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In Situ Hybridization Methods for Mouse Whole Mounts and Tissue Sections with and Without Additional β -Galactosidase Staining

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Abstract

In situ hybridization is a powerful method for detecting endogenous mRNA sequences in morphologically preserved samples. We provide in situ hybridization methods, which are specifically optimized for mouse embryonic samples as whole mounts and section tissues. Additionally, β -Galactosidase (β -gal) is a popular reporter for detecting the expression of endogenous or exogenous genes. We reveal that 6-chloro-3-indoxyl- β -D-galactopyranoside (S-gal) is a more sensitive substrate for β -gal activity than 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal). S-gal is advantageous where β -gal activity is limited including early stage mouse embryos. As a result of the increased sensitivity as well as the color compatibility of S-gal, we successfully combined β -gal staining using S-gal with in situ hybridization using DIG-labeled probes in both whole mounts and sections.

Keywords

β -Galactosidase; In situ hybridization; Mouse; S-gal (6-chloro-3-indoxyl- β -D-galactopyranoside); X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside)

1 Introduction

Expression patterns of endogenous genes change drastically during embryogenesis. Knowledge of tissue- and stage-specific gene expression patterns is essential to speculate about genetic function. In addition, expression of particular genes can be used as markers to identify the presence or absence of a particular lineage, tissue, or cell type. Therefore, methods of mRNA detection in morphologically preserved samples serve as principal tools for analyses of mutations that cause abnormalities during embryogenesis [1, 2]. Generally, in situ hybridization can be performed using radiolabeled nucleic acid and non-radiolabeled (digoxigenin label) nucleic acid as a probe. There has been some debate over the sensitivity between radiolabeled and non-radiolabeled probes. Radiolabeling techniques require special instruments and it takes longer for detection than non-radiolabeled probes. In addition, radiolabeled methods work best for sectioned tissues, and they are difficult to apply to whole mount tissues. Use of digoxigenin-labeled probes can overcome these problems. Here we describe in situ hybridization methods using non-radiolabeled probes for both whole mount

and sectioned mouse embryonic tissues. By following our protocol, it is easy to assess intriguing gene expression patterns during all stages of mouse embryogenesis.

The *LacZ* gene of *Escherichia coli*, which encodes β -gal, has been applied as a reporter gene in the developmental biology field because of its high sensitivity and ease of detection. In the mouse, *lacZ* has been used by a variety of ways: e.g., by knocking it into the targeted gene locus for analysis of gene expression, as a transgenic reporter of tissue specificity, for detection of gene recombination by Cre-mediated recombination, and for detection of mutant ES cell derivatives in chimeric analyses. In these contexts, double staining to detect β -gal activity and mRNA presence via in situ hybridization techniques can provide a powerful strategy to examine the expression pattern of endogenous genes while monitoring β -gal activity in the same sample. However, a similar color is produced by β -gal activity using 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) and in the detection of mRNA using in situ hybridization by DIG-labeled probes using BM purple (AP substrate). Therefore, it is often difficult to distinguish between these two stains in the same sample.

To overcome this difficulty and allow for double staining of β -gal activity and mRNA detection by in situ hybridization techniques, we use a different β -gal substrate, 6-Chloro-3-indoxyl- β -D-galactopyranoside (S-gal), instead of X-gal, to create a different color outcome between the two techniques. S-gal creates a pink/magenta color after the reaction and has been used for detection of β -gal activity [3, 4]. There are several options depending on materials and purposes for detecting the expression patterns of endogenous genes as well as the monitoring β -gal activity in both whole mount tissue and sectioned samples during multiple stages of mouse embryonic development [5]. S-gal is more advantageous than X-gal due to its higher sensitivity and color compatibility as exemplified in Figs. 1 and 2.

2 Materials

2.1 S-gal (6-Chloro-3-Indoxyl- β -D-Galactopyranoside) Staining

1. Stock solution for 25 mg/mL S-gal (catalog number: #C-5000, BIOSYNTH INTERNATIONAL, Inc.) in Dimethyl Sulfoxide (DMSO) HYBRI-MAX (catalog number: #D2650, Sigma) and stored at -20°C . It is vital to protect the stock solution from the light.
2. Rinse buffer for S-gal staining: 0.1 % Sodium Deoxycholate, 0.2 % IGEPAL CA-630 (catalog number: #I3021, Sigma), mM MgCl_2 in 0.1 M Na Phosphate buffer (pH 7.3). 1,000 mL of 0.5 M Na Phosphate buffer (pH 7.3) is prepared by mixing with 158 mL of 1 M NaH_2PO_4 , 342 mL of 1 M Na_2HPO_4 , and 500 mL water.
3. Substrate solution for S-gal needs to be made fresh and should contain 1 mg/mL S-gal from the stock, 5 mM Potassium Ferricyanide, and 5 mM Potassium Ferrocyanide in the rinse buffer.

