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An optimized method for obtaining adult rat spinal cord motor neurons to be used for tissue culture

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HIGHLIGHTS

- Redefined adult rat spinal cord motor neuron extrusion and culture method.
- Method confirms primary adult motor neuron survival beyond 28 days.
- Method is robust, reduces processing time, complexity and cost.
- No requirement for Intracardiac perfusion, or to cool (4 °C) medium post extrusion.
- Results and discussion written for those new to primary culture techniques.

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ABSTRACT

Background: There is a paucity of detailed methods describing how to harvest and process motor neurons obtained from the adult rat spinal cord.
New method: Removal of intra-cardiac perfusion step. The spinal cord is extruded intact from the rat in under 60 s post-decapitation then processed without differentiation of ventral and dorsal regions. The temperature during processing was maintained at room temperature (22 °C) except during the Papain processing step where the temperature was increased to 30 °C.
Results: Cell debris interfered with the counting of cells at the time of plating. Also, cell types could not be identified since they appear rounded structures with no projections. Cell viability counts reduced to 91% and 63% from day 7 to day 14 and days 7–28 respectively. Red blood cell counts in stepped density gradient layers 2 and 3 were low.
Comparison with existing method(s): No requirement for intra-cardiac perfusion. No requirement to cool to 4 °C post harvesting, No requirement for specialized substrates. Reduces processing time by at least 2 h and reduces the potential for processing errors through a reduction in complexity. Procedures are also explained suitable for those new to the culture of primary adult motor neurons.
Conclusions: Cell viability counts indicate that removal of the perfusion step has a minimal effect on the viability of the cultured nerve cells, which may be due to the reduction in the spinal cord harvesting time and the inclusion of Hibernate based media during extrusion and processing.

1. Introduction

In the absence of cell division, adult primary neurons are difficult to maintain in culture for extended periods. As a consequence, it is not uncommon for neuroscientists to use either embryonic neurons, immortalized neural cell lines or in more recent time's stem cells for in vitro experiments. However, these cells can differ in important aspects from the adult primary neuron (Bar, 2000; Cameron and Núñez-Abades, 2000; Carrascal et al., 2005; Gingras

Abbreviations: °C, degrees celcius; ACSF, artificial cerebro-spinal fluid; bFGF, basic fibroblast growth factor; BDNF, brain-derived neurotrophic factor; Ca^{2+}, calcium; cAMP, 8(-4 chlorophenylthio cyclic adenosine 3′5′ monophosphate); CO^2, carbon dioxide; CNTF, ciliary neurotrophic factor; DIV, days in vitro; DNA, deoxyribose nucleic acid; DSHB, Developmental Studies Hybridoma Bank; EDTA, ethylene diamine tetra acetic acid; GDNF, glial-derived neurotrophic factor; HIB-PM, hibernate processing medium; O^2, oxygen; PBS, phosphate buffered solution; PDL, poly-D-lysine; PET, polyethylene terephthalate; Neurobasal CM, neurobasal based conditioned medium; Mg^{2+}, magnesium.

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Table 1
Stage 1 density gradient – prepare in 4 separate 15 mL conical tubes.

Conical Tubes	Optiprep 1.32 g/mLv/v%	OptiPrep1.32 (μL)	Hib-PM (μL)	Total (μL)
Conical 1	17.50%	525	2475	3000
Conical 2	12.50%	375	2625	3000
Conical 3	10.00%	300	2700	3000
Conical 4	7.50%	225	2775	3000

et al., 2007; Jacobson, 1985). Therefore, the use of these cells or cell lines for the investigation of spinal cord injury or degenerative diseases such as Amyotrophic Lateral Sclerosis, Parkinson's disease, and Multiple Sclerosis can be problematic in clinical translation. Neuroscientists are thus, always interested in a well-defined adult primary neuronal cell culture for comparative purposes.

Brewer (1997) succinctly described the sequence of steps required in which to successfully bring adult primary neurons into in-vitro culture, that of; (1) Harvesting, (2) Separation of cells from the extracellular matrix, (3) Removal of growth inhibitory factors, (4) Targeted specificity of cell type, and (5) Provision of an adequate substrate and nutrients to ensure viability. When considered in their entirety, these steps can come together to build a robust protocol. However, in isolation, each step has benefits and consequences that have to be carefully balanced. One such example is between media selection, temperature and time.

Here we describe a protocol to harvest adult rat spinal cords and isolate and process neurons to in-vitro culture. The choice of hydraulic extraction of the spinal cord (De Sousa and Horrocks, 1979; Meikle and Martin, 1981) demonstrates how the risk of ischemia can be balanced to negate the requirement for intracardiac perfusion. We also reiterate the benefits of using Hibernate during processing in ambient air (Brewer and Price, 1996; Brewer and Cotman, 1989). To demonstrate the robust processing methods, the neurons were cultured more than 21 days using the combination C2C12 conditioned media first described by (Montoya-Gacharna et al., 2009). Montoya-Gacharna and associates also used cyclic adenosine-3′,5′-monophosphate (cAMP), which has been previously shown to substantially reduce the need for high quantities of growth factors (Hanson et al., 1998; Meyer-Franke et al., 1998).

