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Application of *lacZ* Transgenic Mice to Cell Lineage Studies

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1. Introduction

Cell lineage analyses trace the hierarchy of cell types derived from a progenitor population. Critical to these analyses is the ability to track reliably all or defined subsets of the clonal descendants of the progenitor population. This necessitates marking the cells with a heritable and cell autonomous marker. Transgenes encoding molecules that can be visualized directly *in situ* without compromising cell differentiation, such as the reporter, β -galactosidase encoded by *lacZ* and chloramphenicol acetyltransferase, encoded by the CAT gene are the most widely used. The *lacZ* can be readily detected using a sensitive histo-chemical assay such that cells in which the β -galactosidase gene is transcrip-tionally active produce a blue stain in tissue sections or in whole mounts (1). Some lineage studies demand the simultaneous detection of the *lacZ* product and other tissue-specific proteins or transcripts. In this chapter, we discuss the experimental strategies in which the *lacZ* transgene can be utilized in the analysis of cell lineages, and we detail assays for detecting β -galactosidase by X-gal histochemistry or immunological localization of the enzyme in combination with mRNA *in situ* hybridization, immunochemistry, and histochemical procedures, such as alkaline phosphatase staining.

1.1. Choosing the Appropriate Transgenic Animals for Lineage Analysis

The expression of the *lacZ* transgene varies according to the nature of the regulatory elements (promoter and enhancer) that drive gene expression, the number of active copies, and the chromosomal domain where the transgene is localized. The choice of transgene is dictated by the specific questions to be addressed. Several examples of transgenes give ubiquitous nonlineage biased expression. For example, in transgenic mice where the *lacZ* gene is regulated by the hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase promoter, the transgene is expressed in all lineages at high levels during development (2). Similarly, the ROSA- β -geo transgene produced by site-directed mutagenesis (3) and the *lacZ* reporter driven by rat β -actin promoter (4) also provide ubiquitous tagging of multiple cell lineages. Ubiquitously expressed transgenes provide the most ideal marker for unbiased cell lineage analyses, provided the lineage progenitors or founder cells to be studied can be isolated as a pure cell population.

³To reduce the background associated with endogenous β -galactosidase activity normally found in bone, kidney, and brain, the histochemical reaction should be carried out at pH 7.4, which is optimal for bacterial β -galactosidase in contrast to the mammalian enzyme, which is most active at a more acidic pH value of 4.0.

⁴Prolonged exposure of embryonic tissues to the fixative diminishes β -galactosidase activity, and enzyme activity becomes undetectable after more than 60 min in paraformaldehyde. When coverslipping sections, use Canada balsam, as other mounting media can cause the blue stain to fade.

⁵If the fixation of the embryo fragment or specimen is insufficient, leakage of the reaction product stains the reaction mixture blue. It is advisable not to leave stained samples in alcohols and solvents longer than necessary, since this may leach out some of the reaction product. When sections are viewed by dark-field microscopy, the *lacZ* stain appears pink and contrasts with surrounding tissues.

Integration of the transgene into a specific chromosome may be useful in some cases for tracing cell lineages. Transgenes that are expressed in the same pattern as neighboring genes at the site of integration provide a ready marker for any tissue-specific pattern of expression. For example, transgenes integrated into the X chromosome may behave like the endogenous X-linked gene; however, the fact that the expression pattern is due to a transgene, not an endogenous gene, must be taken into account when interpreting the results (5,6). In female mice that carry the *lacZ* transgene on only one X chromosome, X-inactivation during embryonic development generates two cell populations, one that expresses the X-linked transgene and another that does not. Since the status of X-inactivation is heritable, descendants of either population are stable for the transgene expression. Several studies on the lineage relationship of cells in tissues, such as the retina, brain, and tongue, have been performed using the mosaicism of transgene expression generated by random X-chromosome inactivation (7–10).

Finally, some transgenes display lineage and stage-specific expression through use of a tissue-specific promoter or as a consequence of their unique integration sites. For example, in *Wnt1-lacZ* transgenic mice, the transgene is regulated by the *Wnt1* 3'-cis-acting enhancer element, which directs specific expression to the dorsal part of the neural tube and subsequently in the neural crest cells derived from this region (11). Another example is R197 transgenic mice, in which the *lacZ* gene is expressed principally in the muscle lineage from early organogenesis stages onward as a consequence of its integration site (12). Provided that the expression of the transgene is tissue specific for a defined period of development, such transgenes offer an excellent tool for tracing the differentiation of cell lineages. A prerequisite for using these mice and any other transgenic lines is to establish the pattern of transmission and ascertain the tissue and temporal specificity of transgene expression.

