

the **heart** of neuroscience

Table of Contents

Introduction.....	4
Neural Stem Cells and Neural Development.....	4
Neural Cell Types in Neurological Diseases.....	7
Neural Cell Culture and Differentiation.....	10
Culturing Human Neural Stem Cells.....	10
Culturing Rat Fetal Neural Stem Cells.....	16
Xeno-free Culture of Neural Stem Cells.....	21
Differentiating Neural Stem Cells into Neurons and Glial Cells.....	24
Differentiating Glial Precursor Cells into Astrocytes and Oligodendrocytes.....	33
Derivation and Culture of Cortical Astrocytes.....	36
Isolation, Culture, and Characterization of Cortical and Hippocampal Neurons.....	39
Derivation of Dopaminergic Neurons (from Human Embryonic Stem Cells).....	45
Derivation and Culture of Dopaminergic Neurons (from Midbrains of Rodents).....	55
Cryopreserving Neural Stem Cells.....	60
Cryopreservation and Recovery of Mature Differentiated Neural Cells.....	63
Cell Analysis.....	65
Cell Viability Assays for Neural Stem Cells.....	65
Markers for Characterizing Neural Subtypes.....	68
Surface Marker Analysis by Flow Cytometry.....	69
Immunocytochemistry.....	72
Electrophysiology.....	75
Molecular Characterization.....	77
PCR Primers for Molecular Characterization of Neural Subtypes.....	77
RNA Isolation and cDNA Preparation from Neural Stem Cells.....	78
Characterizing Neural Cells by qPCR.....	82

Transfection.....85

 Transfecting Neural Cells Using the Neon® Transfection System.....85

 Lipid-Mediated Transfection of Human Astrocytes.....93

Using Neural Cells for Cell Therapy.....99

 Modeling Parkinson’s Disease in Rats.....99

Appendix.....101

 Life Technologies Products.....101

 Resources for More Information.....106

 TechnicalSupport.....108

Introduction

Neural Stem Cells and Neural Development

Overview

It has long been thought that the adult mammalian nervous system was incapable of regeneration after injury. However, recent advances in our understanding of stem cell biology and neuroscience have opened up new avenues of research for developing potential treatments for incurable neurodegenerative diseases and neuronal injuries. Because stem cells have the capacity to self-renew and generate differentiated cells, stem cell replacement therapy for central and peripheral nervous system disorders and injuries strives at repopulating the affected neural tissue with neurons and other neural cells. One of the main strategies towards this end aims to recapitulate the normal development of the nervous system by activating the endogenous regenerative capacity of the neural stem cells or by transplanting neural or embryonic cells.

This chapter defines the key concepts in stem cell biology with respect to the nervous system, presents an overview of neural development, and summarizes the involvement of neural cell types in specific neural diseases.

Stem Cells

The classical definition of a stem cell requires that it has the capacity to self-renew and that it possesses potency. **Self-renewal** is defined as the ability of the stem cell to go through multiple cycles of cell division while maintaining its undifferentiated state (i.e., to generate daughter cells that are identical to their mother). **Potency** is the ability of the stem cell to differentiate into specialized cell types.

Pluripotent vs. Adult Stem Cells

A stem cell can divide to generate one daughter cell that is a stem cell, maintaining its capacity for self-renewal and potency, and another daughter cell that can further divide produce differentiated cells. While some **pluripotent stem cells**, including Embryonic Stem Cells (ESC) and Induced Pluripotent Stem Cells (iPSCs), have the capacity for multilineage differentiation to construct a complete, viable organism (i.e., they are totipotent), **adult stem cells** can generate only one specific lineage of differentiated cells to reconstitute tissues or organs.

Neural Stem Cells

Neural stem cells (NSC) are stem cells in the nervous system that can self-renew and give rise to differentiated progenitor cells to generate lineages of neurons as well as glia, such as astrocytes and oligodendrocytes. This characteristic is known as **multipotency**. NSCs and neural progenitor cells are present throughout development and persist in the adult nervous system. Multiple classes of NSCs have been identified that differ from each other in their differentiation abilities, their cytokine responses, and their surface antigen characteristics.

Rationale for Studying Neural Stem Cells

Neurological disorders, especially neurodegenerative disorders, are at the top of the list of diseases that have been suggested as targets for stem cell therapy. Despite the enthusiasm for the use of stem cells in neurological disorders, a thorough characterization of NSCs and a better understanding of neural patterning and the generation of all three major cell types that constitute the central nervous system (i.e., neurons, astrocytes, and oligodendrocytes), as well as the microenvironments that can support them, is crucial to increase the likelihood of clinical success.

Stem Cells and Cancer

An exciting finding has been the discovery that many cancers may be propagated by a small number of stem cells present in the tumor mass. This was first described in breast cancers and subsequently in a variety of solid tumors. Several reports have suggested that cancer stem cells can be identified in the nervous system as well, and that these cells bear a remarkable similarity to neural stem cells present in early development. Likewise, cells resembling glial progenitors have been isolated from some glial tumors suggesting an intriguing link between developmental and cancer biology

Neural Development

The development of the central nervous system (CNS) is initiated early in the development by the induction of NSCs and neural progenitor cells; this stage in development is called **neural induction**. By studying neural induction and neural development, we can determine the various factors that stimulate or inhibit the differentiation of NSCs and the requirements of these NSCs and their offspring for survival and proper function.

Stages of Neural Development

The nervous system is one of the earliest organ systems that differentiate from the blastula stage embryo. This differentiation can be mimicked in culture and NSCs can be derived from human ESC cultures over a period of 2–3 weeks. *In vivo*, the primitive neural tube forms by approximately the fourth week of gestation by a process termed primary **neurulation**, and neurogenesis commences by the fifth week of development in humans.

Separation of PNS and CNS

During neurulation, the neuroectoderm segregates from the ectoderm and the initially formed neural plate undergoes a stereotypic set of morphogenetic movements to form a hollow tube. The neural crest which will form the peripheral nervous system (PNS) segregates from the CNS at this stage. The neural crest stem cell generates the sympathetic and parasympathetic systems, the dorsal root ganglia and the cranial nerves, as well as the peripheral glia including Schwann cells and enteric glia. In addition to neural derivatives, the cranial crest generates craniofacial mesenchyme that include bone cartilage, teeth, and smooth muscle, while both cranial and caudal crests generate melanocytes. Placodes, which will form a subset of the peripheral nervous system and the cranial nerves, arise at this stage as well. These populations appear distinct from the CNS stem cell though similar media and culture conditions can be used to propagate them for limited time periods.

Stem Cells in Ventricular Zone

Stem cells that will generate the CNS reside in the ventricular zone (VZ) throughout the rostrocaudal axis and appear to be regionally specified. These stem cells proliferate at different rates and express different positional markers. The anterior neural tube undergoes a dramatic expansion and can be delineated into three primary vesicles: the forebrain (prosencephalon), the midbrain (mesencephalon), and the hindbrain (rhombencephalon). Differential growth and further segregation leads to additional delineation of the prosencephalon into the telencephalon and diencephalon, and delineation of the rhombencephalon into the metencephalon and myelencephalon. The caudal neural tube does not undergo a similar expansion, but increases in size to parallel the growth of the embryo as it undergoes further differentiation to form the spinal cord. The ventricular zone stem cells appear homogenous despite the acquisition of rostrocaudal and dorsoventral identity, but differ in their differentiation ability and self-renewal capacity. Specific regions of the brain may have relatively distinct stem cell populations, such as the developing retina and the cerebellum.

Stem Cells in Subventricular Zone

As development proceeds, the ventricular zone is much reduced in size and additional zones of mitotically active precursors appear. Mitotically active cells that accumulate adjacent to the ventricular zone are called the subventricular zone (SVZ) cells. The SVZ later becomes the subependymal zone as the ventricular zone is reduced to a single layer of ependymal cells. The SVZ is prominent in the forebrain and can be identified as far back as the fourth ventricle, but it cannot be detected in more caudal regions of the brain; if it exists in these regions, it likely consists of a very small population of cells. An additional germinal matrix derived from the rhombic lip of the fourth ventricle, called the external granule layer, generates the granule cells of the cerebellum.

Like the VZ, the SVZ can be divided into subdomains that express different rostrocaudal markers and generate phenotypically distinct progeny. Distinct SVZ domains include the cortical SVZ, the medial ganglion eminence, and the lateral ganglion eminence. The proportion of SVZ stem cells declines with development and multipotent stem cells are likely to be present only in regions of ongoing neurogenesis (e.g., anterior SVZ and the SVZ underlying the hippocampus) in the adult CNS. At this stage, marker expression is relatively heterogeneous. Other relatively less characterized stem cells have also been described.

Neural Precursor Cells

Neural stem cells do not generate differentiated progeny directly but rather generate dividing populations of more restricted precursors analogous to the blast cells or restricted progenitors described in the hematopoietic lineages. These precursors can divide and self-renew, but they are located in regions distinct from the stem cell population and can be distinguished from them by the expression of cell surface and cytoplasmic markers and their ability to differentiate. Several such classes of precursors have been identified, including neuronal precursors, bi- and tri-potential glial precursors that generate astrocytes and oligodendrocytes, as well as unipotent astrocyte or oligodendrocyte precursors. Other precursors such as a neuron-astrocyte precursor may also exist and the same precursor may have multiple names. Such precursors can be distinguished from stem cells by their marker expression, ability to differentiate and time of development.

Neural Cell Types in Neurological Diseases

Summary

The table below lists some of the neurological disorders that have been studied and modeled in the laboratory and the cell types involved.

Neural disease	Experimental model	Cell type	Growth factor	Progenitor cell	Marker	Mature marker	Trans-plantation	Reference
Spinal cord injury	Transplantation of OPC into demyelination model	Oligo-dendrocyte	EGF, bFGF, RA	OPC	OLIG1, A2B5, SOX10, NG2	GalC, RIP, O4	Yes	Keirstead et al., 2005
Multiple sclerosis	Demyelinated axons, co-cultured with rat hippocampal neurons	Oligo-dendrocyte	EGF, bFGF, PDGF, RA	OPC	PDGFR, A2B5, NG2	O4, O1, MBP, PLP	No	Kang et al., 2007
	Remyelination models	Oligo-dendrocyte	RA, EGF, bFGF, Noggin, Vitamin C, Mouse laminin	OPC	PDGFR, NG2, OLIG1/2, SOX10	O4, O1, MBP, PL	Yes	Izrael et al., 2007
Amyotrophic lateral sclerosis and spinal muscular atrophy	Transplantation of motoneuron progeny into the developing chick embryo	Motoneuron	BDNF, GDNF, AA, RA, SHH, Noggin	Motoneuron Progenitor	BF1, HOXB4, NKX6-1/6-2, OLIG1/2	NKX6-1, OLIG2, NGN2, ISL1, ChAT, VACHT, HB9, LHX3, HOX	Yes	Lee et al., 2007
	<i>in vitro</i> studies only	Motoneuron	bFGF, RA, SHH, BDNF, GDNF, IGF-1	Motoneuron Progenitor	OLIG1/2, NKX6-1/6-2, NGN2	NKX6-1, OLIG2, NGN2, ISL1, ChAT, VACHT, HB9, Synapsin	No	Li et al., 2005
Parkinson's disease	Not applicable	DA neuron	SHH, FGF8, BDNF, AA, TGF β , TGF-3	DA precursor	PAX2, PAX5, LMX, EN1	MAP2, TH, AADC, VMAT, NURR1, PTX3	No	Perrier et al., 2004
	<i>in vitro</i> drug screening	DA neuron	FGF2 or FGF8, SHH, BDNF, GDNF, cAMP, AA	DA precursor	EN1, OTX2, WNT1, PAX2, GBX2	TH, GABA, EN1, AADC	No	Yan et al., 2005
	Transplantation into the neostriata of 6-hydroxydopamine-lesioned Parkinsonian rats	DA neuron	FGF2, FGF8, SHH, BDNF, GDNF, FBS	DA precursor	EN1, PAX2, OTX2	TH, TUJ-1	Yes	Roy et al., 2006
	Transplantation into the striatum of hemi-Parkinsonian rats	DA neuron	SHH, FGF8, BDNF, GDNF, AA, IGF-1	DA precursor	PAX2, EN1, NURR1, LMX1B	TH, EN1, AADC	Yes	Park et al., 2005

OPC: oligodendrocyte progenitor cells; **DA:** dopaminergic.

Neural disease	Experimental model	Cell type	Growth factor	Progenitor cell	Marker	Mature marker	Trans-plantation	Reference
Glial related diseases	Astrocyte related disease	Astrocyte	Cyclopamine, human astrocyte medium	—	—	GFAP, S100, GLAST, BDNF, GDNF	No	Lee et al., 2006
CNS/PNS diseases	Peripheral and central nervous system neurons	Peripheral sensory neurons	Noggin, NGF	Neural precursor	NCAM, TUJ-1, SNAIL, dHAND, SOX9	Peripherin, BRN3, TH, TRK-A	No	Brokhman et al., 2008
Macular retinal de-generation	Not applicable	Retinal pigmented epithelium	Noggin, Dickkopf-1, IGF-1	Retinal progenitor	RX, PAX6, LHX2, SIX3	RPE-65	No	Lambda et al., 2006
Huntington's disease	—	Striatal medium spiny neuron specification	—	—	Islet1, DARPP-32, mGluR1, and NeuN	—	—	Molero et al., 2009

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Neural Cell Culture and Differentiation

Culturing Human Neural Stem Cells

Summary

Neural stem cells (NSC) are valuable resources because of their ability to differentiate into neurons and glial cells with applications in neuroscience and clinical use for treatment of neurodegenerative disease and neurological disorders. NSC are obtained by isolation from tissue, or differentiated from pluripotent cells. This chapter describes methods for expanding human NSC in cell culture and their subsequent characterization.

Required Materials

Cells • Human neural stem cells (e.g., Cat. no. N7800-100)

Reagents • StemPro[®] NSC SFM (Cat. no. A10509-01)
• β -Mercaptoethanol (Cat. no. 21985)
• GlutaMAX[™]-I (Cat. no. 35050)
• CELLstart[™] CTS[™] (Cat. no. A10142-01)
• Fibronectin (Cat No. 33016-015)
• Water, distilled (Cat. no. 15230)
• Dulbecco's Phosphate-Buffered Saline (D-PBS) without Ca^{2+} and Mg^{2+} (Cat. no. 14190)
• Dulbecco's Phosphate-Buffered Saline (D-PBS) (Cat. no. 14040)
• StemPro[®] Accutase[®] Cell Dissociation Reagent (Cat. no. A11105)
• TrypLE[™] Express Stable Trypsin Replacement Enzyme (Cat. no. 12604-013)

Preparing Media

StemPro® NSC SFM Complete Medium

StemPro® NSC SFM complete medium consists of KnockOut™ D-MEM/F-12 with StemPro® Neural Supplement, bFGF, EGF, and GlutaMAX™-I. Complete medium is stable for 4 weeks when stored in the dark at 2–8°C.

To prepare 100 mL of complete medium:

1. Reconstitute bFGF and EGF with 0.1% BSA solution (in KnockOut™ D-MEM/F-12) at a concentration of 100 µg/mL. You will need 20 µL of each per 100 mL of complete medium. Freeze unused portions in aliquots.
2. Mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Final concentration	Amount
KnockOut™ D-MEM/F-12	1X	97 mL
GlutaMAX™-I Supplement	2 mM	1 mL
bFGF (prepared as 100 µg/mL stock)	20 ng/mL	20 µL
EGF (prepared as 100 µg/mL stock)	20 ng/mL	20 µL
StemPro® Neural Supplement	2%	2 mL
You may observe a white precipitate when thawing StemPro® Neural Supplement; this precipitate will disappear when the supplement is completely thawed or dissolved.		

Preparing Matrix

For culture of adherent cultures, use either Fibronectin or CELLstart™ CTS™ to prepare a matrix for coating your plates.

Coating Culture Vessels with CELLstart™ CTS™

1. Dilute CELLstart™ CTS™ 1:100 in D-PBS with calcium and magnesium (i.e., 50 µL of CELLstart™ CTS™ into 5 mL of D-PBS).

Note: CELLstart™ CTS™ should not be frozen, vortex or exposed to vigorous agitation due to potential gel formation.

2. Coat the surface of the culture vessel with the working solution of CELLstart™ CTS™ (14 mL for T-75, 7 mL for T-25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish).
3. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO₂ for 1 hour.
4. Remove the vessel from the incubator and store it until use. Remove all CELLstart™ CTS™ solution immediately before use, and fill the vessel with complete StemPro® NSC SFM.

Note: You may coat the plates in advance and store them at 4°C, wrapped tightly with Parafilm®, for up to 2 weeks. Do not remove CELLstart™ CTS™ solution until just prior to use. Make sure the plates do not dry out.

**Coating Culture Vessels
with Fibronectin**

1. Dilute Fibronectin in distilled water to make a 1-mg/mL stock solution.
2. Store working solution at -20°C until use.
3. Add stock solution to D-PBS to make a working solution of 20 $\mu\text{g}/\text{mL}$.
4. Add enough working solution to cover the surface of the culture vessel (10 mL for T-75, 2.5 mL for 60-mm dish, 1.5 mL for 35-mm dish).
5. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO_2 for 1 hour.
6. Remove the vessel from the incubator and store it until use. Remove Fibronectin solution immediately before use, and fill the vessel with complete StemPro[®] NSC SFM.

Note: You may store the Fibronectin-treated plates at 4°C , wrapped tightly with Parafilm[®], for up to 2 weeks. Ensure that plates do not dry out. There is no washing step needed and use the dish directly after aspiration.

Culturing Neural Stem Cells

Neural stem cell (NSCs) populations can be expanded from frozen stocks and grown in StemPro[®] NSC SFM complete medium as adherent cultures, or as suspension cultures. In either environment, change the spent culture medium every other day. When the cells in adherent culture reach >90% confluency, they are ready to be passaged. When the neurospheres in suspension culture become > 3.5 mm in diameter, they are ready to be passaged. Cells cultured during expansion can be frozen down to create additional frozen stocks of higher passage number.

**Thawing Frozen Neural
Stem Cells**

1. Prepare 10 mL of 1X KnockOut[™] D-MEM/F-12 and warm to 37°C .
2. Transfer vial of frozen NSC from nitrogen tank to water bath. It is important to make the transfer immediately to prevent crystal formation.
3. Transfer thawed cells into a 15-mL tube and add warmed 1X KnockOut[™] D-MEM/F-12 to 10 mL.
4. Spin down the thawed cells by centrifugation at 1,000 rpm for 4 minutes. Aspirate and discard the supernatant.
5. Resuspend the cells in StemPro[®] NSC SFM complete medium and plate on a CELLstart[™] CTS[™] or Fibronectin-coated plate at high density (1×10^5 cells/ cm^2).

Note: Viability of thawed cells should be ~80% if they were frozen following the cryopreservation protocol described here.

Passaging Neural Stem Cells (Adherent Culture)

1. Aspirate the medium and wash with D-PBS without Ca^{2+} and Mg^{2+} .
2. Add 1 mL of TrypLE™ Express or StemPro® Accutase® to the culture vessel.

Note: The monolayer lifts off from the culture dish within 30 seconds of application of TrypLE™ Express or StemPro® Accutase®.
3. Gently pipette to loosen monolayer into a single cell suspension. Neutralize the treatment by adding 4 mL of medium. Do not treat the cells for longer than 3 minutes after addition of TrypLE™ Express or StemPro® Accutase®.
4. Spin down the cells by centrifugation at 1,200 rpm for 4 minutes. Aspirate and discard the supernatant.
5. Resuspend the cells in StemPro® NSC SFM complete medium.
6. Count the cell number using a hemacytometer.
7. Plate cells in fresh medium on a CELLstart™ CTS™ - or Fibronectin-coated plate at a density of 1×10^4 – 1×10^5 cells/cm², or split the cells at a 1:4 ratio.

Passaging Neural Stem Cells (Suspension Culture)

1. Transfer medium containing neurospheres into a 15- or 50-mL conical tube.
2. Leave the tube at room temperature and allow the neurosphere to settle to the bottom of tube. Alternatively, spin down the cells by centrifugation at 500 rpm ($200 \times g$) for 2 minutes.
3. Aspirate the supernatant carefully, and leave the neurospheres in a minimum volume of medium.
4. Wash the neurospheres with 10 mL D-PBS without Ca^{2+} and Mg^{2+} , aspirate the D-PBS supernatant carefully, and leave the neurospheres in a minimum volume of D-PBS.
5. Add 1 mL of TrypLE™ Express to the spheres and gently triturate neurospheres using a Pasteur pipette to create a single cell suspension.
6. Neutralize the treatment by adding 4 mL of medium.
7. Spin down the cells by centrifugation at 1,200 rpm for 4 minutes. Aspirate and discard the supernatant.
8. Resuspend the cells in StemPro® NSC SFM complete medium.
9. Count cell number using hemacytometer.
10. Seed the cells in fresh medium in a suspension dish (a non-coated flask can be used) at a density of 200,000 cells/mL.

Cryopreserving Neural Stem Cells

1. Aspirate the medium and wash with D-PBS without Ca^{2+} and Mg^{2+} .
2. Add 1 mL of TrypLE™ Express to the culture vessel.
3. Gently pipette to loosen monolayer into a single cell suspension. Neutralize the treatment by adding 4 mL of medium. Do not treat the cells for longer than 3 minutes after addition of TrypLE™ Express.
4. Spin down the cells by centrifugation at 1,200 rpm for 4 minutes. Aspirate the supernatant.
5. Resuspend the cells in StemPro® NSC SFM complete medium at a density of 2×10^6 cells/mL.
6. Prepare freezing medium consisting of 20% DMSO and 80% medium.

Note: Freezing medium (2X) can be prepared on the day of use and stored at 4°C until use.
7. Add a volume of freezing medium equal to the amount of StemPro® NSC SFM complete medium used to resuspend the cells in a drop-wise manner.
8. Prepare 1 mL aliquots (1×10^6 cells) in cryovials and place the vials in an isopropanol chamber.
9. Put the isopropanol chamber at –80°C and transfer the vials to liquid nitrogen storage the next day.

Characterizing NSCs by Immunocytochemistry and PCR

Antibodies for NSC Characterization

Use the antibodies listed in the following table to characterize NSCs by immunocytochemistry. For details on the procedure, refer to Chapter 17, **Immunocytochemistry** (page 71).

Category	Antigen	Type	Working concentration
Neural Stem Cells	Sox1	Rabbit IgG	1:200
	Sox2	Mouse IgG	2 µg/mL
	CD133	Rabbit IgG	1:100
Proliferation	Ki67	Rabbit IgG	1:50
	EdU	Chemical	1:1,000
Isotype Control	Mouse	IgM and IgG	Do not dilute
	Rabbit	IgG	Do not dilute
	Rat	IgM and IgG	1:50

Primers for NSC Characterization

Use the primer sets listed in the following table to characterize NSC by PCR. Refer to Chapter 21, **Characterizing Neural Stem Cells by qPCR**, for details on the procedure (page 81).

Target	Primer	Sequence	T _m	Amplicon size	Intron size
Neural Stem Cells	Sox1-F	GCGGAAAGCGTTTCTTG	53.0	406	No Intron
	Sox1-R	TAATCTGACTTCTCCTCCC	50.2		
	Sox2-F	ATGCACCGCTACGACGTGA	59.3	437	No Intron
	Sox2-R	CTTTTGACCCCTCCCATT	56.0		
	Nestin-F	CAGCGTTGGAACAGAGGTTGG	58.6	389	1142
	Nestin-R	TGGCACAGGTGTCTCAAGGGTAG	60.7		
Endogenous Control	ACTB-F	ACCATGGATGATGATATCGC	58.2	281	135
	ACTB-R	TCATTGTAGAAGGTGTGGTG	54.4		

Culturing Rat Fetal Neural Stem Cells

Summary

Rat neural stem cells (NSCs) serve as a well-established model for investigating human brain development, disease processes, and treatment strategies for debilitating central nervous system (CNS) disorders. This protocol describes the *in vitro* expansion, passaging, and morphology of rat fetal NSCs in adherent or neurosphere suspension cultures.

Required Materials

- | | |
|---------------------------|--|
| Cells | <ul style="list-style-type: none"> GIBCO® Rat Fetal Neural Stem Cells (Cat. no. N7744-100) or homogenous cell preparation from 14–18 days post-coitum rat brain tissue |
| Media and Reagents | <ul style="list-style-type: none"> Dulbecco's Phosphate-Buffered Saline (D-PBS) (Cat. no. 14040) Dulbecco's Phosphate-Buffered Saline (D-PBS) without calcium or magnesium (Cat. no. 14190) StemPro® NSC SFM (Cat. no. A10509-01) StemPro® Accutase® Cell Dissociation Reagent (Cat. no. A11105-01) CELLstart™ CTS™ (Cat. no. A10142-01) Trypan blue (Cat. no. 15250) (included with the Countess®) or the LIVE/DEAD® Cell Viability Assay Kit (Cat. no. L34951) |
| Special Tools | <ul style="list-style-type: none"> Countess® Automated Cell Counter (Cat. no. C10227) or hemacytometer |

Preparing Media

Medium for Expanding Neural Stem Cells

StemPro® NSC SFM complete medium consists of KnockOut™ D-MEM/F-12 with StemPro® Neural Supplement, bFGF, EGF, and GlutaMAX™-I. Complete medium is stable for 4 weeks when stored in the dark at 2–8°C.

To prepare 100 mL of complete medium:

1. Reconstitute bFGF and EGF with 0.1% BSA solution (in KnockOut™ D-MEM/F-12) at a concentration of 100 µg/mL. You will need 20 µL of each per 100 mL of complete medium. Freeze unused portions in aliquots.
2. Mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Final concentration	Amount
KnockOut™ D-MEM/F-12	1X	97 mL
GlutaMAX™-I Supplement	2 mM	1 mL
bFGF (prepared as 100 µg/mL stock)	20 ng/mL	20 µL
EGF (prepared as 100 µg/mL stock)	20 ng/mL	20 µL
StemPro® Neural Supplement	2%	2 mL

You may observe a white precipitate when thawing StemPro® Neural Supplement; this precipitate will disappear when the supplement is completely thawed or dissolved.

Coating Culture Vessels with CELLstart™

For adherent cultures, prepare plates with CELLstart™ CTS™ as described below.

1. Dilute CELLstart™ CTS™ 1:100 in D-PBS with calcium and magnesium (e.g., 50 µL of CELLstart™ CTS™ into 5 mL of D-PBS).

Note: CELLstart™ CTS™ should not be frozen, vortexed, or exposed to vigorous agitation due to potential gel formation.

