purchased from Sutter Instrument Co.. Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$), 1-hexanethiol (HAT), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were bought from Sigma-Aldrich. KO_2, DMSO, NaClO, $FeSO_4$, 2,2'-azobis (2-methylpropionamidine)-dihydrochloride (AAPH), and diethylamine nonoate (DEA NONOate) were acquired from Aladdin Chemistry Co. Ltd. Hoechst 33258, Mito Tracker Deep Red FM, calcein-AM, propidium iodide were provided by Invitrogen Corp. 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES), KCl, HCl, NaOH, 30% H_2O_2 and 98% H_2SO_4 were prepared from Sinopharm Chemical Reagent Co., Ltd.

Apparatus and Instrumentation

Glass capillaries were pulled by a laser puller (Sutter P-2000 micropipette puller). A Hitachi S-4800 scanning electron microscope provided scanning electron microscope (SEM) images at 15 kV accelerating voltage. An ultrathin coating of platinum was ejected onto the unmodified nanopore before SEM analysis. A JEOL JEM-2100 transmission electron microscope was employed to obtain transmission electron microscope (TEM) images, energy-dispersive X-ray (EDX) spectrum, and elemental mappings. X-ray photoelectron spectrometer (XPS) data were collected from a Thermo Scientific ESCALAB 250Xi. Zeta potential was detected by a Malvern Zetasizer Nano ZS90. The static contact angle was caught by a JC2000A contact angle meter (Shanghai Zhongchen Digital Technology Co. Ltd., China). The Sensapex zero drift manipulator was applied for positioning nanoelectrode under the observation by Leica TCS-SP8 confocal scanning microscope.

Fabrication of Hexanethiol-Modified Glass Nanopipettes

To remove organic residue from the capillary glass surface, the borosilicate glass capillaries should be completely immersed in piranha solution (98% H_2SO_4 : 30% H_2O_2 = 3 : 1) for 2 h before pulling. The capillaries were then ultrasound in large quantities of deionized water for another 1 h and dried in an oven at 80℃. Nanopipettes used in the experiment were then fabricated by a Sutter P-2000 laser puller. The shape and tip size of the nanopipette can be adjusted by setting different heating temperatures, speed, delay time, tension value, and other parameters of the laser puller. The parameters used in this work is: HEAT = 280; FIL = 3; VEL = 40; DEL = 180; PULL = 200. The pulled glass nanopipette was coated with gold film by the UV irradiation method.[1] First, ethanol and chloroauric acid were mixed with a volume ratio of 2:3 and the mixture was injected into the nanopipette with a microsyringe. Then, the nanopipette was exposed to a UV light (254 nm, 8W) for 2 h and the excess solution in the nanopipette was removed. Finally, the nanopipette was cleaned with ethanol and dryed at room temperature, followed by being baked in an oven at 100 ℃ for 1 h. For further modification of the above nanopipette with HAT, ethanol solution of 20% HAT was injected into the nanopitte and left for 15 h at room temperature. Subsequently, the nanopiette was washed with ethanol and deionized water and dried at room temperature.

Ionic Current Measurement

The ionic currents of nanopipette was tested employing a HEKA EPC 10 double patch clamp amplifier. The electrolyte solution inside and outside the glass nanopiette were both 10 mM KCl solution (buffered with 0.01 M HEPES, pH 7.4). Two Ag|AgCl electrodes were used to apply a voltage to the nanopipette. One electrode was inserted in the nanopipette as a working electrode, and the other was placed in the external bulk solution as a reference/auxiliary electrode. The voltage scanned from -1.0 V to +1.0 V with a scan rate of 50 mV/s. Each experiment was repeated five times to obtain the average current value. All electrochemical tests were carried out at room temperature of 25 ℃ in a shielding chamber.

•OH radicals were generated by Fenton reaction (see Eq.1) and the concentration of •OH was varied by regulating the amounts of Fe^{2+} and H_2O_2 (Fe^{2+} / H_2O_2 = 1:6). The concentration of •OH generated was considered to be the same as that of Fe^{2+} due to the excess of H_2O_2. Considering the ultrashort lifetime of •OH, the Fenton reagent was freshly prepared for all experiments. In a typical experiment, the nanopipette electrode was first immersed into the Fe^{2+} solution followed by the addition of H_2O_2. After the reaction of the nanopipette with Fenton reagent for a constant time (15 min), the ionic current was collected.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH \quad (1)$$

Cell Culture and Treatment

RAW 264.7 macrophages were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with penicillin (100 units mL^{-1}), streptomycin (100 μg mL^{-1}), and fetal bovine serum (10%, v/v) at 37 °C under a humidified 5% CO_2 atmosphere. Before experiments, cells were transferred to a 35 mm Petri dish with 14 mm bottom well and allowed to adhere and incubate for 24 h before using.

