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Advances in microfluidics-based experimental methods for neuroscience research

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The application of microfluidics to neuroscience applications has always appealed to neuroscientists because of the capability to control the cellular microenvironment in both a spatial and temporal manner. Recently, there has been rapid development of biological micro-electro-mechanical systems (BioMEMS) for both fundamental and applied neuroscience research. In this review, we will discuss the applications of BioMEMS to various topics in the field of neuroscience. The purpose of this review is to summarise recent advances in the components and design of the BioMEMS devices, *in vitro* disease models, electrophysiology and neural stem cell research. We envision that microfluidics will play a key role in future neuroscience research, both fundamental and applied research.

Introduction

In neuroscience, investigations on basic functions of the nervous system form the basis for understanding nervous system disorders and medical treatments. The neuron, the basic element of the nervous system, is highly sensitive and responsive to its surrounding microenvironment. Neurons respond to biochemical signals, including growth factors, enzymes, metal ions and nutrients, and biophysical signals,

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including action potentials and rigidity or roughness of the extracellular matrix (ECM). *In vivo*, these signals vary spatially and temporally. However, in traditional petri dish-type tissue culture systems, neurons are randomly seeded and their cellular interactions also randomly depend on proximity and distribution. Moreover, cells are cultured in homogeneous media, making it difficult to control spatially localized focal stimulation of signals. Recent developments in miniaturization based on microfabrication have appealed to biologists by overcoming these limitations of traditional cell culture methods. Influenced by developments in Si-based microelectronics industry, a new field of micro-electro-mechanical systems (MEMS) has emerged in the past several decades. MEMS technologies have expanded into many different areas of science and engineering. Notably, the biological MEMS (BioMEMS) field was started by developing novel devices and structures for biological applications.[1] The early stages of BioMEMS started from development of new devices for micro-analytical methods for chromatography and electrophoresis. These methods made it possible to achieve high sensitivity and high resolution with small amounts of sample, and the concepts of micro total analysis systems (μTAS) and Lab-on-a-chip (LOC) were born. Beyond the advances in analytical chemistry, BioMEMS combined with microfluidics opened a new paradigm in biological research.

Conventional *in vitro* methods for biological research are based on relatively large plastic wares such as a petri dish or a multiwell plate. Manipulating extracellular environments and controlling single-cell-level treatments of chemicals are difficult. Combined with microfluidics, BioMEMS techniques provide precise spatially and temporally controlled extracellular environments in an *in vitro* system. However, MEMS techniques could not be directly applied to biological research due to the properties of their materials (silicon and glass), as gas permeability is required to culture animal cells. Thus, these materials were replaced with appropriate materials for culturing cells.[2]

Soft-lithography is a widely used method for making micro- and nanoscale structures using elastomeric elements and moulds by photolithography.[3] The elastomeric polymer polydimethylsiloxane (PDMS) is the main component of BioMEMS platforms due to its optical transparency, thermal stability, low cost, biocompatibility, gas permeability and ease of fabrication.[4] Due to BioMEMS with PDMS, LOC has emerged as one of the most rapid advanced technologies during the past decades and opened a new standard in biological research. Many researchers successfully cultured microbes and mammalian cells on microfluidic platforms[5,6] with complex systems comprising valves,[7,8] mixers,[9] and pumps.[10] As with other cell types, various types of neuronal cells have cell–cell and cell–tissue interactions and respond to microenvironments, so many concerns exist in the application of microfluidics to the field of neuroscience.[11] Recently, BioMEMS with microfluidics has been used to achieve biological insight and to manipulate neurons through biochemical and electrophysiological analysis in the field of neuroscience.

In this review, we summarise recent progress and trends in BioMEMS applications for neuroscience research. The article is divided into four categories of important areas: (1) platforms for neuron culture, (2) *in vitro* disease models, (3) electrophysiology and (4) neural stem cell research.

I. Platforms for neuron culture and manipulation

The most representative traditional compartmentalised neuron culture systems have been reported by Champenot[12] and

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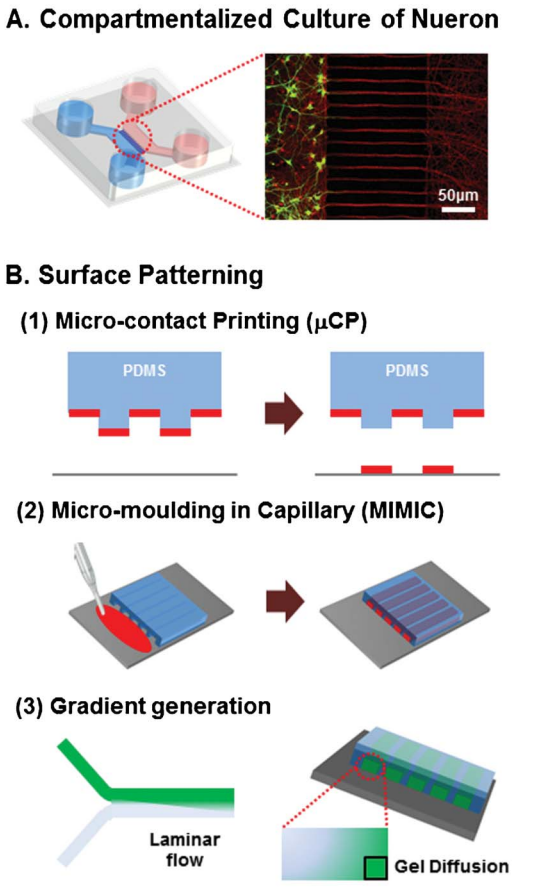

Fig. 1 Methods for isolating and guiding axons using microfabrication techniques. (A) Compartment device consisting of a PDMS mould containing a relief pattern of somal and axonal compartments connected by microgrooves. Confocal micrograph of Tau (green) and MAP5 (red) immunostained neurons (adapted from ref. 15, copyright Nature Publishing Group, 2005). (B) A patterning of axon guiding molecules on substrates is made by μCP, MIMIC, and gradient generation. (C) Axon out growth response on micro/nanotopographic patterns (adapted from ref. 60, copyright Creative Commons Attribution License, 2010 and from ref. 59, copyright Elsevier, 2011).

