Imaging and Labeling Dense Neuronal Spheroids in 3D

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Highlights

When combined with high content confocal microscopy and immunolabeling, tissue clearing with Visikol® HISTO-M™ allows for imaging of the interior environment of microBrain® 3D cortical spheroid models, dramatically improving quantification of cells in neuronal spheroids.

- The large, dense neuronal microBrain® 3D cortical spheroid model from Stemonix® recapitulates *in vivo* structure and function
- High-Content Imaging along with Visikol's® Permeabilization Buffer and tissue clearing by Visikol® HISTO-M™ enables quantification of immunolabeled cells in the neuronal microBrain® 3D

Introduction

The use of *in vitro* three-dimensional (3D) cell cultures has increased dramatically for drug discovery since 3D cell culture models more accurately mimic the *in vivo* environment compared to traditional monolayer cultures[1]. Many models have been developed to recapitulate the *in vivo* structure and function of a variety of tissues including but not limited to breast, cardiac, liver and bone tissues[2]. However, current imaging-based analysis of these 3D cultures relies upon techniques developed for 2D cell cultures. Due to the thickness of these 3D cell cultures – which is typically >100 µm – light scattering does not permit imaging of the center of the spheroid[3]. This technical limitation introduces a sampling bias in imaging analysis, since only the exterior cells can be imaged and analyzed. Tissue clearing has emerged as a technology for overcoming this limitation, enabling researchers to obtain the most useful and accurate survey of the cellular environment of 3D Cell Culture Models (CCMs).

When performing immunolabeling on large, dense 3D CCMs or organoid models utilized in drug discovery and toxicology research, there is a need to for added permeabilization steps – more than what would be necessary for 2D cell culture – as to allow access to the interior of the model for intracellular and intraorganellar antigens[4]. Permeabilization of the model is the first step to investigating intracellular antigens and once researchers have access to the interior of the models, immunolabeled antigens can be added to investigate the activity of the desired proteins that elicit a physiological response.

Three-dimensional tissue models to study the central nervous system (CNS) are of great interest to researchers as they can allow the investigation on the effects of disease, physical injury toxicology, and drug efficacy on nervous tissue *in vitro*[5]. Neuronal spheroidal models recapitulating this native tissue are large and dense, hindering the evaluation of the entire organoid. Since new therapeutic techniques are being developed and their mechanisms unknown, imaging the interior of the spheroid is critical when testing them[6]. Also, imaging the interior of the spheroid model is crucial when evaluating the neural plasticity before and after the addition of a treatment[7]. Domoic acid is a classic neurotoxicant known to cause Amnesic Shellfish Poisoning (ASP) in humans, and has been implicated in a variety of *in vitro* and *in vivo* studies, ranging from developmental neurotoxicity (DNT) to behavioral and pathological neurotoxicology[8,9]. It is an glutamate analog, and is known to affect both Glutamatergic and GABAergic neurons in the brain, both of which are present in the neuronal 3D CCM used herein.

In this study, we use the microBrain 3D® cortical spheroid model from Stemonix® combined with the Visikol® HISTO™ Permeabilization Buffer and Visikol® HISTO-M™ tissue clearing reagent to evaluate the neurotoxicity of domoic acid on these

organoids designed to model the CNS using high content confocal microscopy.

Materials and Methods

Reagents and cell culture

Neuronal microBrain 3D® tissues were obtained from Stemonix®. Antibodies and fluorescent dyes were obtained from Thermo Scientific and Abcam.

Treatment of Neuronal Spheroids

After receipt, the Stemonix microBrain® 3D Assay Ready 384-Well Plate (#BSARX-AA-0384) spheroids were recovered with BrainPhys™ Neuronal Media supplemented with 1X SM1 neuronal supplement, 20 ng/mL BDNF and GDNF, along with Hyclone Penicillin-Streptomycin. The tissues were kept in the incubator at 37°C, 5% CO_2 for 48 hours to condition before experimental treatments. Domoic Acid stock solution was prepared at a concentration of 10 mM in DI H_2O. The stock solution was then diluted into media to the final dosage concentrations of 100 nM, 1 uM, and 10 uM. The spheroids were treated for 24 hours with compound. After exchanging for fresh media, the tissues were then incubated with Live/Dead™ fixable red dead cell stain (1:1000, in PBS, catalog #L23102) to detect non-viable compromised cells with a fluorescence probe.

