Morphological characterization of spiral ganglion neurons grown in the pulsating electromagnetic field

Borić, Tina

Master's thesis / Diplomski rad

2022

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Split, Faculty of Science / Sveučilište u Splitu, Prirodoslovno-matematički fakultet**

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:166:100785

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Foremost, I'm extremely grateful to my mentor, Assist. Prof. Dr. Damir Kovačić, for his support, advice, and inspiring my work.

Very special thanks to Jelena Žarković and Boris Delipetar for their guidance and help during my experimental work.

Furthermore, many thanks to Ana Bedalov and KARMENstudio team for their help with image analysis and data collection.

Lastly, I would like to thank my sister for her extreme support and patience.

Temeljna dokumentacijska kartica

Sveučilište u Splitu
Prirodoslovno – matematički fakultet
Odjel za fiziku
Ruđera Boškovića 33, 21000 Split, Hrvatska

Diplomski rad

Morfološka karakterizacija neurona spiralnog ganglija uzgojenih u pulsirajućem elektromagnetskom polju

Tina Borić

Sveučilišni diplomski studij Fizika, smjer Biofizika

Sažetak:
Neuroni spiralnog ganglija (NSG), smješteni u modiolusu pužnice, prenose slušne signale od slušnih stanica dlačica do kohlearne jezgre u moždanom deblu. Oštećenje ili gubitak neurona spiralnih ganglija i slušnih stanica primarni je uzrok gubitka sluha. Kohlearna implantacija jedan je od najuspješnijih načina obnavljanja osjeta sluha kod ljudi sa slušnim oštećenjem. Jedno od glavnih ograničenja kohlearnog implantata je postojanje anatomske praznine između elektroda i živčanih vlakana. Otkriće metoda kojima bi se moglo utjecati na smjer rasta neurita, u svrhu smanjivanja te anatomske praznine, značajno bi poboljšalo prostornu i vremensku rezoluciju kohlearnog implanta. Cilj ovog rada bio je ispitati mogu li na morfologiju i orijentaciju neurona spiralnog ganglija utjecati vrsta podloge i elektromagnetsko polje. Utvrđeno je da prisutnost elektromagnetskog polja smanjuje radijuse tijela neurona spiralnog ganglija, dok ih supstrati s mikropilarima povećavaju. Osim toga, pokazalo se da vrsta podloge i elektromagnetsko polje značajno utječu na orijentaciju neurona spiralnog ganglija. Dobiveni nalazi poticaj su za daljnja istraživanja ovog područja.

Ključne riječi: Neuroni spiralnog ganglija, elektromagnetsko polje, *in vitro* kulture, morfologija, navođenje neurita

Rad sadrži: 27 stranica, 14 slika, 8 tablica, 28 literaturnih navoda. Izvornik je na engleskom jeziku

Mentor: doc. dr. sc. Damir Kovačić

Ocjenjivači: doc. dr. sc. Damir Kovačić
izv. prof. dr. sc. Larisa Zoranić
doc. dr. sc. Ivana Weber

Rad prihvaćen: 2. 12. 2022.

Rad je pohranjen u knjižnici Prirodoslovno – matematičkog fakulteta, Sveučilišta u Splitu.

Basic documentation card

University of Split
Faculty of Science
Department of Physics
Ruđera Boškovića 33, 21000 Split, Croatia

Master thesis

Morphological characterization of spiral ganglion neurons grown in the pulsating electromagnetic field

Tina Borić

University graduate study programme Physics, orientation Biophysics

Abstract:
Spiral ganglion neurons (SGNs), located in the modiolus of the cochlea, transmit auditory signals from mechanosensory hair cells to the cochlear nucleus in the brain stem. Damage of sensitive hair cells, the most common cause of hearing loss, typically leads to the degeneration of SGNs. A cochlear implant (CI), as the most successful neuroprosthetic clinical intervention, allows patients with hearing loss to bypass damaged hair cells by direct electrical stimulation of SGNs. However, one of the main limitations of cochlear implants is the neuroanatomical gap between electrodes and nerve fibers. Finding ways to influence neurite growth direction to reduce the anatomical gap would highly improve a cochlear implant's spatial and temporal resolution. This thesis investigated the effect of substrate type and electromagnetic field (EMF) on th morphology and orientation of SGNS. We conclude that the EMF may reduce the soma radii of SGNs while the micro pillared substrates have the potential to enlarge them. In addition, we found a significant influence of EMF and substrate type on the orientation of SGNs.

Keywords: Spiral ganglion neuron, electromagnetic field, *in vitro* cultures, morphology, neurite guidance

Thesis consists of: 27 pages, 14 figures, 8 tables, 28 references. Original language:English

Supervisor: Assist. Prof. Dr. Damir Kovačić

Reviewers: Assist. Prof. Dr. Damir Kovačić
Asoc. Prof. Larisa Zoranić
Assist. Prof. Dr. Ivana Weber

Thesis accepted: December 2, 2022.

Thesis is deposited in the library of the Faculty of Science, University of Split.

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1 Introduction

The spiral ganglion is located in the modiolus, the conical structure in the central cochlear axis (Fig. 1). It contains cell bodies of spiral ganglion neurons (SGNs), which are responsible for transmitting signals from the ear to the central nervous system (CNS). The peripheral processes of SGNs, mainly referred to as dendrites, gather auditory information from sensory hair cells located in the organ of Corti [1]. SGN's axons or central processes, which form the cochlear portion of the eight cranial nerve, transmit this information to the cochlear nucleus in the brain stem [1].

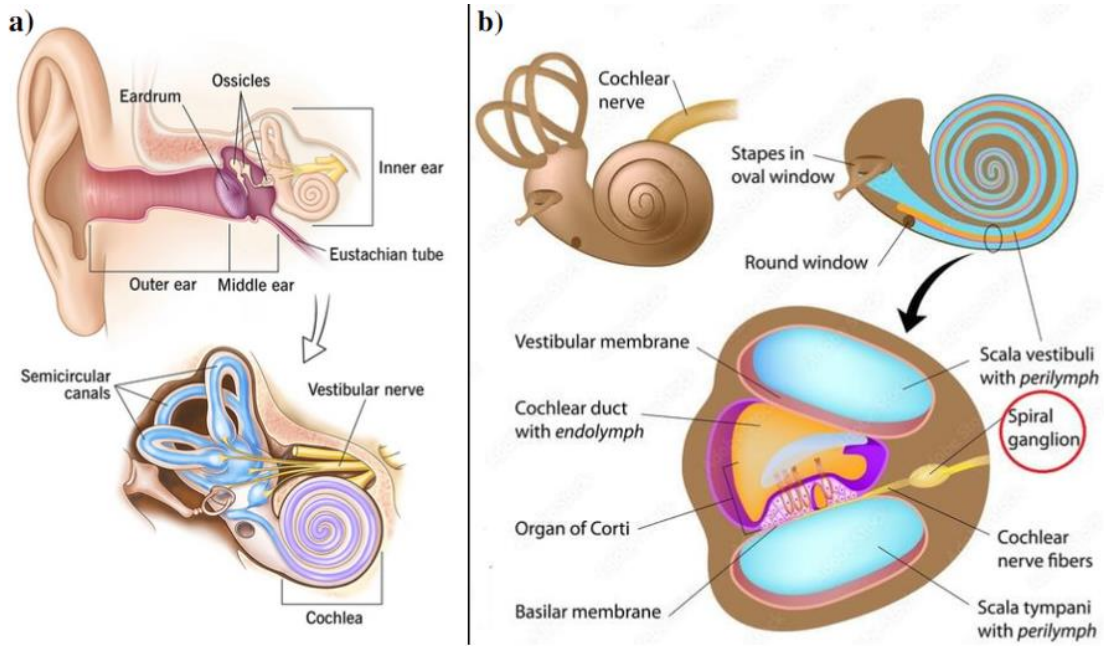

Figure 1 *Overview of the anatomy of the ear (a) and the cochlea (b). The spiral ganglion is shown in an enlarged cross-section [2,3].*

SGNs are divided into two categories based on their morphology, number, and neurite characteristics (Fig. 2). Type I SGNs, which correspond to about 90-95% of the total SGN population, are bipolar and are in synaptic contact with the inner hair cells (IHC) [4, 5]. These hair cells are responsible for the signal transduction and activation of SGNs [5, 6]. Another 5-10% of the total SGN population is made of type II SGNs that are smaller than type I SGNs and are in contact with the outer hair cells (OHC) whose main function is sound amplification [4, 6]. Damage or loss of hair cells, SGNs, or both is the primary cause of hearing loss [1]. Cochlear implant (CI), a neuro-prosthetic device implanted into the inner ear, provides one of the best ways to restore sound sensation in people with impaired hearing [1, 7, 8]. The outer part of CI captures, encodes, and transmits sound, while the implanted part contains a linear electrode that serves as a substitute for IHCs by stimulating the auditory nerve [7, 9].

