OM&P

CULTIVATION OF PRIMARY HIPPOCAMPAL CELL CULTURES FOR THE FUNCTIONAL AND MORPHOLOGICAL MATCHING ORGANIZATION OF SINGLE NEURONS

O.M. Shirokova [1], *R.A. Sokolov* [2], *V.I. Pershin*[1,2], *I.V. Mukhina* [1,2]

[1] Privolzhsky Research Medical University of the Ministry of Health of the Russian Federation, 10/1, Minin and Pozharsky Sq., Nizhny Novgorod 603950, Russia,
[2] Lobachevsky State University of Nizhny Novgorod 23 Prospekt Gagarina (Gagarin Ave.), Nizhny Novgorod, 603950, Russia

Abstract. Correlation microscopy is an invaluable tool for studying the functional and structural features of individual cells. Here we present an approach for the cultivation of primary hippocampal cultures grown on a special substrate that allows drawing a convenient coordinate grid. The metabolic activity and immunocytochemical labeling of neurons were estimated, as well as the patch-clamp method and ultrastructural analysis.

Introduction

Correlation microscopy methods take a leading position in cell biology and are of great practical importance However these microscopic systems too expensive as commercial glass and Petri dishes with a grid. At the same time, functional imaging is considered an essential research tool for biologists. The ability to combine studies on a single cell with the effect of any substances, the search for certain types of neurons or glial cells is only on living objects. Nonetheless to study the cause of certain reactions complex approach is needed. If you visualize the receptors on these cells or check the condition of the organelles, it will become possible to find out the cause of a particular reaction. With the help of an appropriate coordinate grid, it's possible to perform even single-cell genetic studies. Electrophysiological studies revealing membrane depolarization can be substituted with calcium-sensitive indicators. Calcium indicators or genetically encoded calcium indicators are widely used for studying changes of intracellular cytoplasmic or organelle's calcium. The concentration of calcium indirectly reflects the depolarization of the membrane and is a measure of neuronal cell activity. It is also possible to investigate the functional features of glial cells. Nowadays, fluorescence immunolabeling of certain proteins is a common technique and it's especially useful for quantifying receptors or performing measurements subcellular structures in neuron-glia networks, e.g. – synapses and mitochondria.

However, some structural features can be detected only with ultrastructural analysis, e.g. membrane integrity, a simultaneous study of various organelle types, intracellular contacts or classification of the types of synaptic contacts. That's why it was decided to develop the method of cultivating cells for matching patch-clamp method, calcium imaging, immunocytochemistry and ultrastructural analysis of primary hippocampal cell cultures. Cells previously used to be cultivated on the thermoplastic films for electronic microscopy (Agar Scientific, AGL4458), however, such a method wasn't used for the cultivation of neuronal cell cultures. The application of this film as the substrate for cultivation allows an easy-manner investigation of individual cells. Also, usage of such a substrate is convenient for electron microscopy because it is easily separated from the polymerized resin, leaving a pattern on the resin and there is no risk of damaging the diamond knife with glass residues.

Methods

Preparation of thermoplastic film

All experiments were performed using primary hippocampal cell culture prepared from E18 C57bl/6 pregnant mice embryos at an initial density of 1500 cells/mm^2.

The heat-resistant film was cut on the hexagons of the required size. Further, a coordinate

grid of Roman numerals was drawn with a scalpel on the film, four numbers in a row, four rows. Next, films were wiped and placed into an ultrasound bath with EtOH for 2 hours. After that, they were dried and sterilized at a 140oC within 2 hours. Then, the dissociated cells were placed on coated with an anchoring agent (polyethyleneimine, Sigma, USA) and cultured according to the standard protocol [1] for 30 days.

Patch-clamp method.

