1 **Title**

2 Spinal cord injury *in vitro*: Modelling axon growth inhibition

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Teaser

Experimental *in vitro* models continue to be essential in understanding the mechanisms and pathophysiologies in spinal cord injury, and in providing insights for future therapeutics.

Abstract

Over the past three decades, tremendous progress has been made in elucidating mechanisms underlying regenerative failure after Spinal Cord Injury and in devising therapeutic approaches to promote functional nerve regeneration. Various *in vitro* assays have been developed using brain and/or spinal cord neuronal cells to study axon growth in conditions which represent the post injury environment. This review outlines the current models used to dissect, analyse and manipulate specific aspects of spinal cord injury leading to axon growth inhibition.

Keywords: Experimental models; Axon injury; Axon Guidance; Glial Scar; Myelin Degradation Products

Traumatic Spinal Cord Injury (SCI) is a devastating condition, with an incidence of approximately 130,000 survivors reported worldwide each year [1]. The majority of survivors are left paralysed with no restorative treatment available as yet. First described by Ramon y Cajal as ‘abortive regeneration’, it was understood almost a century ago that neurons fail to regenerate after injury. However, continued research in the field began identifying growth inhibitory components in the injured spinal cord, which, if properly modulated, could lead to enhanced regenerative capacity and functional regrowth [2].

Following traumatic injury to the spinal cord, two events take place that have been associated with impaired neurological function and ineffective attempts at axon regeneration: the acute primary mechanical insult and the chronic secondary reactive damage, the hallmark of which is molecular inhibitors [4]. Primary traumatic damage to the spinal cord, usually in the form of crush injury, results in shear stress to the axons of neurons. In addition to causing immediate death of cells in the epicentre of injury site, the initial impact causes local disruption of blood flow and an increased inflammatory response. This response includes the migration and proliferation of meningeal fibroblasts, forming an inhibitory fibrotic scar in the lesion core. Membrane disruption also causes damaged neurons to leak out their contents, including neurotransmitters, which in turn exacerbates tissue damage by increasing calcium influx into the cells. Astrocytes become reactive and produce a glial scar on top of the fibrotic scar preventing further meningeal invasion. In addition, injury to myelin sheaths releases myelin degradation products in the vicinity of the scar. The

injury mechanisms and their effect on the pathophysiology of SCI are discussed in another review [5].

Molecular inhibitors of axon growth have been particularly linked to three main components of the lesion: the fibrotic scar, the glial scar tissue, and the damaged myelin (summarized in table 1 and reviewed in [6]). Within the glial/fibrotic scars, astrocytes and meningeal fibroblasts become reactive and upregulate expression of chondroitin sulphate proteoglycans (CSPG) and Semaphorins. These inhibitory constituents, in addition to myelin degradation products, restrict the innate capacity of axons to regenerate. Figure 1A illustrates a schematic of the primary and secondary injury mechanisms leading to regenerative failure following SCI.

The quest for a cure for SCI, coupled with knowledge of the mechanisms of injury had allowed researchers to identify the potential for using animal models. This transition facilitated the experimentation of anatomical and molecular changes seen after injury. Two main classes of injury: contusion/compression and transection, are the most widely accepted methods by which SCI is modelled *in vivo*. For a general discussion of the models of experimental SCI, the reader is referred to a recent review [7]. Limitations for using these models include the complexity surrounding this type of injury and the inability to study the progression of disease processes, rendering the analysis and interpretation of isolated mechanisms difficult. Other limitations include cost and ethical concerns. While there is an increasing demand for identifying key molecular signals originating from and affecting SCI, there is an increasing availability of techniques to allow researchers to manipulate cells *in vitro*, including tools to isolate and culture neuronal cell types, assays to control and characterize

neuronal growth behaviour, and analytical methods to determine molecular signals pertaining to neuronal development and regeneration. Therefore, depicting the multitudes of mechanisms of axon growth inhibition *in vitro* is an essential and complementing step towards understanding failure of regeneration and eventual identification of potential therapeutics that could be translated to the bedside [8]. In this review we outline the different mechanisms of injury-related axon growth inhibition and common *in vitro* paradigms used to recapitulate them.

