Chapter 7

Preparation of Neural Stem Cells and Progenitors: Neuronal Production and Grafting Applications

Joseph F. Bonner, Christopher J. Haas, and Itzhak Fischer

Abstract

Neural stem cells (NSC) are not only a valuable tool for the study of neural development and function, but an integral component in the development of transplantation strategies for neural disease. NSC can be used to study how neurons acquire distinct phenotypes and how the reciprocal interactions between neurons and glia in the developing nervous system shape the structure and function of the central nervous system (CNS). In addition, neurons prepared from NSC can be used to elucidate the molecular basis of neurological disorders as well as potential treatments. Although NSC can be derived from different species and many sources, including embryonic stem cells, induced pluripotent stem cells, adult CNS, and direct reprogramming of non-neural cells, isolating primary NSC directly from rat fetal tissue is the most common technique for preparation and study of neurons with a wealth of data available for comparison. Regardless of the source material, similar techniques are used to maintain NSC in culture and to differentiate NSC toward mature neural lineages. This chapter will describe specific methods for isolating multipotent NSC and neural precursor cells (NPC) from embryonic rat CNS tissue (mostly spinal cord). In particular, NPC can be separated into neuronal and glial restricted precursors (NRP and GRP, respectively) and used to reliably produce neurons or glial cells both in vitro and following transplantation into the adult CNS. This chapter will describe in detail the methods required for the isolation, propagation, storage, and differentiation of NSC and NPC isolated from rat spinal cords for subsequent in vitro or in vivo studies.

Key words Neural stem cells, Neural precursor cells, Pluripotency, Multipotency

1 Introduction

1.1 Preface

Neural stem cells (NSC) grown in culture are a valuable tool for the study of neural development, neural function, and the development of therapeutic strategies for neural disease. In the fields of neural development and function, NSC can be used to study how neurons acquire diverse, region specific, phenotypes and how interaction between neurons and glia in the developing nervous system shape the structure and function of the CNS. Although NSC are often considered as a therapeutic tool, they can be used as a source of neurons for mechanistic studies that require a population of naïve neurons that have not yet been influenced by the

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CNS environment [1, 2]. NSC can also be used in the study of neurological disorders to elucidate the molecular basis of the disease and how it could potentially be treated. This application has become particularly exciting with the recent progress of obtaining NSC derived from patient cells through the induced pluripotent technology [3, 4].

Although NSC can be derived from different species and many sources, including embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), adult CNS, and direct reprogramming of non-neural cells [5], isolating primary NSC directly from fetal tissue is an important technique with certain advantages. Whether NSC are derived from fetal tissue or pluripotent sources, such as ESC and iPSC, similar techniques are used to maintain NSC in culture and to differentiate NSC toward mature neural lineages. ESC and iPSC cultures produce neurons only after progressing through a sequence of intermediate cell types with concomitant gradual fate restriction, which include the generation of NSC [6]. In this way, pluripotent stem cell research can follow protocols developed for primary NSC culture. This is particularly evident in the derivation of neurons from NSC, where the diversity and complexity of neuronal phenotypes can be difficult to replicate in vitro. This chapter will describe specific protocols for isolating multipotent neuroepithelial cells (NEP) and neural precursor cells (NPC) from embryonic rat CNS tissue (mostly spinal cord). Several laboratories have previously shown that these cell types can be used to reliably produce neurons in vitro [7–9], with NPC also reliably producing neurons after transplantation into the adult CNS [9, 10].

1.2 Terminology

Stem cell research exists at the junction of development, embryology, and cell biology. While this diversity leads to exciting discoveries across multiple areas of research, confusion often arises regarding terminology. Several terms will be briefly defined and discussed for this Chapter. Stem cells are defined by their phenotypic potency and capacity for self-renewal. *Potency* is the range of cells that a particular stem cell can produce and is best tested by clonal analysis. Pluripotent cells can form all the cells of the mature organism, including cells from all 3 germ layers, and theoretically have unlimited capacity for self-renewal. The term embryonic stem cell (ESC) specifically refers to pluripotent stem cells derived from the inner cell mass of a developing embryo. Multipotent cells can produce multiple types of cells but are generally limited to one germ layer. Both their potency and capacity for self-renewal are more limited than pluripotent cells. The term neural stem cells or NSC generally refers to multipotent cells capable of generating neurons, astrocytes, and oligodendrocytes. The terms *precursor* and *progenitor* refer to a class of stem cells that have committed a more restricted lineage profile than NSC, and therefore have a limited potency and limited capacity for self-renewal. Specific examples of NPC present in the developing CNS include neuronal and glial restricted precursors (NRP and GRP, respectively). The term *fetal stem cell* is sometimes

used to describe a stem cells derived from developmental tissue beyond the blastocyst stage (i.e., cells more mature than ESC). This is often a misnomer, especially when applied to rodent systems that have a relatively short fetal stage (embryonic days 17–21 in rats); however some human neural stem/progenitor cells derived from aborted tissue can be accurately described as fetal stem cells. *Embryonic day* is a term that is used to describe the age of developing organisms. For the purposes of harvesting stem cells, the embryonic day is defined as the number of days since mating/fertilization. However, disagreement exists over how fertilization should be assigned a numerical value. Fertilization is alternatively defined as the beginning of embryonic day 0 (E0) or the beginning of the embryonic day 1 (E1). Likewise, fertilization + 12 h is alternatively defined as E0.5 or E1.5. For the purposes of this chapter we will continue to use the nomenclature that we have used in our previous studies, with fertilization being defined as the beginning of E0; however, readers should note that many animal vendors designate fertilization as E1 when selling timed pregnant rats.

1.3 Neural Stem Cells and Neural Progenitors of the Developing Spinal Cord

The developing rat spinal cord has been well described in the literature, both in terms of isolation of NSC and NPC populations for in vitro studies, as well as the use of NSC, NPC, and fetal spinal cord tissue for transplantation studies in vivo [11–13]. At E10.5 the caudal neural tube contains neuroepithelial cells (NEP), a multipotent NSC population capable of generating neurons, astrocytes, and oligodendrocytes. Clonal analysis has confirmed that NEP are a common progenitor for the cellular phenotypes found in the adult spinal cord [14], including motor neurons, interneurons, and sensory neurons [7]. NEP can be identified by immunocytochemistry for Nestin (Fig. 1a) and Sox2. Although NEP have a robust capacity to generate multiple neuronal and glial phenotypes in vitro, they show poor survival following transplantation into the adult CNS, likely due to a lack of trophic support in the mature CNS microenvironment [10]. Other sources of multipotent NSC show improved survival, but will only produce glial progeny in the spinal cord, despite being able to produce neurons in neurogenic regions on the CNS [15, 16]. Thus, NEP are a useful source of multiple neuronal phenotypes for in vitro studies, but must be predifferentiated to a more mature, intermediate stage (e.g., NRP) prior to use in transplantation studies.

