Chapter 6

In Vivo Analysis of Engrafted Adult Hippocampal Neural Progenitors

Matthew J. Robertson, Joseph Peltier, and David V. Schaffer

Abstract

A neural degenerative disease is characterized by the deterioration of neural tissue and subsequent loss of function. The *in vivo* engraftment of neural stem cells is a promising approach to the functional replacement of neural tissue with the ultimate goal of regaining lost function. In addition, by studying the behavior of engrafted neural stem cells in healthy and diseased tissue, insight can be gained into the extracellular and intracellular mechanisms which regulate stem cell behavior *in vivo*. Adult hippocampal neural progenitor cells (AHNPCs) are one potential source of cells that can be used to this goal. In this chapter, we describe some of the *in vivo* techniques necessary to study hippocampal progenitors in the adult rat, including engraftment and analysis by immunofluorescent staining. These techniques are important for studying AHNPCs within the physiologically relevant environment of the adult brain rather than in a culture dish.

Key words: Neural, Hippocampus, Progenitors, Rat, *In vivo*, Adult, Engraftment, Immunostaining, Pulse-chase/BrdU, Neurons, Astrocytes, Differentiation

1. Introduction

Hippocampal progenitors have been successfully engrafted into numerous regions of the nervous system, including the two constitutively active neurogenic regions of the adult brain: the hippocampus and the subventricular zone to olfactory bulb pathway. After engraftment into both regions, cells have differentiated and expressed markers consistent with neurons native to the engraftment site (1, 2). Grafting hippocampal progenitors provides a means to determine their plasticity and identify new regulatory signals when the mechanisms that control their behavior are incompletely understood, in either healthy or damaged/diseased tissue. Furthermore, their ability to

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differentiate and integrate into the nervous system provides hope for potential cell-based therapies for the treatment of neurodegenerative diseases (3).

Grafting studies involve first marking the cells for tracking postengraftment. For simplicity we will discuss using BrdU to mark cells, but other methods are available (see Note 1). After the cells have been marked, they can either be cultured under conditions that induce differentiation prior to engraftment (see Chapter 5, Subheading 3.1.4 for common conditions), or grown in proliferative culture conditions (20-ng/mL FGF-2). Before proceeding, we assume the reader is familiar with the growth and differentiation techniques outlined in Chapter 5. After engraftment, the cells are allowed to acclimate and integrate into the host, from several weeks to several months (1, 2). Longer time points allow the cells to fully differentiate and to functionally integrate. Lastly, the brains are harvested and examined for colocalization of BrdU and neural lineage markers to determine the progenitor cell fate.

2. Materials

2.1. Preparation of Samples for Grafting

1. Dulbecco's Modified Eagle Medium/Nutrient Mix F-12 (DMEM/F-12) with HEPES and L-Glutamine (Invitrogen, Bethesda, MD) supplemented with 1% (v/v) N-2 Supplement (Invitrogen). Store supplemented medium at 4°C for up to 1 month.
2. Accutase (Innovative Cell Technologies, San Diego, CA).
3. Basic fibroblast growth factor (FGF-2, Peprotech, Rocky Hill, NJ). FGF-2 stock solution: dissolve in DMEM/F-12 + N-2 at 0.1 mg/mL, aliquot, and store at –20°C. Once thawed, aliquot can be stored at 4°C for up to 1 week.
4. Sterile cotton-plugged glass Pasteur pipettes.
5. 5-Bromo-2′-deoxyuridine (BrdU, Sigma, St. Louis, MO). Prepare 5 mM stock solution in distilled/deionized water and 0.22 µm syringe filter.
6. Phosphate buffered saline (PBS), pH 7.4 (Invitrogen).
7. Poly-L-ornithine/laminin-coated culture plates (see Subheading 2.1.1).

