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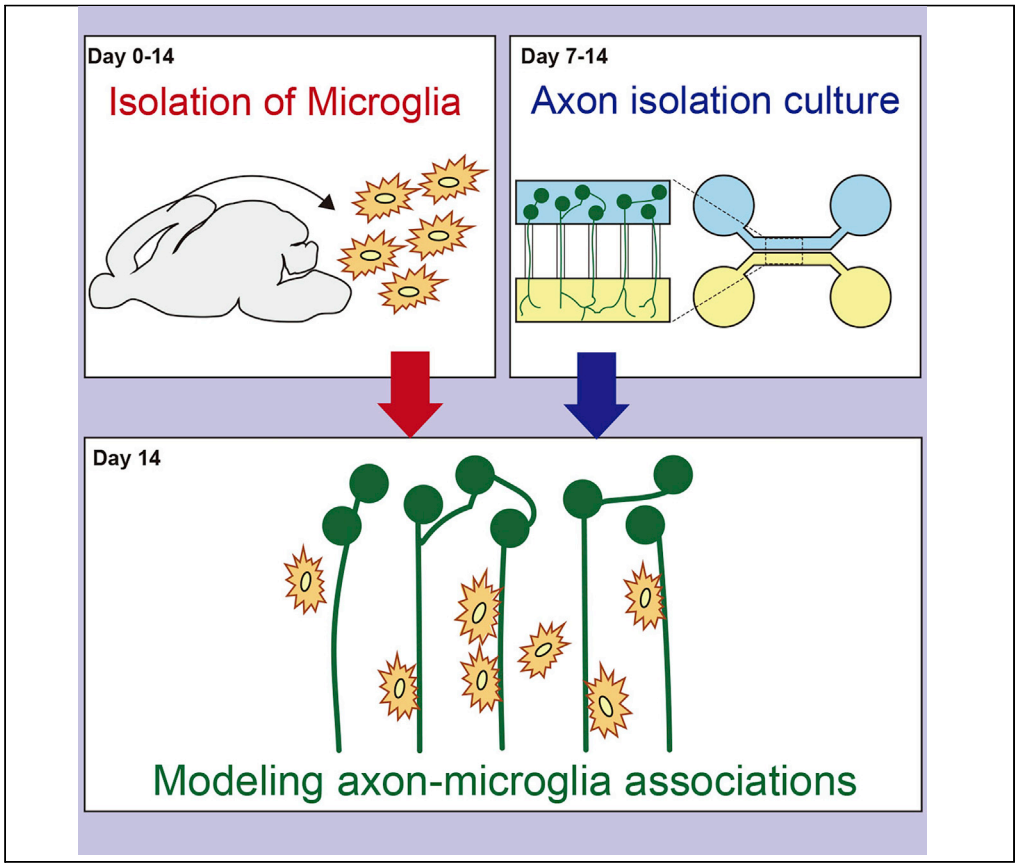
Protocol for Co-culture of Microglia with Axons



Microglia, the resident immune cells of the central nervous system, accumulate along axons and support neuronal survival during the early postnatal period. Although methods have been developed to isolate microglia or neurons, the procedures for co-culture of these cells can inadvertently affect the interactions between them. Here, we describe a protocol to investigate the accumulation of microglia toward neuronal axons using axon isolation culture devices. This approach is useful for modeling neuron-glia associations.

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HIGHLIGHTS

A protocol for accumulation of microglia to axons

Use of axon culture devices to isolate axons from the cell body

Can be adopted to model neuron-glia associations

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Protocol for Co-culture of Microglia with Axons

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SUMMARY

Microglia, the resident immune cells of the central nervous system, accumulate along axons and support neuronal survival during the early postnatal period. Although methods have been developed to isolate microglia or neurons, the procedures for co-culture of these cells can inadvertently affect the interactions between them. Here, we describe a protocol to investigate the accumulation of microglia toward neuronal axons using axon isolation culture devices. This approach is useful for modeling neuron-glia associations.

For complete details on the use and execution of this protocol, please refer to Ueno et al. (2013) and Fujita et al. (2020).

