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Axon pathfinding and targeting: (R)evolution of insights from *in vitro* assays

Oriol Ros¹ & Xavier Nicol^{2*}

¹ Universitat de Barcelona, Department of Cell Biology, Physiology and Immunology. Avinguda Diagonal 643, 08028, Barcelona, Catalonia, Spain.

² Sorbonne Université, Inserm, CNRS, Institut de la Vision, 17 rue Moreau, F-75012 Paris, France

* **Correspondence:** Xavier Nicol, xavier.nicol@inserm.fr

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Abstract

1 Investigating axonal behaviors while neurons are connecting with each other has been a
2 challenge since the early studies on nervous system development. While molecule-driven axon
3 pathfinding has been theorized by observing neurons at different developmental stages *in vivo*,
4 direct observation and measurements of axon guidance behaviors required the invention of *in*
5 *vitro* systems enabling to test the impact of molecules or cellular extracts on axons growing *in*
6 *vitro*. With time, the development of novel *in vivo* approaches has confirmed the mechanisms
7 highlighted in culture and has led *in vitro* systems to be adapted for cellular processes that are
8 still inaccessible in intact organisms. We here review the evolution of these *in vitro* assays, which
9 started with crucial contributions from the Bonhoeffer lab.
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Introduction

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19 The complex connectivity of the nervous system is established during development, when
20 neurons find their post-synaptic partners following a stereotyped pathfinding over space and time.
21 This process generates the patterns of innervation that are the basis of a functional nervous
22 system. Such refined connectivity has amazed scientists since Cajal's first drawings, sparking
23 curiosity on the mechanisms that generate these patterns. Only *in vitro* assays can address
24 disputes, such one between defenders of a pure electrical activity-based mechanism and the
25 supporters of axon pathfinding and wiring based on chemical cues and gradients. Both are today
26 accepted as critical processes shaping neuronal networks. However, the chemotropic theory, first
27 formulated by Cajal in the early 1900s (Ramón y Cajal, 1909) and revived by Sperry six decades
28 later (Sperry, 1963), still lacked strong experimental support in the late 1980s. The complexity of
29 neuronal connections in the brain has fueled the need to find simplified models that reproduce
30 the development of neural networks in a dish, as means to understand the mechanisms of
31 nervous system patterning. Friedrich Bonhoeffer pioneered such studies by developing elegant
32 *in vitro* models to assess the behavior of axonal growth cones. His results revealed simple axonal
33 behaviors in response to molecular cues that explained complex innervation patterns *in vivo*.
34 These experimental models provide a controlled environment for growing axons and expose them
35 to discontinuities or asymmetries, thus enabling researchers to track growth cone behaviors and
36 morphological changes when exposed to these singularities. During the early days of the
37 experimental investigation of Sperry's theory, several *in vitro* assays were developed to identify
38 the source of signals that could favor or inhibit axon outgrowth in a given direction. Bonhoeffer
39 was a pioneer in the development of seminal approaches, including the stripe assay that is still
40 used decades after its creation (Walter et al., 1987a, 1987b). Using this simple assay, he identified
41 cellular components with differential effect on subsets of axons and further identified axon
42 guidance molecules including ephrin-As, in parallel with John Flanagan who also used the stripe
43 assay (Cheng et al., 1995; Nakamoto et al., 1996). In the same period, other assays have been
44 developed, leading to the characterization of families of axon guidance cues. Forward or reverse
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1 genetic screens for axon guidance errors later confirmed the cues identified *in vitro* as key players
2 of the development of neuronal connectivity. The Bonhoeffer lab was involved in this switch
3 towards *in vivo* approaches to investigate axon pathfinding (Baier et al., 1996; Karlstrom et al.,
4 1996; Trowe et al., 1996). The advancement of *in vivo* approaches could have meant the
5 progressive replacement of *in vitro* assays, but adapting manipulations performed in a dish led to
6 spread their use and extend the questions that can be explored using axons *in vitro*. With time,
7 the findings addressing these novel questions have been confirmed *in vivo* and have fueled a
8 new adaption cycle of *in vitro* tests. This loop between *in vitro* and *in vivo* methods enabled
9 researchers to dissect with deeper and deeper understanding the cellular and molecular
10 mechanisms underlying the development of neuronal connectivity (Figure 1). We here review the
11 main *in vitro* systems that identified guidance molecules and mechanisms (Table 1), highlighting
12 their past contributions but also their current use, for applications that are not (yet?) accessible
13 by *in vivo* approaches.
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23 **1. Sharp boundaries and abrupt changes in the culture medium**

