

Electronic Supplementary information (EIS)

Single-Cell ATP Detection and Content Analyses in Electrostimulus-Induced Apoptosis by Functionalized Glass Nanopipette

Dandan Wang^{a,b}, Guohua Qi^{a,c}, Ya Zhou^{a,b}, Ying Zhang^{a,b}, Bo Wang^a, Ping Hu^{a,b},
Yongdong Jin^{*a,b,c}

^a State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China

^b University of Science and Technology of China, Hefei, Anhui 230026, China

^c University of Chinese Academy of Sciences, Beijing 100049, China

* Corresponding Author. E-mail: ydjin@ciac.ac.cn

Table of contents

1. Experimental section.....	S-4
• Reagents and materials	
• Experimental setup and data acquisition	
• Preparation and characterization of laser-pulled nanopore	
• Synthesis of AuNPs	
• Preparation of the NPSAs	
• ATP detection	
• Cell Culture	
• Intracellular ATP measurements	
• Determination of mitochondrial location	
• Extraction and detection of ATP from cells	
• Electrostimulus-induced apoptosis	
2. SEM image of G-nanopore.....	S-10
3. TEM images of NPSA and AuNPs.....	S-10
4. Characterizations of NPSA.....	S-11
5. Control experiments.....	S-11
6. ATP functionalization of NPSA.....	S-12
7. ATP concentration and interaction time response.....	S-12
8. The selectivity of NPSA to ATP.....	S-13
9. Insertion of nanopipette into single cells.....	S-14
10. Cell viability tests.....	S-14

11. Control experiment on pH.....	S-16
12. Equilibrium time in single cells.....	S-16
13. Detection of ATP level in single living-state cells.....	S-
17	
14. ATP detection for cell lysates.....	S-17
15. Intracellular ADP and AMP effect.....	S-
18	
16. Apoptosis induced by electrostimulus.....	S-19
17. Determination of ATP level in single apoptotic-state	
cells.....	S-20
18. References.....	S-20

1. Experimental section

Reagents and materials

Tetrachloroauric acid trihydrate ($\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$), sodium chloride (NaCl), sodium citrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$) were purchased from Aladdin. Ltd. (Shanghai, China). The Dulbecco's modified Eagle's medium (DMEM), antibiotic solution, fetal bovine serum (FBS) and 0.25% trypsin/2.2 mM EDTA solution were purchased from Biological Industries Israel Beit Haemek Ltd. Fluorescence dyes MitoTracker™ Green FM, Calcine-AM and propidium iodide (PI) were purchased from Thermo Scientific. The Tris acetate, DNA sequences, Adenosine triphosphate (ATP), Guanosine triphosphate (GTP), Cytidine triphosphate (CTP) and uridine triphosphate (UTP) were purchased from Sangon Biotech. Co. The sequences of DNAs used here were probe 1: 5'-TCACAGATGAGTAAAAAAAAAAAAA-(CH₂)₃-SH-3', probe 2: 5'-SH-(CH₂)₆-CCCAGGTTCTCT-3' and ATP-aptamer: ACTCATCTGTGAAGAGAACCTGGG-GGAGTATTGCGGAGGAAGGT. ATP Assay Kit was purchased from Beijing Solarbio Science & Technology Co., Ltd. Conductive glass (ITO) was purchased from the Kaiwei (Zhuhai) Optoelectronic Technology Co., Ltd. All the solutions were prepared through the ultrapure water which was using a Millipore Milli-Q water purification system (Billerica, MA), with an electric resistance >18.25 MΩ. All solutions in the experiment were filtered through a 0.22 μm Millipore filter before use.

Experimental setup and data acquisition

All ionic current measurements were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) in voltage-clamp mode using a low-pass Bessel filter of 5 kHz. The signals were digitized with the DigiData 1440A digitizer (Molecular Devices, Sunnyvale, CA, USA) at 100 kHz and viewed with pCLAMP10.7 software (Molecular Devices, Sunnyvale, CA, USA). The nanopipette is fixed to a microscope by a holder (1-HL-U, Axon Instruments, Union City, CA, USA) connected

to an Axopatch 200B low-noise amplifier (Molecular Devices, Sunnyvale, CA, USA) for current measurements. A three-dimensional MPC-200 micromanipulator (Sutter Instruments Co., Novato, CA, USA) equipped with inverted microscope (Leica DMI5000B, Germany) was applied for the precise control of the nanopipette by the operator ROE-200 (Sutter Instruments Co., Novato, CA, USA) to insert into single cells under observation. The current-voltage (I-V) curves were recorded by sweeping the voltage from -0.6 V to +0.6 V (to reduce unnecessary electrode reactions in this interval) with the scan rate of 20 mV s⁻¹, and recorded with a sampling frequency of 100 kHz (as the sampling frequency increases, the fluctuation of the ion current increases). The I-V and I-t plots were analyzed by Clampfit 10.2 software and drawn by OriginLab 9.0.

Preparation and characterization of laser-pulled nanopore

Borosilicate glass capillaries (BF100-58-10, Sutter Instrument Co., Novato, CA, USA), with outer diameter of 1.0 mm and inner diameter of 0.58 mm, were used for the experiments. All glass capillaries were thoroughly cleaned by immersing in freshly prepared piranha solution (3:1 98% H₂SO₄/30% H₂O₂) for ~2 h in order to remove organic impurities on the surface. Then, the capillaries were thoroughly rinsed with deionized water to neutral and were rinsed several times with ethanol, and vacuum dried at 80 °C prior to use. The glass nanopores (G-nanopores) were then fabricated by using a CO₂-laser based micropipette puller system (model P-2000, Sutter Instruments Co., Novato, CA, USA) with two-lines program containing the following parameters, line1: Heat =350, Fil = 3, Vel = 30, Del =220, Pull = 0; line2: Heat =340, Fil = 2, Vel = 27, Del =180, Pull = 250. Scanning electron microscopic (SEM) characterization was performed on a XL30 ESEM instrument.

Synthesis of AuNPs

The AuNPs were prepared based on the previously reported method.¹ Briefly, the 2 mL of 1 wt.% HAuCl₄ was added into the 48 mL of deionized water so that the final HAuCl₄ concentration was 1 mM and heated to boiling under the vigorous stirring, then a 5 mL of 38.8 mM sodium citrate solution was mixed with the boiling solution.

