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Intraspinal transplantation of mouse and human neural precursor cells

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Abstract

This unit describes the preparation and transplantation of human neural precursor cells (hNPCs) and mouse neural precursor cells (mNPCs) into the thoracic region of the mouse spinal cord. The techniques in this unit also describe how to prepare the mouse for surgery by performing a laminectomy to expose the spinal cord for transplantation. Here we show NPCs genetically labeled with eGFP transplanted into the spinal cord of a mouse following viralmediated demyelination can efficiently be detected via eGFP expression. Transplantation of these cells into the spinal cord is an efficacious way to determine their effects in neurological disorders such as multiple sclerosis, Alzheimer's disease, and spinal cord injury.

INTRODUCTION

Neural precursor cells (NPCs), derivatives of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are proposed as cell replacement therapies for neurologic disease such as multiple sclerosis (MS), spinal cord injury, and Alzheimer's Disease (Abdel-Salam, 2011; Giannakopoulou et al., 2011; Pluchino et al., 2010; Ruff et al., 2012; Wilcox et al., 2012). Prior to human transplantation, the functionality, efficacy, and the recipient's ability to tolerate these cells need to be tested in animal models of disease. We have shown that syngeneic mouse NPCs transplanted intraspinally into mice following viral-mediated demyelination are well tolerated and result in recovery of motor skills associated with extensive remyelination (Hardison et al, 2006; Totoiu et al 2004). In contrast, allogeneic NPCs are rapidly rejected under the same transplant conditions, confirming the antigenicity of these cells and indicating that immune suppression to prevent rejection must be considered to ensure longterm survival (Weinger et al 2012). We are currently testing the therapeutic effects of intraspinal transplantation of human NPCs (hNPCs) using a model of viral-induced demyelination (Buchmeier and Lane, 1999; Lane et al., 2006; Lane and Hosking, 2010). This unit provides a method for preparing both mNPCs and hNPCs for intraspinal transplantation (Basic Protocol 1 and Alternate Protocol 1) that includes a test for hNPC multipotency (Support Protocol 1) as well as a method for preparing mice (Basic Protocol 2) and performing the transplant surgery (Basic Protocol 3).

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BASIC PROTOCOL 1: Preparation of hNPCs for mouse intraspinal transplantation

Introductory paragraph

The hNPCs described in this protocol are derived from pluripotent stem cells. There are several published protocols for inducing a neural phenotype in pluripotent stem cells isolated from the preimplantation stage blastocyst (Chambers et al., 2009; Koch et al., 2009). Alternatively, hNPCs can be derived from patient fibroblasts (Yu et al., 2007). Below, this protocol describes the seeding and subsequent isolation of hNPCs from 6 well plates and their preparation for intraspinal transplantation. It is important to be diligent in following these steps to ensure high cell viability and multipotency.

Materials

Reagents and Solutions:

Poly-L-ornithine

Laminin (BD Biosciences, cat. no. 354239)

1x HBSS (Cellgro, cat. no. 21-022-CM)

Complete hNPC medium (see recipe)

Accutase (Invitrogen, cat. no. 11330-032)

0.4% Trypan blue

Equipment:

Tissue culture hood: Class II A/B3

6 well tissue culture plate

Tissue culture incubator, 37°C, 5% CO2, in humidified air

50mL conical tube

Refrigerated Table-top centrifuge, 1000-1500rpm

Hemocytometer with cover slip

Inverted phase/contrast microscope

Light duty wipe (Kimwipe)

1.7mL microcentrifuge tube

Protocol steps

1. Coat 6 well plates with 20μg/ml poly-L-ornithine and 5μg/ml laminin.

   Dilute poly-L-ornithine and laminin to desired dilutions in 1x HBSS and cover each well with at least 1mL. Incubate the plates overnight at 37°C. The following day, aspirate the coating mixture and wash three times with

1x HBSS. Plates can be covered in foil and stored at 4°C for up to a week if they are not going to be used immediately.

2. Plate 1.5–2×10^5 hNPCs per well of the poly-L-ornithine/laminin-coated plates in 2mL of complete hNPC medium.

   Incubate the cells at 37°C for 3–4 days or until they are 80–90% confluent. This will yield approximately 1×10^6 cells per well at approximately 90% confluency. Change the medium daily.

3. Dissociate the hNPCs with Accutase

   Aspirate complete hNPC medium and add 0.5mL accutase per well. Let sit for 10min at room temperature and quench with 0.5mL complete hNPC medium. Optional: you can wash one time with 1x HBSS prior to adding accutase.

4. Pool wells into 50mL conical tube and fill to 30mL with complete hNPC medium.

5. Centrifuge hNPCs at 1500 rpm at 4°C for 5 min.

   Aspirate medium, being careful not to disturb the pellet of hNPCs.

6. Wash cells by bringing up the hNPC pellet in 20mL complete hNPC medium and centrifuging at 1500 rpm at 4°C for 5 min. Repeat twice, counting cells at the second wash.

   After each wash aspirate the medium being careful not to disturb the pellet. Prior to the last wash remove a 10μL aliquot to count cells.

7. Dilute 10μL aliquot of hNPCs with 10μL of 0.4% trypan blue and load 10μL of diluted cells onto the hemocytometer. Count one (or take an average of two) of the 16 squares using an inverted phase-contrast microscope.

   Use the following calculation to determine the concentration of cells: average # of cells in each square × dilution factor (2 in this case) × 1×10^4. This gives the concentration of cells per mL. Multiply by the number of total mLs to determine the total number of cells.

8. After the final spin, decant the medium and leave the tube upside down to allow additional droplets to exit the tube. This also prevents the droplets from mixing with the pellet.

