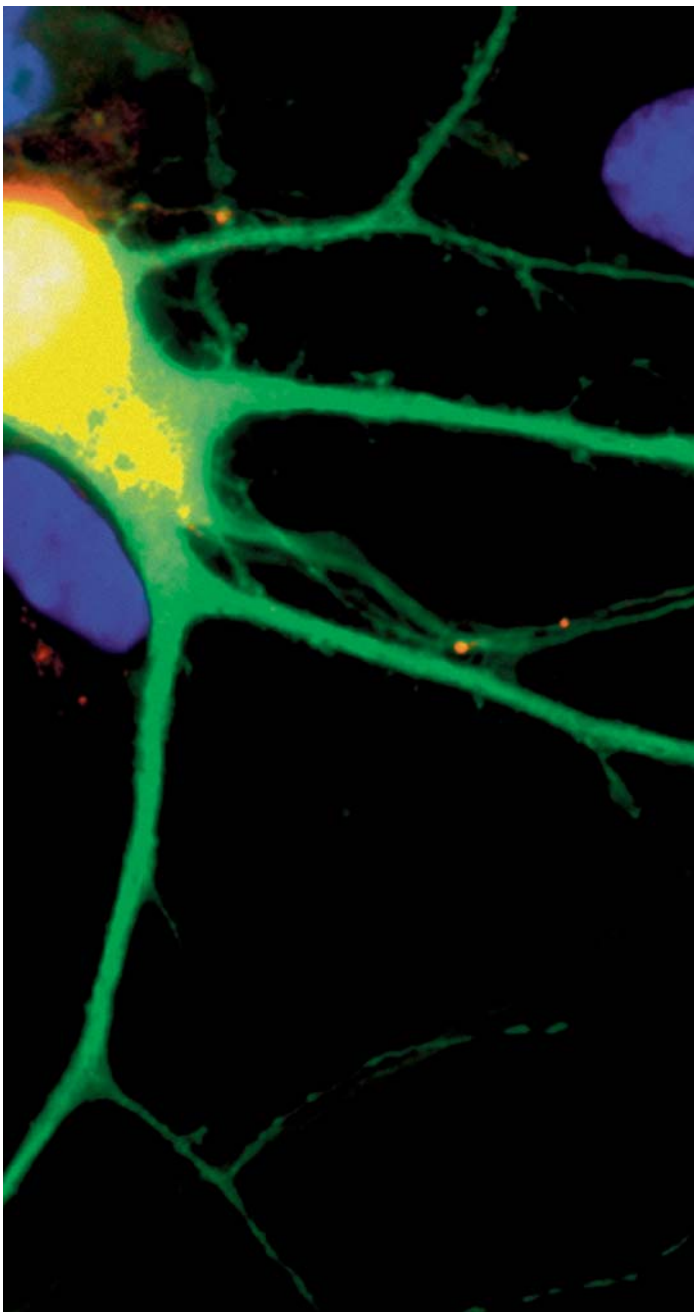


White Paper

High Throughput Nucleofection[®] of Primary Rat Hippocampal Neurons



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Post-mitotic neurons are routinely difficult to transfect. This application note establishes the efficient high throughput transfection of plasmid DNA into primary hippocampal neurons. Using Lonza's 96-well Shuttle[®] System, between 30 and 50% of neurons were successfully transfected and showed normal neuronal development. In addition, quantitative downregulation of target proteins by RNA interference was achieved. In the future, we envision a high throughput analysis of target genes even in primary neurons using the 96-well Shuttle[®] System.

Introduction

Despite the development and adaptation of a variety of methods [1, 2], the transfer of DNA into neurons remained unsatisfactory in regard to either transfection efficiency or cytotoxicity. The first generation of the Nucleofector[®] Technology overcame these limitations and allowed biochemical analyses to be performed in primary neurons, e.g., the assessment of protein levels after RNAi downregulation by Western blot [3].

The 96-well Shuttle[®] System promises several additional unique advantages. In addition to the ability to perform high throughput transfections of siRNA duplexes or plasmid DNA in such cells as primary neurons, it uses significantly lower cell numbers than the conventional Nucleofector[®] Device. Together, these features allow to rule out a number of possible errors (such as variations in culture conditions which typically arise from separate neuron preparations). In this study, we used the 96-well Shuttle[®] System to successfully transfect primary hippocampal rat neurons with various marker constructs. Transfected neurons were subsequently grown in 96-well plates. The morphology of the transfected neurons was assessed by phase contrast and fluorescence microscopy. Finally, individual proteins were successfully downregulated by RNA interference in primary hippocampal neurons.

Material and Methods

Isolation of hippocampal neurons

E17 hippocampal neurons were prepared as described [4]. P0 cultures were prepared accordingly with the following modifications: time of trypsination was expanded to 15 minutes and cells were triturated for 4 minutes with standard size, fire-polished Pasteur pipettes.