2.2 Mouse Embryo Fixation for Whole Mount In Situ Hybridization

1. 4 % paraformaldehyde (PFA) with Phosphate Buffered Saline (PBS) in Diethylpyrocarbonate (DEPC)-treated water.

2. 25, 50, and 75 % methyl alcohol (MeOH) in PBT.

PBT: PBS with 0.1 % Tween 20.

2.3 Embedding Procedure of Mouse Embryo for Section In Situ Hybridization

1. 4 % PFA–DEPC-treated PBS
2. 20 % sucrose ultrapure (catalog number: #802536, MP Biomedicals, LLC.) in PBS. It is necessary to make freshly on the day of use.
3. Optimal Cutting Temperature (O.C.T.) compound (catalog number: #4583, Sakura Finetek USA, Inc.).
4. Liquid nitrogen for making cryo-blocks.

2.4 Labeling of Probe for In Situ Hybridization

The following labeling kit is used for synthesizing RNA probe. DIG RNA Labeling Mix (catalog number: #11277073910, Roche), T3 RNA Polymerase (catalog number: #11031163001, Roche), T7 RNA Polymerase (catalog number: #10881767001, Roche), and SP6 (catalog number: #10810274001, Roche). RNase inhibitor (catalog number: #03335399001, Roche). DNase I (catalog number: #04716728001, Roche).

2.5 Whole Mount In Situ Hybridization for Mouse Embryo

All solutions need to be made fresh on the day of use

1. 6 % Hydrogen peroxide in PBT solution.
2. 10 µg/mL proteinase K (catalog number: #P2308, Sigma) in PBT solution.
3. 2 mg/mL glycine in PBT solution.
4. 0.2 % glutaraldehyde, 0.1 % Tween 20 in 4 % PFA–DEPC-treated PBS solution.
5. Hybridization mix solution (final concentration): 50 % Formamide, 5× SSC (pH 4.5, use citric acid to adjust pH), 50 µg/mL yeast RNA, 50 µg/mL Heparin, and 1 % SDS in DEPC-treated water. Stored at –20 °C.
6. Solution I (final concentration): 50 % Formamide, 5× SSC, and 1 % SDS in water.
7. Solution II (final concentration): 0.5 M NaCl, 10 mM Tris–HCl (pH 7.5), and 0.1 % Tween 20 in water.
8. RNase A 100 µg/mL (catalog number: #10109142001, Roche) in Solution II.
9. Solution III (final concentration): 50 % Formamide and 2× SSC in water.
10. 10× TBST (100 mL): 8 g of NaCl, 0.2 g of KCl, 25 mL of 1 M Tris–HCl (pH 7.5), and 10 mL of 10 % Tween 20 in water. Levamisole (catalog number: #L9756, Sigma, final 2 mM) will be added to 1× TBST.
11. Sheep serum (catalog number: #S22-100 mL, Chemicon).
12. Mouse embryonic powder. Preparation: Dissect E14.5 mouse embryos from 4 to 6 wild-type litters. Fix embryos with acetone, then store at –20 °C for a couple of

days. Next, homogenize these mouse embryos completely by using mortar and pestle until their form becomes a dried brown powder. Store at -20°C .

13. Anti-Digoxigenin-AP, Fab fragments (catalog number: #11093274910, Roche).
14. NTMT buffer (final concentration): 100 mM NaCl, 100 mM Tris-HCl (pH 9.5), 50 mM MgCl_2 , and 0.1 % Tween 20 in water. Levamisole (final 2 mM) will be added to NTMT.
15. BM Purple for AP substrate precipitating, ready-to-use solution for color development (catalog number: #11442074001, Roche).

