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Neural Stem Cells

Methods and Protocols

Edited by

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Preface

Over the last decade, neural stem cell research has provided penetrating insights into the plasticity and regenerative potential of the brain. Stem cells have been isolated from embryonic as well as adult central nervous system (CNS). Many non-CNS mammalian tissues also contain stem cells with a more limited repertoire: the replacement of tissue-specific cells throughout the lifetime of the organism. Progress has been made in understanding fundamental stem cell properties that depend on the interplay of extrinsic signaling factors with intrinsic genetic programs within critical time frames. With this growing knowledge, scientists have been able to change a neural stem cell's fate. Under certain conditions, neural stem cells have been induced to differentiate into cells outside the expected neural lineage and conversely, stem cells from nonneural tissue have been shown to transdifferentiate into cells with distinct neural phenotypes.

At the moment, there is an accelerated effort to identify a readily available, socially acceptable stem cell that can be induced to proliferate in an undifferentiated state and that can be manipulated at will to generate diverse cells types. We are on the threshold of a great new therapeutic era of cellular therapy that has as great, if not greater, potential as the current pharmacologic era, glorified by antibiotics, anesthetics, pain killers, immunosuppressants, and psychotropics. Cellular therapeutics carries the promise of replacing missing neurons, but also may serve to replenish absent chemical signals, metabolites, enzymes, neurotransmitters, or other missing or defective components from the diseased or injured brain. Cellular therapies may provide the best vehicle for delivery of genetic material for treatment of hereditary diseases.

Although a great deal of data has been gathered and insights have been provided by researchers around the world, we are still in the dark about fundamental processes that determine cell fate or that maintain a cell's "stemness." To take some of the mystery out of this field and to provide a practical guide for the researcher, we have collected straightforward methods and protocols used by outstanding scientists in the field. Our primary goal is to facilitate research in neural stem cell biology by providing detailed protocols to both stimulate and guide novices and veterans in this area.

We divided *Neural Stem Cells: Methods and Protocols* into three broad sections. The first section, "Isolation and Culture of Neural Stem Cells" introduces the reader to different sources of stem/progenitor cells and provides a wide range of conditions for their selection, nourishment, growth and survival in culture. The second section, "Characterization of Neural Stem Cells in vitro" is a collection of the cellular, electrophysiological, and molecular techniques required to define the characteristics of neural stem cells in culture. The third section, "Utilization/Characterization of Neural Stem Cells in vivo," is a collection of techniques to identify and characterize endogenous stem cells as well as exogenous stem cells after transplantation into the brain.

At this stage in Neural Stem Cell Biology , we have relied on the available state-of-the-art techniques to define the properties of these cells and to test their inherent plasticity. We hope that this collection of methods and protocols, ranging from simple to sophisticated in complexity, will serve as a handy guide for stem cell scientists. We expect that the user will develop even more advanced techniques and strategies in this field. Like a good cookbook full of recipes and cooking instructions, we are confident that experimentation with these procedures may generate even better results suited to the particular goals of the researcher.

We would like to acknowledge Professor John M. Walker who initially suggested we put together this book and then later advised us throughout the editorial process. We greatly appreciate the suggestions and encouragement from Dr. Mahendra S. Rao. We especially thank Marcia McCall for her caring assistance, attention to detail, and long hours invested into compiling this volume.

Tanja Zigova, PhD
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Contents

Preface v
Contributors xi

PART I ISOLATION AND CULTURE OF NSCs

1 Neural Differentiation of Embryonic Stem Cells
***K. Sue O'Shea* 3**

2 Production and Analysis of Neurospheres from Acutely Dissociated and Postmortem CNS Specimens
***Eric D. Laywell, Valery G. Kukekov, Oleg Suslov, Tong Zheng, and Dennis A. Steindler* 15**

3 Isolation of Stem and Precursor Cells from Fetal Tissue
***Yuan Y. Wu, Tahmina Mujtaba, and Mahendra S. Rao* 29**

4 Olfactory Ensheathing Cells: *Isolation and Culture from the Rat Olfactory Bulb*
***Susan C. Barnett and A. Jane Roskams* 41**

5 Culturing Olfactory Ensheathing Glia from the Mouse Olfactory Epithelium
***Edmund Au and A. Jane Roskams* 49**

6 Production of Immortalized Human Neural Crest Stem Cells
***Seung U. Kim, Eiji Nakagawa, Kozo Hatori, Atsushi Nagai, Myung A. Lee, and Jung H. Bang* 55**

7 Adult Rodent Spinal Cord Derived Neural Stem Cells: *Isolation and Characterization*
***Lamya S. Shihabuddin* 67**

8 Preparation of Neural Progenitors from Bone Marrow and Umbilical Cord Blood
***Shijie Song and J. Sanchez-Ramos* 79**

9 Seeding Neural Stem Cells on Scaffolds of PGA, PLA, and Their Copolymers
***Erin Lavik, Yang D. Teng, Evan Snyder, and Robert Langer* 89**

PART II CHARACTERIZATION OF NSCs IN VITRO
A. CELLULAR TECHNIQUES

10 Analysis of Cell Generation in the Telencephalic Neuroepithelium
Takao Takahashi, Verne S. Caviness, Jr., and Pradeep G. Bhide ... ***101***

11 Clonal Analyses and Cryopreservation of Neural Stem Cell Cultures
Angelo L. Vescovi, Rossella Galli, and Angela Gritti ... ***115***

12 Assessing the Involvement of Telomerase in Stem Cell Biology
Mark P. Mattson, Peisu Zhang, and Weiming Fu ... ***125***

13 Detection of Telomerase Activity in Neural Cells
Karen R. Prowse ... ***137***

14 In Vitro Assays for Neural Stem Cell Differentiation
Marcel M. Daadi ... ***149***

15 Electron Microscopy and Lac-Z Labeling
Bela Kosaras and Evan Snyder ... ***157***

B. ELECTROPHYSIOLOGICAL TECHNIQUES

16 Techniques for Studying the Electrophysiology of Neurons Derived from Neural Stem/Progenitor Cells
David S. K. Magnuson and Dante J. Morassutti ... ***179***

C. MOLECULAR TECHNIQUES

17 Fluorescence *In Situ* Hybridization
Barbara A. Tate and Rachel L. Ostroff ... ***189***

18 RT-PCR Analyses of Differential Gene Expression in ES-Derived Neural Stem Cells
Theresa E. Gratsch ... ***197***

19 Differential Display: *Isolation of Novel Genes*
Theresa E. Gratsch ... ***213***

20 Cell Labeling and Gene Misexpression by Electroporation
Terence J. Van Raay and Michael R. Stark ... ***223***

21 Gene Therapy Using Neural Stem Cells
Luciano Conti and Elena Cattaneo ... ***233***

22 Modeling Brain Pathologies Using Neural Stem Cells
Simonetta Sipione and Elena Cattaneo ... ***245***

PART III UTILIZATION/CHARACTERIZATION OF NSCs IN VIVO

A. ENDOGENOUS POOLS OF STEM/PROGENITOR CELLS

23 Activation and Differentiation of Endogenous Neural Stem Cell Progeny in the Rat Parkinson Animal Model
Marcel M. Daadi* *265

24 Identification of Musashi1-Positive Cells in Human Normal and Neoplastic Neuroepithelial Tissues by Immunohistochemical Methods
Yonehiro Kanemura, Shin-ichi Sakakibara, and Hideyuki Okano* *273

25 Identification of Newborn Cells by BrdU Labeling and Immunocytochemistry In Vivo
Sanjay S. P. Magavi and Jeffrey D. Macklis* *283

26 Immunocytochemical Analysis of Neuronal Differentiation
Sanjay S. P. Magavi and Jeffrey D. Macklis* *291

27 Neuroanatomical Tracing of Neuronal Projections with Fluoro-Gold
Lisa A. Catapano, Sanjay S. P. Magavi, and Jeffrey D. Macklis* *299

B. TRANSPLANTATION

28 Labeling Stem Cells In Vitro for Identification of Their Differentiated Phenotypes After Grafting into the CNS
Qi-lin Cao, Stephen M. Onifer, and Scott R. Whittemore* *307

29 Optimizing Stem Cell Grafting into the CNS
Scott R. Whittemore, Y. Ping Zhang, Christopher B. Shields, Dante J. Morassutti, and David S. K. Magnuson* *319

30 Vision-Guided Technique for Cell Transplantation and Injection of Active Molecules into Rat and Mouse Embryos
Lorenzo Magrassi* *327

31 Transplantation into Neonatal Rat Brain as a Tool to Study Properties of Stem Cells
Tanja Zigova and Mary B. Newman* *341

32 Routes of Stem Cell Administration in the Adult Rodent
Alison E. Willing, Svitlana Garbuzova-Davis, Paul R. Sanberg, and Samuel Saporta* *357

Index ***375***

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I

Isolation and Culture of NSCs

1

Neural Differentiation of Embryonic Stem Cells

K. Sue O'Shea

1. Introduction

Differentiation of pluripotent embryonic stem (ES) cells into specific lineages is an important source of cells for implantation and gene delivery, as well as a useful model to study patterns of differentiation and gene expression during the very early development of the mammalian embryo ***(1)***. Embryonic stem cells are derived from the blastocyst inner cell mass ***(2,3)***, and remain totipotent when grown on the surface of embryonic fibroblasts or on gelatin-coated substrates in the presence of leukemia inhibitory factor (LIF). ES cells appear to have unlimited proliferative capability, and, remarkably, when returned to the inner cell mass after culture and gene manipulation, resume their development and participate fully in the formation of ALL tissue types. Recently, embryonic stem cells have been derived from human blastocysts after in vitro fertilization (IVF) ***(4,5)***. Pluripotent stem cells have also been derived from human primordial germ cells ***(6)***, with obvious clinical applications.

Studies of the differentiation potential of mouse ES cells have taken two major approaches: aggregation-mediated differentiation or direct differentiation. In the first, ES cells are grown in suspension culture in medium without LIF (± serum). After several days in vitro, often in the presence of the morphogen/teratogen retinoic acid, cells aggregate and a layer of endoderm surrounds a mass of differentiating cells, which has been termed an "embryoid body" ***(7)***. Embryoid bodies (EBs) are then plated on adhesive substrates, and after an additional 6–8 d in vitro, multiple differentiated derivatives including myocytes, neurons, endoderm, and keratinocytes form ***(7,8)***. When embryoid bodies are grown in defined medium to select against non-neural cells, the percentage of neural progenitors is greatly increased ***(9)***.

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Suspension culture of ES cells over several days produces a collagen-, fibronectin-rich basement membrane surrounding the aggregated cells, which inhibits diffusion of signaling molecules and growth factors into the interior of the aggregate. However, disaggregation of the EBs and plating as single cells on adhesive substrates with growth factors has improved the recovery of neuronal cells from these aggregates ***(9–11)***. Additional problems are due to the fact that individual cell lineages must be somehow separated from the aggregate, and by the time they can be identified, the earliest stages of differentiation are well past. Differentiation as EB has made it possible to ascertain the developmental potential of gene-targeted ES cells when gene deletion is embryo lethal. Implantation of EB into the CNS of injured or neurological mutant rodents, even though cells are heterogeneous, has successfully replaced both glia ***(12)*** and neurons ***(13)***.

Direct differentiation of ES cells can be accomplished by the forced expression of a developmental control gene such as *myoD* ***(14)***, *neuroDs* ***(15)***, or *Sox2* ***(16)***; or by epigenetic means such as culture in defined medium on adhesive substrates ± specific growth factors ***(9,11,17,18)***; or on bone marrow stromal cells ***(19)***. Unlike other stem cell populations, the technology for transfection and gene expression in ES cells is relatively well developed, so ES cells can be modified to (over)express signaling molecules of interest and receptors (or dominant/negative receptors) for them. Alternatively, putative differentiating agents can be added directly to the culture medium. ES cells have been transfected to express molecules involved in neural induction (e.g., *noggin*, ***20***), neural determination genes (*NeuroD3*; ***15***), or pan neuroepithelium/stem cell restricted genes (*nestin*) ***(21)***, driving expression of neo to create "neural progenitor" cell lines, that can then be tested for their growth factor responsiveness and downstream gene expression patterns.

2. Materials

2.1. Routine ES Cell Culture

Mouse embryonic stem cells are routinely passaged in 25 or 75 mL flasks in D-MEM to which glutamine, β-mercaptoethanol, LIF, and fetal bovine serum are added. The following are used:

1. Plasticware: T75 flasks with filter caps (Costar, cat. no. 3376), T25 flasks with filter caps (Costar, cat. no. 3056), 15 mL centrifuge tubes (Falcon, cat. no. 2095), 50 mL tubes (Falcon, cat. no. 2098), freezing vials (Corning, cat. no. 430659), sterile pipets (10 mL: Fisher, cat. no. 13-678-11E, 2 mL: Falcon, cat. no. 7507), Bottle top filters (500 mL, Corning, cat. no. 431168), 500 mL bottles.
2. Substrate coating: 0.1% gelatin (Sigma, cat. no. 430521) dissolved in sterile water (Sigma, cat. no. W-3500).

3. ES growth medium (*see* **Note 1**): Dulbecco's modified Eagle's medium (D-MEM) (Gibco, cat. no. 11965-092). Fetal bovine serum (FBS) (ES tested) (*see* **Note 2**), leukemia inhibitory factor (LIF) (Chemicon, cat. no. LIF2010), 1000 units/mL. The following are also added: HEPES (Gibco, cat. no. 11344-025), 23.83 g, L-glutamine (Gibco, cat. no. 21051-016), 4 g, 2-mercaptoethanol (Sigma, cat. no. M-7522), 70 μL. Combine these three ingredients, then add D-MEM to 1000 mL. Aliquot in 28 mL volumes in sterile tubes. Store at –80°C.
4. Ca^{2+}/Mg^{2+} free HBSS (Gibco, cat. no. 14180-061) (*see* **Note 3**).
5. Trypsin/EDTA (Gibco 15400-054).
6. Freezing/storage medium: 90% FBS/10% DMSO (Sigma, cat. no. D-2650).
7. Centrifuge with swinging buckets.
8. Cell freezer (–140°C chest freezer or liquid nitrogen storage with canes).
9. CO_2 incubator.
10. Tissue culture hood.
11. Inverted microscope.
12. Coulter counter or hemacytometer.
13. Vacuum pump.

2.2. Neural Differentiation

1. Tissue culture plastic: 60 mm plates (Falcon, Primaria, cat. no. 3803), 12-well plates Costar, cat. no. 3513), six-well plates (Costar, cat. no. 3506), chamber slides (e.g., Lab-Tek, 8 well, cat. no. 154534), nylon fiber mesh, 20 micron (TETKO, cat. no. 3-20/14).
2. Substrates: Poly-ornithine (Sigma, cat. no. P-8638), sterile water (Sigma, cat. no. W-3500), laminin-1 (Gibco, cat. no. 23017-015, Collaborative Research, cat. no. 40232) (*see* **Note 4**).
3. Medium and growth factors: F-12 (Gibco, cat. no. 11765-054), FGF-2 (Gibco 13256-029), 5 ng/mL, D-MEM (Gibco, cat. no. 11965-092), IGF-1 (Gropep, cat. no. IM001), 5 ng/mL, N2 supplement (Gibco, cat. no. 17502-048), NT-3 (R&D Systems, 267-N3-005), B27 supplement (Gibco, cat. no. 17504-044), BDNF (Alomone Labs, cat. no. B-250), neurobasal medium (Gibco, cat. no. 21103-049), pyruvate (Gibco, cat. no. 11360-070).

3. Methods

As a simple alternative to differentiation in embryoid bodies, in the first protocol, ES cells are plated on tissue culture plastic previously ultraviolet irradiated to produce a poorly adhesive substrate (**Fig. 1A**). Under these conditions, ES cells initially attach, then form uniform, unstratified aggregates of cells that resemble neurospheres. Aggregates lift from the surface of the dish and as early as 24 h in vitro express the stem cell/neuroepithelium marker *nestin*. Aggregates are gravity sedimented, then plated at a constant density on polyornithine/laminin-1 coated substrates in an 80/20 mix of N2/B27

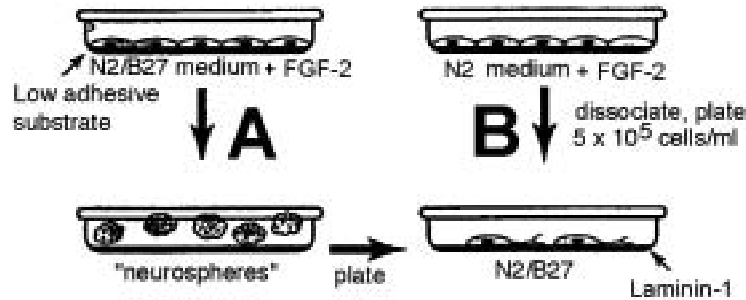

Fig. 1. Schematic illustrating the two culture paradigms: the neurosphere **(A)** and the disaggregation **(B)** differentiation paradigms.

media. Under these conditions, there is robust neural (both neuronal and glial) differentiation that peaks at 8–10 d in vitro. The second protocol (**Fig. 1B**) relies on differentiation in low-density cultures in defined medium with differentiation-promoting agents, and is highly dependent on substrate conditions (laminin-1), but produces a more pure neuronal population of cells.

3.1. Routine ES Cell Culture (*see* Note 5)

ES cells (*see* **Note 6**) are routinely grown on 0.1% gelatin coated 75 mL flasks in D-MEM medium containing LIF, fetal bovine serum, and additives. Under these conditions, ES cells must be passaged at 48 h intervals, and remain largely undifferentiated as assessed by morphology, by immunohistochemical localization of cell type restricted proteins, and by RT-PCR analysis. **Figure 2A** illustrates the typical undifferentiated appearance of D3 ES cells adapted to grow on gelatin. When cells are approximately 70–80% confluent, they are either passaged or frozen in 90% serum, 10% DMSO.

1. To prepare ES culture medium, add 50 mL of ES-tested fetal bovine serum, 28 mL additives (from frozen stock), and 500 mL of D-MEM to a 500 mL bottle top filter attached to a 500 mL glass bottle and gently vacuum filter the medium. Medium should be aliquotted in 100-mL bottles, and stored at 4°C. LIF should be added (1000 units/mL) just prior to use. Do not filter LIF.
2. To split or freeze cells, ES cells are washed to remove serum proteins by a 5 min rinse in Ca^{2+}/Mg^{2+} free HBSS (10 mL) at room temperature, followed by 5 min incubation in 7 mL trypsin/EDTA at 37°C.
3. Enzyme activity is inhibited by the addition of 8 mL of complete (serum-containing) medium (*see* **Note 7**), the flask is tapped gently to release any remaining cells and the contents are transferred to a 15-mL conical centrifuge tube and centrifuged for 3 min.

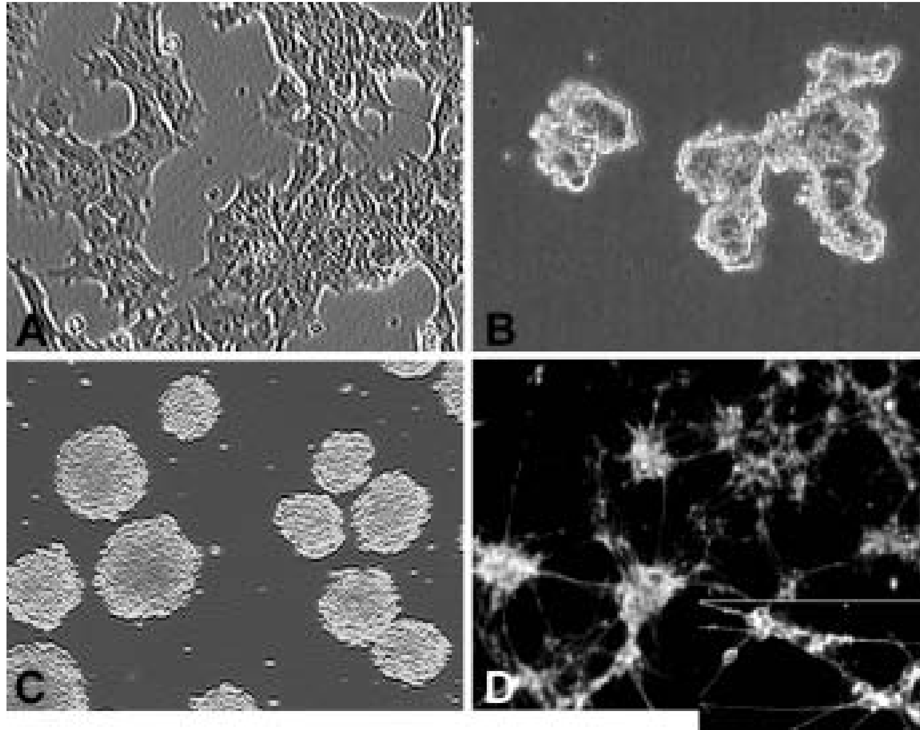

Fig. 2. Neurosphere differentiation cultures. **(A)** ES cells in complete medium growing on gelatin coated substrates illustrating their normal, undifferentiated appearance. **(B)** ES cells growing as poorly attached "clumps" on UV inactivated plastic. **(C)** After 24 h, uniform aggregates lift from the surface and float in the medium. **(D)** After eight days on laminin-1 coated substrates, in N2/B27 medium there is robust neuronal differentiation. Primary antibody = TuJ1; secondary antibody = Cy3.

4. The supernatant is removed and discarded; 1 mL complete medium is added and cells are triturated gently. The cell suspension is divided between two 75 mL gelatin-coated flasks (containing 8 mL complete, LIF+ medium) to passage cells.
5. To freeze cells, the supernatant is completely removed, 1 mL freezing medium is added, and cells are gently triturated. The cells are frozen in a controlled freezing device, or placed directly in a –80° liquid nitrogen cell freezer. Viability of frozen cells is typically 90–95%.

3.2. Initiation of Differentiation

To initiate neural differentiation of ES cells, serum and LIF are removed by overnight culture in N2 medium (F-12 + N2 supplement) to which FGF-2 *(22)*

(5–20 ng/mL) is added. The following day (12–18 h later), cells are removed from their substrate as described above, by a 5 min room temperature rinse in 10 mL Ca^{2+}/Mg^{2+}-free HBSS to remove serum proteins, followed by a 5 min incubation at 37°C in 7 mL trypsin/EDTA. An excess of *defined* medium (8 mL) is added to dilute the enzyme, flasks are tapped gently to remove any remaining adhering cells, and cells are spun, resuspended in 1 mL defined medium, and extensively triturated using a 2 mL pipet (*see* **Note 8**). This step is the first for both differentiation protocols, and is largely problem free (*see* **Note 9**).

Substrate preparation: Substrate preparation is critical and requires a minimum of 3 d. Costar six- or 12-well plates or chamber slides (*see* **Note 10**) can be successfully employed, or acid washed, polyornithine/laminin-1 coated coverslips can be added to wells.

1. Plates are coated initially with 0.01% poly-ornithine (PORN) solution for at least 4 h at room temperature; PORN is removed and plates are UV light sterilized 1–2 h, followed by adsorption of laminin-1 (20 ng/mL in PBS or sterile water) to the surface.
2. Laminin is added at 2 mL (six well); 1 mL (12 well), 200-500 μL per chamber (chamber slides) (*see* **Note 11**); plates are covered with plastic wrap, and polymerized at 4°C for at least 72 h (*see* **Note 12**).
3. Prior to use, the laminin-1 solution is removed and differentiation medium is added immediately, keeping the surface moist.
4. For neurosphere differentiation, 60 mL dishes are exposed to UV light (in the tissue culture hood) for at least 18 h (up to 48 h) to cross-link the proprietary protein surface coatings. After UV light inactivation, plates can be wrapped and stored at room temperature prior to use, but we prefer to prepare them just before each experiment to avoid contamination.

3.3. Differentiation as "Neurospheres"

After overnight incubation in N2/FGF-2, cells are resuspended in differentiation medium as described above. At this step, it is critical to count the cells using either a Coulter counter or hemacytometer, as differentiation is highly dependent on cell density.

1. Cells should be plated at a final density of 5×10^5 cells/mL in 8 mL of 80/20 medium (*see* **Note 13**) on tissue culture plastic (60 mm dishes) previously UV light treated for at least 18 h to render the surface poorly adhesive (*see* **Notes 14,15**). The ES cells will initially adhere in small clumps (**Fig. 2B**), then "neurosphere-like clusters" will lift from the surface, forming small, uniform aggregates of cells (**Fig. 2C**). Occasional aggregates will remain lightly attached to the tissue culture plastic; gentle tapping of the dish will release them.
2. The supernatant is removed from the dishes and transferred to 15-mL conical tubes, then aggregates are either gravity sedimented for 10 min. at 37°C, or gently spun for 3 min.

3. After removing the supernatant, add 1 mL N2/B27 medium. "Neurospheres" are triturated gently then plated at a density of 1000/mL on poly-ornithine/laminin-1 coated substrates in an 80/20 mixture of N2/B27 medium (*see* **Note 16**). Density of the aggregates is critical and should be determined by careful counting.
4. A minimal volume of medium should be used at plating to encourage initial adhesion of the aggregates; 1–1.5 mL (six-well plates), 0.5–0.75 mL (12-well plates); 0.2–0.4 mL (chamber slides). It is also possible to use a cytofuge to "encourage" adhesion to chamber slides.
5. Cells should be placed in a *humidified* CO_2 incubator maintained at 37°C, 5% CO_2.
6. Medium should be changed at 48 h intervals by withdrawing, then replacing, half of the total volume. Cells can be examined at 24 h intervals; at early stages of differentiation, care must be taken not to dislodge them from their substrate either during handling or medium changes.

After 48–72 h in vitro, processes (both neuronal and glial) will extend from the aggregates, with continued growth over an additional 5–14 d in vitro. At that time, cells can be fixed for immunohistochemical localization of cell type specific proteins (e.g. neuronal tubulin [e.g., **Fig. 2D**]), GFAP, or vimentin), RNA can be harvested for PCR, or cells can be removed and resuspended for implantation.

3.4. Neuronal Differentiation

The combination of FGF-2 withdrawal, followed by culture in defined medium on poly-ornithine/laminin-1 coated surfaces in the presence of growth and differentiation factors promotes neuronal differentiation of ES cells. This technique produces cultures highly enriched in neuronal cells (as many as 95%), and is HIGHLY dependent on substrate preparation and cell density.

1. To initiate differentiation, cells are grown overnight in defined medium (N2 + 5–20 ng/mL FGF-2) followed by washing in HBSS, incubation in trypsin/EDTA, centrifugation, and resuspension in 1 mL of N2 medium, as described above.
2. To remove cell clumps, cell suspensions are passed through a 20 micron pore mesh previously cut into 2 cm × 2 cm squares and autoclaved.
3. Cells are counted, then plated at 1×10^5 cells/mL on tissue culture plastic previously coated as described above with poly-ornithine/laminin–1 in N2 medium also containing growth factor cocktails (IGF-1 and BDNF or NT-3) (*see* **Note 17**).
4. A minimal amount of medium should be used at plating to ensure that cells contact the laminin-1 substrate (as described above), and at 24–48 h intervals, cells should be fed by withdrawing, then gently replacing half the volume of medium.

As early as 24 h post-plating, ES cells extend short processes (length of the cell body), and neurofilament protein is expressed in a polar distribution in the

forming neurite. Over the next 48–72 h, cells continue to extend processes, with differentiation peaking at 3–6 d in culture (*see* **Note 18**).

4. Notes

1. Antibiotics, e.g., penicillin/streptomycin, can be added to any of the media. We typically do not include antibiotics, because we want to ensure that the cultures are not contaminated, and we commonly use antibiotics (G418) to select stable transfected cell lines from ES cells. Use sterile techniques.
2. The quality of the fetal bovine serum (FBS) is critical and should be tested for its ability to stimulate ES cell proliferation (e.g., ***23***), then purchased in bulk. Alternatively, university transgenic cores commonly carry out these testing procedures and ES tested sera are available from them. There are a number of additional products available that are serum replacements for ES cells, which work well and could also be employed. We routinely aliquot ES tested FBS into 50 mL tubes and store it frozen at –80°C prior to use.
3. Although we buy 1X culture medium to avoid contamination problems, we buy HBSS and trypsin/EDTA at 10X concentrations. HBSS is diluted in Sigma water (50 mL concentrate in 500 mL water), and trypsin/EDTA is diluted in 1X HBSS (10 mL concentrate in 100 mL 1X HBSS).
4. Laminin-1 obtained from Collaborative Research, or Gibco but not the entactin-free laminin, are effective substrates. Laminin-2 is also effective, as is fibronectin or matrigel. Because of variation between lots and the numerous growth factors, proteases, etc., in matrigel, it should be avoided. Each substrate binds and presents different growth factors, so it is preferable to conduct experiments with a single product.
5. Many detailed descriptions regarding the derivation, passage, and freezing of ES cells are available ***(23,24)***.
6. Many ES cell lines are available, including lines expressing β-galactosidase, EGFP, RFP, etc., and can be used for implantation and tracing of the ultimate disposition of the cells. We have employed D3, E14, R1, ROSA, and ES from the GFP mouse ***(25)*** successfully, although each has slightly different growth and adhesion characteristics. In addition, the many gene targeted lines developed to produce gene "knock-outs" or "knock-ins" can be differentiated to determine the effects the genetic alteration on neural differentiation. It may be necessary to delete both alleles of the gene (–/– cells) by raising the G418 concentration in the passage medium ***(26)***.
7. This medium should contain serum (to inhibit enzyme activity), but can be LIF free for economic reasons.
8. Trituration into a single cell suspension is critical in both differentiation protocols. For neuronal differentiation, the suspension is passed through 20 μm mesh to remove cell clumps; a drop should be added to a Petri dish to check that the cell

suspension is largely aggregate free before plating. Overzealous trituration or trituration in trypsin/EDTA should be avoided as it damages the cell membrane and can cause adhesion defects.

9. We have, however, developed one adhesion-deficient cell line (a stable line in which the CNS specific nestin enhancer drives neo), which requires that trypsin action be "stopped" by the presence of serum in the medium used to dilute the trypsin-EDTA, or cells fail to adhere to the UV inactivated plastic.
10. The smaller the volume of the wells, the more likely differentiating cells will adhere at the edges of the plates and complicate microscopic analysis.
11. Do not be tempted to save here; sufficient coverage is critical to ensure that laminin-1 is not deposited only at the edges of the dish, but that there is uniform coverage. These volumes are minimal for complete coverage.
12. The poly-ornithine/laminin-1 coated plates available from Becton-Dickinson (Biocoat) are adequate for neurosphere differentiation. However, since drying of extracellular matrix molecules causes them to fold, and cell binding domains become occult, commercial plates should not be employed for single cell differentiation. The dish surface should remain moist during the coating process.
13. Although B27 contains small amounts of retinyl acetate ***(27)***, we have found that this combination produces the optimal balance of differentiation and cell survival. The "semi-defined" medium developed by Jennie Mather ***(28)*** is excellent for neuronal differentiation, but contains pituitary extract, which makes it difficult to determine the role of individual signaling molecules or growth factors in neural differentiation.
14. This step is also critical; culture on untreated tissue culture plastic (Petri dishes) commonly used to produce embryoid bodies will produce very large, nonuniform aggregates of cells in which neural differentiation is incomplete. Hanging drop cultures can also be employed.
15. Crosslinking by exposure to UV light inactivates extracellular matrix proteins ***(29)***.
16. Differentiation medium (80/20) is made by preparing 200 mL N2 medium (100 mL F-12, 100 mL D-MEM, 2 mL 10X N2 salts), and 50 mL B27 medium (50 mL Neurobasal, 1 mL 5X B27 supplement). Combine 160 mL of N2 with 40 mL of B27, add 2 mL pyruvate solution.
17. We have tested many growth factor combinations. Exposure of ES cells to noggin protein results in rapid, widespread neuronal differentiation ***(20)***. The recent report that *neural* stem cells differentiate into a cholinergic phenotype following exposure to BMP-9 ***(30)***, into dopaminergic neurons following overexpression of Nurr-1 and contact with type 1 astrocytes ***(31)***, suggest additional growth factor combinations that could be tested in this system. Lineage selection, either positive in which cells are transfected to express a developmental control gene to promote differentiation or negative in which cells NOT expressing a particular gene are killed by high levels of antibiotic has also been employed to create ES cell lines ***(32)***.

18. Neurons formed using these techniques may extend very long processes and contact other neurons, but when analyzed using TEM, typically fail to form mature synaptic profiles.

Acknowledgment

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2

Production and Analysis of Neurospheres from Acutely Dissociated and Postmortem CNS Specimens

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1. Introduction

For most of the history of neuroscience, it was widely believed, despite isolated reports to the contrary ***(1)***, that *de novo* generation of neurons in the mammalian CNS did not persist past perinatal development. It was not until the last decade of the twentieth century that the existence, within the CNS of fully developed mammals, of a persistent germinal zone containing neural stem cells (NSCs) with the capacity to differentiate into both neurons and glia became widely accepted ***(2)***. It is now known that in vivo genesis of new neurons occurs throughout life, and is restricted primarily to the periventricular subependymal zone (SEZ), which generates neurons destined for the olfactory bulb, and the subgranular layer of the hippocampus, which generates neurons destined for the dentate gyrus ***(3,4)***.

In vitro, NSCs can be propagated from a variety of rodent and human tissues including cerebral cortex, SEZ, hippocampus, and spinal cord ***(5–7)***. Clones of single NSCs can be cultivated either as monolayers of substrate-anchored cells ***(6,8)*** or as suspended, spherical structures called "neurospheres" ***(9,10)***. In this chapter, we will describe a method that our laboratory has developed for the generation and study of neurospheres ***(5,11)***, which involves cultivating single-cell suspensions in the absence of cell–cell and cell–substrate interactions. This method is based on the theory that our culture conditions will allow for the clonal expansion of single cells without contamination from neighboring

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cells, and will maintain cells in a primitive ontogenetic state, because substrate attachment is necessary for differentiation to occur.

Neurospheres can be generated from a variety of CNS structures in mice ranging in age from embryonic to adult. However, we have found that SEZ of first postnatal week mice give the best results. Because neurosphere yield is much higher than other structures, it is technically easier to make a discrete isolation of the SEZ as compared to embryonic animals, thereby increasing the signal to noise ratio, and the dissociation procedure seems to be far gentler (as compared to older animals that have already undergone significant myelination) resulting in better cell survival.

We have also demonstrated that it is possible to generate neurospheres from SEZ following extended postmortem intervals using the same methods ***(12)***. However, neurosphere yield declines precipitously if the brain is kept at room temperature. Storing the brain at 4°C dramatically lengthens the neurogenic potential of postmortem tissue such that is possible to cultivate neurospheres almost 1 wk after death.

A major benefit of our protocol is that neurospheres are easily manipulated, and lend themselves to many different analyses both individually and collectively. The plating density that we use is low enough to allow single neurospheres to be quickly removed with a hand-held pipettor. Once isolated, a neurosphere can be used for immunocharacterization, gene analysis, or the generation of secondary neurospheres. Populations of multiple neurospheres are also suitable for these purposes and can, in addition, be used for ultrastructural analysis, long-term cryostorage, or transplantation (*see* **Fig. 4** and **Subheading 3.** of this chapter for methods). Regarding preparation of neurospheres for transplantation, it is possible to start with a variety of transgenic animals or transfected cells that contain marker genes useful for subsequent discernment of donor vs host derived cells ***(13)***.

The methods described here allow cell and molecular analyses of individual clones of cells, neurospheres, derived from neural stem/progenitor cells. Neurospheres can be cultivated from a variety of normal, genetically altered, or pathological tissue specimens, even with protracted postmortem intervals, using the protocols detailed here, for studies of mechanisms underlying neurogenesis, cell fate decisions, and cell differentiation. Neurosphere forming-cells, themselves, hold great promise for the development of cell and molecular therapeutics for a variety of neurological diseases.

2. Materials

2.1. Generation of Neurospheres from Acutely Dissociated SEZ

1. 1X DMEM/F12 medium (Gibco cat. no. 12500-062).
2. Neurosphere cloning medium (StemCell Technologies).

3. 1X DMEM/F12 medium containing 10% fetal bovine serum (FBS: Atlanta Biological).
4. Trypsin/EDTA solution (Gibco cat. no. 15405-012).
5. Antiadhesive solution: Prepare by adding 90–100 mg of poly(2-hydroxyethyl methacrylate: Sigma cat. no. P3183) to 100 mL of 100% EtOH. Shake vigorously overnight at 37°C. The final solution is viscous, and should be stored at 37°C. This solution will turn cloudy as it cools, but will clear again when warmed, or when allowed to dry on culture surfaces.
6. Six-well plates (TPP) coated with antiadhesive: Prepare by adding enough anti-adhesive solution to cover the surface of each well. Immediately aspirate excess, and allow plates to dry at least several hours at 37°C. Once antiadhesive coating has dried, plates can be stored for several months at ROOM TEMPERATURE; however, plates should be sterilized prior to use by washing in PBS containing antibiotic/antimycotic, and/or several minutes of exposure to a germicidal UV lamp.
7. Fire-polished Pasteur pipets: Prepare medium and narrow bore sets by briefly exposing the tip to the flame of a Bunsen burner to narrow the lumen. Add a cotton plug to the proximal end, and autoclave before use.
8. 15 mL Falcon tubes (TPP).
9. Growth factor stock solution: Cultures require supplementation with 20 ng/mL of EGF (Gibco cat. no. 13247-010) and 10 ng/mL of bFGF (Sigma cat. no. F0291) every 2–3 d; since each culture well will contain approx 2 mL of medium, we supplement with 50 μL aliquots of 40X stock (8000 ng of EGF, and 4000 ng of bFGF in 10 mL of DMEM/F12). Stock can be prepared more concentrated if desired, but we do not recommend a less concentrated stock, because the correspondingly larger aliquots will quickly reduce the viscosity of the neurosphere cloning medium.
10. PBS or DMEM/F12 containing antibiotic/antimycotic (Sigma A9909).

2.2. Immunolabeling

1. Standard small-volume pipettor with sterile tips.
2. 12-well culture plates (TPP).
3. 18 mm round coverglass (Fisher cat. no. 12-546) coated sequentially with poly-L-ornithine and laminin: Prepare by incubating coverglass overnight at room temperature in H_2O containing 10 mg/mL of poly-L-ornithine (Sigma cat. no. P4957). Wash 3X with H_2O, and incubate 8–10 h at 37°C in PBS containing 2.5 mg/mL of laminin (Sigma cat. no. L2020). Wash 3X with PBS. Plates can be stored short-term in PBS at 4°C or long-term in PBS at –20°C. Sterilize before use with PBS containing antibiotic/antimycotic, and irradiate with an ultraviolet germicidal lamp.
4. 1X DMEM/F12 medium containing 1% FBS.
5. Inverted phase microscope.

2.3. Ultrastructural Analysis

1. Embedding plastic (Spurr or Epon).
2. 2% agar in H_2O.

3. 90°C water bath.
4. EM fixative: 0.1 *M* sodium cacodylate buffer (EMS 12300) containing 2% paraformaldehyde (Sigma cat. no. P6148), 2% glutaraldehyde (EMS cat. no. 16350), and 0.5% acrolein (EMS).
5. 2% uranyl acetate (EMS cat. no. 22400) in 0.9% saline.
6. 1% OsO_4 (EMS cat. no. 19100) in PBS.
7. Small plastic microcentrifuge tubes.
8. Graded ethanols (50, 70, 80, 90, 100%).

2.4. Gene Analysis

1. Microtip sonicator.
2. 0.6 mL tubes.
3. Standard PCR reagents, including: RNase-free water, RNase inhibitor, and RNase H.
4. Superscript reverse transcriptase (Gibco).
5. Solutions for sterilizing the microtip sonicator: 1 *M* HCl, 1 *M* NaOH, 1 *M* Tris-HCl (Gibco cat. no. 15567-027), and ddH_2O.

3. Methods

3.1. Generation of Neurospheres from Acutely Dissociated SEZ

The following protocol is the standard method our laboratory has developed to produce neurospheres from mouse and adult human brain ***(5,11)***. Any culture dish configuration can be used, but we prefer six-well plates, because they allow for multiple experimental manipulations of subpopulations of the same sample, they lend themselves to rapid visual screening without the optical interference common to plates with smaller well diameter, and potential infections are contained within single wells, and can be removed without sacrificing the entire sample.

1. Decapitate mouse pup, and briefly dip the head in EtOH.
2. Remove the brain, and place it on a clean surface suitable for cutting.
3. With a razor blade, make a coronal block, about 2 mm in thickness, in the area between the rhinal fissure and the hippocampus (*see* **Fig. 1A**). Lay the block flat on the cutting surface and use the razor blade to make two parasagittal cuts just lateral to the lateral ventricles, and a horizontal cut to remove the tissue above the corpus callosum (*see* **Fig. 1B**). This procedure leaves a small, rectangular chunk of tissue surrounding the lateral ventricles containing a high density of NSCs.
4. Wash the tissue chunk for several minutes in medium or PBS containing antibiotics/antimycotics. All subsequent work should be performed with sterile materials in a laminar flow hood.
5. Remove antibiotics/antimycotics, and incubate tissue in trypsin/EDTA solution at 37°C for 5 min.

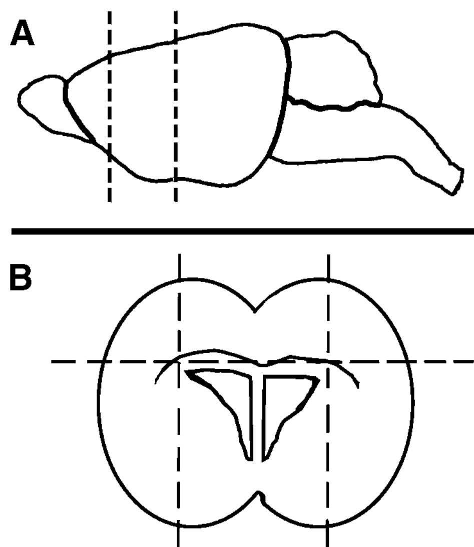

Fig. 1. Schematic of the dissection protocol. Starting with whole brain, make two coronal cuts in the area between the rhinal fissure and the hippocampus (broken lines in **A**). Lay the resulting tissue chunk on its posterior surface, and make two parasagittal cuts just lateral to the lateral ventricles, and one horizontal cut at about the level of the corpus callosum (broken lines in **B**). Make neurospheres by dissociating the central, rectangular piece of tissue containing the lateral ventricles.

6. Gently triturate tissue through a series of descending-diameter, fire-polished Pasteur pipets to make a single-cell suspension.
7. Add several volumes of DMEM/F12 containing 10% FBS. Centrifuge to pellet cells, and wash several times with fresh medium.
8. Count cells using a hemacytometer.
9. In a 15-mL Falcon tube, combine 6 mL of DMEM/F12 medium, 60,000 cells, and 50 μL of 40X growth factor stock. Add neurosphere cloning medium to bring the final volume to 12 mL.
10. Mix for several minutes by repeatedly inverting the tube.
11. Distribute 2 mL to each well of a six-well plate previously coated with antiadhesive. The final cell density will be about 1000 cells/cm^2, although the viscosity of the cloning medium makes precise volumetric measurements difficult.
12. Add 50 μL aliquots of 40X growth factor stock every 2–3 d. Neurospheres will be visible under phase optics after 7–10 d. True neurospheres are characterized by near perfect spherical shape, as well as very sharp, phase-bright outer edges. Importantly, individual cells should not be seen with low-power phase optics (*see* **Note 1**). *See* **Fig. 2**.

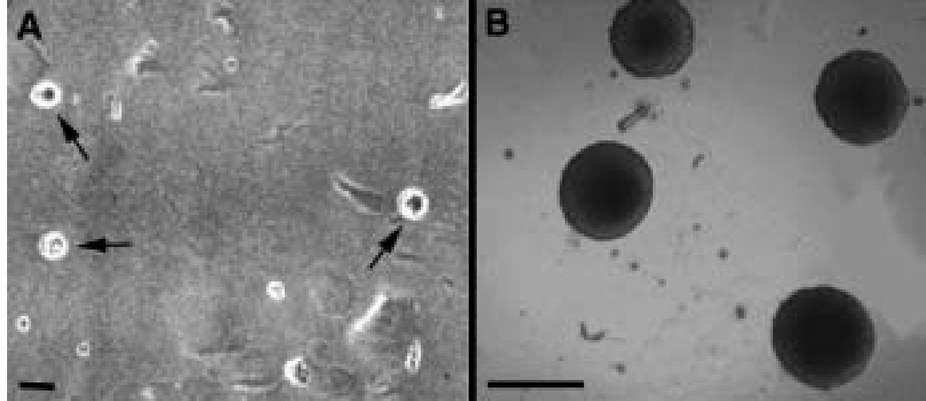

Fig. 2. Phase micrographs of neurospheres in suspension culture. **(A)** Appearance of very young neurospheres five days after plating. Emerging neurospheres are very tight, phase-bright spheres (arrows). **(B)** After 10–14 d, neurospheres are approaching their greatest diameter, and appear as large globes with sharp outer borders. Scale bar in a = 50 μm, and 150 μm in **B**.

3.2. Immunolabeling

The following protocol is our standard method for immunolabeling neurospheres (*see* **Note 2**) after they have attached to a favorable substratum and have begun to migrate and differentiate.

1. Place coated coverslips in 12-well plates, and put a drop (50–100 μL) of medium near the center of each. Keep in a laminar flow hood.
2. Remove the cover from a six-well plate containing neurospheres, and visualize a neurosphere with the inverted microscope (contamination of wells is rare, even though the plate is opened outside of the hood, but wash the microscope and pipettor with EtOH before use).
3. While looking through the microscope, guide the tip of a pipettor set for 2–5 μL to the neurosphere, and aspirate it into the pipet tip.
4. Eject the neurosphere into the drop of medium on the coverslip. Repeat as often as desired. We typically place 2–10 neurospheres on each coverslip.
5. Place 12-well plates in an incubator. Neurospheres should be attached firmly to the coverslip by the next day, and can be fixed then, or cultivated for longer periods of time. If neurospheres are to be cultured for more than 1–2 d, it is important to carefully flood the coverslip with fresh medium after attachment has taken place.
6. Wash, fix, and process coverslips for standard immunolabeling, and/or scanning EM. *See* **Fig. 3.**

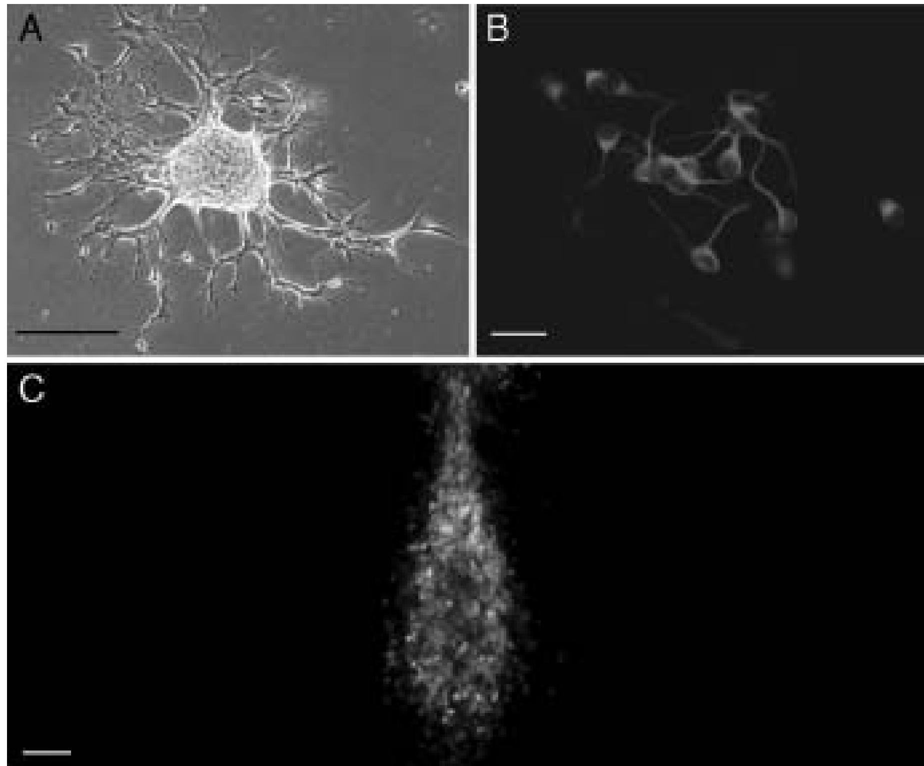

Fig. 3. Characterization and use of neurospheres. **(A)** Phase microscopy of a single neurosphere attached to coverslip. Numerous processes extend in all directions, and individual, phase-bright cells are seen migrating away from the main mass of the neurosphere. **(B)** Immunofluorescence labeling of attached neurosphere cells reveals a population of β-III tubulin positive neurons. **(C)** Dissociated neurospheres derived from a green fluorescent protein (GFP) transgenic mouse are useful for transplantation since constitutive GFP renders the donor cells easily distinguishable from the host tissue, as seen in this fluorescence micrograph of a graft into adult mouse cortex. Scale bar = 150 μm in **A**, 25 μm in **B**, and 50 μm in **C**.

3.3. Ultrastructural Analysis

We have developed a method for generating electron micrographs of suspended neurospheres. Owing to the need for visually tracking the sample during processing, this method does not allow for the ultrastructural analysis of a single, prospectively identified neurosphere, but rather requires that a large

number of neurospheres be processed together before retrospectively choosing individual examples to section and analyze.

1. Liquefy 2% agar by placing in water bath.
2. Use a transfer pipet to pool several hundred neurospheres in a 15-mL Falcon tube.
3. Centrifuge to pellet neurospheres. Aspirate medium, and gently resuspend in EM fixative. Incubate for 30 min at room temperature.
4. Wash 2–3X by gently pelleting and resuspending in PBS.
5. After a final pelleting, aspirate as much PBS as possible. Resuspend in a small (20–50 μL) volume of PBS, and transfer to a plastic microcentrifuge tube. Quickly add an equal volume of melted agar to the neurospheres, and mix gently (work quickly so the agar does not solidify before being mixed with the neurospheres). Place tube at 4°C for 15 min to harden agar.
6. Cut off the tip of the tube with a razor blade, and use a small spatula to pry the agar plug out. Place the plug into OsO_4 for 2 h at room temperature; the osmium will turn the neurospheres brown, and they should then become apparent to the naked eye.
7. Rinse for 30 min at room temperature in H_2O, and place into uranyl acetate for 1 h.
8. Dehydrate through graded ethanols, place into embedding plastic, and section for standard transmission EM. *See* **Fig. 4**.

3.4. Gene Analysis

Gene profiling (*see* **Note 3**) of large numbers of pooled neurospheres can be performed using standard techniques for RNA isolation. Sometimes, however, there may be a desire to examine transcripts present in an individual neurosphere. Because neurospheres consist of, at most, several thousand cells, and because these cells are embedded in a dense extracellular matrix, RNA extraction can be tricky, and the normally low RNA yield can be completely lost if subjected to the additional step of RNA isolation. To address these problems, we have developed a method that combines sonication, and RT-PCR without RNA isolation ***(14)***. All procedures are performed in the same microcentrifuge tube, and the results are much better than those obtained with extraction by either the guanidine cyanide method or freeze-thawing, both of which lead to significant loss of material.

1. Place a single neurosphere in a 0.6 mL tube containing 10 μL of RNase-free water with 5U of RNase inhibitor. Keep tube on ice.
2. Release RNA by sonicating with a microtip sonicator (Kontes) by gently touching the surface of the water for 4–10 sec. Immediately put the tube back on ice.
3. If working with multiple samples, wash the sonicator tip between samples sequentially in ice cold 1 *M* HCl, 1 *M* NaOH, 1 *M* Tris-HCl, pH 7.5, and ddH_2O.

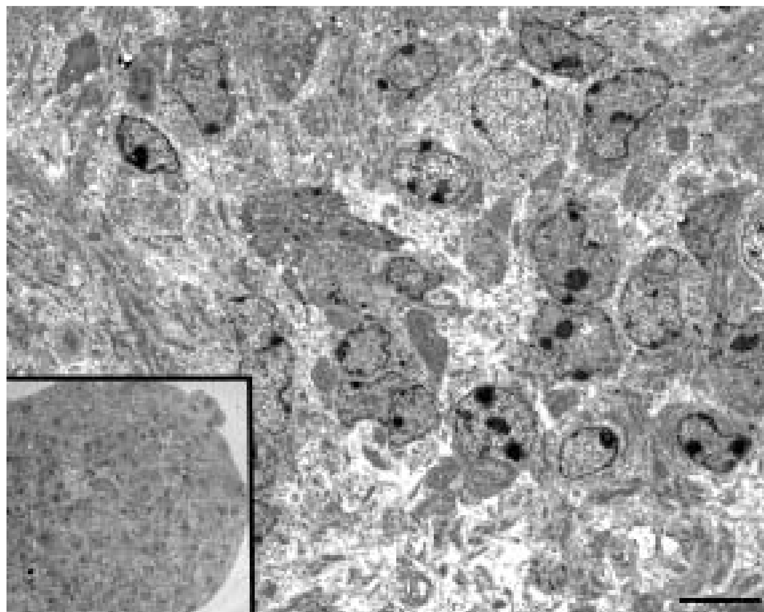

Fig. 4. Ultrastructure of a single neurosphere. Inset contains a lower power micrograph showing most of a single neurosphere derived from a surgical biopsy of adult human SEZ. Ultrastructural studies of neurospheres reveal a diverse population of cells in different states of differentiation from a presumed stem/progenitor cell to differentiated neurons and glia ***(5)***. Scale bar = 10 μm.

4. Perform first-strand cDNA synthesis using Superscript reverse transcriptase (Gibco) according to the manufacturer's instructions.
5. Add 4 U of RNase H to remove the cDNA:RNA hybrid. This solution is now ready to use as template in standard PCR reactions optimized for each primer set.

4. Notes

1. The following are suggestions to address some common problems that can arise when trying to grow neurospheres using our protocol:
   a. False neurospheres: Undissociated tissue pieces can, over time, begin to resemble spheres, which is why it is important to begin with a single-cell suspension. These pieces do not, however, have sharply-defined outer borders, and are only rarely spherical enough to be easily confused with true neurospheres. It should be possible to eliminate these pieces from your culture by more thorough trituration with smaller diameter pipettes. Additionally, you can allow the dissociated cell suspension to sediment for several minutes, which permits the larger, undissociated chunks to settle to the bottom of the

tube; the upper portion can then be transferred to a new tube, added to cloning medium, and plated.

Additionally, single, dissociated cells can clump together to form aggregates that resemble neurospheres. These, too, lack sharp outer edges, and it should be easy to discern individual cells within the mass using phase optics. If aggregation is a problem, try plating at lower cell density.

b. Infection: You may, from time to time, encounter infected wells. Use a repeating pipettor when applying growth factor aliquots, as this will minimize the number of times you need to open the growth factor stock solution.

Remember to sterilize your tools and cutting surface with EtOH and flame before each dissection. Micrococcus infections readily originate from the skin of the donor animal, so take care to thoroughly wash the head in EtOH before removing the brain.

Finally, the antiadhesive plates are a potential source of infection because they must be manipulated extensively before use. Wash each well with an antibiotic/antimycotic solution, and irradiate with a UV germicidal lamp before plating cells.

c. Low neurosphere yield: This protocol typically yields dozens of neurospheres in each well, depending on the age of the animal. If you wish to increase your yield, try making a cleaner dissection by removing more of the tissue surrounding the SEZ; the less of these other tissues (striatum, cortex, etc.), the greater the percentage of plated cells that will generate neurospheres. It is also possible to plate at a higher cell density, but beware that too high a density will increase the likelihood of forming non-clonal aggregates.

During the dissociation, take care not to triturate so harshly as to lyse the cells. Determine empirically the largest bore pipette that results in a single cell suspension. Also, do not over-incubate the tissue in trypsin, as this will eventually lead to cell death.

Finally, it is possible to subclone primary neurospheres by dissociating and recloning them. A single dissociated neurosphere typically will give rise to 5–15 secondary neurospheres. Dissociation can be performed by collecting neurospheres in a tube containing trypsin/EDTA, and triturating with a small-bore pipet. The resulting cell suspension can then be replated in cloning medium as described above.

d. Attachment of cells to the culture dish: Occasionally, cells will attach and differentiate on the bottom of culture dishes that have been coated with anti-adhesive, due perhaps to the presence of cracks or abrasions. These attached cells are apparent with phase optics and can, in sufficient numbers, form a favorable substrate for the attachment and differentiation of neurospheres. If significant numbers of cells are seen attaching to the dish surface, the remaining suspended cells and neurospheres should be collected and transferred to a new plate.

2. Poor attachment of neurospheres to coverslip: It is not uncommon for a small percentage of neurospheres to not readily attach. Allowing more time for

attachment—up to 48 h—often solves the problem. In general, a neurosphere that has not attached after 48 h will never attach. Increasing FBS to 5–10% usually improves attachment; however higher serum also alters differentiation. It may be worthwhile to increase serum concentration to facilitate attachment, and decrease it again before differentiation and migration occurs.

Also, very young neurospheres do not attach readily. Avoid plating neurospheres that have been in culture less than 7 d, and choose only those greater than about 50 μm in diameter.

Finally, apply and aspirate solutions (PBS, fixative, etc.) slowly and gently to avoid dislodging lightly attached neurospheres.

Contamination of 6-well plates: If your plate often becomes infected during the process of removing neurospheres, be sure that you minimize your work directly over the open plate. Maintaining the pipettor at a steep angle as you approach a neurosphere will help. If possible, work in a small room that can be exposed to a germicidal UV lamp for several minutes prior to use.

3. Low RNA yield: This method normally yields enough RNA from a single neurosphere to serve as template for 30–40 PCR runs using primers for high-abundance genes (e.g., housekeeping genes). If you have trouble achieving this level, you may need to adjust the sonication protocol. Sonicating for too long will increase the sample temperature, which increases RNase activity, and can reduce yield; too little sonication will not effectively release RNA, again leading to low yield. If you are still unable to sufficiently increase yield, or if your primer set is designed to reveal low-abundance transcripts, it may be necessary to amplify the sample after sonication.

Addendum

Recent evidence has accumulated suggesting that glial cells have stem cell characteristics in vivo, and may represent the neurosphere-forming cell in vitro. Specifically, certain astrocytes have been shown to undergo mitosis and give rise to neuroblasts in the adult mouse SEZ ***(15)***. Furthermore, work from our laboratory has demonstrated that subpopulations of cultured mouse astrocytes—derived from a variety of CNS regions—can generate neurospheres in a regionally and temporally restricted manner ***(13)***. Astrocytes cultured from cerebral cortex, cerebellum, and spinal cord can generate neurospheres when grown in the presence of growth factors, but only if these cultures are derived from animals younger than about postnatal d 11. Astrocytes cultured from SEZ can generate neurospheres when derived from both perinatal and adult animals.

The following protocol describes our method of generating astrocyte monolayers that can subsequently be used for producing multipotent neurospheres. Once monolayers are established, they can be replated under neurosphere-generating conditions, as described above, where 1–10% of plated cells form neurospheres.

Materials

1. PBS or DMEM/F12 containing antibiotic/antimycotic (Sigma cat. no. A9909).
2. 1X DMEM/F12 medium containing 10% fetal bovine serum (FBS: Atlanta Biological).
3. Trypsin/EDTA solution (Gibco cat. no. 15405-012).
4. 15 mL Falcon tubes (TPP).
5. T-75 tissue culture flasks (TPP).

Methods

1. Decapitate mouse pup, and briefly dip the head in EtOH.
2. Remove the brain, and place it on a clean surface suitable for cutting.
3. Use a razor blade or microknife to isolate your CNS area of interest (e.g. SEZ; *see* **Subheading 3.1.**).
4. Wash tissue briefly in a 15-mL Falcon tube containing medium or PBS with antibiotics/antimycotics.
5. Remove antibiotics/antimycotics, and incubate tissue in 5–10 mL of trypsin/EDTA solution at 37°C for 5 min.
6. Triturate with a 5 mL serological pipet for 2–3 min to break up the tissue into small chunks. It is not necessary to make a single-cell suspension.
7. Add 1–2 mL of FBS to neutralize trypsin, and centrifuge cells to form a pellet.
8. Aspirate supernatant, and wash by trituration with fresh medium. Pellet and repeat 3–4X.
9. Resuspend in DMEM/F12 medium containing 10% FBS, plate in T75 culture flasks (use one flask for each brain), and place in an incubator overnight.
10. Remove the culture supernatant, and replate into fresh T75 flasks (discard original flasks which contain primarily microglia).
11. Replace medium every 2–3 d with fresh DMEM/F12 containing 10% FBS until astrocyte monolayers become confluent.
12. Remove astrocytes from flasks by aspirating culture supernatant and incubating in trypsin/EDTA for 5–10 min.
13. Collect cells in a Falcon tube, add serum to neutralize trypsin, and proceed with step 8 of **Subheading 3.1.** above.

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3

Isolation of Stem and Precursor Cells from Fetal Tissue

Yuan Y. Wu, Tahmina Mujtaba and Mahendra S. Rao

1. Introduction

Generation of neurons, astrocytes, and oligodendrocytes in the nervous system involves a sequential process of differentiation. Initially, multipotent stem cells generate more restricted precursor cells, which go through additional stages of differentiation to generate fully differentiated progeny ***(1)***. Precursor cells at each stage of differentiation can be distinguished from each other on the basis of cytokine dependence, functional properties, and antigen expression. Using markers to antigens expressed on the cell surface, live multipotent stem cells, intermediate precursor cells, and differentiated cells can be isolated at various stages of development.

Neural stem cells are most abundant at early developmental stages with the maximum being present just after neural tube closure, prior to the onset of neurogenesis, and their numbers decline over subsequent stages of development. Stem cells, however, persist throughout development, and significant numbers can be isolated even from the adult cortex. Rat E10.5 caudal neuroepithelial (NEP) cells represent the earliest multipotent neural stem cells identified. The majority of NEP cells express nestin but do not express any markers characteristic of differentiated cells. In the presence of fibroblast growth factor (FGF) and chick embryo extract (CEE), NEP cells can be maintained in an undifferentiated and homogeneous state in culture for over 3 mo. Cultured NEP cells can readily differentiate into neurons, astrocytes, or oligodendrocytes upon withdrawal of CEE and reduction of FGF concentration ***(2)***.

Neuron-restricted precursor (NRP) cells exist in developing rat neural tubes ***(3)*** and in selected regions of the adult brain ***(4)***. NRP cells are most abundant

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at E13.5 around the onset of neurogenesis. NRP cells can be maintained over multiple passages in culture in the presence of FGF and neurotrophin-3 (NT-3). The cells can differentiate into multiple neuronal phenotypes, but cannot differentiate into oligodendrocytes or astrocytes ***(3*,5)**. NRP cells can be identified and isolated by their expression of polysialic-acid NCAM (E-NCAM), a cell-surface antigen ***(3)***.

The tripotential glial-restricted precursor (GRP) cells can be identified as early as E12.0 (but are more abundant at E13.5) in developing rat neural tubes ***(6)***. These cells can be identified and isolated by their expression of the cell surface antigen A2B5 before the expression of any other oligodendrocyte or astrocyte differentiation markers. In the presence of FGF, these GRP cells can be maintained in culture for prolonged periods and can differentiate into oligodendrocytes and two distinct astrocyte populations under specific culture conditions ***(6)***.

In this chapter, we describe the isolation of NEP, NRP, and GRP cells by dissecting out the neural tube at specific stages of development and by using cell surface markers. The first strategy, enzymatic separation, is used to isolate rat E10.5. Another strategy, mechanical dissection, is used for older stage embryos (rat E13.5–E14.0) to isolate NRP and GRP mixed cultures. Then, immunopanning is used to enrich NRP and GRP cells with cell-surface antibodies anti-NCAM and anti-A2B5. In this chapter, rat embryos are employed to describe these procedures. However, the isolation of the corresponding cells in mouse is essentially identical ***(7)***.

2. Materials

2.1. Animals

1. E10.5, E13.5–E14.0 timed-pregnant Sprague-Dawley rats (Simonsen) (*see* **Note 1**).

2.2. Culture Media and Solutions

1. NEP basal medium: The following are added to DMEM/F12 medium (GibcoBRL, cat. no. 11320-033) to give the final indicated concentrations: 1X N2 supplement (100X stock) (GibcoBRL, cat. no. 17502-014), 1X B27 supplement (50X stock, without vitamin A) (GibcoBRL, cat. no. 98-0153SA), 1 mg/mL BSA (Sigma, cat. no. A-2153), 20 ng/mL recombinant human FGF-basic (PeproTech, cat. no.100-18B) (*see* **Note 2**), 1X Penicillin-Streptomycin (100X stock) (GibcoBRL, cat. no.15070-063), sterilize the media by 0.22 μm filter (Corning, cat. no. 28199-788).
2. Fibronectin-coated tissue culture dishes:
   a. 35 × 10 mm tissue culture dishes (Corning, cat. no. 25382-348).
   b. Fibronectin (Sigma, cat. no. F1141) is diluted in distilled, deionized water to a final concentration of 20 μg/mL. Fibronectin solution can be used repeatedly for up to two months if stored at 4°C.

   c. Coat the dish with 1 mL fibronectin solution at 4°C overnight. Rinse the dish with culture medium once before use (*see* **Note 3**).
3. Poly-L-lysine/laminin-coated tissue culture dishes:
   a. Poly-L-lysine (Sigma, cat. no. P-1274) is diluted in distilled, deionized water to a final concentration of 15 µg/mL. The solution can be used repeatedly for up to 3 mo if stored at 4°C.
   b. Laminin (GibcoBRL, cat. no. 23017-015) is diluted in distilled, deionized water to a final concentration of 15 µg/mL. The solution can be used repeatedly for up to 2 mo if stored at 4°C.
   c. Coat the dish with 1 mL poly-L-lysine solution at room temperature for at least 30 min. Remove the poly-L-lysine. Rinse the dish with distilled water and incubate the dish with 1 mL laminin solution at room temperature for 4 h or 4°C overnight. Rinse the dish with culture medium once before use.
4. Enzymatic solution for dissection: The following are added to 10 mL of 1X Hanks' balanced salt solution, without calcium and magnesium (GibcoBRL, cat. no. 24020-117) to give the following final indicated concentrations: 2 mg/mL dispase II (Boehringer Mannheim, cat. no. 165859), 1 mg/mL collagenase type 1 (Worthington Biochemical Corporation, cat. no. 4196) (*see* **Note 4**), sterilize the solution using 0.22 µm syringe filter (Millipore corporation, cat. no. SLGS 025 0S).
5. Growth factor stock solutions for maintaining and differentiating cells:
   a. FGF-basic (Pepro Tech, cat. no. 100-18B): Make 1 µg/mL of stock solution in Leibovitz's L-15 Media (GibocoBRL, cat. no. 11415-064) with 1 mg/mL bovine albumin, Path-O-Cyte 4 (BSA) (INC Biomedicals, Inc. cat. no. 81010).
   b. 1 µg/mL recombinant human NT-3 (Pepro Tech, cat. no. 450-03): Make 10 µg/mL of stock solution in distilled water.
   c. Platelet-derived growth factor BB, human (Upstate Biotechnology, cat. no. 01-309): Make 5 µg/mL of stock solution in 10 mM acetic acid with 1 mg/mL BSA.
   d. Store the growth factors at –20°C as working aliquots. The shelf life of the above stock solutions is 1 yr at –20°C.
6. Phosphate-buffered saline, pH 7.4, without calcium and magnesium (GibcoBRL, cat. no. 10010-023).
7. Trypsin-EDTA, 0.05% (GibcoBRL, cat. no. 25300-054).
8. Tissue culture H_2O (Sigma, cat. no. W-3500).
9. Fetal bovine serum, heat inactivated at 56°C for 30 min (GibcoBRL, cat. no. 26140-079).
10. Dimethyl sulfoxide (DMSO) (Sigma, cat. no. D-2650).

2.3. Antibodies

1. Anti-E-NCAM antibody, obtained from 5A5 hybridoma (Developmental Studies Hybridoma Bank) supernatant, 1 : 1 in NEP basal medium.

2. Anti-A2B5 antibody, obtained from A2B5 hybridoma (ATCC, cat. no. CRL-1520) supernatant, 1 : 1 in NEP basal medium.
3. Anti-nestin antibody, obtained from rat-401 hybridoma (Developmental Studies Hybridoma Bank) supernatant, 1 : 1 in NEP basal medium.
4. Anti-β-III-tubulin, 1 : 500 (Sigma, cat. no. T8660).
5. Goat anti-mouse UNLB-IgM (Southern Biotechnology, cat. no. 1020-01).

2.4. Instruments and Others

1. Micro 3-well spot plate, Pyrex brand (Fisher, cat. no. 21-379)
2. Tungsten needle, bent 90° (Fine Surgical Tool, cat. no. 9718)
3. 5# fine forceps (Fine Surgical Tool, cat. no. 11252-20)
4. Fine scissors (Fine Surgical Tool, cat. no. 14060-09)
5. Short stem Pasteur pipet
6. 100X 15 mm petri dish (Fisher, cat. no. 08757100D).
7. Cryogenic vials (Nalge company, cat. no. 5000-0012).
8. 15 mL sterile conical tubes (ISC Bioexpress, cat. no. C-3317-1).
9. Cell scraper (Fisher, cat. no. 3010).

3. Methods

3.1. Isolation and Culture of NEP Cells

NEP cells are isolated enzymatically from E10.5 caudal neural tubes and plated on fibronectin-coated dishes as adherent monolayer cultures. The entire procedure, from removal of embryos to collection of intact tubes, should not take more that 40 min for one litter of embryos.

3.1.1. Removal and Dissection of Embryos

1. Euthanize E10.5 pregnant rat with CO_2.
2. Make an incision from the lower abdomen upward to the chest. Hold one horn of the uterus and dissect the uterus out. Remove the uterus and place it in a 100 × 15 mm Petri dish containing 20 mL of ice-cold PBS.
3. Transfer the dish to a sterile dissection hood.
4. Under a dissecting microscope, remove the muscle wall of the uterus using forceps.
5. The embryos usually “pop out” from the uterus (*see* **Note 5**).
6. Remove the attached membranes from the embryos.
7. Remove the head and the tail with a bent (90°) tungsten needle. Angle the cut so that little non-neural tissue remains at the ends.
8. Collect the trunk segment and transfer them with a short stem Pasteur pipet to a Micro three-well spot plate containing ice-cold PBS.

3.1.2. Removal of Neural Tubes

1. Rinse the segments with medium several times with sufficient force to remove any attached membranes.

2. Add 1 mL of the enzymatic solution to the well and incubate the neural tubes at room temperature for about 5–10 min (*see* **Note 6**).
3. As soon as the ends of the tubes appear to separate from connective tissue, replace enzymatic solution with 1 mL of NEP basal media supplemented with 10% CEE and wash the tubes with the medium several times.
4. Gently triturate the trunks with a Pasteur pipet until the neural tubes come off from the remaining tissues. The neural tube can be easily distinguished from other tissues by its transparency and tubular appearance.
5. Individual tubes should be collected as soon as they separate from the embryos and transferred to a separate dish containing medium. As little medium as possible should be transferred with the tubes (*see* **Note 7**).
6. In general, the tubes should be clean and have no adherent somites or membranes. If some somites are still attached, remove them with #5 fine forceps.

3.1.3. Dissociation and Plating of NEP Cells

1. Collect the neural tubes with a Pasteur pipet in a 15 mL conical tube.
2. Add 2 mL of trypsin-EDTA and incubate the neural tubes at 37°C for 3–5 min (*see* **Note 8**).
3. Neutralize the trypsin with 5 mL of NEP basal media containing 10% CEE.
4. Spin the neutral tubes down at 300*g* at room temperature for 5 min. Discard the supernatant.
5. Resuspend the cells in 100 μL of NEP basal medium.
6. Dissociate the cells by very gentle trituration 3–5X (*see* **Note 9**).
7. Plate the cells at high density on the fibronectin-coated dishes in NEP basal medium containing 10% CEE.

3.1.4. Maintenance and Testing of Undifferentiated NEP Cells

1. Incubate the NEP cells at 37°C in a 5% CO_2.
2. The properties of NEP cells can be tested by immunocytochemistry. Cells should express nestin (*see* **Fig. 1**) and lack all lineage-specific markers including E-NCAM, A2B5 and B-III tubulin.

3.1.5. Passaging NEP Cells

1. At 80% confluence, split the culture at a dilution of 1:4 (*see* **Note 10**).
2. Remove the culture media.
3. Treat the cells with 1 mL of trypsin-EDTA solution for 2–3 min in a 37°C incubator.
4. Add 3 mL of NEP basal media with 10% CEE to stop trypsinization (*see* **Note 11**).
5. Spin the cells down at 300*g*.
6. Resuspend the cells in 100 μL culture medium.
7. Plate the cells on new fibronectin-coated dishes (*see* **Note 12**).

3.1.6. Freezing NEP Cells

1. When the dish reaches 80% confluency, remove the culture media. Add 1 mL of trypsin-EDTA solution for 2–3 min at 37°C.
2. Add 3 mL of NEP basal media with 10% CEE to stop trypsinization.
3. Spin the cells down at 300*g*.
4. Remove the medium. Gently resuspend the cells in 10% DMSO/80% NEP basal medium/10% CEE (4°C) at 10^6/mL (*see* **Note 13**).
5. Transfer the cell suspension to pre-chilled cryogenic vials. Store the vials at –140°C.

3.2. Isolation and Culture of Neuronal-Restricted and Glial-Restricted Precursors Cells

To obtain NRP and GRP cells, the neural tube is dissected mechanically from an E13.5 or E14.0 embryo. At this stage, the neural tube contains approx 10% NEP cells, 70% NRP cells, and 20% GRP cells. The cells can be plated on poly-L-lysine/laminin- or fibronectin/laminin-coated tissue culture dishes. The entire procedure should take about 1 h for one litter of embryos.

3.2.1. Removal of Neural Tube

1. Remove the E14.0 uterus as described for the E10.5 uterus.
2. Transfer the uterus to a 100 mm Petri dish containing fresh ice-cold PBS. Move the dish to a dissecting hood. Cut throughout the length of the uterus with sterile scissors and remove the embryos.
3. Hold the embryo by the head using a #5 forceps. Under a dissecting microscope, use a second #5 forceps to loosen the spinal cord gently from the surrounding tissue.
4. Begin the dissection at the level of the midbrain where the overlying tissue is minimal.
5. Separate the overlying skin all the way down to the caudal end of the embryo.
6. Separate the connective tissue along the lateral side of the tube until the tube is clearly visible. Proceed along the entire rostrocaudal axis. If necessary, repeat on the other side.
7. Gently insert one forcep under the tube to separate it from underlying connective tissue. Proceed to separate the tube beginning rostrally and extending as far caudally as possible.
8. Cut the tube at the caudal end to free the cord from the remaining trunk.
9. Once the cord is clean, remove the head to free the spinal cord (*see* **Note 14**).

3.2.2. Dissociation of the Spinal Cord Cells

1. Collect about three spinal cords in each 15 mL conical tube (*see* **Note 15**).
2. Add 3 mL of typsin-EDTA and incubate the spinal cords at 37°C for 10 min, shaking the tube occasionally (*see* **Note 16**).
3. Remove the typsin-EDTA. Add 2 mL of NEP basal media.

d. Add 8 mL of NEP basal medium to the panning dish. The dish is now ready for immunopanning.

3.3.2. Panning Procedure

Depletion of undesired cells (when purified A2B5+ cells are required, pan out the NCAM+ cells first and vice versa):

1. Isolate the E14.0 spinal cord cells directly from the embryos (*see* **Subheading 3.2.**) or from cells already in culture.
2. Triturate the cells into single cell suspension and add the cell suspension to the panning dish. Shake the panning dish gently to distribute the cells evenly (*see* **Note 22**).
3. Allow the dish to stand for about 40 min to 1 h at room temperature (*see* **Note 23**). Observe in phase contrast. When the dish is lightly tapped, unbound cells should be seen to move.
4. Remove the supernatant and transfer it to 15 mL tube. Spin the cells down. Resuspend the cells in 100 μL of culture media.

3.3.3. Enrichment of Desired Cells by Immunopanning

1. Repeat the panning procedure in **Subsection 3.2.** with the opposite antibody.
2. Wash the panning dish gently with PBS several times until all unbound cells are removed. This time, the desired cells you want are bound to the dish.
3. Add 8 mL of culture media in the dish. Scrape the cells off the dish with a cell scraper.
4. Spin the cells down. Plate out the cells at desired density on poly-L-lysine/laminin- or fibronectin/laminin-coated tissue culture dishes. The medium is further supplemented with NT-3 (10 ng/mL) for the survival of NRP cells or PDGF (10 ng/mL) for the proliferation of GRP cells.
5. Check the efficiency of the panning. The morphology of NRP cells should be cells with small cell bodies and short processes (**Fig. 2**). The morphology of the GRP cells should be phase bright, bipolar cells (**Fig. 3**). The purity of the enriched cells should be determined by staining with E-NCAM, A2B5, and β-III-tubulin antibodies.

Fig. 3. *(opposite page bottom)* GRP cells exist in E13.5 neural tube and can be purified by immunopanning with A2B5-specific antibodies. GRP cells can be detected in E13.5 rat by monoclonal antibody 4D4, a new characterized antibody that recognizes the same antigen in tissue culture as A2B5 antibody **(A)**. Rat E13.5 spinal cord cells were sequentially immunopanned for NCAM and A2B5 to obtain A2B5+/NCAM- cells. The cells were plated and grown on poly-L-lysine/laminin coated dish for 24 h and observed under phase-contrast microscopy **(B)**. Live staining showed that >90% cells are A2B5+ **(B′)**.

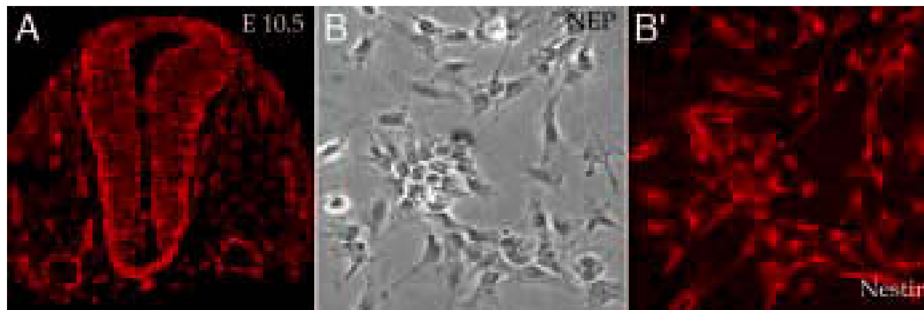


Fig. 1. NEP cells, which are homogenous and nestin positive, are abundant in E10.5 rat neural tube. Multipotent NEP cells exist in E10.5 caudal neural tube and all the cells express nestin (**A**). E10.5 rat NEP cells were dissociated and plated at high density and grown in NEP basal media containing 10% CEE. Cell morphology is observed under phase-contrast microscopy (**B**). When the cells were fixed and labeled for nestin, all of the cells are nestin immunoreactive (**B′**).

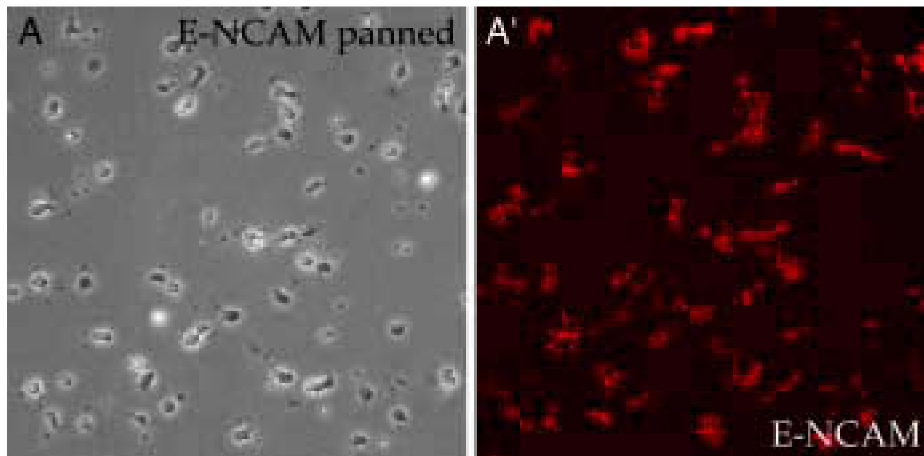
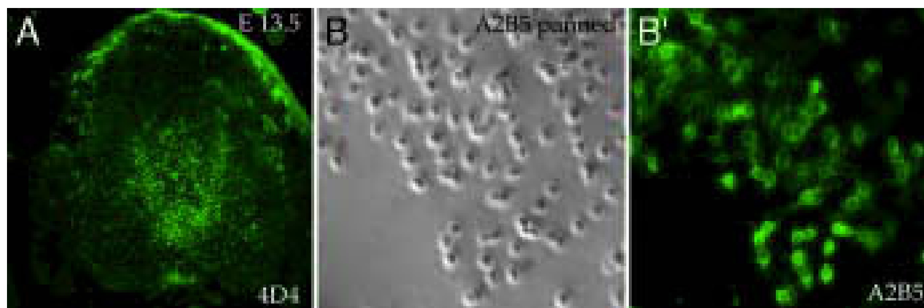


Fig. 2. NRP cells can be purified by immunopanning with the E-NCAM-specific antibodies. Rat E13.5 spinal cord cells were sequentially immunopanned for A2B5 and NCAM to obtain NCAM+/A2B5- cells. The cells were plated and grown on poly-L-lysine/laminin coated dish for 24 h and observed under phase-contrast microscopy (**A**). Live staining showed that >90% cells are NCAM+ (**A′**).



4. Notes

1. Equivalent mouse and human embryonic ages to isolate NEP, NRP and GRP cells are as follow:
   a. In mice, E8.5 for NEP cells and E11.5–E12.0 for NRP and GRP cells.
   b. In humans, embryonic week (EW) 5 for NEP cells, EW8–16 for NRP cells and EW16–24 or later for GRP cells.
2. Since some cultures require the reduction of FGF, NEP media can also be made without FGF and be supplemented with FGF later.
3. Dishes can also be coated at 37°C for 4 h. However, the fibronectin solution cannot be reused.
4. Make fresh enzymatic solution each time before dissection. Higher concentration of papain or dispase alone has also been used successfully by us and others to dissociate neural tube cells, but we prefer the combination of collagenase and dispase for rat neural tubes. Trypsin can also be used to dissect mouse neural tubes.
5. If an embryo does not come out, apply pressure at the other end of the sac gently and squeeze the embryo out.
6. Incubation time is variable depending on the individual researcher.
7. Neural tubes are very sensitive to the enzymes. Even a trace of the enzymes can kill the cells.
8. The NEP cells are very sensitive. Overexposure to trypsin can result in decreased survival.
9. Vigorous mechanical treatment may kill the cells. Small cell aggregates do not affect their attachment to the dish.
10. NEP cells grow rapidly, doubling every 4–6 h in the presence of bFGF and CEE. NEP cells should be passaged every 2 d. Never let cells reach confluence as it causes the cells to differentiate.
11. The most common problem for passaging NEP cells is that cells appear healthy when plated but die overnight or fail to grow due to the residual trypsin. Care needs to be taken to avoid this. Soybean trypsin inhibitor and higher concentration of CEE can be used to neutralize the remaining trypsin.
12. Keep the cells at relatively high densities to ensure a high rate of cell division and to minimize differentiation.
13. Other freezing protocols also work for other labs.
14. The meninges exists as a thin layer and is often overlooked. This could be a possible source of contaminating cells. It is difficult to remove the thin meninges sheet once both ends of the spinal cords are free. Therefore, let the tube remain attached to the head and peel off the meninges while holding on to the head.
15. Do not put too many spinal cords in one tube, otherwise the cells will not dissociate well.
16. Incubating time is variable depending on the individual lab.
17. Do not triturate the spinal cords too harshly. This decreases cell viability.
18. It is hard to replate the frozen NRP cells. If that is the case, use 50% of serum instead of 10%.

19. A 100 mm panning dish can pan cells from about four intact neural tubes. The supernatant preparation is different between labs.
20. The optimal antibody dilution needs to be determined by individual researcher. The concentration of the primary antibodies should be around 5 μg/mL.
21. Do not allow the panning dish to dry between washes.
22. Make sure the cells are dissociated into single cells. Otherwise the purity is reduced dramatically.
23. Do not pan at 37°C. This promotes cell adhesion and allows nonspecific cells to attach to the panning dish.

5. Appendix

5.1. CEE Preparation

5.1.1. Materials

1. Chicken eggs
2. Minimal essential medium (2X) (GibcoBRL, cat. no. 11935).
3. Hyaluronidase, 10 mg/mL stock at –20°C (Sigma, cat. no. H3884).

5.1.2. Procedure for Making CEE

1. Incubate chicken eggs in a humidified incubator at 37°C for 11 d.
2. Wash eggs with 70% ethanol.
3. Macerate approximately 10 embryos at a time by passing them through a 30 mL syringe into a 50 mL Corning tube. This should produce about 25 mL of volume.
4. Add the same volume of EMEM media and incubate at 4°C for 45 min on a rotary wheel.
5. Add sterile hyaluronidase to the final concentration of 2% and centrifuge at 30,000*g* for 6 h at 4°C.
6. Filter the supernatant through a 0.45 μm filter and then through a 0.22 μm filter.
7. Aliquot and store at –80°C. The CEE should last for up to 2 yr if stored as working aliquots at –80°C.

Acknowledgments

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4

Olfactory Ensheathing Cells

Isolation and Culture from the Rat Olfactory Bulb

Susan C. Barnett and A. Jane Roskams

1. Introduction

The ability to produce highly purified populations of individual cell types is crucial for examining the molecular regulation of cell growth, differentiation, and function. Olfactory ensheathing cells (OECs) are also known as olfactory ensheathing glia (OEG) and olfactory nerve ensheathing cells (ONECs). These glia do not fit the classic description of stem cells, but have a number of intriguing developmental characteristics that have recently piqued the interest of the developmental neurobiology community. Their ability to support neuronal regeneration, both within the olfactory system and elsewhere in the central nervous system (CNS) has made them an attractive cellular model for transplantation paradigms. Their ability to switch, in vitro, from a non-myelinating to a myelinating state, has enabled investigators to examine OECs for events leading to the onset of myelination.

In trying to purify OECs and examine their characteristics in a controlled in vitro environment, a number of obstacles have to be overcome. The cellular population of the olfactory bulb consists primarily of oligodendrocytes, astrocytes, OECs, and several types of neurons. The OECs reside in the outer layer of the bulb termed the olfactory nerve layer (ONL) ***(1–3)*** and also ensheath the olfactory nerve as it travels from the olfactory epithelium to the olfactory bulb. It is not clear if the OECs from the olfactory nerve and bulb are phenotypically identical, but they do share many similar properties in vivo. For example, based on antigenic and morphological criteria, both tissues contain two types of OECs, one of which has Schwann cell-like properties and the

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other that has astrocyte-like properties ***(4,5)***. Studies on the OECs from the olfactory bulb have shown that the Schwann cell-like OEC has a spindle-like morphology, expresses the low affinity growth factor receptor (L-NGFr) p75 ***(6)***, and contains diffuse staining for the glial-specific intermediate filament, glial fibrillary acidic protein (GFAP) ***(7)***. The astrocyte-like OEC has a flattened morphology and expresses the embryonic form of neural cell adhesion molecule (E-NCAM), otherwise known as the polysialic form of NCAM ***(8,9)***, contains fibrous GFAP, and expresses low levels of the L-NGFr ***(5)***. In this chapter, we provide a method for culturing OECs from the nerve fiber layer of the rat olfactory bulb. A second method is provided for preparing OECs from the turbinates of the mouse olfactory epithelium.

2. Materials

2.1. Purification of OECs from the Olfactory Bulb

1. Leibowitz medium (L-15, GIBCO) + 25 μg/mL gentamycin (ICN-Flow) (for collecting dissected olfactory bulbs).
2. Hank's balanced salt solution, Ca^{2+}- and Mg^{2+}-free (GIBCO) (for dissociating tissue through needles).
3. Collagenase, stock of 13 mg/mL in L-15 (approx 155 U/mg: ICN Biochemical).
4. Monoclonal antibodies: O4 (Roche Diagnostics) ***(10)***, anti-galactocerebroside (anti-Gal C) ***(11)***.
5. Anti-low affinity nerve growth factor receptor (L-NGFr) (Boehringer Mannheim) A2B5 ***(12)***.
6. Second-class-specific antibodies: anti-mouse IgM-fluorescein (Southern Biotechnology Assoc.), anti-mouse IgG3-phycoerythrin (Southern Biotechnology Assoc.).
7. Poly-L-lysine (PLL; Sigma, <100,000 MW), stock 4 mg/mL, dilute 1:300 in sterile double-distilled water; use at a final concentration of 13.3 μg/mL.

2.2. Generation of Astrocyte Conditioned Media

1. 0.25% trypsin dissolved in L15 (Sigma).
2. SD media: Soybean trypsin inhibitor (0.52 mg/mL), DNAse (0.04 mg/mL), and bovine serum albumin (3 mg/mL) in DMEM (SD) dissolved in L15 (all from Sigma). Store as frozen aliquots at –20°C.
3. DMEM + 10% FCS.
4. Cytosine arabinoside (Ara-C: Sigma) stock of 1 m*M* in DMEM.
5. DMEM-BS: DMEM medium (GIBCO), 4.5 g/L glucose, and supplemented with 25 μg/mL gentamycin (Life Technologies), 0.0286% BSA Pathocyte (ICN), 0.5 μg/mL bovine pancreatic insulin (Sigma), 100 μg/mL human transferrin, 0.2 μ*M* progesterone, 0.10 μ*M* putrescine, 0.45 μ*M*-L-thyroxine, 0.224 μ*M* selenium, and 0.49 μ*M* 3,3′,5-triiodo-L-thyronine (all from Sigma) ***(13)***.
6. Anti-GFAP antibody (Dakopatts).

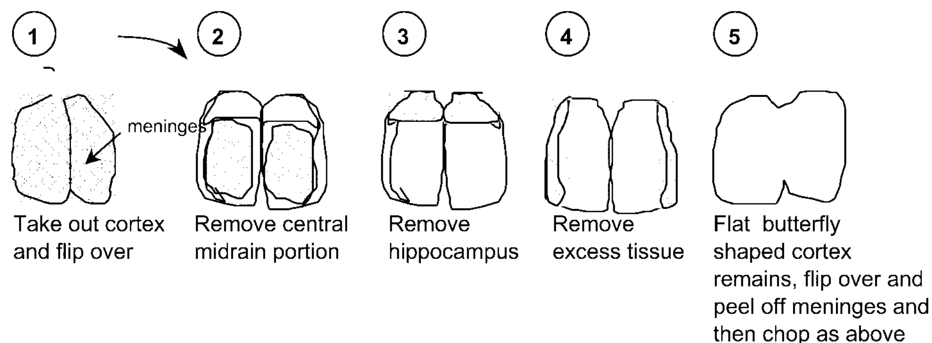

Fig. 1. Dissection of cortex for culturing astrocytes.

7. Coating coverslips/flasks with poly-L-lysine: Pipet enough PLL to coat the appropriate dish/flask and incubate 0.5 h or overnight in a 37°C incubator. Remove all the PLL and leave the dish/flask to air dry in the hood before use. Dried PLL-coated dishes can be stored for at least 1 wk at room temperature.

3. Methods

3.1. Primary Culture of Astrocytes for Generating Astrocyte Conditioned Media (ACM)

1. Remove the cortex from 1 d old or newborn rat pups and place in a small Petri dish containing 1 mL of L-15 + gentamycin.
2. Dissect off the central tissue (midbrain), followed by the edge tissue and hippocampus, until a flat, white, butterfly-shaped piece of cortical tissue remains. Flip this over and peel off the meninges (**Fig. 1**).
3. Chop up the cortex using a sterile scalpel and place in a 30 mL plastic sterile universal container (Bibby-Sterilin). For 15 animals, place the cortices in 2 mL of L-15 and 1 mL of collagenase and incubate at 37°C for 30 min.
4. Add 1 mL of 0.25% trypsin and incubate at 37°C for a further 20 min.
5. Centrifuge the cells at 800*g* for 5 min and then remove the supernatant.
6. Add 1 mL of EDTA and 1 mL of 0.25% trypsin to the pellet and incubate for a further 20 min at 37°C.
7. Add 1 mL of SD media from frozen aliquots, pipet up and down to mix, then centrifuge the cells at 1000*g* for 5 min and remove the supernatant.
8. Add 5 mL DMEM-10%FCS and gently pass through a 5 mL pipet 5X (*see* **Note 1**).
9. Count the cells and plate at 1.5 –2 × 10^7 cells/75-cm^2 flask in 10 mL of DMEM/10% FCS.
10. Feed and maintain cultures in DMEM/10% FCS the next day to remove debris. Keep feeding twice a week. When cells are completely confluent and no more obvious cell division can occur, proceed with purification.