The solution was kept at boiling for 15 min, after which the solution was subsequently cooled to room temperature. The AuNPs prepared were centrifuged at the 12000 rpm for 15 min and resuspended in to the deionized water to further use. The AuNPs were characterized through Hitachi 500 transmission electron microscope (TEM).

Preparation of the NPSAs

We used the freezing method to directly modify the thiolated DNA (probe 1 or probe 2) on the surface of AuNPs.² Specifically, thiolated DNA probe 1 and probe 2 were respectively solved in 20 mM Tris acetate buffer (pH 8.2) with the concentration of 1 mM. 1.5 μ L of DNAs were respectively mixed with 500 μ L of citrate-AuNPs ($\sim$ 10 nM), and the mixtures were then placed in laboratory freezer (-20 $^{\circ}$ C) for 2 h, followed by thawing in room temperature. The DNA-functionalized nanoparticles (probe 1@AuNP, probe 2@AuNP) were purified by centrifugation with the 12000 rpm for 15 min and removal of supernatant for three times, and then were solved in 500 μ L of 20 mM Tris acetate buffer (added 300 mM NaCl, pH 8.2). Solutions of probe 1@AuNP and probe 2@AuNP were mixed with 10 μ L of ATP-aptamer (100 μ M, solved in 20 mM Tris acetate buffer, pH 8.2) and stored in 4 $^{\circ}$ C for 12 h. Nanoparticles aggregated and changed color from red to purple in this process and the NPSAs were formed (Fig. S3d). The samples were centrifuged with 5000 rpm for 10 min, and the precipitates were collected and dispersed in 20 mM Tris acetate buffer (added 300 mM NaCl, pH 8.2). The NPSAs could be stored in 4 $^{\circ}$ C for several weeks, and were injected in borosilicate nanopipette before use. The nanoparticle aggregates were characterized through Hitachi 500 transmission electron microscope (TEM). The UV/Vis absorption measurements were carried out on a Shimazu UV-2500 UV/Vis spectrometer. The zeta potential and size distribution characterizations of different nanoparticles were performed with the dynamic light scattering (DLS, Zetasizer Nano ZS 90) purchased from British Ma Erwen Co., Ltd.

ATP detection

In the experiment, two Ag/AgCl electrodes were applied as working electrode inserted into the functionalized nanopipettes which was backfilled with NPSAs solved in 20

mM Tris acetate containing 150 mM NaCl (pH 8.2), and as reference electrode placed in the bath solution (20 mM Tris acetate, 150 mM NaCl, pH 8.2), respectively. And the current-voltage (I-V) curves were recorded by sweeping the voltage from -0.6 V to +0.6 V with the scan rate of 20 mV s⁻¹. For ATP detection, the nanopipettes were backfilled with pipette solution, and then were immersed into the bath solution containing different concentration of ATP (10 mM, 5 mM, 1 mM, 0.5 mM and 0.1 mM) allowed to stand for 10 min. The current-voltage (I-V) curves again were recorded by sweeping the voltage from -0.6 V to +0.6 V with the scan rate of 20 mV s⁻¹, and were compared with previous ones. All detection experiments were performed at room temperature. To determine the specificity for ATP detection, the nanopipettes were immersed into the bath solution containing 10 mM GTP, CTP and UTP, respectively.

Cell Culture

HeLa cells (cervical cancer), MCF-7 cells (breast cancer), and L929 cells (mouse lung fibroblasts) were bought from the American Type Culture Collection (ATCC, USA). HeLa, MCF-7 and L929 cells were grown in the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

Cells vitality measurement: There were two ways to prove that there was no damage to the cell membrane after the G-nanopore was inserted into the cell. The First one was that, after the G-nanopores were withdrawn from L929 cells, cells were washed with PBS three times and stained with Calcein AM (2 µM) and propidium iodide (PI, 4 µM) for 20 min. After that, the cells were washed three times using the PBS solution (10 mM, pH 7.4) and observed using Leica DMI5000B microscope with a fluorescence detector with 40× objective. The second one was that cells were stained with Calcein AM (2 µM) for 20 min, and real-time monitoring the changes of fluorescence intensity within 20 min after the G-nanopore was inserted into single MCF-7 cell with applying a voltage of 50 mV and within 30 min after it was withdrawn from the cell.

Intracellular ATP measurements

Before detecting ATP in single cells, the I-V curve of the nanopipette from -0.6 V to

+0.6 V with the scan rate of 20 mV s⁻¹ was obtained in the solution (20 mM Tris acetate, 150 mM NaCl, pH 8.2). The nanopipette was anchored on the holder of the three-dimensional MPC-200 micromanipulator equipped on inverted microscope. After the G-nanopores were immersed into the bath solution, the pCLAMP10.7 software was used for real time monitoring the changes of the ion baseline current inside G-nanopores. Applying a positive voltage of 50 mV before immersion of the nanopipette not only helping the nanopipette to insert into the cell, but also protecting the tip from being contaminated by dead cells or cell residue in the medium, and this also could prevent the tips of G-nanopores from being broken as well. When the baseline current of nanopipette instantaneously declined with applying a voltage of 50 mV, it indicated that the nanopipette was just contact with the target cell membrane. After the insertion, the current inside the G-nanopore instantly recovered in small increments (Fig. S9). In order to ensure adequate interaction (contact) of the intracellular ATP with inner wall's NPSAs and to avoid obvious damage to cellular function by applying a large potential, a small voltage of 50 mV was continuously applied for 10 min during the G-nanopore insertion. Then, the nanopipette was withdrawn from the cell and the I-V curves was recorded again by sweeping the voltage from -0.6 V to +0.6 V in the solution (20 mM Tris acetate, 150 mM NaCl, pH 8.2).

Determination of mitochondrial location

Cells were washed with PBS three times and stained with MitoTracker™ Green FM (2 µg/mL) with the function of Targeting mitochondria for 20 min. After that, the cells were washed three times using the PBS solution (10 mM, pH 7.4) and observed using Leica DMI5000B microscope with a fluorescence detector. After the G-nanopores were inserted into different single cells, the tips of the glass nanopipettes were in the fluorescent position (Fig. 3a).

