Video Article

High Resolution Whole Mount *In Situ* Hybridization within Zebrafish Embryos to Study Gene Expression and Function

Babykumari P. Chitramuthu[1,2], Hugh P. J. Bennett[1,2]

[1]Endocrine Research Laboratory and Department of Medicine, Royal Victoria Hospital

[2]McGill University Health Centre Research Institute

Correspondence to: Babykumari P. Chitramuthu at babykumari.chitramuthu@mail.mcgill.ca, Hugh P. J. Bennett at hugh.bennett@mcgill.ca

URL: https://www.jove.com/video/50644
DOI: doi:10.3791/50644

Keywords: Neuroscience, Issue 80, Blood Cells, Endoderm, Motor Neurons, life sciences, animal models *in situ* hybridization, morpholino knockdown, progranulin, neuromast, proprotein convertase, anti-sense transcripts, intermediate cell mass, pronephric duct, somites

Date Published: 10/19/2013

Citation: Chitramuthu, B.P., Bennett, H.P. High Resolution Whole Mount *In Situ* Hybridization within Zebrafish Embryos to Study Gene Expression and Function. *J. Vis. Exp.* (80), e50644, doi:10.3791/50644 (2013).

Abstract

This article focuses on whole-mount *in situ* hybridization (WISH) of zebrafish embryos. The WISH technology facilitates the assessment of gene expression both in terms of tissue distribution and developmental stage. Protocols are described for the use of WISH of zebrafish embryos using antisense RNA probes labeled with digoxigenin. Probes are generated by incorporating digoxigenin-linked nucleotides through *in vitro* transcription of gene templates that have been cloned and linearized. The chorions of embryos harvested at defined developmental stages are removed before incubation with specific probes. Following a washing procedure to remove excess probe, embryos are incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase. By employing a chromogenic substrate for alkaline phosphatase, specific gene expression can be assessed. Depending on the level of gene expression the entire procedure can be completed within 2-3 days.

Video Link

The video component of this article can be found at https://www.jove.com/video/50644/

Introduction

The zebrafish (*Danio rerio*) has emerged as a powerful animal model for the study of vertebrate development, disease, behavior, and in-drug screening[1-3]. Zebrafish embryos can be obtained in large numbers from a single crossing. Fertilization and development occurs *ex utero* and the optically clear embryos develop rapidly. Critical developmental events occur during the first 48 hr post-fertilization (hpf). This includes the appearance of organ primordia and the initiation of cytodifferentiation. Knockdown or over-expression of proteins can be achieved through the microinjection of embryos with antisense morpholino (MO) oligonucleotides or mRNA respectively at the one or two cell stage (0.75 hpf).

The WISH of zebrafish embryos facilitates the study of the spatio-temporal expression of specific genes of interest. Application of the WISH technique following microinjection of embryos with mRNA or MO to over-express or knockdown specific protein levels reveals differential regulation of other genes.

Changes in gene expression patterns can be correlated with phenotypic changes and reveal the function of target genes during early organogenesis. Since probes can be prepared and stored in advance, the ISH technique can be applied to reveal gene expression patterns for at least 20 genes at a time using six-well plates and custom made baskets.

Protocol

1. Whole-mount *In Situ* Hybridization of Zebrafish Embryos

1.1 Preparation of Embryos

1. Collect embryos at the required developmental stages. Please see Kimmel *et al*.5 for embryonic stage description.
2. Remove chorions manually using forceps (Dumont Watchmakers Forceps no. 5).
3. Fix embryos in a 4% solution of paraformaldehyde (PFA) made up in PBS (Phosphate Buffered Saline) overnight at 4 °C.
4. Wash embryos with PBS at least 3x for 5 min each at room temperature.
5. Store embryos in 100% methanol (MeOH) at -20 °C until used for future application of techniques including whole-mount *in situ* hybridization.
6. Freeze staged embryos in liquid nitrogen for RNA extraction and store at -80 °C.
7. Extract RNA from staged embryos and synthesize cDNA for PCR amplification and subsequent cloning.

1.2 Synthesis of Digoxygenin-labeled RNA Probes

1. PCR amplification and gene cloning for probe template. Setup PCR reaction with total volume of 50 µl as shown in **Table 1**. Use more genomic DNA or less cDNA in the PCR reaction as template. Include 10 min extension time at 72 °C following the last cycle to make sure that the PCR reaction products are full length and 3' adenylated in order to facilitate TOPO cloning.

Reagent	**Volume**
Template DNA	100 ng
PCR Buffer (10x)	5 µl
dNTPs (10 mM)	2 µl
Primers (10 mM)	1 µM each
Add water to a final volume of	49.7 µl
Taq Polymerase (5 unit/µl)	0.3 µl
Reaction Volume	**50 µl**

Table 1. PCR amplification reaction.

2. Verify the size of the PCR product using agarose gel electrophoresis. The estimated size of the PCR product for probe synthesis is close to 1 kb.
3. Take special care to avoid nuclease contamination by carrying out the experiment under sterile conditions and using nuclease free water. In the event that multiple products are observed, gel-purify the appropriately sized PCR product before proceeding with cloning using the TOPO TA Cloning Kit. Optimize the PCR reaction to eliminate the appearance of smearing and multiple bands.
4. Perform the TOPO cloning reaction as indicated in **Table 2**. Combine TOPO Vector and PCR Product and incubate at room temperature for 5 min.

