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In vitro models of axon regeneration

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Abstract

A variety of *in vitro* models has been developed to understand the mechanisms underlying the regenerative failure of central nervous system (CNS) axons, and to guide pre-clinical development of regeneration-promoting therapeutics. These range from single-cell based assays that typically focus on molecular mechanisms to organotypic assays aimed at recapitulating *in vivo* behavior. By utilizing a combination of models, researchers can balance the speed, convenience, and mechanistic resolution of simpler models with the biological relevance of more complex models. This review will discuss a number of the models that have been used to build our understanding of molecular mechanisms of CNS axon regeneration.

Keywords

axon regeneration; neurite outgrowth; in vitro models; cell based assays; organotypic assays; high content screening

II- Introduction

Axons regenerate poorly in the central nervous system (CNS) of adult mammals. This is in stark contrast to the robust regeneration observed in the mammalian peripheral nervous system (PNS), or in the CNS of many other organisms. The absence of a robust regenerative response after CNS injury contributes to permanent functional deficits and disability. Consequently, substantial efforts have been directed at understanding the biological basis for

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the disparity in regenerative behavior between axons in the CNS and the PNS, and towards identifying mechanisms for stimulating CNS regeneration.

In the late 1970s, it was observed that that PNS axons, while able to regenerate in PNS tissue, have difficulty regenerating through optic nerve grafts [1,2]. A few years later, Aguayo et al demonstrated that some injured CNS axons are able to regrow long distances within sciatic nerve grafts but fail to reintegrate into CNS tissue [3,4]. These observations led to the conclusion that the PNS microenvironment is permissive for axon regeneration, while factors in the CNS are non-permissive or even inhibit axon regrowth. In addition to these *in vivo* works, several groups demonstrated that certain diffusible (e.g. NGF), cell surface (e.g. L1), and matrix bound factors can positively regulate cell adhesion and axon growth [5–8]. Later studies focused on characterizing the molecular mechanisms that mediate the CNS's inhibitory activity [9–13]. Multiple extracellular molecules with potent inhibitory properties, along with their cognate receptors and intracellular signaling pathways, were described. These include myelin associated proteins, tenascins, and chondroitin sulfate proteoglycans (CSPGs), [10,14–21].

Disrupting extracellular inhibitory signals, either individually or in combination, has thus far failed to produce robust axon regeneration [22–24]. On the other hand, manipulating signaling pathways within CNS neurons could promote significant regeneration [25–28]. These findings underscored another important aspect of regeneration failure, *i.e.* the genetically programmed decline of intrinsic regenerative capacity in CNS neurons. The complex interplay between neuron intrinsic and extrinsic factors suggests that strategies for affecting robust CNS axon regeneration must simultaneously target multiple mechanisms.

A large number of *in vitro* models have been developed to investigate axon regeneration. Cell-based assays are useful for dissecting subcellular and molecular mechanisms, but they do not reflect much of the complex cellular interactions that occur *in vivo*. Conversely, assays utilizing multiple cell types or tissue explants can have stronger biological relevance, but may be more difficult to dissect mechanistically. A broad range of experimental models will be reviewed here, with some discussion about the strengths and weaknesses of each. It should be noted that nerve regeneration is a complex process that may involve the genesis of new neurons, glia, axons, synapses, and connective tissue. This review specifically focuses on *in vitro* models of mammalian axon regeneration.

III. Cell-based models

A variety of *in vitro* assays have been developed to assess the intrinsic regenerative ability of CNS neurons and discover agents that can induce axon growth. Typically, cell preparations are obtained by mechanical dissection, enzymatic dissociation, and centrifugation. Filtration or affinity separation techniques may be utilized to produce relatively homogenous cultures from mixed cell preparations. It is important to note that the dissociation process itself is likely to cause extensive severing of preformed neuronal processes depending on the age and location of isolated cells, and therefore induce an injury response. Proper methodology and controls are necessary to isolate regenerative responses resulting from dissociation from those that are due to manipulations done to the animal, or to perturbagens applied to the

cultured cells. Long standing research efforts and optimization of culture reagents has made it possible to grow dissociated neurons in chemically defined serum-free media. These media preparations can selectively favor the survival of certain cell types (e.g. neurons over glia) [29–32]. This can help to maintain the purity of neuronal cultures and minimize variance in assay readout. The use of cells from mice in which apoptosis is impaired via knockout strategies, can assist in the dissociation of effects on regeneration and survival [33].

III. a. Low-density (sparse) culture models

The main advantage of low-density culture assays is their ability to extract detailed morphological information on individual cells. Another advantage is the relative ease with which they can be adapted to high content screening (HCS) approaches [34]. HCS provides the ability to efficiently acquire morphological measurements for millions or billions of cells treated with different perturbagens. It permits detailed and unbiased investigation of the overall morphological response and population variance for any given treatment. This creates an efficient platform for identifying agents with high cellular efficacy and low toxicity, as reflected by the growing emphasis on phenotypic screening [34].

Most neuronal HCS assays use neurite outgrowth *in vitro* as a surrogate measure for process extension *in vivo*. This is due to multiple reasons including: 1- the time required for cultured neurons to reliably express axonal or dendritic protein markers (several days to weeks) [35,36] is beyond the optimal assay duration (1-5 days), and 2- axonal markers are typically restricted to the neurite at some distance away from the cell body, making it difficult to automatically connect traced processes to the correct cell body [34]. Accordingly, assayed neurons are typically immunostained for β3-tubulin, and growth of putative axons and dendrites is often assessed simultaneously (FIGURE 1). Nevertheless, depending on the exact goals and desired throughput of a study, additional dendrite or axon-specific staining (e.g. Anti-tau or anti-MAP2) may be employed [37,38].

