Neuronal circuits on a chip for biological network monitoring

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Abstract

We report a novel Cultured Neuronal Network on a chip as a viable alternative to current culture methods for the analysis of neuronal network formation and evolution of the structural properties of the network's graph . This innovative microfluidic chip fabricated from Polydimethylsiloxane, vinyl, and glass offers worthy features such as the posibility to develop and produce custom designs effortlessly, easy handling and monitoring, culture medium renewal, and reducing the exposure to contamination. Due to these benefits, longer survival of the neuronal networks are observed in comparison to conventional methods. In addition to the fast and cost-effective manufacturing of the chip, this technology provides a significant step forward in the studies of neuronal network development and many challening applications such as drug testing of in vitro cell culture models .

1 Introduction

One of the remaining questions in Neuroscience is how the Central Nervous system self-organizes into an extremely complex system. Among the several perspectives and approaches undertaken to understand this process, in vitro neural cultures have become a powerful tool which allows a very controlled environment, in contrast to in vivo animal models. Although cultured neuronal networks (CNNs) have their own limitations and are very simplified models, they still retain fundamental structural and functional features that can be extrapolated to in vivo models, as memory or connectivity, and are a significant tool in drug testing.

The importance of this last task triggers the need of producing large amounts of equivalent cultures that can be created in the same conditions and simultaneously controlled. For this purpose, recent advances in integration of microfluidics and micro/nanoengineering have allowed the development of a new concept in cell culturing: the organ-on-a-chip. The aim of this microfluidic device is to contain and replicate an organ or tissue structure with its physiology, dynamics and functionality. In recent years, several organ-on-a-chip devices have been reported with different organs as a target, such as lung, liver, heart, kidney, intestine, among others.

In the case of the neuroscience field, microfluidic devices have been used to improve neuronal cultures especially in the Blood-Brain Barrier models. Microfluidic techniques allow precise control of the cell environment, creation of concentration gradients, the delimitation of the cell seeding area, accurate control of the cell density and the possibility of stimulating specific regions of the culture. Besides, the use of microfluidic based devices enables a cost reduction compared with traditional culturing methods because the reagent volumes (culture medium, cell density, drugs, etc.) are significantly smaller.

This technology has been employed in neuronal cultures as well for synapse studies, designing of predefined neuronal connectivities, analysis of neuronal connectivity, neuron polarity or the study of intracellular dynamics in neuronal networks.

The microfluidics chip designs of these previous CNNs usually incorporate a similar fabrication process and material selection based on soft lithography using polydimethylsiloxane (PDMS) for the microfluidic chambers, often combined with a substrate of microelectrode arrays (MEAs). PDMS offers several advantages

for microchip manufacturing: it is transparent, flexible, gas permeable, biocompatible, autoclavable, cost-effective and it can protect electronic components. However, soft lithography PDMS process has the mayor inconvenience that any change in the microfluidic design needs a new mould and a new pattern, which slows down the optimization process and testing stage of the microfluidic chip. For this reason, it is needed to search and test new materials in the fabrication of microfluidic chips. In this work we present for the first time a new microfluidic device fabricated from PDMS, vinyl and glass for the study of neuronal circuits development using optical microscopic techniques in a microfluidic device and compare the resulting networks with those grown in a Petri dish as previously reported in Refs.. Besides the abovementioned micro-nano fabrication benefits, in this article we report the behaviour of this novel CNN on a chip to monitor biological networks in a most effective way, offering excellent key performance indicators such as avoidance of contamination, efficient culture medium renewal, higher life expectancy of neurons, increasing longer the period of observation, portability and easy handling and monitoring of the neuronal network formation process; which make this new technology for cells and tissue culture engineering a promising alternative to the conventional methods reported in the scientific literature.

2 Materials and methods

2.1 Design

The designed device is a monolithic chip manufactured from glass, vinyl, and PDMS (Fig. 1). Glass provides tightness and easier handling of the chip; vinyl defines the microfluidic chamber pattern and PDMS seals the device on the top and enables a correct fit for the tubing. The chip is integrated inside the microfluidic system through two PVC tubes attached to the inlet and outlet channels.