2.1.1. Laminin-Coated Culture Plates

Reagents

1. Sterile tissue culture water.
2. Sterile PBS, pH 7.4 (Invitrogen, Bethesda, MD).

3. Poly-L-ornithine hydrobromide (Sigma) dissolved in tissue culture water at 10 mg/mL. Sterilize by 0.22 µm syringe filtration, aliquot, and store at –20°C. Avoid repeated freeze/thaw.
4. Natural mouse laminin (Invitrogen). Store at –80°C. Thaw slowly at 4°C before use. Avoid repeated freeze/thaw and vortexing.
5. Plastic wrap.

Preparation of Plates

6. Dilute poly-L-ornithine in tissue culture water to a final concentration of 10 µg/mL and add to tissue culture plates such that plates are well covered (~7 mL for 10 cm plate). Incubate overnight at room temperature. To maintain sterility, leave the plates in a biological safety cabinet with blower off and sash closed. Leave UV light OFF.
7. Rinse plates twice with approximately the same amount of sterile water as used for poly-L-ornithine incubation. Do not allow plates to dry out.
8. Dilute laminin in sterile PBS to a final concentration of 5 µg/mL and add to plates at the same volume as was used for poly-L-ornithine.
9. Incubate at 37°C overnight.
10. Wrap in plastic wrap and store at –20°C for up to 6 months. Freeze plates on a level surface to avoid pooling and dry spots.

2.2. Surgical Grafting and Tissue Sectioning

1. Puralube Vet Ointment (PharmaDerm, Duluth, GA).
2. Sterotaxic instrument (e.g., Benchmark™ Stereotaxic Instrument, myNeuroLab.com, St. Louis, MO).
3. Betadine Vet Solution (Fisher, Pittsburgh, PA).
4. Scalpel, No. 10 blade.
5. 5 µL Hamilton syringe.
6. 26-gauge beveled needle.
7. Dental drill for surgery (Dremel, Racine, WI).
8. Surgical staples.
9. 70% Ethanol.
10. PBS, pH 7.5 (Invitrogen).
11. 0.9% (w/v) NaCl, approximately 150 mL per animal (0.22 µm sterile filtered).
12. Institutionally approved analgesia (e.g., 0.05 mg/kg buprenorphine dissolved in sterile, warm 0.9% NaCl).

13. Peristaltic pump for perfusion (e.g., Carter Manostat, Fisher).
14. Tubing for perfusion, 2.79 mm inner diameter (Fisher).
15. Blunted 17- or 18-gauge needle (or needle hand-pump attachment).
16. Surgical scissors.
17. Large straight forceps.
18. Two small curved forceps.
19. Rongeurs forceps.
20. Spatula.
21. Ice cold 4% (w/v) paraformaldehyde (PFA). While stirring, carefully heat 400 mL water on a hot plate in a fume hood to 60°C. Do not heat above 60°C, because aldehyde will form and cause autofluorescence. While maintaining temperature, add 20 g PFA and stir. As necessary, add NaOH pellets one at a time until paraformaldehyde dissolves. Generally, 1–2 NaOH pellets over the course of 10–15 min will fully dissolve the PFA. There will still be small particulates in suspension. Cool to room temperature. Add 50 mL 10× PBS and pH to 7.4 with HCl. Bring total volume to 500 mL with water and sterile filter to remove particulates. Prepare approximately 300 mL per animal. During perfusions keep the PFA on ice. 4% PFA must be disposed as chemical waste.
22. 20 mL scintillation vials (Wheaton, Millville, NJ).
23. 30% sucrose. Dissolve 30 g of sucrose in sufficient water for 100 mL of final solution. Sterilize the solution with a 0.22 µm filter and store at 4°C.
24. Tissue cryoprotectant: 25% ethylene glycol, 25% glycerol, 50% 0.1 M Na_2HPO_4, pH 7.4. Store at 4°C.
25. 96-well plate for storing sections.
26. Parafilm.
27. Sliding microtome (e.g., Vibratome, St. Louis, MO).
28. Trypan blue stain (Invitrogen).
29. Hemacytometer.

