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Neural Tissue Engineering and Biohybridized Microsystems for Neurobiological Investigation In Vitro (Part 1)

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ABSTRACT: Advances in neural tissue engineering have resulted in the development and implementation of three-dimensional (3-D) neural cellular constructs, which may serve as neurofidelic in vitro investigational platforms. In addition, interfacing these 3-D cellular constructs with micro-fluidic and/or micro-electrical systems has created biohybridized platforms, providing unprecedented 3-D microenvironmental control and allowing noninvasive probing and manipulation of cultured neural cells. Cells in the brain interact within a complex, multicellular environment with tightly coupled 3-D cell-cell/cell–extracellular matrix (ECM) interactions; yet most in vitro models utilize planar systems lacking in vivo–like ECM. As such, neural cultures with cells distributed throughout a thick (>500 μm), bioactive extracellular matrix may provide a more physiologically relevant setting to study neurobiological phenomena than traditional planar cultures. This review presents an overview of 2-D versus 3-D culture models and the state of the art in 3-D neural cell-culture systems. We then detail our efforts to engineer a range of 3-D neural cellular constructs by systematically varying parameters such as cell composition, cell density, matrix constituents, and mass transport. The ramifications on neural cell survival, function, and network formation based on these parameters are specifically addressed. These 3-D neural cellular constructs may serve as powerful investigational platforms for the study of basic neurobiology, network neurophysiology, injury/disease mechanisms, pharmacological screening, or test-beds for cell replacement therapies. Furthermore, while survival and growth of neural cells within 3-D constructs poses many challenges, optimizing in vitro constructs prior to in vivo implementation offers a sound bioengineering design approach.

KEY WORDS: neural engineering, neuroengineering, three-dimensional, 3-D culture, neural culture, axon, co-culture, extracellular matrix, neuron, astrocytes, traumatic brain injury, biohybrid, microsystems

I. INTRODUCTION

Advances in neural tissue engineering may benefit the fidelity of neurobiological investigations in vitro as well as provide new therapies for neurological disorders. This set of articles (Parts 1 and 2) will summarize the development, implementation, and state of the art for three-dimensional (3-D) engineered neural constructs for use both in vitro and in vivo. Collectively, Parts 1 and 2 will address three interrelated applications: (1) 3-D neural cellular constructs as neurofidelic investigational platforms in vitro; (2) tissue engineered constructs for

ABBREVIATIONS

3-D, three dimensional; ECM, extracellular matrix ; 2-D, two dimensional; DRG, dorsal root ganglion; DIV, days in vitro; SEM, scanning electron microscopy; GFAP, glial-fibrillary acidic protein; RMP, resting membrane potentials; NSC, neural stem cell; BMSC, bone marrow stromal cell; CNS, central nervous system; MEA, multielectrode array

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neuroregeneration in vivo; and (3) living biohybridized neural-electrical microsystems in vitro or in vivo. The common feature for all these applications is the use of 3-D tissue engineered neural constructs. These consist of various permutations of living neural cells distributed throughout matrices/scaffolds in vitro, either for final experimental use in vitro, for transplantation in vivo to augment nervous system repair (i.e., axonal regeneration or replacement), or to facilitate functional integration with chronic interface modalities (e.g., electrical, optical, fluidic).

The overarching theme of these articles is the development and implementation of tissue engineered 3-D neural cellular constructs. Design criteria are based on applications ranging from investigational platforms in vitro to neuroregenerative or neural interface modalities in vivo. Three-dimensional neural cell systems present a degree of experimental control not possible in vivo, yet retain the ability to incrementally add complexity at a metered pace for improved interpretability. The engineering of neural constructs in vitro creates many advantages, including spatial and temporal control of cellular composition, matrix/scaffold properties, mechanical environment, and exogenous factors. For cellular composition, neural and non-neural cell types may be selected, and the constructs can be further customized by considering special cell sources (e.g., transgenic labeling of all neurons or a particular neuronal subtype). These cells may be added in a controlled ratio temporally and spatially, and at a prescribed 3-D density. The matrix/scaffold constituent(s) may also be designed, with control over concentration, porosity, mechanical factors (e.g., stiffness), and degree of bioactivity. Matrix/scaffold choice ranges from bioactive mammalian ECM components with specific interactions with neural cells to relatively bioinert hydrogels with more stable physical properties in culture. Moreover, cell orientation throughout the 3-D matrix can be controlled, either in a homogenous fashion, with an anisotropic spatial arrangement, or segregating cell bodies or cell types. These systems can also be designed to control the physical environment of the cells through the application of external forces, either to drive neural cell growth or to model potentially traumatic mechanobiological responses. Finally, the delivery of exogenous compounds including media components and other soluble factors can be orchestrated, with the ability to collect and analyze used media to directly and indirectly assess cell function or responses.

Although many of these attributes are shared with traditional two-dimensional (2-D) cell culture models, it is important to note that cells retained in engineered 3-D systems have the added benefit of enhanced microenvironmental fidelity to the in vivo situation. This degree of experimental control makes 3-D tissue engineered neural constructs superior over 2-D preparations for most facets of neurobiological investigation. Furthermore, the concept of "pre-engineering" these constructs in vitro for a particular neurological disease/injury treatment makes them extremely valuable for developing nervous system repair interventions. However, successful application of tissue engineered constructs depends on a clear understanding of the design parameters and the ramifications for neural cell survival, growth, and functionality. Therefore, in this review, we will discuss the factors governing successful design of 3-D engineered neural constructs for both in vitro and in vivo applications. We will address key design criteria for the following applications: investigational platforms in vitro, engineered constructs for neuroregeneration, and biohybridized neural interface microsystems. Collectively, the ensuing sections will present the major challenges and the key factors for successfully engineering these constructs.

Survival and growth of neural cells within 3-D constructs poses many challenges—some unique to the neural arena. Requisite parameters governing neural cell survival in complex 3-D microenvironments, such as cell-cell support, matrix mechanical factors, haptotaxic/chemotaxic support, and mass transport limitations may be learned from the development of 3-D neural constructs in vitro. Novel constructs may be optimized in vitro prior to transplantation, the cytoarchitecture and performance of which may be designed based on the disease/injury state they are intended to treat. Sophisticated pre-

transplant interface experiments can be performed, with complexity incrementally added to systematically evaluate construct responses to known in vivo conditions (e.g., in vivo–like oxygen tension, inflammatory cytokines) so that robustness and desired function may be maintained. In addition, 3-D neural cellular models are particularly suited to serve as investigational platforms for the study of basic neurobiology, neurophysiology, mechanobiology, pharmacological screening, or test-beds for cell replacement therapies. Many important neurobiological questions require the spatial, temporal, and microenvironmental control afforded by in vitro investigations. However, the vast majority of such investigations have involved neural cells cultured on planar substrates, typically very stiff (e.g., glass or tissue culture plastic), often with a monotypic cell population and initially lacking extracellular matrix (ECM). This creates a microenvironment that is profoundly different from the cellular microenvironment in vivo, which is defined by cytoarchitecture, cell-cell and cell-matrix interactions, each in 3-D, and a mechanical environment relatively similar to a neural cell itself. Many neurobiological or neurophysiological responses will inherently better match in vivo responses by culturing neural cells in 3-D scaffolds. Furthermore, 3-D neural tissue engineering technology may be functionally integrated with engineered nonbiological components for electrical, electro-chemical, or microfluidic control. Such biohybridized neural interface microsystems may serve as powerful platforms to study and control neurophysiological and electrical responses of "small world" neural networks in 3-D. Overall, the use of tissue engineered 3-D neural constructs with engineered microsystems may significantly advance neurobiological and neural systems investigations.

II. THREE-DIMENSIONAL CELL CULTURE MODELS

II.A. 2-D Versus 3-D Culture Models

Although in vitro models are invaluable in the systematic elucidation of cell behavior in a highly controlled setting, the interpretation of cellular responses in traditional planar 2-D models may be confounded by critical deviations in the cellular microenvironment (e.g., access to soluble factors), atypical cellular morphology,[1,2] and altered cell-cell/cell-matrix interactions.[3–6] Culture models in 3-D, in which cells are grown within a scaffold such that the thickness is at least ten cell diameters, allow investigation of cellular behavior in a more physiologically relevant state by mimicking the cytoarchitecture of in situ tissue to a higher degree than cells grown on nonphysiologic rigid surfaces. Moreover, these systems preserve the primary advantages of traditional in vitro systems, such as control of cellular environment, accessibility for repeated imaging, and elimination of systemic effects. A 3-D environment provides a high surface area for growth and migration, which can be tuned to support other cell behaviors, such as differentiation or maturation. The scaffold may act to protect cells from environmental disturbances, such as media changes, and can be designed for physiological structural stability. Scaffolds can also be designed for optimal mass exchange (per network configuration, pore or mesh size) for both nutrient and waste diffusion.

There are fundamental differences between cells cultured in monolayer versus 3-D configurations (see Table 1). There are differences in the types, quantity, and distribution of cell-cell and cell-matrix interactions,[3–6] with cells in a 3-D bioactive matrix contacting ECM proteins and experiencing cell-cell interactions (e.g., receptor-mediated, synaptic) in all spatial directions. For example, the matrix surrounding cells has been shown to have widespread effects on cellular function for a variety of cell types[3,7] including neural cells.[8–10] Cell-cell and cell-matrix interactions are, by definition, constrained in planar cultures: cells in 2-D configurations may experience such interactions in only a single plane, since a majority of the cell surface is exposed. These phenomena, in turn, may have ramifications on morphology,[2] growth and proliferation,[11] viability,[12] gene and protein expression,[13,14] and in the response to biochemical[15] and/or mechanical stimuli.[16] In particular, from a physical perspective, cells grown in 3-D versus 2-D

TABLE 1. A Summary of the Comparisons Between 2-D and 3-D Cultures in Terms of Culture Configuration, Functional Measures, and Applicability to Modeling In Vivo Physiology.

2-D vs. 3-D Cell Culture	Advantages	Limitations
2-D Cultures	Environmental control Cell observation, measurement, and manipulation are easier than 3-D A rich body of literature exists with which to compare results	2-D systems may be inherently unable to depict traits exhibited by in vivo systems, e.g., altered gene expression and growth characteristics due to deficiency in cell-cell/cell-matrix interactions Less compatibility with in vivo systems Altered drug sensitivity Cells have majority of their surface exposed
3-D Cultures	Cells are in proximity with other cells on all sides Neurites are able to extend in all directions More accurate representation of in vivo cytoarchitecture Survival and other cell behavior more in vivo–like	Diffusional transport limitations: O_2 and other essential nutrients may not reach all of the cells; toxic waste products may accumulate within scaffold space Culture dependent alterations in gene expression, cell proliferation, viability, productivity, and product quality due to nutrient deprivation

environments have a starkly different morphology and cytostructure.[2] Cells stay more spherical when embedded within a scaffold than 2-D cultures and, in the case of neurons and other process-extending cells, outgrowth can occur in all directions. The somata and growth cones of neurons in 2-D are unrealistically flattened compared to cells in 3-D or in vivo, which present a rounder, more bulbous shape.[1] Differences also exist in terms of access to soluble factors: in traditional 2-D cultures, the sink-like property of the bathing medium may serve to dilute secreted or released molecules.

In vivo–like cell-cell interactions may lead to increased cellular survival and more realistic gene expression and cellular behavior. Accordingly, differences in gene expression, growth characteristics, and viability are found when comparing cells cultured in 3-D and 2-D to cells in vivo.[7,17] Specifically, cells cultured in a 3-D environment have been shown to better represent in vivo cellular behavior than cells cultured in monolayer, for both neural and non-neural cells (e.g., osteoblastic cells;[11] fibroblasts;[18] breast epithelial cells;[19] and neurons and astrocytes).[12,20] Planar culture of chondrocytes results in de-differentiation, whereas 3-D chondrocyte cultures maintain the expression of chondrogenic markers aggrecan and collagen type II.[21,22] Similarly, cardiomyocytes in 3-D culture maintain more in vivo–like molecular and electrophysiological characteristics than those cultured in monolayers.[23] A growing body of evidence suggests that cells grown in planar cultures do not have the same morphology,[3] proliferation rates,[24,25] migration,[26] gene expression,[27,28] differentiation,[25,29] cellular signaling,[30] or pathological susceptibility[17,24,31] as in 3-D culture or in vivo. For example, it has been shown that dopaminergic neurons harvested from embryonic brain are viable longer when grown in 3-D than monolayer cultures.[32] Also, 3-D cultures have been shown to result in longer neurites, higher levels of survival, and different patterns of differentiation as compared to 2-D monolayers.[33–37] When comparing embryonic mesencephalon tissue in 2-D versus 3-D, more cell death occurred in dissociated monolayer cultures, whereas tissue explants and 3-D cultures in collagen gels survived to a much greater extent.[38] Hippocampal neurons grown on a 3-D aragonite matrix also survived to a greater

extent than 2-D counterparts, and formed higher density networks.[39] Cells cultured in 2-D have also exhibited an increase in sensitivity to chemical treatments independent of changes in surface area,[40] challenging the suitability of 2-D models for studies evaluating pharmacological responses. We have shown differences in response to 2-D versus 3-D neural cultures to mechanical injury, in which 3-D cultures sustained more cell death than 2-D cultures subjected to the same strain and strain rate.[41] Neural culture systems in 3-D may also be more appropriate for electrophysiological studies than planar counterparts. For example, in 3-D culture, Na^+/H^+ exchangers have been shown to have polarized expression to the apical membrane, which is difficult to maintain in monolayer cultures.[42] In addition, neurons in a 2-D culture environment have exaggerated Ca^{2+} dynamics in comparison to 3-D cultures.[43–45] Collectively these findings raise questions about the appropriateness of employing monolayer 2-D culture models to study certain cell, tissue, or systems level phenomena.

However, there are important limitations and design considerations that affect the utility of 3-D in vitro systems. Cells cultured in 3-D may experience diffusional transport limitations, causing essential nutrients to not reach all cells and accumulation of toxic waste products in close proximity to the cells. Such phenomena may result in altered gene expression and a detrimental effect on protein synthesis and function, proliferation and viability, leading to an appreciable deviation from in vivo behavior.[46] Many caveats exist in using reduced models: critical differences in cell phenotype and cell density, ECM constituents, and neuroanatomy, among others, must be acknowledged in interpreting the results attained using 3-D neural cellular constructs. However, carefully designed and optimized 3-D models, with sufficient mass transport and appropriate cell and matrix elements, may more faithfully recapitulate aspects of native tissue than 2-D models. Cells are influenced by complex environmental stimuli, central to which is the local extracellular microenvironment. Inherent differences in cell-cell/cell-matrix interactions coupled with corresponding alterations in cell morphology and alterations in the cellular microenvironment may have a profound impact on intracellular signaling and gene expression. Thus, due to fundamental deviations in planar cell culture from the in vivo environment, cells cultured in 2-D may be inherently unable to recapitulate certain traits exhibited in vivo, whereas properly designed and regulated 3-D systems may represent a step closer to in vivo.