2.6 Section In Situ Hybridization for Mouse Embryo

All solutions need to be made fresh on the day of use

1. 20 $\mu\text{g/mL}$ proteinase K (catalog number: #P2308, Sigma) in PBS solution.
2. 2 mg/mL glycine in PBS solution.
3. 1,000 mL of 0.1 M TEA buffer (pH 8.0): 18.57 g of Triethanolamine hydrochloride (catalog number: #T9534, Sigma) and 2.5 g NaOH in DEPC-treated water.
4. Acetic anhydride (catalog number: #A6404, Sigma).
5. Hybridization mix solution (final concentration): 50 % Formamide, $5\times$ SSC (pH 4.5, use citric acid to adjust pH), 1 mg/mg Ribonucleic acid, transfer (catalog number: #R4018, Sigma), 0.1 mg/mL Heparin, 1 % Blocking reagent (catalog number: #11096176001, Roche), 0.1 % Tween 20, 0.1 % Chaps, 5 mM EDTA (pH 8.0) in DEPC-treated water. Stored at -20°C .
6. TNE buffer (final concentration): 10 mM Tris-HCl (pH 7.6), 500 mM NaCl, and 1 mM EDTA in water.
7. RNase A 100 $\mu\text{g/mL}$ (catalog number: #10109142001, Roche) in TNE buffer.
8. Buffer I (final concentration): 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl in water.
9. Buffer II (final concentration): 2 % Blocking reagent (catalog number: #11096176001, Roche) in Buffer I.
10. Buffer III (final concentration): 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM MgCl_2 in water.
11. Anti-Digoxigenin-AP, Fab fragments (catalog number: #11093274910, Roche).
12. BM Purple for AP substrate precipitating, ready-to-use solution for color development (catalog number: #11442074001, Roche).

3 Methods

For double staining of whole mount/section in situ hybridization and β -galactosidase, mouse embryos need to be stained with S-gal first. Therefore, we first describe the experimental procedures for LacZ staining for whole mount (Subheading 3.1) and sectioned samples

(Subheading 3.2). We describe the preparation of the digoxigenin labeled RNA probes for in situ hybridization (Subheading 3.3) and their use in whole-mount (Subheading 3.4) and section in situ hybridization (Subheading 3.5).

The most important step for performing in situ hybridization is the collection of mouse embryos efficiently in order to avoid degradation of endogenous mRNA. In addition, appropriate fixation and dehydration of the mouse embryos is critical. It is also necessary to treat the embryos with an adequate amount of solution during every procedure. If at any point the tissue dries, it will cause undesirable increase in background during color development.

In regard to later stage embryos, proper penetration of substrates within the embryo is crucial for whole mount in situ hybridization. Based on our experience, we achieve good signal(s) up to stage E10.5. However, past stage E11.5, optimization is necessary by the individual researcher. Some tissues tolerate whole mount staining such as limb bud and lung tissue even after E11.5 stage if they are dissected from the rest of the embryo.

3.1 LacZ Staining of S-gal for Whole Mount Embryo (See Note 1)

1. Dissect mouse embryos in cold DEPC-treated PBS.
2. Fix with 4 % PFA–DEPC-treated PBS at 4 °C as follows:
 - E7.5 or younger—2 min
 - E8.5—5 min
 - E9.5—10 min
 - E10.5 or older—20 min
3. Quickly wash with PBS twice.
4. Wash with rinse buffer for 10 min × 3.
5. Stain with substrate solution at 37 °C for as long as needed to see stain, up to an overnight time period. Keep covered and in the dark during color development.
6. Wash with PBS for 5 min × 2.
7. The embryo can now be fixed in 4 % PFA, unless one intends to continue with in situ hybridizations. If this is the case, continue to Subheading 3.4.

3.2 LacZ Staining of S-gal for Cryo-section (See Note 1)

An example of the result is shown in Fig. 1.

1. Dissect mouse embryos in cold DEPC-treated PBS.
2. Fix with 4 % PFA–DEPC-treated PBS at 4 °C overnight.

¹If the investigator is only staining for LacZ activity and therefore not also performing in situ hybridizations, this protocol can be applied for general LacZ staining by using X-gal instead of S-gal.