1.2. Examples of Three Distinct Patterns of Transgenic Reporter Expression

1.2.1. Ubiquitous Expression—The H253 transgenic line (2) is an example of ubiquitous expression of an X-linked transgene. Crossing homozygous X^*X^* females with X^*Y males (where X^* represents the transgene-bearing X chromosome) produces transgenic embryos (X^*X^* , X^*Y), in which β -galactosidase is expressed at high levels in all tissues and progeny during all stages of embryonic development. This series of matings produced a ubiquitous non-lineage-bias transgenic line that has been used successfully in several lineage studies in postimplantation mouse embryos (2,13–15).

1.2.2. X-Inactivation Mosaicism—The H253 transgenic line (2) can also be used to produce a non-lineage-specific mosaic pattern of expression that reflects the pattern of X-inactivation. Crossing homozygous transgenic H253 female and wild-type (C57BL/6) male mice produces hemizygous H253 female mice (X^*X) and hemizygous male mice (X^*Y). Two distinct patterns of staining are observed, uniform staining (X^*Y) and patchy staining (X^*X). The patchy staining of X^*X hemizygous female mice is due to random X chromosome inactivation. This results in the expression of the *lacZ* marker in only about half the cells. The ability to recognize clonal populations means that these two cell populations (one that does express *lacZ* and the other that does not) have been used in cell lineage analyses to reveal the ancestral history of spatially related clones (7–10).

The mosaicism of transgene expression as a consequence of X-inactivation in females has been used extensively in the analysis of specific cell lineages (7–10,16). The *lacZ*-expressing cells can be distinguished from non-expressing cells by X-gal staining. The composition of the sorted populations may be examined for the expression of cell-specific markers by staining with appropriate antibodies. This system therefore provides an assay for

the measurement of the relative proportion of specific cell types at different stages of maturation.

1.2.3. Restricted Expression—The R197 transgenic line is an example of a transgenic marker for lineage- and stage-specific expression patterns (12). The *lacZ* transgene is segmentally expressed primarily in the myogenic lineage from about 8.5 d onward. Transgenic animals can be crossed with non-transgenic (C57BL/6 X DBA) hybrids or other transgenic mice to produce transgenic embryos for the experiment. A reduction in litter size of F1 hemizygous matings and the absence of the homozygotes after genotyping indicated that homozygotes do not survive to term (*see* Note 1). In this transgenic line, homozygous embryos die during the immediate postimplantation period.

2. Materials

All reagents and solutions used in these protocols should be of molecular biology grade and prepared with sterile distilled water. Reagents for some of the protocols are available commercially as kits, and these are indicated.

2.1. *LacZ* Expression by β -Galactosidase Histochemistry

1. PBS ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free; 1 $\times$).
2. Paraformaldehyde (Sigma-Aldrich, St. Louis, MO, P6148). Prepare as a 4% solution in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS by heating to dissolve, but do not boil. Add two to three drops of 0.05 M NaOH to clear the solution (pH 7.5), which can be stored at 4°C for 2 wk. Ideally however, it should be freshly prepared. (Caution: Paraformaldehyde is toxic and readily absorbed through the skin. It is very destructive to the skin, mucous membranes, eyes, and upper respiratory tract. Therefore, glasses, gloves, and a mask should be worn to avoid contact with the dust and work should be performed in a fume hood.)
3. Glutaraldehyde (25% [w/v]); Sigma-Aldrich G5882).
4. X-gal (4-chloro-5-bromo-3-indolyl- β -D-galactopyranoside; Progen, Richlands, QLD, 2000190). X-gal is prepared as a 4% (40 mg/mL) stock solution in dimethylformamide (DMF; Sigma-Aldrich D4551). Store at -20°C and protect from light.
5. Xylene.
6. Sorensen's phosphate buffer (PB), 0.1 M, pH 7.4: 0.02 M NaH_2PO_4 , 0.08 M Na_2HPO_4 .
7. X-gal staining solution: 0.1% X-gal, 2 mM MgCl_2 , 5 mM EGTA, 0.01% (w/v) sodium deoxycholate, 0.02% (w/v) Igepal CA-630 (Sigma-Aldrich, I-3021), 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ (potassium ferricyanide), and 5 mM $\text{K}_4\text{Fe}(\text{CN})_6$ (potassium ferrocyanide) in 0.1 M pH 7.4, Sorensen's phosphate buffer. Potassium ferricyanide and potassium ferrocyanide can be made up as 500-mM stock solutions and should be stored at room temperature, protected from light for a maximum of 2 wk.
8. Ethanol (70, 80, 90, 100%).

¹The possibility of embryonic lethality should be examined as an essential step for using transgenic mice, particularly when a significant reduction in litter size is observed. To ascertain lethality, pregnant mice can be sacrificed at different ages of gestation so that the litter size and the genotype of the embryos or fetuses can be analyzed.