BEFORE YOU BEGIN

Preparation of DNase Stock Solutions

Timing: Can be made prior to the day of cell culture

1. Calculate required volumes and prepare stock DNase solution. Refer to the buffer recipe in the Materials and Equipment section.

Preparation of Medium

2. Culture medium was prepared as described by the manufacturer's protocol. For microglia culture, DMEM is supplemented with 10% of FBS and Penicillin-Streptomycin (1:100 dilution). For neuron culture, Neurobasal Medium is modified with B27 supplements (1: 50 dilution) and 0.5 mM L-glutamine.

Preparation of Axon Culture Device

Timing: 5 h to 1 day before the culture of cortical neurons

3. Please refer to the manufacturer's protocol for full details on device preparation; Standard Neuron Device, Xona Microfluidics cat# SND450
4. 35 mm dishes are coated with 0.1 mg/ mL poly-L-Lysine/PBS at room temperature (20°C–25°C, RT) for 2 h to overnight(12–24 h).
5. Wash with PBS thrice for 5 min each at RT.

6. Coat dishes with laminin (1:100 diluted with PBS) at RT for 2–3 h.
7. Remove laminin solution by aspiration.
8. Place the sterile axon culture device on the dish.
9. Add 200 μL of neuron culture media to well A (Figures 2 and 3) and allow the media to flow through into well B.
10. Add 200 μL of media to well B, and incubate the device for ~15 min at RT.

⚠ CRITICAL: Using a microscope, verify that the media has indeed passed through the microgrooves (Figure 3). See Troubleshooting 1.

11. Add 100 μL of media to well C, and allow the media to flow through into well D.
12. Keep the device at 37°C in a 5% CO_2 incubator until it is time to prepare the neurons.

Preparation of Blocking Solutions

Timing: Can be made prior to the immunostaining step

13. Calculate required volumes and prepare blocking solution. Refer to the buffer recipe in the Materials and Equipment section.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-Iba1 (1:1,000)	Abcam	Cat# ab5076; RRID: AB_2224402
anti-CD11b (1:1,000)	BD Biosciences	550282; RRID:AB_393577
anti-tau (1:1,000)	Millipore	Cat# MABN471; RRID: AB_2736930
Donkey anti-Rabbit IgG, Alexa Fluor 568	Thermo Fisher SCIENTIFIC	Cat# A10042 RRID: AB_2534017
Donkey anti-Goat IgG, Alexa Fluor 488	Thermo Fisher SCIENTIFIC	Cat# A11055 RRID:AB_2534102
Chemicals, Peptides, and Recombinant Proteins		
Trypsin	Thermo Fisher SCIENTIFIC	Cat# 15090046
DNase I	Sigma	Cat# D5025
HEPES	DOJINDO	Cat# 342-01375
NaCl	Wako	Cat# 191-01665
$CaCl_2$	Wako	Cat# 038-24985
Glycerol	Wako	Cat# 072-04945
PBS	Santa Cruz biotechnology	Cat# sc-24947
poly-L-Lysine	Sigma-Aldrich	Cat# P2636
Laminin	Thermo Fisher SCIENTIFIC	Cat# 23017015
DMEM	Gibco	Cat# 12800-058
FBS	Gibco	Cat# 10437
Penicillin-Streptomycin	Gibco	Cat# 15140122
Neurobasal Medium	Gibco	Cat# 21103049
B-27 Supplement	Gibco	Cat#17504044

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
L-Glutamine	Gibco	Cat# 25030081
Fluorescence Mounting medium	Dako	Cat# S3023
BSA	Merck	Cat# A7906
Triton X-100	Nakarai Tesque Inc.	Cat# 12969-25
butorphanol (Vetorphale®)	Meiji Seika	N/A
midazolam, (Dormicum®)	Maruishi Pharma Inc.	N/A
medetomidine (Domitor®, 0.03 m)	Zenoaq Nippon Zenyaku Kogyo	N/A
Experimental Models: Organisms/Strains		
Mouse: C57BL/6	Japan SLC, Inc.	N/A
Other		
Surgical Blade	FEATHER	No.11
AXIS™ Axon Isolation Device	Merck	Cat# AX45005
Standard Neuron Device	Xona Microfluidics	Cat# SND450
35 mm dish	greiner	Cat# 627160
Falcon Cell Strainers (70 μm)	CORNING	Cat# 352350
50 mL tube	Greiner Bio-One International	Cat# 227261

