On-chip electroporation, membrane repair dynamics and transient in-cell recordings by arrays of gold mushroom-shaped microelectrodes

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This study demonstrates the use of on-chip gold mushroom-shaped microelectrodes (gMµEs) to generate localized electropores in the plasma membrane of adhering cultured neurons and to electrophysiologically monitor the ensuing membrane repair dynamics. Delivery of an alternating voltage pulse (0.5–1 V, 100 Hz, 300 ms) through an extracellularly positioned micrometer-sized gMµE electroporates the patch of plasma membrane facing the microelectrode. The repair dynamics of the electropores were analyzed by continuous monitoring of the neuron transmembrane potential, input resistance ($R_{in}$) and action potential (AP) amplitude with an intracellular microelectrode and a number of neighbouring extracellular gMµEs. Electroporation by a gMµE is associated with local elevation of the free intracellular calcium concentration ([Ca$^{2+}$]) around the gMµE. The membrane repair kinetics proceed as an exponential process interrupted by abrupt recovery steps. These abrupt events are consistent with the “membrane patch model” of membrane repair in which patches of intracellular membrane fuse with the plasma membrane at the site of injury. Membrane electroporation by a single gMµE generates a neuron-gMµE configuration that permits recordings of attenuated intracellular action potentials. We conclude that the use of on-chip cultured neurons via a gMµE configuration provides a unique neuroelectronic interface that enables the selection of individual cells for electroporation, generates a confined electroporated membrane patch, monitors membrane repair dynamics and records attenuated intracellular action potentials.

1. Introduction

Micro-injuries of the cellular plasma membrane and the ensuing repair mechanism dynamics are the subject of intense studies by the bioengineering and biomedical communities. The diverse research efforts can be subdivided into three main categories: (a) the development of microelectronic, microfluidic and micro-optical devices that enable transient electroporation of cell membranes under in vitro conditions. These technologies are mainly tailored towards promoting the transfer of membrane-impermeable molecules from the extracellular space to the cell interior.1–5 (b) The development of medically applied systems to electroporate cancer cell membranes under in vivo conditions in manners leading to cell death and tumor ablation6,7 or for gene therapy.8–10 A vast biomedical interest is also devoted to (c), the understanding of the cells biological mechanisms that underlie the repair of injured plasma membranes.11,12 A great interest in this subject is related to two biomedical fields of study: the first is that of membrane damage where the plasma membrane of various cell types undergoes continuous micro-ruptures due to mechanical stress during normal physiological functions, and in particular as a consequence of enhanced mechanical activity,13 as a result of photo-damage14,15 or toxin application.16 Some of these injuries are the underlying cause of devastating skeletal muscle diseases such as limb-girdle muscular dystrophy type 2B, Miyoshi myopathy17 and inflammatory myopathy in humans (and animal models) carrying mutated genes that are required for membrane repair.18 The second field is that of mechanical injury to neurons such as, for example, in axotomy (for a recent review see Bradke et al.19). Through evolution, elaborate cellular mechanisms have been developed and preserved to effectively repair the unavoidable events of membrane raptures.

This study of the molecular and cellular biological mechanisms that underlie membrane repair mechanisms and the search for drugs that enhance membrane repair includes two consecutive experimental steps: cell poration and monitoring of the repair kinetics. In most studies, membrane injuries are inflicted by scraping the cells from a substrate,20 exposing the cells to a hypo-osmotic solution,21 gently “poking” a cell with a microneedle mounted onto a micromanipulator,22 a localized laser beam23 and by axotomy.19 Focused on-chip electroporation is rarely used as most electroporating devices are designed to electroporate cells in a suspension24,25 or to electroporate large populations of cells under in vivo conditions.26 These methods mostly generate distributed electropores over the plasma membrane of individual cells.
Measurements of the membrane repair kinetics are conducted by optically monitoring the permeation of membrane-impermeable fluorescent molecules into the injured cell\textsuperscript{33,27} or by measuring the elevation and recovery of the $[\text{Ca}^{2+}]$\textsubscript{i} after electroporation.\textsuperscript{28–30} Although these approaches are extremely useful in the elucidation of molecular and cellular biological mechanisms underlying membrane repair, they suffer from a number of problems. The elevation kinetics of the intracellular concentration of a membrane impermeable fluorescent probe is related to the formation of a diffusion barrier at the pore site. However the permeability properties of the barrier are not known.\textsuperscript{11} The interpretation of the membrane repair kinetics by this method may be complicated by enhanced pinocytic activity generated at the site of injury.\textsuperscript{11} The influx and recovery of the $[\text{Ca}^{2+}]$ is a complex process that reflects the effectiveness of a large number of calcium regulatory mechanisms rather than simply reflecting the kinetics of membrane seal formation.\textsuperscript{31} Calcium removal kinetics, which is often used to monitor membrane repair, is complicated in many cell types by extended periods of calcium-induced calcium release from intracellular stores.\textsuperscript{32}