24 **a. Stripe assay**

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27 The hypothesis that concentration gradients of molecular guidance cues orient axon growth and
28 shape the connectivity of the nervous system waited more than 20 years after Sperry's original
29 theory to receive experimental support. One of the reasons was the lack of approaches enabling
30 researchers to discriminate between axon guidance and axon outgrowth. The stripe assay,
31 developed by Friedrich Bonhoeffer in the late 1980s, has been instrumental in identifying cellular
32 and molecular determinants of gradients of axon repellents and attractants. This assay offers
33 alternated stripes of distinct substrates to growing axons from a cultured explant. The stripes were
34 initially generated by a reagent aspirated through membranes with porous bands separated by
35 impermeable lines, but the technique later evolved by using microchannels attached to a coverslip
36 or by stamping the desired molecule with a patterned silicon mold (Fig. 2A). The stripe assay was
37 first developed using stripes of membranes prepared from either the anterior or posterior chick
38 tectum. Thus, Retinal axons cultured on these patterns were given the choice between two growth
39 environments. Uniform substrates coated with membranes from either the anterior or posterior
40 tectum are permissive for retinal axon outgrowth. However, the stripe assay enabled researchers
41 to demonstrate that, when given the choice, axons from the temporal retina favor stripes of the
42 anterior tectum over the posterior tectum (Walter et al., 1987a, 1987b). This observation
43 highlighted that chemical cues present in the environment of growing axons influence their growth
44 and the development of neuronal connectivity. The same assay was critical to identify the
45 molecular determinants of the temporal axon preference for anterior membranes: temporal axons
46 avoid stripes prepared from COS cells transfected with ephrin-A5, a membrane-bound protein
47 expressed in an antero-posterior gradient in the chicken tectum (Drescher et al., 1995). Similar
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1 studies conducted in the Flanagan lab showed that ephrin-A2, another member of the ephrin-A
2 family expressed in an antero-posterior gradient in the developing tectum, contributes to the
3 repellent activity of the posterior part of the retinal axon targets, still using the stripe assay as a
4 crucial *in vitro* experimental approach (Nakamoto et al., 1996). This assay was used to
5 characterize other families of axon guidance molecules, including Semaphorins (Bagnard et al.,
6 1998), together with the guidance properties of molecules initially associated with other cellular
7 process, including RGM (Monnier et al., 2002) and Sonic Hedgehog (Kolpak et al., 2005).
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10 Genetic manipulations confirmed the influence of these guidance molecules in a wide range of
11 organisms, ranging from *C. elegans* to mammals and including insects and fish. These knock-out
12 and knock-down approaches limited the utility of stripe assays to identify molecules involved in
13 axon pathfinding. However, using purified recombinant proteins in stripe assays enabled
14 investigators to address questions that are still not accessible using *in vivo* approaches. For
15 instance, stripe assays allow the study of distinct protein products from the same gene, which are
16 difficult to differentiate using genetic approaches. Thus, distinct axon repellent activities were
17 found for Slit2 and its N-terminal product resulting from proteolytic cleavage (Slit2-N). Dorsal root
18 ganglia axons avoid stripes of either Slit2 or Slit2-N when grown on laminin, whereas a fibronectin
19 substrate converts Slit2-N, but not Slit2, to an attractant molecule (Nguyen-Ba-Charvet et al.,
20 2001). Many open questions in the field of axon guidance are to identify the cellular mechanisms
21 that enable axonal growth cones to integrate several cues and to translate these signals into
22 morphological changes of the axon tip. The stripe assay allows researchers to combine guidance
23 cues and to adjust the concentration of each of these molecules, in order to expose axons to
24 relatively complex environments that are more controlled and reproducible than *in vivo*. Using this
25 approach, Poliak *et al* elegantly provided evidence supporting the notion that synergistic
26 interactions between netrin-1 and ephrin-A5, rather than a simple sum of their respective
27 influence, control axon pathfinding. Indeed, spinal motor axons are able to discriminate between
28 stripes lacking guidance molecules and stripes coated with subthreshold concentrations of netrin-
29 1 and ephrin-A5 (*i.e.* concentrations that do not repel or attract axons when each of these cues is
30 applied alone) (Poliak et al., 2015). Similarly, the ability to control the concentration of the
31 guidance molecule revealed that individual molecules can be either axon repellents or attractants
32 depending on their concentration. This was shown for Sonic Hedgehog using a stripe assay-
33 based approach (Kolpak et al., 2005). The compatibility of the stripe assay with a quantitative
34 control of the substrate composition makes this approach suitable for computational modeling in
35 a way that is still hardly accessible to *in vivo* assays. For instance, a model calibrated using stripe
36 assay data for forward and reverse ephrin-A/EphA signaling and demonstrated that these two
37 pathways are sufficient to recapitulate retinotopic mapping. This computational model
38 successfully recapitulated the phenotypes of animals with altered ephrin-A/EphA signaling,
39 including EphA knock-in mice with increased EphA expression in about half the retinal ganglion
40 cells (RGCs, Gebhardt et al., 2012). A variant of the stripe assay termed the gap assay enables
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1 researchers to evaluate the ability of a growth cone to adapt to guidance molecules, a process
2 technically difficult to investigate *in vivo*. In this assay, axons are first exposed to a guidance
3 molecule before reaching a gap devoid of the investigated cue. Once the gap crossed, the axons
4 are confronted to a second area coated with the same or a different guidance molecule. This
5 approach enabled researchers to demonstrate that axons desensitize at the same time for ephrin-
6 A forward and reverse signaling, in a process termed co-adaptation (Fiederling et al., 2017).
7 Finally, the stripe assay can be combined with the use of neurons differentiated from induced
8 pluripotent stem cells, thus enabling to assay the behavior of human neurons derived from
9 individuals carrying pathogenic mutations. This approach has been used to demonstrate that
10 neurons differentiated from biopsies of an individual with Tuberous Sclerosis exhibit altered axon
11 pathfinding (Catlett et al., 2021).
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17 While the stripe assay was developed to study axon pathfinding-based mechanisms, the assay
18 is highly versatile and its uses have been extended to investigate other cellular processes,
19 including cell migration (for neurons and other cell types) (Vielmetter et al., 1990; Knöll et al.,
20 2007) or axonogenesis (Shelly et al., 2010). However, in the context of axon guidance, this assay
21 focuses on comparing the differential affinity of growing axons for two substrates. It is thus not
22 ideal to determine whether a substrate is mostly an axon repellent, an attractant or a permissive
23 substrate for the extension of neuronal processes. It also exposes axons to sharp borders rather
24 than shallow boundaries that may better resemble gradients of guidance molecules that are found
25 *in vivo* and better match the hypothesis formulated by Sperry and Cajal. It also makes it difficult
26 to identify the morphological or signaling response of a growth cone at the time it is exposed to
27 an axon guidance molecule.
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37 **b. Bath perfusion including collapse assay**

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39 Focusing on the repellent activity of membranes prepared from the brain, Raper and Kapfhammer
40 developed an assay based on the change of growth cone morphology and on the change of
41 growth velocity of extending axons. In this assay, termed “collapse assay”, putative repulsive cues
42 are applied (bath perfused) to the culture medium (Raper and Kapfhammer, 1990). If the reagent
43 has an axon repellent activity, the lamellipodium of the growth cone collapses before the
44 disappearance of the filopodia (Fig. 2B). At later time points, strong repellents induce axon
45 retraction. This assay was immediately used by the Bonhoeffer lab to demonstrate that
46 membranes from both the posterior and anterior tectum repel axons from the temporal retina.
47 These observations demonstrated that membranes from both the anterior and posterior tectum
48 inhibit retinal axon outgrowth, although axons from the temporal retina extend preferentially on
49 anterior rather than on posterior membranes when confronted to both using the stripe assay
50 (Muller et al., 1990). This highlights the distinct advantages of experimental approaches using
51 either the stripe or collapse assays. Like the stripe assay, the collapse assay is adaptable to the
52 use of recombinant proteins, enabling researchers to characterize the impact on axon extension
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1 of identifiable proteins. The collapse assay was used to demonstrate the repellent ability of many
2 axon guidance molecules, including ephrin-As and Sema3A, among many others (Luo et al.,
3 1993; Drescher et al., 1995).