Vectors

Freshly isolated neurons were transfected with the following plasmids: (i) pmaxGFP[®] Vector (Lonza), or (ii) pSuperior vectors (Oligoengine; Seattle, WA; [5]), or (iii) double transfected with pSyn-GFP (eGFP under the control of the neuron-specific synapsin promoter, [6]) and pDsRed monomer-C1 RFP (CMV promoter, BD Pharmingen).

96-well Nucleofection[®]

5×10^4 – 5×10^5 neurons per sample were resuspended in 20 μ l 96-well Nucleofector[®] Solution for neurons containing 0.5 μ g plasmid DNA and transfected according to Amaxa[®] Optimized Protocol. 80 μ l prewarmed DMEM medium was immediately added and gently mixed to retrieve neurons from the cuvette. Neurons were seeded at a density of 4 – 7.5×10^4 cells per well (96-well plate) or at 1×10^5 per coverslip in a 12-well plate. 4 hours after plating, medium was replaced by fresh medium. For optimal culture conditions, E17 neurons were cultured in NMEM ([4]; E17 neurons do not grow well in DMEM). P0 neurons, in contrast, were cultured in DMEM (transfection efficiency is reduced in NMEM medium for P0 neurons) with 5 μ M AraC (added after 1 DIV).

Western blot and immunofluorescence

For Western blots, 1×10^6 P0 transfected neurons (pooled from two samples) were seeded in 6 cm dishes (Nunc), cultured for 4 days and analyzed as described [3] using the respective antibodies (detailed information available upon request from the authors). Western Blots were quantified using the Odyssey Infrared Imaging System (Li-Cor, Bad Homburg, Germany). For immunofluorescence of CDC10, E17 or P0 neurons were cultured for 4 days post Nucleofection[®], fixed and stained with respective antibodies.

Results

Optimized conditions for the 96-well Shuttle[®] System

Lonza tested different 96-well programs together with the optimal 96-well Nucleofector[®] Solution to identify the programs with highest transfection efficiencies and viabilities. Figure 1 shows a typical Nucleofection[®] Experiment performed under optimal Nucleofection[®] Conditions with Program 96-CU-133. Freshly isolated P0 hippocampal neurons were transfected and expression of the transfected proteins was analyzed by fluorescence microscopy after fixation in 96-well plates either after 1 day *in vitro* (DIV) (pmaxGFP[®] Vector) or 7 DIV (Syn-GFP). Typical transfection efficiencies ranged between 30–50% for expressed proteins, depending on the applied program and the overall fitness of the respective culture. Since primary hippocampal neurons are very sensitive, high quality cultures are important for optimal and reproducible results. After 7 DIV, we compared the overall morphology of the cells with untransfected control neurons (Figure 1 C) and detected no obvious differences in neuron health and neurite outgrowth.

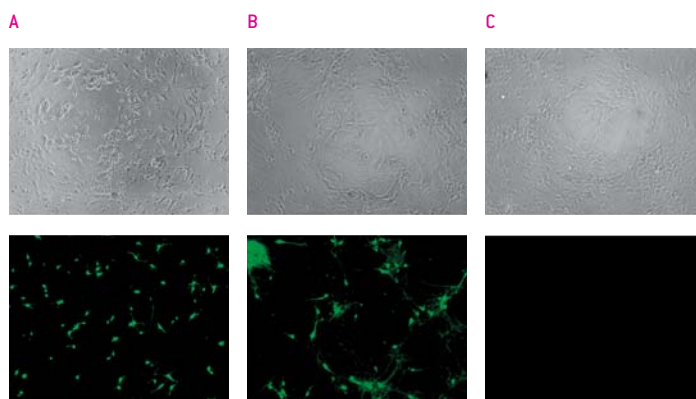


Figure 1: Optimized conditions for the Nucleofector[®] 96-well Shuttle[®] System. Freshly isolated hippocampal neurons (P0) were transfected with Program 96-CU-13 and pmaxGFP[®] Vector (A, 1 DIV) or pSyn-GFP (B, 7 DIV) and cultured in 96-well plates. After 1 or 7 DIV, neurons were fixed and analyzed by light (upper row) and fluorescence microscopy (lower row) and compared to untransfected neurons (C). Transfection efficiency with optimized conditions ranged between 30–50%. Neuron morphology (examined after 7 DIV) was unaltered compared to untransfected neurons.

Normal neuronal development after 96-well Nucleofection[®]

In order to analyze neuronal morphology in more detail, we plated transfected neurons on glass cover slips. Neurons were co-transfected with pSyn-GFP and pDsRed to discriminate

between neurons and glia cells. As shown in Figure 2, transfected 7 DIV neurons display typical characteristics of fully polarized neurons, including an elaborated dendritic network and dendritic protrusions that later develop into typical dendritic spines.

