Electronic Supplementary Information (ESI)

Glucose Level Detection in Single-Cells under Satiety and Starvation States by Enzymatic Functional Glass Nanopore

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1. Experimental section

Reagents and materials.

3-(triethoxysilyl) proylsuccinic anhydride (TESP-SA) was purchased from Bailingwei Tech-nology Co. Ltd. (Beijing, China). 3-N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), zinc chloride ($ZnCl_2$), $Na_2HPO_4{\cdot}12H_2O$, KH_2PO_4 were purchased from Aladdin. Ltd. (Shanghai, China). The cytochrome c (Cyt c), L-ascorbic acid, β-D-glucose were purchased from Sigma Aldrich. The glucose oxidase (GOX), adenosine triphosphate (ATP) were purchased from Sangon Biotech. Co. (Shanghai, China). The Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI 1640), antibiotic solution, fetal bovine serum (FBS) and 0.25% trypsin/2.2 mM EDTA solution were purchased from Biological Industries Israel Haemek Ltd. Fluorescence dyes calcine-AM and propidium iodide (PI) were purchased from Thermo Scientific. 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 cur-rent measurements were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) in volt-age-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 micro-scope 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 with the scan rate of 20 mV s^{-1}, and recorded with a sampling frequency of 100 kHz. 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

Quartz glass capillaries (QF100-70-10, Sutter Instrument Co., Novato, CA, USA), with outer diameter of 1.0 mm and inner diameter of 0.7 mm, were used for the experiments. All glass capillaries were thoroughly cleaned by immersing in freshly prepared piranha solution (3:1 98% H_2SO_4/30% H_2O_2) 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 CO_2-laser based micropipette puller system (model P-2000, Sutter Instruments Co., Novato, CA, USA) with a program containing the following parameters: Heat =700, Fil = 4, Vel = 60, Del =170, Pull = 180. The transmission electron microscopy (TEM) images of the glass nanopores were carried out by using a FEI TECNAI F20 EM with an accelerating voltage of 200 kV equipped with an energy dispersive spectrometer. The tip (~ 2 mm) of the nanopipette as TEM sample was cut off and carefully transferred it to a folding grid.

Preparation of the Cyt c Fe (Ⅱ)

The purchased Cyt c Fe (Ⅲ) needs first to be chemically reduced into Cyt c Fe (Ⅱ) with L-ascorbic acid. Typically, 1.25 μL of L-ascorbic acid solution (200 mM) was added into 25 μL of Cyt c Fe (Ⅲ) (20 mM), during the process the color of the solution instantly changes from orange-red to pink (Fig. S2a). All of them were dissolved in 10

mM PBS buffer solution (pH 7.4). And then the original solution was diluted to 1 mL with 10 mM PBS buffer (pH 7.4) for the UV-visible measurement.

Surface modification of the G-nanopore

The modification of Cyt c Fe (Ⅱ) on the inner surface of G-nanopore was as follows: the TESP-SA (1% v/v in isopropanol) solution was backfilled into the nanotip of nanopipette to react with the silicon hydroxyl groups on the interior surface of G-nanopore for 1 h at room temperature. The nanopipette was then thoroughly rinsed with ethanol for several times and dried at 60 °C in a vacuum oven for 1.5 h. The reduced Cyt c Fe (Ⅲ) (20 mM) was diluted with 10 mM PBS buffer (pH 7.4) containing 200 mM EDC and 50 mM NHS to the concentration of 1 mM, and then injected it into the tip of the nanopipette and followed by a 12 h incubation period in 4 ℃. At this temperature, the oxidation of Cyt c Fe (Ⅱ) to Cyt c Fe (Ⅲ) by oxygen in the air will slow down kinetically. As shown in Fig. S4a, the color and UV-visible spectrum of the pink Cyt c Fe (Ⅱ) solution (at 4 ℃) after exposure to air for 12 h did not change compared to the freshly prepared Cyt c Fe (Ⅱ) solution. In addition, to avoid the partial oxidation of Cyt c Fe (Ⅱ) during the modification process, the glass nanotip was placed in a 20 mL glass bottle throughout the modification process (Fig. S4b). Before putting the glass nanopipette into the glass bottle, the air in the bottle was excluded with N_2. After it was placed in the bottle, the upper end of the glass nanopipette and the mouth of the glass bottle were blocked with hoses to prevent air from entering. After successful modification of Cyt c Fe (Ⅱ), the nanopipette was thoroughly rinsed with 10 mM PBS buffer (pH 7.4) for several times to remove excess Cyt c. Finally, a 10 mg/mL GOx solution (dissolved in 10 mM PBS buffer, pH 7.4) was injected into the nanopipette and it was ready for use. The schematic illustration of the whole modification processes was shown in Fig. S5. During the modification, the liquid solutions were backfilled into the nanotip with a MicroFil^{TM}, and then placed the pipette vertically to spring out the air bubbles and checked under microscope.

