Isolation and culture of adult neurons and neurospheres

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Here we present a protocol for extraction and culture of neurons from adult rat or mouse CNS. The method proscribes an optimized protease digestion of slices, control of osmolarity and pH outside the incubator with Hibernate and density gradient separation of neurons from debris. This protocol produces yields of millions of cortical, hippocampal neurons or neurosphere progenitors from each brain. The entire process of neuron isolation and culture takes less than 4 h. With suitable growth factors, adult neuron regeneration of axons and dendrites in culture proceeds over 1–3 weeks to allow controlled studies in pharmacology, electrophysiology, development, regeneration and neurotoxicology. Adult neurospheres can be collected in 1 week as a source of neuroprogenitors ethically preferred over embryonic or fetal sources. This protocol emphasizes two differences between neuron differentiation and neurosphere proliferation: adhesion dependence and the differentiating power of retinyl acetate.

INTRODUCTION

Here we provide detailed methods for isolation of adult mammalian neurons for use in neuron culture. Although it is relatively easy to isolate embryonic[1,2] or early postnatal[3] neurons before they form synapses, isolation of adult CNS neurons with mature properties is problematic because of tight adhesion of cell bodies and thousands of synapses as well as fragmentation of axons and dendrites that inhibit regeneration of these processes from the isolated somata. Adult neurons also depend on trophic factors for viability[4]. In many cases, it is clear that embryonic neurons behave differently than adult neurons in terms of pharmacology, electrophysiology, development, and regenerative and pathological characteristics. Neuron culture in defined, serum-free medium allows comparisons independent of hormonal, vascular and inflammatory influences that otherwise complicate whole-brain studies. Cultures also offer the advantage of identical replicates for dose–response and time-course studies. In our own studies of the aging brain, the differences between adult and embryonic neurons are dramatic in terms of susceptibility to stressors such as glutamate and A-beta[5], calcium dynamics[6], effects of adult gene haplotypes in mice[7] and mitochondrial function[8]. Others have used the methods described here to study the different effects of estrogen on adult neurons[9,10], transfection of isolated adult spinal cord neurons in relation to spinal cord injury[11], effects of brain-derived neurotrophic factor (BDNF)[12] and pharmacology of a novel nicotinic receptor modulator[13].

The method described employs a density gradient to enrich for neurons over other cell types. Such gradients are often employed in centrifugal separation of blood cells, but can be advantageous for brain cells as well. The following methods were optimized for cell recovery by protease digestion of slices and control of osmolarity and pH outside the incubator with Hibernate medium[14]. These methods enable yields of millions of cortical and hippocampal neurons from each brain in preparations repeated from hundreds of rats[3] as well as human[15] and mouse brain tissue. Although it seems simpler to leave out the density gradient or substitute an albumin gradient, these alternatives produce lower yields of neurons than the method here. The culture medium promotes neuron survival in part by five antioxidants in the B27[2]. Studies of free radical damage and oxidation[16] are possible by switching to a medium containing B27 minus antioxidants[17]. Others have isolated adult neurons with methods that skip the density gradient purification for ease and simplicity[18], but yields and purity are lower. For isolation of neurons from early postnatal animals, similar but simpler procedures suffice[19,20], although one can simply omit the density gradient in the following protocol (Steps 11–15). This protocol updates our prior work with application to mouse brain as well as rat[4,21] and emphasizes only two differences between neuron differentiation and neurosphere proliferation: retinyl acetate in the medium and adhesion dependence.

Adult neurospheres are free-floating clusters of neuroprogenitor cells generated by neural stem cells (NSCs)[22,23]. Adult neurospheres offer a source of neuroprogenitors ethically preferred over embryonic or fetal sources. The method described has been used to study development and differentiation of brain neurons[24,25]. Using different methods with unstated yields, progenitors have been grafted into rat brains with evidence of survival and differentiation[22]. The same lab, using DMEM/F12 with N_2 and serum, isolated only two progenitors per milligram of tissue[26], compared with the yields of thousands of cells per milligram here. These progenitors are being used as grafts to repair damaged brain or spinal cord[27] and to study growth factor dependence for differentiation[28]. Results using a similar culture medium have established the multipotential of these neurospheres to differentiate into neurons, astrocytes and oligodendrocytes[29]. They can also be used as genetic vectors to validate therapeutic targets and mechanisms in animal models[30]. The advantages of physiological oxygen levels near 9% over atmospheric oxygen levels of 21% for enhanced survival of neurons[31,32] have been confirmed for neuroprogenitors[33].

The flow of steps in the procedure is outlined in **Figure 1**. Note that isolation of neurons or neurospheres follows the same procedure until the end.