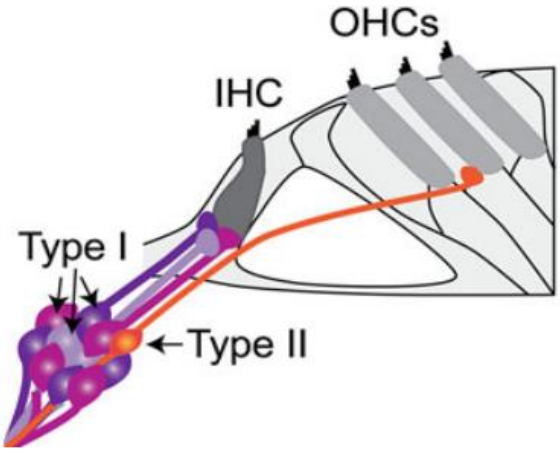

Figure 2 *Schematic view of the two types of SGNs. Type I (purple) and type II (orange) SGNs are connected to the IHCs and OHCs, respectively [1].*

Although CIs are highly successful in hearing restoration, they have several limitations. Foremost, CI cannot restore hearing in individuals with severe damage or loss of SGNs. Additionally, technological limitations in size and number of stimulation channels decrease spatial and temporal resolution, as one electrode stimulates up to a thousand nerve fibers [7, 9]. Therefore, finding ways to influence neurite growth direction toward the electrodes to surpass the anatomical gap and researching methods for regeneration and repair of SGNs are crucial to circumvent these obstacles.

In this experiment, the influence of topographical and electrical cues on SGN morphology and directed neurite growth was studied. SGNs isolated from rat pups were grown under the influence of pulsed electromagnetic field (EMF) on both glass coverslip and STRIPMED micro pillared chip for six days *in vitro* and compared with the control group cultured on the same substrates without stimulation. A coil powered by batteries was used as a source of EMF. Soma radius, orientation, and defletction of neuron orientation from the EF lines were determined from fluorescent images for both the control group and stimulated samples. The parameters were then compared to determine the best conditions for neuron growth and the influence of EMF and substrate topography on neurite orientation.

1.1 *In vitro* neuronal cultures

Cells isolated and then extracted from an animal's nervous system tissue and kept viable in controlled laboratory conditions are referred to as an in vitro neuronal culture. A significant portion of knowledge on SGNs morphology, functions, and response to stimuli comes from in vitro studies of pre-hearing rodent neurons [6, 10]. Two types of cochlear culture preparations are organotypic and dissociated. When organotypic culture is prepared, the entire organ is usually cultured so that the cellular interactions may be studied in conditions similar to those in a living organism [1]. Dissociated cultures usually contain monolayers of individual cells,

previously obtained by chemical and mechanical dissociation of a spiral ganglion, which are then grown on a substrate of interest [1]. Thus, individual cell morphological and electrophysiological properties can be determined from the studies of *in vitro* cell cultures. Since the primary goal of this thesis is to compare the morphological properties of SGNs grown in different conditions, dissociated cultures were chosen as an adequate model. The first step in the preparation of dissociated culture is the dissection of a ganglion [1, 6]. A typical choice of animal species is either rat or mouse pups up to two weeks of age, as rodents are a good model for mammal species. Moreover, the incomplete development of a temporal bone in rodent pups makes dissection easier, and a high number of SGNs may be obtained [1, 6]. Even though dissection happens in the pre-hearing period, it was shown that the neurons are spontaneously active and responsive [1, 10]. This implies that while other parts of an auditory system may not yet be mature, SGNs properties can be studied. After dissection, spiral ganglia is digested enzymatically so that most of the tissue is removed, and cells are then mechanically dissociated using a pipette [1, 6, 10]. Following this process, the cell suspension is seeded on a substrate that is previously treated with adhesive, usually containing laminin, which supports growth and differentiation, and poly-ornithine, which promotes cell adhesion [1, 6, 10]. The composition of a cell culture greatly depends on conditions of extraction from the inner ear; the dissociated SGN cultures usually also contain non-neuronal cells such as Schwann cells, satellite glial cells, and astrocytes [10]. After the required period of incubation, which depends on the experiment's goal, cells are fixated and treated with antibodies so they can be visualized on a fluorescent microscope. Following visualization, different cells found in a culture can be distinguished based on their morphology. The range of ratios of non-neuronal to neuronal cells varies mostly from 1:1 to 12:1, and in some cases, even to 7500:1 [7, 10].

1.2 Morphological properties of neurons

The geometric morphology of a neuron indicates its spatial structure. When the spatial structure is considered, a neuronal cell can be divided into two crucial parts: the soma, the cell body of the neuron, which contains the nucleus and essential components of cell machinery, and neurites or processes crucial for transmitting signals and intercellular communication [11, 12]. Even though neurons possess equal basic structure, their morphology varies greatly when different types of these cells are considered. Morphological characterization is an essential part of neuron analysis as it provides a lin to a structure-function relationship. The cochlear nucleus offers a representative example of this relationship, for which it was shown that the cells belonging to the same morphological classes show similar electrical signatures that can be used to identify them [13]. Electrical, chemical, and topographical cues may be used to influence

neuronal morphology, growth, survival, and axon guidance. The inclusion of neurotrophic factors such as GDNF or neurotrophin 3 in cultures is shown to promote neurite growth [7, 8, 15]. It was also shown that micro-patterned substrates, similar to chips used in this experiment, induce high neuronal alignment, improve axonal development, enhance growth and survival rate, and have a high impact on neuronal morphology [7, 8]. As the pillars on the patterned substrates do not provide electrical stimulation, the long-term survival of neurons cannot be ensured in these conditions [8]. Some parameters used to determine the morphological characteristics of the neurons are the perimeter and area of the soma, neurite length and width, neuron polarity which identifies the number of processes extending from the soma, and branch angles [14]. Neuronal somas can be spherical, oval, or angular and vary from 4 um to 150 um in diameter [12]. Based on their polarity, neurons can be classified as unipolar, pseudounipolar, bipolar, and multipolar (Fig. 3) [1, 12]. A significant percentage of SGNs in adult cochleae are bipolar and their processes are distinguishable by their location; dendrites are connected to the hair cells, while the axons are connected to the brain stem [15].

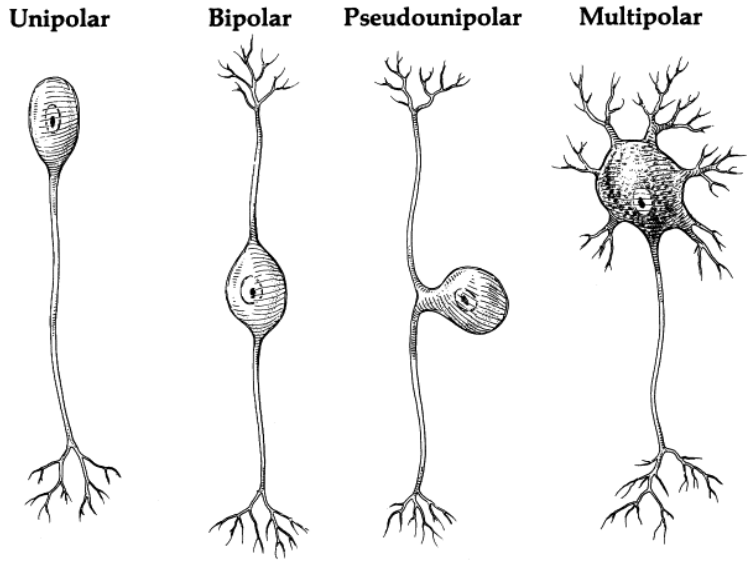

Figure 3 *Different types of neurons distinguished by their polarity [16].*

When conducting research based on neuronal morphology, it is crucial to define quantitative representations of the above-mentioned parameters. Two types of representations widely used are density maps, semi macroscopic approach in which the details are ignored, and the density of neurites or cells is measured, and morphometric statistics, which focuses on identifying and measuring morphology characteristics of individual neurons in the sample [14]. As the goal of this experiment is the comparison of the morphology of individual neurons, morphometric statistics will be used.

1.3 Effects of electromagnetic fields on neurons

Cell division, migration, and differentiation are known to take place in extracellular space where a constant voltage gradient arising from the ionic current flow is maintained [17, 18]. This implies that crucial cell processes are under the influence of endogenous electrical cues. In addition, the excitability of neuronal cells is also controlled by endogenous electrical currents, which influence membrane polarization [19]. It was shown that neurons change the direction of migration by using growth cones which are extensions of developing neurites crucial for determining growth direction and reorientation of a leading process (Fig. 4) [20]. Growth cones have motor and sensory abilities allowing them to recognize and integrate topographical, chemical, or electrical guidance cues into directed migration [17]. Therefore, neuronal cells simultaneously respond to more than one guidance cue.