3.2. Purification of Astrocyte Population

1. To remove top layer of oligodendrocytes, shake the cells on a rocker platform overnight at 37°C (*see* **Note 2**).
2. The next day feed with DMEM-10%FCS containing $2 \times 10^{-5} M$ Ara C, to kill off actively dividing oligodendrocytes and progenitor cells.
3. Feed again the next day with DMEM containing 10% FCS and maintain the cells in this medium until ready to collect conditioned medium DMEM-BS from the confluent astrocytes (*see* **Note 3**).
4. When astrocytes are confluent and pure, add DMEM-BS and leave on for 10 min, then remove and add 10 mL of fresh DMEM-BS for 48 h.
5. Collect the DMEM-BS and replace with DMEM-10%FCS. Spin the ACM at 1000*g* for 5 min.
6. Filter sterilize by passing through a 0.2 µm filter and freeze at –20°C in aliquots of 20 mL. This is your concentrated ACM that is usually added at 1:5 diluted in DMEM-BS to cells. Stocks can be frozen for several months (at –20°C) and once thawed last 1–2 wk at 4°C.

3.3. Dissection and Degradation of Olfactory Bulb

Purified cultures of OECs are generated from neonatal rats (1–7 d old pups). It is possible to use fluorescence-activated cell sorting (FACS) as a purification technique for both younger and adult preparations, but cell numbers may be limited from embryonic tissue. We have used a range of rat strains and routinely use either Sprague-Dawley or Fischer-344 (for in vivo work, inbred strain), which we consider to be the best. For optimal results we use 15–25 animals per preparation.

1. Decapitate the rats according to humane animal care protocols and rapidly remove the skin and skull to reveal the olfactory bulbs (**Fig. 2**).
2. Remove the olfactory bulbs. The bulbs are readily visible and are separated from the brain using the tip of curved forceps to sever connections at the cribriform plate, taking care not to remove any cortical tissue. The bulbs are lightly scooped from the head and placed in a 35 mm Petri dish containing 2 mL of L15 medium + gentamycin (Gibco).
3. Chop the tissue using a scalpel blade with rapid chopping motion moving the dish around in a circle so that all the pieces become evenly cut and the tissue pieces that remain are all roughly the same size of around 0.5 mm^2 (**Fig. 2**). Then transfer tissue using a sterile plastic pipet into a polystyrene 7 mL Bijou (Bibby-Sterilin) containing 500 µL of collagenase (1.33%, ICN Biochemicals in L15 medium) and 1 mL of L15 + gentamycin and incubate for 30 min at 37°C in the incubator (*see* **Note 4**).
4. Following tissue digestion, generate a single cell suspension by sequentially passing the cells 2–4X through a series of decreasing gauge needles starting with a 19G needle followed by 20G and ending with a 23G needle, keeping

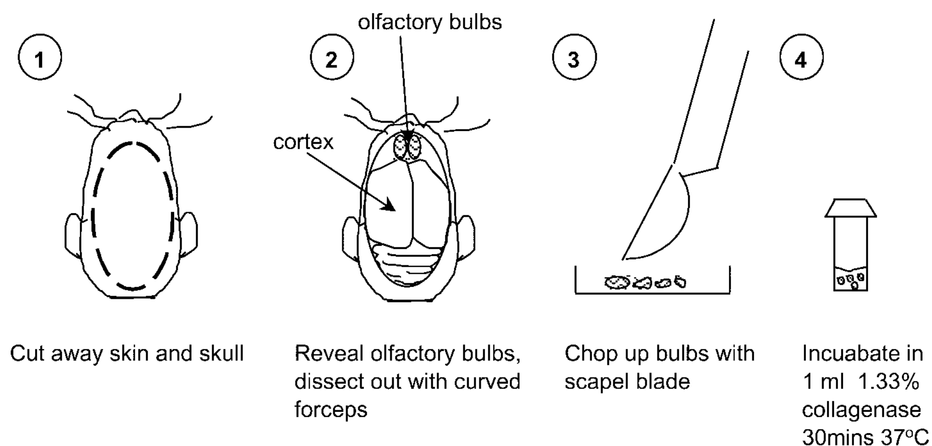

Fig. 2. Dissection of olfactory bulb for culturing OECs.

the cell suspension in the bijou. This must be carried out very gently, as the cells are very fragile. If clumps remain, pass the cells through a 20G needle one additional time.

5. Transfer the final cell suspension into a 15 mL centrifugation tube (Falcon) add 4 mL of HBSS, Ca^{2+}- and Mg^{2+}-free media and spin at 1000*g* for 5 min at room temperature.

3.4. FACS Purification of Olfactory Ensheathing Cells

1. Incubate the olfactory bulb suspension for 1 h at 4°C with hybridoma supernatant from the O4 and Gal C cell lines, both diluted 1 : 1 in L15 or DMEM/1% FCS (*see* **Note 5**).
2. Wash cells once by centrifugation at 1000*g* at room temperature for 5 min in DMEM containing 1% FCS.
3. Transfer the cells into a 15 mL centrifugation tube (Falcon) and add 500 μL of DMEM containing 1% FCS and a 1 : 100 dilution of class specific antibodies IgM-fluorescein and IgG3-phycoerythrein (*see* **Note 6**).
4. Perform secondary antibody incubation for 45 min at 4°C.
5. Wash the cells (*as in* **step 2**, above) twice in DMEM containing 1% FCS, place in snap top 5 mL sterile tubes at 4×10^6 cell/mL and purify using their O4 positive/GalC negative profile (**Fig. 3**).
6. After FACS purification, cells are plated onto PLL-coated coverslips in 30 μL to test for purity. Volumes must be kept low to ensure high cell density, as OECs require close cell contact. Approximately 30 coverslips can be made from 20 animals and for each milliliter of antibody solution we use a cell suspension generated from 10 animals.

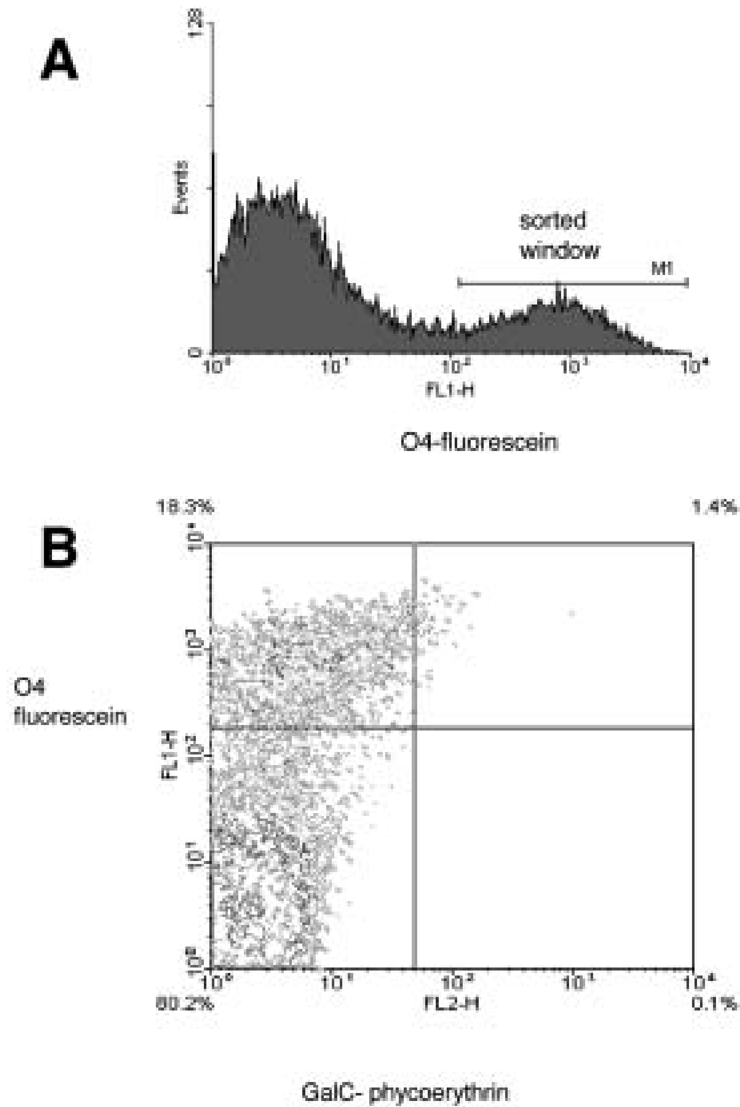

Fig. 3. The one-dimensional profile (upper panel) of the O4 labeled cells (using a Becton Dickinson FACS Vantage cell sorter). The x-axis represents the fluorescent intensity of the labeled cells and the y-axis, the cell numbers. Using a sort window, the average number of cells that are O4 positive ranges from 6-15%. The lower panel represents the two-dimensional plot of O4-fluorescent intensity against GalC-fluorescent intensity (which is much lower). Using this plot we draw the sort window around the population of cells that are O4 positive and GalC negative.

7. Feed cells with ACM diluted 1:5 with DMEM-BS three times a week (*see* **Notes 7** and **8**).

4. Notes

1. This is based on a method of Noble and Murrey ***(14)***. Use 2.5 animals per 75 cm^2.
2. When the astrocyte monolayer is confluent, there are often a top layer of contaminating oligodendrocytes and their progenitors. The astrocytes are the flat fibroblast-like cells, with the contaminating phase bright bi-, tri-, and multiprocess cells of the oligodendrocytes and O-2A progenitors on top.
3. The monolayers should consist of pure type-1 astrocytes, which can be demonstrated by immunolabeling with the intermediate filament glial fibrillary acidic protein (GFAP).
4. One of the antigens that defines the olfactory bulb glial cells is trypsin-sensitive (low affinity nerve growth factor receptor, L-NGFr), so the cells must be dissociated in collagenase alone.
5. To generate hybridoma supernatant from the O4 or GalC expressing cell lines, we grow the cells in DMEM containing 10% FCS until 80% confluent. The medium is gently removed (as the cells grow in suspension) and replaced with DMEM-BS or fresh DMEM-10%FCS. Choice of hybridoma supernatant depends on whether the cells to be immunolabeled are susceptible to differentiation in serum-containing medium. The collection medium is left on the cells for 24–48 h and then collected. The supernatant is centrifuged at 1000*g* for 10 min to remove any cells, and the supernatant is collected and filter sterilized through a 0.2 μm filter. The supernatant is usually collected and pooled to give a stock of around 100 mL, which can then be tested on known positive antigen-expressing cells. Once confirmed positive, the supernatant is frozen and stored in 1–2 mL aliquots. Thawed samples remain positive for several weeks when stored at 4°C. The antibodies are also commercially available (Roche Diagnostics)—use at recommended concentrations.
6. We have found that OECs express the O4 antibody in vivo ***(5)*** that in turn labels the two types of OECs. We also include anti-galactocerobroside (Galc) in the sort, because O4 is also expressed on oligodendrocyte-type 2 astrocyte (O-2A) lineage cells ***(15)***. To remove the committed oligodendrocytes, we create a sort window around the O4 positive GalC negative cells. The efficiency of this procedure may be worked out by calculating the number of O4+ cells on the following day.
7. We have found it best to place dissociated embryonic olfactory bulb tissue in culture and allow them to grow for a week in serum-free modified DMEM [DMEM-BS; ***(13)***] conditioned by type 1 astrocytes (ACM, astrocyte conditioned medium). From 20 animals, it is reasonable to generate 1.5×10^4 viable cells.
8. To characterize the resulting OEC population, a range of antibodies can be used. Expression of a panel of antigens on the OEC can define it as Schwann cell-like or astrocyte-like ***(5,16)***.

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5

Culturing Olfactory Ensheathing Glia from the Mouse Olfactory Epithelium

Edmund Au and A. Jane Roskams

1. Introduction

The majority of studies centered on understanding the in vitro properties of olfactory ensheathing glia (OEG) have utilized OEG prepared from the nerve fiber layer of the embryonic or neonatal olfactory bulb ***(1–3)***, summarized in chapter 4. In fact, a significant fraction of the OEG population is found within the lamina propria of the olfactory epithelium as they ensheath olfactory receptor axons en route to the olfactory bulb (**Fig. 1**). Generating olfactory ensheathing glia from the lamina propria presents a unique set of obstacles—freeing the lamina propria tissue from the neuronal epithelium, the cartilaginous turbinates, and the extracellular matrix (ECM) in which the cells are embedded, in a way that will avoid fibroblast contamination. Thus, the method used to culture epithelium-derived OEGs is quite different from the method for bulb-derived glia, and encompasses steps to minimize cartilage contamination (careful dissection), digest away ECM (using a defined enzyme treatment), and remove fibroblasts, which certainly will find their way into the culture (cytotoxic lysis of contaminating fibroblasts). This method generates a culture of proliferating olfactory ensheathing glia that express, thus far, the same antigenic markers as bulb-derived olfactory ensheathing cells (OECs) ***(4)***. The procedure outlined in this chapter has been shown to eliminate 99% of nonglial cells from the glial culture by passage 3, and to produce a glial culture that is 98–100% positive for the glial markers (glial fibrillary acidic protein) (GFAP), S100Beta and p75, at 4 wk in vitro.

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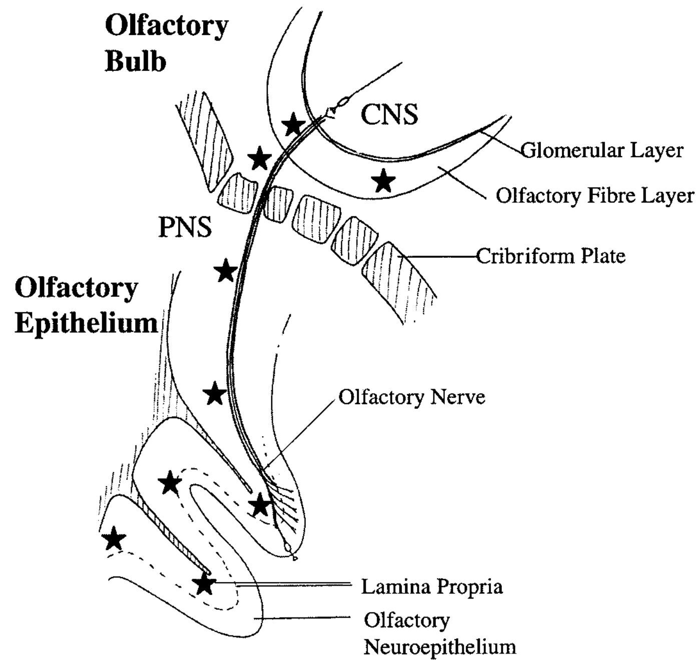

Fig. 1. Distribution of olfactory ensheathing glia. Olfactory ensheathing glia (OEG, depicted by stars) are distributed throughout the olfactory neuraxis and ensheath the olfactory nerve from the olfactory turbinates (in the peripheral nervous system, PNS), through the cribriform plate and around the olfactory bulb (in the central nervous system, CNS).

2. Materials

2.1. Dissection of the Olfactory Epithelium

1. Large pair of surgical scissors (15 cm in length).
2. Fine no. 7 curved forceps (smooth, nonserrated tips, approx 11 cm in length tips).
3. Single edge no. 9 razor blades (one for every three pups used plus one for mincing tissue).
4. Styrofoam slat, 25 × 15 cm and 3 cm thick. Can easily be prepared from the lid of a Styrofoam box.
5. 10 mm Petri dish (Becton Dickinson) containing approx 15 mL DMEM/F12.
6. Small pair of surgical scissors.
7. All equipment is sterilized by bead sterilization and spraying with 70% ethanol prior to use.

2.2. Producing Single Cell Suspension from the Epithelium

1. Two sterile 50 mL conical tubes (Becton Dickinson).
2. Sterile P1000 tip, cut 4 mm from the tip with a sterile pair of scissors to enlarge the opening.
3. Enzyme digestion mix (per pup): 500 µL DMEM/F12 (Gibco), 0.5 mg bovine serum albumin (Sigma), 0.6 mg collagenase D (Roche), 50 µL (30 U/mL) dispase I (Roche), 50 µL (300 µg/mL) hyaluronidase (Sigma), 5 µL (10 U/µL) DNAse (Roche).
4. Cotton-plugged glass Pasteur pipet, 9 in. long (Fisher Scientific).
5. 40 µm nylon cell strainer (Becton Dickenson cat no. 352340).
6. Initial plating medium: MEM-D-valine, 10% fetal calf serum, 1% penicillin/streptomycin (all from Gibco).

2.3. Cytotoxic Elimination of Fibroblasts

1. DMEM/F12 (Gibco).
2. H022 hybridoma medium (ATTC) containing anti-Thy 1.1.
3. Rabbit complement (Sigma) 1 mg/mL.
4. Growth medium: DMEM/F12 , 10% fetal calf serum, 1% penicillin/streptomycin (all from Gibco).
5. 0.25% trypsin/EDTA (Gibco).

2.4. Poly-L-Lysine Coating of Tissue Culture Flasks

Poly-L-lysine coated 175 cm^2 flasks (use one for every five pups used in prep):

1. Precoat T175 flask with 4 mL of poly-L-lysine stock solution (Sigma) 50 µg/mL in 15 m*M* borate buffer solution pH 8.4.
2. Incubate at room temperature for 1 h.
3. Aspirate off poly-L-lysine.
4. Rinse twice with PBS.
5. Let sit in Initial Plating or Growth Medium until ready for use (up to 1 wk).

2.5. Antibodies for Verification of OEG Phenotype

1. Rabbit anti-P75 (lNGFR), Chemicon. The widely published anti-rat lNGFR monoclonal (Boehringer Mannheim) will not work specifically in mouse tissue.
2. Anti-GFAP (Dakopatts).
3. Anti-Beta S100 (monoclonal, polyclonal, Sigma).

3. Method

3.1. Dissection of the Olfactory Epithelium

1. Decapitate P5–P7 mice, five at a time with large surgical scissors and place the heads on the styrofoam slat (*see* **Note 1**).

2. Cut the heads in half sagittally. To do so, place a corner of the razor blade into the back of the head and slice down through the head. Slice five heads in half at a time, perform the dissection, and repeat.
3. Dissect out the epithelium. Place the flat edge of the forcep tips against the top of the palate and the bottom of the skull (along the top and bottom of the epithelium). Make sure the tips do not go further than the cribriform plate. Push down gently, pinch, and peel/tease the epithelium away from the underlying bone and cartilage (*see* **Note 2**).
4. Place the tissue into the 10 mm Petri dish containing 15 mL of DMEM/F12.
5. Pool the dissected tissue from all heads. The remainder of the procedure will be performed in a tissue culture hood.
6. Mince epithelium with small surgical scissors followed by a razor blade. To do so, tip the dish so that all the tissue collects in one area, and mince with small surgical scissors for 2 min. Then lay the dish flat and finely chop the tissue for an additional 2 min with a razor blade.

3.2. Preparing a Single Cell Suspension from the Olfactory Epithelium

1. Pour all of the minced tissue from the dish into a 50 mL conical tube (*see* **Note 3**). Use an additional 10–15 mL of DMEM/F12 to ensure efficient transfer (again, by pouring). Spin at 130*g* for 10 min, room temperature.
2. Aspirate off supernatant. Add 3 mL of fresh DMEM/F12 and triturate (pipeting up-and-down) pellet with the precut P1000 tip 25X. Have the P1000 set at 1000 μL for trituration.
3. Spin mixture at 330*g* for 5 min and aspirate off supernatant. Resuspend pellet by trituration in the enzyme digestion mixture (adjust according to the number of pups used).
4. Incubate mixture for 1 h in a 37°C water bath. Swirl contents manually every 5–10 min.
5. Spin tube at 330*g* for 5 min and aspirate off supernatant. Add 2 mL of fresh DMEM/F12 and triturate pellet 35X with a cotton-plugged Pasteur pipet. Use a pipet-aid (Drummond) at the fast setting, "F" for trituration.
6. Add 10 more mL of DMEM/F12 into tube and swirl gently to bring tissue into suspension.
7. Transfer all digested tissue (by rinsing repeatedly with serum-free DMEM/F12) and the entire slurry of cells through a 40 μm nylon cell strainer into a new 50 mL conical tube.
8. Use another 10 mL of DMEM/F12 to rinse the old conical tube filter this as well.
9. Spin collected cells down at 330*g* for 5 min.
10. Remove supernatant and resuspend cell pellet in 3 mL of initial plating medium.
11. Plate cells into T175 flasks (one T175 flask for every five pups used) precoated with poly-L-lysine and incubate at 37°C with 5% CO_2 for 4 d.

3.3. Cytotoxic Elimination of Fibroblasts

This procedure is usually performed 2–3X on successive passages in order to fully eliminate all fibroblasts from the culture.

1. Aspirate off media and rinse cells with 10 mL of sterile phosphate-buffered saline.
2. Aspirate off PBS and add 1.75 mL of 0.25% trypsin/EDTA (Gibco).
3. Let sit at room temperature for 2 min and take up the cells with 10 mL of PBS into a 15-mL conical tube (Falcon, Becton Dickinson).
4. Spin cells down at 330*g* for 5 min and aspirate off supernatant.
5. Add to pellet (*see* **Note 4**): 1 mL of DMEM/F12, 100 µL of Thy 1.1 hybridoma media, 125 µL (1 mg/mL) of rabbit complement (Sigma).
6. Triturate pellet 10X with a cotton-plugged glass Pasteur pipet (again, use an electric pipet-aid at a setting of "F") and incubate mixture for 30 min at room temperature.
7. Spin down at 330*g* for 5 min and aspirate off supernatant. Resuspend pellet in growth media and plate out cells in poly-L-lysine-coated flasks (*see* **Note 5**).

3.4. Verification of OEG Phenotype

1. Plate OEG onto poly-L-lysine-coated four-chamber slides at a density of 5000 viable cells/well.
2. Remove media and wash 3X with PBS.
3. Fix either with 4% paraformaldehyde (PFA) for 10 min at room temperature or with ice-cold methanol for 10 min at 4°C.
4. Allow cells to settle and expand for 4 d.
5. Store at 4°C for up to 2 wk in PBS (with 0.1% azide), and stain using standard immunofluorescence protocols. GFAP, Beta-S100, and p75 should overlap (degree of staining varies with time in culture) to verify OEG phenotype.

4. Notes

1. For a good cell yield (after 4–5 d, there should be 10–14 × 10^6 cells in each flask), use at least one large litter of mice (10 or more). Mice are generally sacrificed by cervical dislocation prior to decapitation or simply by decapitation alone. (This will depend on the animal care protocols of your institution.)
2. Be careful not to push the forceps too far down or you will be removing optic tissue along with the epithelium. The general idea is to "scoop" the epithelium out of the head with the tips of the forceps.
3. This is to prevent tissue loss. If you transfer the tissue with a plastic pipet, the tissue will tend to stick to the inside walls.
4. Volumes above are for one T175 flask. Multiply the volumes by the number of T175 flasks trypsinized.
5. It may take as many as three passages with complement-mediated lysis to get rid of all fibroblasts in culture. Epithelium-derived olfactory ensheathing glia

expand quickly and can be plated at a dilution of 10^6 per 175 cm^2 flask) and still attain confluency.

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6

Production of Immortalized Human Neural Crest Stem Cells

Seung U. Kim, Eiji Nakagawa, Kozo Hatori, Atsushi Nagai, Myung A. Lee, and Jung H. Bang

1. Introduction

Neural crest cells migrate from the dorsal aspect of the neural tube and differentiate into a variety of cell types in different locations. These cell types include peripheral neurons and glia (Schwann cells), melanocytes, endocrine cells, smooth muscle, skeletal muscle and bone ***(1)***. In vivo lineage tracing and in vitro clonal analyses in avian embryos have indicated that many neural crest cells are multipotent ***(2*,3)** and transplantation and culture studies suggest that the fate of multipotent neural crest cells can be determined by the environment ***(4–6)***.

In the central nervous system (CNS), neurons and glial cells are also generated from a small number of progenitor cells in neural tube. While the existence of multipotent CNS progenitor cells for neurons and glial cells, neural stem cells, has been described in vivo and in vitro, the signals that regulate their proliferation, differentiation, and continuous survival are less well understood. A neural stem cell is defined as a single cell with the ability to proliferate, exhibit self-maintenance or renewal over the life time of the organism, generate a large number of clonally related progeny, retain its multilineage potential over time, and produce new cells in response to injury or disease ***(7–9)***. We have previously reported that immortalized human neural stem cells generated in Harvard/ University of British Columbia are capable of performing all the critical functions previously ascribed to neural progenitor cells ***(10)***.

We have recently generated cell lines of human neural crest stem cells from primary cell culture of human embryonic dorsal root ganglia tissue using a

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retroviral vector containing v-myc oncogene. These cell lines, HNC10, are genetically modified human neural crest stem cells, stem cells of peripheral nervous system, maintained as a stable cell lines in serum-free medium supplemented with basic fibroblast growth factor (bFGF), remain uncommitted, undifferentiated, and multipotent, and they express phenotypes specific for neural crest stem cells such as nestin ***(11,12)***, vimentin ***(13)***, and low affinity nerve growth factor receptor (LNGFR, P75) ***(14)***.

When HNC10 human neural crest stem cells (HNSCs) were grown in serum-containing medium, various differentiated cell types which are known as progeny of neural crest stem cells such as neurons, Schwann cells, adrenal chromaffin cells, and skeletal muscle cells were induced in culture. More than 10–30% of total cells expressed triplet neurofilament proteins (NF-L, NF-M, NF-H), MAP2, tubulin βIII isoform, and peripherin. These phenotypes are specifically and exclusively expressed by mammalian, including human, nerve cells. Schwann cells were identified by the expression of S-100 and PO protein; adrenal chromaffin cells by chromogranin, and skeletal muscle cells by the expression of desmin and myosin, demonstrating that HNSCs grown in serum-containing medium are capable of differentiating into cells with specific phenotypes.

HNC10 cells are self-renewing and multipotent in vitro, giving rise to four different lineages, i.e., neurons, Schwann cells, skeletal muscle cells, and adrenal chromaffin cells. HNC10 cells express phenotypes characteristic for neural crest stem cells, i.e., nestin and p75/LNGFR indicating that HNC10 cells are of neural crest stem cell origin ***(12,19)***. Neural crest stem cells can be distinguished from CNS stem cells by their morphology, by expression of LNGFR, by the progeny that they generate, and by their inability to generate CNS derivatives such as astrocytes ***(20)***. HNC10 cells grown in serum-free medium expressed immunoreactivity for antibodies specific for nestin and p75/LNGFR, while a large number of HNC10 cells grown in serum-containing medium were immunopositive for NF-L, NF-M, NF-H, MAP-2, peripherin, and tubulin β III isoform, cell-type-specific markers for neurons. In addition HNC10 cells grown in serum-containing medium were immunoreactivity-positive for S-100 (for Schwann cells), chromogranin (for adrenal chromaffin cells), and desmin/myosin (for skeletal muscle cells) indicating that HNC10 neural crest stem cells are capable of differentiation into non-neuronal cells such as Schwann cells, adrenal chromaffin cells, and muscle cells. Whether HNC10 cells possess the ability to differentiate into other non-neuronal cell derivatives of neural crest stem cells such as melanocytes, chondrocytes, or osteocytes awaits further studies.

2. Materials

2.1. Dorsal Root Ganglion (DRG) Cell Culture

1. Phosphate-buffered saline (PBS, Sigma, St. Louis, MO).
2. 0.25% collagen (CSL grade, Worthington Biochemical, Lakewood, NJ).
3. DNase I (Sigma).
4. 2.5% trypsin (GIBCO-BRL, Gaithersburg, MD).
5. Dulbecco's modifeid Eagle medium (DMEM) (Stem Cell Tech, Vancouver, Canada).
6. DMEM with 5% fetal bovine serum (Hyclone, Salt Lake City, Utah), 5 mg/mL D-glucose, 20 μg/mL gentamicine (Sigma), and 2.5 μg/mL amphotericine B (Sigma).
7. Nerve growth factor (NGF, Peprotech, Princeton, NJ), 10 μg/mL stock in PBS; epidermal growth factor (EGF, Peprotech), 20 μg/mL stock in PBS; basic fibroblast growth factor (bFGF, Peprotech), 10 μg/mL stock in PBS.
8. Poly-L-lysine (Sigma), 10 μg/mL final concentration in distilled water.
9. Six-well culture plates (Falcon).
10. 50- and 15-mL conical tubes (Falcon).
11. 100- and 60-mm plastic culture dishes (Falcon).

2.2. Retroviral Vector

1. PASK 1.2, an amphotropic replication-incompetent retroviral vector encoding v-myc (transcribed from the retrovirus LTR plus neo transcribed from an internal SV40 early promoter) is produced in our laboratory (**Fig. 1**). This amphotropic vector was generated using the murine retrovirus encoding v-myc, similar to that described for generating murine neural stem cell clone C-17-2 ***(18)***, and used to infect the PA317 amphotropic packaging cell line. Supernatants from the new producer cell line contain replication-incompetent retroviral particles bearing an amphotropic envelope that efficiently infect human cells as indicated by G418 resistance.

2.3. Cytogenetic Analysis

1. Colcemid (Sigma).
2. Ethidium bromide (Sigma).
3. Giemsa stain (Sigma).

2.4. Antibodies for Specific-Cell-Type Markers

1. LNGFR/p75 (1:1 as culture supernatant, mouse monoclonal antibody/mAb, ATCC, Rockville, MD).
2. Biotinylated goat anti-mouse IgG (Vector, Burlingame, CA).
3. Biotinylated goat anti-rabbit IgG (Vector).

Fig. 1. PASK1.2 amphotropic replication-incompetent retroviral vector encoding v-myc.

4. Avidin-biotin complex (ABC,Vector).
5. Diaminobenzidine (DAB).

2.5. Antibodies for Characterization of Specific Cell Types

1. For neurons: neurofilament low-molecular-weight protein (NF-L, 1:1000, mouse mAb, Sigma), NF-M (1:4, mouse mAb, Dr. V. Lee), NF-H (1:1000, mouse mAb, Sigma), microtubule-associated protein-2 (MAP2, 1:1000, mouse mAb, Sigma), β tubulin III isoform (1:1000, mouse mAb, Chemicon, Temecula, CA), and peripherin (1:1000, mouse mAb, Chemicon).
2. For Schwann cells: S-100 protein (1:500, rabbit, DAKO, Carpinteria, CA), P0 myelin protein (1:500, rabbit, Dr. S. Uyemura), and glial fibrillary acidic protein (GFAP, 1:5000, rabbit, DAKO).
3. For myoblast/myotube: desmin (1:1000, mouse mAb, Chemicon) and myosin (1:3000, mouse mAb, Chemicon).
4. For adrenal chromaffin cells: chromogranin (1:1000, rabbit, Dr. R. Angeletti).

3. Methods

3.1. Primary Culture of Human Dorsal Root Ganglion (DRG) Cells

Primary dissociated cell cultures of embryonic human DRG are prepared as described previously ***(15,16)***.

1. Twenty pairs of spinal dorsal root ganglia are isolated from a 15-wk gestation embryo and kept in a 60 mm plastic culture dish containing phosphate-buffered saline (PBS) supplemented with 1000 units/mL of penicilin and 1 mg/mL of streptomycin. Final concentration of antibiotics in PBS is 10-fold of what routinely is used in culture medium (*see* **Note 1**).
2. DRG tissues are transferred to a 15 mL conical centrifuge tube with 3 mL PBS containing 0.25% collagenase and 40 μg/mL DNase type1 and incubate in a shaking water bath for 30 min at 37°C.
3. Remove collagenase solution from the tube without disturbing DRG tissues in the tube bottom, add 3 mL PBS containing 0.25% trypsin and 40 μg/mL DNase type1, and incubate DRG tissues further for 10 min at 37°C.
4. Remove trypsin solution, then add 3 mL of culture medium (consisting of DMEM with 10% fetal bovine serum, 5 mg/mL of D-glucose, 20 μg/mL of gentamicin, and 2.5 μg/mL amphotericine B) to the tube, gently triturate enzyme-digested DRG about 20X. Remove 2.5 mL of the supernatant that contains dissociated single cells into a new 15 mL conical tube, taking care not to include any tissue

Table 1
UBC1 Serum-Free Chemically Defined Medium

N-acetylcysteine	Oleic acid
Albumin	Progesterone
Ascorbic acid	Putrescine
Biotin	Retinol acetate[a]
Catalase	Sodium pyruvate[b]
Cupric sulfate	Sodium selenite
Ethanolamine	Superoxide dismutase
Galactose	Tocopherol acetate
Glutathione/reduced	Transferrin
HEPES	Triiodothyronine
Hydrocortisone	Trolox
Insulin	Vitamin B12
Linoleic acid	Zinc sulfate

[a]Water-soluble forms of retinol acetate and linoleic/oleic acids are used.
[b]Pyruvate should be excluded when DMEM is used as a basal medium.

fragments from the tube bottom. Add 2 mL of culture medium, triturate, remove supernatant, add it to the first, and spin the tube at 300*g* for 8 min (*see* **Note 2**).

5. Aspirate the supernatant and add 3 mL of culture medium containing 100 ng/mL NGF, 20 ng/mL of EGF, and 10 ng/mL of bFGF to the pellet, triturate, and plate at the concentration of 5×10^5 cells/mL in six-well plates. Culture medium is changed twice a week.
6. On d 4, renew culture medium supplemented with 5×10^{-6} *M* of cytosine arabinoside (AraC) and 24 h later change the medium with a fresh culture medium without AraC. AraC treatment should reduce the number of fibroblasts in the DRG culture and enrich with neurons and Schwann cells (*see* **Note 3**).
7. On d 14, replace the culture medium with serum-free culture medium consisting of DMEM containing UBC1 supplements (with human insulin, human transferrin, sodium selenite, progesterone, triiodothyronin, and other nutrients and antioxidants) (*see* **Table 1**) ***(17)***, 5 mg/mL of glucose, 20 μg/mL of gentamicin, 2.5 μg/mL of amphotericin B, and 20 ng/mL of EGF and 10 ng/mL of bFGF. DRG cultures grown for 1–4 wk consist of DRG neurons, Schwann cells, and fibroblasts (**Fig. 2A**) and are used for gene transfer experiments (*see* **Note 4**).

3.2. Retrovirus-Mediated Gene Transfer

Infection of human DRG cells in six-well plates should be performed three times by the following procedures:

1. Add 2 mL of supernatant (4×10^5 CFUs) from the packaging cell line and 8 μg/mL polybrene to target cells in six-well plates and incubated for 4 h at 37°C (*see* **Note 5**).

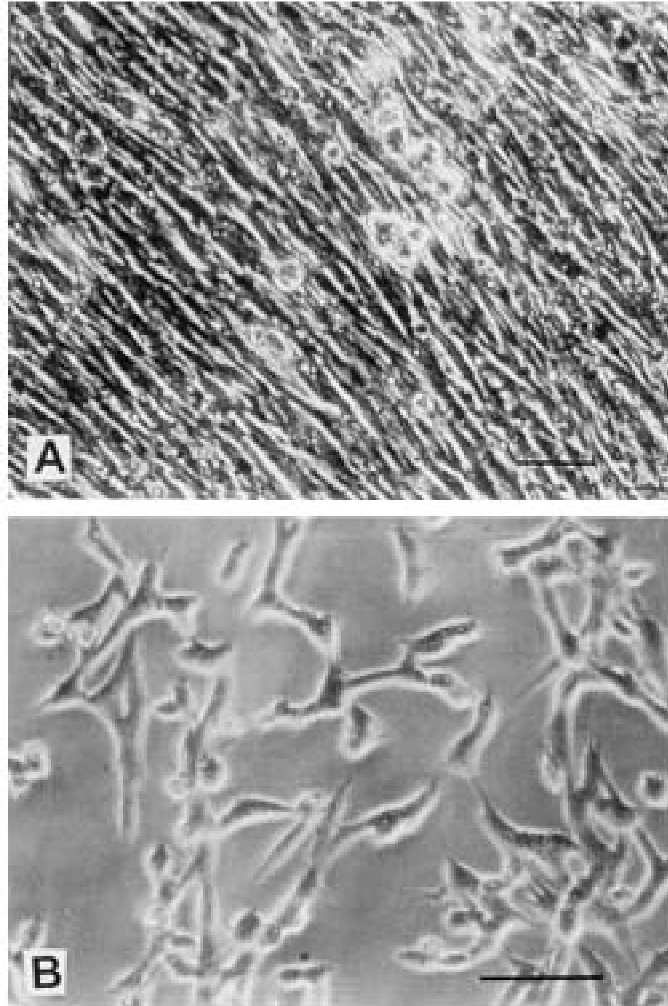

Fig. 2. **(A)** A live cell culture of human dorsal root ganglia isolated from 15 wk gestation embryo grown in vitro for 7 d. The culture contains a large number of neurons, Schwann cells, and neural crest stem cells, and represent a starting material for the generation of human neural crest stem cell lines. Phase contrast microscopy. Scale bar: 20 μm. **(B)** Immortalized human neural crest stem cells, HNC10, are grown in serum-free medium UBC-1 containing 10 ng/mL bFGF. When HNC10 cells are grown in serum-containing medium (10% fetal bovine serum), they differentiate into neurons, Schwann cells, adrenal chromaffin cells, and skeletal muscle cells.

2. Replace infection medium containing retroviral vectors with fresh culture medium; repeat infection process 24 and 48 h later.
3. 72 h following the third infection, select infected cells with 200–500 μg/mL G418 for 14 d. During each culture medium change, supplement the medium wiht G418.
4. At 14 d in culture, terminate G418 treatment and use normal complete medium without G418 is used for further propagation of cultures.
5. At various times during the 3–4 wk in culture, large clusters of cell clones can be identified, isolated, and individually grown in poly-lysine-coated six-well plates.
6. Generate individual clones by limited dilution and propagated them for further study. In our study, six individual clones were isolated and designated as HNC10 human neural crest stem cell lines (**Fig. 2B**).

3.3. Cytogenetic Analysis

Cytogenetic analyses should be performed on established cell lines at various passages.

1. Apply 0.05 μg/mL of colcemid and 5 μg/mL of ethidium bromide together to the cells for 2 h before cell harvest. Detach cells from dishes by brief trypsin treatment.
2. After centrifugation, add a 4:1 mixture of 75 m*M* of potassium chloride and 34 m*M* of sodium citrate to cell pellet for 12 min as hypotonic treatment.
3. Fix the cells in methyl alcohol:acetic acid (3:1). Stain the chromosomes with Giemsa. Three to five karyotypes can be constructed from the each cell preparation.

3.4. Generation of Immortalized Human Neural Crest Stem Cells

1. Isolate and expand six G418-resistant clones. The cloned human neural crest stem cells (HNCSs) are tripolar or multipolar in morphology with 8–10 μm in size (**Fig. 2B).**
2. Cytogenic analysis of HNCSs shows normal karyotype of human cells with a 46XY karyotype without any chromosomal abnormality (**Fig. 3**)
3. Grow the cells on coverslips and process for immunocytochemical staining of cell-type-specific markers. Almost all of the HNCSs are immunopositive for nestin (**Fig. 4A**) and LNGFR/p75 (**Fig. 4B**) indicating that HNC10 cells are indeed neural crest stem cells.

3.5. Immunocytochemical Staining for Specific Markers

Immunochemical determination of cell-type-specific markers in human neural crest stem cell lines should be performed using antibodies specific for neural crest stem cells, peripheral neurons, Schwann cells, and adrenal chromaffin cells (**Table 2**).

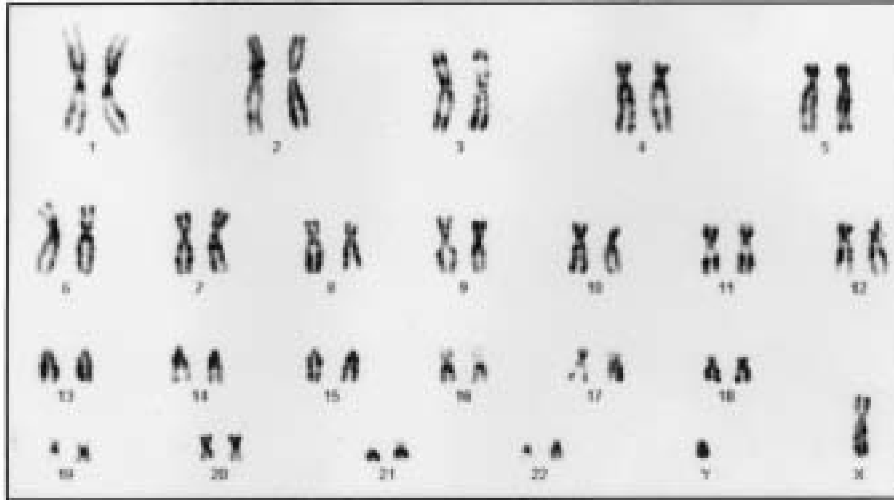

Fig. 3. HNC10 human neural crest stem cells show normal human karyotype of 46XY.

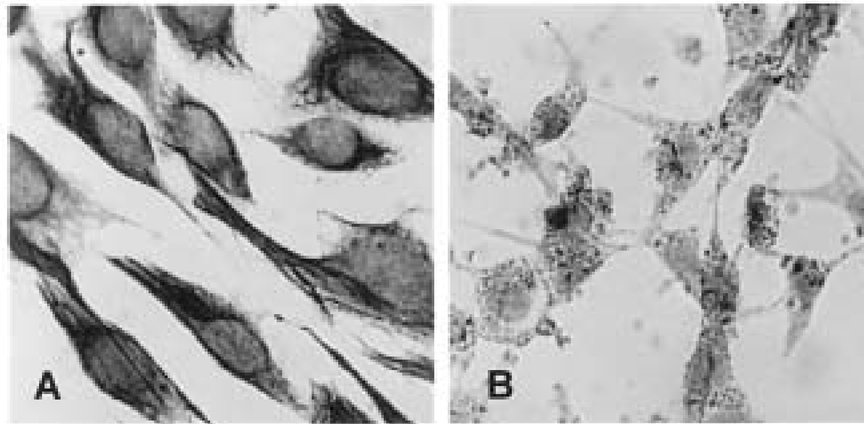

Fig. 4. Two cell-type-specific markers for neural crest stem cells are nestin, intermediate cytoskeletal protein only found in neural stem cells/neural crest stem cells **(A)**, and p75/low affinity neurotrophin receptor (LNGFR) protein **(B)**. Scale bar: 20 μm.

1. Grow HNC10 cells on poly-lysine-coated Aclar plastic coverslips (9 mm diameter) with serum-free medium for 3–7 d and process for immunocytochemistry (*see* **Note 6**).
2. Fix cultures grown in serum-free medium in 4% paraformaldehyde for 3 min, wash twice with PBS, and incubate with an antibody specific for LNGFR/p75 (1 : 1) for 30 min at room temperature (RT).

Table 2
Markers of the Neural Crest Stem Cell Lineage Cells

Protein	Antibody	Cell type	Source
Nestin	Rabbit	Neural crest stem cells	Dr. K. Ikeda
Vimentin	Rabbit	NCSCs	Chemicon
LNGFR/p75	mAb	NCSCs	ATCC
NF-L	mAb NR4	Neurons	Sigma
NF-M	mAb NN18	Neurons	Sigma
NF-H	mAb NE14	Neurons	Sigma
MAP2	mAb AP20	Neurons	Sigma
Peripherin	Rabbit	Neurons/PNS	Chemicon
Tubulin βIII	mAb	Neurons	Chemicon
S-100 protein	Rabbit	Schwann cells	DAKO
P0 protein	Rabbit	Schwann cells	Dr. S. Uyemura
GFAP	Rabbit	Astrocytes	DAKO
Desmin	mAb	Muscle	Chemicon
Myosin	mAb	Muscle	Chemicon
Chromogranin	Rabbit	Adrenal chromaffin cells	Dr. R. Angeletti

3. For cytoplasmic antigen staining of nestin, fix coverslips in cold methanol for 15 min at –20°C, air dry, and then incubate in rabbit polyclonal antibody specific for nestin.
4. Incubate cells with primary antibodies for 1 h at RT, follow with biotinylated secondary antibodies (biotinylated goat anti-mouse IgG or goat anti-rabbit IgG) for 30 min at RT and avidin-biotin complex (ABC) incubation for 30 min at RT. Visualize with AEC chromagen development for 10 min.

3.6. Immunocytochemical Staining for Characterization of Differentiation

In order to induce differentiated cell types in neural crest stem cells, HNC10 cells are grown in serum-containing medium that consists of DMEM supplemented with 10% fetal bovine serum but without bFGF.

1. For immunochemical charaterization of neurons, utilize the following antibodies: NF-L (1:1000), NF-M (1:4), NF-H (1:1000), microtubule associated protein-2 (MAP2, 1:1000), tubulin βIII isoform (1:1000), and peripherin (1:1000).
2. For Schwann cells: S-100 protein (1:500), P0 (1:500), and glial fibrillary acidic protein (GFAP, 1:5000).
3. For myoblast/myotube: desmin (1:1000) and myosin (1:3000).

4. Notes

1. The permission to use embryonic tissue was granted by the Clinical Research Screening Committee involving Human Subjects of the University of British

Columbia, and the embryonic tissues were obtained from the Anatomical Pathology Department of Vancouver General Hospital.

2. During the trituration of enzyme-digested tissues, tissue fragments often stick to the inside of Pasteur pipet. To prevent this, the inside of Pasteur pipet should be coated with silicone. Aspirate Sigmacote solution (Sigma) briefly with Pasteur pipet up to the neck, expel the solution, air dry, and then autoclave. Sigmacote is a special silicone solution in heptane that easily forms a tight, microscopically thin film on glass, is water repellent, and retards tissue attachment.
3. DNA synthesis inhibitors such as AraC (cytosine arabinoside, cytosine β-D-arabinofuranoside) should reduce the number of fibroblasts and other actively proliferating cells. Other inhibitors including fluorodeoxyuridine and mitomycin could be used for the retardation of cell growth. Inhibitors including AraC are usually used 24–48 h.
4. The most simple form of serum-free medium contains three components, insulin (5–10 μg/mL), transferrin (5–10 μg/mL), and sodium selnite (30 n*M*) and is termed ITS. Ready-to-use ITS liquid medium supplement from Sigma and other commercial sources is available. As for the base medium for the serum-free medium, we use Dulbecco's modified Eagle medium (DMEM). Others often use a 50:50 mixture of DMEM and Ham's F-12 medium.
5. Culture supernatant containing viral vector harvested from the packaging cell line could be stored at –70°C and used for transfection experiments, but it is advised to use fresh supernatant each time to obtain good results.
6. Aclar plastic round coverslips (9, 12, and 22 mm diameter) used in our laboratory are custom-made from sheets of clear Aclar fluorocarbon plastic film, thickness of which is identical with No.1 thickness of glass coverslip. Aclar plastic sheets are purchased from Allied-Signal (Pottstown, PA). Unlike glass coverslips, Aclar plastic is non-toxic, non-destructible, easy to handle, and culture cell-friendly.

Acknowledgments

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7

Adult Rodent Spinal Cord Derived Neural Stem Cells

Isolation and Characterization

Lamya S. Shihabuddin

1. Introduction

The generation of neuronal and glial cell types found in the mature central nervous system (CNS) involves the massive proliferation of ventricular zone cells of the fetal brain and spinal cord ***(1)*** that stop dividing early in development and terminally differentiate. Most neurons in the adult CNS are terminally differentiated and last through the life span of the animal. However, recent studies indicate that cell proliferation in the adult CNS is ubiquitous, but is primarily confined to the production of glia, with the exception of discrete regions in the hippocampus and subventricular zone ***(2–4)*** where neurogenesis persists. The discovery that undifferentiated proliferative cells could be isolated from the adult mammalian CNS under favorable cell culture conditions, and induced to differentiate along both glial and neuronal lineages, revolutionized our thinking about the limited ability of the CNS to replace neural cells lost to injury or disease.

Stem cells are able to differentiate into all the different types of cells in a given tissue, while maintaining a pool of themselves ***(5)***. During development of the CNS, in analogy with the hematopoietic system, maturation of neural stem cells involves a continuing loss of potency and restriction of commitment before, finally, a lineage of fully differentiated cells is established. Isolation and long-term culturing of multipotent neural stem/progenitor cells that upon differentiation generate the major building blocks of the CNS—neurons, astrocytes, and oligodendrocytes—have been advanced by the finding that some mitogenic growth factors have proliferative effect on these cells ***(6–8)***.

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Multipotent stem cells that respond to epidermal growth factor (EGF) or basic fibroblast growth factor (FGF-2) have been isolated from both neurogenic ***(9,10)*** and non-neurogenic regions ***(6,11)*** of the adult mammalian CNS using different culture systems ***(12)***. Isolated and cultured stem cells provide an important source of cells for in vitro studies to address issues related to development, as well as transplantation studies to explore their potential as a source of donor cells for therapeutic purposes. Methods for isolation of stem cells from the brain have been described previously ***(13)***. This chapter describes a method for isolating and culturing cells from the adult rat spinal cord, the generation of a clonal population, and the characterization of these cells.

2. Materials

2.1. Isolation and Culturing of Stem Cells

1. Phosphate-buffered saline (PBS; 1X and 10X; Gibco).
2. Dulbecco's Ca^{2+}- and Mg^{2+}-free PBS (D-PBS; Gibco).
3. 0.01% Papain- 0.1% Protease- 0.01% DNase I (PPD) in Dulbecco's modification of Eagle's medium (Cellgro).
4. Dulbecco's modified essential medium (DMEM):Ham's F12 (1:1) medium (Irvine Scientific) containing glucose and L-glutamine with 10% fetal bovine serum (FBS; Sigma).
5. Continuous density Percoll (Amersham, Sigma) gradient 9:1 (vol/vol) with 10X PBS.
6. N2 supplement culture medium (N2 medium), DMEM:F12 with L-glutamine and (1X) N2 supplement (100X, Gibco).
7. Recombinant human basic fibroblast growth factor (FGF-2), 20 ng/mL final concentration (PEPROTECH Inc.).
8. Falcon tissue culture dishes (100 mm and 60 mm).
9. 10 mg/mL poly-L-ornithine hydrobromide (PORN; Sigma) and 5 mg/mL laminin (Gibco) coated culture plates (PORN/Laminin).
10. 15 and 50 mL sterile centrifuge tubes.
11. Sterile filters (0.22 µm, Nalgene).
12. Nylon meshes (pore size 15 µm, Nitex, TETKO, Inc.).

2.2. Isolation of Clonal Cultures

1. Agarose/trypsin: 1 mL of 3% agarose mixed with 2 mL ATV trypsin.
2. Sterile Pasteur pipet.
3. PORN/laminin-coated 96-well plates.
4. Serum-free N2 medium supplemented with 50% conditioned medium from a high-density stem cell culture grown for at least 24 h.

2.3. Passaging, Freezing, and Reculturing of Cells

1. ATV trypsin (Trypsin/EDTA solution, Irvine Scientific).
2. Dulbecco's modified essential medium (DMEM):Ham's F12 (1:1) medium.

3. Dimethylsulfoxide (DMSO; Sigma).
4. 15 mL sterile centrifuge tubes.
5. Cryovials (1.5 mL, Nalgene).
6. Freezing chambers (Nalgene) and liquid nitrogen tank.

2.4. Differentiation and Immunostaining of Stem Cells

1. PORN/laminin-coated glass chamber slides.
2. Serum-free N2 medium containing differentiating agents like serum (0.5%), retinoic acid (0.5 m*M*), or forskolin (10 m*M*).
3. 0.1 *M* Tris-buffered saline (TBS).
4. TBS containing 5% donkey serum and 0.1% Triton X-100 (blocking buffer).
5. 4% paraformaldehyde solution (pH 7.4).
6. Antibodies against lineage specific proteins.
7. Fluorescence-conjugated secondary antibodies.
8. TBS containing DAPI (10 ng/mL) for nuclear counterstain.
9. Antifade mounting medium: 25% glycerol, 10% polyvinyl alcohol and 2.5% 1,4-diazobicyclo-[2.2.2]-octane in 100 m*M* Tris-HCl (PVA-DAPCO).

3. Methods

3.1. Establishment of Stem Cell Cultures from Adult Rat Spinal Cord

Dissect out the adult rat spinal cord, cut it into small pieces, and dissociate the tissue pieces by enzymatic digestion to release cells from connective tissues, and plate (**Fig. 1**). To purify stem cells from debris and enrich for multipotent stem cells based on buoyant density, Percoll density gradient fractionation can be used.

3.1.1. Dissection of Tissue

1. Anesthetize adult female Fischer 344 rats (3–4 mo old) by intraperitoneal injection of anesthesia cocktail: ketamine (44 mg/kg), acepromazine (4.0 mg/kg), and rompun (0.75 mg/kg), and kill by decapitation.
2. Expose the spinal column by making an incision of the skin along the back and clear the rostral opening of the vertebral column from any connective tissue.
3. Cut the vertebral column where it meets the pelvic bone.
4. Fill a 10 mL syringe with sterile 1X PBS and insert the short (1–2 cm) beveled 18 gauge needle into the caudal opening of the central canal, push the needle against the dorsal side of the central canal to form a tight seal, and quickly compress the plunger. The whole spinal cord will be ejected from the rostral opening of the vertebral column.
5. Place in PBS, clean the spinal cord from meninges, dorsal, and ventral roots (*see* **Note 1**). Microdissect sacral, thoracic, lumbar, and cervical areas.

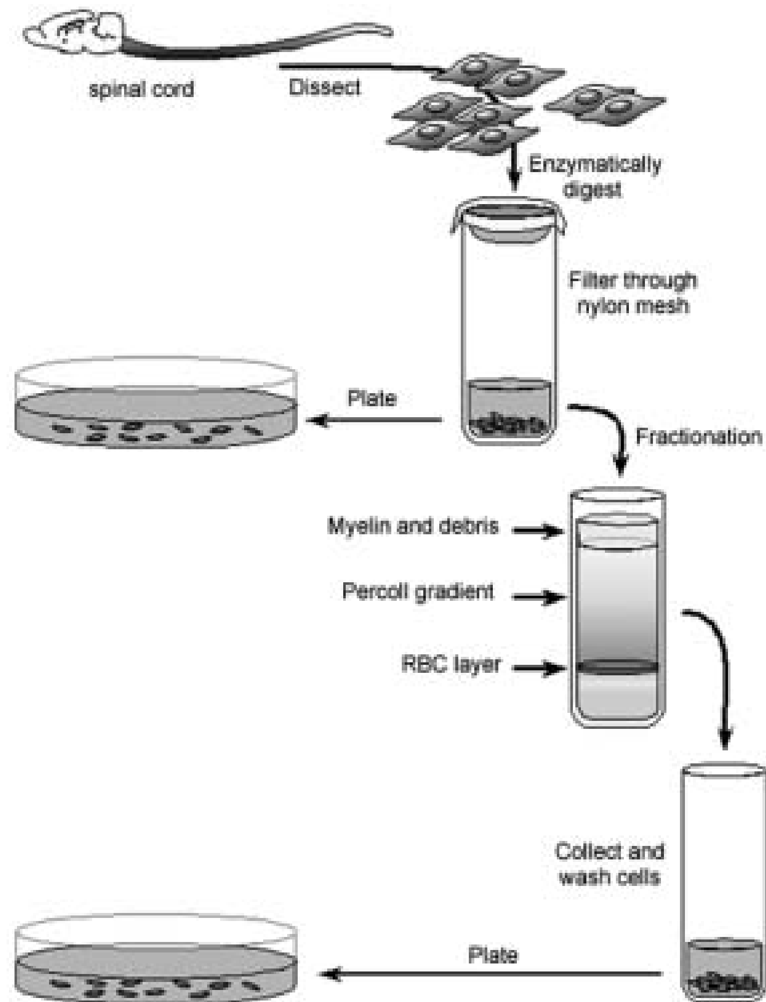

Fig. 1. Schematic diagram of the protocol for isolating stem cells from adult spinal cord. Tissue pieces are subject to enzymatic digestion and mechanical dissociation, filtration to remove large tissue pieces, and then the cell suspension is plated. Alternatively, progenitor population can be enriched and purified from debris by fractionation using a Percoll density gradient, rinsed well then plated (figure prepared by Shannon Macauley).

3.1.2. Enzymatic Digestion

1. Cut the microdissected tissue into small pieces (1–2 mm^3).
2. Wash dissected tissue with 5 mL DPBS 3X and remove the final wash by aspiration (*see* **Note 2**).

3. Resuspend the tissue pieces in PPD solution (approx 1 g tissue cut into small pieces to 20 mL PPD) and incubate for 30–45 min at 37°C with occasional shaking to keep the tissue pieces suspended and triturate with a 5 mL pipet (every 10 min) to break up the large chunks of tissue. Triturate with 5 mL pipet until the cell suspension is free of large tissue pieces (*see* **Note 3**).
4. Remove the remaining tissue pieces by filtration through a nylon mesh. Centrifuge the filtered cell suspension at 1000*g* for 3 min and remove PPD solution by gentle aspiration so that the cell pellet is not disturbed.
5. Suspend cells in DMEM:F12 containing 10% FBS (10 mL/g starting tissue weight) and wash the pellet 3X by centrifugation. Aspirate the medium and resuspend the cells in N2 medium containing 10% FBS (1–2 mL). The cell suspension contains small pieces of tissue, myelin, and red blood cells. Cells can be plated without further purification or can be separated from other cells and debris by Percoll density gradient fractionation.

3.1.3. Percoll Gradient Purification of Stem Cells

A progenitor/stem cell enrichment protocol based on fractionation depending on buoyant density ***(14)***.

1. After washing pellet 3X with DMEM:F12 containing 10% FBS, resuspend cells in 5.5 mL DMEM:F12 containing 10% FBS.
2. Dilute stock Percoll solution 9:1 (vol/vol) with 10X PBS.
3. Mix cells with Percoll (1:1).
4. Transfer the cell suspension to an ultracentrifuge tube (14 × 89 mm, Beckmann) and fractionate by centrifugation for 30 min, 18°C, 20,000*g* (12,700 rpm in SW41Ti Beckmann Rotor in L8-80 M Ultracentrifuge, no brakes).
5. Remove the top myelin and debris layer, and take all layers above the bottom red blood cell (RBC) layer, making sure not to take any RBC, and transfer to a 50 mL centrifuge tube. Immature progenitors/stem cells migrate into the gradient but are enriched at the bottom of the gradient, in a band just above the RBC layer.
6. Dilute 5-fold in cold PBS and wash cells at least 3X by centrifugation at 1000*g* for 3 min. The cell pellet is very small; to avoid losing the cell pellet leave behind approx 1 mL wash liquid in between aspiration and washes.
7. Resuspend the cell pellet in 1 mL of the appropriate plating medium, count cells, and then plate approx 5×10^4 cells/cm^2.

3.1.4. Culturing of Rat Cells as Monolayers ***(15–17)***

1. Resuspend cells in N2 medium containing 10% FBS and plate at least 1×10^4 cells/cm^2 in 10 cm uncoated tissue culture plates (*see* **Note 4**).
2. The next day, change the medium into serum-free N2 medium containing 20 ng/mL FGF-2.
3. Feed cultures every 3–4 d, and if cell density is low, change half of the medium with fresh medium. Add double amount of FGF-2 to have a final concentration of 20 ng/mL.

3.2. Isolation of Clonal Cultures (10,11)

3.2.1. Clonal Cultures Are Isolated to Determine Whether Single Stem Cells Can Generate Multipotent Progeny That Include Both Neurons and Glia

1. Plate cells from bulk culture at clonal density (1 cell/2 cm^2 in a 35 mm Petri dish) onto PORN/laminin-coated dishes in serum-free N2 medium containing appropriate growth factors.
2. To follow a particular cell, mark its position on the dish by scratching the bottom of the plate. Stem cells migrate while proliferating, so always make sure the cells are well separated.
3. Feed cells every 4–5 d with medium containing FGF-2 (20 ng/mL) and supplement with 50% conditioned medium (*see* **Note 5**).
4. When the density of the clones reaches a critical mass (>100 cells/colony), then transfer individual clusters of proliferative cells to 96-well plates (1 clone/well) embedded with agarose/trypsin (*see* **Subheading 3.2.3.**) using a glass pipet, or characterize the clonal population by immunocytochemistry.

3.2.2. Genetic Marking of Cells to Establish Clonal Cultures

1. Infect bulk population of stem cells with a replication-defective retroviral vector of choice expressing a marker gene like green fluorescent protein (GFP), *E. coli Lac Z* gene, or alkaline phosphatase ***(7)*** and the selectable marker gene, neomycin resistant gene. Incubate the cells in medium containing up to 10% virus stocks for 18–24 h.
2. Plate the cells at approx 1% of their initial density in the presence of 100 μg/mL G418 to select for stable transfectant. Usually start with 40 μg/mL G418 and increase the concentration slowly to 100 μg/mL.
3. Feed cells every 3–4 d until proliferative clusters appear. Selection can be stopped when stably transfected cultures are established, but select cells periodically to remove cells that have lost the selectable marker gene.
4. Passage individual clones with agarose/trypsin (*see* **Subheading 3.3.1.**).
5. Confirm the clonality of cultures by determining the integration site of the retroviral genome within the cellular genome using Southern blot analysis. Briefly, prepare genomic DNA by lysis of cells and digest with appropriate digestion enzymes. Resolve digested DNA on agarose gels, transfer to nylon membranes, and probe with ^{32}P-labeled neomycin or transgene-specific probe and visualize using a phosphoimager.

3.2.3. Agarose/Trypsin Method for Transferring Clones

1. Pick clones from plates that have relatively large (>100 cells/clone) and well-separated clones. Mark a clone by marking it on the bottom of the dish.
2. Melt 3% agarose solution (made in PBS) in a microwave oven, cool down, and mix 1 mL agarose with 2 mL ATV trypsin (warmed to 37°C).

3. Remove culture medium and immediately add agarose/trypsin mixture to dish containing the clones, swirl the plate to gently spread over the cells, and allow to solidify 2–3 min.
4. With a sterile Pasteur pipet gently cut around the clones and lift the agarose plugs (with attached cells to them) and transfer them to individual wells of a 24-well plate containing serum-free N2 medium supplemented with 50% conditioned medium. Gently wash the area of the plug with the medium (approx 100 μL) and transfer it to the well containing the cells.
5. Change medium every 3–4 d and allow the cells to proliferate until ready to be passaged.

3.2.4. Isolation of Secondary Clonal Cultures

A defining characteristic of stem cells is self-renewal ***(5)***. Subcloning is done to determine whether a clone that originated from a single cell can be dissociated and replated under the same conditions to yield more than one copy of itself, indicating self-renewing capacity.

For isolation of secondary clones, individual primary clones were dissociated and replated at low clonal density. A single cluster of dividing cells was again transferred to 96-well plates (1 clone/well) using glass pipets, expanded and characterized by immunocytochemistry.

3.3. Passaging, Freezing and Reculturing of Neural Stem Cells

3.3.1. Passaging

1. Add 2.0 mL ATV/trypsin 10 cm plate (add less for small dishes) prewarmed to 37°C. Remove 1 mL and leave 1 mL, swirl plate to distribute the liquid evenly.
2. Let sit for 1 min (*see* **Note 6**). Tap the sides of the plate gently to dislodge the cells.
3. Transfer cells to 15 mL sterile centrifuge tube by using DMEM/F12. Wash the plate once with DMEM/F12 and transfer to the same tube. Centrifuge at 100*g* for 3 min.
4. Remove supernatant slowly so as not to disturb the cell pellet. Resuspend cells in 2 mL serum-free N2 medium.
5. Plate portion of cells (split-ratio will depend on initial cell density and the growth rate of cells) on PORN/laminin coated plates in the same medium containing FGF-2 (*see* **Note 7**).
6. If necessary, freeze cells in liquid nitrogen for long-term storage.

3.3.2. Freezing of Cells

1. Suspend cells in serum-free N2 medium containing 10% DMSO and appropriate growth factors.
2. Aliquot 1 mL in each freezing vial.

3. Put the vials in freezing chambers, and place the chamber in –70°C freezer to allow the cells to freeze slowly.
4. On the next day, transfer the vials to a box kept in liquid nitrogen.

3.3.3. Reculturing of Frozen Cells

1. Remove vials from liquid nitrogen and thaw cells quickly in 37°C water bath.
2. Transfer cells to sterile 15 mL centrifuge tube with DMEM/F12 medium, centrifuge at 1000*g* for 3 min. Remove supernatant.
3. Wash cells once in the same medium. Resuspend the cells in 1 mL of N2 medium.
4. Plate on PORN/laminin-coated plates in serum-free N2 medium containing appropriate growth factors.

3.4. Differentiation and Immunostaining of Stem Cells

3.4.1. Differentiation of Stem Cells

1. Plate cells on PORN/laminin-coated glass chamber slides of 1×10^5 cells/cm^2 (high density) and grow for 24 h in serum-free N2 medium containing FGF-2.
2. Replace the medium with fresh N2 medium without FGF-2 containing differentiating agents like serum, retinoic acid (1 m*M*), or forskolin (10 m*M*) ***(11)***.
3. Change medium every 2 days and allow the cells to differentiate for 6 d. Fix cells with paraformaldehyde and analyze by immunocytochemistry.

3.4.2. Immunocytochemical Analysis of Stem Cells

Staining procedure is carried out at room temperature unless specified otherwise. Rinses are done for 5 minutes each.

1. Grow stem cells in PORN/laminin coated glass chamber slides until 70–80% confluent.
2. Fix cells for 2 min with 2% paraformaldehyde (by removing half of the medium covering the cells and adding an equal amount of 4% paraformaldehade), then for 10 min with 4% paraformaldehyde, and wash 3X in 100 m*M* Tris-buffered saline (TBS). Fixed cells can be stored at 4°C for about a week or processed immediately for immunocytochemistry.
3. Preincubate cells for 1 h in TBS containing 5% donkey serum and 0.1% Triton X-100 (blocking buffer).
4. Incubate with pooled primary antibody (polyclonal and monoclonal) diluted in TBS containing 0.1% Triton X-100 (TBS +). If the antibody recognizes cell-surface molecules, then exclude Triton from the incubation buffer.
5. After 24–48 h at 4°C, wash cells 3X with the blocking buffer and incubate for 2 h in the dark with species-specific secondary antibody conjugated to the desired fluorophores like fluorescein isothiocyanate (FITC), Texas Red, or Cy3 in TBS+. If necessary, the signal for specific antigens can be amplified by using biotin-

streptavidin amplification by incubating cells in biotinylated donkey anti-species antibody diluted in TBS+ for 2 h, washing twice in the same buffer and then incubating in streptavidin conjugated to the desired fluorophore.

6. Wash cells twice in TBS, incubate in TBS containing DAPI (10 ng/mL) for 1 min, and then coverslip in DAPCO-PVA solution. Analyze cell phenotype by confocal or fluorescence microscopy.

4. Notes

1. Clean dissection of tissues increases the purity of the cultures and decreases contamination of non-neuronal cell populations (i.e., connective tissue) that may eventually overtake the cultures.
2. In the initial isolation and first passage of cultures, all buffers and media used contain 2.5 mg/mL Fungizone (Irvine Scientific), 1X penicillin-streptomycin (100X, Gibco) to avoid possible contaminations from dissection.
3. Enzymatic digestion of tissues with PPD for longer than 40 min will lower the yield of stem cells.
4. Survival of rat stem cells grown as monolayers is density dependent, and when plated at less than 1000 cells/cm^2, they may not survive even in the presence of FGF-2 (J. Ray, unpublished observation).When rat stem cells are plated at a high density, proliferating cells can be seen within the first 2 wk, and a mixed population of cells is observed. The cultures contain cells that have stem cell morphology with small, phase-bright cell bodies and short processes, and cells with different morphologies. These morphologies include cells with large, flat, phase-dark cell bodies and long processes.
5. Factors present in conditioned medium will support the survival/proliferation of cells plated at clonal density. Collect the conditioned medium from high-density stem cell culture after at least 24 h incubation, filter sterilize to prevent accidental contamination with residual cells, centrifuge at 1000*g* for 5 min, and keep frozen in aliquots.
6. When passaging, do not trypsinize cultures for longer than 2 min; long exposure to trypsin will result in extensive death of cells. Stem cells detach easily, and short trypsin treatment will enrich passaged cultures with stem cells while leaving behind more differentiated cells.
7. Composition of the substratum is important for the adhesion, survival, proliferation, and differentiation of cells. Factors in serum provide components for cell attachment for the initial plating. After serum withdrawal and the first passage, cells are plated on plates coated with agents like polymers of basic amino acids (PORN) and cells will attach on basis of charge. Also, laminin, a cell-adhesion molecule, can be used in addition to PORN as a substratum.
8. The culturing conditions and requirement of growth factors for the rat, mouse, monkey, and human CNS-derived stem cells are different ***(18–20)***. The best condition for growing adult CNS rat stem cells, irrespective of region, is to culture as monolayers in serum-free N2 medium containing FGF-2 (20 ng/mL) ***(13)***. Growing adult rat spinal cord stem cells on uncoated plates, in serum-free

medium with FGF-2 and EGF, generated small neurospheres, but they grew poorly and could not be expanded more than 4–5 passages ***(21)***.

Acknowledgments

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8

Preparation of Neural Progenitors from Bone Marrow and Umbilical Cord Blood

Shijie Song and J. Sanchez-Ramos

1. Introduction

The ancient Chinese believed bone marrow was the source of brain tissue, as suggested by the maxim "Brain is a sea of marrow" ***(1)***. The existence of stem cells for nonhematopoietic lineages in bone marrow was proposed over 100 years ago, but the isolation and differentiation of marrow stromal cells into osteoblasts, chondroblasts, adipocytes, and myoblasts was only recently demonstrated ***(2)***. Nonhematopoietic progenitors from bone marrow stroma have been referred to as colony-forming-unit (CFU) fibroblasts, mesenchymal stem cells, or bone marrow stromal cells (BMSC). Although BMSC can naturally be expected to be a source of surrounding tissue of bone, cartilage, and fat, several reports demonstrate that these cells, under specific experimental conditions, can differentiate into muscle, glia, and hepatocytes ***(3–5)***. Most recently, BMSC have been shown to develop into cells that express proteins specific for neurons. The first documentation of this phenomenon was performed in vitro ***(6)***. Human or rodent BMSC cultured in the presence of retinoic acid and epidermal growth factor (EGF) or brain-derived neurotrophic factor (BDNF) expressed the mRNAs and proteins for nestin, neuron-specific nuclear protein (NeuN), and glial acidic fibrillary protein (GFAP). When BMSC were co-cultured with rat fetal mesencephalic neurons, the proportion of BMSC that expressed neural markers was increased ***(6)***. Another group of researchers reported that human BMSC could be induced to develop into neuron-like cells that express neuron-specific enolase, NeuN, neurofilament-M, and tau following treatment with dimethylsulfoxide and butylated hydroxyanisole ***(7)***. The differentiation of BMSC into neurons was also demonstrated

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using two in vivo models. Intravenous delivery of genetically marked adult BMSC into lethally irradiated normal adult hosts resulted in donor-derived cells expressing neuronal proteins in the host brain ***(8)***. Another group demonstrated similar results, transplanting adult mouse BMSC into a strain of mice incapable of developing cells of the myeloid and lymphoid lineages ***(9)***. These transplanted BMSC migrated into the brain and differentiated into cells that expressed neuron-specific antigens. Taken together, these findings suggest that bone marrow-derived cells may provide an alternative source of neurons for treatment of neurodegenerative diseases, trauma, and stroke.

More recently, we have demonstrated that human umbilical cord blood cells also contain neural progenitors capable of differentiation into cells that express neuronal and glial markers. With the addition of retinoic acid and nerve growth factor (NGF), we were able to induce a profile of gene expression associated with neurogenesis documented by differential gene expression on DNA microarrays. Proteins indicative of early neural development, such as Musashi-1 and beta-tubulin-III, were expressed as indicated by immunocytochemistry and Western blot analysis ***(10)***.

This chapter describes in detail the materials and procedures utilized to prepare rodent and human bone marrow stromal cells, and human umbilical cord mononuclear cells, for the induction of neural progenitor cells, neurons, and glia.

2. Materials

1. Growth Medium: Dulbecco's modified Eagle medium (D-MEM, Gibco/BRL) supplemented with the following to reach a final concentration: 2 m*M* glutamine (100X stock from Gibco/BRL), 0.001% β-mercaptoethanol, 1 volume per 99 volumes of D-MEM non-essential amino acids (100X stock from Gibco/BRL), 10% donor horse serum (Hyclone) or 10% fetal bovine serum (Hyclone).
2. Medium for selection of neural precursor cells ***(11)***: DMEM/F12 1:1 (Gibco/BRL) is supplemented with the following to reach a final concentration of: 0.6% glucose, 25 μg/mL insulin, 100 μg/mL transferrin, 20 n*M* progesterone, 60 μ*M* putrescine, 30 n*M* selenium chloride, 2 m*M* glutamine, 3 m*M* sodium bicarbonate, 5. m*M* HEPES, 2 μg/mL heparin, 20 ng/mL epidermal growth factor (EGF), 20 ng/mL fibroblast growth factor-basic (FGF-2 or bFGF).
3. Medium for differentiation into neurons and glia: Neurobasal medium (N5) ***(12–14)*** is supplemented with: 5% horse serum, 1% fetal bovine serum (FBS), 100 μg/mL transferrin, 25 μg/mL insulin, 60 μ*M* putrescine, 0.02 μ*M* progesterone, 0.03 μ*M* selenium, 0.5 μ*M* all-trans-retinoic acid, and 10–20 ng/mL BDNF or 100 ng/mL NGF.
4. Antibodies for identification of neural cells: Neuron or glial-specific proteins expressed by BMSC-derived neural cells are most readily visualized by standard immunocytochemistry procedures (*see* Chapter 26). The antibodies and dilutions utilized are listed:

a. Mouse anti-neuronal nuclei (NeuN) monoclonal antibodies (Chemicon) 1:100 in blocking solution with normal horse serum, 1:100 (*see* **Note 1**).
b. Mouse anti-nestin monoclonal antibody (Chemicon) 1:250 in blocking solution with normal horse serum, 1:100.
c. Mouse anti-β-tubulin isotype III monoclonal antibody (Sigma) 1:400 in blocking solution with normal horse serum 1:100.
d. Mouse anti-MAP2 monoclonal antibody (Chemicon) 1:400 in blocking solution with normal horse serum, 1:100.
e. Mouse anti-neurofilament 200 monoclonal antibody (Sigma) 1:40 in blocking solution with normal horse serum, 1:100.
f. Mouse anti-neurofilament 68 monoclonal antibody (Sigma) 1:400 in blocking solution with normal horse serum, 1:100.
g. Mouse anti-human neuronal protein HuC/HuD (anti-Hu) (Molecular Probes) 1:100 in blocking solution with normal horse serum, 1:100.
h. Rat anti-Musashi I monoclonal antibody (Gift from Professor Okano, Japan) 1:500 in blocking solution with normal rabbit serum, 1:100.
i. Rabbit anti-α-internexin polyclonal antibody (Chemicon) 1:500 in blocking solution with normal goat serum, 1:100.
j. Rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (BioGenex) 1:100 in blocking solution (without detergent) with normal goat serum, 1:100.
k. Rabbit anti-human fibronectin polyclonal antibody (Sigma) 1:400 in blocking solution (without detergent) with normal goat serum, 1:100.
l. Secondary Antibodies: horse anti-mouse IgG; goat anti-rabbit IgG; rabbit anti-rat IgG (Vector Laboratories). The concentration of biotinylated anti-immunoglobulins is 1:200 in PBS (*see* **Note 1**). The concentration of fluorescein- (or other fluorescent molecular) labeled secondary antibodies (Vector Laboratories) is 15 μg/mL.

5. Animals: BMSC from C57 mice provide the most consistent results. Other strains or species of rodents, such as transgenic mice- FBV/N or Fisher rats, have been used as a source of bone marrow. The animals are usually between 6–12 wk of age at the time of marrow harvesting.
6. Human bone marrow cells: Human bone marrow cells are difficult to obtain for basic research, because they are a scarce resource for treatment of certain leukemias. An alternative source for BMSC may be the material (bony chips with adherent bone marrow cells, fatty tissue, and debris) retained on the nylon filter which harvested bone marrow is passed through in preparation for bone marrow transplantations. The nylon filters are washed with sterile saline solution 5X and centrifuged to remove bone chips. The supernatant is then treated as described in **Subheading 3.2., step 2**.
7. Human umbilical cord blood (HUCB): We obtain HUCB from CryoCell, Inc. of Clearwater, FL. HUCB can be procured from a number of private or university cord blood banks (*see* **Note 2**). After delivery of the newborn, cord blood is collected from the umbilical cord attached to the placenta, yielding approx

60–90 mL. The cord blood is usually centrifuged to spin out the heavier red blood cells. The remaining cells are suspended in cryopreservative and stored in liquid nitrogen until needed.

3. Methods

3.1. Mouse Bone Marrow Culture (*see* Fig. 1)

1. Sacrifice donor mice by cervical dislocation and wash the extremities with ethanol.
2. With sterile scissors, incise the anterior surface of the skin of the extremities cutting from abdomen to the paws.
3. Trim the muscles away, then remove the femur and tibia.
4. Transfer the bones to a beaker containing normal saline with 0.5% BSA and keep on ice until all the bones are removed.
5. Transfer the femora and tibia to a Petri dish under a microbiological safety hood. Carefully cut off the ends of the femora and tibia and avoid splintering the bone. Insert the tip of a 20–23 gauge needle (attached to a 3 mL syringe) into the proximal end of the femora (and tibia) to flush the marrow through the bone. Collect the flushed material in a 15 mL sterile tube.
6. Spin down the cells at 300*g* for 5 min. Carefully remove the supernatant flushing medium with a Pasteur pipet and resuspend the marrow in 2–3 mL of "Growth Medium." Dissociate cells by mild trituration with a fire-polished Pasteur pipet. Hold the tip of the pipet at a distance of approx 1 cm from the bottom of the tube.
7. After trituration, spin down cells in a centrifuge at 300*g* for 10 min.
8. Suspend the cell pellet in growth medium at 10^6 cells/mL, and plate the cells in culture flasks for 3 d.
9. Replace with the same medium for expanding the cells or with the medium for selecting neural precursor cells using serum-free medium DMEM/F12 1 : 1 for another 3 d.
10. Replace medium with the differentiating medium N_5 (Neurobasal medium) for 4–7 d.
11. Fix cells using 4% paraformaldehyde for immunocytochemical staining (*see* **Subheading 3.6.**), or fix with cold methanol or acetone for optimal immunocytofluorescence staining.

3.2. Human Bone Marrow Cultures

1. To 3 mL of whole bone marrow collected in heparin or EDTA, add 5 mL of phosphate-buffered saline (PBS) and mix well by inversion.
2. To a 15 mL sterile conical centrifuge tube, add 3 mL of Histopaque-1077 (Sigma).
3. Carefully layer 8 mL of the bone marrow–saline mixture onto the Histopaque-1077, centrifuge at 400*g* for 30 min at room temperature.
4. After centrifugation, use a Pasteur pipet to aspirate the upper layer and discard.

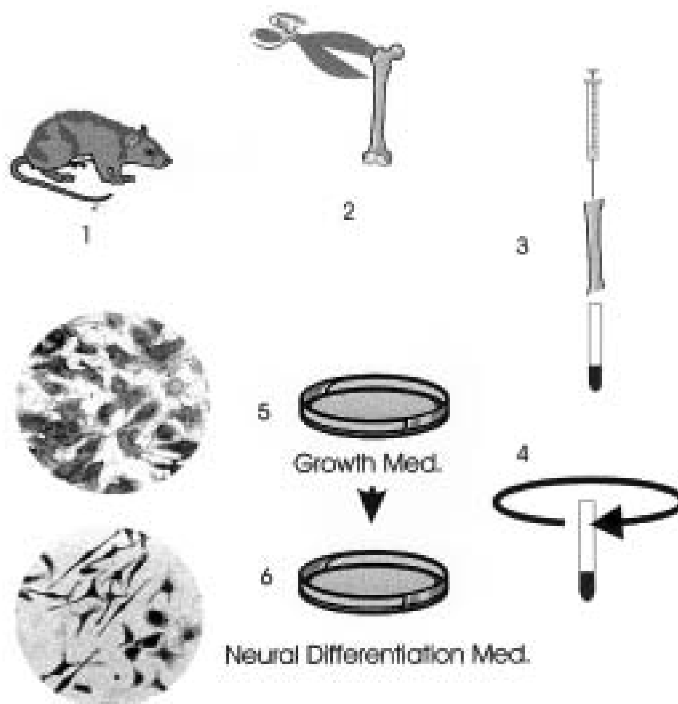

Fig. 1. Preparation of rodent bone marrow cells. (1) After sacrifice of animal, the limbs are cleansed with alcohol prior to incision. After dissecting away muscle, the long bones of the extremities visualized and removed. (2) Both ends of the long bones are cut with scissors so that the marrow can be flushed out. (3) The tip of a 20–23 gauge needle (attached to a 3 mL syringe) is placed into the proximal end of the femora (and tibia) to flush the marrow through the bone. The flushed material is collected in a 15 mL sterile tube. (4) After spinning down the marrow at low speed the supernatant is discarded, the marrow resuspended and plated into culture flasks or dishes. (5) The cells are incubated in "growth medium" (*see* **Subheading 2.1.**) for 2–4 d, followed by change to"neural selection medium" (*see* **Subheading 2.2.**) for 2–7 d. (6) Following addition of "neural differentiation medium" for 2–7 d, cells change phenotype (*see* **Subheading 2.3.**)

5. With a Pasteur pipet, carefully transfer the opaque interface to a sterile, conical centrifuge tube.
6. To this tube add 10 mL of PBS and mix by inversion. Centrifuge at 300*g* for 10 min.
7. Aspirate the supernatant and discard.
8. Resuspend cell pellet with 5 mL of PBS and mix by gentle trituration with a Pasteur pipet.

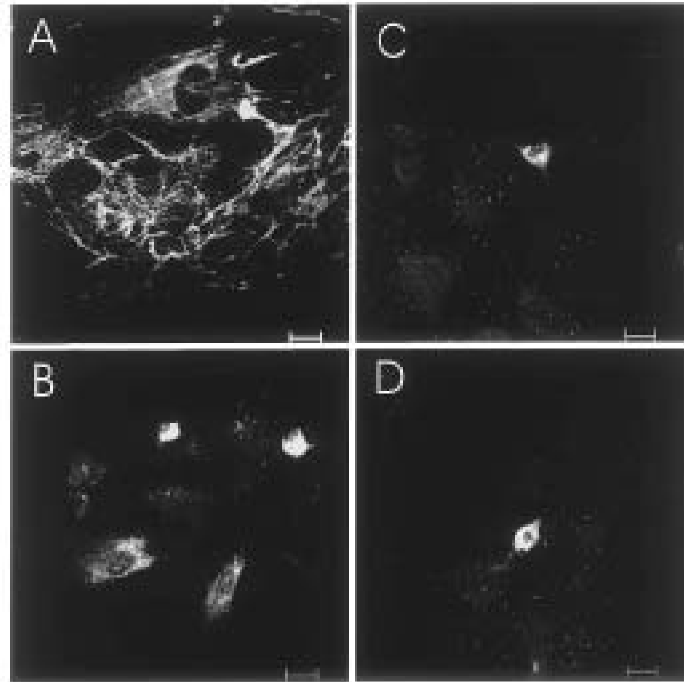

Fig. 2. Demonstration of neural cell markers. BMSC were cultured in presence of RA (0.5 μ*M*) and BDNF (10 ng/mL) for 7 d. Immunofluorescence was used to detect the presence of **(A)** fibronectin, **(B)** nestin, **(C)** GFAP, and **(D)** NeuN. Reproduced with permission from *Experimental Neurology* ***(6)***.

9. Centrifuge at 300*g* for 10 min.
10. Repeat steps 8–10. Discard supernatant and resuspend pellet in 3 mL of growth medium.
11. Distribute to three 75 mm flasks with 10 mL of growth medium per each flask for 3 d.
12. Change the same medium for expanding the cells or use the serum-free medium (DMEM/F12 1 : 1) for selecting neural precursor cells for another 3 d.
13. Transfer cells from the flask with 0.25% trypsin and 1 m*M* EDTA to the six-well plates or chamber slides with differentiating medium for 4–7 d.
14. Fix cells using 4% paraformaldehyde for immunocytochemical processing (*see* **Subheading 3.6.**) or fix with cold methanol or acetone for optimal immunocytofluorescence staining.

3.3. Human Umbilical Cord Cultures

1. Thaw the cryopreserved cells to room temperature.
2. Spin down the cells (300*g* for 10 min) and resuspend in DMEM.

3. Take an aliquot for counting and plate the remainder in 75 mm flasks. Details for remainder of the procedure are the same as for bone marrow stromal cells described in **Subheading 3.2., steps 12–15**.

3.4. Alternative Method for Neural Induction In Vitro

Woodbury and colleagues used a different method for differentiating neural cells from bone marrow ***(7)***. Rat or human BMSCs were maintained in DMEM/20% FBS.

1. Replace the media with preinduction media consisting of DMEM/20% FBS/ 1 m*M* β-mercaptoethanol (BME) for 24 h.
2. Remove the preinduction media and wash the cells with PBS.
3. Use neuronal induction media-DMEM/2% dimethylsulfoxide (DMSO)/200 μ*M* butylated hydroxyanisole (BHA) for 30 min to 6 d.
4. To facilitate long-term survival of BMSC-derived neurons, use long-term induction media (DMEM/2% DMSO/200 μ*M* BHA/25 m*M* KCl/2 m*M* valproic acid/10 μ*M* forskolin/1 μ*M* hydrocortisone/5 μg/mL insulin).
5. Fix the cultures with 4% paraformaldehyde, and use immunocytochemical procedures for identification of neural cells.

3.5. Co-culture of BMSC with Fetal Midbrain Cells

In order to increase the proportion of BMSC that express neural markers, we have co-cultured them with dissociated fetal rat midbrain cells. Detail regarding the preparation of mesencephalic cell cultures from E14 rat or mouse fetal brain is beyond the scope of this book, but the method we use can be found in a previous publication ***(13)***. BMSC are first labeled with an appropriate marker before plating with fetal mesencephalic cells. (*See* Chapter 28 for labeling methods.) As an example, HBMSC labeled with the red fluorescent dye PKH-26 (Molecular Probes, Inc) and co-cultured with fetal mesencephalic cells can be demonstrated by confocal microscopy to express both the neuronal marker NeuN and the PKH-26 (*see* **Fig. 3**).

3.6. Immunocytochemical Staining of Cultures

1. Remove the culture medium and carefully wash cultures 2X with PBS.
2. Fix cultures for 10 min in 4% formaldehyde in PBS at room temperature.
3. Wash cultures 2X in PBS.
4. Incubate cultures with primary antibodies in blocking solution for 24–48 h at 4°C.
5. Remove medium containing primary antibodies (and store at 4°C for later re-use).
6. Warm up cultures to room temperature and wash 3X with PBS.
7. Incubate cultures with second antibody in PBS or blocking solution for 30 min to 2 h (*see* **Note 3**).

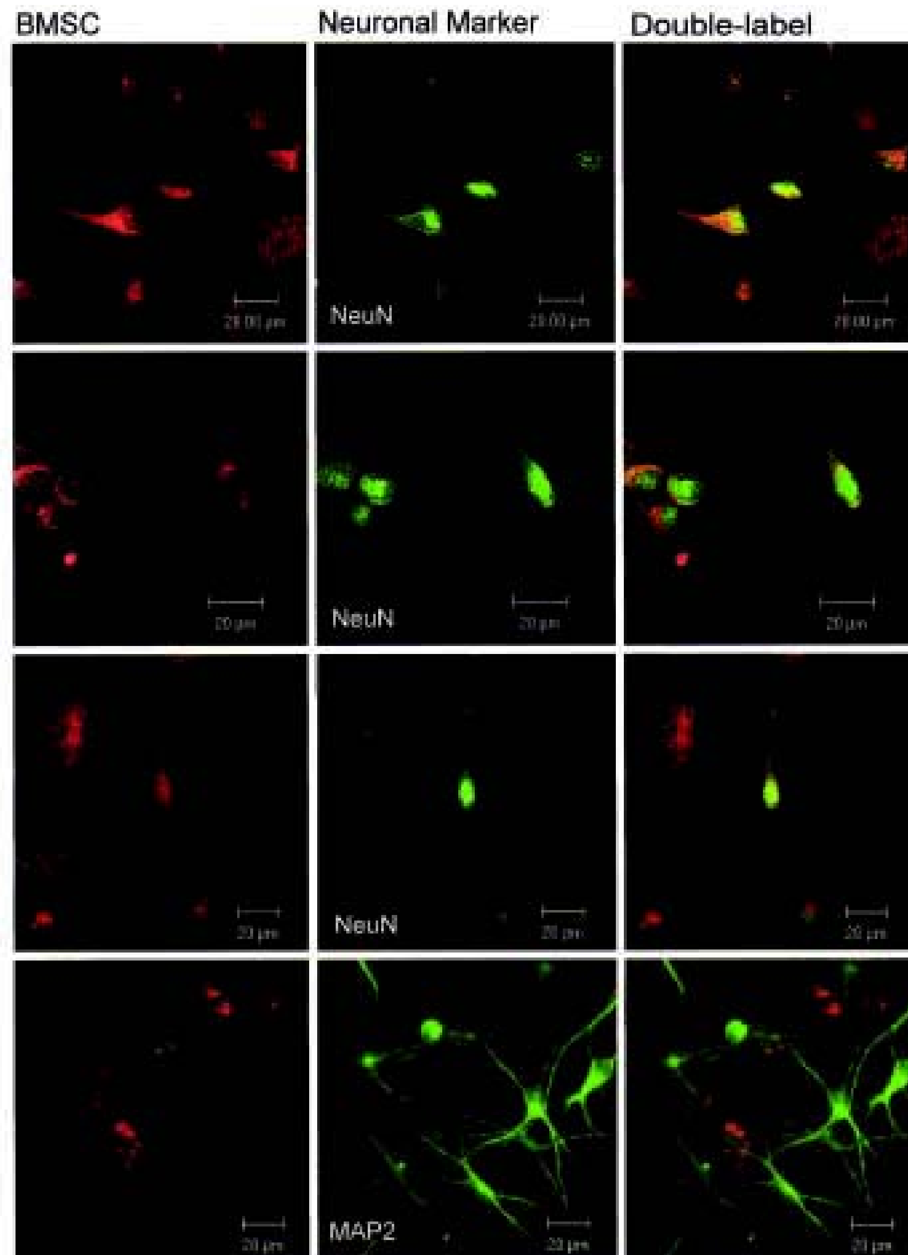

Fig. 3. Demonstration of BMSC expressing NeuN, but not MAP2 in co-cultures containing fetal mesencephalic cells. Mouse BMSC were labeled with the red fluorescent marker PKH-26. They were then co-cultured with fetal midbrain cultures for 1 week. Red fluorescence shows BMSC. Green fluorescence marks cells that express NeuN or MAP2 (bottom row). Double-labeled cells, with a green fluorescent nucleus and red cytoplasm represent BMSC that differentiated into a neuron-like phenotype. Images obtained with a laser scanning confocal microscope (Zeiss LSM 510). Reproduced with permission from *Experimental Neurology* ***(6)***.

8. Wash cultures 3X in PBS.
9. Incubate in H_2O_2 0.3% in PBS for 10 min (*see* **Note 4**).
10. Wash sections in PBS three time and prepare Vectastain reagents at this time (*see* **Note 5**).
11. Incubate cultures in Vectastain reagent preincubated for 30 min at room temperature.
12. Wash sections in PBS 3X.
13. Incubate in developing solution (*see* **Note 6**) for 2–5 min with care not to overdevelop the brown stain.
14. Wash sections in PBS 2–3X.
15. Pool the waste containing diaminobenzidine and treat with hypochlorite.

4. Notes

1. Preparation of blocking solution: 7.1 g $Na_2H_2PO_4$ (final concentration:10 *M*), 0.1 g sodium azide (very toxic) (0.02%), 25 g sucrose (5.0%), 25 g bovine serum albumin (5.0%), 0.5 mL Triton 100 (0.1%). Dissolve ingredients in 450 mL of distilled H_2O, adjust pH to 7.4 using 1 *M* HCl and bring to final volume of 500 mL.
2. For more information regarding collection and banking of HUCB go to www.cordblood.med.ucla.edu.
3. Cultures are routinely incubated with secondary antibody in PBS, but if background staining is high, we incubate the cultures with blocking solution containing serum from the species in which the secondary antibodies were raised.
4. For light microscopic visualization, we use the ABC Vectastain Kit (Vector Laboratories) which utilizes biotinylated secondary antibodies and relies on the high affinity of avidin for biotin. The chromogenic solution contains diaminobenzidine to generate a brown stain.
5. The reagents in the ABC Vectastain kit are prepared (as described in the kit) by adding 9 µL of solution A to 1 mL of PBS, and 9 µL of solution B to 1 mL of PBS. Both solutions A and B are incubated for 40–60 min at room temperature before use.
6. Prepare the development solution containing diaminobenzidine immediately before use. To 10 mL of PBS add 10 mg of diaminobenzidine hydrochloride (caution: toxic, carcinogenic) and 10 µL of 30% H_2O_2.

