Locked nucleic acid-based *in situ* detection of microRNAs in mouse tissue sections

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Here we describe a method for sensitive and specific histological detection of microRNAs (miRNAs) by *in situ* hybridization. The protocol focuses on the use of locked nucleic acids (LNAs), which are bi-cyclic RNA analogs that allow a significant increase in the hybridization temperature and thereby an enhanced stringency for short probes as required for miRNA detection. The protocol is optimized for cryosections in order to study the spatial and temporal expression of miRNAs with high sensitivity and resolution. We detail how to construct probes, set up and conduct an LNA *in situ* hybridization experiment. In addition, we discuss alternative colorimetric strategies that can be used to effectively detect and visualize miRNAs including double staining with other markers. Setting up and conducting the *in situ* experiment is estimated to take ~1 week, assuming that all the component parts are readily available.

INTRODUCTION

The knowledge of spatial and developmental expression of genes is vital information that generates primary insights into their possible developmental functions. One of the most frequently used techniques to study gene expression is *in situ* hybridization. This technology is based on the hybridization of 300–1,500 nucleotide (nt) long RNA probes to their cognate transcripts in whole mount or tissue sections and allows their detection with high cellular resolution[1–4]. One drawback of this method is that shortening the probes greatly reduces their stringency and thus limits their use for *in situ* hybridizations to detect short transcripts. This limitation has hampered cellular studies of differentially spliced exons and small RNAs such as miRNAs.

miRNAs are small, 19–25 nt, highly conserved regulatory RNAs that have emerged as important players in regulating gene expression at the post-transcriptional level[5,6]. miRNA genes generate long transcripts that are processed in defined steps into ~22 nt long miRNA duplexes[7,8]. Expression profiling techniques such as northern blots or microarrays have generated fruitful insights into the tissue and/or developmental specificity of miRNAs[9–11]. However, these methods lack cellular resolution, which in non-homogenous tissues like the brain makes it impossible to determine specifically which cells are expressing the miRNA of interest. Furthermore, a highly expressed miRNA in a sparse population of cells might not be detectable by northern blots.

Other groups including us have successfully used *in situ* probes with a novel modified nucleotide termed LNA to study the expression of mature miRNAs[12–15]. LNAs are bi-cyclic high-affinity nucleotide analogs that significantly improve hybridization