Reagent	**Chemically Competent Cells**	**Electro Competent Cells**
Fresh PCR reaction product	1-4 µl	1-4 µl
Salt Solution	1 µl	
Diluted Salt Solution		1 µl
Water	Up to total volume of 5 µl	Up to total volume of 5 µl
TOPO vector	1 µl	1 µl
Final Volume	**6 µl**	**6 µl**

Table 2. TOPO cloning reaction.

5. Incorporate/introduce the TOPO cloning product into one shot competent cells. This process is called transformation.
6. Incubate the transformed cells at 37 °C for at least 1 hr at 260 rpm to ensure the expression of the antibiotic resistance genes. Plate the transformation mix (50-100 µl) on prewarmed Luria Bertani (LB) agar plates containing 50 µg/ml ampicillin and X-gal. Incubate at 37 °C overnight.
7. Determine the presence of the insert based on appearance of white or light blue colonies. The TOPO vector contains lacz gene which encodes β-galactosidase. In the presence of X-gal, production of β-galactosidase forms blue color. DNA ligated into the plasmid disrupts the formation of functional β-galactosidase and white colonies are produced. White colonies or light blue colonies confirm the presence of the insert.
8. Pick the white colonies from the agar plate using sterile pipette tips and place them in the sterile 10 ml tube containing 4 ml of LB media and culture at 37 °C in the shaking incubator overnight at 200 rpm.
9. Purify plasmid DNA using plasmid DNA kit according to the manufacturers instruction.
10. Linearize cDNA following plasmid purification by digesting 5 µg of cDNA for each probe with the appropriate restriction enzyme that has a unique site located 3' (for sense probes) or 5' (for antisense probes) to the insert.
11. Purify the linearized DNA using the phenol/chloroform extraction method. Add equal volume of phenol/chloroform, vertex vigorously for 30 sec, spin for 3 min at 13,000 rpm at 4 °C. Transfer upper aqueous phase into clean tube and add equal volume of chloroform. Spin for 3 min at 13,000 rpm at 4 °C. Transfer upper aqueous phase into clean tube. Add 10% sodium acetate (3 M) and 2.5x volume of 100% ethanol to precipitate DNA and store at -20 °C for overnight. Spin for 15 min at 13,000 rpm at 4 °C. Remove ethanol and wash the pellet with 75% ethanol. The protocol can be stopped and DNA can be stored at -20 °C and resumed the next day. Spin for 10 min at 10,000 rpm at 4 °C. Remove the supernatant carefully and dry the pellet for 2 min at room temperature. Resuspend the pellet in 10 µl of DEPC treated water and incubate at 55 °C for 20 min. Quantify DNA concentration using the UV spectrophotometer.
12. Assess the purity by agarose gel electrophoresis using 1 µl of DNA on 1% agarose gel and running for about 30 min.
13. Set up RNA labeling reaction. Using an RNase free reaction vial, add 1-5 µg of purified template DNA to sterile, RNase-free, DEPC-treated, double distilled water to a volume of 13 µl. Chill the reaction vial on ice and add the following reagents (*see* **Table 3**):

Reagent	**Volume**
NTP labeling mixture (10x)	2 µl
Transcription buffer (10x)	2 µl

Protector RNase inhibitor	1 µl
RNA Polymerase SP6 **or**	
RNA Polymerase T7	2 µl
Final Volume	**20 µl**

Table 3. RNA labeling reaction.

14. Mix the reagent gently then collect at the bottom by brief centrifugation at 2,000 rpm for 30 sec at room temperature and incubate the reaction mixture for 2 hr at 37 °C.
15. Remove the template DNA by adding 2 µl RNase-free DNase I. Incubate for 30 min at 37 °C and stop the reaction by adding 2 µl 0.2 M EDTA (pH 8.0).
16. Precipitate the reaction products by adding 2.5 µl 4 M LiCl and 75 µl 100% ethanol. Mix the solution and keep at -20 °C for 45 min or stored at -20 °C over-night and the protocol resumed the next day.
17. Centrifuge the suspension for 30 min at 13,000 rpm at 4 °C, then wash the pellet with 500 µl 70% ethanol stored at -20 °C, centrifuge for a further 10 min, dry for 2 min, and resuspend in 50 µl and incubate at 30 min at 37 °C.
18. Verify the probe quality and integrity by running 1-2 ml on 1% agarose gel for about 30 min. This is confirmed by the presence of single product running at the expected size on the gel.
19. Quantify the concentration of the probe by measuring the amount of RNA using spectrophotometer. The yield from this reaction is about 10 µg of RNA (100-200 ng/µl).

1.3 Whole-mount *In Situ* Hybridization

1. **DAY 1**
    1. **Rehydration**
        1. Prepare baskets using nylon mesh (less than 1 mm pore size) and Eppendorf tubes. Construct the baskets by cutting Eppendorf tubes (1.5 ml) to remove the conical ends. To identify individual baskets use different colored tubes and remove the top rims from half of them.
        2. Cut small pieces of nylon mesh to cover the cut ends of the Eppendorf tubes. Stick the cut ends of the tubes to the nylon mesh by heating them on an electrical hot plate (set between medium to high) in a fume hood. Once cooled remove the excess nylon mesh from the baskets and store them in 100% methanol at room temperature.
        3. Prepare successive dilutions of methanol in PBS (75% [vol/vol[methanol, 50% [vol/vol] methanol and 25% [vol/vol] methanol) using the first three wells of the six-well plates followed by 100% PBST(phosphate buffered saline containing 0.1% Tween-20) in the next three wells.
        4. Subject embryos to *in situ* the hybridization procedure using the custom made baskets as shown in **Figurea 1a** and **b**. Use RNase-free solutions up to the hybridization step in 6-well plates (4 ml/well).To achieve this, place up to 6 baskets in the first well. Place basket with rim first followed by baskets without rims in different colors arranged consecutively. Using this arrangement up to 6 gene expression patterns can be determined simultaneously.
        5. Place dehydrated embryos of the same developmental stage to the same basket (*see* **Figure 1**).
        6. Rehydrate embryos by moving baskets from one well in the six-well plate to the next (5 min for each step). Use six wells filled with appropriate buffer. Embryos are subjected to each dilution once. In this way embryos are washed 6x, 5 min/wash. Rehydration of the dehydrated embryos is achieved by replacing methanol with PBS and then by washing 3x with 100% PBST.
    2. **Permeabilization** Digest the rehydrated embryos with proteinase K (10 µg/ml) at room temperature to render them permeable as shown in **Table 4**.

