INVESTIGATION OF THE PREDICTABILITY OF NEURAL CELL SURVIVAL AFTER EXPOSURE TO NON-UNIFORM ELECTRIC FIELDS

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Abstract – **Cortical rat neurons were trapped by negative dielectrophoresis (DEP) using a planar quadrupole electrode structure. The non-uniform field created by this structure was calculated using a finite element software package. By representing the neuron by a single-shell model the membrane potential induced by the electric field can be estimated. It was investigated whether the physiological state of the trapped neurons can be predicted using this estimation. Experimentally, the physiological state of cortical cells trapped at different amplitudes and frequencies of the input signal was determined using a staining method.**

The conclusion is that a rough estimate of the minimum frequency and maximum amplitude can be given in order to predict the status of viability of the cells after being dielectrophoretically trapped.

Key words **– Cortical rat neuron, trapping, Dielectrophoresis, planar micro-electrode plate, membrane breakdown, viability staining**

I. INTRODUCTION

Non-uniform electric fields exert dielectrophoretic forces on polarizable particles [1]. These forces can be used to trap neurons on particular spots, e.g., an electrode tip of a planar multi-electrode array that can be used for stimulation and recording of neuronal activity. A planar quadrupole micro-electrode structure was used for the creation of a non-uniform electric field in order to trap cortical rat neurons in the center of the structure.

Exposing biological cells to large (non-uniform) electric fields, however, causes the membrane potential to rise rapidly above the normal value of about -70 mV. This induced membrane potential is non-evenly distributed over the membrane, i.e., it is the highest at the membrane sites closest to the electrodes and the lowest at membrane sites most remote from the electrodes. A change in membrane structure conformation, and the formation of electropores are the results of the increased membrane potential. It was found that the creation of electropores started at membrane potentials of 0.5-1.0 V for several cell types, except neural cells [2].

For an alternating (sinusoidal) electric field, the generated membrane potential is given by the following equation

$$V_g = \frac{1.5 E_{ext} r_{cell} \cos\alpha}{\sqrt{1+(2\pi f \tau)^2}} \qquad (1)$$

with V_g the generated membrane potential, f and E_{ext} the frequency and electric field strength of the applied external electric field, respectively, α is the angle between the field direction and membrane orientation, r_{cell} is the cell radius, and τ is the relaxation time of the membrane $\tau = r_{cell} C_m (\rho_{int} + 0.5\rho_{ext})$ where C_m is the specific membrane capacitance, ρ_{int} and ρ_{ext} are the specific resistivities of cell interior and exterior, respectively. It is hereby assumed, however, that the cell can be represented by a single-shell model (it is a homogeneous conducting sphere surrounded by a thin membrane, and has a smooth surface), the field is homogeneous, and that the surface admittance and space charge effects do not play a role.

Electropores increase the conductivity of the membrane to counteract the induced membrane potential [3] so as to protect itself from destructive processes. Thus, as soon as pores are created (1) is no longer valid. The area of electroporation is less than 0.1% of the total surface area and the time sequence of the creation of electropores is in the submicrosecond range [4]. Via the created pores, relatively nonspecific molecular exchange between the intra- and extracellular volumes occurs, and a chemical imbalance may result.

Although the mechanism of electroporation is still unclear, it is known that a membrane may recover (reversible electroporation) or break down (membrane rupture or irreversible electroporation) depending on the magnitude and frequency of the electric field. Membrane recovery is, however, not a prerequisite for cell recovery since the chemical imbalance and associated stress may sustain after membrane recovery, which may eventually lead to cell death. Therefore, membrane-based short term tests, like vital stains or membrane exclusion probes, may not necessarily be valid.

