Video Article

Rapid Genotyping of Animals Followed by Establishing Primary Cultures of Brain Neurons

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Abstract

High-resolution analysis of the morphology and function of mammalian neurons often requires the genotyping of individual animals followed by the analysis of primary cultures of neurons. We describe a set of procedures for: labeling newborn mice to be genotyped, rapid genotyping, and establishing low-density cultures of brain neurons from these mice. Individual mice are labeled by tattooing, which allows for long-term identification lasting into adulthood. Genotyping by the described protocol is fast and efficient, and allows for automated extraction of nucleic acid with good reliability. This is useful under circumstances where sufficient time for conventional genotyping is not available, *e.g.,* in mice that suffer from neonatal lethality. Primary neuronal cultures are generated at low density, which enables imaging experiments at high spatial resolution. This culture method requires the preparation of glial feeder layers prior to neuronal plating. The protocol is applied in its entirety to a mouse model of the movement disorder DYT1 dystonia (ΔE-torsinA knock-in mice), and neuronal cultures are prepared from the hippocampus, cerebral cortex and striatum of these mice. This protocol can be applied to mice with other genetic mutations, as well as to animals of other species. Furthermore, individual components of the protocol can be used for isolated sub-projects. Thus this protocol will have wide applications, not only in neuroscience but also in other fields of biological and medical sciences.

Video Link

The video component of this article can be found at http://www.jove.com/video/51879/

Introduction

Rodent models of genetic diseases have proven useful in establishing the physiological functions of normal proteins and nucleic acids, as well as the pathophysiological consequences of defects in these. Examples include mice deficient for proteins involved in key cellular functions, as well as mouse models of disorders such as Alzheimer's disease. However, certain genetic manipulations can lead to neonatal lethality shortly or a few days after birth. In these cases, primary cell cultures are an important tool because live cells can be obtained from the embryonic or neonatal pups before death, they can be maintained for at least a few weeks *in vitro*, and during this time early neuronal development can be followed by biochemical, functional and morphological experiments. For the primary cultures, it can be beneficial to plate the neurons at low density; this makes it possible to visualize the individual somata, dendrites, axonal shafts and nerve terminals at high spatial resolution. However, the survival and differentiation of neurons at low density typically requires that they are plated on a glial feeder layer, co-cultured with glial cells in the absence of physical contact with them, or cultured in medium conditioned by glia [1].

The establishment of low-density neuronal cultures on glial feeder layers can be dependent on fast and reliable genotyping beforehand – within a few hr in contrast to a few days. Speed is especially important when the neuronal genotype needs to be matched to that of a glial feeder layer prepared beforehand. As a more practical example, it may be necessary to decide which pups of which genotype to use in generating cultures, to optimize the efficiency of an experiment.

Here we demonstrate the working protocol that has been used for fast, simplified and reliable mouse genotyping in previous publications [2-6]. Mouse tails and a commercially available kit are used. This protocol includes single-step extraction of nucleic acids from the tissue, and requires neither a nucleic-acid purification step nor use of a termination buffer ('stop solution'). The reliability of this genotyping method is illustrated by presenting the results of a series of tests when differences are introduced with respect to the starting amount of the specimens, the age of the animals and the length of the PCR amplicons. This kit offers the advantages of automated extraction and reliability.

For the sake of being comprehensive, the use of tattooing for long-term identification of the genotyped mice is also demonstrated. Tattooing is achieved by applying tattooing ink to the dermis of the skin (under the epidermis) [7]. A procedure is described for tattooing the paw pads of newborn or 1 day old mice, although tattoos can be applied to other parts of the body, such as tails and toes, and to animals of all ages. In

addition, procedures will be demonstrated for plating and culturing mouse neurons at a low density, based on optimized preparation of different types of glial feeder layers [2,8].

We use a genetic mouse model of the inherited neurological disorder DYT1 dystonia – an autosomal-dominant movement disorder caused by a mutation in the gene *TOR1A* (c.904_906delGAG/c.907_909delGAG; p.Glu302del/p.Glu303del) [9]. The encoded protein, torsinA, belongs to the "ATPases associated with diverse cellular activities" (AAA+) family of proteins, whose members generally perform chaperone-like functions, assisting in: protein unfolding, protein-complex disassembly, membrane trafficking, and vesicle fusion [10-13]. The mutation results in an in-frame deletion of a codon for glutamic acid, and can lead to manifestation of 'early-onset generalized isolated dystonia' [14,15]. However, the pathophysiological mechanisms responsible for this disorder remain poorly understood. In a knock-in mouse model, the mutant allele is *Tor1a*^{tm2Wtd}, mentioned hereafter as *Tor1a*$^{\Delta E}$. Heterozygous ΔE-torsinA knock-in mice are viable and genetically mimic human patients with DYT1 dystonia, whereas homozygous knock-in mice die after birth [16,17], with the latency to postnatal death affected by genetic background [18]. The early death of homozygous knock-in mice necessitates that both the genotyping of animals and the establishment of neuronal cultures are completed rapidly. As another example of genotyping, *Tfap2a* (transcription factor AP-2α, activating enhancer binding protein 2α) will be used. The protein encoded by this gene is important in regulating multiple cellular processes, such as proliferation, differentiation, survival and apoptosis [19].

Protocol

NOTE: All animal procedures performed in this study were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

1. Long-term Identification of Mice Using Tattooing the Paw Pads

1. Immobilize a paw with the paw pad (plantar surface) facing the experimenter. Hold the paw with the thumb and the index finger. Be careful not to pinch the paw.
   NOTE: Stable immobilization is important to ensure that the tattoo pigment is placed into the dermis of the paw pad, and thus is permanent [7].
2. Swab the paw pad with 70% ethanol on a gauze sponge or swab.
3. Apply skin oil to a cotton-tipped applicator, and gently press the tip against the surface of the paw pad several times. Use only a small amount of skin oil; when present in large amounts, it will prevent the tattoo pigment from reaching the skin.
4. Dip the tips of the tattoo needles into the tattooing ink just prior to tattooing. Use clean, aseptic and sharp tattooing needles, to decrease the pain and the possibility of infection, and also to increase the tattooing efficiency.
5. While the paw pad surface is covered with the skin oil, press the tattoo needle tips vertically and lightly against the skin in the center of the paw pad, and inject the ink multiple times. See Table of Materials/Equipment for information about electric tattooing system.
6. Spray 70% ethanol on a gauze sponge, gently press it against the paw to remove extra tattoo pigment on the skin surface, and inspect the quality of tattoo. Check that the middle of paw pad has a dark, round spot that differs from normal skin pigmentation. If the tattoo is not dark or large enough for easy viewing, repeat the prior tattooing steps.

2. Genotyping Newborn Mice Using a Fast PCR Genotyping Kit

1. Disinfect the distal end of a mouse tail with 70% ethanol, cut 5 mm or less of the tail tip and transfer it to a tube of an 8-tube strip of the type used for PCR. Use an un-used razor blade for each pup to avoid cross contamination between specimens. Alternatively, use a pair of scissors, but in this case, carefully and thoroughly remove the remaining tissue on the blades using 70% ethanol. Check for bleeding. If bleeding occurs, apply pressure to the cut portion of the tail with a gauze sponge until bleeding has stopped.
2. Add 200 µl of DNA Extraction Solution to each PCR tube containing a specimen. See Table of Materials/Equipment for its composition and information about the kit.
3. Place the tube strip into a PCR thermal cycler, and start the DNA extraction using the following program: 1 cycle at 55 °C for 10 min, 1 cycle at 95 °C for 10 min, and holding at 4 °C.
   NOTE: This is the same PCR thermal cycler that is later used for PCR.
4. After the DNA extraction is finished, remove the tube strip from the thermal cycler and invert 5 times.
5. Transfer 4 µl of the solution (DNA extract) of each specimen to an un-used tube of an 8-tube strip, and mix with: 10 µl of 2X PCR Ready Mix II, 2 µl of mixed forward & reverse primers (recommended at 0.5 µM; see **Table of Materials/Equipment** for sequences), and 4 µl of nuclease-free H_2O, for each specimen. Spin briefly (*e.g.,* 3 sec) using a table-top centrifuge. Keep the tubes on ice at all times except when handling.
6. Perform thermal cycling. See Table of Materials/Equipment for the thermal program.
7. Detect the amplified DNA products. Load the total reaction solution from the PCR (20 µl) directly into a well of an agarose gel. Load the molecular weight markers into a separate well. Apply an electric field.
8. Acquire fluorescence images of the bands under ultraviolet light.

3. Primary Culture of Mouse Brain Neurons on Glial Feeder Layer

NOTE: The procedures for brain dissection and cell dissociation (3.1) are common to all the subsequent procedures. The procedures for mouse glial cultures (3.2), rat glial cultures (3.3), and mouse neuronal cultures (3.4) are described separately afterwards.

1. Brain Dissection and Cellular Dissocaiation
   1. Sacrifice one mouse or rat pup by decapitation, rapidly remove the brain, and place it into Hanks' solution (see **Table 1** for composition) + 20% fetal bovine serum (FBS) in a 35 mm dish (kept on ice).

2. Cut the brain through the midline into two hemispheres. Remove the region of interest(*e.g.,* cerebral cortex, striatum and hippocampus) from each hemisphere of the brain. Remove the meninges and major blood vessels from the surface. Cut the brain region into 4-10 thin slices using a surgical blade.
3. Rinse the brain slices in a 15 ml centrifugation tube, once with Hanks' solution + 20% FBS, then 3 times with the Hanks' solution (*i.e.*, without serum) (all at 4 °C). To rinse, add 5-10 ml solution, let the brain slices settle at the bottom of the tube, aspirate the solution from the top, and add a fresh solution. Finish the rinsing procedure by aspirating the solution.
4. Filter 2 ml of the trypsin-containing digestion solution (see **Table 1** for composition) using a 3 ml syringe and a 0.2 µm syringe filter, and add the filtrate directly into the tube containing the brain slices. Let the trypsinization proceed for 13 min at RT.
5. Neutralize the trypsin solution first by aspirating most of it and then by adding 7-10 ml of Hanks' solution + 20% FBS (4 °C).
6. Rinse the brain slices, twice with Hanks' solution + 20% FBS, and then three times with the Hanks' solution (*i.e.* without serum) (all at 4 °C). Finish the rinsing procedure by aspirating the solution.
7. Filter 2 ml of the dissociation solution (see **Table 1** for composition) using a 3 ml syringe and a 0.2 µm syringe filter, and add the filtrate directly to the tube with the brain slices.
8. Mechanically dissociate the cells by gently triturating 10-20 times, until visible tissue pieces disappear. Use a cotton-plugged, fire-polished Pasteur pipette and avoid making bubbles during trituration.
9. Wait 3 min for small pieces to settle down.
10. Transfer the majority of the solution (~1.5 ml, leaving some solution at the bottom) to a 15 ml centrifugation tube that contains 3 ml Hanks' solution + 20% FBS solution (4 °C), using the cotton-plugged, fire-polished Pasteur pipette. Do not transfer all the solution because the inclusion of any sediment at the bottom typically results in deterioration of the culture.
11. Centrifuge for 13 min at ~185 g (~1,100 rpm) at 4 °C.
12. Aspirate the supernatant gently, add 1 ml of pre-warmed plating medium (37 °C) to the pellet, and resuspend it by gently pipetting several times using the cotton-plugged, fire-polished Pasteur pipette.
    NOTE: Three different types of plating media are used for different culture purposes: plating medium-1 for mouse glial cells, plating medium-2 for mouse neurons, and plating medium-3 for rat neurons and glial cells (see **Table 1** for compositions).
13. Take out 10 µl of the cell suspension, mix it with 10 µl of 0.4% trypan blue solution, and measure the density of live cells, using either a hemocytometer or an automated cell counter.

2. Mouse Glial Cultures
    1. Wash the coverslips to help establish healthy cultures of mouse cells.
        1. Immerse glass coverslips (round, 12 mm diameter) in 70% nitric acid in a glass Petri dish. Protect from light using aluminum foil, and place it on an orbital shaker O/N.
        2. Rinse the glass coverslips in the Petri dish at least three times with distilled water. Immerse them in distilled water. Place the dish on an orbital shaker O/N.
        3. Dry the coverslips on a Whatman 150 mm filter paper in the biological safety cabinet.
        4. Autoclave the coverslips.
        5. Place the coverslips into 24-well culture dish.