Single Cell Experiment

The zero drift micromanipulator from Sensapex Instrument was employed for locating nanopipette around mitochondrials under the help of fluorescence imaging of mitochondrials by Leica TCS-SP8 confocal scanning microscope and the mitochondrial stain Mito Tracker Deep Red FM. To detect •OH produced by mitochondrial oxidative stress, $A\beta_{1-42}$ was used to stimulate RAW 264.7 macrophages.[2] The freeze-dried powder $A\beta_{1-42}$ oligomer was dissolved in anhydrous DMSO to form 100 μM $A\beta_{1-42}$ solution. The solution was then diluted to 20 μM and incubated with RAW 264.7 macrophages at logarithmic growth for 12 h under 37℃.

Cell Viability

Cell viability was determined by MTT assay. In brief, after Aβ treatment, the cells were incubated in a new culture medium containing 0.5 mg/mL MTT solution for 4 h. After the medium was removed, the formazan blue formed in the cells was dissolved by DMSO. The absorbance was detected at 490 nm. Cell viability was determined by the following formula : cell viability (%) = (absorbance of the experimental group/absorbance of the blank control group) × 100%.

2. SEM image of the unmodified glass nanopore tip

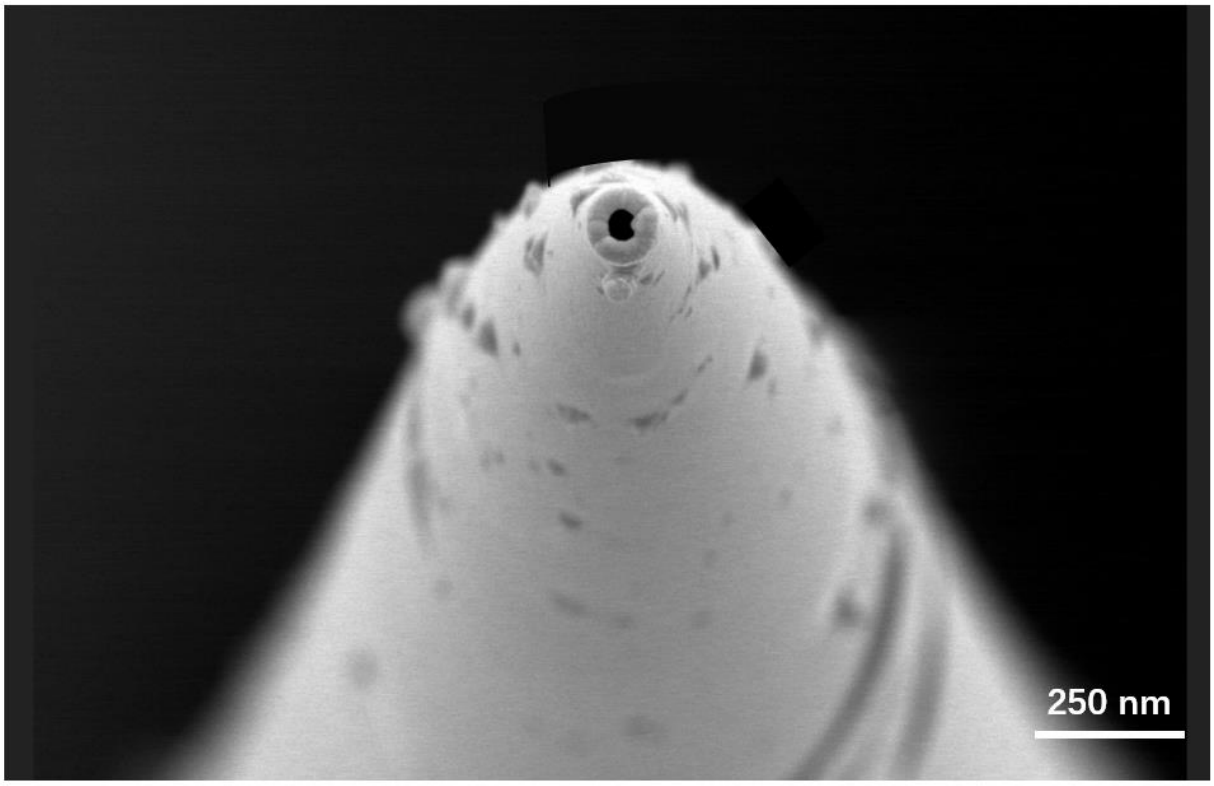

Figure S1. SEM image of the unmodified glass nanopore tip.

