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Advances in ex vivo models and lab-on-a-chip devices for neural tissue engineering

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Abstract

The technologies related to ex vivo models and lab-on-a-chip devices for studying the regeneration of brain, spinal cord, and peripheral nerve tissues are essential tools for neural tissue engineering and regenerative medicine research. The need for ex vivo systems, lab-on-a-chip technologies and disease models for neural tissue engineering applications are emerging to overcome the shortages and drawbacks of traditional in vitro systems and animal models. Ex vivo models have evolved from traditional 2D cell culture models to 3D tissue-engineered scaffold systems, bioreactors, and recently organoid test beds. In addition to ex vivo model systems, we discuss lab-on-a-chip devices and technologies specifically for neural tissue engineering applications. Finally, we review current commercial products that mimic diseased and normal neural tissues, and discuss the future directions in this field.

Keywords

Test beds for neural engineering; Bioreactors; Neural tissue engineering; Lab-on-a-chip; Ex vivo neural models

1. Introduction

This review is a comprehensive attempt to report technologies related to ex vivo models and lab-on-a-chip devices that are used to model regeneration processes in the peripheral nervous system (PNS) and central nervous system (CNS). Sections in this review will discuss the necessity for ex vivo and lab-on-a-chip technologies for neural tissue engineering applications and the advances of these technologies in mimicking brain, spinal cord, and peripheral nerve tissues. In addition, we discuss how the culture systems are progressing from 2D to 3D for regeneration model development and the role biomaterials plays in advancing neural regeneration model systems. For the purpose of this review, injury context will include traumatic brain injury (TBI), spinal cord injury (SCI), and peripheral nerve injury (PNI). Degenerative diseases (e.g., Multiple Sclerosis, Alzheimer's disease), viral and

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microbial diseases (e.g., HTLV 1), genetic/developmental disorders present from birth (e.g., autism, microcephaly), stroke, and models of the blood-brain barrier are not covered. In addition, pharmaceutical test beds are not discussed in this review.

1.1. The need for ex vivo models and lab-on-a-chip devices

Neural tissue damage in the PNS and CNS can cause devastating life-long disability. Neural tissue engineering is an emerging strategy to enhance neural regeneration, facilitate bridging of large tissue gaps, and encourage minimal scar formation. To develop and test regenerative strategies for neural repair, proper preclinical models are required to mimic effectively the neural injury micro-environment. Currently, there are no ideal models to mimic all biological and physiological aspects of neural injury that perfectly translate to an effective clinical application. Such an ideal model should reflect the dynamic clinical and pathological changes and address the requirements of availability and reproducibility [1]. Traditional in vitro cell culture models are associated with several drawbacks, such as oversimplicity and inability to produce extensible and reliable results that accurately predict the in vivo response. In vivo model systems extensively used over the past decades also face disadvantages. Animal models used for neural tissue engineering research provide various types of neural injury models in brain, spinal cord and peripheral nerve for preclinical studies. However, promising regenerative strategies, surgical interventions, and neuroprotective medications, which were identified to be effective in those models, have largely failed in Phase II or III clinical trials [2,3]. Below we discuss a number of reasons for the low reliability of animal models in neural regeneration research. This prevalent issue underscores the necessity to develop more accurate ex vivo models of human neural injury.

1.1.1. Limitations of animal models in studying neural regeneration—The first limitation associated with animal models is that the healing mechanisms are highly affected by the cause, degree and size of injury, which are not easily replicable and quantifiable in animal models. For instance, in the case of fluid percussion and cortical impact TBI, rodent models have been used frequently in tissue engineering research [2]. Although these models are more reproducible compared to other TBI models (e.g., blast models), the degree of cortical damage is highly dependent on both craniotomy position and injury severity [4]. This makes these models inconsistent and difficult for reliably quantifying injury severity. These issues have been extensively discussed and reviewed by Wojnarwicz et al. (2017) and Xiong et al. (2013) [5,6]. In case of SCI, there are important differences between injuries that occur in humans and those created in animal models. Most human cases of SCI consist of a mixture of contusion and persistent compression, whereas the majority of experimental in vivo models are only contusions or transections [7,8]. Although a mixed compressive–contusive lesion can be mimicked using inflatable balloons or *trans*-vertebral screws, the histological and functional outcomes of these models are very inconsistent [9]. In addition, the size of SCI lesion in rodents does not correspond with the size of the lesion in humans, as SCI lesions in humans are approximately 75 times larger than lesions in rat models. Considering this, the average human cell size is not 75 times larger than that of the rat counterparts. Accordingly, the number of cells that need to proliferate and functionally regenerate in human defect is remarkably greater. In case of peripheral nerve injury, severity of the nerve trauma and the size of the nerve gap directly affect wound healing and the

capacity of regeneration [10]. The challenge in this case is the mismatch of the nerve gap size. In rat models, nerve injury gaps from 1 to 1.5 cm are used as critical size defect models, while those in human PNI range from 5 to 30 cm [11].

The second limitation of animals as preclinical models is biological mismatch. We are incapable of truly mimicking human neural injuries in animal models because of the extensive anatomical, functional, molecular, immunological, and pathological differences between humans and frequently studied animals [8,12]. For example, there are remarkable differences in brain structure and function between human and non-human mammals. These include brain geometry, craniospinal angle, gyral complexity, and white to gray matter ratio. This may lead to substantially different responses to TBI from species to species [5]. In the case of SCI, there are significant mismatches in loading conditions of spinal cord between human and rodents. Recently, researchers have demonstrated the key role of the mechanical forces and load distribution in determining the severity of SCI [13]. Another anatomical mismatch is the location of the corticospinal tracts in the spinal cord of rodents versus humans. Due to this neuroanatomical dissimilarity, different degrees of locomotor dysfunction occur as a result of similar defects in the same anatomical regions [14]. Moreover, there are fundamental biological differences in SCI in humans and rats (e.g., the distance to the blood supply), which causes inconsistent results of the cell transplantation efficacy in different species [15]. Likewise, in PNIs, there is a significant difference in general distance between the axonal lesion and cell soma among various species. This causes remarkable differences in the speed and capacity of regeneration. Moreover, while general axonal regeneration may have substantial functional effects in a rat model, such effects in humans are highly dependent on the region of the defect [16].

These differences are not discussed to completely discredit animals as preclinical models, especially since various in vitro models definitely lack the complexity of human physiology. Animal models currently are beneficial to assess systemic effects of newly developed therapeutics and host-graft interactions upon introducing new biomaterials. Ex vivo systems, on the other hand, allow controlled evaluation of specific parameters on cell behavior and tissue regeneration, for example. Although animal models are still critical for clinical translation, improvement of ex vivo models can reduce the number of parameters that have to be tested in animal models, thus lowering the number of animals required. With the rapid progress in the development of complex ex vivo systems and organs-on-a-chip that better mimic physiological conditions of human organs, replacing animal models with these model systems may become a possibility in the future. This necessitates continued research and development of ex vivo models that recapitulate human physiology and address several concerns of animal models such as ethical issues, as discussed below.

1.1.2. Ethical issues and economic concerns of animal models—*“Despite dramatic advances in the molecular pathogenesis of disease, translation of basic biomedical research into safe and effective clinical applications remains a slow, expensive, and failure-prone endeavor”*, said Dr. Francis Collins, the director of the National Institutes of Health in 2016. A systematic review showed that cost and regulatory barriers are often quoted as major reasons for slow process at this level [17]. There are general and growing ethical concerns regarding the use of animals in scientific research [18]. Many countries worldwide

have passed extensive regulations and encouraged scientists to implement the 3Rs (replacement, refinement, and reduction of the use of animals in research). Currently, UK and EU directives include a formal introduction of the 3Rs as guiding principles. In addition, both U.S. and Chinese regulations call for the incorporation of these principles in experimental design [19]. Neuroscience research, including neural tissue engineering, involves extra ethical considerations because of distinct requirements in research and characterization methods such as behavioral analysis [20–22]. Moreover, because of the need to replicate biological and anatomical complexity of the neural tissues, the requirement to utilize larger and higher order mammals is increasing in this area of research. Rodents and other smaller animals are available at a lower cost, however, as discussed previously, they are not perfect models for the complex human nervous system. For higher order mammals, the increased regulatory, space and husbandry burdens make the frequent use of these models more difficult. In particular, non-human primate studies could be very costly. For example, the price of a single purpose-bred macaque (a common non-human primate used in neural research) is about \$30,000 [23]. Additionally, housing and care from specialized facilities range from \$80 to \$110 per day. This cost compounds as animal studies can last many months to years. Further costs are required for the surgical process to induce the injury and to provide post-operative care and medications.

1.2. Injury models for neural tissue engineering applications

The main goal of developing ex vivo models and lab-on-a-chip devices is to provide a platform to mimic biological, biochemical, biophysical, and biomechanical features of an organic system. In neural tissue engineering, such models should be able to mimic the injury microenvironment, replicate the dynamic changes in tissue composition and properties, and encourage similar healing responses. Here, we describe common conditions and cellular events after neural injuries that are fundamental in neural tissue engineering research. Such disease models for neural tissue engineering research are summarized in Table 1.

