

Detecting MicroRNA in Human Cancer Tissues with Fluorescence In Situ Hybridization

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Abstract

The technique of nucleic acid in situ hybridization is an effective method for identifying the existence and abundance of nucleic acids in tissue sections or cytological preparations. Such a method has the advantage of keeping morphological relationships intact while identifying changes at the molecular level. As a noncoding regulatory RNA, microRNA has been found to intricately control many physiological and pathological conditions. We provide here a representative fluorescence in situ hybridization protocol for microRNA detection, and note commonly used alternatives, and some troubleshooting points. The method described is based on formalin-fixed paraffin-embedded oral cancer tissues but should be broadly applicable to similarly processed tissues of other types of cancer.

Key words Squamous cell carcinoma, microRNA, Fluorescence in situ hybridization (FISH), Formalin-fixed paraffin-embedded (FFPE) tissue

1 Introduction

Since first discovered in the nematode *C. elegans* in 1993 [1], microRNA as a category of small regulatory RNA has been associated with numerous physiological and pathological processes in various species, especially in human. Because many microRNAs have been found to play important roles in cancer initiation and progression, there has been an ever-increasing amount of interest in the detection methodology of microRNAs [2].

As a small piece of RNA, all traditional methods for RNA detection, such as Northern blotting, quantitative polymerase chain reaction (qPCR), and microarray, could be and have been attempted for detecting the existence of microRNA or quantifying them in biological samples. However, due to the fact that mature microRNA is only about 22 nucleotides in length, many microRNAs differ from each other by only a few nucleotides. This poses a challenge in terms of specificity and sensitivity in developing a detection method. Some significant modifications have been made to

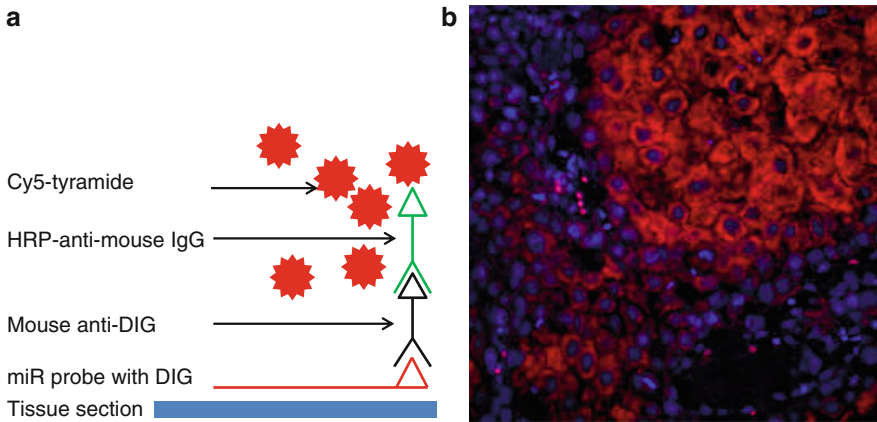


Fig. 1 Key steps in microRNA fluorescence in situ hybridization and typical result. **(a)** Diagram shows a processed microRNA containing tissue section that is first hybridized with DIG-labeled LNA-incorporated probe, followed by mouse anti-DIG, and HRP-conjugated anti-mouse IgG, prior to visualization of the signal by the addition of Cy5-conjugated tyramide (modified from Shi et al. Journal of Oncology, 2012, 903581). **(b)** Shown here is a positive result (*red* staining) from a section of formalin-fixed paraffin-embedded oral cancer tissue that was hybridized with mir-146a probe. *Blue* parts are cell nuclei stained by DAPI. Original magnification 400×

address the uniqueness of microRNA and newer methods have been under development [3].

Cancer tissues are inherently heterogeneous. Oftentimes, carcinoma cell populations are interwoven with nonmalignant stromal cells. So to further delineate the role of microRNA in cancer pathology, it is important to maintain the morphological relationships within the tissue and to differentiate the cancerous components from noncancerous while measuring the expression of microRNAs. In situ hybridization (ISH) is one of the methods to achieve this and has been applied for many years. As there are no antibody-based detection methods to gauge microRNA directly, ISH for microRNA in tissue sections has received a good amount of attention [4].

