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MICROFLUIDICS FOR NANONEUROSCIENCE

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1.1 INTRODUCTION

The nervous system of an organism is like the information technology department of an organization. Each of the billions of building blocks of the nervous system, called neurons, is a multistate device similar to the transistors of a microprocessor. But while transistors are binary state devices, neurons are capable of being in many thousands of states, and this adds many orders of magnitude to the complexity of possible connections within a nervous system. In addition, each neuron has multiple connections with other neurons, and some of these connections are bundled into tracts and nerves that travel within brain and spinal cord, and out to peripheral locations. In computers, disconnection of one network cable, or disabling of the electronic circuits in the server, can seriously compromise the function of the organization. Similarly, traumatic injuries or neurodegenerative processes such as multiple sclerosis, Alzheimer's disease, or Parkinson's disease can significantly impair the functionality of an individual by damaging the neurons, tracts, and nerves. However, unlike computer systems, medical repair processes do not yet exist because we do not yet understand how the system operates in the healthy state. This may change in the near future as cell biologists pursue stem cell interventions to regenerate or remodel

damaged areas of the nervous system. Simultaneously, engineers are teaming up with biologists to design electronic implants and prostheses that can interface with functioning tissue on either side of a damaged connection and act as a bridge to allow restoration of injured neuronal circuits. Pharmaceutical researchers are using nanotechnologies to create novel systems capable of delivering targeted drugs and other agents across the previously impenetrable blood–brain barrier,[1,2] a feature of nervous systems that chemically separates the system from the rest of the organism.

All these advances may be accelerated by knowledge derived from studies of cellular physiology using tools designed to study biological processes at the single cell level. As our ability to fabricate tools on the micro- and nanoscale levels has progressed, we can now study cellular processes at a scale compatible with cell size, and this is revealing new information about their operational responses, including how they respond to physical and chemical cues from their immediate environment. It is important that neuroscience researchers be aware of these new technologies, so that their use can be optimized.

Recent advances in biological applications of micro- or nanotechnology have included novel micro- or nanoscaled carriers for drug delivery,[3–6] quantum dots that operate as nanoscaled sensors at the cellular level,[7–11] and nanoelectrodes.[12] In addition, self-assembled monolayers and scaffolding, as well as carbon nanotubes, have been used as artificial nanotechnology matrices for cell culture.[13–19] In neuroscience specifically, nanoparticles have been used for free radical scavenging in ischemic and neurodegenerative diseases.[20] Scaffolds made of self-assembling nanofibers are being developed to enhance neuroregeneration.[21] The blood–brain barrier has been successfully breached by drugs attached to special nanoparticles.[22] High-resolution studies of the topography and material properties of live nervous system cells are being carried out by atomic force microscopy (AFM) (Figure 1.1).[23,24] Single-molecule tracking using quantum dots has revealed details about the structure and function of membrane receptors.[10,25,26] Finally, nanotubes, nanowires, and nanoneedles are being developed for use as relatively nontraumatic intracellular electrodes.[12,27,28] On a slightly larger scale, microfabrication technology has been used to create microfluidic platforms that have been employed for a variety of nanoneuroscience studies, and these platforms will now be discussed.

Microfluidics refers to a technology that utilizes microscale channels to manipulate fluid and suspended objects in a controlled manner at the nanoliter scale. Most microfluidic chips are designed and constructed using the same techniques as used in the development of microelectronic circuitry. Microfluidics has been advancing rapidly over the past decade and has progressed from basic devices, for example, a channel,[29] a valve,[30] and a pump,[30] to large-scale two-dimensional integration of components,[31] three-dimensional architectures,[32] and nonlinear autoregulatory systems.[32] Simultaneously, the development of the fundamental technology has enabled the advent of a plethora of specialized devices that have miniaturized important macroscale applications such as protein crystallization,[33,34] DNA sequencing,[35] and PCR (polymerase chain reaction), a technique for DNA detection and amplification.[36,37] The same development has also enabled the advent of novel techniques to conduct fundamental research in a scale that was never previously possible.
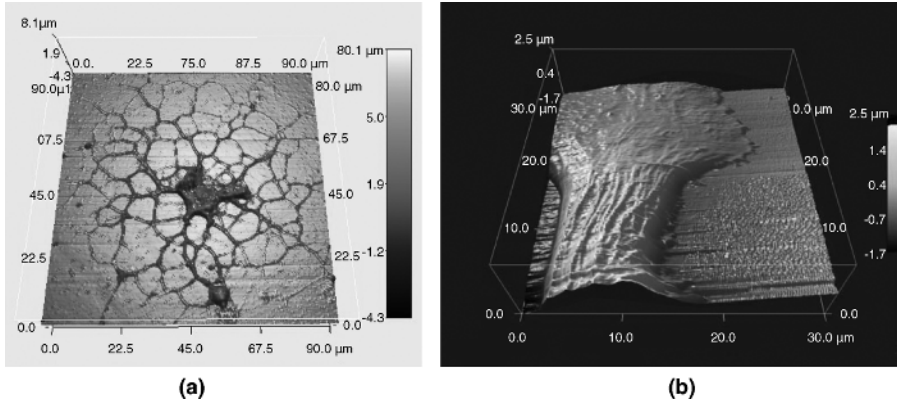
FIGURE 1.1 Atomic force microscopy images of neural lineage cells. (a) Three-dimensional rendering of an oligodendrocyte differentiated from a murine neural stem cell. Fixed sequentially with 100% ethanol and 4% PFA, air dried, and then imaged on an Asylum Research MFP 3D AFM using an Olympus AC160 cantilever in AC mode in air. Note the detailed process formation. Scan size is $90\,\mu m \times 90\,\mu m$. (b) Three-dimensional rendering of a portion of a living astrocyte derived from a human embryonic stem cell on a polyornithine/laminin-coated substrate, imaged in media, in AC mode with an Olympus Biolever, and on an Asylum Research MFP 3D AFM. The image shows cytoskeletal fibrous elements visible through the cell membrane in the proximal thicker area of the cell as they enter a broad, flat attachment area. Scan size is $30\,\mu m \times 30\,\mu m$ (unpublished data, Pamela G. Gross).

More recently, some microfluidic chips incorporate other microtechnology and nanotechnology hardware, such as electrodes,[38–44] magnetic coils,[45,46] and surface-emitting lasers,[47] to enhance their capabilities beyond fluid handling.

Many of the first applications of microfluidic chips involved studying the physics of fluid dynamics at the microscale (characterized by low Reynolds numbers, laminar flow, and fast diffusion), which is quite different from the flow characteristics of bulk fluid at the macroscale (characterized by higher Reynolds numbers, turbulence, and slow diffusion). The unusual behavior of fluid traversing microchannels has allowed creation of new methodologies to manipulate molecules, in order to synthesize novel nanomaterials and chemical/pharmaceutical moieties, and this has been described in other chapters. For biologists, microfluidic platforms have emerged as invaluable tools to study biology at small scales, even down to the single cell level. For neuroscientists, these "lab-on-a-chip" platforms have enabled a novel approach for experiments on the cellular physiology of the nervous system. Their usefulness in deciphering the complicated interactions involved in the differentiation, growth, and maintenance of neurons in health and in disease has become increasingly apparent within the past 5 years, as more research in this field continues to be reported. As more neuroscientists become familiar with this technology, we anticipate a rapid evolution of the field. This chapter will review pertinent contributions in the use of microfluidics to study the physiology and pathophysiology of neurons and their support cells and will hopefully serve as a primer for neuroscientists unfamiliar with this technology, inspiring some to develop new applications of microfluidics to the field of neuroscience.

Microfluidic platforms typically contain a series of chambers and channels that each measure in the range of 1 μm to a few hundred microns and are used to process fluid at a microscopic scale. For *in vivo* applications, microfluidic technology has been integrated with neural implants for precise delivery of solutions.[48] Three-dimensional electrodes with bundled microfluidic channels that can be implanted into severed nerves to guide and monitor their regeneration while allowing infusion of drugs are also under development.[49] However, the most common biological application of microfluidics has been for *in vitro* studies, such as the delivery and processing of biochemical reactants for DNA sequencing[35] and protein analysis,[50] the sorting, counting, and analysis of cells by flow cytometry,[51] the delivery of cell adhesives and cells for substrate micropatterning of cell populations,[52,53] the development of biomimetic three-dimensional tissues, complete with stromal support molecules,[18,19] and the isolation and nurtured maintenance of individual cells to study basic cell physiology and cell–cell interactions on a single (or near-single) cell basis.[54–59] In addition, microfluidic platforms have been used to study the effect of laminar flow and shear forces on the function of endothelial and other types of cells,[60,61] to provide artificial circulation through various organ-simulating cell culture chambers in order to determine the pharmacokinetics of prospective pharmaceutical agents,[62] and to deliver test samples containing potential toxins to cells acting as biosensors (also known as "lab-in-a-cell" technology).[63–65] Finally, microfluidics can be used to study physiology within small organisms, such as the effects of anesthetics on the regrowth of severed axons, or the recovery of axonal synapses after laser ablation in *Caenorhabditis elegans* nematodes that have been captured and immobilized in microfluidic chips.[66,67]

Microfluidic-based cell studies are a useful adjunct to conventional *in vitro* techniques or mini culture systems[68] because microfluidic chambers have the ability to control both the amount of material (media, growth factors, etc.) used for cell study and their exact distribution over well-defined periods. This can permit better control of the experiment by limiting unanticipated extraneous factors and diffusion constraints that can occur in larger systems. The effects of cell population variability will also be more limited in smaller systems and therefore individual differences among similar cells will be less likely to influence results. From an economic standpoint, small culture volumes allow cost savings since the required volume of expensive media, hormones, and growth factors is orders of magnitude less than that used in typical culture flasks. These platforms can also be designed for high throughput and compatibility with automated laboratory equipment such as plate readers. In addition, the hardware is portable and it can be mass produced so inexpensively that it can be very cost-effective to perform massively parallel microfluidic platform-based experiments, in order to confirm results or test the effects of numerous agents simultaneously.

These parallel experiments are necessary to verify results obtained on individual cells since it is known that there can be significant variation in the behavior of particular cells, even if they are cloned from the same precursor cell.[69] Similarly, it will be imperative that the effect of microenvironment parameters such as mechanical forces, shear stress, effective culture volume, and material interfaces be well

understood and controlled before interpreting single cell study results so that these factors do not contribute to misleading conclusions.[70] Nevertheless, observations derived from studies of individual cells in a controlled microenvironment may be much more likely to reveal true cellular physiology responses than those derived from studying the responses of populations of cells simultaneously, as is done with conventional *in vitro* studies.

Although the development of this technology has progressed significantly over the past 5–7 years, its utility as a tool is just beginning to be appreciated by biologists. There are many published reviews on the general topic of microfluidics for biological applications,[69–85] but there have only been a few that have focused on microfluidic applications in neuroscience.[80–82] This chapter will update the reader on the discipline of microfluidics to study the nervous system at the single cell level. Specifically, it will report on microfluidic chips used to decipher physiological processes and responses of cells of neural origin, and it will also focus on examples of systems that combine microfluidic chambers with other technologies for novel research not previously possible.

Section 1.2 will begin with a description of current microfluidic chamber construction techniques, starting with a discussion of the characteristics of the PDMS polymer used in many microfluidic chambers and then moving on to cover step-by-step fabrication processes. Various architectural designs of use for cellular studies will then be introduced, followed by a description of alternative applications of PDMS to create tools that are useful in customizing the substrate of microfluidic chips for specific experiments. Practical limitations of microfluidic techniques will then be discussed to present a balanced view of the topic.

In Section 1.3, gradient-generating designs will be reviewed, along with examples of how they have been used to study cellular responses. Methods of incorporating electrophysiological measurements into chip design, including patch clamping, will be examined and then use of other integrated micro- and nanoscaled analytical devices will be considered. The theory and methodology used for *in vivo* tissue simulation will be evaluated, since the natural behavior of cells is ultimately what most biological research is attempting to discern.

Following this, a literature review of neuroscience research involving microfluidic platforms will be detailed in Section 1.4, starting with cell identification and separation tools, which is essential for researchers requiring specific subpopulations of neural lineage cells. Studies on microfluidic analysis of neuropeptide release will follow, which is of interest to individuals studying synapse formation and function. The use of microchips to study the effects of physical and chemical guidance cues on single cells will then be considered since this is a key to understanding how neural cells interact with their environment and with each other.

