In-vitro Peripheral Nervous System on a chip

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Abstract

Neurological diseases affecting the Peripheral Nervous System (PNS), such as some Neuromuscular Diseases (NMD), often involve the afferent and efferent pathways of the PNS. Studying them requires mimicking the physiological microenvironment. The aim of this research is to use organs-on-a-chip technology to mimic the physiological microenvironment of the PNS, including myelination of Dorsal Root Ganglia neurons (DRGs) and motoneurons (MNs), providing the basis for future steps toward NMD study models. For that purpose, we first studied the viability of SW10 mouse Schwann cells when exposed to the media required for the primary culture of MNs or DRGs, observing SW10 viability decreased. These results suggested that a compartmentalised culture system is required. Schwann cells were also exposed to different biochemical stimuli to induce faster myelination, obtaining Horse Serum as a key component in the culture medium. A compartmentalised microfluidic device was fabricated, obtaining myelination of the PNS, seeding in different compartments Schwann cells with pro-myelinating medium with either primary MNs or DRGs. The afferent pathway of the PNS was successfully mimicked, whereas we faced some difficulties in working with MNs and analysing myelin sheath formation through transmission electron microscopy. To sum up, we have designed a PNS model that still needs to be optimised to better mimic the efferent pathway. This model provides the basis for further research on PNS and offers the possibility of an in vitro tool PNS and NMD diseases models.

1. Introduction and background

Neurological disorders affecting muscles and/or their control through nervous system, known as Neuromuscular Diseases (NMD), comprise several diseases with a different anatomical origin [1]. The prevalence range of 24 of these diseases is a total of 160/100.000 population (ranging from 0.1/100.000 to 60/100.000), reaching similar prevalence values to Parkinson's disease worldwide [1]. Conventionally these diseases have been studied *in vitro* coculturing muscle cells and neurons on the same dish [2,3], culturing tissue slices [4], or culturing neurospheres onto muscle fibres [5]. However, these models do not consider that: i) muscle and neurons are physically separated *in vivo* in and have different microenvironment requirements; ii) both sensory and motor neurons can be altered in particular NMD [6]; iii) glial cells are also affected and involved in several neuromuscular pathologies [2,7].

Organs-on-a-chip represent an alternative approach to overcome these problems. Organs-on-a-chip are microfluidic 2D or 3D cell-culture devices designed to mimic the microenvironment of functional units in organ or tissue level: multicellular architecture, tissue-tissue interfaces (flows and barriers), physicochemical microenvironments (chemical gradients, mechanical strain and electrical stimulation) and pathophysiology. Neuromuscular junction (NMJ) on a chip has frequently been studied by means of microfluidic platforms with two compartments connected through microchannels, either culturing motoneurons and skeletal muscle cells [8], or motoneurons and a myoblasts cell-line [9]. However, few of the currently existing studies suggest the use of a third chamber for myelination and synapsis assessment [10]. In addition, most of them do not consider i) putative glia roles, ii) do not mimic accurately biological or physiological conditions, and iii) to date, a whole motor unit circuit (*Figure 1*) is not reconstructed. In the model, motoneurons (MNs), arising from the Central Nervous System (CNS) spinal cord, innervate skeletal muscle cells connecting through neuromuscular junction ("efferent pathway"), and Dorsal Root Ganglia (DRGs) form a muscle spindle and send the signal back to the spinal cord ("afferent pathway"). Therefore, there is a need of a more accurate study model able to mimic the events taking place in NMD to find new therapies.

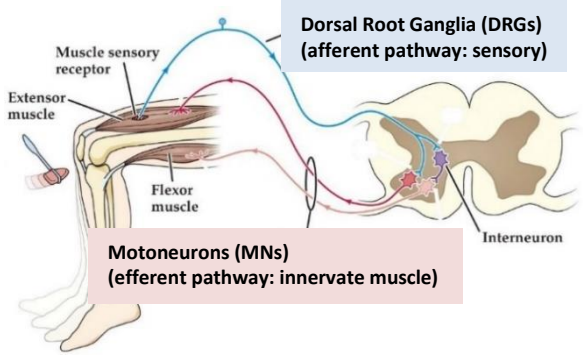

Figure 1. *Motor unit (adapted from Purves et al.*[11]*).*

The aim of this research was to mimic both afferent and efferent pathways of the PNS separately in a microfluidic device, providing preliminary data for further studies on

motor unit study models so as to find new therapies for NMD. For that purpose, the objectives were: to design, fabricate and characterise a microfluidic device; to create a PNS glia GFP positive cell line; to assess its viability when exposed to the media required for the primary culture of neurons (MN or DRGs); to induce its myelination; and to finally mimic the afferent and efferent pathways of the PNS on a chip.

