Chapter 31

Zebrafish Whole-Mount *In Situ* Hybridization Followed by Sectioning

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Abstract

In situ hybridization is a powerful technique used for locating specific nucleic acid targets within morphologically preserved tissues and cell preparations. A labeled RNA or DNA probe hybridizes to its complementary mRNA or DNA sequence within a sample. Here, we describe RNA in situ hybridization protocol for whole-mount zebrafish embryos.

Key words In situ hybridization, Zebrafish, DIG-labeled RNA antisense probe, Pre-hybridization, NBT/BCIP staining, Sectioning

1 Introduction

The zebrafish is increasingly acknowledged for its ability to model human diseases, and as a useful organism in drug discovery and development [1]. Zebrafish models of the Na^+/K^+-ATPase α_2 and α_3 isoforms aim to further highlight the development of the FHM2 and RDP diseases and enable pathology and later drug screenings in larger scale prior to trials on mammalian models.

1.1 Animal Models

The development of gene modified Na^+/K^+-ATPase mouse models has revealed insight into part of the pathology of FHM2 and RDP through a subset of biobehavioral assays and biochemical approaches [2]. Although reverse genetics are feasible in the mouse, it is time and money consuming, and in this context, the zebrafish (*Danio rerio*) has emerged as a model system that allows rapid reverse genetic experiments and robust assays in which a range of hypomorphic phenotypes can be generated in a rapid, cost-effective, and gene-specific manner [3]. Therefore, to assess mechanisms of human diseases in model systems, several advantages are united through the combination of different model organisms, such as mouse and zebrafish, which offer different experimental options.

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1.2 Zebrafish as Model System

Zebrafish have evolved as an excellent embryologically and genetically tractable vertebrate model system. High genetic and organ system homology to humans, external development with optical clarity, small size, short generation time (2–3 months), production of high number of embryos (over 200/female/week), and less expensive maintenance comprise the multifactorial advantages of zebrafish as a model organism over classical vertebrate models [1, 4].

The gross architecture of many zebrafish brain areas, e.g., the retina, the olfactory bulb, the hypothalamus, the cerebellum, and the spinal cord, is similar to human brain structures, although differences between teleosts and mammals do exist [5]. The main neurotransmitter systems, such as the cholinergic, dopaminergic, and noradrenergic pathways are present and have been mapped throughout the zebrafish brain [6]. Indeed, several neurotransmitter systems have been assessed using zebrafish, e.g., the cocaine- and amphetamine-regulated transcript peptidergic system [7], glutamatergic, serotonergic and dopaminergic neurotransmitter systems [8–13].

Indeed, it has served as a model to study several neurological disorders, e.g., Parkinson's disease (PD) [14–16], Alzheimer's disease [17, 18], Huntington's disease [19], and Schizophrenia [20]. Thus, there are several reports supporting that zebrafish models of neurological disorders can serve to reveal the pathogenic mechanisms underlying human diseases [21–24].

Zebrafish have been efficiently used to study ion transport, and zebrafish Na^+/K^+-ATPase models have been previously addressed in different contexts. A recent review suggested zebrafish embryos as a powerful in vivo model for understanding body fluid ion homeostasis and hormonal control in association with four types of ionocyte expressing distinct sets of transporters H^+-ATPase-rich, Na^+/K^+-ATPase-rich, Na^+-Cl^- cotransporter-expressing and K^+-secreting cells [25].

The zebrafish model system offers unique properties and serves to further help unravel the pathologies for the neurological disorders caused by Na^+/K^+-ATPase dysfunction [26]. For instance, zebrafish Na^+/K^+-ATPase has been associated with schizophrenia, where Na^+/K^+-ATPase activity is reduced in animals exposed to NMDA antagonist, MK-801, which mimic schizophrenia via NMDA hypofunction [27].