Our protocol for fluorescence in situ hybridization (FISH) technique to detect microRNA starts with the proper processing of tissues and then proceeds with the deparaffinization, pretreatment of the section, and then hybridization with a labeled probe. Post-hybridization immunohistochemistry (IHC) is used to visualize the positive signals with fluorescence microscopy. A diagram of the key scheme is presented in Fig. 1a. Some parts of the content have been previously published by us in an open access journal paper [5].

2 Materials

All solutions should be prepared with RNase-free Milli-Q-grade water at room temperature, unless otherwise specified.

2.1 Tissue Processing

1. RNase AWAY™ reagent (Life Technologies Corporation).
2. 10 % neutral buffered formalin (NBF): 10 % formalin, phosphate buffer, pH 7.0. To make 1 L of NBF, use 100 mL of formalin and 900 mL of water, 6.5 g of anhydrous disodium hydrogen phosphate (Na_2HPO_4), and 4.0 g of monohydrate sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$). There is no need to adjust the pH. This solution is commercially available (Richard-Allan Scientific, Kalamazoo, MI).
3. Histological grade ethanol, xylene, and paraffin (melting point at 55–57 °C).
4. 4 % paraformaldehyde (PFA).
5. 100 mM Glycine.
6. Tissue cassettes, automatic tissue processor, tissue embedding center, and rotary microtome.
7. Oven or incubator that could be maintained at any temperature from ambient to 65 °C.

2.2 Pretreatment and Hybridization

1. 10× PBS: 0.1 M PBS, pH 7.4. Add 10.9 g of Na_2HPO_4 (anhydrous), 3.2 g of NaH_2PO_4 (anhydrous), and 90.0 g of NaCl to 900 mL water in a glass beaker. Mix and adjust pH to 7.4. Working solution of PBS is used at 1×. Dilute the stock 1:10 with RNase-free Milli-Q-grade water before use and adjust pH if necessary.
2. PBS-T: 0.1 % Tween-20, 1× PBS.
3. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. To make 100 mL of TE buffer, add 1 mL of 1 M Tris-HCl, pH 8.0, and 0.2 mL of 0.5 M EDTA to a beaker and fill up with Milli-Q water to 100 mL (*see Note 1*).
4. Proteinase K solution: 20 µg/mL Proteinase K in TE buffer.
5. Hydrochloric acid (HCl) solution: 24 mM HCl in ethanol. Add 0.2 mL of concentrated (12 M) hydrochloric acid to 100 mL of ethanol.
6. 25 µM Locked nucleic acid (LNA)-based probes (Exiqon, Vedbaek, Denmark) with the trademark of miRCURY LNA™ detection probe (*see Note 2*). Probe sequence for miR-146a is AACCCATGGAATTCAGTTCTCA and it is labeled with digoxigenin (DIG) at the 5' end. The human miR-146a target sequence is UGAGAACUGAAUCCAUGGGUU. Negative control probe sequence is GTGTAACACGTCTATACGCCCA and it is 5'-DIG labeled as well.
7. Sakura Finetek Tissue-Tek Slide Staining Set.
8. Plastic coverslip.
9. Moisture chamber.

10. 20× SSC: 3 M Sodium chloride (NaCl), 0.3 M sodium citrate. Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of Milli-Q-grade water; adjust the pH to 7.0 with a few drops of 1 M HCl, and adjust the volume to 1 L with additional water. Sterilize by autoclaving.
11. 50× Denhart's solution: 1 % Ficoll 400 (w/v), 1 % polyvinylpyrrolidone (PVP) (w/v), 1 % bovine serum albumin (BSA) (w/v). Add 10 g of Ficoll 400, 10 g of PVP, and 10 g of BSA to 900 mL of distilled water, and then fill up to 1 L. Filter the solution prior to storage through a 0.2-μm filter and store at -20 °C. Warm up to room temperature before use.
12. Pre-hybridization solution: 50 % deionized formamide, 2× SSC, 1× Denhart's, 0.02 % sodium dodecyl sulfate (SDS), 0.5 mg/mL yeast tRNA, 0.5 mg/mL salmon sperm DNA.
13. Hybridization solution: 50 % Deionized formamide, 2× SSC, 1× Denhart's, 10 % dextran sulfate, 0.5 mg/mL yeast tRNA, 0.5 mg/mL salmon sperm DNA (*see Note 3*).
14. Soaking solution: 50 % formamide, 1× SSC.
15. Detergent solution: 0.02 % SDS, 1× SSC.

