Novel analysis of neural outgrowth in 3D human brain micro-tissues with application in compound screening

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Abstract

Organotypic three-dimensional (3D) cultures resemble native 3D tissue architecture and are believed to be more representative of real tissues than their 2D (monolayer) counterparts, thus providing higher *in vivo* relevance. Brain organoids attract a lot of interest due to their physiological relevance for the study of neurological development and disease. However, while they show great promise, brain organoids tend to be difficult to grow, and demonstrate variable shape, size, neural network density, and light transmissibility, thus rendering many unsuitable for high-throughput screening. Some of this variability can be attributed to extended maturation times, and the batch-to-batch variability of commonly used extracellular matrix (ECM) mimetics such as Matrigel® or Geltrex™ products.

RealBrain® micro-tissues from Tessara Therapeutics combine high physiological relevance with reproducibility at scale and provide an ideal platform for development of optimized protocols for 3D neurite outgrowth analysis. RealBrain micro-tissues are generated by encapsulating primary or induced pluripotent stem cell (iPSC)-derived neural precursor cells in a proprietary, chemically defined hydrogel that provides the optimal micro-environment to activate endogenous programs of neurodevelopment *in vitro*. The developing cells proliferate, remodel their micro-environment, migrate, self-organize in 3 dimensions, and replace the engineered hydrogel with cell-secreted ECM. Furthermore, the optical clarity of the RealBrain constructs is ideally suited for high-content imaging applications.

In this study, we imaged RealBrain micro-tissues treated with compounds that are reported to either stimulate (Pentadecanoic Acid) or inhibit (Rotenone) neurite outgrowth and stained with fluorescent markers using the ImageXpress® Micro Confocal High-Content Imaging System. We then reconstructed these tissues in 3D and developed a method to quantify the number of neurons and their respective outgrowths using MetaXpress® High-Content Image Acquisition and Analysis Software. With this approach, we confirmed the efficacy of the compounds with the quantification of neurite outgrowth analysis and found that NGF and pentadecanoic acid significantly increase while Rotenone reduces the number of neurites compared to the control. Thus, this type of analysis can be used in multiple applications, such as assessing the effects of compounds on neurons and neural networks.

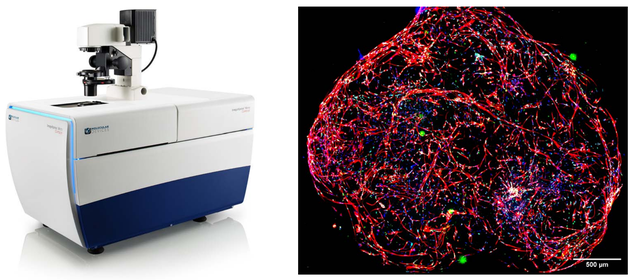

Figure 1. ImageXpress Micro Confocal High-Content Imaging System and RealBrain micro-tissue.

Methods

- We used the ImageXpress Micro Confocal High-Content Imaging System equipped with spinning disk confocal and sCMOS camera to capture the 3D structures of the whole micro-tissues stained with antibodies in different fluorescent channels.
- We used the RealBrain drug screening platform produced by Tessara Therapeutics that features human mimetic 3D brain micro-tissues. The automated manufacturing process from cell encapsulation to mature micro-tissue formation takes 3 weeks and is highly controllable and reproducible. Micro-tissues can be produced in both 96- and 384-well formats.

Imaging and analysis

High-content imaging of 3D human brain micro-tissues

To characterize the 3D brain micro-tissues and investigate their potential in compound screening, we treated the micro-tissues with drugs reported either to inhibit neurite outgrowth (1µM Rotenone) or to promote the neurite outgrowth (1µM Pentadecanoic acid (PD acid)) for 72 hours. The resulting samples were then fixed at end point and labeled with mouse anti-human Beta (III)-Tubulin and chicken anti-human GFAP primary antibodies, which were further stained by anti-mouse Alexa-488 and anti-chicken Alexa-594 together with DAPI. We then leveraged high-content imaging to capture the z stacks of micro-tissues in 3D (Fig. 2). As compared to the control without any drug treatment (Fig. 2A), the micro-tissues treated with 1µM Rotenone demonstrated a significant reduction in the complexity and length of the neurites. In contrast, those treated with 1µM PD acid showed more elaborate networks and longer neurites; both outcomes align with the reported function of the drugs.

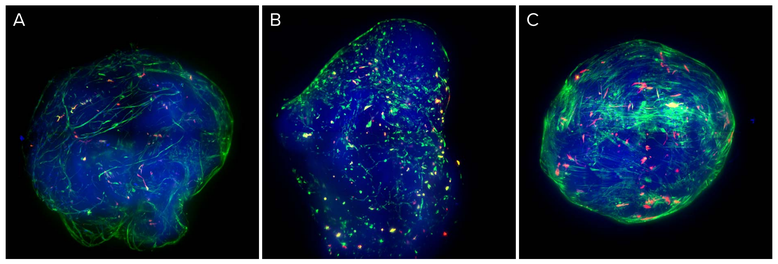

Figure 2. Overlays of 2D projections of fluorescent channels of 3D brain micro-tissues, with tubulin stained with Alexa-488 (green) and GFAP stained with Alexa-594 (Red). (A) The untreated micro-tissue (Control); (B) the micro-tissue treated with 1µM Rotenone, known to inhibit neurite outgrowth; (C) the micro-tissue treated with 1µM Pentadecanoic acid, known to promote neurite outgrowth.

