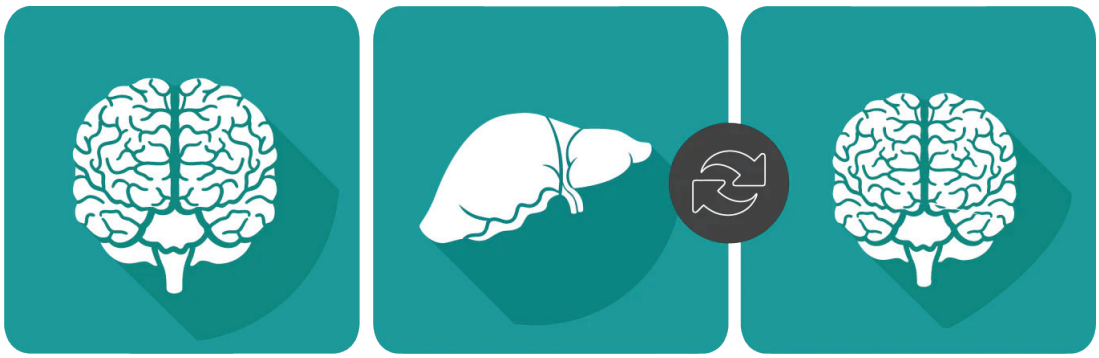
NANOSTACKS™ for CNS Drug Discovery

Human-Relevant In Vitro Models for Toxicity, Seizure Liability & Brain-Liver Crosstalk

NANOSTACKS™
BRAIN



Featuring Application Notes On:

- AI-Integrated MEA for Seizurogenic Risk Prediction
- Human Brain Model with Neurite Outgrowth Assay on NANOSTACKS™
- Modeling the Liver-Brain Axis for Multi-Organ Toxicity

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Application note

Integrating NANOSTACKS™ with microelectrode arrays plates for AI-powered seizure liability screening

Introduction

Accurately predicting a compound's potential to induce seizures remains a significant challenge in pharmaceutical development. Whilst *in vitro* models combining microelectrodes arrays (MEA) allow researchers to assess seizurogenic risk of compounds, the complexity of quantifying neuronal electrical activity, often involving numerous statistical parameters, can render these evaluations heavily operator-dependent. In this application note, we introduce a proof-of-concept AI-driven methodology that leverages the integration of NANOSTACKS™ with microelectrode array (MEA) platforms to predict seizure liability, providing a more reliable and standardised approach to seizurogenicity assessment.

Methods

Cell culture

24-well CytoView MEA Plates (Axion BioSystems) were used as the experimental platform, whilst the Maestro Pro™ multiwell microelectrode array (MEA) and impedance system (Axion BioSystems) was used to read the electrical activity of neurons from the MEA plates. Human iPSC-derived dopaminergic neurons and human iPSC-derived astrocytes (both from FUJIFILM Cellular Dynamics) were cultured following the manufacturer's recommended protocols for seeding and maintenance on MEA substrates. The neuronal-astrocyte coculture model was established by seeding human iPSC-derived dopaminergic neurons directly onto the surface of the MEA where the electrodes are located. After an initial 9-day maturation period, human iPSC-derived astrocytes were introduced using a NANOSTACK™ platform positioned above the neuronal layer. This configuration, within the 24-well MEA plate, allowed paracrine interactions between the astrocytes and neurons (Figure 1). Neurons were maintained in culture for a total of 28 days, with astrocyte addition on Day 9 of the neuronal culture.

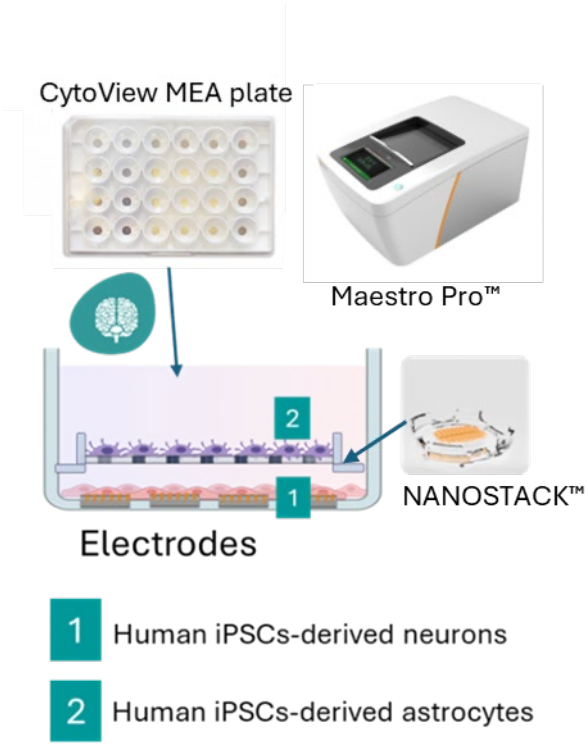

Figure 1. Coculture setup.

Drug treatment

Test compounds were administered following a cumulative dosing protocol. Each drug concentration was incubated for 15 minutes before initiating MEA recordings. After each recording session, the drug concentration was incrementally increased, followed by another 15-minute incubation and subsequent recording. This stepwise approach enabled the assessment of compound effects across multiple concentrations within the same well.

