

Three-Dimensional Neuronal Cultures

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Abstract

In vitro models utilizing neural cells in three-dimensional (3D) cultures may be a more accurate representation of the in vivo environment than two-dimensional (2D) cultures while maintaining many of the benefits. We have developed a 3D neural cell culture system using primary rat cortical neurons embedded throughout Matrigel, a protein-based thermoreversible hydrogel scaffold. Here we present the methodology for reproducing these cultures, as well as selected characterization and analysis techniques. Specifically, we examined viability as a function of cell density and morphology of neurons in both 2D and 3D configurations. We review and discuss the criteria for cell source, cell viability, scaffold selection, and application-specific considerations. The 3D neural models may more accurately represent in vivo neural responses and permit the development of enabling technologies for neurobiological and tissue engineering applications. The engineering of novel culture models is critical for in vitro advances in neuroscience and neural engineering, providing custom and modular design capabilities.

Key terms

cell culture
neuron
neurobiology
scaffold
three-dimensional

11.1 Introduction

11.1.1 2D versus 3D culture models

In vitro models are invaluable tools for studying cell behavior in a highly controlled setting. However, the interpretation of cellular responses in traditional planar, two-dimensional (2D) models may be confounded by atypical cell-cell/cell-matrix interactions and cellular morphology. Three-dimensional (3D) culture models, in which cells are grown within a scaffold such that the thickness is at least 10 cell diameters, maintain many positive aspects of in vitro cultures (e.g., real-time imaging, simplicity), but mimic the cytoarchitecture of in situ tissue to a higher degree than cells grown on nonphysiologic hard surfaces. A 3D 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 permeability (per network configuration, pore, or mesh size) for both nutrient and waste diffusion.

Fundamental differences exist between cells cultured in a monolayer versus 3D configurations in terms of access to soluble factors and the distribution and types of cell-cell and cell-matrix interactions [1–5], which may be constrained in planar cultures (see Table 11.1). Cells stay more spherical when embedded within a scaffold than 2D cultures and, in the case of neurons and other process-extending cells, outgrowth can occur in all directions. In vivo–like cell-cell interactions may lead to increased cellular survival and more realistic gene expression and cellular behavior. For example, it has been shown that dopaminergic neurons harvested from embryonic brain are viable longer when grown in 3D than in monolayer cultures [6]. The 3D cultures have been shown to result in longer neurite outgrowth, higher levels of survival, and different patterns of differentiation as compared to 2D monolayers [7–11]. When comparing 2D versus 3D embryonic mesencephalon tissue, more cell death occurred in dissociated monolayer cultures, while tissue explants and 3D cultures in collagen gels survived to a much greater extent [12]. Hippocampal neurons grown on a 3D aragonite matrix also survived to a greater extent than 2D counterparts, and formed higher-density networks [13]. We

Table 11.1 A Summary of the Comparisons Between 2D and 3D Cultures in Terms of Culture Configuration, Functional Measures, and Applicability to Modeling In Vivo Physiology

2D Versus 3D Cell Culture		
	Advantages	Limitations
2D Cultures:	Environmental control and cell observation, measurement, and manipulation are easier than 3D A rich body of literature exists, to which to outcome measures can be compared	2D systems may be inherently unable to depict traits exhibited by in vivo systems, (e.g., altered gene expression and growth characteristics due to a deficiency in cell-cell and cell-matrix interactions) Less compatibility with in vivo systems Increased drug sensitivity Cells have a majority of their surface exposed
3D 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; accumulation of toxic waste products within scaffold space Culture-dependent alterations in gene expression, cell proliferation, viability, productivity and product quality due to nutrient deprivation

have also shown differences in the response of 2D versus 3D neural cultures to mechanical injury, in which 3D cultures sustained more cell death than 2D cultures subjected to the same strain and strain rate [14].

11.1.2 Cell type and culture configurations

Different types of 3D cultures can be created, including reaggregate or sphere cultures, rotary bioreactor cultures with cell aggregates or microcarriers, hydrogel/scaffold cultures, and organotypic slice cultures. These vary in terms of cell dispersion and preservation of tissue function and organization. Organotypic cultures are slices of tissue with the architecture maintained, thus preserving networks in the cut plane, whereas the former categories generally utilize dissociated cells, which reorganize according to cell type, media conditions, adhesiveness to a substrate or surrounding cells. Regaggregate cultures can be made by rotation-induced reassociation [9] or by providing conditions that promote sphere formation [15]. Although there is some difficulty in controlling extracellular components and the cellular distribution of reaggregate cultures, these can be excellent models to study cell-cell interactions. Cell models have been developed in which neurons are plated above a matrix material [16–18], which recapitulates aspects of 3D morphology; however, cell-cell and cell-matrix interactions are spatially limited. Cells that are grown throughout a 3D scaffold or matrix produce perhaps the most controllable 3D culture models, since cell type(s) can be chosen, as can the extracellular composition [19–22].

11.2 Experimental Design

The overall objective of this study was to establish and characterize primary cortical neurons cultured in a 3D configuration using a Matrigel matrix. We used both multiwell plates (typically 24-well) and custom-made PDMS chambers mounted on to glass cover slips. We varied the cell density to optimize viability for the chambers and culture thickness described later. It is advisable to repeat this characterization before using these cultures. Parallel methods for 2D cultures are presented in some sections for comparison to 3D cultures as needed. As discussed, this system is flexible and can be used to incorporate other features, such as multiple cell types.

