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ISBN

9781071620380

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Publication Date

2022

DOI

10.1007/978-1-0716-2039-7_10

A co-culture system for studying dorsal spinal cord synaptogenesis

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Acknowledgments: Supported in part by grants from the National Institutes of Health R01NS064341, R01DE021847, R01DE029202 (ZDL).

Key words: Pain mechanisms, DRG, dorsal spinal cord, neuronal co-culture, synaptogenesis

Abstract

Studying synapse formation, maintenance and plasticity in adaptation to developmental and pathological changes is critical in our understanding of cellular mechanisms of biological processes and disease states. However, a major barrier in getting cell-type specific detail information in these studies is the complexity of in vivo environment in high-density tissue or organs, such as spinal cord, that is packed with different type of cells, connecting tissues and structural components. In this chapter, we describe a co-culture system in which dorsal root ganglion sensory neurons and spinal cord neurons are cultured in separate compartments without culture medium diffusion between compartments, but allowing sensory neuron axons to outgrowth to adjacent chambers to establish synaptic connections with dendrites of spinal cord neurons. This provides an in vitro environment that mimics the in vivo synaptogenic environment between sensory neurons and spinal cord neurons, and enables manipulation of specific neuronal populations and studying their detail contribution to synaptic formation, maintenance and plastic changes.

1. Introduction

Studying spinal cord synaptogenesis during development and under pathophysiological conditions is critical for our understanding of normal sensory and motor functions as well as relevant disorders, such as chronic pain development and motor function deficits, after peripheral/spinal nerve injury or spinal cord injury. However, it remains a technical challenge to study bio-pathological mechanisms of spinal cord synaptogenesis in vivo due to the complexity of synaptogenic environment and high density of spinal cord tissues. To overcome this barrier, we have fine turned a primary co-culture system of dorsal root ganglion (DRG) neurons and dorsal spinal cord (DSC) neurons to facilitate studies related to sensory neuron synapse formation in a controlled *in vitro* environment (1,2). Although a cell culture system does not wholly replicate the *in vivo* milieu, we believe that this simplified system enables genetic and biochemical manipulations that are critical in studying the roles of individual genes/factors and signaling pathways important for sensory neuron synaptogenesis in a controlled environment that mimics the interaction between DRG neurons and dorsal spinal cord neurons *in vivo*. This protocol was derived from primarily a series of published protocols (3-6) along with some method sections from several papers (7-11).

2. Materials

2.1. Cell culture supplies

1. Calcium Magnesium-Free phosphate buffer (Sigma-Aldrich, St. Louis, MO)
2. Trypsin (Thermo Fisher Scientific, Waltham, MA)
3. DMEM (Thermo Fisher Scientific, Waltham, MA)

4. FBS (Thermo Fisher Scientific, Waltham, MA)

5. Horse serum (Thermo Fisher Scientific, Waltham, MA)

6. Pen/Strep (Thermo Fisher Scientific, Waltham, MA)

7. Trypan blue (Thermo Fisher Scientific, Waltham, MA)

8. Collagenase (Sigma-Aldrich, St. Louis, MO)

9. Poly-D-lysine (Sigma-Aldrich, St. Louis, MO)

10. Natural Mouse Laminin (Thermo Fisher Scientific, Waltham, MA)

11. NB/B27 media (Thermo Fisher Scientific, Waltham, MA)

12. Uridine (Sigma-Aldrich, St. Louis, MO)

13. 5-fluorodeoxyuridine (Sigma-Aldrich, St. Louis, MO)

14. Neurobasal media (Thermo Fisher Scientific, Waltham, MA)

15. Methylcellulose (Sigma-Aldrich, St. Louis, MO)

16. Glass coverslips (Thermo Fisher Scientific, Waltham, MA)

17. Cell culture dish (Corning Life Sciences, Tewksbury, MA)

18. Campenot chambers (Tyler Research Corporation, Edmonton, AB, Canada)

19. Epoxy (Home depot)

20. Small insect pins (Carolina Biological Supply Company, Burlington, NC)

21. Silicone grease (Home depot)

22. 5mL syringe (Thermo Fisher Scientific, Waltham, MA)

