A Reliable, Efficient, and Feeder-Free Method to Generate Brain-Region-Specific Dorsal and Ventral Forebrain Organoids From Human Pluripotent Stem Cells to Model Early Human Brain Development

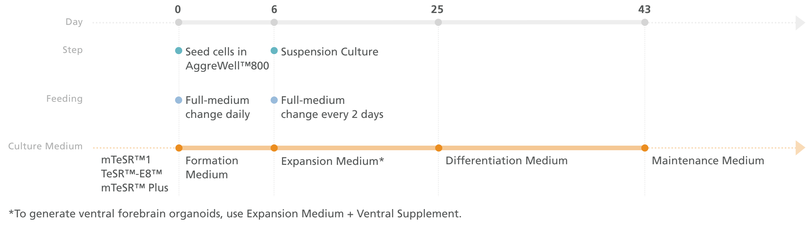
Leon H. Chew[1], Adam Añonuevo[1], Eloi Mercier[1], Jeanne Chan[1], Mark Hills[1], Erin Knock[1], Allen C. Eaves[1,2], Terry E. Thomas[1], and Sharon A. Louis[1]

[1]STEMCELL Technologies, Vancouver BC, Canada; [2]Terry Fox Laboratory, BC Cancer Agency, Vancouver BC, Canada

INTRODUCTION

3D neural cultures derived from human pluripotent stem cells (hPSCs), including embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, have been shown to recapitulate the major features and cytoarchitecture of human brain development. These brain organoid models have enabled scientists to study early fetal human brain development in vitro for the first time. For many of these methods, however, variability in the quality and consistency of organoids generated within and among experiments has hampered their widespread adoption as a research tool. Here we present work demonstrating that the recently developed STEMdiff™ Dorsal Forebrain Organoid Differentiation Kit and STEMdiff™ Ventral Forebrain Organoid Differentiation Kit can be used to robustly generate homogeneous brain-region-specific tissue representing the dorsal neocortex and ventral telencephalon, respectively. The forebrain organoids can be characterized for electrical activity using multielectrode array (MEA) and further used in co-culture to form assemblies or "assembloids" with other 2D or 3D systems. Together, these kits enable the further study of the complexity of brain development and disorders in vitro.

METHODS



*To generate ventral forebrain organoids, use Expansion Medium + Ventral Supplement.

FIGURE 1. Workflow for STEMdiff™ Dorsal Forebrain Organoid Kit and STEMdiff™ Ventral Forebrain Organoid Kit
hPSCs maintained in mTeSR™1 (6 cell lines: 3 ES, 3 iPS), TeSR™-E8™ (3 cell lines: 2 ES, 1 iPS), or mTeSR™ Plus (2 cell lines: 1 ES, 1 iPS) were dissociated and seeded at a density of 3,000,000 cells/well in Formation Medium + 10 µM rho-kinase inhibitor (ROCKi) in AggreWell™800 plates. Cultures were fed 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. Both dorsal and ventral organoids were fed every 2 - 3 days until day 25, at which point organoids were fed with Differentiation Medium until day 43. From day 43 onward, organoids were fed every 2 - 3 days with Maintenance Medium. Organoids were harvested for RNA or fixed in 4% paraformaldehyde for subsequent cryosectioning and immunostaining.

