Cell-type specific transfection of neuronal cells

Data courtesy of Merck & Co., Inc., North Wales, PA, USA and MRC LMCB, University College of London, London, UK

Introduction

In cell biology research, the introduction of nucleic acids and other substrates has become a ground-breaking tool to explore gene function. In example, genomic screening technologies such as RNAi and cDNA screening provide powerful tools for generating gene knockout model systems and studying pathways and gene and protein function in cell culture (1,2). However, the key to successful RNAi experiments is efficient delivery of substrates into cells (3). Non-dividing and differentiated cells including primary neurons are refractory to traditional non-viral methods such as lipid-mediated transfection. In addition, results are unsatisfactory with regard to transfection efficiency and/or cytotoxicity. Viral-mediated gene transfer, although highly efficient, is expensive, time-consuming, and requires safety precautions (4). Advances have been made in the field of electroporation, where a voltage pulse temporarily alters the properties of the plasma membrane, allowing extracellular material to enter the cell (6-9).

High transfection efficiencies can be achieved with conventional electroporation, but often at the expense of cell viability and survival (10-12). One electroporation-based approach for transfection of primary neurons is nucleofection. This is a modified form of cuvette electroporation, using a series of high voltage pulses that enable plasmids to directly enter the nucleus (5, 13). Optimized buffers and cell type-specific pulse protocols minimize damage to the cellular membranes, resulting in higher transfection efficiencies and cell survival than conventional electroporation. The main limitation of cuvette electroporation and transfection of neurons is that cells have to be transfected immediately after isolation, eliminating applications involving mature neurons with developed axons, dendrites, and functional synapses. This obvious lack of simple and efficient methods for transfection of primary neurons has been a bottleneck in neuroscience for years, and despite some advances during later years, there is a great need for a versatile transfection technology for primary neuronal cells in various stages of differentiation.

Figure 1

Neuron-specific transfection in primary rat cultures. (a) Fluorescence image showing GFP-transfection (green) in hippocampal and cortical rat neurons stained for MAP-2 (red). Nuclei (neurons and glia) shown in blue. (b) Neuron viability and GFP transfection efficiency 24 h post-transfection.
The Cellaxess Elektra Discovery Platform utilizes a capillary electromanipulation concept that can be used for introduction of different types of molecules, ranging in size from target-designed dyes to oligonucleotides, siRNA, and plasmids, to adherent cells in 384-well plates. A focused electrical field minimizes electrochemical toxicity and joule heating, and terminally differentiated cells such as primary neurons can be transfected in any developmental state with retained morphology and excellent viability (14). The fully automated platform enables the study of gene function can on a genome-wide scale in cells previously inaccessible to genetic manipulation.

Cell-type specific transfection of neuronal cells

Dissociated embryonic rat neurons [cortex and hippocampus combined] were cultured in poly-D-lysine-coated Cellaxess Elektra 384-well plates and incubated for 24 to 264 h. After 96 h in culture, neurons looked healthy and had a differentiated morphology with visible and extensive neurites. The fraction of neurons in the culture was approximately 50% as measured by nuclear stain and MAP-2. In order to assess how well these cells tolerate Cellaxess Elektra electroporation, a range of pulse protocols were applied, and viability was measured 24 h post-electroporation by means of counting the number of MAP-2 stained neurons compared to mock electroporation (OV). Neuron viability was above 85% in all cases (Figure 1b). The same series of voltages were applied in the presence of plasmid, and GFP expression was studied 24 h post-transfection. MAP-2 staining was used to distinguish neurons from glia.

Transfected neurons expressed high levels of GFP in the cell body as well as in the extensions (Figure 1a). Neuronal transfection efficiencies were determined by dividing the number of GFP positive neurons with the total number of neurons. When using 30 000 cells per well (total number of cells), the best protocols resulted in transfection efficiencies between 40-50% (Figure 1b). The fraction of transfected glia was close to zero, showing that the transfection protocol is specific for neurons. When optimizing parameters for in-situ electroporation several different parameters are important, including cell size, shape, thickness, and origin, making it possible to transfect a specific cell type in mixed cultures. The same range of electroporation protocols was used for embryonic neurons from mouse. GFP expression was found in both cell bodies and extensions (Figure 2a) and transfection efficiencies varied around 30% for the best protocols (Figure 2b). In order to test whether the described protocols for neuron transfection are restricted to cortical and hippocampal neurons, we tested them in cultures from dissociated dorsal root ganglia [containing neurons and Schwann cells] from embryonic rats. Transfections were performed after 96 h in culture, when the cells had developed axonal networks (Figure 3a).