3. Fabrication of hexanethiol-modified nanopore electrode

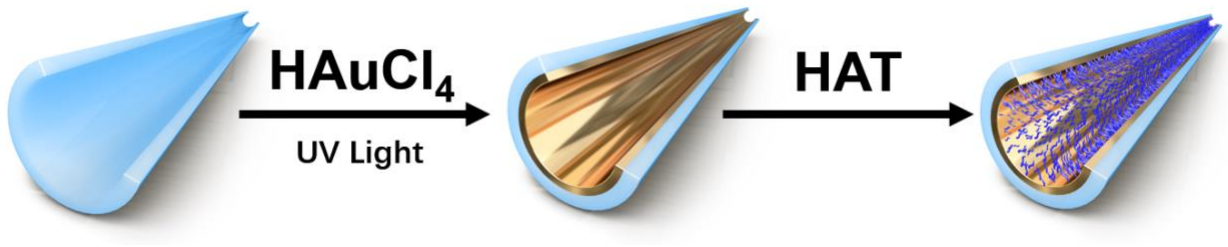

Figure S2. Stepwise modification of HAT functionalized nanopore.

4.TEM and EDX images of Au-free and Au-modified glass nanopore tip

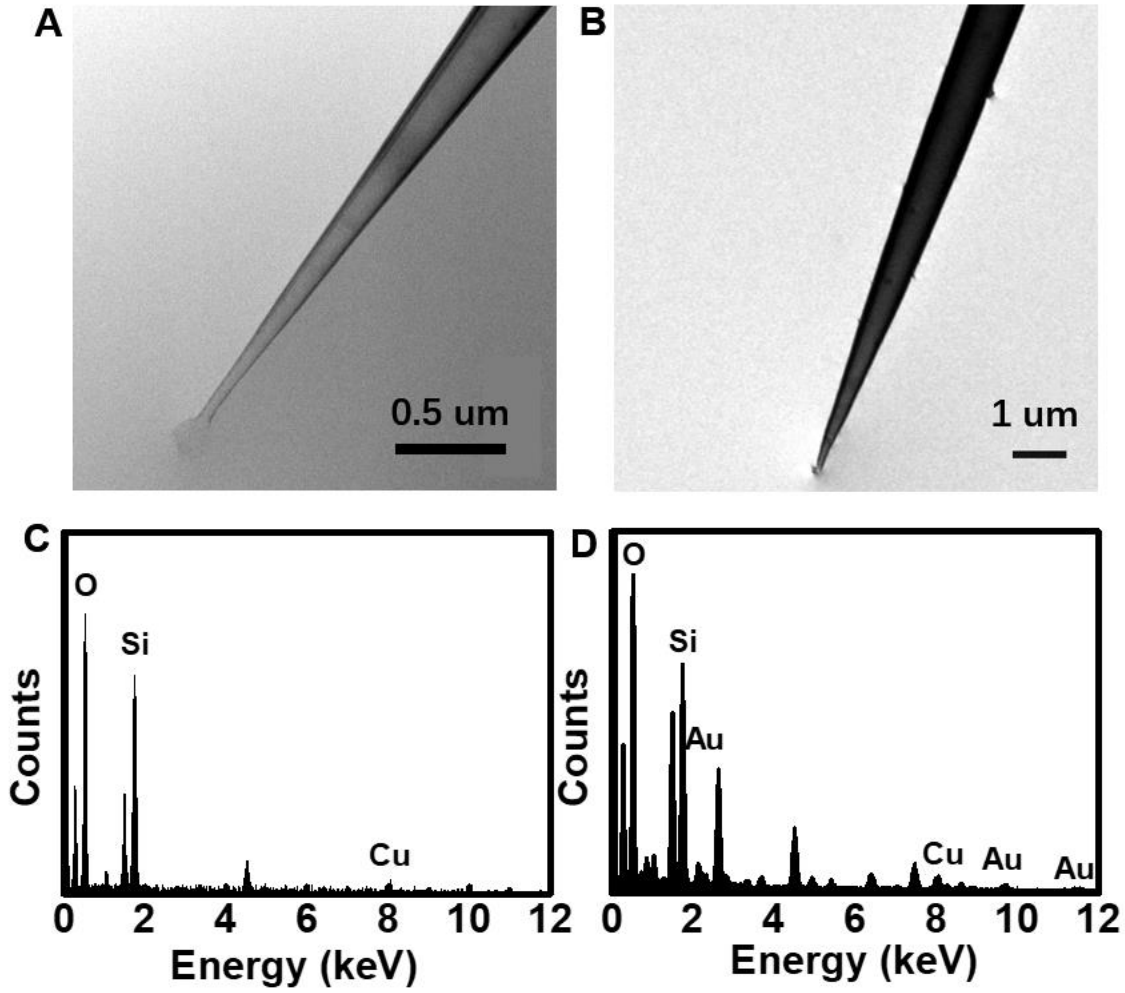

Figure S3. (A,B) medium-magnification TEM images and (C,D) EDX spectra