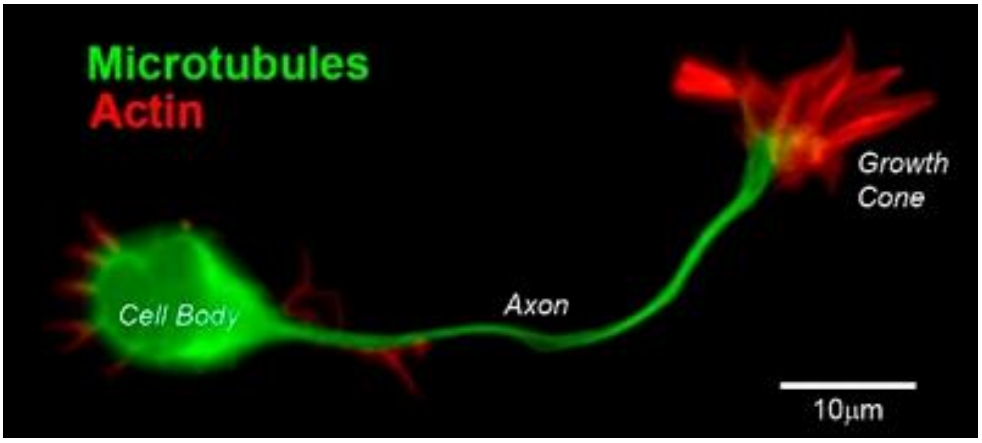

Figure 4 *Mouse spinal commissural neuron with growth cone [18]*

Neurotrophic factors and neurotransmitters released from hair cells, which drive electrical activity in SGNs, are crucial for SGNs survival and development [22]. Although the effects of chemical factors on axonal guidance were mainly studied, there is evidence that EFs may also influence neurite growth and guidance [18]. An extensive set of experimental studies demonstrated the interaction between externally applied electric or electromagnetic fields and neurons [19]. It was shown that direct current (DC) EFs, pulsed EFs, and alternating current fields all influence directional neurite growth. When SGNs, Xenopus spinal neurons, dorsal root ganglia, and hippocampal neurons were exposed to DC EF and uniform pulsed fields, neurites mostly grew towards the negative pole, cathode [20, 23, 24, 25]. Applied fields also induced degeneration of neurites facing the anode and redirected those neurites which were following contact guidance cues [17, 26]. When the EF direction was reversed, the direction of neuron migration was also reversed [20, 24]. When biphasic pulsed EFs were applied, SGN's neurites were shown to turn away from the electrode rings, and specific neurite alignment decreased with increasing distance from the electrodes [26]. As cells may integrate more than one directional cue, culture media, growth factors, and substrate coating may also affect directed neurite growth. Orientation of neurites toward the cathode was shown to be enhanced

by neurotrophin brain-derived factor BDNF and reversed by neurotrophin NT3 [17, 27]. Neurites cultured on laminin-coated substrates may change direction toward the cathode, while those on PDL-coated substrates turned toward the anode [21]. When neurons of the rat hippocampi were placed in the electric field induced by the coil's magnetic field, no change in orientation was reported [28]. Since the effect of EMF on neurons depends on their curvature, length, geometry, interconnection, and other factors, multiple experiments with different parameters should be conducted to determine the actual effect of EMF [28].

These studies indicate that the simultaneous effects of multiple guidance cues may enhance or suppress the impact of a single cue [17]. As the main function of the cochlear implant is stimulation of SGNs, influencing directional neurite growth toward the electrodes of CI may improve its efficacy by increasing the stimulation range or decreasing excitation thresholds [25].

2 Materials and methods

2.1 Sample design

To test the effects of substrate type and stimulation conditions, four samples were analyzed. Neurons were grown in the presence of EMF on STRIPMED micro pillared chips and glass coverslips. The control group included the neurons grown on the same substrates without the presence of EMF. The design is summarized in Table 1. Each group contains one sample.

Table 1 *Summary of the sample design.*

Sample layout		Stimulation	
		No EMF	**EMF**
Substrate	**Glass**	Glass control	Glass EMF
	Chip	Chip control	Chip EMF

2.2 STRIPMED chips

STRIPMED micro pillared chips (Fig. 5) were used as a substrate in the experiment in addition to the glass coverslips. The chips contain 196 electrodes spaced every 100 µm. The width of each electrode is 20 µm. Each chip is divided into four areas containing pillars of different widths. Pillar widths are 1, 1.8, 2.8, and 4.8 µm. The pillar spacing on the chip is 1.2 µm.

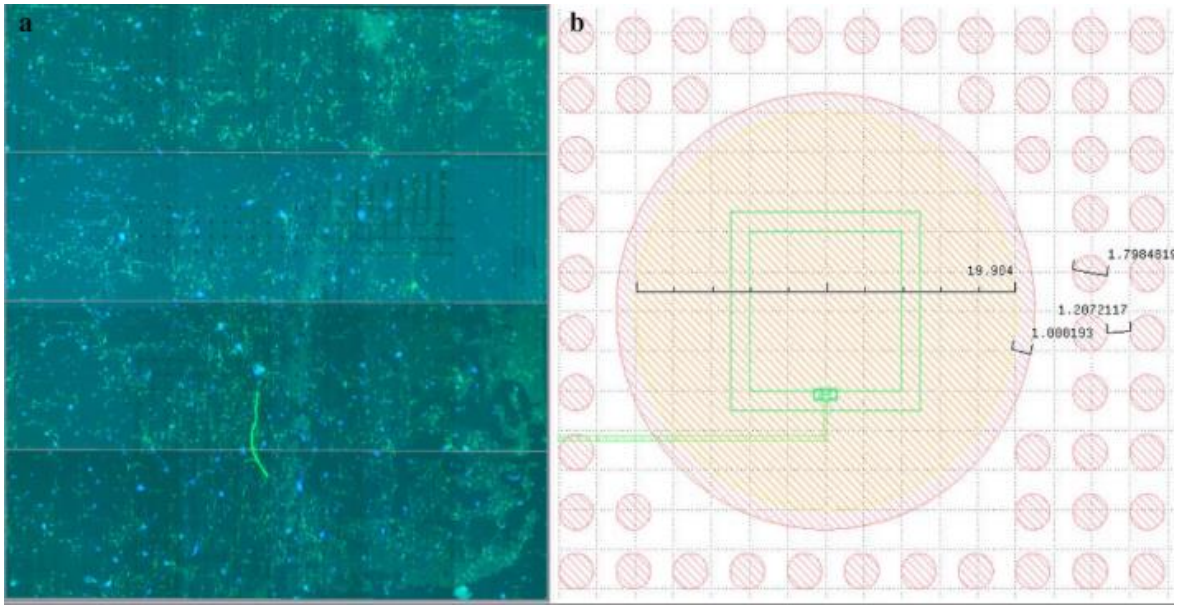

Figure 5 a) *Representative fluorescent microscope image of SGNs cultured on STRIPMED chip.* ***b)*** *Schematic view of the part of the chip with specified spacings and widths.*

2.3 Preparation of STRIPMED chips and glass coverlips

STRIPMED chips were cleaned overnight in acetone. Glass coverslips and chips were then rinsed with sterile deionized water, sterilized with 70% ethanol, rinsed again, and left to dry under sterile conditions. Two substrates, deemed to be control, were placed in 24 well plates for testing. Another two were placed in Petri dishes to allow stimulation with the coil. Substrates were coated with 0.01% poly-L-ornithine, to promote cell attachment and adhesion, and were left at room temperature overnight. All substrates were rinsed with sterile deionized water and additionally coated with 0,03 mg/ml laminin for 2 hours at 37 °C to enhance cell culture. The coating was then rinsed again with sterile deionized water, and substrates were left to dry in sterile conditions.

2.4 Isolation of SGNs

Protocols used are similar as described in [7, 8]. Spiral ganglion neurons were isolated from postnatal six-day-old Sprague-Dawley rat pups. Rat pups were anesthetized on ice and decapitated. Dissecting buffer contained phosphate buffer saline (PBS) supplemented with 0.3% bovine serum albumin (BSA) and 0.6% glucose. Skull was opened along the mid-sagittal plane under the operating microscope, and the brain was removed. The temporal bone was harvested and transferred to the clean dissecting buffer. Then, the otic capsule was dissected, and the cochleae were identified and isolated. The organ of Corti and modiolar cartilage were removed, and the spiral ganglia were collected in the dissecting buffer.