Extraction and detection of ATP from cells

The HeLa Cells were washed with PBS and detached from the flask with 0.25% trypsin. Then the cells were centrifuged at 1000 rpm for 5 minutes and resuspended in 1 mL of DMEM. We used the cell counter to count the number of cells (~5×10⁶ cells). The cells

were centrifuged again and redispersed into 1 mL of ATP exaction buffer. Ultrasonic crushing at 200 W in an ice bath for 1 min, ultrasound for 2 s, and stop for 1 s, and it was centrifuged at 10000 g for 10 minutes. We pipette the supernatant and added 500 μ L of chloroform into it, and it was centrifuged at 10000 g for 3 minutes. Finally, the supernatant was pipette again and was stored on 4 $^{\circ}$ C for the following usages: 1) NPSA-functionalized G-nanopipette ATP detection: in order to obtain a standard curve generated in the presence of cell lysates using the sensing platform we constructed, we diluted the original cell lysate with tris-NaCl buffer (20 mM Tris acetate, 150 mM NaCl, pH 8.2) into the concentrations of 50 cells/mL, 20 cells/mL, 10 cells/mL, 5 cells/mL and 2 cells/mL, respectively. 2) ATP detection for cell lysate with commercial standard ATP assays: all steps were performed according to ATP content detection kit instruction. We measured the absorbance of ATP standard solution (A_{1s}) and cell lysate (A_{1c}) at the wavelength of 340 nm. The reaction solutions were put in a 37 $^{\circ}$ C water bath to react for 3 minutes, and then we immediately measured the absorbance at 340 nm ATP standard solution (A_{2s}) and cell lysate (A_{2c}). Based on the differences between the absorption values before and after the reaction ($\Delta A_s = A_{2s} - A_{1s}$, $\Delta A_c = A_{2c} - A_{1c}$), we could calculate the ATP content in the cell lysate according to the corresponding formula ($C_{ATP} = 0.125 \Delta A_c / \Delta A_s \mu\text{mol}/10^6 \text{ cells}$). The volume of 10^6 cells used was $\sim 10 \mu\text{L}$, from which we can calculate the ATP concentration.

Electrostimulus-induced apoptosis

The cells were cultured on ITO glass for overnight, and then were stimulated with the constant voltages of 1 V for 6 min.³ Specifically, the holder with the Ag/AgCl electrode as work electrode was mounted on three-dimensional MPC-200 micromanipulator equipped on inverted microscope, and the reference electrode was immersed in DMEM medium. When the Ag/AgCl electrode contacted with the ITO glass, a constant voltage of 1 V was applied by Axopatch 200B lasting for 6 min (Fig. S18). Finally, cells were washed with PBS three times and stained with Calcein AM (2 μM) and PI (4 μM) for 20 min. After that, the cells stained were washed three times using the PBS solution (10 mM, pH 7.4) and observed using Leica DMI5000B microscope with a fluorescence

detector.

2. SEM image of G-nanopore

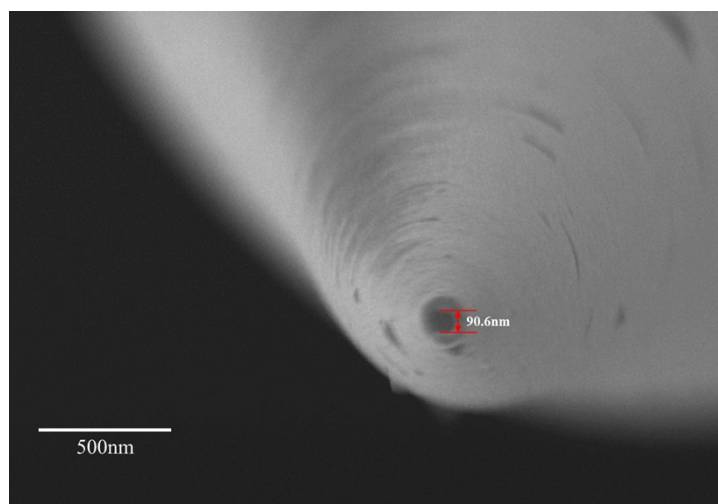


Fig. S1. The SEM image of the borosilicate G-nanopore used in this study.

3. TEM images of NPSA and AuNPs

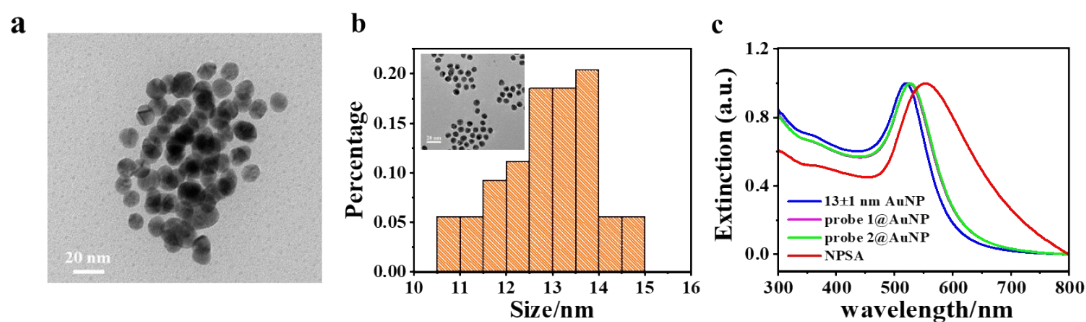


Fig. S2. TEM images of a) NPSA and b) 13 ± 1 nm AuNPs. Scale bar: 20 nm, and size distribution of gold nanoparticles with average diameter of 13 nm. c) UV-vis spectra of 13 ± 1 nm AuNPs (red curve), probe 1@AuNP (blue curve), probe 2@AuNP (yellow curve) and NPSA (green curve).

