

Ex vivo Tissue Culture Protocols for Studying the Developing Neocortex

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[Abstract] The size of the neocortex and its morphology are highly divergent across mammalian species. Several approaches have been utilized for the analysis of neocortical development and comparison among different species. In the present protocol (Note: This protocol requires basic knowledge of brain anatomy), we describe three *ex vivo* neocortical slice/tissue culture methods: (i) organotypic slice culture (mouse, ferret, human); (ii) hemisphere rotation culture (mouse, ferret); and (iii) free-floating tissue culture (mouse, ferret, human). Each of these three culture methods offers distinct features with regard to the analyses to be performed and can be combined with genetic manipulation by electroporation and treatment with specific inhibitors. These three culture methods are therefore powerful techniques to examine the function of genes involved in neocortical development.

Keywords: Human, Mouse, Ferret, Neocortex, Development, Evolution, Neural stem/Progenitor cells

[Background] Several slice/tissue culture methods have been reported for studying brain development *ex vivo* (Noctor *et al.*, 2001; Miyata *et al.*, 2002; Tabata and Nakajima, 2003; Namba *et al.*, 2007 and 2019; Schenk *et al.*, 2009; Betizeau *et al.*, 2013; Lim *et al.*, 2018; Long *et al.*, 2018; Nakagawa *et al.*, 2019; Güven *et al.*, 2020). In combination with *in/ex utero* and *ex vivo* genetic manipulation techniques [e.g., electroporation (Tabata and Nakajima, 2003; Namba *et al.*, 2014), virus transfection (Noctor *et al.*, 2001), and microinjection (Taverna *et al.*, 2012)], the slice/tissue culture methods allow the investigation of cell behavior by time-lapse imaging (Miyata *et al.*, 2001; Noctor *et al.*, 2001; Tabata and Nakajima, 2003; Namba *et al.*, 2011 and 2014; Taverna *et al.*, 2012; Betizeau *et al.*, 2013; Lim *et al.*, 2018; Long *et al.*, 2018; Nakagawa *et al.*, 2019), signaling pathways by studying the effects of pharmacological reagents (Schenk *et al.*, 2009; Long *et al.*, 2018; Kalebic *et al.*, 2019; Namba *et al.*, 2020), and brain tissue development by immunohistochemistry (Schenk *et al.*, 2009; Long *et al.*, 2018; Güven *et al.*, 2020; Namba *et al.*, 2020) (Figure 1). In the present protocol, we describe three different methods to study developing neocortical tissue: (i) organotypic slice culture (Taverna *et al.*, 2012); (ii) hemisphere rotation (HERO) culture (Schenk *et al.*, 2009); and (iii) free-floating tissue (FFT) culture (Long *et al.*, 2018).

Organotypic slice culture has been widely used over the past few decades (Noctor *et al.*, 2001; Miyata *et al.*, 2002; Tabata and Nakajima, 2003; Namba *et al.*, 2007; Taverna *et al.*, 2012) and readily allows time-lapse imaging of labeled cells (Miyata *et al.*, 2001; Noctor *et al.*, 2001; Tabata and Nakajima, 2003; Namba *et al.*, 2011 and 2019; Taverna *et al.*, 2012; Mora-Bermudez *et al.*, 2014; Long *et al.*, 2018; Nakagawa *et al.*, 2019); however, the tissue can become damaged by slicing. The other two methods

may overcome this potential problem. HERO culture was originally described by Schenk and colleagues (Schenk *et al.*, 2009) and has been used for the pharmacological treatment of neocortical tissue (Schenk *et al.*, 2009; Namba *et al.*, 2020). FFT culture has recently been developed and used in our group (Long *et al.*, 2018) to study human neocortical development following pharmacological and genetic manipulation (Long *et al.*, 2018; Kalebic *et al.*, 2019; Kostic *et al.*, 2019; Namba *et al.*, 2020).

