Video Article

In Ovo Electroporation in Embryonic Chick Retina

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Abstract

Chicken embryonic retina is an excellent tool to study retinal development in higher vertebrates. Because of large size and external development, it is comparatively very easy to manipulate the chick embryonic retina using recombinant DNA/RNA technology. Electroporation of DNA/RNA constructs into the embryonic retina have a great advantage to study gene regulation in retinal stem/progenitor cells during retinal development. Different type of assays such as reporter gene assay, gene over-expression, gene knock down (shRNA) etc. can be performed using the electroporation technique. This video demonstrates targeted retinal injection and *in ovo* electroporation into the embryonic chick retina at the Hamburger and Hamilton stage 22-23, which is about embryonic day 4 (E4). Here we show a rapid and convenient *in ovo* electroporation technique whereby a plasmid DNA that expresses green fluorescent protein (GFP) as a marker is directly delivered into the chick embryonic subretinal space and followed by electric pulses to facilitate DNA uptake by retinal stem/progenitor cells. The new method of retinal injection and electroporation at E4 allows the visualization of all retinal cell types, including the late-born neurons[1], which has been difficult with the conventional method of injection and electroporation at E1.5[2].

Video Link

The video component of this article can be found at https://www.jove.com/video/3792/

Protocol

Since some parts of the protocol are performed as previously described in other JoVE videos[2] and papers[3] with minor modification we will not discuss those in detail here.

1. Egg Handling and Needle Preparation

1. Eggs can be stored in a wine cooler at about 13°C for up to 1 week. If the temperature is too high, embryos will start to develop abnormally, while lower temperature causes high mortality. Once you are ready to incubate take out the eggs from the wine cooler and set the eggs vertically with the larger end up. Keep the eggs in the room temperature for at least 2 hours before putting them in the 37.5°C incubator.
2. Incubate the eggs for 96-100 hours which is about embryonic day 4 to obtain embryos that are at the Hamburger and Hamilton stage 22[4-5].
3. Prepare micropipette needles from pulled glass capillary tubes and break the tip under a dissecting microscope with a tweezers to get a tip opening about 0.1 µm in diameter and a 20 mm taper. Needles with larger tips have difficulty piercing the vitelline membrane while smaller tips have difficulty loading and delivering the DNA solution.
4. Attach the needle to a 0.1 ml Hamilton Gastight syringe mounted on a micromanipulator. Use a small piece of Masterplex silicone tube for attaching the needle to the syringe. Since the connections are tight no need to add mineral oil to seal this attachment.
5. Mix 2 µl of reporter plasmid DNA solution with a concentration ranging 3-6 µg/µl and 0.2 µl of fast green (0.025%) on a piece of parafilm. Fast green dye will help to visualize the injection. Slowly, load the needle with the mixture.
6. To free the vitelline membrane from the inner membrane rotate the egg gently about 180° and wait for few minutes then rotate it back to original position and set it for electroporation.
7. Wipe the forceps and egg shell with 70 % ethanol to avoid infection to the embryo.
8. Make a small hole on the egg immediately above the air cell with a pair of size AA forceps. Be careful not to crack the egg shell. Remove small pieces one at a time to make a small window. Carefully remove the inner membrane using the forceps without touching the vitelline membrane.

2. Injection and Electroporation

1. To prevent damage to the brain or the heart, position the needle contra lateral to the main bundle of blood vessels entering the eye and pointing towards the beak.
2. Pierce through the vitelline membrane, sclera, retina and vitreous humor by a sudden mild push of the needle. If the needle pierces through the other end of the eye it should be alright unless it damages any major blood vein.

3. Slowly pull back the needle at the edge of the opening and place it almost tangent to the outer wall of the eyeball.
4. Insert the needle into the sub retinal space between the sclera and retina.
5. Inject the DNA until you can visualize the green solution filling the side of the eyeball and pushing the retina inwards by creating a bulge.
6. If your needle placing is not correct then you will see the DNA solution spreading inside the vitreous humor filling up the middle of the eye ball. Also, if you damage the retina too much then you will see the DNA solution is coming out of the eyeball.
7. Slowly remove the needle and immediately place the electrodes in parallel inside the egg after soaking in PBS. Push down the electrodes to submerge into the amniotic fluid in a way so that the injected eye is located between the electrodes. Avoid touching any major blood vessel or the heart with the electrodes while placing them. The negative electrode should be at the injection side so that DNA can be transported from sub retinal space into the retina towards positive electrode. Electroporate the retina with 5 pulses of 15V for 50 ms with 950 ms intervals.
8. Carefully remove the electrodes and seal the window of the egg with pieces of clear scotch tape.
9. Label and date the injected egg before putting it back to the incubator. Typically, it takes about 3 to 5 minutes to complete the whole electroporation process.
10. GFP expression can be seen as early as 8 hours after electroporation. However, you may wait until the embryo reaches the desired stage before harvesting.

