Video Article

Whole Retinal Explants from Chicken Embryos for Electroporation and Chemical Reagent Treatments

Shahrazad Shirazi Fard¹, Maria Blixt¹, Finn Hallböök¹

¹Department of Neuroscience, Biomedical Center, Uppsala University

Correspondence to: Finn Hallböök at finn.hallbook@neuro.uu.se

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Abstract

The retina is a good model for the developing central nervous system. The large size of the eye and most importantly the accessibility for experimental manipulations *in ovo/in vivo* makes the chicken embryonic retina a versatile and very efficient experimental model. Although the chicken retina is easy to target *in ovo* by intraocular injections or electroporation, the effective and exact concentration of the reagents within the retina may be difficult to fully control. This may be due to variations of the exact injection site, leakage from the eye or uneven diffusion of the substances. Furthermore, the frequency of malformations and mortality after invasive manipulations such as electroporation is rather high.

This protocol describes an *ex ovo* technique for culturing whole retinal explants from chicken embryos and provides a method for controlled exposure of the retina to reagents. The protocol describes how to dissect, experimentally manipulate, and culture whole retinal explants from chicken embryos. The explants can be cultured for approximately 24 hr and be subjected to different manipulations such as electroporation. The major advantages are that the experiment is not dependent on the survival of the embryo and that the concentration of the introduced reagent can be varied and controlled in order to determine and optimize the effective concentration. Furthermore, the technique is rapid, cheap and together with its high experimental success rate, it ensures reproducible results. It should be emphasized that it serves as an excellent complement to experiments performed *in ovo*.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53202/>

Introduction

The retina is part of the central nervous system and it is, with its relative simplicity and well-characterized cellular architecture, a popular model for studying central nervous system development. The eye of the chicken embryo is relatively large in comparison to the rest of the embryo. It is therefore easily accessible *in ovo* for experimental manipulations, such as injections or electroporation, and serves as an excellent tool to gain knowledge about retinal cell and developmental biology *in vivo*. Despite these major advantages, survival of the embryos can be low when experiments are invasive such as with electroporations, repeated injections, or combined experimental manipulations.

Electroporation of DNA plasmids into the chicken embryo *in ovo* is an important and well-established technique¹. It allows for labeling of neurons, tracing of cell fate as well as neuronal tracts in the central nervous system and it allows for ectopic gene expression to analyze protein function *in vivo*. The technique has been used for studies of neural tube², hindbrain³, and retina⁴. Electroporation of embryonic retina *in ovo* has some experimental difficulties that are related to the *in vivo* situation. The position of the eye, due to the cranial folding of the embryo, is relatively close to the heart. This proximity increases the risk of cardiac arrest following electroporation, and the risk increases with the age of the embryo. Moreover, to access the eye, it is necessary to open the embryonic membranes, thereby increasing the risk for bleeding, malformations and subsequent reduced viability. When testing and optimizing a new DNA plasmid often without a known phenotypic outcome, these limitations may decrease the efficacy and power of the method even for an experienced experimentalist. As presented in this protocol, the culture of the whole retinal explant, defined as the whole neural retina with the pigment epithelium removed, is an efficient method that complements the *in ovo* approach.

Intraocular injections of chemical reagents are relatively easy to perform *in ovo*. However, the effective and exact concentration of injected reagents within the neural retina may be difficult to fully control. The injected volume may vary due to leakage and the exact site of injection may affect both the distribution of the reagent within the eye and the diffusion through the vitreous body. The variability will have major implications for the interpretation of the results when *i.e.*, a dose response curve for an enzyme inhibitor is determined; particularly if the effect is small and the temporal window of the effect is narrow. Moreover, only a single eye can be used from each embryo when performing *in ovo* experiments due to potential systemic effects via the blood stream onto the contralateral eye. Age matching is important when studying development and the individual variability between treated and control embryos may lead to additional experimental variability.

For these reasons, an *ex ovo* method based on retinal explants from chicken embryos was developed, in which the neural retina can be exposed to a uniform and controlled experimental condition *in vitro*. The present protocol was developed based on previous protocols⁵⁻⁹. Retinal

explants from stage (st) 20 (embryonic days [E] 3) to st31 (E7) chicken embryos were dissected, cultured and electroporated with a defined DNA plasmid concentration or exposed to a medium containing a defined concentration of a chemical reagent. The protocol presented here has been successfully implemented in recent publications, using several different chemical reagents, including regulators of the DNA damage pathway, such as KU55933, SB 218078, and NSC 109555 ditosylate, and the cell cycle, such as Cdk1/2 inhibitor III^{10,11}.