of (A,C) the unmodified (Au-free) glass nanopore tip and (B,D) the Au-modified glass nanopore tip .

5. TEM elemental mappings of Au membrane-coated glass nanopore

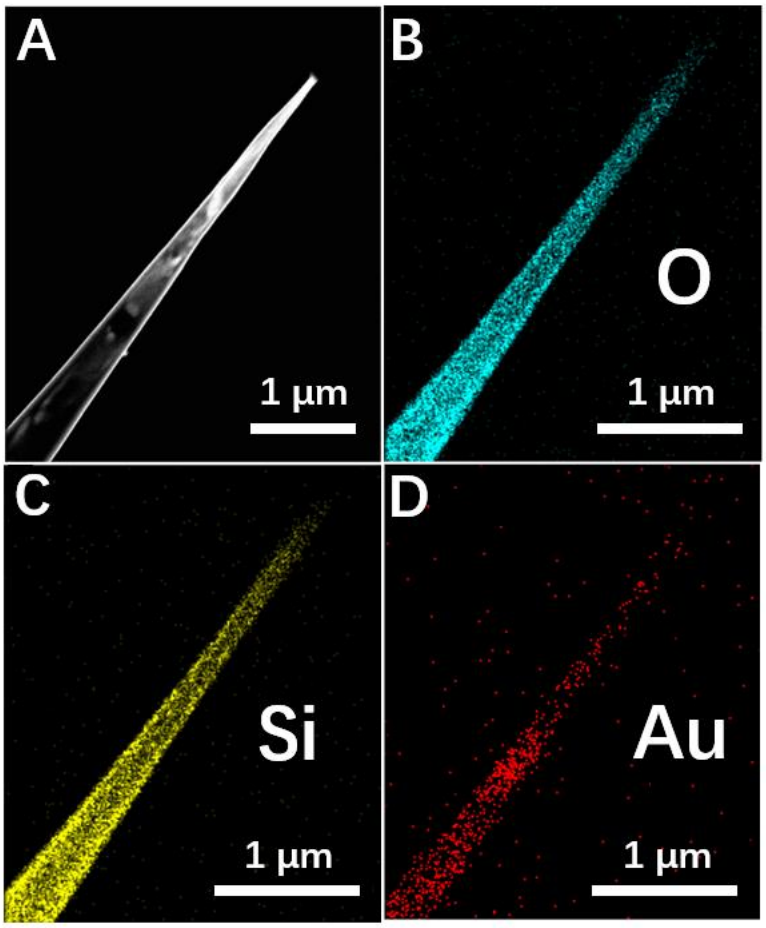

Figure S4. Dark-field TEM image (A) and the corresponding TEM elemental mappings (B-D, O, Si and Au) of Au membrane-coated glass nanopore.