Electrochemical Impedance Spectroscopy (EIS) detection

In order to prove the reliability of the modification method, we modified the Cyt c onto the surface of cleaned ITO electrodes instead of G-nanopore in the same way. Then, the EIS measurements were performed using a 10 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture as a redox indicator. The alternating voltage was set at 5.0 mV, and the frequency scanned from 0.1 Hz to 100 Hz.

Glucose detection

Two Ag/AgCl electrodes, one used as working electrode was inserted into the Cyt c functionalized G-nanopore and the other used as reference electrode was placed in the bath solution (10 mM PBS buffer, pH 7.4), 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 glucose detection, the nanopipettes were backfilled with 10 mM PBS buffer solution containing 10 mg/mL GOX, and then immersed into the bath solution containing different concentration of glucose (10, 5, 2, 1 and 0.5 mM, respectively). All detection experiments were performed at room temperature. For each glucose detection, the G-nanopipette needed to be refreshed by injecting with 1 mM L-ascorbic acid solution stayed for 30 min, and then rinsing thoroughly with 10 PBS buffer (pH 7.4) for several times, and again injecting the 10 mg/mL GOX solution into G-nanopipette for the next detection. To determine the specificity of the method for glucose detection, the nanopipettes were tested by immersing into the bath solution containing 1 mM glucose, ATP, Trypsin, Ca^{2+}, Zn^{2+} and Mg^{2+} solutions, respectively.

Cell Culture

HeLa cells (human cervical cancer cells), H8 cells (human cervical epithelial immortalized cells), were bought from the American Type Culture Collection (ATCC, USA). HeLa cells were grown in the Dulbecco's Modified Eagle's Medium (DMEM) and H8 cells were grown in the Roswell Park Memorial Institute (RPMI 1640) 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% CO_2. The growth environment of HeLa cells under starvation was the same as that of stuffed state

except that 10% FBS was not added.

Cells vitality test

The Fluorescence dyes Calcein-AM and propidium iodide (PI) were used to prove that there was no damage to the cell membrane after the functionalized G-nanopore was inserted into the cell. HeLa cells were stained with these dyes following their Instruction manuals, and images were taken after the G-nanopipette was inserted to or withdrawn from the cells. The cells were observed using Leica DMI5000B microscope with a fluorescence detector with 40× objective. After the G-nanopipette was inserted into the same cell in different sites for 10 times, superimposed images of fluorescence and brightfield were taken continuously with every insertion and withdrawal.

Intracellular glucose measurements

Before glucose measurements, the cultured cells were washed with 10 mM PBS buffer solution (pH 7.4) three times to remove glucose in the environment, and using the 10 mM PBS buffer as bath solution for the subsequent detections. After the G-nanopore was immersed into the bath solution, a constant voltage of 50 mV was applied to help accurately judge whether the nanotip was inserted into the single cell and protect the tip from being contaminated by dead cells or cell residue in the medium, and the membrane test function of pCLAMP10.7 software was used for real time monitoring the change of nanopipette resistance. After reacting a certain period in the single cell (2 min), the nanopore was withdrawn and the I-V curve was measured in the bath solution. Experimentally, immediately after the refreshment of the sensing surface in bath solution (see aforementioned), we repeatedly inserted the G-nanopore into different positions in the same cell for new detection.

$$\text{Cyt c Fe (III)} + e^- \rightarrow \text{Cyt c Fe (II)} \qquad (1)$$

$$2\text{Cyt c Fe (II)} + 2H^+ + H_2O_2 \rightarrow 2\text{Cyt c Fe(III)} + 2H_2O \qquad (2)$$

Scheme S1. Chemical reaction process between Cyt c Fe (Ⅱ) and Cyt c Fe (Ⅲ).