Later in development, at E13.5, the spinal cord contains both neuronal and glial restricted precursors (NRP and GRP, respectively). NRP will only differentiate into neurons [17] and GRP will only differentiate into astrocytes and oligodendrocytes [18]. Thus NRP/GRP cultures are a source for both neurons and glia (Fig. 2). NRP can be derived from ESC, NEP, or directly from the developing spinal cord and retain the ability to generate multiple neurotransmitter phenotypes both in vitro and in vivo [19, 20]. Like NEP, NRP/GRP express the intermediate filament Nestin (Fig. 1b), but NRP can be specifically identified by ENCAM

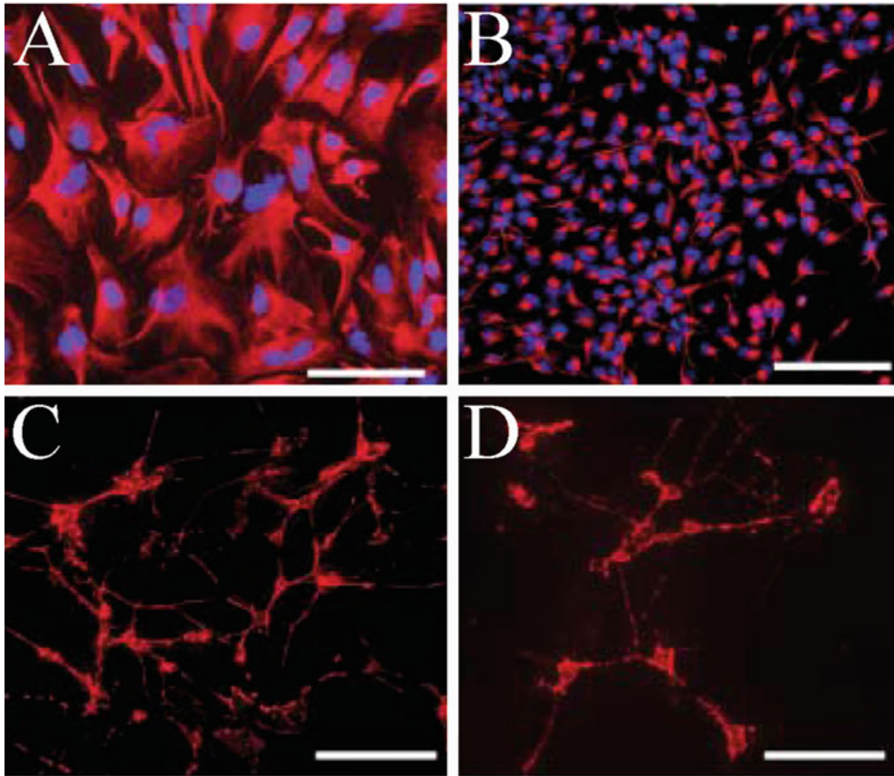

Fig. 1 Neuroepithelial cells (NEP) grow on fibronectin substrates in the presence of bFGF and CEE express the intermediate filament, nestin (**a**, *red*), a neural stem cell marker, throughout the cell body. NRP/GRP grown on poly-L-lysine and laminin substrates in the presence of bFGF and NT-3 also express nestin (*red*, **b**). However, NRP also express ENCAM (**c**) and GRP also express A2B5 (**d**). ENCAM and A2B5 can be used to specifically identify NRP and GRP, respectively, from primary E13.5 spinal cord cultures. Scale bars = 50 μm

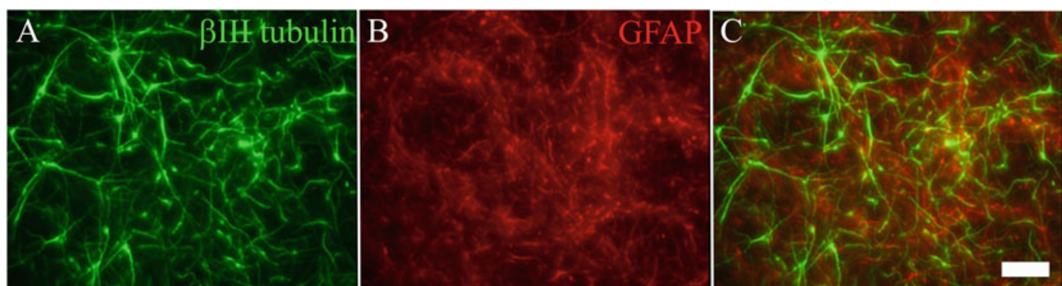

Fig. 2 NRP/GRP grown on PLL/Laminin substrate produce high density βIII tubulin + neurons (**a**) and GFAP+ astrocytes (**b**) when grown in NRP basal medium supplemented with 0.5 % High concentration Matrigel (BD Bioscience, San Jose, CA)

expression (Fig. 1c), whereas GRP can be specifically identified by A2B5 expression (Figs. 1d and 3a). Unlike NEP, NRP survive transplantation in the adult CNS. When transplanted into the intact CNS, NRP will survive and generate neurons [9] (Fig. 4) with appropriate neurotransmitter identities (Fig. 5), per the local

