A modified immunofluorescence *in situ* hybridization method to detect long non-coding RNAs and proteins in frozen spinal cord sections

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Abstract. Immunofluorescence *in situ* hybridization (immuno-FISH) is widely used to co-detect RNAs and proteins in order to study their spatial distribution in cells. The present study used a modified immuno-FISH protocol for the detection of long non-coding RNAs (lncRNAs) and proteins in frozen spinal cord sections. The spinal cords of Sprague-Dawley rats were harvested, frozen and sectioned (10 μm), and oligonucleotide probes and antibodies were prepared. Following antigen retrieval, dehydration, prehybridization, hybridization, post-hybridization and immunofluorescence staining, images were captured. Antigen retrieval was performed by autoclaving or proteinase K treatment, and their effects on the hybridization signal were compared. The same sections were successfully stained by immunofluorescence. Satisfactory fluorescent signals of lncRNA and protein were obtained. The results of the present study suggest that the modified protocol of immuno-FISH for the detection of lncRNAs and proteins in frozen spinal cord sections is effective and time-efficient, and the required reagents are readily available.

Introduction

A number of long non-coding RNAs (lncRNAs) have been identified in the past decade, and previous results link specific lncRNAs to many physiological processes and to various diseases, including cancer and chronic pain (1-3). Investigation into the tissue and subcellular localization of lncRNAs is necessary to determine their function and underlying mechanisms. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is an abundant, ubiquitously expressed lncRNA (4). It has previously been reported that MALAT1 is expressed in the nervous system and regulates lung cancer and glioma (4-6).

In situ hybridization (ISH) is a useful tool for the quantification and localization of specific RNAs within cultured cells or tissue sections. In ISH, an oligonucleotide probe is used to detect the RNA of interest through complementary base pairing (7). Historically, ISH was performed with radioactive probes; however, the handling of radioactive materials has many risks, and the method of image capture was time consuming with this technique (7). These disadvantages were overcome with the advent of fluorescence *in situ* hybridization (FISH), which uses fluorescently labeled probes. The utility of FISH is increased when it is combined with other techniques; for example, immunofluorescence *in situ* hybridization (immuno-FISH) is a combination of FISH and immunohistochemistry that enables the detection of RNAs and proteins in the same samples (8). Variations of the immuno-FISH method have previously been documented. Nehmé *et al* (9) reported that treatment with proteinase K (PK) increased the sensitivity of FISH, but decreased the signal of immunofluorescence staining in a study of 65-kDa glutamic acid decarboxylase mRNA and three proteins [neuronal nuclei (NeuN), FBJ murine osteosarcoma viral oncogene homolog B and tyrosine hydroxylase] in frozen brain sections. Although the author provided a method to correct this problem (9), the method was complicated and its application in studies of noncoding RNA has not been validated. de Planell-Saguer *et al* (10) reported an immuno-FISH method for detecting non-coding RNAs in paraffin-embedded tissues and cultured cells; however, they did not report its application in frozen tissue sections.

In the present study, a modified immuno-FISH protocol was used to investigate the expression and distribution of lncRNA MALAT1 and its association with the protein markers of neurons, microglia and astrocytes in 10-μm frozen spinal cord slices from rats. The modified protocol was also compared with other reported protocols.

Materials and methods

Animals. Adult male Sprague Dawley rats (n=6, 200-250 g, 6-7 weeks old; Shanghai SLAC Laboratory Animal Co., Ltd.,

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Shanghai, China) were housed under a 12-h light/dark cycle, at 23-25˚C and 45-50% humidity and provided with free access to food and water. All surgical and experimental procedures were approved by the Animal Ethics Committee of Fudan University (Shanghai, China).