Section 1.5 will focus on electrophysiology studies that use multielectrode arrays (MEAs) as microfluidic chamber substrates. This is a popular field of endeavor since these two technologies seem to be complementary and can allow studies on action potential characteristics and propagation in single axons. The effect of growth factors on neuronal responses of microfluidically cultured and isolated cells will be covered after this, given its significance in understanding cell differentiation and maturation.

The use of microfluidic chambers for gene therapy studies on neural cells will be subsequently discussed. Although this is a relatively new area of study, preliminary results are very promising and future research will likely take advantage of the unique capabilities that microfluidic chips offer to this field. The final area of research to be covered involves studies based on the microfluidic isolation of axons and neural cell bodies. This approach to neural research is gaining great interest, given the potential applications for those studying neural degeneration and regeneration processes, in addition to those interested in axonal transport mechanisms, and synapse formation and physiology. A general discussion with consideration of future perspectives will complete the chapter. It is hoped that the reader will gain an appreciation for the future potential of these platforms to uncover previously hidden cell-based interactions in the nervous system, and this will stimulate new applications of microfluidics for their specific research programs.

1.2   PDMS MICROFLUIDIC DESIGN AND FABRICATION

1.2.1   Characteristics of PDMS

Initially, most microfluidic chambers were constructed on silicon wafers using "hard" lithography. Since those early studies, "soft" lithography has been developed and various polymers and fabrication techniques have been investigated.[86] Now, soft-sided chambers made of polydimethylsiloxane (PDMS) are gaining increased popularity, especially for biological applications. PDMS is a silicon-type elastomer and can be purchased commercially as Sylgard® 184 by Dow Corning or RTV by General Electric. It can be molded into many different shapes to form valves, chambers, and channels. PDMS is advantageous for biological studies since it is biocompatible, optically transparent down to wavelengths as low as 280 nm, permeable to gases needed for cellular respiration, autoclavable, and naturally inhibitory to cellular adhesion.[87,88] PDMS has therefore proven very handy for cellular studies by allowing long-term cultures, optical microscopy, and fluorescent/chemiluminescent studies, while the cells are still *in situ* in the chip.[64] A final advantage of this material for use with cell culture systems is that PDMS has been shown to be an excellent protective coating for on-chip solid-state analytical devices (such as surface-emitting lasers), since PDMS is optically transparent yet prevents the detrimental effects of ions migrating from the culture medium into sensitive electrical junctions.[47]

Native PDMS is hydrophobic, and this influences many of its surface properties, including its interactions with fluid and molecules that are in contact with it. These properties can be altered by physical and chemical treatments that can change the hydrophobicity of the surface of the PDMS channels and change its adhesive properties if this is desired.[74] For example, the pretreatment of the PDMS channels with bovine serum albumin (BSA) will assist in blocking cell adhesion to its surface.[54] Alternatively, PDMS can be made hydrophilic and supportive of cell growth by treatment with oxygen plasma,[88] or UV/ozone,[89] that acts by changing the moieties on the PDMS surface to increase the number of silanol groups and decrease the number

of siloxane groups. Polyethylene glycol (PEG) can also be used to alter the surface chemistry of PDMS.[35]

The surface interactions of PDMS with adjacent molecules will also depend on local flow conditions. Experimentally, proteins such as collagen and fibrinogen adhere to both hydrophobic and hydrophilic (oxygen plasma-treated) PDMS. But under flow conditions, the oxygen plasma-treated hydrophilic surfaces experienced only temporary adhesion, followed by rapid detachment of any adherent cells, whereas the hydrophobic PDMS channels became permanently clogged with protein and cells.[88] Therefore, systems that are designed to have continuous exposure to protein-laden media and cells will likely benefit from pretreatment of the PDMS with oxygen plasma to increase the functional lifetime of the channels.

1.2.2 PDMS Chip Fabrication Protocol

Microfluidic chip fabrication uses many of the same techniques used in electronic circuit production. The process begins with the creation of an architectural design using a computer-aided design (CAD) software program. The design is printed on a transparency using a high-resolution printer, since the feature size on the final chip will be determined by the resolution of features on the transparency. This transparency acts as a photomask during the next step, in which it is placed over a substrate (silicon wafer or glass) that is precoated with a thin layer of photoresist, a photocurable epoxy. UV exposure polymerizes exposed areas for photoresists such as SU-8, so the developer solution can strip away the unexposed areas because they are not polymerized, while the polymerized exposed structures remain. This type of photoresist is called negative photoresist because the result is the reverse, or "negative," of the mask. On the other hand, photoresists such as 5740, SPR-220, and the AZ family are called "positive photoresists" because the result corresponds to the mask; that is, the result is "positive" to the mask. UV exposure makes a chemical change in positive photoresists that results in the material becoming more soluble, for example, in a strong base. Thus, the developer solution removes the material from the exposed areas, while the structures in the unexposed areas remain. In both cases, the result is a mold where the features are built in photoresist. Since photoresist is softer than silicon, the resulting mold is softer than traditional molds, and so the technique has been named "soft lithography."

In the next step, PDMS is combined with its catalyst in a 10:1 proportion and the mixture is degassed in a vacuum chamber to remove bubbles. It is then poured onto the master, allowed to cure, and then peeled off the mold. Access ports are punched after casting (or silicon tube ports are placed during casting) to create connections to input and drainage tubes. The PDMS slab is then placed onto a substrate such as a silicon wafer or a glass slide to create the final microfluidic platform. The PDMS forms a reversible conformal seal to the substrate, but optional treatment of the PDMS with plasma oxidation of the PDMS surface after curing will render the surface more hydrophilic and allow the PDMS to irreversibly bond to the substrate. After sterilization by means of autoclaving, UV treatment, or immersion in 70% ethanol, the system is ready for use.

Although PDMS can reproduce features down to 10 nm in size,[75] actual fabricated channels in PDMS have not yet achieved a cross-sectional area smaller than 1 μm^2.[74] This is because the feature size of the PDMS is determined by the printed resolution on the photomask, which is determined by the printer used to create it. For example, standard printers that have a resolution of 5080 dpi can reproduce features on the photomask down to 25 μm resolution, whereas photoplotters that print at 20,000 dpi can achieve a resolution down to 8 μm.[75,90] To reproduce smaller features, chrome masks may be used, which are created with e-beam or laser writing and are much more costly. In addition, the relative softness of PDMS makes it difficult to maintain uniformly high quality of the reproduced features when the linear scale is decreased below a few microns.

1.2.3   Architectural Designs of Microfluidic Platforms

The physical behavior of a fluid flowing through microscale channels is very different from the flow characteristics of the same fluid flowing through larger channels. For example, fluid flow through microfluidic channels is laminar, so mixing does not occur between solutes placed at different locations in the channel cross section, except by the slow process of diffusion. Without turbulence, solute gradients will remain relatively intact as fluid traverses down channels of uniform width. If cells are localized at certain areas of this channel, their exposure to specific concentrations of solute can be tightly controlled. In fact, different parts of the cell can even be exposed to different and controlled concentrations of the solute. This laminar flow behavior can also be used to pattern and deposit specific solute concentrations onto the substrate or to pattern cell adhesives and repellents next to each other onto the substrate prior to introduction of cells. Alternatively, if mixed patterns are desired, deliberate oblique grooving of the floor of the channel can be employed to create turbulence in order to mix solutes,[91–93] and nanotopographic features can also be added to the platform substrate to influence cell adhesion.[94]

Most microfluidic chips use some type of dynamic flow conditions, with flow achieved by the use of syringe pumps, gravity-driven reservoirs,[95] electrokinetic control,[96] or other more complicated functional PDMS valve structures. These valves are designed by layering "control channels" that act as bladders across flow channels. Application of pneumatic pressure in the "control channels" can then cause controlled collapse of the underlying fluidic lumen, and this controlled deformation of the flow channel's lumen creates a functional valve.[30] Digital control and sequential coordination of these valves can create peristaltic pumps. Rotary pumps based on similar mechanisms have been designed and used for applications that require repeated cycling of fluid for mixing, such as on-chip PCR, used in amplification and identification of DNA strands in genetic engineering.[97]

Various structures have been devised to immobilize cells within microfluidic chips. These architectures must be able to catch and retain a cell from a passing stream of media, while minimizing damage to the cell. Sieves have been used within the culture chamber to retain cells while also producing a nutrient gradient.[98] Channel walls can be constructed at partial height to create a dam that allows flow from one channel to

another while gently transporting and immobilizing cells for later analysis.[99] Inverted T junctions that have small docks with tiny drain channels at the junction have been used to immobilize single cells and then perform rapid on-chip calcium flux assays.[54] Curved docking areas that can balance the forces exerted on cells (fluid flow versus gravitation) have also been used to isolate individual cells for culture and study of calcium mobilization.[56,57] Gravity-induced flow has been combined with dielectrophoresis to trap and sort cells without physically contacting them.[100] For the capture of cells with variable dimensions such as pancreatic islets (used in diabetes research), designs have combined one semiellipsoidal wall and one movable wall to create an adjustable holding area that will allow studies on the regional effect of infused glucose and drugs.[59] Studies on pairs of cells have used intersecting channels that have been designed to trap pairs of cells from different populations to study intercellular communication via gap junctions between their cell membranes.[55] Finally, the surface of PDMS has been microstructured with arrays of wells and coupled to a microfluidic system to create a test platform for parallel experiments on single cells or small groups of cells.[101] As described above, a significant advantage of microfluidic chambers is that the architectural design and the dimensions of the channels and chambers can be customized for the morphology of the cells to be studied and to the task to be accomplished. As new researchers enter this field, we expect to see a wealth of new designs for novel applications.

1.2.4 PDMS Tools

In many cases, it is desirable to have a microfluidic chip substrate that is patterned with different molecules prior to assembly of the chip. This can be easily achieved by creating a separate PDMS tool that contains the substrate pattern and can be used as a stencil or a stamp. This tool is fabricated using the same techniques as outlined above. After completion of the tool, it can be used for microcontact printing by dipping the patterned area into a fluid with the desired concentration of solute molecules and then transferring this pattern to the substrate. PDMS can also be formed into a two-dimensional stencil sheet that allows patterned deposition of selected proteins or agents onto the underlying substrate, and this PDMS stencil has the advantage of being useful on irregular or curved substrate surfaces. Once the protein pattern has been created on the substrate, the remaining platform can be constructed by applying the matched PDMS chip so that its channels are complementary to it. With this arrangement, future cell attachment and differentiation can be guided, and cocultures can be created in controlled geometric patterns.[102–104] Since this technique can help control the exact position of neurons on a substrate, the resultant controlled neuronal patterns can be very helpful in studying neural networks and interactions occurring within synapses.[69,102,103,105] These techniques have also been combined with selective oxygen plasma treatment to create long-term and short-term cell repellent areas to coculture cells in controlled geometric patterns.[104] In this case, cell repellent polymers were homogeneously deposited on a substrate, and a PDMS stencil was used to selectively protect the repellent from plasma treatment in certain areas. Unprotected areas lost their repellent nature and could then be treated with adhesives like

fibronectin and short-term repellents like BSA to create patterns of relative adhesivity over time that could then be seeded as desired with different cell types.

1.2.5 Practical Considerations and Limitations

As with all new technologies, there are certain practical design considerations and limitations that must be recognized before planning a microfluidic chamber for cell studies. Cell viability has definitely been correlated with channel size and proportions, closed versus open-channel configurations, and static versus dynamic media flow. For example, it has been shown that in contrast to cells grown in conventional tissue culture flasks, the proliferation rates of cells grown in microfluidic channels without media flow depend on the height of the channel.[106] This is likely due to loss of convective movement of cell-expressed inhibitory factors away from cells, rather than lack of nutrients or change in osmolarity or pH of the culture medium. In a static system where there is no flow of media or connection of the media in the channels to a bulk container, secreted factors can only be dissipated by diffusion, and this can be insufficient to remove their often deleterious effects.[106] If continuous or intermittent flow is designed into the system, the flow rate must be optimized to provide nutrients and remove wastes, without producing excessive shear stress that can change morphology or migration of the cells or even detach the cells.[98,107] Similarly, certain secreted factors may be essential for cellular health, and if the flow is too high, then these factors may be washed out.