9. Histolene.
10. Paraffin wax.
11. Aluminium sulfate (Sigma-Aldrich, A7523).
12. Nuclear fast red (0.1% C.I. 60760 Certistain; Merck KGaA,): Prepare by adding 500 mg of nuclear fast red and 25 g of aluminum sulfate to 500 mL of distilled water, heat or stir to dissolve, and filter when cooled to room temperature.
13. Sucrose (0.2 M).
14. Sucrose 30%.
15. OCT (optimal cutting temperature) compound (Tissue-Tek, ProSciTech, Thuringowa).
16. StarFrost glass slides (ProSciTech G311SF).
17. Isopentane (methyl-2-butane).
18. Canada balsam (in Xylene, Merck KGaA).
19. Glycol methacrylate (2-hydroxyethyl methacrylate; Sigma-Aldrich, H8633).
20. Cresyl violet (Sigma-Aldrich).
21. Acetic acid.

2.2. LacZ Expression by β -Galactosidase Immunostaining

1. PBS (Ca^{2+} and Mg^{2+} -free).
2. Paraformaldehyde (4%) in Ca^{2+} and Mg^{2+} -free PBS (*see section 10.2.1*, items 1 and 2, for preparation).
3. Sucrose (0.2 M).
4. OCT compound (Tissue Tek, ProSciTech, IA018).
5. Liquid nitrogen.
6. Avertin: Prepare 100% stock solution by mixing 10 g of 2,2,2 tribromoethyl alcohol (Sigma-Aldrich, T4,840-2) with 10 mL of tert-amyl alcohol ([2-methyl-2-butanol] Sigma-Aldrich 24,048-6). To use, dilute 100% stock to 2.5% in water. Both the 100 and 2.5% stocks are stored wrapped in foil at 4°C. The correct dose of avertin varies with each preparation. Therefore, several mice should be test injected within the range of 0.014–0.018 mL/g body wt.
7. Sodium nitrate (Sigma-Aldrich S5506): Make up as 0.5% solution in $0.5 \times \text{PBS}$.
8. Vibratome (Campden Instruments, Loughborough, Leicestershire, UK).
9. Sodium azide (Sigma-Aldrich S2002).
10. Normal horse serum (Sigma-Aldrich, H0146).
11. Triton X-100.
12. *Escherichia coli* β -galactosidase antibody rabbit IgG (1° antibody; Chemicon International Ltd. Temecula CA, AB1211-5MG).
13. Biotinylated goat antirabbit IgG (2° antibody: Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, PK6101).

14. Texas red conjugated donkey anti-rabbit IgG (2° antibody; Jackson Immuno Research Laboratories Inc., West Grove, PA, 711-075-152).
15. Streptavidin–peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories; Immunodiagnostic, Burlingame, CA, PK6101).
16. Diaminobenzidine (DAB; DakoCytomation, Carpinteria, CA, S3000). Store at -20°C .
17. Hydrogen peroxide (H_2O_2).
18. Streptavidin–FITC complex.
19. Nuclear fast red (0.1%; see **section 10.2.1**, item 12).

2.3. Visualizing lacZ by Immunostaining or Histochemistry in Combination with Alkaline Phosphatase Histochemistry

1. PBS (Ca^{2+} and Mg^{2+} -free).
2. X-gal staining solution (see **section 7.2.4**, item 7).
3. Glutaraldehyde (25%; Sigma-Aldrich G5882).
4. Ethanol (25% [w/v]), 50, 70, 80, 90, 100%).
5. Polyester wax.
6. Xylene.
7. Hydrogen peroxide (H_2O_2).
8. Methanol.
9. Normal goat serum (Sigma-Aldrich G9023).
10. *E. coli* β -galactosidase antibody rabbit IgG (1° antibody; Chemicon International, AB1211-5MG).
11. Bovine serum albumin (Sigma-Aldrich Fraction V A9647).
12. Biotinylated goat anti-rabbit IgG (2° antibody; Vector Laboratories, PK6101): Store at 4°C .
13. Streptavidin–peroxidase complex (Vectastain Elite ABC reagent): Add two drops of reagent A to 5 mL PBS (with Ca^{2+} and Mg^{2+}), mix well, then add two drops of reagent B, mixing immediately, and allow the ABC reagent to stand for about 30 min before use (Vector Laboratories, PK6101).
14. Diaminobenzidine (DakoCytomation, S3000): Store at -20°C .
15. Peroxidase substrate solution: Dissolve one DAB tablet in 10 mL PBS to give 1 mg/mL DAB solution. Mix 2 mL of DAB solution with 15 mL of 3% H_2O_2 (or 16 mL of 30% H_2O_2). The substrate solution is stable for 2 h at room temperature. Unused stock DAB solution can be stored in the dark (wrapped in foil) for up to 5 d at $2-8^{\circ}\text{C}$, or longer at -20°C .
16. Alkaline phosphatase (ALP) buffer: 0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl_2 , pH 9.5. Dissolve 12.1 g/L TRIS-base and 5.84 g NaCl in dH_2O , adjust pH to 9.5 with concentrated HCl, add 50 mL 1 M MgCl_2 , and make up volume to 1 L.
17. NBT/BCIP substrate: Store at -20°C . Prepare NBT/BCIP substrate immediately prior to staining. Add 44 μL NBT to 10 mL alkaline phosphatase buffer, mix

gently, then add 33 μ L BCIP, mix gently (Invitrogen Corporation, Carlsbad, CA: 18280–016).

