

# Single-Cell RNA Sequencing Analysis of Regionally Patterned Human Pluripotent Stem Cell-Derived Neural Organoids

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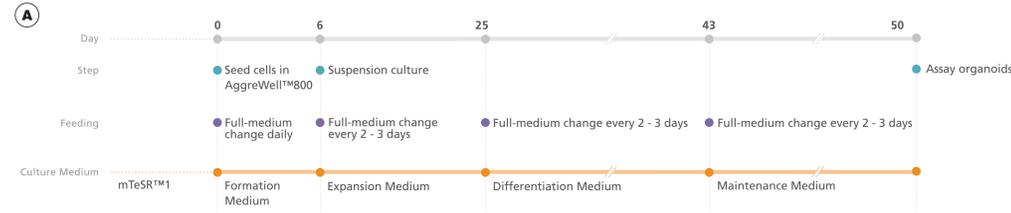
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## INTRODUCTION

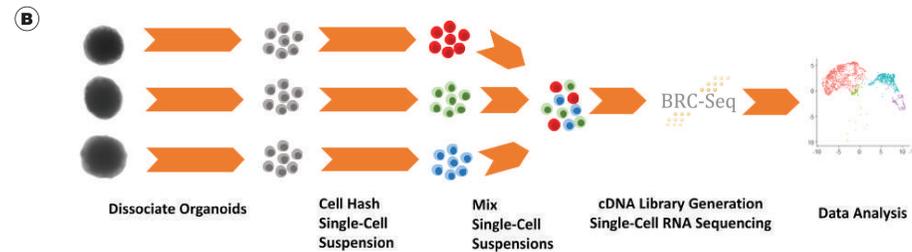
Human pluripotent stem cell-derived 3D neural organoids have been shown to recapitulate the major features and cytoarchitecture of early human brain development. Advancements in the neural organoid culture system from the lab of Sergiu Pașca<sup>1</sup> was optimized and used as the core technology for the STEMdiff™ Dorsal and Ventral Forebrain Organoid Kits to generate brain-region-specific organoids representing the dorsal and ventral forebrain. As a model of the early brain patterned with developmental cues, these neural organoids contain unique and diverse cell types. Here we sought to investigate the cellular diversity of these organoids using single-cell RNA sequencing, which has become the gold standard in elucidating the cellular composition of complex tissues. We generated dorsal and ventral forebrain organoids using the stem cell line H9 that were subjected to single-cell dissociation on day 50 using an optimized dissociation protocol. We further utilized cell hashing to label multiple organoids to explore intra-organoid variability within a culture. Our results show that dorsal forebrain organoids contain a diverse array of cell types including glutamatergic neurons (60%), radial glial cells (20%), and neural progenitors (10%), whereas ventral forebrain organoids contain GABAergic neurons (70%) and ventral progenitors (25%). These results indicate the capacity of STEMdiff™ Dorsal and Ventral Forebrain Organoid Kits to generate highly pure forebrain tissue containing cell types observed during development and devoid of contaminating non-neural cell types. Multiplexed samples also showed low intra-organoid variability. Furthermore, comparative analyses revealed that the organoid cell composition and gene expression of key markers are consistent with those observed in organoids from published protocols and were comparable to publicly available data on developing fetal brain tissue. Our results demonstrate that STEMdiff™ Dorsal and Ventral Forebrain Organoid Kits can generate models of the developing telencephalon and enable further study of the complexity of brain development and disorders in vitro.