23. Glass Pasteur pipette (Thermo Fisher Scientific, Waltham, MA)

24. Filter (40 uM, Thermo Fisher Scientific, Waltham, MA)

25. Blunted 28 or 30 gauge needle (Thermo Fisher Scientific, Waltham, MA. See Note *1*)

26. Hemocytometer (Thermo Fisher Scientific, Waltham, MA)

2.2. Immunofluorescence staining, imaging acquisition and analysis

1. Methanol (Sigma-Aldrich, St. Louis, MO)

2. Dako antibody diluent (now Agilent Technologies, Inc., Santa Clara, CA)

3. Primary antibodies (various vendors based on experimental needs)

4. Alexafluor secondary antibodies (Thermo Fisher Scientific, Waltham, MA)

5. Glass slides (Thermo Fisher Scientific, Waltham, MA)

6. Vectashield DAPI hard mount media (Vector Laboratories, Inc., Burlingame, CA)

7. Confocal microscope (Zeiss International, Oberkochen, Germany)

8. Imaging analyzing software (see Note *13*)

3. Method

3.1. Spinal cord neuron isolation

1. Spinal cord neurons are harvested from E14-E19 mouse embryos by making a lengthwise incision along the ventral side of the spinal vertebrae and collecting the spinal cord in CMF (Calcium Magnesium-Free phosphate buffer) on ice.
2. Dissociate the spinal cord neurons in 5mL 0.25% trypsin for 10 minutes at 37 °C.
3. Inactivate trypsin by adding 10mL DMEM + 10% FBS.
4. Titurate the digested spinal cord to separate spinal cord neurons using two flamed glass Pasteur pipettes with gradually constricted opening tips.
5. Spin down the cell suspension at low speed (1000 RPM) for 5 minutes.
6. Remove supernatant and re-suspend the pellet in fresh DMEM (5mL) + 10%FBS.
7. Pass the re-suspended cells through a 40 μM filter to remove cell clumps.
8. Centrifuge the cell suspension at low speed and re-suspend the pellet in 1mL fresh DMEM + 10%FBS + 10% Horse serum + 1% pen/strep.
9. Count cells using a hemocytometer and Trypan blue at 1:10 dilution. A successful isolation should yield a greater than 10:1 live/dead cell ratio.

3.2. DRG neuron isolation

1. Euthanize mice following IACUC guidelines. DRG neurons should be promptly isolated from freshly euthanized mice to insure high viability and healthy cells.

2. Remove skin along the back of the animal.

3. Make a lateral cut below the shoulder and above the hip and dissect out the vertebral column. This should roughly include segments T7/8 through L4/5.

4. Adult DRG neurons are harvested by first removing the spinal cord via hydraulic extrusion (see Note *1*), which makes isolating the DRGs easier. Spinal cord can also be removed by dissection after laminectomy if the researcher is careful enough not to pull the attached DRGs while removing the spinal cord.

5. A laminectomy is performed to remove the dorsal half of the vertebrae exposing the spinal cavity.

6. Isolate the DRGs from the segments of interest and store in CMF on ice. We routinely recover DRGs from T9 through L5 for our experiments.

7. Dissociate the DRGs in 1.25 mg/mL collagenase for 10 – 15 minutes at 37 °C.

8. Inactivate the collagenase digestion by adding equal or greater volume of DMEM + 10%FBS.

9. Triturate the DRGs using two fire-flamed glass Pasteur pipettes with gradually reduced tip opening.

10. Spin down the cells at low speed centrifugation.

11. Re-suspend the pellet in 1mL DMEM + 10%FBS.

12. Pass the cell suspension through a 40 μM filter.

13. Count the cells using Trypan blue at 1:4 dilution.

14. Cell viability should be above 90%. The 40 μM filter should be able to remove some but not all cellular debris (See Note *2*).

15. Cells are plated onto poly-D-lysine and laminin coated glass coverslips and grown in NB/B27 media and 100 nM uridine/20 nM 5-fluorodeoxyuridine (U/FrdU).

3.3. Co-culture in regular culture dishes (see Note *3*)

In SC/DRG co-cultures, SC neurons are first allowed to grow for 3 days for maturity and neurite sprouting. In our experience, embryonic SC neurons take a few days after plating to sprout neurites while adult DRG neurons are able to sprout neurites overnight. Therefore, DRG

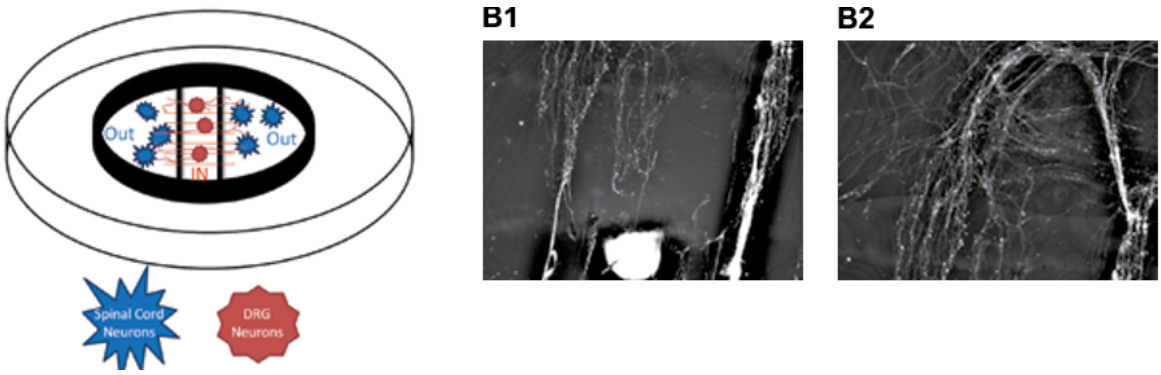
neurons are added to the SC neuron cultures at the time of SC culture media change at Day 3. From Day 3 onwards, neurons are grown in the presence of Uridine/FrdU mitotic inhibitor.