In the present study we examine the potential use of the recently developed gold mushroom-shaped microelectrode arrays\textsuperscript{33–38} to locally electroporate selected adhering cultured neurons and to obtain, in combination with intracellular recordings, kinetic information on the processes underlying membrane repair. Earlier studies from our laboratory emphasized the use of functionalized gM\textsubscript{E}-arrays to monitor extracellularly attenuated intracellular synaptic and action potentials.\textsuperscript{35,36,38} The main principles underlying the neuron-gM\textsubscript{E} interface are: the activation of endocytotic-like mechanisms in which the cultured neurons engulf the gM\textsubscript{E}Es which protrude from the surface of the device (see Fig. 1A for a schematic representation), the formation of a high seal resistance between the plasma membrane and the gM\textsubscript{E}Es and the increased conductance of the plasma membrane facing the gM\textsubscript{E}Es’ caps.\textsuperscript{34}

Here we demonstrate for the first time that a localized electroporating stimulus, applied through a micrometer-sized gM\textsubscript{E} device, generates a neuron-gM\textsubscript{E} configuration that enables transient intracellular recording of action potentials. We show that within this time window the device is effectively electrically-coupled to the inside of the cell and the attenuated recording of the neuron potential corresponds to the repair of the membrane around the interface. After recovery, the neuron-gM\textsubscript{E} configuration reverts back to recording extracellular potentials.

2. Methods

2.1 Fabrication of the gM\textsubscript{E} arrays

gM\textsubscript{E} arrays, for electrical measurements were prepared on glass wafers, as previously described.\textsuperscript{33,38} Briefly, wafers were coated with a Ti (10–15 nm)/Au (45–65 nm) layer by way of evaporation, spin-coated with photoresist S-1813 (4000 RPM) and baked for 30 min (90 °C), after which the first photolithographic process was performed followed by Au/Ti wet etching to define the multi-electrode array. Next, a second lithographic step with a thick photoresist coating was performed to open the holes for the deposition of the gM\textsubscript{E}E stalks. Gold mushrooms were grown by electroplating. Next, a layer of silicon-oxide ($\sim$3000 Å) was deposited by CVD processing. A third layer of photoresist was then applied. A third lithographic step was used to expose the contact pads and the heads of the gold mushrooms followed by wet oxide etching to selectively remove the oxide from the contact pads and the mushroom heads. Wafers were then diced and underwent manual bonding to 62-pad printed circuit boards to which 21 mm glass rings were attached to create a bath for the culturing medium.

2.2 Cell culture

Neurons from the buccal ganglia of Aplysia Californica were isolated and maintained in a culture, as previously described.\textsuperscript{39} Briefly, juvenile Aplysia (1–10 g), supplied from the University of Miami, National Resource for Aplysia, were anesthetized by injecting an isotonic MgCl\textsubscript{2} solution (380 mM) into the animal’s body cavity. The ganglia were dissected and incubated in L-15, supplemented for marine species (ms L-15), containing 1% protease (type IX, Sigma-Aldrich, Rehovot, Israel) at 34 °C for 1.5–2.5 h. Following the protease treatment, the ganglia were dehisheated. Individual neurons were manually pulled out along with their original axons with the aid of a sharp glass microelectrode and were plated on poly-L-lysine functionalized devices. For the present study, the neurons plated on the gM\textsubscript{E} devices were cultured for 48 h–96 h at 24 °C and then used for the electrophysiological experiments. For the current study the gM\textsubscript{E} devices were used once.