Recent studies assessing the functions of α_2 and $\alpha_3 Na^+/K^+$-ATPase by morpholino-induced knock-down of ATP1α_2 and ATP1α_3 have established functions for the $\alpha_2 Na^+/K^+$-ATPase in skeletal and heart muscles as well as astrocytes [28]. The roles in regulation of left-right (LR) patterning in conjugation with Ncx4a [29, 30] for $\alpha_2 Na^+/K^+$-ATPase, and regulation of brain ventricle volume and embryonic motility for $\alpha_3 Na^+/K^+$-ATPase, are likely linked to a depolarization of the resting membrane potential [28, 31].

Detecting specific RNA sequences within cells and tissues by in situ hybridization (ISH) using chemically labeled antisense RNA probes emerged in 1990s [32, 33]. ISH is a valuable method for assessing the distribution of the expression of a gene of interest and developing hypotheses about its functions. The optically clear nature of zebrafish embryos makes ISH more readily applicable to zebrafish embryos, and indeed ISH is one of the most commonly used techniques for zebrafish researchers. Fixed, pre-hybridized zebrafish embryos are hybridized with a digoxigenin uridine-5′-triphosphate (DIG) labeled RNA probe specific to the gene of interest. Fab fragments from polyclonal anti-DIG antibodies, conjugated to alkaline phosphatase are added to bind to DIG. The staining is performed by adding a combination of NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3′-indolylphosphate p-toluidine salt), which then yields an insoluble black-purple precipitate when reacted with alkaline phosphatase.

Here, we outline a step by step protocol, adapted from Thisse and Thisse, 2008 [34], for in situ hybridization on whole-mount zebrafish embryos, also used for detecting *Atp1a2*, *Atp1a3a*, and *Atp1a3b* transcripts [28, 31].

2 Materials

All solutions should be prepared using autoclaved ultrapure water (resistivity levels of 18.2 MΩ cm at 25 °C).

2.1 RNA Probe Synthesis

1. Template DNA harboring RNA probe coding sequence.
2. T3 or T7 RNA polymerase (20 U/μL).
3. 10× transcription buffer.
4. DTT (0.1 M).
5. DIG-RNA labeling mix.
6. RNAse inhibitor (40 U/μL).
7. DNAseI (RNase-free).
8. 0.5 M ethylenediaminetetraacetic acid (EDTA).
9. RNA cleanup column, e.g., SigmaSpin post-reaction cleanup column.
10. RNAlater®.
11. Nuclease free water.

2.2 Egg Collection/ Fixation

1. 1× E3 embryonic medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ in water. Add 100 μL of 1 % methylene blue as a fungicide to 1 L of medium.
2. Pronase (protease from *Streptomyces griseus*): To prepare 1 % (w/v) pronase solution dissolve 1 g of pronase in 100 mL of

1× E3 medium, incubate for 2 h at 37 °C, aliquot and store at –20 °C.

3. 0.003 % 1-Phenyl-2-thiourea (PTU) in 1× E3 medium.
4. Anesthetic solution (0.008 % 3-amino benzoic acid ethylester (*tricaine*)): To prepare the tricaine stock solution dissolve 400 mg tricaine powder in 97.9 mL water. Add 2.1 mL of 1 M Tris–HCl, pH 9.0. Adjust pH to 7.0 and store the tricaine stock in aliquots at –20 °C. To prepare working solution add 2 mL of tricaine stock solution to 98 mL of 1× E3 medium.
5. 4 % paraformaldehyde (PFA) (*see* **Note 1**).
6. Methanol dilutions; prepare 25, 50, and 75 % methanol in 1× PBS.
7. A 100 mL beaker.
8. 100-mm petri dishes (some of them coated with 2 % agarose).
9. 35-mm petri dishes coated with 2 % agarose.
10. 1.5 mL microcentrifuge tubes.
11. Incubator (28.5 °C).

