Clarifying mid-brain organoids: Application of the CLARITY protocol to unperfusable samples

Chiara Magliaro,[1] Arti Ahluwalia[1,2]

[1]Research Center "E. Piaggio", University of Pisa, Pisa; [2]Department of Information Engineering, University of Pisa, Pisa, Italy

Abstract

The aim of this study was to apply a workflow, integrating delipidation methods and advanced 3D imaging techniques for mapping of the global neuronal organization of brain organoids. These are self-organizing constructs *in vitro* generated from human pluripotent stem cells encased in a Matrigel shell, which resemble downscaled structural and functional features of human brains. In particular, we focused on mid-brain organoids, widely considered a promising tool for studying dopaminergic neuron degeneration in Parkinson's Disease. The evaluation of the micro-anatomical alterations at a patient-level will potentially guide future research of this neuropathy, providing meaningful human specific data in line with the European Directives and the 3Rs principles.

Introduction

Brain organoids have enormous potential as downscaled *in vitro* models of the human brain. In particular, human induced pluripotent stem cells-derived organoids are crucial for exploring brain development and pathogenesis in a patient-oriented perspective.[1] Mapping organoid structural organization of the cells is important for predicting alterations in dendritic and axonal arbour associated with neuro-pathology, as well as for inferring how uniquely human features are managed at the cellular level.

In order to image the whole brain organoid micro-structure without cutting it, different optical clearing protocols have been developed in the last decades.[2] Basically, tissue clearing involves exchanging the water in the sample with organic solvents or aqueous solutions with the same refractive index as membrane lipids, such that the samples become essentially transparent, thus extending the depth range of optical microscopes. Clearing techniques such as CLARITY further allow permeability to macromolecules, also providing molecular phenotyping compatibility.[3,4] Although they were originally developed for whole animal perfusion, clearing protocols can be adapted for use on vessel-free, non-perfusable samples.

Here we describe how the integration of de-lipidation methods and advanced imaging could lead to a high-fidelity mapping of the global neuronal organization within the organoids. We applied the workflow to mid-brain organoids, generated for studying dopaminergic neuron degeneration in Parkinson's Disease.

Materials and Methods

Mid-brain organoids were generated as in Berger *et al*.[5] and clarified customizing the CLARITY protocol for unperfusable samples. Briefly, organoids were immersed in 20 mL of hydrogel monomer (4% PFA, 4% acrylamide, 0.05% bis-acrylamide and 0.25% VA-044 thermally triggered initiator) for 7 days at 4°C allowing gel passive diffusion. After hydrogel polymerization at 37°C at vacuum, each sample were immersed in 20 mL of clearing solution (200 mM of boric acid, 4% SDS, pH adjusted to 8.5 adding 1M NaOH dropwise) at 37°C, refreshing every 3 days for 3 weeks. Organoids embedded in the hydrogel and immersed in 20 mL 1X PBS solution were used as controls.

The organoids were immunolabelled with Thy rabbit anti-human (1:1000 for 48 hours at 4°C) and goat anti-rabbit Alexa Fluor 488 (1:500 for 24 h at 4°C) antibodies to identify dopamine-positive neurons. In addition, organoids were immersed in a DAPI solution (1:1000 for 30 minutes) for nuclei identification. The samples were acquired using a Nikon A1 confocal microscope and a 10x objective.

Correspondence: Chiara Magliaro, Research Center "E. Piaggio", University of Pisa, Pisa, Italy.
E-mail: chiara.magliaro@centro3r.it

Key words: Brain organoids; delipidation methods; CLARITY; 3D imaging; neuron morphology.

Conference presentation: this paper was presented at the Second Centro 3R Annual Meeting - 3Rs in Italian Universities, 2019, June 20-21, University of Genoa, Italy.

Received for publication: 28 October 2019.
Accepted for publication: 11 November 2019.

Biomedical Science and Engineering 2019; 3(s3):113
doi:10.4081/bse.2019.113

Results

Figure 1 shows how CLARITY makes the samples permeable to both photons and exogenous macromolecules. In fact, the image stacks of the samples clarified and then acquired with the confocal microscope showed a dataset with a good contrast-to-noise (CNR) and signal-to-noise (SNR) ratio: nuclei are well defined and dopamine-positive neurons can be easily tracked. Staining procedures were not performable on unclarified mid-brain organoids, since the antibodies seem to be stuck within the Matrigel network, *i.e.* a necessary feature of current brain organoid generation protocols, surrounding the sample – image not shown – and therefore cannot penetrate within the 3D constructs.

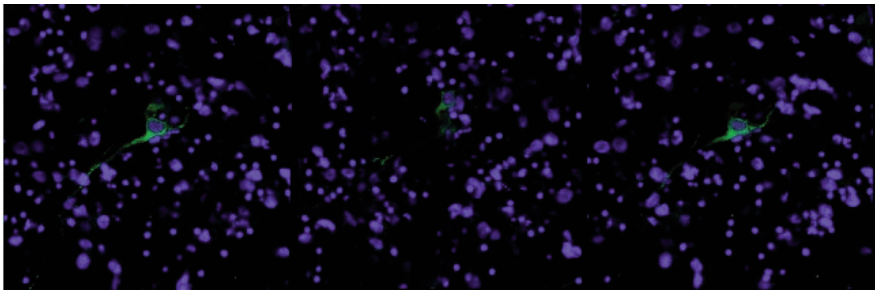

Figure 1. A clarified organoid at different optical sections.

Conclusions

We demonstrated that clarification protocols can be adapted to vessel-free 3D *in vitro* constructs. The confocal datasets obtained are characterized by an improved SNR and CNR, which can facilitate both 3D neuron segmentation and extraction of neuron morphometric features, thus obtaining an unprecedented representation of their 3D cellular structure. A rigorous workflow for establishing the best clearing practise as well as the optimization of the immunolabelling procedures for thick samples in terms of antibody concentration and staining times are on-going to avoid much of the trial and error usually affecting these methodologies.

References

1. Lancaster MA, Renner M, Martin CA, et al. Cerebral organoids model human brain development and microcephaly. Nature 2013;501:373-9.
2. Richardson DS, Lichtman JW. Clarifying tissue clearing. Cell 2015;162:246-57.
3. Chung K, Wallace J, Kim SY, et al. Structural and molecular interrogation of intact biological systems. Nature 2013;497:332-7.
4. Magliaro C, Callara AL, Mattei G, et al. Clarifying CLARITY: quantitative optimization of the diffusion based delipidation protocol for genetically labeled tissue. Front Neurosci 2016;10:179.
5. Berger E, Magliaro C, Paczia N, et al. Millifluidic culture improves human midbrain organoid vitality and differentiation. Lab Chip 2018;18:3172-83.