Pretreatment of the PDMS prior to the introduction of cells can have significant effects on cell culture success. For example, Matsubara et al. showed that different treatments to make the PDMS hydrophilic affected both the morphology and the density of mast cells.[64] Similarly, Prokop et al. found that extracellular matrix deposition and plasma treatment of the PDMS improved subsequent cell cultures.[98]

Other important considerations include recognition of the fact that the tiny volume of microfluidic culture systems confers a much less stable homeostatic system in terms of temperature, carbon dioxide concentration, and humidity control compared to standard Petri or tissue culture flasks. These chips equilibrate much more rapidly with their environment than larger systems given their larger surface area to volume ratio, so each time these chips are removed from the incubator, they are prone to more rapid alteration of their temperature, atmosphere, and humidity. Temperature alone is known to directly influence gene expression, biochemical reactions, and diffusion speed. To maintain a stable system, steps must be taken to minimize losses of environmental stability. Similarly, if media is fed into these chips via tubing that is outside the incubator (i.e., if connected to a syringe pump), or if the tubing is part of a "mini" culture system, it is important that media temperature and CO_2 content do not change during transport through the tubing.[68] Therefore, although these micro-scale systems are technically portable, control systems for their ambient environment may be necessary if they require transport outside the incubator for time lapse imaging or other interventions. We witnessed this effect directly when an isolated axon in a microfluidic chip was observed to shrink back significantly within a few minutes of removal from the incubator and placement onto the cold microscope stage

(unpublished data). Control of humidity is also critical because water diffuses into PDMS according to Fick's law of diffusion.[108] From there, it can evaporate and lead to increased osmolarity of the cell culture medium and premature cell death. Methods to minimize evaporation, such as coating the PDMS with a thin layer of parylene, have been successfully implemented and shown to prolong cell viability.[108]

The last practical consideration in using PDMS as a culture chamber in microfluidic systems involves the high ratio of chamber surface area to the volume of culture medium and number of cells compared to standard culture flasks. This increased surface area to volume ratio can lead to increased interactions between chamber contents (media, cells) and chamber walls. For example, it is known that small hydrophobic molecules may partition into the PDMS and therefore be less bioavailable when studying their effects on cells.[109] This extraction of media solutes by the PDMS can significantly change the concentration of some agents in media within microfluidic channels by many orders of magnitude. The magnitude of this change depends on the partition coefficient of the substance, the pH of the culture medium, and the counterion pairing in the media. Major decreases in the media concentration of neurotransmitters, hormones, and growth factors could change experimental outcomes, and the cost savings for using small volumes of these agents in microfluidic platforms can be lost if much larger quantity of the substance has to used to achieve the same effect. Therefore, many individuals are now experimenting with different surface treatments to decrease the porosity of the PDMS and avoid some of these effects.

In addition to taking up biomolecules, the PDMS may also release potentially toxic agents from its polymer matrix.[110] These can then be concentrated in a proportionally smaller volume of culture medium and may affect more sensitive cells. Certainly, neurons from different sources vary in their hardiness, and culturing sensitive neurons at low density and in serum-free conditions can be difficult, even in the most tightly controlled environments. For example, we have personally had difficulty maintaining the viability of human neural stem cells in PDMS chips, whereas rat dorsal root ganglion cells thrived in the same conditions (unpublished data).

One possible explanation of this phenomenon may be found in the work of Millet et al., who hypothesized that there might be seepage of toxins from the PDMS.[110] In their research, they tried to improve neuron survival in open- and closed-channel microfluidic chips by treating the PDMS with serial solvent-based extraction processes (to remove potentially cytotoxic uncross-linked oligomers and residual platinum catalyst in the PDMS) or with autoclaving (to drive cross-linking and outgas solvents). They found that treatment with extraction improved neuron survival, increased the development of neurites, and lowered platinum levels in the PDMS more than did autoclaving. Specifically, the ratio of neuron survival was 3:28:51 for native PDMS, autoclaved PDMS, and extracted PDMS, respectively. Overall cell viability in low density, small volume, serum-free studies in closed-channel devices was improved from less than 2 days in native PDMS to over 7 days for extracted PDMS. If gravity-driven flow was added, survival could be further increased to over 11 days by improving nutrient delivery and waste removal compared to static systems. These extraction processes will be imperative for future studies on

individual cells or cell-to-cell interactions in low-density cultures in microscale culture volumes.

Perhaps the biggest impediment to the general acceptance of microfluidic platforms as tools for biological investigation will be the initial need for interdisciplinary teams of researchers that include both engineers who can devise and fabricate the chips and biologists who know when and where to best apply the technology. As with all new technology, the developers may not be able to recognize its most useful niche, and the users may not be aware of the technology or have the auxiliary tools and expertise to operate it correctly. However, as more individuals take the steps to experiment with the technology, it would become more commonplace and better utilized.

On the other hand, the novelty of the underlying technology and of the general approach of combining neuroscience with microfluidics offers unique and exciting opportunities to address fundamental problems with new tools in new ways. These technologies thereby carry the immense promise of important breakthroughs and new insights both in fundamental neuroscience and in its extensions to biomedical practice in improving the treatment of many neurological diseases.

1.3   DESIGNS AND DEVICES FOR NEUROSCIENCE APPLICATIONS

1.3.1   Gradient-Generating Designs

As discussed above, the laminar flow that occurs in microscale channels can be used advantageously to create high-resolution gradients of solutes and special factors within the cell culture chamber, in order to assess the effects of these gradients on the behavior of individual cells. These designs employ two or more inputs—one for the studied factor and one for the diluting medium, with each connected to its own syringe pump. The inputs connect to a network of serpentine, interconnected channels that repeatedly split and remix, with each generation of splitting channels increasing in number, until they finally coalesce back into a single larger channel (Figure 1.2). At each branch point, some mixing occurs so that there is a gradient of concentrations of the studied factor(s) that is oriented perpendicular to the flow direction at the final exit channel, and this gradient has a range of resolution spanning from several microns to hundreds of microns. After exiting the gradient-generating device, the established gradient is maintained by laminar flow. By varying the flow rate into one input, dynamic and asymmetric gradients of variable shape (smooth, step, or multiple peaks) can also be created.[52,111,112] These devices have been used to study the effects of IL8 (interleukin 8) on neutrophil chemotaxis,[113]EGF (epidermal growth factor) on breast cancer cell chemotaxis,[114] and various growth factors on neural stem cells.[115]

These gradient-generating devices are commonly used for research on the physiology of neural lineage cells since the devices provide precise control over exposure of growth and inhibitory factors to these cells. In addition, these same devices have been used to etch a controlled gradient into the surface topology of chip substrates by injecting etching reagents, or to lay down gradients of adhesives, self-assembled monolayers (SAMs), and dyes.[111] Finally, these gradient generators have been
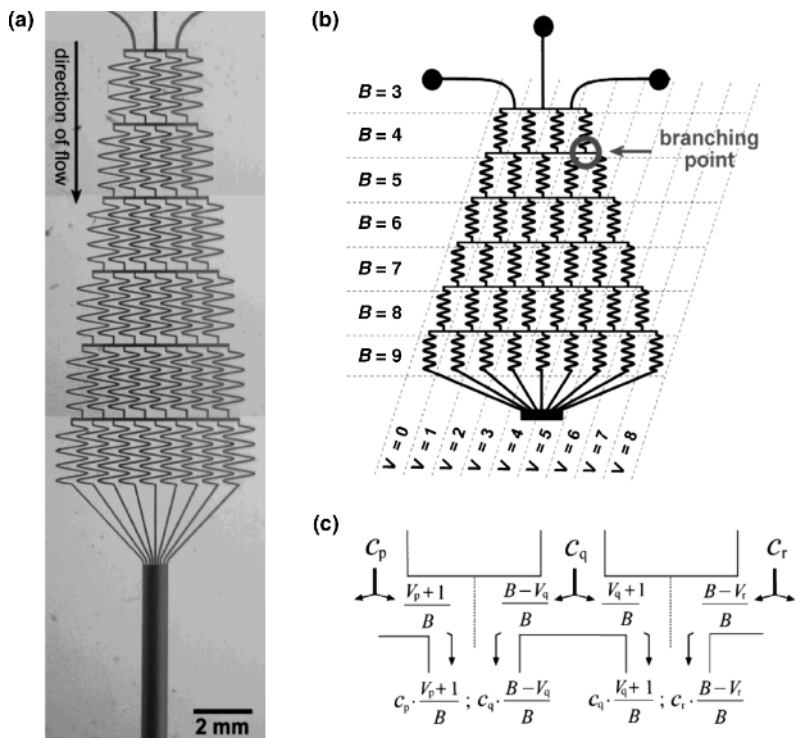
FIGURE 1.2   (a) Photograph showing a microfluidic device used for generating gradients of green and red dyes in solution. The three incoming channels (top part of the photograph) were connected to syringes via tubings (not visible). After combining the streams into a single, wide channel (bottom of the photograph shown in (a)), a gradient was formed across the channel, perpendicular to the direction of flow. (b) Schematic explaining the nomenclature used for the mathematical description of the network. (c) Schematic demonstrating the application of the formulas governing the splitting ratios at the branching points. The dotted lines indicate the boundary between the two combined streams. The concentrations at the end of the serpentine channels can be calculated by multiplying the concentration of the incoming streams (c_p, c_q, c_r) with the corresponding numbers of the splitting ratio ($V_p + 1$)/B, ($B - V_q$)/B, ($V_q + 1$)/B, and ($B - V_r$)/B, as indicated. Reprinted with permission from Ref. 112. Copyright 2001 American Chemical Society.

combined with large chip-based arrays of cell culture chambers (10×10) to simultaneously perform 100 parallel tests on the effect of an agent's various dilutions.[116] Since these chambers each had four individual access ports, repeated growth/passage cycles of the cells could be performed on-board by microfluidic control, so the cell cultures could be maintained over long periods. As demonstrated with the studies described above, these gradient devices are very useful tools for investigations into cellular responses to varying concentrations of specific factors, whether these factors are substrate bound or dissolved in media.

1.3.2   Integrated Electrophysiology

For electrically active cells such as neurons and muscle cells, integrated electrical recording is a very valuable addition to microfluidic platforms. An early example of this was a unique system that was designed as a self-contained, portable unit for field use as a cell-based biosensor. The unit incorporated a hybrid glass/PDMS/silicon chamber for cell culture with integrated microfluidics, a microelectrode array substrate modified with fibronectin and gelatin for cell growth, a temperature regulation system, on-chip electronics for acquisition, analysis and display of action potentials, and a transparent cover that makes the unit amenable to microscopic inspection.[65] This approach of creating a stand-alone unit with its own environmental controls may eventually be required of many platforms in the future; however, most neuronal studies have employed much simpler hardware, typically using commercial MEAs as the substrate for a PDMS microfluidic chip. These will be discussed in more detail in Section 1.4.4.

The "gold standard" for electrophysiological studies has always been patch clamping, and many microfluidic platforms incorporating arrays of patch clamp electrodes have been engineered and successfully demonstrated. Conventional patch clamps use suction to attach the tip of a glass micropipette to a cell membrane, and then break the membrane and record the intracellular potential using a conductive fluid in the micropipette. In early microfluidic designs, these systems used PDMS microfluidic channels to guide cells to pores micromachined into silicon wafers,[117] or they used cell-trapping pores in a horizontal PDMS substrate.[118–120] These pores simulated the tip of a conventional glass micropipette and were used to create a high-resistance seal to the cell wall for subsequent electrical recording.

