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Wholemount *in situ* Hybridization for Spatial-temporal Visualization of Gene Expression in Early Post-implantation Mouse Embryos

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[Abstract] Regionalized distribution of genes plays crucial roles in the formation of the spatial pattern in tissues and embryos during development. *In situ* hybridization has been one of the most widely used methods to screen, identify, and validate the spatial distribution of genes in tissues and embryos, due to its relative simplicity and low cost. However, acquisition of high-quality hybridization signals remains a challenge while maintaining good tissue morphology, especially for small tissues such as early post-implantation mouse embryos. In this protocol, we present a detailed RNA *in situ* hybridization protocol suitable for wholemount early post-implantation mouse embryos and other small tissue samples. This protocol uses digoxigenin (DIG) labeled riboprobes to hybridize with target transcripts, alkaline phosphatase-conjugated anti-DIG antibodies to recognize DIG-labeled nucleotides, and nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) chromogenic substrates for color development. Specific steps and notes on riboprobe preparation, embryo collection, probe hybridization, and color development are all included in the following protocol.

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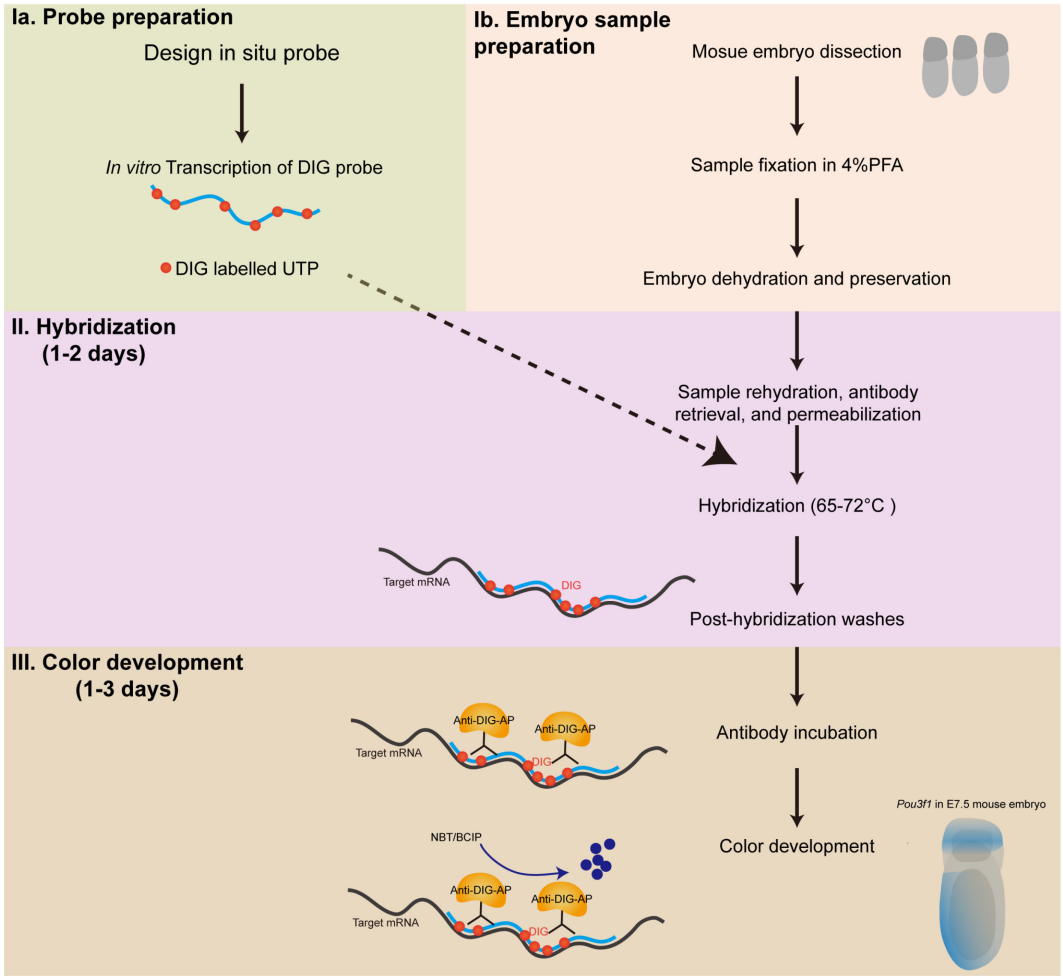
Graphic abstract:



Overview of Wholemount *in situ* Hybridization in Early Mouse Embryos.