MATERIALS

REAGENTS

- Rats or mice (Harlan, Charles River or Jackson Laboratories) (see REAGENT SETUP)
- B27 (Invitrogen, cat. no. 17504) for neurons; B27 minus retinyl acetate (cat. no. 12587-010) for neurospheres; both are 50× concentrates that need to be diluted
- HibernateA/B27 medium (HABG) (see REAGENT SETUP)
- Human basic FGF2 for rat or human neurons (Invitrogen, cat. no. 13256-029) or Neuropro H (BrainBits LLC) or NeuroCult NSC basal medium + supplements (StemCell Technologies) ▲ CRITICAL Growth factors adhere to the walls of containers and denature at low concentrations. Follow the supplier's instructions for storage and dilution. We store 5-µl aliquots at 100 µg ml^{-1} in water at –70 °C. After these have been thawed, the fibroblast growth factor (FGF) is diluted to 1 µg ml^{-1} 0.1% sterile bovine albumin in PBS for immediate dilution into culture medium or use within 1 week if stored at 4 °C.
- Mouse FGF2 for mouse neurons (Invitrogen Biosource, cat. no. PMG0034)
- Laminin (10 µg ml^{-1}; e.g., Sigma, cat. no. L2020)
- Mouse PDGFbb (Invitrogen Biosource, cat. no. PMG0044) or Neuropro M (BrainBits LLC) or NeuroCult NSC basal medium + supplements (StemCell Technologies)
- Gentamycin (10 mg ml^{-1}; Invitrogen, cat. no. 15710)
- L-GIn (Invitrogen, cat. no. 25030; prepare 25 mg ml^{-1} = 171 mM) or Glutamax (200 mM; a stable dipeptide of Ala–Gln; Invitrogen, cat. no. 35050-061) ▲ CRITICAL Store aliquots of 1 ml at –20 °C. Over time, above 4 °C, GIn deaminates to glutamate, an excitotoxic amino acid. Commercial supplies of GIn that we have tried contain measurable amounts of glutamate (0.3–1%) measured by dehydrogenation with conversion of NAD^+ to NADH (Sigma GLN-1). Glutamax has less than 0.1% glutamate.
- HibernateA (HA; BrainBits LLC) (see REAGENT SETUP)
- HA minus calcium (HA-Ca; BrainBits LLC) needed for maximal cell isolation and digestion
- NeurobasalA ('A' for all postnatal and adult tissue; Invitrogen, cat. no. 10888) (salts, glucose, amino acids and vitamins such as DMEM)
- NeurobasalA/B27 medium with L-GIn or Glutamax and growth factors and gentamycin (see REAGENT SETUP)
- Isoflurane (Baxter, Deerfield, IL) as an animal anesthetic (some labs use pentobarbital or carbon dioxide) ! CAUTION Isoflurane is a controlled substance to be acquired through a licensed veterinarian under an approved protocol.
- Papain (Worthington, cat. no. 3119) (see REAGENT SETUP)
- Poly-D-Lys (sterile lyophilized 135 kD; Sigma, cat. no. P6407) (see REAGENT SETUP)
- OptiPrep density 1.32 (Sigma, cat. no. D1556) (see REAGENT SETUP)
- Trypan blue (Sigma, cat. no. T8154)
- 70% Ethanol to wash surfaces and hands before use
- Chloroform (Fisher, cat. no. C607-4)

EQUIPMENT

- 35-mm cell culture dish (e.g., Falcon 3001; Fisher or VWR)
- Ultralow adhesion plastic culture dishes (e.g., Corning 24 well; Corning, cat. no. 3473; or 6 well, cat. no. 3471, Fisher or VWR) (only needed for neurospheres)
- 15- and 50-ml polystyrene or polyethylene terephthalate (PET) centrifuge tubes (Corning, cat. nos. 430055, 430304) ▲ CRITICAL Polyethylene and polypropylene are toxic to neurons.
- 12-mm glass coverslips or other sizes as desired (Assistant Brand; Carolina Biologicals, cat. no. AA-63-3029); autoclave to sterilize ▲ CRITICAL Do not clean with solvents as this is more likely to leave a toxic residue. Adult neurons do not grow well on cell culture plastic.
- Hemacytometer (0.4 mm, 0.1 mm deep; Fisher, cat. no. 02-671-5)
- HEPA filter
- Sterile paper, 1 sheet per tissue, 7 cm (Whatman #2, Fisher, cat. no. 09-810B)
- Sterile syringe filter, 0.2 µm (Fisher Millipore, cat. no. SLGP033RS)
- Sterile syringe, 10 cc (Fisher, cat. no. 14-823-2A)
- Incubator, controlled 5% CO2, 9% O2 gas, humidified (e.g., Forma 3130; Forma, Marietta, OH) ▲ CRITICAL A more common incubator without oxygen regulation will work, but the unphysiologically high oxygen of 20% retards neurite growth[31,32] and neurosphere proliferation[33].
- MacIlwain tissue chopper (Brinkman, Westbury, NY) (see EQUIPMENT SETUP)
- 9-in siliconized Pasteur pipette (see EQUIPMENT SETUP)
- Microscope, inverted, phase contrast with ×10 phase long working distance objective (e.g., Olympus IX71; Olympus, or less expensive, but adequate CKX41)
- Sterile hood for cell culture, HEPA-filtered air (e.g., Envirco ESM-4; Labconco 36214 Type B2)
- Swinging bucket centrifuge for 15-ml tubes, ambient temperature, up to 800*g*
- Water bath, orbital shaker for 30 °C Steps 17 and 18 (Model 3535; Labline, Melrose Park, IL); cell yields are better at this temperature than at 25 or 37 °C.
- Silicoat (Sigma, cat. no. D3879)

REAGENT SETUP

HA Hibernate has the same amino acids, salts, glucose and vitamins as Neurobasal but controls the pH while working outside the CO_2 environment of an incubator. Observe the same storage limitations as for Neurobasal.