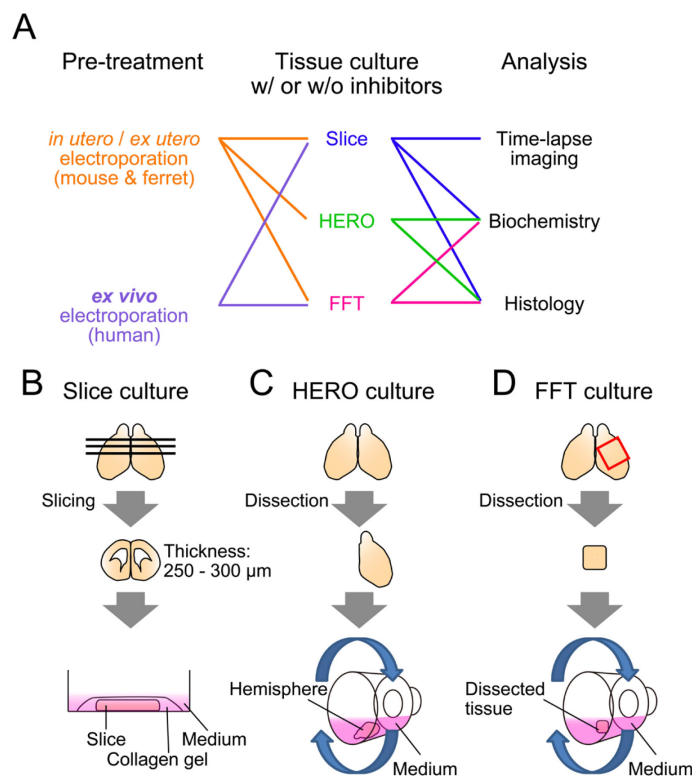


Figure 1. Overview of *ex vivo* slice/tissue culture protocols and their applications. A. Summary of the three methods. B. Slice culture method. C. Hemisphere rotation (HERO) culture method. D. Free-floating tissue (FFT) culture method.

The optimal method for a given experiment depends on the species of the sample, the specific application, and the degree of tissue architecture preservation that is required. The cultured slice/hemisphere/tissue can be subjected to histological and biochemical analyses (Long *et al.*, 2018; Kalebic *et al.*, 2019; Kostic *et al.*, 2019; Güven *et al.*, 2020; Namba *et al.*, 2020). Due to the limitation of sample availability, FFT and organotypic slice culture can lend themselves to the analysis of human neocortical development following manipulation of gene function (Long *et al.*, 2018; Kalebic *et al.*, 2019; Kostic *et al.*, 2019; Namba *et al.*, 2020). Time-lapse imaging (Taverna *et al.*, 2012; Mora-Bermudez *et al.*, 2014; Long *et al.*, 2018) is generally performed in organotypic slice culture. If an experiment requires maintenance of intact tissue architecture, HERO and FFT cultures are the methods of choice.

Materials and Reagents

A. Animals and human samples

1. Ferret embryos (E33-E36)
2. Human neocortical tissue [11 post-conception week (PCW)-14 PCW]
3. Mouse embryos [embryonic day (E) 13.5-E15.5]

B. Common materials and reagents

1. Pasteur pipet (*e.g.*, SARSTEDT, catalog number 86.1171.001)
2. Fine-tip Pasteur pipet (*e.g.*, SARSTEDT, catalog number 86.1175.001)
3. 3.5-cm dishes (for time-lapse imaging, use glass-bottomed dishes, *e.g.*, Thermo Fisher Scientific, Nunc, catalog number: 150680)
4. 6-cm Petri dishes (Greiner, catalog number: 628102)
5. 100× N2 supplement (Thermo Fisher Scientific, Invitrogen, catalog number: 17502048)
6. 100× Penicillin-Streptomycin (Merck, Gibco, catalog number: 15140122)
7. 10 mM HEPES-NaOH (pH 7.3)
8. 50× B27 supplement (Thermo Fisher Scientific, Invitrogen, catalog number: 17504044)
9. Knockout Serum Replacement (KOSR, Thermo Fisher Scientific, Gibco, catalog number: 10828028)
10. 200 mM L-glutamine (Thermo Fisher Scientific, Gibco, catalog number: 25030081)
11. Neurobasal medium (Thermo Fisher Scientific, Gibco, catalog number: 21103049)
12. PBS (made in-house)
13. Rat serum (Charles River Laboratories Japan, catalog number: P00052)

