# Integrated nanoporous electroporation and sensing electrode array for total dynamic time-domain cardiomyocyte membrane resealing assessment

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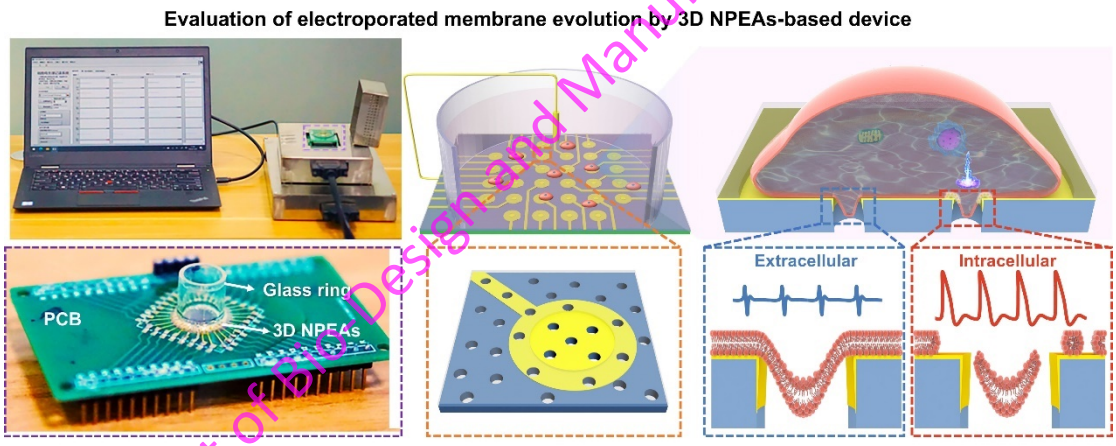
## Abstract

Intracellular electrophysiological research is vital for biological and medical research. Traditional planar microelectrode arrays (MEAs) have disadvantages in recording intracellular action potentials due to the loose cell–electrode interface. To investigate intracellular electrophysiological signals with high sensitivity, electroporation was used to obtain intracellular recordings. In this study, a biosensing system based on a

nanoporous electrode array (NPEA) integrating electrical perforation and signal acquisition was established to dynamically and sensitively record the intracellular potential of cardiomyocytes over a long period of time. Moreover, nanoporous electrodes can induce the protrusion of cell membranes and enhance cell–electrode interfacial coupling, thereby facilitating effective electroporation. Electrophysiological signals over the entire recording process can be quantitatively and segmentally analyzed according to the signal changes, which can equivalently reflect the dynamic evolution of the electroporated cardiomyocyte membrane. We believe that the low-cost and high-performance nanoporous biosensing platform suggested in this study can dynamically record intracellular action potential, evaluate cardiomyocyte electroporation, and provide a new strategy for investigating cardiology pharmacological science.

## Graphic abstract

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## Keywords

Nanoporous electrode array (NPEA), Electroporation, Intracellular potential recording, Cardiomyocyte, Membrane resealing

## Introduction

Regardless of the numerous breakthroughs and progress that have been made in the biomedical field, cardiovascular diseases (CVDs) remain the leading cause of mortality worldwide, accounting for approximately 30% of all deaths [1-7]. Among CVDs, cardiac arrhythmia, which is characterized by irregular electrocardiogram (ECG) and abnormal contraction of the heart, can impose a heavy burden on the heart and even cause sudden death [8-13]. Transmembrane potential analysis, which is closely related to the membrane integrity of cardiomyocytes, is important for exploring the mechanism of cardiac arrhythmias and treatment of CVDs. Currently, many research models, including cell-level action potential and ECG of ex vivo tissues or in vivo large animals, have been reported. Among these models, ECG is considered a suitable in vivo model to study cardiac arrhythmias because of its similarity to human ECG. However, they remain inapplicable for early and extensive investigation because of their low throughput, high cost, roughness, and low-resolution information, which make them unsuitable for in-depth mechanism research [14-19]. Patch clamp, a gold standard accurate technology for electrophysiological detection, can accurately measure transmembrane potentials; however, its low throughput, severe invasiveness, and complex operation restrict the long-term recordings of multiple single cells [20-25]. Voltage-sensitive dyes have high sensitivity and resolution; however, they suffer from phototoxicity and reagent cytotoxicity, making long-term monitoring difficult [26-31].