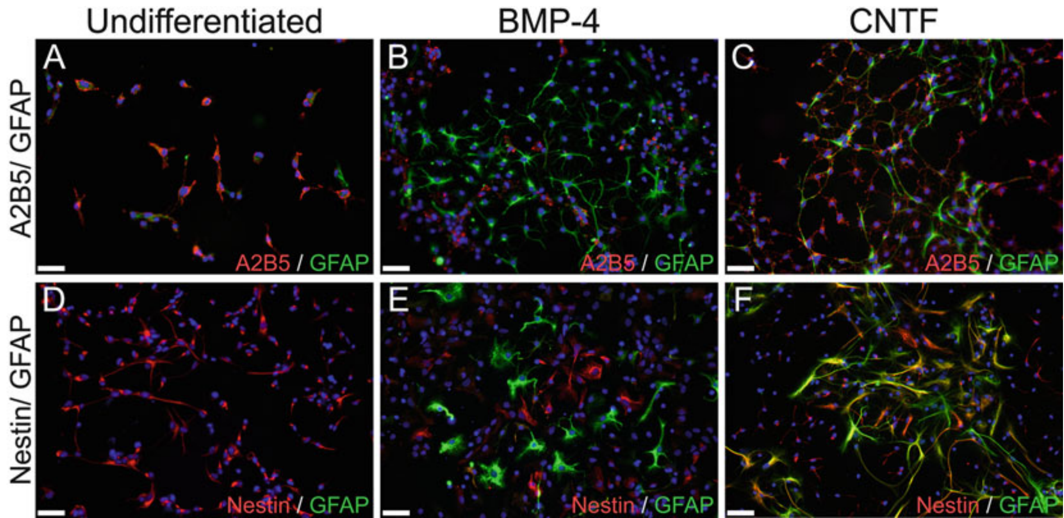

Fig. 3 Enriched GRP grown on PLL substrate express high levels of A2B5 (**a**) and Nestin (**d**). When differentiated with BMP-4 (**b**, **e**) or CNTF (**c**, **f**) GRP produce astrocyte subpopulations characterized by low levels of A2B5 and Nestin, but high levels of GFAP (BMP-4), or intermediate levels of A2B5 and Nestin, and high levels of GFAP (CNTF). Scale bar = 25 μM

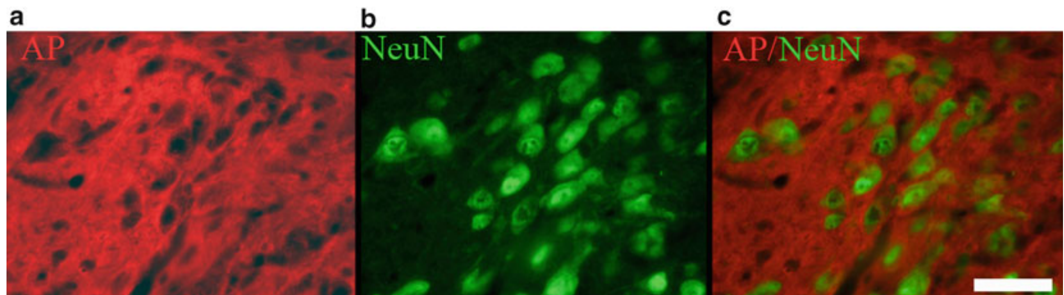

Fig. 4 NRP/GRP derived from AP+ rat embryos survive in the injured cervical spinal cord and produce neurons after differentiation. Immunohistochemistry demonstrates that NRP/GRP express the AP transgene (**a**, **c**) and produce NeuN+ neurons (**b**, **c**) 4 weeks after grafting. Scale bar = 50 μm

microenvironment [19]. However, when purified NRP are transplanted into the injured CNS, their survival and differentiation is poor because the injured spinal cord lacks the appropriate microenvironment to support NRP [8]. We have previously demonstrated that combined grafts of NRP/GRP in the injured spinal cord produce astrocytes, oligodendrocytes, and neurons, indicating that GRP are capable of producing a microenvironment that supports NRP survival and differentiation [21]. NRP will produce both glutamatergic and GABAergic neurons in the injured spinal cord when grafted with GRP. Neurons derived from NRP have been shown to form synapses in vitro [17] and in vivo [22], demonstrating that NRP produce neurons with characteristics of mature, functional neurons.

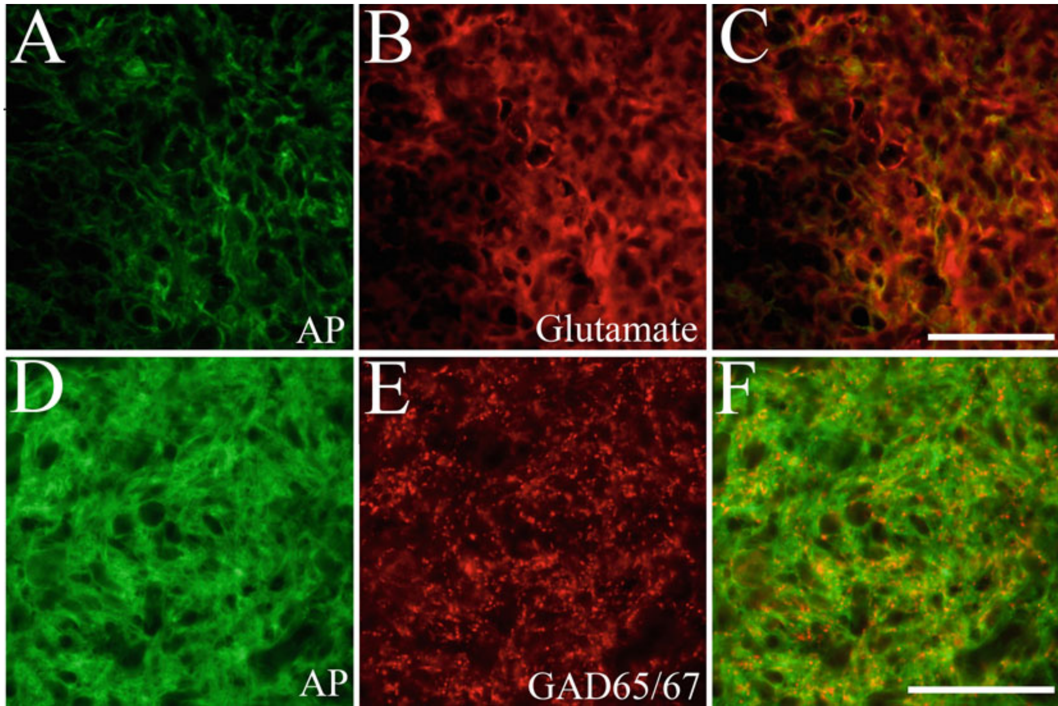

Fig. 5 NRP/GRP derived from AP+ rat embryos were grafted into the injured cervical spinal cord of adult rats. NRP/GRP survive, fill the lesion, and continue to express the AP transgene 6 weeks after grafting in the injured spinal cord (**a**, **d**). The NRP differentiate and express markers for both glutamatergic (glutamate, **b**) and GABAergic (GAD65/67, **e**) neurons. Scale bars = 50 μm

2 Materials

2.1 Preparation of Neuroepithelial Cells (NEP) from E10.5 Rat Spinal Cord

2.1.1 Fibronectin-Coated Dishes

1. Bovine Serum fibronectin.
2. Tissue Culture Water.
3. Hanks Balance Salt Solution without calcium and magnesium.
4. Tissue culture flask or glass coverslips.
5. T-25 tissue culture flask, 35 mm × 10 mm tissue culture dish, or glass coverslips can be used for the culture of NEP (*see* **Note 1**).