9. Use a UV-irradiated sterile light duty wipe to remove excess liquid inside 50mL conical.

   Work quickly and do not let the pellet dry.

10. Resuspend the cells to a concentration of 100,000 cells/μL by adding complete hNPC medium, transfer to a 1.7mL conical tube and store on ice until ready to transplant.

    Because the cells take up volume, add about half of the total calculated volume to the pellet, then add small amounts of medium until the desired concentration is obtained.

Step annotations

SUPPORT PROTOCOL 1 (optional)

SUPPORT PROTOCOL 1: Verifying NPC phenotype of hNPCs: It is important that additional cells are plated in chamber slides that can later be stained at the time of isolation

for transplant to verify that the cells are NPCs. Markers for NPCs include the neural multipotency-associated markers Nestin and PAX6, which are not expressed in embryonic stem cells (ESCs)(Yuan et al., 2011). Additionally, the cells should be OCT4 (POU5F1) negative; OCT4 is a pluripotency marker expressed on ESCs (Boiani and Scholer, 2005). NPCs should also be negative for GFAP (astrocytes), PDGFRalpha (oligodendrocytes) and MAP2 (neurons), as this would indicate further differentiation and loss of multipotency (Chojnacki et al., 2011; Jacque et al., 1978; Shafit-Zagardo and Kalcheva, 1998).

Materials

Reagents and Solutions:

Poly-L-ornithine

Laminin

Complete hNPC medium

1x PBS

1% Paraformaldehyde

1x PBS + 0.5% (v/v) BSA

Goat serum (Vector Laboratories, cat. no. Y0322)

Triton-X100 (Sigma, cat. no. X100)

Antibodies:

- Nestin (Rabbit polyclonal; Millipore, cat. no. ABD69)
- Pax6 (Rabbit polyclonal; Covance, cat. no. PRB278P)
- Goat-anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, cat. no. 111-005-144)
- Goat-anti-Rabbit secondary
  - Alexa 594-conjugated (Invitrogen, cat. no. A11037)
  - Alexa 488-conjugated (Invitrogen, cat. no. A11008)

Dapi Fluormount-G (SouthernBiotech, cat. no. 0100-20)

Equipment:

4 well chamber slides (glass; Lab-Tek; cat. no. 154526)

Tissue culture incubator, 37°C, 5% CO2, in humidified air

Chemical fume hood

Humidified chamber

Coverslips

Inverted fluorescent microscope with 40x objective.

Protocol steps

1. Plate 3×10^4 hNPCs in each well of poly-L-ornithine/laminin-coated chamber slides (see Basic Protocol 1) with 0.5mL complete hNPC medium and incubate for 3–4 days.

   Change the medium daily.

2. Aspirate medium and wash two times with 0.5mL 1x PBS.

3. Fix the cells by treating with 1% paraformaldehyde for 15 minutes at room temperature. Following fixation, aspirate paraformaldehyde and wash two times with 1x PBS.

   Make sure to perform the fixation step in a chemical fume hood as paraformaldehyde is a toxin that may be aerosolized.

4. Block with 0.5mL/well 1x PBS + 0.5% (v/v) BSA + 10% (v/v) goat serum + 0.5% (v/v) Triton-X100 for 1 hour at room temperature.

   The use of serum is necessary to block non-specific binding of the fluorescently-tagged secondary antibody. Use serum from the animal species of the secondary antibody; in this case use goat.

5. Add Nestin antibody in 0.5mL/well in 1x PBS + 0.5% (v/v) BSA + 10% (v/v) goat serum and incubate in a humidified chamber overnight at 4°C.

   The antibodies will be added sequentially, starting with Nestin.

   Dilute Nestin antibody at 1:200. 4 wells are needed; one with Nestin antibody only, one with PAX6 antibody only, one with both, and one with neither primary antibody. All of the wells will receive secondary antibodies. The purpose of the secondary-only control is to determine if there is non-specific binding of the secondary antibodies. For the wells that receive only one primary antibody proceed through the sequential steps as if you were adding both antibodies.

   The humidified chamber can be made by using wet light duty wipes or paper towels in a covered container.

6. After overnight incubation, aspirate and wash wells three times with 1x PBS.

   Be careful to not pipette the 1x PBS too harshly onto the cells as this can cause them to lift off of the plastic. Gently add it to the side of the well.

7. Add secondary antibody in 0.5mL/well in 1x PBS + 0.5% (v/v) BSA for 1 hour at room temperature.

   Dilute secondary antibody 1:1000. Use Alexa594-conjugated goat anti-rabbit for Nestin.

8. After incubation, aspirate and wash three times with 1x PBS.

9. Block for 1hr at room temperature in the humidified chamber with goat-anti-Rabbit IgG in 1x PBS + 0.5% (v/v) BSA.

   Use goat-anti-Rabbit IgG at 1:100. This is to block non-specific binding of the antibody to the first secondary.

10. Add PAX6 antibody in 0.5mL/well in 1x PBS + 0.5% (v/v) BSA + 10% (v/v) goat serum and incubate in a humidified chamber overnight at 4°C.

    Dilute PAX6 antibody at 1:200.

11. After overnight incubation, aspirate and wash wells three times with 1x PBS.
12. Add secondary antibody in 0.5mL/well in 1x PBS + 0.5% (v/v) BSA for 1 hour at room temperature.

    Dilute secondary antibody 1:1000. Use Alexa488-conjugated goat anti-rabbit for PAX6.
13. Carefully remove chambers and coverslip using Dapi Fluormount-G mounting medium.

    Dapi stains cell nuclei and can be visualized with excitation in the ultraviolet range and emission in the blue range.

