Video Article

Efficient Gene Transfer in Chick Retinas for Primary Cell Culture Studies: An *Ex-ovo* Electroporation Approach

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Abstract

The cone photoreceptor-enriched cultures derived from embryonic chick retinas have become an indispensable tool for researchers around the world studying the biology of retinal neurons, particularly photoreceptors. The applications of this system go beyond basic research, as they can easily be adapted to high throughput technologies for drug development. However, genetic manipulation of retinal photoreceptors in these cultures has proven to be very challenging, posing an important limitation to the usefulness of the system. We have recently developed and validated an *ex ovo* plasmid electroporation technique that increases the rate of transfection of retinal cells in these cultures by five-fold compared to other currently available protocols[1]. In this method embryonic chick eyes are enucleated at stage 27, the RPE is removed, and the retinal cup is placed in a plasmid-containing solution and electroporated using easily constructed custom-made electrodes. The retinas are then dissociated and cultured using standard procedures. This technique can be applied to overexpression studies as well as to the downregulation of gene expression, for example via the use of plasmid-driven RNAi technology, commonly achieving transgene expression in 25% of the photoreceptor population. The video format of the present publication will make this technology easily accessible to researchers in the field, enabling the study of gene function in primary retinal cultures. We have also included detailed explanations of the critical steps of this procedure for a successful outcome and reproducibility.

Video Link

The video component of this article can be found at http://www.jove.com/video/52002/

Introduction

Dissociated cell cultures from embryonic chick retinas have been widely used to study various aspects of photoreceptor cell biology, including their survival[2-9], differentiation[10-12], neurite outgrowth[13], and more. The advantages of this system, developed in the 1980s by Ruben Adler and collaborators and perfected by his and other groups[14-20], reside in the intrinsic characteristics of the chick as an animal model[21]. The large size of the chick eye, even at embryonic stages, provides large amounts of material for cultures. Moreover, when cultures are performed using embryonic day (ED) 5 - 6 retinas, 55 - 80% of their progenitor cells differentiate as photoreceptors[14,15,18,22,23], and since approximately 86% of the photoreceptors in this animal are cones[24], these cultures are particularly suitable for studies focusing on this cell type.

We have recently developed and characterized a simple technique that allows for high-efficiency plasmid transfection of the cells in these cultures, thus broadening the usefulness of this system by facilitating genetic missexpression studies[1]. The development of this technique stemmed from a void in the scientific literature of methods that would provide an acceptable level of transfection to allow for the study of gene function in a cell autonomous manner. This is in part because primary neuronal cultures are notoriously hard to transfect[25,26]. Some of the most commonly used techniques previously available for this purpose included chemical transfection methods such as lipofection or calcium phosphate-mediated transfection, which result in efficiencies in the order of 3-5% and can exert considerable toxicity[27-32]. Even though the use of plasmids with an enzymatic reporter system can circumvent the problem of poor transfection efficiency by amplifying the signal, they do not discriminate cell-specific effects, and their results are based on a small cell population that may not be representative of the whole. Another widely used method in the chick, RCAS virus infection, is only applicable to proliferating cells and thus not suitable for this primary retinal culture system[33].

In the current protocol embryonic chick eyes are enucleated at stage 27 (ED 5), the retinal pigmented epithelium (RPE) is removed, and the retinal cup is placed in an electroporation chamber filled with a plasmid-containing solution and electroporated using custom-made electrodes, followed by retinal dissociation and culture using standard techniques[21]. After optimizing this procedure we have been able to consistently achieve transfection efficiencies on the order of 22% of the total number of cells in culture and 25% within the photoreceptor population alone, without compromising the survival and differentiation characteristics of the cultures[1]. Here we provide a detailed protocol outlining all the important steps of this procedure in order to ensure the success and reproducibility of this technique.

Protocol

All procedures described in this work were performed according to the guidelines recommended by the Animal Care and Use Committee at Johns Hopkins University.

