Universiteit
Antwerpen

This item is the archived peer-reviewed author-version of:

Fluorescent in situ hybridization for miRNA combined with staining of proteins

Reference:
Boen Jente, Pintelon Isabel, Gevaert Andreas, Segers Vincent F.M., van Craenenbroeck Emeline.- Fluorescent in situ hybridization for miRNA combined with staining of proteins
Current protocols - ISSN 2691-1299 - 3:9(2023), e880
Full text (Publisher's DOI): https://doi.org/10.1002/CPZ1.880
To cite this reference: https://hdl.handle.net/10067/2002250151162165141

uantwerpen.be
Institutional repository IRUA

CURRENT PROTOCOLS
A Wiley Brand

Template for a Protocol Article

Please submit this Word document to ScholarOne, https://mc.manuscriptcentral.com/currentprotocols Every section must be completed for the manuscript to be reviewed.

ARTICLE TITLE: Fluorescent in situ hybridization protocol for miRNA combined with staining of proteins

AUTHOR(S) AND CONTACT INFORMATION:

J. R.A. Boen: Research group Cardiovascular Diseases, GENCOR Department, University of Antwerp; Laboratory of Physiopharmacology, GENCOR Department, University of Antwerp; Universiteitsplein 1, 2610 Wilrijk; +3232652497; jente.boen@uantwerpen.be

I. Pintelon: Laboratory of Cellular Biology and Histology, University of Antwerp; Antwerp Centre for Advanced Microscopy, University of Antwerp; Universiteitsplein 1, 2610 Wilrijk; +3232653273; isabel.pintelon@uantwerpen.be

*A. B. Gevaert: Research group Cardiovascular Diseases, GENCOR Department, University of Antwerp; Department of Cardiology, Antwerp University Hospital (UZA); Drie Eikenstraat 655, 2650 Edegem, +3238213008, andreas.gevaert@uantwerpen.be

V.F.M. Segers: Laboratory of Physiopharmacology, GENCOR Department, University of Antwerp; Department of Cardiology, Antwerp University Hospital (UZA); Universiteitsplein 1, 2610 Wilrijk, +3232659138, vincent.segers@uantwerpen.be

E. M. Van Craenenbroeck: Research group Cardiovascular Diseases, GENCOR Department, University of Antwerp; Department of Cardiology, Antwerp University Hospital (UZA); Drie Eikenstraat 655, 2650 Edegem; +3238213000, emeline.vancraenenbroeck@uantwerpen.be

*corresponding author

ABSTRACT:

The last decades have illustrated the importance of microRNAs in various biological and pathological processes. The combined visualization of microRNAs using fluorescent in situ hybridization and proteins using immunofluorescence, can reveal their spatiotemporal distribution in relation to the cell and tissue morphology, and can provide interesting insights into miRNA-protein interactions. However, standardized protocols for co-localization of microRNAs and proteins are currently lacking, while substantial technical obstacles still needs to be addressed. Particularly, the incompatibility of protein immunofluorescence protocols with steps required for microRNA fluorescent in situ hybridization, such as proteolytic pretreatments and ethylcarbodiimide post-fixation, as well as hurdles related to low signal intensity of low copy microRNAs remains challenging. Our technique considerably enhances microRNA-based research as current detection techniques lack the ability to elucidate cellular and subcellular localization. Here, we describe an optimized 2-day protocol for combined detection of low abundant microRNAs and proteins in cryosections of cardiac tissue, without the need for protease-dependent pretreatment or post fixation treatment. We successfully demonstrate endothelial-specific localization of low abundant miR-181c-5p in cardiac tissue.

Basic Protocol 1: Fluorescent in situ hybridization protocol for miRNA combined with staining of proteins

KEYWORDS:

Fluorescent in situ hybridization, miRNA, immunofluorescence, protein staining

INTRODUCTION:

MicroRNAs (miRNAs) are key epigenetic regulators, contributing to normal physiology and numerous pathological conditions (Condrat et al., 2020). They are a group of small endogenous non-coding RNA molecules that influence gene expression at the transcriptional and post-transcriptional level. MiRNAs regulate more than 60% of human proteins in a complex spatiotemporal-dependent manner (Catalanotto, Cogoni, & Zardo, 2016; O'Brien, Hayder, Zayed, & Peng, 2018). Because of the extensive research in miRNAs as therapeutic targets or biomarkers, there is a clear need for adequate detection and visualization techniques (Condrat et al., 2020; Okuda et al., 2021; S. S. Zhou et al., 2018). Current techniques that allow quantification of miRNAs, including miRNA real time quantitative polymerase chain reaction (RT-qPCR), microarray analysis and next generation sequencing, all lack the ability to provide spatial localization (Dave et al., 2019; Tian, Wang, & Zhou, 2015). Understanding the spatiotemporal behaviour of miRNAs is important to unravel their function at the cellular and subcellular level (Kasai et al., 2016; Sempere et al., 2010; Song, Ro, & Yan, 2010). Fluorescent in situ hybridization (FISH) for localization and visualization of miRNAs can address these issues. The simultaneous detection of miRNAs with FISH and specific cellular proteins with immunofluorescence (IF) allows visualization of a specific miRNA in a cellular- and tissue-specific manner, and allows detection of spatiotemporal relations with potential target proteins and functional cell state. For example, knowledge on cellular localization of miRNAs can help in the discrimination of different tumor types and in overcoming potential bias in RT-qPCR analyses caused by tissue heterogeneity, which improves the accuracy and applicability of miRNAs as tumor biomarkers (Di Meo et al., 2017; Renwick et al., 2013; Sempere et al., 2010).

The majority of studies using co-detection of miRNAs and proteins finalizes FISH before initiating the IF steps, and struggles with the detection of low expressed miRNAs (Nielsen & Holmstrøm, 2013; Nuovo et al., 2009; Sempere et al., 2010; Shi, Johnson, & Stack, 2012; Zaidi, Enomoto, Milbrandt, & Roth, 2000). In the current protocol, we developed a 2-day protocol for simultaneous detection of miRNAs via FISH and protein markers via IF in frozen cardiac sections (Fig.1). We optimized the protocol for the visualization of low abundant miR-181c-5p in mouse cardiac tissue, with co-detection of isolectin for endothelial cells and DAPI nuclear staining by means of IF. In this way, we were able to determine the cellular localization of low abundant miR-181c-5p in cardiac tissue in greater detail. Scrambled control and nuclear-located U6 snRNA positive control were used for protocol optimization.

