Double Electroporation in Two Adjacent Tissues in Chicken Embryos

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In ovo electroporation is a well established method to introduce transgenes into a number of tissues in chicken embryos, e.g., neural tissue, limb mesenchyme, and somites. This method has been widely used to investigate cell lineage, cell morphology, and molecular pathways by localized expression of fluorescent reporter constructs. Furthermore, gain- and loss-of-function experiments can be performed by electroporating transgenes or gene-silencing constructs. We have developed a new technique to electroporate tissues positioned opposite to each other with different plasmids using an electroporation chamber. As proof of principle, we electroporated the dorsal surface ectoderm with a reporter construct expressing mCherry and the subjacent somites with a reporter construct expressing EGFP. This double-electroporation technique allows investigation of the localization of two different proteins of interest in two adjacent tissues and will be useful to examine the cellular and molecular interaction of neighboring structures during embryonic development.


Key words: Embryonic development; chick; double electroporation; tissue cross talk; avian; transgene; ex ovo; ectoderm; somites

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Introduction

For more than 200 years, the chicken embryo has been an important research model for investigating developmental processes in vertebrates. In its long scientific history, it has helped researchers discover not only milestones in classical embryology, but also many aspects of cellular and molecular mechanisms in embryonic development (Stern, 2005; Stern, 2018). Moreover, it is an important model for studying diseases like heart defects and cancer growth and metastasis (Kain et al., 2014).

In ovo electroporation of chicken embryos has become a well established technique to investigate molecular signaling pathways by introducing, for example, morpholino oligonucleotides to knockdown proteins (Kos et al., 2003; Mok et al., 2018), or to introduce dominant-negative or wild-type transgenes for overexpression into different tissues of chicken embryos (e.g., neural tissue, limb mesenchyme, and somites) (Muramatsu et al., 1997; Momose et al., 1999; Nakamura and Funahashi, 2001; Scaal et al., 2004; Li et al., 2017). Transgenes, which are inserted into suitable expression vectors, are injected into the tissue of interest and transferred into the targeted cells by transient application of a directed electric current across the embryo. The DNA is negatively charged and moves toward the anode (+). The electrical charges lead to reversible depolarization of the cell membrane, resulting in small pores in the membrane. DNA electrophoretically moves into the cells through these pores and is transcribed under control of the enhancer and promoter sequences of the vector (Neumann et al., 1982; Andreason and Evans, 1988). By appropriate positioning of the electrodes relative to the embryo, the tissue to be targeted can be specifically selected.

In the vast majority of studies using electroporation of chicken embryos, transfection has been limited to one tissue of interest only. Many developmental processes, however, are controlled by signaling interaction between two tissues. For instance, in limb development, the surface ectoderm interacts with the limb bud mesenchyme during limb patterning and growth (Altabef et al., 1997; Capdevila and Izpisua Belmonte, 2001; Fernandez-Teran et al., 2013). Tissue cross talk also controls placode development where the mesoderm and mesendoderm induce placode progenitors in the ectoderm (Hintze et al., 2017). In order to understand the molecular interaction of different tissues, it is often desirable to transfect not only one, but two or more different tissues at the same time.

Next to many other examples, the formation and differentiation of the somites are controlled by molecular interactions with the overlying ectoderm, the neural tube, and the notochord. Somites are the primary segments of the early vertebrate embryo. They appear as epithelial spheres developing from the paraxial mesoderm on both sides of the neural tube. In a next
step, they undergo epithelial-mesenchymal transition (EMT) in the ventral subcompartment to form the sclerotome, which gives rise to the axial skeleton. In contrast, the dorsal somite compartment remains an epithelial cell sheet, the dermomyotome. Later on, cells from the dermomyotome also undergo EMT and migrate dorsally toward the surface ectoderm to form dermis and ventrally to form the myotome, which gives rise to the skeletal musculature of the trunk (Christ and Ordahl, 1995; Brent and Tabin, 2002; Gros et al., 2004).

A number of studies have shown that the differentiation of the somites depends on inductive signals from the surface ectoderm. Notably, Wnt signaling from the surface ectoderm has been shown to be required for initial epithelialization of the somites and for epithelial maintenance of the dermomyotome (Linker et al., 2005; Geetha-Loganathan et al., 2006). Genetic targeting of somites and ectoderm will therefore be useful to decipher the cellular and molecular mechanisms involved in this mesodermal-ectodermal cross talk. Here, we present a method to electroporate two different tissues, the somites and the overlying ectoderm, in a single chicken embryo.

Irrespective of double electroporation, efficient electroporation of the differentiated ectoderm in embryos HH-stage 14-16 is difficult to achieve. The plasmid DNA cannot be injected into the epithelial cell layer of the ectoderm, nor has injection into the space between the vitelline membrane and the ectoderm resulted in successful transfection of the ectoderm in these embryos. In this technical protocol, we present a technique to circumvent this problem. We describe in detail a method to electroporate the surface ectoderm and the somites with different vectors in the same chicken embryo.