1. Plate the cells onto a 0.1 mg/mL poly-D-lysine and 0.04 mg/mL laminin coated glass coverslips (see Note *4*).
2. SC neurons are grown in DMEM supplemented with 10% horse serum and 10% fetal bovine serum and 1% pen/strep.
3. After 24 hours, the media are replaced with Neurobasal media supplemented with B27 supplement (NB/B27) (see Note *5*). After 3 days, U/FrdU is added to inhibit non-neuronal cell proliferation (see Note *6*).
4. Cultures are usually ready to use by Day 10-14 when neuronal morphology emerges and significant neurite outgrowth is observed

3.4. Co-culture using Campenot Chamber Cultures

Campenot chambers (Tyler Research, Edmonton) are set up as described by PazyraMurphy & Segal (12). The main benefit of such a setup is to isolate the two different neuronal populations (since there is no media exchange between the inner and outer chambers), so one can selectively treat or manipulate each population of cells for desire studies (see Note *7*).

1. 35 mm cell culture dish lids are coated with 1 mg/mL collagen (see Note *8*). Grooves are scored on the culture dish lid using an insect pin rake at approximately 200 µm intervals (see Note *9*). The grooves provide a physical barrier preventing axons from looping and growing back towards the center chamber and help guide the axons to the outer chambers.



Insert **Figure 1** here

2. Wet grooves with a drop of NB/B27 + 0.6 mg/mL methylcellulose with a sterile pipette.

3. Coat Campenot chamber bottoms with autoclaved silicone grease dispensed from a sterile 5mL syringe and mount onto the scored culture dish lid as shown in Figure 1A, making sure a proper seal is achieved.

4. Add culture media into the middle chamber leaving the outside chambers empty and store the Campenot chamber overnight in an incubator to check if the silicone grease creates a leak-proof seal (see Note *10*).

5. Plate DRG neurons into the middle (rectangular) chamber and fill the outer chamber with culture media. Culture the DRG neurons in NB/B27 media with U/FrdU as discussed in 3.2.15 for around 7 days (see Note *11*).

6. Once DRG axons have started sprouting into the outer chambers (Figure 1 B1, B2), SC neurons are plated into the outer chambers. Chambers are ready to use when sufficient number of DRG axons fully cross the divider between chambers to reach the SC neurons in the outer chambers.

3.5. Immunofluorescence staining

Cells can be stained or imaged directly from the culture plate. For certain microscopes with a short focal length (for certain magnifications) that could not take focused cell images through the bottom of the culture plate, we employed workarounds such as culturing cells on a glass coverslip or looking for a thinner bottom plate such as using a culture dish lid.

For regular co-cultures:

1. Cultured neurons are fixed in -20 °C methanol for 15 minutes (see Note *12*).
2. Samples are then incubated for 24 hours with primary antibodies diluted in Dako antibody diluent (Dako). The antibodies that we used include: chicken Microtubule Associated Protein 2 (MAP2) (Abcam), mouse PSD95 (Pierce), guinea pig VGlut2 (Synaptic Systems) (see Note *13*).
3. Samples are then incubated for overnight in corresponding Alexafluor (Thermo Fisher) 488, 594 and 647 secondary antibodies that are species specific to the primary antibodies.
4. Mount samples onto glass slides using Vectashield DAPI hard mount media (Vector Labs). Images are taken from the selected areas using a 63X objective on a confocal microscope (we used the Zeiss LSM700) (See Note *14*) and analyzed using Volocity 6.0 (Perkin Elmer) (see Note *15*).

For Campenot chamber co-cultures:

1. Remove the Campenot chamber carefully.
2. If possible, wash and carefully remove as much silicon grease as possible.
3. Fix the co-culture cells with methanol as discussed above.
4. Perform immunohistostaining and image acquisition following steps 3 and 4 for regular co-cultures.
5.

4. Notes

1. Insert the blunted-end tip of a 28 or 30 gauge needle attached to a 10mL syringe filled with ice cold PBS into the lambar end opening of the vertebral column.

