TRANSLATIONAL NERVE INJURY-ON-CHIP PLATFORM

- Axotomy and neurite outgrowth kinetics.
- Human motor and sensory neurons-on-chip as a relevant peripheral nerve injury model.
- Discriminate mode of action per cell compartment.
- Segregate therapeutic modality: topical versus systemic.
- Platform compatible with High Throughput Assays and Multielectrode Arrays.

OVERVIEW

Peripheral nerves are made of motor and sensory neurons. Sensory and pain signals are primarily detected by free nerve endings of sensory neurons and transmitted via their axons to their somas in the dorsal root ganglia, and then to dorsal horn neurons. Local integration and modulation of the signal are performed by spinal cord circuits before reaching the brain, the area of consciousness of pain, and central modulation (Figure 1). Motor signals, on the other hand, come from the brain or the local spinal cord reflex circuit before being transmitted to the muscles through specific synapses, the neuro-muscular junctions.

Organs-on-chip (OoC) offer the advantage of isolating neuron somas from their axons, thus reproducing the human anatomical organization which enables recapitulation of translational injury or exploration of treatment paradigms (Taylor et al., 2005). We can easily reproduce the highly segregated peripheral nervous circuits with NETRI's architecture as they enable the isolation of cell bodies, proximal and distal axons into specific compartment. To tease apart each cell type and allow their study separately, we adapted the culture of motor and sensory neurons onto our OoC platform and we used human-induced Pluripotent Stem Cell (hiPSC)-derived neurons to bridge the gap between *in vivo* models and first-in-human studies (Figure 2).

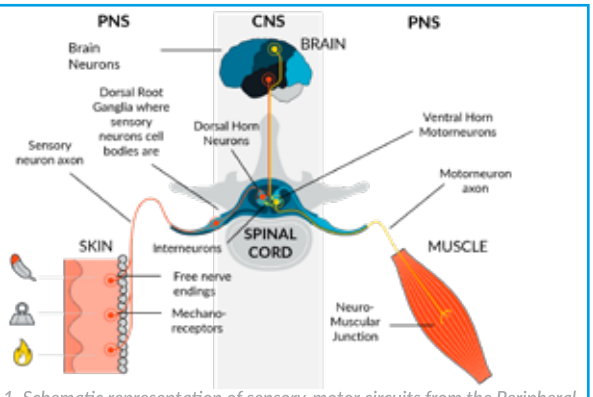

Figure 1. Schematic representation of sensory-motor circuits from the Peripheral Nervous System (PNS) to the Central Nervous System (CNS). Distinct sensory neurons with specific receptors are present in the skin (and all organs). Their cell bodies are located in the Dorsal Root Ganglia (DRG), and they transmit their signals to dorsal horn neurons in the spinal cord. The message is then modulated via interneurons before reaching the brain for consciousness. Motorneuron cell bodies are in the ventral horn of the spinal cord. They receive inputs from the brain or spinal cord circuits and control motor function via signaling at the neuromuscular junction.

Traumatic Nerve Injury Platform

Accidents, surgeries, and compressions (tumor, carpal canal syndrome) are examples of situations in which a traumatic injury to the spinal cord or nerves can induce neuropathic pain in addition to debilitating motor and sensory symptoms. Two main axes of treatments have an important impact on a patient's quality of life: (i) regenerative medicine to reconstruct and regenerate nerves and (ii) efficient pain relief (Lopes et al., 2022; Varshney et al., 2021).

To offer a translational nerve injury platform enabling the discovery of regenerative therapeutics, we used the DuaLink™ Delta Ultra from the Neurofluidics™ line. The microchannel length between channel 2 and 3 (Figure 2) allows the monitoring of neurite regrowth after injury in channel 2. The DuaLink™ Shift architecture offers the same advantages obtained from the microchannels' length (500 µm) and compatibility with MEA readouts.

As a mirror of current *in vivo* models, such as nerve crush injury or nerve ligation that aim to mimic human nerve trauma, we created a repeatable and standardized injury, by cutting motor and sensory axons only using a short, targeted detergent application.

Our devices' microfluidic isolation and architecture offer the advantage of targeting only neurites and not cell bodies like conventional *in vitro* models. We demonstrated the use of our platform by comparing axonal regeneration following treatment with a neurotrophic molecule or with a drug inhibiting neurite outgrowth.

