

Photoablation of Cells Expressing β -Galactosidase

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1. Introduction

Cell ablation can be a powerful technique for studying development. By systematically ablating cells from a developing tissue, one can gain insight into how the cells interact with each other to form the mature structure. Here we describe a new method for performing ablations. This method can be applied to individual cells or whole classes of cells and works well both in vitro and in vivo.

The general procedure is to genetically engineer the cells of interest to express the gene for the enzyme β -galactosidase (β -gal). The β -gal-expressing cells are then labeled with a dye (fluorescein) by treating them with a fluorogenic β -gal substrate, fluorescein-di- β -D-galacto pyranoside (FDG) (Molecular Probes, Inc., Eugene, OR). Once labeled, the cells can be photoablated by illuminating the tissue in the presence of a sensitizing agent, 3-amino-9-ethyl-carbazole (AEC) (Sigma Chemical Co., St. Louis, MO). Light activation of the fluorescein in the presence of the sensitizing agent triggers a chemical reaction that both kills the cells and marks them with a colored product.

This method has been tested on several different β -gal-expressing cell types in the mouse retina and cerebral cortex both in vitro and in vivo and found to ablate >90% of the targeted cells with <2% nonspecific cell death (**1**). Two tests were used to assess cell death. First, cells were scored for the selective uptake of dyes excluded from live cells, ethidium homodimer, and trypan blue (**Fig. 1A–C**) (**2,3**). Second, the tissue containing the β -gal-expressing cells was examined several days after the ablation treatment to assess the loss of the cells (**Fig. 1D**).

This chapter outlines protocols for ablating cells in the mouse retina in vitro and in vivo and in the cerebral cortex in vivo. It also includes methods for examining the effectiveness of the treatment. These protocols should be applicable to most other tissues as well.

2. Materials

1. Saline solutions:

- a. Phosphate-buffered saline (PBS), pH 7.4: 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 , 0.2 g KH_2PO_4 in 1 L distilled water.
- b. L-15 medium (Sigma Chemical Co., St. Louis, MO)
- c. Ringer's: 110 mM NaCl, 2.5 mM KCl, 1 mM CaCl_2 , 1.6 mM MgCl_2 , 10 mM D-glucose, buffered with 22 mM NaHCO_3 , 5% CO_2 /95% O_2 , pH 7.4.

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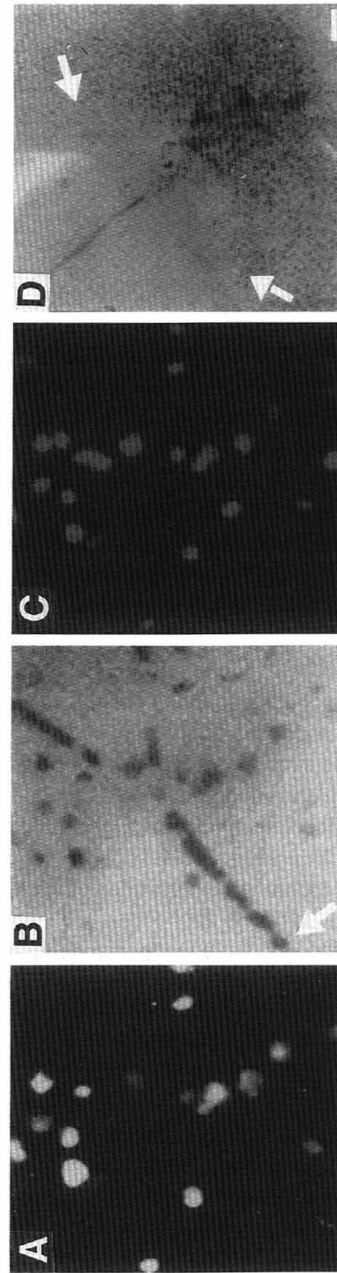


