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Chick Embryo Culture and Electroporation

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Abstract

Important events in embryonic development such as gastrulation, neurulation, and cranial neural crest development occur in ectodermal tissues during vertebrate embryonic development. Although the chicken embryo is a well-established model system in developmental biology, problems of accessibility of the ectoderm for experimental manipulation and an inability to generate gene knockouts previously impeded studies of gene regulation and key processes during chicken gastrulation and neurulation. The technique of in ovo electroporation permits genetic manipulation and provides a powerful animal model. However, the problem of accessibility to the ectoderm in ovo requires an ex ovo whole-embryo culture approach combined with electroporation. This Unit provides convenient and reproducible whole-embryo ex ovo culture and electroporation protocols. These chicken embryo culture protocols can be used not only for gene regulatory experiments, but also for time-lapse imaging of the dynamics of early vertebrate development.

Keywords

chicken embryo; ex ovo culture; electroporation; morpholino; neural crest cells

INTRODUCTION

Development of the head and face are crucial for living animals, and these structures are derived from cranial neural crest and head mesenchyme during embryonic development. Cranial and trunk neural crest cells appear transiently in development, and they emigrate from the dorsal part of the neural tube to migrate throughout the body. These cells have fascinated cell and developmental biologists because neural crest cell development includes fundamental cell behaviors that include cell specification, cell transformation, cell migration, and cell differentiation (Le Douarin and Kalcheim, 1999). The chicken embryo has been a good model system for studying trunk neural crest cell migration pathways, differentiation, and cell lineage mapping by dissecting or grafting cells/tissues in ovo. However, because cranial neural crest specification and its formation occur at gastrulation and neurulation stages when it is difficult to access the early embryonic ectoderm in ovo, it has been difficult to study cranial neural crest development in ovo. To overcome this problem, ex ovo whole-embryo culture is required, and the introduction of electroporation technology a decade ago has provided opportunities to study gene regulatory functions in vivo (Muramatsu et al., 1998; Nakamura et al., 2000). Recently, morpholino oligonucleotides have proven valuable for convenient, specific knockdown of target proteins and loss-of-function experiments, even in animals such as chick (Basch et al., 2006; Taneyhill et al., 2007). Chicken embryos have thereby become a powerful animal model for

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studying early developmental events, including not only cranial neural crest development, but also gastrulation and neurulation.

This unit describes methods for early chicken embryo culture at gastrulation or neurulation stages in Basic Protocol 1, and then for introducing plasmid DNAs and/or morpholino oligonucleotides into the ectoderm for studying early ectodermal events such as gastrulation and cranial neural crest formation in Basic Protocol 2, and finally for time-lapse imaging of whole embryo development and cranial neural crest cell migration in Basic Protocol 3.

BASIC PROTOCOL 1

EX OVO WHOLE-CHICKEN EMBRYO CULTURE

Introductory paragraph—Two different approaches to overcome the problem of accessibility to the ectoderm in chicken embryos at ages prior to Hamburger-Hamilton stage (HH) 7 are available using ex ovo whole-chicken embryo culture: New culture and EC (Early Chick) culture (New, 1955; Chapman et al., 2001). For rapidity and convenience using electroporation, with the option of subsequent time-lapse imaging, the following variation of the excellent EC culture protocol of Chapman (2001) has been developed with additional modifications and improvements.

Materials

Humidified 37°–38°C incubator
Chicken eggs from a commercial supplier, e.g., Charles River Laboratories
70% ethanol
10-cm disposable plastic Petri dishes
35-mm disposable plastic Petri dishes with lids at bottom (Figure 1B)
15-cm glass Petri dishes
Filter paper (Figure 1A, autoclaved)
Disposable transfer pipettes (7.5 ml, VWR 414004-005)
50 ml disposable polypropylene conical tubes
Parafilm (5×5 cm pieces)
Forceps and fine scissors
Razor blade
Kimwipes
Hanks' balanced salt solution (HBSS, see recipe)
Pasteur pipette (5¾ inch or 14.5 cm length, autoclaved)
L-shape bent spoon (approximately 1–3 ml capacity, Figure 1F)
Dissecting microscope (e.g., Stemi SV6, Zeiss)

Incubation of chicken eggs

- 1 Before incubation, each egg is placed with its long axis oriented horizontally, and its top is marked with a line using a pencil to identify the top. Gently rotating the egg horizontally around its long axis a couple of times helps to position the embryo at the top of the yolk.