4 Similar to the stripe assay, the collapse assay lost some of its advantages with the development
5 of the genetic strategies to disrupt gene expression *in vivo*, which enabled the identification of
6 axon guidance molecules within intact organisms. However, dissecting the molecular and cellular
7 mechanisms of axon guidance still strongly benefits from simple *in vitro* approaches like the
8 collapse assay. It has been instrumental in extending the concept of adaptation to guidance
9 molecules first identified using the turning assay (Ming et al., 2002; Fiederling et al., 2017).
10 Additionally, it is extremely useful to study the kinetics of morphological and signaling changes
11 induced by axon repellents. This method has the advantage of being highly compatible with live
12 imaging and is a powerful way to synchronize axonal responses to guidance cues. In addition,
13 acute application of cues is compatible with imaging of biosensors to monitor the changes in
14 intracellular signaling induced by axon guidance molecules. Such a technique is of particular
15 interest when the signal to noise ratio of a biosensor is low, making it difficult to extract data from
16 a single axon. Synchronizing the signaling events by the controlled application of a guidance
17 molecule enables averaging across different axons and eases the discrimination of the signal
18 from the noise inherent to imaging techniques like FRET microscopy. For instance, this approach
19 has been used to monitor subcellular-specific cAMP changes in retinal axons exposed to ephrin-
20 A5 and determine that ephrin-A-induced axon repulsion requires modulation of this second
21 messenger restricted to lipid rafts (Averaimo et al., 2016).

22 Finally, the collapse assay in combination with high resolution time lapse videomicroscopy
23 enables to identify, in a single set of experiments, the distinct steps of axon repulsion and to
24 investigate the signaling pathways associated with each of these steps. Such an approach has
25 been used to highlight that Slit2, but not Semaphorin 3A, induces the elongation of filopodia in
26 dorsal root ganglia growth cones before the disappearance of the lamellipodium. This initial stage
27 of the Slit2-Robo pathway relies on Robo binding to the Ena/Vasp proteins that promote actin
28 polymerization (McConnell et al., 2016). Similarly, live growth cone behaviors observed when
29 tracking the response of RGC axons to ephrin-A5 enabled researchers to determine that the
30 cAMP-synthesizing enzyme adenylyl cyclase 1 does not contribute to the initial growth cone
31 collapse, but is required for the subsequent axon retraction (Nicol et al., 2006).

32 The collapse assay is a useful test to track the dynamic behavior and intracellular signaling of
33 axons exposed to repellent molecules. However, it does not provide a way to mimic the exposure
34 of axons to guidance molecules *in vivo*.

35 2. Gradient-based assays

1 Sharp boundaries mimic the features of the changes in the environment encountered by axons
2 at choice points, where a sudden transition in the extracellular composition modifies growth cone
3 behavior. Nevertheless, some guidance cues are presented in a more subtle fashion, showing
4 shallow borders and increasing concentrations over long distances. This is the case of ephrins in
5 the thalamus/superior colliculus/optic tectum, which give rise to the orderly organization of retinal
6 projections. Gradients of molecules likely also exist at the midline, such as Sonic Hedgehog and
7 netrin-1, although the necessity of floorplate-derived netrin-1 for midline crossing is unclear. A
8 few *in vitro* systems propose strategies to mimic the graded exposure of freely navigating axons
9 to guidance molecules.

14 **a. Collagen/extracellular molecule matrices**

16 The simplest approach to generate a gradient is probably to embed fragments of tissue secreting
17 putative axon guidance cues into a hydrogel. The hydrogel is generated by polymerized
18 extracellular matrix proteins (typically collagen, but several commercial vendors propose
19 alternative formulations). An explant containing neurons that are sensitive to a cue is placed at a
20 distance from the guidance cue expressing tissue (Placzek et al., 1990). The rationale is that
21 secreted proteins progressively diffuse from the source and exert their chemotropic effects on the
22 axons emerging from the neural tissue (Fig. 3A). The tissue secreting the axon guidance cue can
23 be replaced by cell aggregates overexpressing a specific diffusible molecule (Colamarino and
24 Tessier-Lavigne, 1995).

32 Collagen matrices were instrumental in the description of chemotropic guidance in the nervous
33 system and this approach has been used to identify the first described secreted guidance cue,
34 netrin-1 (Kennedy et al., 1994). Extracellular molecular matrices also provide a differential benefit
35 over the methods listed below: the three-dimensional scaffolding of the hydrogel that allows for a
36 more physiological representation of the cellular environment than rigid glass coverslips.

41 The ease of implementation of the method is balanced out by the lack of control of the steepness
42 of the gradient that may vary with different concentrations of hydrogel-forming extracellular
43 proteins, the size of the protein-secreting tissue, the level of expression of the guidance molecule,
44 and the distance between both pieces of tissue. New approaches propose the implementation of
45 gradients inside a matrix by nanoliter deposition of proteins, by means of tightly controlled
46 motorized stage and pumps (Mortimer et al., 2010).

52 **b. Turning assay**

54 Classic experiments with extracellular matrices provide a quick, general view on a determined
55 axon response, but do not offer a detailed vision of the response of the growth cone. The turning
56 assay enables researchers to monitor the behavioral change of an axon exposed to a molecular
57 gradient. This approach was developed to visualize the response of individual axons to polarized

1 shifts in the concentration of intracellular second messengers and allowed for tracking the
2 responses of individual axonal growth cones (Gundersen and Barrett, 1980; Lohof et al., 1992).
3 In the turning assay, chemical solutions are released through the tip of a microcapillary located in
4 the vicinity of a growth cone and positioned away from the axis of axon extension. This allows the
5 generation of a gradient of the loaded molecule with its source on the side of the growing axon
6 (Fig. 3B). Controlled chemical release is made possible by forging a microcapillary to a precise
7 inner diameter and by attaching the micropipette to a positive pressure-generating device gated
8 by an electrical stimulator (Pujic et al., 2008). This set-up has been key to the discovery of
9 polarized growth cone responses to guidance cues and to the description of the asymmetrical
10 signaling cascades involved (Zheng et al., 1994; de la Torre et al., 1997; Ming et al., 1997; Weinl
11 et al., 2003). Furthermore, it enabled researchers to investigate the impact of the substrate on the
12 behavior of axons facing a gradient of a guidance cue. Höpker et al. thus demonstrated that
13 adhesion molecules are signal-carrying substrates influencing the response of axons to a
14 particular cue (Höpker et al., 1999).
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22 Lately, advanced imaging probes and high resolution imaging have enabled researchers to refine
23 the understanding of the cellular mechanisms driving growth cone responses to guidance cues,
24 showing asymmetric signal activation and recruitment of the intracellular machinery, including
25 molecular motors in axons changing the direction of their growth (Leung et al., 2006; Tojima et
26 al., 2007, 2010; Akiyama et al., 2016).
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31 ***c. Patterned microslides for chemotactic assays: Dunn chambers – Ibidi chemotaxis.***