Fig. 1. (See color plate 12 appearing after p. 258.) Assays for cell ablation: ethidium homodimer assay and X-gal assay. (A)–(C) View of a retina whole mount from a transgenic mouse line that expressed β -gal in a population of retinal interneurons. (A) The retina after treatment with FDG/AEC solution. The colored AEC product appeared in the β -gal-expressing cells. (B) The same retina after 5 min of illumination. The colored AEC product appeared in the fluorescein-labeled cells. *Arrow* indicates a blood vessel (to be distinguished from β -gal-expressing cells). (C) The same retina after treatment with ethidium homodimer. Approximately 4 h after ablation treatment, the AEC-labeled cells showed uptake of ethidium homodimer. (D) View of a retina whole mount stained with X-gal 1 wk after ablation treatment was applied to a region of the retina in vivo. *Arrows* indicate the borders of the region, which show a clear absence of X-gal-stained cells.

2. Fluorescein-di- β -D-galactopyranoside (FDG; Molecular Probes) stock solution: 130 mg/mL in dimethyl sulfoxide (DMSO). Store in 5–10- μ L aliquots at -20°C .
3. 3-aminoethyl-9-ethylcarbazole (AEC; Sigma) stock solution: 20 mg/mL in DMSO. Store in 50- μ L aliquots in foil-covered tubes at -20°C .
4. FDG/AEC solution #1: 1 part FDG stock, 1 part AEC stock, 15 parts DMSO, 3 parts L-15 or Ringer's, which gives a final concentration of 6.5 mg/mL FDG, 1 mg/mL AEC in 85% DMSO/15% L-15. Make fresh before use.
5. FDG/AEC solution #2: 1 part FDG stock and 1 part AEC stock, which gives a final concentration of 65 mg/mL FDG and 10 mg/mL AEC. Make fresh before use.
6. Ethidium homodimer (Molecular Probes): 30 $\mu\text{g/mL}$ in PBS. Can be stored at -20°C for <1 yr.
7. Paraformaldehyde fixative: Add 4 g of paraformaldehyde (e.g., Sigma) to 80 mL distilled water with 50–100 μL of 10 *N* NaOH (used to help dissolve the paraformaldehyde). Heat to 60°C , then cool to room temperature, add 10 mL 10X PBS and bring final volume to 100 mL with distilled water. Bring pH to 7.4 with HCl. Can be stored at 4°C for approx 3 d.
8. Glutaraldehyde fixative: 0.5% in 1X PBS buffer. Dilute from a 25% stock (Sigma). Stock can be thawed and frozen many times. Make dilution immediately before use.
9. X-Gal detection buffer (Sigma): 35 mM potassium ferrocyanide, 35 mM potassium ferricyanide, 2 mM MgCl_2 , 0.02% Nonidet P-40, which is also called Igepal CA-630 (made from a 10% stock solution), and 0.01% Na deoxycholate (made from a 10% stock solution) in PBS. X-Gal detection buffer can be stored for <1 yr at room temperature in foil-covered container.
10. X-Gal stock (50X): 40 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) in dimethyl formamide. Store at -20°C in a glass container covered with foil.
11. X-Gal reaction mix: Add 1:49 X-gal stock:X-gal detection buffer. Make fresh before use.

3. Methods

3.1. Photoablating β -Gal-Expressing Cells in Retina Whole Mounts In Vitro

1. Loading β -gal-expressing cells with FDG and AEC: Remove retina from animal and place on a glass cover slip in a small volume of L-15 medium or Ringer's. Use just enough to cover the tissue. Remove all excess liquid to make retina lie flat (*see Note 1*). Apply a 2–3- μL drop of FDG/AEC solution #1 on the tissue, then immediately dilute the FDG/AEC solution with a 50-fold vol (100–150 μL) of L-15. Wash 3X with L-15, using a 100 μL vol each time, then leave the retina in 100 μL L-15 for 10 min (*see Notes 2 and 3*). After 10 min, you should be able to see the β -gal cells labeled with fluorescein. They can be visualized using a standard fluorescence microscope with a fluorescein filter set (e.g., a Zeiss Axiophot with a 100-W mercury short arc lamp and a filter set containing a 450–490-nm exciter filter, a 510-nm dichroic filter, and a 520–560-nm barrier filter.)
2. Photoablating the cells: Illuminate the region of the tissue that contains the β -gal-expressing cells of interest. Focus on the cells with a 10X, 0.3 N.A. objective and illuminate for 3–15 min. Light activation of the FDG triggers a reaction that causes the colorless AEC to form a brown precipitate in the cells. This precipitate should be visible under brightfield illumination (*see Notes 4 and 5*). Once the cells have formed the brown product, return the tissue to a culture dish with fresh media.

