TECHNICAL BULLETIN

# Cryogenic Tissue Processing and Section Immunofluorescence of Cerebral Organoids

## Background

Cerebral organoids provide a physiologically relevant human model for the central nervous system. These 3D cultures enable the study of normal and disease states in human brain development, including models of autism[1] and schizophrenia[2], or brain defects caused by viral infection.[3]

A primary advantage of using human pluripotent stem cells (hPSCs) to create cerebral organoids is the recapitulation of the 3D organization of the developing brain. The central nervous tissue in a cerebral organoid develops a layered structure and pseudo-ventricles, similar to the cerebral structure seen in vivo.[3] In order to capture and analyze this cytoarchitecture, specialized histological methods are required.

There are a number of technical considerations when processing larger 3D tissues that have acquired complex structure, such as cerebral organoids. In addition to careful handling, these organoids require adequate penetration, fixation, and cryoprotection to preserve their histomorphology. Each of these steps can also affect the characterization of phenotypes, through preservation or modification of protein structure that determines epitope availability.

The following protocol describes how mature cerebral organoids can be processed for cryosectioning and immunofluorescence (IF) to minimize tissue damage and preserve important epitopes. Because sample preparation has a large bearing on the quality of downstream staining in any immunostaining protocol, some antibodies may perform better under this protocol with dehydration-based tissue fixatives (Table 1). The antibodies listed in this document (Tables 2 and 3) are confirmed to work with cerebral organoid tissue that has been treated with 4% paraformaldehyde for fixation.

**Table 1.** Advantages and Disadvantages of Chemical Tissue Fixatives

| Fixative Type | Advantages | Disadvantages |
|---|---|---|
| Aldehyde-based<br>e.g. Formaldehyde, Glutaraldehyde | Preservation of secondary and tertiary protein structure | Overfixation can mask epitope |
| Dehydration-Based<br>e.g. Methanol, Ethanol | Precipitation can expose hidden epitopes | Precipitation can destroy some epitopes |

## Materials and Reagents

### Materials Required

- Coplin Staining Jars (e.g., Millipore Sigma Catalog #S5516)
- ImmEdge™ Hydrophobic Barrier PAP Pen (Vector Catalog #H-4000)
- 50 mL conical tubes (Catalog #38010)
- Cryostat
- Microscope slides and coverslips
- Dry ice

### Reagents Required

- 4% Paraformaldehyde (PFA) solution (Alfa Aesar Catalog #J61899)
- Sucrose
- D-PBS (Without Ca++ and Mg++) (Catalog #37350)
- Tween® 20 (Sigma-Aldrich P9416-100ML)
- Gelatin from Porcine Skin (Sigma-Aldrich Catalog #G2500-1KG)
- Sodium azide
- 100% Ethanol
- Thermo Scientific™ Lab Vision™ PermaFluor™ Aqueous Mounting Medium (Fisher Scientific Catalog #TA030FM)
- Bovine Serum Albumin (BSA) (Sigma-Aldrich Catalog #A7906-100G)
- Normal Donkey Serum (NDS) (Millipore Catalog S30-100ML)

**Table 2.** Validated Primary Antibodies

| Antibody | Vendor | Catalog # | Recommended Dilution |
|---|---|---|---|
| CTIP2 | Abcam | ab18456 | 1:2000 |
| FOXG1 | Abcam | ab18259 | 1:2000 |
| Ki67 | Millipore | MAB4190 | 1:2000 |
| MAP2 | Abcam | ab5392 | 1:5000 |
| PAX6 | BioLegend | 901301 | 1:2000 |
| PH3 | Millipore | D6-570 | 1:2000 |
| PVIM | MBL | D076-3 | 1:2000 |
| SOX2 | R&D Systems | MAB2018 | 1:2000 |
| TBR1 | Abcam | ab31940 | 1:2000 |
| TBR2 | Abcam | ab23345 | 1:200 |
| βIII-tubulin/ TUJ1 | STEMCELL Technologies | 60052 | 1:2000 |

**Table 3.** Validated Secondary Antibodies

| Antibody | Vendor | Catalog # | Recommended Dilution |
|---|---|---|---|
| Alexa Fluor® Rt-488 | Jackson ImmunoResearch | 712-545-150 | 1:2000 |
| Alexa Fluor® Ms-594 | Jackson ImmunoResearch | 715-585-151 | 1:2000 |
| Alexa Fluor® Rb-594 | Jackson ImmunoResearch | 711-585-152 | 1:2000 |
| Alexa Fluor® Ms-647 | Jackson ImmunoResearch | 716-605-151 | 1:2000 |
| Alexa Fluor® Rb-647 | Jackson ImmunoResearch | 711-605-152 | 1:2000 |