    2. Label and genotype newborn mice according to steps 1 and 2.
    3. Obtain the brain cells from the mouse pups, according to step 3.1.
        NOTE: Use of the cerebral cortex is typical for preparing the mouse glial feeder layer. However, other brain regions, such as hippocampus and striatum, will work after proper adjustment of cell density due to different numbers and yields of cells from different regions.
    4. Add ~4 ml of pre-warmed plating medium-1 to the final cell suspension (~1 ml). For pre-warming culture media, place the solution in the culture incubator (5% CO_2-95% O_2, 37 °C) O/N, to allow the temperature and pH values to stabilize.
    5. Transfer the cell suspension to an uncoated T25 culture flask, using the cotton-plugged, fire-polished Pasteur pipette. Place the flask in the culture incubator.
    6. At 1 day *in vitro* (DIV), rinse the cultured cells in the T25 flask twice with plating medium-1 (4 °C). Place the flask back into the incubator. Perform rinsing by aspirating the medium inside the flask completely with a Pasteur pipette, adding ~5 ml of fresh medium and gently tilting the flask several times in a swirling motion.
    7. At 6-9 DIV, (*i.e.,* one day before trypsinization and plating on coverslips, in steps 3.2.8-3.2.13), place 100 µl of the coating material with extracellular matrix proteins (see Table of Materials/Equipment for comments about the coating material) on the glass coverslips in a culture dish, and place the culture dish in the culture incubator.
    8. At 7-10 DIV, when the cells are 20-40% confluent (spatially continuous), trypsinize them.
        1. Rinse the flask once, by aspirating all the solution within the flask and adding ~13 ml of Hanks' solution (4 °C), gently tilt the flask several times in a swirling motion, and aspirate the solution completely.
        2. Add 40 µl of DNase solution (final concentration, 750 units/ml) to 4 ml Trypsin-EDTA solution, pass the solution through a 0.2 µm syringe filter, and add the filtrate directly to the cells in the flask.
        3. Let the trypsinization proceed for 13 min at 37 °C in the incubator.
        4. Neutralize the trypsin solution by adding 2 ml of 100% FBS (4 °C) to the flask.
        5. Transfer the trypsinized cells to a 15 ml centrifugation tube using a 5- to 10-ml pipette, add ~4 ml of Hanks' solution + 20% FBS (4 °C), centrifuge at ~185 g and 4 °C for 13 min, and aspirate the supernatant.

    9. Resuspend the pellet in 1 ml of pre-warmed plating medium-1.
    10. Measure the density of the cells according to step 3.1.13.
    11. Aspirate the coating material completely from the glass coverslips, and plate ~50 µl of the resuspended glial cells on coverslips.
        NOTE: These cells will establish the glial feeder layer.
    12. Place the culture dish with coverslips in the incubator.
    13. 20-60 min later, add 1 ml of pre-warmed plating medium-1 to each well, and place the dish back in the incubator. Note: While the plating medium is added, it will be helpful to use another pipette to press down the periphery of the coverslip (where there are no plated cells), so that the coverslip will not float in the medium.

14. At 1 DIV of the glial feeder layer (*i.e.,* on the glass coverslip), replace the medium with 1 ml of pre-warmed plating medium-1, by aspirating all of the solution in a well and then filling it with fresh medium.
15. At 2-3 DIV of the glial feeder layer when the cells are 80-100% confluent, add mitotic inhibitor (10 µl of a mixture of 5-fluoro-2'-deoxyuridine + uridine; final concentrations of 81.2 and 204.8 µM, respectively) to each well, to inhibit DNA replication and therefore to suppress glial-cell proliferation.
16. At 7-9 DIV, when the cells are 90-100% confluent (1-2 hr before plating neurons on the glial feeder layer), replace the culture medium with pre-warmed plating medium-2 (37 °C).
    NOTE: Neurons will be plated on the same day, using step 3.4.

3. Rat Glial Cultures
    1. Coat a T25 culture flask with the same coating material.
       NOTE: This is necessary for later removal of non-adherent cells in step 3.3.5.
        1. Add 2.0 ml of the coating material to the flask, and place the flask in the culture incubator.
        2. After 2-3 hr, aspirate the coating material completely, such that the floor of the flask becomes dry.
    2. Obtain the cells from the rat pups, according to step 3.1.
       NOTE: The CA3-CA1 region of the hippocampus is used for preparing the rat glial feeder layer. However, other brain regions, such as the cerebral cortex and striatum, will work after adjusting for differences in cell density due to different numbers and yields of cells from different regions.
    3. Add ~4 ml of pre-warmed plating medium-3 to the final cell suspension (~1 ml).
    4. Transfer the cell suspension into the coated T25 culture flask, using the cotton-plugged, fire-polished Pasteur pipette. Place the flask in the culture incubator (5% CO_2-95% O_2, 37°C).
    5. At 2-3 DIV, when cells are 20-40% confluent, remove non-adherent or weakly adherent cells, by closing the lid tightly, shaking the flask vigorously ~10 times, aspirating the solution, and adding 4-5 ml of plating medium-3 (at 4 °C). Repeat this procedure once. Examine the cultured cells across the entire flask floor (*e.g.,* using phase-contrast microscope) to confirm that those that appear neuronal (*i.e.*, those for which the outer rim of the cell body appears phase-bright) are completely removed. Repeat the procedure as often as necessary to remove these cells, and then return the flask to the incubator.
       NOTE: The strength and the total number of 'shakes' required may differ among individual experimenters. The important thing is not to keep the total number of shakes to a set number, but to confirm that neuron-like cells are eliminated.
    6. At 6-8 DIV, when cells are 90-100% confluent, passage the glial cells by trypsinizing them as in step 3.2.8.
    7. After centrifugation, resuspend the pellet in 1 ml of plating medium-3 (4 °C), add 10 ml of plating medium-3 (4 °C), transfer the trypsinized cells to an un-coated T75 flask, and culture them in the incubator.
       NOTE: Rat glial cells will be passaged twice; in contrast the mouse glial cells are passaged only once because they become unhealthy after multiple passages. Rat glial cells will be passaged in T75 flasks that are not coated with any coating material. The coating allows rat glial cells to grow too quickly and yield many ciliated cells (putative ependymal cells), which will deteriorate the neuronal culture condition later.
    8. At 17-19 DIV of glial culture in a flask (*i.e.,* 11 days after passaging and one day before trypsinization and plating onto coverslips by steps 3.3.9-3.3.14), place 100 µl of the coating material on unwashed glass coverslips (round, 12 mm diameter) in a culture dish, and place the culture dish in the culture incubator.
       NOTE: For rat glial cultures, a difference was not noticed in the culture results with or without washing the coverslips.
    9. At 18-20 DIV of glial culture in a flask (*i.e.,* 12 days after passaging), prepare the glial feeder layer by trypsinizing the cultured cells, according to step 3.2.8.
    10. During centrifugation, aspirate the coating material completely from the glass coverslips.
    11. After centrifugation, resuspend the pellet in 1 ml of plating medium-3 (4 °C), and measure the cell density, according to step 3.1.13.
    12. Adjust the glial density to 10^4 live cells/ml, and plate 100 µl of the glial cell suspension on the coated glass coverslips.
        NOTE: These cells will establish the glial feeder layer.
    13. Place the culture dish with coverslips into the incubator for 20-60 min.
    14. Add 1 ml of plating medium-3 (4 °C) to each well, and place the dish back in the incubator.
    15. At 3-4 DIV of the glial feeder layer, when the cells on the coverslip are 40-80% confluent, add 1 ml of the pre-warmed growth medium (see **Table 1** for composition) that contains cytosine β-D-arabinofuranoside (AraC, final concentration of 4 µM) to stop glial-cell proliferation. Plate neurons at 7-9 DIV of the glial feeder layer when the cells are 60-80% confluent, using step 3.4.

4. Mouse Neuronal Cultures
    1. Label and genotype newborn mice according to steps 1 and 2.
    2. Use the mouse pups for step 3.1.
    3. Adjust the density of live-cell suspension to ~2.0 x 10^5 cells/ml using pre-warmed plating medium-2 for plating on mouse glial cells, or using plating medium-3 for plating on rat glial cells.
    4. Plate the cells on the glial feeder layer, by gently adding the cell suspension to the culture medium of each well. Note: The volume of the cell suspension to be added will be determined by the target number of cells in a culture well. For example, plate ~60 µl of cell suspension to achieve 12,000 cells/well.
    5. Note that no solution changes are necessary after the neurons are plated. Be careful to avoid evaporation of the solution from the wells over the course of culture, by humidifying the inside of the culture incubator.

Representative Results

As an example of the application of this protocol, representative results are shown for labeling mice by tattooing, reliable genotyping under various experimental conditions, and establishing primary neuronal cultures on glial feeder layers.

Tattooing

Newborn pups were labeled on the paw pads using a tattooing system ('Newborn' in **Figure 1**). The labels remained clearly visible at 3 weeks ('3-week-old') and 32 weeks of age ('32-week-old'). The individual mice can be uniquely identified by a combination of tattoos on the four paws (numbers 1-16) and by the information about the animal cage, or more complex numbering schemes (not shown).

Genotyping

One representative example of genotyping is shown (**Figure 2**). The *Tor1a* gene of wild-type (*Tor1a*$^{+/+}$), heterozygous (*Tor1a*$^{+/\Delta E}$) and homozygous (*Tor1a*$^{\Delta E/\Delta E}$) ΔE-torsinA knock-in mice was amplified from the genomic DNA isolated from tail clips of newborn pups. Genotyping in this specific example is based on the presence of a single 34-base-pair loxP site in the mutant *Tor1a* allele after successful Cre recombination and deletion of a neomycin resistance cassette [16,18].

Genomic DNA was extracted with minimal hands-on time, and many samples were processed simultaneously. The extraction volumes were kept constant, and therefore no adjustment of the volume was necessary to accommodate the changes in conditions tested. The reliability of genotyping was tested under four conditions, as detailed below.

First, the effect of differences in the amount of starting tissue was examined (**Figure 3**). Tails of different lengths were prepared from the heterozygous ΔE-torsinA knock-in mice (*Tor1a*$^{+/\Delta E}$) at weaning age (~3 weeks). Tail lengths of 2 to 5 mm, the range typically recommended in genotyping protocols, gave the same genotyping result of high quality. The two bands correspond to the wild-type and mutated alleles (*Tor1a*$^{+}$ and *Tor1a*$^{\Delta E}$, respectively).

Second, the effects of variability in animal age were examined (**Figure 4**). Tails were obtained from newborn, 3-week-old and ~24-week-old heterozygous ΔE-torsinA knock-in mice (*Tor1a*$^{+/\Delta E}$). Homozygotes were not used because they die several days after birth [16-18]. The two expected bands for wild-type and mutated *Tor1a* genes were visible regardless of animal age (**Figure 4A**). The general applicability of this result was tested using a second gene, *Tfap2a*[19] in wild-type mice (*Tfap2a*$^{+/+}$) (**Figure 4B**).

Third, the effects of differences in length of the PCR amplicons were tested (**Figure 5**). For this purpose, different primer pairs were synthesized for a given gene, such that the amplified DNAs are of different base pair lengths. The *Tfap2a* gene was consistently detected with different amplicon lengths.

Fourth, two DNA extraction methods were compared (**Figure 6**). In one method, PCR strip tubes and the PCR thermal cycler were used (described in step 2). This is an automated, parallel multi-tube extraction method ('Auto' in **Figure 6**). Because multiple tubes can be handled simultaneously in strip format, and a PCR machine is used with a program to operate in four temperature steps, there is no need to transfer the tubes, manually change the temperature during the extraction or use multiple pieces of equipment. Thus it is easy to process many specimens. This was compared with the second method based on manual, single-tube extraction ('Manual' in **Figure 6**). This uses individual PCR tubes and separate non-PCR machines (heat blocks and water baths) to control temperature during DNA extraction. In this case, multiple, single tubes were moved to a new temperature after each step, requiring multiple temperature-controlling apparatuses. In both cases, the *Tor1a* gene was analyzed in wild-type, heterozygous and homozygous ΔE-torsinA knock-in mice (**Figure 6A**), and the *Tfap2a* gene was analyzed in wild-type mice (**Figure 6B**). The two extraction protocols yielded equivalent genotyping results.

In summary, the results presented in **Figures 3** to **6** demonstrate that the genotyping method is robust, achieving reliable and reproducible results in spite of variations in tissue amount, animal age, amplicon length, and the extraction protocol used.

Neuronal cultures

For culturing mouse neurons on the mouse glial feeder layer, the order of the procedures is: (tattooing newborn mice → genotyping for glial culture →) glial culture → tattooing newborn mice → genotyping for neuronal culture → neuronal culture. For cultures established on the rat glial feeder layer, the procedures in parentheses are skipped.