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33 The turning assay generates a gradient in the vicinity of a growth cone, enabling the precise study
34 of a particular axon's response, but it has two major drawbacks: it is complicated to implement,
35 requiring the investment on a couple of expensive units to generate the gradient; and it is time-
36 consuming, allowing the study of a single growth cone at a time. In an effort to increase the yield
37 of each experiment and to be able to investigate guidance in mammalian axons, whose outgrowth
38 is punctuated by long stationary episodes, a series of microslides for chemotactic assays have
39 been designed for the study of axon guidance. Bridge chambers are structured microscope slides
40 presenting two wells separated by a bridge that allows chemical diffusion but impedes fluid flow
41 when a coverslip is attached. Therefore, loading one of the wells with a high concentration of a
42 guidance molecule induces a chemical gradient of the cue across the bridge (Muinonen-Martin et
43 al., 2010), allowing testing of the chemotactic response of the cells found in the bridge or the
44 chemotropic effects on their axons. Another gradient device is the Dunn Chamber, which
45 generates a gradient between two concentric circles engraved on the surface of a microscope
46 slide (Fig. 3C). Using a Dunn chamber allowed researchers to study spinal commissural axons
47 turning towards a Sonic Hedgehog gradient, which depends on a non-canonical signaling
48 cascade involving smoothened and the Src-family kinases (Yam et al., 2009). More recently,
49 using a Dunn chamber strengthened the role of floor-plate-derived netrin-1 as a long-range
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1 attractant of spinal cord commissural axons (Wu et al., 2019). Similarly, Ibidi chambers, a new
2 breed of microslides for chemotactic studies (Zengel et al., 2011) were used to reveal how
3 Synaptobrevin-2 mediates Semaphorin 3A-dependent repulsion, by re-localization and vesicular
4 trafficking of Semaphorin 3A receptors (Zylbersztein et al., 2012). Ibidi chambers encase a series of cell
5 and media reservoirs above a slide generated by plastic molding. These newly developed
6 microslides offer some advantages over the traditional Dunn chamber. Namely, they allow for
7 high magnification imaging using an inverted microscope and easy implementation of high
8 throughput analysis of growth cone behavior, since the gradients are presented linearly
9 throughout the length of the microslide, compared to the concentric presentation of gradients in
10 the Dunn chamber (Muinonen-Martin et al., 2010; Zengel et al., 2011).

16 **d. Microfluidics**

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18 The section above presents machined microslides allowing a controlled chemical flow based on
19 capillarity. Photolithography has enabled the design of intricate patterns of channels into
20 biocompatible silicon molds. This technique allows the generation of a stable linear gradient
21 across a cell-culture chamber. The approach is based on an array of reservoirs containing media
22 with different chemical compositions and microchannels constraining the liquid flow, thus enabling
23 selective medium mixing.
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27 The simplest microfluidic implementations establish a guidance cue gradient inside the
28 microchannels, connecting a “cell culture” well to a “media reservoir”. The axons extending from
29 somas placed in a chamber devoid of any guidance cue grow inside the microchannels and are
30 confronted to increasing molecular concentrations as they grow towards the second chamber
31 (Taylor et al., 2005). Such devices showed that Wnt3a promotes axon outgrowth, as a result of
32 the accumulation and activation of canonical Wnt signaling into the growing axons (Stanganello
33 et al., 2019). Microfluidic chambers have also been used to study transport along the axons, and
34 axon regeneration after axotomy (Encalada et al., 2011; Kim et al., 2012; Castle et al., 2014).
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38 In an effort to distinguish axon guidance from outgrowth, new microfluidic devices establish
39 gradients inside the culture chamber itself, implementing methods to diffuse shear stress induced
40 by medium flow to the culture cells. Two main systems have produced significant results on axon
41 guidance research. The first device (Bhattacharjee et al., 2010) relies on an array of tiny
42 microchannels disposed along either side of the culture chamber, each liberating picoliter
43 amounts of medium with or without guidance cues. This device was able to orient outgrowth of
44 mammalian cortical axons towards netrin-1 gradients. Intracellular signaling is polarized in growth
45 cones with higher phosphorylation of Shootin-1 proximal to the source. Phosphorylated Shootin-
46 1 serves as a mechanical coupler for netrin-1 sensing and axon turning (Bhattacharjee et al.,
47 2010; Baba et al., 2018). The second system using this approach eliminates shear stress by
48 interposing a permeable membrane between the microchannels where fluid mixing takes place,
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1 and the cell culture chamber (Morel et al., 2012). The use of this device revealed polarized
2 expression of GABA_A receptors proximal to a source of GABA on dynamic, mobile growth cones
3 (Morel et al., 2012).

4 **e. Adapted stripe assays for gradient (for repulsion only) – Microprinted gradients and**
5 **patterns.**
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8 The strength of *in vitro* approaches, producing simple answers to a defined, often binary question,
9 is reduced by the fact that *in vivo* environments are complex and developing axons face a variety
10 of combinations of noisy molecular gradients and extracellular matrix proteins. In an effort to
11 replicate the ephrin-A signals that RGC axons face in the visual tectum, the Bonhoeffer lab
12 proposed a variation of the stripe assay, where the printed ephrin-A patterns are presented in a
13 dotted fashion, interrupted by guidance cue-devoid lines of decreasing lengths (von Philipsborn
14 et al., 2006a). The growth cone integrates increasing levels of ephrin-As as it advances on the
15 coverslip, up to a defined point which depends on the position of the RGC within. This setup
16 faithfully reproduced the *in vivo* situation, where RGC axons invade the ephrin-A-expressing
17 tectum with varying invasion distances, that correlate to the soma position in the retina and local
18 ephrin-A levels in the tectum (von Philipsborn et al., 2006b). These studies were made possible
19 by advanced photolithography that allows the generation of micrometer-sized patterns into a
20 silicone stamp, in a technique called microcontact printing. Previous works using this technique
21 established that axons extend preferentially following the tracks of patterned adhesion molecules
22 (Kam et al., 2001; Oliva et al., 2003). Later applications rely on the use of laser-driven protein
23 adsorption onto the glass coverslip to generate complex patterns, an open canvass allowing the
24 design and implementation of multiple and varying adhesion structures to cells (Vignaud et al.,
25 2012). This technique enabled researchers to identify that axons prefer to grow following
26 repetitions of triangular-shaped micropatterns of poly-lysine, and polarize following this pattern
27 (Scott et al., 2012). This approach offers the possibility to print different mixtures of guidance cues
28 with a variety of shapes, having the potential to mimic a multitude of guidance paradigms.
29 Nevertheless, this possibility is yet to be exploited.
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48 **3. Substrate stiffness and mechanosensing**
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50 Although the majority of axon pathfinding studies have focused on chemoaffinity, a key element
51 to transform chemical signals into a change in the direction of axon growth is the regulation of the
52 mechanical forces applied by the growth cone on its substrate, and the ability of its environment
53 to support traction forces (see Franze, 2020 for review). The investigation of the influence of the
54 substrate stiffness on axon guidance has recently expanded, using approaches that either control
55 the stiffness of the 2D or 3D environment of growing axons or directly apply forces on axons
56 exposed to chemical cues.
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a. Manipulating substrate stiffness