    This mounting medium is self-hardening. It will harden overnight at room temperature. Leave the slides at room temperature; do not put the slides in a humidified chamber or at 4°C or the mounting medium will not harden.
14. Visualize slides on an inverted fluorescent microscope.

ALTERNATE PROTOCOL 1 (optional)

ALTERNATE PROTOCOL 1: Preparation of mNPCs for intraspinal transplantation: Preparation of mNPCs for transplantation is similar to hNPCs. The mNPCs described in this unit are derived from the cortex of brains from d1 postnatal C57Bl/6 mice expressing eGFP in all cells. The isolated eGFP-expressing mNPCs (GFP-mNPCs) have been selected to grow serum and growth matrix-free in non-coated plastic flasks. The presence of eGFP allows these cells to be easily tracked in the spinal cord following transplantation (figure 3).

Materials

Reagents and Solutions:

Complete mNPC medium (see recipe)

Trypsin-EDTA (Gibco, cat. no. 25300-054)

Dulbecco's Modified Eagle's Medium (DMEM)

1x HBSS

0.4% Trypan blue

Equipment:

Tissue culture hood: Class II A/B3

T75 flasks

Tissue culture incubator, 37°C, 5% CO_2, in humidified air

50mL conical tube

Refrigerated Table top centrifuge, 1000–1500rpm

Hemacytometer with cover slip

Inverted phase/contrast microscope

Light duty wipe (Kimwipe)

1.7mL conical tube

Protocol steps

1. Plate approximately 2.5×10^6 GFP-mNPCs in a T75 flask with 20mL complete mNPC medium and incubate at 37°C for 3–4 days.

   2.5×10^6 GFP-mNPCs will yield approximately 15–25$\times10^6$ GFP-mNPCs after 3–4 days. *Change the medium every other day.*

2. Dissociate the GFP-mNPCs with 2mL 0.05% Trypsin-EDTA at room temperature for approximately 30 seconds.

   Tapping the side of the flask will facilitate dissociation of the cells.

3. Quench the dissociation with at least 10mL of cold complete mNPC medium or DMEM and transfer to a 50mL conical tube.

4. Centrifuge the cells at 1500rpm at 4°C for 5 minutes.

   Pre-cool the centrifuge since cold temperature when trypsin is present is necessary for optimal cell viability.

5. Wash GFP-mNPCs 3x with 20mL 1x HBSS.

   After each wash decant the medium and prior to the last wash remove a 10μL aliquot to count cells (see Basic Protocol 1 for instructions on counting cells).

6. After the final spin, decant the medium and leave the tube upside down to allow additional droplets to exit the tube. This also prevents the droplets from mixing with the pellet.

7. Use a UV-irradiated sterile light duty wipe to remove excess liquid inside 50mL conical.

   Work quickly and do not let the pellet dry.

8. Resuspend the cells to a concentration of 100,000 cells/μL by adding sterile 1x HBSS, transfer to a 1.7mL conical tube and store on ice until ready to transplant.

   Because the cells take up volume, add about half of the total calculated volume to the pellet, then add small amounts of medium until the desired concentration is obtained.

Basic Protocol 2: Preparation of Mice for Intraspinal Transplantation

Systematic preparation of mice for intraspinal transplantation is necessary to allow for efficient transplantation of cells. Utilizing a team "assembly line" system will maximize efficiency. The mice can be passed to each station consisting of mouse preparation/shaving, laminectomy, injection, and sutures. This will minimize time between cell preparation and injection into mice; prolonged time on ice will reduce cell viability. The following describes the preparation of the mouse from anesthetization through laminectomy.

Materials

Reagents and Solutions:

Ketamine Hydrochloride (Ketaject; Western Medical Supply, cat. no. 4165)

Xylazine Hydrochloride (MP biomedical, cat. no. 158307)

Hair removal cream (Nair)

Soapy water

Iodide solution (Betadine surgical scrub; Fisher cat. no. 19-027132)

Vaseline

70% ethanol (optional)

Sterile saline

Equipment:

Colored tape

Electric hair clipper

Weigh dish

Gauze-tipped applicator

Gauze square

Laminar flow cabinet

Tri-fold paper towels

Fiber optic illuminator (Fisher Scientific, cat. no. 12-562-36)

Dry glass bead sterilizer (Steri 350, Simon Keller AG, cat. no. 06-12287)

50mL glass beaker (optional)

Small graefe forceps (Fine Science Tools, cat. no. 11053-10) Note: Graefe forceps have an array of small teeth at the ends.

Scalpels: #10 and #15 blades

Micro-scissors (World Precision Instruments, cat. no. 555500S)

Sterile gauze-tipped applicator

Sterile gauze square

Protocol steps

1. Put aliquot of hair removal cream, iodide solution, and Vaseline in separate weigh boats to avoid contamination of bottles.

   Iodide solution can be diluted with water if excessively thick.

2. Anesthetize a group of four mice with an intraperitoneal injection of 100mg/kg ketamine + 10mg xylazine in an approximately 150 µl dose per mouse.

Groups of four mice work best since the syringe for cell injection can hold enough for four mice.

Wait at least 7 minutes before touching mice or proceeding to the next step. Prematurely moving to the next step will not allow mice to be fully anesthetized and results in mice moving during surgery. Lightly pinch the rear paw; if mouse jumps the mouse is not ready.

3. Use the electric clippers to shave the hair on the middle of each mouse's back from approximately lumbar vertebrae five to thoracic vertebrae one.

   Shave hair as close to the skin as possible.

4. Use gauze-tipped applicator to apply a generous amount of hair removal cream to the shaved area of the mouse to remove residual hair. Let hair removal cream sit for approximately one minute.

   During this time another group of mice can be anesthetized.