> *CAUTION:* Before starting, clean the bench with 70% ethanol and RNAse ZAP, use autoclaved glassware, and wear gloves during the entire procedure to prevent RNA degradation/contamination. If no RNAse free reagents for buffers are available, autoclave before use. The standard total fluid quantity of buffers or reagents for each slide in each step in this protocol is 200µl/slide, unless specified otherwise. Work at room temperature, unless specified otherwise.

Materials:

C57/BL6J mouse (male, Charles River Laboratories)
70% ethanol (VWR, 93003.1006)
RNAse ZAP (Ambion)
PBS solution 1X (see recipe)
4% paraformaldehyde (PFA) solution (w/v) (see recipe)
30% Sucrose solution (w/v) (see recipe)
Epredia™ Neg-50™ Frozen Section Medium (Thermofisher Scientific,11912365)
Prehybridization buffer (see recipe)
Hybridization buffer (see recipe)
Double digoxigenin (DIG)-labeled MiRCURY LNA detection probes (Qiagen, Table 1).
Saline sodium citrate (SSC) solutions 5X, 4X, 1X, 0.2X (see recipe)
Blocking buffer (10% goat serum, supplied in Alexa Fluor™ 594 Tyramide SuperBoost™ Kit, Invitrogen; B40915)
Mouse-on-mouse Ig blocking reagent (Vectorlabs, MKB-2213-1, 1/50).
Ib4 streptavidin-bound isolectin staining (Vectorlabs, B-1205-.5, 1/500)
DIGX Mouse anti-digoxigenin linker, ready to use (Enzo Life science; ENZ-ABS304-6000)
3% hydrogen peroxidase solution (supplied in Alexa Fluor™ 594 Tyramide SuperBoost™ Kit, Invitrogen; B40915)
Triton™ X-100 0.1% (v/v) (see recipe)
Biotin mouse monoclonal antibody, alexa 488 (BK 1/39) (Santa Cruz; sc-53179; 1/200)

Poly HRP-conjugated goat anti-mouse antibody (supplied in Alexa Fluor™ 594 Tyramide SuperBoost™ Kit, goat anti-mouse IgG, Invitrogen; B40915)
Alexa Fluor™ 594 Tyramide reagent working solution (See recipe)
Tyramide stop reagent working solution (See recipe)
Trueblack solution 1X (see recipe)
VECTASHIELD® non-Hardset™ Antifade Mounting Medium with DAPI (Vector Laboratories)

Surgical board
Surgical tape
Forceps
Surgical scissor
Sterile gauze
Cryosection mold
Liquid nitrogen
Cryotome
SuperFrost® Plus, Menzel Gläser (VWR, 631-9483)
Hydrophobic barrier PAP pen (Merck, Z377821)
Histology chamber
Hybridization oven (Heraeus)
Ice
Ice bucket
RNAse free safe lock microcentrifuge vials, Biopur® (Eppendorf, 022600044)
Heating block
Microcentrifuge
Leica SP8 confocal laser scanning microscope + Leica Application suite X image acquisition software

Protocol steps with *step annotations:*

Tissue collection, fixation and preparation

Ensure that all animal procedures conform with the latest Guide for the Care and Use of Laboratory Animals (National Institute of Health).

1. Collect the murine heart by placing the euthanized C57/BL6J mouse on a surgical board in the supine position with upper and lower extremities extended outward, and subsequently attach them with surgical tape.

2. Spray the fur at the thorax with 70% ethanol to facilitate incision, while avoiding loose hairs of entering the incision wound.

3. Lift the skin up with forceps creating a slight tension and use scissors to make an incision covering the thorax and carefully dissect up into the thoracic cavity without damaging the heart.

4. Remove lungs and surrounding tissue, and clean the chest cavity with sterile gauze to expose the heart.

5. Carefully dissect the heart using scissors and remove fatty tissue.

6. Place the dissected heart immediately in a petri dish filled with RNAse free 1X PBS for washing.

7. Immerse the dissected heart in freshly prepared 4% PFA for 2 h at 4°C.

8. Rinse and transfer the tissue to 30% sucrose solution at 4°C and preserve overnight.

9. Mount the tissue in OCT matrix by covering the bottom of a cryosection mold with OCT matrix (NEG-50).

10. Position the dissected heart in the OCT-filled cryosection mold and add drops of OCT until the whole tissue is covered.

11. Place the cryosection mold in a stainless steel dish filled with liquid nitrogen to solidify.

12. Remove the tissue block when completely solidified and continue with tissue cutting using the cryotome or store embedded tissues at -80°C for long term storage (≤1 year).

13. Cut 10µm cryosections with cryotome and place on Superfrost plus glass slides.

14. Dry slides 30 min at room temperature, and draw a circle with a hydrophobic barrier pen around the tissue sections to minimize reagent spilling.

Prehybridization

15. Prewarm prehybridization buffer at 54°C in hybridization oven.

16. Wash the slides 3 times by pipetting 200µl of 1X PBS on the slides for 3 min each.

17. Perform 2 longer wash steps 5 min each with 1X PBS to remove final leftovers.

18. Add prewarmed prehybridization mix to each slide.

19. Incubate the slides for1 h at hybridization temperature (54°C) in a humidified histology chamber.

Place wet paper towels at the bottom of the histology chamber to create a humidified chamber preventing drying of the slides. Pay attention to always close the lid properly to prevent excess evaporation during the hybridization step. Specialized hybridization cover slips, such as HybriSlip™ (Merck), are commercially available and provide uniform reagent distribution without the risk of evaporation during incubation times at higher temperatures.

Hybridization temperature should be optimized for each probe in advance and is often 30°C lower than the actual melting temperature. Melting temperature of the probe is often provided by the manufacturer.

Prehybridization step is important to reduce background noise.

Hybridization

20. Add the amount of double DIG-labelled LNA detection probes that is required to obtain the optimized probe concentration (150 nM probe) for a final volume of 50µl/slide to RNAse free vials, and denature the probes 4 min at 90°C on a heating block.

Keep detection probes on ice at all times, unless specified differently. The concentration for each probe should be optimized in advance.

21. Spin the vials down briefly in a microcentrifuge and put the probes immediately on ice.

22. Add hybridization buffer to the probe to reach a total volume of 50 µl/ slide.

23. Remove prehybridization buffer from the slides and add 50µl hybridization buffer.

Work as fast as possible to avoid cooling of the slides during handling.