Ivins.[13] The Champenot chamber (made from machined Teflon pieces) was the first compartment device for isolating axons from somas with three compartments.[12] Ivins' chamber used a coverslip as a physical barrier to isolate neurites from the somal side and successfully cultured hippocampal neurons.[13] However, these devices are difficult to fabricate and assemble. They also have limited compatibility with high-resolution optical imaging. Notably, the Champenot chamber has been successfully used to culture peripheral nervous system (PNS) and retinal ganglion neurons. Central nervous system (CNS) neurons have also been cultured but with some difficulty due to the large size of the barriers.

Microfluidic platforms can offer precise spatially and temporally controlled extracellular environments. For applications where precisely engineered parts are required to work with sensitive cells such as primary CNS neurons, microfluidic devices can overcome drawbacks of macro-scale devices such as the Campenot chamber. Taylor *et al.*[14–16] pioneered a microfluidic multi-compartment device incorporating microgrooves (width 10 μm, height 3 μm, length 150–900 μm) to isolate and grow CNS axons (Fig. 1A). They demonstrated long-term culture with CNS axonal injury and validated it as a potential method to screen molecules for regeneration. This platform also provided a co-culture application by co-culturing oligodendrocytes with CNS neurons. This original design was modified for various applications: (1) an open cell culture channel version for obtaining high-density cultures needed for biochemical analysis, (2) an enlarged cell culture area version to acquire sufficient amounts of protein for western blotting and (3) a parallel culture channel version with a design similar to multiwell plates.[17]

Majumdar *et al.*[18,19] combined a microfabricated valve system with a compartment device to co-culture different cell types such as neurons and glia cells, while Pirlo *et al.*[20] fabricated asymmetrical geometries by repeating microwells and microtunnels (the microtunnels prevent migration of cell bodies from microwells). Combining it with a laser cell deposition system, they placed single cells or neuron–glia pairs for polarised cell growth. Arrays of semiconductor nanomembrane (Si and Ge) tubes as a three-dimensional (3D) neuron culture substrate were generated by Williams' group.[21] They observed cells being guided through the nanomembrane tubes. Funnel-shaped asymmetric microgrooves were proposed by Peyrin *et al.*[22] These microgrooves realised unidirectional axon connections. We call this an "axon diode".

Another method for guiding axons is patterning chemical guidance molecules on the cell culture substrate[23] (Fig. 1B). Micro-contact printing (μCP) was first pioneered by Whitesides' group. This method is a non-lithographic techni-

que in which the PDMS stamp is ''inked'' and stamped on the substrate.[24,25] Many groups have used this technique to pattern poly-D-lysine,[26–28] laminin,[29–37] polylysine-conjugated laminin,[38,39] ECM gel or substrates[40–43] and cell adhesion domains[44–48] on cell culture substrates.

Micro-moulding in capillaries (MIMIC) is another non-lithographic technique in which the PDMS mould is placed on the substrate, biomolecules are injected at the open end and a biomolecule-containing liquid fills the channel by capillary action.[49] Jeon's group combined simple pinch valves with a MIMIC system that generated striped inhibitive and permissive biomolecules (aggrecan and laminin) on a substrate to mimic CNS injury.[50] Mai *et al.*[51] made surface-bound proteins (netrin-1 and BDNF) by diffusion in an agarose gel. A vacuum-based MIMIC system was developed by Nevill *et al.*[52] They demonstrated PLL, cGMP and cAMP patterns for directionality of axon polarisation. Whitesides' group generated substrate-bound protein gradients using a laminar flow-based gradient generation device for orientation of axons.[53]

Some groups combined physical and chemical guidance systems. Ravula *et al.*[54] developed a compartmentalised system combined with collagen patterns assembled on microelectrode arrays (MEAs). A compartmentalised device combined with *N*-cadherin patterns was generated by Shi *et al.*[55] They demonstrated patterns as a cue for guiding and accelerating axon growth. Millet *et al.*[56] used a three-compartment device with patterns and gradients of laminin and poly-L-lysine using diffusion and physisorption. A microchannel that had a collagen gel was proposed by Kothapalli *et al.*[57] They generated gradients of neurite guidance cues such as netrin-1. Takayama *et al.*[58] fabricated U-shaped structures for cell trapping and combined them with patterns of cell adhesive and non-adhesive molecules, revealing one-way-structured neural networks. A platform for generation of chemical and topological dual guidance cues was developed by Greene *et al.*[59] They demonstrated it for guiding neurons into user-defined networks (Fig. 1C). Jang *et al.*[60] fabricated a line pattern with nanoscale topographical structures and coated it with laminin to allow the orientation of neurite outgrowth to be controlled (Fig. 1C).

Using soft-lithography, many groups developed platforms for axon guidance using microstructures and patterning chemical guidance molecules. These platforms not only enabled identification of the factors that influence axonal growth, but also provided understanding of how cells respond to factors individually and the combined effect of multiple factors. Due to the evolution of microfluidic techniques, including gradient and co-culture systems, many groups have developed microenvironments that try to mimic the *in vivo* system. An advantage of these platforms is that they can identify the specific parameters of cell functions without interference from an *in vivo* system. Moreover, microscopic observation is possible using fluorescence protein markers in neurons. These techniques can not only serve as a precise control of neuron manipulations, but also provide a foundation for advanced research into disease models, the functions of nervous system and development of neural stem cells. We will discuss recent trends in advanced research in the following sections.

II. *In vitro* disease models

Spinal cord injury

Together with the brain, an important and complex part of the CNS is the spinal cord, whose major function is to serve as a path for motor and sensory information and a centre for co-ordinating certain reflexes. When this complex circuitry is damaged by external force, these functions will be lost. Thus, we call it spinal cord injury (SCI), which consists of primary and secondary injuries. At the primary stage, mechanical trauma causes fracture, contusion, compression, shear, dislocation and laceration of the vertebral column. Days or weeks following the injury, the initial stage triggers a secondary stage that extends from the initial injury site to surrounding regions *via* ischemia, free radicals, lipid peroxidation and inflammation.[61–65] Regeneration and functional recovery of spinal cord neurons are difficult due to growth-inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs)[66,67] and myelin-associated inhibitors (MAG, Nogo and OMgp).[68–71] Setting up an *in vivo* system to investigate the pathology of SCIs is difficult due to their complexity, but *in vitro* models can serve as a simple and selective method, and a convenient technique for analysing their biochemical responses.