Electrophysiological parameters of individual neurons in the networks of primary cell cultures of mouse hippocampus were determined with the patch-clamp technique in the whole-cell configuration. For this purpose, the following equipment was used: EPC 10 USB double patch-clamp amplifiers (HEKA Electronik, Germany) the SliceScope Pro 2000 device (Scientifica, UK), P-97 Flaming/Brown type micropipette puller (Sutter Instrument Co, USA), Masterflex L/S peristaltic pump (Cole-Parmer Instrument, Malaysia), AxioCam ICm 1 photo-video camera (Carl Zeiss, Germany), borosilicate glass capillaries GC120F-7.5 (Harvard apparatus, UK), the software for recording and processing the electrophysiological data – PatchMaster (HEKA Electronik, Germany), MiniAnalysis (Synaptosoft Inc., USA).

To measure neuronal activity, there was used an extracellular solution containing (mmol) 130 NaCl; 2.5 KCl; 1.5 MgCl2; 1.5 CaCl2; 10 glucose; 10 HEPES at 24°C, osmolarity – 300±5 mOsm; pH 7.3–7.4; and intracellular solution (mmol): 130 K-gluconate; 10 HEPES; 2 EGTA; 3 L-ascorbic acid; 2 MgCl2; 1 Na2-GTP; 2 Na2-ATP (pH 7.2; osmolarity – 295±3 mOsm). A similar intracellular solution with QX-314 bromide at 2 mM concentration was used to block sodium currents when recording spontaneous excitatory postsynaptic currents.

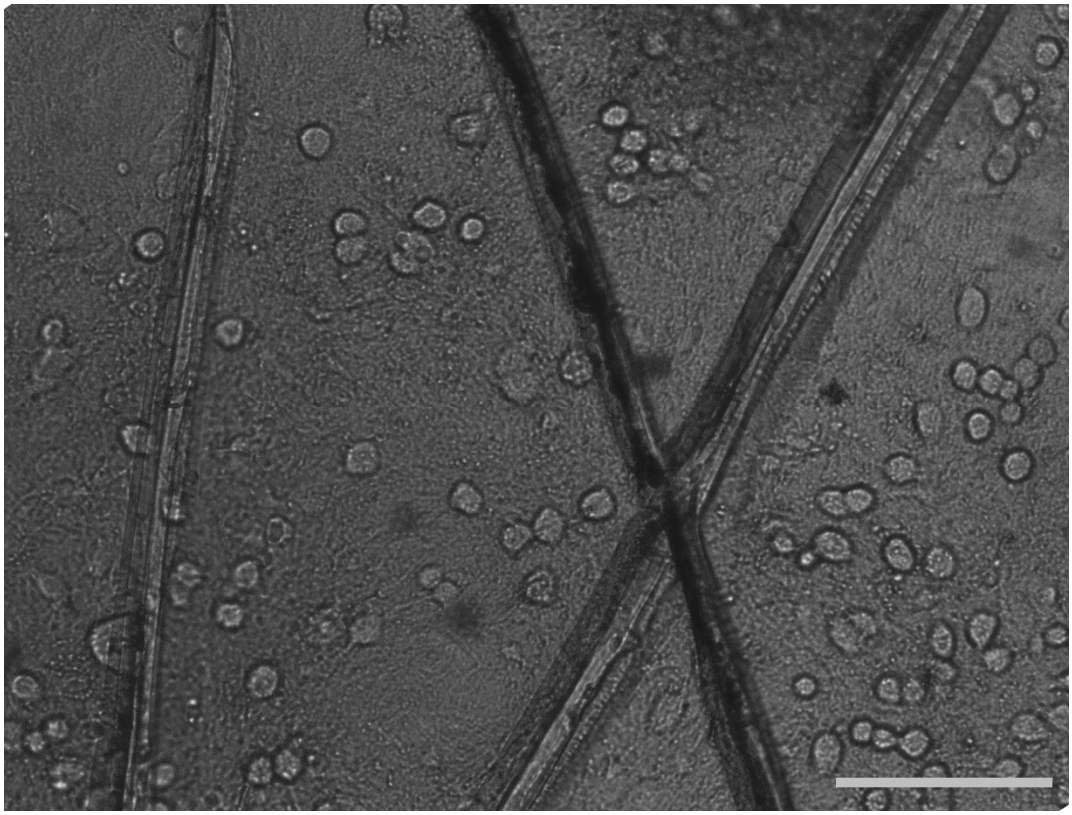

Fig. 1. Wide-field microscopy images of the primary hippocampal cultures on 24 day on development in vitro (24 DIV).Scale bar 100 µm