We examined the supportive effect of the pre-seeded glial feeder layer on neuronal survival and growth. Neurons were obtained from the CA3-CA1 region of hippocampus, the motor region of cerebral cortex, and the striatum of newborn wild-type mice. They were plated at low density on rat glial feeder layer that had been obtained from the CA3-CA1 region of hippocampus and seeded prior to neuronal plating, according to the scheme illustrated in **Figure 7** (simplified summary of the procedures). Low-density cultures are optimal for the imaging of individual dendrites, somata [4,6], nerve terminals [2,3,5,8] and axonal shafts [5] at high spatial resolution. The hippocampal cells (containing both neurons and glial cells) were plated on coated glass coverslips, either with (**Figure 8A**) or without a pre-established glial feeder layer (**Figure 8B**, **C**) and observed with phase-contrast optics. In the presence of a nearly confluent glial feeder layer, the neurons (in each panel, an arrowhead indicates one representative neuron) were relatively dispersed 3 and 7 days after plating (days *in vitro*, DIV) (**Figure 8A**). They also showed signs of good health, such as a clear margin of neuronal somata, extended dendrites, a lack of clustered somata, and a lack of bundled neurites (high-magnification images in insets). At 14 DIV, these neurons formed a dense network characterized by long neurites (dendrites and axons). Note that glial proliferation was inhibited before neurons were plated, by adding growth medium containing the mitotic inhibitor AraC (step 3.3.15).

In contrast, in the absence of the glial feeder layer at the time of plating hippocampal neurons, the growth of the cultured hippocampal neurons was impaired, even in the absence of AraC. At 3 DIV, glial cells (included in the newly plated hippocampal cells) have not formed a confluent sheet (asterisk in top panel of **Figure 8B**). The cultures showed several signs that are not appropriate for high-resolution imaging studies, such as wide areas without underlying glial cells (asterisk), the presence of clustered somata and the presence of bundled neurites in some areas (data not shown). By 7 DIV, glial cells formed a confluent sheet (middle panel of **Figure 8C**). However, neurons were fewer in number than when the neurons were plated on a pre-established glial feeder layer. The surviving neurons also lacked long, network-forming neurites (inset). The glial cells were more heterogeneous in curvature and thickness than those in the feeder layer culture, thus appearing phase-bright. In order to test the effects of suppressing glial proliferation on neurons, we applied AraC-containing growth medium. When AraC was added on 3 or 7 DIV and the cells were cultured until 14 DIV and observed on this day (**Figure 8B** and **C**, bottom panels), the glial cells covered most of the coverslip surface. However, the neurons were still few in number, especially when AraC was applied at 7 DIV. The surviving neurons had only

short processes and were not extensively connected. Thus, the late addition of AraC allowed a uniform glial layer to form but did not promote neuronal viability or neurite extension; rather the uncontrolled glial growth had deleterious effects on neurons.

These data show that the glial feeder layer is critical for the survival and growth of neurons plated at low density, and that the glial layer must be present at the time of neuronal plating rather than later during neuronal culture. Similar results were obtained using cultures of cerebral cortical (**Figure 9**) and striatal neurons (**Figure 10**).

The qualitative observation about neuronal survival was confirmed by quantitative analysis. Specifically, the number of surviving neurons at 14 DIV was counted, based on phase-contrast images of the cultures (**Figure 11**). The number was greatest for neurons cultured on a glial feeder layer (*i.e.*, plated on the glial feeder layer). The number was lower in the case of neurons cultured in the absence of a glial feeder layer. The number was reduced even further when the cells were treated with AraC at a later time. These differences were statistically significant, and were noted in cultures of neurons obtained from all three brain regions.

The types of surviving cells were identified at 14 DIV in the mouse hippocampal cultures plated on the rat hippocampal feeder layer. Double-immunocytochemistry was performed using antibodies against the neuronal marker, microtubule-associated protein 2 (MAP2), and the astrocytic glial cell marker, glial fibrillary acidic protein (GFAP). The cells with extended processes were positive for MAP2, whereas the cells in the underlying glial feeder layer were positive for GFAP (two representative image fields, **Figure 12A**). This staining was not an artifact of a specific combination of primary and secondary antibodies, because the same pattern was obtained when a different set of secondary antibodies was used (**Figure 12B**).

In contrast, when the same staining was performed on the cultures consisting of only a glial feeder layer, *i.e.,* in the absence of added mouse cells (two representative image fields, **Figure 13A**), no cells were positive for MAP2. The cells of the feeder layer were consistently positive for GFAP. For a negative control, both primary antibodies were omitted from the immunocytochemical procedure (**Figure 13B**). No staining was detected in these samples, although cells were present, as evident from the transmitted light optics (differential interference contrast, DIC) and nuclear staining with Hoechst dye. Thus, non-specific staining in the MAP2 and GFAP channels was very weak.

These immunocytochemical data were also analyzed quantitatively (**Figure 14**). In each 8-bit image, intensity was measured and plotted along a line. Overlaid plots reveal MAP2 staining when mouse cells (samples containing neurons) were cultured on a glial feeder layer (Neuron +, glia +, primary antibody (1° Ab) +), whereas such staining was absent in cultures of glial feeder cells alone (Neuron −, glia +, 1° Ab +). In a negative control without primary antibodies, staining was negligible in cultures of glial feeder cells alone (Neuron −, glia +, 1° Ab −). GFAP staining was apparent in the glial feeder layer, regardless of whether mouse cells were plated (Neuron +, glia +, 1° Ab +) or not (Neuron −, glia +, 1° Ab +). Again, staining in the negative control was negligible (Neuron −, glia +, 1° Ab −). These results show that the rat glial feeder layer was composed mostly of astrocytes, and that the neurons were present only when mouse cells were added. They also indicate that the neurons present in these cultures originated solely from mouse.

The Results Section of the online video also shows the DIC and MAP2 images of cultured neurons plated on a glial feeder layer, both prepared from the CA3-CA1 hippocampal region of wild-type mice.

For detailed control of cell culture, it is recommended to measure the density of live cells, both for assessing the general quality of the brain dissection and cellular dissociation steps, and for plating the cells at the pre-determined density. Listed below are four sets of typical density measurements. Note, however, that these numbers can vary depending on culture conditions and reagents. For example, even different lots of serum from the same vendor can affect the results. Thus, these numbers should be considered a general indicator, rather than an absolute guideline.

For cellular dissociation (step 3.1.13), typical values for the live-cell density obtained from one pup are: ~2 x 10^5 cells/ml (mouse motor cortex and mouse CA3-CA1 hippocampus), ~5 x 10^5 cells/ml (mouse cerebral cortex and rat CA3-CA1 hippocampus), and ~1 x 10^6 cells/ml (mouse striatum). In all these cases, the fractions of live cells were >90% (viability, defined as the ratio of the number of live cells to that of the total number of cells). The motor cortex is loosely defined as the region of the cerebral cortex that lies immediately dorsal to the striatum [20]. For hippocampal cultures, the CA3 and CA1 regions of the hippocampus proper are preferred. The whole hippocampus additionally includes the dentate gyrus, the inclusion of which leads to formation of large nerve terminals of granule cells and introduces heterogeneity in synaptic properties [21]. The striatal culture includes the caudate-putamen and globus pallidus, but does not include the nucleus accumbens, or medial or lateral septal nuclei in the nearby structures.

For mouse glial culture (step 3.2.11), the plating density is ~10,000 glial cells on each 12-mm round coverslip, using ~50 µl of cell suspension at a density of ~2 x 10^5 cells/ml. Usually the density measured in the supernatant is relatively constant and does not require adjustment. To convert the density over different areas, the useful information is that the coverslip has a diameter of 1.20 cm and an area of 1.13 cm^2. Thus, ~10,000 cells / coverslip = ~8,800 cells / cm^2 on a coverslip.

For rat glial culture (step 3.3.12), the plating density is ~1,000 glial cells on each 12-mm round coverslip, using ~100 µl of cell suspension at a density of ~1x10^4 cells/ml. Thus, 1,000 cells/coverslip = ~900 cells/cm^2 on a coverslip.

For low-density mouse neuronal culture (step 3.4.4), the plating density ranges between 2,000 and 48,000 cells per well of a 24-well plate. Typical values when plating neurons on rat glial cells are: 10,000-24,000 cells/well for cerebral cortical neurons, 12,000-24,000 cells/well for CA3-CA1 hippocampal neurons and 24,000-48,000 cells/well for striatal neurons. When plating on mouse glial cells, the number is reduced, *e.g.*, 2,000 cells/well for CA3-CA1 hippocampal neurons. As a side note, for plating rat CA3-CA1 hippocampal neurons on a rat glial feeder layer, the plating density is 1,000-6,000 cells/well. For converting the density in a well to the density on a coverslip, the useful information is that the internal well diameter at the bottom is 1.56 cm and its area is 1.91 cm^2. Thus, for example, 12,000 cells/well = 7,100 cells / coverslip = 6,300 cells/cm^2 on a coverslip. These densities were chosen with the aim of culturing relatively sparse neurons for high-resolution cellular imaging. Researchers should choose their numbers that best suit their experimental aims.

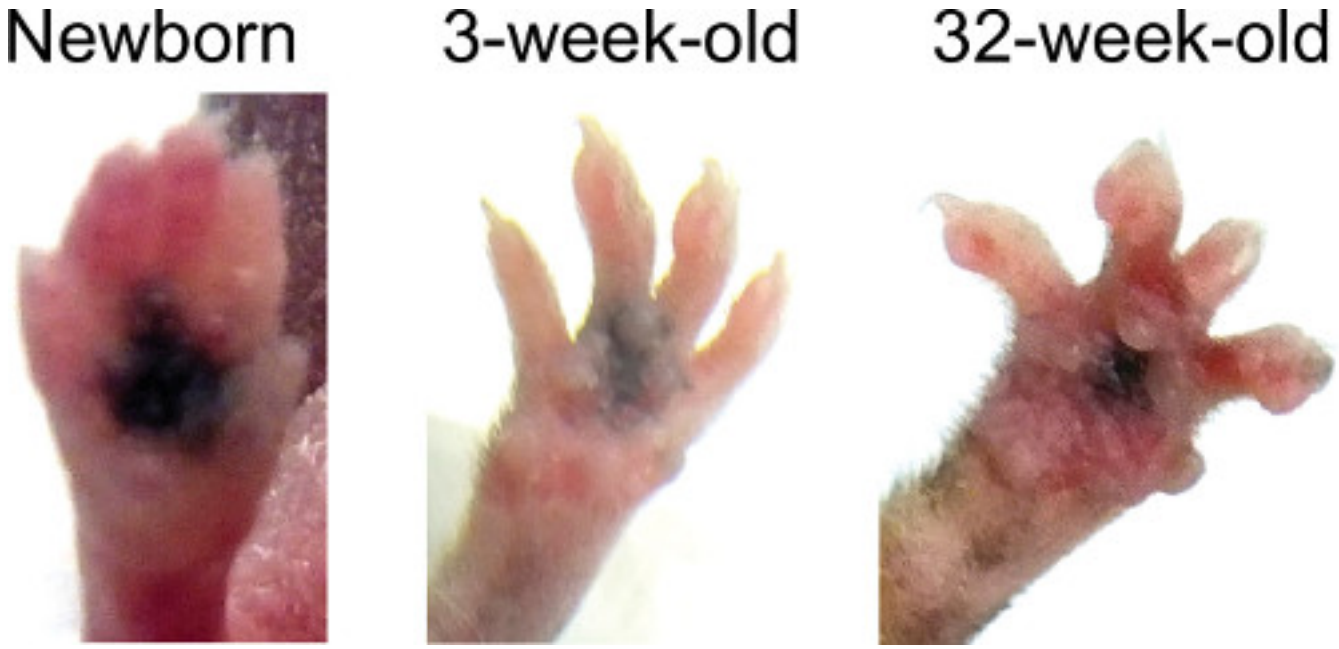

Figure 1. Tattooed paw pads of mice. Newborn mice were labeled by tattooing the paw pads. They were photographed immediately after tattooing (newborn), at 3 weeks of age (3-week-old) and at 32 weeks of age (32-week-old). The labels remained easily visible throughout adulthood. Images were taken from different animals. They are not shown at the same scale. Please click here to view a larger version of this figure.