In vitro approaches to studying SCI models are focused on mechanical injury and axon growth inhibition. The Champenot chamber[12] was used to induce injury by directly cutting off the axons using a water jet that was passed through a 22 gauge needle. Jeon's group used a multi-compartment device to injure axons by vacuum aspiration and monitored regeneration of injured axons[15] (Fig. 2A). They also quantified the inhibitory effect of myelin-associated proteins on injury sites using soluble Nogo-66 and MAG-Fc proteins.[72] Hosmane *et al.*[73] developed a valve-based compressive injury model for single axons. They specified the severity of injury as follows by applying different pressures: mild ($<$55 kPa), medium (55–95 kPa) and severe ($>$95 kPa) (Fig. 2B). A pulsed laser microbeam based injury system was proposed by Hellman *et al.*[74] They integrated a pulsed laser microbeam (duration 180 ps, λ = 532 nm) into a microfluidics-based strip assay platform (Fig. 2C). They induced partial and complete injury of axon bundles by applying laser pulse energies of 400 nJ and 800 nJ, respectively. Vahidi *et al.*[50] introduced simple pinch valves into the MIMIC system and generated a microfluidics-based strip assay for surface-bound inhibitors in SCI. Because CSPGs patterned on PLL-coated substrates restrict axon extension, axons are only extended on PLL patterns. This platform was successfully applied to single drug screening for SCI using chondroitinase-ABC as a target molecule to overcome inhibitory molecules.

Two-dimensional (2D) systems serve as a simple way to understand the pathophysiology of the CNS. Still, microfluidics-based *in vitro* SCI models only use a 2D culture system with surface patterning. Recently, the 3D microenvironment

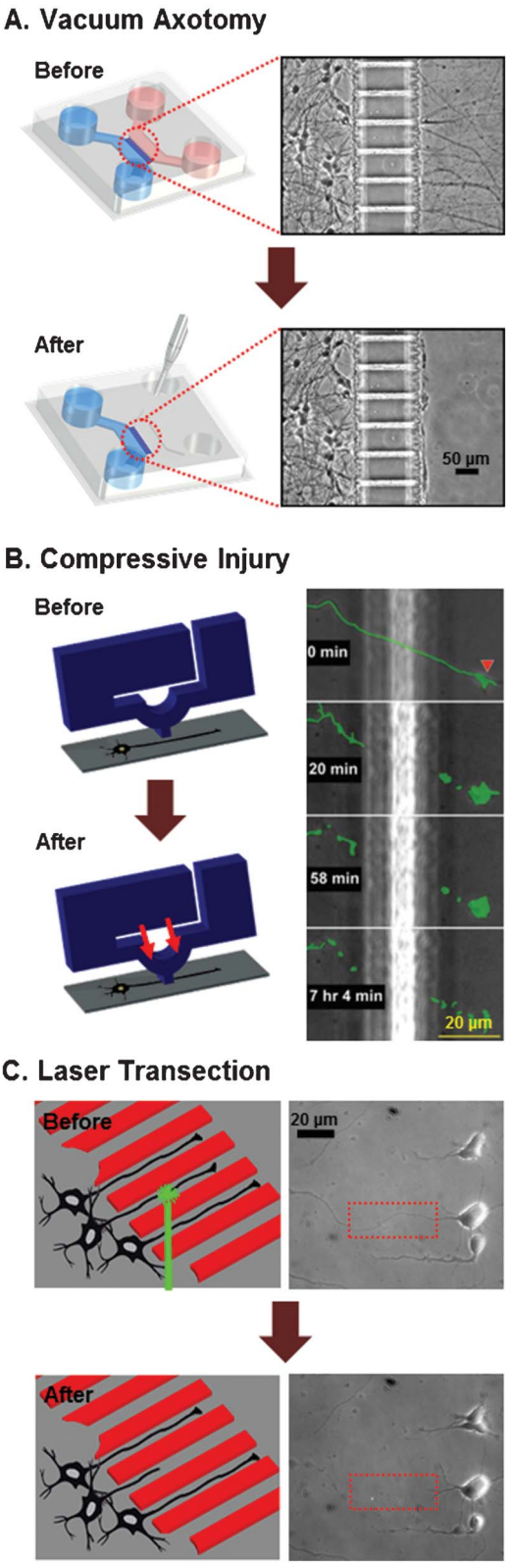

Fig. 2 *In vitro* spinal cord injury models. (A) Schematic of axotomy in the compartment device. Rat E18 cortical neurons cultured >7 days before cutting them by vacuum aspiration (adapted from ref. 16, copyright Nature Publishing Group, 2006). (B) Schematic of compression platform. At medium levels of injury (<68 kPa), axons began to undergo degeneration as shown by axoplasm disruption and nodal swellings (adapted from ref. 73, copyright Royal Society of Chemistry, 2011). (C) Schematic of laser induced axotomy. Before and after images of single axon transection (adapted from ref. 74, copyright Royal Society of Chemistry, 2010).

emerged as an important issue in the field of biology.[75] 3D culture systems have a great potential to serve as a junction between 2D cell culture systems and *in vivo* animal models. Thus, next-generation SCI research will use 3D platforms to focus on not only the behaviour of single cells but also cell–microenvironment interactions.

Neurodegenerative disease

Due to the development of medical treatment, ageing has emerged as an important issue for quality of life. Notably, neurodegenerative disease causes the most severe problems in senior persons and creates high social and economic costs for families and society. The symptoms of Alzheimer's disease (AD), a neurodegenerative disease, are confusion, troubles with language and long-term memory and dementia. The histological hallmark of AD is extracellular accumulation of amyloid plaque (AP) and intracellular neurofibrillary tangles (NFTs),[76] whose main components are amyloid β (Aβ) and tau, respectively. Much research indicates that oligomeric Aβ causes synaptic deficits by influencing intracellular signal pathways.[77–80] Through conventional petri dish based AD research, accurately defining pathology due to uncontrollable axon generation and synapse formation is difficult.