Ionescu-Zanetti et al. improved on this design by incorporating pores on a vertical channel wall of the PDMS to facilitate the use of optical and fluorescent microscopy to monitor the procedure.[121] This vertical approach allows both the cell and the capillary tube leading to the pore to be in the same plane of focus, and, therefore, it permits easier guidance of the selected cell to the pore (using a combination of flow in the cell chamber and suction from the pore). It also permits visual monitoring of the cell condition and position during the recording. Each pore is connected to a capillary tube that applies negative pressure (suction) to attach the cell and break the membrane and to a silver/silver chloride electrode that then connects to a multiplexer circuit to process the recorded signals. Using CHO (Chinese hamster ovary) cells, the seal resistance between pores and cells was an average of 300 megaohms, and the system was able to record currents down to 20 pA. Individual cell trapping could be achieved in less than 3 s, and the seal was stable for 20–40 min (Figure 1.3). This group further updated this system by raising the trapping pore above the chamber floor to avoid deformation of the trapped cell. They also opened the ceiling of main chamber to ensure rapid fluidic access for high-throughput drug profiling on the clamped cell. For these authors, this microfluidic approach to patch clamping represented a much more efficient system for pharmaceutical analysis than traditional patch clamp technology.[122]

Unfortunately, patch clamp resistance seals in the megaohm range as reported above are not ideal, and other groups have been modifying their techniques to
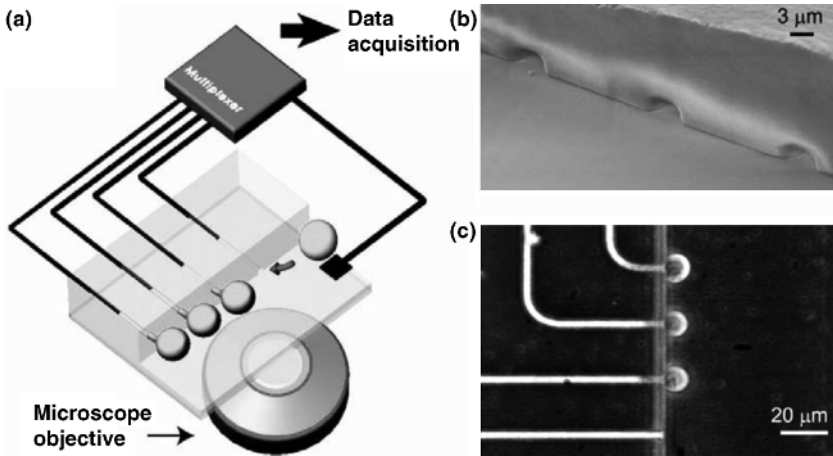
FIGURE 1.3 Patch clamp array on a microfluidic platform. (a) Cell trapping is achieved by applying negative pressure to recording capillaries that open into a main chamber containing cells in suspension. Attached cells deform, protruding into the capillaries. Patch clamp recordings are obtained by placing AgCl electrodes in each of the capillaries, as well as in the main chamber. Signals are fed through a multiplexing circuit and into the data acquisition system. (Multiplexer setup and microscope objective are not to scale.) The device is bonded to a glass coverslip for optical monitoring. (b) Scanning electron micrograph of three recording capillary orifices as seen from the main chamber. The capillary dimensions are $4\,\mu m \times 3\,\mu m$, with a site-to-site distance of $20\,\mu m$. (c) Dark-field optical microscope image of cells trapped at three capillary orifices. Trapping was achieved by applying negative pressure to the recording capillaries. The device consists of 12 capillaries arrayed 6 along each side of the main chamber fluidic channel along a $120\,\mu m$ distance. Reprinted with permission from Ref. 121. Copyright 2005 National Academy of Sciences USA.

improve this. Chen and Folch used e-beam lithography to create a 1 µm cell attachment aperture in their patch clamp chip. This method was combined with standard photolithography using high-resolution photomasks to create larger suction channels. They also used O_2 plasma treatment of their master and PDMS chip to smooth the edges on the aperture, and they achieved reliable gigaohm seals and signal quality that was similar to that obtained with traditional glass pipette patch clamps.[123] Commercial forms of these microfluidic patch clamp technologies will likely be available in the near future.

1.3.3 Other Integrated Sensors and Microfluidic Capabilities

Microfluidic chips have employed many other complementary microtechnologies in recent years for application to biological studies. Although they have not all been used specifically for neuroscience studies, they do have this potential and they are presented here for the interested and motivated reader. For example, as an alternative to using electrodes, charged membrane-permeable, potential-sensitive dyes have been used in a microfluidic device to determine the membrane potential of cells in a rapid, highly

sensitive manner, with minimal consumption of reagents.[124] Various electrical parameters including amperometry, impedance measurement, and potentiometry have also been used to analyze cells and their ionic secretions in microfluidic chambers (see the excellent review on this topic by Bao, Wang, and Lu).[85]

The relative acidity of the contents of microfluidic channels has been monitored by pH-sensitive fluorescently tagged monolayers (SAMs) that are bound to the substrate of the microfluidic platform.[125] An alternative pH meter with higher sensitivity has used a different technology in microliter flow chambers to measure pH changes down to 0.5×10^{-3} pH units. This device has been useful in the study of cellular processes that alter ATP levels, such as receptor activation and signal transduction.[126] The oxygen content in microscale cell cultures is another important parameter that can be monitored by an on-chip oxygen sensor based on fluorescent quenching of ruthenium dye particles encapsulated in the PDMS of the microfluidic culture device.[62] Silicon chips containing multiple microsensors for bulk detection of extracellular pH, oxygen consumption rates, and cell morphological alterations have also been developed.[127] and although not yet applied to microfluidic single cell studies, it is reasonable to expect that they might be adaptable to this purpose in the future.

More advanced technology has also been miniaturized for on-chip use. For example, single nonperfused neurons have been studied with NMR (nuclear magnetic resonance) microcoils, and NMR spectroscopy has been used to determine their metabolite content, but the need for continuous perfusion to prevent cell death was noted.[45] To address this issue, planar NMR probes have been incorporated into microfluidic platforms and preliminary studies on their functionality are underway.[46] Other advanced technologies such as surface-enhanced Raman scattering and confocal microscopy have been combined with microfluidics to study real-time intracellular chemical dynamics of single live cells with high spatial and temporal resolution.[128]

Apoptosis (programmed cell death) is an important cellular process that is well studied by both biologists and pharmaceutical companies since it is critical to understanding how to control cancer and cell growth in general. Microfluidics have been used to study the multiple morphologic and biochemical changes associated with apoptosis at the single cell level.[72] Tamaki et al. noninvasively monitored the change in cytochrome c distribution that occurred during apoptosis of single neuroblastoma–glioma hybrid cells confined in quartz glass microfluidic chambers by using scanning thermal lens microscopy without the need for any labeling materials.[129]

Finally, on-chip single cell genetic evaluation and manipulation will be useful for neuroscience cellular studies. One successful technique for this involved a combined microfluidic/microelectroporation chip that could isolate and temporarily immobilize individual prostate cancer cells in a channel, prior to application of a 10 V, 100 ms electric pulse to puncture the cell membrane and insert green fluorescent protein genes into them.[130] In more recent work, a lower applied voltage of only 0.8 V for 6.5 ms was focused at one location on the membrane of individually trapped HeLa cells and resulted in successful electroporation.[131] Because of the lower voltage requirement and because this design was able to monitor the permeation of the membrane by recording accompanying jumps in electrical current across the cell membrane, it represented a definite improvement over previous technology.

Another example of a platform for genetic studies used microfluidics to isolate cells and then lyse them prior to purifying and recovering their mRNA, the genetic instruction codes that cells use to synthesize proteins.[132] Further development of this technology from this same laboratory has yielded chips capable of performing 72 parallel, 450 pL reverse-transcriptase PCR reactions that could detect mRNA levels down to 34 RNA templates.[133] They have also used microfluidics to synthesize cDNA from subpicogram mRNA templates isolated from single cells[134] and performed gene ligation with plasmids and successfully transformed the plasmid DNA into competent cells.[135] This technology has potential utility for neuronal studies.

1.3.4 Simulating *In Vivo* Tissues with Microfluidics

To draw reasonable conclusions about *in vivo* processes using data derived from our *in vitro* experimental models, these models must simulate real tissues as closely as possible. This is why many researchers have tried to consolidate multiple cell types and extracellular matrix proteins into a three-dimensional architecture. Otherwise, the data may be misleading and oversimplified. For example, most muscle cells grown *in vitro* have different morphology and function compared to those grown *in vivo*. However, one group of researchers found that they could culture cardiac muscle myocytes with more typical morphology if they cocultured fibroblasts alongside of them on linear, intersecting patterns of collagen deposited within microfluidic channels.[136] Similarly, a perfused microfluidic platform built upon a substrate with alternating cell adhesive (matrigel on poly-D-lysine) and cell repellent stripes (poly-acrylamide and polyethylene glycol) was used to grow and fuse myoblasts into realistic multinucleated myotubes.[91]

Based on studies such as these, it is now believed that many environmental parameters can directly influence a cell's cytoskeleton and subsequently alter cell behaviors such as proliferation, motility, and migration, to name a few. To better understand these interactions, the effect of variably sized and shaped microfabricated cell culture wells has been studied. Preliminary work shows that these single cell wells (treated with cell adhesive material via PDMS microcontact printing) have been successful in altering the three-dimensional shape of the cell contained within them.[137] Further study in this field may reveal how cell shape *in vivo* alters cell function.

In addition to controlling individual cell shape, environmental cues from cell attachment matrices containing self-assembling proteins and gel-like substances have influenced the three-dimensional shape of a group of cells *in vitro*.[17] Microfluidics have been used to build up realistic vascular tissue by sequentially depositing layers containing different cell types and extracellular matrices (collagen, matrigel, etc.) within a platform.[18,19] These types of microfluidic platforms have the capability of creating more biomimetic *in vitro* systems, but they may also encounter the same type of limitations on study that using live tissue sections do. Rather than trying to simulate real tissue, it may be more important in the *in vitro* study of individual cell physiology to create a substrate with the right characteristics. For example, to be truly "physiologic," *in vitro* substrates must re-create several characteristics of normal *in vivo* extracellular matrices, including mechanical properties (such as elasticity,

rigidity, and strain), chemical properties (such as ligand density and orientation), and topographic properties (such as surface curvature and fibrous contact guidance).

These characteristics control cell distribution in tissues and guide cell morphology, behavior, gene expression, proliferation, differentiation, and apoptosis, presumably through interactions with transmembrane integrin receptors.[17,138,139] This theory is reinforced by studies that show that neurons grow better on a "soft" bed of astrocytes than on glass.[139] As research in this field progresses, we expect to see more physiologic substrates being incorporated into microfluidic systems.

Researchers investigating neural prosthesis development are very interested in trying to optimize substrate topography and chemistry. Their goal is to find the best substrate that will prevent astrocytes from overgrowing the implanted electrodes since these cells can interfere with signal transmission by insulating the electrodes from the neurons. Results from research on microfluidic substrates for neural growth and on prosthesis optimization will likely benefit both fields. For example, studies have shown that certain cells do prefer certain nanotopographies, as demonstrated by the finding that astroglial cells preferentially attach to pillars over wells and respond to the topography by changing their expression of cytoskeletal proteins such as actin and vinculin.[140]

Independent chemical cues also have different effects on different cell types. For example, substrate-bound peptides with the amino acid sequence IKVAV preferentially promote neural adhesion, whereas the sequence RGD promotes fibroblast and glial cell adhesion.[141] In contrast to the goals of prosthesis development to limit astrocyte attachments, neural stem cell researchers desire a controlled bed of astrocytes to generate a permissive environment for the differentiation of neural stem cells. Research shows that combining topographic cues (in the form of substrate grooves) with chemical cues (in the form of adsorbed laminin molecules) can orient over 85% of astrocytes in the direction of the grooves.[142] This can help to control the differentiation of neural stem cells cultured over the astrocytic bed and hopefully will permit directed axon regeneration in future studies.

A final limitation of developing truly three-dimensional cultures *in vitro* has been the difficulty of maintaining cell viability when cell density approaches the order of magnitude seen in live tissues. Cullen et al. theorized that this might be due to lack of adequate perfusion to supply nutrients and remove waste products.[143] They used microfluidics to create a cylindrical PDMS culture plate that had multiple inlet ports at the base and peripheral (circumferential) outlet ports along the edge. The plate was covered with FEP (fluorinated ethylene propylene) membrane to minimize evaporative losses while allowing gas exchange, and the ports were connected to a syringe pump to keep the culture volume constant, while using forced interstitial convection at various perfusion rates. Cells were loaded into a 500 μm thick 3D matrigel matrix preloaded in the plate. The researchers were able to demonstrate that a perfusion rate of 10–11 μL min^{-1} allowed greater than 90% viability in neuronal or neuronal/astrocyte cocultures, with cell densities that more closely matched the density of the brain than prior successful models (although still lower than that found in the brain cortex). It will likely be imperative that three-dimensional systems incorporate excellent perfusion systems to truly simulate an *in vivo* experience.
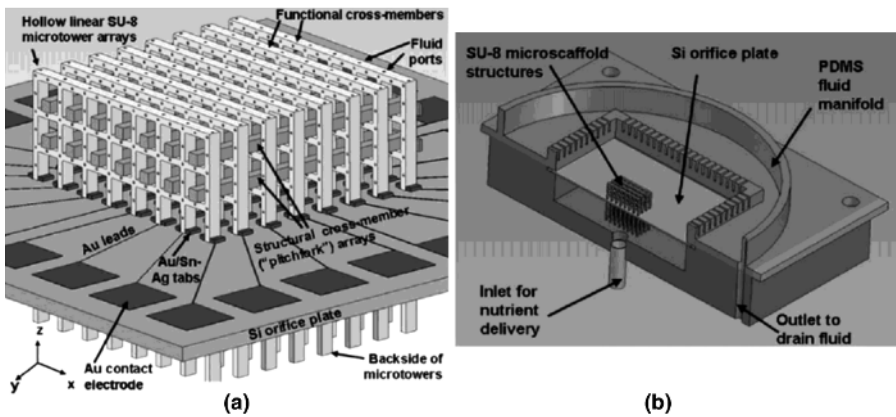
FIGURE 1.4   Schematics showing the microscaffold system. (a) An 8×8 array of hollow microtowers with functional cross-connects along the *x*-direction and structural cross-connects along the *y*-direction. The microtowers in this schematic project 1.5 mm out the topside and backside of the Si orifice plate. (b) A 3D cross section of the microscaffold device encased in the PDMS fluid manifold. The fluid manifold allows continuous perfusion of the cells growing within the active microscaffold. Reproduced with permission from the Royal Society of Chemistry.[144]

Rowe et al. approached the perfusion problem from a structural engineering standpoint and actually engineered a three-dimensional scaffolding system made of SU-8 photoresist and gold electrodes.[144] The design included a system of integral microchannels and ports within the major support struts, to simulate microvascular perfusion, and the entire structure was encased in a PDMS manifold for fluid delivery. Their preliminary results found that their 3D substrate successfully supported neuronal cultures and the resultant neural networks that developed showed more complexity than those grown on two-dimensional electrode arrays. Future studies planned to include electrical recordings from the gold electrodes (Figure 1.4). Regardless of whether future *in vivo*-type culture systems employ three-dimensional support structures built up chemically or structurally, or using a combination of these techniques, they will likely figure prominently in research that is trying to decipher cell behavior in complex geometries and communities.