NERVE CELLS IN CULTURE

Pioneering work by Harrison in the early 1900s has provided insight into the anatomy and physiology of the nervous system whereby neuroscientists were able to grow brain and/or spinal tissue *in vitro* for periods of up to 4 weeks [12]. The ability to maintain and study nerve cells in culture has had a huge impact on our understanding of various parameters of normal and abnormal nervous tissue. Primary cultures of neurons, oligodendrocytes, astrocytes or microglia are readily accessible and are relatively easy to grow on a number of substrates and under different growth conditions including the presence of inhibitory cues. This allows for qualitative and quantitative analysis probing the effect of injury pathologies on single cell types. Moreover, co-cultures of different types of nerve cells or complete nervous tissue (as in organotypic cultures) can help analyze cellular interactions and their impact on the pathophysiology of injury.

To date, different *in vitro* paradigms have been used with neuronal cultures including axon outgrowth assays, growth cone turning, growth cone collapse, and stripe assays

[13,14]. These assays answer critical questions regarding the behaviour of neuronal cells to different stimuli. Parameters of neurite outgrowth could then be analysed *in vitro*, such as neuronal phenotype, cell surface molecules, absolute neurite length and branching, axonal trajectories, and growth cone morphology. For example, studies in developmental neurobiology have adapted these assays to examine different modulators of axon growth. The same models allowed researchers to study inhibitory cues representative of those encountered in the post-injury environment, such as reactive astrocytes, and myelin degradation products, and thus have been fundamental for the understanding of the molecular mechanisms underlying the pathophysiologies of SCI as well as the identification of a growing list of inhibitory molecules expressed in the injured environment of the adult central nervous system (CNS) (reviewed in [6]).

THREE DIMENSIONAL CULTURES

Three dimensional (3D) culture systems offer an intermediary approach between simple monolayer cell culture systems and *in vivo* animal models. Comparing cellular growth in two dimensional (2D) monolayer cultures to 3D matrix cultures has shown clear phenotypic differences, including cell migration, focal adhesions, and neurite and growth cone dynamics [15]. It is more likely that 3D platforms provide a better representation of tissue organization, cell-cell and cell-matrix interactions. 3D platforms are made from either biological matrices; most often components of the extracellular matrix (ECM) like collagen, fibrin, and Matrigel (basement membrane matrix) or polymeric scaffolds, like poly lactic acid, poly lactic-co—glycolic acid, and agarose (reviewed in [16]). One feature of such models is that they can be altered to affect culture conditions to help identify specific molecular signals or detect responses

1 to defined conditions. For example, patterning 3D matrices with effectors of neuronal
2 growth, such neuronal growth factors or ECM molecules, such as laminin peptides,
3 provide superior control over axonal growth and directional guidance [17,18]. This
4 adds a layer of complexity that more closely resembles the *in vivo* environment, and
5 allows for direct comparison of different parameters affecting neuronal growth, while
6 maintaining the flexibility, low cost, as well as high throughput features of
7 conventional 2D cultures.
8

9 **ORGANOTYPIC CULTURES**

10 In contrast to conventional *in vitro* culture systems, organotypic slice cultures are
11 prepared from nervous tissue (brain or spinal cord) without dissociation. They are
12 made up of a heterogeneous population of cells, and hence largely preserve the
13 original cytoarchitecture and maintain neuronal activities and functional synaptic
14 circuitry [19]. Organotypic cultures represent a trade-off between a three-dimensional
15 single cell system and an *in vivo* environment; importantly, individual cells are in
16 close contact and maintain cell adhesion mediated regulatory mechanisms,
17 extracellular architecture as well as transport and diffusion parameters. This is
18 particularly important especially when studying motorneurons, since these are
19 difficult to maintain in single-cell culture systems, or for longer-term assays [20].
20 Organotypic cultures have proven to be useful for *in vitro* studies, as evidenced from
21 their wide use in different applications ranging from neurobiology to neurophysiology
22 (see reviews [19,21]). In the context of injury, this subtype of culture presents a
23 readily manipulated CNS microenvironment to study the different components
24 and effectors of a specific lesion [22,23]. However, there are certain limitations to
25 their use. First, their preparation is technically difficult, as slices must be made of

very thin sections (>500μm) to avoid hypoxia of the central tissue *in vitro*. In addition, the ability to control cell types, ratio of cell types, and extracellular components is not possible in such systems.