6. XPS

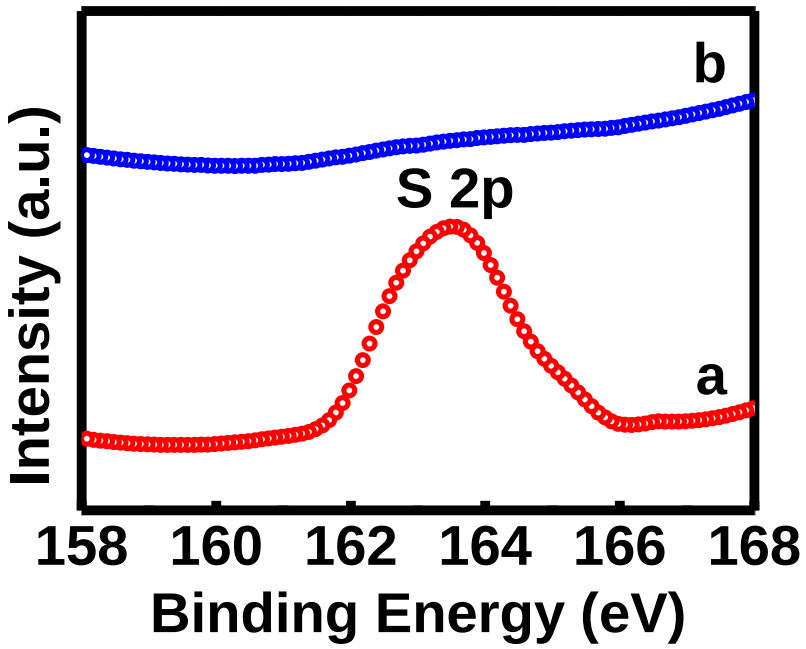

Figure S5. XPS for S 2p obtained at HAT-modified nanopipette electrode (a) before and (b) after treatment with 350 nM •OH .

As displayed in Figure S5, the peak of S 2p disappeared after the reaction of nanopipette with •OH, indicating the successful destruction of hexanethiol modified on the surface of electrode.

7. Reaction time

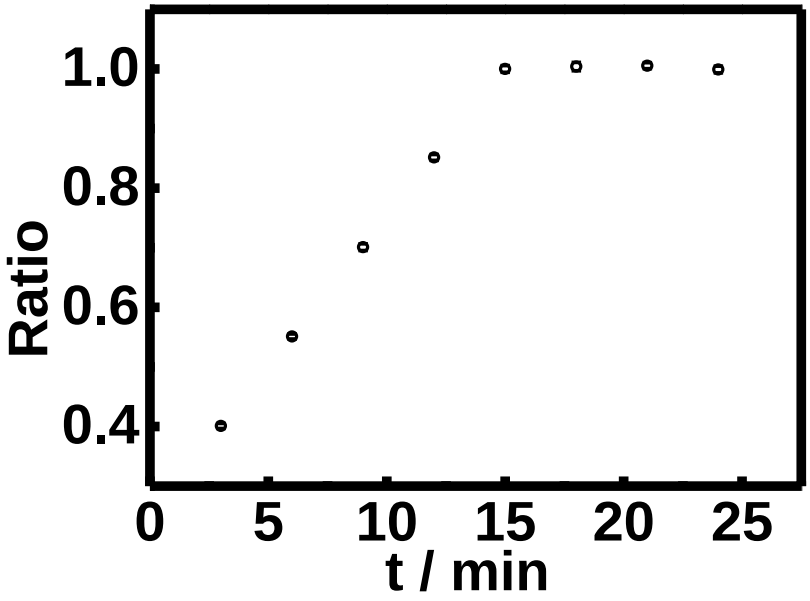

Figure S6. Effect of reaction time on ionic current change ratio at -1.0 V in the presence of Fenton reagent (350 nM Fe^{2+} + 2.1 μM H_2O_2).

As displayed in Figure S6, the current response of nanopipette electrode rose to a maximum at ~15 min and then stabilized. Therefore, we chose 15 min as the optimal reaction time.

