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Compartmentalized Platforms for Neuro-pharmacological Research

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Abstract: Dissociated primary neuronal cell culture remains an indispensable approach for neurobiology research in order to investigate basic mechanisms underlying diverse neuronal functions, drug screening and pharmacological investigation. Compartmentalization, a widely adopted technique since its emergence in 1970s enables spatial segregation of neuronal segments and detailed investigation that is otherwise limited with traditional culture methods. Although these compartmental chambers (*e.g.* Campenot chamber) have been proven valuable for the investigation of Peripheral Nervous System (PNS) neurons and to some extent within Central Nervous System (CNS) neurons, their utility has remained limited given the arduous manufacturing process, incompatibility with high-resolution optical imaging and limited throughput. The development in the area of microfabrication and microfluidics has enabled creation of next generation compartmentalized devices that are cheap, easy to manufacture, require reduced sample volumes, enable precise control over the cellular microenvironment both spatially as well as temporally, and permit high-throughput testing. In this review we briefly evaluate the various compartmentalization tools used for neurobiological research, and highlight application of the emerging microfluidic platforms towards *in vitro* single cell neurobiology.

Keywords: Axon guidance, compartmentalized chambers, micro/nanofluidic platforms, neuron cell culture, neuronal injury, synaptogenesis.

1. INTRODUCTION

The human brain undergoes remarkable self-organization as the morphological changes occur during its development. Understanding the mechanism governing these structural and functional aspects has huge implication from basic biology to treatment of brain disorders. The motive behind advancement of analytical tools and techniques for neurobiology has thus been to facilitate the understanding of the nervous system from molecular scale to systems level. In their natural setting within the complex cytoarchitecture of brain, neurons grow in a densely packed, well-organized, three-dimensional environment supported by signaling factors, proteins, and various neighboring cells. Typically, neurons and their projected extensions spanning over considerable distances are subjected to varying chemical, physical and fluidic microenvironment making *in vivo* investigation a major challenge and sometimes problematic [1]. Isolated primary neurons are widely used for experimental investigation and have served an important role in the understanding of mechanisms governing diverse neuronal functions such as axon guidance and pathfinding [2-10], synaptogenesis [11-15] and plasticity [16-23]. The traditional neuronal cell culture has also been widely utilized to validate the protein functions [24-30] and to screen chemical libraries for identifying targeted drug molecules that treat neurological disorders [31-34]. In comparison to other cell types, neurons usually have polarized morphology with distinct cellular compartments (axon, dendrites and soma) that can cover a wide range of scales; and they are also very sensitive to environmental cues, temperature, growth conditions and can alter their behavior in response to their microenvironment thus making it difficult to achieve results similar to those obtainable from *in vivo* experiments [35]. Conventional neuron cultures do not offer the capability to control and manipulate extracellular environment with sufficient spatial and temporal resolution, which is an absolute requirement for investigating some of the underlying mechanisms involved in spinal cord injury, regeneration, as well as in neurodegenerative diseases. Therefore, there is great need for *in vitro* experimental approaches that can recapitulate the biological complexity *in vivo*, and tools that will enable precise manipulation, high-throughput investigation, thus pushing the boundaries towards single cell and sub-cellular targeted neuropharmacology.

2. NEURON CULTURE AND COMPARTMENTALIZATION

Neurons are very sensitive to their environmental cues. Temperature, composition of culture media, pH, oxygen concentration can alter their behavior in response to the microenvironment. The initial pioneering work with neuronal culture optimization has demonstrated that neurons retain their morphologies in culture and exhibit density-dependent viability [36, 37]. In a standard culture dish based *in vitro* culture the dissociated hippocampal neurons adhere to the protein modified surface within a few minutes and over the next 24-48h continue to mature as they achieve their original morphology. The neuronal polarity is established through a series of structural and functional developmental

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events, early work by Dotti *et al.* has illustrated the five stages of the neuronal polarization process. During the initial stage the seeded neurons adhere to the substrate and are unpolarized; subsequently at stage 2 the neurite processes start extending. The next stage that lasts for 24h is the period during which the neurons express polarization. Initially it is difficult to distinguish the individual neurites explicitly as axons or dendrites. Eventually during the subsequent stage 4, one of the protoplasmic protrusions from the neuron rapidly outgrows in length and prolongs surpassing the other arising minor processes by approximately 10-15 μm, next the dendrites grow and are observed developing. The final stage lasts for about 7 days, the neurons fully mature with extended axon and developed dendritic tree [38]. In an isotropic environment within a culture plate it is difficult to control the growth of neurons in a spatially and temporally well-controlled manner since establishment of neuronal polarity is spontaneous and the outgrowing features have random orientation.

In mature neurons the soma or neuronal cell bodies are typically in the range of 10-20 microns while the axons can extend several hundreds to thousands of microns. Thus, the organelles, proteins, neurotransmitters have to travel a long distance to reach the nerve terminal and synaptic points. Similarly, the neurotrophic factors, extracellular signals, neurotransmitters received from the target cells or other neurons need to be transported to the soma along the axons. The failure in this axioplasmic transportation, either anterograde or retrograde disrupts neuronal function. Extensive studies focused on Parkinson's disease, Alzheimer's disease have suggested the involvement of axonal transport and degeneration on disease onset as well as progression [39, 40]. From a biological perspective, the exact role of isolated neuronal compartment (either the soma or the axon) in disease progression is still unclear. Along with *in vivo* studies, *in vitro* neuronal cultures are being used for these investigations. Yet, there are limited approaches to control growth, orientation and connectivity of neuronal processes within cell culture, and the most widely used technique is compartmentalization. The main advantage of compartmentalization approach is that it enables spatial segregation of neuronal cellular features, improves visualization and enables detailed investigation that is otherwise limited with traditional culture methods. There are several reviews focusing on the application of microtechnologies for neurobiology, here we focus specifically on the compartmentalization and microfluidic aspects [41-45]. These compartmentalization