*Rt = rat; Ms = mouse; Rb = rabbit*

# Preparation of Reagents

## 30% Sucrose Solution

To prepare 1 L of 30% Sucrose Solution:

1. Weigh 300 g of sucrose.
2. Add D-PBS to a final volume of 1 L.
3. Mix thoroughly.

## Gelatin Solution

To prepare 100 mL of Gelatin Solution:

1. Add 10 g of sucrose to 100 mL of D-PBS.
2. Add 7.5 g of gelatin to sucrose solution from step 1.
3. Mix thoroughly.

## PBS-T

To prepare 1 L of 0.1% PBS-T:

1. Add 1 mL of Tween® 20 to 1 L of D-PBS.
2. Mix thoroughly.

## Primary Dilution Buffer

To prepare 100 mL of Primary Dilution Buffer:

1. Add sodium azide to 100 mL of PBS-T to a final concentration of 0.05%.
2. Add BSA to 100 mL sodium azide/PBS-T solution (from step 1) to a final concentration of 5%.
3. Mix thoroughly.

## Blocking Solution

1. Dilute Normal Donkey Serum in PBS-T to a final concentration of 5%.
2. Mix thoroughly.

# Protocol

## Culturing Cerebral Organoids

Culture cerebral organoids using STEMdiff™ Cerebral Organoid Kit (Catalog #08570). For a technical video on culturing cerebral organoids, refer to **https://www.stemcell.com/GrowCO**.

## Fixing Cerebral Organoids

1. Using a cut 1 mL (p1000) pipette tip, transfer organoids to a fresh 50 mL conical tube.

   ***Note:*** *To preserve cytoarchitecture, it is important not to disturb organoids during transfer. Pipette tips can be cut to a larger diameter as needed to accommodate organoid size.*

2. Remove excess medium from the tube. Wash 3X for 10 minutes each with D-PBS. Remove D-PBS.

3. Add fresh 4% paraformaldehyde solution (PFA) at 5 mL per organoid. Incubate overnight (16 hours) at 2 - 8°C.

   ***Note:*** *Detection of cortical layer formation in cerebral organoids via immunofluorescence requires proper and thorough fixation of samples. Therefore, we recommend using freshly prepared 4% PFA solution from frozen stock aliquots.*

4. Remove PFA from conical tube and dispose of in appropriate waste. Wash organoids 3X for 10 minutes each with PBS-T.

5. Store samples in PBS-T at 2 - 8°C for up to 1 week.

## Cryoprotection

To protect against freezing artifacts, equilibrate samples in a 30% Sucrose Solution (see Preparation section), as follows:

6. Remove PBS-T from organoids and discard. Add 30% Sucrose Solution at 5 mL per organoid.

7. Allow to equilibrate overnight at 2 - 8°C.

   ***Note:*** *Time to equilibrate can vary due to organoid size and density. Once organoids no longer float in the 30% sucrose solution it is appropriate to move to the next step.*

## Embedding

8. Warm Gelatin Solution to 37°C in a water bath.

9. Pipette sucrose solution out of conical tube and discard. Add enough Gelatin Solution to completely cover organoids.

10. Incubate at 37°C for 1 hour. This allows the gelatin to penetrate the Corning® Matrigel® droplet and encapsulate the organoid (see Product Information Sheet for STEMdiff™ Cerebral Organoid Kit).

11. Remove organoids from conical tube and transfer to embedding mold.

    ***Note:*** *Gelatin will begin to polymerize and harden at room temperature; it is necessary to quickly transfer organoids to the embedding mold.*

## Snap Freezing

Rapid freezing helps to prevent the formation of ice crystals in the organoids and helps to maintain the native cellular architecture of the sample.

12. Prepare a dry ice/ethanol slurry by adding dry ice to 100% ethanol.

13. Once the mixture stops boiling, add the embedded sample.

14. Keep sample in the cold slurry until completely frozen, then transfer to a -80°C freezer for long-term storage.

    ***Note:*** *If freezing artifacts are still present in samples, snap freezing can also be done via immersion into an isopentane bath chilled with liquid nitrogen.*

## Cryosectioning

15. Remove blocks from the -80°C freezer and allow them to warm in cryostat to sectioning temperature for 30 minutes.

    ***Note:*** *Optimal sectioning temperature for gelatin-embedded organoids is -26 to -30°C.*

16. Thickness can be adjusted to suit imaging system. Typical section thickness is 10 - 20 µm.

    ***Note:*** *Collection of multiple serial sections can allow exploration of different markers in different sections.*

## Immunofluorescence

17. Remove sectioned slides from freezer and allow to dry at room temperature.

18. Outline sections with a PAP pen. When the pen wax is completely dry, proceed to the next step.

19. If necessary, proceed to antigen retrieval, in lieu of step 20, as described below, or proceed to step 20.

## (Optional) Antigen Retrieval

Cross-linkages formed by PFA fixation may interfere or block antibody binding to the epitope of interest (reviewed in Shi et al. 1997).[4] For that reason, detection of certain samples will require an additional antigen retrieval step before incubation with primary antibodies.

