Review

New perspectives on neuronal development via microfluidic environments

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Understanding the signals that guide neuronal development and direct formation of axons, dendrites, and synapses during wiring of the brain is a fundamental challenge in developmental neuroscience. Discovery of how local signals shape developing neurons has been impeded by the inability of conventional culture methods to interrogate microenvironments of complex neuronal cytoarchitectures, where different subdomains encounter distinct chemical, physical, and fluidic features. Microfabrication techniques are facilitating the creation of microenvironments tailored to neuronal structures and subdomains with unprecedented access and control. The design, fabrication, and properties of microfluidic devices offer significant advantages for addressing unresolved issues of neuronal development. These high-resolution approaches are poised to contribute new insights into mechanisms for restoring neuronal function and connectivity compromised by injury, stress, and neurodegeneration.

Introduction

The brain forms through a remarkable process of self-organization. Neuroblasts migrate and terminally differentiate into the neurons and glia of each brain region. They extend multiple protrusions, each of which encounters distinct, complex, and dynamic microenvironments. The emergence of neuronal polarity and synaptic specializations generates nascent networks along which the earliest signals of communication flow. Simple networks elaborate so that they sense, coordinate, and regulate the range of metabolic, endocrine, physiological, and cognitive functions that allow complex behaviors and thoughts. Deciphering the mosaic of signals in the chemical and physical landscape that guide the development of each neuron and direct formation of its axon and dendrites and their synapses and functional domains during this wiring process is a formidable task.

The mature neuron is elongated and highly branched and forms thousands of synaptic connections with other neurons at extensions distant from the soma. Within the complex cytoarchitecture of the brain, neurons and their extensions are densely packed, filling all available space (Figure 1a,b) [1,2]; this impedes subcellular analysis *in vivo*. In dish cultures, neurons develop similar complex morphologies, albeit at lower densities and with relatively less oriented growth (Figure 1c). Consequently, neurites often reside in different chemical, physical, and fluidic microenvironments. Whereas *in vivo* and conventional cell culture approaches have achieved remarkable insights into axonal, dendritic, and synaptic development, discovering how local subcellular signals of individual neurons influence differentiation and function has been elusive. Nevertheless, it is at the subcellular level that dynamic changes during development and throughout life determine information flow and processing (e.g., whether or not specific experiences form or recall memories). Restoration of brain function impaired by damage, stress, or degeneration is especially difficult. Local cues and microenvironments that guide brain wiring during development are often ephemeral and may have been expressed distant from adult termini. Solutions to these issues require new approaches that allow local high-resolution analyses of the interplay between myriad extracellular signals and the intracellular responses that shape the developing brain.

Microfluidic devices (μFDs) – cell-culture environments with channels of micrometer-scale dimensions containing nanoliter volumes – are addressing these needs. Interfacing of engineering technologies with biological methodologies allows the fabrication and application of microfluidic-based systems with new capabilities for maintaining and studying brain cells and circuits in stable microculture (Figure 1d,e). By using replica molding (Figure 2), environments approximating not only single cells but even single neuronal processes can be fabricated in the laboratory setting (Box 1). Advances in chemistry and materials science over the last decade have transformed our ability to control spatial and temporal signals within μFD channels and have facilitated the use of co-cultures and compartmentalization for studying neuronal–glial interactions [3,4], disease progression [5,6], and repair of injury [7–10]. Exquisite spatial and temporal control of cellular microenvironments offers significant advantages for studying neurons and their processes. In this review, we summarize and evaluate new perspectives on neuronal

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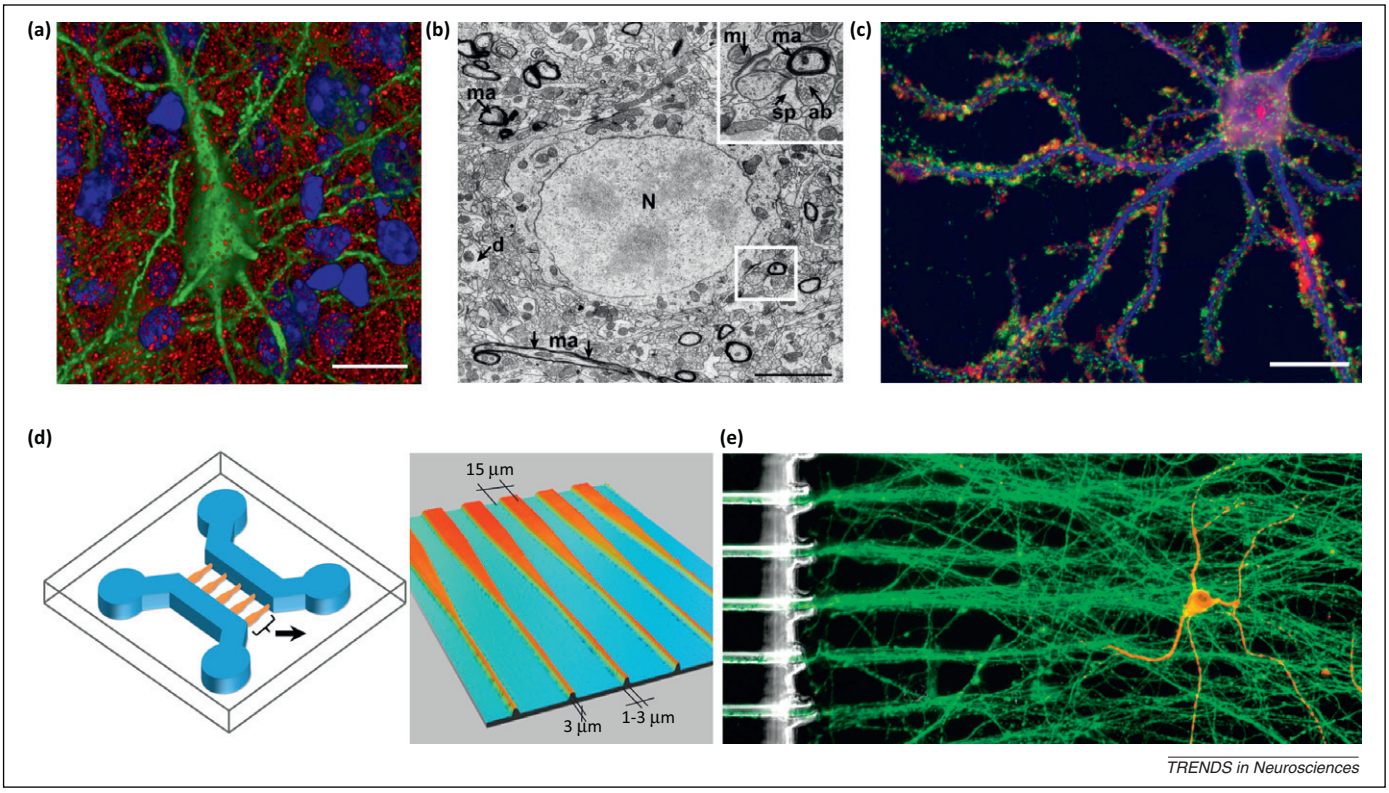