The diameter of the opening might vary depending on the size of the animal. Therefore, trying higher and lower gauge size tips may be necessary to find a tip size that fits snuggly into the opening. We found that using pliers to slightly depress the tip to achieve a more oval shape may help the fitting. These tips can be cleaned and reused.

2. Some cell debris remaining after filtration might include myelinated axon bits, which can be washed off in 12-24 hours upon changing medium once cells have attached.

3. Note that this is a multi-day process. Embryonic spinal cord neurons take longer to establish and produce neurite outgrowth. They also need a different media formulation for the first 24 hours to get established. In our experience and referencing protocols published by other labs, we've found that SC neurons need to be cultured in DMEM + 10% FBS + 10% horse serum for 24 hours before changing to NB/B27 media.

4. The glass coverslips are used to mount the adhered cells to a glass slide for imaging. Alternatively, you can use a glass bottom culture dish.

5. In studying synaptic connectivity, it is important to keep neurons healthy and ensure that they sprout enough neurites to find each other as well as maintain synaptic connectivity. This is especially critical in low-density cultures, including DRG/SC neuron, and Campenot chamber co-cultures since more consistent neuronal health could improve culture consistency, thus improve the assay window and minimize date variation among different sets of cultures. Therefore, it is worthy in initial experiments to compare (e.g. B27 vs B27Plus, Thermo Fisher

Scientific) and select reagents that can better increase culture longevity, improve neurite outgrowth and enhance synaptic connectivity.

6. U/FrdU is required to control the proliferation of non-neuronal cells in both DRG and SC cultures. Uncontrolled proliferation of non-neuronal cells could overwhelm the culture and reduce neuronal survival rates. Spinal cord neurons are very sensitive to mitotic inhibitors for the first few days in culture. On the other hand, DRG neurons can be cultured directly in NB/B27 + mitotic inhibitors U/FrdU and will adhere and produce neurite outgrowth overnight. Thus, in our hands, allowing spinal cord neurons to establish themselves 3 days prior to addition of DRG neurons provides the best compromise.

Getting too little or too much mitotic inhibitor could result in glial cell overgrowth or poor neuronal health and excessive cell death. Newly improved reagents, such as CultureOne (Thermo Fisher Scientific), that can help to suppress glial cell proliferation without affecting neuronal health or accelerating cell death could greatly improve the image analysis and data variation downstream.

7. We also tried the Axis Axon Isolation Device (Millipore-Sigma) but could not get consistent axonal outgrowth across the microgrooves. However, we do believe that it could also be a viable alternative and is worth testing.

8. The cell culture dish bottom is too thick to get focused cell images with our confocal microscope (Zeiss LSM700). We found that the lid is thin enough for downstream imaging applications.

9. To make the rake, use epoxy to glue small insect pins side by side onto a metal stirring spatula to create a handle for the comb tip. Significant force is needed to score the dish so one needs to make sure that the pins are glued firmly together. The grooves are scored directly onto the culture dish lid, thus using a glass culture dish is impractical for this application.

10. In practice, the methylcellulose/silicone layer creates a seal that allows axons to grow through the medium while minimizing media diffusion from the middle chamber to outer chambers. A proper seal is critical in making sure that the middle chamber is fluidically sealed from the outer chambers. Improper seal will result not only in medium diffusion across chambers but also allow SC neurons to flow into the center chamber. If the gaps are big enough, the whole Campenot chamber can become detached from the dish.

11. After around 7 days in culture, one should observe DRG axons crossing or about to cross from the middle chamber to the outer chambers.

12. The fixation protocol is up to preference. We prefer methanol fixation to paraformaldehyde fixatives.

13. We used DRGs from transgenic mice with a pan DRG-RFP reporter to identify DRG neurons in co-cultures. Since DRG neurons are pseudo-unipolar neurons without dendrites, immunoreactivity to dendrite-specific antibody MAP2 could then be used to identify SC neurons. Alternatively, neuronal subtype specific markers can also be used.

14. High Content Analysis Instrument: For our synaptogenesis studies, a critical component is acquiring images of the neurons, their neuritis and associated synapses. Manually finding and taking enough images using a confocal microscope and the subsequent data analysis have proved to be a labor-intensive affair. We would highly recommend using an alternative high content analysis Instrument. Automating data acquisition and analysis takes away user biases and allows the generation of much larger data sets in a timely manner.

15. In addition to Volocity, which is an expensive software that requires a significant learning curve, other software, some of them such as ImageJ (NIH) are free, can also be used.

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Figure legend

Figure 1. Campenot chamber setup (modified from Fig. 3A of Yu et al., 2018 (2) with permission) and DRG neuron axonal outgrowth. A. Diagram of a Campenot Chamber setup. B1. DRG neuron axons, but not soma, crossing the inner chamber wall. B2. DRG neuron axons crossing into the outer chambers.