Reagents. To prepare a 1% sodium pentobarbital solution, 5 g sodium pentobarbital (cat. no. 69020181; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was dissolved in 500 ml distilled (d)H_2O, and the solution was stored at 4˚C in the dark. To prepare 1 l of 4% paraformaldehyde, 40 g paraformaldehyde was added to 1 l of 1X phosphate-buffered saline (PBS) and heated gradually to 60˚C with continuous stirring to dissolve the paraformaldehyde. The pH was subsequently adjusted to 7.4 with NaOH. To prepare a 10 or 30% sucrose solution, 10 or 30 g sucrose (cat. no. 10021418; Sinopharm Chemical Reagent Co., Ltd.) was added to 100 ml dH_2O. To prepare 1 l of antigen unmasking buffer (10 mM sodium citrate), 2.94 g sodium citrate tribasic salt dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$, cat. no. 10019418; Sinopharm Chemical Reagent Co., Ltd.) was added to 1 l dH_2O. The pH was adjusted to 6.0 and the solution was subsequently filtered (pore diameter, 75 μm). To prepare 1 l of 20X saline-sodium citrate (SSC), 175.2 g NaCl and 88.2 g sodium citrate tribasic salt dihydrate were dissolved in 800 ml dH_2O. The pH was adjusted to 7.0, and dH_2O was added to bring the volume to 1 l. To prepare 100 ml of prehybridization buffer, 3 g bovine serum albumin (BSA; cat. no. 69003433; Sinopharm Chemical Reagent Co., Ltd.) was added to 100 ml of 4X SSC. The resulting solution was used to prepare 0.1, 1, 2 and 4X SSC. To prepare 10 ml of hybridization buffer, 1 g dextran sulfate (cat. no. S14047; Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) and 1 ml deionized formamide (cat. no. 30091218; Sinopharm Chemical Reagent Co., Ltd.) were added to 9 ml of 4X SSC. To prepare 100 ml of blocking buffer, 1 g BSA and 0.1 ml Tween-20 were added to 100 ml of 1X PBS. To prepare 50 ml of 4',6-diamidino-2-phenylindole (DAPI) staining solution, 0.5 μl DAPI stock solution was diluted in 50 ml of 1X PBS.

Riboprobes and antibodies. All DNA oligonucleotides, including three probes for rat lncRNA malat1 (Malat1-a, -b and -c) and one probe for GAPDH (positive control; Table I), were designed and synthesized and labeled on the 5'-end with Alexa Fluor 633 by Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Probes were stored at -20˚C in powder form. To prepare a 250 nM working solution, probes were diluted in hybridization buffer. Antibodies against NeuN, Iba1 and GFAP were used as biomarkers of neurons, microglia and astrocytes, respectively (11). The primary and Alexa Fluor 488-conjugated secondary antibodies used for immunofluorescence were purchased from Abcam (Cambridge, MA, USA; Table II) and diluted with blocking buffer to 1:500 and 1:400, respectively.

Tissue preparation. Rats were anesthetized with an intraperitoneal injection of 1% sodium pentobarbital solution (40 mg/kg) and transcardially perfused with 1X PBS and 500 ml of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). The spinal cord was harvested at the cervical level and specimens were immersed in 4% paraformaldehyde for 4 h at 4˚C. The tissues were then sequentially immersed in 10 and 30% sucrose until the spinal cords fell to the bottom. Spinal cords were embedded in optimal cutting temperature compound (cat. no. 4583; Sakura Finetek USA, Inc., Torrance, CA, USA) and mounted on a microtome stage. Horizontal sections (10 μm) were mounted onto pre-warmed poly-l-lysine-coated slides (cat. no. P4981; Thermo Fisher Scientific, Inc.).

Antigen retrieval and dehydration. Following desiccation for 20 min at room temperature, slides were rinsed twice with 1X SSC (5 min/wash). The slides were placed into a Coplin jar filled with antigen unmasking buffer, which was placed into an autoclave and heated to 100˚C for 5 min and subsequently cooled to room temperature. Sections were then washed three times with 1X SSC (5 min/wash). The tissue sections were dehydrated in a graded series of ethanol (50, 70, 90 and 100%; 3 min each) and air-dried for 10 min.

For comparison, some slides were treated with PK instead of treatment with antigen unmasking buffer and autoclaving, according to the method described by Nehmé *et al* (9). For this method, the slides were immersed in a buffer containing 0.1 M Tris-HCl (pH 8), 50 mM EDTA (pH 8) and 10 μg/ml PK (Roche Diagnostics, Basel, Switzerland) for 25 min at 37˚C.

Prehybridization. The prehybridization buffer was warmed to ~47˚C [usually 22-25˚C below the melting temperature (T_m) of probes] and 200 μl warmed prehybridization buffer was pipetted onto each slide and prehybridized for 20 min in a humid chamber (47˚C). The probes (Malat1-a, -b and -c and GAPDH) were diluted with hybridization buffer and denatured at 65˚C for 10 min, then stored on ice.

Hybridization. The prehybridization buffer was removed and 100 μl aliquots of each probe (or same volume of hybridization buffer without probes as negative control) were added to separate slides and hybridized overnight (4-16 h) at the same temperature used in the prehybridization step. From this step on, all slides were kept in the dark.