### Additional Equipment and Reagents Required

- Food steamer (e.g., Hamilton-Beach 37530)
- Heat-resistant plastic Coplin staining jar
- Tri-sodium citrate (dihydrate) 2.94 g

### Preparation of Citrate Buffer

To prepare 1 L of Citrate Buffer:

1. Add 2.94 g of Tri-sodium citrate (dihydrate) to 950 mL of dH20.
2. Adjust pH to 6.0 with HCl/NaOH.
3. Add 0.5 mL of Tween® 20.
4. Top up to 1 L.

### Antigen Retrieval Protocol

1. Place slides in a heat-resistant plastic Coplin staining jar and fill with Citrate Buffer.
2. Place Coplin jar in food steamer and steam for 20 minutes.
3. Carefully remove hot Citrate Buffer and wash slides 3X in PBS-T for 10 minutes each.

## Protocol (Continued)

### Blocking

20. Wash slides with PBS-T at 37°C for 10 minutes to fully remove gelatin from slides.
21. Lay slides flat and pipette enough Blocking Solution to completely cover sections. The borders formed by the wax pen will hold Blocking Solution on the sections of tissue.
22. Incubate at room temperature in a humidified chamber for 1 hour.

### Primary Antibodies

23. Prepare primary antibody mixes in Primary Dilution Buffer. Refer to Table 2 for recommended antibody concentrations.
24. Pour Blocking Solution off of slides and replace with primary antibody mix.
25. Incubate at room temperature in a humidified chamber overnight (16 hours).

### Secondary Antibodies

26. Prepare secondary antibody mixes in PBS-T. Refer to Table 3 for recommended antibody concentrations.

    ***Note:*** *Alexa Fluor® antibodies are very stable, but care should be taken not to leave antibodies in direct light for an extended period of time.*

27. Pour primary antibody mix off of slides and place slides in a Coplin staining jar.
28. Wash slides 3X with PBS-T.
29. Lay slides flat and pipette secondary antibody mix onto slides. Incubate at room temperature in a humidified chamber for 2 hours.

### Coverslips

30. Pour off secondary antibody mix and place slides in a Coplin staining jar.
31. Wash slides 3X with PBS-T for 30 minutes each.
32. Air-dry slides for 5 minutes.
33. Add PermaFluor™ to slides and add coverslip. Store at 2 - 8°C and allow to dry before imaging.

## Expected Results

Organoids cultured with STEMdiff™ Cerebral Organoid Kit can be used to identify discrete features of the developing cortex, including actively proliferating cells at the ventricular border of the progenitor zone, expansion of an intermediate progenitor pool, and the expansion of cortical plate neurons from these progenitor zones.

Each figure below is a fluorescent image of a 16-µm-thick section of a Day 40 cerebral organoid processed using the above protocol, taken at 20X magnification.

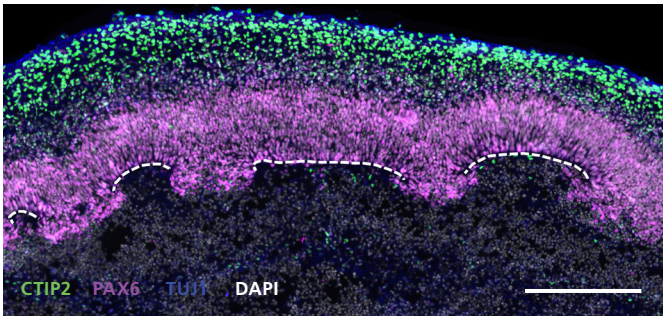


**Figure 1**

Organoid tissue was processed for immunofluorescence and stained for CTIP2 (green), PAX6 (magenta), βIII-tubulin/TUJ1 (blue), and DAPI (gray). Cortical regions are defined by progenitor cells (PAX6+) that are radially organized around a pseudo-ventricle (dashed line). These progenitors give rise to cortical plate neurons indicated by CTIP2 and TUJ1 expression. Scale bar = 500 µm.