2.5 *In vitro* culturing of SGNs

0.25% trypsin-EDTA with the addition of 38 U/ml DNase was used for enzymatic dissociation. Dissociation was performed at 37°C. After 30 minutes, the trypsinization was stopped by the STOP solution. STOP solution contained an equal volume of DMEM: F12 supplemented with 10% of Fetal Bovine Serum (FBS). The tissue was triturated using a pipette tip about 50 times, and excess tissue pieces were allowed to settle at the bottom. The cell suspension at the top was collected and centrifuged for 10 min at 2000 rpm. The pellet was resuspended in 500 µl of culture medium. The culture medium consisted of Neurobasal-A containing 1% Pen-Strep, 0.5mM L-Glutamine, B27 supplement, and 30 ng/ml GDNF. GDNF was chosen as the only neurotrophic factor to avoid interactions among multiple factors and was shown previously to be the most effective in adult SGNs cultures [7]. Cells were counted in a Bürker-Türk chamber and seeded in a 100 µl volume with a density of 40 000 cells per sample. Cells were left in an incubator at 37 °C, 5% CO2, and 85% humidity to settle. After two hours, the rest of the medium was added. The entire culture medium was changed every second day during 6 DIV (days *in vitro*) culturing.

2.6 EMF stimulation

According to Faraday's law of electromagnetic induction, the time-varying current passing through the coil induces the time-varying magnetic field perpendicular to the plane of the coil. As the magnetic field is time-varying, this will cause changes in the magnetic flux ϕ. The change in ϕ will create an induced circular electric field $\vec{E}$, given by $\oint \vec{E} d\vec{l} = -\frac{d\phi}{dt}$, parallel to the plane of the coil and concentric to the coil rings (Fig 6.).

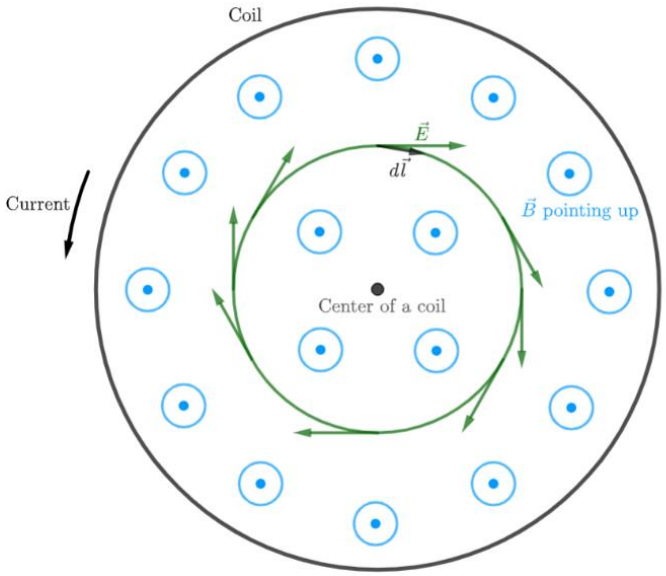

Figure 6 *Time-varying current passing through the coil induces a magnetic field (blue) perpendicular to the plane of the coil, which in turn induces the circular electric field (green) parallel to the plane of the coil.*

A coil with a diameter of 4.1 cm and 80 turns was the source of pulsating electromagnetic field (Fig 7.). The amplitude of the time-varying current and voltage was set at 210 mA and 210 mV, respectively. The period of sawtooth signal was set at 100 ms (Fig. 7). The batteries powered the coil. We analyzed the amplitude, signal shape, and period using an oscilloscope every 60m inutes for 13 hours to examine the battery capacity, efficacy, and stability. The measurements were repeated for different sets of batteries used and various combinations of batteries and coils. It was determined that the batteries used to power the coils could sustain the set amplitude, signal shape, and period for a minimum of twelve hours. To ensure the constant stimulation of the cultures, batteries were replaced every twelve hours. The cells on one glass substrate and one STRIPMED chip, both placed in the Petri dishes, were stimulated for six days in an incubator.

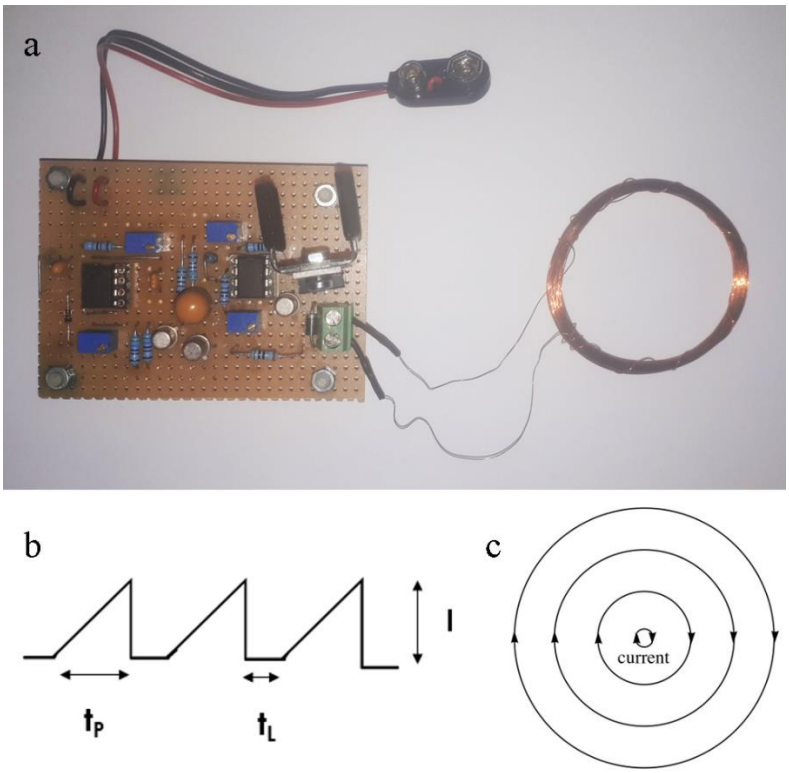

Figure 7 a) *The coil used in the experiment.* ***b)*** *The shape of the signal set in the circuit. The period was* 100 ms*, with* $t_p = 80\ ms$ *and* $t_l = 20\ ms$*. I represents the amplitude of the current set at* 210 mA. ***c)*** *The current passing through the coil.*

2.7 Immunocytochemistry

Immunocytochemistry (ICC) is a laboratory technique that allows the examination of a protein or antigen, usually referred to as a marker, by using antibodies. The type of antibodies used depends on the particular cells that need to be visualized. The primary antibody binds to a marker and then to the secondary antibody, usually containing a conjugated fluorophore. Fluorophores are molecules that can absorb and emit light within a defined range of wavelengths specified by a molecule type. When a light of a specific wavelength illuminates the fluorophore, it will absorb a photon. After the absorption, the energy level of a molecule is raised, and the molecule is in the excited state. In the deexcitation period, part of the energy will be emitted as a photon of a wavelength different from that of the excitation light. As the emitted energy is lower than the absorbed energy, the wavelength of the emitted light will be longer than the wavelength of the absorbed light. The fluorophores can be repeatedly excited until they are irreversibly damaged. According to this mechanism, conjugated fluorophores allow the markers to be observed under a fluorescent microscope

After 6 DIV samples were fixed with 4% paraformaldehyde for 30 min and rinsed with 1X PBS three times. For immunocytochemical analyses, samples were permeabilized with 0.1% Triton X-100 for 5 min and blocked with PBS containing 1% normal goat serum for 1.5 hours at room temperature. Primary staining was performed overnight at 4 °C with 1:500 mouse

monoclonal anti-ßIII tubulin for neurons, in combination with 1:500 rabbit polyclonal anti-S100 for glial cells. After incubation, the samples were rinsed three times with 1XPBS. Secondary staining was performed for 1.5 hours at room temperature using Alexa 488 goat anti-mouse and Alexa 568 goat anti-rabbit, diluted 1:500 in 1X PBS (with 1% normal goat serum). 5 μg/ml DAPI in a ratio of 1:500 was added for nucleus staining, and samples were incubated for 5 min, even though nucleus visualization on a microscope was not performed. After incubation, samples were rinsed five times with 1XPBS and prepared for imaging with Immu-Mount.

2.8 Fluorescent imaging

Cell imaging was performed using Olympus BX61 fluorescent microscope equipped with a monochrome CCD camera (Retiga R6). 10x magnification was used for each image. A snake-like scanning of the whole sample with the motorized stage (Prior Scientific) was set up to achieve the sample imaging obtained with narrow field-of-view objectives. A minimum of 9 consecutive positions in the sample were imaged. Images were later connected with KARMENstudio image processing software.

2.9 Image processing

Images obtained from a fluorescent microscope were processed using KARMENStudio image processing software (Bedalov d.o.o, Kaštel Sućurac, Croatia) containing a broad range of machine learning-based image processing algorithms, optimized for fast and accurate segmentation of neurons. As the density of cells seeded on substrates was too high, it was not possible to use automatic image processing. Instead, about ten clearly visible neurons from each image were randomly chosen and manually contoured. The algorithm recognized manually drawn contours and calculated the most extensive possible radius of a circle that can be inscribed in the soma of each contoured neuron. These approximated soma radii were later used in the statistical analysis. Additionally, neuron orientation and orientation intensity were also calculated.

2.10 Research design

In this research, a complex 2x2 design was used. The influence of two independent variables on three dependent variables was investigated.