2. Coat the surface of the culture vessel with the working solution of CELLstart™ CTS™ (14 mL for a T-75 flask, 7 mL for a T-25 flask, 3.5 mL for a 60-mm dish, 2 mL for a 35-mm dish).
3. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO₂ for 1 hour.
4. Remove the vessel from the incubator and store at 4°C until use. Remove all CELLstart™ CTS™ solution immediately before use, and fill the vessel with complete StemPro® NSC SFM.

Note: You may coat the plates in advance and store them at 4°C, wrapped tightly with Parafilm, for up to 2 weeks. Do not remove CELLstart™ CTS™ solution until just prior to using the coated plates. Make sure the plates do not dry out.

Expanding and Passaging of Rat NSCs

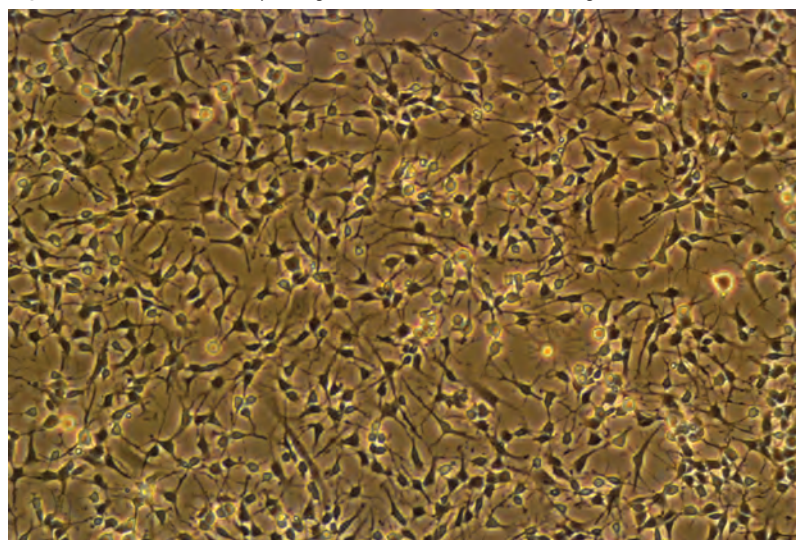
Adherent Cultures 1. Resuspend the rat fetal NSCs as follows:

- For freshly prepared rat fetal NSCs, after rinsing with D-PBS, resuspend in warmed complete StemPro® NSC SFM at a density of 1×10^7 viable cells/mL.
 - For thawed rat fetal NSCs, after determining the viable cell count, resuspend in warmed complete StemPro® NSC SFM at a cell density of 1×10^7 viable cells/mL.
2. Plate rat fetal NSCs onto CELLstart™ CTS™-coated culture vessels at a density of 5×10^4 cells/cm². See the following table for recommended seeding densities for common culture vessels.

Vessel size	Growth area	Volume of media	No. of cells
96-well plate	0.32 cm ² /well	0.1 mL	1.6×10^4
24-well plate	1.9 cm ² /well	0.5 mL	1.0×10^5
12-well plate	3.8 cm ² /well	1 mL	1.9×10^5
35-mm dish	8 cm ² /well	2 mL	4.0×10^5
6-well plate	9.6 cm ² /well	2 mL	4.8×10^5
60-mm dish	19.5 cm ²	5 mL	9.8×10^5
T-25 flask	25 cm ²	5 mL	1.3×10^6
100-mm dish	55 cm ²	10 mL	2.8×10^6
T-75 flask	75 cm ²	15 mL	3.8×10^6

3. Add the appropriate volume of cells to each culture vessel and incubate at 37°C, 5% CO₂ and 90% humidity.
4. Re-feed the rat fetal NSC cultures every 2–3 days with fresh complete StemPro® NSC SFM. The morphology of rat fetal NSCs should exhibit short stellate-like processes with uniform density (see Figure 1 below).

Figure 1 Rat fetal NSCs at passage 3 in adherent culture using StemPro® NSC SFM.



5. When cells reach 75–90% confluency (3–4 days after seeding), the rat fetal NSC cultures are ready to be passaged.
6. Rinse the culture vessel once with D-PBS without calcium and magnesium, then remove the medium.
7. Add pre-warmed StemPro® Accutase® and let the cells detach from the culture surface (within approximately 30 seconds).
8. After detachment, gently pipet the cells up and down to break the clumps into a uniform cell suspension and add four volumes of complete StemPro® NSC SFM to the culture vessel.
9. Disperse the cells by pipetting over the culture surface several times to generate a homogenous cell solution.
10. Transfer the cells to a sterile centrifuge tube and centrifuge at $300 \times g$ for 4 minutes at room temperature. Aspirate and discard the medium.
11. Resuspend the cell pellet in a minimal volume of pre-warmed complete StemPro® NSC SFM and remove a sample for counting.
12. Determine the total number of cells and percent viability using trypan blue stain or the LIVE/DEAD® Cell Vitality Assay Kit.
13. Add enough complete StemPro® NSC SFM to tube for a final cell solution of 1×10^6 viable cells/mL. Incubate at 37°C, 5% CO₂ and 90% humidity. Rat fetal NSC cultures should not be maintained for more than 3 passages.

Important: If you are re-feeding rat fetal NSC in a growth medium other than complete StemPro® NSC SFM, ensure that the medium is supplemented with 10 ng/mL bFGF to maintain the undifferentiated state of the rat fetal NSCs.

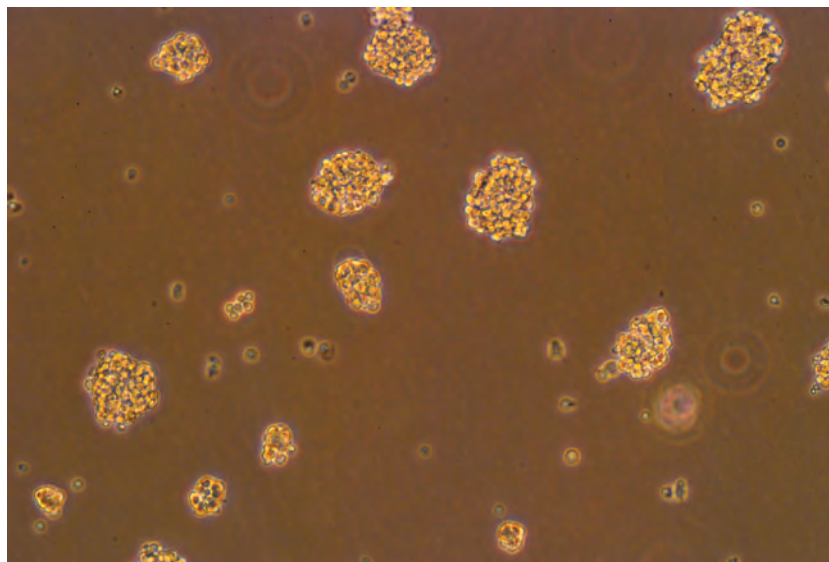
Neurosphere Suspension Cultures

1. Resuspend the rat fetal NSCs as follows:
 - For freshly prepared rat fetal NSCs, after rinsing with D-PBS, resuspend in warmed complete StemPro® NSC SFM at a cell density of 1×10^7 viable cells/mL.
 - For thawed rat fetal NSCs, after determining the viable cell count, resuspend in warmed complete StemPro® NSC SFM at a cell density of 1×10^7 viable cells/mL.
2. Plate the rat fetal NSCs onto uncoated or low-attachment culture vessels at a density of 2×10^5 viable cells/cm². See the table below for recommended seeding densities.

Vessel size	Growth area	Volume of media	No. of cells
96-well plate	0.32 cm ² /well	0.1 mL	6.4×10^4
24-well plate	1.9 cm ² /well	0.5 mL	3.8×10^5
12-well plate	3.8 cm ² /well	1 mL	7.6×10^5
35-mm dish	8 cm ² /well	2 mL	1.6×10^6
6-well plate	9.6 cm ² /well	2 mL	1.9×10^6
60-mm dish	19.5 cm ²	5 mL	3.9×10^6
T-25 flask	25 cm ²	5 mL	5.0×10^6
100-mm dish	55 cm ²	10 mL	1.1×10^7

3. Add the appropriate volume of cells to each culture vessel and incubate at 37°C, 5% CO₂ and 90% humidity.
4. Carefully re-feed the neurosphere suspension of rat fetal NSCs every 2–3 days with fresh complete StemPro® NSC SFM without removing any developing neurospheres. The morphology of the neurospheres should exhibit spherical and transparent multi-cellular complexes (see Figure 2).

Figure 2 Rat fetal NSCs at passage 3 in neurosphere culture using StemPro® NSC SFM.



5. When the neurospheres reach a diameter of 3.5 mm or larger, the rat fetal NSCs are ready to be passaged.
6. Transfer the neurosphere suspension into a sterile centrifuge tube and let the neurospheres settle by gravity or centrifuge at 200 × g for 2 minutes. Aspirate the supernatant carefully to leave the neurospheres in a minimal volume of medium.
7. Rinse the neurospheres once with D-PBS without calcium and magnesium and leave a minimal volume of D-PBS.
8. Add 1 mL of pre-warmed StemPro® Accutase® to the neurospheres and incubate for 10 minutes at room temperature.
9. After incubation, gently pipette the cells up and down to get a single-cell suspension and add 4 mL of complete StemPro® NSC SFM to the tube.
10. Centrifuge at 300 × g for 4 minutes at room temperature, carefully aspirate the supernatant, resuspend in a minimal volume of pre-warmed complete StemPro® NSC SFM, and remove a sample for counting on a hemacytometer or Countess® Automated Cell Counter.
11. Determine the total number of cells and percent viability.
12. Add enough complete StemPro® NSC SFM to the tube for a final cell solution of 1 × 10⁷ viable cells/mL. Incubate at 37°C, 5% CO₂ and 90% humidity. Neurosphere suspension cultures should not be maintained for more than 3 passages.

Important: If you are re-feeding rat fetal NSCs in a growth medium other than complete StemPro® NSC SFM, ensure that the medium is supplemented with 10 ng/mL bFGF to maintain the undifferentiated state of the rat fetal NSCs.

Xeno-free Culture of Neural Stem Cells

Summary

Neural stem cells (NSCs) derived from human embryonic stem cells (hESCs) have the potential to help provide understanding for human neurogenesis and for potential cell therapy applications to treat Parkinson's Disease or spinal cord injuries. Standard methods of culturing NSCs raise concerns about pathogen cross-transfer from non-human sources or contamination with non-neural cells, limiting the efficiency and specificity of the differentiation protocols. These concerns have led to the development of xenofree conditions for maintaining and expanding NSCs, which are described in this protocol.

Required Materials

Cells

- Neural stem cells

Media and Reagents

- CELLstart™ CTS™ (Cat. no. A10142-01)
- Neurobasal® Medium (Cat. no. 21103-049)
- B-27® Supplement XenoFree (Cat. no. A11576SA)
- FGF-basic (AA 10-155), Recombinant Human (Cat. no. PHG0026)
- EGF, Recombinant Human (Cat. no. PHG0311)
- GlutaMAX™-I (Cat. no. 35050)
- TrypLE™ Select, 10X (Cat. no. A12177)
- Dulbecco's Phosphate-Buffered Saline (D-PBS) (Cat. no. 14040)
- Dulbecco's Phosphate-Buffered Saline (D-PBS) without Ca²⁺ or Mg²⁺ (Cat. no. 14190)

Special Tools

- 15-mL conical tube
- Microcentrifuge

Preparing Media and Culture Vessels

CELLstart™ CTS™-Coated Vessels

Prepare culture dishes/flasks with CELLstart™ CTS™ as described below.

1. Dilute CELLstart™ CTS™ 1:100 in D-PBS with calcium and magnesium (e.g., 50 µL of CELLstart™ into 5 mL of D-PBS).

Note: CELLstart™ CTS™ should not be frozen, vortexed, or exposed to vigorous agitation due to potential gel formation.

2. Coat the surface of the culture vessel with the working solution of CELLstart™ CTS™ (2.5 mL for a T-25 flask or 60-mm dish, 1.5 mL for a 35-mm dish).
3. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO₂ for 1 hour.
4. Use the dish immediately after incubation. Aspirate the CELLstart™ CTS™ solution immediately before use.

Note: Prepare a freshly coated culture vessel each time before plating cells. There is no need to rinse the culture vessel before use.

Culture Medium

Prepare 50 mL of culture medium as follows. The growth factors can be added immediately before use. After the medium has been supplemented with growth factors, aliquot the amount needed for the day and store the remaining medium at 4°C. Formulated medium is stable for 2 weeks if properly stored at 4°C.

Component	Amount
Neurobasal® Medium	49 mL
GlutaMAX™-I	2 mM
B-27® Supplement XenoFree	1 mL
bFGF	20 ng/mL
EGF	20 ng/mL

Methods

Thawing and Seeding NSCs

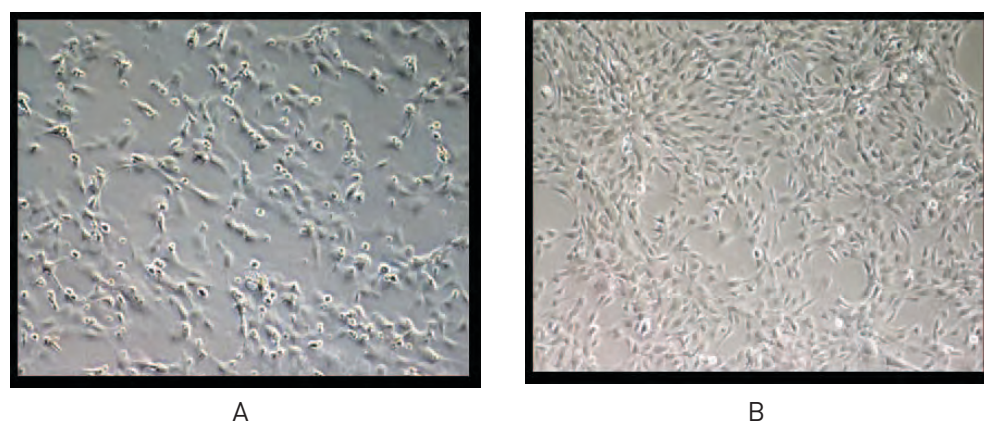
1. Remove a vial of cells from liquid nitrogen and quickly thaw the vial in a 37°C water bath, being careful not to immerse the vial above the level of the cap.
2. When just a small crystal of ice remains, sterilize the outside of the vial with 95% ethanol. Allow the ethanol to evaporate before opening the vial in a cell culture hood.
3. Gently pipet the cell suspension up and down once, and place it into a 15-mL centrifuge tube.
4. Add 10 mL of warm culture medium to the tube dropwise to reduce osmotic shock.
5. Centrifuge the cell suspension at 200 × g for 5 minutes.
6. Remove the supernatant, resuspend the pellet in 5 mL of culture medium, and determine the total number of cells and percent viability.
7. Seed the cells at a concentration of >90,000 cells/cm² onto a dish or flask that has been treated with CELLstart™ CTS™ solution. (Aspirate the CELLstart™ CTS™ solution immediately before using the dish or flask.)
8. Incubate at 36–38°C in a humidified atmosphere (90%) of 5% CO₂ in air.

Culture and Propagation

1. Twenty-four hours after seeding the cells, replace the culture medium.
2. Replace the spent medium every other day with an equal volume of fresh culture medium.

Note: If the medium turns yellow, change the medium daily. Yellow medium will affect the NSC proliferation rate.
3. After 3–4 days, the culture will become semi-confluent.
4. To split the cell culture 1:2, aspirate the medium and wash the cells twice with 5 mL of D-PBS (without calcium and magnesium).
5. Add 1 mL of TrypLE™ Select to dissociate the cells, and incubate for 2 minutes at 37°C.
6. Add 4 mL of culture media to neutralize the TrypLE™ Select activity and pipet up and down 2–3 times to get a uniform cell suspension. Check the cells under a microscope.
7. Transfer the cell suspension to a 15-mL centrifuge tube.
8. Centrifuge the cells at $200 \times g$ for 5 minutes.
9. Aspirate the supernatant and resuspend the cells in 10 mL of culture medium.
10. Split the cell suspension into two fresh T-25 flasks that have been treated with CELLstart™ CTS™ solution. Seed each flask with 5 mL of cell suspension.
11. Incubate the flasks at 37°C in a humidified atmosphere (90%) of 5% CO₂ in air.
12. Grow the cells until semi-confluent, changing the medium once after 12 hours and every two days thereafter.
13. Passage the cells when the culture reaches ~80% confluence.

Figure 1 Phase contrast microphotograph showing NSCs cultured in xenofree media 24 hours post-thaw (Panel A) and semi-confluent NSCs cultured in xenofree media for 3 days (Panel B).



Differentiating Neural Stem Cells into Neurons and Glial Cells

Summary

The protocols in this chapter describe the steps involved in differentiating neural stem cells (NSC) to neurons, astrocytes, and oligodendrocyte lineages *in vitro*. NSCs are self-renewing multipotent stem cells that can be proliferated *in vitro* in supportive culture systems such as Stempro® NSC SFM and can further be differentiated into downstream lineages. The protocols described are primarily optimized with NSCs derived from human embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC). Some optimization in terms of reagent concentration and duration of *in vitro* differentiation is expected for NSCs from other species such as rat or mouse, as well as with NSCs derived from patient-specific iPSCs.

Required Materials

Cells • GIBCO® Human Neural Stem Cells (H9 hESC-Derived) (Cat. no. N7800)

Reagents

- KnockOut™ D-MEM/F-12 (Cat. no. 12660)
- Dulbecco's Modified Eagle Medium (D-MEM) (Cat. no. 11995)
- Dulbecco's Phosphate-Buffered Saline (D-PBS) (Cat. no. 14040)
- StemPro® NSC SFM (Cat. no. A10509-01)
- N-2 Supplement (Cat. no. 17502)
- B-27® Serum-Free Supplement (Cat. no. 17504)
- Neurobasal® Medium (Cat. no. 21103)
- Antibiotic-Antimycotic solution (Cat. no. 15240)
- Fetal Bovine Serum, ES Cell-Qualified FBS (Cat. no. 16141)
- GlutaMAX™-I (Cat. no. 35050)
- FGF-basic (AA 10–155), Recombinant Human (bFGF) (Cat. no. PHG0024)
- EGF, Recombinant Human (Cat. no. PHG0314)
- CELLstart™ CTS™ (Cat. no. A10142-01)
- Geltrex™ Reduced Growth Factor Basement Membrane Matrix (Cat. no. 12760)
- Poly-L-Ornithine (Sigma, Cat. no. P3655)
- Laminin (Cat. no. 23017)
- Dibutyryl cAMP (Sigma, Cat. no. D0627)
- T3 (Sigma, Cat. no. D6397)
- EM grade paraformaldehyde (Electron Microscopy Services, Cat. no. 19208)
- ProLong® Gold antifade reagent (Cat. no. P36930)

Primary Antibodies

Lineage marker	Antigen	Concentration	Subtypes	Reactivity*
NSC	Nestin	1:1,000	Rabbit	Hu, Rt, Ms
	Sox2	1:200	Mouse IgG _{2a}	Hu
Neuron	Dcx	1:400	Rabbit	Hu, Rt, Ms
	MAP2	1:200	Mouse IgG ₁	Hu, Rt, Ms
Glial	A2B5	1:100	Mouse IgM	Hu, Rt, Ms
	CD44	1:50	Mouse IgG	Hu
Oligodendrocyte	GalC	1:200	Mouse IgG	Hu, Rt, Ms
Astrocyte	GFAP	1:200	Rabbit	Hu, Rt, Ms
*Hu = human, Rt = rat, Ms = mouse.				

Secondary Antibodies

Ex/Em* (color)	Alexa® no.	2nd host	2nd against	Cat. no.	Concentration
346/442 (Blue)	350	Goat	Mouse IgM	A31552	1:1,000
		Goat	Mouse IgG	A21049	1:1,000
		Goat	Rat IgG	A21093	1:1,000
		Goat	Rabbit IgG	A21068	1:1,000
		Donkey	Goat IgG	A21081	1:1,000
495/519 (Green)	488	Goat	Mouse IgM	A21042	1:1,000
		Goat	Mouse IgG	A11029	1:1,000
		Goat	Rat IgM	A21212	1:1,000
		Goat	Rat IgG	A11006	1:1,000
		Goat	Goat IgG	A11034	1:1,000
		Donkey	Mouse IgM	A11055	1:1,000
590/617 (Red)	594	Goat	Mouse IgM	A21044	1:1,000
		Goat	Mouse IgG	A11029	1:1,000
		Goat	Rat IgM	A21213	1:1,000
		Goat	Rat IgG	A11007	1:1,000
		Goat	Rabbit IgG	A11037	1:1,000
		Donkey	Goat IgG	A11058	1:1,000
496, 536, 565/576 (Red)	NA	Goat	Mouse IgM	M31504	1:500
		Goat	Mouse IgG	P852	1:1,000
		Goat	Rabbit IgG	P2771MP	1:1,000
*Approximate excitation and emission maxima, in nm; NA = not applicable.					

Preparing Media

StemPro® NSC SFM Complete Medium

StemPro® NSC SFM complete medium consists of KnockOut™ D-MEM/F-12 with StemPro® Neural Supplement, EGF, bFGF, and GlutaMAX™-I. Complete medium is stable for 4 weeks when stored in the dark at 2–8°C.

To prepare 100 mL of complete medium:

1. Reconstitute bFGF and EGF with 0.1% BSA solution (in KnockOut™ D-MEM/F-12) at a concentration of 100 µg/mL. You will need 20 µL of each per 100 mL of complete medium. Freeze unused portions in aliquots.
2. Mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally: If desired, add 1 mL of Antibiotic-Antimycotic solution per 100 mL of complete medium.

Component	Final concentration	Amount
KnockOut™ D-MEM/F-12	1X	97 mL
GlutaMAX™-I Supplement	2 mM	1 mL
bFGF (prepared as 100 µg/mL stock)	20 ng/mL	20 µL
EGF (prepared as 100 µg/mL stock)	20 ng/mL	20 µL
StemPro® Neural Supplement	2%	2 mL
You may observe a white precipitate when thawing StemPro® Neural Supplement; this precipitate will disappear when the supplement is completely thawed or dissolved.		

Neural Differentiation Medium

Neural differentiation medium requires supplementation of Neurobasal® medium with B-27® Serum-Free Supplement and GlutaMAX™-I. Neural differentiation medium is stable for 2 weeks when stored in the dark at 2–8°C.

To prepare 100 mL of neural differentiation medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. If desired, add 1 mL of Antibiotic-Antimycotic solution per 100 mL of medium.

Component	Final concentration	Amount
Neurobasal® Medium	1X	97 mL
B-27® Serum-Free Supplement	2%	2 mL
GlutaMAX™-I Supplement	2 mM	1 mL
If faster differentiation is desired, add dibutyl cAMP (Sigma, Cat. no. 0627) to a final concentration of 0.5 mM at day 7 for a duration of 3 days, as indicated in the differentiation protocols.		

Astrocyte Differentiation Medium

Astrocyte differentiation medium requires supplementation of D-MEM with N-2, GlutaMAX™-I, and FBS. Astrocyte differentiation medium is stable for 4 weeks when stored in the dark at 2–8°C.

To prepare 100 mL of astrocyte differentiation medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. If desired, add 1 mL of Antibiotic-Antimycotic solution per 100 mL of medium.

Component	Final concentration	Amount
D-MEM	1X	97 mL
N-2 Supplement	1%	1 mL
GlutaMAX™-I Supplement	2 mM	1 mL
FBS	1%	1 mL

Oligodendrocyte Differentiation Medium

Oligodendrocyte differentiation medium requires supplementation of Neurobasal® medium with B-27®, GlutaMAX™-I, and T3. Oligodendrocyte differentiation medium is stable for 2 weeks when stored in the dark at 2–8°C.

To prepare 100 mL of oligodendrocyte differentiation medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. If desired, add 1 mL of Antibiotic-Antimycotic solution per 100 mL of medium.

Component	Final concentration	Amount
Neurobasal® Medium	1X	97 mL
B-27® Serum-Free Supplement	2%	2 mL
GlutaMAX™-I Supplement	2 mM	1 mL
T3*	30 ng/mL	0.1 mL
* You can prepare a 30 µg/mL T3 stock solution (1,000X) in distilled water. Filter sterilize the T3 stock solution.		

Preparing Matrix

Coating Culture Vessels with CELLstart™

1. Dilute CELLstart™ CTS® 1:100 in D-PBS with calcium and magnesium (i.e., 50 µL of CELLstart™ CTS® into 5 mL of D-PBS).
2. Coat the surface of the culture vessel with the working solution of CELLstart™ CTS® (14 mL for T-75, 7 mL for T-25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish).
3. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO₂ in air for 1 hour.
4. Remove the vessel from the incubator and store it until use. Immediately before use, remove all CELLstart™ CTS® solution and replace it with complete StemPro® NSC SFM.

Note: You may coat the plates in advance and store them at 4°C, wrapped tightly with Parafilm®, for up to 2 weeks. Do not remove CELLstart™ CTS® solution until just prior to use. Make sure the plates do not dry out.

Coating Culture Vessels with Geltrex™

1. Thaw the Geltrex™ bottle at 4°C overnight to prevent polymerization. The next day, dilute Geltrex™ 1:2 with D-MEM/F-12 at 4°C to make 100X stock solution, using an ice bucket to keep the bottles cold. Quickly prepare 0.5-mL aliquots in 50-mL conical tubes (pre-chilled on ice), and store the tubes at –20°C.
2. Thaw 1 tube of Geltrex™ (0.5 mL, aliquoted as above) slowly at 4°C, and add 49.5 mL of cold D-MEM/F-12 (1:100 dilution). Mix gently.
3. Cover the whole surface of each culture plate with the Geltrex™ solution (1.5 mL for a 35-mm dish, 3 mL for 60-mm dish, 5 mL for a T-25 culture flask).
4. Seal each dish with Parafilm® to prevent drying, and incubate 1 hour at room temperature in a laminar flow hood.
5. Immediately before use, remove all Geltrex™ solution, wash once with D-PBS with calcium and magnesium, and replace pre-warmed complete medium.

Note: You may store the Geltrex™-treated dish at 4°C, wrapped tightly with Parafilm®, for up to 1 month. Do not remove Geltrex™ solution until just prior to use.

Coating Culture Vessels with Poly-L-Ornithine and Laminin

1. Dissolve poly-L-ornithine in cell culture-grade distilled water to make 10 mg/mL stock solution (500X). Aliquot the solution and store it at –20°C until use.
2. Thaw the laminin slowly at 2–8°C and prepare 10 µg/mL working solution in cell culture-grade distilled water. Aliquot the working solution into polypropylene tubes, and store the tubes at –20°C until use. Avoid repeated freeze/thaw cycles.

Note: Laminin may form a gel if thawed too rapidly.