HABG Prepare medium containing 60 ml HA, 1.2 ml B27, 0.176 ml GIn (0.5 mM final) (or 0.15 ml Glutamax). Aliquot 2 ml into each of two 35-mm dishes and store at 4 °C (for use in Steps 6 and 14). Aliquot 5 ml into a 50-ml tube and store at 4 °C (for use in Step 17). You can weigh this tube for later determination of weight of tissue to be placed inside. Aliquot 2 ml into a 15-ml tube and store at 30 °C (for use in Step 19). ▲ CRITICAL This medium can be prepared up to 24 h in advance and stored at 4 °C, then aliquoted on the day of tissue isolation. For cortex, double the volume of medium to accommodate the greater tissue mass.

NeurobasalA/B27 medium with L-GIn or Glutamax and growth factors and gentamycin Prepare medium containing 25 ml NeurobasalA, 0.5 ml B27,

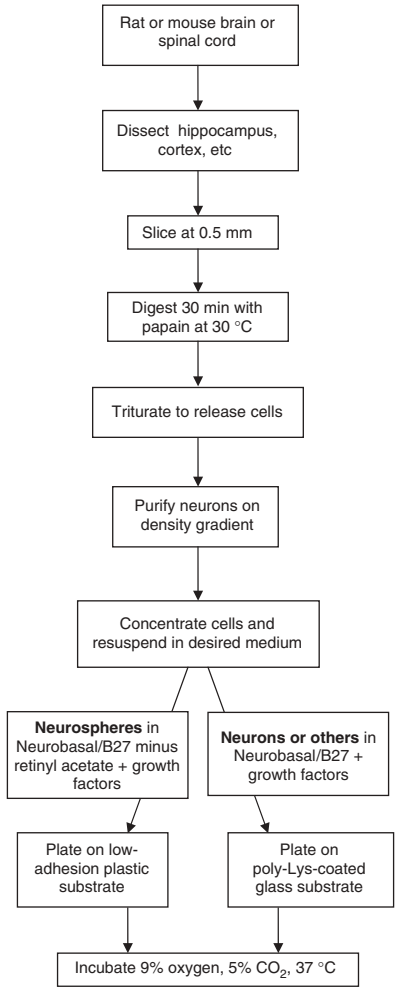

Figure 1 | Flow diagram showing the major steps in the procedure, which are the same for neurons or neurospheres until cells are plated.

0.5 mM GIn or Glutamax, 10 μg ml^{-1} gentamycin. Add the following growth factors: for mouse neurospheres, 5 ng ml^{-1} mouse FGF2 plus 5 ng ml^{-1} mouse PDGFbb; for rat neurospheres, 5 ng ml^{-1} human FGF2. Store at 4 °C. Aliquot 0.4 ml per 2-cm^2 well of two 24-well plates. Place in a CO_2 incubator for use in Step 32A(iii). Make approximately 0.22 ml cm^{-2} substrate of culture dish; one pair of rat hippocampi provides up to 48 slips of 1 cm^2 at 32,000 cells per cm^2; the same density for two pairs of hippocampi from two mice will also provide up to 48 slips. ▲ CRITICAL This medium can be prepared up to 24 h in advance and stored at 4 °C, then aliquoted on the day of tissue isolation. Controlled experiments show that mouse neurons have different growth factor requirements than rat neurons. Other cell types and neurons from other brain tissues may have different growth factor requirements. To our knowledge, rat FGF2 is not available. NeurobasalA/B27 should be prepared fresh every week with growth factors freshly diluted from frozen stock solutions on the day of use. Unopened bottles of NeurobasalA can be stored in the dark at 4 °C for up to 6 months. Opened bottles can be stored in the same way for up to 1 month. B27 will last indefinitely at –70 °C. It should not be refrozen. It can be stored at 4 °C for 1 week.

Papain To lightly digest tissue. On the day of neuron isolation, dissolve 12 mg papain solids per 6 ml HA-Ca, 0.5 mM GIn or Glutamax (approximately 34 U ml^{-1}) at 37 °C for 20–30 min. Filter-sterilize into a 50-ml polystyrene tube. Store on ice until ready to use, leaving for no longer than 3 h. This volume is sufficient for hippocampi from one rat, approximately 85 mg tissue, or hippocampi from two to three mice. For cortex, double the volume of papain. ▲ CRITICAL Do not activate the papain with Cys because this amino acid is excitotoxic to neurons. Worthington papain has proven superior to papain from Sigma. Do not be concerned if solids in the papain do not dissolve completely.

