Chapter 14
In Utero Electroporation: Assay System for Migration of Cerebral Cortical Neurons

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1 Introduction

During the development of the cerebral cortex, the majority of cortical neurons are generated in the ventricular zone (VZ) facing the lateral ventricle and then migrate toward the pial surface to form the highly organized 6-layered cerebral cortex. Detailed profiles of these processes and their molecular mechanisms had been largely unknown because of the absence of an efficient assay system. The in vivo electroporation system was initially devised for use within chick embryos (Funahashi et al., 1999; Itasaki et al., 1999; Momose et al., 1999; Muramatsu et al., 1997), and we and other groups have used that system as a basis for developing an in utero electroporation system, which allows plasmid DNA to be introduced into cortical progenitor cells in developing mouse embryos in the uterus (Fukuchi-Shimogori and Grove, 2001; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001; Takahashi et al., 2002). In utero electroporation of other sites in the brain, including the hippocampus (Navarro- Quiroga et al., 2007), cerebral basal ganglia (Borrell et al., 2005; Nakahira et al., 2006), cortical hem (Takiguchi-Hayashi et al., 2004), and dorsal thalamus (Bonnin et al., 2007), has recently been reported. Introducing green fluorescent protein (GFP) enables the entire processes of migration and layer formation to be visualized (Ajioka and Nakajima, 2005; Sasaki et al., 2008; Tabata and Nakajima, 2002, 2003), and the role of any gene involved in these processes can be easily assessed by overexpressing the proteins or their mutants (Ohshima et al., 2007), or by knocking down the genes by the RNA interference technique (Bai et al., 2003). Furthermore, the Tet-On/Off system and/or other plasmid-vector-based technologies will expand the potential of the analyses. In this section we review the principles and methods of gene transfer into the cortical wall of mouse embryos by means of the in utero electroporation system.
2 Materials

2.1 Instruments

- Electroporator: CUY21E or CUY21SC (Nepa gene, Chiba, Japan)
- Tweezers-type electrode: CUY650P3 for embryonic day (E) 12.5, or CUY650P5 for E13 or older (Nepa gene)
- Aspirator tube assembly (Drummond)
- 1-mm diameter glass capillary tubes (GD-1; Narishige, Tokyo, Japan)
- Optical fiber light
- Sterile gauze: 7.5 × 7.5 cm
- Surgical instruments: fine forceps × 2, surgery scissors × 2, ring forceps, needle holder
- Surgical tape
- Nylon suture
- Silk suture

2.2 Reagents

- HEPES-buffered saline (HBS): Dilute 2 × concentrated HBS (51558, Sigma) with autoclaved water at 1/2
- 0.1% FastGreen solution: Dissolve the powder (F7258, Sigma) with autoclaved water
- Autoclaved phosphate-buffered saline (PBS)
- Diluted Nembutal solution: Dilute Nembutal solution (5% Pentobarbital sodium salt solution) with autoclaved water at 1/10

2.3 Preparation of Plasmid DNA

(See Also Comments 1 and Fig.14.3b)

Plasmid solution prepared by conventional methods, such as with the Qiagen kit, can be used for in utero electroporation. If precipitates form in the plasmid solution, further purification with phenol/chloroform followed by chloroform extraction is required, because such precipitates may cause hydrocephalus. Plasmids are dissolved with HBS. The location of the DNA solution is monitored by adding 0.1% Fast Green solution at 1/10 volume to the aliquot of DNA solution to be injected.

2.4 Preparation of Micropipettes

The micropipettes for injection are made with a puller (PC-10, Narishige, Tokyo, Japan) from 1-mm diameter glass capillary tubes (GD-1; Narishige). The tip of the
micropipettes is cut obliquely with a fine forceps under a dissecting microscope (Fig. 14.1a). The micropipettes are attached to the aspirator tube assembly (Drummond) (Fig. 14.1b).

3 Procedure

1. A pregnant mouse is intraperitoneally injected with a 12μl/g body weight dose of 1/10 diluted Nembutal solution (see Comments 2).
2. Ten minutes later the mouse is placed on its back on a dissecting board, and its limbs are held in place with a surgical tape.
3. After disinfecting the abdominal skin with 70% ethanol, an approximately 2 cm midline skin incision is made extending anteriorly from the level of the midpoint between the most posterior two pairs of nipples using one set of forceps and scissors (Fig. 14.2a).
4. A 2 cm midline incision is then made in the abdominal wall along the linea alba using another set of forceps and scissors. A piece of sterile gauze with a hole
cut in the center is placed over the incision, and one uterine horn is drawn out through the hole in the gauze (Fig. 14.2b).

5. After observing the orientation of the embryos through the wall of the uterine horn, a micropipette is inserted into the lateral ventricle, and 1–2 μl of plasmid solution is injected by expiratory pressure using the aspirator tube assembly (see Comments 3–6, Fig. 14.2c).

