Video Article

Culture of Isolated Floor Plate Tissue and Production of Conditioned Medium to Assess Functional Properties of Floor Plate-released Signals

Camille Charoy[1], Elise Arbeille[1], Karine Thoinet[1], Valérie Castellani[1]

[1]CGphiMC, UMR CNRS 5534, University of Lyon 1

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Abstract

During development, progenitors and post-mitotic neurons receive signals from adjacent territories that regulate their fate. The floor-plate is a group of glial cells lining the ependymal canal at ventral position. The floor-plate expresses key morphogens contributing to the patterning of cell lineages in the spinal cord. At later developmental stages, the floor-plate regulates the navigation of axons in the spinal cord, acting as a barrier to prevent the crossing of ipsilateral axons and controlling midline crossing by commissural axons[1]. These functions are achieved through the secretion of various guidance cues. Some of these cues act as attractants and repellents for the growing axons while others regulate guidance receptors and downstream signaling to modulate the sensitivity of the axons to the local guidance cues[2,3]. Here we describe a method that allows investigating the properties of floor-plate derived signals in a variety of developmental contexts, based on the production of Floor-Plate conditioned medium (FP^{cm})[4-6]. We then exemplify the use of this FP^{cm} in the context of axon guidance. First, the spinal cord is isolated from mouse embryo at E12.5 and the floor-plate is dissected out and cultivated in a plasma-thrombin matrix (**Figure 1**). Second two days later, commissural tissue are dissected out from E12.5 embryos, triturated and exposed to the FP^{cm}. Third, the tissue are processed for Western blot analysis of commissural markers.

Video Link

The video component of this article can be found at https://www.jove.com/video/50884/

Introduction

The floor-plate is a well-known patterning center of the developing spinal cord, playing key roles in the specification of progenitors and postmitotic cell lineages and controlling axon navigation[7,8]. The experimental procedure described here to produce FP^{cm} allows investigators to assess the functional properties of floor plate-derived signals in a various contexts of developmental processes, from cell patterning and survival to cell and axon migration.

To illustrate the use of such FP^{cm}, dorsal spinal cord tissues containing commissural neurons are dissected, dilacerated and stimulated with the FP^{cm}. The tissues can then be processed for Western blot analysis. This allows investigating regulations of the axon guidance machinery by floor-plate released signals. The method of treatment of dilacerated fresh tissue holds the great advantage of preserving the microenvironment of cells within the tissue. Thus the consequences of the treatment by FP^{cm} or any types of treatment are assessed in a more physiological way than in cell and tissue culture conditions.

Protocol

DAY 1

1. Dissection of the Spinal Cord Floor Plate (FP) from E12.5 Mouse Embryos

Note: The entire procedure requires the use of sterile conditions. It is preferable to perform the dissection under a dissection hood to avoid contamination. The surface of the hood should be cleaned with ethanol. All dissection instruments must be sterilized and kept in a sterile Petri dish. The liquids (medium, dissection medium) must be kept closed and put in an ice bath. Each embryo should be collected in individual fresh drop. This is important for tissue preservation. The dissection is performed with Dumont #5 forceps. To transfer the embryos between dishes, grip the umbilical cord or the head without any damage to the hindbrain. It is critical not to damage the spinal cord to successfully complete the dissection. Prepare cold Phosphate Buffered Saline (PBS), cold Hank's Balanced Salt Solution (HBSS)-6.5% glucose and room temperature neurobasal medium.

1.1 Spinal cord dissection

Animals are treated according to the animal care guidelines of the Centre National de la Recherche Scientifique following European directives. The method of euthanasia is cervical dislocation, it consist of applying pressure to the neck and dislocating the spinal column from the brain. This method is recommended for mouse animal models, and can be performed in a fast and efficient way.