Poly-D-Lys Reconstitute at 100 μg ml^{-1} sterile water. Use fresh or store frozen aliquots of 12 ml in 50-ml polystyrene or PET tubes. You will need 0.1 ml per cm^2 of glass substrate to be coated. This is not needed for neurospheres since their attachment is not desired. ▲ CRITICAL Poly-Lys adsorbs to the surface of a glass or plastic container and will not be fully available for coating substrates. Polyethylene or polypropylene containers leach plasticizers that are neurotoxic. Frozen tubes of poly-Lys should not be used after two freeze–thaw cycles. Be sure that frozen poly-Lys thoroughly dissolves after thawing. Unfrozen poly-Lys stored at 4 °C may be used after up to 1 week. The poly-Lys coating is stable up to 2 d for good cell growth. Alternatively, pre-coated substrates are available from BrainBits. Some groups add laminin (10 μg ml^{-1}; e.g., Sigma, cat. no. L2020) to the dried poly-Lys coating. We find no benefit to neuron growth of added laminin.

TABLE 1 | Density gradient preparation.

Layer	OptiPrep 1.32 (μl)	HibernateA/B27 medium (μl)	Total (μl)
Bottom 1	173	827	1,000
2	124	876	1,000
3	99	901	1,000
Top 4	74	926	1,000

OptiPrep density 1.32 On the day of the cell isolation, dilute OptiPrep, as described in **Table 1**, in four sterile 15-ml tubes (1–4). Carefully pipette layer 2 onto the top of layer 1, with minimal disturbance to layer 1. Follow with layer 3 and layer 4 to complete formation of a density gradient. ▲ CRITICAL If you are using mouse, the same volumes can accommodate the cortices from one mouse or the hippocampi from two mice. If you are using rat cortex, prepare two gradients.

Rats or mice The volumes in this protocol are designed for the hippocampi from one rat. We have not found differences between Fisher and Sprague-Dawley rats. ! CAUTION All experiments involving live rodents must conform to national and institutional regulations. This protocol was approved by the Southern Illinois University School of Medicine Laboratory Care and Animal Use Committee.

EQUIPMENT SETUP

MacIlwain tissue chopper On day of neuron isolation, clean a double-edged razor with 70% ethanol and mount in the chopper. Manually cycle chop to position blade flat with surface. Tighten blade and raise manually. Set speed to approximately 1 cycle s^{-1}; set width to 0.5 mm for Step 15.

Nine-inch siliconized Pasteur pipette Coat with Sigma Silicoat (Sigma, cat. no. D3879) diluted 1:20 in chloroform (Fisher, cat. no. C607-4) in a chemical fume hood. Let dry in the hood. Autoclave to sterilize. For each preparation, in a sterile hood, fire-polish the tip of one pipette to no less than 0.6 mm. ! CAUTION Chloroform is a flammable intoxicant.

PROCEDURE

Preparation on the day of cell isolation

1| When preparing neurons, 3–24 h in advance of cell isolation arrange autoclaved glass coverslips in 35-mm sterile plastic dishes, up to four per dish, so that they do not touch each other. One pair of adult rat hippocampi can seed 48 slips of 1 cm^2 at 320 cells per mm^2. Coat slips with 100 μl poly-Lys cm^{-2} substrate. After more than 1 h, or commonly overnight, aspirate approximately 95% of the coating and immediately apply 100 μl sterile water. Aspirate more than 95% of the water and let the rest dry in the hood for more than 1 h. When preparing neurospheres, omit this step.

▲ CRITICAL STEP All operations are performed at room temperature (20–23 °C) in a laminar flow sterile hood unless stated otherwise. Volumes are for one pair of rat or two to three pairs of mouse hippocampi. Do not let these slips dry out or the coating will be toxic to neurons. The poly-Lys coating is stable for up to 3 d for good cell growth. Coating should be smooth without visible signs of aggregation. Alternatively, pre-coated substrates are available from BrainBits LLC.

? TROUBLESHOOTING

Preparation for tissue isolation ● TIMING More than 5 min

2| Anesthetize an adult rat by placing in a container with 2 ml isoflurane (some labs use pentobarbital or carbon dioxide). Approximately 15 s after movement ceases, remove rat and confirm anesthesia by lack of withdrawal reflex from a toe pinch.

3| Decapitate in a guillotine. Disinfect head with 70% ethanol. Dissect the skin over the top of the skull to expose the skull.

4| Insert scissors into spinal canal and carefully cut the calvarium on one side nearly to the front. Avoid damage to the brain. Repeat on the other side.

5| Grab the base of the skull with forceps and lift up and forward to expose the brain.

6| Insert a sterile 4-mm spatula at the front of the brain to sever the olfactory bulb and optic nerves. Gently lift the brain out of the calvarium and transfer to a dissection dish with Hibernate at 4 °C. Be careful to avoid contact with the fur.