6. After soaking the uterine horn with PBS, the head of embryo is pinched with a tweezers-type electrode (CUY650P3 or CUY650P5), and electronic pulses are applied with a CUY 21E electroporator (see Comments 7, Fig. 14.2d). The electroporation conditions are shown in Table 14.1. The voltage should be adjusted so that the actual current would become 40–60 mA.

7. After the procedure is completed on the first uterine horn, it is replaced in the abdominal cavity, and the other uterine horn is exposed and subjected to the same procedure.

Fig. 14.2 In utero electroporation procedure. (a) Anesthetized pregnant mouse placed on a dissecting board. The broken line indicates the site of the incision. (b) One uterine horn is drawn out through a hole in a piece of sterile gauze. (c) Plasmid DNA is injected into one or both lateral ventricles through the uterine wall. (d) Electronic pulses are applied from outside the uterine wall.
When the procedures on the second uterine horn have been completed, the abdominal cavity is filled with PBS.

The abdominal wall and skin are closed with nylon sutures and silk sutures, respectively.

Since the body temperature of the mouse decreases as a result of the exposure of uterus and the effect of anesthetization, the mouse should be warmed with hands or on a plate warmer (see Comments 8).

After surgery, the mice should give birth to the electroporated pups normally. If CAG-promoter-driven expression vector of enhanced GFP (CAG-EGFP) (Niwa et al., 1991) has been injected, the GFP fluorescence would be visible through the skin under a fluorescence stereomicroscope on postnatal day 1.

### 4 Application and Results

#### 4.1 Optimization of Electroporation Conditions

Several conditions must be optimized to make in utero electroporation more efficient.

##### 4.1.1 Number of Pulses

A 4μg/μl dose of CMV promoter-driven luciferase-expression vector (CMV-luc) was injected into the lateral ventricle at E14.5, and one, two, or four electronic pulses were applied. The transfected brains were sampled 24h later, and the efficiency of gene-transfer was evaluated on the basis of luciferase activity (Fig. 14.3a). Application of one pulse was insufficient to achieve significant gene transfer. When more than one pulse was applied, the efficiency of gene-transfer increased almost linearly with the number of pulses. The electric pulses create tiny pores in the plasma membrane, and move DNA toward the anode in the electric field. One pulse might be enough to create the pores, not to drive the DNA toward the anode.

##### 4.1.2 Plasmid Concentration

CMV-luc was injected in dose of 0.5, 2, or 5μg/μl at E15.5 and four pulses were applied. The brains were sampled 24h later, and the luciferase activity of each

<table>
<thead>
<tr>
<th>Table 14.1 The electroporation conditions</th>
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<tr>
<td><strong>Age</strong></td>
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<tr>
<td>E12.5</td>
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<tr>
<td>E13−</td>
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</table>

Pulse-on; the duration of square pulse.
Pulse-off; the interval between pulses.
Fig. 14.3 Optimization of various factors for more efficient in utero electroporation. Effect of number of pulses (a), plasmid concentration (b), and voltage (c) on transfection efficiency. (a) A 4μg/μl dose of CMV-luc was injected into the lateral ventricle at embryonic day 14.5 (E14.5), and different numbers of 30 V pulses were applied. The transfected brains were sampled 24 h later, and luciferase activity was measured. (b) CMV-luc plasmid prepared in various concentrations was injected at E15.5 and four 30 V pulses were applied. and transfected brains were sampled 24 h later. (c) 4μg/μl dose of CMV-luc was injected at E15 and electric pulses of various voltages were applied four times. The brains were sampled at E18. The duration of the square pulses applied in all experiments was 50 ms, and the interval between pulses was 950 ms.
sample was analyzed (Fig. 14.3b). The efficiency of gene-transfer increased almost linearly with the concentration of plasmid DNA injected.

### 4.1.3 Voltage

CMV-Luc was injected in dose of 4μg/μl at E15 and four pulses were applied. The brains were sampled at E18, and the luciferase activity of each sample was analyzed (Fig. 14.3c). Luciferase activity was detected when a voltage of around 30V was applied, and lower voltages did not bring about effective gene transfer. This finding seems to indicate that a high level of energy is needed to produce the pores in the plasma membrane. When a voltage of 35V was applied, the expression of the plasmid that was introduced tended to reduce. It may have resulted from cell death.

