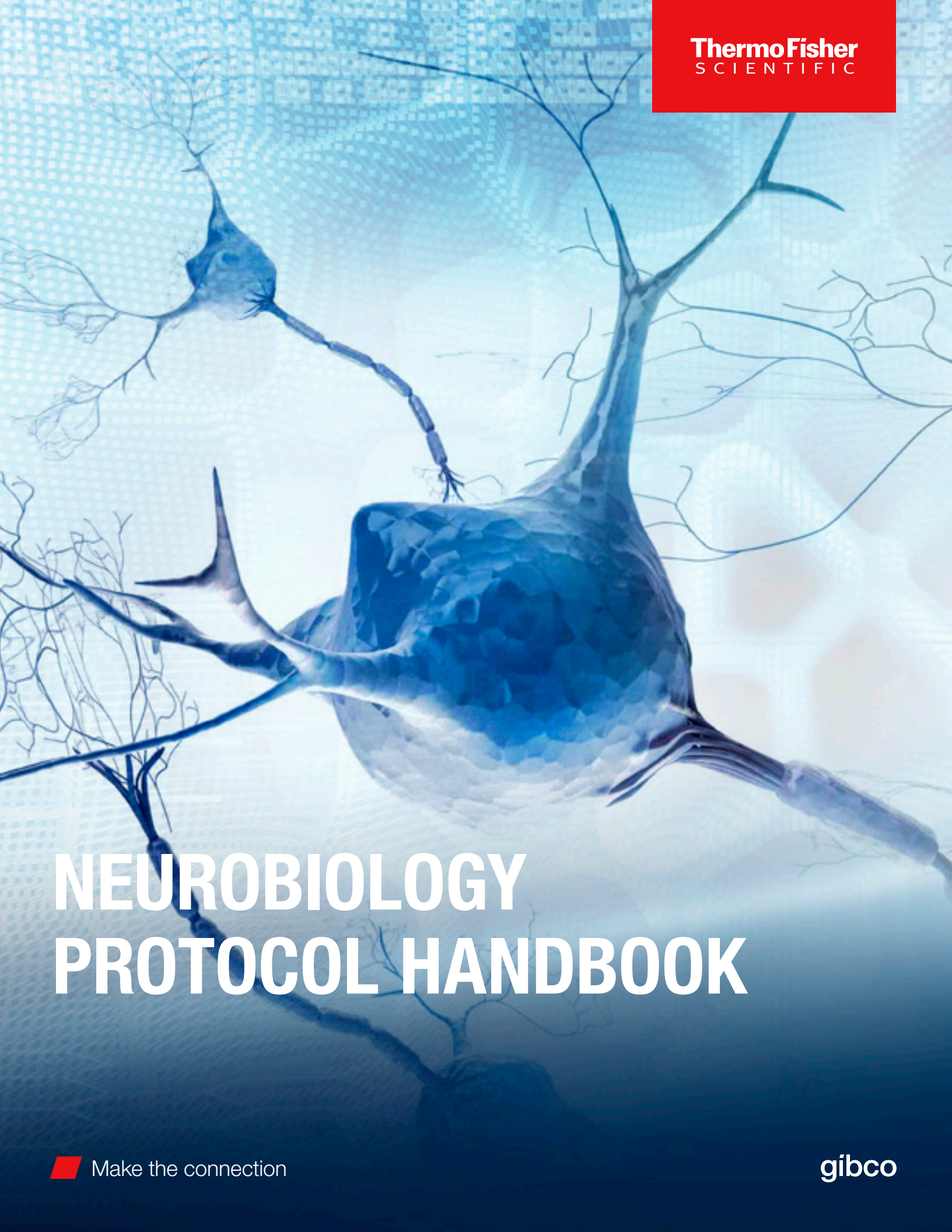

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NEUROBIOLOGY PROTOCOL HANDBOOK

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Introduction

1 Neural development and neural stem cells

Overview

It has long been thought that the adult mammalian nervous system is 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 to repopulate 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 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 describes 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 the mother cell). 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 produce differentiated cells. Some pluripotent stem cells, including embryonic stem cells (ESC) and induced pluripotent stem cells (iPSCs), have the capacity for multilineage differentiation, enabling construction of a complete, viable organism (i.e., they are pluripotent). In contrast, adult stem cells can generate only one specific lineage of differentiated cells to reconstitute tissues or organs.

Neural stem cells

Neural stem cells (NSCs) are stem cells in the nervous system that can self-renew and give rise to differentiated progenitor cells that 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. A thorough characterization of NSCs, 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, are crucial to increase the likelihood of clinical success in the use of stem cells in neurological disorders.

Stem cells and cancer

The discovery that many cancers may be propagated by a small number of stem cells present in a tumor mass is an extremely exciting finding. 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

Establishment of the central nervous system (CNS) is initiated early in development by the induction of NSCs and neural progenitor cells; this stage in development is called neural ectoderm 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

Special notice about B-27 Supplement

For over 25 years, the classic Gibco™ B-27™ Supplement and Gibco™ Neurobasal™ Medium have set the standard for neuronal cell culture reagents. Originally designed for the serum-free culture of primary neurons, B-27 Supplement and Neurobasal Medium have been widely adapted to other applications and cell types, including, among others, PSC-derived neurons and the long-term culture of primary neurons.

To meet the growing needs of researchers, we have optimized the nutritive properties of B-27 Supplement and Neurobasal Medium to better support primary and stem cell–derived neurons. The Gibco™ B-27™ Plus Neuronal Culture System, composed of the Gibco™ B-27™ Plus Supplement and Gibco™ Neurobasal™ Plus Medium, improves upon the classic culture environment through raw material and manufacturing upgrades and minor formulation modifications. Together, these small changes yield big results.

The updated protocols in this handbook include the B-27 Plus Neuronal Culture System, which will:

- **Improve neuronal survival**—Maintaining healthy long-term cultures of primary rodent neurons and stem cell–derived neurons can be challenging, as these cells are quite sensitive and tend to undergo progressive cell death over time. The B-27 Plus Neuronal Culture System improves survival by more than 50%, without the need for additional components such as serum.

- **Improve neuronal functionality**—With increased survival comes a superior initial quality of neurons. Indicators of this are superior neurite outgrowth, increased synaptic complexity, and improvement of electrophysiological activity. The B-27 Plus Neuronal Culture System has been shown to improve functionality of primary and stem cell–derived neurons.

- **Improve neuronal maturity**—During maturation, neurons extend to form highly connected networks, express synaptic markers, and generate spontaneous, networked electrical activity. Robust maturation is necessary for the generation of relevant disease model systems. The B-27 Plus Neuronal Culture System increases synaptic complexity of neurons, leading to more extensive expression of pre- and post-synaptic markers.

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can be derived from human ESC cultures over a period of 1–2 weeks. *In vivo*, the primitive neural tube forms by approximately the fourth week of gestation via a process termed primary neurulation. 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. Neural crest stem cells generate the peripheral nervous system (PNS), which segregates from the CNS at this stage. Neural crest stem cells generate the sympathetic and parasympathetic systems, the dorsal root ganglia and the cranial nerves, and the peripheral glia, including Schwann cells and enteric glia. In addition to neural derivatives, the cranial crest generates craniofacial mesenchyme that includes 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, which are distinct from CNS stem cells, can use similar media and culture conditions for propagation over limited time periods.

Stem cells in the 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 various rates and express different positional markers. The anterior neural tube undergoes a significant 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 and forms the spinal cord. The ventricular zone stem cells appear homogeneous 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 the subventricular zone

As development proceeds, the ventricular zone is 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 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 and uni-, bi-, and tri-potential glial precursors that generate astrocytes and oligodendrocytes. 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.

2 Neural cell types in neurological diseases

This table lists some of the better-known neurological disorders with experimental models, cell types, growth factors, and markers studied.

Neural disease	Experimental model	Cell type	Growth factor(s)	Progenitor cell(s)	Markers	Mature markers	Transplantation	Reference
Spinal cord injury	Transplantation of oligodendrocyte progenitor cells (OPCs) into demyelination model	Oligodendrocyte	EGF, bFGF, PDGF, RA	OPC	OLIG1, A2B5, SOX10, NG2, O4	GalC, MBP, RIP	Yes	Keirstead et al., 2005
Alzheimer's disease	Transgenic models targeting APP, presenilin, tau proteins, tauopathy, atrophy, and neural loss in frontal cortex as well as in other regions	Cholinergic neuron	BDNF	Cholinergic neurons, NMDA receptors	Aβ or tau accumulations in neurons, monitor neurofibrillary tangles or Aβ peptide	Val66Met polymorphism	No	Giri et al., 2016
Multiple sclerosis	Demyelinated axons, co-cultured with rat hippocampal neurons	Oligodendrocyte	EGF, bFGF, PDGF, RA	OPC	PDGFR, A2B5, NG2	O4, O1, MBP, PLP	No	Kang et al., 2007
	Remyelination models	Oligodendrocyte	RA, EGF, bFGF, noggin, vitamin C	OPC	PDGFR, NG2, OLIG1/2, SOX10	O4, O1, MBP, PLP	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	HOXB4, NKX6-1/6-2, OLIG1/2	NKX6-1, OLIG2, NGN2, ISL1, ChAT, VAChT, HB9, LHX3, HOX	Yes	Lee et al., 2007
	In vitro 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

Neural disease	Experimental model	Cell type	Growth factor(s)	Progenitor cell(s)	Markers	Mature markers	Transplantation	Reference
Parkinson's disease	Not applicable	Dopaminergic (DA) neuron	SHH, FGF8, BDNF, AA, TGFβ, TGF-3	DA neuron precursor	PAX2, PAX5, LMX, EN1	MAP2, TH, AADC, VMAT, NURR1, PTX3	No	Perrier et al., 2004
	In vitro drug screening	DA neuron	FGF2 or FGF8, SHH, BDNF, GDNF, cAMP, AA	DA neuron precursor	EN1, OTX2, WNT1, PAX2, GBX2	TH, GABA, EN1, AADC	No	Yan et al., 2005
	Transplantation into the neostriata of 6-hydroxy-dopamine–lesioned Parkinsonian rats	DA neuron	FGF2, FGF8, SHH, BDNF, GDNF, FBS	DA neuron 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 neuron precursor	PAX2, EN1, NURR1, LMX1B	TH, EN1, AADC	Yes	Park et al., 2005
	Transplantation into the striatum of hemi-Parkinsonian rats and MPTP-treated rhesus monkeys	DA neuron	SHH, dual Smad inhibitor, FGF8, CHIR, BDNF, GDNF, dbcAMP, TGFb3	Midbrain floor plate cells	FoxA2, LMX1A, OTX2	TUJ-1, TH, NURR1, FoxA2, PTX3	Yes	Kriks et al., 2011
Glial-related diseases	Astrocyte-related disease	Astrocyte	Cyclopamine, human astrocyte medium	—	—	GFAP, S100, GLAST, BDNF, GNDF	No	Lee et al., 2006
CNS and PNS diseases	Peripheral and central nervous system neurons	Peripheral sensory neuron	Noggin, NGF	Neural precursor	NCAM, TUJ-1, SNAIL, dHAND, SOX9	Peripherin, BRN3, TH, TRK-A	No	Brokhman et al., 2008
Macular retinal degeneration	Not applicable	Retinal pigmented epithelium	Noggin, Dickkopf-1, IGF-1	Retinal progenitor	RX, PAX6, LHX2, SIX3	RPE-65	No	Lamba et al., 2006

Neural disease	Experimental model	Cell type	Growth factor	Progenitor cell	Marker	Mature marker	Transplantation	Reference
Huntington's disease	—	Striatal medium spiny neuron specification GABA neurons	—	—	Islet1, DARPP-32, mGluR1, NeuN	—	—	Molero et al., 2009
Hirschsprung's disease	Engraftment and functional rescue in HSCR mouse model	Enteric neurons	LDN, SB, CHIR, RA, FGF2, EGF, GDNF, AA	Enteric nervous system progenitors	Sox10, CD49d, EDNRB, RET, HOXB2, HOXB5	TUJ-1, PHOX2A, TRKC, 5-HT, GABA	Yes	Fattahi et al., 2016

References

Brokhman I, Gamarnik-Ziegler L, Pomp O, et al. (2008) Peripheral sensory neurons differentiate from neural precursors derived from human embryonic stem cells. *Differentiation* 76:145–155.

Fattahi F, Steinbeck JA, Kriks S, et al. (2016) Deriving human ENS lineages for cell therapy and drug discovery in Hirschsprung disease. *Nature* 531:105–109.

Giri M, Zhang M, and Lu Y (2016) Genes associated with Alzheimer's disease: An overview and current status. *Clin Interv Aging* 11:665–681.

Izrael M, Zhang P, Kaufman R, et al. (2007) Human oligodendrocytes derived from embryonic stem cells: Effect of noggin on phenotypic differentiation *in vitro* and on myelination *in vivo*. *Mol Cell Neurosci* 34:310–323.

Kang SM, Cho MS, Seo H, et al. (2007) Efficient induction of oligodendrocytes from human embryonic stem cells. *Stem Cells* 25:419–424.

Keirstead HS, Nistor G, Bernal G, et al. (2005) Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *J Neurosci* 25:4694–4705.

Kriks S, Shim J-W, Piao J, et al. (2011) Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature* 480:547–551.

Lamba DA, Karl MO, Ware CB, and Reh TA. (2006) Efficient generation of retinal progenitor cells from human embryonic stem cells. *Proc Natl Acad Sci U S A* 103:12769–12774.

Lee H, Shamy GA, Elkabetz Y, et al. (2007) Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons. *Stem Cells* 25:1931–1939.

Lee DS, Yu K, Rho JY, et al. (2006) Cyclopamine treatment of human embryonic stem cells followed by culture in human astrocyte medium promotes differentiation into nestin- and GFAP-expressing astrocytic lineage. *Life Sci* 80:154–159.

Li XJ, Du ZW, Zarnowska ED, et al. (2005) Specification of motoneurons from human embryonic stem cells. *Nat Biotechnol* 23:215–221.

Molero AE, Gokhan S, Gonzalez S, et al. (2009) Impairment of developmental stem cell-mediated striatal neurogenesis and pluripotency genes in a knock-in model of Huntington's disease. *Proc Natl Acad Sci U S A* 106:21900–21905.

Park CH, Minn YK, Lee JY, et al. (2005) *In vitro* and *in vivo* analyses of human embryonic stem cell-derived dopamine neurons. *J Neurochem* 92:1265–1276.

Perrier AL, Tabar V, Barberi T, et al. (2004) Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci U S A* 101:12543–12548.

Roy NS, Cleren C, Singh SK, et al. (2006) Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes. *Nat Med* 212:1259–1268.

Trujillo CA, Schwindt TT, Martins AH, et al. (2009) Novel perspectives of neural stem cell differentiation: From neurotransmitters to therapeutics. *Cytometry A* 75:38–53.

Yan Y, Yang D, Zarnowska ED, et al. (2005) Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. *Stem Cells* 23:781–790.

Neural cell culture and differentiation

3 Recovery, culture, and characterization of cortical and hippocampal neurons

Summary

Primary neuronal cultures are indispensable in the fields of neurobiology and pharmacology. Many researchers prefer to use freshly isolated neuronal cells, as they maintain their functional viability. For convenience, however, an alternate route is to utilize cryopreserved neurons. The ability to recover and culture primary neurons under serum-free conditions can reduce the variation that can arise from the isolation steps and facilitate tighter control of neuronal studies. Some serum-free media and supplements allow for the low-density culturing of neurons, 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 recovery and culture of cryopreserved primary neurons in serum-free media and supplements.

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Required materials

- Gibco™ Primary Rat Cortex Neurons, Sprague-Dawley (Cat. No. A36511 or A36512)
- Gibco™ Primary Rat Hippocampus Neurons (Cat. No. A1084101)
- Gibco™ Primary Mouse Cortical Neurons (Cat. No. A15585 or A15586)
- Gibco™ Primary Mouse Hippocampal Neurons (Cat. No. A15587)
- Gibco™ Poly-D-Lysine (Cat. No. A3890401)
- Gibco™ Distilled Water (Cat. No. 15230162)
- Gibco™ DPBS, calcium, magnesium (Cat. No. 14040141)
- Gibco™ Neurobasal™ Plus Medium (Cat. No. A3582901)
- Gibco™ B-27™ Plus Supplement (50X) (Cat. No. A3582801)
- Gibco™ GlutaMAX™ Supplement (Cat. No. 35050061)

Preparing media

Complete Neurobasal Plus Medium

Complete medium requires supplementation of Neurobasal Plus Medium with B-27 Plus Supplement (50X) and GlutaMAX Supplement. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

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

Component	Final conc.	Amount
Neurobasal Plus Medium	1X	98 mL
B-27 Plus Supplement (50X)	2%	2 mL
GlutaMAX Supplement	0.5 mM	250 µL

Preparing matrix

Coating culture vessels with poly-D-lysine

1. Dilute the poly-D-lysine stock solution 1:40 in DPBS, calcium, magnesium (DPBS +/+), to prepare a 50 µg/mL working solution (e.g., 125 µL of poly-D-lysine stock solution into 5 mL of DPBS +/+).

2. Coat the surface of the culture vessel with the working solution of poly-D-lysine (e.g., 50 µL/well for a 96-well plate).

3. Incubate the vessel at room temperature for 1 hour.

4. Remove the poly-D-lysine solution and rinse the culture surface 3 times with sterile distilled water (e.g., 100 µL/well for a 96-well plate).

   Make sure to rinse the culture vessel thoroughly, as excess poly-D-lysine solution can be toxic to the cells.

5. Remove the distilled water and leave the coated culture vessel uncovered in the laminar hood to dry.

   The culture surface should be fully dry after 2 hours.

   Note: Coated vessels can be used immediately once they are dry, or can be stored dry at 4°C. For storage at 4°C, tightly wrap the vessels with Parafilm™ laboratory film and use within one week of coating.

Cell recovery

Guidelines for recovery of cryopreserved neural cells

- Minimize the duration of exposure of neural cells to cryomedium at 37°C by thawing until only a small ice crystal remains. Avoid longer incubation at 37°C in cryomedium solutions.
- Ensure that the complete medium is added to the neural cells in cryomedium in a dropwise manner, to avoid osmotic shock.

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 medium before using them for transferring cell suspensions, to prevent the cells from sticking to the plastic. Do not centrifuge cells upon recovery from cryopreservation.

1. Remove one 1-mL 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 70% ethanol and tap gently on a surface so that all of the medium collects at the bottom of the vial.

4. Open the vial in a laminar flow hood.

5. Rinse a pipette tip with complete 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 complete medium (pre-warmed to 37°C), 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 medium to the tube (for a total suspension volume of 4 mL).

8. Mix the suspension very gently with a P1000 pipette. Avoid creating any air bubbles.

9. Using a pre-rinsed tip, add 10 µL of cell suspension to a microcentrifuge tube containing 10 µL of 0.4% trypan blue. Mix the cells by gently tapping the tube. Determine the viable cell density using a hemocytometer or the Invitrogen™ Countess™ II Automated Cell Counter.

10. Plate to achieve the seeding density per well recommended for the B-27 Plus system (see Table 3-1 on the next page) in a poly-D-lysine–coated plate or a chambered slide. Bring the cell suspension volume to the desired volume per well by adding complete medium.

Note: Use of a ROCK inhibitor (including Gibco™ RevitaCell™ Supplement) is not recommended, as it tends to rescue the glial population, which will decrease the neuronal purity. When maximum cell viability is desired, add Gibco™ CultureOne™ Supplement (100X), which will suppress the proliferation of the contaminating glial population.

11. Incubate the cells at 37°C in a humidified atmosphere of 5% CO_2 in air.

12. Feed the cells on the next day and every third day thereafter by aspirating half the medium from each well and replacing it with fresh complete medium.

Table 3-1. Recommended seeding densities for primary neurons.

Source	Medium	Seeding density		
		Low (cells/cm²)	Medium (cells/cm²)	High* (cells/drop)
Rat	B-27 classic**	≥40,000	≥90,000	160,000
	B-27 Plus system	20,000	60,000	80,000
Mouse	B-27 classic**	≥60,000	≥100,000	120,000
	B-27 Plus system	30,000	60,000	60,000
Feeding schedule†		1–2 times weekly	Every 3–4 days	Every 2–3 days

* Multi-electrode array (MEA) application.
** B-27 classic: B-27 Supplement with Neurobasal Medium.
† Suggested half-volume change per feed.

Expected results

Following the above protocol guidance and the B-27 Plus Neuronal Culture System protocol (**thermofisher.com/b27plus**) will result in increased neuronal survival, accelerated neurite outgrowth, and improved neural network activity and maturation in primary neurons (Figure 3-1 below and Figure 3-2 on the next page).

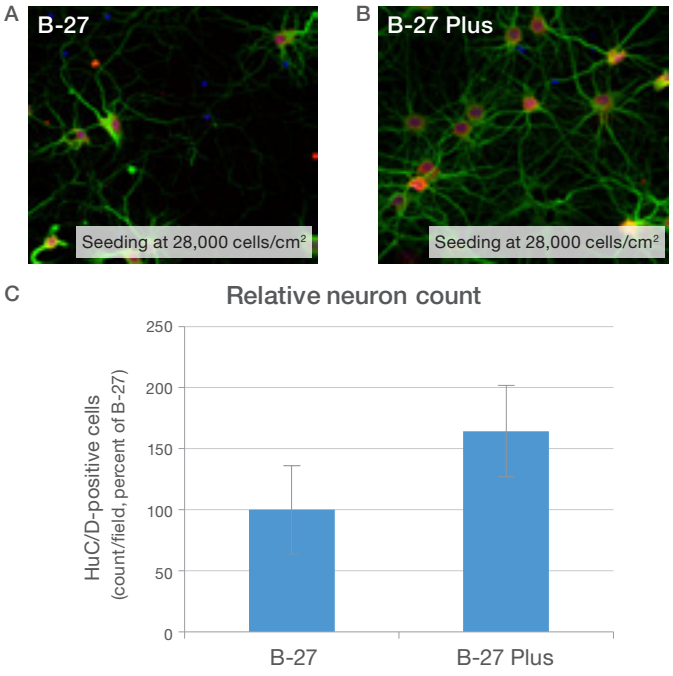

Figure 3-1. Greater viability and quality of primary neurons with fewer cells: mouse cortical neurons on culture day 14. Cells were plated at 28,000 cells/cm² and maintained in either **(A)** "classic" B-27 Supplement and Neurobasal Medium or **(B)** the B-27 Plus Neuronal Culture System (B-27 Plus Supplement with Neurobasal Plus Medium). The number of neurons **(C)** was determined by automated image capture and analysis on the Thermo Scientific™ CellInsight™ CX5 High-Content Screening (HCS) Platform with Thermo Scientific™ HCS Studio™ Cell Analysis Software. Neurons were stained with MAP2 (green) and HuC/D (red) antibodies. Nuclei were labeled with Invitrogen™ Hoechst 33342 (blue). For more information about our antibodies and staining procedure, refer to the Immunocytochemistry chapter (beginning on page 80).

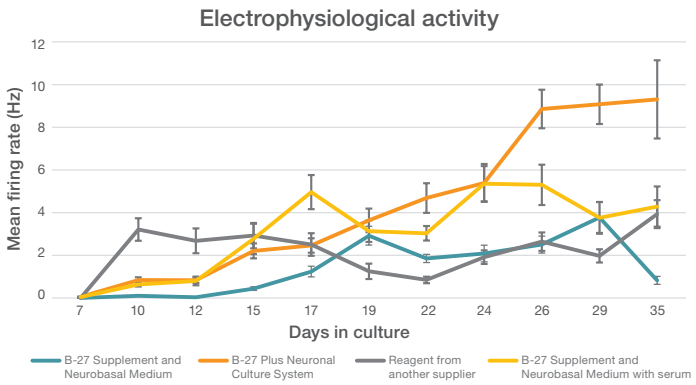

Figure 3-2. The B-27 Plus system promotes better electrophysiological activity than classic B-27 Supplement with serum. The B-27 Plus system (orange) promoted a higher degree of firing activity as the network matured, as quantified via mean firing rate. Excitability of a neural network was quantified by the mean firing rate, defined as the total number of action potentials detected per second.

Troubleshooting

For troubleshooting tips regarding cryopreservation and recovery of mature neurons, see below.

Problem	Possible cause	Solution
Low cell survival	Neurons stressed during recovery from cryopreservation	• Ensure that cells are not left at 37°C for extended periods of time. Be certain to thaw neurons until only a small ice crystal remains. • Ensure dropwise addition of growth medium to the neurons to avoid osmotic shock to the cells. • Limit the number of vials thawed at one time, as longer exposure of cells to cryopreservation media can have a negative impact on post-thaw viability.
Non-reproducible recovery	Failure to prewet plastics	• Ensure prewetting of vials and plastics, including conical tubes and pipette tips, to minimize loss of neurons to the surface of the plastics.

4 Thawing and culture of cryopreserved neurons

Summary

The ability to cryopreserve primary neuronal cells is of great interest to many researchers. Flexibility in experimental workflows allows researchers to bank cells for use in basic research, translational medicine, and cell therapy applications. In this chapter we provide detailed protocols for recovery of cells and culture in complete Neurobasal medium, which provides high post-thaw viability and recovery with superior neuronal quality.

View this protocol online and order products at **thermofisher.com/neuroprotocols**

Required materials

Cells

- Gibco™ Primary Rat Cortex Neurons (Cat. No. A1084001)
- Gibco™ Primary Rat Hippocampus Neurons (Cat. No. A1084101)
- Gibco™ Primary Mouse Cortical Neurons (Cat. No. A15585)
- Gibco™ Primary Mouse Hippocampal Neurons (Cat. No. A15587)

Media and reagents

- Gibco™ Neurobasal™ Medium (Cat. No. 21103049)
- Gibco™ B-27™ Supplement (50X), serum free (Cat. No. 17504044)
- Gibco™ GlutaMAX™ Supplement (Cat. No. 35050061)
- Gibco™ Trypan Blue Solution, 0.4% (Cat. No. 15250061)
- Gibco Poly-D-Lysine (Cat. No. A3890401)
- 48-well plate or 8-chambered slides
- Gibco™ Distilled Water (Cat. No. 15230162)
- Gibco™ DPBS, calcium, magnesium (Cat. No. 14040141)

Preparing media

Complete Neurobasal medium

Complete Neurobasal medium requires supplementation of Neurobasal Medium with B-27 Supplement (50X), serum free, and GlutaMAX Supplement. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

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

Component	Final conc.	Amount
Neurobasal Medium	1X	98 mL
B-27 Supplement (50X), serum free	2%	2 mL
GlutaMAX Supplement	0.5 mM	250 µL

For primary hippocampus neuron cultures, complete Neurobasal 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. Dilute the poly-D-lysine stock solution 1:40 in DPBS, calcium, magnesium (DPBS +/+), to prepare a 50 µg/mL working solution (e.g., 125 µL of poly-D-lysine stock solution into 5 mL of DPBS +/+).

2. Coat the surface of the culture vessel with the working solution of poly-D-lysine (150 µL/cm^2, e.g., 100 µL/well for a 48-well plate).

3. Incubate the culture vessel at room temperature for 1 hour.

4. Remove the poly-D-lysine solution and rinse the culture surface 3 times with sterile distilled water (e.g., 100 µL/well for a 96-well plate).

   Make sure to rinse the culture vessel thoroughly, as excess poly-D-lysine solution can be toxic to the cells.

5. Remove the distilled water and leave the coated culture vessel uncovered in the laminar hood to dry.

   The culture surface should be fully dry after 2 hours.

   Note: Coated vessels can be used immediately once they are dry, or can be stored dry at 4°C. For storage at 4°C, tightly wrap the vessels with Parafilm™ laboratory film and use within one week of coating.

Recovery of neurons

1. Rinse a 50 mL conical culture tube with pre-warmed (37°C) complete Neurobasal medium and leave it in the laminar flow hood prior to thawing the cells.

2. If removing a vial of cells from liquid nitrogen storage, twist cap slightly to release pressure and then retighten cap.

3. Rapidly thaw (<2 minutes) the frozen vial by gently swirling it in a 37°C water bath. Remove the vial from the water bath when only a tiny ice crystal is left (vial should still be cold to the touch).

4. Transfer the vial to the laminar flow hood and disinfect it with 70% isopropyl alcohol. Tap the vial gently on the surface of the hood so that the liquid settles down to the bottom of the vial.

5. Rinse a P1000 pipette tip with complete Neurobasal medium and very gently transfer the cells to the pre-rinsed 50 mL tube (from step 1).

6. Rinse the vial with 1 mL of complete Neurobasal medium (pre-warmed to 37°C) and add to the cells in the 50 mL tube slowly, at the rate of one drop per second. Mix the suspension by gentle swirling after each addition.

   Note: Do not add the entire amount of medium to the tube at once. This may lead to decreased cell viability due to osmotic shock.

7. Slowly add 2 mL of complete Neurobasal medium to the tube (for a total suspension volume of 4 mL). Mix the suspension very gently with the P1000 pipette without creating any air bubbles.

8. To a microcentrifuge tube containing 10 µL of Trypan Blue Solution, 0.4%, add 10 µL of the cell suspension using a pre-rinsed tip. Mix by gently tapping the tube. Determine the viable cell density using a manual counting method (e.g., hemocytometer) .

   Note: Do not centrifuge the cells, as they are extremely fragile upon recovery from cryopreservation.

   Note: It is important to rinse each pipette tip and vial with complete Neurobasal medium before using it for a cell suspension, to prevent the cells from sticking to the plastic.

Culturing neurons

1. Plate ~5 x 10^4 live cells per well in a poly-D-lysine–coated (4.5 µg/cm^2) 48-well plate. Dilute the cell suspension to 500 µL per well by adding complete Neurobasal medium.

2. Incubate the cells at 36–38°C in a humidified atmosphere of 5% CO_2 in air.

3. After 4 to 24 hours of incubation, aspirate half of the medium from each well and replace it with fresh complete Neurobasal medium. Return the cells to the incubator.

4. Feed the cells every third day by aspirating half of the medium from each well and replacing it with fresh medium.

   Note: Do not expose neurons to ambient air at any time.

Typical results

Thawed cortical neurons cultured in complete Neurobasal medium display extensive neurite outgrowth that continues to increase as long as they are kept healthy in culture. In contrast to non-cryopreserved neurons, post-thaw recovered neurons show dead cells due to post-thaw toxicity, and typical cultures contain dead cells that stay attached to the plate surface.

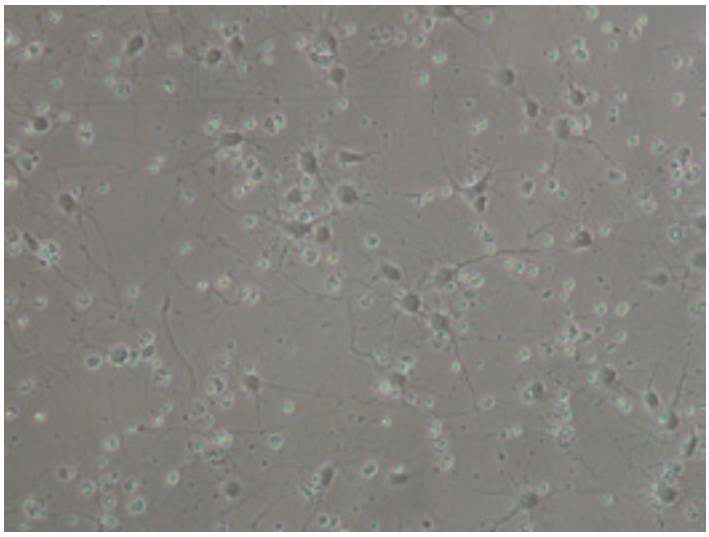

Figure 4-1. Phase-contrast image of primary rat cortical neurons recovered and cultured in complete Neurobasal medium, at day 7.

5 Culturing human fetal neural stem cells

Summary

Neural stem cells (NSCs) isolated from human fetuses are highly valuable resources in neuroscience because of their ability to differentiate into neurons and glial cells. This chapter describes methods for expanding human fetal NSCs in cell culture and their subsequent characterization.

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Required materials

Cells

- Gibco™ StemPro™ Neural Stem Cells (Cat. No. A15654)

Media and reagents

- Gibco™ StemPro™ NSC SFM (Cat. No. A1050901: This kit contains KnockOut™ DMEM/F-12 Basal Medium stored at 4°C; StemPro™ NSC SFM (Neural) Supplement stored at –20°C to –5°C in the dark; and bFGF Recombinant Human and EGF Recombinant Human proteins stored at 4°C, desiccated.)
- Gibco™ GlutaMAX™ Supplement (Cat. No. 35050061)
- Ascorbic Acid (MilliporeSigma, Cat. No. A8960)
- Heparin (MilliporeSigma, Cat. No. H3149)
- Gibco™ Distilled Water (Cat. No. 15230162)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- Gibco™ DPBS, calcium, magnesium (Cat. No. 14040133)
- Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501)

Plasticware

- Thermo Scientific™ Nunclon™ Sphera™ Flasks (Cat. No. 174951 or 174952) (for suspension culture)

Preparing media

Heparin stock

1. To prepare 6,000 U/mL heparin solution (1,000X), add 1.65 mL of distilled water to 10,000 U of heparin and mix until dissolved.

