Video Article

Gene Transfer into Older Chicken Embryos by *ex ovo* Electroporation

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URL: https://www.jove.com/video/4078
DOI: doi:10.3791/4078

Keywords: Molecular Biology, Issue 65, Genetics, Developmental Biology, Gene transfer, gene function, electroporation, chicken, development

Date Published: 7/27/2012

Citation: Luo, J., Yan, X., Lin, J., Rolfs, A. Gene Transfer into Older Chicken Embryos by *ex ovo* Electroporation. *J. Vis. Exp.* (65), e4078, doi:10.3791/4078 (2012).

Abstract

The chicken embryo provides an excellent model system for studying gene function and regulation during embryonic development. *In ovo* electroporation is a powerful method to over-express exogenous genes or down-regulate endogenous genes *in vivo* in chicken embryos[1]. Different structures such as DNA plasmids encoding genes[2-4], small interfering RNA (siRNA) plasmids[5], small synthetic RNA oligos[6], and morpholino antisense oligonucleotides[7] can be easily transfected into chicken embryos by electroporation. However, the application of *in ovo* electroporation is limited to embryos at early incubation stages (younger than stage HH20 - according to Hamburg and Hamilton)[8] and there are some disadvantages for its application in embryos at later stages (older than stage HH22 - approximately 3.5 days of development). For example, the vitelline membrane at later stages is usually stuck to the shall membrane and opening a window in the shell causes rupture of the vessels, resulting in death of the embryos; older embryos are covered by vitelline and allantoic vessels, where it is difficult to access and manipulate the embryos; older embryos move vigorously and is difficult to control the orientation through a relatively small window in the shell.

In this protocol we demonstrate an *ex ovo* electroporation method for gene transfer into chicken embryos at late stages (older than stage HH22). For *ex ovo* electroporation, embryos are cultured in Petri dishes[9] and the vitelline and allantoic vessels are widely spread. Under these conditions, the older chicken embryos are easily accessed and manipulated. Therefore, this method overcomes the disadvantages of *in ovo* electroporation applied to the older chicken embryos. Using this method, plasmids can be easily transfected into different parts of the older chicken embryos[10-12].

Video Link

The video component of this article can be found at https://www.jove.com/video/4078/

Protocol

1. *Ex ovo* Culture

1. Fresh fertilized eggs are laid onto their long side in a forced-draft incubator (BSS160, Ehret, Germany) at 37.5 °C with 60% humidity for incubation. Eggs stored at 12 °C for longer than one week should not be used.
2. After incubation for 2.5 days (about stage HH17), take out the eggs and label the top with a pencil to indicate direction for cracking.
3. Before cracking, pour about 20 ml sterile distilled water into a clean large Petri dish (diameter of 145 mm; Greiner Bio-One GmbH, Germany).
4. Place another small sterile Petri dish (diameter of 94 mm; Greiner Bio-One GmbH) into the large Petri dish.
5. Crack the eggs on the bottom against a sharp metal edge, carefully open the eggs and transfer the entire content of each egg into the small Petri dish.
6. Cover the large Petri dish with a lid and put it into another incubator (BINDER GmbH, Tuttlinge, Germany) for further culture at 37.5 °C with about 60% humidity. After incubation for the desired number of days, the embryos can be used for electroporation.

2. Preparation for *ex ovo* Electroporation

1. Pull glass capillaries with a microelectrode puller (PUL-100 Micropipette Puller; World Precision Instruments, Berlin, Germany) using glass tubes of 1.0-mm diameter (TW100F-4; World Precision Instruments) and break the tip of the glass capillaries into an appropriate diameter.
2. Set up appropriate parameters (i.e., different voltages for the distinct tissues and size of embryos) for electroporation and connect electrodes to an electroporator (CUY21-Edit; Nepa Gene, Chiba, Japan) according to the target tissues and size of embryos.
3. Prepare a plasmid solution containing plasmids in pCAGGS vector encoding a target gene, e.g., cadherin7 (Cad7; pCAGGS-Cad7 with a concentration of 2.0 µg/µl) and a marker gene, e.g., green fluorescent protein (GFP; pCAGGS-GFP with a concentration of 0.25 µg/µl). Then add Fast Green with a final concentration of 0.1% (Sigma) to label the plasmid solution with the green color.
4. Load the glass capillary with the plasmid solution using a mouth pipette.