II.B. Models of Neural Cells in 3-D

Multiple types of 3-D neural cultures have been created, including organotypic slice cultures, cultures on or within biological matrices or hydrogel scaffolds, and reaggregate or sphere cultures. Each approach varies based on degree of control, extent of three-dimensionality, and preservation of tissue function and organization. Organotypic cultures are slices of tissue (e.g., typically less than 500 microns) maintained up to several weeks in vitro. There is no better convergence of in vitro experimental control and maintenance of neural architecture as organotypic cultures. This approach maintains many critical in vivo characteristics, such as neural cytoarchitecture, in-plane neural connections, characteristic electrophysiological responses, ECM, and a representative phenotypic cohort (neurons, astroglia, oligodendrocytes, microglia). However, as an inevitable consequence of excision from the brain, there is an initial period of active degeneration in organotypic cultures, as manifested by robust microglial activity and "thinning out," although electrophysiological properties may develop similarly to that in the intact animal.[47,48] However, the ability to control cell types and matrix properties is also restricted in organotypic cultures.

Alternatively, other 3-D models are developed from the bottom up, typically involving dissociated cells or explanted ganglia, in many cases with controlled scaffolds/matrices. In some cases, cells may reorganize according to cell type, media/fluidic conditions, and adhesiveness to a substrate or surrounding cells. For example, neural cell culture models have been developed consisting of neuronal cells distributed throughout a 3-D matrix.[16,41,49–54] The resulting engineered neural cellular constructs are highly controllable as the cell types, cell distri-

bution, and the matrix constituents are selected a priori. Moreover, such complexity may be incrementally added as these parameters may be systematically varied. In contrast to brain slices, engineered cellular constructs are actively growing over weeks in vitro, and thus are suitable to study developmental phenomena such as neurite outgrowth, synapse formation, and neuronal maturation. Using these 3-D systems, it was demonstrated that dorsal root ganglion cells (DRG) survive and extend neurites through hydrogel matrices in a manner dependent on the physical properties (e.g., agarose pore size,[55] stiffness[1]), ligand concentration (e.g., collagen,[56] RGD peptides in fibrin[57]), and substrate geometry.[58] DRG neurite growth in 3-D matrices was shown to be inhibited by transitions in mechanical properties, as well as chondroitin sulfate interfaces,[59] whereas outgrowth was enhanced in engineered matrices by the presence of specific peptide sequences.[60] Embryonic cortical neurons have been plated within 3-D matrices of collagen and various hydrogels (e.g., poly[N-(2-hydroxypropyl)-methacrylamide],[53] poly(acrylate),[51] agarose,[52] Matrigel,[41] and agarose-collagen[54]). Enhanced survival and neurite outgrowth was observed in collagen (0.4–0.5 mg/mL) as compared to hydrogels lacking ECM ligands, which produced varying degrees of cell viability and a paucity of neurite outgrowth, together indicating that growth and survival of primary cortical neurons are improved by specific cell-matrix interactions.[51–53] Previous efforts using 3-D systems to characterize the influence of matrix mechanical properties and ligand density in neuronal survival and extent of neurite outgrowth has uncovered complex, often synergistic relationships between these parameters (see Section IV.A).

Development of many of the aforementioned neural cell culture systems with cells distributed throughout a matrix has been important in recapitulating 3-D cell-cell and cell-matrix interactions.[49–53,61] However, these studies relied solely on passive diffusion for nutrient delivery and waste removal, necessitating the use of cell densities much lower than those found in the brain. Tradeoffs exist between cell density and the thickness/depth in 3-D culture. For instance, neural cell culture models have been developed to approximate a 3-D morphology/orientation by plating dissociated primary cells above a matrix material.[62–64] Such systems may support high 2-D cell densities, although cell-cell and cell-matrix interactions are constrained, as cells are not distributed throughout the full thickness of the matrix. Reaggregate neural cultures have been developed by rotation-induced reassociation.[65–67] These systems produce 3-D spheres at high cell densities via diffusion with convective enhancement of circulating medium. However, the volume available for growth is inherently limited based on surface area to volume ratios that permit survival, thus limiting the scope of 3-D interactions. Although it is difficult to control ECM components and cellular distribution of reaggregate cultures, these models are extremely useful for studying cell-cell interactions, growth, and function at cell densities that closely match those found in vivo. Until recently, mass transport limitations prevented the development of 3-D neural cell culture models consisting of a high cell density ($\geq 10^4$ cells/mm^3) with greater than millimeter-scale dimensions and full thickness cellular distribution (see Section V.B).[68,69]

II.C. Multi-Cell-Type Composition in 2-D and 3-D

Three-dimensional models consisting of multiple neural cell types more closely approximate the heterogeneity of in vivo tissue while also representing aspects of neural cytoarchitecture to a greater extent than traditional planar cultures.[70,71] With respect to the nervous system, cellular heterogeneity is important to represent such interactions as physical support and metabolic coupling between neurons and astrocytes.[72–74] Multi-cellular composition may be particularly important to modeling nervous system function. In particular, the presence of astrocytes has been shown to significantly influence the biological and electrophysiological properties of neurons. In vivo, astrocytes and neurons have an intricate coupling, as astrocytes provide metabolic support by regulating the blood-brain barrier, providing trophic factors, and maintaining proper glutamate and glutathione metabolism,

thus creating interdependence between these cell types in assuring proper energetics, neuromodulation, and termination of pathologic states such as excitotoxicity.[74] Astrocytes also exhibit a physical role, serving as substrates for neuronal migration and guides for neuronal process outgrowth, as well as forming barriers to segregate axon types. Astrocytes play an important role in neuronal function through tight regulation of the synaptic space by controlling the number and stability of synapses and by ensheathing synapses to maintain an appropriate ionic environment.[75] Co-cultures consisting of neurons and astrocytes are typically 2-D models, although multi-cellular reaggregate cultures have been developed.[76,77] In 2-D neuronal-astrocytic co-cultures, the two cell types often self-arrange into a base layer of astrocytes (which become confluent) with neurons on top.[78] Although this distribution spatially limits neuron-astrocyte and neuron-neuron interactions, such bi-planar models have proven useful in establishing the pivotal role of astrocytes in neuronal survival and synapse formation.[79,80] In addition, the presence of myelinating cells such as oligodendrocytes may be necessary to elicit proper action-potential propagation in certain long axons. Overall, the intimate association of neurons with glial cells in a 3-D environment may result in necessary cell-cell functions to recapitulate phenomena observed in vivo.

III. DEVELOPMENT OF ENGINEERED 3-D NEURAL TISSUE SURROGATES

Morphological and electrophysiological properties of neural cells are substantially influenced by their immediate extracellular surroundings, yet the features of this environment are difficult to mimic in vitro. Therefore, there is a tremendous need to develop culture systems that more closely model the complexity of nervous tissue. To this end, we have engineered novel 3-D neural constructs composed of neurons and/or astrocytes within a bioactive extracellular matrix-based scaffold (Fig. 1). We apply these engineered neural tissue surrogates as in vitro investigational platforms to study and manipulate neurobiological responses within 3-D microenvironments. These models have broad applicability in neurobiological research, with key features including millimeter-scale dimensions, multi-cellular composition (when desired), and the capability for real-time live cell imaging and micro-electrical/micro-fluidic capabilities (see Section V). These microsystems may serve as high-throughput modeling platforms to study appropriate facets of basic neurobiology, neurotoxicity, or injury responses, and thus may be valid in vitro test-beds prior to animal studies.

We focus on neural tissue engineering using differentiated neural cells, with multi-cellular composition achieved by separately isolating individual phenotypes and mixing them in a controlled manner. Alternate approaches utilize neural or other stem cells to achieve phenotypic heterogeneity as well as study the effects of 3-D microenvironments on stem cell biology and differentiation.[38,81,82] Such work is extremely insightful for stem cell biology and tissue engineering, but is not further addressed here. It is noteworthy that our general approach of engineering neural constructs in vitro for a particular in vivo application is valid for constructs using stem cells, and many design criteria will be mutually applicable. An advantage of building 3-D co-culture constructs from dissociated cells is that neurons and glial cells from different transgenic sources can be incorporated. For example, neurons may be isolated from transgenic rodents expressing one fluorescent protein (e.g., green-fluorescent protein) with glia from another (e.g., red-fluorescent protein), permitting identification from one another in culture. This flexibility permits a wide range of applications with different cell types (e.g., inhibitory neurons) and transgenic sources (e.g., knock-outs, knock-ins) to address specific mechanistic questions. Thus, 3-D culture models consisting of multiple neural cell types are capable of maintaining many positive aspects of in vitro modeling while approximating controlled facets of cellular heterogeneity and cytoarchitecture found in the brain. Of note, we previously published detailed methods for the generation of 3-D neuronal cell cultures, including a review of neural cell type and scaffold considerations.[83]

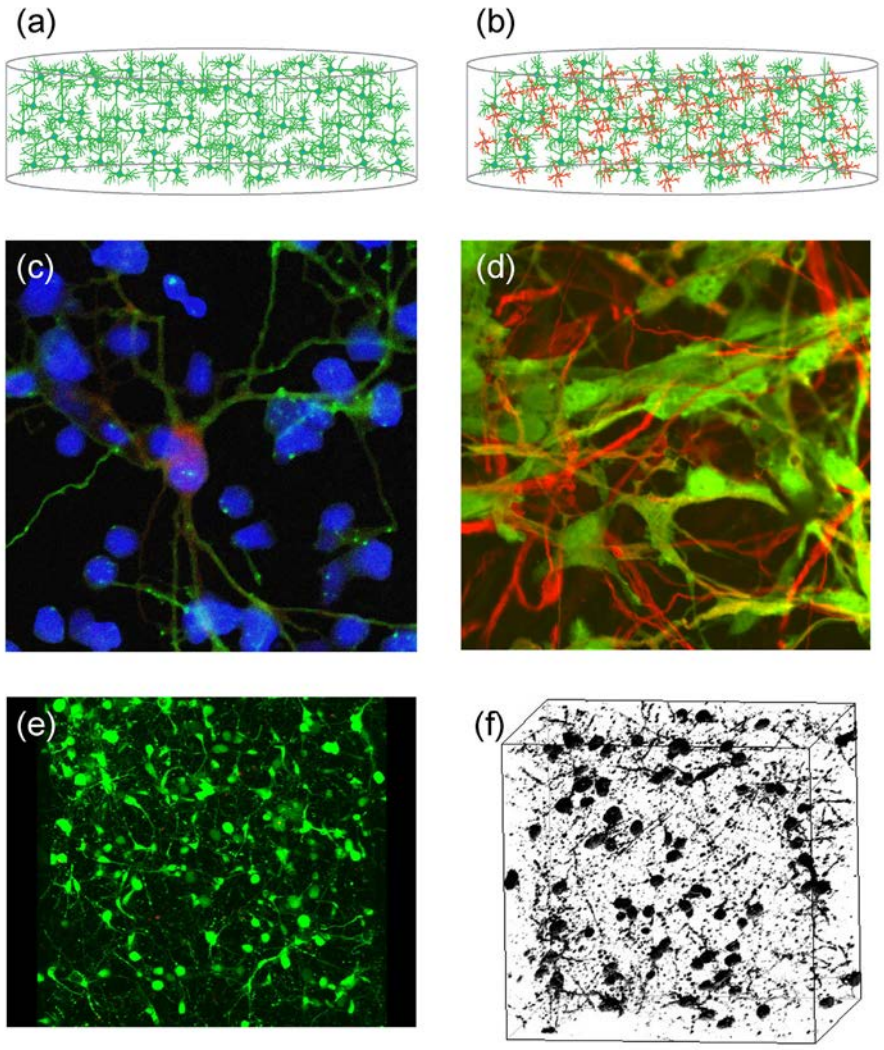

FIGURE 1. Concept: Tissue Engineered 3-D Neural Cell Cultures for Neurobiological Investigations In Vitro. Cellular constructs consist of (a) neurons or (b) neurons mixed with astrocytes, distributed throughout the full thickness of a 3-D matrix/scaffold (>500 μm). (c) Neurons in 3-D culture, shown to express neuronal-specific proteins MAP-2 (green) and tau-5 (red) with nuclear marker Hoechst 33258 (blue), assume complex morphologies with 3-D neurite extension (adapted with permission from Ref. 93, IOP Publishing). (d) These cultures are useful to study neuron-astrocyte interactions in 3-D. (e) Live neurons (green) demonstrating survival and network formation throughout the thickness of the 3-D cultures. (f) Volumetric rendering of live neurons in 3-D culture (adapted with permission from Ref. 41, Mary Ann Liebert, Inc.). Neurobiological studies of neural cells within 3-D matrices provide enhanced fidelity to in vivo while affording all advantages of traditional (planar) in vitro systems.

III.A. Attributes and Design of 3-D Neural Tissue Surrogates

We have developed and optimized a series of 3-D cell culture models to study neurobiological phenomena within 3-D cytoenvironments. These models consist of neurons and glia distributed throughout ECM and/or hydrogel scaffolds. The motivation for the development of these cultures,

as discussed above, is that most studies evaluating in vitro survival, neurite outgrowth, network formation, synaptogenesis, and functionality have been done in planar culture lacking significant amounts of ECM; however, we postulate that the presence of 3-D growth/interactions and bioadhesive ligands are crucial to studying these phenomena in vitro. Using 3-D neural constructs for investigational platforms has the potential to increase our understanding of neuronal and glial cell biology and functional interactions. Notably, these systems may be engineered from the bottom up, with precise control over cellular (e.g., phenotypes, ratios, densities) and scaffold parameters (controlling mechanical properties and extent of bioactive motifs), to assess the influences of specific environmental factors on particular responses. These 3-D cellular systems present a degree of experimental control and precision not attainable in vivo. Specifically, these neural engineered 3-D cell culture systems have the following attributes:

(1) Three-dimensionality: virtually all tissue in the body possesses 3-D architecture. Therefore, reduced models meant to evaluate, and ultimately manipulate, cellular behavior should represent intercellular and cell-matrix interactions in three dimensions (beyond the dimensions of a single cell, which of course is 3-D on the microscale). In our systems, cells were homogenously dispersed throughout matrix to create 3-D cell-cell and cell-matrix interactions throughout thick constructs.

(2) Controlled complexity: the ability to systematically increase the complexity of 3-D engineered microenvironments. Possess precise control of cellular and matrix constituents, and these may be added at a pace appropriate to study the influence of a particular cell type, cell density, or matrix factor.

(a) Cellular composition: control of cell types, ranging from a nearly pure neuronal population to separately harvested neurons and glia mixed in controlled ratios.

(b) Matrix properties: control constituents, concentrations, and mechanical properties. Ranging from bioactive (e.g., laminin-I and collagen-IV) with targeted cell interactions to relatively bio-inert (e.g., hydrogels).

(c) Anisotropy: the ability to engineer anisotropic cell distribution or growth cues.

(d) Cell density: the ability to control 3-D cell density to more realistically approximate that of brain.

(3) Endpoint evaluation: 3-D constructs may be formalin fixed to permit sophisticated immunocytochemical and morphometrical analyses.