24. Hybridize at optimal hybridization temperature (here, 54°C) for 1 h in humidified chamber.

Stringency washes

25. Prepare 5X, 1X and 0.2X SSC buffers and prewarm the 5X and 1X buffer at hybridization temperature (54°C).

Start the preparation and prewarming of the SSC buffers during the 1 h hybridization step to avoid cooling down of the slides.

26. Wash slides 3 times for 5 min each with 5X SSC buffer at hybridization temperature in humidified chamber.

Work fast to prevent the slides and buffer from cooling down.

27. Wash slides 2 times for 5 min each with 1X SSC buffer at hybridization temperature in humidified chamber.

Work fast to prevent the slides and buffer from cooling down.

28. Wash the slides for 5 min with 0.2X SSC buffer at room temperature.

29. Wash the slides for 5 min with 1X PBS at room temperature.

Stringent washes are relevant to remove non-specific binding of the probes.

Blocking

30. Add 200µl of blocking buffer to each of the slides.

Ideally the primary antibody should be raised in species different from the species of your sample tissue to avoid cross-reactivity of the secondary antibody with endogenous immunoglobulins of the sample. If not possible as in this case (a mouse-derived anti-DIG linker used on mouse tissue), high background signal forms a serious stumbling block. A potential solution is the use of a specific anti-mouse blocking buffer in combination with the standard non-specific blocking buffer (10% goat serum) for enhanced blocking to avoid non-specific endogenous binding of the secondary antibody. We added mouse-on-mouse blocking buffer at 1/50 dilution to the standard 10% goat serum blocking buffer.

31. Incubate slides for 40 min at room temperature.

Primary protein markers + anti-DIG linker incubation

32. Remove blocking buffer after incubation.

33. Dilute protein markers, in this case streptavidin-bound isolectin, at optimal concentration (1/500) in ready to use anti-DIG linker solution.

Dilution of each marker should be optimized in advance by testing various dilutions at fixed incubation time and fixed temperature. A guide for dilutions is often provided by the manufacturer. Select the highest dilution that provides an adequate signal strength.

34. Incubate slides overnight at 4°C in a humidified chamber

Washing

35. Wash the slides 3 times for 5 min each with 1X PBS.

Endogenous peroxidase removal

This step can be skipped if your tissue has no or low endogenous peroxidase activity.

36. Add 3% hydrogen peroxide solution to cover the section.

37. Incubate slides for 1 h.

38. Wash slides 3 times for 1 min each with 1X PBS and remove the PBS afterwards.

Secondary antibody incubation

From this step onwards, limit the exposure of the slides to light to avoid photobleaching as recommended by the manufacturer.

39. Dilute secondary antibodies for proteins at optimal concentration in PBS-0.1% triton. In this case, dilute biotin monoclonal antibody in PBS-0.1% triton at 1/200. Mix 50µl of the diluted antibody with 50µl of poly HRP-conjugated goat anti-mouse antibody.

40. Add secondary antibodies for protein markers + HRP-conjugated antibody to the slides (100µl/slide).

41. Incubate the slides 1 h in the dark.

Washing

42. Wash slides 3 times for 2 min each in 1X PBS.

Tyramide signal amplification

43. Prepare tyramide reagents as recommended by the manufacturer.

44. Add 100µL tyramide working solution to the slides.

45. Incubate slides 10 min in the dark.

This incubation period needs to be optimized in a range from 2-15 min to get the optimal signal.

46. Add immediately 100 µl stop reagent working solution to the slides without removing the tyramide working solution.

47. Wash slides 3 times for 1 min each with 1X PBS.

TrueBlack posttreatment

This step can be performed before the prehybridization step (pretreatment) to increase signal strength, on condition that no detergents are required during the further course of the protocol. Otherwise, use posttreatment as recommended in this protocol.

48. Prepare 1X TrueBlack solution.

49. Add 150µl TrueBlack solution to the slides.

Work in small batches of slides to avoid drying out and to stick to the short incubation times.

50. Incubate slides 30 sec.

51. Wash the slides 3 times for 1 min each in 1X PBS.

DAPI staining

52. Mount slides with DAPI vectashield non-hardset (25µl/slide).

53. Incubate slides for 15 min.

54. Store slides or continue immediately with image analysis.

Slides can be stored at -20°C for ≤1 month, however, to minimize signal loss the period before microscopy should be limited. In our lab imaging is performed within 2 days.

Imaging

45. Image slides with confocal laser scanning fluorescence microscope.

REAGENTS AND SOLUTIONS:

Dextran sulfate solution 25% (w/v) (40ml)

- 10g Dextran sulphate sodium salt (Merck; D8906-10G)
- 40ml DEPC treated water (Invitrogen; 4387937)

- Store at 4°C for ≤1 month

Hybridization buffer (100ml)

- 0.2ml of Invitrogen™ UltraPure™ Denhardt's Solution (50X) (Invitrogen; 11518616) (1X final)
- 20 ml of 20X SSC Ultrapure RNAse free (Invitrogen; AM9770) (5X SSC final)
- 40 ml of 25% dextran sulphate solution (see recipe) (10% (w/v) final)
- Adjust to 100ml with DEPC treated water (Invitrogen; 4387937)
- Prepare fresh without storage

Paraformaldehyde (PFA) 4% solution (w/v) (100ml)

- 4g paraformaldehyde (Merck, 818715)
- 100ml 1X PBS solution (see recipe)
- Heat the solution during 10 min while stirring and let the solution cool down at room temperature
- Filter the solution
- Prepare fresh without storage

  PFA is toxic and carcinogenic upon inhalation or skin contact, be careful during handling. The use of a fume hood and sealing of the solution during heating is necessary.

PBS 1X dilution (1L)

- 900ml DEPC treated water (Invitrogen; 4387937)
- 100ml 10X RNAse free phosphate buffered saline (PBS; Invitrogen; AM9625)
- Store up to ≤1 year at room temperature

Prehybridization buffer (100ml)

- 0.2ml of Invitrogen™ UltraPure™ Denhardt's Solution (50X) (Invitrogen; 11518616) (1X final)
- 20 ml of 20X SSC Ultrapure RNAse free (Invitrogen; AM9770) (5X SSC final)
- Adjust to 100ml with DEPC treated water (Invitrogen; 4387937)
- Prepare fresh without storage

Denhardt's solution is used as a blocking reagent to avoid non-specific binding.