2.2.1. Anesthesia

1. Ketamine Hydrochloride, 100 mg/mL (Phoenix Scientific, St. Joseph, MO).
2. Xylazine Hydrochloride, 20 mg/mL (Ben Venue Laboratories, Bedford, OH).
3. 9% (w/v) NaCl in distilled/de-ionized water. Sterilize by 0.22 µm filtration.
4. Anesthetic cocktail: For each 150 g rat, sterilely mix 135 µl ketamine, 75 µl xylazine, 50 µl 9% NaCl, and 240 µl distilled/de-ionized water.

Table 1
Recommended primary antibodies, dilutions, and storage conditions for immunostaining

Name	Species	Supplier	Dilution	Storage
β-Tubulin III	Mouse	Sigma	1:250	Aliquot –20°C
MAP2a+2b	Mouse	Sigma	1:250	Aliquot –20°C
GFAP	Rabbit	Abcam (Cambridge, MA)	1:500	4°C
O4 (see Note 9)	Mouse	Millipore (Billerica, MA)	1:250	4°C
Nestin	Mouse	BD Pharmingen (San Jose, CA)	1:1,000	4°C
BrdU	Mouse	Roche Applied Science (Indianapolis, IN)	1:40	Aliquot –20°C=

2.3. Tissue Section Immunostaining

1. 12- and 24-well plates.
2. Cell culture inserts (net carriers) for 12-well plates (BD Falcon, Bedford, MA).
3. Paint brush, fine point (for transferring and manipulating sections).
4. Tris buffered saline (TBS): Add 6.05 g Tris base and 8.76 g NaCl to 900 mL water. pH to 7.5 with HCl and bring up to 1 L with water. Store at room temperature.
5. Triton X-100 (EMD Biosciences, San Diego, CA).
6. Normal serum (see Note 2).
7. Primary antibodies (see Table 1).
8. Cy3 and Cy5 conjugated donkey anti-rabbit and anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA). Upon receipt, reconstitute in 1:1 glycerol:water according to package insert (volume varies by lot), aliquot, and store at –20°C protected from light. Avoid freeze/thaw.
9. TO-PRO®-3 nuclear stain (Invitrogen). Upon receipt, aliquot and store at –20°C. Protect from light and avoid freeze/thaw.
10. Alexa Fluor® 488 conjugated donkey anti-mouse and anti-rabbit antibodies (Invitrogen). Upon receipt, aliquot and store at –20°C protected from light. Avoid freeze/thaw.
11. 4% (w/v) PFA (see Subheading 2.2, item 21 for preparation instructions).
12. 10× SSC: 3 M sodium chloride and 0.3 M sodium citrate in water. pH to 7.0. Make 50 mL.

13. 50% Formamide solution: Mix 50 mL of formamide in 10 mL of 20× SSC solution and 40 mL of deionized water. Pre-made 50% formamide solution can be stored at –20°C.
14. 2-N hydrochloric acid. Make 50 mL.
15. 0.1 M borate buffer: Add 3.2 g boric acid to 400 mL water. pH to 8.5 with NaOH and bring to 500 mL with water. Store at room temperature.
16. Gelatin-coated slides, 3″×1″ (LabScientific, Livingston, NJ).
17. Microscope cover glasses (24×60 mm, Fisher).
18. ProLong® Antifade Kit (Invitrogen).

3. Methods

3.1. Preparation of Samples for Engraftment

1. One day prior to engraftment, plate cells as described in Chapter 5, Subheading 3.1.1 and supplement the cell culture media with BrdU to a final concentration of 5 μM (1 μL of a 5 mM BrdU stock per mL of culture). Plate enough cells to ensure approximately 10^5 cells per graft (for reference, an 80% confluent 10 cm plate will have approximately 8×10^6 cells).
2. Incubate the cells in DMEM/F-12+N-2+20 ng/mL FGF-2+5 μM BrdU at 37°C in 5% CO_2 atmosphere for 12 h.
3. Aspirate the medium, leaving behind enough so the culture does not dry out. Detach the cells and suspend as described in Chapter 5, Subheading 3.1.1, steps 1–10.
4. Pellet the cell suspension at 200 g for 2 min, and prepare another flame polished pipette (see Chapter 5, Subheading 3.1.1, step 8).
5. Aspirate the supernatant from the cell pellet and add 5 mL of PBS (see Note 3).
6. Suspend the cell pellet as described in Chapter 5, Subheading 3.1.1, step 10.
7. Repeat steps 4–6, but suspend the cells in only 1 mL of PBS.
8. Transfer the cell suspension to a 1.5 mL centrifuge tube.
9. Using a hemacytometer or other preferred means of cell quantification determine the number of cells in the suspension.
10. Pellet the cell suspension at 200 g for 2 min.
11. Carefully aspirate the supernatant from the cell pellet and add sufficient PBS to yield a final concentration of 75,000–100,000 cells per μL. Use a Pipetman for aspiration to avoid disturbing the pellet. The actual cell concentration