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9

Seeding Neural Stem Cells on Scaffolds of PGA, PLA, and Their Copolymers

Erin Lavik, Yang D. Teng, Evan Snyder, and Robert Langer

1. Introduction

Tissue engineering has arisen to address the extreme shortage of tissues and organs for transplantation and repair. One of the most successful techniques has been the seeding and culturing of cells on three-dimensional biodegradable scaffolds in vitro followed by implantation in vivo ***(1,2)***. This technique has been used to treat bladder conditions ***(3)*** and cartilage ***(4)*** and skin ***(5)*** defects, and is being studied for a variety of other applications. While matrices have been made from a host of natural and synthetic materials, there has been particular interest in the biodegradable polymers of poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymers poly(lactic-*co*-glycolic acid) (PLGA) (**Fig. 1**). This particular family of degradable esters is very attractive for tissue engineering because the members are readily available and can be easily processed into a variety of structures, their degradation can be controlled through the ratio of glycolic acid to lactic acid subunits, and the polymers have already been approved for use in a number of applications by the FDA. Furthermore, recent research has shown this family of polymers to be biocompatible in the brain ***(6)*** and spinal cord ***(7)***.

The field of tissue engineering may provide new tools for treating injuries to the CNS. There has been a great deal of interest in applying some of the principles of tissue engineering to study neural processes in vitro in three dimensions to better model in vivo systems ***(8)*** and in treating damage to the CNS ***(9,10)*** utilizing scaffolds alone ***(11–13)*** or with Schwann cells ***(14–17)***, olfactory ensheathing cells ***(18)***, and, now, neural stem cells in these cell–polymer systems ***(19,20)***. Neural stem cells have shown great promise

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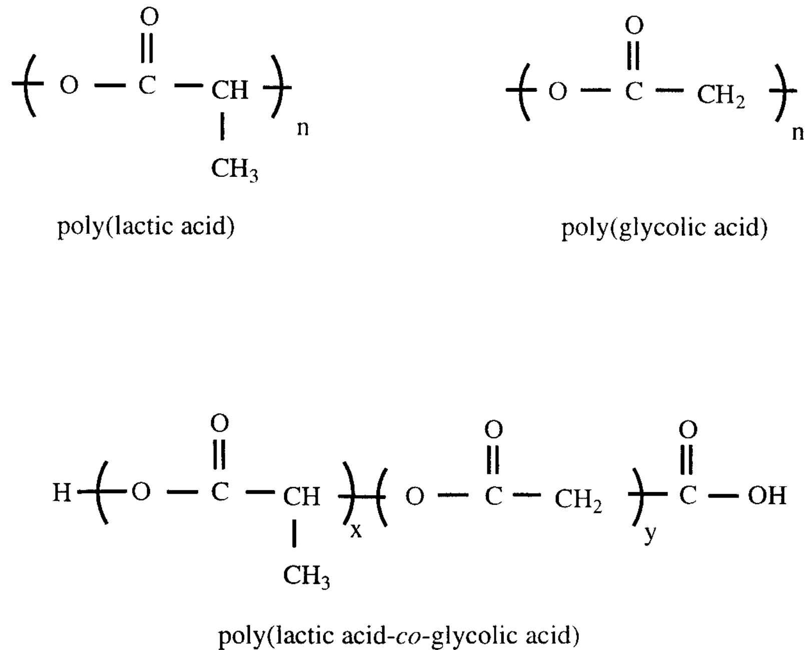

Fig. 1. The chemical structures of PLA, PGA, and PLGA.

in treating a range of defects and, thus, the use of neural stem cell–polymer constructs may be a powerful new technique for in vitro study and in vivo treatment.

The first step in developing a polymer–cell construct is the identification of the appropriate bulk and surface characteristics of the scaffold for the intended application. The bulk characteristics include the mechanical properties as well as the degradation rate of the scaffold. For most applications involving neural stem cell–seeded scaffolds, mechanical strength is not a critical property, but degradation rate is important. The family of poly(glycolic acid), poly(lactic acid) polymers, and their copolymers degrade by acid hydrolysis. The rate of degradation is influenced by the chemical composition (glycolic acid subunits to lactic acid subunits), the molecular weight, and the crystallinity of the polymers. In short, increasing the ratio of glycolic acid units to lactic acid units increases the degradation rate; increasing the molecular weight decreases the degradation rate, as does increasing the crystallinity of the structure.

The surface characteristics play a critical role in determining the interaction between the cells and the scaffold. PGA, PLA, and PLGA all support the growth of neural stem cells without surface modification. However, surface

modification can further control cellular behavior with respect to the surfaces, and may afford an opportunity to achieve more than simple adhesion: controlled differentiation, migration, and axonal guidance. There has been a great deal of work in the field of biomaterials with the development of complex patterned surface structures, but, on a simple level, the surfaces of the degradable polyesters may be altered by the simple adsorption of peptides such as polylysine and proteins such as laminin.

The architecture of the scaffold is also important. There are prefabricated, degradable scaffolds commercially available, in particular meshes made from PGA, PLA, and their copolymers. These meshes may be sterilized, cut, sutured, and manipulated into a variety of shapes suitable to their application (*see* **Note 1**). There are also simple techniques such as salt leaching ***(21)***, which is easily done in the laboratory, to create highly porous scaffolds with a larger range of pore sizes, compositions, and architectures than are currently available commercially.

The method used to seed neural stem cells onto a scaffold depends on the architecture of the scaffold, the attachment capability of the cells, the number of cells available for seeding, their ability to proliferate, and the desired characteristics of the system. The procedures discussed in the following are broad enough to be applicable to many different types of scaffolds and neural stem cell systems.

2. Materials

1. Commercially available sources for scaffolds and polymers: PGA, PLA, and PLGA polymer meshes are available from Albany International Research Corporation (Mansfield, MA, USA). The polymers in the powder form, which can be used to create three-dimensional scaffolds using processes like salt leaching ***(21)***, can be obtained from Boehringer Ingelheim Chemicals, Inc. (Petersburg, VA, USA) under the trade name of Resomer® products.
2. Storage, sterilization, and prewetting of scaffolds: For longer-term storage, the polymers and scaffolds can be aliquotted into any 15 or 50 mL centrifuge tubes. These fit very nicely into Nalgene wide mouth jars (Nalgene Nunc International, Rochester, NY, USA, cat. no. 2121-0005) into which is poured some desiccant, such as Drierite (VWR Scientific Products, West Chester PA, USA, cat. no. 22890-900), which has a blue indicator that slowly turns to purple then pink as water is adsorbed. Ethylene oxide sterilization products, including the ethylene oxide system and refills, are available from Andersen (Andersen Products, Inc., Haw River, NC, USA, cat. no. AN72D and cat. no. AN71).
3. Medium for the C17.2 clone: The neural stem cell used in the development of much of this work was the C17.2 clone, a murine neural stem cell line, courtesy of Dr. Evan Snyder. These materials and techniques have since been used with other populations of neural stem cells with similar success.

C17.2 medium:

DMEM (Gibco Life Technologies, Rockville, MD, USA, cat. no. 11995-065)	500 mL
10% fetal bovine serum (FBS) (Gibco cat. no. 16000-036)	50 mL
5% heat-inactivated horse serum (Gibco cat. no. 26050-070)	25 mL
L-glutamine (Gibco cat. no. 25030-032)	5 mL
Penicillin/streptomycin/fungizone (Gibco cat. no. 15240-062)	5 mL
Sodium pyruvate (Gibco cat. no. 11360-070)	5 mL
Sodium bicarbonate (Gibco cat. no. 11810-025)	18.5 g/10 L of medium

The sodium bicarbonate is most easily added by first making up a solution of 18.5 g/250 mL deionized water and filtering through a sterile filter. In this case, 12.5 mL of the sodium bicarbonate solution is added to the medium. Nothing need be altered or added to the medium to facilitate cell seeding on the three-dimensional scaffolds.

4. Seeding of scaffolds—static and dynamic seeding: All the materials and devices used for seeding are readily found in most cell culture laboratories except the following items: untreated cell culture dishes and orbital shakers that can withstand the taxing environment of the incubator. The purpose of the untreated dishes is that, when seeding cells, one would like the cells to go to the scaffold preferentially over the dish in which the seeding is being performed. One can achieve this either by using treated dishes and washing them first with a sterile solution of BSA or by simply using untreated dishes, which saves time. We use 12-well Falcon untreated dishes (Becton Dickenson, Franklin Lake, NJ, USA, cat. no. 1143).

   With regard to the issue of the orbital shaker, we have found that the Thermolyne orbital shaker is the most robust under the humid, warm atmosphere of the incubator (Rotomotor type 50800).
5. MTT stock solution: MTT powder (Sigma, St. Louis, MO, cat. no. M5655) is dissolved in PBS at a concentration of 5 mg/mL. The solution is sterile filtered through a 0.22 μm filter. It can then be stored at 4°C for frequent use or at –20°C for extended periods.

3. Methods

3.1. Storage of Scaffolds

Both the degradable polyesters and scaffolds made from degradable polyesters need to be stored in an environment free from water (*see* **Note 2**). Both the polymers and the scaffolds of these polymers, which are commercially available, generally arrive in aluminum-backed polyethylene sealed bags which can be stored, unopened, at –20°C until use. Then, the procedure outlined below is recommended to preserve the integrity of the materials, especially for long-term storage.

1. Aliquot the materials into 50 mL centrifuge tubes. In the case of the mesh, it may be cut into the sizes to be seeded with cells using scissors.
2. Place the sealed centrifuge tubes in a large, wide mouthed jar that has approx 100–200 mL of Drierite at the bottom and seal the container.
3. Store the sealed container at –20°C.

To remove samples, allow the entire wide-mouth container to warm up to room temperature on the bench top before opening it to avoid condensation formation on the inside of the container. Once the necessary aliquots are removed, the large container should be sealed and returned to –20°C. For short-term storage, aliquots of the polymers may be stored in a vacuum desiccator.

3.2. Sterilization of Scaffolds for Seeding with Neural Stem Cells

Since PGA, PLA, and their copolymers degrade by acid hydrolysis, they will not survive being autoclaved. Commercially available scaffolds arrive unsterilized. While films of these polymers may be successfully sterilized by UV radiation, the UV light will not necessarily penetrate a three-dimensional scaffold. Therefore, sterilization by ethylene oxide is the most common technique.

3.3. Prewetting of Scaffolds for Seeding

PLA, PLGA, and PGA are all hydrophobic polymers. Therefore, if one takes a scaffold and immerses it directly in medium, one runs the risk of not having the medium penetrate the three-dimensional porous network. Therefore, it is recommended that the scaffolds be soaked in ethanol to prewet the scaffolds, since ethanol wets the polyesters more than water. Soaking the scaffolds in 70% ethanol overnight has also been used in lieu of the ethylene oxide sterilization step effectively. Essentially, the scaffolds are placed in 70% ethanol at room temperature overnight. Prior to seeding, the scaffolds are washed 3X under sterile conditions in PBS and then are seeded with cells as described in the following sections.

3.4. Seeding of Scaffolds: Static Seeding

The easiest method for seeding scaffolds is a static method in which a solution of neural stem cells is prepared, then dripped onto the scaffold in a small dish. After a few hours of incubation to allow for attachment, more medium is added, and the scaffold–cell construct is returned to the incubator. The following protocol was designed for seeding a porous sponge of PLGA, which was 0.75 mm × 1 mm × 6 mm, and was approx 95% porous. The cells used were murine neural stem cells, clone C17.2, courtesy of Dr. Evan Snyder.

In the cell culture hood:

1. Transfer the scaffolds from 70% ethanol to a 60 mm dish containing 5 mL of sterile PBS.
2. Rinse the scaffold 3X in PBS. Allow scaffolds to remain in PBS while preparing the cell solution.
3. Trypsinize cells and perform a cell count.
4. Centrifuge and resuspend cells at a concentration of 7×10^7 cells/mL.
5. Place each scaffold in a well of a 12-well untreated cell culture dish.
6. Carefully add in a drop-wise fashion 0.12 mL of the cell solution onto each scaffold.
7. Place scaffolds in incubator for 3 h at 37°C and 5% CO_2.
8. After 3 h, very gently add 1.5 mL of fresh medium to each well and carefully replace the scaffolds in the incubator for 24 h.
9. After 24 h, aspirate the medium and add fresh medium to each well.
10. Subsequently, change medium approx every 3 d or as needed.

3.5. Seeding of Scaffolds: Dynamic Seeding

Given the option, dynamic seeding and maintenance affords the opportunity for more uniform coverage and better transport of nutrients and gasses throughout the scaffolds, and is certainly the better choice for the seeding of larger scaffolds. While there are a host of ways to seed and maintain scaffolds in a dynamic environment, the following protocol using an orbital shaker in the incubator has been found to be very successful for seeding a variety of scaffolds with neural stem cells and is relatively easy. This protocol uses an excess of cells to obtain well-seeded scaffolds very quickly. One can use a lower cell concentration. As long as the cells attach well to the scaffold, which seems to be the case for a variety of neural stem cells on the PLA and PGA scaffolds, one should get good, uniform cell coverage very easily using this method.

In the cell culture hood:

1. Transfer the scaffolds from 70% ethanol to a dish filled with PBS.
2. Rinse 3X in PBS.
3. Allow the scaffolds to soak in PBS while the cell solution is prepared.
4. Trypsinize the neural stem cells, and resuspend in medium.
5. Centrifuge cells. Perform cell count.
6. Resuspend cells to concentration of 5×10^5 cells/mL.
7. Transfer the scaffolds, one to a well in the 12-well dish.
8. Add 2 mL of the cell solution to each of the wells containing a scaffold.
9. Place scaffolds on orbital shaker set to the lowest speed in the incubator (37°C, 5% CO_2).
10. One day later, aspirate off the old medium and add 2 mL of fresh medium to each well. Replace the scaffolds in the incubator on the orbital shaker.

11. The medium is then changed every few days or as needed, depending on the size of scaffold and number of cells seeded.

3.6. Characterization of Cell Seeding: MTT assay

The MTT assay provides one of the easiest ways to quantify the concentration of live cells growing on a scaffold. The absorbency is read and compared to the absorbency for a set of standards created by using known concentrations of the neural stem cells. The following protocol is simply a modification of standard MTT protocols for use with the scaffolds cultured in the 12-well plates as described in the preceding sections.

1. Rinse sponge 2X with PBS to remove medium with phenol red.
2. Add 0.9 mL of DMEM without phenol red followed by 100 μL of MTT solution to each well of the 12-well plate containing a scaffold. Incubate for 4 h on the orbital shaker at 27°C and 5% CO_2.
3. Transfer the medium from the wells to Eppendorf tubes.
4. Add 0.9 mL of 0.1 *N* HCl/isopropanol to each sponge.
5. Incubate on the orbital shaker for 15 min.
6. Centrifuge the MTT/medium solutions for 5 min.
7. Aspirate off the supernatant and add 100 μL of 0.1 *N* HCl/isopropanol to dissolve formazan crystals. Triturate to facilitate dissolution.
8. Triturate the solution around sponges to facilitate dissolution of formazan crystals.
9. Add the solution from the Eppendorf tubes back to the sponges.
10. Transfer 200 μL of the solution from each sponge to a 96 well plate. Measure the absorbency at 560 nm.

3.7. Characterization of Cell Seeding: SEM studies

There are whole texts that deal with preparation of tissue specimens for scanning electron microscopy (SEM). A particularly excellent text on the subject of biological sample preparation is by Michael Dykstra ***(22)***. SEM is one of the best ways to characterize both the density and nature of neural stem cells on polymer scaffolds. With SEM, one can see cell attachment and spreading, as well as process extension. SEM is therefore highly recommended in any work involving the culturing of neural stem cells on polymer scaffolds (*see* **Note 3**).

4. Notes

1. It is recommended that the scaffolds are trimmed to the appropriate size **before** seeding. In our experience, the implants are more uniform both with respect to their scaffold structure as well as the cell coverage and density when trimmed first.
2. Improper storage (e.g., keeping the scaffolds on the lab bench for extended periods) renders the scaffolds useless due to degradation and the changes in their

properties. It is highly recommended that one ensure that scaffolds are properly maintained to guarantee the quality and reproducibility of the results.

3. The above protocols all assume that the neural stem cells being used are adhesive cultures. Suspension cultures may not lend themselves to these protocols directly, and one may need to modify the procedures, perhaps using a hydrogel in conjunction with the scaffold to localize the cells in the structure. Such composite material structures have been used successfully in other tissue engineering applications.

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II

Characterization of NSCs In Vitro

A. Cellular Techniques

10

Analysis of Cell Generation in the Telencephalic Neuroepithelium

Takao Takahashi, Verne S. Caviness, Jr., and Pradeep G. Bhide

1. Introduction

Neurons and glial cells of the forebrain arise ultimately from a pseudostratified ventricular epithelium (PVE), which lines the ventricular margins of the embryonic cerebral vesicles ***(1)***. Neurons that undergo their terminal mitosis in this epithelium migrate directly to the target structures. Other cells, arising concurrently with neurons, move outward into the cerebral wall, where they constitute a stem cell population that may give rise to both glial cells and neurons over the life of the organism. We describe methods to characterize the proliferative behavior of the PVE of the mouse forebrain. These methods are directly applicable to pseudostratified proliferative epithelial structures that give rise to the CNS and certain somatic structures.

It must be recognized at the outset that the validity of these methods depends upon two critical features of the proliferative behavior of the PVE. First, once a cell undergoes terminal division, it makes its exit from the epithelium immediately. The growth fraction of the PVE is 1.0 at all times. Second, the cells proliferate asynchronously. At any given moment in time, the number of PVE cells negotiating a particular cell cycle phase (e.g., G_1, S, G_2, or M phase) corresponds to the fractional representation of the duration of that cell cycle phase to the total duration of the cell cycle. Thus, if the length of S-phase represents 50% of the total cell cycle length, 50% of the PVE cells must be in S-phase at any given time.

These methods would require critical adaptations for application to nonepithelial proliferative populations.

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The forebrain PVE undergoes two modes of growth over the course of its proliferative life ***(2–4)***. Initially, its generative potential is maximized by a period of exponential expansion of the precursor pool. In this initial phase, both daughter cells resulting from a cell division reenter the cell cycle as proliferative fraction (P or P fraction). At a discrete moment in the course of forebrain development, which is characteristic of each species, the period of exponential expansion yields a state where daughter cells begin to exit the epithelium as cells of the mitotically quiescent fraction (Q or Q fraction). As the proliferative process continues, the magnitude of the Q fraction increases, while that of the P fraction decreases. Ultimately, all daughter cells exit the epithelium, signaling the termination of proliferative activity. Over the proliferative life of the PVE, Q follows an ascending path from 0.0 to 1.0, while P, in complementary fashion, descends from 1.0 to 0.0 (**Fig. 1**). In the neocortical PVE of mouse, this phase is executed in 11 cell cycles, while in monkey it has been estimated to proceed through approx 27 cell cycles ***(3–5)***.

In the course of cell generation and ascent of Q from 0 to 1.0, a stage is reached when both P and Q are at 0.5. At this stage, the number of cells that enter the Q and P fractions is identical, corresponding to a "steady-state condition, " respecting the size of the proliferative pool of cells ***(2)***. The rate of ascent of Q from 0 to 1.0 with each round of cell division, from the first to the last, critically determines the total number of cells produced. A steady and restrained ascent ensures the generation of the greatest possible total cell number, whereas a rapid acceleration of the slope and rise of Q ensures early exhaustion of the precursor pool. Another factor that determines the total number of cells generated is the proportion of the total cell generative interval elapsed (i.e., the percentage of total number of cell divisions executed) by the time Q reaches the steady-state value of 0.5.

In this chapter, we describe methods to characterize growth modes and cell output patterns of the PVE by using S-phase labeling methods. S-phase labeling methods employ analogs of the nucleotide thymidine. Commonly used thymidine analogs are bromodeoxyuridine (BUdR) and tritiated thymidine (^{3}H-TdR). When supplied in abundance exogenously, the thymidine analogs become incorporated into newly synthesized DNA of dividing cells and localize within the nucleus. Once incorporated, the S-phase markers remain permanently incorporated in the DNA, although repeated divisions dilute the label. BUdR and ^{3}H-TdR that become incorporated into the cell nucleus can be identified in histological sections by immunohistochemical and autoradiographic methods, respectively. By making the S-phase markers available for uptake by dividing cells for specified periods of time and analyzing the number and distribution of cells that carry the markers, one can determine the kinetics of cell proliferation, rate of cell output, and patterns of cell migration.

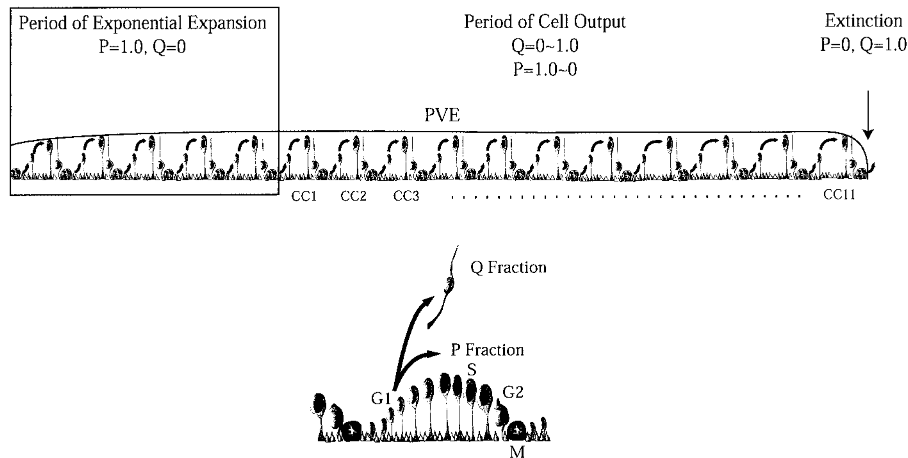

Fig. 1. Neuroepithelial growth patterns. The pseudostratified ventricular epithelium (PVE) undergoes two stages of growth. During the initial period of exponential expansion (rectangle), both daughter cells resulting from a cell division reenter S phase (S) as the proliferative fraction (P fraction; inset). This period is followed by a period of cell output where daughter cells exit the epithelium as cells of the mitotically quiescent fraction (Q fraction; inset). During the period of exponential expansion, P is 1.0 and Q is 0.0. During the period of cell output, the Q fraction increases while the P fraction decreases. Ultimately, the Q fraction reaches 1.0 and the P fraction declines to 0.0 when all daughter cells exit the epithelium, signaling the termination of proliferative activity. In the neocortical proliferative epithelium of mouse, the period of cell output occurs in the course of 11 cell cycles, from cell cycle 1 (CC1) to 11 (CC11). The inset shows progenitor cells executing the different cell cycle phases. A characteristic of the proliferative epithelium is that the position of the progenitor cells corresponds to the cell cycle phase that is being negotiated.

The position a neuron occupies in the precisely interconnected network of the CNS determines that neuron's connections, morphological features, and function. The timing of exit from the neuroepithelium appears to correlate ultimately with the class of cell it is to be and its final position in the vast network. In the second section of this chapter, we will describe S-phase labeling methods to analyze the migratory behavior of postmitotic cells.

2. Materials

2.1. Preparation of S-Phase Markers to Characterize Growth Modes of PVE Cells

2.1.1. Defining ^{3}H-TdR-Only Labeled Cohort of Cells

1. ^{3}H-TdR (methyl tritiated thymidine, specific activity 70–90 Ci/mmol, New England Nuclear Life Sciences Products).

2. BUdR solution for intraperitoneal injection into pregnant rodents (*see* **Note 1**). Weigh BUdR (Sigma Chemical Co.). Add the desired volume of the alkaline saline solution to the BUdR powder and mix thoroughly. Use a wide-mouthed container to prepare the BUdR solution, because you will need to take this solution in a 1 mL hypodermic syringe. It may take 5 min of gentle shaking by hand to dissolve all of the BUdR powder. Enclosing the vial containing the solution in the palm of your hand, or keeping the vial at 37°C for 10 min, helps dissolve the BUdR. When making a 35–50 mg/mL solution for use in rats, the concentration of sodium hydroxide should be increased to 0.1 *N*. Longer periods of mixing at 37°C and repeated use of a Vortex mixer may be necessary. Make sure that the BUdR dissolves completely and that you do not see any white powder in the solution (*see* **Note 2**).
3. Alkaline sodium chloride solution: add 700 μL of 0.1 *N* sodium hydroxide to 10 mL sterile normal saline (0.9% aqueous sodium chloride). (Note: Sodium hydroxide is generally available as a 10 *N* solution or as the concentration standard 6.7 *N* solution.)

2.1.2. Tissue Processing

1. Anesthetic: Ketamine (50 mg/kg body weight) and Xylazine (10 mg/kg body weight).
2. 70% ethanol.
3. Xylene.
4. Paraffin wax for embedding.
5. Super-frost Plus slides (Fisher Scientific).

2.1.3. Immunohistochemistry for BUdR

1. Humidified chamber.
2. Xylene.
3. Ethanol: 100%, 95%, 80%, 70%, 50%.
4. Phosphate buffered saline (PBS) (0.05 *M* sodium phosphate buffer and 0.9% sodium chloride) Prepare a stock solution of 0.4 *M* sodium phosphate buffer as follows: 81 g of Na_2HPO_4, 31.4 g of $NaH_2PO_4 \cdot H_2O$, 2000 mL of distilled water Dissolve completely. Do not heat. Dilute 125 mL of the stock solution in 1000 mL of 0.9% sodium chloride to get PBS.
5. 2 *N* hydrochloric acid. Hydrochloric acid is available as a 10 *N* solution. Dilute in water to make up 2 *N* solution. Store at 4°C.
6. Anti-BUdR incubation solution: 9365 mL of PBS (pH 7.2), 135 μL of anti-BUdR antibody mouse monoclonal (Becton Dickenson), 500 μL of 10% Tween-20. Mix thoroughly.
7. Secondary antiserum solution (biotinylated horse anti-mouse IgG, Vector) For 10 mL of solution, use 10 mL of PBS (pH 7.2), 150 μL of normal horse serum (from kit), 50 μL of secondary antiserum (from the same kit). Mix thoroughly.

8. Vector A-B Elite solution: 10 mL of PBS (pH 7.2), 4 drops of Elite solution A, 4 drops of Elite solution B. Mix thoroughly. This solution should be prepared at least 30 min in advance.
9. Diaminobenzidine (DAB) solution: 20 mL PBS (pH 7.2), 10 mg of diaminobenzidine, 6.6 µL of 30% hydrogen peroxide.
10. Distilled water.

2.1.4. Autoradiography

1. Deionized water.
2. NTB2 nuclear emulsion: Add 1 mL of 10% glycerol to 20 mL of deionized water in an acid-cleaned glass jar. In a photographic dark room under a safety light, add NTB2 emulsion (Eastman Kodak) to bring the volume to 40 mL. This will give a 1:1 mixture of emulsion and water in 0.5% glycerol.
3. Light-proof plastic boxes.
4. D-19 developer (Eastman Kodak).
5. Ektaflow fixer (Eastman Kodak).
6. Basic fuchsin solution: Prepare 0.1% aqueous basic fuchsin (pararosaniline hydrochloride; Fisher Scientific). It may take up to 8 h to dissolve the dye. Stir at room temperature; do not heat. Filter through a Whatman number 1 filter paper (*see* **Note 3**).
7. Ethanol: 95% and 100%.
8. Xylene.

3. Methods

3.1. Estimation of P and Q Fractions

The experiments are based upon a method of labeling with the S-phase markers ^{3}H-TdR and BUdR. The S-phase markers are administered as intraperitoneal injections to pregnant dams at a dose of 5 µCi/g body weight and 50 µg/g body weight, respectively.

3.1.1. Defining ^{3}H-TdR-Only Labeled Cohort of Cells

A "2 h cohort" of cells, synchronized with respect to their positions in the cell cycle is defined by sequential exposure to the two S-phase markers (**Fig. 2**) as follows:

1. ^{3}H-TdR injection at 7:00 AM on any embryonic day.
2. BUdR injection at 9:00 AM, 2 h after the ^{3}H-TdR injection.

In the above injection schedule, the ^{3}H-TdR-only cells are the cells that left S-phase between 7:00 AM–9:00 AM. Any cell that was in S-phase at 7:00 AM and remained in S-phase at 9:00 AM will be labeled with both ^{3}H-TdR and BUdR. Cells that entered S-phase at 9 AM or later will be labeled with BUdR only.

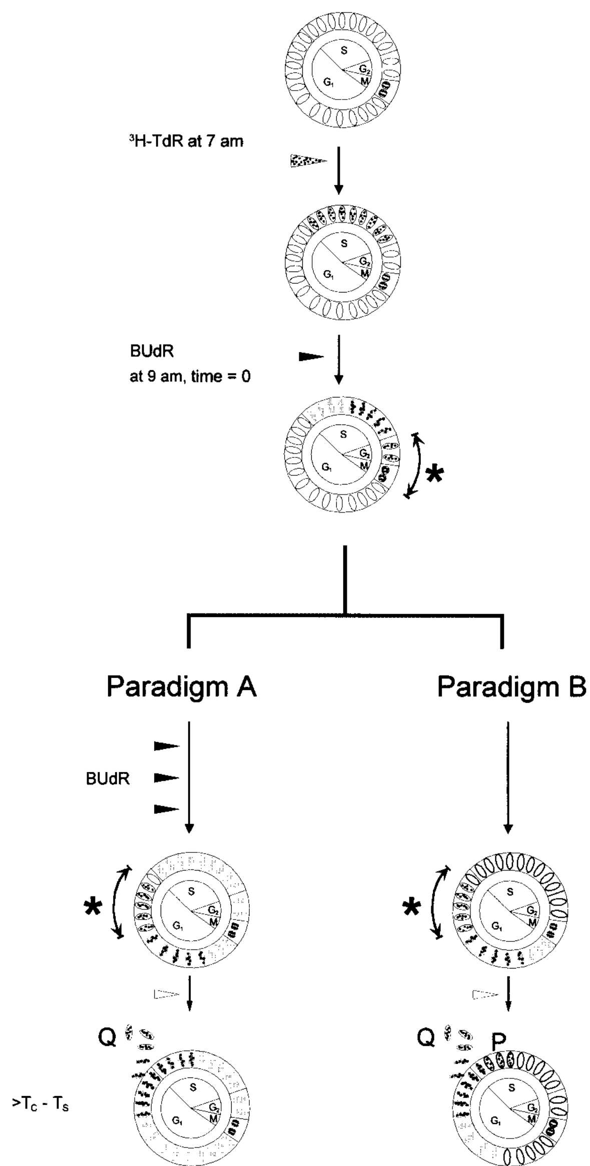

Fig. 2

^{3}H-TdR is reliably available for saturation S-phase labeling for at least 3 h. Therefore, the ^{3}H-TdR-only cells left S-phase 7:00 AM and 9:00 AM (2 h cohort). The interval between the two injections can be modified for a desired cohort size.

3.1.2. Distinguishing Q and P Fraction Cells Within the Cohort

The next step is to identify cells that leave the cycle (Q fraction) from those that reenter it (P fraction) within the ^{3}H-TdR-only labeled 2 h cohort described above. Two separate S-phase labeling paradigms (Paradigms A and B) are used for this purpose. Both paradigms should be performed on the same embryonic day in parallel using one set of pregnant animals for each paradigm. In both paradigms, the timing of termination of the experiment (by sacrificing the animals) should be decided from a set of predetermined cell cycle parameters, i.e., total cell cycle length (Tc) and the length of S phase (Ts). The methods for determining those parameters have been presented in detail elsewhere ***(6)***.

1. *Paradigm A (Q fraction):* After the ^{3}H-TdR injection at 7:00 AM and the BUdR injection at 9:00 AM, a series of additional BUdR injections is given at 3 h intervals. The total duration of cumulative BUdR exposure (i.e., the time between the first BUdR injection at 9:00 AM and the time of termination of the experiment) should be longer than Tc – Ts. Exposure time should also be shorter than Tc, for a given embryonic age and CNS region being analyzed, so as not to allow labeled cells of a lineage to proceed through a second mitosis. For example, for the mouse dorsomedial cerebral wall at E14,Tc and Ts are 15.1 h and 3.8 h, respectively. Therefore, Tc – Ts is 11.3 h and the duration of cumulative BUdR exposure should be longer than 11.3 h but shorter than 15.1 h. All of the

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Fig. 2. *(previous page)* A double-S-phase labeling method for the estimation of P and Q fractions. Two S-phase markers, tritiated thymidine (^{3}H-TdR) and bromodeoxyuridine (BUdR) are administered sequentially to delineate a cohort of dividing cells in S-phase (Common pathway). The ^{3}H-TdR-only labeled cells in the common pathway represent cells that left S-phase between 7:00 AM and 9:00 AM (2 h cohort, double-headed arcs with asterisks). This cohort of nuclei is followed as they go through G2, M, and G1-phases in paradigms A and B. The cells of the labeled cohort are partitioned into Q fraction and P + Q fractions in paradigms A and B, respectively. A series of BUdR injections is administered in paradigm A, whereas no BUdR injection is administered in paradigm B. Paradigms A and B last for a duration that is longer than the difference between total cell cycle length (Tc) and the length of S-phase (Ts). At the end of the labeling periods three types of labeled nuclei will result: Nuclei labeled with ^{3}H-TdR (dots), nuclei labeled with BUdR (gray), and nuclei doubled-labeled with ^{3}H-TdR and BUR (gray with dots). The ^{3}H-TdR-only labeled nuclei (dots) represent the Q-fraction in paradigm A and P + Q fractions in paradigm B.

P fraction cells of the cohort would have reentered S phase of the following cell cycle within the duration of 11.3–15.1 h to become labeled with BUdR. If the exposure were for a period greater than 15.1 h, some of the P fraction cells would have reentered M-phase of the following cell cycle and divided, causing an overestimate of the P fraction cells.

In this paradigm, the P fraction has been "removed" from the initially defined 2 h cohort of cells because the P fraction cells have reentered another S phase and have become labeled with the BUdR supplied by the additional injections. Therefore, the 2 h cohort of ^{3}H-TdR-only cells belongs to the Q fraction.

2. *Paradigm B (P+Q fraction):* In this paradigm, no additional BUdR injection is given after the initial injection at 9:00 AM. The duration of survival after the initial BUdR must be the same as that in paradigm A. Thus, in this case, all of the P fraction cells will have entered S phase, but they will not have incorporated BUdR because no additional BUdR injections were given. Hence, they will remain labeled only with ^{3}H-TdR and will be indistinguishable from the Q fraction cells. Thus, in this paradigm, both the P and Q fraction cells will be labeled with ^{3}H-TdR and the 2 h cohort will be P+Q fraction cells.

3.1.3. Tissue Processing

The next step is to identify the cells that are labeled only with ^{3}H-TdR in histological sections and distinguish them from the cells that are labeled with BUdR or double-labeled with BUdR and ^{3}H-TdR.

1. The pregnant mothers are deeply anesthetized with a mixture of Ketamine (50 mg/kg body weight) and Xylazine (10 mg/kg body weight) administered intraperitoneally.
2. The embryos are removed by hysterotomy and decapitated, and the whole heads are fixed overnight by immersion in 70% ethanol at room temperature. In mouse embryos older than E14, the brains are removed from the heads.
3. The embryonic heads or brains are dehydrated in graded ethanol solutions, cleared in xylene, and embedded in paraffin wax.
4. The tissue is sectioned at 4 μm in the coronal plane. Because 4 μm exceeds the path length of the tritium beta particle, variations in cutting thickness will not influence the apparent number of thymidine-labeled cells.
5. The sections are mounted on Superfrost Plus slides (Fisher Scientifc, Fair Lawn, NJ, USA) and dried at 37°C overnight (*see* **Note 4**).
6. The next step is to visualize BUdR and ^{3}H-TdR labels in the cells in the histological sections. For this, BUdR immunohistochemistry is performed followed by autoradiography to detect the tritium signal.

3.1.4. Immunohistochemistry for BUdR

In the following steps, add (pour) the solutions to the slides to cover every section. Keep slides flat (on strips of glass, for example) at all times. Double-

check that the solutions do not drain off or evaporate or cover the sections unevenly, especially during long incubations. Keep the slides in a humidified chamber or box. Use a plastic box with a lid that can be closed. Wet a few paper tissues with distilled water and keep the wet paper in the closed box. No shaking or agitation is necessary. Make up the solutions in advance of the steps for which they will be necessary.

1. Deparaffinize sections by immersing the slides in xylene. Two changes of 15 min each will be sufficient. Slides can be left in xylene overnight.
2. Rehydrate the sections. 100% ethanol 5 min, twice; 95% ethanol 5 min; 80% ethanol 5 min; 70% ethanol 5 min; 50% ethanol 5 min; PBS.
3. Hydrolyze double-stranded DNA into single strands (the BUdR antibody recognizes the latter only) by incubating in 2 *N* HCl for 1 h at room temperature.
4. Rinse sections in PBS (pH 7.2) for 30 sec.
5. Incubate in the anti-BUdR antibody solution for 30 min at room temperature.
6. Rinse with PBS (pH 7.2) for 5 min, 3X.
7. Incubate in secondary antiserum for 45 min at room temperature.
8. Rinse with PBS (pH 7.2) 5 min, 3X.
9. Incubate in Vector A-B Elite solution for 1 h at room temperature.
10. Rinse with PBS (pH 7.2) 5 min, 3X.
11. Incubate in 0.05% DAB in PBS and 0.1% hydrogen peroxide for approximately 3 min (*see* **Notes 5** and **6**).
12. Rinse thoroughly in PBS and in distilled water.

3.1.5. Autoradiography

Following BUdR immunohistochemistry:

1. Rinse the sections thoroughly in de-ionized water.
2. Dry at 37°C overnight.
3. Coat the slides with NTB2 nuclear emulsion.
4. Store in lightproof plastic boxes at 4°C for 4 weeks.
5. Develop the emulsion in D-19 developer (Eastman Kodak, Rochester, NY, USA; diluted 1 : 1 in water) at 15°C for 5 min without agitation.
6. Rinse for 1 min in distilled water.
7. Fix the emulsion with Ektaflo fixer (Eastman Kodak, diluted 1 : 7 in water) at 4°C (keep fixer on ice) for 5 min.
8. Wash the slides in running tap water for 30 min at room temperature to remove excess fixer.
9. Stain the sections with basic fuchsin for 30–60 sec.
10. Dehydrate the sections as follows: From the stain go directly to 95% ethanol, two rinses of 10 sec each; 100% ethanol two rinses of 10 sec each.
11. Clear the sections in xylene and coverslip.

3.1.6. Quantitative Analysis

The next step is to count the number of ^{3}H-TdR-only labeled cells in the neuroepithelium. For the purpose of counting cells, a sector of the neuroepithelium is defined by superimposing an ocular grid on the image of the neuroepithelium viewed under the microscope. An objective lens of at least 63× magnification, but preferably 100× magnification, should be used so that individual sliver grains can be resolved reliably. The grid should encompass the entire height of the PVE, i.e., from the ventricular surface to the outer limit of the S-phase zone. The ventricular border should form the base of the grid. The grid could be subdivided into uniformly sized "bins" to permit a finer resolution of the position of the P and Q fraction cells within the neuroepithelium.

Sections prepared from paradigms A and B are analyzed to count the total number of cells that are labeled only with ^{3}H-TdR within the sector. The number of cells from paradigm A corresponds to the total number of Q fraction cells (N_Q) arising from the original 2 h cohort. The number of cells from paradigm B corresponds to the cells in both Q and P fraction (N_P+N_Q) arising from the original 2 h cohort. From these numbers, Q fraction is calculated as $N_Q \div (N_P+N_Q)$ and P faction is 1–Q.

3.2. Analysis of the Migratory Patterns of Postmitotic Daughter Cells

In this section, we will describe a method to analyze the position of Q fraction cells from the defined 2 h cohort in the marginal zones of the CNS at various stages of development and maturity (**Fig. 3**). These experiments are designed to track the position of Q fraction of cells in the course of their migration and, after their migrations are completed, redistribution within the mature CNS.

1. A cohort of cells is labeled with ^{3}H-TdR-only as described in Paradigm A on any given embryonic day.

Fig. 3. *(opposite page)* A double S-phase labeling method to study migration of a cohort of postmitotic cells. Cells of the Q fraction (black stippled nuclei; labeled only with ^{3}H-TdR) are identified using the Common Pathway and paradigm A illustrated in Fig. 2. These cells exit from the ventricular zone **(A)**, and migrate **(B)** toward the appropriate position in the developing CNS **(C)**. During migration and redistribution within the CNS, the cells remain labeled only with ^{3}H-TdR and can be identified in histological sections. Thus, a cohort of cells that underwent S-phase over a defined period of time (2 h in this case) can be unequivocally identified during exit from the proliferative epithelium, migration, and arrival at the final destination.

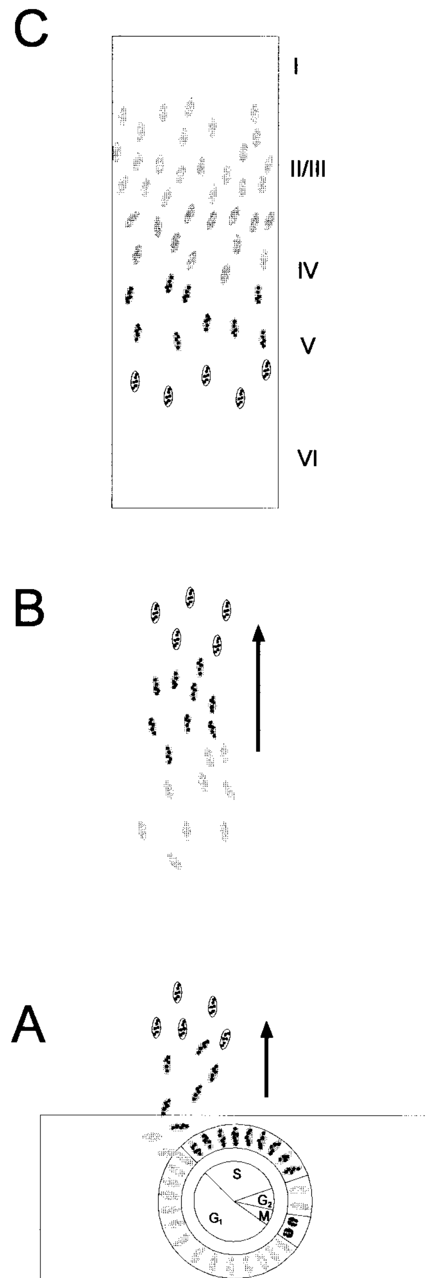

Fig. 3

2. The animals are sacrificed at different periods of development (e.g., day of birth, postnatal week 1, 2, 3, and so forth) and at adulthood.
3. The postnatal animals are perfused with ethanol or 4% paraformaldehyde.
4. Sections of the CNS region of interest are prepared and processed for BUdR immunohistochemistry and ^{3}H-TdR autoradiography, as described above.
5. The position of ^{3}H-TdR-only labeled cells is recorded with respect to anatomical landmarks.

4. Notes

1. BUdR is dissolved at a concentration of 5 mg/mL, in an alkaline solution of aqueous sodium chloride. This concentration is adequate for use in pregnant mice (body weight 30–50 g, depending upon the stage of pregnancy) to label cells in the embryonic CNS. Because pregnant rats are heavier (200–300 g), a concentration of 35–50 mg/mL will be necessary.
2. Use fresh BUdR solution for every injection. However, the solution can be stored in the refrigerator for up to 10 h without deterioration of quality. If the solution is stored in the refrigerator, warm it by holding in the palm of your hand before injecting the mouse to minimize discomfort to the animal that may be caused by injection of cold solution.
3. Do not let basic fuchsin solution stand for more than 3 mo. Protect from light.
4. Beta particles emitted by tritium decay at very short distances away from the source. If sections thicker than 4 μm are used, sampling errors will be introduced in the quantitative analysis.
5. Some batches of DAB appear to interfere with the autoradiographic method by quenching the tritium signal. If this occurs, clear the sections in xylene following BUdR immunohistochemistry and prior to coating the slides with NTB2 emulsion, as follows. After the DAB reaction in the BUdR immunohistochemical procedure, rise the sections in distilled water (twice, 5 min each), dehydrate in ascending concentrations of ethanol, clear in xylene (twice, 10 min each time), and rehydrate in a descending series of ethanol (i.e., 100% ethanol to water). Dry the slides and coat with NTB2 emulsion.
6. The immunohistochemical and autoradiographic methods described above can be used without any modification for tissue fixed in paraformaldehyde and sectioned on a cryostat or a sliding (sledge) microtome. However, if the paraformaldehyde fixed tissue is embedded in paraffin, the following modification will be necessary to the BUdR immunohistochemical protocol. Prior to incubation with 2 *N* HCl **(step 3)**, the sections should be incubated with 0.2% trypsin in 0.1 *M* Tris-HCl buffer (pH 7.5) for 20 min at 37–40°C. Following the trypsin incubation, the sections should be incubated with 2 *N* HCl at room temperature for 30 min. Proceed to **step 4** (BUdR antibody step).

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11

Clonal Analyses and Cryopreservation of Neural Stem Cell Cultures

Angelo L. Vescovi, Rossella Galli, and Angela Gritti

1. Introduction

The mature mammalian central nervous system (CNS) has long been considered incapable of significant cell turnover. This view has changed over the last few decades. Recently, the existence of *de novo* neurogenesis in the adult brain and the presence of stem cells in the mammalian CNS have emerged. The adult brain of both rodents and primates has been shown to embody undifferentiated, mitotically active, precursor cells that are multipotent in nature, and can contribute new, differentiated, neurons and glia to specific regions of the mature brain, such as the olfactory bulb ***(1–5)***, the hippocampus ***(6–8)***, and the cortex ***(9–11)***. While the data clearly suggest the presence of stem cells in the adult CNS in vivo, testing the proliferation, self-renewal, and differentiation capacity of "putative" CNS stem cells relies on the development of methodologies that allow for their isolation and extensive propagation in vitro.

Stem cells function as a reservoir of undifferentiated tissue precursors. These cells are often relatively quiescent or slow proliferating cells, but retain a significant ability to increase their activity and replace dead and/or injured cells, often occurring through the generation of an intermediate, fast-proliferating transit-amplifying cell population. Stem cells are endowed with functional characteristics that also provide a basis for their identification, particularly in the CNS, where specific molecular or antigenic markers have yet to be found. The most widely accepted definition ***(12)*** identifies stem cells as: (a) undifferentiated cells that lack markers of differentiated tissue-specific cells; (b) capable of proliferation; (c) possessing self-renewal capacity; d) able to generate functional differentiated progeny; and (e) able to regenerate the tissue

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after injury. A certain degree of flexibility in the use of these options is also considered a stem cell property.

Because the simultaneous assessment of the above listed functional characteristics is often impossible to obtain, some terms are given greater weight in identifying a candidate stem cell: self-renewal, the capacity to generate a wide array of differentiated progeny, or the ability to regenerate a tissue may be accepted, even alone, to identify a stem cell ***(13)***. The definition of CNS stem cells is hereby applied to neural precursors that have been shown to self-renew extensively, and can be propagated in long-term cultures, displaying a steady capacity to generate neurons, astrocytes, and oligodendrocytes (i.e., are multipotential), as determined by a clonogenic assay.

Clonal analysis and serial subcloning are likely the most difficult and time-consuming task in neural stem cell culturing. Yet, this analysis is essential to demonstrate the self-renewal capacity and the multipotency (i.e., the ability to generate both neuronal and glial cell types) of the candidate cell and to prove its "stem cell" nature. In practical terms, if the progeny of an individual founder cell contains cells that give rise to neurons and glia, and more important, contain one or more cells identical to itself (i.e., able to reproduce multipotent progeny), it can be concluded that the founder cell displays stem cell features. What follows is a detailed discussion of some methods developed and/or improved by our group for the isolation of neural stem cells and the essential issue of their clonogenic capacity.

Once established, stem cell lines can be effectively expanded to obtain a large number of cells that can then be cryopreserved, allowing for the establishment of a reservoir of early cells that can be further expanded to create a homogeneous stock of cells for future experiments. Repeated cycles of freezing and thawing do not affect CNS stem cell functional properties. More important, tissue from which the cell lines have been established can also be cryopreserved and stored if the need to re-establish a specific cell line were to arise. This ability is not particularly relevant for murine cell lines, but it is of fundamental importance for human neural stem cell lines. Our method for the cryopreservation of neurospheres follows.

2. Materials

2.1. Clonal Analysis

2.1.1. Limiting Dilution

1. Glassware: bottles, cylinders, beakers used only for cell culture (*see* **Note 1**).
2. Culture medium: In 375 mL of water, mix 50 mL of 10X DMEM/F12 medium, 10 mL of 30% glucose, 7.5 mL of 7.5% $NaHCO_3$, 2.5 mL of 1 *M* HEPES, 5 mL of 200 m*M* glutamine (Gibco-BRL), 50 mL of 10X hormone mix, 1 mL of

0.2% heparin, 20 μL of EGF or/and 10 μL of FGF2 stock (final concentration: 20 ng/mL of EGF and 10 ng/mL of FGF2).

3. 10X stock solution of hormone mix: Combine 40 mL of 10X DMEM/F12, 8 mL of 30% glucose, 6 mL of 7.5% $NaHCO_3$, 2 mL of 1 *M* HEPES, and 300 mL of water. Add 400 mg of apo-transferrin (Sigma). Dissolve 100 mg of insulin (Roche) in 4 mL of sterile 0.1 *N* HCl, mix in 36 mL of water, add all to the hormone mix solution. Dissolve 38.6 mg of putrescine (Sigma) in 40 mL of water and add to hormone mix solution. Add 40 μL of 2 m*M* progesterone and 40 μL of 3 m*M* sodium selenite. Mix well and filter sterilize. Aliquot in sterile tubes and store at –20°C (*see* **Note 2**).
4. Humidified chamber consisting of glass or plastic chamber with wet gauze on the bottom to hold a 96-well plate.
5. 96-well plate.
6. Plasticware: flasks, 15 mL conical tubes, plastic pipet.
7. P200 Pipetman.
8. Trypan blue.
9. Hemocytometer.
10. Laminin- or Matrigel-coated coverslips.

2.1.2. Manipulation of Individual Cells

1. Heat-polished glass microelectrode pipet (40–70 μm inside diameter).
2. Silicon tubing.
3. Screw driven 500 μL syringe.

2.1.3. Methylcellulose Assay

1. Methylcellulose gel matrix (powder, Dow methocel A4M, premium grade; 4% final concentration) in DMEM/F12 or NS-A growth medium (EUROCLONE).
2. EGF, human recombinant, Peprotech, Rocky Hill, NJ, USA.
3. FGF2, human recombinant, Peprotech, Rocky Hill, NJ, USA.
4. 5 mL syringe.
5. 60 mm Petri dish.
6. Microphotography set up or time lapse cinematography.
7. 5 mL microfuge tubes.

2.1.4. Subcloning Procedure

1. P200 Pipetman.
2. 48-, 24-, or 12-well plates (depending on number of viable cells).
3. Humidified chamber.
4. 5 mL microfuge tubes.

2.2. Cryopreservation

1. Freezing jar.
2. Isopropanol.
3. Freezing medium: culture medium containing 10% dimethylsulfoxide (DMSO).

3. Methods

3.1. Clonal Analysis

3.1.1. Limiting Dilution

1. Warm culture medium to 37°C.
2. Prepare the humidified chamber.
3. Tap sides of flask to dislodge spheres and remove content of the flask to 15 mL sterile plastic conical tubes using a sterile plastic pipet.
4. Pellet cell suspension by centrifugation at 110*g* for 10 min.
5. Remove the supernatant leaving behind about 300 μL. Using a sterilized P200 Pipetman set at 200 μL, vigorously dissociate pellet to a single-cell suspension.
6. Add 10 mL of fresh culture medium and spin cells at 15*g* for 15 min.
7. Remove supernatant and resuspend pellet in 0.5 mL of culture medium.
8. Dilute a 10 μL aliquot in trypan blue and count in a hemocytometer.
9. Resuspend cells in culture medium at a cell density of 5 to 10 cells/mL. Use a dispenser to add 100 μL of this cell suspension to each well, frequently resuspending the starting cell solution. To prepare three 96 well plates, you need 300 cells/30 mL of culture medium.
10. Incubate cells at 37°C in the humidified chamber (*see* **Note 3**). Feed cells with fresh, pre-warmed medium every 4–5 d. For the first feeding, add 100 μL; if further feeding is needed, carefully replace 100 μL of medium in the well with 100 μL of fresh medium.
11. Carefully inspect plates under the inverted microscope to unequivocally identify and mark wells containing single cells. Make sure to use high magnification to assess that a cell is indeed "single." Wells containing two cells or more should not be further considered for the clonal analysis.
12. Inspect the plate once a week; make sure the pH of the medium does not change excessively (*see* **Note 4**). Many of the cells will die and some will differentiate. Only a small percentage will proliferate to form a clonal sphere that could undergo further subcloning. This proliferation will require 10–30 d, depending both on the type of cells and culture conditions.
13. Differentiate cells by transferring the sphere onto a Laminin- or Matrigel-coated coverslip in the absence of growth factors, or perform serial subcloning as described subsequently.

3.1.2. Cloning by Manipulation of Individual Cells

Follow the above protocol and continue:

1. Resuspend cells in culture medium and plate in 35 mm Petri dishes in 2 mL of culture medium at a density of 50 cells/cm^2.
2. After 2–6 h, choose viable cells on the basis of round shape, phase-brightness, hypertrophic appearance, and lack of processes (*see* **Fig. 1A**).
3. Transfer to a 96-well plate (1 cell/well) using a heat-polished glass microelectrode pipet connected by silicon tubing to a screw-driven 500 μL syringe.

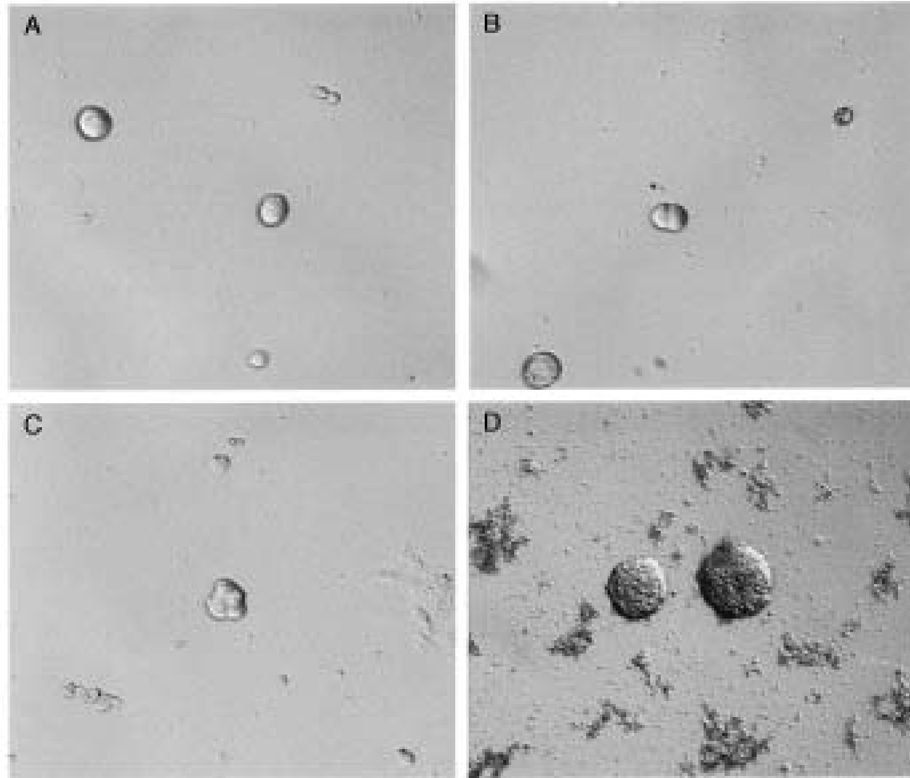

Fig. 1. Neural stem cells of the CNS. Undifferentiated precursor cells can be isolated and cultured from the adult mouse brain and the human embryonic CNS. When plated as single cells in the presence of EGF and/or FGF2 **(A)**, a subset of these cells divide **(B)**, proliferate **(C)**, and give rise to spheres of undifferentiated cells **(D)**. At this point spheres can be further subcloned or collected for cryopreservation.

4. Follow the fate of single cells over time. Clonally derived spheres can be differentiated or can undergo further subcloning by repeating this process or by methylcellulose assay.

3.1.3. Methylcellulose Assay

1. Warm the culture medium to 37°C.
2. Prepare a humidified chamber.
3. Prepare Methylcellulose gel matrix in DMEM/F12.
4. Follow steps 3–8 in **Subheading 3.1.1.**
5. Make sure that the vast majority of the cells are single cells by withdrawing an aliquot and checking it under the microscope.

6. Resuspend single cells in growth medium containing 40 and 20 ng/mL of EGF and FGF2, respectively. Final cell concentration should be less than 200 cells/mL.
7. Aspirate 2.5 mL of the cell suspension into a 5 mL syringe.
8. Aspirate 2.5 mL of the methylcellulose gel matrix into the same syringe.
9. Gently inject the mixture of cells and methylcellulose gel matrix into a 60 mm Petri dish, avoiding bubbling and foaming.
10. Using the same syringe, resuspend the mixture multiple times until a semi-solid homogeneous gel has formed and the single cells are thoroughly dispersed.
11. The day after plating, inspect the plate to identify single hypertrophic cells. Mark their position on the plate with a fine marker and take microphotographs over time (*see* **Note 5**).
12. When clonal spheres have been generated, perform subcloning of individual spheres by this same procedure or by the following method.

3.1.4. Subcloning Procedure

1. Transfer individual clonal spheres to 5 mL microfuge tubes containing 1 mL of appropriate medium (1 sphere/tube) using a sterilized P200 Pipetman set at 180 μL. Rinse tip with medium first, to avoid cell adhesion to tip walls.
2. Centrifuge at 110*g* for 10 min. Remove supernatant leaving behind about 200 μL of medium.
3. Using a sterilized P200 Pipetman set at 180 μL, dissociate spheres to a single-cell suspension (100–150X for human cells, 50–60X for adult mouse cells, and 30–40X for embryonic mouse cells). Rinse tip with medium first, to avoid cell adhesion to tip walls. Press the tip to the bottom or the edges of the well to generate a fair amount of resistance. Avoid foaming and bubbles.
4. Plate all the cell suspension in a clean well of a 48-, 24-, or 12-well plate (depending on the number of viable cells) and incubate cells at 37°C in the humidified chamber (*see* **Note 6**).
5. Within 1 h of plating, count the number of the single cells obtained by dissociation of each clone under the microscope (*see* **Fig. 1A**). A subset of these cells will proliferate (*see* **Fig. 1B**) giving rise to secondary clones. The cloning efficiency can be calculated normalizing the number of secondary clones by the total number of cells in the same well, as assessed by direct observation 1 h after dissociation.
6. Individual secondary clones (*see* **Fig. 1D**) can either be differentiated to assess their multipotentiality, or can be transferred to 5 mL microfuge tubes (1 sphere/tube) to undergo further subcloning.
7. If a clonal cell line has to be generated, secondary spheres derived from a single primary sphere can be pooled, mechanically dissociated to a single-cell suspension, and plated at a cell density of 10,000 cells/cm^2 in the appropriate medium. Subculture until a bulk culture is established.

3.2. Cryopreservation of Neurospheres

3.2.1. Preparation

1. Ensure that freezing jar is at room temperature and filled with isopropanol.
2. Prepare freezing medium: 10% dimethylsulfoxide in culture medium (*see* **Note 7**).
3. Label cryovials with date, cell type, and passage number.

3.2.2. Cryopreservation

1. Collect spheres by gentle pipeting and pellet them by centrifugation at 110*g* for 10 min (*see* **Note 8**).
2. Wash the pellet once with fresh medium and resuspend in 1.5 mL of freezing medium. Swirl gently to resuspend spheres.
3. Transfer cells into labeled 2 mL cryogenic vial(s) and let them equilibrate at room temperature for 15 min.
4. Transfer vial(s) into the freezing jar. Leave the jar at –80°C for a minimum of 4 h to allow a slow and reproducible decrease in temperature (–1°C/min).
5. Transfer vial(s) into a liquid nitrogen tank for long-term storage.

3.2.3. Thawing of Cryopreserved Neurospheres

1. Warm culture medium and water bath to 37°C.
2. Quickly transfer cryovial(s) from liquid nitrogen to 37°C water bath and leave until thawed. Swirl the vial(s) to speed thawing.
3. Wipe entire cryovial with 70% ethanol.
4. Slowly transfer cell suspension from cryovial to 15 mL plastic tube containing 5 mL of warm culture medium.
5. Spin cell suspension for 8 min at 110*g* and remove most of supernatant.
6. Gently resuspend pellet in fresh medium and plate in flask(s) of appropriate size (*see* **Note 9**).

4. Notes

4.1. Clonal Analysis Materials:

1. A set of glassware to be used only for tissue cultures should be prepared by rinsing thoroughly several times with distilled water before being sterilized in an autoclave used for tissue culture purposes only. We suggest that media and all stock solutions be prepared only in sterile disposable tubes and/or bottles.
2. These cultures are extremely sensitive to contaminants present in water or glassware. Distilled, sterile, apirogenic water should be used (use before filter sterilization in sterile, disposable bottles). Alternatively, ultrapure cell culture tissue grade water can be purchased from GIBCO BRL.

4.2. Clonal Analyses Procedure

3. Since small volumes of medium are used in 96-well plates, evaporation of medium is very critical; always hold plates in humidified chambers.
4. Neural stem cell cultures are very sensitive to pH changes. The pH of the culture medium should be around 7.4, which is indicated by a dark orange color. If the color is close to violet, leave the medium to equilibrate in the incubator. If the color is light orange/yellow, check the composition and prepare fresh medium if necessary.
5. Embedding cells in methylcellulose prior to plating is recommended in order to avoid cell aggregation.
6. If you want to generate a clonal cell line, use the limiting dilution protocol, then dissociate single clonal spheres inside their own dish without transferring them. Proceed with plating.

4.3. Cryopreservation Procedure

7. In our experience, glycerol yields very poor results when freezing neural stem cells.
8. Do not let spheres grow too large before harvesting for cryopreservation and do not mechanically dissociate spheres before freezing, which increases the number of dead cells and viability of the culture upon thawing will be very low.
9. The same freezing thawing protocol can also be applied to finely chopped embryonic human tissue. To start a neural stem cell culture from frozen human tissue, upon thawing, follow protocol for dissociation and primary culture ***(14)***.

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12

Assessing the Involvement of Telomerase in Stem Cell Biology

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1. Introduction

Highly proliferative cells including stem cells and cancer cells express high levels of telomerase, an enzyme activity that adds a six-base DNA repeat sequence (TTAGGG) to chromosome ends and thereby prevents their shortening during successive rounds of mitosis ***(1,2)***. Telomerase activity decreases in association with cell differentiation and is generally absent from most somatic cells in the adult; shortening of telomeres in such somatic cells may trigger cell cycle arrest in the G1 phase (cellular senescence). In this way, telomere shortening effectively limits the proliferative potential of cells, functions as a tumor suppressor mechanism and may contribute to the aging process ***(3–6)***. Telomerase consists of an RNA template (TR) and a protein called TERT that possesses reverse transcriptase activity. Several telomere-associated proteins have been identified including TRF1 (telomere repeat-binding factor 1) that may inhibit telomerase activity and promote telomere shortening, and TRF2 which may promote maintenance of telomeres ***(7,8)***. Data obtained during the past several years have provided evidence that telomerase can play important roles in the regulation of cell proliferation, differentiation, and survival. Examples include overexpression of hTERT can immortalize cultured fibroblasts and epithelial cells ***(4)***; telomerase is downregulated during muscle cell differentiation ***(9)***; and TERT promotes cell survival (prevents apoptosis) of developing mouse and rat brain neurons ***(10–12)***.

TERT and telomerase activity levels are high in several types of stem cells including embryonic stem cells ***(13,14)***, hematopoietic stem cells ***(15,16)***, and neural progenitor cells ***(12,17)***. A progressive decrease in telomerase

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levels appears to occur in association with progressive lineage restriction and cellular differentiation, suggesting a role for telomerase in controlling cell fate. Interestingly, studies of cloned cattle in which donor nuclei from adult fibroblasts are injected into oocytes suggest that telomerase activity and telomere length regulation can be "reprogrammed" ***(18)***. It is therefore important from both basic science and clinical perspectives that we better understand the mechanisms controlling telomerase expression and activity, on the one hand, and mechanisms whereby telomerase regulates cell fate, on the other hand. In the present chapter, we detail methods for quantifying telomerase activity, and TERT expression in tissue samples and cultured cells. Protocols for inhibiting TERT expression and telomerase activity are also presented.

1. Materials

2.1. Telomerase Activity Assay

1. CHAPS Buffer: 0.5% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 10 m*M* Tris-HCl pH 7.5, 1 m*M* $MgCl_2$, 1 m*M* EGTA, 5 m*M* β-mercaptoethanol, 1 m*M* PMSF, 1 U/μL RNase Out (Gibco), and10% glycerol.
2. Eppendorf microfuge.
3. BCA Assay (Pierce).
4. TRAP reaction mixture: 20 m*M* Tris-HCl (pH 8.0), 1 m*M* EGTA, 0.005% Tween 20, 1.5 m*M* $MgCl_2$, 63 m*M* KCl, 200 μM dNTP mix, 2 U Taq Polymerase, 10 pmol TS primer (5′-AAT CCG TCG AGC AGA GTT-3′), 10 pmol of CX-ext primer (5′-GGT CCC TTA CCC TTA CCC TTA CCC TTA-3′), and an internal amplification standard (ITAS) that produces a 135 bp product ***(19)***.
5. PTC-20P, MJ Research, DNA Engine.
6. ROX-500 (GeneScan-500Rox size standard, Applied Biosystems).
7. Formamide.
8. ABI Prism 310 capillary electrophoresis unit (Applied Biosystems).

2.2. RT-PCR Analyses of TERT and Telomere-Associated Protein mRNA Levels

1. TRI-zol (Gibco BRL).
2. SuperScript First Strand Synthesis System (Gibco BRL).
3. Reaction mixtures: 1–5 L of the first strand cDNA, PCR Buffer (Gibco) 200 *M* dNTPsmix, 2 U Taq polymerase, 1.5 m*M* $MgCl_2$, 10 p*M* primers.
4. β-actin.
5. Agarose gel.
6. Ethidium bromide.
7. FLA 3000 Imager (Fuji).

2.3. Immunoblot Analysis of TERT Protein Levels

1. SDS-Page (10% acrylamide).
2. PVDF membrane.
3. 5% nonfat milk.
4. TTBS (Tris–Tween buffered saline). The following are dissolved in 1 L of water: 80 g/L NaCl, 29.2 g Tris base, and 0.5 mL Tween-20 (pH 7.5).
5. Peroxidase labeled anti-rabbit or anti-mouse secondary antibody.
6. ECL reagent (Amersham Pharmacia Biotech).
7. Hyperfilm (Amerisham Pharmacia Biotech).

2.4. Immunostaining Methods for Cellular Localization of TERT Protein

1. 4% paraformaldehyde.
2. PBS.
3. 2% Triton X-100.
4. 2% normal goat serum.
5. Biotinylated goat anti-rabbit secondary (Vector Labs).
6. FITC/avidin conjugate (Vector Labs).
7. Anti-fade solution: 10 μ*M* propylgallate in water.
8. ABC reagent (Vector Labs).
9. Nickel enhanced diaminobenzidine solution (Vector Labs).
10. TERT Antibodies: A rabbit polyclonal antibody against human TERT is commercially available from Calbiochem. Results of studies using other TERT polyclonal antibodies have also been reported ***(10,11)***.

2.5. Overexpression of TERT

1. pBabest 12 retro-viral plasmid.
2. SV40 early promoter.
3. Lipofectamine.
4. G-418 antibiotic.

2.6. Suppression of TERT Expression and Activity

1. TERT antisense and control oligonucleotides: An antisense oligonucleotide against mouse TERT (5′-GAGGAGCGCGGG TCATTGT-3′) and the scrambled control oligonucleotide (5′-GGAGGACGCTGCGAGTGTT-3′) are purchased from IDT (Coralville, IA, USA), and are prepared as 1 m*M* stocks in sterile deionized water.

3. Method

3.1. Telomerase Activity Assay

A capillary electrophoresis-based telomeric repeat amplification protocol (TRAP) assay is used to quantify levels of telomerase activity ***(19,20)***. This

assay is performed on lysates of tissues or cultured cells using the following protocol.

1. Tissues or cultured cells are homogenized in CHAPS buffer and incubated for 30 min on ice.
2. Samples are centrifuged for 30 min at maximum speed in a microfuge (Eppendorf) and the supernatant is removed; a 5 μL aliquot is removed for protein determination (BCA assay, Pierce), and the remainder of the sample is stored at –80°C.
3. The reaction is initiated by adding 100 ng of sample protein to a TRAP reaction mixture. The reaction is incubated at 30°C for 30 min to allow telomerase to add telomeric repeats to the TS primer.
4. The telomerase products are amplified by PCR (30 cycles). Each PCR cycle consists of 30°C for 30 min for the first extension cycle, followed by 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec.
5. Two microliters of PCR product is mixed with 1 μL of ROX –500 and 22 μL of formamide and analyzed using an ABI Prism 310 capillary electrophoresis unit (Applied Biosystems).
6. Integrated values are summed for telomerase products containing 5 (one repeat beyond primer dimer size) to 10 telomeric hexamer repeats and calibrated by dividing by the value for the peak area of the internal amplification standard. All assays are performed in triplicate. Experimental values are typically expressed as a percentage of the value obtained using an equivalent amount (usually 100 ng) of HeLa cell extract.

An example of results obtained using this method are shown in **Fig. 1**, and published results using the method can be found in **ref. *12***.

3.2. RT-PCR Analyses of TERT and Telomere-Associated Protein mRNA Levels

For analyses of mRNA levels, total RNA is isolated from samples of tissue or cultured cells using TRIzol reagent according to the manufacturer's protocol (Gibco BRL) and is then subjected to RT-PCR analysis as follows (*see* **Note 1**):

1. The first strand of cDNA is synthesized from 1–2 μg total RNA with the SuperScript First-Strand Synthesis System for RT-PCR using oligo(dT) primers and following recommendations provided by the supplier.
2. Reaction mixture consisting of 1–5 μL of the first strand cDNA, PCR buffer, 200 μM dNTPs mix, 2 U Taq Polymerase, 1.5 m*M* $MgCl_2$, and 10 p*M* primers are denatured at 94°C for 2 min.
3. Samples are then subjected to 33 PCR cycles (each cycle consists of 30 sec at 94°C, 30 sec at 50°C, 45 sec at 72°C), then elongated at 72°C for 10 min. The primers for the internal β-actin control are added to the reaction at the 60°C step of cycle 9.

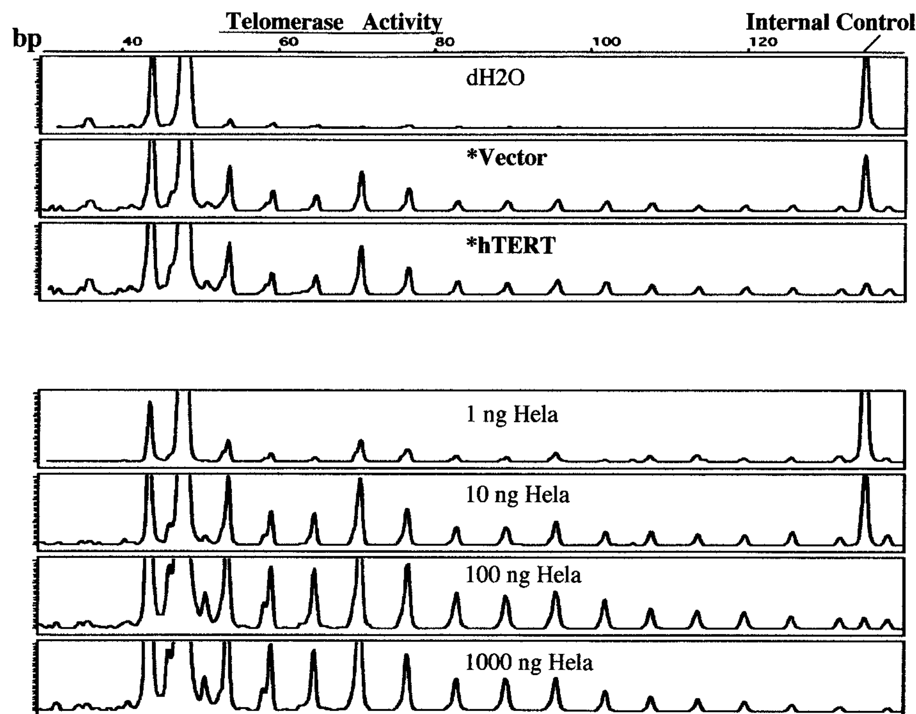

Fig. 1. Capillary electrophoresis-based TRAP assay results for quantification of telomerase activity. Upper. A water control sample, and samples of lysates of HeLa cells transfected with empty vector or with an hTERT expression plasmid. Lower. Samples containing the indicated amounts of HeLa cell protein. The first two peaks in the electrophoretograms represent the primer dimer and the last peak corresponds to the internal amplification standard. Relative telomerase activity is calculated by summing the values for the first 5 telomerase activity peaks and dividing that number by the value for the internal amplification standard peak. bp, base pairs.

The sequences of the primers for these reactions are as follows:

Template	Forward Primer	Reverse Primer
mTERT	5′- CTG CGT GTG CGT GCT CTG GAC -3′	5′- CAC CTC AGC AAA CAG CTT GTT CTC -3′
mTRF2	5′- GCC CAA AGC ATC CAA AGA C -3′	5′- ACT CCA TCC TTC ACC CAC TC -3′
β-actin	5′- TGT GAT GGA CTC CGG TGA CGG -3′	5′- ACA GCT TCT CTT TGA TGT CAC GC -3′
mTRF1	5′- TTC AAC AAC CGA ACA AGT GTC -3′	5′- TCT CTT TCT CTT CCC CCT CC -3′

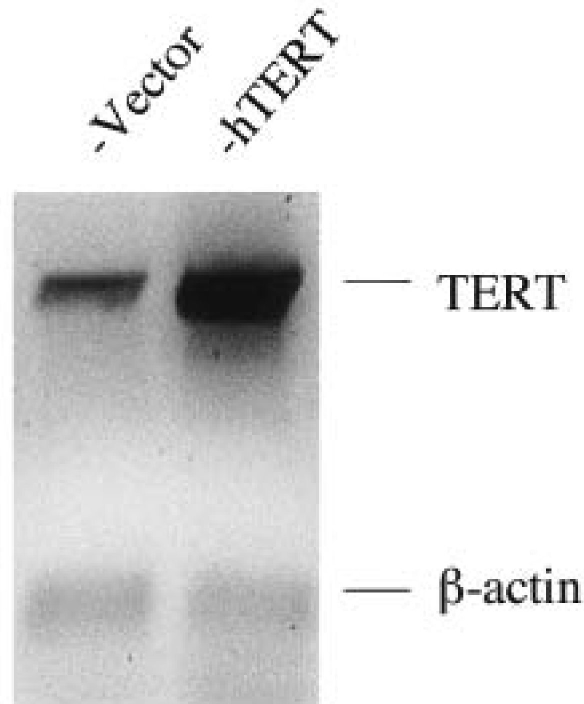

Fig. 2. RT-PCR analysis of TERT mRNA levels in cultured HeLa cells. RNA was isolated from vector-transfected control HeLa cells and cells transfected with a human TERT expression plasmid was subjected to RT-PCR analysis using primers that amplify either hTERT or β-actin mRNAs. See text for details of the methods. The size of the hTERT PCR product is 700 bp and the size of the β-actin PCR product is 200 bp.

4. PCR products are separated by agarose gel electrophoresis (1.5%) followed by staining with ethidium bromide.
5. Images of the stained DNA gels are acquired using a FLA 3000 Imager (Fuji). Densitometric analyses are performed using the software provided by the manufacturer. Values are normalized to the level of actin mRNA in the same sample.

An example of an RT-PCR analysis of TERT mRNA levels in HeLa cells is shown in **Fig. 2**, and published results using the method can be found in **ref. *12***.

3.3. Immunoblot Analysis of TERT Protein Levels

Solubilized proteins from tissues and cultured cells are separated by SDS-PAGE (10% acrylamide) and transferred electrophoretically to a PVDF membrane. The membrane is then processed for immunodetection of TERT protein as follows:

1. The membrane is incubated for 1 h at room temperature in a solution of 5% nonfat milk in TTBS.
2. The membrane is incubated overnight at 4°C in TTBS containing 5% nonfat milk plus primary antibody against TERT. The antibody dilution that results in an adequate signal and a low level of non-specific binding should be established in preliminary studies. Controls for specificity should include preadsorption of the primary antibody with excess antigen.
3. Rinse the membrane 4X with TTBS (5–10 mL/wash).
4. Incubate the membrane for 1 h in TTBS containing peroxidase-labeled anti-rabbit or anti-mouse secondary antibody.
5. Rinse the membrane 4X with TTBS (5–10 mL/wash).
6. Expose the membrane to ECL reagent for 1 min at room temperature.
7. Expose the membrane to Hyperfilm; the time of exposure that results in the best signal to noise ratio depends on a variety of factors and must be determined empirically (typical exposures are from 1–10 min).

Examples of results obtained using this method can be found in **ref. *10***.

3.4. Immunostaining Methods for Cellular Localization of TERT Protein

Tissues in adult mice or rats are fixed by perfusion with a solution of 4% paraformaldehyde in PBS, followed by an overnight incubation at room temperature in the same fixative. Tissue sections are cut at 30 μm on a freezing microtome and are collected in wells of microwell plates containing PBS. Cultured cells are fixed by incubating for 30 min in a solution of 4% paraformaldehyde in PBS [cells can be stored in PBS (4°C) for several weeks (*see* **Note 2**)]. The tissue sections and cultured cells are immunostained using the following protocol:

1. Tissue sections and cells are incubated for 1 h and 5 min, respectively, at room temperature in PBS containing 0.2% Triton X-100 plus 2% normal goat serum.
2. Tissue sections and cells are then incubated overnight at 4°C in PBS containing 2% normal goat serum plus primary TERT antibody at an appropriate dilution (typically between 1 : 1000 and 1 : 10,000 depending upon the antibody titer).
3. Tissue sections and cells are then washed 3X in PBS.
4. Tissue sections and cells are then incubated for 1 h in PBS containing biotinylated goat anti-rabbit secondary antibody.
5. Tissue sections and cells are then washed 3X in PBS.
6. For fluorescence-based detection, tissue sections and cells are then incubated for 30 min in PBS containing FITC/avidin conjugate 4 μL/mL. The sections or cells are then washed in PBS, mounted in an anti-fade solution consisting of 10 μM propylgallate in water, and imaged by conventional or confocal fluorescence microscopy.
7. For peroxidase-based labeling, tissue sections and cells are incubated for 1 h in the presence of ABC Reagent (Vector Labs) and peroxidase is detected using

nickel-enhanced diaminobenzidine solution (Vector Labs). Immunoreactivity is visualized and photographed using bright-field optics.

Examples of results obtained using this method are shown in **Fig. 3**, and published results can be found in **ref. *10***.

3.5. Overexpression of TERT

Clones of cell lines stably overexpressing hTERT can be generated using conventional transfection and antibiotic selection protocols. We have produced clonal lines of pheochromocytoma (PC12) cells overexpressing hTERT using methods similar to those described previously ***(21)***.

1. Cells are transfected with the pBabest2 retroviral plasmid in which hTERT cDNA is under the control of SV40 early promoter ***(22)***. Control cells are transfected with empty vector. Lipofectamine is used to facilitate plasmid uptake.
2. Transfected cells are selected by maintenance in medium containing the antibiotic G418, and single clones are isolated by serial dilution.
3. Clones overexpressing hTERT protein are identified by telomerase activity assay, and by immunoblot and immunocytochemistry using TERT antibody.

Examples of the characterization of cells overexpressing TERT generated using these methods are shown in **Fig. 3**, and published data can be found in **ref. *10***.

3.6. Suppression of TERT Expression and Activity

3.6.1. One Experimental Approach

One experimental approach for studying the function of telomerase is to suppress TERT production by treating cells with antisense oligodeoxynucleotides directed against TERT mRNA ***(10)***. The oligodeoxynucleotides enter the cell, bind specifically to TERT mRNA thereby suppressing translation and reducing levels of TERT protein.

1. Cultured cells (we have employed embryonic mouse brain neurons in primary culture, and human tumor cell lines including SH-SY5Y and HeLa cells in our studies) are switched to a culture medium lacking serum.
2. Antisense oligonucleotides in a 1 m*M* stock solution are diluted into the culture medium to a final concentration of 10–25 μ*M*. Control cultures should receive an equivalent concentration of scrambled control oligodeoxynucleotide, and additional cultures should be treated with vehicle (water) only.
3. Preliminary studies should establish the time course of change in TERT protein levels after exposure of the cells to the antisense oligodeoxynucleotides, which can be accomplished by performing immunoblot and immunocytochemical

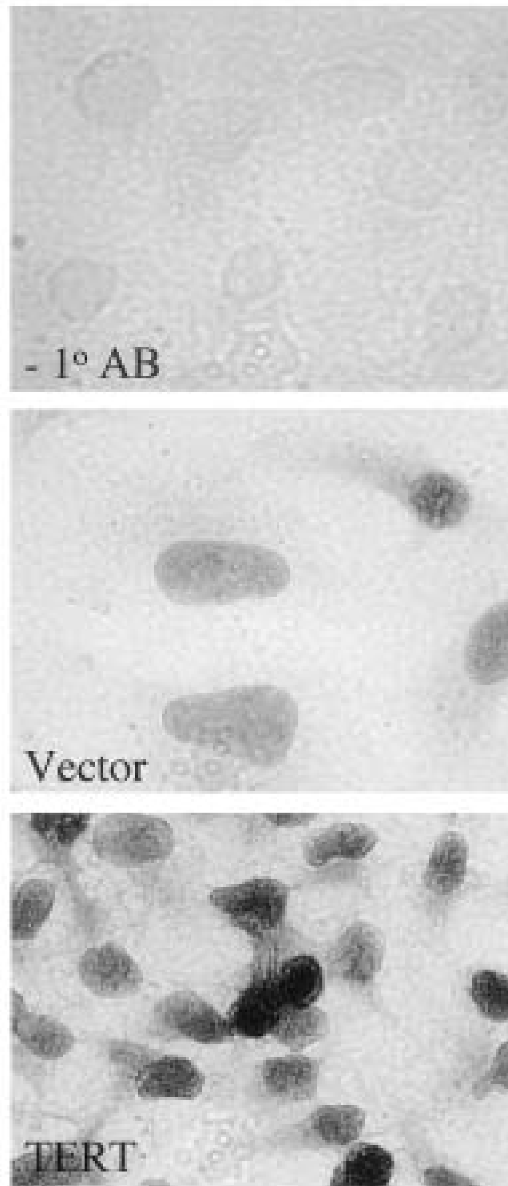

Fig. 3. Immunocytochemical analysis of TERT protein localization in cultured HeLa cells. Vector-transfected control HeLa cells and cells transfected with a human TERT expression plasmid were fixed and processed for immunocytochemistry using a rabbit polyclonal antibody against hTERT (middle and lower panels). Cells in the upper panel were processed without the primary antibody. Note that TERT immunoreactivity is localized primarily in the nucleus, but is also present at lower levels in the cytoplasm.

analyses. A time period of exposure that results in a maximum decrease in TERT protein levels should be chosen for subsequent experiments.

4. After exposure to oligodeoxynucleotides, endpoints of interest are measured to determine whether suppression of TERT expression changes those endpoints. For example, we have used this approach to provide evidence that TERT plays a role in promoting the survival of immature neurons in the developing mouse brain ***(10)***.

3.6.2. A Second Experimental Approach

A second experimental approach involves the use of chemical inhibitors of telomerase enzyme activity. Because of their potential use in cancer therapy, there has been considerable effort placed on identifying chemicals that selectively inhibit telomerase ***(23)***. Examples of inhibitors that we have used to study the roles of telomerase in neural development include the reverse transcriptase inhibitor AZT (3′-azido-3′-dideoxythymidine), 3,3′-diethyloxadicarbocyanine (an agent that binds to dimeric hairpin quadruplexes), and the oligodeoxynucleotide TTAGGG which may bind to the telomerase RNA component and thereby inhibit telomerase activity ***(10,11,24)***. The following is an example of an approach for using chemical inhibitors to study the role of telomerase in regulating the differentiation of cultured neural cells.

1. A toxicity profile of the chemical is established by incubating the cultured cells in the presence of increasing concentrations of the inhibitor for 48–72 h.
2. Cells are incubated in the presence of subtoxic concentrations of the inhibitor for increasing time periods and telomerase activity is measured and compared with control cultures not exposed to the inhibitor or exposed to an inactive analog of the inhibitor. It is important to establish that the inhibitor reduces telomerase activity prior to a change in cell proliferation rate or survival.
3. The effects of inhibitors at concentrations and time points that decrease telomerase activity on endpoints of interest are then determined.

4. Notes

1. In the RT-PCR analyses it is important to establish that the RT-PCR products of the correct size do, in fact, correspond to the mRNAs of interest by excising the bands from the gels and sequencing them. Preliminary analyses will also be required to determine the optimum PCR conditions that result in a level of amplification that falls within the linear range.
2. For immunoblots and immunostaining, it is important that antibodies not be subjected to multiple freeze-thaw cycles; this can be avoided by diluting the stock with an equal volume of glycerol and storing it at –20°C. To reduce the level of non-specific binding of the primary antibody to proteins in immunoblots, the concentration of primary antibody can be decreased and the membranes

can be washed more extensively with buffer after the primary antibody step. Cross-reactivity of antibodies raised against hTERT peptides with tissues and cultured cells from mice and rats should be established. It is important to include a positive control (e.g., HeLa cells) and a negative control (e.g., adult brain tissue) in the analyses.

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13

Detection of Telomerase Activity in Neural Cells

Karen R. Prowse

1. Introduction

Telomeres are specialized nucleoprotein structures that cap linear eukaryotic chromosomes and function to prevent the chromosomes from recombining or unraveling ***(1)***. Telomerase is a ribonucleoprotein (RNP) polymerase that synthesizes telomeric sequence *de novo* onto the 3′ end of chromosomes using a portion of its internal RNA component as a template ***(2,3)***. The regulation of telomerase activity is complex. While most differentiated somatic cells lack telomerase activity, most cancer cells, germline cells, and stem cells express detectable levels of telomerase activity ***(4)***. In those cells that lack telomerase activity, telomeric sequences are incompletely replicated during cell division due to the end replication problem of DNA polymerases ***(5,6)*** and thus the net length of telomeres in the daughter cells is reduced ***(7)***. The progressive loss of telomeric DNA triggers a growth-arrest mechanism in normal somatic cells ***(8)***. However, in cells that maintain telomerase activity, telomeres can be completely replicated with no loss of sequence information during cell division, and proliferation can continue without activating this check point ***(9)***. Therefore, by maintaining the integrity of telomeres, telomerase plays an important role in unlimited cellular proliferation.

The conventional method for detecting telomerase activity ***(10)*** required extracts prepared by hypotonic lysis of large numbers of cells (>10^7), large amounts of radionucleotides, and long autoradiographic exposure times to detect a weak signal. Detection of telomerase activity was improved several years ago by the development of a PCR-based assay ***(11)*** called the Telomeric Repeat Amplification Protocol (TRAP). The TRAP assay utilizes a detergent lysis method for cell extract preparation, and PCR amplification of telomerase-generated products, which is both faster and more sensitive than the conven-

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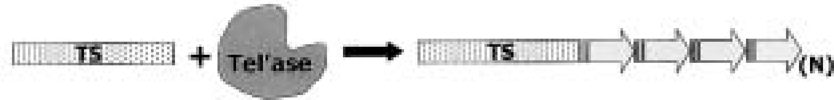
Step 1: Generation of telomerase products



Step 2: PCR amplification

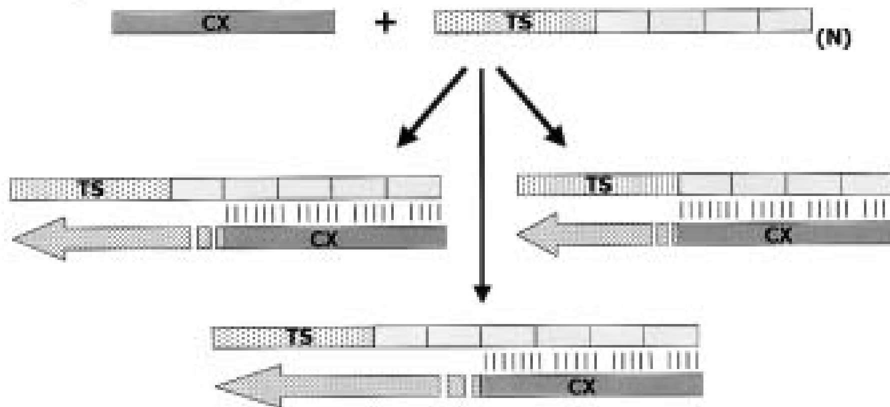


Fig. 1. Schematic of the TRAP assay. In Step 1, telomerase extends oligonucleotide TS by adding variable numbers of telomeric repeats (N). In Step 2, the oligonucleotide CX is added under hot start conditions, and anneals in staggered registers to the telomerase-generated products. Taq polymerase then synthesizes the second strand, and the different products lengths are visualized by separation in polyacrylamide gels.

tional assay. The detergent lysis method allows smaller numbers of cells (>10^4) to be used in making the extract and standardizes the extraction process. The standard TRAP assay is composed of two steps, which are carried out in a single reaction tube (**Fig. 1**). First, telomerase recognizes a single-stranded substrate oligonucleotide (TS) and extends it by adding templated telomeric sequences onto the 3′ end. Second, the oligonucleotide CX is added to the reaction under hot start conditions and serves as a reverse primer for PCR amplification of the telomerase-generated products. The unextended TS oligonucleotides serve as the forward primer for further PCR amplification. A radioactively labeled dNTP is included in the reaction to visualize the PCR products after nondenaturing gel electrophoresis.

A modification of this TRAP assay was made to allow even smaller numbers of cells (≥1) to be tested for telomerase activity ***(12)***. Using this method, a cell sorter is used to isolate the desired numbers of cells and deposit them directly

into a TRAP preparatory buffer containing a higher concentration of detergent, in which the cells are lysed, and telomerase extends the TS oligonucleotide. A different reverse primer (ACX) is end-labeled to increase the specificity and sensitivity of detection of the products and used in the PCR amplification step. The products are then analyzed by nondenaturing gel electrophoresis as in the standard TRAP assay.

The standard TRAP method described in this chapter was used to assess the role of telomerase in the expansion of human and rodent neural cells in culture ***(15)***. Telomerase was found to be expressed at low levels in cortical cell cultures, and activity decreased when the cells showed diminished proliferative capacity ***(15)***. Such studies aimed at discovering the molecular mechanisms involved in neural cell growth and differentiation in vitro will contribute to the understanding of cell renewal and proliferation in vivo, and thus have implications for the role of these cells in possible stem cell therapy.

2. Materials

2.1. Contamination Prevention and Precautions

1. Personal protective gear (gloves, lab coat, disposable sterile sleeves, and surgical mask).
2. Diethyl pyrocarbonate (DEPC, Sigma, St. Louis, MO USA)-treated H_2O.
3. Separate set of pipetmen and aerosol resistant tips.
4. PCR work stations (Research Products International Corp, Mount Prospect, IL, USA).