III.B. Characterization of 3-D Neural Tissue Surrogates

The initial 3-D neural cellular constructs we developed consisted of: (1) primary cerebral cortical neurons and (2) primary cerebral cortical neurons and secondary cortical astrocytes in co-culture (Fig. 1). Neurons and astrocytes were harvested separately from rats or mice, dissociated using standard techniques, and mixed in controlled ratios.[41,54,68,84] These neural cells were homogeneously distributed throughout the full thickness of bioactive scaffolds (typically 500–600 μm thick, up to 1.0 mm in some cases), and were maintained using a defined, serum-free, Neurobasal medium. Initially, the neural cell culture models described here have been developed using Matrigel matrix, a reconstituted basement membrane that is biologically active for neural cells through matrix (laminin, collagen IV, entactin, heparan sulfate proteoglycan)[85] and cytokine-related interactions,[86] which has been shown to promote neurite outgrowth.[87] Culture architecture, viability, morphology, neuronal marker expression/differentiation, neurite outgrowth, neuronal/astrocytic composition, and synaptic protein markers were compared between 3-D and analogous 2-D models. Also, these systems were characterized based on cell density, matrix constituents, and days in vitro (DIV), with comparisons made between neuron-only cultures and neuron-astrocyte co-cultures to assess the influences of astrocytic presence. These 3-D models may more accurately represent in vivo neural responses and develop enabling technologies for neurobiological and tissue engineering applications.

1. Three-dimensionality: interactions and microenvironments

Culture architecture. We employed confocal mi-

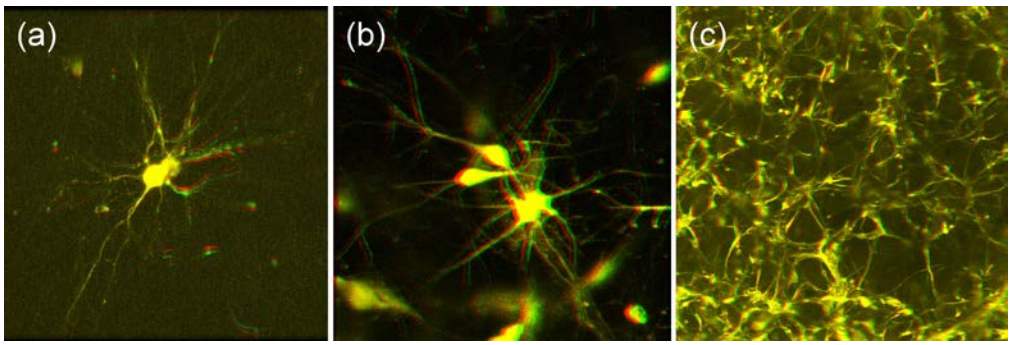

FIGURE 2. Three-dimensionality. Stereo images labeling live neural cells, showing complex neuronal morphologies and 3-D culture architecture (may be viewed with standard 3-D glasses). (a) A transgenic fluorescently labeled neuron showing complex morphology within a 3-D matrix. (b) Viability staining showing live neurons within a 3-D matrix. (c) Viability stain in 3-D neuronal-astrocytic co-cultures demonstrating robust survival at over 60 DIV.

croscopy to ensure that neural constructs maintained a 3-D culture configuration throughout experimentation. Extensive process outgrowth extending from cells was visible under bright field microscopy by 1 DIV and became more pronounced as the culture matured, resulting in the formation of 3-D neural networks by 7 DIV. Cells plated in 3-D extended numerous processes at all orientations, while cells plated in 2-D remained nearly planar, as expected. We verified the desired culture architecture up to 21 DIV for neuron-only cultures, and up to 63 DIV for co-cultures, the longest time-points evaluated for each (Fig. 2). Reconstructions of confocal z-stacks comparing 3-D and 2-D configurations for neuronal cultures and neuronal-astrocytic co-cultures are at http://www.neuro.gatech.edu/groups/laplaca/3Dreconstruction.html (live cells are labeled green and the nuclei of dead cells are labeled red). Confocal microscopy across the full thickness of the constructs revealed that this high viability was consistent across the heights of the cultures.

2. Survival

Survival in neuron-only cultures based on cell density. In order to determine the optimal 3-D cell density for neuronal survival, neurons were plated at various densities (1,250–12,500 cells/mm^3) and viability was assessed at 7 DIV.[83] Interestingly, there was a parabolic relationship between cell plating density and cell viability in the 3-D neuronal cultures (Fig. 3). Cultures plated at lower density (≤2500 cells/mm^3) or higher density (≥6250 cells/mm^3) exhibited poor viability (<50% for each), whereas there was an optimum viability of ~90% for cultures plated at 3750–5000 cells/mm^3 (p < 0.05 versus other cell densities). This suggests an important balance between diffusional requirements (affecting the higher density cultures) and a threshold for cell-cell interactions (both physical and chemical, adversely affecting the low density cultures). Neuronal death in low cell density 3-D cultures was likely independent of mass transport limitations. However, mass transport limitations were likely the predominant reason that 3-D neuronal cultures exhibited poor viability at high cell densities (≥6250cells/mm^3) (see Section V.B). Increasing the cell density in 3-D effectively decreases the available area for diffusion, increases tortuosity factors, and increases the overall rates of nutrient consumption and waste product production, creating system-specific relationships between culture thickness, cross-sectional area for diffusion, cell type, and cell density. However, the optimized cell density range (3750–5000 cells/mm^3) yielded cultures with extensive neurite arborization, robust neuronal survival, and active outgrowth. Overall, viability in 3-D neuronal cultures was highly dependent on cell density, with optimal viability at an initial plating density of 3750 cells/mm^3 for 500-

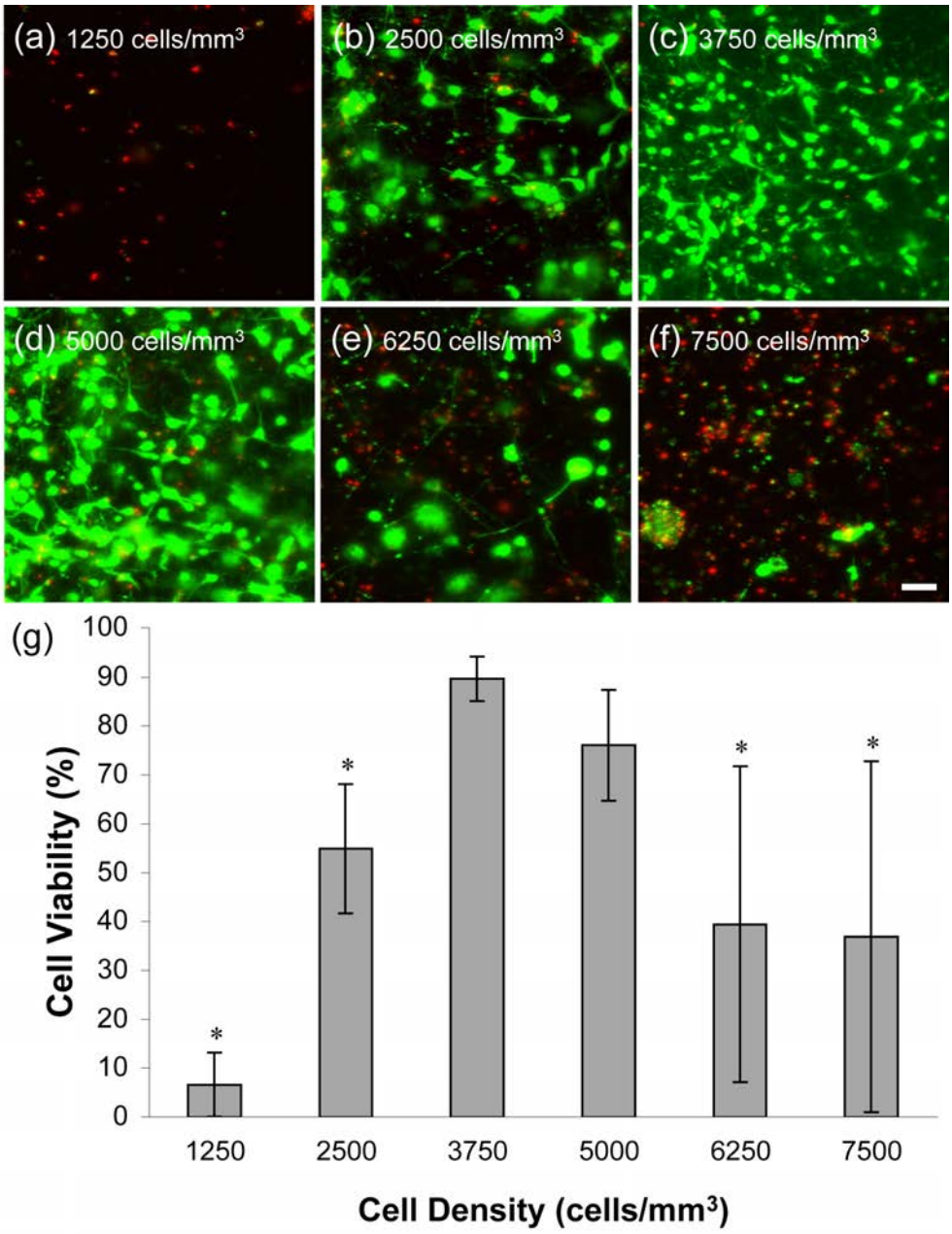

FIGURE 3. Neuronal survival in 3-D depends on cell density. (a–f) Fluorescent confocal reconstructions of representative neuronal cultures plated at various cell densities (scale bar = 50 μm). At 7 DIV, cells were stained to discriminate live cells (green) and the nuclei of dead cells (red). (g) In this culture model, cell density was found to be a significant factor in neuronal survival (data presented as mean ±standard deviation). Neuronal cell densities >5000 cells/mm^3 and <2500 cells/mm^3 result in poor survival. *Groups varied significantly from the peak cell viability attained at a cell density of 3750 cells/mm^3 ($p<0.05$). Thus, there were thresholds in survival of thick, 3-D neural cultures based on cell density when supported by passive diffusion, potentially related to biomass transport limitations at higher cell densities. Reproduced with permission from Ref. 83,*Methods in Bioengineering: 3D Tissue Engineering,* Norwood, MA: Artech House, Inc., 2010. ©2010 by Artech House, Inc.

to 600-μm thick cultures.

Survival in neuron-only cultures based on depth. At 7 DIV, overall cell viability in cultures plated at the optimized 3-D density did not vary statistically from that attained in planar culture (87.1 ± 7.0% in 3-D versus 92.8 ± 4.0% in 2-D).[41] In these 3-D neuronal cultures, the total culture thickness occupied by cells was measured to be 550.9 ± 28.6 μm. Throughout this thickness, there were not statistically significant differences in cell viability or cell density (3608 ± 1280 cells/mm^3) based on depth. Although these data demonstrate consistent neuronal survival and distribution throughout the thickness of the 3-D cultures, neuronal survival was more variable deeper into the constructs, which is possibly indicative of approaching the limits of mass transport with these cultures.

Long-term survival in neuron-only cultures. Using the optimal 3-D cell density of 3750 cells/mm^3 and comparing to 2-D cultures (1250 cells/mm^2), viability was assessed up to 21 DIV.[88] There were no statistical differences in cell viability between the 3-D and 2-D cultures at 7 DIV, 14 DIV, or 21 DIV (Fig. 4). Furthermore, there was not a significant difference between either the 7 DIV to 14 DIV or the 14 DIV to 21 DIV for either configuration; however, there was a significant decrease in culture viability at 21 DIV compared to 7 DIV in both the 3-D and 2-D models ($p < 0.05$). Overall, viability in optimized 3-D neuronal cultures did not differ from viability in 2-D neuronal cultures up to 21 DIV.

Long-term survival in neuronal-astrocytic co-cultures. Based on an adjustment to the optimal cell density established in neuronal cultures, neuronal-astrocytic co-cultures were developed where each cell type was derived from a separate source and mixed in a 1:1 or 1:5 ratio.[88] At either plating ratio, the viability from 7 DIV to 21 DIV remained >95% for 3-D co-cultures and >98% for 2-D co-cultures; however, there was not a statistically significant difference between these groups at any time-point evaluated (Fig. 5).We have cultured 3-D neuronal-astrocytic co-cultures for over 60 days with only a modest decrease in viability (Fig. 2). Thus, astrocytes are robust in 3-D culture, and the presence of glial support appears to improve neuronal survival in 3-D. These data suggest that co-culture conditions (i.e., glial support) may be required for the long-term culture of primary cortical neurons, yet even under those circumstances the culture viability has been shown to steadily decrease.[89]

3. Morphology

Confocal and scanning electron microscopy (SEM) revealed morphological and cytostructural differences between neurons distributed throughout a matrix compared to neurons in planar culture.[41,83,88] In particular, SEM revealed distinct morphological differences between cells plated within a 3-D matrix and cells plated on a 2-D rigid substrate (Fig. 6). Neural cells plated in 3-D presented a bulbous morphology with intercellular contacts in all spatial directions. Neurons in 2-D culture had a flatter base with a restricted cytostructure. In 2-D, neurites departing the soma were mainly in the horizontal plane, and interactions occurred on the substrate plane or up the height of the cell body. In addition, co-cultures in 2-D arranged themselves into a base layer of astrocytes (which approached confluency) with neurons segregated to a second plane above.

4. Cell Composition/Phenotype

Neuron-only cultures. Cellular phenotypic identification was assessed using specific immunocytochemical markers.[41] The level of astrocyte presence in the neuronal cultures was assessed through immunostaining for the astrocyte-specific intermediate filament glial-fibrillary acidic protein (GFAP) in conjunction with Hoechst staining. At 7 DIV, the percentage of GFAP$^+$ cells was 2.9 ± 1.5% in 3-D and 3.2 ± 1.6% in 2-D, without a significant difference between these groups. Additionally, immunocytochemistry revealed both 2-D and 3-D configurations yielded similar percentages of cells staining positive for the maturing isoform of the neuronal cytoskeletal marker tau-5 (~90% each), with no significant differences.