## METHODS

### Neural Organoid Formation



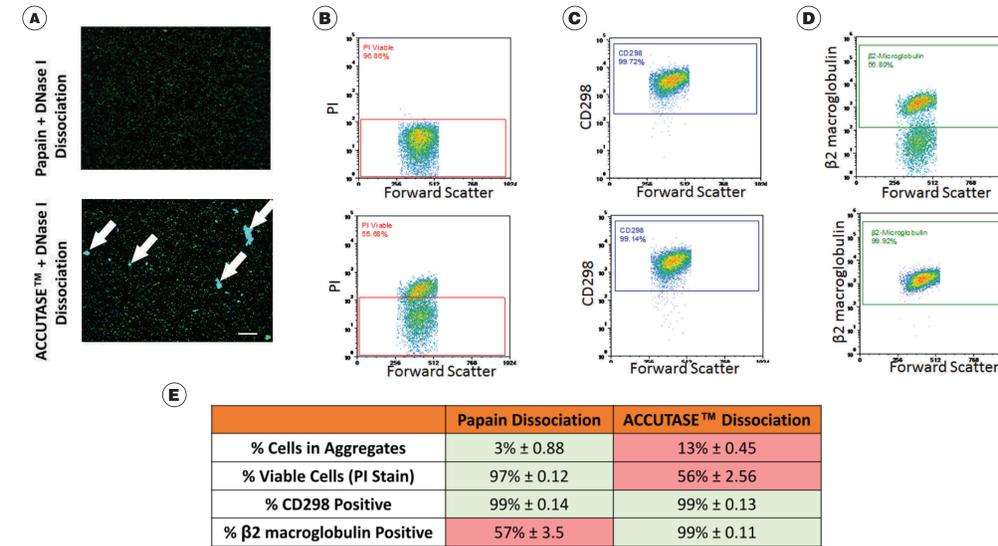
### Single-Cell Dissociation of Neural Organoids and Single-Cell RNA Sequencing



**FIGURE 1. Workflow for Single-Cell RNA Sequencing Analysis of Dorsal Forebrain Organoids and Ventral Forebrain Organoids**

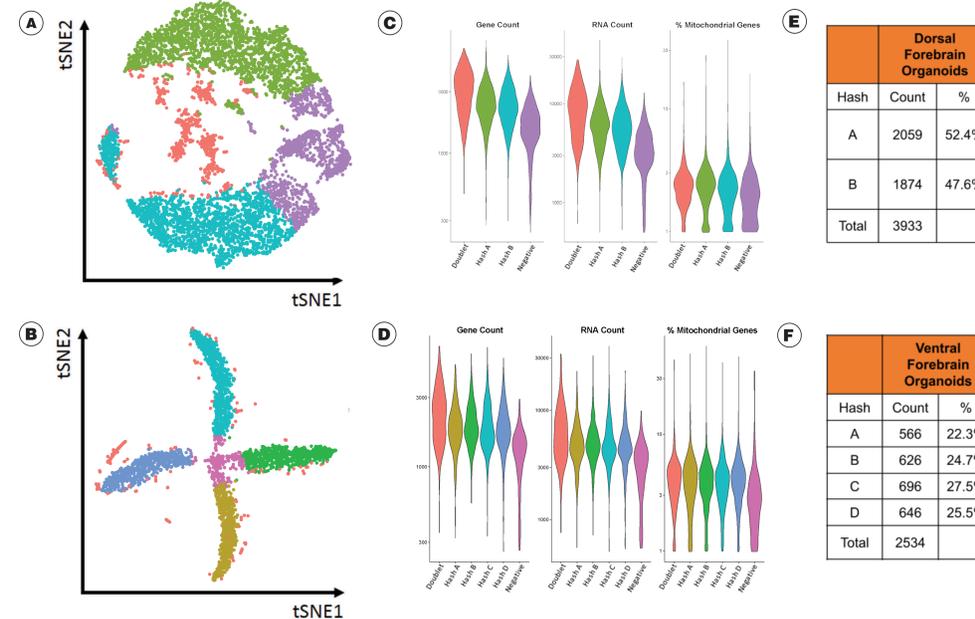
(A) Dorsal forebrain organoids and ventral forebrain organoids were generated using STEMdiff™ Dorsal Forebrain Organoid Differentiation Kit and STEMdiff™ Ventral Forebrain Organoid Differentiation Kit, respectively, using the highly published human embryonic stem cell line H9. In brief, H9 cells maintained in mTeSR™1 were dissociated and seeded at a density of 3 million cells/well in Formation Medium + 10 μM rho-kinase inhibitor (ROCKi) in AggreWell™ 800 plates. Full-medium changes were performed daily with Formation Medium without ROCKi. After 6 days, aggregates were transferred to a 6-well plate in either Dorsal Expansion Medium or Ventral Expansion Medium. For both dorsal and ventral organoids, full-medium changes were performed every 2 - 3 days until day 25, at which point the medium was switched to Differentiation Medium. Full-medium changes were performed every 2 - 3 days until day 43. On day 43, the medium was switched to Maintenance Medium, and full-medium changes were performed every 2 - 3 days until day 50. (B) On day 50, four dorsal forebrain organoids and four ventral forebrain organoids were transferred to individual sterile tubes and rinsed with PBS. To each organoid, 500 μL of dissociation solution (250 U/mL papain or ACCUTASE™, 2000 U/mL DNase I in Hanks' Balanced Salt Solution [HBSS]) was added. Organoids were incubated at 37°C for 30 minutes on an orbital shaker (Infors HT Celltron) set at 90 rpm. After incubation, a 1 mL pipettor was used to triturate each organoid 5 - 10 times. The cell suspension was incubated at 37°C for an additional 10 minutes on an orbital shaker set at 90 rpm. The cell suspension was triturated an additional 5 - 10 times. The dissociation reaction was quenched by adding 2 mL of ovomucoid protease inhibitor solution (10 mg/mL ovomucoid protease inhibitor in HBSS). Cells were centrifuged at 300 x g for 5 minutes and resuspended in HBSS followed by a cell count using AO/DAPI staining and analysis on NucleoCounter® NC-200™ (Chemometec). The cell suspension was passed through a 37 μm strainer to remove any additional aggregates. One million cells were labeled using TotalSeq™-B anti-human oligo-tagged antibody (Biolegend) using a concentration of 1 μg/mL for 30 minutes at room temperature. Cells were rinsed twice with sterile PBS and mixed in equal proportions to obtain > 2500 cells for the dorsal forebrain organoids and > 2500 cells for the ventral forebrain organoids. Cell suspensions were placed on ice and transferred to the University of British Columbia (Canada) BRC-Sequencing Facility for single-cell cDNA library generation and RNA sequencing. Two of the dorsal forebrain organoid samples were excluded from the analysis after failing quality control checks.