! CAUTION Work in a fume hood or other suitable ventilation to avoid self anesthesia.

Tissue isolation ● TIMING **More than 10 min**

7| Rapidly dissect hippocampi or other brain regions or spinal cord from the rodent brain[34–36] into 2 ml HABG at 4 °C in a 35-mm-diameter dish. To dissect the hippocampus, orient the brain with dorsal side up so that the clear midline of two hemispheres is visible and follow Steps 8–15 below.

8| With forceps spread approximately 2 mm and index finger on top of the two edges of the curved forceps, insert down the dorsal midline to approximately half the depth; squeeze to sever the cerebral commissure. Re-insert with 2 mm spread into midline of the midbrain, all the way down to plastic dish as a stable anchor.

9| With other forceps spread, use both tips to gently peel one hemisphere to the side.

10| Insert on this exposed side and sever the hippocampal/septal junction.

11| Use this lesion to grab the meninges and peel back along curve of cortex.

12| Make three cuts between hippocampus and cortex. After each cut, immediately peel off meninges in this area.

13| Slide the hippocampus out, cut the posterior edge and peel off any remaining meninges.

14| Transport by cradling the hippocampus with forceps to a dish with HABG on ice. Do not squeeze to crush the tissue.

15| Repeat Steps 8–14 with the contralateral hippocampus.

Tissue slicing

16| Transfer hippocampi or other brain regions or spinal cord to sterile filter paper pre-wet with HibernateA/B27 on the cooled stage of a MacIlwain tissue chopper. Orient the long axes of the hippocampi perpendicular to the razor blade. Cut 0.5-mm slices of the hippocampi. Immediately wet with HABG and transfer with curved forceps to the 50-ml tube at 4 °C containing 5 ml HABG.
Note: If the tube was pre-weighed, you can weigh this tube again to determine the weight of tissue.

17| Transfer the 50-ml tube with tissue to a shaking water bath at 30 °C. Also place the 50-ml tube with papain with the 15-ml tube with HABG in the bath. Shake for 8 min to equilibrate temperature.

18| Transfer the tissue with a wide-bore pipette to the tube at 30 °C containing papain. Shake for 30 min at a speed just high enough to suspend the tissue (e.g., 170 r.p.m.).

19| Transfer the slices with a wide-bore pipette and as little papain as possible to the 15-ml tube containing 2 ml HABG at 30 °C and allow to sit for 5 min at room temperature.

Release of cells from tissue

20| Using the siliconized 9-in Pasteur pipette with the fire-polished tip, triturate approximately ten times in approximately 45 s. Trituration involves sucking the tissue up into the pipette, without air bubbles, and immediately emptying the contents back into the tube, without air bubbles. Triturating too gently will not disrupt the tissue. Triturating too vigorously will break cells.
▲ CRITICAL STEP Do not introduce bubbles. Pay attention to the trituration time. If a sticky mass of DNA accumulates, the cells were triturated too vigorously or the pipette tip was not smooth enough or was too narrow. The tip of a 1-ml plastic pipette tip is too sharp for optimum release of cells.
? TROUBLESHOOTING

21| Allow the pieces to settle for 1 min and transfer the supernatant to an empty 15-ml tube.

22| Re-suspend the sediment from the first tube in 2 ml HABG. Repeat Steps 20 and 21 two more times, combining the supernatants from each trituration to make approximately 6 ml of suspended cells (12 ml for cortex).

Separation of cells by density gradient centrifugation

23| Carefully apply the cell suspension to the top of the prepared OptiPrep density gradient. The cell suspension should float on top of the gradient. For cortex, two gradients are used.

24| Cenfrifuge the gradient at 800*g* (1,900 r.p.m. in a swinging bucket centrifuge) for 15 min at 22 °C. Also transfer the tube with Neurobasal medium at 4 °C to room temperature ready for Step 30.

25| Aspirate the top 6 ml containing cellular debris. The top 1 ml of the gradient can also be aspirated if oligodendrocytes are not desired.

26| Collect desired fractions with a pipette (**Fig. 2**). Fraction 1 (top 1 ml) is enriched for oligodendrocytes. Fraction 2, the most opaque band, contains cell fragments, neurons and other cells. Fraction 3 (from lower edge of dense band to 0.5 ml from bottom of tube) is enriched for neurons. Fraction 4 (bottom 0.5 ml and loose pellet) contains microglia. For pure neurons, but lower yield, use only fraction 3. For maximum yield of neurons, combine fractions 2 and 3.

27| To dilute gradient material, mix desired cell fractions with 5 ml HABG. For cortex, use 10 ml.

28| Centrifuge the cell suspension containing the desired cell fractions for 2 min at 22 °C at 200*g* (1,100 r.p.m. in a swinging bucket centrifuge).

29| Discard the supernatant, which contains debris. Loosen the pellet of cells by flicking the bottom of the tube with one finger. Immediately re-suspend the cells in 5 ml HABG. Centrifuge the cell suspension for 2 min at 22 °C at 1,100 r.p.m. (200*g*). Place remaining HABG at 37 °C ready for Step 32A(ii).