The independent variables were defined as substrate type (glass coverslips, STRIPMED micro pillared chips) and stimulation conditions (control, EMF). The dependent variables were soma radius, neuron orientation, and deflection of neuron orientation from the EF lines. Soma radius was defined as the most extensive possible radius of a circle that can be inscribed in the neuron soma. Neuron orientation was defined as a deflection angle from the y-axis, and the

deflection of neuron orientation from the EF lines was defined as an angle closed by the tangent of the EF line and the neuron orientation intensity line (Fig. 8).

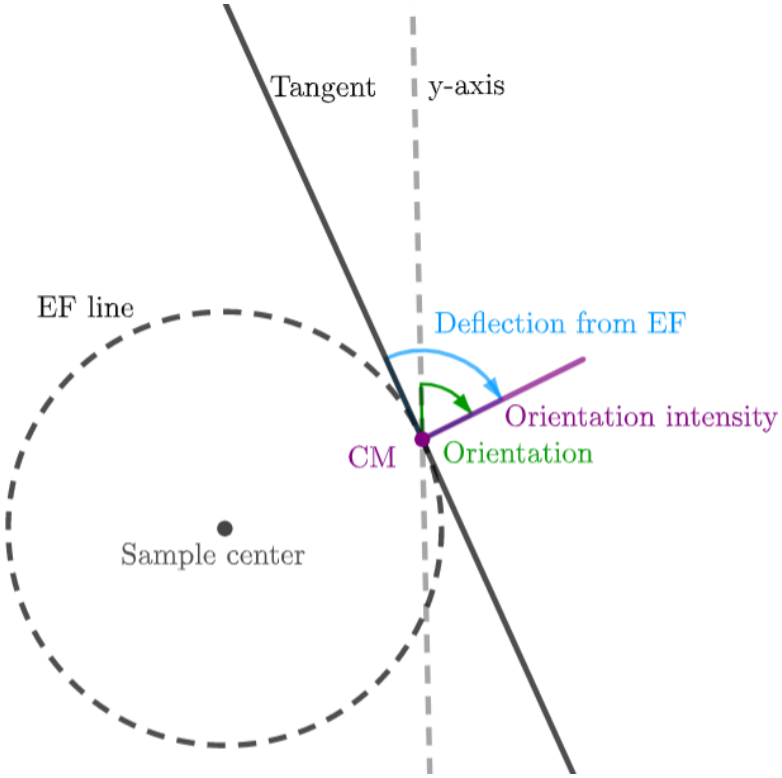

Figure 8 *Neuron orientation is defined as a deflection from the y-axis (green). Orientation intensity and the center of mass of the neuron are shown in purple. The deflection angle of neuron orientation from the EF lines was defined as an angle (blue) closed by the tangent of the EF line and the neuron orientation intensity line.*

2.11 Statistical analysis

As the data distribution did not satisfy parametric assumptions, a nonparametric test for independent groups was used. Precisely, data were transferred to spreadsheets and analyzed using multiple Kruskal-Wallis tests in Microsoft Excel and Python 3, to test if there were any statistical differences between the mean ranks of 4 groups of neurons. All graphs were made using Python programming language. Measures calculated for soma radii are given μm, and the angles are shown in degrees. N represents the number of neurons in each sample. Mdn, min, max, Q1, and Q3 represent the median, minimum, maximum, first quartile, and third quartile of data sets, respectively. The p-value less than 0.05 was considered significant.

3 Results

3.1 Effects of substrate type and stimulation conditions on soma radius

To determine the effect of stimulation and substrate type on the soma radius of SGNs four fluorescent images of samples were analyzed. Representative images of each sample are shown in Figure 9. Fluorescent images were processed using KARMENStudio image processing software. Neurons from each sample were randomly selected and manually contoured. The software was used to calculate the most extensive possible radius of a circle which can be inscribed in the soma of each contoured neuron. Radii were measured in pixels and then converted to μm.

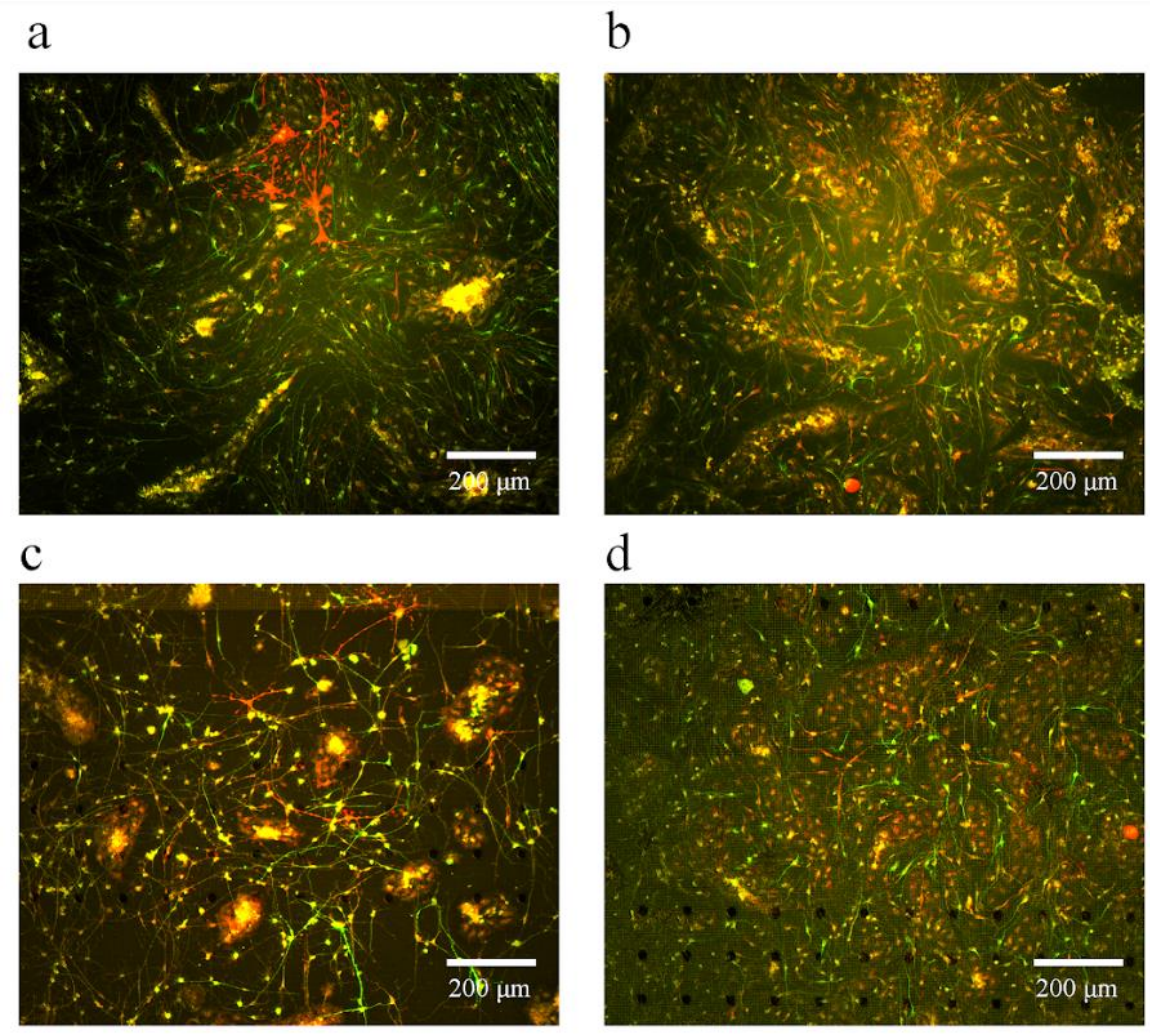

Figure 9 *Representative fluorescent images of the samples. SGNs are shown in green and glial cells in yellow.* ***a)*** *Neurons grown on the glass coverslip without stimulation (glass control).* ***b)*** *Stimulated neurons grown on the glass coverslip (glass EMF).* ***c)*** *Neurons grown on the STRIPMED chip without stimulation (chip control).* ***d)*** *Stimulated neurons grown on the STRIPMED chip (chip EMF).*

Table 2 contains descriptive data on the soma radii of SGNs for each of the four samples.

Table 2 *Descriptive data on neuronal soma radii. All measures except N are shown in μm.*

Stimulation	Substrate	*N*	*Mdn*	*Min*	*Max*	*Q1*	*Q3*
Control	**Glass**	146	5.65	4.01	8.27	4.96	6.46
	Chip	159	6.52	4.01	9.78	5.61	7.44
EMF	**Glass**	125	5.45	3.91	7.39	4.78	6.18
	Chip	120	5.91	3.91	8.26	5.25	6.68

Multiple Kruskal-Wallis tests were performed to determine if there is a statistically significant difference in soma radii of neurons grown in different conditions.

