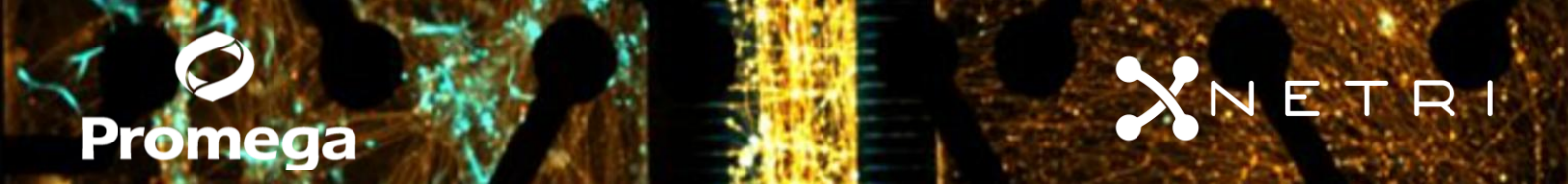


Application Note Promega/NETRI: Versatile cytotoxicity assay in 2D and 3D NETRI microfluidic systems with LDH-Glo™

Héloïse Castiglione[a,b,c] et Thomas Bessy[a], Louise Dubuisson[a], Audrey Azema[a], Florian Mignot[d], Sébastien Chevalier[d], Rania Ben Jouira[d], Alexandre Guichard[a]

[a] NETRI, Lyon, France
[b] SupBiotech, Ecole d'Ingénieurs en Biotechnologies, Villejuif, France
[c] Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA), Service d'Etude des Prions et des Infections Atypiques (SEPIA), Université Paris-Saclay, Fontenay-aux-Roses, France
[d] Promega, Charbonnières-les-Bains, France

Overview

Innovative *in vitro* models are essential for advancing neurobiology and neurotoxicology research. NETRI microfluidic platforms provide versatile solutions tailored to distinct scientific challenges, from central nervous system (CNS) development studies to peripheral nervous system (PNS) pain models. By providing tightly controlled microenvironments, these devices support physiologically relevant culture conditions and promote organized cellular architectures that better reflects *in vivo* physiology.

A common requirement across these models is the possibility to perform sensitive, non-invasive cytotoxicity assays that preserve sample integrity for complementary analyses. The LDH-Glo™ Cytotoxicity Assay (Promega, J2380) meets this need by quantifying lactate dehydrogenase (LDH) released from damaged cells into the culture medium, providing a direct measure of membrane integrity loss. Its luminescent readout ensures high sensitivity even with very small sample volumes, making it ideal for microfluidic systems.

In this Application Note, we illustrate the versatility of this approach through two distinct use cases:

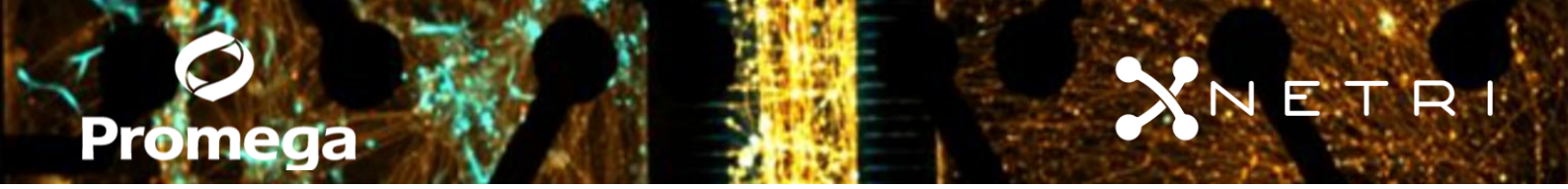
1) 2D peripheral neuron culture on-chip

We first illustrate a standard implementation of the LDH-Glo™ assay in a 2D microfluidic context using the NETRI DuaLink™ platform. Human iPSC-derived sensory neurons were cultured in microfluidic chips, where the compartmentalized architecture enabled independent assessment of drug-induced cytotoxicity in neuronal cell bodies and axonal terminals, providing region-specific toxicity profiles. The LDH-Glo™ assay was performed following the manufacturer's standard instructions, including the use of conventional normalization strategies.

