Determining miRNA expression patterns in *Xenopus*

Authors: Marco Antonaci*, Alice M. Godden*, Grant N. Wheeler

Affiliation: School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, United Kingdom.

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* Contributed equally

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Abstract

Whole Mount in Situ Hybridisation (WISH) is a technique that enables temporal and spatial visualisation of RNA molecules in an embryo or whole tissue by using a complementary labelled probe. MicroRNAs are short non-coding RNAs of 20-25nt length mainly involved in post-transcriptional regulation of gene expression. In this chapter, we describe how to visualise miRNAs in *Xenopus laevis* or *tropicalis* by WISH using two different approaches: LNA-WISH to visualise mature miRNAs and pri-miRNAs-WISH to visualise the immature form of miRNAs, the pri-miRNAs.

1. Introduction:

One of the most important goals in developmental biology is to determine where and when a gene is expressed. *Xenopus laevis* and *Xenopus tropicalis* are powerful model species which have been used to identify several fundamental biological processes in development. This is due to the relative ease from which the eggs can be collected and fertilised, and by the large numbers of eggs that a single frog can lay (over hundreds in a single use). This, combined with the fact that many human diseases can be modelled in Xenopus species (1), make this animal an excellent model to study genetic diseases and basic embryonic development. A well-established technique to visualise the expression of a gene during development is Whole Mount in Situ Hybridisation (WISH). The general idea of WISH is to use a digoxigenin-labelled RNA probe that pairs with the RNA of interest. Following hybridisation with this probe, the sample is incubated with an Alkaline Phosphatase-labelled anti-digoxigenin antibody. The colour reaction occurs by incubating the sample with dyes that upon dephosphorylation will generate a coloured precipitate. This colour reaction therefore only occurs in the regions of the sample where the RNA target is expressed (2, 3). As a "good" probe is usually 250-1000nt in length, and mature miRNAs are around 20-25nt in length (4), alternative approaches have been developed in order to visualise their expression. The first involves the use of Locked Nucleic Acid (LNA) probes to stain mature miRNAs (5, 6). A second approach generates a probe that pairs with the unprocessed form of the miRNA of interest (primary miRNA or pri-miRNA) (7, 8). Pri-miRNAs length can be very different from a minimum range of 80-120nt for isolated miRNAs. Clustered miRNAs, such as the ones in the group miR-17~92 can have primary transcripts longer than 1kb. Probes that binds these transcripts can be used the same way as probes for mRNAs. In this chapter, we will discuss how to carry out an LNA-WISH and a pri-miRNA WISH on *Xenopus* embryos.

2. Materials:

2.1 Collection of Xenopus species embryos:

- PMSG – Intervet
- Chorulon hcG – Intervet
- 1X MMR – 100mM NaCl, 2mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 5mM HEPES pH 7.5 (Note 1)
- 0.1X MMR – 10mM NaCl, 0.2mM KCl, 0.1mM $MgCl_2$, 0.2mM $CaCl_2$, 0.5mM HEPES pH 7.5 (Note 1)
- 0.05X MMR – 5mM NaCl, 0.1mM KCl, 50µM $MgCl_2$, 0.1mM $CaCl_2$, 250µM HEPES pH 7.5 (Note 1)
- 2% L-Cysteine pH 7.5 in MMR (1X for *X. laevis*, 0.1X for *X. tropicalis*) (Note 2)
- Xenopus laevis or tropicalis frozen sperm – Sourced from EXRC, The University of Portsmouth

2.2 Fixation of Xenopus species embryos:

- 10X MEM Salts – 1M MOPS, 20mM EGTA, 10mM $MgSO_4$, pH 7.4 in DEPC-H_2O (Note 3)
- MEMFA – 3.7% Formaldehyde, in 1X MEM Salts (Note 4)
- 100% Ethanol
- DEPC-PBS – 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4 in DEPC-H_2O (Note 1)

