NETRI's APP NOTE

COMPARTIMENTALIZED CULTURE OF PRIMARY OR hiPSC-DERIVED NEURONS USING AN MEA-CAPABLE HIGH-THROUGHPUT ORGANS-ON-CHIP PLATFORM

- Simultaneously record up to 16 replicates of human or rodent neuron activity in compartmentalized microfluidic devices.
- Co-culture different cell types to create highly relevant *in vitro* models throughout neurons-skin, neurons-neurons, or others tissue interfaces.
- Perform selective neuronal modulation by drugs thanks to the fluidic isolation between compartments.
- Capture imagery using automated microscopy for brightfield or fluorescence imaging.

OVERVIEW

The human brain is composed of a complex cellular heterogeneity of approximately 170 billion cells organized in an intricated cellular network. Major neurological disorders involve the central nervous system (CNS) or the peripherical nervous system (PNS) as well as their neuronal communication through neurons and synapses. The complexity of *in vitro* brain modelling and relevant neuronal communication led to failure in most of the late phase clinical trials targeting neurological disorders[1]. Classic high-throughput *in vitro* cell culture tools for CNS and PNS struggle to accurately replicate physiologically relevant connectivity patterns. This is partly due to the absence of geographical and fluidic separation between innervated tissues and neurons or between regions of the brain.

The emergence of organs-on-chip (OoC) in neurosciences could respond to the lack of relevant and complex models. Their aim is to reproduce - *in vitro* – the complexity of *in vivo* molecular and morphological aspects of neurons connections by controlling their microenvironment[2]. Compartmentalized microfluidic devices have been used in neurosciences studies for close to two decades, to isolate neurites from somas[3]. These technologies used microchannels where neurites could grow all the way to the other channels, creating synapses with the other cell type and keeping fluidic isolation between both culture channels. This enables the co-culture of two cell types that do not have the same microenvironment, culture protocols, or culture medium[4,5]. These OoC technologies are thus *in vitro* platforms able to provide unprecedented insights into neuronal mechanisms and limit the use of animal models, whether they be "healthy" or pathophysiological[6,7,8]. They are currently used with conventional tools used in neurosciences research such as microscopy, calcium imaging, and MicroElectrode Array (MEA)[9,10,11].

While conventional OoC solutions enable compartmentalization, they fail to provide high throughput (HTS) functional activity recordings. In this study, we sought to assess the capabilities of NETRI's DuaLink MEA, an HTS compartmentalized microfluidic device outfitted with MEA (up to 16 devices at a time, see Figure 1). We first described the DuaLink MEA's novel set up. We then proceeded to assess the benefits of the device for co-cultures of CNS applications, using rodent hippocampal and cortical cells. We also looked its compatibility with human induced pluripotent stem cells (hiPSCs) growth by culturing hiPSC glutamatergic neurons. Finally, we modulated neuronal activity in each of the above cultures to record electrophysiological changes in the culture using an inhibitory drug -tetrodotoxin (TTX).

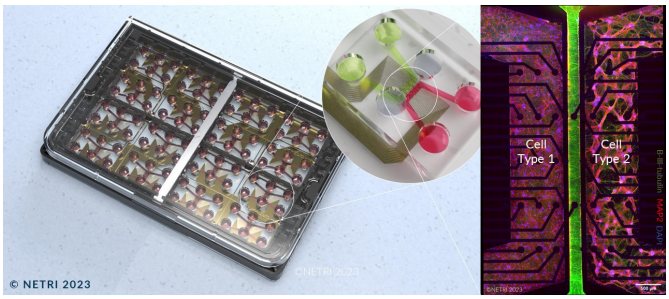

Figure 1: Global representation of NeoBento™ DuaLink MEA (A). The DuaLink MEA enables the co-culture of one or two cell types. Immunofluorescent picture of rodent co-culture of hippocampal neurons (Cell Type 1) and cortical neurons (Cell Type 2) at day 21 marked.