Modeling Wallerian degeneration, an essential process after neural injury, is one focus of neural tissue engineering. Wallerian degeneration occurs after axonal injuries in the CNS and PNS. In the PNS, Wallerian degeneration results in fragmentation of axons at the distal site, where the growth cone collapses [24]. In brain, axonal fragmentation is a common condition that occurs after TBI, because of acute mechanical or chemical insult to the brain. Damage to white matter (where the long axons traverse the brain) post TBI includes traumatic axonal injury and de-myelination, which results in axonal degeneration. Recently, this has been recognized as a predominating mechanism for progressive neurodegeneration after TBI [25,26]. Therefore, TBI models that focus on axonal regeneration are essential. Different models mimic the cause of injuries (mechanical or chemical insult) and mimic microtubule deformation and delayed axonal signaling following injury [27]. In general, there are fewer model systems designed to specifically mimic and assess SCI. This is because the testing devices that are good for modeling brain are also able to model spinal cord after slight modification in the cell type and coating components. These devices can better mimic healthy and injured spinal cords through using spinal cord cells (e.g., spinal cord derived astrocytes and motor neurons) and culturing them with the extracellular matrix (ECM) components present in the spinal cord. Nevertheless, there are test beds that

specifically model the secondary injury that occurs because of SCI. Such models aim to recreate excessive deposition of chondroitin sulfate proteoglycans (CSPGs) that result in formation of inhibitory glial scar [27,28].

PNI disease models also have a certain overlap with CNS injury models. This is partially because many of the devices do not include CNS- or PNS-specific ECM components but rather general adhesive molecules such as poly-L-lysine (PLL) or ECM proteins that exist in both CNS and PNS (e.g., laminin). Spontaneous axonal regeneration, which accrues after PNI and mediates neural repair, has been modeled using simpler organisms such as roundworm *C. Elegans* [29]. Although these studies do provide us with insights into axonal repair that may lead to further understanding of repair mechanisms and development of improved therapeutic strategies, the organisms employed in these studies do not fully recapitulate mammalian cell behavior. To address this, a previously developed microfluidic model has been utilized to perform femtosecond laser-assisted axotomy on mammalian cell axons [30].

2. Ex vivo models for neural tissue

The development of in vitro models of brain, spinal cord, and peripheral nerve is rapidly evolving. In general, these models fall into one of six broad categories: two-dimensional (2D) culture systems, three-dimensional (3D) engineered scaffold systems, ex vivo bioreactor-based models, tissue explants, neurospheres, and organoids. Fig. 1 demonstrates ex vivo models for neural tissue engineering applications. In this section, we review the evolution of in vitro models of CNS and PNS injury (from traditional to modern), highlight common practice and recent progresses of ex vivo models, and discuss advantages and disadvantages of each.

2.1. Traditional culture models

2.1.1. Two-dimensional culture models

CNS: Traditional 2D cell culture used to be extremely prevalent and it is still useful in numerous applications, including modeling aspects of nervous system activities like myelination and neurite outgrowth. For TBI, many different methods of induction of injury have been developed for 2D culture, including scratch/tear [31], fluid percussion [32], stretching [33], and shearing [34]. In case of SCI, development of a glial scar is the most common aspect recapitulated in vitro. This response is characterized by the activation of astrocytes to a reactive state and then depositing of inhibitory ECM made predominantly of CSPGs. Together, reactive astrocytes and their ECM generate a chemical and physical barrier to regeneration [35,36]. One of the simplest models of the glial scar is a co-culture system of astrocytes and fibroblasts in the presence of TGF- β 1. This 2D model robustly generate key features of the fibrotic core of the glial scar, such as fibroblast proliferation, expression of growth inhibitory molecules, and accumulation of CSPGs [37,38]. Another way to mimic the glial scar in vitro is to utilize Neu7 astrocytic cells which abundantly produce CSPGs [39]. While glial scar modeling is more prevalent in 2D culture systems, SCI has also been induced by scratch/tear [40–42] and stretch similarly to those generating TBI [43]. Outside the CNS, PNS 2D culture models are also studied. Explanted dorsal root

ganglia (DRG) are one of the most commonly used cell clusters for modeling PNS [44]. For the purposes of this review, test beds utilizing DRG cultures are classified by culture method: attached to peripheral nerve or spinal cord (explant), on 2D surfaces, or within 3D scaffolds.

PNS: One of the simplest models of peripheral nerve regeneration is plated DRGs or Schwann cells grown in the presence of different growth factors and cytokines [45–47], or ECM components [48–53]. Most studies that generate a peripheral nerve scaffold first test the material, topography, or entire scaffold with DRGs and/or Schwann cells. For further reading, please see reviews by Marquardt and Sakiyama-Elbert and Gu et al. [54,55]. Researchers have also used 2D culture systems to model myelination, an important aspect of peripheral nerve regeneration. Co-cultures of Schwann cells and DRGs have largely resulted in Schwann cell proliferation, alignment, and later development of myelin and myelin sheaths on neuronal fibers [56–58]. Alternatively, motor neurons derived from sciatic nerves have been used in place of DRGs [59]. Similar models have been used for demyelination modeling. For example, high concentrations of Forskolin (40 μ M) can be used to induce robust demyelination in myelinated DRG cultures [60]. Models such as these are able to mimic at least one aspect of peripheral nerve injury in vitro and may serve to test interventions aimed at facilitating re-myelination.

There is a large body of literature over many decades for 2D neural cell culture. 2D culture models provide some level of control over environmental factors and are the easiest models to manipulate and observe. Finally, 2D cell culture allows for flexibility in cell sourcing, as it is amenable to isolated and commercially available cells derived from many donor species. However, the biggest limitation of these systems is lack of complexity in cell-cell and cell-matrix interactions. Accuracy of recapitulating in vivo like cell behavior is questionable in many 2D cell culture contexts. Studies have demonstrated that neural cells cultured in 2D differ in cellular behavior compared to those cultured in 3D and in vivo [61,62].

Additionally, either glass or tissue culture plastic are used for traditional 2D cell culture. These materials are orders of magnitude stiffer than those found in neural tissue in vivo [63]. Substrate mechanical properties influence cell behavior; for example, when astrocytes are cultured in 2D compared to 3D, they adopt an artificial reactive phenotype. These changes then alter cell morphology, gene expression, and proliferation [64,65].

2.1.2. Explant models—Tissue explants and organotypic slice cultures are some of the earliest in vitro models for organ systems and injury or disease (Fig. 2). The earliest known explant culture was conducted by Harrison et al. (1907) when a frog embryo was cultured ex vivo and neurons were seen growing out of the explanted tissue [66].

CNS: Explant models of TBI are numerous and provide a more physiologically accurate representation of injury in vitro than 2D cultures. Researchers induced TBI by crushing the CA3 region of the hippocampus with forceps before culturing in vitro [67]. Another research group obtained human cortical slices from patients with temporal lobe epilepsy and post-mortem donors, and demonstrated that the resection/preparation process elicited degeneration and glial cell reactivity reminiscent of that found after traumatic injury [68]. Contusion TBI is often induced via weight drop apparatuses or cortical impact devices

similar to the previously discussed methods for TBI induction. Other methods of induction of TBI include stretch and fluid percussion. Researchers cultured hippocampal slices on silicone membranes, which were bi-axially stretched to induce dynamic deformation [69,70]. Fluid percussion models are able to replicate recapitulate TBI from explosive blasts. To model this type of injury, researchers utilized a polymer split Hopkinson pressure bar to generate controllable pressure waves which travel through an artificial cerebrospinal fluid filled chamber and are imparted on coronal slices mounted on ballistic gelatin [71].

To model SCI on explants, injury is created via weight drop apparatuses, transection, or chemical administration. Implementation of weight drop as a model of compressive SCI is one of the oldest in vitro models of SCI. In this type of SCI model, a weight is dropped from a varying height onto a spinal cord slice [72,73]. Transection models of injury are very common as they are relatively simple to implement. By slicing spinal cord cultures with scalpels to generate a lesion margin, key aspects of SCI can be recreated [74,75]. Additionally, transection models of SCI have been able to recapitulate lost functional connectivity. Heidemann et al. (2014) demonstrated that after transection of integrated spinal slices from adult animals, slices were not able to regenerate and establish functional connectivity as determined by percentage of synchronized bursts between each slice [76]. Chemical induction of spinal cord lesion represents another means of inducing SCI on explant models. To replicate the environment during secondary SCI in vitro, the addition of pathological medium (standard salt solution, 10 mM H₂O₂, 500 μM sodium peroxynitrite, no glucose and Mg²⁺) and glutamate-analog kainate is used. This method of inducing injury impairs synaptic transmission and causes hyperexcitability, metabolic perturbation, and damage to neuronal networks [77–79]. Once an explant injury model is validated and reproducible, it can then be used to assess interventions such as biomaterials that promote regeneration. Gerargo-Nava et al. (2014) demonstrated an effective ex-vivo 3D model for post-natal motor axon regeneration where they connected a spinal cord slice with ventral nerve root within 3D scaffolds. This model has also been used to investigate regeneration and maturation of Schwann cells [80].

PNS: Researchers typically use crush or transection to create injuries in explanted peripheral nerves. Early studies tended to induce injury using crush. For instance, frog sciatic nerves were isolated with their attached DRGs and injured by looping suture thread around the nerve and tightening to cause a crush injury [80,81]. Later studies favored inducing crush injury using forceps [82,83]. Studies that are more recent have turned towards generation of peripheral nerve injury using transection. Vyas et al. (2010) abutted spinal cord slices and peripheral nerves (medial and ulnar) to generate a re-innervated spinal cord/peripheral nerve explant model [84]. After re-innervation, PNI was induced by cutting the nerve segments with micro-scissors. This construct was later improved to facilitate ease of manipulation, enable high resolution imaging, and permit isolation of nerve and spinal cord segments for separate interrogation [85].