The study involved cells with access resistance below 20 MOhm and leakage current of 100 ... 100 pA. The resting potential for neurons was maintained at 70 mV. The following parameters of the action potential were studied: amplitude (calculated based on the points from the subthreshold potential to the overshoot point); hyperpolarization amplitude (the difference between the lower point of hyperpolarization period after the action potential and the baseline of stimulation step where this potential was generated); threshold potential amplitude (calculated based on the rate of potential increase for each cell). The above parameters were determined based on the first action potential on the threshold stimulus. Spontaneous neuronal activity was recorded in the cell culture for 5 min. Membrane capacity was tested by applying 5 mV pulses and calculated based on changes in current. GraphPad Prism 6 (GraphPad Software Inc., USA) was used for performing statistical analysis and building the graphs.

Ca2+ imaging

Functional activity was monitored by measuring the dynamics of the concentration of intracellular calcium using a specific fluorescent calcium dye Oregon Green BAPTA-1 AM (0.4 μM, Invitrogen, O-6807, USA) that was added to the culture medium for 40 min and confocal laser scanning microscope Zeiss LSM 510 (Germany). Registration of fluorescence intensity changes in response to increased intracellular calcium was made at frequency 2 Hz. Image J ROI manager tool was used to prepare an ROI set of mean gray values of each time series. Subsequently, ROI set were uploaded into open-source java-based application CaSiAn [3]. This analysis-tool provides an intuitive graphical user interface allowing experimentalists to easily process a large amount of Ca2+ signals, interactively tune peak detection, revise statistical measures and access the quantified signal properties as excel or text files. Among all parameters, we analyzed such parameters as frequency and interspike intervals and the number of spontaneously oscillating cells.

Immunocytochemistry

The cultured cells were fixed for 15 min in 4% formaldehyde containing PBS (pH 7.4) with sugar. To increase the permeability of the plasma membranes and the antibody's binding efficiency with intracellular antigens, the cultures were treated with ice-cold methanol for 3 min. Next, the fixed samples were treated with 0.1% Triton X-100 (Sigma) and 2% bovine serum albumin (BSA) for 20 min. The cells were incubated for 2 h at room temperature in PBS containing 1% BSA of the primary antibodies (mouse monoclonal MAP2a+MAP2b (ab36447, Abcam) to stain neurons. After washing, the samples were incubated for 40 min at room temperature with the following secondary antibodies (rabbit anti-mouse conjugated Alexa Fluor 488 (Abcam)). The immunostained cultures were examined under the confocal laser-scanning microscope (Zeiss LSM 510, Germany).

Electron microscopy

For electron microscopic studies, tissue treatment was carried out according to the standard protocol [1]. As a fixative 2.5% glutaraldehyde in PBS (pH = 7.4) was used. Subsequently, cells were immersed in a mixture of 1% of osmium tetraoxide and 1.5% potassium ferricyanide within 1 hour. The sample was dehydrated in alcohols of upward concentration (up to 100% alcohol), then three times in 100% acetone. After dehydration step sample was treated with the mixture (1:1) of 100% acetone and resin mixture the Epon-Araldite within 1 hour. Further tissue was embedded in the mixture Epon-Araldite for an additional 1 hour with subsequent resin polymerization at 45oC and 60oC. Pyramids for ultrathin sections were obtained with sharp edges of glass knives [4]. Ultrathin sections were made with ultramicrotome Leica EMT UC7 (Leica Microsystems, Германия). Ultrathin slices were contrasted with uranyl acetate and lead acetate according to the Reynolds method.