Neuronal-astrocytic co-cultures. The percentage of neurons in co-culture with astrocytes was assessed using immunocytochemical techniques for tau and GFAP in conjunction with Hoechst labeling.[84,88] At 7 DIV, the neuronal component in 3-D co-cul-

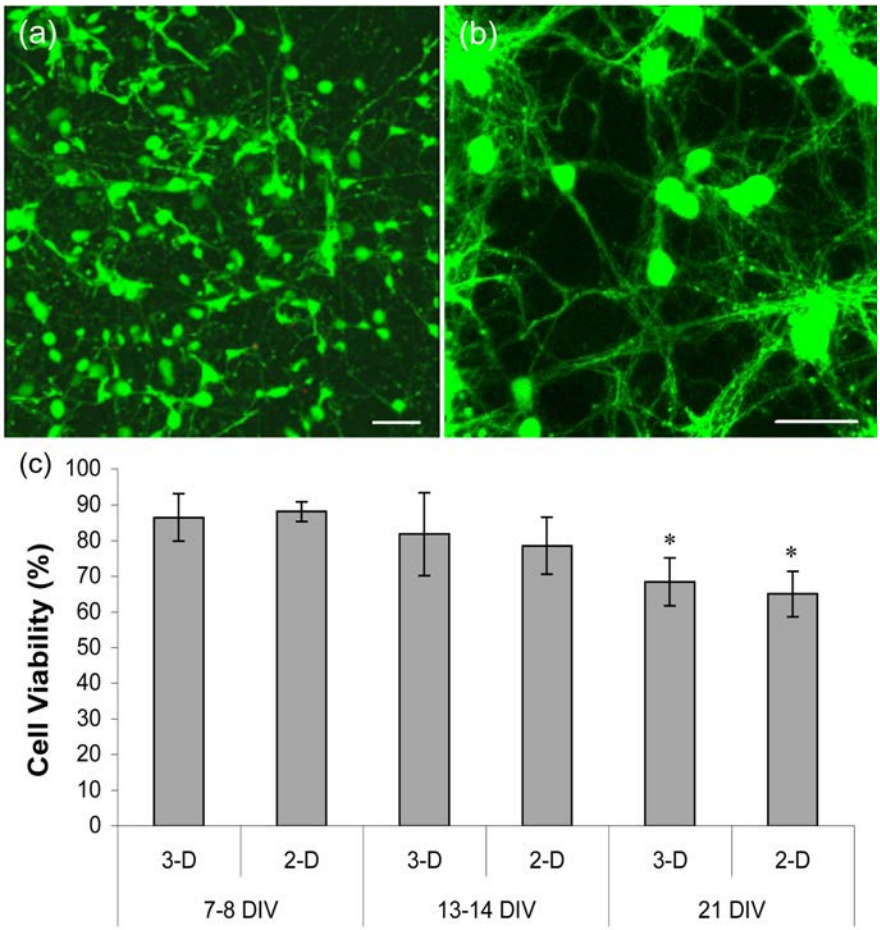

FIGURE 4. Viability in 3-D and 2-D neuronal cultures. Fluorescent confocal reconstructions of representative neuronal cultures at 7 DIV plated (a) in 3-D throughout a matrix or (b) in 2-D above a matrix (scale bars = 50 μm). Live cells are stained green while the nuclei of dead cells are stained red. (c) The percentage of viable cells in 3-D and 2-D neuronal cultures was quantified at 7–8, 13–14, and 21 DIV (mean ±standard deviation). Neurons in 3-D were plated at the optimal cell density for this system (3750 cells/mm^3). There was not a significant difference between 3-D and 2-D viability at any time-point; however, both 3-D and 2-D neuronal cultures had a significant decrease in cell viability at 21 DIV compared to 7–8 DIV (*$p < 0.05$).

tures was 22.0 ± 5.4% (initially 50%), compared to 2-D co-cultures, in which the neuronal percentage was 19.8 ± 7.2% (initially 50%). Thus, for an initial plating ratio of 1:1, there was a similar alteration in the neuron:astrocyte ratio in 3-D compared to 2-D. However, by 21 DIV the percentage of neurons relative to the total cell population in 3-D co-culture had reduced to 9.8 ± 3.7%, possibly a function of continued astrocyte proliferation or an indication of some neuronal death during culture development. Similar neuronal composition percentages were determined by staining for MAP-2, another microtubule-associated protein expressed predominantly in neurons.

5. Neuronal Maturation and Synapse Formation

The continuum transition from an immature to a mature neuronal phenotype is marked by established cellular polarity and electrochemical activ-

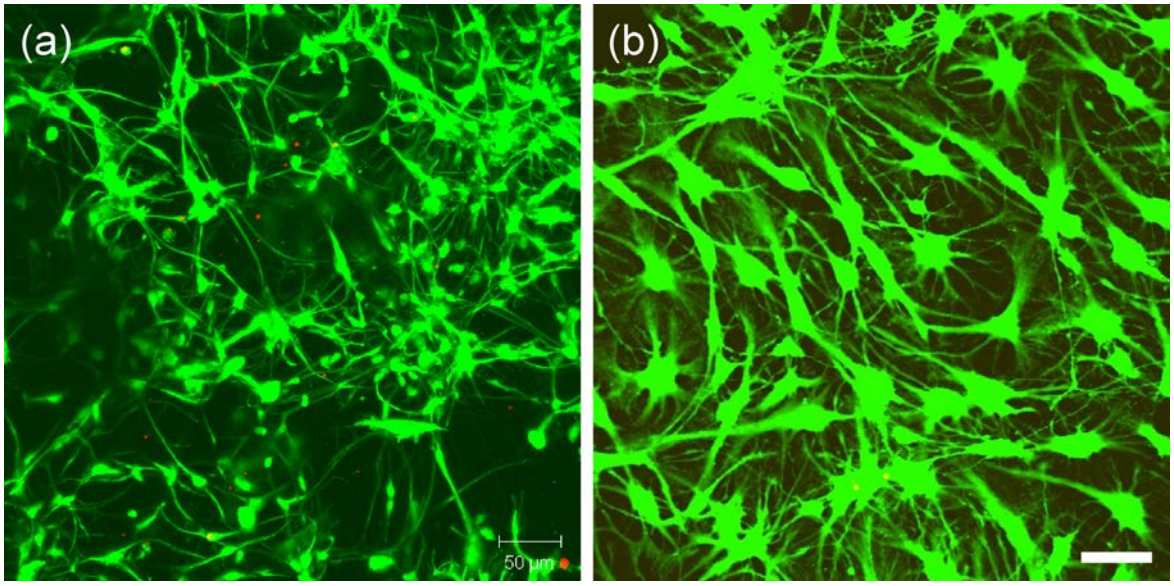

FIGURE 5. Viability in 3-D and 2-D neuronal-astrocytic co-cultures. Fluorescent confocal reconstructions of representative neuronal-astrocytic co-cultures at 21–22 DIV in (a) 3-D and (b) 2-D. Live cells are stained green while the nuclei of dead cells are stained red. The viability for both 3-D and 2-D co-cultures was >95% at all time-points evaluated, with no statistically significant difference in the viability between these culture configurations at any time-point. Scale bar = 50 μm.

ity (i.e., synaptic communication). These events coincide with the widespread expression of mature isoforms of structural proteins as opposed to developmental (immature) isoforms.[79] Immunocytochemistry revealed that neurons within the constructs expressed the neuronal-specific proteins MAP-2 (2A + 2B isoforms) and tau-5, the former being a mature isoform of that microtubule associated protein (Fig. 1). The formation of synapses was assessed via immunocytochemical staining for neuronal cultures and neuronal-astrocytic co-cultures.[88] Immunolocalization of synapsin$^+$ puncta along neuronal processes suggests the presence of active synapses.[64,90,91] The vast majority of cells in neuronal culture expressed the neuronal marker tau, and by 7 DIV these cells presented synapsin distributed in discrete puncta along the processes or somata that increased in number out to 21 DIV. Similarly, synapsin$^+$ puncta were also observed along tau$^+$ somata and processes in co-cultures in 3-D and 2-D, and there was a similar trend of increased synapsin staining as a function of DIV. There was a substantial increase in the number of synapsin$^+$ puncta per neuron in neuronal-astrocytic co-cultures versus the neuronal cultures (Fig. 7). However, there was no change in the formation of synapsin$^+$ puncta based on culture dimensionality. Thus, from 7–21 DIV, astrocytic presence increased the number of synapses per neuron, but this was not affected by planar versus 3-D culture.

Overall, in neuronal and neuronal-astrocytic co-cultures in 3-D and 2-D, cells exhibited the neuronal cytoskeletal marker tau and discrete puncta containing the pre-synaptic vesicle protein synapsin. This immunolocalization was suggestive of the presence of maturing neuronal phenotype and active synapses from 7 to 21 DIV. The methodology used in this study to identify synapses via immunolocalization has previously been correlated with the formation of active synapses via whole-cell patch clamp recordings in similar neuronal culture models.[64,90] Our results, along with those of others, demonstrate that astrocytic presence is not a requirement for synapse formation, although

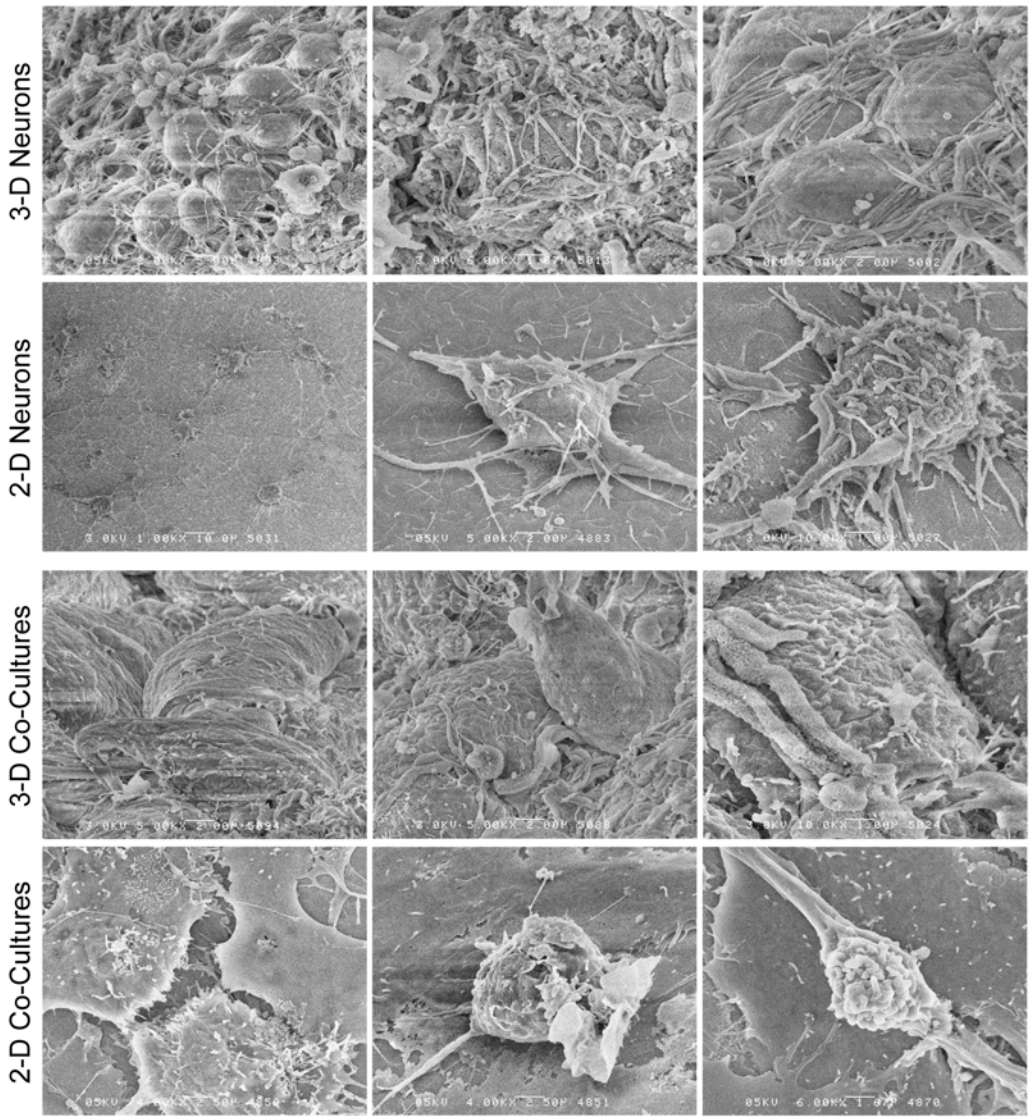

FIGURE 6. Electron micrographs of neuronal cultures and neuronal-astrocytic co-cultures in 3-D and 2-D. Neurons in a 3-D matrix present a rounded morphology with matrix interactions possible in all spatial dimensions, while neurons in 2-D have a flattened morphology. Cells in neuronal-astrocytic co-cultures in 3-D are rounded with an even dispersion of neurons and astrocytes, compared to 2-D where the cell types segregate themselves to a base layer of flattened astrocytes with neurons in a plane above.

evidence suggests that neuronal-glial interactions may be necessary to elicit proper neuronal differentiation and fully functional synapses.[92]

6. Neurite Outgrowth

Three-dimensional neurite outgrowth was evident throughout the matrices in both neuronal and neuronal-astrocytic co-cultures. We semiquantitatively assessed the amount of neurite outgrowth in neuronal cultures in 2-D versus 3-D configurations based on the relative expression of the microtubule-associated proteins tau-5 and MAP-2 (Western

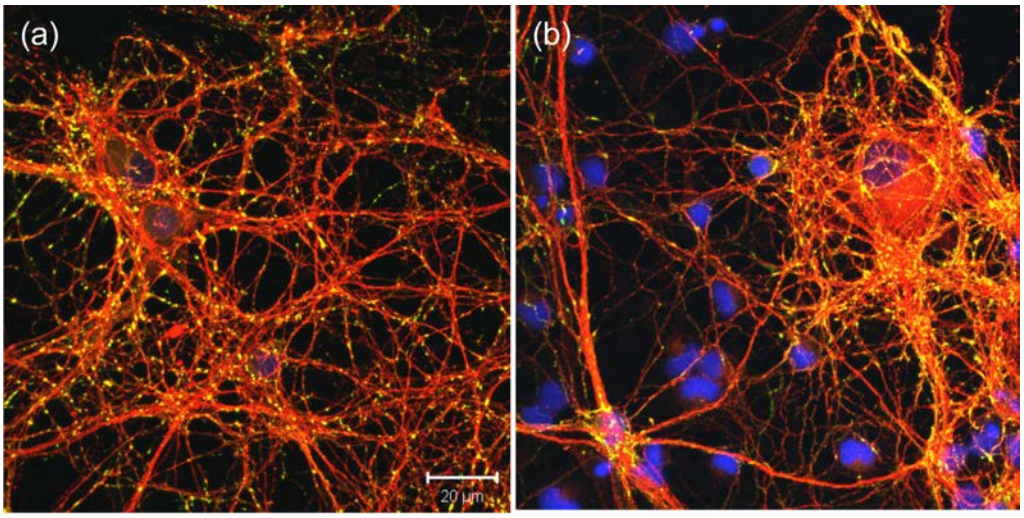

FIGURE 7. Synaptic marker expression. (a) Neuronal cultures and (b) neuronal-astrocytic co-cultures labeled via immunocytochemistry for tau (red), a neuronal marker, and synapsin (green), a marker of presynaptic specializations, and counterstained with the nuclear marker Hoechst 33258 (blue). Synapsin$^+$ puncta can be seen along somas/neurites in both culture systems. There was an increase in synapsin$^+$ puncta per neuron in neuronal-astrocytic co-cultures versus the neuron cultures, signifying an enhanced rate of synapse formation or retention in the presence of astrocytes. However, culture dimensionality did not affect the number of synapsin$^+$ puncta per neuron.

blot normalized based on total cell number).[88] At 7 DIV, although there was no statistical difference in MAP-2 expression between neurons in the two configurations, there was a 1.9-fold increase ($p < 0.05$) in the amount of tau-5 expressed by neurons cultured in 3-D compared to 2-D (Fig. 8). Also, there was not a statistically significant difference in the mean number of neurites departing the soma per neuron for neurons plated in 2-D compared to 3-D configurations (4.6 ± 1.5 and 4.1 ± 1.8 neurites/neuron, respectively).[41] These data suggest similar dendritic outgrowth in 2-D versus 3-D, but a 3-D configuration results in an increase in axonal length/caliber or an enhanced state of neuronal maturation compared to a 2-D configuration. Altogether, these results demonstrate differences in neuronal cytoarchitecture and neurite outgrowth in 3-D versus 2-D cultures. Processes within 2-D cultures were constrained to a nearly planar morphology, while those in 3-D constructs traversed much longer z distances, more representative of in vivo cytoarchitecture.