3. Dilute the poly-L-ornithine stock solution 1:500 in cell culture-grade distilled water to make 20 µg/mL working solution.

4. Coat the surface of the culture vessel (with or without cover slips) with the poly-L-ornithine working solution (14 mL for T-75, 7 mL for T-25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish).
5. Incubate the culture vessel overnight at 4°C or for 1 hour at 37°C.
6. Rinse the culture vessel twice with sterile water.
7. Coat the surface of the culture vessel (with or without cover slips) with the laminin working solution (14 mL for T-75, 7 mL for T-25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish).
8. Incubate the culture vessel overnight at 4°C or for 2 hours at 37°C.
9. Rinse the culture vessel with D-PBS without calcium or magnesium, and store the vessel covered with D-PBS until use. Immediately before use, remove all D-PBS and replace it with complete StemPro® NSC SFM.

Note: You may coat the plates in advance and store them at room temperature, wrapped tightly with Parafilm®, for up to 1 week. Do not remove D-PBS until just prior to use. Make sure the plates do not dry out.

Differentiating Neural Stem Cells

Neural stem cells (NSCs) will proliferate as progenitors a few times even after the complete growth medium is replaced with the appropriate differentiation medium. If the cells reach 90% confluency, it might be necessary to split the cells at a 1:2 ratio. However, do not split the cells once they reach day 9–10 of differentiation when they can get damaged during the passaging process.

Differentiation into

Neurons

1. Plate neural stem cells on a polyornithine and laminin-coated culture dish in complete StemPro® NSC SFM at 2.5×10^4 – 5×10^4 cells/cm².
2. After 2 days, change the medium to neural differentiation medium. Change the spent medium every 3–4 days.
3. If expedited differentiation is desired, add 0.5 mM of dibutyryl cAMP (Sigma, Cat. no. D0627) to the differentiation medium daily starting at day 7 of differentiation for 3 days.

IMPORTANT! Do not expose cells to air at any time after they have differentiated into neurons.

Differentiation into

Astrocytes

1. Plate the NSCs on a Geltrex™-coated culture dish in complete StemPro® NSC SFM at 2.5×10^4 cells/cm².
2. After 2 days, change medium to astrocyte differentiation medium. Change the spent medium every 3–4 days.

**Differentiation into
Oligodendrocytes**

1. Plate the NSCs on a polyornithine and laminin-coated culture dish in complete StemPro® NSC SFM at 2.5×10^4 – 5×10^4 cells/cm².
2. After 2 days, change the medium to oligodendrocyte differentiation medium. Change the spent medium every 3–4 days.

Characterizing NSCs and Differentiated Lineages by Immunocytochemistry

**Preparing
Paraformaldehyde Fixing
Solution****20% paraformaldehyde (PFA) stock solution**

1. Add PBS to 20 g of EM grade paraformaldehyde (Electron Microscopy Services, Cat. no. 19208), and bring the volume up to 100 mL.
2. Add 0.25 mL of 10 N NaOH and heat the solution at 60°C using a magnetic stirrer until the solution is completely dissolved.
3. Filter the solution through a 0.22-μm filter, and cool on ice. Make sure the pH is 7.5–8.0.
4. Aliquot 2 mL in 15-mL tubes, freeze the tubes on dry ice, and store them at –20°C.

4% PFA for fixing

1. Add 8 mL of PBS into each 15-mL tube containing 2 mL of 20% PFA, and thaw each tube in a 37°C water bath.
2. Once the solution has dissolved, the tubes cool on ice.

Fixing Cells

1. Remove culture medium and gently rinse the cells once with D-PBS, without dislodging the cells.
2. Fix the cells with 4% fresh Paraformaldehyde Fixing Solution (PFA) at room temperature for 15 minutes.
3. Rinse 3X with D-PBS containing Ca²⁺ and Mg²⁺.
4. Check for the presence of cells after fixing.
5. Proceed to staining, described below. You may store slides for up to 3–4 weeks in D-PBS at 4°C before staining. Do not allow slides to dry.

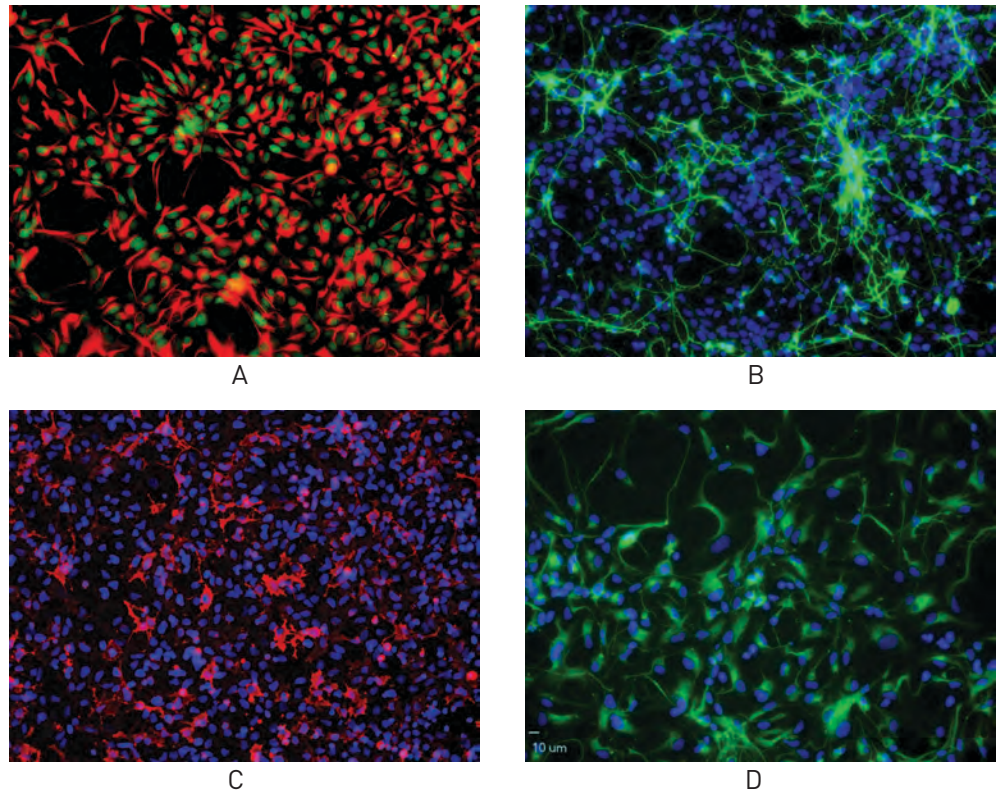
- Staining Cells**
1. Incubate cells for 30–60 minutes in blocking buffer (5% serum of the secondary antibody host species, 1% BSA, 0.1% Triton[®]-X in D-PBS with Ca²⁺ and Mg²⁺).

Note: If you are using a surface antigen such as GalC, omit Triton[®]-X from the blocking buffer.

2. Remove the blocking buffer and incubate the cells overnight at 4°C with primary antibody diluted in 5% serum. Ensure that the cell surfaces are covered uniformly with the antibody solution.
3. Wash the cells 3X for 5 minutes with D-PBS containing Ca²⁺ and Mg²⁺ (if using a slide, use a staining dish with a magnetic stirrer).
4. Incubate the cells with fluorescence-labeled secondary antibody (5% serum in D-PBS with Ca²⁺ and Mg²⁺) in the dark at 37°C for 30–45 minutes.
5. Wash the cells 3X with D-PBS containing Ca²⁺ and Mg²⁺, and in the last wash, counter stain the cells with DAPI solution (3 ng/mL) for 5–10 minutes, and rinse with D-PBS.
6. If desired, mount using 3 drops of ProLong[®] Gold antifade reagent per slide and seal with the cover slip. You may store the slides in the dark at 4°C.

Expected Results

Figure 1 Fluorescence images (20X) of GIBCO[®] hNSCs that have been cultured in StemPro[®] NSC SFM for three passages, and then allowed to differentiate into neurons, oligodendrocytes, or astrocytes. Upon directed differentiation, cells start to lose the undifferentiated NSC marker, nestin, but stain positive for the differentiated cell type markers Dcx, GalC, and GFAP. Cells were stained for the undifferentiated NSC markers nestin (red) and SOX2 (green) prior to directed differentiation (Panel A). Cells were then differentiated into neurons and glial cells, and respectively stained for the neuronal marker Dcx (green) (Panel B), for the oligodendrocyte marker GalC (red) (Panel C), or for the astrocyte marker, GFAP (green) (Panel D). The nuclei were counterstained with DAPI (blue) in panels B–D.



Troubleshooting

The table below lists some causes and solutions to help you troubleshoot your potential differentiation problems.

Possible cause	Solution
Culture medium contains bFGF	Remove bFGF from culture medium
Cell density too high and endogenous bFGF is preventing differentiation	Reduce cell density
Concentration of GlutaMAX™-I Supplement is incorrect	Use the GlutaMAX™-I Supplement at a final concentration of 2 mM
Cells have been passaged too many times	Obtain new GIBCO® human neural stem cells

References

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Elkabetz, Y. and Studer, L. 2008. Human ESC-derived neural rosettes and neural stem cell progression. *Cold Spring Harb Symp Quant Biol.* 73:377–387.

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Differentiating Glial Precursor Cells into Astrocytes and Oligodendrocytes

Summary

Glial precursor cells (GPCs), also known as glial restricted progenitors (GRP) or oligodendrocyte progenitor cells (OPCs), are cells that have the potential to differentiate into oligodendrocytes or astrocytes. The GPC population is derived from tissue or is generated from pluripotent cells by differentiation, which is induced by exogenously applied factors. Here we described a culture system that can be adjusted to favor differentiation into either astrocytes or oligodendrocytes.

Required Materials

Cells

- GIBCO® Rat Glial Precursor Cells (Cat. no. N7746-100)

Media and Reagents

- Neurobasal® Medium (Cat. no. 21103-049)
- D-MEM (Cat. no. 11995)
- GlutaMAX™-I (Cat. no. 35050)
- B-27® Serum-Free Supplement (Cat. no. 17504-044)
- N-2 Supplement (Cat. no. 17502-048)
- T3 (Liothyronine) (Sigma, Cat. no. T6397)
- FBS (Cat. no. 16000)
- Geltrex™ Reduced Growth Factor Basement Membrane Matrix (Cat. no. 12760)
- Laminin (Cat. no. 23017)
- Poly-L-Ornithine (Sigma, Cat. no. P4957)
- Water, distilled (Cat. no. 15230)
- KnockOut™ D-MEM/F-12 (Cat. no. 12660)
- Dulbecco's Phosphate-Buffered Saline (D-PBS) without Ca²⁺ and Mg²⁺ (Cat. no. 14190)
- Dulbecco's Phosphate-Buffered Saline (D-PBS) (Cat. no. 14040)
- StemPro® NSC SFM (Cat. no. A10509-01)

Preparing Media

Preparing Oligodendrocyte Differentiation Medium

Oligodendrocyte differentiation medium uses Neurobasal® medium supplemented with B-27®, GlutaMAX™-I, and T3. Complete medium is stable for 2 weeks when stored in the dark at 2–8°C.

To prepare 100 mL of complete medium, mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Final concentration	Amount
Neurobasal® Medium	1X	97 mL
GlutaMAX™-I Supplement	2 mM	1 mL
B-27® Supplement	2%	2 mL
T3	30 ng/mL	0.1 mL

Preparing Astrocyte Differentiation Medium

Astrocyte differentiation medium uses D-MEM medium supplemented with N-2, GlutaMAX™-I, and FBS. Complete medium is stable for 2 weeks when stored in the dark at 2–8°C.

Component	Final concentration	Amount
D-MEM Medium	1X	97 mL
GlutaMAX™-I Supplement	2 mM	1 mL
N-2 Supplement	1%	1 mL
FBS	1%	1 mL

Preparing Matrix

Matrix for Oligodendrocyte Differentiation

1. Prepare a 1:500 dilution of poly-L-ornithine in distilled water for a final concentration of 20 µg/mL.
2. Add 2 mL of 20 µg/mL poly-L-ornithine solution to a 35-mm dish (0.5 mL for 4-well plate or slide, 0.25 mL for 8-well slide).
3. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO₂ for at least 1 hour.
4. Rinse the culture vessel once with distilled water.
5. Prepare a 1:1,000 dilution of laminin in distilled water for a final concentration of 1 µg/mL.

6. Add 2 mL of 1 µg/mL laminin solution to a 35-mm dish (0.5 mL for 4-well plate or slide, 0.25 mL for 8-well slide).
7. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO₂ for at least 1 hour. Store it at 4°C until use.

Note: You may coat the plates in advance and store them at 2–8°C, wrapped tightly with Parafilm[®], for up to 4 weeks.

Matrix for Astrocyte Differentiation

1. Thaw the Geltrex[™] bottle at 4°C overnight to prevent polymerization. The next day, prepare a 50X stock solution of Geltrex[™] by diluting to a final concentration of 10 mg/mL with D-MEM/F-12. Use an ice bucket to keep the bottle at 4°C. Quickly prepare 1 mL aliquots in 50-mL conical tubes (pre-chilled on ice), and store the tubes at –20°C.
2. Thaw one tube of Geltrex[™] (1 mL aliquot described above) slowly at 4°C, and add 49 mL of cold D-MEM/F-12. Mix gently.
3. Cover the whole surface of each culture plate with the Geltrex[™] solution (1.5 mL for a 35-mm dish, 3 mL for 60-mm dish, 5 mL for a T-25 culture flask).
4. Seal each dish with Parafilm[®] to prevent drying, and incubate 1 hour at room temperature in a laminar flow hood. Store at 4°C until use.
5. Immediately before use, remove all Geltrex[™] solution, wash once with D-PBS with calcium and magnesium, and replace it with pre-warmed complete medium.

Note: You may store the Geltrex[™]-treated dish at 2–8°C, wrapped tightly with Parafilm[®], for up to 1 month. Ensure that plates do not dry out. Do not remove the Geltrex[™] solution until just prior to use.

Differentiation of GRPs

Differentiation to Oligodendrocytes

1. Plate glial precursor cells on poly-L-ornithine and laminin coated plate in StemPro[®] NSC SFM at a density of 2.5×10^4 – 5×10^4 cells/cm².
2. Culture the cells for 2 days, then change the medium to Oligodendrocyte Differentiation Medium.
3. Change the medium every 3–4 days.

Differentiation to Astrocytes

1. Plate glial precursor cells on Geltrex[™] coated plate in StemPro[®] NSC SFM at a density of 2.5×10^4 cells/cm².
2. Culture the cells for 2 days, then change the medium to Astrocyte Differentiation Medium.
3. Change the medium every 3–4 days.

Derivation and Culture of Cortical Astrocytes

Summary

Astrocytes are the most numerous cell type in the central nervous system (CNS). They play critical roles in adult CNS homeostasis, provide biochemical and nutritional support of neurons and endothelial cells that form the blood-brain barrier, perform the vast majority of synaptic glutamate uptake, and maintain extracellular potassium levels. Astroglial dysfunction has been implicated in a number of CNS pathologies. This protocol describes the preparation of primary cortical astrocytes from new-born rats or mice.

Required Materials

Animals • Newborn rats or mice at 1–2 days after birth.

Media and Reagents

- Ether
- 70% ethanol
- Distilled water (Cat. no. 15230-162)
- Acetic acid (Sigma, Cat. no. 34254)
- Collagen (Fisher Scientific, Cat. no. CB-40236)
- 0.05% Trypsin/EDTA solution (Cat. no. 25300-054)
- Hanks' balanced salt solution (HBSS) (Cat. no. 14170-112)
- Gibco® Astrocyte Medium (Cat. no. A12613-01)
- EGF, Recombinant Human (Cat. no. PHG0314)
- Dulbecco's Phosphate-Buffered Saline (D-PBS) without Ca^{2+} or Mg^{2+} (Cat. no. 14190)
- Dibutyryl cyclic-AMP (dBcAMP) (Sigma, D0627)
- Penicillin-streptomycin (Cat. no. 15070-063)
- Trypan Blue Stain (Cat. no. 15250) (included with the Countess® Automated Cell Counter) or LIVE/DEAD® Cell Vitality Assay Kit (Cat. no. L34951)

Special Tools

- Desiccator
- Iridectomy scissors
- 70- μm mesh cell strainer
- Countess® Automated Cell Counter (Cat. no. C10227) or hemacytometer

Preparing Reagents and Media

Astrocyte Medium GIBCO® Astrocyte Medium has been specifically formulated for the growth and maintenance of human and rat astrocytes while retaining their phenotype. The medium has three components: basal medium (D-MEM), N-2 Supplement, and One Shot™ Fetal Bovine Serum (FBS). Epidermal growth factor (EGF) may also be added to enhance astrocyte proliferation.

To prepare 100 mL of complete medium, mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Amount
D-MEM	83 mL
N-2	15 mL
FBS	1 mL
Penicillin-streptomycin	1 mL
<i>Optional:</i> EGF (prepared as 100 µg/mL stock)	20 µL

Collagen Prepare a 50 µg/mL working solution in distilled water with 0.02 M acetic acid and sterilize the solution with a 0.22-µm filter.

Dibutyryl cyclic-AMP (dBcAMP) Prepare a 0.25 M stock solution of dBcAMP in D-PBS, aliquot into sterilized tubes, and store at -20°C.

HBSS Chill on ice prior to use.

Preparing Astrocyte-Enriched Cultures

1. Coat the culture vessels with collagen and let stand for 45 minutes at room temperature. Rinse with D-PBS without calcium or magnesium two times.
2. Anesthetize rat or mouse pups with ether in a desiccator in a chemical fume hood.
3. Remove pups from the hood and spray 70% ethanol over the animal. Decapitate the animals with scissors. Open the skull with iridectomy scissors. Remove the meninges and dissect the brain tissue from the cortices.
4. Put the cortices in a petri dish containing 5–10 mL of ice-cold HBSS. Pool the cortices from two pups in a new petri dish and wash with 5 mL of HBSS.

5. Take the petri dish to a laminar flow hood. Mince the cortices into small pieces with a scissors in a petri dish containing about 5 mL of ice-cold HBSS. Transfer the tissue to a 15-mL sterile tube. Centrifuge the tube at $200 \times g$ for 3 minutes at 4°C and aspirate the supernatant.
6. Resuspend the tissue in 5 mL of 0.05% trypsin and incubate at 37°C for 25 minutes in a shaker bath.
7. Centrifuge the tissue suspension at $200 \times g$ for 3 minutes, aspirate the trypsin solution with a pipette, and rinse the cells 3 times with 3 mL of HBSS.
8. Add 6 mL of astrocyte medium and pipet the cell suspension up and down with a 10-mL pipette to dissociate cells.
9. Filter the cell suspension through a 70- μ m mesh cell strainer into a 50-mL sterile tube. Rinse the mesh with another 4 mL of astrocyte medium (total of 10 mL suspension).
10. Remove 10 μ L of the filtrate for counting on a hemacytometer or the Countess® Automated Cell Counter.
11. Determine the total number of cells and percent viability using trypan blue stain or the LIVE/DEAD® Cell Vitality Assay Kit.
12. Dilute the cell suspension to 5×10^4 viable cells/mL with astrocyte medium and plate the cells into culture vessels at 2.5×10^4 /cm².
13. Incubate the cells in a 37°C incubator with 5% CO₂ and 90% humidity.
14. Change the astrocyte medium the next day and then every other day until cells are confluent.
15. When confluent, feed the cells with astrocyte medium containing 0.25 mM dBcAMP to induce differentiation. (Dilute 0.25 M stock of dBcAMP 1:1,000 in astrocyte medium.)
16. Feed the cultures with dBcAMP two times per week and check for differentiation.
17. Astrocytes are ready for experiments 2–3 weeks after culturing.

Isolation, Culture, and Characterization of Cortical and Hippocampal Neurons

Summary

The ability to culture primary neurons under serum-free conditions facilitates tighter control of neuronal studies. Some serum-free media and supplements allow for the low-density neuronal cultures, which in turn enables the study of individual neurons and synapses. This has not been possible using serum-supplemented media without a feeder layer of glial cells. In serum-supplemented media, glial cells continue to multiply, necessitating the use of cytotoxic mitotic inhibitors. Serum also contains unknown and variable levels of growth factors, hormones, vitamins, and proteins. This chapter details the isolation and culture of neural cells in serum-free media and supplements.

Required Materials

Isolating Rat Brain Cells

- Hibernate®-E Medium (Cat. no. A12476-01)
- B-27® Serum-Free Supplement (Cat. no. 17504)
- GlutaMAX™-I (Cat. no. 35050)
- Hibernate®-E Medium, without Ca²⁺ (BrainBits LLC, Cat. no. HE-Ca)
- Papain (Worthington, Cat. no. LS003119)
- Neurobasal® Medium (Cat. no. 21103-049)
- Trypan Blue Stain (Cat. no. 15250-061)
- Pasteur pipettes
- Hemacytometer, cell counter and trypan blue, or the Countess® Automated Cell Counter (Cat. no. C10227)
- Conical tubes (15-mL, 50-mL)

Culturing Embryonic Neurons

- Poly-D-lysine hydrobromide (Sigma, Cat. no. P-6407)
- 48-well plate or 8-chambered slides
- Distilled water (Cat. no. 15230-162)
- Dulbecco's Phosphate-Buffered Saline (D-PBS) (Cat. no. 14040-141)
- Neurobasal® Medium (Cat. no. 21103-049)
- B-27® Serum-Free Supplement (Cat. no. 17504)
- GlutaMAX™-I (Cat. no. 35050)

Immunocytochemistry

- Goat serum (Cat. no. 16210-064)
- Mouse anti-MAP2 antibody (Cat. no. 13-1500)
- Rabbit anti-GFAP antibody (Cat. no. 08-0063)
- Alexa Fluor® 488 goat anti-mouse IgG (H+L) (Cat. no. A-11029)
- Alexa Fluor® 594 goat anti-rabbit IgG (H+L) (Cat. no. A-11037)
- 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Cat. no. D1306)
- ProLong® Gold antifade reagent (Cat. no. P36930)
- EM grade paraformaldehyde (Electron Microscopy Services, Cat. no. 19208)

Preparing Media

Hibernate®-E Complete Medium

Hibernate®-E is a serum-free, nutrient basal medium for the short-term maintenance of cultured rat neurons and long-term storage of viable brain tissue in ambient CO₂ (0.2%) conditions. The complete medium consists of Hibernate®-E medium supplemented with B-27® Serum-Free Supplement and GlutaMAX™-I. Hibernate®-E complete medium is stable for 2 weeks when stored in the dark at 2–8°C.

To prepare 100 mL of Hibernate®-E complete medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally.

Component	Final concentration	Amount
Hibernate®-E Medium	1X	98 mL
B-27® Serum-Free Supplement	2%	2 mL
GlutaMAX™-I Supplement	0.5 mM	250 µL

Neurobasal® Complete Medium

Neurobasal® complete medium requires supplementation of Neurobasal® medium with B-27® Serum-Free Supplement and GlutaMAX™-I. Complete medium is stable for 2 weeks when stored in the dark at 2–8°C.

To prepare 100 mL of Neurobasal® complete medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally.

Component	Final concentration	Amount
Neurobasal® Medium	1X	98 mL
B-27® Serum-Free Supplement	2%	2 mL
GlutaMAX™-I Supplement	0.5 mM	250 µL
For primary rat hippocampus neuron cultures, Neurobasal® complete medium requires additional supplementation with 25 µM L-Glutamate up to the fourth day of culture.		

Preparing Matrix

Coating Culture Vessels with Poly-D-Lysine

1. Prepare a 2-mg/mL poly-D-lysine stock solution in distilled water.
2. Dilute the poly-D-lysine stock solution 1:40 in D-PBS to prepare a 50 µg/mL working solution (i.e., 125 µL of poly-D-lysine stock solution into 5 mL of D-PBS).
3. Coat the surface of the culture vessel with the working solution of poly-D-lysine (150 µL/cm², i.e., 100 µL per well for a 48-well plate).
4. Incubate the culture vessel at room temperature for 1 hour.
5. Remove the poly-D-lysine solution and rinse 3 times with distilled water. Make sure to rinse the culture vessel thoroughly, because excess poly-D-lysine can be toxic to the cells.
6. Leave the coated vessels uncovered in the laminar hood until the wells have completely dried. You may use the dry plates immediately or store them at 4°C, wrapped tightly with Parafilm[®], for up to one week.

Isolating Neurons

1. Dissect cortex or hippocampus pairs from ten E-18 rat embryo brains. Remove all the meninges thoroughly.
2. Collect all the tissue in a conical tube containing Hibernate[®]-E supplemented with 2% B-27[®] Serum-Free Supplement and 0.5 mM GlutaMAX[™]-I at 4°C.
3. Allow the tissue to settle to the bottom of the tube and then carefully remove the supernatant leaving only the tissue covered by the medium.
4. Enzymatically digest the tissue in 4 mL of Hibernate[®]-E medium without Ca²⁺ containing 2 mg/mL of filter-sterilized papain for 30 minutes at 30°C. Gently shake the tube every 5 minutes.
5. Add 6 mL of complete Hibernate[®]-E medium to the tube and centrifuge for 5 minutes at 150 × g.
6. Remove the supernatant and resuspend the tissue in 5 mL of complete Hibernate[®]-E medium by pipetting up and down with a fire-polished glass Pasteur pipette.
7. Let the tube stand undisturbed for 2 minutes to allow for the cell debris (if any) to settle down.
8. Transfer the cells to a new tube leaving behind all the debris.
9. Count the cells using a hemacytometer, cell counter and trypan blue, or the Countess[®] Automated Cell Counter.
10. Centrifuge the tube for 4 minutes at 200 × g.

11. Remove the supernatant and resuspend the cell pellet in Neurobasal® medium with 2% B-27® Serum-Free Supplement and 0.5 mM GlutaMAX™-I for culturing.

Note: Plate the cells immediately after resuspension. If you need to store the cells longer, store them in Hibernate®-E medium supplemented with 2% B-27® Serum-Free Supplement and 0.5 mM GlutaMAX™-I at 4°C for up to 48 hours. Do not expose the neurons to air at any time.

Culturing Neurons

1. Plate $\sim 1 \times 10^5$ cells per well in poly-D-lysine coated 48-well plate or an 8-chambered slide. Bring the cell suspension volume to 500 μ L per well by adding complete Neurobasal® medium.
2. Incubate the cells at 37°C in a humidified atmosphere of 5 % CO₂ in air.
3. Feed the cells every third day by aspirating half of the medium from each well and replacing it with fresh medium.