2.2 Innovative fabrication

PDMS was fabricated from SYLGARD 184 with a ratio of 10:1.5 (elastomer base: curing agent). The mixture was poured to and cured by moulding and thus obtaining a rectangular block of PDMS with dimensions of 50 x 25 x 2 mm.

Vinyl offers a fast manufacturing of microchips with different layouts. This feature allows us to test different variants for the same application and check which one has greater performance.

Mactac vinyl sheets of 75 µm thick were cut with a cutter Summa S Class in two different layouts (Fig. 1A). A first layout (B) contains a circular area (radius ˜5 mm) that delimits the area where the cells will be seeded, and a second layout (A) that adds to the main circular area two smaller holes intended for inlet and outlet flow channels that facilitates microscopic biological network observation, while avoids a direct incidence of the flow on the adhered cells. Both layouts A and B include 4 circles around the main design as an alignment guide for the vinyl sheets to overlap and thus obtain the required thickness. The microfluidic device is robust enough to preserve the water tightness by itself without the need of external fittings.

2.3 Assembly

For a single microfluidic chip, 1 vinyl sheet with design B and 20 sheets with design A are overlapped to create a final single piece of vinyl 1500 µm thick, which sets the depth of the microfluidic device. Afterward, this piece is stuck onto a glass slide. To avoid leaks between vinyl and glass, pressure and temperature are applied to the piece (47 KPa, 80°C). To complete the assembly process, the chip is sealed with a PDMS block. The PDMS block must be previously cleaned with isopropanol and exposed to an oxygen plasma treatment (50 Watts, 120") to hydrophilize the surface which is essential to seal the PDMS with the vinyl. After the plasma treatment the PDMS block is placed above the vinyl and the glass slide and the complete chip is assembled. This monolithic chip is reusable and autoclavable.

2.4 Microfluidic Platform

During the culture life, the chip is connected to a perfusion pump that provides a continuous workflow of 0.1 µL/min. The pump is charged with a syringe of 1 mL.

2.5 Biological sample obtention

The CNNs grown inside the microfluidic chip are primary cultures of dissociated neurons from the frontal ganglia of *Schistocerca gregaria* locusts. This model is widely used as it shares neuronal basic features with vertebrates while having larger neurons. This last feature makes our model an advantageous alternative to mammals for the study of structural details of the neuronal network. Locusts are stored in a cage at 30°C with 12:12 night-day cycle and daily fed with grass.

CNNs are obtained following the protocol described in Ref.. Each adult locust is dissected to extract its frontal ganglion, which contains approximately 100 neurons. The connectivity tissue of the ganglia is removed by enzymatic and mechanical procedures to obtain a suspension of dissociated neuron somas in Leibovitz's L-15 medium supplemented with L-glutamine (Sigma Aldrich/Merck) and 0.01 % penicillin-streptomycin (Biological Industries, Israel). The medium is furthermore enriched with 5% locust hemolymph as a growth factor for the neurite development. To inhibit the damaging melanin production, the hemolymph is heated at 60°C for 1 h, then frozen at -20°C, and finally filtered and centrifugated before being added to the medium.

2.6 Functionalization & cell seeding

To ensure the correct attachment of the neurons to the chip, the culturing surface is functionalized with a mix of Concanavalin-A (Sigma Aldrich) and poly-D-lysine (0,016 mg/L) (Sigma Aldrich) in a final solution of 70 µL. The functionalization mixture is pipetted into the chip through the inlet hole, and it is maintained at 37°C for 2 hours. After functionalization, the chip is washed 3 times with distilled water and sterilized for 30 minutes under UV light.

Finally, the suspension of disaggregated neuron somas is pipetted into the chip through the inlet hole. After this cell deposition, the chip is left for 2 hours before connecting the microfluidic chip to the microfluidic platform.

2.7 Maintenance & Monitorization

The neurons on a chip are incubated in darkness for 22 days in vitro (DIV) under controlled temperature (29°C) and humidity (70%). After seven days it is needed to refill the microfluidic system with another syringe. The used workflow is estimated from a previous work in the study of neural network formation on top of typical plate cultures.