5. Hold the mouse near the tail and use a wet gauze square to wipe away the hair removal cream by gripping the skin and wiping toward the head.

6. Use a soapy wet gauze square to remove residual hair removal cream and any loose hairs.

7. Use a gauze-tipped applicator to apply a thin layer of iodide solution.

8. Use a gauze-tipped applicator to apply Vaseline to eyes to protect them from drying out during anesthetization.

9. Cut a small piece of colored tape and attach it to the end of the mouse's tail and place a matching piece of colored tape on the mouse cage.

   Since four mice are anesthetized at a time and there are not always four mice in a cage this color-coded tape will ensure the mouse returns to the correct cage. Placing the tape and the very end of the mouse tail will allow for it to come off easily.

10. Carefully move the mice to the laminectomy station inside a laminar flow cabinet.

11. Prepare the laminectomy station. Use tri-fold paper towels as a platform for the mouse during the laminectomy. Set up the fiber optic illuminator so it will point directly at the mouse's back. Sterilize the tools by placing them in glass dry bead sterilizer for 10–15 seconds at approximately 220°C. Let the tools cool to room temperature before using.

    Optional: you can further sterilize and quickly cool the tools after using the glass dry bead sterilizer by placing the tools in a 50mL glass beaker with 70% ethanol. You can put a piece of gauze in the bottom of the beaker to avoid bending or dulling the tips of the micro-scissors on the bottom of the beaker.

12. Position mouse dorsal side up on the tri-fold paper towel. Use forceps to hold the skin at the rostral side of the shaved area and use a scalpel with a #10 blade to make an approximate 1.3cm incision spanning from T8 to T11.

    The head of the mouse should point to the left if you are right handed.

13. While holding the mouse in place near the scapulae with your left hand, gently push the rear of the mouse forward with your right hand to exaggerate the curvature of the spinal cord.

Locate T10, which is the peak of the curvature.

14. Count two vertebrae rostral to T10 and firmly secure the spinal column with the graefe forceps (left hand) by gripping underneath the pedicles at T8.

    The pedicles can be identified as the spiny protrusions along the side of the spinal column.

    Do not grip too tightly as this can cause unnecessary damage to the tissue. The pedicles should gently rest on the tips of the graefe forceps.

    Depending on the age and how much fat the mouse has you may have to move the fat above the spinal thoracic and trapezius muscles out of the way to see the pedicles of T8.

15. While holding the spinal column at T8 use scalpel #15 to score behind and around the pedicles of T10. Carefully scrape the junction between T9 and T10 to reveal the bony top of the lamina.

    The fine curvature of scalpel #15 will allow you precisely score around the pedicles of T10. You will be able to feel the bony protrusions with the side of the scalpel blade.

    Be careful not to score too deeply as you could risk cutting into the spinal cord.

16. Once the pedicles and top of T10 are visible use the micro-scissors to cut away the pedicles.

    Hold the micro-scissors at a 45° angle lateral to the spinal column with the curved end of the scissors pointing dorsally. Carefully slide the scissors under each pedicle and cut with a small snip.

    Make sure not to cut too deeply or too laterally as this might cut the vasculature running along side of the spinal column and cause excessive bleeding. Excessive blood will make it difficult to see the spinal cord for injection. If there is bleeding you can use a sterile gauze-tipped applicator to absorb the blood.

17. Carefully slide the micro-scissors under T10 lamina and cut each side of the lamina with small snips.

    Again be careful not to cut too deeply or laterally. Also be careful to hold the points of the micro-scissors angled dorsal enough so they do not puncture or nick the spinal cord. If the spinal cord is punctured or nicked it will be visible as a dark red or purple mark.

18. The lamina should lift up as you snip the sides, but if not, carefully slide the micro-scissors under T10, cutting away tissue holding down the lamina. Once the tissue is trimmed, carefully lift the lamina.

19. Release the graefe forceps holding T8 and delicately grip the lamina so it can be held up while you trim it off by snipping behind the pedicles of T9.

    This grip and cut is easier if you turn the mouse slightly so the head is away from you and tail is toward you.

20. Cover the exposed lamina with a sterile gauze square while the mouse waits to be injected.

Again, if there is excessive bleeding absorb it with a sterile gauze-tipped applicator.

Make sure the gauze square is not touching the spinal cord as it may stick as blood dries.

If the incision area begins to dry a few drops of sterile saline can be applied.

Basic Protocol 3: Intraspinal Transplantation of mNPCs or hNPCs and Post-Operative Care

The following protocol directly follows the laminectomy described in the previous protocol. When done efficiently in concert with the previous protocol, a team can transplant approximately 40 mice in three hours. While the methods described are for injection of up to 2.5×10^5 mNPCs or hNPCs in 2.5µl (1×10^5 cell/µl) these methods can be used to inject other cell types into the spinal cord at T10.