Neurite outgrowth assays

Neurite outgrowth assays are among the most commonly utilized phenotypic screens relevant to axon regeneration. They have been successfully used to identify genetic regulators of axon regeneration [25,39], perform mechanistic studies of the involved pathways [15,40–42], and identify small molecules that can promote regeneration *in vivo* [43]. Neurons are typically grown on an adherent substrate that may be coated with one or more biological matrix proteins [44]. The exact choice of substrate components depends on the phenomenon under investigation. For example, using a growth-inhibitory substrate (e.g. CSPGs or myelin) may be used to focus on mechanisms that mediate extrinsic inhibition from matrix elements. On the other hand, a more permissive substrate (e.g. laminin) may be used to more easily identify agents that inhibit growth. The substrate proteins are usually loaded on a coat of polylysine. Neurons plated directly on polylysine will adhere and extend neurites, although at a much slower rate than neurons grown on laminin. This may be beneficial in certain situations, particularly where a large effect window is desired for a given assay. Neurons grown on laminin will extend neurites at a high rate, making the window for detecting improvement on this growth relatively small. On the other hand,

laminin can significantly improve neuronal survival and process extension, and has been extensively used in mechanistic studies of neurite outgrowth and axon regeneration.

Neurite outgrowth assays can be used to assess the effect of perturbagens on neurite extension. For chemical screens, cells are usually plated and allowed to adhere before compounds are added to the culture. For genetic screens, cells are often transfected prior to plating [25,39,41]. Recent advances in transfection technologies, however, enable passive transfection after plating [43,45]. Neurite outgrowth assays can also be used to assess latent effects of *in vivo* manipulations performed prior to extracting the cells from the animal. Examples may include pre-conditioning of dorsal root ganglia by damaging the peripheral nerve root, or virally transducing neurons *in vivo. In vitro* axotomy may also be performed where axons of cultured neurons are visually selected and transected [46]. This allows the investigation of events that occur after an injury in individual cells.

Following treatment, cells are incubated for a pre-determined amount of time. The duration depends on a number of factors including: intended perturbation length, basal neurite extension rate, and the required amount of separation between readouts from positive controls and baseline [34]. Larger separations (i.e. bigger effect windows) are desirable for screening [47].

For single endpoint assays, neurons are typically fixed and immunostained for a cytoskeletal marker, such as the neuron-specific βIII-tubulin protein [34]. Staining for βIII-tubulin fills the cell body and neuronal extensions. This makes it possible to automatically image and analyze neuronal morphology, including parameters such as neurite count, length, and branching. Plasma membrane specific dyes can also be used for delineating cell bodies and neurites [48]. Neurite outgrowth can also be measured dynamically in live low-density cultures in a label-free setting, using light microscopy [49]. Alternatively, neurons can be fluorescently labeled using transfection or viral transduction. This permits measurement of neurite outgrowth in live neuron-only cultures as well as in neuron-astrocyte co-cultures [49].

Neurite retraction assays

Neurite retraction assays [50] are a variation on the neurite outgrowth assay. Neurons are cultured for a given amount of time and allowed to extend neurites. Then, neurons are challenged with a substance (e.g. lysophatidic acid) that causes growth cone collapse and neurite retraction [51]. The neurites are allowed time to respond (minutes to hours), after which the cultures are analyzed. The benefit of this approach is that it can investigate the mechanisms leading to collapse and retraction by inducing the process in an entire cell culture. As such, it is a valuable tool for identifying agents that can inhibit retraction in order to favor a pro-regenerative state [41,52–54],

Stripe assays

The stripe assay was originally developed to investigate axonal guidance mechanisms *in vitro* [55,56]. It has been used to study mechanisms governing axon growth and guidance in the contexts of both development and regeneration [57–64]. Assay setup consists of applying stripes of control and test substrates, with the aid of specially manufactured silicon matrices, to a glass or plastic surface in a culture dish [65,66]. Control stripes typically contain a growth

permissive substrate, while the alternating “guidance” stripes contain the substance of experimental interest. A fluorescent label can be added to the base solution of either condition for easy visualization of the stripes. Linear stripes are most common, but zigzag [67] and graded patterns have been used [68]. Laminin can be applied homogenously to the entire substrate to provide a growth-promoting signal. Dissociated neurons are then applied to the dishes and incubated for a predetermined period, after which cultures are fixed and immunostained for analysis using fluorescence microscopy. Alternatively, cultures can be analyzed using time-lapse video with phase microscopy [67]. Results are interpreted based on how frequently axons growing from the control stripes avoid or grow over the experimental stripes [65]. One variation of the stripe assay, called the “step assay”, involves creating stripes with increasing concentration of the guide substance on each subsequent step of the gradient [69]. This enables simultaneous testing of a range of conditions [8]. The stripe assay can be combined with the turning assay to demonstrate the active repulsiveness of a given substance to axons. The turning assay was originally performed by applying a concentration gradient of a test substance to an individual growth cone via a micropipette [70,71]. This was later combined with electrically gated pressure application systems to deliver repetitive pulsatile injections of picolitre volumes of solution containing the guidance cue [72]. Ultimately, finely adjustable low pressure applicator systems eliminated the need for pulsatile application [72,73]. Newer variations involve confronting growing axons with beads coated with a substance and observing growth cone behavior using time-lapse video microscopy [74]. A turning of the growing axon or collapse of the growth cone upon contact with the beads confirms active repulsion by the coating substance.