MATERIALS AND EQUIPMENT

DNase Stock Solution

Reagent	Final Concentration	Amount
DNase I	50 mg/mL	250 mg
HEPES (1 M, pH 7.4)	20 mM	200 μL
NaCl (5 M)	50 mM	100 μL
$CaCl_2$ (0.1 M)	0.1 mM	10 μL
Glycerol	50%	5 mL
ddH_2O	n/a	~4.69 mL
Total	**n/a**	**10 mL**

Stock solution can be stored for one month in −20°C freezer. This solution should be aliquoted for single use to avoid unnecessary freeze-thaw cycles.

Alternatives: DNase I, grade II, from bovine pancreas (cat #10104159001; Merck) can be also used for this protocol.

Blocking Solution

Reagent	Final Concentration	Amount
BSA	5%	2.5 g
Triton X-100	0.1%	50 μL
PBS	n/a	~50 mL
Total	**n/a**	**50 mL**

STEP-BY-STEP METHOD DETAILS

Culture of Microglia

Timing: for steps 1–14, 60–90 min; for steps 15–17, 14 days

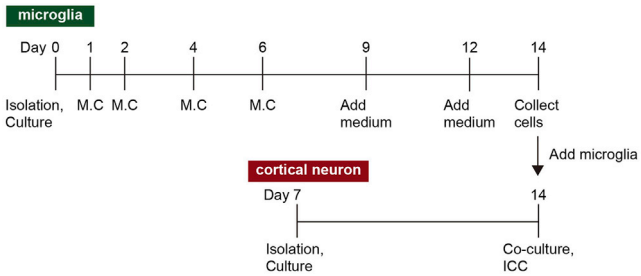

Figure 1. Workflow of Co-culture with Microglia and Axons

This protocol can be adapted for use in culturing microglia (Figure 1). Time course for the collection of microglia depends on the number of cells and the number of experimental groups. Scarify mice after intraperitoneal administration (10 mL / kg body weight) of anesthesia with a mixture of butorphanol (Vetorphale®, 0.5 mg/mL), midazolam, (Dormicum®, 0.4 mg/mL), and medetomidine (Domitor®, 0.03 mg/mL) dissolved in sterilized water. Chop the brain region of interest immediately following euthanasia. The brain and/or chunks should be kept on ice throughout the process. Older pups (~postnatal day 7), as well as newborn pups, could be used to prepare microglia. However, newborn pups (~postnatal day 3) could provide a better yield in the number of microglia (Bordt et al., 2020; Lian et al., 2016). Here, we use postnatal day 3 pups.

1. Aliquot 9 mL of PBS into 50 mL tubes and cool on ice. Keep all dissecting tools cool on ice.
2. Dissect the brain region of interest and chop up with cold fine scissors or blades until no tissue lumps remain. Collect into the prepared 9 mL of ice cold PBS. (Schildge et al., 2013).

   Note: If you are dissecting more than five brains use separate tubes

3. Add 1 mL of 2.5% trypsin/PBS (final concentration 0.25%) and 50 μL of 50 mg/mL DNase into each tube.
4. Incubate for 15 min in a water bath at 37°C.

   ⚠ CRITICAL: Agitate tubes every 5 min to mix tissue.

5. Add 10 mL of DMEM/10% FBS solution into each tube (equal volume of enzyme mix).
6. Mix by pipette (approximately 20 times) using a 10 mL disposable pipette.

   ⚠ CRITICAL: Proper pipetting is important for cell viability. To avoid air bubbles, pipet up and down while leaving 0.5–1 mL of cell suspension in the disposable pipette each time. See Troubleshooting 2.