2.3 Electrophysiology and electroporation

Conventional intracellular recording and stimulation of the cultured Aplysia neurons were used, as previously described.\textsuperscript{34} The microelectrodes were pulled from 1.5/1.02 mm borosilicate glass tubes with filaments and filled with 2 M KCl. Electrode resistance ranged between 4 and 10 MΩ. For the intracellular recording and stimulation the microelectrode tip was inserted into the cell body.

Voltage recordings and electroporation pulses were made using the 62 poly-L-lysine functionalized gM\textsubscript{E}Es, using a Multi Channel Systems (Reutlingen, Germany) AC amplifier (MEA-1060-Inv-BC), with frequency limits of 1–10 000 Hz and a gain of 110–1100. The data shown is of the raw, unprocessed recordings.

2.4 Calcium imaging and image analysis

The calcium indicator Rhod-2 potassium salt (Molecular Probes) was used for intracellular calcium imaging, as previously described by our group.\textsuperscript{40} To that end the cell body was impaled with a 10–15 MΩ microelectrode filled with 2 M KCl and 10 mM Rhod-2 potassium salt. The indicator was loaded into the neurons by pressure injection. The neurons were loaded until a vague indicator signal (a few gray levels) was detectable in the axon. Imaging was performed after the dye had equilibrated throughout the soma (approximately 30 min). The dye was imaged with a 543 nm laser line and collected with a 590/70 band-pass filter using an Olympus microscope IX70 and a Bio-Rad Radiance 2000/AGR-3 confocal imaging system. The objective used was an Olympus plan-Apo 60 × 1.4 NA oil. The images were collected using LaserSharp. The images were
analyzed offline using NIH ImageJ software (Bethesda, MD), and MATLAB (MathWorks, Natick, Massachusetts).

2.5 Computer simulation and offline deconvolution

Computer simulations were done using PSPICE (OrCAD). For the simulations, the parameters used were: (1) the non-junctional membrane resistance ($R_{nj}$) was measured in a large number of neurons with an average value of 25 MΩ. (2) The junctional membrane resistance ($R_{j}$), estimating the $g_{M_{ej}}$ area to be $\sim 14 \mu m^2$ and multiplying the total input resistance ($R_{in}$) with the ratio between the surface area of the neuron and the surface area of the gM$_{µE}$, was estimated to be $\sim 100$ GΩ. (3) The non-junctional membrane capacitance ($C_{nj}$) equals the total
membrane capacitance ($C_m$) and was set to be 600 pF, as measured previously in our laboratory.\(^3\) (4) The estimated junctional membrane capacitance ($C_j$) is calculated as the total membrane capacitance ($C_m$) divided by the ratio between the surface area of the cell and the surface area of the gMjEs and was taken as 0.1 pF. (5) The gMjE resistance ($R_{gMjE}$) in solution was estimated to be 1000 GΩ in accordance with the measured resistance of gold electrodes in physiological solution\(^1\) normalized by the gMjE surface area. (6) The capacitance of the gMjE in solution is estimated by taking the specific capacitance of the gold electrical double layer to be $\sim$50 µF cm\(^{-2}\) and multiplying by the surface area of the gMjE. It ranges between 0.5 and 25 pF. (7) The amplifier input capacitance is 8 pF (Multi Channel Systems, Rutlingen, Germany).

3. Results and discussion

3.1 Localized electroporation of cultured neurons

*Aplysia* neurons were cultured on an array of gold mushroom-shaped microelectrodes (gMjEs) and allowed to adhere to the poly-L-lysine-coated device for 24 h. A glass micropipette, that served for both intracellular current injection (Fig. 1B) and voltage recording (Fig. 1, C1–C5), was inserted into the cell body of a neuron residing on top of a number of gMjEs, as described previously\(^{3,5,36}\) (see also the experimental setup in Fig. 1A). For the experiment, a 100 ms long depolarization current pulse was delivered to the neuron to generate a single action potential (Fig. 1A and B), followed by a 300 ms long hyperpolarizing pulse to measure the cell’s input resistance (Fig. 1B and C). Delivery of an alternating voltage pulse (an electroporating pulse ranging between 500–1000 mV, 100 Hz, 300 ms Fig. 1, C2) through a gMjE residing beneath the cell body resulted in electroporation of the neuron, as indicated by its membrane depolarization, the generation of a train of action potentials and the concomitant reduction of the neuron’s input resistance (Fig. 1, B2). Thereafter, the resting potential, input resistance, spike shape and amplitude gradually recovered indicating that the injured membrane underwent a repair process (Fig. 1, C4, C5).