Materials

Reagents and Solutions:

Sterile 1x HBSS

ddH_2O

70% ethanol

Lactated Ringers (Hospira, cat. no. NDC 0409-7953-03)

Buprenorphine (Buprenex; Western Medical Supply, cat. no. 7292)

Equipment:

Pipette (p200 and p20)

Filter tips

50mL conical tubes

10µL Hamilton syringe with removable plunger (Hamilton Company, cat. no. 7635-01)

Hamilton needles (30 gauge needle, point style 4, 30 degree bevel) (Hamilton Company, cat. no. 7803-07)

Hemostat (Fine Science Tools, cat. no. 13010-12)

Stereotaxic apparatus (Kopf instruments) including: universal holder with needle support foot (cat. no. 1772), electrode holder with removable open side clamp (cat. no. 1773), dual small animal stereotaxic platform (cat. no. 902)

Tri-fold paper towels

Olsen-Hegar needle holder (Fine Science Tools, cat. no. 12502-12)

Sutures (size 5-0, 3/8" circle, 19mm needle, 45cm braided thread)(Ethicon, cat. no. 1676G)

Reflex 7 Wound Clip Applicator (Fine Science Tools, cat. no. 12031-07)

10mL syringe

18G 1/2" needle

Protocol steps

1. Setup stereotaxic apparatus by attaching the electrode holder on the left side of the small animal stereotaxic instrument and the universal holder with needle support foot on the right side of the animal stereotaxic instrument. See figure 2 for apparatus attachment configuration settings.
2. Place tri-fold paper towels as a platform for the mouse on the stereotaxic apparatus in between the two holders.
3. Lightly spray apparatus and paper towels with 70% ethanol to sterilize.
4. Fill three 50mL conical tubes separately with ddH^20, 70% ethanol, or 1x HBSS and place in a test tube holder.
5. Tightly attach the Hamilton needle to the Hamilton syringe and clean syringe and needle by flushing 150–200μl of ddH20 through it five times. In between each flush insert the plunger to push the water through the syringe. This also acts to clean the plunger.

   Using the p200 and filter tips, make sure the tip is firmly inserted in the syringe so all the water goes into the syringe. Because the syringe is only 10μL it will take approximately 10 seconds to flush all the water through the syringe. You can depress the pipette to the second stop to aid in the flow.
6. Repeat the previous step with 70% ethanol five times and then 1x HBSS five times.
7. Load 13–15μL of cell suspension into the Hamilton syringe using a p20 pipette and filtered tip.

   mNPCs and hNPCs are large, causing them to sink to the bottom of the small conical tube. Use a p200 pipette to gently resuspend the cells before loading the Hamilton syringe.

   Before loading the cells loosen the needle nut slightly. This will prevent backpressure, which can cause the cells to leak out the back end of the syringe before you can place the plunger in. As with washes place the pipette tip firmly in the back of the syringe. If the cells are not loading into the syringe, loosen the needle nut a little more. If there is air between the cells and the back end of the syringe once the pipette tip is removed a small slow tightening of the needle nut will move the cells back, eliminating the air. Finally, place the plunger in the back of the syringe, tighten the needle nut completely and move the plunger to the 10μL mark. A small drop of cells should appear at the end of the needle. This can be removed by carefully touching the drop to a sterile gauze square. Be careful that the needle does not touch anything.

   When tightening the needle nut turn the bevel of the needle so it will face to the left when the syringe is loaded on the stereotaxic apparatus.
8. Inspect the syringe for air bubbles or clumps.

If there are air bubbles or clumps discard the cells and reload. While this doesn't happen often when cells are loaded appropriately, this is why a buffer amount of cells is needed when setting up your experiment.

Leave the needle in a horizontal position until you are ready to inject to avoid movement of cells in the syringe.

9. Use the dry glass bead sterilized hemostat to grip the laminectomized mouse at the spinalis dorsi muscle that connects vertebrae T7 and T8.

   Take care not to grip to deeply as this might cause unnecessary tissue damage, or too superficially, as this can cause the mouse to slip out of the hemostat during injection.

10. Clamp the hemostat to the left electrode holder so that the mouse's front paws are in the air and its rear paws are lightly touching the tri-fold paper towels.

11. Attach the syringe to the right universal holder with needle support foot.

    The universal holder should be positioned so the needle is at a 70° angle

    Turn the needle so the numbers are facing toward you and the bevel is facing to the left. The needle should be all the way down into the needle holder. Take care that the plunger does not touch anything on the stereotaxic apparatus as this might cause cell loss from the syringe.

    Make sure the right universal holder is locked after loading the syringe on the stereotaxic apparatus as you will need to loosen this and swing the arm in order to load and unload the syringe.

12. Carefully stabilize the mouse by gently holding the base of its tail to the tri-fold paper towels.

13. Slowly lower the needle until the tip of the needle is just above the dorsal midline and insert the needle approximately 1mm into the spinal cord. The tip of the needle should be in the left gray matter close to the central canal.

    The spinal cord will initially give resistance to the needle. Continue to lower the needle until the needle pierces the spinal cord.

14. Slowly inject 2.5μL of cells at a rate of approximately 1mL/ 5 seconds. After cells have been injected wait approximately 10 seconds and then slowly retract the needle by turning 1/8th of a turn every few seconds.

    Slow injection of the cells and slow retraction of the needle will help prevent efflux of the cells.

15. Loosen the right universal holder and move the syringe away from the mouse so you can easily remove the syringe. Place the syringe horizontal on a sterile surface until the next moue is ready.

    This should be done in between each mouse to avoid clumping of the cells in the syringe. The right universal holder should be loosened and swiveled to allow for easy removal of the syringe.

16. Remove the mouse from the left electrode holder and remove the hemostat from the mouse. Gently and carefully move the mouse to the suture station.

17. After four mice have been completed the syringe and needle should be cleaned with at least two washes each of ddH_2O, 70% ethanol, and 1x HBSS before being loaded with more cells.

This ensures any cell clumps are removed and prevents the plunger from sticking, causing it to bend.

If cell types are being changed five washes of ddH_2O, 70% ethanol, and 1x HBSS should be done.

18. Suture the incision by connecting the superficial fascia on both sides of the incision with two or three knots to cover the exposed spinal cord.

19. Close the skin by applying two or three staples.

Carefully pull the skin away from the mouse to avoid accidentally stapling the muscle. Also, do not staple too tightly, as this can cause the skin to become necrotic as it loses its blood supply.