1.4   NEUROPHYSIOLOGY EXPERIMENTS USING MICROFLUIDIC CHIPS

1.4.1   Cell Separation Tools

One prerequisite for *in vitro* studies on cells of neuronal lineage is starting with the right cells. When harvested from the nervous system of donor animals, samples of cells contain a mixture of large neurons and generally smaller glial support cells. A separation process must then be used to isolate the desired cell type. Wu et al. devised

a method of microfluidic cell separation using the change in spatial distribution that occurs when fluid streams of different viscosity are mixed across an expanding channel.[145] They suspended the mixture of cell types in an aqueous solution of sodium alginate that supported neuronal survival rates of greater than 90% during the separation process. They then mixed a stream of this suspension with an eluent stream traveling at a different flow rate. As the flow rate increases, the viscosity decreases since the alginate polymers become more linearly aligned with the micro-fluidic channel walls. As the flow rate decreases, the viscosity increases since the polymer chain bunches and intertwines with itself. If the eluent stream has a higher viscosity than the stream in which the cells are suspended, then the interface between the two streams is moved in such a way that the cell suspension stream becomes narrower and more closely applied to the channel wall. When this cell suspension stream width approaches a certain minimal value, the larger cells are picked up by the higher viscosity eluent stream and the smaller cells are left behind in the original cell stream. When these streams then encounter a branching point, the cells can be separated. The researchers were able to successfully separate neurons and glia at a rate nearing 100% when flow rates and interface distributions were stringently controlled.

Stem cells that are fated to differentiate into neurons or astrocytes cannot be separated based on their size since they are indistinguishable by their morphology and size. Separation by flow cytometry is based on the elaboration of different antigens that can be fluorescently tagged, but these may not be present early in the differentiation process. Flanagan et al. devised a microfluidic chip that used the dielectric properties of stem cells to characterize their fate bias.[146] Electrodes applied an alternating electric field to cells in a microfluidic channel. At low frequencies, the cells are repelled from the electrodes, but as the frequency of the switching field is increased, they are attracted to the electrodes. The frequency at which they are "trapped" between repulsion and attraction is characteristic for the cell's fate bias, and these differentiat-ing dielectric properties can be demonstrated prior to the development of protein markers. In this study, live cells could be distinguished from dead cells that do not "trap." Astrocytes were trapped at 0.3 MHz, neurons trapped at 5 MHz, and neural stem cells trapped at 1 MHz. In addition, neural stem cells harvested from embryos at earlier developmental ages trap at a higher frequency than those harvested from embryos at later developmental ages, consistent with the fact that more stem cells from young embryos differentiate into neurons and more stem cells from older embryos differentiate into astrocytes. This technique can be used to measure heterogeneity in cell cultures and has several advantages over flow cytometry and FACS since it can be done on small numbers of cells, it can exclude dead cells, it is sensitive to minor differences in cells, and it needs no antibody.

1.4.2 Neuropeptide Release

Neuropeptides are endogenous chemicals that play a major role in the differentiation, maturation, and communication between cells of neuronal lineage. A significant portion of neurophysiology research involves deciphering the factors involved in their

release, uptake, and induced responses in local and distant cells. The effect of neuropeptides on developing neuromuscular junctions has been studied using microfluidic chips. This research is exemplified by the work of Tourovskaia et al. who used PDMS masks to apply micropatterns of cell adhesive and cell repellent molecules to isolate single myotubes in thin parallel lines.[147] A microfluidic device was then placed over this, myoblasts were allowed to attach to the substrate, and streams of media containing agrin were microfluidically perfused perpendicular to the axis of the cells. Agrin is a molecule released by the tip of growing axons when they contact muscle cells. The study showed that focal application of agrin stabilized the acetylcholine receptor aggregations in the myotubes, consistent with its presumed actions involved in the early development of neuromuscular synapses (Figure 1.5).

Indirect measurement of neurotransmitter release from isolated single cells was demonstrated by Huang et al.[148] They used PDMS microchannels to guide single pheochromocytoma PC12 cells into a chamber etched into glass. When this cell was stimulated with nicotine, they could detect dopamine release by amperometric monitoring using a carbon fiber microelectrode. Gold-covered single cell wells in silicon chips were also used to record catecholamine release from adrenal chromaffin cells.[149] PDMS microchannels were used to immobilize PC12 cells and use amperometry to record calcium-induced dopamine and norepinephrine release.[150] In similar work, Sun and Gillis were able to record quantal exocytosis of catecholamines after stimulation of chromaffin cells in microfluidic channels with potassium solution.[151] They recorded amperometric spikes using indium tin oxide (ITO) electrodes when the catecholamines were oxidized on the electrode surface.

When trying to differentiate the complex interactions that occur between different cells in mixed-type or pure-type cultures, it is important to determine the cell's chemical response to stimulation, in addition to its electrical and morphological responses. Although the latter responses have been more readily studied at the single cell or near-single cell level using microfluidics, the analysis of released chemicals at this scale has been more difficult to achieve, given the extremely minute concentrations of the chemicals to be studied, in addition to the fact that the exact chemical species may not be known in advance. Jo et al. designed a microfluidic chip that would allow off-line analysis of neuropeptides released in response to chemical stimulation of neurons with potassium chloride (KCl).[152] They used multichambered microfluidic chips that contained a cell culture chamber functionalized with poly-L-lysine to allow attachment of *Aplysia* bag cell neurons. Valves were used to selectively connect this chamber to three other chambers, each functionalized with a SAM that could adsorb molecules (released neuropeptides) by hydrophobic interactions. The cell culture chamber was exposed to the KCl that activated release of the neuropeptides, and the cell culture fluid was sequentially flushed from the culture chamber into each of the SAM-containing chambers before, during, and after KCL stimulation. Once the connecting valves were closed and the solutions were allowed time to adsorb to the SAM, the PDMS was peeled from the chip, exposing the SAMs for MALDI mass spectrometry measurements and subsequent imaging. The results of the research confirmed that this methodology could successfully detect two different released neuropeptides and that the majority of the released peptides adsorbed onto the SAM
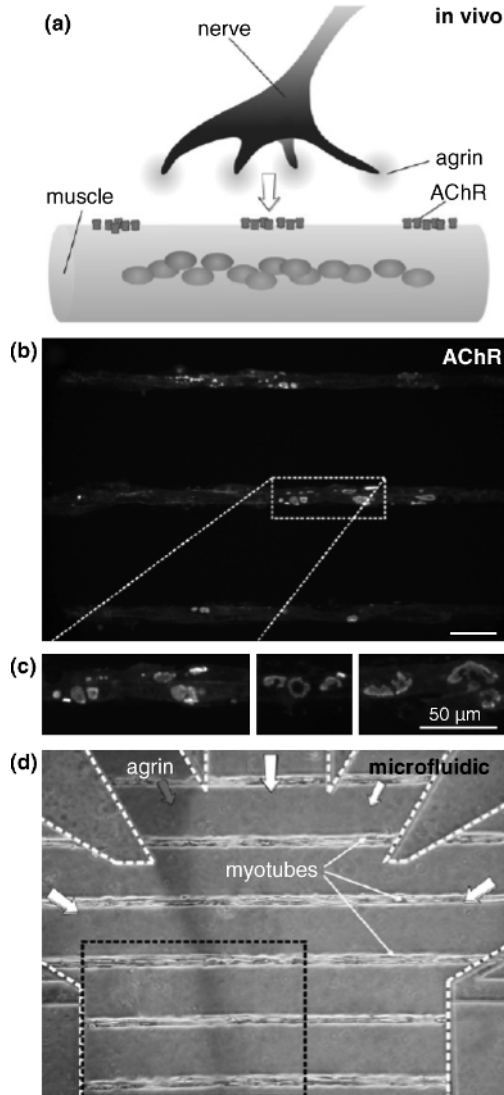
FIGURE 1.5   Synaptogenesis on a chip. (a) During development, neurons release agrin at the site of contact between nerve and muscle. (b) Fluorescence micrograph of a portion of the myotube microarray after staining the AChRs with Alexa Fluor 488-conjugated α-bungar-otoxin (BTX*). Scale bar is 50 μm. (c) Three high-magnification fluorescence micrographs of myotubes stained with BTX*, showing that aneural AChR clusters display intricate shapes similar to those found *in vivo*. (d) Phase contrast micrograph of the microfluidic device containing a ladder micropattern of myotubes during stimulation by a laminar stream of agrin (spiked with Allura red dye for visualization). The black dashed box corresponds to the area shown in (b). Reproduced from Ref. 147. Copyright 2008 Elsevier.

layer and not onto the PDMS walls. This innovative combination of microfluidic control of cell bathing solution, with off-line mass spectrometry, has significant potential to study known and previously unknown chemical responses of neurons spatially and temporally.

Since both the concentrations and the volumes of released neuromediators are so low, microfluidic-based on-chip analysis of these agents can represent an attractive alternative to standard laboratory techniques. Mourzina et al. devised and optimized an on-chip capillary electrophoresis system to separate neuromediators.[89] They experimented with various PDMS treatments and separation buffers to improve electroosmotic pumping and decrease adsorption of the neuromediators onto the PDMS surface. With the addition of field-amplified sample stacking, they were able to achieve separation of fluorescently labeled neuropeptides (including oxytocin, serotonin, glutamic acid, and others) within tens of seconds at 110 pL volume. Field-amplified sample stacking utilizes a principle where the analyte is dissolved in a dilute ionic solution that is sandwiched between higher concentration ionic solutions. Application of fluid flow and electrical current causes formation of a stepped electric field, resulting in migration of the analyte into the boundary area between these solutions for easier separation. This work demonstrates the utility of using microfluidic chips to process biologically relevant samples at minute scales.

Along similar lines, unpublished data from other researchers (Phillips and Wellner) has demonstrated the adaptation of commercial microfluidic micromixer chips to detect proteins such as proinflammatory cytokines in tiny samples of blood or perspiration from patients with depression using recycling immunoaffinity chromatography (online communication at http://www.nibib.nih.gov/HealthEdu/eAdvances/30Jan09). Further refinement of these techniques will likely be forthcoming.

1.4.3 Physical and Chemical Guidance Cues

The ultimate goal of most neuroscience research involves learning ways of preventing degeneration and promoting repair and regeneration of neurons and their processes. One key prerequisite for this is an understanding of how physical and chemical guidance cues affect neurite growth. This is also of great interest to individuals who study neural network design. Many studies in this field use microchannels to isolate and observe axonal responses. An early study demonstrated the relative importance of chemical guidance when neurites from chick spinal neurons that were otherwise physically confined in "v"-shaped channels and pits on a silicon nitride substrate were able to grow out of the channels if the substrate was pretreated with polylysine.[53] The effects of physical cues alone were demonstrated by studying neurite elaboration by cells confined to square bottomed channels (constructed from polyimide walls placed on a glass substrate).[153] Narrow channels (20–30 μm in width) caused fewer neurites to be elaborated from each cell, and each neurite was longer and more likely to be oriented parallel to the channel wall. These changes might be due to inflexibility of the cytoskeleton.