Results

Morphological parameters of cell cultures didn't differ in comparison with the control cells that were grown on coverglass with the same anchoring agent.

It was convenient to monitor the movement of cell bodies, neurite outgrowth, etc., moreover, cells are easily located due to the presence of a coordinate grid. It's strongly recommended

to orient coordinate grid in the same position (Fig. 2F)

Due to the high level of autofluorescence of the film (Fig. 2C), it is strongly recommended to use an upright confocal microscope. For monitoring of functional calcium imaging with subsequent immunocytochemistry or electron microscopy applications, films with cells were oriented strictly in the same position based on the drawn numbers, an area with the lowest density of neurons was selected, and several z-stacks of different fields of view far away from each other were made.

Sulforhodamine is a widely used dye for astrocyte's labeling, however, this approach has several disadvantages [5]. Here, we demonstrated the method for matching Ca 2+ activity and immunocytochemical labeling against microtubule-associated protein 2 (MAP2) in neurons (Fig.2). As can be seen from the figure, several cells were stained with Oregon Green BAPTA-1 AM, but not MAP2+, their calcium activity has also differed it means that with the approach it is possible to separate and analyze different types of cells.

It is acceptable to match functional activity with ultrastructural experiments growing cells on these films, e.g. in this study we matched data obtained by patch-clamp recordings with the ultrastructure of the measuring cell (Fig. 3). Electrophysiological data were compared with other intact cells (Fig. 3E). The following parameters were measured: the amplitude of the action potential, the threshold of depolarization, the amplitude of hyperpolarization, and the half-width of the action potential.

As can be seen, the amplitude of the action potential is slightly lower than the average amplitude of other intact neurons, but the half-width of the action potential is slightly higher than other cells.