3. Replace with 20 % sucrose in PBS at 4 °C overnight. Embryos will float in this solution. They are considered saturated by sucrose when the samples sink down completely.
4. Embed the samples in optimum cutting temperature (O.C.T.) compound on liquid nitrogen, then store at −80 °C (*see* Note 2).
5. Cut 14 µm cryo-sections. We use Superfrost Plus slide glass (catalog number: #12-550-15, Fisher Scientific) for tight attachment of sections to the glass. Then dry the sections completely by using a hair dryer (cooling mode) for 2 min (*see* Note 3).
6. Wash with PBS for 5 min × 3.
7. Wash with rinse buffer for 10 min × 3.
8. Stain with substrate solution at 37 °C overnight. Keep in the dark during color development.
9. Wash with PBS for 5 min × 2.
10. Mount the stained section using glycerol, unless one intends to continue with in situ hybridizations. If this is the case, proceed from **step 9** to continue to **step 1** of “Section in situ hybridization: day 1,” below, in Subheading 3.5.

3.3 Synthesizing Digoxigenin-Labeled RNA Probes for In Situ Hybridization (See Note 4)

1. Template DNA is prepared by linearizing a plasmid, which contains the cDNA of interest and the appropriate restriction enzyme site. The authors typically digest 10 µg of plasmid in 200 µL as a final volume and incubate at 37 °C for overnight.
2. Purify the plasmid by phenol–chloroform extraction three times.
3. Precipitate the digested plasmid by adding 2.5 volume of 100 % ethanol and 1/10 volume of 3 M sodium acetate. Leave at −20 °C for 1 h.
4. Centrifuge (15,000 rpm in a microfuge, 10–15 min) and wash pellet with 70 % ethanol.
5. Add 20 µL of TE (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA).
6. Take 1 µL of the DNA suspension and confirm that it is linearized with agarose gel electrophoresis. Store remaining DNA solution at −80 °C.
7. In vitro transcription is performed by using DIG RNA Labeling Mix, RNase inhibitor, DNase I, and T3/T7/SP6 RNA polymerase.
 - 10× transcription buffer—2 µL
 - Template DNA—2 µL

²It is necessary to freeze the O.C.T. block slowly. The authors put the O.C.T. embedded samples on the 10 cm cell culture dish, floating them on liquid nitrogen. The color of the O.C.T. compound will be changed from clear to white after 5 min. Then, collect these cryo-blocks and store in 50 mL tube at −80 °C.

³For consistent results, the authors prepare cryo-sections on the day of use.

⁴It is critical to perform this section completely RNase free.

- Digoxigenin labeling mix—2 μ L
 - RNase inhibitor—1 μ L
 - RNA polymerase—2 μ L
 - DEPC-treated water—11 μ L
8. Incubate at 37 °C for 2 h.
 9. Add 2 μ L of DNase I and incubate at 37 °C for 30 min.
 10. Add 200 μ L of TE, 600 μ L of ethanol, and 10 μ L of 4 M LiCl.
 11. Leave at –20 °C for 2 h.
 12. Centrifuge (15,000 rpm) and wash with 70 % ethanol.
 13. Add 20 μ L of TE and store at –80 °C.
 14. Check 1 μ L of aliquot via electrophoresis to confirm that digoxigenin-labeled RNA is successfully synthesized.

3.4 Whole Mount In Situ Hybridization

Dissection of mouse embryos for whole mount in situ hybridization

1. Dissect mouse embryos in cold DEPC-treated PBS, or use S-Gal-stained embryos from Subheading 3.1.
2. Fix with 4 % PFA–DEPC-treated PBS at 4 °C overnight.
3. Dehydrate gradually with 25 % MeOH–PBT—50 % MeOH–PBT—75 % MeOH–PBT—100 % MeOH for 10 min each on ice. Larger samples require longer time, around 30 min for each step instead of 10 min.
4. Wash with 100 % MeOH twice then store at –20 °C.

Whole mount in situ hybridization: Day 1 (see Note 4)

1. Rehydrate the mouse embryos with 75—50—25 % MeOH–PBT solutions.
2. Wash with PBT twice.
3. Bleach with 6 % hydrogen peroxide in PBT for 1 h.
4. Wash with PBT (5 min $\times$ 3).
5. Incubate with 10 μ g/mL proteinase K in PBS for 10–20 min depending on the stage and size. Incubation time needs to be optimized by each researcher. Usually, the authors use the proteinase K condition for 5 min (mouse embryonic stage at E6.5–E7.5), 10 min (E8.5–E9.5), and 20 min (E10.5–E11.5).
6. Wash with 2 mg/mL glycine in PBT for 5 min.
7. Wash with PBT twice.
8. Re-fix the samples with 0.2 % glutaraldehyde and 0.1 % Tween 20 in 4 % PFA–DEPC-treated PBS solution for 20 min.

