Protocol for Culturing Rat Microglia

Microglia are the resident immune cells of the central nervous system. They are important for maintaining homeostasis, monitoring tissue debris and pathogens, and eliminating or remodeling synapses. These cells are activated by neural injury and disease states, causing them to undergo morphological and proliferative changes, increase their phagocytic activity, and secrete proinflammatory molecules, which propagate neuroinflammation and neurodegenerative diseases. Utilizing primary microglia cell cultures provides a means to investigate the mechanisms by which these cells contribute to neurodegenerative diseases. This protocol provides step-by-step instructions for culturing microglia from isolated cortical tissue from 5–9 postnatal (P1–P2) rat pups. The protocol can be scaled up for more pups if needed.

Please read the protocol in its entirety before starting.

Note: Aseptic techniques should be used in this protocol to ensure there is no bacterial, fungal, or mycoplasma contamination. The initial dissection and collection of brain tissue can be completed outside of a laminar flow cell culture hood. However, preparation of reagents and cell culture plates, and all steps following tissue harvest, should be conducted within a hood. Likewise, all reagents and materials used should be sterile.

Supplies Required

Reagents

- 70% ethanol
- Cultrex® Poly-L-Lysine (R&D Systems, Catalog # 3438-100-01)
- Deionized distilled H_2O, sterile (ddH_2O)
- DNase I (Worthington Biochemical Corp., Catalog # LK003170), or equivalent
- Fetal Bovine Serum (FBS)
- L-glutamine-penicillin-streptomycin solution (100x), or equivalent
- HBSS, with Ca^{2+} and Mg^{2+}, no phenol red (1x, ThermoFisher Scientific, Catalog # 14025092)
- Minimum Essential Medium Eagle (MEM)
- PBS, sterile (1x): 0.137 M NaCl, 0.05 M NaH_2PO_4, pH 7.4
- Trypan blue (0.4%)
- Trypsin solution from porcine pancreas (10x, Sigma-Aldrich, Catalog # T4549), or equivalent

Materials

- 5, 10, 25, and 50 mL pipettes
- 50 mL conical centrifuge tubes
- Cell culture flasks (T175), sterile
- Cell strainers, 40 µm (ThermoFisher Scientific, Catalog # 22-363-547), or equivalent
- Ice
- P1–P2 rat pups
- Petri dishes, 100 × 20 mm
- Pipette tips
- Syringe filters, 0.22 µm (Cole-Parmer, Catalog # UX-81053-14), or equivalent

Equipment

- 37 °C, 5% CO_2 humidified incubator
- 37 °C water bath
- Centrifuge
- Dissection microscope
- Dissection tools
  - Fine forceps, #5, straight
  - Forceps, #7, curved
  - Micro spatula
  - Surgical scissors, small
- Hemocytometer
- Inverted microscope
- Laminar flow cell culture hood
- Orbital shaker, 19 mm orbit diameter
- Pipette controller
- Pipettes

Reagent Preparation

Note: Prepare all solutions in a laminar flow cell culture hood.

Co-culture Media

1. 1x L-glutamine-penicillin-streptomycin solution, 10% FBS in MEM

DNase I Stock Solution

1. 3 mg/mL DNase I in PBS
2. Filter using a 0.22 µm syringe filter to sterilize.

Note: Store at ≤ –20 °C for up to 2 years.

Procedure

Coating and Preparation of Cell Culture Flasks

Note: Preparation of the culture flasks should be done in a laminar flow cell culture hood.

1. Dilute the Cultrex® Poly-L-Lysine solution with PBS to a final concentration of 100 µg/mL.
2. Coat each T175 cell culture flask with 10 mL of the 100 µg/mL poly-L-lysine solution, and rock the flasks side-to-side.

Note: Other cell culture plates and flasks can be used to either scale up or down these cell cultures. See Table 2 (pg. 34) for the volume of 100 µg/mL poly-L-lysine that should be used for the different sized cell culture plates and flasks.

