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High-resolution whole mount *in situ* hybridization using 3'-DIG labeled miRCURY™ probes

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Fixation and storage of Zebrafish and Mouse embryos

- Remove chorions by pronase treatment (for embryos older than 18 somites) or manually (for earlier stages). We only do manual dechorionation on all stages.
- Fix embryos in 4% paraformaldehyde* (PFA) in PBS overnight at 4°C.
- Transfer embryos into 100% Methanol (MeOH), store them at -20°C (2h to several months).

In situ hybridization, day 1

- Rehydration: Transfer embryos into small baskets and rehydrate by successive incubations in:
  - 75% MeOH - 25% PBS for 5 min
  - 50% MeOH - 50% PBS for 5 min
  - 25% MeOH - 75% PBS for 5 min
  - 100% PBST (PBS/Tween-20 0.1%) 4 x 5 min
- Digest with Proteinase K (10 µg/ml).
  - blastula and gastrula: 30 seconds
  - early somitogenesis: 1 min
  - late somitogenesis (14 to 22 somites): 5 min
  - 24h embryos: 15 min
  - 36h/48h embryos: 30 min
- Refix in 4% PFA-PBS, 20 min.
- Wash in PBST, 5 x 5 min.
- Preabsorb the anti-DIG antibody (Boehringer) in a 1:1000 dilution in PBST-sheep serum 2%-BSA (2mg/mL) for several hours at RT with a batch of previously fixed embryos. Use about 500 embryos for 10 mL of antibody.
- Prepare the Prehybridization and Hybridization mix:
  - **Prehybridization and Hybridization mix (HM):**
  - Formamide 50-65%
  - 5 x SSC
  - Tween-20 0.1%
  - Citric acid to pH 6.0 (460 µL of 1M stock for 50 mL)
  - Heparin 50 µg/mL
  - tRNA 500 µg/mL

Note: Add tRNA and Heparin to the prehybridation and hybridization mix only (not the wash solutions). Vary the formamide concentration according to the desired hybridization stringency.

- Prehybridize embryos in 800 µL of hybridization mix, 2 to 5 hrs at a hyb. temperature which is approx. 20-22°C below the calculated melting temperature (Tm) of the miRCURY™ probe.

- Label 100 pmol of miRCURY™ probe (LNA probe) for miRNA detection using the DIG Oligonucleotide 3'-End Labeling Kit from Roche Applied Science (cat # 3 353 575) according to the manufacturer's instructions with the following modifications: Use 200 U of terminal transferase (0.5 µl) for each end-labeling reaction and incubate the reaction mixture for 30 min at 37 °C. Place on ice and stop the reaction by adding 5 µl of 0.1 M EDTA (pH 8.0). Remove the unincorporated label from the 3'-DIG labeled miRCURY™ probe in a volume of 25 µL using a MicroSpin G-25 column (Amersham Biosciences cat# 27-5325-01) according to the manufacturer's instructions.

  Note: It is important to clean-up the labeled probe before use, since the unincorporated label may result in unspecific background staining in in situ hybridization.

- Remove prehybridization mix, discard, and replace with 200 µL of hybridization mix containing 1-2 µL of the MicroSpin G-25-purified 3'-DIG labeled miRCURY™ probe.
- Adjust the temperature of the waterbath so that the *in situ* hybridization is carried out at a temperature which is ca. 20-22°C below the calculated melting temperature (Tm) of the miRCURY™ probe and hybridize overnight.

In situ hybridization, day 2

Washes:

- 100% HM at the same temperature as above for hybridization (approx 20-22°C below the Tm of the miRCURY™ probe), very brief wash

- 75% HM/25% 2 x SSC at hybridization temp. 15 min
- 50% HM/50% 2 x SSC at hybridization temp. 15 min
- 25% HM/75% 2 x SSC at hybridization temp. 15 min
- 2 x SSC at hybridization temp. 15 min
- 0.2 x SSC, at hybridization temp. 2 x 30 min
- 75% 0.2 (or 0.05) x SSC/25% PBST at RT 10 min
- 50% 0.2 (or 0.05) x SSC/50% PBST at RT 10 min
- 25% 0.2 (or 0.05) x SSC/75% PBST at RT 10 min
- PBST at RT, 10 min
- PBST/2% sheep serum/2mg:ml BSA at RT, several hrs

Incubation with anti-DIG antiserum

Incubate in antibody solution overnight with agitation at +4°C.