AI model training

The electrophysiological data generated from the MEA recordings were used to train a proof-of-concept artificial intelligence (AI) model. The model aimed to differentiate between seizurogenic and non-seizurogenic compounds and assign a probability score reflecting the likelihood of seizure induction at various tested concentrations.

Results

The proof-of-concept AI model analyses the neural electrical activity of neurons on MEA plates and provide a probabilistic assessment of whether the observed neuronal activity patterns within a given time window are consistent with seizure-like activity (Fig. 2). In Figure 2, the number next to bars indicates the number of time segments of neural activity classified as negative (next to blue bars) or positive (next to red bars). The length of the bars indicates the maximum confidence of the AI prediction amongst all time segments classified as negative (blue bars) or positive (red bars). In the figure, concentrations tested are ordered from the highest (concentration 5) to the lowest (concentration 1).

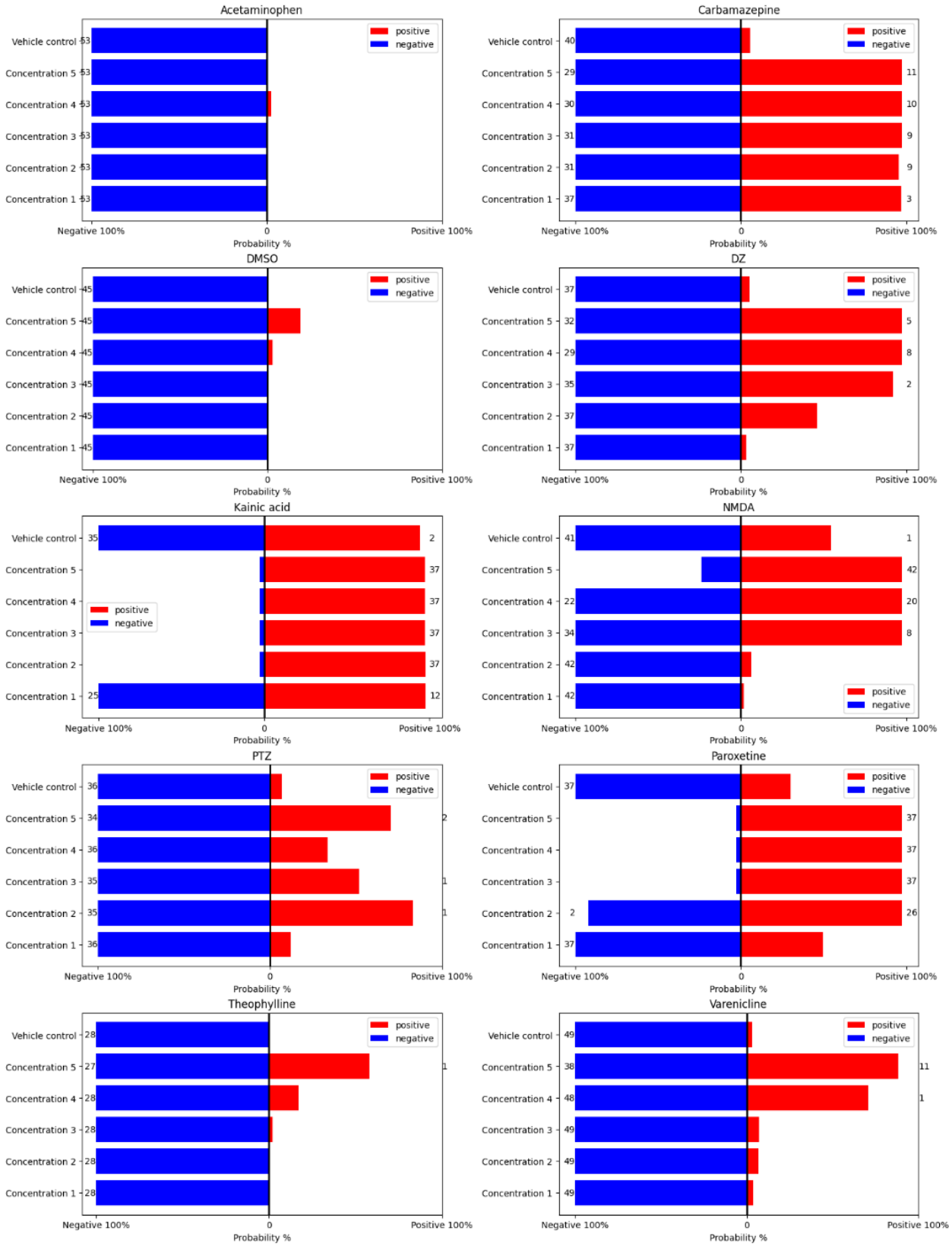

Figure 2. Results obtained from the use of an AI model for seizurogenic potential prediction on a set of compounds tested on a brain *in vitro* model based on the use of NANOSTACKS™ and 24-well CytoView MEA Plates.

Conclusion

This work confirmed the compatibility of the Revivocell NANOSTACKS™ platform with Axion BioSystems 24-well CytoView MEA Plates, supporting the use of these integrated systems for *in vitro* neurotoxicity testing. Additionally, the proof-of-concept AI model developed in this study was capable of assigning seizurogenicity probability scores to a variety of test compounds across multiple concentrations, providing promising early results. Although preliminary, these findings serve as a strong foundation for further experiments designed to expand the potential of the AI model. As this approach continues to evolve, it has the potential to streamline drug testing efforts by enabling more rapid and reliable early-phase assessment of the seizurogenic potential of compounds, ultimately improving the safety profile of new therapeutic candidates.