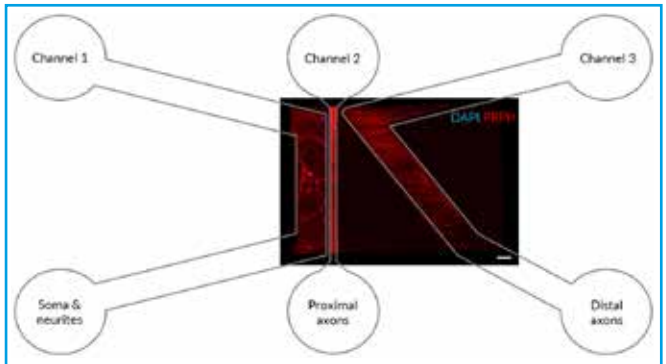

Figure 2. axoCell™ sensory neurons cultured 21 days in DuaLink™ Delta Ultra according to our Operating Protocol - AxoCells™ Sensory Neurons. Neurons were fixed and then stained for Peripherin. For more details, see Operating Protocol - Immuno Staining. Scale bar is 500 µm.

RESULTS

Axotomy Protocol Validation

Our axotomy protocol was first developed on iCell Motor Neurons cultivated in DuaLink™ Delta Ultra (Operating Protocol - iCell Motor Neurons). The axotomy was performed at day 7, when axons have crossed over to channel 2 and exited in channel 3 at the top. Briefly, a 0.5% Triton X-100 solution was applied 30 seconds in channel 2, then washed with cell media (Operating Protocol - Motor Nerve Injury). Destruction of neurites was confirmed by visualization of channel 2 (Figure 3-B). A Live/Dead assay kit was used to assess the viability one day post-injury. No difference was detected between the control condition without axotomy (20.88% +/- 1.24%, n= 5) and the condition with axotomy (19.35% +/- 0.65%, n=5).

Two live dyes were used to validate axotomy performance. In practice, images were acquired before and after the axotomy of the same chip. We compared calcein, an AM-ester fluorescing only after being cleaved by intracytoplasmic esterases, to a Cell Tracking Red dye kit, a membrane stain. The two non-invasive approaches showed that 100% of the neurites were absent 2h after the detergent was added to channel 2 (Figure 3). This technique also allows axonal regrowth to be monitored in real-time and showed that the regrowth of several neurites can be detected as early as 24 hours after axotomy (Figure 4-A) and that around 50% of the chips have axons reaching channel 3 seven days after axotomy (Figure 4-B and 4-C). Due to a higher S/N ratio, calcein live dye was chosen for the remaining experiments.

The same method of localized neuritic lesions was used with adaptations of the exposure days (3 days to 2 days) linked to the specificities of axoCells™ sensory neurons (Operating Protocol - Sensory Nerve Injury).

The methodology has been successfully reproduced in DuaLink™ Shift (confidantial user data), which has the same advantages as the DuaLink™ Delta Ultra: microchannels of suitable lengths to measure axonal outgrowth and fluidic isolation to separate cell bodies from projections.