Saline sodium citrate (SSC) dilutions

For 5X (100ml):

- 25ml 20X SSC Ultrapure RNAse free (Invitrogen; AM9770)
- 75ml DEPC treated water (Invitrogen; 4387937)

For 1X (100ml):

- 5ml 20X SSC Ultrapure RNAse free (Invitrogen; AM9770)
- 95ml DEPC treated water (Invitrogen; 4387937)

For 0.2X (100ml):

- 1ml 20X SSC Ultrapure RNAse free (Invitrogen; AM9770)
- 99ml DEPC treated water (Invitrogen; 4387937)

- Store solutions up to ≤1 year at room temperature

Sucrose solution 30% (w/v)(100ml)

- 30g sucrose (Sigma, S1888)
- 100ml 1X PBS solution (see recipe)
- Mix by stirring
- Filter the solution
- Prepare fresh without storage

Triton-X 100 0.1% (v/v) (100ml)

- 100µl Triton-X100 (Sigma, ref.T8787)
- 99.9ml 1X PBS solution (see recipe)
- Store up to ≤1 year at room temperature

TrueBlack solution 1X (100ml)

- 5ml TrueBlack® Lipofuscin Autofluorescence Quencher 20X (Biotium, 23007)
- 95ml 70% ethanol (VWR chemicals)
- Preparation should be performed under the fume hood when working with ethanol
- Prepare fresh

Tyramide reagent working solution (1X) (10ml)

- 100µl 100X tyramide reagent stock solution (supplied in SuperBoost™ Kit, Invitrogen; B40915)
- 100µl H_2O_2 solution (supplied in SuperBoost™ Kit, Invitrogen; B40915)
- 10ml of 1X reaction buffer (supplied in SuperBoost™ Kit, Invitrogen; B40915)
- Prepare fresh

Tyramide stop reagent working solution (1X) (10ml)

- 0.9ml tyramide stop reagent stock solution (supplied in SuperBoost™ Kit, Invitrogen; B40915)
- 9.1ml 1X PBS solution (see recipe)
- Prepare fresh

COMMENTARY:

Background Information:

In 1969, Gall and Pardue (Pardue & Gall, 1969) visualized DNA fractions on cytological preparations of Xenopus oöcytes based on the principle of nucleic acid hybridization and reannealing kinetics. They denatured DNA in situ, and then formed DNA-DNA hybrids with radioisotope-labelled DNA probes, that can be detected with autoradiography. Since then, in situ hybridization has been applied in numerous fields, including developmental biology, phylogenetics, molecular biology, pathology, microbiology, oncology, cardiology and neurology (Cao et al., 2023; Carr, 1995; Cui, Shu, & Li, 2016; Fitzpatrick, Murali, & Nardi, 2023; Laurent-Huck & Felix, 1991; Luo & Liu, 2019; Pecciarini et al., 2023; T. Zhou et al., 2023). Although FISH has been used to

localize miRNAs in cells (Dixon-McIver et al., 2008; Herzer, Silahtaroglu, & Meister, 2012; Silahtaroglu, 2010), it remains challenging to detect low abundant miRNAs in histological sections (Kasai et al., 2016; Nuovo et al., 2009; Sempere et al., 2010; Zaidi et al., 2000). Also, there is a need for reliable protocols for co-detection of miRNAs by FISH and proteins by IF as this provides detailed information about the cellular and tissue-specific localization of miRNAs and generates interesting insights in spatiotemporal relations with cell state, which is lacking by current miRNA detection techniques (Dave et al., 2019; Tian et al., 2015). However, traditional IF procedures are often incompatible with several steps necessary for miRNA FISH. For instance, the use of proteolytic pretreatments, such as proteinase K, recommended for improved retrieval of miRNAs can hinder detection of certain proteins, while ethylcarbodiimide (EDC) post-fixation alters antigen structure (Lei et al., 2018; Renwick et al., 2013). Thus, it is important to optimize the combination of different FISH and IF steps.

In this protocol, we did not add proteinase K treatment or any other proteolytic treatment, neither did we perform a post-fixation step with EDC, in contrast to most studies (Chaudhuri, Yelamanchili, & Fox, 2013). As the majority of protein antibodies or markers are not tested in combination with proteinase K treatment, which also has a higher tendency for tissue overdigestion, skipping this step allows the use of a broader range of protein markers, while protein marker antigenicity is better preserved (Urbanek, Nawrocka, & Krzyzosiak, 2015; H. Yang, Wanner, Roper, & Chaudhari, 1999). Some studies have indicated that the use of EDC fixation to prevent miRNA release from tissue (Pena et al., 2009) was not needed in cryosections, due to sufficient miRNA preservation (Lu & Tsourkas, 2009).This greatly facilitates co-detection of miRNAs and proteins (Chaudhuri et al., 2013; Nuovo et al., 2009; Zaidi et al., 2000).

FISH of miRNAs is difficult because of the short length of miRNAs (18-24 nucleotides), their low abundancy, and the high sequence similarity between miRNAs (Urbanek et al., 2015). For this reason, miRNAs are hard to detect with traditional longer RNA detection probes, which have lower specificity, sensitivity, and binding affinity for short transcripts, resulting in melting temperatures that are too low to succeed for in situ hybridization (Obernosterer, Martinez, & Alenius, 2007; Urbanek et al., 2015). To address these issues, multiple chemical probe modifications have been developed (Urbanek et al., 2015). In particular, locked nucleic acid (LNA) labelled probes significantly increased the sensitivity and specificity compared to conventional RNA/DNA probes (Song et al., 2010). The low melting temperature and poor mismatch discrimination of standard miRNA-DNA duplexes associated with short miRNA length can be overcome by flexible addition of LNA moieties to nucleotide bases. These LNA structures 'lock' the duplex interaction, while obeying Watson-Crick rules, thereby facilitating base pairing (Silahtaroglu et al., 2007) and preventing degeneration as they are more resistant for enzymatic degradation (Koshkin et al., 1998). Melting temperature increases 2-10°C for each monomer added, while minimizing total probe length, which provides enhanced stringency for low abundant miRNA detection (Nuovo et al., 2009; Obernosterer et al., 2007; Song et al., 2010). The double DIG-labelled LNA probes used in this protocol are purchased from Qiagen, who offer probes for most miRNAs annotated in miRBase, with already pre-defined 'optimal' LNA positioning to ensure sequence specificity while limiting self-annealing or secondary structures. The double DIG-label at 3' and 5' position amplifies signal intensity.