will be lower than the calculated due to residual liquid on the pellet. Ideally, by targeting a higher concentration the final concentration will be approximately 50,000 cells per μL.

12. Take 3 μL of the final cell suspension and dilute 1:10 in PBS. Using the 1:10 dilution, determine the cell concentration in the final suspension. Store the remaining 20 μL on ice for determining cell viability (see Subheading 3.2, steps 17–22).
13. Using a Pipetman, carefully measure the final volume of the cell suspension. Before starting the animal procedure, ensure there is sufficient cell suspension for all the planned engraftments. It is best to have sufficient cell suspension for two to five extra engraftments as a contingency.
14. Close the centrifuge tube and place the cell suspension on ice to maintain cell viability.

3.2. Grafting

This protocol is a general description of how to perform a stereotaxic graft of hippocampal progenitors into an adult rat's brain. To assess cell engraftment, the rat is perfused transcardially so that the cells can be analyzed histologically at time-points after the graft that would permit assessment of proliferation (short survival) and differentiation (longer survival – typically 4 weeks or more).

Similar to engraftment, endogenous hippocampal progenitors can be virally transduced by stereotaxic injection of a retrovirus produced as described in Chapter 7. This virus may contain a gene that will simply mark the progenitors (e.g., GFP) or it may contain some other gene of interest. Rather than injecting cultured progenitors as described in this section, approximately 2–3 μL of purified virus is injected.

Anesthesia

1. Anesthetize the rats using ketamine/xylazine anesthetic cocktail (500 μL of anesthesia per 150 g of body weight) via intraperitoneal injection.
2. Wait several minutes to ensure that the rat is anesthetized. Check reflexes by pulling the hind limb laterally and pinching the hind paw while the animal is on its stomach. If the rat is not fully under it will retract its hind paw.
3. When there is no withdrawal reflex, apply a small drop of Puralube Vet Ointment to each eye to prevent dryness, as the animals are unable to blink while under anesthesia. Proceed to the sterotaxic injection.

Stereotaxic Surgery

4. Assemble the sterotaxic apparatus with the 5 μL Hamilton syringe attached to a 26-gauge beveled needle.

5. Shave the hair on the top of the animal's head.
6. Mount the anesthetized rat in the sterotaxic apparatus, using ear bars and nose bar, as per the manufacturer's directions to ensure the skull is level (bregma and lambda are aligned horizontally). Sterilize the shaved skin with an approved antiseptic such as Betadine.
7. Using a scalpel, make a medial incision stretching from bregma to lambda. Retract the skin and underlying membranes to expose the skull. Align the needle over bregma, and note the coordinates on the stereotaxic instrument. Using a rat atlas such as Paxinos and Watson (4) to determine coordinates for the desired injection site, move the needle to the desired location over the skull, and use the drill to slowly drill through the skull being careful not to disturb the underlying dura or brain tissue (see Note 4).
8. Raise the Hamilton syringe to its highest possible position in the stereotaxic instrument, place the tube containing cells underneath it, and use the Hamilton syringe to gently mix the prepared cells from Subheading 3.1, step 14.
9. Using the syringe, measure sufficient cell suspension for 75,000 cells based on the concentration calculated in Subheading 3.1, step 12 (e.g., for 50,000 cell/μL measure 1.5 μL).
10. Slowly lower the needle to the correct dorsal/ventral sterotaxic coordinate.
11. As smoothly as possible, inject liquid at the rate of 1 μL/min until complete. An infusion pump can be used to ensure a slow, even injection.
12. Raise the syringe 1 mm, wait 1 min, and then raise the syringe slowly until it exits the brain.
13. Remove the rat from the sterotaxic apparatus and close the incision using surgical staples.
14. Subcutaneously inject 1 mL of 0.9% warm NaCl to hydrate the animal and administer your institutionally accepted analgesia (e.g., 0.05 mg/kg buprenorphine dissolved in sterile, warm 0.9% NaCl).
15. Between engraftments rinse the syringe and needle first with PBS, then with 70% ethanol, and finally rinse three times with PBS. Return to step 1 for the next animal.
16. Wait for the cells to exhibit the desired behavior (proliferation or differentiation), often over a period of weeks, then proceed to perfusion.