3.2 Mapping the spatial distribution of the electropores

Plasma membrane electroporation by an alternating current pulse delivered through a gMjE residing beneath the neuron is mainly localized to the membrane patch facing the electroporating-gMjE. This conclusion is based on two types of experiments: mapping the amplitudes of the action potentials measured by a number of gMjEs residing under the cell body and the imaging of the $[\text{Ca}^{2+}]$, during the electroporation. Mapping the field potentials (FPs), generated by an intracellularly evoked action potential, by neighbouring gMjEs spaced at a distance of 20 µm from each other revealed that the larger increase in the FPs amplitude is detected by the electroporating gMjE. In the experiment shown in Fig. 2 for example, a short depolarization pulse, applied through the intracellular glass microelectrode generated a 60 mV, intracellularly recorded action potential (Fig. 2B, leftmost black trace). This potential was recorded as 200–300 µV extracellular field potentials by 5 gMjEs residing underneath the stimulated neuron (Fig. 2B, gMjEs a–e) but not by other gMjEs which reside outside of the stimulated neuron.

![Image](https://example.com/image.png)
perimeters (Fig. 2B, gMµEs f–h). Delivery of an electroporating pulse by gMµE-a increased the potential amplitude recorded by it from 200 µV to ~6 mV (t = 5 s) while the nearby gMµEs (b–c) displayed a decreased amplitude. gMµE-e displayed a minor increase in the field potential amplitude. Concurrently the amplitude of the action potential recorded intracellularly decreased.

With time, the amplitude recorded by the intracellular electrode increased and partially recovered while the amplitudes of the extracellularly recorded potentials by electrode (a–e) diminished (Fig. 2B). These observations are consistent with the view that the electroporating pulse ruptured the plasma membrane adjacent to the electroporating electrode.

The local nature of membrane rupture following electroporation is also shown by the transient elevation of the [Ca²⁺]o, as imaged by the calcium indicator Rhod-2 (Fig. 3). Following the delivery of an electroporating pulse applied through a gMµE (Fig. 3A, arrow), the [Ca²⁺]o was initially locally elevated around the stimulating gMµE (Fig. 3B and 3C, t = 1 s). Thereafter, the [Ca²⁺] concentration increased further around the electrode and was followed by the recovery to the control level52 (Fig. 3B and 3C, t > 22 s).

3.3 Characterization of the action potential picked-up by the electroporating gMµEs

As shown in the experiment depicted in Fig. 2, after electroporation the electroporating gMµE records APs. Although the amplitude of the APs recorded by the gMµE is largely attenuated, their shape is very similar to the intracellularly recorded action potential. The large attenuation is the consequence of the lower seal resistance generated by neurons cultured on poly-L-lysine coated gMµE devices33,34 compared with neurons grown on an engulfment promoting peptide,35,36 as well as the capacitance of the gMµE, which acts as a low-pass filter.35 The application of electroporating pulses by gMµEs functionalized with an engulfment promoting peptide did not change the recorded APs (not shown), most likely due to the Ohmic conductance already present at the interface36 which practically distributes the electroporative current over the large nonfunctional membrane surface area.