3. Incubate the flasks for at least 20 minutes in a 37 °C, 5% CO_2 humidified incubator.
4. Aspirate the poly-L-lysine solution. Wash the flasks two times with sterile ddH_2O. Aspirate to remove all liquids. Allow flasks to dry.

Note: Flasks can be stored at 2–8 °C for up to 2 months.

Dissection of Rat Cortical Tissue

Note: Soak dissection tools in 70% ethanol for at least 10 minutes to sterilize. Allow excess ethanol to drip off and place on a sterile, disinfected surface.

1. Add PBS to three 100 x 20 mm petri dishes and one 50 mL conical tube. Add 8 mL HBSS to a 50 mL conical tube. Place all on ice.
2. Decapitate P1–P2 rat pups at the head/neck junction using the small surgical scissors.
3. Place the heads in the chilled 50 mL conical tube containing PBS. Keep the tube on ice.
4. Transfer a rat head to a chilled 100 × 20 mm petri dish containing PBS.
5. Stabilize the dissociated head using the #7 curved forceps and #5 fine forceps. Moving caudal to rostral, cut through the skull with the small surgical scissors.

Note: Keep cuts shallow to avoid damaging the brain tissue.

6. Peel back the two halves of the separated skull.
7. Remove the whole brain from the head cavity using the micro spatula and place it in a second chilled 100 x 20 mm petri dish containing PBS. Repeat steps 4–7 for the remaining heads.
8. Using the #5 fine forceps and #7 curved forceps, separate the hemispheres along the median longitudinal fissure. Cut off and discard the brainstem, cerebellum, and olfactory bulbs. Place the remaining forebrains in the third chilled 100 × 20 mm petri dish.
9. Under a dissecting microscope, remove the meninges, midbrain, and hippocampus from each forebrain. Place the remaining cortical tissue in the Dissociation Tube, a chilled 50 mL conical tube containing 8 mL HBSS.

Dissociation and Culture of Rat Cortical Cells

Note: From this point forward, any opening of tubes/flasks that contain any tissue, cells, media, or reagents should be done in a laminar flow cell culture hood.

1. Warm the Co-Culture Media in 37 °C water bath. Warm the DNase I Stock Solution and 10x Trypsin solution to room temperature.
2. Add 200 µL of DNase I Stock Solution and 400 µL of 10x Trypsin to the cortical tissue in the Dissociation Tube. Using a 25 mL pipette, gently pipette the tissue up and down to break it into small pieces (~1 mm^2).

Note: The tissue can be cut into smaller pieces using a razor blade. Care should be taken to not over mince the tissue as this can increase the amount of fine debris.

3. Gently swirl the Dissociation Tube for 5 seconds and then incubate for 15 minutes in a 37 °C water bath.
4. Remove the Dissociation Tube from the water bath and let it sit undisturbed at room temperature for 5 minutes to let the tissue pieces settle.
5. Transfer all but 3 mL of the cell suspension to a new 50 mL conical tube, the Collection Tube. Add 2 mL of FBS and swirl to mix. Let sit at room temperature.

Note: Use plastic or siliconized glass to transfer the cell suspension as microglia can adhere to untreated glass surfaces.

6. Add 9 mL of HBSS, 200 µL of DNase I Stock Solution, and 400 µL of 10x Trypsin to the cortical tissue in the Dissociation Tube. Using a 10 mL pipette, gently pipette the tissue up and down to break it into smaller pieces.
7. Gently swirl the Dissociation Tube for 5 seconds. Incubate the tube in a 37 °C water bath for 15 minutes, agitating the contents of the tube by hand every 5 minutes.
8. Remove the Dissociation Tube from the water bath and let it sit undisturbed at room temperature for 5 minutes to let the tissue pieces settle.
9. Transfer the cell suspension to the Collection Tube, pooling it with the cell suspension and FBS solution from step 4. Save the cortical tissue.

