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Three-dimensional neural constructs: a novel platform for neurophysiological investigation

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Abstract

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 a new generation of culture systems that more closely model the complexity of nervous tissue. To this end, we engineered novel electrophysiologically active 3D neural constructs composed of neurons and astrocytes within a bioactive extracellular matrix-based scaffold. Neurons within these constructs exhibited extensive 3D neurite outgrowth, expressed mature neuron-specific cytoskeletal proteins, and remained viable for several weeks. Moreover, neurons assumed complex 3D morphologies with rich neurite arborization and clear indications of network connectivity, including synaptic junctures. Furthermore, we modified whole-cell patch clamp techniques to permit electrophysiological probing of neurons deep within the 3D constructs, revealing that these neurons displayed both spontaneous and evoked electrophysiological action potentials and exhibited functional synapse formation and network properties. This is the first report of individual patch clamp recordings of neurons deep within 3D scaffolds. These tissue engineered cellular constructs provide an innovative platform for neurobiological and electrophysiological investigations, serving as an important step towards the development of more physiologically relevant neural tissue models.

(Some figures in this article are in colour only in the electronic version)

Introduction

Cells are influenced by complex environmental stimuli, central to which is the local microenvironment, as extracellular context profoundly affects cell behavior. For example, the matrix surrounding cells has been shown to have widespread effects on cellular function for a variety of cell types (Cukierman *et al* 2001, Schindler *et al* 2006) including neural cells (Kiryushko *et al* 2004, Loers and Schachner 2007, Venstrom and Reichardt 1993). Cell–cell and cell–matrix interactions are inherently different in the intact organism than in monolayer cell culture; therefore the projection of cell culture-based results to *in vivo* systems is not without numerous caveats. Cells cultured in a 3D environment have been shown to better represent *in vivo* cellular behavior than cells cultured in a monolayer in several cell types (Schindler *et al* 2006, Smalley *et al* 2006). For example, planar culture of chondrocytes results in de-differentiation, whereas 3D chondrocyte cultures maintain the expression of chondrogenic markers aggrecan and collagen type II (Benya and Shaffer 1982, Buschmann *et al* 1992). Similarly, cardiomyocytes in 3D culture maintain more *in vivo*-like molecular and

electrophysiological characteristics than those cultured in monolayers (Bursac *et al* 2003). A growing body of evidence suggests that cells grown in planar cultures do not have the same morphology (Cukierman *et al* 2001), proliferation rates (Hindie *et al* 2006, Willerth *et al* 2006), migration (Friedl *et al* 1998), gene expression (Birgersdotter *et al* 2005, Liu *et al* 2006), differentiation (Chun *et al* 2006, Willerth *et al* 2006), cellular signaling (Pedersen and Swartz 2005), or pathological susceptibility (Behravesh *et al* 2005, Hindie *et al* 2006, Smalley *et al* 2006) as in 3D culture or *in vivo*. These findings raise questions about the appropriateness of employing monolayer culture models to study tissue- or systems-level phenomena.

Some potential limitations of 3D cultures should be noted, however. For example, there are diffusion limitations that increase with density and thickness. In addition, 3D cultures typically are randomly oriented; therefore, tissue architecture is difficult to reproduce. And, not in the least, are the challenges in culturing required to maintain consistent thickness and extracellular support. Nonetheless, the potential advantages warrant the use of 3D cultures that exploit cell–cell and cell–matrix supports rather than cell–glass/plastic support, as cells respond differently depending on the extracellular mechanical properties (Discher *et al* 2005).

In the context of recording electrophysiological activity, alterations in ion channel expression, membrane permeability and the cellular microenvironment (including ion concentrations and pH) can affect the static and dynamic electrophysiological properties of cells. For example, in 3D culture, Na+/H+ exchangers have been shown to have polarized expression to the apical membrane, which is difficult to maintain in monolayer cultures (Castillon *et al* 2002). In addition, neurons in a 2D culture environment have been reported to have exaggerated Ca^{2+} dynamics in comparison to 3D (Desai *et al* 2006, Mao and Kisaalita 2004, Xu *et al* 2006). Therefore, the establishment of a 3D culture system for electrophysiological studies may provide insight into cellular behaviors that more closely mimic those seen in the intact CNS.