Suitable 3-D microenvironments must sufficiently support neurite outgrowth to enable the formation of functional interneuronal synapses. In principle, neurite outgrowth, and hence the extent of network connectivity, is more constrained on planar substrates compared to 3-D matrices. Thus, neurites may extend over several millimeters within 3-D bioactive scaffolds, which is appropriate to model properties of specific neuronal subpopulations where target innervation is distant from the soma. We found that neurons within 3-D constructs assumed complex 3-D morphologies with rich neurite arborization in all spatial dimensions, expressed mature cytoskeletal proteins, and demonstrated network connectivity.

7. Electrophysiology and Synaptic Activity

Within intact cortical tissues, neurons interact closely in all dimensions with glia and ECM; thus, electrophysiological properties of neurons are influenced by a number of factors, including cell-cell and cell-matrix interactions. Using techniques

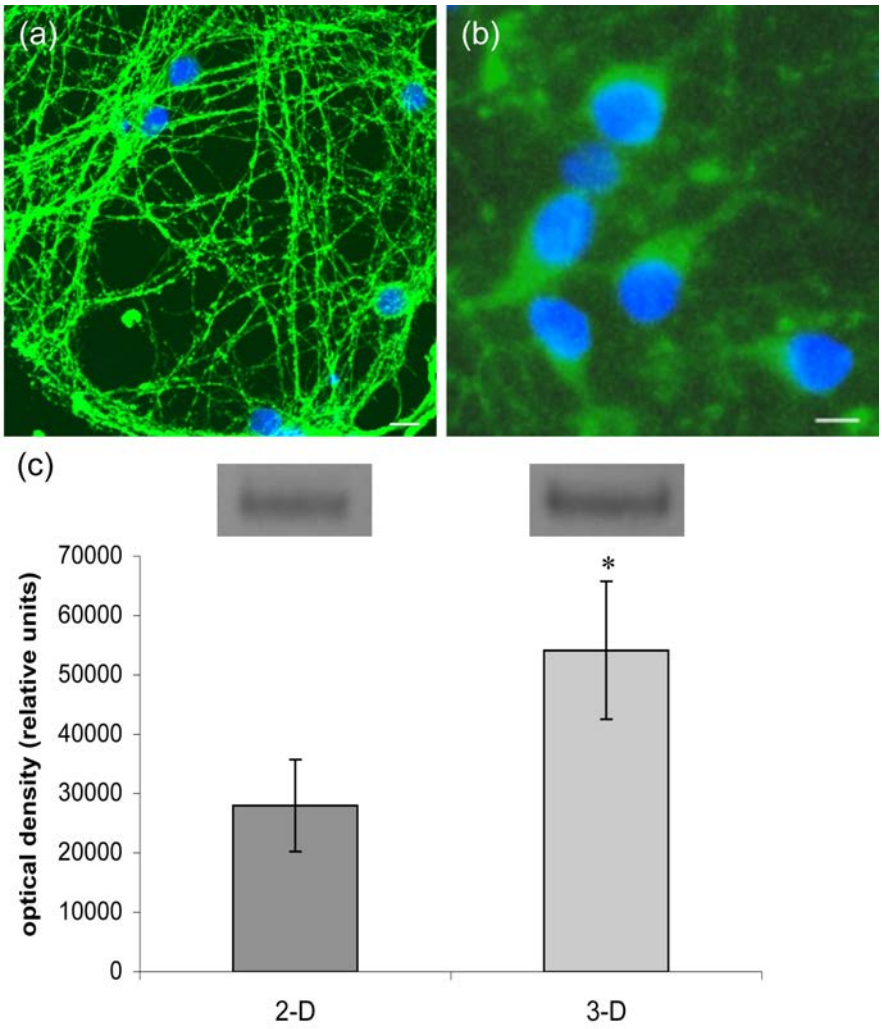

FIGURE 8. Tau expression in neurons cultured in 2-D and 3-D. Fluorescent confocal reconstructions of representative neuronal cultures in (a) 2-D or (b) 3-D immunolabeled for tau-5 (green) to denote neurons with Hoechst counterstaining (blue) to denote nuclei (scale bars = 10 μm; adapted with permission from Ref. 41, Mary Ann Liebert, Inc.). (c) Tau expression, a neuron-specific cytoskeletal protein, was semiquantitatively assessed in 2-D and 3-D via Western blot normalized based on cell number (mean ± standard deviation). There was 1.9-fold increase in tau-5 expression in 3-D compared to 2-D (*$p < 0.05$), suggesting enhanced axonal outgrowth in 3-D.

modified from traditional acute slice whole-cell patch clamp recordings, we assessed neuronal electrophysiology in 3-D versus 2-D co-cultures.[93] We found that neurons within these 3-D constructs exhibited excitable membrane properties including spontaneous and evoked action potentials, along with evidence of functional synaptic currents and network activity. Cells within the 3-D constructs were observed to have typical resting membrane potentials (–56.2 ± 11.5 mV) not statistically different from neurons in similar 2-D cultures (–52.8 ± 6.1 mV). Interestingly, action potentials of neurons within the 3-D constructs had statistically higher spike heights (70.3 ± 9.9 mV) than neurons

TABLE 2. Properties of 3-D Co-Cultures at the Time of Plating Compared to Developed Cultures, After a Dense Network of Processes Has Formed

3-D Co-Culture Remodeling		
	0 DIV	21 DIV
Viability	–	95.4 ± 1.9%
Cell Density (cells/mm^3)	2500	8903.8 ± 1173.4
Diameter (mm)	15.8	7.9 ± 0.5
Neuronal Presence (%)	50	9.8 ± 3.7

in 2-D sister cultures (47.3 ± 10.6 mV; $P < 0.01$). In response to a slow increase in extracellular K^+ concentration through a perfusion system, neurons within the 3-D cultures exhibited repeated spiking with an increased discharge frequency and an increasingly less negative membrane potential. Once the source was turned off, the high K^+ washed out of the culture and membrane potential and firing rate returned to original levels within 3 minutes. Taken together, these data suggest the neurons within the 3-D constructs were spontaneously active, had functional voltage-gated ion channels, and exhibited action potentials similar to those observed in acute slices.

In addition to excitable properties of individual cells, we observed evidence of functional synapses and network activity within the 3-D co-culture constructs (Fig. 9). Fluorescent immunocytochemistry of representative cultures at 21 days in vitro revealed abundant synapsin I expression along with the neuronal marker tau-5. Electrophysiological, spontaneous synaptic activity was evident above baseline noise, as was network-driven burst activity. In addition, through SEM, we observed processes with the characteristic appearance of neurites extending across somata and culminating in synaptic terminals. Together, these observations provide significant evidence of functional synapses and network properties within the 3-D co-culture constructs. These traits make the cultures useful with traditional electrophysiology protocols (i.e., patch clamp) as well as in studies that rely on interfacing neurons with multiple electrodes within a culture.

8. Extracellular Matrix and Remodeling

Neuronal–astrocytic co-cultures. In 3-D co-cultures, there was a significant amount of matrix contraction that was not observed in neuron-only cultures, demonstrating an enhanced matrix-remodeling capability of astrocytes.[94,95] During the first week in vitro, the 3-D co-culture constructs contracted and rose from the culture surface, resulting in a ~50% reduction in culture diameter (to 7–9 mm), a ~25% reduction in thickness (to 500–600 μm), with a concomitant 3.5-fold increase in 3-D cell density (to ~9000 cells/mm3).[93] This contraction was likely due to reactive forces on matrix fibrils resisting tension associated with process outgrowth, a phenomenon observed in similar 3-D collagen (e.g., fibroblasts[18]) and collagen-glycosaminoglycan matrices (e.g., peripheral nerve explants[96]). The 3-D constructs were similar in size, shape, opacity, and mechanical integrity to acute cortical slices from rodent brains, making them transferable and thus amenable to a range of investigation techniques used to investigate slice cultures. Notably, this predictable contraction over the first week in culture permits a concomitant change in construct size and surface area to volume ratio such that diffusion-based mass transport is suitable to support the increased cell density. Conversely, constructs of the same plating geometry consisting of these higher cell densities exhibit widespread cell death and matrix degradation due to limitations in diffusion-based mass transport.[68] Table 2 presents a subset of parameters comparing initial culture conditions (0 DIV) to a later time-point following the development of mature neural networks (21 DIV). Although culture viability remains high, there are

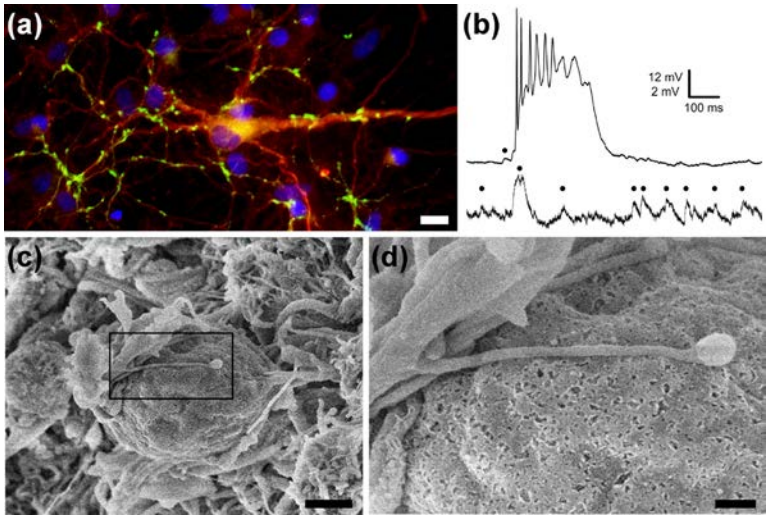

FIGURE 9. Functional synapses and network activity in 3-D neural constructs. (a) Immunofluorescent photomicrograph of a neural construct labeled to denote expression of the neuronal marker tau-5 (red), the synaptic marker synapsin (green puncta), and nuclear marker Hoechst 33258 (blue) (scale bar = 20 μm). (b) Spontaneous synaptic activity was evident above the baseline noise (black circles), as was network-driven burst activity. (c) Scanning electron micrograph of the surface of a 3-D neural construct, showing a matrix-embedded neuron with a rounded morphology and extensive process outgrowth along the neuron, on the matrix surface, and also plunging into the matrix interior (scale bar = 2 μm). (d) A morphologically identified synapse observed on the neuronal somata (scale bar = 500 nm) (boxed region in c). Reproduced with permission from Ref. 93, IOP Publishing.

stark increases in cell density, potentially a by-product of the modest levels of astrocyte proliferation and overall matrix contraction.

Although cell viability remains high, there was a sharp increase in cell density concurrent with a reduction in the neuronal presence. Moreover, dramatic alterations were observed in the culture diameter.

9. Summary—3-D Neural Tissue Surrogate Characterization

Here we have presented a brief review of our work to develop and characterize 3-D neural cell cultures within a bioactive scaffold consisting of extracellular matrix proteins and proteoglycans. These 3-D constructs consisted of either predominantly primary cortical neurons or separately isolated primary cortical neurons and cortical astrocytes mixed in controlled ratios. In both models, there was considerable process outgrowth resulting in the formation of 3-D, interconnected neural networks. In particular, neurons within these constructs exhibited extensive 3-D neurite outgrowth, expressed mature neuron-specific cytoskeletal proteins, and remained viable for several weeks. These neurons assumed complex 3-D morphologies with rich neurite arborization and clear indications of network connectivity, including synaptic junctures. Interestingly, there were no significant differences in culture viability, number of neurites per neuron, or neuron/astrocyte composition when comparing optimized 3-D conditions to similar 2-D cultures. However, there was a ~2-fold increase in axonal outgrowth in 3-D compared to 2-D. Also, there were stark morphological/cytostructural differences in neurons cultured in 2-D, which present a flat morphology, compared to cells distributed throughout a matrix, which present a bulbous morphology with cell-matrix contact in all spatial dimensions. Plating density was a critical parameter for neurons in 3-D, with an optimal cell viability obtained at 3750 cells/mm^3 (based on our starting surface area to volume ratio). Astrocytic

presence significantly improved long-term culture viability in both 2-D and 3-D, as cell viability in neuronal cultures was ~90% at 7 DIV, but <70% at 21 DIV, whereas viability in co-cultures was >95% up to 21 DIV. Also, astrocytic presence enhanced expression of neuronal functional markers via an increased rate of synapse formation and increased number of synapses per neuron. Moreover, we found that neurons in 3-D co-culture have normal resting membrane potentials (average RMP –56 mV), express voltage-sensitive ion channels (Na^+ and K^+ currents), displayed both spontaneous and evoked action potentials (average spike height 70 mV), and exhibited functional synapse formation and network properties. These tissue engineered 3-D cellular constructs provide an innovative platform for neurobiological and electrophysiological investigations, and serve as an important step in the development of more physiologically relevant neural tissue models.

IV. APPLICATIONS OF 3-D ENGINEERED TISSUE SURROGATES

A key future objective in this arena is to engineer increasingly biomimetic 3-D neural cellular constructs to study neurobiological phenomena in vitro in order to improve our understanding of neurological function and ultimately enhance repair/regeneration in the nervous system. The application of 3-D neural constructs for investigational platforms represents a fertile ground to increase our understanding of neuronal and glial cell biology and functional interactions that are currently most easily observed in vitro. Many facets of neurobiological and pathophysiological study may benefit from a 3-D model. However, we focus on neuronal survival, neurite outgrowth/pathfinding, mechanotransduction/trauma, test-beds for cell-cell interactions, and neural network formation.

IV.A. Neuronal Survival and Neurite Outgrowth

In work on 3-D culture development and tissue engineering applications, it is desirable to have a fundamental understanding of interactions between physical and biochemical matrix properties in the ability to support the viability and growth of neural cells. Overall, the matrix mechanical properties are important, and trade-offs exist between stiffness and porosity. However, porosity has absolute limits in relatively bioinert hydrogels (e.g., agarose) but is less of a restrictive factor in bioactive scaffolds (e.g., collagen), where localized enzymatic digestion can facilitate growth cone burrowing. Thus, the effects of matrix mechanical properties ("mechanotaxis"), soluble factors (chemotaxis; diffusible versus immobilized), and ligand density (haptotaxis) on neuronal survival and neurite outgrowth are of critical importance. Moreover, considerable effort has been exerted to understand and mathematically model the mechanics of neuritic growth cone extensions. However, virtually all efforts have utilized extension on planar surfaces, potentially underappreciating the tensile forces required to navigate through a 3-D environment. Previous work has shown that parameters of neurite outgrowth such as growth rate and neurite branching depend on matrix mechanical properties (in 3-D and 2-D)[1,97] as well as ligand presence/density.[49,50,56,57,98] Neuronal survival and neurite outgrowth in 3-D matrices may be influenced by intrinsic (e.g., neuronal maturation, receptor expression) as well as extrinsic (e.g., matrix mechanical properties, ligand concentration) signals. For example, examining the effects of agarose concentration on DRG neurite outgrowth revealed that matrix stiffness and pore size differentially influence the rate and degree of neurite extension, with maximal neurite outgrowth occurring in low concentration (<1.0%) gels.[1,50,55] However, low-concentration hydrogels have been shown to be unsuitable for the survival and neurite outgrowth of cortical neurons,[52,54] underscoring that different intrinsic mechanisms exist between different neuronal subtypes, and thus engineered systems must be optimized for a particular neuronal population. Studies of DRG neurite outgrowth in collagen matrices of varying concentrations (and hence stiffness) have found that neurite extension was maximized in lower (0.6 mg/mL) rather than higher (2 mg/mL) concentration gels.[56] Taken together, these studies suggest that matrix stiffness

may be a good predictor of neurite outgrowth in the absence of ligand presence.[1] However, the addition of significant ligand binding revealed a nonlinear relationship between ligand concentration and neurite outgrowth, where intermediate ligand densities resulted in maximal neurite extension with higher densities inhibiting outgrowth.[54,57] Thus, although optimized neurite outgrowth and survival for a particular neuronal subtype may require a specific range of ligand density, matrix complex modulus, and pore size, with optimized conditions they are likely multifactorial and interrelated.