2. Methodology

2.1. Microfluidic device

The microfluidic cell culture device is fabricated in poly-dimethylsiloxane (PDMS) by standard photolithography and soft lithography techniques as previously described [12]. The design (*Figure 2*) includes three microchambers, connected through microchannels, and supplied by a medium reservoir. The platform characterisation is performed through routine optic microscopy observation, profilometry, Scanning Electron Microscopy, and fluorescein assay to test microchannels connectivity.

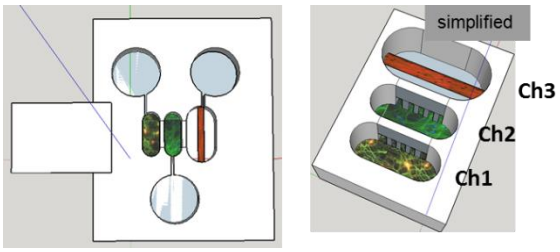

Figure 2. *Design of the microfluidic device (not scaled proportionally).* ***Ch1*** *includes MNs or DRGs somas.* ***Ch2*** *mimics the PNS, including axons of MNs or DRGs and myelinating Schwann cells.* ***Ch3*** *mimics a muscle fibre and its connexion with neurons.*

2.2. Cells

Primary MNs or DRG neurons together with an established Schwann cell line (ATCC, CRL-2766) were used for the experiments. DRGs were harvested from 6-8 weeks old adult mice and MNs were harvested from E12.5 mice.

As indicated, an eGFP transgenic mouse Schwann cell (SC) line was used. SW10 cells were infected with an eGFP positive lentivirus (pLenti-CMV-MCS-GFP-SV-puro plasmid, Addgene) and were purified through Fluorescence Activated Cell Sorting (FACS), obtaining a cell line of GFP positive Schwann cells (SW10-GFP$^+$).

2.3. Glia viability assay

To assess the viability of SC after MN media diffusion, SC were seeded on SC media: DMEM, 1% penicillin-streptomycin, 1% sodium-pyruvate, 10% foetal bovine serum (FBS). Two days later the media was changed to MN media: 95% Neurobasal media, 2% B27, 2% horse serum (HS), 1% penicillin-streptomycin, 0.025% Glutamine, 0.02% GDNF, 0.02% CNTF, 0.5% cytosine arabinoside (AraC) 1.5µM. A Propidium Iodide viability test was performed on Schwann cells as previously reported [13], together with a morphology analysis for a period of 5 days, using as control SW10 cultured with SC media.

In order to verify the viability of Schwann cells with DRG media (DMEM F12 1:1, 1% penicillin-streptomycin, 2% B27), a morphology analysis was performed, with SW10-GFP$^+$ cells, followed by a viability assay through morphology analysis with DRGs media plus different nutrients to find out the essential media requirements.

2.4. Myelin protein production analysis

To find a pro-myelinating medium, different media compositions were tested on SW10-GFP$^+$ cells: **1)** SC media; **2)** SC medium + horse serum (HS); **3)** SC medium + AraC; **4)** SC medium + HS + AraC; **5)** DRG medium + FBS; **6)** DRG medium + FBS + HS; **7)** DRG medium + FBS + AraC; **8)** DRG medium + FBS + HS + AraC; **9)** DRG conditioned medium (DRG medium with the secretome of DRGs obtained over 24h culture and filtered at 0.2µm) + FBS; **10)** DRG conditioned media + AraC. Cells were lysated at 1-4-7 DIV (days in vitro) and protein lysates were analysed through Western Blot.

2.5. Peripheral nervous system (PNS) on a chip

To mimic afferent and efferent pathways of the PNS separately, DRGs or MNs were cultured in *chamber-1* (*Figure 2*) or petri dishes for controls at a density of $2{\cdot}10^4$ cells/cm^2 or $1{\cdot}10^5$ cells/cm^2 respectively. In both cases, once neurons crossed the microchannels, after 12 DIV, SW10-GFP$^+$ cells were cultured on the *chamber-2* with promyelinating medium at a density of $1{,}5{\cdot}10^3$ cell/cm^2. Cells were fixed after 2, 5 or 7 days of coculture (total of 14, 17 and 19 DIV for the primary culture) and immunostainned against TUJ1 and MBP proteins.

A transmission electron microscopy (TEM) analysis was performed on cultures of DRGs and SW10-GFP$^+$ cells seeded on petri dishes fixed after 17 DIV (5 days of coculture) to look for myelin sheath formation.