2. After dissolving, filter-sterilize through a 0.22 µm filter, aliquot 50–100 µL into sterile tubes, and store at –20°C.

Ascorbic acid stock

1. To prepare 100 mM ascorbic acid stock solution (500X), add 17.27 mL of distilled water to 500 mg of ascorbic acid powder and mix until dissolved.

2. After dissolving, filter-sterilize through a 0.22 µm filter, aliquot 100–200 µL into sterile tubes, and store at –20°C.

bFGF and EGF stock

1. Reconstitute bFGF and EGF with 0.1% BSA in DPBS, calcium, magnesium (DPBS +/+, Cat. No. 14040133), or DPBS, no calcium, no magnesium (DPBS –/–, Cat. No. 14190144), at a concentration of 20 µg/mL.

2. Aliquot 50–100 µL into sterile tubes, and store at –20°C.

Complete StemPro NSC SFM

To prepare 100 mL of complete neural stem cell culture medium, mix the components shown in the table on the next page under sterile conditions. KnockOut DMEM/F-12, bFGF, EGF, and StemPro Neural Supplement are components of the StemPro NSC SFM kit. Complete

medium is stable for 4 weeks when stored in the dark at 2°C to 8°C. To make larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
KnockOut DMEM/F-12	1X	96.5 mL
bFGF (prepared as 20 µg/mL stock)	20 ng/mL	100 µL
EGF (prepared as 20 µg/mL stock)	20 ng/mL	100 µL
StemPro Neural Supplement	2%	2 mL
GlutaMAX Supplement	2 mM	1 mL
Heparin	6 units/mL	100 µL
Ascorbic acid	200 µM	200 µL

Culturing neural stem cells

Fetal NSC populations can be expanded from frozen stocks and grown as suspension cultures as plating on matrix would trigger differentiation. The following procedure provides instructions for thawing one vial of StemPro NSCs containing 1 x 10^6 cells in two T-25 flasks (for a total culture area of 50 cm^2).

Thawing frozen neural stem cells

1. Prepare 30 mL of complete StemPro NSC SFM and warm to 37°C.

2. Rapidly thaw (<2 minutes) the frozen vial by gently swirling it in a 37°C water bath. Remove the vial from the water bath when only a tiny ice crystal is left (vial should be still cold to touch).

3. Transfer the vial to the laminar flow hood and disinfect it with 70% ethanol. Allow the ethanol to evaporate before opening the vial.

4. Transfer thawed cells into a 15 mL tube and add complete StemPro NSC SFM, pre-warmed to 37°C, in a dropwise manner to a total volume of 5 mL.

5. Spin down the thawed cells by centrifugation at 300 x *g* for 4 minutes. Aspirate and discard the supernatant.

6. Resuspend the cells in 1 mL complete StemPro NSC SFM.

7. Determine the concentration of viable cells using your preferred method.

8. Resuspend NSCs in pre-warmed complete StemPro NSC SFM to a final concentration of 0.5 x 10^6 cells/cm^2.

9. Transfer 1 mL each of the cell suspensions to uncoated T-25 flasks containing 6 mL of complete StemPro NSC SFM. The total volume will be 7 mL for each T-25 flask (~2 x 10^4 cells/cm^2).

10. Change the spent culture medium after 2 days and every 2–4 days thereafter.

11. Culture the NSCs in suspension for up to 21 days, passaging them every 7 days.

Change medium

1. Prepare 10 mL of complete StemPro NSC SFM per T-25 flask and pre-warm to 37°C.

2. From each culture flask, tilt and pipet the medium containing the suspension cells into a corresponding pre-labeled 15 mL centrifuge tube.

3. Add 2 mL of complete StemPro NSC SFM (pre-warmed to 37°C) to each flask, and place the flasks back into the incubator.

4. Centrifuge the 15 mL tubes with the suspension cells at 300 x *g* for 4 minutes and aspirate the supernatant to ~0.5 cm above the pellet surface without disturbing the pellet. Discard the supernatant appropriately.

5. Retrieve flasks from incubator (from step 3) and transfer them into the laminar flow hood.

6. Add 1 mL of warm medium into each tube (from step 4) and dissociate the cells by gently pipetting up and down. Transfer the cell suspension back to the appropriate flask.

7. Pipet another 4 mL of pre-warmed medium into each tube in a manner that washes the sides of the tube. Pipet the medium up and down the sides several times. Transfer the cell suspension back to the appropriate flask.

8. Mix the cell suspension evenly by gently moving flasks in a left-to-right and then forward-and-backward motion several times.

9. Return flasks to the 37°C incubator with a humidified atmosphere of 5% CO_2 in air.

Passaging neural stem cells

1. Transfer medium containing neurospheres into a 15 or 50 mL conical tube.

2. Leave the tube at room temperature and allow the neurospheres to settle to the bottom of tube. Alternatively, spin down the cells by centrifugation at 200 x *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 DPBS –/–, aspirate the DPBS supernatant carefully, and leave the neurospheres in a minimum volume of DPBS.

5. Add 1 mL of StemPro Accutase reagent to the neurospheres and incubate the tube at 37°C for 10 minutes. Swirl the tube at 5 and 8 minutes to ensure that the cells do not aggregate or settle at the bottom of the tube.

6. Using a P1000 pipette, break up the neurospheres by pipetting up and down 5 times. Place the tube back in the laminar flow hood for another 5–25 minutes.

7. Gently triturate neurospheres using a Pasteur pipette or P1000 pipettor to create a single-cell suspension.

8. Neutralize the treatment by adding 4 mL of complete StemPro NSC SFM.

9. Spin down the cells by centrifugation at 300 x *g* for 3 minutes. Aspirate and discard the supernatant, and seed cells in fresh complete StemPro NSC SFM in a suspension dish at a density of 2 x 10^4 to 5 x 10^4 cells/cm^2.

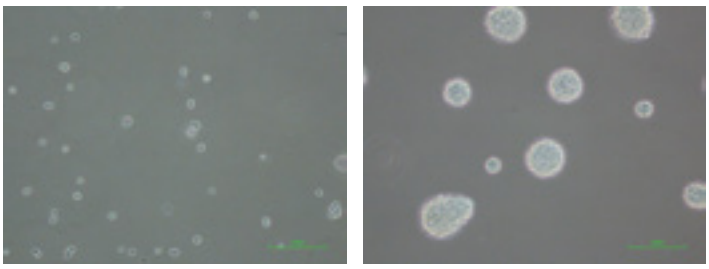

Figure 5-1. Culture of neural stem cells. Phase-contrast image of cells **(A)** 1 day after thawing of StemPro Neural Stem Cells and **(B)** 7 days after thawing of StemPro Neural Stem Cells before harvest.

Cryopreserving neural stem cells

1. Harvest cells using method described above in "Passaging neural stem cells".

2. Resuspend the cells in complete StemPro NSC SFM at a density of 2 x 10^6 cells/mL.

3. Prepare freezing medium (2X) consisting of 20% DMSO and 80% complete StemPro NSC SFM.

   Note: Freezing medium (2X) can be prepared on the day of use and stored at 4°C until use.

4. Add a volume of freezing medium equal to the amount of complete StemPro NSC SFM used to resuspend the cells in a dropwise manner.

5. Prepare 1 mL aliquots (1 x 10^6 cells) in cryovials and place the vials in an isopropyl alcohol chamber.

6. Put the isopropyl alcohol chamber at –80°C and transfer the vials to liquid nitrogen storage the next day.

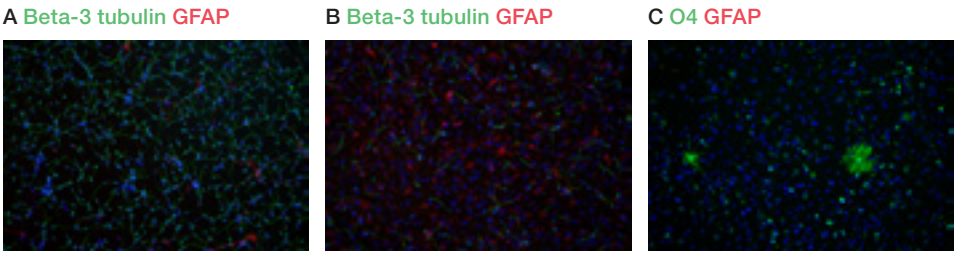

Figure 5-2. Differentiation of StemPro NSCs. After 7 days of directed differentiation, **(A)** neurons, **(B)** astrocytes, and **(C)** oligodendrocytes were labeled with phenotype markers of beta-3 tubulin (neurons), GFAP (astrocytes), and O4 (oligodendrocytes).

6 Culturing rat fetal neural stem cells

Summary

Rat neural stem cells (NSCs) serve as a well-established model for investigating mammalian brain development, disease processes, and for study of 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.

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Required materials

Cells

- Gibco™ Rat Fetal Neural Stem Cells (Cat. No. N7744100) or homogeneous cell preparation from 14–18 days post-coitum rat brain tissue

Media and reagents

- Gibco™ DPBS, calcium, magnesium (Cat. No. 14040141)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- Gibco™ StemPro™ NSC SFM (Cat. No. A1050901: This kit contains KnockOut™ DMEM/F-12 Basal Medium stored at 4°C; StemPro™ NSC SFM (Neural) Supplement stored at –20°C to –5°C in the dark; and bFGF Recombinant Human and EGF Recombinant Human proteins stored at 4°C, desiccated.)
- Gibco™ StemPro Accutase™ Cell Dissociation Reagent (Cat. No. A1110501)
- Gibco™ CELLstart™ CTS™ Substrate (Cat. No. A1014201)
- Gibco™ Trypan Blue Solution, 0.4% (Cat. No. 15250061) (included with the Invitrogen™ Countess™ II Automated Cell Counter) or the Invitrogen™ LIVE/DEAD™ Cell Vitality Assay Kit, C_{12} Resazurin/SYTOX™ Green (Cat. No. L34951)

Special tools

- Invitrogen™ Countess™ II Automated Cell Counter (Cat. No. AMQAX1000) or hemocytometer

Preparing media

Medium for expanding neural stem cells

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

To prepare 100 mL of complete StemPro NSC SFM:

1. Reconstitute bFGF and EGF with 0.1% BSA solution (in KnockOut DMEM/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 DMEM/F-12	1X	97 mL
GlutaMAX 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 CTS Substrate

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

1. Dilute CELLstart CTS Substrate 1:100 in DPBS, calcium, magnesium (DPBS +/+) (e.g., 50 µL of CELLstart CTS Substrate into 5 mL of DPBS).

   Note: CELLstart CTS Substrate 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 Substrate (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% CO_2 for 1 hour.

4. Remove the vessel from the incubator and store at 4°C until use. Remove all CELLstart CTS Substrate 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 laboratory film, for up to 2 weeks. Do not remove CELLstart CTS Substrate 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 DPBS +/+, resuspend in warmed complete StemPro NSC SFM at a density of 1 x 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 x 10^7 viable cells/mL.

2. Plate rat fetal NSCs onto culture vessels coated with CELLstart CTS Substrate at a density of 5 x 10^4 cells/cm^2. 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 x 10^4
24-well plate	1.9 cm^2/well	0.5 mL	1.0 x 10^5
12-well plate	3.8 cm^2/well	1 mL	1.9 x 10^5
35 mm dish	8 cm^2/well	2 mL	4.0 x 10^5
6-well plate	9.6 cm^2/well	2 mL	4.8 x 10^5
60 mm dish	19.5 cm^2	5 mL	9.8 x 10^5
T-25 flask	25 cm^2	5 mL	1.3 x 10^6
100 mm dish	55 cm^2	10 mL	2.8 x 10^6
T-75 flask	75 cm^2	15 mL	3.8 x 10^6

3. Add the appropriate volume of cells to each culture vessel and incubate at 37°C, 5% CO_2, 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 (Figure 6-1 on the next page).

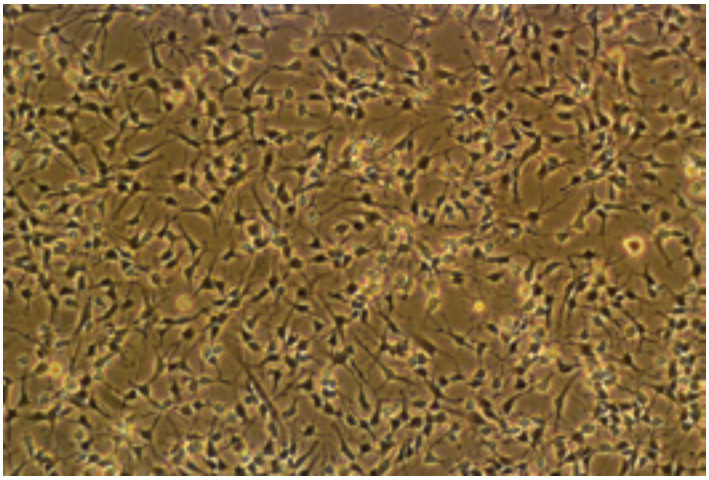

Figure 6-1. Rat fetal NSCs at passage 3 in adherent culture using complete StemPro NSC SFM.

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 DPBS –/–, then remove the medium.

7. Add pre-warmed StemPro Accutase reagent 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 homogeneous cell solution.

10. Transfer the cells to a sterile centrifuge tube and centrifuge at 300 x *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 the tube for a final cell solution of 1 x 10^6 viable cells/mL. Incubate at 37°C, 5% CO_2, and 90% humidity. Rat fetal NSC 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.

Neurosphere suspension cultures

1. Resuspend the rat fetal NSCs as follows:

   - For freshly prepared rat fetal NSCs, after rinsing with DPBS –/–, resuspend in warmed complete StemPro NSC SFM at a cell density of 1 x 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 x 10^7 viable cells/mL.

2. Plate the rat fetal NSCs onto uncoated or low-attachment culture vessels at a density of 2 x 10^5 viable cells/cm^2. See the table on the next page 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 x 10^4
24-well plate	1.9 cm^2/well	0.5 mL	3.8 x 10^5
12-well plate	3.8 cm^2/well	1 mL	7.6 x 10^5
35 mm dish	8 cm^2/well	2 mL	1.6 x 10^6
6-well plate	9.6 cm^2/well	2 mL	1.9 x 10^6
60 mm dish	19.5 cm^2	5 mL	3.9 x 10^6
T-25 flask	25 cm^2	5 mL	5.0 x 10^6
100 mm dish	55 cm^2	10 mL	1.1 x 10^7
T-75 flask	75 cm^2	15 mL	1.5 x 10^7

3. Add the appropriate volume of cells to each culture vessel and incubate at 37°C, 5% CO_2, 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 multicellular complexes (Figure 6-2).

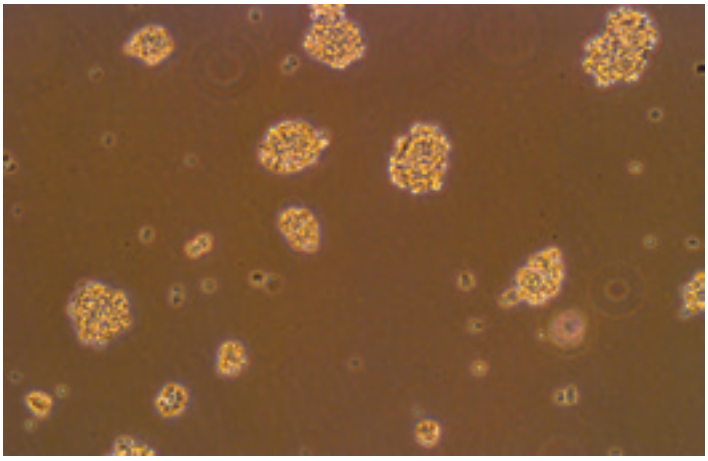

Figure 6-2. Rat fetal NSCs at passage 3 in neurosphere culture using complete StemPro NSC SFM.

5. When the neurospheres reach a diameter of 200 µm 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 x *g* for 2 minutes. Aspirate the supernatant carefully to leave the neurospheres in a minimal volume of medium.

7. Rinse the neurospheres once with DPBS –/– and leave a minimal volume of DPBS.

8. Add 1 mL of pre-warmed StemPro Accutase reagent 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 x *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 hemocytometer or Countess II 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 x 10^7 viable cells/mL. Incubate at 37°C, 5% CO_2, 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.

7 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 describe a culture system that can be adjusted to favor differentiation into either astrocytes or oligodendrocytes.

View this protocol online and order products at **thermofisher.com/neuroprotocol/gpc**

Required materials

Cells

- Gibco™ Rat Glial Precursor Cells (Cat. No. N7746100)

Media and reagents

- Gibco™ StemPro™ NSC SFM (Cat. No. A1050901: This kit contains KnockOut™ DMEM/F-12 Basal Medium stored at 4°C; StemPro™ NSC SFM (Neural) Supplement stored at –20°C to –5°C in the dark; and bFGF Recombinant Human and EGF Recombinant Human proteins stored at 4°C, desiccated.)
- Gibco™ Neurobasal™ Plus Medium (Cat. No. A3582901)
- Gibco™ B-27™ Plus Supplement (50X), serum free (Cat. No. A3582801)
- Gibco™ Astrocyte Medium (Cat. No. A1261301: This kit contains N-2 Supplement (100X) stored at –20°C; Dulbecco's Modified Eagle Medium (DMEM) (1X) stored at 2°C to 8°C; One Shot™ Fetal Bovine Serum, Certified, stored at –20°C in the dark.)
- Gibco™ PDGF-AA Recombinant Human Protein (Cat. No. PHG0035)
- T3 (3,3′,5-triiodo-L-thyronine) (MilliporeSigma, Cat. No. T6397)
- *N*-acetyl-L-cysteine (MilliporeSigma, Cat. No. A8199)
- Gibco™ BDNF Recombinant Human Protein (Cat. No. PHC7074)
- Gibco™ CNTF Recombinant Human Protein (Cat. No. PHC7015)
- Gibco™ GlutaMAX™ Supplement (Cat. No. 35050061)
- Gibco™ Distilled Water (Cat. No. 15230162)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- Gibco™ DPBS, calcium, magnesium (Cat. No. 14040133)
- Gibco™ Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413201)
- Gibco™ Laminin Mouse Protein, Natural (Cat. No. 23017015)
- Poly-L-Ornithine Solution (MilliporeSigma, Cat. No. P4957)

Preparing media

PDGF-AA stock

1. To prepare 10 µg/mL PDGF-AA stock solution (500X), centrifuge the 10 µg vial briefly before aseptically adding 1 mL sterile 4 mM HCl containing 0.1% BSA.
2. Aliquot 50–100 µL into sterile tubes, and store at –20°C.

T3 stock

1. To prepare 30 µg/mL T3 stock solution (1,000X), add 1 mL of 1 N NaOH to 1 mg of T3 powder, and

then dilute further with 32.3 mL of DPBS, calcium, magnesium (DPBS +/+), and mix until dissolved.

2. After dissolving, filter-sterilize through a 0.22 µm filter, aliquot 50–100 µL into sterile tubes, and store at –20°C.

N-acetyl-L-cysteine stock

1. To prepare 5 mg/mL *N*-acetyl-L-cysteine stock, dissolve 50 mg *N*-acetyl-L-cysteine in 10 mL Neurobasal Plus Medium by vortexing.

2. Add 1 N NaOH dropwise to neutralize pH; requires approximately 250 µL until medium returns to original red color.

3. Filter-sterilize through a 0.22 µm filter, aliquot 50–100 µL into sterile tubes, and store at –20°C.

BDNF stock

1. To prepare 10 µg/mL BDNF stock solution (5,000X), aseptically add 1 mL of DPBS +/+ supplemented with 0.1% BSA to 10 µg of BDNF protein, and mix until dissolved.

2. Aliquot 10–20 µL into sterile tubes, and store at –20°C.

CNTF stock

1. To prepare 10 µg/mL CNTF stock solution (5,000X), aseptically add 2 mL of DPBS +/+ supplemented with 0.1% BSA to 20 µg of CNTF protein, and mix until dissolved.

2. Aliquot 10–20 µL into sterile tubes, and store at –20°C.

Complete StemPro NSC SFM

Complete StemPro NSC SFM consists of KnockOut DMEM/F-12 with StemPro Neural Supplement, GlutaMAX Supplement, bFGF, and EGF. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of complete medium:

1. Reconstitute bFGF and EGF with 0.1% BSA solution (in KnockOut DMEM/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 conc.	Amount
KnockOut DMEM/F-12	1X	97 mL
GlutaMAX 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; swirl thawed supplement briefly in a 37°C bath until precipitate dissolves.

3. Add 20 ng/mL PDGF-AA to prewarmed complete StemPro NSC SFM immediately prior to use.

Oligodendrocyte differentiation medium

Oligodendrocyte differentiation medium uses Neurobasal Plus Medium supplemented with B-27 Plus Supplement (50X), *N*-acetyl-L-cysteine, T3, BDNF, and CNTF. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of oligodendrocyte differentiation medium, mix the components shown in the table on the next page under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
Neurobasal Plus Medium	1X	98 mL
B-27 Plus Supplement	2%	2 mL
N-acetyl-L-cysteine	5 µg/mL	100 µL
T3	30 ng/mL	100 µL
BDNF	2 ng/mL	20 µL
CNTF	2 ng/mL	20 µL

Astrocyte differentiation medium

Astrocyte differentiation medium uses DMEM supplemented with N-2 Supplement and FBS. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

Component	Final conc.	Amount
DMEM	1X	97 mL
N-2 Supplement	1%	1 mL
FBS	1%	2 mL

Preparing matrix

Matrix for oligodendrocyte differentiation

1. Add 2 mL of 100 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).

2. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO_2 for at least 1 hour.

3. Rinse the culture vessel 3 times with distilled water.

4. Prepare a 1:100 dilution of laminin in distilled water for a final concentration of 10 µg/mL.

5. Add 2 mL of the 10 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).

6. Incubate the culture vessel at 37°C in a humidified atmosphere of 5% CO_2 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°C to 8°C, wrapped tightly with Parafilm laboratory film, for up to 4 weeks.

Matrix for astrocyte differentiation

1. Thaw a bottle of Geltrex Basement Membrane Matrix at 2°C to 8°C.

2. Dilute Geltrex matrix 1:100 in cold DMEM and coat the bottom of each culture vessel by adding 2 mL of diluted Geltrex matrix 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 36°C to 38°C for 1 hour.

   Note: Dishes coated with Geltrex matrix can be used immediately or stored at 2°C to 8°C for up to one week, sealed with Parafilm laboratory film. Do not allow dishes to dry out. Warm stored Geltrex matrix plates to room temperature for 1 hour prior to use.

Differentiation to oligodendrocytes

1. Plate glial precursor cells on a poly-L-ornithine– and laminin-coated plate in complete StemPro NSC SFM supplemented with 20 ng/mL PDGF-AA at a density of 2.5 x 10^4 to 5 x 10^4 cells/cm^2.

2. On the next day, change the medium to oligodendrocyte differentiation medium.

3. Perform a half-medium change every 2–3 days thereafter. Differentiated oligodendrocytes are typically observed on day 3.

Differentiation to astrocytes

1. Plate glial precursor cells on a Geltrex matrix–coated plate in complete StemPro NSC SFM supplemented with 20 ng/mL PDGF-AA at a density of 2.5 x 10^4 cells/cm^2.

2. On the next day, change the medium to astrocyte differentiation medium.

3. Change the medium every 3–4 days. Differentiated astrocytes are typically observed on days 5–7.

8 Xeno-free culture of neural stem cells

Summary

Neural stem cells (NSCs) derived from human pluripotent stem cells (hPSCs) help provide understanding for human neurogenesis and have potential for cell therapy applications related to Parkinson's disease, spinal cord injuries, and other neurological diseases. 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 xeno-free conditions for maintaining and expanding NSCs, which are described in this protocol. Gibco™ Cell Therapy Systems (CTS™) products help minimize the risk of contamination and variability, and meet your regulatory and quality requirements.

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Learn more about CTS products at **thermofisher.com/cts**

Required materials

Cells

- Neural stem cells

Media and reagents

- Gibco™ CELLstart™ CTS™ Substrate (Cat. No. A1014201)
- Gibco™ CTS™ KnockOut™ DMEM/F-12 (Cat. No. A1370801)
- Gibco™ CTS™ N-2 Supplement (Cat. No. A1370701)
- Gibco™ CTS™ B-27™ Supplement, XenoFree (Cat. No. A1486701)
- Gibco™ CTS™ FGF-Basic Full Length Recombinant Human Protein (Cat. No. CTP0261)
- Gibco™ EGF Recombinant Human Protein (Cat. No. PHG0311)
- Gibco™ CTS™ GlutaMAX™-I Supplement (Cat. No. A1286001)
- Gibco™ CTS™ TrypLE™ Select Enzyme (Cat. No. A1285901)
- Gibco™ CTS™ Dulbecco's Phosphate-Buffered Saline (DPBS), calcium, magnesium (Cat. No. A1285801)
- Gibco™ CTS™ Dulbecco's Phosphate-Buffered Saline (DPBS), without calcium chloride, without magnesium chloride (Cat. No. A1285601)

Special tools

- 15 mL conical tube
- Tissue culture plates and flasks
- Microcentrifuge

Preparing culture vessels and media

CELLstart CTS Substrate–coated vessels

Prepare culture dishes or flasks with CELLstart CTS Substrate as described below.

1. Dilute CELLstart CTS Substrate 1:100 in CTS DPBS calcium, magnesium (CTS DPBS +/+), (e.g., 50 µL of CELLstart CTS Substrate into 5 mL of CTS DPBS +/+).

   Note: CELLstart CTS Substrate 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 Substrate (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% CO_2 for 1 hour.

4. Use the dish immediately after incubation. Aspirate the CELLstart CTS Substrate 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 by aseptically mixing the components in the table below.

Component	Amount
CTS KnockOut DMEM/F-12	48.5 mL
CTS GlutaMAX-I Supplement	0.5 mL
CTS B-27 Supplement, XenoFree	1 mL
CTS N-2 Supplement	0.5 mL
CTS FGF-Basic	20 ng/mL
EGF	20 ng/mL

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

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, remove vial from water bath (<2 minutes) and sterilize the outside of the vial with 95% ethanol. Allow the ethanol to evaporate before opening the vial in a laminar flow 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 x *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 1 x 10^5 cells/cm^2 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°C to 38°C in a humidified atmosphere (90%) of 5% CO_2 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 4–7 days the culture will be 90% confluent and ready for passaging.

4. To passage the culture, prepare a 0.5X solution of CTS TrypLE Select Enzyme in CTS DPBS, without calcium chloride, without magnesium chloride (CTS DPBS –/–).

5. Aspirate the culture medium and wash the cells with CTS DPBS –/–.

6. Add 0.5X CTS TrypLE Select Enzyme to dissociate the cells, and incubate for 2 minutes at 37°C or until the cells start to round up and separate from the culture surface.

7. Add 4 mL of culture medium to neutralize the CTS TrypLE Select Enzyme activity and pipet up and down 2–3 times to get a uniform cell suspension. Check the cells under a microscope.

8. Transfer the cell suspension to a 15 mL centrifuge tube.

9. Centrifuge the cells at 200 x *g* for 5 minutes.

10. Aspirate the supernatant and resuspend the cells in 10 mL of culture medium.

11. Determine the total number of cells and percent viability using your method of choice. Seed the cells at a concentration of 1 x 10^5 cells/cm^2 onto a dish or flask that has been treated with CELLstart CTS solution.

12. Incubate the flasks at 37°C in a humidified atmosphere (90%) of 5% CO_2 in air.

13. Grow the cells until 90% confluent, changing the culture medium once after 12 hours and every 2 days thereafter.

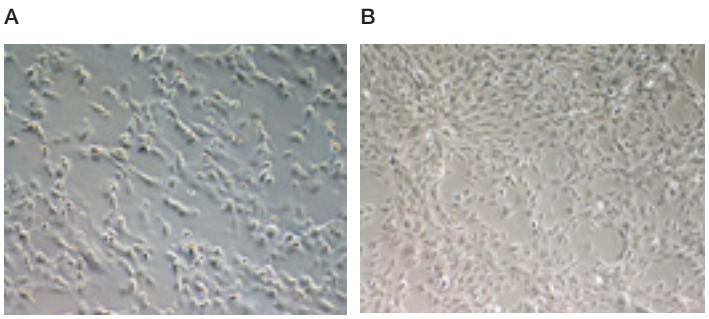

Figure 8-1. Phase-contrast microphotograph showing (A) NSCs cultured in xeno-free medium 24 hours post-thaw and (B) semi-confluent NSCs cultured in xeno-free medium for 3 days.

9 Creation of neural stem cells from human pluripotent stem cells

Summary

The derivation of neural stem cells (NSCs) is the first step in producing various neural cell types from human pluripotent stem cells (hPSCs). We have developed an efficient neural induction medium that can convert hPSCs into NSCs in one week without the need for the tedious and time-consuming process of embryoid body formation. Neural induction medium works with hPSCs cultured either in feeder-containing or feeder-free conditions.

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Required materials

Media and reagents

- Gibco™ PSC Neural Induction Medium (Cat. No. A1647801): This kit contains 1X Neurobasal™ Medium stored at 4°C and 50X Neural Induction Supplement stored at –20°C to –5°C in the dark. After thawing of Neural Induction Supplement, aliquot 0.5 to 1 mL into sterile tubes and store at –20°C to –5°C in the dark. Avoid frequent freezing and thawing.
- Gibco™ Advanced DMEM/F-12 (Cat. No. 12634010)
- Gibco™ Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413301): Thaw Geltrex matrix at 4°C overnight before using. The thawed Geltrex matrix can be kept at 2°C to 8°C for up to 2 weeks.
- Gibco™ Distilled Water (Cat. No. 15230162)
- ROCK Inhibitor Y-27632 (MilliporeSigma, Cat. No. Y0503): Dissolve 1 mg of Y-27632 in 0.625 mL distilled water to make 5 mM stock solution. Sterilize the stock solution by filtering through a 0.22 µm filter. Aliquot 20–50 µL into sterile tubes and store at –20°C to –5°C in the dark for up to 1 year. The thawed Y-27632 solution can be kept at 2°C to 8°C for up to 2 weeks.
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190250)
- Gibco™ Essential 8™ Medium (Cat. No. A1517001)
- Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501): Thawed Accutase reagent can be kept at 2°C to 8°C for up to 2 weeks.
- Dimethyl sulfoxide, Hybri-Max™ grade (DMSO) (MilliporeSigma, Cat. No. D2650)

Special tools

- Thermo Scientific™ Nunc™ Cell Scraper (Cat. No. 179693 or 179707)
- 100 µm strainer
- Thermo Scientific™ Nalgene™ General Long-Term Storage Cryogenic Tubes (Cat. No. 5000-0012 or 5000-1012)
- Thermo Scientific™ Mr. Frosty™ Freezing Container (Cat. No. 5100-0001)
- Microscope marker, such as Nikon™ microscopy object marker (Nikon Instruments Inc., Cat. No. MBW10020) with a Nikon™ microscopy C-OA 15 mm objective adapter (Nikon Instruments Inc., Cat. No. MXA20750)

Preparing media

Neural induction medium

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

Component	Final conc.	Amount
Neurobasal Medium	1X	98 mL
Neural Induction Supplement	1X	2 mL

The complete neural induction medium can be stored at 2°C to 8°C for up to 2 weeks. When feeding cells, warm up the appropriate amount of neural induction medium in a 37°C water bath for 5–10 minutes before feeding.

Neural expansion medium

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

Component	Final conc.	Amount
Neurobasal Medium	0.5X	49 mL
Advanced DMEM/F-12	0.5X	49 mL
Neural Induction Supplement	1X	2 mL

The complete neural expansion medium can be stored at 2°C to 8°C for up to 2 weeks. When feeding cells, warm up the appropriate amount of neural induction medium in a 37°C water bath for 5–10 minutes before feeding.

Neural preservation medium

To prepare 10 mL of neural preservation medium, mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
Neural expansion medium	80%	8 mL
DMSO	20%	2 mL

Neural induction

1. Maintain the high quality of hPSCs including hESCs and hiPSCs by culturing in feeder-free media such as Essential 8 Medium or complete StemPro hESC SFM, or on mouse embryonic fibroblasts (MEFs).

2. When hPSCs reach ~70–80% confluency, split hPSCs into 6-well culture plates to reach 15–25% confluency at day 1 of hPSC splitting.

3. At day 1 of hPSC splitting, aspirate spent medium to remove non-attached cells and add 2.5 mL pre-warmed neural induction medium into each well of 6-well plate. Return plates to an incubator.