Figure 1. Cytoarchitecture of neurons *in vivo* compared with dish or microfluidic-device (μFD) cultures. **(a)** The cytoarchitecture of brain tissue is remarkably complex, as seen in cell packing and interwoven processes of the cerebral cortex *in situ* imaged by array tomography. The 3D space of a mouse cortical neuron (green) is surrounded by other cells (marked by 4′,6-diamidino-2-phenylindole-labeled nuclei, blue) and myriad presynaptic boutons (synapsin-labeled, red). Figure modified from [2] and used with permission. Scale bar, 10 μm. **(b)** Electron micrograph of layer IV of the rat somatosensory cortex reveals densely packed structures with essentially no free space around the cell body (nucleus, N), myelinated axons (ma), axon bundles (ab), dendritic spines (sp), and dendrites (d); mitochrondria (m) are also shown. Scale bar, 5 μm. Original image contributed by Graham Knott. **(c)** At low densities in a conventional dish culture, neurons develop complex cytoarchitectures and connections despite open space between neurites and cell bodies. Presynaptic terminals (synapsin, green) stud the dendritic arbor (microtubule-associated protein 2, MAP2; blue) of a primary hippocampal neuron (33 days *in vitro*); rhodamine–phalloidin labels filamentous actin red. Scale bar, 20 μm. Modified from [80] and used with permission. **(d)** A μFD fabricated to organize cellular connections and control the local fluidic microenvironment. Depicted is a two-channel μFD (left) with tapering interconnects (enlarged on the right) that facilitate directional orientation of neurons for analysis of interactions. Neurons can be cultured in each channel; interactions can be restricted to and guided by the interconnecting tunnels (i.e., interconnects). Cortical neurons were seeded in one channel (left, blue) and striatal neurons in another channel (right, blue). Interactions between channels are restricted to the interconnects (orange, enlarged on the right). Tapered interconnects funnel cortical axons toward the channel on the right, while restricting the reverse migration of afferent axons back through the interconnects. Reproduced and modified from [52] by permission of The Royal Society of Chemistry (RSC). **(e)** Complex and elaborate architectures of neurons can be guided and organized using μFDs. A single striatal neuron (yellow, right channel) is innervated by organized axon bundles from cortical neurons (green) cultured in the adjacent channel and directed via the interconnecting tunnels. Striatal neurons are immunolabeled for MAP2 (red) and cortical neurons are labeled for α-tubulin (green). Reproduced and modified from [52] by permission of the RSC.

development at subcellular, cellular, and tissue levels resulting from the dissemination and adoption of μFDs.

Neuronal differentiation and polarity

Neurons developing *in vitro* undergo sequential differentiation, polarization, and specification of neurites as axon or dendrites, recapitulating developmental stages *in vivo* [11]. In the absence of patterned instructive cues in dish cultures, processes tend to be stellate (Figure 1c) and overlapping. μFDs allow significantly greater control over neuronal development *in vitro*. The chemical and topographic disposition of developmental cues with micropatterning techniques can induce predetermined morphologies [12–18]. Attractive and/or repulsive cues can be patterned with highly controlled geometries. Cues can be patterned singly, within close proximity, in combination with other substrates, or in direct apposition to them (Figure 3a,b). Physical cues on the scale of individual neurites can be fabricated to control neurite navigation [19]. Presentation of spatially defined cues permits the influence of local extracellular signals on neuronal differentiation to be resolved.

The stripe assay, also known as the Bonhoeffer stripe assay, has long been used in dish cultures to interrogate the behavior of the axons of tissue explants in response to neutral versus known or potentially novel guidance cues. Application of the stripe assay demonstrated that the first hippocampal neurite that encounters a locally presented growth-promoting molecule [laminin (LN) or neuron-glia cell adhesion molecule (NgCAM)] is immediately specified to become the axon [14]. A recent modification using sole-source silicone matrices in a complex fabrication protocol extended the stripe resolution to 100 μm [20].

μFDs have been used to refine stripe patterns for subcellular analyses. Stripes can be laid down more easily with greater line resolution (<10 μm) and sharper edges, and are more versatile for multiple chemical depositions compared to the conventional stripe assay protocol. The μFD-based approach identified signals that induce axon initiation during the polarization process [21]. When one neurite of a hippocampal neuron is selectively exposed to a high-resolution stripe of substrate bearing brain-derived neurotrophic factor (BDNF), the local encounter initiates a cascade of events, activating cAMP signaling, directional