Cell Viability

17. To ensure any differences observed after engraftment are not due to cell preparation and handling, it is best to perform a

cellular viability assay (e.g., trypan blue exclusion) before and after the animal surgeries using any extra cells. For this purpose, it is sufficient to use the 1:10 diluted cells from Subheading 3.1, step 12 (see Note 5).

18. Using the 1:10 dilution of cells from Subheading 3.1, step 12 or other appropriate source of cells, transfer 10 μL of suspension to a fresh 1.5 mL microcentrifuge tube.
19. Add 10 μL of trypan blue to the fresh aliquot and mix gently 2–3 times (one part trypan blue solution per one part cell suspension).
20. Incubate at room temperature for 3 min (see Note 6).
21. Using a hemacytometer count the unstained (viable) and stained (nonviable) cells.
22. The fraction of viable cells is calculated as the number of viable cells (unstained cells) divided by the total number of cells (unstained cells + stained cells). Ideally this fraction will be >90% and similar for each sample used for engraftment before and after the animal surgery.

Perfusion

23. Assemble the peristaltic pump for perfusion following the manufacturer's directions. Using water, confirm that the pump is set for a flow rate of 20 mL/min. Attach the outlet of the pump to a needle for inserting into the rat's heart (see Note 7).
24. Attach the 0.9% NaCl solution to the pump intake and clear all water from the pump lines, making sure to rid the lines of air bubbles. During all subsequent perfusion steps, the pump lines should be kept bubble free.
25. Anesthetize the rats as described in steps 1–3.
26. Place the animal on a perfusion table or near a sink for drainage.
27. With the animal on its back and its tail toward you, use scissors to make a small incision at the base of the animal's abdomen through the underlying muscle.
28. Cut along the left side of the animal toward the head stopping at the rib cage. Repeat for the right side. During the cutting gently pull up to ensure that no organs are damaged.
29. Carefully cut the diaphragm away from the rib cage and then cut along the right and left sides of the rib cage. Next, pull the rib cage away from the chest cavity with a large straight forceps.
30. Clamp the descending artery, which runs adjacent to the spinal cord, with a curved forceps to prevent blood flow to the lower body. This will reduce the volume necessary for effective perfusion of the cranium.

31. Hold the heart with a pair of curved forceps and using the scissors make a small incision in the right atrium of the rat's heart for drainage.
32. With the scissors make a small incision into the base of the heart and insert the needle into the rat's left ventricle toward the left atrium. Hold the needle in place by clamping the curved forceps.
33. Perfuse the rat with 0.9% NaCl at a rate of 20 mL/min until the fluid exiting from the right atrium is clear. Generally, 100 mL of saline is sufficient. Properly and safely dispose of the saline and blood, typically via drainage.
34. Transfer the pump's intake to the ice cold 4% PFA. Perfuse approximately 250 mL total at 20 mL/min. When perfusion begins the animal will twitch. Perfusion is complete when the animal is stiff, especially the head and front legs. Because PFA is toxic be sure to wear gloves when handling and avoid inhaling the fumes. Remove the line from the animal.
35. Transfer the pump's intake back to the 0.9% NaCl solution to flush the PFA from the pump tubing.