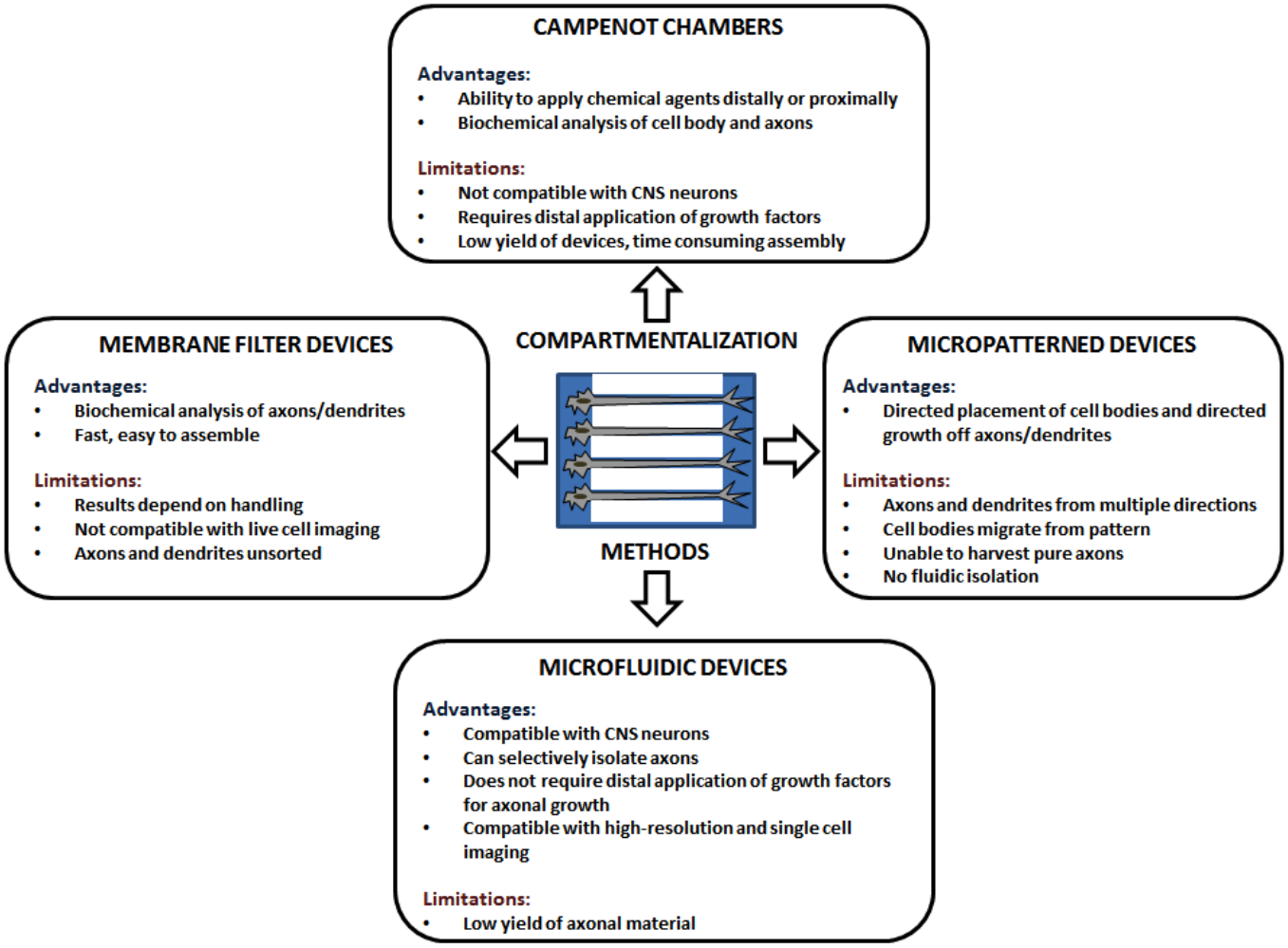


Fig. (1). Different methods to achieve compartmentalization in two-dimensional neuronal cultures.

devices can be generally classified into traditional and emerging microscale devices for the sake of comparison; Fig. **1** depicts the different types of devices that are used with their advantage and limitations.

The conventional compartmentalized devices include Campenot chambers, filter-based devices, while microscale devices are either micropatterned or microfluidic. These devices enable manipulation of the neuronal components and enable selective separation, stretching as well as biochemical analysis that is not possible in a standard culture dish. The following sections provide introduction to the different compartmentalized devices with their application, advantages and limitations.

3. NON-MICROFLUIDIC COMPARTMENTALIZED PLATFORM FOR NEURONAL CELL CULTURE

This category includes the traditional plat forms that were developed to enable physical isolation of the neurons and sub-cellular compartmentalization, but not explicitly addressing all the requirements for single cell investigation. An emerging technique that relies on surface modification by protein micropatterning to achieve site-directed cell placement and growth is also presented.

3.1. Campenot Chamber

Campenot chambers, introduced by Campenot in 1977 have been widely used for studying neurite and axon biology [46]. In this work, the chambers were employed to evaluate the survival and growth of sympathetic neurons to distal nerve growth factor. The basic assembly of the device consisted of a modular Teflon piece having three individual chambers sealed with a layer of grease to a collagen coated tissue culture dish and 20 parallel tracks that were spaced 200μm apart (Fig. **2 A**, **B**). The neurons that were plated in the central chamber of the Teflon piece grew axons along the tracks within two to three days and extended into the adjacent chambers filled with NGF. This simple format Campenot chambers have been used to investigate various neuronal functions such as the transport of signaling factors within the axons, the synthesis of structural protein β-tubulin, and actin [46-50]. These chambers have also been used to

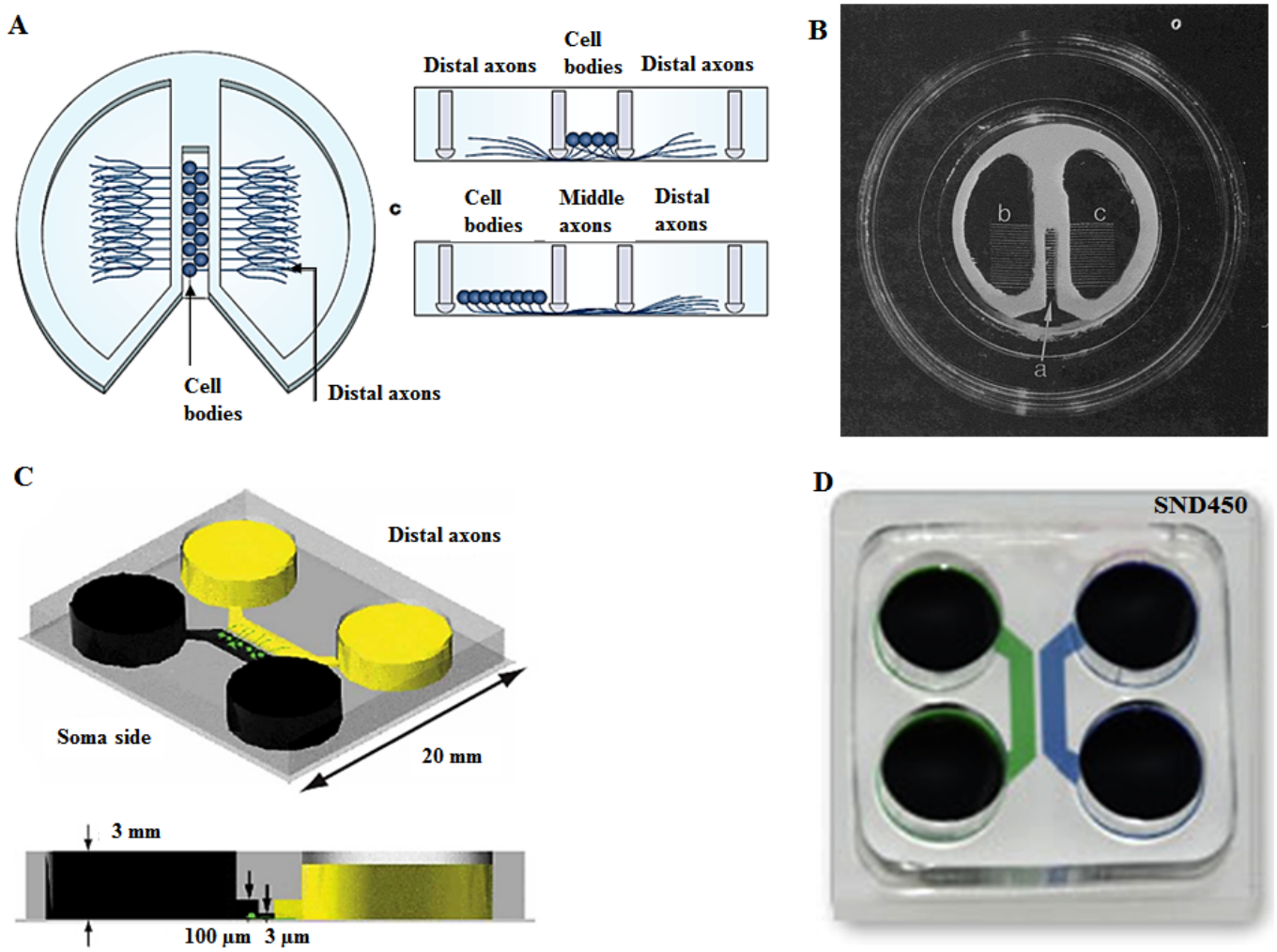


Fig. (2). Traditional compartmentalized chambers **A**) Schematic of a Campenot chamber [113] The neurons are placed in the cell body chamber and extend their axons into the distal chamber **B**) First prototype Campenot chamber [46] **C**) Schematic of a two chamber compartmentalized microfluidic device [119] **D**) Commercial Microfluidic Compartmentalized chamber (Xona Microfluidics LLC).

isolate the axonal extensions for subsequent molecular analysis using PCR [51], Western blot analysis [52]. A modified version of the Campenot chamber consisting of Teflon ring and glass coverslip forming two compartments was used by Ivins *et al.* to study β-Amyloid induced degeneration and apoptosis in cultured hippocampal neurons [53]. The unique design of the device makes it easy to perform biochemical analysis or apply stimulants distally or proximally. Some of the limitations associated with these Campenot type chambers are a) Fluid in chambers leaks out because of the sealing with just a layer of grease; b) Device is difficult to assemble and to integrate with advanced live cell imaging microscopy. c) Most of the applications have been towards PNS neurons that have a dependency on neurotrophic agents but the device has not been effective with CNS neurons. More importantly, the structure of Campenot chambers is on the scale of millimeter, which does not match the scale of cellular features and cannot provide accurate spatial control of the individual neurons.