Moreover, most studies evaluating *in vitro* survival, neurite outgrowth, network formation, synaptogenesis and functionality have been done in planar culture absent of a 3D protein matrix; however, the presence of 3D growth/interactions and bioadhesive ligands is crucial. The application of 3D neural constructs to study such basic neurobiological phenomena represents a fertile ground to increase our understanding of neuronal and astrocytic cell biology and functional interactions that are currently most easily observed *in vitro*. Accordingly, here we describe a novel 3D construct of neurons and astrocytes within a bioactive scaffold consisting of extracellular matrix-derived proteins and proteoglycans. These culture conditions supported considerable process outgrowth, complex 3D morphologies and neural cells remained viable over several weeks in culture. Neurons within these cultures demonstrated spontaneous electrophysiological activity and exhibit features of functional synapse formation and network properties. We propose that these constructs may be a better platform for a variety of neurophysiological applications than traditional 2D monolayer cultures, as they more closely mimic *in vivo* cytoarchitecture and function. Tissue-engineered biomimetic 3D neural constructs may thus serve as useful investigative platforms to study neurobiological phenomena by more accurately representing *in vivo* interactions with the control and precision realized by *in vitro* systems.

Materials and methods

3D neuron-astrocyte co-culture constructs

Neurons were isolated from embryonic day 17–18 Sasco Sprague-Dawley rats (Charles River Labs, Wilmington, MA) or transgenic mice bred in-house expressing green fluorescent protein (GFP; strain C57BL/6-Tg(cAG-EGFP)C14–701-FM1310sb). Cerebral cortices were digested in Hank's buffered salt solution (HBSS) with trypsin (0.25% + 1 mM EDTA) at 37 °C for 10 min, followed by mechanical trituration with DNase I (0.15 mg ml^{-1}). The cells were centrifuged at 1000 rpm for 3 min and resuspended in co-culture medium (Neurobasal + 2% B-27 + 1% G-5 + 500 μM L-glutamine). Astrocytes were separately harvested from postnatal day 1 Sprague-Dawley pups. Isolated cortices were minced, digested in trypsin (0.25% + 1 mM EDTA) for 3–5 min at 37 °C, followed by DNase I (0.15 mg ml^{-1}) treatment and gentle mechanical trituration. Cells were plated in DMEM/F12 with 10% fetal bovine serum. Mechanical agitation was used to detach less adherent cell types at 24 and 72 h and the primary astrocyte culture (>95% type I astrocytes) was passaged upon reaching ~90% confluency for 4 weeks. Astrocytes were used between passages 4 and 12 for plating in 3D culture.

Cultures were plated in custom-made cell culture chambers consisting of a glass cover slip below a silicone-based elastomer mold (Sylgard 184 and 186, Dow Corning; Midland, MI; cross-sectional area = 2 cm^2) (figure 1). Prior to plating, the chambers were pre-treated with 0.05 mg ml^{-1} poly-L-lysine (PLL, Sigma) followed by Matrigel™ (0.5 ml/well at 0.5 mg ml^{-1}, Becton Dickinson Biosciences; Bedford, MA) in a Neurobasal™ medium (each treatment was >4 h). 3D neuronal-astrocytic co-cultures were plated at a 1:1 initial neuron:astrocyte ratio at a density of 2500 cells mm^{-3} in the Matrigel™ matrix (500–800 μm thick; final concentration 7.5 mg ml^{-1}). The Matrigel™ matrix is biologically active for neural cells through matrix components (e.g., laminin, collagen IV, entactin, heparan sulfate proteoglycan) (Kleinman *et al* 1986) and cytokine-related interactions (Vukicevic *et al* 1992a). Following matrix gelation at 37 °C, a co-culture medium was added. The co-cultures were maintained at 37 °C and 5% CO_2–95% humidified air, and the medium was replaced at 24 h and every 2 days thereafter. Experiments were initiated at 21 days *in vitro* to permit neurite outgrowth, network formation and neuronal maturation. All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the Georgia Institute of Technology. All cell reagents were obtained from Invitrogen (Carlsbad, CA) or Sigma (St. Louis, MO) unless otherwise noted.

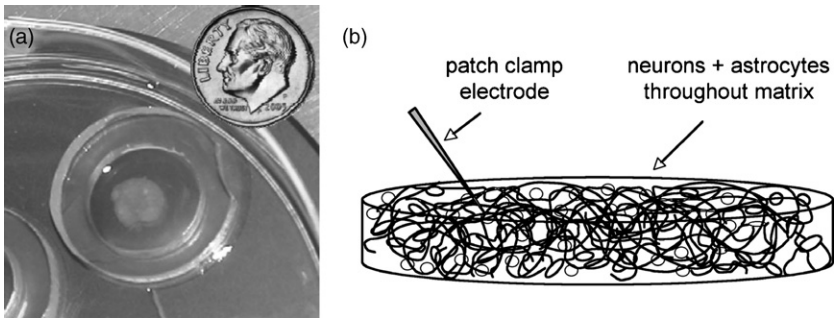

Figure 1. Gross morphology and schematic of 3D neural constructs. Within a few days of plating, 3D constructs contracted and lifted off the culture surface taking on a shape similar to an acute brain slice (a). Schematic representation of 3D neural constructs (b) (not to scale). Neurons and astrocytes were separately isolated, mixed in a controlled ratio, and then homogeneously dispersed throughout the full thickness of a 500–800 μm bioactive matrix. Cell survival and phenotype were evaluated via confocal microscopy, and neuronal electrophysiological properties were assessed using modified whole-cell patch clamp techniques, where neurons deep within the constructs were probed using glass electrodes.