1. Euthanize a pregnant mouse at E12.5 of gestation (E0.5= first day following matting). Place the mouse ventral side up.
2. Soak the abdomen with 70% ethanol. Pinch the skin of the abdomen with Adson forceps and cut through the skin and the peritoneal layers with surgical scissors. Make an incision and use it to cut on both side of the mouse to expose the abdominal cavity.
3. A string of embryos is present on each side of the mouse. Grasp one horn of the uterus in the tissue between two embryos and dissect it out starting from the exterior part. Transfer the intact uterus to a 100 mm Petri dish with cold PBS on ice.
4. Extract the embryos from extra-embryonic tissues. Grasp the placenta tissue (in dark red) with two pairs of forceps and pull softly to tear the tissue and remove the embryos from the uterine sac.

Note: For a better conservation, collect the embryos from their uterine sac one by one.

5. The next steps are done under binocular magnifying glass and dissection hood. Place one embryo in a drop of cold HBSS-6.5% glucose.
6. Using forceps, decapitate the embryo.

Note: Cut between the lower jaw and the hindbrain, the posterior part of the hindbrain is important for the next steps.

7. Place the embryo ventral face down with the anterior part facing the experimenter.
8. Pinch the skin of the embryo with one pair of forceps and with the other pair pull away the skin.

Note: This step has to be repeated until the skin is completely removed from the anterior part of the embryo.

9. Turn the embryo anterior side to the left. With one forceps "pin down" the embryo in the anterior part. With the other forceps grab the skin and pull towards the rostral side of the embryo. The spinal cord is then accessible.
10. Use one side of the forceps to cut the meninges that still wrap the spinal cord. The spinal cord is now open.
11. Turn the embryo anterior side to the right.
12. Detach the tissue from the spinal cord starting from the cervical side of the embryo. Slide the forceps under the spinal cord (the forceps should be held closed to avoid spinal cord damage). The forceps have to be visible under the spinal cord. Small rotatory movements under the spinal cord are sufficient to detach the surrounding tissue and the dorsal root ganglia (DRG).

Note: Leaving attached surrounding tissue add weight to the spinal cord, thus increasing the probability of breaking it during the next step.

13. Place the isolated spinal cord in a fresh drop of cold HBSS-6.5% glucose.
14. Place the spinal cord in a flat position, meninges on the top and anterior side away from the experimenter.
15. "Pin down" the spinal cord using the remaining hindbrain with one forceps, and with the second forceps grab the meninges. Peel off the meninges starting from the rostral side of the spinal cord.

Note: The movement should be slow and constant to avoid any breaking of the spinal cord. Vibratory movements can facilitate the detachment of the meninges.

1.2 FP dissection

1. Pinch the hindbrain with one forceps.
2. The FP is the central transparent part of the tissue. Using a scalpel cut out the floor plate.

Note: To cut both sides with the same quality, start from the rostral side and alternately cut the right side and the left side of the floor plate.

3. Conserve the dissected FP in Neurobasal medium at room temperature.

2. FP Culture

Note: All steps should be performed under sterile conditions in a tissue culture hood. Use fresh medium and freshly thawed supplements and reagents. Prepare sterile glass coverslips, warm neurobasal+B27, plasma, and HBSS-thrombin.

2.1 FP culture

1. Place several sterile coverslips in a 100 mm Petri dish and pipette 20 µl plasma in the center of each coverslip.
2. Transfer two to four FP in each plasma drop and add 20 µl HBSS-thrombin next to the plasma drop and carefully mix. Keep the coverslips for a minimum of ten minutes at room temperature to allow coagulation of the plasma.

Note: Transfer two FPs in case of complete dissection and more in case of partial dissection. Mix slowly with a pipette tip and avoid contact with the FP explants.

The plasma clot retracts a few µm as a sign of the coagulation. The coagulation must not be stopped prematurely, to avoid detachment of the plasma clot.

