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bio-protocol www.bio-protocol.org/e3674 Bio-protocol 10(13): e3674. DOI:10.21769/BioProtoc.3674

Organotypic Slice Culture of the Embryonic Mouse Brain

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[Abstract] Organotypic slice culture is a powerful technique for exploring the embryonic development of the mammalian brain. In this protocol we describe a basic slice culture technique we have used for two sets of experiments: axon guidance transplant assays and bead culture assays.
Keywords: Organotypic, Slice culture, Transplant, Axon guidance, Morphogen, Bead culture

[Background] Organotypic slice culture is a technique that has been widely used in recent years and has been a particularly popular technique in the field of neurodevelopment. It has the great advantage of allowing the culture of developing neural cells *in-vitro* while maintaining the *in-vivo* structure of the tissue. In this protocol we describe the slice culture technique we used for two experiments from our recent paper (Clegg *et al.*, 2019). Firstly we performed an axon guidance transplant assay whereby fluorescently labeled tissue was transplanted into a non-fluorescent host slice in order to observe axon outgrowth. This is a modified version of a protocol previously used to study the development of the corpus callosum, but could be easily adapted to examine other axon tracts such as the thalamocortical tract (Niquille *et al.*, 2009). Secondly, we performed a bead culture assay in which beads soaked in recombinant FGF protein was embedded in the tissue, this allowed the examination of the molecular and cellular response of the tissue to the FGF protein. The use of protein soaked beads in this way provides a method for focal delivery of recombinant protein at a specific position, in contrast to bath application which exposes all tissue to the protein equally. This mimics the situation *in-vivo* where a morphogen (such as FGF17) is expressed at a particular anatomical position and then diffuses through the tissue. This technique could be used to explore the response to a number of different recombinant proteins or pharmacological agents.

Materials and Reagents

Materials

1. 7 ml bijou tubes (Greiner, catalog number: 189170)
2. Pipette tips (Greiner, catalog number: 739288)
3. 1.5 ml microcentrifuge tubes (Greiner, catalog number: 616201)
4. Peel-A-Way® Embedding Molds (Square–S22, Polysciences)
5. Falcon® 60 mm TC-treated Center Well Organ Culture Dish (Corning, catalog number: 353037)
6. 13 mm Nuclepore polycarbonate track-etched membranes (Whatman, catalog number: 110401)
7. Specimen disk

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8. Adhesive

Animals

Mice used in our original study ubiquitously express τGFP and have been described previously (Pratt *et al.*, 2000). All mice were maintained on a CD-1 background (Charles River, strain code: 022). Mice used for timed matings were aged between 6 and 24 weeks.

Reagents

1. Earls balanced salt solution (EBSS) calcium, magnesium, phenol red (Thermo Fisher, catalog number: 24010043)
2. SeaPlaque GTG Agarose (Lonza, catalog number: 50110)
3. Affi-Gel Blue gel beads (Bio-Rad, catalog number: 1537301)
4. MEM (Thermo Fisher, catalog number: 11090081)
5. Neurobasal medium (Thermo Fisher, catalog number: 21103049)
6. 1 M HEPES (Thermo Fisher, catalog number: 15630080)
7. Penicillin-streptomycin (Thermo Fisher, catalog number: 15140122)
8. D-Glucose solution (Merck, catalog number: G8769)
9. Gentamicin 50 mg/ml (Thermo Fisher, catalog number: 15750060)
10. Glutamine 200 mM (Thermo Fisher, catalog number: 25030081)
11. Fetal bovine Serum (Merck, catalog number: F4135)
12. B-27 supplement (Thermo Fisher, catalog number: 17504044)
13. OCT embedding matrix (Cellpath, catalog number: KMA-0100-00A)
14. 1x Krebs buffer (from 10x stock, see recipes)
15. 1x PBS (Thermo Fisher catalog number: 14190094)
16. NaCl (Thermo Fisher catalog number: 10428420)
17. KCl (Thermo Fisher catalog number: 10684732)
18. NaH_2PO_4 (Thermo Fisher catalog number: 10723621)
19. $CaCl_2$ (Thermo Fisher catalog number: 10657662)
20. $MgCl_2$ (Thermo Fisher catalog number: 10386743)
21. Triton X-100 (Merck, catalog number: 10789704001)
22. Bovine serum albumin (Merck, catalog number: A1933)
23. Anti-GFP antibody (Abcam, catalog number: ab290)
24. Goat anti-Rabbit IgG 488 (Thermo Fisher, catalog number: A-11008)
25. DAPI (Thermo Fisher, catalog number: D1306)
26. Vectashield Hardset (VectorLabs, catalog number: H-1400)
27. 10x Krebs Buffer (see Recipes)
28. 1x Krebs with antibiotic (see Recipes)
29. MEM with serum (see Recipes)
30. Neurobasal (see Recipes)