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Application note

Development of an *in vitro* model of the human brain using NANOSTACKS™

1-2-2024

Introduction

The use of multicellular, complex *in vitro* models of the human brain can facilitate the study of physiological and pathological aspects of the human central nervous system (CNS). Additionally, neural *in vitro* models including multiple cell types can be used as platforms for drug testing, in order to assess the neurotoxic potential of novel compounds with increased accuracy compared to traditional monocellular models (1).

Amongst the variety of cells constituting the CNS, neurons and astrocytes play a key role. In particular, the main function of neurons is to carry electrical signals and form a functional neural network, whilst astrocytes provide trophic and metabolic support (2).

The neuroblastoma cell line SH-SY5Y can be used to model human neurons. In particular, SH-SY5Y can be used in an "undifferentiated" form, resembling immature neurons, or in a "differentiated" form, modelling adult neurons (3).

In this work, a NANOSTACKS™-based *in vitro* model of the human brain was developed using both differentiated and undifferentiated SH-SY5Y cells as a neuronal component, whilst an astrocytoma cell line was utilized to model astrocytes.

The neural model based on undifferentiated SH-SY5Y was then used to test the compound methylglyoxal, and a neurite outgrowth assay was performed on the model including differentiated SH-SY5Y cells to test the drug clomipramine.

Methods

Cell seeding on NANOSTACKS™

NANOSTACKS™ including a polyethylene terephthalate (PET) membrane with a pore size of 0.4 µm were used throughout this work. Additionally, in this work the 1321N1 astrocytoma cell line was included to model the glial component of the human brain. However, the 1321N1 cell line was derived from U-138 MG, another astrocytoma cell line. Therefore, throughout this work we identified the cell line 1321N1 with the cell line U-138 MG. Unless otherwise specified, during the course of this work the cell culture medium used was composed of Dulbecco's Modified Eagle's medium (11995065; Thermo Fisher Scientific) including 10% foetal bovine serum, 1X MEM Non-essential Amino Acids Solution (M7145-100ML; Merck) and 1X Penicillin-Streptomycin (P0781; Merck). NANOSTACKS™ were placed in wells of 24-well plates, one NANOSTACK™ per well. Then, SH-SY5Y and U-138 MG cells were seeded at a seeding density of respectively 5 x 10^3 and 6 x 10^3 cells per NANOSTACK™. In particular, during the seeding procedure, a droplet of 75 µL of cell suspension was placed on the membrane of the NANOSTACKS™, and the seeded cells were then incubated at 37 °C, 5% CO_2 for 2 hours. Subsequently, 825 µL of cell culture medium was added to the wells containing the cell-seeded NANOSTACKS™, reaching the working volume of 900 µL.

Compound testing on undifferentiated SH-SY5Y

The day following the cell seeding, SH-SY5Y and U-138 MG seeded on NANOSTACKS™ were combined in a coculture by placing the seeded platforms on top of each other into 24-well plates. The cell culture medium volume per well was increased to 1.5 mL, and the compound methylglyoxal was added at the following concentrations: 0 (vehicle control), 0.01, 0.1, 1, 10 and 50 µM. SH-SY5Y in monocultures on NANOSTACKS™ were used as a control. 2 days later, cell viability of SH-SY5Y in both cocultures and monocultures was assessed by performing a CellTiter-Glo® (G7571; Promega) assay according to the vendor's instructions.

Compound testing on differentiated SH-SY5Y

SH-SY5Y cells were seeded on NANOSTACKS™ on day 0, as previously described. On day 1 and every 2-3 days, cell culture medium was exchanged with a differentiation medium composed of neurobasal medium (21103049; Thermo Fisher Scientific), 1X B27 supplement

(17504044; Thermo Fisher Scientific), GlutaMAX™ (35050061; Thermo Fisher Scientific), 1X Penicillin-Streptomycin, and retinoic acid (R2625; Merck) at a concentration of 10 µM. On day 7, U-138 MG were seeded on NANOSTACKS™ as previously described, at a cell seeding density of 5 x 10^3 cells per NANOSTACK™. On day 8 the differentiation of SH-SY5Y was completed, and U-138 MG and differentiated SH-SY5Y on NANOSTACKS™ were combined in a coculture. The differentiation medium volume per well was increased to 1.5 mL, and the compound clomipramine was added at the following concentrations: 0 (vehicle control), 10, 50 µM. Monocultures of differentiated SH-SY5Y on NANOSTACKS™ were used as a control. On day 10, SH-SY5Y cells were fixed in 4% V/V paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS).

Immunostaining

Differentiated SH-SY5Y were stained according to the following protocol. All steps were carried out at room temperature, unless otherwise stated. Firstly, cells were permeabilised by submerging the NANOSTACKS™ in a 0.1% V/V solution of Triton X-100 in DPBS for 10 min. Subsequently, cells were washed three times for 5 min in DPBS. Then, after a brief submersion in DPBS, cells were submerged for 1 hour in a "blocking solution", composed of 1% W/V bovine serum albumin in a 0.1% V/V solution of Tween-20 in DPBS. After the blocking step, cells were submerged overnight at 4 °C with a 1:1000 dilution of β-Tubulin III antibody (ab18207; abcam) in blocking solution. Then, cells were washed briefly in DPBS 3 times, and subsequently submerged for 1 hour with a 1:1000 dilution of secondary antibody (ab150077; abcam) in blocking solution, shielded from light. Subsequently, cells were washed for 5 min in DPBS, and then submerged with a solution of 1.4 µg/mL Hoechst 33342 (H3570; Thermo Fisher Scientific) in DPBS for 12 min, shielded from light. Then, cells were washed in DPBS 3 times for 5 min, and finally stored in DPBS.