Spot assay

Similar to the stripe assay, the spot assay involves creating a small boundary area containing a bioactive substance within a poly-lysine coated surface and then determining “boundary strength” by measuring the percentage of neurites from cells outside the spot perimeter that cross the spot’s rim [75]. To achieve this, a small drop of solution containing the test substance is allowed to coat the culture surface after it has been coated with poly-lysine and allowed to dry. The solution is often mixed with a dye to facilitate visualization. The surface is then rinsed and dissociated cells are plated onto it. Cultures are maintained for enough time for neurites to grow, after which they are fixed, immunostained, and analyzed [76,77]. A variant of this assay involves spotting a mixed solution of laminin with proteoglycans at different concentrations. The outcome is a circular area with laminin concentration decreasing from center to rim, while proteoglycan concentration decreases from rim to center. Thus, dissociated cells plated inside the spot extend neurites outwards against an increasing gradient of proteoglycan, which can be visualized by immunostaining with anti-proteoglycan antibodies [78].

Examples of low-density primary neuronal culture systems

Several major factors influence the choice of cell type for *in vitro* regeneration assays. The first consideration is, of course, the biological relevance of the cell model, which naturally predisposes the choice towards neurons that are directly involved in the disease phenotype *in vivo*. Another important consideration is the ability to culture the particular neuronal type in a setup that lends itself to the assay design. For example, cells that cannot survive at low

density cannot be used to study neurite outgrowth in individual cells in a homogenous culture, and may require the presence of a supportive cell layer (see neuron-glia co-cultures below). Other considerations include the ease of acquisition, number of cells obtained per animal, and the requirements of the assay based on desired throughput. Low-density culture systems have been developed for a variety of neuronal cells, some of which are outlined below.

Retinal ganglion neuron cultures—Retinal ganglion cells (RGCs) can be purified from dissociated rodent retina using a series of immunopanning steps to remove macrophages and other cells [79]. RGC survival in low-density cultures is enhanced by trophic factors that are normally produced along the visual pathway, such as BDNF and CNTF [80]. RGCs have been used to study pathways that control axon growth [25,81,82]. They have also been used to study growth cone dynamics and to investigate the effects of small molecules on CSPG-mediated axon growth inhibition [21,62,83]. Finally, RGCs have been utilized in genetic screens to identify agents that mediate neuronal death [45].

Cortical neuron cultures—Cortical neuron cultures can be prepared from dissociated embryonic or postnatal rodent sensorimotor cortex [39]. Glia conditioned media is usually required to support neuronal survival, particularly in low-density cultures grown on poly-lysine. Cortical neurons have been successfully used in genetic screens to identify genes that control intrinsic regeneration capacity [39]. The KLF7 transcription factor, a member of a protein family implicated by a screen with cortical neurons, was later shown to promote axon regeneration of CST axons *in vivo* [27].

Hippocampal neuron cultures—Hippocampal neurons can be easily prepared from embryonic rodent brains to yield cultures that are relatively free of non-neuronal cells [84,85]. An overexpression screen using embryonic hippocampal neurons was used to identify KLF4, a member of the KLF family of transcription factors, as a developmental regulator of axon growth [25]. Small molecule screens with these neurons have also identified kinase inhibitors that were later shown to promote regeneration of axons in cortical slice cultures and of CST axons *in vivo* [43,86]. Overexpression, biochemical, and computational studies have found that many of the kinases that regulate neurite outgrowth in other CNS neurons also regulate neurite outgrowth in hippocampal neurons [42,43]. Thus, while there are clear variations in the biology and responses of different CNS neuronal types, there appears to be sufficient conservation in the major pathways that control neurite outgrowth and axon regeneration in CNS neurons to justify the use of these neurons for screening. Additionally, neurite outgrowth is strongly inducible in these cells with relatively consistent behavior in controls when grown in chemically defined media on polylysine [43]. This allows for sensitive detection of neurite outgrowth promoters and accurate comparisons of the relative activities of various perturbagens. Such features are particularly useful for drug discovery and development campaigns, where structure activity relationship (SAR) studies following the initial step of hit discovery require reasonable effect sizes and relatively low variability.

Cerebral granule neuron cultures—Cerebral granule neurons (CGNs) can be purified from dissociated postnatal rodent cerebella by centrifugation on a Percoll gradient [87]. They

are a large population of relatively homogeneous neurons, and respond to inhibitory substrates such as myelin and CSPG [88]. They can be grown in standard cell culture media without the need for trophic factors or glial conditioning. Low-density cultures are possible on growth-supportive substrates (laminin) but tend to have poorer survival on polylysine. CGN cultures have been used in chemical screens to identify small molecules that modulate neurite outgrowth on inhibitory substrates [15,89,90]. They have also been used in genetic screens to identify genes that regulate neurite outgrowth [41,42].