20. Inject 0.5mL of lactated ringers subcutaneously on the rear right flank of the mouse using a 10mL syringe and 18G 1/2" needle.

Lactated ringers is administered to restore fluid lost due to blood loss during the surgery and can counteract the effects of acidosis that may occur during surgery.

21. Inject Buprenorphine at a dose of between 0.05 to 0.1mg/kg on the rear left flank of the mouse using a 10mL syringe and 18G 1/2" needle.

This is generally between 300 and 500μL.

Buprenorphine is a semi-synthetic opioid that is used as a pain management plan to treat acute pain in the mice caused by the surgery.

22. Remove tape tag from the end of the mouse's tail and return the mouse to its cage. Place the mouse on its side to avoid suffocation.

23. Clean the syringe and needle for storage by flushing 5x each with 1x HBSS, 70% ethanol, and ddH_2O.

This is the reverse of the order for cleaning the syringe and needle for use. Cleaning with ddH_20 last eliminates salts from the 1x HBSS in the syringe and needle.

REAGENTS AND SOLUTIONS

Complete hNPC medium

DMEM/F12 + GlutaMax (Gibco, cat. no. 10565-018)

0.5X N2 (Gibco, cat. no. 17502-048)

0.5X B27 (Invitrogen, cat. no. 17504044)

1X Penicillin/Streptomycin (Fisher, cat. no. MT-30-001-CI)

20ng/ml human fibroblast growth factor (hFGF; Millipore, cat. no. GF003)

20ng/ml human epidermal growth factor (hEGF; Invitrogen, cat. no. 13247-051)

Complete mNPC medium

DMEM/F12+Glutamax

10ng/ml Ciproflox (Cellgro, cat. no. 61-277-RF)

50ng/ml Gentamicin (Sigma, cat. no. G1397)

1x Fungizone (Invitrogen, cat. no. 15290018)

1x Penicillin/Streptomycin

1x N2

20ng/ml hEGF

70% ethanol

Diluted with ddH_2O from 100% ethanol

Ketamine + xylazine

Dissolve 0.01g xylazine in 7.1mL sterile 1x HBSS. Use a syringe to obtain 0.9mL ketamine from sealed bottle and dilute in the above HBSS/xylazine solution. Filter solution through 0.22μm sterile filter and wrap in foil to avoid light exposure.

COMMENTARY

Background Information

NPCs have enormous potential for treatment of human neurologic diseases. Indeed, recent studies have demonstrated that transplantation of hNPCs into mice with severe dysmyelination results in the formation of functional myelin (Uchida et al., 2012). Moreover, Rowitch and colleagues (Gupta et al., 2012) have built upon these findings and have shown that engraftment of hNPCs into children with the rare leukodystrophy, Pelizaeus-Merzbacher disease (PMD) results in new myelin formation. These studies add to a growing literature supporting the use of stem cells for treating diseases involving myelin damage including the human demyelinating disease MS (Reekmans et al., 2012). Transplantation of neural progenitor cells promotes clinical recovery, axonal protection and formation of new myelin and impairs the spread of demyelination in both mouse and rat models of neuroinflammatory-mediated demyelination (Ben-Hur et al., 2003; Brustle et al., 1999). Intraspinal transplantation has been shown to be an effective method for delivering both mNPCs and hNPCs. We have shown mNPCs efficiently migrate, proliferate, differentiate, and remyelinate following intraspinal transplantation into mice persistently infected with the neurotropic JHM strain of mouse hepatitis virus (JHMV)(Carbajal et al., 2010; Hardison et al., 2006; Totoiu et al., 2004). These studies have allowed a better understanding of how location, microenvironment, and pathologic conditions influence clinical and histologic outcome of NPC transplantation.

Critical Parameters and Troubleshooting

Cell viability is the most critical aspect of the preparation protocol and can have dramatic effects on the data obtained following spinal cord transplantation. Transplantation of an excessively high number of dead or dying cells may skew the immune response resulting in enhanced recruitment of macrophages to remove the debris. In addition, the presence of a large number of dead cells could result in an increased inflammatory response that can be detrimental to healthy spinal cord tissue. Therefore, maintaining cell viability following dissociation from culture dishes is imperative to ensure the greatest possible experimental success. Using cold medium to quench enzymatic dissociation, centrifuging at 4°C, and keeping cells on ice offers the best short-term solution for greatest viability. If transplants are going to take more than three hours, the procedure should be split into two sessions with freshly isolated cells for each session. Optimal cell viability prior to transplant is 85% or greater; if there is an excessive number of dead cells, fresh cells should be prepared. Besides temperature, another potential cause of cell death is leaving cells in dissociation enzyme too long before quenching. During the 10 minute dissociation incubation, one can periodically view the cells under a phase-contrast microscope to see if cells are ready and at which point the flasks can be gently tapped to aid in cell dissociation.

Other issues that can affect transplant results occur during the preparation of the mouse and subsequent injection, including spinal cord damage and cell efflux. Damage to the spinal cord can occur during laminectomy if the micro-scissors nick the spinal cord. This usually occurs when sliding the micro-scissors under the lamina in order to snip the sides of the lamina. While large cuts into the spinal cord are obvious, small nicks or stabs can be visualized as red or purple mark in the spinal cord. To minimize the chance of this make sure the points of the curved micro-scissors are facing away from the cords. Also, make sure there are no sharp bone fragments left behind after cutting away the lamina as these could cause damage to the spinal cord with movement by the mouse. If micro-scissors are dull, sharp bone fragments are more likely to occur. Moreover, sharp micro-scissors allows for cleaner cuts and this reduces the necessity for excessive snipping and trimming of bone and tissue that can cause bone fragments. Cell efflux can occur as a result of injecting the cells too rapidly or removing the needle from the spinal cord too quickly. The injection and retraction speed listed in the protocol should be followed to avoid these events. It should be noted that hNPCs do not enter the tissue as readily as mNPCs and as a result tend to efflux easier. Extreme caution is needed when transplanting hNPCs that the needle is not retracted too quickly. A 30-gauge needle is described in this protocol; however, a 33-gauge needle will result in less efflux. Not all cells will be capable of passing through a 33-gauge needle without being lysed. Test your cells by running a typical cell suspension through both size needles to ensure there is no loss of cell viability.