4. The morphology of cell colonies should be uniform with a smooth edge after 2 days of culture in neural induction medium (Figure 9-1, panel A). Due to differentiated or partially differentiated hPSCs before neural induction, colonies of non-neural differentiation can be observed (Figure 9-1, panels B–F). Mark all colonies showing the morphology of non-neural differentiation on the bottom of the plate by using a microscope marker.

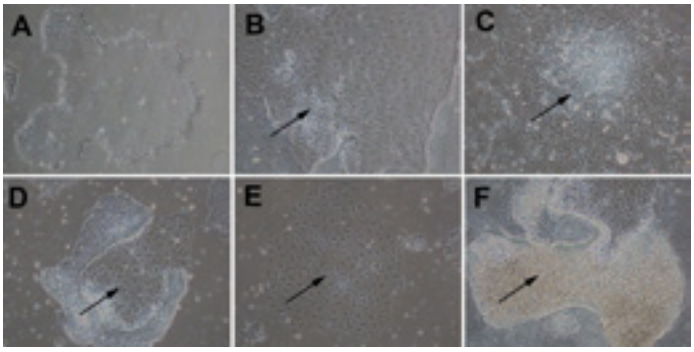

Figure 9-1. Morphology of neural and non-neural differentiated cells at day 2 of neural induction. (A) Normal morphology of cells. **(B–F)** Representative images of non-neural differentiated cells (indicated by arrows) due to the use of poor-quality starter hPSCs.

5. Aspirate spent medium from each well. Remove all colonies of non-neural differentiation by pointing a Pasteur glass pipette to marked colonies to aspirate cells off. Add 2.5 mL pre-warmed neural induction medium per well and return plates to an incubator.

6. At day 4 of neural induction, cells should reach confluency. Mark all colonies of non-neural differentiation by using a microscope marker.

7. Aspirate spent medium from each well. Remove all colonies of non-neural differentiation by pointing a Pasteur glass pipette to marked colonies to aspirate cells off. Add 5 mL pre-warmed neural induction medium per well, and return plates into an incubator.

8. Feed cells every day with 5 mL neural induction medium per well until day 7 of neural induction.

NSC expansion

1. At day 7 of neural induction, NSCs (P0) are ready to be harvested.

2. Dilute thawed Geltrex matrix in Neurobasal Medium (1:100). Add appropriate amount of Geltrex solution into each culture vessel to cover the surface (e.g., 0.5–1 mL for each well of a 6-well culture plate) and incubate at least 1 hour at 37°C.

3. Aspirate the spent medium with a Pasteur glass pipette and rinse cells with DPBS –/– once.

4. Gently add 1 mL pre-warmed StemPro Accutase Cell Dissociation Reagent to each well of 6-well plate.

5. Incubate for 5–8 minutes at 37°C until most of cells are detached from the surface of culture vessels.

6. Use a cell scraper to detach the cells from the surface of the plates.

7. Transfer cell clumps using a pipette, and place cells into a 15 or 50 mL conical tube.

8. Add 1 mL DPBS –/– to each well of a 6-well plate to collect residual cells, and transfer cell suspension to the conical tube.

9. Triturate cell suspension 3 times with a 5 or 10 mL pipette to break cell clumps.

10. Pass cell suspension through a 100 µm strainer and centrifuge cells at 300 x *g* for 4 minutes.

11. Aspirate supernatant, resuspend cells with appropriate amount of pre-warmed neural expansion medium (e.g., 1 mL for all cells from 1 well of a 6-well plate).

12. Determine cell concentration using preferred method.

13. Dilute cell suspension to 4 x 10^5 cells/mL with pre-warmed neural expansion medium.

14. Add ROCK inhibitor Y-27632 solution into cell suspension to reach final concentration of 5 µM.

15. Aspirate Geltrex solution from coated vessels and add appropriate amount of diluted cell suspension into each culture vessel to plate cells at a density of 1 x 10^5 cells/cm^2.

16. Place vessels gently in an incubator and move culture vessels in several quick back-and-forth and side-to-side motions to disperse cells across the surface of vessels.

17. Change neural expansion medium at day 1 of cell plating to eliminate Y-27632. Change neural expansion medium every other day thereafter.

18. Usually, NSCs reach confluency at day 4–5 after plating. When NSCs reach confluency, NSCs can be further expanded in neural expansion medium. For the first 3 to 4 passages, overnight treatment with 5 µM Y-27632 at the day of NSC plating is necessary to prevent cell death.

Cryopreservation of NSCs

1. After determining cell concentration at step 12 in the NSC expansion section above, dilute cell suspension to 2 x 10^6 to 4 x 10^6 cells/mL with neural expansion medium.

2. Add an equivalent volume of neural preservation medium dropwise.

3. Allocate 1 mL of cell suspension into each cryotube and freeze cells at –80°C overnight in Mr. Frosty Freezing Containers.

4. Transfer cells into liquid nitrogen tank on the next day for long-term storage.

Recovery of cryopreserved NSCs

1. Coat culture vessels with Geltrex solution for at least 1 hour before thawing NSCs by following the procedures of step 2 in the NSC expansion section above.

2. Wear eye protection, as cryotubes stored in the liquid phase of liquid nitrogen may accidentally explode when warmed.

3. Wear ultralow-temperature cryogloves. Remove cryotubes of NSCs from the liquid nitrogen storage tank using metal forceps.

4. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.

5. When only an ice crystal remains, remove the vial from the water bath.

6. Quickly remove the sticker or copy the information written on the vial in your notebook. The writing may come off the vial after spraying the outside of the vial with 70% ethanol.

7. Spray the outside of the vial with 70% ethanol and place in a laminar flow hood. Pipette cells gently into a sterile 15 mL conical tube using a 1 mL pipette.

8. Add 1 mL DPBS –/– into the vial to collect residual cells.

9. Use a pipette to remove DPBS–cell mixture from the vial and add it to the 15 mL conical tube dropwise. While adding, gently move the tube back and forth to mix NSCs. This reduces osmotic shock to cells.

10. Centrifuge at 300 x *g* for 5 minutes and aspirate the supernatant.

11. Resuspend the cell pellet in DPBS –/–, centrifuge at 300 x g for 5 minutes, and aspirate the supernatant.

12. Resuspend cell pellet in appropriate amount of pre-warmed neural expansion medium (e.g., 1 mL for all NSCs from 1 vial) and determine cell concentration using preferred method.

13. Dilute cell suspension to 4 x 10^5 cells/mL with pre-warmed neural expansion medium. If the passage number of the NSCs is less than 4, add ROCK inhibitor Y-27632 solution into the cell suspension to reach final concentration of 5 µM.

14. Aspirate Geltrex solution from coated vessels and add an appropriate amount of diluted cell suspension into each culture vessel to plate cells at the density of 1 x 10^5 cells/cm^2.

15. Place vessels gently in an incubator and move culture vessels in several quick back-and-forth and side-to-side motions to disperse cells across the surface of vessels.

16. Change neural expansion medium every other day thereafter until NSCs reach confluency for splitting.

10 Differentiating human pluripotent stem cell–derived neural stem cells into neurons

Summary

Neural stem cells (NSC) derived from human pluripotent stem cells (hPSCs) are self-renewing multipotent stem cells that can be further differentiated into downstream lineages such as neurons and glial cells. The protocols described herein are primarily optimized with NSCs derived from hPSCs using Gibco™ PSC Neural Induction Medium.

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Required materials

Cells

- NSCs derived by PSC Neural Induction Medium

Media and reagents

- Gibco™ Neurobasal™ Medium (Cat. No. 21103049)
- Gibco™ Neurobasal™ Plus Medium (Cat. No. A3582901)
- Gibco™ B-27™ Supplement (50X), serum free (Cat. No. 17504044)
- Gibco™ B-27™ Plus Supplement (50X) (Cat. No. A3582801)
- Gibco™ CultureOne™ Supplement (100X) (Cat. No. A3320201)
- Gibco™ Laminin Mouse Protein, Natural (Cat. No. 23017015)
- *Optional:* Gibco™ Poly-D-Lysine (Cat. No. A3890401)
- Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501): Thawed Accutase reagent can be kept at 2°C to 8°C for up to 2 weeks.
- Gibco™ Distilled Water (Cat. No. 15230162)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- ROCK Inhibitor Y-27632 (MilliporeSigma, Cat. No. Y0503): Dissolve 1 mg of Y-27632 in 0.31 mL distilled water to make 10 mM stock solution. Sterilize the stock solution by filtering through a 0.22 µm filter. Aliquot 20–50 µL into sterile tubes and store at –20°C to –5°C in the dark for up to 1 year. The thawed Y-27632 solution can be kept at 2°C to 8°C for up to 2 weeks.
- L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (MilliporeSigma, Cat. No. A8960): Dissolve 1 g of ascorbic acid 2-phosphate sesquimagnesium salt hydrate in 17.3 mL distilled water, filter-sterilize through a 0.22-µm filter, aliquot 100–200 µL into sterile tubes, and store at –20°C to –5°C in the dark for up to 6 months.

Equipment

- 37°C humidified cell culture incubator with 5% CO_2
- Centrifuge
- 37°C water bath

Consumables

- Corning™ BioCoat™ Poly-D-Lysine Multiwell Plates: 96-well (Fisher Scientific, Cat. No. 08-774-255), 48-well (Fisher Scientific, Cat. No. 08-774-288), 24-well (Fisher Scientific, Cat. No. 08-774-271), 12-well (Fisher Scientific, Cat. No. 08-774-270), or 6-well (Fisher Scientific, Cat. No. 08-774-268), or Gibco Poly-D-Lysine (to prepare poly-D-lysine–coated culture plates)
- 15 mL and 50 mL sterile polypropylene conical tubes
- 5, 10, 25, and 50 mL sterile pipettes

Preparing media

To make 100 mL complete Neuronal Differentiation Medium with CultureOne Supplement, mix the components below under sterile conditions:

Component	Final conc.	Amount
Neurobasal Medium or Neurobasal Plus Medium[1]	1X	96 mL
B-27 Supplement (50X), serum free or B-27 Plus Supplement[2]	2%	2 mL
GlutaMAX Supplement	2 mM	1 mL
CultureOne Supplement (100X)	1%	1 mL
Ascorbic acid (200 mM)	200 µM	100 µL

[1] Make complete medium with either Neurobasal Medium with B-27 Supplement or Neurobasal Plus Medium with B-27 Plus Supplement. Do not interchange Neurobasal Plus Medium with Neurobasal Medium and vice versa.

[2] Supplement can be thawed at 2°C to 8°C overnight or quickly in a 37°C water bath for about 5 minutes, and then aliquoted and frozen at –20°C to –5°C to allow for the preparation of smaller volumes of complete medium. Avoid repeated thawing and freezing.

Note: Complete medium can be stored at 2–8°C in the dark for up to 2 weeks. Warm medium in a 37°C water bath for 5–10 minutes before using. Do not warm medium in a 37°C water bath for >10 minutes, as this may cause degradation of the medium.

Optional: Add growth factors such as 10–20 ng/mL glial cell–derived neurotrophic factor and 10–20 ng/mL brain-derived neurotrophic factor to improve neuron survival.

Optional: Add antibiotics such as gentamicin.

Preparing matrix

To prepare the matrix, follow the process below to apply coatings in the sequence of poly-D-lysine first and laminin second.

Coating culture vessels with poly-D-lysine

1. Dilute the poly-D-lysine solution in sterile DPBS, calcium, magnesium (DPBS +/+) to prepare a 50 µg/mL working solution.

2. Coat the surface of the culture vessel with the working solution of poly-D-lysine (e.g., 50 µL/well for a 96-well plate).

3. Incubate the vessel at room temperature for 1 hour.

4. Remove the poly-D-lysine solution and rinse the culture surface 3 times with sterile distilled water (e.g., 100 µL/well for a 96-well plate).

   Make sure to rinse the culture vessel thoroughly, as excess poly-D-lysine solution can be toxic to the cells.

5. Remove the distilled water and leave the coated culture vessel uncovered in the laminar hood to dry.

   The culture surface should be fully dry after 2 hours.

Note: Plates can be used immediately or stored at 4°C once they are dry. For storage at 4°C, tightly wrap the vessel with Parafilm laboratory film and use within one week of coating.

Coat culture plates with laminin

1. Thaw a vial of laminin stored at –80°C at room temperature.

2. Dilute the thawed laminin solution 1:100 with sterile distilled water.

3. Add laminin solution into poly-D-lysine–coated plates to cover the whole surface, and incubate in a 37°C, 5% CO_2 incubator for 1 hour.

4. Culture plates can now be used. Just prior to use, aspirate the laminin solution from each well. Cells can be plated directly onto the laminin-coated plates without rinsing. Coated plates can also be stored at 2–8°C for up to one week. When storing, seal culture plates with Parafilm laboratory film to prevent drying. Before using, warm up the coated plates stored at 2–8°C at room temperature for 20–30 minutes.

Plate and differentiate NSCs into neurons

1. Dissociate expanded hPSC-derived NSCs in culture with StemPro Accutase Cell Dissociation Reagent or thaw frozen hPSC-derived NSCs.

2. Resuspend dissociated or thawed NSCs with 5–10 mL DPBS, no calcium, no magnesium (DPBS –/–).

3. Centrifuge the cells at 300 x *g* for 5 minutes and aspirate the supernatant.

4. Resuspend NSCs in 1–2 mL of pre-warmed Neuronal Differentiation Medium containing Neurobasal Medium and B-27 Supplement, depending on the number of NSCs.

5. Determine the concentration of viable cells using your preferred method.

6. Dilute the NSC suspension with pre-warmed Neuronal Differentiation Medium containing Neurobasal Medium and B-27 Supplement to an appropriate concentration.

7. Aspirate the laminin solution from poly-D-lysine– and laminin-coated plates.

8. Gently shake the tube containing NSCs and add an appropriate amount of diluted NSC suspension into each well of culture plates to plate NSCs at a density of 5 x 10^4 cells/cm^2 or less.

   Note: If the passage number of NSCs is less than 5, add ROCK inhibitor Y-27632 into Neuronal Differentiation Medium to the final concentration of 5 µM. The treatment with ROCK inhibitor at the time of NSC plating is crucial for cell survival with NSCs less than 5 passages. The optimal plating density may also vary depending on hPSC lines from which NSCs were derived.

9. Move the culture plates in several quick back-and-forth and side-to-side motions to disperse NSCs across the surface, and place them gently in a 37°C CO_2 incubator.

10. Add the same volume of pre-warmed Neuronal Differentiation Medium containing Neurobasal Medium and B-27 Supplement into each well of plates 2–3 days after NSC plating, and return them into a 37°C CO_2 incubator.

    Note: Differentiating neurons detach easily. When removing spent medium, do not touch cells with pipette tips. Also, add fresh medium gently toward the wall of culture plates.

11. Change spent medium every 2–3 days thereafter. When changing medium, remove half of spent medium from each well and add the same volume of pre-warmed fresh Neuronal Differentiation Medium with Neurobasal Medium and B-27 Supplement into each well of plates and return them into a 37°C CO_2 incubator.

12. Maintain neurons differentiated with CultureOne Supplement for 1–5 weeks or longer, depending on NSC lines and the purpose of experiments.

Note: At 1–2 weeks after NSC differentiation, CultureOne Supplement can be withdrawn by adding fresh Neuronal Differentiation Medium without CultureOne Supplement into each well of plates when changing spent medium. However, withdrawal of CultureOne Supplement may increase the chance of cell clumps reforming in the culture due to proliferating progenitor cells.

Note: Neuronal Differentiation Medium containing Neurobasal Plus Medium and B-27 Plus Supplement can be used to replace Neuronal Differentiation Medium containing Neurobasal Medium and B-27 Supplement after 5 days of NSC plating for better survival of differentiating neurons.

Expected results

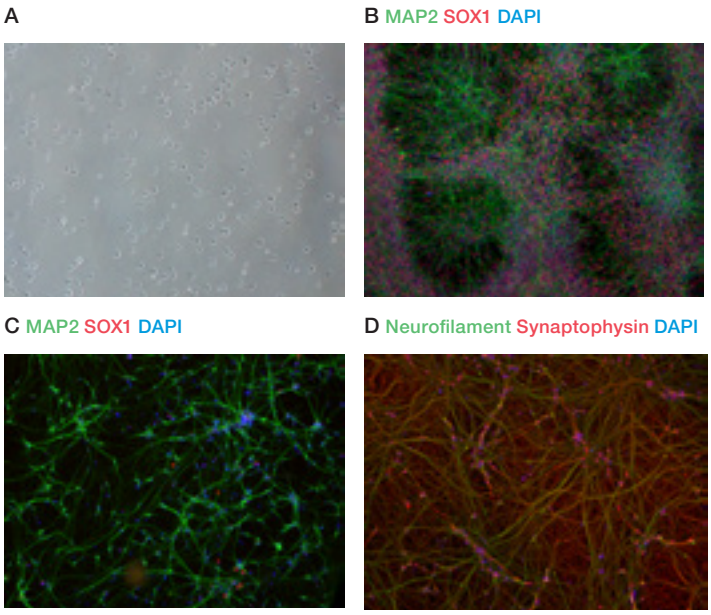

Figure 10-1. Differentiation of hPSC-derived NSCs by PSC Neural Induction Medium into neurons. (A) H9 ESC-derived NSCs were plated at a density of 5 x 10^4 cells/cm^2 in Neuronal Differentiation Medium containing B-27 Supplement and Neurobasal Medium. **(B)** Without CultureOne Supplement, cells at 2 weeks of differentiation were highly dense, formed cell clumps, and contained MAP2-positive neurons (green) and a significant number of SOX1-positive NSCs (red). **(C)** At 2 weeks of differentiation, cultures treated with CultureOne Supplement had an even distribution of MAP2-positive neurons (green) with minimal SOX1-positive NSCs (red) and no cell clumps. **(D)** At 5 weeks of differentiation, differentiated cells treated with CultureOne Supplement expressed the mature neuronal markers neurofilament (green) and synaptophysin (red). The nuclei were counterstained with DAPI (blue) in panels **B–D**.

11 PSC-derived neuron cell culture: Neuronal differentiation, maturation, and maintenance

Summary

Human induced pluripotent stem cell (iPSC)-derived neurons have increasingly become a valuable system for the study of neurological disorders. Improved differentiation protocols, cell reprogramming, and gene editing enable scientists to generate patient-specific, disease-in-a-dish models for disorders such as Parkinson's disease, Alzheimer's disease, and autism, among others. These human models tend to be flexible and scalable, and maintain many of the characteristics found in these disorders, which are key requirements for their use in mechanistic and drug discovery studies.

A critical step in generating useful PSC-derived neurons is neuronal maturation. During maturation, neurons extend neurites to form highly connected networks, express synaptic markers, and generate spontaneous, networked electrical activity. Robust maturation is necessary for PSC-derived neurons to be relevant disease model systems. Typical maturation conditions are inefficient, generating poorly matured neurons with low levels of functionality. Recently we developed a neuronal maturation and maintenance medium, the Gibco™ B-27™ Plus Neuronal Culture System, which includes a user guide specifically for PSC-derived neurons. This next-generation system was designed to improve long-term neuronal survival, maturation, and functionality of neurons in culture.

This protocol details neuronal differentiation, maturation, and maintenance of PSC-derived neurons.

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Generating PSC-derived neurons

There are many ways to generate PSC-derived neurons. The most commonly used methods include the monolayer approach (Gibco™ PSC Neural Induction Medium), rosette formation, and factor-driven induction (Figure 11-1).

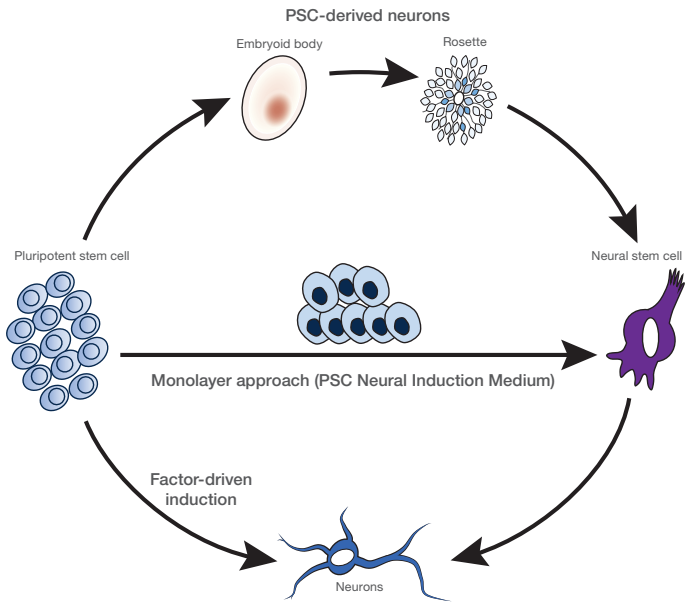

Figure 11-1. Methods for generating PSC-derived neurons. Schematic of the three most common methods used for generating PSC-derived neurons.

The monolayer and rosette differentiation methods mimic *in vivo* development of PSC-derived neurons, and both involve an intermediate step that yields an expandable population of neural stem cells (NSCs) that can be further differentiated into neurons. NSC methods are flexible and may be more relevant to disease modeling.

Another method is factor-driven induction of PSCs into neuronal "iN" cells. This method typically involves overexpression of lineage-specific factors to rapidly induce a neuronal cell fate. The benefits of this system are rapid and highly efficient generation of functional neurons within

14 days. Factor-driven induction can also be used to generate induced neurons from somatic cells.

Neuronal differentiation and maturation

Starting from an NSC population, cells should be differentiated toward a neuronal fate for 3–7 days. Optimal conditions for neuronal differentiation of NSCs depend on the NSC derivation method. For example, NSCs from the monolayer method require different media conditions than NSCs from the rosette method. When differentiating cells have adopted a neuronal-like morphology (Figure 11-2), cells are ready to switch to a B-27 Plus neuronal maturation medium (B-27 Plus NMM) for continued maturation and long-term maintenance.

This protocol provides guidance for differentiating and maturing neurons from NSCs derived by the monolayer method with PSC Neural Induction Medium. Recommended medium conditions for neuronal differentiation of rosette-derived NSCs or the factor-driven method can be found in the appendix of the user guide: **B-27 Plus Neuronal Culture System (PSC-Derived Neuron Applications)**.

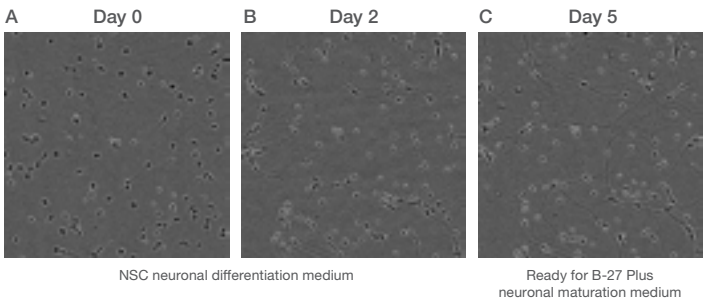

Figure 11-2. NSC differentiation: guidance for switching to the B-27 Plus system for maturation.

Required materials

Media and reagents

- Gibco™ Poly-D-Lysine (Cat. No. A3890401)
- Gibco™ Neurobasal™ Plus Medium (Cat. No. A3582901)
- Gibco™ B-27™ Plus Supplement (50X), serum free (Cat. No. A3582801)
- Gibco™ GlutaMAX™ Supplement (Cat. No. 35050061)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- Ascorbic Acid (MilliporeSigma, Cat. No. A8960)
- Gibco™ Distilled Water (Cat. No. 15230162)
- Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501)
- Gibco™ Neurobasal™ Medium (Cat. No. 21103049)
- Gibco™ B-27™ Supplement (50X), serum free (Cat. No. 17504044)
- Gibco™ CultureOne™ Supplement (100X) (Cat. No. A3320201)

Preparing media

Ascorbic acid stock preparation

1. To prepare 200 mM ascorbic acid stock solution (500X), add 20 mL of distilled water to 579.0 mg of ascorbic acid powder and mix until dissolved.

2. After dissolving, filter-sterilize through a 0.22 µm filter, aliquot 100–200 µL into sterile tubes, and store at –20°C.

B-27 neuronal differentiation medium (B-27 NDM)

Complete medium requires supplementation of Neurobasal Medium with B-27 Supplement (50X), CultureOne Supplement, GlutaMAX Supplement, and ascorbic acid. Complete B-27 NDM is stable for 2 weeks when stored in the dark at 4°C.

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

Component	Final conc.	Amount
Neurobasal Medium	1X	98 mL
B-27 Supplement (50X), serum free	2%	2 mL
CultureOne Supplement (100X)	1%	1 mL
GlutaMAX Supplement	1X	1 mL
Ascorbic acid (200 mM)	200 µM	100 µL

B-27 Plus neuronal maturation medium (B-27 Plus NMM)

Complete medium requires supplementation of Neurobasal Plus Medium with B-27 Plus Supplement (50X), CultureOne Supplement, GlutaMAX Supplement, and ascorbic acid. Complete B-27 Plus NMM is stable for 2 weeks when stored in the dark at 4°C.

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

Component	Final conc.	Amount
Neurobasal Plus Medium	1X	98 mL
B-27 Plus Supplement (50X), serum free	2%	2 mL
CultureOne Supplement (100X)	1%	1 mL
GlutaMAX Supplement	1X	1 mL
Ascorbic acid (200 mM)	200 µM	100 µL

Preparing matrix

To prepare the matrix, follow the process below to apply coatings in the sequence of poly-D-lysine first and laminin second.

Coating culture vessels with poly-D-lysine

1. Dilute the poly-D-lysine solution in sterile DPBS without Ca^{2+} and Mg^{2+} (DPBS –/–) to prepare a 50 µg/mL working solution.

2. Coat the surface of the culture vessel with the working solution of poly-D-lysine (e.g., 50 µL/well of a 96-well plate).

3. Incubate the vessel at room temperature for 1 hour.

4. Remove the poly-D-lysine solution and rinse the culture surface 3 times with sterile distilled water (e.g., 100 µL/well of a 96-well plate).

   Make sure to rinse the culture vessel thoroughly as excess poly-D-lysine solution can be toxic to the cells.

5. Remove distilled water and leave the coated culture vessel uncovered in the laminar hood to dry.

   The culture surface will be fully dry after 2 hours.

   Note: Once the plates are dry, they can be used immediately or stored at 4°C. For storage at 4°C, tightly wrap the vessel with Parafilm laboratory film and use within one week of coating.

Coating culture vessels with laminin

6. Thaw a vial of laminin stock solution at 4°C.

   Note: Upon receipt, laminin stock solution should be aliquoted and stored at –80°C to avoid repeated thawing and freezing.

7. To create a working solution, dilute the laminin stock solution to a concentration of 3 µg/mL in sterile distilled water.

8. Add the laminin solution to poly-D-lysine–coated plates to cover entire surface. For example, to coat a 96-well plate, add 60 µL of the dilute 3 µg/mL laminin solution. Incubate plates in a humidified 37°C, 5% CO_2 incubator for 2 hours, or tightly wrap the plate with Parafilm laboratory film and store overnight at 4°C.

9. Immediately prior to use, aspirate the laminin solution from each well, rinse once with DPBS –/–, and then plate cells.

Differentiating and maturing neurons from NSCs derived using PSC Neural Induction Medium

Plating and differentiating NSCs

1. Dissociate expanded hPSC-derived NSCs in culture with StemPro Accutase Cell Dissociation Reagent, or thaw frozen hPSC-derived NSCs.

2. Resuspend dissociated or thawed NSCs with 5–10 mL DPBS –/–.

3. Centrifuge the cells at 300 x *g* for 5 minutes and aspirate the supernatant.

4. Resuspend NSCs in 1–2 mL of pre-warmed B-27 NDM, depending on the number of NSCs.

5. Determine the concentration of viable cells using your preferred method.

6. Dilute the NSC suspension with pre-warmed B-27 NDM to an appropriate concentration.

7. Remove precoated poly-D-lysine– and laminin-coated plates from the incubator. Just prior to use, aspirate the laminin solution from each well and rinse once with DPBS –/–, before plating cells.

8. Gently mix the tube containing the NSCs and add an appropriate amount of the diluted NSCs in B-27 NDM suspension into each well of culture plate.

9. Move the culture plates in several quick back-and-forth and side-to-side motions to disperse NSCs across the surface, and place them gently in a humidified 37°C, 5% CO_2 incubator for 48 hours.

10. After the 48-hour incubation (on day 2), perform a half-medium change with B-27 NDM.

11. At day 5, differentiating cells will have adopted a neuronal-like morphology (see Figure 11-2 on page 45), and are ready for neuronal maturation.

Maturation and maintenance of PSC-derived neurons

12. Once cells adopt a neuronal morphology (see Figure 11-2 on page 45), remove half the spent medium and replace with an equal volume of pre-warmed, fresh complete B-27 Plus NMM.

13. Change spent medium every 3–4 days thereafter. (For high-density cultures, change medium every 2–3 days). When changing medium, remove half the spent medium from each well and add the same volume of pre-warmed fresh B-27 Plus NMM into each well of plates and return cultures to a humidified 37°C, 5% CO_2 incubator.

14. Change spent medium every 3–4 days thereafter. (For high-density cultures, change medium every 2–3 days).

15. Maintain maturing neurons with B-27 Plus NMM for 3–10 weeks or longer, depending on NSC lines and the purpose of experiments.

    Note: Differentiated neurons detach easily. When removing spent medium, do not touch cells with pipet tip; when adding fresh medium, do it gently toward the wall of the culture plate.

Expected results

Increased neuronal survival with the B-27 Plus system

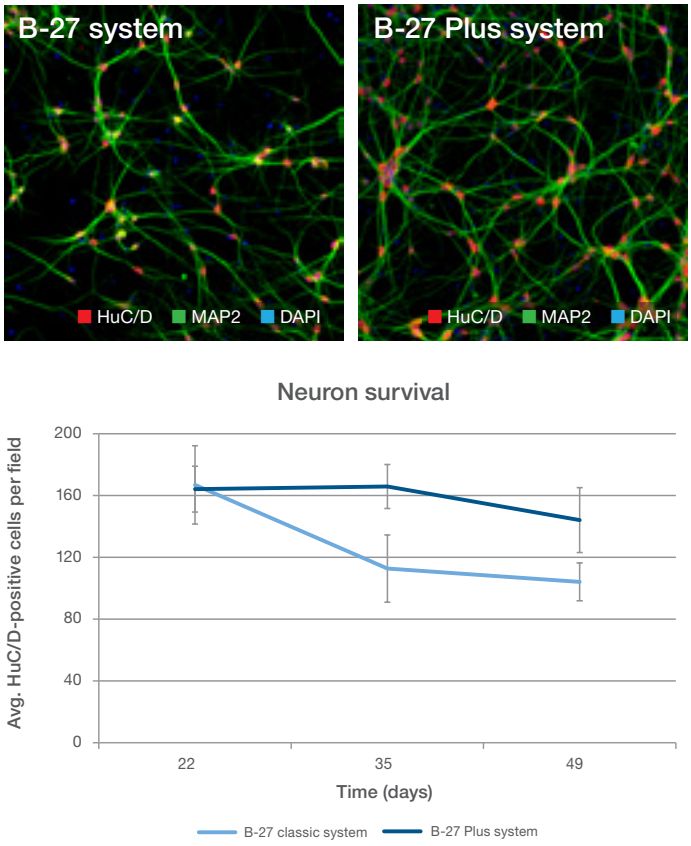

Figure 11-3. The B-27 Plus system increases long-term survival of iPSC-derived neurons. NSCs derived using PSC Neural Induction Medium matured in the B-27 Plus system for 4 weeks, resulting in an approximately 2-fold increase in survival compared to classic B-27 Supplement and Neurobasal Medium.

Enhanced neuronal maturation with the B-27 Plus system

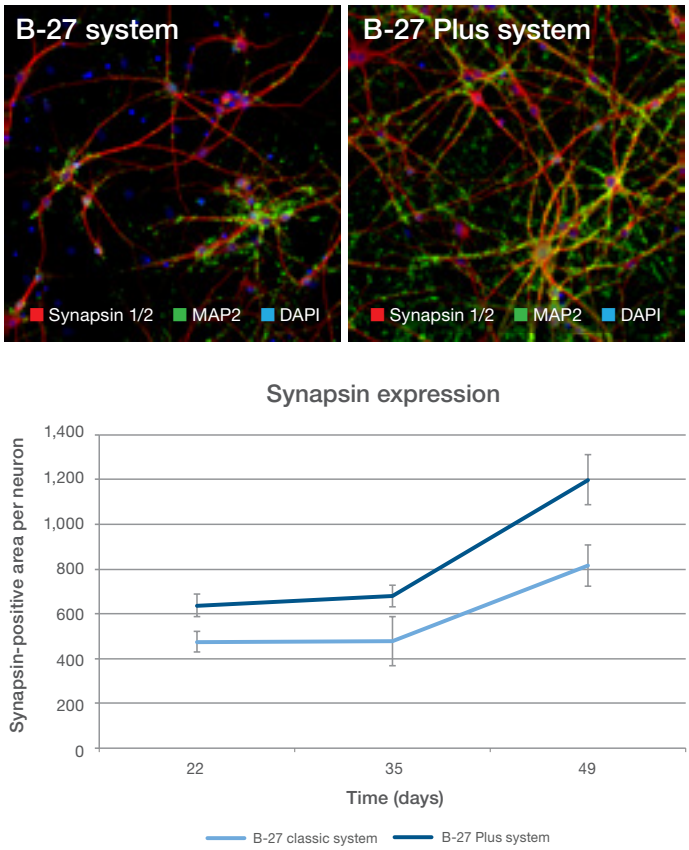

Figure 11-4. The B-27 Plus system enhances synaptic marker expression in iPSC-derived neurons. NSCs that were derived using PSC Neural Induction Medium and then matured in the B-27 Plus system for 7 weeks resulted in significantly higher levels of synapsin 1/2 expression than in the classic B-27 system. Synaptic marker expression is an indicator of functional maturity.