Doral root ganglion neuron cultures—Enhancing sensory axon regeneration after PNS and CNS injury remains a goal of clinicians and scientists [91]. Thus, being able to study sensory neuron cultures obtained from dissociated dorsal root ganglia (DRG) has obvious benefits. A feature that distinguishes DRG neurons from other CNS neurons is that their peripheral axons can regenerate long distances and often re-establish appropriate functional connections after PNS injuries [92,93]. Furthermore, these neurons become “pre-conditioned” during such a process, such that their central axons acquire an enhanced regenerative/sprouting response [94,95]. Using dissociated DRG cultures, many of the mechanisms by which these processes occur have been studied [96–99]. Similarly, researchers have perturbed cells *in vivo* and then investigated how neurite outgrowth is affected on inhibitory substrates (e.g. myelin, CSPGs) *in vitro* [16,100]. Additionally, mechanisms by which certain neurotrophic factors, matrix molecules, and cell surface receptors influence regeneration have been elucidated using DRGs [88,101,102]. A salient benefit to using DRG neurons is that all neurites extended from these cells are technically axons, thereby eliminating the need for additional staining for axon-specific measurements. Drawbacks, however, include the fact that dissections can be time consuming owing to the ganglia’s location within the vertebral column. Furthermore, DRGs contain multiple classes of sensory neurons and these different populations may exhibit different phenotypes in vitro; this may affect assay performance [34,103,104]. Additionally, multiple different enzymatic digestions and immunopanning steps may be needed to obtain enriched neuronal cultures [105,106]. Finally, DRG neurons tend to extend neurites in culture at a much higher rate than other CNS neurons, which can shorten an assay’s duration and compress its dynamic range. This is particularly true for neurite outgrowth assays [34].

Non-primary neuron cultures—Short-term post-mitotic neuronal cultures, derived directly from primary tissue, have the benefit of phenotypic and gene expression profiles that mimic native neurons. Primary neurons are therefore considered more biologically relevant to neurons *in vivo* than immortalized cell lines [34]. Nevertheless, non-primary neuronal cultures offer some advantages. Primary neurons are post mitotic, thus small amounts of cells cannot be amplified to yield sufficient quantities needed for molecular studies or drug discovery campaigns. Thus, expandable cell line models may be desirable in situations where large amounts of cells are required. Examples include the PC12, SH-SY5Y, and NTERA-2 cell lines. The PC12 cell line is derived from a rat adrenal pheochromocytoma [107]. NGF-1 treatment ceases proliferation in PC12 cells and induces them to differentiate and extend neurites. This lead to their use as a model for neuronal differentiation, neuritogenesis, and neurite outgrowth, with some differences noted relative to neurite outgrowth in primary neurons [108]. The human derived cell lines, NTERA-2 and

SY5Y, were clonally isolated from a pluripotent human embryonal carcinoma and a human bone marrow neuroblastoma, respectively [109,110]. Both neuron-like cell lines can be induced with retinoic acid to differentiate and extent neurites in culture. Neurite outgrowth in NTERA-2 differentiated neurons is potently inhibited by CSPGs, making them a useful model for studying mechanisms of CSPG inhibition [14].

Limited access to live primary neurons from human has made it challenging to study the molecular underpinnings of neurological disorders in a patient-specific manner. Pluripotent stem cells (PSC) derived from human embryonic tissues or directly reprogrammed from patient somatic cells (human-induced PSC, iPSC) can, in principle, be differentiated into any cell type, at a scale compatible with high-throughput technologies [111,112]. Neurons differentiated from patient iPSCs may be able to model human neurological disorders in ways that animal model neurons cannot, owing to genetic and/or epigenetic hallmarks of a given disease. To date, human iPSCs have been differentiated into a variety of neuronal cells including glutamatergic, GABAergic, dopaminergic, retinal, sensory, and motor neurons [113–120]. Phenotypic assays using iPSC-derived neurons have been developed and can be readily adapted for neurite outgrowth studies, including high content screening [121,122].

III. b. High-density culture models

High-density cultures can have several advantages over low-density neuronal cultures, especially if material is needed for follow-up molecular studies (e.g. proteomic or transcriptomic analyses). Additionally, high-density cultures may favor the generation of more biologically relevant neuronal phenotypes that are difficult to obtain with very low-density cultures (e.g. dendritic spines, synaptogenesis, neural networks, myelination). Unlike low-density cultures, high-density cultures often cannot produce readouts at the level of individual cells. Neurite outgrowth assays may still be performed using non-low density cultures, by normalizing overall neurite outgrowth to the total number of viable cells [121,123].

Scratch assay

The basic design of the scratch assay consists of plating cells at high-density to form a monolayer, followed by mechanical removal of cells from an area within the culture (scratch), and finally quantification of the occupancy in the scratch area over a given amount of time [124]. A major goal for regeneration research is to identify agents that can stimulate pre-existing axons, destroyed by injury, to regrow and reestablish connectivity. The rate of neurite initiation from neuronal cell bodies, however, can strongly influence overall neurite outgrowth, making it difficult to distinguish between agents that promote initiation and those that promote elongation. The scratch assay has the benefit of more specifically quantifying growth from pre-existing neurites (or axons). Scratch assays were originally used to study ‘contact-inhibition’, cell proliferation, and cell migration [125,126]. However, they have been adapted to study wound healing, polarization, and process extension from cells.

