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Fate Tracing of *neurogenin2*-Expressing Cells in the Mouse Retina Using CreERTM: LacZ

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Abstract

Delineating the final fate of progenitor cells that transiently express a regulatory gene may shed light on how the gene participates in regulating retinal development. We describe the steps in tracing final fates of progenitor cells that once transiently express *neurogenin2* (*ngn2*) during mouse retinal development with the binary, conditional Ngn2-CreERTM—LacZ reporter system. Ngn2-CreERTM mice (Zirlinger et al. Proc Natl Acad Sci USA 99:8084–8089, 2002), in which *ngn2* promoter drives the expression of Cre-estrogen receptor CreERTM (Littlewood et al. Nuc Acid Res 23:1686–1690, 1995; Hayashi and McMahon Dev Biol 244:305–318, 2002), are crossed with Rosa26-LoxP-LacZ reporter mice (Soriano Nat Genet 21:70–71, 1999), in which the expression of *lacZ* requires the removal of “stop” by Cre recombinase (Wagner et al. Transgenic Res 10:545–553, 2001). 4-hydroxytamoxifen (4-OHT), a synthetic ligand with high affinity for ERTM, is administered to double transgenic embryos and/or neonatal mice. Binding of 4-OHT to Cre-ERTM activates Cre recombinase, which then catalyzes the removal of the “stop” sequence from the LoxP-LacZ transgene, leading to *lacZ* expression in cells that express *ngn2*. Retinal tissues are fixed at different time points after 4-OHT treatment and analyzed for LacZ activities by colorimetric reaction. Double-labeling with a cell type-specific marker can be used to define the identity of a LacZ⁺ cell. Combining persisted *lacZ* expression through the life of the cell and the short half-life (0.5–2 h) of 4-OHT (Danielian et al. Curr Biol 8:1323–1326, 1998), this system offers the opportunity to track the final fates of cells that have expressed *ngn2* during the brief presence of 4-OHT administered during retinal development.

Keywords

Retinal neurons; Progenitor cells; Differentiation; Cell fate tracing; Cre-LoxP; Cre-ERTM; ROSA26

1. Introduction

One problem often encountered in studying genes that regulate cell fate specification is their elusive association with the cell type that they regulate, because of their expression often being confined to progenitor/precursor cells and becoming no longer detectable once the cells have differentiated sufficiently to exhibit their terminal identities. With the development of Cre-mediated conditional DNA recombination (1–3), a bipartite genetic-system based lineage-tracing emerged and has been used to produce valuable information on the final fates of progenitor cells that transiently express a gene of interest. We have used the binary, conditional Ngn2-CreERTM—LacZ reporter system to map the fate of *ngn2*-expressing cells in the developing mouse retina (4). In Ngn2-CreERTM mice, generated by Zirlinger et al. (5), the coding sequence of *ngn2* is replaced with CreERTM. CreERTM is a fusion protein of Cre recombinase with the hormone-binding domain of a mutant mouse

estrogen receptor ERTM (6, 7), which fails to bind the naturally released ligand 17 β -estradiol at normal concentrations but retains relatively high affinity for the synthetic ligand 4-hydroxytamoxifen (4-OHT) (6–8). For LacZ reporter mice we used ROSA26, which was created by transgenesis with a construct of LoxP-stop cassette/Neo-LoxP-LacZ (9). In ROSA26 mice, *lacZ* is not expressed and its expression requires Cre-mediated removal of the “stop” sequence from the DNA cassette (2).

Because Ngn2-CreERTM homozygotes often die soon after birth, heterozygous Ngn2-CreERTM ^{+/-} mice are crossed with homozygous ROSA26-LacZ reporter mice. 4-OHT is administered to double transgenic embryos (through pregnant mice) and to neonates to activate Cre recombinase in *ngn2*-expressing cells. The activated Cre recombinase removes the “stop” sequence, a cell-heritable DNA rearrangement event, from the LoxP-stop cassette/Neo-LoxP-LacZ, leading to the persistent activation of the reporter LacZ in the progeny of cells transiently expressing *ngn2*. Since the 4-OHT has a half-life of only 0.5–2 h in vivo (5, 10), the activation of CreERTM is further restricted to a relatively narrow time window after the ligand is injected. Thus, the final expression pattern of the *lacZ* will exclusively identify the progeny of cells expressing *Ngn2-CreER*TM during this time window, and will not include cells that express *Ngn2* at either earlier or later times (5).

2. Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Adhere to policies and regulations when disposing waste materials. We do not add sodium azide to solutions prepared in our own laboratory.

2.1. Mice

Ngn2-CreERTM mice: provided by Dr. David Anderson (Caltech); Rosa26-LoxP-LacZ reporter mice: obtained from the Jackson Laboratory (Stock #003310, 129s-Gt(ROSA)26Sor) (see Note 1).

2.2. Reagents for Genotyping

1. Digestion buffer. Add 5 ml of 5 M NaCl, 2.5 mL of 1 M Tris–Cl (pH 8.0), 50 mL of 0.5 M EDTA (pH 8.0), 5 mg of pancreatic RNase, 12.5 mL of 10% SDS, and adjust volume to 250 mL with H₂O. Store at 4°C.
2. Proteinase K: 20 mg/mL in PBS. Store at –20°C.
3. 10 M ammonium acetate (NH₄Ac).
4. Phenol chloroform (pH 8.0): Phenol:chloroform:isoamyl alcohol mixture(25:24:1) (see Note 2).
5. Ethanol: 100% and 70% ethanol.
6. 5 M betaine.
7. 10× TE. Add 100 mL of 1 M Tris–HCl, pH 7.5, 20 mL of 0.5 M EDTA, pH 8.0, 880 mL of H₂O, and mix. TE: mix 100 mL of 10× TE with 900 mL of H₂O. Aliquot into 15-mL and 50-mL tubes.

¹Several reporter lines are available. The choice of a particular mouse line depends on the detection method an investigator plans to use.

²Commercial sources often provide phenol/chloroform solutions of different pH. Remember to use the pH 8.0 one for DNA purification, as the pH 4.5 one is for RNA extraction.

8. 0.5 M EDTA, pH 8.0. Add 186.1 g of disodium ethylenediamine tetraacetate-2H₂O to 800 mL of H₂O, stir vigorously, and adjust the pH to 8.0 with NaOH. EDTA will slowly dissolve as the pH approaches 8.0. Adjust the volume to 1 L with H₂O and sterilize the solution by autoclaving.
9. TAE buffer (50×). Add 121 g of Tris base into 250 mL of H₂O, stir to dissolve, add 28.6 mL of 1 N acetic acid, 50 mL of 0.5 M EDTA (pH 8.0), and adjust the volume to 500 mL with H₂O.
10. Loading buffer (6×) (Sigma).
11. Ethidium bromide (Sigma).
12. 1% Agarose gels. Add 0.75 g of agarose to 75 mL of 1× TAE buffer and microwave for 2–3 min. Cool the melted agarose for 5 min at room temperature. Add 5 µL of ethidium bromide, mix by swirling, and pour into a mid-size gel casting tray with comb. Let gel solidify at room temperature for about 20–30 min (see Note 3).
13. HotStartTaq Plus Master Mix Kit (1000) (Qiagen) (see Note 4).
14. 1 kb DNA ladder mix.
15. Oligonucleotides. Ngn2-CreERTM: Cre: 5′ atccgaaaagaaacgttga 3′ and 5′ atccaggttacggatagatg 3′ (see Note 5); ngn2: 5′ ctgagactctggagttgaag 3′ and 5′ ctagatacagtcctggcg 3′ (see Note 6). ROSA26: 5′ gcgaagagttgtcctcaacc 3′, 5′ aaagtcgctctgagttgttat 3′, and 5′ ggagcgggagaaatggatgtg 3′ (see Note 7). Internal control GAPDH (glyceraldehyde-3-phosphate dehydrogenase): 5′ catgaccacagtcctcatgcat 3′ and 5′ cctctctgtcctagtcctt 3′ (see Note 8).

