The advantages of CNS-3D Brain Organoids for functional neuromodulation studies

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Background and Purpose

A recent surge in support for microphysiological systems (MPS) in drug development has put a spotlight on human induced pluripotent stem cell (hiPSC)-derived technologies to improve clinical translation. One of the most attractive aspects of hiPSC-derived models is their scale and compatibility with high-throughput screening (HTS), though this requires high sample-to-sample reproducibility to be effective. Traditional neural spheroid models typically combine terminally differentiated neuronal subtypes and glia, enabling precise control of cellular ratios and regional specification of brain identity[1]. Alternatively, neural organoids utilize hiPSCs as starting material thereby permitting differentiation into a variety of neuronal subtypes, though often at the cost of high variability in size and cell composition between organoids. To this end, 28bio has developed CNS-3D Brain Organoids (CNS-3D). This hiPSC-derived cortical organoid technology uses a scalable batch production process which yields a co-differentiated mixture of key neural cell populations aligned with human neurodevelopment[2]. Here we present the advantages of using CNS-3D Brain Organoids and traditional spheroid approaches when performing *in vitro* neuromodulation assays.

Methods

CNS-3D Brain Organoids were generated using a homogenous neural progenitor cell (NPCs) starting material and were seeded into ultra-low attachment 384-well plates. Over the course of the differentiation process, progenitor cells co-differentiated into approximately equal proportions of neurons (of which 90% were glutamatergic, 10% GABAergic neurons) and astrocytes and developed stable spontaneous coordinated network activity. Prefrontal cortex spheroids (PFC spheroids) were generated using healthy, terminally differentiated iPSC-derived neurons (90%) and iCell Astrocytes (10%) from Fujifilm CDI and seeded into ultra-low attachment 384-well plates as previously described[1]. The neural population in these spheroids was comprised of 70% iCell GlutaNeurons, 30% iCell GABAneurons. Following 9 weeks of CNS-3D differentiation and 3 weeks of spheroid formation[1], multiple assays were performed to compare the morphology (brightfield imaging), cellular content (ICC) and spontaneous activity (calcium imaging, FLIPR) of the two distinct models.

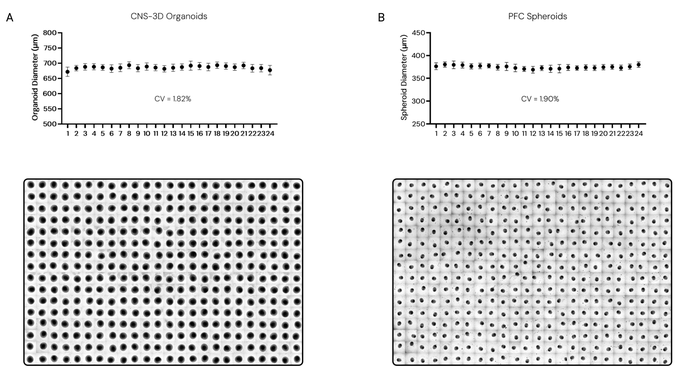


Figure 1: **(A)** CNS-3D Brain Organoids and **(B)** PFC spheroids were produced using identical seeding methods and maintenance protocols. Both models exhibited consistent size distributions (CV <2%) across 384-well plates, with organoid/spheroid size proportional to the initial seeding density (25,000/10,000 cells per well).

Organoid versus Spheroid Characteristics

CNS-3D Brain Organoids and PFC spheroids were compared in terms of cellular content, baseline functional activity, and morphology. CNS-3D Brain Organoids exhibit greater circularity and structural uniformity, along with lower FLIPR peak frequency and higher amplitude, resulting in a high signal-to-noise ratio. In contrast, PFC spheroids showed reduced circularity, potentially reflecting heterogeneous distribution of cell types as observed in ICC. Furthermore, higher frequency, lower amplitude signals may limit assay sensitivity and interpretability.

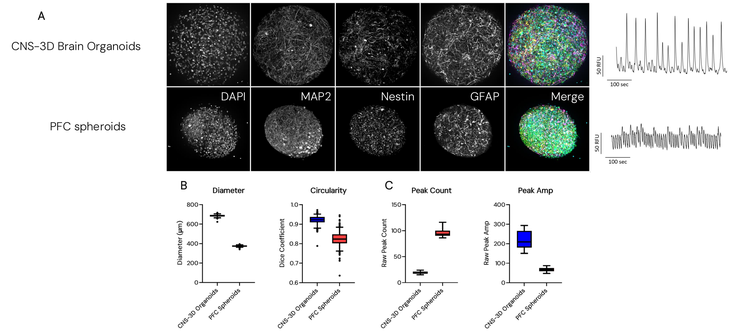


Figure 2: **(A)** Immunostaining for nuclei (DAPI), neurons (MAP2), neural progenitor cells (Nestin) and astrocytes (GFAP) and representative FLIPR waveforms for CNS-3D Brain Organoids and PFC spheroids. **(B)** Diameter and circularity measurements across an entire 384-well. **(C)** Raw FLIPR Peak Count and Peak Amplitude measurements of spontaneous calcium oscillations at baseline.