Processing of Neuronal microBrain 3D® Tissues

Following fixation at room temperature for 30 min with 10% neutral-buffered formalin, spheroids were washed several times with PBS and subsequently transferred to PBS with 0.05% sodium azide at 4°C before proceeding to permeabilization and immunolabeling steps.

Permeabilization and immunolabeling

The fixed spheroids were processed for immunolabeling using the normal Visikol® HISTO™ protocol with slight modifications due to the large size and density of the neuronal microBrain 3D® cell culture model. The spheroids were first incubated in Visikol® HISTO™ Tissue Permeabilization Buffer for 30 minutes at room temperature then washed 3x for 20 minutes in PBS to thoroughly remove any remaining buffer. Subsequently, spheroids were subjected to a dehydration gradient of 50% methanol (in PBS) for 20 minutes, 80% methanol (in DI H2O) for 20 minutes, and finally 100% methanol for 20 minutes. Then the samples were incubated at room temperature in 20% DMSO/methanol for 30 minutes, then in 80% methanol (in H2O) for 20 minutes, 50% methanol (in PBS) for 20 minutes, PBS for 20 minutes, and finally in PBS/1% Triton™ X-100 for 30 minutes. Spheroids were incubated in Visikol® HISTO™ Penetration Buffer for 30 minutes at room temperature with gentle shaking followed by Visikol® HISTO™ Blocking Buffer at 37°C with gentle shaking for 1 hour. The primary antibody dilution was prepared in Visikol® HISTO™ Antibody Buffer which was then passed through a syringe filter (0.45 µm) prior to use. For anti-glutamine synthetase (ab176562), the dilution was 1:100. Samples were incubated at 37°C with gentle shaking for 12 hours.

The spheroids were washed with Visikol® HISTO™ Washing Buffer (1X working concentration) several times at 37°C before adding the secondary antibody. For the secondary antibody (Goat anti-Rabbit AlexaFluor 488, ab150077) a dilution of 1:400 was prepared in Antibody Buffer and filtered with a syringe filter prior to use. The samples for were incubated for an additional 12 hours at 37°C. The spheroids were washed again several times with Washing Buffer, and finally with PBS to remove any traces of detergent/antibody.

Clearing and high throughput imaging of microtissues

Spheroids were treated with a dehydration gradient at room temperature starting with 50% methanol in DI H2O for 20 minutes, then 100% methanol for 20 minutes with gentle shaking. Visikol HISTO-M can absorb up to 10% H2O by volume, so it is imperative to remove as much water from the wellplate in which the 3D cell culture has been processed. If removal of excess water is not feasible, adding a dehydration step may be necessary to ensure effective clearing. After dehydration, Visikol HISTO-M was added to clear the spheroids. Imaging of was conducted in Corning® 96 half-well area HCS plate with a CX7-LZR High Content Screening platform confocal imager (ThermoFisher). Multi-channel z-stacks were collected for each tissue, using 5 µm steps at 20X magnification. Images were processed and cells were and counted using ImageJ.

Results and Discussion

Quantification of Glutamine Synthetase Positive Cells

Glutamine synthetase (GS) is an astrocytic enzyme responsible for catalyzing reversible transformation of glutamine to the neurotransmitter glutamate, along with gamma-aminobutyric acid (GABA). The baseline expression of which is necessary for maintaining a balance of the flux of those neurotransmitters. Domoic acid is a glutamate analog and therefore can be used as an agonist with excitotoxic effects the brain. These effects on both GS expression and overall cell health in the cortical spheroid models were measured using high content imaging coupled with fluorescence immunolabeling and dead cell detection. The results are presented below (Figures 1-3). After 24 hours of treatment at various doses (0.1, 1, 10 µM), it is seen that the number of GS positive cells declines, from ~28%

of the total cell population to ~13%. There is also a commensurate decrease in total cells (along with spheroid size), suggesting the exposure to domoic acid induces more than a downregulation of GS. From the results of the signal area measurement of the dead cells, we see that increasing dosage of domoic acid starts to induce cell death and overall lower viability. The volume of dead cells increases from ~1.5% to 10% and the spheroid diameter decreases from an average of 510 µm to 450 µm. Additionally, the concentration of dead cells seems to be clustered in certain regions of the spheroid, usually polarized to some degree. This effect is attributed most likely to concentration gradients that emerge within the smaller wells of the 384 well plate.

