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High Cell Density Three-Dimensional Neural Co-Cultures Require Continuous Medium Perfusion for Survival

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Abstract—**Three-dimensional (3-D) models of neural cell culture may provide researchers with a more physiologically-relevant setting to study neurobiological phenomena than traditional two-dimensional (2-D) culture models. However, in the development of thick (>500 μm) 3-D cultures, diffusion limited mass transport necessitated the use of cell densities much lower than those found in the central nervous system (CNS). The goal of this study was to evaluate the effects of continuous medium perfusion on the survival of thick, 3-D neuronal-astrocytic co-cultures at cell densities closer to those found in brain tissue. At the cell density and thickness used for these studies, 10^4 cells/mm^3 and 500 – 750 μm, respectively, non-perfused cultures exhibited widespread cellular/matrix degradation and cell death. However, co-cultures perfused at relatively high rates (2.5 – 11.0 μL/min, corresponding to 6 – 27 medium exchanges/day) demonstrated decreased degradation and enhanced viability compared to non-perfused co-cultures. Furthermore, the highest perfusion rate evaluated, 11.0 μL/min, resulted in >90% cell viability and maintenance of culture thickness. Next generation 3-D neural cultures, with cell types and densities better approximating the CNS, may provide enhanced model fidelity and be valuable in the mechanistic study of cell growth, interactions, and the responses to chemical or mechanical perturbations.**

I. Introduction

Experimental neurobiologists are increasingly utilizing more realistic three-dimensional (3-D) cell culture systems over traditional two-dimensional (2-D), monolayer cell cultures. This 3-D context may allow researchers to investigate cellular behavior in a more physiologically-relevant state, while preserving the primary advantages of traditional *in vitro* systems, such as control of cellular environment, accessibility for repeated imaging, and elimination of systemic effects. Cells cultured in a 3-D environment have been shown to better represent *in vivo* cellular behavior than cells cultured in monolayer (e.g., osteoblastic cells [1]; fibroblasts [2]; breast cells [3]; and neural cells [4,5]). Fundamental differences exist between cells cultured in monolayer versus 3-D configurations in terms of access to soluble factors and the distribution and types of cell-cell and cell-extracellular matrix (ECM) interactions [6-9]. In many cases, cell-cell and cell-ECM interactions may be constrained or completely omitted in planar cultures. Furthermore, culture models consisting of multiple cell types closer approximate the heterogeneity of *in vivo* tissue. With respect to the nervous system, cellular heterogeneity is important to represent such interactions as physical support and metabolic coupling between neurons and astrocytes; see [10-12] for reviews.

Neural cell culture models have been developed to approximate a 3-D orientation by plating dissociated primary cells above a matrix material [13-15]. 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. Other systems have been developed with cells distributed throughout a matrix material [16-19]. However, these cultures have relied on passive diffusion for nutrient delivery and waste product removal, necessitating the use of cell densities much lower than those found in the brain. Thus, technical limitations have prevented the development of 3-D neural cell culture models consisting of a high cell density ($\geq 10^4$ cells/mm^3) with full thickness (>500 μm) cellular distribution.

This work describes the development and validation of a novel engineered neural cell culture system consisting of the following attributes: 1) the use of true three-dimensionality for cell-cell and cell-matrix interactions throughout a full thickness of 500 - 750 μm, 2) a heterogeneous cell population consisting of neurons and astrocytes mixed in a controlled ratio, and 3) the ability to increase 3-D cell density to more closely approximate brain. The latter point was accomplished using a custom-built perfusion system to convectively enhance nutrient delivery and waste product removal beyond that of passive diffusion. This 3-D neural cell culture system may provide enhanced physiological relevance with the ability to systematically approach *in vivo* complexity, making this model suitable for the study of a range of neurobiological phenomena.

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II. METHODS

A. 3-D Neuronal-Astrocytic Co-Culture

Primary cortical neurons (E17-18) were plated with secondary cortical astrocytes (harvested at P0, plated in 3-D between passages 4-10). Dissociated neurons and astrocytes were suspended in Matrigel matrix (10^4 cells/mm^3, 1:1 initial neuron:astrocyte ratio). Cultures were placed at 37°C for 30 min to permit matrix gelation and 3-D cell entrapment (Figure 1a). Each culture consisted of ~2 x 10^6 cells in a 500 – 750 μm thick matrix. Co-culture medium was Neurobasal + 2% B-27 + 1% G-5 + 500 μM L-glutamine (Invitrogen).

B. Perfusion

Co-cultures were plated in custom cell culture chambers consisting of PDMS with a 3 x 3 array of circular Au grids (54 μm x 54 μm entry ports) at the base to permit medium entry (Figure 1b). A perfusion pump provided simultaneous infusion and withdrawal at a controlled rate. Perfused cultures received co-culture medium at flow rates of 1.0, 2.5, 6.25, 11.0 μL/min; non-perfused cultures had 50% of the media changed daily (n = 3 – 5 per condition). Cultures were maintained at 37°C in 5% CO_2 - 95% air.