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Recently, extracellular recording microelectrodes have been proposed and widely used to perform action potential recordings in cardiomyocytes because of their noninvasive, dynamic, and large-scale characteristics. However, the loose cell–electrode interface coupling and membrane-barrier lead to weak signal intensity and mismatch the native transmembrane potential [32]. Recent advances in 3D microelectrodes have demonstrated that geometrical structures enhance the interface coupling of membranes and access premium intracellular recordings [33-39]. Furthermore, to perform high-quality intracellular recording, the use of appropriate assisted technology is important. Among the assisted techniques for intracellular recordings, electroporation is widely used because it is convenient and biosafe [40-44]. During the past decades, although many efforts have been devoted to electroporation mechanism research [42, 45-50], they remain insufficient to elucidate the process of membrane perforation and resealing, attributing to the lack of a quantitative and dynamic research platform. Therefore, the development of a noninvasive, dynamic, long-term recording device for membrane evolution and intracellular action potential is crucial for the study of cardiac mechanisms and the screening of arrhythmia drugs [51-54].

In this study, a nanoporous electrode array (NPEA)-based device (Fig. 1) was suggested for dynamic evaluation of electroporated membrane evolution in cardiomyocytes and for achieving intracellular recording in a minimally invasive, long-term, and highly sensitive manner. Cell membrane evolution can also be quantitatively and segmentally

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studied in situ during the whole recording process (nanocrack perforation, stabilization with partial recovery, and gradual resealing) [55]. Compared with planar electrodes, 3D porous electrodes can trap and tightly couple with cultured cardiomyocytes to enhance electroporation, providing a new platform for long-term intracellular recording in the fields of cardiology and pharmacological science.

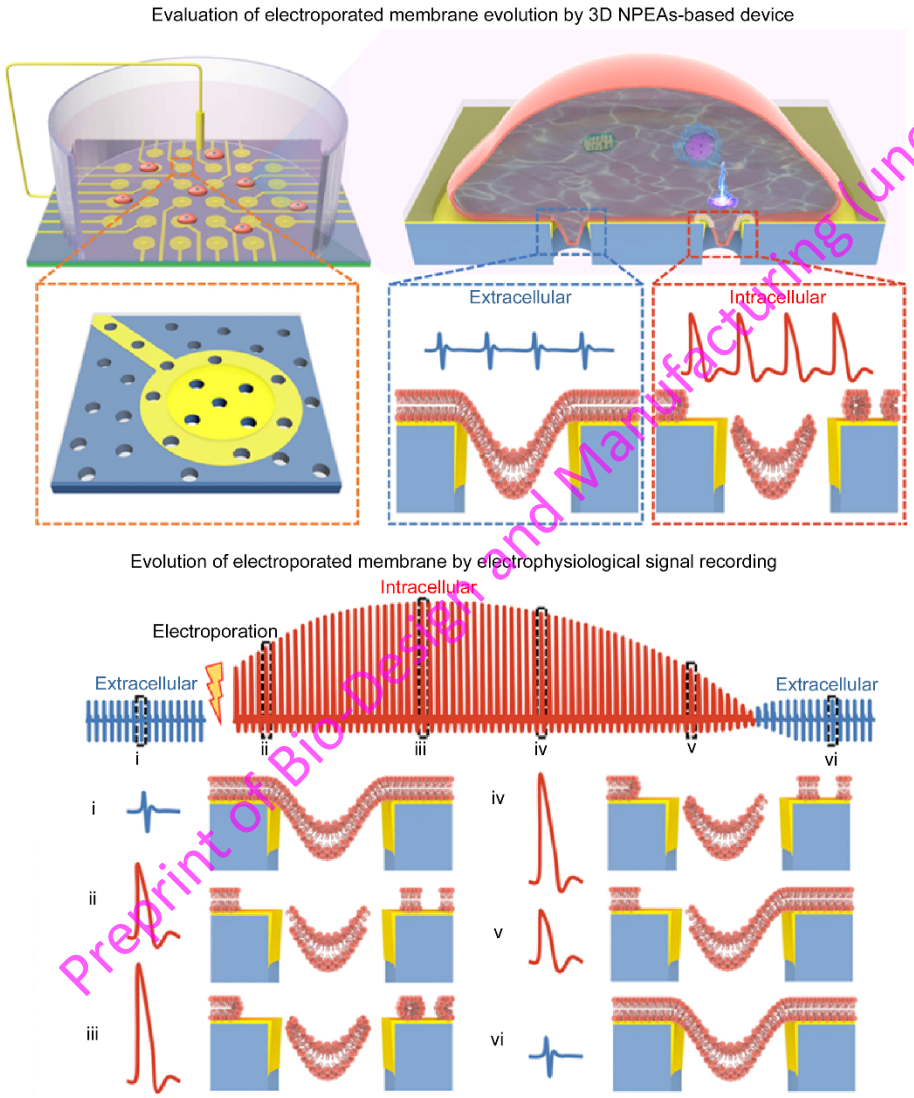


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Fig. 1 Schematic of the NPEA-based sensing device for the dynamic evaluation of electroporated membrane resealing in cardiomyocytes. This integrated electroporating and sensing system can dynamically detect and analyze both extracellular and intracellular signals in real time. After electroporation, electrophysiological signals are converted from extracellular signals to intracellular signals. The amplitude quickly rises, then gradually declines, and finally returns to the extracellular state. NPEA: nanoporous electrode array