Previous experiments have shown that dielectrophoretic trapping of cortical cells at 3 V_{tt}/14 MHz, using a planar quadrupole electrode structure as shown in Fig. 1, did not result in morphological changes of the cells [5]. Morphological aspects included the area taken up by a cell representing cell adhesion, and the number and length of the processes. Further, the number of non-outgrowing and outgrowing cells was determined. During 30 minutes the cells were trapped and up to 5 days after field exposure the cells were monitored at certain time steps. Survival of these cells could be expected, since the induced membrane potential was small due to the relatively high frequency and low amplitude according to (1). The question is, however, whether the viability of the cells after field exposure can be predicted by the theoretical estimation of the induced membrane potential during field exposure.

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Abstract

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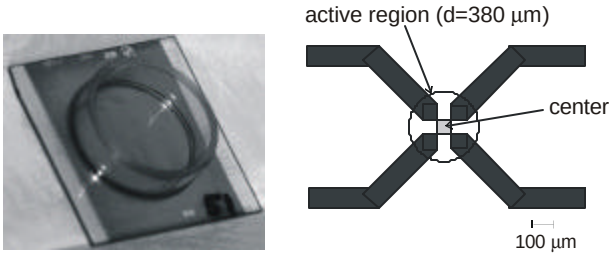
Dielectrophoretically trapped cortical rat neurons were stained with Propidium Iodide and Acridine Orange to investigate viability after 1 day in vitro (DIV). Theoretical estimation of the maximum induced membrane potential under the used field conditions was related to the staining results to investigate the predictability of the state of the cells after exposure to non-uniform electric fields.

II. Materials & Methods

Photolithographic, plasma deposition and etching techniques were used to create a quadrupole micro-electrode structure on a glass plate of 5 x 5 cm. An insulation layer, consisting of a silicon-nitride layer sandwiched between two silicon-oxide layers, was applied to avoid electrochemical processes at the electrode-culture medium interface and to reduce unnecessary heating of the medium. The 'active regions', the regions around and in between the electrode tips were kept free of this insulation layer. The tips were triangularly shaped with angles of 90° and the inter-electrode distance between two diagonally opposing tips was 100 μm. Fig. 1 shows the electrode plate and a layout of the quadrupole electrode structure.



A **B**

Fig. 1. **A** The electrode plate with a glass ring (3 cm diameter) mounted on top, serving as a culture chamber for long term cultures. Small pins are used to electrically connect the electrode structures located inside the culture chamber via the electrode strips at the two sides of the plate, with a signal generator. **B** The quadrupole electrode structure with a diagonal inter-electrode distance of 100 μm. Indicated are the center, and the active region, which is the region kept free of insulation. Six of these electrode structures are located on one electrode plate.

Before the experiment was started the electrode plate was coated with PEI (Poly-Ethylene-Imine 30 ngram/ml, Sigma-Aldrich Chemie Gmbh, Germany) to enhance cell adhesion and prevent cell clustering [6]. Within 30 minutes after plating the cells have already adhered rather well to the substrate using this coating.

Cells obtained exclusively from the telencephalic cortex of newborn rats (P2) were used for the experiments in this study. The brains were taken out after decapitation, and the cortices of the forebrain were carefully collected in a Petri dish and cut into small pieces. After collecting the tissue parts in a tube they were chemically dissociated with the use of trypsine trypsine/EDTA (0.25% trypsine, 1 mM EDTA-4Na, Gibco BRL, USA) for 45 minutes while stored in an incubator (37°C, 5% CO_2) after which the trypsine was removed and 250 μl trypsine inhibitor/R12 (a concentration of 1 mg trypsine inhibitor (Gibco BRL, USA) per 1 ml R12 medium [7]) and 50 μl DNase/R12 (a concentration of 1 μl DNAse (Deoxyribonuclease I, 151 U/μl, Gibco BRL, USA) per 250 μl R12 medium) were added. After precipitating undissociated parts the upper layer of the suspension was transferred into a new tube and centrifuged at 1200 rpm for 5 minutes. The medium above the pellet of cells was replaced by fresh R12 medium and a final suspension of $750*10^3$ cells/ml was prepared. From this suspension a drop of 100 μl was used to cover each of the electrode structures. Reference experiments were performed by placing a drop of cell suspension on the same electrode plate, but far away from the electrodes.