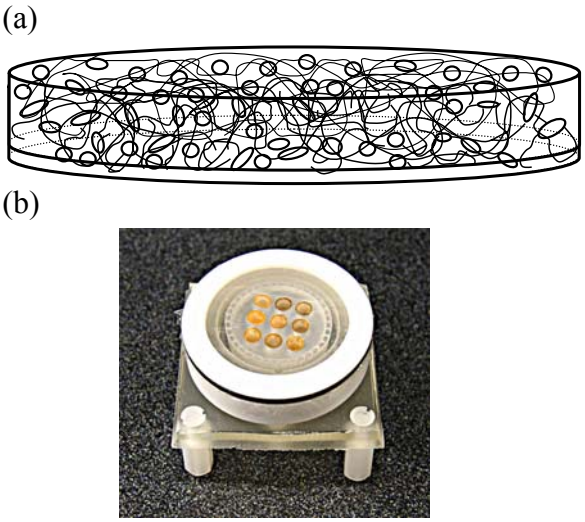

Figure 1: (a) Schematic representation of the 3-D cell culture model used in this study (not to scale). Neuronal-astrocytic co-cultures in 3-D were homogeneously dispersed throughout a 500 - 750 μm matrix. (b) Co-cultures were contained within a custom PDMS mold with perfusion through Au grids at the base.

C. Fluorescent Labeling and Image Acquisition

Cells were labeled using fluorescent probes for distinguishing live and dead cells (LIVE/DEAD Viability/Cytotoxicity Kit; Molecular Probes). At 2 days *in vitro*, cell cultures were rinsed in buffer and incubated with 2 μM calcein AM and 4 μM EthD-1 at 37 °C for 30 min and rinsed in PBS. After viability/cytotoxicity staining, cells were viewed using a Laser Scanning Confocal Microscope (Zeiss 510). Multiple z-stacks (5 - 20 μm plane-to-plane separation) were acquired from the different culture conditions. Confocal images were viewed using LSM Image Browser (Zeiss).

D. Data Collection and Statistical Analysis

Three to five randomly selected regions per culture were analyzed for statistical analysis. Where observed, dead cell nuclei (EthD-1$^+$) and live cells (calcein$^+$) were manually quantified. Data are presented as mean ± standard deviation. Confocal photomicrographs are 50 μm thick reconstructions. General linear model ANOVA was performed followed by Tukey's pairwise comparisons ($p < 0.05$ was considered significant).

III. RESULTS

The effects of continuous medium perfusion on the survival of high cell density, 3-D neuronal-astrocytic co-cultures were assessed in comparison to non-perfused cultures. By 2 days post-plating, there was widespread degeneration in the majority of the non-perfused co-cultures, corroborating previous results demonstrating that this combination of culture thickness and cell density is not suitable to support viable neural cultures reliant upon passive diffusion for nutrient delivery [19]. As degeneration in the non-perfused cultures progressed, there were viable clusters of cells, although the majority of the cells plus matrix were completely degraded (Figure 2). This pattern of degeneration may be caused by unregulated proteolytic activation due to uncontrolled cell death.

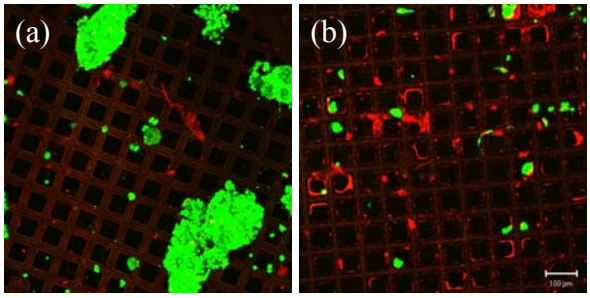

Figure 2: Widespread cell death and matrix degradation was observed in non-perfused, high density 3-D co-cultures.

Continuous medium perfusion was found to attenuate culture degeneration across all flow rates evaluated (1.0 – 11.0 μL/min). Furthermore, culture survival was vastly improved at the higher flow rates evaluated (6.25 - 11.0 μL/min) (Figure 3). Perfused co-cultures were observed to extend processes and form 3-D connections across the full thickness of the cultures.

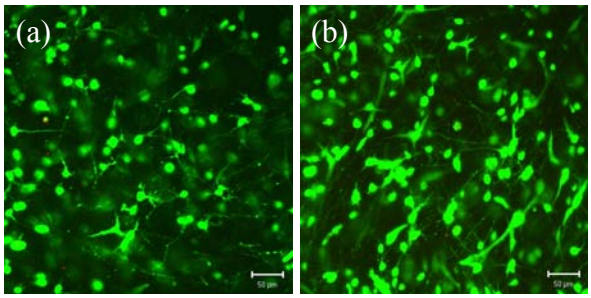

Figure 3: Controlled medium perfusion improves culture survival vs. non-perfused cultures. Cell viability following controlled medium perfusion at (a) 6.25 μL/min and (b) 11.0 μL/min. Reconstructions from the middle of cultures (50 μm thick projection).