Placing eggs with long axis oriented horizontally improves embryo development and survival according to the author's experience.

- 2 Incubate eggs at 37°–38°C in a humidified incubator for approximately 18–24 hours (the exact length of time will depend on egg and incubator conditions) to reach Hamburger-Hamilton stage (HH) 4–5 (Hamburger and Hamilton, 1951).
- 3 After incubation, allow the eggs to cool for 30–45 min in a cold room or refrigerator (4°C), then transfer the eggs back to room temperature before experiments. Clean and sterilize egg shells with 70% ethanol and allow them to dry.

Whole-embryo culture

- 4 Crack the bottom of an egg on the edge of a laboratory bench, then open and release its contents into a 10-cm plastic Petri dish. The embryo should be located on the top of the yolk; if not, rotate the yolk carefully using an L-shaped bent spoon.
- 5 Collect the most-liquid portion of the egg white (termed thin albumen, Figure 1C) using a disposable transfer pipette and save it in a 50 ml conical tube for later use. Repeat this collection of the thin albumen whenever new eggs are opened.

Note that eggs have two types of albumen, thin and thick (viscous), with only the former readily aspirated into the pipette (Figure 1C). For this collection of thin albumen for later use, you can alternatively pre-collect the albumen from 10–15 unincubated eggs ahead of time.

- 6 Gently remove the thick, viscous albumen covering the embryos by blotting it away using a piece of folded Kimwipe, moving from the center to the edge. Removal of this albumen is necessary for adherence of the embryo to filter paper in the next step.
- 7 Place a 2×2 cm piece of filter paper with a ~15 mm diameter hole in the center (see Figure 1A and legend for details) on top of each embryo, covering the vitelline membrane. Wait a couple of seconds to allow it to adhere firmly to the embryo.
- 8 Using fine scissors, cut through the vitelline membrane around the filter paper, taking care not to cut the embryo. With forceps, gently pull the filter paper with the attached embryo away from the yolk in an oblique direction (Figure 1D).
- 9 Place the filter paper with embryo ventral side up (flip it over) into a 10-cm plastic Petri dish filled with Hanks' BSS (HBSS).
- 10 Wash off excess yolk gently from each embryo using a jet of HBSS from a Pasteur pipette. Take care to avoid detaching the embryo from the vitelline membrane.
- 11 Place the filter paper with the embryo dorsal-side down onto Parafilm with a triangular hole covering a 35-mm plastic dish filled with thin albumen (see Figure 1B and figure legend for details). The embryo should be centered over the hole. The thin albumen in the dish should be to the top but not overflowing so that the albumen just moistens the embryo. Avoid bubbles in the dish.

In the original version of EC culture, the embryo with filter paper is placed on an albumen/agar plate ventral-side up and requires that the embryo be turned over (inverted) after HH8 for normal development. However, the present

method does not require this turning of the embryo. A triangular hole works better than a square hole.

- 12 The 35 mm dish is placed inside a 150-mm glass dish with 2–3 Kimwipes moistened with about 25 ml of water on the bottom for humidification (Figure 1E), then covered with the glass lid.
- 13 Place the glass dish in a humidified 37°–38°C incubator.

BASIC PROTOCOL 2

Electroporation of morpholino oligonucleotides or plasmids into chicken embryos

The following protocol provides a reproducible method for electroporation into the ectoderm and/or Hensen's node of gastrulation- or neurulation-stage embryos. After electroporation, the embryos are placed into whole-embryo culture and allowed to develop to HH8–9 for 11–12 hrs or to HH12–13 for 24 hrs.