3.2. Membrane Filter Devices

These devices are based on selective isolation of the soma from the neurites as they grow through the filter pores, the assembly can be similar to a Boyden chamber or simply a membrane sandwich placed on glass coverslip. Several studies have illustrated the use of such membrane filter devices for mRNA isolation and quantification of protein synthesis during development with CNS neurons and Dorsal root ganglion (DRG) explants [54-59]. Torre *et al.* [54] were one of the first to perform detailed study on the use of membrane filters for axon, dendrite isolation from hippocampal neurons. They used a double-surfaced coverslip, the plating surface was porous and permits the passage of neurites but not cell bodies while the second surface, which receives the neurites was attached to the plating surface by a protein matrix. After l0-15 days *in vitro*, the plating surface was stripped off leaving a nearly pure preparation of living axons and dendrites on the second surface. In another interesting approach, Wu *et al.* [55] used modified Boyden chamber to isolate axons from DRG explants growing on the filter and mechanical scraping of the neurons after exposure to NGF gradient.

The membrane filter based isolation of neurites is a fast and easy technique but it is impossible to control the orientation of single cells during growth. For assay involving pseudounipolar DRG neurons that only extend axonal processes in culture, this technique can provide a pure preparation of axons, but when used for culturing neurons with full axon-dendrite polarity (*e.g.* cortical or motor neurons) a mixed preparation of axons and dendrites is obtained. Furthermore, the assembly used for the compartmentalization is not compatible with live cell imaging; besides the process requires mechanical handling making it less efficient as compared to Campenot chamber.

3.3. Micropatterned Devices

This method entails making micropatterns of cell adherent molecules (such as polylysine, laminin *etc.*) on glass, polymer or plastic substrates either by direct microcontact printing, laser patterning, plasma etching or using microfluidics. Micropatterning has been widely applied for *in vitro* neuronal investigation studies such as axon guidance [60-65], neuron polarization [66-69], neuronal networks [70-74] and synapse formation [75, 76]. The major advantage of the micropatterning technique is that it is cheap and easy to develop the protocols in house, secondly the defined patterns allow directed placement of the neurons. Most of the proteins, antibodies retain their biological activity during the stamping or contact printing process. Fig. **3** illustrates two such techniques for generating micropatterns, [62] and illustrative examples demonstrating the capability to generate protein gradient patterns for axonal guidance studies. One of the limitations related to this technique is that it does not allow selective isolation of axons and dendrites, also the cell bodies migrate from the pattern if the adjoining area is untreated, furthermore there is no fluidic isolation between the neurites so it is difficult to investigate axonal transportation or selective influence of neurotrophic factors. In a recent study, Scott *et al.* [69] were able to eliminate some of the above mentioned drawbacks and constrain motility of the soma while causing the axons to grow directionally from the polarized neurons on a poly-D-lysine ratchet pattern, with long chain acrylate-based PEG monolayers preventing non-specific protein adhesion.

4. MICROFLUIDIC COMPARTMENTALIZED DEVICES

Microfluidics is the science and technology of manipulating nanoliter volume of fluid in microscale structures [77]. Over the past two decades, the development in the microfluidic technology has enabled the creation of more advanced platforms for neurobiology [1, 78-81]. Microfluidic devices were initially developed with the aim to miniaturize chemical and bimolecular analysis and achieve enhanced sensitivity, speed as well as resolution over conventional methods [82], and have been increasingly utilized for a wide range of applications, including biological assays [79, 83, 84], chemical synthesis [80], single-cell analysis [85-87] and protein, cell, tissue engineering [88-90]. The rapid expansion of microfluidic technology in biomedical research was driven by its exceptional ability to process small sample volumes and to create a well-controlled reaction microenvironment for precise spatial and temporal manipulations of biological events [81, 91]. Briefly, the prime advantages of microfluidics for neurobiology [92] can be summarized as follows: a) High experimental reproducibility given the ease of device fabrication, assembly, and control over substrate surface chemistry; b) Fluidic flow control not only enables density-controlled neuronal seeding and passaging, but also permits precise isolation of the neurites without damage to the somata. The placement of service microchannels allows localized treatment of specific sub-cellular compartments with neurotrophic factors, small molecules, drugs, or nanoparticles; The treatment regime can be performed either in a continuous or pulsatile manner, at desired concentration; c) The small size of the microchambers allows scaling up the number of compartments that can be included on a single chip enabling high-throughput investigation, at a relatively lower cost than plate based assays. The devices are well suited for long-term culture. d) The microfluidic devices are compatibile with microscopy set-up and enable investigation of cellular dynamics with