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## Experimental methods

### Materials and reagents

Polyethylene glycol terephthalate (PET) membrane (Wuhan Kejin Xinfa Technology, China), S1813 photoresist (Rohm & Haas, USA), SU-8 2005, 2-acetoxy-1-methoxypropane (PGMEA) (Kayaku Advanced Materials, USA), quartz glass, polydimethylsiloxane 184 (PDMS 184) (Dow Corning, USA), conductive silver glue paste (Electrolube, Ashby de la Zouch, UK), 1-d-old Sprague–Dawley rats (Laboratory Animal Center of Zhejiang University China), Dulbecco's modified eagle medium (DMEM), Hank's balanced salt solution (HBSS), trypsin, type II collagenase, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin (Thermo Fisher Scientific, USA), and cell sieve (Sigma) were used without any further purification.

### Fabrication and characterization of the nanoporous electrode array

NPEA was fabricated on a 2 cm×2 cm porous PET membrane with certain diameters using standard microfabrication techniques, photolithography, and magnetron sputtering according to the work of Xu et al. [56]. Typical fabrication procedures are as follows: First, the S1813 photoresist (2-μm-thick) was spined onto the porous PET membrane at 3500 r/min, followed by baking at 110 °C for 1 min. Second, a UV aligner (URE-2000/30L, Institute of Optoelectronic Technology, Chinese Academy of Sciences, China) was used to expose the PET substrate above 130 mJ/cm$^2$, followed by development in MF-319 (Rohm and Haas, USA) for 40 s. Third, the 10 nm Ti/50 nm Au conductive layer was sputtered onto the substrate and then lifted off in acetone. The deposited substrate was then spin-coated again with a 5 μm SU-8 layer to insulate the leads. The effective electrode region was defined again via photolithography, 160 mJ/cm$^2$-exposure, and then rinsed in PGMEA and isopropanol. Finally, the fabricated device was prepared after drying in $N_2$ and hard baking (150 °C, 30 min). The morphologies of the fabricated devices, which were sputtered with a layer of 2 nm of Au, were characterized using scanning electron microscope (SEM) SUPRA 60 (Zeiss, Germany) at a voltage of 10 kV.

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**Assembly of the nanoporous electrode array**

The fabricated NPEA was immobilized on a customized printed circuit board (PCB) using PDMS 184 and electrically connected using conductive silver glue paste. A cell

culture chamber made of a glass ring, 0.8 cm in diameter and 1 cm in height, was then glued to the middle of a chip using PDMS. The reference electrode (Pt wire) fixed to the centrifuge tube cover was electrically connected to the PCB. The device was finally completed by soldering the pin headers on the PCB. The finished device can record the electrophysiological potentials of the cultured cardiomyocytes.

**Primary rat cardiomyocyte culture**

Ventricle tissues from the purchased 1-d-old Sprague–Dawley rats (75% ethanol-sterilized and ice-cold DMEM-rinsed) were sliced (about 1 $mm^3$) in ice-cold HBSS, which contains 0.07% trypsin and 0.05% collagenase type II, for digestion at 37 °C in an incubator (5.0% $CO_2$). The digestion step was repeated several times within 2 h to avoid overdigestion. The isolated cells were then purified as follows: centrifugation at 1000 r/min for 5 min, suspension in DMEM containing 10% FBS, filtering using a 70-μm cell sieve, and purification using a differential adhesion (45 min, twice). In addition, before cell seeding, the devices were first treated with 75% ethanol and UV irradiation and then modified with fibronectin (10 μg/mL) in an incubator (37 °C, 5.0% $CO_2$). Finally, each device was seeded with about $3\times10^5$ cells in 1 mL DMEM with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, which was refreshed every 48 h.

**Electrophysiology recording**

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Electrophysiological recording of cardiomyocytes was performed using our NPEA-based system, which integrates electrical perforation and signal acquisition. First, the device with cardiomyocytes was electrically connected to a module comprising a primary amplifier and pulse signal generator. Then, the module placed in the incubator was connected to another module, which comprised a filter and a secondary amplifier, outside the incubator. Finally, pulse control of electrical perforation and signal acquisition, display, and storage were performed using a computer. Extracellular and intracellular signals (20 kHz) were filtered using a 1–7.5 kHz bandpass. To electrically perforate, 20 consecutive square-wave pulses of 200 μs pulse width and 3 V amplitude were applied to the device in 1 s.