2.3 Immuno-fluorescence

1. Blocking solution: 10 % normal goat serum in PBS.
2. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA).
3. Mouse anti-digoxigenin (Roche Applied Science, Indianapolis, IN).
4. Tyramide Signal Amplification (Cy5-TSA) kits (Perkin Elmer, Waltham, MA, catalog # NEL-705A) (*see Note 4*). The kit contains HRP-streptavidin, blocking reagent, amplification diluent, and Cyanine 5-conjugated tyramide.
5. Working solution of Cy5-TSA. To prepare working solution dilute Cy5-TSA from the kit (*see item 4* of Subheading 2.3) 1:50 with 1× amplification diluent from the kit.
6. ProLong Gold Antifade mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, CA).
7. Fluorescence microscope that is equipped with proper filters for DAPI and Cy5 dyes.

3 Methods

Use caution to maintain an RNase-free environment by wearing gloves and mask during all the procedures wherein RNA has been exposed until the hybridization step is finished (*see Note 5*).

3.1 Processing of Tissues

1. Fixation: Trim surgically removed tissue slice so as not to exceed 0.4 cm × 2.5 cm × 2 cm (in thickness × length × width) to be accommodated by the size of tissue cassette and to ensure the efficiency of downstream processing steps. Immediately fix the tissue in 10 % NBF for 24–72 h (*see Note 6*).
2. Dehydration: Secure tissue slices in a cassette; wrap up tissues with sponge pieces if tissue size is too small to prevent slices from floating away. Immerse tissue-containing cassettes in the following solutions in order: 70 % ethanol for 30 min, 80 % ethanol for 30 min, 90 % ethanol for 30 min, 100 % ethanol for 45 min, and 100 % ethanol for 45 min again.
3. Clearing: Immerse tissue cassettes in xylene for 45 min and another round of xylene for 45 additional minutes.
4. Infiltration: Immerse tissue cassettes in molten paraffin (65 °C) for 45 min and repeat with new molten paraffin for 45 min.
5. Embedding: Put tissue into paraffin block mold with proper orientation using embedding center (Tissue-Tek TEC, Sakura), fill up with molten paraffin, and let solidify on cold plate.
6. Sectioning: Use microtome (Leica RM2125) for sectioning. Set thickness at 5 µm and mount sections on positively charged slides (*see Note 7*). Drain and store the slides at room temperature.

3.2 Deparaffinization of Sections

1. Baking: Heat the sections in a metal rack at 65 °C for 1 h.
2. Dewaxing: Put slides on a histological staining rack and immerse sections in xylene for 10 min; repeat this step in another container of xylene.
3. Rehydration to water: Pass through in serial ethanol solutions (100, 90, 80 %), 1 min each, and into 1× PBS for 5 min.

3.3 Pretreatment of Sections

1. Wash sections in 1× PBS for 5 min, and repeat.
2. Put a section in the horizontal position in an open-lid moisture chamber; such a position is needed whenever a small amount of liquid is applied to the sections. Apply 300 µL of 24 mM HCl solution to each section and incubate for 10 min at room temperature (RT) to quench endogenous peroxidase (*see Note 8*).
3. Wash slides in a container with 1× PBS for 5 min twice.
4. Apply proteinase K solution, 500 µL each; incubate at 37 °C for 10 min.
5. Wash slides in a container with 1× PBS for 5 min twice.
6. Post-fixation: Apply 500 µL of 4 % PFA to each section in the horizontal position; fix for 10 min.
7. Wash slides in a container with 1× PBS for 5 min twice.

