Video Article

A System for Culturing Iris Pigment Epithelial Cells to Study Lens Regeneration in Newt

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Abstract

Salamanders like newt and axolotl possess the ability to regenerate many of its lost body parts such as limbs, the tail with spinal cord, eye, brain, heart, the jaw [1]. Specifically, newts are unique for its lens regeneration capability. Upon lens removal, IPE cells of the dorsal iris transdifferentiate to lens cells and eventually form a new lens in about a month [2,3]. This property of regeneration is never exhibited by the ventral iris cells. The regeneration potential of the iris cells can be studied by making transplants of the *in vitro* cultured IPE cells. For the culture, the dorsal and ventral iris cells are first isolated from the eye and cultured separately for a time period of 2 weeks (**Figure 1**). These cultured cells are reaggregated and implanted back to the newt eye. Past studies have shown that the dorsal reaggregate maintains its lens forming capacity whereas the ventral aggregate does not form a lens, recapitulating, thus the *in vivo* process (**Figure 2**) [4,5]. This system of determining regeneration potential of dorsal and ventral iris cells is very useful in studying the role of genes and proteins involved in lens regeneration.

Video Link

The video component of this article can be found at https://www.jove.com/video/2713/

Protocol

1. Iris Cell Culture

1. Collect eyeballs from 7 newts (anesthetized in 0.1% ethyl 3-aminobenzoate methane sulfonic acid prepared in PBS) and place in calcium magnesium free Hanks solution (CMF).
2. Change gloves and work in the tissue culture hood.
3. Sterilize eye balls in Lugol's-EtOH for 3 seconds.
4. Wash 2 times in CMF.
5. Transfer eyes to Hanks and begin dissection.
6. Dissect iris-corneal complex and remove neural retina.
7. Transfer complexes into a new dish of Hanks.
8. Using #15 scalpel, separate complexes into dorsal and ventral halves.
9. Remove iris fragments (ventral first) and place in a dish containing 1 ml L-15.
10. Add 15% (150 ul) volume of dispase (7.5 units/ml concentration).
11. Incubate at 27°C for 2 hours (wrap dish with parafilm).
12. Isolate IPE cells from stroma (Place trypsin solution at 27°C).
13. Collect IPE cells into a 1.5 ml tube.
14. Centrifuge at 1000 rpm at RT (room temperature) for 2 minutes, remove supernatant.
15. Wash with 1 ml Hanks and centrifuge at 1000 rpm at RT for 2 minutes, remove supernatant.
16. Add 1 ml trypsin solution; incubate for 2 hours at 27°C.
17. Centrifuge at 1000 rpm at RT for 2 minutes, remove trypsin.
18. Add 1 ml L-15 to wash, centrifuge at 1000 rpm for 2 minutes.
19. Add 800 ul L-15, pipet slowly up and down ~ 10 times (more than 12 times reduces adherence).
20. Plate the IPE cells on a 24 well collagen coated plate and incubate at 27°C for 2 weeks (Usually, 4 dorsal and 4 ventral wells are obtained from 7 newts).
21. At this stage cells are suitable for gene transfection or treatment with factors to determine their role in inducing or interfering with lens regeneration.

2. Aggregation of Iris Cells

1. To the plates containing iris cells add 20µl of dispase solution to 400 µl medium, swirl gently.
2. Incubate plates at 27°C for overnight.
3. Pipet gently up and down to dislodge cells
4. Place cells into Eppendorf tube and spin for 2 minutes at 1000 rpm at RT.
5. Remove medium and wash by adding 1 ml complete L-15, spin 2 minutes at 1000 rpm at RT, remove medium.
6. Use 2000-7000 cells per aggregate. Add 200 ul L-15 per aggregate into each tube.
7. Split cells (200 µl) into new eppendorf tubes.
8. Spin at 1000 rpm for 2 min at RT.
9. Incubate for 48 hrs at 27°C.

3. Implantation of Aggregrated Cells

1. Make a slit in the cornea of newt eye and remove the lens.
2. After lens removal, place the IPE cell aggregate just below the corneal tissue on the ventral side of the eye.
3. The newts are kept for a month to let them regenerate the lens.

4. Embedding of Newt Eye

1. Remove the newt eyes implanted with the aggregate and place it in phosphate buffered saline (PBS).
2. Fix in 4% paraformaldehyde at 4°C for at least 4 hrs or overnight.
3. Wash in PBS, 4°C for 30 minutes.
4. Treat it with 0.85% saline: 4°C, 30 minutes.
5. Wash in Saline/Ethanol (1:1): RT for 15 minutes.
6. Wash in 70% Ethanol: RT, 15 minutes. Repeat it once.
7. Wash in 85% Ethanol and 95% Ethanol at RT, 30 minutes each
8. Wash in 100% Ethanol: RT, 30 minutes. Repeat it once.
9. Store at 4°C or continue.
10. Wash in 100% xylene: RT, 30 minutes. Repeat it once.
11. Treat with xylene/paraffin (1:1): 60°C, 45 minutes.
12. Treat with 100% Paraffin: 60°C, 20 minutes. Repeat it two more times.
13. Embed in embedding molds.

5. Sectioning

1. Prepare 15 µm sections of the embedded eye using a microtome.
2. Place the eye sections on a gelatin coated slide and leave it on a slide warmer.