18. Canada balsam (in Xylene, Merck KGaA; 101693).
19. 4% (w/v) paraformaldehyde (see **section 7.2.4**, item 2).
20. X-gal staining solution: 0.1% (w/v) X-gal, 2 mM MgCl_2 , 5 mM EGTA, 0.01% (w/v) sodium deoxycholate, 0.02% (w/v) Igepal CA-630 (Sigma-Aldrich I-3021), 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$ (potassium ferricyanide) and 5 M $\text{K}_4\text{Fe}(\text{CN})_6$ (potassium ferrocyanide) in 0.1 M pH 7.4 Sorenson's PB.

2.4. Visualizing *lacZ* by Histochemistry in Combination with In-Situ Hybridization

1. PBS (without Ca^{2+} - and Mg^{2+}).
2. 4% Paraformaldehyde (Sigma-Aldrich: see **section 7.2.1**, item 2, for preparation).
3. Ethanol (50, 70, 80, 90, 100% v/v).
4. Xylene.
5. Paraffin wax.
6. TESPA- ([3-aminopropyl]triethoxysilane; Sigma-Aldrich A3648) coated slides are prepared as follows. Wash slides in 10% HCl/70% ethanol, followed by 95% ethanol for 1 min each, then air dry. Dip the slides in 2% TESPA in acetone for 10 sec. Wash twice with acetone and then with distilled water before drying at 37°C.
7. DIG RNA labeling kit (Roche 1175025).
8. Triton X-100.
9. Proteinase K (20 mg/mL; 6 μ g/mL).
10. SSC (20 X).
11. SDS (10% w/v).
12. Hybridization buffer: 5 $\times$ SSC, 50% (v/v) deionized formamide, 0.4% (w/v) SDS, 0.1% (w/v) N-lauroylsarcosine, 2% (w/v) blocking reagent (Roche Blocking Reagent 1096176).
13. Heparin sodium salt from porcine intestinal mucosa (50 μ g/mL or 7U; Sigma-Aldrich H9399).
14. Yeast tRNA (20 μ g/mL).
15. NaCl (1 M).
16. Maleic acid (1 M).
17. Sheep serum (Sigma-Aldrich S2263).
18. DIG antibody-sheep conjugated alkaline phosphatase (dilute 1:200 in 150 mM NaCl/100 mM maleic acid/10% sheep serum; Roche 1093274).
19. Alkaline phosphatase buffer (ALP; see **section 7.2.3**, item 16).
20. NBT/BCIP substrate (see **section 7.2.3**, item 17).
21. *E. coli* β -galactosidase antibody rabbit IgG (5 μ g/mL in PBS/20% normal goat serum; Chemicon International AB1211–5MG).
22. Normal goat serum (Sigma-Aldrich G9023).

23. Biotinylated goat anti-rabbit IgG (2° antibody; diluted 1:200 in PBS/0.2%BSA; Vector Laboratories, PK6101) for 60 min at room temperature.
24. Streptavidin- β -galactosidase conjugate (diluted 1:200 in PBS/0.2% BSA; Roche 1112481).
25. X-gal washing buffer (X-gal staining solution without X-gal; *see* **section 7.2.1**, item 7).
26. X-gal staining solution (*see* **section 7.2.1**, item 7).
27. CHAPS(3-[[3-cholamidopropyl]dimethyl-ammonio]-1-propanesulfonate)zwitterionic; Roche 810118).
28. PBT (0.1% Tween-20 in PBS).
29. Methanol (25, 50, 70, 100% v/v).
30. Hydrogen peroxide (H₂O₂).
31. Dimethylsulfoxide (DMSO; Sigma-Aldrich D2650).
32. Glycine.
33. Glutaraldehyde (25% w/v; Sigma-Aldrich G5882).
34. Tween-20.
35. Prehybridization solution consists of the following and should be prepared on the day of use: 50% deionized formamide, 5×SSC, 2% blocking powder, 0.1% Tween-20, 0.5% CHAPS, 50 μ g/mL yeast RNA, 5 mM EDTA, 50 μ g/mL heparin.
36. Bovine serum albumin (Fraction V; Sigma-Aldrich A9467).
37. Mouse embryo powder: This is prepared by homogenizing 12.5–14.5-d mouse embryos in a minimum volume of PBS. Add 4 volumes of ice-cold acetone, mix, and incubate on ice for 30 min. Spin at 10,000 g for 10 min and remove the supernatant. Wash the pellet with ice-cold acetone, and spin again. Spread the pellet out, and grind into a fine powder on a sheet of filter paper. Allow the powder to air dry, and store in an airtight tube at 4°C.
38. NTMT: 100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl₂, 0.1% Tween-20.
39. Levamisole hydrochloride (2 mM in NTMT; Sigma-Aldrich L9756).
40. Color reaction mix consists of the following and should be prepared immediately before use: 4.5 mL NBT, 3.5 μ L BCIP (Invitrogen Corporation: 18280–016) in 1.0 mL NTMT.