1. Ahead of Time: Preparation of Instruments, Reagents and Dishes

1. Preparation of Eggs: Incubate fertilized White Leghorn chicken eggs at 37.5 °C and 60% relative humidity for 5 days in an egg incubator.
   NOTE: An incubator with rocking capacity may be helpful for standardizing and synchronizing embryonic development, but rocking is not strictly necessary for the outcome of this protocol.
2. Plasmid Preparation:
   1. Design plasmid construct for gene overexpression or downregulation (for example through the use of RNAi technology[1,34]) as needed. Include a reporter gene (such as EGFP, RFP or LacZ) in the construct whenever possible.
      NOTE: Some commonly used promoters such as CMV, can become silenced after time. The CAG promoter (chicken beta-actin promoter with CMV enhancer) is a very good alternative, providing efficient long term expression of the gene of interest[35-37].
   2. Prepare a plasmid solution at a concentration of 1.5 µg/µl in sterile PBS. OPTIONAL: Prepare plasmid solution ahead of time and store it frozen until ready to use.
      NOTE: Higher plasmid concentrations do not result in a significant increase in efficiency, whereas using lower plasmid concentrations lower the transfection efficiency[1].
3. Electrodes:
   1. For the anode use a thick gold tipped electrode (see Table of Specific Materials/ Equipment). Wipe clean with 70% ethanol.
      NOTE: Insulation of the gold tipped electrode, leaving approximately 0.5 mm exposed, is advisable to minimize leakage of the electric current. Information for insulating material can be found in Table of Specific Materials.
   2. Make a cathode out of a 2.5 mm square box filament, 4.5 mm wide (normally used in micropipette pullers). With the aid of forceps, undo the square box and straighten filament into a long strip. Then, under dissection microscope and using a ruler as a guide, bend the filament into a square 'U' shape 3 mm long at the base and 2 mm high at one side, with the other side the remaining length of the filament (**Figure 1, C - D**). Using forceps, dip the electrode in 96% ethanol and flame to sterilize. Then attach an electrical lead with a small alligator clip to the longer arm of the electrode.
4. Electroporation Chamber Setup:
   1. Prepare the electroporation chamber by cutting the lid of a sterile 1.5 ml microcentrifuge tube and affixing it, flat side down, to a 100 mm Petri dish using tape (**Figure 1E**).
      NOTE: Other container in the form of a well of similar size would work; however, a microcentrifuge tube lid is easily accessible and autoclavable. The well must be big enough to house a chick eye and cathode electrode, but not so big as to result in a waste of plasmid solution.

2. *Ex ovo* Electroporation Procedure

1. Preparation of Instruments:
   1. Sterilize micro-dissection tools (2 pairs of big curved Moloney forceps for opening egg shells and scooping out embryos, 2 pairs of fine-tip curved Dumont tweezers for removing RPE, one pair of Bonn toothed forceps for removing lens and vitreous) by dipping the tips in 96% ethanol and flaming. Clean dissection scope using wipes dipped in 70% ethanol. Warm a bottle of sterile Calcium-Magnesium-free Hank's Balanced Salt Solution (CMF-HBSS) in a 37 °C water bath. Fill 100 mm sterile Petri dishes to 3/4 full with warm CMF-HBSS.
   2. Place electroporation chamber under another clean dissection microscope and fill with 120 µl of plasmid solution. Connect electrodes to electroporation apparatus, making sure custom-made electrode is connected to the negative pole and gold tipped electrode to the positive pole.
2. Embryo Collection:
   1. Clean the eggs by wiping shells with wipes wetted in 70% ethanol. Maintain sterile procedure throughout the rest of the protocol to avoid carrying contamination into the cultures. Using big curved Moloney forceps carefully open a hole in the egg shell by gently tapping on the rounded tip of an egg (over the air chamber) and then gripping and pulling out shell pieces.
   2. With another pair of forceps remove membranes and collect embryo by scooping it out of the egg (being careful not to damage the embryo). Place it in a dish with CMF-HBSS.
      NOTE: Collect as many embryos as necessary to obtain the desired number of retinal cups for electroporation as specified in 2.4.4
   3. CRITICAL STEP: Stage the embryos according to Hamburger and Hamilton[38] .
      NOTE: To obtain optimum efficiency embryos must be at stage 27 (**Figure 2A**).
   4. Euthanize the embryos by decapitation using Moloney forceps.
3. Obtaining Retinal Cups:
   1. Using fine Dumont tweezers, carefully enucleate the eyes and transfer to a new CMF-HBSS dish.
   2. Starting from the posterior part of each eye, close to the optic nerve head, and using two fine Dumont tweezers, introduce one tip of each pair of tweezers between the neural retina and RPE and carefully dissect out the RPE being cautious to not damage the neural retina (**Figure 2B**).
      NOTE: The lack of Ca^{2+} and Mg^{2+} in the solution will help the RPE detach from the neural retina more easily.
4. Electroporation:

1. Set up electroporator's parameters to deliver 5 square pulses of 15 Volts, 50 msec duration, and 950 msec interval. Place 'U' shaped negative electrode inside electroporation chamber (microcentrifuge lid filled with 120 µl of plasmid-containing solution).
2. Using curved Dumont tweezers transfer one retinal cup to the electroporation chamber (by scooping, not pressing), and place it inside the 'U' shaped electrode, with the posterior part of the retina towards the bottom of the electrode and the lens facing upwards (**Figure 2D**).
3. Place the anode so that it touches the anterior part of the eye, next to the lens, and using the electroporator's foot pedal deliver the electric pulses.
4. Transfer electroporated retinal cup to a new Petri dish with CMF-HBSS and repeat electroporation procedure with each of the remaining retinal cups.
   NOTE: Up to 6 retinal cups can be electroporated with the same plasmid solution without a significant decrease in transfection efficiency.
5. Remove the lens and vitreous body of each electroporated retinal cup by gripping them with Bonn toothed forceps and gently pulling through the pupil opening while holding the eye in place with the back of the curved Dumont tweezers.
6. Proceed to the dissociation and culture of the electroporated retinal cells following standard procedures.
   NOTE: For a detailed protocol on the dissociation and culture of chick retinal cells refer to Vergara and Canto-Soler, 2012, Additional file 4[21]. In this protocol the cells are seeded at low density on polyornithine-coated 6-well plates or 35-mm dishes (6 x 10^5 cells/35 mm-diameter well or dish). The medium used is medium 199 with penicillin/glutamine, 10% fetal calf serum (FCS), and linoleic acid-BSA at 100 µg/ml. The cultures are maintained in a humidified 37 °C incubator with 5% CO_2. Using this procedure, 5 - 6 x 10^6 cells can typically be obtained per each stage 27 electroporated eye.
   NOTE: Ideally, no more than 3 hr should pass between embryo collection and cell plating, to ensure good cell survival and minimize stress. A typical timing for observation and further analysis is after 4 days in culture, since at this point the cells have achieved good morphological and molecular differentiation and survival is still high.

Representative Results

Here we present a simple protocol for plasmid transfection into enucleated chick retinal cups for subsequent dissociated cell culture. Transfection is achieved by electroporation using easy to make custom electrodes (**Figures 1 - 2**). The parameters described in this protocol have been optimized to obtain transfection efficiencies that range between 20 and 27% (with an average of 22%) (**Figure 3D**). Notice that, for the reasons stated above, these results are quantified after 4 days in culture. In this context, transfection efficiency refers to the percentage of cells that express the transgene within the total cell population. If only the photoreceptor population is considered, transfection efficiencies increase to an average of 25% (**Figure 3D**). This is an important feature since these types of cultures are primarily used to study photoreceptors.

Cell cultures that have been electroporated using this technique are indistinguishable from their non-electroporated counterparts in their morphology under DIC illumination, cell survival characteristics, and expression of molecular markers such as visinin (a widely used marker of photoreceptors) and Pax6[1].