Two tests were conducted to find if there is a statistically significant difference in soma radii of neurons grown on the different substrates with and without stimulation. The first test was performed to determine if there is a statistically significant difference in soma radii of neurons grown on the glass and those grown on the chip without stimulation. It was found that substrate type in the absence of stimulation significantly affects neuronal soma radius ($H(1)=34.46$, $p<0.001$). Neurons grown on the chip without stimulation had larger soma radii (Mdn=6.25) than neurons grown on the glass coverslip (Mdn=5.65). The second test was performed to determine if there was a statistically significant difference in soma radii of neurons grown on the glass and those grown on the chip in the presence of stimulation The effect of the substrate type was found to be statistically significant in this condition as well ($H(1)=11.58$, $p<0.01$). Neurons grown on the chip in the presence of stimulation had larger soma radii (Mdn=5.91) than neurons grown on the glass coverslip (Mdn=5.45).

Additional two tests were performed to find if there is a statistically significant difference in soma radii of neurons grown in different stimulation conditions on glass coverslips and STRIPMED chips. The third test was performed to show if there is a statistically significant difference in soma radii of neurons grown on STRIPMED chips with and without stimulation. It was found that soma radii of neurons grown on chips were significantly influenced by the stimulation conditions ($H(1)=14.30$, $p<0.01$). Neurons grown without stimulation had larger soma radii (Mdn=6.59) than those grown in the presence of EMF (Mdn=5.98). The fourth test was performed to show if there is a statistically significant difference in soma radii of neurons grown on glass coverlips with and without stimulation. The statistically significant difference in soma radii of neurons grown in these conditions was not determined ($H(1)=2.11$, $p=0.15$).

Results of Kruskal-Wallis tests are summed in Table 3. H represents the test statistic and df are degrees of freedom for each group.

Table 3 *Summary of results of multiple Kruskal-Wallis tests for soma radii.*

Groups		*H*	*df*	*p*
Substrate type	**Control**	34.46	1	<0.001
	EMF	11.58	1	<0.01
Stimulation conditions	**Glass**	2.11	1	0.15
	Chip	14.30	1	<0.01

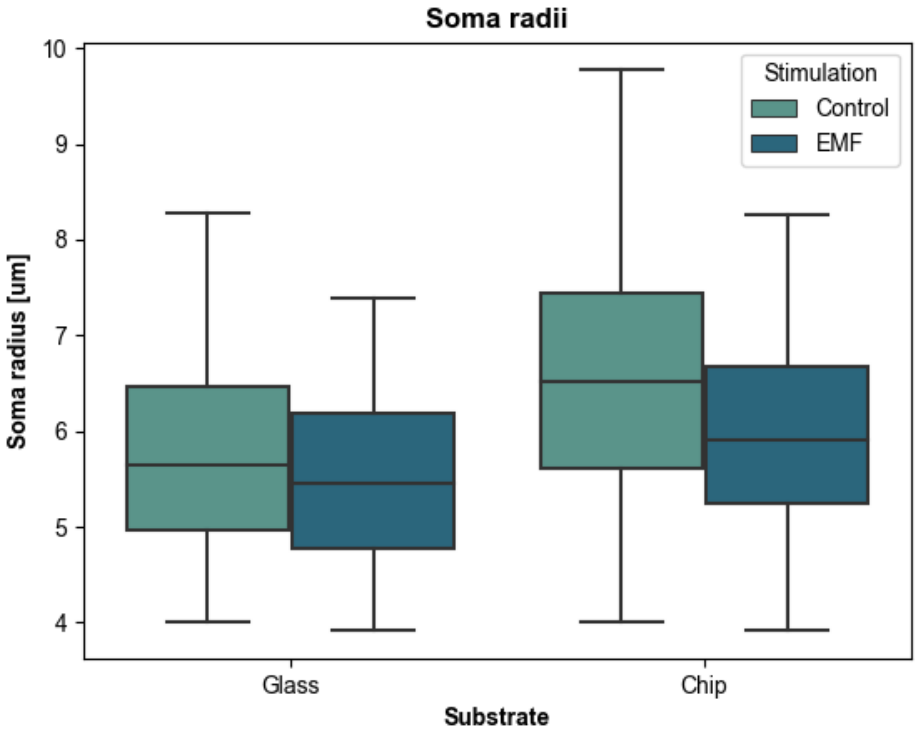

Figure 10 *Box and whisker plots showing the effects of substrate type and stimulation conditions on soma radii of SGNs.*

3.2 Effects of substrate type and stimulation conditions on the neuronal orientation

To determine the influence of stimulation and substrate type on the orientation of SGNs the same neurons as described above were analyzed. The orientation angle, defined as the angle of the deflection from the y-axis (Fig. 8), and orientation intensity for each contoured neuron were calculated using the KARMENstudio software. All measures are reported in degrees.

Table 5 contains descriptive data on neuron orientations for each of the four samples.

Table 4 *Descriptive data on neuron orientations. All measures except N are shown in degrees.*

Stimulation	Substrate	*N*	*Mdn*	*Min*	*Max*	*Q1*	*Q3*
Control	**Glass**	146	105.50	0	177	59.50	128
	Chip	159	93	0	179	48.50	139.50
EMF	**Glass**	125	102	0	177	50	136
	Chip	120	79	0	178	48.75	101.25

Histograms of orientation angles for each sample are shown in Figure 11. Orientations on glass coverslips are highly uniform, except for the peak in the glass control group. For the neurons grown on the chips, with and without stimulation, the highest number of neurons is oriented between 80 and 100 degrees. The specific orientation is more prominent on the chip in the presence of the EMF, possibly because of the larger number of neurons analyzed in this sample.

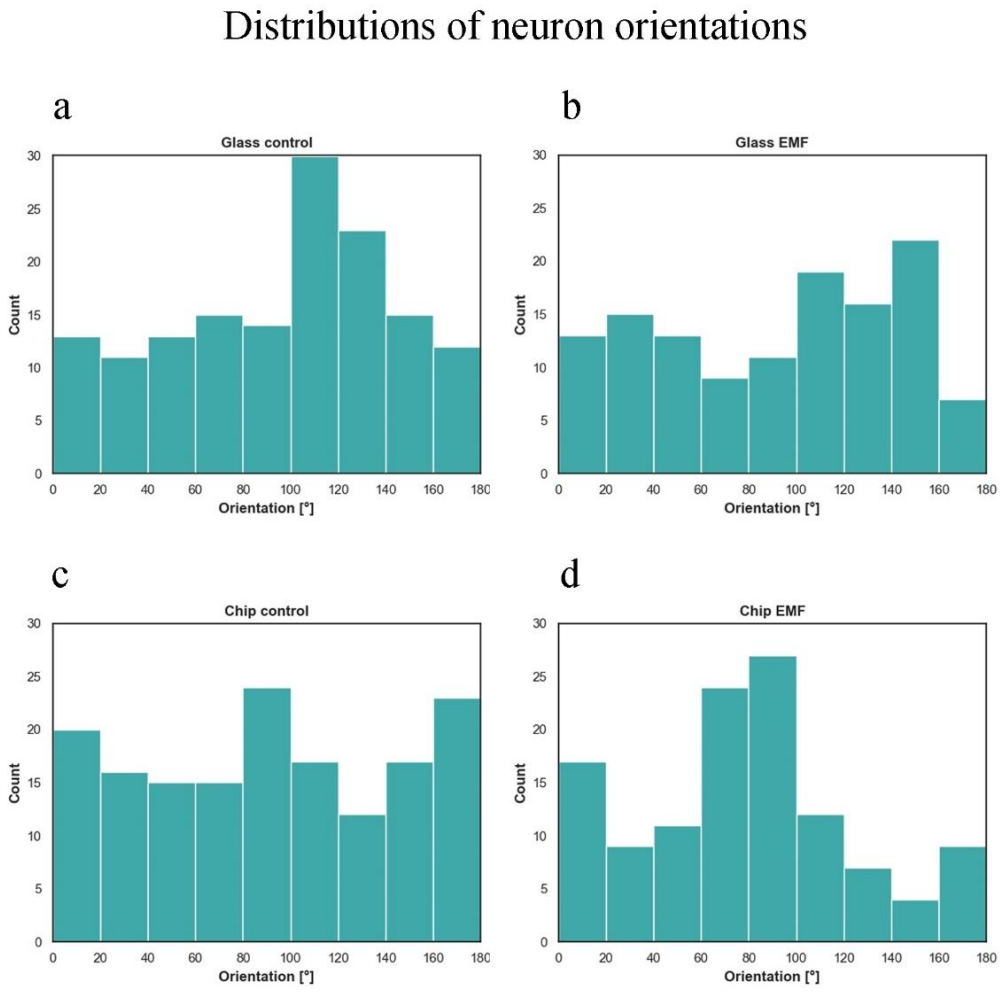

Figure 11 *Histograms of neuron orientation angles for each of the four samples.*

Multiple Kruskal-Wallis tests were performed to show if there is a statistically significant difference in the orientation of neurons grown in different conditions.