PRIMARY TRAUMATIC DAMAGE: MECHANICAL INJURY

In vitro approaches to studying mechanical injury to neurons have evolved with the need to understand how the initial impact leads to various outcomes, and the potential for developing appropriate therapeutics to prevent secondary reactive damage. Various models have been used including axonal transection, compression models, and cell/substrate stretching devices [24-26]. These models offer a high degree of experimental control providing the researcher with the flexibility to create defined mechanical inputs and analyse the resulting cellular outcomes.

In the cell stretch model, cells are grown on flexible substrates that can be mechanically stretched (available commercially as Flexplate®), indirectly impacting shear stress on adherent cells. Adapted by Ellis *et al.*, these flexible substrates fit into the bottom of a pneumatic cylinder and positive pressure pulses are applied through a controller unit (see fig.1C) [25]. With respect to compression models, one example includes an organotypic slice culture consisting of thin cross-sections of whole adult mouse spinal cords. These cultures were exposed to a weight-drop injury (see fig.1B), and assessed for cell death with and without the use of neuroprotective pharmacological compounds [22]. The use of these models is limited because of technical difficulties hindering reproducibility and lack of uniformity across culture substrates. Another model, involving axonal transection, makes use of organotypic

cultures of spinal cords from newborn rats made from longitudinally cut sagittal sections. The advantage of this particular model is that the slice includes several spinal cord segments with maintained neuronal cytoarchitecture and ventral-dorsal polarity [23]. It also employs a fairly uniform mechanism of injury that is highly reproducible. Transverse lesions were made using scalpel blades, and the cultures evaluated for spontaneous neuronal regeneration. The finding that pharmacological agents such as rolipram were able to improve axonal regeneration through the lesion site provides evidence that such models can be used to assess the efficacy of potential therapeutics. More recently, the introduction of tissue-engineered platforms has enlarged our understanding and control of the different parameters of mechanical injuries. For example, LaPlaca *et al*. described a device that delivers a defined shear strain to neuronal cell cultures in a 3D Matrigel matrix. Potential uses for *in vitro* traumatic models include studying the effect of secondary damage triggered by the initial trauma, and methods at preventing or overcoming that (discussed in 'Models of the Glial Scar' below). Other uses include examining short and long-term gene expression following injury.

SECONDARY REACTIVE DAMAGE

The hallmark of the secondary reactive phase is scar formation at the initial impact site. Mature astrocytes often become hypertrophic and adopt a reactive phenotype which expresses inhibitory proteoglycans (CSPGs). Meningeal fibroblasts also become reactive and upregulate expression of Semaphorin3. These scar specific molecules, as well as myelin degradation products (such as MAG and Nogo), are generally organized in a crude gradient around injured neurons, with the lowest concentrations in the penumbra and the highest in the lesion epicentre [27].

MODELS OF THE GLIAL SCAR

To analyze constituents of the glial scar that are inhibitory to axon growth, earlier studies have relied on 'explant scarring', for example by using monolayer neuronal cells grown on explant scars from nitrocellulose sheets inserted into the cortex [28]. This technique isolates scar tissue that forms *in vivo* with little contamination from normal tissue. A second approach created astrocyte/meningeal cell interfaces, and examined the growth of neurons across these interfaces [29]. Analysis of axon outgrowth from these studies showed features suggestive of inhibition such as limited growth, and/or collapsing growth cones. This has since led to the identification of inhibitory molecules in the vicinity of the scar tissue and provided solid grounds for more specific studies aimed at elucidating mechanisms underlying this inhibition [30]. More recently, protein immobilization techniques of 3D gel matrices were used to attach inhibitory proteoglycans to agarose gels, allowing 3D culture of neurons in isolated inhibitory environments similar to but much simpler than those of the glial scar [31]. This model was used to define the relative contribution of specific CSPGs, which could help design more specific therapies. The importance of the aforementioned models is that they incorporate reactive astrocytes or their products, and hence contain constituents both molecularly and spatially comparable to the glial scar *in vivo*. Their limitation, however, is failure to reproduce the scarring process. The latter was achieved *in vitro* by applying biochemical and/or mechanical triggers to co-cultures of astrocytes and meningeal fibroblasts to simulate glial scarring [32,33]. For example, one study employed shear deformation to thick (> 500μm) 3D neuronal–astrocytic co-cultures at a prescribed strain rate and magnitude. Briefly, parallel motion of the top plate of the chamber with respect to the bottom produces a linear shear strain, uniformly deforming the 3D cell matrix, and resulting in a

