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Temporal Control of Gene Silencing by *in ovo* Electroporation

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Summary

The analysis of gene function during embryonic development asks for tight temporal control of gene expression. Classic genetic tools do not allow for this, as the absence of a gene during the early stages of development will preclude its functional analysis during later stages. In contrast, RNAi technology allows one to achieve temporal control of gene silencing especially when used with oviparous animal models. In contrast to mammals, reptiles and birds are easily accessible during embryonic development. We have developed approaches to use RNAi for the analysis of gene function during nervous system development in the chicken embryo. Although the protocol given here describes a method for gene silencing in the developing spinal cord, it can easily be adapted to other parts of the developing nervous system. The combination of the easy accessibility of the chicken embryo and RNAi provides a unique opportunity for temporal and spatial control of gene silencing during development.

Key Words: *in ovo* RNAi; *in ovo* electroporation; long dsRNA; chicken embryo; development; nervous system.

1. Introduction

No matter whether you want to analyze the function of a number of candidate genes that you identified in a screen or whether you want to assess the function of your favorite gene, you may need an *in vivo* system that allows for the rapid

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detection of a possible phenotype during development. The analysis of gene function during development requires tight temporal control of gene silencing. Classic genetic tools will only allow for an assessment of gene function during the initial phase of gene activity. Additional activities during later developmental stages will not be within reach, as the lack of gene function during the early stages will preclude the analysis of all subsequent stages. For this reason, specific gene silencing by RNA interference (RNAi) provides an exceptional tool for loss-of-function approaches during development in vertebrates. Until now, different RNAi strategies have been established for mouse, rat, and chicken embryos ***(1–4***; reviewed in ***5–7)***. However, due to the limited accessibility of mouse and rat embryos during development, RNAi in combination with *in utero* electroporation is very difficult and requires special equipment and expert training. Therefore, the use of mammals as a model organism for developmental studies is limited. In contrast to mammals, the chicken is easily accessible for *in vivo* manipulations during embryonic development. With the establishment of *in ovo* electroporation, as an efficient method for nucleic acid transfer, and *in ovo* RNAi, as a method for gene silencing, the chicken embryo has been turned into a unique model organism for the efficient functional characterization of genes involved in developmental processes ***(4,8–12***; reviewed in ***6)***. *In ovo* RNAi using long dsRNA, shRNA, or siRNA has been used for a variety of functional studies in different parts of the CNS but also in other embryonic tissues ***(4,13–17***; reviewed in ***5)***.

Here we provide a detailed protocol for the silencing of a candidate gene during early development of the spinal cord by *in ovo* RNAi. A particular advantage of *in ovo* RNAi is the fact that long dsRNA can be used for the induction of loss-of-function phenotypes. Unlike adult tissue or cell lines, embryos do not respond to long dsRNA with unspecific inhibition of protein synthesis and apoptosis ***(18,19)***. Therefore, there is no need for lengthy selection of an efficient siRNA or shRNA. Any cDNA fragment or expressed sequence tag (EST) can be used to produce dsRNA by *in vitro* transcription. Since the chicken genome was fully sequenced in 2004, it can be directly compared with the human, mouse, or rat genome, significantly facilitating the identification of orthologs ***(20)***. Therefore, *in ovo* RNAi offers the possibility to study candidate genes identified in other species using commercially available chicken ESTs for the synthesis of the dsRNA.

In the protocol reported here, long dsRNA is injected into the central canal of the developing spinal cord (**Fig. 1a**). Subsequently, the embryo is exposed to an electric field for efficient transfection of selected cell populations (**Fig. 1b**). Depending on the time point and the position of the electrodes, different neuronal populations within the spinal cord (**Figs. 1c, 1d**) but also of the peripheral nervous system can be targeted. Furthermore, this method allows for knockdown of several genes by injecting a mixture of different dsRNAs.