Two tests were performed to find if there is a statistically significant difference in orientations of neurons grown on the different substrates with and without stimulation. The first test was performed to show if there is a statistically significant difference in orientations of neurons grown on the glass and chip without stimulation. It was found that substrate type in the absence of stimulation does not significantly affect the neuronal orientation ($H(1)=0.63$, $p=0.43$). The second test was performed to show if there is a statistically significant difference in orientations of neurons grown on the glass and chip in the presence of stimulation. Influence of the substrate type in the presence of stimulation was found to be statistically significant ($H(1)=4.90$, $p<0.05$).

Additional two tests were performed to find if there is a statistically significant difference in orientations of neurons grown in different stimulation conditions on glass coverslips and STRIPMED chips. The third test was performed to show if there is a statistically significant difference in orientations of neurons grown on STRIPMED chips with and without stimulation. It was found that the orientation of neurons grown on chips was significantly influenced by the stimulation conditions ($H(1)=4.17$, $p<0.05$). The fourth test was performed to show if there is a statistically significant difference in orientations of neurons grown on glass coverslips with and without stimulation. The statistically significant difference in orientations of these neurons was not determined ($H(1)=0.30$, $p=0.58$).

The results of the Kruskal-Wallis tests are summed in Table 6. H represents the test statistic and df are degrees of freedom for each group.

Table 5 *Summary of the results of multiple Kruskal-Wallis tests for neuronal orientation.*

Groups		*H*	*df*	*p*
Substrate type	**Control**	0.62	1	0.43
	EMF	4.90	1	<0.05
Stimulation conditions	**Glass**	0.30	1	0.58
	Chip	4.17	1	<0.05

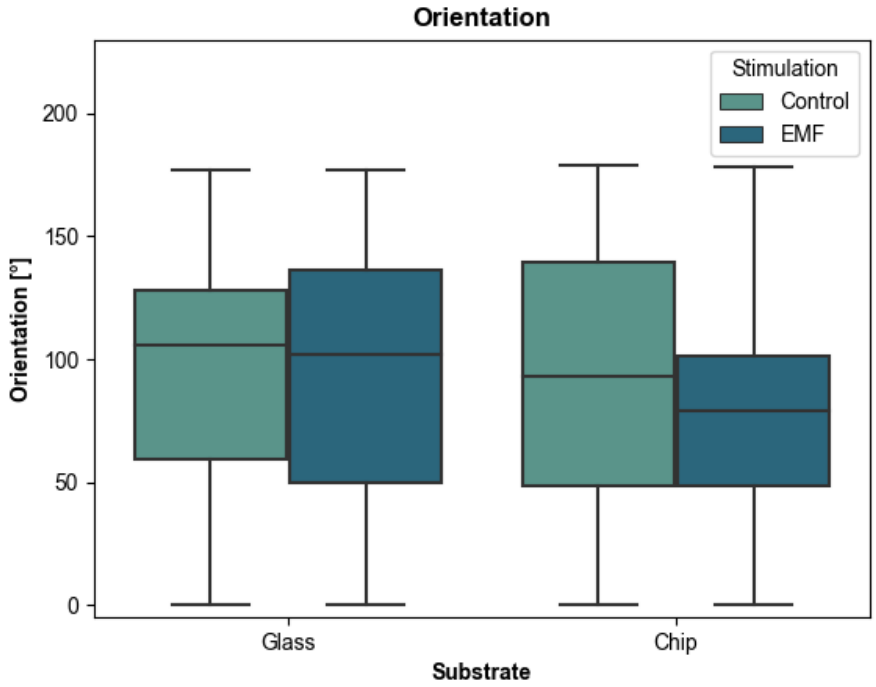

Figure 12 *Box and whisker plots showing the effects of substrate type and stimulation conditions on the orientations of SGNs.*

3.3 Effects of substrate type and stimulation conditions on the neuronal deflection from EF lines

Deflection of neuron orientation from electric field lines was defined as an angle closed by the tangent of the EF line and the neuron orientation intensity line (Fig. 8). To determine the influence of stimulation and substrate type on the deflection angle of SGNs, the same neurons as described above were analyzed.

Table 7 contains descriptive data on deflection angles for each of the four samples.

Table 6 *Descriptive data on deflection angles from EF lines. All measures are shown in degrees.*

Stimulation	Substrate	*N*	*Mdn*	*Min*	*Max*	*Q1*	*Q3*
No EMF	**Glass**	146	99.61	1.29	179.71	48.11	140.51
	Chip	159	98.17	1.11	179.51	55.07	136.98
EMF	**Glass**	125	87.39	5.17	175.77	47.58	138.38
	Chip	120	82.74	0.67	177.5	42.25	124.06

Histograms of deflection angles for each sample are shown in Figure 11. From the histograms, it can be seen that distributions across the different samples are relatively uniform.

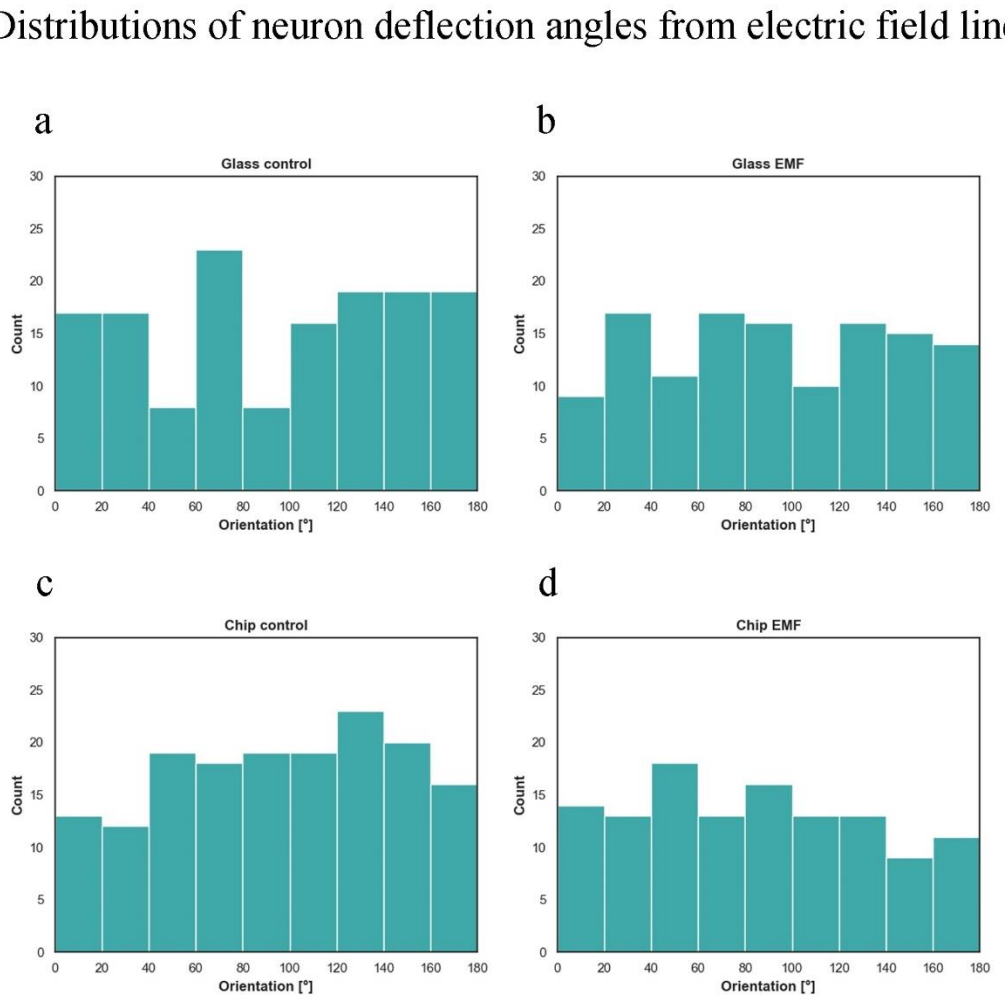

Figure 13 *Histograms of neuron deflection angles for each of the four samples.*

As with orientation and soma radius, multiple Kruskal-Wallis tests were performed to determine if there was a statistically significant difference in deflection angles from the EF lines between the defined groups of neurons. Groups were as defined before: substrate type in the absence of stimulation, substrate type in the presence of stimulation, stimulation conditions on the glass coverslips, and stimulation conditions on the chips. There was no statistically significant difference determined in any of the groups. The summary of the results of the Kruskal-Wallis tests is given in Table 8.

