Protocol for In Situ Hybridization on whole mount zebrafish embryos.

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II. Preparation of antisense DIG labelled RNA Probes

A. Isolation and preparation of the DNA template

This method is used for the preparation of large amounts of antisense RNA probes and can be divided into two steps: preparation of DNA and synthesis of antisense RNA probe.

For the preparation of the DNA template, 5 μg of DNA is linearized in a 2hr digestion using the appropriate restriction enzyme under appropriate conditions of salt and temperature. Care must be taken to ensure that the direction of insert of interest is known to allow for the correct production of the antisense and sense probes. Once the reaction is complete, a phenol/chloroform extraction is used to remove the enzyme. DNA is then purified from the aquaous phase by using Microcon YM-50 columns (Millipore, 42415). After 5 min. centrifugation at 10,000 g (the column should be dry) 100 μl sterile-filtered H_2O is added to the column and it is then centrifuged a second time for 5 min. at 10,000 g (again the column should be dry). The microcon column is put in a new eppendorf, 20 μl of sterile water is added, briefly vortexed and the microcon device is put up side down and centrifuged 1 min at 5,000g. Test 2 μl on a 1% agarose gel to check if the linearization is complete.

Following successful production of template the next step is the in vitro synthesis of the antisense RNA using a 2 hr incubation at 37°C with the following transcription mix:

Linearized DNA	1μg
Transcription buffer (Promega)	4 μl
NTP-DIG-RNA (Boehringer)	2 μl
RNAse inhibitor (35 units/μl, Promega)	1 μl
T3 or T7 RNA polymerase (20 units/μl, Stratagene)	1 μl
Sterile water up to 20 μl total	

Following the initial incubation, the DNA template is digested by the addition of 2 μl RNAse free DNAse (Roche 776785) for 15 min. at 37°C. Stop the digestion reaction by adding 1 μl EDTA 0.5M pH 8.0. Synthesized RNA is then precipitated by the addition of 2.5 μl LiCl 4M and 75 μl cold 100 % Ethanol followed by incubation at -70°C for 10 min. then centrifugation at 4°C for 30 min. at 10,000 g. Finally, wash the pellet with 70% Ethanol, dry and resuspend in 20 μl sterile water. Alternatively, a RNA purification kit can be used. Place a Sigmaspin Post Reaction Purification column (Sigma, S5059) in a microfuge tube and centrifuge 15 seconds at 750 g. Break the base of the column and remove the top, then re-centrifuge for 2 min. at 750 g. Place the column in a new tube, and add the RNA sample on top of the resin. Centrifuge 4 min. at 750 g. Discard the column. Add to the RNA sample 1 μl EDTA 0.5M and 9 μl RNAlater (Sigma, R-0901). Store at 20°C.

Visualize 1/10 of the synthesized RNA on 1% agarose gel to determine that the procedure was successful.

B. PCR generation of template as an alternative to linearization and purification of the DNA template.

The advantage of the PCR amplification method is that it is fast and can be used for large scale in situ analysis, and is a viable method when there are no RNA polymerase promoters available. For example, in the case when the RNA polymerase promoter is determined by the oligonucleotide in 3 of the probe due to the poor incorporation of digoxygenin-3 UTP by the SP6 RNA polymerase. Therefore, only T3 or T7 RNA polymerases will be chosen. The PCR amplification method can also be used when there is no unique sites usable to linearise the DNA with a restriction enzymes in 5 of the cDNA.

For PCR amplification, mix 100 ng purified DNA (or 1 μl of an overnight culture of the bacteria containing the plasmid carrying the cDNA), 0.5 μl primer 1 (0.5 μg /μl), 0.5 μl primer 2 (0.5 μg /μl), 50 μl PCR master mix (Promega, M7505), up to 100 μl sterile water in a 0.5 ml sterile tube. The mixture is denatured at 95°C for 4 min., followed by 35 cycles of 95°C 30 s, 55°C 30 s, 72°C 3 min. (at least 1 min. per kb), then a final extension at 72°C for 7min. The product is then stored at -20°C.

For PCR product purification, place a microcon PCR device (Genomics Millipore, UFC7PCR50) on the provided eppendorf tube. Add 400 μl sterile water and the 100 μl of PCR reaction. Centrifuge for 15-20 min. at 1,000g. The membrane should be dry. Put the microcon device in a new eppendorf, add 20 μl sterile water, briefly vortex and then place the microcon device upside down. Centrifuge 1 min. at 1,000g to recover the DNA.