3.2. Photoablating β -Gal-Expressing Cells in the Retina In Vivo

1. Loading β -gal-expressing cells with FDG and AEC: Inject the eye of an anesthetized mouse with 0.5–1.0 μL of FDG/AEC solution #2. Assuming the vitreal volume is 5–10 μL ,

the solution will be diluted 10–20-fold in the eye. To make the injection, first fill a 10- μ L blunt-tipped 33-gage Hamilton syringe with the FDG/AEC solution. Then create a small hole in the cornea to allow the syringe to enter the eye. This can be done by puncturing the cornea with the tip of a 30-gage needle. Then insert the syringe needle into the hole and inject the dye into the vitreous humor. The fluorescein-labeled cells can be visualized by placing the mouse on the microscope stage so that the microscope objective is focused through the optics of the eye onto the retina. You can reduce the corneal refraction by placing a glass cover slip on the animal's cornea (*see Note 6*).

2. Photoablating the cells: Focus on the labeled cells and illuminate the retina for 8–10 min with the 10X, 0.3 N.A. objective, as described above in **Subheading 3.1**.

3.3. Photoablating β -Gal-Expressing Cells in the Cortex In Vivo

1. Loading β -gal-expressing cells with FDG and AEC: Cut open the skin above the brain area in the anesthetized mouse. Reflect back or remove the bone. Using a 30-gage needle, make tiny perforations in the dura to permit drug entry into the tissue. Apply FDG/AEC solution #1 to the surface of the brain and allow 10 min for penetration.
2. Photoablating the cells: Focus on the labeled cells and illuminate the retina for 8–10 min with the 10X, 0.3 N.A. objective as described in **Subheading 3.1**. (*see Note 7*). Replace the bone and close the skin as described in NIH guidelines for survival surgery.

3.4. Ethidium Assay for Cell Ablation

1. Ethidium homodimer loading: This is for use on live tissue. After ablation treatment, place retina on cover slip and remove all excess liquid to make retina lie flat. Apply 2–3 μ L of ethidium homodimer solution on the retinal surface (to drive the dye into the tissue (*see Note 1*), then immerse the retina in 2–3 mL of ethidium homodimer solution and place at 37°C for several hours (*see Note 8*).
2. Viewing ablated cells: Examine whether the cells that contain the AEC precipitate also show ethidium-labeling. View the AEC-label with brightfield and the ethidium fluorescence with a 630–640-nm excitation filter (*see Note 9*).

3.5. X-Gal Assay for Presence (or Loss) of β -Gal-Expressing Cells

1. Fixing retina: Fix retina flat on a cover slip by immersing it in a small volume (just enough to cover tissue) of 4% paraformaldehyde solution for 5 min. Transfer retina to a small dish (e.g., a well in a 24-well dish) and continue to fix for 10 min.
2. Washing retina: Remove fix and wash 5X, each time for greater than 5 min with 2 mL of PBS.
3. Detecting β -gal activity using X-gal: Remove PBS from last wash and add 0.5–1 mL of X-gal reaction mix, incubate at 37°C for several hours or overnight. Remove X-gal reaction mix and wash 3X, each time with 2 mL of PBS. Examine for the loss of X-gal-stained cells by microscopy using standard brightfield illumination (*see Note 10*).