2) 3D brain organoid culture on-chip

We next present a more advanced application involving 3D hiPSC-derived brain organoids cultured on-chip in NETRI Duplex™ device. In this context, the LDH-Glo™ assay protocol was adapted by NETRI, following the methodology proposed by Castiglione *et al*. (2024) [1], to establish a non-invasive approach compatible with longitudinal studies in complex 3D models.

By presenting both approaches, first a standard implementation of LDH-Glo™ assay in a 2D microfluidic system, followed by an adapted non-invasive strategy for complex 3D organoids, we aim to provide users with a transparent and practical framework. The adapted protocol for 3D cell culture addresses specific needs for longitudinal, non-destructive studies in advanced 3D models, while leaving investigators free to select or refine normalization strategies according to their experimental design.



1) Cytotoxicity assay with the LDH-Glo™ on a NETRI 2D PNS on chip model

Microfluidic culture systems offer unique advantages for *in vitro* neurobiology studies by enabling precise control of microenvironments and compartmentalization. NETRI DuaLink™ platform exemplifies this approach by allowing region-specific analysis, thanks to its physically separated compartments for neuronal cell bodies and distal processes. When combined with the LDH-Glo™ assay, this configuration supports sensitive, non-invasive cytotoxicity measurements in complex culture formats, compatible with long-term experiments and high-throughput workflows. Here, we applied this approach to investigate region-specific neurotoxicity caused by chemotherapy-induced peripheral neuropathy (CIPN) using paclitaxel.

1. Experimental protocol

Microfluidic channels of the DuaLink™ microfluidic device were opened with 70% ethanol and immediately rinsed with PBS. Chips were then coated with 0.1mg/mL poly-D-lysin (PDL) (Thermofischer, A38904) overnight in a cell culture incubator. After 3 washes with PBS, channels were incubated for 2 hours with iMatrix 511-Silk (Anatomic, M511S) diluted 1:100 in the culture medium (SensoMM supplemented with Brainfast Astro Supplement diluted 1:1000). The iMatrix solution was removed prior to cell seeding. Sensory neurons were differentiated from human induced pluripotent stem cells (hiPSC) obtained from CD34+ cord blood cells (Anatomic, RealDRG 1020F1-3M). Cortical astrocytes were differentiated from a hiPSC line (BrainXell, BX-0600). 40k sensory neurons and 20k astrocytes were seeded in the neuronal cell body compartment in 3 µL of SensoMM (Anatomic, 1030) supplemented with Brainfast Astro Supplement (BrainXell, BX-2600) (1:1000). Brainfast Astro Supplement was maintained in the culture medium up until day 9. On day 1, the medium was fully renewed. Subsequently, 75% of the medium was replaced three times per week. Cells were cultured for 21 days before the assay. Cells were treated at day 22 with paclitaxel in 0.01% DMSO (Sigma, T7191) or with 0.01% DMSO. 5 µL of media was collected at 48h and frozen in 45 µL of LDH preservation buffer, following LDH-Glo™ recommendations (200 mM Tris-HCl (pH 7.3), 10% glycerol, and 1% bovine serum albumin (BSA)). Cultures lysed with 0.3% Triton X-100 were used for LDH maximum release value, as recommended in LDH-Glo™ instructions. Samples in the cellular body compartment were further diluted at a 1:5 ratio in LDH preservation buffer. Cytotoxicity was calculated as the percent of maximum LDH release. An ANOVA test was performed with multiple comparison versus the control condition.

2. Results

We first validated the LDH-Glo™ assay using hiPSC-derived sensory neurons cultured in DuaLink™ devices under three conditions: untreated control, 20% DMSO for 45 min (intermediate toxicity), and 0.3% Triton X-100 (maximum LDH release). Samples from each compartment were collected and tested at three dilutions (1/2, 1/5, and 1/10), in addition to the initial dilution from sample collection of 1:10 ratio (**Figure 1**).