Note: If a debris pellet has formed in the Collection Tube, remove it before pooling the cell suspension solutions.

10. Add 10 mL of PBS to the cortical tissue pieces in the Dissociation Tube. Swirl to mix and then let it sit undisturbed at room temperature for 5 minutes.
11. Transfer the PBS to the Collection Tube. Centrifuge the Collection Tube at 300 × g for 5 minutes at room temperature. Carefully discard the supernatant.

Note: Take care when decanting the supernatant as the cell pellet is not very firm at this stage.

12. Wash the cell pellet in the Collection Tube with 25 mL of PBS. Centrifuge at 300 × g for 5 minutes at room temperature. Decant the solution.
13. Resuspend the cells in 5 mL of warmed Co-Culture Media. Prepare two serial 1:5 dilutions of the cell suspension in 0.4% Trypan blue. Count the live cells.
14. Plate ~ 1.2 million cells in prepared T175 culture flasks containing 45 mL of warmed Co-Culture Media. Incubate in a 37° C, 5% CO_2 humidified incubator. This is Day 0 in the culture schedule (see table).

Culture Schedule

Note: Ideally, this cell culture protocol will result in astrocytes reaching confluency on days 9–10, allowing the microglia to be harvested 1–2 days later. However, the progression of the culture can vary based on a number of factors including debris level (more debris can cause the astrocytes to grow more slowly), speed of dissection, and serum variability. Thus, cultures should be monitored so the microglia can be harvested shortly after the astrocytes become confluent. Neuronal development is abundant about 3–4 days after astrocytes reach confluency, so, if microglia are harvested too late, neurons will shake off with the microglia. If the astrocytes are reaching confluency too soon or too late, plate fewer or more cortical cells, respectively, when setting up the mixed culture.

1. Prior to performing media changes, warm Co-Culture Media in a 37 °C water bath.
2. Follow the culture schedule.

Day 0	Plate ~1.2 million cells in prepared T175 culture flasks
Day 1	Cells separate and start to form processes.
Day 2	Astrocytes appear (large, flattened cells).
Day 4	Astrocytes are 2–6% confluent. Perform complete media change with warmed Co-Culture Media.
Day 7	Astrocytes are 60–80% confluent. Perform complete media change with warmed Co-Culture Media.
Day 9–10	Astrocytes reach 100% confluence
Day 11–12	Harvest microglia

Note: Do not feed the culture after Day 7 because microglia, and not neurons, will thrive under conditions of nutrient depletion.

Harvesting Rat Microglia

1. Place T175 flasks on shaker inside a humidified 37° C, 5% CO_2 incubator. Shake for 2 hours at 180 rpm.
2. Collect the Co-Culture Media from a flask and pass it through a 40 µm cell strainer into a 50 mL conical tube. Repeat for all flasks.
3. Centrifuge at 300 × g for 5 minutes at room temperature. Discard the supernatant.
4. Resuspend microglia in desired media. Pool cells if needed.

Note: Protocol will result in about 2–6 million microglia per T175 flask.

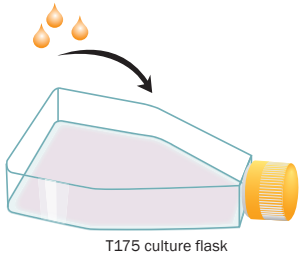
References

1. Ni, M. and M. Aschner (2010). Curr. Protoc. Toxicol. Unit **43**:12.17:1–16.

Protocol for Culturing Rat Microglia

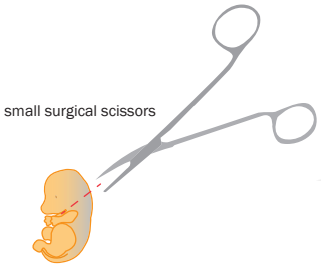
Day 1

Prepare T175 cell culture flasks by coating with Poly-L-Lysine.



Day 2

Decapitate P1–2 rat pups.