Embryonic Stage (hours post fertilization)	Digestion Period (Proteinase K)
Up to 6	15 sec
6-12	30 sec
12-18	3 min
24	12-15 min
48	25-30 min
72	40 min
96-120	50 min

Table 4. Permeabilization reaction.

    3. **Postfixation and PBST Washes**
        1. Fix embryos by incubating in 4% paraformaldehyde (wt/vol) in 1x PBS for 20 min.
        2. Remove residual paraformaldehyde by washing embryos 3x, 5 min/wash, in 1x PBST.
    4. **Acetylation** Prepare the acetylation mixture (125 µl triethanolamine and 27 µl acetic anhydride in 10 ml ddH_2O) and incubate embryos twice for 10 min each. To minimize endogenous phosphatase activity, prepare the acetylation mixture fresh when required. To remove excess reagent, wash embryos twice in PBST (10 min/wash)
    5. **Prehybridization** Prehybridize embryos by transferring embryos from baskets to 1.5 ml sterile Eppendorf tubes and incubating with 200 µl hybridization mixture for 2-4 hr in a 70 °C water bath.
    6. **Preadsorption of Antibody** Remove about 50 embryos from the baskets and transfer them to a 1.5 ml sterile Eppendorf tubes. Incubate with anti-DIG-AP (1:500) antibody using blocking buffer (1x PBST, 2% calf serum [vol/vol], 2 mg/ml Bovine Serum Albumin

[BSA]) at room temperature for 2-4 hr, and then store at 4 °C overnight. Remove from the incubator in the morning and allow embryos to reach room temperature.

7. **Hybridization** Add 100-200 ng of probe to the prehybridized embryos and incubate overnight at 70 °C in water bath.

2. **DAY 2**
    1. **SSC (Saline-sodium Citrate) Washes**
        1. Place 50 ml tubes of the hybridization mixture (HM), 2x SSC and 0.2x SSC in a beaker with water in a 70 °C water bath and prewarm them for at least 20 min. Remove the hybridization reaction mixture containing the probe, add 1 ml of prewarmed hybridization mixture to the tubes and incubate for 15 min at 70 °C. Fill six-well plates with various dilutions of prewarmed HM in 2x SSC by replacing 100% HM through 50 % to 25% HM to remove excess reagents.
        2. While waiting, fill six-well plates with 2x SSC for washes and keep them at 70 °C.
        3. After 15 min place the embryos from the tube to the baskets kept in the six well plate filled with the dilutions of HM with 2x SSC and wash the hybridized embryos by moving the basket from one well to the next (15 min each in a 70 °C water bath). Wash embryos 2x, 15 min/wash, in 100% 2x SSC at 70 °C.
        4. Set another water bath at 65 °C. Fill six-well plates with 0.2x SSC for high-stringency washes to avoid nonspecific hybridization of transcript with the probe.
        5. After the embryos are washed twice with 2x SSC at 70 °C, follow a further two 30 min washes in 0.2x SSC at 65 °C.

    2. **PBST Washes** Prepare six-well plates with successive dilutions of 0.2x SSC in PBST as follows: 75% (vol/vol) 0.2x SSC, 50% (vol/vol) 0.2x SSC, 25% (vol/vol) 0.2x SSC, and the remaining two-wells with 100% PBST. Wash the hybridized embryos by moving the basket from one well to the next (5 min for each step at room temperature using a rocker).
    3. **Preincubation** Preincubate embryos for 3-4 hr at room temperature in blocking buffer (1x PBST, 2% calf serum [vol/vol], 2 mg/ml BSA).
    4. **Incubation with anti-DIG Antibody** Incubate embryos with a solution of anti-DIG-AP antibody (1:5,000) in blocking buffer overnight at 4 °C with gentle agitation.

3. **DAY 3**
    1. **PBST Washes** Prepare the six-well plates with 4 ml/well PBST. Wash incubated embryos by moving the baskets in the six-well plate containing PBST 6x for periods of 15 min. Embryos can be stored at 4 °C and the protocol resumed the next day.
    2. **Prestaining** Prestain the embryos by washing 2x for 15 min with prestaining buffer at room temperature with gentle agitation prior to incubation in the presence of BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium). BCIP and NBT are the substrates used for the color development of alkaline phosphatase activity. Since probes are attached with Alkaline phosphatase the development of purple color indicates the expression of the target gene. Embryos can be stored at 4 °C and the protocol resumed the next day.
    3. **Staining**
        1. Incubate embryos at room temperature in the dark with BCIP and NBT for 30 min.
        2. Monitor the staining reaction every 30 min using a stereomicroscope. Reaction times vary from 15 min-16 hr depending upon the level of gene expression. Reaction times are shorter for highly expressed genes and longer for weakly expressed genes. Sense probes will detect signals if the gene of interest expresses complementary antisense (AS) transcripts.