Limitations of colorimetric-based in situ hybridization can be overcome by the use of fluorescent probes. Because most miRNAs have low expression levels in tissue, signal amplification is necessary to pick up the signal. Tyramide signal amplification (TSA) (Fig. 2) substantially facilitates miRNA in situ detection by increasing signal intensity a 100-fold compared to conventional fluorescent probes. TSA is based on the principle of catalyzed reporter deposition, in which DIG-labelled detection probes are targeted by horseradish peroxidase (HRP)-linked anti-DIG antibodies. Subsequent conversion of inactive fluorescein-conjugated tyramides into tyramide radicals by HRP and hydrogen peroxidase leads to deposition of these radicals and covalent binding with local tyrosine residues (Kerstens, Poddighe, & Hanselaar, 1995; Shi et al., 2012). The use of DIG-labelled probes are preferred over alternative biotin-labelled probes due to their non-mammalian origin, avoiding non-specific signal detection (Nuovo, 2010). Fluorescently labelled probes also enable the simultaneous detection of multiple markers, including proteins.

Critical Parameters:

During protocol optimization, non-specific staining and low signal intensity proved to be the biggest hurdles.

Tissue morphology and permeabilization

We achieved better results and sensitivity with cryosections compared to paraffin embedded sections (Lei et al., 2018). The drawbacks of cryosections are storage at lower temperature (-20°C) and incompatibility with harsh antigen retrieval steps. Regarding cryo-embedding, morphology was better preserved with PFA/30% sucrose/OCT embedding compared to only OCT matrix embedding. In addition, we used formamide-free hybridization buffer to minimize tissue toxicity and maintain tissue morphology (Sinigaglia, Thiel, Hejnol, Houliston, & Leclère, 2018). In our protocol, we did not add proteinase K treatment or any other proteolytic pretreatments as a permeabilization method for detection of intracellular miRNAs as sufficient signal intensity was obtained. The short length of miRNA LNA detection probes also simplifies cellular entrance, thereby circumventing the need for harsh permeabilization methods.

However, in case of issues with low signal intensity, inclusion of this step might be considered as permeabilization is tissue-dependent (H. Yang et al., 1999).

Hybridization and stringency

In this study higher probe concentrations (150nM) improved signal intensity compared to lower concentrations (10nM, 100nM) (Fig.3), and better than increasing stringency washes. Nevertheless, the right temperature and stringency of post-hybridization washes are important, because they determine the balance between miRNA-probe binding and off-target effects (Huber, Voith von Voithenberg, & Kaigala, 2018; Nuovo et al., 2009). Optimization of (pre)hybridization time and temperature also helps to improve signal intensity. The theoretical hybridization temperature is 30°C below melting temperature, but needs to be assessed empirically, because a single degree can significantly affect signal detection. Drying of the slides, as a result of evaporation during (pre)hybridization, significantly increases background signal and should be avoided.

Tyramide signal amplification (TSA)

Besides probe concentration and stringency washes, the TSA incubation time was found to be a critical factor to reduce background fluorescence and increase signal strength of low abundant miRNAs. We noticed that prolonged TSA incubation time (>10 min) induced a blurry background that masked the co-stained protein marker (isolectin) (Fig. 3). The optimal incubation time for TSA in this protocol was 10 min.

Autofluorescence and non-specific endogenous isotope binding

Reducing high background signal often observed in cardiac tissue is challenging. Background fluorescence of cardiac tissue and red blood cells was considerably reduced by including TrueBlack treatment. TrueBlack is a lipofuscin autofluorescence quencher that prevents both lipofuscin and non-lipofuscin associated autofluorescence. Compared to alternative reagents, such as Sudan Black, it induces less background fluorescence, certainly in the far-red wavelengths (Whittington & Wray, 2017). Non-specific binding of anti-mouse secondary antibodies on non-targeted endogenous mouse epitopes, mainly in blood vessels, was extensively blocked by mouse-on-mouse blocking buffer.

Imaging

In the post-analytical phase, an image modality with an appropriate resolution should be applied when it comes to the detection of low abundant miRNAs. Therefore, confocal fluorescence microscopy is preferred over widefield fluorescence microscopy.

Troubleshooting:

Table 2 shows a troubleshooting guide for miRNA FISH with co-staining of proteins via IF, with frequently encountered problems, corresponding causes and possible solutions.

Understanding Results:

The detection of low abundant miRNAs with in situ hybridization is challenging, especially when co-detection of protein markers is required. Therefore, we selected miR-181c-5p, expressed at low levels in cardiovascular tissue, to optimize this protocol for intracellular miRNA visualization by FISH-IF, using co-staining of isolectin as a protein marker for endothelial cells, in combination with DAPI nuclear staining. With the current protocol, we were able to detect low expression levels of miR-181c-5p in cardiac tissue with specific localization in endothelial cells as illustrated by the overlap with isolectin staining (Fig.4). These observations correspond with previous studies (Solly, Psaltis, Bursill, & Tan, 2021; Sun, Sit, & Feinberg, 2014; G. Yang, Wu, & Ye, 2017). Interestingly, miR-181c-5p was not expressed in every blood vessel. The underlying mechanism is still unknown, but it strengthens the concept of spatiotemporal regulation of miRNAs (Dean, Riahi, & Wong, 2015; Fernández-Hernando & Suárez, 2018; Pothof & van Gent, 2011; Shu et al., 2012). This protocol can be used to detect other low abundant miRNAs and provides a template for experimental FISH-IF setup, with miRNA-specific optimization.

We expect that the protocol can be used to detect other low abundant miRNAs, though optimization might be required, dependent on the miRNA and protein targets, with further validation regarding pretreatment and permeabilization steps when non-cardiac tissue or different species are used. As the expression of miRNAs strongly differs between cell types, tissues and species, adequate optimization of probe concentration, stringency washes and hybridization temperature/ incubation period for each miRNA probe is necessary.

For optimization of the protocol we recommend to start with the FISH-part restricted to a positive and negative control only, such as U6 snRNA and scrambled detection probe, to avoid excess spilling of the LNA probe detecting the miRNA of interest. When an appropriate signal-to-noise ratio and signal strength is obtained for the positive control, the protocol can be applied to the miRNA of interest for further optimization with regard to miRNA-specific hybridization conditions, permeabilization, TSA parameters and probe concentrations, until a specific staining with strong signal strength and low background is reached. See Table 2 for more troubleshooting guidelines. Protein IF can be optimized separately and finally combined with the optimized FISH protocol to validate protocol compatibility. Optimization is finished when both miRNA FISH and protein markers show specific, adequate signal strength with minimal background noise.