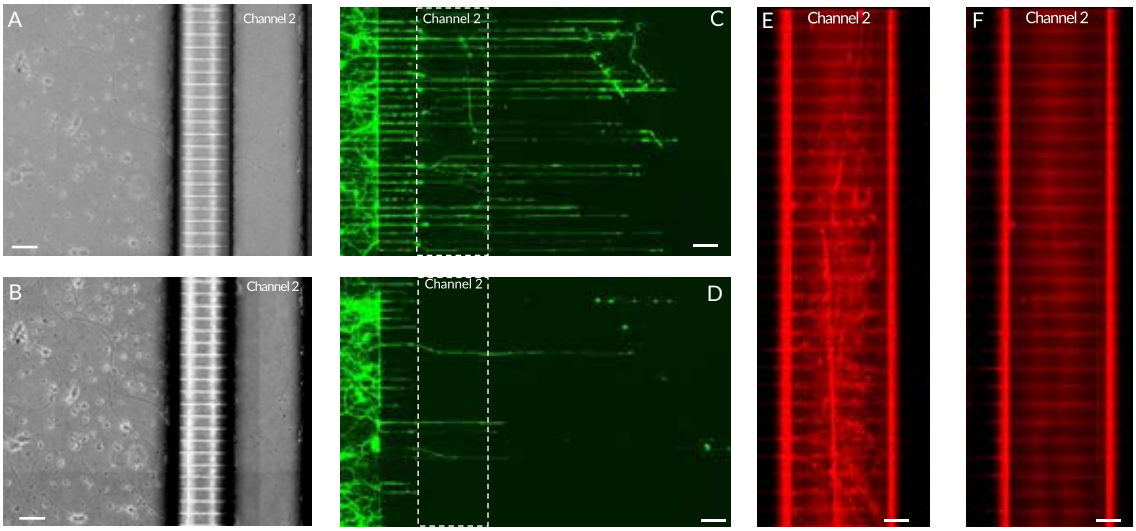

Figure 3. Axotomy protocol development on iCell Motor Neuron.
Transmitted light pictures of the same iCell Motor Neurons chip before (A) and after (B) axotomy at D7. Illustrative pictures of chips stained with calcein live dye (C3100MP 0,1µg/ml, 30 min) at D8 without (C) or with axotomy (D). Validation of axotomy protocol in channel 2 on axoCell™ sensory neurons. Same device stained with Cell Tracking Red Dye kit (ab269446, 1/20, 24h) before (C), 2h (D) after axotomy (Operating Protocol - Sensory Neurons Nerve Injury). Scale bars are 100 µm.

