Published in final edited form as:

Methods Mol Biol. 2012 ; 884: 183–192. doi:10.1007/978-1-61779-848-1_12.

Transfection of Primary Embryonic Chicken Retinal Cells Using Cationic Lipid

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Abstract

Primary neuronal culture and transfection are useful tools in determining gene functions within specific tissue contexts and developmental stages. Chicken embryonic retinal cultures are easily obtainable and often robust as the chicken eye is relatively large compared to mouse eye at similar developmental stages. Various DNA-based constructs have been developed to overexpress or knockdown genes of interest and can be delivered into the cells using lipofectamine, a cationic lipid-based transfection system. Here, we describe a method to culture and transfect primary chicken embryonic retinal cells in order to manipulate genes involved in retinal development. This technique can simultaneously deliver multiple genes without construct-size constraints and permit the usage of tissue or cell type-specific promoters, and is thus a useful approach to explore gene functions during neural retina differentiation.

Keywords

Vertebrate; Retina; Neuron; Progenitor; Primary Cell Culture; Transfection; Gene function; Immunocytochemistry

1. Introduction

Neuronal network formation in vertebrates involves complex orchestration between cell intrinsic factors and extrinsic signals to generate proper composition of various neuronal types from multi-potent progenitors (1–3). Disrupting gene function has been an essential approach to elucidate molecular mechanisms that regulate neuronal fate specification and differentiation. Developmental biologists have often used transgenic technologies to express genes of interest via chosen promoters in model species such as mouse and zebra fish. In addition, homologous recombination-based gene deletion in the mouse germ line has enhanced the understanding of specific gene functions in the nervous system (4–6). Furthermore, Cre-loxP-mediated gene ablation or activation have enabled temporal and cell type-specific perturbation of gene function in the developing and mature nervous system (7, 8). Together, these techniques have greatly advanced our understanding of development within the *in vivo* context. However, the use of these techniques requires generation of transgenic animals and extensive breeding to obtain the required genotypes, which are time

consuming and costly. In case of functional redundancy or complex systems that require disruption of multiple genes, the transgenic or knockout approach can be difficult or impossible to carry out. Thus, simple and efficient means to perform gene perturbation in specific cell types and temporal framework remain important.

Certain molecular mechanisms of the visual system development are evolutionarily conserved from insects to mammals (9, 10). All vertebrate retinas are similarly organized and their ontogeny follows a conserved temporal sequence. The embryonic chicken retina, compared to the rodent retina, is considerably larger at equivalent stages of development and easily accessible. Furthermore, embryological and molecular manipulation tools have been developed to study early chicken eye morphogenesis and retinogenesis (11). In contrast to the mouse retina, the chicken retina is cone-photoreceptor rich. In addition, chicken retinal cells can be easily cultured in vitro and at lower costs. In recent years, the post-hatching chicken retina has been shown to possess self-repair potentials (12). These features of the chicken retina make it a valuable vertebrate model to study neurogenesis and neural repair.

We have taken advantages of the chicken retina to address the role of Pax6 gene in early retinogenesis. Pax6 is an evolutionarily conserved paired homeodomain gene required for retinal development from the fly to humans. The dosage of Pax6 critically influences eye morphogenesis in mouse (13, 14). Conditional ablation of Pax6 in the periphery retina suggests that Pax6 plays a role to maintain the pluripotency of retinal progenitors (15). We observed that the expression level of Pax6 decreases as retinal progenitor cells enter the neurogenic phase. Furthermore, Pax6 protein levels undergo dynamic changes during the neurogenic cell cycle (16). These data indicate that Pax6 protein levels are stringently regulated during retinogenesis. To address the significance of the dynamic Pax6 protein levels in progenitor proliferation and neuronal fate specification, we perturbed Pax6 expression in primary chicken retinal cultures through transfection. Pax6 was overexpressed from a strong universal promoter, the chicken beta actin promoter (CAGp) (17) and down-regulated using U6 promoter-mediated shRNA knockdown (18). These molecular manipulations in conjunction with cell cycle analyses and confocal imaging allowed us to conclude that dynamic Pax6 expression during the neurogenic cell cycle critically influences proliferation and cell fate choices (16).

In this chapter, we describe in detail, the step-by-step procedure for delivering DNA constructs into chicken primary retinal cultures. In brief, developing chicken retinas are dissected and dissociated using enzymes. Retinal cells are cultured as a monolayer and transfected using lipofectamine PLUS with various DNA constructs along with a GFP-expressing plasmid. At different times post-transfection, cultured retinal cells are analyzed by immunocytochemistry using antibodies against GFP-positive transfected cells and other markers for proliferation, neuronal differentiation, or apoptosis to detect and quantify the effects of gene perturbation.

