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Cryopreserved Dissociated Rat Brain Neuronal Cells Are Easily Transfected Using the 4D-Nucleofector® Y Unit

By Anthony Krantis[1], Susan VanderHoek[1], Barbro Tinner-Staines[1], Paul Shock[1] and Scott D'Andrea[2]
[1]QBM Cell Science, Inc., Ottawa, Canada;
[2]Lonza Walkersville, Inc., Walkersville, MD, USA

Introduction

Ready-to-use, batch tested, cryopreserved dissociated neuronal cells combined with high quality, high-throughput adherent neuronal transfection of the 4D-Nucleofector® Y Unit is an effective and time saving approach for researchers employing genetically modified strategies for their neurobiology programs.

Cryopreserved primary neuronal cells dissociated from the rodent brain and periphery have been commercially developed by QBM Cell Science as ready-to-use primary cells and are ideal for a spectrum of studies in basic research and drug development fields. To date, more than 100 peer-reviewed publications prove these cryopreserved dissociated rodent neuronal cells (available exclusively from Lonza) to be an excellent research tool in neurobiology. The scope of applications of these cells is easily surveyed from the general literature as well as industrial monographs (see publications at www.qbmcellscience.com) where they are cited as used to develop cell assay, screening and interrogation technologies. When thawed and cultured, these batch tested, cryopreserved neuronal cells display all of the properties of freshly dissociated neuronal cells with the added advantage that they are guaranteed bacteria and mycoplasma free and show extraordinary inter- and intra-batch consistency. Collaborators employing different techniques can be testing cells from the same batch and experiments can also be revisited on the same batch of cells that can be archived in liquid nitrogen. Our testing shows hat when thawed and cultured, neuronal cells cryopreserved and frozen since 2000 display no deterioration. Thus, cryopreserved rat brain neuronal cells are a convenient and practical substitute for freshly prepared neuronal cells.

The transfection of nucleic acids into cells is now considered crucial for the study of many aspects of neurobiology. However, primary neurons are difficult to transfect, making genetic modification of neuronal cells by delivery of either siRNA or plasmid DNA challenging. The cryopreserved brain neuronal cells are amenable to transfection by a variety of manual and automated techniques where the efficiency of transfection was comparable to freshly prepared neuronal cells with no obvious signs of toxicity post transfection. These studies include the use of virus-based, electroporation, chemical- and photo-transfection methods (Dityateva *et al.*, 2003; Gartner *et al.*, 2006; Gehl, 2003).

A relatively new form of electroporation-based transfection, Nucleofection®– where plasmids are transfected directly into the nucleus (Iversen *et al.*, 2005; Karra and Dahm, 2010) – has gained prominence across many research fields. The advantage of this technique is better transfection rates than classical electroporation or chemical methods, combined with good cell survival and quality of the culture post transfection due to specific programming of electrical parameters and use of specific transfection solutions to optimize the physiological environment and viability of the cells (Zeitelhofer *et al.*, 2007).

The advent of a Nucleofector® System with multi-well plate 'adherent' formats, *i.e.* the 4D-Nucleofector® Y Unit, means that the immature neuronal cells can now be first cultured to allow for normal development of neuronal processes, network connectivity and neurochemistry, then transfected. Here we report the application of adherent Nucleofection® based transfection in cultures of cryopreserved dissociated rat brain neuronal cells.

Methods

Cell Culture

Primary neuronal cells are labor intensive and less convenient than cell lines since the researcher is faced with the tedium, caprice and waste associated with tissue dissection/dissociation first hand. These difficulties are now overcome by ready-to-use, cryopreserved primary neuronal cells, available from Lonza. Shipped frozen, they can be simply thawed and cultured in a variety of single- or multi-well formats to obtain high quality and high yield cultures of dissociated primary neuronal cells.

For the studies described herein, vials of frozen neuronal cells dissociated from rat brain cortex (R-Cx-500) and hippocampus (R-Hi-501) were thawed and plated into 24-well culture plates using a standard protocol. Briefly, 24-well plates were coated with poly-D-lysine/laminin (50:50) overnight at 37°C. Rat hippocampal or cortical neuronal cells were thawed and plated onto the multi-well plates. For comparison, freshly prepared hippocampal cells (from the same dissection batch) were plated and processed in the same way. Freshly isolated hippocampal cells were plated at 150,000/mL. Cryopreserved cells were plated at a higher cellular density of 200,000/mL (hippocampal cells) and 400,000/mL (cortical cells) to account for the reduction in viability due to cryopreservation. After 4 hours, the cultures were changed to PNGM™ Media and then again at 4 days *in vitro* (DIV). Cultures were subjected to an 80% media change every 3 – 4 DIV thereafter.