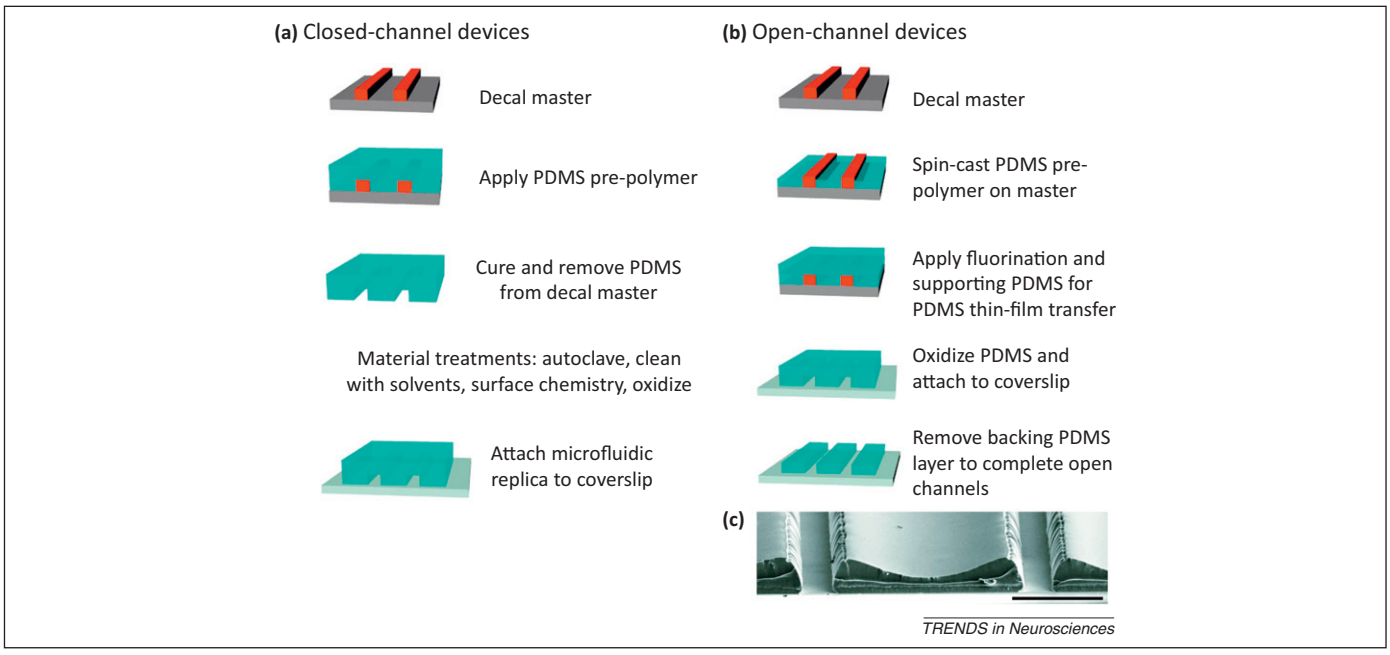

Figure 2. Replica molding allows fabrication of open- and closed-channel microfluidic devices (μFDs). Masters are made by patterning SU-8 photoresist on silicone to generate the decal and a 3D negative of the final structure; treatment with a nonstick layer allows polydimethylsiloxane (PDMS) release [81]. **(a)** For closed-channel devices, bulk PDMS is poured onto the master, cured, cleaned, treated (if appropriate), and assembled onto clean coverslips. Channels or coverslips may be coated with substrate guidance cue(s), such as poly-lysine, and rinsed with media prior to cell culture. **(b)** For one type of open-channel device, PDMS is spin-cast onto the master and cured, fluorinated, and covered with bulk PDMS to support the PDMS thin-film decal during transfer. The multilayer replica is removed, oxidized, and covalently attached to a clean coverslip, after which bulk PDMS is removed. **(c)** Scanning electron micrograph of an open-channel μFD. Scale bar, 100 μm. Reproduced and modified from [64] by permission of The Royal Society of Chemistry.

elongation, and axonal differentiation. Asymmetric reciprocal regulation of cAMP or cGMP evokes axon or dendritic formation, respectively [22]. Thus, upstream effectors that regulate cAMP or cGMP commit naive neurites to their developmental path. Presentation of spatially and chemically distinct substrates to the developing axon in a μFD-based stripe assay revealed that the phosphorylation state of the E3 ubiquitin ligase Smurf1 determines substrate preferences [23]. This approach identified transforming growth factor β (TGF-β) as an extrinsic cue that induces neuronal polarity during patterning of neural circuits [24]; how TGF-β signal transduction engages intrinsic regulators, such as cAMP, is yet to be determined.

Advances in fabrication methods, such as vacuum soft lithography, allow the patterning of multiple geometrically defined instructive cues in a single procedure. Such approaches are refining the stripe assay by enhancing chemical and spatial complexity [25]. These precise patterning methods offer a high degree of control in guiding the formation of neuronal networks with predetermined connectivity [25,26]. Intricate large-scale patterns, such as substrate chemicals in interconnected or juxtaposed shapes, letters, or designs, can be printed to design the arrangement of neurons and their connections [27]. We envision that advances in patterning techniques will permit real-time study of neuronal signaling pathways, including exquisite resolution of subcellular processes, neuronal network communications, and neural repair mechanisms, and the construction of functionally integrated neuronal systems.

Axon guidance and differentiation

After polarity has been established, the axon begins a phase of rapid growth. While traversing its developmental course, the axon passes many cells, extracellular matrices, fluid microdomains, and structures, such as capillaries, before arriving at its target. What cues guide the course of each axon? Studies in culture dishes effectively used the stripe assay to determine axon preference for single substrate molecules; ephrin–Eph receptor signaling identified in this way was instrumental in the elucidation of axon guidance *in vivo* [28]. μFDs allow great flexibility in the design of microenvironments to evaluate responsiveness, sensitivity, and the dynamics of population or individual axonal growth cones towards single or multiple guidance cues [26,29–31]. Furthermore, spatial control of neuronal morphology by μFDs can be exploited to selectively isolate axons and then assess their behavior optically and determine their molecular content (e.g., proteomic profiles or axonal mRNAs) [32–34].

Patterned biochemical substrates

Similar to the stripe assay, patterned lines of binary substrates can align axons within a population. In μFDs, this substrate pattern forms parallel fibers of axonal bundles without axonal intersections. This avoids potential confounds in conventional unpatterned dish cultures, in which many axons overlap. These populations of axons can be used to study organized axotomy and axonal transport and to analyze recovery [7,8,35]. Such patterned neuronal cultures are also useful for titrating substrate concentrations that guide the growth and behavior of single growth cones, and for dissecting axonal segments for molecular analysis [36,37].

To elucidate the mechanism of a putative axon guidance cue, a compartmentalized microfluidic chamber can be positioned onto micropatterned stripes designed to expose

Box 1. Advantages of microfluidic devices for studying neuronal development

μFDs offer unparalleled spatial, chemical, and temporal control of the microenvironments that shape differentiating neurons, facilitating investigation of neuronal development in new ways. Simple to complex μFDs can be fabricated in the laboratory, are available through commercial sources for off-the-shelf use, or can be designed and manufactured for specific research applications. Some resources for μFDs include KNI Foundry at CalTech, Micralyne, Microfluidic ChipShop, Microliquid, Micronit Microfluidics, Stanford Microfluidics Foundry, and Xona Microfluidics.