C. Organotypic slice culture

1. DMEM-F12 (Merck, catalog number: D8900-10X1L)
2. Low-melting agarose (Merck, catalog number: A2790)
3. Sodium bicarbonate (NaHCO₃) (Merck, catalog number: 1063290500)
4. Type IA collagen (Nitta Gelatin, Cellmatrix, catalog number: 631-00651)
5. Tyrode's salt (Merck, catalog number: T2145)
6. Tyrode's solution (see Recipes)
7. 3% (w/v) low-melting agarose (see Recipes)
8. Slice culture medium for mouse and ferret tissue (SCM, see Recipes)
9. Slice culture medium for human tissue (SCM-KOSR, see Recipes)
10. Collagen gel mixture (see Recipes)
11. Reconstitution buffer (see Recipes)
12. 5× DMEM-F12 solution (see Recipes)

Equipment

1. Cell culture hood/biological safety cabinet for human samples
2. Cell culture incubator
3. Dissection microscope (e.g., Olympus, catalog number: SZX10)
4. Dissection scissors (FST, catalog number: 15000-10)
5. Heating plate (VWR, catalog number: 75838-286)
6. Gas mixture (5% CO₂ + 40% O₂ + 55% N₂ or 5% CO₂ + 60% O₂ + 35% N₂)
7. Scalpels (Surgical Disposable Scalpel, Braun, catalog number: 5518032)
8. Spoon (for HERO culture, FST, catalog number: 10370-19)
9. Spoon (for human tissue samples, chemical spoon)
10. Vibratome (e.g., Leica, catalog number: VT1000S)
11. Water bath
12. Whole-embryo culture bottles (Nakayama, catalog number: 010-032-11)
13. Whole-embryo culture system (Nakayama, catalog number: 10-0310)

Procedure

Part I: Organotypic slice culture

Please see Figure 2 for images of the selected procedures.

A. Dissection and slicing

1. Warm 2 × 50 ml Tyrode's solution, SCM (see below) to 37°C.
2. Melt 3% low-melting agarose in PBS and keep at 37°C.
3. Dissect mouse or ferret embryos in Tyrode's solution (3 embryos per experiment).
4. Move the heads to warm Tyrode's solution (37°C) and dissect the brains one by one in a 6-cm Petri dish.
5. Dissect the telencephala and store on a heating plate in Tyrode's solution.
Note: For human tissues, start from this step.
6. Remove the meninges after incubating the telencephala in Tyrode's solution.
7. Embed the telencephala in low-melting agarose (takes about 30 min).
8. Cut the telencephala in PBS with a vibratome into 250-300-µm slices.
9. Dissect the neocortical region of interest using a scalpel, if necessary.

B. Collagen gel embedding and culture

1. Prepare the collagen gel mixture (see below) on ice under a hood.
2. Transfer the slices, pre-rinsed in collagen, to a dish containing collagen (on ice) using a Pasteur pipet and ensure that the slices are fully immersed in the collagen gel mixture by gently pipetting up and down.

3. Transfer the slices and collagen gel mixture (200-300 μ l) to a clean dish.
4. Place the slices in the desired position.
5. Remove the excess collagen using a fine-tip Pasteur pipet (<200 μ l).
6. Polymerize the collagen gel on a heating plate at 37°C for 5 min.
7. Transfer the dishes to a cell culture incubator and incubate the slices for 30-40 min.
8. Add 2 ml SCM (for mouse and ferret) or SCM-KOSR (for human) and continue slice culture for the desired time.

Note: Start inhibitor treatment at this step.

9. Keep the slices in the cell culture incubator at 37°C in an atmosphere of 5% CO₂ + 40% O₂ + 55% N₂ for up to two days.

Part II: Hemisphere rotation (HERO) culture

A. Dissection

1. Dissect the mouse or ferret brain from the head and place into a 6-cm Petri dish containing PBS at room temperature.
2. Remove the meninges in PBS.

Note: You do not need to completely remove the meninges. If you are interested in the lateral neocortex, you can keep the meninges in the medial part.

