Transfection of Schwann cells using a novel electroporation method

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SUMMARY
The molecular mechanisms behind the creation and maintenance of the myelin sheath are not fully known. One approach of elucidating these mechanisms of neuron-glia interactions is to induce RNA interference by transfecting Schwann cells with small interfering RNA. This represses the expression of a gene of interest. By studying the resulting phenotype, the eventual relevance of the product of the gene can be evaluated.

In this study a novel electroporation method, Cellaxess™ (developed by Cellectricon, Gothenburg), was used to transfect primary cultures of Schwann cells. The focus was on optimizing the performance of the electroporating device and obtaining high transfection efficiency together with high cell viability. This was done by transfecting Schwann cells extracted from newborn rat pups with a plasmid containing green fluorescent protein. The results were evaluated with immunocytochemistry and viability staining.

I have shown that adherent primary cultures of Schwann cells can be transfected by electroporation with the Cellaxess™ system. The viability following electroporation was high while the efficiency of the transfection was relatively low.
**INTRODUCTION**

The nervous system is often divided into the peripheral nervous system (PNS) and the central nervous system (CNS). The main cell types that constitute the CNS and PNS are neurons and glial cells. The main role for the glial cells is to provide support and protection to the neurons.

Schwann cells are the only type of glial cell present in the PNS. There are two types of Schwann cells, those that myelinate axons and those that ensheath multiple thin calibre axons or neural cell bodies of ganglia (Jessen, 2004). One Schwann cell myelinates a small portion of one axon, and over one thousand cells are needed to myelinate one axon of the sciatic nerve that can become over 1 m long in a human. Between two adjacent myelin-forming Schwann cells, sodium channels and membrane proteins cluster, creating the node of Ranvier which is a small gap of exposed axon between two Schwann cells (Sherman & Brophy, 2005). Figure 1 shows the axon together with the portions of myelin formed by each Schwann cell.

![Diagram of axon with associated Schwann cells](image)

**Figure 1.** Axon with associated Schwann cells [1]. A cross section [2] illustrates how the Schwann cell wraps around the axon. The node of Ranvier [3] is located between adjacent Schwann cells.

It is believed that the node of Ranvier is formed by proteins in the axon membrane in response to diffusible or membrane-bound factors coming from the Schwann cell (Sherman & Brophy, 2005).

Neuron-glia interactions like those described in the example above are crucial for the development and maintenance of the PNS. Schwann cells are derived from neural crest cells and both the differentiation and the migration to the target axon are directed by extrinsic signals. The signalling occurs in both directions and the neuron and the Schwann cell depend on each other for survival (Jessen & Mirsky, 2005).

The exact molecular events behind the neuron-glia interactions are not known. It is of great interest to elucidate how these mechanisms work and what soluble and membrane-bound factors are involved in different developmental processes, since this may lead to a better understanding of the development of our nervous system and ultimately to cure neurological diseases (Sherman & Brophy, 2006).

One approach to determine if a protein is involved in cell-cell interactions is to repress the expression of the protein and then observe the phenotype of the manipulated cells. This repression can be formed by triggering an antiviral defence system in the cell known as RNA interference (RNAi). RNAi is normally initiated by viral double-stranded RNA or by endogenous micro RNAs (Rana, 2007).

A suppression can also be induced by a transfection of cells with exogenous double-stranded RNA known as small interfering RNA (siRNA). Inserted siRNA induces the assembly of the
RNA-induced silencing complex (RISC). RISC contains one strand of the introduced siRNA together with proteins that bind and cleave RNA. The siRNA strand functions as a guide strand that directs RISC to a complementary target mRNA, which is then cleaved. By designing siRNAs that are complementary to the mRNA from a gene of interest, the expression of the gene can be temporarily suppressed in the cells (Rana, 2007).

In order to confirm that the transfection is successful an indicator is needed. For this purpose a plasmid containing a sequence coding for a membrane-bound farnesylated green fluorescent protein (GFP) can be transfected together with the siRNA. The cells that get the GFP-plasmid start to express GFP and can then be investigated under a fluorescence microscope. Although the GFP-plasmid and the siRNA are administered simultaneously, it is not certain that a cell expressing GFP also was transfected by siRNA. However, it is more likely that a GFP positive cell has been transfected with siRNA. The reason for this is that the GFP plasmid has to reach the cell nucleus, while a siRNA only needs to reach the cytosol in order for it to be expressed.