Protocol

This protocol is performed in accordance with the recommendations in the "Guide for the Care and Use of Laboratory Animals of the Association for research in vision and ophthalmology".

1. Egg Handling and Eye collection

1. Store white Leghorn eggs at 12-14 °C for no longer than 1 week. If available, use a refrigerated wine cooler to store the fertilized eggs.
Note: Higher store temperatures lead to abnormal development of embryos, while lower temperatures increases mortality. Extended storage time increases both mortality and abnormal development.
2. Incubate the eggs in a 37.5 °C and 60% humidified egg incubator with gentle rocking at a frequency of 5 times per 24 hr.
3. Remove the incubated eggs from the egg incubator at the desired embryonic age.
Note: The age of the chicken embryo is determined as the time of incubation and is denoted as embryonic days (E) or as stages (st), according to the series of developmental stages described by Hamburger and Hamilton¹².
4. Place the selected eggs in the laminar flow hood. Wipe the eggshell with 70% ethanol to avoid contamination of the embryo.
Note: Conduct all procedures under aseptic conditions with sterile solutions <http://www.jove.com/science-education/5030/making-solutions-in-the-laboratory> lutions and instruments.
5. Tap the side of the egg firmly against a hard surface to crack open the egg. Place the content in a 100 mm Petri dish. Use a small spoon to transfer the embryo to a 35 mm Petri dish containing pre-warmed (37 °C) 1x PBS to prevent the tissue from drying.
6. Place the 35 mm Petri dish containing the embryonic tissue onto a binocular stereovision dissecting microscope in the laminar flow hood. Decapitate the embryo by pinching the neck with fine forceps and discard the body.
7. Use fine forceps to make an incision through the mouth, ventral to the eye. Starting at the site of incision, tear open the tissue surrounding the entire eye. Pinch off the optic nerve and remove the intact eye from the eye socket. Collect both the left and right eye from the same embryo for use as either the control or the treated eye.
8. Use a small spoon to transfer the eyes to a new 35 mm Petri dish containing pre-warmed (37 °C) 1x PBS.

2. Preparation of Whole Retinal Explants

1. Use fine forceps to remove all tissue around the eye including the scleral anlage.
Note: The tissue will detach easily leaving the retina on the vitreous body and lens with the pigment epithelium intact (**Figure 1A-B**).
2. Use fine forceps to pinch a small incision into the pigment epithelium at the dorsal part of the eye without damaging the neural retina (**Figure 1C**).
3. Use fine forceps to gently tear open the pigment epithelium starting at the site of incision, leaving the lens and the entire retina attached to the vitreous body (**Figure 1D**). Keep the eye in warm (37 °C) 1x PBS to facilitate the removal of the pigment epithelium.
Note: The pigment epithelium may be difficult to remove, in particular along the ciliary body around the pupil, in the anterior region of the eye, and along the choroid fissure. Therefore, some of the pigment epithelium may be left on the neural retina (**Figure 1E-F**).

3. Treatment of Whole Retinal Explants

1. Electroporation of whole retinal explants
Note: Electroporation of whole retinal explants can be performed directly after step 2.
 1. Prepare culture medium containing 1:1 DMEM:F12 Nutrient mix, 10% FBS, 10 U/ml penicillin streptomycin, 5 µg/ml Insulin, and 2 mM L-glutamine.
 2. Cut off a 1.5 ml disposable plastic cuvette (12.5 mm x 12.5 mm x 45 mm) (or any small container capable of holding 300-400 µl solution) approximately 8 mm from the bottom using a fine-bladed saw.
 3. Dilute the DNA plasmid of choice in 1xDPBS to a final concentration of 0.1 µg/µl.
 4. Turn on the electroporator and set parameters to 15 V for st20 – st25 (E3-E4) retina and 20 V for st26 – st27 (E5) retina. Set the pulse-repeats to 5 pulses of 50 msec pulse-length with 1 sec intervals. Use a pulse generator that can be controlled by a foot-pedal on the floor as both hands will be needed to hold the electrodes in place.
 5. Connect two paddle shaped (flat, circular diam. 4 mm) platinum electrodes (**Figure 2A**) to the output socket on the pulse generator.
Note: The platinum electrodes used in this protocol were made by the university workshop. The electrodes were made from 0.1 mm platinum plate "rondelles" (platinum grade: 950 Pt/5 Cu). The connecting 0.8 mm wire was insulated using plastic tubing.
 6. Carefully transfer the whole retinal explant from step 2.3 to the cuvette (or equivalent small container) using a polyethylene Pasteur pipette with the tip cut off to acquire the right tip-opening size for the retinal explant. Avoid using pipette tips as the retina tends to attach to the plastic and tear apart.
 7. Use a 100 µl pipette to gently remove the entire 1x PBS surrounding the retinal explant taking care not to touch the retina to avoid tearing.
 8. Add 100 µl plasmid solution to the cuvette to cover the whole retinal explant. Gently push down the retinal explant with forceps if it floats to the top. Use forceps or electrodes to position the lens to face any one side of the cuvette and the optic nerve to the bottom of the cuvette.
 9. Place the positive electrode in front of the lens and the negative electrode behind the retina (**Figure 2A**), without touching the tissue.
 10. Apply the current by pressing down the foot-pedal. Check for bubbles at the electrodes indicating successful discharge.