7. Centrifuge at 180 × *g* (RCF) for 5 min at 4°C with max acceleration and brake.
8. Discard the supernatant. Add 10 mL of DMEM /10% FBS solution and resuspend cell suspension by gently pipetting.
9. Place a 70 μm filter mesh on top of a new 50 mL tube and filter samples by gravity flow.
10. Centrifuge the flow-through at 180 × *g* for 5 min at 4°C with max acceleration and brake.
11. If the supernatant is cloudy, repeat resuspension and centrifugation (Salazar et al., 2017) .
12. Discard the supernatant. Add 10 mL of DMEM/10% FBS solution and resuspend cell suspension by gently pipetting.
13. Count the number of cells and seed them at 5 × 10^5 cells / mL placing up to 4 mL in a 6-cm dish (2 × 10^6 cells per plate).
14. Incubate the cells in 5% CO_2 at 37°C

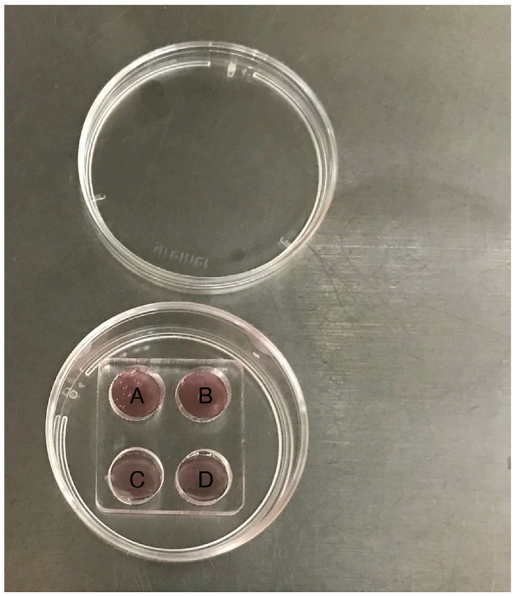

Figure 2. General Setup of the Axon Isolation Culture Device

15. Replace the medium on days 1, 2, 4, and 6 with 4 mL of fresh DMEM/10% FBS solution to remove debris and floating cells.
16. On days 9 and 12, add 0.5–1 mL of fresh DMEM/10% FBS solution.

For issues with microglial viability, please see Troubleshooting 3.

17. On day 14, collect microglia from supernatant and co-culture with the axons. Proceed to step 34 for detailed steps of microglia collection.

Axon Isolation Culture

Timing for steps 18–33: 60–70 min

Using axon isolation culture devices, axons were separately cultured from the cell bodies of the cortical neurons. Here, we used embryonic mice (E17) to prepare cortical neurons. Neonatal mice (~P3) can be used for the culture of cortical neurons. However, older mice tend to provide lower number of cells.

18. Prepare the axon culture device (AXIS™ Axon Isolation Device, Merck; Neuron Device, Xona Microfluidics) following manufacturer's protocol. Please refer to "Preparation of axon culture device" section for additional details (Figures 2 and 3).

⚠ CRITICAL: Do NOT use expired devices.

19. Add 9 mL of PBS into 50 mL tubes and cool on ice. Keep all dissecting tools cool on ice.
20. Dissect the cortex and chop up with cold fine scissor or blades until no tissue lumps remain. Collect into the prepared 9 mL of ice cold PBS.
21. Add 1 mL of 2.5% trypsin (final concentration 0.25%) and 100 µL of 50 mg/mL DNase into each tube .
22. Incubate in a water bath at 37°C for 15 min.

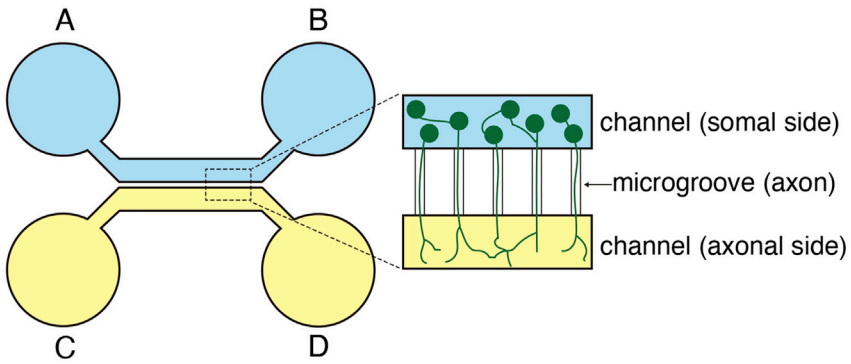

Figure 3. Schematic Images of Axon Isolation Culture

Neurites grow from the somal side (pale blue) toward the axonal side (yellow) and go through the microgroove.