The effect of isolated chemical cues is the subject of many ongoing studies. In one such study, a PDMS device with parallel stripes of channels was used to deliver

poly-L-lysine (PLL) or collagen to a substrate pretreated with sequential 3-amino-propyltriethoxysilane (APTES) and glutaraldehyde.[154] This pretreatment allowed PLL or collagen to bind covalently to the substrate, so it was more stable and structurally homogeneous than those attached by simple protein adsorption. The PDMS was then removed and *Aplysia* neurons were applied and monitored as their neurites developed. Standard electrophysiology and mass spectrometry were used to investigate any differences between neurons cultured on patterned versus uniform layers of protein. The results showed that patterned substrates caused shorter, thicker, less branched, and slower growing neurites and caused changes in the cell's electrical activity compared to neurons grown on uniform proteins.

The effect of substance gradients on neurite extension was also specifically studied by Whitesides and colleagues.[52] This group utilized the serpentine network of microfluidic channels discussed in Section 1.3.1 to study and quantify the effect of different laminin concentrations on neurite sprouting, differentiation (into dendrite or axon), and directionality from hippocampal neurons. They deposited a layer of poly-L-lysine onto channels in a plasma-treated PDMS chip and then created a gradual gradient that ranged from pure BSA to pure laminin. This treated PDMS channel was then cut from its substrate, inverted in a Petri dish, and seeded with hippocampal neurons. Cells that grew alone in the center of the gradient (center of channel) were observed and the length of their neurites was measured to determine axonal specification (axons were typically four times the length of dendrites). The results showed that approximately 60% of axons were oriented within a 120° arc in the direction of increasing laminin concentration, and although the laminin concentration did not guide the axonal growth, it did specify which early neurite would become the axon. In addition, the concentration of laminin required to influence axonal specification was determined. It is of particular importance to understand the *in vitro* effects of laminin since it has been shown to play a critical role in axonal pathfinding in the embryonic CNS *in vivo*.[155]

The effect of the absolute concentration of a substance on axonal growth is just one parameter that has been studied in microfluidic chips. The slope of the substance's concentration gradient also exerts effects on axonal growth on specific types of neurons. Lang et al. demonstrated this with ephrin A5, which is a repulsive axon guidance molecule.[156] They used silicon wafers with etched microfluidic channels to create multiple stripes of varying concentrations of ephrin A5. The molecules in these solutions were transferred to a PDMS stamp and ultimately into polystyrene culture dishes into which chick retinal ganglion cells originating from nasal or temporal locations on the retina were cultured. They tested the effects of both steep gradient variations and shallow gradient variations on axonal growth. They found that axons growing from neurons originating on the nasal portion of the retina do not respond to ephrin A5 gradients at all. However, axons growing from neurons originating from the temporal portion of the retina are inhibited, grow farther into shallow gradients than steep gradients, and halt their growth at a lower total ephrin A5 concentration and total exposure in shallow gradients of this molecule than in steep gradients. This research indicates the complicated inputs involved in the growth of axons of differentiated cells and the usefulness of microfluidics in deciphering the signals controlling axonal
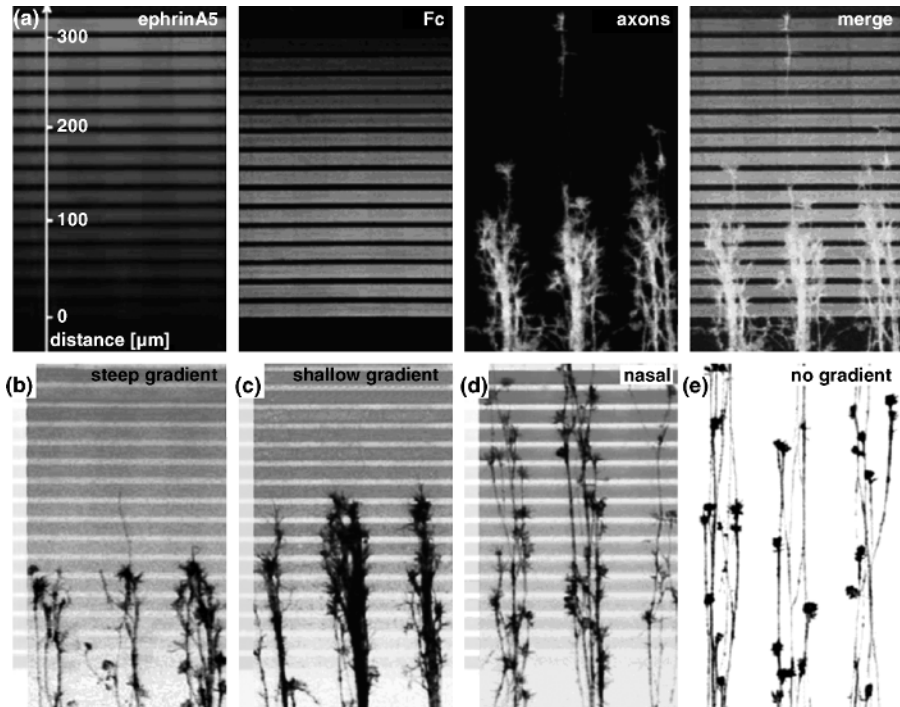
FIGURE 1.6 Temporal RGC axons stop in substrate-bound gradients produced by μFN. (a) Fluorescence images of a stepwise gradient of ephrin A5 spanning a distance of 320 μm, and the corresponding countergradient of Fc and temporal axons stained with phalloidin invading the gradient. In the original article with color images, in (b–e) phalloidin-stained axons are shown in black and antibody-stained Fc in green. (b) Temporal axons stopping in a steep ephrin A5 gradient. (c) The stop zone shifts further into the gradient in a shallow gradient. (d) Nasal axons in a steep gradient do not stop. (e) Temporal axons growing on laminin lanes without underlying gradient. For scale, see (a). With kind permission from Springer Science + Business Media.[156]

responses (Figure 1.6). These researchers published a detailed protocol on their methodology, including the use of a second active protein to set up an overlapping or countergradient.[157]

Similarly, gradient mixers have been used to create substrate-bound gradients with multiple agents of defined concentrations and slopes.[112] Li et al. used a syringe pump connected to a PDMS gradient mixer to combine laminin, chondroitin sulfate proteoglycans (CSPGs), and/or BSA to form various patterns on a glass substrate.[158] After 12 h of adsorption, the PDMS superstructure was removed and DRG neurons were applied. The study showed that cells adhered more strongly to higher laminin and lower CSPG concentrations, neurites grew toward higher laminin and lower CSPG concentrations, and double opposing gradients provided the strongest guidance cues. This research confirmed that neurites can detect and respond to both the slope and the fractional concentration change of substrate-bound gradients.

In reality, it may be an oversimplification to study the effect of individual agents on neurons since it is more likely that they are programmed to respond to complex, interacting, and synergistic forces, including topography, electromagnetic fields, and chemical/biological cues. For example, when adult rat hippocampal progenitor cells were cocultured with astrocytes aligned on laminin-coated substrate grooves, there was enhanced neuronal differentiation and alignment of neurites parallel to the astrocyte processes and substrate grooves. It is presumed that the astrocytes align themselves on the grooves and then secrete soluble factors that are concentrated locally by the topography, resulting in facilitated neuronal differentiation of the progenitor cells.[159]

1.4.4 Electrophysiology and Microfluidics

Studies on neurons frequently involve monitoring of multiple physiologic parameters, including their electrical activity, as well as their morphology and expressed proteins. Research in this field has been made easier by the availability of commercial MEAs, which typically employ a glass substrate with electrically conductive microcontacts and leads made of gold, platinum, or transparent ITO. Heuschkel et al. used an ITO MEA and engineered a microfluidic chamber on its surface by layering negative photoresist and using photolithography to pattern buried microchannels in the resist.[38] Once it was processed and baked, laminin and polyornithine were applied and chick embryonic motoneurons were introduced via the microchannels. As the neurons grew, their electrical activity was monitored via the microelectrodes. Although the experiment was successful and the photoresist was found to be biocompatible, most investigators now use PDMS to fabricate microfluidic channels intended for cell culture.

For example, Morin et al. aligned the wells and channels of a PDMS microfluidic chip to commercial and custom planar microelectrode array substrates.[39] Poly-L-lysine or laminin was applied to the chips prior to addition of chick or murine cortical neurons. These cells remained viable and electrically active for weeks, as demonstrated by optical microscopy and electrical responses to stimulation. Despite a few problems with isolating potentials from single cells, inhomogeneity of the cells in each well (neuron versus glial), and PDMS adhesion to the commercial microelectrode substrate, the authors felt that this system showed potential for the development of neuronal networks. In similar work, Claverol-Tinture et al. used PDMS chips with channels and wells over poly-L-lysine-coated planar ITO microelectrode arrays and manually placed individual neurons in the wells.[40] Once the axons grew, they were able to record single cell spikes from the soma (contained in the well), or the axon (contained in the channels), depending on how the microfluidic channels were aligned on the electrodes (Figure 1.7). In follow-up studies, they were able to achieve a signal-to-noise ratio of 20 dB when recording electrical spike activity of up to 300 μV amplitude from multiple sites on single neurites extending in microchannels.[41]

Thiebaud et al. also developed a PDMS microfluidic chamber that incorporated microelectrode arrays.[42] The first step in the fabrication process involved using a PDMS microcontact stamp to deposit laminin onto an MEA substrate in parallel
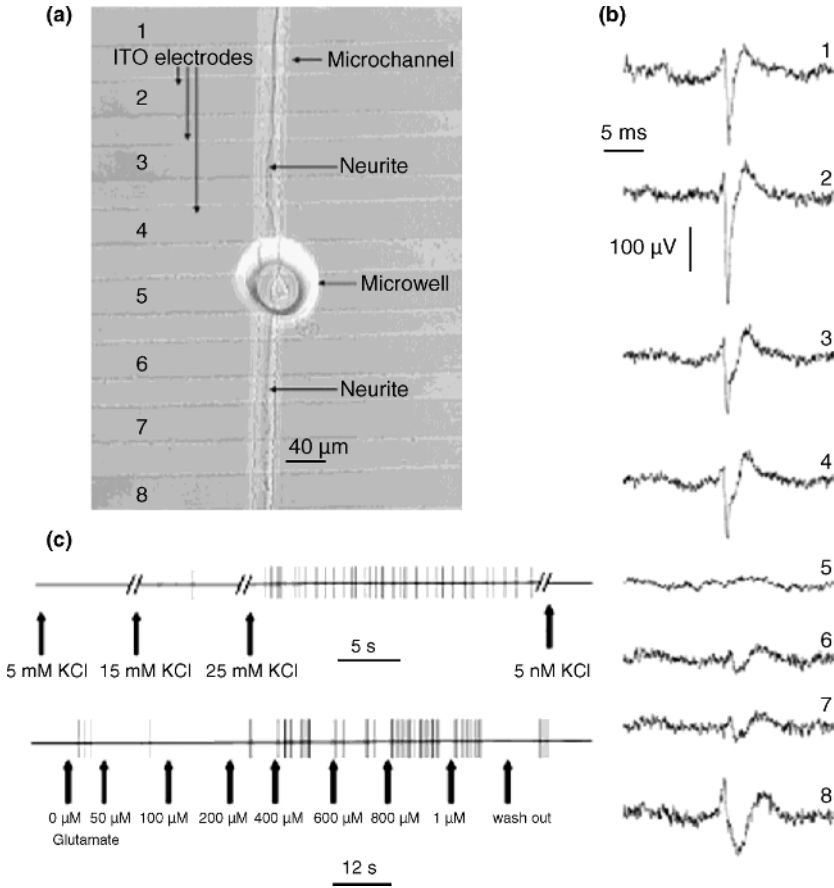
FIGURE 1.7 (a) Phase contrast image of a confined bipolar neuron after 12 days *in vitro* sprouting two neurites along a microchannel. (b) Extracellular potentials associated with an action potential and recorded by the eight microelectrodes shown in (a). (c) Raster plot showing KCl and glutamate dose responses. Reproduced with permission from Ref. 41. Copyright 2007 IEEE.

stripes whose width was consistent with the size of microelectrode arrays. A PDMS microfluidic apparatus with aligned microchannels then delivered culture medium containing neuronal cells to the laminin stripes to establish the culture. Once the cells attached, agents were injected into the parallel channels and delivered to the established lines of cells via laminar flow to study the electrophysiological effects of various pharmaceutical agents on the neurons.