11. Use a 100 μ l pipette to remove all the plasmid mix from the cuvette without damaging the retina. The plasmid mix can be reused three to five times.
 12. Fill up the cuvette with pre-warmed (37 °C) 1x PBS. Use forceps to gently detach the retinal explant from the wall of the cuvette. After electroporation the retina is sometimes covered with bubbles, which makes it adhere to the wall of the cuvette.
 13. Use the polyethylene Pasteur pipette to transfer the whole retinal explant into a 24-well plate containing 1 ml pre-warmed (37 °C) culture medium per well. Incubate one retinal explant per well.
 14. Incubate the retinal explant at 37 °C on a rotator shaker, with a constant speed of 50 rpm, inside a cell incubator with 5% CO₂ for 24 hr. The rotation is to ensure maximum exposure to the medium and to prevent adhesion of the retina to the bottom of the 24-well plate.
 15. Proceed to step 4.
2. Treatment of whole retinal explants with chemical reagents
- Note: Chemical treatment of whole retinal explants can be performed directly after step 2.
1. Prepare culture medium containing 1:1 DMEM:F12 Nutrient mix, 10% FBS, 10 U/ml penicillin streptomycin, 5 μ g/ml Insulin, and 2 mM L-glutamine.
 2. Use a small spoon to transfer the retinal explant from step 2.3 into a 24-well plate containing 1 ml pre-warmed (37 °C) culture medium per well. Incubate one retinal explant per well.
 3. Incubate the whole retinal explant for 60 min at 37 °C on a rotator shaker, with a constant speed of 50 rpm, inside a cell incubator with 5% CO₂ before adding a chemical reagent or vehicle.
 4. Prepare the chemical solution, for example Cdk1/2 inhibitor III, an inhibitor of M-phase progression¹³, at a final concentration of 30 μ M in DMSO.
 5. Remove the 24-well plate, containing the whole retinal explant, from the cell incubator. Add 10 μ l of the chemical reagent or the vehicle (DMSO) directly to the retinal medium, resulting in a final concentration of 300 nM Cdk1/2 inhibitor III in 0.01% DMSO. Manually rotate the 24-well plate to allow for even distribution of the chemical reagent or vehicle in the solution.
 6. Incubate the retinal explants at 37 °C on a rotator shaker, with a constant speed of 50 rpm, inside an incubator with 5% CO₂ for the desired time (up to 24 hr).
 7. Proceed to step 4.

4. Fixation and Freezing of Whole Retinal Explants

1. Remove the 24-well plate containing the treated whole retinal explant from the cell incubator.
2. Use a small spoon to transfer the retinal explant to a 24-well plate containing 1 ml/well ice cold 4% paraformaldehyde in 1xPBS. Incubate on ice for 15 min.
3. Use a small spoon to transfer the whole retinal explant to a 24-well plate containing 1 ml/well ice cold 1xPBS to wash the retina. Incubate on ice for 10 min.
4. Use a small spoon to transfer the whole retinal explant to a 24-well plate containing 1 ml/well ice cold 30% sucrose in 1xPBS. Incubate on ice for 1-3 hr depending on the embryonic day of the retina. For st20 – st25 (E3) retinas, incubate for 1 hr, older retinas need longer incubation time in sucrose.
5. Use a small spoon to transfer the whole retinal explant onto a paraffin film containing approximately 500 μ l cryostat freezing mounting medium. Remove as much sucrose as possible by gently moving the retinal explant in the freezing medium using the small spoon.
6. Transfer the retinal explant to an embedding mold containing freezing medium. Position the eye to facilitate future sectioning. Put the embedding mold containing the whole retinal explant on dry ice until the medium is frozen solid. Store at -80 °C.