Characterizing Neural Cells

Preparing Paraformaldehyde Fixing Solution

20% paraformaldehyde (PFA) stock solution

1. Add PBS to 20 g of EM grade paraformaldehyde, and bring the volume up to 100 mL.
2. Add 0.25 mL of 10 N NaOH and heat the solution at 60°C using a magnetic stirrer until the solution is completely dissolved.
3. Filter the solution through a 0.22- μ m filter, and cool on ice. Make sure the pH is 7.5–8.0.
4. Aliquot 2 mL in 15-mL tubes, freeze the tubes on dry ice, and store them at –20°C.

4% PFA for fixing

1. Add 8 mL of PBS into each 15-mL tube containing 2 mL of 20% PFA, and thaw each tube in a 37°C water bath.
2. Once the solution has dissolved, the tubes cool on ice.

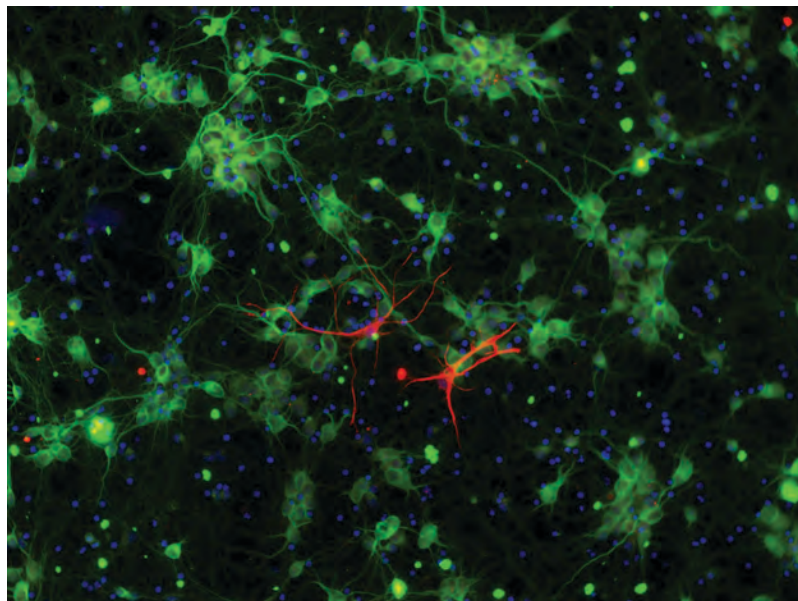
- Fixing Cells**
1. Remove the culture medium and gently rinse the cells without dislodging them twice with D-PBS containing Ca^{2+} and Mg^{2+} .
 2. Fix the cells with 4% fresh Paraformaldehyde Fixing Solution (PFA) at room temperature for 15 minutes.
 3. Rinse the cells three times with D-PBS containing Ca^{2+} and Mg^{2+} .
 4. Permeabilize the cells with 0.3% Triton[®]-X (diluted in D-PBS with Ca^{2+} and Mg^{2+}) for 5 minutes at room temperature.
 5. Rinse the cells three times with D-PBS containing Ca^{2+} and Mg^{2+} .

- Staining Cells**
1. Incubate cells in 5% goat serum diluted in D-PBS with Ca^{2+} and Mg^{2+} for 60 minutes at room temperature.
 2. Remove the 5% goat serum solution and incubate the cells overnight with the primary antibody (Mouse anti-MAP2 at 10 $\mu\text{g}/\text{mL}$ and/or Rabbit anti-GFAP at 4 $\mu\text{g}/\text{mL}$) diluted in 5% goat serum at 4°C. Ensure that the cell surfaces are covered uniformly with the antibody solution.
 3. Wash the cells three times for 5 minutes with D-PBS containing Ca^{2+} and Mg^{2+} (if using a slide, use a staining dish with a magnetic stirrer).
 4. Incubate the cells with fluorescence-labeled secondary antibody (Alexa Fluor[®] 488 goat-anti mouse (H+L) at 10 $\mu\text{g}/\text{mL}$ and/or Alexa Fluor[®] 594 goat-anti rabbit (H+L) at 10 $\mu\text{g}/\text{mL}$) diluted in 5% goat serum solution for 60 minutes at room temperature.
 5. Wash the cells three times with D-PBS containing Ca^{2+} and Mg^{2+} . In the last wash, counter-stain the cells with DAPI solution (3 ng/mL) for 10 minutes.
 6. Rinse the cells with D-PBS, and if desired, mount using 3 drops of ProLong[®] Gold antifade reagent per slide and seal it with the cover slip. You may store the slides in the dark at 4°C.
 7. Observe the cells under the microscope using filters for FITC, Cy5, and DAPI.

Expected Results

The thawed cortical neurons cultured in Neurobasal[®] medium supplemented with 2% B-27[®] Serum-Free Supplement and 0.5 mM GlutaMAX[™]-I show > 90% neuronal population stained with MAP2 antibody and a minimum number of astrocytes. Within 3–4 days in culture, the neurons display extensive neurite outgrowth that keeps on increasing as long as the neurons are kept healthy in culture. Note that results vary if the neurons are cultured in the presence of serum.

Figure 1 Primary Rat Cortical neurons. Immunofluorescence detection of primary neuronal cells stained with mouse anti-MAP2 antibody (Green) and presence of astrocytes as detected by rabbit anti-GFAP marker (Red). Nuclei are stained with DAPI (blue).



Troubleshooting

The procedures are designed for neuronal cells grown in Neurobasal[®] medium supplemented with 2% B-27[®] Serum-Free Supplement and 0.5 mM GlutaMAX[™]-I. Results may differ with culture systems grown in other complete media formulations, which can result in higher numbers of cells other than neurons (i.e., astrocytes).

References

- Brewer, G. J. and Price, P.J. 1996. Viable cultured neurons in ambient carbon dioxide and hibernation storage for a month. *Neuroreport* 7:1509–1512.
- Brewer, G. J., Torricelli, J.R., Evege, E. K., and Price, P.J. 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J Neurosci Res.* 35:567–576.

Derivation of Dopaminergic Neurons (from Human Embryonic Stem Cells)

Summary

Directed differentiation of specific lineages has been a focal point in the field of human embryonic stem cell (hESC) research. Cell replacement therapy using hESCs have the potential for treating Parkinson's disease and other neurodegenerative disorders. This chapter describes the procedure for the derivation of dopaminergic (DA) neurons from hESCs.

Required Materials

- | | |
|---------------------------|--|
| Cells | <ul style="list-style-type: none">• GIBCO® Mouse Embryonic Fibroblasts (MEF), irradiated (Cat. no. S1520-100)• Human embryonic stem cells (hESC) |
| Media and Reagents | <ul style="list-style-type: none">• Dulbecco's Phosphate-Buffered Saline (D-PBS) without Ca^{2+} and Mg^{2+} (Cat. no. 14190)• D-MEM/F-12 with GlutaMAX™-I (Cat. no. 10565-018)• Neurobasal® Medium (Cat. no. 21103-049)• Knockout™ Serum Replacement (Cat. no. 10828-028)• 10% Bovine Serum Albumin (BSA) (Cat. no. P2489)• Fetal Bovine Serum (FBS) (Cat. no. 16000-044)• Dulbecco's Modified Eagle Medium (D-MEM) (Cat. no. 10569-010)• Non-essential Amino Acids Solution (NEAA) (Cat. no. 11140)• B-27® Supplement without Vitamin A (Cat. no. 12587-010)• N-2 supplement (Cat. no. 17502-048)• β-Mercaptoethanol (Cat. no. 21985-023)• Attachment Factor (Cat. no. S-006-100)• Natural Mouse Laminin (Cat no. 23017-015)• StemPro® Accutase® Cell Dissociation Reagent (Cat. no. A11105-01)• Recombinant Human FGF Basic (bFGF) (Cat. no. 13256-029)• FGF-8b Recombinant Human (Cat. no. PHG0271)• B-DNF Recombinant Human (Cat. no. PHC7074)• G-DNF Recombinant Human (Cat. no. PHC7045)• Trypan Blue Stain (Cat. no. 15250-061)• Distilled water (Cat. no. 15230-162)• Poly-L-Ornithine (Sigma, Cat. no. P3655)• Heparin (Sigma, Cat. no. H3149)• Ascorbic Acid (Sigma, Cat. no. A4403)• Dibutyryl cyclic-AMP (dcAMP) (Sigma, Cat. no. D0627)• Recombinant human sonic hedgehog (SHH) (R&D systems, Cat. no. 1314-SH-025) |

- Special Tools**
- StemPro® EZPassage™ Disposable Stem Cell Passaging Tool (Cat. no. 23181-010)
 - Cell scraper (Fisher, Cat. no. 087711A)

Preparing Media

Stock Solutions **Knockout™ Serum Replacement (KSR)**

Thaw a bottle of KSR, prepare 50 mL aliquots, and store at -20°C . Use KSR within a week of thawing.

Recombinant human FGF, basic

Prepare a $10\text{-}\mu\text{g/mL}$ stock solution in D-PBS with 0.1% BSA, aliquot into sterilized tubes, and store at -20°C .

Heparin

Prepare a 2-mg/mL stock solution in D-PBS, aliquot 0.5 mL into sterilized tubes, and store at -80°C .

Ascorbic acid

Prepare a 200-mM stock solution in D-PBS, aliquot 0.5 mL into sterilized tubes, and store at -20°C .

Recombinant human sonic hedgehog

Prepare a 0.2-mg/mL stock solution in D-PBS with 0.1% BSA, aliquot into sterilized tubes, and store at -20°C .

Recombinant human FGF8b

Prepare a 0.1-mg/mL stock solution in D-PBS with 0.1% BSA, aliquot into sterilized tubes, and store at -20°C .

Recombinant human BDNF

Prepare a $25\text{-}\mu\text{g/mL}$ stock solution in D-PBS with 0.1% BSA, aliquot into sterilized tubes, and store at -20°C .

Recombinant human GDNF

Prepare a $20\text{-}\mu\text{g/mL}$ stock solution in D-PBS with 0.1% BSA, aliquot into sterilized tubes, and store at -20°C .

Dibutryl cyclic-AMP (dcAMP)

Prepare a 1-mM stock solution in distilled water, aliquot 0.5 mL into sterilized tubes, and store at -20°C .

Poly-L-Ornithine

Prepare a 10-mg/mL stock solution in distilled water, aliquot 0.5 mL into sterilized tubes, and store at -20°C .

Mouse Embryonic Fibroblast (MEF) Medium

To prepare 100 mL of MEF medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally.

Component	Amount
D-MEM	90 mL
FBS	10 mL

Human Embryonic Stem Cell (hESC) Medium

To prepare 100 mL of hESC medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. hESC medium lasts for up to 7 days at 4°C.

Component	Amount
D-MEM/F-12	79 mL
Knockout™ Serum Replacement	20 mL
NEAA	1 mL
Basic FGF Solution	40 µL
β-Mercaptoethanol*	182 µL
*Add β-Mercaptoethanol (final 0.1 mM) at the time of medium change.	

Neural Induction Medium

To prepare 100 mL of neural induction medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. Neural induction medium lasts for up to 7 days at 4°C.

Component	Amount
D-MEM/F-12	98 mL
N-2 Supplement	1 mL
NEAA	1 mL
Basic FGF Solution	200 µL
Heparin Solution	100 µL

Neural Expansion Medium

To prepare 100 mL of neural expansion medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. Neural expansion medium lasts for up to 7 days at 4°C.

Component	Amount
D-MEM/F-12	96 mL
N-2 Supplement	1 mL
B-27 [®] Supplement	2 mL
NEAA	1 mL
Basic FGF Solution	200 µL
Heparin Solution	100 µL

DA Neuronal Differentiation Medium

To prepare 100 mL of DA neural differentiation medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. DA neural differentiation medium lasts for up to 7 days at 4°C.

Component	Amount
Neurobasal [®] Medium	96 mL
L-Glutamine	1 mL
B-27 [®] Supplement	2 mL
NEAA	1 mL
GDNF Solution*	100 µL
BDNF Solution*	100 µL
Ascorbic Acid Solution*	100 µL
dcAMP Solution*	100 µM
*Add GDNF, BDNF, ascorbic acid, and dcAMP at the time of medium change.	

Preparing MEF Culture Vessels**Gelatin Coating Culture Vessels**

1. Cover the whole surface of each culture vessel with Attachment Factor solution (1 mL for each well of a 6-well plate, 2 mL into each 60-mm dish, or 4 mL into each 100-mm dish) and incubate for 1 hour at room temperature. Wash once with distilled water before plating the MEF.

Note: AF is sterile 1X solution containing 0.1% gelatin, available from Invitrogen.

Thawing MEFs

1. Wearing eye protection and ultra low-temperature cryo-gloves, remove the vials of irradiated MEF from the liquid nitrogen storage tank using metal forceps.

Note: Transfer the vials into a container with a small amount of liquid nitrogen if the vials are exposed to ambient temperature for more than 15 seconds between removal and step 3.
2. Briefly roll the vials containing MEF between your hands for about 10–15 seconds to remove frost and swirl them gently in a 37°C water bath. Do not submerge the vials completely.
3. When only a small amount of ice remains in the vials, remove them from the water bath. Spray the outside of the vials with 70% ethanol before placing them in the cell culture hood.
4. Pipet the thawed cells gently into a 15-mL conical tube using a 1-mL pipette.
5. Rinse the cryovial with 1 mL of pre-warmed MEF medium. Transfer the medium to the same 15-mL tube containing the cells.
6. Add 4 mL of pre-warmed MEF medium dropwise to the cells. Gently mix by pipetting up and down.

Note: Adding the medium slowly helps cells to avoid osmotic shock.
7. Centrifuge the cells at $200 \times g$ for 5 minutes.
8. Aspirate the supernatant and resuspend the cell pellet in 5 mL of pre-warmed MEF medium.
9. Remove 10 μ L of cell suspension and determine the viable cell count using your method of choice.

Note: We recommend using the Countess[®] Automated Cell Counter for easy and accurate cell counting and viability measurements.

Plating MEFs

1. Centrifuge the MEFs at $200 \times g$ for 5 minutes and aspirate the supernatant.
2. Resuspend the cell pellet in MEF medium to a concentration of 2.5×10^6 cells/mL.
3. Aspirate the Attachment Factor solution from the coated culture vessels and wash the plates once with D-PBS.
4. Add the appropriate amount of MEF medium into each culture vessel (2.5 mL into each well of 6-well plate, 5 mL into each 60-mm dish, or 10 mL into each 100-mm dish).
5. Into each of these culture vessels, add the appropriate amount of MEF suspension (0.1 mL into each well of 6-well plate, 0.2 mL into each 60-mm dish, or 0.6 mL into each 100-mm dish). The recommended plating density for GIBCO[®] Mouse Embryonic Fibroblasts (Irradiated) is 2.5×10^4 cells/cm².
6. Move the culture vessels in several quick back-and-forth and side-to-side motions to disperse cells across the surface of the wells and dishes. After plating the cells, place the vessels in a 37°C incubator with a humidified atmosphere of 5% CO₂. Use the MEF plates and dishes within 3–4 days of plating.

Thawing and Plating hESCs

1. Wearing eye protection and ultra low-temperature cryo-gloves, remove a vial of hESCs from the liquid nitrogen storage tank using metal forceps.
2. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
3. When only a small amount of ice remains in the vial, remove it from the water bath. Spray the outside of the vial with 70% ethanol before placing it in the cell culture hood.
4. Transfer the cells gently into a sterile 15-mL conical tube using a 1-mL pipette. Rinse the vial with 1 mL of pre-warmed hESC medium to collect the remaining cells in the vial and add them dropwise to the cells in the 15-mL conical tube.

Note: Adding the medium slowly helps cells to avoid osmotic shock.

5. Add 4 mL of pre-warmed hESC medium dropwise to the cells in the 15-mL conical tube. While adding the medium, gently move the tube back and forth to mix hESCs.
6. Centrifuge the cells for 5 minutes at $200 \times g$.
7. Aspirate the supernatant and resuspend the cell pellet in 5 mL of pre-warmed hESC medium.
8. Label the culture vessel containing inactivated MEFs with the passage number of the hESCs from the vial, the date and your initials.
9. Aspirate the MEF medium from the culture vessel containing the MEFs and gently add the resuspended hESCs into the vessel.
10. Move the culture vessel in several quick back-and-forth and side-to-side motions to disperse the cells across the surface of the vessel. Place the vessel gently into a 37°C incubator with a humidified atmosphere of 5% CO₂.
11. Replace the spent medium and examine the cells under a microscope daily. If feeding cells in more than one vessel, use a different pipette for each vessel to reduce the risk of contamination.

Note: hESC colonies may not be visible in the first several days.

12. Observe the hESCs every day and passage the cells whenever the colonies are too big or too crowded. The ratio of splitting depends on the total number of hESC in the culture vessel (approximately 1:2 to 1:4 for hESCs at the first time of recovery).

Passaging hESCs

General Guidelines

- In general, split cells when the first of the following events occurs:
 - MEF feeder layer is two weeks old.
 - hESC colonies are becoming too dense or too large.
 - Increased differentiation occurs.
- The split ratio varies, but it is generally between 1:4 and 1:6.
- Occasionally hESCs grow at a different rate, requiring the split ratio to be adjusted. A general rule is to observe the last split ratio and adjust the ratio according to the appearance of the hESC colonies.
- If the cells look healthy and colonies have enough space, split them using the same ratio as the previous passage. If the cells are overly dense and crowded, increase the split ratio; if the cells are sparse, decrease the ratio.
- Generally, hESCs need to be split every 4–10 days based upon their appearance.

Passaging hESCs

1. Two days prior to passaging your hESC culture, prepare fresh MEF culture vessels.
2. Remove the culture vessel containing hESCs from the incubator. Mark differentiated colonies under a microscope using a microscopy marker and remove them by aspirating with a Pasteur pipette in the culture hood.
3. Add an appropriate amount of pre-warmed hESC medium into each culture vessel (2 mL for each 60-mm dish or 4 mL for each 100-mm dish).
4. Roll the StemPro® EZPassage™ Disposable Stem Cell Passaging Tool across the entire vessel in one direction (left to right). Rotate the culture vessel 90 degrees and roll the tool across the entire dish again.
5. Using a cell scraper, gently detach the cells off the surface of the culture vessel. Gently transfer the cell clumps into a 15- or 50-mL conical tube using a 5-mL pipette.
6. Rinse the culture vessel with an appropriate amount of pre-warmed hESC medium (1 mL for each 60-mm dish or 2 mL for each 100-mm dish) to collect remaining cells.
7. If some cell clumps are too big, pipet the cell solution up and down several times using a 5-mL pipette to break the cell clumps into smaller pieces.
8. Aspirate the MEF medium from each MEF culture vessel and replace it with an appropriate amount of pre-warmed hESC medium (5 mL for each 60-mm dish or 10 mL for each 100-mm dish).
9. Gently shake the conical tube containing the hESCs to distribute the cell clumps evenly and add an appropriate amount of hESC suspension into each MEF culture vessel.

Note: The volume of hESC suspension added into each dish depends on the ratio of splitting (see General Guidelines, above).

10. Move the culture vessels in several quick back-and-forth and side-to-side motions to disperse the hESCs across the surface of the vessels. Place the culture vessels gently in a 37°C incubator with a humidified atmosphere of 5% CO₂.
11. Replace the spent medium daily. hESCs need to be split every 4–10 days based upon their appearance.

Differentiating hESCs

Making Embryoid Bodies

(EBs)

1. Culture the hESCs on MEF feeder cells until they are 90–100% confluent.
2. Roll the StemPro® EZPassage™ Disposable Stem Cell Passaging Tool across the entire vessel in one direction (left to right). Rotate the culture vessel 90 degrees and roll the tool across the entire dish again.
3. Using a cell scraper, gently detach the cells off the surface of the culture vessel. Gently transfer the cell clumps into a 50-mL conical tube using a 5-mL pipette.

Note: Do not break the cells clumps into smaller pieces.
4. Add 1 mL of pre-warmed hESC EB medium into each well of 6-well plate, 2 mL into each 60-mm dish, or 3 mL into each 100-mm dish to collect remaining cells and add them to the 50-mL conical tube containing the hESC.
5. Centrifuge the cells for 5 minutes at $200 \times g$.
6. Aspirate the supernatant from the hESC pellet. Gently re-suspend the pellet with an appropriate amount of EB medium (15 mL for all the cells from one 60-mm dish or 40 mL for all cells from one 100-mm dish).
7. Transfer the cell clumps to an uncoated T-75 flask for a couple of hours. This allows the fibroblasts to differentially attach to the flask.
8. After a few hours, set the T-75 flask down at a tilted angle to allow the EBs to settle in one corner of the flask. Aspirate the EB medium and replace it with 40 mL of fresh EB medium. Transfer the cell clumps to a fresh T-75 flask and incubate them in a 37°C incubator with a humidified atmosphere of 5% CO₂.
9. Feed the EBs with EB medium every day for 4 days. When feeding, set the flask down at a tilted angle so that the EBs settle in one corner of the flask. Aspirate almost all spent EB medium, replace it with pre-warmed EB medium, and return flask to the incubator.

Note: Due to DNA release from dead cells, cell clumps may stick together. In this case, gently pipet the EBs up and down 2–3 times using a 5-mL pipette. This will help you clean the dead cells off the EB surface. If the EBs attach to flask, use a 5-mL pipette to blow the attached EBs off the bottom of the flask.

Differentiating EBs (Rosette Formation) and Midbrain Specification

1. After culturing the EBs in EB medium for 4 days, transfer the EBs from one T-75 flask into a 50-mL centrifuge tube and centrifuge for 3 minutes at $200 \times g$.
2. Aspirate the EB medium and resuspend the EBs in 10 mL of pre-warmed neural induction medium.
3. Centrifuge the EBs for 3 minutes at $200 \times g$.

4. Aspirate the supernatant and resuspend the EBs in 40 mL of pre-warmed neural induction medium. Transfer the EBs into a fresh T-75 flask and incubate the EBs in neural induction medium for 2 days in a 37°C incubator with a humidified atmosphere of 5% CO₂.

After the EBs float in the neural induction medium for 2 days, they are ready to be differentiated.

Note: If the EB attach to the flask, use a 5-mL pipette to blow the attached EBs off the bottom of the flask.

5. Dilute laminin in D-PBS to 20 µg/mL and coat ten 100-mm culture dishes using 2.5–3 mL of laminin for each dish. Incubate the laminin-coated culture dishes in a 37°C incubator for several hours.

Note: Laminin may form a gel when thawed too rapidly. To avoid this, thaw slowly in the cold (2°C–8°C). Once thawed, aliquot into polypropylene tubes and store at –5°C to –20°C. Do not freeze and thaw laminin repeatedly.

6. After incubation, aspirate the laminin and add 10 mL of pre-warmed neural induction medium into each 100 mm dish.
7. Transfer the EBs from the T-75 flask into a 50-mL tube and centrifuge for 3 minutes at 200 × g.
8. Aspirate the supernatant and resuspend the EBs in 10 mL of pre-warmed neural induction medium.
9. Gently shake the 50-mL tube containing EBs to distribute the EBs evenly and add 1 mL of EB suspension into each laminin-coated culture dish.
10. Move the culture dishes in several quick back-and-forth and side-to-side motions to disperse the EBs across the surface of the dishes. Place the dishes gently in a 37°C incubator with a humidified atmosphere of 5% CO₂.
11. Feed the EBs every other day with fresh pre-warmed neural induction medium until early rosettes form (approximately 2–3 days).
12. To direct the neural precursors to the midbrain fate, feed the differentiating EBs every other day with neural induction medium containing 100 ng/mL FGF-8b and 200 ng/mL sonic hedgehog (SHH) for 5–6 days.

Note: Plate the EBs at a density of 200–250 per one 100-mm dish. Generally, all EBs from hESCs cultured in one 100-mm dish can be plated into eight to ten 100-mm dishes. The variation is from the confluence of hESCs and efficacy of EB formation

Isolating DA Progenitors

1. Label all differentiating colonies containing rosettes using a microscope marker.
2. Using a 200-µL pipette tip pointing to the center of each marked colony, blow off the cells in rosettes.
3. Use a 10-mL pipette to transfer the detached cell clumps into a 50-mL centrifuge tube.

Note: You can combine the cell clumps from five 100-mm dishes into one 50-mL tube.
4. Centrifuge the cells for 3 minutes at 200 × g.

5. Aspirate the supernatant and resuspend the cell clumps in 40 mL of neural expansion medium containing 100 ng/mL FGF-8b and 200 ng/mL SHH.
6. Transfer the cell clumps to a T-75 flask and place the flask in a 37°C incubator with a humidified atmosphere of 5% CO₂.

The rosettes will roll up to form neurospheres after about 1 day in the incubator.

7. Replace half of the neural expansion medium containing 100 ng/mL FGF-8b and 200 ng/mL SHH with fresh medium every other day.

Note: Contaminating non-neural cells tend to attach to the flask. When changing the medium, set the flask down at a tilted angle to allow the neurospheres to settle in one corner of the flask. Aspirate half of the neural expansion medium and use a 10-mL pipette to transfer the neurospheres with the rest of the spent neural expansion medium to a fresh T-75 flask. Add 20 mL of pre-warmed fresh neural expansion medium to the flask and incubate in a 37°C incubator with a humidified atmosphere of 5% CO₂.

You can perform this procedure several times to purify the neural cells.

- DA Neuron Differentiation**
1. Coat the surface of the culture vessel (with or without cover slips) with poly-L-ornithine working solution at 20 µg/mL in distilled water (14 mL for T-75, 7 mL for T-25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish) and incubate the vessel overnight at room temperature.
 2. Wash the poly-L-ornithine-coated vessel 4 times with distilled water, and then coat it with laminin working solution at 10 µg/mL in D-PBS without calcium or magnesium (14 mL for T-75, 7 mL for T-25, 3.5 mL for 60-mm dish, 2 mL for 35-mm dish). Incubate the culture vessel for 3 hours at 37°C.
- Note:** You may coat the culture vessels in advance, replace the laminin solution with D-PBS without calcium or magnesium, and store them wrapped tightly in Parafilm for up to 1 week. Make sure that the culture vessels do not dry out.
3. After the neurospheres float in neural expansion medium for 6–8 days, transfer them into a 15-mL tube and centrifuge for 5 minutes at 200 × g.
 4. Aspirate the supernatant and incubate the neurospheres in pre-warmed StemPro® Accutase® Cell Dissociation Reagent for 10 minutes at 37°C.
 5. Gently pipet the cell clumps up and down to break the larger clumps into a single cell suspension.
 6. Centrifuge the cells for 5 minutes at 200 × g and aspirate the supernatant.
 7. Resuspend the cells in 10 mL of pre-warmed neural differentiation medium.
 8. Repeat steps 6 and 7.
 9. Aspirate the laminin from the coated culture vessels and plate the dissociated DA progenitors.
 10. Incubate the cells in a 37°C incubator with a humidified atmosphere of 5% CO₂ and replace the spent medium with fresh neural differentiation medium every other day.
 11. You can evaluate DA neuron differentiation 3–4 weeks after plating.

