

Materials needed

10 mL and 60 mL syringes (*BD #309604, BD #301035*)
24-well plate (*BD Falcon #353847*)
Cell culture inserts, 3 µm pore size PET (*BD Falcon #353096*)
Conical centrifuge tubes, 15 mL and 50 mL (*BD Falcon #352196 and Fisher #553913*)
Coverslips, 12 mm No.1, 0.13-0.17 mm thickness (*Carolina Biologicals #633029*)
Dissection tools (big scissors, small scissors, No. 2 forceps, two No. 5 forceps, spatula (or hippocampal tool), scalpel)
Petri dish, 10 cm (*BD Falcon #351029*)
Petri dish, 35 mm (*BD Falcon #351008*)
Sterile transfer pipette (*Samco #202-1S*)
Surgical blade, No. 10 (*Feather 2976#10*)
Syringe filters, 0.22 µm (*Millipore #SLGP033RS*)

Chemicals needed

2-mercaptoethanol ≥99% (*Sigma #M7522*)
B-27 serum-free supplement (*Invitrogen #17504-044*)
Cytosine beta-D-arabinofuranoside (Ara-C, *Sigma #C1768*)
Deoxyribonuclease I (DNAse I) from bovine pancreas (*Sigma #D5025*)
Dulbecco's phosphate-buffered saline, no calcium, no magnesium (*Invitrogen #14190*)
Glutamax-I supplement (*Invitrogen #35050-061*)
Hanks' balanced salt solution (HBSS) (*Invitrogen #14170*)
HEPES buffer (1 M) (*Sigma #0887*)
Monosodium glutamic acid (MSG, *Sigma #G5889*)
Neurobasal-A medium (*Invitrogen #10888*)
Poly-D, L-lysine hydrobromide (PDLL) (*Sigma #P9011*)
Sodium pyruvate (100 mM) (*Invitrogen #11360*)
Trypsin inhibitor from soybean (*Sigma #T9003*)
Trypsin, 2.5% (10X) (*Invitrogen #15090*)

➤ **Make sure to use good sterile technique throughout!**

Prepare in advance

1. Make 0.1 M **borate buffer**, pH 8.5 by dissolving 3.09 g of boric acid (H₃BO₃) in 500 mL of water. pH with NaOH. Filter sterilize with 0.22 µm pore-size filter. This borate buffer can be stored at 4°C for several months.
2. In the hood, prepare 1 mg/mL **PDLL** in 0.1 M borate buffer. Make aliquots

and store in the freezer.

3. Prepare **HBSS** by adding 5 mL sodium pyruvate and 5 mL HEPES buffer to 500 mL of HBSS (inside the hood). Use this solution during the dissection whenever HBSS is mentioned. The HBSS can be stored in the fridge for several weeks.
4. Thaw **DNase** on ice and dissolve in ice cold sterile water to 20 mg/mL. Aliquot and store at -20°C. DNase is very temperature sensitive, so make sure to keep it cold until you use it.
5. Dilute **2-mercaptoethanol** (0.01 mL) in sterile D-PBS (5.71 mL) to 25mM. Aliquot and store in freezer.
6. Make a 20mM stock solution of **AraC** in double-distilled water. Sterile filter. Aliquot and store at -20°C.
7. Thaw **B27** and make 1 mL aliquots. Store at -20°C.
8. Make 25 mM stock of **MSG** using double distilled water. Filter sterilize, aliquot and store at -20°C.
9. **GlutaMax** is supplied as a 100X (200 mM) solution. Make aliquots inside the hood and store at -20°C.

Etch coverslips

1. Put ~500 coverslips in a glass container. Rinse 1x with double distilled water.
2. Add 3.7% HCl (made by diluting concentrated, fuming HCl 1:10) to coverslips.
3. Shake coverslips on a horizontal shaker for 15-20 minutes vigorously enough that all coverslips get exposed HCl.
4. Rinse 3 times with double distilled water.
5. Rinse 1 time with 70% ethanol. Leave the coverslips in ethanol until the next step.
6. Spread coverslips out on a piece of filter paper in the cell culture hood.
7. UV and dry for 30 min. Collect coverslips in a 15 cm Petri dish between cut-out circles of filter paper.
8. Store coverslips in a sterile environment for future use.