The data reported in this study correspond to 2 cultures grown under the same conditions. Culture visualization and Network process monitorization are performed using a charge-coupled device camera (DSFi1, Nikon) mounted on a phase-contrast microscope (Eclipse TiS, Nikon) with a 10x air objective (Achromat, ADL, NA 0.25) and an automated motorised XYZ stage controller. Mosaic images are captured in a daily basis with the large image method implemented in NIS-Elements software, which does automatic blended stitching with an overlap of 25%.

2.8 Image segmentation & Structural network analysis

During the culture lifespan, the neurons grow new connections and self-structure themselves into a complex network, whose structural evolution is the objective of the study. Therefore, from the large-scale photographs acquired during the monitoring days, we extracted the connection scheme and mapped it into a graph using an image segmentation algorithm coded in MatLab to identify both the neurons and their connections. The whole procedure is illustrated in Figure 2. Panels A1-3 show the growth evolution of a small part of the whole culture between day 1 and 15. Panels B1-3 show the result of the image segmentation algorithm after detecting neurons (and their aggregates) in red colour while the segmented neurites are marked in green. This information is used to construct a connectivity graph (panels C1-3) where nodes (green circles) are single neurons or aggregates and links (blue connecting lines) are either direct paths between nodes or through a branching path.

Our aim is to compare the evolution of CNNs cultured on a chip to those cultured on Petri dishes as previously reported in Refs.. The network evolution involves the creation of new links connecting nodes over

time. We monitor the progress of several commonly studied graph parameters such as the average clustering coefficient (C), which accounts for how densely connected is each local vicinity of the network, and the shortest path length (L), computed in the largest connected component, that quantifies how far are the nodes in the topological sense. These two last parameters are used as an indicator of the balance between the local and long-distance connectivity in the network.

3 Results & Discussion

Typical CNNs experiments in Petri dishes usually concentrate the cell population in an approximately circular area (radius ˜5 mm), which had been previously coated for cell adhesion. However, this procedure does not allow an accurate selection of the area to be functionalized, which finally results in a certain degree of cell dispersion and the reduction of the cell concentration in the area of interest. The custom design of our chips permits different shapes and sizes to develop the desired areas to grow cells. The work area contention is achieved by designing and fabricating just a circular area connected to the inlet and outlet entries.

We conducted two culture on chips experiments along this study. In our experiments it was successfully observed that the microflux drag force does not affect the cells adhesion and their uniform distribution. As expected, neurons moved slightly to form neuronal aggregates, but they were heavily attached to the surface, and no cell loss or detachment due to a failing in the coating or the flow were observed.

Continuous flow can be regulated to supplement the amount of fresh medium wanted. Although the flow was able to drag small particles and debris, larger dissociation particles as void ganglia capsules were not removed. The flow also washed off particles and toxic metabolites products of cellular activity, as melanin, particularly important in cell cultures from insects when hemolymph is added to the medium and whose accumulation can be cytotoxic due to phenol oxidase activity and can damage the cells. Consequently, the CNNs developed in the chip survived longer, at least 22 days, than the ones grown on Petri dish (up to 14-18 days) , where the medium is not changed. In fact the CNN was still connected in a good conditions until that day.

Another point to analyse was the homogeneity of the flow distribution. It has not been observed greater process of detachment of the cells in the most peripheral areas of the chip, and in addition, network processes have also been formed in these areas of the chip, so it seems that the flow has been homogeneous in all the culturing volume.

In experiments where the culture status evaluation and measurement are done by means of optical microscopy, the microfluidic device design must fulfil several requirements. In addition to be transparent enough, the chip dimensions must fit the microscope holding plate. Also, the overall thickness must allow to focus the culturing surface, and therefore must fit the range imposed by the microscope focal.

Once both requirements are met, the new neuron-on-a-chip culture is connected to a microfluidic system with hemolymph enriched medium, as explained above. The chip is monitored for at least 18 days following the described observation protocol.