2.3 Hybridization

1. 1× PBS-T: 1× PBS (phosphate-buffered saline) with 0.1 % Tween 20 (v/v).
2. Proteinase K (20 mg/mL).
3. 20× SSC: 0.3 M sodium citrate, 3 M NaCl, pH 7.0. Make 2× and 0.2× SSC dilutions in water.
4. Hybridization mix minus (HM–): 50 % formamide, 5× SSC, 0.1 % Tween 20, adjust pH to 6.0 with 1 M citric acid.
5. Hybridization mix plus (HM+): HM$^-$ with 500 μg/mL RNA from Torula yeast, 50 μg/mL heparin.
6. Blocking buffer: 2 % sheep serum (v/v), 2 mg/mL bovine serum (BSA) in 1× PBS-T.
7. HM- dilutions in 2× SSC (75, 50, 25 %).
8. 100 % 2× SSC.
9. 100 % 0.2× SSC.
10. Sheep anti-digoxigenin-AP Fab fragments.
11. Alkaline Tris buffer: 0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl, and 0.1 % Tween 20 (v/v) (*see* **Note 2**).
12. Labeling solution: 1:50 dilution of NBT/BCIP stock solution (solution of 18.75 mg/mL nitro blue tetrazolium chloride and 9.4 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate, toluidine-salt in 67 % (DMSO) (v/v)).
13. Stop solution: 1× PBS, pH 5.5, 1 mM EDTA, 0.1 % Tween 20 (v/v).
14. Hybridization oven (70 °C).

2.4 Sectioning

1. Ethanol solutions; 70, 96 and 99 %.
2. 5 % agarose.
3. 2-Hydroxyethyl methacrylate (Technovit 7100).
4. Dehydrating machine Citadel 1000.
5. Rotatory microtome Microm HM 355 with Ralph glass knives.

3 Methods

3.1 Probe Synthesis

1. Generate a template DNA harboring the coding sequence for antisense RNA probe. Sense RNA probe can also be generated as a control. This can be done by amplifying the sequence to be targeted from cDNA and subcloning into a vector with a T3 or T7 RNA polymerase promoter located at 3′ end (for antisense probes) or 5′ end (for sense probes). Another way to generate a template would be PCR amplifying this sequence and including T3 or T7 RNA polymerase promoter in the 5′ end of the reverse primer (for antisense probes) or the forward primer (for sense probes). Table 1 lists primers used to generate the ISH probes as well as the ISH probe sequences.
2. Prepare the mix indicated in Table 2 below and incubate for 2 h at 37 °C.
3. Add 2 μL RNAse free DNAse I and 18 μL nuclease-free water. Mix and incubate for 30 min (min) at 37 °C.
4. Stop the reaction by adding 1 μL sterile 0.5 M EDTA and 9 μL nuclease-free water.
5. Purify the probe using an RNA cleanup column, e.g., SigmaSpin post-reaction cleanup column.
6. Add 1 μL sterile 0.5 M EDTA and 9 μL of RNAlater to the sample; this protects the RNA from degradation. Store at −80 °C.

3.2 Preparation of Zebrafish Embryos

1. Collect zebrafish eggs and place them in a 100 mL beaker covered with a minimal amount of 1× E3 medium.
2. To remove the chorion, add pronase (1 % w/v) to a dilution of 1:10 depending on the medium volume in the beaker. Incubate for 5 min at 28.5 °C swirling occasionally (*see* **Note 3**).
3. Gently rinse the eggs three times with 1× E3 medium. Avoid the contact of the embryos with the medium/air interface.
4. Place the embryos into an agarose coated petri dish and grow them to the desired stage at 28.5 °C.
5. To prevent pigmentation, change the buffer to 0.003 % PTU solution (*see* **Note 4**) at the end of gastrulation (defined by the 50 %-epiboly stage, which begins at 5.25 h of development at 28.5 °C).

Table 1
List of primers used to generate the ISH probes, and well as the sequence of the ISH probes

