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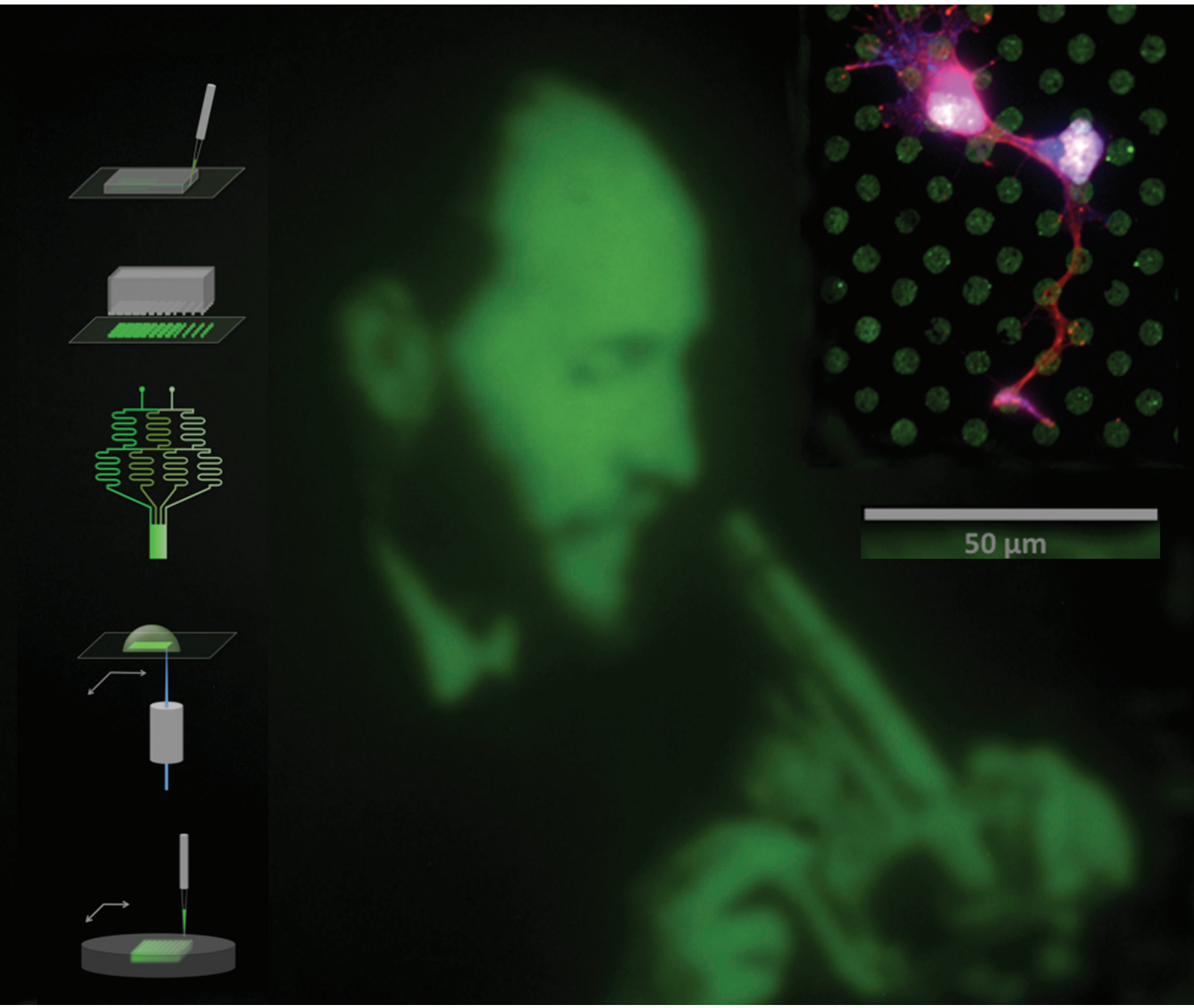

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Engineered cell culture substrates for axon guidance studies: moving beyond proof of concept

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Promoting axon regeneration following injury is one of the ultimate challenges of neuroscience, and understanding the mechanisms that regulate axon growth and guidance is essential to achieve this goal. During development axons are directed over relatively long distances by a precise extracellular distribution of chemical signals in the embryonic nervous system. Multiple guidance proteins, including netrins, slits, semaphorins, ephrins and neurotrophins have been identified as key players in this process. During the last decade, engineered cell culture substrates have been developed to investigate the cellular and molecular mechanisms underlying axon guidance. This review is focused on the biological insights that have been achieved using new techniques that attempt to mimic *in vitro* the spatial patterns of proteins that growth cones encounter *in vivo*.