RESULTS

A. DuaLink MEA design allowing co-culture

The DuaLink MEA is composed of 16 chips nested in a NeoBento™ HTS format on an Axion Biosystems bespoke MEA Surface. Both the NeoBento™ and the Axion Surface compose the NeoBento™ MEA HTS format. Each device has three compartments linked with microchannels tunnels, enabling axonal connectivity while maintaining fluidic isolation (Figure 2).

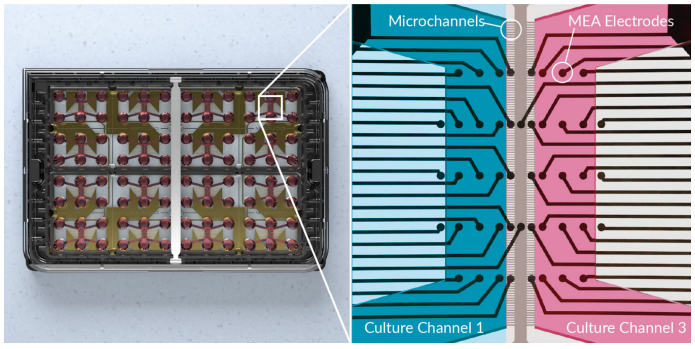

Figure 2: Schematic representation of the DuaLink MEA in a NeoBento™. The DuaLink MEA is composed of three channels linked with microchannels tunnels. Two channels are used to culture cell types (Culture Channel 1 and 3) and a small channel in the middle (Channel 2) improves fluidic isolation. MEA Electrodes are underneath the device and in direct contact with neurons.

Neurites in the channel 1 (Ch1) can grow throughout the microchannels up to the channel 3 (Ch3), creating synaptic connections with the other cell type. The DuaLink MEA architectures are made of Poly-dimethyl-siloxane (PDMS), essential to oxygenate the neuronal culture and maintain long-term culture without pumping apparatus. The PDMS architecture is irreversibly bonded to an SU8-coated PET sheet that harbors the PEDOT-plated gold electrodes and reference electrodes. Neurons are seeded directly in contact with the electrodes.

With the DuaLink MEA, 42 electrodes are active to record all parts of the neuronal network: 15 electrodes in both channels 1 and 3,5 electrodes in both inter microchannels (1µ2 and 2µ3) and 2 electrodes in channel 2 (Ch2). Thanks to the position of electrodes on the sheet, it is possible to record all parts of neurons (axons, dendrites, and synapses), independently. The electrodes under the microchannels make it possible to isolate axons and record action potential propagation from one channel to the other specifically (Figure 3).

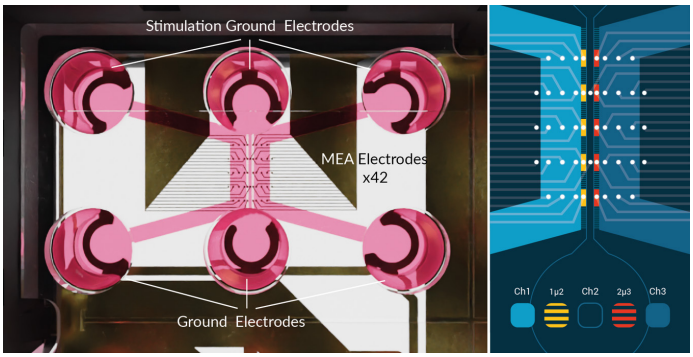

Figure 3: The DuaLink MEA is composed of 42 symmetrically distributed electrodes, with 15 electrodes in the channel 1 (Ch1) and channel 3 (Ch3), 5 electrodes in the microchannels between the channels (1µ2 and 2µ3) and 2 electrodes in the channel 2 (Ch2). The stimulation ground electrodes and ground electrodes are placed in the reservoirs of the device.

Electrophysiological recordings of the neuronal cultures were performed using an Axion Maestro Pro. Recording results were then extracted with NETRI's Uplink software, linked to Axion's software by Axion's APIs. NETRI Uplink enables the collection of every electrophysiological metric calculated by Axion software, for all the 16 chips in a NeoBento™ MEA and in all possible compartment's configurations, in one highly sortable excel document (Figure 4).