**Signal processing and statistical analysis**

The LabVIEW software was used to process the signals by displaying data, removing noise, and extracting electrophysiological parameters. In the program, noise removal was performed according to the "db06" wavelet basis, and the adaptive threshold was defined using the average of the top 10 peaks in this data segment. For electrophysiological action potential spikes, the peak points were determined using the LabVIEW program's peak detection function. For the extracellular cells, valley points were also determined using the peak detection function, but for the intracellular cells, the beginning points were determined using the dynamic threshold. Other characteristic

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points can be determined using the peaks, valleys, and beginning points, and then used for calculating electrophysiological parameters. All results and error bars are expressed as mean±SD. An ordinary one-way analysis of variance (ANOVA) was performed to analyze statistical significance using Origin 2018, and significant differences were considered at $P<0.05$.

## Results and discussion

### Establishment and characterization of an intracellular electrophysiological recording system

The intracellular electrophysiological recording system comprised an NPEA device and a homemade system integrated with electroporation and signal analysis functions. The NPEA was fabricated using a standard microfabrication technique (Fig. 2a). Porous PET membranes were used as the optimum substrate for NPEA chips, which are rich in native porous structures of different sizes and densities. Steps of photolithography, sputtering with 10 nm Ti/50 nm Au, and stripping with acetone were performed to define the electrodes, tracks, and pads on the PET substrate. Finally, another 5-μm SU-8 layer was patterned to define the 30-μm electrode and insulate the leads. Compared with traditional silicon-based semiconductor processing technology that relies on etching, the above steps greatly simplify the process steps of the three-dimensional chip, improve efficiency, and save costs. As shown in Figs. 2b–2d, the 2 cm×2 cm NPEA

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device had 32 electrodes, and the distance between the centers of adjacent electrodes was 300 μm, which is beneficial for reducing the mutual interference between adjacent electrodes. Considering that the diameter of adherent cardiomyocytes was 10–15 μm, the nanoporous electrode (30 μm in diameter) can sufficiently adhere to a single cardiomyocyte. Conversely, many studies suggest that the nanopore structure promotes interface coupling between the cell membrane and electrode, further reducing the interface impedance and enhancing the intracellular signal [57, 58]. Meanwhile, the nanopore's edges have a significant relationship with the cell membrane's curvature, and the curvature is beneficial for the coupling of the electrode and cell, and can delay its resealing [57-60]. The resealing time of the cell membrane is closely related to the detection window of intracellular electrophysiology. Thus, it is important to study the effect of the nanopore diameter of the electrode on membrane resealing to reveal the mechanism of intracellular electrophysiological detection. As shown in Figs. 2e–2g, SEM was employed to characterize the nanopores with different diameters (220, 450, and 1000 nm), indicating that the nanopores with different diameters possess uniform, intact porous structures. Noteworthy, the number of nanopores was inversely proportional to the diameter, and overlap occurred when the number of nanopores was large, which might affect the intensity of the intracellular electrical signal.

Furthermore, to evaluate the electrophysiological recording performance of NPEA devices with different nanopore sizes, cardiomyocytes were cultured on the assembled

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system (Fig. 2h), which comprised an NPEA device and a homemade analyzing system. The NPEA device was assembled using a glass ring, a PCB base, and an NPEA chip, which was used for cardiomyocyte culturing and assessment (Fig. 2i). The homemade analyzing system is illustrated in Fig. S1 (Supplementary Information), which includes the electroporation, signal amplifying and filtering, data processing, and LabVIEW control modules. To maintain cardiomyocytes in a stable culture and assessment environment, the NPEA device was placed in a 37 °C, 5% $CO_2$ incubator and connected to an external laptop via a cable for long-term assessment (Fig. S2 in Supplementary Information). The homemade LabVIEW program can control electroporation, record intracellular electrical signals, and evaluate the resealing of the cardiomyocyte membrane (Fig. S3 in Supplementary Information). The corresponding SEM image of cardiomyocytes coupled to the NPEA device is also shown in Fig. S4 (Supplementary Information), indicating that cardiomyocytes with good morphology are closely diffused and bound to the NPEA device.