Improved physiological activity with the B-27 Plus system

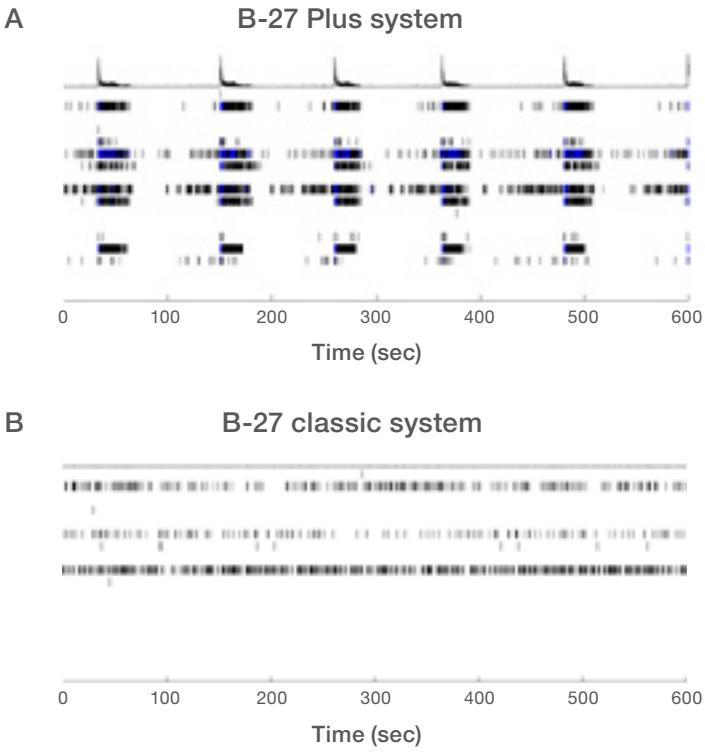

Figure 11-5. The B-27 Plus system improves functional activity in neurons from monolayer NSCs derived using PSC Neural Induction Medium. Multi-electrode array (MEA) data recorded as raster plots from NSCs matured in the B-27 Plus system for 31 days **(A)** showed high levels of spontaneous, synchronous network bursting activity compared to the classic B-27 culture system **(B)**. This networked activity was maintained for over 4 weeks.

12 Differentiating PSCs to midbrain dopaminergic neurons

Summary

Midbrain dopaminergic (mDA) neurons derived from human pluripotent stem cells (hPSCs) provide an excellent alternative to primary human neurons for disease modeling of Parkinson's disease and drug screening. During brain development, mDA neurons are derived from distinct populations of cells termed midbrain floor plate (mFP) cells. In this protocol, we describe how to (1) specify hPSC to mFP cells, (2) expand and cryopreserve specified cells, and (3) revive and mature cells to mDA neurons.

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Required materials

Cells

- Gibco™ Human Episomal iPSC Line (Cat. No. A18945) or equivalent PSCs cultured in Gibco™ Essential 8™ or Essential 8™ Flex Medium (see Cat. No. information below)

Media and reagents

- Gibco™ PSC Dopaminergic Neuron Differentiation Kit (Cat. No. A3147701: Contains Floor Plate Specification Supplement (20X) stored at –20°C to –5°C in the dark; Floor Plate Cell Expansion Kit, consisting of Floor Plate Cell Expansion Base stored at 4°C and Floor Plate Cell Expansion Supplement (50X) stored at –20°C to –5°C in the dark; and Dopaminergic Neuron Maturation Supplement (50X) stored at –20°C to –5°C in the dark.)
- Gibco™ Essential 8™ Medium (Cat. No. A1517001), contains Basal Medium and Supplement; or Essential 8™ Flex Medium Kit (Cat. No. A2858501), contains Flex Basal Medium and Flex Supplement
- Gibco™ Vitronectin (VTN-N), Recombinant Human Protein, Truncated (Cat. No. A14700)
- Gibco™ Neurobasal™ Medium (Cat. No. 21103049) (base medium for specification)
- Gibco™ DMEM/F-12, GlutaMAX™ Supplement (Cat. No. 10565018) (base medium for maturation)
- Gibco™ Laminin Mouse Protein, Natural (Cat. No. 23017015)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- Gibco™ Distilled Water (Cat. No. 15230162)
- Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501)
- ROCK inhibitor Y-27632 (MilliporeSigma, Cat. No. Y0503)
- Dimethyl sulfoxide, Hybri-Max™ grade (DMSO) (MilliporeSigma, Cat. No. D2650)
- Gibco™ Human Dopaminergic Neuron Immunocytochemistry Kit (Cat. No. A29515) (for image-based analysis of intermediate floor plate progenitors and mature dopaminergic neurons)

Equipment and plasticware

- Thermo Scientific™ Nunclon™ Sphera™ Flasks (Cat. No. 174951 or 174952) (for suspension culture)
- Thermo Scientific™ Nunclon™ Sphera™ Dishes (Cat. No. 174932) (for suspension culture)
- Gibco™ Poly-D-Lysine (Cat. No. A3890401) (to prepare poly-D-lysine and laminin double-coated culture plates)
- Thermo Scientific™ Mr. Frosty™ Freezing Container (Cat. No. 5100-0001)
- Thermo Scientific™ Nalgene™ General Long-Term Storage Cryogenic Tubes (Cat. No. 5000-0012 or 5000-1012) (for banking floor plate progenitor cells)
- 37°C humidified cell culture incubator with 5% CO_2
- Liquid nitrogen storage
- Centrifuge
- 37°C water bath
- 15 and 50 mL sterile polypropylene conical tubes
- 5, 10, 25, and 50 mL sterile pipettes

Preparing media

Specification medium

Floor Plate (FP) Specification Supplement (20X) can be thawed at 4°C or room temperature and dispensed into aliquots if desired. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

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

Component	Final conc.	Amount
Neurobasal Medium	1X	95 mL
FP Specification Supplement (20X)	1X	5 mL

Expansion medium

Floor Plate (FP) Cell Expansion Supplement (20X) can be thawed at 4°C or room temperature and dispensed into aliquots if desired. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

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

Component	Final conc.	Amount
FP Cell Expansion Base	1X	98 mL
FP Cell Expansion Supplement (50X)	1X	2 mL

Maturation medium

Dopaminergic (DA) Neuron Maturation Supplement (20X) can be thawed at 4°C or room temperature and dispensed into aliquots if desired. Complete medium is stable for 2 weeks when stored in the dark at 2°C to 8°C.

To prepare 100 mL of DA neuron maturation medium, mix the following components under sterile conditions. For larger volumes, increase the component amounts proportionally.

Component	Final conc.	Amount
DMEM/F-12	1X	98 mL
DA Neuron Maturation Supplement (50X)	1X	2 mL

ROCK inhibitor stock solution

To prepare 10 mM ROCK inhibitor Y-27632 solution, add 10 mg of Y-27632 to 3.125 mL of distilled water. Mix well until dissolved.

After dissolving, filter-sterilize through a 0.22 µm filter, aliquot 20–50 µL into sterile tubes, and store at –20°C in the dark for up to 1 year. Thawed Y-27632 solution can be kept at 4°C for up to 4 weeks.

Preparing matrix

Vitronectin-coated plates

1. Prepare a 1:50 dilution of vitronectin solution in DPBS, no calcium, no magnesium (DPBS –/–) for a final concentration of 10 µg/mL.

2. Add 1 mL of the diluted vitronectin solution to each well of a 6-well plate.

3. Incubate the coated plates at room temperature for 1 hour. The culture vessel can now be used or stored at 4°C wrapped in Parafilm laboratory film for up to one week. Do not allow the vessel to dry.

4. Before use, pre-warm the culture vessel to room temperature for at least 1 hour before aspirating and discarding the vitronectin solution.

   Note: It is not necessary to rinse off the culture plate after the removal of the vitronectin solution.

Laminin-coated plates

1. Thaw the required volume of 1.0 mg/mL laminin stock solution (stored at –80°C) slowly at 4°C.

2. Prepare a 1:100 dilution of laminin solution in water for a final concentration of 10 µg/mL.

3. Add 1 mL of the diluted laminin solution to each well of a 6-well plate.

4. Incubate the coated plates at 4°C overnight or at 37°C for 2 hours. The culture vessel can now be used or stored at 4°C wrapped in Parafilm laboratory film for up to one week. Do not allow the vessel to dry.

5. Before use, pre-warm the culture vessel to room temperature for at least 1 hour before aspirating and discarding the laminin solution.

   Note: Laminin may form a gel when thawed too rapidly. Thawing the stock solution in the cold (4°C) prevents this. Thawed stock solution can be stored at 4°C for up to 1 month.

Poly-D-lysine and laminin double-coated plates

1. Coat each well of a 6-well plate with 1 mL of poly-D-lysine working solution (100 µg/mL).

2. Incubate the coated plates at room temperature for 1–2 hours.

3. Remove the Poly-D-Lysine solution and rinse 3 times with distilled water.

4. Prepare a 15 µg/mL working solution of laminin in sterile distilled water.

5. Remove distilled water rinse and leave the coated culture vessel uncovered in the laminar hood to dry.

6. Add 1 mL of the 15 µg/mL laminin working solution to each well of a 6-well plate.

7. Incubate the coated plates overnight at 4°C or at 37°C for 2 hours.

8. Before use, pre-warm the culture vessel to room temperature for at least 1 hour before aspirating and discarding the laminin solution.

   Note: You can use the coated culture plate immediately or store it at 4°C wrapped in Parafilm laboratory film for up to one week. Do not allow the plate to dry.

Specification (PSC to mFP)

Set up hPSC culture (day –1)

1. Prepare vitronectin-coated plate and complete Essential 8 Medium.

2. Plate a high-quality PSC culture from frozen vial or ongoing culture on vitronectin plate in Essential 8 Medium to target 20–40% confluency on the next day.

3. If plated as single cells, supplement medium with 10 µM ROCK inhibitor Y-27632 to inhibit cell death.

   Note: Depending on cell line used, culture kinetics are different. Optimization is needed to find the right seeding density to get 100% confluency after 6–7 days of specification. With H9 ESC lines, 30,000 cells/cm^2 seeding density resulted in 20–40% confluency on the next day.

Specification (day 0–day 10)

1. Start specification by changing medium with FP specification medium (day 0). Aspirate the spent Essential 8 Medium containing the ROCK inhibitor and replace it with pre-warmed FP specification medium.

2. Incubate at 37°C in a humidified atmosphere of 5% CO_2 in air.

3. Replenish culture with fresh medium at day 3, 5, 7, and 9. Medium consumption will be increased over time, so use 2X volume for later points (day 7 and 9) to compensate.

Expansion

Harvest FP progenitor cells (day 10, 12, and 16)

1. Prepare laminin-coated plate and FP cell expansion medium.

2. Aspirate the spent medium from the specification culture plate and rinse the wells with DPBS, calcium, magnesium (DPBS +/+) to remove any remaining medium.

3. Aspirate the DPBS and add an appropriate volume of StemPro Accutase Cell Dissociation Reagent to fully cover the surface (1 mL per well of a 6-well plate, or 1 mL per 10 cm^2 of surface area).

4. Incubate the vessel at 37°C, 5% CO_2 for ~5–15 minutes, continually observing the wells for cell detachment.

5. After several minutes or when some colonies start detaching (whichever happens first), gently tap the bottom of the vessel several times. Most colonies should freely come into suspension. If all colonies do not detach, wait 1–2 minutes, and then tap the vessel again to detach the remaining colonies.

6. Transfer the cell clumps to a sterile 50 mL culture tube.

7. Rinse the wells of the specification culture plate twice with DPBS +/+, using 4x the volume of StemPro Accutase reagent used in each well (4 mL per well of a 6-well plate). After each rinse, collect the cell clumps in the same 50 mL culture tube to ensure the recovery of all colonies.

8. Centrifuge the cell suspension at 300 x *g* for 3 minutes at 4°C to pellet the cells. Carefully aspirate the supernatant, leaving the cell pellet in the culture tube.

Replate FP cells (day 10 and 12)

1. Gently flick the bottom of the tube to dislodge the cell pellet, and resuspend the cells in a sufficient volume of complete expansion medium plus 5 µM ROCK inhibitor Y-27632.

2. Use 1:2 split ratio (i.e., one plate to two plates) for FP passage 0 (FPp0) (day 10) and use 1:4 split ratio for FP passage 1 (FPp1) (day 16).

   Note: Overnight treatment with the ROCK inhibitor is required upon passaging. The ROCK inhibitor is removed from the culture the following day when the spent medium is replaced with FP specification medium.

3. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO_2.

4. The next day, replenish culture with fresh medium and every other day thereafter.

Cryopreserve FP passage 2 (FPp2) cells (day 16)

1. Prepare freezing medium at 2X concentration (80% FP expansion medium + 20% DMSO) and chill at 4°C before use.

2. Calculate the volume of cells in the FPp2 cell suspension that corresponds to the number of cells you want to cryopreserve, and transfer to a sterile tube.

3. Dilute the cells to 2X the intended final frozen concentration using FP expansion medium at 4°C.

4. In a dropwise manner, add the same volume of 2X freezing medium (chilled to 4ºC) as the cell suspension while gently rocking the tube back and forth.

5. Aliquot 1 mL of the cell suspension into each cryogenic vial, place the vials in a Mr. Frosty Freezing Container with isopropyl alcohol, and freeze them at –80°C overnight.

6. The next day, transfer the frozen vials to liquid nitrogen (vapor phase) for long-term storage.

Recover frozen FPp2 cells (day 16)

1. Remove the cryogenic vial of FPp2 cells from the liquid nitrogen storage and immediately immerse it in a 37°C water bath without submerging the cap. Swirl the vial gently.

2. When only an ice crystal remains (~1–2 minutes), remove the vial from the water bath and spray the outside of it with 70% ethanol to decontaminate.

3. Pipet the cells gently into a sterile 15 mL conical tube using a 1 mL pipette.

4. Add 1 mL of DPBS –/– into the vial to collect the remaining cells and transfer the cell suspension dropwise to the 15 mL conical tube. While adding, gently move the tube back and forth to mix the cells and prevent osmotic shock.

5. Add an additional 3 mL of DPBS –/– to the cells to have a 5 mL suspension.

6. Remove a small volume of cell suspension and perform a viable cell count.

7. Centrifuge the cell suspension at 300 x *g* for 3 minutes to pellet the cells. Carefully aspirate the supernatant, leaving the cell pellet in the culture tube.

8. Gently flick the bottom of the tube to dislodge the cell pellet and resuspend the cells to 1.0×10^6 viable cells/mL in FP expansion medium plus 5 µM ROCK inhibitor Y-27632.

Sphere formation of FPp2 (day 16–day 21)

1. On day 16, harvest or thaw FPp2 cells as described, then remove a small volume of cells and perform a viable cell count.

2. Resuspend the FPp2 cells to 1.0 x 10^6 viable cells/mL in FP expansion medium plus 5 µM ROCK inhibitor Y-27632.

3. Transfer cell suspension to a non–tissue culture treated vessel and adjust the volume of the cell suspension to the size of vessel.

4. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO_2.

5. Perform a complete medium change by the centrifugation method on the next day and every other day thereafter. Transfer the spheres to a 15 mL conical tube and then centrifuge at 200 x *g* for 2 minutes. Aspirate the supernatant and discard.

6. Resuspend the spheres in fresh FP expansion medium without the ROCK inhibitor, and then transfer to original flask.

7. Pipet the sphere suspension up and down several times to prevent them from merging with each other before plating.

Maturation

Dissociate spheres (day 21)

1. Prepare double-coated culture plates and DA maturation medium.

2. Transfer the sphere suspension from culture vessel to a sterile 15 mL conical tube. Allow spheres to settle to the bottom of the tube (~2–5 minutes) before proceeding to the next step.

3. Carefully aspirate the spent medium, leaving the spheres at the bottom of tube in a minimal volume (~100 µL) of the remaining medium. Resuspend the spheres in 5 mL of DPBS –/–.

4. Repeat steps 2 and 3, leaving the spheres at the bottom of tube in a minimal volume (~100 µL) of DPBS.

5. Add 1 mL of StemPro Accutase Cell Dissociation Reagent to the spheres and incubate for 30 minutes at 37°C. Every 10 minutes, gently swirl the cell suspension to ensure that spheres are exposed to the StemPro Accutase reagent evenly.

6. While the spheres are incubating with the dissociation reagent, aliquot the amount of complete DA maturation medium needed for the day and warm at 37°C.

7. Gently pipet the cell suspension up and down with a P1000 pipette until all of the spheres are dispersed into a single-cell suspension.

8. Remove a small volume of cell suspension to perform a viable cell count using an automated cell counter (e.g., Invitrogen™ Countess™ II Automated Cell Counter) or a hemocytometer.

9. Centrifuge the cell suspension at 300 x *g* for 3 minutes to pellet the cells. Carefully aspirate the supernatant, leaving the cell pellet in the culture tube.

Plate and mature FP cells to DA neurons (day 21–day 35)

1. Resuspend the cell pellet to a single-cell suspension in DA maturation medium plus 5 µM ROCK inhibitor Y-27632.

2. Seed the double-coated culture plates with the dissociated cells at a seeding density of 1.0 x 10^5 to 2.0 x 10^5 cells/cm^2 in DA maturation medium plus 5 µM ROCK inhibitor Y-27632.

3. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO_2.

4. On day 22 of differentiation (first medium change), add the same volume of fresh DA maturation medium (without the ROCK inhibitor) as the existing culture volume (e.g., 2 mL for each well of a 6-well plate).

5. For subsequent feeds (every 2~3 days), aspirate half of the spent medium and replace it with fresh DA maturation medium.

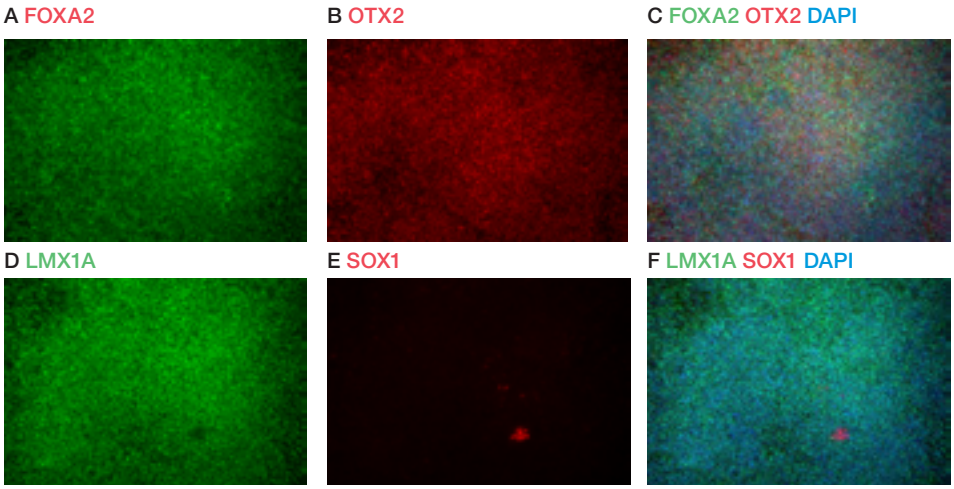


Figure 12-1. Marker expression of induced FP progenitor cells. hPSCs were treated with FP specification medium for 7 days, and the cells were analyzed for the key phenotypic markers of the human DA neuron lineage using the Human Dopaminergic Neuron Immunocytochemistry Kit (Cat. No. A29515). **(A–C)** After FP specification of hPSCs, the cells express FP marker FOXA2 (green) and rostral marker OTX2 (red). **(D–E)** The specified FP cells are positive for the DA progenitor marker LMX1A (green), but negative for the neural stem cell marker SOX1 (red).

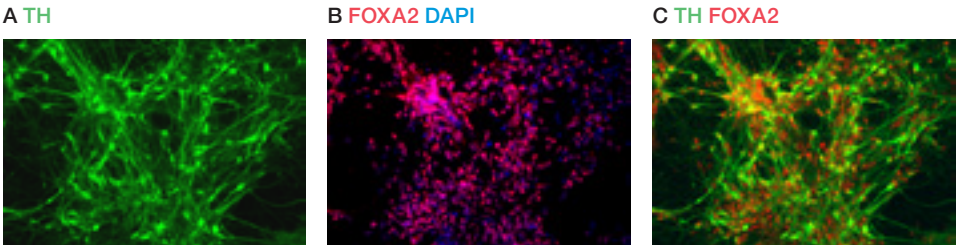


Figure 12-2. Representative images of mature DA neurons after 14 days in DA maturation medium. The majority of the TH-expressing neurons also coexpressed FOXA2. **(A)** Anti-TH antibody (green). **(B)** Anti-FOXA2 antibody (red) and Invitrogen™ NucBlue™ reagent (a DAPI nuclear DNA stain) (blue). **(C)** Merged image with anti-TH and anti-FOXA2 antibodies (green and red).

13 Culture of cortical astrocytes

Summary

Astrocytes constitute a critical mass of the central nervous system (CNS), in addition to oligodendrocytes and neurons. They are involved in adult CNS homeostasis, 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 newborn rats or mice, or from human fetal brain tissue.

View this protocol online and order products at **thermofisher.com/neuroprotocol/astro**

Introduction

Astrocytes outnumber neurons by up to tenfold, and have critical roles in adult CNS homeostasis (Pekny and Nilsson, 2005). They provide biochemical and nutritional support of neurons and endothelial cells that in turn form the blood–brain barrier, perform synaptic glutamate uptake, and maintain extracellular potassium (Rothstein et al., 1996; Rothstein et al., 1994). Astroglial dysfunction has been implicated in a number of CNS pathologies including amyotrophic lateral sclerosis (ALS) and ischemic neuronal death (Maragakis and Rothstein, 2006; Takano et al., 2009). Transplant-based astrocyte replacement therapy has been shown to be a promising therapeutic strategy against neuronal death (Lepore et al., 2008) and in lessening the disease impact in ALS. Although there are few known differences between cortical and hippocampal astrocytes, it has been reported that astrocytes from different regions of the brain show different sensitivity to ischemic injury (Xu et al., 2001; Zhao and Flavin, 2000).

Sources of primary cortical astrocytes

Gibco™ Rat Primary Cortical Astrocytes are isolated from the cortices of fetal Sprague-Dawley rats at embryonic day 19 (E19) of gestation. The cells are isolated from tissue under sterile conditions, and placed through one round of enzymatic dissociation and expansion in astrocyte growth medium (85% DMEM containing 4.5 g/L glucose and 15% FBS). The cells are cryopreserved at passage 1 (P1) in 90% astrocyte growth medium plus 10% DMSO. Each vial of Rat Primary Cortical Astrocytes contains 1 x 10^6 cells/mL that can be expanded in culture for at least one passage.

Gibco™ Human Astrocytes are derived from human brain glial progenitors. They have star-like morphology and express glial fibrillary acidic protein (GFAP) at high percentages. Human Astrocytes are supplied cryopreserved at a concentration of ≥1 x 10^6 cells/mL in Gibco™ Astrocyte Medium without EGF and with 10% DMSO.

Characteristics of Gibco Rat Primary Cortical Astrocytes

- Isolated from the brain cortex of fetal Sprague-Dawley rats at E19 of gestation
- Exhibit ≥70% viability upon thawing
- Stain >80% positive for the astrocyte-specific marker GFAP
- Stain ≤10% positive for neuron- and oligodendrocyte-specific markers galactocerebroside (GalC) and doublecortin (DCX)
- Exhibit a doubling time of approximately 9 days at P2
- Expandable in culture for at least one passage

Characteristics of Gibco Human Primary Cortical Astrocytes

- Derived from progenitor cells isolated from human fetal brain
- Limited expansion capacity in culture

Required materials

Cells

- Gibco™ Human Astrocytes (Cat. No. N7805100) or Gibco™ Rat Primary Cortical Astrocytes (Cat. No. N7745100)

Media and reagents

- Gibco™ Astrocyte Medium (Cat. No. A1261301: This kit contains N-2 Supplement, 100X, stored at –20°C; Dulbecco's Modified Eagle Medium (D-MEM) (1X), stored at 2°C to 8°C; and Gibco™ One Shot™ Fetal Bovine Serum, Certified, stored at –20°C in the dark.)
- Gibco™ Penicillin-Streptomycin (5,000 U/mL) (Cat. No. 15070063)
- Gibco™ EGF Recombinant Human Protein (Cat. No. PHG0314)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- Gibco™ Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413201)
- Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A1110501), pre-warmed to 37°C
- Gibco™ Trypan Blue Solution, 0.4% (Cat. No. 15250061) (included with the Invitrogen™ Countess™ II Automated Cell Counter) or Invitrogen™ LIVE/DEAD™ Cell Vitality Assay Kit, C_{12} Resazurin/SYTOX™ Green (Cat. No. L34951)
- Gibco™ B-27™ Plus Neuronal Culture System (Cat. No. A3653401: This kit contains B-27 Plus Supplement (50X), stored at –20 to –5°C in the dark; and Neurobasal Plus Medium, stored at 2–8°C in the dark.)
- Gibco™ poly-D-lysine (Cat. No. A3890401)
- Gibco™ GlutaMAX™ Supplement (Cat. No. 35050061)

Equipment and plasticware

- Invitrogen™ Countess™ II Automated Cell Counter (Cat. No. AMQAX1000) or hemocytometer
- 37°C incubator with humidified atmosphere of 5% CO_2
- Uncoated, tissue-culture treated flasks, plates, or petri dishes
- Disposable, sterile 15 mL or 50 mL conical tubes, pre-rinsed with medium

Preparing reagents and media

Complete astrocyte medium

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 (DMEM), 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 astrocyte medium, mix the following components under aseptic conditions. For larger volumes, increase the component amounts proportionally.

Component	Amount
DMEM	88 mL
N-2 Supplement	1 mL
FBS	10 mL
Penicillin-Streptomycin	1 mL
Optional: EGF (prepared as 100 µg/mL stock)	20 µL

Complete neuronal medium

The B-27 Plus Neuronal Culture System is a next-generation media system that provides the highest rate of *in vitro* survival of primary rodent and human stem cell–derived neurons. Composed of B-27 Plus Supplement (50X) and Neurobasal Plus Medium, this system is an evolution of the most cited neuronal cell culture products, Gibco™ B-27 Supplement and Gibco™ Neurobasal Medium. It features an optimized formulation, upgraded manufacturing process, and more stringent quality control of raw materials and final product. These improvements enable the highest neuronal cell survival, improved electrophysiological activity, and enhanced functional maturity compared to other neuronal cell culture media systems.

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

Component	Amount
Neurobasal Plus Medium	98 mL
B-27 Plus Supplement	2 mL
GlutaMAX Supplement	0.25 mL

Prepare plasticware coated with Geltrex matrix (human astrocytes only)

Before thawing or passaging Gibco Human Astrocytes, prepare culture vessels coated with Geltrex matrix as described below.

Note: Rat astrocytes do not require the use of Geltrex matrix–coated plates.

1. Thaw a bottle of Geltrex Basement Membrane Matrix at 2°C to 8°C overnight.
2. On ice, prepare a stock solution of Geltrex matrix diluted 1:1 in DMEM. Store in aliquots at –20°C until needed.
3. Dilute the stock solution 1:100 in DMEM and coat the bottom of each culture vessel (200 µL of Geltrex matrix per cm^2 of culture vessel).
4. Incubate the culture vessel at 36°C to 38°C for 1 hour.

Dishes coated with Geltrex matrix can be used immediately or stored at 2°C–8°C for up to one week, sealed with Parafilm laboratory film. Do not allow dishes to dry out.

Note: Warm stored Geltrex matrix plates to room temperature for 1 hour prior to adding astrocytes.

Note: When you are ready to add cells, aspirate the Geltrex matrix solution and rinse once with DPBS with calcium and magnesium before adding the cell solution.

Thawing cryopreserved rat or human primary cortical astrocytes

Before thawing or harvesting Gibco Human Astrocytes, prepare culture vessels coated with Geltrex matrix as described previously.

Important: Astrocytes readily stick to plastics. Prewet all plastics with complete astrocyte medium.

Note: We recommend seeding cells at 2 x 10^4 cells/cm^2 for rat astrocytes, or 4 x 10^4 cell/cm^2 for human astrocytes. This is equivalent to 180,000 or 360,000 cells in 2–3 mL in one well of a 6-well plate.

1. Remove a vial of cells from liquid nitrogen storage and immediately thaw by swirling in a 37°C water bath. Remove the vial when the last bit of ice has melted, typically <3 minutes. Do not submerge the vial completely, thaw for longer than 3 minutes, or create bubbles in the cell suspension, as this will decrease cell viability.

2. When thawed, disinfect the outside of the tube with 70% isopropyl alcohol and transfer the tube to a laminar flow hood.

3. Precondition (prewet) a 15 mL centrifuge tube with warm complete astrocyte medium. Discard the medium.

4. Using a prewetted sterile pipette tip, slowly transfer the thawed cells (~1 mL) to the preconditioned centrifuge tube.

5. Add 1 mL of medium to rinse the cryovial. Add this dropwise to the centrifuge tube while swirling.

6. Add 3 mL of additional warm complete astrocyte medium slowly for a total of 5 mL.

7. To remove cryoprotectant (DMSO) from the cells, centrifuge the tube at 250 x *g* for 5 minutes. Remove and discard the supernatant above the cell pellet.

8. Prewet a sterile pipette and suspend the cells in 2–3 mL of warm complete astrocyte medium.

9. Determine the viable cell count using your method of choice (e.g., Countess II Automated Cell Counter) to seed at the correct density.

   Note: If recovery seems poor, count the cells before and after centrifugation with the next vial to determine if cells are lost due to centrifugation.

10. Adjust the cell density with warm complete astrocyte medium for correct plating density.

11. For human astrocytes, remove a Geltrex matrix–coated plate from 2°C to 8°C storage and warm to room temperature for 1 hour. Remove the medium by tipping slightly to aspirate the Geltrex matrix solution.

    Note: Do not allow the plate surface to dry out before plating the cells. (Rat astrocytes do not require Geltrex matrix–coated plates.)

12. Immediately plate the cells at 2 x 10^4 cells/cm^2 for rat astrocytes, or 4 x 10^4 cells/cm^2 for human astrocytes. This is equivalent to 180,000 or 360,000 cells in 2–3 mL in one well of a 6-well plate.

13. Incubate the cells at 36°C–38°C in a humidified atmosphere (90%) of 4–6% CO_2 in air. Allow the cells to adhere for at least 24 hours.

    Note: Change the medium every 2 days.

Expanding rat primary cortical astrocytes

1. Remove the spent growth medium from the culture dish containing the cells, and store in a sterile tube to use as a washing solution.

2. Rinse the surface of the cell layer once with DPBS without Ca^{2+} and Mg^{2+} (approximately 2 mL DPBS per 10 cm^2 culture surface area) by adding the DPBS to the side of the vessel opposite the attached cell layer, and rocking back and forth several times.

3. Aspirate the DPBS and discard.

4. To detach the cells, add 3 mL of pre-warmed StemPro Accutase Cell Dissociation Reagent per T-75 flask; adjust volume accordingly for culture dishes of other sizes.

5. Incubate for up to 30 minutes at 37°C. Rock the cells every 5 minutes, and check for cell detachment and dissociation toward single cells under the microscope.

6. Once you observe cell detachment, gently pipette up and down to break clumps into a single-cell suspension. Stop the cell dissociation reaction by an adding equal volume of the spent medium from step 1. Disperse the medium by pipetting over the cell layer surface several times.

7. Transfer the cells to a new 15 mL or 50 mL pre-rinsed conical tube, and centrifuge at 250 x *g* for 5 minutes at room temperature. Aspirate and discard the supernatant.

8. Gently resuspend the cell pellet in pre-warmed complete astrocyte medium and remove a sample for counting.

9. Determine the total number of cells and percent viability using your method of choice. If necessary, add warm complete astrocyte medium to the cells to achieve the desired cell concentration and recount the cells.