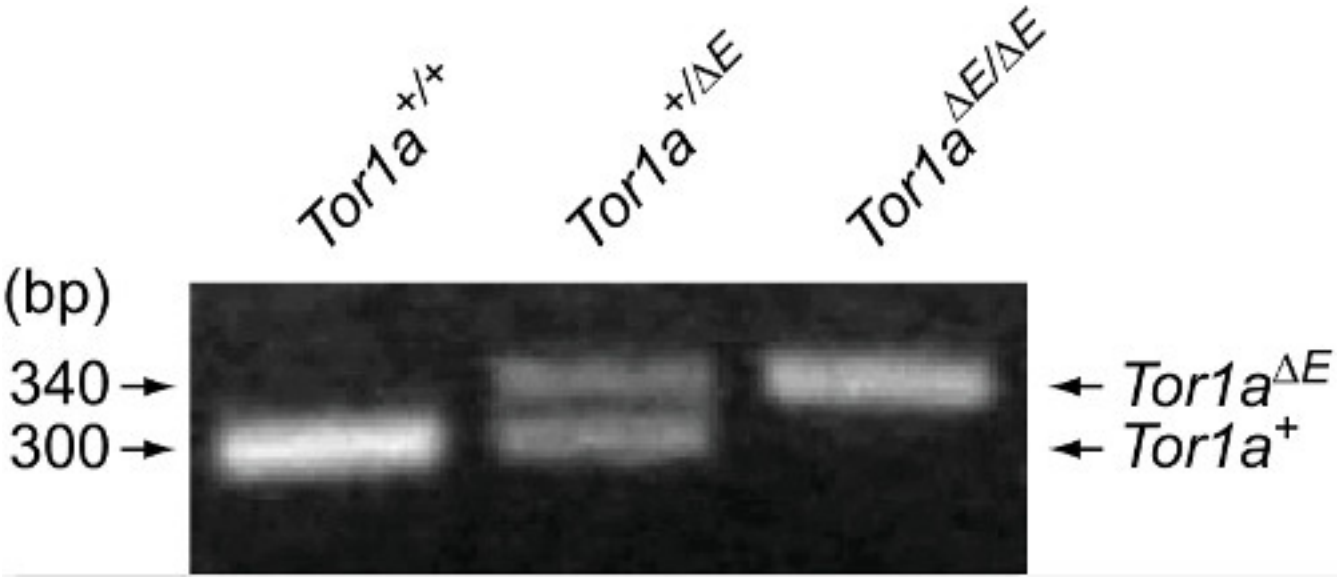

Figure 2. Genotyping of wild-type (*Tor1a*$^{+/+}$), heterozygous (*Tor1a*$^{+/\Delta E}$) and homozygous (*Tor1a*$^{\Delta E/\Delta E}$) ΔE-torsinA knock-in mice. The *Tor1a* gene alleles were analyzed in wild-type and mutant forms, using DNA extracted from the tails of newborn pups. Tail tips were ~4 mm in length. DNA was stained using SYBR Safe DNA Gel Stain. See the Table of Materials/Equipment for the information about the primers and thermal cycling program for *Tor1a* gene. Please click here to view a larger version of this figure.

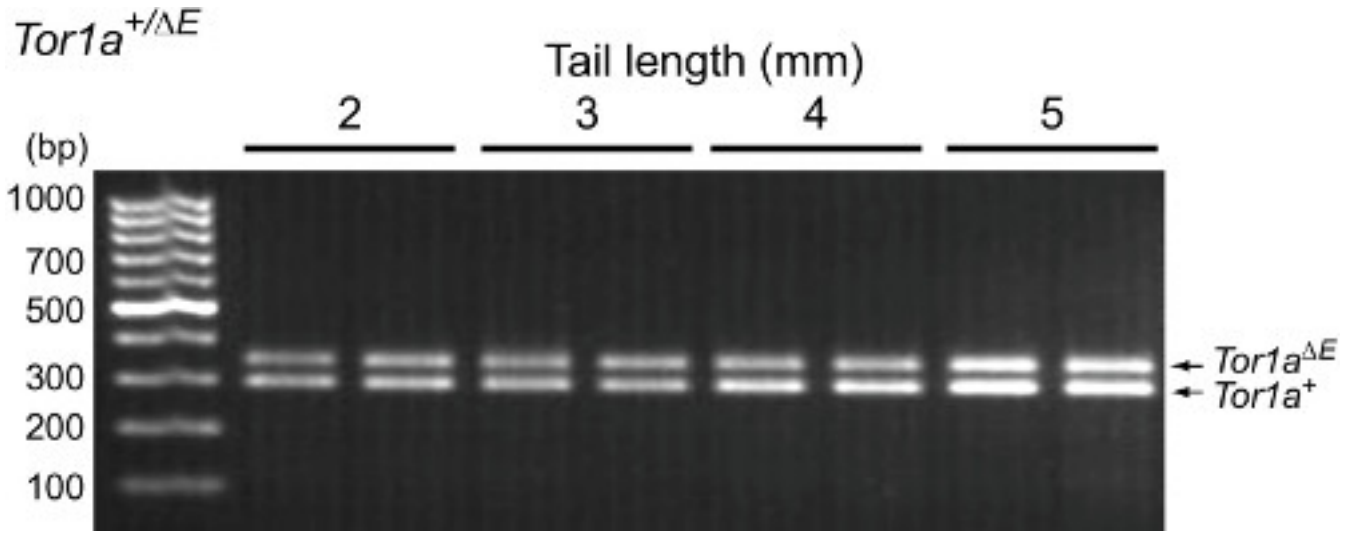

Figure 3. Detection of genomic DNA in the context of variation in the amount of starting material. Tail tips of different lengths were used. Tested animals were 3-week-old, heterozygous ΔE-torsinA knock-in mice (*Tor1a*$^{+/\Delta E}$). Lanes represent the tail lengths of 2, 3, 4 and 5 mm, in duplicate (from left). The tail tips were obtained from different mice. Molecular-weight size markers in the leftmost lane represent DNA lengths in steps of 100 base pairs. Please click here to view a larger version of this figure.

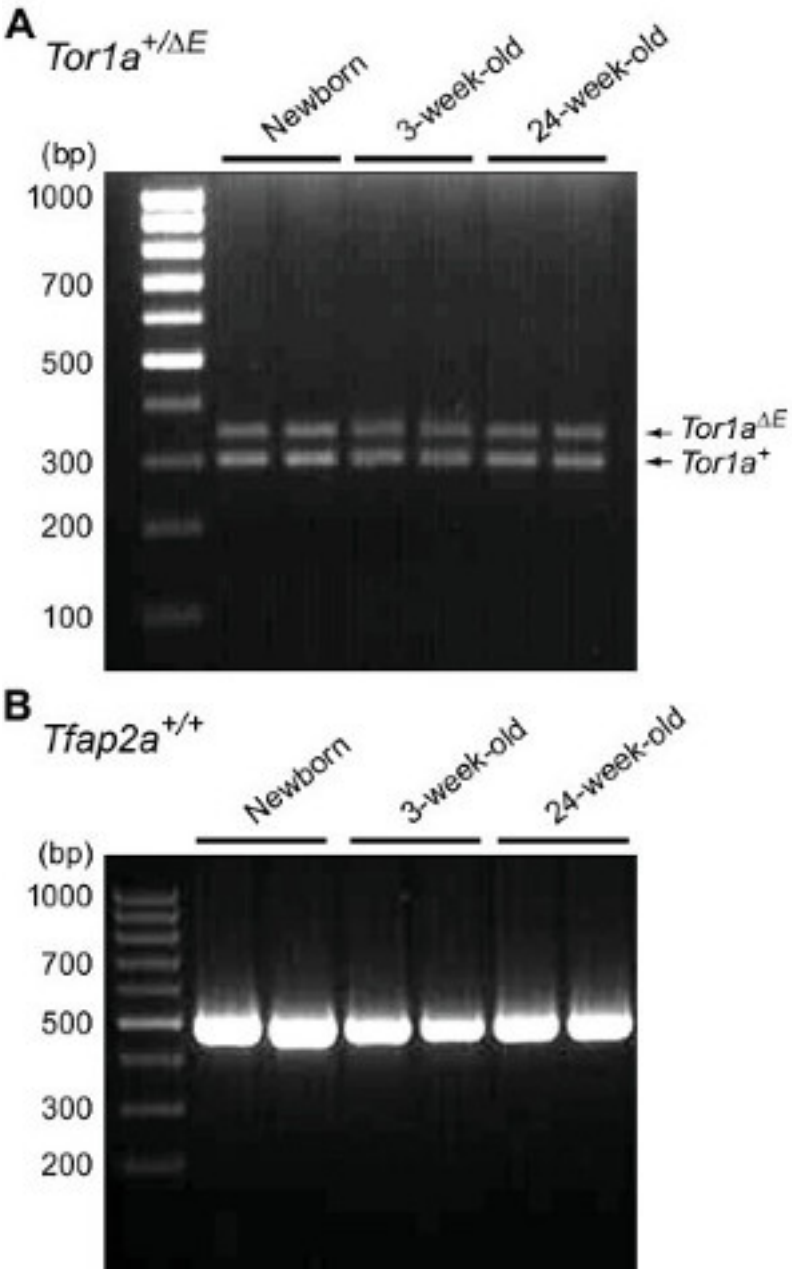

Figure 4. Detection of genomic DNA in the context of variation in animal age. (A) *Tor1a* gene. Lanes represent tail tips obtained from heterozygous ΔE-torsinA knock-in mice (*Tor1a*$^{+/\Delta E}$) at the following stages: newborn, 3-week-old, and ~24-week-old (in duplicates from left). **(B)** *Tfap2a* gene. Lanes represent tail tips obtained from wild-type (*Tfap2a*$^{+/+}$) mice at the following stages: newborn, 3-week-old, and ~24-week-old (in duplicates from left). Both genes were amplified from tails ~4 mm in length. The expected PCR fragment is 498 bp. The PCR program was the same as that used for *Tor1a* gene. Please click here to view a larger version of this figure.

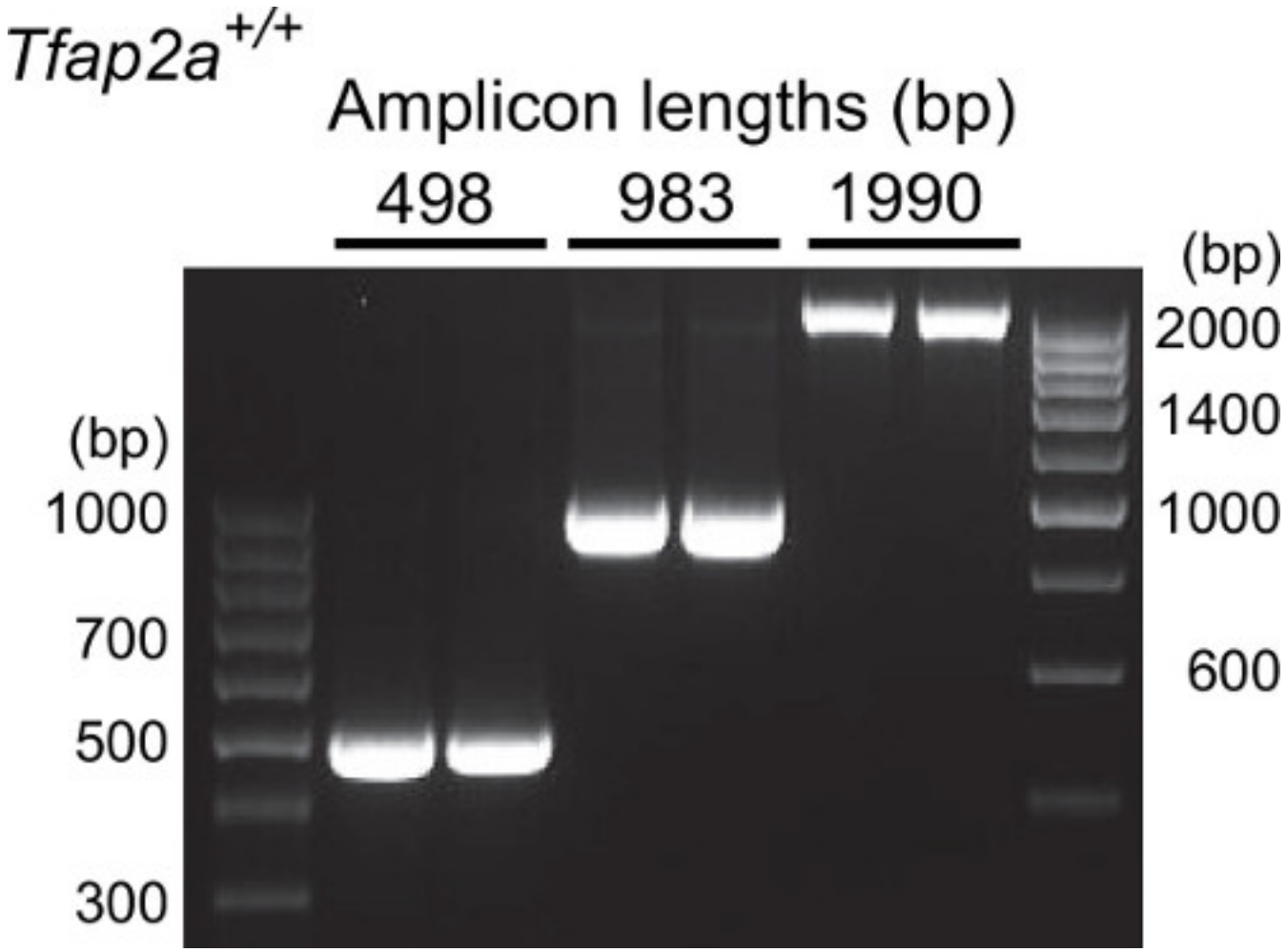

Figure 5. Detection of genomic DNA in the context of variation in length of the PCR amplicon. The *Tfap2a* gene was analyzed with PCR amplicon lengths of 498 bp (left lanes), 983 bp (middle lanes) and 1990 bp (right lanes) in duplicates. The gene was amplified from tails of 3-week-old mice. Molecular-weight size markers in the leftmost and rightmost lanes of the gel represent DNA lengths in steps of 100 and 200 base pairs, respectively. See the **Table of Materials/Equipment** for information about the primers for different amplicons. Please click here to view a larger version of this figure.

