HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2019 January 01.

Published in final edited form as:

Methods Mol Biol. 2018 ; 1715: 101–111. doi:10.1007/978-1-4939-7522-8_8.

In Vivo Electroporation of Developing Mouse Retina

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Abstract

In vivo electroporation enables the transformation of retinal tissue with engineered DNA plasmids, facilitating the selective expression of desired gene products. This method achieves plasmid transfer via the application of an external electrical field, which both generates a transient increase in the permeability of cell plasma membranes, and promotes the incorporation of DNA plasmids by electrophoretic transfer through the permeabilized membranes. Here we describe a method for the preparation, injection, and electroporation of DNA plasmids into neonatal mouse retinal tissue. This method can be utilized to perform gain of function or loss of function studies in the mouse. Experimental design is limited only by construct availability.

Keywords

Electroporation; Gain of function; Gene expression; In vivo; Loss of function; Plasmid; Subretinal injection; Retina

1 Introduction

The functional characterization of genes that are expressed during mammalian retinogenesis, and may regulate retinal development presents several technical challenges. Gene targeting for the purpose of engineering constitutive or conditional loss of function knockouts requires a potentially prohibitive investment in both time and resources. Additionally, retina expressed genes may have essential roles in extraocular tissues, resulting in embryonic mortality in knockout models, precluding ocular analysis. There may also be extraretinal phenotypes that can confound the analysis and interpretation of retinal gene function. The use of conditional knockout Cre-lox models can significantly limit both mortality and extraocular effects, but remains both resource and time intensive. Though knockout models can provide informative loss of function data for a gene of interest, the ability to ectopically express a gene in a controlled gain of function experiment can reveal additional functional effects. Gain-of-function studies are especially valuable for the elucidation of cell fate specification and/or terminal differentiation roles in the retina, wherein activation of gene expression may reprogram retinal progenitor cells altering their course of development.

Electroporation is a method that can be used for the rapid and efficient incorporation of DNA plasmids into the neonatal mouse retina [1–9]. The historical development of electroporation began with the discovery that the application of short electrical pulses was sufficient to promote a transient increase in plasma membrane permeability [10]. This

induced membrane permeability could be exploited to facilitate the transfer of materials across membranes [10–13]. Further development revealed that electroporation could be utilized as a method of gene transfer into mammalian cells [14]. Continuous technical refinement established electroporation as a robust and reproducible method for in vivo gene transfer in multiple vertebrate species including the mouse [15]. Here we describe a method by which electroporation can be used for in vivo gene transfer in the developing mouse retina.

A DNA solution comprised of isolated and purified engineered plasmid expression constructs is injected into the developmentally transient subretinal space in neonatal mice. Successful injection places the DNA solution between the retinal pigment epithelium and the mitotically active apical neuroretina. Electrical charges are applied using a tweezer-type electrode, thereby facilitating the correct directional orientation of the electrical field such that electrophoresis of the negatively charged DNA moves into the retina and away from the pigmented epithelium. The strength and robustness of gene expression will vary based on differences in plasmid promoter design. We have identified robust plasmid incorporation and gene expression as early as 48 h post electroporation. There is no significant lateral migration of developing cells in the retina. Consequently, electroporation results in distinct electroporated and nonelectroporated regions in the retina. Non-electroporated regions may serve as internal histological controls where appropriate.

Electroporation offers several key advantages over alternative methods for the transformation of retinal tissue. Currently, the size of a gene delivered by retroviral-mediated transduction is limited by the packaging constraints of the viral capsid [16]. Such constraints on gene size are not a factor using electroporation. Furthermore, multiple plasmids can be mixed together in injection solutions to perform combinatorial gene delivery [8, 9]. Plasmid design will vary from study to study. Broadly, retinal electroporation can be used to express a gene of interest under a ubiquitous promoter, such as CAG. Furthermore, plasmids can be designed to disrupt gene function by expressing engineered shRNA constructs or CRISPR/Cas9 plasmids [1, 17]. Loss of function can be further mediated by electroporation of plasmids expressing Cre recombinase into floxed mouse lines [8]. Selective retinal gene expression can be achieved through the use of cell-class specific gene promoters [2]. Visualization of electroporated cells is typically achieved using bicistronic constructs expressing a fluorescent reporter gene such as GFP, or by co-electroporating a reporter gene expression plasmid.