11.3 Materials

11.3.1 Harvest

- Time-pregnant (embryonic day 18 (E18)) Sasco Sprague-Dawley rats (Charles River)
- Surgical tools
- Autoclave
- Laminar flow hood
- 4–6 100-mm Petri dishes
- Sterile Hanks Balanced Salt Solution (HBSS, Gibco)
- Ice

- 50-mL centrifuge tube
- Fume hood
- Isoflurane
- Anesthetization chamber
- Guillotine
- Sterile dissection hood
- 70% ethanol
- 15-mL centrifuge tube
- 2-mL L-15 supplemented with 2% B-27
- Aluminum foil

11.3.2 Dissociation

- Cortical hemispheres
- 5-mL trypsin (0.25% + 1mM EDTA) (Invitrogen) and 400- μ L deoxyribonuclease I (DNase, 1.5 mg/mL, Sigma)
- 37°C water bath
- Ice
- Ca²⁺- and Mg²⁺-free Hanks Balanced Salt Solution (CMF-HBSS)
- 50-mL centrifuge tube
- Plugged Pasteur pipette or serological pipette
- DMEM/F12 (Invitrogen) + 10% fetal bovine serum (FBS, Invitrogen)
- Neuronal medium: 100-mL neurobasal medium + 2 mL B-27 + 250- μ L Glutamax (0.5-mM final Glutamax concentration)
- 200–400- μ L DNase
- Vortex
- Flame-narrowed Pasteur pipette
- Centrifuge
- 190- μ L HBSS
- 200- μ L trypan blue
- Hemocytometer

11.3.3 Plating

- Neuronal medium: 100-mL neurobasal medium + 2-mL B-27 + 250- μ L Glutamax
- Poly-D-Lysine (PDL, stock solution 1 mg/mL, dilute to working concentration of 0.05 mg/mL in deionized water (di-H₂O) prior to use (1:20 dilution))
- Autoclave
- 70% EtOH
- UV
- Incubator (37°C, 5% CO₂, 95% RH)
- Laminar flow hood
- Protein precoat (e.g., Ln, Fn, Cn, Matrigel)

- Feeding media
- DNase (7.5 $\mu\text{g}/\text{mL}$) in HBSS
- HBSS

11.3.4 Assessment

- Viability/cytotoxicity
- Immunocytochemistry
- Microscope
- Hemocytometer

11.4 Methods

11.4.1 Harvest

11.4.1.1 Notes

1. Time-pregnant (embryonic day 18 (E18)) Sasco Sprague-Dawley rats (Charles River).
2. This procedure may also be used for a harvest from E17 rat pups.
3. All surgical tools should be sterilized by an autoclave prior to dissection.
4. Dissection is performed within a horizontal laminar flow hood.
5. Prior to dissection, fill 4–6 100-mm Petri dishes with 15 mL each of sterile Hanks Balanced Salt Solution (HBSS, Gibco) and place on ice
6. If cortices will be stored rather than dissociated immediately, then prior to dissection, fill a 50-mL centrifuge tube with 25 mL of sterile HBSS, and place on ice (for rinse steps). If cortices will be dissociated immediately following harvest, see Section 11.4.2.1.

11.4.1.2 Procedure

1. In a closed chamber with a hose inlet, anesthetize the dam using 3–5% isoflurane in oxygen for approximately 5 minutes, until unconscious and unresponsive to tail pinch.
2. Rapidly decapitate using a guillotine.
3. Transfer the carcass to a sterile dissection hood.
4. Lay the carcass ventral side up and rinse the abdomen thoroughly with 70% ethanol.
5. Cut beginning at the lower abdomen extending rostrally to expose the uterus.
6. Remove the uterus and place in a Petri dish with ice-cold HBSS.
7. Remove each fetus from the amniotic sacs and transfer to a new Petri dish containing ice-cold HBSS.
8. Decapitate each fetus and remove brains by cutting and peeling back the top portion of the skull.
9. Transfer brains to a new Petri dish containing ice-cold HBSS.
10. Cortical isolation:
 - a. Remove the hindbrain and perform a midsagittal cut.
 - b. Remove the midbrains and place hemispheres with lateral surfaces facing down.

- c. Remove the olfactory bulbs and turn over hemispheres such that the medial surface faces down.
 - d. Detach the meninges and again turn the hemispheres over such that the lateral surface faces down.
 - e. Remove the cortical region from the remaining midbrain and cut the hippocampal region from the cortex (hippocampal regions may also be saved, dissociated, and cultured).
 - f. Transfer the cortical regions to a 15-mL centrifuge tube (4–6 cortical hemispheres per tube) and place on ice.
11. Immediately proceed with the dissociation procedure or follow the storage procedure outlined in the next step.
 12. Storage procedure:
 - a. Rinse twice with a cold HBSS (removing small debris)
 - b. Add 2-mL L-15 supplemented with 2% B-27.
 - c. Wrap the tube in aluminum foil and place the tube on its side at 4°C, ensuring that all cortices are submerged.
 - d. For optimal viability, dissociate within 2 days (>90% cells viable).