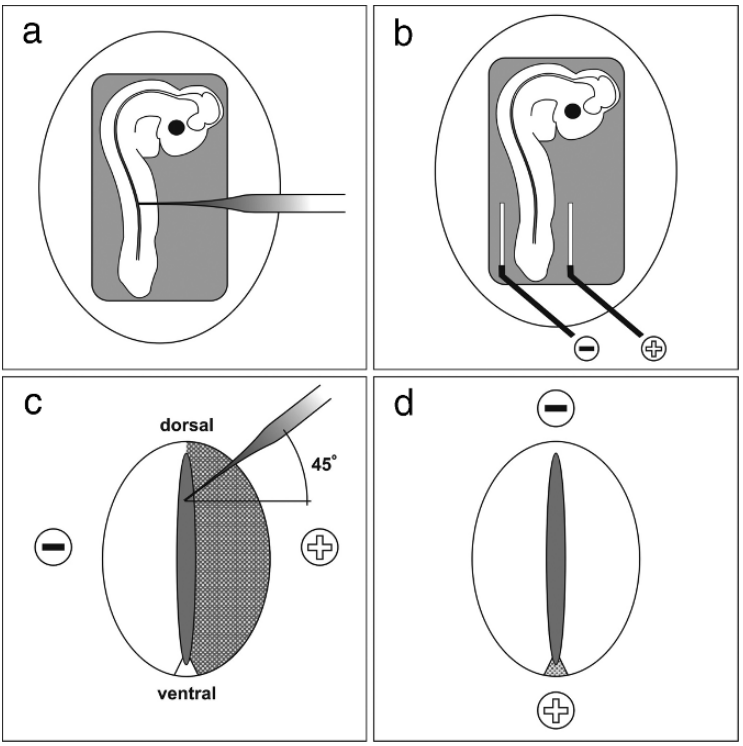

Fig. 1. *In ovo* electroporation. (a) The chicken embryo is made directly accessible through a window in the eggshell for the injection of nucleic acids into the central canal of the spinal cord. (b) The electroporation permeabilizes the cell membrane and therefore allows for the efficient uptake of RNA or DNA. Depending on the position of the electrodes with respect to the embryonic body axis, different tissues can be targeted: A parallel position of the electrode to the spinal cord results in a unilateral transfection [checkered area in (c)]. Within the applied electric field, the injected nucleic acids migrate toward the anode due to the negative charge of RNA and DNA. Therefore, the untransfected side of the spinal cord [left side in (c)] can be used as an internal control. The capillary should be kept at a 45° angle for injection. Placing the electrodes over the dorsal (cathode) and the ventral (anode) midline of the spinal cord results in efficient targeting of floor-plate cells [checkered area in (d)].

Thus, *in ovo* RNAi represents an efficient and inexpensive method to alter the expression of specific genes in a temporally and spatially controlled manner.

2. Materials

2.1. Preparation of dsRNA by in vitro Transcription

1. Heating block at 95 °C.
2. Equipment for gel electrophoresis.

3. *Bam*HI restriction endonuclease (10 U/μL; Roche, Basel, Switzerland).
4. *Sac*I restriction endonuclease (10 U/μL; Roche).
5. RNasin (40 U/μL; Promega, Madison, WI).
6. SP6 and T7 RNA polymerases (15 U/μL; Promega).
7. RNase-free DNaseI (10 U/μL; Roche).
8. 5X transcription buffer (Promega).
9. 100 m*M* rNTPs (25 m*M* of each rNTP; Roche).
10. 100 m*M* DTT (Promega).
11. 0.5 *M* EDTA (pH 8.0).
12. 7.5 *M* ammonium acetate.
13. Phenol–chloroform–isoamylalcohol (25:24:1 vol/vol/vol; pH 7.6–8.0).
14. Acidic phenol–chloroform–isoamylalcohol (25:24:1 vol/vol/vol; pH 4.0).
15. Chloroform–isoamylalcohol (24:1 vol/vol).
16. 100% ethanol.
17. DEPC-treated double-distilled water (ddH_2O) (1:1,000 vol/vol).
18. 70% ethanol in DEPC-treated ddH_2O.
19. Phosphate-buffered saline (PBS, DEPC-treated, 1:1,000 vol/vol): 137 m*M* NaCl, 2.7 m*M* KCl (*see* **Note 1**), 8 m*M* Na_2HPO_4, 1.5 m*M* NaH_2PO_4 (pH 7.4).
20. RNaseZAP (Sigma-Aldrich, St. Louis, MO).

2.2. Windowing the Eggs

1. Fertilized Hisex eggs were obtained from a local hatchery.
2. Preincubator: 38–39 °C, 45% humidity (JUPPITER 576 SETTER+HATCHER; F.I.E.M., Guanzate, Italy; *see* **Note 2**).
3. Incubator: 38–39 °C, 45% humidity (Heraeus/Kendro Model B12, Kendro Laboratory Products, Hanau, Germany; *see* **Note 2**).
4. Egg-Lume Candler (Brinsea Products Ltd., Sandford, UK).
5. Heating plate, 80 °C, to melt paraffin.
6. Soldering iron.
7. Scalpel for drilling holes.
8. 10-mL syringe with needle (Sterican 100, Ø 18G, B. Braun Melsungen AG, Melsungen, Germany).
9. Small scissors for cutting the eggshell (Fine Science Tools Inc., Foster City, CA).
10. Paraffin (Paraplast Tissue Embedding Medium, Oxford Labware, St. Louis, MO).
11. Coverslips, 24 × 24 mm (VWR International AG, Dietikon, Switzerland).
12. Kleenex.
13. Scotch tape.
14. 70% ethanol.