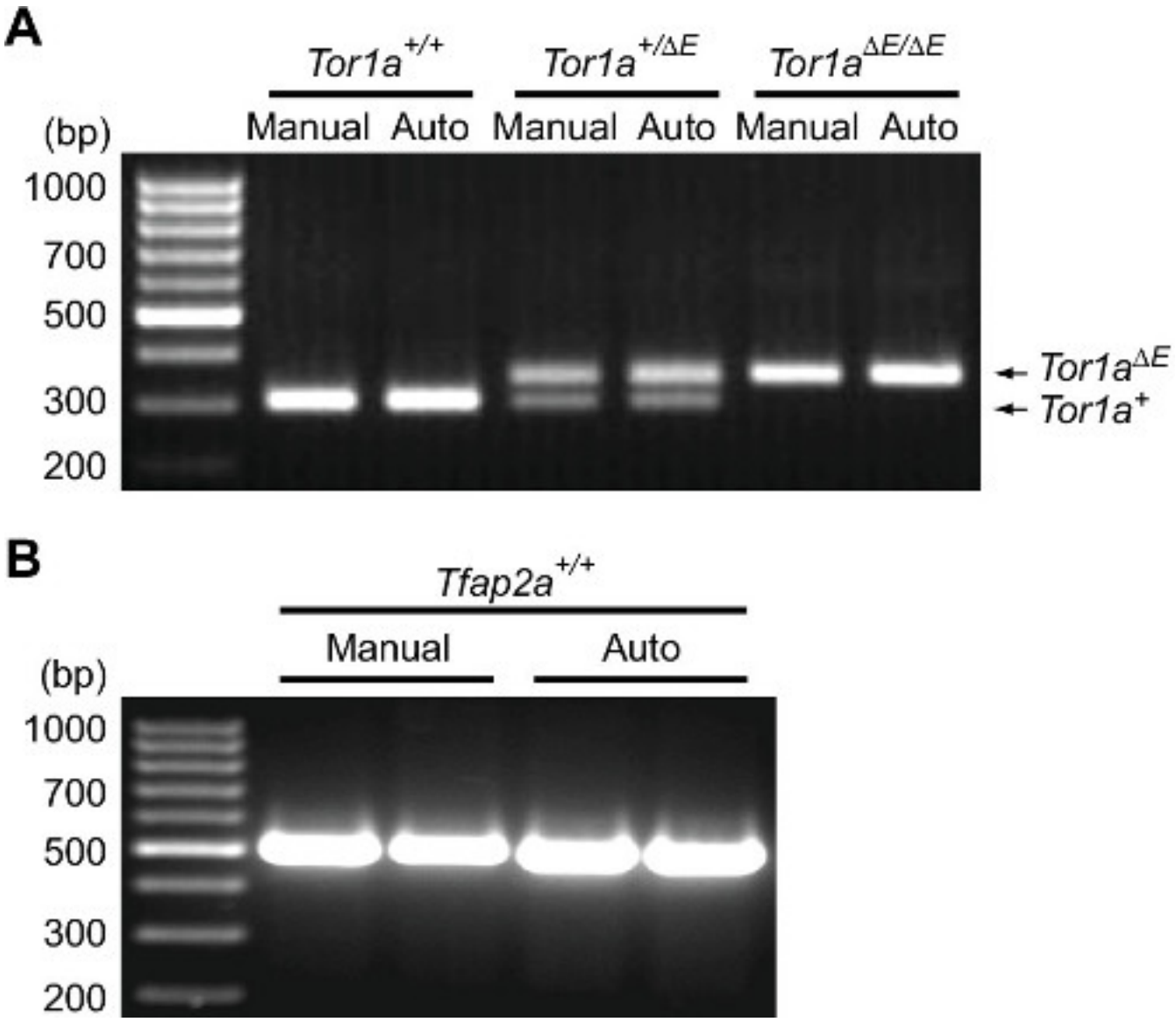

Figure 6. Detection of genomic DNA in the context of variation in DNA extraction method. Outcomes for the manual, single-tube extraction (Manual) and the automated, parallel multi-tube extraction methods (Auto) are compared. The latter method was used throughout this report. Genes were amplified from tails ~4 mm in length, collected from newborns. **(A)** *Tor1a* gene in the newborn pups of ΔE-torsinA knock-in mice. Lanes represent DNAs extracted from wild-type (manual and automated), heterozygous (manual and automated), and homozygous mice (manual and automated) (from left). **(B)** *Tfap2a* gene in 3-week-old wild-type mice. The expected PCR fragment is 498 bp. Please click here to view a larger version of this figure.

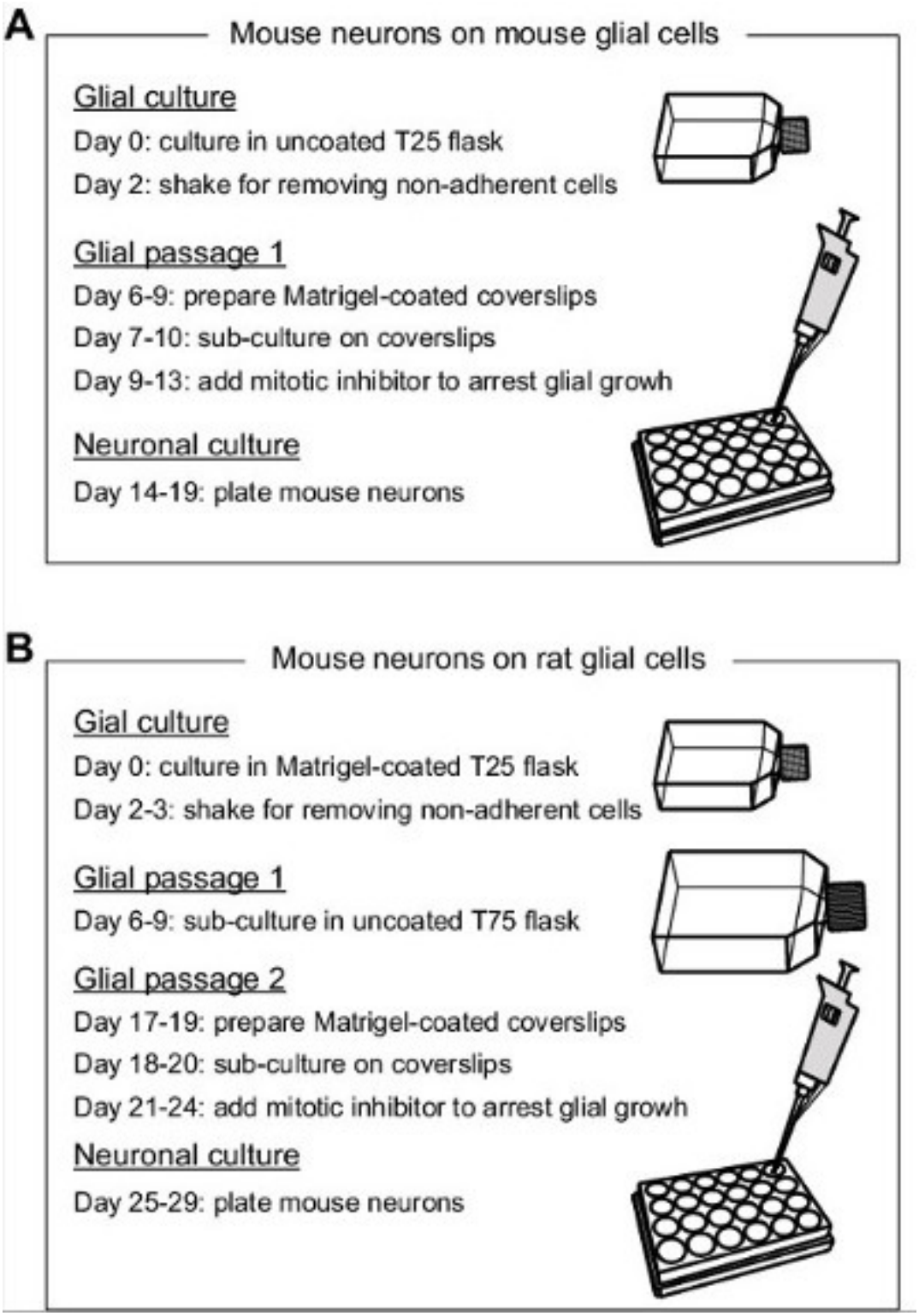

Figure 7. Simplified, schematic illustration of procedures for plating mouse neurons on mouse (A) and rat (B) glial feeder layers. The numbers of days (days *in vitro*) refer to the cumulative days after the glial cells are first plated in culture flasks. They serve only as a rough estimate. For practical details, see the Procedures section. Please click here to view a larger version of this figure.

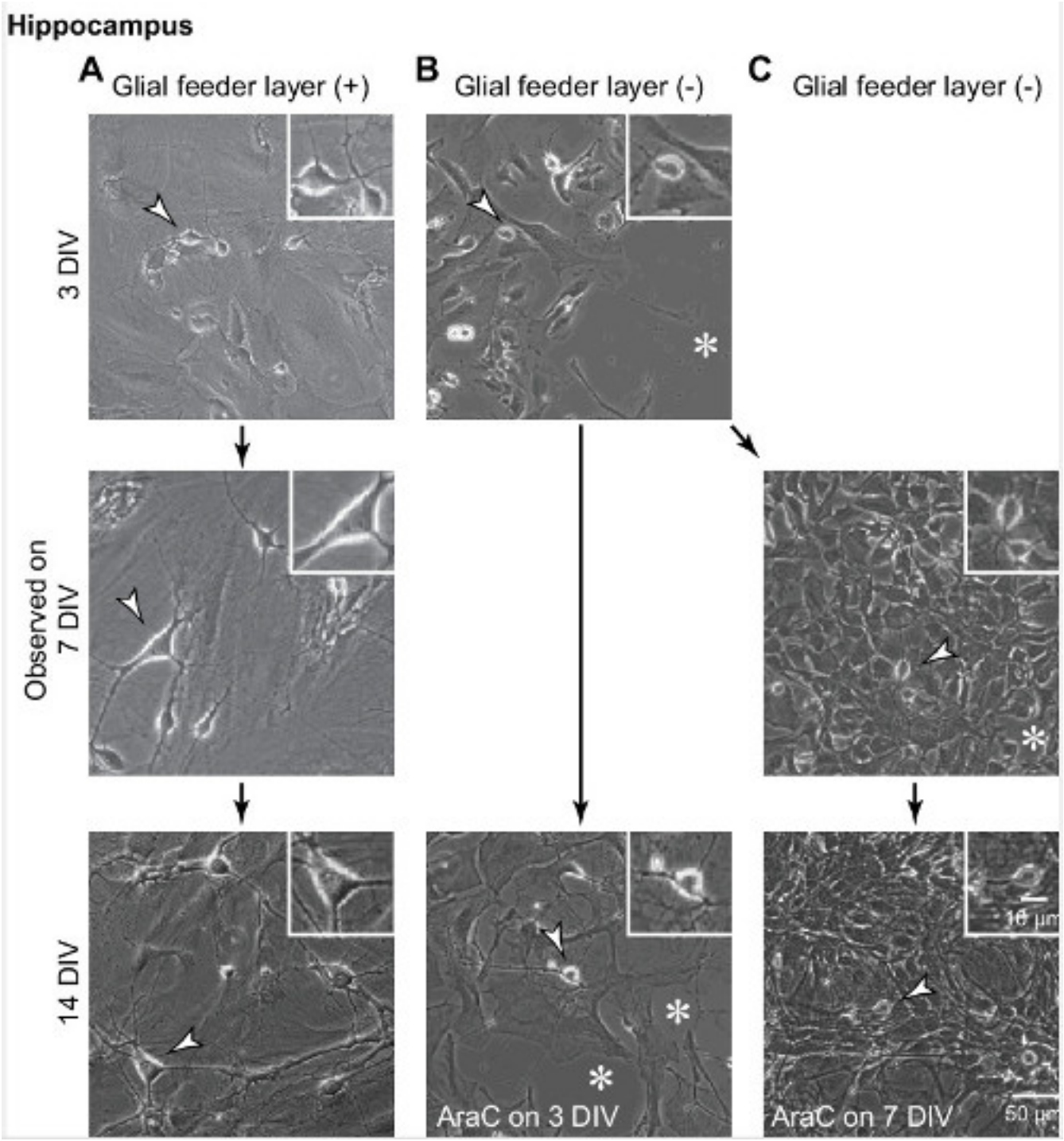

Figure 8. Supportive effect of glial feeder layer on growth of hippocampal neurons in a low-density culture. Neurons were obtained from the CA3-CA1 region of the hippocampus of newborn, wild-type mice. **(A)** Mouse hippocampal cells were plated on coated glass coverslips, which had been pre-seeded with a rat glial feeder layer obtained from the CA3-CA1 region of hippocampus. Neurons were dispersed evenly in a healthy manner. Cultures were observed at 3, 7 and 14 DIV. **(B, C)** Mouse hippocampal cells were plated on coated glass coverslips, which had no pre-established feeder layer. Cultures were observed at 3 DIV (top panel in B) and 7 DIV (middle panel in C) without AraC treatment. In other sets of cultures, the cells were treated with AraC at 3 DIV (bottom panel in B) and 7 DIV (bottom panel in C), and observed at 14 DIV. All images represent the sister cultures obtained from the same pup, whose neurons were plated on the same day. See text for details. For each panel, an example of a neuron is indicated by a white arrowhead and magnified in an inset. Neurons have cell bodies whose perimeter appears bright when viewed by phase-contrast optics, and they also have thick processes. Asterisks indicate areas without glial cells. The cultures were imaged live on an inverted microscope using phase-contrast optics with 20X objective lens (numerical aperture of 0.45) without an intermediate lens (*i.e.,* at 1x). Please click here to view a larger version of this figure.