biomechanically controlled traumatic injury model [34]. This model was used to induce cell death and reactive astrogliosis, thereby mimicking a reactive injury site. Evaluation of neural stem cell survival and the validity of a therapeutic scaffold were then carried out. Another approach using the cell stretch culture system describes a model of the glial scar, whereby the use of mechanical stretching by abrupt deformation of silastic culture plates introduced astrogliotic changes to astrocytes and meningeal co-cultures. This is evidenced from the expression of biochemical markers specific of SCI [32]. A recent model describes a 3D culture system, whereby TGFβ1 triggers the astrogliotic changes. The value of this model lies in the ability to monitor reactive changes to astrocytes in culture and to carry out spatiotemporal analyses [35]. Aided by knowledge of the mechanisms governing glial scar formation and the ability to recapitulate its effect *in vitro*, the previous studies have succeeded in creating well-characterized models of the glial scar. One must stress however, the importance of recognizing potential pitfalls arising from the use of tissue culture models, including but not limited to genetic and phenotypic instability of cultured cell types, as well as functional differences from their *in vivo* counterparts.

MODELS OF AXON GUIDANCE

Physical and chemical cues interact on the molecular level to guide cell attachment and directional axon growth and migration. On the one hand, mechanical interaction with the surrounding ECM components initiates a cascade of events leading to neurite growth during development and cessation of growth after injury. This feature was tested *in vitro* by using different substrates, both natural and synthetic, and by changing physical topographies in both monolayer and 3D culture systems (see 'Topographic Micropatterning & Microfluidics' below and review in [16]). On the

other hand, chemical cues are diffusible and substrate bound factors that guide the advancing neurites through a complex milieu. Studies of the latter involved creating gradients of molecular cues and studying axonal responses such as adhesion to underlying substrates, number of neurites and growth cone morphology [36].

The pipette/growth cone turning assay has been widely used to study axonal responses to gradients of diffusible cues in their immediate environment. The turning assay offered many advantages over conventional outgrowth assays by giving researchers the capacity to control and study interacting signals. This helped identify trajectories of axonal projections, growth cone dynamics and downstream molecular signals [13,37]. For example, turning assays of DRG neurons using MAG as guidance cue identified a novel signalling mechanism involving integrin receptors [38]. On the other hand, axonal responses to insoluble cues have also been studied (see fig.1D) including those in stripe assays where molecules of interest such as neuronal growth factors, proteoglycans, or MAG, are patterned in stripes alternating with permissive coatings on various surfaces [39-41]. This approach also gives better control over the immediate microenvironment and specifies axonal trajectories. Analyses of parameters of neurite outgrowth such as cell-ECM interactions, growth cone collapse, neurite length, and axonal branching then identified interactions within these microenvironments that eventually lead to inhibition. In another approach, Tom *et al*. described a two dimensional *in vitro* assay that mimics the proteoglycan gradient representative of the *in vivo* glial scar by growing neurons on aggrecan-laminin spot gradient substrates [42]. Neurons maintained attachment to the underlying substrate but had limited growth within the proteoglycan core with dystrophic endballs typical of lesioned axons. The use of this model identified the dynamic behaviour of these

dystrophic endings supporting the notion that injured axons maintain their capacity to grow, and shedding new light onto the regenerative capacity of the spinal cord. More recently, this model was used to identify a novel receptor and downstream signalling mechanism for proteoglycans [11].

Better representation of the mechanisms underlying axon guidance and its response to various molecular cues present in both permissive and inhibitory environments can help develop strategies for future therapies. Their limitations include the lack of physiological similarities between these simplified *in vitro* systems and the *in vivo* environment as well as the lack of interaction with cellular tissue components. It is therefore only logical to combine different classes of molecular cues in complex cellular microenvironments to study their effects alone and in combination [43]. How this can be therapeutically translated is envisaged from the development of structural and molecular anisotropy in tissue-engineered designs. This may lead to better regeneration by exploiting the sensitivity of neurons to directional growth [18,44].