The main advantages of microfluidic devices are as follows:

(i) **High reproducibility:** Small variations on the microenvironmental scale could affect significant variables at the subcellular level; therefore, high reproducibility is essential. Replica molding facilitates the fabrication of highly reproducible, disposable μFDs of moldable gels and polymers in a hood or on a bench (Figure 2) [84]. PDMS, an advanced silicone material, is the most widely used fabrication material; it can be molded easily to form simple, complex, or multilayered channel systems. A large number of PDMS devices can be made from a single master, reducing the time and cost of fabrication.

(ii) **Easy assembly:** PDMS-based μFDs are easily sterilized through conventional means and can be reversibly or irreversibly sealed to glass, polystyrene, or silicone wafers to produce a 3D microenvironment [64].

(iii) **Fluidic control:** Control of the fluidic environment is critical for establishing the local chemical and fluidic features of subcellular domains. μFDs allow control of the chemical substrate features and fluidic surround with spatial and temporal dynamics and precision [32,49].

(iv) **Material versatility:** Probing of specific developmental issues may benefit from μFDs fabricated from different moldable polymers with distinct physical and/or chemical features. A variety of moldable polymers can be used [84,85]. The choice of material is influenced by fabrication requirements and the experimental design.

(v) **Design flexibility:** Channel design is established by photolithography, which uses light to selectively transfer the design pattern via a photomask to a light-sensitive chemical photoresist on a substrate. This process either engraves the pattern or allows deposition of material in the pattern on the substrate beneath the photoresist. This allows microfabrication of a range of designs from simple channels to highly complex fluid networks at a resolution down to ~500 nm. This design flexibility means that μFDs are broadly applicable to specific problems in neuroscience, facilitating the study of subcellular regions, individual cells, neuron pairs, and networks of neurons in highly controllable ways [35,64,86].

(vi) **Experimental feasibility:** The intrinsic properties of μFDs make them amenable for cell culture, imaging, and biochemical analysis [60,63,64,87]: local fluid exchange, optical transparency and imaging compatibility, high gas permeation, low water permeability, thermal stability, and the ability to physically confine neurons to control connectivity [88–90]. Substrate properties that can be altered by design include hydrophobicity, stiffness, topography, functionalization with bound biomolecules, and substrate patterning.

subcellular regions of neurons to defined substrates. For example, micropatterned stripes were used to resolve the mechanisms of axonal avoidance of semaphorins (Sema3f) [38]. Materials can be applied throughout the μFD or just only in subcompartments for parsing of global versus local signaling mechanisms. Somata can be restricted to a channel used for seeding cultures (Figure 1d). Axons grow down interconnecting tunnels to an adjacent, fluidically isolated channel [10,39]. The use of stripes of N-cadherin or LN within the channels can restrict the patterned growth substrates to axons. Selective inhibition of fibroblast growth factor receptors (FGFRs) of fluidically isolated axons reduced the rate, but not the direction, of axonal growth [39]. FGFR inhibition restricted to the soma had no effect on the axonal growth rate or navigation. Thus, the growth cone behavior and axon morphology are mediated locally by FGFRs on the axon or growth cone rather than by soma.

Factors that accelerate axon elongation have been selectively probed using μFDs with complex substrates in an effort to identify common targets that mediate axon growth inhibition and to develop interventions that enhance the axon regenerative capacity of mature neurons. Dorsal root ganglia neurons were cultured so that elongating axons isolated in channels were exposed to permissive–inhibitory substrate borders, including chondroitin sulfate proteoglycan (CSPG), an inhibitor of axon elongation via non-muscle myosin II (NMII). Local application of blebbistatin, which is thought to increase microtubule extension toward the leading edge of the growth cone, markedly accelerated axon growth over the inhibitory CSPG substrate [40]. Because mature central neurons fail to regenerate axons after injury owing in part to diminished intrinsic axonal growth capacity, NMII may be a target for modulation of regenerative capacity. These findings demonstrate the power of spatial control offered by μFDs to reveal local regulatory mechanisms for guidance, growth, and inhibition of axons [41]. μFDs thus allow local resolution of the roles of specific substrates and regulatory mechanisms within subcellular domains of axons.

Biochemical gradients

In addition to patterned lines of substrate, developing neurons respond to biochemical gradients. External gradients, which may be both long-range diffusive cues and short-range contact cues [42], guide axonal growth and trajectory. Patterning of neural circuits is determined by cues that are non-uniform and transient and change during critical developmental periods. Compared to binary patterns, which provide sharp contrasts in surface cues, gradients offer subtle, yet meaningful contrasts of environmental signals that promote and/or repel axon growth [29,43]. μFDs permit patterning of microscale substrate and diffusive gradients far more controlled and refined than is possible in dishes, and thus achieve a scale and complexity that more closely approximate nature [44].

Gradients of potentially instructive molecules can be controlled through μFD-based laminar flow and presented either tethered to the substrate or as diffuse cues [29,43–46]. Substrate-tethered gradient cues can be deposited in single or multiple layers using laminar flow (Figure 3a,b). When early post-natal rat hippocampal neurons were cultured on LN substrate gradients, the behaviors of individual axons within the population could be tracked and outcomes scored. On average, 68% of axons showed a preference for increasing LN concentrations, whereas 10% migrated away from LN, 5% were at right angles, and 17% were indifferent to it [43].