Commercial microelectrode arrays have also been integrated into microfluidic devices and used to study the effect of temperature changes on the electrical activity of a subpopulation of cold-sensitive cells derived from the dorsal root ganglion.[44] In this research, a microfluidic chip was used to deliver polylysine over the array prior to the application of the cell suspension. There were two inlets to the chip—one

providing culture medium at $45°C$, and the other providing medium at $4°C$. By varying the flow rate of each input, they could rapidly (<1 s) switch the culture medium temperature flowing over cells from 35 to $16°C$. Using a multichannel multiprocessor recording system, they recorded action potentials from the array of electrodes and found that certain cells consistently changed their firing rate from a mean of 0.028 spikes per second to a mean of 0.94 spikes per second in response to a switch to a colder temperature. These cells had the same morphology as the others that were not sensitive to temperature changes. This system demonstrated that the combined use of two unique microtechnologies (microfluidics and microelectrodes) could characterize and identify a special subpopulation of cells based on their electrophysiological responses.

In a follow-up study, these researchers modified their microfluidic design by adding small reservoirs with flexible membrane covers to the side channels.[43] This essentially introduced a "switch" that could rapidly perturb the relative flow from each input and vary the temperature flowing over the cells over a 50 ms pulse. Future physiologically relevant research will benefit from this potential to rapidly and transiently deliver agents to cultured cells on a timescale that more closely matches a cell's innate responsiveness capabilities.

1.4.5 Growth Factor Effects

As discussed in Section 1.3.1, microfluidic channels can be arranged to create gradient generators to study the effects of special factors on cell physiology. One research study examined the effect of various concentrations and combinations of growth factors (epidermal growth factor, fibroblast growth factor 2, and platelet-derived growth factor) on neural stem cell proliferation and differentiation.[115] Chung et al. first demonstrated that they could successfully culture human neural stem cells in chambers that were precoated with poly-L-lysine and laminin, and kept under constant low flow ($0.1\,\mu L\,min^{-1}$) of culture medium for at least 7 days. This low flow would help minimize autocrine and paracrine effects of secreted factors. When growth factor gradients were added, they demonstrated that neural stem cells proliferated in direct proportion to the growth factor concentration, whereas astrocytes differentiated in inverse proportion to the growth factor concentration, and all cells demonstrated increased migration toward areas with higher concentrations. The use of microfluidic gradients in this study presented a significant advantage over routine *in vitro* culture techniques and helped to elucidate the action of these specific factors on these cells.

Wittig et al. also investigated the use of microfluidic channels to deliver graded concentrations of growth factors.[160] They applied a reusable PDMS microfluidic culture medium and a factor delivery apparatus onto a standard poly-L-lysine/laminin-coated Petri dish. Two channels delivering different additives in media coalesced in a "Y" shape that was placed adjacent to a neonatal spiral ganglion explant culture area. The base of the "Y" allowed growing neurites to sample two different media choices and then to decide which arm of the "Y" they preferred to grow into. They demonstrated that neurites preferred to grow toward culture medium containing neurotrophin-3 and they anticipated that this approach could be very useful in studying

the various effects of growth and inhibitory factors on proliferation, neurite extension, and cell migration.

In addition to allowing precise delivery of controlled concentrations of growth factors spatially, microfluidic platforms with control valves have also been used to control the timing of the delivery of these substances to cells to determine the differentiation of cells. Nakashima and Yasuda used a microfluidic control valve to release nerve growth factor (NGF) through nanopores to control the differentiation and axonal growth of adrenal pheochromocytoma cells.[161] They were able to switch the microvalve on and off with controlled frequency and duty cycles to guide cellular differentiation. They anticipated that the addition of an electrode to monitor real-time cellular response to their pulsed release of growth factors would be very useful in studying the physiological responses of cells during axonal regeneration processes.

1.4.6 Gene Therapy

An alternative method of delivering growth factors to cells involves the use of microfluidic methods to genetically manipulate neurons by directly delivering the DNA codes for a given growth factor to them. Houchin-Ray et al. cultured neurons on a substrate that had been microfluidically patterned with a mixture of lipoplexes and plasmid DNA that coded for NGF and green fluorescent protein.[162] They were able to achieve a transfection efficiency of 25% using a vector concentration 10 times less than typically used in culture media. Neurons cultured on the patterned areas had improved survival and enhanced neurite outgrowth, indicating that the NGF DNA had been incorporated into the cells and expressed. They also determined that pretreatment of the PDMS microfluidic chip with pluronic (an amphiphilic copolymer with surfactant properties) improved transfection rates since it made the PDMS less likely to bind the cationic lipid/DNA complexes. The NGF concentration gradient could be adjusted by changing the plasmid density in the solution and the size of the microchannels in the PDMS chip used to pattern the substrate. The potential application of patterned gene delivery and expression to specific cells in culture has significant promise for studies of neural physiology, cell-to-cell interactions, and regenerative medicine.

Microfluidic chips that allow fluidic isolation of parts of neurons (Section 1.4.7) have enabled selective delivery of nonviral DNA to either neurites or the cell body of neurons to study the differences in processing that occurs at these locations. This is important for eventual *in vivo* studies since access to both the soma and the axon may not be possible given the long lengths of many axons. Since many studies are aimed at axon regeneration after spinal cord injury, treatments may need to be specialized for the site that is accessible. Bergen and Pun illustrated this point with their research that microfluidically delivered DNA in culture medium to the isolated neurites or to the isolated soma of PC12 neuron-like cells.[163] The DNA was attached to either a lipid-based carrier (lipofectamine) or PEI (poly(ethylenimine)), a polymeric nanoparticle. In general, both had 4–5 times the uptake if delivered at the soma, compared to the neurite, although the lipid-based carriers had an uptake that was 5–7 times that for the PEI carrier. Uptake seemed to be mediated by vesicle formation. When delivered to the isolated neurites, the lipoplexed DNA could be taken up, but not transported,

whereas the PEI-based DNA was taken up and retrogradely transported in a saltatory fashion, but never made it into the soma. Therefore, gene expression occurred only if the gene-complexed carrier was delivered directly to the soma, and the transfection efficiency was much higher for the lipofectamine than for the PEI. This research sheds light on the difficulties inherent in repairing a damaged spinal cord.

1.4.7 Axonal Isolation

As demonstrated in the above literature review, microfluidic chambers have been used to isolate small numbers of cells of neuronal lineage and to study their individual responses to stimuli, often with the aid of on-board analytical devices. However, none of these studies were able to fluidically isolate cell segments (like axons) from their soma, or from neighboring cells of various lineages. The only method to achieve this in the past involved the use of Campenot chambers that used nerve growth factor or brain-derived neurotrophic factor to "artificially" stimulate axonal growth from macro-scaled cultures across grease layers.

However, Jeon's group at the University of California at Irvine has successfully designed and implemented a microfluidic platform that incorporates tiny grooves of adequate size and length to allow fluidic isolation of axons from the regular cell culture chamber, so that their physiology can be studied independently.[164] Specifically, they designed a PDMS chip with two chambers that were separated by a series of grooves that each had dimensions of 10 μm width, 3 μm height, and 150 μm length. These grooves were used to guide neurite growth from a cell culture chamber into a second chamber that could be used to study the isolated axons. The narrowness of the grooves prevented cell bodies from penetrating them, and the length of the grooves prevented the typically shorter dendrites from emerging into the axonal isolation chamber. A key design element of the tiny grooves was their high resistance to fluid transport that allowed them to achieve temporary (15 h) fluidic isolation of the somal compartment from the axonal compartment by applying a slightly higher hydrostatic pressure in the somal compartment. On doing this, there was one-way flow only, going from the somal to the axonal compartment, in a very slow and restricted manner. This allows independent chemical manipulation of the axon, without any direct effects on the soma, unless the axon directly transports the agents to the soma in a retrograde fashion.

These researchers used a patterned poly-L-lysine substrate to keep the axons aligned in parallel stripes, so that they could be more easily identified along with their respective cell body.[164–166] They used several alternative tools for the patterning, including a PDMS mold with tiny channels that could wick the agent in by capillary action (micromolding in capillaries), a PDMS stamp to transfer the agent by micro-contact printing, and a PDMS mask to selectively protect and preserve a uniform, pre-applied, dried agent during plasma etching.

In follow-up research, Jeon's group lengthened the microgrooves to 450 μm to allow longer studies (14 days) that still isolated axons from dendrites (Figure 1.8).[164,165] They used this design to investigate axonal injury, regeneration, and interactions with cocultured oligodendrocytes during myelination. In addition, they proved that their isolated compartments could permit detection of purely axonal
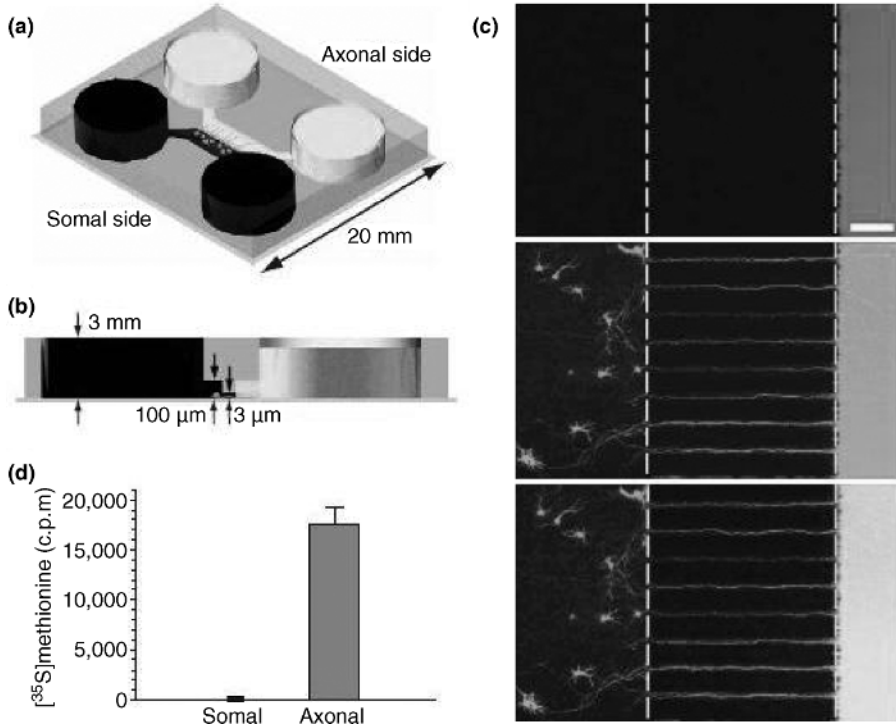
FIGURE 1.8 The microfluidic-based culture platform directs axonal growth of CNS neurons and fluidically isolates axons. (a) The culture chamber consists of a PDMS mold containing a relief pattern of somal and axonal compartments (1.5 mm wide, 7 mm long, 100 μm high) connected by microgrooves (10 μm wide, 3 μm high). The optically transparent PDMS adheres to a polylysine-coated coverslip. Rat CNS neurons (medium gray spots) are added to the somal-side reservoir and are drawn into the somal channel (black) by capillary action. Within 3–4 days, axonal growth is guided into the axonal side (light gray) through the microgrooves. (b) A volume difference between the somal side and the axonal side (∼50 μL) allows chemical microenvironments to be isolated to axons for over 20 h owing to the high fluidic resistance of the microgrooves. Similarly, the volume difference can be reversed to isolate a chemical microenvironment to the somal side. (c) Fluidic isolation of Texas red dextran (top panel) to the axonal compartment demonstrates that axonal or somatic microenvironments can be independently manipulated using this culture platform. Axonally restricted application of CellTracker Green (middle panel) backtracked neurons from their isolated axons. The bottom image is the merged figure. Scale bar, 100 μm. (d) Counts of radioactivity in samples from somal and axonal compartments after [^{35}S]methionine was localized to the axonal compartment for over 20 h. Counts in the somal compartment (3.7 c.p.m. ± 1.5 s.e.m.) were similar to background levels. Error bars, s.e.m. ($n = 3$). Reprinted with permission from Ref. 165. Copyright 2005 Macmillan Publishers Ltd.

mRNA, and they demonstrated changes in gene expression in the soma in response to axonal chemical and physical manipulations. This chip has now become an important tool used in research programs at different academic settings, given its potential to reveal details of neuronal pathophysiology in neurodegenerative diseases and traumatic nerve damage.