Transfection

We used the 4D-Nucleofector® Y Unit – configured for 24-well adherence culture formats – using the AD1 4D-Nucleofector® Y Kit with pmaxGFP™ Vector. Expression of GFP fluorescent protein was evaluated at 4 hours, 24 hours to 19 days post Nucleofection® by inverted fluorescence microscopy. The Nucleofector® Solutions were prepared following the protocol provided. Typically, for 5 mL of supplemented AD1 solution, 250 µL of 1 µg/µL pmaxGFP™ Vector was added. Media was carefully removed from the wells and 350 µL of the solution-DNA mix added. The 24-well dipping electrode array was carefully placed into the wells making sure there were no air bubbles under the electrodes. The plate was positioned into the 4D-Nucleofector® Y Unit for Nucleofection®. Post transfection, the Nucleofector® Solutions from the wells were removed and replaced with fresh 37°C PNGM™ Media. This was followed by a 50% media change at 4 hours post transfection. The cells were fixed at various time points post transfection using our standard procedure.

Morphological Assessment

Fluorescent micrographs were used to qualitatively assess viability, transfection efficiency, and general morphology of cryopreserved neuronal cells following Nucleofection®, compared to non-transfected cryopreserved neuronal cells and freshly prepared neuronal cells prior to and post Nucleofection®. Cultures were fixed and stained for immunohistochemical examination directly in the wells and examined using inverted microscopy. Immunohistochemical identification of cell types within the cell cultures was performed using commercially available antibodies. The same general procedures were followed; primary antibody diluted to working concentration was applied to the wells overnight at 4°C. After rinsing in 10 mM phosphate-buffered saline (PBS), secondary antibody at its appropriate working dilution was applied for 30 minutes. Wells were again rinsed in PBS. Neuronal cells were examined using the general neuronal marker anti-PGP9.5 (1:1600, Rb; Abcam). Astrocytes were identified using anti-GFAP (Abcam). Alexa Fluor® 594 anti-Rb antibodies were used as secondary antibody.

Results and Discussion

The frozen cryopreserved neuronal cells can be thawed and cultured with ease on coated multi-well plates. When thawed and cultured using the optimized protocol provided, these batch tested cells display

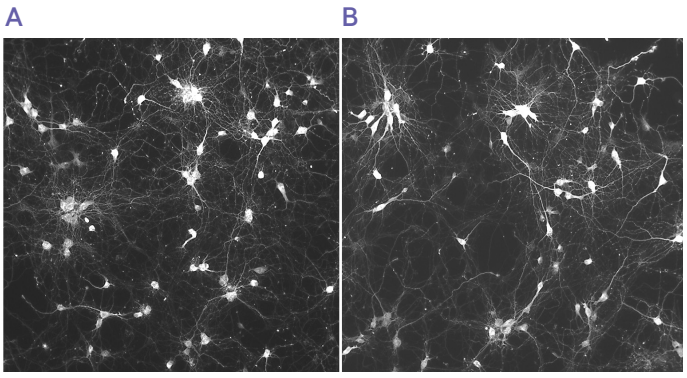

Figure 1.
Neural networks in non-transfected cultures prepared from (A) fresh and (B) cryopreserved rat hippocampal neuronal cells in 24-well plates. After 7 DIV they were immunostained with the specific neuron marker anti-PGP9.5.