Explant models are the closest approximation to the in vivo setting by preserving the native heterogeneous cell populations, spatial distribution, network connections, and extracellular environment and architecture. By utilizing explant models, neuronal effects can be better isolated, though not fully removed from systemic circulation and immunological responses.

This allows for a more simplified environment to investigate complex cell interactions that occur within the tissue. Finally, explant models from adult donors provide a more clinically relevant test bed.

However, there are distinct limitations to the use of explant in vitro models. First, the use of explant models still requires animals or donors to generate in vitro models. Isolation, preparation and culture of neural explants can also be challenging. Further, the physical process of explantation may be traumatic to the tissue, thus creating artificial phenotypes or injury-like responses before culture begins, which may confound results. Size is also a limitation as thicker samples may result in hypoxic conditions and core necrosis. Additionally, explants are difficult to fully maintain over long periods [86]. Although human explants are possible, the availability of these samples is limited as nervous system explants are typically harvested post-mortem. In some cases, explants can be performed during the donor's lifetime, e.g., during surgery for temporal lobe epilepsy, but this severely limits the population of donors to a specific subset whose pathology may impact in vitro studies [87]. Overall, tissue explants are currently the best approximation of the complete in vivo environment, but still do not completely replicate normal and injured CNS and PNS environments.

2.2. Modern three-dimensional engineered scaffolds as ex vivo models

2.2.1. Biomaterials for ex vivo models—As an intermediate between 2D and explant cultures, 3D biomaterial based models serve as a bottom-up approach to generate ex vivo models. 3D models aim to recreate multiple facets of the in vivo environment including mechanical properties, architecture, and protein content but provide the next level of control over system fabrication. This allows for further isolation of mechanisms of injury or regeneration compared to explant models while providing more accurate physiological cues compared to 2D culture. There is a large body of literature regarding biomaterials and 3D engineered scaffolds for assessment of neural tissue cell function and behavior. These materials are typically used as a proof of concept before proceeding to pre-clinical assessment in vivo [88].

Biomaterials currently used as scaffolding materials for neural regeneration (and a few partly used as test beds) are generally classified as natural (e.g., hyaluronic acid (HA) [89], collagen [64,90], silk fibroin [63,91], elastin [92], fibrin [93,94], decellularized peripheral nerve ECM [95], agarose [96], alginate [97], Matrigel® [98], chitosan [99], serum albumin [100]), synthetic (e.g., poly (L-lactic acid) [101], polyethylene glycol (PEG) [102], polycaprolactone (PCL) [103,104], polyurethane [105], polyamide [106], polypyrrole (conductive polymer) [107]), or composite (e.g., agarose/CSPGs [108], PEG/PuraMatrix™ [109], dextran/laminin peptide [110], poly-L-lysine/chitosan [111], Graphene/PCL [112], HA/collagen/laminin [113], PEG/HA/collagen [114]). These biomaterials are summarized in Table 2.

These materials can be tuned to mimic ECM features by adding architectural, mechanical and biochemical cues, to match better the target neural tissue (bottom-up approach). Provision of appropriate microarchitecture facilitates neural growth and organization. Various microarchitectures have been designed and studied for neural regeneration, such as

aligned PCL fibers with different diameter for peripheral nerve studies [103,104]. Mechanical properties of the 3D culture systems have immense impact on cell behavior. Natural cell alignment and spatial arrangement, and morphology can be modified by changing the mechanical properties of the substrate [65,115]. Biochemical mimicking of the ECM is another strategy for improvement of cell migration, differentiation and proliferation. A hydrogel that mimics ECM can be fabricated by combining natural ECM components, such as proteoglycans (e.g., glycosaminoglycans) and fibrous proteins (e.g., collagen I, collagen IV, elastin, fibronectin and laminin) [116,117] or developed from decellularized nerve tissue [95].

Another approach for using biomaterials for fabrication of regenerative neural ex vivo models involves bottom-up assembly of integrated material and cell layers. In a more traditional top-down approach, cells are seeded on bio-engineered scaffolds, whereas in a bottom-up approach, stacked cell layers are used as 3D building blocks, nano-fibrous scaffolds infused with cells are used to provide nano-scale mimics of ECM, or self-assembled peptide hydrogel/cell composites are fabricated as injectable solutions to fill the irregular-shape cavities in brain and spinal cord. In one example, electrospun fibers from biocompatible polymers, such as poly-L-lactic acid (PLLA), have been used to guide neural cell morphology in an ex vivo platform. Although this approach faces limitations such as difficulty in handling and processing the electrospun fibers, recently this approach has been investigated increasingly for building neural tissue engineering constructs [101].

Although many biomaterials have been developed and studied, there are only a few model systems designed to mimic the neural injury microenvironment. Compared to healthy nerve, injured neural tissue exhibits significant alterations in protein expression (e.g., excessive deposition of CSPGs in the CNS) and fragmentation of ECM proteins [118–120]. Although researchers have assessed astrocyte proliferation and inhibitory extracellular matrix deposition in vitro prior to in vivo evaluation, these studies have not been specifically designed to model the injury microenvironment and scar formation. We believe the next generation of ex vivo injury models will require combining biological knowledge about the injury environment with advanced biomaterials strategies to integrate biochemical, mechanical and topographical features that mimic injured nerve ECM.

2.2.2. Three-dimensional culture models—3D culture models utilize similar cell types as 2D cell culture models, but rather than on a flat surface, the cells are cultured within appropriate biomaterials in a 3D construct such as a hydrogel. A number of platforms are designed to broadly understand neural behavior in the CNS or model the glial scar that occurs after both TBI and SCI [121]. It is also important to note that most 3D cell culture is used to assess cytotoxicity and neurite outgrowth in response to a therapeutic method or drug. While these types of publications provide valuable information pertaining to cell behavior with certain biomaterials, these are not discussed in this review, as their goal is not to develop an in vitro model of the CNS or PNS (Fig. 3). Current 3D models for stroke or ischemia are focused on recapitulating the blood-brain barrier and thus not covered, see reviews in Refs. [122–124].

CNS: Current 3D engineered scaffold models of TBI are largely based on shear deformation, compression, and pressure induced damage. Cell shearing devices developed by LaPlaca et al. (2005) are able to impart linear displacement of the top portion of a 3D culture (Matrigel®) while the bottom is fixed in a base [125]. The researchers found astrocytic and neuronal cell death was dependent on strain rate and strain, where higher strains and strain rates induced further cell death. This system also has been used to test the effect of material properties on injury type [96], strain deformation on astrogliosis [126], and cell membrane permeability [98]. Although Matrigel® culture systems are successfully implemented, the fact that it derives from tumor tissue raises some concerns for in vivo similarity [127]. Compression TBI is commonly induced on 3D cultures via weight drop or compression apparatuses [90,128]. Weight-drop contusion injuries can be inflicted on cultures by dropping a defined weight from a prescribed height, thus allowing for gradation of injury. In one model, researchers dropped a 10.9 g weight from varying heights onto bioengineered silk/collagen scaffold brain mimics. The authors confirmed that this type of injury led to impact-dependent neuronal damage, hyperactivity, and increased glutamate release that are all similar to responses seen in other in vitro and in vivo models. More recently, Bar-Kochba et al. (2016) reported a novel TBI model that employed a specialized cell compression device mounted atop a confocal microscope stage [90]. Authors noted the real-time induction of morphological changes in neurites within the collagen-based scaffold consistent with those seen in vivo after TBI. TBI has also been induced using pressure bursts applied to a 3D alginate gel modified with the ECM adhesive peptide sequence, RGD. Just recently, in one study a controlled system has been fabricated to induces TBI via air pressure bursts with a high level of control and reproducibility of injury production [129].

Only a few models of inducing SCI on 3D culture systems have been reported. Recently, researchers developed a collagen tube scaffold infiltrated with neural cells to model spinal cord tissue and used forceps to induce an injury that mimicked SCI. Compression resulted in key hallmarks of SCI, including increased GFAP expression, upregulation of vimentin, and increased astrocytic proliferation near the injury site [130]. Another recent study inserted cycling glass probes into a collagen-based CNS mimicking 3D culture to model the effects of micro-motion induced by neural implants on glial scar formation [131]. While not directly mimicking SCI, it may serve as a very relatable test bed. In penetrating cases of SCI, objects such as bullets or bone fragments impinge on the spinal cord and may move in response to respiration, body movement, and surgical intervention. Glial scar test beds are ex vivo models that recreate aspects of the glial scar, which normally occurs after injury in vivo. One way the glial scar can be mimicked is similar to a method found in 2D culture, activation of astrocyte cultures [64,132]. For instance, primary astrocytes cultured in 3D collagen hydrogels were stimulated to be reactive by adding TGF- β 1. Treated astrocytes became more hypertrophic, increased expression of activation markers, and had increased deposition of CSPGs. Another model proposed by East et al. (2012) aimed to model the cellular interface at a CNS lesion. In this model, a custom two-part mold was used to simultaneously cast astrocyte-seeded collagen and DRG-seeded collagen to create a cellular interface without generating a mechanical interface [132]. Authors demonstrated that astrogliosis occurs at the cellular interface as there was an increase in astrocyte volume and GFAP expression compared to controls. Additionally, neurites were significantly less able to

cross the interface and instead neurites preferred to grow parallel to the interface. By layering different concentrations of agarose in a mold, researchers generated a mechanical mismatch interface to model the edge of the glial scar. Using the same model, the chemical interface of inhibitory CSPGs was also recreated [93].