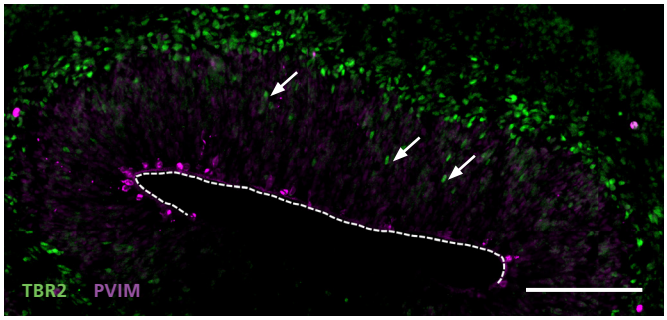


**Figure 2**

Organoid tissue was processed for immunofluorescence and stained for TBR2 (intermediate precursors, green) and phosphorylated vimentin (PVIM, dividing cells, magenta). Cells actively divide at the apical border of cortical regions along the border of the pseudo-ventricle (dashed line). A population of these dividing cells will express TBR2 and then migrate (arrows) from the progenitor zone to form a layer of intermediate progenitors. Scale bar = 100 µm.

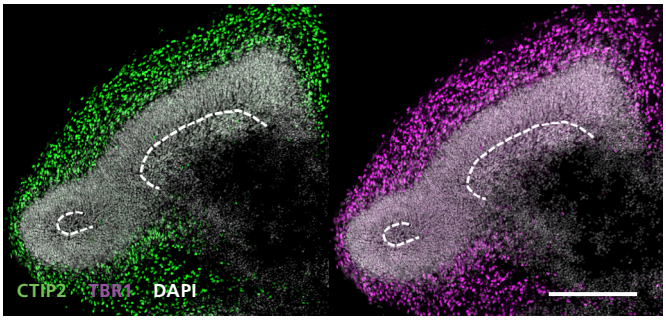


**Figure 3**

Organoid tissue was processed for immunofluorescence and stained for CTIP2 (green), TBR1 (layer 5/6 cortical neurons, magenta), and DAPI (white). Deep layer neuronal markers CTIP2 and TBR1 are expressed in cells around presumptive progenitor zones (white) toward the outside or apical surface of organoids. Scale bar = 100 µm.

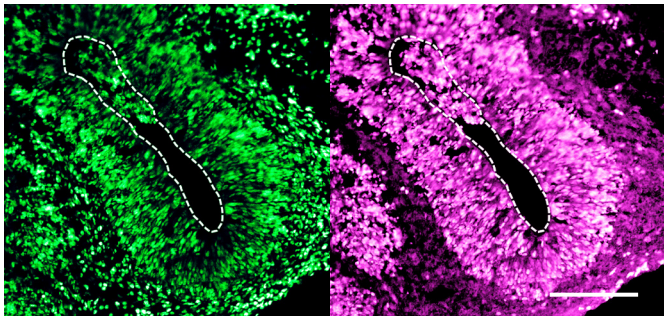

**Figure 4**

Organoid tissue was processed for immunofluorescence and stained for FOXG1 (forebrain cells, green) or SOX2 (neural progenitors, magenta). Organoids derived from STEMdiff™ Cerebral Organoid Kit generate forebrain-type tissue as indicated by FOXG1 expression. Neural progenitors expressing SOX2 are radially arranged around a pseudo-ventricle area (dashed line). Scale bar = 100 µm.

## Discussion

The intricate structure of the central nervous system requires sensitive histological technique to faithfully reproduce this structure for viewing under a microscope. This cryosectioning protocol allows the user to process 3D-cultured tissue with little damage or distortion in the final sample. Sections of 3D cerebral organoids processed as IF micrographs allow for the visualization of tissue-level phenotypes not readily apparent in 2D cultures. For example, the evaluation of defects in cortical layering is not possible with conventional 2D culture.

The ability to generate neural organoids from stem cells has become a powerful tool for the study of human neural tissue and its organization. Together, these techniques enable the study of new questions and hypotheses about nervous system function and dysfunction. Cortical layering defects have been implicated in neuropsychiatric disorders such as schizophrenia.[2,5] In addition, dendritic spine density in layer V neurons is greatly affected in mouse models of Rett Syndrome.[6] Designing therapies in human models of these diseases will be accelerated through the cryosectioning and staining of individual patient-derived organoids.

## References

1. Forsberg S et al. (2018) Transl Psychiatry 8(1): 14.
2. Stachowiak E et al. (2017) Transl Psychiatry 7(11): 6.
3. Lancaster M et al. (2013) Nature 501(7467): 373–9.
4. Shi S et al. (1997) J Histochem Cytochem 45(3): 327–43.
5. Garey L (2010) J Anat 217(4): 324–33.
6. Stearns N et al. (2007) Neuroscience 146(3): 907–21.