4. Characterizations for NPSA

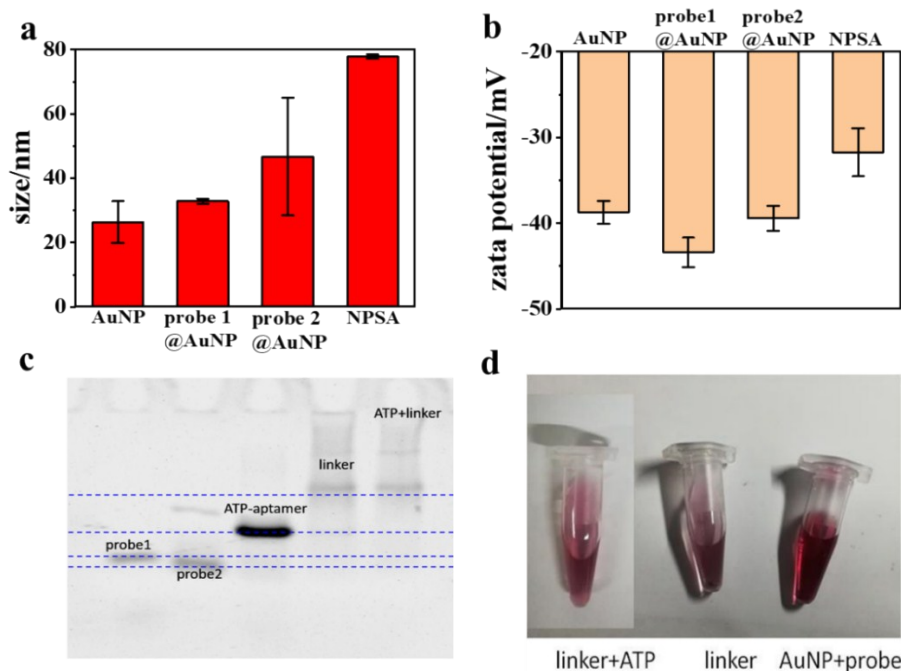


Fig. S3. a) Size distribution and b) zeta potential of 13 ± 1 nm AuNPs, probe 1@AuNP, probe 2@AuNP and NPSA. c) 20 % native polyacrylamide gel electrophoretic analysis of different DNA probes, NPSA and NPSA+ATP. d) The color changes of probe@AuNPs and NPSA and after addition of ATP into NPSA

5. Control experiments

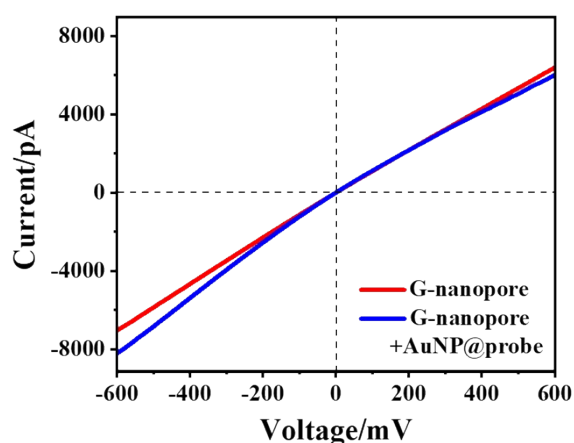


Fig. S4. I-V curves obtained of the nanopipettes filled with probe@AuNPs (blue curve) dissolved in 20 mM Tris acetate (in 150 mM NaCl, pH 8.2) compared with bare nanopipettes (red curve).

6. ATP functionalization of NPSA

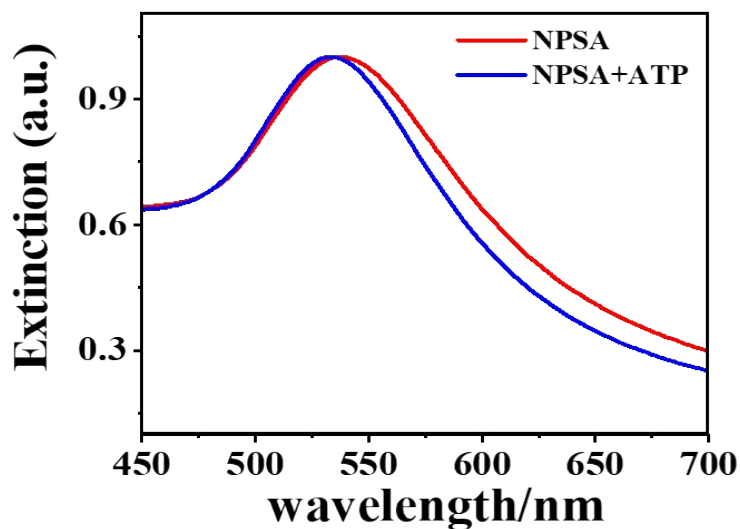


Fig. S5. UV-visible spectra of NPSA (red) before and after the addition of 10 mM ATP (NPSA+ATP, blue).

7. ATP concentration and interaction time response

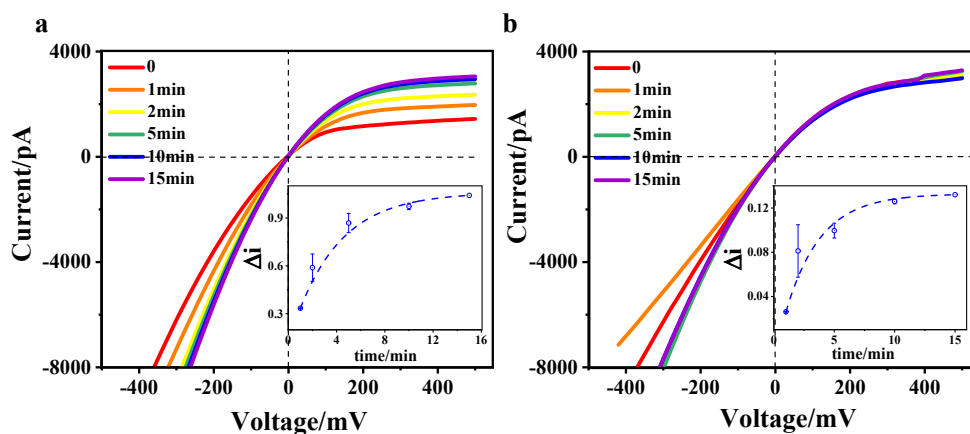


Fig. S6. Time evolution of current changes in the I-V curve of functionalized G-nanopore in the presence of 5mM ATP (a) and 0.5mM ATP (b). The inset plots show the relationship of current change ratio Δi at 500mV with the interaction time of ATP.