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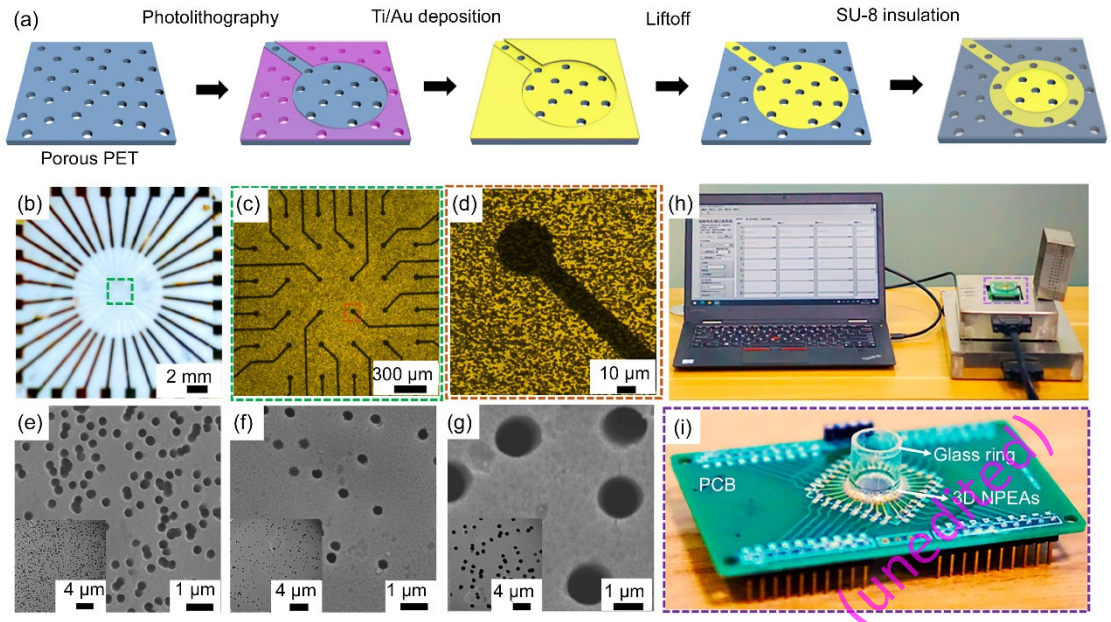


Fig. 2 Production process and characterization of 3D NPEA. **a** Flowchart for fabricating 3D NPEA, which was patterned on a porous PET substrate according to a standard microfabrication technique. Photograph (**b**) and optical microscope images (**c**, **d**) of the 3D NPEA. The distance between adjacent electrodes (the green dash box in Fig. 2b) was 300 μm, and the diameter of the working electrode (the orange dash box in Fig. 2c) was 30 μm. SEM images of nanopores with 220 nm (**e**), 450 nm (**f**), and 1000 nm (**g**) in diameter on working electrodes. Insets are the corresponding SEM images of a porous working electrode site. **h** Photograph of the self-developed system for dynamic evaluation of electroporated membrane evolution in cardiomyocytes. **i** Photograph of the 3D NPEA-based device, which corresponds to the purple region in Fig. 2h. NPEA: nanoporous electrode array; PET: polyethylene glycol terephthalate; SEM: scanning electron microscope

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**Total time-domain assessment of cardiomyocyte membrane resealing**

Cell membrane electroporation is a technique for establishing access to intracellular electrophysiology, which introduces transient pulse voltage to lower cell–electrode interface impedance and breakdown cell membrane dielectric, inducing nanocracks on the membrane [43]. Because of the self-healing properties of the cell membrane, the nanocracks on the membrane reseal after electroporation. Additionally, ion channels on the cell membrane play an important role in transmembrane potential regulation [61]. Therefore, the assessment of membrane status is important for recording intracellular electrophysiology and studying the mechanism of ion channel drug intervention. Although conventional MEA can evaluate cell membrane status by recording extracellular potential, the cell membrane barrier of the cell–electrode interface remains a hindrance. The natural barrier causes a large sealing impedance and weakens the action potential signal acquisition. In this study, NPEA performed electroporation and intracellular electrophysiological recordings, and cell membrane resealing was evaluated via real-time assessment of action potential.

Extracellular and intracellular action potential recordings for membrane dynamic evaluation are shown in Fig. 3a. This suggests that cardiomyocytes can first generate low-quality extracellular potentials, quickly convert them to intracellular potentials with maximum amplitude upon 3 V electroporation, and finally return to the extracellular state. The conversion between extracellular and intracellular signals demonstrated nanocrack generation and resealing on the cell membrane. From the