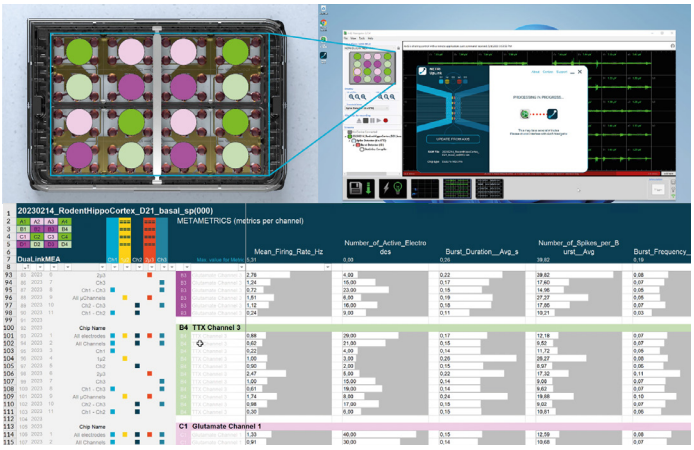

Figure 4: Screenshots of the UpLink software used to extract electrophysiological metrics. These metrics are organized in an Excel file per NeoBento™ with all compartment's configurations.

B. Rodent co-culture viability and maturity in the DuaLink MEA

Hippocampal and cortical rodent neurons were sampled on E18 rat embryos. Hippocampal neurons were seeded in Ch1, whereas cortical neurons were seeded in Ch3, previously coated with Poly-D-lysine (PDL) and laminin. Media changes were performed three times a week to maintain for 21 days to reach neuronal maturity.

Neurons were stained with anti-β-III-tubulin and DAPI to improve morphological visualization, alongside anti-MAP2, a dendritic marker, used to visualize the maturity of the culture (Figure 5-A). Brightfield contrast pictures were taken at every media change, showing the evolution of neurons growth, and electrophysiological recordings were performed after 7, 14 and 21 days of culture. The 5 min raster plots presented in Figure 5-B and Figure 6 provide a visualization of neuronal activity and neuronal synchronicity in each compartment, by marking every action potential (spikes) with a dot as a function of time.

Synchronicity of the co-culture appears at D14 and becomes more and more refined until D21 (Figure 6). The mean firing rate (MFR, corresponding to the number of spikes per seconds) increased with time in both the channels and the microchannels. Interestingly, functional activity could be detected notably earlier in microchannels when compared to the channels (about 0.8 in microchannels compared to 0.3 spikes/s in channels at D14) (Figure 7). It is important to note that the 16 DuaLink MEA devices present on a single plate can be recorded simultaneously using the Axion Maestro Pro. These parameters, showing the maturity of neuronal network, are used as benchmarks to then proceed to pharmaceutical drug testing (see Section D).

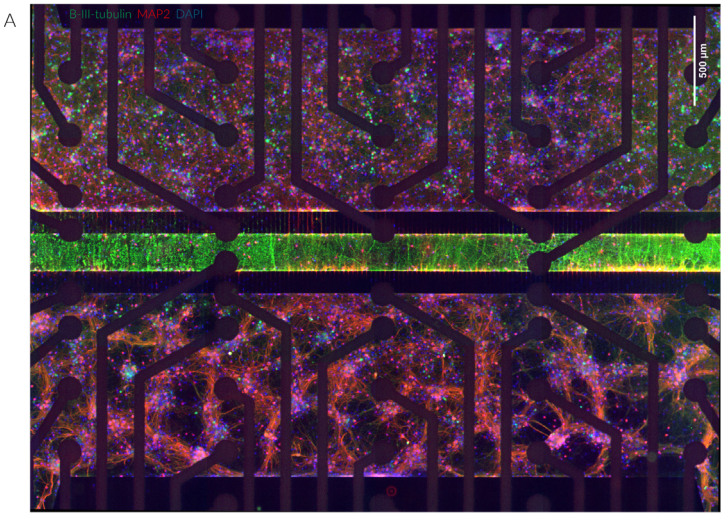

Figure 5: Immunofluorescence image of rodent hippocampal neurons (Ch1) and cortical neurons (Ch3), in a DuaLink MEA (DAPI in blue, β-III-tubulin in green, and MAP2 in red, scale bar is 500 µm) (A). Raster plot of co-culture in a DuaLink MEA at D21. Each dot corresponds to a detected spike as a function of time (600 seconds of recording) (B).