8. Apply 300 μL of 100 mM glycine, and incubate for 10 min to neutralize any residual PFA.
9. Wash slides in a container with 1 \times PBS for 5 min twice.
10. Wash slides in a container with 2 \times SSC wash for 5 min.
11. Apply 100 μL of pre-hybridization solution, cover with plastic coverslip, and incubate in moisture chamber filled with Kimwipes, which were soaked in soaking solution, at 50 °C for 2 h (*see Note 9*).

3.4 Hybridization and Stringent Wash

1. After pre-hybridization time is up, gently discard the solution by tipping over the slide to a waste can. Wipe clean slide area outside tissue section.
2. Without any wash, add 100 μL of hybridization solution containing probe at 25 nM (1 μL of 25 μM DIG-labeled probe to 1,000 μL) to sections. Cover with plastic coverslip, and incubate overnight (18 h) at 50 °C in moisture chamber (*see Note 10*).
3. Start stringent washes. Discard hybridization solution and plastic coverslip by immersing the slides individually into a container of 2 \times SCC. Use a water bath shaker for gentle shaking, 30 rpm.
4. Wash in 2 \times SSC at 37 °C, for 15 min.
5. Wash at 2 \times SCC at 50 °C for 15 min, with gentle shaking.
6. Wash in 1 \times SSC at 37 °C for 15 min.
7. Wash in 1 \times SSC at 50 °C for 15 min, with gentle shaking.
8. Wash in detergent solution at 37 °C for 15 min.
9. Wash in detergent solution at 50 °C for 15 min, with gentle shaking.
10. Wash in PBS-T at RT for 5 min; repeat three times.

3.5 Post-hybridization Immunofluorescence

1. Serum blocking: Add 300 μL of blocking solution per section and incubate at RT for 1 h.
2. Without washing, add 300 μL of mouse anti-DIG diluted 1:250 in blocking and incubate at RT for 0.5 h.
3. Wash in PBS-T for 5 min; repeat three more times (*see Note 11*).
4. Add 300 μL of HRP-conjugated goat anti-mouse IgG 1:500 dilution in 1 \times PBS and incubate at RT for 0.5 h.
5. Wash in PBS-T for 5 min; repeat three more times.
6. Add working solution of Cy5-TSA, 200 μL per slide; incubate at RT for 10 min.
7. Wash in PBS-T for 5 min; repeat three more times.
8. Wash in 1 \times PBS for 5 min.

9. Air-dry for 10 min and then mount glass coverslip using ProLong Gold. Let solidify overnight and seal with nail polish before observation under a fluorescent microscope.
10. Document the results with a camera-equipped fluorescent microscope. Typical results: Technical negative control slides should produce no signal on Cy5 channel while positive staining locates in the cytoplasm of selective cell populations. DAPI from the mounting medium stains all cell nuclei (Fig. 1b).

4 Notes

1. Proteinase K solution is stable over a broad pH range (4.0–12.5), but the optimum is pH 8.0. Proteinase treatment is very critical in unmasking the nucleic acid targets. Concentration and incubation time with different tissues may vary. Pepsin or collagenase may be used as alternatives.
2. A probe is a labeled nucleic acid whose nucleotide sequence is complementary to target nucleic acid, which dictates the specificity of hybridization. While other choices are possible, LNA-based probe is the default type of probe for microRNA detection and has been widely used because of its supremacy in specificity and sensitivity [4]. As for tracer labeling, biotin-labeled probe is another choice; however certain human tissues are rich with endogenous biotin, which could lead to significant background staining. Further, blocking of such endogenous biotin is not easily achievable. In contrast, DIG as a hapten is from a plant source; there is no similar analog in animal tissues, so anti-DIG antibodies have much less background binding.
3. Pre-hybridization and hybridization solutions usually have the same components except that the latter will be added with probes before use. Factors affecting hybridization include temperature, pH, concentration of monovalent cations, and presence of organic solvents such as formamide. The use of formamide significantly reduces the melting temperature of nucleic acid hybrids, therefore making it possible for hybridization to happen at a much lower temperature than melting temperature (T_m). The best condition for each probe and the probe concentration may vary and should be optimized.
4. Tyramide as a substrate of HRP has greatly enhanced the sensitivity of immunohistochemistry that uses HRP-labeled antibody [6, 7]. But because of its high sensitivity, the suppression of endogenous peroxidase sometimes becomes challenging.
5. An important consideration is to use diethylpyrocarbonate (DEPC)-treated or otherwise made RNase-free water in all