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Introduction

Understanding the fundamental molecular mechanisms underlying axon guidance remains a challenging biological problem. The wiring of the nervous system is an autonomous process that creates a circuit several orders of magnitude more complex than the most sophisticated microchip ever produced. The formation of this overwhelmingly complex structure is ultimately governed by stochastic molecular interactions that repeatedly yield the same electrical design. Since the beginning of the last century, neuroscientists have made tremendous progress in identifying the molecules critical for directing extending axons to their synaptic targets.[1–3]

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From a clinical perspective, obtaining a better understanding of axon guidance mechanisms is opening new avenues for the development of therapies in regenerative medicine. The possibility of reconnecting the nervous system after trauma or overcoming the impairments caused by a degenerative disease is the basis of substantial contemporary research that typically engage increasingly interdisciplinary teams. In particular, the combination of expertise in molecular biology with techniques in micro-fabrication is yielding an impressive array of new technologies and biomimetic materials.

Engineered culture substrates can now be tailored to investigate the response of growing axons challenged with highly detailed spatial distributions of molecules. Not surprisingly, these new technologies have played a major role in the progress toward understanding the effects of tropic and trophic factors. They have facilitated studying the behavior of neurons in controlled microenvironments and contributed to the identification of new guidance cues.

The goal of this article is not to review the latest technologies for studying axonal guidance, but rather to consider the biological insights that have been achieved as a result of the application of these new methods. The technical details of the most important methodologies,[4–6] such as protein patterning,[7,8] approaches to study diffusible axon guidance cues, such as the Campenot[9] and Dunn[10] chambers, 3-dimensional functionalization of hydrogels,[11] and microfluidics[12] have been extensively reviewed.

We begin with a brief and simple introduction to axon guidance to present the basic mechanisms and some of the ligands demonstrated to contribute to axon pathfinding. We provide a brief historical overview of pioneering studies that have driven the field and consider their main discoveries. The essential biological contributions of the different technologies are presented, grossly divided by categories. We close with a personal view of the progress achieved so far in the area and the present and future challenges.

Santiago Costantino

Santiago Costantino received his PhD in ultrafast lasers from the Physics Department of the University of Buenos Aires, under the supervision of Oscar E. Martínez, in 2003. He moved to Canada for his postdoctoral training in microscopy and neuroscience at McGill University. He established his biophotonics lab at the Maisonneuve-Rosemont Hospital, Montreal University, in 2007. He is now an assistant professor and his current research spans microengineering, image analysis and the development of medical tools for vision health. In 2009 he received a career award from the FRQS. He is married to Claudia and has two wonderful children, Manuela and Tomás.

Axon pathfinding and guidance cues

An axon pursuing its target faces enormous challenges in the developing embryo. The distance to its final destination can be relatively large and, to get there, it must correctly interpret a multitude of cues embedded in a highly rich and dynamic environment. Multiple studies have established that although synaptic activity refines neuronal circuits once they have been established, extracellular molecular cues are the critical stimulus that initially directs axons to their targets. Axons appear to use three main strategies to reach their goal: they extend early during development when distances are smaller, they utilize intermediate targets that break up long complex trajectories into smaller more manageable steps, and axons that extend later in development often migrate using cell–cell interaction either along radial glia or fasciculating with and following earlier pioneer axons.

The growth cone at the tip of an axon is a motile structure that is sensitive to guidance cues in its environment.[13,14] The outer domain of a growth cone is composed of filopodia and lamellipodia, highly dynamic membrane protrusions at the leading edge of many motile cells.[15] Filopodia are thin finger-like extensions of actin bundles that can probe the surrounding environment. Lamellipodia are flattened veils of membrane, with a dense actin meshwork between the filopodia. It has been shown that the contact of the tip of a single filopodium with an appropriate extracellular target is sufficient to cause a growth cone to turn,[16,17] indicating that receptors for guidance cues are present at the tips of growth cone filopodia. Disruption of these structures causes errors in axon guidance.[18,19] Hence, as growth cones probe their local environment by extending and retracting filopodia and lamellipodia, guidance in one direction or another occurs through selective stabilization of these F-actin-based membrane protrusions on one side, coupled with the withdrawal and collapse of the trailing edge on the opposite side.

Although multiple families of axon guidance cues have been identified and their number continues to increase, the diversity of known cues is small in light of the immense complexity of the nervous system. Multiple extracellular matrix (ECM) components influence axon extension during neural development.[20,21] Among these, the laminin family is notable. Many types of neurons, derived from either the central nervous system (CNS) or peripheral nervous system, readily extend axons on laminin. Laminins are a major component of basement membranes, a layer of ECM at the base of epithelia.[21] Multiple proteins interact with laminins. Particular important laminin receptors are the integrins, a large family of receptors for ECM proteins.[22] Laminin is known to promote axon regeneration in the peripheral nervous system following injury[23] and depletion of laminin from preparations of peripheral nerve myelin substantially reduces its capacity to

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promote axon growth. Laminin-1 is very commonly used as a permissive substrate that promotes axon outgrowth in cell culture. It has been shown to exert a modulatory influence, changing the response of growth cones to gradients of other guidance molecules.[24,25]