Table 2 summarizes the different *in vitro* paradigms currently employed to study and simulate mechanisms of axon growth inhibition.

THE FUTURE OF *IN VITRO* MODELS OF AXON GROWTH INHIBITION

TOPOGRAPHIC MICROPATTERNING & MICROFLUIDICS

Although conventional cell-culture models have had a great impact on our understanding of axon growth in response to injury, one major disadvantage remains our inability to precisely control cell microenvironments. The use of micropatterned substrates is rapidly making its way into models of nerve injury and regeneration (for

a discussion, see [16]). With the use of soft lithography, substrates can now be modified to incorporate physical cues, in the form of grooves and ridges [45-47]. These micropatterns are aimed at mimicking *in vivo* physical stimuli that guide axonal migration. An advantage of using these models is that they allow the compartmentalization of axonal outgrowth, which in turn enhances the analysis of neuronal architectures in response to different substrates.

The field of microfluidics, which incorporates microfabrication techniques into the study of biological systems, offers additional control of the distribution and organization of added reagents and substrate constituents. One clear advantage in using microfluidic devices is the ability to manipulate axonal growth, and to modulate reactions with various chemical cues by generating gradients across chambers [48]. The applicability of microfluidic platforms for studies of neuronal injury has recently been explored [49,50]. These studies demonstrated the ability to accurately and selectively injure axons and analyze their biochemical responses, with potential applications in drug discovery and design strategies for tissue-engineered constructs. For a detailed discussion of the use of microfluidics in neuronal studies, the reader is referred to [51].

The future lies in combining methodologies to add a level of complexity to these models by specifying structural and molecular cues, whitle retaining their analytical values. In one instance, Figure 2A is a schematic representation of how microfluidics and micropatterning can be used to incorporate topographical features necessary for guiding neuronal growth in hydrogels, as well as gradients of chemical guidance cues, including attractants and repellents [52]. It is also important to note that modulators of

neuronal polarity and cytoskeleton machinery that are key to migrating axons can be studied using micropatterned surfaces. Primary cells in culture, such as cortical, spinal and dorsal root ganglion are essentially injured cells, stripped of their axons and replated on *in vitro* surfaces. This requires cells to re-organize their cytoskeletal structures to initiate axon extension. Much can be learnt from understanding these processes *in vitro*, and incorporating them into therapeutic strategies. For example, one study looked into the morphology, motility, and cytoskeletal dynamics of axonal extensions after localized transection *in vitro* [53]. Another study demonstrated that laminin gradients are essential in specifying neuronal polarity, and hence indirectly resulting in better directional growth, and migration [39]. This finding found its way into the development of an experimental treatment based on the incorporation of the laminin epitope [54]. We therefore believe it is necessary to incorporate such mechanistic studies when modelling nerve injury, as this will help us understand with great reproducibility both intracellular and extracellular mechanisms governing axon guidance.

GENOMIC INPUT IN *IN VITRO* MODELS & STEM CELL NICHES

In vivo studies allow analysis of transcriptional changes in response to nerve injury. However, the complex *in vivo* interactions make it difficult to interpret these findings as they are are mostly representative of postmortem tissue, and are not necessarily specific to the axon, which in turn could invalidate conclusions based on these analyses [55]. *In vitro* models, on the other hand, provide the tools necessary to study cell-specific transcriptional changes in response to controlled inputs. Results from these genomic analyses can be incorporated into computational models that simulate biological interactions, and yield arrays of genetic and protein expression, which

could be translated into physical models. One important application of *in vitro* transcriptomic models is to incorporate cells from different lineages (neurons, astrocytes, and oligodendrocytes) in matrices modified with tools to up-regulate or down-regulate expression of genes of interest [43,56]. Furthermore, these models will enhance our understanding of the properties of neural stem cells with a view to their therapeutic application in neural repair. This can also be expanded to simulate stem cell niches *in vitro* [57,58]. The notion of such a system would be to use information from mechanistic and transcriptomic studies, and construct stem cell based biomimetic matrices with factors that will control their differentiation into specific neural lineages. One study showed that by using biomimetic approaches, one is able to promote neural stem cell differentiation into neuronal lineages and thus enhance functional recovery after SCI [54]. Another potential application could include stem cell based matrices with modifications resulting in various phenotypes that are temporally separated, allowing a high throughput analysis of the mechanisms and pathophysiology of injury, and may as well act as models for drug screening. Figure 2B is a schematic of a bioengineered stem cell niche that progresses into a spinal cord surrogate.