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whole recordings, the extracted signals can be analyzed in detail (Fig. 3b). The initial extracellular action potential was 375.68 μV at about 52.8 s (i) and was then converted to intracellular potential after electroporation. The intracellular action potential started from 1092.53 μV at 31.5 s (ii) to 3697.37 μV at 75.8 s (iii), quickly reached a maximum of 4197.65 μV at 101.3 s (iv), and then slowly decreased (3483.30 μV at 140.2 s (v), 2506.79 μV at 171.6 s (vi), and 1220.01 μV at 202.3 s (vii)). Finally, it returned to the extracellular state (221.84 μV at 246.6 s (viii)). The whole converting process (Figs. 3b and 3c) of the electroporated cell membrane reveals its reversibility, suggesting its potential application in evolution assessment by continuous electrophysiological recordings in a dynamic and quantitative manner. Additionally, a device with a good spatial and temporal resolution of action potential is expected to be used in the development of cardiac drugs (Fig. S5 in Supplementary Information). The drug test results showed that our porous device is sensitive to such drug screens. Furthermore, based on its high spatiotemporal resolution characteristics, this device may also be used to monitor neuroelectrophysiological signals.

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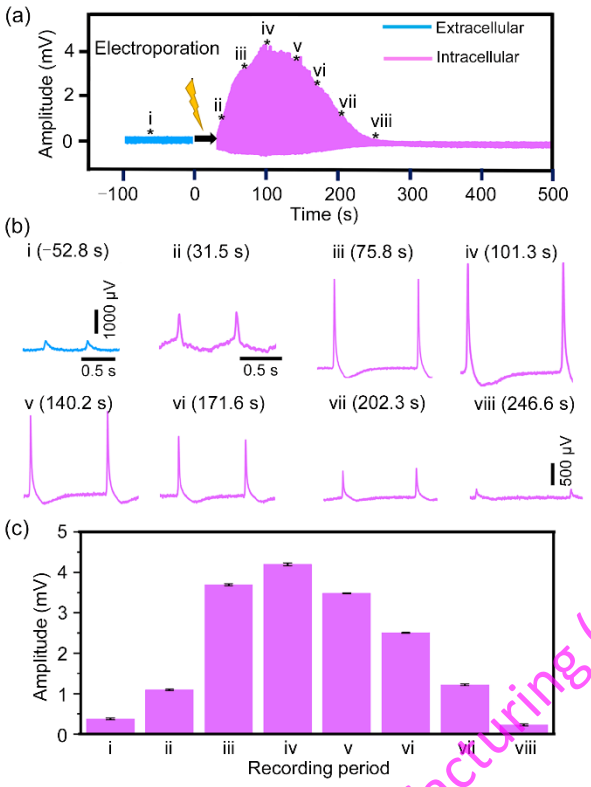


Fig. 3 Dynamic assessment of the electroporated membrane evolution in cardiomyocytes through continuous electrophysiological recordings. Typical extracellular (blue) and intracellular (rose red) recordings (**a**) and corresponding enlargements (**b**). **c** Amplitude data at different recording periods

**Optimization of intracellular electrophysiological recording using a nanoporous electrode array**

To confirm the electrophysiological recording performance of NPEA, electroporated cardiomyocyte membranes were assessed in real time using MEA (planar, 2 cm×2 cm) and NPEA (porous) (Fig. 4a). By statistically analyzing the intracellular amplitudes (Fig. 4b) and signal-to-noise ratios (SNRs) (Fig. 4c) of the nanoporous and planar electrode recordings, the average amplitude and SNR for the nanoporous electrode were 1.68 mV and 26.85 dB, respectively, which are much higher than that of the planar

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electrode (0.57 mV and 20.73 dB, respectively). The SNR of the extracellular raw noise levels was also plotted (Fig. S6 in Supplementary Information), indicating that the porous device is also sensitive in the extracellular cell. In addition, the amplitude and its variation in a short time before (increase stage) and after (decrease stage) electroporation were also studied using different microelectrodes (Figs. 4d and 4e). The time point for electroporation was set to 0 s. Figure 4d shows that the amplitude and its variation of both 3D porous and planar electrodes increase slightly over time before electroporation, but the nanoporous electrode had a slightly higher value near the electroporation, indicating that the nanoporous electrode is more sensitive to recording than the planar electrode. Simultaneously, after electroporation, the amplitudes gradually decreased, suggesting slow resealing of the cell membrane. Conversely, the amplitude variation rates first reached a negative peak and then slowly increased to approximately 0, indicating cell membrane recovery (Fig. 4e). Additionally, the amplitude for the nanoporous electrode was higher than that for the planar electrode, suggesting a higher sensitivity of the nanoporous electrode. On the basis of the above results, NPEA showed excellent performance in dynamically recording electroporated membrane evolution.