Table 7 *Summary of the results of multiple Kruskal-Wallis tests for deflection angle.*

Groups		***H***	***df***	***p***
Substrate type	**Control**	0.02	1	0.90
	EMF	1.63	1	0.20
Stimulation conditions	**Glass**	0.01	1	0.91
	Chip	3.45	1	0.06

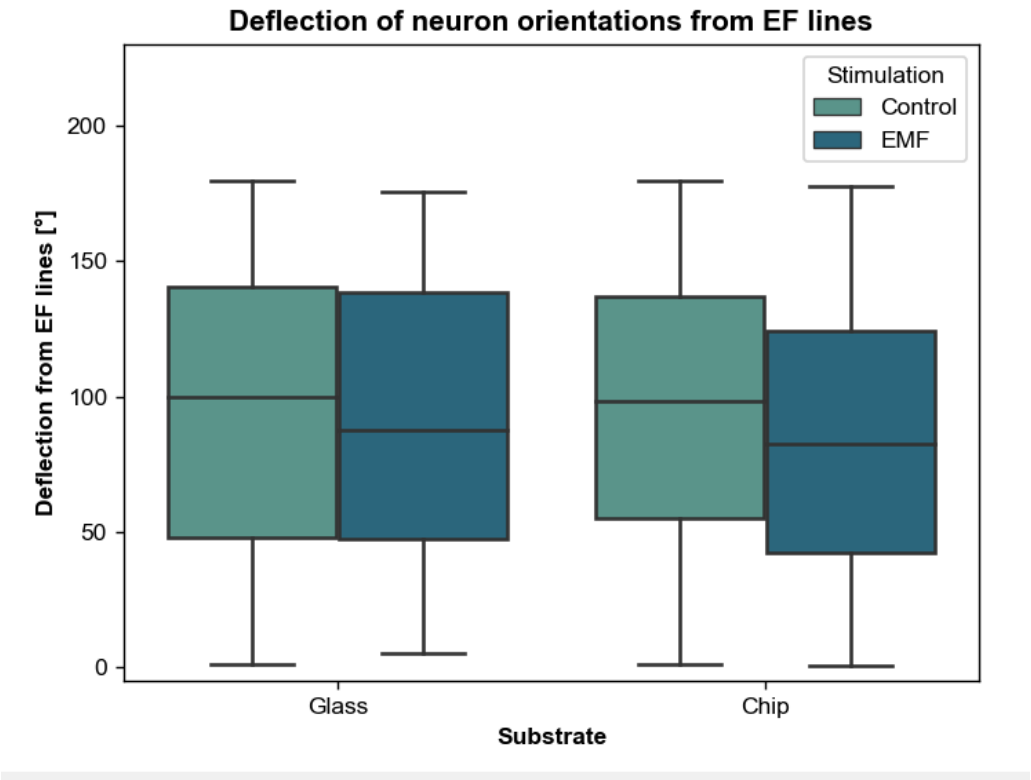

Figure 14 *Box and whisker plots showing the effects of substrate type and stimulation conditions on the deflection angles from the EF lines of SGNs.*

4 Discussion

In this research, we examined the impact of stimulation conditions and substrate type on SGN's soma radius, orientation, and deflection from EF lines. It was shown that substrate type, both in the presence and the absence of the stimulation significantly affects neuronal soma radius. Neurons grown on chips had a larger soma radius than those grown on the glass coverslips in both cases. The stimulation conditions were shown to significantly affect the soma radius when the neurons were grown on the chip, while the differences in radii were deemed insignificant for the neurons grown on the glass coverslips. However, in both cases, the medians of soma radii were smaller when neurons were exposed to the EMF. When considering neuron orientation, it was determined that substrate type had a significant effect when the neurons were exposed to the EMF, while the effect of the substrate type was deemed insignificant for the control group. It was shown that the large number of neurons grown on the chip in the presence of EMF was oriented in a specific direction, while those grown on the glass coverslip had a highly uniform distribution of orientations. Additionally, it was determined that stimulation conditions did not have a significant effect on neuron orientations on glass coverslips, while their effect was deemed significant for SGNs grown on the STRIPMED chips. In this case, the distribution of neuron orientations on the chip in the control group was less uniform when compared to the glass substrate but more uniform when compared to the orientations of neurons grown on the chip in the presence of EMF. The influence of substrate type and stimulation conditions on neuronal orientation deflection from EF lines was determined to be insignificant for each of the above-described cases. From the histograms and box and whisker plots, it can be seen that distributions of angles, their medians, and their spreads are highly uniform in all of the samples.

In the previous research, it was determined that electrical and topographical cues might be used to influence neuronal morphology [7, 8, 17]. Micro-patterned substrates were shown to promote neuron growth and improve axonal development [7, 8]. Additionally, the externally applied electric fields were shown to promote a specific type of growth in different types of neurons. In a large number of experiments, when cells were seeded on laminin-coated substrates, it was shown that neurite growth was enhanced towards the negative pole, cathode, and decreased towards the positive pole, anode [20, 23, 24, 25]. As micro-patterned substrates were shown to promote SGNs growth overall, it was expected to notice their positive influence on the dimensions of the cell body. The negative influence of EMF on neuronal soma radius was somewhat unexpected, as the above-mentioned result indicates that there may be a possibility of positively influencing growth using different types of EF field. However, the influence of EF on neurons depends on a large number of factors, and there wasn't any research that examined the morphology of SGNs exposed to circular EF. To confirm and better

understand obtained results, a larger number of experiments with different sets of initial parameters should be conducted.

Both micro-patterned substrates and electric fields were shown to have a high impact on neuronal alignment [7, 8, 20, 23, 25, 25]. Applied fields were also shown to redirect those neurites that were following previously set contact guidance cues [17, 26]. This implies that the electric fields might have a more substantial influence on the neuron alignment than other guidance cues. The SGNs examined in this experiment showed higher alignment on the chip than on the glass coverslips both with and without stimulation, which confirms results obtained in the previous research [7, 8]. As the applied field was circular, its effect on vertical deflection was not expected. This is confirmed when glass coverslips are considered, however, there is a significant difference in the orientations of neurons on the chip. As the chips contain electrodes in addition to pillars, there is a possibility of the interactions of electrodes and externally applied field that might influence the SGNs orientation.

As the neurons were shown to follow electrical guidance cues, it was expected that SGNs exposed to circular EF would align their neurites with EF lines. However, there was no statistically significant difference determined in the deflection angles from EF lines. Distributions of deflection angles were similar for all the samples. It is important to note that the electric field inside a coil is only circular in ideal conditions. In this case, the shape of the electric field could be influenced by the different materials placed inside the coil, such as a Petri dish, and different densities of neuronal cultures across the samples. All of the samples were placed in the same incubator, so there is a possibility that electric fields interacted, which should also modify the assumed circular shape. Additionally, since the high density of cells was seeded on all of the substrates, the large number of glial cells might have disrupted the effect of the externally applied EF field. Also, a large number of neurons in the samples were interconnected and, therefore, could not be analyzed.

One of the advantages of this experiment is the simultaneous analysis of multiple cues on neuron morphology and alignment. Also, all neurons were obtained from the same litter of rat pups, so there should be no initial differences between them.

Potential limitations are visible in selecting an appropriate sample, which significantly reduces the possibility of generalizing the results to the general population. Furthermore, as the normality was not assessed, multiple nonparametric tests were used, which indicates lower statistical power. Manually contouring of selected neurons may also have led to mistakes in soma radii and orientations.

In future experiments, it is recommended to use lower densities of cultures so that there is a lesser number of glial cells in the cultures, and the interaction between neurons and glial cells is minimized. Cultures should be grown for less DIV. This would also reduce the number of glial cells in the samples and would result in shorter neurites. This setup would allow the

examination of most of the neurons on the substrates using the computer algorithm, which would significantly reduce the possibility of human error in contouring and choosing the appropriate sample. Multiple densities and DIV should be tested to confirm the results. The effects of circular EF field should be investigated on glass coverslips first, to understand them better before introducing other guidance cues such as micro-pillared substrates. The specific shape of the electric field inside the coil should be determined as accurately as possible to define better quantitive measures of neuronal alignment with EF lines. Also, the effects of different shapes and amplitudes of the current and voltage on neuronal morphology, survival, growth, and alignment should be investigated.

5 Conclusion

The way topographical, chemical and electrical cues influence morphological properties of neurons is a growing area of research. Inducing the desired morphologies and specific alignments in SGNs using bio-compatible techniques might improve the overall effectivness of cochlear implants. In this experiment, the effects of substrate type and electromagnetic stimulation conditions on neuronal soma radius, orientation, and neuronal deflections from EF lines were investigated. It was shown that somas of the neurons grown on micro pillared substrates had larger radii as opposed to those grown on glass coverslips. Neurons grown in the electromagnetic field on both substrate types were shown to have smaller soma radii than the control group. The substrate type was shown to significantly influence the neuronal orientation in the presence of electric field. Also, the stimulation conditions were shown to significantly affect the neuronal orientation on the chips. No significant effect of substrate type and stimulation conditions on neuronal deflections from EF lines was determined. The experimental setup had several limitations such as the inability to analyze all of the neurons in the sample, and the manual selection and contouring of the cells. It is recommended to investigate this further and conduct additional experiments in the future, with better set initial parameters and a larger sample size, to describe the effects of the electromagnetic field on the spiral ganglion neurons more accurately.

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