3. Methods

3.1. Histochemical Staining for *lacZ* with X-Gal

3.1.1. Small Embryos (7.5–11.5 D pc) and Fragments of (12.5–18.5 D pc) Embryos

1. Fix small embryos or fragments of older embryos in 4% paraformaldehyde/0.2% glutaraldehyde for 5–15 min.
2. Wash the embryos in PBS (2 $\times$ 5 min), and stain overnight in X-gal staining solution at 37°C in the dark.

3. Wash the embryos in 70% (10 min) ethanol and dehydrate in 70, 80, and 90% ethanol (10 min each), then 100% ethanol (3×10 min).
4. Clear the embryos in xylene or histolene (3×10 min) and impregnate in paraffin wax (3×20 min) before embedding in fresh wax.
5. Cut 5–10 μ m sections and counterstain with nuclear fast red (*see* Notes 2–6).

3.1.2. Older Embryos and Adult Organs—Larger specimens can be handled in two ways, depending on the thickness of the tissue. Frozen sections can be stained or, alternatively, very small tissue pieces can be stained then embedded in paraffin wax and sectioned. Paraffin embedding is described in **section 7.3.1.1** (*see* Note 7).

1. Fix dissected adult tissues or embryos in fresh 4% paraformaldehyde/0.2% glutaraldehyde in 0.1 M phosphate buffer. Determine the time required by the size of the tissue to be fixed based on penetration rates of 1 mm/h at room temperature. (Perfusion can also be carried out if desired, *see* **section 7.3.2.2**).
2. Wash (3×2 min) in phosphate buffered saline (PBS).
3. Leave in 30% sucrose at room temperature overnight.
4. Fill Tissue-Tek cryomolds with OCT medium and place a piece of tissue in each labeled cryomold. Minimize the number of bubbles and avoid bubbles in contact with the tissue.
5. Fill a beaker to about one third full with isopentane and float the beaker in liquid nitrogen. When a crust of frozen isopentane is visible, lower the cryomolds into the isopentane one at a time to freeze (1–2 min) using long forceps. Place samples on dry ice until all are processed then store samples at -80°C . (*see* Note 8)
6. Section samples on cryostat to desired thickness e.g.: 8–10 μ m onto StarFrost coated slides.
7. Rinse slides very carefully with PBS to remove Tissue-Tek. Place on the smallest box or tray that will fit the number of slides to be stained.
8. Very gently rinse in washing buffer 3×20 mins.
9. Put a few drops of *lacZ* substrate on each slide and add coverslips. Seal box with parafilm and aluminium foil and incubate at 37°C overnight.
10. Gently rinse off *lacZ* substrate with PBS and follow with 2×10 min PBS washes.
11. Counterstain with nuclear fast red 1–3 minutes.
12. Quickly dip in water.
13. Dip in 70% ethanol.

²Enzyme activity in embryos from preimplantation up to about 11.5 d pc can be directly analyzed in whole mounts. However, for embryos older than 11.5 d, penetration of the substrate in whole embryos becomes limiting; and this poses a problem for accessibility of staining reagent to internal tissues, especially after a substantial amount of insoluble reaction product has been deposited in more superficial *lacZ*-expressing tissues. Dissection of older embryos (>11.5 d) into smaller fragments facilitates penetration of the substrate and greatly improves the staining of deeper tissues.

⁶To enhance the discrimination between expressing cells, non-expressing cells, and cells expressing endogenous β -galactosidase-like activity, it is useful to target the *lacZ* product to a particular cellular compartment, such as the nucleus. The nuclear localization signal (nls) from the early region of the simian virus (SV40) genome has been used to provide effective nuclear localization of β -galactosidase (nls*LacZ*; [17,18]).

⁷For more detailed histological studies, organs can be cut by a scalpel blade into small parts, which are then dehydrated in alcohol and embedded either in paraffin wax or glycol methacrylate. Wax sections are counterstained in 0.1% nuclear fast red. Methacrylate sections are counterstained in 0.1% cresyl violet in 1% acetic acid (pH 3.3) for 50 min at 40°C .

⁸Specimens go white in about 30 s when freezing them in isopentane.