PNS: There are relatively few peripheral nerve models and, to our knowledge, no mammalian models that induce PNI in a 3D in vitro context. One peripheral nerve model employs aligned electrospun polycaprolactone (PCL) fiber scaffolds with a combination of neuronal and Schwann cell components. Daud et al. (2012) demonstrated that this PCL base scaffold could support glial and neuronal cell survival and growth [103]. This system allowed for Schwann cell and neurite adhesion, alignment, migration, and close association. Allodi et al. (2011) proposed another 3D peripheral nerve model in which DRGs and spinal cord slice explants were embedded in a 3D collagen hydrogel [133]. Both motor and sensory neurons extended neurites into the collagen matrix. Another model strives to recreate motor peripheral nerves from the point it exits the spinal cord to its termination in the target muscle. Gingas et al. (2008) developed a 3D model of motor peripheral nerves using a co-culture system. Primary fibroblasts and Schwann cells were cultured within a 3D collagen-chitosan scaffold, and motor neurons were seeded on top. This system allowed the deposition of ECM similar to that found in vivo. The authors demonstrated that this model supported long-term survival of motor neurons without additional trophic support. Neurons readily migrated and grew into the scaffold, matured, and underwent robust myelination. Furthermore, neurites extended deeper into the scaffold when trophic factors were added beneath the collagen-chitosan material. By generating a gradient, guidance of motor axons to a target muscle was mimicked in vitro [134].

3D in vitro test beds are more physiologically relevant than 2D alternatives by providing a more in vivo-like cellular and extracellular architecture. Cells cultured in 3D test beds are surrounded by cells on all sides (as opposed to only one side on 2D surfaces) and grow/migrate in any direction. Additionally, 3D test beds can be tailored to reflect more accurately the native ECM in terms of components and mechanical microenvironment. Together, this can contribute to more in vivo-like cell phenotype and behavior. Similar to 2D test beds, 3D in vitro test beds provide improved control and reproducibility, is higher throughput, lower cost, and reduce complexity compared to explant-based test beds. Current 3D model systems are still limited. In general, nutrient introduction and waste removal are dependent on diffusional transport that can be limited by culture size. This can be improved using bioreactors (discussed later) designed for larger or longer-term cultures. Potential deprivation or inconsistency in nutrient delivery may cause variations in cell viability, proliferation rates, behavior, and gene expression depending on culture conditions. In 3D culture models, functional evaluation may be difficult and methods for doing so are not well established compared to 2D alternatives. Although many studies have implemented ECM-based systems, these systems may not necessarily reflect the nervous system environment. For example, collagen is extensively used in CNS modeling despite being in relatively low concentrations in brain and spinal cord [119,135]. Most current models lack effective recapitulation of native ECM and architectural features, such as the perineuronal nets (CNS) and basal lamina tubules (PNS). In addition, similar to 2D in vitro test beds, 3D in vitro test

beds suffer from similar cell sourcing issues and have limited employment of human cells. Finally, one of the most noticeable limitation is the lack of true injury mimicking in vitro test beds for spinal cord and peripheral nerve. There has been minimal to no induction of a traumatic injury, such as transection or crush/contusion, on 3D spinal cord and peripheral nerve constructs. This currently limits the physiological relevance of these 3D in vitro test beds.

2.3. Bioreactor model systems

Bioreactors are becoming more important in ex vivo cultures because they can control the biological and physical conditions of the cell/tissue culture at the macro- and, more recently, micro-scale [136]. Bioreactors control chemical conditions, temperature and flow rate of media, and apply mechanical and electrical stimuli to cultures to better mimic in vivo conditions.

Traditionally, bioreactors have been used to obtain large cell numbers for cell-based therapies, and more recently have been used for culturing 3D cell-seeded scaffolds for tissue engineering applications. In neural tissue engineering, conventional bioreactors such as stirred suspension systems have been used for neural and stem cell expansion for cell therapy applications [137]. Routinely, bioreactors mimic the dynamics of the natural body environment by stirring and circulating the fluid in vitro. This conventional system, although simple, is still beneficial for the modern applications in neural tissue engineering. Teixeira et al. (2016) designed computer controlled stirring jars to investigate how using stem cell secretome products induce neural regeneration [138]. The authors showed that stirred suspension bioreactors have a number of advantages, including amenability to large-scale cell expansion, continuous removal of metabolites and inhibitory factors and replenishment of fresh growth factors, and tight control of process variables such as pH, temperature and dissolved oxygen concentration.

Although bioreactors are being used to accelerate procedures and scale up cell culture, recently some devices have been developed to mimic the 3D natural microenvironment of the CNS and PNS to serve as in vitro test beds. For example, Sun et al. (2008) designed a customized bioreactor to simulate peripheral nerve regeneration in conduits for different injury gap sizes [139]. The closed loop system consisted of scaffold, a medium reservoir, and a peristaltic pump connected together by silicone tubes. Using this bioreactor, researchers were able to study scaffolds within guidance conduits varying from 10 to 80 mm in length. The authors showed Schwann cell adhesion and alignment on the longitudinal axis in conduits which mimics the natural 3D condition in this bioreactor system (Fig. 4, A) [139]. Another bioreactor system designed by Xu et al. (2014) accelerated peripheral nerve regeneration and axonal outgrowth [140]. This bioreactor consists of two parallel chambers that can be extended in length by utilizing computer-controlled micro-motion to implement custom axon stretch profiles and improve in vitro nerve regeneration (Fig. 4, B).

Application of mechanical and electrical stimulation is one of the remarkable features of recent bioreactor designs. These stimuli can be applied independently or combined in various forms and intensities, depending on the tissue type and the goals of the study. Electrical stimulation of neural cells/tissues has been performed broadly using conductive

polymers and/or simply applying electrodes into the cell culture area, without specifically incorporating the stimulation system into the bioreactor structure (controlling temperature, flow, etc.). It has been shown that replicating the changes in the endogenous electrical field after injury can trigger regeneration and accelerate healing [141,142]. Regeneration and outgrowth in response to an electrical field have been shown in DRGs, isolated motor, and sensory neurons [143–145]. However, optimization of the electrical stimulation regime for neural tissue regeneration will be essential to develop a reliable bioreactor model to mimic the endogenous electrical field in native tissue.

Bioreactors provide a unique opportunity to study the interaction between biological, mechanical, and electrical stimuli in different culture systems such as 2D, 3D and explant cultures. Bioreactors are capable of continual operation, good temperature control, and simplicity of construction. Bioreactors also have fair operating costs in comparison to in vivo studies. For instance, a cost for a large animal study (e.g., sheep) including 10 animals, which can be quite limited in terms of number of treatment performance, is equal to a four-station bioreactor system including software, which allows unlimited number of studies. Conventional bio-reactors face challenges such as size and scale up limitations, which make miniaturized lab-on-a-chip systems more attractive. Moreover, bioreactors are not optimum systems for developing computational models for linking in vitro and ex vivo cultures to in vivo outcomes. These systems are limited in predicting in vivo outcomes because of inhomogeneous variables in their larger chambers [146,147].

2.4. Neurosphere and organoid models

2.4.1. Neurosphere culture models—Neural spheroids are clusters of non-adherent cells containing differentiated cells and stem/progenitor cells capable of self-renewal and can be an effective tool to investigate the capability of stem/progenitor cells to self-renew, differentiate, and proliferate naturally or after injury [148,149]. Although not as extensively studied, neurospheres represent an important class of in vitro model that could provide valuable information regarding regeneration, development, and cell behavior in response to neural trauma (Fig. 5).

CNS: One type of neurosphere-based test beds for injury investigation relies on neurosphere cultures used in combination with in vivo models of injury [150,151]. These types of models require generating brain injury in vivo, harvesting brain tissue at different time points after injury, and assessing effects of injury on stem cell populations [150]. Another type of in vitro model of brain injury utilizing neurospheres is co-culture of neurospheres and organotypic brain slices (reviewed by Humpel, 2015 [152]). One study shows an endogenous population of glial cells exhibited stem like properties after severe TBI as these cells produced multipotent neurospheres [153]. Studies of spinal cord neurospheres have been geared towards studying spinal stem cell niches and understanding the characteristics and markers of these cells [36,154–156]. The effects of spinal stem cell donor age has also been investigated [155]. To the best of our knowledge, models utilizing neurospheres to mimic spinal cord tissue are lacking.

PNS: Neurospheres derived from the PNS are less predominant than CNS alternatives. PNS based neurospheres have been generated from various peripheral sources including sciatic nerve, de-differentiated Schwann cells [157], enteric ganglia [158], and dorsal root ganglia [159]. Thus far, peripheral nerve based neuro-spheres have focused on generation and determination of factors involved in stem cell differentiation, self-renewal maintenance, and proliferation. However, similar to neurosphere models for SCI, there is a lack of peripheral nerve injury mimicking neurosphere in vitro test beds.

In vitro test beds based on neurosphere culture have a number of advantages such as flexibility of cell sourcing and long-term survival in culture. Neurospheres allow for the investigation of injury on stem cell populations and neural stem cell progeny in a more controlled and defined 3D environment [160]. However, inconsistency is the main limitation to neurosphere culture. Neurospheres are notoriously sensitive to culture methods and techniques, cell density, environmental conditions, age of donor, concentrations of factors, passage number, and time of dissociation, which can all affect properties of the neurospheres (e.g., cell type profiles, neurosphere dimension). This variability makes studying specific events and interpretation of the data challenging [148].