In the cell body compartment, the 1/2 dilution exceeded the upper point in the range (1,400,000 RLU) for maximum LDH release, while the 1/5 dilution fell within the dynamic range and allowed clear discrimination of intermediate toxicity. In contrast, the axonal compartment

exhibited markedly lower luminescence values, consistent with reduced cytoplasmic content. Intermediate toxicity was not detected in axonal samples, possibly due to higher resistance of axonal terminals to DMSO or a limited dynamic range at the tested dilutions. Increasing assay sensitivity by testing undiluted samples could improve detection in this compartment.

Overall, the LDH-Glo™ assay demonstrated high sensitivity, requiring only 5 µL of sample to quantify cytotoxicity. This enables routine, high-throughput, and non-invasive monitoring of neuronal health in microfluidic systems. Furthermore, applying different dilution strategies per compartment enhances the ability to detect region-specific cytotoxicity.

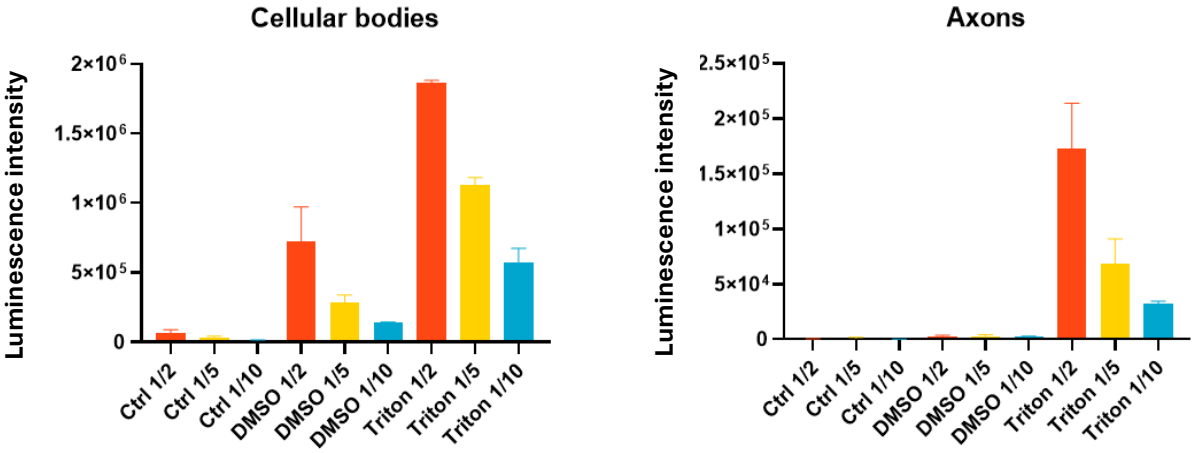


Figure 1 : *Dilution test of LDH positive and negative control samples in two different microfluidic compartments: the cellular body (left) and axonal endings (right) compartments.*

To illustrate the relevance of this approach, we applied the LDH-Glo™ assay to assess the dose-dependent cytotoxicity of paclitaxel, a chemotherapeutic agent known to induce CIPN. hiPSC-derived sensory neurons were co-cultured with astrocytes in DuaLink™ devices in a model used

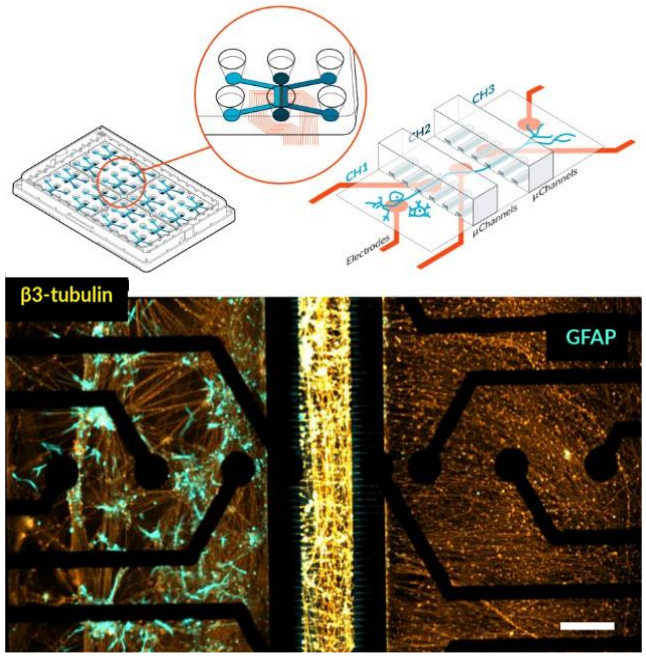


Figure 2 : *PNS model for electrophysiological assessment of CIPN with a sensory neuron astrocyte coculture. Scheme illustrating the architecture of the microfluidic device and the cell organisation in the different compartments (top). Immunofluorescence image illustrating the coculture with sensory neurons stained with b3-tubulin (yellow) and astrocytes with GFAP (cyan).*