To test these factors, we developed an additional 3-D cell culture system consisting of primary rat cortical neurons distributed throughout thick (>500 μm) gels consisting of type IV collagen (Col) conjugated to agarose.[54] Neuronal viability and neurite outgrowth were evaluated over a range of agarose (1.0–3.0%) and collagen (0–600 μg/mL) concentrations in order to optimize culture parameters in this 3-D engineered system. In the acute period following cell seeding, agarose percentage, and hence matrix complex modulus, dictated neuronal viability, with softer gels resulting in reduced survival. For instance, in unmodified agarose (no collagen), 1.5% gels supported viable cultures with significant neurite outgrowth, which was not found at lower (≤1.0%) concentrations. The collagen content of collagen-agarose matrices significantly influenced the matrix complex modulus, and in these matrices at 2 DIV for a fixed agarose percentage, neuronal survival did not vary based on collagen concentration, but neurite outgrowth was enhanced at the midrange collagen assayed (Fig. 10). This trend continued at 7 DIV, with neurite outgrowth demonstrating a nonlinear relationship with respect to collagen concentration, but survival not being influenced by collagen concentration. These results suggest that neuronal viability and neurite outgrowth in 3-D depend on interrelated biochemical and physical properties, with initial neuronal survival dependent on matrix complex modulus and neurite outgrowth varying with ligand density. Overall, optimized agarose percentages (1.25–1.50%) with a photoimmobilized ECM (collagen = 30–300 μg/mL) permitted development of thick, 3-D neuronal cultures with a network of interconnecting neurites through all spatial dimensions, with neurite outgrowth, but not survival, statistically equivalent to a positive control Matrigel matrix.

Thus, our results and others suggest a complex relationship between matrix mechanical properties and ligand density on cerebral cortical neuronal survival and neurite outgrowth through 3-D hydrogel matrices. Converging observations suggest that cortical neurons will survive in relatively soft gels provided the ligand density is sufficiently high (up to mg/mL concentrations of relevant matrix proteins). Stiffer gels will maximize survival and neurite outgrowth absent ligands or at low ligand densities (approximately 10–100 μg/mL, but will vary based on the specific matrix protein) until pore size becomes too constrictive to permit neurite extension. A key challenge is to develop comprehensive relationships between these extrinsic 3-D matrix factors (e.g., mechanical properties, charge, haptotaxic cues, chemotaxic gradients, etc.) based on intrinsic neuronal properties (individual neuronal subtype, maturity, and/or injury) due to the observation that different neuronal subtypes have different optimized gel parameters that will maximize survival and neurite outgrowth.

Our model system is advantageous in that it is relatively simple, and increasing levels of complexity (including other ECM ligands and additional cell types) can systematically be added to further manipulate culture properties. This may be a useful platform to elucidate the relative contributions of microenvironmental cues in axonal pathfinding. For instance, these matrices may be engineered to simultaneously vary and/or provide gradients in mechanical properties, internal ligand presence/density/stiffness, and potentially provide chemotaxic signals. Matrix mechanical, haptotaxic, and chemotaxic cues have been investigated in planar[97,99–105] as well as 3-D environments.[54,56,106–108] Additionally, geometric guidance cues such as surface (substrate) curvature are increasingly being recognized as affecting neurite outgrowth directionality, as shown on 2-D or quasi-planar surfaces.[109–112] Precise individual and synergistic effects of growth-

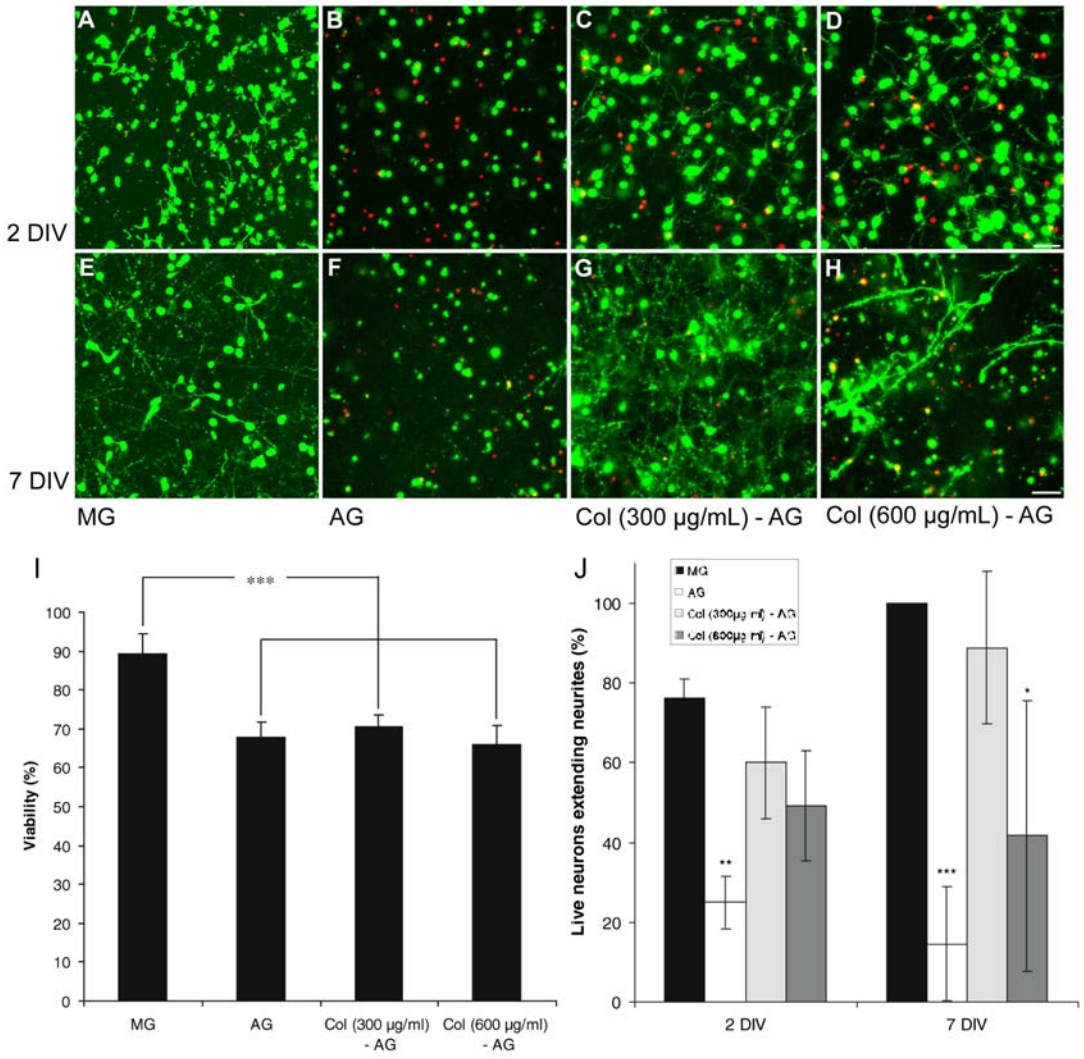

FIGURE 10. Neuronal viability in collagen-agarose. Survival was evaluated for neuronal cultures in (A, E) Matrigel ("MG"), (B, F) agarose ("AG"), and collagen ("Col") cross-linked to agarose, (C, G) Col(300 µg/mL)-AG, and (D, H) Col(600 µg/mL)-AG at 2 DIV (A–D) and 7 DIV (E–H). Live cells are stained green and the nuclei of dead cells stained red (scale bar = 50 µm). (I) At 7 DIV, there were no statistical differences in neuronal survival between AG, Col(300 µg/mL)-AG, and Col(600 µg/mL)-AG cultures; however, the AG and Col-AG groups had significantly lower neuronal viability than MG cultures ($p < 0.001$). (J) Neurite outgrowth in neuronal cultures in MG, AG, Col(300 µg/mL)-AG, and Col(600 µg/mL)-AG varied with Col content. At 2 DIV there were statistically significant decreases in the percentage of live neurons extending neurites in AG and compared to MG ($p < 0.05$); statistically there was no difference between MG, Col(300 µg/mL)-AG, and Col(600 µg/mL)-AG. At 7 DIV the percentage of neurons extending neurites was statistically less than MG for cultures in AG ($p < 0.001$) and Col(600 µg/mL)-AG ($p < 0.01$); statistically there was no difference in neurite outgrowth between MG and Col(300 µg/mL)-AG ($p > 0.05$). Data presented as mean ± standard deviation. Adapted with permission from Ref. 54, Springer.

promoting and/or inhibitory ligands have also been investigated in sophisticated planar micropatterned systems.[113,114] The ability to systematically vary all these parameters independently in a true 3-D environment has been elusive. Moving forward, the influence of multiple additional factors on neurite outgrowth may be isolated within engineered 3D matrices, such as multicellular composition,[93,115] microfluidically induced gradients,[68,69] and trauma-induced intrinsic and extrinsic alterations such as neuronal injury and reactive astrocytosis.[20,84,116] These neurobiological systems will further demonstrate the dependence of culture environment and architecture on neuronal regeneration and have implications for the development of tissue equivalents for both in vitro and in vivo applications.

IV.B. Mechanotransduction and Mechanotrauma

Perhaps one of the most critical needs for mechanically characterized 3-D neural constructs is the study of mechanotransduction and mechanotrauma. To study the effects of physical forces on cells under both physiologic and pathologic states requires the use of experimental systems that mimic the cytoarchitecture and microenvironment found in vivo. In the realm of neurotrauma, in vitro models have been developed to evaluate the injury response in brain tissue slices, thus preserving the 3-D configuration.[117–119] However, most in vitro models developed to study the response of neural cells to mechanical deformation utilize planar cell culture, and include mechanical stretch[120–122] and hydrodynamic shear stress[123,124] systems. Differences in cell morphology and the spatial distribution of cell-cell/cell-ECM interactions in planar versus 3-D cultures may be important, especially in models of traumatic cellular injury, as bulk deformation is translated to cells through physical coupling, which may be unrealistically represented in 2-D culture.

Receptor-mediated cell-ECM interactions are particularly crucial in many homeostatic cellular processes, including proliferation, migration, and differentiation,[125,126] and may be affected during various pathological states.[127,128] However, the role of cell-ECM adhesions in acute and chronic responses following traumatic neural loading is currently not known. In many non-neural systems, cells communicate with their immediate physical environment through such interactions and alter intracellular biochemical signaling in response to mechanical cues, a process referred to as mechanotransduction.[129–131] Transmembrane proteins such as integrins transduce mechanical stimuli from the ECM to the cytoskeleton and activate intracellular signaling molecules regulating the activity of enzymes (e.g., proteases, phosphatases, kinases) that can modify cytoskeletal organization and may result in changes in gene expression.[130,132–136] However, the mechanisms involved in transduction of nonphysiological mechanical deformation are not fully understood. Non-homeostatic (i.e., pathological) mechanotransduction may play a role in cellular dysfunction in response to mechanical forces through integrin-mediated signaling events, increasing intracellular Ca^{2+} concentration, causing membrane disruption via protein phosphorylation, and/or activating cytoskeletal-cleaving enzymes, all of which are components of the secondary sequelae associated with traumatic brain injury.[137] CNS neurons are normally protected from high rate strain and may not have inherent mechanisms to homeostatically transduce mechanical stimuli that other cells (e.g., vascular smooth muscle cells, osteoblasts) possess, and may therefore have a low threshold to mechanical forces, causing abnormal biochemical responses leading to cell dysfunction and possibly death.

Factors affecting the translation of forces from bulk tissue (macro) deformation to cellular (micro) deformation may differ markedly in 3-D versus 2-D configurations. In particular, cell morphology, matrix mechanical properties, and cell-matrix/cell-cell interactions will play differential roles in the transfer of strain to the cells, and/or differential susceptibility to localized stress formations. Neural cell morphology, more heterogeneous in 3-D, will affect the complexity of the strain field to which a cell is exposed,[41] often leading to simultaneous combinations of compressive, shear, and tensile forces. In a 2-D orientation, it is not possible to

simulate complex 3-D strain combinations; in fact, most utilize a single mode of deformation. Also, the bioactivity and physical properties of a matrix material may affect the transfer of bulk deformation to cellular deformation. Properties such as viscoelasticity (i.e., viscous and elastic moduli) and porosity may affect the fidelity of the deformation transfer to cells contained within a 3-D matrix, and such properties may be tailored to approximate that of brain tissue.[138–140] The quantity and spatial distribution of cell-matrix and cell-cell interactions may also affect the magnitude and locations of stresses developed on and within cells. Differences in the types, quantity, and distribution of cell-cell and cell-matrix interactions between cells in 2-D versus 3-D have previously been characterized for various cell types,[3,5] supporting the assertion that neurons in a 3-D bioactive matrix will contact matrix factors and experience cell-cell interactions (e.g., receptor-mediated, synaptic) in all spatial directions. The skewed, one-sided distribution and types of cell-cell/cell-matrix interactions found in 2-D cultures may affect pathological mechanotransduction mechanisms. Thus, 3-D models of neural mechanotransduction/trauma may better recapitulate the relevant cell biomechanics. Furthermore, models amenable to systematic control of cell culture parameters (e.g., cell composition, matrix constituents) provide a framework for the elucidation of the roles of specific factors in deformation transfer to cells and associated acute responses.