## RESULTS

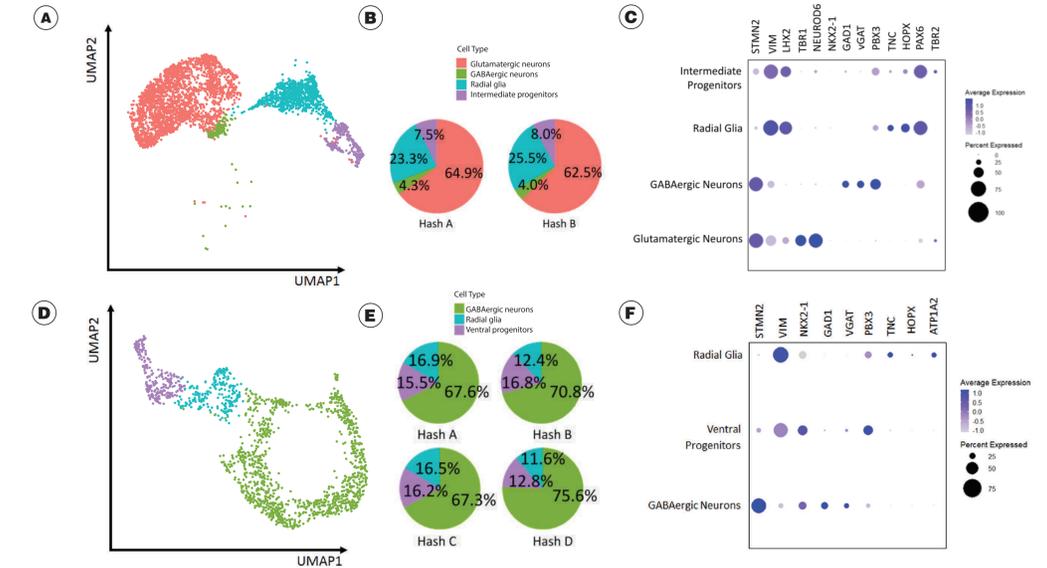


**FIGURE 2. Papain-Dissociated Neural Organoids Generate Highly Viable Single-Cell Suspensions and Stain Positive for CD298**

(A) Representative image obtained from NucleoCounter® NC-200™ of single-cell suspension stained with acridine orange (green) and DAPI (blue). ACCUTASE™ dissociation of organoids resulted in higher numbers of undissociated aggregates. Scale bar = 200 μm. (B) Propidium iodide (PI) staining was used to evaluate cell viability; papain-dissociated cell suspensions exhibited low PI staining while ACCUTASE™-dissociated cell suspensions exhibited higher levels of PI staining. Evaluation of cell surface epitopes for TotalSeq™-B anti-human antibodies for (C) CD298 and (D) β2 macroglobulin. It was determined that both papain- and ACCUTASE™-dissociated cells stained for CD298 while papain dissociation decreased β2 macroglobulin staining. (E) Summary of performance results for cell suspensions from papain-dissociated vs. ACCUTASE™-dissociated organoids (n=3, average +/- SEM).