14. Dip in 90% ethanol.
15. Immerse for 1 min in 100% ethanol, then repeat in fresh ethanol.
16. Immerse for 2 min in histolene, then repeat in fresh histolene.
17. Coverslip using Canada balsam mounting media.

3.2. Immunostaining for *lacZ*

3.2.1. Embryos (7.5–18.5 D pc)

1. Dissect embryos in 4% paraformaldehyde (in Ca^{2+} and Mg^{2+} -free PB) and fix for 4 h.
2. Wash the embryos in 0.2 M sucrose (1 h), then immerse in OTC briefly and immediately before freezing slowly in liquid nitrogen vapor, followed by immersion in liquid nitrogen.
3. Cut 60- μm thick sections using a cryostat and store at 4°C until required, then go to **section 3.2.3**, step 1.

3.2.2. Adult Mice and Body Organs

1. Anesthetize adult mice with 2.5% avertin (0.017 mL/g of body weight) and perfuse via the left ventricle with 0.5% w/v sodium nitrate in half-strength phosphate-buffered saline.
2. Dissect the required organ, and postfix with the same sodium nitrate fixative (2 h).
3. Section the tissue with a vibratome at 50–100- μm thickness.
4. Store sections for immunohistochemistry in PBS/0.02% sodium azide at 4°C until required. Then go to **section 3.2.3**, step 1.

3.2.3. Detection

1. Wash sections from **sections 3.2.1.** and **3.2.2.** selected for detection of β -galactosidase in PBS/10% normal horse serum/0.02% Triton X-100 at room temperature (12 h).
2. Incubate sections with *E. coli* β -galactosidase antibody (diluted 1:1000 in PBS/10% normal horse serum/0.02% Triton X-100) overnight at room temperature.
3. Wash sections in PBS (3×10 min), and then incubate with 2° antibodies either biotinylated goat anti-rabbit IgG (diluted 1:400 in 0.02% Triton X-100 in PBS) or Texas red donkey anti-rabbit IgG (diluted 1:500 in 0.02% triton X-100 in PBS) for 2 h.
4. Remove unbound antibody by washing in PBS (10 min).
5. If using biotinylated goat anti-rabbit IgG, incubate the sections in streptavidinperoxidase complex (diluted 1:100 in 0.2% Triton X-100) for 2 h.
6. Visualize the immunochemical reaction by washing sections with diaminobenzidine (0.5 mg/mL) in the presence of 0.01% H_2O_2 until the brown color is apparent.
7. If Texas red–conjugated donkey antirabbit IgG is used, then incubate the sections in streptavidin–FITC (diluted to 1:100 in 0.02% Triton X-100 in PBS) for 2 h.
8. Wash sections in PBS (3×10 min), and view either by light or fluorescence microscopy. Sections may be counterstained with 0.1% nuclear fast red.

3.3. Visualizing *lacZ* by Immunostaining or Histochemistry in Combination with Alkaline Phosphatase Histochemistry

3.3.1. Simultaneous Immunological Detection of *lacZ* and Alkaline Phosphatase on Sections

This protocol allows for the staining of *E. coli* β -galactosidase antibody and NBT-BCIP histochemical detection of alkaline phosphatase. It is designed to detect alkaline phosphatase in primordial germ cells in *lacZ*-expressing embryos. All staining should be done at room temperature unless otherwise indicated and on 6–8 μ m sections.

1. Dissect out embryos in PBS medium and stain either the tail, head, yolk sac, or a nonessential body part with X-gal staining solution to screen for transgenic embryos.
2. Fix embryos in 0.5% glutaraldehyde in PBS for 5–15 min, depending on the size of the embryos. As a general rule:

a.	<8.5 d pc	5 min
b.	9.5 d pc	5–10 min
c.	10.5–12.5 d pc	10–15 min
d.	13.5–15.5 d pc	15 min
e.	>16.5 d pc	20–30 min
3. Wash embryos 2 $\times$ 10 min with PBS. The embryos then can be either stored in 70% ethanol for a short period of time or dehydrated and embedded as described in **section 7.3.3.1, step 4**.
4. Dehydrate embryos through 70, 80, 90, and 100% ($\times$ 3) ethanol according to the following times:

a.	<8.5 d pc	2–5 min
b.	9.5 d pc	5 min
c.	10.5–12.5 d pc	10 min
d.	13.5–15.5 d pc	10–15 min
e.	>16.5 d pc	20–30 min
5. Transfer embryos to fresh polyester wax for 10–15 min. Repeat with fresh wax 2 $\times$ 10 min each. Embed in fresh polyester wax at room temperature, and section with either a cryostat or a microtome kept at 15°C (*see* Note 9).
6. Dewax sections in xylene (5 min), and rehydrate the sections by incubating them in a 37°C oven for 30 min followed by 2-min washes in 100% ethanol (2 $\times$ at 37°C), 90, 70, 50, and 25% ethanol. Finally, wash the sections for 5 min in dH₂O.
7. Quench the endogenous peroxidase activity with 0.3% H₂O₂ in 40% methanol for 20 min or 3% H₂O₂ in H₂O for 5 min.
8. Wash the sections with PBS (3 $\times$ 3 min).
9. Block non-specific binding by overlaying sections with 5–10% normal goat serum for 20 min.