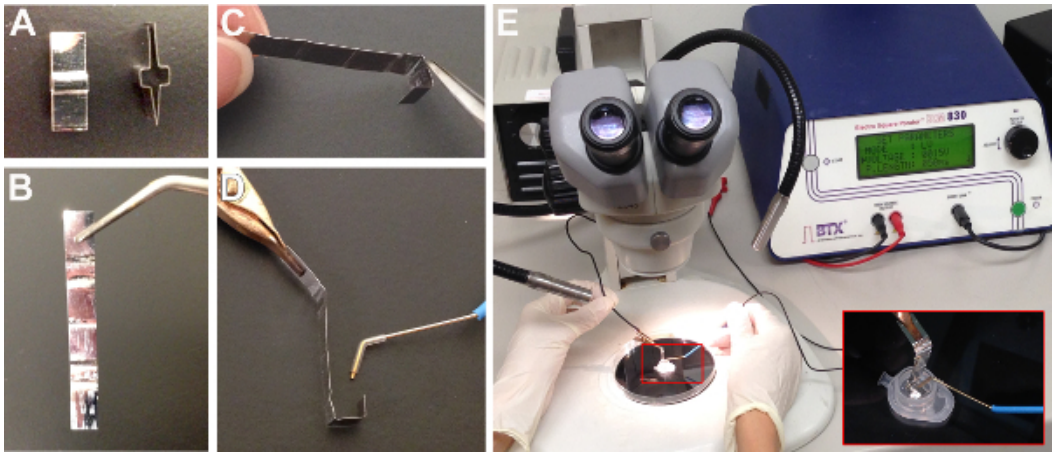

Figure 1. Making of the Electrodes. (A - D) The cathode is made out of a square box filament **(A)**, which is first straightened **(B)** and then bent with forceps into a square 'U' shape **(C - D)**. The anode is a thick gold tipped electrode **(D)**; Notice the insulation around the anode electrode, leaving only 0.5 mm of the tip exposed. **(E)** Electroporation setup. Inset shows higher magnification of boxed area. Please click here to view a larger version of this figure.