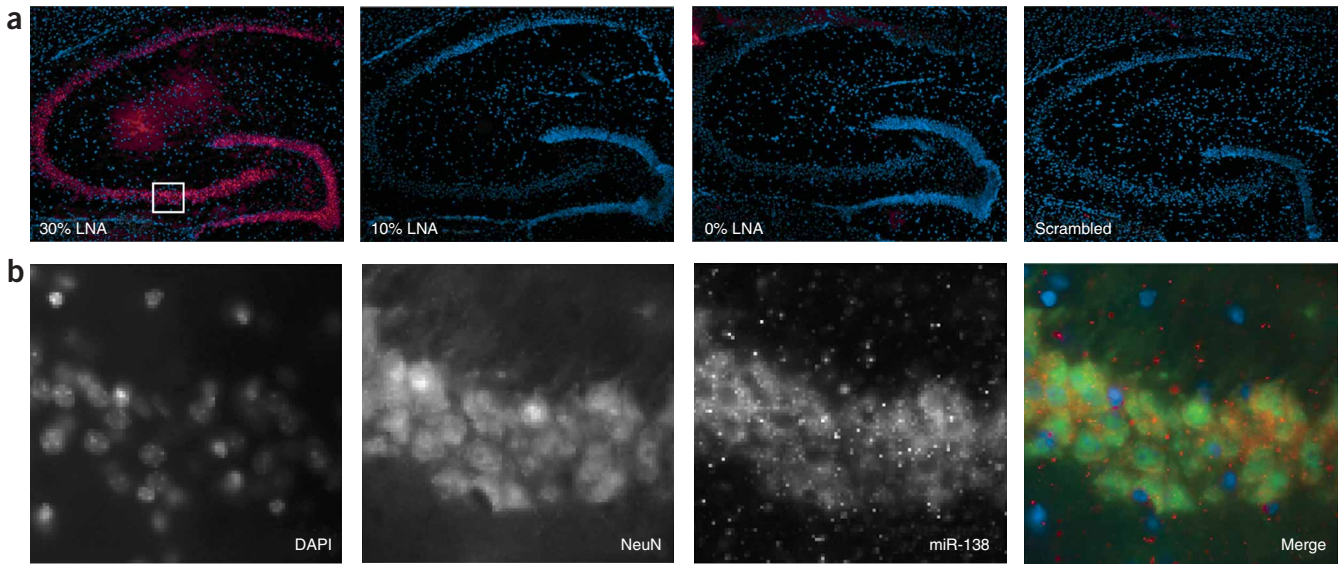

Figure 1 | miR-138 *in situ* staining of mouse hippocampus. (**a**) Mouse brain sections hybridized with miR-138 LNA probes containing 30%, 10% and 0% LNA (red) as well a scrambled miR-138 30% LNA probe at 60 °C. Pictures are at five times resolution. DNA was counterstained with DAPI (blue). The white box depicts the region that is magnified in **Figure 2**. (**b**) Mouse hippocampus sections hybridized with miR-138 (red; center right) at 60 °C and double-stained with mouse anti-NeuN (green; center left: using Invitrogen, Clone A60). DNA was counterstained with DAPI (blue; far left). A merge of the different color channels is depicted (far right). Pictures were imaged at 50 times magnification and 512 × 512 pixels resolution on a Zeiss LSM 510 Meta confocal microscope.

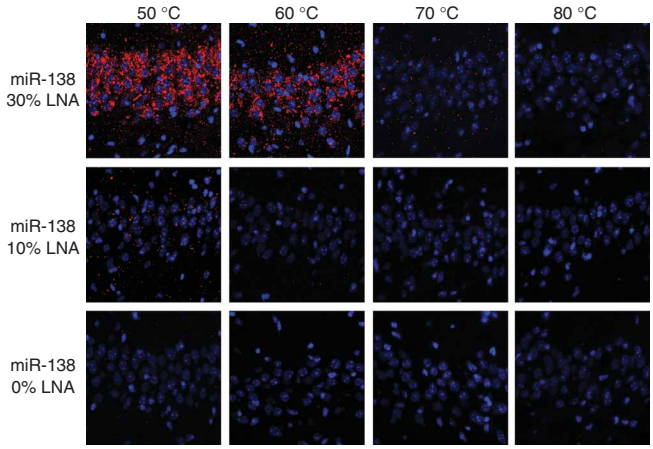

Figure 2 | LNA-dependent hybridization stringency of miR-138. Mouse hippocampus sections hybridized at different hybridization temperatures (50, 60, 70 and 80 °C) with miR-138 LNA probes containing 30%, 10% and 0% LNA. Note the specific Fast red staining of miR-138 in the neurons at 50–70 °C with the high LNA content probe (30%) and that only 50 °C with 10% LNA gave any detectable signal; all other conditions were devoid of signal. Pictures were imaged at 25 times magnification and 512 × 512 pixels resolution on a Zeiss LSM 510 Meta confocal microscope. DNA was counterstained with DAPI (blue).

properties of DNA oligonucleotides[16–18]. It was shown in northern blot analysis that the substitution of every third nucleotide position by LNA yielded an increased sensitivity (by at least tenfold) of miRNA detection compared to normal DNA probes[14]. A similar LNA design is used for the synthesis of the Exiqon A/S (Denmark) miRCURY probes (around 30% LNA) that we have used for this *in situ* protocol[19]. Each incorporated LNA monomer increases the melting temperature (T_m) of a DNA/RNA hybrid by 2–10 °C. The increased thermal stability of the LNA-DNA/RNA duplex makes it possible to make probes as short as ~20 nt with high melting temperatures (≥70 °C), providing the basis for high stringency required for *in situ* hybridizations. However, one limitation with the use of LNA probes is their low signal strength. LNA *in situ* hybridizations on cultured cells have previously been performed with directly conjugated fluorescent dyes but could only detect abundant miRNAs[19].