2.3. In ovo *Injection and Electroporation*

1. Spring scissors and forceps (Fine Science Tools Inc.).
2. Electroporator (Electro Square Porator ECM830, BTX Instrument Division, Harvard Apparatus Inc., Holliston, MA; *see* **Note 3**).

3. Platinum electrodes (4-mm length, 4 mm between anode and cathode; BTX Instrument Division, Harvard Apparatus Inc.; *see* **Note 3**).
4. Needle puller (PC-10, Narishige Co., Ltd., Tokyo, Japan).
5. Borosilicate glass capillaries (outer Ø/inner Ø: 1.2 mm/0.68 mm; World Precision Instruments, Sarasota, FL).
6. Polyethylene tubing (Ø 1.24 mm).
7. 0.2-μm filter (Sarstedt, Sevelen, Switzerland).
8. Reporter plasmid: EGFP under the control of the chicken β-actin promoter.
9. Trypan Blue solution 0.4% (Invitrogen, Carlsbad, CA).

3. Methods

3.1. Synthesis of Long dsRNA

A cDNA fragment of a candidate gene cloned into a standard plasmid containing SP6 (or T3) and T7 promoters flanking the insert can be used for the synthesis of long dsRNA by *in vitro* transcription. Here we synthesized dsRNA from a 678-bp cDNA fragment (1,620–2,298 bp) encoding Axonin-1 cloned in the pSP72 vector using SP6 and T7 promoters flanking the insert (*see* **Note 4**).

1. Linearize 10 μg of the plasmid with 20 U *Bam*HI and *Sac*I restriction endonuclease, respectively, for 1 h at 37 °C.
2. For *in vitro* transcription, mix 2 μg of the linearized plasmid with 0.8 μL of 100 m*M* rNTPs, 0.5 μL of RNasin, 2 μL of SP6 or T7 RNA polymerase, 4 μL of 5X transcription buffer, and 2 μL of 100 m*M* DTT, and add DEPC-treated ddH_2O to a total volume of 20 μL (*see* **Note 5**).
3. Incubate the *in vitro* transcription mixture for 2 h at 37 °C (T7 RNA polymerase) and 40 °C (SP6 RNA polymerase), respectively (*see* **Note 6**).
4. Remove the DNA template from the *in vitro* transcription mixture by adding 2 μL RNase-free DNaseI, and incubate at 37 °C for 1 h (*see* **Note 6**).
5. Add 20 μL of DEPC-treated ddH_2O and mix well.
6. Add a mixture of 2 μL 0.5 M EDTA and 22 μL 7.5 M ammonium acetate. Mix well.
7. Purify the synthesized ssRNA with 1 vol of acidic phenol–chloroform–isoamylalcohol; subsequently, extract 1 vol of chloroform–isoamylalcohol.
8. Precipitate with 2.5 vol of 100% ethanol for at least 1 h at –80 °C.
9. Centrifuge for 30 min at 4 °C and 20,000 x g.
10. Wash the RNA pellet with 70% ethanol and spin down.
11. Air-dry the pellet.
12. Dissolve the ssRNA in 20 μL of DEPC-treated PBS (*see* **Notes 6** and **7**).
13. Mix equal ng amounts of antisense and sense ssRNAs (*see* **Note 8**).
14. Heat the mixture for 5 min at 95 °C, and allow for it to cool down slowly to room temperature by switching off the heating block (*see* **Note 6**).
15. Confirm the proper annealing by gel electrophoresis (*see* **Note 6**).
16. Store the dsRNA at –80 °C until further use (*see* **Note 17**).

3.2. Windowing the Eggs

For access to the embryo, the eggs are windowed on the third day of incubation (**Figs. 1a** and **2**).