3.4 The dynamics of membrane repair after electroporation

To gain insight into the dynamics of the membrane repair process after electroporation, we next monitored the relationships between the resting potential and cell input resistance during electroporation and the ensuing membrane repair process. The input resistance was estimated by applying constant hyperpolarizing current square pulses (as shown in Fig. 1 B, C). Fig. 4 depicts six experiments in which a neuron was electroporated. The recovery time of the resting potentials (Fig. 4, A–F, upper blue traces) and input resistance (Fig. 4, A–F, lower red traces) ranges between tens of seconds (Fig. 4A) to a number of minutes (Fig. 4, E and F). The recovery kinetics varied substantially between individual experiments. In some, the recovery was characterized by a rapid phase that smoothly subsides to a slow one (Fig. 4A). In others, the neurons show a smooth exponential recovery of the input resistance and the transmembrane potential which was interrupted by a sudden recovery step (Fig. 4, arrows). Some neurons show a full recovery (Fig. 4, A and D) while others show a partial recovery (Fig. 4, B and E). A recovery of the input resistance to a value higher than before electroporation was also observed (Fig. 4F) and may be explained by the theory suggested by Idone et al., by the removal of leaky membrane patches through endocytosis.11 In all the experiments the input resistance and resting potential made a recovery of 78.8% and 83% from 25.3 ± 10.7 MΩ and 56.3 ± 5.5 mV to a steady state level of 19.95 ± 7.9 MΩ and 46.7 ± 10.6 mV of their original values within 0.5–10 min after membrane-electroporation (n = 7).

3.5 Analog electrical circuit simulation of the electroporation and recovery process

Our observations clearly indicate that gMµE induced electroporation is generated locally within the plasma membrane facing the electrode (Fig. 2 and 3). Two modes of membrane rupture could account for the local decrease in the plasma membrane resistance. The first is the generation of distributed hydrophilic nanopores, as described previously,33,44–46 and the other is the formation of a single pore generated by the combined forces of the electroporating pulse and the localized mechanical tension generated at the site of gMµE-membrane interface. This latter

Fig. 3  Localized transient elevation of the free intracellular calcium concentration following the delivery of an electroporating pulse through a gMµE. (A) A cell body loaded with the calcium indicator Rhod-2 (red channel) and gold electrodes (green channel), superimposed on a transmitted light image (grey) depicting cell features. The yellow arrow marks the gMµE through which an electroporating pulse was delivered. (B) The [Ca²⁺]o is initially locally elevated around the stimulating gMµE electrode, as indicated by the elevation in the Rhod-2 fluorescence at t = 1 s. Calcium elevation throughout the cell cytosol follows (t > 22 s).
mode of membrane poration mimics the established method of sharp glass microelectrode insertion into cells routinely used by generations of electrophysiologists. The current study cannot differentiate between these mechanisms. Nevertheless, the conductance generated by an electroporating pulse can be treated as a single equivalent pore with a diameter of 900 nm (see the calculations based on the input resistance below). Similar values are obtained, assuming that this pore represents a set of hydrophilic nanopores with diameters of 1–10 nm and spaced at an average inter-pore interval of ∼60 nm.45,46 The membrane patch facing the ∼14 µm² gMµE may harbor between 2700 and 3700 nanopores that are estimated to generate a conductance equivalent to a single pore with a diameter of up to 500 nm.

The abrupt events of increased input resistance and transmembrane potential recovery occurring within minutes after electroporation are consistent with the proposed membrane ‘patch mechanisms’ in which Ca²⁺ influx through the injury site triggers the fusion of pre-existing intracellular vesicles within the plasma membrane and among themselves in the vicinity of the membrane pores. These vesicle assemblies then fuse with the plasma membrane, ‘patching’ the pore.47 The cases in which we observed a smooth exponential recovery are more consistent with a second model assuming that calcium-induced vesicle exocytosis reduces the plasma membrane tension and thereby enables pore repair by constriction and bilayer resealing.48 It is conceivable that the partial recovery (48 ± 0.6%) of the resting potential and input resistance that unfolds within a number of minutes after pore formation reflects the ionic conductance of the recruited membranes forming the diffusion barrier patch. It is also conceivable that the diffusion barrier is initially formed by calcium-dependent protein cross-linking49 and is only later backed up by additional membrane repair processes.11

Interestingly the recovery of the input resistance and resting potential of axotomised Aplysia neurons follows similar kinetics to that described in the present study.19,50–53

To quantitatively estimate the dimensions of an equivalent membrane pore generated by the electroporating pulse and the parameters which permit the recording of attenuated intracellular action potentials, we used an analog electrical circuit which depicts the structural and physical relations between a single cultured Aplysia neuron and two gMµEs (Fig. 5). The simulation includes the generation of intracellular action potentials by current injection into the model neuron (Fig. 6A, upper panel) and the delivery of intracellular hyperpolarizing pulses by current injection through the intracellular microelectrode (Fig. 6B, upper panel). Simulated voltage recordings are made by an intracellular electrode and by two gMµEs. One gMµE is used for both the electroporation and voltage recording.