8. Selectivity experiment

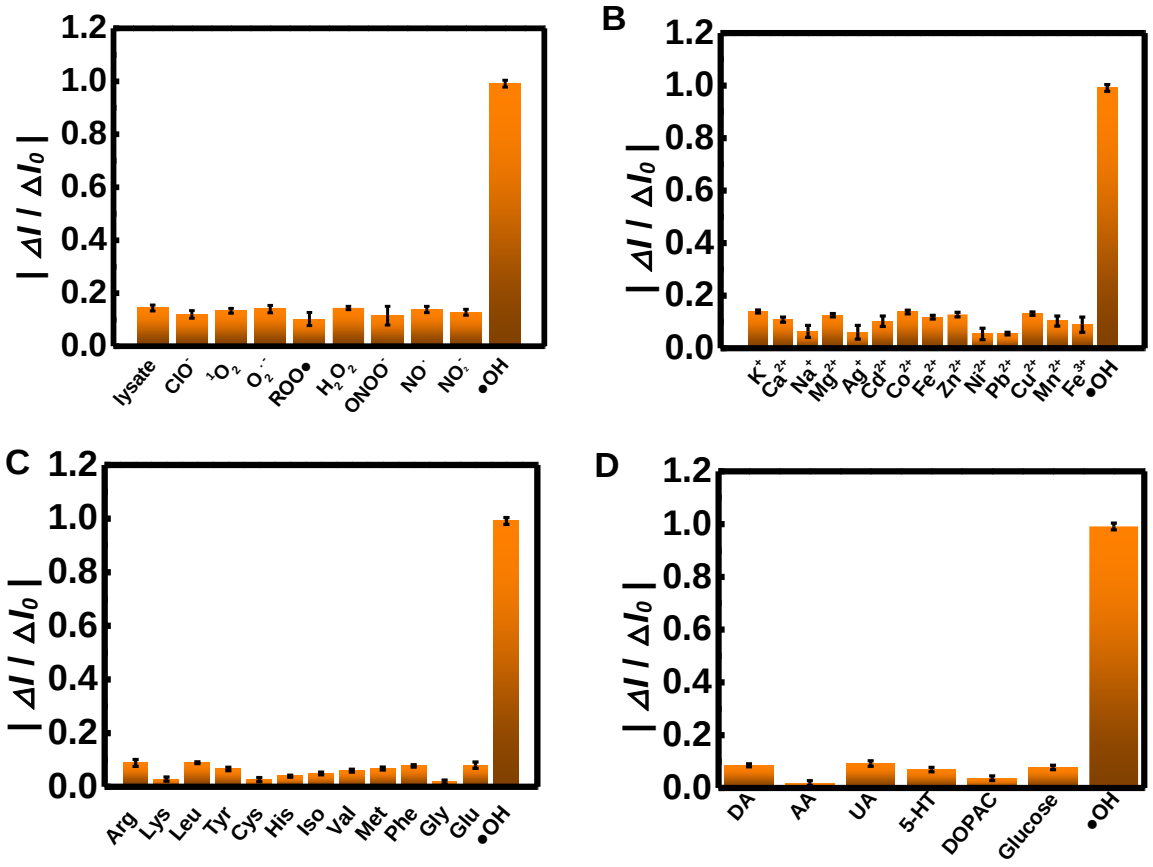

Figure S7. Selectivity experiments for the nanopipettes toward (A) other ROS/RNS and cell lysate (0.1mM for other ROS/RNS; 5 nM for •OH). (B) matal ions (1 mM for K^+, Ca^{2+},Na^+, Mg^{2+}; 10 μM for other metal ions; 5 nM for •OH). (C) amino acids (10 μM for all the tested amino acids, 5 nM for •OH). (D) other biological molecules (20 μM DA, 50 μM AA, 20 μM UA, 20 μM 5-HT, 50 μM DOPAC, 1 mM glucose). Current change ratio represents [($I-I_0$) / I_0] induced by interferences when the modified nanopipettes were immersed in 10 mM HEPES solution (pH 7.4).