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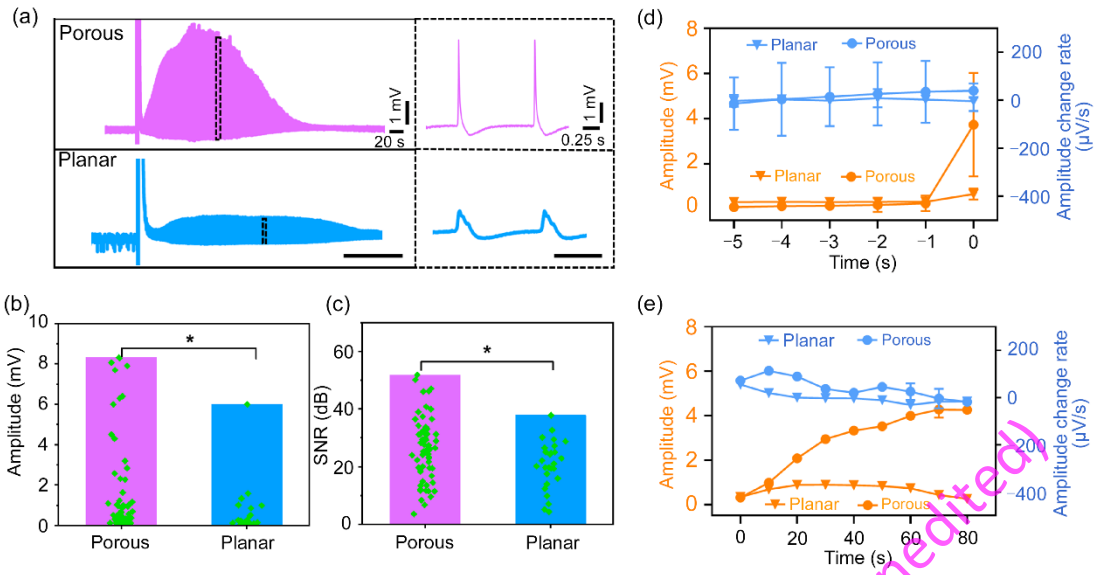


Fig. 4 a Electrophysiological recording using electrodes with different shapes (rose red for 3D NPEA and blue for planar MEA). Enlarged signals show the typical shape of the intracellular action potential. Statistical measurements of the intracellular amplitude (**b**) and SNRs (**c**) recorded using different MEAs. An ordinary one-way ANOVA was used to analyze statistical significance, $^{*}P<0.05$. Evolution of the amplitude (orange) and its rate of change (blue) of the action potential before (**d**) and after (**e**) electroporation. The time required to reach the maximum was set to 0 s, and the error bars are presented as mean±SD ($n$=3). NPEA: nanoporous electrode array; MEA: microelectrode array; SNR: signal-to-noise ratio; ANOVA: analysis of variance; SD: standard deviation

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The successful monitoring of membrane resealing demonstrated that NPEA can dynamically assess cell membrane status by recording intracellular electrophysiological signals. Research shows that nanopores on the electrode are helpful for electroporation and resealing by enhancing the coupling between the cell membrane and electrode [57,

58, 62, 63]. The interface between the electrode and cell implies much information for intracellular recording via signal transmission and electroporation. The electric-field models of the NPEA and MEA were established using COMSOL, as shown in Figs. S7–S9 (Supplementary Information). The NPEA had stronger electric field penetration, especially in the nanoporous area, which is almost five times that of MEA, and the magnitude distribution was more uniform. A possible reason for this phenomenon may be that the pore structure with broadband characteristics and low losses can be regarded as an isotropic structure, and its electromagnetic characteristics are consistent for waves in different directions. Compared with planar structures, porous structures can improve their relative dielectric constant and other electromagnetic characteristics, resulting in a more concentrated electric field intensity. As long as the number of pores with a certain diameter on the electrode is large, the electric field intensity can be sufficiently concentrated. Noteworthy, the more uniform the pore distribution, the more conducive it is to the concentration of the electric field, but the pore distribution with low uniformity also has this tendency to cause the electric field to concentrate. To further optimize the recording of electrophysiological signals, nanopores on electrodes with different diameters (220, 450, and 1000 nm) were used to record the electrophysiological potential (Fig. 5a). By statistically analyzing the intracellular potential amplitudes (Fig. 5b), SNRs (Fig. 5c), and durations (Fig. 5d) collected by the nanoporous electrodes with diameters of 220, 450, and 1000 nm, the maximum

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potential amplitude was 2.83, 8.33, and 2.62 mV, the average SNR value was 18.84, 26.74, 17.28 dB, and the maximum duration was 599.74, 570.46, and 350.93 s, respectively. The results above show that the intracellular potential recorded using the 3D porous device with a 450-nm diameter has a larger percentage in high amplitude and SNR, although its duration is a little short. A possible reason for this may be the better performance of nanoporous devices with more pores and longer circumferences, which can supply more sites for cardiomyocytes to improve sealing and electroporation. However, if the pore diameter is too small, the cell membrane cannot protrude into the nanocracks, thus increasing signal loss and preventing electrodes from accessing the interior.