Neurite outgrowth assay

To assess the effect of clomipramine on SH-SY5Y seeded on NANOSTACKS™, a neurite outgrowth assay was performed according to the following protocol. Firstly, n = 3 images of random fields at a resolution of 20X were acquired per NANOSTACK™ using a fluorescent microscope. Then, using the software FIJI (version: 1.54f) a square-shaped region of interest (ROI) was identified and maintained for each image, and the total length of neurites within the ROI was quantified. The area within the ROI positive to Hoechst was also quantified, obtaining the total nuclear area per ROI. The total length of neurites per ROI was then normalised with the total nuclear area per ROI.

Statistical analysis

Each datapoint reported in this application note was derived from measures obtained from a minimum of n = 3 cell-seeded NANOSTACKS™.

Results

Undifferentiated SH-SY5Y were cocultured with U-138 MG on NANOSTACKS™, and the drug methylglyoxal was tested on the model, using a monoculture of undifferentiated SH-SY5Y on NANOSTACKS™ as a control. In particular, methylglyoxal was used at concentrations 0.01, 0.1. 0.5, 1, 5 and 10 mM, and 48 h later the viability of undifferentiated SH-SY5Y was analysed (Fig. 1A-B). The IC50 associated to the coculture model (0.78 mM) was 27.9 % higher than the IC50 obtained from the monoculture (0.61 mM, fig. 1A), indicating that U-138 MG astrocyte-like cells exert a protective effect on undifferentiated SH-SY5Y. In particular, the viability of undifferentiated SH-SY5Y was found to be lower in monocultures relatively to cocultures at concentrations of Clomipramine of 1, 5 and 10 mM (Fig. 1B), and the difference was statistically significant.

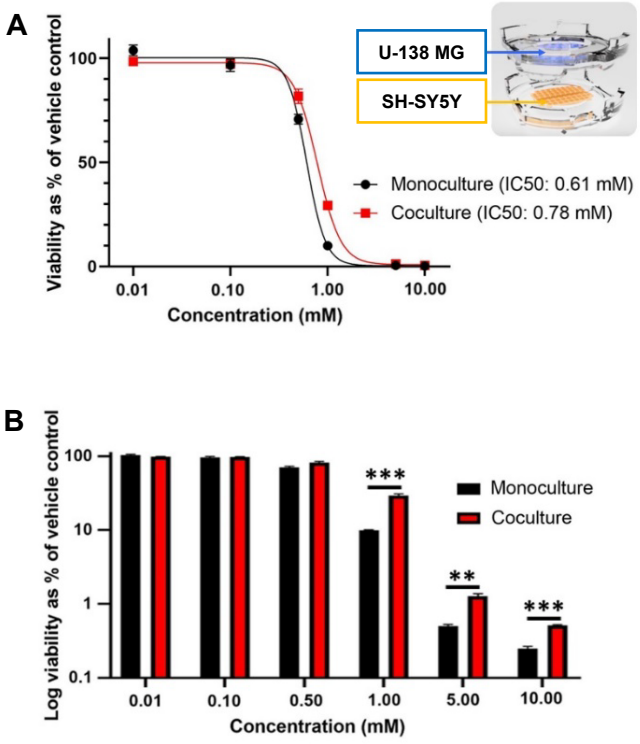

Figure 1. *Viability of undifferentiated SH-SY5Y upon drug testing with methylglyoxal.* ***A:*** *Dose response curves of coculture and monoculture models.* ***B:*** *Bar graph indicating viability of undifferentiated SH-SY5Y in monocultures and cocultures. Data are reported as mean ± SEM.*