Cell viability

The cell cultures were incubated with 4 μM ethidium homodimer-1 (EthD-1) and 2 μM calcein AM (both from Molecular Probes, Eugene, OR) at 37 °C for 30 min and then were rinsed with 0.1 M Dulbecco's phosphate-buffered saline (Invitrogen). The percentage of viable cells was calculated by counting the number of live cells (fluorescing green by AM-cleavage) and the number of cells with compromised membranes (nuclei fluorescing red by EthD-1) ($n = 18$).

Immunocytochemistry

The cultures were fixed in 3.7% formaldehyde (Fisher, Fairlawn, NJ) for 30 min, rinsed in PBS and permeabilized using 0.3% Triton X100 (Kodak, Rochester, NY) + 4% goat serum (Invitrogen) for 20 min. Primary antibodies were added (in PBS + 4% serum) at 18–24 °C for 4 h to the following intracellular proteins: (1) tau-5 (MS247P, 1:200, NeoMarkers, Fremont, CA), a microtubule-associated protein expressed predominantly in neurons; (2) MAP-2 (rabbit polyclonal antibody; AB5622; 1:200; Chemicon); (3) synapsin I (A6442, 1:200, Molecular Probes), a synaptic vesicle protein localized in presynaptic specializations (Fletcher *et al* 1991) ($n \geqslant 5$ for each). Secondary fluorophore-conjugated antibodies (FITC or TRITC-conjugated IgG (Jackson Immuno Research, West Grove, PA) or Alexa 488 or 546-conjugated IgG (Molecular Probes) were added (in PBS + 4% serum) at 18–24 °C for 2 h. Counterstaining was performed using Hoechst 33258 (1:1000, Molecular Probes).

Fluorescence microscopy and image processing

The cells were viewed using confocal laser-scanning microscopy (LSM 510, Zeiss, Oberkochen, Germany) using 10x, 20x or 63x objectives. Confocal z-stacks were acquired across the full thickness of the constructs (up to 800 μm thick). The images were analyzed using LSM Image Browser (Zeiss). Post-processing was performed in ImageJ (U. S. National Institutes of Health, Bethesda, MA) with built-in custom libraries and with the VolumeJ plugin. Briefly, z-stacks ($512 \times 512 \times 18$; an optical slice thickness of 4.3 μm) were converted from RGB to 8-bit grayscale. An intensity threshold was taken for each stack (to accentuate thin dendrites) using the middle-z-axis plane as a reference point. The histograms were summed along the z-axis and normalized (full-range grayscale). The stacks were linearly interpolated in the z-direction. The images were volume rendered with a ray-casting algorithm using a Levoy depth-cueing classifier (makes voxels more opaque the closer their intensity is to a user-defined threshold and the higher their surface gradient with voxel brightness decreasing with distance). Color mapping was determined from a grayscale intensity-ramped index volume created with the same interpolation parameters used on the stacks. The volume-rendered images were despeckled using a median filter with 2-pixel radius. The histograms for volume-rendered images were then normalized.

Scanning electron microscopy

Cell morphology and the spatial distribution of cell–cell and cell–matrix interactions were qualitatively assessed through low voltage, high resolution scanning electron microscopy (LVHR-SEM). The co-cultures were fixed using 2.5% EM-grade glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.4) at 4 °C for 24 h, washed in di-H_2O, and then post-fixed with 1% OsO_4 in a 0.1 M cacodylate buffer (pH 7.4) for 1 h followed by several rinses. The co-cultures were dehydrated using ethanol (30, 50, 70, 3 × 100%, 15 min each), wrapped in a parafilm, and loaded into a Polaron E-3000 critical point dryer (CPD) and exchange with CO_2 was made while monitoring the exhaust gas rate. The CPD was thermally regulated to the critical temperature and pressure and, following phase transition, the CO_2 gas was released at a constant flow rate. Prior to LVHR-SEM, all specimens were sputter coated with 3 nm of chromium.