3. Gene Transfer into Chicken Optic Tectum by *ex ovo* Electroporation

1. After *ex ovo* culture, e.g., by incubation day (E) 6, the chicken embryos are used for *ex ovo* electroporation.
2. Carefully tear the vitelline and amnion membranes over the optic tectum with fine forceps and add 3 drops of sterile 0.9% Sodium Chloride solution on the surface of the tectum.
3. Inject the plasmid solution into the cavity of the tectum by glass capillary using the mouth pipette.
4. Place the electrodes with a tungsten needle cathode and a rectangle plate anode (CUY 661-3x7, Nepa Gene) beside the tectum, and immediately apply electric pulses (six pulses, 25 V, 60 ms pulse length, 100 ms intervals in each case) produced by the electroporator to the tectum.
5. Cover the large Petri dish with the lid and return it into the incubator. After incubation for appropriate days (e.g., 2 or 3 days after electroporation), the tectum are collected and fixed for immunostaining (**Figure 1**) and biological detection.

4. Representative Results

Successful overexpression of the exogenous proteins of Cad7 and GFP by *ex ovo* electroporation is shown in **Figure 1** as an example. When Cad7 plasmid together with GFP plasmid was electroporated into the tectum at E6, two or three days after electroporation the embryos were collected and fixed. At E8, the GFP protein is strongly expressed in the tectum in whole mount images (**Figure 1A-C**). Furthermore, in the sections of the tectum at E9, GFP protein (green in **Figure 1D**) and Cad7 protein (red in **Figure 1E**) are coexpressed (yellow in **Figure 1F**), suggesting that *ex ovo* electroporation is a successful method for gene transfer into the older chicken embryos *in vivo*.

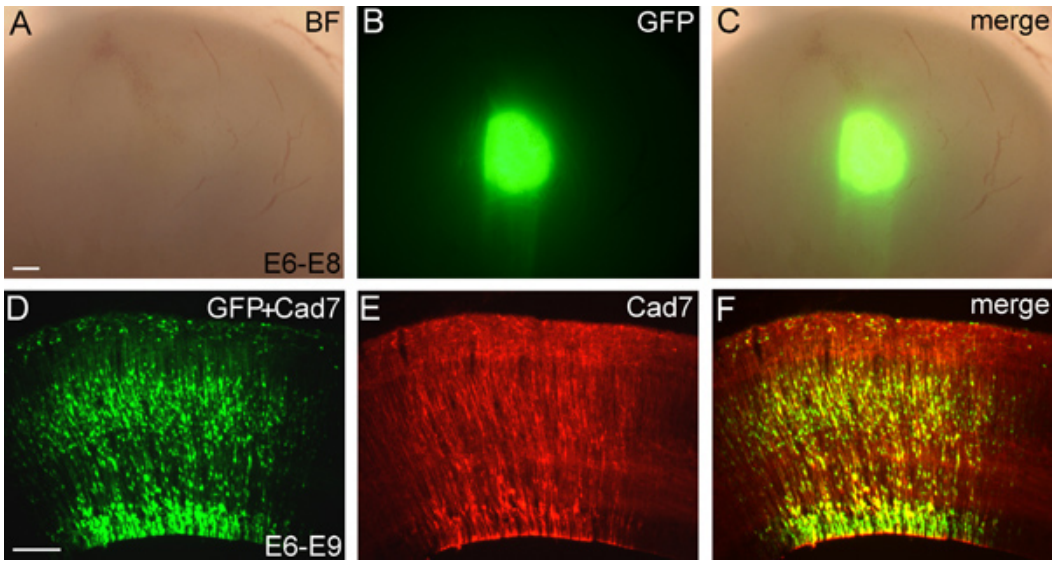

Figure 1. When the plasmids encoding GFP and Cad7 are cotransfected into the chicken tectum at E6, two or three days after *ex ovo* electroporation, GFP protein (green) is strongly expressed as shown in wholemount images (A-C) at E8. In sections of the tectum at E9, GFP protein (green in D) and Cad7 protein (red in E) are coexpressed (yellow in F). BF, bright field image. Scale bar: 200 μm in A for A-C; 100 μm in D for D-F.

Discussion

This protocol provides a guide for gene transfer into older chicken embryos (e.g, into the optic tectum at E6) by *ex ovo* electroporation. This method extends the use of electroporation to older chicken embryos and can be easily applied to many laboratories for studying gene function *in vivo* at later stages. Embryos from E4 to at least E7 can be successfully electroporated by this method and the electroporated embryos can be survival until E15[11]. Besides the brain, this method can be also used for gene transfer into the chicken limb system[11].

Special attention should be paid to by *ex ovo* electroporation. For example, in order to obtain high survival of the embryos by *ex ovo* culture, eggs used should be fresh and stored less than one week at 12 °C; the small Petri dishes should be sterile and egg cracking performed at around E2.5. For *ex ovo* electroporation, the size and placement of electrodes should be correctly chosen according to the stage and targeted part of the embryos. When two plasmids are cotransfected, the injected plasmids should be in an appropriate ratio of the concentration (e.g., 1:8 between the marker GFP gene and targeted gene) in order to be sure that the GFP marked cells are almost coexpress the targeted gene.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was supported by a grant from the German Research Foundation (DFG; LU1455/1-1).

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