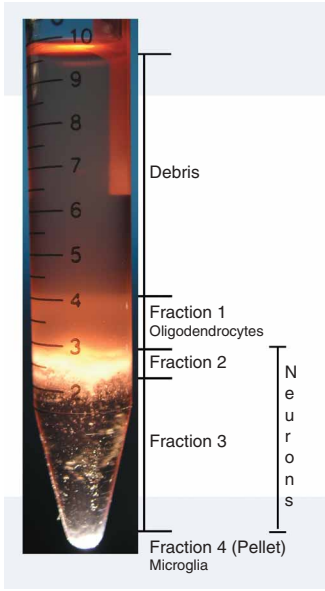

Figure 2 | OptiPrep gradient separation of cortical neurons pooled from the brains of two 6-month-old C57/BL6 mice. Fractions are numbered as described in Step 26. Note the prominent dense band at fraction 2 that contains debris and neurons.

30| Aspirate the supernatant and discard. Loosen the pellet of cells by flicking the tube and re-suspend the cells in 3 ml 22 °C NeurobasalA/B27 with L-GIn or Glutamax and growth factors and gentamycin. For cells derived from the cortex, re-suspend in 8 ml NeurobasalA/B27 medium containing L-GIn or Glutamax and growth factors and gentamycin. After the aliquot in Step 31, securely cap this container and store at 22 °C until Step 32A(ii).

31| Aliquot 10 µl of cell suspension into a small tube containing 10 µl trypan blue. Mix and apply approximately 10 µl to each side of a hemacytometer. Observe under ×10 phase contrast microscopy. Count phase bright spherical cells in the large center square on both sides of the hemacytometer. Add these two numbers. This sum times 10^4 is the cell concentration (cells per milliliter). If fraction 2 was collected, the background of the cell suspension still contains large amounts of debris, making counting of the phase bright spherical cells difficult. This debris will be reduced in Step 32A(ii). Yield for hippocampi from one rat is approximately 1.8 million cells, more than 60% of which survive and regenerate as neurons in culture. Typical cell yields are listed in **Table 2**. Plated cells may be immunostained for neurons, astroglia, oligodendrocytes and microglia[4].

? TROUBLESHOOTING

Cell plating

32| Plate and culture cells. Follow option A for neurons or other differentiated cells and option B for neurospheres.

(A) For neurons or other differentiated cells

(i) Plate cells at the desired concentration (i.e., 90–640 cells per mm^2, most commonly 320) in 50–150 µl NeurobasalA/B27 using a 200-µl or 1-ml pipette tip onto sterile, coated glass coverslips from Step 1.
▲ **CRITICAL STEP** After every 3 min of loading slips, cover and place slips in the CO_2 incubator to avoid toxic elevation of pH in ambient CO_2. To avoid spilling the cell suspension over the edge of a slip, do not let one slip touch another.

(ii) To remove excess debris, 1 h after plating and incubation, aspirate medium and immediately but gently add 1 ml 37 °C HABG to the 35-mm dish with four plated slips. Aspirate this rinse and immediately add a second 1-ml HABG. Repeat aspiration and rinse one more time (total = three times). Leave the last rinse on to keep the slips covered.

TABLE 2 | Yield of dissected tissue, isolated cells and plated cells [mean + S.E. (*n*)].

Tissue source from one brain		Weight (mg)	Isolated cells × 10^6	% Live neurons plated
Rat[a]	9-month hippocampus	86 + 11 (10)	1.89 + 0.18 (10)	94 (at 13 d)
	24-month hippocampus	85 + 13 (10)	1.93 + 0.15 (10)	73 (at 13 d)
Rat	9-month cortex	794 + 41 (10)	11.1 + 2.3 (10)	
	24-month cortex	793 + 59 (10)	10.5 + 2.4 (10)	
Mouse[b]	6-month hippocampus	22 + 7 (4)	1.75 + 0.25 (4)	
Mouse	6-month cortex	202 (2)	6.90 (2)	

[a]Fisher 344 rat. [b]C57/BL6 mouse.

(iii) Immediately after Step 32A(ii), with sterile forceps, transfer each slip to a well in a 24-well plate containing 0.4 ml NeurobasalA, B27, Glutamax and appropriate growth factors at 37 °C and equilibrated with 5% CO_2. Return culture to the incubator. Although three or four 12-mm diameter slips can be cultured in a 35-mm dish, we usually transfer slips to a 24-well plate to facilitate handling of multiple time points or concentrations. Also, individual cultures in a 24-well plate are less likely to spread contamination if one slip carries an infectious agent.

(iv) When culturing for the next 5–60 d, refresh nourishment and remove waste by removing one half of the medium on day 4 or 5 and replacing it with an equal volume of medium now containing the original growth factor concentration(s). Repeat this medium change every 7 d. **Figure 3** shows typical mouse hippocampal neurons after 9 d in culture. Different cell types can be identified by specific immunostains[7]. Typical yields after 8–12 d are 80% neurons, 10% oligodendrocytes, 5% astroglia and 5% microglia[6]. There is more debris at the start that is consumed by the microglia. After 1 week, very little debris remains (**Figure 3**).