2.1.2 Chick Embryo Extract (CEE)

1. Fertilized chicken eggs.
2. Eagle's Minimal Essential Medium.
3. 20 ml syringes.
4. 60 ml syringes.
5. 50 ml conical tubes.
6. Hyaluronidase 1 mg/ml solution.
7. 0.45 μm sterile syringe filter.
8. 0.22 μm sterile syringe filter.

2.1.3 NEP Medium

1. DMEM/F12.
2. Bovine Serum Albumin (BSA).
3. B-27 (50× stock solution).
4. Pen/Strep (100× stock solution).
5. L-Glutamine (100× stock solution).
6. Fungizone (1,000× stock solution).
7. N2 supplement (100× stock solution).
8. NEP Complete Medium.
9. NEP Basal Medium.
10. Chick Embryo Extract (CEE).
11. bFGF.

2.1.4 NEP Dissection (All Materials Must Be Sterile)

1. Anesthetic.
2. E10.5 timed-pregnant rat.
3. Scissors.
4. 100 mm × 20 mm cell culture dishes.
5. DMEM/F12.
6. Tungsten needles.
7. Collagenase I/dispase II solution.
8. NEP complete medium.
9. Pasteur pipette.
10. #5 forceps.
11. 0.05 % Trypsin/EDTA.
12. Soybean trypsin inhibitor.
13. Fibronectin-coated T-25 flask or equivalent.

2.1.5 Passaging NEP Cells

1. 3 T-25 flasks, 1T-75 flask, or equivalent.
2. HBSS without calcium or magnesium.
3. 0.05 % Trypsin/EDTA.
4. Soybean trypsin inhibitor.

2.1.6 Freezing NEP

1. NEP Basal.
2. DMSO.
3. CEE.
4. Cryovials.

2.1.7 Thawing NEP

1. NEP basal medium.
2. NEP complete medium.
3. Fibronectin-coated culture dishes, T-25 flask, T-75 flask, or equivalent.

2.2 Preparation of Neuronal Restricted Progenitors and Glial Restricted Progenitor from E13.5 Rat Spinal Cord

2.2.1 Preparation of Poly-L-Lysine and Laminin Coated Dishes for NRP/GRP Culture

1. Poly-L-Lysine (PLL).
2. Tissue Culture Water.
3. 0.22 μm sterile syringe filter.
4. Natural Mouse Laminin.
5. Sterile Phosphate Buffered Saline (PBS).

2.2.2 NRP/GRP Medium

1. DMEM/F12.
2. BSA.
3. B-27 (50× stock solution).
4. Pen/Strep (100× stock solution).
5. N2 supplement (100×).
6. NRP/GRP complete medium.
7. NRP basal medium.
8. bFGF.
9. NT-3.

2.2.3 NRP/GRP Dissection

All materials must be sterile.

1. Anesthetic agent.
2. 70% ethanol.
3. 100 mm × 20 mm cell culture dish.
4. DMEM/F12.
5. E13.5–E14.0 timed-pregnant rat.
6. Microscissors.
7. #5 fine forceps.
8. Collagenase I/dispase II solution.
9. 50 ml conical tube.

2.2.4 NRP/GRP Dissociation

1. 0.05 % Trypsin/EDTA.
2. Soybean trypsin inhibitor.
3. NRP/GRP basal medium.

2.2.5 NRP/GRP Culture

1. NRP/GRP complete medium.
2. Poly-L-Lysine and Laminin T-75 tissue culture flask, or equivalent.

2.2.6 Enrichment of NRP/GRP

1. Anti-A2B5 IgM antibody (DSHB).
2. Anti-ENCAM IgM antibody (DSHB).

Table 1
A partial list of useful antibodies and dilutions

Antigen	Host species	Recommended dilution	Vendor	Product ID	Use
Nestin	Mouse IgG1	1:1,000	BD Pharmigen	556309	NEP, NRP/GRP
Sox2	Mouse IgG2A	1:200	R&D	MAB2018	NEP
A2B5	Mouse IgM	1:2	DSHB		GRP
ENCAM	Mouse IgM	1:2	DSHB		NRP
BIII Tubulin	Mouse IgG2a	1:500	Covance	MMS-435P	Neurons
BIII Tubulin	Rabbit	1:1,000	Covance	Prb-435P	Neurons
GFAP	Mouse IgG1	1:1,000	Millipore	MAB3402	Astrocytes
GFAP	Rabbit	1:2,000	Millipore	AB5804	Astrocytes
RIP	MouseIgG1	1:10,000	Millipore	MAB1580	Oligodendrocytes
O4	Mouse IgGM	1:50	R&D	MAB1326	Oligodendrocytes

Developmental Studies Hybridoma Bank (DSHB)

3. Goat anti-Mouse IgM, unlabeled (Millipore, AP128).
4. Sterile, bacteriological polystyrene petri dishes.
5. Freshly dissected NRP/GRP.

2.2.7 Freezing NRP/GRP

1. NRP/GRP Freezing Medium.
2. NRP/GRP Basal.
3. DMSO.
4. CEE.
5. NT-3.
6. bFGF.

2.3 Characterizing NEP and NRP/GRP Cultures In Vitro with Immunocytochemistry

Antibodies and dilutions

3 Methods

3.1 Preparation of Neuroepithelial Cells (NEP) from E10.5 Rat Spinal Cord

NEP are a multipotent cell type capable of producing neurons, astrocytes, and oligodendrocytes and NEP can self-renew in vivo. Therefore, NEP are a true NSC population and represent an intermediate step between less mature cells, such as ESC, and mature neural phenotypes. NEP are grown in an adherent culture on

fibronectin-coated dishes rather than as neurospheres, which helps to maintain the uniformity of the culture and provides the opportunity for accurate observation of cultures with simple phase microscopy. NEP identity can be confirmed using antibodies for Nestin and Sox2. Isolation of NEP from the neural tubes of rats requires a combination of physical and chemical dissection techniques and should take 1–2 h per litter. We recommend having at least two technicians prepared to conduct the dissection, as rodent litters may vary between 2 and 15 embryos. Also take this into account when preparing medium, culture dishes, etc. (*see* **Note 2**).

3.1.1 Fibronectin-Coated Dishes

NEP can be expanded and maintained in their multipotent state on fibronectin-coated dishes. The following protocol describes how to use bovine fibronectin for coating. After coating, fibronectin-coated dishes are stable for 2 months at 4 °C.