2.2. Cell Lysate Preparation for the Standard TRAP Assay

1. Phosphate-buffered saline (PBS), Ca- and Mg-free.
2. CHAPS lysis buffer: A 10 mL stock can be prepared as follows, aliquoted into 1 mL portions, and stored at 4°C for several months. All stock solutions listed below should be made with DEPC-treated H_2O. Mix 100 µL of 1 *M* Tris-HCl, pH 7.5, 10 µL of 1 *M* $MgCl_2$, 20 µL of 0.5 *M* EGTA, 500 µL of 10% (w/v) 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS, Pierce Chemical Co, Rockford, IL, USA), 1 mL of 100% sterile glycerol, and 8.36 mL of DEPC-treated H_2O. Before using a 1 mL aliquot, add 1 µL of 0.1 *M* phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis, MO, USA, dissolved in 100% ethanol and stored at –20°C) and 0.35 µL of 14.4 *M* β-mercaptoethanol (BioRad, Hercules, CA, USA). Final concentrations: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* $MgCl_2$, 1 m*M* EGTA, 0.5% CHAPS, 10% glycerol, 0.1 m*M* PMSF, 5 m*M* β-mercaptoethanol.
3. Protein Assay Dye Reagent (BioRad, Hercules, CA, USA).
4. Spectrophotometer.
5. Disposable 1 mL plastic cuvets.
6. Bovine serum albumin (BSA) (Sigma), dissolved in distilled H_2O at 1 mg/mL.

2.3. Standard TRAP Assay

1. Oligonucleotide TS (5′-AATCCGTCGAGCAGAGTT-3′), HPLC-purified and resuspended in 10 m*M* Tris-HCl, pH 7.5, at 0.1 μg/μL. Aliquot small amounts into Eppendorf tubes, flash freeze in liquid nitrogen or on dry ice, and store at –20°C. Do not freeze-thaw more than 5X.
2. Oligonucleotide CX (5′-$[CCCTTA]_3$CCCTAA-3′), HPLC-purified and resuspended in 10 m*M* Tris, pH 7.5, at 0.1 μg/μL. Aliquot small amounts into Eppendorf tubes, flash freeze in liquid nitrogen or on dry ice, and store at –20°C. Do not freeze-thaw more than 5X.
3. DNase-free RNase (Boehringer Mannheim, Mannheim, Germany).
4. Deoxynucleotide mix: Combine equal amounts of 10 mM dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim, Mannheim, Germany) to give a final concentration of 2.5 m*M* of each dNTP. Aliquot small amounts into Eppendorf tubes, flash freeze in liquid nitrogen or on dry ice, and store at –20°C. Do not freeze-thaw more than 5X.
5. Taq Polymerase, 1 U/μL (Boehringer Mannheim, Mannheim, Germany).
6. 10X TRAP buffer: A 10 mL stock can be prepared as follows, aliquoted into 1 mL portions, and stored at –20°C. All stock solutions listed below should be made with DEPC-treated H_2O. Mix 2 mL of 1 *M* Tris-HCl, pH 8.3, 150 μL of 1 *M* $MgCl_2$, 6.3 mL of 1 *M* KCl, 1 mL of 0.1 *M* EGTA, 50 μL of Tween 20 (Boehringer Mannheim, Mannheim, Germany), and 500 μL of 20 mg/mL BSA. Final concentration in 10X buffer: 200 m*M* Tris-HCl, pH 8.3, 15 m*M* $MgCl_2$, 630 m*M* KCl, 10 m*M* EGTA, 0.05% Tween 20, 1 mg/mL BSA.
7. [α-^{32}P]dCTP, 10 μCi/μL, 3000 Ci/mmol (Amersham Corp., Arlington Heights, IL, USA).
8. Thermal cycler and 0.5 mL thin-walled PCR tubes.
9. Polyacrylamide vertical gel apparatus, 17 × 14 cm, 1.5 mm spacers.
10. 10X TBE solution.
11. 40% acrylamide/ 2% bis-acrylamide solution (Boehringer Mannheim, Mannheim, Germany).
12. TEMED.
13. 10% ammonium persulfate solution.
14. Nondenaturing loading dye: 50% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol FF in 1X TBE.
15. Gel dryer.
16. PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

2.4. TRAP Assay for Single Cells or Small Numbers of Cells

1. FACS (Becton Dickinson).
2. Oligonucleotide TS (5′-AATCCGTCGAGCAGAGTT-3′), HPLC-purified and resuspended in 10 m*M* Tris-HCl, pH 7.5, at 0.1 μg/μL. Aliquot small amounts into Eppendorf tubes, flash freeze in liquid nitrogen or on dry ice, and store at –20°C. Do not freeze-thaw more than 5X.

needed for the TRAP assay should be used solely for the TRAP assay and should be kept separately.

3. A set of pipetmen should be designated for TRAP usage and should be cleaned regularly with 10% bleach. The use of aerosol resistant pipet tips is strongly recommended.
4. It is advisable to use physically separate areas to perform the three stages of TRAP analysis (lysate preparation, reaction set up, and product analysis). Commercially available PCR workstations can be used, if desired, and regular cleaning of all areas with 10% bleach is recommended. Contamination from RNases and PCR products can occur from any materials shared between areas, including the investigator, tip boxes, pipets, tubes, racks, solutions, etc., so care should be taken to keep materials separate.

3.2. Cell Lysate Preparation for Standard TRAP Assay

1. The isolation protocols of various neural cells are described in detail in Part I of this book. Once the cells of interest have been obtained, they may be frozen and stored for later analysis as long as **steps 2** and **3** are performed first.
2. Rinse isolated cells in PBS to remove any culture medium or tissue fluids, and pellet the cells in a microcentrifuge at 5,000*g* for 5 min. Remove supernatant and resuspend cells in fresh PBS.
3. Count the number of cells. In general, 10^6 cells are used per sample (*see* **Note 1**). Pellet the appropriate number of cells in a 1.5 mL Eppendorf tube at 5,000*g* for 5 min, and remove all traces of the supernatant. At this point, the cells may be frozen in liquid nitrogen and stored at –80°C, if desired.
4. Re-suspend the cell pellet by repeated pipeting in ice-cold CHAPS lysis buffer using 50 μL per 10^6 cells (*see* **Note 2**), and incubate samples on ice for 30 min.
5. Centrifuge samples at 15,000*g* for 30 min at 4°C.
6. Carefully remove supernatant making sure not to disturb the cell pellet, and place lysate in a fresh Eppendorf tube. Reserve 2 μL from each sample for measuring the amount of protein. Extracts may be frozen in liquid nitrogen and stored at –80°C for at least a year without loss of telomerase activity.
7. Use the BioRad Protein Assay solution to quantitate the amount of protein in each extract. Prepare a standard dilution series using CHAPS lysis buffer and 0, 1, 2.5, 5, 10, 15, and 20 μg of BSA in a total volume of 50 μL. Mix 48 μL of CHAPS lysis buffer with the reserved 2 μL of extract. Add 1 mL of 1:5 diluted BioRad Protein Assay solution to each standard or sample, vortex, and incubate 5 min at room temperature. Place in 1 mL plastic disposable cuvettes and read absorbance at 595. Plot a standard curve (A_{595} versus μg of BSA) and determine the regression line formula. Calculate the concentration of each extract. Typically, extracts contain between 1 and 10 μg/μL. Dilute extracts with CHAPS lysis buffer to 1 μg/μL.

3.3. Standard TRAP Assay

Several controls should be included with each experiment to ensure the specificity and accuracy of the assay. Because telomerase is an RNP, pretreatment of an aliquot of the CHAPS extracts with RNase will destroy the internal template RNA of the telomerase enzyme and thus prevent the formation of telomerase-generated products during the TRAP reaction ***(11)***. Samples should always be tested with and without pretreatment with RNase. In addition, an aliquot of CHAPS lysis buffer in place of extract should be tested as a second negative control. Finally, an extract should be made from a telomerase-containing cell line and tested as below as a positive control for the assay conditions. A dilution series (e.g., 5 μg, 1 μg, 0.2 μg of total protein) from this cell line extract can be used as a rough estimate of the amount of telomerase activity in the neural cell samples.

1. Thaw all reagents and extracts on ice.
2. For each reaction, use the following volumes of reagents: 5 μL of 10X TRAP buffer, 1 μL of 2.5 m*M* dNTPs, 1 μL of 0.1 μg/μL TS oligo, 1 μL of 1U/μL Taq polymerase, 0.4 μL of [α-^{32}P]dCTP (10 μCi/μL, 3000 Ci/mmol), and 35.6 μL of depc-treated H_2O. It is convenient and advisable to make one mixture of the reagents for the appropriate number of samples and then aliquot 44 μL per 0.5 mL PCR tubes. (Final concentrations or amounts: 1X TRAP buffer, 50 μ*M* dNTPs, 100 ng TS oligo, 1U Taq polymerase, 4 μCi of [α-^{32}P]dCTP) (*see* **Note 3**).
3. Pretreat 5 μg (5 μL) of each extract in a separate tube with 0.5 μL (5U) of DNase-free RNase for 5 min at room temperature.
4. Add 5 μg (5 μL) of each RNase-treated or 5 μg (5 μL) of each untreated extract, or 5 μL of lysis buffer or cell line extract, individually to the PCR tubes containing the 44 μL of reaction mix (*see* **Note 4**).
5. Incubate for 15 min at room temperature (*see* **Note 5**). While incubating, layer 75 μL of mineral oil carefully on top of the solution in each tube.
6. Transfer the tubes to a thermal cycler and heat for 10 min at 94°C (*see* **Note 6**).
7. Keeping the heating block at 94°C, remove one tube at a time to add 1 μL of 0.1 μg/μL CX primer (*see* **Note 7**) to the aqueous layer, and return tube to the heating block.
8. Set the PCR machine to run for 27 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec (*see* **Note 8**). Let the last step of the last cycle run for 10 min at 72°C. The reactions may be stored at –20°C until analyzed.
9. Add 5 μL of non-denaturing loading dye to each sample and mix, being careful to avoid disturbing the mineral oil. About half of each reaction volume (25 μL) should be analyzed by electrophoresis in 0.6X TBE on 10% polyacrylamide

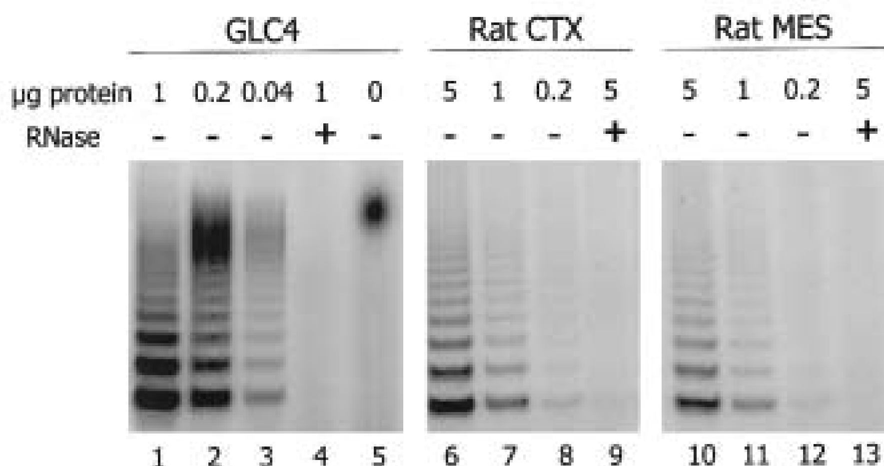


Fig. 2. TRAP assay of rat neural cells. Neural cells isolated from rat cortex and rat mesencephalon and grown in culture ***(15)*** (generously provided by Clive Svendsen, University of Wisconsin-Madison) were assayed by TRAP analysis as described. Lanes 1 to 4, telomerase-positive control lung tumor cell line GLC4; lane 5, CHAPS lysis buffer negative control; lanes 6 to 9, rat cortex; lanes 10 to 13, rat mesencephalon. The µg amount of total protein extract tested is shown above each lane. RNase, + and – indicate whether the extract was pretreated with RNase to destroy telomerase activity.

non-denaturing gels at 450 V for 45 min or until the bromophenol blue is at the bottom of the gel. Save the remainder of the reaction at –20°C for additional gel analysis if necessary.

10. Dry the gel on Whatman paper and expose to a PhosphorImager plate. After visualization using ImageQuant software, the products appear as a ladder of bands with a 6 base pair periodicity, beginning with the smallest product band of about 40 bp (*see* **Notes 7** and **9**). An example of a typical TRAP assay of rat neural cells is shown in **Fig. 2**.

This method describes an assay to simply and reliably detect the presence or absence of telomerase activity in neural cells. A protocol for quantitation of the relative amount of telomerase activity in each sample has been described in detail previously ***(13)*** and will not be repeated here.

3.4. TRAP Assay for Single Cells or Small Numbers of Cells

1. Thaw all reagents on ice.
2. For each reaction, use the following volumes of reagents: 12.5 µL of 2X Preparatory buffer, 0.5 µL of 2.5 m*M* dNTPs, 1 µL of 0.1 µg/µL TS oligo, and 11 µL of depc-treated H_2O. It is convenient and advisable to make one mixture of

the reagents for the appropriate number of samples and then aliquot 25 μL per MicroAmp tube. (Final concentrations or amounts: 1X Preparatory buffer, 50 μm dNTPs, 100 ng TS oligo) (*see* **Note 10**).

3. Set up at least six MicroAmp tubes for any desired number of cells and add 1 μL of RNase to three of the tubes. Use a FACS to obtain the exact numbers of desired cells and sort directly into the prepared tubes. Prepare several tubes extra and add 5 μL of sorting buffer without cells to carry through the assay as negative controls.
4. Incubate tubes for 60 min at 30°C in the Perkin Elmer 9600 thermal cycler, or water bath. This allows the cells to lyse and telomerase to extend oligo TS.
5. Meanwhile, label the ACX oligonucleotide as follows: Combine 10 μL of 0.1 μg/μL ACX oligo, 10 μL of 10X PNK buffer, 4 μL of 10 U/μL PNK, 10 μL of [γ-^{32}P]ATP (10 μCi/μL, 3000 Ci/mmol), and 66 μL of DEPC-treated H_2O. Incubate at 37°C for 30 min and heat inactivate for 15 min at 65°C. Store on ice until use. This reaction labels enough ACX oligo for 10 assays. Set up more reactions as necessary to obtain the appropriate amount of labeled ACX oligo (*see* **Note 11**).
6. At the end of the 60 min incubation from **step 4**, add an additional 12.5 μL of 2X Preparatory Buffer, 2 μL of 1 U/μL Taq DNA polymerase, and 10 μL of end-labeled ACX reverse primer to the reaction tubes.
7. Set the tubes in the PCR machine and set it to run for 32 cycles of 94°C for 30 seconds and 60°C for 30 sec. No mineral oil is needed since the Perkin Elmer 9600 PCR machine has a heated cover. The reactions may be stored at –20°C until analyzed.
8. Add 5 μL of non-denaturing loading dye to each sample and mix. About half of each reaction volume (25 μL) should be analyzed by electrophoresis in 0.6X TBE on 10% polyacrylamide non-denaturing gels at 450 V for 45 min or until the bromophenol blue is at the bottom of the gel. Save the remainder of the reaction at –20°C for additional gel analysis if necessary.
9. Dry the gel on Whatman paper and expose to a PhosphorImager plate. After visualization using ImageQuant software, the products appear as a ladder of bands with a 6 base pair periodicity, beginning with the smallest product band of about 50 bp (*see* **Note 12**).

4. Notes

1. In immortal tumor cell lines where telomerase activity is plentiful, as little as 10^4 cells may be used to make the extract ***(11)***. In human neural cells, the level of telomerase activity is low ***(15)***, and 10^6 cells should be the standard number of cells used to make the extract.
2. The amount of CHAPS lysis buffer used to make the cell extract can vary between 20 μL and 2000 μL per 10^6 cells ***(11,14)***. The optimal volume for a given cell type should be determined empirically.
3. The amount of [α-^{32}P]dCTP used can be increased to increase sensitivity, if desired.

4. The amount of protein assayed can be adjusted up or down as necessary. Testing a range of proteins amounts, beginning with 5 μg and decreasing by 5-fold (5, 1, 0.2 0.04 μg) is recommended to establish the appropriate concentration for the cell type of interest.
5. The incubation time at room temperature for the telomerase portion of the reaction can be lengthened to increase the sensitivity of the assay.
6. Heating the extract to 94°C will inactivate the telomerase activity ***(11)***.
7. Recent improvements in the TRAP assay relieve the necessity of performing the PCR amplification under stringent hot start conditions to prevent primer dimer formation ***(13)***. An anchored return primer, called ACX (5′-GCGCGG [CTTACC]$_3$CTAACC-3′), was developed which has a 6 nucleotide sequence added to the 5′-end and a new permutation of the complementary telomeric sequence ***(13)***. This primer can replace oligo CX in the reaction, if desired. Telomerase product bands generated using ACX are 50 bp and longer. Another advantage to using ACX is that if rare primer dimers form, the artifacts can be distinguished from the genuine telomerase product bands since they lack the normal 6 nucleotide periodicity ***(13)***. Finally, because the 3′-anchor of ACX prevents elongation of telomerase products during PCR amplification, the length of the PCR products produced will accurately reflect the processivity of telomerase. However, if hot start conditions are utilized, oligo CX gives completely satisfactory results and is shorter and thus cheaper to synthesize.
8. The number of PCR cycles may be increased to increase the sensitivity of the assay.
9. If no product bands are observed in the positive control lanes, there could be several problems. First, make sure that all reagents were included in the assay and that the protocol was followed. Second, check that the Taq polymerase is active by performing a control PCR with primers and a template that have been shown to give amplified products previously. If the Taq polymerase is active, contamination by RNases is a likely problem and new solutions and extracts should be prepared. If product bands are visible in the CHAPS lysis buffer control lanes, primer dimer formation or PCR product contamination may have occurred. Repeat experiment using new solutions and extracts. If RNase control lane shows product bands, check to make sure that the RNase is still active. If the RNase is active, primer dimers may have formed or the reaction or solutions were contaminated with PCR products. Repeat experiment with new reagents.
10. The elevated concentration of Tween 20 in the preparatory reaction (0.5% as opposed to 0.005% in the TRAP assay conditions) was sufficient to lyse the cells, allowing telomerase, if present, to extend the TS sequences ***(12)***. The concentration of BSA is also 10-fold more than in the TRAP assay conditions to provide enough protein to stabilize the telomerase activity.
11. The end-labeling reaction was designed to ensure maximum efficiency of ACX labeling to enhance the sensitivity of detection of amplified de novo telomere repeats ***(12)***.

12. The single cell assay is more prone to artifact formation than the TRAP assay ***(12)***. It is recommended that if less than 10 cells are assayed per tube, at least 8 assays should be performed so that statistical significance can be tested.

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14

In Vitro Assays for Neural Stem Cell Differentiation

Marcel M. Daadi

1. Introduction

Neural stem cells (NSCs) are an undifferentiated population of cells residing in the tissue lining the ventricular system of both the embryonic and adult mammalian central nervous system (CNS) ***(1–3)***. When isolated under the appropriate genetic or epigenetic conditions, NSCs have the ability to self renew and produce clinical amounts of cells that may be well characterized in vitro and in vivo, cryopreserved, or ready for therapeutic use. Upon transplantation into the adult CNS, NSCs integrate seamlessly within the host tissue and differentiate according to the local microenvironment into any one of the principal CNS cell types (neurons, astrocytes, or oligodendrocytes). These attributes make NSCs a good source of cells for cell replacement therapy for neurological disorders. However, this source of neural tissue will not reach the clinical stage unless it is well characterized both in vitro and in animal models.

As we continuously perpetuate NSCs in vitro, one or more of their properties may change. Therefore, in order to ensure product consistency, it is necessary to cryopreserve and characterize a reference stock of cells. Many different assays and techniques may be used to define and characterize NSCs. There is an extensive list of commercially available markers that may be used to characterize NSCs by means of simple immunocytochemistry or fluorescence-activated cell sorting. Other techniques include microarray technology to establish gene or protein expression profiles that may also be used to identify the NSC line. Together, these assays will ensure that the cells are stable and maintain the desired characteristics. Functional assays may be used to further define the NSCs and determine suitability for their intended purpose. Ultimately, these assays will correlate with the animal model data to support the intended therapeutic use.

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We have been using different bioassays to test whether or not a specific NSC line has the potential to express different neurotransmitter phenotypes. Particularly, we investigated the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in the synthesis of the neurotransmitter dopamine, as a functional correlative marker. Based on the outcome of this assay and the in vivo transplantation studies, the NSC line is either selected or not for potential use in Parkinson's disease cell replacement therapy.

2. Materials

1. Medium for culture of NSCs: The culture medium is a serum free medium composed of 1 : 1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and F12 nutrient supplemented with hormone mixture. The media components are: DMEM (Gibco, cat. no. 430-2100), Ham's F12 nutrient (Gibco, cat. no. 430-1700), Glutamine (Gibco, cat. no. 320-5030), Insulin (Sigma, cat. no. I-5500), Putrescine (Sigma, cat. no. P-7505), Progesterone (Sigma, cat. no. P-6149), Sodium Bicarbonate (Sigma, cat. no. S-5761), Glucose (Sigma, cat. no. G-8270), Transferrin (Sigma, cat. no. T-2252), HEPES buffer (Sigma, cat. no. H-3375), Selenium (Sigma, cat. no. SQ-133), epidermal growth factor (EGF, Collaborative Research, cat. no. 01-40001), and basic fibroblastic growth factor (bFGF, Upstate Biotechnology, cat. no. 01-106).
2. Cell-culture plasticware VWR (Brisbane, CA, USA).
3. Medium for culture of astroglial cell layer: DMEM : F12 culture medium supplemented with 10% (v/v) fetal bovine serum was used to establish astrocyte culture. For the astroglial culture, the dissection and preparation protocol we used is similar to the one reported by Cole and de Vellis ***(4)***.
4. Antibodies for characterization of the stem cell progeny:
   a. Polyclonal anti-microtubule associated protein-2 (anti-MAP-2, 1 : 100, Boehringer Mannheim)
   b. Monoclonal anti-β-tubulin (type III, 1 : 1000, Sigma).
   c. Polyclonal antibody against GFAP (1 : 100, Sigma).
   d. Polyclonal anti-galactocerebroside (anti-GC, 1 : 1000, Chemicon).
   e. Monoclonal anti-bromodeoxyuridine (BrdU, 1 : 5, Amersham).
   f. Polyclonal anti-γ-amino-butyric acid (GABA, 1 : 5000, Sigma).
   g. Polyclonal anti-tyrosine hydroxylase (1 : 1000, Pel-Freez).
   h. Polyclonal antisera to: Neuropeptide Y (anti-NPY, 1 : 500), somatostatin (anti-Som, 1 : 500), substance-P (anti-Sub P, 1 : 100), and methionine-enkephalin (anti-Met-Enk, 1 : 100), glutamate (anti-Glu, 1 : 500) were from Diasorin Inc.
   i. Secondary antibodies raised in goat against mouse and rabbit immunoglobulins, conjugated to the fluorophore rhodamine isothiocyanate (RITC, 1 : 200) or fluorescein isothiocyanate (FITC, 1 : 100) (Jackson Immunochemicals).
5. DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride) (Roche Molecular Biochemicals).
6. FluorSave Reagent (Calbiochem).

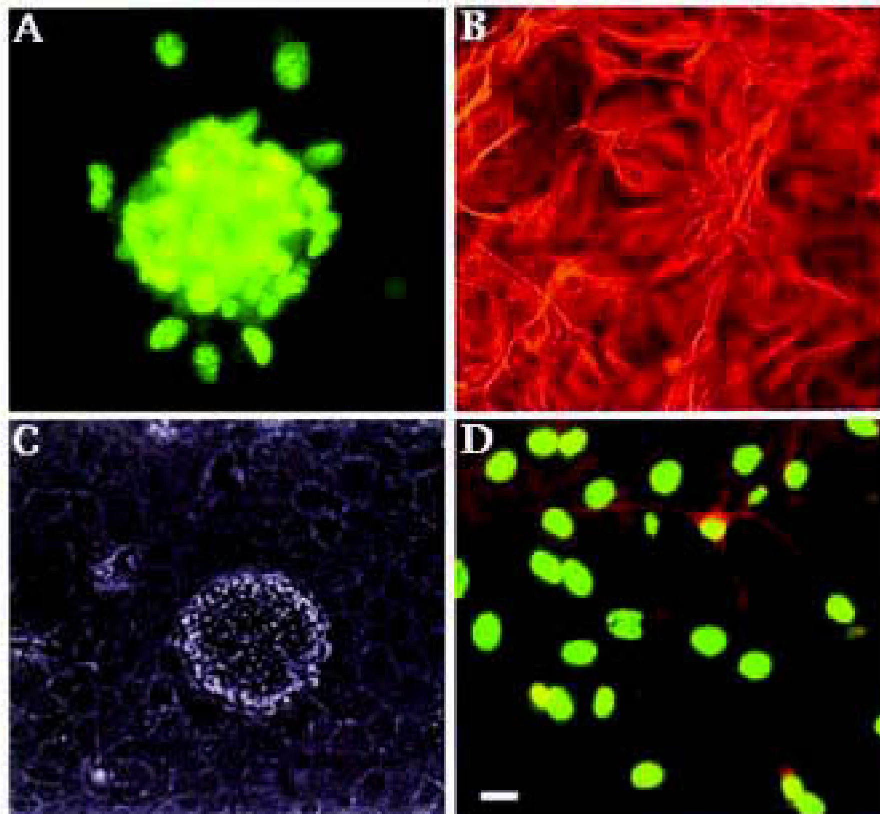

Fig. 1. Co-culture of NSCs with astroglia: **(A)** The majority of NSC progeny incorporated BrDU after 24 h exposure. **(B)** An example of an astroglial monolayer immunostained with GFAP and ready for co-culture. **(C)** Photo shows co-culture of NSC progeny with the astroglial monolayer. **(D)** Photo shows stem cell progeny that are BrdU immunoreactive (green), dispersed on the top of the astroglia and expressing mature neurotransmitter phenotype (Substance-P, red) after 7 d in vitro. The bar represents 20 μm in **A**, **B**, **D**, and 30 μm in **C**.

3. Methods

3.1. Isolation of Neural Stem Cells

The isolation of stem cells from the nervous system is described in detail in the first section of this book and will not be elaborated on in this chapter. However, it is worthy of mention that according to the media components and growth factors used, specific cell populations will be selected with well-defined characteristics acquired throughout serial passaging (*see* **Note 1**). We use chemically defined medium (**Table 1**) containing mitogenic growth factors

astroglia, or treated them with glial-derived conditioned medium (CM) to test for the induction of new neurotransmitter phenotype in the neuronal population. To ensure consistency and stability of the stem cell line, this procedure is systematically carried out at each passage and the numbers of neurons, astrocytes, oligoendrocytes, and neuronal subpopulations expressing specific neurotransmitter phenotype are measured.

3.2.1. Co-culture of Neural Stem Cells with Astrocytes

As mentioned above, we have been utilizing the direct interaction with astroglial microenvironment to test for the potential of NSC to express new neurotransmitter phenotypes. The isolation of pure astrocytic cultures is carried out as described by Cole and deVellis ***(4)***. Striatal tissue derived from post-natal d 1 mice is used to generate astrocyte cultures. After mechanical dissociation, the cells are suspended in DMEM/F12 media supplemented with 10% fetal bovine serum (FBS), and plated at a concentration of 1.25×10^4 cells/cm^2 culture flask. Once a confluent monolayer is established, cells are enzymatically dissociated (Trypsin-EDTA) and plated at 200,000 cells/cm^2 onto poly-L-ornithine-coated glass coverslips in 24-well culture dishes. After 3–4 d, cell confluency is established. NSCs are then resuspended in fresh medium and plated on the glial cell layer. To be able to distinguish NSC progeny from the astroglial cells, we labeled them with the DNA replication marker, BrdU, in the S-phase (*see* **Note 4**). BrdU is added at a concentration of 1 µ*M* to 6 d NSC culture for 24 h. Clonally derived NSCs (in the shape of sphere of cells) are then washed free from mitogens and BrdU prior to co-culture with astrocytes.

After 7 d in vitro, cultures are processed for single or double immunocytochemistry (for BrdU and each of the neurotransmitter antibody listed in **Subheading 3.**) as follows:

1. All the coverslips are fixed with 4% paraformaldehyde (with 0.1% glutaraldehyde for anti-GABA or glutamate) for 30 min.
2. Wash for 10 min, 3X with PBS.
3. Incubate in the primary antibodies in PBS/10% normal goat serum/0.3% Triton X-100 for 2 h at 37°C, except for anti-BrdU (*see* **Note 5**).
4. Wash for 10 min, 3X with PBS.
5. Apply rhodamine- and/or fluorescein-conjugated secondary antibodies prepared in PBS for 30 min at room temperature.
6. Wash coverslips 3X (10 min each) in PBS then rinse them with water and place them on glass slides.
7. Cover the slides with large coverslip using Fluorosave as the mounting medium.
8. The number of neurons, astrocytes, and oligodendrocytes is then determined for each NSC clone under a Nikon optiphot photomicroscope and their NSC origin is confirmed by the nuclear immunostaining of BrdU. New neurotransmitter

phenotype expression is systematically evaluated in all cultures and recorded for each cell passage.

3.2.2. Induction of TH Expression in the NSCs

This bioassay was used to test the potential of a stem cell line to adopt a dopaminergic fate ***(5)***. Thus, this assay constitutes a first selective criterion for a cell line of potential therapeutic use for Parkinson's disease.

1. Dissociated NSCs are suspended in control medium and plated at a density of 2.5×10^5 cells/cm^2 on PLO-coated glass coverslips in 24-well Nunclon culture dishes with 0.5 mL/well.
2. After 2 h, cultures are treated with the appropriate differentiation inducing agents: bFGF, 20ng/mL + 75% (v/v) glial conditioned medium (*see* **Note 5**).
3. Twenty four h later, cultures are fixed and investigated for induction of TH gene expression using the immunocytochemical technique, described above.
4. Cultures are also left for 7 d in vitro (DIV) and half of the culture medium is replaced after 3 and 5 DIV before processing for TH immunocytochemistry.
5. The total number of live cell nuclei stained with DAPI and the percentage of TH immunoreactive neurons is determined for each culture at each passage.

4. Notes

1. The growth medium is critical in the development of a stable NSC line. Variations may exist from lot to lot, due to something as simple as water purity. Thus, it is necessary to set up criteria for testing raw material, and for qualifying each of the media components (DMEM/F12, hormone mixture and growth factors) for their consistency in growth promotion.
2. Optimal growth medium is a perpetual endeavor. However, changes in cell line characteristics may be due to cross-contamination with other cell lines, to sterility, or frequently, to mycoplasma contamination. Therefore, cell lines should be routinely karyotyped or subjected to isoenzyme analysis for species identity, and tested for mycoplasma. Kits are available and several companies offer testing services for reasonable prices. The use of the fluorescent dye DAPI (Roche, cat. no. 236276) is the simplest and quickest way to test for mycoplasma contamination.
3. Plating and adherence properties of dissociated neural stem cells may be inconsistent on PLO-coated glass coverslip, possibly due to coverslip quality, coating process, bad batch of coating substrate, bad plates, mycoplasma infection, and so forth. Coverslips may float after adding PLO to the well. Pushing them with a pipet to the bottom of the well will ensure that the entire surface of the coverslip is covered with PLO. We have experienced consistent results with Nalge Nunc glass coverslips (cat. no. 174950).
4. When using BrdU in the last 24 h of NSC culture, there may be a post-mitotic subpopulation of NSC progeny that will not incorporate the DNA marker, and thus be undetectable after co-culture. To overcome this problem, BrdU may be

added earlier to 2–4 d NSC cultures. Alternatively, neural stem cells may be derived from a different species allowing for the use of species-specific markers or from transgenic mice like the GFP and ROSA26 that ubiquitously express the markers green fluorescent protein and beta galactosidase respectively (The Jackson Laboratory, stock sumbers 003291 and 002292).

5. BrdU immunocytochemistry works best when the antibody is incubated overnight with no acid treatment.
6. The preparation of CM from glial cell lines is critical for successful induction of TH gene expression in stem cell progeny. Glial cell culture need to be more then 95% confluent and appearing as a healthy monolayer of cells, with no aggregate before beginning CM preparation. We use CM at 75% (v/v) of the total volume of plating medium.

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15

Electron Microscopy and Lac-Z Labeling

Bela Kosaras and Evan Snyder

1. Introduction

Knowing the morphology,
the physiology can be understood better,
because, after all, the morphology carries the function;
there is no function without structure at all.

The recognition of the potential use of stem cells in the body gives immense hope for the restoration of cell function lost to degenerative disease, injury, or genetic disorders. To prove that transplanted stem cells are actually surviving and functioning, it is necessary to label them for tracking with any of several detection methods.

A great advantage of molecular genetic technique is that it allows a selected gene or genes to be transferred into cells that previously did not express them. Transfer of such a foreign gene into, for example, neural stem cells in vitro allows the foreign gene's protein product to serve as a "reporter" that marks such neural stem cells when they are grafted into an unmarked host. Widely used "reporter" proteins are chloramphenicol acetyltransferase (CAT), luciferase (Luc), and especially β-galactosidase (β-Gal). A caveat is that such genes are subject to unpredictable down-regulation, to an extent that may limit their reliability as reporters.

The lac- operon of the bacterium *Escherichia coli* consists of three linearly positioned genes, *lac-Z*, *lac-Y*, and *lac-A* (**Fig. 1**). The *lac-Z* gene encodes the "reporter" enzyme protein, β-galactosidase, that cleaves off the terminal galactose residues that are β-1,4-linked to a monosaccharide, oligosaccharide, or glycopeptide.

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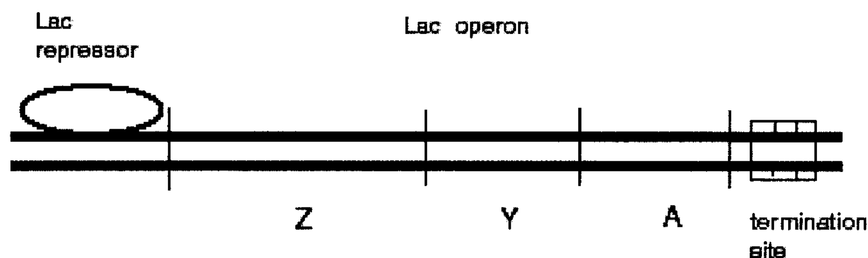

Fig. 1. The Lac operon of *Escherichia coli*, showing the three genes linearly, upstream the lac repressor, and downstream the termination site.

Purified potential transgenes are carried into foreign cell nuclei as components of specially constructed vectors derived by using plasmids ***(1–3)*** or lambda phages ***(4–12)***. A circular vector, constructed with the SV40 promoter and enhancer gene, an ampicillin cassette, and the *lac-Z* "reporter" gene, is diagrammed in **Fig. 2**.

The efficiency of uptake of the vector by foreign cells can be enhanced by any of several methods, including electroporation, or exposure to cationic lipids (lipofectin), calcium phosphate, or DEAE-dextran, depending on whether a transient or a stable transfection is the goal ***(1,2,13–15)***. The simplest method, though it gives a reasonably effective, stable transfer of the transgene, is to use calcium phosphate to transfect cells with the constructed retrovirus vector ***(1–3,16,17)***. The transfection of neural stem cells with the bacterial "reporter gene," *lac-Z*, enables us to detect and distinguish these cells from host cells in mammalian grafting experiments by using histochemical and immunohistochemical techniques.

An early breakthrough in enzyme histochemical methodology was the use of indigo dyes ***(22)*** for esterase detection in tissue sections. A detailed series of follow-up studies on esterase histochemistry by Holt and colleagues ***(23,24)*** led to the introduction of indolyl dyes as substrates that give an insoluble blue reaction product, indigo blue. Detection of β-galactosidase enzyme activity in tissue sections was demonstrated by Rutenburg et al. ***(25)*** using halogen-containing naphthol derivates, which also give insoluble reaction precipitates. These studies provided a solid background for Pearson et al. ***(26)***, who modified Holt's indolyl glycosidic chromogen substrate and found that 5-bromo-4-chloroindol-3-yl-β-D-galactopyranoside gave the best results for detection of mammalian β-galactosidase enzyme activity in histological sections.

The β-galactosidase enzyme cleaves the D-galactose from this substrate and the residual backbone, the indolyl portion, gets oxidized to indoxyl, which self-couples to form a reaction product that is a very fine precipitate with blue-

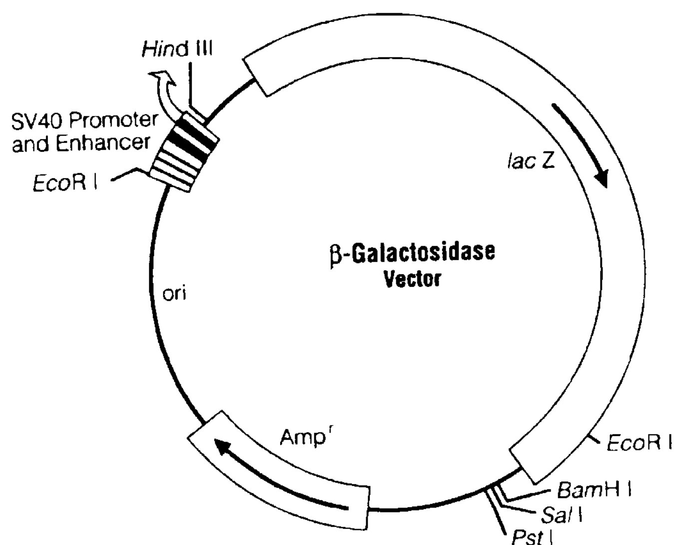

Fig. 2. The constructed vector that contains the *lac-z* "reporter" gene.

green indigo color, the indigo blue (*see* **Fig. 3**). The reaction product shows minimal diffusion from the sites of enzyme activity in tissue sections. Further useful modifications were introduced by Lojda ***(27)***. The β-galactosidase enzyme, besides its pH optimal requirement, needs $MgCl_2$ as an activator and co-factor in the incubation medium ***(28)***.

A ferro/ferricyanide mixture was used by Holt ***(29)*** to speed the oxidation of the indoxyl to indigo and contribute the deep blue color, Prussian blue ***(28,30,31)***. The addition of Prussian blue not only deepens the overall color intensity but also makes the complex more insoluble in water. The complex precipitate, with the ferro/ferri ions, has high scattering effect on an electron beam, and this electron opacity makes possible the detection of the β-galactosidase activity by transmission electron microscopy.

Still, this relatively simple method that is widely used for localizing the bacterial β-galactosidase enzyme as a marker in neural stem cells is subject to major errors that can lead to either underestimation or overestimation of the distribution and fate of grafted neural stem cells. Even in a laboratory that has considerable prior experience with the X-gal method, the technical steps should be reassessed whenever new personnel take over the techniques, the commercial manufacturers introduce changes (announced or unannounced) into their methods for preparing key reagents, a previously unfamiliar region of the nervous system is under investigation, or a new host species or source

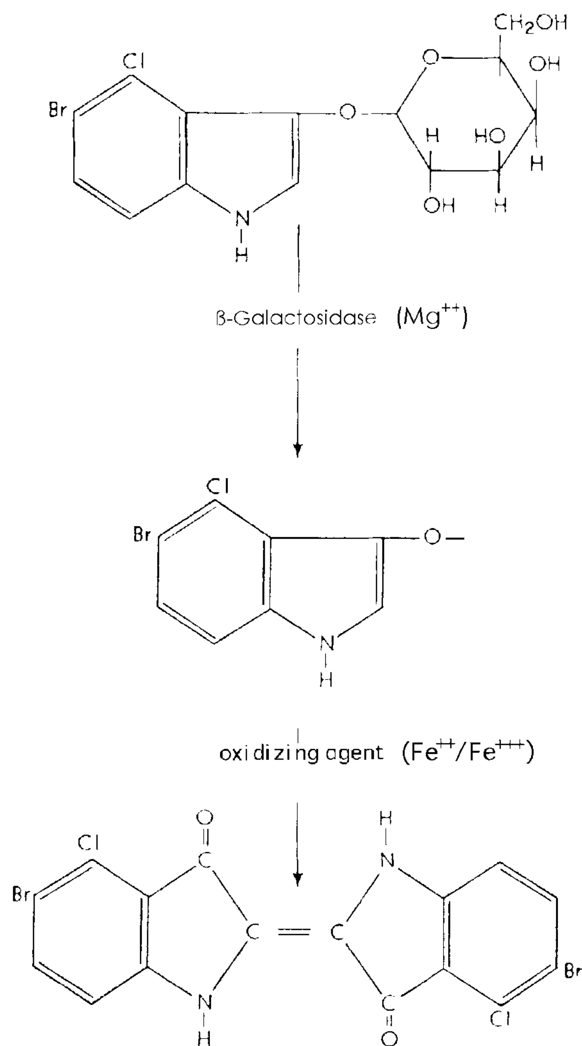

Fig. 3. The figure shows the substrate and the reaction product after β-galactosidase cleaves off the galactose and the two indoxyl moieties bind together through carbon-carbon bonds to form an insoluble colored product, indigo blue.

of neural stem cells is studied. Interpretations and conclusions should be based on consistent results obtained with several independent methods. For example, the bacterial β-galactosidase can also be visualized as a protein moiety by immunohistochemistry, independent of its enzymatic activity.

Under appropriate conditions, the immunoreaction is a highly specific reaction between antigen and antibody. The sites of such reactions can be visualized

microscopically by a number of different techniques (such as sandwich-DAB, and tag-labeling, mainly with fluorescent substrates). The β-galactosidase enzyme is readily detected by this approach ***(21,32,33)***. The great advantage, compared to the X-gal histochemical reaction, is that the immunoreaction detects the β-galactosidase protein itself, not its enzymatic activity, and, thus, is independent of the potential down-regulation of enzyme activity that may occur as grafted neural stem cells undergo various patterns of differentiation. Also, immunoreactivity is generally more sensitive than enzyme histochemistry. The use of diaminobenzidine (DAB) for visualizing the immunoreaction also allows this technique to be extended to the electron microscopic level, because the osmicated DAB produces electron dense precipitates ***(34)***.

Other agents can be introduced into neural stem cells prior to grafting them and can serve to distinguish donor cells from host cells. A widely used agent is bromodeoxyuridine, BrdU, incorporated into DNA of S-phase proliferating stem cells during their last round of cell division in vitro prior to grafting. BrdU is antigenic, and antibodies suitable for immunoreactivity are available to detect this DNA component by both light and electron microscope ***(35–38)***. The protocol for the BrdU immunohistochemistry is very similar to the β-galactosidase protocol; of course, the appropriate antibody must be used. Another important thing to consider is that BrdU is incorporated into the DNA double helix of the cell. In this case, the BrdU has been hidden from the access of the anti-BrdU antibody and likely the histone proteins also cover the molecules. To make these molecules accessible to the antibody, the DNA double helix should be denatured and single stranded ***(37–39)***.

2. Materials

2.1. Transfection of Cells with the "Reporter" Bacterial lac-Z Gene

It is self-explanatory that everything that comes into contact with the cells must be sterilized or handled under sterile conditions (sera, supplements of any kind, e. g., antibiotics, L-glutamine, trypsin, and so forth). The glassware should be handled separately and only used for tissue culture work, avoiding any toxic chemical contamination of the cells. The water is preferred to be bi-distilled.

1. Cell culture dishes (Corning cat no. 3001, 35 × 10 mm).
2. Retrovirus vector containing the *lac-Z* transgene.
3. Packaging cell line.
4. The target cells (neural stem cells or other cells intended to use).
5. Falcon tubes.
6. HBS (Hepes-buffered saline): 137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 6 m*M* dextrose, 21 m*M* Hepes, pH 7.05.

7. 2 *M* $CaCl_2$.
8. Pipets.
9. Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO).
10. Glycerol, autoclaved.
11. 10% calf serum, (GIBCO) + 100 U/mL of penicillin, 100 µg/mL of streptomycin, 2 m*M*/mL of L-glutamine.
12. Acrodisc filter, 0.45 µm (Gelman Sciences), product no. 4184.
13. Mitomycin C, Aldrich, cat. no. 85,549-9 or Sigma, cat. no. M 4287.
14. 2 µg/mL polybrene, Sigma cat. no. M 0503; Aldrich, cat. no. 10,768-9.
15. The polybrene stock solution is (800 µg/mL) is made up in bi-distilled water, filter sterilized, and stored at –20°C.
16. Geneticin (G418) (GIBCO).
17. Tissue culture incubator.
18. Tissue culture hood.

2.2. X-gal Histochemical Reaction

1. X-gal substrate (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside, Promega, cat. no. V3941). Dissolve X-gal substrate in *N*,*N*-dimethylformamide at 50 mg/mL concentration in glass covered with aluminum foil and store at –20°C. Use the X-gal substrate at 1 mg/mL concentration in the histochemical procedure.
2. Potassium ferrocyanide (Sigma).
3. Potassium ferricyanide (light sensitive, protect with foil).
4. 1.0 *M* $MgCl_2$ (Sigma).
5. 0.5 *M* EGTA (Sigma).
6. 10% desoxycholate (Sigma).
7. 10% NP-40 (Sigma).
8. 0.1 *M* PIPES buffer, pH 7.4 or PBS pH 7.6 (Sigma).
9. Rinse solution: PBS pH 7.6, 2 m*M* of EGTA, 2 m*M* of $MgCl_2$.
10. X-gal detergent solution: PBS pH 7.6, 2 m*M* EGTA, 2 m*M* $MgCl_2$, 0.01% Na-desoxycholate, 0.02% NP-40.
11. X-gal reagent solution: PBS pH 7.6, 5 m*M* K-ferrocyanide, 5 m*M* K-ferricyanide, X-gal substrate 1 mg/mL.
12. Foil.
13. Refrigerator.
14. Staining dishes.
15. Plastic forceps (metal forceps cannot be used in X-Gal reagent solution).

2.3. Immunohistochemistry

1. 4% paraformaldehyde in 0.1 *M* PBS, pH 7.4. It is made up from powder form. Heat the distilled water to 70°C (do not boil), then add 1 *N* NaOH drop by drop, while stirring, until clear. When solution is cool, add PBS. Paraformaldehyde is toxic and may be carcinogenic; therefore, prepare the solution under a hood and wear gloves and mask. Disposal should follow chemical safety rules.

2. 30% sucrose in 0.1 *M* PBS.
3. 0.1 *M* PBS, pH 7.4.
4. Dry ice.
5. OCT for encapsulating the sample for cryostat cutting.
6. Triton X-100.
7. 0.3% H_2O_2 (freshly made up from the concentrate solution). The concentrated solution is a strong oxidizer; wear gloves working with it.
8. 2 *N* HCl, the concentrated HCl is toxic and produces poisonous gas; wear mask and gloves while working with it under a chemical hood.
9. 0.1 *M* borate buffer, pH 8.3.
10. Fetal bovine serum (FBS) (GIBCO).
11. Donkey serum (DS) (Jackson ImmunoResearch Laboratories, Inc.).
12. Blocking solution: 10% FBS, 5% DS, 0.3% Triton X-100 in PBS.
13. Rabbit anti-β-galactosidase (polyclonal antibody) (Chemicon).
14. Mouse anti-BrdU (monoclonal) antibody (Roche).
15. AffiniPure Donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.).
16. AffiniPure Donkey anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.).
17. Vectastain Elite ABC Kit (Standard) (Vector).
18. DAB Substrate for Peroxidase (Vector). DAB is carcinogenic; wear gloves when working with it.
19. Neutral red for counterstaining.
20. Hematoxylin for counterstaining.
21. Ethanol dilution series for dehydration.
22. Xylene or Histoclear. Xylene is flammable and toxic; work with it under a chemical hood.
23. Mounting media (Cytoseal or Permount). Mounting media are usually xylene-based substances; work under a chemical hood wearing gloves and mask.

2.4. Tissue Preservation and Processing for EM Study

1. 2% paraformaldehyde (Electron Microscopy Sciences). Preparation as above (*see* **Subheading 2.3.**).
2. 2.5% glutaraldehyde, EM grade (Electron Microscopy Sciences) 25% glutaraldehyde in ampule, as stock solution. Store it in refrigerator at 4°C. It should be handled similar to paraformaldehyde.
3. 1% OsO_4. Make 4% OsO_4 stock solution in distilled water and store it in refrigerator at 4°C (Electron Microscopy Sciences). The OsO_4 is highly toxic, work with it under chemical hood. Store solution in a tightly sealed, double container in refrigerator. It is very volatile and can cause cornea damage.
4. 0.1 *M* phosphate buffer, pH 7.4.
5. Ethanol (Fisher).
6. Propylene oxide (Electron Microscopy Sciences). Propylene oxide is flammable and toxic; work with gloves under chemical hood.

7. Epon or Araldite (Electron Microscopy Sciences). Epon and Araldite are irritants and the accelerator component is toxic; use gloves and work under a chemical hood.
8. Uranyl acetate (Electron Microscopy Sciences). The uranyl acetate powder is depleted; however, it should be handled as radioactive (specific activity: 1.04–4.01 μCi/g). Waste should be stored and disposed of accordingly. Use gloves.
9. Perfusion pump.
10. Needles, gauge #18.
11. Tubes.
12. Glass strips (Ted Pella, Inc., or Electron Microscopy Sciences).
13. Knife maker (LKB Instruments Inc.).
14. Grids (Electron Microscopy Sciences).
15. Ultramicrotome (Reichert/Leica) or access to one.
16. Electron microscope (JEOL, ZEISS) or access to one.

3. Methods

3.1. Neural Stem Cell Labeling

Calcium phosphate precipitates DNA. This complex is sticky and adheres to the cell membrane. The presence of calcium promotes the hydrophobic interactions of the molecules. After the precipitate has attached to the cell membrane, it is engulfed by endocytosis into the cell. The transfected gene is integrated permanently and efficiently into the chromosome DNA. This protocol is based on Graham et al. ***(16)***, Parker and Stark ***(17)***, and Cepko ***(18)*** modifications.

3.1.1. Transfection for Viral Production

1. Plate packaging cells to about 10–20% confluency the day before of transfection.
2. In the laminar flow hood, place 10 μg of the retroviral vector DNA into 0.5 mL of filter-sterilized HBS and mix by pipeting (*see* **Note 1**).
3. Add 32 μL of 2 *M* $CaCl_2$ while gently shaking the tube. Tap the tube for about 30 sec.
4. Incubate at room temperature for 45 min. A fine blue precipitate should develop (*see* **Note 2**).
5. Remove medium from cells and gently add the HBS-DNA solution into the center of the dish.
6. After 20 min of exposure to the DNA, replace the culture medium and return the cells to the incubator.
7. After 4 h, remove the medium and add 2.5 mL of HBS containing 15% glycerol at room temperature.
8. Return the dish to the incubator for 3.5 min (*see* **Note 3**).
9. Remove glycerol-HBS quickly and rinse with 5 mL of DMEM.
10. Repeat rinse with DMEM and add 5 mL DMEM+10% calf serum.

11. Remove the medium 18–24 h later and filter through a 0.45 μm filter. The harvested virus can be stored at –80°C, though it can be used for an infection immediately. (It can also be concentrated and titered out on fibroblast before use.)
12. Add 5 mL of culture medium to the cells, and continue to cultivate for 1–2 d.
13. To obtain stable transfected producer clones, split transfected cells 1:20 or 1:40 into medium containing a selective drug (like G418).
14. Every 2 d refresh the selection medium.
15. One should see resistant colonies after 7–10 d of cultivation. Sub-cultivate well-isolated colonies after 10–14 d (use sterile disks or cylinders for isolation).
16. Assay the colonies for virus titer, or use another assay to determine the expression level.
17. When a good producer clone is identified, freeze a large stock of vials (*see* **Note 4**).
18. After a producer clone is chosen, some tests for the integrity of the viral genome should be performed on the infected cells (e.g., Southern or Northern blots).

3.1.2. Co-cultivation Method for Infection of the Target Cells

1. Plate virus producer cells (ψ2-BAG, CRIP-myc) and allow them to become nearly confluent (*see* **Note 5**).
2. It is often desirable to inhibit replication of producer cells so that cultures of infected target cells will not be contaminated with producer cells or virus after the initial co-cultivation. Cell division of producer cells can be blocked by 10 μg/mL Mitomycin C (*see* **Note 6**).
3. Target cells are plated onto producer cells and incubated for several days in the presence of 2 μg/mL polybrene (*see* **Note 7**).
4. If the cells are non-adherent, they can be washed off the producer monolayer and sub-cultivated in selective conditions (G418) (*see* **Note 8**).

3.2. Detection of Neural Stem Cells Labeled with the* lac-Z *Gene

3.2.1. Histochemical Reaction: Desired Properties

The inserted *lac-Z* gene, expressed in the neural stem cells, encodes the β-galactosidase enzyme. Reliable detection of the ß-galactosidase enzyme in histological sections is a simple histochemical procedure, though several problems must be overcome:

1. In addition to preserving the structure of the tissue, the enzyme activity of the β-galactosidase protein must be retained.
2. Diffusion of the enzyme to abnormal intracellular sites must be minimized.
3. A substrate must be chosen that will be converted by the enzyme into a strongly colored reaction product detectable by naked eye or by light microscope.
4. The reaction product should precipitate at the intracellular site of enzyme activity and should not diffuse subsequently from this site.

5. The reaction product should precipitate fine enough to indicate precisely the intracellular site of the enzyme protein.

3.2.2. Fixation for Light Microscopy

To prevent diffusion of the enzyme from grafted cells, we obtain the best results by flushing blood from the vessels by perfusion of phosphate-buffered saline (PBS), at room temperature, through the heart, followed immediately by perfusion of cold (4°C) fixative. The fixative of choice is 2% paraformaldehyde in 0.1 *M* PIPES buffer (*see* **Note 9**), containing 2.5 m*M* of $MgCl_2$ and 2.5 m*M* of EGTA ***(19,20)***. The fixative has a remarkable effect on the result of the X-gal reaction ***(21)***.

3.2.3. Fixation for Electron Microscopy

To obtain satisfactory structural preservation of the nervous tissue at the ultrastructural level, the fixative should contain glutaraldehyde and paraformaldehyde. Both reagents, but particularly glutaraldehyde, tend to increase the background of the X-gal reaction, so it is advisable to hold the glutaraldehyde at relatively low concentrations (1–2.5%). The buffer is 0.1 *M* PIPES buffer (*see* **Note 9**) containing 2.5 m*M* of $MgCl_2$ and 2.5 m*M* of EGTA, as recommended for light microscopy study ***(19,20)***.

3.2.4. X-Gal Enzyme Histochemical Reaction

The reaction can be performed on cryosections mounted on glass slides or on vibratome sections floating in the solutions. If the samples are to be used for electron microscopy study, to avoid the potential freezing artifacts we cut 100–200 μm thick vibratome sections and process them through the steps described below.

1. Put the sections in rinsing solution for 10 min.
2. Repeat the wash with rinsing solution for 10 min.
3. Put the sections in X-gal detergent solution for 10 min. (Sections used for EM study should avoid detergent treatment for better structural preservation.)
4. Prepare the X-gal reagent solution (*see* **Notes 10–13**) while the sections are in the detergent solution.
5. Incubate the sections in the X-gal reagent solution for 4 h in oven at 37°C. Check the sections for the blue reaction (intensely blue staining of cells) either by naked eye or by observing them under "wet" light microscope (*see* **Fig. 4**). If the reaction is present, it can be terminated by the next step. If the reaction product is weak, the incubation can be prolonged for a couple of hours more or overnight, but keep in mind that the chance of having nonspecific precipitation in the section is increased (*see* **Notes 15–17**).

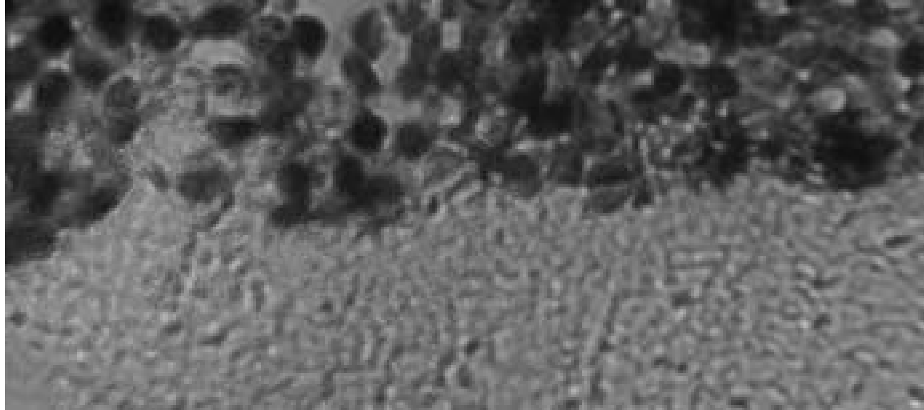

Fig. 4. The engrafted neural stem cells appear in deep blue color (here they shown in lighter and darker black color) after the X-Gal histochemical reaction. This big aggregate of cells can be noticed by the naked eye in the section.

6. Stop the reaction by washing the sections in rinsing solution for 3X 10 min each.
7. The sections can be counterstained with neutral red, if desired.
8. Dehydrate and clear sections.
9. If thick vibratome sections intended for EM study were put through **steps 1–7**, omit **steps 8** and **9**. Go to **Subheading 3.3.** to continue.

3.2.5. Protocol for Anti-β-Gal Immunoreaction

1. Air-dry cryosections at room temperature.
2. Wash out OCT with PBS for 10 min.
3. Incubate sections in 0.3% Triton X-100 in PBS for 30 min at room temperature.
4. Quench endogenous peroxidase activity in 0.3% H_2O_2 solution in PBS for 30 min at room temperature.
5. Incubate sections with blocking solution for 30 min at room temperature.
6. Incubate sections with the primary antibody, rabbit anti-β-galactosidase overnight at 4°C.
7. Wash sections with PBS, 4X for 10 min each.
8. Incubate sections with the secondary antibody, AffiniPure donkey anti-rabbit IgG, for 3 h at room temperature.
9. Wash sections with PBS, 4X for 10 min each.
10. Prepare the ABC solution and let it set for 30 min prior to use.
11. Incubate sections with ABC solution for 1 h at room temperature.
12. Wash with PBS three times for 10 min each.
13. Incubate sections with DAB solution 3–10 min by checking the reaction product under microscope.

14. Wash the sections thoroughly in PBS.
15. Neutral Red counterstaining can be applied.
16. Dehydrate sections.
17. Clear sections and mount with Cytoseal.

3.2.6. Immunohistochemical Reaction for BrdU

1. The sections should be treated with 2 *N* HCl for 1 h at room temperature.
2. To restore the pH of the sections, incubate them in 0.1 *M* of borate buffer (pH 8.3) twice for 10 min each.
3. Wash the sections with PBS twice for 10 min each.
4. Apply the primary antibody that is anti-BrdU and then follow the procedure as in **step 5** above.

If immunoreaction with DAB is detected, the sections can be processed for EM study as well (*see* **Subheadings 3.2.5.** and **3.3.1.**).

3.3. Processing Tissue for EM Examination

3.3.1. Postfixation and Embedding the Tissue

1. After performing the X-gal histochemical reaction on thick vibratome sections (*see* **Subheading 3.2.4., step 10**), examine and select appropriate sections to be processed for EM study.
2. Post-fix the thick sections (100–200 μm) with 1% osmium tetroxide in 0.1 *M* of phosphate buffer.
3. Stain with 1% uranyl acetate, followed by standard dehydration and embedding procedures in any of the commonly used embedding media (Araldite, Epon, and so forth) (*see* procedure in **Subheading 3.4.**, below). There are no restrictions among the standard embedding media with respect to possible deleterious effects on detection of the X-Gal reaction precipitate.

3.3.2. Cutting Ultrathin Sections

Conventional 1 μm thick sections are cut to orient the area of interest to trim the EM block for cutting ultrathin sections. The optimal environment for this procedure is a room of draft-free, dust-free, with minimal vibration tendency. The cutting is carried out by standard procedures: the thin sections are floated on water surface and collected on copper grids.

As discussed above (*see* **Subheading 3.2.4.**) the final precipitate of the X-Gal reaction is a Prussian blue complex containing a heavy metal (Fe^{2+}/Fe^{3+}). The Fe atoms confer electron density, and, therefore, the sections need no further contrast with either of the conventionally applied reagents, uranyl acetate or lead citrate. In fact, if uranyl acetate or lead citrate is applied to the sections, the X-Gal reaction product will become so masked as to be difficult to detect.

3.4. EM Procedure

1. Fixative: 2% paraformaldehyde and 1% glutaraldehyde in 0.1 *M* PIPES buffer at pH 7.4 with $MgCl_2$ (2 m*M*), EGTA (2 m*M*) and 4.1% sucrose. It is better to prepare the fixative immediately before use.
2. Perfusion: The deeply anesthetized animal (mouse) is perfused through the heart with buffer at room temperature to flush out the blood, followed with 100–200 mL of fixative at 4°C over about 20–30 min by perfusion pump. The head is fixed further by immersion in the same fixative overnight. Then, the brain is removed from the skull and washed in buffer.
3. The selected brain region is sectioned with a Vibratome at a thickness of 100–200 μm.
4. The thick sections are collected and processed through the X-Gal histochemical procedure by floating them as described in the X-Gal histochemical reaction protocol (*see* **Subheading 3.2.4.**). Check the sections after 4 h. If intensely blue-labeled cells have appeared, the reaction should be stopped by transferring the sections through several changes of the rinse solution. Otherwise, the reaction can be continued overnight, though, it is better to avoid the longer incubation time because of the nonspecific precipitations.
5. After the reaction has been stopped by washing the sections in buffer, the sections are postfixed in 1% osmium tetroxide in 0.1 *M* phosphate buffer in refrigerator at 4°C for 1 h. After washing the sections again in phosphate buffer, they are stained in 1% uranyl acetate in refrigerator at 4°C for 30 min, then dehydrated in graded ethanols and propylene oxide series.
6. Infiltrate and embed the thick sections in Epon or Araldite or an Epon-Araldite mixture.
7. Embed the sections in Epon or Araldite in capsules.
8. Cure the blocks in an oven at 60°C for 24 h to harden.
9. Cut 1 μm sections and stain them with alkaline toluidine blue for light microscopic study for orientation.
10. Cut thin sections by ultramicrotome and place them on copper grids.
11. The thin sections are not stained further either with uranyl acetate or lead citrate.
12. Observe the sections by transmission electron microscope.

3.5. EM Criteria for Valid X-Gal Reactivity

The X-Gal reaction product is an electron-dense, rather ellipsoid-shaped, very fine granular precipitate. Sometimes crystal-like, fine needle formations can be observed within the precipitate. The reaction product is typically seen in the perinuclear region, where a small saccular distension occurs ***(19,20,32,33)***. It is also detected in the endoplasmic reticulum, in either of two forms: 1) as a precipitate just as described in the perinuclear region, and 2) as a precipitate filling out the irregularly-shaped sacs or tubules of the endoplasmic reticulum

as electron-dense elongated rod-shaped precipitates. The components, particles of the precipitate, have the same very fine granular, ovoid shapes as in the perinuclear region. Similar precipitates of small sizes are also found in dendrites, axons, and synaptosomes (**Fig. 5**), where they are typically close to or surrounded by membrane.

4. Notes

1. Use good quality transparent plastic tubes for clear visibility of the precipitate. Carefully check the pH of the HBS solution. pH is critical. The working pH range is 7.05–7.12.
2. If the precipitate has large clumps, it will not work well and should be redone.
3. The time should be tested for every packaging line.
4. After identifying a good producer clone, it is wise to freeze several vials to be safe from any cell culture disasters or from a gradual loss of titer for unknown reasons.
5. The stage of confluency should be determined empirically for the given target cells. More confluent cells may produce more virus.
6. Producer cells can be incubated with 10 µg/mL Mitomycin C for 3 h in their medium, rinsed several times with fresh medium, and then can be used for co-cultivation.
7. Make sure the co-cultivation medium is optimal to support cell division of the target cells, while allowing for virus production by producer cells. Allow at least two target cell cycles to elapse during the co-cultivation period (you can dilute the infective medium with the one optimal for the target cells or change the medium during infection 2–4X).
8. As long as the entire culture is not confluent it can be subjected to drug selection by replacing the medium with selection medium. If the culture is confluent, split the cells and then carry out the drug selection.
9. Phosphate buffer can be substituted for the PIPES buffer without noticing any significant disadvantage.
10. The reaction intensity is influenced by the concentration of the X-gal substrate (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). We usually use a concentration of 1 mg/mL, although the staining intensity can be enhanced by increasing the X-gal substrate concentration up to 4 mg/mL. At higher concentrations non-specific reaction product is prone to form.
11. Similarly, the concentration of the K-ferro-/ferricyanide solution can be increased up to 30–35 m*M*, accelerating the oxidation of the indoxyl; however, it may enhance production of false-positive, particularly if the incubation time is prolonged overnight.
12. Chelators in the fixatives, buffers and other solutions seem to reduce background reaction by excluding Ca^{2+} ions from the reaction site.
13. The pH should be kept on the alkaline side. Bacterial β-galactosidase enzyme activity is optimal between pH 7.0–8.0 ***(44)***. If the pH drops into the acidic range,

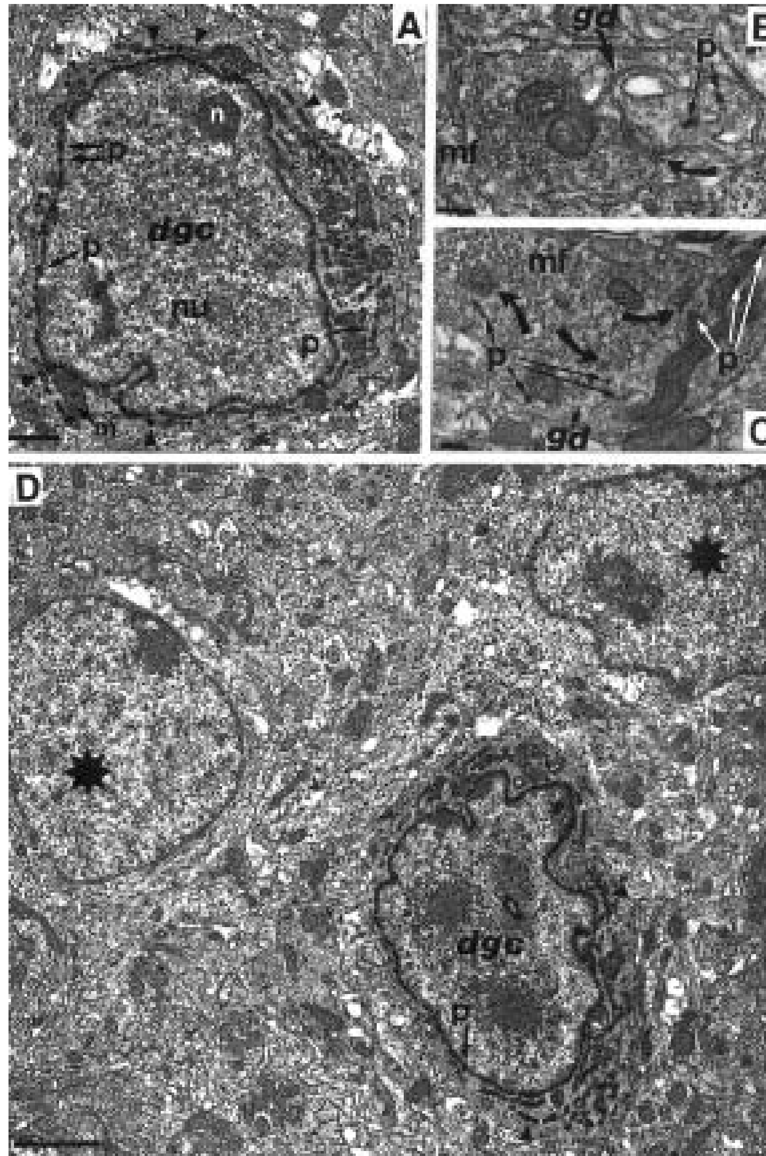

Fig. 5. This plate shows the donor-derived and host cells in the cerebellar cortex. **(A,D)** Neural stem cell (dgc) having significantly darker appearance with the X-Gal precipitates around the perinuclear region (p) and in the endoplasmic reticulum tubules. Host cells are labeled with asterisks. **(B,C)** The X-gal precipitates can also be detected in synaptic boutons and dendrite profiles (p).

endogenous β-galactosidases may produce a false reaction, because this is the optimal (pH 3.5–5.5) for the endogenous enzyme. A high concentration of CO_2 in the incubation environment will tend to lower the pH.

14. The incubation time is also a critical factor, since longer incubation times increase the probability of false-positives triggered by endogenous iron and a pH shift to the acidic range. Hold the incubation time to 4 h whenever possible. If the incubation is allowed to run overnight, watch carefully for a non-specific reaction product creating a false-positive result.
15. The tissue redox status can influence the outcome by allowing non-enzymatic oxidation of the substrate and production of a false color reaction.
16. During long incubation times, oxygen absorbed into the reagent solutions from air also can oxidize substrate and result in a false-positive reading.

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II

Characterization of NSCs In Vitro

B. Electrophysiological Techniques

16

Techniques for Studying the Electrophysiology of Neurons Derived from Neural Stem/Progenitor Cells

David S. K. Magnuson and Dante J. Morassutti

1. Introduction

The use and study of neural stem cells (NSCs) both in vitro and in vivo has increased exponentially over the past 6–8 years, with the majority of laboratories working toward the use of these cells therapeutically. The bulk of the characterization of NSCs has relied on immunocytochemical techniques, sometimes combined with morphological analysis. However, evidence for the ability of many types of NSCs to generate functionally mature neurons capable of forming normal and appropriate synapses is still largely lacking.

We have approached the electrophysiological study of NSC-derived neurons in two distinct fashions, in vitro and in vivo. Electrophysiological examination of engrafted cells is problematic, and relies heavily on recording from large numbers of graft-derived neurons in brain slice preparations and on the successful immunocytochemical identification of recorded cells in fixed and sectioned tissue recovered from the prepared slices. Their study in vitro is less problematic, and is very similar to techniques used for the examination of primary cultures, but several technical decisions must be made that will in part determine the types of data that are collected. These choices are largely dictated by what aspects of the electrophysiology of NSCs are of greatest interest, namely, whole cell voltage clamp to examine the complement of channels present or whole cell current clamp to examine action potential generation in individual cells and synaptic activity in circuits within the population of cultured neurons. Results obtained using the in vivo methods are found in Morassutti ***(1)*** and Magnuson ***(2,3)***. The in vitro results are found in Liu ***(4)***.

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2. Materials

2.1. Electrophysiology of Transplanted NSCs

1. Pentobarbital (60 mg/kg; Sigma) and syringes.
2. Ibotenic acid (5 μg/μL) in HBSS (Sigma) or artificial cerebrospinal fluid (ACSF; see below).
3. Stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA).
4. Dremmel drill with a burr bit.
5. 26 gauge, 10 μL Hamilton syringe, with a 90° bevel and positive displacement inner stylet using Hamilton Syringe (*see* Chapter 29).
6. 1.2 mm outside diameter (OD) borosilicate micropipets pulled and beveled at 55° to an inside diameter (ID) of 50–100 μm.
   Using glass micropipet (*see* Chapter 29):
   a. Electrode puller (Sutter Instruments P87, Novato, CA, USA, or equivalent).
   b. Electrode beveller (World Precision Instruments, Sarasota, FL, USA, or equivalent).
   c. General Valve Picospritzer or equivalent.
7. NSCs suspended in HBSS or artificial cerebrospinal fluid.

It is ideal to have 50 μL of 0.5–2 × 10^5 cells per μL (*see* Chapter 29). Optimal cell survival is obtained when the cells are grown as small 10–50 cell aggregates so that the preparation does not require trypsinization prior to transplantation ***(1)***.

2.2. Slice Preparation and Electrophysiological Recording

1. Vibroslice (Campden Instruments, Leicester, U.K.) or Vibratome (TPI/Vibratome, St. Louis) or equivalent.
2. Ketamine (35 mg/kg; Sigma) and syringes.
3. Halothane (Sigma).
4. Filter paper circles (Whatman #2).
5. Dissecting instruments including #10 scalpel, small stainless spatula, forceps, and stiff-backed razor blades.
6. Submersion-style tissue chamber (RC-13, Warner Instruments, Hamden, CT, USA), continuously perfused.
7. Artificial cerebrospinal fluid (ACSF): 118 m*M* NaCl, 3.0 m*M* KCl, 1.0 m*M* NaH_2PO_4, 0.81 m*M* $MgSO_4$, 2.5 m*M* $CaCl_2$, 10 m*M* glucose, 24 m*M* $NaHCO_3$. Bubbled with 95% O_2/5% CO_2 at room temperature.
8. Incubating chamber; a tea strainer and a 250-mL beaker filled with ACSF (continuously gassed) or equivalent (*see* **Note 1**).
9. 1.2 mm OD borosilicate glass micropipet blanks (Warner Instruments or equivalent).
10. Potassium acetate (KAc, 2 *M*), 1.5 mL centrifuge tubes and 28 or 34 gauge Microfil needle, 67 mm long (World Precision Instruments) and syringes (*see* **Note 4**).

11. 100 µL of 1% biocytin (Sigma) in potassium acetate (2 *M*) made fresh each day (*see* **Note 4**).
12. Gold electron microscope grids (G50 square mesh in Gold, SPI Supplies, West Chester, PA, USA) and 1–1.5 mm long pieces of platinum wire, 0.25 mm diameter (World Precision Instruments).
13. Sutter Instruments electrode puller.
14. Standard dissecting microscope with trans-illumination.
15. Micropositioners (two, SD Instruments, Oregon, MX-6600 or equivalent).
16. Electrophysiological rig. For example, Axoclamp 2B microelectrode amplifier, Digidata 1200 data acquisition system, PClamp 7.0 or 8.0 (all by Axon Instruments, Foster City, CA) and a PC computer.
17. L-glutamate (1 m*M*) in 0.4% NaCl for pressure ejection onto the slice.
18. 4% paraformaldehyde (100 mL), cryostat or other sectioning instrument, histology and immunostaining supplies.

2.3. Electrophysiology of NSCs in Culture

1. 24-well standard tissue culture plates with 13 mm round tissue culture coverslips (Thermanox, Fisher Scientific).
2. E-C-L cell attachment matrix for coverslips (Upstate Biotechnology, Lake Placid, NY, USA).

OR

3. 30 mm round standard tissue culture dishes.
4. 15 µg/mL poly-L-ornithine (Sigma) and either 1 µg/mL fibronectin (Intergen, Purchase, NY, USA) or 1.5 µg/cm^2 mouse laminin (Sigma).
5. Growth factor containing media of choice and differentiating media of choice.
6. Upright or inverted compound microscope equipped with contrast enhancing optics of choice: Hoffman Modulation Contrast (HMC), Nomarski or phase-contrast optics.
7. Stage or table mounted micropositioners (SD Instruments).
8. Stage mounted recording chamber (Warner Instruments RC13) or stage adapter for 30 mm round dish (from microscope manufacturer or custom made; aluminum or Plexiglas).
9. Stage mounted syringe/needle and source of 95% O_2/5% CO_2 to be gently blown into 30 mm round dish for static bath recording.
10. Electrophysiology rig including intracellular/whole-cell patch amplifier, digitizer, and data acquisition system. For example, AxoClamp 2 series or Axopatch series amplifier, Digidata data acquisition system, PClamp 7.0 or 8.0 data acquisition software (all by Axon Instruments) and a PC computer.
11. Digital (video) camera mounted on microscope to allow photomicrographs to be taken of cells before recordings are made, to allow for positive identification of recorded cells following immunohistochemical processing. Example; Pixera 120es camera (Pixera Corporation, Los Gatos, CA, USA).

3. Methods

3.1. Transplantation

1. Sprague–Dawley rat, anesthetized with pentobarbital and mounted in a stereotaxic frame (Kopf).
2. Region of interest is exposed by a burrhole and the dura is opened.
3. Ten microliters of HBSS or ACSF containing ibotenic acid (5 μg/μL), and 0.5–2.0 × 10^4/μL NSCs is loaded into the Hamilton syringe, 2–4 μL to be injected stereotaxically into each area of interest. Or 1.2 mm OD borosilicate glass micropipets are pulled and bevelled at 55° to an ID of 50–100 μm. Pipet is attached to a Picospritzer (General Valve; see Chapter 29). NSCs are prepared for transplantation as described by Whittemore et al. (Chapter 29) and loaded into the micropipets for transplantation. The ibotenic acid is added only if a concomitant lesion is desired in a striatal target ***(1)***.
4. Post-operative animals are given fluids (1 cc/10 g of Ringers lactate or 0.9% NaCl subcutaneously) at the end of the procedure, and are warmed by placing cages on heating blankets overnight or until they recover from the procedure.

3.2. Slice Preparation and Electrophysiology of Transplanted Cells

1. Animals are injected with ketamine (35 mg/kg) and exposed to 1.5% halothane in 100% oxygen for 3 min and decapitated.
2. The brain is rapidly removed to chilled and gassed ACSF, by first doing a craniotomy to remove the entire dorsal aspect of the skull. A scalpel is then used to make transverse cuts to separate the bulk of the brain from the most anterior cortex and olfactory bulbs, and also from the medulla and cerebellum. A stainless-steel spatula is used to roll the brain out of the skull, freeing it from any adhering dura and cranial nerves.
3. After 90 sec, the brain is removed to level surface (counter-top or instrument tray) with filter paper that is moistened with chilled and gassed ACSF. The brain is blocked by hand using stiff-backed razor blades.
4. The tissue block is glued (cyanoacrylate, Crazy Glue) to the plastic cutting block of a Vibratome (Oxford Instruments) or Vibroslice (Campden Instruments, available from World Precision Instruments) and submerged in chilled and gassed ACSF.
5. Slices are prepared (12–15 slices, 300–500 μm thick) and transferred to an incubating chamber at 33°C for 30–60 min.
6. One brain slice containing the grafted tissue is transferred to the recording chamber and submerged in flowing, gassed ACSF (1.5–3.0 mL/min).
7. Gold electron microscope grids are placed on the slice, onto which small pieces of platinum wire are also placed to hold the slice firmly to the bottom of the chamber. When transilluminated, areas of lesioned brain are opaque while areas of grafted tissue are translucent or mottled in appearance.
8. Grafted neurons are recorded intracellularly by lowering glass microelectrodes (120–200 M filled with 2 *M* KAc) into the area of interest using a micropositioner.

9. If desired, 1% biocytin can be included in the KAc. Neurons are labeled intracellularly with 1% biocytin in the recording electrodes, and intracellular filling can be assisted by passing 500–800 msec hyperpolarizing current pulses. Only the tips of the intracellular electrodes need to contain biocytin (*see* **Note 4**), the rest of the electrode is filled with KAc via a 30 g needle/syringe.
10. Slices are fixed in 4% paraformaldehyde in 0.1 *M* PBS for 1 h, and are cryoprotected by placing in 10% sucrose in 0.1 *M* PBS for 48 h at 4°C. The slices are quick-frozen onto liver tissue that has a precut surface and is already mounted on a cryostat chuck. This allows serial 14–20 µm sections to be taken from the entire slice without losing any tissue. Intracellularly recorded cells are positively identified by treatment with streptavidin-Texas Red (1:100, Amersham) for 1 h at 37°C, and may be double labeled as desired.

3.3. Examination of Synaptic Activity in Grafted Neurons

1. A second glass microelectrode is lowered into the superfusate and onto the surface of the slice. This electrode can be a standard intracellular electrode that has been beveled at 55°C to an ID of 2–5 µm, or it can be a patch-clamp style electrode with a resistance of 2–10 M when filled with 0.9% saline.
2. This electrode should be filled with either L-glutamate (1 m*M* in 0.9% saline) or a high K+ ACSF (20 or 40 m*M* KCl, substituted for NaCl).
3. The solution is ejected using short pressure pulses either directly onto intracellularly recorded neurons or into nearby graft or host tissue (up to 1 mm away). In our case, functional synapses between precursor-derived grafted neurons were easily demonstrated, however synapses between host and graft could not be found ***(3)***.

3.4. Preparation of NSCs, In Vitro: Coverslips

1. Prepare NSCs from desired source and culture using standard techniques with mitogens as desired.
2. Following a second passage or a cloning procedure, replate cells at $1–2 \times 10^4$ cells per well on 13 mm round coverslips coated with E-C-L cell attachment matrix in 24-well tissue culture plates. Grow cells on coverslips in control media containing the mitogen for 3 d or until approx 50% confluent (*see* **Note 2**).
3. Switch media to include a differentiating factor (2% fetal calf serum) without added mitogens until recording.
4. Transfer coverslips to a flowing (0.5 mL/min of ACSF at room temperature) recording chamber mounted to the stage of an inverted or upright compound microscope equipped with some type of contrast-enhancing optics (we chose Hoffman Modulation Contrast or HMC) for either whole-cell current or voltage-clamp recording. Recordings are made using standard electrophysiological techniques.

3.5. Preparation of NSCs, In Vitro: Plastic Dishes

1. Following a second passage or a cloning procedure, NSCs are replated at $1–2 \times 10^4$ cells/cm^2 onto 30 mm round dishes coated with 2.5 µg/cm^2 E-C-L

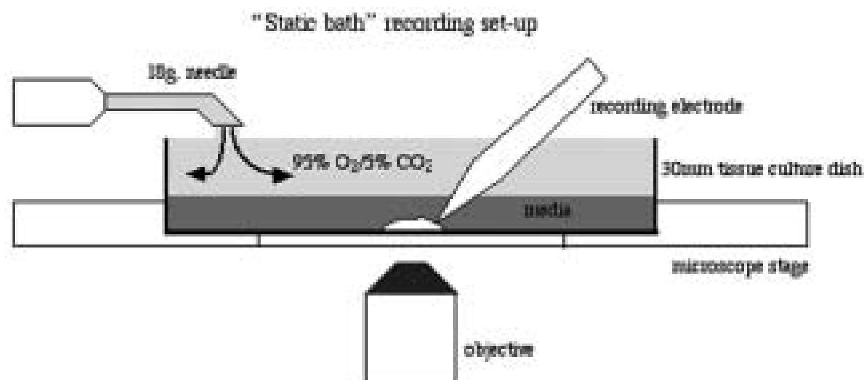

Fig. 1. Electrophysiology of NSCs in culture. This figure shows one technique that allows recordings to be made from NSC-derived neurons growing on tissue culture plates. The cells are maintained in either tissue culture media or artificial CSF during the recording and are transferred to the stage of an upright or inverted compound microscope equipped with contrast enhancing optics. The desired pH and oxygen of the bathing solution is maintained by gently blowing 95% O_2/5% CO_2 over the surface of the solution during the recording procedure. The stage and gases can be warmed if desired, but we have found that room temperature is sufficient for most recording situations.

or 15 μg/mL poly-L-ornithine with either 1 μg/mL fibronectin or 1.5 μg/cm^2 mouse laminin.

2. Grow for 3–4 d in media with mitogen (until 50–60% confluent) before switching to a differentiating medium with fetal calf serum.
3. Dishes are transferred to the stage of an upright or inverted compound microscope equipped as described above, and recordings are made using standard whole-cell current or voltage clamp techniques. These recordings are best made in a static, non-flowing bath (*see* **Note 3** and **Fig. 1**), since adding and removing fluid from an open tissue culture dish while recording is difficult.

4. Notes

1. We have found that preparing slices that include grafts is difficult during the first week post-grafting because of inflammation and the likelihood that the graft will not stay in the slice once it is cut. Even after 1 wk, slices must be handled very carefully to ensure that the graft–host interface is not stressed and that the graft stays in the slice. Slices can be transferred with little damage by using a wide-mouthed pipet that is made by breaking most of the thinned end of a Pasteur pipet off and fire polishing. Turn the pipet around and use the end that usually goes into the bulb as the open end to suck up the brain slice. Brain slices with or without grafts should be incubated at room temperature for 30–60 min before being transferred to the recording chamber. Although there are

commercially available incubating chambers, we have found that a household tea strainer (plastic sieve with a handle) designed to hold loose tea leaves in a cup or mug, is very suitable for holding brain slices in ACSF. We use a 250 mL beaker, filled to within 1 cm of the top with ACSF that is being slowly bubbled with 95% O_2/5% CO_2 via small diameter polyethylene tubing. Place the tea strainer in the beaker and transfer the slices to the strainer using a wide-mouthed pipet.

2. For our experiments using NSCs, in vitro, grown on coverslips for whole-cell recording, we chose to use young adult and adult Sprague–Dawley rats as the source of NSCs, which were isolated from the spinal cord and subventricular zone, respectively. The mitogens used were EGF or FGF2, or a combination of the two. Although we did not use cloning procedures, no recordings were made until after at least two passages. Recordings could be made successfully as soon as 4 h after plating; however, we found it best to plate the cells at a relatively low concentration and leave them in mitogen containing media for a few days before switching to media containing a differentiating factor (fetal calf serum). This protocol gave us what appeared to be healthier cells, and also allowed for recovery of recorded cells for histological examination.
3. For the preparation of NSCs on plastic dishes, we found that, as with coverslips, cells in dishes should be grown for 3–4 d in mitogen (until 50–60% confluent) before switching to a differentiating medium with fetal calf serum. For recording, dishes are transferred to the stage of an upright or inverted compound microscope equipped as described above. These recordings are best made in a static bath as shown in **Fig. 1**. To allow recordings to be made in a static bath without compromising the pH of the media or ACSF, a bent 18 gauge needle attached to a source of 95% O_2/ 5% CO_2 is placed 1–1.5 cm over the solution and the gasses can be gently blown over the surface of the solution (100–200 mL/min; **Fig. 1**). This technique will allow recordings to be made over a period of 1–3 h at room temperature without deterioration of the cells or culture media. Media-filled culture dishes, without cells, can be moved from an incubator to the recording stage to establish the gas flow rate that best maintains the correct media or ACSF pH.
4. The identification of recorded cells can be accomplished in two ways, by intracellular labeling [with biocytin, for example; Huang ***(5)***] or by making photomicrographs of recorded cells before each recording and after fixation and immunolabeling. We have found that switching from media containing one or more mitogens to one containing fetal calf serum to induce differentiation when the cells are still fairly sparse (less than 50% confluent) can allow positive identification of cells using before-and-after photomicrographs. We use a Nikon upright microscope equipped with HMC optics and an inexpensive Pixera digital video camera. For the best results, each cell chosen for recording is photographed before the patch electrode is lowered into the solution and again after fixation/staining. The location of the cell on the coverslip is noted, sometimes with respect to a scratch placed on the bottom or edge of the coverslip.

When using biocytin to label intracellularly recorded neurons, it must be made up fresh each day. Adding some KOH to the KAc to bring it to pH 10 will allow the biocytin to dissolve more easily than at lower pH, and will also enhance the filling of cells when using hyperpolarizing current pulses since biocytin is negatively charged above pH 7. Only the tips of the electrodes need to be filled with biocytin, and this is accomplished by placing the electrodes, tips up, into 100 μL of the biocytin solution (1% in KAc or intracellular solution in a 1.5 mL centrifuge tube) until the tip has back-filled. The rest of the electrode is filled with either KAc (sharp electrodes) or intracellular solution (whole cell). Filling cells during whole-cell recording does not require current pulses, since the exchange between the electrode and the cytoplasm is much better than during intracellular recording with sharp electrodes. Biocytin is often difficult to get into solution, so either a vortex mixer needs to be used (for 2–3 min) or the sealed centrifuge tube containing 100–200 μL of the biocytin solution can be placed in a sonicator bath for a few minutes.

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II

Characterization of NSCs In Vitro

C. Molecular Techniques

17

Fluorescence *In Situ* Hybridization

Barbara A. Tate and Rachel L. Ostroff

1. Introduction

Many interesting new hypotheses about neural stem cells are now being tested. For example, understanding the potential of stem cells from non-neuronal tissue types to differentiate into neural tissue is of great interest. Definitive proof of some of these hypotheses requires overcoming current technical limitations and difficulties. One of the technical challenges of research involving neural stem cells is identification of transplanted cells *in situ*. Unequivocal identification of transplanted cells after integration into host brain tissue can be extremely difficult. Genes inserted into donor cells to code for marker proteins may be downregulated over time, or may be downregulated after stem cells differentiate into mature neural cells. Cells prelabelled with Bromodeoxyuridine (BrdU) or a tracer dye may continue to divide, thus diluting the label until daughter cells carry so little label that it is below detectable levels. Cross-species transplantation (mouse into rat or human into rodent, for example) requires identification of donor cells by antibodies that are absolutely species specific, which is sometimes difficult to achieve.

One successful method of identifying donor cells following integration into host brain involves *in situ* hybridization of probes specific to genes of the transplanted cells. For instance, a probe can be used *in situ* that is against the gene for a marker protein labeling the donor cell. Because the probe is against the gene, such identification of the donor cells does not require the expression of either the mRNA or the protein of the marker, thus overcoming the difficulty that expression may be downregulated upon transplantation or differentiation. However, low-copy-number genes may be difficult to visualize as the signal to noise ratio may be too low to give convincing results. Therefore, use of probes

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against repeated sequences may be much more successful in achieving good results. Finally, it is often desirable to be able to identify not only a cell as donor vs host, but also the ultimate, differentiated fate of donor stem cells in host tissue. Therefore, a combination of FISH and fluorescent immunohistochemistry is often used to identify both the origin and the fate of cells in the brain (*see* **Note 1**). Fluorescent tags on both the probe used *in situ* and the antibody used for immunohistochemistry, especially in combination with confocal microscopy, permits unambiguous determination of probe and antigen colocalization (*see* **Note 2**).

Protocols for both *in situ* hybridization and immunohistochemistry are readily available in primary articles, textbooks, manuals, and Web sites. In addition, the individual reagents and even kits containing all necessary reagents for carrying out both immunohistochemistry and *in situ* hybridization are commercially available. We review here the variations on types of probes and labeling techniques that can be used for *in situ* hybridization, as well as tissue preparation. In addition, we include a protocol for labeling a DNA probe followed by a protocol to use the probe in fluorescent *in situ* hybridization.

2. Materials

2.1. Buffers and Solutions

1. Phosphate-buffered saline (PBS): To 800 mL double distilled water add 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4. Dissolve, adjust the pH to 7.4 with HCl, and bring up to 1000 mL with water.
2. 20X SSC: To 800 mL of double distilled water, add 175.3 g NaCl and 88.2 g of sodium citrate. Dissolve and adjust the pH to 7.0 with 10 *N* NaOH. Bring to 1000 mL with water.
3. Paraformaldehyde (PFA): This solution must be prepared under fume hood while wearing appropriate safety attire. For 20% stock, add 40 g of paraformaldehyde to 150 mL of PBS. Heat to 70–80°C, then add 1 *N* NaOH dropwise until the solution clears. When cooled, pH to 7.4. Add PBS to 200 mL. This stock can be kept for several weeks. Dilute with PBS to 4% before using.

2.2. Probes

If the sequence of the gene of interest is known, synthetic oligonucleotide probes can be produced. More often templates for probes are produced by isolation of a sequence of interest from the native DNA and cloning of the fragment into plasmids to produce sufficient quantities of DNA for labeling. Plasmids containing sequences of interest are available from commercial sources (see Linscott's Directory at linscottsdirectory.com, or any of a number of vendors that specialize in molecular biology), from other investigators, or

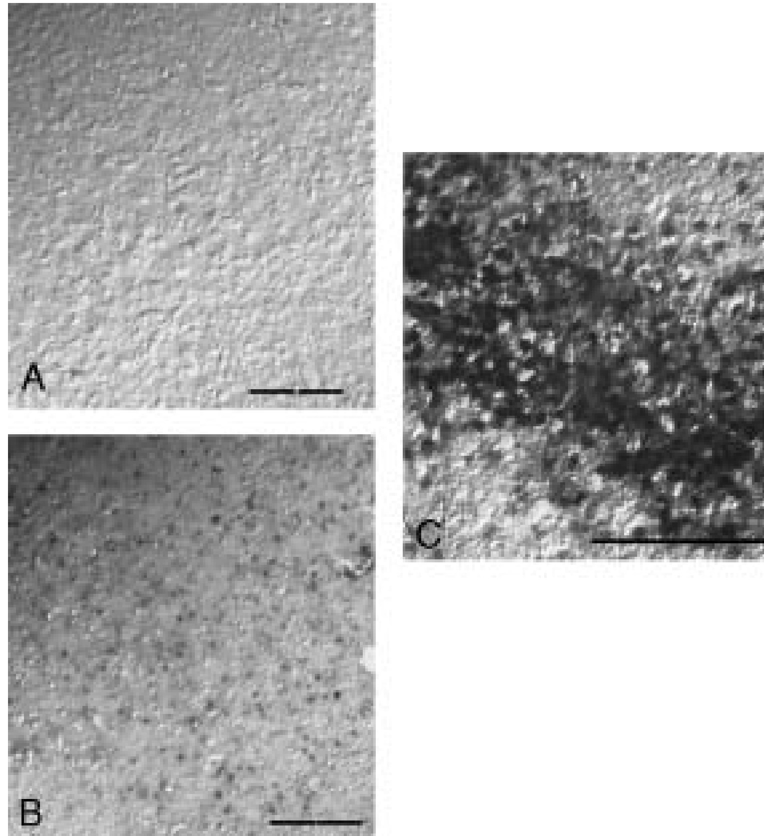

Fig. 1. Newborn mice were given transplants of cells from a clonal mouse neural stem cell line derived from male neonatal brain tissue ***(4)***. Cells were grafted into both lateral ventricles, allowing them access to the subventricular germinal zone, a location that can yield cells to most of the brain. Animals were sacrificed by cardiac perfusion one month later. Animals were perfused with 4% paraformaldehyde (PFA). Brains were post fixed in 4% PFA, then cryoprotected in 30% sucrose, and stored frozen until cryosectioned (20 μm sections). Sections were then processed for *in situ* hybridization with a probe to the mouse Y-chromosome. The Y-probe was labeled with digoxigenin-dUTP (DIG High Prime Labeling kit, Boehringer Mannheim). Following hybridization, the probe was visualized with an antibody to digoxigenin conjugated with alkaline phosphatase (Boehringer Mannheim). The positive control in the *in situ* reaction was an unengrafted male mouse **(A)**; the negative control was an unengrafted female mouse **(B)**. **(C)** Tissue from the olfactory bulb of the grafted female mouse showing many male donor cells integrated into the host tissue.

can be produced in the lab. Once the probe is obtained, it must be labeled prior to use in the *in situ* hybridization reaction.