**Transfection by electroporation**

Electroporation is a method where transient pores in the cell membrane are created by the application of a brief electrical field. Through the pores, small molecules like DNA, siRNA, peptides and dyes, can be introduced into the cell. The electrical field is either generated by a discharging capacitor or by a square-wave pulse generator. The latter is preferred since it offers increased reproducibility and a better control of the parameters building up the electroporation pulse.

**Evaluation**

*Immunocytochemistry*

Immunocytochemistry is carried out by exposing a cell culture or single cells to an antibody raised to bind to an antigen specific for the cell type of interest. The antigen is often a protein that can be membrane-bound or present in the cytosol or nucleus. To help the antibody to bind cytosolic and nucleic antigens it is necessary to treat the cells with detergent to make the membrane permeable. In addition the cells need to be fixed to avoid rupture of delicate structures during the different steps of the procedure. The fixation is done by adding paraformaldehyde, which creates cross-links between proteins and DNA.

To investigate the presence of a certain cell type in the sample, the antibody bound to the antigen needs to be detected. For this purpose indirect immunocytochemistry can be used. In this method a secondary antibody directed against the primary antibody is conjugated to fluorochromes, enzymes or colloidal gold particles. Fluorochromes are visible under fluorescence microscopes where they emit light of different wavelengths. (Fritschy & Härtig, 2001).

*Viability – Propidium iodide/Hoechst 33258*

Propidium iodide (PI) is a fluorescent stain that stains DNA by intercalating between base pairs. PI is unable to cross the membrane of living cells and therefore only stains the dead cells. Hoechst 33258 also stains DNA but is more lipophilic and thus easily crosses membranes and stains both living and dead cells.
**Aims**

The aims for this study were to optimize a protocol for transfection of adherent primary cultures with the Cellectricon Cellaxess™ with respect to transfection efficiency and cell viability. Two protocols have been suggested by the manufacturing company (Cellectricon) and in these two protocols the voltage is varied to find the optimal setting for successful transfection. For these experiments a GFP-plasmid was used as the sole transfection agent.
RESULTS

Culturing Schwann cells

Schwann cells were prepared from the sciatic nerve of newborn rats. To separate the Schwann cells from endogenous fibroblasts a new technique described by Jirsová et al. (1997) was used. This method is called cold jet. The technique utilizes the fact that fibroblasts and Schwann cells react differently to cold cell culture medium. When exposed to cold media, the Schwann cells shrink and detach rapidly from the underlying layer of fibroblasts while the fibroblasts remain attached. When transferred to a new coated flask the Schwann cells will reattach rapidly since no proteolytic cleavage of adhesion receptors has taken place. This makes it possible to separate Schwann cells from fibroblasts. This method was quite simple to perform, but to obtain enough cells without fibroblasts was difficult and took time.

To further help with keeping the Schwann cell cultures as pure as possible Forskolin was added to the culture media. Forskolin is an adenyl cyclase activator that leads to an increase in intracellular levels of cyclic AMP. Forskolin stimulates Schwann cell proliferation and represses the proliferation of fibroblasts (Dumaz, 2005; Monje, 2006).

Transfections

Cellectricon Cellaxess™

A new electroporation device, Cellaxess™ (developed by Cellectricon, Gothenburg), was used for the experiments. The advantage with this system is that it makes it possible to transfect adherent cells at any time during development, directly in the culture dish using the normal cell culture medium for transfection. The transfection efficiency of the Cellaxess™ system for primary Schwann cells cultures was investigated. The effect of different parameters such as pulse length, pulse interval and voltage were evaluated. Figure 2 below displays the Cellaxess™ system in detail.

Preparation of the GFP-plasmid

The GFP-plasmid already existed in an E. coli bacterial strain in the lab. After freeze-thawing a new tube of bacteria I obtained plasmids by preparing the bacteria with a plasmid purification kit.
**Evaluation**

*Efficiency – Immunostaining*

To evaluate efficiency immunocytochemistry were used. I treated the cultures with antibodies specific for Schwann cells and observed the cells under a fluorescence microscope.

*Viability – PI/Hoechst*

The viability was assessed by staining the transfected cultures with PI and Hoechst and counting the living and dead cells under a fluorescence microscope.