2.3. Materials for Tissue Preparation

1. 4-OHT solution (10 mg/mL). Dissolve 25 mg of 4-OHT with 500 µL ethanol first, then mix dissolved 4-OHT with 2 mL of autoclaved sun flower seed oil (Sigma, Cat#: S5007) to 10 mg/mL, and sonicate for approximately 20 min. Aliquot out, wrap with foil, and store in −20°C. Thaw the solution completely before using (see Note 9).
2. Specimen molds (Tissue-Tek).
3. Superfrost/Plus glass slides (Fisher Scientific).
4. 22 × 50 mm cover glass.

³1% agarose gel works well for separating DNA fragments of >500 bp. If the size of PCR products is smaller than 500 bp, use a 2% or 3% agarose gel.

⁴Investigators may choose any commercially available PCR reaction mixture. If a laboratory wishes to prepare its own, following this formula: 2 µL of 10× PCR Buffer (usually comes with Taq polymerase), 2 µL of 2 mM dNTP, 1 µL of 25 mM MgCl₂ (final concentration to 1.5–2.5 mM), 1 µL of DMSO, 4 µL of 5 M betaine, 2 µL of oligonucleotides, 1 µL of Taq polymerase (20,000 U/mL), and 7 µL of H₂O (to a total of 20 µL).

⁵Cre oligonucleotides: Cre Expression Vector PTN75 CRE (NCBI: AB542060.1), sequence site: 1,871–2,500, a total length of 630 bp.

⁶Ngn2 oligonucleotides: mouse *ngn2*, NCBI: NM_009718, sequence site: 328–1,106, a total length of 779 bp.

⁷Information on the primers and PCR conditions to verify the Rosa26-loxP-LacZ reporter genotypes is provided by Jackson laboratory.

(http://jaxmice.jax.org/protocolsdb/f?p=116:2:1946125058970626::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:4615,003309).

⁸GAPDH (glyceraldehyde-3-phosphate dehydrogenase) oligonucleotides: NCBI: NM_008084, sequence site: 566–1,098, a total length of 533 bp.

⁹4-OHT does not dissolve in sun flower seed oil readily. The cloudy suspension needs to be sonicated in a sonicator bath or with sonicator probe until the cloud disappears.

5. X-Gal solution (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside). Add 10 mL of dimethyl sulfoxide (DMSO) to 500 mg of X-gal to make 50 mg/mL solution. Aliquot out, wrap with foil, and store at -20°C .
6. X-gal rinsing buffer: 0.2 M sodium phosphate, pH 7.3, 2 mM magnesium chloride, 0.02% NP-40, and 0.01% sodium deoxycholate.
7. X-gal staining buffer: X-gal rinsing buffer plus 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide. Wrap the container with foil and store in 4°C . Before staining, add X-gal to a final concentration of 1 mg/mL (see Note 10).
8. ImmEdge Pen (Vector, cat#H-4000).
9. PBS (Phosphate Buffered Saline). Dissolve the following in 800 mL of H_2O : 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 , 0.24 g of KH_2PO_4 . Adjust pH to 7.4 and the final volume to 1 L with H_2O . Sterilize by autoclaving.
10. 0.2 M phosphate buffer (PB) (pH 7.4). Dissolve 6.4 g NaH_2PO_4 and 21.8 g Na_2HPO_4 into 1 L of H_2O .
11. PBST: 0.1% (V/V) Tween-20 in PBS.
12. 4% Paraformaldehyde solution. Dilute 16% paraformaldehyde solution (EMS) with 0.2 M PB (see Note 11).
13. 1% Triton-PBS: 1% (V/V) of Triton-X100 in PBS. 0.2% Triton-PBS: 0.2% (V/V) of Triton-X100 in PBS.
14. 20% Sucrose: 20% (W/V) sucrose in PBS.
15. OCT:Sucrose solution. Mix 1 volume of OCT with 2 volumes of 20% Sucrose (see Note 12).
16. Maleic Acid buffer (100 mM Maleic Acid, 150 mM NaCl, pH 7.5): Dissolve 11.61 g of maleic Acid and 8.76 g of NaCl into 900 mL of H_2O . Adjust pH to 7.4 with 7.5 g solid NaOH, plus 30–50 drops of 10 N NaOH. Bring the volume to 1 L with H_2O , and autoclave.
17. Blocking reagent. Stock solution (10%): dissolve blocking reagent (Roche) with maleic acid buffer for a 10% (w/v) concentration, autoclave, aliquot into 50-mL tubes, and store in -20°C . Working solution (2%): diluted with maleic acid buffer.
18. Mounting solution: Vector Mount AQ.