DNA probes can be labeled by a number of techniques, including random priming, nick translation, or polymerase chain reaction (PCR). In each technique, the probe is labeled by incorporation of labeled nucleotides into the DNA strands. Reagents for each type of labeling can be purchased from commercial suppliers. For instance, digoxigenin (DIG), biotin, or fluorescein labeling kits for random priming labeling of DNA probes can be purchased from Roche Molecular Biochemicals (biochem.roche.com). DIG labeled and biotin labeled probes are visualized in tissue following hybridization by detection with antibodies to DIG or biotin, or by avidin binding to biotin. Therefore, the visualization of the probe requires additional steps after hybridization whereas fluorescein labeled probes can be seen under fluorescence microscopy immediately following the completion of the *in situ* hybridization protocol. However, labeling a probe with DIG or biotin and detection of the label by immunostaining with a fluorescently labeled secondary antibody adds additional sensitivity to the procedure by amplification of the signal for one molecule of probe to several molecules of secondary antibody.

All three labeling methods can be adjusted to alter the length of the labeled probe. The goal is to have a probe that is small enough to allow good penetration into brain tissue but long enough to reduce non-specific hybridization. Random priming and PCR result in a mixture of probe lengths, which may work in favor of hybridization.

RNA probes can also be labeled and used for FISH. The labeled probes must be produced by in vitro transcription from template DNA. RNA is inherently more prone to degradation, and therefore more difficult to work with than DNA. Reagents and glassware must be kept RNAse free. An advantage of RNA probes is that RNA–RNA hybrids are more stable than DNA–RNA or DNA–DNA hybrids ***(3)***.

2.3. Tissues

Fresh frozen, fixed or embedded tissue have all been used for *in situ* hybridization. The challenge is to preserve morphology while preventing extensive crosslinking of proteins surrounding nucleic acids that may inhibit hybridization. Tissue is usually permeabilized with detergents early in the *in situ* protocol to allow penetration of the probe. In addition, tissue should be collected onto specially treated glass slides (for instance, SuperFrost Plus slides) to prevent the tissue from falling off during the harsh conditions of the *in situ* procedure but to decrease non specific probe binding that may occur on gelatin coated slides. If immunohistochemistry is to be done on the same

sections to look for colocalization of probe target and an specific antigen, the immunohistochemical staining is usually done prior to the *in situ* procedure. Sample methods for labeling probe and *in situ* hybridization are presented below.

3. Methods

3.1. Preparation of Probe by DIG Labeling

1. Add 14 µL sterile double distilled water to 2 µL DNA (2.85 µg probe DNA isolated from plasmid).
2. Denature DNA by heating in boiling water for 10 min, then very rapidly chilling in ice/ethanol bath.
3. Add 4 µL DIG-High Prime random priming reagent (Roche Molecular Biochemicals). Mix and incubate at 37°C overnight.
4. Stop reaction with 2 µL of 0.2 *M* EDTA (pH 8.0) and by heating to 65°C for 10 min.
5. Add 2.5 µL of 4 *M* LiCl and 75 µL of ethanol at –20°C. Mix well.
6. Keep at –20°C for 2 h.
7. Centrifuge at 12,000*g* for 15 min.
8. Remove supernatant and wash pellet in 50 µL cold 70% ethanol.
9. Remove supernatant and air-dry the pellet. The pellet now contains DIG labeled probe.

3.2. Tissue Preparation

1. Cryosection 10–20 µm brain sections of 4% paraformaldehyde perfused mouse brain onto SuperFrost Plus slides. Use sections immediately or store at –20°C.
2. Wash sections in phosphate buffered saline. Perform immunohistochemistry at this point using fluorescein labeled secondary antibodies. Following immunohistochemistry, keep sections in the refrigerator, in the dark until *in situ* is performed.

3.3. In Situ Hybridization

1. Rinse slides in 0.2% Triton X-100 in phosphate buffered saline (PBS) for 5 min (*see* **Note 1**).
2. Wash in PBS 15 min.
3. Treat with 0.2 *N* HCl for 20 min at room temperature, in a humidified chamber.
4. Wash in PBS for 15 min.
5. Digest with 100 µg/mL proteinase K in 0.1 *M* Tris-HCl, 0.05 *M* EDTA, pH 8.0 at 30°C in humidified box for 15 min.
6. Wash with 0.1% glycine in PBS for 2 min.
7. Wash in PBS for 15 min.
8. Post fix in 4% paraformaldehyde in PBS for 15 min.
9. Wash in PBS for 15 min.

10. Perform pre-hybridization step. Incubate tissue in 2X SSC, 0.1% N-lauroyl-sarcosine, 0.2% SDS, 50% formamide (deionized) in sterile dd water for 30 min at 37–47°C (optimized for probe) in a humidified chamber (2X SSC: 300 m*M* NaCl, 30 m*M* sodium citrate, pH 7.0).
11. Apply the probe to the tissue and denature both probe and target DNA simultaneously. Probe should be at a concentration of 100 pg/µL in 20% dextran sulfate in 2X SSC hybridization buffer (see above). Apply to tissue, quickly coverslip slides and seal the edges of the coverslips with rubber cement and incubate for 10 min at 95°C.
12. Put slides containing probe on ice for 5 min.
13. Incubate slides overnight in a humidified chamber at 37–47°C (optimized for probe used).
14. Very carefully remove coverslips and wash sections at 37°C with prewarmed solutions, 30 min per wash as follows: 2X SSC, 1X SSC, 0.1X SSC (*see* **Note 2**).
15. Block in 150 m*M* NaCl, 100 m*M* Tris-HCl, pH 7.5, containing 2–10% serum of antibody species.
16. Incubate in anti-DIG-Rhodamine at 2 µg/mL diluted in for 1 h at room temperature.
17. Wash 3X for 10 min each in 150 m*M* NaCl, 100 m*M* Tris-HCl, pH 7.5, 0.5% Tween-20.
18. Coverslip with aqueous mounting medium and keep slides in dark (*see* **Note 3**).

4. Notes

1. Always run positive and negative control tissue sections.
2. One common problem with *in situ* hybridization procedures is high background or significant non-specific staining. If background or non-specific staining is a problem, first check to see if it is due to non-specific binding of the probe or non-specific binding of the antibody used to visualize the probe. Run control slides with no probe but all other steps of the procedure. Probe concentration can be adjusted if the problem is non-specific binding of probe. In addition, the wash step after hybridization can be increased in length and the temperature at which they are carried out can also be increased.
3. It should be determined that the probe is well labeled prior to using it in the procedure. For reasons that are not always clear, some probes work better in the procedure than others. Make sure the starting material is of good quality (sufficiently purified from plasmid DNA and not degraded prior to labeling). Alternative labeling procedures may be necessary, i.e., use nick translation instead of random priming.

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18

RT-PCR Analyses of Differential Gene Expression in ES-Derived Neural Stem Cells

Theresa E. Gratsch

1. Introduction

Reverse transcription and the polymerase chain reaction (RT-PCR) provides a very sensitive method to identify known genes that are both upregulated and downregulated during neuronal differentiation. First strand cDNAs are generated in the initial reaction with reverse transcriptase in the presence oligo-dT primed RNAs. The cDNA provides the template for amplification of gene specific targets with the appropriate primers. The purity of the RNA is extremely important to the success of these procedures. The presence of chromosomal DNA in the RNA preparation will influence the reliability of both RNA concentration and the PCR products generated from these RNA templates in the reverse transcription reactions. Many parameters in PCR have to be optimized for each gene specific primer pair and template; they include primer and $MgCl_2$ concentrations, primer annealing temperatures, and the number of PCR cycles ***(1,2)***.

Although many neuronal genes have been identified, the cascade of gene expression that directs neuronal differentiation is not well understood. We have expressed (transfected) several early neurogenic genes (*noggin*, *chordin*, *NeuroD1*, *2*, and *3*) in mouse embryonic stem (ES) cells, to assess the ability of each to direct neuronal differentiation and to identify their downstream targets ***(3,4)***. The number of genes to examine by RT-PCR can be overwhelming. In order to focus on genes that are differentially expressed in each of the transfected ES cells, a modified reverse Northern technique was employed. The procedure can be used to screen and identify genes that are differentially expressed in a particular transfected cell population. Twenty cDNA products

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(ranging in size from 300 bp to 1 kb) were selected from our primer panel and cloned into pBS SK (pSK). The cDNAs selected can be customized for any research project. The RT-PCR is then carried out using the selected gene specific primers. These clones provide a stable source of gene specific probes and targets for *in situ*, Northern, and Southern hybridizations.

2. Materials

2.1. RNA Extraction and Purification

1. Trizol Reagent, (Life Technologies, Rockville, MD, USA).
2. Chloroform.
3. Isopropanol.
4. 70 and 75% Ethanol in DEP-C H_2O.
5. DNaseI [10 U/μL], and10X Buffer, (Genehunter Corp., Nashville, TN, USA).
6. Phenol : chloroform, 3 : 1 v/v.
7. 3 *M* NaOAc, pH 5.2.
8. 100% ethanol.
9. DEP-C H_2O/RNase free H_2O.
10. 1.5 mL microtubes.
11. Agarose.
12. 10X TBE: Tris base 108 g, boric acid 55 g, 0.5 *M* EDTA (pH 8) 40 mL to 1 L with ddH_2O.
13. Ethidium bromide (EtBr) (10 mg/mL).
14. TE buffer: 10 m*M* Tris-HCl, 1 mM EDTA.

2.2. Reverse Transcription

1. Total RNA, 1–5 μg per reaction.
2. DEP-C H_2O (*see* **Note 1**).
3. Oligo-$dT_{12–18}$ primer [0.5 μg/mL] (Invitrogen Life Technologies). 0.1 *M* dithiothreitol (DTT).
4. 10 m*M* dNTP mix, 10 m*M* each of dATP, dCTP, dGTP, dTTP.
5. Superscript II [200 U/μL] and 5X First strand buffer (Invitrogen Life Technologies).

2.3 Polymerase Chain Reaction

1. RT template, 1/10 total volume of the RT reaction.
2. 200 μL l thin wall PCR tubes.
3. Taq DNA polymerase (5 U/μL) and 10X Taq DNA pol. buffer (Invitrogen Life Technologies, Rockville, MD).
4. 50 m*M* $MgCl_2$ (Invitrogen Life Technologies).
5. 10 m*M* dNTP mix, same as above.
6. Gene specific primers, 10–20 p*M*/reaction (*see* **Note 4**).
7. Thermocycler (Perkin Elmer Model 2400).
8. 100 bp or 1 kbp plus DNA ladders, (New England Biolabs, Beverly, MA, USA and Invitrogen Life Technologies).

9. DNA gel loading buffer: 40 % glycerol, 0.1 *M* EDTA (pH 8.0), and 0.5% SDS and a pinch of bromophenol blue.

2.4. Purification and Cloning of PCR Products

1. DNA gel extraction/purification kit, (Invitrogen Life Technologies or Qiagen, Valencia, CA, USA).
2. pBS SK cloning vector (Stratagene, LaJolla, CA, USA).
3. Restriction enzymes, BamHI, EcoRI, HindIII and XbaI (New England Biolabs and Invitrogen Life Technologies).
4. T4 DNA ligase [1 U/μL] and 5X T4 DNA ligase buffer (Invitrogen Life Technologies).
5. Calf intestinal alkaline phosphatase (CIAP)[22 U/μL], 10X dephosphorylation and enzyme dilution buffer (Invitrogen Life Technologies).
6. DH5α *E. coli* competent cells (Invitrogen Life Technologies).
7. SOC medium (Invitrogen Life Technologies).
8. LB agar and LB broth (2,5).
9. Ampicillin (20 mg/mL).
10. Colony lysis buffer: TE buffer with 0.1 % Tween.
11. T3 and T7 primers.
12. Standard PCR reagents, *see* **Subheading 2.3.**
13. 250 μ*M* dNTP mix.
14. Plasmid DNA prep kit (Qiagen).

2.5. Reverse Northern Blots

1. Purified gene-specific cDNAs.
2. Agarose, 10X TBE and EtBr.
3. Nylon membranes (Stratagene).
4. Standard RT reagents, see above.
5. ^{33}P-dATP or -dCTP, 3000 Ci/mmol, (New England Nuclear).
6. 20X SSC: NaCl 175.3 g, sodium citrate 88.2 g, dissolved in 800 mL ddH_2O. Adjust pH to 7.0 with 10 *N* NaOH, to 1 L with ddH_2O and autoclave.
7. 10X SSPE: 175.3 g NaCl, 27.6 g NaH_2PO_4, 7.4 g EDTA, in 800 mL ddH_2O, pH to 7.4 with NaOH, then bring total volume to 1 L and autoclave.
8. 10% SDS.
9. 50X Denhardt's solution: 5 g Ficoll, 5 g polyvinyl pyrolidone, 5 g BSA (fractionV), to 500 mL with ddH_2O, filter and store in 10 mL aliquots at –20°C.
10. EDTA.
11. Salmon sperm DNA, 10 mg/mL.
12. 3 MM Whatman filter paper and cassette.
13. Kodak X-OMAT film and cassette (Fisher Scientific).

3. Methods

3.1. RNA Extraction and Purification

This procedure involves the extraction of total RNA from undifferentiated and neural differentiated mouse ES cells, followed by the removal of chromo-

somal DNA by treatment with DNase I. The presence of chromosomal DNA in the RNA preparation will interfere with the PCR and can yield products independent of mRNA expression. Precautions must always be taken when working with RNA (*see* **Note 1**). There are a variety of reagents and kits that are available for the isolation of total RNA. We prefer the Trizol Reagent and follow the manufacturer's protocol.

1. To harvest ES cells for RNA, remove the culture medium and discard. Add 500 μL of Trizol to the cells in a 35 mm dish (5×10^5 cells), generally we have six-well, 35 mm plates. Gently rock the plate so the Trizol covers the cell monolayers, and with a pipet transfer the cell lysates to 1.5 mL tubes and place on ice immediately. If there are more plates to harvest, be sure that all cell lysates are kept on ice until all plates have been harvested. At this point, the samples can be stored at –70°C for at least 1 mo. To finish the extractions at a later time, thaw tubes on ice and then proceed with the protocol.
2. Incubate tubes at room temperature for 5 min. Then add 100 μL of chloroform to each tube (chloroform: isoamyl alcohol can also be used), shake tubes by hand for 15 sec, incubate samples at room temperature for 3 min. Centrifuge samples at 12,000*g* (all centrifugations are done at 14,000 rpm unless otherwise stated) for 10 min at 4°C.
3. Carefully pipet off the upper, aqueous phase (500 μL) into a fresh 1.5 mL tube. Add 250 μL of isopropanol to each, mix by inversion (there should be a small white pellet), and incubate at room temperature for 10 min.
4. Centrifuge 4°C for 10 min. Pour off the isopropanol from the side opposite the pellet. (The pellet will be clear and gel-like, but usually visible.)
5. To wash the pellet, add 500 μL of 75% EtOH (in DEP-C H_2O), and centrifuge at 4°C for 10 min.
6. Pour off the EtOH, keeping the tube inverted, place on a clean Kimwipe, and let the remainder of the EtOH drain for about 10–15 min. Do not let the pellet dry completely because it will be difficult to resuspend. Alternatively, after the wash EtOH is poured off, recentrifuge the samples for 1 min. Carefully pipet off the residual EtOH (100 μL); do not disturb the pellet.
7. Resuspend RNA pellets in 20–100 μL of DEP-C H_2O and keep on ice. For 5×10^5 cells (one 35 mm dish), the pellet is resuspended in 20 μL and yields 1–3 μg RNA/μL. Determine the [RNA] by UV spectrophotometry (*see* **Note 2**).
8. Electrophorese 2 μL of each RNA in a 1% agarose gel with 0.3 μg of EtBr/mL and 1X TBE (standard agarose gel). To each 2 μL of RNA sample, add 3 μL of loading buffer and heat denature at 65°C for 10 min. Put tubes on ice and load directly. Run gel for 2 h at 100V. Examine the gel on an UV transilluminator and photograph. **Figure 1** demonstrates both good and degraded RNA preps.
9. The remainder of the RNA from **step 7** is treated with DNase I. To the 18 μL of RNA add the following: 32 μL of DEP-C H_2O, 5.7 μL of 10X DNase reaction buffer and 1 μL of DNase I [10 U/μL] (Genehunter Corp., Nashville, TN, USA), total volume is 56.7 μL. Incubate at 37°C for 30 min.

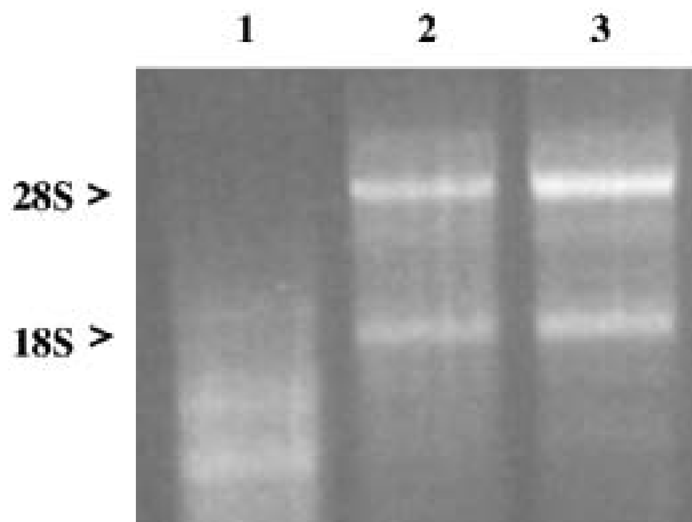

Fig. 1. 1% agarose RNA gel. After DNase I treatment, 1 μL of the purified RNAs are electrophoresed in a 1% agarose/TBE gel in the presence of EtBr. Lane 1 is an RNA preparation that was degraded in the purification and thus is not a good template for RT. Lanes 2 and 3 represent RNA preparations that are not degraded and will be good templates for RT. The 2 most prominent bands represent the 18 S and 28 S ribosomal RNAs. In general the 28 S ribosomal band should be about twice as intense as the 18 S ribosomal band. If there is an increase in intensity of the 18 S ribosomal band, then the RNA preparation is being degraded.

10. Add 40 μL of phenol : chloroform (3 : 1), vortex briefly, place on ice 10 min, centrifuge 4°C for 5 min. Transfer the upper, aqueous phase (50 μL) to a fresh 1.5 mL tube and precipitate the RNA with 5 μL of 3*M* NaOAc (pH 5.2) and 200 μL of 100% EtOH, incubate at –70°C for at least 2 h. Precipitation can go overnight.
11. To pellet the RNA, centrifuge at 4°C for 30 min. Pour off the EtOH and wash the pellet with 500 μL of 70% EtOH (in DEP-C H_2O), and centrifuge at 4°C for 10 min. Pour off the EtOH wash and dry pellet as in **step 6**.
12. The RNA is resuspended in 10–50 μL of DEP-C H_2O. Electrophorese 1–2 μL of each RNA in 1% agarose gel, as above. Obtain A260 reading for 1 μL of the sample and calculate [RNA].

3.2. Reverse Transcription

Reverse transcriptase (RT) will use the purified RNA as a template to synthesize a first strand cDNA in the presence of an oligo d-T primer. The amount of total RNA used in the RT reactions can range from 1 to 5 μg, but the same amount of RNA must be used in each reverse transcription. To verify that the RNA is free of chromosomal DNA, it is important to always include

a RT (–) control. This reaction will include the RNA and all the components except the RT.

1. Denature the RNA template as follows: combine 1–5 μg of RNA (1 μg), 0.5 μL of oligo d-T [0.5 μg/μL], and DEP-C H_2O in a 1.5 mL tube to a total volume of 13 μL. Boil tubes for 1 min and place immediately on ice.
2. To the denatured RNAs add: 1 μL of 0.1 *M* DTT, 4 μL of 5X first strand buffer, 1 μL of 10 m*M* dNTP mix, and 1 μL of Superscript II RT [200 U/μL]. Master mixes should be made as much as possible to decrease pipeting errors (*see* **Note 3**). Incubate at 48°C for 1 h. Superscript II RT has activity at temperatures from 37–55°C. Higher temperatures increase specificity and keep the RNA denatured during the reaction.
3. The RT is heat inactivated by boiling tubes for 2 min or 75°C for 10 min, then place tubes on ice. The RT templates are ready to use in PCR.

3.3. Polymerase Chain Reaction (PCR)

The RT reactions serve as templates with gene specific primers to amplify selected targets in PCR. Many parameters in PCR have to be determined for each gene specific primer pair and template; they include primer and $MgCl_2$ concentrations, primer annealing temperatures, and cycle number (*see* **Notes 4** and **5**). See **Fig. 2** for a rapid approach for the determination of PCR cycle number. Another concern is PCR contamination. A reagent (buffer, H_2O, dNTPs, and so forth) can be contaminated with a DNA template. This is caused by nonsterile techniques such as not changing pipet tips between tubes. Therefore, it is essential that a reagent only (– RT template) control be included with each PCR.

The RT templates are undifferentiated and neural differentiated mouse ES RNAs. We have primers designed to identify stem cells (Rex-1, Oct-4), early neurons (nestin, Sox-1, NeuroD1,2,3, Math-1, Mash-1, Zic-2), epidermis (keratin, BMP-2 and -4), mesoderm (*brachyury*), inhibitors (REST), and a positive control (β-actin). The positive control primers should represent a gene that is expressed at the same level by all cells. In order to focus on only the genes that are differentially expressed in a particular cell population, we have developed a reverse Northern approach to eliminate RT-PCR for genes from our gene specific primer panel that are not differentially expressed (*see* **Subheading 3.5.**).

1. All PCRs are set up in 200 μL, RNase/DNase-free, thin wall tubes.
2. For each 100 μL reaction combine: 2 μL of the total RT reaction, 10 μL of 10X Taq DNA polymerase buffer, 3–5 μL (1.5–2.5 m*M*) of 50 m*M* $MgCl_2$, 1 μL of 10 m*M* dNTP mix, 2 μL (10–20 p*M*) of each primer (i.e., β-actin forward and

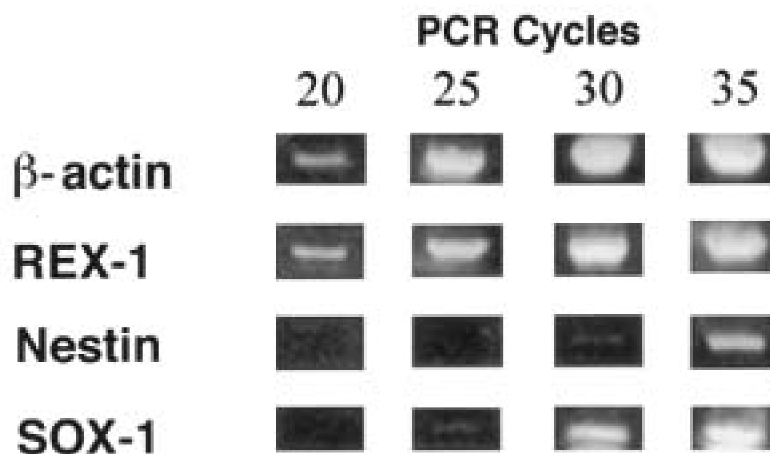

Fig. 2. Optimization of the number of PCR cycles. After the PCR has been optimized to yield the correct size product with gene specific primers, (β-actin, REX-1, Nestin and SOX-1), the number of PCR cycles must be optimized. Standard PCR (100 μL/reaction) is done to determine the number of cycles within the linear range of amplification for each of the genes specific primers. At intervals of 20, 25, 30, and 35 cycles, the thermocycler is paused and 20 μl aliquots are removed from the reactions and electrophoresed in 1.5% agarose gels. The optimized number of cycles selected for the primers is as follows: β-actin = 20 cycles, REX-1 = 25 cycles, Nestin = 35 cycles, and SOX-1 = 30 cycles.

β-actin reverse), 0.5 μL of Taq DNA polymerase (5 U/μL), bring total volume to 100 μL with RNase/DNase-free H_2O. Make sure tops are closed tightly and place tubes in thermocycler.

3. Always test the RT templates first with the + control primer pair (i. e., β-actin primers), before other gene specific primers. Standard PCR conditions are: 94°C/3 min (initial denaturation), 94°C/30–60 sec (denaturation/cycle), 50–60°C/30–60 sec (annealing, primer dependent), 72°C/1–2 min (extension, products synthesized 500 bp/30 sec) for 20–35 cycles (*see* **Note 4**), 72°C/10 min (final extension), and 4°C hold.
4. Electrophorese the β-actin PCR products (expected size 500 bp) in 1–1.5% agarose gel with 1X TBE and EtBr. To 20 μL of PCR product add 5 μL of DNA loading buffer, load into gel. Also include a DNA ladder so that the size of the products can be determined. The gel is run at 100 V for 2 h (**Fig. 3**).
5. Examine the gel on an UV transilluminator and photograph. There should be a PCR band at 500 bp in all reactions except the RT– and the PCR reagent controls. Because all RTs were done with an equal amount of RNA, the intensity/amount of the products should be the same with the control primers (*see* **Note 6**).

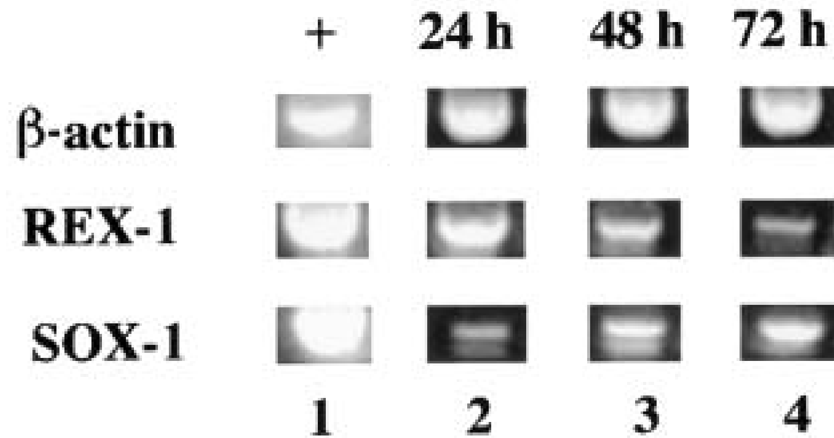

Fig. 3. RT-PCR Analyses of Gene Expression. RT-PCR products (20 μL) are electrophoresed in 1% agarose gel. The products were derived from differentiated cells at 0, 24, and 48 h. The neural marker SOX-1 expression is up-regulated over time, while the stem cell marker REX-1 is down-regulated. The control, β-actin product does not change.

3.4. Purification and Cloning of RT-PCR Products/cDNAs

In our system, we can examine the neurogenic potential of individual genes, but not all neuronal genes will have the same downstream targets. In order to focus on genes that are differentially expressed between the undifferentiated and neuronally differentiated ES cells, we selected 20 cDNAs (ranging in size from 300 bp to 1 kbp) from our primer panel that would be useful to clone into pSK. These clones provide a stable source of gene-specific probes and targets for *in situ*, Northern, and Southern hybridizations. To generate cDNAs for cloning, "new" primers are designed that contain unique restriction enzyme sites added to the 5′ ends of the original forward and reverse primers (*see* **Note 7**). Select an RT template that gives an abundant PCR product in the above RT-PCR. The PCR is repeated with the "new" primers under the previously optimized PCR conditions. This is a very straightforward and efficient approach, as 20+ cDNAs can be cloned and isolated in a week.

3.4.1. Gel Purification of cDNAs

To gel purify the cDNAs, there are several kits available. We prefer to use the DNA gel extraction kits from either Invitrogen Life Technologies or Qiagen, both are quick protocols that yield greater recovery of the cDNAs.

1. Pour a standard 1% agarose gel in TBE and use a comb with wells that will be able to hold 75 μL.
2. Combine 60 μL of PCR with 15 μL of loading buffer. Load all of the sample, and electrophorese at 100 V for 2 h. Examine the gel on an UV transilluminator.
3. Remove the gel from the tray and excise the bands of the correct size cDNAs, then place each in a 1.5 mL tube. Extract and purify the DNA from the gel slice according to the manufacturer's protocol.
4. Check the recovery of the cDNAs, run 5 μL (100–200 ng) from a total volume of 50 μL of each in a 1% agarose gel.
5. Set up restriction enzyme (RE) double digests with purified cDNAs. Combine 40 μL of the purified cDNA with 6 μL of 10X RE buffer, 0.5 μL (10 U/μL) of the appropriate RE (i.e., *BamH*I and *EcoR*I) in a total reaction vol. of 60 mL. Always refer to the manufacturers recommended reaction conditions. Incubate at 37° overnight.
6. At the same time, set up the same double digest with the cloning vector (4 μg of pBS SK) in a total reaction volume of 40 μL.
7. After incubation, the digested, purified cDNAs are put through another gel extraction and purification exactly as in **steps 1–4**. Note that the size of the products on the gel won't change after digestion because only a few nucleotides on each end have been removed. At this point the BamHI/EcoRI cDNAs are ready to ligate.

3.4.2. CIAP Reaction with the BamHI/EcoRI Digested pSK

1. Add 5 μL of 10X dephosphorylation buffer directly to the double digest, then mix 2 μL of CIAP with 3 μL of enzyme dilution buffer and add the 5 μL to the reaction.
2. Incubation temperature and time are restriction enzyme dependent, RE that leave 5′ overhang incubate at 37°C for 30 min, blunt or 3′ overhang incubate at 50°C for 1 h. The CIAP is heat inactivated at 75°C for 15 min.
3. Next, a phenol:chloroform extraction is done to remove CIAP. Add P : C v/v (50 μL), vortex 30 sec, centrifuge at room temperature for 3 min. Recover the aqueous phase and precipitate the DNA with 5 μL of 3*M* NaOAc (pH 5.2) and 200 μL of 100% EtOH. Incubate at –70°C/1 h or –20°C overnight.
4. Recover the BamHI/EcoRI/CIAP pSK by centrifuging at 4°C/30 min. Pour off the EtOH after centrifugation.
5. Add 500 μL 70% EtOH and centrifuge 4°C/10 min, discard the EtOH, keep the tube inverted and let dry 10–15 min (*see* **Subheading 3.1.6.**).
6. The DNA is resuspended in 50 μL of ddH2O, let sit at room temperature for 15 min.
7. To check the recovery of the purified DNA, run 5 μL in a 0.8% agarose gel as described above. Prior to ligation, dilute the vector to (5–10 ng/μL).

3.4.3. Ligation and Transformation of the BamHI/EcoRI cDNAs

To ligate the *BamH*I/*EcoR*I cDNAs into pSK *BamH*I/*EcoR*I/CIAP set up the following ligations:

1. L1: 0.5 µL of pSK, 6.5 µL of cDNA, 2 µL of 5X T4 DNA Ligase Buffer, 1 µL of T4 DNA Ligase (1 U/µL), TV=10 µL.
2. L2: 0.5 µL of pSK, 2.5 µL of cDNA, 1 µL of 5X T4 DNA ligase buffer, 1 µL of T4 DNA ligase, TV=5 µL.
3. L0: 0.5 µL of pSK, 6.5 µL of H_2O, 2 µL of 5X T4 DNA ligase buffer, 1 µL of T4 DNA Ligase, TV=10 µL. The L0 reaction is a control for self-ligation of the vector alone (*see* **Note 8**).
4. Incubate the ligations in a water bath at 4°C overnight.
5. Transformation: Thaw DH5 α E. coli competent cells on ice. The total volume of each ligation is brought to 50 µL with H_2O.
6. Place four tubes (15 mL Falcon 2059) on ice, one for each ligation and a positive control.
7. Aliquot 50 µL of competent cells in each tube, then add 5 µL of each ligation to the appropriate tube (the remainder of the ligations are stored at –20°C and can be used for up to 1 wk).
8. For the positive control, add 2 ng of pSK to 50 µL of competent cells. Keep the tubes on ice for 30 min, heat shock cells at 42°C/45 sec (critical step), then return to ice for 2 min.
9. Add 600 µL SOC (warmed to room temperature) to each tube and place tubes in a shaker incubator at 37°C/1 h, 220 rpm.
10. Plate the cells on LB agar with ampicillin (40 µg/mL). For L1 and L2, add 100 µL and 400 µL/plate for each. To the L0 add 100 µL/plate, and for pSK add 10 µL/plate.
11. Let the plates air dry about 30 min then place inverted in a 37°C incubator overnight (*see* **Note 9**).
12. Set up replica plates of single, isolated colonies from the L1 and L2 plates ***(5)***.
13. Incubate plates inverted at 37°C overnight.

3.4.4. Colony Lysis PCR

Colony lysis PCR is done to quickly screen colonies for cDNA inserts. This takes advantage of the T3 and T7 primer binding sites, which flank the multiple cloning site in pSK. The cDNA insert is amplified in PCR with the T3 and T7 primers.

1. Aliquot 50 µL of colony lysis buffer into a 1.5 mL tube for each colony to be analyzed. A sterile pipet tip is used to pick each colony (a small amount of bacteria just visible on the end of the tip is more than enough). Make sure the bacteria are mixed well with the colony lysis buffer.

2. Incubate the tubes in boiling H_2O for 10 min.
3. Centrifuge at room temperature for 10 min, and transfer the clear supernatant to a fresh tube. This is the template for PCR.
4. For PCR combine the following in a 200 μL tube: 8.4 μL of H_2O, 2 μL of 10X Taq DNA polymerase buffer, 1.5 μL of 50 m*M* $MgCl_2$, 1.6 μL of 250 m*M* dNTP mix, 2 μL (10 p*M*) of the T3 and T7 primers, 2 μL of colony lysate, and 0.5 μL of Taq DNA polymerase (5 U/μL), TV=20 μL. To decrease pipeting errors, make a master mix that includes all of the reagents except the primers and templates/lysates.
5. The PCR conditions are 94°C/3 min, 94°C/30 sec, 45°C/1 min, 72°C/2 min for 35 cycles, 72°C/7–10 min, 4°C hold.
6. Analyze 20 μL of each in a 1% agarose gel, at 100V for 2 h. The PCR product will be 120 bp (this represents the pSK MCS) if there is no insert. The product size is 120 bp larger than the original PCR product if an insert is present (i.e., original β-actin product is 500 bp, the product on this gel will be 620 bp, *see* **Fig. 4**). Those colonies that contain inserts are selected for DNA plasmid column preps.
7. DNA column preps are done using the Qiagen column prep kits and protocol.
8. The final DNA plasmids (i.e., pSK/β-actin and so forth) are sequenced to verify the correct cDNA. DNA plasmids can be stored indefinitely at –20°C.

3.5. Reverse Northern Hybridizations

3.5.1. Reverse Northern Protocol

We have adapted the reverse Northern protocol to allow us to screen several genes that may be differentially expressed between RNA populations. The pSK/gene specific cDNAs and the T3/T7 PCR provide an unlimited source of target cDNAs to be hybridized with first strand cDNA probes generated from the undifferentiated and neural differentiated ES cell RNAs. The use of this approach prior to RT-PCR eliminates genes from our panel that are not differentially expressed. Thus, the focus for RT-PCR is directed only to those genes that are differentially expressed, and can eliminate many reactions.

1. Electrophorese 50–100 ng of each gel purified PCR cDNA in a standard 1% agarose gel. It is not necessary to run a DNA ladder on this gel because the size of the PCR products should have been checked for correct size following gel purification. Be sure that an equal amount of DNA is loaded for each sample in order to make an accurate comparison between hybridization signals.
2. The gel is run at 100 V for 2–3 h. Photograph the gel on an UV transilluminator to verify equal loading of samples.
3. A standard Southern blot transfer is set up and the cDNAs are transferred to a nylon membrane (Duralon memebrane) in 10X SSC overnight. (Be sure to cut a corner of the gel so that you know the orientation of the samples.) To prepare the membrane for hybridization, follow standard protocol and manufacturer's instructions (Stratagene) ***(5)***.

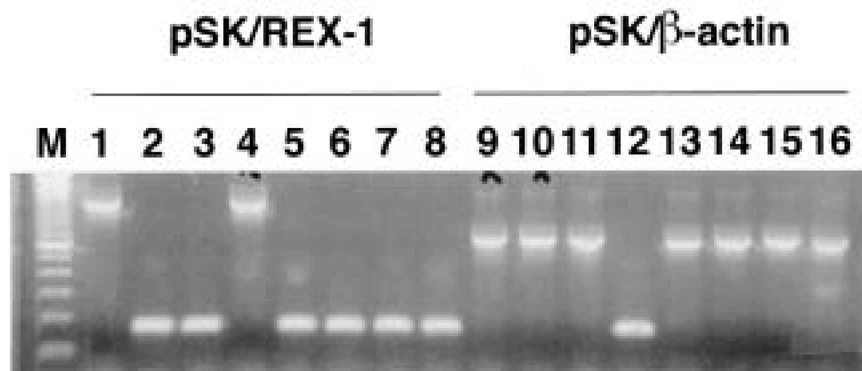

Fig. 4. Colony Lysis PCR. Transformed colonies were selected from pSK/REX-1 and pSK/β-actin for colony lysis PCR analyses. The size of the purified products for β-actin and REX-1 are 500 bp and 930 bp, respectively. Therefore, the colony lysis PCR product should be 120 bp larger. For each reaction 20 μL of the PCR product are electrophoresed in a 1% agarose gel. Lanes 1 and 4, are pSK/REX-1 colonies that contain the correct size cDNA insert (1.1 kb product), while lanes 2, 3, 5, 6, 7, and 8 do not (120 bp product). Lanes 9–11 and 13–16, are pSK/β-actin colonies that contain the correct size cDNA insert (620 bp).

4. Mark the position of the wells on the membrane with a pencil prior to removing filter from the gel. After transfer, check the gel on an UV transilluminator to verify complete transfer of the cDNAs.
5. To prepare 100 mL of the prehybridization/hybridization solution, combine the following: 50 mL of deionized formamide, 25 mL of 20X SSPE, 10 mL of 50X Denhardt's solution, 1 mL of 10% SDS, and 14 mL of sterile H_2O. The prehybridization solution can be stored at –20°C for 1 month.
6. The filter should be placed in a secure container to avoid leakage of the radioactive probe. The membranes are placed in seal-a-meal bags and then in a second sealed plastic container. Alternatively, hybridization bottles can be used.
7. Add 10 mL of prehybridization solution to the membranes (11 cm × 7 cm).
8. Denature salmon sperm DNA (10 mg/mL) by boiling for 10 min, then place on ice and add 100 μL/membrane to the prehybridization solution. The bags are sealed and placed in the second container.
9. Incubate at 42°C for 2–3 h with rotation, as the fluid needs to move freely over the membrane, so the probes will have access to the target cDNAs.

3.5.2. Synthesis of ^{33}P-dATP Probes

Total RNAs from the undifferentiated and neuronally differentiated ES cells serve as the templates in the synthesis of ^{33}P-dATP probes. The total RNA does not have to be DNased for this procedure.

1. Combine the following: RNA (5–10 μg), DEP-C H_2O, and 1 μL of oligo-dT primer to a TV=25.5 μL.
2. Boil 1 min and quick cool on ice.
3. Add to the denatured templates: 10 μL of 5X first strand buffer, 2.5 μL of 0.1 *M* DTT, 6 μL of 0.5 m*M* dNTP mix (-dATP), 5 μL of ^{33}P-dATP (3000 Ci/mmol), 1 μL of MMLV RT (200U/μL) (*see* **Note 10**).
4. The labeling reaction is incubated at 42°C/1 h, then an additional 1 μL MMLV RT is added and incubation continued for another hour.
5. The RT is heat inactivated by boiling 2 min.
6. The tubes are placed on ice, and total volume brought to 160 μL with TE buffer.
7. The probes are purified on micro bio-spin P-30 chromatography columns (Biorad) according to manufacturer's instructions.
8. Determine incorporation of ^{33}P-dATP ***(2,5)***. Our typical results are in the range of $5 \times 10^5 - 1 \times 10^6$ cpm/μL. The probes can be stored at –70°C for a few weeks.
9. Hybridization is carried out overnight at 42°C. The probe is boiled for 10 min ($2–3 \times 10^6$ cpm/mL for each filter) and added directly to the prehybridization solution. The bag is then resealed and placed in the second container. Once the radioactive probe has been added, be sure the containers are sealed completely.
10. After overnight hybridization, the solution is discarded in the radioactive waste and the membranes are washed 4X. Wash 2X with 2X SSC at room temperature for15 min (discard washes in radioactive waste), wash 1X with 1X SSC/0.1% SDS at 42°C/30 min, final wash 0.1X SSC at room temperature for 15 min.
11. Let membrane air dry on the bench top. Expose membrane to film, exposure time varies from overnight to 3 d (**Fig. 5**).

4. Notes

1. The precautions for working with RNA are listed in many references ***(2,5)***. Be sure to always wear gloves and use only sterilized tips and glassware at minimum. It is also a good idea to have reagents that are only used with RNA. In all RNA protocols, DEP-C H_2O is always used in reagents, reactions, and always to resuspend RNAs.
2. UV spectrophotometry is done to determine the [RNA] or [DNA]. Make a 1 : 100 dilution of each sample in TE and obtain A_{260} and $A_{260/280}$ ratio. The A_{260} is used to determine concentration, for RNA(A_{260} × 40 μg/mL × dilution factor), and DNA (A_{260} × 50 μg/mL × dilution factor). The $A_{260/280}$ ratio indicates the purity of the sample, and should be in the range of 1.7–2.0. A ratio less than 1.7 indicates that the prep contains contaminants that could interfer with the RT-PCR.
3. Master mixes contain all the common reagents for a particular reaction, except templates and primers. It is a good rule to make at least enough of the master mix for one additional reaction (i.e., if you have nine reactions, then the master mix is made for 10 reactions). This decreases the number of possible pipetting errors. Be sure to mix well prior to use.

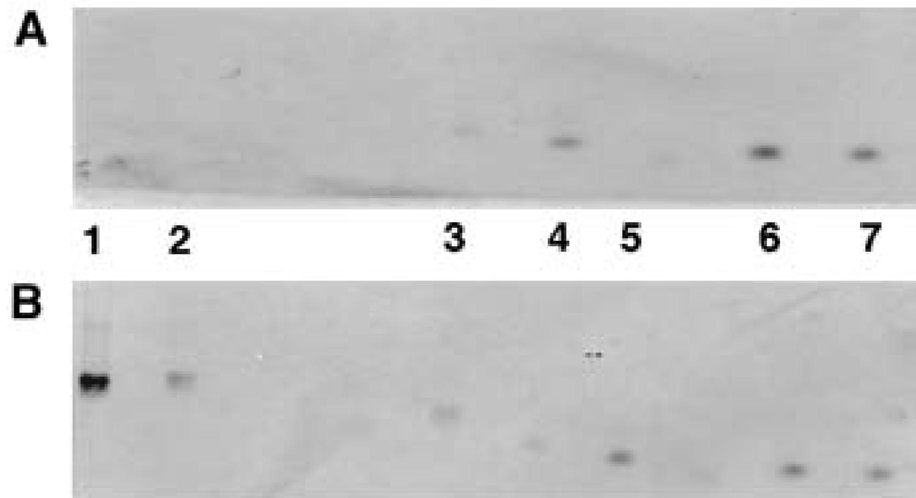

Fig. 5. Reverse Northern Hybridization. Ten cDNAs (50 ng/each) were selected and electrophoresed in a 1% agarose gel. The cDNAs were transferred to a nylon membrane in 10 X SSC overnight. The membranes were hybridized o/n at 42°C with α^{33} P-dATP probes generated from undiffernitiated ES cell RNAs (A) and neuronally differentiated ES RNAs (B). After the membranes were stringently washed, they were exposed to film for 2 d. Seven of the ten cDNAs gave hybridization signals. Lane 7 represents the β-actin control cDNA. Lanes 1, 2, 4 and 5 represents genes which appear to be differentially expressed and thus are selected for RT-PCR analyses.

4. Gene specific primer design and PCR optimization have already been covered extensively ***(1,2,5)***. **Figure 2** shows a PCR optimization gel.
5. Here are three web sites that are useful:
   a. PCR Jump Station, http//highveld.com/redirect/ad15.html, this site has links for protocols, and problem solving.
   b. NCBI: www.ncbi.nlm.nih.gov/: At this site, nucleotide sequences can be obtained from Genbank for primer design and restriction enzyme maps.
   c. Web Cutter 2.0: www.firstmarket.com/cutter/cut2.html, this site will generate a restriction map for any DNA sequence, which is useful for the selection of unique RE sites for PCR primers and cloning.
6. A PCR product with the RT- template indicates that there is still some chromosomal DNA present in the prep. When a product of the same size is amplified with all of the templates including the PCR reagent control, it indicates that some component in the reaction is contaminated with an extraneous DNA template. The best way to resolve the contamination problem is to discard the reagents that were used and start with fresh reagents. If more than one PCR product is present,

then the reaction conditions need to be optimized for the primer pair (i.e., alter annealing temperature, [primer], $MgCl_2$). If none of the templates yield PCR products with the control primer pair (i.e., β-actin), it is possible one of the reagents could have been left out of either the RT reaction or PCR (i.e., RT, Taq DNA polymerase, dNTPs). Another possibility is that the RNA sample could be degraded. To troubleshoot, repeat the PCR with only a couple of the templates. If a product is obtained, then some component of the PCR was not present in the initial PCR. If there still is no product, electrophorese 2 μL of the RNAs in a 1% agarose gel to check for RNA degradation (*see* **Fig. 1**). If the RNA is not degraded, repeat both the RT reaction and PCR (other remedies may be to increase the amount of RNA in the RT reaction or the amount of template in the PCR). However, if the RNA is degraded, it will be necessary to obtain new RNA samples. If the experimental primers (i.e., Oct-4, Sox-1, and so forth) did not yield PCR products, the results may be correct, as long as the PCR conditions have been previously optimized and the above positive and negative controls gave the expected results. Templates that are very G:C rich can sometimes be difficult to amplify, in this case add DMSO (10%) to the PCR. DMSO acts as additional denaturant.

7. A restriction map for each PCR product must be generated, in order to select unique restriction enzymes sites (those that do not cut within the PCR product) to add onto the 5′ ends of the primers (*see* **Note 5**). Try to use the same RE sites as much as possible with different primer pairs, if there are several cDNAs that will be cloned. This simplifies the cloning, as it limits the number of vectors that have to be prepared for ligations. For example, we have cloned 20 cDNAs using either BamHI/EcoRI or XbaI/HindIII sites. Additionally, these sites can also be used to linearize the plasmids for +/– RNA probe synthesis with T3 or T7 RNA polymerase. For this protocol, any standard vector can be used to clone the cDNAs as long as T3 and T7 promoters flank the MCS.
8. The general rule for ligation reactions is that the insert (cDNA) is always present at a higher concentration than the vector (pSK). The recommended molar ratio is 3 : 1(insert : vector) ***(5)***.
9. Record the number of colonies on each plate if feasible. Some plates may be covered with colonies (to numerous to count). The L0 plate should have far fewer colonies than the L1 and L2 plates. An increase in colonies on the L0 plate indicates that the CIAP reaction was not very efficient, as the vector was able to self ligate at a high frequency. If colonies are only on the positive control plate, and there are no colonies on the L1 and L2 plates, the transformation protocol and reagents worked fine, but there is a problem with the ligations. Repeat the ligation reaction and transformation.
10. This reaction has been done with α-^{35} S-dATP, but the α-^{33} P-dATP probes give a better hybridization signal and requires a shorter exposure time. When working with radioactive isotopes take all precautions to avoid contaminating the work area.

Acknowledgment

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19

Differential Display

Isolation of Novel Genes

Theresa E. Gratsch

1. Introduction

Differential display is an efficient and reproducible method for the detection of differential gene expression between a variety of cells and/or tissue populations ***(1)***. The approach is based on reverse transcription with oligo-dT anchored primers and PCR in the presence of the original anchor primer and an arbitrary 13-mer ***(2*,3)**. Three oligo-dT anchored primers and 80 arbitrary 13-mers are used to amplify all mRNAs present in particular cells or tissues. Both primers contain *Hind*III sites on their 5′ ends to facilitate the cloning of the differentially expressed cDNAs. The sensitivity of RT-PCR allows for the amplification of rare messages. Additionally, several differentially expressed cDNAs can be isolated within a week. They are large enough to identify a specific mRNA, and small enough to be separated by size in standard polyacrylamide sequencing gels. The cDNAs range in size from 200 to 800 bp and represent the 3′ ends of mRNAs expressed in a particular cell or tissue type.

We have used this method to isolate genes that are both upregulated and downregulated in neuronal differentiation. Simultaneously, genes that represent stem cell markers and inhibitors of neuronal differentiation can also be isolated. The novel cDNAs are used to probe a mouse cDNA library in order to isolate a full-length clone. Additionally, these cDNAs are used as templates to synthesize digoxygenin riboprobes for *in situ* hybridization or radioactive probes for Northern and Southern hybridization studies.

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1. Materials

2.1. RNA Extraction and Purification

1. Trizol (Invitrogen Life Technologies, Rockville, MD, USA).
2. All other materials are listed in Chapter 18 on RT-PCR, **Subheading 2.1.**

2.2. Reverse Transcription Reaction (DD-RT)

1. Purified RNA, 200 ng for each RT reaction.
2. Anchored oligo-dT primers, H-T_{11}A, H-T_{11}C, H-T_{11}G (Genehunter Corp., Nashville, TN, USA).
3. 5X first strand Buffer, (Invitrogen Life Technologies).
4. MMLV reverse transcriptase (200 U/μL) (Invitrogen Life Technologies).
5. 0.1 *M* DTT (Invitrogen Life Technologies).
6. 250 μ*M* dNTP mix-stock solution is 100 m*M* dNTPS (Invitrogen Life Technologies).
7. DEP-C H_2O.

2.3. Differential Display PCR (DD-PCR)

1. RT template (2 μL).
2. RNase/DNase-free H_2O (ddH_2O).
3. Taq DNA polymerase (5 U/μL); 10X PCR buffer; 50 m*M* $MgCl_2$ (Invitrogen Life Technologies).
4. 25 μ*M* dNTP mix.
5. Arbitrary primers, H-AP 1-80 (Genehunter Corp.).
6. ^{33}P-dATP, 3000 Ci/mmol, (New England Nuclear, Boston, MA, USA).

2.4. DD-PCR Sequencing Gel Electrophoresis

1. 6% urea/polyacrylamide gel in TBE Buffer, Gel-Mix 6 (Invitrogen Life Technologies).
2. 10X TBE buffer (for 1 L): 108 g Tris, 55 g boric acid, 3.7 g EDTA, to 1 L with ddH_2O.
3. DNA loading dye, 95% formamide, 10 m*M* EDTA, and a pinch of xylene cyanol and bromophenol blue dyes.
4. Standard sequencing gel electrophoresis unit (40 × 30 cm), and a constant current power supply (Fisher Scientific).
5. 3MM Whatman filter paper.
6. Gel dryer.
7. X-ray film and cassette.

2.5. Reamplification of DD-PCR Products

1. Needle (18 gauge) and scalpel or razor blade.
2. RNase/DNase-free H_2O.
3. Taq DNA polymerase (5 U/μL), 10X Taq DNA polymerase buffer, 50 m*M* $MgCl_2$ (Invitrogen Life Technologies).

4. 250 μ*M* dNTP mix (Invitrogen Life Technologies).
5. The original H-T_{11}A,C,G primer and H-AP 1-80 primer (Genehunter Corp.).
6. Template/gel slice containing band to be reamplified.

2.6. Northern/Reverse Northern Hybridizations

1. NEB blot labeling kit (New England Biolabs).
2. Duralon nylon membranes (Stratagene, LaJolla, CA, USA).
3. Agarose and 10X TBE buffer.
4. Deionized formamide.
5. 37% formaldehyde solution.
6. α-^{33}P-dATP, 3000Ci/mmol (New England Nuclear).
7. 20X SSC: NaCl 175.3 g, sodium citrate 88.2 g, dissolve in 800 mL of ddH_2O. Adjust pH to 7.0 with 10*N* NaOH, to 1 L with ddH_2O and autoclave.
8. 20X SSPE: NaCl 175.3 g, NaH_2PO_4 27.6 g, EDTA 7.4 g in 800 mL H_2O, pH to 7.4 with NaOH, bring volume to 1 L and autoclave.
9. Salmon sperm DNA (10 mg/mL).
10. 10% SDS (sodium dodecyl sulfate).
11. 50X Denhardt's solution: Ficoll 5 g, polyvinyl pyrrolidone 5 g, BSA (fractionV) 5 g, to 500 mL with ddH_2O, filter and store in 10 mL aliquots at –20°C.
12. 0.5 *M* EDTA pH 8.
13. RNA loading buffer (for 10 mL): 2.5 mL glycerol, 0.5 mL 10% SDS, 0.5 mL of 0.5 m*M* EDTA to 10 mL with sterile H_2O.
14. Formaldehyde gel loading buffer (for 10 mL): 7.1 mL formaldehyde, 0.8 mL sodium phosphate (pH 7.0), 2.1 mL sterile H_2O, and a pinch of bromophenol blue.

2.7. Cloning DD-PCR Products/cDNAs

1. DNA gel extraction/purification kit (Invitrogen Life Technologies).
2. HindIII restriction enzyme (10 U/μL).
3. pBS SK cloning vector (Stratagene).
4. T4 DNA Ligase (1 U/μL) and 5X ligase buffer (Invitrogen Life Technologies).
5. DH5α *E. coli* competent cells (Invitrogen Life Technologies).
6. SOC medium (Invitrogen Life Technologies).
7. LB agar and LB broth.
8. Ampicillin (20 mg/mL).

3. Methods

3.1. RNA Extraction and Purification

1. To extract and purify the RNAs follow the protocol exactly as described in Chapter 18 on RT-PCR (*see* **Note 1**).
2. Dilute each of the RNAs to (100 ng/μL) in DEP-C H_2O. Each RT reaction requires 200 ng of RNA. The RNA dilutions are not reused, so dilute only enough RNA for the present reactions. To compare two RNA templates (undifferentiated

and neuronally differentiated ES cell RNAs) with all three anchored oligo-dT primers, you will need a total of 600 ng/RNA. Always make a fresh RNA dilution for each round of reverse transcription.

3.2. Differential Display Reverse Transcription (DD-RT)

Total RNA (Dnase I treated) will serve as the template in RT reactions with three anchored oligo-dT primers (H-T11A, H-T11C, H-T11G) ***(1)***. Label all tubes clearly, for each RNA sample you will need three tubes (i.e., ES-A, ES-C, ES-G) (*see* **Note 2**). Make a master mix with all of the DD-RT reagents, except the RNAs and primers (always make extra).

1. Set up the RT reactions in 200 μL tubes. All reagents are thawed on ice. Combine the following for each: 2 μL (200 ng) of RNA, 8.4 μL of DEP-C H_2O, 1 μL of 0.1 *M* DTT, 4 μL of 5X first strand buffer, 1.6 μL of 250 μ*M* dNTP mix, 2 μL of H-T11M primer (2 μ*M*), TV=19 μL. Always include an RT control for each RNA (*see* **Note 3**). The reactions are carried out in a thermocycler under the following conditions: 65°C/5 min (denaturation), when temperature comes down to 37°C, pause the machine and add 1 μL of MMLV RT to each tube, then continue incubation 37°C/60 min (first strand synthesis). The RT is heat inactivated at 75°C/5 min. The templates are ready to use in DD-PCR. The reaction yields enough templates for at least 9 reactions. The RT templates are stored at –70°C.

3.3. Differential Display PCR (DD-PCR)

PCR is carried out with the original H-T11A/C/G primer, an arbitrary 13-mer primer (i.e., H-AP 1–80) and the above RT templates to amplify a series of mRNAs. The DD-PCR products are radioactively labeled with ^{33}P-dATP (*see* **Note 4**).

1. I usually do not set up more than 24 reactions at a time, so for six templates, four arbitrary 13-mers are selected, i.e., H-AP 26, 27, 28, 29. Make a master mix of all the PCR reagents (RT template and primers). It is critical that the original PCR tubes are labeled clearly with original template and primer pairs (i.e., ES-A26, ES-C26, ES-G26, N-A26, N-C26, N-G26, and so forth) and recorded (*see* **Note 2**). For each DD-PCR combine the following reagents in a 200 μL RNase/DNase-free PCR tube: 9 μL of ddH_2O, 1 μL of 50 m*M* $MgCl_2$, 1.6 μL of 25 μ*M* dNTP mix, 2 μL of H-AP 26 (2 μ*M*), 2 μL of H-T_{11}A (2 μ*M*), 2 μL of RT template, 0.2 μL of ^{33}P-dATP, 3000Ci/mmol, and 0.3 μL of Taq DNA polymerase. Repeat for each template and primer pair. Be careful to add the correct H-T_{11}M primer to the corresponding template (i.e., H-T_{11}A added to ES-A26) or the reaction will not yield DD-PCR products.
2. The conditions for DD-PCR are: 94°C/3 min, 94°C/30 sec, 42°C/2 min, 72°C/2 min for 40 cycles, 72°C/7 min and 4°C hold. DD-PCR samples can be stored at –20°C for 1 wk. The remainder of the RT templates can be stored at –70°C.

3.4. DD-PCR Sequencing Gel Electrophoresis

The ^{33}P-dATP labeled DD-PCR products are separated on standard 6% polyacrylamide sequencing gels with 1X TBE (*see* **Note 5**).

1. The sequencing gel is pre-run for 30 min at 55 W. Prior to starting the pre-run, flush the urea out of the wells and then load 3 μL of DNA loading dye into a few wells spread across the gel (*see* **Note 6**).
2. During the pre-run, prepare the PCR products for electrophoresis. Mix 3.5 μL of each DD-PCR with 2 μL of DNA loading buffer. The samples are heat denatured at 80°C/2 min immediately before loading. Load 4 μL of each sample onto the gel; urea should be flushed out of each well prior to loading sample. In order to reproduce results (i.e., differentially expressed bands), always load equal amounts of samples. The samples are loaded so that the undifferentiated ES and neuronally differentiated (N) templates with the same primer pair are run next to each other, i.e., ES-A26, N-A26, ES-C26, N-C26, ES-G26, N-G26. If duplicate reactions are done, then run them next to each other.
3. The gel is run for about 3.5 h [when the slower moving dye front (xylene-cyanol) is at the bottom of the gel] at 55 W constant power.
4. The gel is then blotted to a sheet of Whatman filter paper and then covered with clear plastic wrap. The gel is dried under vacuum at 80°C/1 h.
5. Place dried gel in cassette and expose to film overnight. Be sure to mark the exact orientation of the film on the gel, use a needle to mark the alignment. This is critical for the recovery of the differentially expressed cDNAs. Leave the plastic wrap over the gel during the exposure because sometimes portions of the gel remain damp and cause the film to stick to the gel. An overnight exposure (16 h) is usually sufficient to observe clear, distinct bands on the autoradiogram (**Fig. 1A**).

3.5. Reamplification of DD-PCR Products/cDNAs

1. Align the dried gel and autoradiogram with the needle marks made in **Subheading 3.4., step 5**, and at one end, staple them together so they maintain the correct alignment.
2. Using a needle mark, punch holes in the gel to identify the position of any differentially expressed bands on the gel. For each band of interest label a 1.5 mL tube with the correct template and DD primer pair (i.e., N-A26), often there is more than one band in a particular lane, in which case use 1, 2, 3, and so forth (i. e., N-A26-1). I also record the relative position (top, middle, bottom) of each band on the gel, bands at the top of the gel represent the largest DD cDNAs. Select at least one band that is abundantly expressed with both templates (undifferentiated and neuronally differentiated RNAs). This common band is the positive control for the reamplification and subsequent analyses (i.e., hybridization studies). The bands of interest are excised from the dried gel with a scalpel or razor blade and the gel/paper slice placed into a labeled tube. Store the bands at –20°C. Do not proceed to **step 3** until the DD-PCR bands are reproducible.

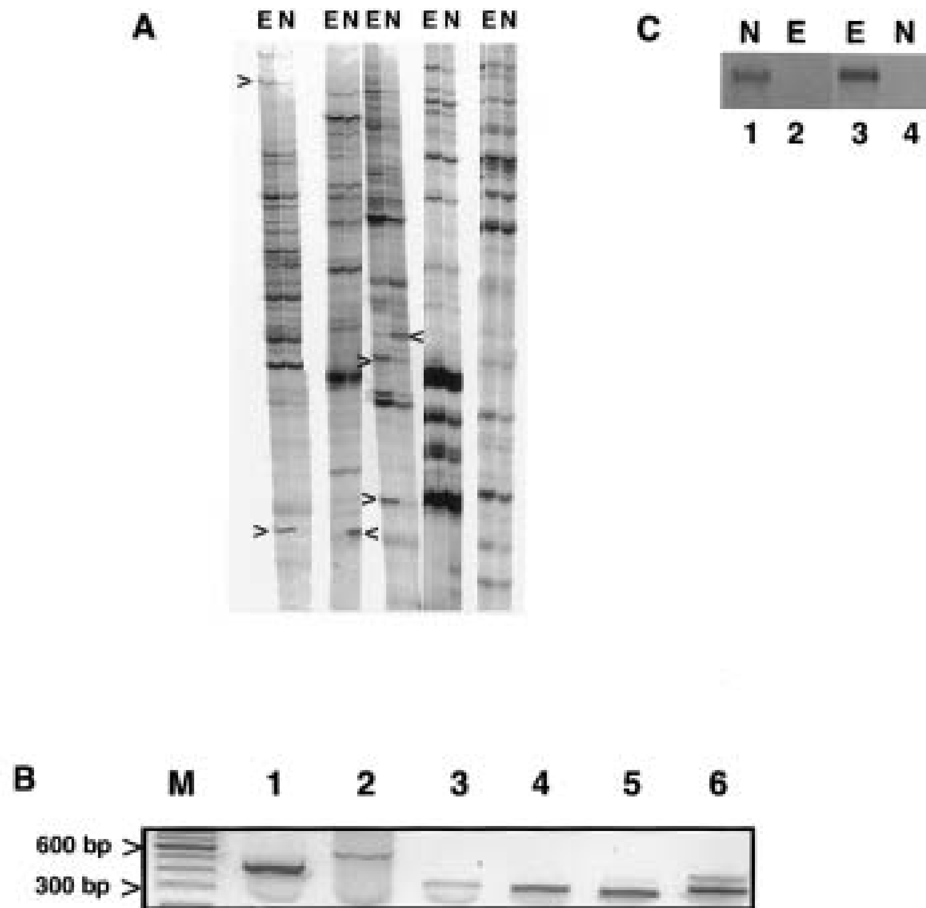

Fig. 1. Isolation, Reamplification and Northern Hybridization of DD-PCR products. **(A)** DD-PCR Sequencing Gel. Undifferentiated ES cell RNAs (E) and neuronally differentiated ES cell RNAs (N) are used as templates in DD-RT-PCR with the same H-T_{11}M and H-AP primers. 4 μL of each DD-PCR product are electrophoresed in 6% polyacrylamide sequencing gels. The gels are dried and exposed to film over night. The arrows indicate bands that are differentially expressed between the two populations of mRNAs. The reactions were repeated to verify the differentially expressed bands. **(B)** Reamplification of DD-PCR cDNAs. Differentially expressed cDNAs were excised directly from the gels and served as templates in PCR with the original primer pair. 30 μL of each reamplified PCR product was electrophoresed in 1.5% agarose gels. Lane M is a 100 bp DNA ladder to size the PCR products. Lanes 1–6 represent reamplified products. The size of the PCR products should correspond to their position on the DD-PCR sequencing gel, i.e., bands located at the top of the gel should be larger than those further down on the gel. Lane 6 also demonstrates that one band can represent more than one PCR product. **(C)** Northern Hybridization. To verify that a particular cDNA is differentially expressed between the two populations of mRNAs, a Northern blot is done. 10 μg of each RNA (undifferentiated and differentiated) is electrophoresed

3. The DD cDNAS are reamplified in another round of PCR with the gel slice and the original primer pair. The templates are the gel slices. Cut off 1/4 of each gel slice and place the filter paper in a 200 μL PCR tube. The following reagents are added to each tube: 17 μL of dd H_2O, 4 μL of 10X Taq DNA polymerase buffer, 3 μL of 50 m*M* $MgCl_2$, 3.5 μL of 250 μ*M* dNTP mix, 4 μL of original H-T11M primer, 4 μL of original H-AP primer, and 0.5 μL of Taq DNA polymerase (5 U/μL). The original PCR conditions are used for reamplification.
4. Electrophorese 30 μL of each PCR product in a standard 1.5% agarose gel, use a 100 bp DNA ladder to size the products (**Fig. 1B**). The gel is run at 100 V for 2 h, and then photographed as usual. The size of the cDNAs should correspond to their relative position on the DD sequencing gel. The cDNAs are gel purified as described in Chapter 18, **Subheading 2.4.1.** Repeat the reamplification PCR in **step 3** using 5–10 μL of the gel-purified cDNAs as templates. Repeat the agarose gel purification. The purified cDNAs can be cloned, act as templates in probe synthesis for Northern and *in situ* hybridizations, and as target DNAs in reverse Northern blot hybridizations to verify differential gene expression.

3.6. Northern and Reverse Northern Hybridizations

The Northern and reverse Northern hybridizations are used to verify and eliminate cDNAs that are not differentially expressed between the embryonic stem (ES) and neuronal differentiated ES cells. Differential display can yield a large number of cDNAs to be analyzed. The reverse Northern approach allows you to screen a large number of cDNAs at the same time; some of the cDNAs will not be differentially expressed (i.e., false positives) and can be eliminated. Positive cDNAs are then used as probes in Northern hybridizations with undifferentiated and neuronally differentiated ES cells to verify differential expression. The reverse Northern hybridization protocol has already been described in Chapter 18, **Subheadings 3.5.1.–3.5.2.** A Northern hybridization protocol is described below.

1. The RNAs are electrophoresed in a 1% agarose formaldehyde gel. The gel is prepared in a fume hood as follows: dissolve 1.5 g of agarose in 120 mL of sterile H_2O, let cool slightly, add 3 mL of sodium phosphate (pH 7.0), and 27 mL of 37% formaldehyde. The formaldehyde gel running buffer is prepared as follows:

Fig. 1. *(continued)* in a 1% agarose/formaldehyde gel. The RNAs are then transferred to nylon membranes. ^{33}P-dATP labeled probes of the differentially expressed cDNAs (N 144 and E 86) are hybridized at 42°C overnight. After hybridization the filters are washed stringently and exposed to film for 1 week. Lanes 1 and 2 were hybridized with a probe N 144. Lane 3 and 4 were hybridized with an E 86 probe. N 144 was isolated from the neuronally differentiated ES cells and E 86 was isolated from undifferentiated ES cells.

215 mL of 37% formaldehyde, 24 mL of 1.0 *M* sodium phosphate (pH 7.0), and sterile H_2O to make 1200 mL. To prepare the RNA combine the following: one volume of RNA (5–10 μg), an equal volume of formaldehyde gel sample buffer, and two volumes of deionized formamide. The templates are heat denatured at 65°C for 10 min, then add 5 μL of RNA loading buffer. Load samples into the gel and run at 70 V for 3–4 h.

2. The transfer and preparation of the membrane for hybridization are done by standard protocols ***(4*,5)**.
3. The pre-hybridization/ hybridization solution, temperature, and procedure are described in Chapter 18, **Subheading 3.5.1.** However, the probes that will be used are the differentially displayed purified cDNAs.
4. For the Northern hybridization, the ^{33}P-dATP probes are synthesized with a random primer labeling kit (NEBlot kit). The reaction is set up according to the manufacturer's instruction. The probe purification and the determination of the incorporation of α-^{33}P-dATP is done as described in Chapter 18, **Subheading 3.5.2.** For hybridization, 3–4 × 10^6 cpm/mL of the cDNA probe is boiled for 10 min and added directly to each filter. The filters are hybridized overnight at 42°C.
5. The membranes are washed, dried, and exposed to film as described in Chapter 18. **Subheading 3.5.2.** (*see* **Note 7**). Generally, the film is exposed for 48 h to 1 wk, and is dependent on the abundance of the target mRNA in the sample RNA.
6. Differentially expressed cDNAs that are verified by Northern hybridizations are cloned and further characterized.

3.7. Cloning DD-PCR cDNAs

1. The DD cDNAs are cloned in the same manner as the RT-PCR products are in Chapter 18, **Subheading 3.4.** Follow the protocol from **steps 1–4**. The only difference is that the cDNAs and pSK to be used in cloning are digested with *Hind*III only. This is because both the H-T_{11}M and H-AP primers used in DD-PCR contain *Hind*III sites at their 5′ ends. The ligations, transformations and colony lysis PCR are carried out exactly the same.
2. The pSK clones that contain the DD cDNAs can be sequenced and used to generate probes for *in situ* hybridizations. Novel cDNAs can be used to probe a specific cDNA library to isolate a full-length clone.

4. Notes

1. The purity of the RNA is critical for reliable and reproducible results. The presence of chromosomal DNA in the RNA prep can result in false bands on the differential display gel. To be certain the preps are free of DNA, I test the RNA as template in PCR with the control, β-actin primers. Each RNA (200 ng) is diluted in a total of 20 μL of DEP-C H_2O. Then, 2 μL serves as the template in a standard PCR with the β-actin primers (*see* Chapter 18, **Subheading 3.3., step 2**). The PCR products are electrophoresed in a 1% agarose gel. If DNA is

not present in the RNA prep, then no products are present. If the PCR did yield products, the DNase I reaction should be repeated.

2. The Genehunter Corporation designed all of the primers (H-T11A, H-T11C, H-T11G, and H-AP 1-80). It is important to label all tubes clearly, in terms of template and primer(s), because the different primer combinations used in DD-RT-PCR can generate a large number of reactions.
3. The bands on the differential display gel must be reproducible. Therefore, either duplicate reactions (both RT and PCR) are done simultaneously, or the reactions can be repeated at another time ***(1,6)***.
4. Be aware that ^{35}S dATP sometimes leaks out of the thin wall PCR tubes and can contaminate the thermocycler ***(6)***.
5. The 6% polyacrylamide sequencing gels can be prepared in the lab, or many companies supply premixed gel solutions ***(4,5)***. I use Gel-Mix 6 (Life Technologies). Instructions for preparing the gel mix and the mechanics of pouring a sequencing gel are described in *Short Protocols in Molecular Biology*, p. 7–42 ***(4)***.
6. During the pre-run, DNA loading buffer is added to various wells across the gel. The dye fronts in the buffer give a good indication of how the samples will run at different positions on the gel.
7. In order to optimize hybridization signals, the temperatures during the hybridization and washes may need to be increased for certain probes.

Acknowledgment

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20

Cell Labeling and Gene Misexpression by Electroporation

Terence J. Van Raay and Michael R. Stark

1. Introduction

Recent advances in molecular biology have led to the identification of several genes expressed at key stages of neural development. In order to evaluate gene function, scientists have typically used transgenic model systems or retroviral gene delivery systems. However, these methods are not always amenable to all experimental paradigms. Therefore, scientists have looked to develop alternative, non-viral methods of gene transfer that allow a wider range of approaches to study gene function, regulation, and expression ***(1)***. Recently, scientists have developed the technique of in vivo electroporation to misexpress genes in chick ***(2–13)*** and, to a limited extent, in mouse ***(2,14,15)*** embryos. Electroporation is an extremely effective method of targeting cells for gene misexpression, and can also be used to label a subset of cells within the neuroepithelium with a marker gene such as GFP.

The purpose of this chapter is to highlight the basic technique of in vivo electroporation, and emphasize variations that allow for broad or targeted gene misexpression. In addition, we introduce methods of targeting embryonic neuroepithelial precursor cells in vitro.

2. Materials

2.1. Electroporation Apparatus

For the experiments described here, we used a BTX T820 ElectroSquare-Porator™ (Genetronics, Inc.; current model is ECM 830) in combination with BTX Genetrode 508 electrodes or fine tungsten electrodes.

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2.2. Expression Plasmid

1. Eukaryotic expression vector (2 μg/μL minimum concentration) with gene of interest (in this case GFP) under the control of a strong promoter such as CMV, RSV, or SV40. The high DNA concentration improves gene transfer efficiency.
2. 5% Fast green (Fisher cat. no. F-99) solution in H_2O.
3. Sterile 15% sucrose solution.

2.3. In Ovo Chick Embryo Electroporation

1. Fertile chicken eggs.
2. Humidified incubator, 38°C (G.Q.F. Manufacturing, model 1550E).
3. Sterile PBS.
4. Pen/Strep (Gibco cat. no. 15070-063).
5. Pelikan India ink (Snowstar® Ltd.: www.snowstar.com—Pelikan Fount India).
6. Syringes: 10 mL and 1 mL.
7. Hypodermic needles: 1 in. 18 gauge, 5/8 in. 22 gauge.
8. Transparent sealing tape (i.e., Scotch tape).
9. Small surgical scissors.
10. Picospritzer injector (General Valve Corp.) with 1.5 mm injection handle.
11. 1.5 mm fine drawn glass pipets.
12. 10 μL Wiretrol calibrated micropipets (Drummond Scientific; Fisher cat. no. 21-175B).
13. Fine forceps.
14. Micromanipulator(s).
15. Black electrical tape.
16. Banana plug fittings and attachment cords.
17. Tungsten wire (Goodfellow cat. no. W005137), sharpened by etching ***(16)***. Briefly, wire sharpening can be achieved by repeatedly dipping the tip of a tungsten wire into hot fused sodium nitrite. Care should be taken to perform sharpening in a fume hood continually heating the sodium nitrite to keep it from solidifying. Alternatively, etching can be performed using a 12 V or variable transformer to pass a current through a saturated sodium nitrite or sodium nitrite/potassium hydroxide solution (i.e., 71 g $NaNO_2$, 34 g KOH in H_2O). Briefly, a carbon rod is attached to one lead (preferably the positive electrode) and is partially submersed into the sodium nitrite solution. The tungsten wire is attached to the other lead and is dipped repeatedly into the solution, creating a current and resulting in rapid etching of the wire. The sharpened tungsten wire, a 1 mL syringe, a 22 gauge needle, and a banana plug are used to make the tungsten electrode (**Fig. 1A,B**). To do so, carefully thread the tungsten wire through the needle to the desired length (approx 0.5 cm), leaving about 10 cm of wire available to run through the 1 mL syringe and attach to the banana plug. Assemble the electrode to resemble that shown in **Fig. 1A,B**, ensuring a good connection is made between the wire and the banana plug. Attach the banana plug to the syringe using electrical tape. Finally, carefully paint the needle and

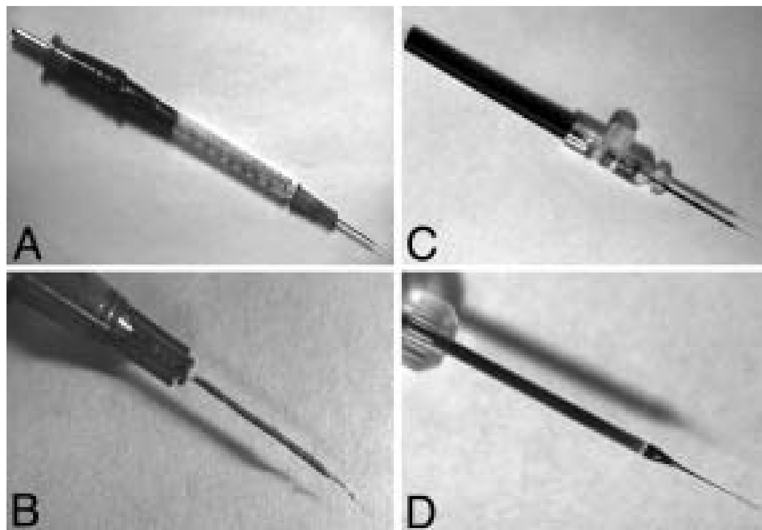

Fig. 1. Electrode and injection needles. **(A)** The negative electrode contains a sharpened tungsten wire passed through a 1 mL syringe containing a 5/8 inch 22 gauge needle (see text for details). **(B)** Higher magnification of electrode showing sharpened tungsten wire. **(C,D)** A 1.5 mm finedrawn glass pipet is backfilled with appropriate DNA/dye solution using a Wiretol calibrated micropipet. **(D)** Higher magnification of the finedrawn end of the glass pipet shown in **(C)**.

part of the tungsten wire with fingernail polish or similar insulating varnish to insulate all but the tip of the negative tungsten electrode.

2.4. In Vitro Embryonic Neural Tube Electroporation

1. E9.5 mouse embryos (*see* **Note 1**).
2. Sterile PBS.
3. Collagenase/Dispase solution (10 mL; make fresh): 1.0 mg/mL Collagenase Type I (Worthington Biochemical Corp, Lakewood, NJ, USA, cat. no. 4196), 2.0 mg/mL Dispase II (Boehringer Mannheim, Indianapolis, IN, USA, cat. no. 165 859), 1X Hanks' Balanced Salt Solution (Gibco BRL Gaithersburg, MD, USA, cat. no. 14180-053) in sterile water. Filter through a 0.22 μm syringe filter, dispense into 500 μL aliquots and store on ice.
4. Trypsin-EDTA.
5. Chick Embryo Extract (CEE; good for 1–2 years at –80°C):
   a. Incubate chicken eggs at 37°C in humidified incubator for 11 d.
   b. Rinse eggs with 70% ethanol, dissect out embryos, and place in sterile Modified Eagle's Medium with Glutamine and Earle's salts (MEM 2X; Gibco cat. no. 11935) at 4°C.

c. Macerate approx 10 embryos at a time by passing them through a 30 mL syringe into a 50 mL Falcon Tube. This produces approximately 25 mL.
d. Add an equal volume of MEM and shake at 4°C for 45 min.
e. Add 100 μL of 10 mg/mL sterile Hyaluronidase (Sigma cat. no. H3884) per 50 mL of embryo/MEM mix.
f. Centrifuge for 180,000*g*·h (e.g., 30,000*g* × 6 h).
g. Filter supernatant through a 0.45 μm filter and then through a 0.22 μm filter.
h. Aliquot and store at –80°C.

6. NEP Basal media (90 mL; store at 4°C, good for 1–2 weeks): 86 mL DMEM/F12 (Gibco cat. no. 11320), 1 mL N2 Supplement (Gibco cat. no. 17502), 2 mL B27 Supplement (Gibco cat. no. 17504), 1 mL Pen/Strep (Gibco cat. no. 15070), 100 mg BSA (Sigma cat. no. A-2153).
7. NEP Complete Media (make fresh): 90% NEP Basal media, 10% CEE, 20 ng/mL of human bFGF (Pepro Tech, Hocky Hill, NJ, cat. no. 100-18B).
8. Six-well tissue culture dishes.
9. 0.1% Fibronectin (Sigma cat. no. F-1141).
10. Sterile water.
11. Sterile dissecting hood.
12. Fine dissecting forceps.
13. CO_2 incubator.
14. Nine-well spot plate (Fisher cat. no. 13-748B).
15. Sterile glass pipets.
16. 15 mL Falcon tubes (Falcon cat. no. 352096).

3. Methods

3.1. DNA Injection and Electroporation Apparatus

The methodology described here is similar to what others have reported for *in ovo* electroporation (*see* **Subheading 1.** for references). It is important to prepare a pure solution of the desired gene expression construct, and resuspend at a minimum concentration of 2 μg/μL in TE or water. Subsequently adding fast green (0.1–0.5% final concentration) will allow for easy visualization of the DNA solution upon injection. Addition of sucrose to the DNA solution will slow the dispersion rate and cause the solution to settle. The electroporation parameters described here have proven most appropriate in our hands. However, these parameters may need to be adjusted for individual applications. For most applications, conditions range as follows: pulse length = 10–20 msec; voltage = 15–50 V; number of pulses = 5. It is important to set up the electroporation and injection station prior to any planned experiment to ensure that the necessary equipment is in place. In addition, we found that having multiple electrode combinations available during experimentation provides versatility.

3.2. In Ovo *Chick Embryo Electroporation*

In most geographical areas, fertile white leghorn chicken eggs can be obtained locally. Alternatively, SPAFAS eggs (Charles River Spafas, Inc.) can be obtained commercially, but at greater expense. Any standard humidified egg incubator should suffice. However, cleaning and sterilization of the incubator will help viability.

1. Incubate fertile chicken eggs horizontally at 38°C for 48 h (or to desired developmental stage) in a humidified incubator.
2. Remove eggs, and sterilize outer shell by wiping gently with 70% ethanol.
3. Carefully poke a small hole in the narrow end of the egg with an 18 gauge needle attached to a 10 mL syringe, and remove 3 mL of albumin. If yolk is accidentally withdrawn into the syringe, discard the egg, as the embryo may not develop properly.
4. Seal the needle hole with clear tape, and apply additional tape to the top-most surface of the eggshell to cover a 1.5 cm^2 area. With small surgical scissors, carefully poke through the top-most surface of the eggshell (covered with tape), and cut a circle approx 1 cm in diameter. Usually the embryo will be revealed on the yolk surface near where the eggshell was cut. A larger hole may be needed to accommodate embryo manipulation and electrode placement.
5. To prevent drying and infection, apply a few drops of sterile PBS containing Pen/Strep (1% v/v) to the surface of the embryonic area. To visualize the embryo clearly, inject a small amount of 10% India Ink in PBS into the sub-blastodermal cavity under the embryo. Do this using a 1 mL syringe and a 22 gauge needle (bent in the middle to 90°) poked into the yolk lateral to the embryo.
6. Carefully remove the vitelline membrane over the area to be electroporated using a fine glass needle or fine forceps.
7. If microelectroporation is planned using a negative tungsten electrode as the driving electrode, place the positive reference electrode near the embryo using a micromanipulator. It is important to note that gene transfer will occur directionally toward the positive electrode. We use one of the two Genetrode electrodes (BTX) for this purpose. Alternatively, for broader electroporation, both Genetrode electrodes can be positioned using a micromanipulator. In this case, an electrode should be placed on both sides of the embryo.
8. Carefully inject the DNA into or near the area to be targeted using a pulled glass pipet and a Picospritzer microinjector (**Fig. 1C**,**D**; **2A**). The glass pipet can be back-filled with DNA using Wiretrol calibrated micropipets or a Hamilton syringe.
9. By hand, quickly place the negative tungsten electrode near the target tissue, and activate the electroporator before the DNA can disperse (**Fig. 2B**). Optimal conditions used here were: pulse length = 20 msec; voltage = 15–30 V; number of pulses = 5. Alternatively, the negative tungsten electrode can be placed prior

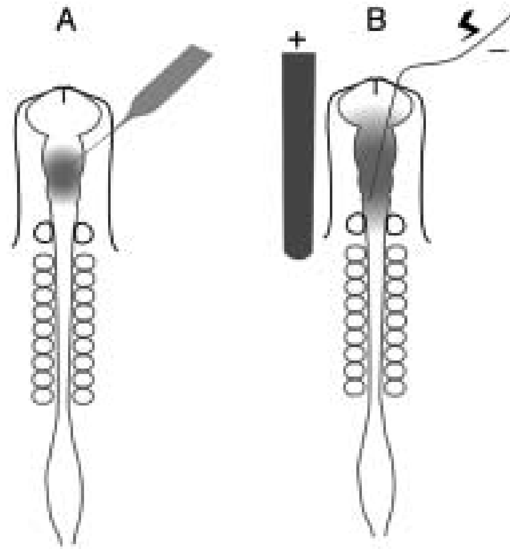

Fig. 2. Electroporation procedure. **(A)** The DNA/dye solution is injected into the neural tube at desired stage and position (HH Stage 10; Hindbrain region is shown). **(B)** The positive electrode is placed adjacent to the region of interest while the sharpened tungsten wire (negative electrode) is inserted directly into the neural tube. Current is then applied (see text).

to DNA delivery using a micromanipulator. Tissue survival and overall development is enhanced significantly if the tissue near both electrodes is completely submersed in liquid during electroporation. Small bubbles should be produced from the electrodes, indicating a current was generated. In addition, a slight darkening of the fast green may occur.

10. Remove the electrodes and completely seal the eggshell window with clear tape. Return the egg(s) to the incubator and allow development to proceed 1–3 d.
11. GFP expression can be observed immediately by whole-mount fluorescent microscopy (**Fig. 3A–C**). After confirming expression, embryos can be fixed in 3% formaldehyde, and prepared for *in situ* hybridization or for sectioning as needed (*see* **Note 4**).

3.3. In Vitro Neuroepithelial Precursor Cell Electroporation

1. Approximately 24 h prior to experiment, coat tissue culture dishes with 0.002% fibronectin in sterile water and incubate overnight at 4°C. Remove and recycle fibronectin (can be reused up to 4X), carefully rinse with sterile PBS and store at 4°C in PBS until ready to use.
2. Prepare DNA solution to 1–4 μg/μL in 0.1–0.5% fast green and 5% sucrose and store on ice.

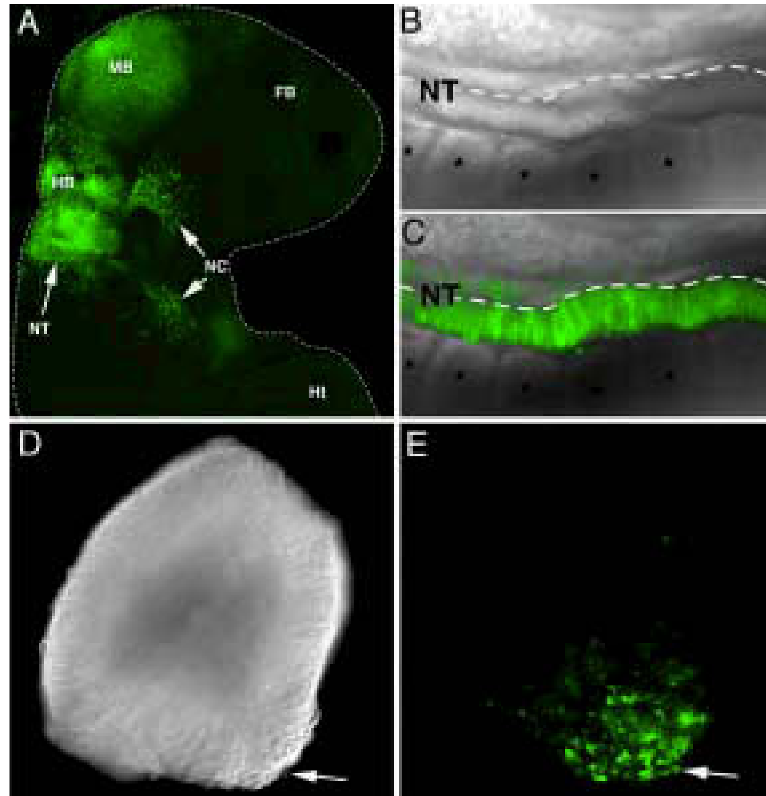

Fig. 3. Electroporation results. **(A)** 3 µg/µL of a DNA vector containing GFP driven by a SV40 promoter was injected into the hindbrain region of a HH stage 10 embryo. Electroporation was carried out with 5 pulses of 10 msec each at 15 V. 24 h post electroporation, the embryo was harvested, fixed, and observed under a fluorescent microscope. GFP labeled cells can be observed throughout the midbrain (MB), hindbrain (HB) and rostral neural tube (NT). In addition, GFP labeled cells are also observed in migrating neural crest cells (NC). FB, forebrain; Ht, heart. **(B)** Dorsal-lateral view of a brightfield image of a neural tube electroporated with a GFP construct as described in **(A)**. The dashed line demarcates the dorsal midline of the neural tube. **(C)** Fluorescence image overlaid on the brightfield image. GFP positive cells are observed predominantly in only one half of the neural tube. The asterisks denote the somites. **(D)** Brightfield image of a mouse E9.5 neural tube 24 h after electroporation. Electroporation was carried out with five pulses of 10 msec each at 20 V. During the 18 h period in suspension culture, the neural tube becomes spherical in shape. **(E)** Confocal fluorescence image of neural tube shown in **(D)**. Numerous GFP labeled cells can be observed in the area of electroporation (arrow).

3. Harvest E9.5 embryos, leaving all extraembryonic membranes intact, from timed pregnant mice in sterile PBS. From this point, all work should be done in a sterile dissecting hood.
4. Using a dissecting microscope, remove all extraembryonic membranes as well as the amnion and move the embryo to a clean dish containing sterile PBS. Repeat for all embryos.
5. Remove the head just caudal to the otic vesicles and remove as much of the soft tissue as possible. Clip the very end of the tail. This procedure removes as much of the non-neural tissue as possible, as well as the cephalic regions, which are not to be included as neuroepithelial precursor cells. Clipping the tail will allow for more complete enzyme penetration. Do this for all the embryos before proceeding to **step 6**.
6. Put approx 3–4 embryos into 500 μL of the Collagenase/Dispase solution and store on ice.
7. Once all of the embryos are in the Collagenase/Dispase solution, put the tubes at 37°C for 10 min (*see* **Note 2**).
8. Remove embryos from Collagenase/Dispase solution, quickly rinse in PBS and place in one well of a nine-well spot plate containing NEP Complete media, mix and move to fresh well containing NEP Complete media to dilute out the Collagenase/Dispase solution.
9. Working with a single embryo at a time in one well containing NEP Complete media, triturate with a sterile glass pipet several times. The neural tube should easily separate away from the surrounding somites and ectoderm. Using fine dissecting forceps, carefully remove any remaining non-neural tissue and place the purified neural tube into fresh NEP complete media. Repeat for all embryos.
10. Place approx three neural tubes per well in a six-well tissue culture dish containing 2 mL of sterile PBS (*see* **Note 3**).
11. Pipet 10 μL of DNA solution directly over the neural tube. The sucrose will keep the solution concentrated around the neural tube.
12. Place electrodes on either side of neural tube and electroporate as follows: pulse length = 10 msec; voltage = 20 V; number of pulses = 5.
13. Allow neural tubes to recover for approx 10 min, then transfer to a clean tissue culture dish with fresh NEP complete media and incubate at 37°C, 5% CO_2 for 4–18 h. The neural tubes will become spherical from growing in suspension and initial gene expression can be observed approx 8 h after electroporation (**Fig. 3D**,**E**).
14. Remove neural tubes and place in 15 mL sterile Falcon tube, remove media and add 500 μL of Trypsin/EDTA. Carefully watch as neural tubes start to dissociate. Neutralize Trypsin by adding 1.5 mL of CEE. Spin down cells at 250*g* for 5 min at room temperature. Remove supernatant and add NEP complete media. Resuspend and triturate cells to completely dissociate them. Plate on fibronectin coated tissue culture dishes at desired density. Incubate at 37°C, 5% CO_2. After confirming expression, cells can be fixed in 3% formaldehyde, and prepared for immmunocytochemistry as needed (*see* **Note 4**).

4. Notes

1. If E8.5 embryos are desired, they can be isolated in a similar way, but they are much more sensitive to mechanical manipulation. In addition, Trypsin should not be used to dissociate the cells. Instead, the neural tubes should be triturated using a pipet to obtain single cell suspensions. For older embryos (>E9.5), the Collagenase/Dispase step will not work. Instead, the neural tube can be isolated mechanically by first separating the neural tube, with some attached mesenchyme, from the embryo. Leaving the neural tube attached at the hindbrain until the very end will aid in handling. There is a tough meninges layer tightly associated with the neural tube that needs to be removed. Starting from the rostral end this can be peeled back, removing the mesenchyme tissue with it and leaving a very clean neural tube.
2. The exact time for this incubation needs to be determined for every lot of Collagenase and Dispase. In general, test an embryo after 5, 7, and 10 min at 37°C. Longer incubation times may be necessary, and the neural tubes are fairly resistant to the digestive enzymes, compared to the surrounding tissue. Beware of prolonged incubations as they will have detrimental effects on the quality of the neural tissue.
3. Electroporations will work equally well in NEP complete media if this is desired.
4. Fixation of tissue or cells with MeOH will ablate or severely reduce GFP expression. In this case antibodies against GFP (Boehringer Mannheim cat. no. 1814 460) can be used to identify GFP labeled cells.