    4. **Stop Reaction** After the desired staining intensity is reached, stop the reaction by transferring baskets containing the embryos to wells containing the stop solution (1x PBS, pH 5.5, 1 mM EDTA, 0.1% Tween). This will avoid further development of the color. Rinse embryos for 10 min 2x on a rocker with gentle agitation at room temperature.
    5. **Postfixation**
        1. Incubate the embryos in paraformaldehyde 4% (wt/vol) in PBS for 20 min.
        2. Wash the embryos (5 min/wash) three times with PBST in order to remove residual paraformaldehyde. Store the fixed embryos in stop solution in the dark at 4 °C.

    6. **Mounting, Observation, and Image Acquisition**
        1. Transfer the embryos to a six-well plate containing 100% glycerol using the minimum possible volume of PBS. Place the six-well plate on a rocker and gently agitate overnight at room temperature in the dark.
        2. Place embryos in 100% glycerol and then observe the signal under a stereomicroscope.
        3. Acquire Images and save as Tiff file format or any other type which permits the production of high quality images through the use of image software such as Photoshop (**Figures 3-6**).

2. Double *In Situ* Hybridization

1. To achieve double labeling the method described above from 1.1 - 1.3.1.7 is used but includes simultaneous incubation of both digoxygenin and fluorescein labeled probes during hybridization.
2. Follow the same procedure for post hybridization washes namely SSC and PBST washes (steps 1.3.2.1 and 1.3.2.2) and blocking incubations (step 1.3.2.3). Perform antibody and staining reactions sequentially as described below.
3. Incubate embryos with AP-coupled-anti-digoxigenin antibody (1:5,000) at 4 °C with gentle agitation.
4. Wash and incubate with BCIP-NBT (steps 1.3.3.1 - 1.3.3.6).
5. Following detection, wash embryos twice with PBST at room temperature for 20 min.
6. Incubate embryos at 65 °C in PBST with 1 mM EDTA for 30 min to inactivate the anti-digoxigenin antibody.
7. Incubate embryos in 100% methanol followed by rehydration in 75%, 50%, and 25% methanol with PBST and back to PBST for 1 hr.
8. Incubate embryos overnight with AP-coupled fluorescein (1:2,000) in blocking buffer at 4 °C with gentle agitation.

9. Wash embryos with PBST (step 1.3.3.1) and pre-stain embryos 2 times for 15 min with pre-staining buffer (0.1 M Tris-HCl, pH 8.2) at room temperature with gentle agitation prior to incubation in the presence of fast red. Protocol can be stopped and embryos can be stored at 4 °C with gentle agitation and resumed the next day.
10. Dissolve one Fast Red tablet in 2 ml of staining buffer immediately before use.
11. Start the staining reaction by placing embryos in the staining buffer with Fast Red reagent.
12. Stop the staining reaction when the desired staining intensity is reached. It may take from a few hours at room temperature to one or two days overnight when staining at 4 °C. Stop the reaction immediately if the staining intensity does not develop further or starts to show non-specificity when compared to the sense control.
13. Follow the procedures outlined above for the remaining steps namely post fixation, mounting, observation and image acquisition (steps1.3.3.5 and 1.3.3.6).

Representative Results

Using the protocol with 50 embryos per basket (per gene/per experimental condition) the expression pattern of that gene can be achieved in one experiment. Almost all the embryos show similar expression patterns for a particular gene. Representative examples of the *in situ* hybridization staining are shown in **Figures 3-6**.

Both the sense and anti-sense riboprobes were synthesized from the cDNAs corresponding to PC5.1, PC5.2[6], SCL/tal-1[7], gata-1[8,9], flk/ kdrl[10], shh[11], dlx2[12], fkd7/foxa1[13], insulin[14,15], and trypsin[16] have been constructed by amplifying a segment of the full-length mRNA using Taq DNA polymerase, and cloned into pCRII-TOPO (Invitrogen). The authenticity of individual amplicons was confirmed by sequencing. After verification of the clone orientation, corresponding antisense riboprobes were synthesized using the protocols[17] with modifications[18,19] as described here using either the Sp6 or T7 RNA polymerases. Steps involved in whole-mount *in situ* hybridization are illustrated briefly in **Figure 2**. Purple staining observed after hybridization with riboprobe of interest represents both the abundance and the sites of expression of particular gene. For instance PC5.1 shows very discrete expression within the anterior and posterior part of otic vesicle and lateral line primordium at 24hpf and is strongly localized within the anterior and posterior lateral line neuromasts by 72 hpf **(Figure 3).** In contrast PC5.2 shows ubiquitous expression within the CNS with distinct regionalization within the somites, otic vesicle and pronephric duct and is highly expressed within the liver and intestine at 96 hpf (**Figure 4**). Absence of gene expression when using respective sense riboprobes serves as a negative control. (**Figures 3b** and **4b**). Expression analyses of blood markers showed staining of SCL/tal-1 within the bilateral cranial cells and in the intermediate cell mass (ICM). Although the staining for gata-1 is also seen within the ICM the expression pattern is different. The expression of flk/ kdrl was observed within the hindbrain, main and intersegmental vessels and in the ICM **(Figure 5).** The staining for shh was observed in the notochord **(Figure 5).** The expression of dlx2 at 34 hpf is localized in the telencephalon, diencephalon, hypothalamus and cranial ectomesenchymal arches and the fkd7/foxa1 expression at 24 hpf is seen mainly in the floor plate and hypochord. Expression patterns of the pancreatic markers showed insulin staining in the endocrine pancreas and trypsin in the exocrine pancreas **(Figure 6)**. These results showing expression pattern with high resolution represents the success of the outlined protocol for the detection of the expression of both high and low abundant genes. For further clarity images can be taken using confocal microscopy after mounting the embryos on a microscopic slide[20]. Some of the examples of the results obtained when the experiment was undertaken under sub-optimal conditions during optimization of the protocol are shown in **Figure 7.** High background signal with poor *shh* expression was noticed under inadequate permeabilization and hybridization between 55-60 °C.