A sinusoidal input signal of 3 V_{tt} and 5 V_{tt} at three different frequencies (10 and 100 kHz, 1 MHz) was used for the creation of the non-uniform electric field. An extra experiment at 3 V_{tt} 14 MHz was performed in order to determine the compatibility with previous results. The field was applied for 30 minutes. After this time period the neurons were placed in an incubator (37 °C, 5% CO_2). Three hours after the experiment 2 ml of R12 medium was supplied to the culture chamber. For each field setting six experiments were performed and the results of these experiments were averaged.

After 1 day-in-vitro (DIV) cell viability or its absence was indicated by Acridine Orange and Propidium Iodide staining (10 ml PI (40 μg/ml) + 14 PBS + 24 μl AO (5 μg/ml)). Viable cells were colored green by AO, dead cells were colored red by PI. Digital images were taken using fluorescence microscopy (Nikon Inverted Microscope, Diaphot-TMD) and a digital camera (Zeiss Axiocam). After enhancing the contrast of the image the red and green area within the center of the electrode structure were determined. A same-sized area was taken for the reference situation.

Using the finite element method (finite element software package ANSYS) the electric field created by the electrode structure was determined for both amplitudes. The maximum field strength was used to calculate the (maximum) induced membrane potential according to (1).

III. Results

Theoretical results

Fig. 2 shows the field profile at z=0 μm (at the bottom of the culture chamber) as calculated with the finite element method. The maximum electric field was 43.9 kV/m and 73.2 kV/m for an input signal of 3 V_{tt} and 5 V_{tt}, respectively.

The following parameter values were assumed (or measured) for the neuron: radius r=5 μm, interior conductivity (cytoplasm) σ_{int}=0.75 S/m, interior permittivity $\varepsilon_{int}=80*\varepsilon_0$ (with ε_0 the permittivity of free space), membrane capacitance c_m=1.8 μF/cm^2, and the conductivity of the medium in which the neurons were suspended σ_{med}=1.6 S/m.

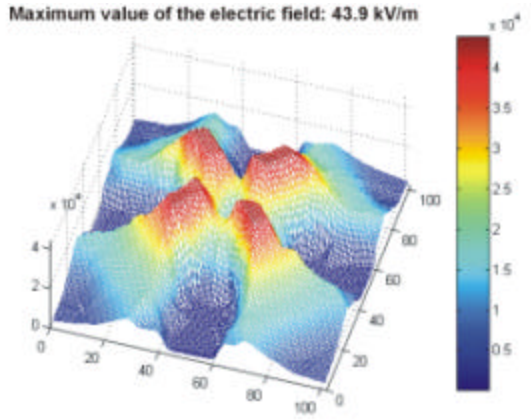


Fig. 2. The electric field in the horizontal plane (-200 μm<x,y<200 μm) at z=0 μm for an input signal of 3 V_{tt}.

Fig. 3 shows the calculated maximum induced membrane potentials resulting from the maximum electric field strengths for the two signals used, as a function of frequency.

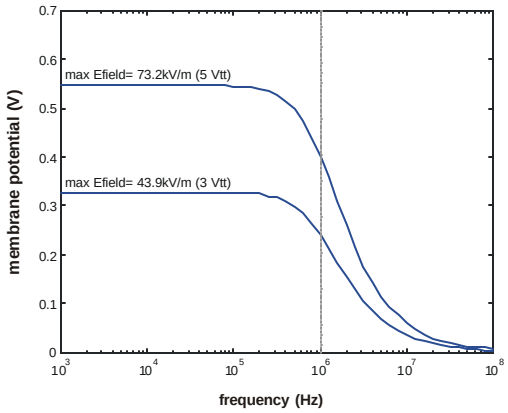
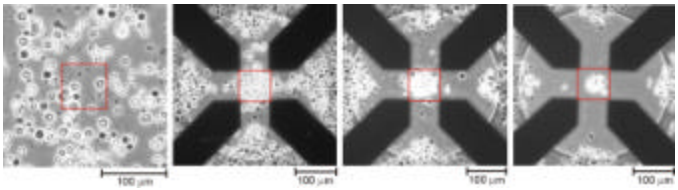


Fig. 3. Maximum induced membrane potential according to (1) for the two input signals used.