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21

Gene Therapy Using Neural Stem Cells

Luciano Conti and Elena Cattaneo

1. Introduction

Cell replacement and gene transfer approaches for the diseased or injured CNS have provided the basis for the development of potentially powerful new therapeutic strategies for a broad spectrum of brain diseases. Transplantation of cells engineered to produce growth factors or molecules with antitumor effects shows the potential of grafted cells as vectors for protein delivery. However, for any possible clinical application, evidence that the gene transfer is completely innocuous and safe for the recipient must be obtained. To this regard, special cautions must be taken not to interfere with other brain functions apart from the one to be targeted. The grafted cells must integrate without inducing further damage to the recipient brain, with no apparent systemic or local side effects, such as uncontrolled growth, production/release of harmful compounds, or inflammatory response.

Alongside the capacity of grafted cells to properly integrate in the host tissue, the capability to achieve long-term delivery of proteins of therapeutic interest in the brain represents an important requirement for obtaining effective advances in ex vivo gene therapies. The development of safe retroviral transduction procedures coupled with the use of stable promoters (such as the retroviral long terminal repeats or the cytomegalovirus promoter) has proven to be the most effective for ex vivo gene therapy purposes ***(1)***. Furthermore, a major requirement for gene therapy is to keep transgene expression in target cells under "outside" control. Recent studies in the development of systems for pharmacologically controlled gene expression have proven the tetracycline-based regulatory systems to offer tight control over the level and timing of transgene expression, thus representing an efficacious therapeutic tool ***(2)***.

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The first approaches to ex vivo gene therapy in the brain were attempted by Rosenberg et al. ***(3)*** and Fisher et al. ***(4)*** using fibroblasts, which were genetically modified to secrete nerve growth factor (NGF) or L-DOPA, respectively. Despite the relevant results obtained, the use of engineered fibroblasts presents several disadvantages. In fact, they do not exhibit migratory ability, thus locally limiting the delivery of the therapeutic compound. Furthermore, they represent a "compact foreign body" in the host brain tissue potentially leading to inflammatory response. The use of encapsulated heterologous engineered cells partially overcomes these disadvantages ***(5)***. This method drastically reduced the occurrence of inflammatory response, but does not allow a wide delivery of the transgenes, and does not provide cellular and anatomical corrections to diseased neural tissue. On the other hand, the use of engineered astrocytes may achieve wide delivery of transgenes. Astrocytes are particularly well suited because of their CNS origin, their efficient secretory mechanisms, and their role as neuronal support. Ridet et al. ***(6)*** demonstrated that astrocytes derived from adult cerebral cortex could be efficiently transduced by an adenoviral vector encoding human tyrosine hydroxylase (hTH) under the negative control of the tetracycline-based regulatory system (tet-off). Grafted astrocytes were shown to migrate, synthesize large amounts of active hTH, and release L-DOPA, thus representing an efficient, pharmacologically regulated transgene expression.

More recently, a breakthrough in gene therapy approach came from the identification and isolation of neural stem cells (NSC) and methods for their long-term expansion (***7***,***8***, reviewed in ***9***) in serum-free medium supplemented with growth factors ***(10)***. An alternative method for long-term expansion of NSC employed the immortalization strategy ***(11,12)***. Oncogenes such as *myc* and large-T antigen have been shown to exhibit immortalizing abilities without fully transforming the cells. The discovery of mutant alleles of particular oncogenes ***(13)*** and strategies for the pharmacologically regulated expression of oncoproteins have also been employed in order to generate safe cell lines for gene therapy purposes ***(14,15)***.

In recent years, there has been encouraging progress in using NSC, both growth factor-expanded and immortalized; and the possibility to isolate, expand, and/or immortalize NSC of human origin ***(15–19)*** has further pushed the therapeutically encouraging premises. This feature is a step forward, since NSC transplantation studies have shown the ability of the grafted NSC to survive well after grafting into adult recipients without any sign of tissue disruption or tumor formation, and with real advantages for wider and more stable distribution of a soluble ligand in the diseased brain ***(20)***. The ability of NSC to migrate extensively when transplanted into the adult CNS may

enable such cells to deliver therapeutic agents widely throughout this tissue, as described by Benedetti et al. ***(21)***. Immortalized and growth factors-expanded NSC ***(22–25)*** were used as vehicles for the delivery of the immunomodulator interleukin (IL)-4 by unilateral injection into the rat striatum 5 d after injection of the syngeneic GL261 glioma cell line. Animals receiving IL-4-producing NSC survived better than animals receiving no treatment. Furthermore, it was found that IL4-engineered NSC exhibit a marked migratory capability along the same routes used by the tumor cell, thus representing a therapeutic delivery system that could distribute its activity throughout the CNS in a manner resembling the distribution of the cancer cells themselves.

Beside their great potential to survive and give rise to post-mitotic neurons in vivo, together with the possibility to be modified for intracerebral long-term delivery of molecules of therapeutic interest, both growth factor-expanded and immortalized NSC exhibit some flaws. On the one hand, growth factor-expanded NSC do not express potentially hazardous oncogenes; on the other hand, they do exhibit a longer doubling time and a lower degree of control in the reproducibility of gene transfer procedure, when compared to immortalized cell lines. More importantly, both of them, when transplanted into brain, migrate and integrate along with the endogenous mature cells ***(26,27)*** but demonstrate a prevalence to differentiate to glial cells ***(26–29)***. Development of strategies aimed at improving the rate of neuronal over glia differentiation by genetically engineering NSC represents one of the major challenges for the proper employment of these cells in cell replacement gene therapy ***(20)***.

In this chapter, methods will be presented for immortalizing and engineering NSC for gene therapy purposes. The foundational rationale behind immortalization is to block the progression of a developmental program by forcing the cells to remain in a continuous cell cycle. Immortalization can be achieved by a number of manipulations, although the most common is the introduction of exogenous cDNA coding for oncogenic proteins. Importantly, a common property of murine and human NSC lines generated via retroviral transduction of immortalizing oncogenes, is that they behave like established lines, but show no signs of transformation either in vitro or in vivo ***(21,26,27)***.

Beside the use of temperature sensitive oncogenes, new strategies, such as the use of the Cre-*lox*P reversible immortalization procedure or the tetracycline regulated expression (*see* Chapter 22), are now beginning to be considered to completely avoid the presence of the immortalizing oncogene in the grafted cell lines for the safest employment in human gene therapy protocols. Furthermore, the tetracycline-based systems offer tight control over the level and timing of transgene expression, and have been shown to function both in vivo and in vitro.

2. Materials

2.1. Growth and Transfection of Packaging Cell Line

1. Normal tissue culture equipment, i.e., incubators with CO_2, laminar (vertical) biohazard flow hood.
2. Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, cat. no. 41966) containing 0.11 g/L of sodium pyruvate (Life Technologies, cat. no. 11840), 2 m*M* of L-glutamine (Life Technologies, cat. no. 25030), 3.7 g/L of sodium bicarbonate (Life Technologies, cat. no. 11810) and 50 units/mL of penicillin–streptomycin (Life Technologies, cat. no.15140).
3. Fetal bovine serum (Life Technologies, cat. no. 10108).
4. 15 mL conical tubes.
5. 0.22 μm and 0.45 μm filters.
6. DNA retroviral vector carrying an immortalizing oncogene together with a selection cassette.
7. Chloroquine (Sigma, cat. no. C6628). Chloroquine stock is 50 m*M*; for 7 mL media + 3 mL DNA, add 5 μL).
8. 2 *M* $CaCl_2$ (Sigma, cat. no. 4160).
9. Stock solution of Na_2HPO_4 dibasic (5.25 g in 500 mL of water).
10. Make 2X HBS: 8.0 g NaCl, 6.5 g HEPES (sodium salt; Sigma, cat. no. H-7006) 10 mL of Na_2HPO_4 stock solution. pH to 7.0 using NaOH or HCl. Bring volume up to 500 mL and sterilize by filtering. Check pH again. The pH is very important, it must be exactly 7.0. Because pH is so important, make three batches: pH 6.95, pH 7.00, pH 7.05. Test each solution and use the one that yields the best precipitate. All reagents should be at room temperature prior to use.

2.2. Dissociation of Human Fetal-Derived Telencephalic Tissue

1. Normal tissue culture equipment.
2. Hank's Balanced Salt Solution with no calcium and magnesium (HBSS; Life Technologies, cat. no. 14170).
3. Protease (Sigma, cat. no. P3417).
4. Enzyme inhibitor: trypsin inhibitor (1 mg/mL; Life Technologies, cat. no. 170075) and bovine serum albumin (BSA) (1 mg/mL; Sigma, cat. no. A9647).
5. Poly-D-lysine (Sigma, cat. no. P7280).
6. Dulbecco's Modified Eagle's Medium (DMEM)/F12 (1:1) (Life Technologies, cat. no. 11039).
7. N2 supplement (Life Technologies; cat. no. 17502).
8. Growth factors containing serum free medium (GF-SFM). It is composed of: DMEM/F12 (1:1), N2 supplement, 20 ng/mL of epidermal growth factor (EGF) (Life Technologies, cat. no. 13247), 20 ng/mL of fibroblast growth factor-2 (FGF-2) (Life Technologies, cat. no. 13256), and 1% BSA.
9. Fetal bovine serum (Life Technologies, cat. no. 10108).
10. Bovine serum albumin (Sigma, cat. no. A9647).

2.3. Infection of NSC

1. Normal tissue culture equipment.
2. Poly-D-lysine (Sigma, cat. no. P7280).
3. Frozen or fresh retroviral vector supernatant.
4. Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, cat. no. 41966) containing 0.11 g/L of sodium pyruvate (Life Technologies, cat. no. 11840), 2 m*M* of L-glutamine (Life Technologies, cat. no. 25030), 3.7 g/L of sodium bicarbonate (Life Technologies, cat. no. 11810), and 50 units/mL of penicillin-streptomycin (Life Technologies, cat. no. 15140).
5. N2 supplements (Life Technologies; cat. no. 17502)
6. Growth factors containing serum-free medium (GF-SFM). It is composed of DMEM/F12 (1:1), N2 supplement, 20 ng/mL of epidermal growth factor (EGF) (Life Technologies, cat. no. 13247), 20 ng/mL of fibroblast growth factor-2 (FGF-2) (Life Technologies, cat. no. 13256), and 1% BSA.
7. Fetal bovine serum (Life Technologies, cat. no. 10108).
8. Bovine serum albumin (Sigma, cat. no. A9647).
9. Neomycin analog G418 (Life technologies; cat. no. 11811).
10. Nunc tissue culture plastic dishes and flasks.
11. Polybrene (Sigma, cat. no. H9268). Polybrene is prepared as stock solution at 5 mg/mL and stored at –20°C.

2.4. Infection of Immortalized NSC

1. Normal tissue culture equipment.
2. Frozen or fresh retroviral vector supernatant.
3. Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, cat. no. 41966) containing 0.11 g/L of sodium pyruvate (Life Technologies, cat. no. 11840), 2 m*M* of L-glutamine (Life Technologies, cat. no. 25030), 3.7 g/L of sodium bicarbonate (Life Technologies, cat. no. 11810), and 50 units/mL of penicillin–streptomycin (Life Technologies, cat. no. 15140).
4. Fetal bovine serum (Life Technologies, cat. no. 10108).
5. Nunc tissue culture plastic dishes and flasks.
6. Polybrene (Sigma, cat. no. H9268). Polybrene is prepared as stock solution at 5 mg/mL and stored at –20°C.

3. Methods

3.1. Growth and Transfection of Packaging Cell Lines

When passaging packaging cell lines, it is important to never let cells reach confluence. This will reduce transfection efficiency in the short term. For maximally healthy cells, a split of 1:4 or 1:5, of a 70–80% confluent 10 cm plate into a new plate every 2–3 d should provide optimal cell conditions. Three million cells on a 10 cm plate is a good starting point for seeding cells

prior to transfection; it is important to titer slightly up and slightly down to maximize transfection efficiencies. Efficiencies of 50–60% of the cells should be achieved. The highest transfection efficiencies are obtained with cells that are 70–80% confluent at the time of transfection.

Important note: Viral supernatants produced by these methods contain potentially hazardous recombinant virus. The user of these systems must exercise due caution in the production, use, and storage of recombinant retroviral virions. During infection, all liquids and items in contact with virus are considered bio-hazardous, treated with bleach, and autoclaved.

1. 18–24 h prior to transfection, plate cells at 3 million cells per 10 cm plate in 7 mL of DMEM 10% FBS. Gently shake forward and backward, then side to side, in order to distribute cells evenly about the plate. Allow cells to attach. At 2/3 confluence, a 10 cm plate should provide 5 million cells. It is at this sub-confluent stage that cells are most transfectable and will best survive the rigors of transfection giving the highest titer virus possible.
2. About 5 min prior to transfection, add chloroquine to each plate to 25 μM. Chloroquine acts to inhibit lysosomal DNases by neutralizing vesicle pH. DNA delivered by Ca_2PO_4 transfection is thought to transit through lysosomes.
3. To a 15 mL tube, add (per 10 cm plate, with all reagents at room temperature):
   a. 15–30 μg of DNA (DNA is added in a drop to side of tube).
   b. 1340 μL of dd H_2O (wash the DNA to bottom of tube with water).
   c. 150 μL of 2*M* $CaC1_2$.
   d. Mix thoroughly with finger tapping. 1500 μL total volume. Scale volume and DNA/reagent amounts.
   e. Add 1.5 mL of 2X HBS quickly then bubble vigorously with automatic pipetor (keep eject button depressed) for 3–15 sec (actual length of bubbling time depends on each batch of 2X HBS).
4. Add HBS/DNA solution dropwise onto media (gently and quickly) by spreading across cells in media. Observe the cells under a microscope; you should observe evenly distributed small dark particles.
5. Gently shake plates a few times to distribute Ca_2PO_4/DNA complexes.
6. Put plates in 37°C incubator and let the cells grow for 24 h.
7. Change media to 5 mL of fresh DMEM, 10% FCS and leave for further 24 h. Virus is more stable if incubation is carried out at 33°C, although 37°C is acceptable. Do not leave Chloroquine on cells more than 24 h because it is toxic.
8. Pipet supernatant from transfected cells into 15 mL tubes and centrifuge at 300*g* for 5 min to pellet cell debris. Filtering through 0.45 μm filter removes cells as well. Supernatant can be frozen at –80°C for later infection, although titer drops by one-half for each freeze-thaw cycle.

If the transfected DNA carried a reporter gene, transfection efficiency can be eventually monitored. The transfected cells can be selected by adding the

specific agent in order to obtain a stable producer cell line, avoiding performing the transfection procedure every time and ensuring a ready-to-use high titer retroviral supernatant.

3.2. Retroviral Mediated Immortalization of NSC

In this paragraph, procedures to immortalize different sources of NSC are described (*see* **Notes 1** and **2**).

3.2.1. Immortalization of Primary NSC

1. Perform an enzymatic dissociation of the tissue for 6 min at 37°C with gentle agitation in HBSS containing 3 mg/mL of protease.
2. Wash the tissue with warm HBSS and then wash with warm enzyme inhibitor. If working with embryonic (d 12–16) murine brain tissue, the enzymatic dissociation can be avoided. The mechanical dissociation described below is enough.
3. Triturate brain tissue fragments approximately 15X with a 10 mL pipet, and then collect them by centrifugation at 800*g* for 3 min.
4. Aspirate supernatant and leave the tissue on ice for 10 min.
5. Re-suspend the pellet in 1 mL of DMEM/F12 (1 : 1) 10% FBS and triturate with pipets of decreasing bore size to yield a single-cell suspension.
6. Plate the cells on poly-D-lysine-coated tissue culture plastic in DMEM/F12 (1 : 1) 10% FBS, at a density of 8×10^6 cells per 10 cm dish.
7. After 12/16 h replace the medium with GF-SFM and grow for 4 d at 37°C.
8. Infect the cultures by incubation for 24 h at 33°C with a mixture of one volume of retroviral vector supernatant (DMEM 10% FBS; 4×10^5 cfu/mL) and two volumes of GF-SFM to which 5 μg/mL of polybrene have been added.
9. Replace medium with GF-SFM and incubate at 37°C, or at 33°C if temperature-sensitive oncogene has been employed.
10. Two days after infection, start G418 selection. We recommend a starting dilution of 200 μg/mL, and weekly change of 2/3 of the medium with fresh GF-SFM containing 250 μg/mL of G418. During the first week of G418 selection, a consistent cell death will occur. Colonies should appear 2–3 wk after infection.
11. Clones can be isolated, expanded as single-cell clones in coated or uncoated plasticware using GF-SFM, or serum-supplemented medium (requirement for growth factors should be assayed), and passaged using trypsin-EDTA depending on the replication time of the specific clone. Expanded cells can be cryopreserved in a freezing solution consisting of FBS and 10% dimethyl sulfoxide (if the cells are of human origin, use a freezing solution consisting of 50% FBS, 40% GF-SFM, and 10% dimethyl sulfoxide). Expression of the immortalizing oncogene's mRNA and/or oncoprotein should be confirmed by RT-PCR amplification and/or Western blot/immunocytochemistry, respectively. Monoclonality should be confirmed by identification of a single and identical genomic insertion site by Southern blot analysis.

3.2.2. Immortalization of Growth Factor-Expanded NSC

NSCs have been shown to be long-term expanded in vitro in presence of EGF or FGF-2 on non-coated plastic. In this condition, rodent NSCs, as well as their human homologs, grow in suspension-forming aggregates of variable sizes called "neurospheres" ***(8,9)***.

1. 24 h before infection, collect "neurospheres" by centrifugation (900*g* for 10 min), dissociate to single cells and plate at a final density of 20,000/cm^2 in GF-SFM (DMEM : F12 N2 supplemented medium containing 20 ng/mL of EGF, 20 ng/mL of FGF-2 and 1% bovine serum albumin) poly-D-lysine (20 μg/mL) treated 10 cm dishes.
2. Replace medium with retroviral vector supernatant (DMEM 10% FBS; 4 × 10^5 cfu/mL) supplemented with polybrene (5 μg/mL) and incubate at 33°C for 24 h.
3. Replace medium with GF-SFM and incubate at 37°C for 24 h.
4. Replace medium with retroviral vector supernatant (DMEM 10% FBS; 4 × 10^5 cfu/mL) supplemented with polybrene (5 μg/mL) and incubate at 33°C for 24 h.
5. Replace medium with GF-SFM and incubate at 37°C for 48 h.
6. Two days after infection, start selection with the appropriate drug (*see* **Subheading 3.2.1.**).
7. Clones should be visible after 2–3 wk and expanded, cryopreserved, or characterized as described (*see* **Subheading 3.2.1.**).

3.3. Engineering of NSC to Express Therapeutic Genes

In this section, procedures to deliver therapeutic genes in growth factor-expanded and immortalized NSC by retroviral transduction procedures are described. Since, in most cases, the therapeutic gene consists of a secreted molecule, it is important to precisely evaluate the amount of transgene produced by using, whenever possible, an ELISA assay (*see* **Notes 3** and **4**).

3.3.1. Engineering of Growth Factor-Expanded NSC

1. The day of infection, collect "neurospheres" by centrifugation (900*g* for 10 min), dissociate to single cells and plate at a final density of 40,000/cm^2 in non-treated 25 cm^2 flasks in 15 mL retroviral vector supernatant (DMEM 10% FBS; 4 × 10^5 cfu/mL) supplemented with polybrene (8 μg/mL).
2. Centrifuge cells at 1,000*g* at room temperature for 60 min and incubate at 33°C for 2 h.
3. Collect the cells by centrifugation (900*g* for 10 min) and re-suspend in GF-SFM.
4. Incubate for 24 h at 37°C.
5. Repeat steps 1–3 and incubate at 37°C.

6. Two days after infection, start selection with the appropriate drug. If using G418, a starting dilution of 150 µg/mL is recommended. Replace weekly 2/3 of the medium with fresh GF-SFM containing 200 µg/mL of G418. Resistant cells should appear 1 week post infection.
7. Cells can be expanded and cryo-preserved as described (**Subheading 3.2.**).
8. Expression of the delivered gene's mRNA and/or protein should be confirmed by RT-PCR amplification and/or Western blot/immunocytochemistry, respectively. If the delivered gene codes for a secreted protein, quantitative analysis of the synthesized protein should be evaluated by ELISA.

3.3.2. Engineering of Immortalized NSC Lines

Immortalized rodent NSC lines, in contrast to human ones, have been shown to be cultured in the presence of serum, without the requirement of EGF or FGF-2. In this section, we will refer to the former. However, the same procedure can be used for cell lines of human origin simply by using growth factor-supplemented, serum-free medium.

1. The day before infection, plate the cells on poly-D-lysine coated tissue culture plastic in DMEM 10% FBS, at a density of 5×10^5 cells per 10 cm dish.
2. Infect the cultures by incubation for 24 h at 33°C with 10 mL of retroviral vector supernatant (DMEM 10% FBS; 4×10^5 cfu/mL) in the absence of polybrene.
3. Replace medium with fresh retroviral vector supernatant and incubate at 33°C for 24 h.
4. Repeat **step 3**.
5. Plate the cells at a very low density (about 20 cells for a 10 cm plate) in DMEM 10% FBS and start selection with the appropriate drug. Colonies should appear after 10 d.
6. Clones can be isolated, expanded, and assayed for transgene expression as described in **Subheading 3.2.**

4. Notes

1. Immortalization of NSC of human origin can be achieved by using the same retroviral transduction procedure described for rodent NSC (*see* **Subheadings 3.1.** and **3.2.**). In this case, to produce retroviral particles able to infect cells of human origin, amphotropic packaging cell lines, such as the GP+envAM12 must be used. When using an amphotropic (infective but replication-defective) retroviral vector to generate the immortalized cell lines, there is the remote possibility that the cells themselves could become producers of amphotropic retroviral particles carrying potentially oncogenic products. For this reason, it is of primary importance to assay the supernatant of immortalized cell lines for the presence of these particles. The absence of such biohazardous agents could be directly tested by incubating other human cells (such as HeLa cells) in culture with the supernatant from immortalized cells and starting selection with the selective agent. No clones should be obtained from these cultures.

2. Different oncogenes have been used to immortalize murine NSCs from diverse brain regions and donor ages, v-*myc* and the temperature sensitive allele of the large-T antigen (tsA58U19) being the most extensively used ones. In this respect, human neural material appears to differ from that of rodent origin. In particular, large-T antigen seems unsuccessful in inducing immortalization of human NSC ***(18,30)***, probably due to a more stringent control of cell cycle progression in these cells compared to the murine ones. On the contrary, *myc* acts via stimulation of growth factor regulated genes, overcoming the tight cell cycle regulation. Notably, the only two cell lines of human origin reported to date ***(15,18)*** have been generated with the retroviral transduction of the v-*myc* (*gag-myc* fusion) oncogene. In this respect, it is also worth noting that the division of immortalized human CNS stem cell lines seems to be more dependent on growth factors, and is fully reversible upon cessation of mitogen stimulation. In fact, as observed in grafting experiments performed in rodent brain, integrated v-*myc* transduced human NSCs downregulate their v-*myc* expression by themselves (the exact mechanisms are not yet addressed), stop dividing (due to the limited presence of EGF/FGF-2 in the normal brain), and show no sign of transformation, overgrowth, disruption, or tumorigenesis.
3. In order to obtain a more fine control of the oncoprotein presence both in vitro and in vivo, for the cell line generated by Sah et al. ***(15)***, the v-*myc* expression was driven by a modified, Tet-off regulatable CMV promoter. Upon addition of tetracycline to the culture medium, production of the immortalizing protein in this system is pharmacologically shut off. This, in addition to the "natural" tendency of cells to downregulate v-*myc* upon the withdrawal of growth factors, constitutes a further safe mechanism for a more impressive use of these cell lines in therapeutic protocols.
4. The use of genetically modified NSCs to produce neurotrophic factors has been largely employed and described as a useful therapeutic approach to neurodegenerative disease ***(20,27)***. More generally, it is difficult to imagine that the introduction into the CNS of a constitutively produced compound that was not part of normal CNS function would be completely harmless. To this respect, once again the Tet systems represent an efficacious therapeutic tool for pharmacologically controlled gene expression.

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22

Modeling Brain Pathologies Using Neural Stem Cells

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1. Introduction

The study of the mechanisms underlying neurodegenerative diseases is a highly demanding goal, complicated by the complexity and heterogeneity of the nervous system and by the long period of time over which these pathologies develop in humans. The use of "simplified" in vitro cell models is therefore often mandatory and useful to investigate aspects of the pathology. Yet, it is a matter of debate how truly informative is this so-called "reductionist approach" to the pathology, especially in the case of complex diseases like those affecting the central nervous system. It is undeniable that in vitro cell cultures lack that unique microenvironment affecting cell behavior and survival in vivo and, of course, extrapolation of results obtained in vitro to the in vivo situation is difficult. Nevertheless, it is just the potential absence of any kind of complicating factors that make it possible to observe and measure the many fundamental biological processes that occur, for example, in the presence of a mutant protein involved in a disease. Another advantage of in vitro models is that they are extremely easy to handle and surely represent the first choice when initially screening for therapeutic compounds.

Genetically determined diseases are by far the most suitable pathologies to be modeled. Once the genetic defect underlying the disease has been identified, it may be easily reproduced in transgenic models. With this approach, our understanding of human neurodegenerative diseases has progressed tremendously over the last decade, especially in the case of Huntington's disease (HD), several spinocerebellar ataxias, dentatorubralpallidoluyasian atrophy (DRPLA), and spinocerebellar muscular atrophy (SBMA). In vivo models have been also developed in the attempt to mimic other pathologies such

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as Parkinson's disease, amyotrophic lateral sclerosis (ALS), and Alzheimer's disease, for which, besides the more common, non-genetically determined forms, familial forms with precise pathogenic mutations have been identified as well.

Several different cell types have been genetically engineered to mimic pathologies in a cell culture dish. Ideally, the introduction of the gene responsible for a pathology into an isolated and selected neuronal population that is affected in the disease should represent the in vitro model closest to the disease. This goal can be achieved by using cultures of primary cells isolated from the relevant brain region. However, the use of primary cultures suffers from major problems inherent in the reduced survival of neurons in vitro, and the difficulty of delivering transgenes into them in a stable manner. In addition, primary cell cultures may lose tissue-specific characteristics after a relatively short period of time.

Tumoral cell lines (mainly neuroblastoma and pheochromocytoma cells) have been successfully used in place of primary neurons for their facility to be engineered and maintained in culture. While lacking most of the features of primary neurons, these cells can be induced to differentiate into mature neurons with appropriate reagents. Another advantage in the use of these cells is the homogeneity of cell population, at least in theory, since most of them are clonally derived. However, their tumoral origin makes these cell lines extremely prone to genetic instability and, consequently, non-homogeneously responsive to treatments or other experimental conditions.

In this regard, a good compromise between easy handling and maintenance of neuronal features and genetic background is represented by the neural stem cells (NSC). Custom-made cell lines may be obtained from NSC isolated from different cerebral areas and manipulated to express genes involved in diseases. Neurospheres of growth factors-expanded NSC (GF-NSC) may be similarly engineered via retroviral vectors carrying the pathological transgenes (*see* Chapter 21 for the engineering of GF-NSC). Upon treatment with appropriate factors, immortalized NSC may also differentiate to some extent into neuronal-like cells with at least some of the features of the brain area of origin ***(1)***, making the in vitro model more closely mirror the in vivo background of any specific brain pathology. When transgenic animal models exist for a pathology, NSC bearing the pathological transgene may also be directly isolated and immortalized from transgenic brains, rather than being engineered in vitro ***(2)***. This approach has the additional advantage of relying on a previously controlled phenotype (the specific phenotype of the donor transgenic animal) without the uncertainties related to positional effects of the transgene stable integration in the cell genome.

While techniques for the isolation, characterization, and stable transgene expression in NSC have been extensively discussed in other chapters of this book, we will focus here on procedures to obtain inducible NSC models of inherited brain pathologies. The possibility to induce, in a tightly controlled manner, the expression of a pathogenic transgene in NSC may open the way to a deeper understanding of the pathology, especially when the transgene produces a subtle phenotype. Cellular events induced by the mutant protein may be followed from their first appearance and directly correlated to the pathology. It may become easier to study the reversibility of cell dysfunctions and the effects of specific drugs at various stages of the pathogenic event.

When modeling brain pathologies in vitro, it has to be clear what phenotypes will be measured to validate the model. As a matter of fact, most of the neuropathological features observed in the brain in vivo will not be reproducible in an in vitro cell model. Yet, aspects of the pathological mechanisms acting in the affected cells may become measurable. Apoptosis is one of these mechanisms, being a hallmark of most neurodegenerative diseases ***(3,4)***. It is not possible here to define all the requirements of a cytotoxicity assay, so we will discuss the most used techniques for the assessment of cell viability and apoptosis. Analyses and confirmation of immunohistochemical markers, neurotransmitter phenotypes, and electrophysiological tests are dealt with in other chapters of this book.

2. Materials

2.1. Generating TetON Inducible Cell Lines

2.1.1. Generating rtTA-positive NSC

1. Standard tissue culture equipment.
2. Complete growth medium: Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, cat. no. 41966) containing 0.11 g/L of sodium pyruvate (Life Technologies, cat. no. 11840), 2 m*M* L-glutamine (Life Technologies, cat. no. 25030), 3.7 g/L of sodium bicarbonate (Life Technologies, cat. no. 11810), and 50 units/mL of penicillin–streptomycin (Life Technologies, cat. no. 15140), and supplemented with 10% fetal bovine serum (Life Technologies, cat. no. 10108).
3. RetroPack PT67 cell line (Clontech, cat. no. K1060-D).
4. pRevTet-ON vector (Clontech, cat. no. 6159-1).
5. Neomycin analog G418 (Life Technologies, cat. no. 11811).
6. 0.45 μm Millex-HV filters in Durapore, 25 mm (Millipore, cat. no. SLHV R25 LS).
7. Polybrene (Sigma, cat. no. H9268). Polybrene is prepared as stock solution at 5 mg/mL and stored at –20°C.
8. Trypsin–EDTA solution (Sigma, cat. no. T3924).
9. Cloning rings (PGC Scientific, cat. no. 62-6150-40, -45).

2.1.2. RT-PCR Screening

1. RNase-free Eppendorf tubes.
2. RNase/DNase-free PCR tubes.
3. RT lysis buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0. Just before use, add 10 U/µL of RNaseOUT (Life Technologies, cat. no. 10777-019) and DTT from a 1 *M* stock solution to a final concentration of 2 m*M*.
4. SuperScript II (Life Technologies, cat. no. 18064-014).
5. Random primers.
6. Rat GAPDH primers (forward: 5′-tccatgacaactttggcatcgtgg-3′; reverse: 5′-gttgct gttgaagtcacaggagac-3′), PCR grade.
7. rtTA primers (forward: 5′-tgcttaatgaggtcggaatcgaa-3′; reverse: 5′-acgcg gacccactttcacat-3′), PCR grade.
8. PCR Mix: 1X PCR buffer with 1.5 m*M* $MgCl_2$ (provided with the Taq polymerase), 0.2 m*M* dNTPs, forward and reverse primers (rtTA or GAPDH primers) to a final concentration of 0.4 µ*M* each, 0.2 µg/µL BSA (molecular biology grade), and Taq DNA polymerase 2.5 U/sample (Life Technologies, cat. no. 10342-020).
9. Thermocycler GeneAmp 9700, Perkin-Elmer.
10. Ultrapure agarose.
11. Ethidium bromide solution: dissolve 10 mg of ethidium bromide in 1 mL of H_2O. Caution must be exerted when handling ethidium bromide as it is a powerful mutagen. Always wear gloves and avoid contamination of surfaces.
12. Standard equipment for horizontal gel electrophoresis.
13. UV transilluminator.

2.1.3. Transient Expression of Inducible Luciferase and Luciferase Assay

1. Standard tissue culture equipment.
2. Tet System Approved Fetal Bovine Serum (Clontech, cat. no. 8630-1).
3. Tet-free medium: Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, cat. no. 41966) containing 0.11 g/L sodium pyruvate (Life Technologies, cat. no. 11840), 2 m*M* L-glutamine (Life Technologies, cat. no. 25030), 3.7 g/L sodium bicarbonate (Life Technologies, cat. no. 11810) and 50 units/mL penicillin–streptomycin (Life Technologies, cat. no. 15140) and supplemented with 10% Tet System Approved Fetal Bovine Serum.
4. Trypsin–EDTA solution (Sigma, cat. no. T3924).
5. pRev-TRE-Luc Vector (included in the RevTet-On System Kit, Clontech, cat. no. K1627-1).
6. Lipofectamine2000 (Life Technologies, cat. no. 11668-027).
7. Phosphate buffered saline.
8. Doxycycline (Sigma, cat. no. D9891).
9. 5X luciferase lysis buffer (100 mL): dissolve 1.5 g Tris base and 0.348 g CDTA (1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetracetic acid, Sigma, cat. no. D-1383) in

30 mL H_2O. Add 50 mL of glycerol and mix. Adjust pH to 7.8 with H_3PO_4. Add 2.5 mL of Triton X-100 and bring to the final volume of 100 mL with H_2O. The solution may appear milky and can be stored at –20°C for several months.

10. 1X luciferase lysis buffer: dilute 5X luciferase lysis buffer in H_2O (the final concentration of the buffer will be 25 m*M* Trisphosphate, pH 7.8, 2 m*M* CDTA, 10% glycerol, 0.5% Triton X-100) and add DTT to 2 m*M* final concentration from 1 *M* stock solution. Add DTT just before use.
11. Luciferase assay buffer: 20 m*M* Tricine, 0.1 m*M* EDTA, 1.07 m*M* $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$ (Sigma, cat. no. M0125), 2.67 m*M* $MgSO_4$, pH 7.8. Filter sterilize the buffer and store at 4°C.
12. Luciferase assay reagent: just before use add the following components to 1X luciferase assay buffer: DTT to a final concentration of 33.3 m*M*, coenzyme A (Na salt; Sigma, cat. no. C3144) to a final concentration of 270 μ*M*, ATP (Sigma, cat. no. A2383) to a final concentration of 530 μ*M*, luciferin (K salt; Promega, cat. no. E1602) to a final concentration of 470 μ*M*. It is advisable to prepare stock solutions for all the reagents to be added to the luciferase assay buffer. DTT, coenzyme A, and ATP can be dissolved in H_2O and stored in small aliquots at –20°C for several months. Luciferin should be dissolved in luciferase assay buffer and stored in small aliquots in light-tight vials at –80°C. Once the luciferase assay reagent has been reconstituted, keep it on ice and protect from light. Bring to room temperature just before use.
13. Röhren tubes (SARSTEDT, cat. no. 55.476).
14. Luminometer (LUMAT LB 9501, Berthold).

2.1.4. Production of Inducible NSC Expressing a Pathogenic Transgene of Interest

1. Standard tissue culture equipment.
2. Tet System Approved Fetal Bovine Serum (Clontech, cat. no. 8630-1).
3. Tet-free medium.
4. RetroPack PT67 cell line (Clontech, cat. no. K1060-D).
5. Transgene of interest cloned in pRevTRE vector (Clontech, cat. no. 6137-1).
6. 0.45 μm Millex-HV filters in Durapore, 25 mm (Millipore, cat. no. SLHV R25 LS).
7. Polybrene (Sigma, cat. no. H9268). Polybrene is prepared as stock solution at 5 mg/mL and stored at –20°C.
8. Trypsin–EDTA solution (Sigma, cat. no. T3924).
9. Hygromycin B (Boehringer Manneheim, cat. no. 843555).
10. Cloning rings (PGC Scientific, cat. no. 62-6150-40, -45).
11. Doxycycline (Sigma, cat. no. D9891).
12. RIPA buffer: 0.15 *M* NaCl, pH 7.2, 1% (w/v) Nonidet P-40, 1% (w/v) Na-deoxycholate, 0.1% (w/v) SDS, 2 m*M* EDTA.
13. Protease inhibitor cocktail (Sigma, cat. no. P8340).
14. Equipment for SDS-PAGE electrophoresis and Western blotting.

2.1.5. Dose-Response Curves

1. Standard tissue culture equipment.
2. Tet System Approved Fetal Bovine Serum (Clontech, cat. no. 8630-1).
3. Tet-free medium.
4. Doxycycline (Sigma, cat. no. D9891).
5. RIPA buffer.
6. Protease inhibitor cocktail (Sigma, cat. no. P8340).
7. BCA-200 Protein Assay kit (Pierce, cat. no. 23226).
8. Equipment for SDS-PAGE electrophoresis and Western blotting.

2.2. Assays for Neuronal Cell Death

2.2.1. Electronic Cell Counting

1. Standard tissue culture equipment.
2. Tet System Approved Fetal Bovine Serum (Clontech, cat. no. 8630-1).
3. Tet-free medium.
4. Doxycycline (Sigma, cat. no. D9891).
5. PBS.
6. Trypsin–EDTA solution (Sigma, cat. no. T3924).
7. 0.9% NaCl solution, filter sterilized. Alternatively, a commercially available electrolyte solution (ISOTON II, Coulter) can be used.
8. Cell-counting pots.
9. Coulter Counter.

2.2.2. MTT Assay

1. Standard tissue culture equipment.
2. Tet-free medium.
3. Doxycycline (Sigma, cat. no. D9891).
4. DMEM without phenol red.
5. MTT solution (5 mg/mL): dissolve 5 mg/mL of MTT (Sigma, cat. no. M2128) in 10 mg/mL DMEM without phenol red (Sigma, cat. no. D2902). Filter sterilize and keep the solution sterile. This solution is stable in the dark at 4°C up to 3 weeks. MTT powder must be stored at 4°C.
6. Propan-2-ol.
7. 0.04 *M* HCl in propan-2-ol.
8. Horizontal shaker.
9. MicroELISA reader (ELx800, Packard, Biotek Instruments, Inc.).

2.2.3. DNA Laddering

1. PBS.
2. DNase-free Eppendorf tubes.
3. Proteinase K digestion buffer: 100 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, 1% SDS, 10 m*M* NaCl. Autoclave. Add 1 mg/mL of Proteinase K (Life Technologies, cat. no. 25530-015) before use.

4. Phenol/chloroform/isoamylalcohol (25 : 24 : 1) saturated with 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA (Sigma, cat. no. P3803).
5. 3 *M* sodium acetate: dissolve 40.8 g of sodium acetate in H_2O and adjust pH to 5.2 with 3 *M* acetic acid. Add H_2O to 100 mL. Autoclave.
6. Ethanol.
7. 70% ethanol.
8. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA. Autoclave.
9. RNase A (Sigma, cat. no. R6513). Prepare a 10 mg/mL stock solution in autoclaved H_2O. Heat to 100°C for 10 min to inactivate any contaminating DNase activity, unless differently suggested by the manufacturer. Store the solution in small aliquots at –20°C.
10. Ultrapure agarose.
11. Ethidium bromide solution: dissolve 10 mg of ethidium bromide in 1 mL of H_2O. Caution must be exerted when handling ethidium bromide as it is a powerful mutagen. Always wear gloves and avoid contamination of surfaces.
12. Standard equipment for horizontal gel electrophoresis.
13. UV transilluminator.

3. Methods

3.1. Generating TetON Inducible Cell Lines

Several systems have been developed and are commercially available to tightly control expression of transgenes in eukaryotic cells. A description of all these systems is beyond the scope of this book and we will just focus here on one possible approach that makes use of a modification of the original tetracycline regulatory system ***(5)***, now commercially available (TetON System, Clontech). A brief description of this system is summarized in **Fig. 1**, and for further information we refer the reader to the copious literature on this topic. The procedure consists of two major steps summarized in **Fig. 2**. In the first step, NSC (either immortalized or growth factors-expanded) are infected with a retroviral vector transducing a reverse tetracycline-transactivator (rtTA, see legend to **Fig. 1**). RtTA-positive clones are accurately selected and tested for their ability to induce the expression of a transiently transfected reporter gene (luciferase) in a tightly regulated manner. This step leads to the generation of well-characterized inducible rtTA-expressing subclones, suitable for the insertion of a variety of genes under the control of a tetracycline-responsive promoter (TRE). In the second step of the strategy the rtTA-positive cells are finally infected with the gene of interest and screened again for tight inducibility of the transgene.

The use of retroviral vectors to deliver both the rtTA and the pathogenic gene of interest allows a more stable chromosomal insertion of the transgene and is crucial for a successful engineering of GF-NSC (which are not amenable to cell transfection). For details on the procedures to prepare retroviral medium

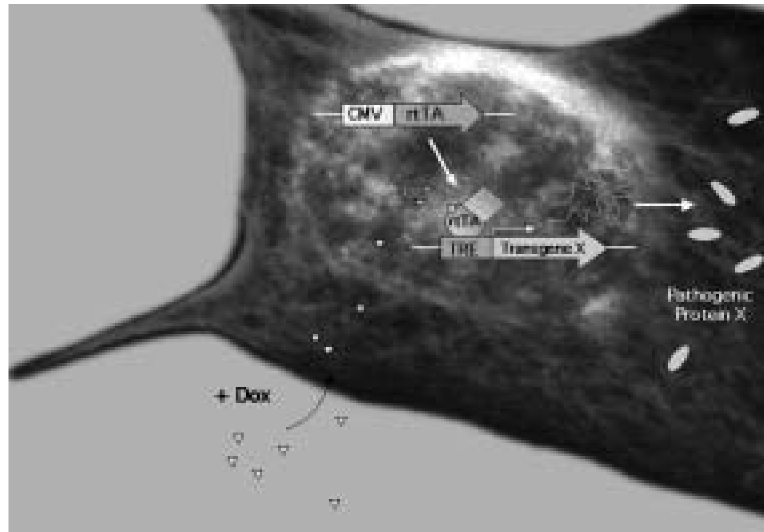

Fig. 1. Schematic representation of the TetON System *(5)*. The system is based on the tetracycline-regulated operon Tn10 transposon of *E. coli*. The tetracycline-controlled transactivator (rtTA), a transcriptional activator, is cloned downstream to the CMV promoter and expressed in NSC. The pathogenic transgene of interest is introduced into the same cells under the control of a specific tetracycline-responsive element (TRE) and an inactive minimal CMV promoter. In the absence of doxycyline the transgene is silent. When doxy is administered to the cells, the rtTA becomes active, binds the TRE and activates transcription of the pathogenic transgene, allowing to follow the progression of cellular dysfunction.

from packaging cell lines and engineering of GF-NSC, please refer to Chapter 21 (*see also* **Note 1**).

3.1.1. Generating rtTA-Positive Clones

1. Transfect the packaging cell line (Retropack PT67, Clontech) with the pRev-TetON vector and select clones stably producing retroviral particles with 400 μg/mL of G418.
2. Seed NSC at a density of 40% in 35 mm dishes 12–18 h before infection.
3. Filter freshly collected viral supernatant from packaging cells through a 0.45 μm filter (*see* **Note 2**). Dilute viral supernatant 1:2 with fresh complete medium (*see* **Note 3**).
4. Add polybrene to the viral medium to a final concentration of 4 μg/mL to allow a better adhesion of retroviral particles to the surface of target cells.
5. Replace cell medium with the viral medium and perform infection for 6 h.
6. Replace viral medium with fresh complete medium.

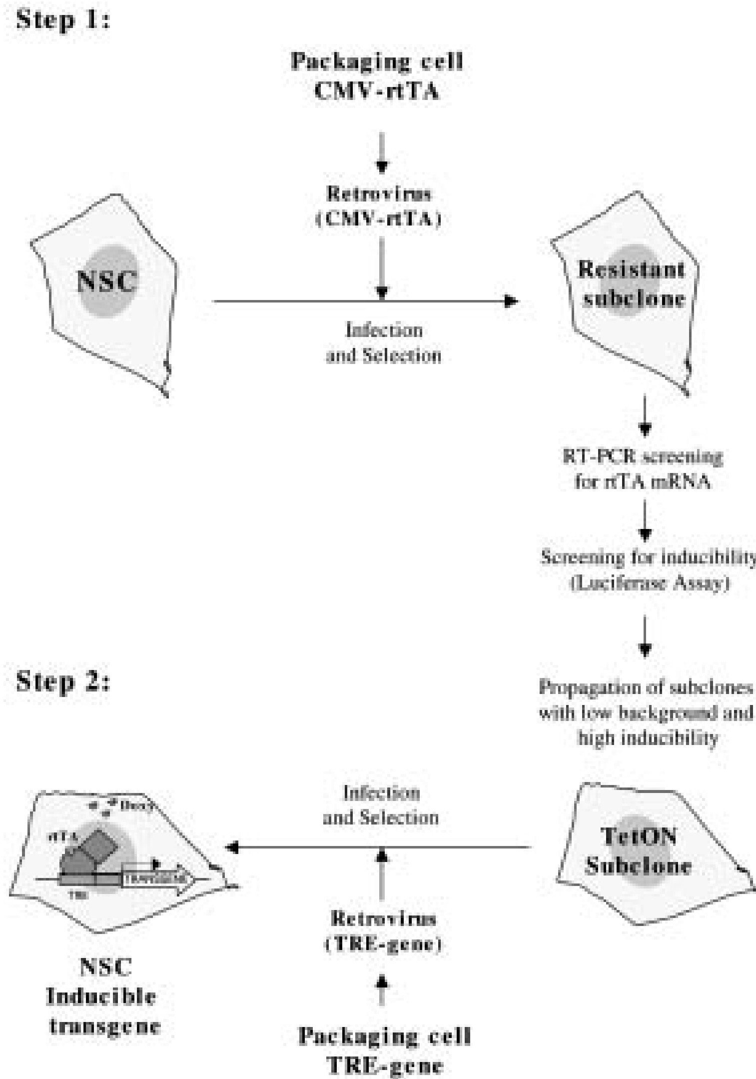

Fig. 2. Generation of TetON inducible NSC. Step 1: the transcription activator rtTA is delivered to NSC by retroviral transduction. After antibiotic selection, infected subclones are first screened for the presence of rtTA mRNA by RT-PCR. Positive clones are further screened for rtTA activity in transcriptional activation. Luciferase is used as reporter gene and its activity is measured upon addition of doxy to the cells. rtTA subclones with low level of basal activity and high level of doxy-induction are selected. Step 2: the pathogenic transgene of interest, under control of the TRE, is delivered to the rtTA subclones by retroviral transduction. After antibiotic selection, resistant subclones are screened for inducible expression of the transgene. Subclones with low basal expression of the transgene and high level of induction upon addition of doxy are selected.

7. Trypsinize cells as for routine subculture 48 h after infection and transfer them into two 150 mm dishes (for easier clone pick-up) containing 18 mL of complete medium.
8. Apply antibiotic selection (400 µg/mL of G418 for infection with pRevTetON). Keep cells under selection until single resistant clones appear on the dishes. Change medium (supplemented with antibiotic for selection) after the first 2–3 d of selection and then every 4 d.
9. Pick up single antibiotic-resistant clones (cloning rings or pipetman taking-up are both suitable techniques for picking up clones) and transfer each of them into two wells of a 96-well plate containing 100 µL/well of fresh medium with the antibiotic for selection.
10. Let the clones grow until they become 90% confluent before proceeding to the screening for the presence of rtTA in one well. For each clone, the second well is used for individual expansion of the positive clones.

3.1.2. RT-PCR Screening for the Presence of rtTA mRNA in Resistant Subclones

Although the infection procedure leads to a very high rate of insertion of both the transgene of interest and antibiotic-resistant gene, not all clones that are resistant to the antibiotic selection will also express a functional rtTA. Screening clones by Western blotting using antibodies for rtTA (Clontech) is possible but not easy to accomplish, especially when a large number of clones have to be screened to select for tight inducibility or when antibiotic selection is not possible. In addition, commercially available antibodies for rtTA do not perform well, at least in our hands. We therefore developed an alternative quick strategy based on RT-PCR to check for the presence of rtTA mRNA in selected clones (*see* **Note 4**).

1. Trypsinize cell clones grown in 96-well plates with 50 µL of trypsin.
2. As soon as cells detach from the plate, block trypsin with 100 µL of complete medium. Pipet up and down a few times to dissociate clumps of cells.
3. Transfer cell suspension into RNase-free Eppendorf tubes and centrifuge at 1000*g* for 5 min to pellet cells.
4. Remove the supernatant and completely dissolve the cell pellet in 50 µL of RT lysis buffer. No clumps must be present. Keep the samples on ice if RT-PCR is carried out immediately; alternatively, store the samples at –80°C until use.
5. Use a 2–5 µL aliquot of the cell lysate directly for reverse transcription (RT) with random primers. In our experience SuperScript II works much better with crude cell lysate than MoMuLV reverse transcriptase.
6. Perform RT following SuperScript II manufacturer's instruction.
7. Use 5 µL of RT reaction for PCR amplification with tTA primers or GAPDH primers (RT-PCR control) in a final volume of 50 µL of complete PCR mix.

8. Perform PCR using the following cycling parameters (for GeneAmp 9700, Perkin-Elmer): 95°C for 7 min; 95°C for 30 sec, 52°C for 30 sec, 72°C for 1 min (for 40 cycles); 72°C for 7 min.
9. Run 10 μL of PCR product on 1% agarose gel containing 0.5 μg/mL ethidium bromide and visualize amplification products at UV light. With the primers described in **Subheading 2.**, the rtTA product will appear as a band of 579 bp while the GAPDH product will be 377 bp. Smearing and a strong diffuse band at low molecular weight may appear in the samples due to the presence of large quantities of DNA and RNA in the crude cell lysate used for RT-PCR. However, this does not usually affect amplification and recognition of the rtTA product. If PCR fails to amplify GAPDH, reduction of the starting cell material should be considered to diminish interference by components of the cell lysate.
10. Discard rtTA-negative clones.

3.1.3. Screening of rtTA Inducible Clones by Transient Expression of Inducible Luciferase

Selected subclones expressing the messenger RNA for rtTA should be further checked for inducibility and levels of background expression by transient transfection with a vector (pRev-TRE-Luc or others) in which the reporter gene luciferase is cloned downstream to the tetracycline-responsive element (TRE). Luciferase activity in non-induced cells—no doxycycline (doxy) administered—gives a measure of the basal level of expression of the transgene (leakage of the system), which may greatly vary from subclone to subclone. The ratio between luciferase activity in lysates from cells exposed to doxy (induced) and luciferase activity in noninduced cells gives a measure of the fold of induction of the transgene in each clone.

1. Grow rtTA-positive clones in Tet-free medium for at least three days before transfecting/infecting them with pRev-TRE-Luc.
2. For each rtTA-positive subclone: plate each subclone in a row of a 12-well plate (four wells) at the cell concentration of 1–1.5 × 10^5 cells/well. The number of cells to be plated may vary for different cell lines; however, cells should be plated at a density of 85%.
3. 12–15 h after plating, transfect cells with pRev-TRE-Luc using Lipofectamine 2000 according to the manufacturer's instruction.
4. Replace transfection medium with fresh Tet-free medium.
5. Add 1 μg/mL of doxy to the third and fourth well of each row to induce luciferase expression.
6. Let cells grow for 48 h.
7. Remove medium and wash the cells with PBS.
8. Completely remove PBS. Wells must be completely dry since traces of Ca^{2+} may inhibit luciferase activity.

9. Add 120 μL of 1X luciferase lysis buffer/well.
10. Incubate cells for 5 min at room temperature. At this step, cells will appear as "ghosts" when observed under microscope.
11. Carefully transfer lysates to Eppendorf tubes avoiding cell debris and immediately place tubes on ice. Cell lysates may be kept on ice for 2–3 h before performing luciferase assay or stored at –80°C without significant loss in luciferase activity.
12. Prepare luciferase assay reagent, keep it on ice and protect from light until use. Just before use, bring it to room temperature.
13. Turn on the luminometer 20–30 min before starting the measurement of luciferase activity. Set the instrument and perform wash cycles according to the manufacturer's instruction. Set an injection volume of 100 μL. Place a vial containing the luciferase assay reagent in the luminometer.
14. Transfer 20 μL of cell lysate into a 5 mL Röhren tube and immediately measure luciferase activity. The luminometer will automatically inject 100 μL of luciferase assay reagent into the tube.
15. For each measurement subtract the activity measured for 20 μL of luciferase lysis buffer (blank).
16. Discard clones with high levels of background expression (high level of luciferase activity in the absence of doxy) or with low level of induction (*see* **Note 5**).

3.1.4. Production of Inducible NSC Expressing a Pathogenic Transgene of Interest

Once rtTA clones with tight inducibility have been identified, they can be transduced with a retroviral vector containing the gene of interest under control of a TRE-minimal promoter (pRevTRE, Clontech). In the case of NSC, retroviral delivery is ideal. For standard molecular biology techniques to clone the gene of interest into the pRevTRE vector, see Sambrook et al. ***(6)***.

1. Transfect Retropack PT67 cells with pRevTRE vector containing the gene of interest and perform selection using 200 μg/mL of hygromycin. For details on these procedures, see Chapter 21.
2. Plate rtTA inducible clones in 35 mm dishes at a cell density of 40% in Tet-free medium.
3. Perform infection as described in **Subheading 3.1.1., steps 3–8**.
4. Apply antibiotic selection (200 μg/mL of hygromycin B). Keep cells under selection until single resistant colonies appear on the dishes. Change medium (supplemented with antibiotic for selection) after the first 2–3 d of selection and then every 4 d.
5. Pick up single clones using cloning rings and transfer them into 96-well plates containing 100 μL/well of fresh medium supplemented with hygromycin B. Let the clones grow until they become almost confluent.
6. Trypsinize cell clones with 50 μL of trypsin solution.
7. As soon as cells detach from the plate block trypsin with 100 μL of Tet-free medium. Pipet up and down a few times to dissociate clumps of cells.

8. For clone subculturing, remove 20 μL of the cell suspension and transfer it in 24-well plates containing 1 mL of Tet-free medium with the antibiotic for selection.
9. For clone screening, divide the remaining cell suspension into two wells of a 24-well plate containing 1 mL of Tet-free medium. Add 1 μg/mL of doxy to one of the wells (for induction of the transgene). Let cells grow to confluence.
10. Lyse cells in 50 μL of ice-cold RIPA buffer (or other convenient lysis buffer) containing 1X protease inhibitors cocktail. Scrape cells and collect them in Eppendorf tubes.
11. Pellet cell debris at 10,000*g* for 15 min and store the supernatant at –20°C until use.
12. Perform SDS-PAGE electrophoresis and Western blotting on cell lysates comparing expression of the protein of interest in non-induced and Doxy-induced condition.
13. Select clones with the lowest basal background and the highest expression of the protein of interest after doxy induction (*see* **Note 6** and **7**).

3.1.5. Characterization of the Inducible Clones: Dose-Response Curves

Clones with low basal background of transgene expression and a good level of inducibility usually respond to doxy induction in a dose-dependent manner. However, this may not be always true. After identification of putative tightly inducible clones, it is advisable to perform dose-response experiments to test effective concentrations of doxy and related levels of transgene induction. The optimal concentration may vary with different cell lines, but full activation of gene expression in TetON cell lines can be obtained with 0.1–1 μg/mL of doxy.

It may also be extremely useful to determine a range of nonsaturating doxy concentrations for studies on the effects of the concentration of the protein of interest on the cell phenotype, or when physiologic levels of expression of the protein are required.

1. Plate six aliquots of 1–1.5 × 10^5 cells/well in six-well plates containing 2 mL of Tet-free medium.
2. Add Doxy to final concentrations of 0, 0.05, 0.1, 0.25, 0.5, and 1 μg/mL from serial dilutions of 1 mg/mL stock solution.
3. Let cells grow for 48 h.
4. Lyse cells in 100 μL of ice-cold RIPA buffer containing 1X protease inhibitors cocktail. Scrape cells and collect them in Eppendorf tubes.
5. Pellet cell debris at 10,000*g* for 15 min.
6. Transfer supernatant into a new Eppendorf tube and keep on ice or store at –20°C till use.
7. Determine protein concentration in the sample with the BCA reagent (Pierce) according to manufacturer's instruction.
8. Perform SDS-PAGE and Western blotting on 20–50 μg of protein sample.

3.2. Markers and Assays for Neuronal Cell Death

3.2.1. Electronic Cell Counting

Rapid and accurate counts of cells can be obtained using a Coulter Counter, named after the scientist who developed the technique *(7)*. Briefly, cells or other particles suspended in an electrolyte solution are drawn through a small orifice in an electrical insulator crossed by the current established by two immersed electrodes. Each particle that enters the orifice displaces a corresponding volume of electrolyte and generates a short voltage pulse that is proportional to the particle size. Voltage pulses are then converted into "spherical equivalents" corresponding to the number of cells present in the suspension.

1. Plate 4×10^4 cells into each well of a 24-well plate in complete medium without phenol red. Set up a plate for each point of a time-course experiment if cell survival must be evaluated upon induction of the transgene.
2. For cell counts, remove medium and wash plates twice with PBS.
3. Add 250 µL of trypsin solution/well and allow cells to completely detach.
4. Pipet up and down to dissociate clumps. Alternatively use syringe with 21G needle and syringe cells for a few times.
5. Dispense 9.8 mL saline into the counting pot and add 0.2 mL of the cell suspension just before counting.
6. Mix by gently rocking the counting pot. Do not invert or shake, as this will generate air bubbles that will interfere with the count.
7. Turn on the Coulter Counter at least 15 min prior to use. Set the instrument and perform flushing operation according to manufacturer's instruction.
8. Count samples.

3.2.2. MTT Assay

The MTT assay *(8)* is a quantitative colorimetric assay that measures viability and proliferation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes to convert, in living cells, the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product that is insoluble in water. The amount of formazan produced is directly proportional to the cell numbers (*see* **Note 8**). The technique is quick and it allows the processing of a large number of samples with a high degree of precision using a microELISA reader (*see* **Note 9**).

1. Seed cells onto 96-well microplates and expose them to the toxic condition (induction of the pathogenic transgene and/or other stress stimuli).
2. Prepare MTT stock solution of 5 mg/mL in DMEM without phenol red and filter through a 0.22 µm filter to sterilize the solution (*see* **Note 10**).

3. Add 10 μL of the MTT solution to each well containing 100 μL of cell medium.
4. Incubate in a humidified incubator at 37°C for 1–4 h.
5. Add 100 μL of 0.04 *M* HCl in propan-2-ol to each well.
6. Mix on a horizontal shaker for 2–5 min to completely dissolve the blue formazan crystals.
7. Carefully clean the bottom of the microplate from dust and/or fingerprints.
8. Read plate on a microELISA reader using a test wavelength of 570 nm and reference wavelength of 630 nm. Read the plates within 30 min.

Alternative protocol:

1. Prepare 1 mg/mL of MTT solution in DMEM without phenol red.
2. Completely remove growth medium from cells.
3. Add 100 μL of MTT solution for each well.
4. Incubate in a humidified incubator at 37°C for 1 h.
5. Completely remove the MTT solution taking care of not removing formazan crystals and leave the plate upside-down on tissue-paper for 2–5 min or until the wells are completely dry.
6. Add 100 μL/well of propan-2-ol (*see* **Note 11**).
7. Mix on a horizontal shaker for 2–5 min.
8. Read plate on a microELISA reader using test wavelength of 570 nm and reference wavelength of 630 nm. Read the plates within 30 min.

3.2.3. Detection of Apoptosis by DNA Laddering

A hallmark of apoptosis in internucleosomal fragmentation of nuclear DNA is a result of induced nuclease activity ***(9)***. In contrast, nuclear DNA of necrotic cells remains intact or becomes degraded resulting in DNA fragments of heterogeneous length. Analysis of cell DNA on agarose gel clearly allows us to distinguish between the two conditions.

1. Grow cells on 100 mm culture dishes and expose them to the toxic condition (induction of the pathogenic transgene and/or other stress stimuli).
2. Remove medium and scrape cells in 500 μL of PBS.
3. Centrifuge the suspension at 250*g* for 5 min and remove the supernatant.
4. Completely resuspend the pellet in 400 μL of proteinase K digestion buffer. Do not vortex.
5. Incubate at 56°C for 4 h on a rotor shaker.
6. Extract the sample with an equal volume of phenol/chloroform/isoamylalcohol (25 : 24 : 1).
7. Mix by repeated inversion and centrifuge at 2500*g* for 10 min to resolve upper aqueous and lower organic phases.
8. Transfer the upper phase to a fresh tube and repeat **steps 6** and **7**.

9. Transfer the upper phase to a fresh tube and precipitate DNA with 1/10 volume of 3*M* sodium acetate and 2 volumes of ethanol at –20°C for 1 h.
10. Centrifuge at 12,000*g* at 4°C for 30 min.
11. Wash the pellet with 500 μL of 70% ethanol and centrifuge at 12,000*g* for 15 min.
12. Air-dry the pellet and gently resuspend the pellet in 30 μL of TE buffer (pH 8.0). Do not vortex.
13. Add RNase A to a final concentration of 0.25 mg/mL and incubate at 37°C for 1 h to digest RNA in the sample.
14. Extract sample with phenol/chloroform/isoamylalcohol (25 : 24 : 1) and precipitate DNA as in **steps 9–11**.
15. Air-dry the pellet and gently resuspend in 30 μL of TE buffer (pH 8.0).
16. Measure DNA concentration at 260 nm in a UV-spectrophotometer.
17. Load 10 μg of DNA onto 1.5% agarose gel and run the gel for 2 h at 70 V.
18. Stain the gel with 2 μg/mL of ethidium bromide for 30 min.
19. Destain the gel for 30 minutes in 1 m*M* of $MgSO_4$ before observing under UV light. DNA from apoptotic cells will appear as a ladder of discrete bands of different length produced by the internucleosomal cleavage of nuclear DNA.

4. Notes

1. Although we routinely use non-diluted viral supernatant for infection of NSC and have no effects on cell growth and survival, growth of other target cells may be strongly affected by media conditioned by the packaging cell lines. For this reason, we suggest using diluted viral supernatant for the infection procedure or modify the composition of the growth medium for packaging cells.
2. Use cellulose acetate or polysulfonic low-protein-binding filters to filter viral supernatant. Nitrocellulose filters bind the proteins of the retroviral envelope and destroy the viral particles.
3. For a high-titer viral medium it is advisable to select and use pools of packaging cell clones stably producing retroviral particles.
4. Although the RT-PCR procedure on crude cell lysates here described may lead to amplification of non-transcribed plasmid DNA as no DNase digestion is performed, in our experience, more than 95% of PCR-positive clones do produce mRNA for rtTA. Consequently, this procedure is highly efficient to predict the presence of rtTA and extremely quick and easy when screening a large number of clones. Traditional RNA extraction using TRizol reagent (Sigma) may be performed on clones grown at confluence in 24-well dishes but we found that is not necessary in most cases.
5. Luciferase activity measured in the absence of doxy induction should be within a range of 5–10 fold above blank. Higher levels of background activity may be expected in transiently transfected cells. However, when the transcription unit controlled by the TRE-minimal promoter is stably integrated into the cell genome, regulation of transcription may be deeply influenced by "positional effects," with the possibility of lower or higher background depending on the

integration site. Therefore, inducibility may greatly vary from clone to clone, and in stable versus transiently engineered TetON subclones.

6. Basal background expression of the transgene and the level of inducibility are strictly dependent on "positional effects." Integration of the transgene in proximity of enhancer sequences may result in very high level of basal expression even in the absence of doxy. There may also be transcriptional read-through from outside promoters. In order to isolate clones with tight regulation of the transgene and minimum or absent level of background, it becomes necessary to screen a large number of clones. In our experience (and with NSC), screening of up to two hundred or more clones is necessary to get 5–8 tightly regulated inducible clones.
7. Responsiveness of inducible clones to Doxy may vary with time and passages in culture. Inactivation of the TRE-minimal promoter by methylation has been described, although in rare cases. We recommend freezing several aliquots of selected clones at early passages and periodically checking for level of transgene induction when cells are maintained in culture for several passages. In the case of loss of induction, the culture may be started again from frozen aliquots.
8. A linear relation between cell number and MTT formazan exists up to 1×10^6 cells. When the experiment requires the use of a larger number of cells, a standard curve for the cell number should be constructed. If necessary, plating cells on 24-wells and scaling up the protocol should be considered.
9. Although it is widely assumed that MTT is reduced by mitochondrial activity, it has been demonstrated that most of the compound is reduced in the endosomal/lysosomal compartment ***(10–11)***. MTT is membrane impermeable and is taken up by cells through endocytosis. After reduction in the endosomal/lysosomal compartment in a few hours, it is transported to the cell surface through exocytosis. The rate of exocytosis of formazan crystals is strictly dependent on the cell type. On the basis of these considerations, caution must be exerted when extrapolating informations on cell survival from MTT data as these may also mirror the metabolic state of the cells and the activity of the endosomal/lysosomal compartment. It is sometimes advisable to compare MTT data with data generated through other cell survival assays (cell counter).
10. It is strongly advisable to prepare enough solution to treat all the samples that have to be compared, since great variability may be introduced using different stock solutions for the same experiment. It is also strongly recommended that the stock solution be kept sterile.
11. DMSO may be used instead of propan-2-ol to solubilize formazan crystals, as far as it is used for all the samples and experiments that have to be compared to each other.

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III

Utilization/Characterization of NSCs In Vivo

A. Endogenous Pools of Stem/Progenitor Cells

23

Activation and Differentiation of Endogenous Neural Stem Cell Progeny in the Rat Parkinson Animal Model

Marcel M. Daadi

1. Introduction

The ability to target biologically active molecules to precise locations in the central nervous system (CNS) is a promising therapeutic treatment. It overcomes many problems encountered by systemic delivery. Among these problems are the inability to penetrate the blood-brain barrier (BBB), global actions, and metabolism by peripheral organs, which may result in undesirable side effects. Although direct administration of biological substances to the brain overcomes these problems, there are certain limitations. These limitations primarily include long-term delivery and limited diffusion. The later may be caused by limited extracellular space, cellular metabolism, substance liability, or "the sink effect," which results from rapid turnover of the cerebrospinal fluid (CSF). To overcome these problems, scientists have been innovative in coming up with effective delivery systems for both clinical use and basic research. Among delivery systems particularly suited for regenerative medicine are genetically engineered cells, biomaterials, and osmotic pumps. The osmotic pump (Durect, Cupertino, CA, USA) consists of a semipermeable hard outer shell, a compressible inner bag that will contain the biological factor, and a high-osmolarity substance between the two. The diffusion of the interstitial water across the outer shell increases the volume and gradually compresses the inner bag. The result is a continuous infusion of substances prepared at specific concentrations into a localized area of the brain.

The discovery of stem cells and their progeny within the subependymal layer (SEL) of the adult brain ***(1–5)*** opens new avenues of potential therapeutic

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intervention. Their location within the SEL lining the ventricular system of the CNS makes them accessible for manipulation. Many laboratories have successfully been able to activate these endogenous neural stem cells ***(6,7)*** and manipulate them in animal models of neurological disorders ***(8)***.

In the present chapter, we will focus on the use of Alzet osmotic pumps to activate and mobilize stem cell progeny into the striatal parenchyma of 6-OHDA lesioned rats and induce them to differentiate into tyrosine hydroxylase (TH) expressing cells.

The experimental protocol is as follows: The rats are first subjected to behavioral analysis for at least 2 wk, then unilaterally lesioned by injecting the 6-OHDA into the substantia nigra. After 2 wk, rats are screened for the completeness of the striatal dennervation using the apomorphine rotational behavioral test. Activation of stem cells is achieved by intracerebroventricular (ICV) delivery of EGF for 14 d. The differentiation of stem cell progeny is then induced by intraparenchymal infusion of bFGF and glial-derived conditioned media (CM) into the striatum (**Fig. 1**). After 4 wk animals are tested for any behavioral improvement and a first group of animals is sacrificed at 5 wk post-treatment.

2. Materials

2.1. Osmotic Pump Preparation

1. Alzet pump model 2002: rate 0.5 μL/h, 14 d duration (Durect).
2. Vinyl catheter tubing (Small Parts, Inc., Miami Lakes, FL, USA).
3. 5–10 mL syringe, hemostat, scissors, 96-well plates, 15 mL Falcon tubes (VWR, Brisbane, CA, USA).
4. Gentamycin, rat serum albumin (Sigma, St. Louis, MO, USA).
5. Physiological Saline (Baxter Healthcare Corporation, Round Lake, IL, USA).
6. Growth factors: epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Upstate Biotechnology, Lake Placid, NY, USA).

2.2. Osmotic Pump Implantation

1. Animals: Adult female Sprague–Dawly rats (200–250 g) (Harlan Sprague Dawley, Indianapolis, IN, USA).
2. Ketamine (Ketaset, Bristol Laboratories, Syracuse, NY, USA).
3. Xylazine (Rompum, Miles Laboratories, Shawnee, KS, USA).
4. Acepromazine maleate (TechAmerica Group, Elwood, KS, USA).
5. Stereotaxic apparatus and electrode holder (David Kopf Instruments, Tujunga, CA, USA).
6. Sterile dissecting instrument: forceps, hemostatic forceps, scalpel, scissors, and safety glasses (Roboz surgical instrument, Rockville, MD, USA).
7. Dental cements (Henry Schein, Inc., Melville, NY, USA).
8. Steel drilling bit, small pin vice, and stainless-steel screws (Small Parts, Inc., Miami Lakes, FL, USA).

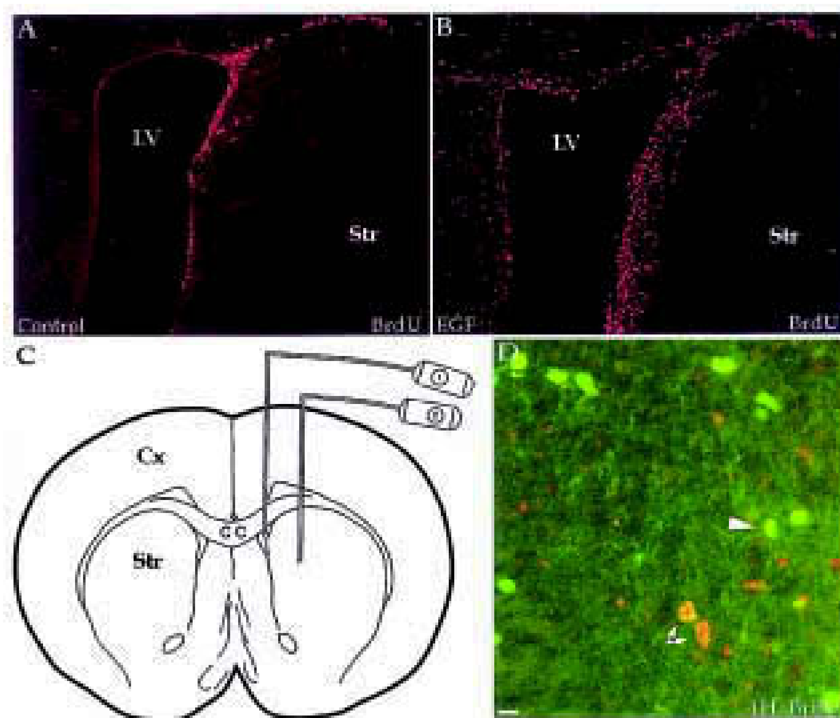

Fig. 1. *In vivo* manipulation of stem cell progeny to express TH. **(A)** and **(B)** are representative pictures taken from frontal striatal sections immunostained with anti-BrdU and showing the outcome of EGF infusion into the lateral ventricle (treatment 1 in **C**). ICV infusion of EGF increased the number of cells that had incorporated BrdU in their nuclei (red labeling). **(C)** diagrammatic representation of the cannula sites: two weeks after the 6-OHDA lesion, the infusion cannula of an Alzet osmotic pump (model 2002, infusion time 14 d, 0.5 μL/h outflow rate) was stereotaxically implanted into the lateral ventricle for the first treatment (1=EGF, stem cell activation) and into the striatum for the second treatment (2=bFGF+CM, differentiation of stem cell progeny). **(B)** 30 μm frontal sections through the striatum were processed for TH/BrdU double-immunocytochemistry. **(D)** The small double arrows show examples of TH-immunoreactive cells (red labeling) located within the rostral migratory stream in the rostral part of the striatum. Arrowhead shows an example of a newly generated cell (BrdU+, green) in the same region of the rat brain. The bar represents 50 μm in A, B and 20 μm in D.

3. Methods

In a previous in vitro study *(9)*, we have shown that the combination of bFGF and glial-cell-derived CM induces TH gene expression in stem cell progeny derived from the embryonic EGF-responsive stem cells and the adult

mouse SEL. We hypothesized that in vivo manipulation of the surrounding environment of the CNS stem cell progeny may favor the repopulation and functional support of the injured or diseased brain region. Using the 6-OHDA rat Parkinson animal model, we tested whether or not infusion of FGF2 and glial-cell-derived CM via osmotic pumps into the forebrain induces the SEL-derived stem cell progeny to express TH in vivo. In this chapter, we describe a means to achieve this goal.

3.1. Osmotic Pump Preparation

1. Clean surface area with 70% ethanol.
2. Place pumps in a sterile 96-well plate with open end facing upward.
3. Place the pump cannula, the connecting medical vinyl tubing, and the brain cannula into a sterile Petri dish.
4. Using hemostat and scissors, snap off white caps from pump cannula.
5. At one end of the connecting tubing, attach the pump cannula to the tubing with an overlap of 3 mm. At the other end of tubing, attach the brain cannula.
6. To fill the pump and tubing, fill a 1 mL syringe with appropriate infusate solution (*see* **Note 1**).
7. The infusate is made up with physiological saline solution containing gentamycin sulfate at 0.1 mg/mL, rat serum albumin at 1 mg/mL, and growth factors EGF or bFGF at 25 μg/mL. Concentrations were adjusted when CM was to be infused so that the final concentration of CM in the pump is 75% (v/v).
8. Attach a blunt-end needle to the syringe and push solution until it comes out of the cannula tip.
9. Put blunt-end needle tip into the open end of the pump and slowly fill to the top.
10. Fill the rest of the apparatus (tubing connected to cannulae) with the same solution.
11. Connect pump to pump cannula.
12. Make sure no air bubbles are visible in the tubing.
13. Aliquot 4 mL of sterile physiological saline into sterile 15 mL Falcon tubes.
14. Immerse the filled pumps in the aliquot of physiological saline. Ensure they are completely covered.
15. Incubate for 4 h at 37°C to activate the pump (*see* **Note 2**).

3.2. Osmotic Pump Implantation

The 6-OHDA lesion of the rats was carried out as previously described ***(10)*** and will not be detailed here. Two weeks after the 6-OHDA lesion, both sham and lesioned animals are subjected to pump implantation.

1. Surgery area must be cleaned with 70% ethanol.
2. Rat is anesthetized by intramuscular injection of a mixture of ketamine (62.5 mg/kg body weight), xylazine (3.25 mg/kg), and acepromazine (0.62 mg/kg).

3. Once asleep, clean the head with 70% ethanol and cut hair in front and back of the ears.
4. Put animal in ear bars of the stereotaxic apparatus.
5. Place teeth bar at 3.3 mm below intra-aural level, rest the rat nose on the nose bar and secure firmly.
6. Make one central incision with sterile scalpel to expose the skull.
7. Remove muscle layer on skull by scraping gently.
8. Clean skull with 70% ethanol.
9. Put a screw on the contralateral side to the intended brain cannula site. To put the screw in the skull, drill a hole and turn the screw in for one and one-half turns to avoid damaging the underlying cortex.
10. For pump placement, loosen the skin below the shoulder with hemostats.
11. Bring pump out of 37°C, and immediately insert it under the skin of the animal.
12. Put the pump into place and attach brain cannula into stereotaxic holder.
13. Find bregma and measure desired coordinates. The following are measured according to Paxinos and Watson Atlas ***(11)***:

Lateral ventricle (LV) (*see* **Note 3**)		striatum	
anterior/posterior:	–0.8 mm	anterior/posterior:	–0.8 mm
lateral:	–1.4 mm	lateral:	–2.25 mm
dorsal/ventral:	–4.5 mm	dorsal/ventral:	–4.5 mm

14. Mark on the skull the exact position where the brain cannula will be inserted.
15. Drill a hole.
16. Insert brain cannula.
17. Cement cannula in place with dental cement and liquid.
18. Do not remove animal from the stereotaxic apparatus until the cement is completely dry.
19. After surgery record each animal's weight.
20. Put each animal in a cage under a heat lamp until recovered from anesthesia.
21. Following surgery, animals are given analgesic, food, and water.
22. First treatment: stem cell activation is achieved by EGF infusion for 14 d (**Fig. 1A–C**). To label the newly generated cells, BrdU was injected intraperitoneally at 100 mg/kg of body weight 3X/d during the last 4 d of EGF infusion (*see* **Note 4**).
23. Second treatment: differentiation of stem cell progeny is achieved by FGF2 and CM infusion into the striatal parenchyma (**Fig. 1C**,**D**).
24. After the 2 wk delivery period, the pumps are removed from the animals under anesthesia.
25. After a survival period of 5 wk, animals are perfused, brains removed, cut, and processed for various neural markers and BrdU immunocytochemistry (*see* **Note 5**) (**Fig. 1**).

4. Notes

1. When preparing the pumps, avoid any air bubbles either in the pump or in the tubing. These bubbles may block the infusion process. To avoid air bubbles, ensure there are none in the syringe and when filling the pump or tubing slowly inject the substance.
2. To verify that your pump is working, look for an infusate drop at the tip of the cannula after the incubation/activation period at 37°C.
3. When placing the cannula in the LV, watch for reflux of the CSF for confirmation of successful cannulations.
4. Different paradigms of BrdU labeling may be adapted to the study objective and experimental design. Keep in mind that the approximate cell cycle length of the constitutively proliferating cells is 12.7 h ***(5)***.
5. For ideal BrdU immunostaining, sections need to be incubated in 1*M* HCl for 30 min. This acid treatment often destroys the TH antigen. We found that processing the sections for TH first then for BrdU immunoreactivity works best. For immunocytochemistry, sections are incubated with the primary antibody(ies) prepared in PBS+0.3% Triton+10% normal serum overnight at 4°C in a moist chamber, washed 3X with PBS and then incubated with the appropriate secondary antibody(ies) at room temperature for 1 h.

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24

Identification of Musashi1-Positive Cells in Human Normal and Neoplastic Neuroepithelial Tissues by Immunohistochemical Methods

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1. Introduction

For years, it was generally believed that the normal mammalian postnatal brain had little capacity to produce new neuronal cells or to repair itself after injury ***(1)***. However, recent progress in neurobiology has led to a new understanding of the development and cell lineages of mammalian brain cells. Studies have demonstrated that neurons and glial cells are derived from common precursor cells, called neural stem cells (NSCs) ***(2,3)***, and that NSCs are present in the periventricular zone of fetal, postnatal, and adult mammalian brains ***(2–14)***.

Our knowledge about mammalian NSCs has been mainly derived from vigorous studies of rodents by cellular, molecular, and immunohistochemical methods ***(2–6)***. Now, with the emergence of regenerative medicine, there is an increasing interest in characterizing human NSCs ***(7–14)***. To pursue this problem, the identification of human NSCs in vivo is one of the most useful approaches, and several phenotypic markers that can be used to detect human NSCs by immunohistochemical methods have been isolated ***(7,8)***. One of these markers is Musashi1, an evolutionarily well-conserved RNA-binding protein ***(4–8,15,16)***. Musashi1 is a member of the Musashi family of proteins, which likely play a central role in the posttranscriptional gene regulation that is essential for the self-renewal of NSCs as well as for proper neuronal and glial development. Our previous immunohistochemical results showed that Musashi1 is expressed in NSCs and neuronal progenitors located in the ventricular and subventricular zones of embryonic, postnatal, and adult rodent

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brains ***(4–6)***. Moreover, Musashi1 is also expressed in astroglial progenitor cells and mature astrocytes, but not in cells committed to the oligodendrocytic lineage ***(4–6)***. A recently generated rat monoclonal Musashi1 antibody (clone 14H1) cross-reacts with the human Musashi1 homolog, and recognizes it well in formalin-fixed paraffin-embedded archival tissues, when a microwave antigen retrieval method is used ***(6,8)***. Using this antibody, we recently showed that the expression patterns of Musashi1 in normal developing and adult human brains correspond well with those reported for rodents ***(7,8)***. These findings indicate that Musashi1 could be one of the best markers for human fetal and adult NCSs, neuronal progenitor cells, and astrocytic lineage cells, for the purpose of detection in routine clinical diagnosis ***(7,8)***.

Interestingly, some human brain tumors, such as glioblastomas, astrocytomas, and oligoastrocytomas contain Musashi1-positive and GFAP-negative tumor cells ***(8)***. Furthermore, the expression level of Musashi1 in these brain tumors is positively correlated with the malignancy and proliferative activity of the tumor cells. These results indicate that Musashi1 is a versatile marker, not only for NSCs or progenitor cells in normal human brains, but also for the diagnosis of gliomas, both in establishing their cellular origin and in evaluating their malignancy. These features are likely to be useful in designing future therapeutic strategies ***(8)***.

In this chapter, we describe the immunohistochemical methods used to detect Musashi1-positive cells in human normal and neoplastic neuroepithelial sections prepared by the most wide-spread formalin-fixation method and embedded in paraffin wax. The protocol involves the preparation of sections, application of the antigen retrieval method ***(8,17)***, and Musashi1 single immunostaining by the avidin-biotin-complex (ABC)-peroxidase method, which is the most common immunohistochemical technique used in human pathological studies ***(8,18)***. Moreover, we also describe a double-immunohistochemical method to detect Musashi1 and other neuronal and glial phenotypic markers in the same sections at the same time, which can provide even more details about cellular origins and phenotypes ***(8,18)***.

2. Materials

2.1. Equipment

1. 1000 mL glass beaker.
2. Coloration vessel and stainless-steel rack for glass slides.
3. Liquid barrier (e.g., ImmEdge Pen, Vector Laboratories, Burlingame, CA, USA, cat. no. H-4000).
4. Glass slides.
5. Humidified incubation chamber.

2.2. Reagents

1. 1X phosphate-buffered saline (PBS). Prepare a 10X concentrated stock solution as follows: Dissolve 80 g of NaCl, 2 g of KCl, 29 g of $Na_2HPO_4 \cdot 12H_2O$, and 2 g of KH_2PO_4 in 800 mL of distilled water. Adjust the pH to 7.4 with HCl, then add distilled water to 1 L. Store at room temperature.
2. 0.2% (v/v) Tween® 20 (ICN Pharmaceuticals Inc, Aurora, OH, USA) in 1X PBS (PBS-T). Use only freshly prepared buffer and keep it at room temperature.
3. Citrate buffer (10 m*M*, pH 6.0). Prepare a 0.1 *M* stock solution as follows: Dissolve 19.2 g of citric acid (anhydrous) (Sigma, cat. no. C-0759) in 800 mL of distilled water. Adjust the pH to 6.0 with NaOH, then add distilled water to 1 L. Store at room temperature.
4. 30% (w/w) hydrogen peroxide solution (Sigma, cat. no. H-1009). Store at 4°C.
5. 3-Amino-propyltriethoxy-silane (APS, Sigma, cat. no. A-3648). Store at 4°C.
6. Tris-HCl buffer (50 m*M*, pH 7.2). Prepare a 1 *M* stock solution as follows: Dissolve 121.1 g of Tris base (TRIZMA BASE, Sigma, cat. no. T-1503) in 800 mL of distilled water. Adjust the pH to 7.2 by adding concentrated HCl. Allow the solution to cool to room temperature before making the final pH adjustment. Add distilled water to 1 L. Store at room temperature.

2.3. Antibodies

All the antibodies should be freshly diluted in blocking solution (*see* **Subheading 2.4.**) just before use. Do not store them as diluted solutions.

1. Primary antibodies
   a. Rat anti-Musashi1 monoclonal antibody (MAb) (clone 14H1) ***(6)***.
   b. Mouse anti-Hu proteins MAb (clone 16A11, supplied by Dr. M. F. Marusich, University of Oregon) ***(19,20)***.
   c. Mouse anti-GFAP MAb (clone G-A-5, Boehringer Mannheim Biochemical, Indianapolis, IN, USA).
2. Secondary antibodies
   a. Rabbit affinity-purified biotinylated anti-rat IgG (Vector, cat. no. BA-4000).
   b. Goat affinity-purified biotinylated anti-mouse IgG (Vector, cat. no. BA-9200).

2.4. Blocking Solutions, ABC Kit, and Enzyme Substrates

2.4.1. Blocking Solutions

1. Blocking solution A: 5% (v/v) normal rabbit serum (Vector, cat. no. S-5000) and 1% (w/v) bovine serum albumin (Sigma, cat. no. A-7030) in 1X PBS.
2. Blocking solution B: 5% (v/v) normal goat serum (Vector, cat. no. S-1000) and 1% (w/v) bovine serum albumin (Sigma) in 1X PBS.

Use only freshly prepared solution A and B. Store at 4°C before use.

2.4.2. ABC Kits

1. VECTASTAIN Elite ABC Standard Kit (Vector, cat. no. PK-6100).
2. VECTASTAIN ABC-AP Standard Kit, (Vector, cat. no. AK-5000).
   Prepare the ABC reagents 30 min before use, following the manufacturer's instructions. Store at 4°C.
3. DAB substrate solution: Combine 10 mg of 3, 3′-diaminobenzidine (DAB) (Sigma, cat. no. D-5905), 6.67 µL of 30% hydrogen peroxide, and 20 mL of 0.05*M* Tris-HCl buffer (pH 7.2) immediately before use. Store at room temperature and protect from light as much as possible.
4. Alkaline phosphatase substrate solutions (AP-substrates) 5-bromo-4-chloro-3-indolyl phosphate/4-nitro blue tetrazolium chloride (BCIP/NBT, Alkaline Phosphatase Substrate Kit IV, Vector cat. no. SK-5400). Prepare the AP-substrate working solution immediately before use, following the manufacturer's instructions. Store at 4°C and protect from light as much as possible.

3. Methods

3.1. Preparation of Sections and Prestaining Treatment for Musashi1 Immunohistochemistry

Normal human brain tissue and brain tumor sections that have been fixed in 10% formalin and embedded in paraffin wax are used for immunohistochemistry according to the routine processing procedure for detecting Musashi1 immunoreactivity (*see* **Note 1**).

1. Prepare APS-coated glass slides by dipping slides carried in a stainless-steel rack into 2% APS-acetone solution for 30 sec. Wash the slides in distilled water for a few seconds, then dry them completely in an oven at 60°C (approx 1 h).
2. Cut 5 µm-thick sections on a microtome, mount them on APS-coated slides, and air-dry them in an incubator overnight at 37°C (*see* **Note 2**).
3. Deparaffinize the samples in three changes of xylene for 5 min each. Hydrate the sections gradually through a graded series of alcohol dilutions: wash in 100% methanol 3X for 5 min each, 90% methanol for 5 min, 80% methanol for 5 min, then 70% methanol for 5 min.
4. Rinse sections in PBS-T 3X for 5 min each (*see* **Note 3**).
5. Retrieve antigens by treating the sections with citrate buffer and microwaving as follows (*see* **Note 4**) ***(8,17)***.
   a. Place sections carried in a stainless-steel rack into a 1000 mL beaker.
   b. Add citrate buffer to the beaker to completely submerge all parts of the steel rack (approx 1 L of buffer is needed). If part of the steel rack is exposed, microwaving will cause sparks. Mark the liquid level on the beaker.
   c. Microwave for 3 min (power level: 600 W).
   d. Repeat the microwaving step 4X (total microwaving time: 15 min). Check the amount of buffer after each microwaving step and make up for the loss of volume by adding distilled water to the point marked in **step b**.

   e. Allow the sections to cool in the buffer to room temperature (approx 30 min). Avoid rapid cooling by ice or refrigeration.
6. Wash samples in PBS-T 3X for 5 min each at room temperature.
7. If sections are to be stained by the ABC-peroxidase method, treat them with 2% hydrogen peroxide in methanol for 30 min at room temperature to quench the endogenous peroxidase activity (*see* **Note 5**).
8. Wash in PBS-T 3X for 5 min each at room temperature.
9. Blot the excess PBS-T and circle the sections with a liquid barrier.
10. Cover sections with PBS-T. If you want to take a break in the staining procedure, it is possible to store the sections in PBS at 4°C for about 24 h at this step.

3.2. Musashi1 Staining Procedure by the ABC-Peroxidase Method

Musashi1 immunoreactivity in human neuroepithelial tissues can be detected by either the ABC-peroxidase or ABC-alkaline phosphatase staining methods. However, the ABC-peroxidase method usually results in a lower background and higher contrast of Musashi1 immunostaining. Thus, the ABC-peroxidase method is recommended for Musashi1 immunostaining, as indicated in this section. All the incubation steps are carried out in a humidified chamber, with sufficient amounts of reagent to cover the specimens. The tissue sections should not be allowed to dry out at any time during the following procedure.

1. Incubate the sections for 20 minutes with blocking solution A (approx 150 μL per slide is usually adequate) (*see* **Note 6**).
2. Blot the excess blocking solution A.
3. Incubate the sections with 14H1 MAb diluted 1:500 in blocking solution A overnight at 4°C (approx 100 μL per slide is usually sufficient).
4. Wash in PBS-T 3X for 5 min each at room temperature.
5. Incubate the sections with rabbit affinity-purified biotinylated anti-rat IgG diluted 1:200 for 1 h at room temperature (approx 150 μL per slide is usually sufficient).
6. Wash in PBS-T 3X for 5 min each at room temperature.
7. Incubate the sections with ABC-peroxidase reagent for 30 min at room temperature.
8. Wash in PBS-T 3X for 5 min each at room temperature.
9. Incubate the sections in DAB substrate solution until the desired staining intensity develops (many sections should be developed within a few minutes) (*see* **Note 7**).
10. Stop the enzymatic reaction with tap water.
11. If desired, dip the slides in hematoxylin for 5 min to counterstain. Wash in tap water several times.
12. Dehydrate the samples through a graded series of alcohol dilutions and xylene as follows: soak in 70%, 80%, 85%, and 90% methanol for 1 min each, 100% methanol three times for 1 min each, then xylene 3X for 1 min each.
13. Wipe off excess xylene and add permanent mounting medium. Cover samples with a glass coverslip, and observe by light microscopy.

3.3. Indirect Double Immunostaining of Musashi1 with Neuronal or Glial Markers

Indirect double immunostaining of Musashi1 with other neuronal or glial phenotypic markers is done by combining the ABC-peroxidase and ABC-alkaline phosphatase staining methods. In this section, we describe the protocol for the double immunostaining of Musashi1 with Hu (a neuronal marker) or GFAP (an astrocytic marker) in human neuroepithelial sections (*see* **Note 8**).

1. Prepare sections following the protocol given in **Subheading 3.1.**
2. Incubate the sections for 20 min with blocking solution B at room temperature (approx 150 μL per slide is usually sufficient) (*see* **Note 6**).
3. Blot the excess blocking solution B.
4. Incubate the sections with anti-GFAP MAb diluted 1:60 or anti-Hu MAb diluted 1:400 in blocking solution B overnight at 4°C (approx 100 μL per slide is usually sufficient).
5. Wash in PBS-T 3X for 5 min each at room temperature.
6. Incubate the sections with goat affinity-purified biotinylated anti-mouse IgG diluted 1:200 in blocking solution B for 1 h at room temperature (approx 150 μL per slide is usually sufficient).
7. Wash in PBS-T 3X for 5 min each at room temperature.
8. Incubate the sections with ABC-AP reagent for 30 min at room temperature.
9. Wash in PBS-T 3X for 5 min each at room temperature.
10. Incubate the sections in AP substrate until the desired staining intensity develops (10–20 min in most cases).
11. Stop the enzymatic reaction with tap water.
12. Mount the samples in 100% glycerol and examine the results of the first staining. Photograph if desired.
13. Wash in PBS-T 3X for 5 min each at room temperature.
14. Stain for Musashi1 by the ABC-peroxidase method following the protocol in **Subheading 3.2.** (*see* **Note 9**).

4. Notes

1. Samples that have been stored for a long time under inappropriate conditions, or are over-fixed in formalin can show strong background staining in some cases. Frozen sections can be also used for Musashi1 immunohistochemistry.
2. Glass slides coated with poly-L-lysine (PLL) can also be used for Musashi1 staining. However, sections on PLL-coated slides peel off more easily during the microwave treatment than do those on APS-coated slides. Thus, we recommend using APS-coated glass slides.
3. In place of PBS-T, it is possible to permeabilize the sections in PBS containing Triton-X. However, the repeated rinsing of sections in PBS-T reduces background staining.