In high-density neuronal cultures, the scratch serves two important functions: 1- It clears out an area where regrowth of axonal projections can be measured and quantified, and 2- it

provides an *in vitro* axonal injury paradigm. This model has been used to identify phosphatases that suppress axon regeneration after injury [127].

In relationship to glia, scratch assays can be used to model reactive gliosis and changes in astrocytes induced by trauma [128,129]. In particular, the involvement of signaling molecules (e.g. Cdc42) that regulate reactivity in astrocytes and neurite outgrowth in neurons have been elucidated with this system [130,131].

Radial assay

The radial axon growth assay is a special application of high-density cultures where the cell bodies are sequestered into a dense cluster from which axons grow radially outward [132]. This is achieved by applying a droplet of a highly concentrated suspension of dissociated neurons near the center of a well within a 1 mm diameter region followed by adding the appropriate amount of culture media. The resulting arrangement readily permits experimental axotomy and imaging of various axonal segments.

Fluidic Chambers

Fluidic technologies provide a platform for specifically isolating, treating, lesioning, and analyzing axons [133,134]. This is achieved by culturing neurons in one compartment, and allowing the axons to grow into a second, fluidically isolated, compartment. The use of fluidically isolated chambers began with the ‘Campenot’ chambers, which are composed of a Teflon divider adhered with silicon grease to a petri dish [135,136]. Campenot chambers require the use of a stimulating agent (e.g. NGF or BDNF) to induce the growth of neurites from cells within the Teflon divider into the outer chamber, which restricts the range of applications. Microfluidic devices were later developed to overcome this restriction [134]. Microfluidic chambers take advantage of liquid behavior in very small volumes to create chambers that are fluidically isolated without being entirely physically separate [137]. Typically, such devices consist of two or more small chambers connected by microgrooves of varying lengths (usually 0.5 mm or longer). A minute volume difference between the chambers creates a hydrostatic pressure differential, and the high fluidic resistance of the microgrooves allows a very small but sustained flow towards the chamber with less fluid [134]. This unidirectional flow fluidically isolates the chambers. Consequently, neurons can be plated into the higher volume chamber and their axons will extend through the microgrooves towards the lower volume chamber. This enables selectively exposing axons to perturbagens in the axonal compartment without directly exposing the cell somas and dendrites. Conversely, the flow may be reversed after axons reach the “axonal compartment” by reversing the volume differential, therefor allowing selective treatment of cell bodies in isolation from the axons in the axonal compartment. The length of the microgrooves can be adjusted depending on the duration of the experiment and the rate of axon extension of the studied neurons. This can help to ensure that only axons and not dendrites make it to the axonal compartment [138]. In related applications, microfluidic chambers can be used to create interconnected co-cultures with individual synapsis formed at defined locations. Such setups are useful for studying trafficking and other processes that occur along the length of axons connecting two cell populations [139].

Neuron-glia co-cultures

While neuron-only models are useful for dissecting neuron-intrinsic regenerative responses, they are not informative about extrinsic effects on axon regeneration from other cell types. Extrinsic factors from non-neuronal cells contribute to the limited capacity for axons to regrow [140,141]. Astrocytes undergo morphological changes and upregulate expression of CSPGs in response to in vivo and in vitro injuries (reactive gliosis) [142–144]. The astrocytic response is required for wound healing, but negatively influences axon regeneration in the chronically injured CNS [145,146]. Accordingly, it is important that agents for promoting neurite outgrowth don't contribute to the detrimental aspects of astrogliosis. Enriched astrocyte cultures have traditionally been obtained through passaging mixed glial cultures multiple times [147,148]. Now, primary astrocytes can be efficiently purified from rodent cortex using a astrocyte-specific antibodies or genetic labels in concert with cell sorting [149,150]. These cells have been utilized in phenotypic assays to examine the response of astrocytes to various perturbations [151–153].

Assays that utilize neurons cultured directly on astrocytes can provide a composite readout on the effect of perturbagens or astrocyte stressors on neurite outgrowth. While this setup may potentially be of higher relevance to what occurs *in vivo*, it provides less insight on individual cell-specific mechanisms. In the case of chemical perturbagens, these can be added to the co-cultures, or to either cell type prior to combining the cultures. In the case of genetic perturbagen, cells can be transfected prior to co-culturing, or neuron-selective transduction methods can be used (ex. AAV virus). Nevertheless, even in the case of cell-specific initial perturbation, the final outcome will be the result of complex neuron-astrocyte interactions, and will require follow up experiments to dissect mechanistically. Various protocols for culturing hippocampal or cortical neurons on astrocytes have been developed and can be used to study neurite outgrowth [75,154,155] or neurotoxicity [49,156]. Three dimensional co-cultures have also been used, in attempt to better mimic the environment of axons growing *in vivo* [157,158]. A stretch injury model has also been used to induce glial activation in co-cultures with DRG or cortical neurons [89,144,159]. Single-cell axotomy can also be performed in co-cultures using a 2-photon laser to study morphological changes taking place after injury [160].

IV. Explant/Organotypic models

In organotypic cultures, most cell-cell and cell-matrix interactions are not disrupted by dissociation procedures. Thus, organotypic or explant cultures are, in principle, a middle ground between dissociated cells and *in vivo* studies. Explant studies have been instrumental in enhancing our understanding of axon guidance [161]. While the initial preparation may require fewer steps than a typical dissociation protocol, downstream portions of the experimental protocol can be lengthier, owing to increased time requirement for immunostaining and imaging procedures. Additionally, analysis of neurite outgrowth can be complicated by tissue architecture.