2 Materials

This protocol describes a procedure for the purification and concentration of plasmid DNA with the objective of obtaining an injectable DNA solution at a concentration of 5 μg of DNA per μL of solution. The protocol then describes the injection of this purified DNA solution into the subretinal space and the application of electrical pulses for the purpose of transforming the retinal tissue. All prepared solutions should be made with ultrapure water (ASTM Type I standard) and be prepared such that they follow any applicable guidelines for their use in animal procedures. All steps may be performed at room temperature unless

otherwise indicated. All governmental and institutional waste disposal regulations should be followed when disposing laboratory waste products.

2.1 Plasmid Preparation

1. Phenol: Phenol that is Tris-equilibrated 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
2. Chloroform: >99% chloroform anhydrous.
3. 3 M Sodium acetate: 3 M sodium acetate molecular biology grade, pH 5.5.
4. Ethanol: Absolute ethanol, molecular biology grade 200 Proof.
5. 80% v/v Ethanol: 8 parts of absolute ethanol is diluted (v/v) with 2 parts ultrapure water.
6. 1× PBS: Phosphate buffered saline pH 7.4.
7. 1% Fast Green FCF: Dissolve 1% w/v Fast Green FCF into 1% (v/v) >99.7% acetic acid in ultrapure water.
8. Plasmid DNA purification kit (Qiagen Maxi-Kit or similar).

2.2 Subretinal Injection of DNA

1. Sharp beveled 30 gauge needle: Precision glide beveled 30 gauge needles (BD).
2. 70% Isopropyl alcohol prep: Isopropyl alcohol prep pads Curity 70% Isopropyl alcohol (Covidien).
3. 33 gauge blunt ended needle: Exmire microsyringe, needle outer diameter 0.52 mm, inner diameter 0.13 mm (Ito Corporation, Shizuoka, Japan).
4. Stereomicroscope.
5. Hemostat: Surgical tool used for paw pinch tests.
6. Ice in a box.
7. Thin latex barrier to prevent direct contact of mouse pups with the ice.

2.3 Electroporation

1. Electroporator Model ECM 830 (BTX-Harvard Apparatus) (*see* Note 1).
2. Tweezertrodes: 10 mm tweezer electrode diameter Stainless steel (BTX-Harvard Apparatus).
3. 110 V Heat lamp (Braintree Scientific).
4. Clean tissue-lined container.
5. Mouse pups postnatal day 0 (P0) to P4.

[1]The specific make and model of the electroporation system is subject to system availability and/or the preferences of the research group performing the studies. Electroporators marketed by manufacturers different from that outlined in this protocol are perfectly acceptable given that they can achieve the pulsing paradigm outlined in Subheading 3.3, **step 2**.

2.4 Fixation and Cryoembedding

1. 4% paraformaldehyde (PFA): Add 4% (w/v) paraformaldehyde into 1× PBS pH 7.4, add 20 μL 10 N NaOH and incubate at 65 °C until dissolved. Cool to room temperature and adjust pH to 7.4 and filter pass to remove any particulate.
2. 30% Sucrose: Add 30% (w/v) sucrose into 1× PBS pH 7.4 and stir over moderate heat until dissolved. Cool to room temperature and filter pass before use.
3. O.C.T. compound: Tissue-Tek (Sakura Finetek USA).
4. Optional Fluorescent stereomicroscope or inverted fluorescent microscope.
5. Molds for O.C.T. embedding.
6. Dry ice chilled methylbutane to freeze the O.C.T. filled molds.
7. Cryotome.
8. −80 °C freezer.

3 Methods

The following methods include a description of a surgical procedure where DNA solution is injected into the subretinal space of neonatal mice followed by the application of an electrical field. All animal procedures must be approved and performed under the specific guidelines of any and all applicable governmental and institutional regulators. Injections are performed with the aid of a stereomicroscope, the make and model of which may vary based on the preferences of the research group. Variations to the methods may be required based on the specific animal research policies at any given institution.

3.1 Plasmid Preparation

1. Aliquot 100 μg of plasmid DNA solution. Plasmids should be cultured, isolated and purified such that the stock concentration ranges from 2 to 5 μg/μL with sufficient volume for isolation of 100 μg of DNA. Acquisition of this amount of plasmid DNA typically necessitates preparation with a Plasmid Maxi- Kit or similar method.
2. Dilute the volume of DNA to 100 μL for ease of calculation and manipulation.
3. Add 67 μL of phenol to the 100 μL diluted plasmid DNA preparation in order to obtain a 60% (v/v) DNA–40% phenol ratio (*see* Note 2). Mix the tubes thoroughly by inversion; do not mix the samples by pipetting or by vortexing as this may cause excessive shearing of the plasmid DNA.
4. Spin the DNA–phenol mixture for 5 minutes (min) in a microcentrifuge designated for the use of organic solvents at 16,000 × *g* at room temperature.