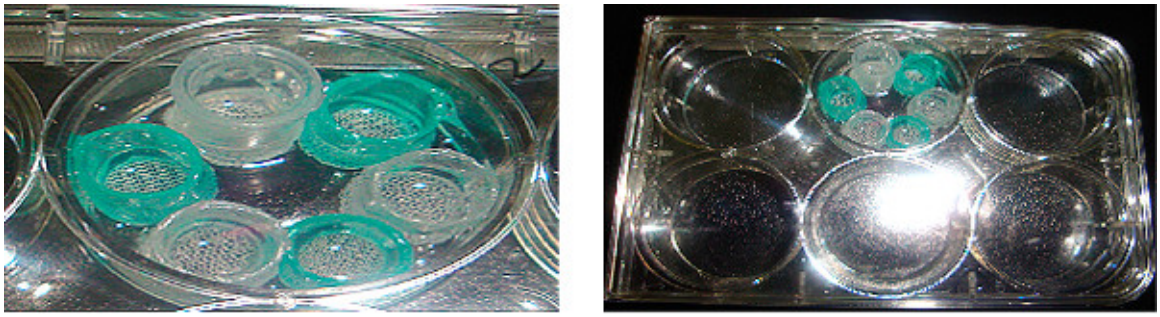

Figure 1. Preparation and use of Eppendorf-nylon mesh baskets to hold staged embryos. One well can hold six Eppendorf-nylon mesh baskets. The image shows the 6-well sterile plates containing six Eppendorf-nylon mesh baskets used for successive dilutions of methanol in PBS.

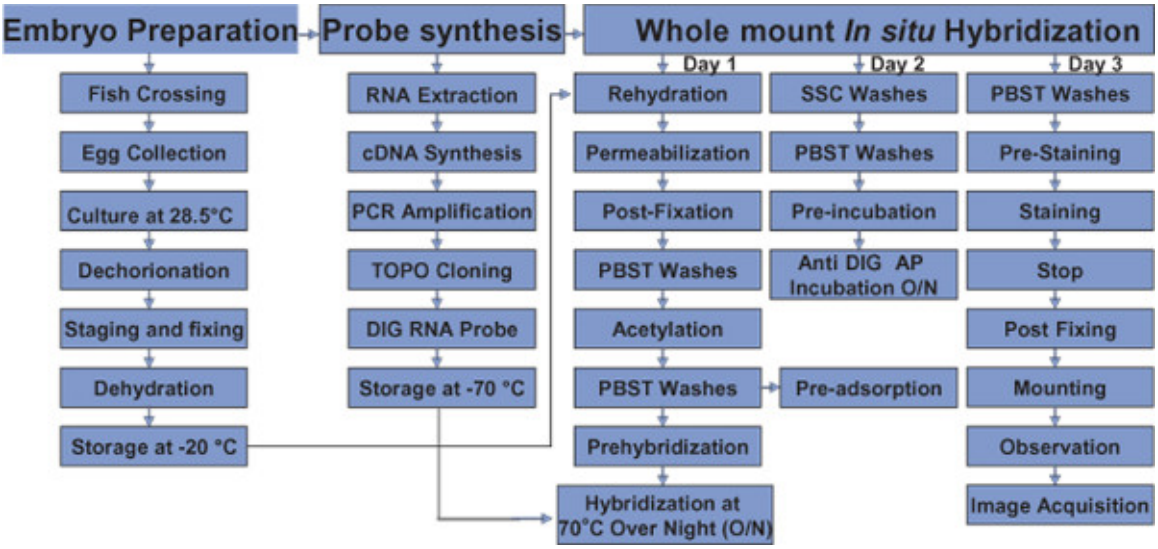

Figure 2. Diagram showing the steps involved in the whole-mount *in situ* hybridization technique. After fixing staged embryos can be stored in methanol at -20 °C until used. Probes can be synthesized in advance and kept at -80 °C. Storing probes in small aliquots is advisable if they are to used over long periods. In general whole-mount *in situ* hybridization experiments can be completed in 3 or 4 days. The staining reaction for weakly expressed genes will take up to 16 hr at room temperature or longer when the staining reaction is undertaken at 4 °C Click here to view larger figure.

