COST EMF - MED (Action BM1309):
European network for innovative uses of EMFs in biomedical applications

STSM Report:

Studying the effects of electric field stimulation on the fate of neuron cell cultures

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Abstract:

This report describes the main scientific activities carried out during the present STSM in the frame of COST action EMF-MED. The goal of this STSM was to study the performance of different setups aimed to apply electrical stimulation to neurons in different scenarios. The objective of the mentioned electrical stimulation being to improve and to accelerate the regeneration of injured axons as a model of central nervous system injury. The results of the performed experiments indicate that the conceived devices are suitable platforms to study the effects of electrical stimulation and results suggest that electrical stimulation could have beneficial effects in the regeneration of neurons.

A. Purpose of the STSM

The main goal of this STSM was to setup and test the performance of two experimental platforms aimed to study the effects of electric field stimulation in the regeneration of neurons in an in vitro model. The objective of the present study is to gather information for the development of a future implantable device for the enhancement of nervous system regeneration in adult mammals.

Two different approaches were tested in parallel:

1. A first setup consisting in a scaffold tunnel-like platform where neurons were seeded and electrically stimulated. Dorsal Ganglion Roots (DRGs) were cultured in combination with Schwan cells. Fig. 1 depicts the complete system prepared for stimulating DRGs. The scaffolds were placed in Polydimethylsiloxane (PDMS) chamber that included platinum wires for the electrical stimulation. The chambers were connected to a custom made current source based on operational amplifiers and biphasic electric pulses were generated using an Agilent 33220A arbitrary waveform generator commanded by a Labview software.

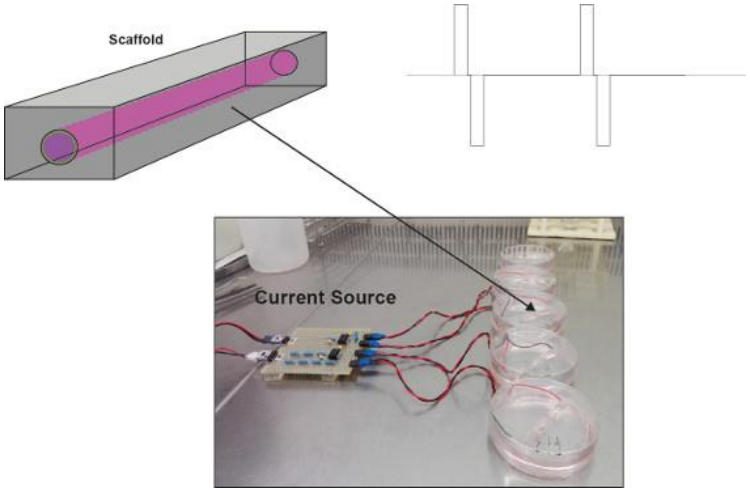

Fig. 1 Schematic of the system for the electrical stimulation of cells in scaffold.

2. The second setup consisted of a microfluidic chamber-like structure including channels through which axon elongation of individual neurons can be essayed. In Fig. 2 a 3D schematic is shown. The micro chamber was fabricated in a clean room facility by soft lithography. PDMS was patterned using a SU-8 master fabricated using standard procedures. The system consists of three isolated chambers and micro channels that connect them.

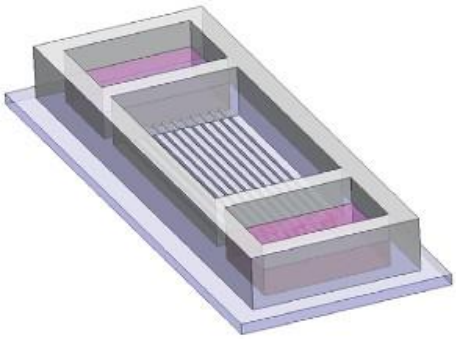

Fig. 2 Microchamber for the culture of individual neurons

B. Work Description

Once the devices were prepared in advance in the Home laboratory, the idea of this STMS was to test their performance with cells.

- **Task A:** Optimization of electric field conditions in scaffold.

Given the fact that neurons would be co-cultured with Schwan cells (SC) during electrical stimulation, the first task consisted in testing the impact of electrical stimulation to these cells. 10 days previous to experiments SC cells were cultured in the scaffolds following the protocols already developed in the Host institution. This allowed cells to form a tube-like layer covering the inner walls of the scaffolds [1].

Electrical stimulation was performed during 24 or 48 hours with different electric field intensities.
Proliferation/viability assays were performed to assess the state of cells.