6. Staining

1. Place slides in xylene for 10 minutes. Repeat it once more.
2. Hydrate in: 100%, 95%, 80%, 70%, 30% ethanol for 1 minute each
3. Rinse in DI water for a minute.
4. Wash in PBS for 15 minutes.
5. Wash in PBST (0.2% Triton-X 100 in PBS) for 15 minutes.
6. Wash in PBS for 15 minutes.
7. Place in blocking solution (10% goat secondary antibody) for an hour.
8. Place in primary antibody for overnight.
9. Wash primary antibody in PBS for 15 minutes.
10. Wash in PBST for 15 minutes.
11. Wash in PBS for 15 minutes.
12. Place in secondary antibody for 2 hours in dark (1:100 dilution in PBS).
13. Wash in PBS, PBST, and PBS for 15 minutes each and then mount.
14. Observe the slides under a fluorescence microscope.

7. Representative Results:

This procedure of culturing newt iris cells has been utilized to study the regeneration potential of the dorsal and the ventral IPE cells. Moreover, it is also possible to study specific genes that contribute towards the lens regeneration mechanism in newt eye. When the cells have been cultured for 2 weeks (**Figure 1**) they can be transfected by genes to examine their function in lens regeneration. Of particular interest are genes that might induce the ventral iris. Since the ventral iris cells cannot transdifferentiate to lens (**Figure 2**) the inductive function of a candidate gene can be studied. In the past, using this technique we have shown that when *six-3*was over expressed in the presence of retinoic acid lens induction was observed from the ventral iris [6]. In **Figure 3**we can see that the ventral iris aggregate gave rise to a fully grown and differentiated lens (arrowhead), not different than the host lens from the dorsal iris (arrow).

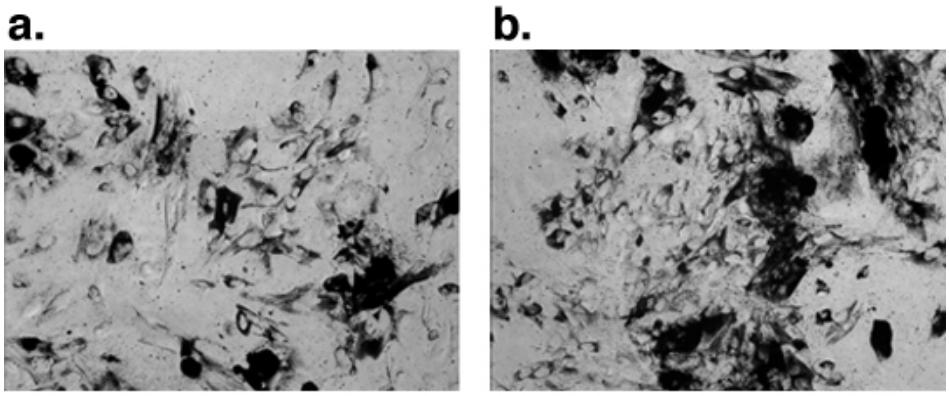

Figure 1. a) Dorsal iris pigmented epithelial cells cultured *in vitro* for a period of 2 weeks. b) Ventral iris pigmented epithelial cells cultured *in vitro* for a period of 2 weeks. Note that pigmentation persists to this stage in both dorsal and ventral iris cells.

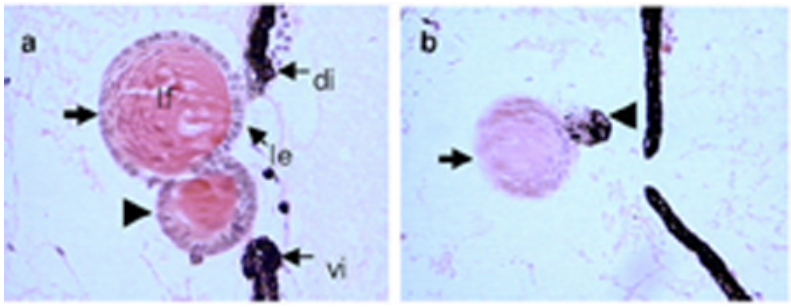

Figure 2. Regeneration ability of cultured IPE cells. a) Lens induction from dorsal IPE cell aggregate (arrowhead). Host lens induction from dorsal iris (arrow), di: dorsal iris, vi: ventral iris, le: lens epithelium, lf: lens fibers. b) Absence of lens induction from ventral IPE cell aggregate (arrowhead). Host lens induction from dorsal iris (arrow).

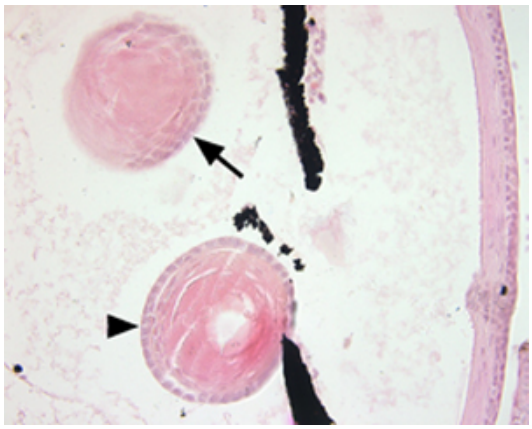

Figure 3. Lens induction from ventral aggregate transfected with six-3 and treated with retinoic acid. Host lens induction from dorsal iris (arrow). Lens induction from ventral aggregate (arrowhead).

Discussion

This protocol has established an *in vitro* system to study lens regeneration mechanisms in newts. Since the aggregates (either dorsal or ventral faithfully follow their *in vivo* behavior during regeneration this technique can alleviate the tremendous effort required for transgenesis in newts and can be used for gain of function as well as loss of function experiments [7,8,9]. Also, the aggregates or the irises as a whole can easily be treated with growth factors and examine their effects as described in this protocol. For example the role of BMP pathway has been studied using this technique [6].

Disclosures

No conflicts of interest declared.

Acknowledgements

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