Keywords: Wholemount *in situ* hybridization, Mouse embryo, Gene expression visualization, Hybridization

[Background] Wholemount *in situ* hybridization has been widely used to explore gene expression distribution in both tissues and sections (Hauptmann and Gerster, 1994; Nieto *et al.*, 1996). In the field of developmental biology, information on the spatial and temporal distribution of gene expression revealed by *in situ* hybridization has facilitated the identification of master regulators of embryogenesis. In our recent study, we reported that *Pou3f1* is an important regulator of mouse neuroectoderm development by combining wholemount *in situ* hybridization and multiple functional analyses (Zhu *et al.*, 2014). We optimized a wholemount RNA *in situ* hybridization protocol that uses digoxigenin labeled RNA probes and an anti-digoxigenin antibody conjugated with alkaline phosphatase to detect the enrichment of *Pou3f1* in the anterior embryonic region of the mouse gastrula, which indicated potential biological functions of *Pou3f1* in embryonic ectoderm development. Thereafter, more lineage regulators of the mouse gastrulation have been revealed and validated using this optimized protocol (Yang *et al.*, 2018

and 2019; Peng *et al.*, 2016 and 2019). The current protocol exhibits strong experimental robustness and displays application potential in a wide range of biological studies. Thus, we summarize the protocol here, in the hope its application can facilitate the study of gene expression.

The wholemount RNA *in situ* hybridization assay starts with the preparation of digoxigenin labeled RNA probes corresponding to target gene transcripts by using an *in vitro* transcription system and digoxigenin labeled dNTP mix. Pre-fixed embryo samples are treated with H_2O_2 and protease K for antigen retrieval and permeabilization. Embryos are then incubated with RNA probes and hybridized overnight. Several rounds of stringent wash are performed to remove unbound RNA probes. Subsequently, an antibody that recognizes digoxigenin is added to the reaction system and incubated overnight. Color development is performed to visualize the signal, and samples can be stored in a 50% Glycerol/PBS solution.

Further extensions based on the current protocol can be explored in the future, including, but not limited to, replacing digoxigenin labeled RNA probes with multiple fluorescent RNA probes, replacing the AP-conjugated DIG antibody with fluorescent conjugated antibodies, and even combining this method with protein immunofluorescence staining. However, for that to occur, essential optimization and adjustment of experimental conditions should be carefully performed. Noticeably, multiple alternative methods have been established these days, such as RNAscope (Wang, 2012). We recognize that the current protocol may exhibit a relative low detection sensitivity in comparison with RNAscope. Nevertheless, the outstanding properties of experimental robustness, no requirement for specialized instruments, and extremely low economic cost undoubtedly make our protocol an excellent option for the rapid screening and validation of gene expression in multiple fields of biological research.

Materials and Reagents

Note: All materials and reagents should be prepared in a DNase and RNase free environment unless otherwise described.

1. Pipette tips: 10 µl, 20 µl, 200 µl, 1,000 µl Microvolume tips (Axygen®, catalog numbers: TF-300-R-S, TF-20-R-S, TF-200-R-S, TF-1000-R-S)
2. Eppendorf tubes (Axygen®, catalog number: MCT-150-C)
3. 35 mm × 10 mm dish (Corning, catalog number: CLS430165)
4. 24-well plate (Corning, catalog number: 3524)
5. Paraformaldehyde (PFA; Sigma-Aldrich, catalog number: P6148-1kg)
6. DPBS (Gibco, catalog number: 14190144)
7. Tween-20 (Sigma-Aldrich, catalog number: P9416-100ML)
8. Invitrogen UltraPure™ SSC, 20× (Thermo Fisher Scientific, catalog number: 15557044)
9. Yeast RNA (Sigma-Aldrich, catalog number: 10109223001)
10. Heparin (Sigma-Aldrich, catalog number: H3149-500ku)
11. RiboLock RNase Inhibitor (Thermo Fisher Scientific, catalog number: Eo0382)
12. ScriptMAX Thermo T7 Transcription Kit (Toyobo, catalog number: TYB-TSK-101)