2. Materials

2.1. Chicken Primary Culture

1. Fertilized white leghorn chicken eggs.

2. Humidified chicken egg incubator (Lyon Electric).
3. Tissue culture incubator supplied with 5% CO₂.
4. 1× Retinal culture medium (RCM) 50 ml: in a tissue culture hood, mix 1 ml of fetal bovine serum (FBS), 0.1 ml of chicken serum (ChS), 0.5 ml of 1 M HEPES buffer pH 7.0, 0.5 ml of 100× penicillin/streptomycin, 22.9 ml of F12, and 25 ml of Dubecco Modified Eagle's Medium (DMEM) in a 50-ml sterile conical tube. Store at 4°C and warm to 37°C prior to use.

2× RCM 25 ml: mix 1 ml of FBS, 0.1 ml of ChS, 0.5 ml of 1 M HEPES buffer, 0.5 ml of 100× penicillin/streptomycin, 10.4 ml of F12, and 12.5 ml of DMEM in a 50-ml sterile conical tube. Store at 4°C and warm to 37°C prior to use.
5. Stock solutions:
 - a. 10 and 1× Phosphate buffered saline (PBS).
 - b. 1× Dulbecco's Modified Eagle Medium (DMEM).
 - c. 1× Hank's buffered saline solution with (HBSS+) and without magnesium and calcium (HBSS-).
 - d. 100 mg/ml Trypsin type XII in HBSS-.
 - e. 1 mg/ml Soybean Trypsin Inhibitor in DMEM.
 - f. 1 g/ml DNaseI in DMEM.
 - g. 10 mg/ml poly-D-lysine in sterile tissue culture grade water.
6. Surgical tools: two #5 Inox forceps, curved scissors, spring scissor, perforated spoon, medium sterile transfer pipette.
7. Egg holder: aluminum foil shaped like a culture dish.
8. Dishes for dissection: 100, 60, and 35 mm sterile culture dishes as needed.
9. Sterile 1.5-ml Eppendorf tubes and microcentrifuge.
10. Culturing slides: 150-mm dish and 8-well Lab-Tek permnox slides (Nunc/Nalge).

2.2. Transfection Reagents

Lipofectamine PLUS (Invitrogen, Carlsbad, CA) and Opti-MEM (Invitrogen).

2.3. immunocytochemistry

1. Fix solution: 4% paraformaldehyde in 1× PBS.

Pre warm 300 ml of ddH₂O in a 1 L beaker to 60°C with a stirrer bar, weigh 20 g of paraformaldehyde and add to the heated water, cover with plastic wrap and stir, the solution will be cloudy with some precipitates. Using a small transfer pipette, add 10 N NaOH, a drop at a time until paraformaldehyde is completely dissolved.

Add 50 ml of 10× PBS, mix, and measure the pH. Titrate the pH using NaOH or HCl to get a pH of 7.4. Filter the solution through funnel with a Whatman filter paper into a graduated cylinder and bring the volume up to 500 ml with ddH₂O and mix thoroughly. Aliquot into 50 ml sterile conical tubes and put on ice to use immediately or store in −20°C.

2. Wash solutions: 1× PBS; 1× PBT (1× PBS with 0.1% Tween-20).
3. Blocking solution: in 50 ml sterile conical tube, add 5 ml of FBS, 0.5 ml of Triton-X, 0.5 ml of 2% sodium azide in water, 1 ml of serum from the secondary antibody host animal and bring the volume up to 50 ml with DMEM.
4. Primary antibody: Rabbit anti-activated caspase-3 antibody (Upstate), Rabbit anti-Pax6 antibody (Chemicon), and mouse anti-GFP antibody (Molecular Probes/Invitrogen).
5. Secondary antibody: Alexa 488 anti-mouse IgG and Alexa 596 anti-rabbit IgG (Molecular Probes/Invitrogen).
6. Stock DAPI solution: 100 µg/ml 4',6-diamidino-2-phenylindole (DAPI) in H₂O (Roche).
7. Slide mounting: Gel mount medium and glass coverslip.