Anticipated Results

The method of preparing and isolating NPCs for intraspinal transplantation should yield highly viable cells. When syngeneic mNPCs are transplanted, they migrate, proliferate, and differentiate into myelinating oligodendrocytes in the demyelinated spinal cord. Transplanted mNPCs under demyelinating conditions differentiate and a majority of them stain positive for immature (PDGFRalpha) and mature (GSTpi) oligodendrocytes (Carbajal et al., 2010). As described, eGFP expressed by genetically engineered mNPCs can be used as a marker to evaluate transplant efficiency. Note that to ensure that the eGFP signal is marking only the transplanted cells, co-labeling for endogenous phagocytic cells such as Iba1 (microglia/macrophages) and CD11c (dendritic cells) can be done to verify eGFP signal is not from resident cells that engulfed eGFP (Carbajal et al., 2010).

Time Considerations

It is important to time the plating of hNPCs or mNPCs so their expansion to obtain enough cells for transplantation coincides with the transplant date. For example, in our viral model of demyelination, cells are transplanted at the peak of demyelination, typically day 14 post-infection. Coating of plates should be done 4–5 days prior to the day of transplantation and plating of cells 3#x02013;4 days prior. Preparation of cells for transplantation, including washing, counting, and resuspension takes approximately one hour. To save time, preparing anesthesia and counting cells can be done during wash spins. The entire transplant from preparing the mouse to suturing the mouse is done most efficiently as a team. A skilled four-person team can transplant 40 mice in approximately three hours.

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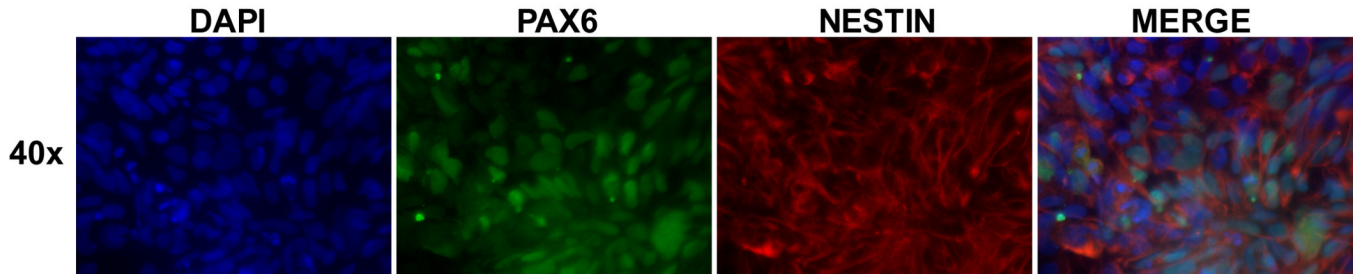

Figure 1.
Fluorescent microscopy of hNPCs at time of dissociation for preparation for intraspinal transplantation. PAX6 and Nestin are markers for multipotent neural precursor cells and Dapi stains cell nuclei. Images are shown at 40x magnification.