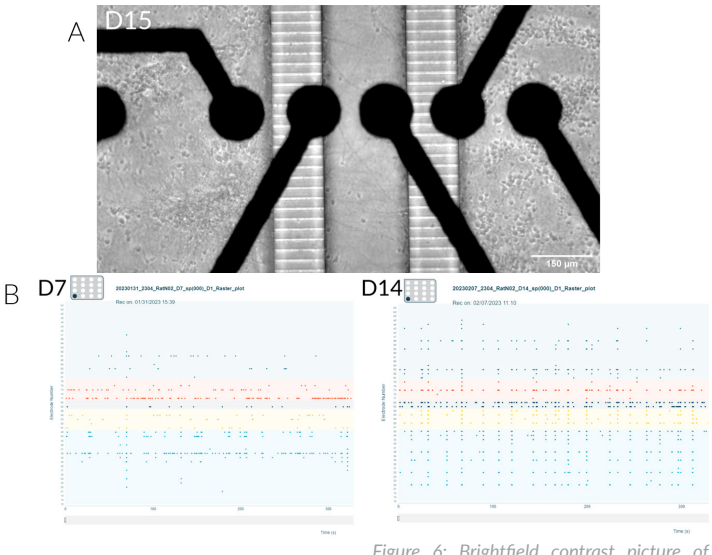

Figure 6: Brightfield contrast picture of rodent co-culture at D15. Hippocampal neurons are seeded in Ch1 and cortical neurons in Ch3 with axonal projections in Ch2 (A). Raster plots (300 seconds) of D7, D14 and D21 recordings of rodent co-culture. Raster plots inform on the network activity, with the culture becoming synchronized from D14 and mature at D21 (B).

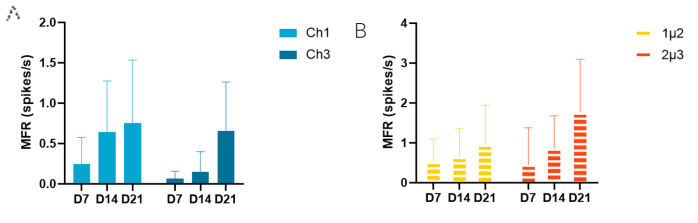

Figure 7: Plot of functional activity (MFR (spikes/s)) of rodent co-culture at D7, D14 and D21 in the culture channels (A) and in the microchannels (B). n=16 for each condition.

C. Human glutamatergic neurons iPSC-derived culture viability and functional activity in the DuaLink MEA

Human glutamatergic neurons derived from induced pluripotent stem cells were purchased from BrainXell (Madison, USA). Neural progenitors were seeded only in Ch1 of the DuaLink MEA pre-coated with PDL and geltrex. Glutamatergic neurites in Ch1 can grow up to Ch3. The fluidic isolation between the channels 1 and 3 enabled the creation of a somas compartment (Ch1) and of an axonal compartment (Ch3).

As described earlier, the neurons were stained with DAPI and an anti-β-III-tubulin antibody, but also with anti-vGlut1, a specific marker for glutamatergic neurons, to identify and confirm the success of the differentiation. Media changes were performed twice a week for up to 21 days to reach neuronal maturity (Figure 8). Brightfield contrast pictures were taken every two days showing the evolution of neurons growth, and electrophysiological recordings were performed after 7, 13, 19 and 21 days of culture.

As expected with glutamatergic neurons, the 5 min raster plots (Figure 9) show synchronicity as early as D13, becoming more refined by D19 (Figure 9). As described before, and across the 16 recorded chips, the activity in microchannels was detected earlier than in the channels, with about 4 spikes/s in channels at D19 compared to 18 spikes/s at D13 (Figure 10), demonstrating the maturity of glutamatergic neurons. It is important to note that these activity levels are comparable to those measured by the cell suppliers themselves.