DRG explants

Whole ganglia explants have been used extensively for identifying growth factors and matrix derived factors that influence axon growth and regeneration [70,162,163]. A major advantage of this model stems from the fact that the ganglia can be rapidly isolated from organisms (typically, mouse, rat, or chicken) at various developmental stages and directly cultured [164]. The cells are subjected to minimal trauma (i.e. axotomizing of peripheral and central branches) relative to the harsh enzymatic and/or mechanical stress of dissociated cell preparations. Once the ganglia adhere to the culture substrate, neurites extend radially from the explant [165]. Because the explants are large, they can be handled with fine forceps or pipette tips and placed into specific regions within a culture dish. This makes the model particularly useful for carrying out variants of the stripe or spot assays experiments described above [166,167]. DRGs can be obtained from specific spinal levels following particular *in vivo* manipulations (e.g. preconditioning lesion), and placed in culture to investigate downstream effects on neurite outgrowth within a chemically controlled environment [95,168].

Despite the tractability of this model, there are several drawbacks. For instance, given that neurons are retained within the DRG capsule, routine immunohistochemical studies of sensory neuron cell bodies and their proximal processes may not be possible. The extended neurites generally fasciculate and some may never directly contact a defined culture substrate. Additionally, the relatively large size of the explants makes them susceptible to fluid movement, which could shear neurites if the cultures aren't handled with extreme care. Finally, it is important to note that DRG explants contain large numbers of satellite and supporting cells that migrate out from the capsule. Thus, distinguishing between direct and indirect effects on neurons may be challenging.

Retinal explants

The retina is an extension of the CNS and resembles other CNS tissues in terms of structure, function, and response to injury [169]. Thus, the optic nerve, which contains retinal ganglion cell axons, is effectively CNS tissue [170]). The retina is an attractive *in vitro* model system because the tissue can be easily obtained and cultured [171–173]. Furthermore, retinal explants can yield tissue slices of relatively uniform thickness that survive well in culture and produce quantifiable neurite outgrowth [174]. Early work with retinal tissue was focused on exploring the role of molecular gradients in modulating CNS axon growth [175–177]. Culture systems using retinal explants with strips cut out of the optic tectum (hence, strip assay), and later stripes of defined matrix molecules (hence, stripe assay), were successfully used to elucidate receptor-ligand partners in axon guidance [65,173,178–180]. Co-cultures of retinal explants and tectum have also been employed to study changes in neuron's intrinsic regenerative capacity [181]. Retina explants were also utilized to study the effect of Schwann cells on CNS axon growth and regeneration [182–184]. Retinal explants have been useful for elucidating the dependence of axon growth on both electrical activity and neurotrophic factors [82,185]. More recently, several studies using retinae demonstrated similarities between the growth of blood vessels and axons [186–188]. Finally, retinal explants have been used to examine CNS axon regeneration in response to perturbation with small molecules or extracellular matrix elements [189–191].

Brain and Spinal Cord Slice cultures

Slice cultures (from brain, spinal cord or both) serve as contextual regeneration models because axons grow in a glial-rich environment. With tools like genetic fate mapping and viral vectors, it is possible to observe growing axons without classical antero- or retrograde tracers [86,192]. In recent years, cortical slice cultures have been used to study axon regrowth following manipulations of regeneration associated transcription factors [193]. They have also been used to study how kinase inhibitors affect axon growth [86]. Recently, spinal cord slice cultures were used to study axon growth and remyelination, as well as astrocyte migratory and growth-supportive behavior [194–196]. Several groups have reported co-culturing cortex and spinal cord tissue to investigate the regrowth of cortex derived axons into spinal cord tracts [197,198].

While some of the native context of neurons is preserved in organotypic cultures, the extraction and culture process is itself an injurious procedure that can induce widespread cell death and other alterations within the slice [199]. For example, it has been demonstrated that some aspects of reactive gliosis are manifested in hippocampal slice cultures [200]. This is important because it demonstrates that the neuronal environment within a slice culture is not equivalent to the intact *in vivo* setting. Additionally, analyzing axons or neurite outgrowth is complicated by non-uniform tissue thickness and tortuous growth paths, whereby confocal microscopy is required to visualize axons throughout the slice. Thus, appropriate sampling and analysis tools must be used in order to generate reliable data [86,193,201].

V. Special Considerations in High Content Screens

Information obtained from screens using “real” primary neurons is highly valuable and ultimately worthwhile. However, high content screens on primary neurons must be carefully designed and executed—the list of challenges is long. Major issues include:

1) Variability in day-to-day and week-to-week performance of the assays, owing to subtle effects of cell density on neurite outgrowth, lot-to-lot variability of cell culture reagents purchased from vendors, and other less obvious variables.

2) Selecting the “ideal” primary neuron for screening is fraught with challenges, not the least of which stem from grant/manuscript reviewers with little or no screening experience. In an ideal situation, researchers would obtain many millions of appropriately aged and homogeneous neurons that are highly relevant to the *in vivo* population being modeled. For most types of primary neurons, this is simply impossible. For example, obtaining large numbers of adult neurons from the brain, spinal cord, or retina is difficult or impossible. Dissociating nervous tissue when the neurons are more than 1-2 days post-generation typically leads to an injury response that may have substantial effects on their transcriptional and epigenetic state. As examples, while young cerebellar or hippocampal neurons are relatively homogenous, cortical or spinal cord neurons are clearly not. Dorsal root ganglion neurons and retinal ganglion cells, while relevant to specific important research questions, have multiple subpopulations and are difficult to obtain in large numbers.

