Thinking in 3D: Electromagnetic Radiation Exposure in a Novel 3D *in vitro* Model

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Introduction

Widespread use of non-ionizing electromagnetic (EM) radiation in medical, military, industrial, and commercial technologies necessitates understanding the threshold of damage from pulsed radiofrequency (RF), a global public health concern.

High-power EM radiation is used every day for cooking, air traffic control, surveillance, global positioning system (GPS), mobile communications, weather/marine radars, and in medical applications such as tissue/tumor ablation, ultrasound, and magnetic resonance imaging (MRI) scans, to name a few.

For studies of radio frequency (RF) radiation bioeffects, realistic three dimensional (3D) *in vitro* brain models that incorporate the geometry and architecture of the human head/brain require further development. This study aims to develop a novel, cell-based, *in vitro* model incorporating a 3D network of living human brain cells that can be used to study bio-effects of RF radiation and thermoelastic phenomena.

Materials and Methods

2D Expansion: ReNcell VM Human neural progenitor cell line (EMD Millipore) (passage 8-9) was expanded for 3 days in ReNcell NSC Maintenance media (Sigma Aldrich) supplemented with 20ug/mL of human endothelial growth factor (hEGF) and human fibroblast growth factor (hFGF). It is recommended not to passage beyond passage 10.

Differentiation: ReNcells have the capability to differentiate into neurons and glial cells in the absence of specific growth factors: human Fibroblast Growth Factor (hFGF) and human Epidermal Growth Factor (hEGF). ReNcells are seeded in the presence of these growth factors for 3 days to allow for expansion. On Day 4, media is changed, and growth factors were either added or omitted from the media, allowing cells to either remain undifferentiated or differentiate into neurons and glial cells (Figure 3). Differentiation occurred as early as 6 days following removal of growth factors.

Fluorescent staining & Imaging: Cells were stained for anti-human Nestin, SRY-box 2 (SOX2) labelling neural progenitor cells, βIII-Tubulin for differentiated neurons and Glial fibrillary acid protein (GFAP) for glial cells. Next, 2D imaging of differentiation of neural progenitor cells to neurons and glial cells confirmed by fluorescent microscopy using BioTek Cytation5 (Figures 4-5). Brightfield images of hydrogel beads were produced using Nikon TS2 (Figure 3).

Hydrogel Bead Formation: ReNcells were incorporated into a hydrogel matrix (TheWell Bioscience) to create 3D beads (Figure 1). For optimal imaging, hydrogel beads loaded with ReNcells were cultured in 8-well chamber slides. After 4 days, culture media was changed, and growth factors were omitted allowing for differentiation within the hydrogel beads (Figure 3). The cell density, droplet size and culture conditions were optimized for maximal viability and proliferation.

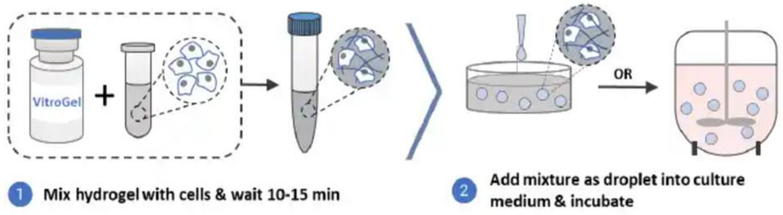


Figure 1. **Hydrogel bead formation procedure** (TheWell Bioscience). Image from TheWell Bioscience https://www.thewellbio.com/product/3d-cell-culture-vitrogel-hydrogel-matrix/

	6 well plate	24 well plate	8 well chamber slide
# of 10µL hydrogel beads/well	60	24	6
# of 12µL hydrogel beads/well	50	20	5
# of 15µL hydrogel beads/well	40	16	4
Volume media/well (µL)	3000	750	400

Table 1. Hydrogel parameters to accommodate for different size wells.

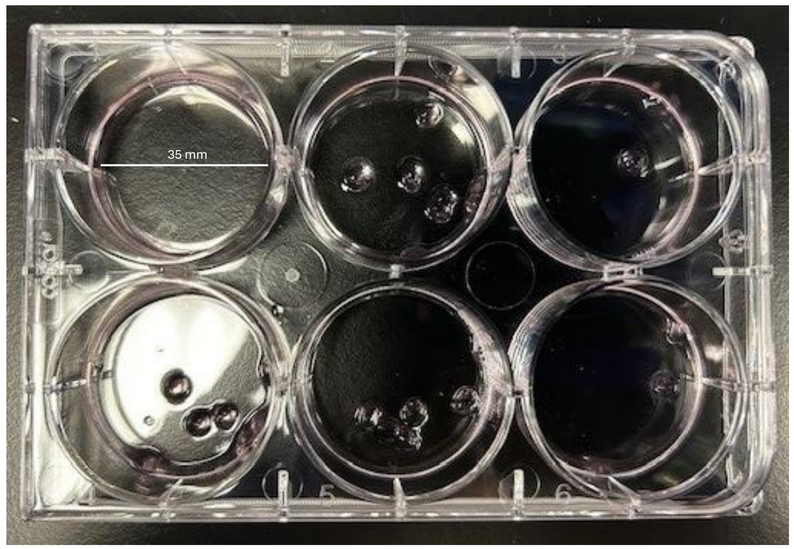


Figure 2. 15uL Hydrogel beads loaded with 1x 10^6 undifferentiated ReNcells plated in a 6 well plate. Six ReNcell-loaded Hydrogel beads were seeded into each well. Media was removed from the wells for visualization purposes.