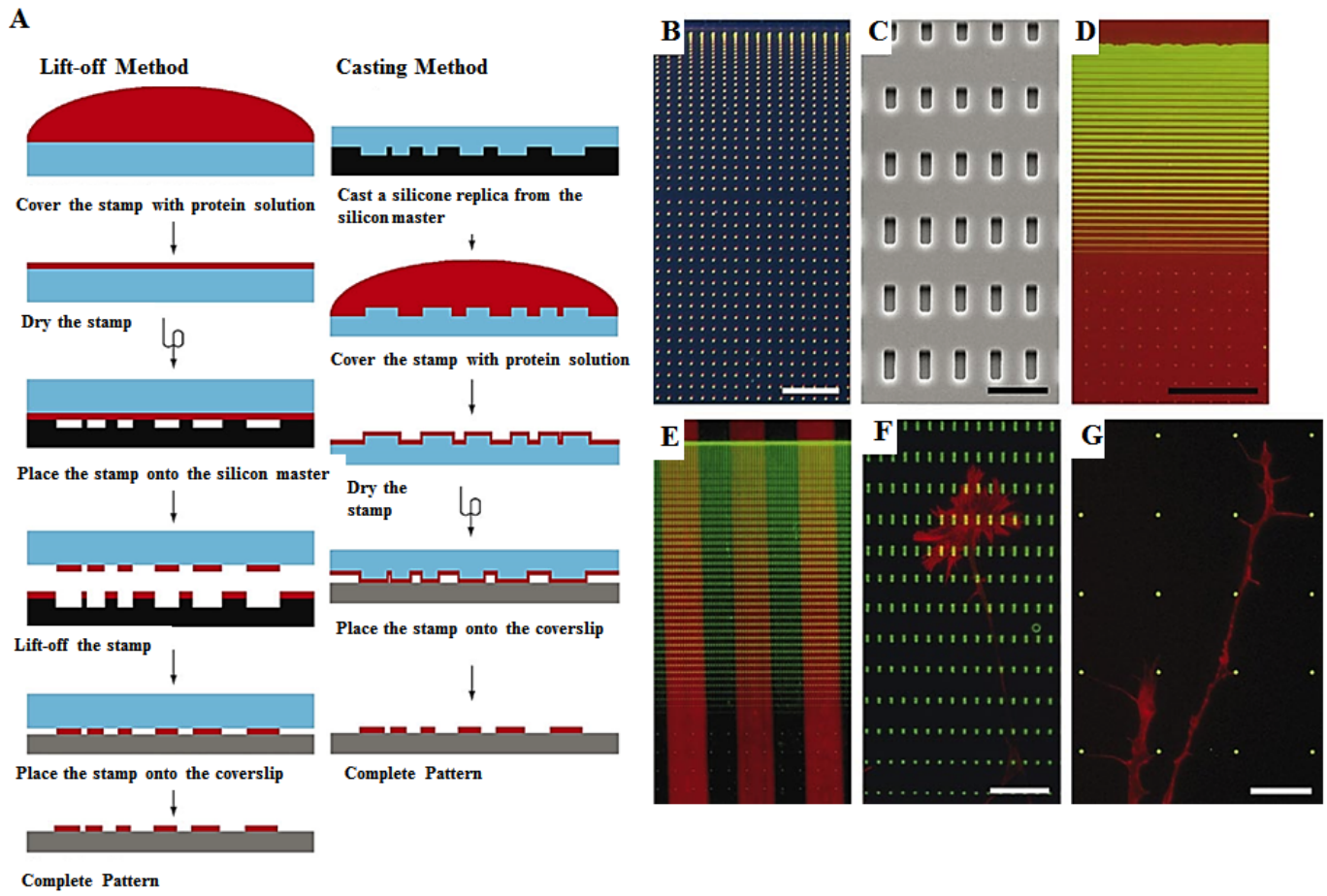


Fig. (3). Microcontact printing technique and application towards axon guidance [adapted and modified from 62]. **A**) Two different approaches to print protein patterns (red) onto glass coverslip (gray) using either a silicon master (black) or a PDMS elastomer stamp (blue). **B**) A silicon master with progressively spaced dots providing a graded pattern. **C**) Scanning electron microscopy image showing dots etched into a silicon master (~650 nm deep). **D**, **E**) Fluorescence microscopy image showing contact printed protein patterns displaying gradient, EephrinA5 (green), laminin (red). In E, laminin parallel stripes. **F**, **G**) Neuronal growth cones navigating along the protein gradient patterns, ephrinA5 (green), phalloidin-stained axonal actin (red). Scale bars: b, c, d 100 µm; e, 5 µm; f, g, 15 µm.

single cell resolution. While microfluidics can enable good control over cell culture certain aspects need to be addressed for achieving good results. The shear stress generated within confined microchannels during high-pressure fluid flow can stress the cells, and cause, damage or retraction of the neuritic processes and detachment [93, 94]. Usually operating at low flow rates using programmed syringe pumps or slow dispensing with micropipettes can avoid these issues; Other issues that need to be considered by new users include material selection, poly (dimethylsiloxane) (PDMS) a widely used material is transparent, and has high gas permeability, also its surface chemistry can be modified to control liquid surface-tension within the channels [95-97].

Typically, the devices are fabricated using a technique called soft-lithography, a modified fabrication strategy borrowed from the semiconductor industry now employed for making microfluidic devices [98]. A standard fabrication workflow for making a microfluidic device involves three steps (Fig. **4**): a) Design and fabrication of template, a designated pattern is fabricated either by photolithography using photoresist material such as SU-8 on a silicon wafer (or micromachined on a metal plate). b) Pattern transfer, the patterned silicon wafer is then used as a template to fabricate devices in polymeric material such as PDMS using a simple process called replica molding. c) Assembly and culture, finally the microfluidic device can be assembled with ports and modified to be used for cell culture and subsequent biological assays. This cheap and easy to implement fabrication route can enable even a standard biological laboratory to make their own devices in house, with limited engineering facility and microfabrication knowledge.

A variety of materials have been used for microfluidic cell culture including silicon, glass, quartz, polystyrene and poly (dimethylsiloxane) (PDMS). Silicon and glass substrates can be functionalized to make them biocompatible for neuron culture, also with the added advantage being that these substrates can be integrated with additional features such as microelectrodes for live cell recording. PDMS is widely used for neurobiology application given its advantages of biocompatibility, thermal stability, optical transparency, elasticity, ease of fabrication and low cost [84, 92, 95-97, 99].

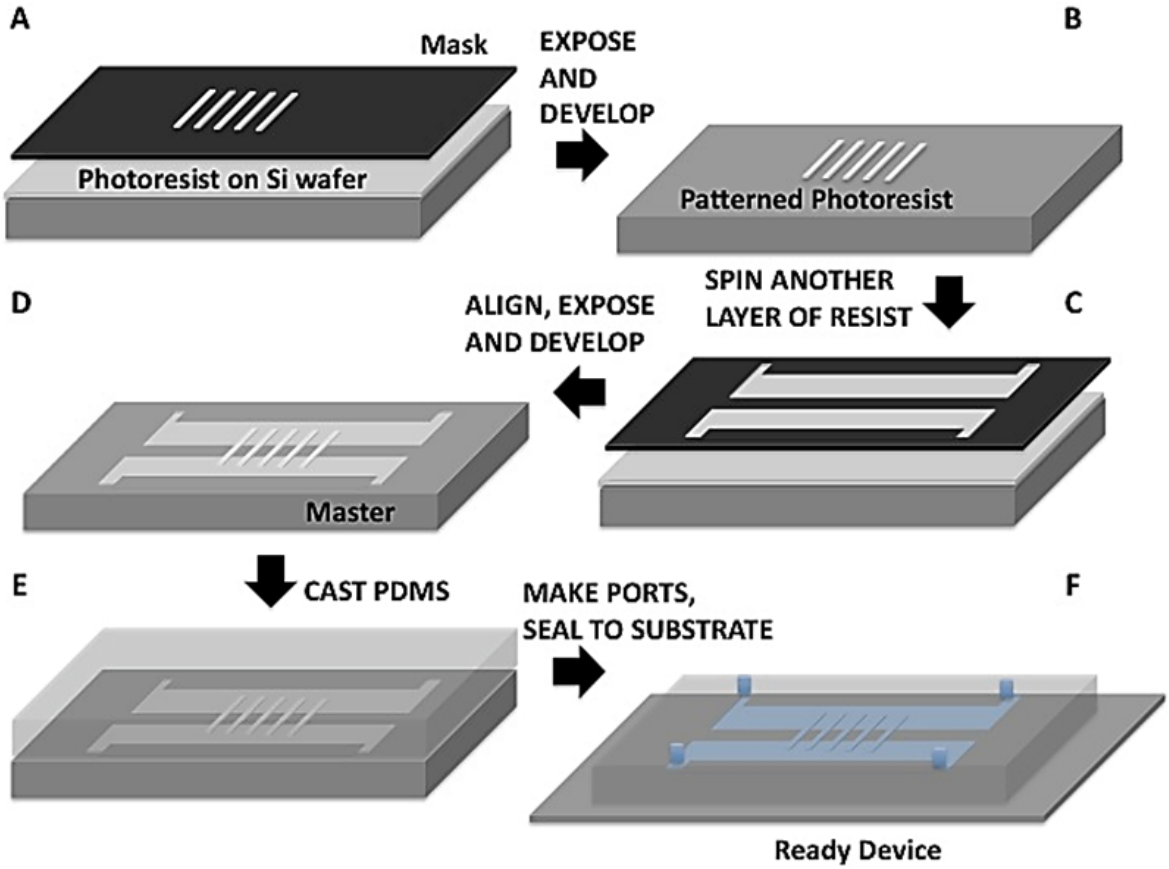


Fig. (4). Soft-lithography process for fabricating compartmentalized microfluidic devices (Modified from [119]). **A**, **C**) layer of photoresist, SU-8, is deposited on top of a Si wafer, a photomask is then used to exposure specific patterns. **B**, **D**) The master-mold with the micropatterns is developed. **E**) Master with raised microstructures features. PDMS is poured over the master and cured. **F**) The device is peeled, washed, and then sealed over a clean glass coverslip.