The percentage of viable cells was quantified based on perfusion rate (Figure 4). Flow rates in the range of 2.5 – 11.0 μL/min were found to significantly improve culture viability compared to non-perfused cultures ($p < 0.005$). However, a lower flow rate (1.0 μL/min) did not improve survival compared to non-perfused controls. Furthermore, the highest perfusion rate of 11.0 μL/min presented a statistically significant increase in culture viability versus the other flow rates evaluated ($p < 0.05$).

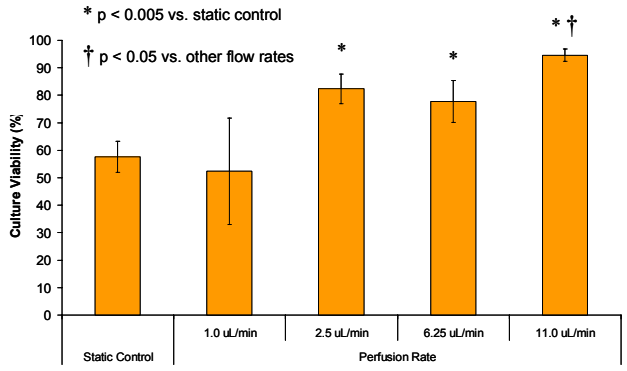

Figure 4: The percentage of viable cells increased with increasing perfusion rate. Media perfused at a continuous rate of 11 μL/min maintained higher culture viability than other flow rates ($p < 0.05$).

IV. Discussion and Conclusions

We have developed a system for the support of thick, high cell density, 3-D neuronal-astrocytic co-cultures using continuous medium perfusion. Non-perfused co-cultures at this thickness and cell density were found to undergo widespread culture degradation by 2 days post-plate, likely signifying that cellular metabolic needs were not met. However, continuous medium perfusion attenuated this culture degeneration, with medium flow rates in the range of 2.5 – 11.0 μL/min significantly improving culture viability. Furthermore, the highest medium flow rate of 11 μL/min (corresponding to ~16 mL/day or ~27 exchanges/day) was found to maintain culture viability to a greater extent than the other flow rates evaluated.

The availability of nutrients via intercellular mass transport must surpass specific metabolic thresholds for 3-D culture systems to support viable cells. The mass transport of this system may be influenced by the culture thickness and the cell density, in addition to the diffusion/consumption characteristics of a particular compound (amongst potentially other factors). Cultures perfused at sufficiently high rates remained healthy while non-perfused cultures experienced widespread degradation and cell death, suggesting that diffusion barriers in nutrient delivery were the predominant reason non-perfused cultures exhibited poor viability. Increases in 3-D cell density likely increases the overall rates of nutrient consumption and waste product production. Additionally, a relatively high 3-D cell density reduces the interstitial diffusion rate such that the nutrient requirements effectively increase. Analogously, mass transfer by passive diffusion becomes inefficient in removing the catabolic waste and thus requires a convective enhancement. Hence, convective circulation creates a more favorable cellular microenvironment by providing sufficient nutrients with concurrent, efficient waste removal, thus augmenting culture viability.

The methodology employed in these studies for the quantification of live and dead cells via fluorescent labels may result in significant underestimation of cell death in the non-perfused cultures. It was observed that, even at the relatively early time-point employed in these studies, that the overall degradation in the non-perfused cultures resulted in loss of cells, hence these dead cells were not directly quantified. Future studies may utilize other biomarkers of cell death, such as those released into the medium by dead or dying cells (e.g., lactate dehydrogenase).

The results of these studies require follow-up based on higher flow rates and longer experimental time-points. Higher flow rates may further improve culture survival; conversely, the ability of the cells to regulate their microenvironment may decrease as convection increases. Also, this work evaluated survival at a relatively early time-point. However, in many cases, neurons require weeks in culture to become functionally mature, thus future work will include evaluating the effects of perfusion on longer-term co-culture viability and functionality. Furthermore, although this work was able to design a system for the support of thick cell cultures at cell densities beyond the support of passive diffusion, the 3-D cell density used in these studies remain far from the densities of 10^5 - 10^6 cells/mm^3 reported in various cortical regions [20,21]. However, given higher perfusion rates and smaller average distances between the cells and fluid entry ports, it may be possible to approach these cell densities *in vitro*.

In vitro models of neural cells have proven effective in the systematic identification of a range of neurobiological phenomena. The 3-D model presented here maintains the advantages of traditional *in vitro* systems while more closely approximating the *in vivo* environment

than many previous models. Specifically, the 3-D neural cell culture model presented here recapitulates many aspects of the *in vivo* environment including 3-D cytoarchitecture, distribution of cell-cell/cell-matrix interactions, and multicellular composition in addition to having a cell density approaching that of brain tissue.

To our knowledge, this is the first report of thick, 3-D neural co-cultures at cell densities beyond those supported by passive diffusion. Next generation engineered 3-D cell culture systems, with extracellular matrix constituents and high cell density, heterogeneous cell populations at ratios inspired by those of brain tissue, may provide a powerful experimental framework for the systematic elucidation of neurobiological function in a controlled setting. Such models may more accurately represent *in vivo* neural responses and develop enabling technologies for neurobiological and tissue engineering applications.

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