13. Methanol (*e.g.*, Sensi Chemical)
14. Formamide (*e.g.*, Sensi Chemical)
15. Proteinase K solution (Invitrogen, catalog number: AM2548)
16. Glutaraldehyde (Sinopharm Chemical, catalog number: 30092436)
17. DIG RNA Labeling Mix (Sigma-Aldrich, catalog number: 11277073910)
18. Anti-Digoxigenin AP antibody (Roche, catalog number: 11093274910)
19. NBT/BCIP stock solution (Sigma-Aldrich, catalog number: 11681451001)
20. Glycerol (Sigma-Aldrich, catalog number: G9012-100 ml)
21. QIAquick Gel extraction kit (QIAGEN, catalog number: 28704)
22. MEGAclear™ Kit (Ambion, catalog number: AM1908)
23. DNase I (RNase-free) (New England Biolabs, catalog number: M0303S)
24. 30% H_2O_2 (w/w) in H_2O (Sigma-Aldrich, catalog number: H1009-100ML)
25. UltraPure 0.5 M EDTA, pH 8.0 (Invitrogen, catalog number: 15575020)
26. Albumin, Bovine Serum, Fraction V, Crystalline (Sigma-Aldrich, catalog number: 9048-46-8)
27. NaCl (Sigma-Aldrich, catalog number: S5886-1KG)
28. Tris base (Sigma-Aldrich, catalog number: TRIS-RO)
29. Magnesium chloride ($MgCl_2$; 1.00 M ± 0.01 M; Sigma-Aldrich, catalog number: M1028-100ML)
30. KOD FX neo (Toyobo, catalog number: KFX-201)
31. UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen, catalog number: 10977015)
32. 4% PFA (see Recipes)
33. PTW buffer (see Recipes)
34. 20 mg/ml Yeast RNA (see Recipes)
35. 50 mg/ml Heparin (see Recipes)
36. Hybridization solution (see Recipes)
37. 10× TBST stock (see Recipes)
38. Blocking buffer (see Recipes)
39. NTMT buffer (see Recipes)
40. 4% PFA/0.1% glutaraldehyde (see Recipes)
41. 6% H_2O_2/PTW solution (see Recipes)

Equipment

1. Thermal cycler (Applied Biosystems, model: 9700)
2. Thin-walled PCR tubes with caps (Axygen®, catalog number: PCR-02-L-C)
3. NanoDrop 2000 (Thermal Scientific)
4. Hybridization incubator (SciGene, model: 2000)
5. Olympus SZX10/16 microscope

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Procedure

Note: All steps should be performed in a DNase and RNase free environment unless otherwise described.

A. Collection of Sample/Embryo

1. Carefully collect tissue samples/mouse embryos (Figure 1) in a 35 mm dish or 24-well plate with DPBS (Downs and Davies, 1993; Piliszek *et al.*, 2011; Pereira *et al.*, 2011).

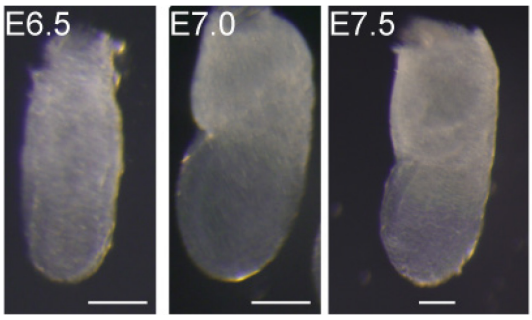

Figure 1. Representative images of the unstained collected mouse gastrula at E6.5, E7.0, and E7.5 stages.

Images were acquired with a Olympus SZX10/16 microscope. Scale bars: 100 μm.

2. Fix embryos in 4% PFA (see Recipes) at 4°C overnight.
3. Transfer the embryos into a graded series of methanol (25% Methanol/DPBS; 50% Methanol/DPBS; 75% Methanol/DPBS; 100% methanol) at room temperature (RT). Embryos are dehydrated for 5 min in each condition. Sufficient volume should be applied to completely submerge the embryo samples.

   Note: Prepare graded series of methanol buffer right before use.

   Pause point: The dehydrated embryos could be stored in 100% methanol at -20°C for up to 1 week.

B. Preparation of digoxigenin labeled RNA Probes

1. Primer design for target cDNA sequence cloning:

   For direct transcription of the PCR product *in vitro*, a minimal T7 promoter sequence (5-TAATACGACTCACTATAGGGAGA-3) should be added to the 5' terminal of the primer. The length of probe sequence should be 250-1,500 bases; probes with 600-900 bases exhibit the highest sensitivity and specificity.
2. PCR amplification and purification of probe DNA:

   A cDNA pool with high enrichment of target transcripts was used as PCR template for amplification of probe DNA. DNA polymerases such as KOD FX Neo with high fidelity characteristics are recommended. The exact PCR conditions should be adjusted according to the selected probe. After agarose gel separation, excise target DNA fragments precisely and

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perform gel extraction following the manufacturer's instructions (Figure 2). Determine the concentration of acquired DNA using NanoDrop 2000.

Note: For in vitro transcription from plasmid, the plasmid should be linearized with appropriate restriction enzyme digestion and purified with a commercial kit.