3. Transfer each coverslip in a 24-well plate and add 500 µl of Neurobasal+B27. Incubate 48 hr at 37 °C.

DAY 3

3. FP Conditioned Medium (FP^{cm}) Harvesting

Note: Harvesting should be performed under sterile conditions in a tissue culture hood. If the supernatant is contaminated or if floor plates are floating (meaning they might have died during the culture period) then do not harvest the FP^{cm} from this well.

3.1 FP^{cm} harvesting

1. Harvest carefully the medium from each well.

Note: Do not pipette the polymerized plasma-thrombin mix or any FP fragments.

2. Store 100 µl aliquots at -80 °C.

DAY 4

4. Dissection of Spinal Cord Commissural Neurons from E12.5 Mouse Embryos

Note: This preparation does not need to be performed under sterile conditions. Follow the procedure described above to collect the embryos. Prepare cold PBS, cold HBSS-6.5% glucose and cold neurobasal.

4.1 Spinal cord dissection

1. Using forceps, decapitate the embryo.

Note: Cut between the lower jaw and the hindbrain, the posterior part of the hindbrain is important for the next steps.

2. Place the embryo ventral face down in a fresh drop with the anterior part facing the experimenter.
3. Pinch the skin of the embryo with one forceps and with the other one pull away the skin.

Note: This step has to be repeated until the skin is completely removed from the anterior part of the embryo.

4. Turn the embryo anterior side to the left. With one forceps "pin down" the embryo in the anterior part. With the other forceps, grasp the skin and pull towards the rostral side of the embryo, to expose the spinal cord.
5. Use one side of the forceps to cut the meninges that still wrap the spinal cord. The spinal cord is now exposed and open.
6. Position the embryo anterior side to the right.
7. Detach the tissue from the spinal cord starting from the cervical side of the embryo. Slide the forceps under the spinal cord (keep the forceps closed). Forceps must visible under the spinal cord. Small rotatory movements under the spinal cord are sufficient to detach the adjacent tissue and the dorsal root ganglia (DRG).
8. Transfer the isolated spinal cord in a new drop of cold HBSS-6.5% glucose.
9. Place the spinal cord in a flat position, meninges on the top and anterior side away from the experimenter.
10. "Pin down" the spinal cord using the remaining hindbrain with one forceps, with the second forceps grab the meninges. Peel off the meninges starting from the rostral side of the spinal cord.

4.2 Commissural Neuron dissection

1. Pinch the hindbrain with one forceps.
2. The Commissural neurons are located in the most lateral part of the spinal cord (the dorsal side). Cut out this lateral part with a fine scalpel.

Note: The dorsal part of the spinal cord can be distinguished from the ventral part by difference of cell density, the ventral part being more opaque.

Note: To cut both sides with the same quality, start from the rostral side and alternately cut a small length of the right side and of the left side.

3. Conserve the dissected tissue in Neurobasal medium on ice for immediate use.

5. Treatment of Commissural Tissue with the FP^{cm}

Note: This procedure does not require sterile conditions. The tissue should be collected in individual 1.5 ml tubes. The frozen FP^{cm} should be used only once. Avoid multiple refreezing. Prepare warm FP^{cm} (37 °C).

5.1 Treatments of commissural neurons

1. Dilacerate the commissural tissue with small scissors.

Note: Cut the tissue as small as possible, to potentiate the accessibility of the cells.

2. Distribute fairly the tissue between the different experimental conditions control treatment and FP^{cm} treatment.
3. Centrifuge the tissue fragments at 800 x g for 1 min at 4 °C.

4. Check the repartition between tubes.

Note: If necessary redistribute and centrifuge again until fragments are equally distributed.

5. Remove the supernatant.
6. Add the warm FP^{cm} and control treatments to the tissues.

Note: The volume must be adjusted to the amount of the pellet and should be at least twice the volume of the pellet.

7. Incubate at 37 °C for 30 min.

Note: Gently shake every 10-15 min

8. Centrifuge at 800 x g for 1 min and remove the treatment.
9. Add the lysis buffer

Note: The volume must be adjusted to the amount of the pellet and should be at least twice the volume of the pellet.