Using micro- or nanopillar arrays of different stiffness to measure forces or control topographical cues of cultured cells has been available for many years. However, applying this technology to growing axons has remained a challenge. A few groups recently succeeded in applying this technique to neuronal cells, suggesting that information about axon pathfinding might be obtained using this approach in the future (Moore et al., 2009; Bugnicourt et al., 2014; Fan et al., 2021). However, the main technique that has been used to control the stiffness of the axonal environment *in vitro* is to generate polyacrylamide or collagen hydrogels of controlled rigidity, quantified by measuring the Young modulus. The stiffness of a polyacrylamide hydrogel can be controlled by adjusting the acrylamide:bis-acrylamide ratio to influence the crosslinking of the gel (Koser et al., 2016; Thompson et al., 2019). The elasticity of collagen gels is adapted using collagen crosslinking with genipin in a concentration-dependent and incubation-duration-dependent manner (Sundararaghavan et al., 2008; Yunoki et al., 2013). These approaches have been used to demonstrate that different neuronal subtypes favor distinct degrees of stiffness for the extension of their axons. Whereas axons of motor neurons favor stiffer substrate, axons of forebrain neurons grow faster on softer gels (Nichol et al., 2019). These observations match the stiffness of the *in vivo* environment of these axons: the central nervous system is softer than peripheral tissues (Franze, 2013). To mimic durotactic axon guidance, Koser et al. developed a gel exhibiting a soft to stiff gradient (Fig. 4A) to show that RGC axons turn towards the softer substrate *in vitro*, mimicking their behavior *in vivo* (Koser et al., 2016).

Although measuring and manipulating the stiffness of the axon environment has been successfully achieved *in vivo* in developing *Xenopus laevis* (Koser et al., 2016; Thompson et al., 2019), such approaches remain limited to very few accessible model systems and organisms, making *in vitro* approaches essential to investigate the impact of durotactic axon pathfinding. Many *in vitro* studies investigating the impact of the substrate stiffness on axons focused on outgrowth (with the noticeable exception of Koser et al., 2016, although the technology is mature enough to investigate the axonal response to guidance molecules on a range of substrates of various elasticity).

b. Applying forces to growth cones

Investigating the interactions between chemotaxis and durotaxis has been approached using the direct application of mechanical forces on a focal region of axonal growth cones. First achieved by directly holding the axon with a microelectrode or an elongated glass pipette coated with collagen or concanavalin A (Bray, 1984; Chada et al., 1997), they evolved to use microbeads trapped using different means (Fig. 4B and 4C), including mechanical handling with a pipette or a microneedle (Schaefer et al., 2008; Decourt et al., 2009), immobilization on a substrate (Moore et al., 2009), optical trapping in a focal laser beam (Bard et al., 2008; Moore et al., 2009, 2012)

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or a magnetic field to control a magnetic bead (Kilinc et al., 2014). Yet, these approaches are challenging to apply on growing axons and very few studies have combined them with the study of axon guidance molecules. However, by immobilizing netrin-1-coated beads on coverslips, Moore et al. demonstrated that this axon attractant is not able to orient axon outgrowth if not associated with mechanotransduction. Investigating this process further, the same group measured the growth cone-generated forces using netrin-1-coated beads immobilized with an optical trap. These forces largely exceed those observed in axons not exposed to the guidance cue (Moore et al., 2009). These observations have been confirmed by the later identification of a signaling pathway converting the netrin-1-dependent chemical signal into mechanical forces (Toriyama et al., 2013).

There are limited studies combining chemotaxis and mechanotransduction, due to the challenge associated to combining the investigations from these two fields. However, the availability of techniques offering such a possibility might lead to more frequent attempts to better understand how chemical cues influence the forces required to orient axon elongation.

4. **Organotypic ex vivo cultures**

One of the greatest challenges of *in vivo* research is the difficulty to understand processes occurring within inaccessible structures immersed deep inside tissues. On the other hand, the biggest drawback of *in vitro* cell assays is the growth of axons in an unnatural context. Organotypic cultures aim at overcoming both these short-comings by transferring the growing axons and their target tissues onto a surface suitable to live cell imaging, offering a natural environment for growth cones to navigate in an easy to image and manipulate platform (Fig. 5A).

Early approaches using organotypic cultures focused on understanding the guidance of axons from the visual thalamus towards the visual cortex (Molnár and Blakemore, 1999). This *in vivo* imaging study followed the growth of thalamic axons in the cortex and the reconstruction of specific target innervation, pointing at a chemotropic origin of the connectivity. This coculture system also highlighted the appropriate development of the cortex *ex vivo* and the transfer of crucial features of brain development to an *in vitro* set-up (Yamamoto et al., 1989; Bolz et al., 1992). However, this approach was also useful to prove that some of the features of neuronal connectivity were not encoded by the local environment, pointing at structural and temporal constraints influencing the development of axon targeting (Molnár and Blakemore, 1991).

For some *in vivo* preparations, the guidance phenotype can be observed within a single structure containing both the cell body of the studied neuron and a critical choice point for the navigation of the developing axon. This is the case of commissural neurons in the spinal cord, whose must navigate towards, across and beyond the floor plate in the three coordinated axes. The open book is an ingenious preparation that unfolds the spinal cord along the ventral midline and allows researchers to trace axonal trajectories in a single plane (Fig. 5B, Wilson and Stoeckli, 2014).