Derivation and Culture of Dopaminergic Neurons (from Midbrains of Rodents)

Summary

Dopaminergic (DA) neurons are located in the ventral midbrain (VM). The ability to isolate precursor cells and neurons from the VM provides a powerful means to characterize their differentiation properties and to study their potential for restoring dopamine neurons degenerated in Parkinson's disease (PD). This chapter describes methods to differentiate precursor cells derived from embryonic ventral mesencephalon into DA neurons.

Required Materials

Embryos • Embryonic (E14) rats or embryonic (E13) mice

Media and Reagents • Water, distilled (Cat. no. 15230-162)
• Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (Cat. no. 14170-112)
• Dulbecco's Phosphate-Buffered Saline (D-PBS) without Ca^{2+} and Mg^{2+} (Cat. no. 14190)
• D-Glucose (Sigma, Cat. no. G8270)
• Penicillin-Streptomycin (Cat. no. 15070-063)
• Ascorbic Acid (Sigma, Cat. no. A4034)
• StemPro[®] Accutase[®] Cell Dissociation Reagent (Cat. no. A11105-01)
• Trypan Blue (Cat. no. 15250-061)
• Natural Mouse Laminin (Cat. no. 23017-015)
• Poly-L-Ornithine (Sigma, Cat. no. P4957)
• L-Glutamine (Cat. no. 25030-081)
• Neurobasal[®] Medium (Cat. no. 21103-049)
• B-27[®] Serum-Free Supplement (Cat. no. 17504-044)
• Heat-inactivated Fetal Bovine Serum (FBS) (Cat. no. 10438)

Special Tools • Microdissecting instruments (sterilized)
– Small dissecting scissors
– Medium dissecting scissors
– Dumont forceps, straight
– Dumont forceps, angled or curved
– Curved microdissecting scissors
– Spatula, Moria perforated spoon with holes (e.g., Moria MC17)
• Dissecting microscope (e.g., Leica MZ6 or Zeiss Stemi 2000)
• Curved scalpel blade (e.g., BD Bard-Parker no. 23 or 24)

Preparing Reagents

Poly-L-Ornithine Stock Solution

Make a 10-mg/mL stock solution of poly-L-ornithine in distilled water. Filter-sterilize using a 0.22- μ m filter and store for up to 12 months at -20°C .

Ascorbic Acid Stock Solution

Make a 200-mM stock solution of ascorbic acid in D-PBS. Filter-sterilize using a 0.22- μ m filter. Protect from light and store for up to 12 months at -20°C .

Dissection Buffer

For 100 mL of dissection buffer, aseptically mix the following components. The buffer can be stored at 4°C for 1 week. Add ascorbic acid solution before use.

Component	Amount
HBSS	98 mL
D-Glucose	360.3 mg
Penicillin/Streptomycin	2 mL
Ascorbic Acid Solution	0.1 mL

Preparing Media

Differentiation Medium

For 100 mL of differentiation medium, aseptically mix the following components. The medium can be stored at 4°C for 1 week and add ascorbic acid solution before use.

Component	Amount
Neurobasal [®] Medium	98 mL
L-Glutamine	1 mL
B-27 [®] Supplement	2 mL
FBS	1 mL
Ascorbic Acid Solution	0.1 mL

Preparing Matrix

Matrix for Midbrain Neural Cell Culture

1. Prepare a 1:500 dilution of poly-L-ornithine in distilled water for a final concentration of 20 µg/mL.
2. Add 2 mL of 20 µg/mL poly-L-ornithine solution to a 35-mm dish (0.5 mL for a 4-well plate or slide, 0.25 mL for a 8-well slide).
3. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO₂ for at least 2 hours.
4. Rinse the culture vessel once with distilled water.
5. Prepare a 1:100 dilution of laminin in distilled water for a final concentration of 10 µg/mL.
6. Add 2 mL of 10 µg/mL laminin solution to a 35-mm dish (0.5 mL for a 4-well plate or slide, 0.25 mL for a 8-well slide).
7. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO₂ for at least 2 hours. Store at 4°C until use.

Note: You may coat the plates in advance and store them at 2–8°C, wrapped tightly with Parafilm®, for up to 4 weeks.

Isolating and Culturing Precursor Cells from the Ventral Midbrain

Except for the initial steps of collecting the uterine horns, work under sterile conditions in a laminar flow hood, or add antibiotics (penicillin/streptomycin at standard concentrations) to reagents. Perform the steps in a timely manner, and keep the tissue cooled on ice and immersed in ice-cold buffers throughout the procedure.

Collecting Embryos

1. Using aseptic technique, collect the uterine horns from a time-pregnant rat (staged to E14 of gestation) or mouse (staged to E13 of gestation).
2. Submerge uterine horns in a 100-mm petri dish containing ice-cold, sterile HBSS, and carefully rinse 2–3 times with 15 mL ice-cold, sterile HBSS.
3. Transfer the uterine horns to a clean 100-mm petri dish containing dissection buffer.
4. Under a dissection microscope placed in a laminar flow hood, dissect each embryo from the uterine sac and remove the amniotic membranes.
5. Use a Morian-type perforated spoon to transfer the embryo to a clean sterile petri dish containing ice-cold dissection buffer.
6. Confirm the gestational age by measuring and recording the crown rump length of the embryos (10–12 mm for E14 rat or E13 mouse embryos). Exclude any malformed or otherwise damaged embryos.

- Dissecting Brains**
1. Decapitate each fetus using microdissection scissors or a scalpel.
 2. Hold the tissue with forceps near the forebrain or hindbrain region to avoid damage to the midbrain region of interest. Carefully dissect and remove the overlying scalp tissue to isolate the brain.
 3. Place the isolated brain in a clean 60-mm petri dish containing dissection buffer on ice.
 4. Stabilize the brain with forceps near the forebrain or hindbrain regions, carefully remove and discard the fore- and hindbrain regions using a scalpel or microscissors. Make the rostral cut close to the forebrain vesicles and thalamic region, and the caudal cut at the isthmus region.

**Dissecting the Ventral
Midbrain**

1. Steady the obtained midbrain tube with forceps exclusively at the posterior midbrain region marked by the convex curvature at the dorsal midline.
2. Use small microscissors or the very tip of a curved scalpel blade to gradually dissect open the midbrain tube along the dorsal midline.
3. Carefully open the now characteristically butterfly-shaped tissue flap.
4. Use forceps to thoroughly remove any remaining overlying meningeal tissue.
5. Trim the outermost (most dorsal) areas of the midbrain tube by dissecting away two thirds of the tissue on each side (approximately lateral/posterior to the sulcus limitans as an anatomical landmark).
6. Transfer the resulting tissue piece (~0.3 mm × 1.0 mm in dimension) into a conical tube containing cold dissection buffer kept on ice. Use ~0.2 to 0.5 mL of buffer volume for each piece of VM tissue.

- Dissociating Cells**
1. Wash the pieces of VM tissue in cold dissection buffer (e.g., 15 mL of buffer in a 15-mL conical tube) by letting the tissue pieces sink to the bottom of the conical tube. Aspirate the medium, and fill the tube with fresh buffer.
 2. Aspirate the buffer and add 1 mL StemPro® Accutase® for every 10 pieces of VM tissue. Incubate the tissues 3–15 minutes at 37°C. Observe the digestion process and determine the optimal duration by test dissociation and homogenization. Avoid over-digestion,
 3. Using fire-polished Pasteur pipets with decreasing diameter, gently dissociate the tissue pieces by pipetting the tissue up and down for a total of ~20 times. Alternatively, you may dissociate and homogenize the tissue by first using a pipettor with a 1000-μL tip, followed with a 200-μL tip. Avoid excessive formation of air bubbles during mechanical dissociation of VM tissue, as it reduces cell viability.
 4. If large pieces of tissue remain in the solution, selectively homogenize the pieces separately.
 5. *Optional:* Pipet the cell suspension through a cell strainer cap or through a 35- to 70-μm mesh. To minimize loss of cells from this filtering step, flush the filter membrane with a small volume of medium after the cell suspension is passed.
 6. Centrifuge the cell suspension at 4°C for 3–5 minutes at 200 × g. Aspirate the supernatant.

7. Resuspend the cells with differentiation medium. Use 200 μ L of differentiation medium for every 10 pieces of midbrain originally isolated.
8. Using aliquot of the cell suspension, determine the cell concentration and viability by dye exclusion method (Trypan Blue). Use the quantity of live cells counted for calculating the cell concentration. Cell viability needs to be >80%, and should ideally range from 95–100%.

DA neurons are among the most fragile cells in the solution. While the cultures will contain neuronal cell types after relatively harsh treatment, the number of DA neurons will be low.

9. Keep the cell suspension on ice or at 4°C until use.

Culturing Midbrain Neural

Cells

1. Aspirate the laminin from the poly-L-ornithine and laminin-coated culture plate and plate midbrain neural cells in differentiation medium at a density of 2×10^5 – 5×10^5 cells/cm².
2. Culture the cells in an incubator, changing medium every other day.
3. Culture the cells for 3–10 days, then check for DA neurons by immunocytochemical staining using antibodies against the neuronal marker β -III-tubulin, and the dopaminergic markers tyrosine hydroxylase (TH).

Cryopreserving Neural Stem Cells

Summary

There are numerous protocols available for cryopreserving neural stem cells (NSCs) derived from human embryonic stem cells; the primary objective of these methods are the recovery of the cells post-thaw and the retention of their multipotent properties. This chapter describes a standardized cryopreservation protocol that does not alter the viability and sublineage differentiation capacity of the preserved cells.

Required Materials

Cells

- Neural Stem Cells (NSCs)

Media and Reagents

- KnockOut™ D-MEM/F-12 (Cat. no. 12660)
- StemPro® NSC SFM (Cat. no. A10509-01)
- FGF-basic (AA 10–155), Recombinant Human (bFGF) (Cat. no. PHG0024)
- EGF, Recombinant Human (Cat. no. PHG0314)
- TrypLE™ Select (1X) (Cat. no. 12563-029)
- Dulbecco's Phosphate-Buffered Saline (D-PBS) (Cat. no. 14190-144)
- DMSO (Dimethylsulphoxide) (Sigma, Cat. no. D2650)

Tools and Equipment

- Sterile 15-mL conical tubes
- Tabletop centrifuge
- Syringe filter
- Cryovials
- Cryo 1°C Freezing Container (Nalgene, Cat. no. 5100-0001)

Preparing Media

StemPro® NSC SFM Complete Medium

StemPro® NSC SFM complete medium consists of KnockOut™ D-MEM/F-12 with StemPro® Neural Supplement, EGF, bFGF, and GlutaMAX™-I. Complete medium is stable for 4 weeks when stored in the dark at 2–8°C.

To prepare 50 mL of StemPro® NSC SFM complete medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. If desired, add 0.5 mL of Antibiotic-Antimycotic solution per 50 mL of complete medium.

Component	Final concentration	Amount
KnockOut™ D-MEM/F-12	1X	48.5 mL
GlutaMAX™-I Supplement	2 mM	0.5 mL
bFGF	20 ng/mL	1 µg
EGF	20 ng/mL	1 µg
StemPro® Neural Supplement	2%	1 mL
You may observe a white precipitate when thawing StemPro® Neural Supplement; this precipitate will disappear when the supplement is completely thawed or dissolved.		

Freezing Medium

To prepare 10 mL of freezing medium, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. Filter sterilize the freezing medium and store at 2–8°C until use.

Component	Final concentration	Amount
StemPro® NSC SFM Complete Medium without bFGF and EGF (see above)	90%	9 mL
DMSO	10%	1 mL

Cryopreserving Neural Stem Cells

Guidelines for Cryopreserving Neural Stem Cells

- Cryopreserve NSCs when they are 80–90% confluent (2–4 days after seeding).
- Freeze NSCs at a concentration of 2×10^6 – 2.4×10^6 viable cells/mL and a volume of 1 mL/vial.
- Use a freezing medium composed of 90% complete StemPro® NSC SFM without the growth factors (i.e., bFGF and EGF) and 10% DMSO.
- Do not incubate the NSCs in TrypLE™ Select for more than 2 minutes to avoid cell death.
- Pre-label all cryovials with the following information: cell line, passage number, concentration, date of freezing, and your initials.

- Freezing Neural Stem Cells**
1. When NSCs are 80–90% confluent (2–4 days after seeding), aspirate the complete StemPro® NSC SFM from the culture vessel.
 2. Wash the cells twice with D-PBS. Aspirate the D-PBS and discard.
 3. Add 1 mL of pre-warmed TrypLE™ Select to the culture vessel and incubate at 37°C for 2 minutes.

Note: Do not incubate the NSCs in TrypLE™ Select for more than 2 minutes to avoid cell death. Neutralize TrypLE™ Select by adding complete StemPro® NSC SFM immediately after the incubation period (see below).
 4. Detach the NSCs from the culture vessel by pipetting off the cells or by tapping the culture vessel against the heel of your hand.
 5. Stop the TrypLE™ Select treatment by adding 5 mL of complete StemPro® NSC SFM.
 6. Gently pipet the NSCs up and down to get a single cell suspension and transfer the cell suspension into a sterile 15-mL conical tube.
 7. Centrifuge the NSCs at $200 \times g$ for 5 minutes. Aspirate the supernatant and discard.
 8. Resuspend the cell pellet in a minimal volume of pre-warmed complete StemPro® NSC SFM and remove a sample for counting.
 9. Determine the total number of cells using your method of choice.
 10. Gently aspirate the medium from the conical tube and drop-wise add pre-chilled (4°C) freezing medium to resuspend the cells at a concentration of 2×10^6 – 2.4×10^6 viable cells/mL.
 11. Transfer 1 mL of the NSC suspension in freezing medium into each pre-labeled, pre-chilled (4°C) cryovial.
 12. Transfer the cryovials to the Cryo 1°C Freezing Container and place the container into a –80°C freezer. This procedure ensures that the cells freeze slowly.
 13. The next day, transfer the cells into a liquid nitrogen.

Cryopreservation and Recovery of Mature Differentiated Neural Cells

Summary

Primary neuronal cultures are indispensable in the field of neurobiology and pharmacology. Many researchers favor freshly isolated neuronal cells as they maintain their functional viability, but for convenience, an alternate route is to cryopreserve fresh cells for later use. This chapter describes the generation of cryopreserved stocks from the freshly isolated neural cells, and thawing procedures for recovering the stocks.

Required Materials

- | | |
|---------------------------|--|
| Rat Brain Cells | <ul style="list-style-type: none">• Homogenous cell preparation from E18 rat brain tissue as described in Chapter 9, Isolation, Culture, and Characterization of Cortical and Hippocampal Neurons (page 40). |
| Media and Reagents | <ul style="list-style-type: none">• Neurobasal[®] Medium (Cat. no. 21103-049)• B-27[®] Serum-Free Supplement (Cat. no. 17504-044)• GlutaMAX[™]-I (Cat. no. 35050-061)• Trypan Blue (Cat. no. 15250-061)• Synth-a-Freeze[®] Cryopreservation Medium (Cat. no. R-005-50) |
| Equipment | <ul style="list-style-type: none">• Cryogenic Vials• Isopropanol Chamber• Freezer, -80°C• Liquid nitrogen freezer• Water bath set to 37°C |

Cryopreservation

- | | |
|------------------------------|---|
| Freezing Neural Cells | <ol style="list-style-type: none">1. Isolate and prepare a suspension of rat brain cells in Neurobasal[®] medium supplemented with 2% B-27[®] as described in Chapter 9, Isolation, Culture, and Characterization of Cortical and Hippocampal Neurons (page 40).2. Count the cell number using a hemocytometer.3. Centrifuge the cells at $200 \times g$ for 4 minutes. Aspirate the supernatant.4. Resuspend the cell pellet in cold Synth-a-Freeze[®] at a concentration of 2.0×10^6–1.0×10^7 cells/mL.5. Make 1 mL aliquots of the cells in pre-labeled, pre-chilled cryovials and place the vials in an isopropanol chamber at 4°C for 10 minutes. |
|------------------------------|---|

6. Transfer the isopropanol chamber to -80°C for overnight.
7. Transfer the frozen vials to the vapor phase of liquid nitrogen storage until use is required.

Cell Recovery

Recovering Frozen Neural Cells

Handle cells gently, because they are extremely fragile upon recovery from cryopreservation. It is important to rinse pipette tips and vials with complete Neurobasal[®]/B-27[®] medium before using them for transferring cell suspensions to avoid the cells sticking to the plastic. Do not centrifuge cells upon recovery from cryopreservation.

1. Remove one vial of frozen cells from liquid nitrogen.
2. Thaw the vial in a 37°C water bath with gentle swirling.
3. Wipe down the vial with ethanol and tap gently on a surface so that all of the medium collects at the bottom of the tube.
4. Open the vial in a laminar flow hood.
5. Rinse a pipette tip with medium and very gently transfer the cells from the vial to a pre-rinsed 15-mL tube.
6. Rinse the vial with 1 mL of pre-warmed complete Neurobasal[®]/B-27[®] medium, and transfer the rinse to the 15-mL tube containing the cells at a rate of one drop per second. Mix by gentle swirling after each drop.
7. Slowly add 2 mL of complete Neurobasal[®]/B-27[®] medium to the tube (for a total suspension volume of 4 mL).
8. Mix the suspension very gently with P-1000 pipette. Avoid creating any air bubbles.
9. Add 10 μL of cell suspension to a microcentrifuge tube containing 10 μL of 0.4% Trypan blue using a pre-rinsed tip. Mix the cells by gently tapping the tube. Determine the viable cell density using a hemocytometer.
10. Plate $\sim 1 \times 10^5$ cells per well in poly-D-lysine coated 48-well plate or an 8-chambered slide. Bring the cell suspension volume to 500 μL per well by adding complete Neurobasal[®]/B-27[®] medium.
11. Incubate the cells at 37°C in a humidified atmosphere of 5 % CO_2 in air.
12. Feed the cells every third day by aspirating half of the medium from each well and replacing it with fresh medium.

Cell Analysis

Cell Viability Assays for Neural Stem Cells

Summary

The LIVE/DEAD® Viability/Cytotoxicity Assay Kit provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead neural stem cells (NSCs) with probes that measure two recognized parameters of cell viability: intracellular esterase activity and plasma membrane integrity.

The polyanionic dye calcein AM is well-retained within live cells, producing an intense uniform green fluorescence in live cells (excitation/emission ~495 nm/~515 nm), while ethidium homodimer-1 (EthD-1) enters cells with damaged membranes to produce a bright red fluorescence in dead cells (excitation/emission ~495 nm/~635 nm).

Protocols are provided for fluorescence microscopy or microplate analysis of adherent cells, or flow cytometry analysis of cells in suspension.

Required Materials

- | | |
|---------------------------|---|
| Cells | <ul style="list-style-type: none">• Adherent or suspended NSCs |
| Media and Reagents | <ul style="list-style-type: none">• LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Cat. no. L-3224)<ul style="list-style-type: none">– Calcein AM– Ethidium homodimer-1 (EthD-1)• Dulbecco's Phosphate-Buffered Saline (D-PBS) (Cat. no. 14040) |
| Special Tools | <ul style="list-style-type: none">• Fluorescence microscope |

Note: Calcein and EthD-1 can be viewed simultaneously with a conventional fluorescein longpass filter. The fluorescence from these dyes may also be observed separately; calcein can be viewed with a standard fluorescein bandpass filter and EthD-1 can be viewed with filters for propidium iodide or Texas Red® dye.

Preparing Reagents

Prepare the reagents in the LIVE/DEAD® Viability/Cytotoxicity Assay Kit as follows:

1. Remove the stock solutions provided in the kit from the freezer and allow them to warm to room temperature.

2. Add 20 μL of the supplied 2 mM EthD-1 stock solution (Component B) to 10 mL of sterile, tissue culture–grade D-PBS. Vortex to ensure thorough mixing. This prepares a $\sim 4 \mu\text{M}$ EthD-1 solution.
3. Combine the reagents by adding 5 μL of the supplied 4-mM calcein AM stock solution (Component A) to the 10 mL of EthD-1 solution in D-PBS. Vortex the resulting solution to ensure thorough mixing.

Note: This reagent mixture is suitable for most neural cells. For cells with higher esterase activity, you might need to start with a lower calcein AM concentration. For further information, refer to the user manual provided with the LIVE/DEAD® Viability/Cytotoxicity Assay Kit.

The resulting working solution of $\sim 2 \mu\text{M}$ calcein AM and $\sim 4 \mu\text{M}$ EthD-1 is ready to be used. The final concentration of DMSO is $\leq 0.1\%$, a level generally innocuous to most cells.

Note: Prepare a freshly coated culture vessel each time before plating cells. There is no need to rinse the culture vessel before use.

Methods

Determining the Viability of Adherent Cells

Adherent NSCs may be cultured on sterile glass coverslips or in a multiwell plate.

1. Aspirate the medium supernatant and wash the cells gently with the same volume of D-PBS prior to the assay to remove or dilute any serum esterase activity.

Note: Serum esterases could cause some increase in extracellular fluorescence by hydrolyzing calcein AM.

2. **Fluorescence microscopy:** Transfer an aliquot of the cell suspension to a coverslip and allow the cells to settle on the surface at 37°C in a covered petri dish. Then add 100–150 μL of prepared LIVE/DEAD® reagent to the coverslip, so that all cells are covered by solution.

Microplate reader: Add an aliquot of the cell suspension to each microplate well in a sufficient volume to cover at least the bottom of each well. Then add an approximately equal volume of prepared LIVE/DEAD® reagent.

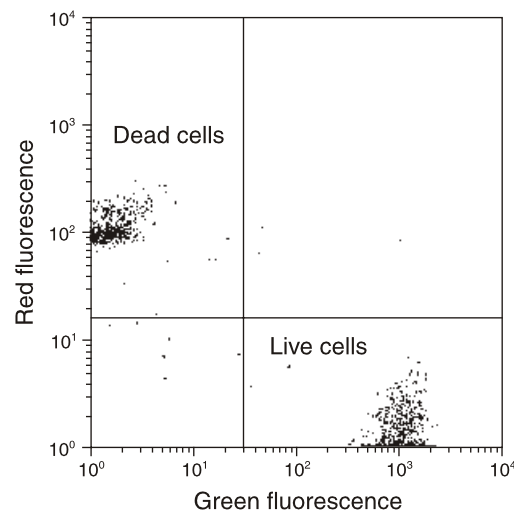
3. Incubate the cells at room temperature for 10–30 minutes. Measure fluorescence using the appropriate excitation and emission filters.
4. Analyze the sample under a fluorescence microscope or using a fluorescence microplate reader.

Determining Viability of Cells in Suspension with Flow Cytometry

Allow all the reagents to come to room temperature before proceeding.

1. Make an 80-fold dilution of calcein AM (Component A) in DMSO to make a 50 μM working solution (e.g., add 2 mL of calcein AM to 158 mL DMSO).
2. Prepare a 1-mL suspension of cells with 0.1×10^6 to 5×10^6 cells/mL for each assay. Cells may be in culture medium or buffer.
3. Add 2 μL of a 50- μM calcein AM working solution and 4 μL of the 2-mM EthD-1 stock to each milliliter of cells. Mix the sample.
4. Incubate the cells for 15–20 minutes at room temperature, protected from light.
5. As soon as possible after the incubation period (within 1–2 hours), analyze the stained cells by flow cytometry using 488 nm excitation and measuring green fluorescence emission for calcein (i.e., 530/30 bandpass) and red fluorescence emission for EthD-1 (i.e., 610/20 bandpass).
6. Gate on cells to exclude debris. Using single color-stained cells, perform standard compensation. The population should separate into two groups: live cells will show green fluorescence and dead cells will show red fluorescence (Figure 1).

Figure 1 Flow cytometry viability assay using the LIVE/DEAD® Viability/Cytotoxicity Kit. A 1:1 mixture of live and ethanol-fixed human B cells was stained with calcein AM and EthD-1 following the protocol provided. Flow cytometry analysis was performed with excitation at 488 nm. The resulting bivariate frequency distribution shows the clear separation of the green fluorescent (530 nm) live cell population from the red fluorescent (585 nm) dead cell population.



Markers for Characterizing Neural Subtypes

Summary

After cells are isolated from tissue or differentiated from pluripotent precursors, the resulting population needs to be characterized to confirm whether the target population has been obtained. This chapter lists cell-type specific antibody markers commonly used for immunocytochemical (ICC) and flow cytometric analysis of neural subtypes.

Cell-type Specific Antibodies for Characterizing Neural Subtypes

Cell type	Antigen	Type	ICC dilution*
Neural stem cells	Sox1	Goat IgG	10 µg/mL
	Sox2	Mouse IgG	2 µg/mL
	Nestin	Mouse IgG	1:500
	CD133	Rabbit IgG	1:100
Neuronal progenitors Neurons (Pan)	MAP2	Mouse IgG	1:200
	HuC/D	Mouse IgG	10 µg/mL
	NF	Mouse IgG	1:100
	NCAM	Mouse IgG	1:50
	βIII tubulin	Mouse IgG	1:2,000
	Dcx	Rabbit IgG	1:200
Dopaminergic neurons	TH	Rabbit IgG	1:1,000
Motoneurons	Isl1	Mouse IgG	1:50
	HB9	Mouse IgG	1:50
GABAergic/Glutaminergic neurons	GABA	Rabbit IgG	1:2,000
Cholinergic neurons	ChAT	Goat IgG	1:100
Astrocyte progenitors Astrocytes	CD44	Mouse IgG	1:50
	GFAP	Rat IgG	1:100
	GFAP	Rabbit IgG	1:200
Oligodendrocyte progenitors Oligodendrocytes	GalC	Mouse IgG	1:200
	NG2	Mouse IgG	1:200
	A2B5	Mouse IgM	1:2,000
	O4	Mouse IgM	1:50
Proliferation	Ki67	Rabbit IgG	1:50
	EdU	Chemicals	1:1,000
Isotype control	Mouse	IgM and IgG	Use as is
	Rabbit	IgG	Use as is

* These are recommended starting concentrations for ICC applications; optimal working concentrations must be determined empirically.

Surface Marker Analysis by Flow Cytometry

Summary

Flow cytometry is a technique for counting particles using electronic detection apparatus, and is often used to collect quantitative information about cell populations. The technique involves labeling cells with a fluorescent marker, and suspending cells in a stream of fluid which passes through, and is measured by a fluorescence measuring station.