11.4.2 Dissociation

11.4.2.1 Notes

1. This procedure is typically for 2–3 brains (4–6 cortical hemispheres); the expected yield is 3–5 million cells/cortical hemisphere.
2. Prior to dissociation, transfer 5-mL trypsin (0.25% + 1mM EDTA) (Invitrogen) and 400- μ L deoxyribonuclease I (DNase, 1.5 mg/mL, Sigma) to a 37°C water bath.
3. Keep the tissue/cells on ice throughout the procedure (except the trypsin step) and perform the rinses with ice-cold Ca²⁺- and Mg²⁺-free Hanks Balanced Salt Solution (CMF-HBSS).
4. Prior to dissociation, fill a 50-mL centrifuge tube with 25 mL of sterile CMF-HBSS and place on ice (for rinse steps, it may be same tube prepared prior to dissection).
5. Rinses may be performed using a plugged Pasteur pipette or serological pipette
6. Prepare DMEM/F12 (Invitrogen) + 10% fetal bovine serum (FBS, Invitrogen) and place on ice (to deactivate trypsin)
7. Prepare the neuronal medium: 100-mL neurobasal medium + 2-mL B-27 + 250- μ L Glutamax (0.5-mM final Glutamax concentration) and place it on ice.

11.4.2.2 Procedure

1. Acquire the cortical (or hippocampal) region from E18 rat fetuses (cortices will be in HBSS or L-15 + B-27; see Section 11.4.1).
2. Rinse twice with CMF-HBSS (remove small debris).
3. Add 5-mL trypsin (0.25% + 1mM EDTA) prewarmed to 37°C, manually agitate tube once to mix, and place the tube in a water bath:
 - a. At 5–7 minutes, manually agitate the tube to mix and check clumping.
 - b. Check periodically; stop trypsinization if the tissue is one large clump.

- c. Stop at ~10 minutes (time can be up to 15 minutes, depending on the trypsin strength):
 - Remove the trypsin, being careful not to disturb the tissue.
 - Rinse with cold DMEM/F12 + 10% FBS and gently wash twice with CMF-HBSS.
 - Add 1.8-mL CMF-HBSS + 200–400- μ L DNase (final concentration 0.15–0.30 mg/mL).
 - Vortex for 30 seconds and/or triturate 5–10 times with a flame-narrowed Pasteur pipette to break up tissue clumps (any clumps remaining after 10 triturations may be removed and discarded).
 - Centrifuge cells at 200 rcf for 3 minutes.
 - Aspirate supernatant and resuspend the pellet in 2 mL of the neuronal medium (to redissociate cells, use a serological pipette, a flame-narrowed Pasteur pipette, or revortex).
 - Dilute the cells to the desired density.
4. Ensure that the cell solution is well mixed (gently triturate 3–5 times) and remove a small sample to dilute and count. Typically use a 1:40 dilution consisting of 10- μ L cell solution + 190- μ L HBSS and 200- μ L trypan blue (let the diluted cell solution sit for 30 seconds prior to the trypan blue addition).
5. Ensure that the diluted cell solution is well mixed (gently triturate) and transfer 10 μ L to each chamber of a hemocytometer.
6. Count the cells on the hemocytometer to determine the total cell number and percent viability via a trypan blue exclusion.
7. The total cell yield should be 3–5 million cells/cortical hemisphere. Do not proceed if the yield is substantially less than this amount.
8. If the neuronal viability is less than 90%, then do not proceed.
9. Dilute with the neuronal medium to desired density: sample 2D cell density: 4×10^5 cells/mL, and sample 3D cell density: 7.5×10^6 cells/mL.

11.4.3 Plating

11.4.3.1 Solutions

1. Neuronal medium: 100-mL neurobasal medium + 2-mL B-27 + 250- μ L Glutamax (0.5 mM final Glutamax concentration).
2. Poly-D-Lysine (PDL, stock solution 1 mg/mL, dilute to working concentration of 0.05 mg/mL in di-H₂O prior to use (1:20 dilution)).

11.4.3.2 Surface prep (specific to plating surface)

1. Sterilize (e.g., autoclave, 70% EtOH, UV, flame (glass only)).
2. PDL treatment (0.05 mg/mL):
 - a. Add 250 μ L/cm² and place in incubator (37°C, 5% CO₂, 95% RH) for 4–12 hours.
 - b. Aspirate PDL, gently rinse once with sterile di-H₂O, and let the excess evaporate by leaving the lid open under a laminar flow hood for ~2 minutes.
 - c. Optional protein precoat (e.g., Ln, Fn, Cn, Matrigel): 0.5 mg/mL, 250 μ L/cm², and place in an incubator (37°C, 5% CO₂, 95% RH) for 4–12 hours.

11.4.3.3 Procedure

1. Add cells:
 - a. 2D final density: 50,000–125,000 cells/cm², volume = 250 μ L/cm².
 - b. 3D final density: $3.75\text{--}5.00 \times 10^6$ cells/cm³, add appropriate volume for desired thickness, final Matrigel concentration = 7.5 mg/mL.
2. Place the cultures in the incubator (37°C, 5% CO₂, 95% RH).
3. Feed/change the media:
 - a. 2D: change the media at 24 hours postplating and feed every 3–4 days thereafter by replacing 50% media.
 - b. 3D: add the feeding media 30 minutes postplate and feed at 24 hours and every other day thereafter by replacing 50% media.
4. Remove DNA fragments (2D only, optional):
 - a. At 24 hours postplate, aspirate the medium and treat with DNase (7.5 μ g/mL) in HBSS for 5 minutes.
 - b. Aspirate DNase and rinse twice with HBSS.
 - c. Add the neuronal medium and return the cultures to incubator.