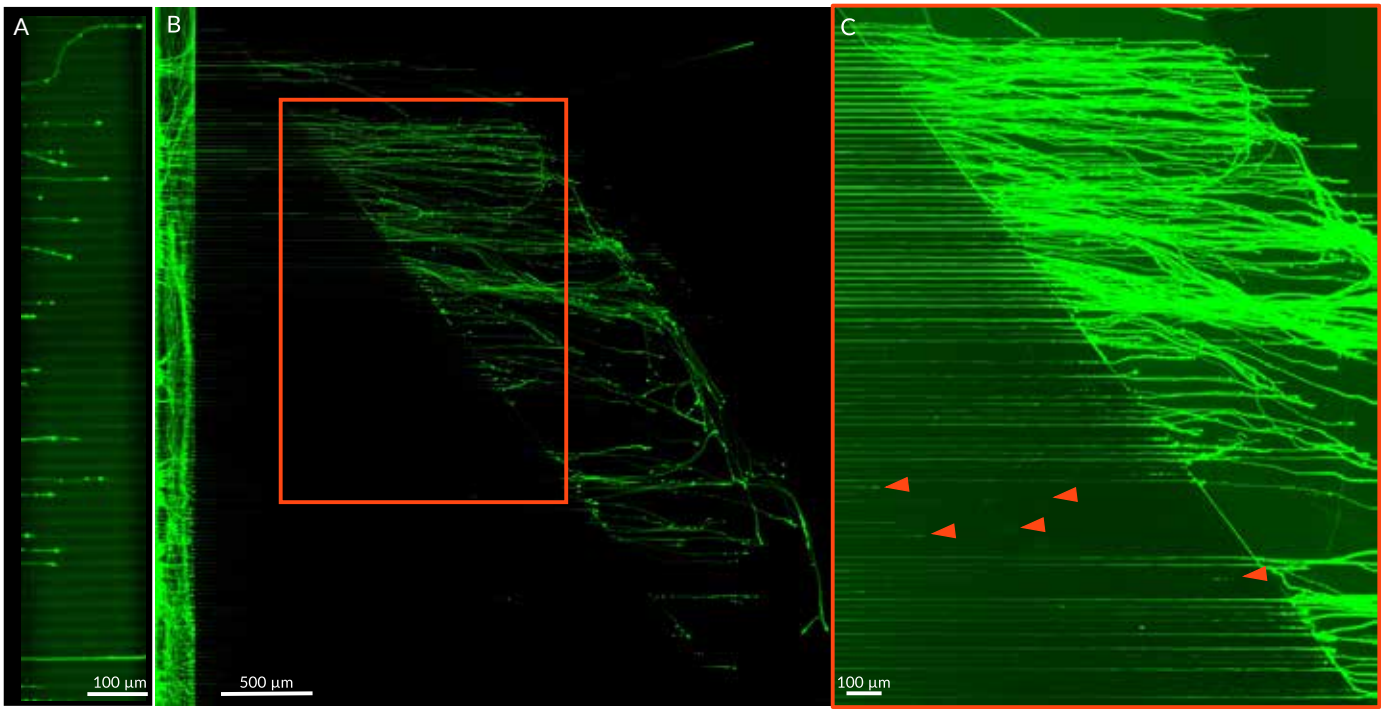

Figure 4. Regrowth 24h (A) and 7 days after axotomy (B & C), C is a magnified and enhanced contrast view of B, calcein live dye.
Arrowheads indicate neurite growing in microgrooves. Axotomy was performed 7 days after seeding.

Motor Nerve Injury platform

To demonstrate the potential of our platform to study neurotrophic or neurodegenerative molecules, we tested two mechanistic drugs: nocodazole and staurosporine. Nocodazole destabilizes microtubules, preventing neurite outgrowth, and is cytotoxic at high concentrations (Vasquez et al., 1997). Staurosporine is a wide-spectrum protein kinases inhibitor that demonstrated potential for enhanced neurite outgrowth in several neuronal models (Wakita et al., 2014). Both drugs were added to the media in the axotomy channel and regrowth was observed 3 days later using calcein live dye.

The cytotoxic effect of nocodazole could be seen on the iCell Motor Neurons at 10 µM, with no neurite outgrowth parameters measurable. A tenfold lower concentration however did not affect viability, impacting neurite outgrowth only. Indeed, at 1 µM Nocodazole, the reduction of three parameters could be evaluated: the number of axons, their maximal length, and the total sum of length (Figure 5). The mean length is not representative here as it only includes one or two axons measured for some chips.

Staurosporine, applied at 50 nM on iCell Motor neurons, increased the number of axons and the sum of length compared to the vehicule condition. We confirmed its positive effect on neurite outgrowth parameters in our conditions, validating that it can be used as a positive neurotrophic control for neurite outgrowth assays.

Sensory Nerve Injury platform

We cultivated the axoCells™ sensory neurons according to our Operating Protocol - AxoCells™ Sensory Neurons. For this sensory model, the two same drugs were added to the axotomy channel and regrowth was observed two days later (due to the faster growth kinetics of the cells).

At the highest concentration tested (1 µM), nocodazole completely inhibited neurite outgrowth preventing metrics' extraction (Figure 6). We tested a ten-fold lower concentration and showed that 0,1 µM nocodazole reduces the number of axons and the total sum of length, without affecting cell viability. Nocodazole was more potent on axoCells™ sensory neurons compared to iCell Motor Neurons.

Similarly, the effect of staurosporine varied whith the cell type used. Staurosporine has been shown to enhance neurite outgrowth in a dose and time-dependent manner (Sherman & Bang, 2018). Applied to axoCells™ sensory neurons, it did not show a significant impact at the tested concentration (50 nM), most likely due to their fast growth kinetics.

Our platform can detect differences in efficacy depending on the cell model used. This demonstrates the importance of complete characterization of the biological material used in OoC platforms, before trying to do any kind of clinical extrapolation.