RESULTS

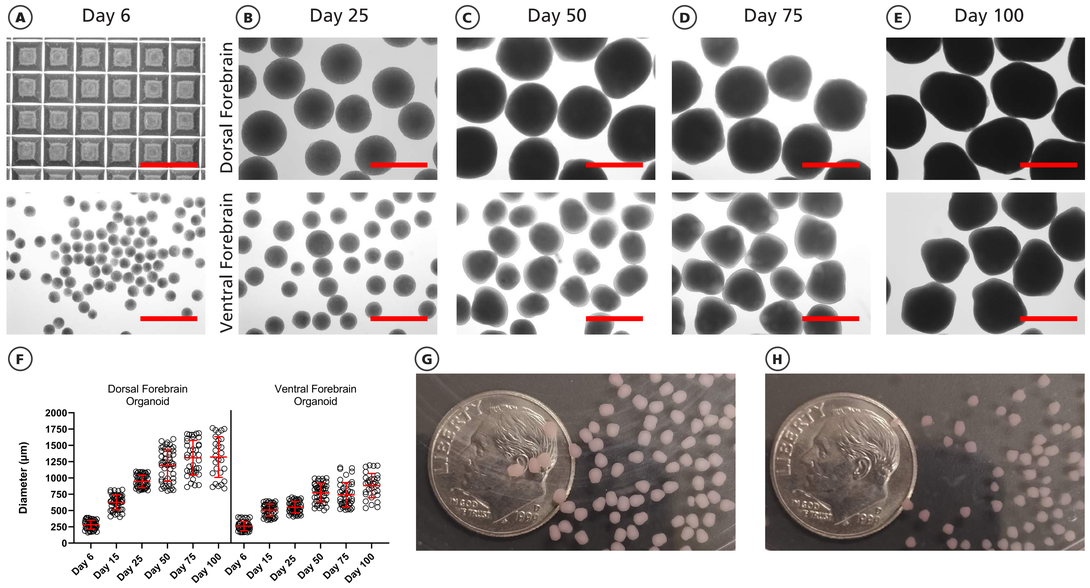


FIGURE 2. STEMdiff™ Dorsal Forebrain Organoid Kit and STEMdiff™ Ventral Forebrain Organoid Kit Support the Generation of Homogeneous Organoids
(A) Representative morphology of neural aggregates formed in AggreWell™800 exhibit uniform size and shape at day 6 (top: aggregates in AggreWell™800, bottom: aggregates in suspension). Representative morphology of hESC H9-derived organoids at days **(B)** 25 **(C)** 50 **(D)** 75 and **(E)** 100. Top row: dorsal forebrain organoids, bottom row: ventral forebrain organoids. Scale bar = 1 mm. **(F)** Both dorsal and ventral forebrain organoids exhibit homogeneous size over multiple cell lines (average ± SD, using 11 cell lines with 3 - 5 organoids counted per cell line and timepoint). **(G)** Dorsal forebrain organoids and **(H)** ventral forebrain organoids compared to an American dime for scale.

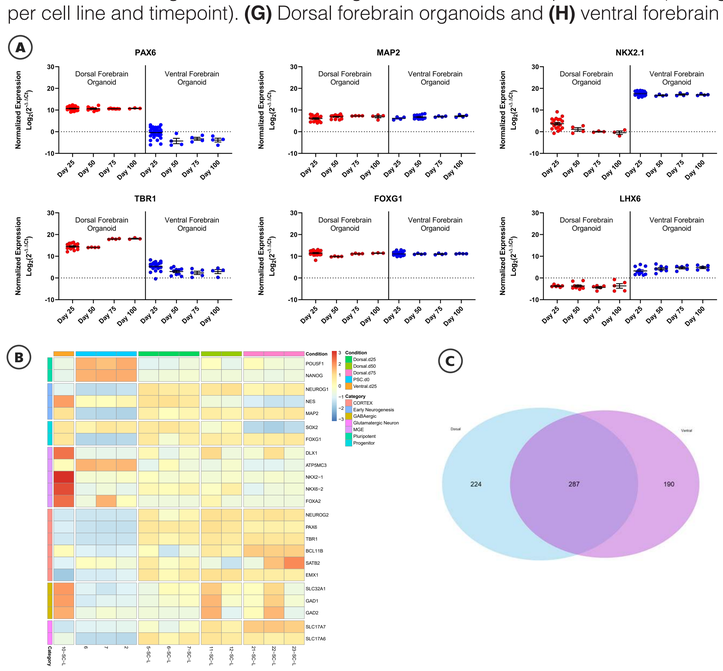


FIGURE 3. Dorsal and Ventral Forebrain Organoids Express Appropriate Markers of Their Respective Tissue Over Time
RNA from a single organoid was harvested at each respective timepoint and subsequently assayed either using RT-qPCR or bulk RNA-seq. **(A)** RT-qPCR analysis shows upregulation of PAX6 and TBR1 in the dorsal forebrain organoids while NKX2.1 and LHX6 were upregulated in the ventral forebrain organoids. Both MAP2 and FOXG1 were not differentially expressed between dorsal and ventral forebrain organoids. Average ± SEM; n = 3 - 20 organoids per timepoint using 11 hPSC lines; Data are normalized to 18S/TBP and compared to an undifferentiated hPSC control. **(B)** Heat map of select genes shows that dorsal forebrain organoids have an increasing amount of cortex- and glutaminergic neuron-specific genes from day 25 to 75. Day 25 ventral forebrain organoids exhibit high expression of markers of the MGE and GABAergic neurons. **(C)** Venn diagram comparing day 25 dorsal forebrain and ventral forebrain organoids shows a high degree of overlap of markers that were differentially upregulated in these organoids compared to hPSCs. Dorsal forebrain organoid-specific genes are related to pallium development of the neocortex whereas ventral forebrain organoid-specific genes are related to subpallium development of the ventral telencephalon.