⁹When infiltrating with polyester wax, the specimens may float, which is normal, but they sink as they are infiltrated with wax. If, after the first 15 min of incubation in wax, the specimens have not sunk, it is still advisable to go to the next wax change.

10. Drain excess serum and incubate slides with *E. coli* β -galactosidase antibody (diluted 1–5 $\mu\text{g/mL}$ in PBS) for 60–75 min.
11. Wash sections in PBS + 0.2% BSA (3×10 min).
12. Incubate sections with biotinylated goat anti-rabbit IgG (2° Ab diluted 1:200 in PBS, 0.2% BSA, 3% normal goat serum) for 30 min.
13. Wash sections with PBS + 0.2%, BSA (3×10 min).
14. Incubate sections for 30 min with streptavidin peroxidase complex.
15. Wash sections with PBS + 0.2% BSA (3×10 min).
16. Incubate sections with peroxidase substrate solution (1 mg/mL DAB + 0.0225% H_2O_2) for 2–7 min or until desired staining intensity develops.
17. Wash sections for 5 min with dH_2O .
18. Incubate sections with PBS (10 min).
19. Incubate sections with alkaline phosphatase buffer, pH 9.5 (2×10 min).
20. Incubate sections in NBT/BCIP substrate for 10–15 min in the dark at 37°C , or until desired intensity develops.
21. Wash the sections with dH_2O (5 min).
22. Dehydrate and mount in Canada balsam.

3.3.2. Simultaneous Whole-Mount Histochemical Staining for β -Galactosidase and Alkaline Phosphatase

1. Fix 7.5–11.5 d mouse embryos or older embryo (12.5–18.5 d) fragments and organs in 4% paraformaldehyde, 0.5% glutaraldehyde in PBS for 5–10 min.
2. Wash embryos in PBS (2×10 min). Incubate embryos for 10 min in alkaline phosphatase buffer.
3. Incubate embryos in NBT/BCIP substrate at 37°C for 10–20 min. Check intensity every 5 min.
4. Wash embryos extensively in X-gal washing buffer (staining solution without X-gal; 2×10 min).
5. Incubate embryos overnight in X-gal staining solution at 37°C .

3.4 Visualizing *lacZ* by Histochemistry in Combination with In-Situ Hybridization

3.4.1. Simultaneous Detection of Oct 4 mRNA and β -Galactosidase Protein on Paraffin Sections

1. Fix 7.5–11.5-d embryos or fragments and organs of older embryos (12.5–18.5 d) in 4% paraformaldehyde overnight at 4°C , then wash in PBS (2×10 min). Dehydrate the embryos according to the schedule given in section 7.3.3.1, step 4; wash in xylene (3×10 min) followed by paraffin wax (3×15 min); then embed the embryos in fresh paraffin wax before storing at 4°C . Cut sections at $8 \mu\text{m}$, and mount on TESPA-coated slides.
2. A 600 bp Oct4 RNA digoxigenin probe was generated by in-vitro transcription with T3 (antisense strand) and T7 (sense strand) RNA polymerase (DIG RNA labeling kit, Roche, cat no. 1175025).

3. Dewax the embryos in xylene (5 min), rehydrate ($100\% \times 2$, 90, 80, 70, 50, dH₂O; for 1 min each), and then wash the sections in PBS (2 min).
4. Refix the sections in 4% paraformaldehyde (10 min), then wash with PBS (2×5 min).
5. Treat the sections with 0.3% Triton X-100 in PBS (15 min), then wash in PBS (2×5 min).
6. Incubate the sections in 37°C prewarmed proteinase K (20 mg/mL; 5 min), wash in PBS (3×10 min), then postfix the sections in 4% paraformaldehyde (15 min).
7. Prehybridize the sections for 3 h in hybridization buffer ($5 \times$ SSC, 50% deionized formamide, 0.4% SDS, 0.1% N-lauroylsarcosine, 2% blocking reagent).
8. Dilute the probe in hybridization buffer containing 50 µg/mL heparin and 20 mg/mL yeast tRNA to a final concentration of 0.05 ng/mL. Heat the probe at 80°C (10 min) and immediately store on ice until addition to the slides. Incubate with sections with 500 µL of probe at 55°C in a humidified chamber overnight.
9. Wash the sections at room temperature in $2 \times$ SSC/0.2% SDS (2×10 min); then at 58°C in $0.5 \times$ SSC/0.2% SDS (2×30 min) followed by 150 mM NaCl/100 mM maleic acid (pH 7.5; 10 min).
10. Block the section in 150 mM NaCl/100 mM maleic acid (pH 7.5) containing 10% sheep serum (30 min), then incubate with diluted alkaline phosphatase-conjugated sheep anti-DIG antibody (1:200 dilution) for 2 h.
11. Wash the sections in 150 mM NaCl/100 mM maleic acid (pH 7.5; 2×15 min), then in alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5, 10 min), and incubate with NBT/BCIP substrate. Allow the color to develop in the dark for 90 min at room temperature.
12. Wash the sections in PBS (3×10 min), block with 20% normal goat serum in PBS (30 min), then incubate with 5 µg/mL *E. coli* anti-β-galactosidase antibody overnight at 4°C.
13. Wash the sections in PBS containing 0.2% BSA ($3 \times$ in), then incubate with biotinylated goat anti-rabbit IgG (2° antibody; diluted 1:200 in PBS/0.2% BSA) for 60 min at room temperature.
14. Wash the sections in PBS containing 0.2% BSA (3×10 min), then incubate with streptavidin-β-galactosidase conjugate for 60 min (1:200 dilution).
15. Wash the sections in PBS (2×10 min), then in X-gal washing buffer (10 min), and incubate in X-gal solution at 37°C (20 min) in the dark.
16. Dehydrate the sections, and mount in Canada balsam.