Whole-cell patch clamp

For all patch clamp recordings, borosilicate glass electrodes with filaments (external diameter 1.5 mm; inner diameter 0.75 mm; Sutter Instruments, Novato, CA) were pulled on a laser puller (P-2000; Sutter Instruments) with a 7–10 MΩ resistance. The electrodes were back-filled with

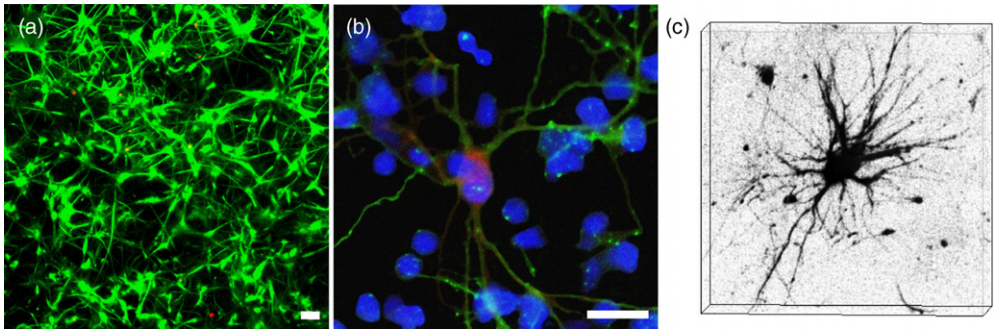

Figure 2. Neural cell survival and morphology within 3D neural constructs. Fluorescent confocal reconstructions of 3D neural constructs stained to discriminate live cells (green) and the nuclei of dead cells (red) (a). Neural constructs presented >95% viability over at least 21 days *in vitro* (a). Neurons within these constructs assume 3D morphologies with neurite extension across all three spatial dimensions (b), (c). These neurons expressed the neuronal-specific proteins MAP-2 (green) and tau-5 (red), the former being a mature isoform of microtubule-associated protein, with nuclear marker Hoechst 33258 (blue) (b). Neurons isolated from transgenic animals expressing fluorescent proteins served to label live neurons and permitted identification of 3D neurite outgrowth (region dimensions = 264.5 μm × 264.5 μm × 99.0 μm) (c). Scale bars = 20 μm.

internal solution (140 mM K-gluconate, 10 mM HEPES, 5 mM KCl, 0.1 mM K-EGTA, pH 7.3; 315–325 mOsm). The external solution used was artificial cerebral spinal fluid (aCSF) containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 26.2 $NaHCO_3$, 11 mM glucose (pH 7.4 by bubbling with CO_2/O_2 (95/5) mixture; 320 mOsm) made daily and gravity perfused. A separate perfusion tube was used to add the high K^+ solution (20 nM in external solution) which flowed into the bath solution at 1–2 ml min^{-1}. The normal perfusion system was still flowing during high K^+ influx and efflux. An AxoPatch 1D (Axon Instruments) patch-clamp amplifier coupled with a Digidata acquisition PCI card, and WinWCP software (University of Strathclyde, Glasgow, U. K.; http://www.bio-logic.info/electrophysiology/winwcpandwinedr.html) was used for data acquisition, recording and stimulating. Recordings were displayed on Tektronix Oscilloscopes (TDS 3014B and 7603) and analyzed offline in WinWCP and Igor Pro (WaveMetrics).

Data analysis

For viability and immunocytochemical assays, three to five randomly selected regions per culture were counted and averaged. The standard deviations were calculated when appropriate. The statistical values were calculated by paired, 2-tailed *t*-tests with a p-value < 0.05 considered significant.

Results

Neurobiological characteristics of 3D neural constructs

We developed a novel neural 3D co-culture construct to be used as a physiologically relevant *in vitro* model of neural tissue. These constructs consist of cerebral cortical neurons and astrocytes homogeneously distributed across the thickness of a bioactive scaffold and maintained using a defined serum-free neurobasal-based medium (see figure 1(b) for a schematic). During the first week *in vitro,* the 3D neural 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 3D cell density (to ∼9000 cells mm^{-3}). This contraction was likely due to reactive forces on matrix fibrils resisting tension associated with process outgrowth, a phenomenon observed in a similar 3D collagen, e.g. fibroblasts (Grinnell 2000), and collagen-glycosaminoglycan matrices, e.g., peripheral nerve explants (Spilker *et al* 2001). Following this contraction, the constructs increased matrix density and opacity (figure 1(a)), and, as a result, the cells could be visualized to a depth of approximately 100 μm with differential interference contrast (DIC) and near-infrared optics. Notably, this controlled contraction over the first week in culture results in 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. Interestingly, high-density constructs plated with a larger volume exhibit widespread cell death and matrix degradation due to limitations in diffusion-based mass transport (Cullen *et al* 2007c).