CONCLUSIONS

Because there are no viable therapies to promote functional nerve regeneration, spinal cord injury represents a challenging area of research. Experimental models of this injury are essential in studying mechanisms of inhibition as well as therapeutic targets by which to overcome this inhibition. The role of *in vitro* models of nerve injury has been steadily growing with the introduction of novel approaches to recapitulate *in vivo* environments, including the various physical and biochemical complexities. Moreover, the advent of new research in stem cell niches, microfluidics and functional biomaterials, holds great promise for researchers in the field by expanding the capabilities and *in vivo* characteristics replicated in *in vitro* models.

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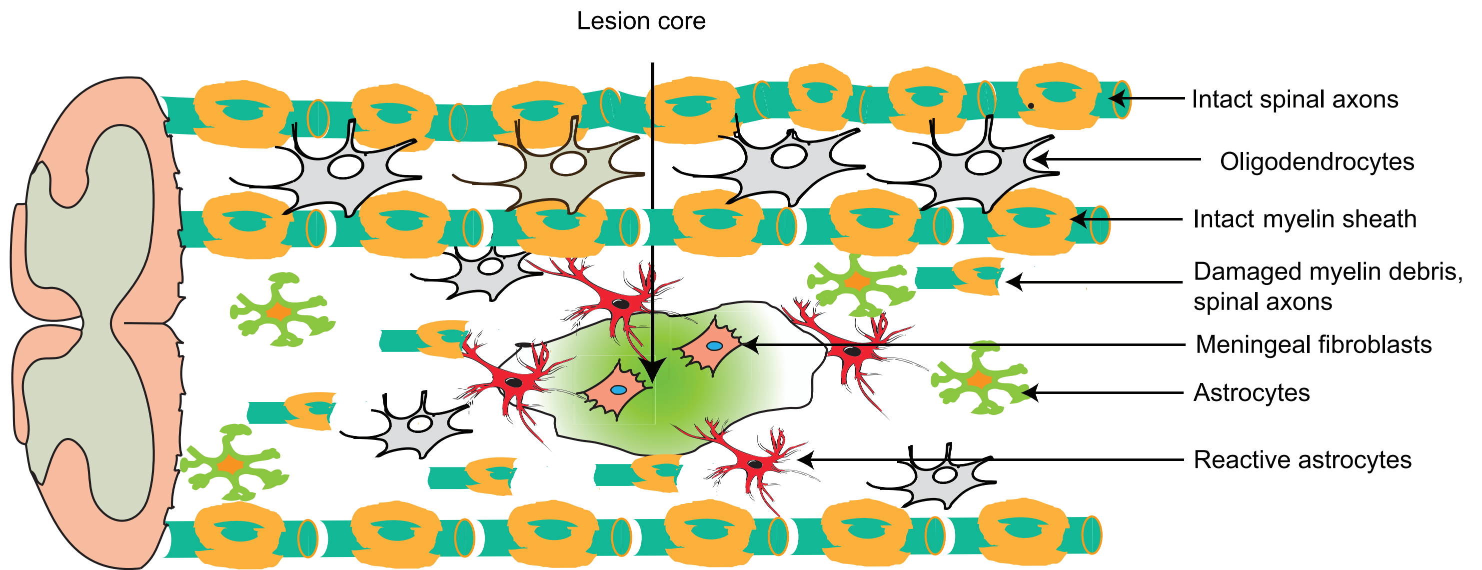
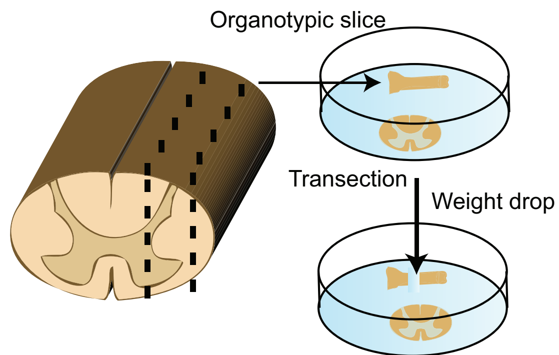
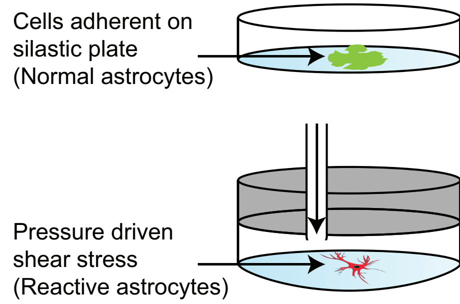
Figure 1: A. Schematic of the spinal cord lesion site and inhibitory constituents restricting axon regeneration (Adapted from Yiu and He) [30]. B. Organotypic spinal cord culture model. Cross section is of longitudinal slice of spinal cord spanning multiple segments. Slices are grown in culture dishes, and later exposed to transection or weight drop injury. C. Cell stretch injury model. Astrocytes are grown on flexible silastic substrates, to which a pressure driven shear stress is delivered via a controlled pump (Adapted from [25]). D. Axon guidance platforms: Nerve cells are grown on one side and exposed to molecular inhibition in the form of damaged white matter (top panel), or to micropatterned substrates (lower panel). The trajectory of axonal migration is analyzed.