3. Methods

3.1. Chicken Primary Retinal Culture (Fig. 1a)

The following procedures are performed in a dedicated surgical area and a tissue culture hood in an aseptic manner:

1. Incubate two dozen fertilized chicken eggs in the humidified chicken egg incubator at 38.5°C for 120 h (5 days). Turn off the rotator an hour before dissection (see Note 1).
2. Dissect HH stage 27 (19) chick embryo: using the curved scissors, cut away a piece of the eggshell exposing the embryo. Place the egg on an egg holder and cut through the membranes around the embryo with the curved scissors while using the forceps to hold the embryo. Scoop up the embryo using a perforated spoon and transfer the embryo into a 10-cm Petri dish containing HBSS+. Dissect away the membranes and release the embryo into the solution (see Note 2).
3. Dissect the central retina: using the small spring scissors to cut and remove 40% of the anterior half of the eye, including the ciliary margin and the lens. Using forceps to detach the remaining 60% of the retina away from the retinal pigmented epithelium (RPE). Carefully trim away any remaining RPE still attached to the central retina. Using a sterile pipette to transfer the dissected retina to a clean 35-mm culture dish containing ~2 ml of 1× RCM prewarmed to 37°C (see Note 3).
4. Dissociate retina:

Before starting the dissociation, make a humidified chamber for the LabTek culture slides using a 150-mm dish with a clean moist paper towel wetted with sterile water. Each 150-mm dish will hold up to 4 slides. Pre-coat the culture slide wells with 400 μ l of 10 μ g/ml poly-D-lysine solution in water (1:1,000 dilution of the stock solution with sterile water). Incubate at 37°C for minimum of 15 min. Aspirate right before plating.

All retinal cell centrifugation are carried out at $327 \times g$ for 2 min with a microcentrifuge.

All solutions must be at room temperature before use. This process can dissociate retinas collected from up to a dozen embryos per tube.

- a. Transfer the retinas into a 1.5-ml Eppendorf tube using a medium sterile transfer pipette.
- b. Spin and wash with HBSS– three times with 1 ml of solution for each wash. For the last wash, take out as much of the solution as possible and add 1 ml of HBSS–.
- c. Add 100 μ l of 100 mg/ml Trypsin typeXII to each tube, mix by tapping the tube, and incubate at 37°C for 10 min.
- d. Add 100 μ l of 1 mg/ml soybean trypsin inhibitor, mix, and spin.
- e. Withdraw 500 μ l of solution without disrupting the cell pellet (see Note 4).
- f. Add 500 μ l of diluted DNaseI (50 μ l of stock DNaseI at 1 g/ml in 450 μ l of HBSS+) and carefully triturate the cell suspension with a P1000 pipette until no cell clumps are observed.
- g. Spin and replace with 1 ml of $1\times$ RCM.
- h. Count the cells by using a hemocytometer under a microscope (see Note 5).
- i. Dilute dissociated cells to 4,000 cells/ μ l in $1\times$ RCM (see Note 6).
- j. Plate 400 μ l of cell suspension per well in the 8-well LabTek to achieve a cell plating density of 20,000 cells/mm². Allow the cells to settle at room temp for 30 min and then place the LabTech culture slides in the 37°C 5% CO₂ incubator for 3 h (see Note 7).

3.2. Transfection (Fig. 1b)

The preparation of DNA/PLUS reagent/Lipofectamine solution should be carried out approximately 30 min before the cells are ready. This recipe is for one well only. To accommodate multiple wells, multiply the volumes for a single well and make sure there are excess reagents, e.g. for three wells make $3.5\times$ the amount.

1. Set up DNA and PLUS solution. Mix the following:
2 μ l Pax6i shRNA or Pax6 overexpression vector DNA at 500 ng/ μ l.

1 µl CAGpGFP vector at 500 ng/µl.

20 µl Opti-MEM.

4 µl Lipofectamine PLUS reagent.

Mix the DNA and Opti-MEM prior to the addition of the PLUS solution.

Mix the PLUS solution prior to use.

Add PLUS reagent directly to the DNA/Opti-MEM solution and mix.

Incubate for 15 min at room temperature (RT).

2. In a separate tube, mix 1 µl Lipofectamine directly into 20 µl of Opti-MEM and mix.
3. Add the lipofectamine mix (step 2, 21 µl total) directly to the DNA/PLUS solution (step 1, 27 µl total) and incubate for 15 min at RT.
4. Wash cells once with Opti-MEM and add 160 µl of Opti-MEM.
5. Add all of the lipofectamine/PLUS/DNA (48 µl total volume) dropwise on the cells and incubate in 5% CO₂ incubator at 37°C for 3 h.
6. Add 200 µl 2× RCM to the cells and incubate for additional 24 h.
7. Replace with 400 µl 1× RCM and incubate for additional 48 h. At this point if the transfection was successful, GFP-positive cells can be observed under a fluorescent dissection scope.

3.3. Immunocytochemistry

All wash steps need at least 3 min of incubation (see Note 8).