1. Dilute fibronectin in Tissue Culture Water at 20 μg/ml.
2. Add enough fibronectin solution to the culture dish to just cover the bottom of the dish.
3. Place the dish on a rocker at 4 °C overnight.
4. Following day rinse flasks 2× with HBSS without calcium and magnesium.
5. Cover bottom of flask with HBSS for storage.
6. Coated dishes can be stored for up to 2 months.
7. Rinse flasks with warm (37 °C) tissue culture medium before use.

3.1.2 Collagenase I/Dispase II Solution

A solution of collagenase I/dispase II will be used to dissociate the neural tubes during the NEP dissection and may be used during the NRP/GRP dissection. It should always be made fresh the day of the dissection.

1. Combine 1 mg/ml collagenase I (Worthington) and 2 mg/ml Dispase II (Roche) in Hanks Balanced Salt Solution (HBSS) without calcium and magnesium.
2. Pass through a sterile 0.22 μm filter.

3.1.3 Chick Embryo Extract (CEE)

CEE is a critical component of the NEP medium and freezing medium for both NEP and NRP/GRP. CEE can be prepared well in advance and stored at −80°. In our experience the effectiveness of CEE varies by vendor. We recommend using the CEE from US Biological (San Antonio, TX, USA); otherwise follow the steps below to produce CEE.

1. 36–40 fertilized chicken eggs are incubated for 11 days at 37.5 °C.
2. Wash eggs with 70 % ethanol.
3. Remove embryos in small batches, decapitate with sterile forceps and rinse 3× in petri dish with ice-cold sterile Eagle's

Table 2
NEP Media

NEP Basal Medium	
Component	**Concentration**
DMEM/F12	94.9 %
Bovine Serum Albumin (BSA)	1 mg/ml
B-27 (50× stock solution)	2 %
Pen/Strep (100× stock solution)	1 %
L-glut (100× stock solution)	1 %
Fungizone (1,000× stock solution)	0.1 %
Pass medium through 0.22 μm sterile filter	
N2 supplement (100× stock solution)	1 %
NEP Complete Medium	
Component	**Concentration**
NEP Basal Medium	90 %
Chick Embryo Extract (CEE)	10 %
bFGF	20 ng/ml

Minimal Essential Medium (MEM) until all embryos are harvested.

4. Embryos are macerated by passage through a 20 ml syringe into 50 ml conical tubes.
5. Ten embryos produce about 25 ml of homogenate; add equal volume of MEM.
6. Rock the tubes at 4 °C for 1 h.
7. Add sterile hyaluronidase (1 mg/25 ml of chick embryo).
8. Centrifuge for 6 h at 30,000 × *g*.
9. Collect the supernatant into a 60 ml syringe and pass through a 0.45 μm filter.
10. Collect the filtrate into another 60 ml syringe and pass through a 0.22 μm filter.
11. Aliquot and store at –80 °C.

3.1.4 NEP Medium

NEP medium should be prepared under sterile conditions in a tissue culture hood to prevent contamination. The materials required are listed in Table 2 and should be prepared in DMEM/F12 for growth of cells in 5–10 % CO_2 environment. NEP basal medium can be used for manipulating cells during the dissection and other

procedures, but NEP complete medium (NEP basal + bFGF and CEE, *see* Table 2) should be used for culturing cells. Complete medium should only be prepared as needed to save costs.

3.1.5 NEP Dissection

1. Anesthetize the dam with an appropriate agent, such as Euthasol.
2. When the dam is anesthetized, swab the abdomen with 70 % ethanol for 10 s.
3. Perform a laparotomy and remove both uterine horns.
4. Euthanize the dam with a thoracotomy.
5. Remove a sac from the uterine horn and rinse 1× in 70 % ethanol 2× in DMEM/F12.
6. Place in the lid of a sterile 100 mm × 20 mm cell culture dish partially filled with DMEM/F12 (*see* **Note 3**).
7. If the embryo is the correct gestational age it should be "C" shaped and it should be expelled from the sac when sac is cut. Count somites to insure correct gestational age (13–20 somites).
8. Dissect trunk segments of neural tube with surrounding somites and tissue (last ten somites) using tungsten needles.
9. Trunk segments are then incubated for approximately 7 min at room temperature with 1–2 ml of collagenase I/dispase II (*see* **Note 4**).
10. After the neural tubes begin to separate, carefully aspirate collagenase I/dispase II and replace with 2 ml NEP complete medium (*see* **Note 5**).
11. Carefully triturate with a Pasteur pipette to release neural tubes from the somites and connective tissue.
12. Remove any remaining somites with #5 forceps and/ or tungsten needles.
13. Remove the supernatant and incubate the neural tubes in 2 ml 0.05 % Trypsin/EDTA at 37 °C for 5 min.
14. After 5 min add an equal volume (2 ml) of soybean trypsin inhibitor.
15. Triturate the cells to dissociate the neural tubes.
16. Spin at 150–300 × *g* for 5 min.
17. Resuspend cells in 5 ml NEP complete medium and plate on a fibronectin-coated T-25 flask.
18. Incubate cells at 37 °C and 5 % CO_2.

3.1.6 Passaging NEP Cells

1. Grow cells to 80–90 % confluency before splitting.
2. Plan to split cells into three times the surface area (i.e., 1T-25 flask to 3T-25 flasks or 1T-75 flask).

Table 3
NEP freezing medium

Component	Concentration
NEP Basal	80 %
DMSO	10 %
CEE	10 %
Pass medium through 0.22 μm sterile filter	

3. Remove medium and rinse 2× with warmed HBSS.
4. Add 0.05 % Trypsin/EDTA (enough to cover base of culture dish).
5. When cells have lifted add equal volume of soybean trypsin inhibitor.
6. Add 10 ml of complete medium and spin at 150–300 × *g* for 5 min.
7. Decant supernatant and resuspend pellet in complete medium and plate on fibronectin-coated dishes.

3.1.7 Differentiation of NEP to NRP/GRP

1. Passage cells as described in Subheading 3.1.6 and plate on Poly-L-Lysine (PLL) and Laminin (LN) substrates (same substrate as NRP/GRP).
2. Remove CEE from culture medium to allow NEP to differentiate.
3. Add bFGF (30 ng/ml) to NEP complete medium to bias cells towards GRP.
4. Add bFGF (10 ng/ml) and NT-3 (10 ng/ml) to NEP complete medium to bias cells towards NRP.

3.1.8 Freezing NEP (See Table 3)

1. Cells should be approximately 90 % confluent.
2. Remove medium.
3. Rinse 2× with warmed HBSS to remove any dead or floating cells.
4. Incubate at 37 °C with 0.05 % Trypsin/EDTA.
5. Once cells have lifted, add an equal amount of soybean trypsin inhibitor.
6. Decant cells into a conical vial.
7. After cells are decanted, wash the flask 2× with 5 ml NEP complete medium to resuspend any cells that were left behind in the previous step. Decant and add to the same conical vial as the cell suspension.
8. Spin at 150–300 × *g* for 5 min.