3) Even when working with a fairly homogenous population of neurons, there is large and non-Gaussian variability in the lengths of neurites produced by individual cells. The inherent problem of the heterogeneous response of primary neurons to neurite outgrowth promoting factors was illustrated by J. Raper and colleagues [202] and is observed today in all HCA assays of neurite outgrowth. Strong growth promoters like laminin and L1cam may increase mean neurite outgrowth by over 50% compared to non-biological substrates like poly-lysine. This leads to assays with small signal-to-noise ratios and Z'-factors that are considerably lower than biochemically based assays. Identifying and ranking robust hits is therefore challenging.

4) Assay design can have significant impact on assay sensitivity. For instance, with immature cells like E18 hippocampal neurons or E18 DRG neurons growing on highly permissive substrate like laminin, neurites will grow rapidly and start contacting other cells or neurites in the culture. This has two consequences for the assay. The first is that analysis of neurite length at the cell level becomes impossible, since image analysis software cannot assign neurites to the soma of origin. The second consequence is less obvious. If basal neurite growth is rapid, it becomes difficult to detect an increase in growth rate. This "ceiling" effect is well known in biological assays [203]. We have overcome these issues in several cases by using culture conditions that are less permissive for neurite outgrowth, such as using polylysine without laminin as a substrate.

5) Lack of effective controls is also a major hindrance to assay development. Without robust positive and negative controls, most screening centers will not accept a project, since it is impossible to calculate assay performance metrics and normalize data across plates and experiments. In the early 2000's, there were almost no compounds or genes that could robustly enhance neurite outgrowth. For some cell types (DRG neurons, sympathetic neurons, retinal ganglion cells), soluble growth factors could serve this purpose. But for other cells, such as cerebellar, cortical, or hippocampal neurons, no positive controls existed, especially for overexpression and knockdown screens. Our lab currently uses several treatments as positive controls (e.g. doublecortin for overexpression screens and a kinase inhibitor, ML7, for compound library screens) for several different neuronal types.

The advent of RNAseq has provided a means for investigators to compare the transcriptional landscapes of neurons *in vivo* and *in vitro*. The field is still relatively immature, with only about 200 papers mentioning RNAseq and neuron in PubMed in January, 2016. However, in the next two to three years there will likely be massive amounts of information that researchers can mine to evaluate the similarity of neurons used in screening assays to those in various developmental or disease states. Most importantly, it will allow investigators to assess whether neurons suitable for screening (e.g. E18 hippocampal neurons or iPSC derived human neurons) express genes and pathways relevant to neurons that are less suitable for HCS (e.g. RGCs or layer 5 corticospinal tract neurons). With this knowledge, massive libraries could first be screened with the HCS-suitable neurons to identify hits, and

then these hits can be validated in small-scale secondary assays with the non-HCS-suitable neurons, *in vitro* or even *in vivo*.

VI. Summary and Discussion

It has been known for thousands of years that the CNS does not experience sufficient regeneration in most cases to restore function following injury [204]. Experiments into why regeneration is so poor in the CNS were carried out by Cajal and colleagues at the turn of the 20th century [93,205]. In the almost 100 years that have passed since then, scientists have been extracting individual components of the nervous system and studying their behavior in attempts to deconstruct the phenomena that control regeneration. Such approaches, and the *in vitro* models that resulted from them, have produced an extensive body of knowledge with molecular-level resolution.

These studies began with whole tissue explants, where CNS components were extracted from a live organism and maintained in culture. Examples include retinal, DRG, cortical, and spinal cord tissue explants. By mixing and matching tissues from various CNS and PNS origins, strip assays were born. These led to the discovery of numerous matrix, secreted, or membrane-bound factors that influence axon growth, pathfinding, and regeneration. Strip assays were later refined into stripe and spot assays. These permitted more detailed investigation of the effects of purified or mixed bioactive molecules, over homogenously or gradient coated surfaces. Relatively recent advances in cell culture technologies have made it possible to maintain purified primary neuron-only cultures *in vitro*. Improved media formulations can support the survival of low-density neuronal cultures for days or weeks. Such setups permit detailed molecular and morphological investigation of neuron-specific responses, even at the level of individual neurons.

From whole tissue explants to single cells, every model comes with its own set of strengths and weaknesses. With the possibility of culturing almost every neuronal tissue or cell type, the choice of *in vitro* model can be prioritized by biological relevance at every level. For example, one may choose cortical neurons to study CST regeneration, DRG neurons to study sensory axon regeneration, RGC neurons to study optic nerve regeneration, etc. Sufficient conservation in the mechanisms that control neurite outgrowth across different cell types may permit some flexibility with cell choice, particularly where strong technical considerations factor into assay design (e.g. using hippocampal neurons instead of cortical neurons for large scale screening campaigns focused on spinal cord injury). It is possible that iPSC derived neurons will become major tools for such studies in the foreseeable future, especially for HTS screening. It may take years, however, for the field to agree on robust and comprehensive markers to define “signatures” for different kinds of neurons (e.g., cortical and spinal motor neurons) and the culture conditions required to produce them from iPSC cells. In the end, a carefully selected battery of *in vitro* regeneration models, with varying levels of complexity, will likely constitute the best approach for understanding CNS regeneration biology. It is also of particular value for prioritizing therapeutic strategies before embarking on the expensive and time consuming *in vivo* studies.