2.3 Whole Mount in Situ Hybridisation:

- PBST – 0.1% Tween 20 in 1X PBS
- 75% Ethanol in DEPC-PBST
- 50% Ethanol in DEPC-PBST
- 25% Ethanol in DEPC-PBST
- Proteinase K Solution - 20µg/ml Proteinase K in DEPC-PBST (Note 2)
- RNase A Solution - 1µg/ml RNase A in 2X SSC (Note 2)
- Fixing Solution – 3.7% Formaldehyde in DEPC-PBST (Note 4)
- 2X SSC – 0.3M NaCl, 30mM Na-Citrate, pH7 in dH_2O (Note 5)
- 0.2X SSC – 30mM NaCl, 3mM Na-Citrate, Ph 7 in dH_2O (Note 5)
- Hybridisation Buffer – 50% Formamide, 25% 20X SSC, 1mg/ml Torula RNA, 100µg/ml Heparin, 1X Denharts, 0.1% Tween 20, 0.1% Chaps, 10mM EDTA in DEPC-H_2O (Note 6)
- Probe Buffer – 1µg/ml DIG-labelled probe in Hybridisation buffer (Note 6 and 7)
- MAB – 0.1M Maleic Acid, 150mM NaCl in dH_2O (Note 1)
- Blocking Solution – 2% Boheringer Blocking Reagent (BBR) in MAB
- Antibody Solution – 1:3000 α-DIG (Anti-Digoxigenin-AP Fab fragments, Sigma-Aldrich, REF: 11093274910) in Blocking Solution (Note 2)
- NTMT – 100mM Tris-HCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$, 1% Tween 20 (Note 2)
- NBT – 50mg/ml nitro blue tetrazolium in 70% dimethylformamide (DMF) (Note 8)

- BCIP – 50mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in dimethylformamide (DMF) (Note 8)
- Colour Solution – 0.09% NBT, 0.35% BCIP in NTMT (Note 2 and 8)
- Imaging plate – 2% Agarose in dH_2O (Note 9)
- 100% Methanol (Note 10)

3. Methods:

3.1a Collection of Xenopus laevis eggs:

Females of *Xenopus laevis* are primed with an injection of 200U (200µl) PMSG onto one lymph sac 3 to 7 days before eggs collection (Note 11). Frogs are not fed during this time. Induction is then performed ~16h before eggs collection with an injection of 500U (500µl) of Chorulon onto one lymph sac. Once the first eggs are naturally released, frogs are gently squeezed in order to collect eggs in a petri dish.

3.1b Fertilisation of Xenopus laevis eggs:

Xenopus laevis frozen sperm is collected from the -80°C in dry ice and thawed for 30sec in a 37°C water bath doing an "8" movement (Note 12). Then, 125µl of 0.1X MMR is added to the frozen sperm and gently mixed with a pre-cut tip, the mixture is then pipetted on the eggs. The eggs are then incubated at 18°C for 10min. After this incubation, the dish is flooded with 0.1X MMR and incubated for 20min more at 18°C. At this points, fertilised eggs will turn with the animal pole (pigmented side) on the top and vegetal pole (non-pigmented side) on the bottom. The embryos are then de-jellied by removing 0.1X MMR and adding 2% Cysteine for 7min. During this incubation, it is useful to gently swirl the embryos. At the end of the 7min, cysteine is removed by washing the embryos a minimum of three times with 1X MMR and then a minimum of three more times with 0.1X MMR.

3.2a Collection of Xenopus tropicalis eggs:

Females of *Xenopus tropicalis* are primed with an injection of 10U (100µl) Chorulon onto one lymph sac 24h-72h before eggs collection. Frogs are not fed during this time. Induction is then performed ~5h before eggs collection with an injection of 200U (200µl) of Chorulon onto one lymph sac. Once the first eggs are naturally released, frogs are gently squeezed in order to collect eggs in a petri dish.

3.2b Fertilisation of Xenopus tropicalis eggs:

Xenopus tropicalis frozen sperm is collected from the -80°C and thaw for 30sec in a 37°C water bath doing an "8" movement (Note 12). Then, 125µl of 0.1X MMR is added to the frozen sperm and gently mixed with a pre-cut tip, the mixture is then pipetted on the eggs. The eggs are then incubated at 25°C for 20min. After this incubation, the dish is flooded with 0.05X MMR and incubated for 30min more at 25°C. At this points, fertilised eggs will contract the pigmentation on the animal pole, making it darker than the one of unfertilised eggs. The embryos are then de-jellied by removing 0.05X MMR and adding 2% Cysteine for 7min at 25°C. At the end of the 7min, cysteine is removed by washing the embryos a minimum of three times with 0.1X MMR and a minimum of three times with 0.05X MMR.