A multi-compartment device can serve as a solution for neuroscience researchers. The device of Jeon's group is widely used by various researchers to investigate the pathology. Poon *et al.*[81] demonstrated that the formation of Aβ oligomers causes synaptic dysfunction by affecting neurotrophin (brain-derived neurotrophic factor, BDNF) signalling. They showed that retrograde trafficking of tropomyosin-related kinase B (TrkB), a receptor of BDNF, is impaired by Aβ exposure. Recent research revealed that Aβ oligomers also impair mitochondrial transport.[82] As the ''power plant'', mitochondria is one of the most important intracellular organelles. Kim *et al.*[83] quantified mitochondrial transport by measuring the morphology, locations and movements of mitochondria using compartmentalised and surface-patterned culture platforms (Fig. 3). They revealed that Aβ exposure decreases mitochondrial movement considerably. Another histological hallmark of AD is NFTs, abnormal protein aggregations of the tau. When tau molecules are hyperphosphorylated, they pair with each other, which leads to the formation of NFTs and disintegration of the axonal transport system.[84] Stoothoff *et al.*,[85] using the device of Jeon's group, revealed that the 4-repeat tau-containing isoform has a greater influence on the trafficking of mitochondria to axons than the 3-repeat isoform.

The symptoms of another neurodegenerative disease, Parkinson's disease (PD), are movement disorders and dementia. The histology of PD is defined by intracellular aggregates of Lewy bodies (LBs), the main component of which is α-synuclein (α-syn), which causes synaptic deficits by an oligomeric state.[86] Volpicelli-Daley *et al.*[87] used C-terminally

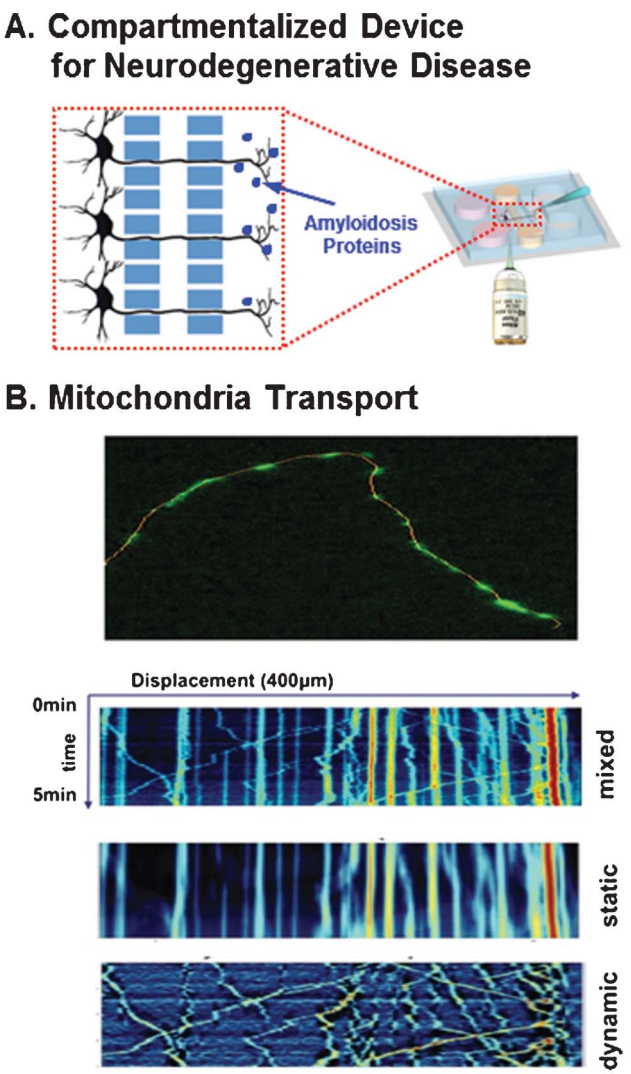

Fig. 3 (A) Applications of compartment device for neurodegenerative disease research. (B) Demonstration of image processing. (Top) mito-GFP signal along an axon (green) and extracted axon centreline (red). (Bottom) Space–time diagram showing fluorescence along the axon centreline. Automatically decomposed into static component and dynamic component. Static and rapidly moving mitochondria are represented as vertical lines and diagonals, respectively (adapted from ref. 83, copyright American Chemical Society, 2012).

myc-tagged α-syn-1-120 preformed fibrils (pffs) at the somal side of the device of Jeon's group, and then concluded that propagation of α-syn aggregates induced synaptic dysfunction and cell death through anterograde direction by pffs.

In the neurodegenerative disease research field, determining the precise pathology of AD and PD is difficult using these amyloidosis proteins related to cell cytotoxicity due to their inadequate interactions with the extracellular and intracellular components such as metal ions.[88–92] Next-generation neurodegenerative disease research will focus on revealing cytotoxic effects with various components and accurate pathologic models of AD and PD.

Stroke

Together with the spinal cord, the brain is the main component of the CNS. When blood vessels in the brain are damaged, essential nutrients such as glucose and oxygen cannot be supplied to the brain tissue. Due to this oxygen–glucose deprivation (OGDs) in brain tissue, neuronal cells die as a result of reduced ATP levels, ionic imbalances and glutamate-mediated cell toxicity. We call this a stroke.[93,94] Stroke consists of a primary and a secondary stroke. At the primary stage, OGDs causes necrotic cell death with membrane potential loss and depolarisation. After hours or days of primary stroke, the initial stage triggers a secondary stage that extends from the initial site to surrounding regions through apoptotic cell death.[95]

Traditional methods for stroke research apply OGDs to the whole culture system by bubbling O_2–N_2 gas mixtures into the culture medium[96,97] (Fig. 4A). Applying hypoxic conditions to a specific region is difficult with these methods. Using the microfluidic system, some groups achieved precise spatially and temporally controlled dissolved oxygen (DO) levels in the medium (Fig. 4B). They used a previously mentioned characteristic of PDMS: gas permeability. A microfluidic device with a PDMS membrane and microchannels used O_2–N_2 gas mixtures to generate oxygen gradients.[98–102] These methods can generate DO levels above the normal O_2 concentration. Other groups used oxygen scavengers instead of O_2–N_2 gas mixtures.[103,104] They used the phenomena of oxygen transport thorough PDMS membranes to control DO levels in culture media. These techniques are still used in microbiology,[100,103] kidney cells,[101] and basal epithelial cells.[98,104] Eddington's group applied a microfluidics-based oxygen control system to brain tissue culture.[102] They achieved stable and homogeneous DO levels in the brain slice and rapid switching of DO levels in a hippocampal slice.