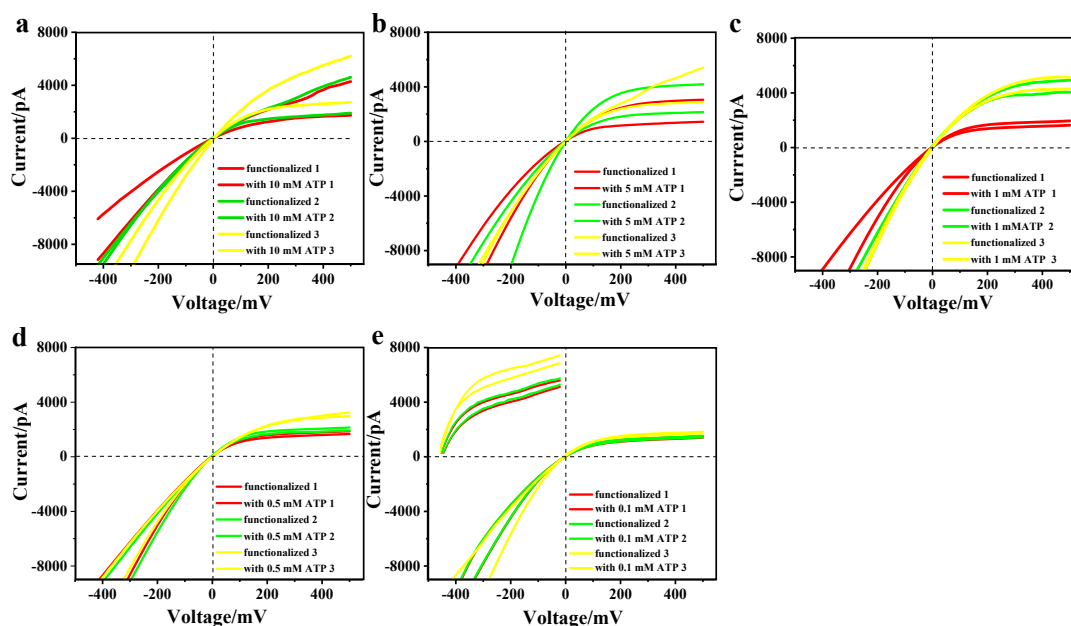


Fig. S7. Typical I-V curves obtained at the NPSA-functionalized nanopipettes treated with different concentrations of ATP: a) 10 mM, b) 5 mM, c) 1 mM, d) 0.5 mM, and e) 0.1 mM, in 20 mM pH 8.2 Tris acetate (with 150 mM NaCl).

8. The selectivity of NPSA to ATP

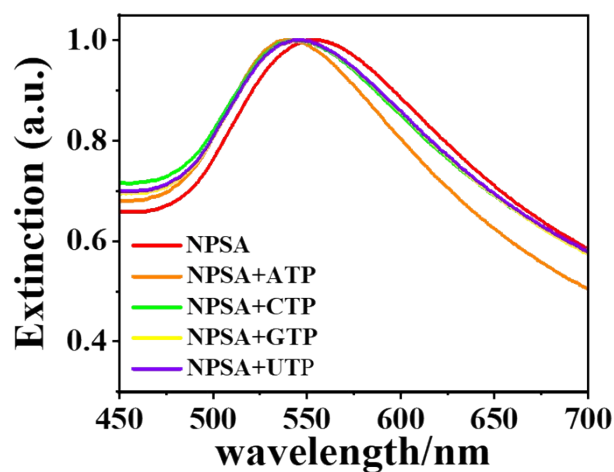


Fig. S8. UV-visible spectra of NPSA (red curve) and after the addition of ATP (blue curve), CTP (green curve), GTP (yellow curve) and UTP (pink curve), respectively.

9. Insertion of nanopipette into single cell

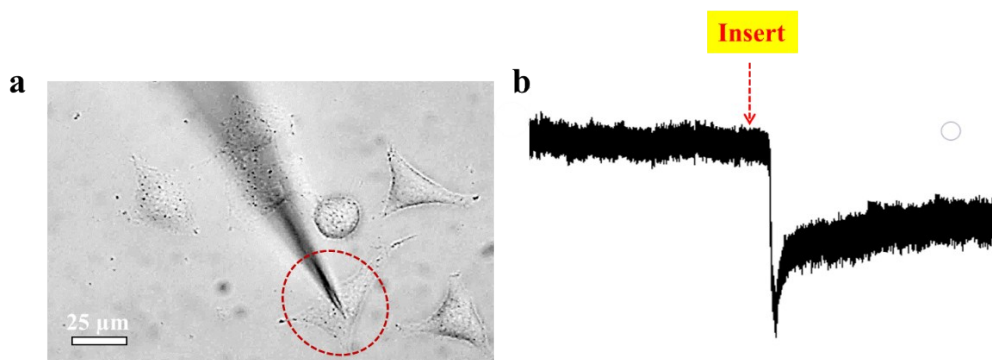


Fig. S9. a) The bright image shows the insertion of the NPSA-functionalized G-nanopipette into a single cell. b) An abrupt change in baseline current occurred when a G-nanopore was inserted into the single MCF-7 cell at the voltage of 50 mV.

For intracellular analysis with nanopipettes, it was critical to ensure the glass nanopipette had been successfully inserted into the single cell according to real-time observation of the current change inside the G-nanopore. Before the nanopipette was inserted into single cell, a positive voltage of 50 mV was applied not only helping the nanopipette to insert into the cell, but also protecting the tip from the contamination of dead cells or cell debris in the medium, and this could prevent the tip of the nanopipette to be broken as well. When the baseline current of nanopipette instantaneously declined, it indicated that the nanopipette was just contact with the target cell membrane, and after the insertion, the current inside the G-nanopore instantly recovered in small increments (Fig. S9)

10. Cell viability tests

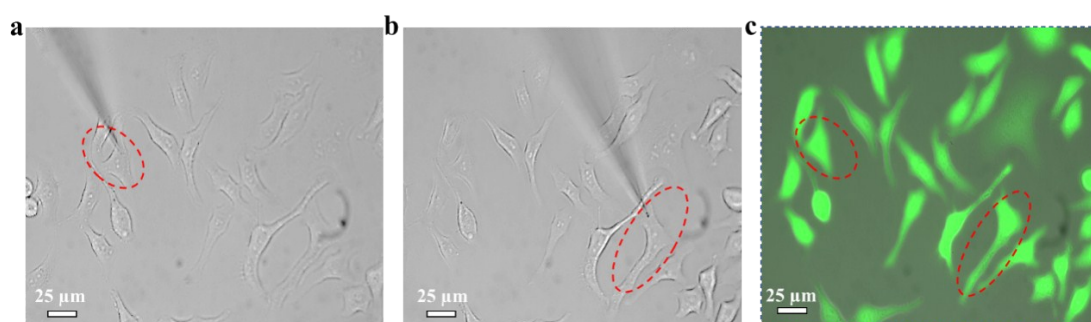


Fig. S10. a-b) Bright-field images after the nanopipette were inserted in L929 cells. c) Superposition of bright-field image with fluorescence image of Calcein-AM/PI stained cells after the nanopipettes were withdrawn from L929 cells.