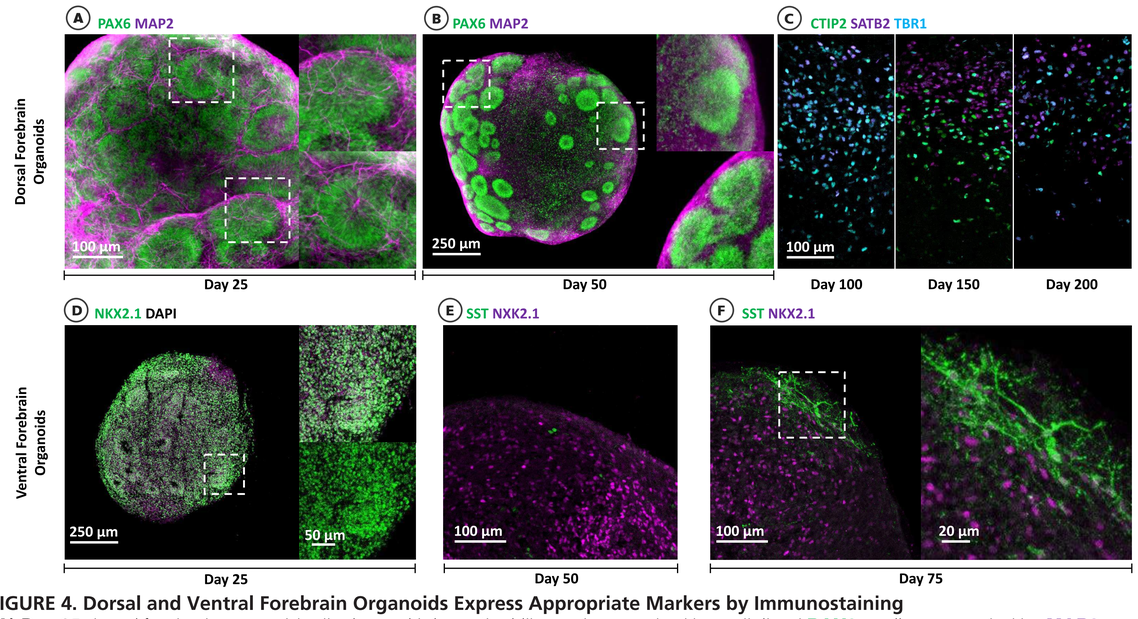


FIGURE 4. Dorsal and Ventral Forebrain Organoids Express Appropriate Markers by Immunostaining
(A) Day 25 dorsal forebrain organoids display multiple cortical-like regions marked by radialized **PAX6+** cells surrounded by **MAP2** neurons. **(B)** Day 50 dorsal forebrain organoids continue to display multiple cortical-like regions marked by **PAX6** and **MAP2**. **(C)** Dorsal forebrain organoids cultured for 100 - 200 days show increasing separation of deep-layer neurons (**CTIP2**, **TBR1**) from upper-layer neurons (**SATB2**). **(D)** Ventral forebrain organoids at day 25 exhibit a high level of expression of **NKX2.1.** **(E)** and **(F)** Somatostatin (**SST**)-positive GABAergic interneurons are not observed at day 50 but can be seen by day 75.