Anti-DIG antibody solution
Pre-adsorbed anti-DIG, 1:5000 dilution (final concentration) in PBST
2% sheep serum
2mg/mL BSA

Zebrafish *in situ* hybridization, day 3

Washes

- Remove antiserum, discard, and then wash extensively:
- PBST at RT, very brief wash
- PBST at RT, 6 x 15 min
- Staining buffer (100 mM Tris HCl pH9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20), at RT 3 x 5 min
- Staining:
- Incubate embryos in staining solution at RT and monitor with a dissecting microscope.

Mouse *in situ* hybridization, day 3

Washes:

- Remove antiserum, discard, and then wash extensively:
- PBST at RT, very brief wash
- PBST at RT, 5 x 1 hour
- PBST at +4 °C, 2 days by exchanging the PBST buffer at every 2 hours

Staining buffer (100 mM Tris HCl pH9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20), 3 x 5 min

- Staining blue

Incubate embryos in staining solution at RT and monitor with a dissecting microscope.

NBT 50 mg/mL - 225 µL
BCIP 50 mg/mL - 175 µL
Staining buffer - 50 mL

(NBT stock: 50 mg Nitro Blue Tetrazolium in 0.7 mL of Dimethyl-formamide anhydride + 0.3 ml H2O. BCIP stock: 50 mg of 5-Bromo 4-Chloro-3-Indolyl Phosphate in 1mL anhydrous Dimethyl-formamide).

- Stop the reaction by removing the staining solution and washing the embryos in stop solution

PBS pH5.5
EDTA 1mM

- Store the embryos in stop solution at +4°C in the dark.

Mounting

- For observation using a dissecting microscope, mount embryos directly in stop solution and methylcellulose.
- For observation using a compound microscope, mount embryos in 100% glycerol.
- For embryos at early development stage (up to 18h), dehydrate in 100% methanol, clear for a few minutes in methylsalycilate, and mount in Permount.
- (What we mostly do) Wash embryos 3x 5 min in PBST. Dehydrate by successive incubations in:

75% PBS - 25% MeOH for 5 min
50% PBS - 50% MeOH for 5 min
25% PBS - 75% MeOH for 5 min
100% MeOH for 5 min
100% MeOH for 5 min
Murray's (benzylalcohol:benzylbenzoate 1:2) for 5 min
Murray's (benzylalcohol:benzylbenzoate 1:2), store at 4°C

Reagents and chemicals

PFA: paraformaldehyde (Sigma)
10 x PBS
Tween-20 (Sigma P1379)
PBST: PBS containing 0.1 % Tween-20
MeOH: methanol
Proteinase K (Boehringer 1000144)
Anti-DIG antibody - alkaline phosphatase Fab fragment (Boehringer 1 093 274)
BSA fraction V protease free (Sigma A-3294)
Formamide (deionized, high purity grade)
20 x SSC
Heparin at 5 mg/mL (Sigma H3393)

RNAse free tRNA (Sigma R7876, 50 mg/mL resuspended in H_2O and extensively extracted several times in Phenol/$CHCl_3$ to remove protein)
Citric acid 1M
Normal Sheep serum (Jackson ImmunResearch 013-000-121)
Tris HCl pH9.5 1M
$MgCl_2$ 1M
NaCl 5M
NBT 50 mg/mL (made from powder, Sigma N6876)
BCIP 50 mg/mL (made from powder, Sigma B8503)
PBS pH5.5
EDTA 0.5M
Glycerol 100%
Methylsalicylate (Sigma M6752)
Permount (Fisher SP15-100)

*Please note: For optimal fixation it may be critical to use fresh formaldehyde solutions. Fresh 4% solutions can be made from 16%, methanol free, formaldehyde or from solid paraformaldehyde (4% w/v).

For preparation of buffers please refer to :
Molecular cloning : a laboratory manual / Sambrook, Joseph; Russell, David W. --
3rd ed. -- New York: Cold Spring Harbor Laboratory, 2001.

This protocol is adapted from:
Thisse, C., Thisse, B., Schilling, T. F., and Postlethwait, J. H. (1993). Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. Development 119, 1203-1215

Please refer to:
Thisse B, Heyer V, Lux A, Alunni V, Degrave A, Seiliez I, Kirchner J, Parkhill JP, Thisse C. Spatial and temporal expression of the zebrafish genome by large-scale in situ hybridization screening. Methods Cell Biol. 2004;77:505-19.