Neurite outgrowth analysis workflow

Developing neurons produce new projections as they grow and integrate into a neural network. As a result, assessing the extent of neurite outgrowth is an important quantification of neural network complexity. Such quantification requires a tightly orchestrated and integrated workflow that renders visualization and analysis of these complex biological samples, at scale. Thus, we leveraged the Custom Module Editor (CME) from MetaXpress High-Content Image Acquisition and Analysis software and developed a workflow to extract the information of number of neurites, number of cell bodies and number of nodes (Fig. 3).

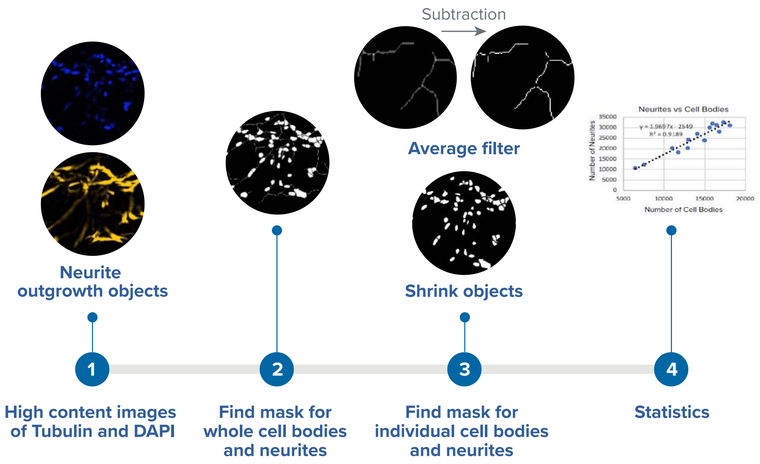

Figure 3. The neurite outgrowth analysis workflow comprises 3 major steps. The first step uses the Neurite Outgrowth Objects module from CME to generate the whole mask for the cell bodies and neurites. The second step extracts the separate masks for individual cells bodies and neurites. The last step exports the numbers for the statistics.

Results

Neurite outgrowth analysis with application in drug screening

The total number of neurites, total number of cell bodies, and total number of nodes (the crossing of two neurites) per micro-tissue are counted and displayed in Fig. 4A and Fig. 4B. With a sample size of seven for each of the treatments, we observe on average 1.13, 0.91 and 0.18 neurites per cell bodies for 1µM PD acid, Control and 1µM Rotenone. The total neurite length versus the micro-tissue volume are plotted in Fig. 4C, where three clusters are separated, and the separation is dominated by the total neurite length per micro-tissue.

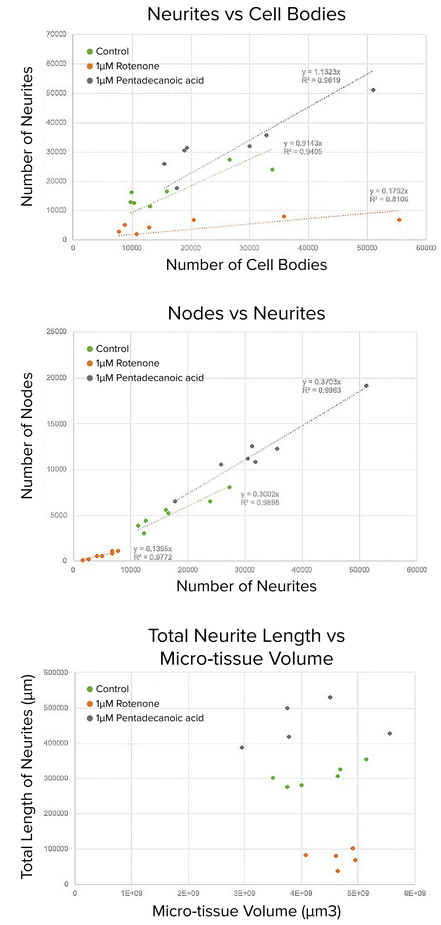

Figure 4. Statistics of neurite outgrowth analysis. (A) Total number of neurites versus total number cell bodies per micro-tissue. (B) Total number of nodes versus total number of neurites per micro-tissue. (C) Total neurite length versus the micro-tissue volume.

Conclusion

A robust approach for neurite outgrowth analysis

- We implemented high-content imaging of Tessara's 3D brain micro-tissues with about 1mm z-depth and three fluorescent channels using the ImageXpress Micro Confocal High-Content Imaging System.
- We leveraged CME's built-in modules to find and modify objects and images, customize analysis algorithms, and gain deeper insights.
- We also successfully developed an algorithm to analyze the neurites, nodes, and cell bodies from the human brain micro-tissue.
- This innovative combination of high-content imaging with 3D human brain micro-tissues was applied to drug screening and revealed greater mechanistic insights as an industrial drug-screening platform.

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