2. Theoretical calculation for charge density

A voltage was applied through a glass nanopore to induce a flow of counter charges inside the surface electric double layer (EDL).[1,2] Ionic current I could then be produced, which could be calculated with the following equation:

$$I=\int_0^R 2\pi r u(r)[\rho_+(r)-\rho_-(r)]\,dr. \quad (1)$$

where $u(r)$ and $\rho(r)$ are the velocity distribution and ion concentration profile in the radial direction, respectively, R is the pore radius, and r is the local radius. The velocity distribution can be described with the Poiseuille's law:

$$u(r)=\frac{1}{4\eta}(R^2-r^2)\frac{\Delta P}{L} \quad (2)$$

where L is the pore length of effective sensing area (~ 2 μm), ΔP is the applied voltage, and η is the viscosity of water $\eta = 1.007\times10^{-3}$ Pa s. The ion concentration profile in the radial direction could be determined with the following equation：

$$\rho_{\pm}(r)=\rho_0 exp[\pm\frac{e_0\Phi(r)}{kT}] \quad (3)$$

where ρ_0 is the density of cations and anions in the neutral electrolyte, respectively, e_0 is the proton charge, k is the Boltzmann constant, and T is the absolute temperature. $\Phi(r)$ is the dimensionless electrical potential (1 unit corresponds to RT/F), which is governed by the Poisson-Boltzmann equation as follows:

$$\nabla^2\Phi(r)=\sin h[\Phi(r)]/\lambda^2 \quad (4)$$

Where λ (the Debye length that represents the EDL thickness) is defined as:

$$\lambda=\varepsilon RT/2C_0F^2 \quad (5)$$

Where F is the Faraday constant, ε and R are the dielectric permittivity of electrolyte solution and universal gas constant. Suppose the inner surface of the nanopore is charged homogeneously; the boundary condition for the above equation could be given by Gauss's law:

$$\nabla\Phi(r) = F\sigma/\varepsilon RT \qquad (6)$$

Where σ is the charge density on the inner surface on the nanopore. The Cyt c-modified G-nanopore filled with 10 mg/mL GOX solution was treated with 10 mM glucose (dissolved in 10 mM PBS buffer), and the ion current I at the voltage of -500 mV reduced from 6.3 nA to 4.5 nA after 2 min. The negative charge density σ changed from -2.585 C/m^2 to -2.571 C/m^2, therefore the transport rate of glucose was 72.55 nmol.m^{-2}.min^{-1} within 2 min for this reaction. It would have at least 72.55 nmol.m^{-2}.min^{-1} of glucose to participate in the reaction within 2 minutes. According to the chemical formula, the conversion rate of Fe (Ⅱ) to Fe (Ⅲ) was ~145 nmol·m^{-2}·min^{-1} within 2 min.

3. The comparison of our work with other literatures

Table S1: Comparison of our work with other reports on glucose detection.