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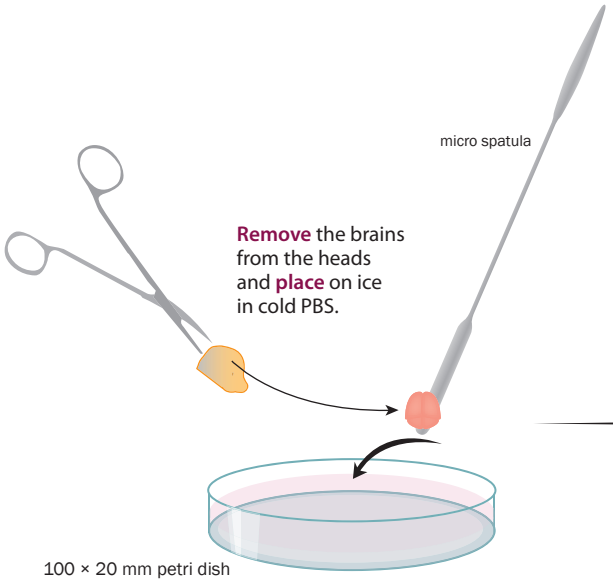
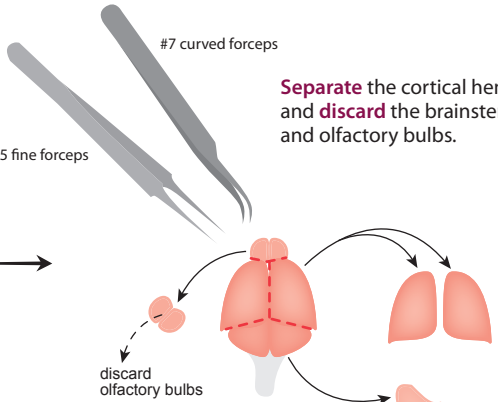


Remove the brains from the heads and **place** on ice in cold PBS.



Separate the cortical hemispheres and **discard** the brainstem, cerebellum, and olfactory bulbs.

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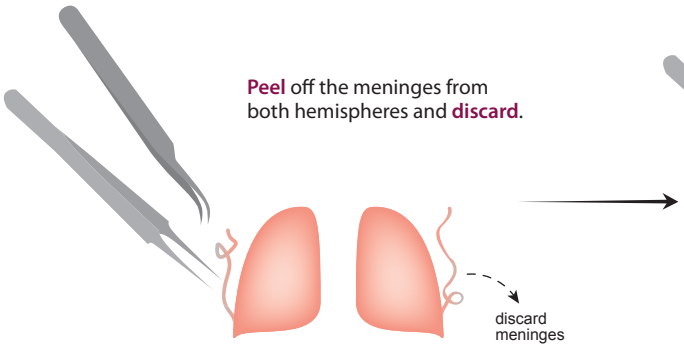
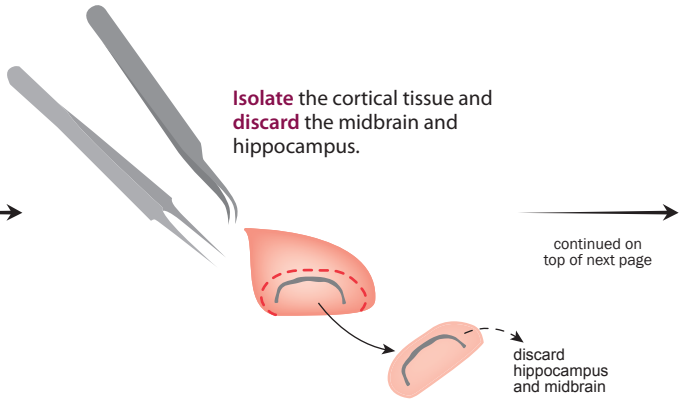


Peel off the meninges from both hemispheres and **discard**.



Isolate the cortical tissue and **discard** the midbrain and hippocampus.