Here we provide a detailed step-by-step protocol for the application of *in situ* hybridizations with LNA probes on mouse cryosections (**Figs. 1** and **2**). The advantage of using sections over whole-mount preparations is that the diffusion times are significantly shorter, reducing any signal spreading, which gives higher sensitivity and cellular resolution. Furthermore, the protocol is designed for digoxigenin (DIG)-conjugated LNA probes and can be used with two different alkaline phosphatase color reactions, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) and Fast Red, to enhance the signal strength. The NBT/BCIP colorimetric method generates a blue precipitate that can be detected with light microscopy and is highly sensitive. Fast Red is a less sensitive substrate but is fluorescent, which makes it suitable for double staining in conjunction with detection of proteins, cell type-specific markers or putative target genes (**Fig. 1b**). We have successfully used the protocol on sections of various tissues like brain, liver and whole mouse embryos[15]. Moreover, we have used LNA probes against different regions of the long nascent miRNA transcript, making it possible to detect the different miRNA maturation stages with cellular resolution[15], a type of study that was previously possible only at organ resolution using northern blots. The described protocol is easy to set up and is reduced to a few essential steps that have been proven to be crucial. The critical steps of the protocol are outlined as a flowchart in **Figure 3** with approximate time estimates being included.

MATERIALS

REAGENTS

- Sucrose (Fluka, 84097)
- Paraformaldehyde (PFA) (Sigma, P-6148) ! CAUTION Allergenic, use gloves.
- Formamide (Riedel, 33272) ! CAUTION Allergenic, work in fume hood.
- Diethylpyrocarbonate (DEPC) (Fluka, 32490) ! CAUTION Carcinogenic, work in fume hood.
- Triethanolamine (Fluka, 90279)
- Conc. HCl (Riedel, 30721) ! CAUTION Corrosive.
- Acetic anhydride (Sigma, A6404)
- 50× Denhardt's (Sigma, D-2532)
- Yeast tRNA (Sigma, R-6750)
- Salmon sperm DNA (Sigma, D-91565)
- Blocking reagent (Roche, 109676)
- Anti-digoxigenin antibody (Roche, 1093274)
- Levamisol (Sigma, L-9756) ! CAUTION Carcinogenic, use gloves.
- BCIP (Roche, 1383221)
- NBT (Roche, 1383213) ! CAUTION Carcinogenic, use gloves.
- Tissue Tek OTC (Sakura, 4583)
- Fast Red Substrate (Dako, K0699) ! CAUTION Carcinogenic, use gloves.
- 1 M Tris pH 8.2
- 10% (w/w) CHAPS (Sigma, C5070-5G)
- 10% and 20% (w/w) Tween (Fluka, 93773)
- 5 M NaCl (Merck, 1.06404.1000)
- 1 M $MgCl_2$ (Fluka, 2320946)
- PBS
- 20× SSC
- Dry ice

EQUIPMENT

- Super Frost Slides (Henzel Glaeser, 51800AMNZ)
- Coverslips (Menzel-Glaeser, 24×60 mm)
- Hybridization oven (Biometra Compact Line OV04) ▲ CRITICAL Heat the hybridization oven to the desired hybridization temperature during Step 15.
- Water bath (GFL, 1002)
- Glass beakers ▲ CRITICAL Bake at 180 °C to destroy RNases.
- Slide carousel ▲ CRITICAL Bake at 180 °C to destroy RNases.
- Slide mailers ▲ CRITICAL Bake at 180 °C to destroy RNases.
- RNase-free chamber (Nunc Bio-Assay Dish, 245×245×25, 240835)
- Hybridization chamber: add 3MM paper soaked with 5× SSC/50% formamide to a Nunc Bio-Assay dish (245×245×25, 240835) during Step 14 to make a hybridization chamber
- Humidified chamber: add 3MM paper soaked in tap water to a Nunc Bio-Assay Dish (245×245×25, 240835) during Step 22 to make a humidified chamber
- Confocal microscope (Zeiss Axioplan-2)
- Digital camera (Coolsnap HQ, Photometrics)
- Imaging Software (Adobe Photoshop 7.0)

REAGENT SETUP

DEPC-treated PBS (2 liters) Add 2 ml of DEPC to 2 liters of PBS and autoclave. ! CAUTION DEPC is carcinogenic until autoclaved; use gloves and work in a fume hood.