11.4.4 General assessment: staining, imaging, and data acquisition

11.4.4.1 Notes

1. Cultures should exhibit minimal clustering, meaning that there should be some small multicell clusters, but most neurons should not be in direct soma-soma contact.
2. Cultures may be assayed at different time points based on experimental objectives; however, more neuronal maturation is observed post-first week in culture.
3. Viability should be done with the entire culture and imaged and quantified using a confocal microscope.
4. Immunostaining can be performed with the whole 3D culture, assuming that culture density allows antibody penetration. Thick (1–2 mm) cultures can be fresh-sectioned using a vibrotome.
5. Otherwise, cryofix and section the culture on a cryostat or a microtome at a 30–40- μ m thickness.
6. Besides viability and phenotypic analysis, electrical activity or other outcome measures may be considered (e.g., metabolic state, network properties, and gene and protein expression over time).

11.4.4.2 Procedures and criteria

1. Cell viability: >90% (assessed by viability/cytotoxicity assay):
 - a. Incubate with a 4- μ M ethidium homodimer-1 (EthD-1) and 2 μ M calcein AM (Molecular Probes) at 37°C for 30 minutes.
 - b. Rinse with 0.1M Dulbecco's phosphate-buffered saline (DPBS, Invitrogen).
 - c. The percentage of viable cells is 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).

- d. In 3D neuronal cultures, the viability was assessed at cell densities of 1,250; 2,500; 3,750; 5,000; 6,250; and 7,500 cells/mm³ at 7 DIV ($n = 6, 10, 10, 10, 6, 6$, respectively).
 - e. In neuronal cultures at a density of 3,750 cells/mm³, viability was assessed at 7–8, 13–14, and 21 DIV ($n = 5, 6, 4$, respectively) and compared to viability in 2D cultures ($n = 5, 6, 5$, respectively) (data not shown).
2. Cell type: >95% neuronal (assessed by immunocytochemistry) (data not shown):
 - a. Fix cells or sections in 3.7% formaldehyde (Fisher, Fairlawn, New Jersey) for 30 minutes, rinse in PBS, and permeabilize using 0.3% Triton X100 (Kodak, Rochester, New York) + 4% goat serum (Invitrogen) for 20 minutes.
 - b. Add the primary antibodies (in PBS + 4% serum) at 18–24°C for 4 hours.
 - c. After rinsing, add the appropriate secondary fluorophore-conjugated antibodies (FITC or TRITC-conjugated IgG, Jackson Immuno Research, West Grove, Pennsylvania, or Alexa 488 or 546-conjugated IgG, Molecular Probes) (in PBS + 4% serum) at 18–24°C for 2 hours.
 - d. Primary antibodies that can be used are as follows:
 - Glial fibrillary acidic protein (GFAP) (AB5804, 1:400; MAB360, 1:400, Chemicon, Temecula, California), for contaminating astrocytes.
 - Tau-5 (MS247P, 1:200, NeoMarkers, Fremont, California), a microtubule-associated protein expressed predominantly in neurons.
 - Synapsin I (A6442, 1:200, Molecular Probes), a synaptic vesicle protein localized in presynaptic specializations.
 - Counterstain with performed Hoechst 33258 (1:1000, Molecular Probes).
 3. Cell density: perform cell counts in 3–5 random microscopic fields of view and divide by the total volume of field of view. Verify the correspondence with the initial plating density.
 4. Electrical activity:
 - a. A 3D MEA biochip (Ayanda Biosystems, Lausanne, Switzerland) with an 8 × 8 matrix of microelectrode hillocks can be used to assess extracellular field recording parameters such as the spike rate and burst rate [23]. In our hands, we observed that the spike rate as well as the burst rates increased rapidly in the first week in culture, leveling off during the second week, suggesting an adaptation or saturation. This was followed by a gradual and slight drop in the third week suggesting pruning and/or culture degradation. This activity was responsive to agents known to interfere with synaptic transmission, confirming a biological response (data not shown).
 - b. A patch clamp can also be used to assess the electrical properties of 3D neuronal cultures either when cells are cultured atop a 3D matrix [17, 24] or by penetrating the 3D culture [25]. Note that more synapsin positive cells are present with neurons are cocultured with astrocytes, and thus, electrical activity may be quite different between mono- and multitypic cultures (data not shown).
 5. Cell morphology and cell distribution with scanning electron microscopy:
 - a. Low-voltage, high-resolution scanning electron microscopy (LVHR-SEM) was used to qualitatively assess cell and culture morphology.
 - b. Cultures were plated on pretreated silicon wafers and fixed using 2.5% EM-grade glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) at 4°C for 24 hours.

- c. Cultures were washed in di-H₂O and then postfixed with 1% OsO₄ in 0.1M cacodylate buffer (pH 7.4) for 1 hour and then rinsed.
- d. A graded series of ethanol (30, 50, 70, 3 × 100%, 15 minutes each) was used to substitute culture fluids prior to wrapping individual gels in parafilm.
- e. Samples were loaded into a Polaron E-3000 critical point dryer (CPD) and an exchange with CO₂ was made while monitoring the exhaust gas rate. The CPD was thermally regulated to the critical temperature and pressure and, following the phase transition, the CO₂ gas was released at a constant flow rate. Prior to LVHR-SEM, all specimens were sputter-coated with 3 nm of chromium.