1 The open book preparation has been instrumental in understanding commissural neuron
2 navigation, and the mechanisms behind differential axonal responses before and after crossing
3 the ventral midline (Zou et al., 2000; Philipp et al., 2012; Alther et al., 2016; Ros et al., 2018).

4 Organotypic cultures allow the easy manipulation of tissues *in vitro*, or the mixing of genotypes,
5 enabling the investigation of innervation patterns in genetic backgrounds that are lethal *in vivo*.
6 This approach was used to show that Sema3C is not involved in the specific innervation of septal
7 GABAergic afferents in the hippocampus: septal GABAergic neurons specifically innervate
8 hippocampal GABAergic interneurons, independently of Sema3C expression in the hippocampus
9 or upon Sema3C blockade with Neuropilins (Rubio et al., 2011). Similarly, the blockade of ephrin-
10 A signaling with EphA3-Fc, and the use of tissues from Adenylyl Cyclase-1 mutants were used to
11 demonstrate the requirement of cAMP signals in RGC axons for the refinement of visual
12 projections in the superior colliculus (Nicol et al., 2006, 2007).

13 Although the development of conditional knock-outs enabled to investigate the phenotype
14 induced by overall lethal gene deletion in an intact organism, organotypic cultures are a versatile
15 platform to investigate axon guidance and the connectivity on a variety of systems. Coupled to
16 improved imaging techniques (Dumoulin et al., 2021) and refined control of cell activity with
17 optogenetics (Malyshevskaya et al., 2013), organotypic cultures may offer new insights into the
18 mechanisms of axon navigation and the development of the nervous system connectivity.

31 **Conclusion**

32 *In vitro* assays have largely contributed to the birth and expansion of the axon guidance field.
33 However, the development of genetic strategies enabling a variety of experimental approaches
34 *in vivo*, including large screens or conditional knock-out animals, has reduced the power of culture
35 assays and replaced *in vitro* tests in a range of applications. Indeed, the study of axons in a dish
36 suffers from several drawbacks, often including a 2D substrate provided to the axon, whereas a
37 3D environment shapes the growth cone differently compared to a flat substrate (Santos et al.,
38 2020). The substrate provided to axonal growth cones in *in vitro* approaches profoundly diverges
39 from the extracellular matrix and molecules encountered by axons in intact organisms. The
40 diffusion of secreted proteins *in vivo* is difficult to evaluate carefully but is likely to largely differ
41 from the spread of molecules in a culture medium or in a gel. Nevertheless, *in vitro* experimental
42 approaches still provide pieces of information that are inaccessible in an intact organism. These
43 advantages include a high subcellular and temporal resolution, two critical points for the study of
44 a dynamic process like axon pathfinding. Imaging axons in a dish also enables to perform
45 functional imaging and to use reporters for signaling pathways that do not benefit from biosensors
46 with high signal-to-noise ratios, like the popular calcium sensors. Furthermore, improved imaging
47 and cell manipulating techniques providing enhanced resolution in space and time give new
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impetus to *in vitro* approaches. Finally, controlling the concentration and timing of guidance molecules in the environment a growth cone opens the possibility to develop computational models that can, once calibrated, be tested to reproduce the behavior of an axon *in vivo* and suggest mechanistic insights to explain the wiring of the nervous system. Overall, a combination of both *in vitro* and *in vivo* experimental procedures supporting each other provides a body of information that enables to overcome the limitations of each of these approaches and has the potential to offer deeper insights into the mechanisms of axon guidance than single-sided approaches.

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Figure legend

Figure 1: Back and forth feedback between *in vitro* and *in vivo* approaches to understand axon pathfinding in the retino-collicular system. Early *in vitro* work (stripe and collapse assays) identified the repellent activity of membranes from the posterior superior colliculus for axons from the temporal retina (Walter et al., 1987b), and enabled to pinpoint ephrin-As as critical molecular determinants of this repulsion (Drescher et al., 1995). These observations were confirmed *in vivo* with the exuberant axonal arbors of temporal retinal axons in ephrin-A knock-out animals (Feldheim et al., 2000). Subsequent *in vitro* studies highlighted that a cAMP modulation restricted to lipid rafts is induced by ephrin-A5 in retinal axons (Averaimo et al., 2016). Altering cAMP signaling specifically in lipid rafts in retinal axons *in vivo* confirmed that this subcellular signal is required for the wiring of retino-collicular projections (Averaimo et al., 2016), setting the stage for a new *in vitro/in vivo* cycle to better understand the mechanisms underlying axon pathfinding.

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Figure 2: Sharp boundaries and abrupt changes in the culture medium. (A) Stripe assay. Stripes are prepared by injecting solutions of a guidance molecule into a mold that adhere to a coverslip (left). Explants of the neuronal tissue to test are placed on the prepared stripes (middle) and growing axons extend on the stripes containing the preferred substrate (right). (B) Collapse assay. Axons are grown *in vitro* (left) and the culture medium is replaced by a medium containing the guidance molecule to test (middle). If axons are repelled, growth cones exhibit a drastic change in morphology, with a loss of the filopodia and lamellipodia (right)

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Figure 3: Gradient-based assays. (A) Collagen-embedded gradients. An explant of neuronal tissue (brown) is embedded at a distance of an aggregate of cells expressing an axon guidance molecule (green, left). While the neurons from the explant extend axons, the aggregate secretes the guidance cue to generate a gradient within the collagen gel (middle). The axons grow preferentially towards or away from the aggregate, inducing an asymmetry in the culture if they are sensitive to the tested molecule (right). (B) Turning assay. Neurons are plated on a flat substrate, extending an axon. A micropipette loaded with a concentrated solution of a guidance molecule is positioned in the vicinity of the growth cone (left). The solution is slowly released from the micropipette, generating a gradient of the tested molecule (middle). If the axon is sensitive to this guidance cue, the growth of the axon is oriented towards or away from the micropipette (right). (C) Dunn Chamber. The device consist of a slide engraved with a center well surrounded by a circular reservoir, linked together by a thin bridge enabling slow medium diffusion and mixing. A guidance molecule is loaded in one of the wells while the other is filled with culture medium only. A glass cover on which neurons have been cultured is placed upside-down on top of the chamber (left). If the cultured axons are sensitive to the tested molecule, they orient according to the gradient created by the chamber (right).

