Video Article

Tissue-specific miRNA Expression Profiling in Mouse Heart Sections Using *In Situ* Hybridization

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Abstract

micro-RNAs (miRNAs) are single-stranded RNA transcripts that bind to messenger RNAs (mRNAs) and inhibit their translation or promote their degradation. To date, miRNAs have been implicated in a large number of biological and disease processes, which has signified the need for the reliable detection methods of miRNA transcripts. Here, we describe a detailed protocol for digoxigenin-labeled (DIG) Locked Nucleic Acid (LNA) probe-based miRNA detection, combined with protein immunostaining on mouse heart sections. First, we performed an *in situ* hybridization technique using the probe to identify miRNA-182 expression in heart sections from control and cardiac hypertrophy mice. Next, we performed immunostaining for cardiac Troponin T (cTnT) protein, on the same sections, to co-localize miRNA-182 with the cardiomyocyte cells. Using this protocol, we were able to detect miRNA-182 through an alkaline phosphatase based colorimetric assay, and cTnT through fluorescent staining. This protocol can be used to detect the expression of any miRNA of interest through DIG-labeled LNA probes, and relevant protein expression on mouse heart tissue sections.

Video Link

The video component of this article can be found at https://www.jove.com/video/57920/

Introduction

micro-RNAs (miRNAs) are short (18–25 nucleotides), single-stranded, noncoding RNAs that function as negative regulators of gene expression at the post-transcriptional level by inhibiting messenger RNA (mRNA) translation and/or promoting mRNA degradation[1]. miRNAs are transcribed from introns or exons of coding or noncoding genes and are cleaved in the nucleus by DROSHA, to precursor miRNAs (pre-miRNAs), which are short stem-loop structures of 70 nucleotides[2]. Following cytoplasmic export, pre-miRNAs are further processed by DICER into mature miRNAs that span 18–25 nucleotides[3,4]. Subsequently, the RNA-induced silencing complex (RISC) incorporates these miRNAs as single-stranded RNAs, which allows for their binding to the 3' untranslated region (3'-UTR) of their target mRNAs to suppress their expression[3,5].

Within the last three decades, since they were first identified, miRNAs have emerged to master regulators of gene expression, whose own expression levels are tightly controlled[6]. Roles for miRNAs have been described in organ development[7,8,9,10,11,12], maintenance of homeostasis[13,14], as well as disease contexts that include neurological[15,16,17,18,19], cardiovascular[20], autoimmune conditions[21,22], cancers[23,24], and others[25]. The increasing appreciation for the relevance of miRNA expression patterns has brought forward the need for reliable detection methods of miRNA transcripts. Such methods include Real Time PCR, microarrays, Northern Blotting, *in situ* hybridization and others, which vary in the sensitivity, specificity, and quantitative power, predominantly due to the fact that miRNA transcripts are comprised of short and highly homologous sequences[6].

We recently reported an important role for miRNA-182 in the development of the myocardial hypertrophy[26], a condition describing the structural adaptation of the heart in response to elevated hemodynamic demands[27,28]. Cardiac hypertrophy is characterized by the increase in the myocardial mass, which, if associated with maladaptive remodeling[29], can lead to increased risk for heart failure, a condition accounting for 8.5% of all deaths attributable to cardiovascular disease[30].

Here, we describe our protocol that combines *in situ* hybridization with a digoxigenin-labeled (DIG) Locked Nucleic Acid (LNA) probe and immunostaining for the concurrent detection of miRNA and protein molecules on mouse heart tissue sections, in our model of cardiac hypertrophy.

Protocol

Mouse heart tissue samples for this study were obtained in compliance with the relevant laws and institutional guidelines and were approved by Yale University Institutional Animal Care and Use Committee.