10. Plate cells in an uncoated tissue-culture treated flask, plate, or Petri dish at a seeding density of 2×10^4 cells/cm^2.

11. Incubate cells at 37°C, 5% CO_2, and 90% humidity, and change growth medium every 4–5 days.

12. Astrocytes are ready for experiments 2–3 weeks after culturing.

Expanding human primary cortical astrocytes

For replating human astrocytes, prepare culture vessels coated with Geltrex matrix as described above.

Equilibrate stored plates to room temperature for 1 hour prior to use.

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 DPBS without calcium, magnesium, or phenol red.

4. Aspirate DPBS and add StemPro Accutase reagent to the cells, following the StemPro Accutase reagent instructions.

5. Incubate for 5–10 minutes at 36°C–38°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 StemPro Accutase reagent activity.

7. Transfer the cells to a 15 or 50 mL tube.

8. Rinse culture vessels with 1 mL of complete astrocyte medium and add it to the tube.

9. Centrifuge the tube for 5 minutes at 250 x *g*.

10. Aspirate and discard the supernatant.

11. With a prewetted pipette, suspend the pellet in 2–3 mL warm complete astrocyte medium.

12. Count the live cells using a method of choice.

13. To replate human astrocytes, remove a Geltrex matrix–coated plate from 2°C to 8°C storage and warm to room temperature for 1 hour. Tip slightly to aspirate the Geltrex matrix solution.

    Note: Do not allow the plate to dry out.

    Note: Rat astrocytes do not require Geltrex matrix–coated plates.

14. Dilute the astrocytes to the desired concentration in complete astrocyte medium. We recommend 4×10^4 cells/cm^2 (360,000 cells in 2–3 mL in one well of a 6-well plate).

15. Immediately seed the cells on Geltrex-coated plates and incubate at 37°C, 5% CO_2 in air, and 90% humidity.

16. Change the medium every 2 days with fresh complete astrocyte medium.

Plating primary cortical astrocytes for support of neurons

Astrocytes are often grown as feeders to provide metabolic, trophic, or synaptic support for neurons. Such co-cultures are typically treated with anti-mitotic drugs to prevent overgrowth of the glia. Although cytosine arabinoside (AraC) is a commonly used anti-mitotic, it can be toxic to neurons (Wallace and Johnson, 1989). Fluorodeoxyuridine (FUdR) is less toxic, but it is still important to choose the lowest dose that is effective in arresting proliferation. This is often in the range of 10–50 µM. Uridine (50 µM) is typically given as well to allow RNA synthesis. Note that feeders should be plated and allowed to attach for at least 24 hours prior to plating neurons.

1. Prepare dishes coated with 100 µg/mL poly-D-lysine.

2. Passage rat or human primary astrocytes as described above.

3. Seed astrocytes in complete astrocyte medium at high density, roughly 4×10^4 cells/cm^2. The correct seeding density must be determined empirically for the chosen substrate and the requirements of the assay.

4. Twenty-four hours later, wash the cells twice with the unsupplemented Neurobasal Plus Medium component from the B-27 Plus Neuronal Culture System, and then replace with complete neuronal medium.

5. Seed neurons in complete Neurobasal Plus Medium at the desired density for downstream applications.

6. Two to three days after seeding neurons, change half of the medium with complete Neurobasal Plus Medium containing 10–50 µM FUdR and 50 µM uridine.

7. After 3–4 days, perform a half-volume change with fresh Neurobasal Plus Medium without FUdR or uridine. Continue feeding cells with half-volume changes 2–3 times per week.

References

Lepore AC, Rauck B, Dejea C, et al. (2008) Focal transplantation-based astrocyte replacement is neuroprotective in a model of motor neuron disease. *Nat Neurosci* 11:1294–1301.

Maragakis NJ and Rothstein JD (2006) Mechanisms of disease: Astrocytes in neurodegenerative disease. *Nat Clin Pract Neurol* 2:679–689.

Martin DP, Wallace TL, and Johnson EM Jr. (1990) Cytosine arabinoside kills postmitotic neurons in a fashion resembling trophic factor deprivation: evidence that a deoxycytidine-dependent process may be required for nerve growth factor signal transduction. *J Neurosci* 10:184–93.

Pekny M and Nilsson M (2005) Astrocyte activation and reactive gliosis. *Glia* 50:427–434.

Rothstein JD, Martin L, Levey AI, et al. (1994) Localization of neuronal and glial glutamate transporters. *Neuron* 13:713–725.

Rothstein JD, Dykes-Hoberg M, Pardo CA, et al. (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 16:675–686.

Schutte RJ, Xie Y, Ng NN, et al. (2018) Astrocyte-enriched feeder layers from cryopreserved cells support differentiation of spontaneously active networks of human iPSC-derived neurons. *J Neurosci Methods* 294:91–101.

Takano T, Oberheim N, Cotrina ML, and Nedergaard M (2009) Astrocytes and ischemic injury. *Stroke* 40:S8–S12.

Wallace TL and Johnson EM Jr. (1989) Cytosine arabinoside kills postmitotic neurons: evidence that deoxycytidine may have a role in neuronal survival that is independent of DNA synthesis. *J Neurosci* 9:115–24.

Xu L, Sapolsky RM, and Giffard RG (2001) Differential sensitivity of murine astrocytes and neurons from different brain regions to injury. *Exp Neurol* 169:416–424.

Zhao G and Flavin MP (2000) Differential sensitivity of rat hippocampal and cortical astrocytes to oxygen-glucose deprivation injury. *Neurosci Lett* 285:177–180.

14 Cryopreserving neural stem cells

Summary

There are numerous protocols available for cryopreserving neural stem cells (NSCs) derived from human embryonic stem cells. The primary objectives 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 optimizes survival of NSCs post-thaw, while maintaining sublineage differentiation capacity of the preserved cells.

View this protocol online and order products at **thermofisher.com/neuroprotocol/cryo**

Required materials

Cells

- Neural stem cells (NSCs)

Media and reagents

- Gibco™ StemPro™ NSC SFM (Cat. No. A1050901: This kit contains KnockOut™ DMEM/F-12 Basal Medium stored at 4°C; StemPro™ NSC SFM Supplement stored at –20°C to –5°C in the dark; and bFGF Recombinant Human and EGF Recombinant Human proteins stored at 4°C, desiccated.)
- Gibco™ GlutaMAX™ Supplement (Cat. No. 35050061)
- *Optional:* Gibco™ Antibiotic-Antimycotic (100X) (Cat. No. 15240062)
- Gibco™ TrypLE™ Select Enzyme (1X), no phenol red (Cat. No. 12563011)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- 100% isopropyl alcohol
- Gibco™ Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413301)

Cryopreservation and recovery solution options:

- Gibco™ PSC Cryopreservation Kit (Cat. No. A2644601): Includes PSC Cryomedium and RevitaCell™ Supplement (100X), or Gibco™ CTS™ Synth-a-Freeze™ Medium (Cat. No. A1371301)

Tools and equipment

- Sterile 15 mL conical tubes
- Tabletop centrifuge
- Thermo Scientific™ Nalgene™ General Long-Term Storage Cryogenic Tubes (Cat. No. 5000-1020)
- Thermo Scientific™ Mr. Frosty™ Freezing Container (Cat. No. 5100-0001)
- 37°C water bath

Preparing media

Complete StemPro NSC SFM

Complete StemPro NSC SFM consists of KnockOut DMEM/F-12 with StemPro NSC SFM Supplement, EGF, and bFGF, combined with GlutaMAX Supplement. Complete medium is stable for 4 weeks when stored in the dark at 2°C to 8°C.

To prepare 50 mL of complete StemPro NSC SFM, aseptically mix the following components. For larger volumes, increase the component amounts proportionally. If desired, add 0.5 mL of Antibiotic-Antimycotic (100X) solution per 50 mL of complete medium.

Component	Final conc.	Amount
KnockOut DMEM/F-12	1X	48.5 mL
GlutaMAX Supplement	2 mM	0.5 mL
bFGF	20 ng/mL	1 µg
EGF	20 ng/mL	1 µg
StemPro NSC SFM Supplement*	2%	1 mL

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

Freezing and recovery media

Xeno-free PSC Cryomedium (a component in Cat. No. A26444601) and animal origin–free CTS Synth-a-Freeze Medium (Cat. No. A1371301) have shown utility for cryopreservation of NSCs. Once thawed, these cryomedia are stable for up to 6 months when stored at 2°C to 8°C protected from light.

RevitaCell Supplement is provided as a 100X solution for addition to growth medium (complete StemPro NSC SFM) for the first 18–24 hours post-thaw to assist in minimizing apoptosis and necrosis. RevitaCell Supplement is a chemically defined recovery supplement containing a specific ROCK inhibitor coupled with molecules that have antioxidant and free radical scavenger properties. Upon thaw, RevitaCell Supplement should be efficiently mixed by gentle inversion and dispensed into usage-size aliquots. Aliquots should be stored frozen at –20°C to –5°C protected from light. Avoid long-term (>5 days) storage at 2°C to 8°C.

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 1 x 10^6 to 2.4 x 10^6 viable cells/mL and a volume of 1 mL/vial.
- Xeno-free PSC Cryomedium or CTS Synth-a-Freeze Medium can be used for cryopreservation of NSCs. Prechill cryomedium prior to use in cryopreservation procedures to minimize toxicity of DMSO within these formulations.
- Do not incubate the NSCs in TrypLE Select Enzyme for more than 5 minutes to avoid cell death.
- Pre-label all cryovials and Mr. Frosty Freezing Containers (filled with 250 mL of 100% isopropyl alcohol and prechilled to 4°C) prior to addition of cells.

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 once with DPBS, no calcium, no magnesium (DPBS –/–), according to the volumes shown in Table 14-1 on the next page. Aspirate the DPBS and discard.

3. Add room temperature TrypLE Select Enzyme, according to the volumes shown in Table 14-1 on the next page, to the culture vessel and incubate at 37°C for 2–5 minutes.

   Note: Do not incubate the NSCs in TrypLE Select Enzyme for more than 5 minutes to avoid cell death. Neutralize TrypLE Select Enzyme by adding complete StemPro NSC SFM immediately after the incubation period (according to the instructions 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. Quickly transfer the cell suspension to a 15 mL conical tube containing the appropriate volume of complete StemPro NSC SFM (see Table 14-1 on the next page) to neutralize the TrypLE Select Enzyme.

6. Mix the cell suspension by gentle inversion 3 times and remove a small aliquot for assessment of cell count and viability using the Invitrogen™ Countess™ II Automated Cell Counter or traditional hemocytometer.

7. Centrifuge the NSCs at 200 x *g* for 5 minutes.

8. Gently aspirate the medium, being careful to avoid the cell pellet, and add prechilled (4°C) freezing medium dropwise to the cells while moving the conical tube back and forth; gently resuspend the cells to a final concentration of 1 x 10^6 to 2.4 x 10^6 viable cells/mL.

9. Transfer 1 mL of the NSC suspension in freezing medium into each pre-labeled, prechilled (4°C) cryovial.

10. Transfer the cryovials to the Mr. Frosty Freezing Container and place the container into a –80°C freezer. This procedure ensures that the cells freeze slowly at approximately –1°C/minute.

11. The next day, transfer the cells into liquid nitrogen. Note that it is important to avoid longer storage at –80°C.

Table 14-1. Reagent volumes (per well or per dish).

Culture vessel (surface area)	DPBS –/– for wash	TrypLE Select for dissociation	StemPro NSC Medium for neutralization
6-well (10 cm^2)	2 mL	1 mL	3 mL
12-well (4 cm^2)	1 mL	0.4 mL	1.2 mL
24-well (2 cm^2)	0.5 mL	0.2 mL	0.6 mL
35 mm (10 cm^2)	2 mL	1 mL	3 mL
60 mm (20 cm^2)	4 mL	2 mL	6 mL
100 mm (60 cm^2)	12 mL	6 mL	18 mL

Guidelines for recovery of neural stem cells

- Minimize the duration of exposure of NSCs to cryomedium at 37°C by thawing until only a small ice crystal remains. Avoid longer incubation at 37°C in cryomedium solutions.
- Ensure dropwise addition of growth medium (complete StemPro NSC SFM) to the NSCs in cryomedium to avoid osmotic shock.
- When using RevitaCell Supplement, do not include additional ROCK inhibitors such as Y-27632 or Thiazovivin to the growth medium for recovery.
- Within 18–24 hours post-thaw, aspirate growth medium supplemented with RevitaCell Supplement and replenish with fresh StemPro NSC SFM in the absence of RevitaCell Supplement for the remainder of culture.

Recovery of cryopreserved neural stem cells

1. Coat the culture vessels with the appropriate substrate on which to culture your NSCs. Recommended substrates include Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413301), Gibco™ CTS CELLstart™ Substrate (Cat. No. A1014201), or Gibco™ Laminin Mouse Protein, Natural (Cat. No. 23017015). Aspirate coating matrix immediately prior to seeding of recovered NSCs.

   Note: Do not allow matrices to dry out.

2. Prepare recovery medium by supplementing complete StemPro NSC SFM with RevitaCell Supplement at 1X final concentration (e.g., add 100 µL RevitaCell Supplement to 10 mL of complete StemPro NSC SFM).

3. Quickly thaw NSCs in a 37°C water bath until a small ice crystal remains.

4. Transfer the vial to the laminar flow hood and disinfect it with 70% ethanol. Allow the ethanol to evaporate prior to opening the vial.

5. Gently triturate cells and transfer to a 15 mL conical tube.

6. Add 5 mL of complete StemPro NSC SFM dropwise per 1 mL of cell suspension, while shaking the tube back and forth to avoid osmotic shock.

7. Mix the cell suspension by gentle inversion 3 times.

8. Centrifuge the NSCs at 200 x *g* for 5 minutes. Aspirate the supernatant and discard.

9. Resuspend the cell pellet in recovery medium (e.g., StemPro NSC SFM supplemented with 1X RevitaCell Supplement) and perform cell count using the Countess II Automated Cell Counter or traditional hemocytometer.

10. Aspirate matrix solution from precoated plates and seed NSCs at desired plating concentration for downstream assay in recovery medium (e.g., StemPro NSC SFM supplemented with 1X RevitaCell Supplement). For NSCs an initial seeding density of ~20,000–50,000 viable cells/cm^2 is recommended.

11. 18—24 hours post-seeding, aspirate recovery medium and replace medium with complete StemPro NSC SFM. Refresh medium every other day thereafter.

Expected results

H9 ESC-derived NSCs cultured in complete StemPro NSC SFM were cryopreserved according to the instructions provided above and evaluated for viability. While percentage viability direct post-thaw is commonly used as a metric to assess the performance of a cryomedium solution (Figure 14-1), following cryopreservation and recovery of NSCs, additional cell death is not apparent immediately post-thaw. Additional loss of cell viability occurs over the first 24 hours post-thaw due to the processes of apoptosis and necrosis (Baust et al., 2000 and 2001) and is a direct reflection of the stress on the NSCs during the cryopreservation and recovery processes. To assess the impact of these processes, NSCs were examined under a phase-contrast microscope and assayed for viability (Figure 14-2). The cryomedium is shown to provide >70% viability direct post-thaw of cryopreserved NSCs, and RevitaCell Supplement was shown to significantly improve post-thaw recovery of NSCs cryopreserved in CTS Synth-a-Freeze Medium or PSC Cryomedium.

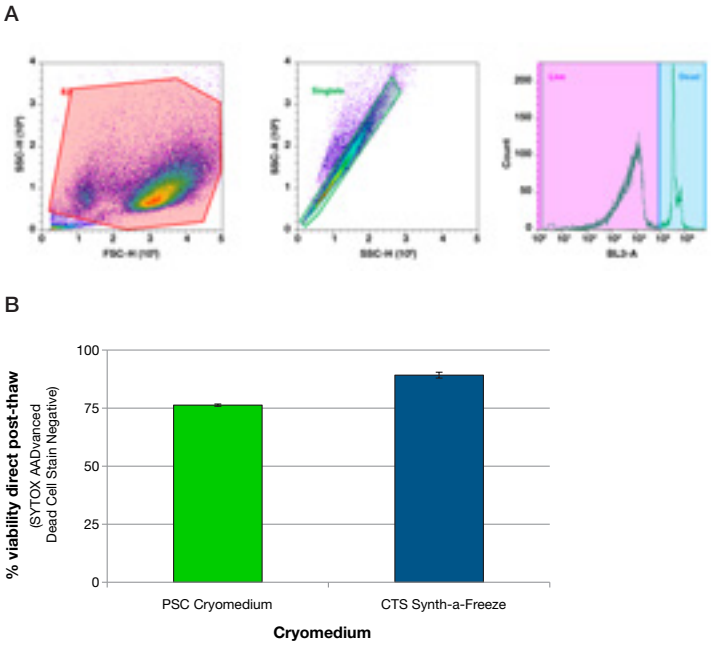
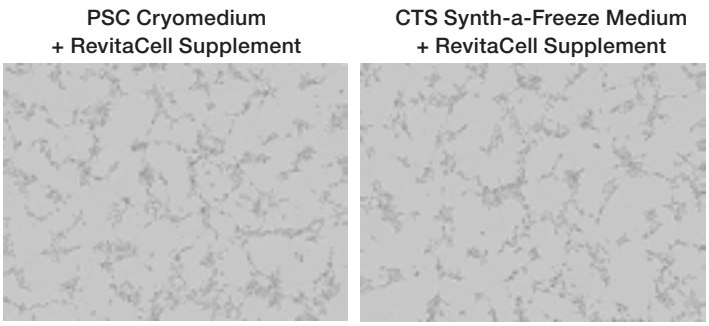


Figure 14-1. Direct post-thaw viability assessment of cryopreserved H9 ESC-derived NSCs. Post-thaw viability was assessed by Invitrogen™ SYTOX™ AADvanced™ Dead Cell Stain Kit (Cat. No. S10349) using the Invitrogen™ Attune™ NxT Flow Cytometer. Experiments for recommended cryomedia included **(A)** a gating strategy for cryopreservation and **(B)** a direct post-thaw viability percentage. The cryomedium is shown to provide >70% viability direct post-thaw of cryopreserved NSCs.

A



B

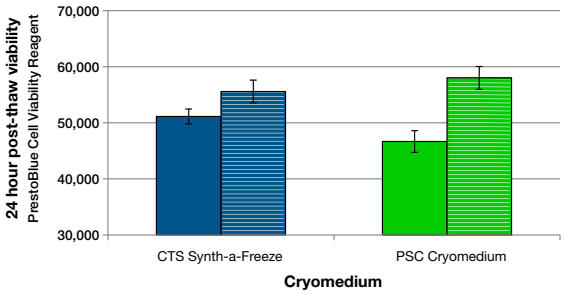


Figure 14-2. 24-hour post-thaw viability assessment of cryopreserved H9 ESC-derived NSCs. To assess 24-hour post-thaw recovery, NSCs plated on Geltrex matrix–coated plates were examined via **(A)** phase-contrast imaging and **(B)** Invitrogen™ PrestoBlue™ Cell Viability Reagent (Cat. No. A13261). RevitaCell Supplement was shown to significantly improve post-thaw recovery of NSCs cryopreserved in CTS Synth-a-Freeze Medium or PSC Cryomedium; solid bars indicate recovery in medium alone, while hatched bars indicate recovery in medium plus RevitaCell Supplement.

Troubleshooting

For troubleshooting tips regarding cryopreservation and recovery of NSCs, see below.

Problem	Possible cause	Solution
Low cell survival rate	NSCs stressed during cryopreservation	• Ensure that NSCs are not overly confluent prior to cryopreservation; target 80–90% confluency at time of harvest. • Ensure that TrypLE Select Enzyme is not left on the NSCs for >5 minutes. • Ensure Mr. Frosty Freezing Container contains the appropriate amount of 100% isopropyl alcohol. Be certain to refresh the container with fresh isopropyl alcohol every 5 freeze cycles to ensure proper cooling rate of about –1°C/minute. • Ensure that Mr. Frosty Freezing Container, cryovials, and cryomedium solutions are prechilled to 4°C prior to use. • Ensure dropwise addition of prechilled cryomedia to the NSCs to avoid osmotic shock. • Limit the number of samples you are cryopreserving to minimize the toxicity of DMSO on your NSCs. Placing vials on ice can assist in minimizing damage during the vialing process. • Do not disturb the Mr. Frosty Freezing Container for up to 4 hours post-placement in the –80°C freezer.
	NSCs stressed during recovery from cryopreservation	• Ensure that cells are not left at 37°C for extended periods of time. Be certain to thaw NSCs until only a small ice crystal remains. • Ensure dropwise addition of growth medium to the NSCs to avoid osmotic shock to the cells. • Limit the number of vials thawed at one time, as longer exposure of cells to cryopreservation media can have a negative impact on post-thaw viability. • Ensure addition of RevitaCell Supplement to recovery medium for the first 18–24 hours post-thaw to maximize cell survival.
Non-reproducible cryopreservation and recovery	Inconsistent cell confluency	• Always harvest cells at comparable confluency.
	Inefficient mixing of cryomedium or RevitaCell Supplement	• Upon thaw, ensure efficient mixing of PSC Cryomedium, CTS Synth-a-Freeze Medium, and RevitaCell Supplement by gentle inversion.

References

Baust JM, VanBuskirk RG, Baust JG, et al. (2000) Cell viability improves following inhibition of cryopreservation-induced apoptosis. *In Vitro Cell Dev Biol Anim* 36(4):262–270.

Baust JM, Vogel MJ, VanBuskirk RG, et al. (2001) A molecular basis of cryopreservation failure and its modulation to improve cell survival. *Cell Transplantation* 10:561–571.

Cell analysis

15 Cell viability assays for neurons and neural cells

Summary

The Invitrogen™ LIVE/DEAD™ Viability/Cytotoxicity Kit provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead neurons and 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 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 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.

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Required materials

Cells

- Adherent or suspended NSCs or neurons

Media and reagents

- LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells (Cat. No. L3224)
  - Calcein AM
  - Ethidium homodimer-1 (EthD-1)
- Gibco™ DPBS, calcium, magnesium (Cat. No. 14040141)

Special tools

- Fluorescence microscope
- Invitrogen™ Attune™ NxT Flow Cytometer (if using flow cytometry)

Note: Calcein AM and EthD-1 can be viewed simultaneously with a conventional fluorescein longpass filter. The fluorescence from these dyes may also be observed separately; calcein AM 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 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 DPBS, calcium, magnesium (DPBS +/+). Vortex to ensure thorough mixing. This prepares a ~4 µM 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 DPBS +/+. 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 Kit.

   The resulting working solution of ~2 µM calcein AM and ~4 µM EthD-1 is ready to be used. The final concentration of DMSO is ≤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 neurons or NSCs may be cultured on sterile glass coverslips or in a multiwell plate. A protocol for a 96-well plate is described below.

1. Aspirate the spent medium from the wells and rinse the cells gently with 200 µL of DPBS +/+ 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. Add 200 µL of working solution of LIVE/DEAD reagent.

3. Incubate for 30 minutes at 37°C.

4. Rinse cells with 200 µL of DPBS +/+ prior to analyzing cells either by fluorescence microscopy or by plate reader.

Determining viability of cells in suspension with flow cytometry using the Attune NxT Flow Cytometer

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 x 10^6 to 5 x 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 mL 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 AM (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 15-1).

Typical results

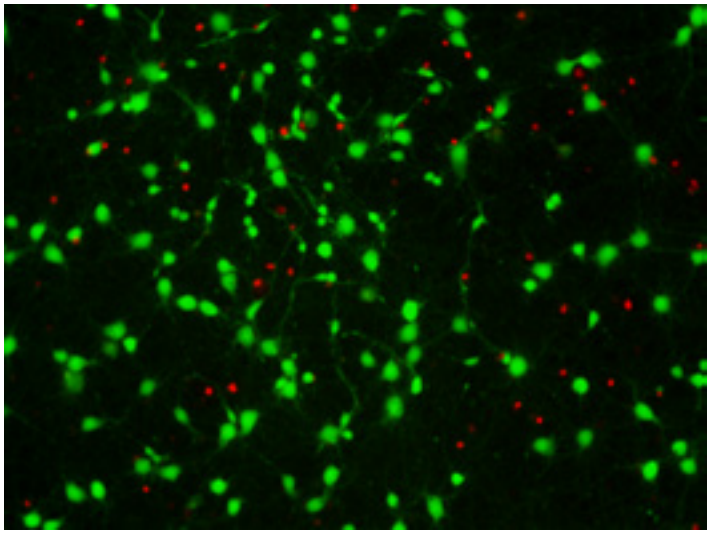

Figure 15-1. Primary rat hippocampal neurons showing live (green) and dead (red) cells using the LIVE/DEAD Viability/Cytotoxicity Kit.

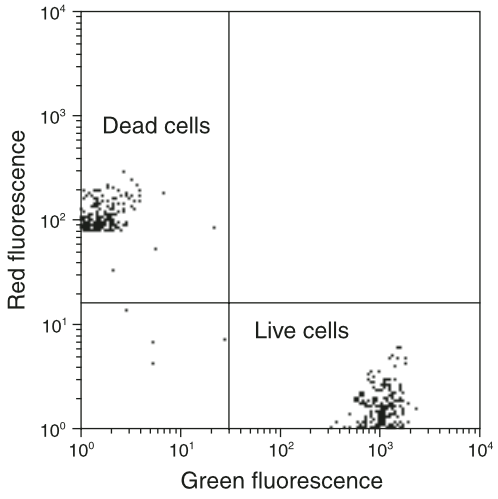

Figure 15-2. Flow cytometry viability assay using the LIVE/DEAD Viability/Cytotoxicity Kit. A 1:1 mixture of live and ethanol-fixed human B cells was stained with calcein AM and EthD-1 following the protocol provided. Flow cytometry analysis was performed with excitation at 488 nm. The resulting bivariate frequency distribution shows the clear separation of the green fluorescent (530 nm) live cell population from the red fluorescent (585 nm) dead cell population.

16 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.

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Cell type–specific antibodies for characterizing neural subtypes

Cell type	Antigen	Antibody Cat. No.
Neural stem cells	Sox1	PA5-23351
	Sox2	PA1-094, PA5-85144
	Nestin	MA1-110
	CD133	PA5-38014
	Pax6	42-6600, MA1-109
Neuronal progenitors Neurons (pan)	MAP2	MA5-12823, 13-1500, MA5-12826
	HuC/D	A21271, A21272
	Neurofilament H, M, L	MA1-2010, 34-1000, 13-0400, 13-1300
	NCAM	701379, MA5-11563, PA5-78402
	βIII tubulin	MA1-118, 480011
	Dcx	48-1200
Dopaminergic neurons	TH	P21962, 701949
	Nurr1	MA1-195
	OTX2	701948, MA5-15854
	LMX1a	710980
Motoneurons	ISL1	MA5-15515, MA5-15516, PA5-27789
	HB9	PA5-23407
GABAergic neurons	GABA	PA5-32241
	GAD2/GAD65	PA5-22260
Glutaminergic neurons	vGLUT1	48-2400
	vGLUT2	MA5-27613
	NMDAR1	32-0500, PA3-102
	NMDAR2B	71-8600, PA5-85362, PA3-104
Cholinergic neurons	ChAT	PA1-4738, PA5-29653

Cell type	Antigen	Antibody Cat. No.
Astrocyte progenitors Astrocytes	CD44	701406, MA5-13887, MA5-13890, PA5-21419
	GFAP	MA5-12023, PA1-10019, PA1-10004, PA5-16291
	S100beta	PA5-78161
	Glutamine synthetase	710963
Oligodendrocyte progenitors Oligodendrocytes	GalC	PA5-42652, PA5-27352
	NG2	37-2300, 37-2700, 41-2000
	A2B5	MA1-90445
	Olig2	MA5-15810
	MBP	PA5-78397, PA1-10008
	Olig1	PA5-21613, MA5-23954, MA5-22956, PA5-80870
Microglia	IBA1	711504, MA5-27726, PA5-27436
	CD11b	14-0112-82, 14-0118-82, CD11b00
	CD68	MA5-13324, 11-0689-42
	HLA-DR	11-9956-42
	CX3CR1	702321, 711353, PA5-19910
	TREM-2	PA5-46980, 702886

17 Flow cytometry analysis

Summary

Flow cytometry is a cell analysis application that allows for single-cell multiparametric quantitative analysis of millions of cells in a short time. This provides a more comprehensive systemic analysis and understanding. Flow cytometry accomplishes this by using lasers of multiple wavelengths to excite fluorescent molecules. These fluorescent molecules can be antibody conjugates, fluorescent cellular dyes, and fluorescent proteins. The laser excites these fluorescent molecules to emit signals of varying wavelengths that are further filtered by wavelength and captured for quantitation. This ultimately allows for a more comprehensive surveying of the expression and density of particular proteins, nucleic acids, and other cell characteristics of a cell population. Flow cytometry allows for flexibility of interrogation, from the simple screening of cells transfected with fluorescent reporter proteins all the way to multiparametric analysis of different cellular traits such as cell health, metabolism, DNA changes, cytokine formation, and other cell phenotypes, in a large heterogeneous cell population of millions of cells.

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Required materials

Cells

- Cells in suspension
- Adherent cells, trypsinized and in suspension buffer
- Dissociated tissue cells

Reagents and equipment

- 0.1% BSA in buffer (e.g., PBS, HBSS) or cell culture medium (staining buffer)
- Antibodies (primary and secondary, as needed based on experiment)
- Cell health dye reagents (e.g., Invitrogen™ LIVE/DEAD™ reagents)
- Flow cytometer (e.g., Invitrogen™ Attune™ NxT Flow Cytometer with acoustic focusing technology)

Titrating antibodies

Determining the optimal concentration of antibody for flow cytometry

1. Dilute labeled antibodies for the appropriate antigens to be detected in staining buffer. Make dilutions of all antibodies: undiluted, and 2-, 5-, 10-, 20-, 40-, 80-, and 100-fold dilutions.
2. Prepare the cells that express the antigen to be analyzed.
3. Count the number of cells.
4. Use 1 x 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 x *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. If desired, cells labeled with an isotype control can be used.

8. Mix well and incubate cells for 25–30 minutes.

9. Wash with 3 mL of staining buffer. Discard the supernatant and resuspend the cells in 0.5 mL of staining buffer.

10. 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.

Note: We recommended that you always use a dead cell stain to identify dead cells in any immunophenotyping experiment, as dead calls may nonspecifically bind antibodies and give false readings.

One-step staining with fluorescent labeled antibodies

1. For adherent cells, trypsinize and then add staining buffer. Transfer the cells to a conical tube and centrifuge at 300 x *g* for 5 minutes. Discard the supernatant.

2. Add the appropriate amount of diluted fluorescent conjugated primary antibodies to the cell pellet.

3. Resuspend the cell pellet by gentle mixing and incubate for 25–30 minutes.

4. Wash the cells with 3 mL staining buffer. Centrifuge the cells at 300 x *g* for 5 minutes.

5. Discard the supernatant and resuspend the cells with 0.5 mL staining buffer.

6. *Optional:* Filter the cell suspension through a fine mesh filter before analysis or sorting the cells by flow cytometry.

Two-step staining with biotinylated antibodies

1. For adherent cells, trypsinize and add staining buffer. Transfer the cells to a conical tube and centrifuge at 300 x *g* for 5 minutes. Discard the supernatant.

2. Add 5 µL of appropriately diluted biotinylated primary antibody.

3. Resuspend the cell pellet by gently mixing and incubate for 25–30 minutes.

4. Wash the cells with 3 mL staining buffer. Centrifuge the cells at 300 x *g* for 5 minutes.

5. Discard the supernatant. Add diluted streptavidin conjugated to a fluorescent tag.

6. Mix well and incubate the cells for 25–30 minutes.

7. Wash the cells with 3 mL staining buffer. Centrifuge the cells at 300 x *g*, 4°C for 5 minutes.

8. Discard the supernatant and resuspend cells with 0.5 mL of staining buffer.

9. Filter the cell suspension through a fine mesh filter before analysis or sorting the cells by flow cytometry.

18 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 subcellular localization of proteins.

