Protocol: Primary neuron culture for live-imaging of axonal cargoes

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Dissection, mouse, primary neuron, live-imaging

Abstract

This protocol describes the preparation and culture of mouse primary cortical neurons for live-imaging experiments. Cortices were dissected from mouse embryos at day 15.5. Cortical neurons were isolated by digestion with 0.25% Trypsin and trituration with a serological pipette. Neurons were plated on glass-bottom imaging dishes in Attachment Media. After 5 hours in culture, Attachment Media was replaced with Maintenance Media, and AraC was added on the next day to prevent glia cell proliferation. Neurons were transfected 16-24 hours before imaging using Lipofectamine 2000.

Materials

Dissecting microscope

Durmont #5 Mirror Finish Forceps (Fine Science Tools, Cat# 1125123)

Micro spring scissors (Fine Science Tools)

Hemocytometer or automated cell counter (Countess 3, Thermo Fisher)

35 mm glass-bottom imaging dishes (MatTek, Cat# P35G-1.5-20-C)

15 mL conical tubes

10 cm cell culture dish

Reagents

Poly-L-lysine, mol wt 70,000 – 150,000 (Sigma, Cat# P1274-100MG)

10x HBSS (Thermo Fisher, Cat# 14185052)

1M HEPES (Thermo Fisher, Cat# 15630080)

2.5% Trypsin (Thermo Fisher, Cat# 15090046)

Minimum Essential Medium, MEM (Thermo Fisher, Cat# 11095072)

Horse Serum, heat inactivated (Thermo Fisher, Cat# 16050122)

D-(+)-Glucose Glucose solution, 45% (Sigma, G8769-100ml)

Sodium Pyruvate, 100 mM (Thermo Fisher, Cat# 11360070)

Trypan Blue Stain 0.4% (Thermo Fisher, Cat# T10282)

Neurobasal Medium (Thermo Fisher, Cat# 21103049)

B-27 Supplement (Thermo Fisher, Cat# 17504044)

GlutaMAX (Thermo Fisher, Cat# 35050061)

Penicillin-Streptomycin (Thermo Fisher, Cat# 15140122)

AraC (Sigma, Cat# C6645)

Lipofectamine 2000 Transfection Reagent (Thermo Fisher, Cat# 11668019)

Hibernate E Low Fluorescence Imaging Medium (BrainBits, Cat# HELF)

Safety warnings

Take necessary precautions with sharp objects during dissection. Follow institutional recommendations for disposal of animal tissue and biohazardous materials.

Day before dissection:

- Coat glass-bottom imaging dishes with PLL: hydrate 100 mg PLL (Sigma) in 50 mL 0.1 M borate buffer, pH 8.5. Store PLL stock solution (2 mg/mL) in 1 mL aliquots at -80°C. On the day before neuron dissection, dilute PLL in ddH2O 1:20 to a final concentration of 100 µg/mL. Add 1 mL PLL to each glass-bottom imaging dish (MatTek) and incubate overnight at 37°C. Only coat the glass center with PLL.
  Note: for easy handling, we find it helpful to place imaging dishes in 10 cm or 15 cm cell culture dishes.
- Prepare HBSS, attachment media and maintenance media: for 500 mL 1x HBSS, combine 50 mL 10x HBSS with 5 mL 1M HEPES, fill up to 500 mL with ddH2O and filter-sterilize. Store 1x HBSS at 4°C and use within one month. For 50 mL attachment media, combine 5 mL heat-inactivated horse serum, 500 µL 100 mM sodium pyruvate, and 660 µL 45% Glucose; add MEM up to 50 mL. For 50 mL maintenance media, combine 500 µL GlutaMAX, 500 µL Penicillin/Streptomycin,

660 µL 45% Glucose, 1 mL B-27, and add Neurobasal up to 50 mL. Store attachment media and maintenance media at 4°C.

Note: Maintenance Media should be used within 7 days. Attachment media can be kept at 4°C for 3-4 weeks.