3.3 Fixation of Xenopus species embryos:

Fertilised embryos develop at 16-21°C (*X. laevis*) or 25°C (*X. tropicalis*). Once they reach the desired stage, embryos are collected and fixed in MEMFA for 2h at RT with gentle rocking, or ON at 4°C on gentle rocking. Once fixed, embryos are washed three times with 100% Ethanol. At this point, it is possible to proceed with the WISH protocol, or the embryos can be stored at -20°C indefinitely.

3.4 LNA-WISH

All steps are carried out at RT and with gentle rocking, unless specified (Note 13, 14, 15 and 16.).

- Wash with 75% Ethanol in DEPC-PBST for 5min (Note 17 and 18);
- Wash with 50% Ethanol in DEPC-PBST for 5min;
- Wash with 25% Ethanol in DEPC-PBST for 5min;
- Wash twice with DEPC-PBST for 5min;
- Treat with Proteinase K solution, guidelines for the incubation time on Table 1. No rocking;
- Wash twice with DEPC-PBST for 5min;
- Treat with fixing solution for 20min;
- Wash twice with DEPC-PBST for 5min;
- Treat with pre-heated Hybridisation buffer (54°C) until the embryos settle to the bottom of the vial. No rocking;
- Replace the Hybridisation buffer with some new Hybridisation buffer and incubate at 54°C for at least 3h;
- Incubate with the LNA-probe solution ON at 54°C (Note 19);
- Wash with Hybridisation buffer once for 10min at 54°C;
- Wash three times with 2X SSC for 20min at 54°C;
- Wash twice with 0.2X SSC at 54°C;
- Wash with MAB for 10min;
- Block in Blocking solution for at least 1h;
- Incubate in the Antibody solution ON at 4°C (Note 20);
- Wash twice with MAB for 5min;
- Wash six times with MAB for 30min;
- Wash with MAB ON at 4°C;
- Wash three times with MAB for 5min;
- Wash twice with NTMT solution for 10min;
- Incubate in Colour solution protected from light. At this point, keep the embryos in colour solution until the control LNA-probe starts developing colour. It is possible to replace the Colour solution if the solution starts changing colour from pale yellow to purple. This will speed the reaction and reduce the background staining;
- Stop the solution by washing three times with PBST (Note 21);
- It is possible to remove some of the background by incubating the embryos ON at 4°C in 100% Methanol. Embryos can be stored at -20°C indefinitely;
- Rehydrate by washing twice with PBS for 10min;
- Acquire images on a 2% Agarose plate with PBS.

3.7 Pri-miRNA WISH

All steps are carried out at RT and with gentle rocking, unless specified (Note 13, 14, 15 16 and 22).

- Wash with 75% Ethanol in DEPC-PBST for 5min (Note 17 and 18);
- Wash with 50% Ethanol in DEPC-PBST for 5min;
- Wash with 25% Ethanol in DEPC-PBST for 5min;
- Wash twice with DEPC-PBST for 5min;
- Treat with Proteinase K solution, guidelines for the incubation time on Table 1. No rocking;
- Wash twice with DEPC-PBST for 5min;
- Treat with fixing solution for 20min;
- Wash twice with DEPC-PBST for 5min;
- Treat with pre-heated Hybridisation buffer (60°C) until the embryos settle to the bottom of the vial. No rocking;
- Replace the Hybridisation buffer with some new Hybridisation buffer and incubate at 60°C for at least 1h;
- Incubate with the probe solution ON at 60°C;
- Wash with Hybridisation buffer once for 10min at 60°C;
- Wash three times with 2X SSC for 20min at 60°C;
- Treat with RNAse A solution for 30min at 37°C;
- Wash with 2X SSC for 10min;
- Wash twice with 0.2X SSC at 60°C;
- Wash with MAB for 10min;
- Block in Blocking solution for at least 1h;
- Incubate in the Antibody solution ON at 4°C (Note 20);
- Wash twice with MAB for 5min;
- Wash six times with MAB for 30min;
- Wash with MAB ON at 4°C;
- Wash three times with MAB for 5min;
- Wash twice with NTMT solution for 10min;
- Incubate in colour solution protected from light. Keep checking the embryos every 20-30min until a signal is seen. It is possible to replace the colour solution if the solution starts changing colour from pale yellow to purple. This will speed the reaction and reduce the background staining;
- Stop the solution by washing three times with PBST (Note 21);
- It is possible to remove some of the background by incubating the embryos ON at 4°C in 100% Methanol;
- Rehydrate by washing twice with PBS for 10min;
- Acquire images on a 2% Agarose plate with PBS.