Netrins are a small family of laminin-related proteins that direct axon outgrowth during embryogenesis.[26,27] They are bifunctional, attracting some axons and repelling others. Although netrins 1–4 are secreted and are often studied as diffusible cues, *in vivo* they are tightly associated with the ECM and recent studies have focused on the contribution of adhesion to netrin function. For example, these studies indicate that netrins must be anchored or immobilized in order influence axon growth.[28–30]

Slits were initially identified as axonal repellents at the embryonic CNS midline.[31] In addition, Robos, which are receptors for Slits, also exert a neuromodulatory function. For example, Robo contributes to silencing of netrin–1–DCC signaling in commissural axons once they reach the ventral midline of the embryonic spinal cord.[32] Functional contributions of Slits and Robos have now been demonstrated at many phases of brain development.[33]

Semaphorins are a large family of secreted and membrane-associated proteins. The first evidence that semaphorins function as axonal chemorepellents was provided by the demonstration that semaphorin–3A could collapse sensory ganglion growth cones *in vitro*.[32] Although they are best understood for their role as repellents that affect axon steering, fasciculation, and branching,[34–36] like many axon guidance cues, they are bifunctional and also promote the growth of some axons.[37,38]

Graded expression of ephrins across the tectum, and complementary gradients of their receptors, the Eph tyrosine kinases, play key roles directing the projection of the retina to the tectum.[39] EphA receptor expression by retinal ganglion cells is also graded and ephrin-As in the tectum direct the topographic projection of retinal ganglion cells (RGC) axons along the tectal anterior/posterior axis. Complementing this, graded expression of EphB receptors by retinal ganglion cells and ephrin-Bs in the tectum direct the formation of lateral to medial projections into the tectum.[40,41]

Finally, the neurotrophins are a small gene family of secreted growth factors, associated with synaptic plasticity, axon growth and survival, but when presented as a gradient, they can act as guidance cues.[42,43] In particular, nerve growth factor (NGF)[44,45] and brain-derived neurotrophic factor (BDNF)[46] can influence axon extension by regulating signal transduction mechanisms that direct actin remodeling.[47,48]

Although we focus here on axon guidance by patterns of substrate bound proteins, we acknowledge that not all such guidance cues encountered *in vivo* are bound to substrates or extracellular matrix, nor are they necessarily always proteins. Guidance can involve proteoglycans or growth factors and other soluble molecules, and even non-molecular interactions, such as stereotropism, where migrating cells will follow structural discontinuities in their local environment.

Brief historical perspective

Engineered cell culture substrates attempt to mimic the graded expression of proteins that axons encounter *in vivo*. Discoveries made by Roger Sperry in the early 1940s provided strong evidence that chemical gradients direct axon growth.[49] As a result, it became essential to identify the key molecular players, characterize their distribution *in vivo*, and attempt to reproduce these distributions experimentally *in vitro*. With the goal of studying how neurons respond to the identified guidance cues, studies sought to measure cell survival, axon extension, growth cone turning and branching, all of which have begun to provide new insight into how cells interpret spatial information. As a first order approximation, graded distributions can be depicted by their mean concentration and slope, and the majority of studies so far have aimed to address these two parameters.

The first studies to apply substrate engineering as a tool for neuroscience appeared in the late seventies. Work by Letourneau and colleagues illustrated the use of electron microcopy calibration grids to both deposit metals on coated surfaces and to function as masks for UV irradiation for neuronal culture substrates. Cell adhesion on patterned dishes could quantitatively demonstrate *in vitro* that growth cones avoided non-permissive areas of the substrate when choosing the pathway along which to extend.[50,51] Furthermore, the adhesive guidepost hypothesis was tested using substrates with laminin coated squares separated by non-adhesive regions of variable width.[52]

A series of pioneering studies that used substrate engineering to investigate axon growth was carried out by Bonhoeffer and colleagues more than two decades ago.[53] In contrast to destructive methods that generated non-permissive regions by denaturing proteins with UV light, their ''stripe assay'' allowed substrates to be patterned by depositing different solutions of membrane fragments in separate lanes on Nuclepore filters that were then used to support neurite outgrowth. Mimicking choices made by these axons *in vivo*, they found that temporal retinal axons demonstrate a preference to grow on membranes derived from the anterior rather than posterior tectum. They showed *in vitro* that this preference disappears for tectal membranes derived from chick brains that were more than two weeks old, coinciding with the completion of the development of retinotectal projections *in vivo*. On the basis of these studies they concluded that a repellent molecule in the membrane solutions was selectively recognized by temporal axons.[54] This tectal repellent (ephrin-A5) was subsequently shown to be expressed in a spatiotemporal graded pattern during development[55] and then found to be a ligand for Eph-related tyrosine kinases.[56] A detailed protocol for generating such stripes to test the response of growing axons, as well as migrating cells, is available.[57]

In a series of early studies, Gundersen, Park, and their colleagues used brass blocks to make patterns of substrate bound NGF to demonstrate that growth cones of one-week-old ganglia were responsive to such patterns, but not two-week old