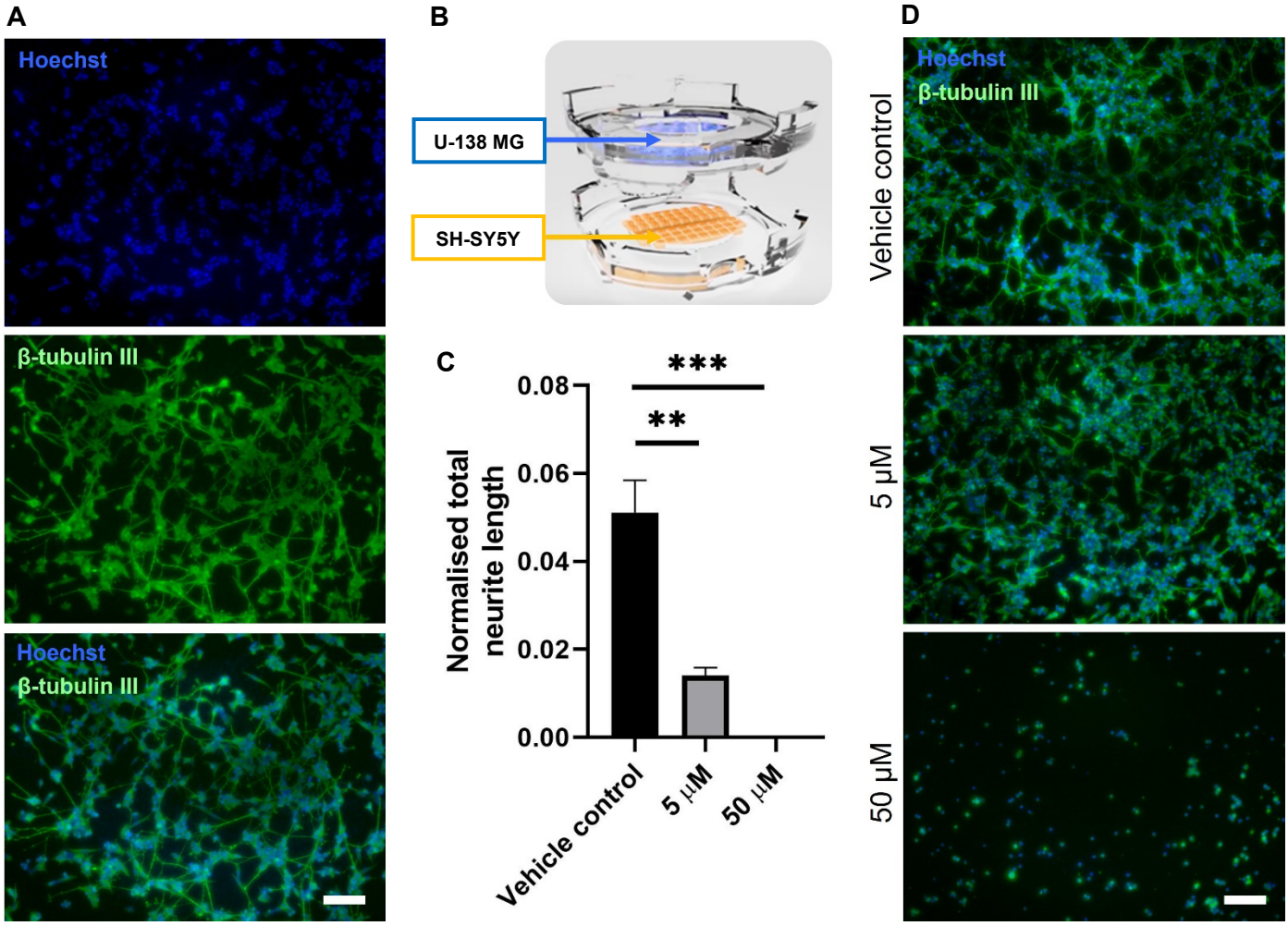

Figure 2. *Clomipramine testing on a neural model based on differentiated SH-SY5Y and U-138 MG.* ***A:*** *Representative images of Hoechst (blue) and β-tubulin III (green) immunostaining of differentiated SH-SY5Y included in the model, associated with the vehicle control of the clomipramine testing, 2 days after drug testing. Magnification: 20X. Scale bar: 100 µm.* ***B:*** *Scheme of the coculture.* ***C:*** *Neurite outgrowth assay results reported as mean ± SEM.* ***D:*** *Representative images of immunostaining of differentiated SH-SY5Y with β-tubulin III (green) and Hoechst (blue), associated with the vehicle control (top) and with concentrations of clomipramine of 5 µM (middle) and 50 µM (bottom). Magnification: 20X. Scale bar: 100 µm.*

In addition to the development of a model based on undifferentiated SH-SY5Y, a differentiation protocol was performed to differentiate SH-SY5Y towards a more mature neuronal phenotype. Then, the differentiated SH-SY5Y cells were cocultured with U-138 MG on NANOSTACKS™, and the drug clomipramine was tested on the model at a concentration of 5 µM and 50 µM (Figure 2). Upon completion of the compound testing, immunostaining of differentiated SH-SY5Y revealed the presence of neurites extending from cell bodies in the vehicle control and 5 µM conditions, whilst neurites were not observable in the 50 µM condition (Fig. 2D). Quantification of neurite outgrowth indicated that the length of the neurites normalised to nuclear area was higher in the vehicle control than in the 5 µM and 50 µM conditions (Fig. 2C), and the difference was statistically significant ($p < 0.05$).

Conclusions

This work demonstrated that the astrocytoma cell line U-138 MG and the neural cell line SH-SY5Y are compatible with NANOSTACKS™, and can be combined to build a coculture model of the human brain. The model can also be used for toxicity testing, as demonstrated in this work using the compounds methylglyoxal and clomipramine. In the model inclusive of undifferentiated SH-SY5Y, the presence of U-138 MG reduced the neurotoxicity of methylglyoxal relatively to the SH-SY5Y monoculture control. This result is in line with the work of De Simone *et al.* (4), although in their study a different astrocytoma cell line, D384, was used in place of U-138 MG.

In this work SH-SY5Y were also used in a differentiated form, providing a model of human neurons closer to the phenotype of primary adult cells (3). The neural phenotype of differentiated SH-SY5Y was confirmed upon immunostaining of

β-tubulin III, which revealed a network of neurites. Finally, the model including differentiated SH-SY5Y was compatible with neurite outgrowth assays, demonstrating the neurotoxicity of clomipramine. This work therefore demonstrates the feasibility of performing imaging-based assays to assess the neurotoxicity of compounds on NANOSTACKS™-based multicellular *in vitro* models.