3. Representative Results

In our study, we use various plasmid constructs to study the regulation of gene expression that involved retinal cell development. In this video pCAG-GFP (transfection control) was used to follow a successful injection and electroporation. However, any plasmid construct with reporter gene (GFP, RFP etc.) can be used. Even though GFP expression can be seen as early as 8 hours after electroporation, we typically start harvesting the egg on day 6 (E6) and onwards. Electroporated retinas were dissected out of the embryo and analyzed under fluorescent dissection microscope before embedding and sectioning. Typically, reporter gene expression can be seen at least in a quarter of the retina after a successful electroporation (Fig 1). The transfected retinal tissues were further analyzed through sectioning for clear visualization of cell morphologies. Immunohistochemistry using cell type specific markers (Brn3a, Pax6 etc.) allowed characterization of cell-specific GFP expression (Fig 2).

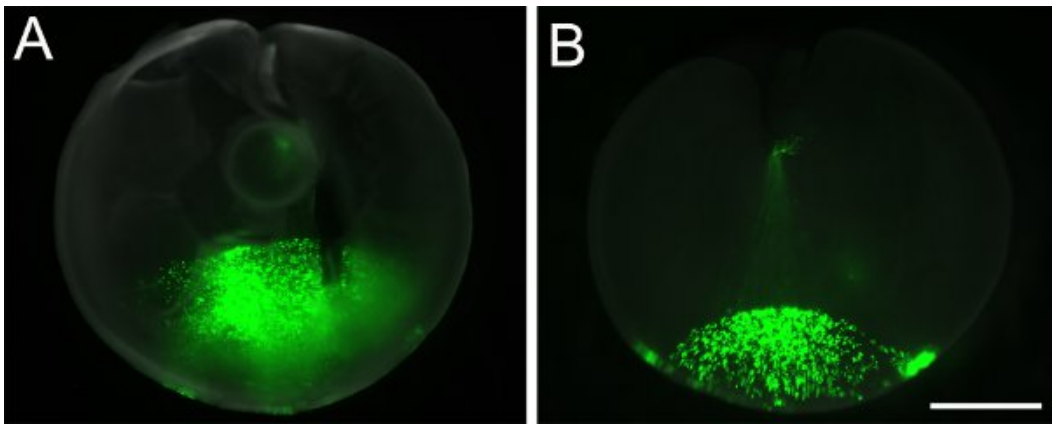

Figure 1. Successful electroporation of reporter plasmid results positive GFP expression. Chicken embryonic retinas were injected and electroporated at embryonic day 4 and harvested at embryonic day 6. At least 25% of the retina was successfully transfected (A=Top view, B= Bottom view). Scale Bar = 1mm.