Check the PCR amplification by loading 1/10 on 1% agarose gel.

Synthesis of antisense RNA

To 2.5 μl DNA (100-200 ng) add 2.5 μl of the following mix: 1 μl transcription buffer (Promega, P118B), 0.5 μl DTT (Promega, P117B), 0.5 μl NTP-DIG-RNA (Roche, 1277073), 0.25 μl RNAsin inhibitor (Promega, N251X), 0.25 μl RNA polymerase (T7 polymerase: Promega, P207B; T3 polymerase: Promega, P208C). Mix and incubate 2 h at 37°C. Add 2 μl RNAse free DNAse I (Roche, 776785) and 18 μl sterile water. Mix and incubate 30 min. at 37°C. Stop reaction by adding 1 μl sterile EDTA 0.5 M and 9 μl sterile water. Purification of the RNA template is on a Sigmaspin Post Reaction Purification column (Sigma, S5059) as described above.

III. Preparation of embryos

Eggs are collected from single mating pairs about 1 h after laying. They are cleaned and unfertlized eggs are discarded. Embryos are allowed to develop in regular fish water until the end of gastrulation. For embryos older than 24 h, in order to prevent pigmentation, fish water is replaced at the end of gastrulation (10 hpf) by a solution 0.0045% 1-phenyl-2-thiourea (P-7629 Sigma) in 1x Danieau medium (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM Ca(NO3), 2, 5 mM HEPES, pH 7.6).

Chorions are removed by pronase treatment (Sigma, P-6911) according to the online Zebrafish book protocol (http://zfin.org/zf_info/zfbook/chapt4/4.1.html) prior to fixation in 4% paraformaldehyde. Alternatively chorions can be removed after fixation using sharp forceps.

Embryos are fixed at the appropriate stage in 4% paraformaldehyde (PFA, Sigma, P-6148) in 1x PBS overnight at 4°C. Paraformaldehyde powder is dissolved in 1 x PBS by heating on a hot plate with agitation using a magnet stirrer to 95°C (do no boil). Once the powder is completely dissolved, the solution is cooled on ice.

Fixed embryos are dehydrated in 100% Methanol (MeOH) for 15 min. at room temperature, then stored at -20°C (for at least 2 h and up to several months) prior to proceeding with in situ hybridization experiments.

IV. Reagents and buffers for the in situ hybridization

10 x PBS (Dulbecco, Sigma D-5652)

Methanol (MeOH)

Tween 20 (Sigma, P-1379)

Proteinase K (Boehringer, 1000 144)

Anti DIG antibody-alkaline phosphatase Fab fragment (Boehringer, 1 093 274)

BSA fraction V protease free (Sigma, A-3294)

Formamide : high purity grade, (Sigma or Carlo Erba, n° 452286), deionized by adding and stirring slowly 2 x 15 min. with 10g/l Serdolit MB-3 (Serva, 40721). The solution is filtered to remove the resin and stored in the dark at 4°C.

20 x SSC

Heparin at 5 mg/ml (Sigma, H-3393)

RNAse free tRNA (Sigma, R-7876): 50 mg/ml resuspended in water and extensively extracted several times in Phenol/CHCl3 to remove protein.

Citric acid 1M

Normal Sheep serum (Jackson Immunoresearch, 013-000-121)

Tris HCl pH 9.5 M

MgCl2 1M

NaCl 5M

NBT (Nitro Blue Tetrazolium) 50 mg/ml (made from powder, Sigma N-6876. 50 mg NBT dissolved in 0.7 ml anhydrous dimethylformamyde and 0.3 ml H2O). Store in the dark at 20°C.

BCIP (5-Bromo 4-Chloro 3-Indolyl Phosphate) 50 mg/ml (made from powder, Sigma B-8503. 50 mg dissolved in 1ml anhydrous dimethylformamyde). Store in the dark at 20°C

Embryo Storage buffer : PBS pH5.5 (Na2HPO4 1.08 g/l ; NaH2PO4 6.5 g/l ; NaCl 8.0g /l ; KCl 0.2 g /l), EDTA 1mM, Tween 20 0.1%.