1. Incubate the fertilized eggs in a preincubator at 39 °C (*see* **Notes 9** and **10**).
2. After three days of incubation, place the egg on the side for at least 30 min before opening, to allow the embryo to reposition on top of the yolk.
3. Mark the position of the embryo on the egg shell with a pencil using an Egg-Lume Candler held against the blunt end of the egg.
4. Wipe the eggshell with 70% ethanol to avoid contamination.
5. Make small holes at the blunt end of the egg and at the corners, outlining the planned window using a scalpel (**Fig. 2a**).
6. Carefully remove 3 mL of albumin through the hole at the blunt end of the egg using a syringe (**Fig. 2a**; *see* **Note 11**).
7. Seal the hole at the blunt end and any possible cracks in the shell by applying melted paraffin.
8. Put a piece of Scotch tape onto the shell to prevent small pieces of the eggshell from falling into the egg (**Fig. 2a**).
9. Cut the outlined window into the eggshell (*see* **Note 12**).
10. Seal the egg by applying melted paraffin to the edges of the window using a brush and a coverslip (**Fig. 2b**; *see* **Note 13**).

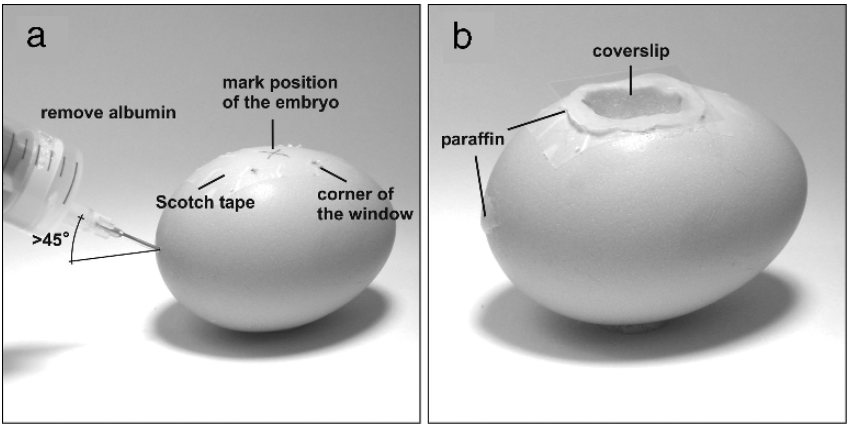

Fig. 2. Windowing the egg. (a) Before egg windowing, the position of the embryo is marked with a pencil on the shell. Subsequently, small holes are drilled at the blunt end of the egg and at the corners outlining the window. Albumin (3 mL) is removed through the hole at the blunt end (*see* **Note 11**). The syringe is kept at a 45º angle to avoid damage to the embryo and the yolk. A piece of Scotch tape prevents pieces of the shell from falling inside the egg while cutting the window with small scissors. (b) For further incubation, the window is sealed with melted paraffin and a coverslip.

11. Put the egg back in the incubator. Make sure that the position of the egg is the same as before windowing to keep the embryo accessible through the window (*see* **Note 17**).

3.3. In ovo *Injection and Electroporation*

1. Autoclave the tools and wipe the working space with 70% ethanol.
2. Reopen the sealed egg by pressing the soldering iron briefly onto the coverslip and removing it carefully.
3. Stage the embryo according to Hamburger and Hamilton ***(21)***. Embryos should be between stages 17 and 19.
4. Remove the extraembryonic membranes covering the embryo with forceps and scissors in order to have direct access to the embryo (**Fig. 3a**).
5. Break off the needle tip to obtain a tip diameter of approximately 5 µm.
6. Sterile PBS containing the dsRNA derived from AXONIN-1 (200–400 ng/µL) and an EGFP reporter plasmid (20 ng/µL) with 0.04 % (vol/vol) Trypan Blue are injected into the central canal of the spinal cord at the level of the hind limbs using a glass capillary connected to a piece of tubing (**Figs. 1c** and **3b**). The injection is controlled by mouth, and the maximal injection volume is achieved when the blue dye reaches the brain vesicle (arrowhead in **Fig. 3b**).
7. Add a few drops of PBS before electroporation to lower the electric resistance and to prevent overheating of the embryo.
8. The electrodes are placed in a parallel manner along the anterior-posterior axis of the spinal cord (**Fig. 3c**).
9. Electroporate the embryo by applying five pulses of 26 V and 50-ms duration each (*see* **Note 14**).
10. After the electroporation, add a few drops of PBS to cool the embryo.
11. Rinse the electrodes with plenty of distilled water to remove denaturated proteins from the surface (*see* **Note 15**).
12. Reseal the egg with a glass coverslip and a soldering iron (*see* **Note 13**).
13. For further incubation, the egg is returned to 39 °C until stage 25 is reached, i.e., approximately two additional days of incubation ***(21)*** (*see* **Note 17**).