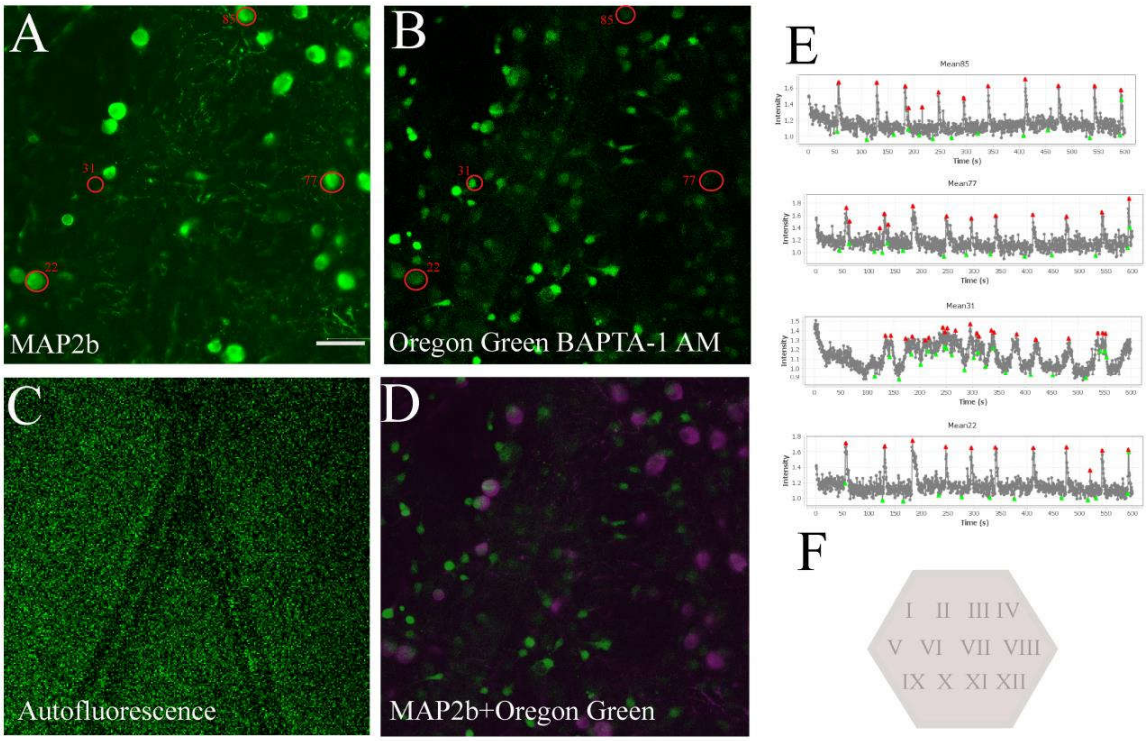

Fig. 2. A - Immunocytochemistry labeling of neurons, scale bar 50 µm; B – Representative image of cell culture stained with Ca 2+ indicator Oregon Green BAPTA-1 AM; C – autofluorescence of the film with the coordinate grid; D – Matching of neurons stained with Oregon Green and MAP2 antibody; E – Representative Ca2+ traces; F – schematic representation of the film with the coordinate grid