EDTA 0.5M

Glycerol 99% pure (Sigma, G-6279)

V. In situ hybridization protocol

This protocol is adapted from Thisse et al, 1993; Thisse and Thisse, 1998; Thisse et al, 2001.

Day 1:

Transfer embryos (of the same developmental stage) into small baskets made of a metal or nylon mesh (mesh opening 100150 μm) fused at the bottom of a plastic tube and placed in 24 or 6 well tissue culture plates. Baskets are made with 2 ml eppendorf tubes or 50 ml conical centrifuge tubes (Fig. 1A) cut with a cutter or a saw in order to produce a cylinder of plastic about 1.5 - 2 cm high, with a diameter of 1.2 cm (small baskets), or 3 cm (large baskets). A stainless steel mesh (for large baskets) or nylon mesh (for small baskets) is fused at the top of the tube as follows: on a hot plate, place a small piece of aluminum foil, then put the metal or nylon mesh on the foil and press the plastic tube onto the mesh (Fig. 1B), until the fusion of the plastic glues the mesh to the tube. Once fused, rapidly remove from the hot plate. The aluminum foil, glued to the basket cools down quickly and can be removed easily. Small baskets (convenient for treatment of up to 50 embryos) are usable in 24 well plates, large baskets made with 50 ml conical centrifuge tubes (for 500 to 1,000 embryos) can be used in 6 well plates (Fig. 1C).

1. Rehydratation:

Embryos stored in 100% methanol are rehydrated by successive incubations (moving baskets from well to well) in the following solutions:

75% MeOH - 25% PBS for 5 min.

50% MeOH - 50% PBS for 5 min.

25% MeOH - 75% PBS for 5 min.

100% PBT (PBS/Tween 20 0.1%) 4 x 5 min.

2. Digest with proteinase K (10 μg/ml)

This step permeabilizes the embryos permitting access of the RNA probe. The digestion time is dependent on the developmental stage. For blastula, gastrula and somitogenesis stages (up to the 18 somites stage) : 30 seconds to 1 min. is sufficient. For 24 h old embryos digest for up to 10 min. and for older embryos (from 36 h to 5 days old embryos) digest for 20 - 30 min. Proteinase K digestion is stopped by incubation in 4% paraformaldehyde in 1 x PBS 20 min., followed by washes in 1 x PBT, 5 x for 5 min.

3. Prehybridization:

Embryos are transferred to 1.5 ml eppendorf tubes (up to 50 embryos per tube). At this step embryos of different developmental stages can be pooled and treated together until the end of the in situ hybridization experiment. Prehybridization is performed by incubation in 700 μl of Hybridization Mix (HM) for 2 to 5 h at 70°C in a waterbath. Prehybridized embryos can then be directly hybridized or stored in hybridization mix at 20°C (up to several weeks)

Hybridization Mix (HM) :

Formamide 50%

5 x SSC

Tween 20 0.1%

Citric acid to adjust HM to pH 6.0 (460 μl of 1M Citric acid for 50 ml of mix)

Heparin 50 μg/ml

tRNA 500 μg/ml

4. Hybridization :

Remove and discard the prehybridization mix. Replace with 200 μl of hybridization mix containing about 100 ng of antisense DIG labelled RNA probe. Hybridize overnight in the eppendorf tube at 70°C in a waterbath.

5. Preadsorbtion of anti-DIG antibody

In addition to the embryos used for the in situ hybridization, a batch of embryos are treated the same way excluding the hybridization step, and are used for the preadsorbtion of the anti-DIG antibody. 1,000 embryos are used for 20 ml of anti-DIG antibody diluted at 1/1000 in PBT-sheep serum 2%-BSA (2mg/ml). Antibody is preadsorbed for several hours at room temperature under gentle agitation on a test Tube rocker (Thermolyne, vari-mix). Embryos used for preadsorbtion are removed and the preadsorbed antibody is stored at 4°C until its use on day 2.