- **Task B:** Electrical stimulation of Dorsal Ganglion Roots (DRGs) in scaffold.

Similar to previous task, 10 days previous to experiments SC cells were cultured in the scaffolds. The day of experiments, DRGs were extracted from rats and co-cultured in the scaffold with SC. Cells were stimulated during 7 days and results were assed only 48 h after the end of stimulation.

- **Task C**: Optimization of culture conditions for the micro chamber setup.

This task consisted in the observation of the response of dissociated neurons coming also from DRGs in the micro chamber system. No electrical stimulation was performed as the objective was just to study if cells were capable of normally grow in the device.

C. Results

A- Response of Schwan cells to electrical Stimulation:

The goal of the first set of experiments was to fix the limits of electric current intensity applied to cells that would preserve their viability and proliferative capability. Four different current intensities were assayed following a fixed stimulation protocol consisting in 100 µs biphasic pulses applied in bursts at a fixed frequency. After the period of stimulation cells were left for an additional 24-hour period before assessing viability. LIVE/DEAD® Viability/Cytotoxicity Kit (Thermofischer) was used to differentiate between live (green) and dead (red) cells. After staining, scaffolds containing Schwan cells were imaged in a confocal microscope. Fig.3 shows representative micrographs for the control condition, where cells were not stimulated, and the condition corresponding to the maximum current applied to cells. It can be observed a slightly increase in cell mortality in the case of electrically stimulated cells indicating that the corresponding intensity applied to cells may have harmful effects to cells. In the case of the other intensities applied cells preserved a good viability comparable with the levels observed in controls. Interestingly, as observed in Fig.3, it is suggested a possible change in the shape of Schwan cells due to stimulation. This should be studied in a more detailed way in future experiments.

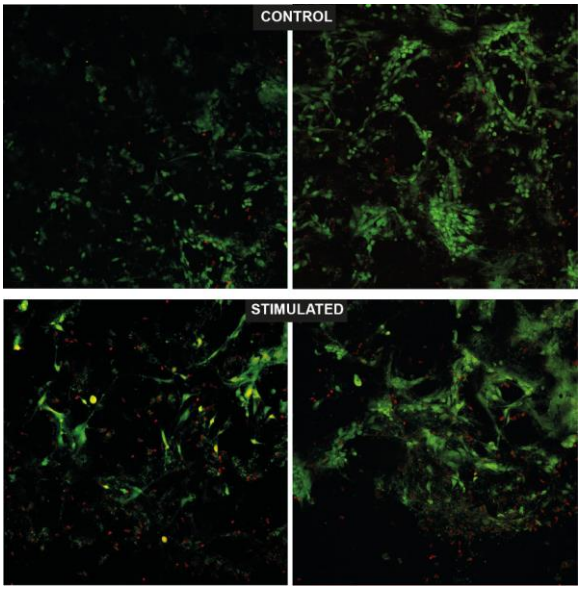

Fig. 3 Confocal images of Schwan cells in the scaffold after electrical stimulation. Alive cells are stained in green while dead cells are marked in red.

B- Axon elongation of DRGs submitted to electrical stimulation in co-cultured with Schwan cells:

Once the limits for the range of electric current intensities applied were fixed using Schwan cells, the next step was performing the complete experimental procedure including the DRGs. DRGs extracted from young rats were placed in the scaffold system containing the Schwan cells and were stimulated with two different electric current intensities. Ten days after the begging of the experiment (1day of initial culture + 7 days of electric stimulation + 2 days of post-stimulation culture) DRGs were fixed and immunostained using Tuj 1 (Neuron-specific class III beta-tubulin) marker in combination with Hoescht, subsequently the complete scaffold system was imaged in a confocal microscope. Fig.4 shows a reconstruction of confocal images along the scaffold for the control condition (no stimulation) and the two intensities applied.

