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Original Article

An *in vitro* microfluidic culture device for peripheral neurotoxicity prediction at low concentrations based on deep learning

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ABSTRACT — In this study, a microfluidic culture device and related evaluation methods were developed using deep learning to construct a rapid assessment platform for peripheral neuropathy caused by typical anticancer drugs. Primary rodent dorsal root ganglia were cultured in a microfluidic culture device that separated the cell body and neurites, and morphological changes in the neuritis were analyzed using immunofluorescence imaging. Successful culture of separated neurites in the microfluidic device for more than 1 month indicated that this test process, including culture, drug stimulation, and fluorescence observation, results in a viable outcome. In addition, cultured samples were treated with several anticancer drugs known to cause peripheral neurotoxicity (i.e., vincristine, oxaliplatin, and paclitaxel), and morphological changes in the neuritis were analyzed using deep learning for image analysis. After training, artificial intelligence (AI) could identify morphological changes in the neurites caused by each compound and precisely predict toxicity, even at low concentrations. For the test compounds, AI could also precisely detect neurotoxicity based on neurite images, even at low concentrations. Our results suggest that this microfluidic culture system is useful for *in vitro* toxicity assessment.

Key words: Microfluidic device, Chemotherapy-induced peripheral neuropathy, Neurite morphology, Deep learning for image analysis

INTRODUCTION

Chemotherapy-induced peripheral neuropathy (CIPN) is a major common adverse event associated with chemotherapy. CIPN is primarily associated with neurological abnormalities linked to pain, loss of sensation, and motor functionality, ultimately leading to a decreased quality of life (Cavaletti *et al.*, 2011; Starobova and Vetter, 2017; Eldridge *et al.*, 2020). The diagnosis and treatment of CIPN remain challenging because its clinical presentation and molecular mechanisms are heterogeneous, and international guidelines have not reached a clear consensus yet (Colvin, 2019; Loprinzi *et al.*, 2020). An accurate assessment is essential to improve knowledge about CIPN incidence. Recently, several *in vitro* cell models of rat and mouse dorsal root ganglia (DRG) sensory neurons have been developed to study CIPN at the mechanistic level (Malgrange *et al.*, 1994; Yang *et al.*, 2009; Guo *et al.*, 2017). Drug-induced neurotoxicity to DRG neurons as a result of administering commonly used chemotherapeutic agents (e.g., taxanes, vinca alkaloids, and bortezomib) has been evaluated based on cell viability analysis and mor-

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phology (Fukuda *et al.*, 2017). For example, both bortezomib- and vincristine-treated neurons reportedly showed decreased neurite outgrowth without increased cell death (Geisler *et al.*, 2019), while cisplatin and oxaliplatin treatment induced cell death (Ta *et al.*, 2006). Therefore, cultured DRG neurons can serve as a reliable and robust *in vitro* model for mechanistic and therapeutic CIPN studies.

However, traditional neurite outgrowths and cell viability assays are mainly used for determining drug dose limitation after CIPN symptom appearance during chemotherapy. Identifying morphological changes in the neurites is important for CIPN prediction and prevention; however, it is nearly impossible to observe such changes using traditional assays (e.g., the MTT assay, the TUNEL assay, and the Neurite Outgrowth assay) (Fukuda *et al.*, 2017). With the development of deep learning technology, artificial intelligence (AI) has enabled faster and easier extraction of information from images (Ravindran, 2022). In this study, an assessment platform for DRG neurotoxicity prediction was established based on deep learning for image analysis. Herein, a cyclo olefin polymer (COP)-based microfluidic device was constructed for DRG culture, which provided advantages such as stable microstructures, reduced chemical compound absorption, and mass production suitability (Yamanaka *et al.*, 2021). Deep learning for image analysis was used to evaluate morphological changes in neurites exposed to chemotherapeutic agents known to cause peripheral neuropathy. Moreover, the potential of deep learning for drug-induced nerve abnormality prediction was demonstrated, even at low concentrations.