Brain Isolation and Dehydration

36. Fill a 20 mL scintillation vial with a 4% PFA.
37. Using a large pair of scissors detach the head.
38. Cut along the midline of the head pulling back the skin with a pair of scissors.
39. Insert the rongeurs forceps into the foramen magnum. Remove skull fragments until the brain is exposed.
40. Gently, using the scissors, remove the meninges overlying the brain, and then with a spatula transfer the brain to the 20 mL vial containing 4% PFA.
41. Incubate brain in 4% PFA for 24 h at 4°C.
42. Dispose of all other tissue as per institutional biosafety requirements.
43. Replace the PFA with a 30% sucrose solution. Initially the brain will be buoyant, and as the sucrose diffuses into the tissue, it will sink to the bottom of the vial. This process can be accelerated by changing the 30% sucrose solution every 12–24 h. Once the brain has sunk proceed to sectioning. During this process store the brain at 4°C, and be sure to minimize the storage time to prevent bacterial growth.

Sectioning

44. Performing the sectioning is dependent on the application and resources available. We generally use a freezing, sliding

microtome and take 40 μm coronal or transverse sections. We prefer to store the sections at –20°C in a 96-well plate format. The plate is filled with 200 μL per well of tissue cryoprotectant (25% ethylene glycol, 25% glycerol and 0.1 M phosphate buffer), and the plate edges are sealed with Parafilm. Under these conditions, the sections can be stored for extended time periods, such as a year.

3.3. Immunostaining

Pretreatment required to expose the BrdU epitope for antibody staining can alter other epitopes, and thereby compromise immunostaining of some proteins (e.g., nestin and GFP). Therefore, if unsure of whether BrdU pretreatment destroys an epitope of interest, it is generally best to first stain for all desired markers followed by BrdU staining. It is helpful to use 12-well net carriers for all rinse steps, since the carriers allow for easy transfer between rinses. However, for antibody incubations transfer the sections to a smaller 24-well plate to help minimize antibody consumption. Transfer and manipulation of sections are easily accomplished using a fine pointed paint brush. Additionally, all steps are performed under gentle shaking, and each net carrier and well in a 24-well plate can hold approximately five sections for the volumes provided.

Antibody Staining

1. Place a net carrier in a 12-well plate and fill wells with 2 mL TBS (pH 7.5).
2. Using a paint brush, transfer desired tissue sections to 12-well plate nets.
3. Shake for 10 min at room temperature and then transfer the section-containing nets to a fresh well of TBS.
4. Repeat 2 mL TBS rinse at room temperature two additional times to remove cryoprotectant. If staining for O4, see Note 8 before proceeding.
5. Block in TBS with 0.3% Triton X-100 and 5% normal serum (TBS-ST) for 2 h at room temperature (see Note 2).
6. Transfer sections to a 24-well plate and incubate with 0.5 mL of primary antibody diluted in TBS-ST for 72 h at 4°C (see Table 1 for suggested dilutions for common markers). Afterwards transfer section back to a 12-well plate with net carriers. Use a paint brush to transfer sections. Thoroughly wash the paint brush with ethanol and rinse between staining sets to prevent antibody cross-contamination. Alternatively, a bent, disposable P200 pipette tip may be used to transfer sections.
7. Rinse twice in 2 mL TBS for 10 min per rinse at room temperature.
8. Rinse once in 2 mL TBS-ST for 10 min at room temperature.

9. Transfer the sections to a 24-well plate and incubate in 0.5 mL of 1:250 diluted secondary antibody in TBS-ST for 1 h at room temperature. While incubating, cover with aluminum foil to protect from light. Care should be taken in all subsequent steps to minimize light exposure and avoid photobleaching. Afterwards, transfer sections back to a 12-well plate with net carriers. Use a paint brush to transfer sections, and use care to prevent antibody cross-contamination.
10. Rinse three times in 2 mL TBS for 10 min per rinse at room temperature.
11. Postfix sections with 2 mL 4% PFA for 1 h at room temperature.
12. Rinse four more times in 2 mL of TBS for 10 min per rinse at room temperature.