Required Materials

- | | |
|-------------------------------|---|
| Cells | <ul style="list-style-type: none">• Cells in suspension |
| Reagents and Equipment | <ul style="list-style-type: none">• 0.1% BSA in PBS (Staining Medium)• Fluorescently labeled antibody• Flow cytometer |

Titration Antibodies

Determining the Optimal Concentration of Antibody for Flow Cytometry

1. Dilute labeled antibodies for the appropriate antigens to be detected in Staining Medium. Make dilutions of all antibodies at x1, x2, x5, x10, x20, x40, x80 and x100.
2. Prepare the cells that express the antigen to be analyzed.
3. Count the number of cells.
4. Use 1×10^6 cells for each dilution. Smaller numbers of cells ranging from 50,000 to 100,000 may work as well.
5. Centrifuge cells at $300 \times g$ for 5 minutes at 4°C and discard the supernatant.
6. Add 5 µL of antibody from each dilution into separate sample tubes containing cells.
7. Prepare negative controls of cells that have not been stained with antibody, and cells stained with an isotype control.
8. Mix well and incubate cells on ice for 25–30 minutes.
9. If primary antibodies are not directly conjugated to fluorescent tags, carry out the second step incubation with secondary antibody tagged to a fluorescent tag.

10. Wash with 10 mL of Staining Medium. Discard the supernatant and resuspend the cells in 0.5 mL of Staining Medium.
11. Analyze the cells by flow cytometry.

Note: Use the same cell number in every experiment. Starting with larger numbers of cells is preferred since setting up parameters during flow cytometry analysis takes time and collecting >10,000 events produces more reliable data.

One-Step Staining with Fluorescently Labeled Antibodies

One-Step Staining with Fluorescently-labeled Antibody

1. Trypsinize cells and add Staining Medium. Transfer the cells to a conical tube and centrifuge at $300 \times g$, 4°C for 5 minutes. Discard the supernatant.
2. Add 5 μL of diluted primary antibody conjugated to a fluorescent tag to the cell pellet.
3. Flick the tube to resuspend the cell pellet. Mix well and incubate on ice for 25–30 minutes.
4. Wash the cells with 10 mL of cold Staining Medium. Centrifuge the cells at $300 \times g$, 4°C for 5 minutes.
5. Discard the supernatant and resuspend the cells with 0.5 mL of Staining Medium.
6. Filter the cell suspension through FACS filter tubes before analysis or sorting the cells by flow cytometry.

Note: For negative controls, prepare cells that have not been stained with antibody, and cells stained with an isotype control.

Two-Step Staining with Biotinylated Antibodies

Two-step Staining with Biotinylated Antibody

1. Trypsinize cells and add Staining Medium. Transfer the cells to a conical tube and centrifuge at $300 \times g$, 4°C for 5 minutes. Discard the supernatant.
2. Add 5 μL of appropriately diluted biotinylated primary antibody.
3. Flick the tube to resuspend the cell pellet. Mix well and incubate on ice for 25–30 minutes.
4. Wash the cells with 10 mL of cold Staining Medium. Centrifuge the cells at $300 \times g$, 4°C for 5 minutes.
5. Discard the supernatant. Add diluted streptavidin secondary antibody conjugated to a fluorescent tag.

6. Mix well and incubate the cells on ice for 25–30 minutes.
7. Wash the cells with 10 mL of cold Staining Medium. Centrifuge the cells at $300 \times g$, 4°C for 5 minutes.
8. Discard the supernatant and resuspend cells with 0.5 mL of Staining Medium.
9. Filter the cell suspension through FACS filter tubes before analysis or sorting the cells by flow cytometry.

Note: For negative controls, prepare cells that have not been stained with antibody, and cells stained with an isotype control.

Immunocytochemistry

Summary

Immunocytochemistry is a technique used to assess the presence of a specific protein or antigen in cells by use of a specific antibody that binds to it. The antibody allows visualization of the protein under a microscope. Immunocytochemistry is a valuable tool to study the presence and sub-cellular localization of proteins.

Required Materials

Cells	Primary Rat Cortex Neurons (Cat. no. A10840-01) or Primary Rat Hippocampus Neurons (Cat. no. A10841-01)
Media and Reagents	- Neurobasal[®] Medium (1X), liquid (Cat. no. 21103-049) - B-27[®] Serum-Free Supplement (50X), liquid (Cat. no. 17504-044) - GlutaMAX[™]-I Supplement (Cat. no. 35050-061) - Trypan Blue Stain (Cat. no. 15250-061) - Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid (with calcium and magnesium) (Cat. no. 14040) - Goat serum (Cat. no. 16210-064) - MAP2, Mouse Monoclonal Antibody (Cat. no. 13-1500) - Rabbit anti-GFAP (Glial Fibrillary Acid Protein) (Cat. no. 08-0063) - Alexa Fluor[®] 488 goat anti-mouse IgG (Cat. no. A11029) - Alexa Fluor[®] 594 goat anti-rabbit IgG (Cat. no. A11037) 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Cat. no. D1306) - ProLong[®] Gold antifade reagent (Cat. no. P36930) - Paraformaldehyde (4%) - Triton[®]-X
Special Tools	- Multi-chambered slides - Fluorescence microscope

Methods

Treating Surfaces with Poly-D-Lysine

Treat the multi-chambered slides used in immunocytochemistry analysis with poly-D-lysine prior to analysis, as follows:

1. Prepare a 2-mg/mL stock of poly-D-lysine in nuclease-free water. Prepare aliquots and store at -20°C .
2. Prepare a working solution of the poly-D-lysine stock from step 1 in D-PBS (with calcium and magnesium) at a concentration of 50 $\mu\text{g/mL}$.
3. Add 150 μL per cm^2 of poly-D-lysine in D-PBS to each chamber of a multi-chambered slide (e.g., add 150 μL per chamber for an 8-chambered slide, 300 μL per chamber for a 4-chambered slide).
4. Incubate slide at room temperature for 1 hour in a tissue-culture hood.
5. Aspirate the poly-D-lysine solution, and rinse 3 times with nuclease-free water.

Note: Rinse thoroughly, since extra poly-D-lysine can be toxic to the cells.

6. Leave the plates uncovered in the hood until the wells are completely dry. Plates can be used when dry or can be covered with Parafilm[®] and stored at 4°C for up to two days.

Maintaining Neuronal Cultures

1. Thaw cryopreserved primary rat cortex cells according to the instructions provided with the cells.
2. Plate the cells onto a multi-chambered slide that has been treated with poly-D-lysine. Seed 1×10^5 cells per chamber in 500 μL of medium.
3. Incubate the slide at 37°C in a humidified atmosphere of 5% CO_2 in air.
4. After 24 hours of incubation, aspirate half of the medium from each well and replace with fresh medium. Return the slide to the incubator.
5. Feed the cells every third day by aspirating half of the medium from each well and replacing with fresh medium.

Immunocytochemistry Analysis

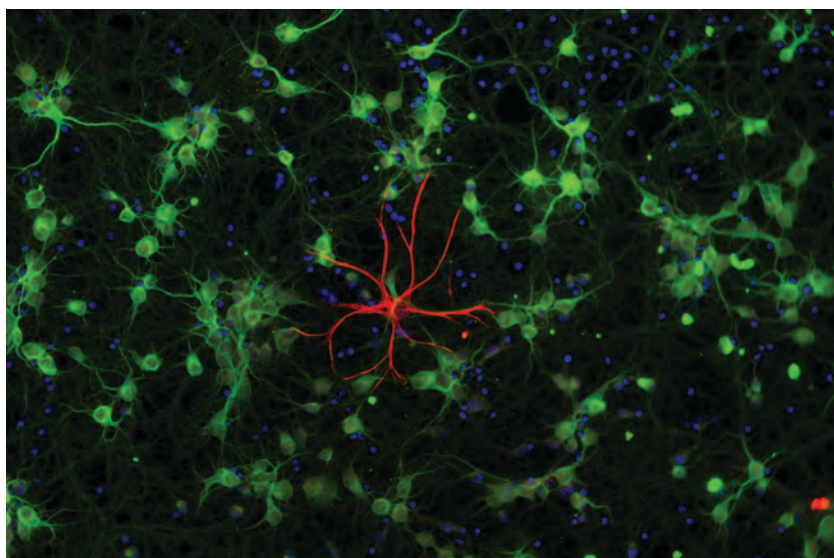
1. Before proceeding, prepare a solution of 5% goat serum in D-PBS with calcium and magnesium. This solution will be used to coat the cells before antibody detection and to dilute the antibody. Prepare enough solution to completely coat the cells twice.
2. When you are ready to perform the immunocytochemistry procedure, aspirate the supernatant from each chamber and rinse the cells twice with D-PBS with calcium and magnesium.
3. Treat the cells with 4% paraformaldehyde for 20 minutes to fix them.
4. Rinse the cells three times with D-PBS with calcium and magnesium.

5. Permeabilize the cells with 0.3% Triton[®]-X (diluted in D-PBS with calcium and magnesium) for 5 minutes at room temperature.
6. Rinse the cells three times with D-PBS with calcium and magnesium.
7. Add enough 5% goat serum solution from step 1 to the cells to coat them, and incubate for 60 minutes at room temperature.
8. Remove the solution from the wells and coat the cells with primary antibody (mouse anti-MAP2, 10 µg/mL, and/or rabbit anti-GFAP, 4 µg/mL) diluted in 5% goat serum solution.
9. Incubate the coated cells at 2–8°C overnight.
10. Rinse the cells three times with D-PBS with calcium and magnesium.
11. Treat the cells with a secondary antibody (Alexa Fluor[®] 488 goat-anti mouse (H+L), 10 µg/mL, and/or Alexa Fluor[®] 594 goat-anti rabbit (H+L), 10 µg/mL) diluted in 5% goat serum solution.
12. Incubate for 60 minutes at room temperature.
13. Rinse the cells three times with D-PBS with calcium and magnesium.
14. Stain the cells with a DAPI solution (3 ng/mL) for 10 minutes.
15. Mount the cells with ProLong[®] Gold Antifade Reagent and observe them under the microscope using filters for FITC, Cy5, and DAPI.

Typical Results

Thawed cortical neurons cultured in Neurobasal[®] Medium supplemented with B-27[®] Serum-Free Supplement and GlutaMAX[™]-I Supplement show a >90% neuron population with a minimum number of astrocytes when stained with MAP2 antibody. Within 3–4 days in culture, the neurons display extensive neurite outgrowth that keeps on increasing as long as they are kept healthy in culture. Results vary if neurons are cultured in the presence of serum.

Figure 1 Primary rat hippocampus neurons. Immunofluorescence detection of primary neuronal cells stained with mouse anti-MAP2 antibody (green) and astrocytes stained with rabbit anti-GFAP antibody (red). Nuclei are stained with DAPI (blue).



Electrophysiology

Summary

The following protocol describes how to perform fluo-4-based measurements of cytosolic calcium changes in neural stem cells in response to neurotransmitter applications.

Required Materials

Cells	Neural stem cells, cultured on poly-D-lysine coated 96-well plate or other culture vessel
Reagents	- Hanks' Balanced Salt Solution (HBSS) (Cat. no. 14025-134) - Fluo-4, AM (Cat. no. F14201) - Pluronic® F-127 (Cat. no. P-3000MP) - DMSO (Dimethylsulphoxide) (Sigma, Cat. no. D2650) - Neurotransmitters or ligands (e.g., acetylcholine, glutamate)
Tools and Equipment	- Inverted microscope (e.g., Nikon T2000) - Illumination system (e.g., Sutter Instruments Lambda DG-4) - Digital camera (e.g., Hamamatsu ORCA-ER)

Preparing Reagents

Fluo-4 AM Loading Solution Fluo-4 AM loading solution consists of 3 μ M fluo-4 AM (reconstituted in DMSO) and 0.1% Pluronic® F-127 in Hanks' Balanced Salt Solution (HBSS). Use the fluo-4 AM loading solution as soon as possible after preparation to avoid decomposition with subsequent loss of cell loading capacity.

1. To reconstitute fluo-4 AM, add 44 μ L of DMSO to one vial of fluo-4 AM (50 μ g) and vortex thoroughly. You may store the fluo-4 AM reconstituted in DMSO protected from light, frozen, and desiccated for up to one week.
2. Add 9 μ L of Pluronic® F-127 to the reconstituted fluo-4 AM and vortex thoroughly.

Note: Because fluo-4 AM is relatively insoluble in aqueous solutions, addition of the low-toxicity dispersing agent Pluronic® F-127 facilitates cell loading. However, Pluronic® F-127 may decrease the stability of AM esters, so it should only be added to working stocks (i.e., the loading solution).

3. Add 50 μ L of the ~860 μ M fluo-4 AM/ Pluronic® F-127 solution to 14.3 mL of HBSS.

Loading NSCs with Fluo-4 AM Loading Solution

1. Wash the NSCs with 100 μ L of Hanks' Balanced Salt Solution (HBSS).
2. Load the NSCs with 100 μ L of fluo-4 AM loading solution per well of a 96-well plate. You may adjust the volume as appropriate to other culture vessels.
3. Incubate the NSCs in the dark at room temperature for ~60 minutes.
4. Wash the fluo-4-loaded NSCs with 100 μ L of HBSS and maintain at room temperature in the dark until data acquisition

Data Acquisition

1. Place the 96-well plate containing the fluo-4-loaded NSCs in an inverted microscope (e.g., Nikon T2000) for visual inspection and fluorescent imaging.
2. To acquire and analyze data, define regions of interest around a random series of cells using your software of choice (e.g., MetaFluor, MDS Analytical Technologies).

Note: The NSCs should display a typical neuronal morphology with dendritic and axonal processes clearly recognizable by cellular polarity and proportionate size.
3. Identify 50–100 neurons for data acquisition and analysis in each well examined.
4. Excite the NSCs with 488-nm light (e.g., Lambda DG-4 light source) and collect images from 520-nm emitted light with a CCD or digital camera (e.g., ORCA-ER).
5. Challenge the cells in one well with a neurotransmitter or other ligand. For example, add 20 μ L of 3 mM acetylcholine to achieve a final concentration of 500 μ M acetylcholine in the well.
6. Collect the data using the appropriate software (e.g., MetaFluor, MDS Analytical Technologies).
7. Repeat the procedure for each neurotransmitter or ligand of interest in separate wells. Use the following final concentrations for each well: 500 μ M glutamate, 500 μ M dopamine (add 500 μ M ascorbic acid with dopamine to prevent dopamine oxidation), 500 μ M γ -aminobutyric acid, and 500 μ M ATP.

Data Analysis

1. Integrate the acquired fluo-4 520-nm emission signal for each region of interest, normalize to the first ten data points (F/F_0) and then plot against time.
2. Set the response criteria. For example, a NSC might be considered responsive to a given neurotransmitter or ligand if the resulting normalized signal rises more than 10% within 60 seconds following neurotransmitter addition compared to the baseline signal. The number of NSCs that exhibit clear changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) depends on the neurotransmitter and differentiation state of the NSCs.

Molecular Characterization

PCR Primers for Molecular Characterization of Neural Subtypes

Summary

After cells are isolated from tissue or differentiated from pluripotent precursors, the resulting population needs to be characterized to confirm whether the target population has been obtained. The table below lists PCR primers that can be used in quantitative polymerase chain reactions (qPCR) to measure the expression levels of specific genes for characterizing neural stem cells (NSCs) and their sublineages.

Target	Primer	Sequence	T _m (°C)	Amplicon size (bp)	Intron size (bp)
Neural stem cells	SOX1-F	GCGGAAAGCGTTTCTTG	53.0	406	No Intron
	SOX1-R	TAATCTGACTTCTCCTCCC	50.2		
	SOX2-F	ATGCACCGCTACGACGTGA	59.3	437	No Intron
	SOX2-R	CTTTTGACCCCTCCCATTT	56.0		
	NESTIN-F	CAGCGTTGGAACAGAGGTTGG	58.6	389	1,142
	NESTIN-R	TGGCACAGGTGTCTCAAGGGTAG	60.7		
Oligodendrocytes	MAG-F	TCTGGATTATGATTTAGCC	49.7	366	159
	MAG-R	GCTCTGAGAAGGTGTACTGG	54.7		
	OSP-F	ACTGCTGCTGACTGTTCTTC	55.1	283	5,714
	OSP-R	GTAGAAACGGTTTTACCAA	50.8		
Astrocytes	ALDH1L1-F	TCACAGAAGTCTAACCTGCC	55.5	398	21,837
	ALDH1L1-R	AGTGACGGGTGATAGATGAT	54.4		
	GFAP-F	GTACCAGGACCTGCTCAAT	55.0	321	2,989
	GFAP-R	CAACTATCCTGCTTCTGCTC	55.3		
Neurons	MAP2-F	CCACCTGAGATTAAGGATCA	55.1	482	11,798
	MAP-R	GGCTTACTTTGCTTCTCTGA	55.0		
	ChAT-F	ACTGGGTGTCTGAGTACTGG	55.0	451	7,692
	ChAT-R	TTGGAAGCCATTTGACTAT	54.9		
Endogeneous control	ACTB-F	ACCATGGATGATGATATCGC	58.2	281	135
	ACTB-R	TCATTGTAGAAGGTGTGGTG	54.4		
GABAergic/Glutaminergic neurons	GAD1-F	GTCGAGGACTCTGGACAGTA	55.3	357	12,277
	GAD1-R	GGAAGCAGATCTCTAGCAAA	54.9		
Serotonergic neurons	SLC6A4-F	GCCTTTTACATTGCTTCCTA	54.8	447	2,251
	SLC6A4-R	CCAATTGGGTTTCAAGTAGA	55.2		
Cholinergic neurons	ChAT-F	ACTGGGTGTCTGAGTACTGG	55.0	451	7,692
	ChAT-R	TTGGAAGCCATTTGACTAT	54.9		
Dopaminergic neurons	TH-F	TCATCACCTGGTCACCAAGTT	56.0	126	656
	TH-R	GGTCGCCGTGCCTGTACT	60.0		

RNA Isolation and cDNA Preparation from Neural Stem Cells

Summary

A rapid method of analysis for determining the identity of neural stem cells (NSCs) and their sublineages involves the early detection of differentiation markers tracked at the RNA level. This protocol follows methodologies described in the PureLink™ RNA Mini Kit manual for isolating total RNA from neural stem cells (NSCs), followed by cDNA synthesis using Superscript® III reverse transcriptase. The following protocol gives you a step-by-step procedure for template preparation required for RT-PCR or qPCR.

Required Materials

Cells

- Neural stem cells

Reagents and Equipment

- PureLink™ RNA Mini Kit (Cat. no. 12183-018A)
 - RNA Lysis Solution
 - Wash Buffer I
 - Wash Buffer II
 - RNase-free water
 - RNA spin cartridges
 - Collection tubes
 - RNA recovery tubes
- Superscript® III First Strand Synthesis SuperMix (Cat. no. 18080-400)
 - Superscript® III / RNaseOUT™ Enzyme Mix
 - 2X First-strand Reaction Mix
 - Annealing Buffer
 - 50 mM Oligo(dT)₂₀
 - Random hexamers (50 ng / mL)
- β-Mercaptoethanol (Cat. no. 21985-023)
- TrypLE™ Express Stable Trypsin Replacement Enzyme (Cat. no. 12604-013)
- Dulbecco's Phosphate-Buffered Saline (D-PBS) without Ca²⁺ and Mg²⁺ (Cat. no. 14190)
- Ribonuclease H (RNase H) (Cat. no. 18021-071)
- 10X BlueJuice™ Gel Loading Buffer (Cat. no. 10816-015)
- Table-top centrifuge

RNA Isolation

Isolating RNA

Important: Perform all steps on ice unless noted otherwise. For all incubations, heat the thermocyclers in advance. Pre-chill all reagents and thaw all frozen reagents and cells immediately prior to use. Use RNase-free pipette tips with aerosol barriers.

1. Prepare RNA Lysis Solution by adding 10 μ L β -mercaptoethanol per mL of RNA Lysis Solution.
2. Remove media from T-25 flasks, rinse once with Dulbecco's phosphate-buffered saline (D-PBS) and treat cells with 1 mL of pre-warmed TrypLE™ reagent for 10 minutes at 37°C.
3. Harvest the cells and place them into 15-mL centrifuge tubes. Take 100 μ L of the sample and obtain a viable cell count.
4. Centrifuge the cells in a tabletop centrifuge for 7 minutes at $100 \times g$. Discard the supernatant.
5. Freeze the cells overnight in a -70°C freezer.
6. Allow the cell pellet to thaw. Add 0.5 mL of RNA Lysis Solution for each T-25 flask harvested for the pellet (0.5 mL per 2×10^6 – 5×10^6 cells). Pipet the cells ~20 times until the pellet is disrupted.
7. Transfer 0.5 mL of cell lysis solution to 1.5-mL RNase-free microcentrifuge tubes and centrifuge at room temperature for 2 minutes at $12,000 \times g$ (12,000 rpm).
8. Add 0.5 mL of 70% ethanol to each tube, and vortex the suspension 5–10 times.
9. Apply a 600 μ L aliquot of sample to the RNA Spin Cartridge. Centrifuge at room temperature for 15–30 seconds at $12,000 \times g$, then discard the flow-through. Continue applying 600 μ L aliquots of the same RNA sample to the spin cartridge until the entire sample has been processed.
10. Add 700 μ L Wash Buffer I to the spin cartridge and centrifuge at room temperature for 15–30 seconds at $12,000 \times g$. Discard the flow-through and the tube. Place the spin cartridge into a clean 2 mL RNA Wash Tube.
11. Add 500 μ L Wash Buffer II (containing ethanol) to the spin cartridge and centrifuge at room temperature for 15–30 seconds at $12,000 \times g$. Discard the flow-through. Centrifuge for 1 minute to dry the cartridge.
12. Place the cartridge into an RNA Recovery Tube. Add 40 μ L of RNase-free water to the cartridge, and let it stand for 1 minute. Centrifuge the cartridge at room temperature for 2 minutes at $12,000 \times g$. Add an additional 40 μ L of RNase-free water to the cartridge and repeat the step. Yield should be about 60–300 μ g total RNA.

Note: Always allow time for the RNase-free water to percolate into the cartridge bed. Do not spin the cartridge immediately because it may result in partial recovery and alter the yield of RNA. To recover more RNA, add an additional 40 μ L of RNase-free water to the cartridge and repeat the last step for a third time.

- Determining RNA Quality** 1. Measure ratio of absorbance at 260 nm and 280 nm by analyzing 1 μL of the RNA sample using a NanoDrop[™] spectrophotometer. Conduct readings three times, and use the average as the final value. Wipe down the analysis stage with a lab tissue wetted with DEPC water before and after measuring each RNA sample. The $A_{260/280}$ of pure RNA is ~2.

Note: The yield and quality of the isolated RNA depends on the type and age of the starting material, in addition to how the material was collected and preserved.

2. Prepare the RNA samples for RNA gel analysis as follows:

Component	Amount
RNA sample	1 μL
2X BlueJuice [™] gel loading buffer	1 μL
DEPC-treated water	8 μL

3. Mix the components and load the samples onto individual wells of an agarose gel. Use 10 μL of 0.1 kb and 1 kb molecular weight markers to estimate the molecular weight size of ribosomal RNA bands. Use 10 μL DEPC water for empty wells. Run samples for 30 minutes, visualize the bands on an UV light box, capture the gel image, and perform band intensity measurements.

RNA Storage Store RNA samples at -70°C or process it further for cDNA synthesis.

cDNA Preparation

First-Strand cDNA Synthesis

This protocol follows the methodologies described in the instructions for Superscript[®] III First Strand Synthesis SuperMix.

- Mix and briefly centrifuge each component before use. Pre-heat the thermocycler to 65°C .
- Combine the following components on ice in a 0.2-mL thin-walled PCR tube. Use a volume containing up to 1 μg of total RNA for the reaction.

Component	Amount
Annealing buffer	1 μL
Random hexamer(50 ng/ μL)	1 μL
RNA (1 μg)	x μL
DEPC-treated water	to 8 μL

- Incubate the reaction in the thermocycler at 65°C for 5 minutes, and then immediately place on ice for at least 1 minute. Collect the contents of the tube by brief centrifugation.

4. While the tube is on ice, add the following components to the tube:

Component	Amount
2X First-Strand Reaction Mix	10 μ L
SuperScript [®] III/RNaseOUT [™] Enzyme mix	2 μ L

5. Vortex the sample briefly, and collect the contents by brief centrifugation.
6. Incubate the tube at 25°C for 10 minutes.
7. Incubate the tube at 42°C for 50 minutes.
8. Terminate reaction by incubating at 85°C for 5 minutes, then chill the tube on ice.
9. Add 1 μ L of RNase H to the sample, and incubate at 37°C for 20 minutes.
10. Store the cDNA samples at -20°C.

Characterizing Neural Cells by qPCR

Summary

Quantitative polymerase chain reaction (qPCR) is one of the most accurate and sensitive methods for studying gene regulation, and can be used to measure the expression levels of specific genes in neural stem cells (NSCs). These genes can be used to characterize the NSCs and their respective sublineages.

Here we provide guidelines and a general protocol for performing qPCR using the Applied Biosystems 7300 Real-Time PCR System and Platinum® SYBR® Green qPCR SuperMix-UDG with ROX Reference Dye.

Required Materials

- | | |
|---------------------------|---|
| Starting Material | <ul style="list-style-type: none"> • cDNA generated from total RNA isolated from neural stem cells (NSCs) (see Chapter 20, RNA Isolation and cDNA Preparation from Neural Stem Cells, page 77) |
| Media and Reagents | <ul style="list-style-type: none"> • Platinum® SYBR® Green qPCR SuperMix-UDG (Cat. nos. 11733-038, 11733-046) • SuperScript® VILO™ cDNA Synthesis Kit (Cat. nos. 11754-050, 11754-250) • TRIzol® Reagent (Cat. nos. 15596-018, 15596-026) • Custom primers (www.invitrogen.com/oligos) |
| Special Tools | <ul style="list-style-type: none"> • Applied Biosystems 7300 Real-Time PCR System or similar instrument • 0.2-mL microcentrifuge tubes or 96-well or 384-well PCR plates • Vortex mixer • Microcentrifuge |

Methods

- | | |
|----------------------------------|---|
| Template Preparation | For qPCR, prepare a 1:10 dilution series of cDNA generated from 10 pg–1 µg of total RNA using the protocol described in Chapter 20, RNA Isolation and cDNA Preparation from Neural Stem Cells (page 77). |
| Real-Time PCR Instruments | Platinum® SYBR® Green qPCR SuperMix-UDG can be used with a variety of real-time instruments, including but not limited to the following Applied Biosystems instruments: 7300 and 7500 Real-Time PCR Systems; PRISM® 7000, 7700, and 7900HT; and GeneAmp® 5700. Optimal cycling conditions will vary with different instruments. |

Primer Design

Primer design is one of the most important parameters when using a SYBR® Green qPCR detection system. We strongly recommend using a primer design program such as OligoPerfect™, available at www.invitrogen.com/oligos, or Vector NTI Advance® software. When designing primers, the amplicon length should be approximately 80–250 bp. Optimal results may require a titration of primer concentrations between 100 nM and 500 nM. A final concentration of 200 nM per primer is effective for most reactions.