MATERIALS AND METHODS

Device fabrication

Ushio Inc. constructed the microfluidic device as previously described (Yamanaka *et al.*, 2021). In brief, vacuum ultraviolet (VUV) photobonding from an excimer light at a 172-nm wavelength was used to generate functional groups (i.e., hydroxy and carboxyl groups) for directly combining two COP material layers under heat treatment. The microfluidic device comprised four individual microfluidic cell culture channels (Fig. 1A-a). The narrow middle slot of the channel is 1000 μm in width, 165 μm in length, and 40 μm in height, with an open-top channel (1000 μm in width and 6 mm in length) and two circular holes (2 mm in diameter) at both ends that open into a medium rectangular reservoir (15 mm in width, 8 mm in length, and 5 mm in height). The maximum volume contained in each channel is 1 mL. COP material (Zeonex 690R, Zeon, Tokyo, Japan) was individually injected into the two molds. The components were irradiated with VUV from an excimer lamp (172 nm; Ushio Inc., Tokyo, Japan) at 25°C after removing the structured COP components from the molds. The component surfaces were assembled using a heat press at < 132°C. Finally, ethylene oxide gas (Japan Gas Co. Ltd., Kanagawa, Japan) was used to decontaminate the device.

Cell culture

Before cell seeding, the microfluidic device was coated with 0.02% Poly-L-lysine (P4707, Sigma–Aldrich) for overnight at 4°C. After washing with phosphate-buffered saline (PBS), the device was coated with 2.5 μg/mL laminin 511 (381-07363, Wako) for 30 min at 37°C.

DRG neurons were harvested as previously described (Ta *et al.*, 2006). All procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and were approved by the Tohoku Institute of Technology Animal Care and User Committee. In brief, timed pregnant female Wistar rats were anesthetized with isoflurane and decapitated. E15 rat pups were removed from the uterus. Spinal ganglia were carefully removed and immediately seeded onto the circular hole at one end of the microfluidic channel (one ganglion/channel). The cultures were grown in a neurobasal medium (21103049, Gibco) containing B27 supplement (17504044, Gibco), supplemented with 100 ng/mL nerve growth factor (NGF, 01–125, Millipore), for the first 2 weeks. The medium was then replaced with DMEM/F12 medium (11320033, Gibco), supplemented with 15% FBS, 50 μg/mL L-ascorbic acid, and 50 ng/mL NGF. This protocol yielded a stable neurite outgrowth population in the microchannel.

After another 6 weeks in culture, three typical anticancer drugs were administered to the cultures at two different concentrations each: paclitaxel at 1 μM (paclitaxel) and 100 nM (paclitaxel low), vincristine at 30 nM (vincristine) and 3 nM (vincristine low), and oxaliplatin at 100 μM (oxaliplatin) and 10 μM (oxaliplatin low). Acetaminophen (10 μM) and sucrose (100 μM) were added as two negative drugs to the cultures, and DMSO (0.1%) was added as a control drug to the cultures. The drug exposure lasted for 24 hr at 37°C.

Immunocytochemistry

The sample cultures were fixed with 4% paraformaldehyde in PBS on ice (4°C) for 10 min. Fixed cells were incubated with 0.2% Triton-X-100 in PBS for 5 min, then with preblock buffer (0.05% Triton-X and 5% FBS in PBS) at 4°C for 1 hr, and finally with preblock buffer con-