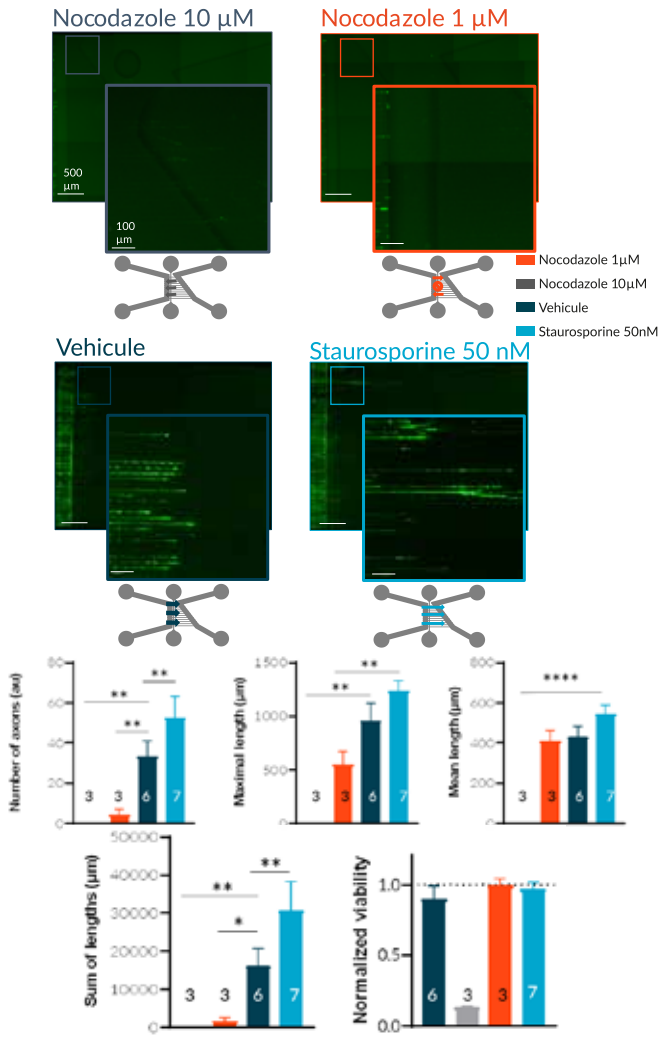

Figure 5. Illustrative pictures of iCell Motor Neurons in DuaLink Delta Ultra stained with calcein live dye, 3 days of exposure post-injury with nocodazole 1 µM and 0.1 µM, vehicule or staurosporine 50 nM applied in channel 2 post-axotomy. Schematics of expected results are underneath each condition. iCell Motor Neurons regrowth quantification post-injury. Quantifications were performed using ImageJ. Graphs and statistical analysis were generated using GraphPad Prism. Independent t-test with Welch's correction (p-value ≤ 0.05, ** ≤ 0.01).*

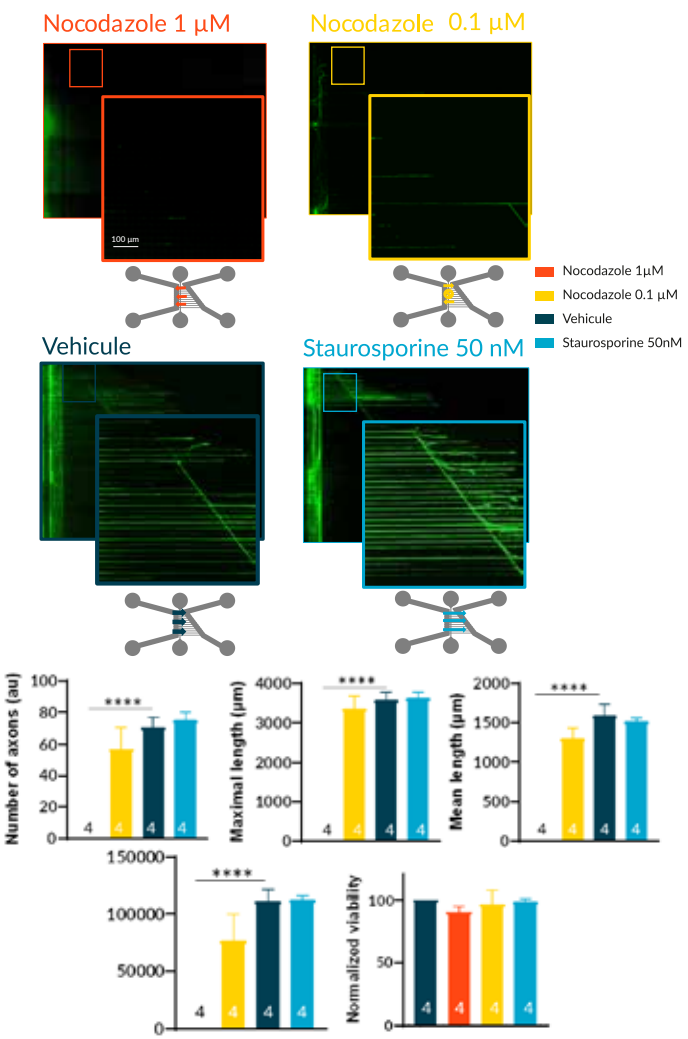

*Figure 6. Illustrative pictures of axoCells™ sensory neurons in DuaLink Delta Ultra with calcein live dye, 2 days of exposure post-injury with nocodazole 1 µM and 0.1 µM, vehicule or staurosporine 50 nM applied in channel 2 post-axotomy. Schematics of expected results are underneath each condition. Graphs show the quantification of cell viability and regrowth kinetics after compounds addition. The statistical analysis was performed using Graph Pad Prism with Independent t-test with Welch's correction. **** p-value<0.0001.*