References

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Application note

NANOSTACKS™ LIVER-BRAIN

Modeling the liver-brain axis using NANOSTACKS™

Introduction

The liver plays an important role in the metabolism of compounds such as pharmaceutical drugs (1). The chemical modifications of compounds occurring during their metabolization lead to the generation of metabolites, which can exert toxic effects on human organs (2). Therefore, when assessing the toxicity risk of a drug on a particular target organ, it is advantageous to use *in vitro* models including a liver cellular component to simulate the metabolization of the compound.

In addition to its role in drug metabolism, the liver interacts with other organs of the human body such as the brain, particularly in pathological conditions such as Alzheimer's Disease (3) and Parkinson's Disease (4). To further improve our knowledge of such pathologies and develop novel therapies, the use of an *in vitro* model of the liver-brain axis would be advantageous.

In this work, we developed an *in vitro* model of the liver-brain axis using NANOSTACKS™. In particular, the neuronal cell line SH-SY5Y and an astrocytoma cell line were included to model respectively neurons and astrocytes, whilst the hepatic cell line HepaRG® was included to model the liver component. The model was characterised and then used as a platform to assess the neurotoxicity of the drug paclitaxel.

Methods

Collagen coating of NANOSTACKS™

NANOSTACKS™ were coated with collagen I (A10483-01; Gibco) by submerging their porous membrane with 60 µL of collagen I solution at a concentration of 27.5 µg/mL for 1 hour at room temperature. Collagen-coated NANOSTACKS™ were then washed 3 times for 5 min with distilled water, then dried overnight at room temperature.

HepaRG® culture on NANOSTACKS™

During the thawing and seeding procedures, HepaRG® cells (cryopreserved differentiated HepaRG® cells, HPR116010; Wepredic) were cultured using a cell culture medium termed "thawing medium", obtained by adding HepaRG® Thawing/Plating/General Purpose Medium supplement with antibiotics (ADD670C; Wepredic) to Williams E Medium with Glutamax™(32551020; Thermo Fisher Scientific) according to the vendor's instructions. Cells were seeded on collagen-coated NANOSTACKS™ placed on wells of 24-well plates at a seeding density of 72 x 10^3 cells per NANOSTACK™. In particular, during the seeding procedure, a droplet of 75 µL of cell suspension was placed on the membrane of the NANOSTACKS™, which were then incubated at 37 °C, 5% CO_2 for 2.5 hours. Subsequently, 825 µL of thawing medium were added to the wells containing the cell-seeded NANOSTACKS™, reaching the working volume of 900 µL. The next day, and every 2-3 days, cell culture medium was changed using a medium termed "maintenance medium" obtained by adding HepaRG® Maintenance/Metabolism Medium Supplement with antibiotics (ADD620C; Wepredic) to Williams E Medium with Glutamax™ according to the vendor's instructions.

Cell viability and CYP activity assays on HepaRG®

Cell viability was assessed by performing a CellTiter-Glo® (G7571; Promega) assay, and CYP activity was assessed by performing a P450-Glo™ CYP3A4 Assay (V9001; Promega) following the instructions of the vendor. Both assays were performed in 24-well plates, making sure that sufficient volumes were added to the wells to completely submerge the NANOSTACKS™ at any point when cell submersion was necessary. The cell culture medium used throughout the assays was termed "induction medium" and was obtained by adding HepaRG induction medium supplement with antibiotics (ADD640C; Wepredic) to Williams E Medium with Glutamax™ according to the vendor's instructions.

Drug testing on the liver-brain axis model

On day 0, SH-SY5Y and U-138 MG cells (formerly known are 1321N1 cells) were seeded on NANOSTACKS™ at seeding densities of respectively 6.5 x 10^3 cells per NANOSTACK™ and 9 x 10^3 cells per NANOSTACK™, whilst HepaRG® cells were seeded on NANOSTACKS™ at a seeding density of 72 x 10^3 cells per NANOSTACK™ as previously described, with the following modifications with regards to the culture of SH-SY5Y and U-138 MG: the cell culture medium used during the seeding procedure and at day 0, termed “neural medium”, was composed of Dulbecco’s Modified Eagle’s medium (11995065; Thermo Fisher Scientific) including 10 % foetal bovine serum, 1X MEM Non-essential Amino Acids Solution (M7145-100ML; Merck) and 1X Penicillin-Streptomycin (P0781; Merck), and upon cell seeding the medium required to reach the working volume of 900 µL was added 2 hours later. On day 1, NANOSTACKS™ including the 3 cell types were combined to initiate the triculture, using maintenance medium as cell culture medium at a working volume of 1.5 mL for both tricultures and for monocultures of SH-SY5Y used as control, and including paclitaxel dissolved in dimethyl sulfoxide at concentrations: 0 (vehicle control), 0.0001, 0.001, 0.01, 0.1, 1, 10, 50 µM. On day 3, the viability of SH-SY5Y cells was assessed by performing a CellTiter-Glo® assay as previously described, using neural medium whenever the protocol required the use of cell culture medium.

Statistical analysis

Each datapoint reported in this application note was derived from measures obtained from a minimum of n = 3 cell-seeded NANOSTACKS™.