Protocol and Results

In order to target adjacent embryonic tissues with two different plasmids, we use a modified form of the Early Chick (EC) culture (Chapman et al., 2001) and a custom-made electroporation.
chamber (Fig. 1) (Voiculescu et al., 2008; Endo, 2012) to first electroporate the ectoderm and then consecutively electroporate the somites.

We present a detailed step-by-step protocol of the complete double-electroporation procedure in the ectoderm and somites.

**Prepare Before the Experiment**

- Incubate eggs at 37.5°C and at 55% humidity until they reach the required age; for double electroporation of somites and ectoderm, we incubate eggs for 50–55 hr until they reach HH-stage 14–15.
- Prepare 0.2% Ham’s agarose plates for ex ovo culture as follows: Heat Ham’s F12 w/L-glutamine (51651C-1000 ml; Sigma-Aldrich Chemie GmbH) in a water bath to 55°C and mix with 2% universal agarose (Bio-Budget) to a final concentration of 0.2%. Fill 3 ml each in 3.5 cm petri dishes and let it set at room temperature, resulting in a layer of 5 mm. The plates can be stored in a humidified chamber at 4°C for one week.
- Cut out rings of Whatman paper (Gel Blotting Paper, thickness 0.34 mm, Carl Roth GmbH) 2.8 cm outer and 1.5 cm inner diameter.
- Cut out square pieces of Whatman paper (Gel Blotting Paper, thickness 0.34 mm, Carl Roth GmbH) with the size of the area to be electroporated. For our purpose, it will cover the embryo over a length of 3–4 somites and a transverse width of the embryo (approx. 1 x 2 mm). Keep the paper dry in a 3 cm petri dish.
- Glue the lid of a 1.5 ml Eppendorf tube upside down in the middle of a 6 cm petri dish so it can be used as a little bowl in which the pieces of Whatman paper will be soaked in DNA.
- Draw injection needles from borosilicate glass capillaries (O.D. 1.5 mm; I.D. 1.10 mm; Science Products GmbH) on a Sutter P-97 Puller.
- Prepare a sufficient amount of plasmid DNA (e.g., with the EndoFree Plasmid Maxi Kit, Qiagen) and concentrate to 5 μg/μl.

**On the Day of the Experiment**

- Take eggs out of the incubator and allow them to cool for 30 min at room temperature.
- In the meantime, prepare plasmid solution to be electroporated into the ectoderm at a concentration of 5 μg/μl DNA (here we used pCAGS–mCherry). For ectoderm electroporation, we use a square piece of Whatman paper as a carrier of the DNA solution. We cut it into rectangular pieces that cover the ectoderm over the length of 3 somites, as described above. For application of the DNA to the paper squares, we put 3–4 paper squares into the cavity of an inverse lid of a 1.5 ml Eppendorf tube, which is positioned in the middle of a 6 cm petri dish to form a humidified chamber. The petri dish is filled with 1 x PBS surrounding the lid. A few drops (approx. 0.5 μl) of DNA are dropped onto each filter paper square. The coating of the filter paper with DNA has to be done freshly for every embryo; don’t reuse the filter paper.
- Fill the electroporation chamber with 1 x PBS. The electrode in the chamber must be connected to the anode (+) (Fig. 1).
- Crack the eggshell by tapping it carefully against the edge of a glass petri dish. Then hold the cracked surface downward and release the content carefully into the glass petri dish without breaking the vitelline membrane. The yolk must stay intact and the embryo must be positioned on top of the yolk; it helps to put some water into the dish before cracking so that the egg yolk stays intact (Fig. 2A).

**Fig. 3.** Illustration of the double-electroporation procedure. A,A’: A filter paper soaked in plasmid DNA is placed onto the ectoderm. B,B’: An electrode connected to the cathode is placed directly on top the filter paper and current is applied. C,C’: After a recovery phase of about 4–5 hr, the second plasmid is injected into the somites. D,D’: The anode is placed within the 1 x PBS solution overlying the injected somites and current is applied.
• Place a Whatman paper ring gently onto the vitelline membrane so that the embryo lies in the inner aperture and the outer margin approximately follows the marginal vein that surrounds the embryo as described for the EC culture protocol (Chapman et al., 2001) (Fig. 2B,C).

• Cut the extraembryonic membranes along the outer margin of the paper ring with fine scissors (Fig. 2D).

• Lift the embryo, which should stick to the paper, with fine forceps. Dab excess yolk off the paper ring using a swab (Pur-Zellin; Hartmann) (Fig. 2E).

• Place the embryo sticking to the paper ring dorsal side up into the electroporation chamber, so that the embryo floats on the surface of the 1 x PBS; avoid submerging it in the PBS (Fig. 2F).