DEPC-treated water (1 liter) Add 1 ml of DEPC to 1 liter of distilled water and autoclave. ! CAUTION DEPC is carcinogenic until autoclaved; use gloves and work in a fume hood.

4% PFA for fixation Add 4 g PFA to 100 ml boiling PBS + 10 µl 10 N NaOH. Shake until solved, chill on ice and store at 4 °C. Use within 3 days of preparation. ! CAUTION PFA is highly allergenic; use gloves and work in a fume hood.

Acetylation solution Prepare 590 ml of DEPC-treated water in a beaker during Step 10. Add 8 ml of triethanolamine and 1,050 µl conc. HCl (min. 37%). Mix gently with a magnetic stirrer. During the last wash step (Step 10), add 1.5 ml acetic anhydride; wait until it has fully dissolved before using in Step 11.

Hybridization solution 20 ml (final concentration given in parentheses) 10 ml formamide (50%), 5 ml 20× SSC (5×), 2 ml 50× Denhardt's (5×), 250 µl 20 mg ml^{-1} yeast RNA (200 µg ml^{-1}), 1,000 µl 10 mg ml^{-1} salmon sperm DNA (500 µg ml^{-1}), 0.4 g Roche blocking reagents and 1.75 ml DEPC-treated water. Can be stored for months at −20 °C. ▲ CRITICAL Denature salmon sperm DNA at 96 °C for 5 min before adding to the hybridization solution.

Denaturating hybridization solution Prepare as hybridization solution but add 500 µl of 10% CHAPS, 100 µl of 20% Tween and 1,150 µl (instead of 1.75 ml) of DEPC-treated water. ! CAUTION Use gloves and work in a fume hood to protect you from hazardous formamide and the hybridization solution from RNases.

5× SSC/50% formamide In a 50 ml falcon tube mix 20 ml formamide, 10 ml 20× SSC and 10 ml DEPC-treated water.

Solution B1 (0.1 M Tris pH 7.5/0.15 M NaCl): 100 ml 1 M Tris pH 7.5 and 30 ml 5 M NaCl. Make up to 1 liter with sterile water.

Blocking solution (20 ml): 2 ml FCS and 18 ml B1. For higher stringency, include 100 µl 10% Tween.

Solution B3 (0.1 M Tris pH 9.5/0.1 M NaCl/50 mM $MgCl_2$; 1 liter): 200 ml 0.5 M Tris pH 9.5/0.5 M NaCl, 50 ml 1 M $MgCl_2$ and milliQ water. ▲ CRITICAL Use a 0.45 µm filter (Nalgene 290-4545) to filter the solution, otherwise the $MgCl_2$ will precipitate.

NBT/BCIP developer solution 3.4 µl 100 mg ml^{-1} NBT, 3.5 µl 50 mg ml^{-1} BCIP, 2.4 µl 24 mg ml^{-1} levamisol, 5 µl of 10% Tween and 986 µl B3.

Fast Red solution Add one tablet of Fast Red to 2 ml substrate buffer. Let it stand in the dark for 8 min. Vortex the solution until the tablet is completely dissolved. A 2 ml staining solution will be sufficient for 8–9 slides. Pour the solution into the supplied substrate container and put on a provided filter top and apply the Fast Red to the slides. ▲ CRITICAL This solution needs to be prepared within 20 min before usage; oxidized red tablets produce a greatly increased background signal and should be avoided.