4.1. Application of Microfluidics and Compartmentalized Devices towards *In vitro* Disease Models

The application of microfluidic devices enables us to overcome most of the limitations in traditional compartmentalized devices. The integrated polymeric microfluidic device is easy to assemble and does not need any hydrophobic seal (such as grease in Campenot chamber). The devices have been used for both PNS and CNS derived neurons with good success. The flexibility with fabrication enables both open-channel and closed-channel devices that allow multiplexed chemical stimulation capability or integration of external recording probes. The standard feature of most compartmentalized microfluidic devices is parallel multi-groove topography, the ends of these groove channels open into a larger channel that serves either to seed the neurons or stimulate the growth of axons. Jeon's group was the first to demonstrate the microfabricated compartmentalized device for neuronal cell culture [100]. Their two-compartment device was fabricated using soft lithography technique, the bottom of a 150 μm wide barrier that isolated the chambers had 120 micron-size grooves (10 μm wide, 3 μm high, and 150 μm in length). The individual grooves were uniformly spaced 50 μm apart to prevent any structural collapsing on assembly. The embryonic neurons were plated in the growth compartment, within 4 days neurites were seen extending through the grooves and into the adjacent compartment. These micron sized grooves counteracted diffusion given the high resistance created by the hydrostatic pressure difference between the two compartments. An example of such a device used for another application by the same group is shown in Fig. **2C**, also a commercial version of the device now available from Xona Microfluidics *LLC*. is shown in Fig. **2D**.

There have been a few variations to the original two chamber microfluidic compartmentalized device design, a modified three chamber design [101, 102]; devices that enable electrode placement [103, 104]; circular layout [105-107]. Taylor *et al.* [102] published a three compartment microfluidic local perfusion (μLP) chamber that was able to fluidically isolate neuronal populations and promote neurite outgrowth through more than 100 parallel microgrooves. They could independently manipulate the two populations at the same time (Fig. **5**). For temporal control in these experiments, Taylor *et al*. used a 3-inlet perfusion channel. The addition of a microperfusion channel orthogonally to the microgrooves allowed addition of various drugs and neurotransmitters to the dendrites and axons in spatially and temporally controlled manner. Using this device they showed that localized treatment of the dendrites with glutamate lead to increased calcium locally as well as in the somatic region. They also investigated the effect of pulsed

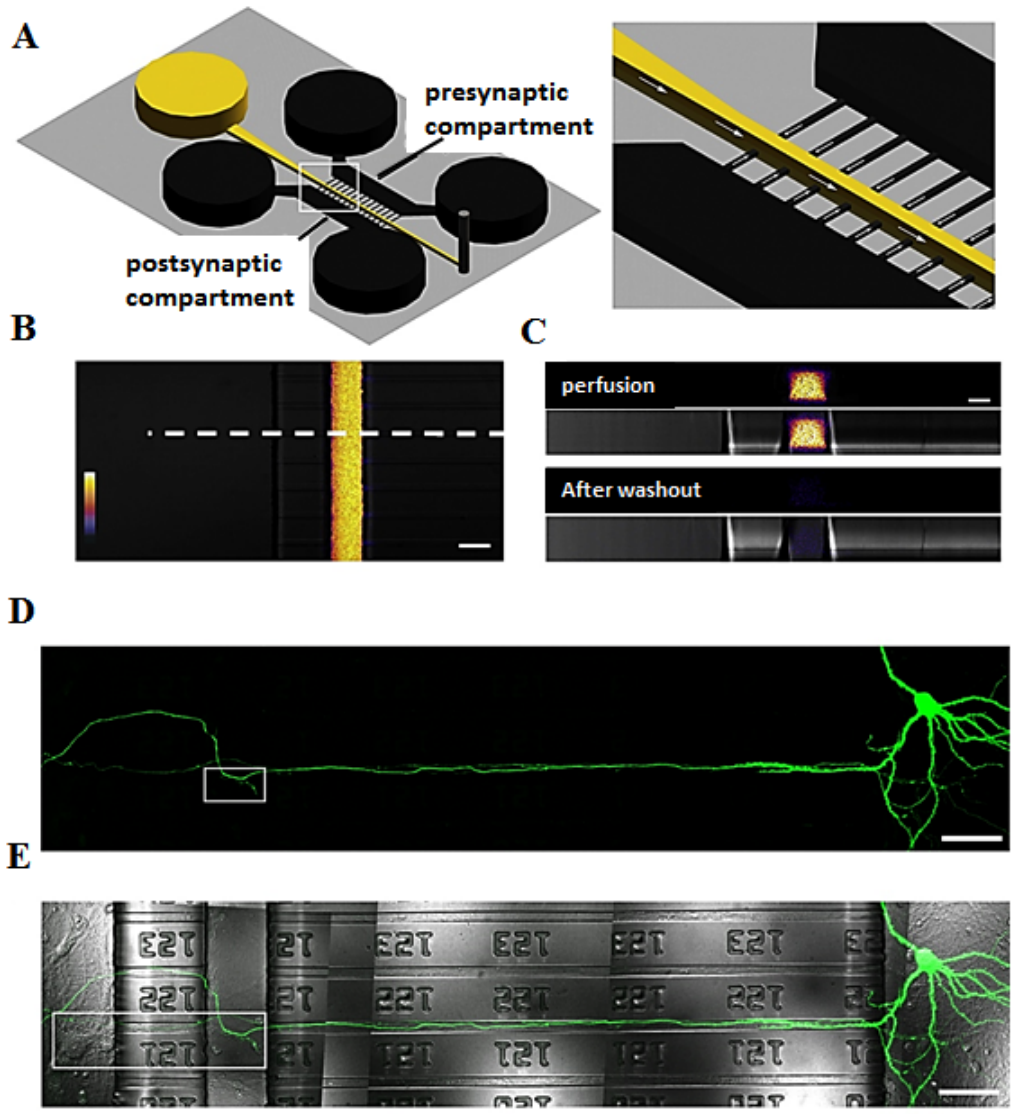


Fig. (5). Example of a three chamber compartmentalized microfluidic device that enables manipulation of synapses [102]. **A**) Schematic of an integrated local perfusion chamber and channel (yellow) The enlarged image on the right shows the direction of fluid flow within the perfusion channel and the microgrooves. **B**) A merged fluorescence and DIC image of the device showing the perfusion of the dye Alexa Fluor 488. Scale bar = 50 μm. **C**) Z axis profile of the dye within the channel during the perfusion and after wash out, taken along the line showing in B) Scale bar = 30 μm. **D**) A fluorescent image of GFP-labeled neuron extending an axon from the seeding chamber through the microgrooves into the perfusion channel. Scale bar = 50 μm. **E**) Merged images (DIC, GFP) clearly showing the location of the extended axon through the microgrooves into the perfusion channel.

glutamate stimulation on the dendrites, and found an increased pCREB within the somata as compared to continuous stimulation. Local perfusion of DHPG a metabotropic receptor (mGluR1) agonist to the isolated synaptic regions caused an increase in Arc transcription within the nucleus and Arc mRNA within the treated dendrites. This works provides a seminal example of the growing influence of compartmentalized microfluidic devices to locally manipulate single neurons, and sub-cellular targeting to study the signaling pathways.

While compartmentalization enables efficient segregation of the cellular parts, precise placement of the cells within the device has been challenging. Dinh *et al.* [108] developed a compartmentalized neuron arraying device that enabled single neuron patterning with precision and addresses this issue. The device design enabled developing liquid meniscus at the micropillars and selective protein patterning, thus restricting the neuronal culture only to the arraying region and outgrowth channels. SH-SY5Y neuroblastoma cell line or dopaminergic Lund human mesencephalic (LUHMES) neuronal precursors were seeded by the differential flow trapping. The patterning efficiency of this device was greater than 75% and almost 85% of the outgrowth channels were occupied by the extending neurites.

Such microfluidic design strategies for compart-mentalization and cell placement are essential to conduct high-throughput single neuron level experimentation. In the following section we present various *in vitro* neurobiology

assays that have been possible using microfluidic compartmentalized devices. As we focus on the single-cell investigation, the organotypic slice culture [109] and whole organism screening on microfluidic chips [110, 111] that can be classified as compartmentalized devices have not been included in this review.