2.4.2. Organoid culture models—Organoids are 3D heterogeneous cell clusters that are spatially organized. These culture models target to recreate specific functions of the organ origin [161,162]. As organoids are fairly recent developments within the fields of neuroscience and biomedical engineering, these tools have not been adapted for the study of traumatic nervous system injury. While not yet used for traumatic injuries, organoid models that mimic neural tube have been developed with mouse embryonic stem cells in 3D hydrogels such as laminin/enactin, polyethylene glycol, and Matrigel® [163,164]. Recently, Kawada et al. (2017) introduced a model system for axonal fascicles that can also be used as an axonal degeneration model. The authors produced nerve organoids from human stem cell-derived motor neurons inside a spheroid chamber and directed the axonal growth through a single microchannel. After 20–30 days of axon fascicle formation, the authors demonstrated electrical activity of the axons [165].

Organoids have only been generated as fetal tissue correlating to a fetus in the third trimester [166,167]. Thus far, this technology has not reached the point of recapitulating adult tissue, thus limiting the current application as a clinical model of nervous system trauma. It is important to note that there are differences in the regeneration ability between embryonic, neonatal, and adult neurons. For example, neurogenesis occurs more robustly in the human brain during early embryonic and postnatal stages whereas in adult tissue, only a few neurogenic zones remain active [168]. On the other hand, gliogenesis (i.e., astrocyte and oligodendrocyte proliferation) in the human brain is not active until the late embryonic stage and persists through adulthood [170]. These differences between embryonic and adult tissue in turn impacts the content of the local microenvironment before and after injury. For instance, CSPG expression has been demonstrated to increase in adult versus neonatal tissues after SCI [169]. This increase in CSPG expression after injury in adult tissues is linked to inhibition of neural repair in CNS [170]. In addition to inhibitory components, nerve growth factor (NGF) receptor mRNA is expressed at different levels in various areas of the human nervous system during development and adulthood [171]. NGF receptors on

Purkinje cells (located in the cerebellar cortex of the brain) and CNS neurons are abundant in fetal cerebellum, however, receptor expression decreases with subsequent development of the fetus and is absent in the adult cells [172]. In another example, the level of NGF mRNA in total rat brain is reduced to 90 and 80% of the levels seen in the adult brain at 47 weeks and 17 months, respectively [173].

As mentioned previously, utilizing human derived cells for ex vivo modeling can be challenging and in some cases fraught with ethical concerns. This is especially concerning when developing organoid systems that rely on pluripotent stem cell populations. In response, the field has begun to move towards the employment of human-derived induced pluripotent stem cells (iPSCs) to generate neural organoids. However, to the best of our knowledge, these iPSC-derived organoids have been not used as a regenerative model. For example, cerebral organoids derived from two separately sourced iPSC lines have been reported by Gabriel et al. (2017). Although the authors did not investigate traumatic neural injury, they instead aimed to utilize human cerebral organoids to elucidate underlying mechanisms of how the Zika virus causes microcephaly [174]. Bershteyn et al. (2017) also recently published on similar cerebral organoids developed from iPSCs derived from human fibroblasts. In this case, cerebral organoids were used to further understand cerebral malformation and mechanisms that result in Miller-Dieker syndrome [175]. These systems could be adapted in the future for ex vivo regeneration models.

Organoids have the potential to generate miniaturized neural tissues that have more similar network organization, development/maturation, and cytoarchitectural organization compared to other in vitro models such as 2D and 3D cultures. Additionally, organoids and spheroids can be patient specific, allowing for future modeling and drug screening in personalized medicine. However, current organoid cultures have clear limitations that need addressing before future use as nervous system trauma models. Heterogeneity and variability remain a serious limitation. Another limitation is the little control over spatial orientation or cell types that arise. Additionally, while parts of the culture may resemble structures, adjacent regions may not be correctly spatially organized as organoid culture lacks an embryonic axis which drives higher order organization [161,166,176]. Similar to other types of in vitro test beds, organoids lack vasculature and are limited in their ability to grow to large sizes. In addition, for clinical relevance, organoids should be matured sufficiently in vitro to mimic adult tissue. Currently, studies using organoids for spinal cord and peripheral nerve tissue applications are lacking. With a better understanding of differentiation cues and culture methods, organoids and neurospheres will become a more applicable model for nervous system trauma.

3. Lab-on-a-chip devices

Lab-on-a-chip devices, for neural tissue engineering applications, are microsystems that enhance neural proliferation, migration and differentiation, and monitoring of these processes. Compared to conventional model systems (e.g., 2D and 3D cultures systems and bioreactors), microfluidic devices offer multiple advantages. These advantages include improved control over channel geometry and dimensions, scalability, reduced system size, cost effectiveness due to low volume of reagents needed, and a precise spatiotemporal

control over physicochemical cues across the channels [177–179]. To this end, use of microfluidic platforms has dramatically increased in recent years for in vitro investigation of various biomedical research areas ranging from modeling healthy and pathological tissues and stem cell differentiation to developmental biology and drug testing [180–182]. Microfluidic devices offer unique benefits to neuroscience and neural tissue engineering research, since channels can be designed to allow control of axonal directionality and growth and precise application of injury at a single axon level [183]. In addition, by having dissimilar channel widths across different areas within the device, separation of somal area from axonal area can be achieved, and this unique feature can be utilized to assess axon-specific molecules via immunofluorescence and mRNA isolation [184] as well as to test drug sensitivity in somal versus axonal areas [185]. For example, researchers designed a microfluidic device with radial patterns of six separate axonal compartments with a single central somal chamber, which enables different treatments of axons in parallel as well as specific interactions between axons and glial cells [186].

Microfluidic devices can be designed with a number of engineering features to help replicate the injured and regenerative microenvironment of nerve tissue, making them useful neural regeneration research platforms. These engineering design parameters include micropatterns, culture dimensions, chemical gradients, mechanical features, electrical stimulation, and optical stimulation (Fig. 6).

Micropatterns:

Use of micropatterned microfluidic chips for neural research began in the 1970s with the Campenot device [187]. In this device, a petri dish was partitioned into three distinct chambers by a Teflon divider across a series of parallel scratches on the dish. Ganglion-derived neurons were seeded in the central chamber, and gradients of nerve growth factor (NGF) were applied across the side chambers. This allowed for assessing the effect of local NGF concentration on axonal extension and degeneration, and showed that the presence of NGF at the distal site of the axons was critical to continued neural growth. Since the introduction of Campenot devices, micropatterned microfluidic devices to guide axon extensions along a specific direction have been widely documented. A notable system was published in 2003, where axons from cortical neurons were allowed to extend along the micro-grooves in the neuritic compartment while neuronal cell bodies were contained within the somal compartment [188]. The authors showed that the containment of fluids within the two compartments was achieved simply by differences of volume in the media ports, and this was used to control local application of insults to the cells in a soluble form specifically to either axons or soma. In another study, Gladkov et al. (2017) created micropatterns of various asymmetric shapes to control direction of axonal extensions [189] (Fig. 6, “Micropatterning”). The authors showed increased axonal extensions from neurons seeded in the “source” chamber, whereas they observed fewer axonal extensions from the neurons seeded in the “target” chamber. These observations suggested that axons from the “source” chamber were “funneled” by the patterns in a single direction, facilitating larger numbers of axons, whereas the axons from the “target” chamber were diverted/blocked by the geometric features. The authors assessed the effect of different geometrical bottlenecks on axonal extensions and analyzed synaptic connections across the two chambers via

electrophysiological measurements. These microfluidic designs can be used to model unidirectional neuron connectivity in CNS tissue [189].

Culture Dimensions:

Most of the microfluidic devices reported for neural research, including the ones described in the Micro-patterns section above, are fabricated using polydimethylsiloxane (PDMS) elastomer and thus can only accommodate 2D cell culture. However, there exist microfluidic devices where cells are embedded in 3D matrices [190–192]. In one study by Yang et al. (2015), a three-channel PDMS microfluidic device was fabricated, where human neural stem cells were embedded in 3D collagen hydrogel in the center channel flanked by GDNF-overexpressing human mesenchymal stem cells (hMSCs) seeded on an oxygen plasma-treated PDMS surface [192]. The authors showed that 3D culture, compared to 2D culture, improved differentiation of human neural stem cells into functional dopaminergic neurons in response to paracrine signaling from hMSCs. In a different study by Adriani et al. (2017), the authors created a 3D neurovascular chip, where neurons and astrocytes were embedded in collagen I hydrogels and endothelial cells were seeded on a collagen-coated channel (Fig. 6, “Culture Dimensions”) [190].