[2]All use of organic solvents should be performed under appropriately ventilated hoods. Disposal of organic solvents should follow all governmental and institutional regulations.

5. Collect the 100 μL of aqueous supernatant and transfer to a fresh 1.5 mL centrifuge tube. The organic layer may be discarded. Add 67 μL of chloroform to the 100 μL of aqueous solution in order to obtain a 60% (v/v) DNA–40% chloroform ratio (*see* Note 2).

6. Spin the DNA–chloroform mixture for 5 min in a microcentrifuge designated for the use of organic solvents at 16,000 × *g* at room temperature.

7. Collect the 100 μL of aqueous supernatant and transfer to a fresh 1.5 mL centrifuge tube. The subnatant organic layer may be discarded. Add 10 μL of 3 M sodium acetate to the 100 μL of DNA solution, mix gently by inversion and then add 250 μL of 100% ethanol. Mix the solution thoroughly by inversion. Avoid pipetting or vortexing as this may cause excessive plasmid shearing. The plasmid DNA should precipitate out of solution during the mixing (*see* Note 3).

8. Spin the DNA–ethanol solution in a microcentrifuge at 4 °C for 10 min at 16,000 × *g*.

9. A large white pellet should be visible at the bottom of the centrifuge tube. Pour off the aqueous solution with care so as not to lose the DNA pellet. Add 350 μL of 70% ethanol to the tube and gently invert the tube to rinse the DNA pellet (*see* Note 4).

10. Spin DNA–ethanol solution in a microcentrifuge at 4 °C for 5 min at 16,000 × *g*.

11. Pour off the 70% ethanol solution with care so as not to lose the DNA pellet. Air-dry the pellet. Excess ethanol may be aspirated off by pipetting to speed the process. Do not excessively dry out the DNA pellet because this will make it difficult to resolubilize (*see* Note 5).

12. Dissolve the DNA pellet into 20 μL of 1× PBS to achieve a final concentration of 5 μg/μL of plasmid DNA (*see* Note 6).

13. Add 2 μL of Fast Green FCF (1% stock) in order to achieve a final dye concentration of 0.1%. Fast Green FCF serves as an injection tracer facilitating observation of the spread of the injection solution into the subretinal space (*see* Note 7).

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[3]Due to the relatively large amount of DNA (100 μg) in the solution, the precipitation of the DNA should be readily observable after mixing by inversion. Failure to see a cloudy/stringy white precipitate should be taken as a sign that an error may have occurred earlier in the process, and that the plasmid DNA may have been lost in washes or transfers.

[4]The pellet may become dislodged from the bottom of the tube following inversion, this is not problematic. Simply ensure that the pellet is resubmerged in the 70% ethanol by gently tapping the tube before the following centrifugation step.

[5]The importance of not overdrying the DNA pellet cannot be emphasized enough. Excessively dried pellets will not dissolve readily into the 1× PBS injection solution. Attempts to improve solubilization by mixing the pellet into solution through pipetting or vortexing may cause plasmid shearing which can impact plasmid performance following electroporation. The pellet will appear white and opaque following the alcohol wash step and will become transparent as it dries. Transparency will be observed first at the outer edges of the pellet with the center mass of the pellet remaining opaque. Upon removal of the excess ethanol the pellet should monitored while drying. Once the outside edges of the pellet begin to clear the pellet is ready to be dissolved into 1× PBS. Do not wait for the center mass to clear as this will overdry the pellet.

[6]The final concentration of plasmid required for optimal electroporation efficiency may need to be determined empirically for any given construct. We generally observe excellent electroporation efficiency injecting CAG promoter based plasmids at a concentration of 5 μg/μL. However, injection concentrations ranging from 1.25 to 6 μg/μL have yielded successful electroporation results.

[7]The use of an injection tracer is of value primarily within albino mouse strains. If electroporation is being performed on pigmented mice, the inclusion of Fast Green FCF may not be of significant value as a tracer and can be omitted at the experimenter's discretion.