Number	Method	Detection limit	Concentration range	Sample	Quantify (Yes/No)	Reference [3-11]
1	Electrochemical	0.86 mM	0.05-30 mM	serum	Yes	*Biosens. Bioelec.,* 2012, **37**,30–37
2	Electrochemical	0.1 mM	0.1-10 mM	serum	Yes	*Biosens. Bioelec.,* 2017, **98**, 449–456
3	Colorimetric	22.2 mg/dL	9–1350 mg/dL	saliva	Yes	Biosens. Bioelec., 2015, **67**, 763–768
4	Fluorescence	8.9 μM	10-500 μM	serum	Yes	*J. Am. Chem. Soc.,* 2015, **137,** 1290−1295
5	Fluorescence	1.5 μM	9−9 μM	serum	Yes	*Anal. Chem.,* 2014, **86,** 5323−5329
6	SERS	70 nM	0.5-5 mM	urine and serum	Yes	*Anal. Chem.,* 2016, **88,** 7191−7197
7	SERS	Not mentioned	0.1-5 mM	cells	No	*Talanta,* 2018, **179**, 200–206
8	Nanopore	Not mentioned	0-10mM	No	Yes	*Electroche. Commun.,* 2013, **36,** 71–74
9	Nanopore	1 nM	1nM-10 μM	saliva	Yes	*Anal. Chem.,* 2019, **91,** 14029−14035
10	Nanopore	0.025mM	0.5-10 mM	single cell	Yes	Our work

(Note: Although the detection limit of our method was not the lowest compared to other methods for glucose detection, the sharp tip of glass nanopore could quantitatively detect glucose concentration in a single cell under the premise of maintained cell membrane integrity and cell viability.)

4. TEM of the glass nanopore

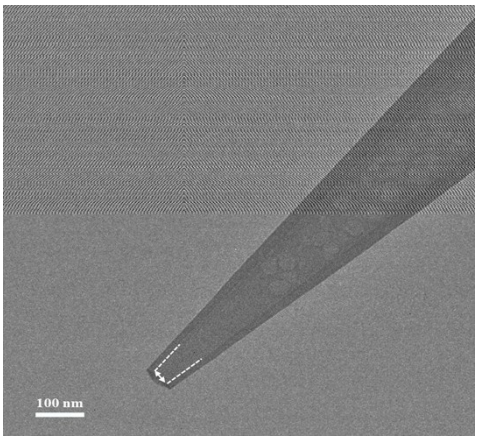

Fig. S1: The TEM image of bare glass nanopore with a diameter of ~40 nm.

5. Characterizations for Cyt c

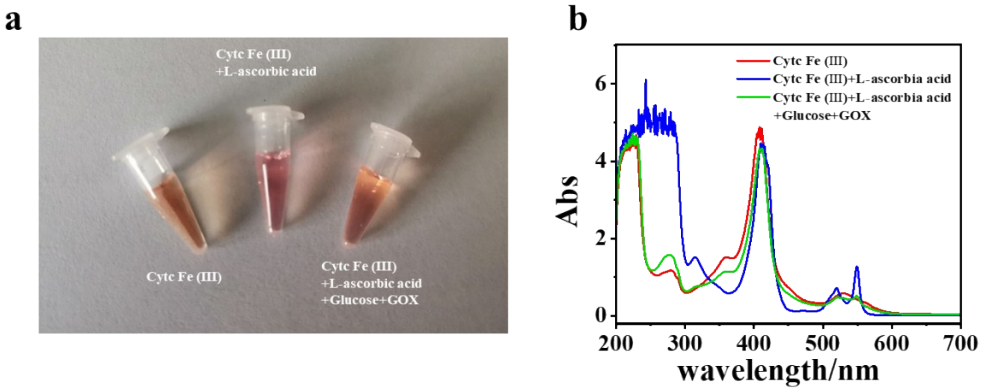

Fig. S2: a) Photos and b) corresponding UV-visible spectra characterizations of Cyt c (Fe (Ⅲ)) and the reduced Cyt c (Fe (Ⅱ)).