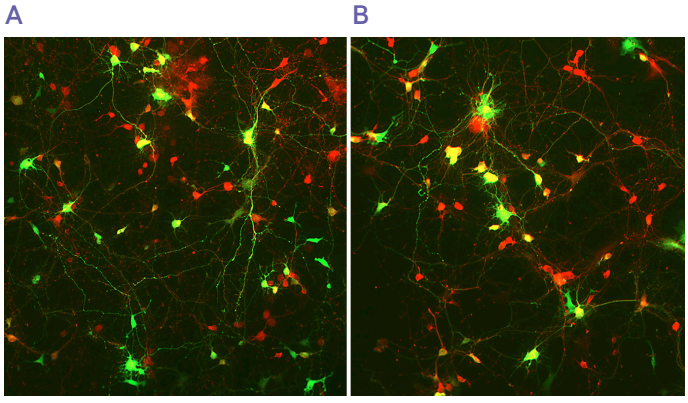

Figure 2.
Comparison of transfection in cultures of cryopreserved neuronal cells dissociated from different rat brain regions. Cryopreserved cortical cells (A; program EH-166) or cryopreserved hippocampal cells (B; program ED-158) were transfected at 5 DIV and analyzed 24 hours post Nucleofection®. The overlayed images of typical cultures display rich neuropil with comparable numbers of maxGFP™ positive cells (green) relative to PGP9.5 positive neurons (red).

the properties of freshly dissociated neuronal cells. The time course for morphological differentiation of cryopreserved dissociated neuronal cells following plating on coated 24-well plates was characterized over 7 days in culture (Figure 1). They displayed typical neuronal neurite networks, cell morphology and neurochemistry. Neurons could be easily distinguished from astrocytes.

We found the quality of transfection between cultures prepared from freshly dissociated cortical and hippocampal neuronal cells versus neuronal cells cryopreserved from the same batch then cultured, were comparable. Both neurons (Figure 2) and glia (Figure 3) are transfected. For both fresh and frozen cells, good quality transfection could be achieved from 3–11 DIV. However, best results were achieved using cultures at 5 DIV, with fixation 24 hours post Nucleofection®

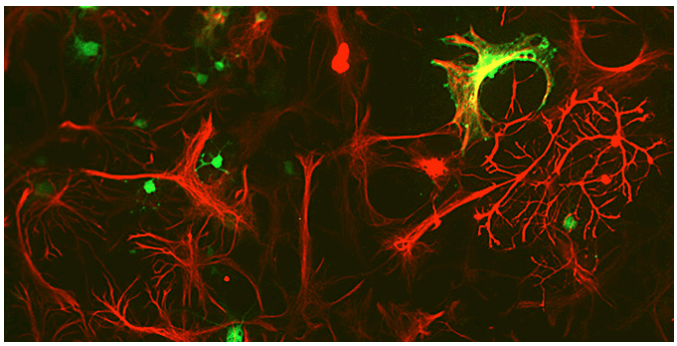

Figure 3.
Cultures of cryopreserved rat cortical cells transfected 11 DIV (program ED-158). In this overlayed image, glial cells (red) can be seen together with maxGFP™ positive neurons (green). Some transfected glia can also be seen (yellow-green).

(Figure 4). Nucleofection® Conditions were critical as shown in Figure 5. Programs ED-158 and EH-166 were equivalent and gave the optimal results.

The cultures transfected in adherence with the 4D-Nucleofector® Y Unit displayed neural networks and cell morphology comparable to the non-transfected controls. Close examination of the transfected cultures showed that neuropil disruption appeared equivalent between cultures of fresh versus cryopreserved cells. Cultures which were transfected >5 DIV displayed highly differentiated networks, with high quality transfection. However, the number of transfected cells was less. Cultures transfected at 5 DIV but examined 4 hours post Nucleofection®, showed few or weakly transfected cells.

Taken together, these data show optimal conditions for transfection of cryopreserved neuronal cells by Nucleofection® require cultures to be 5 DIV with 24 hours for expression of signal. Nucleofector® Programs ED-158 and EH-166 were found to be best for both cryopreserved rat hippocampal and cortical neuronal cells, and for freshly cultured neuronal cells.

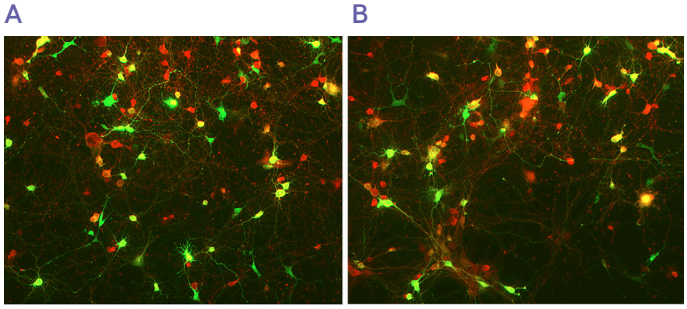

Figure 4.
Comparison of transfection of freshly prepared and cryopreserved rat hippocampal cells. Neuronal cell cultures were transfected at 5 DIV and fixed 24 hours post Nucleofection® (program EH-166). Transfection of freshly prepared cells (A) can be directly compared neuronal cells from the same dissection-dissociation batch that were first cryopreserved, frozen, then thawed and cultured (B).