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Equipment

1. Pipettes
2. Laminar flow tissue culture hood
3. Tissue culture incubator (37 °C, 5% CO_2)
4. Dissecting microscope (Euromex, catalog number: DZ1100)
5. Leica VT1000 S vibratome
6. Water bath
7. Fisherbrand microspatula with flat-ended blade (Thermo Fisher, catalog number: 21-401-20)
8. Forceps, size 5 (Fine Science Tools, catalog number: 11251-20)
9. Microscissors, 5 mm cutting edge (Fine Science Tools, catalog number: 15003-08)
10. Scissors, 9 cm (Fine Science Tools, catalog number: 14060-09)
11. 45° Sterile blade (Altomed, model: A10136)
12. Cryostat (Leica, catalog number: CM3050 S)
13. Super-frost Plus slides (Thermo Fisher, catalog number: J1800AMNZ)
14. Hydrophobic barrier pen (VectorLabs, catalog number: H-4000)

Procedure

A. Organotypic slice culture

1. Oxygenate EBSS by bubbling 100% oxygen through the solution until a color change (pink to orange) is observed. EBSS should then be placed on ice to cool.
2. Cull pregnant dams at the desired embryonic stage by dislocation of the neck or an overdose of anesthetic.
   Note: Slice cultures can be performed from embryonic day (E) 13.5 onwards. For axon guidance assays a stage should be selected during which axon outgrowth of the tract under investigation is occurring, e.g., E17.5 for the corpus callosum.
3. Remove the uterus and place in a dish of pre-cooled oxygenated EBSS on ice, in the tissue culture hood, use scissors and forceps to remove embryos from the uterus. Using scissors, cut the head of the embryo from the body, and transfer to the second dish of EBSS.
4. Use forceps to peel away the skin from the head to reveal the skull, cut down the midline of the skull using microscissors and peel back the skull to reveal the brain. Carefully detach the brain from the base of the head using forceps.
   Note: Brains should be dissected out and placed in EBSS within 1 h of the pregnant dam being culled.
5. Collect brains in 7 ml bijou tubes containing oxygenated EBSS and place tubes on ice.
   Note: Brains can be held at this stage for up to 3 h to allow time for genotyping (if necessary). It is recommended to add glucose to the EBSS (concentration 0.1%) if planning to hold brains at this stage.