2.4. Equipment and Instruments

1. 37°C oven.
2. $55\text{--}60^{\circ}\text{C}$ water bath.
3. Dissecting microscope.
4. Dissection instruments: curved and straight fine forceps, microsurgery scissors.
5. Microwave.

¹⁰X-gal staining buffer is light sensitive. Protect it from the light with foil and store 4°C . Before staining, add X-gal to 1 mg/mL final concentration.

¹¹4% paraformaldehyde: dilute 10 mL of 16% paraformaldehyde solution with 30 mL of 0.2 M PB, mix well, and leave it on ice for 30 min before using.

¹²OCT:Sucrose (1:2). In a 500-mL clear container, combine one bottle of OCT with two bottles of 20% sucrose solution (using the OCT bottle), and mix well. Allow the air bubbles to dissipate before using.

6. PCR machine.
7. Electrophoresis unit.
8. Microcentrifuge for 1.5-mL tubes.
9. Sonicator.
10. Syringes with a 29/30 gauge needle.
11. Cryostat.
12. Research microscope with camera.
13. UV light/camera system for taking pictures of DNA on agarose gel.

3. Methods

3.1. Genotyping

1. Collect 0.5–1 cm of mouse tail and place it in a 1.5-mL micro-centrifuge tube (see Note 13).
2. Add 200 μ L of the digestion buffer and 20 μ L of Proteinase K solution.
3. Mix well and incubate in a 55–60°C water bath for 3 h or overnight.
4. Take samples out and leave it at room temperature for 30 min to equilibrate to room temperature.
5. Add 200 μ L phenol/chloroform, mix gently by inverting the tube several times, followed by centrifugation 12,000 g (12,000 rpm) for 10 min (see Note 14).
6. Transfer 100 μ L of the top aqueous phase (containing DNA) to a new 1.5-mL tube (see Note 15).
7. To the DNA add 100 μ L of phenol/chloroform to extract one more time.
8. Centrifuge at 12,000 g (12,000 rpm) for 10 min. Transfer ~90 μ L of the top aqueous phase (containing DNA) to a new 1.5-mL tube.
9. Add 1/10th volume of 10 M ammonium acetate.
10. Add 2.5 volume of 100% ethanol, swirl gently, and leave on ice for 30 min, then centrifuge 12,000 g (12,000 rpm) for 12 min.
11. Discard the supernatant and wash pellet two times with 0.5 mL of 70% ethanol with centrifugation 12,000 g (12,000 rpm) for 5 min each (see Note 16).
12. Air-dry the DNA for 3–5 min and dissolve the DNA in 60–100 μ L of H₂O (or TE) (see Notes 17 and 18).

¹³The mice tail snips can be stored at –20°C.

¹⁴Don't vortex the tube after phenol/chloroform is added to avoid shearing of genomic DNA. Instead, mix the content by inverting the tube several times. The phenol/chloroform is toxic. The worker should wear the glove and masks, operate in a fume hood, and dispose waste properly.

¹⁵After centrifugation, hold the tube at the angle similar to its position in the centrifuge to avoid mixing of its contents. Slowly take out 100 μ L of the top transparent, aqueous phase with a 200- μ L micropipette into a new 1.5-mL microcentrifuge tube.

¹⁶Care should be taken when discarding the 70% ethanol in the tube after centrifugation. We recommend slow aspiration to avoid accidentally losing the DNA pellet.

¹⁷Do not overdry DNA pellet. Overdried DNA pellet is difficult to dissolve. The DNA can be dissolved in H₂O if used immediately; otherwise dissolve the DNA in TE.