Dissection of cortical neurons

- In the morning of the day of dissection: wash PLL-coated imaging dishes twice with sterile ddH2O. Add 2 mL attachment media per imaging dish and leave dishes at 37°C in cell culture incubator. Warm required amount of attachment media and 1x HBSS (4.5 mL for one dissection) in 37°C water bath. Aliquot maintenance media into 10 cm cell culture dish to equilibrate in 37°C / 5% CO2 cell culture incubator. Let 2.5% trypsin aliquots thaw at room temperature.
- Sacrifice pregnant mouse, dissect embryos, and place embryonic brains in HBSS on ice. Using a dissecting microscope, remove meninges from brain hemispheres with fine forceps. Isolate cortices using fine forceps and small spring scissors. Transfer dissected cortices into a 15 mL conical tube filled with 5 mL HBSS and keep on ice until all cortices are collected.

  Note: Use clean and sterile equipment for all dissection steps to prevent bacterial contamination of neuron cultures.

  Note: We find that using ice-cold HBSS helps preventing the tissue from getting sticky during the dissection. If HBSS gets too warm during the dissection, replace with fresh cold HBSS.

- Perform all following steps under a sterile tissue culture hood. Once all cortices are collected, remove HBSS from 15 mL conical tube and add 4.5 mL warm (37°C) HBSS and 0.5 mL 2.5% trypsin. After adding trypsin, invert the tube to mix. Then incubate for 10 minutes in a 37°C water bath.
- Remove HBSS-trypsin solution with a 5 mL serological pipette. Wash thrice with 7 mL attachment media: add attachment media, then wait until cortex tissue has settled at the bottom of the conical and remove attachment media with a serological pipette to repeat the washing step.

  Note: we do not recommend using a vacuum aspirator for removing HBSS and attachment media, instead use a 10 mL serological pipette.
- Add 5 mL attachment media after the last washing step, then triturate cortices by pipetting up and down forcefully with a 5 mL serological pipette 10 – 15 times. Trituration is complete when no tissue clumps are visible and attachment media turns turbid. Let media with triturated tissue settle for 1-2 minutes, then transfer top 4.5 mL to a new tube to remove any remaining cell clumps.
- Mix 10 µL cell suspension with 10 µL 0.5% trypan blue in an Eppendorf tube, then count cells using a hemocytometer or an automated cell counter.
- Dilute cortical neurons to 1,000,000 cells/mL. For transfection and live-imaging, plate 200,000 cells per live-imaging dish. Place imaging dishes in 37°C cell culture incubator.

  Note: Take up cells in a 200 µL pipette, then plate drops of cells in different areas to distribute neurons evenly across the live-imaging dish.

- After 3-4 hours, use an aspirator to remove all attachment media and replace with 2 mL pre-equilibrated maintenance media per imaging dish. Cells should be attached to the glass-bottom dish at this point.

  Note: maintenance media must always be pre-equilibrated to 5% CO_2 in 37°C incubator before adding to cells.

Neuronal cell culture

- On the day following the dissection, dilute AraC to 10 µM in maintenance media and bring to 37°C. Add 200 µL maintenance media + AraC to each imaging dish for a final AraC concentration of 1 µM.
- Every 3-4 days, remove 600 µL maintenance media from each dish and replace with 750 µL fresh, pre-equilibrated maintenance media.

  Note: cultured neurons are sensitive. Try to keep time outside the cell culture incubator to a minimum. If possible, use a separate incubator for primary neurons and keep openings to a minimum.

Transfection

- Transfect primary neurons on DIV6-7, ~ 16 hours before live-imaging.
- Replace conditioned media with fresh, pre-equilibrated maintenance media (2 mL per imaging dish). Save old media = conditioned media in a 10 cm cell culture dish at 37°C in the cell culture incubator.

- For each imaging dish, prepare two tubes with transfection reagents. In tube 1, add plasmid DNA to 200 µL Neurobasal medium. In tube 2, add Lipofectamine 2000 to 100 µL Neurobasal medium.

  Note: it is important to use non-supplemented Neurobasal and not maintenance medium to set up the transfection reaction.

  Note: the amount of Lipofectamine 2000 and plasmid DNA depends on the specific construct(s) used and may require optimization. We find that for transfection with one plasmid, 0.4 µg DNA and 1 µL Lipofectamine 2000 works well in most cases.
- Combine contents of tube 1 + 2 and mix by gently pipetting up and down 4-5 times. Incubate mix at room temperature for 10 minutes
- Add Lipofectamine-DNA mix to imaging dishes dropwise. Then incubate for 45 minutes at 37°C in cell culture incubator.
- Remove all transfection media and replace with conditioned media collected earlier. Return cells to incubator and image on the next day.