**Investigating the efficiency of transfection of Schwann cells with a GFP-plasmid**

To investigate transfection efficiency, Schwann cells were electroporated using two different protocols showed in table 1.

**Table 1.** Protocols\(^1\) used for electroporation of Schwann cells.

<table>
<thead>
<tr>
<th>Voltage (V)</th>
<th>Pulse length (ms)</th>
<th>Interval between pulses (s)</th>
<th>Number of pulses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol A</td>
<td>220</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>260</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Protocol B</td>
<td>100</td>
<td>25</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>25</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>25</td>
<td>0.1</td>
</tr>
</tbody>
</table>

\(^1\) For each electroporation event in both protocols 20 µl of 200 ng/µl DNA solution were dispensed with a speed of 50 µl/min.

As seen in table 1 the voltage was varied in both protocols while pulse length, interval between pulses and the number of pulses was kept constant. Following electroporation, the cells were incubated for 24 hours. The cells were then fixed and labelled with a Schwann cell specific S100 antibody. The cells were then photographed using a fluorescence microscope. Figure 3 shows transfected Schwann cells stained with S100 antibodies.
Figure 3. The picture shows cells that have been transfected by electroporation (green) and stained with antibodies against S100 (Schwann cell marker) (red). [1] shows a transfected Schwann cell (green and positive for S100), [2] shows a transfected cell not positive for S100, most likely a fibroblast, and [3] shows a Schwann cell that has not been transfected.

The efficiency was estimated by counting the number of transfected Schwann cells and comparing this with the total amount of Schwann cells present in each picture. In table 2 the results of transfections with protocol A is summarized. A one-way ANOVA show that the mean values were not significantly different (table 2 and 3).

Table 2. Mean efficiency with standard deviation of transfections with the three voltage settings in protocol A.

<table>
<thead>
<tr>
<th>Voltage (V)</th>
<th>Mean efficiency (%)</th>
<th>Standard deviation</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>220 V</td>
<td>1.86</td>
<td>2.57</td>
<td>8</td>
</tr>
<tr>
<td>260 V</td>
<td>2.58</td>
<td>2.29</td>
<td>4</td>
</tr>
<tr>
<td>300 V</td>
<td>6.94</td>
<td>6.19</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3. Mean efficiency with standard deviation of transfections with the three voltage settings in protocol B.

<table>
<thead>
<tr>
<th>Voltage (V)</th>
<th>Mean efficiency (%)</th>
<th>Standard deviation</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 V</td>
<td>2.46</td>
<td>2.50</td>
<td>3</td>
</tr>
<tr>
<td>120 V</td>
<td>6.64</td>
<td>9.54</td>
<td>6</td>
</tr>
<tr>
<td>140 V</td>
<td>4.48</td>
<td>4.03</td>
<td>6</td>
</tr>
</tbody>
</table>

Cell viability following electroporation
Some cells die during an electroporation procedure. In order to find out how the cell viability was affected by the electroporation, Schwann cells were transfected according to the protocols showed in table 1. After electroporation, the cells were incubated for 24 h and then stained with propidium iodide and Hoechst. Pictures were taken of the electroporated areas and viability was evaluated by counting the dead cells in the area and then compare them to the total number of dead and living cells. Tables 4 and 5 show the viability for transfections with protocol A and B respectively.
Table 4. The mean value of cell viability with standard deviation of Schwann cells transfected according to protocol A.

<table>
<thead>
<tr>
<th>Voltage (V)</th>
<th>Mean viability (%)</th>
<th>Standard deviation</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>220 V</td>
<td>91.5</td>
<td>7.2</td>
<td>8</td>
</tr>
<tr>
<td>260 V</td>
<td>69.1</td>
<td>15.9</td>
<td>4</td>
</tr>
<tr>
<td>300 V</td>
<td>94.5</td>
<td>1.76</td>
<td>3</td>
</tr>
</tbody>
</table>

A one-way ANOVA found the mean value differences in table 4 to be significantly different.

Table 5. The mean value of cell viability with standard deviation of Schwann cells transfected according to protocol B.