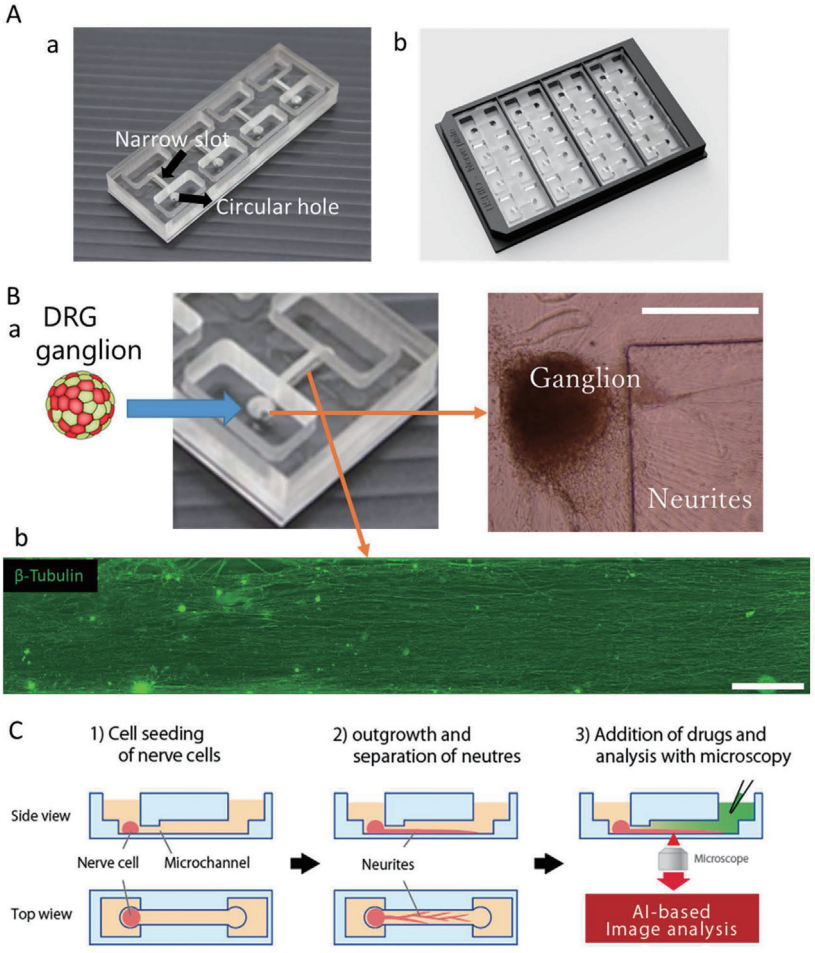


Fig. 1. COP-based microfluidic device application for cell culture. A) Microfluidic device overview. B) A representative image of a DRG cultured in the device. a) Phase contrast image of a DRG in the seeding hole after 6 weeks of culture. Scale bar = 500 μm. b) Whole length immunofluorescence image sample of neurite outgrowth in a microfluidic channel after 6 weeks of culture. Scale bar = 500 μm. C) A schematic image of the experimental processes from cell culture to immunofluorescence observation.

taining a specific primary antibody, mouse anti-β-tubulin III (1:1000, T8578, Sigma–Aldrich), at 4°C for 24 hr. The samples were then incubated with a secondary antibody, anti-mouse 488 Alexa Fluor (1:1000 in preblock buffer, ab150113, Abcam), for 1 hr at room temperature. Stained cultures were washed twice with preblock buffer and rinsed twice with PBS. A confocal microscope (Eclipse Ti2-U, Nikon) was used to capture local images and an All-in-One fluorescence microscope (BZ-X, Kenyence) was used to capture whole microchannel-length images of the immunolabeled samples. ImageJ software (NIH) was used to adjust image intensity.

Deep learning for image analysis and neurotoxicity prediction

First, whole microchannel-length immunofluorescence images were subdivided into 50 × 50 pixel images, and only the images in which axons were reflected were extracted to create the axon image dataset. The training image dataset for AI analysis consisted of DMSO (n = 3 wells), acetaminophen (n = 3 wells), oxaliplatin (n = 4 wells), and vincristine (n = 4 wells). The untrained image dataset consisted of sucrose (n = 3 wells), oxaliplatin low (n = 3 wells), vincristine low (n = 3 wells), paclitaxel (n = 3 wells), and paclitaxel low (n = 3 wells).