BrdU

13. Pretreat for BrdU by incubating sections in 2 mL of 50% formamide solution at 65°C for 2 h using shaker or water bath.
14. Rinse once in 1 mL 1× SSC solution (diluted in deionized water) for 10 min at room temperature.
15. Incubate sections in 2 mL of 2N HCL at 37°C for 30 min using a shaker or water bath.
16. Rinse in 2 mL 0.1 M borate buffer for 10 min at room temperature.
17. Rinse sections six times in TBS (2 mL rinse for a total of 1.5 h).
18. Return to steps 5–10 to perform the primary and secondary antibody staining. The primary antibody is anti-BrdU diluted 1:40 (see Table 1). Using a paint brush, transfer sections to a 24-well plate for antibody incubations to conserve antibody. Thoroughly wash with ethanol and rinse the paint brush between staining sets to prevent antibody cross-contamination. Alternatively, a bent, disposable P200 pipette tip may be used to transfer sections.
19. *Optional*: During the last secondary antibody incubation, DAPI or another nuclear stain, such as TO-PRO®-3, can be used.
20. Mount sections by floating in TBS and transferring with a paint brush to a gelatin-coated slide. Allow the slides to dry in the dark for approximately 30 min (tissue sections will turn translucent when dry).
21. Follow instructions in ProLong® Antifade Kit to prepare antifade/mounting medium. Pipette a line of ~200 µl antifade/mounting medium lengthwise along the edge of the slide,

and slowly lay a coverslip over the slide, starting at the edge with the antifade/mounting medium. Proceed slowly and with care to avoid bubbles. Antifade/mounting medium should evenly coat the slide.

22. Dry the slide at room temperature in the dark for at least 2 h (overnight drying is acceptable). Seal the slide with clear nail varnish. Apply the varnish carefully to avoid covering the samples. Dry at room temperature in the dark for approximately 30 min.

4. Notes

1. It is important to be able to track the cells once they have been engrafted. Depending on the experiment, it is common to use a viral vector (e.g., a retroviral or lentiviral vector) to establish a GFP- or LacZ-expressing cell line. Establishment of this marked cell line via transduction of adult hippocampal neural progenitor cells (AHNPCs) is detailed in Chapter 7. These cells can subsequently be engrafted and tracked without BrdU labeling.
2. Normal serum is serum derived from the species in which the secondary antibody was raised. Use of nonmatched secondary antibodies and blocking serum can lead to nonspecific, background staining.
3. The PBS used during engraftment can be supplemented with growth factors such as FGF-2 or other small molecules as desired. It is best to perform the rinses using the final formulation of PBS used during the engraftment. This ensures that the desired final concentration of supplement is present, since there will always be residual liquid in the cell pellet after each pellet and aspiration step.
4. For sterotaxic coordinates we have used Paxinos and Watson (4). Furthermore, we have found greater accuracy measuring from the dura for the dorsal/ventral positioning of the needle, rather than from the skull surface. For hippocampal engraftments, we have used the following sterotaxic coordinates: AP −3.5, ML ±3.0, and DV −4.0.
5. Cells from Subheading 3.1, step 12 may also be plated on CultureSlides and stained to confirm BrdU incorporation. Use the BrdU staining protocol outlined in Subheading 3.3, steps 13–21 but adjust volumes appropriate to the CultureSlide being used.
6. Within 3–5 min of mixing cells with trypan blue be sure to determine the viability, because longer incubation periods will lead to cell death.

7. A blunted 17- or 18-gauge needle can be used, but we have found that the needle attachment for a hand or bicycle pump works very well.
8. If immunostaining for O4, do not permeablize with Triton X-100. O4 is a surface marker which can be disrupted by Triton X-100. For section staining, follow Subheading 3.3, steps 1–12 without Triton X-100, and then continue with permeablization and costaining at step 5.
9. The mouse anti-O4 antibody is an IgM. Confirm that the intended secondary antibody will recognize IgM before use.

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