11.4.5 Data analysis

Outcome measures were analyzed using quantitative microscopy to verify viability and phenotype and to examine morphology. After viability and immunocytochemistry assays, cells were viewed using fluorescent microscopy techniques on an epifluorescent microscope (Eclipse TE300, Nikon, Melville, New York) or a confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany). For epifluorescent microscopy, images were digitally captured (DKC5T5/DMC, Sony, Tokyo, Japan) and analyzed using Image-Pro Plus (Media Cybernetics, Silver Spring, Maryland). Confocal images were acquired across the full thickness of the cultures and were analyzed using the LSM Image Browser (Zeiss). Three to five randomly selected regions per culture were digitally captured and counted for statistical analysis of viability. Data are presented as mean ± standard deviation. All confocal photomicrographs from 3D cultures are 100- μ m-thick reconstructions (from 500–750- μ m-thick cultures). A general linear model ANOVA was performed followed by Tukey's pairwise comparisons (p -value < 0.05 was considered significant).

11.5 Anticipated Results and Discussion

11.5.1 Characterization of cell morphology and viability in 3D neuronal cultures

By following these procedures, it is possible to generate 3D primary neuronal cultures that are viable for several weeks and maintain typical neuronal morphology and function. SEM was used to qualitatively assess cell and culture morphology in 2D and 3D neuronal cultures (Figure 11.1). Cells cultured on tissue culture plastic remained relatively flattened as compared to 3D neuronal cultures, presumably due to the properties of Matrigel, allowing for rounded cells with a more elaborate process extension. In order to determine the optimal cell density for neuronal viability in 3D culture, neurons were plated at various densities. Cell viability followed a parabolic relationship with cell density, with an optimum viability of ~90% at 3,750 cells/mm³ (Figure 11.2). Throughout various 3D platings, it was observed that cell culture chambers of smaller cross-sectional area (~0.3 cm²) did not support viable cells at the culture thicknesses utilized in this study (data not shown). This observation suggests an interaction between culture thickness and the cross-sectional area for diffusion that exists independent of cell density.

As highlighted in this chapter, 3D neural cultures can be created with primary neurons and a Matrigel matrix. It is anticipated that the needs of many applications can be

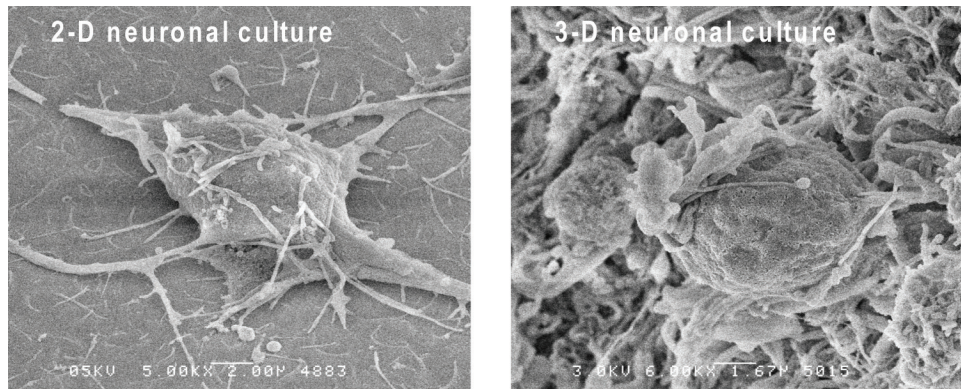


Figure 11.1 Electronmicrographs of neuronal cultures in 2D (left) and 3D (right). Neurons in 2D have a flattened morphology, while neurons cultured in a 3D matrix of Matrigel present a rounded morphology with matrix interactions possible in all spatial dimensions. (The authors thank Dr. Robert P. Apkarian, the director of the Integrated Microscopy and Microanalytical Facility at Emory University, for his assistance in electron microscopy.)

met with these procedures, but specific scenarios may require different protocols. There are several potential aspects of the methodology that are prone to variability and worth some discussion. Troubleshooting suggestions are offered below to stimulate innovative solutions to common as well as potential problems. For example, one must optimize the cell density and overall culture shape according to the application, keeping in mind that shape and size of the culture will likely change the surface area-to-volume ratio, and therefore some intralaboratory troubleshooting, outside of that described here, may be necessary. This general methodology provides an experimental framework on which to build, including varying the cell type, adding additional cell types, modifying or changing the scaffold, or interfacing with other systems, such as electrodes, fluidic support, or other cell systems.

11.5.2 Cell considerations

The 3D cultures allow tissue-like, multidimensional cell-cell/cell-matrix contacts. Slices and other explants are valuable for some applications, but 3D cultures allow the construction of a defined system with respect to cell type, cell ratios, and orientation (whether random for a more uniform response, or patterned in some way, allowing for a designed culture architecture). Cells from transgenic animals can also be introduced, allowing the probing of mechanisms or identification of a cell subpopulation. Extracellular proteins and other constituents can be added to bind to or enter cells. Synthetic components like electrodes or artificial microvessels can be easily introduced, as the cultures can be “cast” around micromachined devices. The choice of cell type is important and the advantages and disadvantages for adult versus fetal cells, primary cells versus cell lines, and human versus animal should be considered. The selection of media formulation is equally important; serum-containing media are not as well characterized as serum-free defined media, yet contain many essential components. Conditioned media are an option for some culture conditions as well, yet consistency in composition should be verified. In choosing and optimizing a protein-based scaffold, appropriate adhesion receptors should be assessed. Also, will the chosen cell type pro-