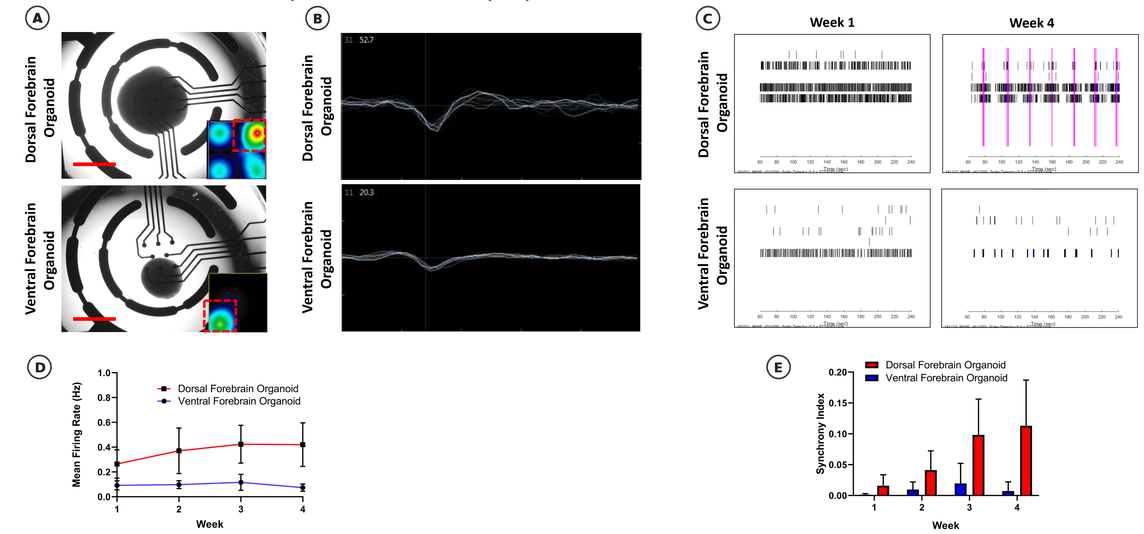


FIGURE 5. Dorsal Forebrain Organoids But Not Ventral Forebrain Organoids Display Early Network Bursting Activity
Day 50 dorsal and ventral forebrain organoids were plated on an Axion electrode array (CytoView MEA 96) coated with 0.1% polyethyleneimine in borate buffer and 20 µg/mL CellAdhere™ Laminin-521 and measured once per week for 4 weeks. **(A)** Representative bright field image of dorsal and ventral forebrain organoids on electrode array. Representative spike activity heat map is shown. Scale bar = 1 mm. **(B)** Representative wave form of electrode marked in (A). **(C)** Raster plot of spike activity shows increasing network bursting for dorsal forebrain organoids at week 4, which is not observed in the ventral forebrain organoid. **(D)** Mean firing rate (average ± SEM; 3 - 6 organoids per timepoint) shows increasing mean firing rate for dorsal forebrain organoids but not ventral forebrain organoids over 4 weeks of measurements. **(E)** Synchrony index (average ± SEM; 3 - 6 organoids per timepoint) exhibits increasing synchrony of firing for dorsal forebrain organoids compared to ventral forebrain organoids.

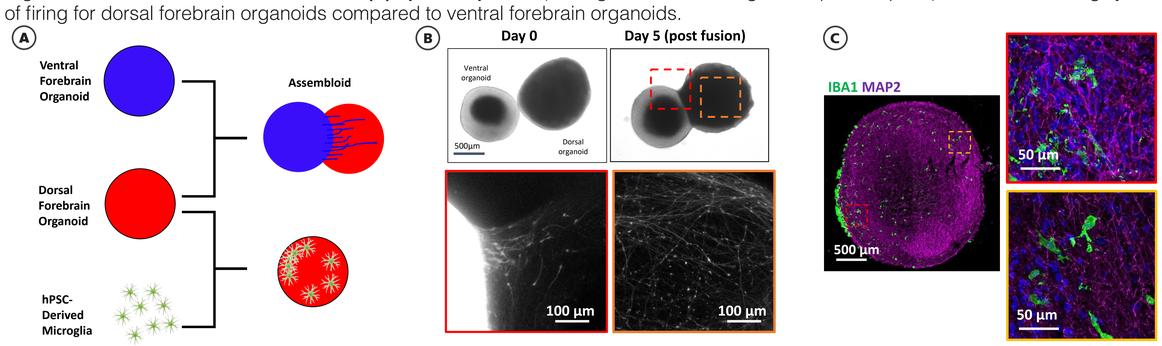


FIGURE 6. Brain-Region-Specific Organoids Can Be Used in Co-Culture to Form Assembloids
(A) Schematic depicting the formation of a dorsal (red) and ventral (blue) forebrain organoid assembloid and a dorsal forebrain organoid and microglia co-culture. **(B)** A day 30 dorsal forebrain organoid and ventral forebrain organoid (expressing endogenous GFP) were allowed to fuse together over 5 days. On day 5, GFP-expressing cells from the ventral forebrain organoid had migrated into the dorsal forebrain organoid (red and orange boxes). **(C)** A day 200 dorsal forebrain organoid was incubated with 250,000 microglia generated using STEMdiff™ Microglia Differentiation Kit. Within one week, **IBA1**+ cells with stereotypical microglia morphology have been incorporated into the dorsal forebrain organoid.

Summary

- STEMdiff™ Dorsal Forebrain Organoid Differentiation Kit and STEMdiff™ Ventral Forebrain Organoid Differentiation Kit generate tissue resembling the dorsal forebrain (PAX6, TBR1) and ventral forebrain (NKX2.1, LHX6), respectively
- Dorsal forebrain organoids were observed to exhibit network bursting earlier than ventral forebrain organoids
- Dorsal and ventral forebrain organoids can form assembloids to study interneuron migration and interactions with microglia

Features of STEMdiff™ Forebrain Organoid Kits:

- RELIABILITY: Optimized and matrix-free formulations for patterning of brain-region-specific organoids improves reproducibility across experiments and cell lines
- SCALABILITY: High-throughput organoid generation in AggreWell™800
- MODULARITY: Ease of use in generating co-culture models (assembloids)