for MEA-based electrophysiological characterization of the CIPN effect of such drugs [2] **(Figure 2).** Since paclitaxel is reported to exert differential toxic effects on axons and neuronal cell bodies [3, 4], we evaluated the local effect of different paclitaxel doses: 50 nM, 200 nM, and 1,000 nM, alongside a vehicle control.

Our results show that paclitaxel induced a mild cytotoxicity of ~3% of maximum LDH release, compared to ~1% for vehicle-treated controls, highlighting the high sensitivity of the assay **(Figure 3).** The toxicity dynamics were similar in both compartments, but effects were detectable at lower concentrations in the cell body compartment, confirming the importance of compartment-specific analysis. This approach can reveal distinct regional vulnerabilities to neurotoxic compounds, and testing additional compounds with preferential effects on cell bodies or axons could further illustrate this specificity.

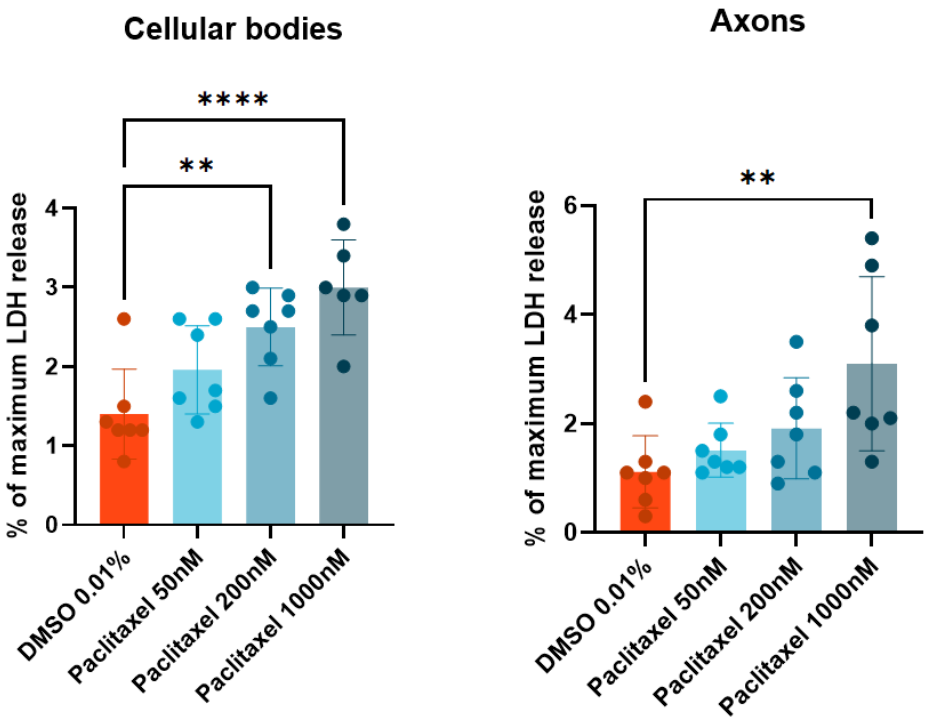


Figure 3 : *Quantification of LDH release as a percentage of the maximum release in the cellular bodies compartment (left) and axonal endings compartments (right) of DMSO or paclitaxel treated cocultures.*

2) Cytotoxicity Evaluation in a NETRI Brain Organoid-on-Chip Model Using the LDH-Glo™ Cytotoxicity Assay

Brain organoids-on-chips are promising 3D *in vitro* models that recapitulate key aspects of human brain development, cellular organization, and physiology. Their growing use in preclinical research and neurotoxicity testing creates a pressing need for sensitive, reliable, and non-destructive cytotoxicity assays that preserve organoid integrity for complementary and longitudinal analyses.