⚠ CRITICAL: Agitate tubes every 5 min to mix tissue.

23. Add 10 mL of DMEM/10% FBS solution into each tube (equal volume of enzyme mix).
24. Centrifuge at 180 × *g* for 4 min at 4°C with max acceleration and brake.
25. Discard the supernatant, then add 10 mL of DMEM/10% FBS solution.
26. Mix by pipette (approximately 10 times) using a 10 mL disposable pipette tip.

⚠ CRITICAL: Proper pipetting is important for cell viability. To avoid air bubbles, pipet up and down while leaving 0.5–1 mL of cell suspension in the pipette each time.

27. Place a 70 μm filter mesh on top of a fresh 50 mL tube and filter the cell suspension by gravity flow.
28. Centrifuge the flow-through at 180 × *g* for 4 min at 4°C, with max acceleration and brake.
29. If the supernatant is cloudy, repeat resuspension and centrifugation.
30. Discard the supernatant. Add 10 mL of neuron culture medium, and resuspend the cell pellet by gently pipetting.
31. Count the number of the cells, and seed 5 μL of cell suspension (5×10^6 cells per mL) according to the manufacturer's protocol.
32. Incubate the cells in 5% CO_2 at 37°C
33. In approximately one week, neurites grow from the somal side toward the axonal side through the microgrooves (Figure 3).

For issues with neuronal viability, please see Troubleshooting 3.

Co-culture of Microglia with Axons

Timing for steps 34–41: 60–90 min

Microglia are collected from the supernatant and added to the cultured axons.

34. On day 14 of microglia culture, collect microglia from supernatant and co-culture with the axons. To collect microglia, collect all the conditioned culture media from microglia culture dishes into fresh 50 mL tubes.
35. Add 0.5 mL of fresh microglia culture media to the culture dishes.
36. Tap the dishes and gently apply the 0.5 mL media to wash the cell layer and collect any remaining microglia. Collect the supernatant including floating cells, and add to the same 50 mL tubes containing media collected at step 34.

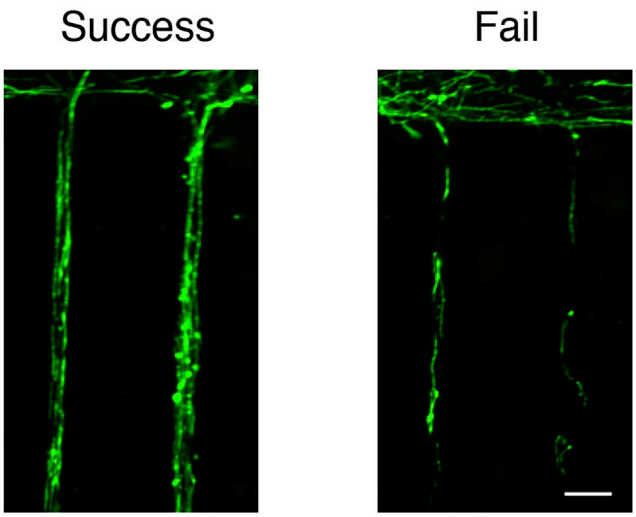

Figure 4. Depiction of a Successful or Failed Axon Isolation Culture

GFP-expressing axons after device removal. When the axons are properly cultured along the microgroove, the line of axons are easily detected (left). In contrast, when the axon culture or device removal fail, the lines of axons are broken (right). Scale bar, 20 μm.