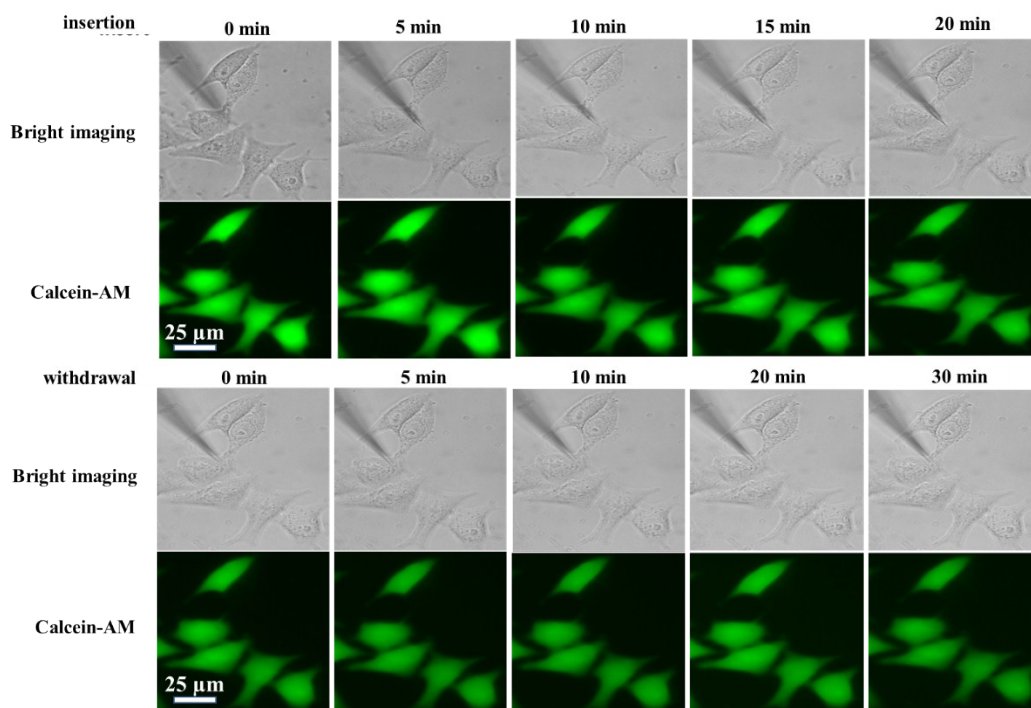


Fig. S11. Bright-field and fluorescence images of the Calcein-AM stained MCF-7 cells before and after the nanopipette was inserted and withdrawn from the cells.

We employed fluorescence probes CalceinAM and propidium iodide (PI) to stain the L929 cell and proved the membrane integrity after the insertion of the nanopipette by strong fluorescence intensity (Fig. S10). We stained the MCF-7 cells with CalceinAM, and then monitored the changes of fluorescence intensity in real time after the G-nanopore was inserted into and withdrawn from the single cell (Fig. S11), which could further validate the cellular viability. From Green fluorescence intensity of Calcein AM, it was found that the membrane integrity did not change significantly, proving that the insertions in 20 min with applying a voltage of 50 mV and withdrawal in 30 min barely influenced the function of the living cell.

11. Control experiment on pH

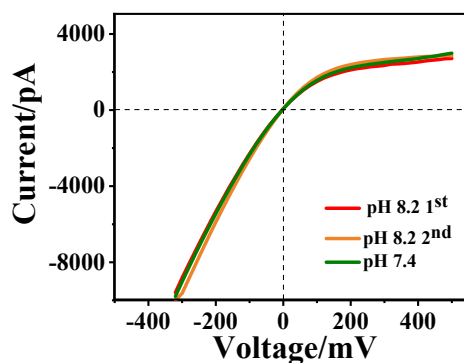


Fig. S12. a) I-V curves of G-nanopore recorded in pH 7.3 Tris acetate solution (green curve), and in 20 mM Tris acetate (in 150 mM NaCl, pH 8.2) before (red curve) and after (orange curve) pH 7.3 solution treatment.

12. Equilibrium time in single cells

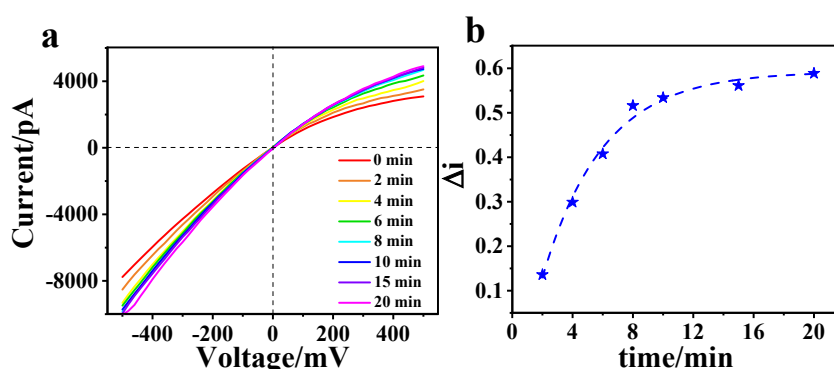


Fig. S13. a) The changes of I-V curves recorded in 20 mM Tris acetate (in 150 mM NaCl, pH 8.2) with extending the reaction time of intracellular ATP and NPSA-functionalized G-nanopore. b) The change of Δi with the extension of reaction time.

13. Detection of ATP level in single living-state cell

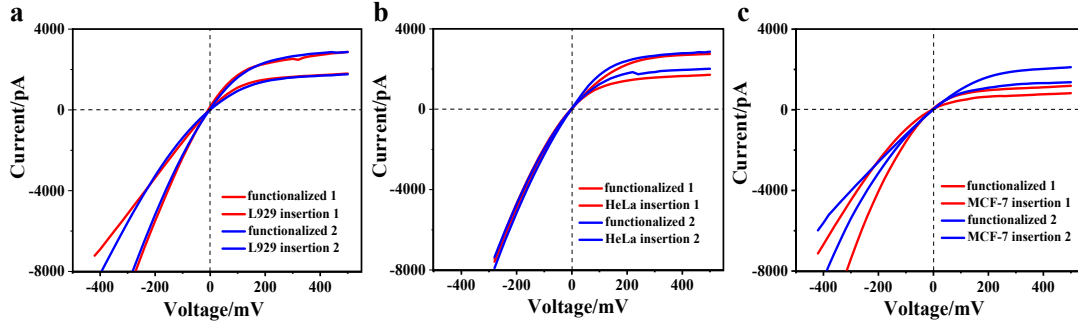


Fig. S14. I-V curves of the NPSA-functionalized G-nanopipettes before and after the insertion into different cells: a) L929, b) HeLa and c) MCF-7 cells, respectively.