We previously demonstrated that the application of shear deformation to 3-D neuronal or neuronal-astrocytic co-cultures causes cell death and network degeneration (Fig. 11) in a strain-rate dependent manner.[41,84] However, the underlying mechanisms that translate bulk tissue deformation to cellular dysfunction remain poorly understood, but may be dictated by cell orientation, cell-cell connections, and cell-matrix interactions. Therefore, we investigated the roles of cell-matrix interactions in neuronal response to traumatic loading, using the collagen-agarose system previously described.[54] Here, a bioactive scaffold was engineered by controlling matrix mechanical properties and ligand density to find optimal ranges for neuronal survival and neurite outgrowth. This cell culture model was then used to study the response to high rate bulk shear deformation to the 3-D neuron-containing engineered matrices. This study revealed that the post-loading viability decreased with increasing collagen concentration and with increasing complex modulus, indicating that both factors may work in concert to translate bulk shear deformation to cells. These results suggest interrelated roles for matrix mechanical properties and receptor-mediated cell-matrix interactions in transduction of high-rate deformation influencing neuronal viability. The physical properties of the matrix material, including complex modulus and pore characteristics (e.g., the mean pore size for 1.5% AG is 150 nm—see Ref. 50) influence the degree to which cells were contained within the matrix material, and as such they may dictate whether forces deforming the gel (i.e., bulk shear deformation) were translated to and thus deform the cells within the gel. Additionally, receptor-mediated cell-matrix interactions may also influence the translation of forces from bulk matrix deformation to cellular deformation. Cell-ECM adhesions may increase the fidelity of deformation transfer from matrix to cellular deformation, and serve as areas where forces are acutely translated to create local stress concentrations. This stress distribution may influence the probability of structural failure, and resulting biophysical disruptions of the plasma membrane, for example, may alter ionic homeostasis,[123,141] initiating abnormal neuronal signaling and leading to cell death.[137] Additionally, cell surface receptor-mediated mechanotransduction may contribute to biochemical alterations following high-rate deformation, possibly initiating neuronal dysfunction and/or death. While mechanotransduction is generally considered in a homeostatic context (e.g., cells relying on mechanical stimulation to sense environmental cues), it may also contribute to neuronal dysfunction by altering the balance of phosphatase/kinase activity[142–144] and activating Ca^{2+}-dependent proteases that degrade cytoskeletal elements.[145–147] Future studies will aim to determine the contribution of specific receptor-mediated cell-ECM interactions to altered, and potentially detrimental, intracellular signaling events following

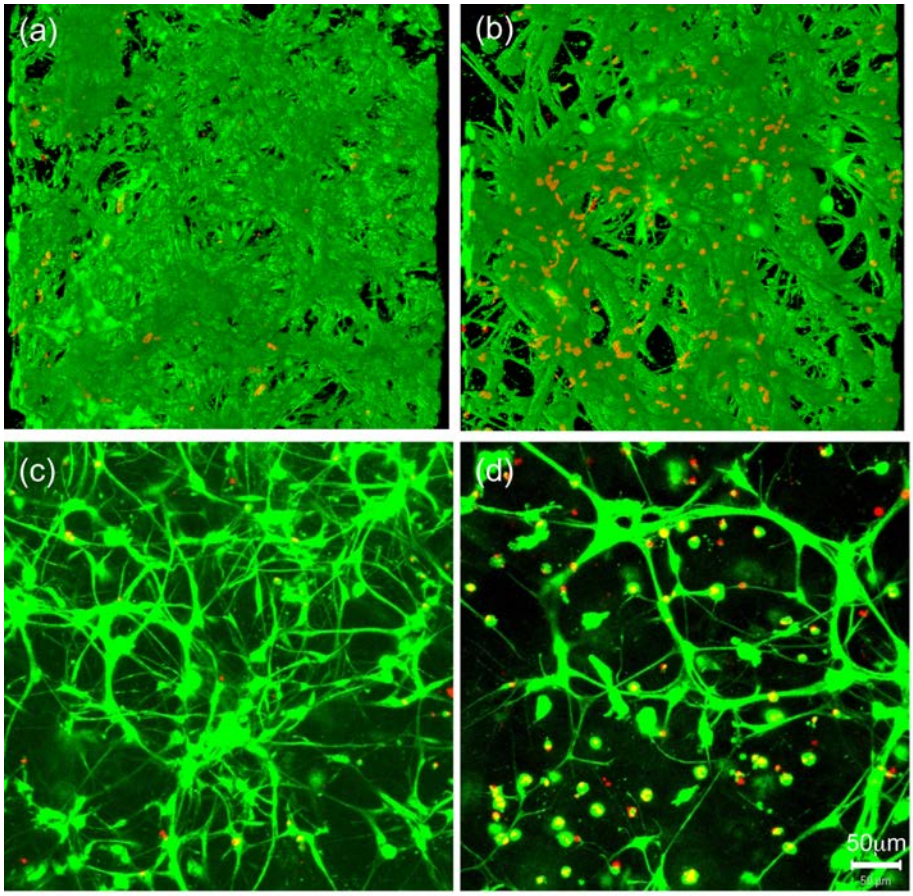

FIGURE 11. 3-D neural co-cultures as an in vitro model of traumatic brain injury. (a–b) Full thickness volumetric rendering and (c–d) optical slices showing live cells (green) and dead cells (red) in 3-D neuronal-astrocytic co-cultures. High strain rate shear deformation (0.50 strain, 30 s^{-1} strain rate) causes significant cell death and neural network degradation at 48 hours post-injury (b, d) compared to uninjured controls (a, c). (c, d) Adapted with permission from Ref. 84, Elsevier.

high-rate loading. This model system may be further exploited for the elucidation of mechanotransduction mechanisms and cellular pathology following mechanical insult; however, clearly such mechanisms and loading thresholds on a per-cell basis require the use of 3-D culture models capable of representing the relevant factors.

IV.C. Evaluating Transplant Strategies

Neural models designed to evaluate interfaces between various cell culture populations have generally been developed as simple bi-planar cultures. These include neural stem cells (NSCs) delivered above confluent astrocyte monolayer[148] or more complex preparations with cells placed above tissue slices.[149] Although this work has provided valuable information, fundamental differences between these test environments and cell-cell and cell-matrix interactions within a 3-D environment should be noted. Also, the local microenvironment may differ as secreted and/or soluble factors may be diluted into the culture medium, whereas in 3-D they may be maintained locally or become matrix bound. Here, key responses may require three-dimensionality, as they may be dependent on ECM changes or cytokine-ECM interactions.

Thus, we utilized mechanically injured 3-D neuronal-astrocytic co-cultures as an in vitro testbed to optimize NSC survival upon delivery within this microenvironment.[116] We found that co-delivery with a methylcellulose-laminin scaffold attenuated NSC cell death, suggesting a protective role of pro-survival ligands that may improve outcome. This in vitro model was a simplified yet versatile system to evaluate 3-D interactions between NSCs and an injured/reactive microenvironment.

IV.D. 3-D Cell Patterning and Microchannels

Neural systems anatomy in vivo is marked by the organization of neuronal nuclei connected by long axonal tracts. To mimic this complex 3-D cytoarchitecture, an important goal is to recapitulate aspects of neuroanatomy by creating segregated neuronal nuclei connected by discrete axonal tracts/fascicles within 3-D matrices. In 2-D preparations, discrete neuronal somata have been connected by axonal tracts due to micropatterning/stamping,[122,150,151] microfluidics,[152–154] and axonal "stretch-growth."[155–157] However, in the brain, these neuronal populations are arranged in 3-D space, and axons must traverse a 3-D environment to reach appropriate targets. As described previously, factors influencing axonal extension in 3-D matrices have been investigated in order to determine the relative contributions of chemotaxic gradients, matrix mechanical properties, and ligand type/density in a microenvironment more representative of the in vivo situation.[54,56,106,107] However, to advance these concepts, the differential and synergistic effects of matrix physical and biochemical factors providing 3-D directional specificity for axonal growth has yet to be fully described. The elucidation of these factors would enable cell patterning and neuronal network formation in 3-D, as well as significantly adding to the repertoire of neural tissue engineers in regenerative medicine applications. However, to date no clear strategy has emerged capable of driving directed axonal growth linking discrete populations of neurons in vivo or in a 3-D microenvironment in vitro. However, notable recent progress has been made in this endeavor, and directed 3-D neurite outgrowth in 3-D hydrogel matrices has been achieved utilizing engineered microchannels created via laser photoablation[158] or laser micropatterning of bioactive peptide sequences.[159] These approaches have created 3-D neuronal networks with structured connectivity useful for experiments dependent upon or benefited by a 3-D microenvironment.

IV.E. Considerations

Suitable 3-D microenvironments should allow sufficient diffusion of nutrients to keep the culture viable for long-term studies, while sufficiently supporting neurite outgrowth, network connectivity, and the formation of functional synapses. Although we attained excellent long-term survival using Matrigel matrix, alternative biomimetic scaffolds with specific ligands cross-linked to hydrogel backbones, such as methylcellulose[160] or agarose,[54] may allow more control over the presentation of extracellular matrix ligands while continuing to provide the support necessary for neuronal function and are worthy of consideration. These 3-D neural cultures that incorporate bioactive ECM constituents more representative of those found in the developing or mature brain (e.g., laminin, hyaluronan, or proteoglycans) may further increase the survival, function, and relevance of cultured neural cells. Thus, future efforts should be aimed at engineering scaffolds that are more defined and allow more complete control over the cellular context while continuing to provide the support necessary for neuronal function. Next-generation engineered 3-D neural cell culture systems inspired by the CNS, which include heterogeneous cell populations, high cell densities, and ECM constituents may provide a powerful experimental framework for the systematic elucidation of neurobiological function in a controlled setting.

V. BIOHYBRID NEURAL INTERFACE MICROSYSTEMS IN VITRO

V.A. Overview

Microsystems may be engineered to directly interface with living neural cells. These systems, typi-

cally with micron-scale features, must be neurocompatible and are specialized to noninvasively probe and/or control neural cell function. Such biohybridized neural interface microsystems may include microfluidic and microelectrical capabilities to enhance control and assessment of the local microenvironment. These engineered microsystems may also be coupled with real-time live-cell imaging (confocal/2-photon) to enable the quantitative and noninvasive probing of neural cells in a sustained fashion. Thus, these microsystems have tremendous potential as investigative platforms to study and manipulate neurobiological responses/functionality within 3-D microenvironments. Such incremental complexity and precise microenvironment control are extremely powerful. Importantly, information from in vivo findings will aid in determining critical parameters to accurately model a particular outcome in vitro. These microsystems may serve as high-throughput modeling platforms to study appropriate facets of basic neurobiology, neurotoxicity, or injury responses and thus may reduce the use of animals in research. In particular, advanced neurophysiological and pathophysiological studies may benefit from engineered microsystems with capabilities to control mass transport, drug delivery, neural network architecture, and neural interface modalities within 3-D microenvironments.

V.B. 3-D Microfluidic Platforms

Neural cells in vivo interact within a complex, multi-cellular environment with tightly coupled 3-D cell-cell/cell-matrix interactions. Thick 3-D neural cultures at cell densities approaching that of brain rapidly decay, however, presumably due to diffusion-limited interstitial mass transport. More biologically accurate 3-D cell-cell and cell-matrix interactions may be represented by thick, high cell density neural preparations, but these parameters are in direct competition with providing a sufficient supply of nutrients and oxygen to the cells. The most common systems to address this issue are spinner flasks and rotating vessels, with both systems enhancing diffusion via increased fluid flow around a 3-D construct rather than using perfusion for direct forced convection through a construct. In non-neural systems, perfusion bioreactors have been developed for a range of tissue engineering applications. In general, the goals of these systems are to enhance survival in thick and/or high cell density systems [e.g., thick osteoblast constructs;[161] high density (10^5 cells/mm^3) hepatoblastoma cell line;[162] hepatocytes[163]], as a mechanism to improve cell seeding into porous scaffolds (e.g., bone marrow stromal cells (BMSCs)[164]), to introduce hydrodynamic shear stress to enhance cell differentiation or improve productivity (e.g., BMSCs;[165,166] bone marrow–derived osteoblasts;[167,168] bovine chondrocytes,[169] or some combination of these general parameters (e.g., hepatocytes;[170] chondrocytes;[171] NIH/3T3 cells[172]).

The development of large volume (~200 μL), thick (>500 μm) 3-D cultures has necessitated the use of cell densities much lower than those found in the CNS as diffusion-limited mass transport leads to poor survival in high cell density 3-D cultures. To address this issue, we developed and validated a custom-designed bioreactor perfusion platform that utilizes forced intercellular convection to enhance mass transport (Fig. 12), thereby continuously delivering nutrients and withdrawing waste from thick constructs.[68,69] This enabled the creation of relatively thick, 3-D neural cultures at cell densities closer to those found in brain tissue.

We had initially demonstrated that in thick (>500 μm) 3-D neural cultures supported by passive diffusion, cell densities $\leq 5.0 \times 10^3$ cells/mm^3 were required for survival (see Section III). In 3-D neuronal and neuronal-astrocytic co-cultures with increased cell density ($\geq 10^4$ cells/mm^3), continuous media perfusion at 2.0–11.0 μL/min improved viability compared to nonperfused cultures ($p < 0.01$), which exhibited widespread cell death and matrix degradation (Fig. 13). Additionally, the highest perfusion rates evaluated (10.0–11.0 μL/min, corresponding to 25–27 medium exchanges per day) maintained overall culture viability at >90% in both neuron-only and neuronal-astrocytic co-cultures, demonstrating that convective enhancement of nutrient delivery substantially increased survival in high cell density 3-D neural cultures. This range of perfusion rates produced neural cultures with

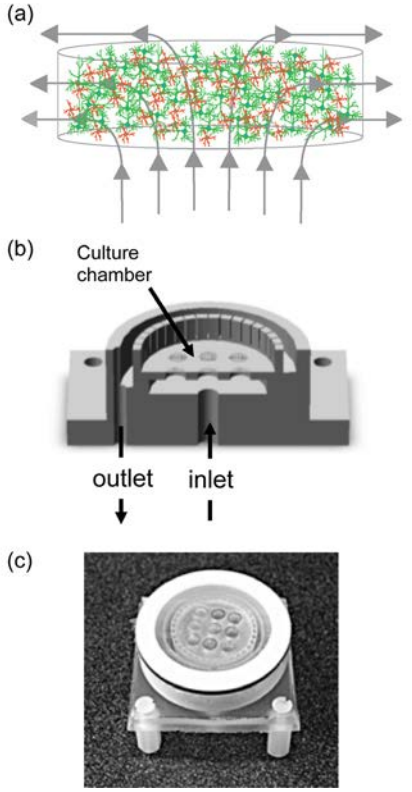

FIGURE 12. Micro-perfusion bioreactor. (a) Schematic of the global, induced flow through 3-D neural constructs. Cells in 3-D (neuronal cultures or neuronal-astrocytic co-cultures) were homogeneously dispersed throughout a 500- to 750-μm-thick matrix (not to scale). Media issuing closer to the perimeter of the engineered construct begin turning at lower elevations and vectoring towards the exit microchannels. Media issuing near the center penetrate deeper into the construct and start turning at higher elevations. (b) Cross-section view of the PDMS perfusion platform showing centrally formed, perfused culture chamber (580 μL total volume); inlet and outlet ports. (c) Perfusion platform with attached FEP membrane (permitting gas exchange without loss of water vapor) in a Teflon holder that encapsulates the device. Adapted with permission from Ref. 68, IOP Pubishing.

widespread process outgrowth and the formation of 3-D connectivity. Interestingly, in cultures perfused at 2.00–6.25 μL/min, survival was dependent on proximity to the perfusion source ($p < 0.05$). At perfusion rates of 10.0–11.0 μL/min, survival did not depend on the distance from the perfusion source, and resulted in a preservation of cell density $>10^4$ cells/mm^3 with >90% viability. These experimentally derived parameters for 3-D cell culture density still remain far from the densities of 10^5–10^6 cells/mm^3 reported in various cortical regions,[173,174] but with a heterogeneous neural cell population and a perfusion system, it may be possible to approach this cell density in vitro. Indeed, in a recent follow-up study, we were able to attain cell densities up to 5×10^4 cells/mm^3, approaching the cell density of regions of the cerebral cortex.