Neurons and astrocytes were harvested separately and mixed in a controlled ratio. This created the ability to mix neurons expressing GFP with wild-type astrocytes, which greatly enhanced the ability to distinguish neurons for electrophysiological assessment (figure 1(b)). In the present study, the constructs were generated at a 1:1 neuron-to-astrocyte ratio and were found to have an overall culture viability of >95% (95.4 ± 1.87%) at 21 days *in vitro* (figure 2(a)). Confocal microscopy across the full thickness of the constructs revealed that this high viability was consistent across the heights of the cultures. At 21 days *in vitro*, neurons constituted 9.75 ± 3.65%, indicating that astrocyte proliferation occurred over the culture period. It is possible that there was also neuronal cell death, however, given the high viability at 21 days *in vitro*, we attribute the shift in cell type ratio primarily to astrocyte proliferation. Using these methods, we have maintained viable 3D neural constructs for over 60 days *in vitro*.

Extensive process outgrowth extending from cells was visible under bright field microscopy by 7 days and became more pronounced as the culture matured.

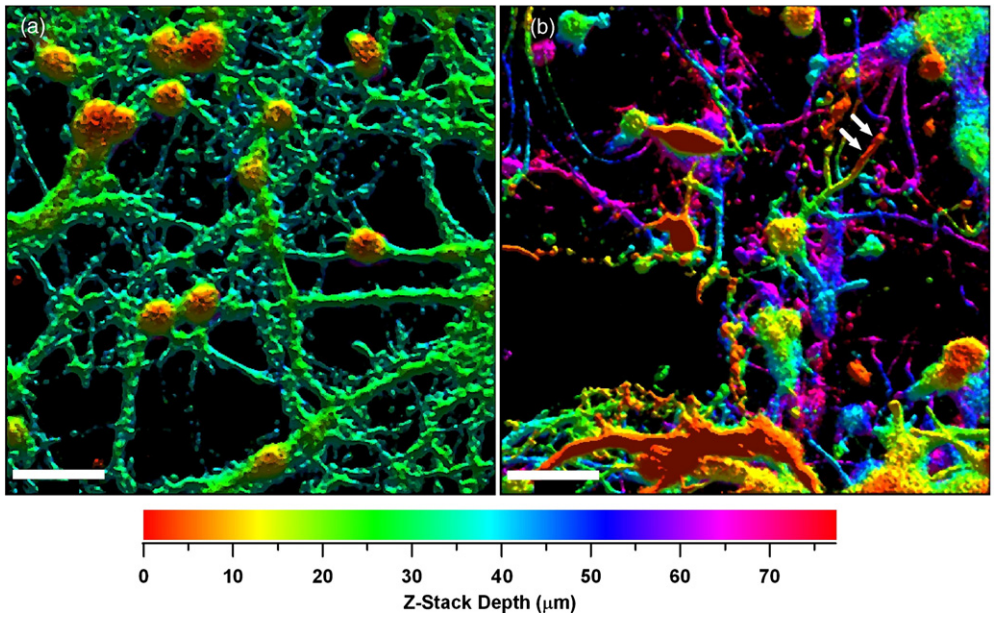

Figure 3. Comparison of 2D (a) and 3D (b) cultures. Volume-rendered z-stacks are color coded for depth from the objective lens. While most of the 2D culture neurites were restricted to a planar region, in the 3D cultures neurites traverse much larger z-distances (see arrows). Scale bars = 50 μm.

Immunocytochemistry revealed that neurons within the constructs expressed the neuronal-specific cytoskeletal proteins MAP-2 and tau-5 (mature isoform) (figure 2(b)). Morphologically, neurons within the constructs exhibited spherical somata with processes extending throughout the 3D space (figure 2(c)). Processes within similar 2D cultures were constrained to an approximately planar region, while those in 3D constructs traversed much longer z-distances (figure 3), more representative of *in vivo* cytoarchitecture.

Electrophysiological properties of neurons in 3D neural constructs

The 3D constructs were similar in size, shape, opacity and mechanical integrity to acute cortical slices from rodent brains. This enabled the transfer of the constructs to a custom perfusion chamber, permitting the use of a modified acute brain slice patch clamp rig for electrophysiological recording. We found unique challenges to recording from cells within 3D cultures including an increased susceptibility to mechanical vibrations than acute slices, which required additional anchoring. In addition, the construct was more adhesive and denser than acute slices from young rodent brains. Consequently, it was more difficult to penetrate the extracellular matrix with an electrode; however, by maintaining strong positive pressure on the electrode and a diagonal approach angle, successful recordings were achieved. While there was some difficulty establishing intracellular access for sub-surface cells, this challenge was not unique to our constructs and was overcome with our modified approach. Similar challenges have been reported with 3D collagen I cultures (O'Shaughnessy *et al* 2003); however, in those cultures, the cells were seeded on top of partially formed gels and hence the cells were recorded from at or near the surface of the gel. In contrast, we were able to access cells substantially below the surface. The ability to access cells at increasing depths may be improved by altering the concentration or constituents of the matrix. However, a significant reduction in the matrix concentration decreased the mechanical stability of the constructs, and resulted in more fragile cultures and increased their susceptibility to mechanical vibration during recording.