The microfluidic system stroke model is still challenging, and previously described techniques will play an important role in advancing research into the pathology of primary and secondary stroke.

III. Potential use of integrated microfluidic platforms for electrophysiology of neurons

To understand the function of the nervous system, the most important characteristic of neurons is the electrical signal such as action potentials. The traditional patch clamp assay can measure ion channel currents in neurons using a glass micropipette containing a small electrode and conductive solution.[105] This assay serves as a powerful tool to investigate the physiological role of single ion channels in neurons. However, this method can only measure one neuron at a time, so applying it to the LOC system is problematic.

Some groups solved problems of high-throughput assays using a microfabrication technique. Klemic *et al.*[106] first made a planar PDMS patch electrode casting from a micropipette and a micromachined silicon wafer. This technique served as a simple and easy way to fabricate and also realised high-

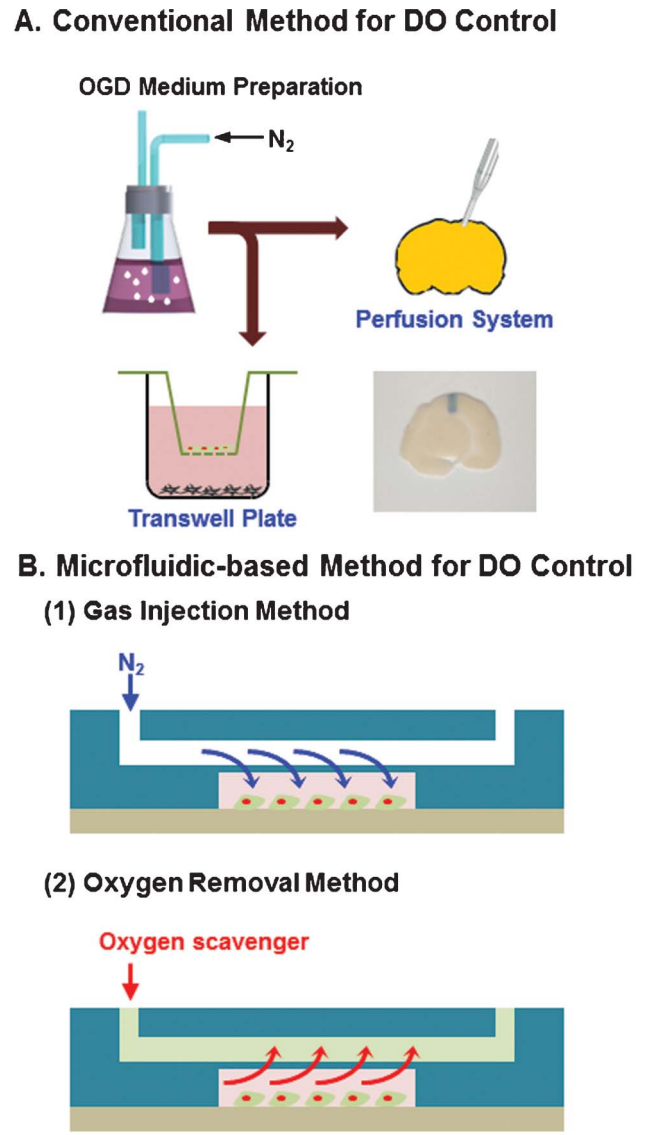

Fig. 4 *In vitro* stroke models. (A) Conventional stroke research applied OGDs on whole culture systems by bubbling O_2–N_2 gas mixtures into the culture media (adapted from ref. 97, copyright Elsevier, 2010). (B) Microfluidic techniques serve precise spatially and temporally controlled dissolved oxygen (DO) levels in the media using gas injection and O_2 removal methods.

throughput recording. After this innovative technique, this technology provided the foundation for manufactured patch clamp machines and was shared with various companies, including Axon Instruments, Flyion, Cytocentrics, Sophion, Nanion and Molecular Devices.[107] Manufactured patchclamp systems not only overcame limitations of previous patch clamp methods, but also incorporated automation. Recently, a new patch clamp concept was developed by Spira's group.[108] They demonstrated "in-cell" recording and stimulation by extracellular mushroom-shaped microelectrodes. Compared with traditional and previous multi-patch clamp assays, this method does not require dialysis of cells due to "self-impaling" of their electrodes.

Another tool to study the function of the nervous system is microelectrode arrays (MEAs) equipped with numerous electrodes. MEAs combined with microfluidic techniques emerged as the most representative technique to study the electrophysiology of the neural network and provided insight into the functional organization. MEAs can stimulate large numbers of neurons and record electrical signals with large numbers of electrodes. Various types of MEAs are already commercially available from companies included Multi Channel Systems, Ayanda Biosystems, Alpha Med Scientific and Axion Biosystems.[109] These MEAs platforms offer a better understanding of the fundamentals of neuroscience and of signal propagation in neural networks, and while this technique can be applied to both *in vivo* and *in vitro* systems, *in vivo* applications will not be discussed because the focus of this review is about *in vitro* systems.

Many researchers have successfully cultured neurons,[110–112] stem cells,[113] and brain slices[114] on MEAs. However, these platforms formed random networks because of homogeneous surface treatment with cell attachment molecules. To investigate the precise electrophysiology of neuronal networks using MEAs, cells should be cultured on the electrode for accurate stimulation and recording. The previously mentioned μCP method is widely used for patterning of cell adhesion molecules on the MEAs[27,28,35] (Fig. 5A). Erickson *et al.*[115] made caged MEAs using a parylene structure for trapping soma. This method identified electrically connected cells and neuron to electrode specificity.