4.1.1. Axon Guidance

In its natural microenvironment an axon from a polarized neuron navigates a complex landscape as it grows towards its target, based on guidance cues received by its growth cone. These cues can be short-range or long-range, chemo-attractive of repulsive and may involve guidance molecules, morphogens, growth factors, and cell adhesion proteins [112, 113]. These cues are received by the growth cones found at the tips of growing axons; these are converted into amplified internal signals that lead to asymmetric physiological changes and axonal extension. In a standard culture dish based assay it is difficult to mimic the *in vivo* gradient microenvironment that is essential to study axonal migration and turning behavior. Microfluidic devices allow control of fluidic interfaces and generating molecular gradients in a systematic and repeatable manner, thus making it possible to create axonal guidance in a controlled microenvironment [64, 114, 115]. A hybrid system involving microfabricated Campenot chamber combined with micropatterned surface was developed by Shi *et al*. [64] to investigate the effect of fibroblast growth factor receptor (FGFR), as well as adhesion protein N-cadherin signaling on axon guidance and growth. The rostral cervical motor neurons that were derived from embryonic stem cells and placed in one chamber extended their axons through the microchannel barrier into the second chamber patterned with N-cadherin on laminin. Their work indicated that the local crosstalk between N-cadherin and FGFR stimulated axonal outgrowth, but did not influence the axon guidance along the N-cadherin.

While most of the studies on growth cone have focused on promoting axon growth by directly manipulating the growth cone under stimulant gradient, Hur *et al*. [114] used a two-compartment microfluidic chamber to study the effect of non-muscle myosin II (NMII) inhibition on axonal growth. They concluded that pharmacological inhibition using blebbistatin or knockdown of NMII gene markedly accelerates axon growth over permissive as well as non-permissive substrates, which include inhibitors such as chondroitin sulfate proteoglycans and myelin-associated inhibitors. The local blockade of NMII activity on the axonal side of the device was sufficient to trigger growth cones to grow across the substrates.

Manipulating the growth cones and obtaining quantitative insight is arduous using standard techniques or within traditional *in vitro* culture. The examples presented above provide a good overview of integrating microfluidic compartmentalization for systematic study of axonal guidance and local growth cone modulation.

4.1.2. Neurite Transportation, Neurodegenerative Diseases

The prime attribute of neurodegenerative diseases such as Alzheimer's and Parkinson's has been defect in axonal transportation and/or synaptic deficits due to aggregation of extracellular amyloid plaque, or intracellular neurofibrillary tangles or Lewy bodies [116-118]. The use of traditional culture dish is not suitable for studying these mechanisms given the random orientation of the axons, overlapping synapse formation and difficulty in recovering the axonal material for analysis. Compartmentalized microfluidic devices enable localization of individual neuron with spatial segregation of the soma and growing axons. With limited background noise from other neurons and powerful imaging system it has been possible to study axonal trafficking. Modifying the original two channel compartment design initially developed by Jeon group [119] other groups have demonstrated single-molecule imaging of retrograde axonal transport of NGF [120] and dendrite-to-nucleus signaling of BDNF [121, 122]. In structural biology much of the current understanding about the microtubule assembly in axons and transportation of endosomes is based on biochemical assays and electron microscopy imaging, but this does not recapitulate the real situation in live neurons. Mudrakola *et al.* [123] utilized the two compartment microfluidic platform in combination with photosensitive quantum dot imaging probes and pseudo total internal reflection fluorescence imaging system to track endosome transportation and dynamic path switching along microtubules. Furthermore, such microfluidic compartmentalized devices have also been used to investigate the defects in axonal transportation caused by the two pathophysiological molecules β-amyloid peptide (Aβ) and Tau protein [124-126].

4.1.3. Neural Injury

CNS and PNS injury can lead to irreversible damage and permanent loss of function, regeneration is difficult at axonal level due to growth inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs) [127] and myelin-associated inhibitors [128, 129]. Animal experiments and *in vivo* models of injury provide limited information at neuronal level. The present single cell axotomy technique requires micromanipulators and micron sized glass tips. The procedure is slow, and also increases possibility of contamination, tip breaking and accidental neuronal death.. The ability to spatially segregate axons on microfluidic devices makes it possible to investigate *in vitro* traumatic axonal injury and study the biochemical changes, gene expression profile and phenotypic changes. Two microfluidic models have been emerged that can cause chemical injury [106, 119, 130, 131] as well as physical injury [132, 133] to the growing neurons.

Taylor *et al*. [119] utilized their previously published two chamber compartmentalized microfluidic device [100] for studying CNS axon injury and regeneration. Dissociated CNS neuron cells were loaded in the culture platform, and after 4 days the axons extended through the microchannels into the axonal compartment, without dendrites. Vacuum aspiration was employed to axonal channel for 5 seconds to perform axotomy. Furthermore, RNA expression change in FBJ murine osteosarcoma viral oncogene homolog (*Fos* or *c-fos*) an immediate early gene, was determined. The vacuum axotomy procedure resulted in an increase in the expression of RNA by about 200% within 15 minutes, and 320% within 2h after the lesion. The microfluidic device was further used to screen candidate molecules for axonal regeneration. A combination of neurotrophin-3 and brain-derived neurotrophic