Characterization of Neural Cell Morphology with MAP2 Immunolabeling

Immunolabeling for MAP2 on treated and non-treated cortical spheroid models was also performed to characterize the effect that domoic acid treatment has on overall neuronal morphology (Figure 4). It is interesting to note that the expression levels decrease substantially with increased toxicant concentration, which suggests neurons exposed to the excitotoxic conditions for long enough may experience some destabilization of structure within these models.

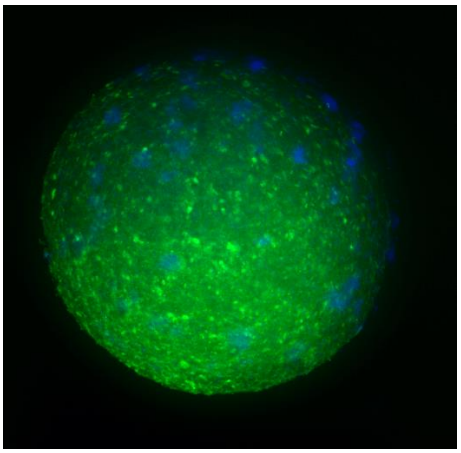

Figure 1. Z-Projection Image of microBrain® 3D cortical spheroid labeled with anti-Glutamine Synthetase (Abcam, ab176562) counterstained with DAPI.

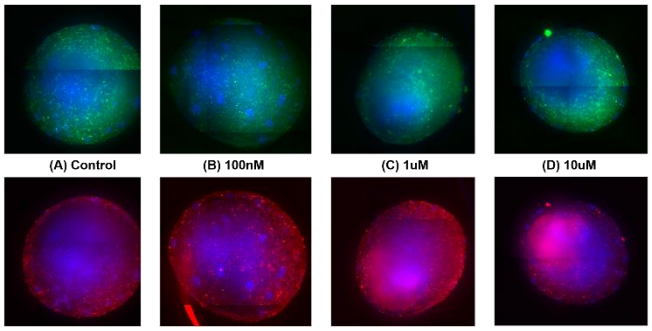

Figure 2. Projections of cortical spheroid models labeled with anti-Glutamine Synthetase (top row), and Live/Dead Fixable dead cell stain (bottom row, Thermo, L23102), counterstained with DAPI.

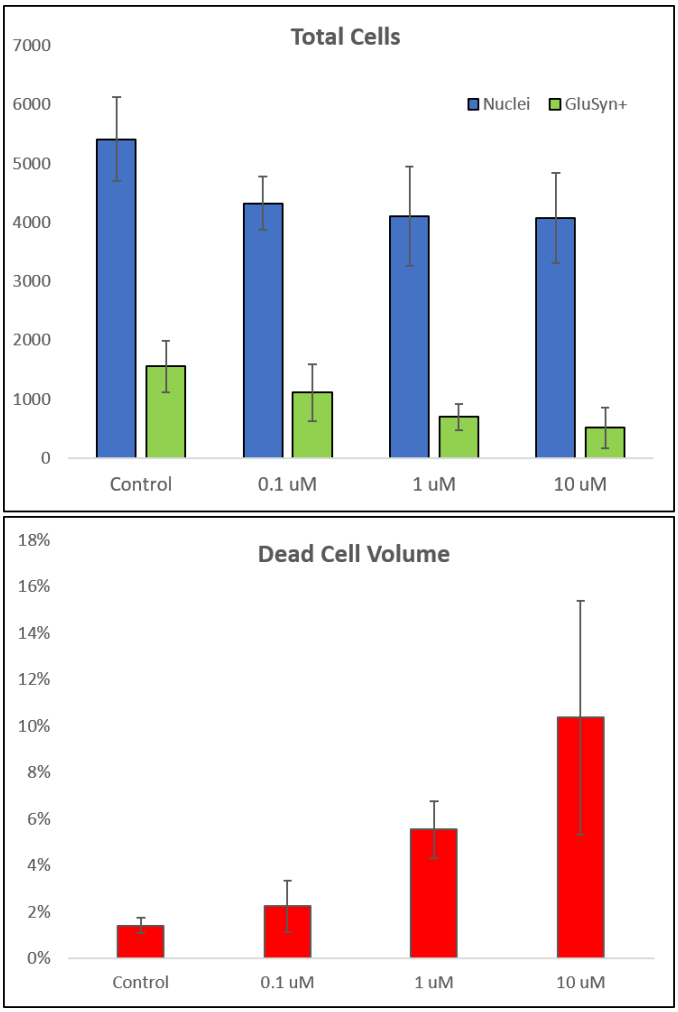

Figure 3. Graphical results from image quantification: Cell counting (top) and normalized dead cell area (bottom) are shown. The bar graphs are averages with standard deviation error bars. The dead cell area was quantified by measuring the total signal volume for the LiveDead Red probe and normalizing by the total spheroid volume.