37. Centrifuge at 300 × *g* for 5 min at 4°C with max acceleration and brake.
38. The remaining cells in the pellet are microglia. Discard the supernatant and resuspend the cells in 1–5 mL of neuron culture medium. Reconstituting at a high density of cells could be better since it allows you to dilute cells at an identical density after the cell counts.
39. Carefully remove the silicon-based axon culture device from the dish (Methods Video S1).

⚠ CRITICAL: Using an inverted microscope (CKX41, Olympus, or other), verify that axons kept along the microgroove line (Figure 4).

40. Seed microglia into an axon isolated culture dish at 1–2 × 10^4 cells /cm^2.
41. Incubate the cells in 5% CO_2 at 37°C for 6 h.

Immunostaining

Timing for steps 42–52: 1.5–2 days

Visualize microglia and axons by immunofluorescent staining.

42. Remove the culture medium with a pipette.

Note: Do NOT use aspirator to remove the medium.

43. Fix the cells with 4% Paraformaldehyde/PBS at room temperature (20°C–25°C, RT) for 30 min, without shaking.
44. Wash with PBS three times, each time for 5 min at RT, while gently shaking.
45. Blocking: Remove PBS and add Blocking solution containing 0.1% Triton X-100, 5% BSA in PBS.
46. Incubate at RT for 1 h while gently shaking.
47. Remove the blocking solution and add primary antibodies such as anti-CD11b (alternatively, anti-Iba1) and anti-tau, diluted in blocking solution (1:1,000).
48. Incubate at 4°C, overnight (12–24 h) while gently shaking.

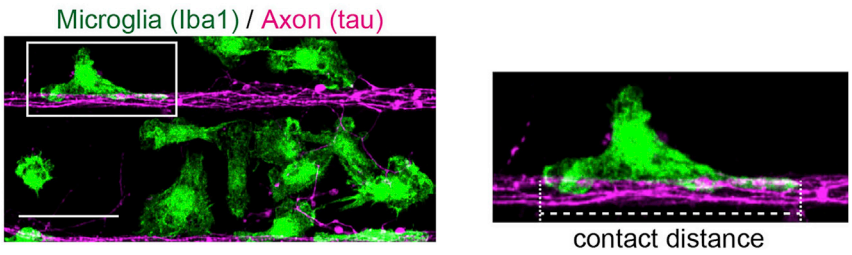

Figure 5. Definition of Contact Distance

Microglia were stained with anti-Iba 1 antibody (green) and axons were stained with anti-tau antibody (magenta). The "contact" distance is measured by the distance of the microglial signal (green) covering the axons (magenta). Scale bar, 20 μm.

49. Wash with PBS three times, each time for 5 min at RT, while gently shaking.
50. Remove the PBS and add secondary antibodies diluted in 5% BSA in PBS (1:1,000).
51. Wash with PBS three times, each time for 5 min at RT, while gently shaking.
52. Mount with mounting medium and coverslip with appropriately sized glass covers.

For issues with immunofluorescent staining, please see Troubleshooting 4.

EXPECTED OUTCOMES

By following the above protocol, we have obtained microglial cell counts of 1–5 × 10^4 cells using a P3 mouse brain. We also obtained 2.5–5 × 10^6 cortical neurons using E17 mouse cortex.

The contact distance between microglia and axons was measured using ImageJ software (National Institutes of Health) and normalized to the total number of microglia. "Contact" in this context refers to microglial (green) coverage on axons (magenta), as demonstrated in Figure 5. The expected average distance between the points of contact is 29.9 ± 3.19 μm.

The number of CD11b- or Iba1-positive microglia attached to the axon was counted and normalized to the total number of Iba1-positive microglia.

This protocol could also be helpful to assess the contact points between neurons and other type of glia such as astrocytes and oligodendrocytes.

LIMITATIONS

Purity of Microglia

Collecting microglia from conditioned culture media has been widely used for the culture of microglia (Lian et al., 2016; Tanaka et al., 2009). However, the purity of microglia collected by this method mostly depends on the tapping and pipetting step (step 36). Generally, strong tapping and pipetting increase the yield of microglia, but reduce the purity. The typical purity of microglia, using our methods, is approximately 84.2% ± 5.4% of CD11b-positive cells (confirmed by immunocytochemistry).