LNA probe design For mature miRNAs, design LNA probes complementary to the full ~19–23 nt long miRNA. We have used probes with ~30% LNA content and the position of LNA incorporations was determined by the supplier (Exiqon, miRCURY probes). However, by designing probes targeting unpaired regions upstream or downstream of the miRNA, we have shown that LNA probes can be used to analyze miRNA processing transcripts (**Fig. 4**) (ref. 15). In general, we suggest designing LNA probes in accordance with the recommendations outlined in **Box 1**. The epitope used in this protocol is DIG and probes can either be ordered from Exiqon to be directly 3′- or 5′-DIG labeled or synthesized without a DIG label (Exiqon, SIGMA-PROLIGO) and 3′-DIG nucleotides added afterwards by a terminal transferase reaction (Roche DIG High-Prime, 1585606). We have not found any difference in sensitivity for either of the two labeling positions. However, for the detection of low-expressed miRNAs, probes with both 3′- and 5′-DIG labels might be used to enhance the detection signal. See ? TROUBLESHOOTING

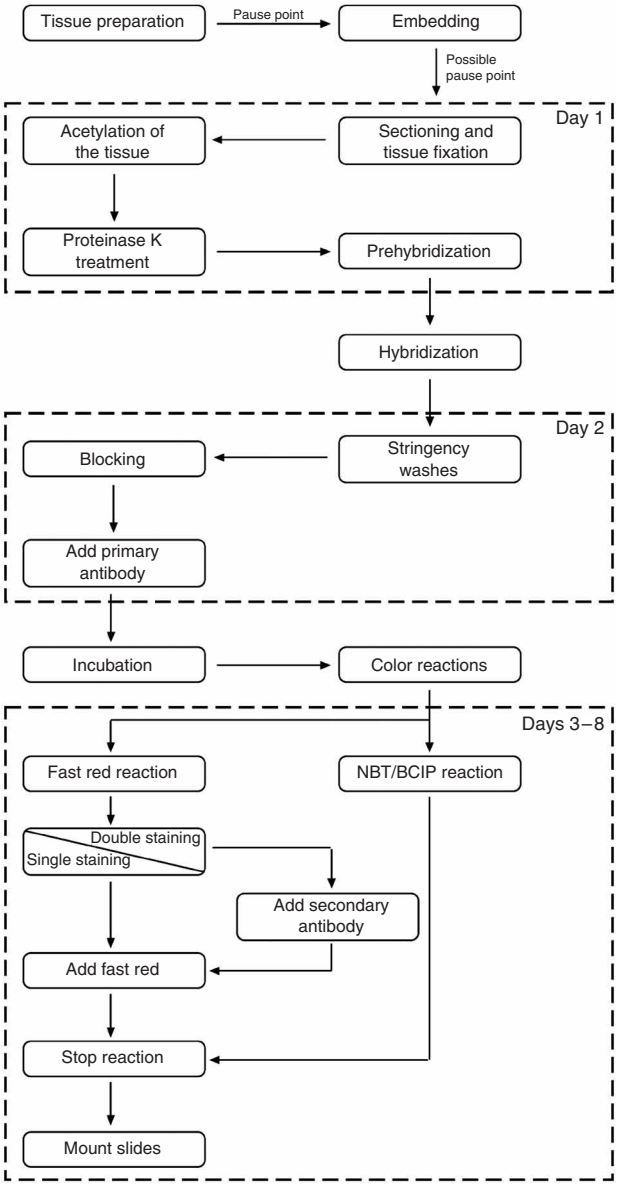

Figure 3 | *In situ* hybridization experiment flowchart. The flowchart illustrates the main points of the *in situ* hybridization procedure (see the ● TIMING section).

PROCEDURE

Tissue preparation

1| Dissect and pre-fix the tissue(s) of interest or embryos for 30 min up to 2 h in 4% PFA at room temperature (25 °C). For detailed fixation times, please see **Table 1**. After fixation, place the specimen in PBS with 30% sucrose, to minimize freeze fracturing of the tissue.
? TROUBLESHOOTING

2| Incubate overnight at 4 °C.