A major challenge associated with these 3D models lies in the normalization of biochemical assays, such as LDH assay [5]. In 3D organoids, standard approaches such as cell counting or quantification of total intracellular protein or DNA require sample dissociation and/or cellular lysis and are therefore inherently destructive, making them incompatible with repeated measurements on the same sample over time. Normalization to total intracellular content or total

LDH in parallel reference wells, commonly used in 2D cultures, is also problematic for organoids, as their heterogeneity can lead to imprecise results.

To address this need, the LDH-Glo™ assay protocol was adapted by NETRI by applying the conditioned medium-based normalization methodology relying on total secreted protein proposed by Castiglione and colleagues (2024) [1]. This strategy is conceptually aligned with secretome-based approaches and is consistent with a subsequent study in brain organoids reported by Fertan *et al.* (2023) that proposed LDH normalization based on glucose consumption as an indirect readout [6].

Our strategy based on total secreted proteins enables normalization without organoid lysis, preserving sample integrity and allowing longitudinal measurements of individual organoids. LDH measurements were thus performed on a per-organoid basis, providing sample-specific data while maintaining the feasibility of repeated analyses over time.

It should be emphasized that this approach, while practical and biologically relevant, has inherent limitations. Secreted protein levels do not necessarily scale linearly with cell number and can vary depending on the physiological state of the cells or the effects of compound exposures. Similarly, invasive normalization methods based on intracellular protein content are also influenced by cellular stress or differentiation status. Consequently, all normalization strategies have limitations, and users should feel free to adapt the approach according to their experimental design and objectives.

Here, this conditioned medium-based normalization workflow was applied to assess neurotoxicity in hiPSC-derived cortical organoids on-chip chronically exposed to two reference compounds: valproic acid, known to induce developmental neurotoxicity, and vanillin, reported as non-neurotoxic. Adaptations of the LDH-Glo™ assay protocol are indicated in the text and further detailed in the accompanying footnotes.

1. Experimental protocol

Cerebral cortical organoids derived from hiPSCs were cultured following an adapted protocol from Xiang and colleagues (2017, 2019) [7, 8], and maintained on-chip in the Duplex™ device. On-chip cortical organoids were chronically exposed from day 18 (D+18) to day 24 (D+24) of differentiation to two reference compounds:

- Valproic acid (VPA, 10 mM), a compound known for its developmental neurotoxicity properties, used here at a supra-physiological concentration to induce high toxicity [9–11]
- Vanillin (100 nM), a compound considered as non-neurotoxic at this dose [12, 13]

At D+24, conditioned medium from the brain organoid-on-chip cultures was collected for further analysis of LDH activity. For cryopreservation, samples of conditioned medium were mixed at a 1:1 ratio with the LDH preservation buffer, and then frozen at -20 °C until analysis. As recommended in the LDH-Glo™ instructions, the LDH preservation buffer was composed of 200 mM Tris-HCl (pH 7.3), 10% glycerol, and 1% BSA. For LDH assay normalization, total secreted protein concentration was first determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, 23225), according to the manufacturer's recommendations [a]. For each BCA assay, a BSA standard curve was generated. Conditioned medium samples (previously mixed at a 1:1 ratio with preservation buffer) were further diluted at 1/4 in sterile water to ensure measurements

[a] Adaptation from NETRI (Castiglione *et al.,* 2024) for LDH normalization based on total secreted protein concentration.

remained within the linear range of the BCA assay for protein concentration determination [b]. Next, protein concentration was determined using the BCA assay and LDH activity was measured using the LDH-Glo™ Cytotoxicity Assay, based on the water-diluted samples. As a positive control of LDH activity, a cortical organoid was dissociated and lysed in 1 mL of RIPA Lysis and Extraction Buffer (ThermoFisher Scientific, 89900) supplemented with protease and phosphatase inhibitors (A32959) for 15 minutes at 4°C, followed by gentle mechanical dissociation through pipetting [c]. A negative control consisting of fresh culture medium was included in the assay. Finally, the resulting LDH activity, expressed as luminescence signal, was normalized to total protein concentration in the corresponding conditioned medium.