Post-hybridization. Following hybridization, the slides were rinsed with the following solutions: 4X SSC (twice at 5 min/wash), 2X SSC (5 min), 1X SSC (5 min), and 0.1X SSC (5 min) at the same temperature as in the hybridization step. The slides were then rinsed with 1X PBS for 5 min at room temperature.

Immunofluorescence staining. Following *in situ* hybridization, the sections were blocked in blocking buffer for 30 min at 37˚C. From this step on, sections were kept in a humid chamber to protect the tissue sections from drying out. The blocking buffer was removed and 200 μl primary antibody diluted in blocking buffer was added (Table II), and the sections were incubated overnight at 4˚C in a humid chamber. Sections were rinsed three times with 1X PBS (5 min/wash), then incubated with corresponding secondary antibody (200 μl diluted in blocking buffer) for 2 h at 37˚C. Slides were subsequently rinsed three times with 1X PBS (5 min/wash). At this point, all FISH and immunofluorescence staining steps were complete. However, if the nucleus needed to be observed, nuclear staining with DAPI was performed. Finally, a few drops of antifade (cat. no. P36965;

Table I. Riboprobes used in the present study.

Name	T_m, ˚C	Sequence, 5'-3'	Length, bp	GC, %
Malat1-a	66.1	GGGCCGTTATAAGAGTCGACTGTCGCATGTACGAAGGCATGAG	43	53.5
Malat1-b	66.1	GCGGTTCGTTGGAGGAAGCTAGGAAGAAGGAGCCGAAATGATG	43	53.5
Malat1-c	62.4	GGCTGGTAGTTTATTCTTTTCCCCCTCCCTTAACAAGACTTG	42	45.2
GAPDH	64.0	CTTGTGACAAAGTGGACATTGTTGCCATCAACGACCCCTTCATTG	45	46.7

Malat, metastasis-associated lung adenocarcinoma transcript; T_m, melting temperature; bp, base pairs.

Table II. Antibodies used in the present study.

Name	Primary/secondary	Company	Cat. no.	Conjugated	Host	Dilution
Anti-NeuN	Primary	Abcam	Ab177487	No	Rabbit	1:500
Anti-Iba-1	Primary	Abcam	Ab5076	No	Goat	1:500
Anti-GFAP	Primary	Abcam	Ab7260	No	Rabbit	1:500
Anti-rabbit IgG	Secondary	Abcam	Ab150129	Alexa fluor 488	Donkey	1:400
Anti-goat IgG	Secondary	Abcam	Ab150073	Alexa fluor 488	Donkey	1:400

NeuN, neuronal nuclei; Iba-1, ionized calcium binding adaptor molecule 1; GFAP, glial fibrillary acidic protein; IgG, immunoglobulin G.

Thermo Fisher Scientific, Inc.) were added to the slides and a coverslip was placed. The slides were sealed with nail polish.

Image acquisition and analysis. Images were captured with a confocal laser-scanning microscope (FluoView™ FV1000; Olympus Corporation, Tokyo, Japan) equipped with a digital camera and image analysis system (FV10-ASW 4.0; Olympus Corporation). All images were analyzed using Image J 1.44 (National Institutes of Health, Bethesda, MD, USA). Brightness and contrast were adjusted. Positive cells and the background surrounding positive cells were selected as regions of interest. The ratio of integral optical density (IOD) between the positive cells and background signal was calculated using Image J 1.44. All statistical analyses using Student's t-test and histograms were completed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

All three probes are effective for immuno-FISH. Satisfactory fluorescent signals were obtained from all three malat1 probes (Malat1-a, -b and -c; Fig. 1A-C). During FISH, GAPDH served as a positive control and sections without probes (hybridization buffer only) served as negative controls (Fig. 1D and E). The IOD ratio of Malat1-a, -b, -c group was respectively compared with negative control using Student's t-test. All three Malat1 probes were demonstrated to be effective (n=6, $P<0.05$); however, the Malat1-b probe exhibited the highest signal amplitude (Fig. 1F). Therefore, Malat1-b was used for subsequent experiments.

Antigen retrieval by autoclaving and PK treatment has similar effects on FISH. Two antigen retrieval methods, autoclaving and PK treatment, were compared. Using the same imaging parameters, both methods achieved notable FISH signals (Fig. 2). The IOD ratio of positive cells to background signal was calculated for each group, and did not differ significantly between the two groups (Fig. 2C).