3.2 Subretinal Injection of DNA

1. Hypothermic anesthesia will be used to anesthetize neonatal (postnatal day (P)0) mice prior to subretinal injection of DNA and electroporation (*see* Note 8). Pups are placed on a bed of ice for several minutes with constant monitoring (Fig. 1a). A thin latex barrier should be placed between the pups and the ice to prevent freeze damage to the skin. Pups must not be buried in the ice as this may increase mortality. The duration of ice exposure that any given pup may require to ensure appropriate hypothermic analgesia will vary from animal to animal. Typically, 5 min is sufficient but mice should be carefully monitored as individuals may respond very quickly or may require longer exposure. A paw pinch test using a hemostat may be conducted to check for a withdrawal reflex, when the withdrawal reflex is not identifiable the pup is ready for the procedure.

2. Prior to injection of DNA into the subretinal space, the eye must be opened using sharp beveled 30 gauge needle. Swab the eye to be injected with a 70% isopropyl alcohol prep, and with the assistance of a stereo microscope identify the fused junctional epithelium where the eyelids of the eye to be injected come together.

3. Using the sharp beveled 30-gauge needle, carefully open the eye by cutting along the fused junctional epithelium (Fig. 1b). This can be achieved by gently slicing along the junction with the sharp edge of the needle. Do not use a sawing, poking or stabbing motion to open the eye. Avoid applying excessive force; too much pressure may result in the penetration of the underlying eye. Furthermore, avoid cutting past the range of the fused eyelid junction. This may result in significant bleeding which will obscure the eye increasing the difficulty of the following injection.

4. Because the injection needle is blunt ended, it is necessary to make a small incision in the sclera at the point of injection to facilitate insertion of the injection needle into the eye. Use the tip of a 30-gauge beveled needle to make an incision in the sclera adjacent to the corneal limbus at the site where the blunt ended needle will penetrate the eye. Do not penetrate too deeply in order to avoid damaging the lens with the sharp tip of the beveled needle.

5. Draw 0.6 μL of DNA solution into the 33 gauge blunt ended Exmire microsyringe injection needle. The gradients incorporated into the barrel of the syringe may be used to measure the volume drawn (*see* Note 9). Insert the needle into the incision generated in the previous step. The opposing scleral wall will provide resistance once the needle has passed through the vitreous humor and retina. Care should be taken to avoid penetrating or scratching the lens as the needle is passed through the eye. A ruptured lens may contribute to ocular dysplasia as the eye develops making retinal analysis difficult or impossible.

[8]Hypothermia is generally acceptable for procedures requiring <10 min in mouse pups up to age P5. Once practiced the process of eye opening, incision, injection and electroporation takes approximately 3 min, which will not allow the pup enough time to recover.

[9]Although only 0.3 μL will be injected into the subretinal space, excess injection solution should be drawn into the needle for each injection to ensure that the air which is present in the barrel of the metal needle portion of the syringe and which gets drawn into the syringe preceding the injection solution does not get injected into the eye. This can result in the creation of an air bubble in the subretinal space, which may cause retinal detachment and dysplasia.

6. Slowly inject 0.3 μL of DNA solution into the subretinal space (Fig. 1c). The experimenter should execute a controlled and even ejection of solution from the syringe so that the DNA solution does not overflow the subretinal space and become deposited into the vitreous chamber of the eye. Successful subretinal placement of the DNA solution will produce an even spread in a portion of the subretinal space which will be readily observable by the Fast Green FCF injection tracer (Fig. 1d). The experimenter will be able to discriminate areas of retina with and without underlying Fast Green FCF stained solution. Gradual rotation of the head of the pup under the microscope should help make these regions discernable.

3.3 Electroporation

1. Electroporation is performed on injected pups using a tweezertrode. The tweezertrode should be immersed in 1× PBS prior to application on injected pups. Wetting the tweezertrode with 1× PBS is performed to maximize the electrical conductivity from the tweezertrode to the pup. The tweezertrode may then be applied to the head of the injected pup (Fig. 1e). The electrodes should be oriented such that the positive pole electrode is placed over the injected eye and the negative pole electrode is placed over noninjected eye. This placement ensures that the electrical field is oriented such that electrophoresis will drive the injected DNA solution from the subretinal space into the retina.
2. Apply the electrical pulses using the pulse generator (electroporator). For electroporation of neonatal (P0–P4) mouse pups the electrical paradigm should be set as follows: 5 square pulses, each pulse is set at 80 V and 50 ms in duration. There should be a 950 ms interval between pulses.
3. Using a 70% isopropyl alcohol prep clean the area around the injected/electroporated eye. The electroporated pups must now be allowed to recover from the hypothermic anesthesia (Fig. 1f). Place the pups in a clean, tissue-lined container beneath a heat lamp (*see* Note 10). Allow the pups to remain under the heat lamp until full mobility and normal pink coloring of the pups has returned to each animal. The duration of time under the light may vary for each individual animal. Upon full recovery, return the electroporated pups to their mother and monitor over the next 1–2 days for signs of distress.