Notes

1. MMR, DEPC-PBST and MAB can be prepared as stock solution of 10X and stored at 4°C
2. 2% Cysteine, MEMFA, Proteinase K solution, RNase A solution, fixing solution, Antibody solution, NTMT and colour solution should be prepared fresh
3. MEM salts are photosensitive and should be stored at 4°C wrapped in aluminium foil
4. MEMFA and fixation solution contain formaldehyde, which is toxic, handle with care
5. SSC can be prepared as stock solution of 20X and stored at 4°C
6. Hybridisation buffer and probe buffer contain formamide, which is toxic, handle with care. Both can be prepared in advance and stored at -20°C
7. LNA-probes need to be prehybridised against a mixture of different stage embryos up to 6 times before first use in order to minimise background staining. Both LNA-probe and pri-miRNA probe can be used multiple times, and stored at -20°C. Usually, probes that have been used at least 1-2 times, show cleaner staining
8. Colour solution, BCIP and NBT contain dimethylformamide, or DMF. All are toxic, handle with care
9. A thicker agarose layer on the plate will show a nicer blue background during the acquisition of the picture
10. Methanol is toxic, handle with care
11. An alternative to the use of PMSG is to prime *X. laevis* with a diluted Chorulon, injecting 50U/frog of Chorulon in the same way
12. For more detailed information on how to use frozen sperm to fertilise Xenopus eggs, see https://xenopusresource.org/using-frozen-sperm-4
13. Fixed embryos are particularly fragile, while changing solution, it is better to leave a bit of the previous solution but not to ruin the embryos, rather than removing all of it
14. Embryos should never be let to dry
15. The whole experiment can be performed in glass vials, but is also possible to use normal 2ml tubes and, before the colour reaction, transfer the embryos in glass vials. This way it will be possible to look at the embryos during the colour reaction.
16. Usually, 10-15 embryos per sample are a good number for each WISH
17. Usually, 0.5-1ml of solution is enough to completely cover all the embryos
18. While rocking, glass vials can be kept standing. This can be optimal since, if they gets stock on the lid of the vial, they won't be in contact with the solution and will dry out
19. LNA-probes should be used 4-6 times before they lose their non-specific staining. Before starting an experiment it should be better to perform mock LNA-WISH with that specific probe, in order to obtain the best results
20. After the incubation with primary antibody, the use of DEPC-treated solutions is no longer necessary
21. It is possible to stop and restart the colour reaction by washing three times with PBST and leaving the embryos ON at 4°C. The day after, is possible to incubate again twice with NTMT and then start again the colour reaction

22. For the list of primers used to produce xtr-miR-219 pri-miRNA probe, see Godden *et al* (7).

Table 1. Time in minutes of incubation with Proteinase K solution. This is a guideline, every time a new batch of Proteinase K is prepared, it should be tested with known probes on different stages of development. The staging is performed according to the standard Nieuwkoop and Faber staging system .

Stage (NF)	1-10	10.5-12	13-16	17-20	21-25	26-30	31-33	34-36	37-40	41-45
Time (min)	0.5	1	2	3	4	5	6	8	18	20

Figure 1. Example of LNA-WISH and pri-miRNA WISH. **A)** LNA-WISH on: from top to bottom, expression of xtr-miR-30-5p, xtr-miR-124 and control miRNA at tailbud stages. **B)** LNA-WISH for xtr-miR-19a on neurula stages. **C)** pri-miRNA-WISH on xtr-miR-219. On the left side WISH on xtr-miR-219 anti-sense probe at NF stage 7 (top) and NF stage 4 (bottom). On the right side, WISH on xtr-miR-219 sense probe at NF stage 7 (top) and NF stage 4 (bottom).