6. The modification of Cyt c inside nanopore

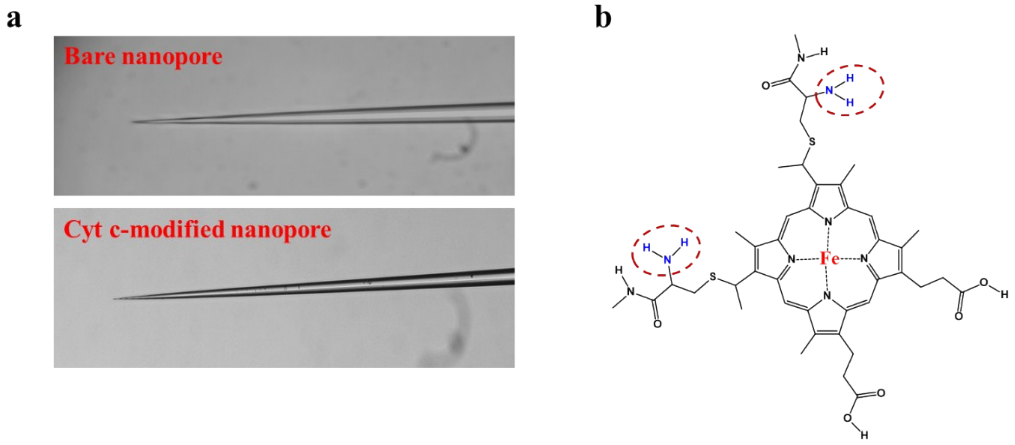

Fig. S3: a) Bright-field images of bare glass nanopore (top) and the Cyt c-modified glass nanopore (bottom). b) The chemical structure of the heme moiety of Cyt c.

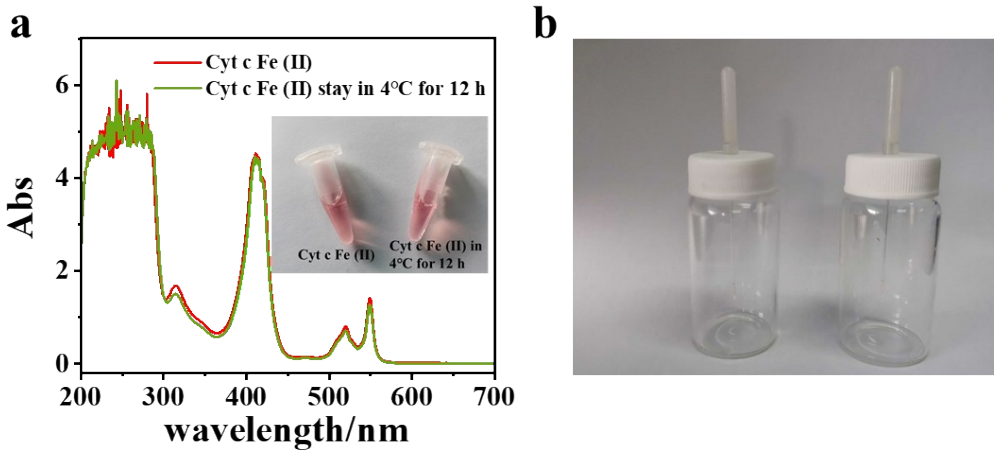

Fig. S4: a) UV-visible spectra and color (inset plot) characterizations for Cyt c Fe (Ⅱ) solution freshly prepared and placed at 4 ℃ for 12h respectively. b) The relatively sealed experimental container used for Cyt c Fe (Ⅱ) modification on inner surface of glass nanopore.

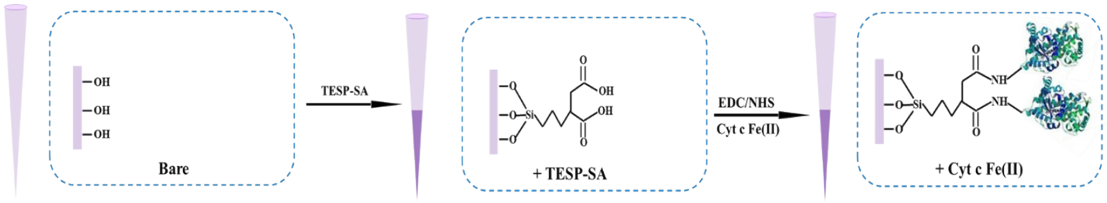

Fig. S5: Schematic illustration of the modification process of the inner surface of the nanopipette before it is ready for glucose sensing.