1. Solution Preparation

1. Prepare RNase free ddH_2O, by treating 5 L of ddH_2O with 5 mL of 0.1% diethylpyrocarbonate (DEPC) overnight (O/N), at room temperature (RT). Autoclave (121 °C) to deactivate the DEPC. Use DEPC-treated ddH_2O for the preparation of downstream solutions as indicated.
   CAUTION: DEPC is a known carcinogen, only handle in the fume hood.
2. Prepare 1x Phosphate Buffered Saline (PBS) solution by dissolving 5 PBS tablets in 1 L of DEPC-treated ddH_2O. Autoclave (121 °C).
3. Prepare 0.1% non-ionic detergent/PBS (PBST) by diluting 1 mL of non-ionic detergent per 1 L of 1x PBS.
4. Prepare 90%, 70%, 50% and 30% ethanol in DEPC-treated ddH_2O.
5. Prepare a 10 mg/mL stock solution of Proteinase K (ProK) in DEPC-treated ddH_2O. Aliquot and store at -20 °C. Prepare a 20 µg/mL working solution of ProK by diluting 100 µL of ProK stock solution in 50 mL of DEPC-treated ddH_2O. Make fresh before use.
6. Prepare 4% paraformaldehyde (PFA) by adding 2 g of PFA in 50 mL of 1x PBS. Heat at 65 °C with occasional shaking, until dissolved. Aliquot and store at -20 °C.
   Caution: PFA is a known carcinogen, only handle in the fume hood.
7. Prepare a 3 M NaCl solution by adding 87.8 g to 500 mL of ddH_2O. Autoclave (121 °C).
8. Prepare a 1 M $MgCl_2$ solution by adding 20.3 g to 100 mL of ddH_2O. Autoclave (121 °C).
9. Prepare 1 M Tris pH 8.0 by dissolving 121.1 g in 800 mL of ddH_2O. Adjust the pH to 8.0 with a few drops of 1 N HCl, bring the volume to 1 L and autoclave (121 °C). Prepare a working solution of 50 mM Tris pH 8.0 by diluting 2.5 mL of 1 M Tris pH 8.0 in 47.5 mL of ddH_2O.
10. Prepare 1 M Tris pH 9.5 by dissolving 60.6 g in 400 mL of ddH_2O. Adjust the pH to 9.5 with a few drops of 1 N HCl, bring the volume to 500 mL and autoclave (121 °C).
11. Prepare 0.13 M 1-Methylimidazole pH 8.0 by diluting 1.6 mL of 1-Methylimidazole to 130 mL of DEPC-treated ddH_2O. Adjust the pH to 8.0 with approximately 450 µL of 12 N HCl. Add 16 mL of 3 M NaCl and bring the volume to 160 mL. Make fresh before use.
    Caution: 1-Methylimidazole is harmful to mucous membranes, eyes, and skin, only handle in the fume hood.
12. Prepare 0.16 M N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) by diluting 176 µL of EDC to 10 mL of 0.13 M 1-Methylimidazole. Adjust the pH to 8.0 with approximately 100 µL of 12 N HCl. Make fresh before use.
    CAUTION: EDC is harmful to mucous membranes, eyes, and skin, only handle in the fume hood.
13. Prepare 1% H_2O_2 by diluting 1.5 mL of 30% H_2O_2 in 50 mL of 1x PBS. Make fresh before use.
14. **Prepare 20x SSC pH 7.0 by dissolving 175.3 g of NaCl and 88.2 g of sodium citrate ($Na_3C_6H_5O_7$) in 800 mL of ddH_2O. Adjust the pH to 7.0 with a few drops of 1 N HCl, bring the volume to 1 L and autoclave (121 °C).**
    1. Prepare a working solution of 2x SSC pH 7.0 by diluting 20 mL of 20x SSC pH 7.0 in 180 mL of ddH_2O.
    2. Prepare a working solution of 1x SSC pH 7.0 by diluting 2.5 mL of 20x SSC pH 7.0 in 47.5 mL of ddH_2O.
    3. Prepare a working solution of 0.2x SSC pH 7.0 by diluting 5 mL of 2x SSC pH 7.0 in 45 mL of ddH_2O.
15. Prepare 1x hybridization solution by diluting 0.5 mL of 2x microRNA ISH buffer in 0.5 mL of DEPC-treated ddH_2O. Make fresh before use.
16. Prepare 200 mM stock solution of Levamisole by adding 250 mg to 5 mL of ddH_2O. Aliquot and store at -20 °C.
17. Prepare blocking solution for step 5 (10% sheep serum/1% BSA/0.2 mM Levamisol) by diluting 1 mL of sheep serum in 9 mL PBST, and adding 0.1 g of BSA. Add 10 µL of Levamisole before using. Make fresh before use.
18. Prepare blocking solution for step 6 (10% goat serum/1% BSA) by diluting 1 mL of goat serum in 9 mL of PBST, and adding 0.1 g of BSA. Store at 4 °C and use within a week.
19. Prepare prestaining solution by diluting 3.3 mL of 3 M NaCl, 5 mL of 1 M $MgCl_2$,10 mL of 1 M Tris pH 9.5, 100 µL of Tween-20, 100 µL of Levamisole in 81.5 mL of ddH_2O. Make fresh before use.
20. Use nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indoly phosphate (BCIP) tablets to prepare 20 mL of Alkaline Phosphatase (AP) substrate solution according to the manufacturer's instructions. Add 20 µL of Levamisole. Make fresh before use and protect from light.
21. Prepare KTBT solution by adding 4 g of Tris, 4.4 g of NaCl and 375 mg of KCl in 500 mL of ddH_2O. Autoclave (121 °C).
22. Optional: Prepare a DAPI working solution (1 µg/mL) by diluting 50 µL of DAPI stock solution (1 mg/mL) in 50 mL of 1x PBS.
    NOTE: Standard solutions such as 3 M NaCl, 1 M $MgCl_2$, 1 M Tris-HCl, nuclease-Free ddH_2O, 1x PBS and 20x SSC can be alternatively purchased. For this protocol, 50 mL glass Coplin jars or 20 mL polypropylene slide mailer jars have been used.