Representative Results

This protocol describes the preparation (**Figure 1A-F**) and culturing of whole retinal explants from chicken embryos. This protocol has been successfully used for whole retinal explants from embryos of st20 (E3) to st31 (E7).

Electroporation of DNA plasmids into whole retinal explants allows for labeling and tracing of retinal progenitor cells or over-expression of different gene products. For electroporation experiments, the pigment epithelium was carefully removed from the enucleated eye of a st25 (E4 ½) embryo. The whole retinal explant was then placed in a cuvette containing the DNA plasmid solution, electrodes were submerged (**Figure 2A**) and a current was applied. After 24 hr of culturing, GFP positive cells were visible in a large part of the intact retina (**Figure 2B**). After sectioning, single GFP+ cells were easily detected along the apico-basal axis of the retina (**Figure 2C**). Electroporation resulted in a large retinal area taking up the plasmid and expressing the reporter gene. The success rate, measured by the number of retinas with an electroporated area larger than 1/5 of the total retina, was 75% (62 out of 83 retinas) for whole retinal explant electroporation, compared to 14% (37 out of 263 eyes) for *in ovo* electroporation. Electroporation experiments on whole retinal explants have successfully been performed on st20 (E3) to st27 (E5) embryos.

The whole retinal explants can be cultured for approximately 24 hr (**Figure 3A and B**). Longer incubation times may lead to developmental delay, morphological malformations, apoptosis and eventually disintegration of the retina (**Figure 3C**). When culturing the chicken retinas as whole explants the architecture of the retina remains intact. This includes distribution of the different phases of the cell cycle along the apico-basal axis. During normal development the cells undergo S-phase on the basal side, followed by mitosis on the apical side of the retina^{14,15}. A section from a whole retinal explant, cultured for 24 hr, was immunostained with a Phospho-Histone 3 (PH3) antibody, labeling cells in late G2/M-phase (**Figure 3D**). The PH3 positive cells were found on the apical side of the retina, consistent with normal retinal development. An active cyclin B1-Cdk1 complex in the nucleus will initiate M-phase transition¹³. Blocking the Cdk1-kinase activity will inhibit down-stream events that are necessary for cell cycle propagation into mitosis. Stage 29 (E6) whole retinal explants were incubated with the Cdk1/2 Inhibitor III for 4 hr and PH3 immunoreactivity was analyzed. The Cdk1/2 inhibitor III reduced the number of PH3 positive cells (**Figure 3E**), indicating an efficient block of G2/M-phase transition. The reduction of the number of mitoses after treatment with the Cdk1/2 inhibitor verified that the treatment was effective.

Figure 1. Removal of the pigment epithelium. An embryonic day 6 (st29) chicken eye was dissected and prepared for culturing as whole retinal explant. **(A)** View of the anterior eye with pupil and lens and the choroid fissure facing down. The pigment epithelium is intact but the scleral anlage and surrounding connective tissue has been removed. **(B)** View of the posterior part of the eye. The optic nerve exit and choroid fissure are facing down. **(C)** Incision in the pigment epithelium. **(D)** Most of the pigment epithelium has been removed. **(E)** Some pigment epithelium is left at along the rim of the ciliary body and **(F)** the choroid fissure. LE: lens and ciliary body, CF: choroid fissure, ON: optic nerve exit. Scale bar is 0.5 mm. [Please click here to view a larger version of this figure.](#)

Figure 2. Electroporation of whole retinal explants. (A) Schematic presentation of the platinum electrodes and how they are positioned on either side of the whole retinal explant that is submerged in DNA plasmid solution in the truncated cuvette. (B) Whole retinal explant after 24 hr of culturing, and (C) sectioned retina. ON: optic nerve exit. Scale bar is (A) 4 mm, (B) 0.5 mm, (C) 50 μ m. [Please click here to view a larger version of this figure.](#)

Figure 3. Cultured whole retinal explants were fixed, frozen, cryosectioned, and labeled by immunohistochemistry. (A) A st29 whole retinal explant cultured for 24 hr. Fluorescence micrographs of nuclear staining after (B) 24 hr and (C) 48 hr. (D) A PH3 antibody was used to label cells in late G2/M-phase. (E) The relative density (PH3+ cells/mm², mean $\pm$ SD) and fluorescence micrographs of PH3+ cells at st29 after Cdk1/2 inhibitor III treatment for 4 hr compared to vehicle. st: Hamburger and Hamilton stages, Student's t-test, ** p < 0.01, n $\geq$ 4 treated eyes, 4 sections per eye, h: hours. Scale bar is (A) 0.5 mm, (B-E) 10 μ m. [Please click here to view a larger version of this figure.](#)

Discussion

In this work a detailed protocol for dissection, electroporation or chemical treatment, and culturing of whole retinal explants from chicken embryos is presented. This protocol is easy, quick and allows for both a high success rate and reproducible results.