2. Materials and methods

A detailed description of all steps in this protocol follows in a format that can be directly applied in the laboratory for the extraction of rat spinal cord motor neurons and their long-term culture.

2.1. Pre-surgical preparation

Prepare the C2C12 muscle conditioned medium in advance (>14 days) from the C2C12 myoblast cell line as previously described by (Montoya-Gacharna et al., 2009, 2012). Briefly, myoblasts are cultured in Dulbecco's modified Eagle medium (DMEM) containing L-Glutamine 20 mM (Sigma), Gentamicin 10 μg/mL and 10% fetal bovine serum (Sigma) reaching 30% confluence. Cells are differentiated into myocytes by replacing the current culture medium with new culture medium containing 10% horse serum (Sigma). After differentiation (i.e. in 3 days), wash the cells in warm phosphate buffered saline (PBS). Then, culture in a serum-free medium containing Neurobasal-A™ (Gibco), 2% B27™ supplement 50x (Gibco), Glutamax 0.5 mM (Invitrogen) and Gentamicin (Invitrogen) 10 μg/mL for 2 days. The supernatant (the conditioned medium) is then collected, filtered through a 0.22 syringe filter and stored at −20 °C until required.

At least 24 h before spinal cord harvesting, sterilize glass 12 mm coverslips. Then, within a laminar flow hood insert them into 24-well culture plates (Corning). Apply to the surface of individual coverslips 100 μL sterile poly-D-lysine (PDL) (50 μg/mL) (Sigma). Note: There is variation in methods of this procedure http://protocolsonline.com/recipes/stock-solutions/polylysine-coated-tissue-culture-surfaces/ (Accessed 4/10/2015). In this protocol apply PDL, and then transfer the culture plates to a culture oven at 37 °C for 1 h. Excess PDL solution is aspirated, and the surface of the coverslip rinsed twice with sterile water. Leave these culture plates within the laminar flow hood under Ultraviolet light overnight to dry off.

Prepare 24 h prior to use, 150 mL of Hibernate™ based processing medium (Hib-PM) containing Hibernate-A (Gibco), 2% B27 supplement 50x (Gibco), Glutamax 0.05 mM (Sigma) and Gentamicin 10 μg/mL (Invitrogen) (Brewer and Torricelli, 2007):

1. Transfer 50 mL of Hib-PM into a 50 mL conical tube mark, "Hib-PM" processing
2. Transfer 7 mL of Hib-PM to each of 3 × 15 mL conical tubes, mark as "Hib-PM" processing
3. Leave in the laminar flow hood 15 mL of Hib-PM in a 15 mL conical tube, mark as "G1."

Store the remaining Hib-PM with the four conical tubes marked Hib-PM processing (1 × 50 mL, 3 × 15 mL) at 4 °C wrapped in foil. Mark ×4 15 mL conical tubes D1 through to D4. Then, prepare stage 1 of the Brewer and Torricelli Optiprep™ 1:32 (Sigma) density

Table 2
Neurobasal conditioned medium (NEUROBASAL CM) (Maintain at 4 °C).

Product	Concentration Required	Qty Per 100mLs	From
Neurobasal A	65%	68 mL	Gibco
C2C12 Conditioned Media	33%	30 mL	Sigma
B27 (50×)Supplement	2%	2 mL	Gibco
Glutamax 100 mL stock = 200 mM	0.5 mM	250 μL	Sigma
BDNF Stock aliquot 100 ng/200 μL (Refer Reagents)	1 ng/mL	200 μL	Sigma
GDNF Stock aliquot 100 ng/200 μL (Refer Reagents)	1 ng/mL	200 μL	Sigma
[a]CNTF Stock aliquot 1000 ng/200 μL (Refer Reagents)	10 ng/mL	200 μL	Sigma
[a]bFGF Stock aliquot 200 ng/200 μL (Refer Reagents)	2 ng/mL	200 μL	Sigma
Gentamicin 10 mg/mL	10 μg/mL	100 μL	Invitrogen
8(-4 Chlorophenylthio cyclic adenosine 3′5′ monophosphate) cAMP 100 mg = MW 493.7	125 μM	618 μL	Sigma

[a] CNTF and bFGF added to the medium to support the survival of nerve cells post-axotomy (Grothe et al., 1991; Sendtner et al., 1991). Note: bFGF at a higher dosage >20ng/mL has the potential to isolate progenitors found in the adult mammalian spinal cord (Shihabuddin et al., 1997).