3. Results

3.1. SW10 viability assay

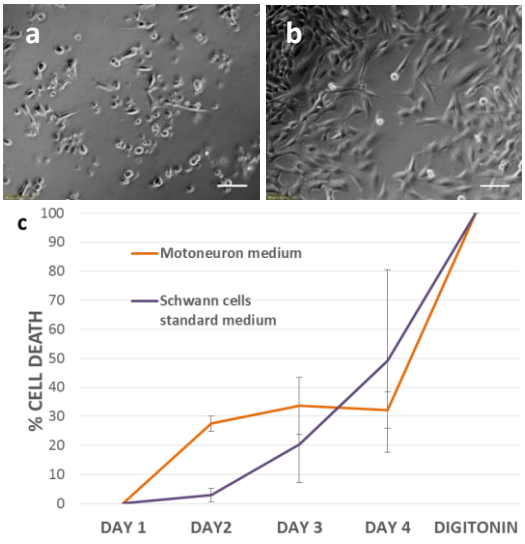

Figure 3. *Viability test performed with SC in MN media. Scale bar: 100µm.* ***a)*** *SW10 cells cultured in MN media after 2DIV.* ***b)*** *Cells cultured in SW10 culturing SC media after 2DIV.* ***c)*** *Propidium iodide analysis over 4 days, where average and standard deviation are shown for n=3.*

The viability test performed showed that MN media induced higher cell death in SW10 cells, as compared to its own standard medium, especially in the first 24h (*Figure 3*). Results of the viability test performed with SW10-GFP$^+$ cells in DRG media show that they do not survive (*Figure 4b*). When culturing on DRG media with different nutrients from the SW10 culturing standard media, it can be seen that they only survive when adding FBS (*Figure 4e*).

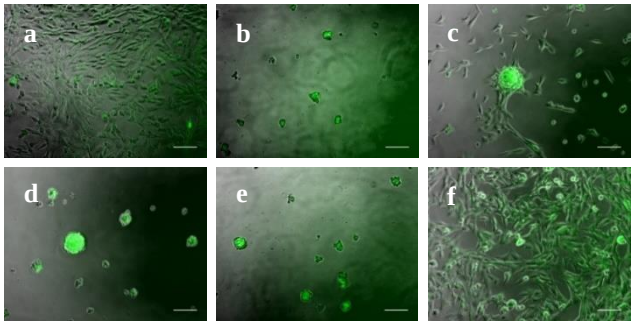

Figure 4. *Viability test performed with SW10-GFP$^+$ cells in DRGs medium. Scale bar: 100µm.* ***a)*** *Cells cultured on SW10 culturing standard media.* ***b)*** *Cells cultured on DRGs media.* ***c)*** *Cells cultured on DRG media +1% standard media.* ***d)*** *Cells cultured on DRG media + 1% Sodium pyruvate.* ***e)*** *Cells cultured on DRG media + 4,5mg/mL glucose.* ***f)*** *Cells cultured on DRG media + 10% FBS.*

3.2. Myelin protein production analysis

The promyelinating medium test tried in SW10 cells show that all media tested containing AraC 1.5µM induced cellular dead after 2DIV (results not shown) and all conditions that included HS in the media induced higher MBP production levels, when compared to same media without HS and to DRG conditioned media used (*Figure 5*).

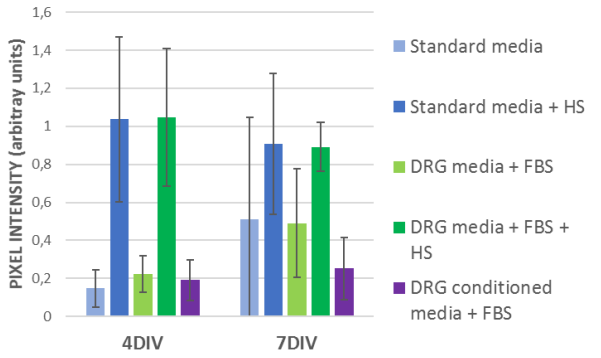

Figure 5. *Quantification of Western blot results. Myelin base protein (MBP) expression levels produced by SW10-GFP$^+$ cells under different experimental media conditions. MBP pixel intensity arbitrary units average and standard deviation are shown on tested conditions at 4 and 7 DIV for n=3.*

3.3. Peripheral nervous system (PNS) on a chip

The afferent pathway of the PNS was successfully mimicked, obtaining myelinated DRG axons by Schwann cells, as well as myelin sheaths (*Figure 6*). Apposed membranes were also detected in TEM, but classic myelin formation was not observed (*Figure 7*).

The efferent pathway of the PNS was not successfully mimicked as MN died after 14 DIV (2DIV coculture), not providing enough time to induce myelination under tested conditions (not shown).