¹⁸This method of genomic DNA isolation from mouse tail snips works well in our hand. However, investigators may choose to use a commercially available genome DNA isolation kit.

13. Set up PCR (final volume 20 μ L). For Cre/ngn2/GAPDH: add 10 μ L of 2 $\times$ HotStartTaq Plus Master Mix, 7 μ L of H₂O, 1 μ L of each of the two oligonucleotides, and 1 μ L of genome DNA. Mix. Modification for PCR of LacZ: 6 μ L H₂O and 3 μ L oligonucleotide mix.
14. PCR condition: 95°C for 5 min, 56°C/60°C for 1 min, 72°C for 1 min, 34 cycles of “95°C for 15 s, 56°C/60°C for 1 min, 72°C for 1 min” 72°C for 5 min, and 12°C for holding. Modification for PCR of LacZ: annealing at 65°C (see Note 19).
15. Analyze PCR products with agarose gel electrophoresis and taking pictures under UV light (see Note 20).

3.2. Mouse Breeding and Sample Collection

All experimental procedures involving the use of animals must be approved by institutional animal use and care committee.

1. Breed Ngn2-CreERTM mice and select the heterozygous after genotyping to establish a colony.
2. Verify Rosa26-LoxP-LacZ (+/+) reporter mice by PCR genotyping and breed to establish a colony (see Note 21).
3. Cross heterozygous Ngn2-CreERTM mice with Rosa26-LoxP-LacZ homologous mice to generate embryos that are Ngn2-CreERTM (+/-)/Rosa26-LoxP-LacZ (+/+) (Fig. 1). The pregnant mice and the neonatal mice are used for experiments (see Note 22).
4. Time pregnancies. The age of embryos is determined by designating as embryonic day 0.5 (E0.5) when the vaginal plug is observed at midday (see Note 23).
5. Inject 200–500 μ L of 4-OHT (10 mg/mL) intraperitoneally (5 mg/20 g weight) with 29 gauge needle attached to a syringe into pregnant mice with embryos at a desirable age, from E10 to E20, or neonatal mice between postnatal day 1 (P1) to day 14 (P14). Include multiple (3–5) mice for each developmental stage (see Note 24).
6. At P17–P20, sacrifice the mice with CO₂. Collect tail snips for genotyping (see Note 25).

¹⁹Choose annealing temperature by considering both the T_m (melting temperature) of the oligonucleotides and the results of trial tests. Usually, annealing temperature is around 4–6°C below the T_m of the nucleotides. The T_m of the oligonucleotides described in this chapter is ~60°C, but results from out trail tests with gradient temperature showed that 56°C is optimal for ngn2 and GAPDH, and 60°C for Cre, and 65°C for LacZ.

²⁰Anticipated Ngn2-CreERTM DNA genotyping results: heterozygote, PCR positive for Cre and for Ngn2; homozygote, PCR positive for Cre only; WT, PCR positive for Ngn2 only. Anticipated ROSA-LacZ DNA genotyping results: heterozygote, PCR products of 340 bp and 650 bp; homozygote, 340 bp only; WT, 650 bp only. Labeling the mice according to the PCR results with ear marker or ear wholes punch for the colonies' amplification.

²¹The 129 s-Gt(ROSA)26Sor mice from Jackson Lab are from cryopreserved embryos and have at least 1129 s-Gt(ROSA)26Sor transgenic (<http://jaxmice.jax.org/strain/003310.html>). Upon receiving them, genotyping is needed to identify the positive ones, which can be amplified to get the more 129 s-Gt(ROSA)26Sor(+/-) mice. Crossbreeding these 129 s-Gt(ROSA)26Sor(+/-) mice is carried out to generate a sufficient number of homozygous reporter mice.

²²Crossing heterozygous Ngn2-CreERTM mice will generate 1/4 homozygous, 1/2 heterozygous, and 1/4 of wild type.

²³The age of pregnancies is important for data analysis and interpretation. We check the mice two times a day to determine whether the vaginal plug is present because the vaginal plug may remain only for 16–48 h.