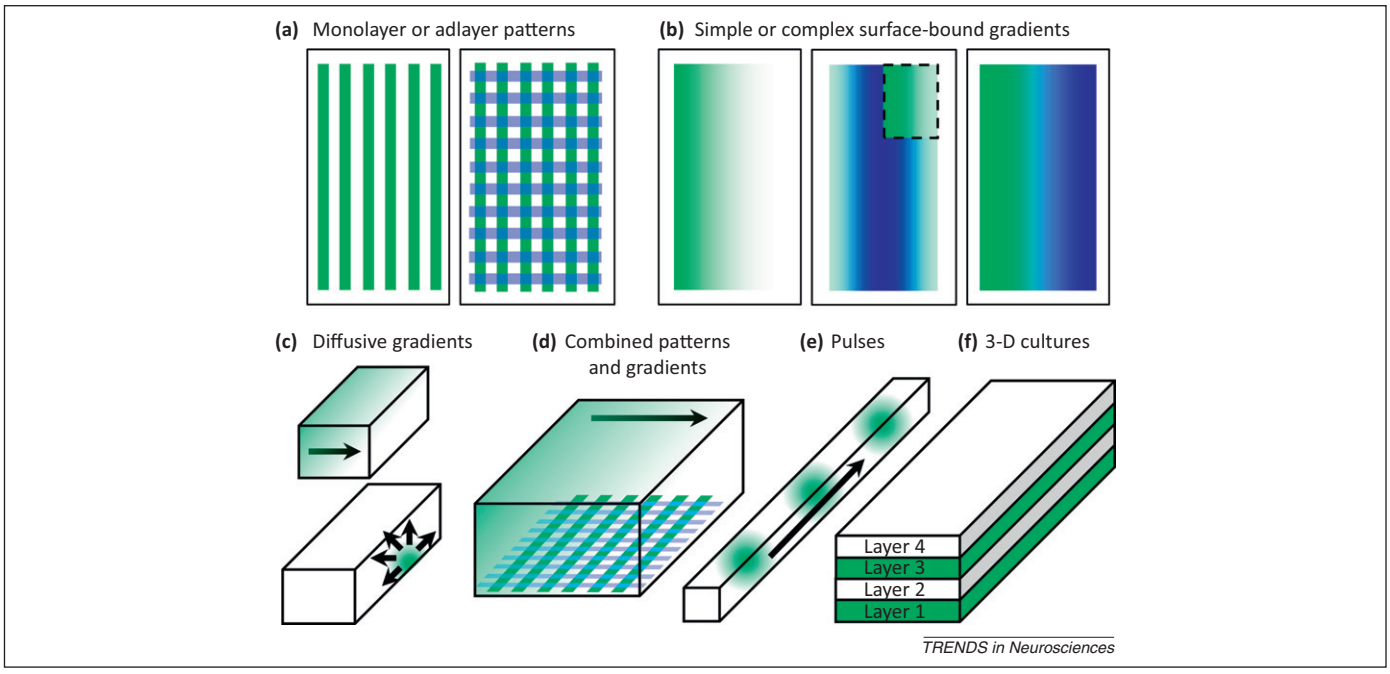

Figure 3. Microfluidic devices (μFDs) allow a high degree of spatial and temporal control of neuronal microenvironments. **(a)** Binary patterns of substrate molecules can be defined with a high degree of spatial resolution to generate monolayer stripes of one or two cues patterned in apposition (left). Adlayers of binary protein patterns can also be formed as culture substrates (e.g., laminin, poly-lysine, neurotrophins) (right). **(b)** Using laminar flow, surface-bound gradients of guidance cues can be patterned onto planar culture substrates. Gradients can be of single (left) or multiple cues aligned onto each other (middle), or the gradients can be laid down with opposing slopes of increasing concentration (right) [43]. The inset shows a cut-away of the top layer of the two-layer pattern. **(c)** Diffusive gradients established via different 3D fluidic channel domains. Upper left: The arrow shows the direction of diffusion from high to low concentration; the source can be from adjacent laminar-flow streams or gels [43,48,82]. Lower right: A focal source of a chemical factor can be introduced via a small inlet channel or jetting stream [83]. **(d)** Fluid-phase and surface-bound cues can be used simultaneously. **(e)** Chemical factors can be introduced into channels with pulsatile temporal control to deliver dynamic patterns of chemical agents to cells and subregions within [49]. **(f)** 3D gel structures can be fabricated via μFDs. Layers can alternately incorporate neurons, then diffusive growth factors, or various combinations for axonal growth towards a lateral or vertical target region [67].

Fluid-phase gradient cues within μFDs can be tuned on the fly through microchannel mixers, jets, or valves to expose cells to transient or stable diffusive gradients (Figure 3c–f). Such approaches are fully compatible with real-time, high-resolution microscopic imaging. Microfluidic jets can be used to create chemical gradients within culture wells for testing the influence of an array of candidate agents on axonal outgrowth, and even the dynamics of vanguard filopodia [47]. Large-scale diffusive, fluid-phase gradients of putative axon guidance factors (e.g., netrin) were generated microfluidically in cultures of cortical neurons from embryonic mice. Although the majority of neurons (~73%) extended axons towards increasing netrin concentrations, axons of the minor population showed degrees of aversive growth trajectories, suggesting heterogeneity in the responding population [47]. This study revealed a distributed response of axons to fluid-phase netrin gradients. How this distribution of axon directionalities in populations in which cells are in contact may differ from isolated neurons remains to be resolved.

A hybrid microfluidic–collagen gel gradient generator was developed to screen neurite responses to growth factor gradients [48]. This high-throughput analysis of putative neuronal guidance cues confirmed netrin-1 (attractive) and slit-2 (repellant) as regulators of axonal guidance, and also revealed that homogenized mouse brain pulp contains significant guidance cues for directing the axonal growth of embryonic hippocampal neurons. Thus, μFDs minimize experimental confounds inherent *in vivo* and, compared with dish cultures, facilitate the analysis of signaling cues that govern axonal growth and guidance.

Dendritic signaling and synapse formation

Dendrites are conduits of information influx and integration from the synapse to the nucleus and back. Interconnected compartments within μFDs (Figures 1d and 4) also facilitate controlled probing of dendrites and subregions. Ultra-thin fluid streams of chemoattractants can be positioned or moved across neurons in μFDs to administer neurotransmitter pulses [49,50], mimicking synaptic release. The ability to control microenvironments of dendrites has allowed studies to address fundamental questions pertaining to mechanisms of synapse formation and regulation [46,49].