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ganglia.[58,59] Later, the development of micro-contact printing (μCP)[60] and its use in cell biology[61,62] substantially increased the capacity to present complex patterns of substrate bound protein to cells in culture. The growth cones of mammalian neurons are typically in the range of approximately 10–20 μm wide and the capacity of μCP to engineer cell culture substrates with micron resolution therefore has the potential to probe the mechanisms that neurons use to explore the microenvironment at an unprecedented level of detail. Such techniques have allowed the investigation of cell adhesion sites,[46,63] the production of graded distributions of printed protein dots[64,65] and the generation of continuous gradients.[29]

Stripe assay

As stated above, the stripe assay was originally developed to study axonal guidance mechanisms implicated in the development of the visual system.[53,54] The implementation of the assay requires the fabrication of striped carpets on glass, plastic or Nuclepore membranes using a specially manufactured matrix of channels that are filled with protein solution (see Fig. 1A). Although the patterns that can be obtained with this technology are simple and not particularly versatile, it is one of the most widely used for neuroscience research of all technologies reviewed here. This technique succeeded to become a standard assay in larger studies, where the molecular biology and signaling pathways of axon guidance are analyzed in depth, providing information that is complementary to more classical molecular biology approaches like western blotting, directed mutagenesis, antibody and drug inhibition, and transgenic animal models. Importantly, quantification of the results obtained with this assay is relatively straightforward and provides an accurate way to determine how axons respond to the pattern. It has been used as a basis for the discovery of guidance cues, to investigate outgrowth and regeneration, and to dissect signal transduction mechanisms underlying axon guidance.

As an example, this protocol was used to investigate the role of serum response factor (SRF) in neurite outgrowth and axon guidance.[66] In this study, hippocampal neurons derived from an SRF-deficient mouse grown on stripes of ephrin-A5 were found to be less sensitive to this repulsive cue, providing evidence that SRF regulates the guidance of mossy fiber axons. This assay was also used to examine the function of EphA in axon guidance from the vomeronasal organ (VNO) to the olfactory bulb.[67] The differential expression of ephrin-A5 in the VNO and the graded expression of EphA6 in the accessory olfactory bulb were studied. The stripe assay revealed that VNO axons would preferentially grow on lanes containing EphA6. The stripe assay was also used to demonstrate the preferential growth of thalamic and cortical axons on stripes containing recombinant limbic system-associated membrane protein (LAMP). In a loss of function analysis, when antibodies against LAMP were added, the preference disappeared.[68]

Myelin inhibits axon regeneration in the CNS and the stripe assay has been used to challenge neurons with myelin and