CONCLUSION

By combining NETRI's engineering, biological & digital expertise, we validated our axotomy protocol on two types of hiPSC-derived neurons from distinct suppliers. Moreover, the methodology was successfully replicated on all DuaLink NeuroFluidics™ architectures (DuaLink Delta, DuaLink and DuaLink Shift, NETRI's internal data and users' confidential data). Last, we demonstrated the physiological relevance of our platform by comparing axonal regeneration following treatment with a neurotrophic molecule and a drug inhibiting neurite outgrowth.

Our Nerve Injury-on-chip platform offers pharmaceutical companies and researchers a new translational model of traumatic nerve injury to study the efficacy and mode of action of novel therapeutic modalities.

Here we show that compartmentalized microfluidics can be used to reproduce traumatic injury and segregate motor from sensory components. This methodology paves the way to study complex cell communication during regenerative processes by adding glial cells (e.g. satellite cells, Schwann cells) or target organs (e.g. skin, muscle). Furthermore, with NETRI's NeuroFluidics™ MEA Line, the electrophysiological digital signature of axonal regrowth can now be resolved in the DuaLink Shift MEA.

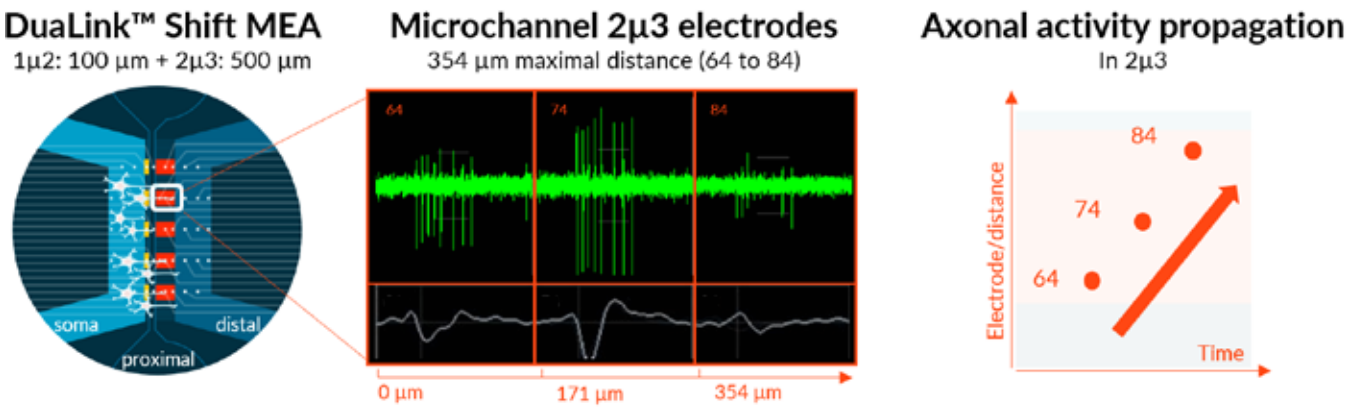

Figure 7. The DuaLink Shift MEA can be used to follow electrical activity associated with axonal regrowth.
Left: the architecture is composed of 100 µm and 500 µm microchannels, letting only distal axons reach the channel 3.
Middle: traces from Axis Navigator representing the axonal activity of three electrodes in the same microchannel (20 µV and 100 ms per division). The distances from proximal to distal are indicated, underneath the example of spikes detected.
Right: UpLink raster plot zoom, representing the spikes detected from the same axons projecting from electrode 64 to 84.

RESOURCES

Available upon request

- Operating Protocol_iCell Motor Neurons – DR_3B_064-V1
- Operating Protocol_Motor Nerve Injury - DR_3B_092
- Operating Protocol_Sensory Nerve Injury - DR_3B_095
- Operating Protocol_AxoCells™ Sensory Neurons DR_3B_057
- Operating Protocol_Immuno Staining DR_3B_059

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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!

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