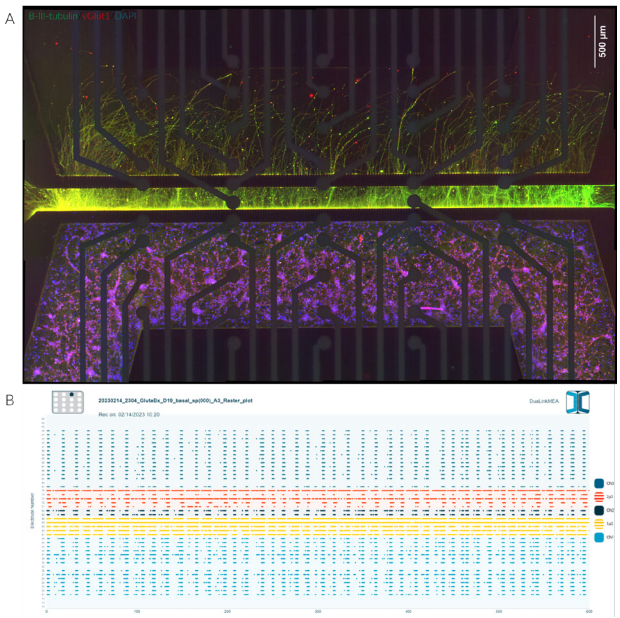

Figure 8: Immunofluorescence image of human glutamatergic neurons (Ch1) and glutamatergic neurites (Ch3), in a DuaLink MEA (DAPI in blue, β-III-tubulin in green, and vGlut1 in red, scale bar is 500 µm) (A). Raster plot of human glutamatergic neurons in a DuaLink MEA at D21 (600 seconds of recording) (B).

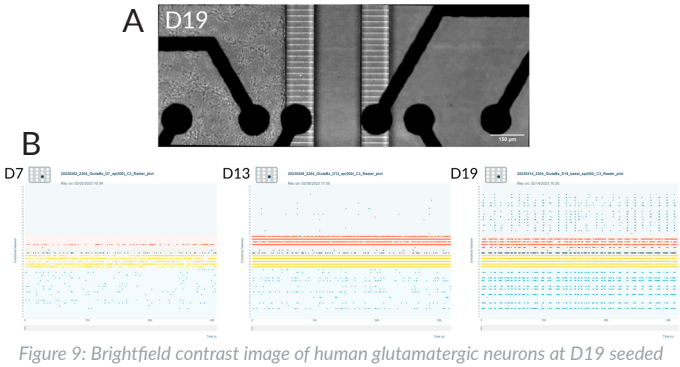

Figure 9: Brightfield contrast image of human glutamatergic neurons at D19 seeded in Ch1 (A). Raster plots (300 seconds) of D7, D13, D19 and D21 recordings of human glutamatergic neurons. Raster plots inform on the network activity, with the culture becoming more synchronized and mature from D19 (B).

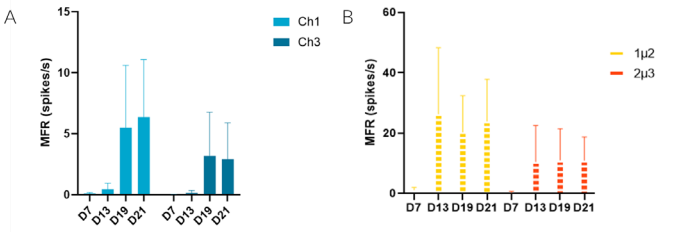

Figure 10: Plot of functional activity (using MFR (spikes/s)) of human glutamatergic neurons at D7, D11, D19 and D21 in the channels (A) and in the microchannels (B). n=8 for each condition.

D. Manipulation of spontaneous electrophysiological activity with pharmacological compounds

a. In rodent hippocampal-cortical co-culture

Thanks to the DuaLink MEA architecture, it is possible to precisely modulate the neuronal functional activity, as demonstrated here by using Tetrodotoxin (TTX), a voltage-gated sodium channel blocker that blocks neuronal communication. TTX was added after 21 days of culture to either the channel 1, (thus the hippocampal compartment) or to the channel 3 (cortical compartment). Electrophysiological recordings were performed both before and few minutes after addition of TTX.