Figure 2. A. This model is adapted from the work of Vickerman et al. [52]. It makes use of a microfluidic culture platform consisting of a bulk phase/mould base made of a fibrous collagen hydrogel, in which axon specific channels are inlaid. They can also be modified to provide topographical support to cell adhesion and migration. This particular design includes the use of chambers containing chemorepellents or chemoattractants applied at one end to generate a gradient, hence guide neuronal growth across the chambers. B. Using stem cell niches as models of axon injury. Stem cells are grown in microenvironments embedded with reservoir systems to program their fate into different neuronal lineages. The end result is a spinal cord surrogate.

Figure1







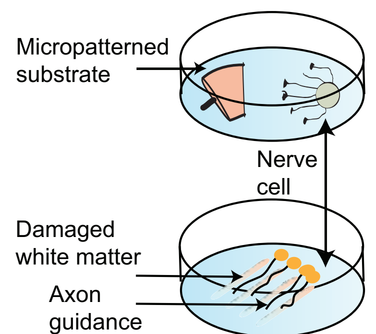
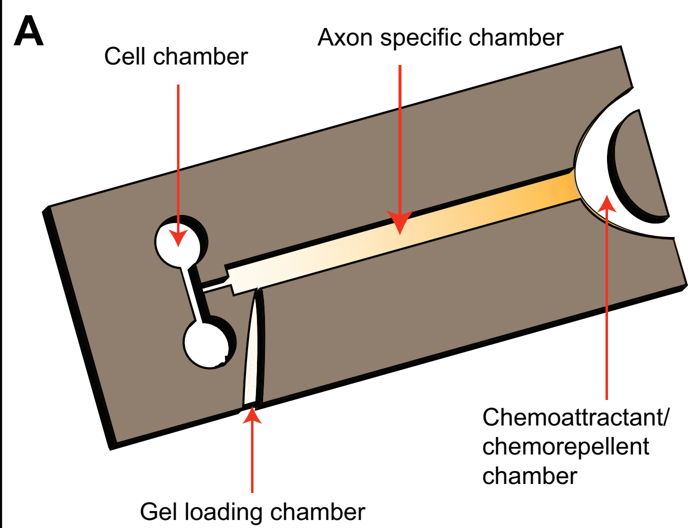