solution preparation and to exercise caution to maintain an RNase-free work environment during all procedures. Avoid RNase contamination by wearing disposable mask and gloves during the entire reagent preparation and other material preparation processes since skin contact and salivary fluid shed from talking are rich sources of RNases. Treat with RNase AWAY™ reagent all surfaces that may come in contact with RNA-containing specimen. RNase-free environment is not necessary after hybridization. Some protocols use RNase treatment as a negative control for RNA hybridization, but we do not recommend it.

6. Formalin fixation time was often a controversial topic. Considering the fact that this fixative penetrates tissue at a speed of 0.5 mm/h and protein cross-linking happens more slowly, a minimum of 24 h is required for a typical tissue slice; but overfixation may cause difficulty in unmasking the targets for ISH and IHC [8, 9]. Frozen sections cut by cryostat could be fixed for 30 min in 10 % NBF and used for FISH as well.
7. The thickness of paraffin sections affects the results of ISH; thicker sections would provide more targets for probes to bind but may compromise the revelation of a clear morphological structure. The use of commercially available positively charged microscope slides gives improved cellular adhesion. This is to prevent that the lengthy incubation and extensive washing steps in FISH procedures may detach tissue sections from slide surfaces. Poly-L-lysine- or amino silane-coated glass slides work equally well for this purpose and could be self-prepared. Proper baking before the dewaxing is also very important in retaining the section on slides.

Processing tissues for formalin-fixed paraffin-embedded (FFPE) materials is a routine part of a standard histology lab. The majority have been automated by using tissue processor. Our model of this equipment is Leica TP1020. Making perfect sections from paraffin blocks is often challenging to beginners, so it is better to have this part performed by a well-trained histotechnologist. The quality of microRNA preservation in FFPE material has been a matter of concern since previously mRNA has been shown to be poorly preserved in such materials. Improper fixation or processing would certainly degrade all biomolecules within tissue specimens. However, recently some labs have verified that microRNA survives even better than mRNA in FFPE material, probably because of its short size and association with complicated protein complex [10, 11]. In addition to successful microRNA FISH on FFPE specimens, we have been able to isolate decent quality RNA for microRNA quantitative PCR via laser capture microdissection on FFPE materials.

8. Alternatively, use 3 % hydrogen peroxide (H_2O_2) in methanol incubation for 10 min. However, 24 mM HCl solution for suppressing endogenous peroxidase may have the additional benefit for improving signal-to-noise ratio because of partial hydrolysis of target sequence and extraction of proteins.
9. A pre-hybridization is always necessary to prevent nonspecific binding of probes, which would lead to background noise in staining.
10. The DIG-labeled LNA-incorporated mir-146a probe from Exiqon has a T_m of 85 °C for RNA target. Hybridization temperature is a critical factor affecting specificity and sensitivity of FISH; it has been recommended to be 30–45 °C lower than T_m with 50 % formamide present in hybridization solution and optimized with other factors upon specific probes [12]. Also, the choice of LNA-based probes in microRNA ISH has become a norm because LNA-modified oligos have a better mismatch discrimination [4, 13].
11. Substituting 0.1 % Triton X-100 for 0.1 % Tween-20 in the PBS-T buffer for wash may help decrease the background.

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Nucleic Acid Detection

Methods and Protocols

Kolpashchikov, D.M.; Gerasimova, Y.V. (Eds.)

2013, XIV, 320 p. 85 illus., 58 illus. in color., Hardcover

ISBN: 978-1-62703-534-7

A product of Humana Press