3.4.2. Whole-Mount In-Situ Hybridization Combined with X-Gal Staining—All steps are performed at room temperature unless otherwise stated.

1. Fix embryos in 4% paraformaldehyde for 5 min.
2. Stain embryos in X-gal staining solution for 1 h.
3. Refix embryos in 4% paraformaldehyde overnight.
4. Wash embryos in PBT (2×10 min).

5. Wash embryos in 25, 50, and 75% methanol/PBT (5 min), then in 100% methanol (2×10 min). Embryos can be stored in 100% methanol at -20°C .
6. Fix the embryos in methanol:DMSO (4:1) for 1 h, then bleach the embryos in methanol:DMSO:30% H_2O_2 (4:1:1) for 1 h.
7. Rehydrate embryos in 75, 50, and 25% methanol/PBT (5 min), then wash the embryos in PBT (2×5 min). It is important to poke a few holes with a small needle into these embryos prior to washing to facilitate the flow of solutions.
8. Treat the embryos with proteinase K (6 mg/mL; 5 min).
9. Wash the embryos in 0.2% glycine in PBT (2×5 min).
10. Refix the embryos in 4% paraformaldehyde/0.2% glutaraldehyde in PBS (30 min).
11. Wash embryos in PBT (2×5 min).
12. Wash embryos in 1 mL of prehybridization solution.
13. Prehybridize embryos in 1 mL of prehybridisation solution at 65°C (3 h). Embryos can be stored at this point at -20°C in prehybridization solution.
14. Incubate embryos in 1 mL of hybridization solution at 60°C overnight.
15. Posthybridize wash with each of the following for 5 min at 60°C :
 - a. 100% Prehybridization solution.
 - b. 75% Prehybridization solution: 25% $2 \times \text{SSC}$.
 - c. 50% Prehybridization solution: 50% $2 \times \text{SSC}$.
 - d. 25% Prehybridization solution: 75% $2 \times \text{SSC}$.
16. Wash with each of the following for 30 min at 60°C :
 - a. $2 \times \text{SSC}$: 0.1% CHAPS.
 - b. $0.2 \times \text{SSC}$: 0.1% CHAPS ($\times 2$).
17. Wash embryos in PBT (2×10 min).
18. Preblock embryos in 10% normal sheep serum/1% BSA in PBT at 4°C (3 h).
19. Preabsorb the antibody at 4°C (3 h) with 3 mg mouse embryo powder, 500 μL of 10% normal sheep serum, 1% BSA in PBT, and 1 μL of anti-DIG antibody, rocking the mixture gently. Spin the solution at 2,000 g for 5 min and remove the supernatant (preabsorbed antibody).
20. Incubate the embryos in preabsorbed antibody overnight at 4°C with constant rocking.
21. Wash embryos in 0.1% BSA in PBT (5×1 h).
22. Wash embryos in 2 mM levamisole in NTMT (2×10 min).
23. Wash embryos in NTMT (10 min).
24. Incubate embryos in the color reaction mixture until sufficient coloration is visible in the specimen.
25. Refix embryos in 4% paraformaldehyde overnight, wash in PBS, and examine (*see* Notes 10 and ¹¹).

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¹⁰It is important that the embryos are not overfixed with paraformaldehyde for X-gal staining prior to the immunohistochemistry, because it may result in some loss of antigenicity.

¹¹Each of these procedures contains multiple wash steps that require the transfer of embryos. If the dish containing the embryos is placed on a black background, it is easier to see the embryos during transfer.