As with other cell culture platforms, MEAs platforms are enclosed in a chamber for long-term observation (Fig. 5B). Egert *et al.*[114] used a chamber made from silicone rubber and centrifuge tube segment and cultured rat hippocampal slice on MEAs for 4 weeks. A PDMS based microfluidic chamber with MEAs was proposed by Berdichevsky *et al.*[116] They cultured hippocampus slices and recorded the development of spontaneous activity in the culture platform for 4 weeks.

Combined with the microfluidic platform, many applications were realised, such as a co-culture system (Fig. 5C). Takeuchi *et al.*[117] co-cultured superior cervical ganglion (SCG) neurons and ventricular myocytes (VMs) separately to mimic the cardiac system. They used a PDMS chamber with two compartments to introduce synapse formation between neuron and myocytes. They measured signals evoked from neurons and confirmed interactions between neurons and myocytes by the frequency and numbers of pulses. Musick *et al.*[118] made electrically and fluidically active 3D MEAs for neural recording by fabricating, aligning and stacking patterned thin films. This system is more likely to be a valid *in vivo* system and is appropriate for a new paradigm of MEAs.

Another advantage of microfluidic systems is that they enable high-throughput experiments (Fig. 5D). High-throughput platforms are realised by direct insertion of microwells on MEAs electrodes[119,120] and parallel fabrication of multiple chambers.[109] Also, a synapse microarray was proposed by Shi *et al.*[121] This platform allowed high-throughput screening to find small molecules that promote synaptogenesis. Their

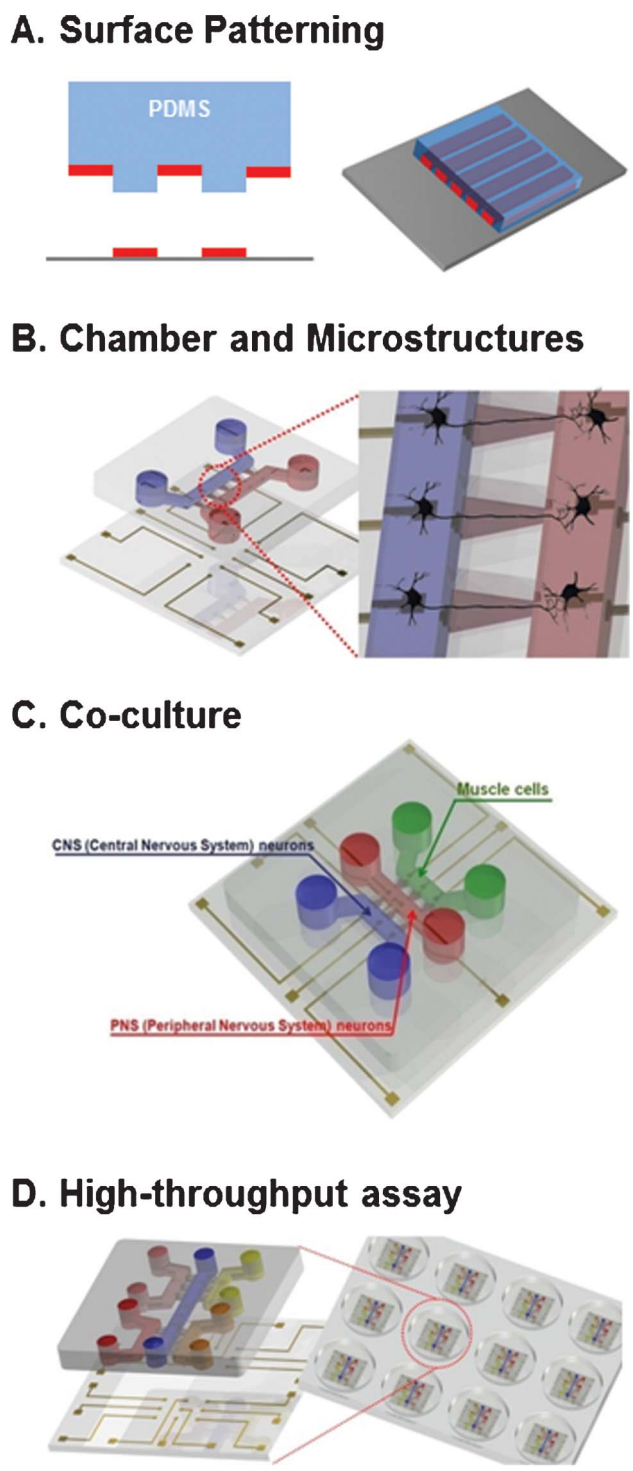

Fig. 5 Integrated microfluidic platforms with MEAs for neuroscience. (A) Surface patterning for accurate stimulus and recording, (B) chamber and microstructures for long time observation, (C) co-culture system and (D) high-throughput assays.

platform consists of a compartmentalised platform and arrayed microwells. They cultured hippocampal neurons and HEK293 cells, which have neuroligin-1 (NGL1) for synapse formation, to create synapse arrays in the arrayed microwell region. These high-throughput systems have great potential to explore drug screening assays as pharmacological and toxicological experiments.[122]

Neural electrophysiology has evolved from patch clamping to MEAs. These systems allow recording of large areas and high-throughput experiments. From the beginning of the 21st century, a combination of microfluidic device and microelectrode arrays has facilitated the development of multifunctional integrative platforms for electrophysiological research in neuroscience. Since microfluidics can manipulate axons and co-culture with glial cells, combining this technique with MEAs enables the creation of a system similar to an *in vivo* system and high-throughput research with high efficiency. In the future, to mimic the real brain, 3D electrical stimulation recording systems should be developed for whole-brain research.