1. List of primers and probes	
Primers used for generating probes	
AS *Atp1a2*	T7, 5'-TAATACGACTCACTATAGGG-3' DR-atp1a2-probeR, 5'-AAGCAGAGGATGGCACCGAT-3'
AS *Atp1a3a*	DR-atp1a3-p-R-T7, 5'-TAATACGACTCACTATAGGGGATTGCTCCGATCCACAGCA-3' DR-atp1a3-probeF, 5′-AACCACGCAGGACAATGATG-3′
AS *Atp1a3b*	DRatp1a3b-probeF, 5′-TCTCGCCCCACAATTTCCTA-3′ atp1a3b-probeRT7, 5′-TAATACGACTCACTATAGGGAGCCACCAAAATCCACCAGA-3′
Probe	*Sequence*
Atp1a2 (**316 bp**)	5′-GGAAAGGGTACGGACATGAAAGCAGCCCGGAGGCGGCACCAACGGGGGGAAAGA GGAAGAAGAAGGATAAAGATTTAGATGAGCTGAAGAAAGAGGTGTCACTGGATGAT CATAAGCTGACTCTGGATGAGCTCAGTACTCGTTATGGAGTTGACCTTGCCAGAGGT TTGACCCATAAGAGAGCGATGGAAATCCTGGCGCGTGACGGTCCAAATGCGCTGACC CCTCCGCCCACCACCCCGGAGTGGGTGAAGTTCTGCAAGCAGCTGTTTGGAGGATTC TCCATCCTGCTCTGGATCGGTGCCATCCTCTGCTT-3′
Atp1a3a (**292 bp**)	5′-AACCACGCAGGACAATGATGATAAGGAATCTCCCAAAAAGGGAAAGGGAGGGAA AGACCTTGATGACTTGAAGAAGGAAGTGCCACTGACAGAGCATAAGATGTCGATTG AGGAAGTGTGCCGAAAGTACAACACTGACATCGTACAGGGTCTGACTAATGCCAGG GCTGCAGAGTACCTCGCTCGGGATGGTCCCAATGCTCTCACCCCTCCACCCACCACC CCGGAGTGGGTCAAATTCTGCCGACAGCTCTTCGGAGGTTTCTCCATCCTGCTGTGG ATCGGAGCAATC-3′
Atp1a3b (**330 bp**)	5′-TCTCGCCCCA CAATTTCCTATAATACTCTT TGCATGTATT TTCACACGGC TGTGTACATA AGTCTACTA TATATCTAAAACCGGCAGCT GCATAACCAT CATGTAGGTT GGAAAGGAGC TTTTGTCGCT ATAAAGGGAA TATGTACGCT TGCCTATTTA AACAAAAGAC TGTCCCTCCA TAAGCCCATG CCTTTCCCTC CAGTCACACA CACACAGTTC ACACAGAACA CCTCTACACA TACTCTAGAC TGCGTTTGAC ATAATAGTTT CTCGTTTTCA TGTTTTGTTC TTTAATTGAG CTCTTTAATC TCTGGTGGAT TTTGGTGGCT-3′

Table 2
List of components for RNA synthesis

Component	Amount per reaction	Final
PCR product or linearized plasmid DNA		100–200 ng
Transcription buffer (10×)	0.5 µL	1×
DTT (0.1 M)	0.5 µL	10 mM
DIG RNA labeling mix (10×)	0.5 µL	1×
RNAse inhibitor (40 U/µL)	0.25 µL	10 U
T3 or T7 RNA polymerase (20 U/µL)	0.25 µL	5 U
H_2O	Up to 5 µL	

6. If the embryos are older than 24 h post-fertilization (hpf), anesthetize them in 0.008 % tricaine.
7. Fix dechorionated embryos in 4 % PFA, overnight (ON) at 4 °C in 35 mm petri dishes.
8. Collect the embryos in 1.5 mL microcentrifuge tubes, 30–50 embryos per tube, (the rest of the protocol can be performed in these tubes) and dehydrate in 100 % methanol for 3× 5 min at room temperature (RT).
9. Place the embryos to -20 °C in fresh 100 % methanol for at least 2 h. Embryos can be stored in methanol at –20 °C for several months.