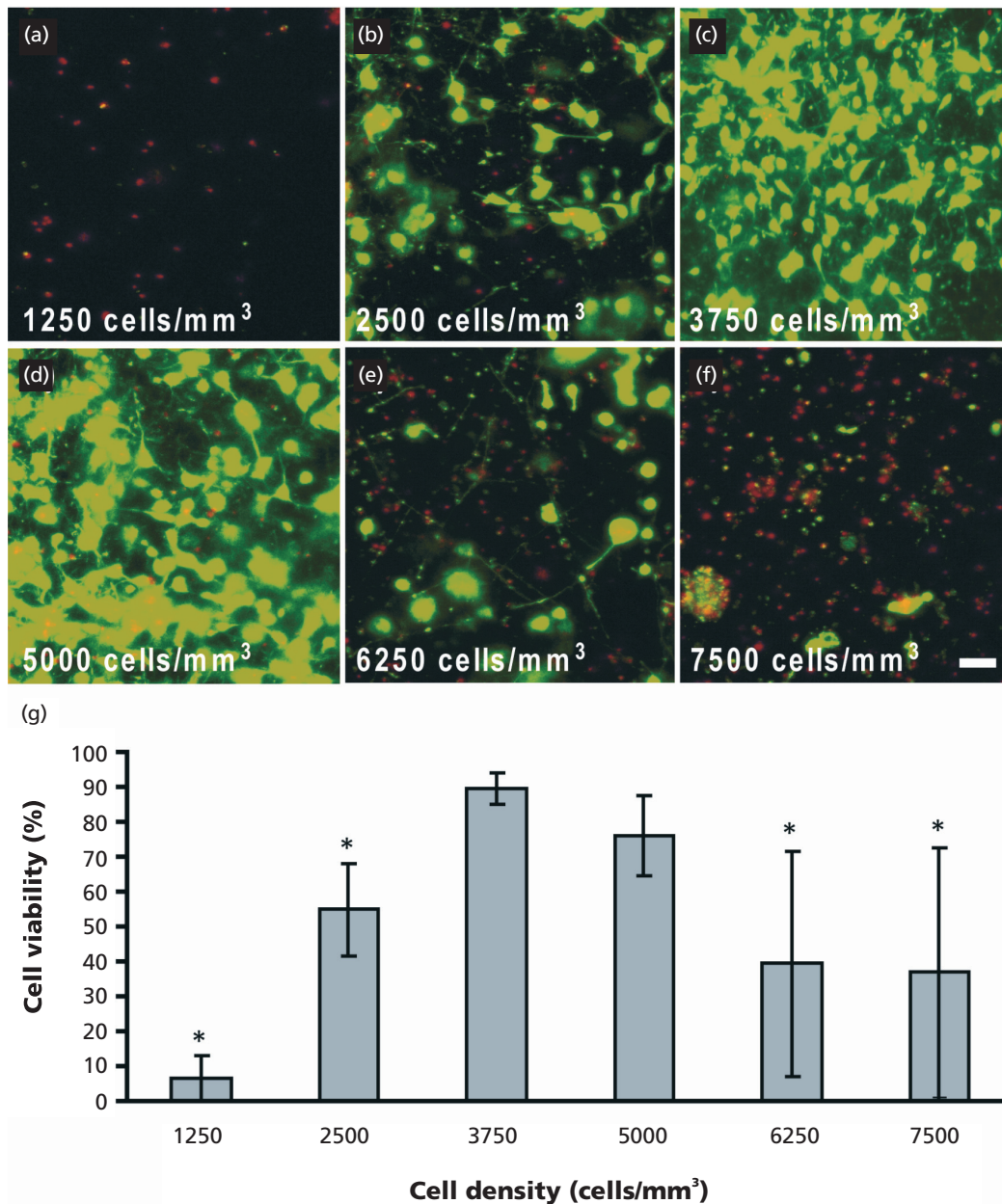


Figure 11.2 Neuronal survival was assessed as a function of 3D cell density. (a–f) Fluorescent confocal reconstructions of representative neuronal cultures plated at various cell densities. At 7 DIV, cells were stained to discriminate live cells (green) and the nuclei of dead cells (red). Scale bar = 50 μm . (g) The percentage of viable cells in 3D neuronal cultures at 7 DIV as a function of cell density (cells per mm^3). In this culture model, cell density was found to be a significant factor in neuronal survival. *Groups varied significantly from the peak cell viability attained at a cell density of 3,750 cells/mm^3 ($p < 0.05$).

duce, secrete, and adhere to endogenously made adhesive proteins? What is the minimum and maximum time in culture required for a particular application? Cell-produced enzymes and adhesive proteins may alter a biological scaffold, perhaps replacing it in

some cases. The addition of other cell types is another important consideration. The addition of astrocytes, microglia, and endothelial cells may all change the response, yet are ultimately necessary if trying to mimic brain tissue. Optimal cell type ratios should be experimentally determined, with clear outcome criteria established.