The available options to isolate microglia use cell-type specific markers such as CD11b and Cx3cr1 (Bohlen et al., 2019; Bordt et al., 2020). We have confirmed that the above method could be used in microglia isolated from the cortices of Cx3cr1 +/GFP mice (Jung et al., 2000) by using cell sorting devices (BD FACSAria III, BD Biosciences) as well as microglia from wild-type mice. In our protocol, GFP-positive cells were isolated using florescence-activated cell sorting (FACS) after cell resuspension (step 12).

The Age of Mice

The number of both microglia and neurons yielded has been shown to decrease with age (Lian et al., 2016). Therefore, applying this protocol for use in culturing adult cells, could be difficult.

Time Course of Accumulation of Microglia

The axon culture isolation device is useful to assess neurite outgrowth (Park et al., 2006; Taylor et al., 2005). Here, we use this system to assess the contact between axons and microglia. The method described herein can be available to assess the early time course of microglial accumulation at the axons. However, long-term culture promotes axonal branching, making it difficult to assess the contact points between the microglia and axons, thus would reduce the accuracy of the quantification of cell proximity. The development of novel devices, for instance a device with wide distances between axons, might be more helpful to assess the neuron-microglia association in other events requiring long-term culture. Alternatively, a three-compartment system (e.g., NEUROFLUIDICS, Cat #NB6L3XSBS, NETRI) would also be useful; however, it would have its own complications.

Alteration of Microglia Morphology under In Vitro Culture Condition

Cultured microglia show different morphology compared with microglia in vivo, though it is not clear whether the morphological feature can be linked to an "activated" state in vitro. In this study, we co-culture microglia with neurons and they exist close to the axons. Therefore, the close proximity to axons might affect their morphology.

TROUBLESHOOTING

Problem 1

Flow-through of the media to the opposite side and through all the microgrooves is incomplete (refer to step 10 in "Before You Begin").

Potential Solution

Incubate the device for an additional 15 min.

Pipette up and down alternating between well A and B several times until the media flows through the microgroove.

Additional incubation (~24 h) might be required.

Problem 2

Large cellular aggregates remain after pipetting (refer to step 6 in Culture of Microglia). This might occur after severe cell damage when DNA causes cells and other debris to aggregate into large clumps. Images of non-dissociated aggregates are available in an established protocol (Salazar et al., 2017).

Potential Solution

Time spent on tissue dissection should be shortened. Ideally a maximum of 12 pups are suggested for efficient dissection.

Make fresh or increase DNase concentration/quantity.

Extend enzyme reaction time. We have not detected any obvious effect on the results by adjusting the enzyme incubation time to 30 min. However, excess incubation could also cause damages to the cells and over incubation should be avoided.

Gently pipet up and down to avoid excess cellular damage, however it is necessary to fracture the minced tissue into a cellular suspension with sufficient pipetting speed. Medium gets cloudy and tissue clumps disappeared when the cells were suspended correctly.

Problem 3

Low survival rate (refer to step 16 in Culture of Microglia and step 33 in Axon Isolation Culture)

Potential Solution

Gentler pipetting (slower, less frequent, less agitation, using a large orifice pipette tip, etc.).
Shorten the time to prepare cortical neurons.
Keep tissues on ice until they are incubated with enzyme.
Use milder enzyme (e.g., papain) for the cell preparation.
Use younger age mice.
Use a reduced concentration of antibiotics.

Problem 4

Non-specific fluorescence signal after immunostaining (refer to steps 43–52 in Immunostaining). If the antibody or the detergent is too concentrated, non-specific binding occurs, resulting in a ubiquitous signal.

Potential Solution

Lowering the dilution of the primary (~1:5,000) or secondary (~1:2,000) antibodies, would be helpful.
Use appropriate species serum for the secondary antibody in the blocking solution.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Toshihide Yamashita (yamashita@molneu.med.osaka-u.ac.jp).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100111.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Y.F. and T.Y. conceived and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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