Using techniques modified from traditional acute slice whole-cell patch clamp recordings, we successfully recorded from cells within the 3D co-culture constructs (figure 4(a)). Neurons within the 3D constructs exhibited excitable membrane properties including spontaneous and evoked action potentials. The cells within the 3D constructs were observed to have typical resting membrane potentials (-56.2 ± 11.5 mV; $n = 28$) which were not statistically different from neurons in similar 2D cultures (-52.8 ± 6.1 mV; $n = 12$; t-test $p = 0.35$). In addition, action potentials of neurons within the 3D constructs had large action potential amplitudes (70.3 ± 9.9 mV) similar to mature neurons. Neurons within the 3D cultures exhibited depolarization and repetitive spiking with an increased discharge frequency in response to an increase in extracellular K^+ concentration (figure 4(b)). The membrane potential and firing rate returned to the original levels within 3 min after K+ washout (data not shown). Together, these data suggest that the neurons within the 3D constructs express functional voltage-gated ion channels and exhibited action potentials similar to those observed in acute slices, with the added ability to control cell density and types.

We also observed evidence for functional synapses and network activity within the 3D co-culture constructs. Fluorescent immunocytochemistry and confocal microscopy of representative cultures at 21 days *in vitro* revealed abundant

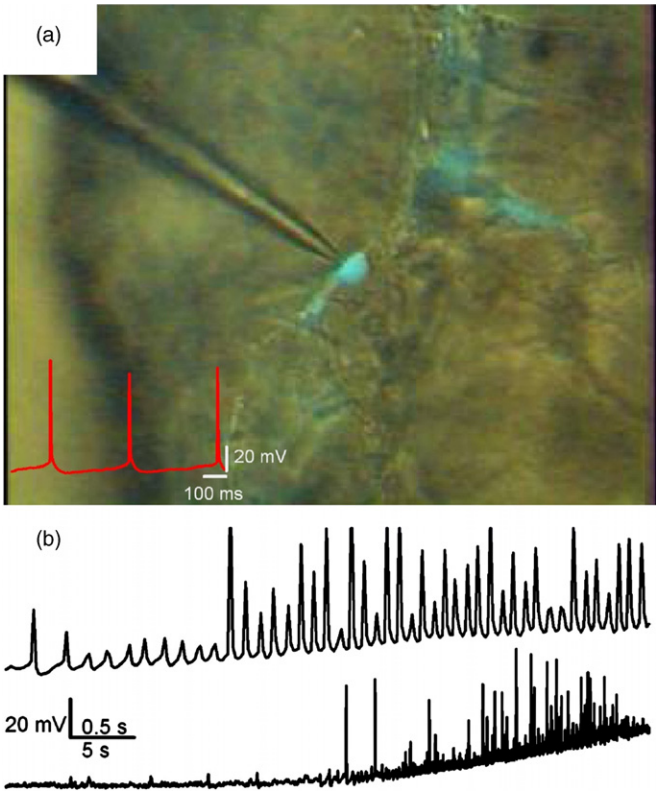

Figure 4. Whole-cell patch clamp recording in 3D neural constructs. Patch clamp electrode inserted into a GFP^+ neuron within a 3D construct (a). Using modified techniques normally used with acute slice whole-cell patch clamp recording, we were able to record from cells deep within the 3D co-cultures. GFP^+ neurons were cultured with wild-type astrocytes to facilitate identification of neurons within the culture. Spontaneous action potentials from a neuron within a 3D co-culture (inset). Active response of a neuron to a 20 pA s^{-1} current ramp (100 pA maximum) ((b), top) and to high, extracellular K^+ ((b), bottom). Extracellular K^+ perfusion (20 mM) evoked an increase in spiking and an elevated resting membrane potential.

synapsin I expression (figure 5(a); green puncta) along with the neuronal marker tau-5 (red). Electrophysiologically, spontaneous synaptic activity was evident (figure 5(b); black circles), as was network-driven burst activity. Additionally, through scanning electron microscopy, we observed processes with the characteristic appearance of neurites extending across somata and culminating in synaptic terminals (figures 5(c), (d)). Together, these observations provide significant evidence of functional synapses and network properties within the 3D co-culture constructs.

Discussion

We have engineered 3D co-culture constructs composed of neurons and astrocytes within a bioactive matrix as an *in vitro* model of neural tissue. Cells within these constructs exhibited *in vivo*-like somatic morphologies with extensive 3D process outgrowth and high viability over several weeks. Specifically, neurons within these constructs assumed complex 3D morphologies with rich neurite arborization, expressed mature cytoskeletal proteins and demonstrated network connectivity via synaptic junctures. Although morphology and neuronal marker expression are valid indicators of phenotypic maturity, the ultimate attribute to healthy neurons is the ability to generate action potentials and form synapses. Neurons within these 3D constructs exhibited spontaneous excitable properties along with evidence of functional synaptic currents and network activity. These traits allow the cultures to be used in studies of intercellular properties given the ability to interface with multiple electrodes within a culture. Additionally, these 3D constructs are similar in size and opacity to acute brain slices, making them transferable and thus amenable to a range of investigation techniques.