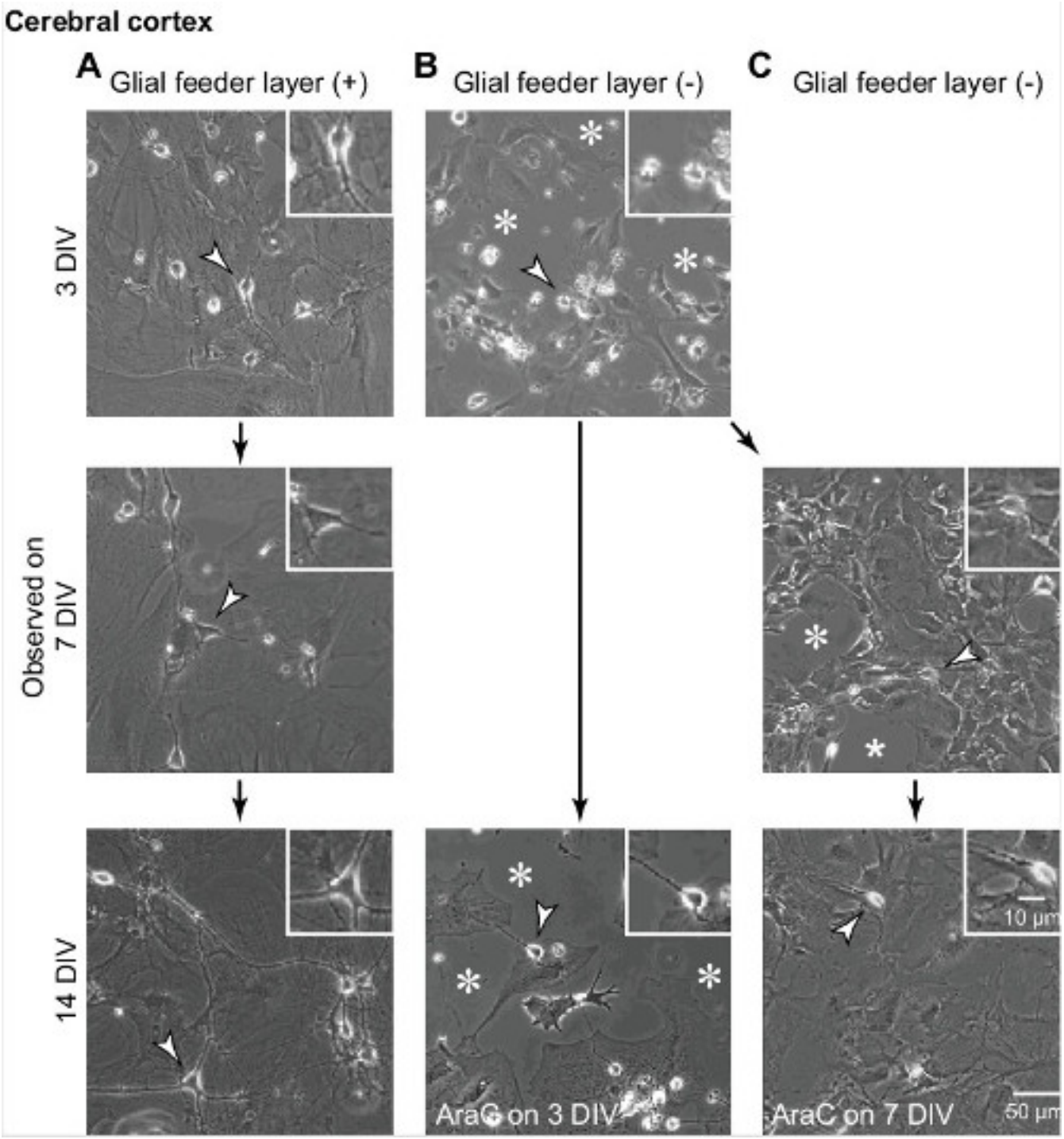

Figure 9. Supportive effect of glial feeder layer on growth of cerebral cortical neurons in a low-density culture. Similar results as in **Figure 8**, but with neurons obtained from the motor region of cerebral cortex. The conditions under labels **A-C** correspond to those in **Figure 8**. Please click here to view a larger version of this figure.

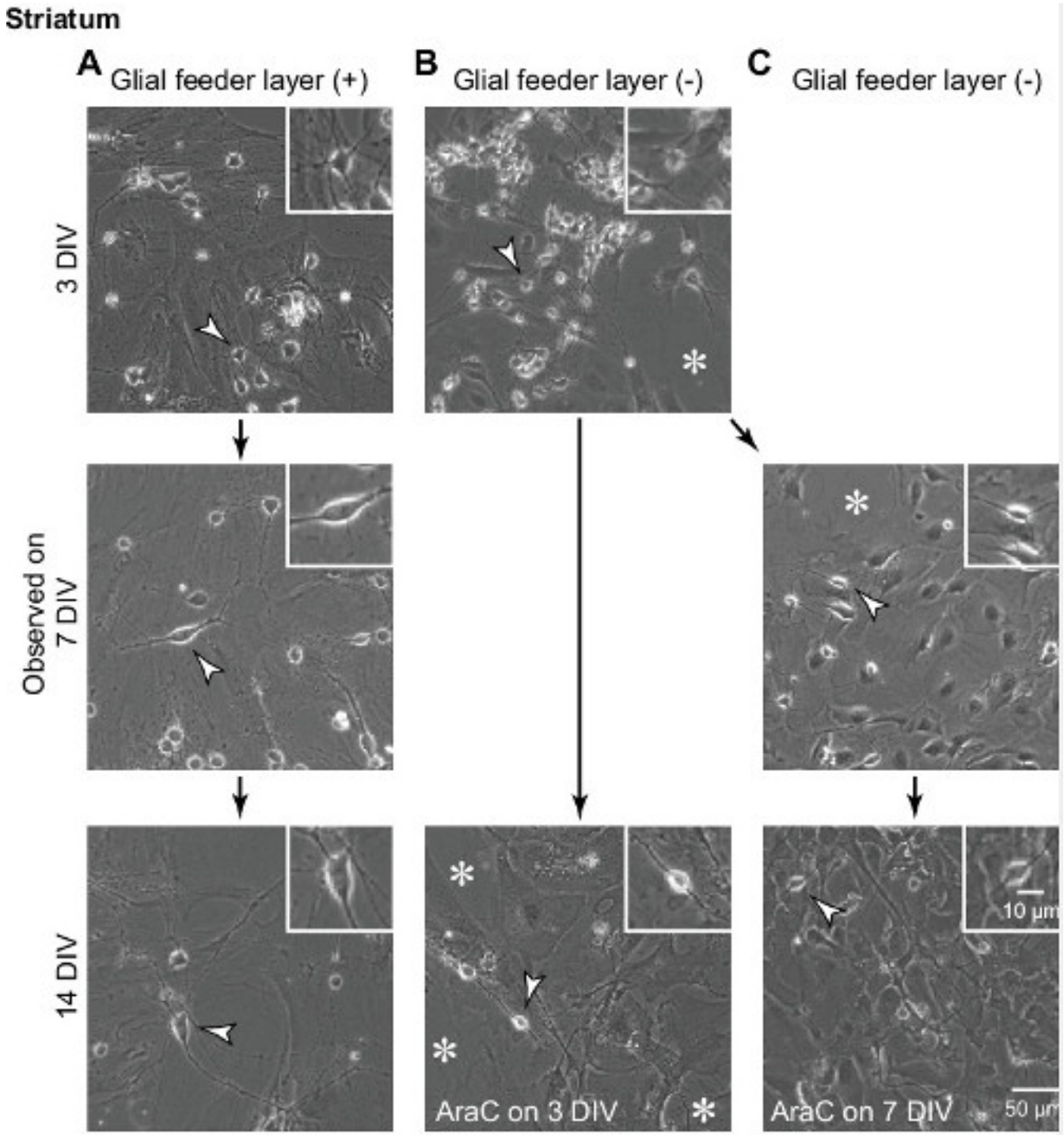

Figure 10. Supportive effect of glial feeder layer on growth of striatal neurons in a low-density culture. Similar results as in **Figures 8** and **9**, but with neurons obtained from the striatum. The conditions under labels **A-C** correspond to those in **Figure 8**. Please click here to view a larger version of this figure.