Obviously, complex relationships exist between overall culture dimensions (e.g., thickness and volume) and cell density for 3-D cultures reliant on passive diffusion for mass transport. For instance, neurons can be successfully cultured at $5–10 \times 10^5$ cells/mm^3 if they are essentially planar at 1–2 cell layers thick.[175] However, the thicker the construct, the lower the cell density will need to be, eventually attaining our 500-μm to 1.0-mm cultures, which need to be less than 5000 cells/mm^3 for survival. In addition, the surface area to volume ratio (incorporating thickness) will be a critical factor for a given 3-D culture configuration. More data are required to empirically derive the relationships between cell density, culture thickness (and/or cross-sectional area for diffusion), and survival for a given neural cell type. However, this information would be extremely useful to establish parameters governing the survival of neural tissue engineering constructs for survival in the body, based on relevant oxygen levels and vascular proximity.

Overall, we demonstrated that forced interstitial convection for nutrient delivery was sufficient to meet the cellular metabolic requirements throughout thick, high cell density 3-D neural constructs. This bioreactor perfusion system therefore may be a valuable platform for a range of neurobiological as well as neural tissue engineering applications. Not only does increased 3-D cell density with multicellular composition reconstitute more in vivo–like cell-cell interactions, but this custom neural bioreactor system may be utilized to apply

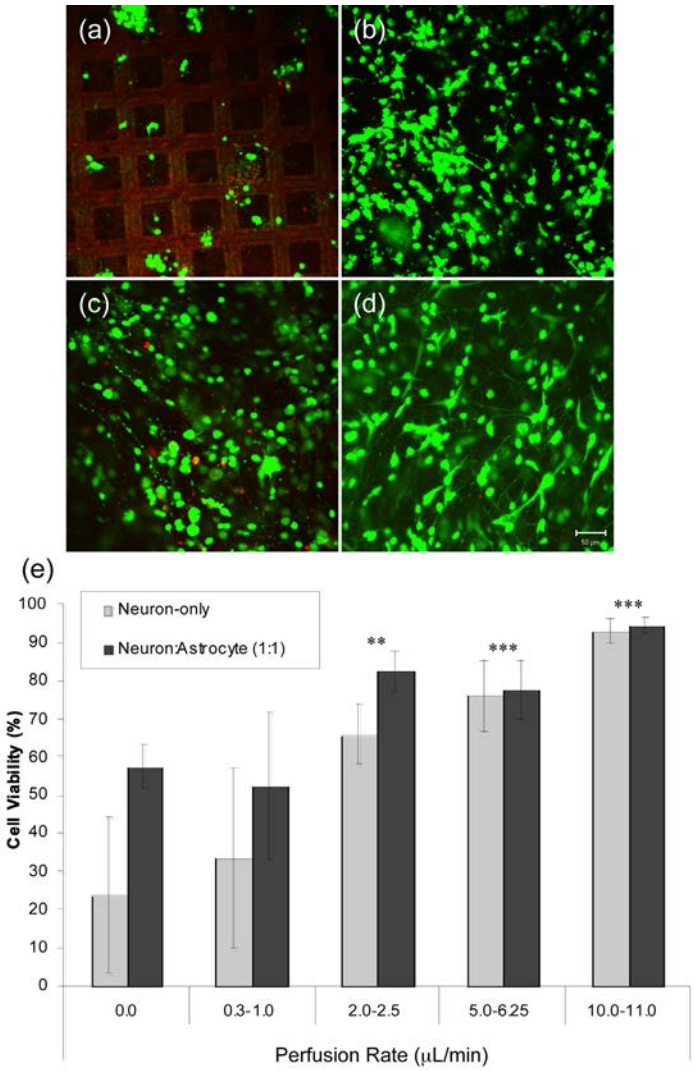

FIGURE 13. Neuronal and neuronal-astrocytic survival with continuous media perfusion. Representative confocal reconstructions of 3-D neural cultures stained to denote live cells (green) and the nuclei of dead cells (red). Increased cell density (10^4 cells/mm^3), thick (≥500 μm) 3-D neural cultures were plated with or without continuous media perfusion. Neuronal cultures in 3-D at 2 DIV either (a) nonperfused or (b) perfused at a rate of 10.0–11.0 mL/min. Neuronal-astrocytic (1:1 ratio) co-cultures in 3-D at 2 DIV either (c) nonperfused or (d) perfused at a rate of 10.0–11.0 mL/min. Passive diffusion was insufficient to support thick 3-D neural cultures at such high cell densities, as nonperfused cultures had widespread cell death and matrix degradation. Relatively high perfusion rates of 10.0–11.0 mL/min (corresponding to 25–27 medium exchanges per day) maintained culture viability at >90% in both neuron-only and neuronal-astrocytic co-cultures, demonstrating that convective enhancement of nutrient delivery can increase survival in high cell density neural cultures. (e) Specifically, there was significant improvement in survival for perfusion rates of 2.0–2.5 μL/min (**, $p < 0.01$), 5.0–6.25 μL/min (***, $p < 0.001$), and 10.0–11.0 μL/min (***, $p < 0.001$) compared to nonperfused cultures in both neuron-only cultures and neuronal-astrocytic co-cultures (data shown as mean ± standard deviation). Scale bar = 50 μm; reconstructions were from the middle of the cultures (50 μm thick z-stack). Reproduced with permission from Ref. 68, IOP Pubishing.

variable flow profiles for nutrient/drug delivery or controlled release of neuromodulatory compounds. Also, perfusate may be captured and analyzed to probe cellular responses.

V.C. Neural-electrode interface in vitro

Neurobiological-electrical interface platforms are being developed and implemented to extracellularly probe and modulate the function of neuronal populations for both in vitro and in vivo applications. In vitro, these specialized systems permit continuous monitoring of neuronal activity over weeks or months and are in widespread use in neural cell cultures and brain slices. Commercially available multielectrode array (MEA) systems are made for in vitro experimentation and consist of rigid electrode-bearing surfaces arranged in a planar or a dimple-shaped 3-D orientation.[176–179]

To integrate this technology with 3-D neural cell cultures, a critical step was to demonstrate neuronal and astrocytic survival in intimate proximity to 3-D electrically active substrates. Here, we cured the 3-D neuronal-astrocytic co-culture constructs described previously (see Section III) around high aspect ratio tower arrays fabricated using SU-8.[180–182] Our objective was to study 3-D culture interactions and distribution (cell and matrix) at the tower interface, which in this case was a surrogate for fully functional MEMS-based hardware such as 3-D electrode arrays (Fig. 14). To specifically assess neuronal interactions with the towers, we harvested neurons from GFP+ transgenic rodents and plated them to the active substrate. We then used live-cell imaging techniques to track neural growth with respect to the towers over weeks in culture. Interestingly, we found that the cellular distribution around the towers changed with time, with cells being more uniformly distributed at 1 DIV, and increasing their density in contact with the tower and bottom surfaces with progressively increasing DIV. These 3-D neural cellular constructs formed an interconnected 3-D network around and across the towers, and retained a high viability over three weeks. At this time-point, immunocytochemistry was performed to assess cell phenotype in contact with the towers, revealing a high density of both neurons and astrocytes in direct contact. This analysis revealed robust neural cell growth, network formation, and a high cell density of neurons with supporting astrocytes in intimate contact with high aspect tower arrays, which are therefore suitable for future studies assessing electrophysiological behavior of these 3-D neural networks over time in culture.

Collectively, these in vitro neural interface microsystems are designed to permit microelectrical interfaces (3-D cultures cured around microfiber/tower electrodes), microfluidic capabilities (forced interstitial convection to drive nutrients in, waste produces out, with perfusate collection for protein analyses), and real-time live-cell confocal and/or 2-photon microscopy, and are powerful platforms for neurophysiological and neural systems investigation. This technology permits noninvasive assessment of neural cells within 3-D tissue surrogates to capture network-level signal propagation or rapid neurobiological phenomena, thus addressing critical gaps in our understanding.

VI. SUMMARY AND CHALLENGES

Cellular models consisting of neurons and astrocytes distributed throughout a 3-D matrix more closely approximate the heterogeneous composition and architecture of native tissue and thus may exhibit more in vivo–like behavior than previous in vitro models consisting of 2-D monotypic populations. Three-dimensional culture models consisting of multiple neural cell types are capable of maintaining many positive aspects of in vitro modeling while approximating the cytoarchitecture of the brain to a greater extent than traditional planar cultures. These 3-D models potentially provide enhanced physiological relevance and will be valuable in the mechanistic study of cell growth, interactions, and the responses to chemical or mechanical perturbations. Although typical in vitro cell preparations (e.g., planar cultures) have yielded important neurophysiological information, the conditions used in such studies vary significantly from those found in the highly complex system of the whole organism, and therefore may influence indi-

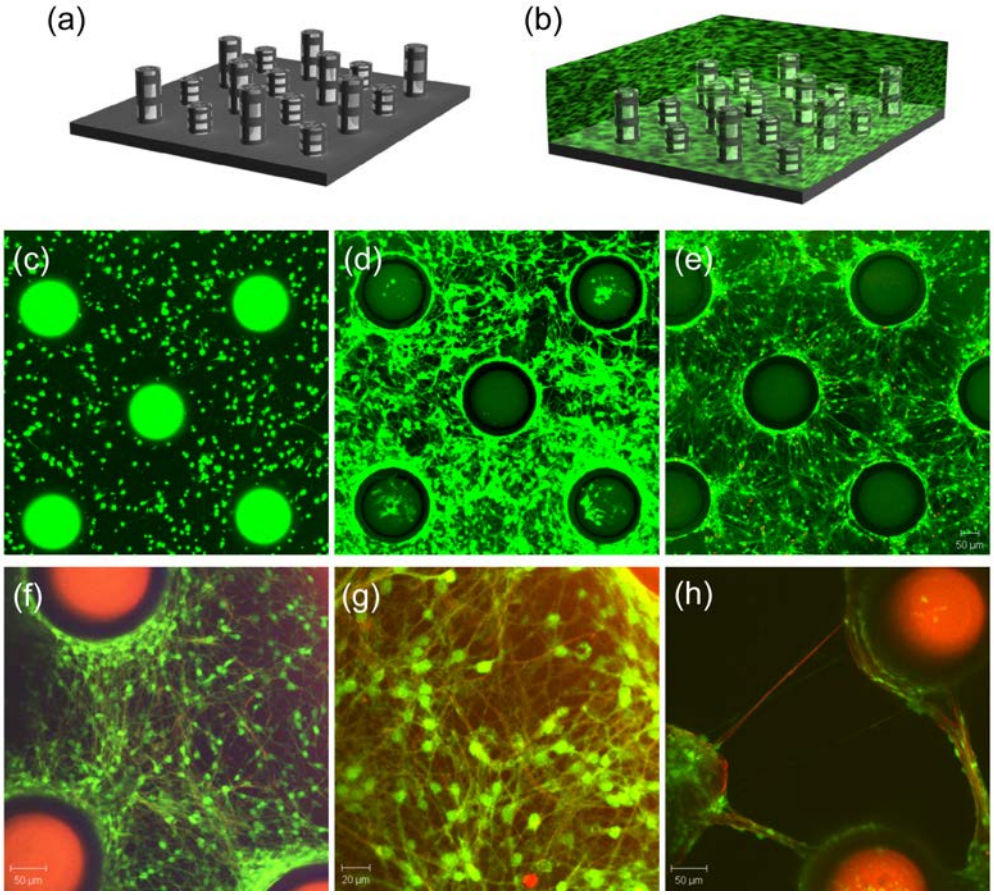

FIGURE 14. Biohybridized neural interfaces. (a–b) 3-D neural cell cultures formed around 3-D microtowers (250 μm tall, 200 μm diameter) as enabling methodology to create biohybridized neural interface microsystems. (c–d) Live neurons (green fluorescent protein; GFP^+) forming 3-D networks around microtowers in culture. (c) At 1 DIV, neurons are homogenously distributed throughout the matrix (>500 μm thick). (d) However, by 13 DIV, there was increased cell density around the towers and the culture thickness had decreased to approximately 300 μm. (e) Live (green) and dead (red) neural cells at 23 DIV, demonstrating viable neural networks adhering to the microtowers. (f–g) Immunocytochemistry labeling astrocytes ($GFAP^+$, red) with GFP^+ neurons. Neurons and astrocytes were in intimate contact with each other and the microtowers in the bottom 300 μm of the cultures. (h) In some cases, neural cells and processes had coalesced into tracts spanning neural populations on microtowers (top 200 μm shown here). With microelectrical capability, these microsystems may serve as powerful in vitro platforms to continuously monitor the electrical activity and dynamics of "small world" neuronal networks in 3-D matrices.

vidual cellular functions. The differences between 3-D, ECM-based cultures and monolayer cultures should not be ignored, as they may impact cellular responses to manipulation significantly. The 3-D neural constructs presented here represent an important step towards more physiologically relevant neural tissue models. This innovative investigational platform has many distinct advantages for neurobiological and electrophysiological applications. These models should have broad applicability in neurobiological research, serving as an engineered tissue surrogate with millimeter-scale dimensions,

multicellular composition, real-time live cell imaging, and microelectrical/microfluidic capabilities.

One of the major challenges in neuroscience is to translate basic findings in neurobiology to real clinical solutions. Robust 3-D culture models that can be reproduced may help to accelerate neurobiological research as well as neuropharmaceutical development. While culture models are not a replacement for animal or human research, they are ingrained in the beginnings of basic neuroscience research and yet in many respects are not ideal models. For example, we often model adult disease processes using fetal cells; we model complex neurobiological processes using neuron-only cultures; and we apply drugs without considering systemic effects. While much is to be learned using established fetal cultures (and cell-lines), there is a tremendous need to increase complexity in a rational, systematic way, without compromising feasibility and reproducibility. The challenges are to (a) choose the most appropriate building blocks to create complex 3-D neural cultures and (b) to define and measure physiological homeostasis and functional fluctuations. These challenges stem from specific needs: (a) current in vitro systems are limited and (b) many processes and mechanisms cannot be isolated in vivo, given the complexity. Realistic neural tissue equivalents are expected to be invaluable tools for neuroscience and neurotherapeutics. For example, successful 3-D neural platforms can be adopted for assessing disease mechanisms via microsampling and microbased assays, optimizing drug delivery, and preclinical testing for mono- and combination therapies in a semi-high-throughput fashion.

In vitro models utilizing neural cells in 3-D cultures may be a more accurate representation of the in vivo environment than 2-D models while maintaining many of the benefits. We developed a novel 3-D neuronal-astrocytic co-cultures system, establishing parameters resulting in the growth and vitality of mature 3-D networks, potentially providing enhanced physiological relevance and providing a robust platform for the mechanistic study of neurobiological phenomena. The engineering of novel culture models is critical for in vitro advances in neuroscience and neural engineering, providing custom and modular design capabilities. Three-dimensional models may offer enhanced physiological and pathological relevance due to culture architecture and heterogeneous properties. These 3-D neural constructs may serve as a useful in vitro test-bed, and engineered microsystems like these improve our ability to noninvasively probe neural cells while providing unprecedented control of the local microenvironment.

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