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Figure 4: Substrate stiffness and mechanical forces applications. (A) Stiffness gradient. A solution of soft polymerizing gel is loaded between two coverslips on top of a preloaded gel of stiffer polymerizing gel (left). Once the gels have polymerized, one of the coverslip is peeled off, leaving a substrate with a gradient of stiffness on the remaining coverslip. A neuronal explant is cultured on top of this gradient (middle). The growing axons orient themselves according to the gradient if sensitive to stiffness (right). (B) Bead held by a pipette. A collagen- or concanavalin A-coated bead is attached to a glass pipette and positioned to a growth cone in culture. Once the bead and the axon are in contact, the pipette is used to pull the bead and apply a force on the axon (left). The turning behavior of the axon in response to the applied force is monitored under a microscope (right). (C) Optical trap. In this approach the bead is maintained within a focal laser beam. Displacing the beam is used to pull the growth cone (left). The turning behavior of the axon is monitored under a microscope (right).

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Figure 5: Organotypic cultures. (A) Coculture. An explant containing the neurons that will extend axons to test (here retinal explants) and an organotypic slice of the target tissue (here a

collicular slice) are prepared. Once assembled the axons exhibit axon pathfinding behaviors that resemble their developmental sequence *in vivo*. (B) Open-book. The spinal cord is harvested from a developing embryo. A cut is performed along the dorsal midline, exposing the floor plate (grey). A lipophilic dye is injected in the dorsal spinal cord to label commissural neurons. The behavior of these axon can then be monitored while crossing the ventral midline.

Table 1: Features of *in vitro* systems for axon guidance investigation.