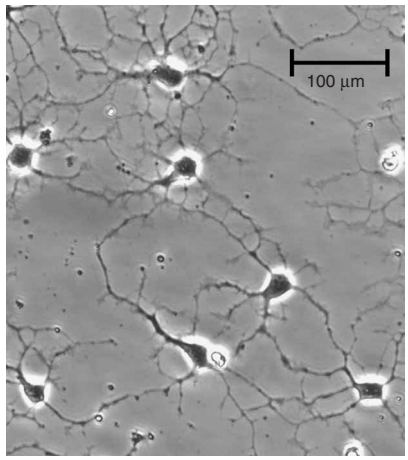

Figure 3 | Hippocampal neurons in culture after isolation from a 6-month-old C57/BL6 mouse. Cells were plated at 32,000 cells per cm^2 and regenerated for 9 d in culture.

? TROUBLESHOOTING

(B) For neurospheres

(i) For propagation, dilute cell suspension to 150,000 cells per ml Neurobasal, 2% B27 minus retinyl acetate, 0.5 mM Glutamax, 10 ng ml^{-1} FGF2 (optional 10 ng ml^{-1} epidermal growth factor; mouse neurospheres need 10 ng ml^{-1} PDGFbb also). Plate 0.2 ml cm^{-2} ultralow adhesion substrate (3,000 cells per mm^2). For isolated clones of neurospheres, dilute cell suspension to 250 cells per ml of the same medium and similarly plate 0.2 ml cm^{-2}.

▲ **CRITICAL STEP** You can grow neurospheres on regular tissue culture polystyrene, but considerable differentiation into neurons and astrocytes will occur. Do not plate onto substrates coated with poly-Lys or laminin as this will result in firm adhesion, differentiation and lack of neurospheres. B27 without retinyl acetate retards differentiation.

(ii) Culture at 37 °C, in a 5% CO_2, 9% O_2 gas, humidified incubator. **Figure 4** shows the appearance of several neurospheres grown from mouse cortex after 7 d in culture. After 7 d, neurospheres can be collected for further proliferation or differentiation as follows. First disperse loosely adherent neurospheres in each well using a 1-ml pipette.

(iii) Collect media with floating cells into a 15-ml centrifuge tube and rinse well with 0.2-ml warm HA-Ca.

(iv) Collect cells by centrifugation for 1 min at 22 °C at 200*g* (1,100 r.p.m. in a swinging bucket centrifuge).

(v) Discard supernatant and flick the tube to disperse the pellet of cells.

(vi) Re-suspend the cells in 0.2 ml Hibernate-Ca + papain.

(vii) Return re-suspended cells plus papain to the original culture well.

(viii) Incubate at 37 °C, ambient CO_2 (not 5%), for 10 min.

(ix) Disperse cells by two to three cycles of gentle trituration using a fire-polished Pasteur pipette.

(x) Aliquot 40 μl using a 1-ml pipette and mix with 40 μl trypan blue.

▲ **CRITICAL STEP** Using a narrow-tip pipette will exclude clusters and result in undercounting total cells.

(xi) Load hemacytometer with 1-ml pipette and observe clusters. If more than 10% of cells are in clusters return to Step 32B(ix). Count phase bright spherical cells as in Step 31.

(xii) Remove papain by centrifugation and re-suspend cells at desired concentration in original medium + growth factors. To passage cells, re-plate at 3,000 cells per mm^2 uncoated plastic substrate. To differentiate cells, plate onto glass substrate coated with 100 μg ml^{-1} poly-D-Lys. For neurons, plate in NeurobasalA, B27, 0.5 mM Glutamax at 40–400 cells mm^{-2} substrate. For astrocytes, plate in NeurobasalA, 10% horse serum, 2 mM GIn at 100–300 cells mm^{-2}.

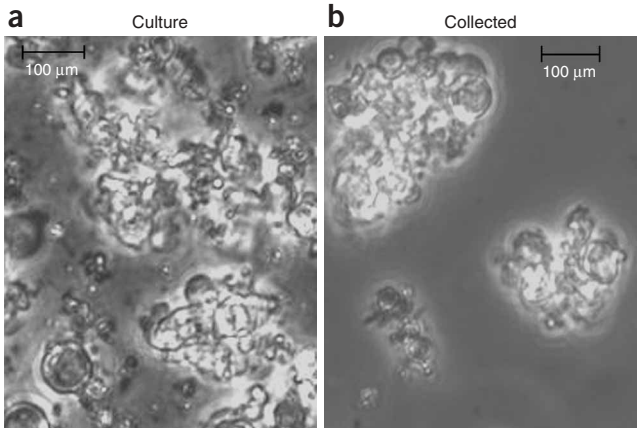

Figure 4 | Cortical neurospheres from a 6-month-old C57/BL6 mouse. Cells were plated at 300,000 cells per cm^2 and cultured for 7 d. (**a**) Undisturbed culture; (**b**) After collection and replating. Scale bar = 100 μm.