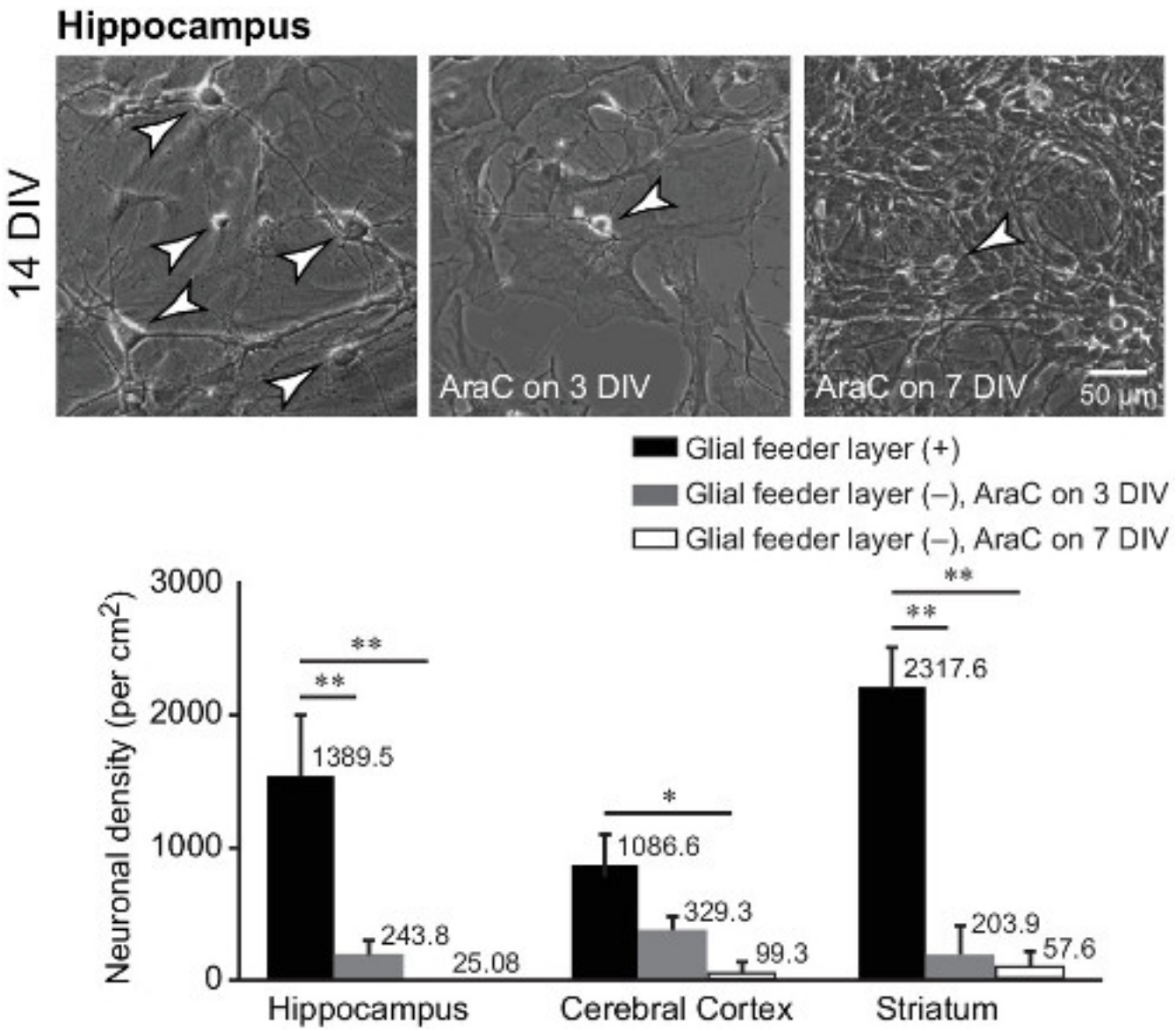

Figure 11. Supportive effect of glial feeder layer on neuronal survival. The number of surviving neurons was counted in the experiments shown in **Figure 8**, using images acquired by phase-contrast microscopy. Images for 14 DIV are shown here again, but with arrowheads pointing to examples of counted neurons. The bar graph shows the number of neurons per image field (449.0 µm x 335.5 µm) (mean ± standard error of the mean, n = 7-24 fields for each culture condition). Asterisks indicate statistically significant differences of 'Glial feeder layer (-), AraC on 3 DIV' and 'Glial feeder layer (-), AraC on 7 DIV' from 'Glial feeder layer (+)' (* $p < 0.05$, ** $p < 0.01$, unpaired Student's *t*-test). The numbers above the bars indicate the neuronal density measured in montages of multiple image fields. Images were acquired using a 20X objective lens. To avoid acquisition bias, all images were acquired along a full vertical strip, from the top to the bottom of a coverslip and passing through the approximate center of the coverslip. Individual images had some overlap (*e.g.,* 1/6 to 1/4 of an image field at the top and bottom). Two methods were used for the analysis. In one, neurons were counted in alternating images to ensure that individual neurons were not counted more than once. The resulting numbers were used to generate the bar graph. In the second method, a single montage was created from the individual images. They were stitched using the Microsoft Image Composite Editor or manually using Photoshop. The montages also excluded multiple counts of the same neurons. The resulting numbers are listed above the bars. In both methods, wide areas at the coverslip periphery that did not contain any cells were excluded. Cells were counted as neurons if they had cell bodies that appeared bright by phase-contrast microscopy, as well as extended cellular processes. Please click here to view a larger version of this figure.

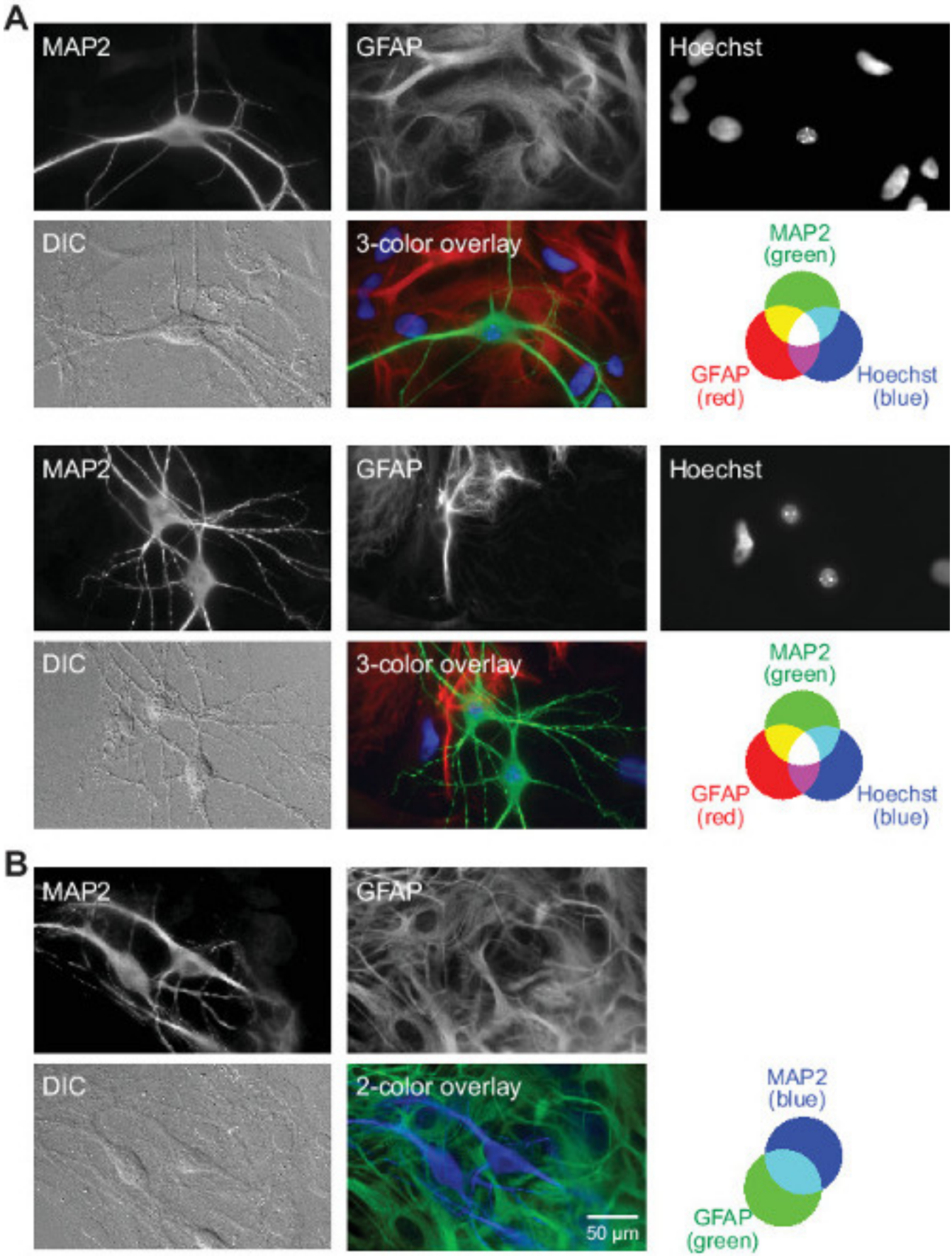

Figure 12. Immunocytochemical identification of cell types in neuronal cultures. The culture condition corresponds to the bottom-left image of **Figure 8A**. Neurons were obtained from the CA3-CA1 region of the hippocampus of wild-type mice, and plated on a glial feeder layer generated from the CA3-CA1 region of hippocampus of wild-type rats. The cultured cells were analyzed by immunocytochemistry for the neuronal marker MAP2, and the astrocytic glial cell marker GFAP. Differential interference contrast (DIC) microscopy was used to reveal the general morphology of the imaged fields, 14-17 days after the neurons were plated on the glial feeder layer. **(A)** Three-color staining,

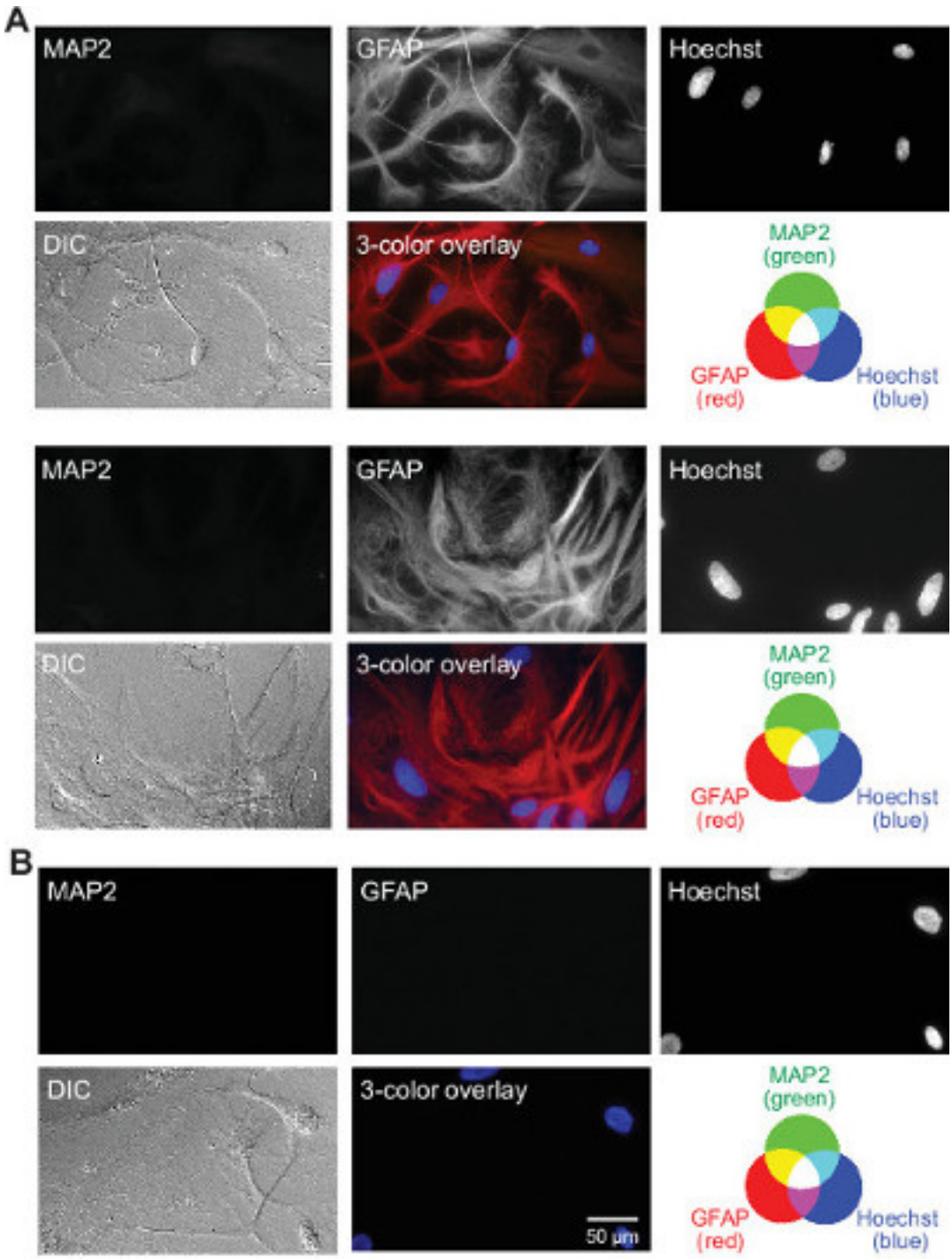

Figure 13. Immunocytochemical identification of cell types in glial feeder layer cultures. Sister cultures of the rat glial feeder layers as in **Figure 12**, but without the plating of mouse neurons. **(A)** Staining using the immunocytochemical procedures described in **Figure 12A**. Two representative image fields are shown. **(B)** Staining of the glial feeder layer using the immunocytochemical procedures described in A, but with the primary antibodies omitted. All MAP2 images in **Figures 12A** and **13A, B** were acquired under the same imaging conditions, and are shown at the same image contrast to allow comparison of intensity. The same procedures were used for GFAP images in **Figures 12A** and **13A**, **B**. The glial feeder layer culture was imaged on the same day as the cultures in **Figure 12**. Thus the glial feeder layers in **Figures 12** and **13** were cultured *in vitro* for the same amount of time. Please click here to view a larger version of this figure.