Figure2



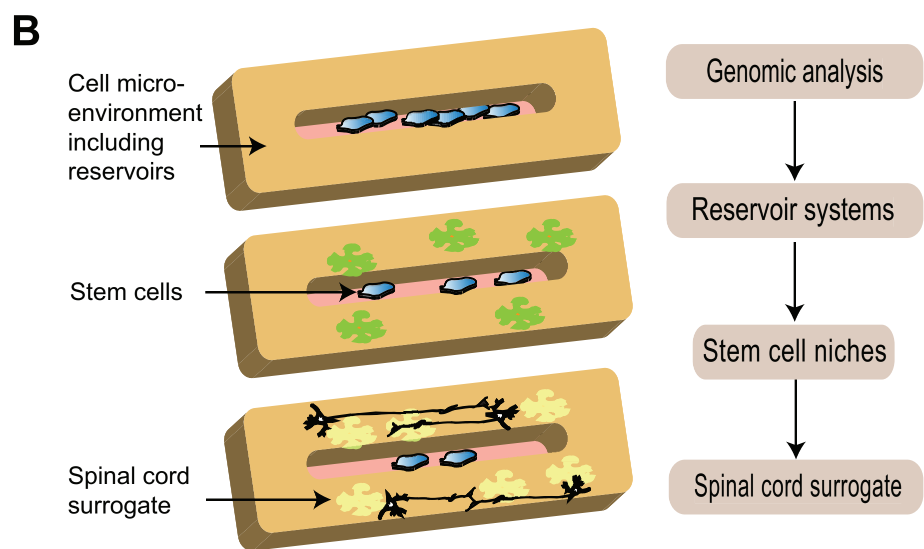


Table1

Table 1: Axon growth inhibition following Spinal Cord Injury.

Classes of Inhibitors	Receptor[1]	Mechanism of Inhibition	Temporal Distribution	Spatial Distribution after SCI	Ref.
Myelin derived: Nogo, MAG[2]	NgR1, β1-Integrin*, PirB*	Receptor mediated RhoA activation; Dynamic alteration of components of the cytoskeleton Increase [Ca2+]i	Immediately after injury; sub-acute	Disruption of myelin sheaths following traumatic injury results in release of soluble fragments of myelin debris in and around the injury site	[6,9,10]
Astrocyte-derived: CSPG[3]	PTPσ*	Possible masking of cell surface adhesion molecules Activation of RhoA/ROCK pathway Increase [Ca2+]i	7-14 days post injury	CSPGs are closely associated with extracellular matrix deposition with the highest concentration in the lesion core	[6,11]
Meningeal fibroblast-derived: Semaphorins	NP-1/Plex1	Activation of RhoA/ROCK pathway Disruption of cytoskeletal dynamics and cell adhesion mediators causing growth cone collapse	14 days post injury	Distribution similar to CSPGs	[6]

[1] This refers to the main receptor, or receptor complexes involved in modulating the inhibitory function of these molecules
[2] Myelin Associated Glycoprotein
[3] Chondroitin Sulfate Proteoglycans
* Newly identified receptors

Table2

1 Table 2: Summary of *in vitro* reproduction of axon growth inhibition

Type of injury	Mode of injury	Description	Inhibitory environment	Inhibitory molecules	Ref.
Cellular trauma	Shear stress	Substrate deformation of astrocyte-neuronal co-cultures	Reactive astrocytes	CSPGs, Semaphorins	[32]
	Axonal transection	Traumatic axon damage	Damaged axons; myelin debris	?	[26]
	Contusion	Weight drop impacting crush injury on organotypic cultures	Damaged axons; myelin debris; reactive astrocytes	CSPGs	[24]
Glial scar (Explant scars)	Lesioned cortices	Neuronal cultures on extracts of damaged white matter	White matter debris; reactive astrocytes	MDP[1]; CSPGs	[59,60]
	Nitrocellulose sheets in the lesion site	Neuronal cultures on substrates from nitrocellulose sheets recovered from lesioned brains	Reactive astrocytes; myelin debris	CSPGs; MDP	[28]
		Substrates preconditioned with reactive astrocytes		CSPGs	[61]
		Growth cone turning/collapse assays	Damaged white matter	MDP, Semaphorins	[59,62]
Axon guidance		Substrate bound cues		CSPGs, MAG, Nogo, Semaphorins	[41,63]
		Micropatterned substrates from postnatal spinal cords	Postnatal spinal cords		[64]

2 [1]MDP: Myelin Degradation Products