2. Tissue Preparation

NOTE: The mouse heart sections used here were prepared by Yale Pathology Tissue Services, from Formalin-Fixed/Paraffin-embedded tissue, cut at 5 µm and positioned on charged slides.

1. **Tissue rehydration.**
    1. Warm the slides at 60 °C for 10 min, in the hybridization oven until paraffin is visibly melting.
    2. Incubate the slides in a glass Coplin jar containing 50 mL of commercially available clearing agent (see **Table of Materials**) for 10 min, twice.
    3. Incubate the slides in a glass Coplin jar containing 50 mL of 100% ethanol for 2 min, twice, followed by 90% ethanol for 2 min, 70% ethanol for 2 min, twice, 50% ethanol for 2 min and 30% ethanol for 2 min.
    4. Incubate the slides in a glass Coplin jar containing 50 mL of DEPC-treated ddH_2O for 5 min.
    5. Incubate the slides in a glass Coplin jar containing 50 mL of 1x PBS for 5 min.
2. **Tissue de-proteinating.**

1. Incubate slides in a glass Coplin jar containing 50 mL of 20 µg/mL ProK working solution, at 37 °C for 15 min in the hybridization oven.
   CAUTION: Under-digestion may result in poor or no signal development, while over-digestion may result in tissue degradation and development of background staining. Optimization may be required for this step (1–20 µg/mL ProK, 5–20 min incubation, RT-37 °C).
2. Wash the slides in a glass Coplin jar containing 50 mL of 1x PBS for 5 min, at RT, twice.

3. **Tissue fixation.**
   1. Use a hydrophobic pen to draw a water repellent circle around the tissue on each slide.
   2. Apply 500 µL of 4% PFA solution to the length of each slide and incubate slides horizontally for 10 min, at RT.
   3. Wash the slides in a glass Coplin jar containing 50 mL of 1x PBS for 5 min, at RT, twice.
   4. Wash the slides in a glass Coplin jar containing 50 mL of 0.13 M 1-methylimidazole for 10 min, at RT, twice.
   5. Apply 500 µL of 0.16 M EDC solution to the length of each slide and incubate slides horizontally for 1 h at RT, in a humidified chamber.
   6. Rinse the slides in a glass Coplin jar containing 50 mL of 50 mM Tris pH 8.0 at RT.
   7. Rinse the slides in a glass Coplin jar containing 50 mL of 1x PBS at RT, twice.
      NOTE: Both PFA and EDC fixation steps are required for miRNA crosslinking and should not be omitted.[31]

4. **Endogenous peroxidase block.**
   1. Incubate slides in a glass Coplin jar containing 50 mL of 1% H_2O_2 for 30 min at RT.
   2. Rinse the slides in a glass Coplin jar containing 50 mL of 1x PBS at RT, twice.