The longitudinal observation of the development of the CNN, as reflected in Fig. 3, seems to follow a similar path as reported in our previous works. Isolated neurons in the first day in vitro (Fig.2 A1) started to grow neurites and being linked to their neighbouring neurons around 3-6 days of culture, getting closer among to form clusters. At DIV 7 (Fig.2 A2), connections among farther neurons are observed and junctions previously formed are more entrenched. In this step we observed an increase in the number of connections and a larger network is settled. After this stage the networks grow, forming bigger clusters, until the CNNs settled as a consolidated and mature network at day 15 of culture (Fig.2 A3). No significant changes in the network were observed beyond this point under our observation until day 22.

In order to properly quantify the different network states with the tools of graph theory, the resulting graphs from the segmented mosaics (Fig.2 B1-3) were mapped into adjacency matrices (Fig.2 C1-3) whose elements a_{ij} are equal to 1 if nodes i and node$j$ are connected and 0 otherwise. Results of the evolution and development of the graph of the two CNNs grown on chips have similar trends to those grown on Petri

dishes with similar initial density. As shown in Fig.3A, the number of connected nodes, nodes having at least 1 connection, increases until the 6-7 days in vitro, as a result of the neurite growth and connections being made. After this stage, the network development is steady, and in fact, there is a slightly decrease of the number of connected nodes because when connected neurons form clusters, the segmentation algorithm identifies them as a single node.

Other network properties we focused on were the longitudinal progression of the averaged clustering coefficient (C) and of the shortest path length L, normalised by the size of the largest connected component SGC[1], L / SGC (Fig. 3B). Regarding the normalized shortest path, high values were observed at the start of the experiment. Around 4-6 days in vitro, this value decreases, as links between nodes are formed. Beyond that, L/SGC keeps a low constant value in the more mature stage. Clustering coefficient shows near 0 values at the start, as expected as there are no connections between neurons. In the critical stage of network formation, around 3-6 days in vitro, C increases revealing the new connections between neurons. When the mature network is settled, the C value keeps constant at a relatively high value indicating the presence of loop circuits in the network.

Both values, low shortest path values, and the high clustering coefficient are studied frequently as characteristics of a small world structure, where the high tendency to form clusters of nodes in highly interconnected subgroups and short distance between them contribute to an optimal functionality in the network based in high efficiency. These results are very similar to the ones obtained in Petri dishes mentioned above, with no significant alteration, so we can confirm that the use of our chips to grow optimal functional CNNs with the desired parameters not only does not affect their development but they promote a longer network survival.

Concluding remarks

As a first attempt to study the performance evaluation of this novel CNN on a chip, we can conclude that the growth of cultured neuronal networks was very efficient promoting the formation of neuronal network structures with Small-world properties. One of the most important benefits of the CNN on a chip reported in this article is the simplicity to fabricate custom designs leading the adaptation of the CNNs. We observed that these CNNs on a chip is a reliable and helpful system due to easy handling, renewal of culture medium, avoidance of contamination, control of the culture conditions and whose portability makes straightforward the follow-up of the chip bioreactor chamber where the biological network is formed. Moreover, we found longer survival of the neuronal cultures which opens the possibility to use this CNN on a chip for testing drugs and toxicity in the future, as well as to evaluate different configurations of neuronal circuits on a chip, to extend to biological networks of other type of neurons, or even monitor and test diverse inspired tissues and biopsies on a chip.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

5 References

Figure legends

Figure 1. Images of the microfluidic chip layout and construction. A) Exploded view of the chip with the different layer which form the monolithic chip. B) Plan and lateral view of the microfluidic chip. C) Flow diagram of the fluidic chamber. D) Overview of the microfluidic platform integrated by a perfusion pump that connected to the neuronal circuits on a chip (Created with Biorender.com).