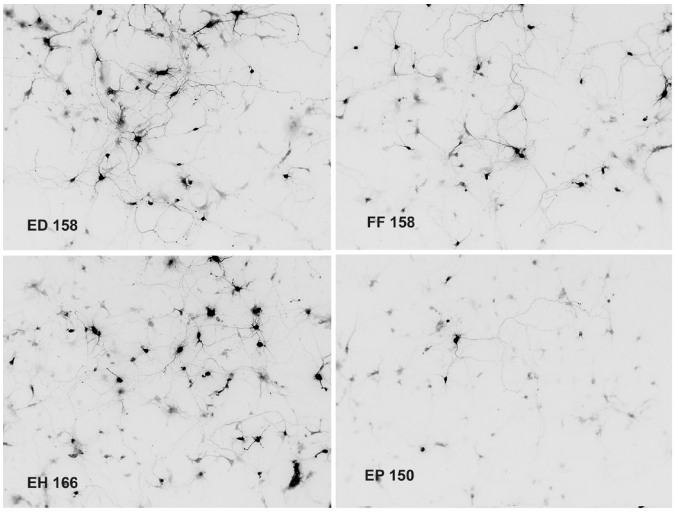

Figure 5.
The effects of different Nucleofector® Programs on transfection of cryopreserved rat hippocampal neuronal cells. Cells were transfected at 5 DIV using different Nucleofector® Programs and examined 24 hours post Nucleofection®. The montage shows inverted GFP fluorescence images from wells of plates subjected to different Nucleofector® Programs.

The quality of transfection using these Nucleofector® Programs with cultures of 5 DIV or 11 DIV was striking. The GFP expression was intense in the cell soma and in the outermost extensions of dendrites and axons and it was straightforward to visualize a high amount of spines on transfected neurons (Figure 6).

The ready-to-use advantage of batch tested cryo-preserved dissociated rodent neuronal cells, combined with the high quality, high-throughput transfection of these cells in culture using the 4D-Nucleofector® Y Unit, offers a convenient, effective and time saving approach for researchers employing genetically modified strategies for their neurobiology R&D programs.

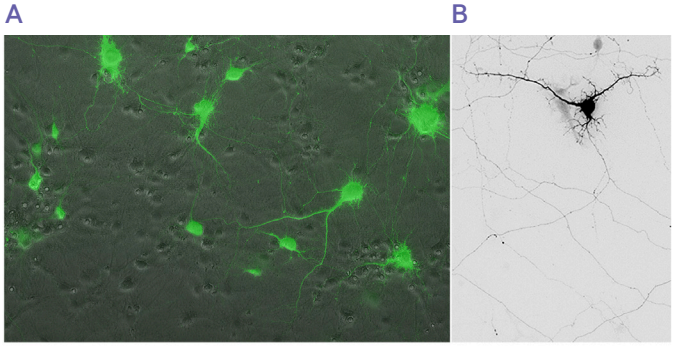

Figure 6.
High quality transfection by Nucleofection®. maxGFP™ Expression was intense in the cell soma and in the outermost extensions of dendrites and axons shown here in phase contrast (A) and inverted fluorescence (B) images from different cultures of cryopreserved hippocampal neuronal cells transfected 11 DIV and photographed 24 hours post Nucleofection®. Spines and processes on transfected neurons are easily observed and axons can be followed long distances.

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Contact Us

North America
Customer Service: +1 800 638 8174 (toll free)
order.us@lonza.com
Scientific Support: +1 800 521 0390 (toll free)
scientific.support@lonza.com

Europe
Customer Service: +32 87 321 611
order.europe@lonza.com
Scientific Support: +49 221 99199 400
scientific.support.eu@lonza.com

International
Contact your local Lonza Distributor
Customer Service: +1 301 898 7025
Fax: +1 301 845 8291
scientific.support@lonza.com

Lonza Cologne GmbH – 50829 Cologne Germany

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