3. Hybridization

1. Preheat hybridization solution (500 µL per slide) at 50 °C in the hybridization oven, until the target temperature, is reached (10–15 min).
2. Prepare the hybridization chamber by placing 2 pieces of filter or tissue paper in the bottom of the chamber and wetting the paper with 50% formamide/1x SSC.
   Caution: Formamide is harmful to mucous membranes, eyes and skin, only handle in the fume hood.
3. Apply 200 µL of hybridization solution to the length of each slide. Incubate slides horizontally for 1–3 h at 50 °C, in a humidified chamber.
4. Prepare the probe solution by mixing 0.5 µL stock probe (25 µM) in 250 µL ofhybridization solution (final concentration 50 nM) in a 1.5 mL tube.
   NOTE: Different miRNAs are expressed at different levels, so optimization regarding the probe concentration may be required for this step (1–50 nM), to avoid no signal or high background development.
5. Apply 250 µL of probe solution to the length of each slide and place an RNase free coverslip on top. Avoid the formation of bubbles.
6. Carefully seal the hybridization chamber with parafilm and incubate O/N at 50 °C.
   NOTE: The hybridization temperature should be set approximately 30 °C below the RNA melting temperature (Tm) of each probe. Optimization may be required. Here, 50 °C is used as the hybridization/wash temperature for miRNA-182, adjust accordingly for different probes.

4. Stringency Washes

1. Prewarm 200 mL of 2x SSC at 50 °C in the hybridization oven, until the target temperature is reached (20–40 min).
2. Wash the slides in a glass Coplin jar containing 50 mL of 2x SSC at 50 °C for 5 min, or until the coverslips loosen.
3. Wash the slides in a glass Coplin jar containing 50 mL of 2x SSC at RT for 5 min, twice.
4. Wash the slides in a glass Coplin jar containing 50 mL of 0.2x SSC at RT for 5 min.
5. Wash the slides in a glass Coplin jar containing 50 mL of PBST at RT for 5 min.
6. Wash the slides in a glass Coplin jar containing 50 mL of 1x PBS at RT for 5 min.
   NOTE: Use of RNase-free conditions is no longer necessary since the RNA-RNA hybrids are considered stable.

5. DIG Antibody Detection

1. Use a hydrophobic pen to re-draw a water repellent circle around the tissue on each slide if necessary.
2. Apply 500 µL of blocking solution to the length of each slide. Incubate slides horizontally for 30 min at RT, in a humidified chamber.
3. Prepare the DIG antibody solution (1:1,000) by diluting 0.5 µL of DIG antibody in 500 µL ofblocking solution in a 1.5 mL tube.
   CAUTION: Optimization may be required for this step (1:500–1:2,000).
4. Apply 500 µL of DIG antibody solution to the length of each slide. Incubate slides horizontally for 1 h at RT, in a humidified chamber.
5. Wash the slides in a glass Coplin jar containing 50 mL of PBST at RT for 5 min, thrice.
6. Wash the slides in a glass Coplin jar containing 50 mL of prestaining solution at RT for 5 min, twice.
7. Incubate slides in a polypropylene slide mailer jar containing 20 mL ofAP substrate solution at 37 °C, for 6–24 h, protected from light.
   Caution: Color development should be checked every 2 h. Optimization may be required for this step.
8. Wash the slides in a glass Coplin jar containing 50 mL of KTBT solution at RT for 5 min, twice.
9. Wash the slides in a glass Coplin jar containing 50 mL of 1x PBS at RT for 5 min.

6. cTnT Antibody Detection

1. Use a hydrophobic pen to re-draw a water repellent circle around the tissue on each slide if necessary.
2. Apply 500 µL of blocking solution to the length of each slide. Incubate slides horizontally for 1 h at RT, in a humidified chamber.
3. Prepare the cTnT antibody solution (1:100) by diluting 2 µL of cTnT antibody in 200 µL ofblocking solution in a 1.5 mL tube.
4. Apply 200 µL of cTnT antibody solution to the length of each slide. Incubate slides horizontally O/N at 4 °C, in a humidified chamber.
5. Wash the slides in a glass Coplin jar containing 50 mL of 1x PBS at RT for 5 min, thrice.

6. Prepare the anti-rabbit-568 antibody solution (1:500) by diluting 0.5 µL of anti-rabbit-568 antibody in 250 µL ofblocking solution in a 1.5 mL tube.
7. Apply 250 µL of anti-rabbit-568 antibody solution to the length of each slide. Incubate slides horizontally for 1 h at RT, in a humidified chamber.
8. Wash the slides in a glass Coplin jar containing 50 mL of 1x PBS at RT for 5 min, thrice.
9. Optional: Apply 250 µL of DAPI working solution to the length of each slide, and incubate slides horizontally for 1 min at RT, protected from light.
10. Wash the slides in a glass Coplin jar containing 50 mL of 1x PBS at RT for 5 min, twice.

7. Mounting and Imaging

1. Mount the slides with 2–3 drops of mounting medium and a glass cover slip. Allow the slides to dry flat, O/N, at 4 °C, protected from light.
2. Proceed to epifluorescent or confocal microscopy.