4. Antigen retrieval is not always necessary for single Musashi1-immunostaining of tumor sections, especially glioblastomas, which often show intense Musashi1 immunoreactivity. However, for staining other types of samples and for double-immunohistochemical staining, the antigen-retrieval technique will result in more intense Musashi1 signals and better contrast.
5. For the staining of normal human brain sections, a lower concentration (0.1–1%) of hydrogen peroxidase in methanol and a shorter time (5–10 min) can be used to quench the endogenous peroxidase activity. However, for staining tumor sections, even using the shorter time of treatment and lower hydrogen peroxidase concentration may result in high background staining, because tumor samples often contain abundant red blood cells within the tumor vessels.
6. Ideally, it would be best to use normal blocking serum of the same species in which the secondary antibody was raised.
7. The reaction time required for the DAB treatment may differ depending on the level of antigen. For many normal sections, it will take a few minutes. However, in some tumor sections, an immediate DAB-mediated reaction will often be observed. Using cooled DAB solution or decreasing the DAB concentration will slow down the reaction.
8. The ABC-alkaline phosphatase method is also available for Musashi1 immunostaining. However, Musashi1 signals obtained by the ABC-alkaline phosphatase method are often lower than those obtained by the ABC-peroxidase method. If the ABC-alkaline phosphatase method is selected, the enzymatic activity should be detected with a reddish substrate (for example, Vector® Red Alkaline Phosphatase Substrate Kit I, Vector cat. no. SK-5100) to obtain a better contrast in the staining. The Musashi1 signals obtained with BCIP/NBT staining are sometimes associated with a high background and poor contrast.
9. Indirect double immunostaining should start with the ABC-alkaline phosphatase method. If detection of Musashi1 by the ABC-peroxidase method is performed first, higher background signals are often seen during the subsequent ABC-alkaline phosphatase staining step.

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25

Identification of Newborn Cells by BrdU Labeling and Immunocytochemistry In Vivo

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1. Introduction

5-Bromodeoxyuridine, variously abbreviated as BrdU, BUdR, and BrdUrd, is a halogenated thymidine analog that is permanently integrated into the DNA of dividing cells during DNA synthesis in S-phase. BrdU can be immunocytochemically detected in vitro and in vivo, allowing the identification of cells that were dividing during the period of BrdU exposure. In vivo, it has been used to identify the "birthdate" of cells during development, to examine the fate of postnatally generated cells, and to label cells prior to transplantation for subsequent identification.

BrdU can be used in situations where tritiated thymidine would otherwise be used. BrdU is preferable for some applications and limited in others, when compared to tritiated thymidine. BrdU can be very quickly detected; BrdU immunocytochemistry is highly reproducible and takes about 1 d, while tritiated thymidine autoradiography is more variable and typically requires at least 2 wk of exposure. In addition, fluorescent BrdU labeling can be imaged on confocal microscopes, allowing one to positively identify multilabeled cells. BrdU labeling reveals details about nuclear morphology that tritiated thymidine autoradiography does not. In addition, BrdU can be detected in 40–50 μm sections, unlike tritiated thymidine, which can only detect labeled cells in the most superficial 3–5 μm of sections. On the other hand, counting grains on tritiated thymidine autoradiographs can allow the tracing of the number of divisions a cell has gone through since the tritiated thymidine incorporation. Differences in the degree of immunocytochemical BrdU label-

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ing, while sometimes apparent, are not reliable markers of the number of divisions a cell has gone through.

Iododeoxyuridine (IrdU) is a halogenated thymidine analog similar to BrdU and is less commonly used in neuroscience research. The majority of antibodies detect both BrdU and IrdU, but using antibodies that can distinguish between the two can enable measurement of cells' cycle times or identification of cells born at different times ***(1)***.

Owing to its bromine side group, BrdU is potentially more toxic to experimental cells and animals than tritiated thymidine, a factor that should be taken into consideration when administering BrdU. Negative effects of BrdU on the survival of labeled cells have been reported ***(2)***, and there are reports that embryonic BrdU treatment can yield reduced brain and body weight ***(3)***. However, BrdU-labeled cells can undergo apparently normal division, differentiation, tangential and radial migration, and process integration both in developmental studies ***(4)*** and in studies of adult neurogenesis ***(5–7)***. With these caveats in mind, one should minimize the amount of BrdU used, to reduce cell toxicity and experimental artifacts.

In vitro, there is evidence that BrdU can integrate into apoptotic cells, presumably as a result of DNA damage and repair. The appearance of nuclear BrdU labeling in dying cells in these in vitro studies is typically that of minor nuclear speckling rather than the diffuse labeling of the entire nucleus seen in mitotic cells. However, in vivo , even at doses significantly higher than typically used, BrdU does not integrate in detectable levels into adult cells induced to undergo necrotic or apoptotic death or early postnatal cells undergoing apoptotic death (Magavi and Macklis, unpublished observations). In these in vivo experiments, adult mice received intracortical ibotenic injections, inducing the apparently necrotic death of cortical neurons and glia. BrdU was injected at equal intervals, and sections were examined at 3, 6, and 24 h after induction of necrosis. Sections were processed for BrdU immunocytochemistry and stained with the nuclear dye Hoechst 33258, which reveals condensed nuclei of dying cells. Daily BrdU doses ranging from 1,000 to 3,200 mg/kg revealed no dying cells that integrated detectable levels of BrdU (n = 10,000 cells observed). Postnatal (d 0 or P0) mice, which normally contain many neurons undergoing developmental apoptosis, were similarly treated with 350 mg/kg BrdU every 1.5 h for 6 h (1,400 mg/kg total dose) and labeled for both BrdU and cleaved caspase-3, a protein involved in apoptosis that labels apoptotic cells. None of the apoptotic cells were BrdU-labeled (n = 5,000 cells observed). Thus, it is highly unlikely that apoptotic cells would be mistaken for newly born cells in vivo.

BrdU labeling can be used to assess proliferation of a population of cells or to assess the survival of labeled cells. When combined with phenotypic

markers, it can be used to establish the fate of cells labeled during the period of BrdU administration. To specifically assess effects on proliferation, animals are commonly injected with BrdU intraperitoneally and sacrificed 2 h later ***(6)***. After longer periods, cells may die or migrate away from the region of interest, affecting cell counts. Survival of BrdU-labeled cells can be assessed analyzing tissue after longer time periods; in such experiments, the possible effects of migration must also be considered.

2. Materials

2.1. BrdU Administration

1. 5-Bromodeoxyuridine (Sigma, cat. no. B5002).
2. Acrodisc Syringe Filter (Gelman Laboratory, cat. no. 4192).
3. Sterile saline, pH 7.35.
4. ddH_2O.

2.2. BrdU Staining

1. Antibody to BrdU. Harlan # MAS250p, clone BU1/75 rat anti-BrdU antibody is available through Accurate Antibodies. Rat-derived antibodies are relatively uncommon, making it easier to perform double or triple labeling using antibodies derived from other species.
2. Fluorescent secondary antibody. Alexa 488 goat anti-rat antibody, Molecular Probes cat. no. A11006 (*see* **Note 1**).
3. Phosphate-buffered saline (PBS): 2.625 g/L Na_2HPO_4, 11.5 g/L NaH_2PO_4, 9 g/L NaCl. Dissolve compounds in double-distilled water (ddH_2O) and adjust pH to 7.35. PBS is stable indefinitely.
4. Heparin in PBS: Dissolve heparin, sodium salt (Sigma, cat. no. H3393) in PBS to make a 10 units/mL solution.
5. 4% paraformaldehyde in PBS. Make an 8% solution of paraformaldehyde prills (Electron Microscopy Sciences, cat. no. 19202) in ddH_20. Paraformaldehyde does not enter solution easily. To dissolve it more quickly, add 4 mL of 1 *M* NaOH for each 100 mL of solution, and heat the solution to 50°C, while stirring constantly. Add an equal volume of 2X PBS to make a 4% solution of paraformaldehyde in PBS. Adjust the pH to 7.35 and filter the solution through Fisherbrand Qualitative P8 filter paper, cat. no. 09-795C.

   Paraformaldehyde is stable for several weeks. Under this protocol, BrdU staining is incompatible with glutaraldehyde fixation.
6. Blocking solution (*see* **Note 2**). 8% goat serum (Gibco), 0.3% bovine serum albumin (Sigma), 0.3% Triton X-100 (Sigma). Dissolve serum and triton in PBS.
7. Netwells, optional. Costar Netwells, VWR cat. no. 29442-138, can be used to conveniently move sections quickly from one solution to another. We use six-well Netwell plates when moving sections through inexpensive solutions such as PBS, blocking solution, and Hoechst 33258 dye.

3. Method

3.1. BrdU Administration

BrdU can be administered via intraperitoneal (IP) injection, intraperitoneal infusion, oral ingestion, or intraventricular infusion. The particular characteristics of each technique are described below.

1. The most common method of BrdU administration is through intraperitoneal (IP) injection. IP injection is technically simple and easily temporally controlled. A single BrdU injection labels cells for approx 5–6 h ***(8)***, though this may vary depending on the initial dose given. Thus, labeling cells over an extended period requires repeated injections, a potentially time-consuming and invasive procedure. BrdU doses ranging from 50 up to 200 mg/kg are common for rodents. BrdU injected into pregnant dams will label dividing cells in the developing embryos. To prepare a BrdU solution for injection, dissolve BrdU in saline and adjust pH to 7.35. Filter sterilize through a 0.2 μm filter and store in a sterile vial suitable for storing injectable solutions. BrdU is stable in solution for months when stored at 4°C.
2. Intraperitoneal infusion: Osmotic minipumps can be inserted either subcutaneously or intraperitoneally for a longer-term more-stable dosage of BrdU. Although we have not used this technique, it seems likely that a dose of 150 mg/kg per day in saline solution would be appropriate.
3. Oral ingestion: Animals can be provided BrdU in their drinking water. This technique of BrdU administration is completely noninvasive and requires no animal handling, making it especially useful for extended BrdU administration. However, it does not allow precise control over the exact time and level of BrdU administration, and it may introduce some variability into experiments. Animals drink different amounts of water depending on ambient temperature, and animals may also drink only at particular times of day, affecting the number of cells that are labeled. In addition, long-term BrdU exposure may lead to metabolic changes that reduce BrdU absorption in a species- and strain-specific manner ***(9)***. In our experience, dividing cells in the brains of C57/Bl6 mice can be labeled reliably for at least several weeks. Mice drink relatively consistently throughout the day, in a manner roughly proportional to their body weight and room temperature. We recommend a dose of 225 mg/kg per day. Mice ingesting approx 300 mg BrdU/kg per day display as many or more labeled, dividing cells than mice injected twice daily with 120 mg/kg BrdU. Mice ingesting approx 150 mg/kg of BrdU per day display similar numbers of BrdU+ cells, however, labeled cells are less intensely stained and are sometimes difficult to detect. Mice ingesting doses of BrdU above 300 mg BrdU/kg per day had similar numbers of BrdU-labeled cells, but these were even more brightly labeled. It seems likely that BrdU delivered orally yields lower plasma levels of BrdU than the same amount of BrdU delivered intraperitoneally. BrdU is easily dissolved in drinking water and is stable for at least 1 wk at room temperature.

4. Intraventricular infusion: It is possible to label dividing cells near the ventricles using BrdU administered through an intraventricular cannula. A BrdU solution can be combined with other reagents of interest, such as growth factors, and infused directly into the ventricles. This method delivers BrdU to a limited region of the brain. Cells near the boundary of the ventricles or in the anterior hippocampus are preferentially labeled using this technique. Dividing cells in regions of the brain further from the ventricles are not labeled. Rats receiving an intraventricular infusion of 120 μg of BrdU per day have significant numbers of BrdU-positive cells in the ipsilateral ventricle ***(10)***, and we find that a significant number of cells are labeled in the mouse at a dose of 100 μg BrdU per day. We find strong BrdU labeling in the ipsilateral ventricle and anterior hippocampus, and less labeling in the contralateral ventricle and posterior hippocampus. There is little labeling of dividing cells not directly adjacent to the ventricles.

3.2. Staining Techniques

1. BrdU staining is relatively simple and reproducible for those comfortable with immunocytochemistry. BrdU can be stained under a variety of conditions. However, it is necessary to denature nuclear DNA into single-stranded form so antibodies can bind the BrdU. DNA can be denatured using either acid, microwave treatment ***(11)***, rapid heating ***(12)***, or DNase treatment ***(13)***. Treating floating sections with 2 *M* HCl for 2 h at room temperature is a simple and consistent method for preparing sections for BrdU treatment. The HCl treatment used in this protocol destroys some antigens—in particular, cell surface markers (*see* **Note 1**).
2. Transcardially perfuse with 10 unit/mL heparin followed by 4% paraformaldehyde/PBS. Under this protocol, BrdU staining is incompatible with glutaraldehyde fixation (*see* **Note 3**).
3. Postfix whole brains in paraformaldehyde solution overnight. Cut 40 μm sections using a vibratome. Store sections in PBS; the BrdU antigen will remain stable for several months. Storing sections in a 0.01% NaN_3/PBS solution will enhance their stability.
4. Wash floating sections in PBS for 3 min.
5. Dip sections in ddH_2O 1X 3 sec. This removes the excess PBS, which could buffer the HCl.
6. Incubate sections in 2 *M* HCl at room temp for 2 h on a rocker. Be sure that sections are not floating on top of the solution (*see* **Note 4**). HCl exposure denatures DNA, exposing the BrdU. BrdU antibodies only recognize BrdU in single-stranded DNA; attempting to stain for BrdU without denaturing the DNA will not work. Other protocols use microwave, heat, or DNase antigen exposure to denature the DNA, though HCl exposure has worked most consistently in our hands. Sections will shrink and become whitish, then regain their normal appearance after washing in PBS and blocking solution. We find that sections are actually easier to handle after HCl exposure.

7. Wash sections 4X 8 min in PBS at room temp on a rocker to reequilibrate the sections to pH 7.35. Sections will lose their whitish, shrunken appearance and regain their normal appearance.
8. Wash sections in blocking solution for one hour at 4°C on a rocker.
9. Incubate sections in anti-BrdU antibody dissolved in blocking solution diluted at 1:400 overnight at 4°C. Approximately 70–80 μL of solution per section is sufficient, though using less may be possible. We incubate sections in 24-well plates, typically placing four or five sections in 400 μL of antibody in a single well. Plates need not be sterile for antibody staining and can be reused if well cleaned.
10. Wash sections 3X 5 min in PBS.
11. Incubate sections in Alexa 488 anti-rat secondary antibody at 1:250 in blocking solution for 2 h.
12. Wash sections 3X 5 min in PBS.
13. Briefly dip sections (approx 1 sec) in ddH_2O to remove excess PBS before mounting.
14. Mount sections on gelatin-coated slides and coverslip with Fluoromount or PBS. Fluoromount flattens sections, producing crisp pictures, and it stabilizes fluorescence well. Refrigerated Fluoromount-mounted sections kept in the dark will retain their fluorescence for months, if not years.

    Mounting sections in PBS with minimal drying preserves the three-dimensional structure of tissue and makes it much easier to distinguish the depth of particular features. This is important when performing multiple labeling with cytoplasmic markers and nuclear markers such as BrdU, since nuclei of glia of other cells can be closely opposed to other cells and mistaken for their nuclei. PBS often yields less-crisp-appearing images, because more of the section is out of the objective's depth of focus. Coverslips can be removed from PBS-mounted sections allowing the sections to be stained for additional markers, if the need arises. To mount sections in PBS, place a drop of PBS on the sections, gently place the coverslip, and then remove excess PBS using bibulous paper. The coverslip will stop sliding easily when you have removed enough PBS. Use nail polish to seal the edges and prevent the PBS from evaporating. PBS-mounted sections can later be dehydrated and mounted in Fluoromount.

BrdU-positive cells will have nuclear labeling but no cytoplasmic labeling. Brain sections containing the hippocampus or subventricular zone of BrdU-treated animals can be used as positive controls, since these portions of the brain contain populations of constituitively dividing cells.

4. Notes

1. HCl exposure is compatible with many immunocytochemical analyses, including those for NeuN, Hu, Dcx, TOAD-64, Map-2, GFAP, IGF-I receptor, and RIP antigens. HCl exposure destroys other antigens, such as MBP, A2B5, and O4. Some secondary antibodies, including Molecular Probes Alexa 546 secondary

antibodies, are stable through HCl exposure. This allows one to complete staining for a sensitive antigen and then expose the sections to HCl for BrdU staining, in order to double-label sections. BrdU has been reported to quench Hoechst staining ***(14)***, but this quenching appears undetectable in tissue sections under the staining protocol that follows.

2. Because blocking solution contains serum, it can be contaminated. Blocking solution is stable for 1 wk at 4°C and for several months frozen. If the solution becomes cloudy, it has become contaminated and should not be used. Adding 0.025% sodium azide, a bacteriostatic preservative, can reduce the risk of contamination, but it may interfere with certain types of staining.
3. Improperly perfused animals will have high levels of background staining and may be difficult to section. Often, commonly used secondary antibodies against IgG groups will bind to blood or immune cells, staining the vasculature in the brain. Perfusing with more heparinized PBS solution can reduce background due to vasculature.
4. Often, "floating" sections (a misnomer, since it is necessary that they sink) will float on the surface of the solution they are in, exposing only one side of the section to the solution and leading to inconsistent staining. This is easily avoided by gently shaking the solution until the sections sink, or simply dunking them using a fine paintbrush.

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26

Immunocytochemical Analysis of Neuronal Differentiation

Sanjay S. P. Magavi and Jeffrey D. Macklis

1. Introduction

Fully understanding the phenotype of neurons in vivo involves examining their morphology, immunocytochemically analyzing their protein expression, examining their afferent and efferent integration into neuronal microcircuitry, and functionally examining their activity. This task is significantly more difficult when one is attempting to determine whether multipotent precursor cells, often referred to as stem cells, differentiate into neurons in vivo. Transplanted or endogenous precursor cells often produce relatively small numbers of new neurons in the adult brain, making electron microscopy or electrophysiological analysis extremely challenging, and functional analysis difficult. Studying such cells usually depends heavily on immunocytochemical approaches. In this chapter, we will review a range of immunocytochemical techniques for identifying whether transplanted or endogenous neural precursors have differentiated into mature neurons. We provide immunocytochemical protocols for the migratory neuronal marker Doublecortin (Dcx), the early expressed marker Hu, and mature neuronal marker NeuN. In Chapters 25 and 27, we provide protocols for identifying newborn cells using the mitotic label bromodeoxyuridine (BrdU) and for examining axonal projections using the retrograde label FluoroGold.

The underlying assumption behind immunocytochemical cell identification approaches is that particular cell types uniquely express particular proteins. Thus, because antibodies can be produced that specifically recognize such proteins, we can identify the phenotype of cells by assessing whether they express particular proteins. The first issue in neuronal phenotype analysis to

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consider is the choice of antibodies. Obviously, it is important to examine the literature and choose antibodies that specifically recognize proteins expressed only by neurons. Sometimes, it is only discovered after some time that a particular antibody is not specific for a particular phenotype. For example, neuron-specific enolase (NSE) can label glia in addition to neurons. Therefore, despite its name, NSE is no longer an appropriate marker to use for neuronal identification. It is necessary to review thoroughly the relevant literature when choosing markers for neuronal identification.

Even if one is using antibodies that are normally specific to proteins expressed by neurons, it is still theoretically possible that multipotent cells under analysis could pathologically misexpress both neuronal and non-neuronal proteins during the process of differentiation. Thus, it is necessary to assess whether the cells of interest both (1) express more than one neuronal marker and (2) do not express markers of potential alternative cell phenotypes, such as astroglia and oligodendroglia. Only cells that express multiple neuronal markers and do not express glial markers can be considered neurons.

A further understanding of the development and phenotype of neurons formed from transplanted or endogenous precursors can be gained by using neuronal markers whose expression is temporally regulated. By showing the development of new neurons, one can also exclude the possibility that preexisting neurons were artifactually labeled with markers intended to identify transplanted or endogenous precursors. For example, we used such an approach of progressive developmental markers to examine induced cortical neurogenesis using immunocytochemical markers Dcx, Hu, and NeuN ***(1)***. The Dcx enabled us to identify that at least some of the newborn cells arose from the subventricular zone (SVZ) and migrated into neocortex, while Hu and NeuN allowed us to demonstrate that they survived for many months and differentiated into mature neurons.

In addition to the broader issues concerning antibody labeling, there are a number of technical issues to consider. The first is simplest; it is necessary to confirm that there is no artifactual cross-reactivity between the antibodies employed and other proteins. Secondary antibodies used to visualize labeling can nonspecifically bind to brain tissue, though generally at low levels, and in a fairly reproducible manner. It is thus important to perform relevant control experiments, such as omission of the primary antibody, to clearly understand the level and appearance of background staining. Background staining in vivo can sometimes resemble specific labeling, and it may take substantial experience to differentiate between the two. Background staining can be reduced by incubating the tissue in the serum of the species that the secondary antibodies are derived (“blocking”). Blocking tissue sections in 5–10% serum

or 0.3–0.5% serum albumin solution for 1 h is often sufficient to substantially reduce background staining. Background staining can be further reduced by using 5–10% serum or 0.3–0.5% serum albumin in the primary and secondary antibody solutions.

When using multiple immunohistochemical labels simultaneously in order to double or triple label sections, it is necessary to ensure that the secondary antibodies used do not cross-react with unintended primary antibodies, best achieved by using (1) primary antibodies developed in different species and (2) highly cross-adsorbed secondary antibodies that are species-specific. Highly cross-adsorbed secondary antibodies are generally most specific for their antigens, but even these need to be tested to ensure their specificity. For example, if one were labeling sections with both a mouse-derived NeuN antibody and a rat-derived BrdU antibody, it would be important to ensure that the anti-mouse secondary antibody intended to bind the NeuN primary antibody does not bind to the rat-derived BrdU primary antibody.

Another issue arises when attempting to identify the phenotype of cells labeled with a marker that does not indicate the morphology of the cell. Cells labeled with BrdU, which only labels the nucleus, or fluorescence *in situ* hybridization (FISH), which produces punctate nuclear staining, can be confused with cells located immediately above or below, as reported by Kuhn et al. ***(2)***. Drying and mounting tissue can cause the tissue to shrink to as little as one quarter its original thickness, making it difficult to identify the limits of a cell along the *z* axis. This difficulty can be overcome using confocal microscopy, which provides a very narrow focal depth, and enables very high-resolution images along the *z* axis of tissue. Examining images obtained at various depths through the tissue can enable confirmation that both the cell nucleus and phenotypic markers are co-localized. By using confocal microscopy to analyze serial sections through a cell and produce three-dimensional reconstructions of cells, it is possible to unequivocally demonstrate that two labels are co-localized.

One can also increase resolution in the *z* axis by mounting tissue in PBS without drying it significantly. Tissue that has not been dehydrated does not shrink, making it much easier to resolve the depth of individual cells by routine microscopy with high numerical aperture, narrow depth-of-focus objectives. Co-localization must still be confirmed by confocal microscopy, but the PBS mounting technique can greatly reduce the number of false-positives taken in for confocal examination.

Counterstaining with the nuclear dye Hoechst 33258, which labels all DNA, or an equivalent nuclear marker, can assist in confirming that particular nuclei belong to particular cells. This technique can also assist in identifying the

morphology of neuronal vs non-neuronal nuclei. Neurons generally have much larger, rounder nuclei than glial cells, with dispersed heterochromatin, giving insight into their identity. However, nuclear characteristics alone are not sufficient to identify neurons, since dividing or recently mitotic cells can also have large nuclei. A protocol for Hoechst staining follows the immunocytochemistry protocol.

The majority of the following protocol is general for Dcx, Hu, and NeuN. The only differences are in the concentrations of the primary antibodies used and the type of secondary antibodies used to label the primary antibodies.

2. Materials

2.1. Immunocytochemical Cell Identification

Dcx is a microtubule-associated protein that is expressed exclusively in migrating and differentiating neurons. It is downregulated with maturation. Dcx is expressed in the soma and leading processes of migrating neurons, and in the axons of differentiating neurons ***(3,4)***. Hu is an RNA-binding protein that begins to be expressed soon after neuronal differentiation and continues to be expressed through adulthood. It, too, is expressed in the nucleus and soma of neurons ***(5,6)***. NeuN is expressed exclusively in mature neurons ***(7)***. It is highly expressed in the nucleus of neurons and more weakly in the cytoplasm of the soma. Long axonal or dendritic processes are not visible with this marker.

1. Antibody to Dcx: J. Gleeson, UCSD or C. Walsh, Harvard Medical School; a rabbit IgG antibody.
2. Antibody to Hu: Monoclonal antibody facility, University of Oregon, MAb 16a11; a mouse IgG antibody.
3. Antibody to NeuN: Chemicon cat. no. MAB377; a mouse IgG antibody.
4. Fluorescent secondary antibodies: Alexa 546 goat anti-mouse IgG (H+L), highly cross-adsorbed antibody, Molecular Probes cat. no. A11030. Alexa 546 goat anti-rabbit IgG (H+L), highly cross-adsorbed antibody, Molecular Probes cat. no. A11035.
5. Phosphate-buffered saline (PBS): 2.625 g/L Na_2HPO_4, 11.5g/L NaH_2PO_4, 9 g/L NaCl. Dissolve compounds in double-distilled water (ddH_2O) and adjust pH to 7.35. PBS is stable indefinitely.
6. Heparin in PBS: dissolve heparin, sodium salt, in PBS to make a 10 units/mL solution. Store at 4°C. Heparin solution is stable for at least 6 mo.
7. 4% Paraformaldehyde in PBS: make an 8% solution of paraformaldehyde prills (Electron Microscopy Sciences, cat. no. 19202) in ddH_2O. Paraformaldehyde does not enter solution easily. To dissolve it more quickly, add 4 mL of 1 *M* NaOH for each 100 mL of solution, and heat the solution to 50°C, while stirring constantly. Add an equal volume of 2X PBS to make a 4% solution of paraformaldehyde in PBS. Adjust the pH to 7.35 and filter the solution through

Fisherbrand Qualitative P8 filter paper. Paraformaldehyde is stable for several weeks.

8. Blocking solution: 8% goat serum, 0.3% bovine serum albumin, 0.3% Triton X-100. Dissolve sera and Triton X-100 in PBS (*see* **Note 1**).
9. Netwells, optional: Costar netwells can be used to conveniently move sections quickly from one solution to another. We use six-well Netwell plates when moving sections through inexpensive solutions such as PBS, blocking solution, and Hoechst 33258 dye.
10. Fluoromount (BDH Laboratory Supplies. Poole, UK).

2.2. Hoechst Dye Staining

1. PBS, as above.
2. Hoechst 33258, also known as bisbenzimide (Sigma cat. no. B2883).

3. Methods

3.1. Immunocytochemistry Protocol

1. Transcardially perfuse with 10 units/mL of heparin followed by 4% paraformaldehyde/PBS. High quality perfusion will reduce background staining (*see* **Note 2**).
2. Postfix whole brains in paraformaldehyde solution overnight. Cut 40 μm sections using a vibratome. Store sections in PBS: the NeuN and Hu antigens will remain stable for several months. Storing sections in a 0.01% NaN_3/PBS solution will enhance the stability of sections.
3. Wash floating sections in PBS for 3 min.
4. Incubate sections in blocking solution for 1 h at 4°C on a rocker. Be sure that the sections are not floating on the surface of any of the solutions throughout this protocol (*see* **Note 3**).
5. Incubate sections in blocking solution containing either: anti-Dcx antibody at 1:100; anti-Hu antibody at 2 μg/mL; or anti-NeuN antibody at 1:400. Incubate sections overnight at 4°C with rocking. 70–80 μL of solution per section is sufficient, though using less may be possible. We incubate the sections in 24-well plates, usually putting four or five sections in 400 μL of antibody solution in a single well. The plates need not be sterile for immunostaining, and can be reused if cleaned well. During incubation, the wells should be covered with parafilm to prevent evaporation. Antibody concentrations should always be titrated for the particular experiment in question (*see* **Note 4**).
6. Wash sections 3X 5 min in PBS.
7. Incubate sections in secondary antibody at 1:750 in blocking solution for 2 h. for Dcx : Alexa 546 anti-rabbit IgG; for Hu : Alexa 546 anti-mouse IgG; for NeuN : Alexa 546 anti-mouse IgG.
8. Wash sections 3X 5 min in PBS.
9. Briefly dip sections (approx 1 sec) in ddH_2O to remove excess PBS before mounting.

10. Mount sections on gelatin-coated slides and coverslip with Fluoromount or PBS. Fluoromount clears and flattens the sections, producing crisp images, and it stabilizes the fluorescence well for long periods. Refrigerated, Fluoromount-mounted sections kept in the dark will retain their fluorescence for months, if not years. Alternatively, mounting sections in PBS, without drying them excessively, preserves their three dimensional structure. Evaporation of the PBS can be prevented by sealing the edges of the coverslip with nail polish (*see* **Note 5**).

3.2. Hoechst Dye Staining

Hoechst staining allows one to identify the nuclei of cells, making it a useful fluorescent counterstain. Hoechst 33258, also known as bisbenzimide, intercalcates into A-T rich regions of DNA. It appears blue-white under fluorescence microscopy.

1. Perfuse animals, postfix brains, and cut brains as described in **Subheading 3.1., steps 1–3**.
2. Incubate floating sections in a 2.5 μg/mL solution of Hoechst in PBS at room temperature for 4.5 min with rocking.
3. Wash sections 3X 5 min in PBS.
4. Mount sections as described above.

4. Notes

1. Since blocking solution contains serum, it can be contaminated. Blocking solution is stable for 1 wk at 4°C and for several months frozen. If the solution becomes cloudy, it has become contaminated and should not be used. Adding 0.025% sodium azide, a bacteriostatic preservative, can reduce the risk of contamination, but it may interfere with certain types of staining.
2. Improperly perfused animals will have high levels of background staining and may be difficult to section. Often, commonly used secondary antibodies against IgG groups will bind to blood or immune cells, staining the vasculature in the brain. Perfusing with more heparinized PBS solution can reduce background due to vasculature.
3. Often, "floating" sections (a misnomer, since it is necessary that they sink) will float on the surface of the solution they are in, exposing only one side of the section to the solution and leading to inconsistent staining. This problem is easily avoided by gently shaking the solution until the sections sink or by simply dunking them using a fine paintbrush.
4. One should titrate primary and secondary antibody concentrations to obtain the best possible staining. It is important to titrate antibodies so that one obtains bright staining without producing undue background or wasting costly antibodies. Laboratory-to-laboratory variations, such as the intensity of fluorescence illumination or specific filter set on microscopes, can make identically stained sections appear either overexposed or dim.

5. Mounting sections in PBS with minimal drying preserves the three-dimensional structure of tissue and makes it much easier to distinguish the depth of particular features. This is important when performing multiple labeling with cytoplasmic markers and nuclear markers such as BrdU, since nuclei of glia cells can be closely opposed to other cells and mistaken for their nuclei. PBS often yields less crisp appearing images, because more of the section is out of the objective's depth of focus. Coverslips can be removed from PBS-mounted sections allowing the sections to be stained for additional markers, if the need arises. To mount sections in PBS, place a drop of PBS on the sections, gently place the coverlip, and then remove excess PBS using bibulous paper. The coverslip will stop sliding easily when you have removed enough PBS. Use nail polish to seal the edges and prevent the PBS from evaporating. PBS-mounted sections can later be dehydrated and mounted in Fluoromount.

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27

Neuroanatomical Tracing of Neuronal Projections with Fluoro-Gold

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1. Introduction

The study of neuronal connectivity requires the ability to trace axons from the neuronal cell body to its axon terminal (anteriograde tracing) and from the terminal back to the soma (retrograde tracing). Such neuroanatomical tracing is frequently used to identify neurons on the basis of their pre- or post-synaptic connections. Neuroanatomical tracing has become particularly important in the field of nervous system regeneration and repair, allowing investigators to follow the axon projections of newly born, transplanted, or axotomized neurons in lesioned or neurodegenerative environments. To allow further study of neurons identified and labeled in this way, it is particularly important that tracers are compatible with other tissue processing such as immunocytochemistry.

Fluoro-Gold (FG; Fluorochrome Inc., Denver, CO, USA) is a fluorescent retrograde marker, commonly used for neuronal labeling and neuroanatomical tracing. Like other retrograde markers, FG is taken up by axon terminals, or through injured axons, and retrogradely transported to neuronal cell bodies, thus specifically labeling neurons that project to the region of application. The main advantages of FG, as compared to other retrograde markers, are (1) its ability to be visualized without additional processing; (2) its extensive labeling of distal dendrites; and (3) its stability for relatively long periods of time after application and under a variety of fixation and histochemical conditions.

FG is taken up by intact axon terminals and injured axons, but not by intact axons at nonterminal sites ("axons of passage") ***(1)***. Intact axonal uptake occurs via endocytosis at nerve terminals ***(2)***. Following retrograde transport, FG labels neuronal soma and dendrites. In the soma, FG is associated with vesicles

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in the cytoplasm, the plasma membrane, and the nucleolus ***(1)***. It does not diffuse from labeled neurons, and it is not transported trans-synaptically ***(1)***.

Because of its stability, FG may be applied to axon terminals or cut nerves by a relatively wide variety of methods. FG may be administered directly to the target region via pressure injection (typically <1 μL); FG can be iontophoresed (+5 to +10 μA/10 min) from small-tipped pipets that can be used to record physiological activity from cells before labeling ***(3)***; and FG may be placed in the target region in crystalline form. FG also may be applied indirectly (and nonspecifically) to axon terminals by intraperitoneal (IP) or intravenous (IV) injection ***(4)***. FG widely labels axon terminals in the peripheral nervous system following IP or IV injections. However, it does not cross the blood-brain barrier (BBB), and therefore does not label neurons that project to areas within the CNS protected by the BBB ***(1,4)***.

A significant limitation of FG as a marker of specific projection neurons is its tendency to diffuse from the site of injection in neural or muscle tissue. This diffusion can lead to nonspecific labeling of neurons that project to regions neighboring the intended target region ***(5)***.

FG is delivered to the cell body by fast axonal transport ***(6)***. The time required for retrograde labeling depends on distance and on degree of dendritic filling desired. FG labeling of cell bodies has been detected as soon as 2 h after injection ***(7)***. Typically, minimum survival time for axonal and cytoplasmic labeling is 1–2 d. Longer survival periods result in more complete labeling of high-order neurites, up to hundreds of microns beyond the soma ***(8)***. FG labeling is stable for weeks following injection, and persists up to 2 mo in some reports ***(1)***.

FG is functional at a wide range of concentrations (1–10% w/v). Lower concentrations (2–4%) are recommended for most procedures. Higher concentrations and larger injection volumes cause more intense retrograde labeling, but also induce more necrotic damage at the injection site ***(1)***. FG labeling in the neuronal cell body is non-toxic at recommended concentrations.

FG has been reported to locally label non-neuronal cells in vivo. Almost all cells at the injection site become labeled. Rinamin et al. ***(9)*** found phagocytic microglia and other macrophages containing FG following degeneration of retrogradely labeled axotomized neurons, presumably due to ingestion of dying FG-labeled neurons. Similarly, Streit et al. ***(10)*** reported FG-containing glia following toxic injury to motor neurons, but not in control (nontoxic) conditions. Injection of large volumes of FG into the lateral ventricle in rats labels periventricular astrocytes and ependymal cells ***(1)***.

FG can be visualized directly by fluorescence microscopy using an ultraviolet filter (excitation, 323 nm; emission, 408 nm). Color varies slightly with changes

in pH from gold (neutral and basic pH) to blue (acidic pH) ***(1)***. The ability to detect FG without additional histochemical processing allows for identification and dissection of FG-labeled regions under UV light ***(11)***. Visualization by fluorescence microscopy is limited by FGs tendency to photobleach, although its photostability far exceeds commonly used chromophores such as AMCA or Cy-3. Ju and Han ***(12)*** suggest that photobleaching is largely due to water in tissue sections, and may be avoided or minimized by the use of alternative mounting media.

Several methods have been developed to convert FG's fluorescence into more stable, electron-dense products for electron microscopy (EM). FG can be converted directly to an electron-dense diaminobenzidine (DAB) reaction product by photooxidation to allow for EM visualization ***(13)***. The development of antisera against FG ***(14)*** has also allowed FG to be detected with DAB or immunogold-silver ***(8)***. In these protocols, FG is detected not only in lysosomes, but also dispersed throughout the cytoplasm and distal dendrites, two to three branch points beyond the soma ***(8)***. FG is reported to be stable in a variety of fixatives, or in the absence of fixation, and is not affected by most standard immunohistochemical procedures.

We have used Fluoro-Gold to label both callosal projection neurons and cortico-thalamic projection neurons in vivo in adult and neonatal mouse neocortex ***(15–20)*** and projection neurons in the song networks of the avian forebrain ***(21)***. The following protocol outlines our methods for labeling corticothalamic neurons in adult mice, and can be adapted to retrogradely label different populations of projection neurons.

2. Materials

1. Fluoro-Gold Fluorochrome Inc., Denver, CO, USA [(303) 832-1212].
2. Fluoro-Gold solution. Make a 2% (w/v) solution of Fluoro-Gold in double distilled water (*see* **Note 1**).
3. Nanoject Variable (Drummond Scientific Company). The Nanoject Variable is a digitally controlled oocyte pressure injector that is especially useful for injecting nanoliter volumes of solutions extracellularly into the brain, via small diameter pulled glass micropipets.

3. Method

In this protocol, we inject Fluoro-Gold into the thalamus using a posterior approach, inserting the glass micropipet at a 30° angle from the vertical axis in order to avoid injury to more anterior cortex, which was important for the specific experiments we were conducting.

1. Anesthetize and prepare the mouse for surgery in compliance with institutional and NIH guidelines.

2. Make a midline incision in the scalp from 2 mm posterior to the interaural line to 1 mm posterior to the eyes. Expose the lambda cranial suture and regions up to 5 mm lateral to it.
3. Mount the mouse in a stereotactic frame. It is critical that the skull is mounted in a stable horizontal position. The orientation of the skull can be confirmed by measuring the height of the lambda and bregma sutures and confirming that they are equal.
4. Make a small craniotomy with a scalpel (a drill may be used for animals with thicker skulls, such as rats) using the following coordinates: medial boundary, 0.5 mm lateral to midline; lateral boundary, 2 mm lateral to midline; posterior boundary, 1 mm anterior to lambda; and anterior boundary, 2 mm anterior to lambda. One should be careful to avoid damaging vasculature in order to minimize bleeding. It is not necessary to replace the removed bone fragment after surgery with such small craniotomies (*see* **Note 2**).
5. Make three injections into the thalamus. Insert pulled glass micropipet at a 30° angle from the vertical axis at the following coordinates:

	1	2	3
Anterior to lambda	1.5 mm	1.5 mm	1.5 mm
Lateral to midline	0.8 mm	1.2 mm	1.6 mm
Depth	3.6 mm	3.7 mm	3.7 mm

These coordinates place the pipet tip in the following locations, targeting the thalamic nuclei VLc, VPLo, Area X, and vLo, which receive projections from motor cortex:

Anterior to lambda	3.24 mm
Posterior to bregma	0.97 mm
Lateral to midline	0.9, 1.2, 1.6 mm
Depth	3.04 mm

6. Inject 32 nL of FG solution and wait 1 min. Retract the glass micropipet 100 µm, inject another 32 nL and wait an additional minute. Retract the pipet slowly. Waiting for the FG to diffuse and withdrawing the pipet slowly minimizes the amount of FG that potentially effluxes along the micropipet's path. Repeat at each injection site (*see* **Note 3**).
7. Suture the incision area and allow the mouse to recover. We allow 7 d for retrograde transport of FG to neuronal cell bodies in adult animals (*see* **Note 4**).
8. Transcardially perfuse with 10 units/mL heparin/PBS solution followed by 4% paraformaldehyde in PBS solution. Postfix brains overnight in 4% paraformaldehyde at 4°C. Section brains at 40 µm using a vibrating microtome (Vibratome); mount sections on gelatin-coated slides using Fluoromount (BDH); and examine by fluorescence microscopy under a filter with a 350–380 nm excitation spectrum.

4. Notes

1. FG is soluble in saline solution as well, but it precipitates out of PBS, forming a suspension. FG solution stored in the dark at 4°C is stable in solution for 6 mo. FG crystals are stable for years when protected from light and stored at 4°C.
2. Cranial bleeding can usually be stopped using gel-foam absorbable gelatin sponges (Harvard Scientific, cat. no. 59-9863).
3. The inner diameter of the glass micropipettes should be as small as possible; an outer diameter of 40 μm is sufficient. It is also possible to inject Fluoro-Gold using a Hamilton syringe. However, the large bore of the metal needle is likely to injure a much larger region of the brain, and is likely to result in additional efflux along the injection path. In addition, it is difficult to accurately inject the small volumes required to target single areas of the rodent brain using a syringe and needle.
4. FG may transport in shorter periods of time. Allowing longer times for transport can result in brighter nuclear labeling and increased labeling of dendrites.

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III

Utilization/Characterization of NSCs In Vivo

B. Transplantation

28

Labeling Stem Cells In Vitro for Identification of Their Differentiated Phenotypes After Grafting into the CNS

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1. Introduction

Stem cells have unlimited therapeutic potential for restoring CNS function lost secondary to trauma or degenerative disease. To date, stem cell grafts have only in a few instances partially ameliorated functional deficits in experimental models ***(1,2)***, and substantial preclinical studies remain to be undertaken before clinical application can be considered. Most important, regardless of the injury model investigated, successful utilization of stem cell grafts requires a functional change after grafting. However, unequivocal demonstration that the engrafted stem cells have survived integrated into the host CNS and differentiated with a phenotype consistent with those functional changes is also of paramount importance. This latter issue is not a trivial one and must be approached very carefully. In this chapter, we will summarize the various methods that can be used to label stem cells in vitro prior to grafting. We will delineate the advantages and problems with each approach, and also discuss the most appropriate ways to document their differentiated phenotype(s) in vivo. Unfortunately, there is no single method that works best for all cell types. Optimal labeling methods have to be empirically determined for each cell type and application. What we present here are the critical variables that have to be established. Excellent comprehensive reviews and comparative experimental studies examining this issue have been previously published ***(3–5)***. The reader is referred to these publications for details of the types of cells that were labeled, and more in depth information on the specific properties of each label.

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2. Labeling Stem Cells In Vitro

Choosing an appropriate label depends on a number of variables:

1. Is a nuclear, membrane, or cytoplasmic label desired?
2. Are the engrafted cells expected to continue dividing and if so for how long?
3. Is cell lineage-specific expression of the label desired?
4. How long will the cells be required to express the label in vivo?
5. Does the label alter the physiology of the cells?
6. What detection strategy will be used for both the label and any cell specific antigen that might be concurrently used?
7. What are the technical limitations of the investigator?

The answers to these questions will dictate the type(s) of labels that can and cannot be used for a given application.

Table 1 lists various types of labels that have been utilized for CNS grafting. All are suitable for stem cell grafting, but each has advantages and disadvantages. In general, the membrane, nuclear, and cytoplasmic labels listed in **Table 1** are very easy to use, but are neither as definitive nor as long-lasting as reporter genes. Conversely, reporter genes require expertise with viral vectors and a higher biosafety level of containment. All of the membrane, nuclear, and cytoplasmic labels listed in **Table 1** can be added directly to the culture medium of growing stem cells, and the concentrations and labeling parameters are indicated. All of these labels will be diluted out as the cells divide in vitro, so it is imperative to label the cells as close to the time of engraftment as possible. As will be discussed subsequently, this is not the case with the reporter genes.

The advantage of using CFSE and CellTracker™ orange is that these labels are cell membrane permeable but are modified intracellularly to a membrane impermeant form. Thus, in principle, these labels cannot be transferred to host cells. The problem with CFSE is that it bleaches very rapidly upon exposure to light. CellTracker™ orange has only recently been developed but appears to label cells up to 4 mo post-engraftment ***(6)***. If it is essential that cell morphology be identified in vivo in the absence of additional antibody staining to delineate cell phenotype (*see* **Subheading 3.**), then either cytoplasmic or membrane labels must be used. While the membrane labels DiI and PKH26 are very bright fluorescent labels, membrane does turn over, and, with extended times both in vitro and in vivo, these labels tend to end up in lysosomal vesicles and cell morphology becomes difficult to distinguish. Nuclear labels obviously will not provide any information about cell shape. Excellent results have been obtained for all of these labels in grafting experiments, in which the cells do not remain *in situ* for more than 1 mo. However, with the exception of BrdU and ^{3}H-thymidine, none of these labels can be used for chronic grafting

Table 1
Approaches to Label Stem Cells for CNS Grafting

Labeled compartment	Label	Concentration(s)	Labeling conditions	Detection
Nuclear	bisbenzimide[a]	10 μg/mL	3 min–1 hr	UV fluorescence
	BrdU[b]	0.5–10 μ*M*	4 h–overnight	Immunohistochemistry[i]
	^{3}H-thymidine	1 μCi/mL	48 h	Autoradiography
Cytoplasmic	Fluorescent beads	0.1–5.0%	5 min–6 h	fluorescence[i]
	Fast Blue/True Blue	20 μg/mL	15 min–3 h	UV fluorescence
	Fluoro-Gold	1–10 mg/mL	5 min–2 h	UV fluorescence/immunohistochemistry[i]
	Gold particles	0.01–2.5%	1 h	silver precipitation/electron microscopy
	RDA[c]	50 μg/mL	30 min	Rhodamine fluorescence[j]
	CFSE[d]	0.05–5.0 μ*M*	30 min	FITC fluorescence
	CellTracker™ Orange	50%	15 min	Rhodamine fluorescence[j]
Membrane	DiI[e]	25–40 μg/mL	10–40 min	Rhodamine fluorescence[j]
	PKH26	1 μ*M*	3–8 min	Texas Red fluorescence[j]
	PHA-L[f]	0.2–20 mg/mL	10–40 min	Immunohistochemistry[i]
Reporter genes	*lacZ*[g]			Enzyme histochemistry/ immunohistochemistry
	EGFP[h]			FITC fluorescence/ immunohistochemistry
	Alkaline phosphatase			Enzyme histochemistry
	Luciferase			Enzyme assay/ immunohistochemistry

[a]Also called Hoechst's 33342.
[b]5-bromo-2′-deoxyuridine.
[c]Rhodamine dextran amine.
[d]Carboxyfluoresceine succinimidyl ester.
[e]1,1'dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate.
[f]*Phaseolus vulgaris* leucoagglutinin.
[g]*E. coli* β-galactosidase.
[h]Enhanced green fluorescent protein.
[i]The filter set used depends on which fluor is chosen for use.
[j]Optimal filters for rhodamine, Texas Red, or CY3 have similar excitation and emission spectra and can be used for all three fluors.

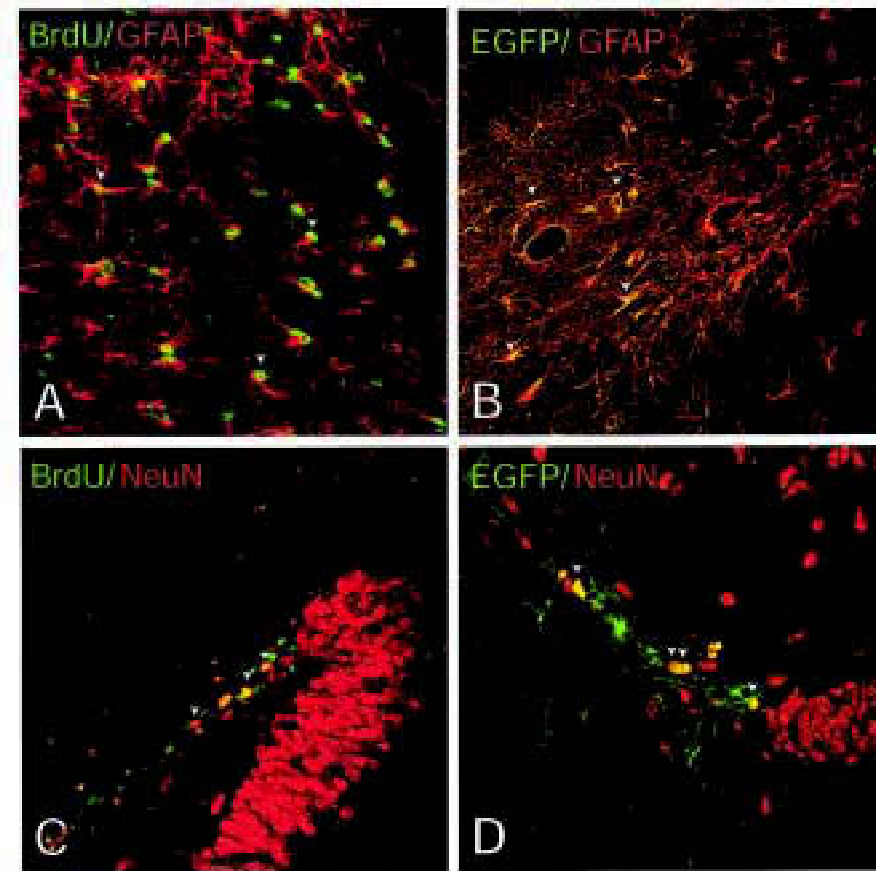

Fig. 1. Detection of BrdU- and EGFP-labeled E14 rat cortical stem cells after engraftment into the adult rat CNS. **(A,B)** BrdU- **(A)** and EGFP- **(B)** labeled stem cells double-stained with GFAP showing extensive astrocytic differentiation 2 mo after engraftment into the ventral white matter of the adult rat spinal cord. Note that BrdU labels nuclei while EGFP is expressed in the cytoplasm and enables cell morphology to be determined. **(C,D)** BrdU- **(C)** and EGFP- **(D)** labeled stem cells double-stained with the neuronal nuclear marker NeuN showing extensive neuronal differentiation 2 mo after engraftment into the granule cell layer of the adult rat dentate gyrus in the hippocampal formation. In all panels, arrows indicate representative double-stained cells.

experiments because the labels tend to leak out of the cells. If the grafts are expected to remain in vivo for longer than 6 wk before analysis, either BrdU, ^{3}H-thymidine, or one of the reporter genes should be utilized. Otherwise, it becomes extremely difficult to unequivocally state that a label-expressing cell is of graft origin. For example, **Figs. 1A,C** show BrdU-labeled cortical

stem cells 2 mo after engraftment into the adult rat spinal cord. Note the clear nuclear labeling of the engrafted stem cells. For both BrdU and ^{3}H-thymidine, the cells have to be actively dividing, as these labels incorporate into the DNA of dividing cells. Slowly proliferating populations of stem cells may have to be labeled for extended periods of time, if a high percentage of cells is not labeled with the standard overnight incubation. BrdU can be detected with an antibody but ^{3}H-thymidine requires autoradiographic detection and as such is technically more difficult to work with.

2.1. Establishing Nontoxic Concentrations of Labels

All cell types are differentially sensitive to the various labels. Moreover, a stem cell may be more or less sensitive to a particular label while proliferating than after differentiation, and this too has to be empirically determined. When evaluating a label, concentrations bracketing those that have been used by other investigators should be evaluated, with the percentage of labeled cells and the survival of those proliferating cells as the crucial variables. This evaluation can easily be done by counting the cells in a hemocytometer with trypan blue (*see* **Subheading 3.4.** in Chapter 29). In the case of stem cells that will differentiate in vivo, it is imperative that the stability of the label and the toxicity be established in differentiated cells in vitro prior to undertaking grafting. Differentiated cells cannot be counted in a hemocytometer, but must be immunostained with appropriate cell-specific antigens and the number of specific cell types counted. This staining is essential because one differentiated cell type may be differentially sensitive to a particular label. For example, we demonstrated that BrdU-labeling does not affect the number of neurons, astrocytes, or oligodendrocytes observed after differentiation of E14 cortical stem cells in vitro ***(7)***, whereas fluorescent microspheres are tolerated by proliferating RN33B cells, but are toxic to differentiated cells ***(4)***.

In addition, because all of these labels have the potential to be transferred to endogenous cells ***(4)***, it is essential that a lysed cell control graft is performed. Cells, at the identical concentration to that used for grafting, can be freeze-thawed twice, checked with a hemocytometer and trypan blue to ensure that all cells are dead, and identically engrafted.

2.2. Labeling with Reporter Genes

Using reporter genes requires that the investigator have some expertise with molecular biology and/or cell culture experience with various virus vectors. Thus, this approach is technically more challenging. The advantage of using these reporter genes is that they can be stably integrated into the stem cell genome so that they cannot be transferred to endogenous cells. That, combined with their continued expression for long periods of time, makes them an

excellent choice of label for chronic grafting experiments. A number of reporter genes have been successfully used to label grafted cells. The two most commonly used are *E. coli* β-galactosidase (*lacZ*) and EGFP, but both alkaline phosphatase and luciferase have also been used. The advantage of EGFP over *lacZ* is that it is constitutively fluorescent and can be seen without additional identification steps. In practice, however, it is much better to use antibody detection of EGFP expression in grafts because the antibody staining markedly amplifies the fluorescent signal and enables better detection of the signal, and is especially true when trying to identify axons and dendrites of differentiated neurons in vivo. One should always use FITC-conjugated secondary antibodies as EGFP fluoresces in the FITC wavelength. While *lacZ* requires additional detection methods, its advantage is that either histochemical or antibody detection can be used. The latter is more sensitive, but histochemical detection can be seen at the light and EM levels and does not require fluorescence microscopy. *lacZ* can also be detected by EM immunohistochemistry ***(8)***. The one concern with histochemical detection of the *lacZ* reporter gene is that there is endogenous β-galactosidase activity in the mammalian CNS. The pH of the histochemical detection solutions must be very carefully controlled to eliminate that endogenous activity ***(9)***. Alkaline phosphatase shares many of the properties of β-galactosidase as a reporter gene, and is also optimally detected with antibodies. Luciferase is a much less widely used reporter gene for grafting studies. Its main advantage would be in quantifying graft survival by enzyme assay in unfixed tissue homogenates, as the assay is extremely sensitive; however, it can also be immunohistochemically detected. All of these reporter genes will show cell morphology as they are cytoplasmic markers, unless a nuclear localization signal is included in the reporter gene construct.

There are a number of ways to deliver reporter genes to stem cells. These genes can be cloned into eukaryotic expression vectors and transfected into the proliferating stem cells, which is relatively easy, but the frequency of transfection is often quite low. However, inclusion of a selectable marker, such as the neomycin- or hygromycin-resistance genes, allows selection of expressing cells. Alternatively, the reporter gene can be delivered by viral infection, either with adenovirus, adeno-associated virus (AAV), retrovirus, or lentivirus. Both retroviral and lentiviral-delivered reporter genes will be stably integrated into the host genome and passed on to all cell progeny as they divide. Thus, labeled cells can be expanded to high numbers, and if EGFP or *lacZ* is used as the reporter gene, enrichment by fluorescent activated cell sorting (FACS) is possible. **Figs. 1B,D** show E14 rat cortical stem cells, labeled with an EGFP retrovirus 2 mo after engraftment into the adult spinal cord **(B)** or hippocampus **(D)**. Note that EGFP is expressed in the cytoplasm and delineates cell morphology. The advantage of lentiviral vectors is that they will

infect nondividing cells; the disadvantage is that they may require biosafety level 3 containment for their construction. Both adenovirus and AAV very efficiently infect dividing or nondividing cells, but both remain episomal and therefore infected cells cannot be passaged and expanded before grafting. Moreover, adenovirus is quite toxic and optimal infection parameters need to be carefully established. The growth of these viruses is now routine in many laboratories, and most of these reporter gene constructs are readily available, some even commercially.

2.3. Alternative Labeling Strategies

There are a number of alternative ways to label engrafted cells. Again, all have their advantages and disadvantages.

2.3.1. Gender-Specific Grafts

Embryonic male tissues have been grafted into female CNS and the grafted cells detected using Y-chromosome-specific DNA probes ***(10,11)***. The problem with this approach is that it requires *in situ* hybridization (*see* Chapter 17) to detect the engrafted cells, a technique that gives no indication of cell morphology, and is also very difficult to use in conjunction with immunohistochemistry to determine the differentiated phenotype of the cells.

2.3.2. Transgenic Animals

Transgenic mice expressing EGFP have been developed ***(12)***, and dorsal root ganglion cells from these animals engrafted into the adult rat CNS ***(13)***. These grafts were xenografts that required immunosuppression (*see* **Subheading 2.3.4.**), but this study shows the potential of this approach. Additional transgenic animals expressing EGFP and other reporter genes under the control of constitutive or cell-specific promoters will continue to be developed and can be used in a variety of grafting paradigms.

2.3.3. Allogeneic Grafts

Allogeneic grafts are grafts between different strains of the same species for which strain-specific antibodies exist. For example, embryonic CNS from mice that express the Thy-1.1allele of the CNS glycoprotein Thy-1 have been grafted into the CNS of Thy-1.2 expressing hosts, and the grafted cells detected with Thy-1.1-specific antibodies ***(14)***. In principle, strains that differ at class I or II major histocompatibility (MHC) antigen loci could be used to identify grafted stem cells. However, CNS neurons do not express these antigens, and astrocytes and oligodendrocytes require induction for expression, greatly restricting its utility for CNS grafts. The concern with allografts is that despite the perceived immunological privilege of the CNS, allogeneic grafts can be

rejected. In our laboratory, we have found lower survival of engrafted stem cells with allogeneic stem cell grafts, and whenever possible use syngeneic stem cells and graft recipients.

2.3.4. Xenografts

Numerous investigators have grafted CNS tissues across species, especially human tissues into adult rodent recipients ***(15–17)***. Such grafts can be detected with species-specific antibodies, which can also be done with mouse cells grafted into rat host CNS ***(18)***. While this detection is essential to evaluate the potential of the human cells to differentiate after CNS grafting, all xenografts are problematic as they require immunosuppression to block rejection or the use of immunocompromised hosts. Immunocompromised animals are expensive to buy and house, and it is difficult to maintain laboratory animals long term on immunosuppressive regimens. It can certainly be done, but is not a recommended approach for graft detection, unless there are no other alternatives.

3. Identifying Grafted Stem Cell Phenotype In Vivo

While it is essential to be able to identify surviving grafted stem cells, it is usually necessary to identify the type of cell into which grafted stem cells have differentiated. Identification requires a labeling approach where the marker used to label the cells, as well as a cell-specific antibody, and indirect immunofluorescence, are used to determine the lineage fate of the engrafted cells. This is most easily done immunohistochemically using two antibodies that are made in distinct species followed by detection with species-specific secondary antibodies that are conjugated to different wavelength fluors. **Table 2** lists commonly used antibodies for identifying undifferentiated stem cells, neurons, astrocytes, and oligodendroctyes. We have indicated at least one supplier of each antibody, which we have used ourselves, but many companies make these antibodies, and they can be purchased from any of those vendors. Note that immunohistochemical detection of BrdU-labeled stem cells requires 2 *N* HCl treatment of the tissue sections. Many antigens do not survive this treatment, and if BrdU is the label chosen, the investigator should be aware of this. In our experience, those antibodies marked with a superscript “b” are problematic after the harsh acid treatment needed to remove the chromatin and expose the BrdU in the DNA.

We strongly recommend using Fab′ fragments for secondary antibodies, as intact IgG or IgM antibodies bind nonspecifically to activated microglia and macrophages that are always found around grafted cells, especially in allografts or xenografts.

Table 2
Antibodies Used for Detection of Differentiated CNS Cells

Cell Types	Antibodies (species made in)	Source[a]
Neural stem cells	Nestin (mouse)	Development Studies Hybridoma Bank
Neurons	βIII-tubulin (mouse)	Sigma
	Microtubule associated protein 2a,b (mouse)	Sigma
	67-kDa neurofilament protein (NFL; mouse)	Sigma
	110-kDa neurofilament protein (NFM; mouse)	Chemicon
	210-kDa neurofilament protein (NFH; mouse)	Sigma
	Neuron specific enolase (NSE; rabbit)	Stress-Gen Biotechnologies Corp.
	NeuN (mouse)	Chemicon
Oligodendrocytes	rabbit anti-NG2	Chemicon
	O1 (mouse)[b]	Boehringer Mannheim
	O4 (mouse)[b]	Boehringer Mannheim
	galactocerebrosidase (mouse)[b]	Boehringer Mannheim
	Rip (mouse)[b]	Chemicon
	APC (mouse)	Calbiochem
	MBP (mouse)	Chemicon
	mAb328 (mouse)	Chemicon
Astrocytes	Glial fibrillary acidic protein (GFAP; mouse)	Boehringer Mannheim

[a]These vendors are ones that we have used routinely. However, other vendors make similar antibodies that may work equally well or better.
[b]These antibodies have proven problematic in combination with BrdU immunohistochemistry.

3.1. Protocol Optimization for Antibody Detection

All antibodies have different conditions under which they optimally detect antigens in fixed tissues. Conditions such as the fixative used, the thickness of the section, whether paraffin or cryosections are being used, and antibody concentration all markedly affect the strength of the signal, and the signal to background ratio. As with much of this chapter, we cannot go through specific protocols because they will be different for each antibody and must be empirically determined. Rather, we present the variables that have to be optimized and the steps to be taken to ensure appropriate specificity.

1. After choosing an antibody, look at the manufacturer's specification sheet to make sure that it detects the species with which you are working, and that it works with immunohistochemistry.

   The best way to ensure utility is to find a reference in the literature that uses that specific antibody. All of the antibodies in **Table 2** *are appropriate for*

immunohistochemical detection in rat CNS cryosections, although we cannot attest to their suitability in paraffin sections.

2. Run a dilution series of the primary antibody on control tissue to establish optimal dilutions.

   This dilution is readily done for the cell specific markers listed in **Table 2**. *We routinely run dilution series of 1*:50, 1:100, 1:200, and 1:400 dilution. A second narrower dilution series might have to be run to determine the optimal concentration. The dilution of secondary antibody to use in this optimization depends on the antibody used. For AMCA- and FITC-conjugated secondary antibodies, we routinely use a 1:100 dilution, Texas Red 1:200, and 1:400 for Cy3 and Cy5.
3. Once a primary dilution is determined, repeat the dilution series with the secondary antibody, with starting and ending concentrations bracketing the initial concentration.

   The reason for this step is to lower the background as much as possible while retaining a strong signal.
4. This process should be repeated for all antibodies. If BrdU is utilized to label engrafted cells, immunohistochemical parameters will have to be optimized on grafted animals.

   For these studies, which can be done while optimizing graft parameters (see Chapter 29), a 1 or 2 wk survival time is sufficient.
5. For visualization of double-labeled cells, an initial screen with fluorescence microscopy can give an indication of whether the cells express specific markers. However, for unequivocal documentation of grafted cells exhibiting a specific phenotype, confocal microscopy is essential.

4. Summary

Labeling stem cells for CNS grafting is an empirical process. Specific protocols cannot be given that will work for all cell types and all applications. We have provided the range of conditions under which various labels have been successfully used in CNS grafting studies, and delineated the parameters that have to be empirically established. Given a clear understanding of the limitations of the respective labels, and the expected outcome of the grafting experiment, these labeling guidelines should enable any investigator to develop a successful labeling approach. Our own personal bias is to use labels that cannot be transferred to host cells. We prefer BrdU, or more often, retrovirally delivered EGFP or *lacZ*. However, each investigator will have to decide what is optimal for their own cell population and experimental design.

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29

Optimizing Stem Cell Grafting into the CNS

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1. Introduction

The potential of stem cells to be used for repair of the injured or degenerating CNS is unlimited because of their capacity to differentiate into neurons, astrocytes, and/or oligodendrocytes. In addition, the fact that stem cells can be expanded in vitro prior to engrafting means that large numbers of clonally derived cells can be obtained. Finally, proliferating stem cells readily incorporate exogenous genes after transfection with eukaryotic expression vectors or infection with viral vectors, enabling their use for ex vivo gene delivery. However, the very process of engrafting into the CNS itself creates damage that can be detrimental to the survival of the engrafted cells, which is especially true when grafting into very small structures such as the spinal cord. Thus, the potential therapeutic efficacy of stem cell grafts will depend on grafting methods that both optimize graft survival and minimize the extent of the graft-induced lesion. Both of these variables are dependent on the type of system used to deliver the cells. We have compared a number of different configurations and sizes of micropipets for grafting into the adult rat spinal cord, and discuss our approaches to optimize the survival and integration of these grafts.

2. Materials

1. 0.4% trypan blue in 0.85% NaCl, sterile.
2. Hank's balanced salt solution (HBSS), sterile.
3. Hemocytometer.
4. Beckman GRP or equivalent refrigerated centrifuge.
5. TW150-4 glass pipets (World Precision Instrument, Inc.) or its equivalent.

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6. Flaming Brown micropipet puller (Model P-80 PC, Sutter Instrument Co.) or its equivalent.
7. Model 1300M micropipet beveler (World Precision Instrument, Inc.) or its equivalent.
8. Diamond lapping film (World Precision Instrument, Inc.) or its equivalent.
9. Bransonic B-2200R-1 sonicator (Branson Cleaning Equipment Co.) or its equivalent.
10. Picospritzer® II (Parker Instrumentation) or its equivalent.

3. Methods

3.1. Micropipet Size and Configuration

While it is intuitively obvious that using smaller micropipettes will cause less damage to host tissues, it was not until 1994 that this was experimentally demonstrated ***(1)***. However, the choice of an appropriately sized micropipet depends also on the size of the stem cells to be engrafted. For example, a single cell suspension would readily flow through a 40 μm outer diameter (OD) micropipet, while a suspension of neurospheres would require a much larger tip diameter if spheres were to be engrafted intact. We compared various OD tips as well as the configuration of the tip (broken, beveled, fire polished; **Fig. 1A–C**) on the deformation of the spinal cord before the tip penetrated the cord parenchyma, an indication of the degree of damage to host tissue. We also examined the effects of grafting with a 10 μL Hamilton syringe, as many investigators use these for grafting into the CNS. Note that the aperture in the Hamilton syringe is not at the bottom of the syringe (arrowhead in **Fig. 1E**), necessitating an extra 300 μm penetration before cells can be engrafted. The data in **Table 1** demonstrate that (1) Hamilton syringes are not recommended as they cause substantial deformation of the CNS (we also observed a 10% lower cell viability with Hamilton syringes), (2) beveled tips are better than broken or fire polished tips, and (3) one should use the smallest pipet tip appropriate for the size of the stem cells that are to be engrafted. We routinely use 50 μm OD beveled pipets for grafting single stem cell suspensions (*see* Chapter 28). If intact spheres of stem cells are to be grafted, the size of the micropipet will have to be empirically determined with the cell viability after ejection from the micropipet being the crucial variable for determining tip diameter (*see* **Subheading 3.5.**). Whatever the ultimate tip diameter used, beveled micropipets should always be used.

3.2. Pulling, Beveling, and Calibrating Micropipets

There are a number of manufacturers of thin-walled borosilicate capillary tubes, pipet pullers, and pipet bevelers. The manufacturers we use have been indicated, but any equivalent can be used. The parameters for pulling appropriate size pipets will vary with both the type of tube and the puller used.

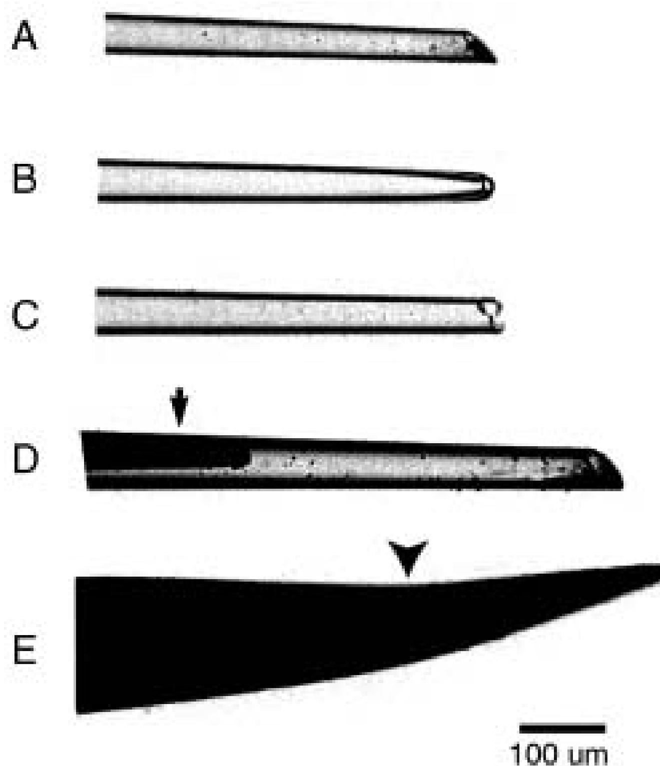

Fig. 1. Micropipet tip configurations. **(A)** beveled, **(B)** fire-polished, **(C)** broken, **(D)** beveled with tungsten filament partially lowered (arrow), **(E)** tip of 10 μL Hamilton syringe. The arrowhead indicates the center of the opening in the syringe. Note that it is located 300 μm from the end of syringe tip.

1. Pull the pipets, put under on a microscope equipped with a micrometer, and break at the appropriate size using #5 forceps.
2. Grind the tip to 55° on diamond lapping film and check its size under the microscope with the micrometer. (*see* **Note 1**.)
3. Clean the tip by dipping in a sonicator containing 100% EtOH for 10–20 sec.
4. Use a fine tipped permanent marker to mark the capillary tubes at 1 mm intervals (*see* **Note 2**).

3.3. Injection System

There are both manual and electronic means by which to transplant cells into the CNS. Many investigators use a Hamilton syringe or glass micropipets attached to a Hamilton syringe and manually dispense the desired volumes. For reasons discussed above, we do not recommend grafting with Hamilton

Table 1
Micropipet Parameters

Pipet size (OD)	Tip type	Spinal cord deformation (mm)
25 μm	Beveled	0.24 ± 0.02
40 μm	Broken	0.65 ± 0.03
40 μm	Beveled	0.35 ± 0.03
90 μm	Broken	0.93 ± 0.04
90 μm	Beveled	0.72 ± 0.04
90 μm	Fire polished	1.08 ± 0.03
1 μL Hamilton syringe		>1.00[a]

[a]Unlike with the pulled glass pipets, the spinal cord remained deformed after the syringe tip entered the spinal cord.

syringes. It is also very difficult to deliver precise small volumes manually, and the Hamilton syringe/glass micropipet should only be used when large volumes are to be engrafted. Other systems involve motorized syringes connected to micropipets that can be precisely driven. The problem with this delivery system is that it relies only on pressure for delivery, and if the resistance of either the delivery micropipet or the host tissue changes, the volume of cells engrafted cannot be accurately controlled. A more accurate way to engraft cells is to use a picospritzer. While this is a more expensive approach, it delivers a fixed volume in a preset time period and then the pressure returns to ambient. This method also suffers from the problem of injection volume being influenced by changes in tip and tissues resistances, but this can be controlled (*see* **Subheading 3.6.**), and we do all of our grafts using a picospritzer. Regardless of the injection system decided upon, it should be mounted in a three-dimensional micromanipulator, and the head or spinal cord rigidly fixed in position to allow accurate and reproducible graft placement.

3.4. Modified Injection System

One of the complications of using small OD diameter pipets is that they have the potential to become clogged as the cells settle. To obviate this problem, we have fabricated a simple device that prevents cell clogging. Inside of the glass micropipet is a ferromagnetic stainless steel sliding rod (6 mm long, 0.8 mm in diameter) to which is attached a 25-mm-long 20-μm-diameter tungsten filament. It is moved up and down by means of a U-shaped magnet attached to a one-dimension micromanipulator parallel to the glass micropipet (**Fig. 2**). When the filament is lowered, it occupies the opening of the micropipet and prevents cells from clogging the tip. When the filament is raised just

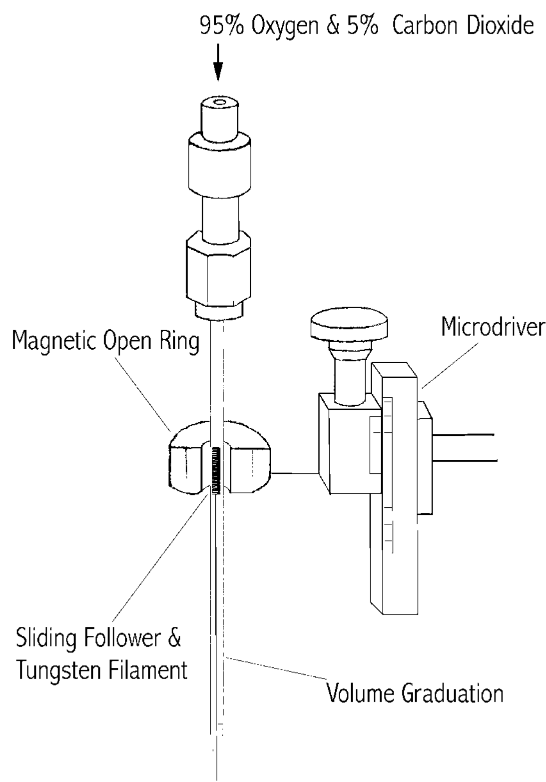

Fig. 2. Tungsten filament to prevent micropipet clogging. An additional microdriver is attached to the micromanipulator that controls the micropipet movement. Note that the micropipet and the microdriver should move in identical directions at all times. Attached to this microdriver is an magnetic open ring that controls the vertical displacement of the tungsten filament that is placed within the micropipet. When the filament is lowered, it prevents cells from aggregating at the narrow beveled opening of the micropipet. When the filament is raised, stem cells can be ejected.

prior to engraftment, it not only clears the tip opening, but also loosens any aggregated cells that may have packed in the tip. The inclusion of the tungsten filament does not alter the viability of the injected cells.

3.5. Quantifying Cell Number and Viability

Before grafting a suspension of cells, both the number and percentage of viable cells needs to be determined. This is easily done for single cell suspensions, but is more difficult to determine for cells grown and engrafted as spheres. For single cell suspensions, cell number and viability are counted in a

hemocytometer using trypan blue. Live cells exclude the dye and appear phase bright, while dead cells take up the dye and appear dark blue.

1. Remove the stem cells from the tissue culture plates or flasks, place in a sterile 15 mL conical tube, and pellet in a refrigerated centrifuge (2 min, 4°C, 500*g*) (*see* **Note 3**).
2. Resuspend the cells in HBSS to approximately 2×10^4 cells/μL for counting and viability assessment with a hemocytometer (*see* **Note 4**).
3. Recentrifuge the cells as described above and suspend in ice cold HBSS to desired final concentration. Store on ice.
4. After all engrafting procedures are completed, a second check of cell viability should be done (*see* **Note 5**).

If one is engrafting intact spheres, there is no good method to get an accurate count of the number of cells. Dissociating intact spheres into single cells often results in the loss of many cells. However, once a population of spheres has been characterized for its average diameter (see below), the spheres should be dissociated and counted with a hemocytometer to obtain an approximate cell number; both live and dead cells should be counted. The spheres can be pelleted by centrifugation (2 min, 4°C, 500*g*), resuspended in a small volume of HBSS, and counted in a hemocytometer, but these will be of varied sizes. However, using a microscope micrometer, one can get a sense of the range and median diameters of the spheres and use that median to characterize the cultures. Variables such as the optimal number and size of the spheres for engraftment and the size of the micropipet used for grafting will have to be empirically determined. The latter two parameters must be established first and can be done in vitro with the viability of the cells after injection the critical outcome measure, which can be measured by trypan blue exclusion as described above. Once these parameters have been established, the sphere density that is optimal for grafting can be again empirically established. It may be that despite good viability in vitro, very large spheres do not survive well after engraftment. The preliminary grafting experiments should address this issue.

3.6. Engraftment Parameters

Three parameters need to be established for optimal stem cell grafting: cell density, the volume to be grafted, and the number of grafts to be made. Our experience is that cell densities of 50,000–100,000 cells/μL are optimal for single cell suspensions. Higher densities are very viscous and have the potential to clog the micropipet, and lower densities do not provide an adequate number of cells for grafting. Nikkah et al. ***(1)*** showed that cell survival and integration are better with multiple grafts of lower volume than single large grafts. Such an engraftment strategy will also enable more area to be covered

by the grafted cells. However, single injections of volumes as large as 10 μL into the intact adult rat spinal cord are not detrimental to locomotor function. Fluids will readily diffuse throughout the spinal cord parenchyma. A 1 μL injection of India ink into the central gray matter of the adult rat spinal cord can be detected 2.9 ± 0.3 mm from the injection site in the parenchyma and >7 mm away in the central canal. A similar injection of 10 μm diameter fluorescent beads (which approximates the size of a stem cell) are found only within 0.9 mm of the injection site. Therefore grafting into a large structure will necessitate multiple cell grafts. We routinely graft 0.5–1.0 μL/graft site unless the grafts are made into a lesion cavity, in which case volumes up to 10 μL can be accommodated. The number of grafts to be made and their spatial arrangement will be determined by the target site.

The volume delivered by any injection system is a function of tip and host tissue resistance. Therefore, it is imperative to use the 1 mm markings on the micropipet (*see* **Subheading 3.2.1.**) to calibrate the engrafted volume. Because the internal diameter of the micropipet is known, the actual volume injected using a given set of parameters can be precisely determined. If that volume is less or more than desired, the injection parameters can be adjusted accordingly.

4. Notes

1. This process usually takes approx 2 min for 40 μm and 10 min for 90 μm micropipets.
2. These marks will be used subsequently to calibrate the injection system.
3. The final desired cell density, 50,000–100,000 cells/μL (*see* **Subheading 3.5.**) is too concentrated to count with a hemocytometer.
4. Once enough preparations have been done, a good estimate of total cell number can be made. We routinely obtain approximately 10^6 viable cells from each 100 mm plate of stem cells ***(2)*** at a viability of 85%.
5. We typically observe a 30% drop in stem cell viability over the course of a 6 h grafting session, even when the cells are stored on ice. More significant decreases in cell viability would suggest that the later occurring grafts in those experiments will likely be suboptimal.

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30

Vision-Guided Technique for Cell Transplantation and Injection of Active Molecules into Rat and Mouse Embryos

Lorenzo Magrassi

1. Introduction

Direct surgical access to the mammalian embryo is made difficult by its protected position, enclosed in the uterus within the decidua and the embryonic membranes. This condition historically favored the development of experimental embryology in submammalian species like newts, frogs, and chicken, whose embryos are simpler to access ***(1)***. However, the potential relevance for human health of testing developmental hypotheses on mammals stimulated the development of *in utero* surgical approaches. Direct surgical manipulation of mammalian fetuses is now possible in many species, including human, but only relatively late in development. Grafting of hematopoietic progenitors ***(2)***, and treatment of complex central nervous system (CNS) malformations, like myelomeningocele ***(3,4)***, have successfully been performed in humans, but their practical utility is still controversial ***(5)***. Lamb and rabbit are among the best models for fetal surgery, but, again, successful manipulations are restricted to the last two thirds of pregnancy, when most of the embryonic processes are completed and the development of the brain is quite advanced ***(6,7)***. Moreover, our knowledge of the biology and genetics of these species is considerably less advanced compared to that available for mice and rats. Lambs are also significantly more expensive to maintain, but they remain, at the moment, the best animal model for developing complex surgical techniques with potential clinical applications ***(6)***. However, for basic neuroscience research, the advantages of using rats and mice are self-evident. In these species, both closed transuterine approaches ***(8,9)*** and open techniques

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(10) that require hysterectomy have been developed. The open technique requires the exposure of the embryo, after section of the uterus and embryonic membranes. In the original description of the technique by Muneoka et al. ***(10)***, at the end of the planned surgical manipulations, the membranes and the uterine wall (that quickly retracts after any incision) were left open. Only the abdominal wall required suturing after filling the cavity with warm saline. In their experience, most of the embryos developed normally until the end of gestation when they were collected by cesarean section. In our experience, this approach, although it does permit very good anatomical control of any surgical manipulation, results in an extremely low number of living fetuses at the end of pregnancy. Furthermore, it is often difficult to convince a foster mother or the convalescent natural mother to raise the operated pups that were still alive at the time of cesarean section.

In this chapter, we describe the vision-guided technique for injecting developing rodent embryos in mid to late developmental stages [rat from embryonic day (E)15 to E19, mouse from E13 to E18]. This technique has been mainly used to target the central nervous system, but can be easily adapted to any part of the embryo (liver, heart, limb buds, and so forth) that can be recognized through the intact uterus and embryonic membranes by transillumination. The injection-based technique allows the introduction of cell suspensions for grafting purposes ***(9)*** and molecules with biological activity ***(11)***. The size of the particles delivered inside the embryo is limited by the maximum diameter of the injecting micropipet. The external diameter of the tip must be less than 50 μm to avoid any significant damage to the embryo and surrounding membranes. When injecting into the brain, a maximum volume of 2 μL can be injected; higher volumes will leak outside the embryo into the amniotic fluid. In rat fetuses, visualization of the internal structures by transillumination is initially (before E14.5) limited by the thickness of the decidua surrounding the embryonic membranes. The decidua during the first 14.5 d of rat development is thick and diffuses light, making the embryonic structures indistinguishable from the background. Only around E14.5 in Sprague–Dawley rats has the decidua grown sufficiently thin opposite the site of the implanted placenta to open an optimal corridor for observation and injection. At earlier stages of development, blind injection into the placenta has been used to deliver cells or retroviruses to the developing embryo ***(8,12,13)***. Nowadays, ultrasound-based techniques ***(14)*** are available to guide injections into embryos before decidual thinning ***(15)***. At later stages (after E19), injections into the brain again become difficult due to the thickening of the skin and the growth of the membranous skull, which not only obscures the brain but also resists perforation by the micropipets commonly used for the injection. Visualization and resistance to puncture, however, are not the only problems:

operation during late gestation days often results in delayed parturition, sometimes causing death of the fetuses before or during delivery and increasing perinatal death. This chapter will describe a detailed protocol for cell transplantation into the developing nervous system of rats and mice. This protocol can be easily adapted to the injection of viruses, nucleic acids, or any other biologically active molecules. All the procedures described are used on a regular basis in our laboratory ***(16,17)***, and have been selected over the last 10 yr for their reliability and simplicity.

2. Materials

1. Appropriately labeled cells for transplantation (*see* **Note 1**).
2. Pregnant rat (E15–E19) or pregnant mouse (E13.5–E18) (*see* **Note 2**).
3. Diazepam (Valium Injectable, Roche, Nutley NJ, USA) open fresh ampule every time.
4. Ketamine (Ketalar, Parke Davis, Morris Plains NJ, USA), keep at 4°C up to 6 mo.
5. 25 gauge, hypodermic needle.
6. 1 mL sterile disposable syringe.
7. Povidone-iodine solution (Betadine, Purdue Frederick Co., Norwalk CT, USA).
8. One 10 × 15 cm disposable surgical drape sheet (3M Health Care, St. Paul, MN, USA).
9. One pair of large (2–3 mm tip) blunt forceps for the manipulation of the internal organs.
10. One pair of medium (1 mm tip) forceps with teeth (1 × 2) for manipulation of the abdominal wall.
11. Dissection scissors.
12. Surgical silk suture (3-0).
13. 35 mm plastic Petri dishes (sterile).
14. Fiber optic illuminator (150 W, halogen) equipped with a 46 cm self-retaining flexible light guide.
15. Rubber tubing (approx 50 cm), micropipet connector, and suction mouthpiece; those found in the canister pack of microsampling pipets are good (Corning 70995 as sold by Sigma).
16. Appropriately pulled glass micropipets (*see* Note 2).

3. Methods

3.1. Labeling Cells Before Transplantation

The single most important point in any transplantation experiment is the ability to follow the transplanted cells and their progeny for the entire duration of the experiment (this often means months). Detection of the label must also be simple, unambiguous, quick, and inexpensive. It is useful to use more than one marker to take advantage of the peculiarities of each method and double check for specificity of cell identification.

Both genetic and nongenetic methods of cell labeling have been used in the past. Many nongenetic methods are subject to failure, particularly at the level of the single cell resolution. However, these methods are simple, do not require a special strain of donor animals, and allow transplantation among syngenic animals. Fluorescent markers, like fluorescent dyes and fluorescent proteins, allow rapid selection of positive sections containing fluorescent cells, and the elimination of those without any fluorescence. In a project that involves serial sections of all transplanted animals, this elimination is no minor advantage and commonly allows scale down of analytical effort by one order of magnitude. Saving time by discarding "negative" sections almost invariably corresponds to an increase in the quality of the analysis that can be carried out on the selected sections.

3.1.1. Genetic Methods of Labeling

Genetic methods of labeling, by definition, preclude the use of syngenic animals, and are more often used in the context of intersex or interspecific chimeras. The simplest marker to score in mouse to rat embryo transplantation experiments is the pattern of chromatin condensation. Mouse nuclei have more abundant and larger chromatin clumps compared to rat cells ***(22)***. This difference is sometimes difficult to distinguish, and it is more clearly visible on sections stained with DNA-specific dyes that are sensitive to the condensation status of the chromatin (e.g., DAPI, Hoechst 33358, Feulgen). Male to female grafts have also been used, but scoring requires *in situ* hybridization with Y-specific probes. Moreover, it is impossible to sex the host fetuses inside the uterus. Thus, in approximately half of the offspring, the transplanted cells will not be distinguishable, because they will have the same sex as the host.

Transgenic animals widely expressing reporter genes that do not interfere with animal viability are also extremely good markers for transplantation. But again, transplantation into syngenic animals is ruled out. One of the most widely used transgenic mice in *in utero* transplantation experiments is 129/sv-TgR(Rosa26)26Sor, originally derived by retroviral insertion of a modified *lacZ* gene fused with the neomycin resistance gene *(βGEO)* into chromosome six of mouse embryonic stem (ES) cells ***(23)***. This transgene, crossed to multiple genetic backgrounds, is now available from Jackson Lab (Bar Harbor, ME, USA). A problem with TgRosa26 is that the levels of βGEO expression are widely variable in postnatal animals depending on the specific cell line considered. Moreover, often, after either histochemical or immunohistochemical staining, the only sign of transgene expression is a small cytoplasmic dot of concentrated β-galactosidase activity that can be missed, especially if careful cell by cell tridimensional reconstruction is not performed. Another problem with Rosa26, that is common to all systems that use β-galactosidase expression

to trace donor-derived cells after transplantation into the rat brain, is the presence of natural β-galactosidase background activity in many rat neuronal populations ***(24)***. This activity makes problematic the use of histochemistry as the sole means of transplant-derived cell identification.

A more recently developed transgene that, in our opinion, has many advantages over TgRosa26 carries the "enhanced" green fluorescent protein (EGFP) under the control of the chicken β-actin promoter and cytomegalovirus enhancer ***(26)***. All cells of these mice express EGFP at a level that cells transplanted into nonfluorescent hosts can be easily distinguished by their fluorescence. The green fluorescent protein is uniformly distributed inside the cell, and gives a complete visualization of any cellular process (axon, dendrites, filopodia, growth cones, and so forth). These animals [C57BL/6-TgN(ACTbEGFP)1Osb] are now available from Jackson Lab. A distinctive advantage of β-gal over GFP is that either X-gal or Bluo-gal, two common substrates used to reveal β-gal activity at the light-microscopy level, can also be visualized at the electron-microscopy level after standard processing. Furthermore, the distinction of GFP-labeled cells at the electron-microscopy level requires indirect immunohistochemistry with the use of a GFP-specific antibody (Clontech Laboratories, Palo Alto, CA, USA); however, the antibody is not completely reliable on tissue sections.

3.1.2. Nongenetic Methods of Cell Labeling

Nongenetic methods of cell labeling. Among the nongenetic methods, one of the earliest used and most popular, at least in the past, is cell labeling with fluorescent dyes. These labels are often lipophilic dyes that become trapped into the lipid compartments of the cell, and are slowly subject to dilution by membrane shedding, vesicular traffic, and cell division. The most popular dyes in this class are:

1. 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR, USA).
2. 3, 3′-dioctadecyloxacarbocyanine perchlorate (DiO) (Molecular Probes).
3. PKH 26 (Sigma, St. Louis, MO, USA).
4. PKH2 (Sigma).

These dyes have very similar biological compatibility, but differ in their absorption and emission spectra; DiI and PKH26 are maximally excited by green light (rhodamine filter set) and fluoresce in orange-red, while DiO and PKH2 require blue light (fluoresceine filter set) for excitation and fluoresce in green. The absorption peak of DiI in methanol is 549 nm, while that of DiO is 484 nm, and their emission peaks are, respectively, 565 nm and 501 nm. However, broad excitation spectra and fluorescence emission changes due to

local conditions result in overlapping of the green and red signals with DiI fluorescing also after blue light excitation and DiO also after green excitation. The same problem is true for PKHs dyes. This fluorescence leakage gives rise to problems when immunofluorescent staining for further characterization of the labeled cells is attempted, unless the secondary antibody is labeled with an UV excitable fluorophore. Another source of uncertainty due to the use of fluorescent dyes is the possibility of undissolved dye flecks, or dye leaking from dying or dead cells, or from phagocytosed grafted cells becoming incorporated into host cells leading to their erroneous identification as donor-derived cells ***(18)***. The main advantage of these dyes is that any cell can be quickly labeled with minimal toxicity immediately before grafting. After grafting, especially at short survival times, it is often possible to visualize the entire dendritic and axonal arborizations. Other fluorescent-based methods depend on the internalization of dyes like 5-chloromethylfluoresceine diacetate (Molecular Probe) that remain membrane permeable and not fluorescent until subjected to the action of intracellular aspecific esterases that free the fluorescent molecule. The fluorescent compound that has decreased membrane permeability is also a mild thiol reactive reagent that reacts with sulfhydryl groups inside the cell, thus increasing its retention. Similar molecules reactive with amines and not with thiols, e.g., 5- (and -6) carboxy-2′,7′ -dichlorofluorescein diacetate, succinimidyl ester (Molecular Probes) can be passively loaded into neural cells ***(19)*** and used after enzymatic conversion to trace them for days.

Labeling cells by incorporation of DNA base analogs may give important indications on the mitotic history of the cells after the transplant. Labeling DNA with modified base analogs that can be visualized by autoradiography (^{3}H-thymidine) or immunohistochemistry (5-bromo-2′-deoxyuridine, BrdU) gives the advantage/disadvantage that the label is incorporated only by cells exposed to the base analog during the S-phase of the cell cycle. Incorporation due to DNA repair processes is usually too low to be detectable by standard techniques. After incorporation, further DNA synthesis and cell division will dilute the label, allowing the distinction of cells that do not divide much after grafting ***(9)***.

Another DNA-dependent labeling method that can be useful for intermediate term labeling depends on the vital DNA intercalating dye 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole (Hoechst 33342, Sigma, St. Louis, MO, USA). This dye enters the living cells where it intercalates into the DNA minor groove, resulting in long-term fluorescent labeling of the cell nucleus ***(20)***. Hoechst labeling may be stable for weeks after transplantation, but slowly leaks out of the cell, and its dilution may also be accelerated by cell division. Furthermore, transfer of Hoechst from donor to host cells, especially glial cells, is also possible ***(21)***.

3.2. Transuterine Cell Grafting

We suggest planning everything in a way to perform all grafts in less than 20 min. Leaving the uterus outside the abdomen longer often results in a significant decrease of pups born alive.

1. Anesthetize the mother of the host fetuses [ketamine HCl (50 mg/kg) and Diazepam (2.5 mg/kg given ip] (*see* **Note 4**).
2. Wait 5 min or until the level of anesthesia is deep. Check by pinching one foot. The animal should not react or react very slowly, and with retraction of the limb.
3. Immobilize the hind limb of the animal in a stretched position (tape down the foot to a dissection table, or if preferred, use halters) (*see* **Note 5**).
4. Disinfect the skin over the abdomen with a surgical grade disinfectant (e.g., betadine) and cover the abdomen with a sterile pad (*see* **Note 6**).
5. Through a premade rectangular window in the pad, cut the abdomen, using scissors, for about 2.5 cm starting 8–10 mm above the superior margin of the vagina and following the midline (**Fig. 1A**) (*see* **Note 7**).
6. Exteriorize one uterine horn (**Fig. 1B**) count the embryos and draw a simple sketch of their position (*see* **Notes 8** and **9**).
7. Bring a self-retaining, flexible fiber optic light guide into contact with the uterine wall (**Fig. 1C**) and turn off the ambient light.
8. Prepare drops of the appropriate volume (0.5–2 μL) of the solution to be injected on the bottom of a Petri dish and close the cover.
9. Mount a micropipet on the holder connected to a tube and a suction mouthpiece (*see* **Note 10**).
10. With blunted-tip forceps, manipulate one uterine swelling containing an embryo until the head becomes visible through the uterine wall and embryonic membranes (**Fig. 1D**) (*see* **Note 11**).
11. Suck one drop from the Petri dish into the micropipet in a way that the capillary section of the pipet remains filled with fluid. (This avoids the fluid getting lost into the pipet or into the tube connected to it.)
12. Puncture with a firm and continuous movement the uterine wall, the embryonic membranes and the embryo itself until the target is reached (**Fig. 1E**). Immediately inject the solution by gently blowing into the mouthpiece (*see* **Note 12**).
13. Stop blowing when you can still see a small amount of the solution close to the tip of the micropipet and retract it out of the embryo and the uterus (**Fig. 1F**).
14. Repeat **steps 11–14** until you finish injecting the embryos you planned to inject in one horn (*see* **Note 13**).
15. Put the uterine horn with the injected embryos back into the abdomen and exteriorize the other. Repeat all steps from 7–15 until you finish with the second uterine horn (*see* **Note 14**).
16. Return the second uterine horn into the abdomen and suture the muscle layers and fasciae together.
17. Once the muscles are closed, suture the skin (*see* **Note 15**).