14. ATP detection for cell lysates

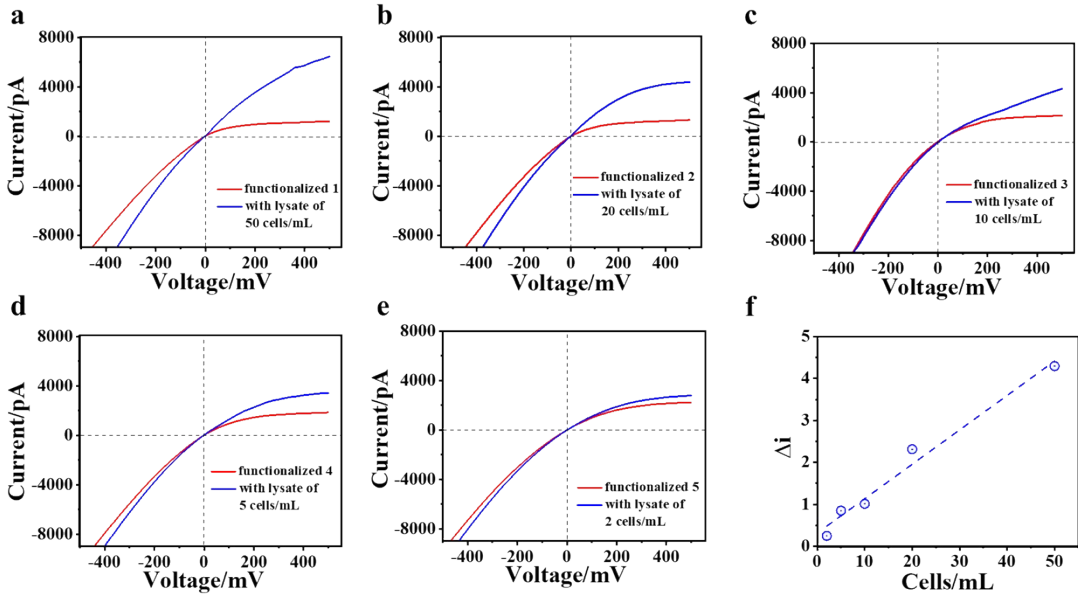


Fig. S15. I-V curves of the NPSA-functionalized G-nanopipettes obtained in 20 mM tris acetate, 150 mM NaCl, pH 8.2 before and after the addition of cell lysates of varied concentrations: a) 50 cells/mL, b) 20 cells/mL, c) 10 cells/mL, d) 5 cells/mL and e) 2 cells/mL, respectively. f) Linear relationship between Δi and the concentration of cell lysates.

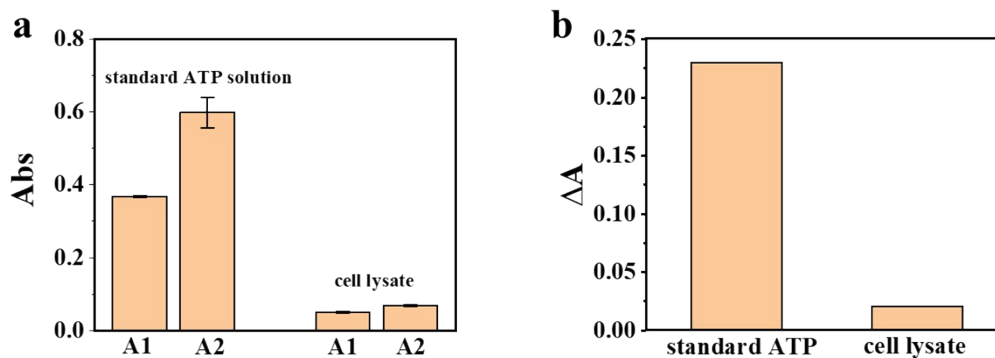


Fig. S16. ATP detection for cell lysate using commercial standard ATP assays. a) The absorption value and b) absorption differences (ΔA) at a wavelength of 340 nm of standard ATP solution and cell lysate before (A1) and after (A2) reacting in a 37 ° C water bath for 3 minutes.

15. Intracellular ADP and AMP effect

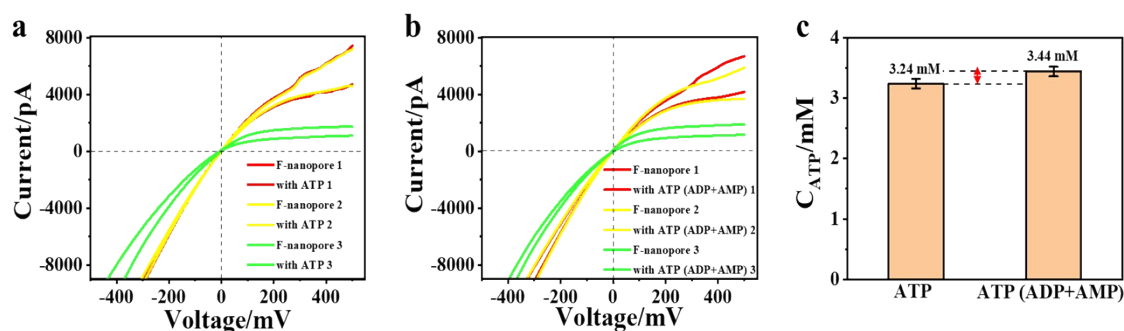


Fig. S17. I-V curves of functionalized G-nanopore obtained in 20 mM Tris acetate, 150 mM NaCl, pH 8.2 solution containing a) 3.33 mM ATP and b) 3.0 mM ATP+0.3 mM ADP+0.03mM AMP (F-nanopore: functionalized G-nanopore). c) Theoretical ATP concentrations measured in two solution systems.