9. Wash with PBT twice.
10. Incubate with hybridization mix at 70 °C for 1 h.
11. Replace the hybridization mix containing 1 µg/mL digoxigenin labeled RNA probe.
12. Incubate at 70 °C overnight (*see* Note 5).

Whole mount in situ hybridization: Day 2

1. Wash with Solution I at 70 °C for 30 min × 2, changing solution at the end of each timepoint.
2. Wash with 1:1 mixture of Solution I and Solution II at 70 °C for 10 min.
3. Wash with Solution II 5 min × 3.
4. Incubate with 100 µg/mL RNase A in Solution II at 37 °C for 30 min × 2.
5. Rinse with Solution II briefly.
6. Wash with Solution III for 5 min.
7. Wash with Solution III at 70 °C for 30 min × 2.
8. Wash with TBST for 5 min × 3.
9. Block the samples with TBST containing 10 % sheep serum at 4 °C for 2 h.
10. During **step 9**, it is necessary to prepare the antibody solution. First, incubate 0.5 mL of TBST containing 3 mg of mouse embryonic powder at 70 °C for 30 min. Then cool the solution on ice for 30 min. Add 5 µL of sheep serum and 0.7 µL of anti-digoxigenin-AP antibody. Shake gently at 4 °C for 1 h. Spin down briefly and collect the supernatant into a new tube. Dilute this supernatant solution with TBST containing 1 % sheep serum to 2 mL as a final volume.
11. Replace the blocking solution with the antibody solution.
12. Incubate at 4 °C overnight.

Whole mount in situ hybridization: Day 3

1. Wash with TBST (5 min × 3).
2. Wash with TBST (1 h × 5).
3. Wash with NTMT (10 min × 3).
4. Replace with BM purple AP substrate solution.
5. Incubate the samples until the color has developed to the desired extent. Keep in the dark during color development (*see* Note 6).

⁵The probe can be recycled once hybridization is done. The authors usually use RNA probes at least three times to obtain the same levels of in situ signals. Store the recycled RNA probes at –80 °C.

⁶After color begins to appear, the color development can be confirmed under the microscope. If the color of the BM purple AP substrate solution changes from yellow to dark purple, you can replace the new substrate solution to enhance further color development.

6. Wash with PBT (5 min $\times$ 2).

Examples of the results are shown in Figs. 3 and 4 for mRNA staining alone and Fig. 2a for lacZ and mRNA staining.

3.5 Section In Situ Hybridization Using Cryo-section

The following method is also possible with paraffin tissue section (i.e., without lacZ stained samples). In the case of paraffin-embedded section, it is necessary to perform deparaffinization first. The authors usually perform the deparaffinization in the following manner. Xylene (5 min $\times$ 3 times)—100 % EtOH (5 min $\times$ 2 times)—70 % EtOH (5 min $\times$ 2 times)—Water (5 min $\times$ 3 times). Then move to **step 1** of “Section in situ hybridization: day 1,” below.

If one is not using LacZ-stained samples, then the following sections must be prepared. If one is using LacZ-stained samples, proceed to **step 1** of “Section in situ hybridization: day 1,” below.

1. Dissect mouse embryos in cold DEPC-treated PBS.
2. Fix with 4 % PFA–DEPC-treated PBS at 4 °C overnight.
3. Replace with 20 % sucrose in PBS at 4 °C overnight.
4. Embryos will float in this solution. They are considered saturated by sucrose when the samples sink down completely.
5. Embed the samples in optimum cutting temperature (O.C.T.) compound on liquid nitrogen, then store at –80 °C (*see* Note 2).
6. Cut 14 μ m cryo-sections.
7. Start to section in situ hybridization (Day 1).

Section in situ hybridization: Day 1 (see Note 4)—It is critical to perform the experiment completely RNase free.

1. Wash the section with PBS for 10 min.
2. Incubate with 10 μ g/mL proteinase K in PBS for 15 min.
3. Re-fix with 4 % PFA solution for 10 min.
4. Wash with 2 mg/mL glycine in PBS for 5 min.
5. Wash with PBS for 5 min.
6. Wash with TEA for 5 min.
7. Incubate with TEA containing 0.25 % acetic anhydride for 10 min (*see* Note 7).
8. Wash with 4 $\times$ SSC for 10 min.

⁷Acetic anhydride is unstable in aqueous solution. The authors usually prepare the TEA containing 0.25 % acetic anhydride solution just before starting this process.