3. Remove the medulla and cerebellum.

B. Culture

1. Warm the SCM to 37°C.
2. Add 1.5 ml SCM to a whole-embryo culture bottle.
3. Transfer the hemisphere(s) to the whole-embryo culture bottle (1-3 hemispheres per bottle) using a spoon.

Note: Start inhibitor treatment at this step.

4. Place the hemispheres in the whole-embryo culture incubator at 37°C, in an atmosphere of 5% CO₂ + 40% O₂ + 55% N₂ for mouse tissue and 5% CO₂ + 60% O₂ + 35% N₂ for ferret tissue, with continuous rotation at 6 rpm for up to two days.

Part III: Free-floating tissue (FFT) culture

A. Dissection and culture of mouse and ferret neocortex

1. Dissect the brain from the head and place in a 6-cm Petri dish containing PBS at room temperature.
2. Remove the meninges in PBS.
3. Dissect the neocortical region of interest using a scalpel and dissection scissors. The size of the tissue is approximately 20-50 mm².

4. Warm the SCM to 37°C.
5. Add 1.5 ml SCM to a whole-embryo culture bottle.
Note: Start inhibitor treatment at this step.
6. Transfer the tissue to the whole-embryo culture bottle (1-2 tissue pieces per bottle) using a spoon.
7. Place the tissue in the whole-embryo culture incubator at 37°C, in an atmosphere of 5% CO₂ + 40% O₂ + 55% N₂ for mouse tissue and 5% CO₂ + 60% O₂ + 35% N₂ for ferret tissue, with continuous rotation at 6 rpm for up to three days.

B. Dissection, pre-incubation, and culture of human neocortex

1. Remove the meninges in PBS.
2. Dissect the neocortical region of interest using a scalpel and dissection scissors. The size of the tissue is approximately 20-50 mm².
3. Warm the SCM to 37°C.
4. Add 1.5 ml SCM-KOSR to a whole-embryo culture bottle.
5. Transfer the tissue to the whole-embryo culture bottle (1-2 tissue pieces per bottle) using a spoon.
6. Pre-incubate the tissues in the whole-embryo culture incubator at 37°C in an atmosphere of 5% CO₂ + 60% O₂ + 35% N₂ with continuous rotation at 6 rpm for 3-5 h.
7. Remove the SCM-KOSR and add 1.5 ml fresh SCM-KOSR to the bottle.
Note: Start inhibitor treatment at this step.
8. Place the tissues in the whole-embryo culture incubator at 37°C in an atmosphere of 5% CO₂ + 60% O₂ + 35% N₂ with continuous rotation at 6 rpm for up to three days.