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Required materials

Cells

- Gibco™ Primary Rat Cortex Neurons (Cat. No. A1084001) or Gibco™ Primary Rat Hippocampal Neurons (Cat. No. A1084101)

Media and reagents

- Gibco™ DPBS, calcium, magnesium (Cat. No. 14040141)
- Gibco™ Goat Serum, New Zealand Origin (Cat. No. 16210064)
- Primary antibodies to proteins of interest
- Secondary antibodies corresponding to primary antibody source species
- Invitrogen™ 4′,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Cat. No. D1306) or Invitrogen™ NucBlue™ Fixed Cell ReadyProbes™ Reagent (Cat. No. R37606)
- Invitrogen™ ProLong™ Gold Antifade Mountant (Cat. No. P36930)
- Paraformaldehyde (4%) in DBPS
- Triton™ X-100 surfactant

Special tools

- Multi-chambered slides
- Fluorescence microscope

Cell type–specific antibodies for characterizing neural subtypes

Cell type	Antigen	Antibody Cat. No.	Source	Reactivity	Dilution	Notes
Neural stem cells	Sox2	MA1-014	Mouse IgG2a	Hu	1:200	Fig. 18-2
	Nestin	MA1-110	Rabbit	Hu, Rt, Ms	1:100	Fig. 18-4
		PA5-11887	Rabbit	Hu	1:200	
	Pax6	42-6600	Rabbit	Hu, Rt, Ms	1:200	
Neuronal progenitors Neurons (pan)	MAP2	13-1500	Mouse IgG1	Hu, Rt, Ms	1:400	Fig. 18-3
		PA5-17646	Rabbit	Hu, Rt, Ms	1:250	Fig. 18-1
	HuC/D	A21271	Mouse IgG2b	Hu, Rt, Ms	1:200	
	Neurofilament	MA5-14981	Rabbit	Hu, Rt, Ms	1:100	
	Beta III tubulin	480011	Mouse IgG	Hu, Rt, Ms	1:1,000	
	Dcx	48-1200	Rabbit	Hu, Rt, Ms	1:400	Fig. 18-2
Dopaminergic neurons	TH	P21962	Rabbit	Hu	1:1,000	
GABAergic neurons	GABA	PA5-32241	Rabbit		1:5,000	
	GAD2/GAD65	39-8200	Mouse IgG1	Hu, Rt, Ms	1:100	Fig. 18-3
Glutamatergic neurons	vGLUT1	48-2400	Rabbit	Hu, Rt, Ms	1:500	Fig. 18-3
	vGLUT2	42-7800	Rabbit	Hu, Rt, Ms	1:100	
	NMDAR1	32-0500	Mouse IgG2a	Hu, Rt, Ms	1:100	Fig. 18-3
Astrocyte progenitors Astrocytes	CD44	A25528	Mouse IgG	Hu	1:50	
		MA5-13890	Mouse IgG2a	Hu	1:1,000	Fig. 18-4
	GFAP	PA1-10019	Rabbit	Hu, Rt, Ms	1:1,000	
		13-0300	Rat	Hu, Rt, Ms		Fig. 18-1
Oligodendrocyte progenitors Oligodendrocytes	GalC	Millipore MAB345	Mouse IgG	Hu, Rt, Ms	1:200	Fig. 18-2
	A2B5	43-3110	Mouse IgM	Hu, Rt, Ms	1:100	
		MA1-90445	Mouse IgM	Hu, Rt, Ms	1:100	Fig. 18-4
Proliferation	Ki67	14-5698-82	Rat	Hu, Rt, Ms	1:100	

Secondary antibodies

Ex/Em* (color)	Alexa Fluor Dye No.	Host	Reactivity	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	Rabbit IgG	A11034	1:1,000
		Donkey	Goat IgM	A11055	1:1,000
590/617 (red)	594**	Goat	Mouse IgM	A21044	1:1,000
		Goat	Mouse IgG	A11032	1:1,000
		Goat	Rat IgM	SA5-10012**	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.
** The Invitrogen™ Goat Anti-Rat IgM Cross-Adsorbed Secondary Antibody, DyLight™ 594 (Cat. No. SA5-10012) provides a high-intensity, photostable DyLight™ fluorescent replacement for the discontinued Invitrogen™ Alexa Fluor™ 594 goat anti–rat IgM secondary antibody.

Methods

Immunocytochemistry analysis

1. Before proceeding, prepare a solution of 5% goat serum (or other serum appropriate for the secondary antibodies) in DPBS, calcium, magnesium (DPBS +/+). 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 three times.

2. When you are ready to perform the immunocytochemistry procedure, aspirate the culture medium from each chamber and gently rinse the cells twice with DPBS +/+, without dislodging the cells.

3. Treat the cells with 4% paraformaldehyde in PBS for 20 minutes to fix them.

4. Rinse the cells 3 times with DPBS +/+.

5. Proceed to staining, or you may store samples for up to 3–4 weeks in DPBS +/+ at 4°C. Do not allow cells to dry.

6. Permeabilize the cells with 0.3% Triton™ X-100 surfactant (diluted in DPBS +/+) for 5 minutes at room temperature.

   Note: If you are using a surface antigen such as GalC, omit permeabilization step and do not include Triton X-100 surfactant in the blocking buffer.

7. Rinse the cells 3 times with DPBS +/+.

8. Add enough 5% serum solution from step 1 to the cells to coat them, and incubate for 60 minutes at room temperature.

9. Remove the solution from the wells and coat the cells with primary antibody diluted in 5% serum solution.

10. Incubate the coated cells at 2°C to 8°C overnight.

11. Rinse the cells 3 times with DPBS +/+.

12. Treat the cells with a secondary antibody diluted in 5% serum solution.

13. Incubate for 60 minutes at room temperature.

14. Rinse the cells 3 times with DPBS +/+.

15. Stain the cells with a DAPI solution (3 ng/mL) for 10 minutes.

16. Rinse the cells with DPBS +/+, and if desired, mount the cells with ProLong Gold Antifade Mountant. Observe the cells under the microscope using filters that correspond to the secondary antibody excitation/emission spectra.

Typical results

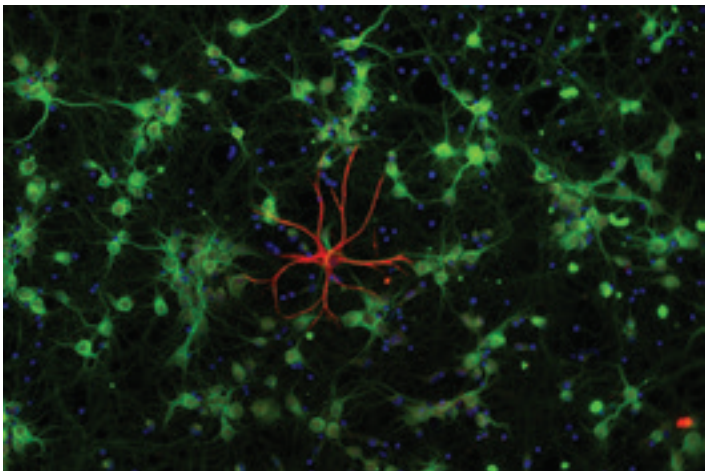


Figure 18-1. Primary rat hippocampal neurons. Immunofluorescence detection of primary neuronal cells stained with mouse anti-MAP2 antibody (green) and astrocytes stained with rabbit anti-GFAP antibody (red). Nuclei are stained with DAPI (blue).

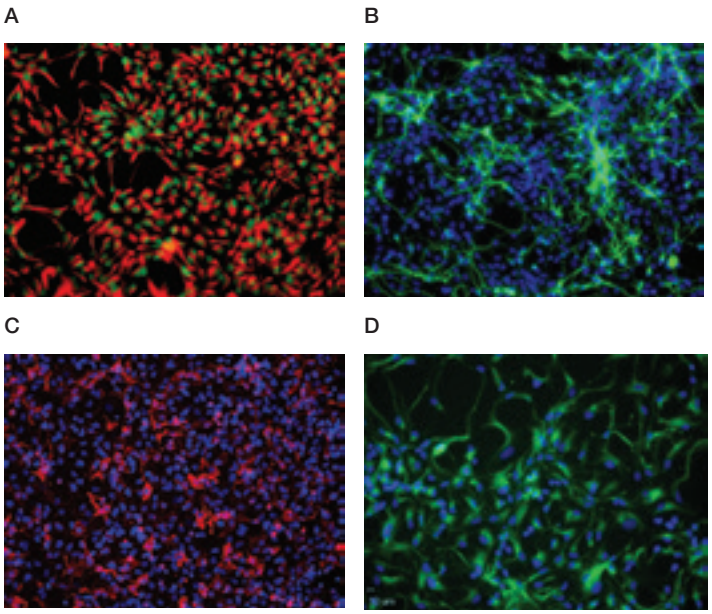


Figure 18-2. Fluorescence images (20x) of Gibco™ hNSCs that have been cultured in complete Gibco™ StemPro™ NSC SFM for three passages, and then allowed to differentiate into neurons, oligodendrocytes, or astrocytes. Upon directed differentiation, cells start to lose the undifferentiated NSC marker, nestin, but stain positive for the differentiated cell–type markers Dcx, GalC, and GFAP. **(A)** Cells were stained for the undifferentiated NSC markers nestin (red) and SOX2 (green) prior to directed differentiation. Cells were then differentiated into neurons and glial cells, and respectively stained **(B)** for the neuronal marker Dcx (green), **(C)** for the oligodendrocyte marker GalC (red), or **(D)** for the astrocyte marker, GFAP (green). Differentiation to **(B)** neurons and to **(C)** oligodendrocytes was observed at day 7, and to **(D)** astrocytes at day 21. The nuclei were counterstained with DAPI (blue) in panels B–D.

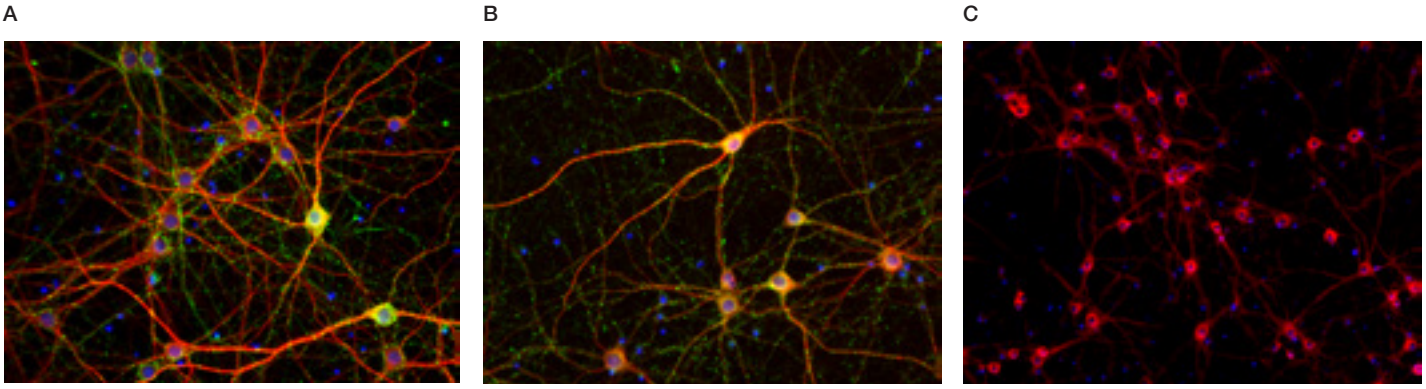


Figure 18-3. Fluorescence images of neurons cultured in Gibco™ Neurobasal™ Plus Medium supplemented with Gibco™ B27™ Plus Supplement (50X). (A) Neurons stained with anti-GAD65 (green) and anti-MAP2 (red), and **(B)** anti-VGLUT1 (green) and anti-MAP2 (red). **(C)** Neurons stained with anti-NMDAR1. Nuclei were counterstained with DAPI in all images.

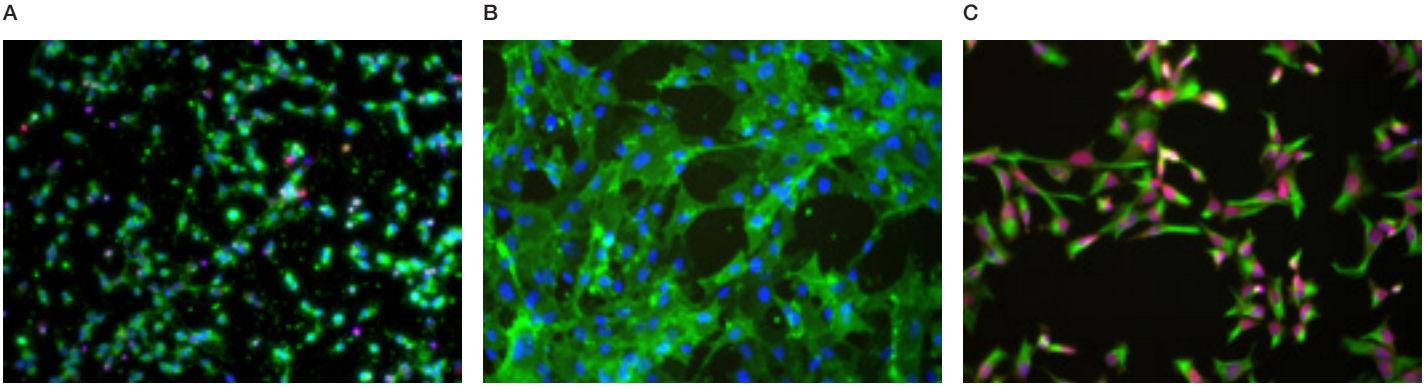


Figure 18-4. Fluorescence images of precursor cells prior to differentiation. (A) Rat glial precursor cells stained with anti-A2B5 (green). **(B)** Human iPSC-derived neural stem cells stained with anti-CD44 (green). **(C)** Human iPSC-derived neural stem cells stained with anti-nestin (green). Nuclei were counterstained with DAPI in all images.

19 Quantitative image analysis

Summary

Cellular imaging technology has long contributed to significant advances in cell biology research. In fact, the ability to visualize individual cells was a founding moment in the field of cell biology. As technology has continued to develop, cellular imaging progressed from a useful qualitative method to a powerful and essential quantitative analysis tool for cell-based assays. Two technological developments that have synergistically assisted the emergence of quantitative image analysis are automated fluorescence microscopes and powerful image analysis software. The automation of microscopes has enabled accurate and consistent exposure times and precise image acquisition, which are two critical factors for intensity-based comparisons of cellular markers. The development of user-friendly image analysis software with an array of dynamic image analysis algorithms and informatics tools enables scientists to make hundreds of parametric measurements and are adaptable to a broad range of cell types and applications. Quantitative image analysis is now an accurate and efficient method for cellular analysis.

This convergence of technological advancements has resulted in a new area of quantitative image analysis called high-content analysis (HCA) or high-content screening (HCS). HCA can be described as a set of analytical methods using automated microscopy, multi-parameter image processing, and visualization tools, to extract quantitative data from cell populations. The high-throughput and data-rich nature of HCA makes it a valuable method for biological research, cell characterization, and drug discovery studies. This chapter will present examples of implementing HCA for cell type characterization and measurement of cell type purity along a neuronal differentiation workflow. Examples of HCA data visualization will also be illustrated.

Thermo Scientific™ HCS Studio™ Cell Analysis Software

The Target Activation BioApplication is a general-purpose assay for intensity-based measurements of molecular localization with broad applicability across multiple disciplines. This assay calculates measurements of fluorescent indicators of choice on a cell-by-cell basis in up to six channels, where a channel represents a fluorophore or a specific exposure condition. An object mask is generated for channel 1 primary objects (e.g., nuclei, cell bodies, organelles), and is used to define the measurement area for fluorescent intensity in downstream channels. The software allows you to visualize object-intensity histograms for each image and enables you to set object intensity–based thresholds for each channel. Population and subpopulation analysis features allow you to quantify the percentage of positively stained cells for each channel as well as subpopulations of co-stained cells.

The Neuronal Profiling BioApplication enables quantification of morphological changes in neurons, allowing control over selecting neurites based on morphological as well as intensity differences. Selection of neurons is possible through the use of nucleus, cell body, and neurite object identification parameters and identifying subpopulations of neurons through quantification of multiple biological characteristics.

Implementation of HCA for measuring neuronal differentiation efficiency

The Gibco™ PSC Dopaminergic Neuron Differentiation Kit (Cat. No. A3147701) enables the differentiation of pluripotent stem cells (PSCs) to midbrain dopaminergic neurons. The workflow for this kit is a three-step process (Figure 19-1). hPSCs are first induced in Floor Plate Specification Medium into midbrain-specified floor plate progenitor (FP) cells. Next, FP cells are expanded as adherent cultures in Floor Plate Cell Expansion Medium and then cultured in suspension to form spheres. Finally, the spheres are differentiated into mature dopaminergic neurons in Dopaminergic Neuron Maturation Medium.

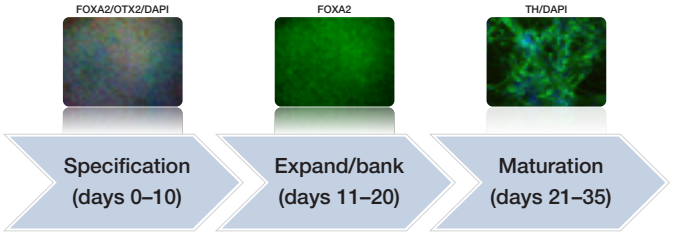


Figure 19-1. Simplified diagram of workflow from midbrain-specified FP cells through expansion/banking to differentiated, mature dopaminergic neurons.

To assess the efficiency of differentiation at key points along this protocol, the Gibco™ Human Dopaminergic Neuron Immunocytochemistry Kit (Cat. No. A29515) was used for optimal image-based analysis of three key markers of neural differentiation: OTX2 and FOXA2 for characterization and quantification of FP progenitor cells (Figure 19-2), and tyrosine hydroxylase (TH) for identification of matured dopaminergic neurons (Figure 19-3).

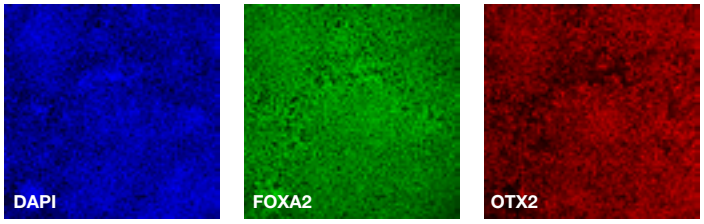


Figure 19-2. FP progenitor cell characterization.

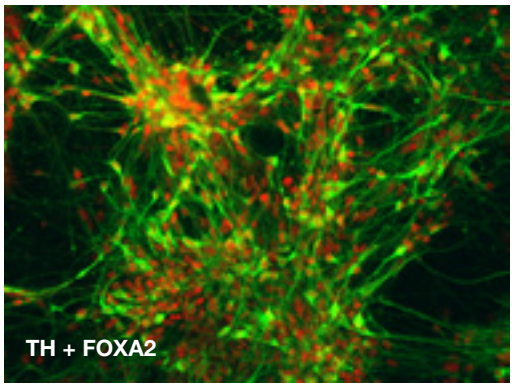


Figure 19-3. Midbrain dopaminergic neurons.

The image acquisition and analysis presented in this chapter was performed using the Thermo Scientific™ CellInsight™ CX5 High-Content Screening (HCS) Platform (**thermofisher.com/hcs**). The CellInsight CX5 platform includes the HCS Studio Cell Analysis Software, which contains over 20 pre-established and highly customizable assays.

Quantification of percentage of FP cells using HCA

The efficiency of the specification step can be measured by quantifying the percentage of FP progenitor cells, using the Target Activation image analysis assay. For this example, the objective of the Target Activation assay is to measure the percentage of cells that are expressing both FOXA2 and OTX2. Channel 1 DAPI-stained nuclei are used to generate a primary object mask (Figure 19-4). Channels 2 and 3 are, respectively, the FOXA2 and OTX2 stains where intensity measurements will be performed. The primary object mask is duplicated for channels 2 and 3, where average intensity measurements are made for each object. Intensity-based thresholds are then set for each of channels 2 and 3. Once the thresholds are set, the software can calculate the percentage of co-stained cells. For the image set in Figure 19-4, the Target Activation assay calculated that 92.3% of the cells are co-stained for FOXA2 and OTX2. Figure 19-5 illustrates the data visualization features of the HCS Studio software. Figure 19-5, panel A, demonstrates setting thresholds for each individual channel. Figure 19-5, panel B, demonstrates the data visualization of the co-stained subpopulation of cells.

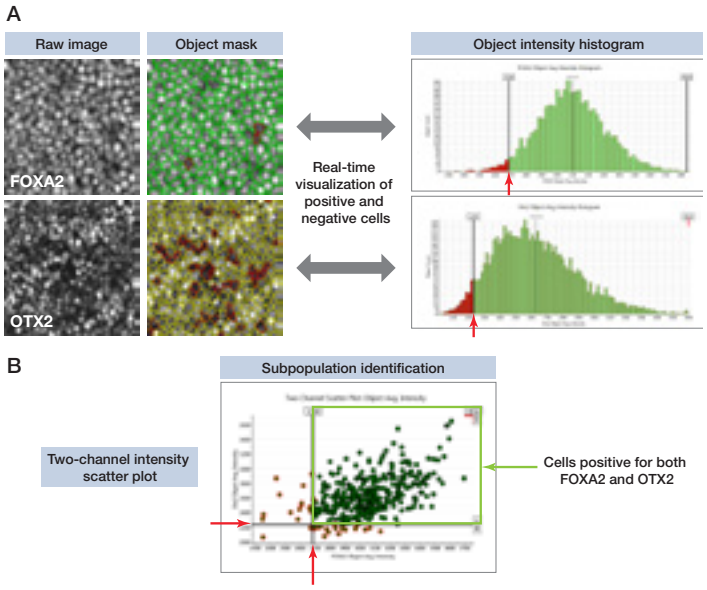


Figure 19-5. Object intensity threshold setting and subpopulation data visualization.

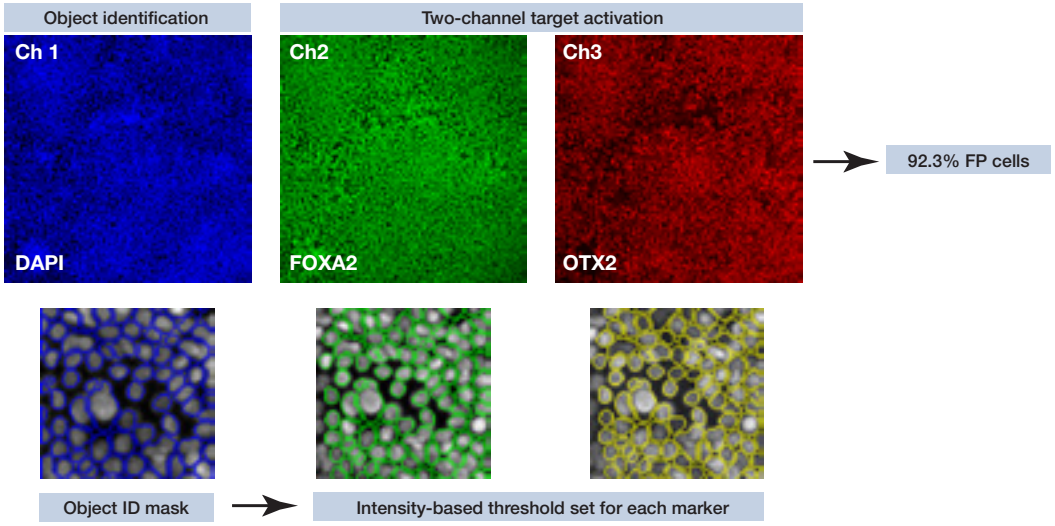


Figure 19-4. Quantification of percentage of FP cells.

Quantification of percentage of dopaminergic neurons using HCA

Upon completion of the maturation step, the percentage of dopaminergic neurons can be quantified using the Neuronal Profiling assay (Figure 19-6). In channel 1, a mask is generated to identify and count DAPI-stained nuclei. For channel 2, a mask is generated to identify and count TH-positive cell bodies. The software can then calculate the percentage of TH-positive cells for each image set. For the image set in Figure 19-6, the neuronal profiling assay calculated 31.3% dopaminergic neurons.

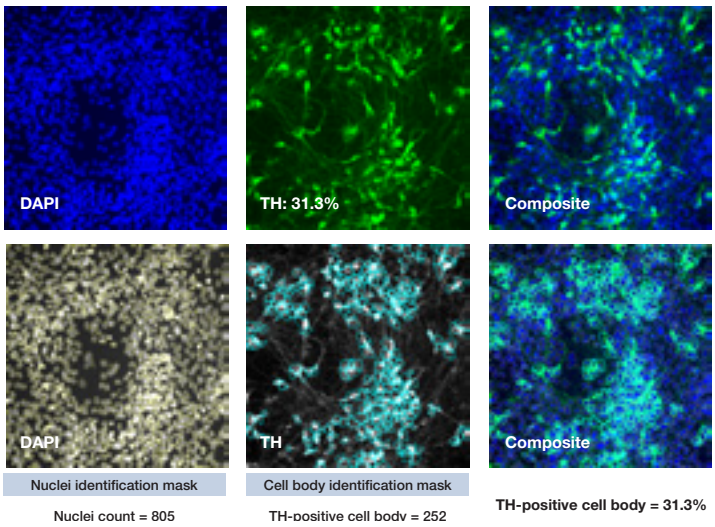


Figure 19-6. Quantification of dopaminergic neurons using the Neuronal Profiling assay.

20 Intracellular calcium assay using fluo-4

Summary

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

View this protocol online and order products at **thermofisher.com/neuroprotocol/fluo4**

Required materials

Cells

- Neural stem cells or primary neurons, cultured on poly-D-lysine–coated 96-well plate or other culture vessel

Reagents

- Invitrogen™ Live Cell Imaging Solution (Cat. No. A14291DJ)
- Gibco™ Glucose Solution (Cat. No. A2494001)
- Invitrogen™ Fluo-4 Calcium Imaging Kit (Cat. No. F10489)
  - Fluo-4 AM dye in DMSO
  - PowerLoad™ Concentrate (100X concentrate)
  - Neuro Background Suppressor
  - Probenecid (100X concentrate)
- Neurotransmitters or ligands (e.g., acetylcholine, glutamate)

Tools and equipment

- Fluorescence inverted microscope
- Invitrogen™ EVOS™ FL Imaging System or EVOS FL Auto Imaging System (**thermofisher.com/evos**)
- Invitrogen™ EVOS™ Light Cube, GFP (Cat. No. AMEP4651)

Preparing reagents

Fluo-4 AM loading solution

Fluo-4 AM loading solution consists of fluo-4 dye (Component A), PowerLoad™ Concentrate (Component B), and probenecid (Component D) prepared in Live Cell Imaging Solution (LCIS). An optional background suppressor dye (Neuro Background Suppressor, Component C) is also included in the kit.

1. Prepare 100X probenecid by adding 1 mL of LCIS to the probenecid vial (Component D). Freeze any unused portions.
2. Prepare 10 mL of fluo-4 AM loading solution by adding the following solutions to a 15 mL vial, in order:
   a. Add 10 µL of fluo-4 AM Dye solution (Component A) to 100 µL of PowerLoad solution (Component B). Mix well and add 8.7 mL of LCIS.
   b. Add 100 µL of 100X probenecid (to a final concentration of 1X) and 100 µL of 200 g/L Glucose Solution (to a final glucose concentration of 10 mM).
   c. *Optional:* Add 1 mL of 10X Neuro Background Suppressor (Component C). If the suppressor is not used, add 1 mL of LCIS.

Loading cells with fluo-4 AM loading solution in a 96-well plate

1. Wash the cells with 100 µL of LCIS.
2. Load the cells with 100 µL of fluo-4 AM loading solution per well of a 96-well plate. Adjust the volume as appropriate for other culture vessels.
3. Incubate the cells for 30 minutes, and then at room temperature for 30 minutes. Cells are ready for imaging.

If the Neuro Background Suppressor is **not** used, remove the fluo-4 AM loading solution and replace with LCIS containing 1X probenecid.

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, an 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.

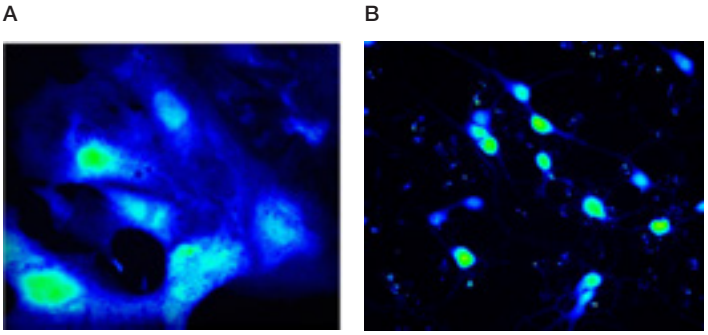


Figure 20-1. Detection of intracellular calcium using fluo-4 AM. **(A)** Calcium waves in beating cardiomyocytes. **(B)** Calcium spikes in firing neurons.

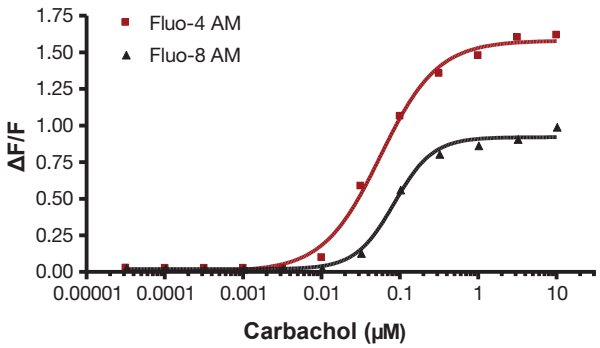


Figure 20-2. Comparison of fluo-4 and fluo-8. Similar EC_{50} values are observed with fluo-4 and fluo-8 AM dyes for a dose response of carbachol on M1 CHO-K1 cells, but the ΔF/F (signal to background) is higher for fluo-4 AM.

21 Measuring membrane potential using the FluoVolt kit

Summary

The Invitrogen™ FluoVolt™ membrane potential dye represents the next generation in voltage-sensitive probes and brings together the best characteristics of the fast- and slow-response membrane potential probes: it has a sub-millisecond response time to changes in membrane potential and displays a high magnitude of response. The following protocol describes how to perform FluoVolt dye–based measurements of changes in membrane potential.

View this protocol online and order products at **thermofisher.com/order/catalog/product/F10488**

Required materials

Cells

- Neural stem cells, cultured on poly-D-lysine–coated 96-well plate or other culture vessel

Media and reagents

- Invitrogen™ FluoVolt™ Membrane Potential Kit (Cat. No. F10488)
  - FluoVolt Dye (1,000X concentrate)
  - PowerLoad™ Concentrate (100X)
  - Neuro Background Suppressor (10X concentrate)
- Invitrogen™ Live Cell Imaging Solution (LCIS) (Cat. No. A14291DJ)
- Invitrogen™ EVOS™ FL Imaging System or EVOS™ FL Auto Imaging System (**thermofisher.com/evos**)
- Invitrogen™ Valinomycin (Cat. No. V1644)
- *Optional*: Gibco™ Glucose Solution (Cat. No. A2494001) (for preparing 20 mM glucose stock in LCIS)

Preparing reagents

FluoVolt loading solution

The protocol below provides instructions for performing the membrane potential assay using cells grown in a 35 mm dish with 2 mL of culture medium.

To a 15 mL tube, add the following reagents in the order listed below to prepare fresh FluoVolt loading solution:

Component	Amount
100X PowerLoad Concentrate	100 µL
FluoVolt dye, 1,000X	10 µL
Swirl to mix.	
LCIS or physiological buffer of choice	10 mL
Optional: Glucose Solution (200 g/L)	100 µL
Invert the tube to mix.	

10 mM glucose stock in LCIS

Dilute the stock Glucose Solution (200 g/L) 1:100 into LCIS for a final glucose concentration of 10 mM. Keep this solution clean and free of contaminants to prevent bacterial, fungal, or yeast growth once glucose has been added.

Loading NSCs with FluoVolt membrane potential dye

1. Remove medium from adherent cells and wash cells twice in physiological buffer of choice or LCIS.

2. Add 2 mL of FluoVolt loading solution (page 92) to cells, and incubate cells at room temperature for 15–30 minutes.

3. Remove FluoVolt loading solution, and wash cells twice in physiological buffer of choice or LCIS.

4. Add 2 mL of physiological buffer of choice or LCIS. Cells are now ready for live-cell imaging.

   Optional: To suppress background fluorescence, add 1:10 diluted Neuro Background Suppressor solution.

Image cells loaded with FluoVolt dye

Standard FITC settings should be used to visualize the membrane staining of FluoVolt dye. Short exposures (2 milliseconds or less) are possible with pixel 2 x 2 binning or greater, but will depend strongly on hardware configurations to measure rapid or successive depolarizations. To confirm positive responses from the dye, treat cells with 10 µM valinomycin (a potassium ionophore) for 30 minutes, and then add an equal volume of isotonic potassium chloride (KCl) solution to depolarize the cells.

Note: Isotonic KCl is composed of 140 mM KCl, 2.5 mM NaCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 20 mM HEPES, 20 mM glucose and adjusted to pH 7.4 with NaOH.