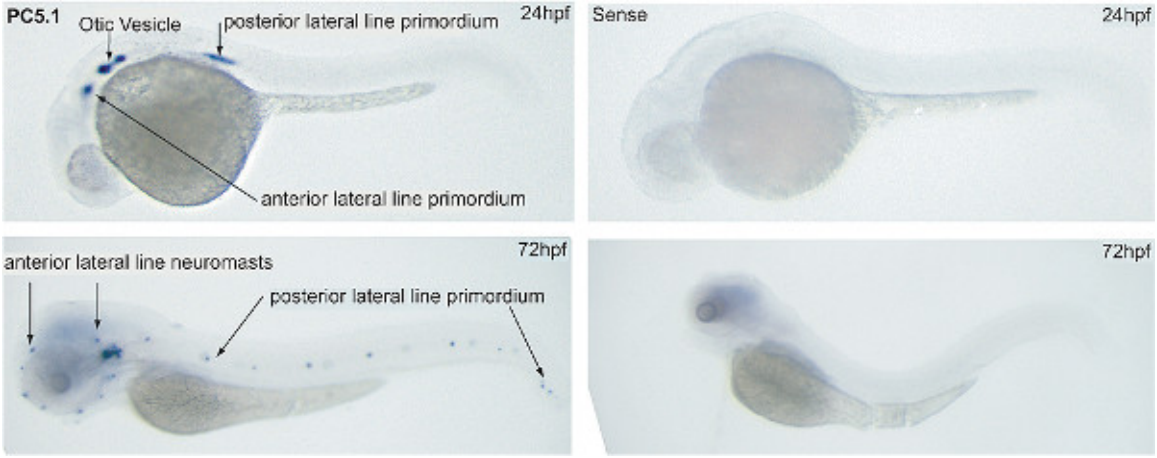

Figure 3. The expression of PC5.1 by whole-mount *in situ* hybridization analyses was carried out using 24 hpf and 72 hpf staged embryos. (a) Lateral view of 24 hpf embryos show very discrete expression of PC5.1 within the anterior and posterior part of the otic vesicle and lateral line primordium using PC5.1 antisense riboprobe. At 72 hpf specific staining was observed within the anterior and posterior lateral line neuromasts. **(b)** Absence of gene expression using PC5.1 sense riboprobe serves as a negative control. Click here to view larger figure.

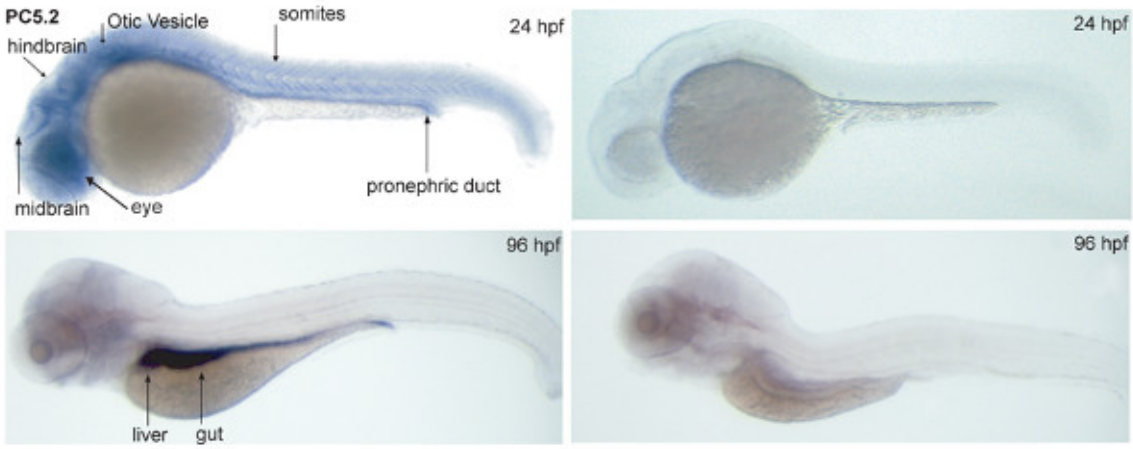

Figure 4. At 24 hpf and 96 hpf whole-mount *in situ* hybridization analyses of PC5.2 was carried out using PC5.2 anti-sense and sense riboprobes. (a) Lateral view of 24 hpf embryos show ubiquitous expression within the CNS, somites otic vesicle and pronephric duct. Specific staining in the liver and gut is seen at 96 hpf using PC5.2 antisense riboprobe. **(b)** Absence of gene expression using PC5.2 sense riboprobe serves as a negative control. Click here to view larger figure.