**Figure 2**

Neuron-specific transfection in primary mouse cultures. (a) Fluorescence image showing GFP-transfection (green) in hippocampal and cortical mouse neurons stained for MAP-2 [red]. Nuclei [neurons and glial shown in blue. (b) Percentage of GFP-positive neurons 24 h post-transfection.

**Figure 3**

Transfection of DRG neurons. (a) Fluorescence images showing GFP-transfection (green) in DRG cultures from embryonic rats. Nuclei shown in blue.
In order for a high-throughput transfection technique to be truly useful, it is of key importance that it exhibits stability within plates as well as between plates. Therefore, a relative large number of plates with combined hippocampal and cortical neurons were transfected. Figure 6a shows data from a total of 20 plates transfected in the course of two weeks. The cells were from different preparations, and were prepared as well as plated by different operators. Cells were plated at different densities. Plates were transfected on different days after plating, typically 4-7 days after plating, and transfection efficiencies were typically evaluated between 24-96 house post transfection. Figure 6b and 6c shows the mean transfection efficiency for each row and column within a plate, respectively. The coefficient of variation for all 20 plates was 9%, and the row and column CVs were 7% and 8%, respectively. Given the typical intrinsic variation in GFP transfections, the large variation in experimental conditions and the fact that this is primary neuronal cells, the stability in transfection efficiency is excellent.

Inter- and intra-plate reproducibility

Figure 4

Transfection at different developmental stages. Fluorescence images showing GFP-transfection (green) in hippocampal and cortical rat neurons stained from MAP-2 (red) in cells cultured for 24 h (a) and 264 h (b). Nuclei shown in blue.

Transfection at any developmental stage

The described electroporation protocols can be used to transfet primary neurons in any developmental stage as long as the cells are allowed to adhere to the culture plate before the process is started. This makes it possible to study neurons before polarization and axon/dendrite formation, but also to investigate neurons with well-established neuronal networks and synapses. Figure 4a shows neurons transfected with GFP plasmid after 24 h in culture, and figure 4b shows neurons that have been allowed to differentiate for 264 h before GFP transfection.

Density-independent transfection

The very low electrochemical toxicity and the fact that no trypsinization or re-plating steps are needed during Cellaxess Elektra transfection ensure that cell consumption is significantly reduced compared to other electroporation techniques (4), something that is important to take into account when screening a large number of genes in cell types that are challenging or expensive to prepare. A variety of cell densities (embryonic rat neurons, cortex and hippocampus combined) were plated and cells were incubated for 96 h before transfection in order to test whether cell numbers can be even further reduced. All densities tested (3 000-30 000 cells per well) resulted in transfection efficiencies above 30%, and when using 10 000 cells per well or more efficiencies were consistently above 50% (Figure 5a). This shows that it is possible to further reduce the number of cells needed for large scale experiments.

Substrate-independent transfection protocols

The Cellaxess Elektra transfection protocols are cell specific but not substrate specific, making it possible to co-transfect with both plasmids and siRNA or to change substrates without re-optimization steps. When adding siRNA against GFP together with the plasmid, GFP expression was significantly reduced 72 h post-transfection compared to when using scrambled siRNA and plasmid at 350 V (Figure 5b). The number of viable neurons 72 h post-electroporation was unaffected compared to non-electroporated controls (Figure 5c), showing that differentiated neurons are viable for several days after transfection.
For transfection with a plasmid vector coding for CopGFP (Eurogen, Moscow, Russia), a source plate (AbGene, Surrey, UK) with plasmid diluted in Cellaxess Elektra Accelerator Solution was prepared and loaded onto the instrument together with the cell plate, a storage plate for recycled medium, and a trough containing fresh cell culture medium. For siRNA experiments, siGFP (cat AM4635) or scrambled siRNA (cat AM2646) from Ambion (Life Technologies, Paisley, UK) at a final concentration of 100 nM was diluted together with pMOWS GFP plasmid. The Cellaxess Elektra protocol for transfection was started through the Commander software. The automated transfection process was composed of the following work steps; 30 μl of the culture medium was transferred to the storage plate, leaving 10 μl of residual medium. Then, 40 μl Cellaxess Elektra Accelerator Solution with plasmid was added, and the electroporation protocol was carried out. 15 μl of the Accelerator Solution was removed and 25 μl of the recycled culture medium was added back to the cells. Finally, 15 μl fresh medium was added to each well, and the cell plate was returned to the incubator. When optimizing Cellaxess Elektra transfection, 16 different pulse protocols were applied in a single 384-well plate (24 wells per condition), making the optimization procedure very efficient.