9. Incubate with 2× SSC with 50 % formamide for 30 min at 65 °C.
10. Add 100 µL of hybridization mix containing 1 µg/mL digoxigenin labeled RNA probe (*see* Note 8).
11. Cover the section with cover glass or parafilm.
12. Incubate the section at 65 °C for overnight (*see* Note 9).

Section in situ hybridization: Day 2

1. Wash with 2× SSC with 50 % formamide at 65 °C for 20 min × 3 (*see* Note 10).
2. Wash with TNE buffer at 37 °C for 5 min.
3. Incubate with 20 µg/mL RNase A in TNE buffer at 37 °C for 30 min.
4. Wash with TNE buffer at 37 °C for 5 min.
5. Wash with 0.2× SSC at 65 °C for 20 min × 3.
6. Wash with Buffer I for 5 min.
7. Blocking with Buffer II for 1 h.
8. Incubate with 1:1,000 dilution of anti-digoxigenin-AP antibody in Buffer II at 4 °C overnight.

Section in situ hybridization: Day 3

1. Wash with Buffer I for 15 min × 2.
2. Wash with Buffer III for 5 min.
3. Add 200–300 µL of BM purple AP substrate solution to the section.
4. Incubate the section until color has developed to the desired extent. Keep in the dark during color development (*see* Note 6).
5. Wash with PBS for 5 min × 2.

Examples of the results are shown in Fig. 5 for mRNA staining alone and Fig. 2b for lacZ and mRNA staining.

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⁸It is necessary to wipe the excess amount of 2× SSC with 50 % formamide solution from around the section, before adding the hybridization mix containing RNA labeling probe. Without this step, final concentration of the RNA probe is reduced and it may lower the detection of signals.

⁹The authors use a plastic container with an airtight seal and add paper towels containing 2× SSC with 50 % formamide on the bottom. This plastic box needs to be sealed completely during hybridization to prevent evaporation.

¹⁰In order to avoid any damage to the section, it is necessary to remove the cover glass or parafilm as follows: the authors carefully sink the slide glass into a beaker containing enough pre-warmed 2× SSC with 50 % formamide solution (65 °C) to cover the sample. After 1–2 min, the cover glass or parafilm will spontaneously detach from the slide glass.

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Fig. 1.

Comparison with X-gal and S-gal staining on time course dependency. S-gal and X-gal staining pattern at each time point. *Upper panels* are 30 min, *middle panels* are 2 h, and *lower panels* are overnight (O/N) color development. *Two left panels* are day 7.5 embryos each set has one embryo carrying *ROSA26* (*left*) and one embryo as a negative control (*right*). The *two right panels* are sagittal sections of the mouse head for day 16.5 embryos carrying *P0-Cre* and *ROSA26*-reporter

Fig. 2.

Dual detection of β -gal activity and gene expression on whole mount and cryo-sectioned materials. (a) S-gal staining (magenta) and in situ hybridization signals (purple) were monitored for E11.5 mouse embryo. (b) S-gal staining and in situ hybridization signals were monitored by magenta and purple color in surrounding mesenchyme of incisor region at E14.5. Left panel is S-gal staining and right panel is double staining with S-gal and *Msx1* in situ hybridization

Fig. 3. Example for whole mount in situ hybridization at early stage of mouse embryonic development. Anterior and posterior marker gene expression analysis of *mouse* embryos at E7.5. Marker gene expression pattern of *Shh*, *Cer1*, *Foxa2* (upper panels) and *Brachyury*, *Wnt3a*, *Tbx6* (lower panels) respectively

Fig. 4.

Example for whole mount in situ hybridization at late stage of mouse embryonic development. Whole mount in situ hybridization was performed using *Shh*, *Patch1*, *Patch2*, and *P21* at E13.5 and E14.5. *Shh*, *Patch1*, and *Patch2* start to express in the prospective palatal rugae at E13.5 but there is no expression of *P21* on the palatal rugae at E13.5 and E14.5. *Patch1*, *Patch2*, and *P21* are strongly expressed in the edge of the palate at E13.5

Fig. 5.

Example for section in situ hybridization by paraffin section. Section in situ hybridization was performed using *Shh* specific RNA probe. Sagittal paraffin section of craniofacial region at E18.5. Note that *Shh* expression is specifically detected in hair follicle (*upper panel*) and pre-ameloblasts (*lower panel*)