Chemical Gradients:

As alluded to above, one of the major benefits of microfabricated devices is establishment of chemical gradients. Li et al. (2012) seeded hippocampal neurons on the somal compartment of a microfluidic device and axons were encouraged to extend towards another compartment by varying hydrostatic pressure between the two channels [185]. Afterwards, a neurotoxin (acrylamide) was applied to either the somal or the axonal compartment, and regeneration was observed in the presence or absence of neuroprotective drugs. In a different study, a micro-fluidic chip with three V-shaped channels circularly arranged was created, and in the center of the device existed a loading port to insert 3D collagen hydrogel [193]. Gradients of chemoattractant netrin-1 or chemorepellent slit-2 were established orthogonal to the cell channel, where DRG neurites extended into the collagen gel. Through mathematical modeling and immunofluorescence, the authors analyzed gradient establishment across the device over the course of 3 days and axonal turning in response to netrin-1 or slit-2 gradients. Another advantage of microfluidic devices is that researchers can simultaneously create chemical gradients of multiple factors for use in high-throughput cell-based studies, such as those in developmental biology. In one study, axonal migration of embryonic neurons in response to gradients of sonic hedgehog (Shh) and/or netrin-1 was assessed [194]. The microfluidic device used in this study consisted of pre-mixer networks to establish wide range of concentration gradients applied to neurons cultured in the adjacent channel. Initially, the authors showed that shallow gradients of both Shh and netrin-1 exist in vivo samples. Based on this observation, the authors established a physiologically relevant fractional concentration gradient of these morphogens and measured angles of axon turning as an indicator of axonal response to the gradients. Interestingly, the authors found that axons responded to lower gradients when both molecules were present compared to higher gradients of a single molecule needed to elicit axonal response. They also demonstrated that downstream signaling by phosphorylation of Src-family kinase in the growth cone occurred only in the presence of both morphogens. In another study, motor neuron differentiation of

embryonic stem cell-derived embryoid bodies in collagen hydrogels was studied in response to gradients of both retinoic acid and Shh [195]. The authors showed that differentiation of embryoid bodies was most prominent in the areas of highest concentration of both morphogens. This device can also dynamically alter concentration gradients, which can be used to study patterns of growth cone migration (Fig. 6, “Chemical Gradients”).

Mechanical Features:

One of the key factors in designing engineered neural tissues is matching mechanical properties to those of native tissues. By exploiting establishment of chemical gradients across the channels, microfluidic devices can be used to generate a gradient of mechanical properties and to assess cell behavior. Using a plant-derived natural cross-linker, genipin, that renders cross-linked hydrogels fluorescent [196], researchers established mechanical stiffness gradient across an H-shaped collagen gel microfluidic device and showed preferred axonal extension from DRGs down the mechanical gradient [197]. The same group later established gradients of laminin bioactive sequences, IKVAV and YIGSR, by preparing two different collagen pre-gel solutions with or without the peptides [198]. Using DRGs, the authors showed that axons preferred to extend up the YIGSR gradients, but not as strongly up the IKVAV gradients, suggesting differential preferences of axons on laminin-derived peptide sequences. These studies not only demonstrate versatility of microfluidic devices in neural research but also practical considerations for designing engineered neural tissues for regenerative medicine and disease modeling. In addition, cells cultured in the microfluidic devices can be mechanically stimulated by virtue of the elastic properties of PDMS. In a study by Tang-Schomer et al. (2010), PDMS-based microchannels were fabricated, in which soma and axons were isolated in separate compartments [27]. By rapidly applying air pulses only to the axon chamber, the substrate was deflected causing the axons to stretch; this allowed for selective mechanical perturbation of the axons without damaging the cell soma. Using this approach, the authors were able to assess the effect of tensile stretch on the temporal progression of microtubule deformation and delayed axonal signaling following injury (Fig. 6, “Mechanical Features”). Another PDMS-based model, developed by Hosmane et al. (2011), separates the cell soma and axons into distinct channels and utilizes microfluidic valve-based compression injury pads to apply mechanical damage only to the axons [199]. The authors showed axonal degeneration versus regeneration of primary hippocampal neurons depending on the intensity of the compression. In the future, these PDMS-based devices can be coated with brain-specific ECM proteins to provide a CNS-like microenvironment.

Electrical Stimulation:

Besides chemical and mechanical features of the cellular microenvironment, electrical signals are important factors present in the regenerative environment of the neural tissues. Electrical field is a critical cue that naturally exists in tissues, especially in the injury microenvironment to enhance regeneration via distressing voltage, ion and pH gradients [142]. Recently, lab-on-a-chip devices utilized microarray electrodes not only for monitoring and measuring the electrophysiological changes, neural signaling and action potentials [200,201], but also for electrical stimulation [202]. These devices have been modified to incorporate microfluidic probes for electrical recording and constant monitoring to fully

assess functional recovery of axons after injury [203]. Hallfors et al. (2013) developed a microfluidic compartmentalized cell culture platform incorporating liquid metal microelectrodes to effectively stimulate target neurons (Fig. 6, “Electrical Stimulation”) [204]. The authors fabricated a single microgroove connecting the two channels to only stimulate axons. To evaluate and optimize electrical stimulation, the authors used calcium indicator dye (Fluo-4) to record neuronal activity in response to stimulation of the cells with 0.6 mA current for 200 μ s and 10 Hz. The results show that the liquid metal electrodes induced a significantly larger calcium response compared to the bath electrodes. In another study, Morin et al. (2006) developed a PDMS microfluidic device laid over planar microelectrode arrays (MEA) [205]. Cells were seeded into the culture ring glued onto the surface of the MEAs and stimulated using five biphasic symmetric square waves (total duration 10 ms) followed by 950 ms of rest. Authors reported larger patterns on the MEA allowed straightforward analysis of the signals from cells in response to electrical stimulation [205]. Most MEA electrodes were consistently responsive to electrical stimulation, whereas some remained consistently silent. These results indicate that the distribution of the neuronal and glial cells inside the channels appeared to be strongly non-homogeneous, and that neurons likely had a limited mobility inside the channels once the glial cells colonized. Overall, we believe there are limited studies incorporating electrical stimulation into lab-on-a-chip devices, which is an opportunity for future exploration.

Optical Stimulation:

Electrodes on the chips can also be used to optically stimulate neurons in the device with either one- or two-photon lasers [206]. In a device created by Jang et al. (2016), a PDMS microfluidic device previously described by other authors [184] was coupled with microelectrode arrays that were placed underneath the device to obtain separate readings from soma and axons (Fig. 6, “Optical Stimulation”) [206]. By applying a Ti:sapphire laser and visible light source, the authors measured neuronal responses to one- or two-photon stimulation. Feng et al. (2015) developed a microfluidic platform with integrated power splitting waveguides for optogenetic neural cell stimulation [207]. A liquid-core/PDMS-cladding waveguide with a power splitter design was integrated with a neural cell culture chamber to provide a simple way to precisely localize optical stimulation. The authors used calcium imaging of neurons during laser light stimulation [207]. Optical stimulation in ex vivo neural regeneration models has only recently been explored, thus providing future opportunity to develop better platforms for studying light- and laser-based therapies [208].

In the following sections, we briefly discuss microfluidic devices designed to model specific units within CNS and PNS that will lead to novel regenerative strategies. For a more comprehensive review of microfluidic chips for modeling of non-traumatic CNS and PNS diseases and drug testing, readers are directed to articles by Choi et al. (2017), Neto et al. (2016), and Yi et al. (2015) [209–211].

CNS:

In TBI, leaky blood vessels lead to ischemia in the brain and hinder functional recovery, and thus angiogenesis is fundamental for tissue repair and regeneration [212–214]. The neurovascular unit (NVU) in CNS maintains homeostasis among blood vessels, neurons and

glial cells [212], and is the niche for neural stem (progenitor) cells in adult brains [215]. Crosstalk between vascular cells and neural cells in the NVU is critical for angiogenesis, neurogenesis, and synaptogenesis that lead to improved functional recovery after traumatic injuries [216,217]. To develop novel regeneration strategies, therefore, in vitro models of the NVU that fully recapitulate the physiological microenvironment are needed. Recent publications describe microfluidic models of the NVU where co-cultures of vascular cells and neural cells were established and vascular permeability in different conditions was assessed [190,191,218–222].

Microfluidic devices specifically developed to recapitulate spinal cord microenvironment are not widely seen, possibly due to difficulties in harvesting spinal cord-specific neuronal cells compared to brain. Therefore, microfluidic chips that model the brain microenvironment in vitro can possibly recreate the spinal cord environment. Further, researchers can use these devices to mimic healthy and injured spinal cords by adding other types of cells such as astrocytes or coating the channels with ECM components that are excessively deposited after injury (e.g., CSPGs). One example is a device made by Vahidi et al. (2008), where strips of permissive (poly-L-lysine/laminin) and inhibitory (aggrecan) stimuli were present to assess neurite extension [223]. In this study, axons of rat primary cortical neurons extended exclusively on the permissive lanes (i.e., poly-L-lysine/laminin). On the other hand, on non-permissive lanes (i.e., aggrecan), axons extended only in the presence of the enzyme chondroitinase ABC, which degrades aggrecan. This approach to creating devices containing non-permissive ECM regions allows scientists to mimic the CNS glial scar environment that naturally occurs after injury.

PNS:

Microfluidic devices can be used to mimic neuromuscular junctions (NMJs). Versatility in design and confining multiple compartments within a single device allows recreating distal connection between motor neurons and peripheral tissues such as muscles. In addition, use of microfluidics allows assessment of the effects of neurotrophic factors such as GDNF on soma versus axons and its effects on muscle innervation [224].

In one study, the neural compartment was filled with photo-excitable embryonic stem cells differentiated into motor neuron neurospheres and a remote muscle unit was recapitulated by C2C12 skeletal muscle cell-derived muscle strips [225]. The two compartments are physically separated, thus mimicking distances between motor neurons and the muscles that the motor neurons innervate, and formation of the NMJ between axons extended from the motor neuron neurospheres and the skeletal muscle units can be visualized. In another study, a two-channel device connected with microgrooves has been used to demonstrate proof-of-concept level of NMJ recapitulation [226]. In this device, DRGs were cultured in one channel (mimicking spinal cord) and muscle tissue was incorporated into another channel (mimicking muscle). The authors observed innervation of muscles by DRG neurons extending across the microgrooves between the two channels. Although functional outcomes (e.g., muscle contraction) were not assessed, this study provides yet another example of co-culturing cells from two different organs to create complex tissue structures such as the NMJ.