Embedding

3| The next day, remove the specimen from the PBS/sucrose solution and place it in a freeze mold. Add tissue Tek OCT to cover the tissue and remove any air bubbles with a pipette tip. Try to arrange the tissue in an appropriate

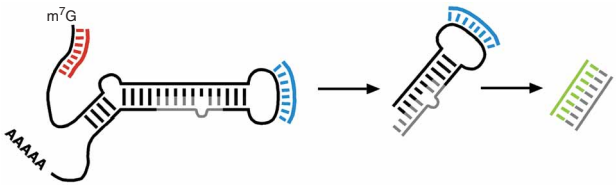

Figure 4 | Detection of miRNA maturation stages by LNA probes. Sequences upstream and downstream of the precursor miRNA as well as the hairpin are colored in black. The mature miRNA is shown in gray. Short ~20 nt long LNA-DNA probes can be designed to bind upstream or downstream the hairpin precursors to single-stranded regions to detect primary transcripts (red). Complementary probes to the hairpin sequence are suitable for pre-miRNA detection (blue) and LNA-DNA probes can be designed to target the mature miRNA (green).

BOX 1 | DESIGNING LNA-BASED PROBES TO DETECT MIRNAS

(1) Identify unpaired regions in the primary or pre-miRNA by using secondary structure prediction programs such as the RNAfold program[20]. In our experience, probes targeting regions of extensive base-pairing will not hybridize efficiently.
(2) Within those regions, design ~23 nt long probes with a GC content of around 40–60%.
(3) Apply local sequence alignment search tools (e.g., SWIFT, BLAST[21,22]) to scan the transcriptome for potential probe-binding sites. Discard probes with an overall sequence identity of more than 60% to other RNAs as these are likely to bind nonspecifically.
(4) Design a 30% LNA scrambled version of the probe as a negative control. Note that it is important to design this probe so that the sequence combination used does not bind within the transcriptome.

orientation, making use of the marks at the bottom of the freeze mold. Also try to place the tissue in the center and at the bottom of the mold; this is done to facilitate the sectioning step.

4| Place the block on top of dry ice and wait until the tissue Tek OCT has become white and frozen.
■ **PAUSE POINT** The frozen block can be stored at −80 °C for at least a month. However, with increased freezing time, the *in situ* signal strength decreases and also the Tissue Tek OCT dries out, which directly affects sectioning.

Sectioning and tissue fixation

5| Take out the freeze molds to be sectioned and place them in the cryostat and leave them there for at least 30 min to equilibrate to the sectioning temperature, which is normally around −15 to −20 °C.
▲ **CRITICAL STEP** To minimize the risk of degradation of RNA, Steps 5–18 are to be considered as RNase free, which means that the glassware should be baked; gloves and filter tips should be used. Furthermore, do not exceed a total of 3 h drying after sectioning to avoid degradation of the RNA.

6| Section 10 µm thick slices in the cryostat and collect them on Superfrost PLUS slides. Remember to include one extra slide for the scrambled probe (negative control). Note that if the sections are thicker than 15 µm, longer proteinase K (PK) treatments might be needed (Step 13).
▲ **CRITICAL STEP** The cryostat temperature is very important for successful sectioning and the optimal temperature depends on the tissue being cut. If the temperature is changed, allow the specimen about 1 min to equilibrate to the newly set temperature before resuming sectioning.
? TROUBLESHOOTING

7| Let slides dry at room temperature for at least 30 min.

8| Fix the dried slides in 4% PFA for 10 min at room temperature.

Acetylation of the tissue

9| Wash slides two times for 3 min in 1× PBS in a slide mailer at room temperature. During the washing steps, prepare the acetylation solution (see REAGENT SETUP).

10| Transfer the slides to a metal slide carousel submerged in 1× PBS in a beaker and wash for 3 min. During this step, add 1.5 ml acetic anhydride to the acetylation solution (see REAGENT SETUP).

11| Immerse the slides in the beaker of acetylation solution and stir gently for 10 min.
▲ **CRITICAL STEP** Acetylation positively charges amino groups of proteins, thus reducing background binding of the negatively charged probe to the tissue sections.

12| After the acetylation step, transfer the slide carousel to a beaker with 1× PBS solution and wash for 5 min at room temperature without agitation.

TABLE 1 | Pre-fixation times for different tissues/organs.

Tissues	PFA pre-fixation times
E7 – E12	15 min
E12 – E15	30 min
E15 – E18	1 h
Heads, E19 – P1	2 h
Large adult organs (liver, brain, etc.)	2 h
Small adult organs (pancreas, heart, etc.)	15 min to 1 h

E denotes embryonic and P post-natal days. In general, the indicated durations of fixation should be seen as suggestions and further optimizations might be needed.