Materials

HH4–5 embryo mounted on filter paper (from Protocol 1)

Electroporator (CUY21, Protech International, Inc., USA)

Electrode chamber with a negative electrode (CUY700-P20E, Protech International, Inc., USA, Figure 2C)

Positive electrode (CUY701-P2L, Protech International, Inc., USA, Figure 2C)

Foot switch (C200, Protech International, Inc., USA)

HBSS (see recipe)

Forceps

Femtotip II microinjection capillaries (Eppendorf)

DNA plasmids (see recipe)

Standard control morpholino or custom morpholino oligonucleotides (GeneTools, LLC, see recipe)

Dissecting microscope (e.g., Stemi SV6, Zeiss)

Glass disposable Pasteur pipettes (5¾ inch or 14.5 cm length, autoclaved)

Electroporation into the ectoderm of chicken embryos

1. Fill the electroporation chamber (Figure 2) with 37.5–40 ml HBSS.
Adjust the amount of HBSS depend on the size of the electroporation chamber.
2. From step 10 of Basic Protocol 1, place the embryos ventral-side up into the electroporation dish directly without washing away the yolk.
3. Carefully remove excess yolk only from the top of the ectoderm using a gentle jet of HBSS from a Pasteur pipette within the electroporation chamber.
4. Inject 0.8–1.0 µl morpholino oligonucleotide and/or plasmid solution using a Femtotip II capillary needle into the space between the embryonic ectoderm and the vitelline membrane.

The ectoderm side of the embryo should face down; insert the glass micropipette carefully into the appropriate area of the embryo, starting from extra-embryonic

tissue and then penetrating the embryo. When electroporation is performed into the endoderm, the morpholino and/or plasmid DNA should be placed onto the endoderm immediately prior to electroporation. In this case, the electroporation chamber should be positive, and the other electrode should be negative.

5. Apply two-three pulses of 10 V, 25 ms each, at 250 ms intervals using the foot switch.

Place the positive electrode directly on the embryo. The distance between the two electrodes should be maintained at 5.5–6.5 mm. The depth of the Hanks BSS can be used to measure this distance if the depth of the HBSS solution is determined in advance. Perform the electroporation promptly after addition of the morpholino/plasmid DNA (within a few seconds) before it diffuses away from the original site.

6. After electroporation, wash off excess yolk from the embryo in the electroporation chamber using a gentle jet of HBSS from a Pasteur pipette. Take care to avoid detaching the embryo from the vitelline membrane.
7. Return to step 11 of Basic Protocol 1. The embryos should be allowed to incubate for at least 2 hours at room temperature to allow recovery by the embryo before incubation at 37°–38°C in a glass dish.
8. The embryos are then cultured until the appropriate stage or used for time-lapse imaging as follows.

You can check the transfection efficiency using a fluorescence microscope. EGFP expression from plasmids or FITC-labeled morpholino transfection can usually be detected within 1.5–2 hours after electroporation.

BASIC PROTOCOL 3

Time-lapse movies

The following protocol provides methods for producing time-lapse movies of whole-embryo development or of cranial neural crest cell migration using whole-embryo culture. When combined with gene knock-down/over-expression using electroporation, this procedure allows direct visualization of the effects of loss-of-function or gain-of-function analyses of genes and proteins of interest genes during early embryonic development, such as on gastrulation or on cranial neural crest formation and subsequent cell migration.

Materials

Chicken embryos at HH4–7 on filter paper (from Protocol 1)

Inverted microscope (e.g., Axiovert 40C, Zeiss) equipped with a humidified CO₂ chamber, CCD camera and time-lapse recording software (e.g., Infinity 2 cool scan camera time-lapse system, Lumenara, Canada)

MatTek glass-bottom dish (P50G-1.5-14-F, MatTek Corporation)

Parafilm (5×5 cm)

5× and/or 10× objective lens

1. For time-lapse imaging, the embryo is placed in the center of a MatTek dish with the thin albumen filling the bottom well and then covered with Parafilm (5×5 cm).
2. Set the dish on the microscope stage of a time-lapse system and focus periodically.
3. Capture an image each 5–10 min for general embryonic development and for cranial neural crest cell migration (see examples in movie 1 and movie 2).

REAGENTS AND SOLUTIONS

Hanks' BSS/HEPES with MgCl₂ and CaCl₂, 1000 ml (pH 7.5)

100 ml 10× Hanks' BSS (Invitrogen, 14185-052)

Distilled H₂O to 1000 ml

Autoclave 20 min at 120°C

After cooling down to room temperature, add sterile 1 ml 1 M MgCl₂ (final concentration ~1 mM), 0.5 ml 2 M CaCl₂ (final ~1 mM) and 1 ml 1 M HEPES (final ~1 mM)

Adjust pH to 7.5 by adding a few drops of 10 N NaOH using a plastic pipette with stirring

Store up to 1 year at room temperature or 4°C

Morpholino oligonucleotides

Morpholino oligonucleotides should be custom-synthesized with either a fluorescein or lissamine label for electroporation by a commercial provider such as Gene Tools, LLC.