Patterns of Functional Activity

Each model was next evaluated for consistency in spontaneous neural activity. CNS-3D Brain Organoids exhibit distributed well-to-well variability that likely reflects inherent biological heterogeneity and more complex cortical biology. In contrast, PFC spheroids show pronounced sensitivity to edge effects, leading to spatially driven variability that can compromise data consistency and reduce usable wells.

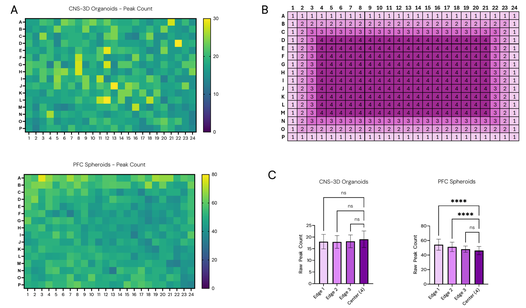
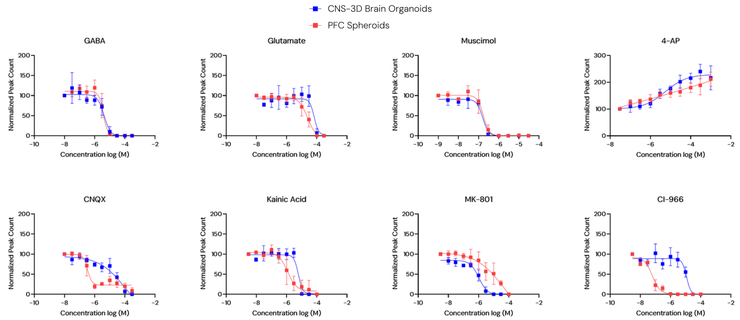


Figure 3: **(A)** Heatmap of raw peak count measurements for both CNS-3D Brain Organoids (top) and PFC spheroids (bottom). **(B)** The schematic used to analyze the edge effects starting on the outside row 1 through the center (labeled 1-4). **(C)** Analyzed edge effects compared from the outer 3 rows to the center of the 384w plate. Statistical significance was assessed by 1-way ANOVA followed by Tukey's post-hoc testing ns = not significant, **** $p < 0.0001$

Functional Response to Various Neuromodulators

Pharmacological responses to eight small molecule neuromodulators were used to assess differences between the two models. Both CNS-3D Brain Organoids and PFC spheroids show clear functional changes to GABA and glutamate agonists (GABA, glutamate, and muscimol) and channel blockers (4-AP). Differential functional responses to NMDA and AMPA receptor agonists (CNQX, MK-801, and kainic acid) are consistent with the relative makeup of glutamate receptor subtypes found in CNS-3D. In contrast the functional response to the GABA reuptake inhibitor (CI-966), which acts on endogenous GABA, is likely due to the higher proportion of terminally differentiated GABAergic neurons in the PFC spheroids. Overall, CNS-3D Brain Organoids exhibit more pharmacologically distinguishable response profiles compared to the more linear, and less discriminative response in PFC spheroids.



Conclusions & Future Directions

CNS-3D Brain Organoids enable human-relevant biological complexity, while maintaining high consistency, supporting their use as a versatile and robust functional screening technology. Specific findings include:

- CNS-3D showed a higher circularity when compared to the PFC spheroids, potentially due to the progenitor cell population encouraging a more homogenous cellular structure.
- CNS-3D exhibited higher signal (Peak Amplitude) to noise relative to PFC spheroids.
- PFC spheroids showed significant edge effects, common to many HTS assays, potentially impacting downstream assay interpretability and limiting well usage.
- Well-to-well functional variability is distributed evenly across CNS-3D Brain Organoids, an expected result of the complex co-differentiation protocols.
- Differential pharmacological responses reflect underlying biological differences in each model.

References

[1] Strong, C.E., Zhang, J., Carrasco, M. et al. Functional brain region-specific neural spheroids for modeling neurological diseases and therapeutics screening. Commun Biol 6, 1211 (2023).

[2] Wang Q, Cohen JD, Yukawa T, Estrella H, Leonard C, Nunes J, Choi C, Lewis L, Baker KS, Kuga K, Dragan YP, Wagoner MP, Mishra N. Assessment of a 3D neural spheroid model to detect pharmaceutical-induced neurotoxicity. ALTEX. 2022;39(4):560-582. doi: 10.14573/altex.2112221. Epub 2022 Apr 19. PMID: 35502629.