Utilizing advanced cryopreservation techniques to produce assay ready primary mouse neurons

Navjot Kaur[1], Leisha Kopp[2], Justin Young1, SongTe Kim[1], Anne Dudley[1] and Alex Hannay[1].
[1]Life Technologies, Primary and Stem Cell Systems, Frederick, MD; [2]Life Technologies, Madison, WI

ABSTRACT

Primary neural cells offer an indispensible tool for dissecting basic neurobiology with prospective applications in cell therapy and drug discovery. Researchers face some difficulties when they start to utilize neuronal cells, namely the labor intensive nature of serial preparation and cultivation of primary cells. Cryopreserved neurons offer some clear advantages over freshly dissociated cells: they provide flexibility to the culturing process and represent a "cell bank" that eliminates the need for timed pregnancy animals each time cell culture is initiated. We have developed protocols to generate cryopreserved neurons isolated from cerebral cortex and hippocampus of C57/Bl6 mouse at embryonic day 17 which maintain high viability ranging from 50% to 80% and very low glial cell background as compared to other commercially available sources. Upon thaw, the cells displayed a neuronal morphology with both dendritic and axonal processes clearly recognizable by cellular polarity and proportionate size. The neurons survived for at least 3 weeks in culture and showed retention of normal neuron-specific markers during this period. The functional presence of a canonical set of neurotransmitter receptors was tested by challenging the cells with saturating concentrations (500 μM) of endogenous ligands such as acetylcholine, glutamate, γ-aminobutyric acid (GABA) and ATP. Cells exhibited clear changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) with varying frequency depending on the neurotransmitter. These data indicate that the cells harvested from mouse brains can be effectively cryopreserved and efficiently thawed preserving the prototypical neuronal properties. These ready-to-use cells provide optimal performance with high purity, minimal lot-to-lot variability, and easy availability.

MATERIAL & METHODS

All reagents were purchased from Life Technologies, unless otherwise noted.

Recovery and Culturing of Primary Neurons: Mouse C57 BL/6 mice Embryonic day 17 Cryopreserved cortical (Cat # A15585/A15585) or hippocampal neurons (Cat # A15587) were thawed quickly in a 37°C water bath, diluted in pre-warmed media and the percentage viability was measured by Trypan blue assay. The cells were cultured in Neurobasal® medium (Cat # 21103) supplemented with 2% B-27® (Cat # 17504) and 0.5 mM GlutaMAX™-I (Cat # 35050) and plated on poly-D-lysine coated dishes.

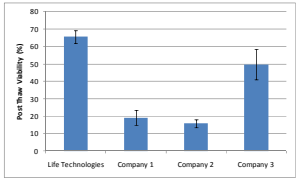
Immunocytochemistry: Cells were cultured for one week, fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton-X. Cells were stained with mouse anti-MAP2 antibody (Cat # 13-1500) and Alexa fluor® 488–labeled anti-mouse secondary antibody (Cat # A-11029). Astrocytes were stained with rabbit anti-GFAP antibody (Cat # 18-0063) and Alexa fluor® 594–labeled rabbit secondary antibody (Cat # A-11037). Oligodendrocytes ere stained with mose anti-GalC antibody (Millipore, Cat # MAB342). Nuclei were stained with DAPI (Cat # D3571).

Neurite Outgrowth Assay: Neurite outgrowth was assessed by Neurite Outgrowth Staining Kit (Cat # A15001). Dyes (1X) were added directly in the culture media. Staining reagents were replaced with background suppression dye after incubation for 15 min at 37°C. Cells were imaged using inverted fluorescence microscope using standard FITC or TRITC filter sets.

Intracellular Calcium Measurement: Neuronal cells were cultured in 96 well poly-D-lysine coated plates and loaded with 3 μM fluo-4 AM (Cat # F14217) + 9% (w/v) Pluronic® F-127 (Cat # P6866). Cells were challenged separately with saturating concentration of acetylcholine, glutamate, γ-aminobutyric acid, and ATP. An excitation light of 488 nm (Lambda DG-4) was used and data were collected at 520 nm (ORCA-ER).

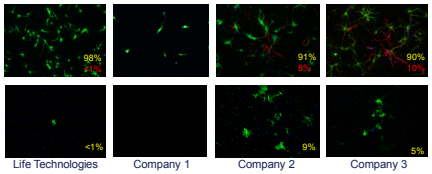
Gene Transfer: Cells were cultured for 7 days and transfected with EGFP control using Lipofectamine®2000 transfection reagent (Cat # 11668), or transduced with 20%(v/v) BacMam GFP transduction control (Cat # B10383) overnight. The cells were analyzed at 24-48 hrs post transduction.