Bone innervation has also been modeled using microfluidic chips by Pagella et al. (2014) [227]. The authors co-cultured trigeminal ganglia and incisor tooth or molar tooth germ cells on commercially-available microfluidic chips; ganglia were seeded in one channel and the molar tooth germ cells in the other channel, separated by microgrooves [227]. The axons extended from ganglia along the microgrooves into the tooth channel. In another study, a previously established device [184] was modified to co-culture DRG explants and MC3T3-E1 osteoblasts [228]. The authors further upgraded their culture condition by seeding osteoblasts on a collagen coating or embedding the cells into an RGD-alginate matrix. The authors showed DRG axonal extensions and synapsin expression reaching the osteoblast compartment.

Overall, the studies described above illustrate the versatility of microfluidic devices for studying CNS and PNS regeneration in controlled ex vivo systems.

4. Commercially available model systems

Many microfabricated and microfluidic devices are currently on the market for use in tissue engineering, regenerative medicine research, and drug discovery and testing. A detailed review of microfluidic devices designed for drug testing and diagnostics is described by Zhang et al. (2017) [229]. In this section, we specifically discuss commercially available microfabricated devices for neural applications. Currently, four biotechnology companies specialize in designing and/or manufacturing microfabricated and microfluidic devices for neural tissue applications (Table 3). With the exception of AxoSim, which utilizes 3D hydrogels, and Mimetas, which uses standard well plate formats, all the other companies use PDMS as their base platform; all these lab-on-a-chip devices are targeted for applications such as axonal separation from the soma, myelination via co-culture, neuronal rewiring, and harvest of axon or soma-specific proteins and nucleic acids.

Xona Microfluidics [230] is one of the first biotechnology companies in this category, founded in 2008 from studies conducted in Dr. Noo Li Jeon's lab at the University of California-Irvine [30,184,188]. This company specializes in two-channeled devices with varying lengths of microgrooves in between, allowing for separation of the neuronal cell body and dendritic extensions from axons and subsequent protein or RNA harvest from specific parts of the cells of interest. Vacuum-mediated axotomy can also be performed with these devices. Xona's devices are fabricated in PDMS, and in 2010, they announced a supply agreement deal with Milli-poreSigma for their AXIS Axonal Isolation Devices. To this end, many other labs have used Xona devices for their publications [227,231–235]. MicroBrain Biotech SAS [236] is a France-based biotech company specializing in PDMS-based microfluidic chips for brain studies. These microfluidic devices include 'axon diodes,' which are funnel-shaped microchannels between two channels that achieve not only somal separation from axons but also axonal extension from one channel but not the other [237]. Further modification of the device design has aimed at orienting neuronal networks [238,239]. MicroBrain BT's devices can also be used for disease modeling, such as that of Alzheimer's, to assess the effect of beta-amyloid on neural connections [240]. Ananda Devices [241] also sells PDMS-based microfluidic chips for not only neural applications but also for cellular and small organisms' migration, invasion, adhesion and differentiation. As

for their Neuro Device, application ranges from axonal separation from soma, studying rewiring neuronal circuits, AFM-mediated axonal injury, and myelination of axons via co-culture with oligodendrocytes or Schwann cells [242–245]. AxoSim is a biotechnology company from Dr. Michael Moore's lab at Tulane University. In contrast to the three previously mentioned companies, AxoSim specializes in 3D hydrogel devices that can be cultured inside a Transwell insert [246]. AxoSim's unique design allows them to fabricate devices with a 3D hydrogel of choice for assessing nerve regeneration in three dimensions. This is useful for neural regeneration research because important biomaterials for neural repair, such as photo-crosslinked hyaluronic acid-based hydrogels [247], can be incorporated into these systems. Devices from Dr. Moore's technique have been used to study neurite outgrowth in multiple different 3D hydrogels [109,248]. Finally, Mimetas has developed OrganoPlate®, a standard 384-well plate system with 96 sets of 2- or 3-channel microfluidic devices. One of the unique features of this system is Phaseguide™ technology, in which small meniscus pinning barriers allow separation of hydrogel channels from media without a need for walls [249]. OrganoPlates can be used to assess 3D neuronal network formation in both neuron monocultures and glial-neuron co-cultures [250], and 3D differentiation of human neuroepithelial stem cells into dopaminergic neurons [251]. In addition, this platform can be used to develop other in vitro model systems such as cancer drug testing [252], intestinal epithelial tubes [253], or perfusable 3D vasculature [254]. In addition, various companies (e.g., Micronit, uFluidix, thinXXS, microLIQUID, IDEX Health & Science, Micalyne, Microfluidic Chipshop) design customized devices for almost any application. As such, the use of microfabricated and microfluidic devices for research on basic cell biology to tissue engineering and drug discovery is on the rise. Scientists and engineers are encouraged to utilize these state-of-the-art lab-on-a-chip systems as physiologically relevant culture models to assess cell and tissue behavior.

5. Current challenges and future directions

Natural, synthetic and composite biomaterials are used broadly for assessment of the therapeutic efficacy in vivo and 3D neural culture. Since biomaterials are highly tunable in mechanical and chemical properties, we believe they can be further utilized for creating ex vivo injury model systems of CNS and PNS tissue. Further, although significant advances in neuroscience research using microfabricated and microfluidic devices have been made, most studies to date lack proper ECM coating or components. Many of the microfabricated devices described in this review employed poly-L-lysine or other adhesive coatings to promote cell adhesion. Some studies used hydrogels made from type I collagen [190–192,197,198,255], which is a physiologically relevant protein to study PNS injuries. However, collagen is not as appropriate for studying brain and spinal cord regeneration because CNS tissue is primarily composed of hyaluronic acid (HA) [119]. Some micro-fabricated devices using HA-based hydrogels and a mixture of HA and other ECM proteins have been created [247,256]. However, to study healthy and injured brain and spinal cord more thoroughly, different device designs with 3D ECM-based hydrogels are required. Furthermore, many of these studies employ rat-derived cells; to understand injury mechanisms fully and to develop novel therapeutics for humans, investigations with human-derived neural and glial cells need to be widely performed. Co-culture of neurons and glial

cells as well as other stromal cells may further enhance physiological relevance of these model systems, getting closer to true organs-on-a-chip such as those devices mimicking the NVU or NMJ. Finally, these devices can be further modified to incorporate micro-probes for electrical recording and constant monitoring to fully assess functional recovery of axons after injury [203].

Moreover, these portable microfabricated devices and ex vivo models can be carried into space to assess the effects of gravity and magnetic field of the earth on changes in complex biological systems. In this regard, National Aeronautics and Space Administration (NASA) and US National Laboratories support programs to study microgravity and space microenvironment for both fundamental and applied science investigations. For instance, AxoSim Technologies is working on a project titled “3D neural microphysiological system for investigating myelination processes in microgravity,” which uses human nerve-on-a-chip devices to study disorders affecting myelination in space. As such, lab-on-a-chip and advanced ex vivo models will compensate for shortages and drawbacks associated with the traditional in vitro systems and animal models, and we foresee these platforms becoming reliable standard test beds that can revolutionize laboratory research and drug discovery in pharmaceutical companies.

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Fig. 1. Ex vivo models for neural regeneration and engineering.

Ex vivo models for neural regeneration and engineering fall into one of 6 broad categories: 2D cell culture based systems [48], bioengineered 3D scaffolds [257], neurosphere based culture [258], tissue explants [259], organoid tissues [166], and culture utilizing bioreactor systems [139]. All of these models strive to mimic aspects of the in vivo environment of brain, spinal cord, and peripheral nerve after injury while providing a higher through-put and cost-effective alternative to in vivo studies.

Fig. 2. Tissue explant models.

Representative images of explant based in vitro models for peripheral nerve (A–C), spinal cord (D–G), and brain (H–L) injury. **A)** A mammalian nerve repair model generated by transecting explanted peripheral nerve and abutting nerve segments. **B)** and **C)** GFP labeled axons bridge the repair site [84]. **D)** A spinal cord transection model of SCI to test regenerative therapies. **E)** A cell devoid region (scale bar = 250 µm) and a reactive edge containing increasingly GFAP positive hypertrophic astrocytes (arrowheads denote examples) and, **F)** activated lectin positive microglia (arrows denote examples) (scale bar = 50 µm), and **G)** neuronal regeneration was limited in the region space without intervention (scale bar = 25 µm) [74]. **H)** A crush model of TBI on hippocampal explants. **I)** injury resulted in a region devoid of axonal (SMI-31), and **J)** dendritic (MAP2) markers as indicated by the arrows. **K)**, and **L)** Regeneration after 7 days across the lesion area, denoted by the arrows (scale bars in I–L are 75 µm) [67].

Fig. 3. 3D bioengineered ex vivo models.