9. Decant supernatant and resuspend cells in freezing medium (3 ml per confluent T-75 flask).
10. Aliquot 1 ml each into 2 ml cryovials.
11. Place cells in −80 °C overnight.
12. Transfer to liquid nitrogen following day.

3.1.9 Thawing NEP

1. Prior to thawing cells, warm (37 °C) 10 ml of NEP basal medium and 15 ml of NEP complete medium.
2. Take cryovial from liquid nitrogen and bury it in bucket of wet ice.
3. Thaw aliquot in 37 °C water bath until cells just begin to thaw.
4. Add 1 ml warmed medium drop by drop to cryovial.
5. Add the 2 ml suspension drop by drop to 10 ml warmed medium.
6. Rinse cryovial once with 1 ml warmed medium.
7. Spin at 150–300 × *g* for 5 min.
8. Decant supernatant.
9. Resuspend cells in 15 ml NEP complete medium and plate in a fibronectin-coated T-25 flask.

3.2 Preparation of Neuronal Restricted Progenitors and Glial Restricted Progenitor from E13.5 Rat Spinal Cord

NRP/GRP are prepared as a mixed culture of cells that are collectively capable of producing neurons, astrocytes, and oligodendrocytes, but NRP will only produce neurons whereas GRP will only produce astrocytes and oligodendrocytes. Therefore, NRP/GRP are not a true NSC population but represent a necessary intermediate step between multipotent NSC and mature neural or glial phenotypes. NRP/GRP are grown in an adherent culture on dishes coated with Poly-L-Lysine and Laminin. NRP can be identified by expression of embryonic neural cell adhesion molecule (E-NCAM) and GRP can be identified by expression of A2B5. Preparation of NRP/GRP from E13.5 embryos requires a primary physical dissection followed by chemical and mechanical dissociation and culturing. The full dissection should take 1–2 h and, as with NEP dissection, we recommend having at least 2 technicians prepared to conduct the dissection, as rodent litters may vary between 2 and 15 embryos.

3.2.1 Preparation of Poly-L-Lysine and Laminin Coated Dishes for NRP/GRP Culture

Preparation of Poly-L-Lysine/Laminin (PLL/LN) coated dishes is a two step process requiring 2 days to complete. The coating should be carried out at 4 °C on a rocker. Coated dishes are stable for 4 weeks after coating.

1. Prepare 10× (150 μg/ml) stock of PLL in Tissue Culture Water, freeze, and store at −20 °C.

2. PLL (Day 1):
   (a) Dilute stock solution of PLL with Tissue Culture Water to 15 μg/ml.
   (b) Filter PLL mixture with 0.22 μm syringe filter and 60 ml syringe.
   (c) Coat bottom of T-75 flask with 15 ml of 15 μg/ml PLL solution.
   (d) Place overnight on rocker at 4 °C.
3. Laminin (Day 2)
   (a) Laminin stocks must be kept at −80 °C and should be thawed for 2–4 h at 4 °C (*see* **Note 6**).
   (b) Aspirate PLL and wash 2× with PBS prior to adding Laminin.
   (c) Prepare 15 μg/ml solution of mouse Laminin in PBS. Do Not Filter.
   (d) Add 15 ml of 15 μg/ml Laminin in PBS to T-75 flask and coat on rocker overnight at 4 °C.
   (e) On third day, rinse 1× with PBS.
   (f) Add 15 ml of HBSS and store at 4 °C for up to 1 month.

3.2.2 NRP/GRP Medium

NRP/GRP medium should be prepared under sterile conditions in a tissue culture hood to prevent contamination. The materials required are listed in Table 4 and should be prepared in DMEM/F12 for growth of cells in 5–10 % CO_2 environment. NRP/GRP basal medium can be used for manipulating cells during the dissection and other procedures, but NRP/GRP complete medium (NRP/GRP basal + bFGF and NT-3, *see* Table 4) should be used for culturing cells.

3.2.3 NRP/GRP Dissection

1. Anesthetize the dam with an appropriate compound, such as Euthasol.
2. When the dam is anesthetized, swab the abdomen with 70 % ethanol for 10 s.
3. Perform a laparotomy and remove both uterine horns.
4. Euthanize the dam with a thoracotomy.
5. Place the entire uterus in a sterile 100 mm × 20 mm cell culture dish with DMEM/F12 on ice.
6. Remove a sac from the uterine horn and rinse the sac 1× in 70 % ethanol and 2× in DMEM/F12.
7. Place in the lid of a sterile 100 mm × 20 mm cell culture dish partially filled with DMEM/F12 (*see* **Note 7**).
8. Using microscissors, open the sac and release the embryo.

Table 4
NRP/GRP media

NRP/GRP basal medium	
Component	**Concentration**
DMEM/F12	96 %
BSA	1 mg/ml
B-27 (50× stock solution)	2 %
Pen/Strep (100× stock solution)	1 %
Pass medium through 0.22 μm sterile filter	
N2 supplement (100×)	1 %
NRP/GRP complete medium	
Component	**Concentration**
NRP basal medium	100 %
bFGF	20 ng/ml
NT-3	10 ng/ml

9. Measure the crown to rump distance to determine the embryonic age, the embryo should be between 8.5 and 10 mm long for an E13.5–E14 spinal cord.
10. Using a pair of microscissors, remove the head at the cisterna magna (*see* **Notes 8** and **9**).
11. Place the embryo with the ventral surface down.
12. Incise the midline skin over the neural tube (i.e., from the dorsal surface) using a pair of microscissors.
13. Firmly hold the embryo down with jeweler's forceps. Place the forceps in an area of the embryo that is not needed as it will be damaged (*see* **Note 10**).
14. Using sharp #4 or #5 forceps, dissect the spinal cord with associated meninges and DRGs from the embryo.
15. Peel off the meninges and DRGs as far caudally as possible. Place any cleaned sections of the spinal cord in a 50 ml conical tube of ice-cold DMEM/F12.
16. The lumbar cord may require the use of collagenase I/dispase II solution to remove meninges; if so place the lumbar cord in collagenase I/dispase II for 9 min (*see* **Note 11**).
17. Remove cord from collagenase I/dispase II and place in 100 mm × 20 mm dish with DMEM/F12.

18. Finish removing the meninges and place the cleaned spinal cord into 50 ml conical tube with DMEM/F12. Place up to three cords in one 50 ml conical tube.