²⁴Working 4-OHT solution is prepared fresh each time. 4-OHT is absorbed by abdominal omentum majus and distributed to the entire body. Studies show that 4-OHT-mediated Cre recombination is dose dependent (7). Thus, sufficient amount of 4-OHT (no less than 3 mg/20 g body weight) should be injected into each animal to activate *lacZ* expression. Otherwise, X-gal staining may be too weak to be detected. A pregnant mouse receives 500 μ L of 4-OHT (10 mg/mL) and a neonatal mouse (P1–P14) receives 100–200 μ L of 4-OHT.

²⁵We sacrifice all 4-OHT-treated mice at P17–P20, when the retina has structurally and functionally matured.

7. Enucleate the eyes with a pair of curved forceps and place the eyes in a dish with ice-cold PBS.
8. Remove the cornea, the lens, and the vitreous. Fix the eye cup with ice-cold 4% paraformaldehyde for 30 min (see Note 26).
9. Rinse the eye cup twice with PBS, followed by two washes with X-gal rinsing buffer for 10 min each. Add X-gal staining buffer and incubate overnight with gentle shaking in dark (see Note 27).
10. After three washes with PBS, post fix the sample for 30 min with 4% paraformaldehyde, followed by three washes with PBS.
11. Embed the samples with OCT: sucrose (1:2) in specimen molds, and freeze the sample with liquid nitrogen or dry ice. Store the frozen samples at -80°C (see Note 28).
12. Section the sample with a cryostat, and collect 5–12- μm retinal cryosections onto Superfrost/Plus glass slides.
13. Look for blue colored (LacZ⁺) cells under a microscope (Fig. 2).
14. Score the number of LacZ⁺ cells, the total number in a retinal section as well as in each individual retinal nuclear layer (i.e., the outer nuclear layer, the inner nuclear layer, and the ganglion cell layer).
15. If needed, continue with immunohistochemistry staining to confirm and/or determine the identities of the LacZ⁺ cells. An example image of such double-labeling is shown in Fig. 2.

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²⁶Before fixing, place the enucleated eye balls immediately into a 35-mm dish with ice-cold PBS. Punch a hole on the cornea with 29/30 gauge needle attached to a syringe and cut the cornea out behind the limbus with microsurgery scissors under a dissecting microscope. Remove the lens and vitreous, and transfer the eye cup (retina + RPE) immediately into a small vial with fixation solution.

²⁷X-gal is light sensitive. Stock solution and samples in staining solution should be covered with foil. If staining is not satisfactory, replenish with fresh X-gal staining solution and continue the incubation for color development. Include positive control to rule out that negative results are due to improper X-gal detection. The postfix after staining helps to maintain the blue color and tissue histology during subsequent processes.

²⁸Samples are frozen with OCT:Sucrose (1:2) using either dry ice or liquid nitrogen. In case of liquid nitrogen, freeze the samples slowly to avoid cracking of the block and for easy cryosectioning.

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Fig. 1.
The overall experimental scheme. LacZ is only expressed [LacZ(+)] in cells that once expressed *ngn2* in the Ngn2-Cre(+)/lacZ-LoxP(+) double transgenic mice.

Fig. 2.

The distribution and identifies of LacZ⁺ cells in mouse retinas with 4-OHT administered at different time. (a) P18 retina with 4-OHT administered at P4. The majority of LacZ⁺ cells (blue; arrowheads) are localized within the outer nuclear layer, indicating that most of the progenitor cells expressing *ngn2* at P4 will late differentiate into photoreceptors. (b) P17 retina with 4-OHT administered at E15. The Arrow points to a LacZ⁺ cell also positive for a ganglion cell marker Brn3A, indicating this ganglion cell came from a cell expressing *ngn2* at E15. (c) P16 retina with 4-OHT administered at E18. The Arrow points to an amacrine cell (LacZ⁺/AP2α⁺) derived from a progenitor expressing *ngn2* at E18. ONL outer nuclear layer, INL inner nuclear layer, GCL ganglion cell layer.