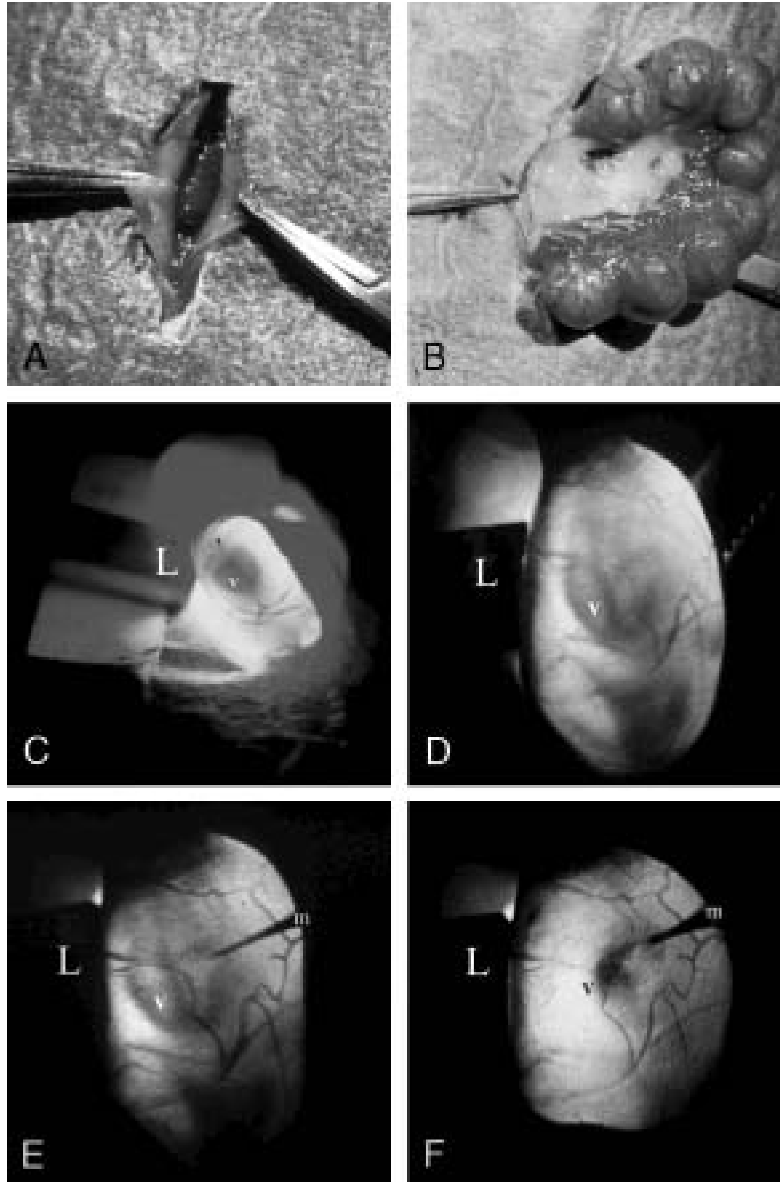

Fig. 1. **(A)** The abdomen has been draped with a disposable surgical pad. All surgical procedures are performed through a rectangular opening of approximately 4 × 25 mm. Two "mosquitoes" are used to spread the incision, the uterus may be seen in the depth of the incision. With a little practice, retraction of the incision margins by "mosquitoes" becomes unnecessary.

18. Remove the pad covering the abdomen and disinfect the skin (*see* **Note 16**).
19. Place the animal back in its cage (*see* **Note 17**).

4. Notes

1. Trypan blue (final concentration 0.01%) may be added to the solution where the cells were resuspended for transplantation. This addition allows checking for the position of the transplant in the embryo and monitoring the injection. However trypan blue per se may exercise a toxic effect on some cells and reduce the rate of successful grafts.
2. Both rats and mice can be transplanted using the above-described technique. Sprague–Dawley rats are our preferred choice owing to the robustness of the strain and the large size of the litter resulting in a good number of transplantable fetuses. We have tried mice of various strains (C57Bl/6, BALB/c, 129/Sv). However, although transplantation per se is not more difficult than in the rat, the yield of surviving animals is lower in mice. On average, about 70% of transplanted rat embryos survive the procedure and are delivered alive. On the contrary, the yield of successfully transplanted mice embryos reaching maturity is often less than 30% or even lower.

Fig. 1. *(continued)* **(B)** The right uterine horn was exposed through the incision and it is now ready for grafting. **(C)** A fiber optic light with a tip diameter of 2–4 mm is moved close to one embryonic chamber. Inside the uterus the head of the embryo (fronto-polar view) is clearly visible. Turning off the light in the room helps visualizing the embryos. Internal markers for the injection are the hemispheres with the lateral ventricles. On a posterior view (occipito-polar) the fourth ventricle and the outlet of the aqueduct are clearly visible. L: fiber optic light, v: ventricle. **(D)** Magnified surgical view. Vessels of the visceral yolk sac are visible between the uterine wall and the head of the embryo. **(E)** The glass micropipet electrode prefilled with 1–2 μL of cell suspension made dark by the addition of trypan blue (0.01%) is brought into the field and by a rapid and continuous advancement the uterine wall, the fetal membranes and cranium are punctured. When the tip of the needle is inside the developing brain, the solution is expelled from the pipette by gentle but steady pressure. Trypan blue may be deleterious to the cells. We suggest using it only for the initial trials to obtain an immediate feedback of the quality of the transplant. L: fiber optic light, v: ventricle, m: micropipet. **(F)** Once the fluid is almost completely expelled from the micropipet we stop blowing and quickly withdraw it. The injected fluid leaked from the injection site in the cortex into the left lateral ventricle whose outline is partially drawn (ventriculographic effect) by the trypan blue stained solution. v: marks the position of the uninjected right ventricle that is now out of focus due to the elastic recoil of the uterus after releasing the pressure exerted by the forceps and the penetrating micropipette. L: fiber optic light, m: micropipet.

3. Pipets are preferably made from hematocrit tubes. Special glass sometimes used to pull microelectrodes for neurophysiology is also good, but unnecessarily expensive. The micropipet will be pulled on a conventional puller, like those commonly used for electrophysiology. A wide variety of micropipet pullers are available. Any apparatus that allows regulation of the temperature of the glass and the speed of pulling in order to modify the length and tapering of the stretched region can be good. The stretched part of the micropipet should be about 1.5 cm. If longer, the capillary may be too flexible and thin, and may break easily when it is pushed through the uterine wall. However, the tip should not be tapered too strongly or the rapidly increasing external diameter of the capillary will damage the tissues of the embryo. The internal diameter of the needle at the tip should be approximately 30 μm; this "large" diameter will be obtained by breaking the tip of the capillary against a smooth surface just before aspirating the solution containing the cells for transplantation. Inspection of the tip under a microscope equipped with a graticule eyepiece is suggested for the neophyte. With a little practice, the breaking procedure will become remarkably reproducible and inspection unnecessary. Irregularities due to the breakage of the glass are not deleterious for grafting, and smoothing of the tip irregularities on a microforge is not necessary. In case of clogging of the needle, it is better to discard the needle and start with a new one. Clogging of the tip of the needle, either due to the presence of multicellular aggregates or to the high viscosity of the cell suspension to be grafted, is common. We suggest preparing about 10 needles per pregnant mother to be transplanted before starting the procedure. Pipets do not need to be pulled the same day they are to be used. Pipets can be maintained with the tip unbroken for months in a closed box (we suggest suspending the pipets by placing them on a strip of plasticine fixed to the bottom of the box). If the micropipets will be used for transplantation, there is no need to siliconize them.
4. We prefer a combination of ketamine HCl (50 mg/kg) and Diazepam (2.5 mg/kg) given ip to the mother approx 5 min before the operation. With this drug combination, ventilation support to the animal is unnecessary; however, respiratory arrest in supersensitive subjects may sometimes occur. We suggest using a 26G needle for the injection, piercing the middle third of the abdomen close to the midline in order to avoid the uterine horns, which at this gestational age are usually more lateral.
5. The anesthetized mother is laid supine. We prefer to fix the lower limbs in a stretched position in order to obtain a more flattened abdominal wall, without fixing the upper limbs to favor normal breathing movements.
6. It is unnecessary to shave the abdominal wall, disinfecting it with a surgical scrub solution (e.g., Betadine) is sufficient.
7. We cut the abdominal wall for about 2.5 cm following the midline, starting 8–10 mm above the superior margin of the vagina. We prefer to use scissors, but a scalpel can also be used. The muscles should be incised along the linea alba to avoid unnecessary bleeding (**Fig. 1A**).

8. We explore the abdominal cavity looking for the uterine horns, with the regularly spaced swellings each corresponding to an embryo. Sometimes, especially at early gestational ages, the two horns may be difficult to identify among the intestine. A good method is to look in the pelvis, just behind the bladder for the vagina and the beginning of the horns, and then following each horn up to the ovarian end. All manipulations should be done with care to avoid damage to the uterine wall; we use blunt forceps with a clasping surface of 2.5 mm. Once the horn is identified, the body of the horn must be exteriorized taking care not to damage the mesometrium where the vascular supply of the uterus is contained. The exteriorized uterine horn must be comfortably left on gauze or a surgical paper towel in register with the abdominal incision (**Fig. 1B**), leaving the uterus in a clean environment and avoiding the possibility of contamination by hair entering the abdomen during the maneuvers necessary for the injections.
9. At this point, we usually draw a quick scheme of the number and position of the embryonic chambers, which is useful for counting the embryos unambiguously and planning the injections. This scheme is also useful if transplants with multiple donor cells are performed, and the experimenter plans to collect the injected fetuses by caesarean section before delivery. However, the resorption of some of the embryos may sometimes make the position of the injected embryos in the scheme difficult to recognize.
10. The glass micropipet is mounted on a pipet holder connected with a 40 cm tube ending in a mouthpiece. Filling of the pipet can be done retrograde by sucking from the tip a drop (any volume between 0.5–2 μL will be good) of the solution. Multiple drops of the appropriate volume must be prepared laying in a row on to the bottom of a small Petri dish just before use.
11. By exerting a gentle pressure with the forceps, the head of the floating embryo is moved as close as possible against the uterine wall, opposite to the light source and facing the surgeon (**Fig. 1D**).
12. With the head in position, the glass micropipet will be inserted into the uterine wall, aiming at one of the brain structures which can be easily recognized inside the head of the embryo (**Fig. 1E**). After insertion of the micropipet, the solution will be pressure injected until almost the entire fluid contained in the glass capillary is expelled. Then, the capillary is rapidly withdrawn and the pressure on the uterine walls exerted with the forceps released (**Fig. 1F**).
13. A few drops of amniotic fluid may sometimes flow from the site of the injection, but this is not usually a problem unless the leakage is massive, which then often results in resorbtion of the fetus.
14. Leaving the uterus outside the abdomen longer often results in a significant decrease of pups born alive. The uterine horns may be kept moist by gentle irrigation with saline from time to time while they are exposed. When more than 12 embryos are present in the uterus, it may be wise to inject only some of them, leaving the others uninjected. It is often more difficult to inject embryos close to the vaginal end of the uterus because the uterine horn is thicker, and close to its anchored origin. If we need to leave uninjected embryos, the embryos

sitting close to the vaginal end of the uterus will be the natural choice. Unless the fetuses are recovered by caesarean section before delivery, there is no reliable method to distinguish the order and position of each pup when they were embryos inside the uterus. This limits the possibility of injecting different embryos in the same pregnant mother with different cells, unless the grafted cells will be distinguishable by a reliable marker, or they will develop into a specific phenotype. Leaving uninjected embryos will increase the rate of animals negative for the transplant at the time of analysis after birth. We tried several procedures to induce readsorption of the uninjected embryos, but the injected embryos are often secondarily affected and we do not recommend any attempt along this line.

15. Closure of the abdomen may be done with continuous sutures; muscle and fascia should be kept separated from the skin. We routinely use 3-0 silk for all sutures. It is not necessary to remove stitches; the animal will do it by itself in a few days.
16. If the animals are not immunosuppressed, they do not need antibiotics. Disinfection of the wound at the end of the operation is sufficient.
17. After surgery, the animals should be left quiet and caged singularly. A delay in the expected delivery time is not uncommon, with prolongation of gestation by one day beyond the expected term.

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31

Transplantation into Neonatal Rat Brain as a Tool to Study Properties of Stem Cells

Tanja Zigova and Mary B. Newman

1. Introduction

Development of the brain is a complex set of events beginning at the time that the fate of embryonic cells is decided. Once chosen, these cells proceed on their migratory track, which brings them to the brain regions in which they reside and settle down. Here, the cells differentiate into array of distinct cell types, and establish a network of functional connections. How these migrating cells find their home and the complexity of events underlying the determination of their phenotypic fate are only partially understood.

The existence of two germinal zones, one giving rise to neural cells prenatally (ventricular zone, VZ) and the second one postnatally (subventricular zone, SVZ) provides evidence that at those particular time points these brain regions have all prerequisite signals for newborn cells to make their decisions, move out from the place of genesis and follow their own developmental pattern. These two important developmental windows serve as an ideal opportunity for investigators to answer questions about properties and behavior of newly born cells. In vitro studies can only partially imitate the complex brain environment; in vivo transplantation experiments can utilize the environmental complexity of either the prenatal (***4***,***6***,***7***, *see* also Chapter 30), or the early postnatal developing brain ***(2***,***16***,***20***,***21)***.

This chapter shows how transplantation of various cell types (stem/progenitor cells and cell lines) can be utilized to study the potential of grafted cells in the signal-rich (instructive) environment of the developing brain. The SVZ of the neonatal brain provides a plausible environment for endogenous precursors (through extracellular matrix and cell surface molecules, along with the radial glia to facilitate migration). This region can support healthy survival

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of transplanted neural stem/progenitor cells, and at the same time, may provide insights into the cell's migratory abilities, differentiation potential, and functional capacities (in case grafted cells can read the cues operating within this region).

This chapter focuses on grafting into two regions of the developing brain: (1) the subventricular zone (SVZ), specifically, the anterior part (SVZa) that serves as a postnatal source of neuronal progenitors for the olfactory bulb ***(12,13)***, and (2) the striatum, which is developed prenatally ***(15)***, and is considered a non-neurogenic region after the animal birth. Here, we present a detailed description of the transplantation technique for the neonatal hosts, including pretransplantation and post-transplantation phase. The chapter gives account of pregrafting procedures including isolation/preparation and labeling of embryonic ventricular zone cells, subventricular zone-derived cells, and postmitotic hNT neurons. Our previous reports utilizing neonatal transplantation paradigm to study survival, differentiation, and migration of transplanted cells are referenced throughout the chapter.

2. Materials

2.1. Animals

We use neonatal (0–2 d old) Sprague–Dawley (SD) rat pups of either sex for grafting (*see* **Note 1**). As a source of the ventricular zone progenitor cells we utilize 16–17 d old SD embryos (E16–E17). Neonatal (postnatal d 0–2, P0–P2) rat pups from the same strain serve for isolation of the subventricular zone (SVZ) progenitor cells (*see* **Note 2**).

2.2. Pregrafting: Cell Isolation, Estimation of Cell Viability, and Cell Labeling

2.2.1. Isolation and Dissociation of Progenitor Cells

1. Dissecting microscope (Wild M3B), fiber optics (Fiber-Lite Pl-900, Dolan-Jenner Industries).
2. Dissecting tools (Fine Science Tools, Inc.,): scalpels #10, fine surgical scissors, microdissecting knife, Dumont forceps #5, straight forceps, fine spring scissors.
3. Chloral hydrate (400 mg/kg) or Equitesin (3.5 mL/kg) to anesthetize pregnant dams.
4. Gauze squares, cotton swabs, 70% EtOH.
5. Tissue culture dishes (Corning, 35 and 100 mm) to collect brains for dissection.
6. Nutrient Mixture Ham's F-10 (Sigma), Hank's balanced salt solution (HBSS; Gibco).
7. Trypsin (Sigma), DNase (Sigma).

8. The incubation medium is prepared from 10 mL of HBSS containing 0.1% trypsin and 0.01 DNase.
9. The dissociation medium contains 0.04% DNase in 10 mL of HBSS.
10. Fetal bovine serum (FBS, Gibco/Life Technologies).
11. Warm water bath (37°C).

2.2.2. Preparation of hNT Neurons

1. Liquid nitrogen tank to store cryopreserved hNT neurons.
2. Water bath (37°C).
3. DMEM, FBS (Gibco/Life Technologies), Gentamicin (Sigma).
4. Sterile fire-polished Pasteur pipets, sterile serological pipets (Fisher Scientific, 1, and 10 mL), automatic pipeter.
5. Vacuum.
6. Centrifuge (Marathon 8K, Fisher Scientific).

2.2.3. Estimation of Cell Viability

The most common viability test is the trypan blue exclusion (described in Chapter 29) The assessment of the viability of cell suspension is determined by a double-labeling procedure using fluorescein diacetate (FDA) and propidium iodide (PI) ***(11)***. This method identifies the viable, FDA-labeled cells fluorescing bright green, while dead cells, stained by PI, are bright red. To perform this staining we need:

1. 15 and 50 mL tubes (Falcon) and 2 mL cryovials (Corning).
2. 0.1 *M* phosphate buffer, pH 7.4.
3. Acetone (Aldrich Chemical Company).
4. Fluorescein diacetate (FDA, Molecular Probes); stock solution contains 5 mg of FDA in 1 mL of acetone (keep sealed with parafilm). Refrigerate.
5. Propidium iodide (PI, Sigma); stock is prepared from 1 mg of PI in 50 mL of 0.1 *M* phosphate buffer. Refrigerate.
6. Working stock solution of FDA contains 5 μL of stock FDA in 1 mL of 0.1 *M* phosphate buffer. This solution needs to be prepared prior to staining.

2.2.4. Cell Labeling

2.2.4.1. PKH 26 Labeling

1. PKH 26, fluorescent lipophilic dye (Sigma) ***(10)***.
2. Sterile Hank's balanced salt solution (HBSS, Gibco/Life Technologies).
3. Normal horse serum (Chemicon).
4. DMEM (Gibco).
5. 15 mL tubes.
6. Centrifuge.
7. Fluorescent microscope (Olympus BX 60).

2.2.4.2. BrdU Labeling

This labeling is described in detail in Chapters 10 and 25. Therefore, we will only refer to those cases related to prelabeling of donor tissue frequently used in our laboratory prior to transplantation into neonatal rodent brain.

1. For labeling of progenitor cells by systemic BrdU administration we need:
   a. BrdU (Sigma), NaOH, NaCl. The stock solution of bromodeoxyuridine (BrdU, Sigma) contains 5 mg of BrdU/mL 0.007 *N* NaOH in 0.9% NaCl ***(2,21,22)***.
   b. Syringes (1, 5 mL), needles.
   c. Sterile gauze squares, 70% EtOH to clean the surgery area and mother's belly prior to embryo excision.
   d. Sterile instruments for cesarean section including scissors, forceps, small curved forceps.
   e. Petri dishes to collect embryos.
   f. All dissecting tools, microscope, and sterile cold media and HBSS described in the **Subheading 2.2.1.**
2. For labeling of progenitor cells by direct injection of BrdU into the subventricular zone, we use the same stock solution as for systemic administration ***(3,21)***. In addition we need:
   a. Crushed ice for hypothermic anesthesia.
   b. Set-up to target the SVZ (*see* transplantation procedure in the **Subheading 3.8.**).
3. Progenitor cells in culture dish are labeled by 20 µ*M* solution of BrdU for 24–48 h before transplantation.

2.3. Grafting

1. Surgical instruments: Dumont forceps #5, straight forceps, fine spring scissors straight clamps (28 mm), scalpel #10.
2. Cotton swabs, 70% EtOH to clean surgical area.
3. Molded Sylgard (or contoured styrofoam slat) to keep animal in a fixed position during transplantation (*see* **Note 3**).
4. Micromanipulator/holder for Hamilton syringe (10 µL) or Picopump (WPI 820) with glass pipets (*see* **Note 4**).
5. Surgical microscope.
6. Surgical glue Nexaband (Formulated cyanoacrylate, Veterinary Products Laboratories, AZ).
7. Crushed ice for hypothermic anesthesia (*see* **Note 5**).
8. Lamp to warm up pups after surgery.

2.4. Postgrafting Period

2.4.1. Marking for Identification

Toe removal after surgery is the method of choice for easy identification of identical groups. For this we need clean razor blade or scalpel and cauterizing device to stop excessive bleeding.

2.4.2. Histological Analyses: Perfusions and Tissue Processing

1. Perfusion minipump allowing the slow/controlled distribution of perfusate/fixative over extended periods of time.
2. 4% paraformaldehyde (Formaldehyde, Aldrich Chemical Co.) in 0.1 *M* phosphate buffer, pH 7.4.
3. Cryostat to prepare frozen sections.
4. Materials for immunocytochemical staining (primary and secondary antibodies, blocking sera, and so forth (*see* other chapters in **Part III**).

3. Methods

3.1. Isolation of Telencephalic Ventricular Zone Cells

1. Anesthetize pregnant (E16–17) dams with ip injection of chloral hydrate (400 mg/kg) or Equitesin (3.5 mL/kg).
2. Perform laparotomy, remove embryos from the uterus (for details see Chapter 3) and place them in cold, sterile Ham's F10 medium. Continue dissection under the sterile hood.
3. Separate the pup's head from the body using fine scissors. Remove skull and attached membranes using a pair of fine forceps. Transfer the clean brain into the fresh, cold media, and with small scissors, dissect a small piece (approx 1 mm^3) of tissue from the dorsal part of the telencephalon (use both hemispheres for dissection) approx 0.2–0.5 mm lateral to the midline. The tissue pieces contain a thick zone of proliferating cells surrounding the lateral ventricle (**Fig. 1**).

3.2. Isolation and Preparation of Subventricular Zone Progenitor Cells

1. Place pup (0–2 d old) on ice for 5 min.
2. Clean the head with a cotton swab soaked in EtOH.
3. Decapitate with a razor, and quickly remove the whole brain under a dissecting microscope (technique varies from person to person).
4. Collect brains in a culture dish filled with cold Ham's F10 (*see* **Note 6**). Continue dissection under the sterile hood.
5. With a sharp scalpel or razor blade, divide the brain sagittally into two hemispheres (**Fig. 2**).
6. Cut 1–2 mm thick parasagittal sections from the midline through the olfactory bulbs.
7. Using a microknife, microdissect the anterior part of the subventricular zone, which can be identified by its transparency and position compared to surrounding structures (**Fig. 2**). Place collected tissue pieces into 2 mL of cold HBSS. Keep on ice during the entire dissection procedure (*see* **Note 7**).

3.3. Dissociation of Tissue (VZ, SVZ) Before Transplantation

1. Take the collected tissue and transfer it into the incubation medium (*see* **Subheading 2.2.1.**) for 20 min in 37°C water bath.

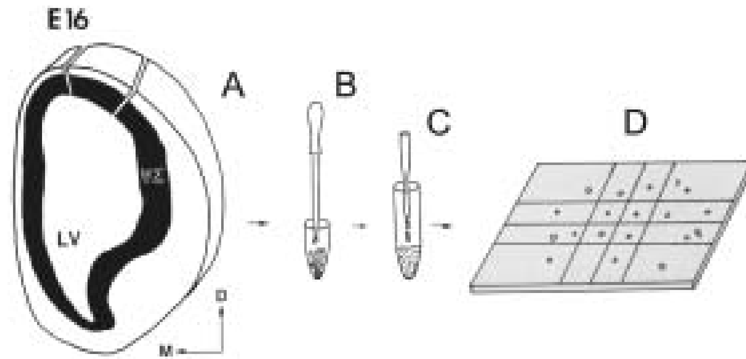


Fig. 1. Procedure for preparation of embryonic ventricular zone progenitor cells before transplantation. **(A)** The picture shows the cross section through the E16 telencephalon. Ventricular zone (VZ, black) surrounding the lateral ventricle (LV) contains proliferating cells. Using a microknife or scissors a small piece of tissue is dissected from the dorsal part of the telencephalon. Arrows in the right corner indicate the orientation of the hemisphere during microdissection (D, dorsal; M, medial). **(B)** Tissue pieces are collected, trypsinized and dissociated mechanically using fire-polished Pasteur pipets. **(C)** Single cell suspension could be stained with fluorescent dye (to label every cell) or BrdU (to tag proliferating cells). **(D)** Using hemocytometer the viability of cell suspension could be determined either through simple Trypan blue exclusion or through double-labeling with FDA/PI.

2. Remove incubation medium and wash tissue using pipet with 1 mL of dissociation medium (*see* **Subheading 2.2.1.**) 5X 5 min each change.
3. Mechanically dissociate tissue by gentle tritutation with a set (3–5) of fire polished Pasteur pipets of decreasing diameter.
4. Transfer dissociated cell suspension into the Ham's medium containing 10% fetal bovine serum (total volume 10 mL in 15 mL tube).
5. Centrifuge for 5 min at 100*g*.
6. Remove the supernatant and resuspend cell pellet with 1 mL of the fresh medium/FBS mixture for estimation of cell viability and determination of desired numbers of cells for transplantation (*see* **Note 8**).

3.4. Preparation of hNT Neurons for Transplantation

1. Thaw frozen hNT cells in the water bath (37°C) and transfer them under sterile hood into 15 mL tube containing a mixture of DMEM and 10% FBS (9 mL of DMEM and 1mL of FBS).
2. Centrifuge (200*g*/7 min) and remove the supernatant.
3. Resuspend cells in 1 mL of the same fresh media mixture for viability testing, prelabeling and adjustment of final cell density for transplantation.

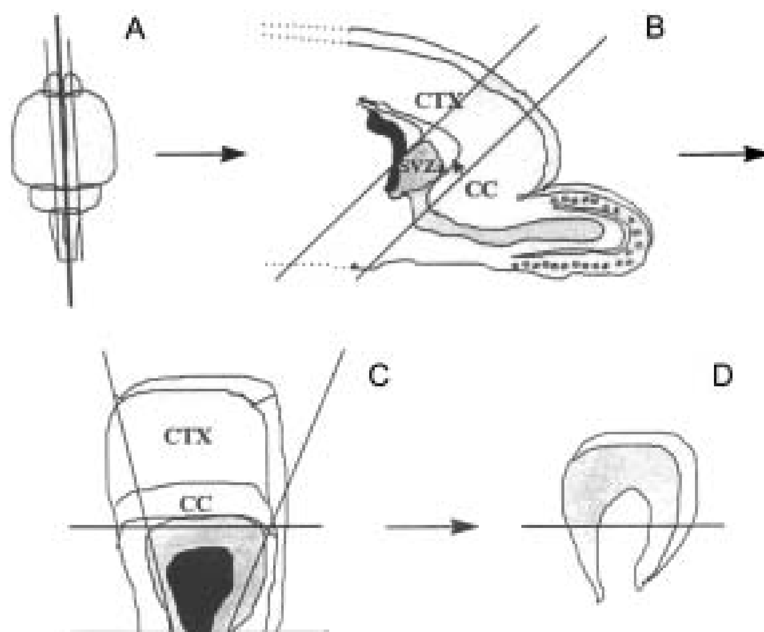


Fig. 2. Microdissection of the SVZa. **(A)** Divide the brain through the midline into two hemispheres (thicker line in the sagittal plane). From each hemisphere cut the parasagittal section (thinner line) through the olfactory bulb about 1–2 mm from the midline and flip the slices on the flat surface to determine the position of the subventricular zone in relation to the lateral ventricle. **(B)** Dissect the region (two parallel cuts) containing the anterior portion of the subventricular zone (SVZa). **(C)** Place the piece on the cut surface closest to the olfactory bulb. This will allow you to see the inside part of the lateral ventricle (black). If necessary, increase the magnification of the microscope and carefully remove the tissue (CTX, CC) surrounding the subventricular zone (grey). **(D)** Remove/shorten the lateral parts of the microdissected region to ensure that the piece will contain only SVZa progenitor cells.

3.5. Viability of Cells

1. Add 100 µL of working FDA stock solution to 30 µL of PI stock solution (*see* **Subheading 2.2.3.**).
2. Add aliquot (20–30 µL) of cell suspension (from 1 mL of resuspended cell suspension) to 130 µL of FDA/PI.
3. Place on ice or in refrigerator for about 5 min.
4. Count under dual fluorescent filter (FITC and rhodamine) the ratio between living (green) and dead (red) cells (*see* **Note 8**).

5. Prepare the required concentration of cell suspension for grafting. Cells need to be again recentrifuged and resuspended in the calculated volume of cold media or HBSS and stored on ice during the entire transplantation procedure (*see* **Note 8**).
6. Determine the viability of remaining cell suspension after transplantation.

3.6. Control Experiments with Dead Cells

In some transplantation experiments designed to test the ability of cells to migrate through the various brain regions, experiments with dead cells were included. For this control study, we microwaved the cell suspension for 30–60 sec, and an aliquot was labeled with trypan blue (**Fig. 3**). These cells were then transplanted into the same brain regions as living cells. Other control experiments using freezing/thawing procedures to kill cells were used to compare the properties of dead and living cells in various transplantation paradigms ***(6,9)***.

3.7. Cell Labeling

3.7.1. PKH26

To identify transplanted cells within the host brain, we routinely stain cell suspensions with membrane-bound fluorescent lipophilic dye PKH26 ***(21,24)*** and visualize the stain under rhodamine filter (**Fig. 4**).

1. Take an aliquot of freshly prepared cell suspension in approx 0.5 mL HBSS (10^6 cells).
2. Centrifuge cells in 15 mL tube at 100*g* for 7 min.
3. Make fresh dye solution [1 m*M* stock dye solution (1:250) prepared with diluent C; e.g., 2 µL of dye/500 µL of diluent C].

Fig. 4. *(opposite page bottom)* Transplantation of prelabeled cells into the neonatal subventricular zone (SVZa) and striatum. **(A)** Schematic drawing of the sagittal section through the neonatal (P0–P1) rat brain. Injections are placed into the anterior part (arrow) of the subventricular zone (yellow). Corpus callosum overlying the SVZ is blue. **(B)** Fluorescently labeled (PKH-26) telencephalic ventricular zone cells (red) injected into the anterior part of the subventricular zone (SVZa) of the neonatal pup. CC, corpus callosum. **(C)** Schematic drawing of the sagittal section through the neonatal striatum (green). Arrow indicates the injection placement. **(D)** BrdU-labeled SVZa-derived progenitor cells transplanted into the striatum of a newborn rat (arrowheads). Many of these cells were found in the vicinity of blood vessels (*). (Reproduced with permission from **ref. *2***).

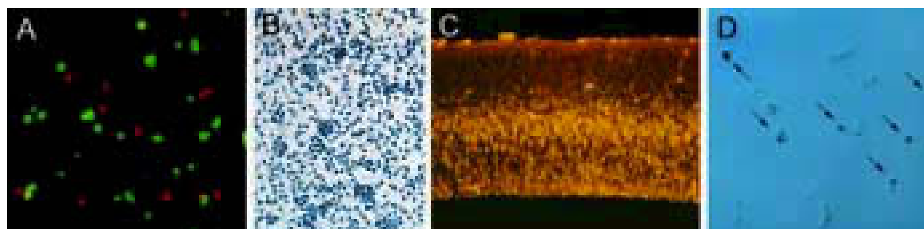
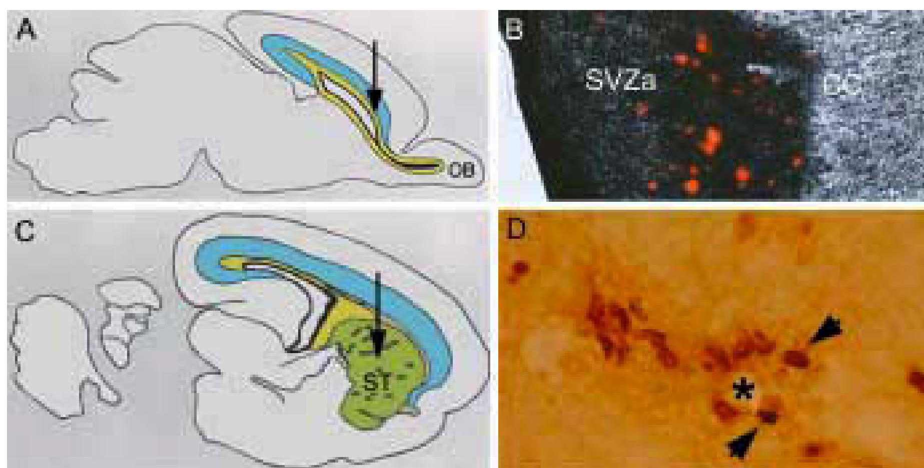


Fig. 3. Viability assessment and BrdU cell labeling prior to transplantation. **(A)** To assess the number of viable cells prior to transplantation we use FDA/PI staining ***(21,25)***. The majority of cells are stained by FDA, which fluoresces green and is taken up by living cells; the PI is red and passively accumulates in dead cells. **(B)** To study the migratory behavior of injected cells into the neonatal brain we often prepare a control cell suspensions of nonviable cells. After dissection and dissociation, the cells are microwaved (*see* **Subheading 3.6.**) and trypan blue exclusion assay is performed. Note that every cell in this preparation is nonviable (blue). **(C)** Fluorescent photomicrograph illustrating numerous BrdU-positive cells (arrows) within the proliferating E16 telencephalic ventricular zone. Pregnant mothers received two ip injections of BrdU stock solution (*see* **Subheading 2.2.4.2.**) on embryonic d 15 and 16. After dissection these BrdU-prelabeled tissue pieces were dissociated into single cell suspension and transplanted into neonatal rat brain. **(D)** Bright-field photomicrograph showing short-term culture of BrdU exposed progenitor cells (arrows). Mild trypsinization is used to lift cells from the culture dish. After proper washing, viability assessment and preparation of the desired cell concentration, cells are transplanted into the neonatal rat brain.



4. Centrifuge at 100*g*/3–4 min and remove supernatant.
5. Tap gently to resuspend cells in remaining fluid.
6. Add 0.5 mL of diluent C and shake gently to mix.
7. Add 0.5 mL of dye solution and incubate for 3 min with occasional swirling.
8. Stop labeling by adding 4 mL of horse serum, wait 1 min.
9. Wash by adding 10% horse serum with DMEM (4 mL) and mix.
10. Spin at 100*g* for 7 min, remove supernatant and resuspend to desired final concentration for grafting (*see* **Note 8**).

3.7.2. Bromodeoxyuridine (BrdU)

3.7.2.1. Labeling of Donor SVZa and VZ Cells by Systemic BrdU Administration In Vivo

1. Donor pups receive either two (0.3 and 0.5 mL, respectively) ip injections of BrdU stock solution (*see* **Subheading 2.2.4.2.**) within a 17 h period or three BrdU ip injections (0.25 mL each) within a 24 h period before removal of selected region. An hour after the last BrdU injection, the progenitor cells from subventricular zone should be isolated, dissociated, and prepared for transplantation as described above.
2. To identify the cells originating from the telencephalic proliferative region, pregnant dams receive two ip injections (3–5 mL each) of BrdU stock solution (*see* **Subheading 2.2.4.2.**) within an 18 h period. The first injection is given on E15 and the second injection 1 h prior to cesarean section on E16. Timing for BrdU administration was designed to follow the onset of cortical neurogenesis ***(14)***.

3.7.2.2. Labeling of Donor Subventricular Zone Cells by Direct Injection of BrdU

Additionally, we may prelabel donor cells by injection of BrdU stock solution (3–4 μL) directly into the subventricular zone (for coordinates *see* **Subheading 3.8.**) 1 h prior to its dissection, followed by dissociation and transplantation. This alternative method is time consuming, requires experimenter's skill, and experience to properly target the required area of the subventricular zone.

3.7.2.3. Prelabeling with BrdU In Vitro

In some cases, we utilize cultured progenitor cells for transplantation into neonatal brain. This procedure requires preparation of tissue culture ***(7)***, which is beyond the scope of this chapter. Briefly:

1. Expose cultured progenitor/stem cells to 20 μ*M* solution of BrdU for 24–48 h.
2. To lift cells replace media with the 0.25% solution of trypsin.
3. Stop trypsinization by adding 5 volumes of DMEM containing 10% FBS.
4. Centrifuge at 100*g*/5 min.
5. Dilute to desired final concentration for grafting (*see* **Note 8**).

3.8. Transplantation Procedure

1. Anesthetize pups by hypothermia for 5 min on crushed ice (*see* **Notes 2** and **5**).
2. Stabilize the pup on a Sylgard mold (or equivalent) under surgery microscope.
3. Make a midline skin incision from just behind the eyes over the length of the cranium.
4. With fine forceps gently pull away the skin and underlying connective tissue and keep it away from exposed skull with a pair of small clamps.
5. Adjust the final head positioning (*see* **Note 9**) so that the anterior–posterior movement of the pipet or Hamilton syringe would trace the midline.
6. Clear the skull surface and make a small diameter craniectomy (0.5–1.0 mm) on the right side of the skull using fine forceps (*see* **Note 10**).
7. Using a surgery microscope, slowly advance the pipet or Hamilton syringe through the opening in the skull until it reaches the dorsal surface of the brain.
8. Slowly lower the pipet into the SVZa by moving the pipet 1 mm to the right of the midline, 2 mm anterior to the bregma, and 2 mm deep to the pial surface ***(20,21,24)***. For striatum, use coordinates ranging from 1.2 to 2.3 mm mediolateral and from 0.7 to 2.0 mm anteroposterior measured from bregma ***(2)*** (*see* **Note 11**).
9. Slowly inject the cell suspension (0.25 μL/min, 1–4 μL total) and leave the pipet in the same position for 1–2 min before beginning a slow needle withdrawal (1 mm/min).
10. After pipet removal, clean the surface of the brain, remove clamps, reposition the skin overlying the injection place, align the edges of the incision, and seal the skin with a surgical glue (*see* **Note 12**). Place the pup under the heating lamp until it recovers and return it to its mother (*see* **Notes 13–15**).

3.9. Posttransplantation Period

3.9.1. Marking Animals for Easy Identification After Surgery

In deeply anesthetized neonatal pups, the toe is removed with a clean razor blade or scalpel, and the remaining part should be cauterized to stop bleeding and minimize the risk of infection. Usually, bleeding stops by itself before the pups are returned to their mother's cages.

3.9.2. Histological Analyses

Depending on the specific questions to be addressed through the transplantation, the final posttransplantation survival time must be planned ahead (*see* **Note 15**). One has to keep in mind that in the brain's environment cells are exposed to multiple signals, and their development may not be exactly the same as during their normal development. Therefore, various survival times should be planned, rather than just one interval. For progenitor cells and cell lines, we preferentially select 1 wk intervals (until 1–2 mo), so the cells have enough time to complete their development or accomplish their migration into other brain areas.

The methods for brain/tissue processing after animal sacrifice will also very much depend on the issues to be addressed. In our laboratory, we prefer to use transcardial perfusions for all neonates (*see* **Note 16**) and young adults ***(17–24,26)***. Cryostat thaw-mounted (18–20 µm) sections are ideal for all immunohistochemical stainings mentioned in other chapters of this book (for BrdU see Chapters 10 and 25; for cell-type specific antibodies see Chapters 26 and 28 and referenced in our original articles.

4. Notes

1. The choice of this species as an experimental host has been favored for reasonable maintenance cost, easy manipulation/handling, and lower immunological rejection after xenografting.
2. The optimal age for grafting is within the first week after birth, preferentially on postnatal d 0–2. The age of the experimental animals should be determined consistently by designating embryonic day (E) 0 as the day the vaginal plug was found, and postnatal day (P) 0 as the day of birth, considered equivalent to E22. When animals are bred in home colonies, it is fairly simple to determine prenatal and postnatal ages. Otherwise, one has to rely on suppliers that provide timed-pregnant animals. If timed-pregnant animals are ordered, it is best to order them at least 1 wk before delivery, in order to give them enough time to adjust to a new environment and alleviate stress. All pregnant dams are placed into clean cages (one mother/cage) where they deliver. To minimize the chances of the mother's neglect or cannibalism, it is very important to pet and hold the mothers (with gloved hands) every day before delivery. During delivery, the mothers should not be disturbed. To avoid introduction of new scents, new bedding should not be given at least three days before delivery of a litter or prior to transplantation. After delivery, the behavior of mother and pups should be carefully observed.
3. Molded Sylgard board or a contoured styrofoam slat provide a very useful base for proper animal placement during surgery. Usually, the body size is a stable parameter within the litter, and small adjustments/modifications of the base can be easily done prior to surgery.
4. To deliver small volumes of cell suspension, we use either a glass micropipet attached to a pneumatic picopump or a 10 µL Hamilton syringe. In our hands, either application caused no or only minimal trauma to the developing rat brain. For additional details and optimal grafting conditions, see Chapter 29.
5. Hypothermic anesthesia is frequently used for newborn and very young animals ***(8)***. Neonatal pups are buried under the crushed ice for approx 4–5 min (1 min/g bw) to allow breathing/ventilation. In our experience, this anesthesia is sufficient for the entire implantation procedure, which does not take longer than 7–10 min. In some cases, additional ice chunks could be placed on the back of animal to maintain anesthesia for a short while. For longer surgeries in neonates, Stoelting

Co. developed a miniaturized hypothermic stereotaxic apparatus that allows precise stereotaxic surgery and extended hypothermic anesthesia (20–30 min) in very young animals.

6. To speed up the whole process, it is possible to collect at least four brains in the Petri dish filled with cold Ham's F10 before going to the microdissection step.
7. For transplantation, we usually collect subventricular zone tissue from 10–15 pups or ventricular zone tissue from 10–12 embryos.
8. Besides rapid staining procedure using 0.4% trypan blue, we frequently use FDA/PI double-immunofluorescence ***(21,25)*** for both progenitor cells and postmitotic hNT neurons to assess the viability of the single cell suspension (**Fig. 3**). A viability of 80–95% for SVZa, 90–95% for VZ cells, and 65–80% for hNT neurons was obtained from cell pellet resuspended in 1 mL of standard media. FDA/PI staining could also be used to assay the viability and morphological appearance of living (nonfixed) cultures ***(25)***. To adjust the final concentration of cells for transplantation, we divide the total number of viable cells (in 1 mL) by the desired cell concentration; for example, if we have 3,000,000 cells and need to deliver 30,000 cells per μL, we need to resuspend these cells in 100 μL of cold media/HBSS.
9. Besides SVZa and striatum, we have also succesfully targeted olfactory bulb ***(17–19)*** and cerebellum (Zigova et al., unpublished) using modifications in this methodology. For precise injections into the neonatal brain, there is also a miniaturized device (Stoelting Co.) for stereotaxic placement of cell suspensions. Detailed description of a grafting technique into the adult and neonatal brain using this device has been reported by Cunningham et al. ***(5)***.
10. As the cranium of neonatal pup is still underdeveloped and ossification is not yet complete, we prefer to use pointed forceps to make the opening in the skull, instead of the dental drill frequently used in adult rats ***(22,23)***.
11. To determine the injection coordinates for neonatal striatum, we used Altman and Bayer's atlas ***(1)***.
12. Minimal introduction of foreign scents during surgery, careful cleaning of blood after surgery, as well as warming the body to blood temperature will substantially lessen the chances of the mothers' unusual behavior. Surgical incisions closed with surgical glue instead of suturing did not cause any maternal neglect or cannibalism.
13. The pups revive under the heating lamp quite fast. Usually, it takes 4–7 min before their body starts warming up, turns reddish, and they start regular breathing pattern. In case the body (especially the abdominal region) becomes dry and the newborn does not begin breathing within 7 min, we first open its mouth and pull out the tongue using a blunt forceps. Then, to initiate the breathing reflex, place a piece of tubing over the nose and mouth of animal. Experimenter blows into the other end of the tubing to get air into the animal's lungs.
14. Never place pups that are not warmed properly back with their mothers; this may cause rejection by the mother. In some cases, we have successfully used another mother with a litter of comparable age to adopt the neglected pup.

15. After transplantation, the whole litter stays with mother depending on required/studied posttransplantation interval/survival. If posttransplantation survival is longer than 30 d, animals from the same litter have to be gender-separated.
16. Transcardial perfusions in neonates are performed in a manner similar to the adult perfusion techniques, but scaled down in the volume of perfusate and needle size. The volume of the perfusate has to be adjusted according to animal weight (10–50 mL). The size of the blunted needle as well as the pump speed need to be reduced considerably in comparison to adult animals.

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32

Routes of Stem Cell Administration in the Adult Rodent

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1. Introduction

Arguably one of the most exciting developments in science in the last decade is the discovery and isolation of neural stem cells (NSCs), not only from the embryonic ***(1,2)*** but also from the adult human brain ***(3,4)***. With the ability of a NSC to proliferate, self-renew, and generate a large number of clonally related progeny of a neuronal, astrocytic, or oligodendrocytic lineage, these cells promise to revolutionize the treatment of neurological disease. The potential of these cells to correct genetic diseases, such as those resulting from inborn errors of metabolism, is staggering. Studies in myelin-deficient rat ***(5)*** and *shiverer* mouse ***(6)*** demonstrated that it is possible to correct a myelin deficiency. Direct transplantation of NSCs into the spinal cord in the first study resulted in remyelination of the cord for up to 3 mm around the implant site. In the second study, the NSCs were implanted intracerebroventricularly and then integrated throughout the brain, predominantly differentiating into oligodendrocytes, which elaborate the myelin. Therefore, the cells are able to react to environmental cues within the host environment and differentiate accordingly, suggesting that NSCs are able to migrate to the damaged region of the host brain. Studies such as these not only demonstrate the utility of the cells for treatment of developmental disorders, but the knowledge gleaned also helps us to better understand the normal development of the nervous system.

The utility of NSC extends beyond the treatment of developmental or genetic diseases. It is possible that these cells could provide an unlimited supply of

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human neural cells for the treatment of diseases of the adult or aging brain as well. However, the environment of the adult brain is not the same as that of the neonate. Aside from specific regions, such as the subventricular zone or the dentate gyrus, in which neurogenesis continues even in the adult, the neural circuitry has long been established. Important developmental/environmental cues that guide the fate of NSCs in the young animal may not be present in the older one. In initial studies of mouse NSC transplantation into the injured brain, some of the cells developed into neurons, suggesting that instructive cues are present in the injured or diseased brain that allow the cells to develop along the appropriate phenotypic lines ***(7)***. Thus, it may be possible for the stem cells to differentiate into the needed cell population in the adult brain.

There have been a number of recent reports demonstrating that stem cells capable of differentiating into neural cells exist outside the CNS. For example, two groups recently reported the production of multipotential neural stem cells from skin ***(8,9)***. It was recently shown that NSCs produce non-neural hematopoietic cells ***(10)***. Furthermore, after systemic delivery of nonhematopoietic stem cells from bone marrow (or bone marrow stromal cells), these cells have been found in the brain ***(11)***. These cells have also been found to differentiate into astrocytes ***(12–14)***. Two papers in a recent issue of *Science* reported on transplants of whole bone marrow that produced cells expressing neuronal markers ***(15,16)***. Furthermore, when these cells are transplanted into a rat model of stroke ***(17)*** or traumatic brain injury ***(18)***, they can ameliorate behavioral deficits and may promote the proliferation of endogenous stem cells within the subventricular zone ***(19)***.

In our laboratory, we have been examining the ability of stem cells derived from non-neural sources (umbilical cord blood) to integrate, differentiate, and ameliorate deficits in rodent models of injury (stroke and spinal cord contusion) and disease (amyotrophic lateral sclerosis). The first paper, a collaborative effort between research groups at University of South Florida and Henry Ford Hospital in Detroit ***(20)***, shows significant improvements in motor function after iv administration of cord blood cells that are maintained 30 d after transplantation. Part of our program has been to compare whether there are differences in the ability of these cells to provide functional benefits depending upon the route of delivery—intraparenchymal vs vascular. In this chapter, we will describe the surgical procedures for cell implantation and the issues involved in the transplantation procedures themselves. Other chapters in this book (*see* **Part I. Isolation and Culture of Neural Stem Cells**) describe in detail the methods for the preparation of stem cells; we will not repeat that here.

2. Materials

2.1. Cell Preparation

1. Stem cells.
2. Media for cell suspension.
3. DiI (Molecular Probes Inc., Eugene, OR, USA); Cell Tracker CM-DiI (C-7000).
4. Dimethyl sulfoxide (DMSO; Sigma, cat. no. D-2650).
5. Cholera toxin subunit B (FITC conjugated, Molecular Probes).
6. Microcentrifuge tubes.
7. 15 mL conical tubes.
8. 0.22 μm non-pyrogenic filter (Corning/Costar 8110).
9. Syringes.
10. Pipeter and tips.
11. Trypan blue, 0.4% (Sigma, cat. no. T-8154 or Gibco, cat. no. 15250-061).
12. Distilled water.
13. Hemocytometer.
14. Inverted microscope.
15. Water bath.
16. Centrifuge.

2.2. Transplantation

1. Sterile physiological saline (0.9%).
2. Syringes (1 mL, 3 mL, 10 mL).
3. Betadine.
4. Equithesin or Isoflurane.
5. Kopf Stereotaxic instrument.
6. Cotton swabs and gauze.
7. Silk suture 3-0 and 5-0.
8. Gelfoam.
9. Lidocaine.
10. Surgical instruments—scalpel, scissors, needle, retractors, forceps, hemostats, needle drivers, drill bit, spatula, dental pick, ronguers.
11. Dental drill.
12. Absorbent table cover.
13. Ethyl alcohol.
14. Vetbond.
15. Hydrogen peroxide.

2.3. Post Operative Requirements

1. Immunosuppressant.
2. Antibiotic.
3. Acetaminophen.

4. Heating pad.
5. Nest building material for mice.

3. Method

3.1. Cell Preparation

Detailed descriptions of the cell preparation (isolation, propagation, or differentiation) have been thoroughly discussed in other chapters. Regardless of the process employed to prepare the cells, we then prelabel the cells for later identification, and that procedure is as follows.

We label our cells with either 20 μg/mL FITC conjugated Cholera toxin B (Ctb) or 2 μ*M* DiI (2.103 μg/mL) (*see* **Fig. 1**). Both fluorescent dyes are obtained from Molecular Probes. The labeling procedure for the Ctb was adapted from Harder et al. ***(21)*** and Janes et al. ***(22)***.

1. The lyophilized compound is reconstituted at 2 mg/mL (or 0.2% concentration) with distilled water and aliquoted into 10 μL aliquots (20 μg). It is then simply a matter of adding 1 aliquot to 1 mL of transplant media to obtain the final concentration of label.
2. The cells are incubated for 15 min at room temperature and then washed 3X in media.
3. The viability and cell number are assessed prior to transplantation using the trypan blue dye exclusion method.
4. Cell concentration is adjusted as desired.

The labeling procedure for DiI is similar. The dye is supplied as a concentrate with 50 μg of compound in 24.5 μL of DMSO.

1. Reconstitute the DiI with 25.5 μL DMSO for a final concentration of 1 μg/μL.
2. Aliquot the DiI at 2.1 μL/vial which, when added to 1 mL of media, is 2 μ*M*.
3. Incubate for 15 min.

3.2. Transplantation

3.2.1. Brain

3.2.1.1. Striatum

Transplanting cells into the striatum is the most straightforward of those protocols in which cells are transplanted directly into the parenchyma. The only technical difficulties that are likely to arise are related to the extent of damage that may occur with various lesioning procedures used to damage the striatum prior to transplantation. It is not uncommon for striatal atrophy to occur after excitotoxic or ischemic injury, which may decrease striatal volume.

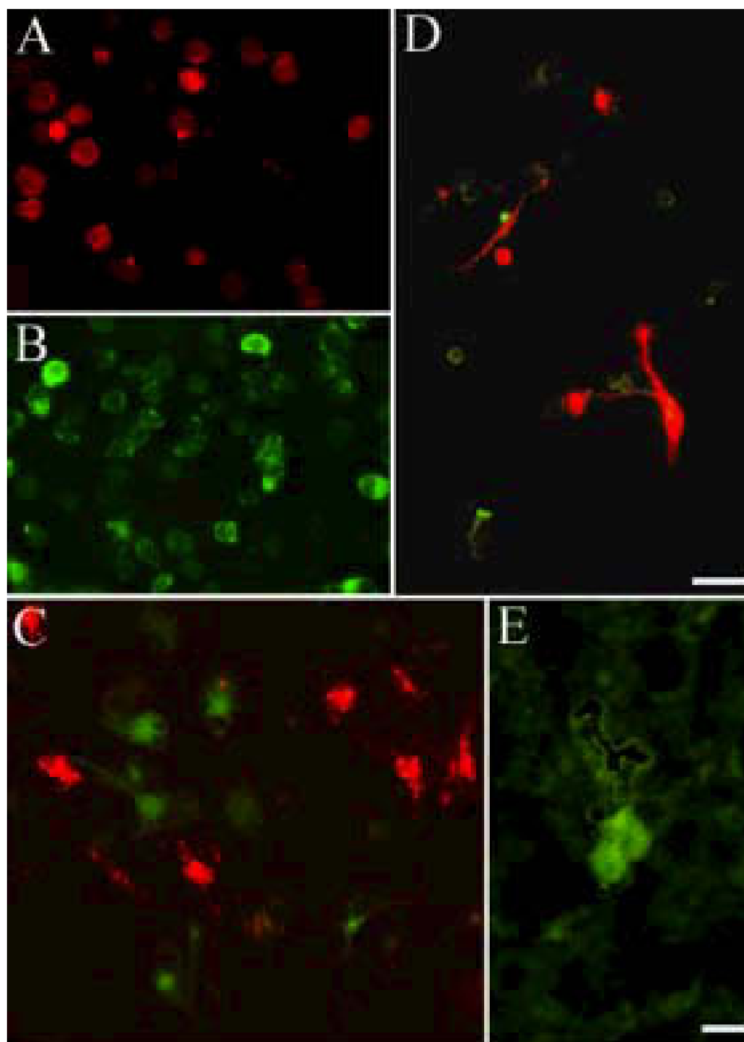

Fig. 1. **(A)** NT2N cells immediately after incubation with DiI for 15 min. **(B)** NT2N cells immediately after incubation with Ctb. Both dyes label the cell membranes of almost all the cells in suspension. **(C)** NT2 cells after 1 d in vitro (DIV). Half the cells were labeled with DiI and other half with Ctb and then placed in culture together to determine whether the dyes could be transferred between adjacent cells. At 1 DIV, there were no double-labeled cells in culture. **(D)** NT2 cells were prepared as described in **(C)**. After 3 DIV, there was still no transfer in the dyes between the separately labeled cells. **(E)** Cholera toxin labeled umbilical cord blood cells found in rat lung 3 weeks after intravenous deliver of stem cells. Scale bars = 25 μm in **A–D**. Scale bar in **E** = 10 μm.

The general procedure for stereotaxic surgery is similar regardless of the region in which the cells are implanted and is as follows:

1. Anesthetize the animal with Equithesin (**Note 1**), shave the top of the head, place in the stereotaxic instrument, and apply Betadine to the shaved area.
2. Make an incision along the midline of the cranium, clean connective tissue from the top of the head, and locate bregma.
3. Using bregma as a landmark, drill a hole in the cranium at the appropriate anterior/posterior, medial/lateral location. Check the burr hole to ensure there are no bone fragments or spurs that may interfere with the needle trajectory through the hole into the brain.
4. Load the cells into a 10 μL Hamilton syringe fitted with a 26 gauge needle.
5. Make a small hole in the dura for the needle to pass through with a dental pick; this decreases the damage that can result from compression of the brain.
6. Lower this needle through the hole until the tip is touching dura. This is considered the zero point in the dorsoventral direction.
7. Slowly lower the needle into position, wait for 2 min to allow the tissue to regain its normal orientation, then deliver the cell suspension at a rate of 0.5 μL/min (*see* **Note 2**).
8. Leave the needle in place for 5 min.
9. If a second cell deposit is to be made, reposition the needle at an adjacent site and repeat the process.
10. Leave the needle in place 5 min, then slowly withdraw.
11. Place Gelfoam in the hole and close the incision with wound clips or sutures.

3.2.1.2. Hippocampus

There are more technical difficulties in placing transplants directly into the hippocampus than into the striatum. First, there are number of blood vessels on the top of the brain that interfere with access to the hippocampus. To address this issue, a 2 mm × 4 mm rectangular bone flap is carefully removed by drilling away the perimeter of the bone flap with a dental burr until the majority of the flap is free, then gently elevating the flap with forceps. We have found it useful to make this craniotomy large enough to obtain good visualization of the cortical surface, allowing us to localize the veins, make minor adjustments in needle placement if necessary, or gently push a vessel out of the way during needle insertion. However, care must be taken while removing this flap not to disrupt any of these veins. In the event that bleeding does occur, a small piece of Gelfoam or a cotton swab soaked in saline can be applied briefly to the craniotomy to stop bleeding from both emissary veins and the cranium. Once the edges of the craniotomy are cleaned with fine ronguers, an "x" is cut in the dura using the tip of a number 11 scalpel to fashion a larger hole in dura; this minimizes any resistance to the needle's passage.

A second issue is that the hippocampus is not attached to the overlying cortex or the thalamus beneath. In addition, the thin layer of the alveus on the ventricular surface of the hippocampus can make transplantation problematic, with the needle either slipping off of the hippocampus or simply compressing it. In either case, the result is a ventricular transplant. To address this issue, we use a sharper beveled needle than is used for the striatal transplant. The tip of the needle is sharpened to a point with a fine whetting stone under a dissecting microscope so that the needle can penetrate the tissue more easily.

3.2.1.3. Cortex.

Transplantation into the neocortex of the rat presents a number of technical difficulties. The cerebral cortex of the rat is approximately 1 mm thick throughout. Insertion of a 30 gauge transplant needle perpendicular to the cortical surface may seem the most direct and expedient method to adopt, but neural tracing studies clearly indicate that the only way to accurately place the tracer in layer 3 or 4 of somatosensory cortex without significant solution reflux is with ionotophoretic injection through glass micropipets with a tip diameter of 10–20 μm ***(23–26)***.

Transplantation of cells within the neocortex usually cannot be accomplished with such small volumes or with drawn glass tips of such small diameter. Nor is the approach described for striatal injection (a 10 μL microsyringe fitted with a 26 gauge thin wall needle) satisfactory. In an effort to improve this method without resorting to drawn glass pipets, we have adapted the striatal delivery system to making cortical transplantation by inserting the needle at an angle, rather than perpendicular, to the cortical surface (**Fig. 2**). This adaptation results in accurate placement of the transplant within the cortex (*see* **Note 3**). The forces on the cortical tissue during tissue penetration result in little tissue deformation. Additionally, some protection of the transplanted tissue is provided because the transplant is placed in a "pocket" within the cortex that is covered with at least a half thickness of intact cortical tissue.

1. Anesthetize the rat and place in a stereotaxic apparatus capable of tilting the injection apparatus to an angle of 20°–45°.
2. Prepare a bone flap as described for hippocampal transplants. Clean the edges of the craniotomy with fine ronguers.
3. Load the microsyringe with cells for transplantation and mark the site where the injection needle contacts the surface of the brain. If the target is the motor cortex of the rat, care must be taken that the injection clears the sagittal sinus (**Fig. 2**).
4. Nick the dural surface at the site where the injection needle will enter the cortex with a 26 gauge hypodermic needle.

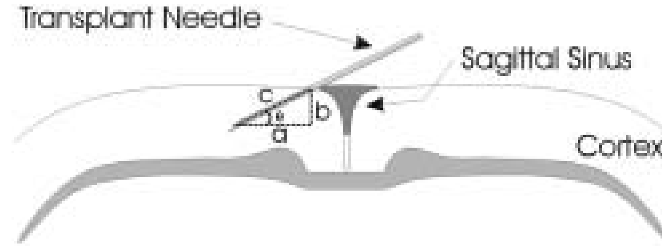

Fig. 2. Needle trajectory for a cortical transplant.

5. Slowly advance the injection needle to the precalculated coordinates, making sure that it is free of the edges of the craniotomy at all times.
6. Leave the needle in place for 5 min to allow the cortical tissue to adjust.
7. Inject the cells at a rate of 0.01–0.1 μL/min.
8. Leave the needle in place for an additional 5 min.
9. Withdraw the needle slowly.
10. We routinely place a piece of Gelfoam soaked in saline over the craniotomy.
11. Close the scalp with discontinuous sutures or with Michel clips.

3.2.2. Spinal Cord

Transplants into the spinal cord are performed in a two step procedure. The first day, we gain access to the spinal cord by drilling small holes in the vertebra (*see* **Note 4**). We then wait 24–48 h and evaluate the animals to make sure that they still have intact motor function. On the second surgery day, the cells are implanted directly into the spinal cord.

3.2.2.1. Preparing the Transplant Site

1. Anesthetize the animals with Equithesin (3.5 mL/kg, i.p.; *see* **Note 5**).
2. Immobilize the anesthetized animal on a small platform.
3. Make an incision in the skin over the vertebra. In both our rat contusion model, and the SOD1 mouse model of ALS, the transplants are placed in the lumbar spinal cord.
4. Remove the erector spinae muscle from the dorsum of the vertebra.
5. Make a hole by gently twirling a 0.8 mm Dremel engraving bit between two fingers. The holes are made 0.5–0.7 mm from the vertebral midline at the level of L_4–L_5.
6. Suture the incision.
7. Monitor the animals to ensure that no damage has been inflicted by these procedures (**Note 6**).

3.2.2.2. Implanting the Cells

One to two days after the burr holes have been drilled or the laminectomy performed, the animals are transplanted.

1. Anesthetize the animals with Equithesin (3.5 mL/kg, ip).
2. Immobilize the anesthetized animal on a small platform.
3. Remove the sutures from the earlier incision and open the incision.
4. Make a hole in the dura mater through which the needle is to pass.
5. Load the cells into a 10 μL Hamilton syringe with a 31-gauge needle.
6. Mount the syringe in a stereotaxic frame to ensure a steady, straight angle of entry.
7. Lower the needle through the prepared site into the ventral horn (1–1.3 mm below the dura).
8. After waiting for 2 min, inject 1 μL of the cell suspension or media into the transplant site over a 5 min period.
9. Wait 5 min.
10. Slowly withdraw the needle.
11. If a laminectomy was performed, cover the spinal cord with Gelfoam (absorbent gelatin).
12. Close the incision. In mice, we do this with Vetbond (tissue adhesive).

3.2.3. Vascular

Introduction of stem cells through the vascular system is a novel, and potentially important method of allowing the stem cells access to organs of the entire body. Stem cells, regardless of origin, appear to preferentially target areas of damage and enter these sites ***(6,12,14,20,27)***. The carotid artery, or femoral, jugular, and tail veins are all potential sites to introduce stem cells into the vascular system (*see* **Fig. 3A**). The choice of vascular route is based more on technical or practical issues than efficacy (*see* **Note 7**). Once the choice has been made, all vascular surgeries are performed using blunt dissection techniques in order to minimize trauma to the vessels and surrounding tissue (*see* **Note 8**).

3.2.3.1. Tail Vein

Perhaps, the most convenient and least traumatic route of vascular access is through the tail vein. The blood to the rat's tail is supplied through bilateral dorsal arteries and is returned by bilateral tail veins (*see* **Fig. 3B**). The tail vein lies laterally, just beneath the surface of the rather thick tail skin, and inferior to the small transverse processes of the caudal vertebrae.

1. Load a 1 mL syringe with the cells to be transplanted (a total volume of 0.25 mL works well for us) and attach a 25 gauge need to the syringe. A larger volume of cells is loaded to allow us to fill the syringe and clear it of air (**Note 9**).
2. Place the rat in a rat restrainer.
3. Hold the rat's tail under warm, flowing water (approximately 42°C) for 2 min. The tail vein on both sides will dilate and should be clearly visible beneath the skin on the lateral surface of the tail. Water should flow over the entire tail to dilate as much of the tail vein as possible.
4. Dry the tail and wipe with alcohol.
5. Hold the syringe so that the bevel of the syringe needle is facing up.
6. Insert the needle into the skin, about 6 cm from the base of the tail (*see* **Note 10**). The needle is initially directed at a 30° angle into the skin, and then threaded a short distance into the vein by decreasing the angle in relation to the tail.
7. During this procedure, gently pull the plunger back just a bit. When the needle enters the tail vein, venous blood will enter the hub of the needle and will also be visible in the syringe.
8. Without moving the syringe, depress the plunger to deliver the contents of the syringe into the tail vein (*see* **Note 11**).
9. Once the cells have been delivered, remove the needle and quickly place pressure over the site of skin penetration to stop the flow of blood. Apply pressure until hemostasis is achieved, usually about 30 sec.
10. Release the animal from restraint and place back in its cage.

3.2.3.2. Jugular Vein

1. Isolate and ligate one branch of the external jugular vein above the junction of the anterior jugular, cephalic, and acromiodeltoid veins (*see* **Fig. 3C**, **Note 12**).
2. Place a second suture on the vessel past where the hole is to be made and tie it loosely.
3. Puncture the vessel using a 25 gauge needle.
4. Insert a 31 gauge needle attached to a Hamilton syringe with the cell suspension already loaded through the hole and into the lumen of the vessel past the second suture.
5. Tighten the suture over the needle.
6. Deliver the cells into the vessel over 2 min.
7. While maintaining tension on the suture, withdraw the needle and permanently tie the suture.
8. Close the incision.

3.2.3.3. Femoral Vein

The procedure for the femoral vein cannulation is much the same as for the jugular vein. The animal is placed on its back, and an incision is made in the skin to expose the muscles of the medial aspect of the thigh. The femoral vein lies together with the femoral artery, superficially overlying the boundary

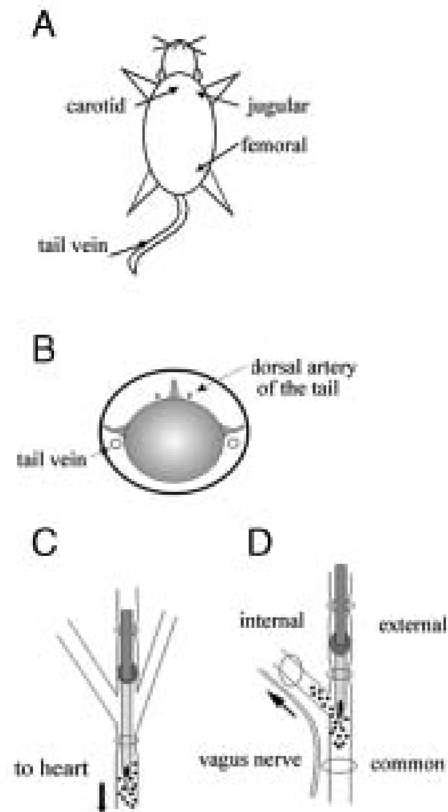

Fig. 3. Vascular routes of cell delivery. **(A)** The location of common vascular delivery sites—common carotid artery, jugular, femoral and tail veins. **(B)** Schematic diagram illustrating the location of the tail vein. **(C)** Illustration of the cannulation of the jugular vein. **(D)** Cannulation of the carotid artery.

between the adductor and extensor muscles and passing under the inguinal ligament to become the external iliac vein of the pelvis. Using blunt dissection, the femoral artery and vein are isolated from each other and the surrounding tissue. The vein is then prepared in the same way as the jugular vein.

3.2.3.4. Arterial

For this procedure, the cells are delivered retrogradely through the external carotid artery into the common carotid (*see* **Fig. 3D**).

1. Anesthetize the animal.
2. Make an incision over the sternohyoideus muscles.

3. Using blunt dissection, isolate the common, external, and internal carotid arteries from the vagus nerve and surrounding tissues in the neck.
4. Place two sutures (5-0 silk) on the external carotid, and permanently tie the one closest to the base of the skull.
5. Place the second tie next to the junction with the common carotid. Do not tie.
6. Place temporary sutures or vascular clamps on the common carotid and the internal carotid to prevent blood loss once a hole is made in the external carotid.
7. Make a hole in the external carotid using a 25 gauge needle.
8. Insert a 31 gauge delivery needle into the lumen of the common carotid.
9. Tie a half knot around the needle with the suture at the junction of the external and common carotid arteries.
10. Remove the clamps on the common and internal carotid.
11. Inject the cells.
12. Leave the needle in place for 2 min, then remove the delivery needle.
13. Completely tie the suture at the junction of the external and common carotid.
14. Close the incision.

3.3. Post-Operative Care

For the most part, the post-operative care of the mice or rats should be fairly standard. One of the most critical concerns after surgery is body temperature; anesthesia and the incision both may contribute to lowering body temperature. After surgery, the first priority should be to place the animal on a heating pad and monitor it frequently. Mice in particular are very sensitive to temperature changes, and are subject to both hypo- and hyper- thermia. Nest building material is provided to assist mice in maintaining their body temperature and increase their sense of security. In addition, the animals are placed on a prophylactic antibiotic therapy for 5 d post surgery to minimize the risk of surgery-related infections. Acetaminophen is also placed in the water for one week. Finally, the animals should be monitored for dehydration. Signs of dehydration include dry excrement, non-elastic skin, etc. Dehydration can be remedied either by twice daily oral administration of water (1 mL per time), substitution of a soft food, or subcutaneous injections of physiological saline.

In addition to standard post-surgical care, transplanted animals in our care also receive immunosuppressant drugs to minimize graft rejection (**Note 13**). We usually use cyclosporine [10 mg/kg/day ip for rats and mice or in some cases 25 mg/kg/day orally for the mice ***(28)***]. It is not yet clear whether this treatment is necessary. It can be argued that embryonic stem cells or stem cells from umbilical cord blood are non-immunogenic because they have relatively few surface markers, but it has yet to be shown that these grafts can be maintained in vivo long-term without immunosuppression.

4. Notes

1. After administration of Equithesin (3.5 mL/kg, ip), a rat or mouse remains anesthetized for approximately 1–1.5 h. This anesthetic is generally well tolerated by the rats in particular and to a lesser extent by mice. Should it be necessary to supplement the initial dose, administer 0.05 mL to the rat or 0.001–0.002 mL for a 20–23 g mouse. After 3–5 min, depth of anesthesia should have increased again. Respiratory problems can occur under both Equithesin and Isoflurane anesthesia. These problems are more easily rectified with gas anesthesia by simply decreasing the amount of anesthetic being delivered. Should problems persist with gas anesthesia or injectable anesthetic, a number of alternatives exist. The simplest method is to stimulate the animal's breathing center with smelling salts (ammonia inhalant of 15% ammonia and 35% alcohol). Three to five ammonia inhalations (each separated by 15–20 sec) should be accompanied by gentle compression of the rib cage. Usually the animal is revived quickly, appearing to sneeze. If there is no response, it may be necessary to administer a respiratory stimulator, such as Doxapram hydrochloride (1–2 drops under the tongue followed by 0.1 mL im). If these measures fail, it may be necessary to perform mouth to mouth resuscitation on the animal using a small tube to blow through. Gentle chest compression may also help.
2. For all surgeries in which the cells are implanted directly into either the brain or spinal cord, there is an issue of seepage of the cell suspension occurring along the needle at the site of penetration. This issue is usually a function of the cells being injected too quickly and can be easily remedied by injecting the cells more slowly. Once the entire volume of cells has been administered, the needle should also be left in place, 2 min for striatal transplant, and up to 10 min for hippocampal or cortical transplants, before being withdrawn.
3. For the cortical transplants, some basic geometry is required to calculate the insertion point and depth of insertion to reach the desired placement of the needle tip (**Fig. 2**). The formula to determine the depth of transplantation, b, from the cortical surface, and for a given angle $\boldsymbol{\Theta}$ is:

$$c(\cos\boldsymbol{\Theta}) = b.$$

   Similarly, the formula to determine the lateral extent of the transplant needle tip from the cortical insertion point is:

$$c(\sin\boldsymbol{\Theta}) = a.$$

   At times it may be easier to find the transplant target site, determine a preferred course for the needle and calculate the angle of insertion, $\boldsymbol{\Theta}$, with the formula:

$$\sin\boldsymbol{\Theta} = a/c.$$

4. When single bilateral injections are made in the mouse spinal cord, we prefer to hand drill burr holes through the vertebra. In our hands, we get a lower incidence

of hemorrhaging. If multiple injections are done or we are transplanting in the rat, then we perform a laminectomy. The procedure is similar through the removal of the erector spinae muscle. After that, the spinous process is cut, the lamina is cut on each side, and the top of the vertebra is removed.

5. For short-term surgeries that do not require a stereotaxic instrument, the gas anesthetic Isoflurane may be used. The depth of anesthesia is easily maintained, and the animals recover from anesthesia faster.
6. If hemorrhaging occurs as a result of cutting the dura and pia mater, the extent of damage should be determined with a microscope or other optic magnification. If the hemorrhage is extensive, surgery should be stopped and the animal must be sacrificed. In the case of minimal damage, surgery can be continued. The animal should be monitored extensively post-surgery to ensure that there is no surgery-related decrement in motor performance.
7. In the mouse, the femoral and tail veins are too small to practically inject the cells. In the rat, it may be more appropriate to transplant an animal that has had a middle cerebral artery occlusion (MCAO) to induce stroke, not through the jugular vein but through either the femoral or tail vein. The reason for this preference is that 24 h after MCAO, the neck region may be swollen and the other blood vessels around the carotid may be more reactive than they would be in a naïve animal. Similarly, in an animal with a spinal cord injury that is dragging its hind limbs around the cage, a femoral artery injection may increase the risk of infection around the incision site.
8. The procedure for cannulating both veins and arteries are much the same in the rat and the mouse. The main difference beyond the obvious size distinction is the consistency (strength, elasticity) of the tissue; mouse tissue is not as sturdy as rat and requires a more delicate touch.
9. One of the biggest concerns with vascular administration of the cells is not introducing an air embolus into the bloodstream. Extra care must be taken in loading the syringe and needle to ensure there are no air bubbles and further, when the needle is placed in the vein or artery, the tip of the needle should be placed in the vessel so that the surgeon can see the suspension as it leaves the needle tip. If, in spite of all precautions, an air bubble is injected, if the surgeon is able to see the tip and is delivering the suspension slowly, then it is possible to draw the air bubble back into the syringe. In most cases, a small air bubble can be tolerated in the rat without complications, but this situation is more critical in the mouse.
10. The tail vein is largest at the base of the tail and smallest at the tip of the tail. However, we choose our initial entry site (6 cm from the base), so that we can make a second or even third (rarely) attempt to enter the tail vein more rostrally should the first attempt fail. Should these three attempts fail, the tail vein on the other side of the tail is also available.
11. There should be no resistance felt when depressing the plunger to deliver the cells into the tail vein. A small swelling may be visible where the needle lies in the tail vein as the bolus is delivered, usually indicating that the injection is

proceeding faster than the capacity of the vein. However, if a prominent swelling is seen as the injection is being made, or if resistance to depressing the plunger is felt, the needle is no longer in the vein and the injection is being made in the substance of the tail.

12. The arteries are stronger and more elastic than the veins and maintain their size and shape even with extensive working. The veins, on the other hand will collapse with extensive manipulation. The best approach to isolating the vessels is to manipulate them only as much as is necessary to isolate them from surrounding nerves or connective tissue. Even so, removing excess connective tissue from the top of the vessel is desirable since it will make it much easier to make a clean hole in the vessel and place the needle directly into the lumen. If this removal is not done, the needle can end up in the connective tissue or in the wall of the vessel. If, upon delivery of the cell suspension, the vessel/connective tissue bubbles up or the delivery is not smooth and easy, then the needle is not in the lumen of the vessel. It may be possible to minimize this constrictive effect by applying a few drops of lidocaine before the vessel is isolated from the surrounding tissue.
13. One of the most troublesome post-operative issues involves the long-term use of cyclosporin. Some of the most common side effects of this treatment include dental problems that interfere with normal feeding, higher risk of opportunistic infections, and gastrointestinal problems. These latter problems can range from diarrhea to bloating and constipation, and can occur in both rats and mice. However, we have found that in the SOD1 mouse, in particular, the gas and constipation can be lethal within 3–5 d of onset. During this time, animals are anorexic, their fur becomes disheveled, and their abdomen bloated. Some researchers have reported delayed gastric emptying and delayed colonic transit time in ALS patients ***(29)***. There are also reports of small bowel bacterial overgrowth ***(30)*** that could contribute to bloating. We have found some efficacy in reversing this problem through the administration of red wine (3–5 drops twice daily on the tongue of the affected mice). The mechanism underlying this effect is not yet clear, but may be related to antioxidant properties of the wine ***(31)***, modulation of intestinal motility ***(32)***, or its antibacterial activities ***(33)***. We are also exploring other pharmacological methods to speed up the transit of nutrients through the gastrointestinal tract.

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Index

A

Alkaline phosphatase, 72, 191, 279, 309, 312
Alzet pump (*see also* Osmotic pump), 266
Antiadhesive solution, 17, 19, 24
Antibiotics, 10, 127, 256, 359
Antigen retrieval method, 274
Apomorphine test, 266
Apoptosis (*see also* Neuronal cell death), 247
Astrocyte monolayer, 25, 26, 47
A2B5, 30, 32, 33, 35, 36, 42, 288
Autoradiography, 105, 109, 112, 330

B

β-galactosidase, 10, 162–163, 167–168, 309
β-III-tubulin, 7, 9, 32, 33, 35, 36, 56, 58, 63, 81, 150, 315
Bone marrow (bone marrow stromal cells, BMSC), 79–87
  co-culture of bone marrow with fetal midbrain cells, 85
  isolation, 82–84
  neural induction in vitro, 85
Bone morphogenetic protein (BMP), 9, 11,
Brain-derived neurotrophic factor (BDNF), 5, 79, 84, 152
Bromodeoxyuridine (BrdU, BUdR, BrdUrd), 104–105, 108–109, 112, 150, 153, 161, 168, 267, 269–270, 285-289, 309, 330, 350
  administration
    intraparenchymal injection, 350
    intraperitoneal infusion, 286
    intraperitoneal injection, 105–108, 269, 286, 350
    intraventricular infusion, 287
    oral ingestion 286
Buoyant density 71

C

Cell
  generation, 101–112
  proliferation, 101–112
  viability, 323–324, 325, 343, 346, 347–348, 353
Chick embryo extract (CEE), 29, 33, 38, 39
Cholera toxin B, 360–361,
Chromogranin, 56, 58, 63
Ciliary neurotrophic factor, 152
Clonal analysis, 115–122
  limiting dilution, 116-119
  methylcellulose assay, 117, 119–120
  subcloning procedure, 117, 120
Colcemid, 57, 61,
Cryopreservation, 115–122

D

Desmin, 56, 58, 63,
Differential display (DD), 213–221

cloning DD-PCR (polymerase chain reaction) products/ cDNAs, 215, 220
differential display PCR (DD-PCR), 214, 216,
DD-PCR sequencing gel electrophoresis, 214, 217
Northern/reverse Northern hybridizations, 215, 219–220
reamplification of DD-PCR products, 214, 217–219
reverse transcription reaction (DD-RT), 214, 216
RNA extraction and purification, 214–216
Differential gene expression, 197–211
polymerase chain reaction, 198–199, 202–203
purification and cloning of PCR products, 199, 204–207
reverse Northern blots, 199, 207–211
reverse transcription, 198, 201–202
RNA extraction and purification, 198–201
DiI (fluorescent dye, 1,1′- dioctadecyl -3,3,3′,3′-tetramethylindocyanine perchlorate), 308–309, 329, 360–361
Dimethyl sulfoxide (DMSO), 6, 31, 69, 73, 85, 211, 239, 261, 359
Dispase, 38
Doublecortin (Dcx, Migratory neuronal marker), 288, 291–296

E

Epidermal growth factor (EGF, *see* Growth factors)
Electron microscopy (EM, *see also* LacZ), 157–172
β-gal immunohistochemistry, 162–163, 167–168
tissue preservation and processing for EM, 163–164, 166, 168–172
transfection of cells with the reporter bacterial lac-Z gene, 161–162, 164–165
X-gal histochemical reaction, 162, 165–167,
Electrophysiology, 179–186
recordings from cultured neural stem cells (NSCs), 181, 183–186
recordings from slice preparations, 180–181, 182–183
recordings from transplanted NSCs, 180–183
Electroporation, 223–231
apparatus, 223, 226
DNA injection, 226
expression plasmid, 224
in ovo, chick embryo, 224–225, 227–228
in vitro, embryonic neural tube, 225–226, 228–231
Embryoid body, 3
Embryonic stem (ES) cells, 3–12
ES cell culture 4–5, 6–7
growth medium, 5
neural differentiation, 5, 7–8,
neuronal differentiation, 9–10
Endogenous pools of NSCs, in general,
activation of NSCs, 265–271
differentiation of NSCs, 265–271, 291–297
identification of newborn cells, 101–112, 283–290

Musashi-1 immunohistochemistry, 273–281
neuroanatomical tracing of neuronal projections (*see also* fluorogold), 299–304
Engineering
growth-factor expanded NSCs, 240
immortalized NSC lines, 241
Enhanced green fluorescent protein (EGFP, reporter gene), 309–310, 316, 331
Enzymatic separation, 30, 31, 70
Epithelium, pseudostratified ventricular (PVE), 101, 103, 110
estimation of P and Q fractions, 105–108
growth modes, 103–104,
Ethidium bromide, 57, 61, 126, 130, 201, 248, 251
Exponential expansion, 103

F

FACS (*see* Fluorescence activated cell sorting)
Fibronectin, 10, 30, 36, 81, 184
Fluorescence-activated cell sorting (FACS), 45–46, 140, 312
FISH (*see* fluorescence *in situ* hybridization)
Fluorescein diacetate/propidium iodide (*see* Cell viability), 343, 347–348
Fluorescence *in situ* hybridization (FISH), 189–194
in situ hybridization, 193–194
preparation of probes by DIG labeling, 193
preparation of tissue, 192–193
probes, 190–192
Fluorogold (FG, retrograde marker), 299–303

G

Galactocerebroside, anti GalC, 42, 45, 46, 150
Gene misexpression (*see* electroporation)
Giemsa stain, 57, 61
Glial fibrillary acidic protein (GFAP, an astrocytic marker), 9, 42, 47, 49, 51, 53, 63, 79, 81, 150, 151, 278, 288, 315
Glial-restricted precursors (GRP), 30
Green fluorescent protein (GFP) transgenic mouse, 10, 21, 72, 155, 223, 229, 231
Growth factors
basic fibroblast growth factor (bFGF), 5, 7, 9, 30, 35, 56, 57, 59, 68, 72, 73, 117, 120, 150, 152, 266–268
epidermal growth factor (EGF), 57, 59, 79, 117, 120, 150, 152, 266–269
nerve growth factor (NGF), 57
platelet-derived growth factor, 31, 36
stock solution 17, 19, 31,
Guanidine cyanide method, 22

H

Hoechst dye
33258, bisbenzimide, DAPI, Feulgen, nuclear stain, 285, 293, 295–296, 309
33342, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2.5′-

bi-1H-benzimidazole), 309, 330-331
hNT cells (*see also* NT2N cells), 343, 346, 353, 361
hTERT (*see also* TERT), 125
Hu (early neuronal marker, RNA-binding protein), 81, 275, 276, 288, 291-296
Hyaluronidase 39, 51

I

Immortalization
f human neural crest stem cells (*see also* Neural crest stem cells), 61
of NSCs, 239-240
growth factor-expanded, 240
retroviral mediated, 239
Immunopanning, 30, 36–39
Iododeoxyuridine (IrdU, halogenated thymidine analog), 284
Isolation of stem and precursor cells from fetal tissue, 29–39
dissociation and plating of neuroepithelial (NEP) cells, 30, 33, 34
freezing NEP cells, 34, 35
maintenance and testing of undifferentiated NEP cells, 30, 33, 35
passaging NEP cells, 30–31, 33–34, 35, 39
removal of neural tubes, 33

K

Karyotype, 61, 62,

L

Labeling
cytoplasmic, 309
membrane (*see also* DiI, PKH26), 309
nuclear (*see also* Hoechst, BrdU, ^{3}H-thymidine), 309
reporter genes (*see also* lacZ, EGFP, enhanced green fluorescent protein), 309
LacZ (reporter gene), 72, 161–163, 165–168, 309, 311–313
Laminin, 5, 6, 8, 10, 11, 17, 31, 36, 68, 118, 184
Leukemia inhibitory factor (LIF), 5, 6, 7, 10
Low affinity nerve growth factor receptor (LNGFR, p75), 42, 47, 56, 57, 63

M

Mechanical dissection, 30, 70
Medium
serum-free chemically defined, 59
Microtubule-associated protein (MAP2), 58, 63, 81, 315
Musashi-1 (RNA-binding protein, marker for NSCs or progenitor cells), 81
immunohistochemistry
double immunostaining with neuronal or glial markers, 278–279
prestaining treatment, 276–277
staining procedure, 277
Migratory patterns, 110–112
MTT assay (*see also* Neuronal death), 95
Myosin, 63

N

NEP basal medium, 30, 226
Nestin, 4, 5, 32, 33, 56

Neural crest stem cells, 55–64
cytogenetic analysis, 57, 61
dorsal root ganglion cell culture, 57, 58–59
generation of immortalized human neural crest stem cells, 61
immunocytochemical staining for specific markers, 57, 61–64
retroviral-mediated gene transfer, 59–61
retroviral vector, 57
Neural determination genes
neuroD3, 4, 197
developmental control genes *myo D*, *neuro DS*, *Sox2*, 4
induction genes, noggin, 4, 197
Neural stem cells
co-culture with astrocytes, 153–154
induction of tyrosine hydroxylase expression, 154-155
in vitro assays for differentiation, 149–155
Neuroanatomical tracing (*see* Fluorogold)
Neuroepithelial cells (NEP), 29
Neuroepithelium, telencephalic, 101
Neurofilament proteins, 56, 58, 81, 315
Neuronal cell death (assays, markers)
DNA laddering 250–251, 259–260
electronic cell counting, 250, 258
MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), 95, 250, 258–259, 261
Neuron-restricted precursors (NRP), 29
Neurospheres, 8–9, 15–26,
generation from acutely dissociated subependymal zone, 16–17, 18–20
gene analysis, 18, 22–23
false, 23
immunolabeling, 17, 20–21
postmortem tissue as a source, 16
ultrastructural analysis, 17–18, 21–22
Neurotrophin-3 (NT-3), 9, 30, 31, 35, 36
NT2 cells (human teratocarcinoma cell line, precursor cells for NT2N neurons, hNTs), 361
NT2N cells (neurons derived from NT2/D1 clone through retinoic acid exposure, also called hNT cells), 361

O

O4 (oligodendrocyte marker), 42, 47, 288, 315
Olfactory ensheating glia (OEG), 49–54
cytotoxic elimination of fibroblasts, 51, 53
dissection of the olfactory epithelium, 50–52
single cell suspension, 51–52
verification of OEG phenotype, 51, 53
Olfactory ensheating cells (OEC), 41–47
dissection and degradation of olfactory bulb, 44–45
FACS purification of OEC, 45–47
preparation of astrocyte conditioned media, 42–44
purification of OEC from the olfactory bulb, 42
Osmotic pump (Alzet)
implantation, 266, 268–270
preparation, 266, 268

P

Papain, 38
Parkinson animal model, 265–266
Peripherin, 58
PKH26, fluorescent dye, 85, 86, 309, 329–330, 343, 348–350
Poly-L-lysine 31, 33, 36, 42, 43, 52, 53, 57, 62
Poly-ornithine 5, 8, 9, 17, 68, 69, 72, 73, 74, 184
Polysialic acid NCAM (E-NCAM, cell surface antigen), 30, 31, 32, 35, 36

R

Retrovirus-mediated gene transfer, 59–61
Rosa 26 (*see also* Transplantation, other labeling strategies, Transgenic animals), 10, 155, 331–332

S

Scaffolds, degradable, 89–96
  polyglycolic acid (PGA), polylactic acid (PLA) and their copolymers poly(lactic-co-glycolic acid, PLGA), 89–91
  seeding
    static, 93–94
    dynamic, 94–95
  sterilization, 93
  storage, 92–93
Serum-free media components, 152
S-100, 49, 53, 56, 58, 63, 81
Soybean trypsin inhibitor, 38, 42
S-phase labeling methods (*see also* bromodeoxyuridine, tritiated thymidine), 101–112
Spinal cord derived neural stem cells 67–76
  differentiation, 74
  enzymatic digestion, 70–71
  immunocytochemical analysis, 74–75
  isolation, 68, 72–73
  passaging, freezing, reculturing, 68, 73–74
  Percoll gradient purification, 71
  tissue dissection, 69–70
Synthetic materials (*see* scaffolds)

T

Telomerase activity assays, 125–134
Telomerase assays, 137–147
  standard TRAP (telomeric repeat amplification protocol) assay, 140, 142–144
  TRAP assay for single cells or small numbers of cells, 140–141, 144–147
TERT (telomere reverse transcriptase), 125
  cellular localization of TERT protein-immunostaining, 127, 131–132
  immunoblot analysis, 127, 130–131
  overexpression of TERT, 127,132
  RT-PCR analyses of TERT and telomere-associated protein mRNA levels, 126, 128–130
  suppression of TERT expression and activity, 127, 132–134
Tetracycline-based regulatory system, 233–235
Transfection of packaging cell line, 236–239
Transplantation of NSCs

brain target sites
  adult brain, 357–374
  embryonic brain, 327–340
  neonatal brain, 341–356
NSCs labeling
  identification after grafting, 307–318
  in vitro, 308–311, 329–330, 348–350
  with reporter genes, 311–313, 331
  other strategies (allogeneic grafts, gender-specific grafts, transgenic animals, xenografts), 313–314, 331–332
optimizing NSCs grafting, 319–326
  cell number and viability, 323–324, 347–348
  engraftment parameters, 324–325
  injection system, 322–323
  micropipet preparation, 320–322
routes of NSCs delivery
  intraparenchymal
    cortex, 363
    hippocampus, 362–363
    spinal cord, 364–365
    striatum, 351, 360–362
    subventricular zone, 351
  vascular
    arterial, 367–368
    femoral vein, 366–367
    jugular vein, 366
    tail vein, 365–366
    transuterine, 332–333
  post transplantation care
    neonates, 351-353
    adults, 359–360, 368–371
TRAP (*see* Telomerase assays)
Tritiated thymidine, ^{3}H-TdR, 102–103, 105–108, 110–112, 309, 310, 311, 330
Trypan blue (*see also* Cell viability), 117, 118, 319, 323–324, 333, 335, 346, 360
Trypsin/EDTA solution, 5, 6, 9, 10, 11, 17, 18, 24, 26, 31, 33, 34, 35, 43, 51, 68, 72, 73, 84, 153, 230, 247, 249, 250, 256, 342
Tyrosine hydroxylase (TH), 150, 154–155, 266–268

U

Umbilical cord blood (UCB) cells, 84–85

V

Vimentin, 9, 56

Y

Y chromosome, 191, 313

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Neural Stem Cells

Methods and Protocols

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Over the last decade, advances in neural stem cell research have raised the hope that one day cellular therapy will not only replace missing neurons, but also replenish absent chemical signals, metabolites, enzymes, neurotransmitters, or other missing or defective components from the diseased or injured brain. In *Neural Stem Cells: Methods and Protocols,* internationally recognized experts from academic, clinical, and pharmaceutical laboratories describe in great detail the most frequently used cellular, molecular, and electrophysiological methods to isolate, characterize, and utilize neural stem cells. These readily reproducible techniques introduce the various sources of stem/progenitor cells, provide a wide range of conditions for their culture, and make it possible to define their properties in culture. Additional techniques are designed to help researchers identify endogenous stem cells as well as exogenous stem cells after transplantation in the brain. The protocols range from the simplest methods of isolation and characterization of neural cell properties to such very sophisticated methods as characterizing gene expression, telomerase assays, and cell cycle kinetics. Each is written by an investigator who has used the method extensively, and includes step-by-step instructions, tips on avoiding pitfalls, and invaluable notes that make all the difference to successful experimental outcomes

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