3.2.4 NRP/GRP Dissociation

1. After the dissection is complete, centrifuge pooled cords at 150–300 × *g* for 10 min
2. Decant the supernatant from the 50 ml conical vial containing the cords.
3. Add 1 ml of 0.05 % trypsin–EDTA and incubate for at 37° for 10–20 min.
4. Using a sterile plugged 1,000 μl pipet tip, set at 800 μl, gently triturate cells about ten times until chunks of tissue look dissociated by eye.
5. Add equal volume 1 mg/ml soybean trypsin inhibitor to quench trypsin, and gently triturate with 10 ml pipet.
6. Do not over-triturate or many cells will die.
7. Pass cells suspension through a 40 μm filter- Cell strainer (BD Falcon 352340) into a fresh 50 ml tube.
8. Rinse the filter with another 10 ml NRP Basal Medium.
9. Centrifuge cell suspension at 150–300 × *g* for 5–10 min to get rid of the trypsin (*see* **Notes 12** and **13**).
10. Carefully decant the supernatant and add 1 ml of NRP basal medium.
11. Dilute 10 μl cells suspension 1:1 in Trypan blue and count with hemocytometer.
12. Discard the supernatant (*see* **Note 14**).

3.2.5 NRP/GRP Culture

1. Resuspend pellet with NRP/GRP complete medium (NRP/GRP basal medium, supplemented with 20 ng/ml bFGF and 10 ng/ml NT3) in PLL/LN-coated flask.
2. Recommended density: 1.5–3 × 10^6 cells/T-75 flask, or 5 × 10^5 to 1 × 10^6 cells/T-25 flask.
3. Incubate cells at 37 °C and 5 % CO_2.
4. NRP/GRP complete medium should be replaced every other day.

3.2.6 Differentiation of NRP/GRP Towards Neuronal Lineage

1. Maintain NRP/GRP on Poly-L-Lysine and Laminin substrate.
   (a) Remove bFGF.
   (b) Add retinoic acid (1 μM) to promote neuronal differentiation and NT-3 (10 ng/ml) to promote neuronal survival.

OR

2. Maintain NRP/GRP on Poly-L-Lysine and Laminin substrate.
   (a) Remove bFGF.
   (b) Add 0.5 % High concentration Matrigel (Fig. 2).

3.2.7 GRP Enrichment and Differentiation Toward Glial Lineage

1. Maintain NRP/GRP on Poly-L-Lysine (100 μg/ml) substrate.
2. Remove NT-3.
3. Passage cells for 7–10 days prior to freezing to enrich for GRP.
4. Thaw enriched GRP on Poly-L-Lysine substrate (Fig. 3a, d).
5. Remove bFGF and NT-3.
6. Add BMP-4 (10 μg/ml; Fig. 3b, e) OR CNTF (10ug/ml; Fig. 3c, f).

3.2.8 Enrichment of NRP/GRP

NRP/GRP are isolated as a mixed culture, but some procedures may require enriched NRP or GRP. Pure NRP cultures are difficult to produce, especially in large numbers because NRP are generally more sensitive to manipulation than GRP. Despite these difficulties, enrichment for NRP is possible. Nearly pure populations of GRP can be produced reliably and with high quantities of cells by exploiting the greater ability of GRP to self-renew [23]. The difficulties in sorting NRP and GRP are due, in part, to the limitations of A2B5 and ENCAM IgM primary antibodies. IgM antibodies are, generally speaking, less suited to immunopanning and fluorescent activated cell sorting (FACS) than IgG antibodies.

Immunopanning is a procedure that uses an antibody covered substrate to bind a subset of cells to the substrate. Immunopanning is often conducted in a 2-phase procedure where cells are first negatively sorted (unwanted cells are bound to the substrate and discarded) followed by a positive selection (desired cells are placed on a new substrate, bound and collected). Thus, when sorting NRP/GRP both A2B5 and ENCAM substrates will always be prepared, only the order will be changed depending on the cell population that is desired.

1. *Step 1: Substrate Preparation*: Start of day1: Incubate two bacteriological petri dishes overnight at 4 °C with 10 ml of goat anti-mouse IgM (10 μg/ml) diluted in Tris–HCl 50 mM ph 9.5 (*see* **Note 15**).
2. Start of day 2: Remove the goat anti-mouse antibody.
3. Rinse the dish with HBSS without magnesium and calcium 3×.
4. Coat 1 dish with mouse-anti-ENCAM IgM hybridoma diluted 1:1 with HBSS without magnesium and calcium.

5. Coat the other dish with mouse-anti-A2B5 IgM hybridoma diluted 1:1 with HBSS without magnesium and calcium.
6. 2 h later remove the panning antibody solution.
7. Rinse the dishes 3× with HBSS without magnesium and calcium.
8. Store coated dishes with HBSS until ready for use.
9. Proceed to the negative selection.
10. *Step 2: Negative selection*: Begin with freshly isolated NRP/GRP suspended in 10 ml NRP/GRP complete medium (from Subheading 3.2.4).
11. The negative selection will remove undesirable cell types. Plate cells on dish coated with antibody specific to the undesirable cell type (i.e., if purified NRP are desired, plate the mixed NRP/GRP on the A2B5 coated dish).
12. Gently swirl the cells to insure the cells and evenly dispersed and there are no clumps.
13. Incubate the cells at *room temperature* for 1 h (*see* **Note 16**).
14. Gently tap the petri dish to remove any nonspecifically bound cells.
15. Remove the cell suspension and place in a 15 ml conical tube.
16. Discard the bound fraction.
17. Spin the cell suspension at 150–300 × *g* for 5 min.
18. Resuspend the cells in 10 ml NRP/GRP complete medium.
19. *Step 3: Positive selection*: The positive selection will bind the desirable cell type to the plate. Plate cells on dish coated with antibody specific to the desirable cell type (i.e., if purified NRP are desired, plate the mixed NRP/GRP on the ENCAM coated dish).
20. Add cell suspension to ENCAM coated dish.
21. Gently swirl the cells to insure the cells and evenly dispersed and there are no clumps.
22. Incubate the cells at *room temperature* for 1 h (*see* **Note 16**).
23. Gently tap the petri dish to remove any nonspecifically bound cells.
24. Remove the cell suspension and discard.
25. Wash the ENCAM plate 2× with NRP/GRP basal medium to remove any nonspecifically bound cells.
26. Use a cell scraper to remove the bound cells.
27. Resuspend the cells in 10 ml NRP/GRP complete medium.
28. Plate the cells on Poly-L-Lysine/Laminin coated dishes (or other desired substrate).