When neuronal axons are damaged, the distal portion experiences a process called Wallerian degeneration, in which the proximal portion of the axon shrinks back toward the cell body. Regeneration of damaged axons is possible but seems to be hampered by many complicated and interacting factors, including glial scar tissue that prevents the regrowing axons from finding their target cells. One active inhibitory component of the scar tissue is a group of molecules composed of core proteins with carbohydrate side chains, known as CSPGs. Neuroscientists studying axonal regeneration have postulated that treatments that limited the production of these molecules at sites of nerve damage might permit axonal regrowth and reestablishment of normal function. Jeon's group tested this hypothesis in their microfluidic chip by isolating axons in a chamber with pre-applied stripes of alternating inhibitory and permissive molecules.[167] The inhibitory stripes contained these aggrecan proteins to mimic glial scars that are generated after spinal cord injury. These inhibitory areas were effectively neutralized when chondroitinase was added to the axonal chamber of the microfluidic chip. This molecule acts to dissolve the carbohydrate glycosaminoglycan side chains of the CSPGs, but left the core protein intact. Since the addition of the chondroitinase allowed axons to cross onto the CSPG stripe, they concluded that the core protein of the CSPG was not inhibitory, but the carbohydrate side chains were the active inhibitory component of the CSPG molecule (Figure 1.9).

Other proteins inhibitory to axon growth have also been tested by this research laboratory. Park et al. used their axon isolation chip to test the effects of two myelin-associated proteins on axon regeneration.[168] After the axons grew into the isolation chamber (about 7 days), they were severed with vacuum aspiration and their regeneration was monitored in the presence and absence of various concentrations of NOGO-66 and MAG protein. Each of these proteins decreased the length of regenerated axons by 75–80% compared to controls. Higher concentrations of NOGO lead to increased inhibition of regeneration, with the effect saturating at a concentration of 10 nM.

The effect of toxins on the electrophysiology and morphology of neurons has been studied using similar axonal isolation microfluidic chips that are combined with MEA substrates. Ravula et al. mated a PDMS superstructure onto a glass substrate with a patterned MEA and recorded the spontaneous and stimulated electrical activities from neurons that they cultured in this platform.[169] They were able to record action potentials from both the soma and the isolated axons when they added potassium chloride. When the sodium channel blocker tetrodotoxin was added to the axonal compartment, only axonal action potentials ceased. In follow-up work, Ravula et al. tested the effects of other chemicals on the electrophysiology of fluidically isolated neuronal soma and axons. They found that low-dose vincristine caused no effect if directly applied to the soma of the neuron, but axonal application sequentially caused a decreased excitability of the axon, an initial increase in excitability of the soma, and
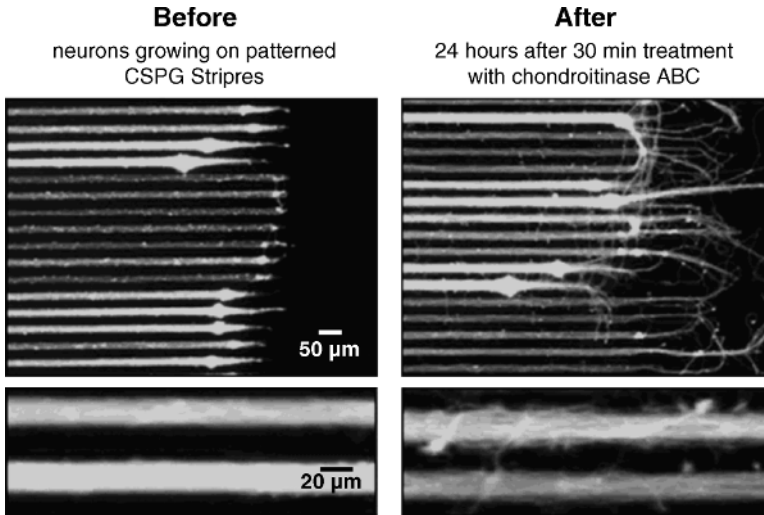
Before

neurons growing on patterned
CSPG Stripres

After

24 hours after 30 min treatment
with chondroitinase ABC

FIGURE 1.9   Effect of chondroitinase ABC treatment on axons growing in microfluidic strip assay device. 10 DIV cortical neurons growing on aggrecan–PLL patterned strips on glass. Axons avoid inhibitor-coated areas until a drug is applied to help axons overcome the inhibition or break down the surface-bound inhibitors. Axons avoid aggrecan strips and are confined to PLL strips during entire experimental period. Striking changes in growth cone morphology and axonal projections were observed when the pattern was treated with chondroitinase ABC, an enzyme that removes the CSPGs' side chains without degradation of the core protein. After 30 min ChABC treatment, axons are observed to randomly extend across the pattern. Higher resolution micrographs indicate that aggrecan inhibition is overcome not only in the growth cones but also in the middle of the stripes. Reprinted from Ref. 167. Copyright 2008 Elsevier.

an eventual degeneration of the axon.[170] Higher dose vincristine caused degeneration if applied to either compartment. The change in electrophysiology occurred approximately 6 h after vincristine exposure and was an earlier indicator of degeneration than were morphological changes. The degradation of the electrical response to a depolarizing dose of potassium chloride is first noted in the distal axon and then progresses proximally. Eighteen hours after exposure, morphological degeneration begins and progresses at a rate of 1–2 mm per day. All the above studies indicate that microfluidic axonal isolation chips, with or without electrical monitoring ability, hold promise for high-throughput screening of many pharmaceutical agents that have effects on neuronal health and might be useful in enhancing axon regeneration.

1.5   DISCUSSION AND FUTURE PERSPECTIVES

Neuroscientists have typically been very open to the use of cutting edge technology for the study of neuronal physiology and have incorporated this technology into their daily research. The challenges of applying novel micro- and nanofabricated hardware to classic neurophysiology experiments will hopefully be matched by their potential

yield. The strength of microfluidics is in the highly efficient utilization of the reductionist approach in well-defined, tightly focused environments and problems. Another advantage of microfluidics is the integrability with multiple methods of interrogation, including chemical, optical, and electrical methods. Microfluidic platforms have the ability to deliver individual cells to specific locations, and then allow study of the effects of temporally and spatially controlled environmental perturbations on isolated parts of the cells. Arrays of these platforms can be arranged to allow large parallel experiments that can multiply experimental yield. When analytical components (such as NMR coils, pH, and electrical and chemical sensors) are incorporated into the microfluidic chips, real-time information on individual live cells can be recorded and followed. It is possible that the conclusions drawn from studies of conventional cell culture populations may not be borne out at the single cell level. Determining which *in vitro* cellular behaviors are most consistent with *in vivo* realities will then become imperative since it is likely that the choice of culture techniques may influence experimental results.

Initiating a microfluidic-based research program is much simpler than one might think. Most clean room fabrication facilities at universities already have the technology to create the master for the PDMS platform since it is the same as that used to create silicon wafer-based electronics. If there is no access to these services locally, commercial businesses and some universities (such as the University of California at Irvine Integrated Nanosystems Research Facility Foundry[171]) do offer these services for a fee. Once the master is available, the supplies and the equipment to cast the PDMS chips are common and inexpensive. For example, PDMS (sold as Sylgard® 184) is available from Fisher Scientific for about $60 for a 1.1 pound kit, which will be sufficient for dozens of chips. Vacuum pumps (to degas the PDMS prior to pouring), and ovens (to bake the mold), will improve the quality of the casting, but these are not necessary for chips that lack very small features. The only other tools that may be necessary are punches to gain access to the channels in the chip, and syringe pumps, if called for in the design. Therefore, once a master is obtained, the ability to produce multiple chips is within easy reach to life scientists.

For those who prefer to purchase their PDMS chips, complete microfluidic fabrication foundries (such as those located at Stanford University and California Institute of Technology) offer commercial services to build chips based on original designs sent by researchers. Plasma treatment equipment confers the advantage of temporarily altering the surface of the PDMS to make it more hydrophilic and able to irreversibly bond to the substrate. However, reversible bonds are sufficient for most low-pressure cell culture applications, and, if desired, the PDMS surface can be made hydrophilic by simple exposure to media containing 10–15% serum.

Commercial multielectrode arrays and supporting electronic modules are available from many sources and can be used as the substrate for microfluidic chips. Substrates with specialized nanotopographic features can be produced by most clean room fabrication facilities. Patterned cell adhesives are easily obtained using dedicated PDMS stencils or stamps. Three-dimensional scaffolding such as collagen sponges from BD Biosciences or Inamed Biomaterials can be used within PDMS growth chambers for studies on tissue engineering within microfluidic chips.

Interdisciplinary collaborations between neuroscientists and engineers may offer the best chance for success for life scientists who plan to initiate a microfluidic research program. In this setting, the engineers are often happy to provide technical advice and hardware in exchange for biological applications for their technology. This may be especially fruitful for each member of the team since federal funding agencies are recognizing the importance of interdisciplinary work for innovative groundbreaking fundamental research and industrial applications.

As we look toward the future, material science issues may become a critical factor for the progress of microfluidic neuroscience. The literature cited above validates the potential for microfluidics, but issues of possible chemical interactions between the PDMS and the cells or the culture medium will have to be resolved. It is known that many materials that are used in standard *in vitro* studies may interact with culture contents. For example, proteins are known to adsorb onto polystyrene culture flasks but the relative ratio of the culture medium volume to the polystyrene surface area usually makes the impact of this phenomenon negligible for most applications. However, molecular and fluid interactions with PDMS on tiny culture volumes will be more likely to play a role in influencing results. Potential toxic effects of the PDMS on sensitive neurons will also need to be controlled. It may be that PDMS will have to be layered with other polymers, like parylene, to limit its interaction with media. Or PDMS may have to be replaced with other polymers that are not as "porous" to water and solutes.

Finally, environmental controls may have to be built into the chips so that their temperature, osmolarity, and atmosphere are preserved during manipulation and imaging of the chip. Given the platform's small volume and lower homeostatic reserve, the heat, humidity, and carbon dioxide levels established in an incubator may otherwise change very quickly as the chip is removed for microscopic examination or media changes. The larger ratio of surface area to volume of these small culture chambers may also lead to a greater tendency toward evaporation and osmotic concentration of the media. All these issues can result in significant effects on the cell's physiological responses and viability. So, to take advantage of the platform's ability to precisely control the milieu of the cells it contains, we may first need to engineer a controllable environment for the platform itself. Despite these challenges, the application of microfluidic technology to appropriate *in vitro* neuroscience research has the potential to offer new insights and to augment ongoing conventional *in vitro* and *in vivo* investigations.

As with all technology, there are certain limitations and challenges to microfluidic studies, as outlined in Section 1.2.5. The "ground-up" approach to studying cellular interactions may yield truly novel results, but caution must be exercised before generalizing information from single cell studies to either homogeneous or heterogeneous populations of cells. Moreover, as demonstrated in the field of tissue engineering, generalizing conclusions from any two-dimensional cell culture (whether microfluidic or conventional in scale) to *in vivo* settings may also be misleading. But, as long as we keep our perspective, we can combine the results obtained from both conventional and alternative methodologies to obtain a greater understanding of the processes we are trying to decipher.

In conclusion, the science of studying individual neuronal cells is still in its infancy, but we now have the tools needed for this endeavor in the form of microfabricated microfluidic channels and electronic sensors that provide the platform for cell proliferation, separation, differentiation, and monitoring. Newer designs also allow isolation of individual axons on these cells, which will enable study of localized axonal physiology and its effects on the cell body. Combining microfluidics with other nanotechnologies will enable truly novel experiments never previously possible. As this chapter outlines, there are many applications of microfluidics to neuroscience, and individuals who can make use of this technology may pioneer entire new areas of research, including studies of individual axons, individual synapses, or the interactions of single neurons with other isolated CNS cells (microglia, oligodendrocytes, and astrocytes).

Advances in other complementary microtechnologies, such as noninvasive single cell electrical recording, monitors for other key markers of cellular physiology (such as pH and ionic currents), tools for NMR spectroscopy, mass spectrometry, and designs to allow on-chip genetic manipulation and evaluation, should allow rapid advances to be made toward understanding neuronal physiology. Once we understand how these cells operate under controlled conditions, we may be able to use this information to gain control of cell responses in disease, and design treatments and possible cures for patients suffering from neurologic diseases.

ACKNOWLEDGMENT

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