Noteworthy examples illustrate the merits of this approach for resolving questions of subcellular regulation of dendritic function. How signals at dendrites change synaptic state is a long-standing question. With the aim of resolving mechanisms of BDNF induction of transcription-mediated synapse strengthening, μFDs were used to compartmentalize and fluidically isolate dendrites from the somata of rat embryonic cortical neurons [51]. Restricted dendritic or somatic exposure to BDNF and pharmacological agents revealed a novel, dendrite-localized neurotrophin signaling pathway not discernible in dish cultures, in which dendrites and axons cannot be isolated. This study discovered that dendrite-to-nucleus induction of

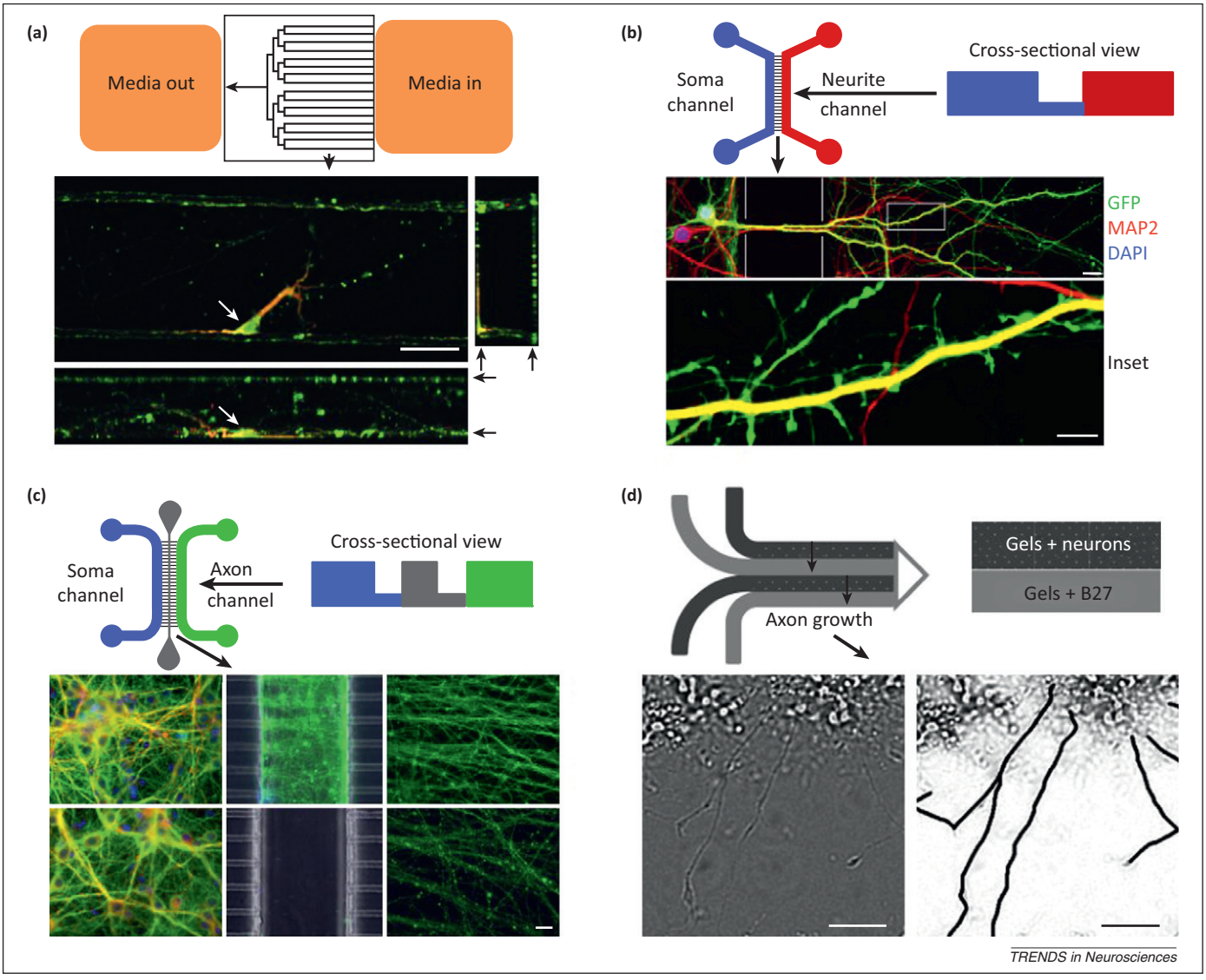

Figure 4. Microfluidic devices (μFDs) offer design flexibility for probing developing neurons. Top-down and some cross-sectional schematics of four μFD types appear above images of the neurons within them. **(a)** Ultra-low densities of primary post-natal rat hippocampal neurons differentiate and extend complex processes in μFDs fabricated with extracted polydimethylsiloxane (E-PDMS). An individual neuron is identified (cell body, white arrow). Dendrites (labeled for microtubule-associated protein 2, MAP2; red) and axons (labeled for tau, green) navigate all surfaces of the microfluidic channels, especially corners, where they contact at least two surfaces. Black arrows (right) mark the top and bottom of the channel for compressed side-view or end-view images. Image orientations: top left, XY, top-down view; bottom, XZ, side view; top right, YZ, end view. 7 DIV, scale bar, 50 μm. Reproduced and modified from [64] by permission of The Royal Society of Chemistry (RSC). **(b)** Regulation of communication via dendrite-to-soma signaling can be studied using a multi-domain μFD. This μFD has two channels that communicate fluidically via a series of parallel interconnecting tunnels. One channel (left, blue) is where the neurons are seeded. As they develop, the neurons extend their processes through the interconnects into the second channel (right, red). The interconnects restrict somal migration while allowing the processes to extend down the interconnects into the neurite channel, where they differentiate into dendrites with spines. Fluorescence microscopy reveals cellular components, including the dendritic cytoskeleton (MAP2, red) of GFP-expressing cortical neurons (GFP diffuses throughout the cytoplasm, green) and the nucleus (DNA marker, 4′,6-diamidino-2-phenylindole; blue). Because dendritic shafts co-localize MAP2 and GFP, they fluoresce yellow. Spines (bulbous, GFP-filled protrusions from the dendritic shaft) develop in the right channel, where processes are fluidically isolated from the soma. This precise subcellular fluidic exposure and cellular manipulation is not achievable in dish cultures. Scale bars, 20 μm (top) and 2 μm (bottom). Modified from [51] and used with permission. **(c)** Spatially controlled axotomy and degeneration of central neurons in a compartmentalized μFD. This μFD has three channels, one in which primary mouse cortical neurons are seeded (left middle and bottom, schematic blue channel), and two in which axons extend (center middle and bottom, schematic gray channel; right middle and bottom, green). Axons extend through interconnects and traverse the central channel (middle, control; bottom, experimental) and a second set of interconnects before emerging in parallel to invade the right channel (middle and bottom). The central channel is the surgical suite (bottom center), where detergent treatment transects axons synchronously and in geometric register. Fluidic isolation protects somata (bottom left) from damage. Axonal channels (schematic green channel) of control cultures contain healthy axons (middle right) versus axons undergoing Wallerian-like degeneration on the distal side of the transection (bottom right). This design provides a model system for studying axonal degeneration and death mechanisms versus regeneration processes with high spatial and temporal control of axonal states and molecular pathways. Neurons are immunostained for β-III-tubulin (green) and F-actin (red). Scale bar, 20 μm. Modified from [54] and used with permission. **(d)** Creation of cortical lamina *in vitro*. Multi-layered μFDs (top left) were used to study alternating layers of embryonic rat cortical neurons (dark grey layer) and trophic factors (i.e., B27, light grey layer), as illustrated in the schematic on the top right. Axons, the longest neurites, traverse from one layer into an adjoining layer (left), as shown in axonal traces (bottom right), generating a laminar structure of cortical neurons. Scale bars, 50 μm. Modified from [67] and used with permission.