IV. Application of microfluidics to neural stem cell biology

Due to the complexity and integrity of the brain, an essential step for studying neurodegenerative diseases is to reconstruct the *in vivo* microenvironment *in vitro*. In conventional petri dish culture, neurons show random outgrowth such that it is difficult to identify neuronal connectivity and to control the spatio-temporal gradient of biomolecules. The ability to control the microenvironment surrounding neuronal cells, such as crosstalk in cell–cell and cell–ECM microfluidic platforms, can provide an *in vivo*-like niche for neural stem cells (NSCs) that can be differentiated into components of the nervous system. Jeon's group has worked intensively to answer several neurobiological questions by providing appropriate experimental platforms that can resolve many limitations in conventional tissue culture. Jeon's microfluidics-based experimental platforms have offered precise spatio-temporal control of cellular microenvironments such that we could explore various neuronal events. By combining microfluidic technology and the neurobiology, they could overcome various technical problems in neurobiology such as culturing CNS neurons,[15] isolating axons,[14,15] patterning cultured neurons,[123] controlling neurite outgrowth to mimic axonal injury[50,74] and observing local protein synthesis in axons,[124] axonal regeneration[17,125] and axonal transport.[83]

Most engineered microfluidic platforms utilised to study CNS neurons have used primary neuronal and glial cells, which have limitations in terms of the number of cells and the variability among individual animals. Stem cell technology allows the generation of uniform populations of neuronal and glial cells. Therefore, the combinatory use of NSCs by employing NSCs could be a potential candidate system to study many neurodegenerative diseases *in vitro*. Jeon's group has pioneered the use of NSCs in microfluidic platforms. By using a pyramidal network of microchannels, widely known as "Jeon's Christmas tree device" and often reproduced, Chung *et al.*[126] studied how the gradient of soluble factors affects the

proliferation and differentiation of human neural stem cells (hNSCs). They generated a stable gradient of soluble cocktails such as epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2) and platelet-derived growth factor (PDGF) to determine the fate of hNSCs. After a week of culture in a microfluidic device, hNSCs differentiated into astrocytes. The proliferation and differentiation of NSCs showed clear linear dependence on the growth factor concentration. They also developed an experimental platform that enabled us to apply biochemical gradients to cultured human neural progenitor cells (hNPCs) in a petri dish.[127] This platform could be potentially useful for performing routine bioassays with no modification of established tissue culture protocols. They further studied the response of mouse embryonic stem cells (mESCs) to a dynamically controlled gradient of bone morphogenic protein 4 (BMP-4).[128] As shown in Fig. 6A and B, a microfluidic device with a vacuum network provides an intact area of stem cells under a stable biochemical gradient. Cells experience a non-steady state microenvironment over time in a static culture, as in a petri dish. Hence, they continuously deplete nutrients from the medium and secrete diffusible signalling molecules and waste to surrounding cells. A uniform response of mESCs to applied BMP-4 was observed in a microfluidics-based perfusion culture system (Fig. 6C). In addition, the amount of BMP-4 required to induce the expression of BRE-lacZ was significantly lower in this system than in conventional static culture systems. These results imply that the microfluidics-based perfusion culture improves the differentiation of mESCs. Their microfluidics-based perfusion culture platform offers advantages over static culture systems because it is able to maintain steady-state conditions.

We are able to perform pathological/mechanistic studies of healthy and diseased states of the nervous system using induced pluripotent stem cells. Therefore, it is strongly believed to be a promising strategy to develop an *in vitro* model system representing many neurodegenerative diseases using patient-derived NPCs. We suggest interdisciplinary work that combines neural stem cell biology with microfluidic technology. The experimental platforms developed by this

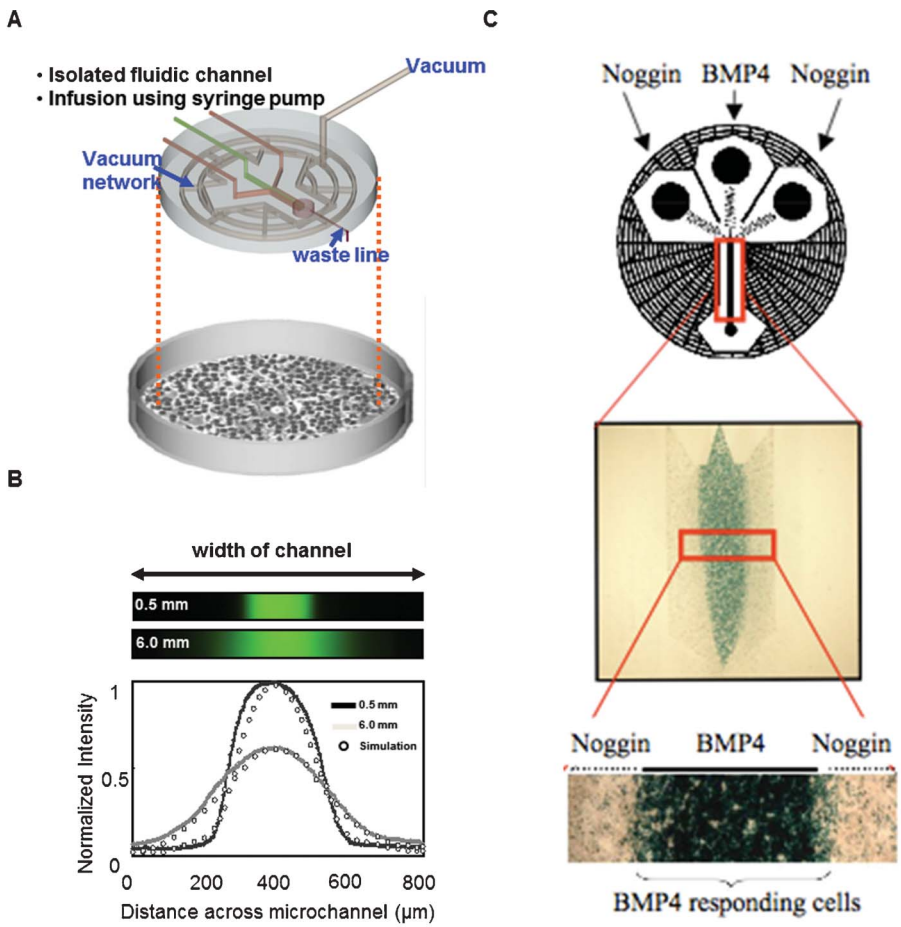

Fig. 6 Microfluidic platform combined with cultured neural stem cells. (A) A schematic diagram showing experimental setup. Isolated fluidic channels are used for generating a biochemical gradient toward cultured stem cells in a petri dish. These channels for generating gradient are intact when a microfluidic device is sealed onto a petri dish because they are isolated from the surrounding vacuum network. (B) The gradient profile demonstrated with 10 kDa FITC–dextran with different width of microchannels. (C) Noggin and BMP-containing media is perfused through the inlets and forms the gradient of BMP. After 24 h of exposure to BMP-4, ESC responses toward various BMP gradients were monitored (adapted from ref. 128, copyright Artech house, 2009).

approach need to be compatible with live cell imaging, molecular biology techniques and electrophysiological techniques. In summary, these efforts provide a better understanding of neurodegenerative diseases by dissecting out signalling and molecules for therapeutic targets.