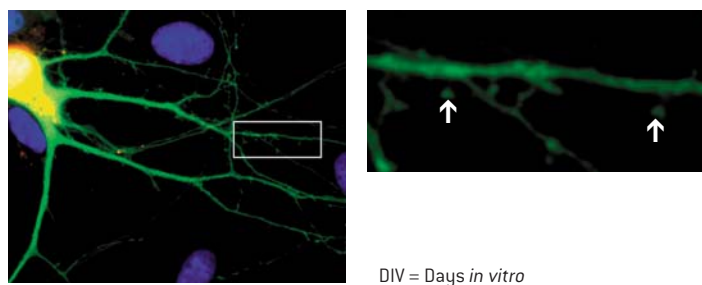


Figure 2: Normal neuronal development after 96-well Nucleofection®. P0 neurons transfected with pSyn-GFP and pDsRed were plated onto glass coverslips and examined after 7 DIV for GFP (green) and RFP (red) expression. Transfected neurons show an extensive dendritic network and, as shown in the magnification, develop dendritic protrusions that already resemble mature mushroom shaped dendritic spines (arrows). Surrounding, untransfected glia cells are shown by DAPI staining (blue).

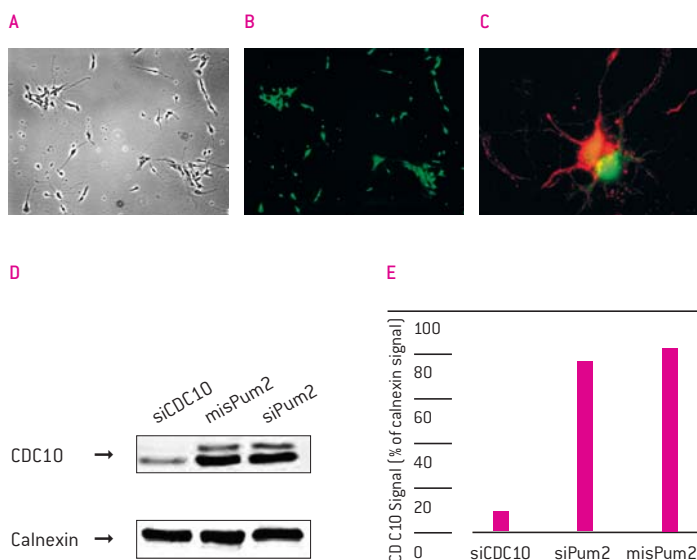


Figure 3: Quantitative downregulation of target proteins using RNA interference. Hippocampal neurons (E17) were transfected with the shRNA vector pSuperior targeting CDC10. **A** and **B**: Efficient Nucleofection® of pSuperior is shown by eGFP expression after 1 DIV. **C**: Immunostaining of CDC10 (red fluorescence) shows reduced endogenous CDC10 protein levels in transfected neurons (green) after 4 DIV compared to untransfected cells (red). Western blot analysis (**D**) and quantification (**E**) of CDC10 downregulation. In contrast to control transfections (siPum2, misPum2), CDC10 expression is significantly reduced in neuronal cultures transfected with pSuperior vectors targeting CDC10.

Quantitative downregulation of target proteins using RNA interference

Neurons were transfected with pSuperior vectors generating specific shRNAs [detailed information available upon request from the authors]. In Figure 3, a pSuperior plasmid directed against CDC10 [7] was efficiently transfected into freshly isolated E17 neurons (as shown by GFP expression after 1 DIV, Figure 3 A and B). After 4 DIV, downregulation was analyzed by fluorescence microscopy and Western blot. Figure 3 C shows efficient downregulation of endogenous CDC10 levels in siCDC10-transfected cells (right cell) compared to untransfected neurons (left cell). To quantify this effect, neurons were lysed and extracts were analyzed by quantitative Western blot (Figure 3 D and E). After 4 days in culture, siCDC10-transfected neurons show a substantial downregulation of CDC10, but not of calnexin, used as reference protein. In contrast, mocktransfected neurons show no significant change in protein expression.

Conclusion and Outlook

With the Nucleofector® 96-well Shuttle® System, a reproducible high throughput expression of various transgenes can be achieved in hippocampal neurons. Furthermore, a future high throughput analysis of RNA interference can now be envisaged in sensitive primary cells, such as neurons and glia cells. Another tempting application will be the use of inducible gene expression systems, e.g., TetON/TetOFF or cre/lox. This will allow for the temporal control of gene expression during maturation of primary neurons. Downregulation of gene expression on a systematic level at various stages of development or maturity will yield important insights into neuronal function and underlying mechanisms.

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