Fig. 4 Membrane repair dynamics as revealed by measurements of the trans-membrane potential and the cell input resistance following electroporation. Six different examples of the recovery of the cell resting potential (A–F, upper blue panels) and input resistances (A–F, bottom red panels) reveal varying time courses and natures of recovery. Out of the six examples only two cells fully recovered the resting potential and input resistance (A, D) within 35–400 s. All other neurons reached a steady state input resistance and membrane potential of 78.8% and 83% of their original values after 250–400 s. Note that the recovery process combines monotonic and abrupt steps consistent with the membrane patch repair models. The incomplete recovery may suggest that the repair patch consists of a leaky membrane.
Before electroporation, now records an action potential of 6 mV. Assuming that the extracellular resistance between the two gMµEs (R_l) from an estimated value of 100 GΩ^{35,36} to 10 MΩ. The junctional membrane resistance above gMµE1 (the junctional membrane resistance R_{j1}) was not altered. This led to a reduction of the neuronal input resistance which can be calculated using:

\[ R_{\text{in}} = \frac{R_{es} \times R_{j1}}{R_{es} + R_{j1}} \approx 7 \text{MΩ} \]  \hspace{1cm} (1)

The calculated neuronal input resistance is therefore reduced from 25 MΩ prior to electroporation to ~7 MΩ after electroporation. The hole size that would correspond to such a change can be calculated according to:

\[ R_{\text{hole}} = \sqrt{\frac{\rho_{\text{solution}} \times l_{\text{membrane}}}{\pi \times R_{j1}}} \]  \hspace{1cm} (2)

Assuming that the conductance of the physiological solution (\(\rho_{\text{solution}}\)) is 100 Ω cm and the thickness of the plasma membrane (\(l_{\text{membrane}}\)) is 30 nm we estimate that the diameter of the hole (2\(R_{\text{hole}}\)) formed in the plasma membrane should be ~900 nm. Using a series of values for \(R_{j1}\), representing the junctional membrane resistance immediately after electroporation and through membrane recovery, we simulated the change in voltage picked up by the glass micropipette (Fig. 7A) and gMµE1 (Fig. 7B).

To reduce the input resistance from 25 MΩ to ~8 MΩ of a cell with radius of 50 μm², we calculated that the junctional membrane resistance has to be 10 MΩ (Fig. 7C) which corresponds to a hole of ~900 nm. Under such conditions the action potential amplitude recorded with an intracellular electrode is reduced from ~60 mV to ~35 mV (Fig. 6A). While the electroporating gMµE, which did not record any signal before electroporation, now records an action potential of ~6 mV. Assuming that the extracellular resistance between the two gMµEs is 2.2 MΩ^{54} and the seal resistance (R_{seal}) between the gMµE and cell membrane is 100 MΩ^{33} the nearby gMµE (gMµE2) also peaked at an attenuated action potential of ~1 mV.

4. Conclusion and future work

The study of membrane repair mechanisms and the identification of pharmacological means to facilitate it is of clinical significance due to a number of hereditary diseases related to the failure of membrane seal formation. An on-chip gMµE provides an advantageous tool to study membrane repair, as it provides a unique configuration whereby the electroporating electrode can be used to monitor the dynamics of membrane repair. The transfer of this method to vertebrate skeletal and cardiac muscles is possible as we have already shown that vertebrate cells (including a line of cardiomyocytes) and primary neurons engulf the electrodes. In addition, the electroporation protocol can be further developed to enable electroporation and a gigaohm seal formation with the plasma membrane of cultured neurons and muscles.

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Fig. 7 The effect of junctional membrane resistance on the voltage recorded by a glass micropipette electrode (A), gMmE (B), neuron input resistance (C) and overall coupling between the gMmE device and cell membrane potential (D). Empirically observed or estimated parameter ranges immediately after electroporation and throughout the membrane repair process are marked in grey.

References