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Figure 1

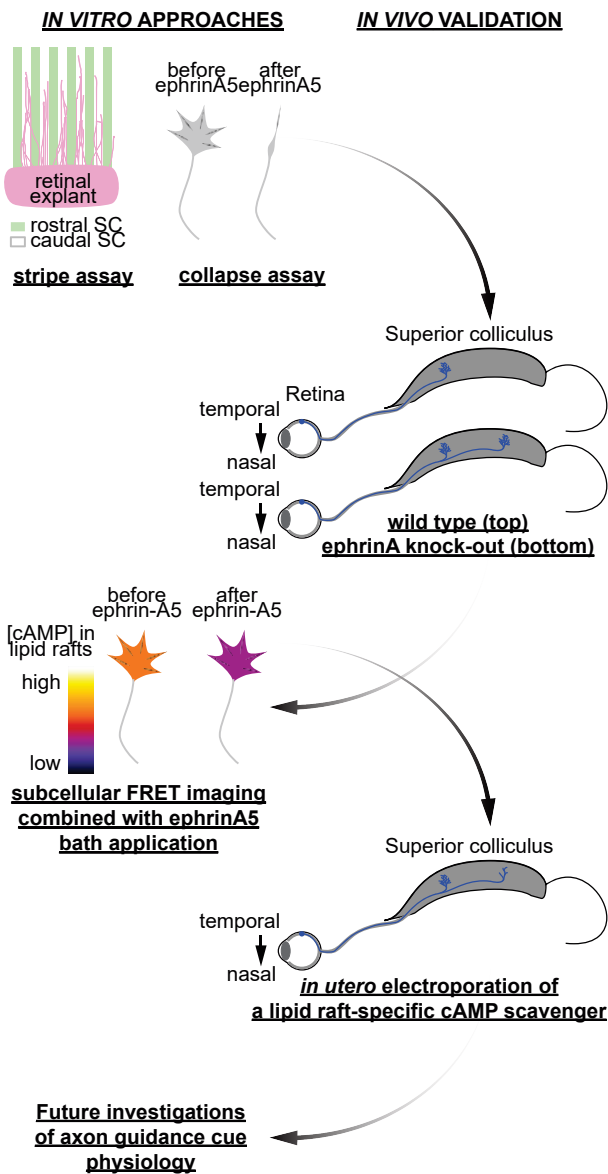


Figure2

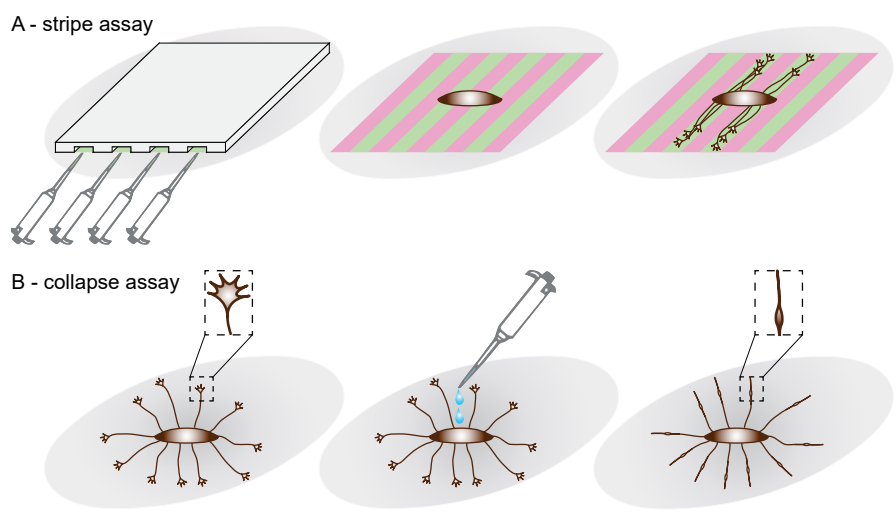


Figure3

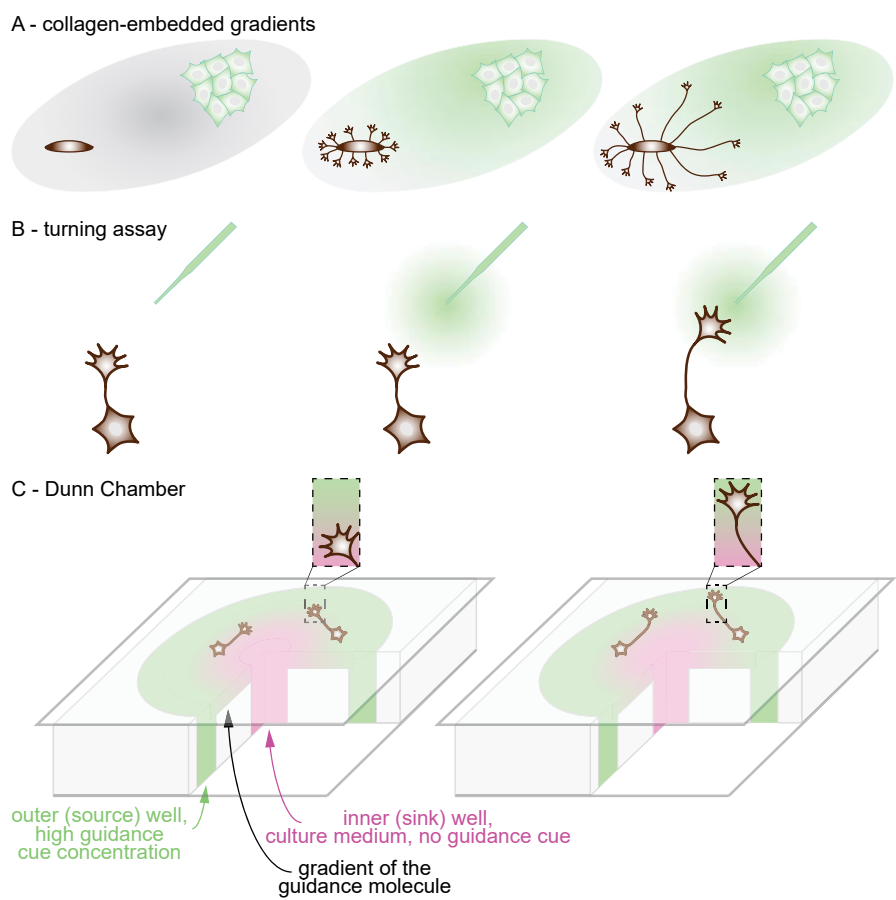


Figure4

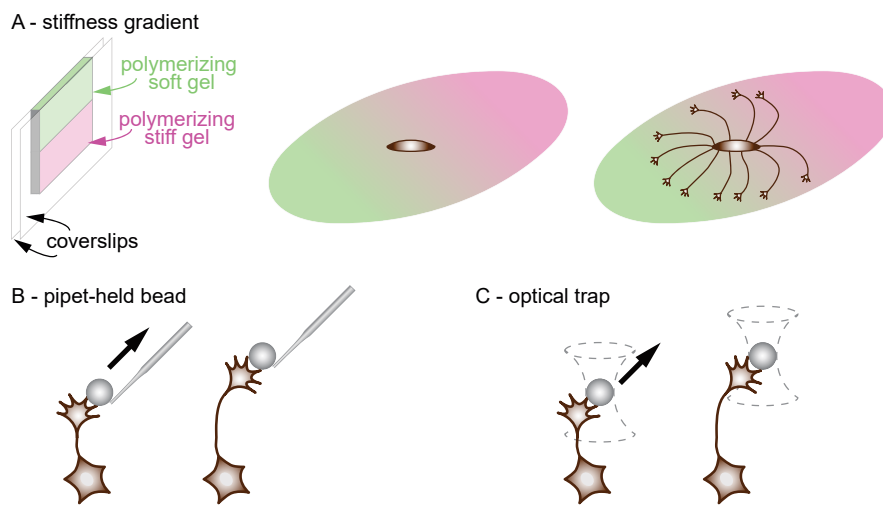


Figure5

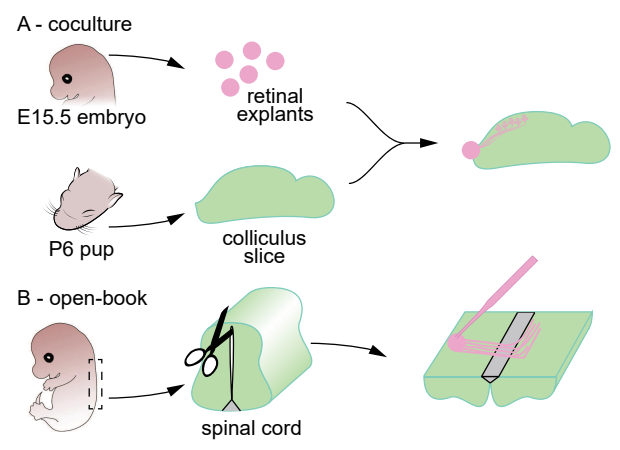


Table1

Assay	Main features	Limits	Commercial availability
Sharp changes in the axon environment			
Stripe assay (membrane based)	- Compare two axon environments (relative attraction/repulsion)	- Requires further experiments to determine the protein responsible of the guidance effect - Difficult to distinguish attraction from repulsion	Yes (M. Bastmeyer lab)
Stripe assay (recombinant protein)	- Compare two axon environments (relative attraction/repulsion) - Adjustable protein concentration - Possibility of combinatorial approaches	- Difficult to distinguish attraction from repulsion	Yes (M. Bastmeyer lab)
Collapse assay	- Easy implementation (Bath perfusion of membranes or molecules) - No need of specific equipment - High throughput approach may reduce noise in live imaging applications	- Use restricted to axon repellents	No
Gradient based assays			
Collagen/ECM Matrices	- Easy to implement - High throughput - 3D axonal environment	- Difficult to standardize experimental setup - Amplitude of the effects may vary with tissue sizes and distance between explants - Generates fluorescent background	No
Turning Assay	- Precise monitoring of axon responses	- Low throughput method - Not viable for mammalian axons - Axon growth on a 2D surface	No
Patterned Microslides and microfluidics	- Inexpensive - High throughput, high resolution may be achieved. - Allows following mammalian axon turning.	- Implementation not always straightforward - Axon growth on a 2D surface	Yes
Microprinted Gradients and Patterns	- Flexible design - Reproducible concentrations and patterns	- Expensive - Axon growth on a 2D surface	Yes
Substrate stiffness assays			
Gels of controlled stiffness	- Inexpensive (if the stiffness is not measured) - No need of specific equipment (if the stiffness is not measured) - Possibility to generate gradients of stiffness	- Approximate stiffness - Does not enable to measure forces	No
Beads held with microelectrodes or micropipette	- Inexpensive - No need of specific equipment	- Does not enable to measure the applied forces - Low throughput method	No
Beads controlled with a magnetic field or an optical trap	- Possibility of measuring the applied forces - Precise control of the applied forces	- Requires expensive and dedicate equipment - Low throughput method	No
Ex vivo systems			
Open book	- Allows 2D visualization of 3D axon behaviors - Single tissue - Environment close to physiological	-Requires good dissection skills -Limited guidance paradigms	No
Co-cultures (retina-colliculus, septum-hippocampus)	- Bypasses intermediate target influence on axon behavior - Multiple emitting and target structure possibilities - Environment close to physiological	- Requires good dissection skills - Needs to find optimal tissue orientation and culture timing - Multiple inputs in target tissue make difficult to determine the guidance factors	No