3.3 Pre-hybridization

1. Rehydrate the embryos by 5 min washes in serial dilutions of methanol in 1× PBS: 75, 50 and 25 % methanol (v/v).
2. Wash 4×5 min in 1× PBS-T.
3. Permeabilize embryos in proteinase K solution (10 µg/mL in 1× PBS-T) at RT for a period of time depending on the stage of the embryo:
   (a) 0–18 somite — 0 min.
   (b) 18 somite to 30 hpf — 5 min.
   (c) 36 hpf — 10–15 min.
   (d) 48 hpf — 35–40 min.
   (e) 60 hpf — 45 min.
   (f) 72 hpf to 6 dpf — 60 min.
4. Stop the proteinase K digestion by replacing proteinase K solution with 4 % PFA solution, for 20 min at RT.
5. Discard the 4 % PFA and wash the embryos 4×5 min in 1× PBS-T to remove the traces of PFA.

6. Wash the embryos 5 min in HM–.
7. Replace HM– with 700 μL of HM+ and incubate the embryos in an hybridization oven set to 70 °C for 2–5 h. Embryos in HM+ can be stored at –20 °C for several weeks.

3.4 Hybridization and Staining

1. Discard HM+ and replace it with fresh HM+ containing 30–50 ng antisense DIG-labeled RNA probe (*see* **Note 5**), incubate the embryos at 70 °C ON (*see* **Note 6**).
2. Pre-warm the following buffers at 70 °C prior to use: HM–, HM– dilutions in 2× SSC (75, 50, 25 %), 100 % 2× SSC, 100 % 0.2× SSC.
3. Remove the probe (probe can be saved at –80 °C for several usages), add HM– and incubate at 70 °C for 10 min with gentle agitation. Gradually change HM– to 2× SSC by 10 min washes at 70 °C with gentle agitation: 75, 50 and 25 % HM– in 2× SSC, and 100 % 2× SSC.
4. Wash 2 × 30 min in 0.2× SSC at 70 °C.
5. Perform the following washes at RT. Gradually change 0.2× SSC to 1× PBS-T by 10 min washes with gentle agitation: 75, 50 and 25 % 0.2× SSC in 1× PBS-T, and 100 % 1× PBS-T.
6. Incubate the embryos in blocking buffer at RT for 3–4 h.
7. Replace the blocking buffer with fresh blocking buffer including anti-DIG antibody (1:5000 to 1:10,000) and incubate ON at 4 °C with gentle agitation.
8. Remove the antibody solution and wash the embryos 6 × 15 min in 1× PBS-T at RT.
9. Wash 3 × 5 min in alkaline Tris buffer.
10. Replace the alkaline Tris buffer with 700 μL of labeling solution (1:50 dilution of NBT/BCIP stock solutions). Protect embryos from exposure to light and incubate at RT until desired staining intensity is reached.
11. Observe the embryos occasionally for staining and when the desired staining intensity is reached (*see* **Notes 5** and **6**), stop the reaction by replacing the labeling solution with stop solution.
12. Wash 3 × 15 min in stop solution.
13. For embryos at early stages (younger than 15-somite stage), in order to clear the yolk that darkens upon staining and affects visualization of the tissues on it, embryos can be incubated in an acidic buffer (1× PBS, pH 3.0) for 5 min.
14. Transfer the embryos to 35 mm petri dishes with 100 % glycerol carrying a minimal amount of stop solution. Store at 4 °C in dark.

3.5 Sectioning

1. Wash the embryos in 1× PBS-T to remove glycerol.
2. Embed and orient the embryos in 5 % agarose, so they could be cut transversely with respect to the length direction.
3. Dehydrate the agarose block including the embryos in ascending alcohol solutions 1½ h in 70 % EtOH, 1¼ h in 96 % EtOH, and 1¼ h in 99 % EtOH.
4. Infiltrate the agarose block for 2 h in 2-hydroxyethyl methacrylate, then followed by infiltration in 2-hydroxyethyl methacrylate. After addition of hardener, the time of workability with samples is approximately 5–8 min and the specimens will cure within a couple of hours at RT.
5. Take a 4- and 20-μm-thick section on a rotatory microtome equipped with Ralph glass knives for each 200 μm, collecting sections in a systematic, uniformLy and random manner.
6. Image and analyze the sections using light microscopy.

A representative image, whole-mount and sections of it, of a zebrafish embryo stained by this protocol for *Atp1a3a* transcripts [26] is illustrated in Fig. 1.