<table>
<thead>
<tr>
<th>Voltage (V)</th>
<th>Mean viability (%)</th>
<th>Standard deviation</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 V</td>
<td>98.2</td>
<td>0.52</td>
<td>3</td>
</tr>
<tr>
<td>120 V</td>
<td>80.0</td>
<td>13.5</td>
<td>4</td>
</tr>
<tr>
<td>140 V</td>
<td>89.1</td>
<td>4.6</td>
<td>4</td>
</tr>
</tbody>
</table>

A one-way ANOVA found the mean value differences in table 5 not to be significantly different.

Visual assessment of the Schwann cell content revealed that it was around 10 %. Also fibroblasts had been transfected, but no quantitative analysis was made regarding efficiency or viability.
DISCUSSION
I have shown that it is possible to transfect adherent primary cultures of Schwann cells. In general the cell viability following electroporation was high, but only using protocol A I could see any difference between voltages. However, I cannot draw any conclusions of this since the protocol is considered optimal only when high efficiency is coupled to high viability.

No reasonable efficiency was achieved as a result of these experiments. It has not been possible to see any differences between the protocols or between the settings within a protocol. The reason for this seems to be the low concentration of Schwann cells in the culture which mainly depends on problems with the “Cold jet” technique. I found out that if the spraying with cold media was done too extensively, also the fibroblasts detach from the flask, which gave lower Schwann cell purity.

Electroporation
Since the electroporation device was a prototype I experienced a variety of issues regarding handling and performance of the apparatus. One major problem was that there were sometimes no transfection occurring. I was able to find out that this in part was due to the concentration of GFP-plasmid being too small.

A lot of error messages from the equipment disrupted the electroporations. The error occurring was that the apparatus were not able to tell which voltage that had been delivered to the cell culture. This of course was a big issue since the aim of the study was to optimize the protocol by varying the voltage.

Future experiments
For future experiments the same approach can be used. I believe that higher efficiencies can be obtained if cultures with a higher concentration of Schwann cells are used. To obtain this I would be more cautious when purifying the cultures with cold jet. I would also use another media when culturing Schwann cells as suggested by Haastert et al. (2007).

In addition I would like to increase the sample size (i.e. the number of transfected cells) to give the results more statistical significance.

The time frame didn’t allow for the improvements mentioned above. If the project had been continued I believe that the results would have revealed a greater difference between the settings of the protocols. This would also make it possible to draw conclusions of which settings is optimal to obtain high efficiency together with high viability when transfecting Schwann cells.
MATERIALS AND METHODS

Cell culturing

Dissection

The study was approved by the regional ethics committee for research on animals (Uppsala, Sweden) and carried out in accordance with the policies of the Society for Neuroscience. Sciatic nerves from newborn Sprague Dawley rats were used to extract Schwann cells for transfection. The pups were decapitated and washed in cold HEPES buffered Leibovitz’s L15 medium (L15; Invitrogen). Sciatic nerves were removed under a dissection microscope and washed in ice-cold L15 medium.

Primary Schwann cell cultures

For the primary cultures the sciatic nerves were placed in a solution of 0.125% trypsin (Invitrogen) and 2 mg/ml collagenase (Sigma) in L15 (37 °C) for 15 min. They were then mechanically dissociated through a Pasteur pipette. Dissociated cells were washed in L15 supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen), to stop the action of the trypsin and collagenase, and centrifuged at 160 × g. Pellets were washed in cold L15 to remove debris and centrifuged again. The pellet containing the cells was re-suspended in DMEM medium (Sigma) supplemented with 0.3% glutamine (Invitrogen), 1% penicillin-streptomycin (Sigma) and 1:1000 Forskolin (Sigma). The cells were plated in poly-L-lysine (Sigma) coated 250 ml flasks (Nunc) and kept at 37 °C in a humidified atmosphere of 5% CO₂ and 95% O₂.

Purification of Schwann cells – Cold jet

After 2 days in culture, the flasks were removed from the incubator and the medium of the cultured Schwann cells was discarded. 5 ml of cold (4 °C) L-15 (Invitrogen) medium was instead added to the flask. After a few seconds the medium from the flask was collected and put into a 15 ml tube (Falcon) and another 5 ml of cold L-15 medium was added to the flask. With a 2 ml glass pipette the cold medium was sprayed over the cells 12-15 times and then collected and put back into a 15 ml tube (Falcon). The L-15 medium now containing Schwann cells was centrifuged for 5 min at 160 × g. After centrifugation the supernatant was discarded and the pellet resuspended in DMEM (Sigma) with 10 % FBS (Invitrogen) and 1:1000 Forskolin (Sigma). The cells were then seeded with a concentration of 30,000 cells per 300 µl of medium on poly-L-lysine (Sigma) coated 13 mm diameter glass cover slips in 4-well plates (Falcon).