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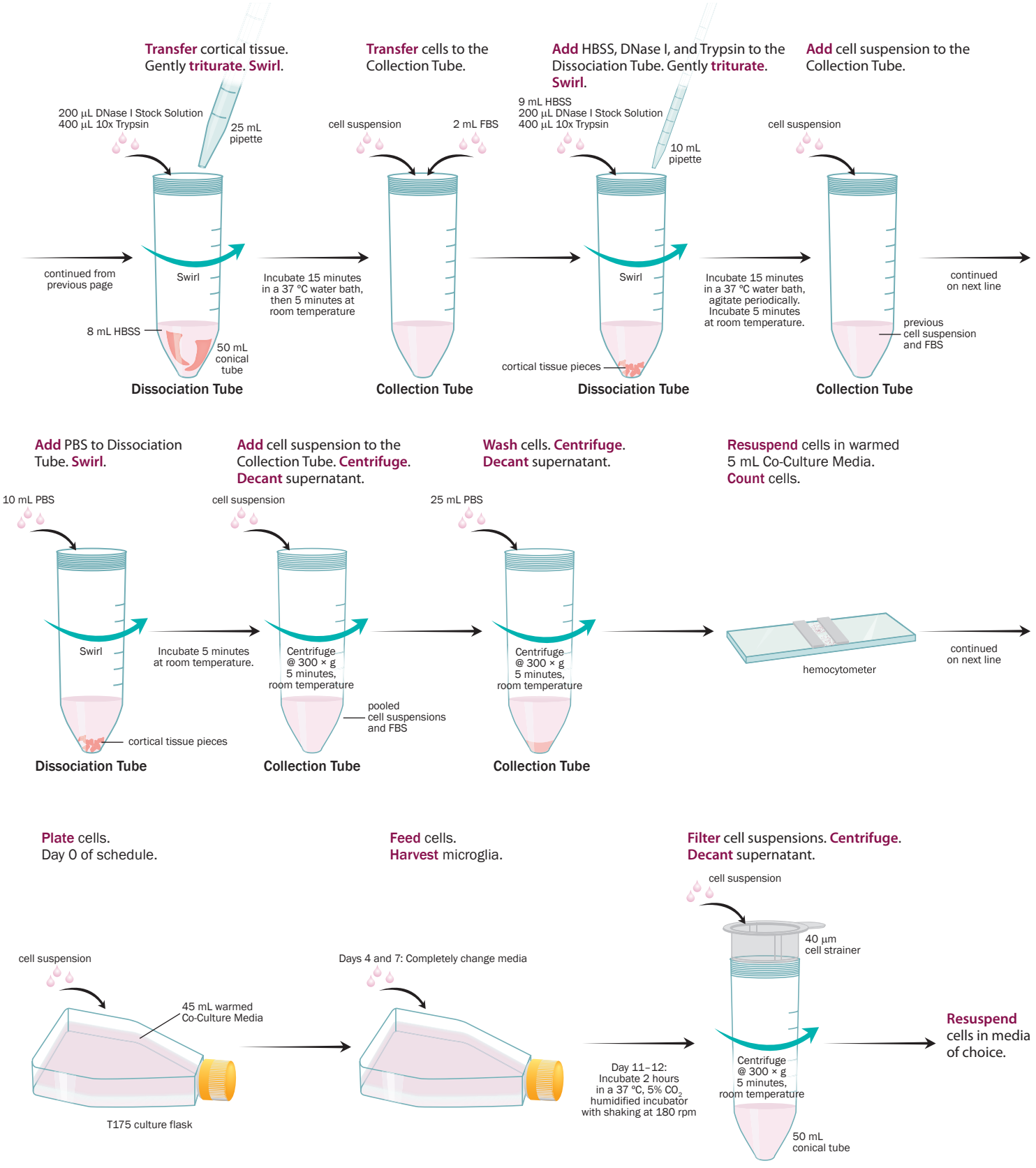
Protocol for Isolating Rat Microglia, continued