### 4.2 Tracing the Lineage of GFP-Positive Cells

CAG-EGFP (5μg/μl) (Niwa et al., 1991) was injected into both hemispheres at E12.5. After fixing the embryos at E14.5, the brains were dissected out and examined for GFP fluorescence under a fluorescence stereomicroscope (Fig. 14.4a). Fluorescence was observed in the lateral region of the hemisphere onto which the anode had been placed and in the medial region of the opposite hemisphere. GFP-positive cells were observed in the VZ 12h after electroporation. Accumulation of GFP-positive cells in the subventricular zone (SVZ) and scattered distribution of GFP-positive cells in the intermediate zone (IZ) were observed 1.5 days after

![Fig. 14.4](image_url)  
GFP expression after electroporation. E12.5 mouse embryos were subjected to in utero electroporation and fixed 2 days later. The brains were removed and examined under a fluorescence stereomicroscope (a). Changes in the position and morphology of GFP-positive cells after in utero electroporation (b–d). E14.5 (b,c) or E15 (d) mouse embryos were transfected with CAG-EGFP and then fixed 1.5 days (b), 3 days (c), and 6 days (d) after electroporation. Abbreviations: VZ; ventricular zone, SVZ; subventricular zone, IZ; intermediate zone, CP; cortical plate, MZ; marginal zone. Scale bar, 50μm
electroporation (Fig. 14.4b). By 2.5 days, the number of GFP-positive cells in the IZ had increased, but only a few GFP-positive cells were found within the cortical plate (CP) at that time. By 3 days, many GFP-positive cells assuming bipolar morphology with their leading process pointing toward the pial surface were found in the CP (Fig. 14.4c). At 6 days after electroporation, the major population of GFP-positive cells was observed beneath the marginal zone (MZ) (Fig. 14.4d), and the cells were extending primitive dendrites into the MZ.

5 Comments

1. The efficiency of electroporation is highly dependent on the DNA concentration. The higher the concentration of DNA used, the higher the level of expression became (Fig. 14.3b). When CAG-EGFP is used, a concentration of 1 \( \mu g/\mu l \) is sufficient to visualize the migrating neurons. Overexpression of unstable or large proteins requires higher concentrations, such as 5 or 10 \( \mu g/\mu l \). When a mixture of two different plasmids is injected, all of the transfected cells should contain both plasmids.

2. Sometimes the anesthesia is inadequate to carry out surgery at the dose of Nembutal described in Procedure Step1. In this case, half the original dose should be applied intraperitoneally.

3. Do not grasp the uterus too tightly. Hook the uterus with ring forceps first, and then draw the uterus out with the fingers.

4. Fast Green enables the distribution of the DNA solution in the lateral ventricle to be seen through the uterine wall.

5. Pushing the back of the head of the embryos so that the head is pressed against the uterine wall facilitates the injection process. The micropipette should be inserted with momentum but should not deeply.

6. We avoid injecting the embryos closest to the vaginal duct, because of the possibility of inducing abortion if they are damaged.

7. The plasmid can be introduced into a larger region of the cortex by placing the cathode on the chin of the embryo and the anode at the center of the injected hemisphere.

8. A long operation time decreases the embryo survival rate. The abdominal cavity should be open for no more than 30 min.

6 Troubleshooting

Gene transfer by electroporation has the major advantages of being a simple procedure and achieving a high level of expression of the gene introduced. However, the risk of damages to the brain may be higher than with other gene transfer systems. The troubleshooting was listed below (Table 14.2).
Table 14.2 Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
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<tbody>
<tr>
<td>Dam dies</td>
<td>Operation time too long</td>
<td>Not every embryo needs to be injected. Select embryos to inject and record their location</td>
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<td></td>
<td>Excessive bleeding due to abortion</td>
<td>See “high abortion rate” below</td>
</tr>
<tr>
<td>High abortion rate</td>
<td>Operation time too long</td>
<td>See above</td>
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<tr>
<td></td>
<td>Severe embryo injury</td>
<td>Handle embryos gently. We recommend getting used to handling embryos by starting with E14 or E15 embryos, the ages at which they are easiest to inject</td>
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<td></td>
<td>Disruption of blood flow to the uterus</td>
<td>Make larger skin and abdominal wall incisions. Make sure blood flow is not obstructed by twisting of blood vessels during the operation</td>
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<td>Delayed recovery from the anesthesia</td>
<td>Keep mouse warm during and after the operation</td>
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<tr>
<td>Hydrocephalus</td>
<td>Embryo injury</td>
<td>See solution to “high abortion rate”</td>
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<td></td>
<td>Poor DNA quality</td>
<td>Remove all precipitates by centrifugation or by phenol/chloroform treatment</td>
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<td></td>
<td>Voltage too high</td>
<td>Determine appropriate voltage</td>
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<tr>
<td></td>
<td>Volume of DNA injected too large</td>
<td>Do not inject more than 2μl of DNA into one lateral ventricle of an E15 embryo</td>
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<td></td>
<td>Tip of the micropipette blunt</td>
<td>Cut tip obliquely</td>
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</tbody>
</table>

References