7. The stability of the functionalized nanopore

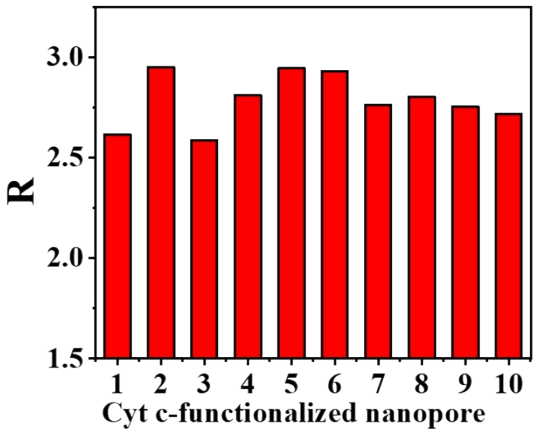

Fig. S6: The R values of ten different G-nanopores prepared by the same modification with 1 mM Cyt c Fe(Ⅱ).

8. Cycle experiment

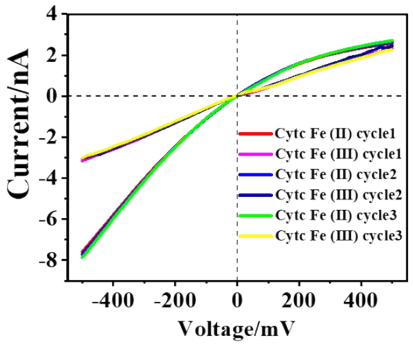

Fig. S7: I-V curves obtained from the Cyt c-functionalized G-nanopore treated with 10 mM glucose and L-ascorbic acid through three cycles.

9. Specificity detection

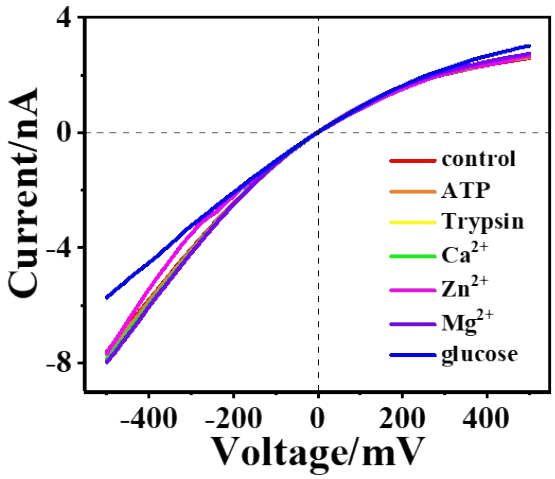

Fig. S8: I-V curves obtained in 10 mM PBS buffer (pH 7.4) containing ATP, Trypsin, Ca^{2+}, Zn^{2+}, Mg^{2+}, respectively, with all concentrations of 1 mM.

10. Cell viability tests

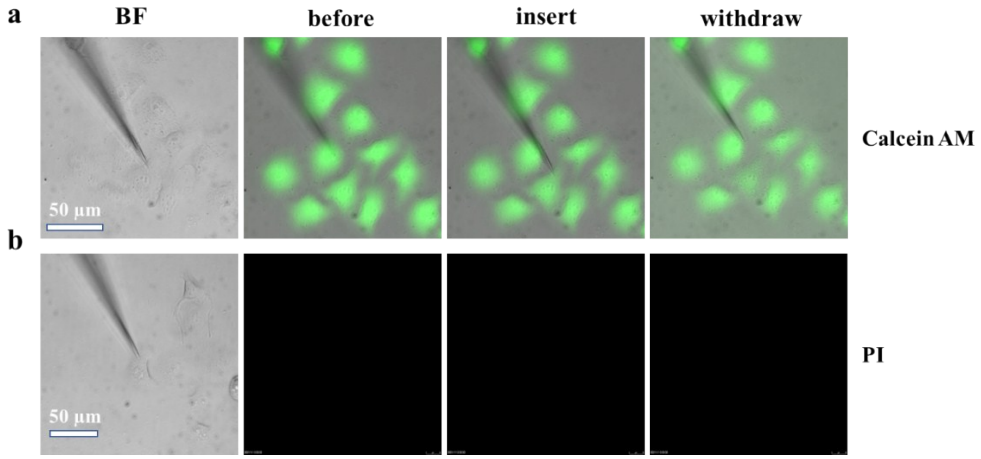

Fig. S9: Bright-field and fluorescent images of a) Calcein-AM and b) PI stained HeLa cells before and after the insertion and withdrawal of the nanopipette (the scale bar: 50 µm, BF: bright field).