Modified immuno-FISH is effective. The Malat1-b probe was used to detect the expression of malat1 (Fig. 3; labeled red). Using immuno-FISH, malat1 and three different proteins were successfully co-detected. NeuN-positive cells were double labeled with malat1 (green-red merge; Fig. 3A), while cells positive for glial fibrillary acidic protein and ionized calcium binding adaptor molecule 1 (Iba-1) were a single color (green; Fig. 3B and C). These results indicated that Malat1 is expressed in neurons but not in microglia or astrocytes.

Discussion

Immuno-FISH is a reliable method for the double staining of RNA and cellular proteins (10). It is typically used to detect the localization and abundance of target RNA expression at the histological level (9). LncRNAs are among the most abundant classes of non-coding RNAs (12). Many lncRNAs have been implicated in the functioning of the nervous system and development of disease (13). In the present study, a modified protocol of immuno-FISH was developed. Using this protocol, high-quality fluorescent signals and histological data were obtained that provided information about the expression and distribution of lncRNA malat1 and three different proteins in the spinal cord.

Pretreatment of tissue sections is a critical step for obtaining satisfactory fluorescence signals, and this step is modified in different protocols. The purpose of pretreatment is to ensure

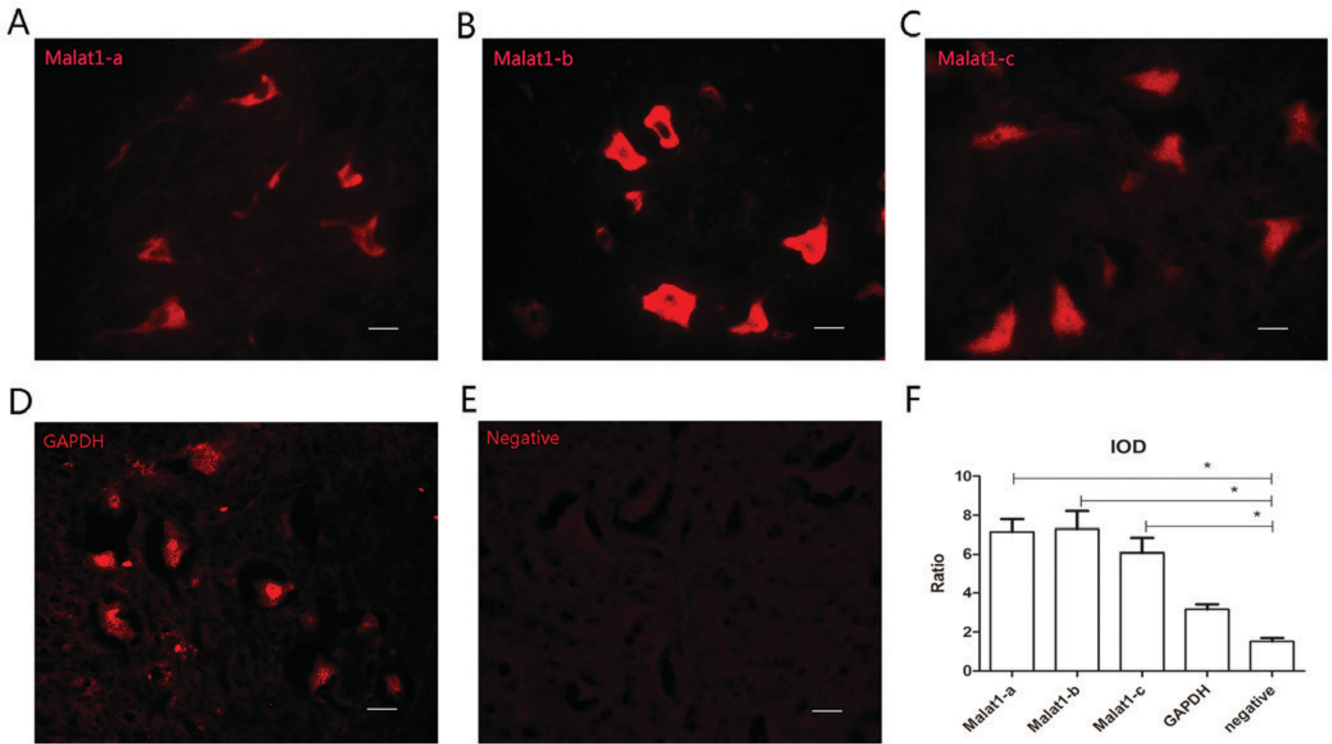

Figure 1. FISH with four probes (Malat1-a, -b and -c and GAPDH) and a negative control (buffer only). Representative images of the (A) Malat1-a, (B) Malat1-b and (C) Malart1-c probes. Representative images of the (D) GAPDH (positive) and (E) negative controls. (F) Mean IOD ratio of each group. Scale bar, 20 μm; magnification, x100. Data are presented as the mean ± standard deviation. *P<0.05 as indicated. FISH, fluorescence *in situ* hybridization; Malat, metastasis-associated lung adenocarcinoma transcript; IOD, integrated optical density.