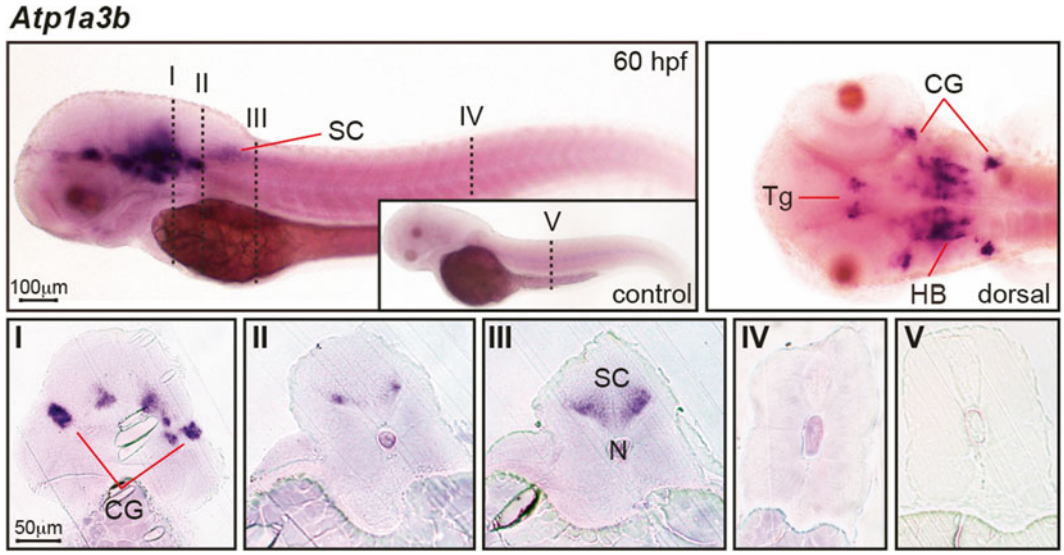

Fig. 1 Expression of *Atp1a3b* mRNA in zebrafish embryos. *Atp1a3b* mRNA expression analyzed by whole-mount in situ hybridization in 60 hpf zebrafish embryos; the *inset* shows sense probe hybridized control embryo. *Atp1a3b* is expressed in specific brain regions. The *numbered vertical dashed lines* show the positions of the transverse sections shown below in *sections I–V*. The abbreviations used are: *CG* cranial ganglia, *HB* hindbrain, *N* notochord, *Tg* tegmentum, *SC* spinal cord. Scale bars represent 100 μm in whole-mount images and 50 μm in sections

4 Notes

1. Prepare the PFA solution in a ventilated hood. Add 4 g PFA into 80 mL of 1× phosphate buffered saline (PBS) that is heated approximately to 60 °C (be careful not to boil) on a stir plate. Keep stirring while slowly raising the pH by adding 1 N NaOH drops until the solution clears (PFA will not dissolve unless the pH is basic). Once the PFA is dissolved, adjust the volume of the solution to 100 mL with 1× PBS and the pH to approximately 7.4 by diluted HCl. Cool the solution and filter it using a Millipore 0.22 μm filtration system. The solution can be aliquoted and frozen at –20 °C or stored at 4 °C for up to 1 month.
2. For DIG system applications of Roche NBT/BCIP stock solution (*see* below), $MgCl_2$ is not included in this buffer as this might lead to spotty background after the detection procedure.
3. This step is not needed if the protocol will be performed on embryos at a stage upon hatching (48–72 h post-fertilization (hpf)).
4. If the embryos are not raised in PTU, embryos can be bleached by incubation in 3 % H_2O_2/0.5 % KOH at RT until the pigmentation disappears. This procedure can be applied after fixing the embryos in 4 % PFA and before dehydrating in methanol. Upon bleaching, wash the embryos in 1× PBS and proceed with the methanol dehydration step.
5. The probe concentration can be optimized if embryos are over/under stained.
6. The reaction time varies depending on the expression level of the assessed gene in the range of 15 min up to 10 h.

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