Table 5
NRP/GRP freezing media

NRP/GRP freezing medium	
Component	**Concentration**
NRP/GRP Basal	80 %
DMSO	10 %
CEE	10 %
NT-3	20 ng/ml
bFGF	30 ng/ml
Pass medium through 0.22 μm sterile filter	

3.2.9 Freezing NRP/GRP (See Table 5)

1. Cells should be approximately 80 % confluent.
2. Remove medium.
3. Rinse 2× with warmed HBSS to remove any dead or floating cells.
4. Incubate at 37 °C with 0.05 % Trypsin–EDTA.
5. Once cells have lifted, add an equal amount of soybean trypsin inhibitor.
6. Transfer cells into a conical vial.
7. Add 5–10 ml of complete medium, count cells.
8. Spin at 150–300 × *g* for 5 min.
9. Decant supernatant and resuspend cells in freezing medium (3 ml per confluent T-75 flask) (*see* **Note 17**).
10. Aliquot 1 ml each into 2 ml cryovials.
11. Place cells in −80 °C overnight (*see* **Note 18**).
12. Transfer to liquid nitrogen following day.

3.2.10 Thawing NRP/ GRP

1. Prior to thawing cells, warm (37 °C) 10 ml of NRP basal medium and 15 ml of NRP complete medium.
2. Take cryovial from liquid nitrogen and bury it in bucket of wet ice.
3. Thaw aliquot in 37 °C water bath until cells just begin to thaw.
4. Add 1 ml warmed medium drop by drop to cryovial.
5. Add the 2 ml suspension drop by drop to 10 ml warmed medium.
6. Rinse cryovial once with 1 ml warmed medium.
7. Spin at 150–300 × *g* for 5 min.

8. Decant supernatant.
9. Resuspend cells in 15 ml NRP/GRP complete medium and plate in a PLL/LM coated T-75 tissue culture flask.

3.3 Characterizing NEP and NRP/GRP Cultures In Vitro with Immunocytochemistry

NEP and NRP/GRP can be easily identified in culture with immunocytochemistry. NEP express Nestin and Sox2, NRP express Nestin and ENCAM, and GRP express Nestin and A2B5. E-CAM and A2B5 are cell surface antigens and should be performed as a live stain. Other markers, such as those for mature neurons (BIII tubulin), astrocytes (GFAP), and oligodendrocytes (O4), can be performed on fixed cells.

3.3.1 Staining Live Cells

1. Decant medium.
2. Wash with 37 °C HBSS 2×.
3. Add 37 °C complete medium with primary antibody (Table 1).
4. Incubate at 37 °C and 5 % CO_2 for 30 min.
5. Decant medium.
6. Wash with warm HBSS 2×.
7. Add warmed complete medium with secondary antibody (1:400).
8. Incubate at 37 °C and 5 % CO_2 for 30 min.
9. Wash with 37 °C HBSS 2×.
10. Add 4 % PFA for 10 min.
11. Wash 3× with PBS.
    (a) Option 1: Counterstain with DAPI (1:1,000), coat with aqueous cover slipping medium and add coverslip.
    (b) Option 2: Store in PBS at 4 °C for additional staining at a later time.
    (c) Option 3: Perform another stain as described in the following section.

3.3.2 Staining Fixed Cells

1. Decant medium.
2. Add 4 % Paraformaldehyde (PFA) for 10 min.
3. Wash with PBS for 5 min 3×.
4. Block for 30 min in 5 % milk or 5 % normal serum at room temperature.
5. Treat with 0.2 % Triton in PBS if needed.
6. Add primary antibody (Table 1) in PBS with 2 % milk or serum.
7. Incubate for 30 min at room temperature.
8. Wash with PBS for 5 min 3×.
9. Add secondary antibody in PBS with 2 % milk or serum (*see* **Note 19**).

10. Incubate for 30 min at room temperature.
11. Wash with PBS for 5 min 3×.
12. Counterstain with DAPI (1:1,000).
13. Coat with aqueous cover slipping medium and add coverslip.

3.4 Summary

Neural stem cells and neural progenitor cells can be a useful source of neurons for in vitro and in vivo studies of neural function. Both NSC, such as neuroepithelial cells (NEP), and NPC, such as neuronal and glial restricted progenitors (NRP/GRP), can be directly isolated from the developing mammalian spinal cord or from pluripotent sources. The techniques discussed here should provide the ability to dissociate culture, freeze, thaw, and differentiate NEP or NRP/GRP towards mature neuronal and glial phenotypes.

4 Notes

1. Smaller culture dishes are recommended due to the small yield from NEP dissection.
2. Dams often produce fewer pups as a result of the first pregnancy. If using an in-house breeding colony using the second or third pregnancy may produce more pups per litter.
3. Using DMEM/F12 without phenol red may make the neural tubes easier to visualize.
4. Inspect the neural tubes under a dissecting scope and stop the collagenase1/dispase II reaction when there is evidence that the tubes and connective tissue are separating.
5. Alternatively, tubes can be moved with a pipette to a fresh vial of NRP complete medium.
6. If using small amounts of Laminin for coating coverslips, Laminin can be thawed, aliquoted, and refrozen. We do not recommend multiple freeze–thaw cycles.
7. Using the lid rather than the base provides better angles for the dissection because the lid sidewalls are lower.
8. Including more rostral sections of the embryo will produce cultures with serotonergic neurons from the raphe nucleus.
9. Other protocols recommend removing the head as the last step and instead using the head to hold the embryo during the dissection.
10. If the embryo will not remain on the ventral surface, ensure that the head has been completely removed and remove the tail if necessary.
11. To prevent overtreatment with collagenase I/dispase II, monitor the cords every few minutes for signs that the meninges are separating.

12. Usually 1,000 rpm for bench top centrifuges capable of accepting 50 ml conical tubes, but consult your centrifuge manufacturer for specific settings.
13. Lower speeds and longer times may be used if desired.
14. Beginners may wish to decant the supernatant to another conical tube. If cell yields are lower than expected, some cells may be found in the supernatant. This would indicate that the pellet was disturbed after centrifugation and should be handled more cautiously.
15. Using tissue culture dishes will promote nonspecific adhesion during the selection steps.
16. Try not to move the culture dish during the incubation period.
17. A2B5 and ENCAM are IgM antibodies and require anti-IgM specific secondary antibodies.
18. Using sterile DMSO can eliminate the need to filter freezing medium.
19. Specially designed cooling containers can improve cell survival in the freezing stage (e.g., Nalgene #5100-0001).

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