3.4. Analysis of the Phenotype and Electroporation Efficiency

For beginners we recommend assessing the efficiency and reproducibility of the *in ovo* electroporation by analyzing EGFP expression directly *in ovo* under the stereomicroscope (**Fig. 3d**). Thus, embryos for further analyses can be preselected (*see* **Note 16**).

The efficiency as well as the specificity of gene silencing by *in ovo* RNAi can be demonstrated by a variety of approaches. Immunohistochemistry on cryostat sections ***(4,13,22)*** and Western blot analysis ***(23,24)*** are common ways to show downregulation of the targeted protein. If antibodies against the targeted

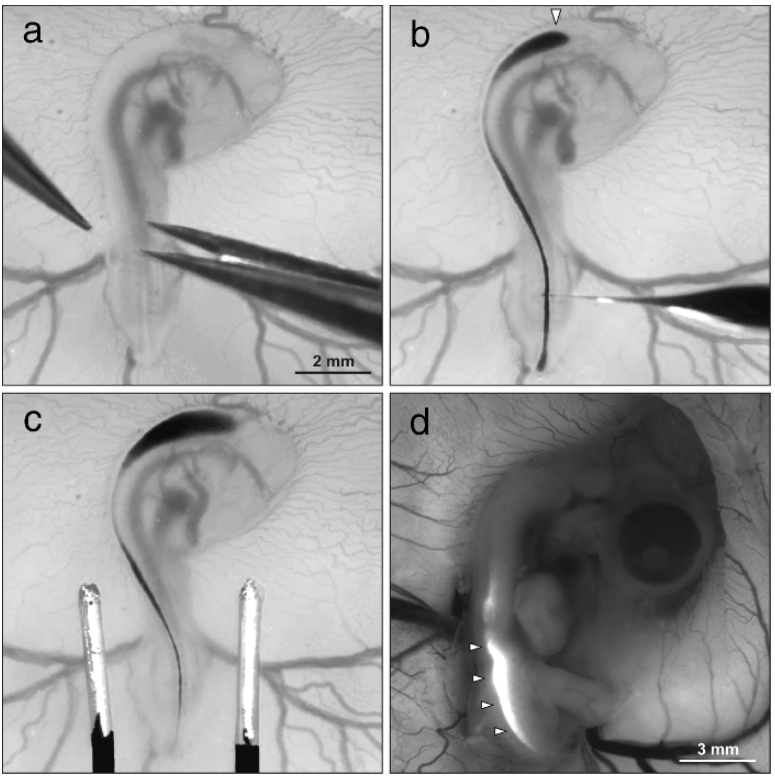

Fig. 3. Injection and electroporation of the embryo. (a) The extraembryonic membranes covering the embryo are carefully removed before injection using forceps and spring scissors. The injection mixture containing dsRNA derived from the gene of interest, an EGFP plasmid for transfection control, and Trypan Blue is injected into the central canal with a glass capillary. The maximal injection volume is achieved when the Trypan Blue has reached the brain vesicle [arrowhead in (b)]. (c) After retraction of the injection needle, the electrodes are placed in a parallel manner along the embryonic axis. For stage 18 embryos, five pulses of 26 V with 50-ms duration are applied for efficient transfection (*see* **Note 14**). (d) The successful transfection can be verified by the expression of EGFP (indicated by arrowheads) under a stereomicroscope equipped with fluorescence optics two days after electroporation.

protein are not available, a decrease in the mRNA can be assessed by *in situ* hybridization using either whole-mount embryos ***(16,25)*** or cryostat sections ***(13)***. Alternatively, semi-quantitative RT-PCR can be used to detect a decrease in the mRNA ***(26)***.

Loss-of-function phenotypes can be analyzed in a variety of ways. To study cell differentiation or cell migration, immunohistochemistry for known

markers may be a good start ***(15)***. Changes in morphology and cell positions, expression patterns, etc. can easily be detected. To visualize axonal trajectories, staining and/or dye tracing in slices or whole-mount preparations are used to account for their three-dimensionality. For an initial assessment and to localize a specific phenotype within the peripheral nervous system, we recommend a neurofilament staining of whole-mount preparations ***(24)***. Alternatively or subsequently, mechanisms involved in wiring the nervous system can be analyzed in vibratome slices by dye tracing or immunohistochemistry ***(4,14)***. For example, we studied molecular mechanisms underlying the pathfinding behavior of commissural axons in the spinal cord using dye tracing in open-book preparations ***(4,13,27)***.