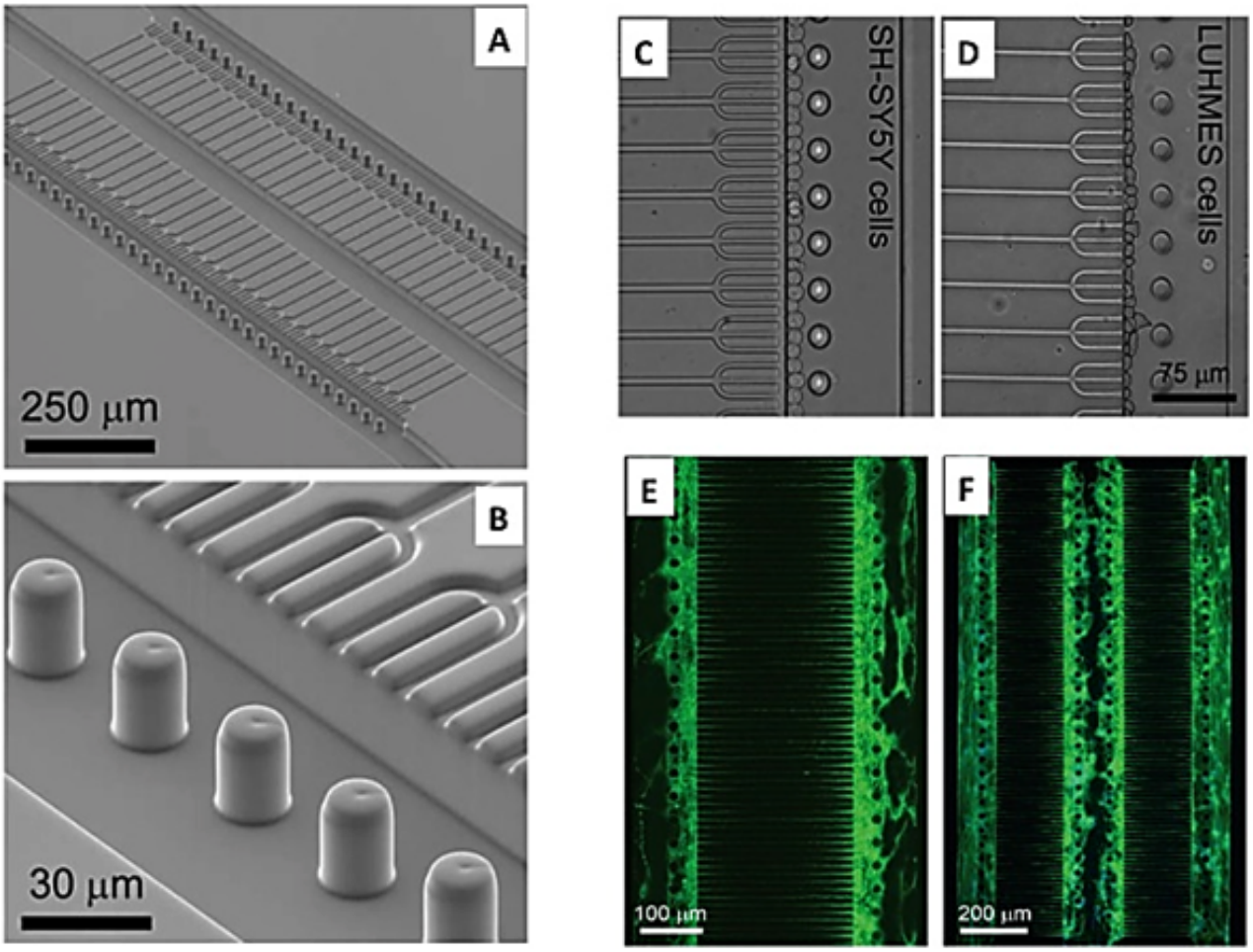


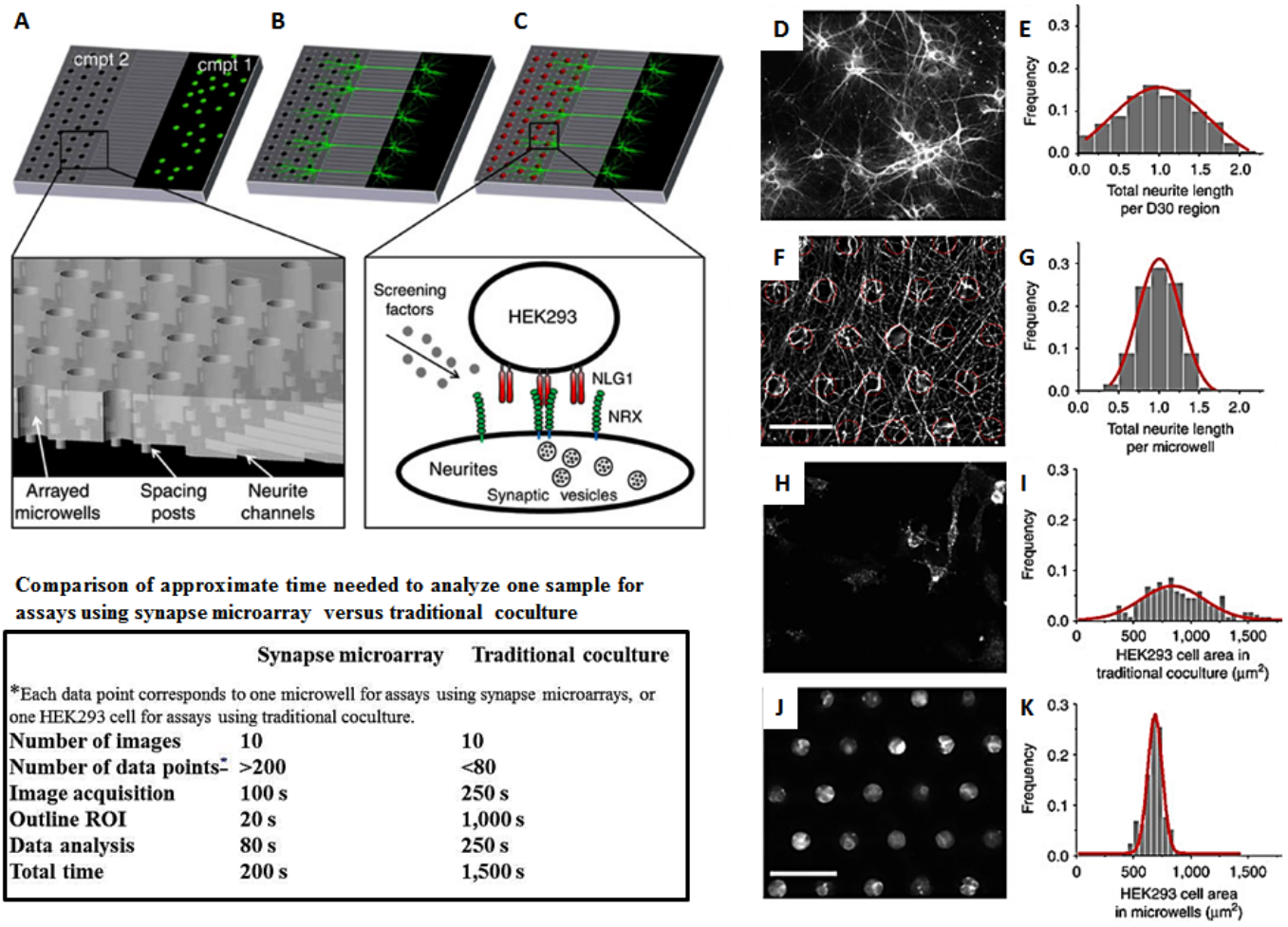
Fig. (6). Compartmentalized microfluidic device for rapid and precise neuronal arraying [108]. **A**, **B**) Scanning Electron Microscope images of the compartmentalized neuron co-culture array showing trapping sites and meniscus-pinning micropillars. **C**, **D**) Bright-field images showing trapped neuronal SH-SY5Y cells and LUHMES cells, taken 4 h after arraying. **E**, **F**) SH-SY5Y co-cultures within the 2-compartment and 3-compartment device, β(III)-tubulin immunostaining performed after 5 days.

factor was applied for 5 days to locally treated the lesioned axons. The isolated axons treated with neurotrophin showed pronounced increase in axonal branching and growth as compared with the control group. In another study Vahidi *et al*. [134] demonstrated a simple pinch valve into the MIMIC system combined with a strip assay incorporated into the device to act as surface-bound inhibitors mimicking spinal cord injury. This platform was successfully applied for drug screening; Chondroitinase ABC was applied to the axons that could then overcome the aggre can inhibitory effects.

In another study, Hosmane *et al.* [132] developed a valve based microcompression platform for inducing injury to micron-scale segments of the individual CNS axons. They tested the effect of acute compression (short 5 s) under different compressive loads (0–250 kPa) and monitored the axons for 12 hours after injury. Under mild compression (< 55 kPa) there was continued growth in 73% of the axons, and slight degeneration. At moderate (55–95 kPa) and severe (> 95 kPa) levels of injury, the number of growing axons dramatically reduced to 8% and below 5% respectively. They also observed that the axons were able to regrow faster and extend 40% more than the uninjured axons even in the absence of exogenous stimulating factors.

4.1.4. High-throughput Compartmentalized Platforms

One of the emerging areas for microfluidic devices is high-throughput screening that allows studying basic biological mechanisms at a larger scale, with reduced cost and time, making it viable for drug development applications. The state-of-art microfluidic devices employ multiple sensors, valves and multiphase flows for achieving high-throughput, and are more feasible for screening large chemical libraries. Such approach is also needed for single cell neuro-pharmacological studies. In one of the first demonstrations, Shi *et al*. [135] developed a compartmentalized microfluidic platform that enabled screening small molecule library and identify compounds that enhance synaptogenesis (Fig. **7**). Synaptic function is affected in many brain diseases, the present assays make it difficult to automate and quantify the synaptic development, and technologies that can enable performing synapse assays in a high-throughput manner can



Comparison of approximate time needed to analyze one sample for assays using synapse microarray versus traditional coculture

	Synapse microarray	Traditional coculture
*Each data point corresponds to one microwell for assays using synapse microarrays, or one HEK293 cell for assays using traditional coculture.		
Number of images	**10**	**10**
Number of data points*	**>200**	**<80**
Image acquisition	**100 s**	**250 s**
Outline ROI	**20 s**	**1,000 s**
Data analysis	**80 s**	**250 s**
Total time	**200 s**	**1,500 s**