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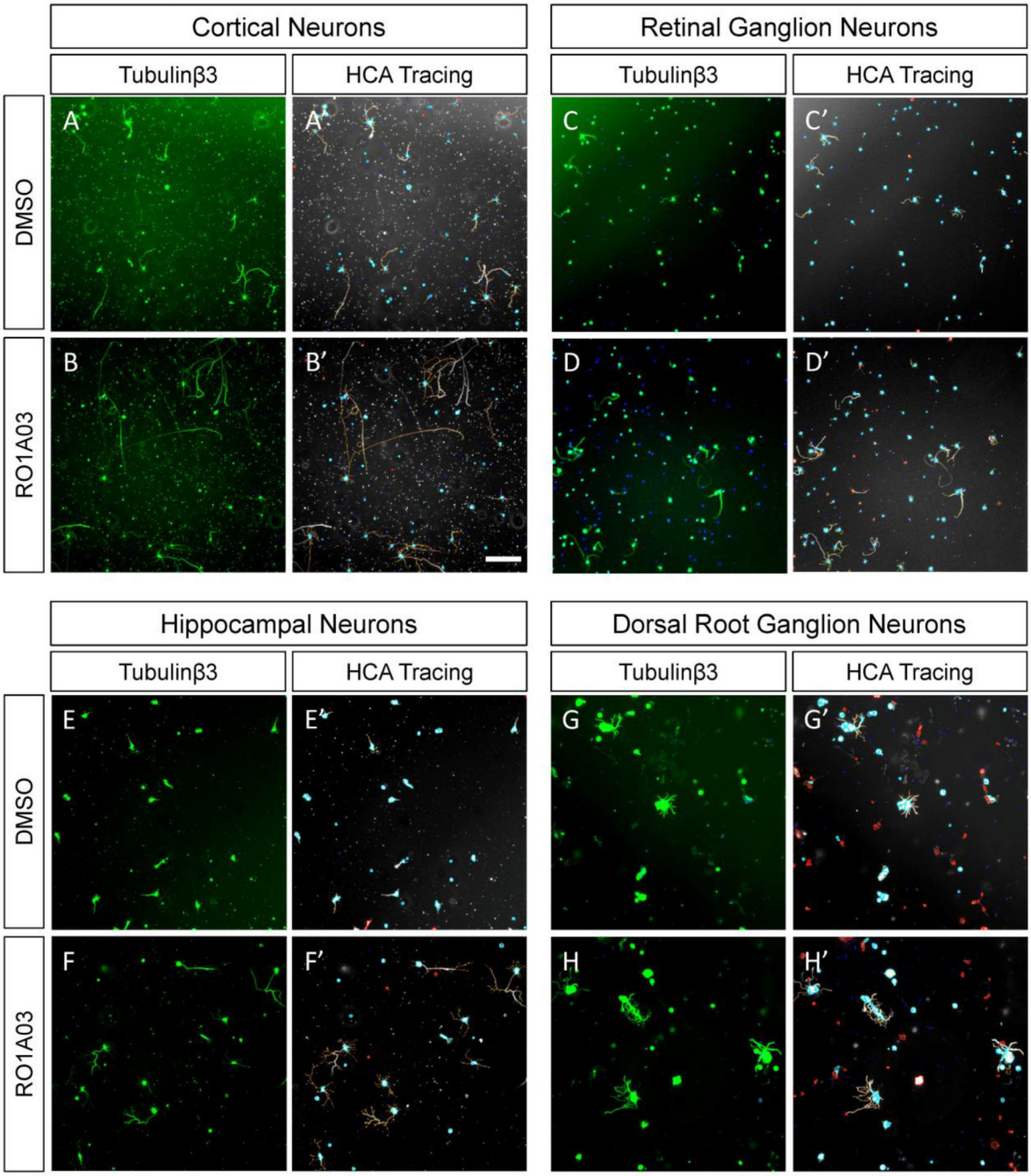

Figure 1. Examples of cell-based in vitro models of axon regeneration from neurite outgrowth experiments

Micrographs show various types of primary neurons cultured in the absence (A-A', C-C', E-E', G-G') or presence (B-B', D-D', F-F', H-H') of a neurite growth promoting kinase inhibitor, RO1A03. A-H) Show anti-β3 Tubulin immunostaining used to visualize the neuronal cytoskeleton (green). A'-H') Automated tracing of neuronal cytoskeleton (grey) obtained using the Cellomics Neuronal Profiler bioapplication shows cell bodies of valid neurons (blue), neurites (orange and purple), and cells rejected by the tracing algorithm (red) based on morphological parameters. A-B) Postnatal (P3) rat cortical neurons 2 DIV. C-D)

Postnatal (P21) retinal ganglion cells 3 DIV. E-F) Embryonic (E18) rat hippocampal neurons 2DIV. G-H) Adult (10w) mouse dorsal root ganglion neurons 1 DIV. Images are single fields obtained with Cellomics VTI (5× objective) from cells cultured in 96-well plates. Scale bar is 200μm.