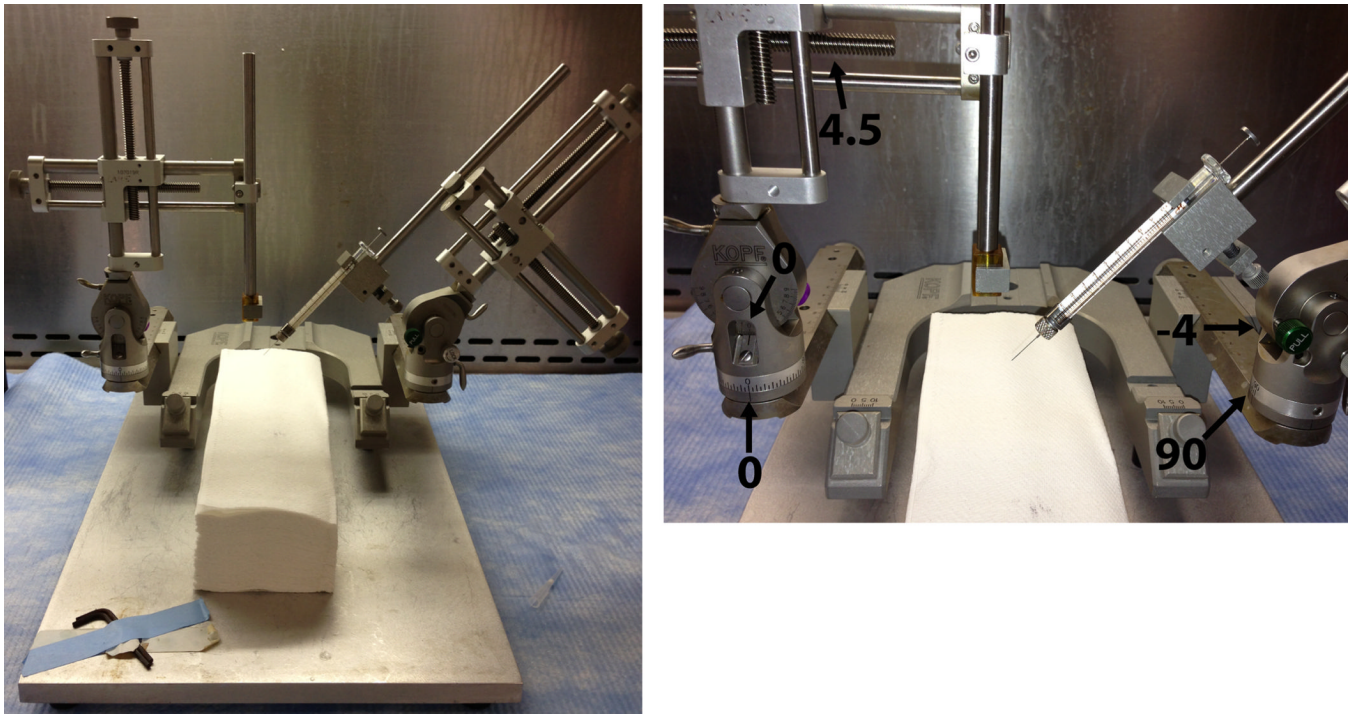

Figure 2.

Setup of stereotaxic apparatus with universal holder with needle support foot on right and electrode holder with removable open side clamp on left of dual small animal stereotaxic platform and tri-fold paper towels. The image shows the standard settings for the stereotaxic apparatus to align the needle with the spinal cord at a 70° angle.

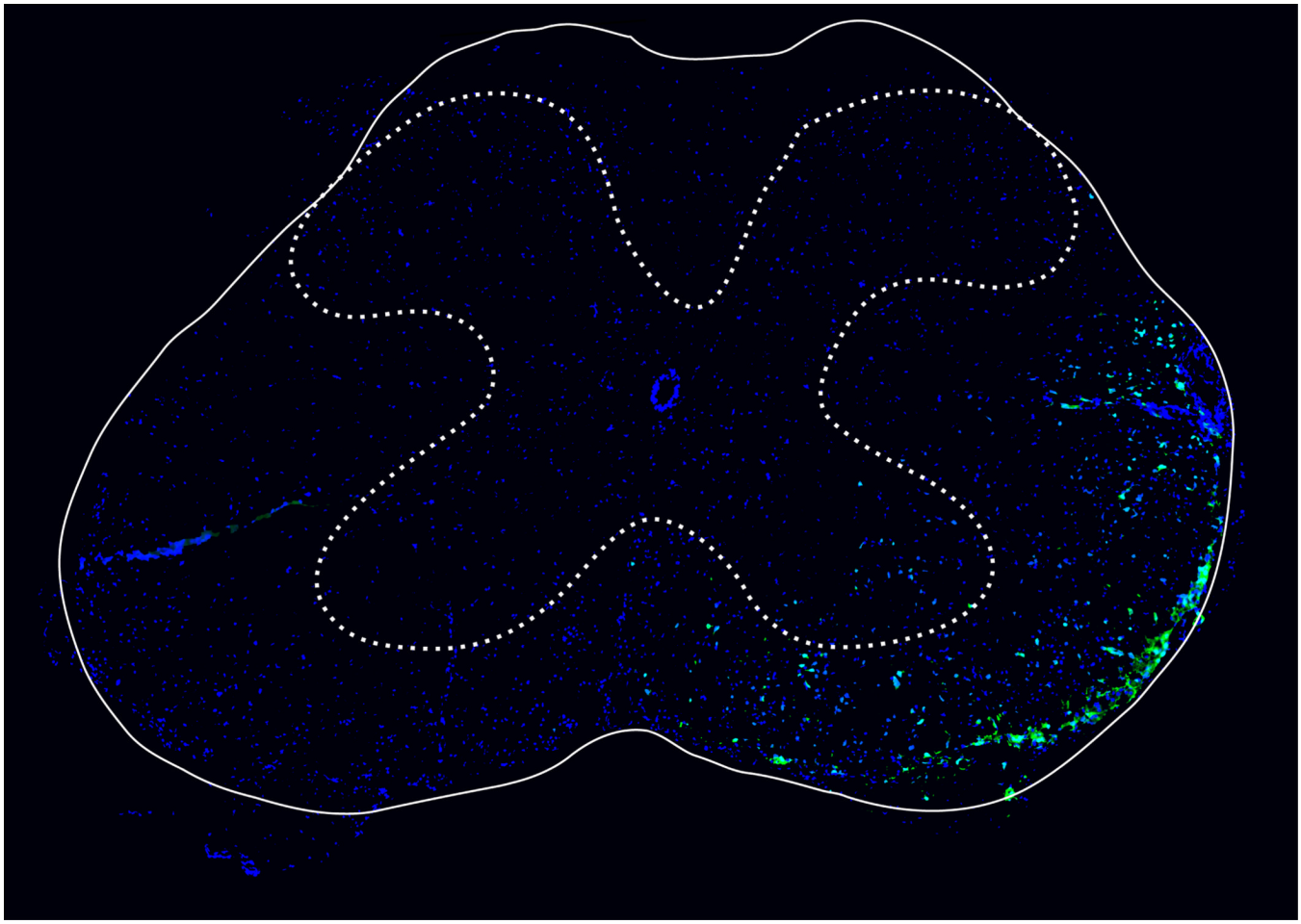

Figure 3.
Coronal section of a C57Bl/6 mouse transplanted intraspinally with GFP-NPCs. Mouse was sacrificed at day 21 post-transplant, 4% paraformaldehyde perfused and 10 micron coronal sections were cut. Transplanted GFP-NPCs survived and migrated into the white matter. Engrafted GFP-NPCs are green and Dapi-stained cell nuclei are blue.