Time Considerations:

The miRNA FISH protocol with combined staining of protein markers described in this study can be completed within two days.

CONFLICT OF INTEREST STATEMENT:

A.B.G reports lecture/advisory board fees paid to his institution by Abbott, AstraZeneca, Boehringer Ingelheim, Novartis, and Menarini, not related to the work.

DATA AVAILABILITY STATEMENT:

Data available on request from the authors.

ACKNOWLEDGMENTS:

This work was supported by BOF-DOCPRO4 PhD fellowship (grant number 180182) and a Geconcerteerde onderzoeksactie grant (GOA, PID36444) from University of Antwerp, and by research grants of the Fund for Scientific Research Flanders (Application number G021420N; VLIR/iBOF Grant 20-VLIR-iBOF-027 (to VFS). E. Van Craenenbroeck and V Segers are supported by a senior clinical investigator grant from the Fund for Scientific Research (FWO) Flanders (1804320N and 1842219N).

LITERATURE CITED:

Cao, G., Xuan, X., Li, Y., Hu, J., Zhang, R., Jin, H., & Dong, H. (2023). Single-cell RNA sequencing reveals the vascular smooth muscle cell phenotypic landscape in aortic aneurysm. *Cell Commun Signal, 21*(1), 113. doi:10.1186/s12964-023-01120-5

Carr, J. (1995). 4 - In Situ Hybridization. In D. L. Wiedbrauk & D. H. Farkas (Eds.), *Molecular Methods for Virus Detection* (pp. 75-101). San Diego: Academic Press.

Catalanotto, C., Cogoni, C., & Zardo, G. (2016). MicroRNA in Control of Gene Expression: An Overview of Nuclear Functions. *International Journal of Molecular Sciences, 17*(10), 1712. doi:10.3390/ijms17101712

Chaudhuri, A. D., Yelamanchili, S. V., & Fox, H. S. (2013). Combined fluorescent in situ hybridization for detection of microRNAs and immunofluorescent labeling for cell-type markers. *Frontiers in Cellular Neuroscience, 7*, 160. doi:10.3389/fncel.2013.00160

Condrat, C. E., Thompson, D. C., Barbu, M. G., Bugnar, O. L., Boboc, A., Cretoiu, D., . . . Voinea, S. C. (2020). miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. *Cells, 9*(2), 276. doi:10.3390/cells9020276

Cui, C., Shu, W., & Li, P. (2016). Fluorescence In situ Hybridization: Cell-Based Genetic Diagnostic and Research Applications. *Frontiers in Cell and Developmental Biology, 4*. doi:10.3389/fcell.2016.00089

Dave, V. P., Ngo, T. A., Pernestig, A.-K., Tilevik, D., Kant, K., Nguyen, T., . . . Bang, D. D. (2019). MicroRNA amplification and detection technologies: opportunities and challenges for point of care diagnostics. *Laboratory Investigation, 99*(4), 452-469. doi:10.1038/s41374-018-0143-3

Dean, Z. S., Riahi, R., & Wong, P. K. (2015). Spatiotemporal dynamics of microRNA during epithelial collective cell migration. *Biomaterials, 37*, 156-163. doi:10.1016/j.biomaterials.2014.10.022

Di Meo, A., Saleeb, R., Wala, S. J., Khella, H. W., Ding, Q., Zhai, H., . . . Yousef, G. M. (2017). A miRNA-based classification of renal cell carcinoma subtypes by PCR and in situ hybridization. *Oncotarget, 9*(2), 2092-2104. doi:10.18632/oncotarget.23162

Dixon-McIver, A., East, P., Mein, C. A., Cazier, J. B., Molloy, G., Chaplin, T., . . . Debernardi, S. (2008). Distinctive patterns of microRNA expression associated with karyotype in acute myeloid leukaemia. *PloS One, 3*(5), e2141. doi:10.1371/journal.pone.0002141

Fernández-Hernando, C., & Suárez, Y. (2018). MicroRNAs in endothelial cell homeostasis and vascular disease. *Current Opinion in Hematology, 25*(3), 227-236. doi:10.1097/moh.0000000000000424

Fitzpatrick, M. J., Murali, M. R., & Nardi, V. (2023). Molecular Diagnostics of Plasma Cell Neoplasms. *Surgical Pathology Clinics, 16*(2), 401-410. doi:10.1016/j.path.2023.01.005

Herzer, S., Silahtaroglu, A., & Meister, B. (2012). Locked nucleic acid-based in situ hybridisation reveals miR-7a as a hypothalamus-enriched microRNA with a distinct expression pattern. *Journal of Neuroendocrinology, 24*(12), 1492-1504. doi:10.1111/j.1365-2826.2012.02358.x

Huber, D., Voith von Voithenberg, L., & Kaigala, G. V. (2018). Fluorescence in situ hybridization (FISH): History, limitations and what to expect from micro-scale FISH? *Micro and Nano Engineering, 1*, 15-24. doi:https://doi.org/10.1016/j.mne.2018.10.006

Kasai, A., Kakihara, S., Miura, H., Okada, R., Hayata-Takano, A., Hazama, K., . . . Hashimoto, H. (2016). Double In situ Hybridization for MicroRNAs and mRNAs in Brain Tissues. *Frontiers in Molecular Neuroscience, 9*. doi:10.3389/fnmol.2016.00126

Kerstens, H. M., Poddighe, P. J., & Hanselaar, A. G. (1995). A novel in situ hybridization signal amplification method based on the deposition of biotinylated tyramine. *Journal of Histochemistry and Cytochemistry, 43*(4), 347-352. doi:10.1177/43.4.7897179

Koshkin, A. A., Singh, S. K., Nielsen, P., Rajwanshi, V. K., Kumar, R., Meldgaard, M., . . . Wengel, J. (1998). LNA (Locked Nucleic Acids): Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. *Tetrahedron, 54*(14), 3607-3630. doi:https://doi.org/10.1016/S0040-4020(98)00094-5

Laurent-Huck, F. M., & Felix, J. M. (1991). 10 - Measurement of Oxytocin and Vasopressin Gene Expression by in Situ Hybridization. In P. M. Conn (Ed.), *Methods in Neurosciences* (Vol. 5, pp. 159-182): Academic Press.