Conclusion

Applications of BioMEMS for neuroscience are in a remarkable developmental process. The fusion of MEMS and biology has solved previous bottlenecks of conventional petri dish-based culture systems. Many researchers determined important mechanisms of biological phenomena in the field of neuroscience by taking advantage of new techniques. In the conventional petri dish culture system, cells are randomly seeded onto the homogeneous substrate and pool. Because cells respond to their local microenvironments like cell to substrate, to cell and to biomolecule interactions, biologists could not obtain a clear dynamic cellular response observed in *in vivo* systems through petri dish culture. Thanks to MEMS techniques, microstructures can achieve the isolation and growth of CNS neurons with co-culturing of other types of cells. BioMEMS also realised the axonal guidance system with biochemical surface micropatterns, microtopography and gradients of soluble and insoluble (surface) biomolecules. Due to these manipulations of cell culture systems, BioMEMS techniques have appealed to neuroscientists. Moreover, BioMEMS with microfluidics have been used to develop *in vitro* microenvironments that potentially mimic part of the complexity that arises when studying *in vivo* systems, and have been used to study disease models, electrophysiology and neural stem cell biology. These efforts have helped to probe the mechanisms of biological phenomena in the field of neuroscience. For example, they have enabled the identification of early signals that initiate axon specification[129] and of protein expression machinery in neuronal axons.[124]

In the infancy state of BioMEMS, most research used 2D systems due to their simplicity, but 2D systems could not reconstitute the mechanical and biochemical microenvironments of *in vivo* systems.[75] Obviously, the CNS does not consist of single-layered organs, but 3D tissues. Recently, a great need has arisen to understand the precise functions of neural networks, pathophysiology and disease prevention. To overcome the limitations of 2D systems, 3D cell culture systems were developed with their needs. In the 3D culture system, cells were grown within ECM gels to mimic tissue-specific microenvironments, enhanced expression of differentiation and improved tissue organization.[130] BioMEMS can be offered as an innovative tool to build up the formation of 3D culture systems for neuroscience. In the 1998, Borkenhagen *et al.*[131] developed 3D patterned laser immobilisation of laminin oligopeptides in agarose gels and observed the growth of neurites through the 3D paths. A decade later, due to the advances in microscopes and materials, Seidlits *et al.*[132] made a path of IKVAV peptides (from laminin) by photo-cross-linking in an hyaluronic acid hydrogel. They demonstrated the growth of dorsal root ganglion (DRG) neurons and hippocampal neural progenitor cells selectively and migrations through the IKVAV 3D paths.

In the near future, more advanced 3D culture systems will open a new paradigm in neuroscience research, ''Organs-on-a-Chip'', which will not only mimic organ microarchitectures but also construct organ-specific biochemical and mechanical environments. For example, hepatocytes are surrounded by many other types of cells, including endothelial cells, fibroblast and Kupffer cells. If hepatocytes are cultured alone *in vitro*, they lose all liver-specific functions within a few of days. For this reason, many researchers have used microfabrication techniques to enhance liver-specific functions in co-culture systems.[133] As with liver cells, neurons are surrounded by many other cell types and are connected by a neural network. If neurons are cultured without microenvironmental cues and a proper 3D network, we cannot determine the precise mechanisms of biological phenomena in the field of neuroscience. The organ-on-a-chip concept has already been applied to human organs including the lung,[134] liver,[135] and kidney.[136] In the field of neuroscience, the concept of a brain-slice-on-a-chip seems to be closely related to the organ-on-a-chip idea. Hutzler *et al.*[137] demonstrated a field-effect transistor (FET) array capable of recording the electrical signals on the surfaces of thin hippocampal slices. Williams' group demonstrated a microfluidic chamber for culturing brain slices with local perfusion of different biochemical environments that can stimulate multiple different areas of the slice biochemically.[138]

We are convinced that the organ-on-a-chip concept will serve the golden age of the field of neuroscience, making it possible to understand precisely human pathophysiology and the prevention of diseases for the process of drug development using novel *in vitro* disease models, which will serve as replacements for *in vivo* animal models. Also, applications in neuroelectrophysiology will play a key role in understanding functions of neuroscience, rules of nervous systems and mechanisms of learning and memory.

Abbreviations

Aβ	Amyloid beta
AD	Alzheimer's disease
AP	Amyloid plaque
BDNF	Brain-derived neurotrophic factor
BioMEMS	Biological micro-electro-mechanical systems
BMP-4	Bone morphogenic protein 4
CNS	Central nervous system
CSPGs	Chondroitin sulfate proteoglycans
DO	Dissolved oxygen
ECM	Extracellular matrix
EGF	Epidermal growth factor
FET	Field-effect transistor

FGF2	Fibroblast growth factor 2
ESCs	Embryonic stem cells
LBs	Lewy bodies
LOC	Lab-on-a-chip
MEAs	Microelectrode arrays
MIMIC	Micro-moulding in capillaries
μCP	Micro-contact printing
μTAS	Micro total analysis systems
NPCs	Neural progenitor cells
NSCs	Neural stem cells
NTFs	Neurofibrillary tangles
OGDs	Oxygen–glucose deprivations
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PDMS	Polydimethylsiloxane
Pffs	Preformed fibrils
PNS	Peripheral nervous system
SCI	Spinal cord injury
TrkB	Tropomyosin-related kinase B

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