Representative Results

miRNA *in situ* hybridization was optimized on mouse heart sections using a scramble miRNA and U6-snRNA, which served as negative and positive controls respectively. Positive staining is indicated in blue, while the negative staining is indicated by the lack of color development (**Figure 1A-1B**). Cardiomyocyte specific expression of miRNA-182 was assessed in heart sections from control and PlGF overexpressing mice. The mice carrying the PlGF transgene under a αMHC promoter develop cardiac hypertrophy, secondary to increased angiogenesis, within 6 weeks of transgene activation[26]. We performed *in situ* hybridization and found increased expression of miRNA-182 in the hearts of PlGF mice, compared to controls (**Figure 2A-2H**), indicated by the blue staining. To determine which cell types express miRNA-182, we then performed immunostaining for cTnT on the same sections. We found miRNA-182 co-localized with the cTnT-positive cardiomyocyte cells, as well as DAPI stained nuclei, in both control and PlGF mouse heart sections (**Figure 2C-2H**), indicated by the red and blue staining respectively. These images were with a 20X objective.

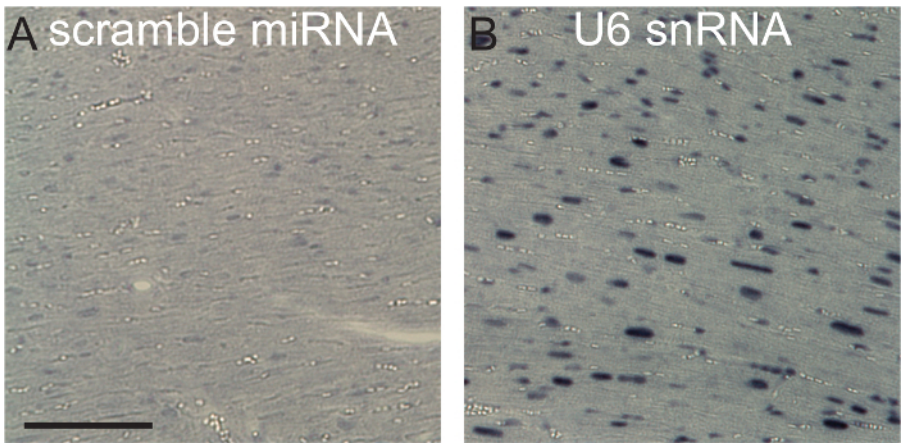

Figure 1: Negative and Positive *in situ* hybridization staining. (A) *In situ* hybridization for scramble miRNA (25 nM) in control mouse heart sections. **(B)** *In situ* hybridization for U6-snRNA (25 nM) in control mouse heart sections. Scale bar = 100 µm. Please click here to view a larger version of this figure.

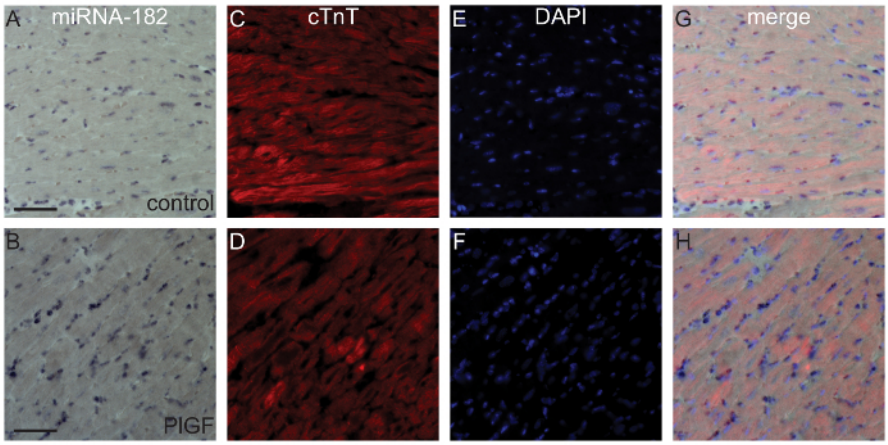

Figure 2: Cardiomyocyte specific expression of miRNA-182 in control and PlGF mouse hearts. (A-B) *In situ* hybridization for miRNA-182 in control and PlGF mouse heart sections. **(C-D)** Immunostaining for cTnT in control and PlGF mouse heart sections that have been previously stained for miRNA-182. **(E-F)** DAPI counterstaining for nuclear localization in control and PlGF mouse heart sections that have been previously stained for miRNA-182. **(G-H)** Merged images of miRNA-182 (deep blue), cTnT (red) and DAPI (light blue) staining for the control and PlGF mouse heart sections. Scale bar = 50 µm. Please click here to view a larger version of this figure.