c-Fos expression by BDNF is: (i) Ca^{2+}-independent, (ii) not dependent on tropomyosin-receptor-kinase (Trk, a tyrosine kinase) activity in dendrites, (iii) mediated by mitogen-activated protein kinase kinase (MAPKK or MEK1/2), wherein dentritic signal processing differs from MEK5-mediated axonal retrograde signaling, and (iv) dependent on intra-dendritic mRNA translation for immediate early-gene expression [51]. These elements distinguish dendritic pathways from retrograde neurotrophin signaling in axons.

Discrimination of mediators of axodendritic synaptic differentiation and degeneration in neurons of the corticostriatal pathway is challenging *in vivo*. Exogenous cues are difficult to control precisely in conventional dishes because of plume dynamics, diffusion from the source micropipette, and static–fluidic conditions. Use of μFDs that control neurite directionality allows analysis of *en passant* axodendritic connections and corticostriatal synapses. Tapered interconnecting channels funnel cortical axons towards an adjacent chamber containing striatal neurons (Figure 1d,e) [52]. This microfluidic approach circumvents many of the confounds of conventional methods by facilitating identification of specific types of neurons within co-cultured populations and segregation of two different populations [53] while permitting ample *en passant* axodendritic interactions. Furthermore, the environment of the μFD promotes striatal neuron differentiation that results in longer dendrites, greater spine density, increased phosphorylated extracellular signal-regulated kinase (p-ERK) activation, and spontaneous Ca^{2+} oscillations synchronous with cortical neuron activity.

Analysis of neuronal stimulus–response characteristics

μFDs allow precise controlled delivery of neuromodulators, imaging reagents, or inhibitors to cultured neurons compared with conventional approaches. They permit focal stimulation of dendrites, visualization and manipulation of synapses and single-molecule axonal transport, monitoring of real-time responses to specific axon guidance cues, analysis of molecular changes, and investigation of traumatic and neurodegenerative conditions [4,47,49,54–56]. Large-order arrays of neurons can be probed to determine the proportion of the population responsive to specific neuromodulators. This design facilitated molecular profiling and resolved the responses of over 2900 olfactory sensory neurons to four different odorant molecules [57]. Absolute spatial and fluidic control permitted discrimination of 15 classes of possible combinatorial responses. This type of population analysis of stimulus–response characteristics should be applied to developing neuronal networks, for which it should facilitate an understanding of components and circuits.

Neurochemical identification of substances released by neurons, including discrimination of those released locally at specific substructures, can be accomplished by coupling sampling via μFDs with analytical chemistry [58]. With the ability to perform spatially targeted sampling of low-density primary neurons [59], media containing local cellular secretions can be collected and processed by mass spectrometry [60]. Competitive signals and confounding variables from surrounding tissues and cells are diminished. Thus, neurochemical analysis of secreted molecules in volume- and analyte-limited samples can be achieved. Due to these advantages, an increasing number of studies are utilizing μFDs as tools for analyses of neuropeptides, mRNA, and subcellular pH [60–63].

Advancing developmental neurobiology at cell population and tissue levels

To more fully understand the signals that shape the forming brain, cultures will need to incorporate multiple cell types in 3D. The central nervous system comprises heterogeneous populations of neuronal, glial, microglial, and endothelial cells that mutually interact. Recapitulation of the microenvironment of the brain in the simplest form should yield topographical neural structures. This requires detailed characterization of critical environmental cues. To construct organized 3D cultures, cell-compatible features must be incorporated into the material properties, fabrication design, and imaging processes. For example, in microchannels of the highly biocompatible silicone gel, solvent-extracted polydimethylsiloxane (PDMS), rat hippocampal neurons prefer extracted PDMS over glass substrates, and neurites show a strong affinity for topographic cues where walls meet at right angles (Figure 4a) [64]. Furthermore, when presented with competitive substrate cues, hippocampal neurons unexpectedly prefer physical contact guidance cues over chemical ligands [65,66]. Thus, cellular tension versus chemical substrate cues must access different, competitive signaling pathways.

Microfluidic channels can be combined with layered hydrogel scaffolds to more closely approximate the physical 3D structure of the developing brain (Figure 4d). An agarose scaffold can be fabricated with four hydrogel layers on the scale of layers within the cerebral cortex. Layers with embedded neurons intercalate adjacent layers that encapsulate growth factors (serum, B27 supplement, and nerve growth factor, NGF), which are released diffusively and guide developing neurons [46,67]. As a result, embedded neurons, axonal growth, and synapse formation within the 3D matrices self-organize into laminar brain tissue, which is useful for studying corticogenesis *in vitro*.