Resuspend to make a 1–2 mM stock solution using distilled H₂O

Store up to 2 years at –80°C

Before the experiment, incubate the stock solution at 65°C for 5 min, and then cool to room temperature

A final concentration of 0.5–0.75 mM morpholino usually provides adequate transfection efficiency with limited damage to embryos

To improve the efficiency of morpholino transfection, a non-expression plasmid DNA (e.g., an empty plasmid vector purified by CsCl ultracentrifugation) must be included as a carrier in the final working solution (final plasmid concentration 3–5 mg/ml). The fast green dye used in other protocols is not required for visualization because of the yellow color of the FITC label on the morpholino.

Plasmid DNAs

Plasmid DNAs can be prepared using commercial kits (e.g., QIAGEN, following the manufacturer's protocol) but must additionally be purified using CsCl (Heilig et al., 1998). Store the plasmid DNA at 10 mg/ml in PBS. For working, 1–5 mg/ml concentration is suitable with 0.001–0.005% Fast Green (Sigma) for visualization. The pCAGGS vector that combines a chicken beta-actin promoter with CMV-IE enhancer (Momose et al., 1999) is the strongest expression vector in this system in the author's knowledge (for information about other vectors, see Yasuda et al., 2000).

COMMENTARY

Background Information

Ex ovo chicken whole-embryo culture has been crucial for the study of early embryonic development, including gastrulation and neurulation. After the introduction of New culture using a glass ring (New, 1955), several methods with various modifications are now available. The choice of which whole-embryo culture method to use is important, not only to permit direct live-tissue imaging by time-lapse microscopy, but also for studying gene regulation and function by a combination with electroporation technology.

Historically, electroporation technology was first introduced in ovo for studying neural development a decade ago (Muramatsu et al., 1998; Nakamura et al., 2000). Endo et al (2002) then applied the technology to early embryonic ectoderm combined with ex ovo whole-embryo culture. However, the method was developed using quail embryos, and it proved to be difficult to apply to chicken embryos (Endo et al., 2002). In order to analyze epiblast development, Voiculescu et al (2008) developed a robust and reproducible protocol using modified New culture.

The protocols provided here focus on HH4–5 stage embryos and subsequent neurulation, as well as on cranial neural crest formation and subsequent cell migration. In contrast to classical New culture, the EC culture protocol developed by Chapman et al (2001) was another important advance, which used Whatman filter paper as a substrate. However, this technique is accompanied by problems such as head defects (microcephaly, Voiculescu et al., 2008; Chapman et al., 2001). Interestingly, the author found that this alternative EC culture methodology introduced in the Chapman paper (2001) can be modified to work effectively without the problem of head defects, similar in this regard to New culture but much more time-efficient and convenient. This method was then combined with electroporation technology and with time-lapse imaging protocols to provide methods for experimentation to determine mechanisms of early development and roles of specific genes.

These protocols can also be applied to analyzing cranial neural crest development and migration. During chicken embryonic development, the cranial neural crest is first specified at HH4 (Basch et al., 2006). It forms at the dorsal neural tube at stage HH7 by indicated by the earliest marker, SNAI2 (Nieto et al., 1994; Endo et al., 2002). After this initial formation, cranial neural crest cells begin migrating into the embryonic body at HH8+ to HH9 from the dorsal neural tube of the midbrain region. The migrating cells subsequently differentiate into neurons, glial cells, melanocytes, and mesenchymal cells to generate craniofacial bones and connective tissues (Le Douarin and Kalcheim, 1999).

Critical parameters and Troubleshooting

This section describes several critical parameters and troubleshooting to avoid abnormal embryonic development and reduced embryo survival.