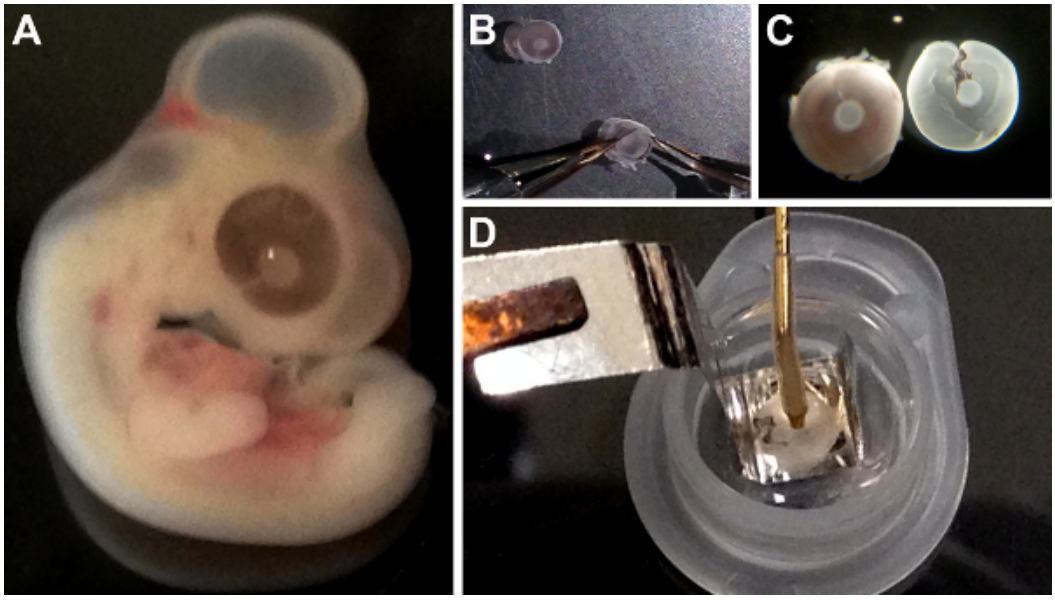

Figure 2. Preparation of Retinal Cups for Electroporation. (A) Chick embryo at Hamburger and Hamilton embryonic stage 27. **(B)** The RPE is carefully removed from the enucleated eyes using sharp Dumont tweezers. **(C)** The retinal cup on the right is devoid of RPE and ready for electroporation. **(D)** The cathode electrode is positioned inside an electroporation chamber made out of a 1.5 ml microcentrifuge tube lid filled with plasmid solution. The retinal cup is carefully transferred into the cathode facing upwards, and the anode electrode is placed on top, next to the lens. Please click here to view a larger version of this figure.

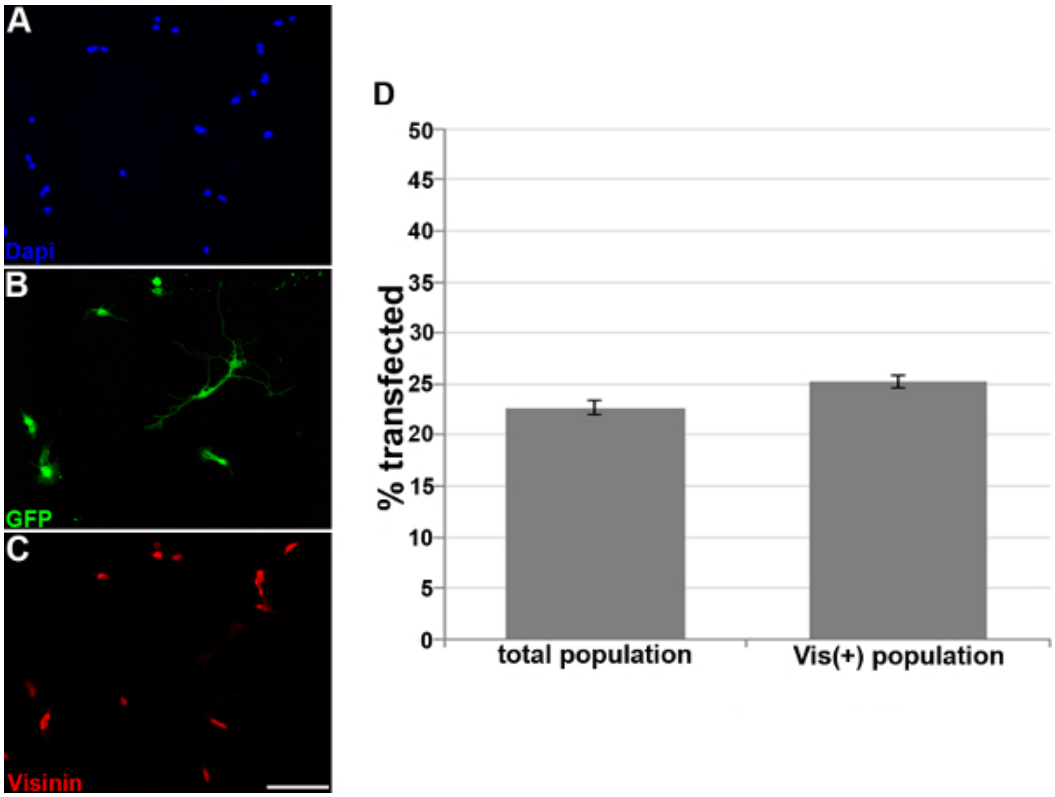

Figure 3. Efficiency of Transgene Expression in Cultured Cells after Electroporation. (A - C) Retinal cups were electroporated with a GFP expression plasmid, dissociated and cultured for 4 days. Fluorescence micrographs show DAPI stained nuclei **(A)** and GFP expressing retinal cells **(B)**. Anti-Visinin antibody staining was performed to identify photoreceptor cells **(C)**. **(D)** Graph illustrating transfection efficiency achieved by *ex ovo* electroporation, represented as the percentage of GFP expressing cells among the total cell population (DAPI positive), or among the photoreceptor population in particular (Visinin positive). Results represent the mean ± S.E.M. of 5 independent experiments. Scale bar in **(C)** represents 100 µm and applies to **(A - C)**. Please click here to view a larger version of this figure.