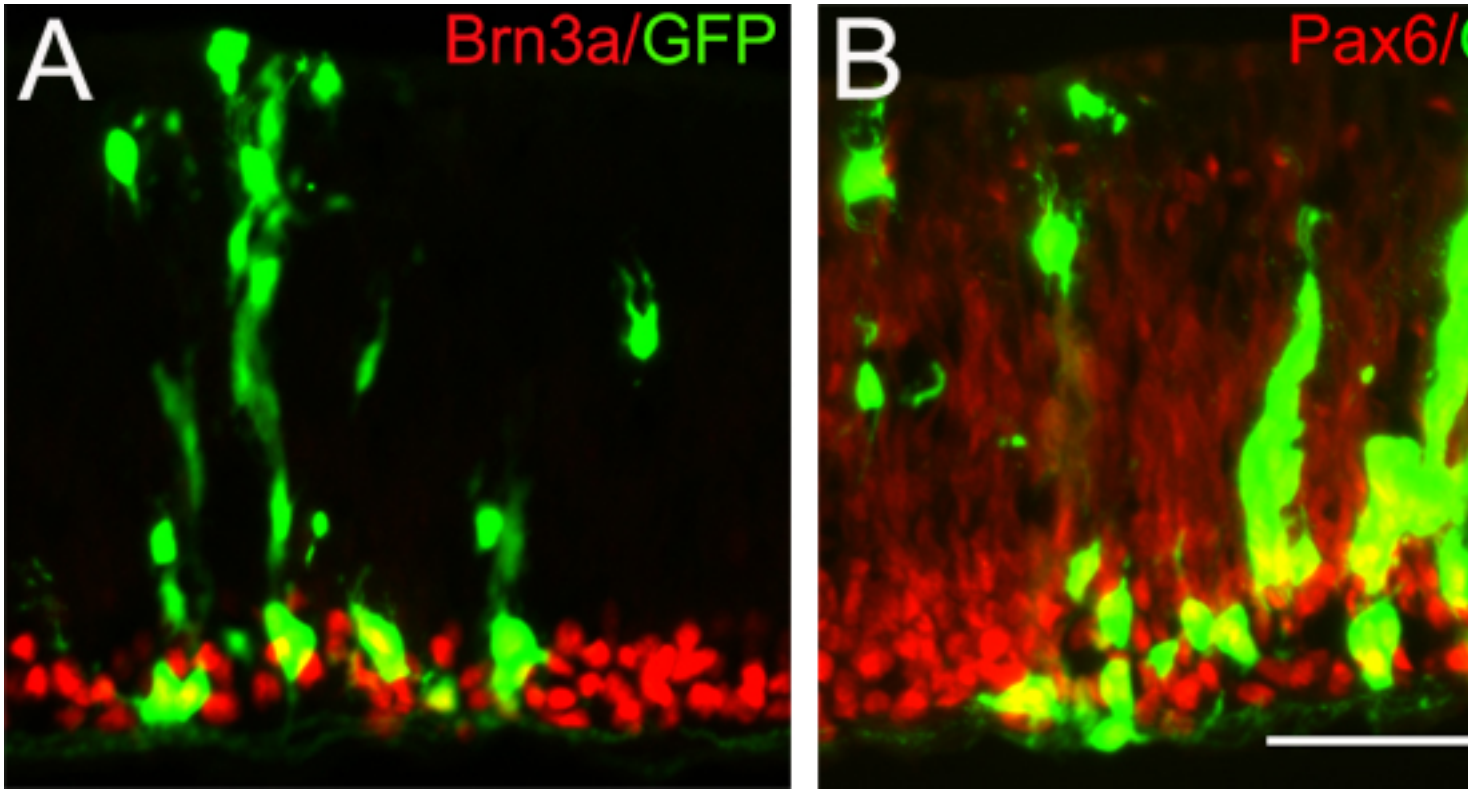

Figure 2. Characterization of GFP expressing retinal cells using immunohistochemistry. GFP expressing retinal tissues at E7 stage were fixed and sectioned. These sections were then stained with various cell specific markers. Brn3a was used to determine ganglion cells (A) while Pax6 was used to determine horizontal, amacrine and ganglion cells (B). Scale bar = 50 µm.

Discussion

Targeted retinal injection and *in ovo* electroporation in embryonic retina at E4 stage can specifically target retinal progenitor cells resulting in the ability to visualize all six major retina cell types at the single cell level. *In ovo* electroporation at HH10 (~E1.5) targeting the optic vesicle is able to transfect cells that develop to form the eye. However, these cells have a very high turnover at this time and this method is not specific for retinal cells. It may be that the high cell turnover rate prevents sustained stable expression. By E4, the embryo is developed enough that the major structures of the eye are all formed but young enough that the majority of cells in the retina are still retinal stem cells. This method can also be applied to gain/loss of function studies where a gene of interest can be targeted to study normal development and/or disease of the retina.

After we published this technique in our previous paper[1], several scientists in this field contacted us for further assistance on this technique. So we decided to produce this video for visualization. In addition, the advances in our technique are described in this video.

To perform this technique successfully, following critical factors are worth describing.

The most critical factor is to place the needle precisely in the subretinal space. Firstly, one needs to be careful to align the needle contra-lateral to the main bundle of blood vessels entering the eye and pointing towards the beak. This position is almost parallel to the heart and reduces the chance to damage the brain and the heart. When piercing through vitelline membrane, sclera, retina and vitreous humor, the needle should not travel far and pierce through any blood vein. If the needle is sharp enough then this can be done very easily with few practices. Next step is to slowly pulling back the needle and position it at the edge of the opening of the retina. There is always a chance to pull it out completely. If that should happen, then one can try again to put the needle tip back at the opening. Placing the needle in the subretinal space (between sclera and retina) requires practice and patience. In the beginning, it looks very difficult; however with some practice it becomes easy. While injecting the DNA solution inside the subretinal space, it is very important to observe the bulge formation. Afterwards, the green solution starts filling the outline of the eye, indicating a successful injection. If the solution diffuses away or starts filling into the middle of the eye, that indicates an incorrect injection which will not yield a successful electroporation.

The second factor is the fabrication of an optimal needle which is made via heat-pulling a glass capillary tube and breaking the tip. Needles with large tips have difficulty piercing through the vitelline membrane which increases the chance of damaging the retina. Usually sharpest needle tips are best for this purpose. However, needle with very small tips have difficulty loading and delivering the DNA and have increased chance to break down inside the eyeball. For this reason we make needles with a tip opening at about 0.1 µm in diameter and a 20 mm taper[1]. Also, while loading the DNA mixture from a droplet on a piece of parafilm, it is very important not to load the last bit of liquid as it increases the chances of getting air inside the needle.

Finally, placing of the electrodes is very critical to achieve successful electroporation. The electrodes should be placed in parallel so that the developing eye is situated between the electrodes. Extra caution should be taken from touching any major blood vessels or the heart with the electrodes. Damaging any of these may result death to the embryo even after a successful injection. Furthermore, the two eyes are differently

oriented. So, it has to be kept in mind to change the electrode orientation (positive vs. negative) so that the negative electrode should be always at the injection side to allow the DNA diffuse into the retinal cells under the electric current.

Disclosures

All of the animal experiments were approved by the Institutional Animal Care and Facilities Committee at Rutgers University.

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