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Short communication

Strategies and techniques for development of 3D neuroblastoma cell models

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Introduction

SH-SY5Y human neuroblastoma cells represent one of the most widely utilized *in vitro* models in neuroscience research due to their capacity to differentiate into mature, nerve-like cells. Upon differentiation, these cells develop elongated neurites and complex branching structures that closely mimic human neuronal architecture and network connectivity.

In contrast, the development of three-dimensional (3D) models enables the visualization and study of neuronal cells within an architecture that more closely resembles their native biological microenvironment. Recent advances in imaging, biomaterials, and manufacturing technologies have facilitated the generation of high-fidelity 3D neural models that better recapitulate cell–cell and cell–extracellular matrix interactions compared with conventional two-dimensional cultures. As a result, 3D models provide a more physiologically relevant platform for investigating neuronal development, differentiation, and disease-associated pathological processes. Furthermore, they offer significant advantages for evaluating the efficacy, penetration, distribution, and toxicity of novel therapeutic agents and advanced drug delivery systems, as they more accurately mimic the barriers and microenvironment encountered in *vivo* (Consales et al., 2021). Characterization of these models is crucial for assessing their structural stability, reproducibility, and biological relevance. Therefore, the aim of this study was to develop and compare three different methods for generating 3D neuroblastoma cell models and to evaluate their structural integrity, morphology, and cellular organization.

Materials and methods

Method 1: Ultra-low attachment (ULA) microplates

A single-cell suspension was seeded at a density of 5,000 cells/well (200 µl total volume) in a 96-well ULA plate. The plate was centrifuged at 100 rpm for 2 minutes to initiate cellular aggregation. Cultures were incubated at 37 C˚ with 5% CO_2. Spheroid maintenance was sustained via a periodic 50% medium exchange until mature spheroids with well-defined structures were formed within 7 to 8 days.

Method 2 title: Hanging drop method

Scaffold-free spheroids were generated by pipetting droplets of a cell suspension (250,000 cells/mL, 5,000 cells/drop) onto the inner surface of a culture dish lid. Phosphate-Buffered Saline (PBS) was added to the dish base to prevent droplet evaporation before inverting the lid. Following a 2-to-4-day incubation under standard conditions (37 C˚, 5% CO_2), the aggregated spheroids were

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harvested and transferred to ULA plates for downstream analysis.

Method 3 title: Alginate hydrogel encapsulation

Prior to encapsulation, a 2% (w/v) sodium alginate solution and a 100 mM calcium chloride ($CaCl_2$) cross-linking solution were sterilized using a 0.22 µm filter. A high-density cell suspension (1,000,000 cells/mL) was mixed 1:1 (*v/v*) with the 2% alginate solution to yield a final mixture of 1% alginate (500,000 cells/mL). The mixture was dispensed dropwise in 30 µl aliquots into a Petri dish containing 5 mL of the sterile $CaCl_2$ bath. The resulting beads were incubated for 10 minutes to complete ionic cross-linking. After polymerization, the supernatant was aspirated, the beads were rinsed with PBS, and then transferred to a 12-well plate (4 beads/well) in 1.5 mL of complete culture medium. Cultures were maintained via a 50% medium refresh every 3 days.

Spheroid formation for all three methods was monitored by optical microscopy throughout the 7-day cultivation period, and cell viability was subsequently assessed on day 7 using the trypan blue exclusion assay.

Results and discussion

The choice of 3D culture methodology directly dictated cell viability and structural integrity due to the distinct mechanical handling and mass transport dynamics inherent to each platform. Optical microscopy performed every second day revealed distinct growth patterns among the three 3D culture methods, demonstrating that culture methodology directly influenced spheroid morphology, structural integrity, and cell viability. In the scaffold-free liquid-overlay approach using ULA microplates, cells rapidly self-assembled into compact, highly spherical, and morphologically uniform spheroids, with progressive increases in density observed throughout the cultivation period (Barbosa et al., 2022). This method achieved the highest cell survival rate (70%), likely due to the expansive and stable culture volume, which ensured continuous nutrient diffusion and gas exchange while minimizing physical handling and shear stress during initial low-speed centrifugation. In the hanging drop system, spheroids also formed through cellular aggregation and compaction; however, greater variability in size and morphology was observed during the early stages of development. The lower viability (39%) associated with this method can be attributed to the strict volumetric limitations of the droplets, which promote nutrient depletion, metabolic waste accumulation, and potential hypoxic conditions during the initial 2–4 days of culture. Furthermore, evaporation during early aggregation stages may impose additional osmotic stress on the cells. In contrast, alginate hydrogel encapsulation promoted the formation of the largest spheroids, with a continuous increase in spheroid diameter throughout the cultivation period. However, these spheroids appeared less compact and exhibited lower cellular density than those generated using scaffold-free approaches. Correspondingly, alginate encapsulation resulted in the lowest cell viability (12%), highlighting the physiological limitations imposed by chemical cross-linking and matrix density. Exposure to 100 mM $CaCl_2$ during gel formation may induce osmotic stress and perturb calcium homeostasis, while the dense 1% cross-linked polymer network restricts oxygen and nutrient diffusion, contributing to localized hypoxia and reduced cellular survival (Barbosa et al., 2022).

Conclusion

Overall, the scaffold-free ULA microplate method provided the most favorable balance of spheroid morphology, structural integrity, and cell viability, supporting the generation of reproducible and physiologically relevant 3D neuroblastoma models for disease modeling and therapeutic evaluation.

References

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