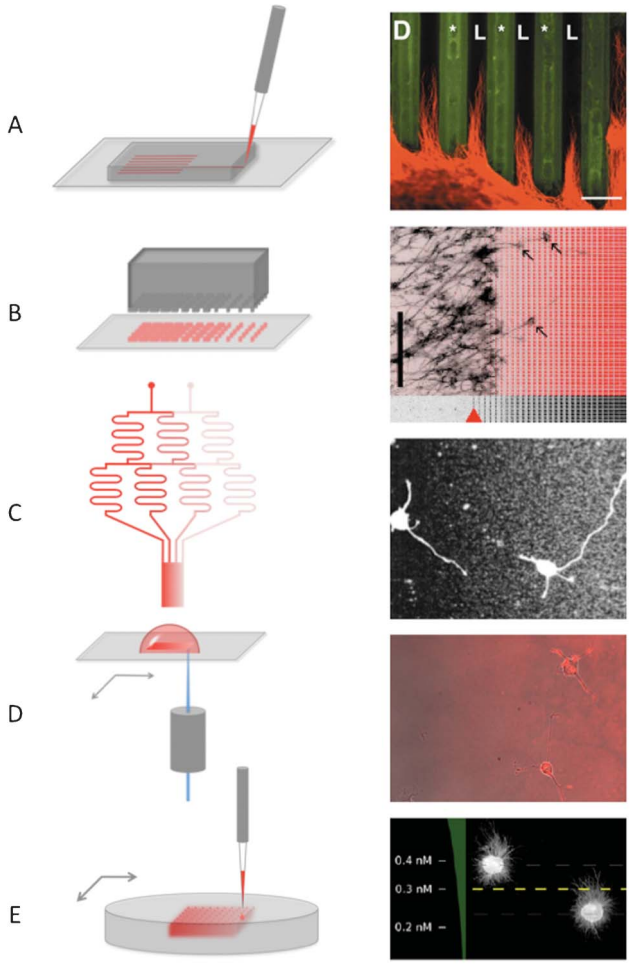

Fig. 1 Engineered culture substrates: (A) stripe assay: a silicone matrix attached to a coverslip containing multiple channels is filled with a solution of guidance proteins using a syringe or a vacuum system. On the right, stripes of permissive laminin alternating with axon growth inhibitor Sema4D. Reprinted with permission from ref. 71. Copyright 2006, Society for Neuroscience. (B) Microcontact printing: PDMS stamp with micrometer-scale patterned surface is used to transfer compounds to a cell culture surface. On the right, nasal RGC growth cones stop extending when exposed to an ephrin gradient. Reprinted with permission from ref. 64. Copyright 2003, The Company of Biologists. (C) Microfluidics devices: a PDMS block engraved with channels and reservoirs is sealed with a coverslip. The serpentine mixer repeatedly mixes and divides solutions to produce concentration gradients. On the right, rat hippocampal neurons exposed to a laminin gradient. Reprinted with permission from ref. 95. Copyright 2002, National Academy of Sciences. (D) Laser-assisted patterning: a laser excites and crosslinks molecules on a cell culture substrate. Protein patterns are made by scanning the surface and varying the laser intensity. On the right, RGC-5 cells guided on a laminin-1 gradient. Reprinted with permission from ref. 111. Copyright 2012, Creative Commons. (E) Patterning 3D hydrogels: nanoliter droplets of proteins are deposited on a collagen matrix using pump and a motorized stage. Reprinted with permission from ref. 121. Copyright 2010, National Academy of Sciences.

investigate the underlying mechanism.[69] The authors used cortical neurons grown on membranes derived from the hippocampus at different developmental stages (from post-natal day 0 (P0) to day 60 (P60)). Newborn rat entorhinal cortex neurons, which project to the hippocampus, preferentially

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extended on early postnatal membranes. The study also demonstrated increased axon elongation on adult membranes when myelin was inactivated with a monoclonal antibody or when the membranes were depleted of myelin. Nevertheless, when presented with a choice between P0 and P > 15 membranes, axons extended preferentially on P0 membranes regardless of myelin treatment, consistent with the P0 membranes either containing a growth promoting factor that the older membranes lack, or that multiple inhibitory cues are present in the older membranes.

The role of axon growth inhibition by glycosaminoglycans, such as chondroitin sulfate proteoglycans (CSPG), which are major inhibitors in myelin, has been investigated using the stripe assay and retinal neurons.[70] This study documented a dose dependent inhibition of axon growth by CSPGs and that incubation of retinal explants with inhibitors of downstream effectors of CSPG signal transduction reduced the effect.

As last examples, two studies used this assay to address semaphorin function as an inhibitor associated with CNS lesions. One group reported that Sema4D is upregulated in CNS lesions and used the stripe assay to demonstrate a growth inhibitory role for postnatal sensory and cerebellar granule cell neurons.[71] The second study used stripe assays to provide evidence for protein synthesis contributing to the repellant response of embryonic spinal motor neurons to stripes of Sema3F.[72]

Microcontact printing

This technology represents an evolution of the stripe assay. While the basic idea is the same, there is substantially more flexibility in the spatial patterns that can be produced, both in terms of resolution and shape. In μCP, stamps with micrometer-scale patterned surfaces are used for transferring compounds to a cell culture surface (see Fig. 1B). Polydimethylsiloxane (PDMS) is the most commonly used material to fabricate these stamps, because it can be easily molded using a master and it results in a polymer that provides a close conformal contact between the stamp and the surface. The stamp is subsequently inked in a solution that is imprinted on the surface. Although several low-cost and rapid alternatives have been proposed,[73–76] soft lithography still usually relies on the use of photolithography to generate the master. Once the master is available, the subsequent procedures can generally be performed outside a clean room. Excellent recent technical and applications reviews,[77–79] and also exhaustive protocols[80] are available.

Initial studies with neurons showed that μCP could be used to promote axon extension along intricate patterns[81–84] that typically involved printing combinations of laminin and poly-D-lysine (PDL). An intriguing study examined synapse formation between axons and neurites using a mix of ECM molecules that was patterned as a grid on a polystyrene surface.[85] Rat embryonic cortical neurons following 12 days *in vitro* were analyzed by double and triple patch clamp measurements with cells on neighboring grid nodes. Cell-to-cell contacts appeared to be reduced by a factor of 3 in the grid pattern as compared to on homogeneous substrates, but the rate at which two cells form a contact remained the same. Cell pairs that were not synaptically coupled did not avoid contact and overlapped to a similar extent as coupled pairs. This provided evidence that recognition between pre- and postsynaptic cells is required for the establishment of a synaptic contact, which was supported by the observation of significant enrichment of reciprocal contacts between excitatory and inhibitory neurons.

Investigating axon guidance by repellent cues, μCP was used to generate a pattern where the spatial density of small dots or lines was varied to generate a global protein gradient.[64] Patterns of ephrin-A were fabricated to study how neurons decide where to stop in a gradient of a repellent molecule. As opposed to nasal retinal RGC axons, temporal axons invaded the patterns but only advanced a certain distance before they stopped. Interestingly, the position of this stop zone in the pattern could be changed by varying the slope of the pattern and it was found that high slopes caused neurons to stop earlier, at a point in the gradient containing a relatively lower concentration of protein. This behavior was also observed when stamps were inked with different concentrations of proteins, the stop zone advanced deeper into the patterns of lower concentration. This suggests that neurons somehow manage to sum the amount of protein on the pattern for determining when to arrest extension. A notable limitation of μCP is that while it is possible to change the size, dot concentration and density of patterned elements, the method does not provide a straightforward means to change the local concentration of protein within the pattern stamped onto the substrate.