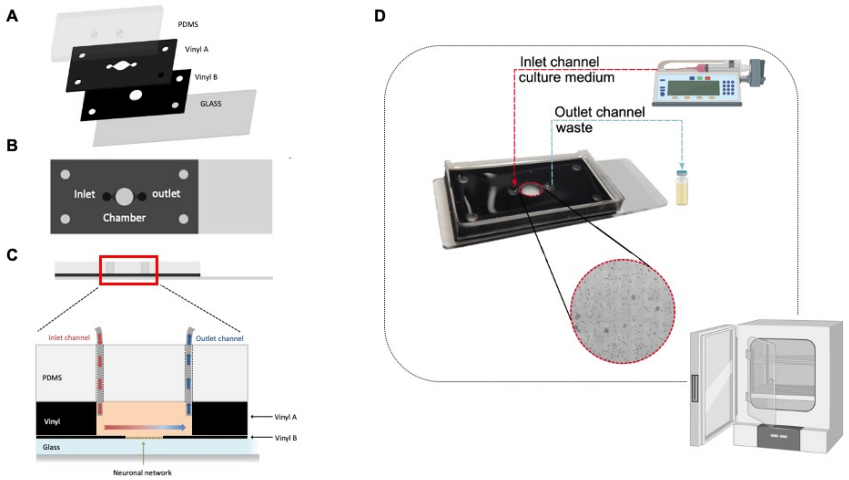

Figure 2. Image segmentation processing steps and extraction of the network graph in the development of a CNN on a chip. (A1-3) Cuts of the mosaic image taken at different days in vitro during the evolution of the CNN. (B1-3) Output of the segmentation algorithm of the region of interest. (C1-3) Graph representing the projection of the full graph into the cluster graph.

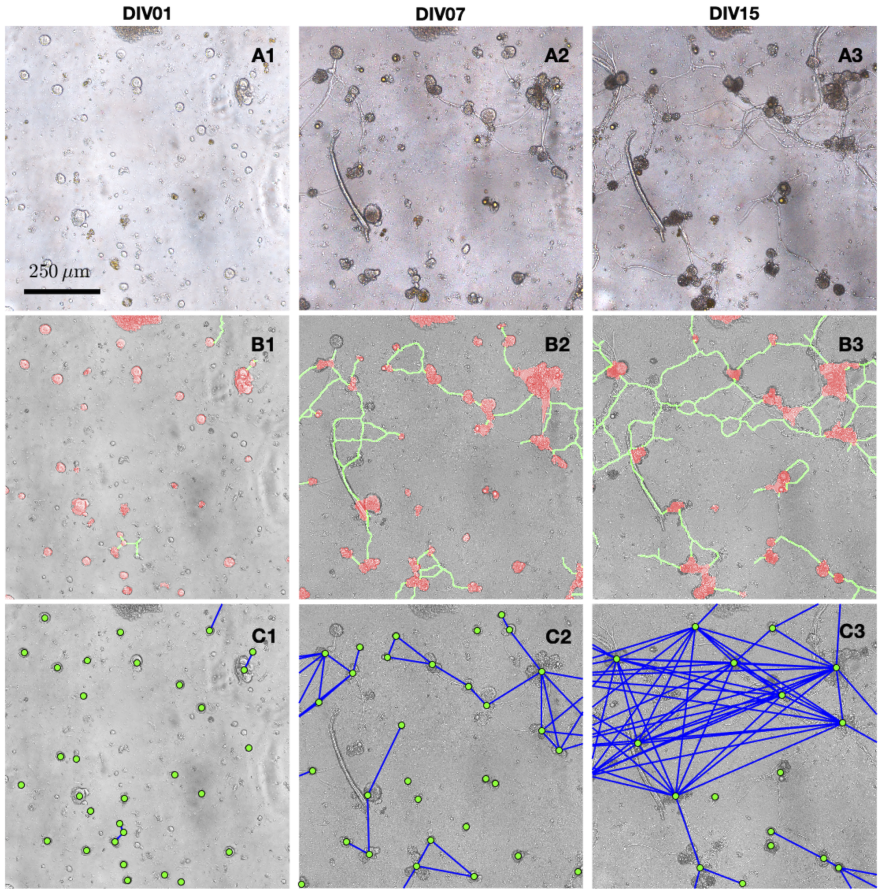

Figure 3. Network properties of the neuronal cultures. (A) Mean values of the number of connected nodes in the CNN as a function of the age of the culture. (B) Mean shortest path length L normalised by the size of the largest connected component GSC (red) and mean clustering coefficient C (blue). Each point is the average over 2 chip experiments.