Plasmid preparation

To prepare the GFP-plasmids, 2 ml of a solution of E. coli bacteria (10⁶ CFU/ml) containing pEGFP-F (BD Biosciences, Cat #6074-1) were incubated overnight at 37 °C in 300 ml of LB (8 g/l yeast extract (Merck), 12.8 g/l peptone (Merk) and 4 g/l NaCl in H₂O). After incubation 100 ml of the E. coli suspension was processed with a Jet Star Plasmid Purification Maxi Kit (Genomed, Cat No. 222010). The purified plasmids were resuspended in Neurobasal medium (Invitrogen) and the DNA concentration was determined by a spectrophotometer (Nanodrop). The final concentration of GFP-plasmid was approximately 1200 ng/µl.

Electroporation

Transfection of adherent Schwann cell cultures was accomplished using Cellaxess™ electroporation technology (Celllectronic, Sweden), combined with a pEGFP-F vector

¹ CFU = colony-forming unit, i.e. the number of viable bacteria in the solution.
delivery. The electroporation capillaries were filled with Neurobasal medium, containing 200 µg/ml pEGFP-F vector. The 4-well plate (Falcon) containing the cells was removed from the incubator and directly placed in the Cellaxess safety box. The electroporation head with its capillary was positioned in the wells to reach the targeted cells, using an inverted microscope. Table 1 shows the two protocols used for the electroporations.

After 30 minutes at 37 °C in a humidified atmosphere of 5% CO₂ and 95% O₂, the medium was changed to new DMEM (Sigma) supplemented with 10 % FBS (Invitrogen) and 1:1000 Forskolin (Sigma). The transfected cultures were then kept for 24 h at 37 °C in a humidified atmosphere of 5% CO₂ and 95% O₂ before evaluating viability and efficiency.

Viability test
For viability evaluation the medium in the wells was removed and replaced with 300 µl of L-15 with 1:1000 Propidium Iodide (1 mg/ml) and 1:100 Hoechst 33258 (10 mg/ml). The live and dead cells were then counted under a fluorescence microscope.

Immunocytochemistry

Fixation – Primary antibody
The cells in the 4-well dishes were fixed for 15 min in Stefanini’s fixative solution, consisting of 2% formaldehyde (w/v) and 15% of a saturated picric acid solution (v/v) in phosphate buffered saline (PBS; 137 mM, NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4). After fixation the cells were washed 3 times in PBS. After washing, the cells were treated with rabbit anti-cow S100 (1:300, DAKO Z0311, Denmark) in blocking solution (PBS with 0.25% Triton-X-100 and 0.25% Bovine Serum Albumin) for 2 h in room temperature. Then the samples were rinsed 3 times 10 minutes in washing solution before mounted on glass microscope slides in 30 µl DTG with DAPI (Sigma). A cover glass were placed on top of the cover slips and sealed with nail polish.

The primary antibody (rabbit anti-cow S100) used in these experiments is against S100 proteins, which belong to the family of EF-hand calcium-binding proteins. S100 proteins regulate intra-cellular Ca²⁺-homeostasis and are present in Schwann cells (Gonzalez-Martinez et al., 2003).

Secondary antibody – Mounting
After rinsing, the cells were treated with donkey anti-rabbit-IgG-RRX² (1:400, Jackson Immunoresearch) in blocking solution for 45 minutes in room temperature. Once again the cells were rinsed 3 times 10 minutes in washing solution before mounted on glass microscope slides in 30 µl DTG with DAPI (Sigma). A cover glass were placed on top of the cover slips and sealed with nail polish.

The secondary antibody (donkey anti-rabbit-IgG-RRX) is conjugated with a red fluorochrome since the transfected cells are expressing green-emitting GFP and are treated with blue-emitting DAPI.

² RRX = Rhodamine Red-X, i.e. the fluorophore conjugated to the secondary antibody.
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REFERENCES