More recently and using a similar approach, but employing gradients of combinations of laminin and poly-L-lysine, rat cortical neurons were found to extend processes up the gradients, but the neurites were significantly longer with increasing concentration of protein.[86] Gradients with lower slopes reduced the difference in directional growth. Changing the width of the printed protein spots did not result in statistically significant differences. When exclusively axons, and not other neurites, were considered, a different pattern of spot size and slope was the most attractive, guiding 84% of the axons, consistent with axons and dendrites interpreting spatial cues differently.

Microfluidics

Microfluidic devices to study the response of neurons challenged with molecular gradients are a compelling option for understanding axon guidance *in vitro*. These techniques have the capacity for seamless integration of long-term culture with highly controlled measurements and chemical delivery. They can create substrate bound protein gradients of high dynamic range and they are unique in readily providing for precise control of timing. Furthermore, the cellular micro-environment can be changed during the experiment and it is possible to generate temporal concentration gradients of

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soluble proteins, such as waveforms or pulses. The most widely used approach to microfluidics is soft lithography, in which a polymer (often PDMS) is poured onto a silicon mold featuring channel and reservoir geometries, and is then cured and demolded. PDMS is transparent from ultraviolet to the near infrared, biocompatible, and non-toxic, which makes it ideal for cell culture, but natural materials such as collagen and other hydrogels have also been utilized in microfluidic devices.[87,88] Microfluidic devices may be passive, or flow can be driven by external pumps or capillary action, and their use for cell culture systems has been thoroughly reviewed.[89–91]

The serpentine premixer is an example of a microfluidic device that relies on both flow and diffusion to produce sophisticated concentration gradients.[92] It typically consists of two inlets for solutions that will repeatedly mix and divide in serpentine channels (see Fig. 1C). Detailed protocols for their fabrication[93] and review articles[94] are readily available.

In initial applications to studying axon guidance, the serpentine mixer was used to fabricate substrate bound gradients of laminin by allowing the protein to adsorb to the chamber walls.[95] Although the absolute concentration of bound protein was not determined in this study, the relative steepness of the various gradients was measured. It was observed that laminin alone was sufficient to promote directed growth of neurites of rat hippocampal neurons when they were challenged with a steep gradient.

A more systematic follow-up study challenged rat dorsal root ganglion (DRG) neurons with multiple-component gradients of laminin-1, BSA as a neutral control, and inhibitory CSPG.[96] Gradients of all three molecules rendered the expected guidance effects when presented to cultured cells one by one. This study also assessed the influence of opposed gradients of laminin and CSPG, and found that they resulted in somewhat stronger guidance, suggesting a synergistic effect that promoted axon turning. Consistent with this, parallel gradients of laminin and CSPG neutralized guidance. The authors also provided evidence that the effect of single-component gradients depended on both slope and absolute concentration.

Microfluidics has also been used to demonstrate that linear gradients of soluble BDNF promoted axon turning toward decreasing concentrations of protein, in embryonic *Xenopus* spinal neurons.[97] In this study, neurons responded to the gradient's slope, rather than the absolute concentration of protein. In addition, more sophisticated gradient profiles were achieved by increasing the complexity of the mixer. In particular, attractive gradients of substrate-bound laminin combined with a repellent gradient of soluble BDNF could be shown to act synergistically, while pointing these proteins gradients in opposite directions yielded random directional growth. Nevertheless, the synergy depended on the concentration of the cues presented.

Microfluidic networks have also been used as a way to ink proteins on μCP stamps. Using a serpentine mixer, where one of the walls consisted of a solid slab made of PDMS, the microfluidic network was incubated allowing physical adsorption of the proteins in the channels onto the PDMS surface. This surface was then rinsed and used as a stamp to transfer the adhered proteins. The parallel channels were filled with solutions of increasing concentrations of ephrin-A5 to analyze the response of chick embryonic RGC axons.[98] Concentration gradients of different slopes were tested and temporal axons were found to extended longer in gradients with shallow slopes, consistent with what had been found on dotted substrates.[64] Nasal axons were not found to respond to these gradients, either when dotted, or to protein lanes made using microfluidic networks, in contrast to what had been observed using the stripe assay,[56] illustrating the challenge of interpreting findings obtained using different *in vitro* assays.

A similar approach was used to covalently bind BDNF and netrin-1 to epoxy-coated glass substrates in a graded fashion.[29] Microfluidic channels were made in an agarose stamp, which was then pressed against a glass cell culture surface, and the channels filled with increasing concentrations of the proteins. Diffusion out of the channels through the hydrogel rendered continuous gradients of protein bound to the substrate once the agarose slab was removed. Embryonic rat hippocampal neurons grown on these netrin-1 gradients exhibited a preference to initiate and turn toward higher concentrations protein, as had been demonstrated for gradients of netrin-1 applied in solution.[99] Using this method, no significant changes were detected with respect to the absolute concentration of netrin-1 or the gradient slope. In contrast, gradients of BDNF induced different responses from neurons growing on regions of the pattern composed of high *versus* low concentration, since axons exhibited a strong tendency to turn towards areas of mid-level concentration. Furthermore, this response could be switched between attraction and repulsion by manipulating the concentration of intracellular cAMP in the neurons.