Results

Upon seeding, HepaRG® cells formed a confluent layer (Fig. 1D) and were viable throughout the entire experiment up to day 9 (Fig. 1A), thus indicating that NANOSTACKS™ are compatible with HepaRG® cells. The CYP3A4 activity of HepaRG® cells was also maintained throughout the entire experiment (Fig. 1B). Upon normalisation of the data obtained from the CYP3A4 activity assay with the data obtained from the viability assay, no statistically significant difference was found between different timepoints.

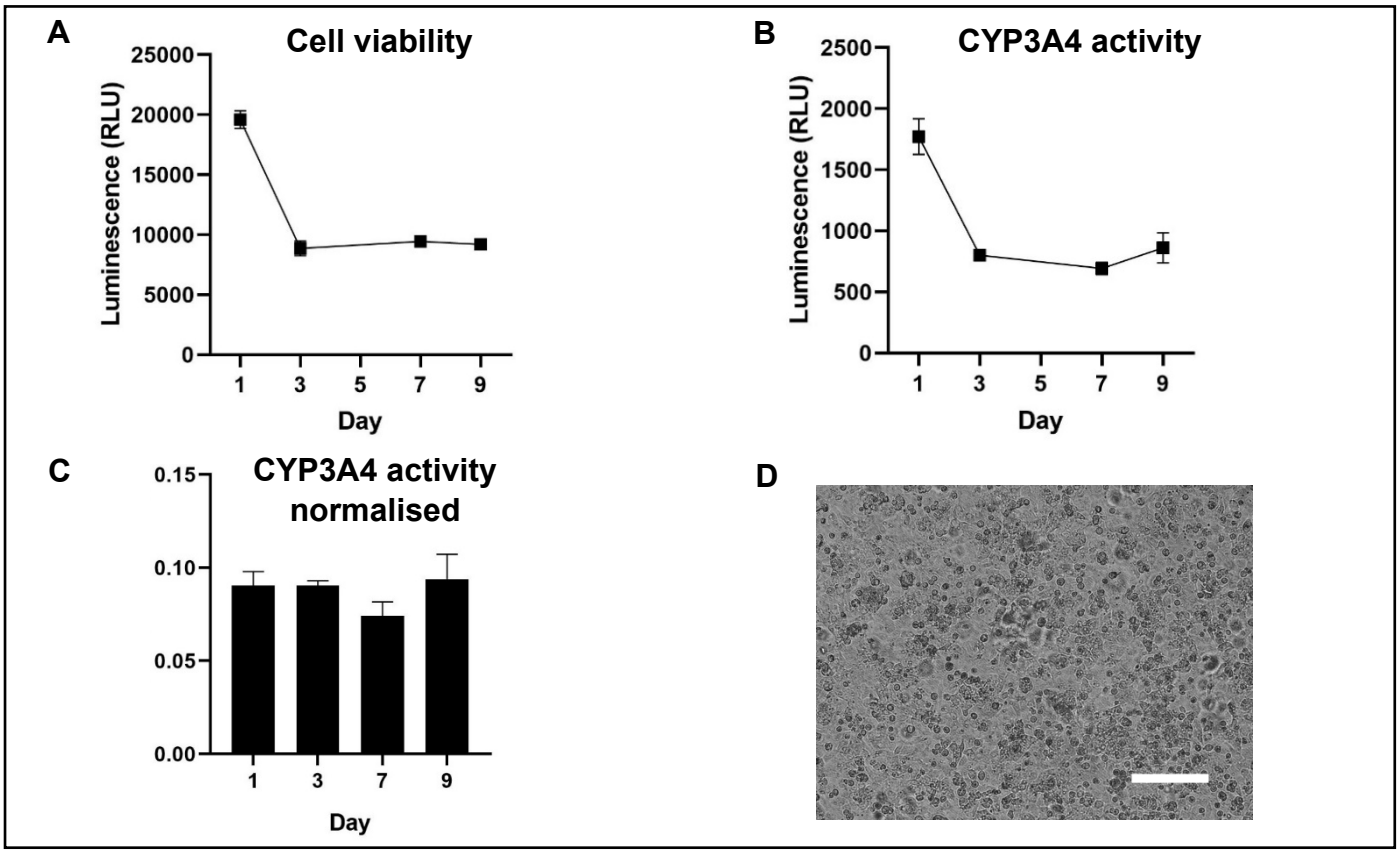

Figure 1. A: *Viability of HepaRG® cells expressed in relative lights units (RLU).* ***B:*** *CYP activity of HepaRG® cells expressed in RLU.* ***C:*** *CYP activity normalised with viability data.* ***D:*** *Representative widefield image of HepaRG® cells on NANOSTACKS™ on day 1. Magnification: 10X. Scale bar: 250 µm. Data are expressed as mean ± SEM.*

HepaRG®, U-138 MG, and SH-SY5Y cells were combined on NANOSTACKS™ to obtain a liver-brain model (Fig. 2B). The effect on SH-SY5Y viability of the drug paclitaxel was tested on both the liver-brain model and on a monoculture of SH-SY5Y on NANOSTACKS™ (Fig. 1C). In particular, the concentration of paclitaxel used in this experiment ranged between 0.0001 µM and 50 µM, and the cells were exposed to the compound from day 1 to day 3, when the viability assays were performed and widefield images were acquired (Fig 2A). The IC50 value obtained from the dose response curve associated to the liver-brain model was 0.0684 µM. The IC50 associated to the triculture was 26.8 % lower than the IC50 associated with the SH-SY5Y monoculture, which was 0.0935 µM.