All the experiments were also repeated using TTX's solvent as vehicle control to discriminate for the effect of the drug specifically. As indicated by the raster plots (Figure 11-A-B), all neuronal networks showed functional activities. Colors correspond to each channel (see legends below the figure), and each point corresponds to a single spike detected during the recording.

By adding TTX in the hippocampal compartment (Ch1), we observed a diminution of 96.6% (± 1.6%) in MFR in the same channel. When the drug was added to Ch3, the MFR in this channel decreased by 98.7% (± 2.4%) (Figure 11-C). In both cases, the changes in activity in the opposite channel was comparable to the control: TTX affects the neurons in its application channel, and not the neurons in the opposite one, thus demonstrating the fluidic isolation between the channels (Figure 11). These results were confirmed with other electrophysiological metrics, such as synchrony (Figure 11-D).

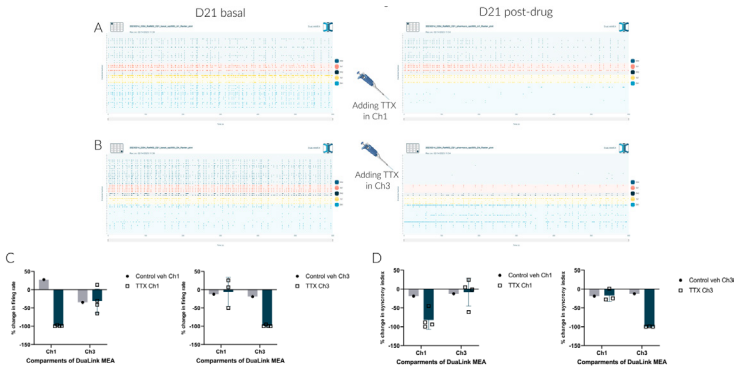

Figure 11: Raster plots of rodent co-culture activity at D21 before and after adding TTX in Ch1 (A) and in Ch3 (B). Plot of % of change in MFR (spikes/s) from baseline with addition of the control vehicle or TTX in Ch1 and Ch3 (C). Plot of % of change in synchrony index from baseline with addition of the control vehicle or TTX in Ch1 and Ch3 (D).

b. In Glutamatergic neurons iPSC-derived

We also used TTX in glutamatergic neurons monoculture after 19 days of culture. Again, TTX was added either to the Ch1 or the Ch3. As shown previously, the TTX affected the neuronal activity only when it was added directly in the soma compartment (diminution of 98.8% in firing rate, see Figure 12-A to C). The effect of TTX is reversible as expected, with the activity going back to baseline levels at day 21.

When added in the axonal compartment, no significant change of activity was recorded compared to the control.
This once again highlights the efficacy of the fluidic isolation and demonstrates that the capacity to target either the somas or the axons (or synapses) specifically is essential to gain insights into the mode of molecules.

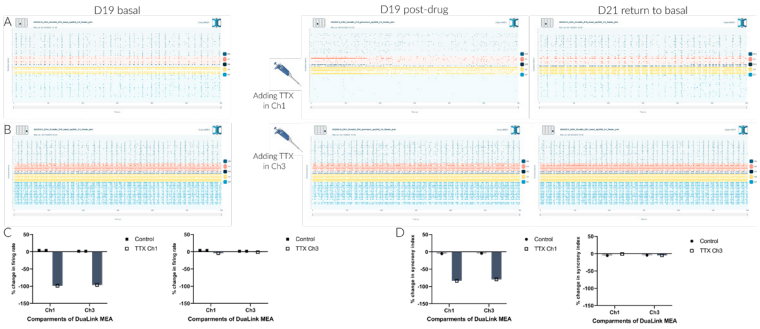

Figure 12: Raster plots of human glutamatergic neurons activity at D21 before and after adding TTX in Ch1 (A) and in Ch3 (B). Plot of % of change in MFR (spikes/s) from baseline with addition of the control vehicle or TTX in Ch1 and Ch3 (C). Plot of % of change in synchrony index from baseline with addition of the control vehicle or TTX in Ch1 and Ch3 (D).