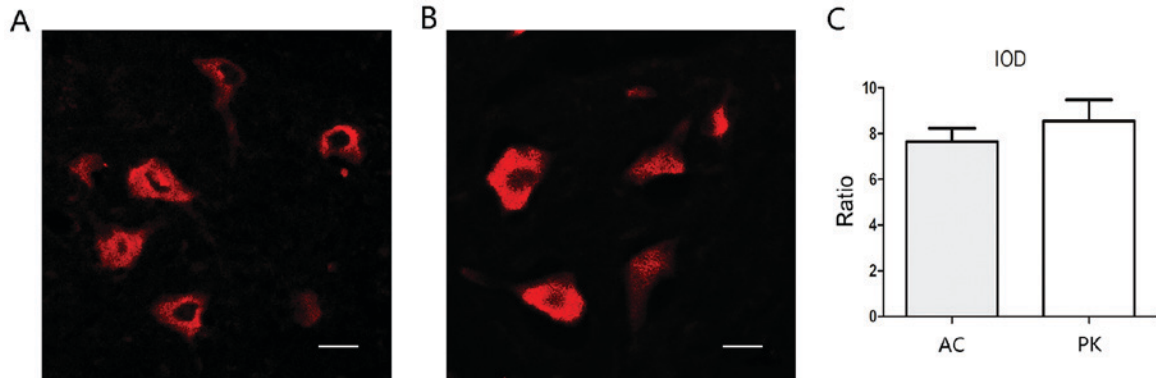

Figure 2. Comparison between autoclaving and PK treatment for antigen retrieval. Representative sections that underwent antigen retrieval by (A) autoclaving and (B) PK treatment. (C) Mean IOD ratio of each group. Scale bar, 20 μm; magnification, x400. Data are presented as the mean ± standard deviation. PK, proteinase K; IOD, integrated optical density; AC, autoclaving.

signal specificity and minimize non-specific background signals (14,15). Acetylation, which negatively charges sections to reduce the adsorption of negatively-charged probes, is widely used as a pretreatment step to reduce background staining (10,16,17). However, in some protocols, acetylation has been reported as a dispensable step (10,18). As such, in the present study, sections were not treated with acetylation, which simplified the protocol and saved time.

In traditional RNA FISH procedures, PK treatment may be used to improve sensitivity, as it denatures many proteins in the membrane and within RNA-protein complexes, making it easier for the probe to cross membranes and bind to target RNA (9). However, previous results have indicated that PK may also denature proteins of interest, which makes it incompatible with immunofluorescence (9). In the present study, sodium citrate-based antigen unmasking and autoclaving were used for antigen retrieval, and high-quality FISH and immunofluorescence signals were obtained. High temperatures may also denature membrane proteins and RNA-protein complexes, unmasking the antigenic sites of target proteins and thus improving antibody-antigen reactions (19). In addition, high pressure prevents solutions from boiling, which otherwise causes tissue sections to peel away from slides.

The reagents used in the current protocol were readily available, relatively non-toxic and inexpensive. Researchers may prepare the antigen unmasking, prehybridization, and