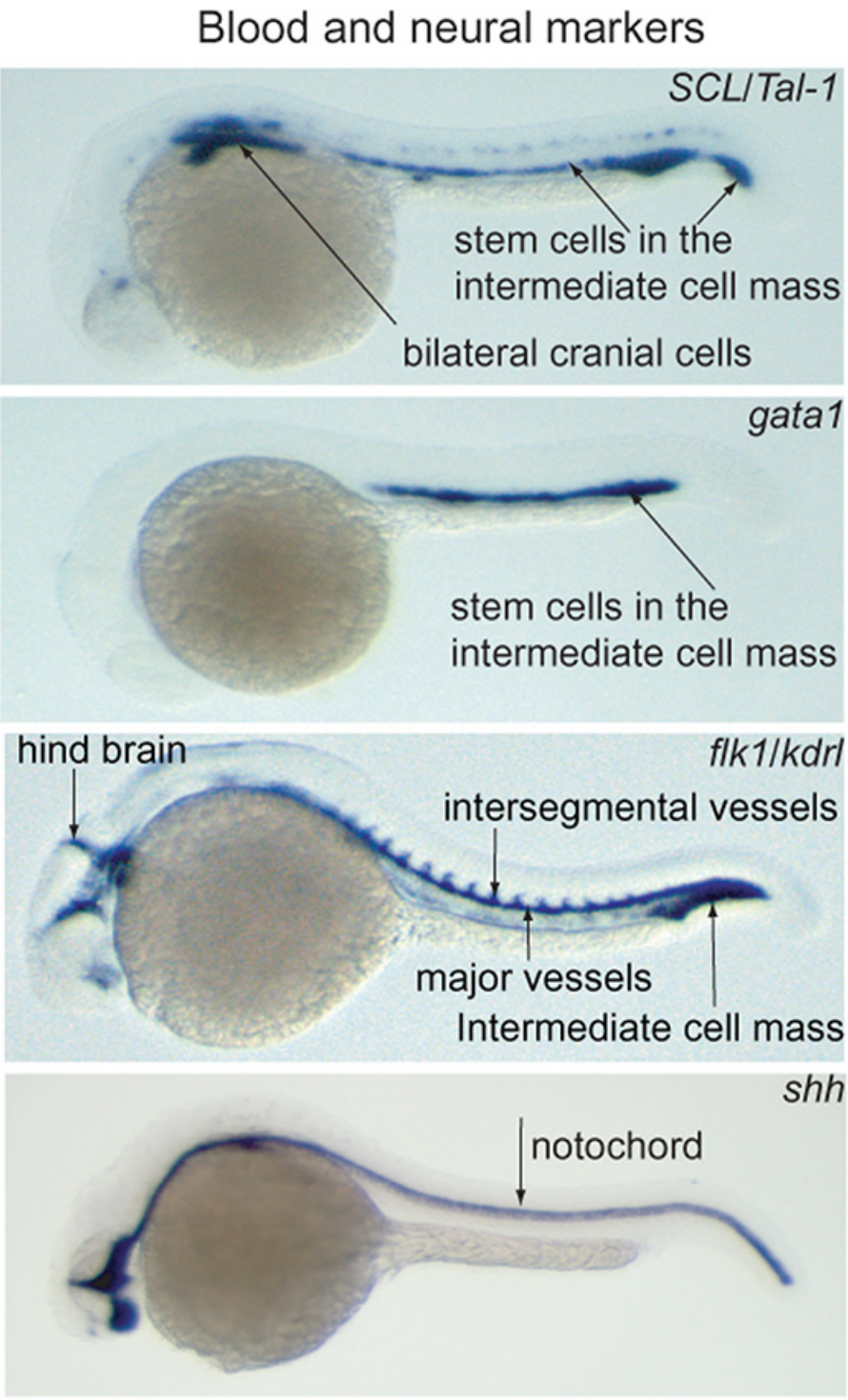

Figure 5. Whole-mount *in situ* hybridization analyses at 24 hpf using SCL/tal-1 riboprobe showed staining within the bilateral cranial cells and in the intermediate cell mass (ICM); gata-1 riboprobe staining is seen in the ICM; the flk/ kdrl expression within the hindbrain, main, and intersegmental vessels and in the ICM. The staining for shh was observed in the notochord.