An AI system for peripheral neurotoxicity prediction was developed by transferring learning an image recognition model, GoogLeNet. AI was trained with DMSO and

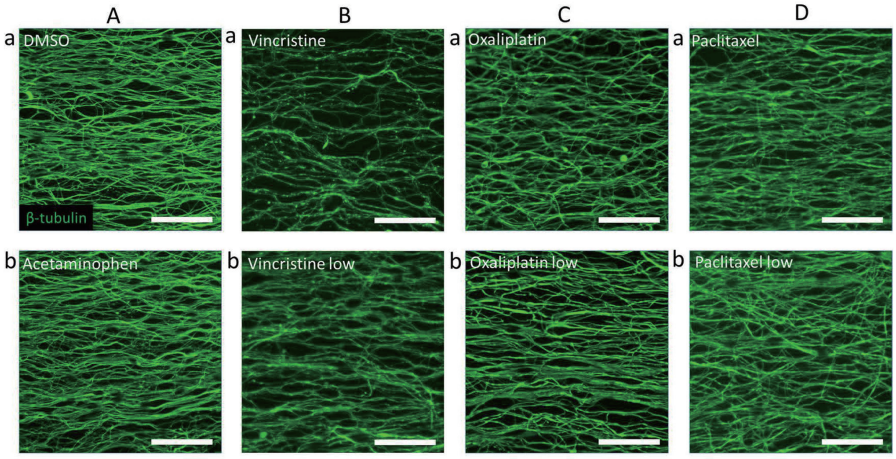


Fig. 2. Representative local immunofluorescence image samples of neurites in a microfluidic channel after drug administration. Scale bar = 50 μm. A) a) 0.1% DMSO (control) and b) 10 μM acetaminophen (negative). B) a) 30 nM vincristine and b) 3 nM vincristine (vincristine low). C) a) 100 μM oxaliplatin and b) 10 μM oxaliplatin (oxaliplatin low). D) a) 1 μM paclitaxel and 100 nM paclitaxel (paclitaxel low).

acetaminophen as negative control compounds and oxaliplatin and vincristine as positive control compounds. The leave-one-out method was used to evaluate the performance of a classification algorithm. Only one well was excluded from the training dataset as validation data, and the trained images from the other wells were used to develop an AI for each excluded well.

The prediction accuracy for the training compounds was calculated by averaging the prediction probabilities of unlearned wells that were excluded from training. The prediction accuracy for the unlearned compounds was calculated by averaging the prediction results of multiple created models. The positive probability of a single well was calculated by dividing the percentage of positive results with the number of segmented images.

Statistical analysis

One-way analysis of variance (ANOVA) was used to perform multiple group comparisons followed by Dunnett's test or Holm's test, which were used to calculate significant differences between each concentration.