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6. Embed brains individually in approximately 5 ml of low melting point agarose in square embedding molds. Agarose should be held at a temperature of 42 °C using a water bath prior to use. Place mold on ice once the tissue is positioned (Figure 1A).
   Note: Before positioning the brains at the desired plane, stir the agarose surrounding the tissue repeatedly in order to remove any carried over EBSS from the tissue. This prevents tissue separating from the agarose during sectioning (Figure 1A).
7. Once set, remove agarose from mold and trim using a razor blade until the agarose block containing the tissue is < 1 cm^3 (Figure 1B). Attach the agarose block to the specimen disk using adhesive (Figure 1C).
   Note. The plane of section should be determined by the brain region or axon tract under investigation, for corpus callosum axon guidance assays a coronal sectioning plane was used and tissue sections containing the telencephalon were collected.
8. Section tissue on the vibratome at a section thickness of 400 µm in ice-cold 1x Krebs buffer.
   Note: While sectioning, the vibratome should be set to a frequency setting of 8 and a speed setting of 4. If tissue starts to break up reduce speed to 3.
9. Collect tissue sections in 1x Krebs containing 100 mM HEPES, 0.5 mg/ml gentamicin and 1% penicillin-streptomycin.
   Note: Transfer of tissue sections should always be done with blunt-ended microspatulas, brushes should not be used as tissue can stick to bristles causing damage.
10. Add 1 ml MEM with serum to the well of the organ culture dish. Place a Whatman nuclepore membrane onto the MEM at the center of the well (shiny side facing the liquid).
11. Carefully transfer a single tissue section to the center of the membrane making sure not to submerge any part of the membrane (Figures 1D and 1E). Incubate the culture for 1 h at 37 °C in 5% CO_2.
12. Aspirate MEM and replace with 1 ml Neurobasal medium containing 2% B27 supplement, 1% penicillin-streptomycin, 0.5% glucose and 2 mM glutamine.
13. Incubate at 37 °C in 5% CO_2 for 12-72 h and fix with 4% paraformaldehyde in PBS overnight at 4 °C.

B. Axon guidance transplant assays
   1. After aspirating MEM in Step A12, use a 45° blade or microscissors to dissect tissue from the donor section (Figure 1F).
      Note: Cutting the tissue explant into an asymmetrical shape will make it easier to maintain orientation during transplant.
   2. Cut a similarly sized piece of tissue from the host section and remove using a pipette.
   3. Using a pipette transfer the donor tissue to the host section and maneuver into position using forceps (Figure 1G).
      Note: To ensure donor axon outgrowth into host tissue, the donor explant must be flush against the host tissue with no visible gap.

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4. Add neurobasal medium and culture for up to 72 h as above to allow for axon outgrowth.
5. Fix using 4% paraformaldehyde.
6. Cryoprotect by immersing sections in 30% sucrose in PBS overnight, or until the tissue has sunk, and embedded in 50% OCT embedding matrix; 50% Sucrose/PBS for cryosectioning. Frozen sections can then be processed for immunofluorescence.
7. If donor tissue expresses GFP or another fluorescent marker, immunofluorescence can then be performed to detect donor axon growth in host tissue.
   Note: While it may be possible to detect fluorescently labeled axons without any further staining, immunofluorescence will give a clearer signal for imaging.
8. Stained sections can be imaged using epifluorescence or confocal microscopy.

C. Bead culture assays
1. Affi-Gel blue gel beads (50 µl) are pre-soaked in recombinant protein or 5 mg/ml bovine serum albumin (BSA) overnight at 4 °C.
   Note: The concentration of recombinant protein used to soak the beads should be determined for each protein used. For our experiments using FGF8 and FGF17, a concentration of 100 µM was used.
2. After aspirating MEM in Step A12, a pipette can be used to place a single bead onto the tissue section, forceps can then be used to embed the bead into the tissue (Figure 1H).
   Note: Pre-soaked beads can be pipetted into a 60 mm Petri dish filled with PBS to allow easier selection of single beads.
3. Add neurobasal medium and culture for up to 72 h as above.
4. Fix using 4% paraformaldehyde in PBS overnight at 4 °C.