The cells *in vivo* are intimately connected to each other and the surrounding matrix to form a complex microenvironment that is not reproduced in traditional culture systems. For instance, 3D cell–cell and cell–extracellular matrix junctures are critical to many neurobiological responses, but such interactions are absent or constrained in most *in vitro* models. Suitable 3D microenvironments must support neural cell attachment while allowing sufficient diffusion of nutrients to keep the culture viable for long-term studies. In addition, the microenvironment should sufficiently support neurite outgrowth and the formation of functional synapses. Other 3D primary neural cell culture systems have incorporated scaffolds of collagen (Coates *et al* 1992, O'Connor *et al* 2000a), agarose (Luo and Shoichet 2004, Yu *et al* 1999) or fibrin (Willerth *et al* 2006) with varying viability. Survival of primary neurons in agarose is poor beyond 10 days (O'Connor *et al* 2001), however neurite outgrowth can be improved by the addition of collagen type IV (Cullen *et al* 2007a) or laminin (Yu *et al* 1999). Collagen I matrices, either alone or mixed with agarose, have also shown some improvement for the growth of neurons, astrocytes and neural progenitor cells (O'Connor *et al* 2000b).

Although these agarose- and/or collagen-based scaffolds are attractive to simplify the extracellular environment, these scaffolds alone may not be the most appropriate for neural cells. Collagen matrices are commonly used as a scaffold for many other cell types, and although collagen matrices are active for neural cells through ligand-based interactions, there is little or no collagen in the brain. Thus, collagen alone may not be ideal to support longer term neuronal viability. In the present study, we attained excellent long-term survival using the Matrigel^{TM} matrix, a reconstituted basement membrane which is biologically active for neural cells through matrix, e.g., laminin, collagen IV, entactin, heparan sulfate proteoglycan (Kleinman *et al* 1986), and cytokine-related interactions (Vukicevic *et al* 1992b). Despite the clear benefits of Matrigel^{TM} for a 3D culture system, alternative biomimetic scaffolds with specific ligands cross linked to hydrogel backbones, such as methylcellulose (Stabenfeldt *et al* 2006) or agarose (Cullen *et al* 2007a), 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. The next generation of 3D 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