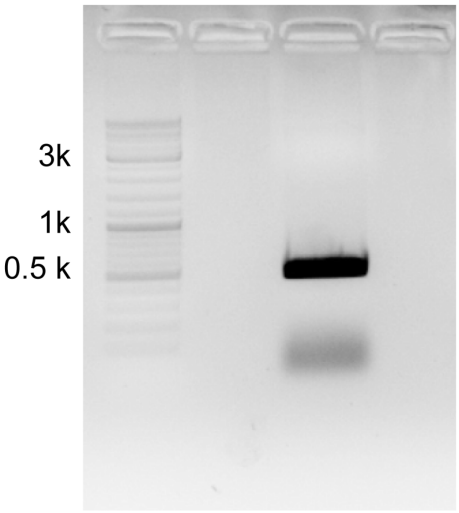

Figure 2. Specific probe DNA (here for *Tal1* gene) amplified through PCR.
To determine PCR specificity, the PCR product is subjected to agarose gel electrophoresis. A specific DNA band can be observed, excised, and purified for further usage.

3. Transcription of the DIG probe:
   a. Prepare the following reaction system:

Component	For 1 µg DNA
DNA	1 µg
10× transcription buffer	3 µl
DIG-nucleotide mix	2 µl
RiboLock RNase Inhibitor	1 µl
T7 RNA polymerase	2 µl
Water	to 30 µl
Total	30 µl

   b. Incubate the reaction at 37°C for 3 h in the thermocycler, with lid temperature no higher than 55°C.
   c. To remove the template DNA, add 0.5 µl DNase I to the reaction mix directly and mix well, then incubate the mix at 37°C for 15 min.
   d. Purify the acquired RNA transcript with MEGAclear™ Kit following the manufacturer's instructions or perform phenol:chloroform extraction followed by alcohol precipitation manually. The RNA probes can be directly dissolved in nuclease free water.

C. Sample rehydration, antigen retrieval, and permeabilization

1. Rehydrate the embryos in graded methanol/PTW buffer (see Recipes) (75%, 50%, and 25% methanol in PTW) for 2-4 min in each concentration, allowing embryos to settle down to the bottom between changes. Wash embryos with PTW for 10 min twice.
   Note: Prepare graded series of methanol buffer right before use.
2. Incubate the embryos in 6% H_2O_2/PTW solution at RT for 10 min, and then wash twice with PTW buffer.
3. Dilute proteinase K in PTW buffer at a final concentration of 10 µg/ml proteinase K in the reaction mix. Remove PTW buffer thoroughly and incubate embryos in 10 µg/ml proteinase K reaction mix at RT. The reaction duration varies for different embryo stages. To specify, for embryos ranging from E7.0 to E9.0 embryos, the appropriate reaction duration should be 7-20 min, but longer times should be pre-tested for more advanced embryos. A pre-experiment for optimization of the conditions is strongly recommended.
4. Remove proteinase K buffer carefully and rinse with PTW buffer twice.
5. Post-fix the digested embryos in 4% PFA/0.1% glutaraldehyde fixation mix (see Recipes). Incubate the embryos for 20-30 min at RT.
6. Remove the fixation buffer, and carefully wash with PTW buffer twice.

D. Hybridization of RNA probes to the embryos

1. Wash the embryos with hybridization solution warmed at 68°C twice; add the hybridization solution (see Recipes) and allow embryos to equilibrate until they sink to the bottom.
2. Incubate for 2-6 h at 65-72°C. The optimal temperature varies between different RNA probes. Usually, a 68°C hybridization temperature works for most probes we have tested.
3. Remove the hybridization solution and replace with the probe diluted in hybridization solution (200-500 ng/ml). Incubate the embryos at their corresponding temperature overnight.
4. Re-collect the probe. Probes in hybridization solution can be re-used 6-8 times. Re-collected probes can be stored at -20°C for up to two months.
5. Wash embryos with hybridization buffer warmed at 70°C for 30 min three times.
6. Wash embryos with 50% hybridization buffer/50% TBST buffer at 70°C for 20 min.
7. Wash embryos with TBST buffer (see Recipes) on a rocker platform at RT three times.

E. Antibody incubation and digoxygenin detection

1. Prepare blocking buffer (see Recipes) and incubate with embryos for 2-3 h at RT.
2. Prepare antibody incubation reaction solution with 1:2,000 diluted anti-digoxigenin AP antibody in blocking buffer. Incubate on a rocker platform at 4°C overnight.
3. Discard the antibody solution and wash the embryos with TBST buffer for 30 min three times. If required, wash the embryos with an extended overnight wash to reduce background signals.
4. Wash the embryos twice with freshly made NTMT buffer (see Recipes).
5. Discard the NTMT buffer and incubate embryos with NBT/BCIP solution (1:50 in NTMT buffer).