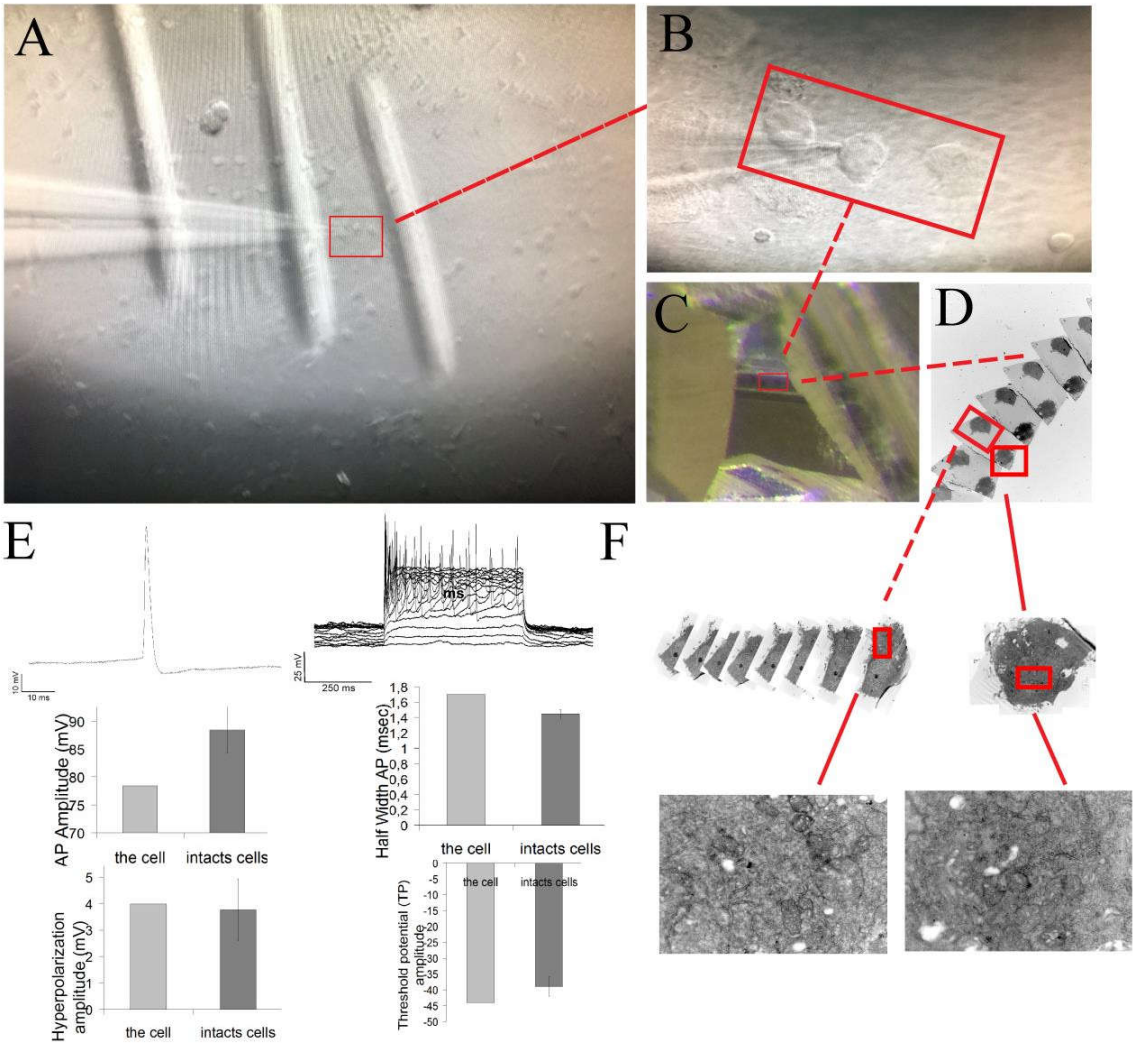

Fig. 3. A – View of the cell during the registration of bioelectrical activity by patch-clamp method B – The same image upscaled C – Polymerized area of resin with three cells in pyramid D – Series of ultra-thin sections. E – electrophysiological activity parameters of the measured neuron. F – ultrathin section of target-cell (left) and control cell (right)

After recording electrophysiological activity cell cultures were fixed for subsequent ultrastructural analysis. Since polymerization of resin thermoresistant film was separated and coordinate grid traces of the coordinate grid were printed on the resin. Due to Since the cells are osmiophilic, they can be easily identified in the ultratome. The pyramid is initially made a little more with standard blades. The final size of the pyramid is turned by glass knives. Further, it is possible to investigate using the method of standard transmission microscopy or to produce serial sections for subsequent reconstruction. As the ultrastructural analysis of the neuron showed, with the activity compared with the comparison cell (right) (Fig. 3 F), there were no significant changes in the membrane structure, ribosome density, or ER organization. The formation of endoplasmic reticulum was not found. However, in the body of this cell, both intact mitochondria and destroyed ones, as well as mitochondria with expanded cristae, were observed.

Discussion

The advantages of this method include low cost and easy separation of the film during sample preparation for ultrastructural analysis. It allows investigating functional cell activity and its molecular environment, e.g. for extracellular matrix labeling. This approach could be used as an alternative way of identification of neurons and glial cells or it may help to discover ultrastructural correlation with the bioelectrical activity of neurons and other cell types.

However, there are some disadvantages. Firstly, grid drawing, sterilization, and handling of these films during the preparation of primary cell cultures – highly time-consuming processes. Secondly, this method allows studying neurons only using low-density and middle-density cell cultures. Thirdly, the autofluorescence of this film is high. In case of usage inverted microscopes, it's necessary to mount film on coverslips; however, it is not convenient to work with such systems due to extremely high fragileness of coverslips

Acknowledgement

The reported study was funded by RFBR according to the research project № 18-34-00877.

References

SHIROKOVA O.M., FRUMKINA L.E., VEDUNOVA M.V., MITROSHINA E.V., ZAKHAROV Y.N., KHASPEKOV L.G., MUKHINA I.V. Morphofunctional Patterns of Neuronal Network Developing in Dissociated Hippocampal Cell Cultures. Sovremennye tehnologii v medicine 2013; 5(2): 6–13

ШИРОКОВА О.М. и др. Нейротропное действие карбамилированного дарбэпоэтина на модели первичной культуры гиппокампа //Современные технологии в медицине. – 2019. – Т. 11. – №. 4.

Moein et al. (2018), CaSiAn: a Calcium Signaling Analyzer tool (Bioinformatics, bty281, open access)

ROGACHEVSKII, V. V. A method of mesa trimming with glass knives for obtaining large series of ultrathin sections. Cell and Tissue Biology, 2013. 7(5), 487–495.

HÜLSMANN, SWEN, et al. Limitations of Sulforhodamine 101 for brain imaging. Frontiers in cellular neuroscience 11 (2017): 44.