If instead, a membrane capacitance of 0.9 μF/cm^2 is assumed [8], the relaxation time τ decreases and the 'low pass frequency' increases (from 1 to 2 MHz). For cells with a larger diameter the membrane potential increases, e.g., for a diameter of 6 μm the maximum membrane potential becomes 0.40 V and 0.66 V for 3 and 5 V_{tt} field application, respectively.

Experimental results

Fig. 4 shows the cortical cells trapped in the center of the electrode structure for each frequency used at 5 V_{tt} after 30 minutes of field application. The number of cells trapped in the center of the electrode structure is frequency and amplitude dependent [9]. At 5 V_{tt} the center was almost completely covered with cells for 10 and 100 kHz. At 1 MHz the group of cells was more compact and covered about 60% of the center area. At 3 V_{tt} the center was covered for 90% at 100 kHz, 1 and 14 MHz (comparable to Fig. 4B). At 10 kHz about 60% of the area was covered in a rather random way. In the reference situation about 25% of the square area was covered.

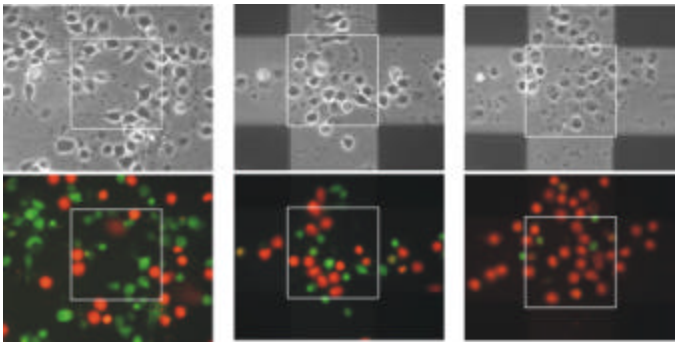


A **B** **C** **D**

Fig. 4. **A** Reference cells (not exposed to the electric field) Cells trapped at 5 V_{tt} and a frequency of **B** 10 kHz, **C** 100 kHz, and **D** 1 MHz at t=30 minutes.

Fig. 5 shows the situation after 1 day-in-vitro (1DIV) for three situations. The lower images show the staining results. The bars of the graphs in Fig. 6 and 7 represent the absolute area of green and red within the square area for 3 V_{tt} and 5 V_{tt}, respectively.

In the reference situation almost half of the cells was dead. After the dissociation procedure a part of the cells had died already and therefore cultures with some dead cells were inevitable.



A **B** **C**

Fig. 5. **A** Reference cells at 1 DIV **B** Trapped cells at 1 DIV (input signal of 3 V_{tt}/100 kHz) **C** Trapped cells at 1 DIV (input signal of 5 V_{tt}/100 kHz) The lower images show the staining results: green is 'viable', red is 'dead'.

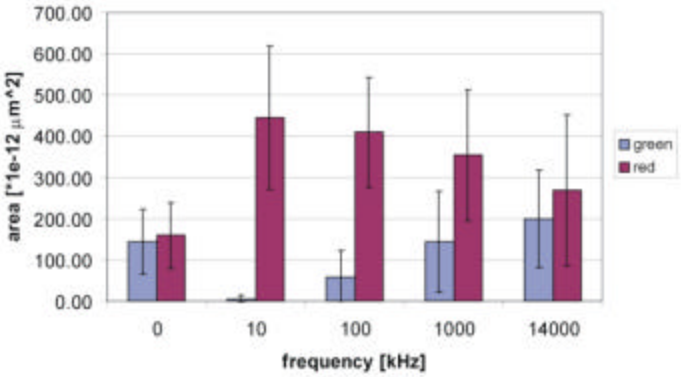


Fig. 6. Area of green (left bar) and red (right bar) for an input signal of 3 V_{tt} for four different frequencies after 1 DIV. At 0 Hz no field was applied (reference situation).