4. Notes

1. Different recipes exist for PBS, and the addition of KCl turned out to be crucial for optimal survival rates.
2. The incubation time and developmental progress of the embryo are dependent on the temperature and humidity. Any incubator set at a temperature of 38–39 °C can be used, as long as high humidity (at least 45%) and good air circulation can be achieved. The use of two incubators, one for preincubation and one for treated embryos, is recommended to minimize contamination and to reduce detrimental effects on the treated embryos due to frequent opening and closing of the incubator.
3. Alternatively, any other electroporator that generates square wave pulses can be used (for a comparison of the different electroporators that are commercially available, *see* **Ref. *8***). For different target tissues, different electrodes have to be chosen to get best results: For *in ovo* electroporations of the developing spinal cord, we use wire electrodes ***(4,13)***. Commercially available electrodes can be found at http://www.btxonline.com/products/electrodes/inovo. Alternatively, for a widespread transfection, platelet electrodes can be used ***(28)***. For a small transfection area, a needle electrode can be placed directly into the tissue ***(17,29,30)***.
4. In addition to long dsRNA, short interfering RNAs (siRNA) and short hairpin RNAs (shRNA) have been applied successfully for RNAi in chicken embryos ***(14–17,22,31)***. In contrast to siRNAs selected by various available algorithms, long dsRNA always effectively silenced target genes in our hands. Long dsRNA is processed by Dicer to give rise to a large number of siRNAs and therefore will always produce many effective ones, making lengthy (and expensive) selection processes unnecessary. Furthermore, long dsRNA can easily be produced by *in vitro* transcription from a cDNA fragment or EST without further cloning steps or expensive synthesis of siRNAs. Chicken ESTs can be obtained from Geneservices Ltd. at http://www.geneservice.co.uk. To exclude any off-target effects

(silencing of nontarget genes), we recommend using two different nonoverlapping dsRNA fragments, as it is highly unlikely that they would both have the same off-target effects. In contrast to mammalian cell lines and nonembryonic tissue, long dsRNA can be applied to embryonic tissue without induction of unspecific effects ***(4,18,19)***. No general inhibition of protein synthesis or induction of apoptosis has been reported in mouse oocytes, embryo-derived cell lines, and chicken embryos ***(4,15,32,33)***.

5. For *in vitro* transcription, RNase-free tips, tubes, and DEPC-treated ddH_2O have to be used. Before starting, clean the workspace with RNaseZAP.
6. Collect 1-µL samples after each step and keep them to control the quality of ssRNA and dsRNA. For this purpose, load the ssRNAs collected after each step and the dsRNA on a 1% agarose gel. Lanes 1 and 2 (one sense and one antisense) contain the linearized plasmids and the ssRNA synthesized by *in vitro* transcription. Lanes 3/4 and 5/6 are sense and antisense ssRNA after DNaseI treatment and purification, respectively. In lane 7 the resulting dsRNA after annealing is loaded. When lanes 5 and 6 are compared to lane 7, the band shift due to the difference in migration between ssRNA and dsRNA should be detected. Furthermore, both ssRNA and dsRNA should give distinct bands. If a smear indicating degradation of the RNA is obtained, the dsRNA should not be used for *in ovo* RNAi.
7. Make sure that the pH and salt concentration of the buffer used to dissolve the ssRNA are in the physiological range and do not have any effect on the development and survival rate of the embryo. Do not use any buffers containing Tris or glycerol.
8. The concentration of the dsRNA for *in vivo* injections should be in the range of 200–400 ng/µL.
9. Eggs should be stored at 15 °C for a maximum of one week before incubation. When the eggs are stored for longer periods, normal development of the embryo is unlikely.
10. To reach 45% humidity, it is usually sufficient to place a tray of distilled water containing 0.1 g/L of copper sulfate at the bottom of the incubator. Copper sulfate decreases the risk of contamination.
11. The holes at the corners are required to allow entry of air and detachment of the embryo from the shell during removal of albumin. Insert the syringe at a steep angle (about 45°; **Fig. 2a**) to avoid damage to the embryo and the yolk that is not compatible with survival.
12. Keep the scissors as horizontally as possible to prevent any damage to the embryo.
13. Make sure that the window is properly closed to prevent dehydration during further incubation. Dehydration will cause the embryo's death. If the paraffin is cooled down too quickly, heat the coverslip briefly with the soldering iron while pressing it down so that the coverslip seals properly along all the edges. Alternatively, the window can be sealed with Scotch tape. Although sealing with coverslips instead of tape is more time-consuming, it facilitates reopening the

window and the development of the embryo can be directly observed through the coverslip. The window can easily be reopened by brief heating of the coverslip using a soldering iron.