Finally, a microfluidic device that generated gradients with negligible flow was used to probe the effect of netrin-1 on mammalian cortical neurons.[100] This system, allowing a high-throughput analysis, illustrated the heterogeneity of cell responses to gradients. A majority of cells extended axons that turned towards higher concentrations of netrin-1. Netrin-1 also increased the rate of axon extension, and the direction of axonal growth was assessed as a function of the area of the growth cone exposed to the gradient.

Laser-assisted patterning

Lasers have been used to fabricate functionalized cell culture platforms by either crosslinking small molecules or proteins to a surface, or by inactivating proteins that are already substrate-bound.[101–106] In general, these technologies allow near-diffraction-limited resolution and, since the patterns are not limited by diffusion or mechanical constrains, the protein arrangements can be made of arbitrary forms and across a wide dynamic range of concentration. For fabrication, either a laser scans the surface with varying intensity and dwell time,[101,103] or an illumination pattern is generated using a mask[107,108] or *via* optical image formation[109,110] (see Fig. 1D).

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Using these techniques, surface-bound laminin, and also a gradient of a short peptide fragment of laminin-1 (19-mer peptide containing the sequence IKVAV) were demonstrated to be sufficient to direct axon growth .[102] Utilizing a Cd/He UV laser and the peptide conjugated to a benzophenone as a crosslinker, gradients were fabricated by moving the laser focus along the surface and changing the scan velocity. Embryonic chick DRG axons were then used to study the dynamics of axon elongation on gradients with low and high slopes. These investigators provided evidence that the growth rate of axons slowed on first encounter with the patterned substrate, but then resumed once the axon became aligned up the gradient.

With a similar approach, but replacing gas by lower-cost and more versatile diode lasers, and using readily available chemical reagents, such as biotin and antibodies conjugated to fluorescent dyes as crosslinkers, the same type of gradients can be produced.[101] Gradients of full-length laminin-1 were shown to influence the direction of initial neuritogenesis, but not guidance using the RGC5 neuronal cell line[111] in a study that implemented a large-scale image analysis algorithm to significantly increase the number of neurons examined.

A similar patterning method was used[112] to compare several mathematical models that describe neurite outgrowth and their differentiation into axons. Using thin lanes of PDL on which single rat hippocampal neurons were attached, and measuring the growth rate of neurites extending in opposite direction from the same soma, they compared averaged length, velocity and acceleration for 18 h in culture. Among the different theoretical models tested, none could reproduce all of the aspects of neurite outgrowth documented in culture, but the systematic analysis carried out illustrated the advantages of each model to describe the process.

The application of a two-photon absorption approach significantly accelerated this technology and was used to show that a series of aligned triangular shapes of PDL was sufficient to promote directional growth from base to apex.[103] This polarization depended on the size of the triangles; small triangles (~10μm) induced an asymmetric growth towards the apex, but neurons growing on larger triangles (>50μm) extended their axons equally in both directions. Embryonic rat hippocampal neurons extended longer neurites on patterns of low concentration PDL and live cell imaging revealed growth that stalled at the triangle borders, but in the backward direction only. In this study, the authors showed that exciting the sample with a femtosecond laser significantly speeds up the fabrication process. In addition to the increased cost of the equipment required, this new approach needs very precise control of the axial position of the focal volume and optical modulators to accurately vary the protein concentration on the surface.

Patterning 3D hydrogels

Experiments utilizing 2D cell cultures have provided the basis for much of our current interpretation of biological phenomena. Nevertheless, cellular behavior can be different when comparing 2D cell culture surfaces with native 3D tissues.[113] As a consequence, biologists and bioengineers alike are actively investigating 3D tissue-engineered scaffolds to better represent native cellular environments in cell culture.[114,115] Producing such scaffolds containing a 3D gradient supported by adequate mechanical and chemical characteristics for neuronal cell survival and axon extension represent perhaps the ultimate *in vitro* guidance assay, although success in generating such cultures will impose several major additional challenges in terms of imaging and quantification. Several types of matrices and gradients have been developed with tissue regeneration in mind,[116–118] nevertheless, only a few studies have focused on using such methods to study cellular mechanisms.