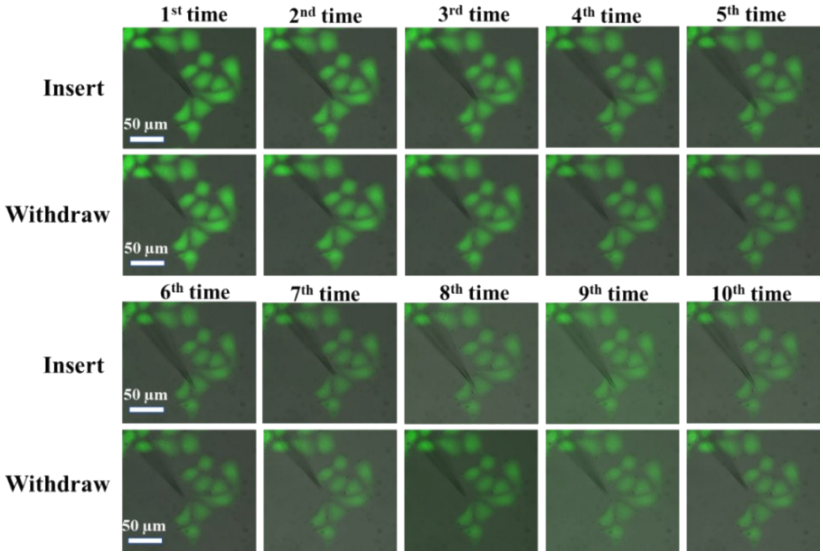

Fig. S10: Superimposed fluorescence and bright-field images of Calcein-AM/PI stained HeLa cells after 10 times repeated insertion into and withdrawal from the same cell. Scale bar: 50 µm.

11. Effect of intracellular H_2O_2 on the sensor

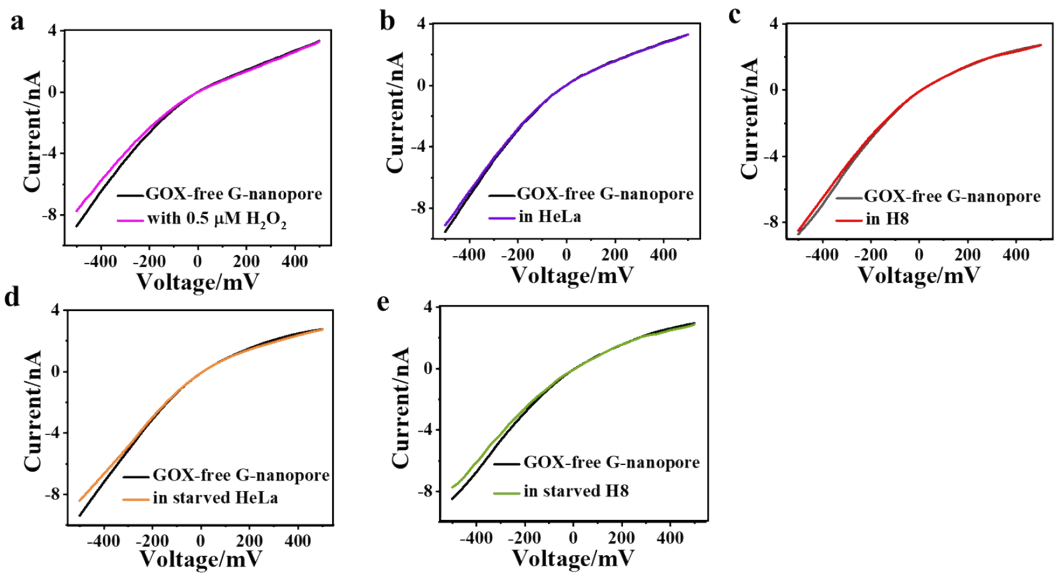

Fig. S11: a) I-V curves obtained at GOX-free Cyt c-modified G-nanopore a) before and after the treatment with 0.5 μM H_2O_2, and before insertion into and withdrawn from single b) HeLa cells, c) H8 cells, d) HeLa cells in starvation and e) H8 in starvation; the nanopores were inserted in cells for 20 min with a constant voltage of 50 mV.