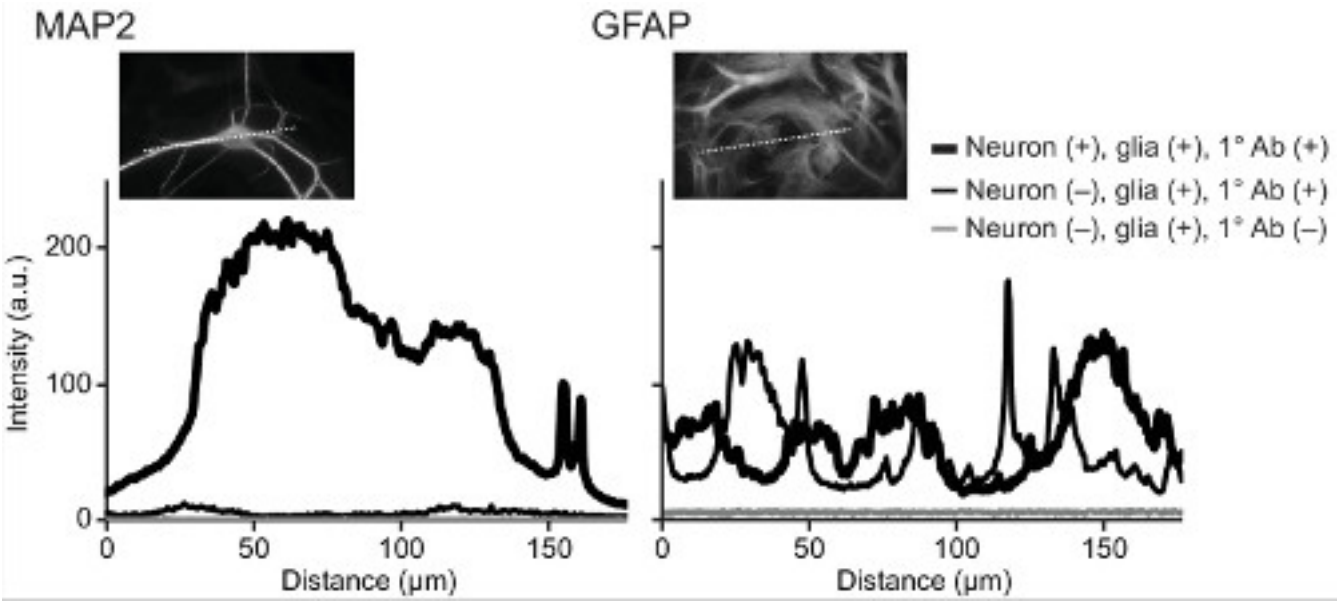

Figure 14. Intensity of immunocytochemical staining. Lines were drawn on the images shown in **Figures 12** and **13**, and the intensity along these was plotted. Insets show images in the top row of **Figure 12A**. The three conditions were: 1) plating of mouse cells (containing neurons) on a rat feeder layer and staining with the primary antibodies (Neuron +, glia +, 1° Ab +), 2) glial feeder layer only (no neuronal plating) and staining with the primary antibodies (Neuron −, glia +, 1° Ab +), and 3) glial feeder layer only (no neuronal plating) and staining without primary antibodies (Neuron −, glia +, 1° Ab −). Neuronal staining (MAP2) was present only when mouse cells were plated (conditions 1 vs. 2), and glial staining (GFAP) was present in both sets of cultures (conditions 1 vs. 2). The immunocytochemical staining was specific to both primary antibodies (conditions 1 vs. 3). Please click here to view a larger version of this figure.

SOLUTIONS
TAE buffer (50x solution)
Tris base, 486 g
Glacial acetic acid, 114.2 ml
0.5 M EDTA, pH 8.0, 200 ml
Add distilled water to bring total volume to 2 L
Make up in a chemical fume hood.
Dilute 50-fold before use.
Hanks' solution
Hanks' Balanced Salts, without calcium chloride, magnesium sulfate and sodium bicarbonate (for 1 L)
$NaHCO_3$, 350 mg (final concentration 4.17 mM)
HEPES, 2.38 g (final concentration, 10 mM)
Adjust to pH 7.4 using 5 M NaOH.
Adjust osmolarity to 310 mOsm using sucrose (Osmolarity tends to ~290 mOsm without any adjustment).
Add distilled water to bring total volume to 1 L
Digestion solution (for trypsin treatment of brain tissue)
NaCl, 800.6 mg (final concentration, 137 mM)
KCl, 37.3 mg (final concentration, 5 mM)
$Na_2HPO_4 \cdot (7H_2O)$, 187.6 mg (final concentration, 7 mM)
HEPES, 595.8 mg (final concentration, 25 mM)
Glucose, 97.3 mg (final concentration, 5.4 mM)
Adjust to pH 7.2 using 5 M NaOH.
Osmolarity tends to ~310 mOsm without any adjustment.
Add distilled water to bring total volume to 100 ml.

Right before usage, add 20 mg of trypsin (final concentration of 10 mg/ml) and 20 µl of DNase (final concentration of 750 units/ml) to 2 ml of digestion solution.
Dissociation solution (for mechanical dissociation of brain tissue)
Hanks' solution
$MgSO_4 \cdot (7H_2O)$, 295.1 mg (final concentration, 11.97 mM)
Adjust osmolarity to 310 mOsm using sucrose.
Total volume is 100 ml.
Right before usage, add 20 µl of DNase (final concentration of 750 units/ml) to 2 ml of dissociation solution.
Plating medium-1 (for mouse glial cells)
Dulbecco's Modified Eagle Medium (DMEM), 449.5 ml
MITO+ Serum Extender, 0.5 ml
FBS, 50 ml
Total volume is 500 ml.
Plating medium-2 (for mouse neurons)
Neurobasal-A, 485 ml
B27, 10 ml
GlutaMAX-I, 5 ml (final concentration, 2 mM)
Total volume is 500 ml.
Plating medium-3 (for rat neurons and glial cells)
Minimum Essential Media (MEM, without phenol red)
Glucose, 2.5 g (final concentration, 27.8 mM)
$NaHCO_3$, 100 mg (final concentration, 2.38 mM)
Transferrin, 50 mg
FBS, 50 ml
GlutaMAX-I, 5 ml (final concentration, 2 mM)
Insulin, 12.5 mg
Total volume is 500 ml.
Growth medium (for rat neurons and glial cells)
MEM (without phenol red)
Glucose, 2.5 g (final concentration, 27.8 mM)
$NaHCO_3$, 100 mg (final concentration, 2.38 mM)
Transferrin, 50 mg
FBS, 25 ml
GlutaMAX-I, 1.25 ml (final concentration, 0.5 mM)
B27 or NS21 {Chen, 2008 #2399}, 10 ml
Cytosine β-D-arabinofuranoside (AraC), 0.56 mg (final concentration, 4 µM)
Total volume is 500 ml.
General comments
Our culture media do not contain antibiotics because they could exert cytotoxic effects on cultured glial cells and neurons (reference 70, 71). This makes it especially important to adhere to sterile procedures in culture-related work.
See the **Table of Reagents** for the detailed sources of chemicals.

Table 1. Solutions

Discussion

The protocol presented here includes procedures for tattooing to label/identify mice, for genotyping mice from tail tips, and for culturing mouse brain neurons at low density. In one round of experiments using 6-8 pups, these procedures typically require ~0.5 hr, ~4 hr and ~2 hr, respectively, at a total of 6-7 hr. This makes it practical for a single experimenter to complete all the procedures necessary from the time of the pups' birth to the plating of neuronal cultures – in less than a single working day (with the exception of prior preparation of glial feeder layers).

Tattooing

Long-term identification of animals is necessary for the purposes of breeding and scientific studies such as analyses of histology, cell function and animal behavior. Tattooing newborn animals is advantageous because it can be carried out rapidly and lasts for much of the animal's life [7,22-25]. Tattooing on the paw pads can be better than toe clipping [23] with respect to preserving testable behaviors, for example in suspension or gripping [22,26], although some studies have not noted such deficiencies (*e.g.,* [25]). There were no instances of mothers rejecting or cannibalizing pups after tattooing. For other methods of long-term identification of animals, see recent reviews [7,23,24].

Genotyping

A critical feature in our protocol is the use of a fast genotyping method. Although similar genotyping methods were described in previous reports (*e.g.,* [27,28]), the system described here has at least two improvements. First, it can tolerate certain variations in the amount of starting tissue, age of animals, and amplicon length. Thus, successful genotyping can be achieved using mice as young as 1 day and as old as 6 months old, and can be carried out using a broad range of primer pairs. Second, this method does not require a stop solution for the DNA extraction step. This eliminates excessive tube handling and pipetting, and allows parallel multi-tube automation of the process with a PCR machine. The number of specimens can be scaled up (*e.g.*, 96 samples) yet processed easily and simultaneously. Also the specimen type is not limited to tail clips; other sample types that could be used include ear punches, toe clippings, whole early embryos, and placenta tissues [2-4,29,30]. Different animal species can also be used. Thus the genotyping procedures are reliable (repeatable with low intra-assay variance) and reproducible (low inter-assay variance between runs or between laboratories), and have the advantage of high scalability.

Note that some caution is required for achieving the expected quality of results. First, during DNA extraction, a large variation in the protocol can degrade the results. For example, a significant reduction in the volume of DNA Extraction Solution (*e.g.,* from 200 to 100 µl in step 2.2) still allows genotyping, but it can reduce the reliability and result in variation in PCR results. Under such conditions, it is recommended to reduce the volume of the DNA extract from 4 to 2 µl, and increase the volume of H_2O from 2 to 4 µl to compensate for the volume change during PCR reactions (step 2.5). Second, at the end of DNA extraction, the solution can be used for PCR immediately without centrifugation in most cases, although the tail will retain its overall structure and will not be completely decomposed. However, the described inversion of the tubes is essential for the purpose of dissociating genomic DNA from the tissue (step 2.4). It is also important to use the top, clear part of the solution, excluding the debris at the bottom of the tube, because inclusion of the latter will lead to inconclusive PCR results. These steps will be effective with minimal time and effort. Equally good results can be obtained by actively vortexing the specimen (in place of the inversions) followed by centrifugation and usage of the supernatant. Third, after the DNA extraction, the DNA can be stored for long-term (*e.g.,* >two weeks). It is recommended to centrifuge the solution using a microcentrifuge (*e.g.,* 3,000-13,000 g at 4 °C for 2 min), transfer the supernatants to new tubes, and store them at -80 °C. When the stored extract is to be used for genotyping, briefly centrifuge the specimen after thawing, and use the supernatant.

Neuronal cultures

Another critical feature of our protocol is the use of a glial feeder layer to establish neuronal cultures at a low plating density. Typically, in primary cultures of mammalian brain neurons, the cells are plated with a relatively high density, on coated coverslips without a glial feeder layer (*e.g.,* [31-39]). When the plating density of neurons is reduced using this method, the initial lack of glial sheet leads to poor neuronal growth and dendritic extension (panels **B**, **C** in **Figures 8-10**), as reported previously [40-42], probably due to poor glial growth [43,44].

Successful, low-density neuronal cultures can be generated by at least three broad types of methods. In one approach, Neurobasal Medium is used as a culture medium. This makes it possible to culture neurons in the absence of a glial feeder layer, and can be used for low-density neuronal cultures [45,46]. In a second approach ('Banker-type' and its modification), glial cells are co-cultured with neurons in the same well but are physically separated from them. In this context the glial cells provide 'trophic support' to neurons without contacting them directly [1,41,42,47-49]. In the third approach, neurons are plated on a pre-established glial feeder layer that supports neuronal growth (*e.g.,* [50-53]) (**Figures 8-10**). Specifically, mouse brain neurons are plated on a glial feeder layer prepared from mice [8,34,54] or rats [41,55,56].

We prefer the last of the three approaches to low-density culture, because the Neurobasal Medium can affect neuronal survival [57], the astrocytic glial cells will be essential in regulating neuronal functions [8,58], and the physical contact between neurons and glial cells may be important. The procedures for culturing the mouse and rat glial cells are similar [59,60], but we have modified them slightly to accommodate for the more rapid *in vitro* growth of rat vs. mouse glial cells. The procedures for the rat cell culture were modified from [61-63]. The use of a glial feeder layer for low-density neuronal cultures makes it necessary to perform rapid genotyping, in order to match the genotype of the feeder layer to that of the neurons within the period between the pups' births and the neonatal deaths or the end of culture window (1-2 postnatal days).

Low-density culture on a glial feeder layer is also an important method when one tries to culture neurons of small nuclei in the brain (*e.g.,* the norepinephrine-releasing locus coeruleus, and the dopamine-releasing substantia nigra). Those nuclei contain only a small number of neurons and would inevitably yield low-density cultures [64-67].

Primary cultures are routinely prepared in our laboratory from the CA3-CA1 region of hippocampus, the motor region of the cerebral cortex and the striatum of mice. The same procedures can be used to prepare neuronal cultures representing other brain regions, for example the whole hippocampus (including the dentate gyrus) and the whole cerebral cortex. Rat neurons from brain regions such as the hippocampus and the locus coeruleus are cultured in similar fashion, using the rat glial feeder layer. These cultured neurons are usually used at 1-4 weeks *in vitro*,

with the exact age of the culture depending on the purpose of the experiment. It is of note that some neurons cultured on a glial feeder layer can survive for more than 10 weeks [34,68].

One potential problem of low-density neuronal cultures is that the neuronal properties can be different from those in high-density cultures or in neurons *in situ*. Comparison of these systems provides an interesting opportunity to study how neuronal development and maturation are affected by multiple factors, such as the neuronal density, the soluble factors secreted from neurons, neuron-to-neuron contact, and glial-neuronal interactions.

In summary, the tattooing-based mouse labeling is long lasting, the genotyping method is rapid, and the culture method allows for plating mouse neurons at low density. The protocol described here can be applied in its entirety to other animal species harboring other genetic mutations. Moreover, individual steps of the protocol can be used for other purposes. Thus the protocol can be used in a wide array of applications based on experimenters' needs.

Disclosures

The author (Zhengmin Huang) is the president of EZ BioResearch LLC that produces reagents described in this article.

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