Extracellular scaffolds, such as collagen type I, can be imprinted by dripping droplets of protein solutions in specific locations with a nanoliter precision pump and a motorized stage[43] (see Fig. 1E). Using this technique, the number of droplets per area and their concentration define the geometry of the gradient. Horizontal diffusion accounts for the continuous shape of the gradient while vertical diffusion provides a graded distribution in the third dimension. This method was employed to confirm theoretical models of gradient sensing by growth cones, using NGF as a guidance cue for dorsal root ganglion explants.[119] The model utilized considers the stochastic interactions of ligands and receptors as a fundamental constraint, so as to establish how the mean concentration and slope of gradients affect growth cone decisions. By measuring the asymmetric growth of neurites extending from early postnatal rat DRG explants the authors provided evidence supporting a Bayesian model and illustrated an extreme sensitivity to gradients as low as 0.24% of 2 pM NGF. It is notable to consider that a 0.3% change over 10 μm at 2 pM corresponds to an absolute change of ~1 molecule per millimeter per 1,000 μm^3 (approximately the volume of a growth cone). This same 3D printing technology was used to demonstrate that the outgrowth response to gradients of NGF was dependent on the rostrocaudal origin of the DRG cells.[120]

In an interesting study, such patterns were used to challenge the idea that growth cones generate immediate turns when responding to a gradient. Instead, the authors propose that a modulation of growth rate contributes to axon turning.[121] Using an automated method to identify growth cones exiting from DRG explants they studied axon turning and asymmetric growth,[119] and identified relatively weak turning responses at the axons' tips in samples where neurite growth is strongly biased up the gradient. The authors concluded that the growth process does not necessarily involve a growth cone making a comparison in the extent of ligand-receptor binding between its sides and immediately turning as a result. They propose that the growth cone makes a comparison along its front-to-back axis and then modulates its speed; at each time step the growth cone also turns slightly, but the direction of this turn is hypothesized to be random and unrelated to the direction of the gradient.

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Table 1 Engineered culture substrate methods to study axon guidance

Methods	Type of gradients	Guidance cues	Models	References
Stripe assay	surface-bound, discontinious	SRF, ephrin-A5, ephA, ephA6, LAMP, CSGP, sema3f, sema4D	hippocampal neurons, VNO, cortical neurons, RGCs, spinal motor neurons, cerebellar granule cells	66–72
Microcontact printing	surface-bound, dotted and continious	laminin-1, PDL/PLL, ECM, ephrin-As	DRG cells, hippocampal neurons, cortical neurons, RGCs	64, 81–86
Microfluidics	soluble and surface-bound, continious	laminin-1, CSPG, BDNF, ephrin-A5	DRG cells, spinal motor neurons, RGCs, hippocampal neurons, cortical neurons	29, 95–98, 100
Laser-assisted patterning	surface-bound, dotted and continious	laminin-1, PDL	DRG cells, RGCs, hippocampal neurons	101–103, 111–112
3D hydrogels	continious	NGF, netrin-1, slit-2	DRG cells	119–122

Finally, a microfluidic network was used to produce 3D gradients by injecting ice-cold liquid collagen in a chamber and connecting channels to subsequently deliver guidance cues to only one side of the chamber.[122] In this system, both diffusion and flow account for the shape of the gradient. This system was tested with netrin-1 diffusing from three different sources as a model of chemoattraction, and with slit-2 as a model of chemorepulsion applied to neurons derived from hippocampus or dorsal root ganglia. Different gradient concentrations were tested, revealing responses across a dynamic ranges of concentrations, with some contrast to what has been concluded previously using substrate bound 2D patterns.[29]

Conclusions

Substrate engineering represents a unique opportunity to probe how extending axons interpret the spatial cues in their local environment. These technologies allow exquisite precision in the presentation of the individual molecular components that are essential to this process. Protein patterns fabricated with subcellular resolution on increasingly biomimetic substrates together with high-content studies can substantially increase sample sizes and improve the statistical reliability of *in vitro* guidance experiments. Engineered substrates have allowed the identification and functional characterization of new molecules that regulate axon guidance, the investigation of unknown synergistic molecular interactions and the description of several signaling pathways. They have been used in a wide variety of neuronal tissues and species, and at different developmental stages to perform a systematic characterization of growth cone navigation along protein patterns (see Table 1).

Beyond the quantitative descriptions of the response of growth cones to gradients that have been achieved, major opportunities are now open to further investigate the axonal response. It is commonly accepted that extending axons break down their journeys into relatively smaller trips, reaching intermediate targets before their final destination. An exciting avenue for future studies involves reconstructing the complex spatial distribution of multiple cues *in vitro* using engineered substrates. Such multicomponent substrates will provide unique tools for obtaining a greater understanding of the spatial and temporal mechanisms that underlie the formation of neural circuits. Studying the signaling mechanisms that regulate this process is essential, but has proven to be technically challenging when limited by traditional approaches. They represent, from our personal perspective, a research direction where lab-on-a-chip neuroscience has the potential of achieving breakthrough discoveries.

The field has advanced substantially since the 1980s and exciting applications are expected using the novel substrate engineering techniques that have recently become available. Furthermore, during the last decade a large number of interdisciplinary teams have been created, as well as the first graduate programs in biophotonics and neuroengineering. This will surely lead to a new generation of investigators that will drive neuroscience research with the unique perspective of cross-disciplinary backgrounds.

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