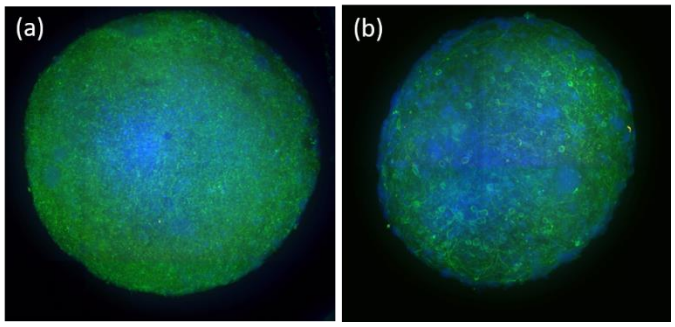

Figure 4. microBrain 3D model labeled with anti-MAP2 (green, Abcam, ab183830) counterstained with DAPI for (a) control and (b) 10 µM domoic acid-treated cortical spheroids.

Conclusions

- Using Visikol® HISTO™ Permeabilization Buffer and Visikol® HISTO-M™ coupled with confocal imaging, the dense microBrain 3D® cortical spheroid models can be immunolabeled visualized successfully.
- Visikol® HISTO-M™ is compatible with immunolabeling and fluorescent staining of microBrain 3D® tissues.
- Neurotoxic effects on *in vitro* neuronal models can be measured with immunofluorescence-based endpoints.

References

1. LaBarbera, D. V., Reid, B. G., & Yoo, B. H. (2012). The multicellular tumor spheroid model for high-throughput cancer drug discovery. *Expert opinion on drug discovery*, *7*(9), 819-830.
2. Elliott, N. T., & Yuan, F. A. N. (2011). A review of three-dimensional in vitro tissue models for drug discovery and transport studies. *Journal of pharmaceutical sciences*, 100(1), 59-74.
3. Kabadi, P. K., Vantangoli, M. M., Rodd, A. L., Leary, E., Madnick, S. J., Morgan, J. R., & Boekelheide, K. (2015). Into the depths: Techniques for in vitro three-dimensional microtissue visualization. *Biotechniques*, *59*(5), 279.
4. Jamur, M. C., & Oliver, C. (2010). Permeabilization of cell membranes. *In Immunocytochemical methods and protocols* (pp. 63-66). Humana Press.
5. Dingle, Y. T. L., Boutin, M. E., Chirila, A. M., Livi, L. L., Labriola, N. R., Jakubek, L. M., ... & Hoffman-Kim, D. (2015). Three-dimensional neural spheroid culture: an in vitro model for cortical studies. Tissue Engineering Part C: Methods, 21(12), 1274-1283.
6. Wardill, H. R., Mander, K. A., Van Sebille, Y. Z., Gibson, R. J., Logan, R. M., Bowen, J. M., & Sonis, S. T. (2016). Cytokine-mediated blood brain barrier disruption as a conduit for cancer/chemotherapy-associated neurotoxicity and cognitive dysfunction. International journal of cancer, 139(12), 2635-2645.
7. Mattson, M. P. (2004). Pathways towards and away from Alzheimer's disease. Nature, 430(7000), 631.
8. Pulido, Olga M. (2008). Domoic Acid Toxicologic Pathology: A Review. Marine Drugs, 6(2), 180-219.
9. Hogberg, H.T., Sobanski, T., Novellino, A., Whelan, M., Weiss, D.G., Bal-Price, A.K. (2011). Application of micro-electrode arrays (MEAs) as an emerging technology for developmental neurotoxicity: Evaluation of domoic acid-induced effects in primary cultures of rat cortical neurons. NeuroToxicology, 32, 158-168.

Getting Started

1. Visit www.visikol.com to learn more about using Visikol HISTO-M with microtissues.
2. Consult with our scientists to develop a customized labeling and imaging workflow.
3. Collaborations begin with pilot projects which can then be scaled up according to your requirements.
4. We work end-to-end to help you take advantage of 3D High Content Imaging assays in your workflow.