2. Results

Cortical organoids chronically exposed to VPA 10 mM from D+18 to D+24 resulted in a significant increase in LDH activity compared with the non-exposed control organoids **(Figure 4A)**, consistent with expected high cytotoxic effects for this exposure. In contrast, chronic exposures with vanillin 100 nM did not induce any significant detectable increase in LDH release compared with non-exposed controls, confirming its non-neurotoxic profile **(Figure 4A)**. To further illustrate these neurotoxicity results, immunofluorescence staining analyses are provided as example, demonstrating optimal cytoarchitectural organization in non-exposed organoids and marked structural alterations in organoids exposed to a neurotoxic compound **(Figure 4B)**. As expected, LDH activity was elevated in lysed organoids (LDH positive control), which exhibited a more than twelve-fold increase in LDH activity compared with control organoids; while no detectable luminescent signal was observed in the fresh culture medium (LDH negative control) **(Figure 4A)**.

These results demonstrate that the LDH-Glo™ Cytotoxicity Assay is a sensitive and reliable method for quantifying cytotoxicity in a NETRI brain organoid-on-chip model. Combined with the conditioned medium-based normalization strategy, it is adapted to non-invasive and longitudinal monitoring of individual organoids over time. Importantly, the observed responses are consistent with the expected neurotoxic profiles of the reference compounds tested, effectively discriminating between toxic and non-toxic conditions under chronic exposures.

[b] Adaptation from NETRI (Castiglione *et al.,* 2024). Conditioned medium samples stored in preservation buffer were further diluted in water to ensure compatibility with the linear range of the protein assay (BSA calibrating curve), as baseline protein content from the culture medium and additional BSA contained in the preservation buffer increase total protein concentration.

[c] Adaptation from NETRI (Castiglione *et al.,* 2024). The LDH-positive control was adapted from the LDH-Glo™ assay recommendations. Instead of Triton X-100 treatment to induce maximal LDH release from cultured cells, complete lysis of an entire organoid was performed to quantify total intracellular LDH (including non-secreted LDH), providing a technical maximum control for the assay under 3D conditions.