RESULTS

Application of the microfluidic device in cell culture

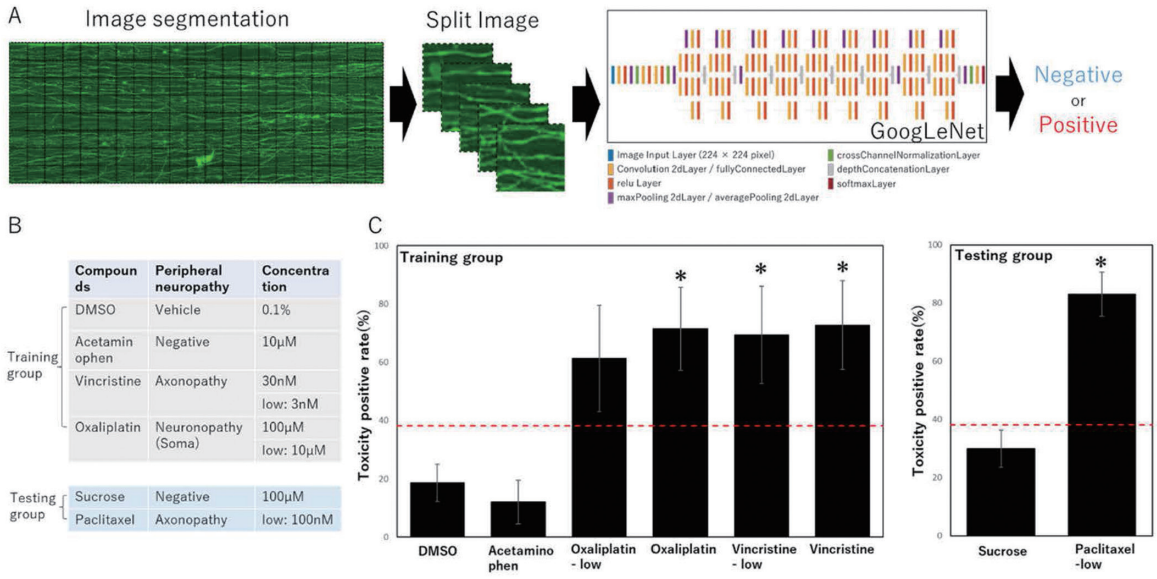
The microfluidic device was developed by directly combining COP materials, providing high mechanical stability and a scaled parallel evaluation platform (Fig. 1A-b). A DRG ganglion was seeded onto the circular hole at one end of the microfluidic channel, and no cell damage or detachment was observed during culture (Fig. 1B-a). After immunostaining, a clear neurite image was obtained for the whole microchannel length (Fig. 1B-b). Neurites grew sufficiently to occupy almost the whole microfluidic channel area, and the axon elongated unidirectionally along the horizontal direction. Therefore, the COP-based microfluidic device allows performing experimental processes from cell culture to immunofluorescence observation (Fig. 1C).

Morphological characteristics of DRG neurons

Fig. 2 shows local immunofluorescence images of cultured DRG neurons in the microfluidic channel after drug administration. After vincristine administration, morphological abnormalities, including decrease in the number of axons and axonal fragmentation (Fig. 2B-a), were observed. However, such morphological changes were not observed when a low concentration of vincristine was administered (Fig. 2B-b). Also, after paclitaxel and oxaliplatin administration (Fig. 2C, 2D), no significant morphological changes in neurite images were observed. Since the neurite density remained high under all conditions, a large dataset scale could be achieved for AI analysis.

Neurotoxicity prediction using deep learning for image analysis

An AI system for peripheral neurotoxicity prediction was developed and trained with neurites image datasets as described above (Fig. 3A and B). Fig. 3C shows the prediction rate of toxicity positivity for each compound



	Compounds	Peripheral neuropathy	Concentration
Training group	DMSO	Vehicle	0.1%
	Acetaminophen	Negative	10μM
	Vincristine	Axonopathy	30nM
			low: 3nM
	Oxaliplatin	Neuronopathy (Soma)	100μM
			low: 10μM
Testing group	Sucrose	Negative	100μM
	Paclitaxel	Axonopathy	low: 100nM

Fig. 3. Neurotoxicity-positive AI prediction based on deep learning for neurite image analysis. A) Dataset treatment for deep learning and the AI analysis process. B) Detailed list of drug administration in the present study. C) The neurotoxicity positive rate predicted by AI analysis for each drug. Data are expressed by means ± standard errors. The toxicity positive cutoff line (red line in the graph) was determined as the average positive rate of two negative control compounds (i.e., DMSO and acetaminophen) plus two times the standard deviation, which is 42.4%. ANOVA was used to perform statistical analysis followed by post hoc Dunnet's test or Holm's test. * $p < 0.05$ vs. negative.

in the training and testing groups. In the training group, the control compound DMSO (17.8%) and the negative compound acetaminophen (13.5%) showed a low toxicity positive rate. A predicted positive line was set based on these two compounds, which is the average value of their positive rates plus two times the standard deviation value (42.4%; the red line in Fig. 3C). As a result, both compounds (i.e., oxaliplatin and vincristine) were positive for toxicity even at low concentrations. The prediction accuracy was then tested using two unlearned compounds in the testing group. Sucrose was detected as a toxicity negative drug, while paclitaxel was detected as a toxicity positive drug at low concentrations.