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6. Observe the signal frequently during the first two hours of NBT/BCIP solution incubation.
   Note: Refresh the NBT/BCIP solution if the solution turns red.
7. Stop the reaction by rinsing the embryos in TBST approximately three times until an obvious signal appears.
8. Fix the embryos in 4% PFA buffer overnight.
9. Transfer the embryos into 50% glycerol/PBS, and record representative images of embryos samples (Figure 3).
10. The post-fixed embryos can be store at 4°C for more than one year.

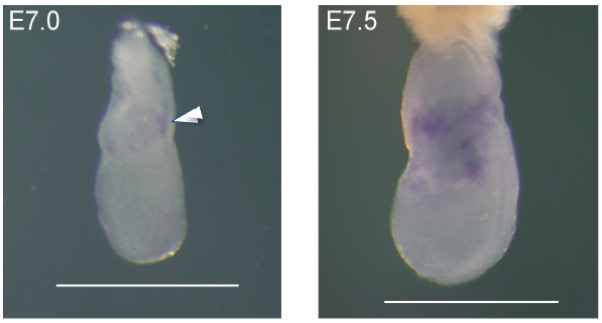

Figure 3. Representative images of wholemount mouse early embryo *in situ* hybridization results of *Tal1* gene.
The images list embryos at E7.0 and E7.5 stages, from which *Tal1* starts to be expressed in extraembryonic mesoderm cells, as indicated by the triangle at E7.0, and peaking at E7.5. Both embryos were stained with the same probe against the *Tal1* transcript. The images were taken using an Olympus SZX10/16 microscope. Scale bars: 500 μm.

Recipes

1. 4% PFA
   4 g of paraformaldehyde in 100 ml of DPBS, thoroughly dissolve.
   Adjust pH to 7.4-7.6 using 1 M NaOH solution and store in 4°C for up to one week.
   Note: Take care to avoid direct contact with PFA powder and solution.
2. PTW buffer
   Calcium and magnesium free DPBS with 0.1% Tween-20.
   Store at room temperature for up to one week.
   Note: Take care to avoid direct contact with Tween-20 solution due to potential harm to skin.
3. 20 mg/ml Yeast RNA
   Dissolve 20 mg of Yeast RNA in 1 ml of nuclease free water and mix thoroughly.
   Store at -20°C for up to one month.
4. 50 mg/ml Heparin
   Dissolve 50 mg of Heparin in 1 ml of nuclease free water and mix thoroughly.
   Store at -20°C for up to one month.

5. Hybridization solution

   Store at -20°C for up to one month.

Component (stock conc.)	**Final conc.**	**Volume to add**
Formamide	50%	25 ml
SSC (20×, pH 5.3 adjusted with citric acid)	1.3× SSC	3.25 ml
EDTA (0.5 M, pH 8.0)	5 mM	0.5 ml
Yeast RNA (20 mg/ml in H_2O)	50 µg/ml	125 µl
Tween-20	0.002	100 µl
Heparin (50 mg/ml in H_2O)	100 µg/ml	100 µl
UltraPure™ DNase/RNase-Free Distilled Water		Replenish to 50 ml
Total	50 ml	

6. 10× TBST stock

   Store at 4°C for up to one month.

Component	**Mass**
NaCl	4 g
KCl	0.1 g
1 M Tris-HCl pH 7.5	12.5 ml
Tween-20	5.5 g
H_2O	Replenish to 50 ml
Total	50 ml

7. Blocking buffer

   1 mg/ml BSA in 1× TBST

   Store at 4°C for up to one week

8. NTMT buffer

   Prepare right before use; store at room temperature for up to 2 days.

Component (stock concentration)	**Final concentration**	**Volume to add**
2.5 M NaCl	0.1 M	1 ml
2 M Tris-HCl (pH 9.5)	0.1 M	1.25 ml
1 M $MgCl_2$	0.05 M	1.25 ml
Tween-20	1%	0.25 ml
H_2O		~21.25 ml
Total		25 ml

9. 4% PFA/0.1% glutaraldehyde

   Dilute 25% glutaraldehyde in freshly prepared 4% PFA to a final concentration of 0.1%. Prepare right before use.

   Note: Take care to avoid direct contact with PFA and glutaraldehyde solution.

10. 6% H_2O_2/PTW solution

    Dilute 30% H_2O_2 stock buffer in freshly prepared PTW buffer to a final concentration of 6%. Prepare right before use.

Note: Take care to avoid direct physical contact with H_2O_2 solution.

Acknowledgments

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Competing interests

The authors declare no conflicts of interest or competing interests.

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