Despite extensive research on central nervous diseases for decades, several of these disorders represent unmet medical needs. Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, amyotrophic lateral sclerosis, and spinal muscular atrophy all exhibit unique neurological pathologies, and the mechanisms for neuronal loss are complex. Genomic screening can aid in the understanding of these, as well as in the generation of novel drug targets, but has been hampered by the lack of efficient transfection techniques for primary neuronal cells. The Cellaxess technology and the neuron specific transfection protocols described make it possible to explore gene function in a variety of adherent neuronal cell types at any density and in every stage of differentiation. Gene function can be studied on a genome-wide scale in cells previously inaccessible to genetic manipulation. Together with the development of the iPSC (induced pluripotent stem cells) technology, which gives rise to the possibility of generating relevant neurons affected by disease, the Cellaxess Elektra opens up a whole new range of opportunities in the field of neuronal target discovery.

**Cellaxess Elektra Transfection**

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**Cell Culture**

Fresh embryonic day 18 rat or mouse brains (combined hippocampus and cortex or dorsal root ganglion) from Brainbits LLC (Springfield, IL) were triturated with small- and large-bore pipets in Hibernate medium (Brainbits LLC). After trituration, undispersed pieces were allowed to settle, and the supernatant was transferred to new tubes and centrifuged at 200 g for 1 min. The pellet was resuspended in NbActiv4 (Brainbits LLC) and cells were counted and plated in Cellaxess Elektra 384-well plates coated with poly-D-lysine. Cultures were maintained at 37°C and 5% CO2.
Reproducibility within and between Plates. (A) Mean transfection efficiency for a total of 20 plates in hippocampal and cortical rat neurons. [B] and [C] shows the mean transfection efficiency versus row and column, respectively.
Immunocytochemistry

24-96 hours post-transfection, cells were fixed, permeabilized, and stained. Briefly, fixation and nuclear staining was performed for 15 min using 40 μL/well of 4% paraformaldehyde and 10 μg/mL Hoechst 3342 (Invitrogen, Eugene, OR) in phosphate-buffered saline (PBS), pH 7.4. Cells were rinsed two times with PBS followed by permeabilization for 20 min in 40 μL/well of 0.25% Triton-X100 and 1% goat serum [Santa Cruz Biotechnology, Santa Cruz, CA]. Permeabilization buffer was removed and cells were incubated for 1 h at 37 °C in 20 μL/well mouse anti-MAP-2 primary antibody [Santa Cruz Biotechnology] in permeabilization buffer. The plates were washed two times with PBS and 0.25% Triton-X and incubated for 1 h with 20 μL/well Texas Red X-conjugated goat antimmouse secondary antibody [Santa Cruz Biotechnology] diluted in permeabilization buffer. The fixed and stained cell plates were washed two times with PBS and 0.25% Triton-X100 and left in 50 μL/well PBS.

Imaging and analysis

Fluorescence images were acquired using an inverted LSM 700 confocal microscope [Carl Zeiss Microlmaging GmbH, Germany]. The Plan-Achromat 20x/0.8 M27 dry objective was used, with the pinhole set to 1 Airy unit. The three channels were acquired in sequential mode: Hoechst was excited at 405 nm and detected using a SP490 nm emission filter, GFP was excited at 488 nm and detected with a SP555 nm emission filter, while Texas Red was excited at 555 nm and its emission was detected above 559 nm. For plasmid transfection efficiency measurements, the plates were analyzed by an ImageXpress Micro [Molecular Devices, Sunnyvale, CA]. We captured four images per well in three channels: (a) nuclei (Hoechst); (b) neuron cell body and neurites (MAP-2); and (c) green fluorescent protein (GFP). Images were processed and analyzed using the MetaXpress software [Molecular Devices, Sunnyvale, CA]. For siRNA experiments images were acquired on the Opera LX confocal microscope [PerkinElmer, Waltham, MA] and GFP intensity in the neuritis was analyzed using the machine learning algorithm in the Columbus software [PerkinElmer] and normalized to total cell number as measured by MAP-2 positive cells.

References