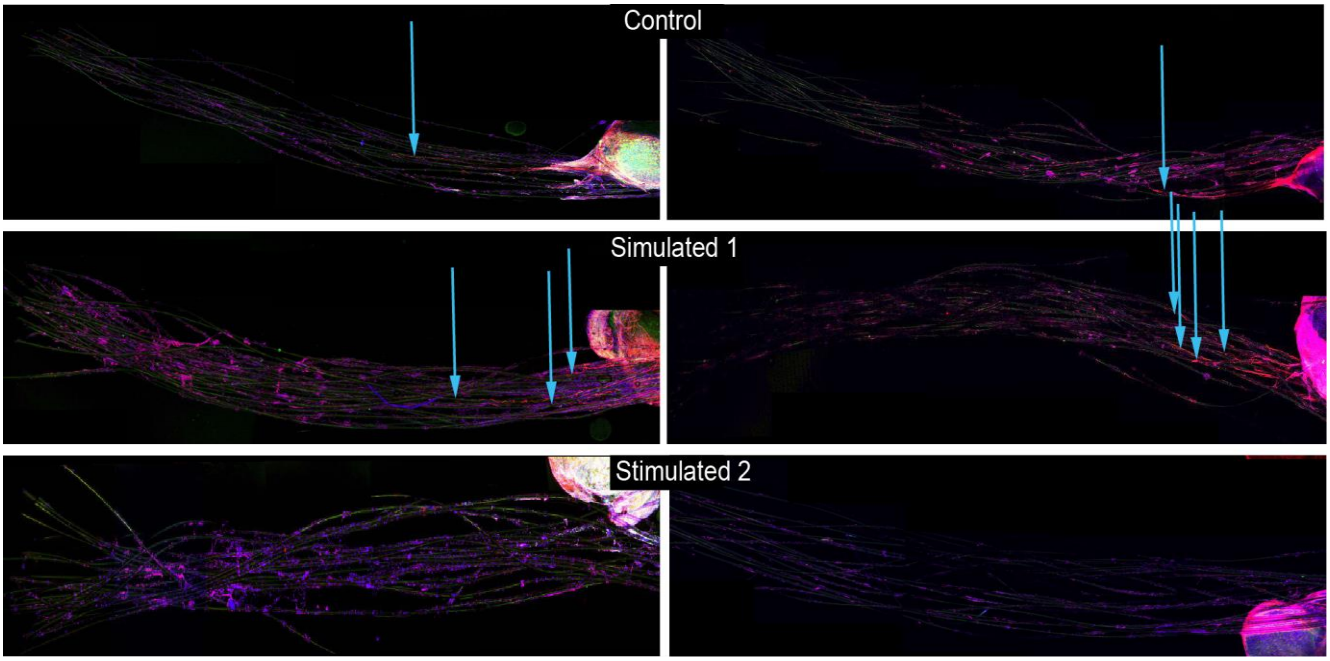

Fig. 4 Confocal reconstruction pictures from the scaffold. The DRGs were placed on the right of the structure. Hoestch staining (in violet) was used to detect the presence of Schwan cells, Tuj-1 (in red) was used to mark the elongated axons, finally, in green the autofluorescence of the scaffold is observed. Blue arrows indicate the presence of axons.

According to these results, it is suggested that compared to the control, where very few axonal projections were observed, in the first stimulated condition (stimulated 1) the number of axonal projections was significantly increased. On the contrary, in the second condition (stimulated 2), where the current applied was three times higher than in the other one, the axonal projections were inhibited. Additionally, in both stimulated conditions, it is observed that the number of Schwan cells was increased.

These preliminary experiments demonstrate the feasibility of the system to be used in future electrical stimulation studies that will be performed in collaboration between the three participating groups.

C- Individual neurons in micro chamber:

The first test performed with neurons in culture in the micro chamber showed that cells are capable of growing in the device. However, some aspects of the system should be optimized in order to its definitive use in electrical stimulation experiments. Concretely, the system should be perfectly sealed in order to prevent cells to escape the channels. Additional tests for optimizing these culture conditions are seek to be performed in the Host institution during the next months.

D. Future collaboration with host institution

This STSM was the perfect opportunity to start a collaboration aimed to be extended in time. The preliminary results obtained encouraged us to go further in this study. Additional experiments will be performed with the devices developed in the frame of this STSM what will require a continuous communication channel opened between the different participants in this project. Future visits from members of the different laboratories are also foreseen. Furthermore, some of the ideas addressed during this collaboration could be part of future funding demands in the frame of European or other competitive project calls.

E. Expected Publications

If the results from subsequent experiments needed to confirm the first results described herein are satisfactory, the study performed would be object of at least one scientific journal publication. Also the results assessed could be part of presentation in scientific conferences.

Confirmation by the host institution of the successful execution of the STSM:

We confirm that Tomas Garcia Sanchez has performed the research work as described above.

Contact Person of Host Institution	Name of researcher
Victoria Moreno Manzano	Tomas Garcia Sanchez
Signature	Signature