PK treatment

13| Transfer the slides from the beaker into a slide mailer containing 75 ml 1× PBS. Add 37.5 µl PK stock solution (10 mg ml^{-1} in DEPC-treated water, yielding a final concentration of 5 µg ml^{-1}) and perform PK treatment at room temperature for 5 min.

▲ CRITICAL STEP The PK treatment increases the permeability and thereby the hybridization efficiency of the fixed sections. Increased PK treatment increases sensitivity but affects the tissue morphology.

? TROUBLESHOOTING

14| Wash the slides three times for 3 min in 1× PBS at room temperature. During the washing steps, prepare the hybridization chamber (see EQUIPMENT).

Pre-hybridization

15| Place the slides horizontally in the hybridization chamber and add 700 µl of hybridization buffer to each slide. Incubate slides at room temperature for 4–8 h.

Hybridization

16| For each slide, prepare 150 µl denaturizing hybridization buffer and add 0.1 µl (1 pM) of the LNA DIG-labeled probe.

17| Denature probes by heating them up to 80 °C for 5 min. Thereafter, quickly place them on ice.

18| Pipette the probe mix carefully onto the tissues and apply glass coverslips. Hybridize slides at 50–60 °C overnight.

Stringency washes

19| Soak the slides in pre-warmed 60 °C 5× SSC and carefully remove coverslips. Note that after the hybridization step, RNase-free conditions are no longer required.

20| Move the slides to a 0.2× SSC slide mailer. Incubate slides in 0.2× SSC at 60 °C for 1 h.

21| Finally, incubate slides in B1 solution (see REAGENT SETUP) at room temperature for 10 min.

Immunohistochemistry

22| Prepare a humidified chamber by placing a tap water-soaked 3MM paper at the bottom of the chamber.

23| For each *X* slides, make *X* times 500 µl blocking solution (see REAGENT SETUP).

24| Remove surplus B1 solution and place the slides horizontally in the chamber. Add 500 µl of blocking solution to each slide and leave them for 1 h at room temperature.

25| Dilute the anti-DIG-alkaline phosphatase antibody 1:2,000 in blocking solution (500 µl per slide) and pipette carefully onto the sections. If multiple labeling is being performed in order to detect proteins, then the appropriate primary antibodies should also be included. Incubate at 4 °C overnight.

Color reactions

26| This protocol can be used with different alkaline phosphatase substrates such as NBT/BCIP (option A) or the Fast Red Substrate (option B). NBT/BCIP produces a purple blue precipitate and supports long exposure times and a high sensitivity due to low background signals. Fast Red generates a red fluorescent precipitate that can be detected using standard fluorescent microscopy and is suitable for double staining with antibodies, thus providing the possibility to correlate the miRNA expression with proteins of choice. However, one drawback is that Fast Red generates a yellowish background after some hours of exposure and thus cannot be used to detect lowly expressed miRNAs.

(A) NBT/BCIP reaction

(i) Place the slides into a slide mailer and wash three times for 5 min in B1 solution at room temperature.
(ii) Equilibrate slides for 10 min in B3 solution.
(iii) For each slide, prepare 150 µl of developer solution (see REAGENT SETUP).
(iv) Place slides in a humidified chamber and pipette carefully the developer solution on the slides and place a parafilm or glass coverslip on top.
(v) Develop at room temperature in the dark ranging from 10 min up to 4 days, depending on the miRNA expression levels. The reaction can be monitored using a light microscope, terminate when a strong blue staining is observed.
(vi) Stop the color reaction by washing the slides for 3× 10 min in 1× PBT.
(vii) Mount the slides in glycerol or any water-soluble mounting media.
(viii) Visualize the staining by using standard light microscopy.

(B) Fast Red reaction

(i) Place the slides into a slide mailer and wash for 3× 5 min in B1 solution.

(ii) If the fluorescent detection of a protein is not required, proceed directly to Step 26B(iii). If fluorescent detection of a protein is required, incubate slides for 1 h at room temperature in the dark with an appropriate green fluorescent secondary antibody such as Alexa 488 (Molecular Probes) diluted 1:500 in blocking solution. Repeat the washes detailed in Step 26B(i) before proceeding with Step 26B(iii).