Discussion

The most critical step for the success of this protocol is selecting the appropriate stage of the embryos. In previous publications, a range of embryonic stages is given for these cultures, typically defined by the days of incubation or embryonic days (ED); thus it is usually assumed that using ED 5 to ED 6 embryos will yield equivalent results. However we have found that on stage 27 (ED 5), transfection efficiency will be around 22% of the total cell population, as stated above; yet efficiency will decrease to 16% if using stage 28 embryos (ED 5.5), and to 12% at stage 29 (ED 6)[1]. Other critical steps include maintaining sterile conditions throughout the process; acquiring the necessary manual dexterity to avoid damaging the retina during dissection and electroporation; and avoiding long gaps from the time of embryo collection to the time of cell plating.

Another important consideration to ensure good transfection efficiencies is the concentration of the plasmid-containing solution: the recommended 1.5 µg/µl is the lowest concentration that we tested that did not result in a decrease in efficiency. This concentration translates into a high amount of plasmid material when considering that the retinal cups are bathed in that solution. Using the smallest size well that will accommodate the eye (such as a microcentrifuge tube lid) can minimize this problem. In addition, several eyes can be electroporated using the same solution; we have not seen a reduction in efficiency when electroporating up to 6 retinal cups consecutively in the same plasmid solution, and more could possibly be performed but we cannot attest to the difference in efficiency thereafter.

Previously published protocols have typically provided low efficiency of gene transfer for this type of culture, and thus they often had to rely on techniques that measure the product of enzymatic reactions on a cell lysate in order to increase sensitivity. Such an approach has the disadvantage of not allowing for cell-type discrimination and of relying on a low number of cells being responsible for the observed outcome, which may not always be reflective of the behavior of the larger cell population. Another way to circumvent the problem of low efficiency is to perform gene transfection *in vivo*, prior to eye enucleation and culture. This is a viable approach but it can usually be applied only to specific experimental paradigms, since both plasmid electroporation and RCAS virus infection (a common gene transduction vector in the chick) typically need to be performed at earlier developmental stages, creating a time lag between transfection and culture. This is an important consideration, because during that time lag the cells are exposed to the effects of both the extracellular microenvironment as well as intracellular factors, having significant implications in the interpretation of the experimental results. In contrast, the substantial increase in gene transfer efficiency achieved with the *ex ovo* electroporation approach allows for the study of gene function in a cell-intrinsic manner. Moreover, the advantage of this high transfection rate can be further increased when used in conjunction with automated high-throughput cell analysis[1].

At the initial stages of the development of this technique, we maintained electroporated RPE-devoid eye cups in culture for 24 hr to evaluate the efficiency of electroporation achieved with different conditions[1]. Although we did not attempt to culture them for longer period of times, our experience indicates that the protocol described herein could also be applied to studies relying on retinal organotypic explants, provided appropriate long-term explant culture conditions are used.

Finally, it would be possible to expand the applicability of this *ex ovo* electroporation approach to other animal models. For example in our experience transfection of mouse eyes at postnatal day 1 or 4 using this protocol yielded qualitatively good results in explant cultures, but when dissociated cell cultures were attempted transfection efficiency was on the order of 6%, similar to that of other techniques. Thus, this could be a simple alternative to other protocols for this animal model, but it would need to be optimized for the particular system if high efficiencies are required. Of note, as stated above, embryonic stage at the time of electroporation was key to the high efficiency outcome obtained in the chick, so this parameter should be carefully considered when applying the technique to other models.

In conclusion, the *ex ovo* electroporation technique can expand the applicability of *in vitro* retinal systems as powerful tools to complement *in vivo* studies, offering new possibilities for retinal research.

Disclosures

The authors declare that they have no competing financial interests or other conflicts of interest.

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