The harvesting of cells or tissue regions from whole tissue inherently involves some injury in the process of slicing or dissociation. While this is often seen as a limitation of *in vitro* systems, it is important to choose cells that maintain a high viability during harvest (otherwise there is a risk of selecting populations other than those desired) and to have the ability to measure recovery from the potential trauma of such manipulation. This being said, cells should be treated as fragile and care should be taken to be consistent and avoid unnecessary handling.

As mentioned earlier, cell density is critical to culture behavior. The human cortex has approximately 10^5 neurons/mm³, with thousands of synapses, both locally and over long distances. This is in contrast to culture conditions, especially 2D cultures, in which most synapses are with neighboring cells. In addition, most of the central nervous system is composed of interneurons with thousands of synapses to other neurons. Some of these neurons as well as the projection neurons synapse with distant neurons, further supporting the need for 3D culture conditions, where the average neuron-to-neuron distance is much greater than 2D cultures, in which neurons are constrained to synapse with neighboring cells. Several methods for increasing cell density to approach that of the brain have been explored. Of note, layered 3D neuronal networks using silica beads allow elaborate networks to form, the density of which is dependent on bead size [26]. In our laboratory, we have designed a convective microperfusion chamber to permit survival of high density cultures [27, 28].

11.5.3 Scaffold considerations

The neural cell culture models described here have been developed using the Matrigel matrix, a reconstituted basement membrane that is biologically active for neural cells (e.g., collagen, laminin) [29] and cytokine-related interactions [30] and has been shown to promote neurite outgrowth [31, 32]. Matrigel is prone to degradation, however, especially in the presence of astrocytes; therefore, enzyme-specific inhibitors are a possible addition to the culture medium. Possible scaffold materials (both protein-based and synthetic) are presented in Table 11.2. The choice of scaffold is quite important and may affect gene expression [33] and other functions. There are several criteria that should be met for 3D culture scaffold choice (see also [34]).

Scaffolds for 3D cultures must have a strong but porous structure to which the cells can adhere, but also must be able to extend processes (neurons) and migrate (microglia), as well as allow for the adequate diffusion/perfusion of medium components. The scaffold should have a defined composition such that the cells are the only black box in the system. Ideally, the scaffold should have adjustable mechanical properties (e.g., via concentration adjustment) such that the properties of neural tissue can be approximated. If the desire is to recreate the ECM as realistically as possible, then the defined composition should include components that are prevalent in the specific ECM (e.g., hyaluronidic acid, HSPGs, collagens). The ECM of the brain contains few collagen/fibrillar components, so we must question making a biomimetic brain using traditional culture scaffolds like collagen I. Given the gelation and mechanical properties of collagen I, however, it may

Table 11.2 A Selection of Materials That Have Merit for Consideration as Scaffolds for 3D Cultures

Scaffold	Source	Description	Composition	Gelling Method	Pros	Cons
Matrigel	BD 354263	ECM from Murine EHS tumor cells	Laminin, collagen IV, HSPG, entactin/nidogen	Thermal	Primary cells survive well in it	Cost; incompletely defined composition
PuraMatrix	BD 354250	Synthetic peptide nanofiber scaffold	RADA16	Ionic strength	Very high water content; no factors added; can be modified to include binding motifs	Low pH; gelling method limits functionality; modified forms would be costly to produce
ECM gel	Sigma E1270	ECM from Murine EHS tumor cells (effectively the same as standard Matrigel)	Laminin, collagen IV, HSPG, entactin	Thermal	Primary cells survive well in it	Cost; incompletely defined composition
MaxGel	Sigma E0282	In vitro–produced nondenatured human ECM	Collagens, laminin, fibronectin, tenascin, elastin, PGs, and GAGs	Thermal	Nondenatured proteins more in vivo–like	No 3D application documented
HydroMatrix	Sigma A6982	Synthetic peptide nanofiber scaffold	Not specified	Thermal or ionic strength	Same as PuraMatrix	Same as PuraMatrix
Extracel	Glycosan	Chemically defined ECM	Hyaluronan and gelatin (thiol-modified)	Thiol-reactive crosslinker (PEGDA)	Defined composition	Not tested with primary neural cultures
Extracel-HP	Glycosan	Chemically defined ECM modified to allow for slow-release of factors	Hyaluronan, HA, heparin, and gelatin (thiol-modified)	Thiol-reactive crosslinker (PEGDA)	Defined composition; thiolated heparin which ionically bind growth factors	Not tested with primary neural cultures
Extracel-SS	Glycosan	Chemically defined ECM modified to allow for easy cellular retrieval	Hyaluronan and gelatin (thiol-modified)	PEGSSDA (reducible PEGDA)	Defined composition; hydrogel can be dissolved by treatment with mild reducing agent	Not tested with primary neural cultures

Others materials are also presented in the text. Application-specific criteria should be applied, including cell type, cell surface adhesion receptors, stability and degradation potential, porosity, and cytocompatibility.

be considered as a structural component (natural or denatured), used in combination with Matrigel [35] or other proteins or peptides. Related to this issue of protein scaffold choice, the cell adhesion surface receptors for any particular cell type should play a prominent role in scaffold selection. The issue of using synthetic versus natural components for a 3D culture scaffold is important and the possibility of developing bioartificial, or combination, scaffolds may be attractive in some instances. *N*-(2-hydroxypropyl) methacrylamide polymer HPMA + collagen I hydrogels, for example, have been shown to have potential as a 3D neural scaffold [22]. Other synthetic scaffold materials include poly(ethylene oxide) (PEO), poly(glycolic acid) (PGA), poly(L-lactic acid) (PLLA), polycaprolactone, polyester urethane, methylcellulose, poly(acrylonitrile) (PAN), poly(vinyl chloride) (PAN/PVC), and polysulfone (PS). The cytocompatibility of the material and the breakdown products should be carefully assessed (see [36, 37] for more on cytocompatibility assays). Biological scaffolds include

Matrigel, as well as chitosan [38], collagens, fibronectin, laminin, fibrin, hyaluronic acid derivatives, and decellularized tissue (see Table 11.2).