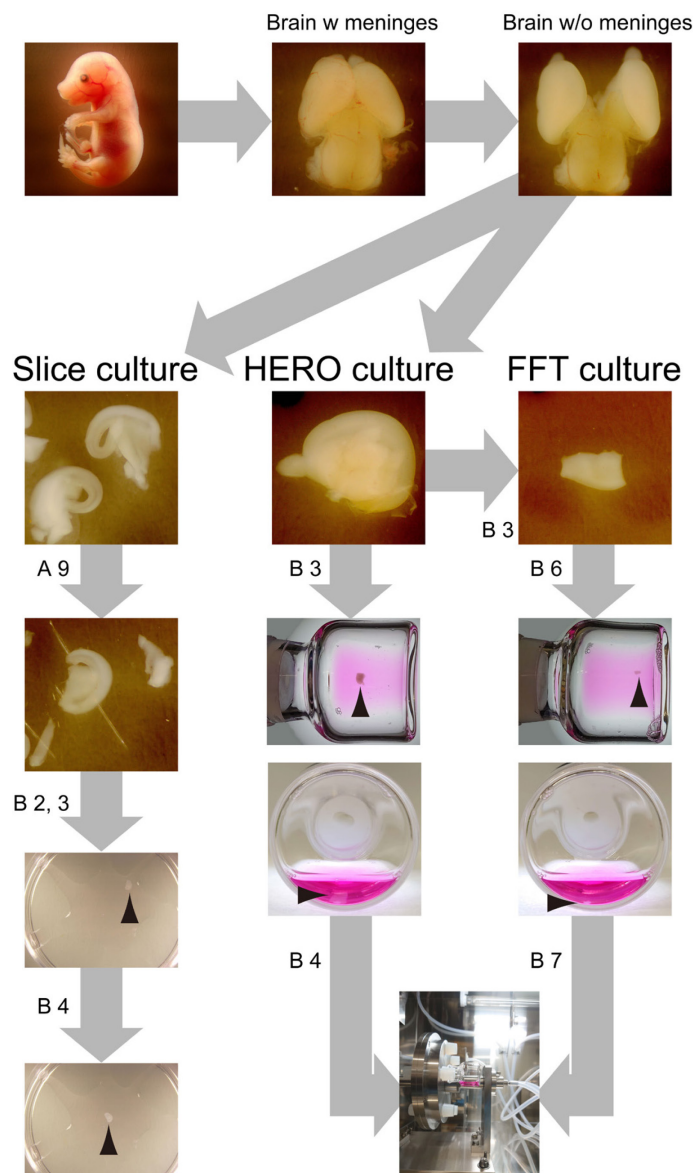


Figure 2. Images illustrating key steps of the *ex vivo* slice/tissue culture protocols. Representative images of the slice, hemisphere rotation (HERO), and free-floating tissue (FFT) culture methods. The combination of letters and numbers corresponds to the steps of each protocol. Arrowheads indicate the neocortical slices/tissues/hemispheres.

Recipes

1. Tyrode's solution
 - a. Dissolve Tyrode's salt and sodium bicarbonate (NaHCO_3 , 1 g for 1 L) in sterile water
 - b. Add 13 ml 1 M HEPES-NaOH (pH 7.3) for 1 L
 Sterile-filter the solution
2. 3% (w/v) low-melting agarose

Low-melting agarose (3 g)

Sterile PBS (100 ml)

3. Slice culture medium for mouse and ferret (SCM) tissue, 100 ml
Neurobasal medium (84 ml),
Rat serum (10%, vol/vol) (10 ml)
200 mM L-glutamine (1 ml)
100× Pen-strep (1 ml)
100× N2 supplement (1 ml)
50× B27 supplement (2 ml)
1 M HEPES-NaOH (pH 7.3) (1 ml)
Store aliquots at -20°C
4. Slice culture medium for human tissue (SCM-KOSR), 100 ml
Neurobasal medium (84 ml)
KOSR (10%, vol/vol) (10 ml)
200 mM L-glutamine (1 ml)
100× Pen-strep (1 ml)
100× N2 supplement (1 ml)
50× B27 supplement (2 ml)
1 M HEPES-NaOH (pH 7.3) (1 ml)
Store aliquots at -20°C
5. Collagen gel mixture, 2.5 ml
Type IA collagen (1.25 ml)
Distilled water (0.5 ml)
5× DMEM-F12 solution (0.5 ml)
Reconstitution buffer (0.25 ml)
6. Reconstitution buffer (100 ml)
NaHCO₃ (262 mM, 2.2 g in 100 ml)
1 M NaOH (5 ml for 100 ml)
1 M HEPES-NaOH (pH 7.3) (20 ml for 100 ml)
Add distilled water to 100 ml
Sterile-filter the solution and store at 4°C in air-tight tubes
7. 5× DMEM-F12 solution (200 ml)
Add 1 bottle DMEM-F12 to 200 ml distilled water

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Competing interests

The authors declare no competing interests.

Ethics

All animal experiments (mice and ferrets) were performed in accordance with the German Animal Welfare legislation (“Tierschutzgesetz”). All procedures regarding the animal experiments were approved by the Governmental IACUC (“Landesdirektion Sachsen”) and overseen by the Institutional Animal Welfare Officer(s). The mouse embryo shown in Figure 2 was collected with approval from the University of Helsinki.

Fetal human brain tissue (PCW 10-14) was obtained from the Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, Universitätsklinikum Carl Gustav Carus of the Technische Universität Dresden, with approval from the local University Hospital Ethical Review Committees and informed written maternal consent, and from the Human Development Biology Resource (HDBR), with the human fetal material being provided by the Joint MRC/Wellcome Trust (MR/R006237/1) Human Developmental Biology Resource (<http://www.hdbrr.org/>).

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