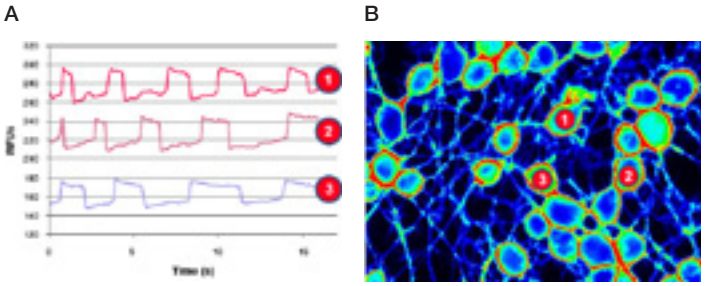


Figure 21-1. Imaging of differentiated NG-108 cells loaded with FluoVolt membrane potential dye. Cells were imaged with 10-millisecond illumination pulses and images acquired with 2 x 2 binning. The three selected traces **(A)** show fluorogenic responses from the dye as selected cells **(B)** spontaneously depolarize and repolarize in culture.

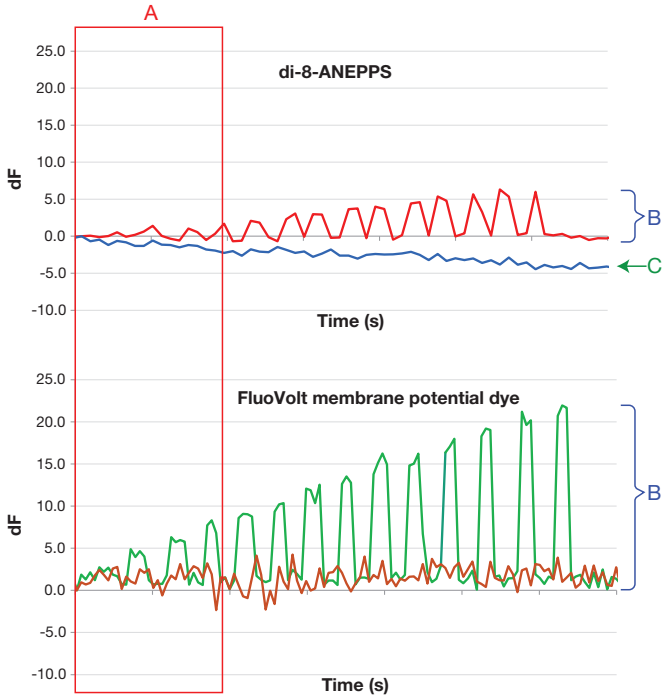


Figure 21-2. FluoVolt membrane potential dye vs. di-8-ANEPPS. (A) Weaker signals, detected earlier by the FluoVolt membrane potential dye. A series of 10 mV steps in voltage clamp from –100 to +30 mV. Changes as small as 10 mV can be detected with the FluoVolt membrane potential dye. **(B)** Greater magnitude of response displayed with the FluoVolt membrane potential dye. **(C)** Unclamped control cell loaded with di-8-ANEPPS shows photobleaching.

Molecular characterization

22 PCR primers for molecular characterization of neural subtypes

Summary

After cells are isolated from tissue or differentiated from pluripotent precursors, the resulting populations should be characterized to confirm the presence of the desired population of cells. Further analysis can help define molecular mechanisms underlying biological differences between different groups of cells (e.g., donors, treatments). 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), mature neurons, and neural subtypes as well as the neuronal supporting cell types, including oligodendrocytes and astrocytes.

Over 1.8 million predesigned Applied Biosystems™ TaqMan® Gene Expression Assays covering more than 30 species are available on our website (**thermofisher.com/taqmangeneexpression**). Alternatively, custom primers and arrays can be searched by disease, pathway, or biological process.

View this protocol online and order products at **thermofisher.com/qpcr**

Target	Gene	Assay ID	Amplicon size (bp)
Neural stem cells	*SOX1*	Hs01057642_s1	96
	SOX2	Hs01053049_s1	91
	NES	Hs04187831_g1	58
Oligodendrocytes	*MAG*	Hs01114387_m1	74
	OSP	Hs00194440_m1	60
Astrocytes	*ALDH1L1*	Hs01003842_m1	55
	GFAP	Hs00909233_m1	57
Neurons	*MAP2*	Hs00258900_m1	98
	CHAT	Hs00758143_m1	65
Endogenous control	*ACTB*	Hs01060665_g1	63
GABAergic	*GAD1*	Hs01065893_m1	100
Serotonergic neurons	*SLC6A4*	Hs00984349_m1	58
Cholinergic neurons	*CHAT*	Hs00758143_m1	65
Dopaminergic neurons	*TH*	Hs00165941_m1	63

23 RNA isolation and cDNA preparation from neural cells

Summary

A rapid method of analysis for determining the identity of neural stem cells (NSCs), mature neurons (and specific subtypes), and glial cells involves the early detection of differentiation and lineage-specific markers tracked at the RNA level. This protocol follows methodologies described in the Invitrogen™ PureLink™ RNA Mini Kit manual for isolating total RNA from neuronal cell types, followed by cDNA synthesis using Invitrogen™ SuperScript™ IV Reverse Transcriptase. The following protocol gives you a step-by-step procedure for template preparation required for RT-PCR or RT-qPCR.

View this protocol online and order products at **thermofisher.com/neuroprotocol/rna**

Required materials

Cells

- Neuronal cell types of interest

Reagents and equipment

- PureLink RNA Mini Kit (Cat. No. 12183018A)
  - RNA Lysis Buffer
  - Wash Buffer I
  - Wash Buffer II
  - RNase-free water
  - RNA spin cartridges
  - Collection tubes
  - RNA recovery tubes
- Gibco™ 2-Mercaptoethanol (Cat. No. 21985023)
- Invitrogen™ SuperScript™ IV First-Strand Synthesis System (Cat. No. 18091050)
  - SuperScript IV Reverse Transcriptase
  - 5X SSIV Buffer Mix
  - 10 mM dNTP Mix
  - 0.1 M DTT
  - 50 µM oligo(dT)$_{20}$
  - Random hexamers (50 ng/µL)
  - Ribonuclease inhibitor
  - Ribonuclease H (RNase H)
- 70% ethanol
- Gibco™ TrypLE™ Express Enzyme (1X), no phenol red (Cat. No. 12604013)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- Invitrogen™ Ribonuclease H (RNase H) (Cat. No. 18021071)
- Invitrogen™ BlueJuice™ Gel Loading Buffer (10X) (Cat. No. 10816015)
- Tabletop centrifuge
- DEPC-treated water

RNA isolation

Isolating RNA

Important: Perform all steps on ice unless noted otherwise. For all incubations, heat the thermocyclers in advance. Prechill all reagents and thaw all frozen reagents and cells immediately prior to use. To prevent RNase contamination, wear disposable gloves while handling all materials and use of sterile disposable plasticware and RNase-free pipette tips with aerosol barriers is recommended. Always wear appropriate personal protective equipment when working in a laboratory environment.

1. Prepare RNA Lysis Solution by adding 10 µL 2-mercaptoethanol per mL of RNA Lysis Solution.

2. Remove the medium from T-25 flasks, rinse once with DPBS, no calcium, no magnesium (DPBS –/–), and treat cells with 1 mL of pre-warmed TrypLE Express Enzyme for 10 minutes at 37°C.

3. Harvest the cells and place them into 15 mL centrifuge tubes. Take 100 µL of the sample and obtain a viable cell count.

4. Centrifuge the cells in a tabletop centrifuge for 7 minutes at 100 x *g*. Discard the supernatant.

5. Freeze the cells overnight in a –80°C freezer; frozen cell pellets can be stored at least a month if desired. Alternatively, freshly isolated cell pellets can be processed directly by going to step 6.

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 x 10^6 to 5 x 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 x *g*.

8. Add 0.5 mL (1 volume) of 70% ethanol to each tube, and vortex the suspension 5–10 times.

9. Apply a 600 µL aliquot of sample to the RNA Spin Cartridge. Centrifuge at room temperature for 15–30 seconds at 12,000 x *g*, then discard the flow-through. Continue applying 600 µL aliquots of the same RNA sample to the spin cartridge until the entire sample has been processed.

10. Add 700 µL Wash Buffer I to the spin cartridge and centrifuge at room temperature for 15–30 seconds at 12,000 x *g*. Discard the flow-through and the tube. Place the spin cartridge into a clean 2 mL RNA Wash Tube.

11. Add 500 µL Wash Buffer II (containing ethanol) to the spin cartridge and centrifuge at room temperature for 15–30 seconds at 12,000 x *g*. Discard the flow-through.

12. Repeat step 11.

13. Centrifuge for 1 minute to dry the cartridge.

14. Place the cartridge into a clean RNA Recovery Tube. Add 40 µL 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 x *g*. Add an additional 40 µL of RNase-free water to the cartridge and repeat the step. Yield should be about 60–300 µg 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.

Determining RNA quality

1. Measure ratio of absorbance at 260 nm and 280 nm by analyzing 1 µL of the RNA sample using a Thermo Scientific™ NanoDrop™ spectrophotometer. Conduct readings 3 times, and use the average as the final value. Wipe down the analysis stage with a lab tissue wetted with DEPC-treated water before and after measuring each RNA sample. The A_{260}/A_{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
BlueJuice Gel Loading Buffer (10X)	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-treated water for empty wells. Run samples for 30 minutes, visualize the bands on a UV light box, capture the gel image, and perform band intensity measurements.

RNA storage

Store RNA samples at –80°C or process them further for cDNA synthesis.

cDNA preparation

First-strand cDNA synthesis

This protocol follows the methodologies described in the instructions for the SuperScript IV First-Strand Synthesis System.

1. Mix and briefly centrifuge each component before use. Pre-heat the thermocycler to 65°C. Note that oligo(dT)$_{20}$ may be substituted for random hexamers primer solution.

2. 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
10 mM dNTP Mix	1 µL
Random hexamers (50 ng/µL)	1 µL
RNA (1 µg)	*x* µL
DEPC-treated water	to 13 µL

3. 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. Vortex and briefly centrifuge the 5X SSIV Buffer Mix.

5. Prepare the reverse transcriptase reaction mix in a separate tube using the components listed below:

Component	Amount
5X SSIV Buffer Mix	4 µL
100 mM DTT	1 µL
Ribonuclease Inhibitor	1 µL
SuperScript IV Reverse Transcriptase	1 µL

6. Cap the tube, mix, and then briefly centrifuge the contents.

7. Add the reverse transcriptase reaction mix to the annealed RNA.

8. Incubate the tube at 50°C for 10 minutes.

9. Terminate reaction by incubating at 80°C for 10 minutes, then chill the tube on ice.

10. Add 1 µL of RNase H to the sample, and incubate at 37°C for 20 minutes.

11. Store the cDNA samples at –20°C or proceed to PCR amplification.

24 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 a wide variety of neuronal cell models. Understanding gene patterns can provide significant insight into diverse biological processes, including the regulation of differentiation and maturation, the impact of different cell-to-cell interactions, and mechanisms of disease and aging.

Here we provide guidelines and a general protocol for performing qPCR using the Applied Biosystems™ 7300 Real-Time PCR System and TaqMan® Gene Expression Master Mix.

View this protocol online and order products at **thermofisher.com/neuroprotocol/qpcr**

Required materials

Starting material

- cDNA generated from total RNA isolated from neural cells (see "RNA isolation and cDNA preparation from neural cells" on page 9796)

Media and reagents

- TaqMan Gene Expression Master Mix (Cat. No. 4369016)
- Applied Biosystems™ TaqMan® Gene Expression Assay (FAM) (Cat. No. 4331182; and see "PCR primers for molecular characterization of neural subtypes" on page 95)
- Invitrogen™ Nuclease-Free Water (not DEPC-Treated) (Cat. No. AM9938)

Special tools

- 7300 Real-Time PCR System or similar instrument
- 96-well or 384-well PCR plates (check the adapter type installed in your real-time PCR machine and choose a compatible plate format; for more information, refer to the **TaqMan Gene Expression Master Mix protocol**)
- Vortex mixer
- Microcentrifuge for 1.5 mL tubes
- Applied Biosystems™ MicroAmp™ Optical Adhesive Film (Cat. No. 4311971)

Methods

Template preparation

For qPCR, prepare cDNA generated using the protocol described in "RNA isolation and cDNA preparation from neural cells" (on page 96).

Real-time PCR instruments

TaqMan Gene Expression Master Mix can be used with a variety of real-time PCR instruments, including but not limited to the following Applied Biosystems™ instruments: 7300 and 7500 Real-Time PCR Systems; ABI PRISM™ 7000, 7700, and 7900HT systems; and GeneAmp™ 5700 system. Prefixed cycling conditions will be used across instruments.

Note: Because TaqMan Gene Expression Master Mix is not supported for use with Fast Mode thermal cycling conditions, select the Standard Mode thermal cycling condition.

Prepare the PCR assay mix (genes)

We recommend performing four replicates of each reaction. In this protocol, we describe conditions for a 20 µL reaction size. For more information, visit **thermofisher.com/qpcr**

Component	Amount
TaqMan Gene Expression Master Mix (2X)	10 µL
Taqman Gene Expression Assay	1 µL
Nuclease-Free Water (not DEPC-Treated)	8 µL
Total volume	**19 µL**

- Calculate the volume of each component of the PCR assay mix by multiplying the volume of each component by the number of replicates for each sample.
- Include excess volume for the loss that occurs during reagent transfers.
- Use 10 to 100 ng of cDNA per replicate.
- Cap the tubes and vortex briefly to mix the solutions.
- Centrifuge the tubes briefly to spin down the contents and eliminate any air bubbles from the solutions.

Prepare the PCR assay plate

1. Transfer 19 µL of each reaction mixture to each well of an optical plate.
2. Transfer 1 µL of cDNA template to each well. Each well will have a total volume of 20 µL for the amplification.
3. Cover the plate with a MicroAmp Optical Adhesive Film.
4. Centrifuge the plate briefly to spin down the contents and eliminate air bubbles from the solutions.

Run the PCR assay

1. Place the reaction plate in the instrument.
2. Confirm or select sample volume of 20 µL.
3. Under the Thermal Cycler tab, select standard mode specified in the following table.

Step	UDG* incubation	AmpliTaq Gold, UP enzyme activation	PCR	
			Cycle (40 cycles)	
	Hold	Hold	Denature	Anneal/ extend
Time	2 min	10 min	15 sec	1 min
Temp	50°C	95°C	95°C	60°C

* UDG: uracil-DNA glycosylase.

Note: The 2-minute, 50°C step is required for optimal UDG enzyme activity. The 10-minute, 95°C step is required to activate the AmpliTaq Gold™, UP enzyme.

4. Start the run.

Analyzing results

The general process for analyzing the data from gene expression assays involves:

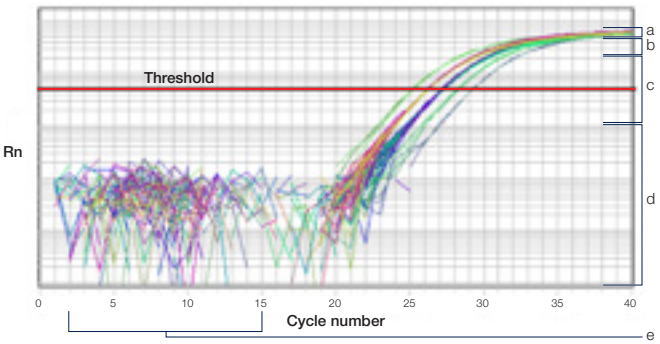
1. Viewing the amplification plots for the entire plate.

2. Setting the baseline and threshold values to determine the threshold cycles (C_t) for the amplification curves.

   Note: When using Applied Biosystems™ real-time PCR instruments, you can use the Sequence Detection System (SDS) software to either automatically calculate or manually set the baseline and threshold for the amplification curves.

3. Using the relative standard curve method or the comparative C_t method to analyze your data.

   Note: After analysis, verify that the baseline and threshold were called correctly for each well by viewing the resulting amplification plots, and adjust the values manually if necessary. For more information, refer to the **TaqMan Gene Expression Master Mix protocol**.



a. Plateau phase
b. Linear phase
c. Exponential (geometric) phase
d. Background
e. Baseline

Figure 24-1. Typical amplification curve.

Transfection

25 Lipid-mediated transfection of neuronal cells

Culture conditions

The following table summarizes the culture conditions for various Gibco™ 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 Gibco™ StemPro™ NSC SFM[1]	• Adherent culture on Gibco™ Geltrex™ matrix or CELLstart™ CTS™ Substrate–, fibronectin-, or poly-L-ornithine–coated culture vessels • 37°C, humidified atmosphere of 5% CO_2 in air • Exchange spent medium every other day
Human Astrocytes	Complete Gibco™ Astrocyte Medium[1]	• Adherent culture on Geltrex matrix–coated tissue culture vessels • 37°C, humidified atmosphere of 5% CO_2 in air • Exchange spent medium every 3–4 days
Rat Fetal Neural Stem Cells	Complete StemPro NSC SFM[1]	• Adherent culture on Geltrex matrix or CELLstart CTS Substrate–, fibronectin-, or poly-L-ornithine–coated culture vessels • 37°C, humidified atmosphere of 5% CO_2 in air • Exchange spent medium every 3–4 days
Rat Primary Cortical Astrocytes	Complete Astrocyte Medium[1,2]	• Adherent culture on standard culture vessels • 37°C, humidified atmosphere of 5% CO_2 in air • Exchange spent medium every 2–3 days
Rat Glial Precursor Cells	Complete StemPro NSC SFM[1], supplemented with 10 ng/mL PDGF-AA	• Adherent culture on CELLstart CTS Substrate– or poly-L-ornithine–coated culture vessels • 37°C, humidified atmosphere of 5% CO_2 in air • Exchange spent medium every other day

1 See "Preparing media" on page 115 for instructions on preparing complete StemPro NSC SFM and complete Astrocyte Medium.

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

Summary

The following protocols provide instructions for lipid-mediated transfection of plasmid DNA, siRNA, or mRNA into various neural cells using Invitrogen™ Lipofectamine™ 3000, Lipofectamine™ 2000, Lipofectamine™ Stem, Lipofectamine™ RNAiMAX, and Lipofectamine™ MessengerMAX™ Transfection Reagents.

- Lipofectamine 3000 reagent is a next-generation, broad-spectrum reagent for the delivery of plasmid DNA into immortalized neural cells with low cytotoxicity.
- Lipofectamine 2000 reagent is a broad-spectrum, animal origin–free formulation recommended for the delivery of plasmid DNA into primary neurons with low cytotoxicity.
- Lipofectamine Stem reagent is a versatile transfection reagent optimized to deliver multiple payloads (small and large DNA plasmids, mRNA, and Cas9 protein complexes) into stem cells, including neural stem cells. Visit **thermofisher.com/lipofectaminestem** to learn more about stem cell transfection.
- Lipofectamine RNAiMAX reagent is a leading RNAi transfection reagent optimized for the transfection of siRNA and Invitrogen™ Stealth RNAi™ duplexes into all neuronal-based cell types, immortalized and primary.
- Lipofectamine MessengerMAX reagent is an mRNA transfection reagent that offers an alternative strategy to transfect astrocytes, neural stem cells, neurons, and other primary cells. If you are not satisfied with the results obtained transfecting plasmid DNA, we highly recommend this as an alternative for superior transfection results. Visit **thermofisher.com/messengermax** to learn more about the benefits of mRNA transfection.

View this protocol online and order products at **thermofisher.com/transfection**

Best practices

The following guidelines are meant to improve your transfection workflow and its subsequent results.

- While our standard protocol has been significantly simplified to reduce the number of optimization parameters, we recommend that you use best practices to further optimize your specific experimental protocol.
- While not always necessary, if you use antibiotics during transfection, test your conditions thoroughly. Note that adding antibiotics to media during transfection may result in cell death.
- Maintain the same seeding conditions between experiments. When using immortalized cell lines, try to use post-thaw cells between passages 4 and 25; ensure the cells are healthy, and are between 70–90% confluent before transfection. In some instances, certain cells achieve better results at lower confluency. In addition, be sure to maintain consistent confluency across each well.
- Use Gibco™ Opti-MEM™ I Reduced Serum Medium to dilute the payload (DNA, siRNA, or mRNA) and the appropriate transfection reagent. Transfections can be performed in either the presence or absence of serum. If serum-free medium is required, be sure to test it for compatibility with the transfection reagent.

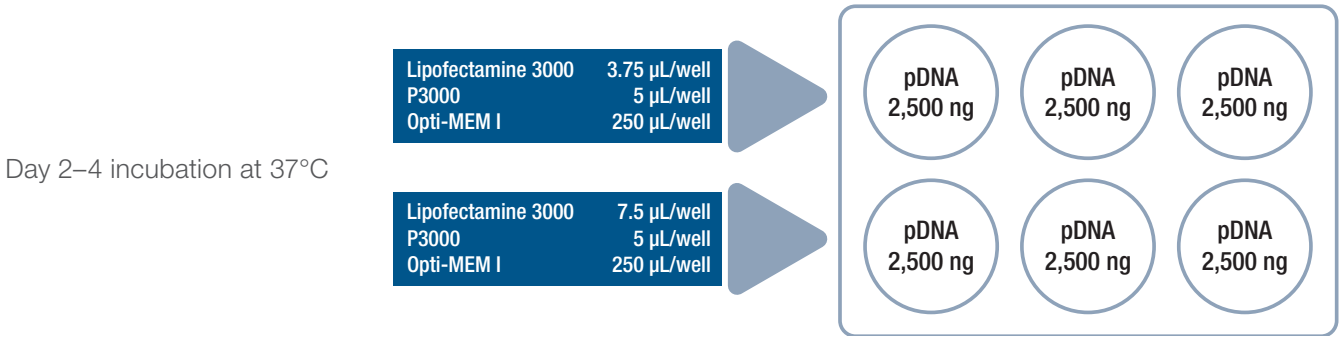
Optimization: a how-to example

Optimizing your transfection experiment is one of the most important steps in achieving the best results. The final results can be heavily influenced by the number of cells, cell confluency, payload quality, incubation time, culturing medium, and other factors.

The example below helps you plan initial optimization experiments using the standard transfection protocols as the starting points. It varies reagent doses and incubation times, but allows for adjustment of other parameters, including payload amounts, as needed.

Example

Plate format	6-well	
Cells	SH-SY5Y: 70% confluent	
Payload	Plasmid DNA: 2,500 ng	Constant
Recommended reagent doses*	Lipofectamine 3000 reagent: 3.75 µL/well and 7.5 µL/well	Variable
Reagent 2	P3000 reagent: 5 µL/well	Constant
Medium	Opti-MEM™ I Reduced Serum Medium: 250 µL/well	Constant
Incubation time	2 days, 3 days, or 4 days at 37°C	Variable



* The recommended doses for Lipofectamine 3000 reagent were identified through extensive testing for the plasmid DNA (pDNA) payload. It was determined that these two doses were the most effective across dozens of cell types.

Transfecting plasmid DNA (immortalized neuronal cells)—protocol

Materials needed

- Plasmid DNA of interest (0.5–5 µg/µL)
- Invitrogen™ Lipofectamine™ 3000 Transfection Reagent (Cat. No. L3000008)
  - Lipofectamine 3000 reagent
  - P3000 reagent
- Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985062)
- Microcentrifuge tubes

Note: The following protocol has two reagent doses (dose 1 and dose 2) that are needed for the initial optimization only.

Step	Description	Component	6-well format*	
			Dose 1/well	Dose 2/well
1	Seed cells to be 70–90% confluent at transfection	Adherent cells	0.25–1 x 10^6	0.25–1 x 10^6
2	Prepare reagent mix: Dilute Lipofectamine 3000 reagent in Opti-MEM I Reduced Serum Medium	Opti-MEM I medium	125 µL	125 µL
		Lipofectamine 3000 reagent	3.75 µL	7.5 µL
2a	Vortex reagent mix		2–3 seconds	
3	Dilute DNA: Prepare master mix of DNA by diluting DNA in Opti-MEM I medium, then add P3000 reagent	Opti-MEM I medium	125 µL	125 µL
		DNA	2.5 µg (2,500 ng)	2.5 µg (2,500 ng)
		P3000 reagent (2 µL/µg DNA)	5 µL	5 µL
4	Add diluted DNA (step 3) to each tube of reagent mix (step 2) in a 1:1 ratio	Reagent mix	125 µL	125 µL
		Diluted DNA	125 µL	125 µL
4a	Incubate DNA–reagent complex		10–15 minutes at **room temperature**	
5	Add DNA–reagent complex to cells			
6	Incubate transfected cells		Incubate cells for 2–4 days at 37°C	
7	Analyze the transfected cells			

* See the experimental scaling table below to adjust for other plate formats.

Experiment scaling

Culture vessel	Confluency	Reagent mix 1 (step 2)			Diluted DNA (step 3)		
		Dose 1	Dose 2	Opti-MEM I	P3000	DNA	Opti-MEM I
6 well	0.25–1 x 10^6	3.75 µL	7.5 µL	125 µL	5 µL	2.5 µg (2,500 ng)	125 µL
24 well	0.5–2 x 10^5	0.75 µL	1.5 µL	25 µL	1 µL	0.5 µg (500 ng)	25 µL
96 well	1–4 x 10^4	0.15 µL	0.3 µL	5 µL	0.2 µL	0.1 µg (100 ng)	5 µL

Transfecting plasmid DNA (primary neuronal cells)—protocol

Materials needed

- Plasmid DNA of interest (100 ng/µL or higher)
- Invitrogen™ Lipofectamine™ 2000 Transfection Reagent (Cat. No. 11668030)
- Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985062)
- Microcentrifuge tubes

Note: The following protocol has four reagent doses (dose 1–4) that are needed for the initial optimization only.

Step	Description	Component	6-well format*			
			Dose 1/well	Dose 2/well	Dose 3/well	Dose 4/well
1	Seed cells to be 70–90% confluent at transfection	Adherent cells	0.25–1 x 10^6	0.25–1 x 10^6	0.25–1 x 10^6	0.25–1 x 10^6
2	Prepare reagent mix: Dilute Lipofectamine 2000 reagent in Opti-MEM I Reduced Serum Medium	Opti-MEM I medium	150 µL	150 µL	150 µL	150 µL
		Lipofectamine 2000 reagent	6 µL	9 µL	12 µL	15 µL
2a	Vortex reagent mix		2–3 seconds			
3	Dilute DNA in Opti-MEM I Reduced Serum Medium	Opti-MEM I medium	150 µL	150 µL	150 µL	150 µL
		DNA	2.5 µg (2,500 ng)	2.5 µg (2,500 ng)	2.5 µg (2,500 ng)	2.5 µg (2,500 ng)
4	Add diluted DNA (step 3) to each tube of reagent mix (step 2) in a 1:1 ratio	Reagent mix	150 µL	150 µL	150 µL	150 µL
		Dilute DNA	150 µL	150 µL	150 µL	150 µL
4a	Incubate DNA–reagent complex		5 minutes at **room temperature**			
5	Add DNA–reagent complex to cells					
6	Incubate transfected cells		Incubate cells for 1–3 days at 37°C			
7	Analyze the transfected cells					

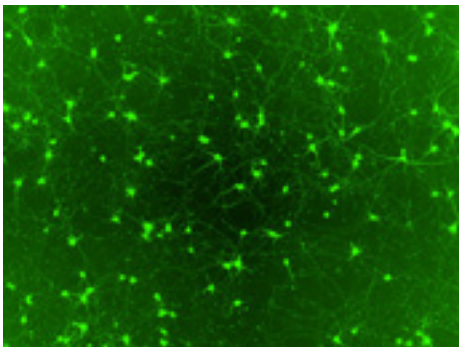
* See the experimental scaling table below to adjust for other plate formats.

Experiment scaling

Culture vessel	Confluency	Reagent mix (step 2)				Diluted DNA (step 3)	
		Dose 1	Dose 2	Dose 3	Dose 4	DNA	Opti-MEM I
6 well	0.25–1 x 10^6	6 µL	9 µL	12 µL	15 µL	2.5 µg (2,500 ng)	150 µL
24 well	0.5–2 x 10^5	2 µL	3 µL	4 µL	5 µL	0.5 µg (500 ng)	50 µL
96 well	1–4 x 10^4	1 µL	1.5 µL	2 µL	2.5 µL	0.1 µg (100 ng)	25 µL

Expected results

A



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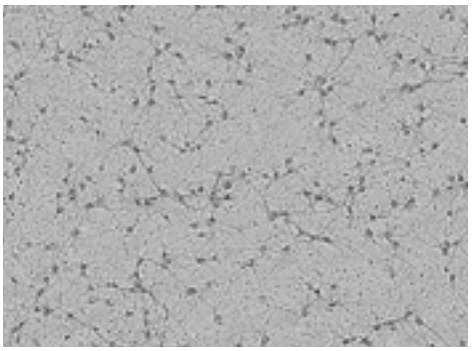


Figure 25-1. Plasmid DNA delivered by Lipofectamine 2000 reagent in primary cortical neurons cultured in the Gibco™ B-27™ Plus Neuronal Culture System (freshly isolated E18 rat; 6 days *in vitro*). (A) GFP expression and **(B)** brightfield images, analyzed 48 hours posttransfection.

Transfecting siRNA (immortalized neuronal cells and primary neuronal cells)—protocol

Materials needed

- Invitrogen™ *Silencer*™ Select siRNAs (**thermofisher.com/sirna**)
- Invitrogen™ Lipofectamine™ RNAiMAX™ Transfection Reagent (Cat. No. 13778075)
- Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985062)
- Microcentrifuge tubes

Step	Description	Component	6-well format* Dose/well
1	Seed cells to be 60–80% confluent at transfection	Adherent cells	0.25–1 x 10^6
2	Prepare reagent mix: Dilute Lipofectamine RNAiMAX reagent in Opti-MEM I Reduced Serum Medium in one microcentrifuge tube	Opti-MEM I medium	150 µL
		Lipofectamine RNAiMAX reagent	7.5 µL
2a	Vortex reagent mix		2–3 seconds
3	Dilute *Silencer* Select siRNA in Opti-MEM I medium in one microcentrifuge tube	Opti-MEM I medium	150 µL
		siRNA (10 µM)	30 pmol
4	Add diluted siRNA (step 3) to each tube of reagent mix (step 2) in a 1:1 ratio	Reagent mix	150 µL
		Diluted siRNA	150 µL
4a	Incubate siRNA–reagent complex		5 minutes at **room temperature**
5	Add siRNA–reagent complex to cells		
6	Incubate transfected cells		Incubate cells for 1–3 days at 37°C
7	Analyze the transfected cells		

* See the experimental scaling table below to adjust for other plate formats.

Experiment scaling

Culture vessel	Confluency	Reagent mix (step 2)		Diluted siRNA (step 3)	
		Dose	Opti-MEM I	siRNA	Opti-MEM I
6 well	0.25–1 x 10^6	7.5 µL	150 µL	30 pmol	150 µL
24 well	0.5–2 x 10^5	1.5 µL	50 µL	5 pmol	50 µL
96 well	1–4 x 10^4	0.5 µL	25 µL	1 pmol	25 µL

Transfecting mRNA (immortalized and primary neuronal cells)—protocol

Materials needed

- mRNA of interest (0.5–5 µg/µL)
- Invitrogen™ mMESSAGE mMACHINE™ T7 ULTRA Transcription Kit (Cat. No. AM1345)
- Invitrogen™ Lipofectamine™ MessengerMAX™ Transfection Reagent (Cat. No. LMRNA008)
- Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985062)
- Microcentrifuge tubes

Note: The following protocol has two reagent doses (dose 1 and dose 2) that are needed for the initial optimization only.

Step	Description	Component	6-well format*	
			Dose 1/well	Dose 2/well
1	Seed cells to be 70–90% confluent at transfection	Adherent cells	0.25–1 x 10^6	0.25–1 x 10^6
2	Prepare reagent mix: Dilute MessengerMAX reagent in Opti-MEM I Reduced Serum Medium	Opti-MEM I medium	125 µL	125 µL
		MessengerMAX reagent	3.75 µL	7.5 µL
2a	Vortex reagent mix		2–3 seconds	
2b	Incubate reagent mix		10 minutes at **room temperature**	
3	Dilute mRNA in Opti-MEM I medium	Opti-MEM I medium	125 µL	125 µL
		mRNA	2.5 µg (2,500 ng)	2.5 µg (2,500 ng)
4	Add diluted mRNA (step 3) to each tube of reagent mix (step 2) in a 1:1 ratio	Reagent mix	125 µL	125 µL
		Diluted mRNA	125 µL	125 µL
4a	Incubate mRNA–reagent complex		5 minutes at **room temperature**	
5	Add mRNA–reagent complex to cells			
6	Incubate transfected cells		Incubate cells for 2–4 days at 37°C	
7	Analyze the transfected cells			

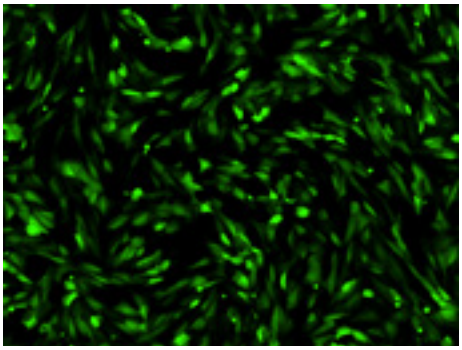
* See the experimental scaling table below to adjust for other plate formats. Visit thermofisher.com/lipofectaminestem for additional information on setting up mRNA transfection in NSCs using Lipofectamine Stem Transfection Reagent.