POST THAW VIABILITY



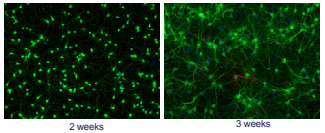
Life Technologies' GIBCO primary mouse neurons demonstrate higher post thaw viability. Cryopreserved mouse neurons from competitors' showed lower post thaw viability while Life Technologies' mouse neurons consistently yield >50% viability as tested with trypan blue staining. Calculated means and standard errors were tested for significance by Student's two tailed *t*-test ($P < 0.005$, n=3).

PURITY OF NEURONS



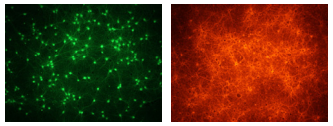
Life Technologies' GIBCO primary mouse neurons have superior neuronal phenotype.
Mouse cortical neurons were cultured for 7 days and stained with mouse anti-MAP2 antibody and Alexa fluor® 488–labeled anti-mouse secondary antibody (Top panel, green). Astrocytes were stained with rabbit anti-GFAP antibody and Alexa fluor® 594–labeled rabbit secondary antibody (Top panel, red). Oligodendrocytes were stained with mouse anti-GalC antibody and Alexa fluor® 488–labeled anti-mouse secondary antibody (Bottom panel, green). Nuclei were stained with DAPI. Percentage neurons *vs.* glial cells are indicated in images. Life Technologies's cryopreserved cells showed a much greater percentage of neurons.

LONG TERM CULTURE



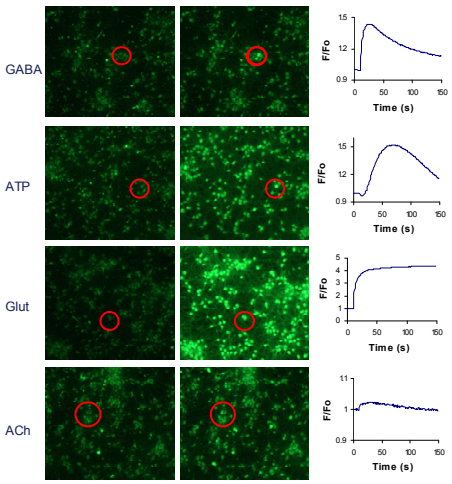
Life Technologies's GIBCO primary mouse neurons remained healthy and viable even after three weeks. Mouse cortical neurons were cultured in Neurobasal® medium supplemented with 2% B-27® and 0.5 mM GLUTAMAX™-I. Neurons showed normal neuronal phenotype demonstrated by immunostaining the cells with anti-MAP2 and anti-GFAP antibodies at week 2 (left) and week 3 (right).

NEURITE OUTGROWTH



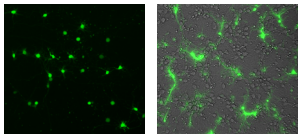
Life Technologies' GIBCO primary mouse neurons show extensive neurite outgrowth. Cell viability and neurite outgrowth was visualized in the same sample using the Neurite Staining Outgrowth kit. Mouse cortical neurons were cultured for 14 days and stained. The cell-permeant green fluorescent Cell Viability Indicator brightly stained the cell bodies of live cells and only faintly stains the extending processes (Left). In contrast, the orange-red fluorescent Cell Membrane Stain brightly stains the outer surfaces of both the cell bodies as well the neurite extensions (Right).

ASSESSEMENT OF PHYSIOLOGICAL FUNCTIONS



Neurotransmitter-induced changes in intracellular calcium levels showed normal neuronal properties. Life Technologies' GIBCO primary mouse cortical neurons were cultured on to poly-D-lysine coated 96 well plate for 7 days. The left column represents the cells before addition, the right column after addition of saturating concentrations of neurotransmitters. The graphs indicate the change in fluorescence from Fluo-4 plotted against time for the cell circled in red for each row.

GENE TRANSFER



Cryopreserved mouse hippocampal neurons were able to be transfected and transduced effectively. Mouse Hippocampal Neurons labeled with EGFP control using Lipofectamine® 2000 (Left), and with BacMam-GFP Transduction Control (Right). Cells were cultured on to poly-D-lysine coated plates for 7 days in Neurobasal® medium supplemented with 2% B-27® and 0.5 mM GLUTAMAX™-I. Image analysis was performed 24h post gene transfer.

SUMMARY

Life Technologies' cryopreserved primary mouse cortical and hippocampal neurons provide:

- A convenient and ready-to-use alternative to freshly isolated neurons.
- Experimental flexibility; eliminates need for animal facility and associated resources.
- Decreased inherent lot-to-lot variability.
- Superior post thaw viability and purity.
- Maintenance of neuronal cell functional characteristics.

PRODUCT INFORMATION

Primary Mouse Cortex Neurons: 1 x 10^6 cells/vial	A15585
Primary Mouse Cortex Neurons: 4 x 10^6 cells/vial	A15586
Primary Mouse Hippocampus Neurons: 1 x 10^6 cells/vial	A15587

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