Electroporation of whole retinal explants produces large areas of cells that express the gene construct of interest. It is easy to correctly position the electrodes and to expose a specific portion of the retina to a defined DNA plasmid concentration. This approach allows for a reliable method to investigate gene function in the retina with a typically higher success rate compared to electroporations *in ovo*. The whole retinal explant also makes it possible to expose the retina to a medium containing a defined concentration of a chemical reagent giving a uniform distribution of the reagent in the neural retina. The protocol allows for combined treatments where the retina is first electroporated and then treated with substances. The influence of variability between individual embryos may be minimized, since both the treated and control eye can be collected from the same embryo and be treated independently.

The protocol has been optimized for whole retinal explants from st20 (E3) to st27 (E5) embryos for electroporation and st20 (E3) to st31 (E7) embryos for chemical substance treatment. At younger stages the retina/optic cup is too small and fragile to effectively handle and the *in ovo* electroporation is most likely more efficient. Electroporations on whole retinal explants was not performed on stages older than st27 (E5). It is likely possible to adapt this protocol to explants from older embryos. That would include obtaining larger cuvettes, larger wells for incubation and increased volume of retinal culture medium. However, by E12-14 the pigment epithelium and the outer segment of the photoreceptor cells become closely associated, and removal of the pigment epithelium may damage the retina. The retina should be intact during the entire experimental procedure and incubation.

With this dissection and culturing protocol, the structural integrity of the retinal architecture remains intact during 24 hr, based on the expression of cell type specific markers. As mentioned, we have studied cell cycle progression in the explants and the process of interkinetic nuclear migration of retinal progenitor cells looks normal. If the protocol is performed swiftly there are only small effects on the developmental progression in the explant. However, the development may be slightly decelerated and an explant that has been cultured for 24 hr may exhibit a developmental age that is 1-2 hr younger than the normal counterpart. It is advised to compare the explant to age-matched normal retina and it is advised to use the contralateral eye as an experimental explant control. It should be pointed out that removal of the retinal pigment epithelium may affect the development of the outer photoreceptor segments. It should also be pointed out that an explant retina represents an injured retina with axotomized retinal ganglion cells.

This protocol is simple and therefore reduces experimental errors but the *ex ovo* situation demands several experimental controls. A specific developmental process that is subject to be studied using this *ex ovo* protocol may also be studied in parallel and be compared to the normal *in ovo* situation. The appropriate experimental controls must always be included, such as vehicles used to solubilize substances. When using signal pathway blockers, use several different inhibitors for the same process or use different inhibitors that target enzymes at more than one level of the specific pathway that is studied. For electroporation, a GFP-expressing DNA plasmid is useful as a positive control. If such plasmid does not produce any reporter gene expression, it is most likely due to that the anode (+) and cathode (-) are incorrectly positioned. The DNA plasmid will then migrate away from the retina. When testing novel DNA plasmids it is therefore recommended to include a DNA plasmid that expresses a different fluorescent protein as an internal electroporation control. The electroporation parameters described in this protocol have been optimized to produce a high number of reporter gene expressing cells. Decreasing the voltage or the number of pulses reduces the number of transfected cells, which may produce false negative results. However, increasing the voltage or the number of pulses may lead to damage of the tissue and increased cell death. Three sets of custom made platinum electrodes have been used for all electroporations during more than two years without showing any reduced capacity. The electrodes must be carefully cleaned and the electrode surface be polished. Commercial 4 mm paddle electrodes are available.

This protocol focuses on electroporation of DNA plasmids and chemical treatment of whole retinal explants. It is possible to further expand this protocol to include other experimental procedures, such as a knock-down of gene expression with the use of morpholino oligomers. The whole retinal explant procedure opens up for the possibility to perform short-term experiments in an easy, quick and highly reproducible way. It reduces costs, time, and the number of animals needed for each experiment and most importantly it increases the possibility to get solid data.

Disclosures

The authors declare that they have no competing financial interests.

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