10. Pipette up and down to resuspend the pellet.

Note: A vortex can also be used.

11. Incubate 15 min on ice.
12. Centrifuge at 14,000 x g for 15 min at 4 °C.
13. Keep the supernatant and measure the amount of protein with a Bradford assay[9].
14. Add 6x Laemmli Buffer.
15. Incubate at 95 °C for 5 min to denature the protein content.

Note: Samples can be used immediately or can be stored at -80 °C.

16. Assess the effects of the treatment by Western blot[10].

Note: At least 50 µg of total proteins should be loaded for each lane.

Representative Results

Commissural tissues were treated by the FP^{cm} and the samples were processed for analysis of receptor levels in Western blot. Application of the FP^{cm} was shown to increase the levels of a guidance receptor, PlexinA1, which mediates the sensitivity of commissural axons to the midline repellent Semaphorin3B after crossing (**Figure 2**). This revealed that signals released by the FP regulate PlexinA1 levels[4,6].

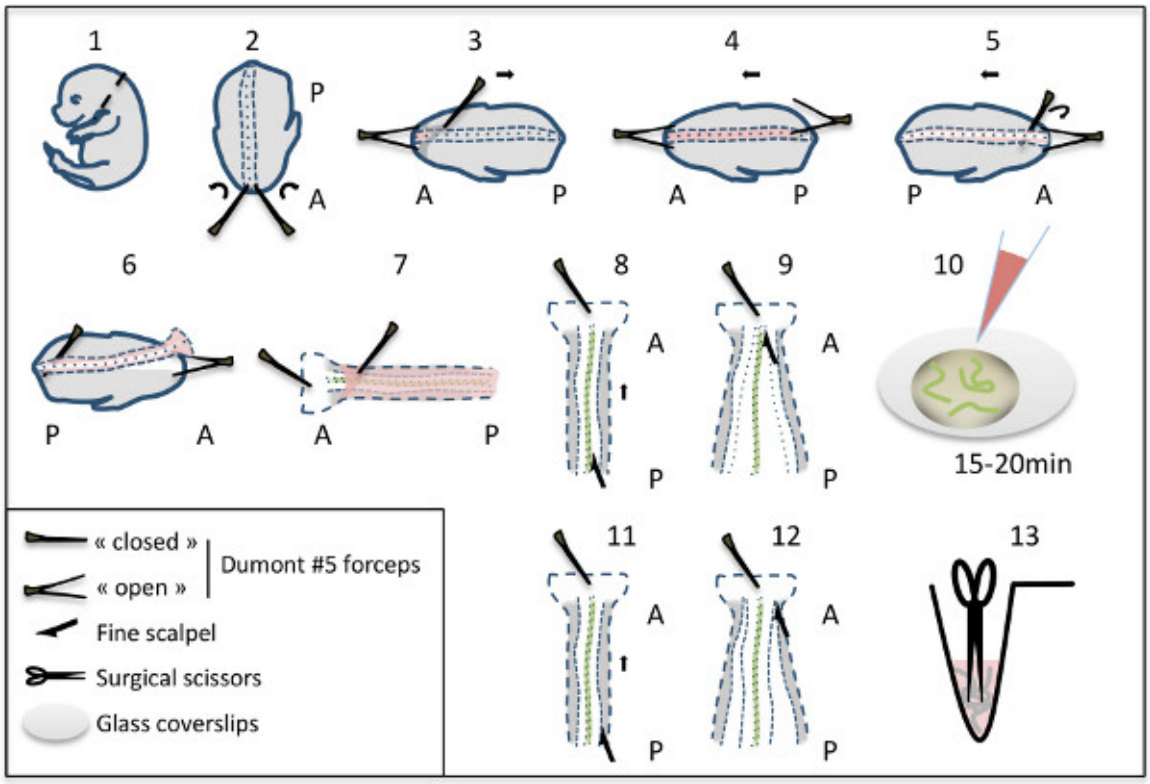

Figure 1. Schemes of the dissection procedure to isolate the Floor Plate. The different steps of the dissection and the culture procedure are illustrated. 1) cut off embryo head; 2-3) remove the skin; 4) open the meninges; 5-6) remove the spinal cord from the embryo; 7) remove the meninges; 8-9) dissect out the floor plate; 10) add the thrombin to the FP-plasma mix and wait 10 to 15 min at room temperature before adding the culture medium. 11-12) dissect out the dorsal neurons; 13) dilacerate the tissue . A: anterior pole of the embryo. P: posterior pole of the embryo. The arrows depict the direction of the movement. Click here to view larger figure.

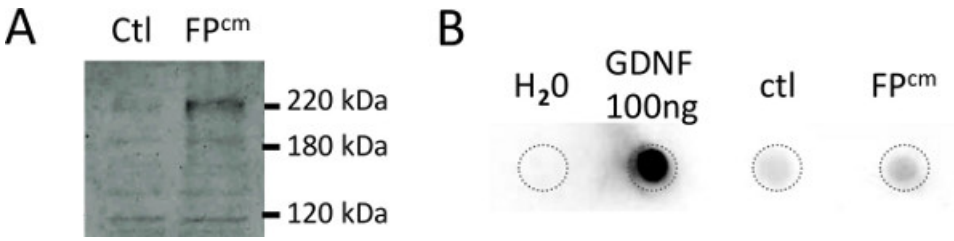

Figure 2. Representative result. A) Dorsal spinal tissue were collected, dilacerated and stimulated with the Floor plate conditioned medium and control medium. The samples were lysed and processed for Western blot. Proteins were probed with antibodies anti-PlexinA1, and β-actin. The photo illustrates the increase of PlexinA1 levels in the sample stimulated with the floor-plate conditioned medium. **B)** The dot blot shows the expression of gdnf in FP^{cm} compared to it's control medium.

Discussion

The production of floor-plate conditioned medium provides an efficient and easy way for assessing the biological properties of floor plate released signals.