12. Detection of intracellular glucose

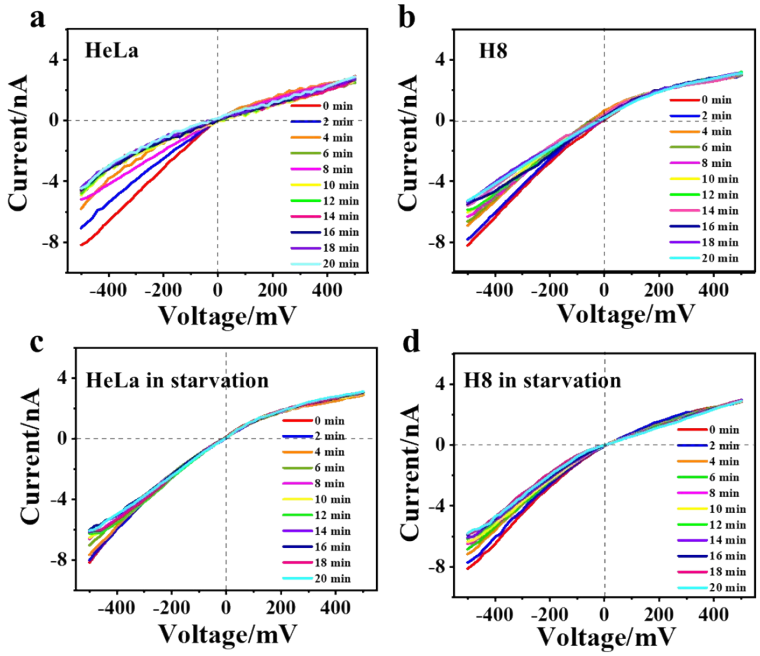

Fig. S12: Continuous intracellular I-V curve recordings obtained at Cyt c functionalized G-nanopore of four cell groups a) HeLa and b) H8, c) HeLa

in starvation and d) H8 in starvation after 10 times repeated insertions into the same cell within 20 min.

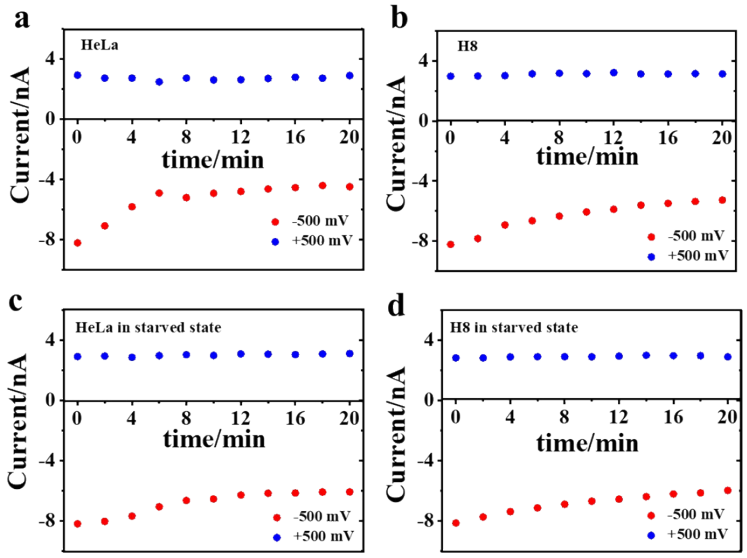

Fig. S13: Continuous intracellular glucose measurements of a) HeLa, b) H8, c) HeLa in starvation and d) H8 in starvation by monitoring I-V curves and recording the current changes at - 500 mV (blue symbol) and + 500 mV (red symbol), respectively, within 10 min.

13. References:

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