ROX Reference Dye

ROX Reference Dye is recommended to normalize the fluorescent reporter signal for instruments that are compatible with that option. ROX is supplied as a separate tube in Platinum® SYBR® Green qPCR SuperMix-UDG at a 25 µM concentration. Use the following table to determine the amount of ROX to use with a particular instrument.

Instrument	Amount of ROX per 50 µL reaction	Final ROX concentration
Applied Biosystems 7300, 7000, 7700, 7900HT, and 7900HT Fast	1.0 µL	500 nM
Applied Biosystems 7500	0.1 µL	50 nM

Protocol for qPCR

For protocols for specific instruments, visit www.invitrogen.com/qpcr. In this section we provide a step-by-step protocol for qPCR on the 7300 Real-Time PCR System (Applied Biosystems) in 20-µL assays.

1. Program your real-time instrument as shown below. Optimal temperatures and incubation times may vary.
 - 50°C for 2 minutes hold (UDG incubation)
 - 95°C for 2 minutes hold
 - 40–50 cycles of:
 - 95°C, 15 seconds
 - 60°C, 30 seconds

Melting Curve Analysis: Program the instrument for melting curve analysis to identify the presence of primer dimers and analyze the specificity of the reaction. A typical melting curve program is listed below (see your instrument documentation for details):

- 95°C for ~30 seconds
- 45°C for ~30 seconds
- 99°C for ~30 seconds

with a 2% ramp rate with data collection from 45–99°C

Note: For the following steps, do not touch the bottom of each tube, and be sure to use powder-free gloves to handle all reagents and plasticware.

- For each reaction, add the following components to a 0.2-mL microcentrifuge tube or each well of a PCR plate. Volumes for a single 20- μ L reaction are listed. For multiple reactions, prepare a master mix of common components, add the appropriate volume to each tube or plate well, and then add the unique reaction components (e.g., template). For no-template controls, add an equivalent volume of water in lieu of template.

Component	Amount
Platinum® SYBR® Green qPCR SuperMix-UDG	10 μ L
ROX Reference Dye (amount specified for AB 7300 system)	0.4 μ L
Forward primer, 10 μ L	0.4 μ L
Reverse primer, 10 μ L	0.4 μ L
Template cDNA (1:10 dilution series from 10 pg to 1 μ g total RNA)	1–2 μ L
DEPC-treated water	to 20 μ L

- Cap or seal the reaction tube/PCR plate, and gently mix. Make sure that all components are at the bottom of the tube/plate; centrifuge briefly if needed.
- Place the reactions in a preheated real-time instrument programmed as described in Step 1. Collect the data and analyze the results using the instrument software.

Figure 1 qPCR detection of Nestin transcripts in human embryonic stem cell-derived NSCs.

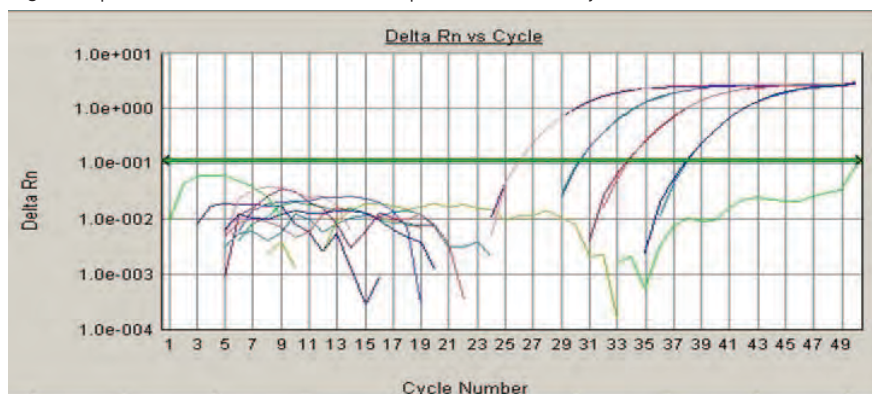
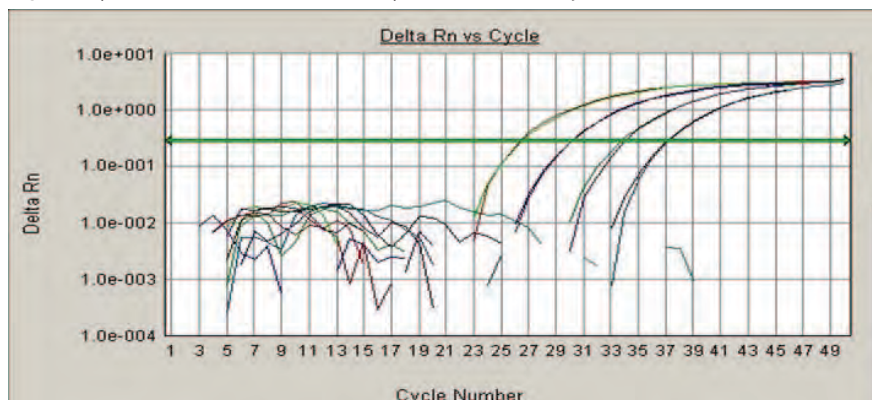


Figure 2 qPCR detection of Sox1 transcripts in human embryonic stem cell-derived NSCs.



Transfection

Transfecting Neural Cells Using the Neon® Transfection System

Summary

The Neon® Transfection System is a benchtop electroporation device that uses the pipette tip as an electroporation chamber to efficiently transfect mammalian cells including primary cells and stem cells.

Instructions for using the Neon® Transfection System for transfecting of neural cells are described below. For detailed instructions on using the Neon® Transfection System, refer to the manual supplied with the product or download the manual from www.invitrogen.com. For detailed information on culture conditions for various neural cell lines, refer to the instructions supplied with the specific cell line you are using.

Required Materials

- Neural cell line of interest
- Growth media and growth factors appropriate for your neural cell line
- Plasmid DNA of interest (1–5 µg/mL in deionized water or TE)
- Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid without Ca^{2+} and Mg^{2+} (Cat. no. 14190-144)
- Neon® Transfection system (Cat. no. MPK5000)
- Neon® Kit, 10 µL (Cat. no. MPK1096) or Neon® Kit, 100 µL (Cat. no. MPK10096)
- Appropriate tissue culture plates and supplies

Culture Conditions

The following table summarizes the culture conditions for various neural cell lines, including neural stem cells. For detailed instructions on culturing and passaging these cells, refer to the instructions supplied with the specific cell line you are using.

Cell type	Media	Culture conditions
Human Neural Stem Cells	Complete StemPro® NSC SFM	- Adherent culture on CELLStart™-, fibronectin-, or poly-L-ornithine-coated culture vessels 37°C, humidified atmosphere of 5% CO₂ in air - Exchange spent medium every other day
Human Astrocytes	Complete GIBCO® Astrocyte Medium	- Adherent culture on Geltrex™-coated tissue culture vessels 37°C, humidified atmosphere of 5% CO₂ in air - Exchange spent medium every 3–4 days
Rat Fetal Neural Stem Cells	Complete StemPro® NSC SFM	- Adherent culture on CELLStart™-, fibronectin-, or poly-L-ornithine-coated culture vessels 37°C, humidified atmosphere of 5% CO₂ in air - Exchange spent medium every 3–4 days
Rat Primary Cortical Astrocytes	Complete GIBCO® Astrocyte Medium*	- Adherent culture on standard culture vessels 37°C, humidified atmosphere of 5% CO₂ in air - Exchange spent medium every 2–3 days
Rat Glial Precursor Cells	Complete StemPro® NSC SFM, supplemented with 10 ng/mL PDGF-AA	- Adherent culture on CELLStart™- or poly-L-ornithine-coated culture vessels 37°C, humidified atmosphere of 5% CO₂ in air - Exchange spent medium every other day

*For increased proliferation of rat astrocytes, you can supplement complete GIBCO® Astrocyte Medium (D-MEM with 1X N-2 Supplement and 10% OneShot™ FBS) with 20 ng/mL EGF. Adding EGF to human astrocyte cultures can increase proliferation, but may result in morphological or phenotypic changes.

Preparing Media

Complete StemPro® NSC SFM

To prepare 100 mL of complete StemPro® NSC SFM, aseptically mix the components listed in the table below. Complete medium is stable for up to 4 weeks when stored in the dark at 4°C.

Component	Concentration	Amount
KnockOut™ D-MEM/F-12	1X	97 mL
GlutaMAX™-I Supplement	2 mM	1 mL
bFGF	20 ng/mL	2 µg
EGF	20 ng/mL	2 µg
StemPro® NSC SFM Supplement	2%	2 mL

Complete GIBCO® Astrocyte Medium

To prepare 100 mL of complete GIBCO® NSC SFM, aseptically mix the components listed in the table below. Complete medium is stable for up to 2 weeks when stored in the dark at 4°C.

Component	Concentration	Amount
D-MEM	1X	89 mL
N-2 Supplement	1X	1 mL
FBS	10%	10 mL
Note: Adding EGF at a final concentration of 20 ng/mL can increase proliferation, but may result in morphological and phenotypic changes in human astrocytes.		

Transfection Protocol

Use this procedure to transfect plasmid DNA into hNSCs in a **24-well format** using the 10-µL Neon® Kit. All amounts and volumes are given on a per well basis.

1. Cultivate the required number of cells in the appropriate growth medium (see table below) such that the cells are 70–90% confluent on the day of the experiment.
2. On the day of the experiment, harvest and wash cells in phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} .
3. Resuspend the cell pellet in Resuspension Buffer R (included with Neon® Kits) at the appropriate final density (see the following table).
4. Prepare 24-well plates by filling the wells with 0.5 mL of the appropriate growth medium without antibiotics and pre-incubate plates at 37°C in a humidified 5% CO_2 incubator. If using other plate formats, adjust the volume accordingly.
5. Turn on the Neon® unit and enter the following electroporation parameters in the **Input** window. Alternatively, press the **Database** button and select the appropriate transfection protocol (if you have already added the electroporation parameters for your cell type). For detailed instructions, refer to the manual supplied with the Neon® unit.

Cell type	Cell density	Pulse voltage (V)	Pulse width (ms)	Pulse number	Neon® tip
Human Neural Stem Cells	1×10^7 cells/mL	1400	20	2	10-µL
		1600	20	1	
		1700	20	1	
Human Astrocytes	1×10^7 cells/mL	1100	30	1	10-µL
		1200	40	1	
Rat Fetal Neural Stem Cells	1×10^7 cells/mL	1300	20	2	10-µL
		1500	10	3	
		1600	10	3	
Rat Primary Cortical Astrocytes	0.5×10^7 cells/mL	1400	20	2	10-µL
		1400	30	1	
		1700	20	1	
Rat Glial Precursor Cells	1×10^7 cells/mL	1300	10	3	10-µL
		1500	20	1	

6. Fill the Neon[®] Tube with 3 mL of Buffer E. (Use Buffer E2 if you are using the 100- μ L Neon[®] Tip.)
7. Insert the Neon[®] Tube into the Neon[®] Pipette Station until you hear a click, indicating that the tube has locked in position.
8. Transfer 0.5 μ g of plasmid DNA into a sterile, 1.5-mL microcentrifuge tube.

Note: The quality and concentration of DNA used for electroporation plays an important role for the transfection efficiency. We strongly recommend using high quality plasmid purification kits such as PureLink[™] HiPure Plasmid DNA Purification Kits to prepare DNA.

9. Add 1 mL of cells (resuspended in step 3) to the tube containing the plasmid DNA and gently mix.
10. Insert a 10- μ L Neon[®] Tip into the Neon[®] Pipette.
11. Press the push-button on the Neon[®] Pipette to the first stop and immerse the Neon[®] Tip into the cell-DNA mixture. Slowly release the push-button on the pipette to aspirate the cell-DNA mixture into the Neon[®] Tip.
12. Insert the Neon[®] Pipette with the sample vertically into the Neon[®] Tube placed in the Neon[®] Pipette Station until you hear a click, indicating that the pipette has locked in position.
13. Ensure that you have entered the appropriate electroporation parameters and press **Start** on the Neon[®] touchscreen.

The Neon[®] device delivers the electric pulse according to the parameters entered in step 5 and the touchscreen displays **Complete** to indicate that electroporation is complete.

14. Remove the Neon[®] Pipette from the Neon[®] Pipette Station and immediately transfer the samples from the Neon[®] Tip into the prepared culture plate containing the appropriate pre-warmed complete growth medium without antibiotics.

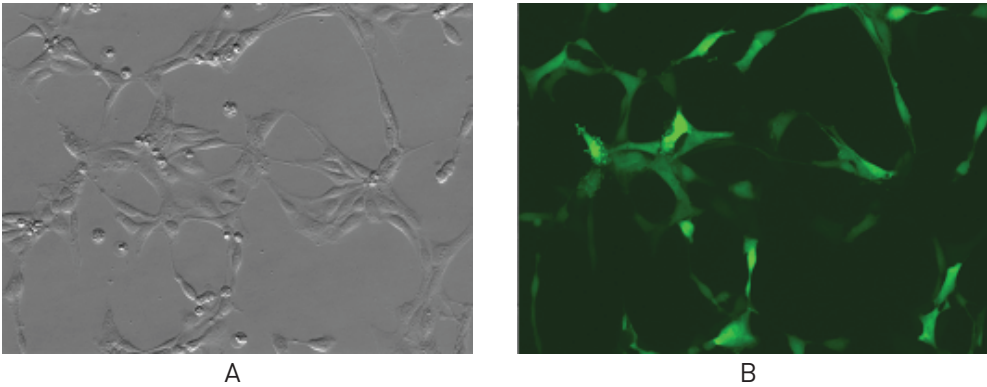
Discard the Neon[®] Tip into an appropriate biological hazardous waste container.

15. Repeat Steps 10–14 for the remaining samples.
16. Gently rock the plate to assure even distribution of the cells. Incubate the plate at 37°C in a humidified 5% CO₂ incubator.
17. Assay the samples to determine the transfection efficiency (e.g., fluorescence microscopy or functional assay).

Expected Results

Human Neural Stem Cells

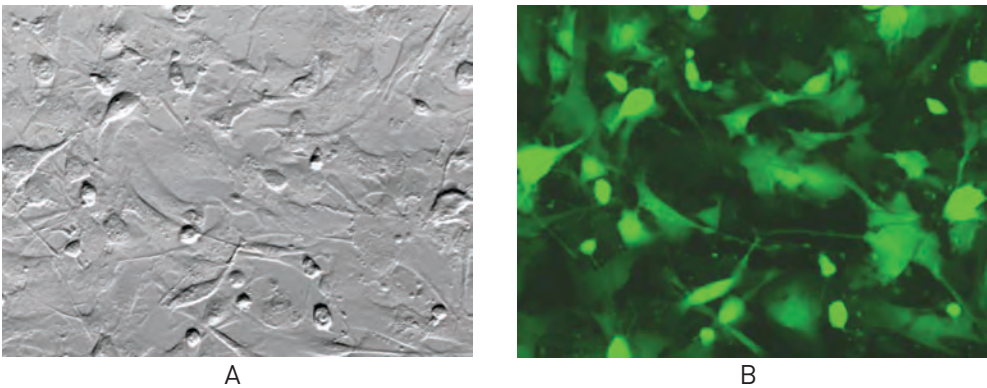
GIBCO® Human Neural Stem Cells (Cat. no. N7800-100), cultured in StemPro® NSC SFM complete medium, were transfected with 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EGFP) using the Neon® Transfection system with the parameters listed in the following table. 48 hours post-transfection, the cells were analyzed by light (Panel A) and fluorescence microscopy (Panel B).



Cell density	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon® tip
1 × 10 ⁷ cells/mL	1400	20	2	82%	95%	10-µL
	1600	20	1	84%	95%	
	1700	20	1	87%	96%	

Human Astrocytes

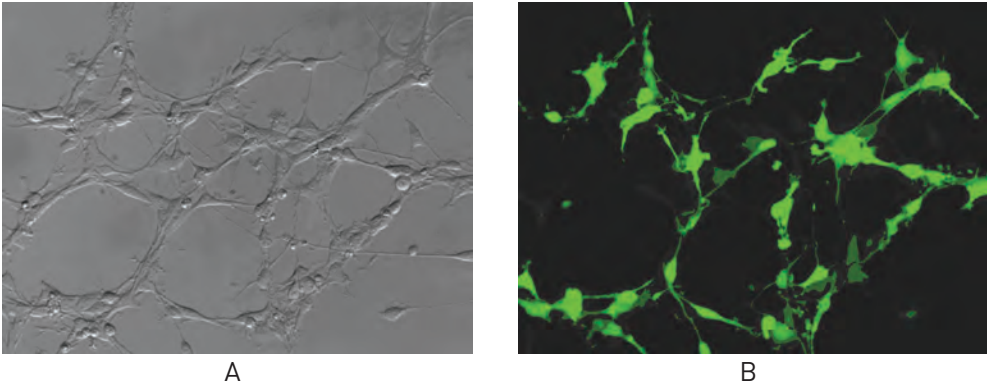
GIBCO® Human Astrocytes (Cat. no. N7805-100) were transfected using the Neon® Transfection Device and 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EGFP); 24 hours post-electroporation, the cells were analyzed by light (Panel A) and fluorescence microscopy (Panel B).



Cell density	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon® tip
1 × 10 ⁷ cells/mL	1100	30	1	92%	97%	10-µL
	1200	40	1	93%	97%	

Rat Fetal Neural Stem Cells

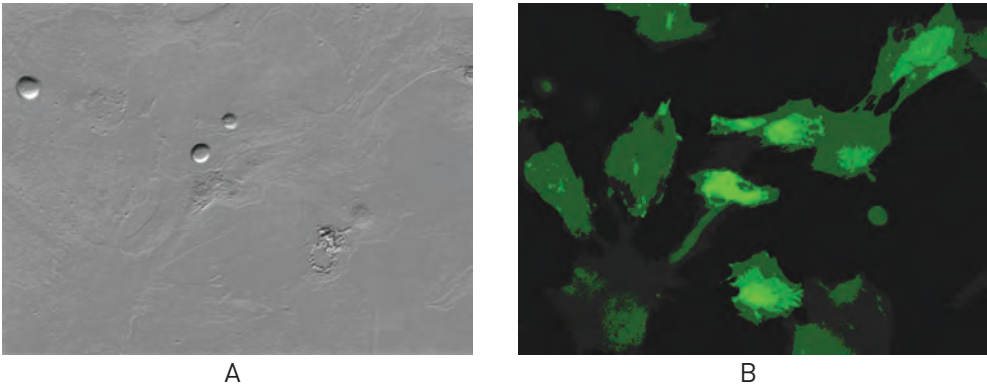
GIBCO® Rat Fetal Neural Stem Cells (Cat. no. N7744-100) were transfected using the Neon® Transfection Device and 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EGFP); 24 hours post-electroporation, the cells were analyzed by light (Panel A) and fluorescence microscopy (Panel B).



Cell density	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon® tip
1 × 10 ⁷ cells/mL	1100	30	1	92%	97%	10-µL
	1200	40	1	93%	97%	

Rat Primary Cortical Astrocytes

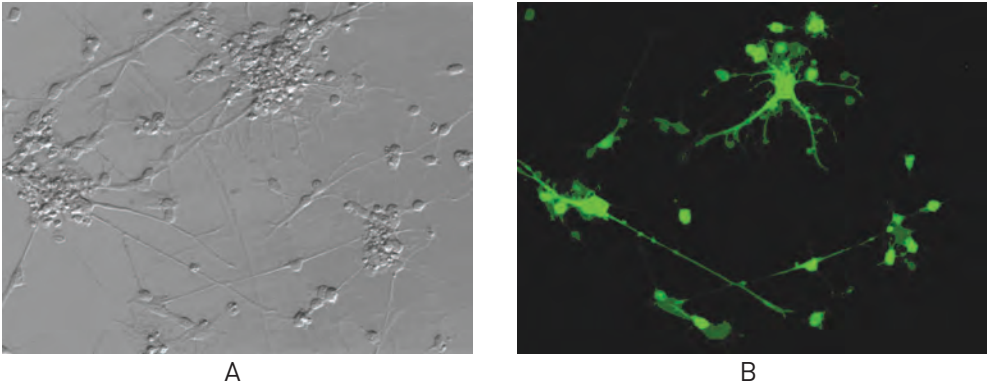
GIBCO® Rat Primary Cortical Astrocytes (Cat. no. N7745-100) were transfected using the Neon® Transfection Device and 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EGFP); 24 hours post-electroporation, the cells were analyzed by light (Panel A) and fluorescence microscopy (Panel B).



Cell density	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon® tip
0.5 × 10 ⁷ cells/mL	1400	20	2	69%	87%	10-µL
	1400	30	1	71%	89%	
	1700	20	1	71%	90%	

Rat Glial Precursor Cells

GIBCO® Rat Glial Precursor Cells (Cat. no. N7746-100) were transfected using the Neon® Transfection Device and 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EGFP); 24 hours post-electroporation, the cells were analyzed by light (Panel A) and fluorescence microscopy (Panel B).



Cell density	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon® tip
1 × 10 ⁷ cells/mL	1300	10	3	49%	78%	10-µL
	1500	20	1	44%	64%	

Troubleshooting

For troubleshooting tips regarding the culture and passaging of your cells, refer to the manual provided with the cells. For troubleshooting tips regarding the Neon® Transfection System, see below.

Problem	Possible cause	Solution
Connection failure	No Neon® Tip is inserted or the Neon® Tip is inserted incorrectly	Make sure that the Neon® Tip is inserted into Neon® Pipette correctly as described. There should be no gap between the tip and the top head of the pipette.
Arcing (sparks)	Air bubbles in the Neon® Tip	Avoid any air bubbles in the Neon® Tip while aspirating the sample.
	High voltage or pulse length settings	Reduce the voltage or pulse length settings.
Low cell survival rate	Poor DNA quality	• Use high quality plasmid DNA for transfection (use high quality plasmid purification kits such as PureLink™ HiPure Plasmid DNA Purification Kits, Cat. no. K2100) to prepare DNA. • Resuspend the purified DNA in deionized water or TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at a concentration between 0.5–5 µg/µL. • Check the purity of the purified DNA preparation by measurement of the A260/280 ratio. The ratio should be at least 1.8 for electroporation. • Do not precipitate DNA with ethanol to concentrate DNA. Concentrated DNA by ethanol precipitation shows poor transfection efficiency and cell viability due to salt contamination.
	Cells are stressed or damaged	• Avoid severe conditions during cell harvesting especially high speed centrifugation and pipette cells gently. • Avoid using over confluent cells or cells at high densities as this may affect the cell survival after electroporation. • After electroporation, immediately plate the cells into prewarm culture medium without antibiotics.
	Multiple use of the same Neon® Tip	Do not use the same Neon® Tip for electroporation for more than 2 times because the repeated application of electric pulses reduce the tip quality and impair their physical integrity.
Low transfection efficiency	Poor plasmid DNA quality or the plasmid DNA is low	• Use high quality plasmid DNA for transfection. • Start with 0.5 µg plasmid DNA per sample.
	Incorrect cell density	Use the recommended cell densities of 1×10^5 cells per 10 µL per sample (i.e., 1×10^7 cells/mL).
	Incorrect electroporation parameters	Use the recommended voltage, pulse width, and pulse number. We recommend optimizing the electroporation parameters using the preprogrammed 24-well optimization protocol available on the Neon® unit.
	<i>Mycoplasma</i> contaminated cells	Test cells for <i>Mycoplasma</i> contamination. Start a new culture from a fresh stock.
Nonreproducible transfection efficiency	Inconsistent cell confluency or passage number	Always use cells with low passage number and harvest cells with comparable confluency levels.
	Multiple use of the same Neon® Tip or the same Neon® Tube	• Do not use the same Neon® Tip for more than 2 times because the repeated application of electric pulses reduce the tip quality and impair their physical integrity. • Do not use the same Neon® Tube for more than 10 times. • Always use a new Neon® Tip and Neon® Tube for different plasmid DNA samples to avoid any cross-contamination.

Lipid-Mediated Transfection of Human Astrocytes

Summary

Astrocytes are by far the most numerous cell type in the central nervous system (CNS) and have critical roles in adult CNS homeostasis. They provide biochemical and nutritional support of neurons and endothelial cells which form the blood-brain barrier, perform the vast majority of synaptic glutamate uptake, and maintain extracellular potassium levels (Rothstein et al., 1996; Rothstein et al., 1994). Although there are few known differences between cortical and hippocampal astrocytes, it has been reported that astrocytes from different regions of the brain show a differential sensitivity to ischemic injury (Xu et al., 2001; Zhao & Flavin, 2000).

The following protocols provide instructions for lipid-mediated transfection of plasmid DNA or siRNA into GIBCO® Human Astrocytes using the Lipofectamine™ LTX Reagent or the Lipofectamine RNAiMAX Reagent. Lipofectamine™ LTX Reagent is a proprietary, animal-origin free formulation for the transfection of DNA into eukaryotic cells with low cytotoxicity. Lipofectamine™ RNAiMAX is a proprietary formulation specifically developed for the transfection of siRNA and Stealth™ RNAi duplexes into eukaryotic cells.

Required Materials

- GIBCO® Human Astrocytes (Cat. no. N7805-100)
- GIBCO® Astrocyte Medium (Cat. no. A12613-01)
Note: The medium kit includes N-2 Supplement, 100X (Cat. no. 17502-048), Dulbecco's Modified Eagle Medium (D-MEM) (1X), liquid (Cat. no. 10569-010), and One Shot™ Fetal Bovine Serum (FBS), Certified (Cat. no. 16000-077).
- Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid without Ca²⁺ and Mg²⁺ or phenol red (Cat. no. 14190-144)
- Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X), liquid with Ca²⁺ and Mg²⁺ (Cat. no. 14040-133)
- Geltrex™ Reduced Growth Factor Basement Membrane Matrix (Cat. no. 12760)
- StemPro® Accutase® Cell Dissociation Reagent (Cat. no. A11105)
- Opti-MEM® I Reduced Serum Medium (Cat. no. 31985-062)
- Appropriate tissue culture plates and supplies

For transfecting plasmid DNA

- Plasmid DNA of interest (100 ng/μL or higher)
- Lipofectamine™ LTX Reagent and PLUS™ Reagents (Cat. no. 15338-100)

For transfecting siRNA

- Silencer® Select siRNAs (see www.invitrogen.com for ordering information)
- Lipofectamine™ RNAiMAX Transfection Reagent (Cat. no. 13778-075 or 13778-150)

Important Guidelines for Lipid-Mediated Transfection

Follow these important guidelines when performing lipid-mediated transfections of human astrocytes.