CONCLUSION

These results showed the ability, in a co-culture context, to independently manipulate and examine detailed subcellular mechanisms like synaptogenesis or plasticity, as well as the effects of therapeutic or toxic compounds. Our findings highlight the potential of MEA-capable compartmentalized microfluidics for investigating neuronal communication in neurodegenerative diseases. By extension, we can apply these findings to PNS applications such as pain, innervated skin, or innervated gut. This approach enables a nondestructive assessment of network-level functional responses in the CNS and the PNS applications, providing valuable insights for pre-clinical pharmaceutical assays and studying compound-induced effects on advanced multi-cell-type models.

RESOURCES

Available upon request

- Operating Protocol DuaLink MEA – 3B_073.04
- Operating_Protocol - Immuno Staining DR_3B_059

1. *Pievani, M., Filippini, N., Van Den Heuvel, M. P., Cappa, S. F. & Frisoni, G. B. Brain connectivity in neurodegenerative diseases - From phenotype to proteinopathy. Nat Rev Neurol 10, 620–633 (2014).*
2. *Wu, Q. et al. Organ-on-a-chip: Recent breakthroughs and future prospects. Biomed Eng Online 19, 1–19 (2020).*
3. *Park, J. W., Vahidi, B., Taylor, A. M., Rhee, S. W. & Jeon, N. L. Microfluidic culture platform for neuroscience research. Nat Protoc 1, 2128–2136 (2006).*
4. *Zahavi, E. E. et al. A compartmentalized microfluidic neuromuscular co-culture system reveals spatial aspects of GDNF functions. J Cell Sci 128, 1241–1252 (2015).*
5. *Chen, L. J. et al. Microfluidic co-cultures of retinal pigment epithelial cells and vascular endothelial cells to investigate choroidal angiogenesis. Sci Rep 7, 1–9 (2017).*
6. *Low, L. A., Mummery, C., Berridge, B. R., Austin, C. P. & Tagle, D. A. Organs-on-chips: into the next decade. Nature Reviews Drug Discovery vol. 20 345–361 Preprint at https://doi.org/10.1038/s41573-020-0079-3 (2021).*
7. *Miny, L., Maisonneuve, B. G. C., Quadrio, I. & Honegger, T. Modeling Neurodegenerative Diseases Using In Vitro Compartmentalized Microfluidic Devices. Front Bioeng Biotechnol 10, 1070 (2022).*
8. *Nikolakopoulou, P., Rauti, R., Maoz, B. M. & Herland, A. Recent progress in translational engineered in vitro models of the central nervous system. Brain 1–33 (2020) doi:10.1093/brain/awaa268.*
9. *Peel, S. et al. Lab on a Chip Introducing an automated high content confocal imaging approach for Organs-on-Chips. (2018) doi:10.1039/c8lc00829a.*
10. *Wan, J. et al. Emerging Roles of Microfluidics in Brain Research: From Cerebral Fluids Manipulation to Brain-on-a-Chip and Neuroelectronic Devices Engineering. Chem Rev (2022) doi:10.1021/acs.chemrev.1c00480*
11. *Halliwell, R., Salmanzadeh, H., Coyne, L. & Cao, W. S. An Electrophysiological and Pharmacological Study of the Properties of Human iPSC-Derived Neurons for Drug Discovery. (2021).*

Based on 10 years of scientific research, NETRI has developed a unique know-how in designing organs/organoids-on-Chip by integrating disruptive building blocks into the same microfluidic devices, while maintaining industrial production standards compatible with pharma industry equipments & requirements.

Thanks to our patented technologies, we are capable of manufacturing prototypes and validating their biological function using primary animal or human induced pluripotent stem cells differentiated in our chip. Our unique infrastructure allows us also to scale up chip production for mass production.

Need more information about NETRI's products and services or how Organs-on-Chip revolutionize pre-clinical trials and human *in-vitro* models? Contact us!

sales@netri.com **+33 4 87 75 63**

www.netri.com
NETRI SAS - 321 Avenue Jean Jaures 69007 Lyon - FRANCE
Bat.A - 213 Rue de Gerland 69007 Lyon - FRANCE
Phone: +33 4 87 65 75 63 - Email: contact@netri.com