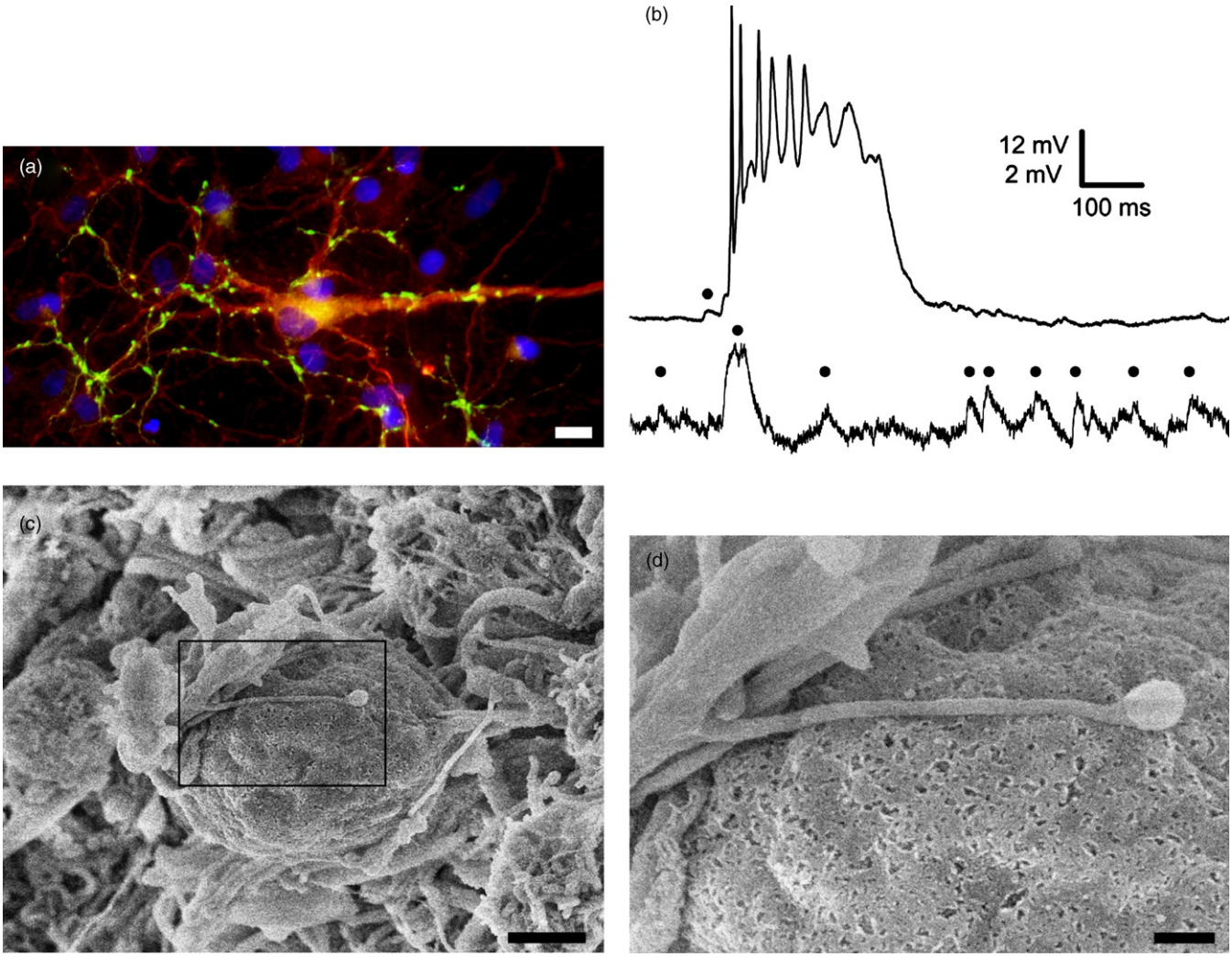

Figure 5. Evidence of functional synapses and network activity in 3D neural constructs. Immunofluorescent photomicrograph of a representative neural construct labeled to denote the expression of the neuronal marker tau-5 (red), the synaptic marker synapsin (green puncta), and nuclear marker Hoechst 33258 (blue) (scale bar = 20 μm) (a). Spontaneous synaptic activity was evident above the baseline noise (black circles) as was network-driven burst activity (b). Scanning electron micrograph of the surface of a 3D 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) (c). A morphologically identified putative synapse can be observed on the neuronal somata (scale bar = 500 nm) ((d), boxed region in (c)).

increase the survival, function and relevance of cultured neural cells. Importantly, 3D culture systems have the distinct advantage of being engineered from the bottom-up, with control over cellular parameters such as phenotype, ratio and density. In addition, by tailoring the 3D scaffolds as discussed above, the influences of specific environmental factors on basic neurobiological functions such as survival, process outgrowth and electrophysiological functionality may also be assessed.

Our constructs were developed using neurons and astrocytes separately harvested from the cerebral cortex and mixed in a controlled ratio. Previous reports demonstrate that the presence of astrocytes significantly influences the biological and electrophysiological properties of neurons. For instance, astrocytes serve as substrates for neurite outgrowth, provide trophic factors and metabolic precursors, and regulate neuromodulation, synaptic efficacy, and synapse number (Tsacopoulos and Magistretti 1996, Ullian *et al* 2001). The intimate interaction between neurons and astrocytes across three dimensions is essential for recapitulating phenomena observed *in vivo*. Additionally, since transformed cell lines have been shown to have incomplete synapse formation (Pancrazio *et al* 1999), altered ion-channel expression (Pancrazio *et al* 1999) or abnormal resting membrane potentials (< -40 mV) (Desai *et al* 2006, Wu *et al* 2006), primary cell cultures incorporating both neurons and astrocytes may be requisite for robust electrophysiological studies. An additional advantage of building 3D co-culture constructs from dissociated cells is that neurons and glial cells from different transgenic sources can be incorporated. In this work, we have used neurons isolated from GFP+ transgenic mice mixed with wild-type astrocytes, which allows easier identification of neurons. Although our focus has been on characterizing neuronal properties within these 3D constructs, it is also noteworthy that this system may be utilized to study astrocytic responses in a 3D setting (Cullen *et al* 2007b). This flexibility permits a wide range of applications with

different cell types and transgenic sources to address specific mechanistic questions.

In summary, the differences between 3D, ECM-based cultures and monolayer cultures cannot be ignored and may impact cellular responses significantly. Physiologically relevant models of neural tissue require prioritization among complexity, control and reproducibility. 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 individual cellular functions. 3D 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 (Bass *et al* 1971, Williams and Herrup 1988). The 3D neural constructs presented here represent an important step toward more physiologically relevant neural tissue models. This innovative investigational platform has many distinct advantages for neurobiological and electrophysiological applications.

Acknowledgments

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