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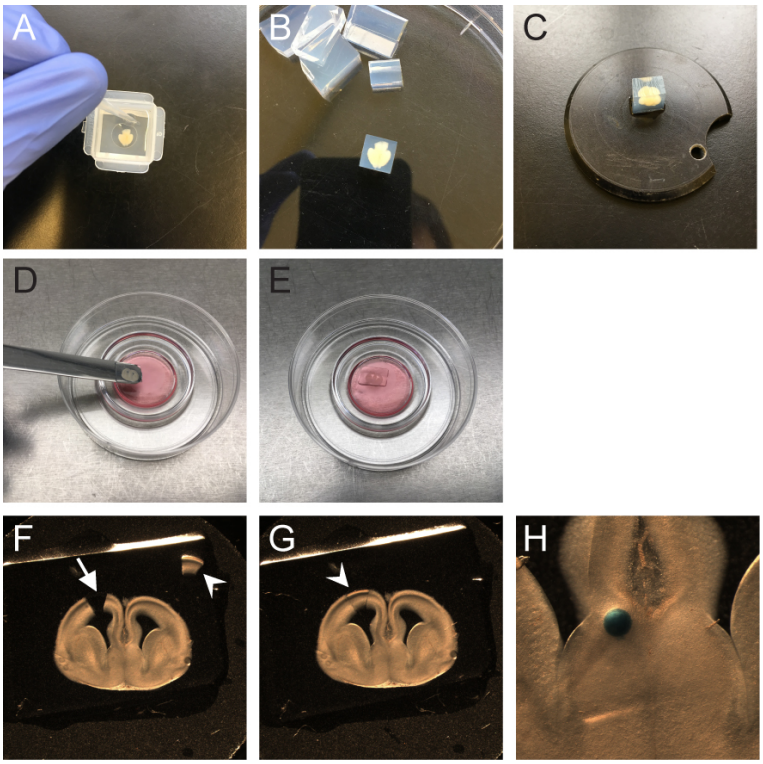

Figure 1. Preparation of tissue sections for culture. A. The dissected brain should be embedded in low melting point agarose, which should be stirred vigorously using a pipette tip. B. Once set, agarose should be trimmed as shown. C. Trimmed agarose block attached to specimen disk using a strong adhesive. D and E. After vibratome sectioning tissue sections should be transferred to floating Whatman Nuclepore membranes using a flat-ended spatula. F and G. For transplant cultures tissue should be cut out from the host section in an asymmetric shape (in this example a 'wedge' shape) to make preservation of tissue orientation easier (arrow, F). A similarly shaped piece of tissue from the donor section can then be positioned in the hole left in the host section (arrowhead F and G). H. For bead cultures pre-soaked Affi-Gel blue gel beads can be embedded in tissue as shown.

D. Immunofluorescence

1. Cryoprotect tissue using 30% sucrose in PBS overnight, or until the tissue has sunk, and embedded in 50% OCT embedding matrix; 50% Sucrose/PBS for cryosectioning.
2. Section tissue using a cryostat at a thickness of 10 µm and collect sections on Superfrost Plus slides.
3. If using an antibody for a morphogen (such as FGF8) perform a methanol fixation step by immersing slides in ice-cold 100% methanol for 30 min.
4. Permeabilize sections by washing with 0.1% Triton X-100 in 1x PBS for 5 min.
   Note: All wash steps should be performed at room temperature in a Coplin jar or similar and with agitation.
5. Draw around tissue sections using a hydrophobic barrier pen.

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6. Block sections using a blocking solution of 1% bovine serum albumin in PBS/0.1% Triton X-100, 200 µl per slide, for 1 h at room temperature.
7. Apply primary antibody diluted in blocking solution, 200 µl per slide, overnight at 4 °C.
   Note: Antibody concentration should be determined by the user, for GFP immunofluorescence used in Figure 2 the anti-GFP antibody (see reagents) was diluted 1 in 300.
8. Wash with PBS/0.1% Triton X-100 for 5 min.
9. Apply the appropriate fluorescent conjugated secondary antibody diluted in blocking solution, 200 µl per slide, for 1 h at room temperature.
   Note: Secondary antibody should be raised against the species of the primary antibody used in Step D7. For the GFP immunofluorescence used in Figure 2, a donkey anti-rabbit 488 antibody was used diluted 1 in 200.
10. Wash with PBS/0.1% Triton X-100, 5 min.
11. Counterstain with DAPI diluted 1 in 1,000 in PBS, 10 min at room temperature.
12. Wash with PBS, 5 min.
13. Coverslip slides using Vectashield Hardset and leave to dry at room temperature for 1 h.
14. Once dried, slides can be imaged using epifluorescence or confocal microscopy.