Lei, Z., van Mil, A., Xiao, J., Metz, C. H. G., van Eeuwijk, E. C. M., Doevendans, P. A., & Sluijter, J. P. G. (2018). MMISH: Multicolor microRNA in situ hybridization for paraffin embedded samples. *Biotechnology reports (Amsterdam, Netherlands), 18*, e00255-e00255. doi:10.1016/j.btre.2018.e00255

Lu, J., & Tsourkas, A. (2009). Imaging individual microRNAs in single mammalian cells in situ. *Nucleic Acids Research, 37*(14), e100-e100. doi:10.1093/nar/gkp482

Luo, X., & Liu, J. (2019). Fluorescence In Situ Hybridization (FISH) Analysis of the Locations of the Oligonucleotides 5S rDNA, (AGGGTTT)3, and (TTG)6 in Three Genera of Oleaceae and Their Phylogenetic Framework. *Genes, 10*(5), 375. Retrieved from https://www.mdpi.com/2073-4425/10/5/375

Nielsen, B. S., & Holmstrøm, K. (2013). Combined MicroRNA In Situ Hybridization and Immunohistochemical Detection of Protein Markers. In J. Moll & R. Colombo (Eds.), *Target Identification and Validation in Drug Discovery: Methods and Protocols* (pp. 353-365). Totowa, NJ: Humana Press.

Nuovo, G. J. (2010). In situ detection of microRNAs in paraffin embedded, formalin fixed tissues and the co-localization of their putative targets. *Methods, 52*(4), 307-315. doi:https://doi.org/10.1016/j.ymeth.2010.08.009

Nuovo, G. J., Elton, T. S., Nana-Sinkam, P., Volinia, S., Croce, C. M., & Schmittgen, T. D. (2009). A methodology for the combined in situ analyses of the precursor and mature forms of microRNAs and correlation with their putative targets. *Nature Protocols, 4*(1), 107-115. doi:10.1038/nprot.2008.215

O'Brien, J., Hayder, H., Zayed, Y., & Peng, C. (2018). Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Frontiers in Endocrinology, 9*, 402. doi:10.3389/fendo.2018.00402

Obernosterer, G., Martinez, J., & Alenius, M. (2007). Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. *Nature Protocols, 2*(6), 1508-1514. doi:10.1038/nprot.2007.153

Okuda, Y., Shimura, T., Iwasaki, H., Fukusada, S., Nishigaki, R., Kitagawa, M., . . . Kataoka, H. (2021). Urinary microRNA biomarkers for detecting the presence of esophageal cancer. *Scientific Reports, 11*(1), 8508. doi:10.1038/s41598-021-87925-1

Pardue, M. L., & Gall, J. G. (1969). MOLECULAR HYBRIDIZATION OF RADIOACTIVE DNA TO THE DNA OF CYTOLOGICAL PREPARATIONS. *Proceedings of the National Academy of Sciences, 64*(2), 600-604. doi:doi:10.1073/pnas.64.2.600

Pecciarini, L., Brunetto, E., Grassini, G., De Pascali, V., Ogliari, F. R., Talarico, A., . . . Cangi, M. G. (2023). Gene Fusion Detection in NSCLC Routine Clinical Practice: Targeted-NGS or FISH? *Cells, 12*(8). doi:10.3390/cells12081135

Pena, J. T. G., Sohn-Lee, C., Rouhanifard, S. H., Ludwig, J., Hafner, M., Mihailovic, A., . . . Tuschl, T. (2009). miRNA in situ hybridization in formaldehyde and EDC–fixed tissues. *Nature Methods, 6*(2), 139-141. doi:10.1038/nmeth.1294

Pothof, J., & van Gent, D. C. (2011). Spatiotemporal aspects of MicroRNA-mediated gene regulation. *Advances in Experimental Medicine and Biology, 722*, 75-85. doi:10.1007/978-1-4614-0332-6_5

Renwick, N., Cekan, P., Masry, P. A., McGeary, S. E., Miller, J. B., Hafner, M., . . . Tuschl, T. (2013). Multicolor microRNA FISH effectively differentiates tumor types. *The Journal of Clinical Investigation, 123*(6), 2694-2702. doi:10.1172/JCI68760

Sempere, L. F., Preis, M., Yezefski, T., Ouyang, H., Suriawinata, A. A., Silahtaroglu, A., . . . Korc, M. (2010). Fluorescence-based codetection with protein markers reveals distinct cellular compartments for altered MicroRNA expression in solid tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research, 16*(16), 4246-4255. doi:10.1158/1078-0432.CCR-10-1152

Shi, Z., Johnson, J. J., & Stack, M. S. (2012). Fluorescence <i>In Situ</i> Hybridization for MicroRNA Detection in Archived Oral Cancer Tissues. *Journal of Oncology, 2012*, 903581. doi:10.1155/2012/903581

Shu, J., Xia, Z., Li, L., Liang, E. T., Slipek, N., Shen, D., . . . Steer, C. J. (2012). Dose-dependent differential mRNA target selection and regulation by let-7a-7f and miR-17-92 cluster microRNAs. *RNA Biology, 9*(10), 1275-1287. doi:10.4161/rna.21998

Silahtaroglu, A. N. (2010). LNA-FISH for Detection of MicroRNAs in Frozen Sections. In J. M. Bridger & E. V. Volpi (Eds.), *Fluorescence in situ Hybridization (FISH): Protocols and Applications* (pp. 165-171). Totowa, NJ: Humana Press.

Silahtaroglu, A. N., Nolting, D., Dyrskjøt, L., Berezikov, E., Møller, M., Tommerup, N., & Kauppinen, S. (2007). Detection of microRNAs in frozen tissue sections by fluorescence in situ hybridization using locked nucleic acid probes and tyramide signal amplification. *Nature Protocols, 2*(10), 2520-2528. doi:10.1038/nprot.2007.313

Sinigaglia, C., Thiel, D., Hejnol, A., Houliston, E., & Leclère, L. (2018). A safer, urea-based in situ hybridization method improves detection of gene expression in diverse animal species. *Developmental Biology, 434*(1), 15-23. doi:https://doi.org/10.1016/j.ydbio.2017.11.015

Solly, E. L., Psaltis, P. J., Bursill, C. A., & Tan, J. T. M. (2021). The Role of miR-181c in Mechanisms of Diabetes-Impaired Angiogenesis: An Emerging Therapeutic Target for Diabetic Vascular Complications. *Frontiers in Pharmacology, 12*. doi:10.3389/fphar.2021.718679