9. Microscopic images of cells

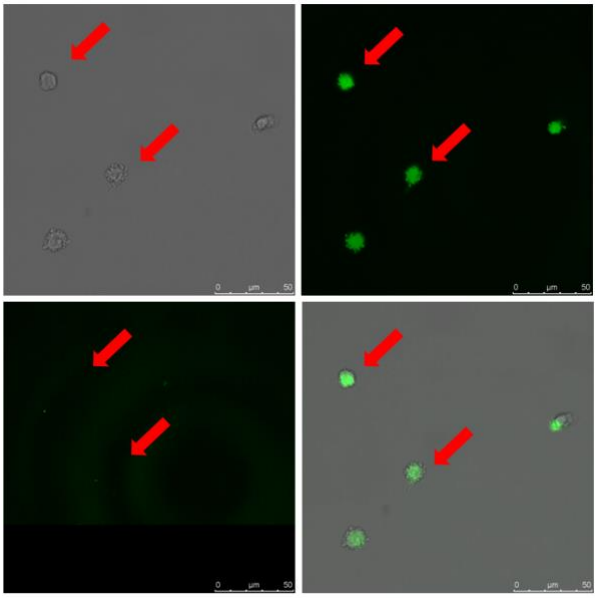

Figure S8. Bright-field (BF) and fluorescence imaging of macrophages without insertion or penetrated by nanopipette electrodes (indicated by arrows). The cells were stained with Calcein-AM and propidium iodide (PI) before imaging. The unmarked cells are controls (no insertion). Merge = merged image of BF, Calcein-AM, and PI images. Scale bar: 50 μm. (Calcein-AM: a fluorescent dye that can stain the living cells, PI: Propidium Iodide, a fluorescent dye that can enter the cell to stain the cell nucleus when the cell membrane is broken).

HAT modified nanopores were inserted into the RAW 264.7 macrophages and then withdrawn. Calcein-AM and PI were then added to the medium at a final concentration of 3 μg/ml following the manufacturer's protocols. After incubation under cell culture condition for 15 min, the residual dyes were washed off and the microphotographs were taken with the confocal laser scanning microscope.

10. Effect of DMSO on Aβ-stimulated cells

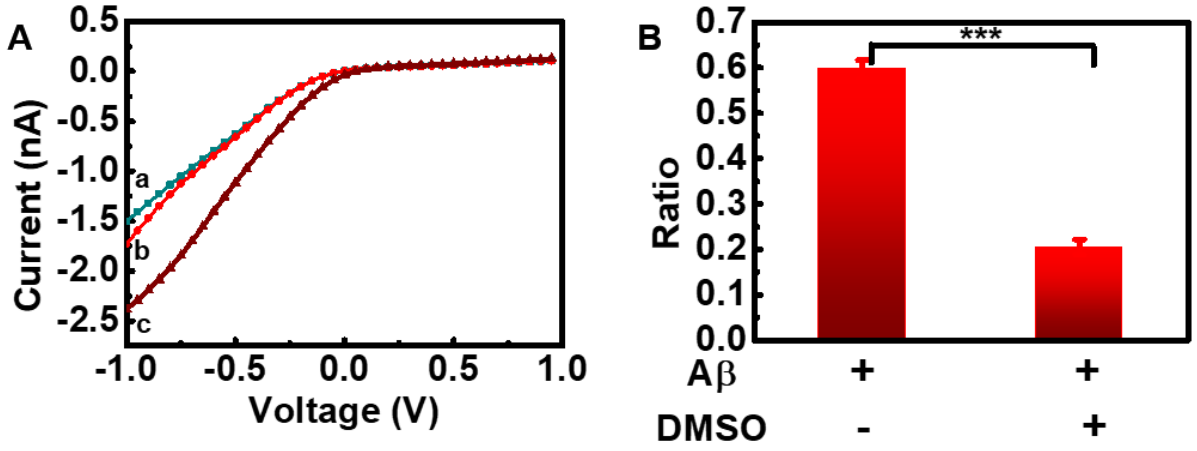

Figure S9. (A) *I-V* curve of functionalized nanopore recorded before (a) and after being inserted into the cytoplasm of the Aβ-stimulated cells without (c) and with (b) 0.1% DMSO treatment in advance and (B) the corresponding •OH concentration changes. The results are presented as mean ± standard deviation (n=3). Significant differences (***$p<0.001$) are performed by Student's t-test.

Table S1 Comparison of the performance of different methods for the determination of •OH

Method	Liner range (μM)	Limit of detection (μM)	Single cell	In-situ detection	Refs
EPR	/	0.01-1	No	No	3
HPLC	0.1-100	0.1	No	No	4
Square wave voltammetry (FcHT electrode)	5.0×10^{-3}-45.0×10^{-3}	0.37×10^{-3}	No	No	5
Electrochemical impedance method (indium tin oxide electrode)	0.9×10^{-3}-21.9×10^{-3}	0.9×10^{-3}	No	No	6
Ionic current recification (1-hexanethiol nanoelectrode)	1.0×10^{-3}-250.0×10^{-3}	1.0×10^{-3}	Single cell	In-situ	This work

Contents:

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