1. **Abnormal embryonic development before EC culture:** First, check that water is present in the water pan and verify the temperature at each shelf of the incubator with a thermometer. Air circulation by using a fan may help achieve uniform temperature in the incubator. Second, the proportion of embryos that survive depends primarily on the original quality of the eggs, and it can vary from year-to-year even from the same supplier. In our experience, Charles River Laboratories supplies relatively reliable embryonic eggs in good condition, especially compared with eggs from a local private farm supplier.
2. **Detachment of embryo from the vitelline membrane before/during EC culture:** After incubation, eggs should be cooled in a cold room or refrigerator. This cool-down step is the most critical point for success in whole-embryo culture to ensure embryo adherence to the vitelline membrane. Overflow of albumen also causes the detachment of embryo during embryo culture, resulting in damage to embryonic development.
3. **Abnormal embryonic development without the detachment during EC culture:** According to the author's experience, HBSS supplemented with 1 mM CaCl_2 , 1 mM MgCl_2 and 1 mM HEPES, pH 7.5 should be used instead of the simple saline solution described originally by Chapman et al. (2001). Using simple saline, PBS, or even PBS with Ca^{++} and Mg^{++} is deleterious to embryo survival.

4. **Lower embryonic survival ratio after electroporation:** First, check the quality of the plasmid DNA. A CsCl purification step to obtain high-quality plasmid DNA is critical for gene transfection efficiency and embryo survival. Second, check that you are using HBSS (see recipe) for electroporation. Third, check the distance between the two electrodes, which should be 5.5–6.5 mm. Fourth, allow a couple of hours for embryo recovery at room temperature post-electroporation.
5. **Abnormal embryonic development during time-lapse movie:** The various elements described in the protocol are important for embryo survival. Because the distance between the objective lens and the embryo is limited, the amount of albumen needs to be reduced to a minimum. However, the ratio of embryos that survive will also decrease with less albumen, requiring empirical determination of the balance between these requirements. An additional factor is the evaporation of albumen in the time-lapse system, which should be performed within a humidified chamber. Check the system frequently for focus and evaporation; it may be necessary to use a custom-designed cover to reduce evaporation.

Anticipated Results

For chicken whole-embryo culture, chicken embryos will develop from HH4–5 to HH8–9 over a period of 12 hours; they can develop further to a maximum of stage HH 17 over a total of 48 hours (Figure 3A and B). After 24 hours, the survival ratio is usually 80–100%, and after 48 hours it is 50–80%.

For electroporation, transfected regions can be controlled by the site of morpholino and/or plasmid DNA introduction into embryos (Figure 3C and D). An internal control within the same embryo can be provided by placing morpholino and/or plasmid DNA on one side and using the other side as the internal control. The survival ratio after electroporation is lower than without electroporation.

For time-lapse movies, as can be seen in movie 1, HH3–4 embryos develop to stage HH8 normally. As shown in movie 2, cranial neural crest cell migration from neural tube can be visualized directly using bright-field microscope illumination. These approaches can be combined to characterize effects of gene transfection in time-lapse movies.

Time Considerations

Egg pre-incubation: 18–24 hours

Preparation of embryos for whole-embryo culture: 1–2 min per embryo

Whole-embryo culture: 0–48 hours incubation, depending on the purpose of the experiment

Electroporation: 1–2 min

Setting up a time-lapse movie: 5–10 min

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. Whatman 3MM CHR filter paper cut by scissors to a 2 cm×2 cm square, with the center removed using a 6.0 mm diameter paper hole puncher. The paper provides support and tension for the vitelline membrane and the embryo. **B.** A 35-mm disposable plastic tissue culture dish with its lid facing upward at its bottom covered firmly by two layers of Parafilm; a triangular (5–7 mm) hole is cut in its center using a razor blade. **C.** Thin and thick (viscous) albumen of the yolk. **D.** An embryo mounted on the filter paper is detached from the yolk using forceps pulled in an oblique direction (red arrow). **E.** Embryos on 35-mm

dishes filled with thin albumen were then placed in a 150 mm humidified glass Petri dish. **F.** Photograph of L-shape bent spoons.

Figure 2.

A. Schematic top view of electroporation chamber with a negative electrode. **B.** Side view of electroporation chamber showing negative and positive electrodes. **C.** Photograph of an electroporation chamber and electrode used for electroporation.

Figure 3.

A. HH17 chicken embryo on the filter membrane after 48 hours culture from HH4. **B.** Higher magnification view of the embryo in panel A. **C–D.** HH8 embryos transfected with FITC-labeled control morpholino after 10.5–11 hours culture from HH4–5.