● TIMING

The entire procedure from animal anesthesia to plating can be performed in 4 h. We typically perform isolation and plating from two structures or two ages of animals in 8 h.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Solution
1	The cell yield is normal but the cells die after 1 d	Most problems with cell death after 24 h or failure of cells to adhere and flatten onto the substrate are a result of poor coating of the glass substrate. Commonly, unbound poly-Lys was not rinsed away or the poly-Lys was not thoroughly dissolved either from the original container or after thawing. Bubbles sticking to the side of the tube are a sign of undissolved poly-Lys. To test for a substrate problem, pre-coated substrates are available from BrainBits LLC. Primary neurons are more sensitive to this problem than other cell types. Trouble-shooting for other laboratories has revealed a problem with some lots of pre-coated substrates. Adult neurons do not grow well on plastic. Another possibility is that the plated cells dried out or were exposed to room air too long so that the plating medium becomes alkaline as CO_2 outgases from the medium
20,31	The cell yield is low	**Table 2** presents some typical yields of adult rat and mouse brain preparations of hippocampal and cortical tissue (minus hippocampus). If the cell yield is low, the most likely problem is triturating too vigorously or through a pipette with too small or too sharp an opening (see REAGENTS and Step 20)
32	The cell yield is normal; cells are alive (phase bright), but cells have not sprouted processes after 4 d	If the cells are phase bright or dark spheres less than 5 μm diameter, they are dead or dying. Be sure that cells were not exposed to air in Step 32(iv) for more than a few seconds and never dried out. Check the incubator temperature, humidity and gas concentrations. If they are larger phase bright spheres that have not sprouted processes after 4 d, then either the substrate is bad (Step 1; the most common problem) or the medium, especially growth factors, is bad or outdated
32	After 4–5 d, the cells began to regenerate processes, but now appear to be overwhelmed by contamination	Likely contaminants include bacteria and fungi. The fungal cells can be yeast-like (10-μm diameter, with smaller budding structures). Yeast usually enter cultures from human breath or coughing. More common are filamentous fungi that form macroscopic mats of black or blue-green hyphae. Fungal spores are ubiquitous in lab air, but should be removed by the HEPA filter of the sterile hood and reduced by a HEPA filter in the incubator. If macroscopic evidence of fungal growth is seen inside the incubator, for example growing on a drop of spilled medium or in the water tray, the entire incubator must be cleaned to avoid continuous infection. Bacterial contamination is quickly recognized as acidic metabolic products quickly turn the medium yellow. Bacteria are small rods or spheres of approximately 1-μm diameter. With appropriate controls of medium only or substrate plus medium without cells, contam-ination can be traced to the medium or poly-Lys. We do not recommend use of anti-fungals such as Fungizone or other antibacterial agents because of demonstrated effects on neuron physiology. It is better to identify the source of contamination and develop better sterile technique than to develop anti-microbial resistance

ANTICIPATED RESULTS

This protocol has been used in a number of studies by the authors to compare properties of neurons isolated from old rat brains, near their median lifespan (24-month-old Fisher rats), with middle-age and embryonic neurons. We find that old neurons can be isolated with the same yield as middle-age neurons, but the old neurons are more susceptible to stressors such as glutamate and A-beta[5], with enhanced caspase activation and apoptosis[37]. The adult neurons are active electrophysiologically[38]. The interplay of inflammatory responses to tumor necrosis factor-α (TNFα) and age in isolated microglia and neurons contributes to age-related susceptibility to A-beta[17]. Old neurons also fail to upregulate glucose uptake in response to glutamate and A-beta[39]. Despite normal appearances, the old neurons display dysregulated calcium dynamics in response to a pulse of glutamate, which can be restored by pre-treatment with estrogen[6]. We have recently focused on fluorescent measures of mitochondrial function *in situ* in neurons[8], which shows that mitochondria in old neurons have a partially depolarized resting mitochondrial membrane potential and a higher rate of generation of reactive oxygen species that predispose them to death by glutamate toxicity.

Most recently, we have been comparing neurons from wild-type and transgenic mice (La Ferla's 3xTg[40]) to determine the relative effect of mouse aging on the observed cognitive decline with neurons under identical culture conditions, uncomplicated by aging blood vessels and hormone levels.

By clonal analysis of brain neuroprogenitors, these multipotent stem cells were shown to differentiate into neurons, astrocytes and oligodendrocytes[41]. The ability of all regions of the CNS to form neurospheres in the absence of substrate adhesion and their regionally different growth factor requirements are well documented[42,43]. Certain other potent developmental factors such as retinoic acid also promote differentiation[44–46]. Together, these results suggest epigenetic regulation of development, but also the reversal, de-differentiation and/or interconversion of progenitors, neurons and astrocytes, depending on growth factors, morphogens and adhesion[47].

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