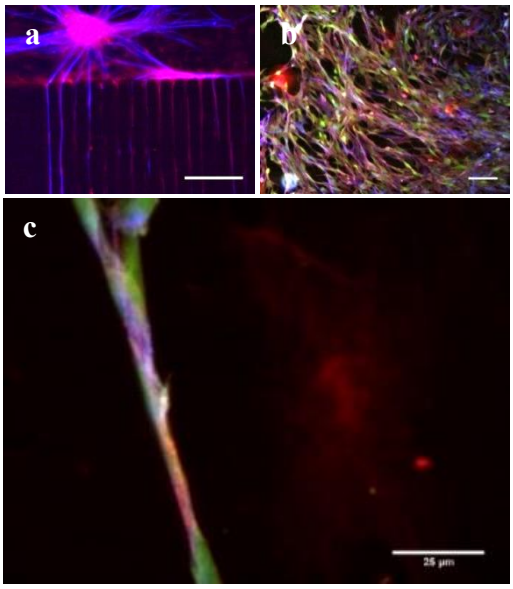

Figure 6. *Immunostaining images of afferent PNS-on-a-chip after 19DIV (7 days cocultured). MBP was stained in red and TUJ1 in blue.* ***(a)****DRGs seeded in chamber-1* cross *microchannels towards chamber-2),* ***(b)*** *where Schwann cells (SW10-GFP$^+$) myelinate the axons, (scale bar 100µm).* **(c)** In few cases, a myelin sheath formation was observed *(scale bar 25µm).*

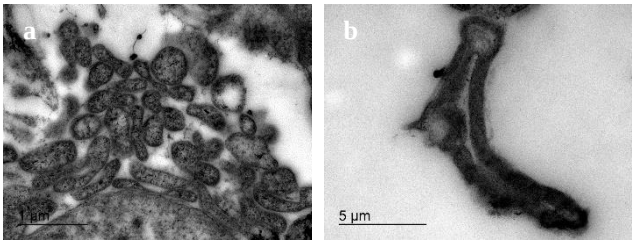

Figure 7. *TEM analysis results of the DRGs and SW10 coculture after 17 DIV (5 days of coculture).* ***a)*** *Transversal cut of the axons (scale bar 1µm).* ***b)*** *Longitudinal cut of an axon (scale bar 5µm).*

4. Discussion

Organs-on-a-chip present a promising alternative to conventional *in vitro* study models. The platform designed here offers the opportunity to culture in 2D different cell types, connecting them through microchannels and mimicking the Nervous System microenvironment. Additionally, the compartmentalisation of the device and the fluidic resistance diminishes the media diffusion from one chamber to the other, avoiding possible cell damage caused by undesired components in the media, as shown in *Figure 3* and *Figure 4*.

Results from the myelination induction study performed here show that DRG media + FBS + HS could be an alternative media to induce faster myelination (*Figure 5*), providing suitable conditions to DRGs, and the minimum FBS required to Schwann cells (*Figure 4*). However, AraC,

suggested as inhibitor to decrease proliferation, is toxic at a concentration of 1.5µM (*Figure 3*).

PNS-on-a-chip was successfully mimicked for the afferent pathway, obtaining myelination as well as formation of myelin sheaths (*Figure 6*). Although, appossed membranes were observed in TEM, classical myelin sheath was not observed (*Figure 7*).

We are currently working on fine tuning the parameters to establish the model with the efferent pathway using same system described for the afferent pathway. In addition to present data, several studies coculturing primary MNs and primary Schwann cells achieve myelination and synapse formation in long term cultures [2,14,15]. Some studies that coculture DRGs and primary Schwann cells also report successful myelination [2,16]. However, working with cell lines together with primary cells represents a challenge due to different proliferation rate as well as media requirements. Most studies that culture MN or DRGs with primary SC, perform cocultures [2,14–16]. Compared to them, the study model presented here separates physically somal and axonal compartments, offering the possibility to: i) have different medium supply for each compartment; ii) stimulate or assess differentially axonal and somal compartments; iii) adapt the study model for NMD, mimicking better physiological conditions, where somas are clustered and away from axons, distal to muscle fibers.

Our model represents first steps towards a complete miniaturisation of a PNS sensorimotor unit on a chip. We believe this is a key element in order to obtain a fully functional *in vitro* model that will facilitate the study and characterisation of both the afferent and efferent pathways of the PNS and their roles in Neuromuscular Diseases.

5. Future steps

The work here presented provides the basis for further steps toward NMD study models on microdevices. Future work for this research includes to successfully recreate the efferent PNS pathway on a chip. Afterwards, muscle cells will be included to complete the motor unit model.

Acknowledgements

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