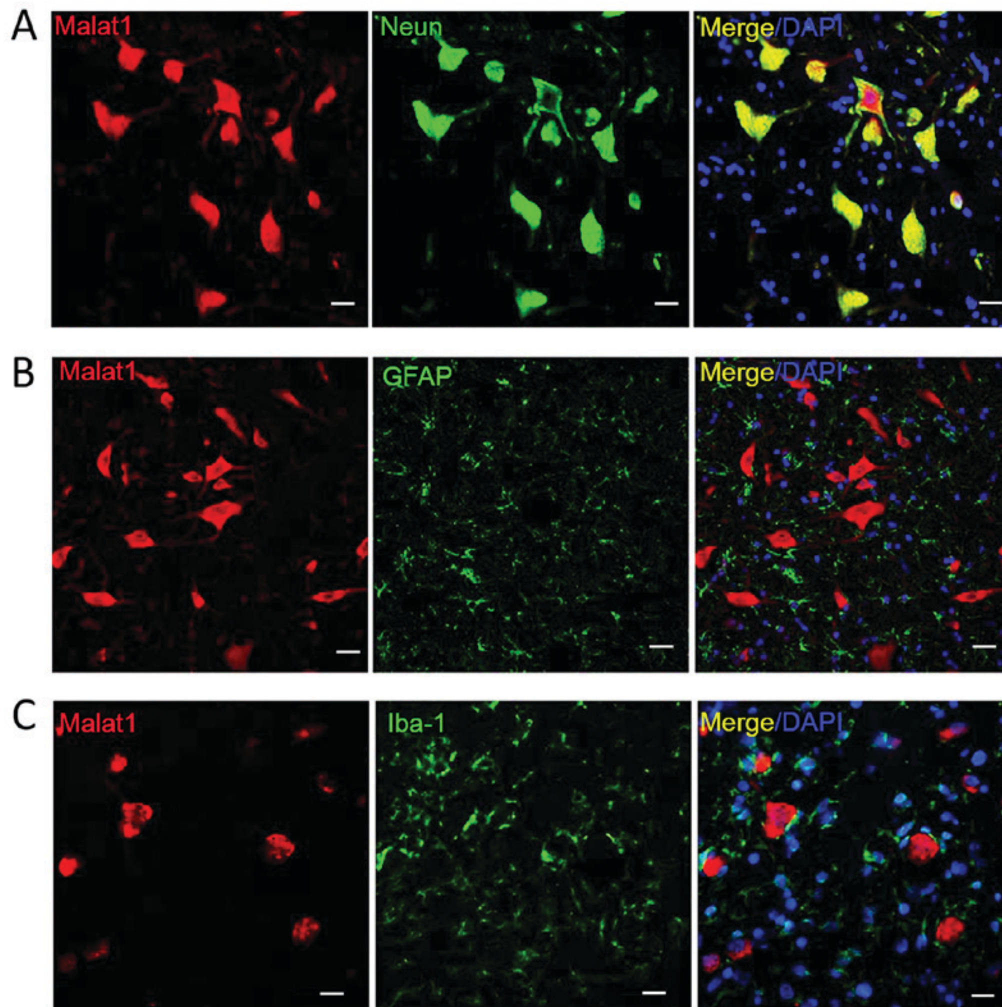

Figure 3. Double labeling for Malat1 and protein markers. Co-detection of Malat1 with (A) NeuN, (B) GFAP and (C) Iba-1. Only NeuN-positive cells were double labeled. Scale bar, 20 μm, magnification, x100. Malat, metastasis-associated lung adenocarcinoma transcript; NeuN, neuronal nuclei; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adaptor molecule 1; DAPI, 4',6-diamidino-2-phenylindole.

hybridization buffers using common reagents following the simple formulas listed above. Other reported hybridization buffer solutions include lauroyl sarcosine (18), Denhardt's solution, transfer RNA or dithiothreitol (9), though these may be difficult to obtain and/or store.

Another advantage of the current protocol was that it was time-efficient. All of the steps undertaken in the present study were performed within 24-32 h. The variation in the time required to complete the protocol was mainly due to the antibodies used for immunofluorescence, as different antigen-antibody reactions required different incubation periods. For instance, it was possible to label NeuN in 2 h, whereas Iba-1 required >6 h at 4˚C. Thus, an overnight reaction at 4˚C is preferred in the majority of immunohistochemistry procedures (3,9,20). In instances where the protocol is applied using other antibodies and the antibody-antigen reaction is not satisfactory, longer reaction times at 4˚C should be considered.

Malat1 is an abundant lncRNA that has been demonstrated to regulate the progression of many diseases (4). In the present study, the distribution of malat1 in the spinal cord of rats was assessed using a simple immuno-FISH protocol, and it was demonstrated that malat1 was expressed in neurons but not in microglia or astrocytes. Despite the lack of a signal amplification method, satisfactory fluorescence signals were obtained. However, the abundance of some lncRNAs is low, for example BACE1-AS and lnc-DC (21,22), and in these cases signal amplification treatments, including tyramide amplification and specially designed riboprobes, may be necessary to obtain detectable fluorescence.

In conclusion, the present study performed a simple immuno-FISH protocol for the detection of malat1 lncRNA and protein markers of neurons, microglia and astrocytes in frozen spinal cord sections of rats. Advantages of the modified immune-FISH protocol include the ready availability of reagents and general speed of the method. This protocol may be adapted for other tissues and RNAs, and may also be used for FISH or immunofluorescence alone.

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