Fig. (7). Schematic of synapse microarray technology and culture results [135]. **A**) Neuronal cells are loaded and cultured in compartment 1 (cmpt 1). **B**) Isolated neurites outgrow through the microchannels and extend to the synapse compartment 2 (cmpt2). **C**) NLG1 expressing HEK293 cells are seeded into the microwell through holes. **D**) Representative fluorescence image of βIII-tubulin stained neurites in a traditional neuron culture. **E**) Distribution of total neurite length (normalized to mean) measured within randomly chosen 30 μm diameter regions in a traditional culture (n = 247). **F**) Fluorescence image of βIII-tubulin stained neurites within the synapse microarray. The microwells are circled in red, scale bar, 100 μm. **G**) Distribution of total neurite length (normalized to mean) as measured in the microwells (n = 270). **H**) Image showing random HEK293 cell spreading in traditional cultures (staining for HA–nLG1). **I**) Histogram of HEK293 cell area coverage in traditional cultures (n = 222). **J**) HEK293 cells exhibit well-defined morphologies in the synapse microarray (staining for HA–nLG1), scale bar, 100 μm. K) Histogram of HEK293 cell area coverage in the synapse microarray format (n = 286).

facilitate rapid identification of drug leads. The synapse microarray platform consists of a two main compartment device connected *via* several parallel microgrooves (10 μm wide and 3 μm high) that effectively isolate the axons from neuronal somata. Dissociated neurons that are plated and cultured in compartment 1 (Fig. **7A**) extend their axons through the microgrooves into the compartment 2 (Fig. **7B**) that is covered by a thin (80 μm) PDMS membrane held 3 μm above the substrate. The space underlying the membrane allow axonal growth, membrane also contains an array of through-holes or microwells that hold the neuroligin-1 (NLG1) expressing HEK293 cells. The platform design enabled synapse formation in a regular array format, by precisely controlling the position of NLG1expressing cells and providing space for the neurites to grow freely around them. This technique reported a ten-fold increase in the sensitivity as compared to the traditional assays, while decreasing the time required for capturing synaptogenic events by an order of magnitude. Using this device the researchers screened a small chemical library and identified novel histone deacetylase (HDAC) inhibitors that promote synapse formation through NLG1.

Microfluidics chamber combined with other micro-structures such as microwells that compartmentalize individual neurons can be aptly used for high-throughput single-cell level screening of compounds. Folch *et al*. [136] developed a microfluidic device (Fig. **8**) that allowed screening over 20,000 single cells at the same time. The microfluidic device was used to load thousands of dissociated single olfactory sensory neurons (OSN) within a microwell array, and provide stimulation, calcium responses from all the trapped neuronal cells were recorded simultaneously. Importantly, this microfluidic system can also be used for both the

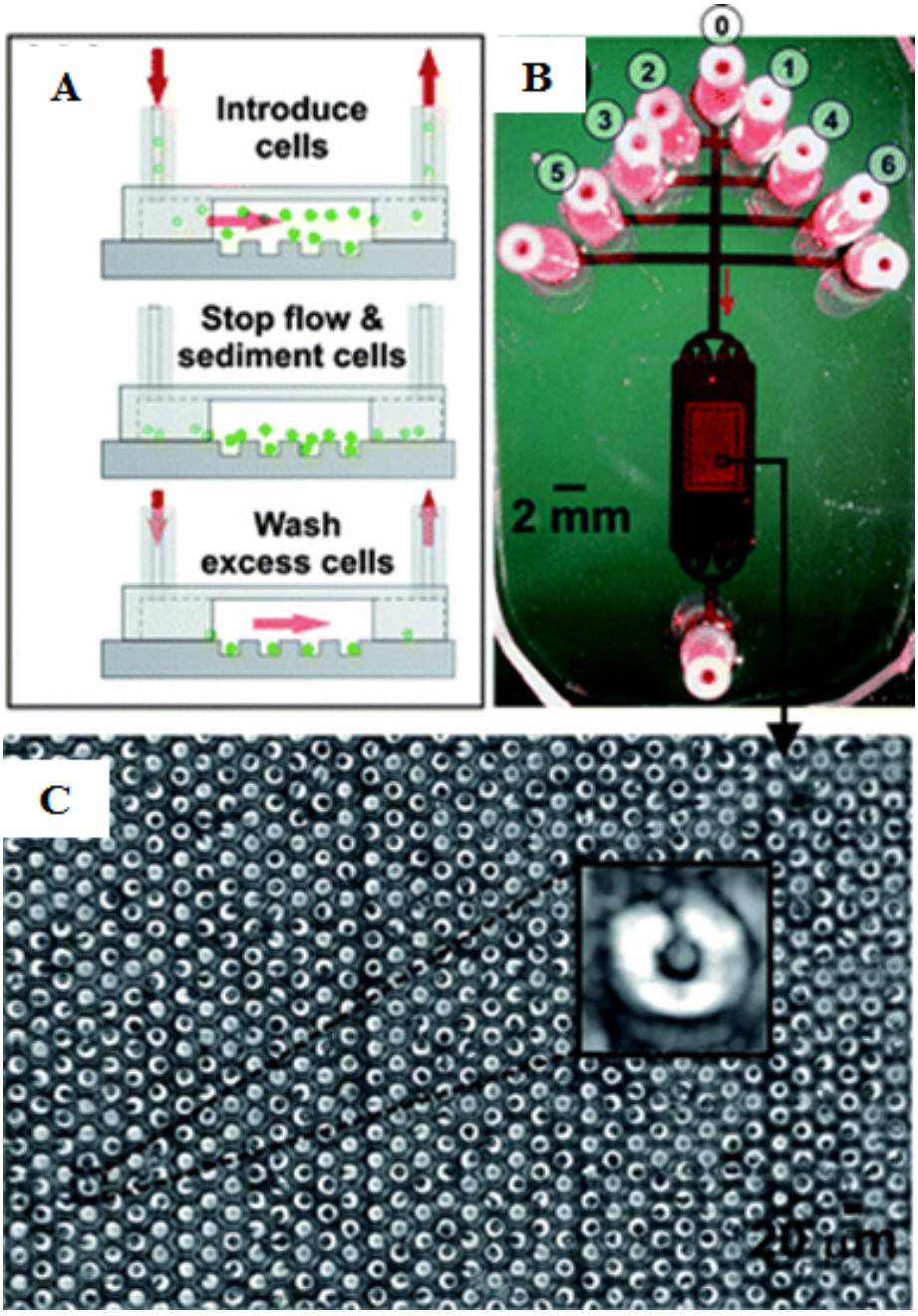


Fig. (8). Microfluidic device integrated microwell based platform for screening Olfactory sensory neuron (OSN) response [136]. **A**) Schematic showing the cell loading procedure. OSNs are gently flown into the assembled device (top). Fluid flow is stopped to allow the cells to settle under gravity into the microwells (middle). The non-adhered cells are washed off (bottom). **B**) A fully assembled device with microwells on the bottom layer. The device has several inlets (numbered) and one outlet. **C**) Close-up of the microwells with OSNs trapped. Inset showing a single OSN within a microwell. Scale bar, 20 µm.

detection of the OSN populations that are intermittently responsive as well as broadly responsive to various odorants that have unrelated structure.

CONCLUSION

The ability to control the microenvironment and direct the growth of neurons in a spatial and temporal manner makes microfluidic compartmentalized devices a versatile tool for neurobiological research. As reviewed here, the application of these devices has enabled understanding the basic mechanism involved in axonal growth, transportation, synaptogenesis and regeneration; this level of detailed investigation was not possible previously using the standard *in vitro* methods. Many outstanding questions in neurobiology still remain unaddressed. The versatility offered by microfluidics combined with capability to compartmentalize single neurons makes it ideal to be paired with super-resolution

imaging techniques (PALM, STORM). Such investigations can provide novel information at sub-cellular scale, shedding new light on transportation, transmission defects responsible for degenerative diseases, and in neuropharmacological research. Additionally, we envision that these devices will be combined with techniques such as RNAi, optogenetics, further broadening their impact on neuropharmacological research.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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