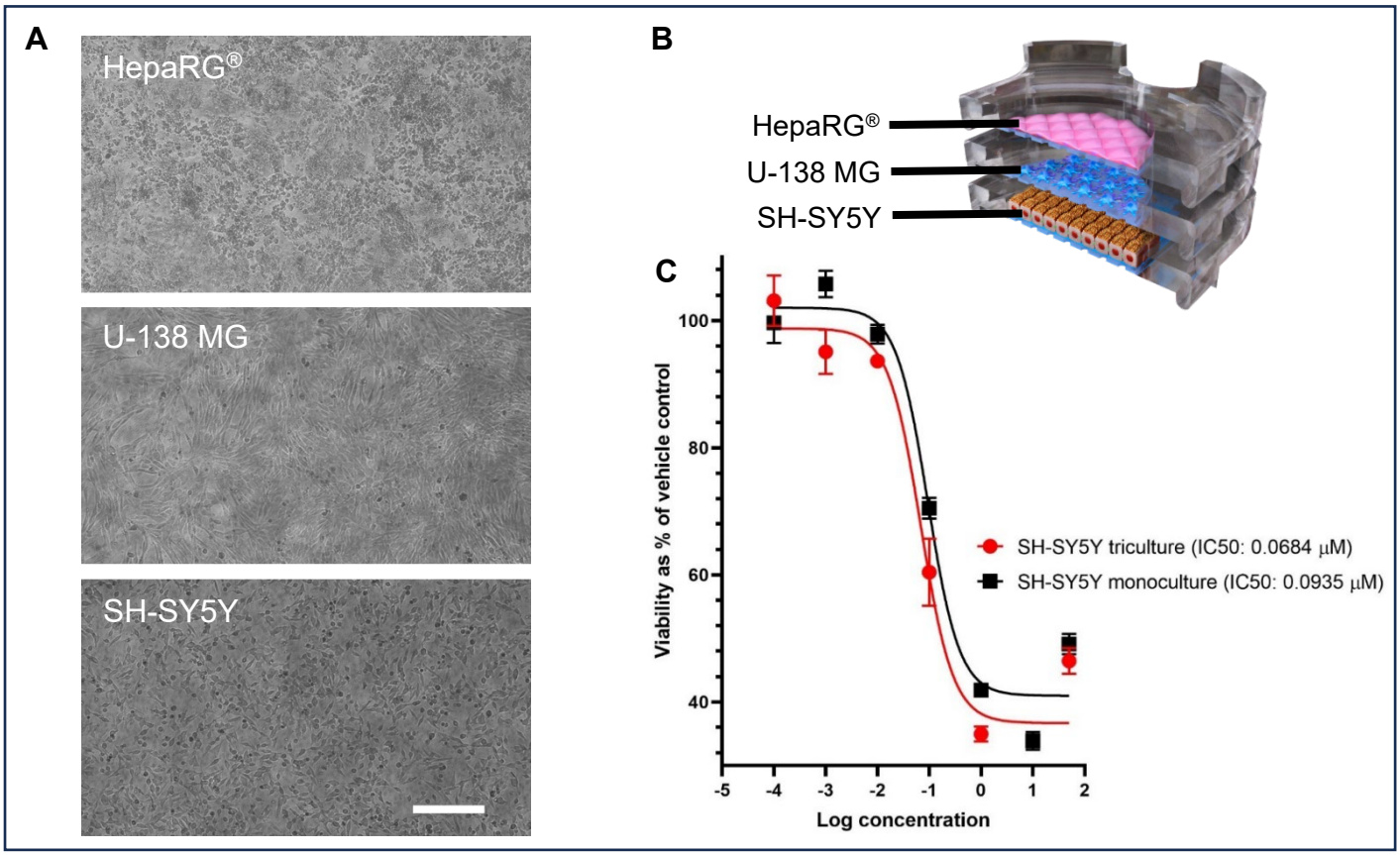

Figure 2. A: *Representative widefield images of HepaRG®, U-138 MG and SH-SY5Y cells in triculture on NANOSTACKS™ on day 3, associated with the vehicle control. Magnification: 10X. Scale bar: 250 µm.* ***B:*** *Triculture scheme.* ***C:*** *Paclitaxel dose-response curve and IC50 values for the triculture and the monoculture setups. Data are expressed as mean ± SEM.*

Conclusions

Differentiated HepaRG® cells adhered to NANOSTACKS™ and maintained their viability for 9 days of cell culture on the platforms. Additionally, HepaRG® cells were metabolically active, as CYP3A4 was detected across all timepoints. Therefore, HepaRG® cells can successfully be incorporated into NANOSTACKS™.

HepaRG® were cocultured with the neural cell lines SH-SY5Y and U-138 MG, recapitulating the liver-brain axis. The drug paclitaxel was tested on the liver-brain model, and the IC50 associated with the viability of SH-SY5Y cells in the triculture model was 26.8 % lower than the IC50 associated with the SH-SY5Y monoculture, suggesting that the liver component increased the model's sensitivity.

This work demonstrates the feasibility of using a NANOSTACKS™-based *in vitro* model of the liver-brain axis for drug testing. However, this model could also be used to study the liver-brain crosstalk in physiological and pathological conditions.