Day 2:

1. Washes:

Embryos are removed from the eppendorf tube and placed (see Fig. 1D) in baskets made from 2 ml eppendorf tubes placed on a styrofoam float (16 x 9 x 1.5 cm with space for 50 small baskets) in a plastic box (21 x 10 x 7 cm) containing 200 ml of 100% HM wash solution at 70°C (Hybridization Mix used in washes do not contain tRNA and Heparin). Embryos will stay in these baskets on the styrofoam float until the staining step on day 3. After a quick wash, the styrofoam float carrying the 50 baskets is placed successively in another plastic box containing 200 ml of prewarmed wash solution and incubated at 70°C in a shaking waterbath (with about 40 strokes per minute). The successive steps and washing solutions are:

15 min in 75% HM / 25% 2 x SSC at 70°C

15 min in 50% HM / 50% 2 x SSC at 70°C

15 min in 25% HM / 75% 2 x SSC at 70°C

15 min in 2 x SSC at 70°C

(these steps gradually facilitate the change from the hybridization mix to 2 x SSC)

Two washes of 30 min each in 0.2 x SSC (these are high stringency washes that remove non specifically hybridized probes)

Following the high stringency washes, embryos are progressively moved from 0.2 x SSC to 100% PBT by the following incubations (in 200 ml) at room temperature with slow agitation using an horizontal orbital shaker (about 40 rpm).

10 min. in 75% 0.2 x SSC / 25% PBT

10 min. in 50% 0.2 x SSC / 50% PBT

10 min in 25% 0.2 x SSC / 75% PBT

10 min in PBT

2. Incubation with anti-DIG antiserum:

1. Block for 3 to 4 h at room temperature in blocking buffer made in PBT containing 2% Sheep serum and 2mg/ml BSA.

2. Incubate in 200 ml of antibody solution diluted at 1/10,000 in blocking buffer overnight at +4°C under slow agitation (30 to 40 rpm on the horizontal orbital shaker).

Day 3:

1. Washes:

Remove the antiserum and discard. After a brief wash in PBT wash extensively 6 x for 15 min in PBT at room temperature under slow agitation (30 to 40 rpm on the horizontal orbital shaker). After the last wash, and before moving into the staining buffer, embryos are dried by placing the styrofoam float carrying the 50 baskets on an absorbing paper to remove remaining PBT (this is to avoid formation of a precipitate in the staining buffer). Embryos are then incubated at room temperature in the alcaline Tris buffer (100 mM Tris HCl pH 9.5, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween 20) changed 3 x at 5 min. intervals.

2. Staining:

Embryos are removed from the baskets and incubated in the staining solution at room temperature (in the dark) in a multiwell plate.

Staining solution (to keep out from the light) :

NBT 50 mg/ml	225 μl
BCIP 50 mg/ml	175 μl
Alcaline Tris buffer	to 50 ml

The staining reaction is monitored regularly under a dissecting microscope with light from the top and with the plate on a white background.

When the signal is perceived as sufficient (and before apparition of background reaction time in a range of 15 min for genes strongly expressed, 1 to 1.5 h for most genes and up to 5 h for genes that are weakly expressed). The staining reaction is stopped by transferring embryos into a 1.5 ml eppendorf tube, staining solution is removed and discarded and embryos are washed several times at room temperature with the stop solution:

Stop solution:

PBS 1x pH5.5

EDTA 1mM

Tween 20 0.1%

Labelled embryos are stored in the stop solution (+4°C in the dark). Labelling stays unchanged for months under these conditions.

VI. Recording Results

Labelled embryos are mounted in 100% glycerol. Due to the photosensitivity of the yolk cell, embryos at early developmental stages are first treated for 5 min in an acidic buffer (either PBS with pH< 3.5 or glycine buffer pH 2.2). This treatment prevents the photoreactivity of the yolk proteins, and even under intense light the yolk cell remains unstained. However, this acidic treatment affects embryo morphology. Therefore while very convenient at early developmental stages (when only a few structures are formed, and when the photolabelling of the yolk is a limiting factor for the observation), this acidic treatment should not be used for embryos older than the 15 somite stage. Embryos are observed in glycerol between slide and coverslip (using bridges made of 4 coverslips of thickness 1.5). Low magnification pictures are taken with a Leica M420 Macroscope (which offers a large field of view and a long working distance. Its vertical beam path provides for parallax-free imaging, resulting in high accuracy, top imaging, fidelity and faithful photography) or with microscope (Leica DM RA2) with differential interference contrast (DIC) using numeric camera (coolsnap CCD, Roper Scientific). Digitalized pictures are saved as TIFF files, then adjusted for contrast, brightness and color balance using a Photoshop software and stored as such or after conversion to the .jpeg format to reduce the files size.