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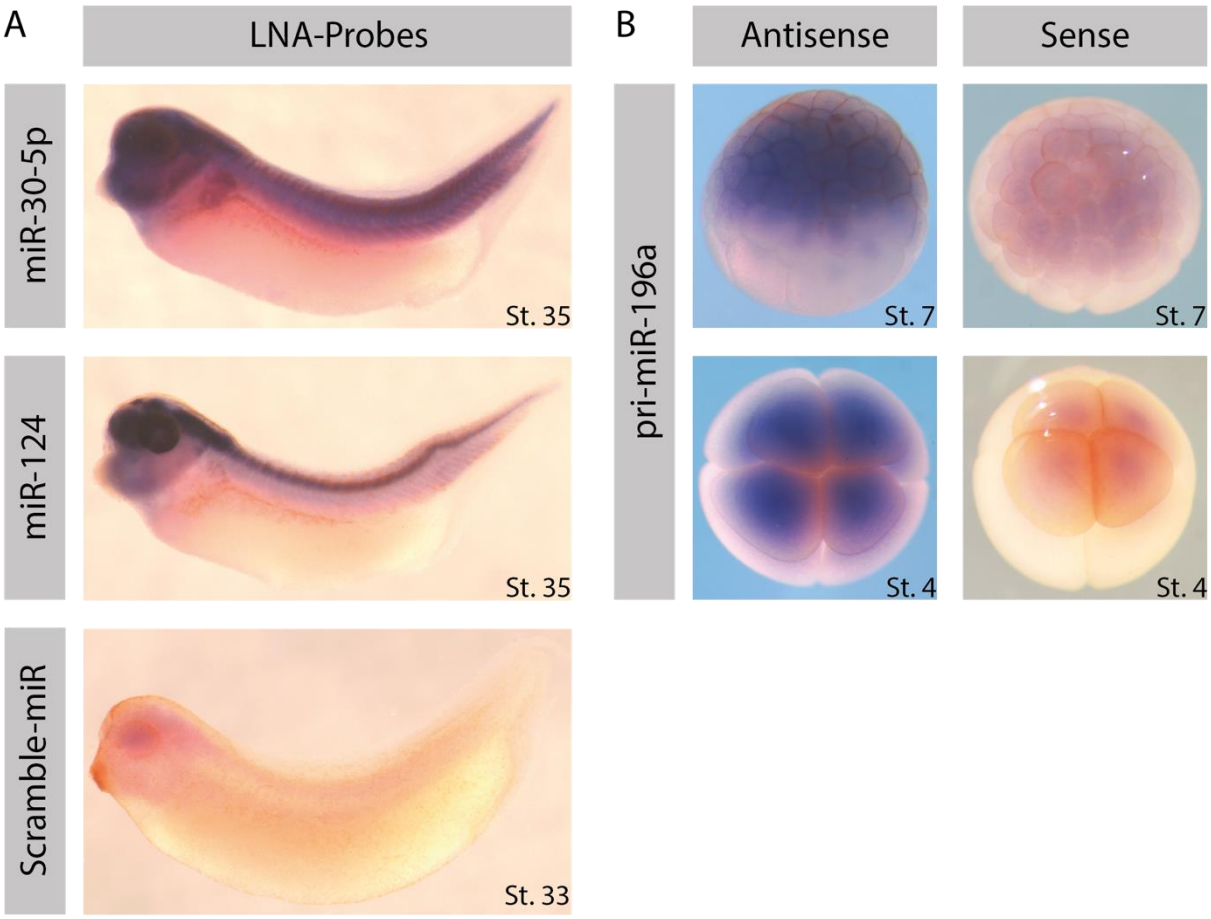
A
LNA-Probes
miR-30-5p
St. 35
miR-124
St. 35
Scramble-miR
St. 33
B
Antisense
Sense
pri-miR-196a
St. 7
St. 7
St. 4
St. 4