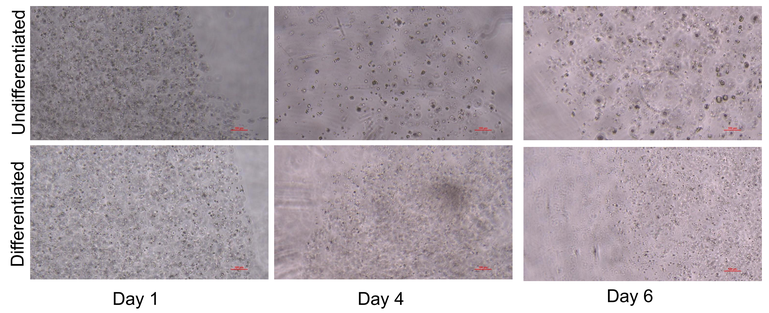


Figure 3. Hydrogel beads loaded with undifferentiated ReNcells (top row) and differentiated ReNcells (bottom row). Days in culture conditions listed under images. All images taken at 10x magnification with Nikon brightfield microscope.

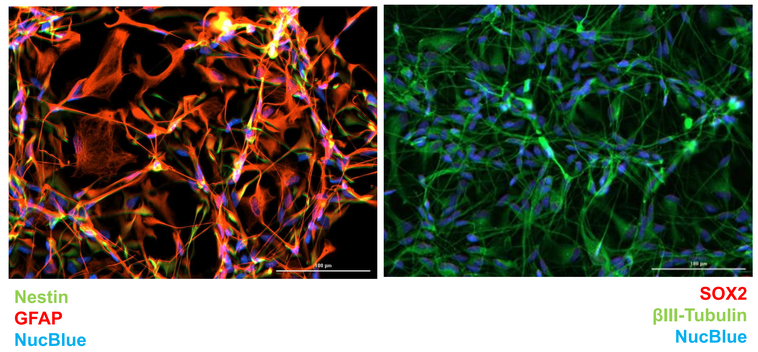
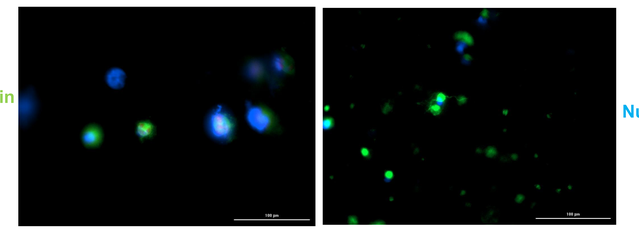
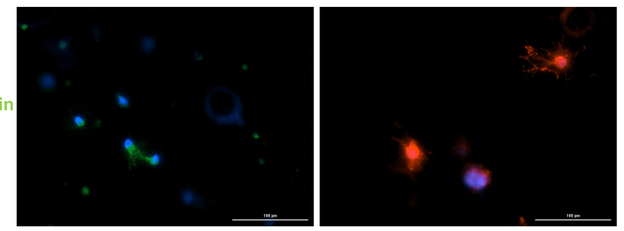


Figure 4. 2D staining of differentiated ReNcells (Day 6) using 2 different mixes. Undifferentiated neural progenitor cells labelled with Nestin and SOX2. Differentiated neurons were labelled with βIII-Tubulin and glial cells labelled with GFAP. NucBlue is a nuclear stain. All images taken at 20x magnification using BioTek Cytation5.

Undifferentiated ReNcells in Hydrogels



Differentiated ReNcells in Hydrogels



Conclusions

Differentiation of ReNcells was verified in 2D cultures by staining of markers of undifferentiated cells (Nestin and SOX2) or differentiated cells (βIII-Tubulin (neurons) and GFAP (glial cells) (Figure 4)). The optimal seeding concentration of neural progenitor cells for use in 3D hydrogel beads is 1-1.5x10^6 cells/mL. 10-15uL bead size is ideal to maintain viable cells within the core of the hydrogel bead. Various plate sizes can be used for this platform and can be optimized for specific use according to table 1. We did observe that at 20uL bead size the bead was too dense and dropped from the pipette tip causing eruption and release of the cells from the hydrogel. Utilization of this 3D model of living brain cells can provide a high-fidelity platform by which to elucidate the molecular, cellular, and neurophysiological effects of RF exposure. This model will serve useful in other studies involving treatment or exposure of complex signaling networks.

Partners/Collaborators

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Navy Medicine Research & Development, with its insight into challenges faced by Sailors and Marines, is uniquely qualified to lead U.S. Navy research efforts to ensure healthy, fit, and lethal service members.
Human subject research protections: The study protocol was approved by the Naval Medical Research Command Institutional Review Board in compliance with all applicable Federal regulations governing the protection of human subjects.