Song, R., Ro, S., & Yan, W. (2010). In situ hybridization detection of microRNAs. *Methods in molecular biology (Clifton, N.J.), 629*, 287-294. doi:10.1007/978-1-60761-657-3_18

Sun, X., Sit, A., & Feinberg, M. W. (2014). Role of miR-181 family in regulating vascular inflammation and immunity. *Trends in Cardiovascular Medicine, 24*(3), 105-112. doi:10.1016/j.tcm.2013.09.002

Tian, T., Wang, J., & Zhou, X. (2015). A review: microRNA detection methods. *Organic & Biomolecular Chemistry, 13*(8), 2226-2238. doi:10.1039/c4ob02104e

Urbanek, M. O., Nawrocka, A. U., & Krzyzosiak, W. J. (2015). Small RNA Detection by in Situ Hybridization Methods. *International Journal of Molecular Sciences, 16*(6), 13259-13286. doi:10.3390/ijms160613259

Whittington, N. C., & Wray, S. (2017). Suppression of Red Blood Cell Autofluorescence for Immunocytochemistry on Fixed Embryonic Mouse Tissue. *Current Protocols in Neuroscience, 81*, 2.28.21-22.28.12. doi:10.1002/cpns.35

Yang, G., Wu, Y., & Ye, S. (2017). MiR-181c restrains nitration stress of endothelial cells in diabetic db/db mice through inhibiting the expression of FoxO1. *Biochemical and Biophysical Research Communications, 486*(1), 29-35. doi:10.1016/j.bbrc.2017.02.083

Yang, H., Wanner, I. B., Roper, S. D., & Chaudhari, N. (1999). An optimized method for in situ hybridization with signal amplification that allows the detection of rare mRNAs. *Journal of Histochemistry and Cytochemistry, 47*(4), 431-446. doi:10.1177/002215549904700402

Zaidi, A. U., Enomoto, H., Milbrandt, J., & Roth, K. A. (2000). Dual Fluorescent In Situ Hybridization and Immunohistochemical Detection with Tyramide Signal Amplification. *Journal of Histochemistry and Cytochemistry, 48*(10), 1369-1375. doi:10.1177/002215540004801007

Zhou, S. S., Jin, J. P., Wang, J. Q., Zhang, Z. G., Freedman, J. H., Zheng, Y., & Cai, L. (2018). miRNAS in cardiovascular diseases: potential biomarkers, therapeutic targets and challenges. *Acta Pharmacologica Sinica, 39*(7), 1073-1084. doi:10.1038/aps.2018.30

Zhou, T., Xiang, Y., Liu, S., Shao, Z., Liu, Y., Ma, H., . . . Chai, H. (2023). Insights into simultaneous nitrogen and phosphorus removal in biofilm: The overlooked comammox Nitrospira and the positive role of glycogen-accumulating organisms. *Science of the Total Environment*, 164130. doi:10.1016/j.scitotenv.2023.164130

FIGURE LEGENDS:

Figure 1. Flowchart with timing of the 2-day protocol for combined miRNA fluorescent in situ hybridisation (FISH) and protein marker staining via immunofluorescence (IF). (HRP= horseradish peroxidase; TSA= tyramide signal amplification, DIG=digoxigenin).

Figure 2. Overview of the principle of tyramide signal amplification (TSA), which enables detection of low-copy miRNAs by increasing signal intensity compared to traditional fluorescent probes. (HRP= horseradish peroxidase, DIG= digoxigenin, F= fluorophore, FISH= fluorescent in situ hybridization).

Figure 3. Upper pictures represent different incubation periods (10, 15, 20 min) for tyramide signal amplification (TSA) with co-staining of isolectin (green), miR-181c-5p (red) and DAPI (blue). Longer incubation periods result in higher background and lower intensities of isolectin staining. 10 min incubation provides the optimal signal, and demonstrates the endothelial localization of miR-181c-5p. Bottom images show different miRNA probe concentrations (10, 100, 150nM) with co-staining of miR-181c-5p (red) and DAPI (blue). Higher probe concentrations result in improved miRNA staining intensity, with best result obtained with probe concentration of 150nM. Figures were taken at 63X magnification.

Figure 4. **A-C)** FISH-IF of miR-181c-5p, scramble negative control and nuclear expressed U6 snRNA positive control in red with co-staining of nuclear DAPI (blue) in murine cardiac tissue. **D-E)** Co-staining of miR-181c-5p (red) with endothelial isolectin staining (green) and DAPI (blue). The arrows indicate the subtle overlay of green and red color representing endothelial localization of miR-181c-5p in cardiac tissue. Figures were taken at 63X magnification.

TABLES:

Table 1: Probe sequences of DIG-labeled LNA-detection probes

Detection probe	**Sequence**
Scramble (negative control)	5DIG/GTGTAACACGTCTATACGCCCA/3DIG
U6 snRNA (positive control)	5DIG/CACGAATTTGCGTGTCATCCTT/3DIG
Mmu-miR-181c-5p	5DIG/ACTCACCGACAGGTTGAATGTT/3DIG

Table 2: Troubleshooting parameters with potential solutions for FISH-IF

Observation	*Cause*	*Possible solution*
Increased background signal	Drying of the slides during incubation	Cover slides with coverslips
	Endogenous isotope binding	Mouse-on-mouse blocker
	Autofluorescence	TrueBlack treatment
	Probe concentration/protein marker concentration too high	Lower concentration
	Stringency washes not optimized	Increase salt concentration, temperature or number of post-hybridization stringency washes
	Antibody /probe incubation	Reduce incubation time
	TSA incubation	Reduce incubation time

	Blocking buffer incubation	Increase incubation time or try different blocking buffers
	Endogenous peroxidase	Be sure to block all endogenous peroxidase if working with HRP-conjugated antibodies, try different incubation times and peroxide concentration
Weak signal	**Pre-analytical**	
	Embedding method	PFA/30% sucrose/OCT embedding provides more preserved morphology than OCT embedded cryosections, and better sensitivity compared to paraffin embedded sections
	Analytical	
	Stringency washes not optimized	Lower stringency
	(Pre)Hybridisation time	Try overnight hybridisation
	(Pre)Hybridisation temperature	Empirically optimize hybridisation temperature
	Probe concentration/Antibody concentration	Increase concentration
	Antibody incubation period	Try longer incubation time of antibodies
	TSA incubation	Try longer incubation time of TSA amplification
	Post-analytical	
	Imaging modality	Confocal laser scanning microscope improves signal sensitivity