The plasma-thrombin matrix provides excellent condition for tissue survival. Nevertheless, this enriched environment might be a limitation for some types of experiments. Thus, the floor plate tissue can also be cultivated in agarose matrix. The quality of the floor plate conditioned medium can be assessed by the detection of known floor-plate released signals, such as gdnf (**Figure 2**), Shh and Netrin[11]. Their presence in the conditioned medium can be assessed by Western blot or dot blot.

In this procedure cells are studied in their native microenvironment. This prevents further information on the type of cells in the tissue that react to the treatment. As the floor plate is not a homogeneous structure, the concentration of active components such as Wnts might be different between the cultures depending on the dissected area along the rostro-caudal axis.

Biochemistry on fresh dilacerated tissue holds the advantage to study cells in their native microenvironment. Thus, cell behaviors are investigated in conditions that recapitulate as closely as possible their native context[12]. The dorsal spinal cord does not contain only commissural neurons so the effects of FP^{cm} can also be assessed in cultured commissural neurons.

The medium can be applied to various types of cell and tissue cultures. A large range of parameters can be assessed, from cell identity, cell survival, cell death, and cell differentiation. Samples can be processed for proteomics.

An important parameter of the preparation of the dilacerated fresh tissue is the repartition of the tissue for the control and experimental treatments. It is important to collect the tissues first in a single tube and to dilacerate the tissue collectively prior to separate the samples. This limits the difference of protein concentration detected after lysis between samples.

Note that HBSS-glucose can be stored at 4 °C for up to one month. Neurobasal can also be kept for one month at 4 °C. B27 aliquots can be stored at -20 °C for long term and at 4 °C for up to one week. Plasma aliquots should be store at -20 °C and can be refrozen after use. Thrombin aliquots can also be stored at -20 °C and cannot be refrozen.

Disclosures

The authors have nothing to disclose

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