Representative 3D bioengineered in vitro test beds for neural engineering. **A**), **B**), and **C**) depict a post-SCI cellular interface model. At the interface, astrocytes (GFP) became increasingly reactive and hypertrophic with an increase in GFAP positive staining (GFAP)) and deterred neurons (BIII tubulin) from crossing the interface. (Scale bars in **C** denote 150 μ m) [132]. **D**), **E**), and **F**) depict a model which aimed to generate a 3D in vitro mimic of peripheral nerve regeneration by seeding fibroblasts (left) and fibroblasts and Schwann cells (right) with motor neurons (NF-M) on a collagen based sponge. By adding a neurotrophin gradient to mimic motor neuron regeneration to a target this 3D system could support robust motoneuron growth and extension into the sponge (bottom panel) compared to no gradient (top panel) (The scale bars in **E** and **F** = 100 μ m) [134]. **G**), to **K**) are a schematic and results of a bioengineered 3D brain mimicking test bed being subjected to weight drop TBI. The injured samples had altered baseline electrophysiological response and total power of the electrophysiological response. A dynamic release of glutamate was observed [128].

Fig. 4. Bioreactors used as ex vivo models for neural regeneration.

A) A closed loop system, consisting of scaffold, a medium reservoir, and a peristaltic pump that were connected together by silicone tubes. This customized bioreactor simulating peripheral nerve regeneration in conduits for different gap size [139]. **B)** A bioreactor system consists of two parallel chambers that are extended to conduct controlled trials for optimized neuronal culture. This bioreactor accelerates peripheral nerve regeneration and axonal outgrowth by utilizing computer controlled micro-motion [140].

Fig. 5. Neurosphere in vitro models.

A) Micrograph and **B)** Fluorescence images of neurospheres generated from the injury site after stab wound TBI (scale bar = 50 μ m in A and 20 μ m in B) [149]. **C-E)** Characteristics of progeny of neurospheres derived from lesioned cortex after 10 days in culture show neurospheres can differentiate into GFAP, β III Tubulin, and O4 positive cells, confirming the stem-like properties of the neurospheres and their ability to generate multiple cell types (scale bar = 20 μ m) [149]. **F)** GFP positive neuro-spheres (top) and GFP positive dissociated stem cells (bottom) were implanted in a transection lesion of an explanted spinal cord slice (scale bar = 400 μ m). **G)** Neurospheres did not facilitate synchronous electrical activity of the lesioned sides, and **H)** did not give rise to mature neurons (NeuN) (bottom) like dissociated stem cells (top) [260].

Fig. 6. Engineering design parameters for neural microfluidic chips.

Micro-Patterning: Microgrooves between channels can be added to facilitate separation of axons from soma and dendrites [189]. *Culture Dimensions:* Microfluidic culture of neural cells can be performed in 2D PDMS-based chips with cell adhesive coating (redrawn and adapted from Ref. [184]), or within 3D hydrogels [190]. *Chemical Gradients:* Establishment of chemical gradients similar to those found in vivo can be performed in microfluidic devices [195]. *Mechanical Features:* Microfluidic valve-based compression injury pads are used to apply mechanical damage only to the axons [199]. *Electrical Stimulation:* Electrodes can be incorporated into the device design for electrical recording and stimulation [204]. *Optical Stimulation:* Electrodes in the chips can also be used to optically stimulate neurons in the device with either one-photon or two-photon stimulation [206].

Table 1

Injury models for neural tissue engineering application.

Injury Type	Injury Induction (examples and references)	Ex-Vivo	Lab-on-a-chip	Cell/Tissue Types (examples and references)
TBI	• Scratch/tear [31]	✓	✓	• 2D culture of primary embryonic cortical neurons and glia [31]
	• Fluid percussion [32,71]			• 2D NT2-N cell line culture [34]
	• Stretching [33,69,70,234,235]			• 3D culture of primary cortical neurons ± astrocytes [90,96,98,128,129]
	• Shearing [34,96,98,125,126]			• 8–10 day rat hippocampal slices [70]
	• Extraction [68]			• Resected adult human temporal neocortex [87]
	• Weight drop/Compression/Crush [67,90,128,199,244]			• Neonatal rat hippocampal neurons and glial cells [185]
SCI	• Air pressure bursts [129]			
	• Chemical/Cytokine treatment [185,191]			
	• Oxygen-glycose deprivation [191,261]			
	• Scratch/tear [40–42]	✓	✓	• Whole neonatal rat spinal cords [78]
	• Stretch [43]			• Longitudinal neonatal mouse spinal cord slices [74]
	• Weight drop/Impact/Crush [72,73,130,199]			• Longitudinal slices of the spinal cord from neonatal mouse [73,75]
PNI	• Transection [74–76]			• Cross sectional slices of the spinal cord from 3 to 6 week rat [73,75]
	• Chemical induction [77–79]			• 3D collagen tubes or sheets seeded with adult rat neuroblasts and/or astrocytes [130]
				• 2D cultured primary embryonic rat spinal cord neurons [42]
				• Co-culture of a neonatal rat astrocyte and fibroblasts [43]
	• Compression/Crush [80,81,244]	✓	✓	• Neonatal mouse ulnar and median nerves [85,263]
	• Transection [82–85]			• Adult mouse sciatic nerves [83]
Glial scar	• Laser axotomy [226,262]			• Frog sciatic nerves [83]
	• Vacuum aspiration axotomy [184]			• Neurons isolated from marine mollusk <i>Aplysia</i> California [264]
				• <i>C. Elegans</i> [262]
				• Dorsal root ganglia and cortical neurons from 16 to 18 day rat [226]
	• Activation of cultured astrocytes (i.e. by co-culture or cytokines) [37,38,64,132]	✓	✓	• 2D co-culture of rat Neu7 cells and embryonic chick dorsal root ganglia [39]
	• Cellular interface culture [132]			• 2D culture of adult rat dorsal root ganglia [265]
				• 3D culture of neonatal rat astrocytes [64]

Injury Type	Injury Induction (examples and references)	Ex-Vivo	Lab-on-a-chip	Cell/Tissue Types (examples and references)
	<ul style="list-style-type: none"> Fabricated mechanical interface [108] Deposited/incorporated CSPGs [108,223,265] Hydrogen peroxide administration [223] Culture Neu7 astrocytic cells [39] 			<ul style="list-style-type: none"> 3D culture of primary neonatal rat astrocytes and adult rat dorsal root ganglia [132] 3D culture of dorsal root ganglia of chicken embryo [108] 2D co-culture of astrocytes and meningeal fibroblasts of neonatal rat [37] Cortical neurons isolated from rat embryos [223]
Demyelination/Myelination	Forskolin application [57,60]	✓	✓	<ul style="list-style-type: none"> Neonatal rat or mouse Schwann cells and dorsal root ganglia [57,58] Co-culture of the mouse Schwann cells and primary motor neurons [59] 2D culture of adult rat dorsal root ganglia [60]
Stem/progenitor activity after traumatic injury	<ul style="list-style-type: none"> In vivo injury prior to culture Cortical impact [150] Oxygen-glucose deprivation [266] Chemical exposure [151,267] 	✓	–	<ul style="list-style-type: none"> Neurospheres derived from rat subventricular zone at different ages [150] Hippocampal derived neurospheres from neonatal rat brain [266] Neurospheres derived from cochlear nerves of adult mouse [267]
Wallerian degeneration	<ul style="list-style-type: none"> β-amyloid peptide application [240,268] Hydrogen peroxide administration [224] Detergent administration [264] 	–	✓	<ul style="list-style-type: none"> Embryonic cortical neurons [268]

Embryonic: in the embryonic stage; **Neonatal:** newly born (up to 3 days); **Adult:** older than 6 weeks.

Table 2

Biomaterials currently used as scaffolding materials for neural regeneration.

Material Class	Examples Used in Literature
Natural	<ul style="list-style-type: none"> • hyaluronic acid (HA) [89] • collagen [64,90] • silk fibroin [63,91] • elastin [92] • fibrin [93,94] • decellularized peripheral nerve ECM [95] • agarose [96] • alginate [97] • Matrigel® [98] • chitosan [99] • serum albumin [100]
Synthetic	<ul style="list-style-type: none"> • poly (L-lactic acid) [101] • polyethylene glycol (PEG) [102] • polycaprolactone (PCL) [103,104] • polyurethane [105] • polyamide [106] • polypyrrole (conductive polymer) [107]
Composite	<ul style="list-style-type: none"> • agarose/CSPGs [108] • PEG/PuraMatrix™ [269] • dextran/laminin peptide [110] • poly-L-lysine/chitosan [111] • Graphene/PCL [112] • HA/collagen/laminin [113] • PEG/HA/collagen [114]

Table 3

Lab-on-a-chip device companies specializing in neural applications.

Company	Description	Device	Year Founded
Xona Microfluidics	<ul style="list-style-type: none"> • PDMS-based (XonaChips™) • Varied lengths of microgrooves between channels for separation of soma and axons • Not a 3D culture platform		2008
MicroBrain Biotech SAS	<ul style="list-style-type: none"> • PDMS-based • Axon diodes allow soma separation from axons, control of axonal extension directionality • Not a 3D culture platform		2014
Ananda Devices	<ul style="list-style-type: none"> • PDMS-based • Neuro Device is capable of axonal separation from cell soma, AFM-mediated axonal injury, and co-culture with other cells (e.g., Schwann cells) • Not a neuro-specific device company • Not a 3D culture platform		2015
AxoSim	<ul style="list-style-type: none"> • Hydrogels of choice can be used • Culture inside a Transwell® insert possible • Not a fluidic device: perfusion not possible		2014

Company	Description	Device	Year Founded
Mimetas	<ul style="list-style-type: none">• OrganoPlate®: 96 2- or 3-channel sets in a standard 384-well plate• 3D culture possible• Phaseguide™ technology allows barrier-free separation between channels• Currently only 400 µm channels in market; custom design available		2013
[252]			