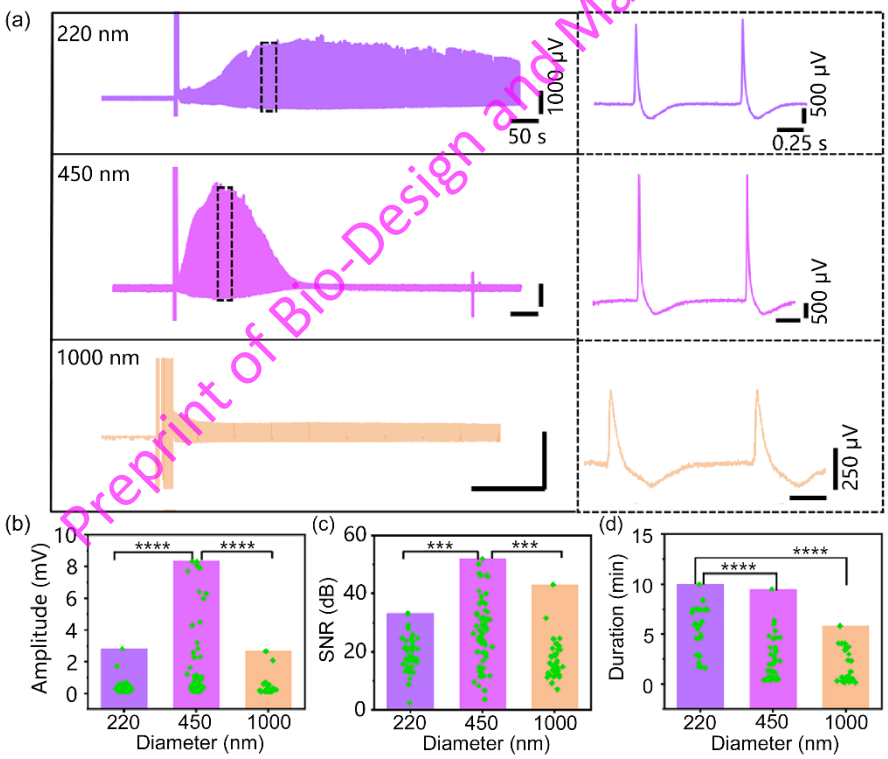


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Fig. 5 **a** Electrophysiological recording using NPEA with different pore diameters (purple for 220 nm, rose red for 450 nm, and light yellow for 1000 nm). The enlarged signal shows the typical shape of the intracellular action potential. **b**–**d** Statistical measurements of the intracellular action potential amplitude, SNRs, and durations recorded using NPEA with different diameters. An ordinary one-way ANOVA was used to analyze statistical significance. $^{***}P<0.001$; $^{****}P<0.0001$. NPEA: nanoporous electrode array; SNR: signal-to-noise ratio; ANOVA: analysis of variance

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## Conclusions

In this study, an electrophysiological sensing device based on NPEA for dynamic evaluation of electroporated membrane evolution, which can dynamically detect and analyze both extracellular and intracellular signals with high sensitivity and throughput for a long time, was developed. During the entire recording process, the signals can be analyzed quantitatively and segmentally according to their amplitudes and change rates of amplitude, which can conveniently represent cell membrane evolution. In summary, the low-cost and high-performance sensing platform suggested in this study can dynamically record the intracellular action potential and evaluate cardiomyocyte electroporation, providing a novel strategy for studying the evolution of electroperforated membranes and understanding the electroporation mechanism in depth.

However, there are still some other works that need to be done in the future, such as (i) selecting a 3D nanodevice with other nanostructures (morphology, size, material, etc) to enhance the cell–electrode coupling and achieve an intracellular recording with long-term and high sensitivity and (ii) enlarging the application field (drug screening, intracellular delivery, etc) of the self-developed sensing device through other models (other cell types and neuron).

**Supplementary Information**

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**Author contributions**

WQS, YL, and CLQ performed all experimentals and conducted data analysis. ZHZ carried out the electric field model simulation of different electrode arrays. ZCT took some data analysis in the stage of replying to the reviewers' comments, and redrew a figure. YXP, CT, and XWW revised the manuscript and provided project guidance. In particular, CT and XWW conceived the whole reply idea of this work and pointed out the direction of the reply. All authors read and approved the final manuscript.

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## Declarations

### Conflict of interest

The authors declare that they have no conflict of interest.

### Ethics approval

All protocols comply with the charter of the Ethical Review Committee of Laboratory Animal Welfare of Zhejiang University, China.

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