Day -1: Coat coverslips and cell culture inserts (or culturing dishes)

1. Dilute PDLL in 0.1 M borate buffer to a final concentration of 0.1-0.5 mg/mL.
2. Put a coverslip in each well of a 24-well plate. Shake plate gently to make sure that coverslips lie flat.
3. Put cell culture inserts in wells that do not contain coverslips.
4. Using a pipette, add ~85 µL (one or two drops) of PDLL to each coverslip.
5. For cell culture inserts, put some solution inside the insert and some on the bottom of the well so that the insert membrane is coated with PDLL on both

sides.

6. Leave plate in incubator or in other sterile environment overnight.

Day 0: Dissection

1. Prepare:
 - a. Two ice buckets – one for chilling solutions, one for chilling dissection plates.
 - b. Dilute 0.5 mL trypsin with 4.5 mL HBSS inside the hood. Put this in 37°C waterbath to activate.
 - c. Dissolve 10 mg trypsin inhibitor in 10 mL of HBSS. Filter sterilize with 0.22 µm filter. Put this in 37°C waterbath.
 - d. Plating medium. Combine and filter sterilize with 0.22 µm filter:
 - i. B27 (1 mL)
 - ii. Glutamax (125 µL)
 - iii. MSG, 25 mM (50 µL)
 - iv. 2-mercaptoethanol (50 µL)
 - v. Neurobasal-A (50 mL)
 - e. Thaw 50 µL DNase I on ice.
 - f. Add HBSS to 35 mm dishes (use 2 dishes per pup + 1 extra dish). Add enough to just cover the bottom. These dishes will be used to perform the dissections in. We use two dishes per brain: one in which to do the initial dissection and removal of meninges, and the other to do finer trimming in. The dissected brain tissue can then be transferred to a communal dish. Chill on ice.
 - g. Spray down the dissection bench with 70% ethanol. Lay out tools, transfer pipette, and waste bag for pup carcasses.
 - h. Rinse coverslips and cell culture inserts three times with PBS. Add 1 mL plating medium to each well. Put the 24-well plate back in the incubator while preparing the neurons.
2. Firmly pack a 10 cm Petri dish with ice, using the cover of the dish to tamp the ice down. Perform dissections on this ice platform.
3. Put one of the 35 mm Petri dishes with HBSS on the ice in the 10 cm dish. After dissecting the brain from the skull, place it in the HBSS-containing dish. Dissect the desired brain region, and transfer it to a second, fresh HBSS dish and trim away meninges and other undesired tissue. This transfer to fresh HBSS acts as a rinse to help get rid of contaminants and factors released by dying cells during dissection which might impair neuron survival. Move trimmed regions to a third dish, where dissections from the other pups will be collected as well. Cut the tissue into smaller pieces.
4. After all dissections are complete, collect all tissue pieces and transfer them

- to the trypsin tube, transferring as little of the dissection medium as possible.
5. Add 50 μ L DNase I to the tissue. Incubate 15 min at 37°C. Swirl the tube every few minutes during the incubation to allow trypsin access to as much of the tissue as possible.
 6. Remove the tube containing trypsin and pieces of tissue from the waterbath. Spray it with 70% ethanol and wipe down before moving it into the culturing hood.
 7. Let the tissue pieces settle to the bottom of the tube. Remove as much medium as possible by aspirating. Add 5 mL of the trypsin inhibitor (spray tube with ethanol before bringing it into the culture hood). Incubate 5 min at 37°C.
 8. Aspirate the inhibitor solution and wash with the remaining 5 mL of inhibitor. Remove the inhibitor solution wash.
 9. Resuspend neurons by triturating in 1 mL HBSS. Avoid introducing bubbles. For good survival it is important to use the fewest strokes possible to dissociate the tissue. Triturating over 25 times seems to reduce neuron survival.
 10. Count the cells. Plate 30,000-100,000 per well, depending on how dense you want your cultures. We commonly plate 40,000 cells per coverslip and 60,000 per cell culture insert. For immunocytochemistry, 30,000 neurons should provide a good neuron density. There is no need to shake or tilt the plate to spread the neurons.
 11. Put neurons in the incubator until ready to use.
 12. It is good practice to check on your neurons regularly, every few days or at least once a week. In addition to making sure the cultures are not contaminated, this will allow you to get a feel for how healthy neurons look during development,

Day 3: Add Ara-C

At 3 days in vitro, add Ara-C to the wells to a final concentration of 1 μ M. Do not add Ara-C to the wells containing cell culture inserts. Gently rock the plate to make sure the drug disperses.

Day 6: Remove Ara-C

After six days the Ara-C is inactive. Move the inserts into the Ara-C treated wells.