Discussion

miRNA transcript detection can be achieved through different techniques that vary in sensitivity, specificity and quantitative power. Here, we demonstrate the coupling of miRNA *in situ* hybridization with immunostaining and describe a protocol that allows for concurrent assessment of the expression levels of miRNA and protein molecules, on the same heart sections. We first show how to perform *in situ* hybridization of DIG-labeled LNA miRNA probes on paraffin embedded heart sections. Next, we describe how to perform immunostaining for cTnT on the same sections. Finally, we demonstrate how to merge the resulting color and fluorescent images. This protocol has been optimized for Formalin Fixed (4 °C, O/N)/Paraffin embedded mouse heart tissues, with the use of DIG-labeled LNA miRNA probes and cTnT. Further optimization may be required for different tissues, probe or antibody types, as outlined below.

RNase-free conditions should be carefully implemented throughout the steps that lead up to probe hybridization, as RNase contamination can severely compromise the outcome of the experiment. All solutions should be made with DEPC-treated ddH_2O, and all Coplin jars should be treated with an RNase decontamination solution. Additionally, the following considerations should be taken into account when working with different DIG-labeled LNA miRNA probes. The hybridization temperature depends on the melting temperature (Tm) of each probe. As a general rule, a temperature that is 30 °C below the given RNA Tm or 20 °C below the given DNA Tm should be used for probe hybridization. Furthermore, the ideal probe concentration should be optimized. It usually lies between 1–50 nM. Lastly, the probe containing hybridization solution may be heated to 95 °C for 5 min to denature any secondary structures, if this is a concern. An important step to test the specificity of a probe, especially when used for the first time, is to include a negative (scrambled) as well as positive (for example U6) control probe, to ensure experimental success (**Figure 2**). Similar controls (for example a CD31 endothelial antibody) should be used during the immunostaining step.

A common problem in *in situ* hybridization experiments is the development of staining artifact/background signal. To avoid or minimize artifact staining steps, the tissue should be protected from drying out throughout all steps, specifically during long incubations. We recommend covering the slides with RNase-free coverslips during the hybridization step, particularly when high temperatures are used, and the use of humidifying chambers. We also suggest the use of a 568 or 594 fluorophore to detect cTnT or the protein of interest during the immunostaining step. This will eliminate any autofluorescence that often arises with the use of 488 fluorophores used on formaldehyde fixed tissues.

Another variable we recommend paying attention to is the duration of AP staining development, which depends on the levels of the specific miRNA present in the tissue. We suggest checking AP staining progression every 2 h, for the first experiment. Further optimization may include changing the temperature of incubation (RT-37 °C) parameter. Lastly, if background begins to develop before the true miRNA probe staining, or if the AP staining solution changes color to blue-brown, we suggest washing the slides in PBST twice, and replacing the staining solution with a freshly made one.

Finally, although this protocol has been optimized for Formalin-Fixed/Paraffin-embedded mouse heart tissues, it may be adapted for alternatively processed tissues (PFA fixed/OCT embedded) and/or the use of the different probe or antibody types. A limitation of combining *in situ* hybridization and immunostaining that should be considered is the failure of several antibodies to bind to their respective epitopes, due to the possible destruction of the latter ones at the high temperatures used during the hybridization and stringency washing steps. Further limitations of both *in situ* hybridization and immunostaining techniques most often refer to the power of these techniques to detect very low concentrations of miRNA or protein molecules respectively. Signal amplification kits are available to use for either DIG or antibody detection when miRNA or protein abundance is a concern. Such kits also provide a fluorescence alternative for the detection of miRNAs, which, when coupled with fluorescent immunostaining can simplify image acquisition. Alternative methods, such as Real-Time PCR and Western Blotting that have greater sensitivity, can also address low abundance issues. However, contrary to *in situ* hybridization/immunostaining protocol, these techniques provide no information on the tissue-specific localization of the miRNA/protein molecules.

To summarize, we describe a protocol that provides simultaneous detection of miRNA and protein molecules on the same tissue, which we believe will be an invaluable tool in miRNA research on mouse models.

Disclosures

The authors have nothing to disclose.

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