• Make a small hole in the vitelline membrane with a tungsten needle over the area to be electroporated, put a small drop of 1 x PBS (approx. 5–8 μl) on the ectoderm, and place the filter paper soaked with the DNA solution directly on the ectoderm (Fig. 3A; see also Supp. Movie 1).

• Immediately place the cathode (-), a pointy platinum wire Ø 0.4 mm, directly on top of the wet filter paper soaked with the DNA (Fig. 3B).

• Apply 20 pulses at 20 volts, 20 ms width, 200 ms space (the voltage may vary according to the size of the chamber and the distance of the embryo to the electrode in the chamber). Formation of bubbles indicates flow of the current.

• After electroporation, remove the filter paper with the DNA and place the embryo onto a petri dish filled with a 5 mm layer of 0.2% Ham’s agarose and a drop of Ham’s medium in the center of the dish where the embryo will be placed (Fig. 2G,H). The electroporation procedure of the ectoderm is also shown in Supplementary Movie 1.

• To allow the embryo to recover from the first electroporation procedure, place the plates in a wet chamber and incubate for 4–5 hr at 37.5°C.

• At this point, the procedure may be interrupted if necessary to be continued the next day. For this, the plates containing the embryo are stored in the wet chamber at 18°–20°C. At this temperature, the metabolism is slowed down and the embryos will not develop further until the protocol is continued the next day.

• Before continuing, check embryos for fluorescence of the transgene in the ectoderm and survival; choose successfully electroporated embryos (Fig. 4A,B).

• Carefully lift the embryo together with the paper ring from the petri dish and place it, again dorsal side up, in the electroporation chamber. In contrast to ectoderm electroporation, the embryo must now be covered by a layer of 1 x PBS sufficiently deep to allow submerged positioning of the electrode.

• Inject the somites subjacent to the electroporated ectoderm with 5 μg/μl plasmid DNA (here we used pCAG-EGFP) + 1 μl Fast Green (Fig. 3C). The injection procedure follows in principle the technique described...
by Scaal et al. (2004), but it is recommended to inject the somites individually in lateral to medial direction.

- Connect the electroporation chamber to the cathode (-) and place the pointy anode (+) over the injected somites of the embryo (Fig. 3D). The electrode must be submerged in the PBS film and at sufficient distance, 2 mm, to the dorsal surface of the embryo.
- Apply 15 pulses at 15 volts, 20 ms width, 200 ms space (the voltage may again vary according to the size of the chamber and the distance of the embryo to the electrode in the chamber). Formation of bubbles indicates flow of current.
- In the same way as before, place the embryo with the paper ring back on the 0.2% Ham’s agarose plate, remove excess PBS from the plate to avoid embryo separating from the filter paper ring, and incubate at 37.5°C for 3–4 hr.
- Check for green fluorescence in the somites and red fluorescence in the overlying ectoderm cells. Fix successfully electroporated embryos with 4% PFA in PBS solution (see Fig. 4).

**Discussion**

The double-electroporation technique presented here allows the electroporation of two closely adjacent embryonic tissues, surface ectoderm and somites, with different vectors and without unwanted cotransfection of either tissue. Moreover, our technique has mastered the challenge that ectoderm and somites need to be electroporated in opposite directions: For ectoderm electroporation, dorsal-to-ventral electrophoresis is required; for somite electroporation, ventral-to-dorsal electrophoresis must occur. This technique could be applied to other tissues with comparable requirements as well (e.g., to study neural tube–somite interactions by electroporation of the neural tube in lateral direction and the adjacent somite cells in medial direction) and should be also useful in optimizing electroporation strategies in ectodermal-mesenchymal systems like limb bud mesenchyme-ectoderm interactions and head mesenchyme-ectoderm interactions.

Using a filter paper soaked with DNA solution on top of the dorsal ectoderm prevents too fast diffusion of the DNA and leakage of the DNA into other tissues, especially the somites, and provides uniform expression in a large number of ectodermal cells. In contrast, efforts to pipette a drop of highly viscous DNA mix thickened with carboxymethyl cellulose (CMC) on top of the dorsal ectoderm prior to electroporation proved to be unsuccessful, as the DNA diffuses quickly, therefore preventing the localized transfection of ectoderm.

It is important to give the embryo time to recover before initiating the second round of electroporation. We recommend waiting until the transgene in the ectoderm is expressed. In our case, robust expression of mCherry in the ectoderm is seen after about 4 hr. The time may vary according to the plasmid used (expression of EGFP can be seen already after about 3 hr) and may also vary in different tissues. If the electroporation procedure in the somites is performed immediately after the first electroporation, a massive loss of transgene expression is observed in the overlying ectoderm. The recovery phase between the two rounds of electroporation is presumably required to prevent reverse electrophoresis of the primarily applied DNA. Another possibility is that the intermediate tissue recovery phase is required to avoid tissue damage.

In summary, the electroporation technique described here allows simultaneous expression of transgenes in tissues that are positioned opposite to each other and will help researchers to transfected diverse regions in chicken embryos by double electroporation.

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**References**