3.4 Fixation and Cryoembedding

1. The end point for each electroporation experiment is subject to the requirements of the specific study and will vary accordingly. Reporter expression off of CAG based plasmid constructs can be visualized within 2 days of electroporation, however the expression profile for any given plasmid construct may vary and should be determined empirically.

[10]Alternatively the pups may be allowed to recover by placing them on a slide warmer. If a slide warmer is used the experimenter should ensure that an appropriate pad is placed between the pups and the metal surface of the slide warmer.

2. Euthanize the electroporated animals at the desired timepoint. All methods utilized for the euthanasia of animals should adhere to all governmental and institutional guidelines. Dissect out the electroporated eye and fix in 4% paraformaldehyde with rotational agitation at 4 °C for approximately 50 min. The optimized fixation time for any given endpoint can vary and should be determined by the individual research group.

3. Carefully dissect out the retina from the fixed eye in cold 1× PBS by removing the sclera, cornea, lens, choroid and retinal pigmented epithelium. If a fluorescent reporter has been used as part of the experimental design, the dissected retinas can be analyzed under a fluorescent stereomicroscope or inverted fluorescent microscope to grossly determine the electroporation efficiency. Dissected retinas can then be transferred into 30% sucrose solution and incubated overnight with rotational agitation at 4 °C.

4. Half of the sucrose solution can be removed and replaced with O.C.T. cryo-embedding media. The 50% sucrose/50% O.C.T. solution can be incubated with rotational agitation at 4 °C until the solution reaches a homogeneous consistency. The retinas can then be embedded into O.C.T. filled molds and frozen over dry ice chilled methylbutane. The embedded retinas can be stored at −80 °C. Retinas may now be sectioned on a cryotome and captured onto glass slides (*see* Note 11).

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[11]The specific make and model of cryotome used for sectioning may vary based on availability or the preferences of the experimenters. Section thickness may range from 15 to 25 μm and will also be determined by the experimenter subject to the requirements of the specific study. It also is recommended that sections be captured on positively charged slides for better tissue adherence. Slides containing captured sections may be stored between −20°C and −80°C depending on the duration of storage necessitated and the requirements of the specific research group.

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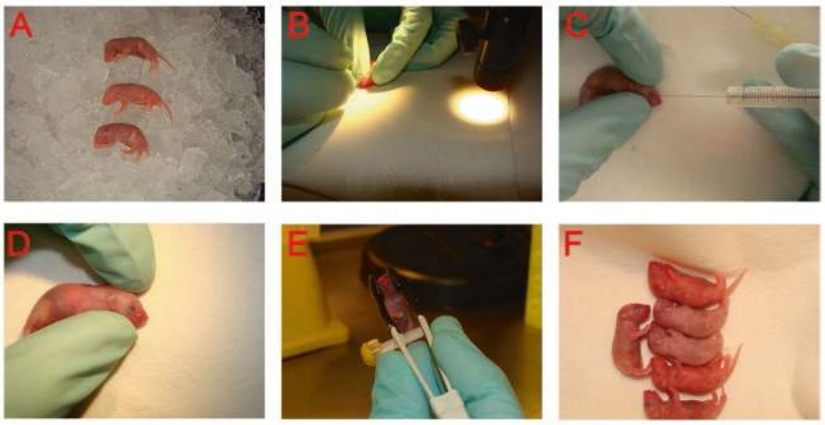

Fig. 1.

Images of key steps in protocol. (**a**) Ice anesthesia of neonatal pups. (**b**) Surgical opening of the eye and scleral incision. (**c**) Manual injection of DNA/Fast Green solution. (**d**) Efficient spread of Fast Green in subretinal space following successful injection. (**e**) Electroporation with tweezertrodes. (**f**) Recovery following surgery under heat lamp