Large, interconnecting populations of dissociated cells (e.g., neurons, glia, microglia) [3,68] and organotypic tissue slices can be achieved through multi-channel μFDs. Organotypic slices from different brain regions with different

Box 2. Potential factors limiting neuronal development in microfluidic devices

Whereas μFDs generally are considered highly biocompatible, some reports suggest adverse effects of materials or the environment on cell growth or viability [64,74]. Influences that could potentially limit or bias viability, development, or function, and thus need controls, are as follows:

- **(i) Shear stress:** Fluid flowing through microchannels can present cells with a range of shear forces due to flow velocities and channel architectures, stresses that could bias neuronal development and function [91,92].
- **(ii) Gas solubility, permeability, and diffusibility:** Defined cell-culture media formulations were developed with levels of antioxidants and buffers for specific concentrations of gases, such as O_2 and CO_2. Materials, channel dimensions, and flow regimes could potentially alter gas concentrations available to cells and chemical properties of the culture media [77].
- **(iii) Absorption:** Porous polymers have the ability to absorb chemicals from the fluid, and thus alter fluidic composition within the μFD or its channels [74,75].
- **(iv) Adsorption:** Fluids or media components may adhere differentially to the surface(s) of the μFD, and thus may become depleted [76].
- **(v) Desorption:** Chemicals or elements of the material may have the capacity to dissolve in the media and bind to and/or accumulate within cells, and thereby influence cell function [74].
- **(vi) Evaporation:** Water can evaporate through porous materials, increasing media osmolarity [93].

culture requirements can be grown so that they interconnect through microchannels, allowing synchronized neural activity between them [69]. These complex tissue-level constructs can be used to precisely manipulate neural circuits to advance our understanding of the development of neural plasticity, circadian-clock coupling, and neuropathologies.

The application of μFDs to the study of developmental interactions of neurons, glia, and cells of interacting brain regions is in its infancy. This approach has the power to interrogate cell-to-cell contact and communication, while achieving differential fluidic exposure, sample manipulation, and collection [26–70]. For example, Alzheimer's disease-like pathological states occur in only a subset of neuronal networks grown within μFDs [5]; similar approaches could be applied to probe developing neuronal networks. Thus, the flexibility of μFD design makes it adaptable for dispersed neurons in compartmentalized microdomains and 3D tissue constructs for addressing a multitude of developmental questions.

Technical considerations

Increased experimental complexity and microenvironmental manipulation in μFDs require stringent evaluation and controls. Greater scrutiny is required by both reviewers and readers in assessing the implications of μFD-based studies. Diligence and care are necessary to prove that observations and conclusions are free of unintended variables or experimental confounds. Even culture media can bias experimental results, demonstrating influences of common reagents on experimental outcomes. Defined formulations for culture media are widely accepted for sustaining primary neurons without the need for complex additives, such as fetal serum. However, defined media formulations are under renewed scrutiny for the influences that albumin, selenium, and antioxidants impose on developing neurons [71–73].

μFD materials themselves may contribute to media biases through absorption, adsorption, and leaching (Box 2) [74–76]. As a result, materials used to fabricate μFDs and the physical influences they exert may affect the biological processes of the cells within them. Because μFDs can be fabricated to a wide range of dimensions and architectures with different materials, potential confounds may depend on process, scale, and material [77]. These subtle variables may explain why some laboratories experience confounds whereas others do not. For example, some researchers have found that mild washing of PDMS with alcohol is less effective at removing unpolymerized oligomers [74], whereas rigorous solvent extraction of PDMS is effective at removing oligomers and improving neuronal viability at low cell densities [64].

Innovative design improvements undoubtedly will minimize or eliminate undesirable effects of the μFD environment. Because fluid flow can influence neuronal growth and function, methods have been developed to minimize neuron exposure to the shear stresses of fluidic perfusion while providing the desired instructive gradient guidance cues for axons [47]. Specific requirements of different biological models may also explain differences in outcomes between groups using μFDs. For example, locust neurons in μFDs require different modifications to culture conditions than rat neurons do [78]. Whereas species-based differences are not surprising, significant culture-induced differences have been found. Conditions have been developed that enhance mRNA profiles of cultured astrocytes from 10% to nearly 80% of mRNAs expressed *in vivo* [79]. This remarkable advance in refining glial cell-culture conditions predicts that we may yet discover different, more optimal, requirements for specific neuronal types or brain regions.

Exposing fragile and sensitive neurons to new materials or physical conditions requires careful characterization and validation of the material and device for experimental compatibility before adopting new techniques with confidence. Physical, chemical, and flow properties of μFDs may influence experimental design and applications to developmental neurobiology (Box 1). Adhering to these principles will drive studies to improve fabrication processes and the material composition, and enable μFDs to further advance developmental neuroscience.

Concluding remarks

The emergence of μFDs that allow the manipulation of microenvironments surrounding neuronal substructures endows neuroscientists with a new tool set that elevates the sophistication of neuroscience *in vitro* to a fundamentally new level of refinement. No single method or device can yet fully recreate the natural environment of the brain to allow studies of neurons under the range of contexts and dynamics in which they develop and function. Nevertheless, remarkable insights have been gained from these reduced neuronal preparations. From decoding neuronal polarity to probing axonal guidance and dendritic function, μFDs are advancing neuroscience on multiple fronts. Thus, as μFDs are refined to address unanswered questions (Box 3), we anticipate that they will reveal new insights into how emergent developmental processes shape the nervous system.

Manipulation of the microenvironment of developing neurons and monitoring of changes in cell structure and function have never been achievable at the resolution provided by μFDs. As for other new technologies, exploitation of the full potential of μFDs requires care, skill, insight, and creativity. The unparalleled ability to control

Box 3. Outstanding questions

Outstanding questions that can be addressed using μFD technologies include the following:

- How are the self-organizing events of early neuronal morphogenesis coordinated through extrinsic cues?
- What mechanisms and extracellular factors govern asymmetric modulation of selective protein translation and protein degradation during neuronal polarization?
- How does establishment of neuronal polarity regulate dendrite development?
- What molecular mechanisms regulate intracellular signaling and trafficking between dendrites and the nucleus to modulate gene expression and then alter dendritic functions selectively?
- What cellular processes regulate dendritic growth and branching?
- How do nascent dendritic filopodia form synapses?
- How is synaptogenesis between different types of neurons orchestrated?

local chemical, physical, and fluidic environments while accessing and probing microdomains of developing neurons offers extraordinary opportunities.

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