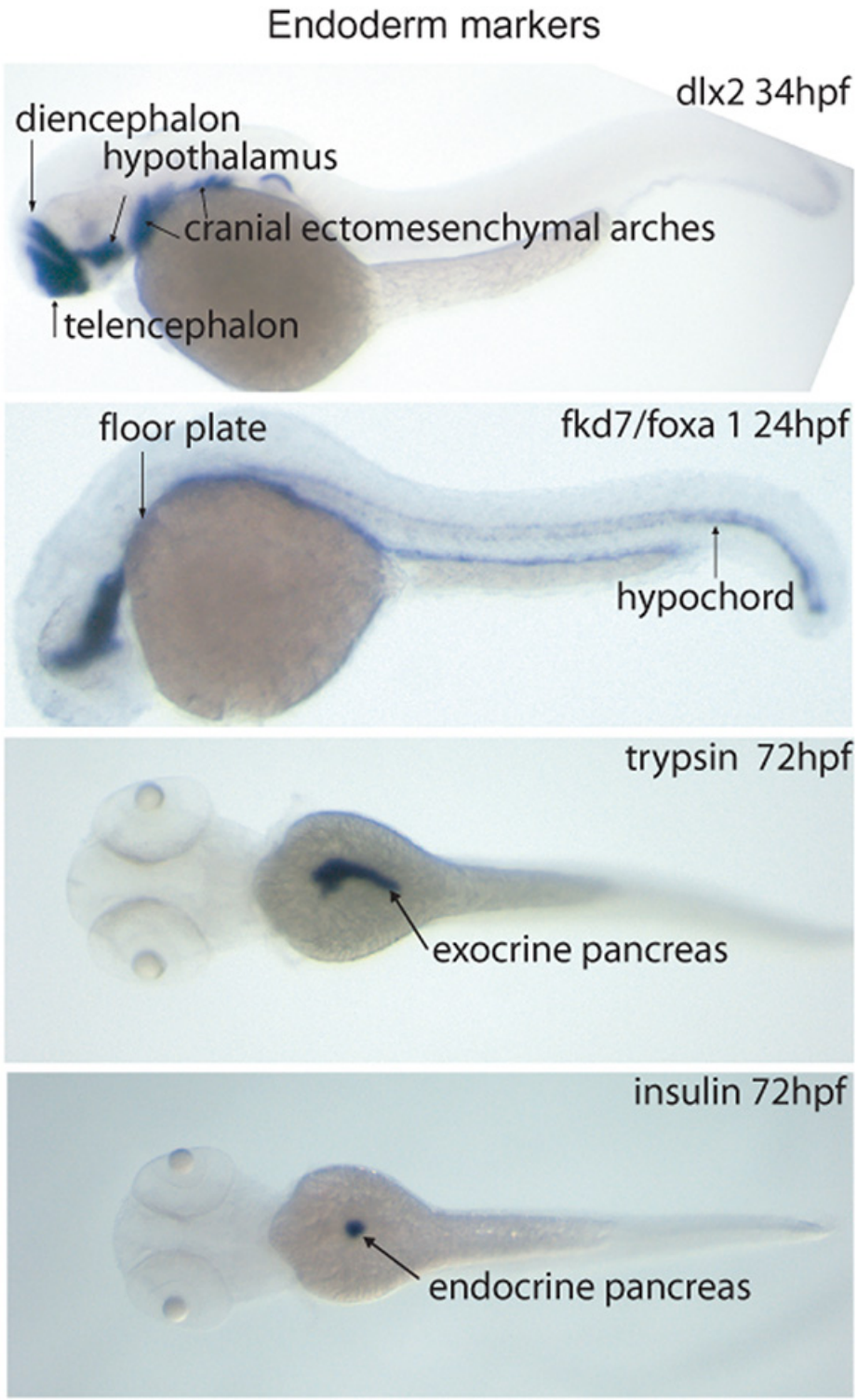

Figure 6. Whole-mount *in situ* hybridization analyses at 34 hpf using dlx2 riboprobe revealed expression of dlx2 in the telencephalon, diencephalon, hypothalamus, and cranial ectomesenchymal arches; the fkd7/foxa1 expression at 24 hpf is seen mainly in the floor plate and hypochord; insulin staining is observed in the endocrine pancreas and trypsin expression in the exocrine pancreas.

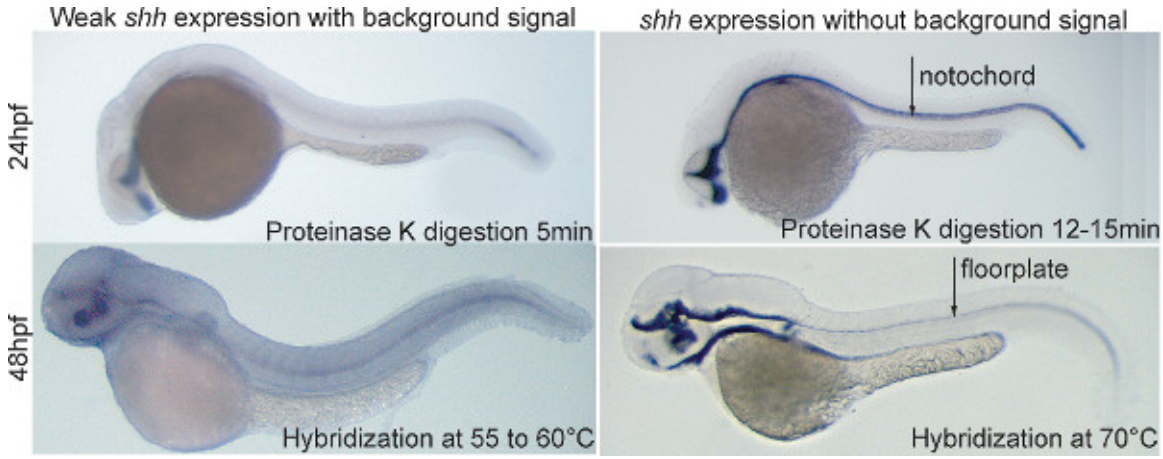

Figure 7. Adequate permeabilization and appropriate hybridization temperature are critical for clear *in situ* hybridization signals. Whole-mount *in situ* hybridization analyses at 24 hpf and 48 hpf using an shh riboprobe revealed expression within the notochord and floorplate without background signal when embryos are permeabilized by proteinase K digestion for 12-15 min and hybridized at 70 °C. Incomplete expression patterns and high background signals were observed when the embryos were subjected to inadequate digestion (5 min proteinase K treatment) or hybridized at low temperature (60 °C). Click here to view larger figure.

Discussion

We have developed improved methods to visualize RNA with high resolution. The *in situ* hybridization procedures are carried out using simple custom-made baskets with porous bottoms **(Figure 1)**. Embryos are processed using RNase-free solutions in 6-well plates at room temperature under sterile conditions.

To segregate embryos baskets are made from different colored Eppendorf tubes. Baskets with or without a rim are used for easy orientation and to identify the embryos within the basket as corresponding to a particular probe.

Prehybridization at 65 °C and hybridization at 70 °C with 50% deionized formamide was found to be particularly important to obtain high resolution signals. In addition incubating embryos twice with alkaline Tris buffer for 15 min post PBST washes after incubation with anti-DIG antibody **and** prior to incubation in the presence of BCIP and NBT facilitates the appearance of high quality clear signals. In our hands we experienced that the permeabilization and fixing of embryos for the optimal time and hybridization at 70 °C were very critical in obtaining clear signals. Inadequate permeabilization produced high background signals. Extended permeabilization or inadequate fixing resulted in the degradation of embryos whereas extended fixing inhibited signal detection. Hybridization between 55-60 °C also produced high background signals **(Figure 7).**

The sense probe will detect a signal for the AS transcript if the gene of interest encodes antisense (AS) transcripts. After the staining reaction is stopped placing embryos in methanol for 30 min or longer converts the signal to a true purple color, and removes non-specific staining. Fixed embryos can be stored in the dark in stop solution at 4 °C for several months.

The advantages and disadvantages of WISH

WISH is an efficient technique to characterize both the temporal and spatial expression of target genes during embryogenesis and larval stages. This protocol also permits visualization of RNA expression of two genes or co-localization of RNA and protein expression at the same time using appropriate modifications depending upon the experimental objectives. This high resolution protocol can be used to detect RNA expression in many tissues and cell types. It can be used for the detection of very low abundant RNA species with minimal background signals. However, to visualize accurate expression of genes within interior compartments of tissues requires *in situ* hybridization using tissue sections.

Disclosures

The authors declare that they have no competing financial interests.

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