Even though at first sight no cells collapsed while the field was applied, electropores were created and although membrane recovery might have taken place, the cells were not able to recover completely in most cases. This is suggested by the large red area together with a small or no green area for almost all cases, except for 3 V_{tt} at 1 and 14 MHz and 5 V_{tt} at 14 MHz. In Fig. 6 most clearly a decrease in red area and an increase in green area is seen with increasing frequency.

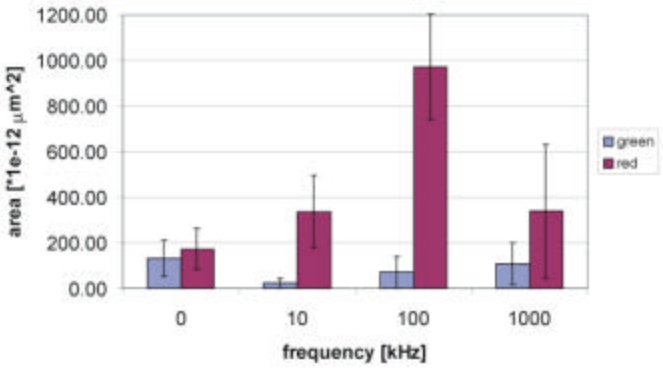


Fig. 7. Area of green (left bar) and red (right bar) for an input signal of 5 V_{tt} for three different frequencies after 1 DIV. At 0 Hz no field was applied (reference situation).

IV. DISCUSSION

Assuming that the critical range of 500 mV to 1 V for the induced membrane potential is also applicable for neural cells, then we would expect that for an input signal of 3 V_{tt} no damage would occur. For 5 V_{tt} this potential is above 500 mV for frequencies up to 500 kHz and thus cell death was expected here.

However, for 3 V_{tt} up to 1 MHz red rules over green in Fig. 6, suggesting that a membrane potential of 300 mV should be avoided at frequencies below 1 MHz. A 'low pass frequency' of 1 MHz is in accordance with the calculated relaxation time. Taking 300 mV as the maximum membrane potential for a neuron to survive it would be expected that at 5 V_{tt}/1 MHz no green area would be present. According to the estimation the induced membrane potential is 400 mV at that point. But, although the standard deviation is rather large, a green area cannot be ignored (see Fig. 7). This means that for higher frequencies a larger membrane potential can be tolerated by the cortical cells compared to low frequencies. Thus with decreasing frequency the tolerance level also decreases. By investigating Fig. 6 and 7 this decreasing tolerance was also found comparing the 10 and 100 kHz results:

- According to Fig. 6 a green area was present at 100 kHz, while at 10 kHz almost no green area was present.
- According to Fig. 7 the total area was significantly larger at 100 kHz than at 10 kHz (5 V_{tt}), while the initial area (at t=30 minutes) covered with cells did not differ significantly. It seems that adhesive properties of the membrane were even influenced by the frequency of the field adding to the destruction of the cell with the result that almost no cells were left inside the center of the electrode structure after 1 day.

Thus, the frequency or 'pulse width' of the signal seems to have an effect on the viability of the trapped cells. Physiologically this probably means that pore density or pore diameter is influenced by the pulse width, i.e. the longer the period of the signal the more non-recoverable damage is done by large pore diameters or a large number of pores [3].

In summary, it can be stated that cell viability is not directly coupled to the estimation of the induced membrane potential according to (1). Even if the induced membrane potential is estimated to be below the critical level of 500 mV this does not directly guarantee cell survival. It was found that this critical level is mainly determined by the frequency of the applied field, i.e., with decreasing frequency the critical level decreases.

Cell survival, however, can most probably be guaranteed when the frequency is set above the 'low pass frequency' and the field induces a membrane potential below about 400 mV for cortical cells.

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