Experiment scaling

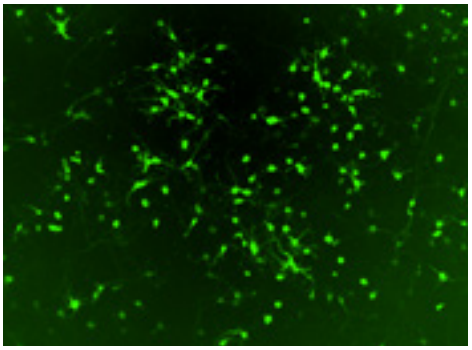
Culture vessel	Confluency	Reagent mix 1 (step 2)			Dilute mRNA (step 3)	
		Dose 1	Dose 2	Opti-MEM I	mRNA	Opti-MEM I
6 well	0.25–1 x 10^6	3.75 µL	7.5 µL	125 µL	2.5 µg (2,500 ng)	125 µL
24 well	0.5–2 x 10^5	0.75 µL	1.5 µL	25 µL	0.5 µg (500 ng)	25 µL
96 well	1–4 x 10^4	0.15 µL	0.3 µL	5 µL	0.1 µg (100 ng)	5 µL

Expected results

A GFP expression 62%



B GFP expression 76%



C GFP expression 92%

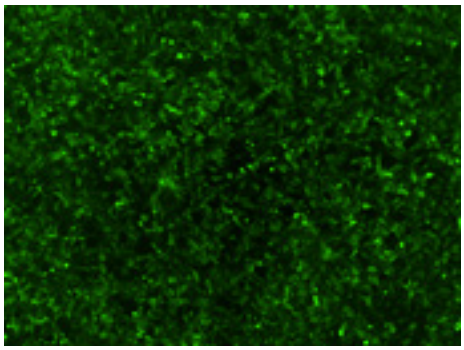


Figure 25-2. Lipofectamine MessengerMAX reagent was used to deliver mRNA encoding GFP (500 ng/well). Cells used included **(A)** primary cortical neurons (freshly isolated from E16 mouse; 5 days *in vitro*), **(B)** SK-N-SH cells (neuroblastoma) in a 24-well format, and **(C)** Gibco™ Human Neural Stem Cells (H9-Derived, Cat. No. N7800100) (hNSCs; 250 ng/well of mRNA was used in a 48-well format). GFP expression (noted above) was analyzed 24 hours posttransfection.

Transfecting plasmid DNA (neural stem cells)—protocol*

Materials needed

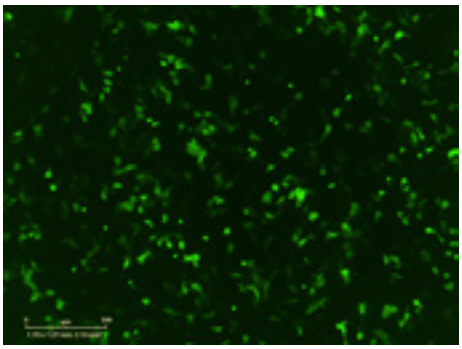
- Invitrogen™ Lipofectamine™ Stem Reagent (Cat. No. STEM00003)
- Gibco™ StemPro™ NSC SFM (Cat. No. A1050901)
- Microcentrifuge tubes
- Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985062)

Step	Description	Component	24-well format*			
			Dose 1/ well 1	Dose 2/ well 2	Dose 3/ well 2	Dose 4/ well 2
1	Seed cells to be 30–60% confluent at transfection	Adherent cells	2.5 x 10^4–7.5 x 10^4			
2	Prepare reagent mix: Dilute **2 volumes** of Lipofectamine Stem Reagent in Opti-MEM I Reduced Serum Medium	Opti-MEM I medium	25 µL	25 µL	25 µL	25 µL
		Lipofectamine Stem reagent	1 µL	1 µL	2 µL	2 µL
3	Dilute DNA: Dilute **2 volumes** of DNA in Opti-MEM I Medium	Opti-MEM I medium	25 µL	25 µL	25 µL	25 µL
		DNA	250 ng	500 ng	250 ng	500 ng
4	Add diluted DNA to diluted Lipofectamine Stem Reagent to each tube in a 1:1 ratio	Reagent mix	25 µL	25 µL	25 µL	25 µL
		Dilute DNA	25 µL	25 µL	25 µL	25 µL
4a	Incubate DNA–reagent complex		10 minutes at **room temperature**			
5	Add DNA–reagent complex to cells	DNA–lipid complex	50 µL	50 µL	50 µL	50 µL
		Final DNA used	250 ng	500 ng	250 ng	500 ng
		Final Lipofectamine Stem reagent used	1 µL	1 µL	2 µL	2 µL
6	Incubate transfected cells		Incubate and monitor cells for 1–2 days at 37°C			
7	Visualize/analyze transfected cells		The following day, overlay an additional 0.5 mL StemPro NSC SFM per well, if NSCs are going to be transfected for 48 hours			

* Visit thermofisher.com/lipofectaminestem for additional information on setting up mRNA or ribonucleoprotein (RNP) transfections in NSCs with Lipofectamine Stem reagent.

Expected results

A



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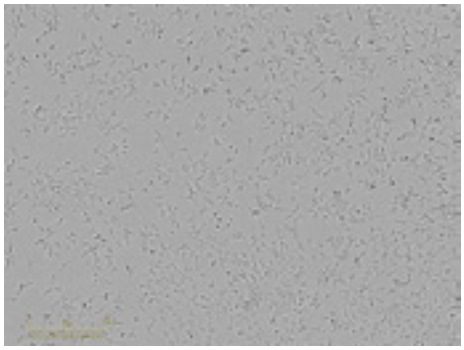


Figure 25-3. Plasmid DNA delivered by Lipofectamine Stem reagent in NSCs. (A) GFP expression demonstrating 60% transfection efficiency and **(B)** brightfield image. NSCs are shown 24 hours posttransfection with 500 ng of a 6 kb EF1α-GFP plasmid and 1 µL of Lipofectamine Stem reagent in StemPro NSC SFM on Gibco™ Geltrex™ matrix.

26 Transfecting neural cells using the Neon Transfection System

Summary

The Invitrogen™ Neon™ Transfection System is a benchtop electroporation device that uses a 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 neural cells are described below. For detailed instructions, including several cell-specific protocols, refer to **thermofisher.com/neon**

For detailed information on culture conditions for various neural cell lines, refer to the instructions supplied with the specific cell line you are using.

View this protocol online and order products at **thermofisher.com/neuroprotocol/neon**

Required materials

Cells

- Neural cell line of interest

Media and reagents

- Growth medium and growth factors appropriate for your neural cell line
- Plasmid DNA of interest (1–5 µg/mL in deionized water or TE buffer)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- Invitrogen Neon™ Transfection System (Cat. No. MPK5000)
- Invitrogen™ Neon™ Transfection System 10 µL Kit (Cat. No. MPK1096) or Neon™ Transfection System 100 µL Kit (Cat. No. MPK10096)
- Appropriate tissue culture plates and supplies
- Gibco™ StemPro™ NSC SFM (Cat. No. A1050901: This kit contains KnockOut™ DMEM/F-12 Basal Medium stored at 4°C; StemPro™ NSC SFM Supplement stored at –20 to –5°C in the dark; and bFGF Recombinant Human and EGF Recombinant Human proteins stored at 4°C, desiccated.)
- Gibco™ Astrocyte Medium (Cat. No. A1261301: This kit contains N-2 Supplement (100X) stored at –20°C; Dulbecco's Modified Eagle Medium (DMEM) (1X) stored at 2°C to 8°C; and One Shot™ Fetal Bovine Serum, Certified, stored at –20°C in the dark.)

Preparing media

Use complete StemPro NSC SFM for human neural stem cells or rat fetal neural stem cells and complete astrocyte medium for human astrocytes, rat primary cortical astrocytes, or rat glial precursor cells. Also see “Culture Conditions” on page 103 of the previous chapter for additional information.

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	Final conc.	Amount
KnockOut DMEM/F-12	1X	97 mL
GlutaMAX 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 astrocyte medium

Use for human astrocytes, rat primary cortical astrocytes, or rat glial precursor cells

To prepare 100 mL of complete astrocyte medium, 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	Final conc.	Amount
DMEM	1X	89 mL
N-2 Supplement	1X	1 mL
FBS	10%	10 mL
EGF	20 ng/mL	2 µg

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 human NSCs 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 (see table on the next page) in the appropriate growth medium 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 DPBS, no calcium, no magnesium (DPBS –/–).

3. Resuspend the cell pellet in Resuspension Buffer R (included with Neon kits) at the appropriate final density (see the table on the next page).

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 x 10^7 cells/mL	1,400 1,600 1,700	20 20 20	2 1 1	10 µL
Human astrocytes	1 x 10^7 cells/mL	1,100 1,200	30 40	1 1	10 µL
Rat fetal neural stem cells	1 x 10^7 cells/mL	1,300 1,500 1,600	20 10 10	2 3 3	10 µL
Rat primary cortical astrocytes	0.5 x 10^7 cells/mL	1,400 1,400 1,700	20 30 20	2 1 1	10 µL
Rat glial precursor cells	1 x 10^7 cells/mL	1,300 1,500	10 20	3 1	10 µL

1. Fill the Neon Tube with 3 mL of Buffer E. (Use Buffer E2 if you are using the 100 µL Neon Tip.)

2. Insert the Neon Tube into the Neon Pipette Station until you hear a click, indicating that the tube has locked in position.

3. Transfer 0.5 µg 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 Invitrogen™ PureLink™ HiPure plasmid DNA purification kits to prepare DNA.

4. Add 1 mL of cells (resuspended in step 3) to the tube containing the plasmid DNA and gently mix.

5. Insert a 10 µL Neon Tip into the Neon Pipette.

6. 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.

7. 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.

8. 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.

9. 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.

10. Discard the Neon Tip into an appropriate biological hazardous waste container.

11. Repeat steps 5–10 for the remaining samples.

12. Gently rock the plate to assure even distribution of the cells. Incubate the plate at 37°C in a humidified 5% CO_2 incubator.

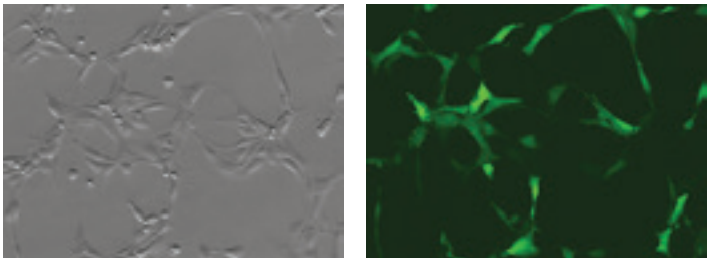
13. Assay the samples to determine the transfection efficiency (e.g., fluorescence microscopy or functional assay).

Expected results

Human Neural Stem Cells

Gibco™ Human Neural Stem Cells (H9-Derived) (Cat. No. N7800100), cultured in complete StemPro NSC SFM, were transfected with 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EmGFP) using the Neon Transfection System with the parameters listed in the following table. 48 hours posttransfection, the cells were analyzed by light **(A)** and fluorescence microscopy **(B)**.

A **B**

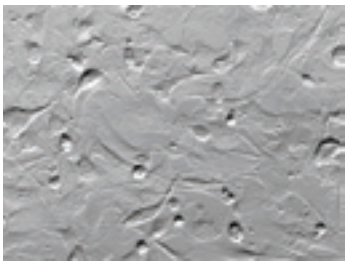


Cell density (cells/mL)	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon Tip
1×10^7	1,400	20	2	82%	95%	10 µL
	1,600	20	1	84%	95%	
	1,700	20	1	87%	96%	

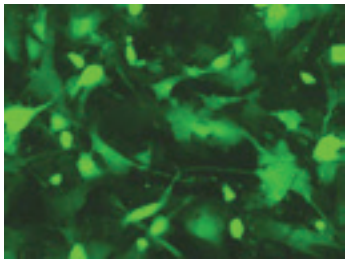
Human Astrocytes

Gibco™ Human Astrocytes (Cat. No. N7805100) were transfected using the Neon Transfection System and 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EmGFP); 24 hours post-electroporation, the cells were analyzed by light **(A)** and fluorescence microscopy **(B)**.

A



B

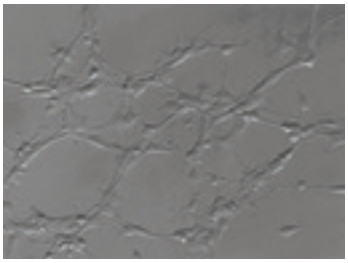


Cell density (cells/mL)	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon Tip
1 x 10^7	1,100 1,200	30 40	1 1	92% 93%	97% 97%	10 µL

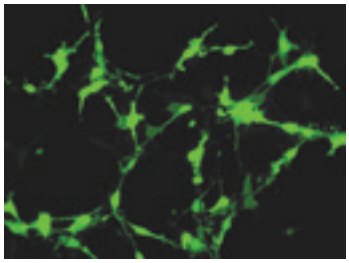
Rat Fetal Neural Stem Cells

Gibco™ Rat Fetal Neural Stem Cells (Cat. No. N7744100) were transfected using the Neon Transfection System and 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EmGFP); 24 hours post-electroporation, the cells were analyzed by light **(A)** and fluorescence microscopy **(B)**.

A



B

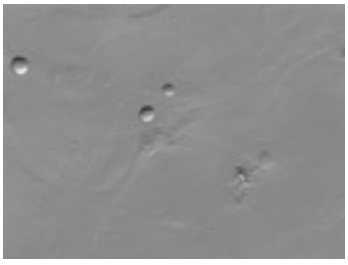


Cell density (cells/mL)	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon Tip
1 x 10^7	1,100 1,200	30 40	1 1	92% 93%	97% 97%	10 µL

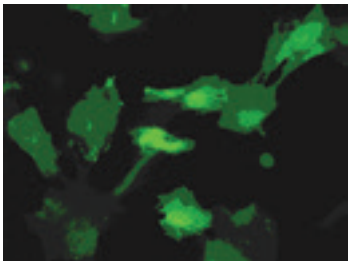
Rat Primary Cortical Astrocytes

Gibco™ Rat Primary Cortical Astrocytes (Cat. No. N7745100) were transfected using the Neon Transfection System and 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EmGFP); 24 hours post-electroporation, the cells were analyzed by light **(A)** and fluorescence microscopy **(B)**.

A



B

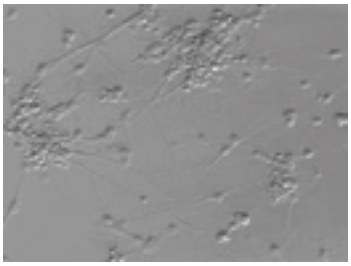


Cell density (cells/mL)	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon Tip
0.5×10^7	1,400	20	2	69%	87%	10 µL
	1,400	30	1	71%	89%	
	1,700	20	1	71%	90%	

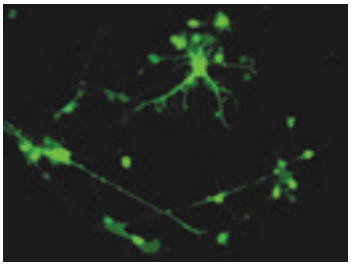
Rat Glial Precursor Cells

Gibco™ Rat Glial Precursor Cells (Cat. No. N7746100) were transfected using the Neon Transfection System and 0.5 µg of a plasmid encoding the Emerald Green Fluorescent Protein (EmGFP); 24 hours post-electroporation, the cells were analyzed by light **(A)** and fluorescence microscopy **(B)**.

A



B



Cell density (cells/mL)	Pulse voltage (V)	Pulse width (ms)	Pulse number	Transfection efficiency	Viability	Neon tip
1×10^7	1,300	10	3	49%	78%	10 µL
	1,500	20	1	44%	64%	

Troubleshooting

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

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) 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 A_{260}/A_{280} ratio. The ratio should be at least 1.8 for electroporation. • Do not precipitate DNA with ethanol to concentrate DNA. DNA concentrated 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 pipet 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 pre-warmed culture medium **without antibiotics**.
	Multiple use of the same Neon Tip	• **Do not** use the same Neon Tip for electroporation more than 2 times, because the repeated application of electric pulses reduces the tip quality and impairs its physical integrity.

Problem	Possible cause	Solution
Low transfection efficiency	Poor plasmid DNA quality or the plasmid DNA amount 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 x 10^5 cells per 10 µL per sample (i.e., 1 x 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.
	Mycoplasma-contaminated cells	• Test cells for *Mycoplasma* 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 reduces the tip quality and impairs its 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.

Appendix

A1 Thermo Fisher Scientific products

Overview

Thermo Fisher Scientific offers products that are designed to meet your neural cell culture needs, including Gibco™ cell culture media, reagents, sera, and growth factors. All cell culture media products available from Thermo Fisher Scientific are tested for contamination to help ensure their quality, safety, consistency, and regulatory compliance.

Ordering information

Cells

Product	Quantity	Cat. No.
Human		
Human Astrocytes	1 mL	N7805100
Human Episomal iPSC line	1 x 10^6 cells/vial	A18945
StemPro Neural Stem Cells	1 x 10^6 cells	A15654
Mouse		
Primary Mouse Cortical Neurons	1 x 10^6 cells	A15585
	4 x 10^6 cells	A15586
Primary Mouse Hippocampal Neurons	1 x 10^6 cells	A15587
Rat		
Primary Rat Cortex Neurons, Sprague-Dawley	1 x 10^6 cells	A36511
	4 x 10^6 cells	A36512
Primary Rat Cortical Neurons	1 x 10^6 cells	A1084001
	4 x 10^6 cells	A1084002
Primary Rat Hippocampal Neurons	1 mL	A1084101
Rat		
Rat Fetal Neural Stem Cells	1 mL	N7744100
Rat Glial Precursor Cells	1 mL	N7746100

Cells

Product	Quantity	Cat. No.
Rat Primary Cortical Astrocytes	1 x 10^6 cells	N7745100

Media and supplements

Product	Quantity	Cat. No.
Advanced DMEM/F-12	500 mL	12634010
	10 x 500 mL	12634028
Astrocyte Medium	500 mL	A1261301
B-27 Plus Neuronal Culture System	1 system	A3653401
B-27 Plus Supplement (50X)	10 mL	A3582801
B-27 Supplement (50X), serum free	10 mL	17504044
	100 mL	17504001
CTS B-27 Supplement, XenoFree	10 mL	A1486701
CTS DPBS, calcium, magnesium	1,000 mL	A1285801
CTS (Cell Therapy Systems) DPBS, without calcium chloride, without magnesium chloride	1 L	A1285601
CTS GlutaMAX-I Supplement	100 mL	A1286001
CTS KnockOut DMEM/F-12	500 mL	A1370801
CTS N-2 Supplement	5 mL	A1370701
CultureOne Supplement (100X)	5 mL	A3320201
Dulbecco's Modified Eagle Medium (DMEM), high glucose, pyruvate*	500 mL	11995065
DMEM/F-12, GlutaMAX supplement	500 mL	10565018
	10 x 500 mL	10565042
Essential 8 Medium	500 mL	A1517001
Essential 8 Flex Medium Kit	1 kit	A2858501
Fetal Bovine Serum (FBS), embryonic stem cell–qualified, US origin*	100 mL	16141061
	500 mL	16141079
GlutaMAX Supplement	100 mL	35050061
	20 x 100 mL	35050079

Media and supplements

Product	Quantity	Cat. No.
Goat Serum, New Zealand origin*	100 mL	16210064
	500 mL	16210072
Hibernate-E Medium	500 mL	A1247601
N-2 Supplement (100X)	5 mL	17502048
	50 mL	17502001
Neurobasal Medium	500 mL	21103049
Neurobasal Plus Medium	500 mL	A3582901
Opti-MEM I Reduced Serum Medium	100 mL	31985062
	500 mL	31985070
	10 x 500 mL	31985088
PSC Dopaminergic Neuron Differentiation Kit	1 kit	A3147701
PSC Neural Induction Medium	500 mL	A1647801
RevitaCell Supplement (100X)	5 mL	A2644501
StemPro NSC SFM	1 kit	A1050901

Substrates, matrices, and bioscaffolds

Product	Quantity	Cat. No.
CELLstart CTS Substrate	2 mL	A1014201
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	1 mL	A1413301
	5 mL	A1413302
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	1 mL	A1413201
	5 mL	A1413202
Laminin Mouse Protein, Natural	1 mg	23017015
Poly-D-Lysine	100 mL	A3890401
Vitronectin (VTN-N) Recombinant Human Protein, Truncated	1 mL	A14700
	10 mL	A31804

Reagents

Product	Quantity	Cat. No.
2-Mercaptoethanol (55 mM) (β-Mercaptoethanol)	50 mL	21985023
Antibiotic-Antimycotic (100X)	20 mL	15240096
	100 mL	15240062
	20 x 100 mL	15240112
CTS Synth-a-Freeze Medium	50 mL	A1371301
CTS TrypLE Select Enzyme	100 mL	A1285901
DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride)	10 mg	D1306
DMSO, Anhydrous (dimethylsulfoxide)	10 x 3 mL	D12345
Glucose Solution	50 mL	A2494001
FluoVolt Membrane Potential Kit	1 kit	F10488
Lipofectamine 3000 Transfection Reagent	0.1 mL	L3000001
	0.75 mL	L3000008
	1.5 mL	L3000015
	5 x 1.5 mL	L3000075
	15 mL	L3000150
Lipofectamine 2000 Transfection Reagent	0.3 mL	11668030
	0.75 mL	11668027
	1.5 mL	11668019
	15 mL	11668500
Lipofectamine Stem Transfection Reagent	0.1 mL	STEM00001
	0.3 mL	STEM00003
	0.75 mL	STEM00008
	1.5 mL	STEM00015

Reagents

Product	Quantity	Cat. No.
Lipofectamine MessengerMAX Transfection Reagent	0.1 mL	LMRNA001
	0.3 mL	LMRNA003
	0.75 mL	LMRNA008
	1.5 mL	LMRNA015
	15 mL	LMRNA150
Lipofectamine RNAiMAX Transfection Reagent	0.1 mL	13778100
	0.3 mL	13778030
	0.75 mL	13778075
	1.5 mL	13778150
	15 mL	13778500
Live Cell Imaging Solution (LCIS)	500 mL	A14291DJ
NucBlue Fixed Cell ReadyProbes Reagent	1 kit	R37606
Penicillin-Streptomycin (5,000 U/mL)	100 mL	15070063
Probenecid, Water Soluble (Cat. No. P36400)	10 x 77 mg	P36400
ProLong Gold Antifade Mountant	2 mL	P10144
	10 mL	P36930
	5 x 2 mL	P36934
PSC Cryopreservation Kit	50 mL	A2644601
StemPro Accutase Cell Dissociation Reagent	100 mL	A1110501
Trypan Blue Solution, 0.4%	100 mL	15250061
TrypLE Express Enzyme (1X), no phenol red	100 mL	12604013
	500 mL	12604021
	20 x 100 mL	12604039
	5 L	12604054
TrypLE Select Enzyme (1X), no phenol red	100 mL	12563011
	500 mL	12563029

Reagents

Product	Quantity	Cat. No.
Trypsin-EDTA (0.05%), phenol red	100 mL	25300054
	500 mL	25300062
	20 x 100 mL	25300120
Valinomycin	25 mg	V1644

Growth factors, purified proteins, and antibodies**

Product	Quantity	Cat. No.
BDNF Recombinant Human Protein	5 µg	10908010
	10 µg	PHC7074
CNTF Recombinant Human Protein	20 µg	PHC7015
EGF Recombinant Human Protein	5 µg	10605HNAE5
	10 µg	PHG0314
	25 µg	PHG0315
	5 x 5 µg	10605HNAE25
	50 µg	10605HNAE50
	100 µg	PHG0311
	5 x 50 µg	10605HNAE250
	1 mg	PHG0313
FGF-Basic Full Length CTS Recombinant Human Protein	100 µg	CTP0261
	1 mg	CTP0263
Goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate	500 µL	A-11029
Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 594 conjugate	500 µL	A-11037
MAP2 Antibody (M13)	100 µg	13-1500
PDGF-AA Recombinant Human Protein	10 µg	PHG0035

Buffers and balanced salt solutions

Product	Quantity	Cat. No.
CTS (Cell Therapy Systems) DPBS, calcium, magnesium (CTS Dulbecco's Phosphate-Buffered Saline (DPBS))	1,000 mL	A1285801
CTS DPBS, without calcium chloride, without magnesium chloride (CTS Dulbecco's Phosphate-Buffered Saline without Ca^{2+} or Mg^{2+})	1 L	A1285601
DPBS, calcium, magnesium (Dulbecco's Phosphate-Buffered Saline with Ca^{2+} and Mg^{2+}, DPBS +/+)	100 mL	14040141
	500 mL	14040133
	1,000 mL	14040117
	10 x 500 mL	14040182
	6 x 1,000 mL	14040216
DPBS, no calcium, no magnesium (Dulbecco's Phosphate-Buffered Saline without Ca^{2+} or Mg^{2+}, DPBS –/–)	500 mL	14190144
	1,000 mL	14190136
	5 L	14190342
	10 x 500 mL	14190250
	6 x 1,000 mL	14190235
	10 L	14190359
	20 L	14190367
HBSS, calcium, magnesium, no phenol red* (Hanks' Balanced Salt Solution with Ca^{2+} and Mg^{2+} and without phenol red)	500 mL	14025092
	1,000 mL	14025076
	10 x 500 mL	14025134
	6 x 1,000 mL	14025126
HBSS, no calcium, no magnesium* (Hanks' Balanced Salt Solution without Ca^{2+} or Mg^{2+})	100 mL	14170120
	500 mL	14170112
	10 x 500 mL	14170161

Assay kits

Product	Quantity	Cat. No.
Fluo-4 Calcium Imaging Kit	1 kit	F10489
Human Dopaminergic Neuron Immunocytochemistry Kit	40 reactions	A29515
LIVE/DEAD Cell Vitality Assay Kit, C_{12} Resazurin/SYTOX Green	1 kit	L34951

Assay kits

Product	Quantity	Cat. No.
LIVE/DEAD Viability/Cytotoxicity Kit, for mammalian cells	1 kit	L3224
mMESSAGE mMACHINE T7 ULTRA Transcription Kit	10 reactions	AM1345
Neon Transfection System 10 µL Kit	25 x 2 reactions	MPK1025
	96 x 2 reactions	MPK1096
Neon Transfection System 100 µL Kit	25 x 2 reactions	MPK10025
	96 x 2 reactions	MPK10096
Platinum SYBR Green qPCR SuperMix-UDG	100 reactions	11733038
	500 reactions	11733046
PureLink RNA Mini Kit	10 preps	12183020
	50 preps	12183018A
	250 preps	12183025
TaqMan Gene Expression Assay (FAM)	S (250 reactions/250 µL), inventoried	4331182
TaqMan Gene Expression Master Mix	1 x 5 mL	4369016

Plasticware

Product	Quantity	Cat. No.
Nalgene General Long-Term Storage Cryogenic Tubes (1.0 mL capacity)	Case of 500	5000-1012
Nalgene General Long-Term Storage Cryogenic Tubes (1.2 mL capacity)	Case of 500	5000-0012
Nalgene General Long-Term Storage Cryogenic Tubes (1.5 mL capacity)	Case of 500	5000-1020
Nunc Cell Scrapers (23 cm handle, for use with 25 to 80 cm^2 flasks)	Case of 250	179693
Nunc Cell Scrapers (32 cm handle, for use with 75 to 175 cm^2 flasks)	Case of 250	179707
Nunc Cell-Culture Treated Multidishes, 4 well	Case of 120	176740
Nunc Edge 2.0 96-Well Treated Plate, Lid	Case of 50	167425
Nunc Lab-Tek II Chamber Slide System, 4 well	Pack of 16	154526PK
Nunc MicroWell 96-Well Optical-Bottom Plates with Polymer Base, cell culture, black	Case of 30	165305
Nunclon Sphera Flasks (T-25 Cell Culture Flask)	6 units/bag, 18 units/case	174951

Plasticware

Product	Quantity	Cat. No.
Nunclon Sphera Flasks (T-75 Cell Culture Flask)	4 units/bag, 24 units/case	174952
Nunclon Sphera Dishes (Multidish 6-Well)	1 unit/pack, 7 units/case	174932
T-25 Nunclon Sphera EasYFlask	Case of 18	174951
T-75 Nunclon Sphera EasYFlask	Case of 24	174952

Instruments and software

Product	Quantity	Cat. No.
Real-Time PCR Systems	**thermofisher.com/qpcr**	
Attune NxT Flow Cytometer	**thermofisher.com/attune**	
CellInsight CX5 High-Content Screening (HCS) Platform	**thermofisher.com/hcs**	
Countess II Automated Cell Counter	**thermofisher.com/countess**	
EVOS FL Auto Imaging System	**thermofisher.com/evosflauto**	
EVOS FL Imaging System	**thermofisher.com/evosfl**	
HCS Studio 2.0 Cell Analysis Software, client installation	1 each	SX000041A
Neon Transfection System	1 each	MPK5000

Accessory products

Product	Quantity	Cat. No.
BlueJuice Gel Loading Buffer (10X)	3 x 1 mL	10816-015
Custom primers	**thermofisher.com/oligos**	
Distilled Water	100 mL	15230170
	500 mL	15230162
	1,000 mL	15230147
EVOS Light Cube, GFP	1 each	AMEP4651
MicroAmp Optical Adhesive Film	25 covers	4360954
	100 covers	4311971
Mr. Frosty Freezing Container (1.0 to 2.0 mL tube capacity)	1 each	5100-0001

Accessory products

Product	Quantity	Cat. No.
Nuclease-Free Water (not DEPC-Treated)	1 x 100 mL	AM9938
	5 x 100 mL	AM9939
	10 x 50 mL	AM9937
	1 x 500 mL	AM9930
	1 x 1,000 mL	AM9932
	4 x 1,000 mL	4387936
Ribonuclease H (RNase H)	30 units	18021014
	120 units	18021071
Silencer Select siRNAs	**thermofisher.com/sirna**	
SuperScript IV First-Strand Synthesis System	50 reactions	18091050
	200 reactions	18091200

* For *In Vitro* Diagnostic Use.
** For additional antibodies, see pages 74–75 and 79–80.

A2 Resources for more information

Books

- **Neural Stem Cell Assays**, edited by Navjot Kaur and Mohan Vemuri, Wiley Press, 2015.
- **Developmental Biology, 9th edition**, edited by Scott F. Gilbert Sinauer Associates, 2010.
- **Neural Stem Cells, 2nd edition**, edited by Leslie 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

- *Neuron*, **cell.com/neuron**
- *Development*, **dev.biologists.org**
- *The Journal of Neuroscience*, **jneurosci.org**
- *European Journal of Neuroscience*, **onlinelibrary.wiley.com/journal/10.1111/%28ISSN%291460-9568**
- *Developmental Neurobiology*, **onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291097-4695**
- *Nature Reviews Neuroscience*, **nature.com/nrn/index.html**
- *Cell Stem Cell*, **cell.com/cell-stem-cell/home**

Organizations

- American Academy of Neurology, **aan.com**
- Society for Neuroscience, **sfn.org**
- European Neuroscience and Society Network, **lse.ac.uk/collections/ENSN**
- Federation of European Neuroscience Societies, **fens.mdc-berlin.de**
- Japanese Neuroscience Society, **jnss.org/en/**
- International Society for Stem Cell Research, **isscr.org**

Government sites

- National Institute of Neurological Disorders and Stroke (NINDS), **ninds.nih.gov**
- National Institute of Mental Health (NIMH), **nimh.nih.gov**
- Food and Drug Administration (FDA), **fda.gov**
- National Institute of Child Health and Human Development (NICHD), **nichd.nih.gov**
- NCBI PubMed, **ncbi.nlm.nih.gov/pubmed**
- National Institutes of Health Entrez Databases, **ncbi.nlm.nih.gov/Database/index.html**
- National Library of Medicine's MEDLINEplus, **ncbi.nlm.nih.gov/Database/index.html**

Websites

- The Dana Foundation, **dana.org**
- Neuromuscular Disease Center at Washington University School of Medicine, St. Louis, **neuromuscular.wustl.edu**
- Neuroscience Information Framework, **neuinfo.org**

A3 Technical support

Web resources

Visit the **thermofisher.com** website for:

- Technical resources, including manuals, vector maps and sequences, application notes, Safety Data Sheets (SDSs), FAQs, formulations, citations, and handbooks.
- Complete technical support contact information
- Access to the Thermo Fisher Scientific online catalog
- Additional product information and special offers
- Warranty information

Contact us

For more information or technical assistance, please consult our website at **thermofisher.com/contactus**

SDS

SDSs are available at **thermofisher.com/sds**

Certificate of Analysis

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

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