16. Apoptosis induced by electrostimulus

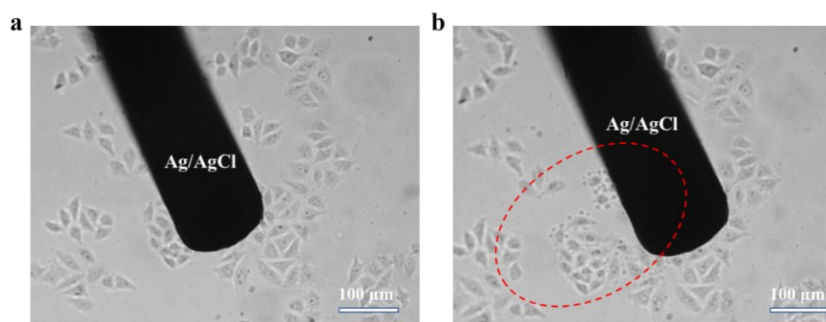


Fig. S18. Bright-field images of HeLa cells a) before and b) after the electrical stimulation. The ES treatment induces cell apoptosis when applied the voltage of 1V for 6min.

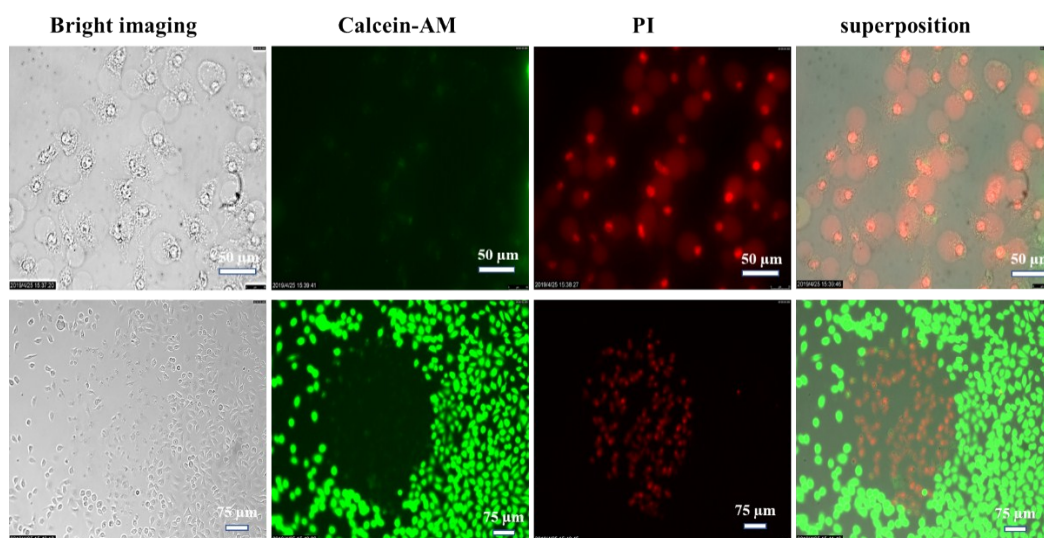


Fig. S19. Bright-field and fluorescence images of Calcein-AM/PI stained cells after the ES treatment, showing ES-induced apoptosis when applied the voltage of 1V for 6min.

A constant voltage of 1 V was applied for 6 min to stimulate the cells, and many apoptotic bodies appeared around the stimulated cells, as shown in Figure S15. We confirmed that only the cells stimulated by electricity were in a state of death by staining them with fluorescent dyes CalceinAM and PI to certain the integrity of cytomembranes (Fig. S19).

17. Determination of ATP level in single apoptotic-state cell

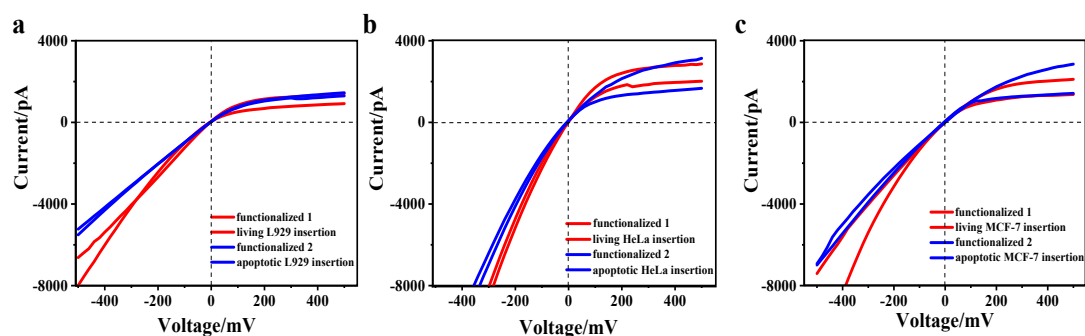


Fig. S20. The comparison of changes in I-V curves after the NPSA-functionalized G-nanopipette was inserted in living-state cells and apoptotic-state cells: a) L929, b) HeLa and c) MCF-7 cells, respectively, for 10min.

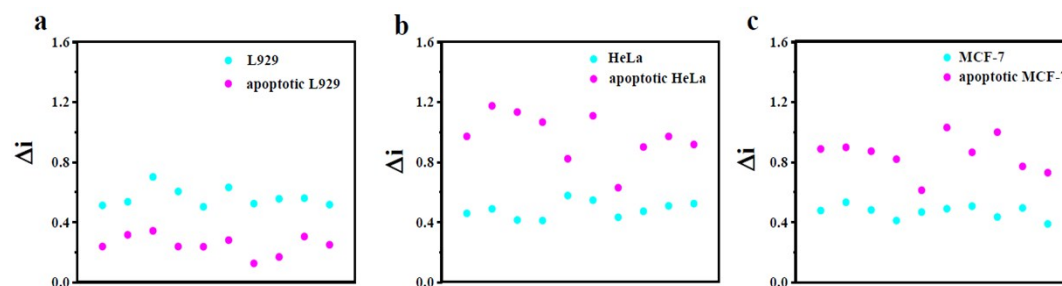


Fig. S21. The comparisons on current change ratio Δi distributions of living-state cells (indigo dots) and apoptotic-state cells (pink dots) for a) L929, b) HeLa and c) MCF-7 cells.

18. References:

1. J. Liu and Y. Lu, *Nat. Protoc.*, 2006, **1**, 246.
2. B. Liu and J. Liu, *J. Am. Chem. Soc.*, 2017, **139**, 9471-9474.
3. G. Qi, H. Li, Y. Zhang, C. Li, S. Xu, M. Wang and Y. Jin, *Anal. Chem.*, 2019, **91**, 1408-1415.