- Maintain human astrocytes on Geltrex™-coated plates.
- Adding antibiotics to media during transfection may result in cell death. If you wish to use antibiotics during transfection, test your conditions thoroughly.
- Maintain the same seeding conditions between experiments. Use low-passage cells; make sure that cells are healthy and greater than 90% viable before transfection.
- Transfections can be performed both in the presence or absence of serum. Test serum-free media for compatibility with Lipofectamine™ LTX or Lipofectamine™ RNAiMAX Reagent.
- Using PLUS™ Reagent enhances transfection performance in human astrocytes.
- We recommend Opti-MEM® I Reduced Serum Medium to dilute the DNA and Lipofectamine™ LTX Reagent or the siRNA and Lipofectamine™ RNAiMAX Reagent before complexing.

Preparing Geltrex™-Coated Plates for Human Astrocytes

Before thawing or passaging GIBCO® Human Astrocytes, prepare culture vessels coated with Geltrex™ as described below.

1. Thaw a bottle of Geltrex™ Basement Membrane Matrix at 4°C overnight.
2. On ice, prepare a stock solution of Geltrex™ diluted 1:1 in D-MEM. Store in aliquots at –20°C until needed.
3. Dilute the stock solution 1:100 in D-MEM and coat the bottom of each culture vessel (200 µL of Geltrex™ per cm² of culture vessel).
4. Incubate the culture vessel at 37°C for 1 hour. Dishes coated with Geltrex™ can be used immediately or stored at 4°C for up to a week, sealed with Parafilm®. Do not allow dishes to dry. When you are ready to add cells, aspirate the Geltrex™ solution and rinse the plates once with D-PBS with Ca²⁺ and Mg²⁺ before adding the cell solution.

Preparing Media

Aseptically mix the following components for preparing 100 mL of complete GIBCO® Astrocyte Medium. Complete Astrocyte Medium is stable for 2 weeks when stored at 4°C protected from light.

Component	Amount per 100 mL	Amount per 500 mL
D-MEM	89 mL	445 mL
N-2 Supplement	1 mL	5 mL
FBS	10 mL	50 mL
Note: Adding EGF at a final concentration of 20 ng/mL can increase proliferation, but may result in morphological and phenotypic changes in human astrocytes.		

Handling and Harvesting Human Astrocytes

1. Warm Complete Astrocyte Medium and StemPro® Accutase® Cell Dissociation Reagent in a 37°C water bath before use.
2. Transfer conditioned medium from the cells to a new tube; this will be used to stop the enzyme reaction in step 6.
3. Wash cells once with 1X D-PBS without calcium, magnesium, or phenol red.
4. Aspirate D-PBS and add StemPro® Accutase® to the cells.
5. Incubate for 5–10 minutes at 37°C. Rock the cells every ~5 minutes and check under a microscope for detachment and dissociation toward single cells.
6. When the cells have detached, add an equal volume (1:1) of conditioned medium (from Step 2) to slow the Accutase® activity.
7. Transfer the cells to a 15-mL or 50-mL tube.
8. Rinse culture vessels with complete medium and add it to the tube.
9. Centrifuge the tube for 4 minutes at 200 × g.
10. Aspirate and discard the supernatant.
11. Gently resuspend the pellet in Complete Astrocyte Medium.
12. Count the live cells using a method of choice.
13. To replate human astrocytes, remove a Geltrex™-coated plate from 4°C storage and tip slightly to aspirate the Geltrex™ solution. Rinse the plate once with D-PBS with calcium and magnesium. Do not allow the plate to dry out.
14. Immediately seed the astrocytes at the desired concentration (we recommend $\geq 2 \times 10^4$ cells/cm²).
15. Incubate the cells in an incubator at 37°C in a humidified atmosphere (90%) of 5% CO₂ in air. Change the medium every 2–3 days with fresh Complete Astrocyte Medium.

Transfecting Plasmid DNA into Human Astrocytes Using Lipofectamine™ LTX Reagent

Use this procedure to transfect plasmid DNA into GIBCO® Human Astrocytes using the Lipofectamine™ LTX Reagent in a **24-well format** (for other formats, see **Scaling Up or Down Transfections**, below). All amounts and volumes are given on a per well basis.

1. The day before transfection, prepare Human Astrocytes that have recovered from cryopreservation and have reached 80% confluency. Use StemPro® Accutase® to detach the cells and count the cells. Plate 5×10^4 cells per well in 0.5 mL of complete growth medium. Cell density should be 80–90% confluent on the day of transfection.
2. For each well of cells to be transfected, dilute 0.5 µg of DNA into 100 µL of Opti-MEM® I Reduced Serum Medium without serum.
3. Using PLUS™ Reagent: Mix PLUS™ Reagent gently before use, then add 0.5 µL PLUS™ Reagent (a 1:1 ratio to DNA) directly to the diluted DNA. Mix gently and incubate for 5–15 minutes at room temperature.
4. For each well of cells, dilute 1.5–3.0 µL of Lipofectamine™ LTX into the above diluted DNA solution, mix gently and incubate for 25 minutes at room temperature to form DNA-Lipofectamine™ LTX complexes.
5. Remove growth medium from cells and replace with 0.5 mL of complete growth medium. Add 100 µL of the DNA-Lipofectamine™ LTX complexes directly to each well containing cells and mix gently by rocking the plate back and forth.
6. Complexes do not have to be removed following transfection. Incubate the cells at 37°C in a CO₂ incubator for 18–24 hours post-transfection before assaying for transgene expression.

Scaling Up or Down Transfections

To transfect Human Astrocytes in different tissue culture formats, vary the amounts of Lipofectamine™ LTX Reagent, DNA, cells, medium and PLUS™ Reagent used in proportion to the relative surface area, as shown in the table (amounts given on a per well basis).

Culture vessel	Surface area per well*	Volume of plating medium	Cells per well	Volume of dilution medium†	DNA	Lipofectamine™ LTX Reagent	PLUS™ Reagent
96-well	0.3 cm ²	100 µL	1.0×10^4	20 µL	100 ng	0.3–0.6 µL	0.1 µL
48-well	1 cm ²	200 µL	2.0×10^4	40 µL	200 ng	0.6–1.2 µL	0.2 µL
24-well	2 cm ²	500 µL	5.0×10^4	100 µL	500 ng	1.5–3.0 µL	0.5 µL
12-well	4 cm ²	1 mL	1.0×10^5	200 µL	1 µg	3.0–6.0 µL	1.0 µL
6-well	10 cm ²	2 mL	2.5×10^5	500 µL	2.5 µg	7.5–15.0 µL	2.5 µL

* Surface areas may vary depending on the manufacturer.

† If the volume of Lipofectamine™ LTX Reagent is too small to dispense accurately, and you cannot pool dilutions, predilute Lipofectamine™ LTX Reagent 10-fold in Opti-MEM® I Reduced Serum Medium, and dispense a 10-fold higher amount (should be at least 1.0 µL per well). Discard any unused diluted Lipofectamine™ LTX Reagent.

Transfecting siRNA into Human Astrocytes Using Lipofectamine™ RNAiMAX Reagent

Prepare siRNAs

1. Resuspend the *Silencer*® Select siRNAs with nuclease-free water. A convenient stock concentration is 100 μ M, which can be diluted to meet downstream experimental needs.
2. Validate the concentration of the siRNA by measuring absorption at 260 nm using a spectrophotometer and adjust with water if necessary. Keep aliquots frozen at -20°C .
3. Dilute stock siRNAs of 100 μ M to a working concentration of 10 μ M.
4. From the working stock dilute siRNAs in 20 μ L of Opti-MEM® I per well to achieve a final concentration of 30 nM or your desired concentration (1 nM–100 nM) in tubes or plates. When applicable, make master mixes for replicates to minimize variability (at least one well overage).

For example with a 10 μ M siRNA stock, mix 0.39 μ L of siRNA (3.9 pmols) + 19.61 μ L of Opti-MEM®-I per well or 1.56 μ L of siRNA + 78.44 μ L of Opti-MEM®-I for the master mix.

5. Plate siRNAs in a Geltrex™-coated plate using the proper method for coating as provided by the manufacturer

Prepare cells

Culture cells according to manufacturer's cell protocol. Cells were in culture for about a week. On the day of transfection, harvest cells according to the protocol. Count and dilute cells to the proper density. We recommend performing an initial optimization experiment to determine your optimal cell density. Based on our optimization, we found 4,000 cells per well to be an optimal cell density.

Transfect cells

We recommend performing an initial optimization experiment to determine the optimal amount of transfection agent to add that balances good knockdown and low toxicity. Based on our optimizations, we found 0.15 μ L per well of Lipofectamine™ RNAiMAX to be the best condition.

1. Dilute 0.15 μ L per well of Lipofectamine™ RNAiMAX in Opti-MEM® I for a total volume of 10 μ L per well in a polystyrene 12 $\times$ 75-mm tube or a conical centrifuge tube. Make a master mix of sufficient volume to treat all wells to be transfected plus an extra 10% for pipetting variability. Mix the Lipofectamine™ RNAiMAX–Opti-MEM® I mixture by gently flicking the bottom of the tube.
2. Combine 10 μ L of the Lipofectamine™ RNAiMAX–Opti-MEM® I mixture per 20 μ L of diluted and pre-plated siRNA. Mix by tapping the plate back and forth. Incubate this mixture for 10 minutes at room temperature.
3. After the incubation, add 80 μ L of human astrocytes that have been diluted to the proper density to each well. The final volume per well should be 110 μ L per well.
4. Place the plate in a 37°C incubator under normal cell culture conditions. Remove the cells and assay for the expression levels of the gene of interest at the desired time point (typically 24–48 hours post-transfection).

References

- Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., *et al.* 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 16:675–686.
- Rothstein, J.D., Martin, L., Levey, A.I., Dykes-Hoberg, M., Jin, L., *et al.* 1994. Localization of neuronal and glial glutamate transporters. *Neuron* 13:713–725.
- Xu, L., Sapolsky, R.M., and Giffard, R.G. 2001. Differential sensitivity of murine astrocytes and neurons from different brain regions to injury. *Exp Neurol*. 169:416–424.
- Zhao, G., and Flavin, M. P. 2000. Differential sensitivity of rat hippocampal and cortical astrocytes to oxygen-glucose deprivation injury. *Neurosci Lett*. 285:177–180.

Using Neural Cells for Cell Therapy

Modeling Parkinson's Disease in Rats

Summary

In animals with a unilateral dopaminergic (DA) lesion, there is an imbalance of motor activity. Complete DA lesion can be induced by unilateral intracerebral stereotactic injection of 6-hydroxydopamine in the medial forebrain bundle (MFB). This model is useful in the study of DA replacement therapy. This chapter describes methods to induce a rat Parkinson's disease model with complete unilateral DA lesion.

Required Materials

- | | |
|---------------------------|---|
| Rats | <ul style="list-style-type: none">• Sprague-Dawley rats (200–250 g) |
| Media and Reagents | <ul style="list-style-type: none">• Sterile saline• Isoflurane• Betadine• 70% Ethanol• 6-hydroxydopamine (Sigma-Aldrich, Cat. no. H116)• Apomorphine (Sigma-Aldrich, Cat. no. A4393)• Amphetamine (Sigma-Aldrich, Cat. no. A1263) |
| Special Tools | <ul style="list-style-type: none">• Stereotactic frame• Animal balance• Isoflurane inhalation chamber• Electric razor• Scalpel• Tissue forceps• Scissors• 10 μL Hamilton syringes and needles• Dental drill• Sutures or staples |

Preparing Reagents

Preparing 6-hydroxydopamine (6-OHDA) Solution

Make a 2-mg/mL solution of 6-hydroxydopamine (6-OHDA) in saline. Store protected from light up to 12 months at -20°C .

Performing the DA Lesion Using a Rat Model

Preparing the Animal

1. Weigh a rat and place it into an isoflurane chamber and apply oxygen and isoflurane until the animal is deeply anesthetized.
2. Position the rat in a stereotactic frame and fix the plastic tube connected to the anesthesia machine to the nose of the rat using surgical tape. Maintain isoflurane at ~1.5% with an oxygen flow of 2–3 liters/minute.
3. Shave the top of the rat's head with an electric razor. Clean the skin with betadine and 70% ethanol.
4. Perform a midline incision with a scalpel and identify the bregma at the intersection of the coronal and the sagittal sutures.
5. Adjust the incisor bar in the rat until the heights of lambda and bregma skull points are equal.
6. Calculate the stereotactic coordinates for injection. For a MFB lesion, coordinates are in reference to the bregma: Anteroposterior (A/P) -2.2 mm; mediolateral (M/L) 1.5 mm.
7. Drill a burr hole at the target site using a dental drill.

Administering 6-OHDA

1. Fill a 10- μL Hamilton syringe with 5 μL 6-OHDA solution. Attach the syringe to the holder on the stereotactic frame.
2. Lower the needle of the Hamilton syringe so that it is 8 mm from the dura.
3. Inject the 6-OHDA solution at a rate of 1 μL /minute.
4. Leave the needle in place for 5 minutes and withdraw the tip slowly.
5. Close scalp margins with sutures or staples. Remove the rat from the stereotactic frame and place it in its home cage. Put food on the floor of the cage and monitor the animal's weight for 3 days after surgery.

Evaluating the Behavior

At 10–14 days after injection of 6-OHDA, the rats that exhibit at least 210 contralateral rotations over 30 minutes when challenged with the dopamine receptor agonist apomorphine (0.2 mg/kg, i.p.), or 630 ipsilateral rotations over 90 minutes when challenged with the DA-releasing substance amphetamine (5 mg/kg, i.p.) are suitable for future study.

Appendix

Life Technologies Products

Overview

Life Technologies provides you with all of your neural cell culture needs through its GIBCO® Cell Culture Media and offers products including reagents, media, sera, and growth factors to support the growth of a range of neural cell lines. All cell culture media products available from Life Technologies are tested for contamination and guaranteed for their quality, safety, consistency, and regulatory compliance. For more information on Invitrogen and GIBCO® products, refer to www.invitrogen.com.

Cells

Product	Quantity	Cat. no.
Rat		
GIBCO® Rat Fetal Neural Stem Cells	1 mL	N7744-100
GIBCO® Rat Fetal Neural Stem Cell Kit, includes StemPro® NSC SFM	1 kit	N7744-200
GIBCO® Rat Primary Cortical Astrocytes	1 mL	N7745-100
GIBCO® Rat Glial Precursor Cells	1 mL	N7746-100
Primary Rat Cortex Neurons (1 × 10 ⁶ cells/mL)	1 mL	A10840-01
Primary Rat Cortex Neurons (4 × 10 ⁶ cells/mL)	1 mL	A10840-02
Primary Rat Hippocampus Neurons	1 mL	A10841-01
Human		
GIBCO® Human Astrocytes	1 mL	N7805-100
GIBCO® Human Astrocytes Kit, includes GIBCO® Astrocyte Medium	1 kit	N7805-200
GIBCO® Human Neural Stem Cells (H9-Derived)	1 mL	N7800-100
GIBCO® Human Neural Stem Cells (H9-Derived) Kit, includes StemPro® NSC SFM	1 kit	N7800-200
Mouse		
GIBCO® Mouse Embryonic Fibroblasts (MEF), irradiated	1 mL	S1520-100

Media

Product	Quantity*	Cat. no.
Dulbecco's Modified Eagle Medium (D-MEM), high glucose	500 mL	11995-065
GIBCO® Astrocyte Medium	500 mL	A12613-01
Hibernate®-A	500 mL	A11473-DJ
Hibernate®-A	500 mL	A12475-01
KnockOut™ D-MEM/F-12	500 mL	12660-012
Neurobasal™ Medium (1X), liquid (without Phenol Red)	500 mL	12348-017
Neurobasal™ Medium (1X), liquid	500 mL	21103-049
Neurobasal™-A Medium (1X), liquid (without Phenol Red)	500 mL	12349-015
Neurobasal™-A Medium (1X), liquid	500 mL	10888-022
Opti-MEM® I Reduced Serum Medium (1X), liquid	100 mL	31985-062
StemPro® NSC SFM	1 kit	A10509-01
Synth-a-Freeze® Cryopreservation Medium	50 mL	R-005-50
* Some of the products are also available in different quantities and packaging sizes.		

Sera and Serum-Replacement Products

Product	Quantity*	Cat. no.
Fetal Bovine Serum, ES Cell-Qualified One Shot™ (US)	50 mL	16141-002
Fetal Bovine Serum, ES Cell-Qualified FBS (US)	100 mL	16141-061
Fetal Bovine Serum (FBS), Certified	500 mL	16000-044
Goat serum	100 mL	16210-064
Heat-inactivated Fetal Bovine Serum (FBS)	500 mL	10438-026
Knockout™ Serum Replacement	500 mL	10828-028
* Some of the products are also available in different quantities and packaging sizes.		

Substrates, Matrices, and Bio-scaffolds

Product	Quantity	Cat. no.
CELLstart™ CTS™	2 mL	A10142-01
Fibronectin, Human Plasma	5 mg	33016-015
Fibronectin, Bovine Plasma	1 mg	33010-018
Geltrex™ LDEV Free Reduced Growth Factor Basement Membrane Extract	5 mL	A11343-01
Geltrex™ Reduced Growth Factor Basement Membrane Matrix	1 mL 5 mL	12760-013 12760-021
Natural Mouse Laminin	1 mg	23017-015

Supplements

Product	Quantity*	Cat. no.
B-27 [®] Serum-Free Supplement (50X), liquid	10 mL	17504-044
B-27 [®] Supplement (50X)	10 mL	0080085-SA
B-27 [®] Supplement Minus A0 (50X), liquid	10 mL	10889-038
B-27 [®] Supplement Minus Vitamin A (50X), liquid	10 mL	12587-010
B-27 [®] Supplement XenoFree (50X)	10 mL	A11576-SA
Bovine Serum Albumin (BSA)	150 mg	15561-020
G-5 Supplement (100X), liquid	1 mL	17503-012
GlutaMAX [™] -I	100 mL	35050-061
L-Glutamine 200 mM (100X), liquid	100 mL	25030-081
MEM Non-essential Amino Acids Solution (NEAA), 10 mM	100 mL	11140-050
Myelin Basic Protein (MBP)	10 mg	13228-010
N-2 Supplement (100X), liquid	5 mL	17502-048
Pluronic [®] F-127	1 mL	P-3000MP
StemPro [®] Neural Supplement	10 mL	A10508-01
β-Mercaptoethanol (1,000X), liquid	50 mL	21985-023
* Some of the products are also available in different quantities and packaging sizes.		

Reagents

Product	Quantity*	Cat. no.
Antibiotic-Antimycotic solution (100X), liquid	100 mL	15240-062
Gentamicin/Amphotericin Solution, 10 vials/pkg	10 × 1 mL	R-015-10
Lipofectamine [™] LTX Reagent and PLUS [™] Reagents	1 mL	15338-100
Lipofectamine [™] RNAiMAX Transfection Reagent	1.5 mL	13778-150
Penicillin-Streptomycin, liquid	100 mL	15070-063
Ribonuclease H (RNase H)	120 units	18021-071
StemPro [®] Accutase [®] Cell Dissociation Reagent	100 mL	A11105-01
TrypLE [™] Express Stable Trypsin Replacement Enzyme	100 mL	12604-013
TrypLE [™] Select, 10X	100 mL	A12177-01
Trypsin/EDTA solution, 0.05%	100 mL	25300-054
Trypan Blue Stain	100 mL	15250-061
TRIzol [®] Reagent	200 mL	15596-018
* Some of the products are also available in different quantities and packaging sizes.		

Growth Factors & Purified Proteins

Product	Quantity	Cat. no.
α -Synuclein Recombinant Human	200 μ g	PHB0044
Acidic Fibroblast Growth Factor (aFGF) Recombinant Human	10 μ g	13241-013
B-DNF Recombinant Human	10 μ g	PHC7074
BMP-4 Recombinant human	5 μ g	PHC7914
BMP-7 (inactive) Recombinant Human	10 μ g	PHC7104
Brain-Derived Neurotrophic Factor (BDNF) Recombinant Human	5 μ g	10908-010
CNTF Recombinant Human	20 μ g	PHC7015
EPO Recombinant Human	500 IU	PHC2054
Epidermal Growth Factor (EGF), Recombinant Human	10 μ g	PHG0314
Epidermal Growth Factor (EGF), Natural Mouse	100 μ g	53003-018
FGF-basic Recombinant Human	10 μ g	13256-029
FGF-basic (AA 10–155), Recombinant Human (bFGF)	10 μ g	PHG0024
FGF-8b Recombinant Human	100 μ g	PHG0271
G-DNF Recombinant Human	100 μ g	PHC7041
G-DNF Recombinant Human	5 μ g	PHC7044
G-DNF Recombinant Human	10 μ g	PHC7045
NT-3 Recombinant Human	5 μ g	PHC7034
NT-4 Recombinant Human	5 μ g	PHC7024
Nerve Growth Factor 2.5S (NGF 2.5S) Natural Mouse	10 μ g	13257-019
Nerve Growth Factor 7S (NGF 7S) Natural Mouse	100 μ g	13290-010
Neurturin Recombinant Human	10 μ g	PHC7064
PDGF-BB Recombinant Human	100 μ g	PHG0041
PDGF-BB Recombinant Human	1 mg	PHG0043
PDGF-BB Recombinant Human	10 μ g	PHG0045
PDGF-BB Recombinant Human	50 μ g	PHG0046

Buffers and Balanced Salt Solutions

Product	Quantity	Cat. no.
Dulbecco's Phosphate-Buffered Saline (D-PBS)	500 mL	14040-133
Dulbecco's Phosphate-Buffered Saline (D-PBS) without Ca^{2+} and Mg^{2+}	500 mL	14190-144
Hanks' Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+}	500 mL	14170-112

Accessory Products

Product	Quantity	Cat. no.
Countess [®] Automated Cell Counter	1 each	C10227
Fluo-4, AM	10 × 50 µg	F14201
LIVE/DEAD [®] Cell Vitality Assay Kit	1 kit	L34951
LIVE/DEAD [®] Viability/Cytotoxicity Assay Kit	1 kit	L-3224
Neon [®] Transfection system	1 each	MPK5000
Neon [®] Kit, 10 µL	192 reactions	MPK1096
Neon [®] Kit, 100 µL	192 reactions	MPK10096
Platinum [®] SYBR [®] Green qPCR SuperMix-UDG	100 reactions 500 reactions	11733-038 11733-046
ProLong [®] Gold antifade reagent	10 mL	P36930
PureLink [™] HiPure Plasmid Miniprep Kit	100 preps	K2100-03
PureLink [™] HiPure Plasmid Midiprep Kit	25 preps	K2100-04
PureLink [™] HiPure Plasmid Maxiprep Kit	25 preps	K2100-07
PureLink [™] RNA Mini Kit	50 preps	12183-018A
Qubit [®] 2.0 Fluorometer	1 each	Q32866
Qubit [®] 2.0 Quantitation Starter Kit	1 kit	Q32871
StemPro [®] EZPassage [™] Disposable Stem Cell Passaging Tool	10 units	23181-010
Superscript [®] III First Strand Synthesis SuperMix	50 reactions	18080-400
Superscript [®] III First Strand Synthesis SuperMix for qRT-PCR	50 reactions	11752-050
SuperScript [®] VILO [™] cDNA Synthesis Kit	50 × 20 µL 250 × 20 µg	11754-050 11754-250
Water, distilled	6 × 1 L	15230-001
10X BlueJuice [™] Gel Loading Buffer	3 × 1 mL	10816-015

Resources for More Information

Books

Developmental Biology, 9th edition, edited by Scott F. Gilbert Sinauer Associates, 2010.

Neural Stem Cells, 2nd edition, edited by Lesile P. Weiner, Humana Press, 2008.

Neural Development and Stem Cells, 2nd edition, edited by Mahendra S. Rao, Humana Press, 2006.

Protocols for neural cell culture, 4th edition, edited by Laurie C. Doering, Humana Press, 2010.

Protocols for neural cell culture, 3rd edition, edited by Sergey Fedoroff and Arleen Richardson, Humana Press, 2001.

Journals

Journal Neuron, www.cell.com/neuron

Journal Development, www.dev.biologists.org

Journal of Neuroscience, www.jneurosci.org

American Academy of Neurology, www.aan.com

European Journal of Neuroscience,
www.onlinelibrary.wiley.com/journal/10.1111/%28ISSN%291460-9568

Journal of Neurobiology, www.interscience.wiley.com/jpages/0022-3034

Developmental Neurobiology,
www.onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291097-4695

Nature Reviews - Neuroscience, www.nature.com/nrn/index.html

Organizations

Society for Neuroscience, www.sfn.org

European Neuroscience and Society Network, www.lse.ac.uk/collections/ENSN

Federation of European Neuroscience Societies, www.fens.mdc-berlin.de

Japanese Society for Neuroscience, www.jnss.org/english/index_e.html

Government Sites

National Institute of Neurological Disorders and Stroke (NINDS), www.ninds.nih.gov

National Institute of Mental Health (NIMH), www.nimh.nih.gov

Food and Drug Administration (FDA), www.fda.gov

National Institute of Child Health and Human Development (NICHD),
www.nichd.nih.gov

NCBI PubMed, www.ncbi.nlm.nih.gov/pubmed

National Institutes of Health Entrez Databases,
www.ncbi.nlm.nih.gov/Database/index.html

National Library of Medicine's MEDLINEplus,
www.ncbi.nlm.nih.gov/Database/index.html

Websites

The Dana Foundation, www.dana.org

Neuroanatomy and Neuropathology on the Internet, www.neuropat.dote.hu

Neuromuscular Disease Center at Washington University School of Medicine, St. Louis,
www.neuromuscular.wustl.edu

Neuroscience Information Framework, www.neuinfo.org

Blog and News Sites

Parkinson's Disease: Blog - Business Exchange,
www.bx.businessweek.com/parkinsons-disease/blogs

Neurology News & Neuroscience News from Medical News Today,
www.medicalnewstoday.com/sections/neurology

ScienceDaily: Neuroscience News,
www.sciencedaily.com/news/mind_brain/neuroscience

Alltop - Top Neuroscience News, www.neuroscience.alltop.com

Regulations for Cell Therapy

EMA, <http://www.ema.europa.eu/ema>

FDA, www.fda.gov/BiologicsBloodVaccines/CellularGeneTherapyProducts

Technical Support

Web Resources

Visit the Invitrogen website at www.invitrogen.com for:

- Technical resources, including manuals, vector maps and sequences, application notes, Safety Data Sheets (SDSs), FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

For more information or technical assistance, call, write, fax, or e-mail. Additional international offices are listed on our website (www.invitrogen.com).

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SDS

Safety Data Sheets (SDSs) are available at www.invitrogen.com/sds.

Certificate of Analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to www.invitrogen.com/support and search for the Certificate of Analysis by product lot number, which is printed on the product packaging (tube, pouch, or box).

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