The *porosity* is critical to cell performance within a 3D culture. The scaffold should be permeable to low molecular weight molecules, gases, and nutrients, and avoid waste buildup or trapping. Sufficient pore volume should exist to permit cell-cell interactions and migration, as well as neurite extension. In addition, the surface area must permit cell attachment and physiological spreading. Scaffolds should balance ligand density with stiffness and pore size, a relationship that may vary from material to material. For example, a decrease in pore size was observed in agarose gels when coupled to laminin peptides [39].

The *stiffness* and *pore size* of a scaffold are related and this relationship will differ from system to system. Distance between cross-links in the polymer chains will positively correlate with the pore size of the hydrogel. For example, in agarose gels, the average pore size was found to decrease exponentially as the concentration (i.e., the stiffness) increased [40]. The elastic modulus of poly(2-hydroxyethyl methacrylate (pHEMA) hydrogels was not, however, affected by increased porosity until pores became interconnected [41]. This *tortuosity*, or path windiness among the pores, will also play an important role in cell behavior, as neurites extend and synapses form. The *charge* of the gel will also affect cell function and the porosity. For example, a positive charge was shown to facilitate neurite outgrowth, while a negative charge inhibits outgrowth [40].

There are many methods for measuring porosity and other scaffold properties. The permeability coefficient can be used to estimate pore size. A slice of the gel with a known thickness is placed in a water column and is subject to a known hydraulic pressure [7]. The water collection over the unit time is used to measure porosity. Porosity may also be examined using scanning or transmission electron microscopy, but the preparation procedure may affect pore size, thus introducing an artifact into the system. Electron microscopy under physiological conditions or in combination with other methods should always be considered. In addition, hydrogels may swell while under culture conditions. Therefore, the characterization of any hydrogel should include the measurement of fluid uptake and formulation adjustment, as this may affect *osmolarity* of the extracellular fluid. The *stability* of a scaffold is vital to consistent culture conditions, whether for short-term cultures or cultures designed to support cells for weeks or months. Scaffolds can be affected by enzymatic degradation or mere culture erosion over time. This can be used as an advantage, however, as neurite outgrowth of photoencapsulated cells within polyethylene glycol has been shown to be controllable by varying the degradation rate [42].

11.5.3.1 Gelation method

The method for gelation is an important design feature, especially for those cultures in which cells are entrapped or “cast” at the same time as scaffold gelation. Many different methods are possible, perhaps with temperature phase transition being the most common (see Table 11.2). Extreme heat or cold, however, should be avoided, as proteolytic processes and immediate early genes may be activated and the transient insult may affect cell behavior. A change in pH transition, ionic strength, and photo cross-linking procedures are also common in the literature and may be considered for particular applications. Caution must be used when considering any gelation methods in terms of

potential toxicity from unpolymerized monomers, change in osmotic pressure during polymerization, heat production, or free radical production.

11.6 Application Notes and Commentary

One of the major challenges in neuroscience is to translate basic findings in neurobiology to real clinical solutions. Robust 3D culture models that can be reproduced will help to accelerate neuroresearch in general and neuropharmaceutical progress in particular. While culture models are not a replacement for animal or human research, they have long been engrained in the beginnings of basic neuroscience research and yet are archaic in many respects. 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 choose the most appropriate building blocks to create complex 3D neural cultures, and to define and measure physiological homeostasis and functional fluctuations. These challenges stem from specific needs: current *in vitro* systems are limited and 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 3D neural platforms can be adopted for assessing disease mechanisms via microsampling and micro-based assays, optimizing drug delivery to the brain, and preclinical testing for mono- and combination therapies in a semi-high-throughput fashion.

11.7 Summary Points

1. Electron microscopy revealed distinct morphological differences between planar cultures, which present a flat morphology, compared to cells distributed throughout a matrix, which present a bulbous morphology with cell-matrix contact in all dimensions.
2. Viability in 3D neuronal cultures was highly dependent on cell density (with optimal viability at 3,750 cells/mm³ for 500- μ m-thick cultures) up to 21 days *in vitro* (DIV).
3. Functional measures, such as intracellular and extracellular electrophysiology can be performed on 3D cultures.
4. Phenotypic markers can be assessed as a function of time in culture to monitor maturity and other changes.
5. The cell type presented here is primary fetal rat cortical cells. Other cell types or additional cell types can be incorporated into the cultures as needed, but density and ratio must be optimized for viability and function.
6. The scaffold used in these methods is Matrigel. While it has many advantages and most cell types encountered by our laboratory thrive in it, it is prudent to consider alternatives as cultures become more sophisticated.
7. The 3D neural culture models potentially provide enhanced physiological relevance over 2D planar cultures and will be valuable in the mechanistic study of cell growth,

cell-cell/cell-matrix interactions, as well as the responses to chemical or mechanical perturbations.

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