DISCUSSION

In vitro cell models have commonly been used to explore the symptoms and underlying mechanisms of CIPN (Guo *et al.*, 2017; Fukuda *et al.*, 2017). Recent developments in biomaterial-based microphysiological systems provide an efficient platform for screening and neurotoxicity assessment of drug candidates as an alternative to animal models (Wang *et al*., 2020; Campisi *et al*., 2022; Virumbrales-Muñozabc and Ayusoab, 2022). Compared to commonly used materials (e.g., polydimethylsiloxane, polystyrene, polymethyl methacrylate, and other photoresists), the COP-based device has several advantages, such as chemical/physical stability and high optical clarity (Kristiansen *et al.*, 2022). The COP-based device maintains normal stem cell growth without undesired cellular damage (Yamanaka *et al.*, 2021). In this study, a COP-based microfluidic device for primary rodent DRG cultures was developed and its potential as an *in vitro* peripheral neurotoxicity assessment platform with an AI analysis method using neurite images was demonstrated.

Primary rodent DRG neurons are commonly used to study CIPN induced by platinum derivatives, vinca alkaloids, and toxoids (Guo *et al.*, 2017; Fukuda *et al.*, 2017). The DRG culture model is a heterogeneous system containing multiple cell types residing in a DRG, thereby representing a more physiologically relevant model than highly purified neuronal cell lines or human iPSC-derived neurons. However, this feature also brings difficulties in evaluating the effects of anticancer drugs based on DRG neuron-specific morphological changes. In the present study, the microfluidic device allowed only neurite elongation into the narrow channel. Therefore, we could only focus on the morphological changes in the neurites, which are thought to be more susceptible than neuronal cell bodies to agents that cause CIPN.

Vincristine at a 30–40-nM concentration reportedly induces axonal degeneration in cultured primary DRG neurons after a 24-hr exposure (Guo *et al.*, 2017; Geisler *et al.*, 2019); this result is consistent with that of our study (Fig. 2B-a). Conversely, oxaliplatin administration over 33.2 μM reportedly significantly decreased neurite outgrowth in DRG explants (Ta *et al.*, 2006), which was not observed in the present study. Indeed, different compounds can act via distinct mechanisms as the cell body is required for oxaliplatin-induced axon degeneration (Geden and Deshmukh, 2016; Simon *et al.*, 2016). Therefore, reproducing similar results was challenging using the present microfluidic channel, in which the neurites were separated from the cell body. However, after deep learning for image analysis, oxaliplatin toxicity could still be predicted using neurite images at relatively high accuracy.

One notable advantage of AI image analysis is that high accuracy for the toxicity positive prediction of vincristine and oxaliplatin was demonstrated at a relatively low concentration. In previous studies, DRG cultures showed no significant changes, even under similar conditions (Guo *et al.*, 2017; Geisler *et al.*, 2019). However, deep learning for image analysis reflected the detailed characteristics of even slight morphological changes; thus, deep learning for image analysis may help understand the underlying mechanisms of CIPN and may be used for CIPN prevention at an early stage.

The prediction accuracy of our AI analysis method was also tested with unlearned compounds, including paclitaxel, a widely used anticancer drug known to induce CIPN. The most toxic dose of paclitaxel is 1 μM, as previously reported (Nicolini *et al.*, 2003). The neurotoxic effect of paclitaxel has mainly been demonstrated in DRG explants with necrosis. Neurite outgrowth reportedly had no significant changes after a 24-hr paclitaxel exposure at a concentration of 10 nM–1 μM (Scuteri *et al.*, 2006), similar to our results (Fig. 2D). Using AI image analysis, paclitaxel toxicity was successfully detected based on neurite images, even at a low concentration. Taken together, our COP-based microfluidic device combined with AI image analysis is an effective *in vitro* toxicity assessment platform for peripheral neuropathies.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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