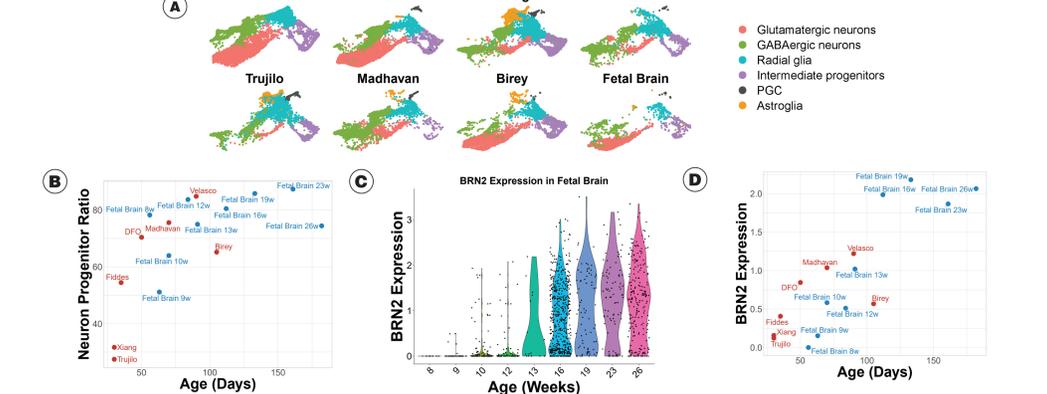


**FIGURE 3. Quality Assessment of Cell Hash Single-Cell Data**  
tSNE plot displaying distribution of cell hash single cells from (A) dorsal forebrain organoids and (B) ventral forebrain organoids. Quantification of distribution of cell counts for oligo-conjugated antibody labeling for (C) dorsal forebrain organoids and (D) ventral forebrain organoids. Negatives and doublets were discarded. Cells with 1500 to 5000 genes and less than 5% of mitochondrial genes were retained for analysis. (E) Total cell number contributed by each specific oligo-conjugated antibody-labeled organoid. For dorsal forebrain organoids, 12% (708/5845) were found to be doublets and 21% (1204/5845) were negative for cell hashing. (F) For ventral forebrain organoids, 7% (207/2996) were found to be doublets and 8.5% (254/2996) were negative for cell hashing.



**FIGURE 4. Single-Cell Characterization of Dorsal Forebrain Organoids and Ventral Forebrain Organoids**

(A) UMAP plot identifying cell populations in dorsal forebrain organoids. (B) Distribution of cell types identified in dorsal forebrain organoids. (C) Genes expressed in cell types identified in dorsal forebrain organoids. Neurons and progenitors were identified by STMN2 and VIM respectively. Specific cell markers were used to refine the classification further (see Introduction). (D) UMAP plot identifying cell populations in ventral forebrain organoids. (E) Distribution of cell types identified in ventral forebrain organoids. (F) Genes expressed in cell types identified in ventral forebrain organoids.



**FIGURE 5. Comparison of STEMCELL Technologies Dorsal Forebrain Organoids to Published Datasets**

(A) UMAP plot identifying cell populations from forebrain organoids in publicly available datasets (GSE98201, GSE130238, GSE106245, GSE110006, GSE129519, GSE93811, and GSE104276). (B) Comparison of neuron to progenitor ratio in brain organoids increases over time and matches what is observed in fetal brain datasets. (C) BRN2 expression increases over time gestation in fetal brain datasets. (D) BRN2 (Pou3f2) expression in neurons increases over time in brain organoids and matches what is observed in fetal brain datasets. The transcription factor Pou3f2 plays a critical role in early neuron development in stem cells.

## Summary

- A papain + DNase I-based dissociation protocol was optimized to obtain a viable single-cell suspension for single-cell RNA sequencing applications
- Day 50 dorsal forebrain organoids contain four major cell types (glutamatergic neurons, GABAergic neurons, astroglia, and intermediate progenitors) while ventral forebrain organoids contain three major cell types (GABAergic neurons, astroglia, and intermediate progenitors), with the majority of cells being neurons
- Dorsal forebrain organoids display similar cell types to published datasets and express appropriate neuronal phenotype, with their expression levels progressing similarly to fetal brain development

References  
1. Yoon SJ et al. (2019) Nat Methods 16: 75–8.

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