1. Aspirate culture medium and wash cells with 1× PBS.
2. Fix cells with ice-cold 4% paraformaldehyde/1× PBS for 15 min at RT.
3. Wash cells with 1× PBS three times and take off the culture well away from the slide at the end of the last wash.
4. Incubate with blocking solution for 60 min at RT.
5. Incubate with primary antibody solution for 60 min at RT (e.g. to make 1 ml of primary antibody solution, add 4 µl of rabbit anti-activated Caspase-3 and 5 µl of mouse anti-GFP antibody in 1 ml of Blocking solution).
6. Wash with 1× PBT three times.
7. Incubate with secondary antibody solution for 60 min at RT (e.g. to make 1 ml of secondary antibody solution, add 2 µl of Alexa 488 conjugated anti-mouse antibody and 2 µl of Alexa 598 conjugate anti-rabbit antibody in 1 ml of blocking solution).
8. Incubate with 1× PBT containing 1 µg/ml DAPI for 10 min at RT (add 1 µl of stock 100 µl/ml DAPI solution per 100 µl of 1× PBT)

9. Wash with 1× PBT three times and remove excess solution.
10. Mount with gel mount medium and glass cover slip. Dry overnight at RT in the dark.
11. Visualize antibody labeling signals using fluorescent microscopy (Fig. 2).

4. Notes

1. Chicken eggs must be rotated to prevent the embryo from sticking to the eggshell and causing deformation and abnormal development. The automatic rotator should be turned off prior to dissection so that the embryo remains at the top of the egg yolk. This will make it easier to find the embryo once the eggshell is opened.
2. If you maintain the orientation of the egg when removing it from the incubator, the embryo would be on the top center, right below the eggshell. Open a window wide enough (about 2 cm in diameter) for insertion of the perforated spoon to scoop up the embryo. Take care not to damage the embryo or disrupt the yolk by gently cracking the eggshell at an off-center position using the tip of the curved scissors, and then cut around the center using the curved scissors. Using the forceps to grab hold of the membrane close to the embryo but not the actual embryo itself. After cutting the membrane around the embryo, lift the embryo onto the perforated spoon and transfer to the dish. At this point, the embryo is surrounded by membranes. To expose the embryo, use two pairs of forceps and gently pull the membranes apart. Stage the embryos according to Hamburger and Hamilton (19) and select stage 27 embryos according to the specific external morphological features including the beak, branchial arches, and limb buds.
3. Removal of the anterior portion of the eye can facilitate the clean dissection of the retina from the RPE. There are many ways one can detach the RPE away from the retina. One effective way to remove the RPE is by inserting the forceps in between the RPE and the retina and allowing the forceps to open. However, the retina tends to be tightly adhered to the RPE at the ventral optic fissure. Further cutting along the optic fissure can help to remove the residual RPE.
4. After the trypsin incubation, the cells will be clumpy and sticky due to releasing of DNA from certain lysed cells. Therefore, DNaseI digestion, which requires Mg^{2+} , is necessary to result in single cell suspension.
5. At this point, if clumps of cells are observed, repeat dissociation from the three times HBSS– wash step.
6. The cells are diluted to 4,000 cells/ μ l of 1× RCM because the culturing surface area of the 8-well Lab-Tek is 0.8 cm^2 or 80 mm^2 , requiring 1.6 million cells in a working volume of 400 μ l to reach the plating density of 20,000 cells/ mm^2 .
7. Make sure to resuspend the cells before each time you take an aliquot of the cells. In order to achieve even plating of cells, avoid moving the culture dishes/slides in circular motions. At the end of the incubation time for plating, check to

see if the cells have adhered to the dish by gently shaking the culture dish under an inverted microscope. Most cells that settle at the bottom should not move.

8. We put enough solution to cover the cells and aspirate the solutions from a fixed position (i.e. top left corner of each well) each time to minimally disturb the cells.

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Fig. 1.

Flow charts of primary chicken retinal cell transfection. **(a)** Procedures to establish dissociated primary embryonic retinal monolayer cultures. **(b)** Steps used in retinal cell transfection and immunocytochemical analysis.

Fig. 2.

Fluorescent micrograph images of primary chicken retinal cells after transfection and 72-h culture. HH stage 27 retinal cells were transfected in vitro with (a) no DNA, (b, d) GFP-expressing plasmid and Pax6shRNA construct, and (c) GFP-expressing plasmid and a Pax6-expressing construct. Cells were colabeled for Pax6, GFP, and DAPI in (a–c) and for caspase-3, GFP, and DAPI in (d). Scale bars = 100 m.