14. The electroporation settings should be chosen according to the embryonic stage (also *see* **Ref. *12***): For stage 18 embryos, 5 pulses of 26 V and 50 ms are applied. The voltage should be adjusted to the embryonic stage and the tissue that is electroporated:

Day of Incubation	Embryonic Stage	Target Tissue	Settings
2	12–14*	Spinal cord and neural crest derivates (for example, dorsal root ganglia)	18 V
3	18–20**	Spinal cord and floor plate	26 V

* Red ink is applied to visualize the embryo. Blue ink should not be used, because it interferes with detection of the Trypan Blue that is used to control injection volume and injection site.

** Electroporation at stage 20 or later with the settings mentioned here will prevent transfection of lateral motor neurons.

During electroporation, contact between the electrodes and the major blood vessels as well as with the embryo has to be avoided to prevent severe damage to, or the death of, the embryo. Keep the electrodes away from the heart.

15. Remaining proteins on the electrodes interfere with efficient electroporation.
16. The transfection efficiency depends on the time point of the injection, the concentration of the injected nucleic acids, and the electroporation settings. *In ovo* electroporation with the given settings at embryonic stage 18 resulted in a transfection efficiency of approximately 60% of cells within the electroporated area ***(4)***.
17. Troubleshooting list:

Protocol Step	Potential Problem	Troubleshooting: See Note
Synthesis of long dsRNA	Degradation or bad quality of dsRNA	5, 6
Windowing the eggs	Low survival rate	9, 11, 12, 13
	Contamination	2, 10
	Delay in development	2, 9, 13
In ovo injection and electroporation	Low survival rate	1, 3, 7, 8, 13, 14
	Contamination	2, 10
	Inefficient transfection	14, 15, 16

References

1. Calegari, F., Marzesco, A. M., Kittler, R., Buchholz, F., and Huttner, W. B. (2004). Tissue-specific RNA interference in post-implantation mouse embryos using directional electroporation and whole embryo culture. *Differentiation* **72,** 92–102.
2. Takahashi, M., Sato, K., Nomura, T., and Osumi, N. (2002). Manipulating gene expressions by electroporation in the developing brain of mammalian embryos. *Differentiation* **70,** 155–162.
3. Bai, J., Ramos, R. L., Ackman, J. B., Thomas, A. M., Lee, R. V., and LoTurco, J. J. (2003). RNAi reveals doublecortin is required for radial migration in rat neocortex. *Nat. Neurosci*. **6,** 1277–1283.
4. Pekarik, V., Bourikas, D., Miglino, N., Joset, P., Preiswerk, S., and Stoeckli, E. T. (2003). Screening for gene function in chicken embryo using RNAi and electroporation. *Nat. Biotechnol*. **21,** 93–96.
5. Baeriswyl, T., and Stoeckli, E. T. (2006). *In ovo* RNAi opens new possibilities for temporal and spatial control of gene silencing during development of the vertebrate nervous system. *J. RNAi Gene Silencing* **2,** 126–135.
6. Bourikas, D., and Stoeckli, E. T. (2003). New tools for gene manipulation in chicken embryos. *Oligonucleotides* **13,** 411–419.
7. Prawitt, D., Brixel, L., Spangenberg, C., Eshkind, L., Heck, R., Oesch, F., Zabel, B., and Bockamp, E. (2004). RNAi knock-down mice: An emerging technology for post-genomic functional genetics. *Cytogenet. Genome Res*. **105,** 412–421.
8. Krull, C. E. (2004). A primer on using *in ovo* electroporation to analyze gene function. *Dev. Dyn*. **229,** 433–439.
9. Momose, T., Tonegawa, A., Takeuchi, J., Ogawa, H., Umesono, K., and Yasuda, K. (1999). Efficient targeting of gene expression in chick embryos by microelectroporation. *Dev. Growth Differ*. **41,** 335–344.
10. Muramatsu, T., Mizutani, Y., Ohmori, Y., and Okumura, J. (1997). Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos *in ovo*. *Biochem. Biophys. Res. Commun*. **230,** 376–380.
11. Nakamura, H., and Funahashi, J. (2001). Introduction of DNA into chick embryos by *in ovo* electroporation. *Methods* **24,** 43–48.
12. Itasaki, N., Bel-Vialar, S., and Krumlauf, R. (1999). "Shocking" developments in chick embryology: Electroporation and *in ovo* gene expression. *Nat. Cell Biol*. **1,** E203–E207.
13. Bourikas, D., Pekarik, V., Baeriswyl, T., et al. (2005). Sonic hedgehog guides commissural axons along the longitudinal axis of the spinal cord. *Nat. Neurosci*. **8,** 297–304.
14. Bron, R., Eickholt, B. J., Vermeren, M., Fragale, N., and Cohen, J. (2004). Functional knockdown of neuropilin-1 in the developing chick nervous system by siRNA hairpins phenocopies genetic ablation in the mouse. *Dev. Dyn*. **230,** 299–308.
15. Chesnutt, C., and Niswander, L. (2004). Plasmid-based short-hairpin RNA interference in the chicken embryo. *Genesis* **39,** 73–78.