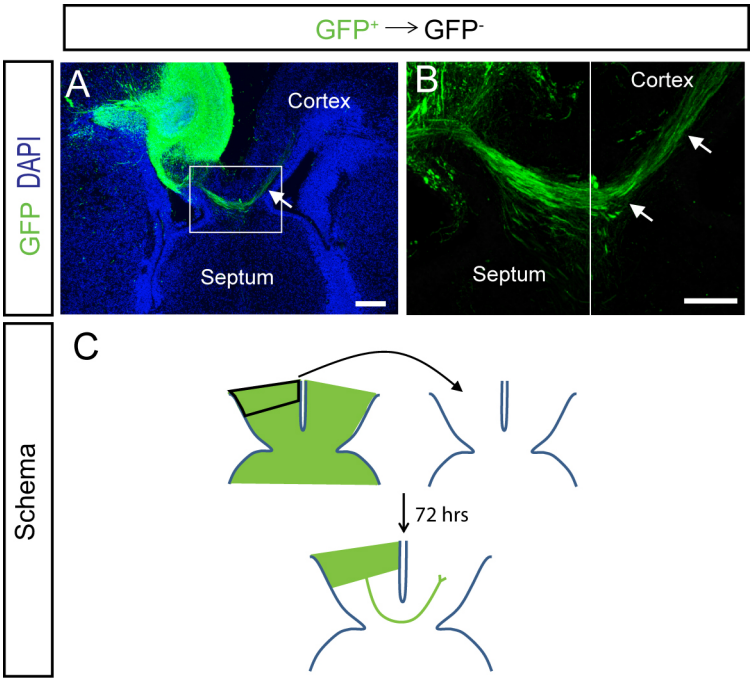

Figure 2. Axon guidance transplant assay. A and B. An example of a corpus callosum axon guidance assay after immunofluorescence for GFP. An explant of cortical tissue from a GFP expressing donor section has been transplanted into a non-GFP expressing host section. After culture GFP expressing callosal axons have grown from the donor explant and crossed the host midline to reach the opposite cerebral hemisphere (arrows A and B). A and C. Schematic diagram illustrating the transplant experiment. B is a higher magnification image of the boxed region in A. Scale bars = 200 µm.

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Recipes

1. 10x Krebs Buffer

NaCl	73.6 g	126 mM
KCl	87 g	2.5 mM
NaH_2PO_4	1.66 g	1.2 mM
$CaCl_2$	3.68 g	2.1 mM
$MgCl_2$	2.44 g	1.2 mM
ddH_2O	1 L	

2. 1x Krebs with antibiotic
   1x Krebs 49 ml
   1 M HEPES 0.5 ml
   Pen-Strep 0.5 ml
   Gentamicin 0.1 ml
3. MEM with serum
   MEM 44 ml
   FBS 5 ml
   50% Glucose 0.5 ml
   Pen-Strep 0.5 ml
4. Neurobasal
   Neurobasal 47.5 ml
   B-27 supplement 1 ml
   50% Glucose 0.5 ml
   Pen-Strep 0.5 ml
   Glutamine 0.5 ml

Acknowledgments

This work was supported by funding from the Wellcome Trust, the Biotechnology and Biological Sciences Research Council and The Simons Initiative for the Developing Brain.

Competing interests

The authors declare no competing financial interests.

Ethics

All mice were bred in-house according to Home Office UK legislation and licenses approved by the University of Edinburgh Ethical Review Committees and Home Office. Animal husbandry was in accordance with UK Animals (Scientific Procedures) Act of 1986 regulations.

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