(iii) Place the slides horizontally in the chamber and equilibrate slides with 700 µl 1 M Tris pH 8.2 for 10 min at room temperature.

(iv) Add five drops of the Fast Red solution (see REAGENT SETUP) to each slide and place a glass coverslip on top.

(v) Develop at room temperature in the dark for 30 min up to 5 h, depending on expression levels. A red precipitate might appear but if not incubate until background or any unspecific staining on the glass is evident.

(vi) Stop the color reaction by washing 3× 10 min in 1× PBT.

(vii) Mount the slides in VECTA-Shield or any similar water-based fluorescent mounting medium. If you want to visualize the nucleus, include DAPI in the mounting media[15].

(viii) Use a fluorescence microscope to see the Fast Red staining, where it can be visualized with the Cy-3 filter.

● **TIMING**

Preparatory steps (Steps 1–4)

Dissect out embryos or organs of choice, fix and place in 30% sucrose overnight. Embed your specimen in Tissue Tek OCT, freeze and store at −80 °C. Bake glassware for day 1 of the *in situ* procedure.

***In situ* hybridization (Steps 5–26)**

Day 1: Section, fix, acetylate, proteinase K treatment, prehybridization and hybridization of slides

Day 2: Stringency wash, block and apply primary antibody on slides

Days 3–8: Wash, add secondary antibody (optional) and initiate color reactions. Develop and mount slides

? TROUBLESHOOTING

If there is no signal after developing the slides, then you might modify the following protocol steps:

Probe labeling (REAGENT SETUP)

The LNA-DNA probes used for the protocol are either 3′- or 5′-DIG labeled. This single DIG labeling affects the signal intensity due to a low number of bound antibodies. To increase the number of epitopes on the miRNA probe, use a 5′-DIG-labeled LNA-DNA probe and add an additional DIG label at the 3′ end with terminal transferase. However, this additional labeling might change the specificity of the probe as extra nucleotides are added.

Pre-fixation (Step 1)

Too long fixation times might reduce the signal, especially for tissues like early staged embryos, or small parts of organs. Over-fixation strongly affects the accessibility of the probe. To circumvent this problem, decrease fixation times.

Cryostat temperature (Step 6)

The cryostat temperature is crucial for the generation of good sections. If the sections jump up on the slide, then the sectioning temperature is too low. If the slices get compressed during sectioning, then the cryostat temperature is too high.

PK treatment (Step 13)

This step makes the tissue more permeable and accessible for the *in situ* probe. The protocol described here uses a relatively mild PK treatment to preserve epitopes that might be used for detecting proteins and the histological characteristics of the tissues analyzed. However, if there is no signal, then the enzyme concentration can be increased up to tenfold and the reaction time prolonged to a maximum of 15 min. From experience, it is advisable to prolong the reaction time rather than increasing the enzyme activity. Thereby, the risk of uneven reactions decreases and it is easier to terminate the reaction.

Red grains after the Fast Red reaction (Step 26B)

During optimization of the *in situ* protocol, both the Fast Red (K0699) and the Liquid Permanent Red Substrate System (K064011) from DAKO were tested. The Fast Red system turned out to give a better signal to noise ratio for the detection of miRNAs. However, store the tablets at –20 °C in the dark, do not use oxidized tablets (red color) and filter before you apply the Fast Red to the slides.

ANTICIPATED RESULTS

After a successful *in situ* experiment, a distinct cytoplasmic staining will be evident that is not visible for the scrambled probe (**Fig. 1a**). In the case of NBT/BCIP staining, a deep blue precipitate will appear. The Fast Red colorimetric system will give a red

fluorescent signal that can be detected in the Cy3 (red) channel. A typical background staining for both colorimetric systems appears as an even, weak staining covering the whole section including the nuclei and the extracellular matrix. Endogenous alkaline phosphatase activity can account for another common background staining. In this case, the scrambled probe (negative control) will give the same signal as the miRNA-specific probe.

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