16. Katahira, T., and Nakamura, H. (2003). Gene silencing in chick embryos with a vector-based small interfering RNA system. *Dev. Growth Differ*. **45,** 361–367.
17. Toyofuku, T., Zhang, H., Kumanogoh, A., et al. (2004). Guidance of myocardial patterning in cardiac development by Sema6D reverse signalling. *Nat. Cell Biol*. **6,** 1204–1211.
18. Svoboda, P. (2004). Long dsRNA and silent genes strike back: RNAi in mouse oocytes and early embryos. *Cytogenet. Genome Res*. **105,** 422–434.
19. Gan, L., Anton, K. E., Masterson, B. A., Vincent, V. A., Ye, S., and Gonzalez-Zulueta, M. (2002). Specific interference with gene expression and gene function mediated by long dsRNA in neural cells. *J. Neurosci. Meth*. **121,** 151–157.
20. Hillier, L. W., Miller, W., Birney, E., et al. (2004). Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature* **432,** 695–716.
21. Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol*. **88,** 49–92. Reprinted as Sanes, J. R. (1992). On the republication of the Hamburger–Hamilton stage series. *Dev. Dyn.* **195,** 229–272.
22. Rao, M., Baraban, J. H., Rajaii, F., and Sockanathan, S. (2004). *In vivo* comparative study of RNAi methodologies by *in ovo* electroporation in the chick embryo. *Dev. Dyn*. **231,** 592–600.
23. Bourikas, D., Baeriswyl, T., Sadhu, R., and Stoeckli, E. T. (2005). *In ovo* RNAi opens new possibilities for functional genomics in vertebrates. In *RNA Interference Technology—From Basic Research to Drug Development* (K. Appasani, ed.), Cambridge University Press, Cambridge, UK, pp. 220–232.
24. Stepanek, L., Stoker, A. W., Stoeckli, E., and Bixby, J. L. (2005). Receptor tyrosine phosphatases guide vertebrate motor axons during development. *J. Neurosci*. **25,** 3813–3823.
25. Dai, F., Yusuf, F., Farjah, G. H., and Brand-Saberi, B. (2005). RNAi-induced targeted silencing of developmental control genes during chicken embryogenesis. *Dev. Biol*. **285,** 80–90.
26. Sato, F., Nakagawa, T., Ito, M., Kitagawa, Y., and Hattori, M. A. (2004). Application of RNA interference to chicken embryos using small interfering RNA. *J. Exp. Zoolog. A Comp. Exp. Biol*. **301,** 820–827.
27. Perrin, F. E., and Stoeckli, E. T. (2000). Use of lipophilic dyes in studies of axonal pathfinding *in vivo*. *Microsc. Res. Tech*. **48,** 25–31.
28. Matsuda, T., and Cepko, C. L. (2004). Electroporation and RNA interference in the rodent retina *in vivo* and *in vitro*. *Proc. Natl. Acad. Sci. USA* **101,** 16–22.
29. Oberg, K. C., Pira, C. U., Revelli, J. P., Ratz, B., Aguilar-Cordova, E., and Eichele, G. (2002). Efficient ectopic gene expression targeting chick mesoderm. *Dev. Dyn*. **224,** 291–302.
30. Luo, J., and Redies, C. (2004). Overexpression of genes in Purkinje cells in the embryonic chicken cerebellum by *in vivo* electroporation. *J. Neurosci. Meth*. **139,** 241–245.

31. Nakamura, H., Katahira, T., Sato, T., Watanabe, Y., and Funahashi, J. (2004). Gain- and loss-of-function in chick embryos by electroporation. *Mech. Dev*. **121,** 1137–1143.
32. Billy, E., Brondani, V., Zhang, H., Muller, U., and Filipowicz, W. (2001). Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc. Natl. Acad. Sci. USA* **98,** 14428–14433.
33. Stein, P., Zeng, F., Pan, H., and Schultz, R. M. (2005). Absence of non-specific effects of RNA interference triggered by long double-stranded RNA in mouse oocytes. *Dev. Biol*. **286,** 464–471.