4. Notes

1. This flattening of the tissue may be necessary only for the retina and may be skipped when loading dyes into other tissues. Retina whole mounts are notoriously difficult to penetrate with dyes, presumably because of the inner limiting membrane.
2. Note the high concentrations of DMSO used to dissolve the FDG and AEC. These are the concentrations we used in our initial work with this method. Although no DMSO-induced cell death was detected in any of the tissues we examined (*1*), high DMSO concentrations have been reported to be teratogenic in some systems (*see Note 4*). Recently, we have had

success with much lower concentrations (1.5 mg/mL FDG, 0.035 mg/mL AEC in 2.5% DMSO/97.5% Ringer's) (4).

3. It is worth mentioning that this procedure works well for loading β -gal-expressing cells with FDG *in tissue*. We have had much less success loading cultured cells using a variety of procedures, including osmotic shock and varying the concentrations of FDG and DMSO.
4. It is difficult to provide a general set of conditions for effective and selective ablation, because cell types vary in their β -gal enzyme activity, position within the tissue, and so forth. For this reason, we are simply reporting conditions under which the ablation treatment was effective and produced little or no nonspecific cell death. These conditions can then serve as a guideline.

A convenient feature of the method is that AEC forms a visible precipitate during the ablation. Thus, one can vary conditions and use the formation of the AEC product as an assay for cell death. If the β -gal-expressing cells are relatively superficial in the tissue, the formation of the AEC precipitate can be watched as it occurs during the treatment. Alternatively, it can be seen by sectioning the tissue after treatment. We suggest a 4% paraformaldehyde fixation, followed by cryostat-sectioning. The AEC label fades within a few hours of fixation, so it is critical to examine the tissue quickly.

5. Note that fluorescein can cross membranes and accumulate in the tissue. In our experiments, the concentration of fluorescein in the tissue surrounding the β -gal-expressing cells was extremely low compared with the concentration inside the β -gal-expressing cells; thus, we saw no cell death in neighboring cells. However, fluorescein leak is a potential problem. It is a function of several factors, including promoter strength and cell density, so the extent of leak will vary with different β -gal-expressing cells. The effects of leakage can be minimized or avoided by illuminating the cells shortly after the application of FDG—before appreciable leak occurs. The leakage of fluorescein *after* illumination poses no threat, because fluorescein itself is nontoxic.
6. A drop of 1% atropine sulfate (Steris Laboratories, Phoenix, AZ) can be used to dilate the pupil to facilitate viewing the fluorescein-labeled cells.
7. One limitation of this technique is that cells located deep in tissue may be difficult to target using a fluorescence microscope as a light source. The reason for this is that the peak excitation wavelength of fluorescein is absorbed by endogenous tissue chromophores, such as flavins and hemoglobin, which prevent the light from reaching deep into tissue (5,6). Thus far, we have successfully targeted cells as deep as halfway through the cortical plate, approx 300–400 μ m below the surface. Cells located deeper in the brain might be targeted with fiber optics inserted into the ventricles.

Light accessibility is not limiting for embryos of transparent organisms, such as zebrafish. In preliminary experiments with zebrafish embryos, β -gal-expressing cells in deep structures, such as the heart, were readily ablated (1).

8. Ethidium homodimer labeling of AEC-labeled cells can take several hours. It is not clear whether or not this means that the cells take several hours to die. Rather, the slow labeling may reflect slow penetration of ethidium homodimer into the tissue (*see Note 1*). Support for the latter possibility was obtained from ablations performed in zebrafish, where uptake of ethidium homodimer into AEC-labeled cells occurred within minutes.
9. The 630–640 nm bandpass filter is used to view the ethidium fluorescence rather than the standard rhodamine barrier filter (590-nm long-pass), because fluorescein fluorescence can sometimes be detected through the rhodamine filter. The 630–640-nm filter completely blocks the fluorescein emission.
10. This assay should be used >36 h after ablation (when ablations are performed *in vivo*), because β -gal activity can remain for several hours to a day after cells are physiologically dead.

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