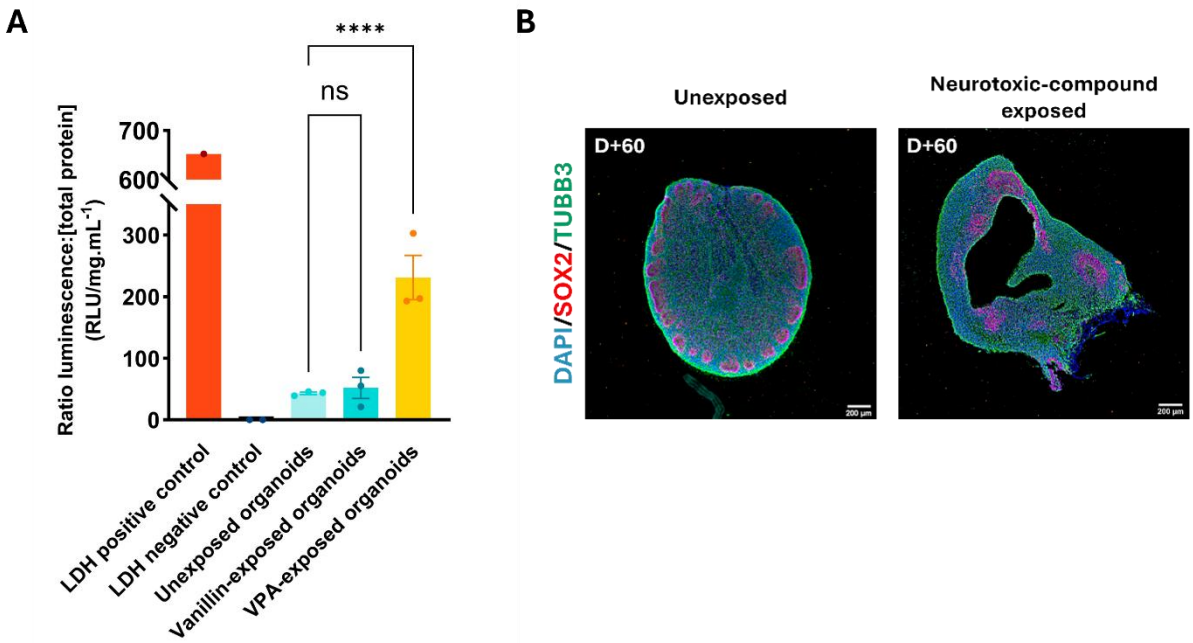


Figure 4. *(A) LDH activity values in the conditioned medium of brain organoids-on-chips at day 24 of culture, including unexposed control organoids, organoids chronically exposed from day 18 to day 24 to vanillin 100 nM or valproic acid (VPA) 10 mM. An LDH-positive control (complete organoid lysis to measure total LDH) and an LDH-negative control (fresh culture medium) were included in the assay. LDH activity was measured by luminescence (RLU) and normalized to the total protein concentration in the conditioned medium (mg·mL $^{-1}$). Mean LDH/[total protein] ratios (RLU·mg $^{-1}$·mL) are shown (mean ± SEM) (n = 3 organoids per condition). Statistical analyses were performed using one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons versus the non-exposed control group, ****p < 0.0001 (comparisons: Vanillin 100 nM vs. Control: ns, VPA 10 mM vs. Control: ****). (B) Immunofluorescent staining of unexposed and neurotoxic compound-exposed organoid examples (SOX2: red, TUBB3: green, Leica Thunder microscope, 10X).*

Conclusion

The LDH-Glo™ Cytotoxicity Assay proves to be a highly sensitive and non-destructive method for evaluating cytotoxicity in advanced neural models integrated within NETRI microfluidic platforms. In 2D applications, such as hiPSC-derived sensory neurons on-chip, the assay enables compartment-specific toxicity profiling and reliably detects paclitaxel-induced cytotoxicity under standard 2D conditions. In more complex 3D systems, including brain organoid-on-chip models, the assay is equally applicable, with specific optimizations introduced to address normalization challenges inherent to heterogeneous organoids. The conditioned medium-based normalization strategy enables non-invasive longitudinal monitoring, while preserving organoid integrity. Importantly, these adaptations are proposed to facilitate 3D applications; however, users remain free to implement alternative normalization approaches according to their experimental design.

Together, these results validate the LDH-Glo™ assay as a robust and adaptable solution for predictive neurotoxicity testing and preclinical safety assessment in NETRI organ- and organoid-on-chip models.

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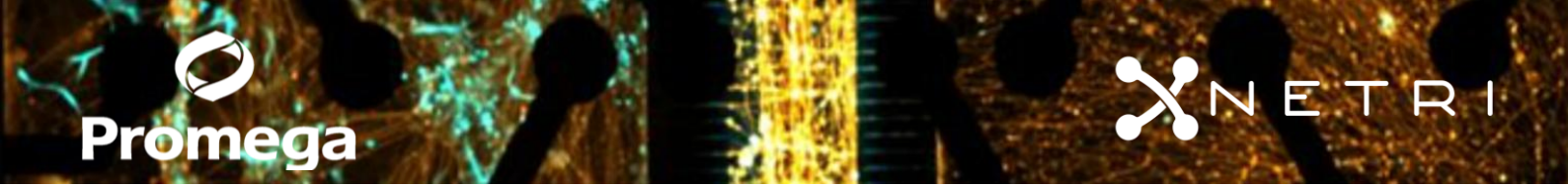

Contact & Resources

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For further information, see Technical Manual TM548, available at: LDH-Glo™ Cytotoxicity Assay | LDH Assay | LDH Release | J2380 | Promega

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For further information, see all our resources, available at: https://netri.com/resources-hub/

or contact our experts, at : sales@netri.com

PROMEGA CORPORATION
• 2800 WOODS HOLLOW ROAD • MADISON, WI 53711-5399 USA
• TELEPHONE 608-274-4330
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NETRI SAS, Bâtiment Bioserra 3, 76 rue Georges Gouy, 69007 Lyon, France
SAS with capital of €3,266,992, registered with the Lyon Trade and Companies
Register under no. 840 248 744
contact@netri.com