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Original Article

An optimized protocol for whole mount in situ hybridization of mouse brain

Wei Wu, Bingmin Luo, Zhongju Xiao

Department of Physiology, School of Basic Medical Science, Southern Medical University, Key Laboratory of Psychiatric Disorders of Guangdong Province, Guangzhou 510515, PR China

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Abstract: In this study, we describe an optimized protocol of whole mount in situ hybridization (WMISH) which is a preferred method for transcript distributions detection in whole embryos, tissues and organs, for the detection of c-fos mRNA in fresh frozen brain tissue. Therefore, critical steps of WMISH were optimized including proteinase K digestion time, composition of hybridization buffer as well as durations of incubation and washing steps. The expression of c-fos mRNA and protein was detected in mouse brains with the modified WMISH protocol. As a result, the expression patterns of c-fos mRNA and protein in mouse brains were successfully detected with great specificity and low background signal.

Keywords: Whole mount in situ hybridization, c-fos mRNA, mouse brain

Introduction

Since in situ hybridization (ISH) technique was applied as a means of the detection of specific RNA or DNA sequences in the cytological preparations, a lot of researches and technological breakthroughs have promoted the application of ISH to a broader range [1]. The ISH technique not only processes the ability of precise temporal and spatial localization to target RNA by using complementary RNA (cRNA) probes, which also named as antisense, in the hybridization with mRNA in target tissue, but also can identify different classes of RNA that visualized inside cells. In addition, ISH can be applied for the spatial and temporal distribution analysis of transcripts in the tissues, quantitation of gene expression levels or copy number and tracking the physical location of mRNAs or chromosomal segments inside the cell nucleus [2-4]. All of these confirm that ISH is a useful method for biological study.

Whole mount in situ hybridization (WMISH) was first applied in vertebrate embryos in 1990s [5]. After that it was successfully applied for the detection of expression patterns of temporally and spatially restricted gene in the researches focusing on the embryonic development of various species, such as imaginal discs, salivary glands, testes, Xenopus, zebrafish, cow, mouse, etc. [6], as WMISH can detect the expression patterns of genes in tissues or embryos and analysis the position and intensity of mRNA expression with high sensitivity and specificity [7].

Meanwhile, the combination of ISH and immunohistochemistry (IHC) which has the advantage in the detection of specific brain proteins, can preserve anatomical construction and characterize distinct neuronal populations, verify the expression pattern of genes and detect the molecule expression in many experimental models. Additionally, this combination method can also visualize multiple targets located in one or different subcellular compartments [8, 9].

However, even though the combination of ISH with IHC is a common method now, in some cases, they are not compatible. Therefore, we describe here an optimized protocol for c-fos mRNA and protein in whole mouse brains.

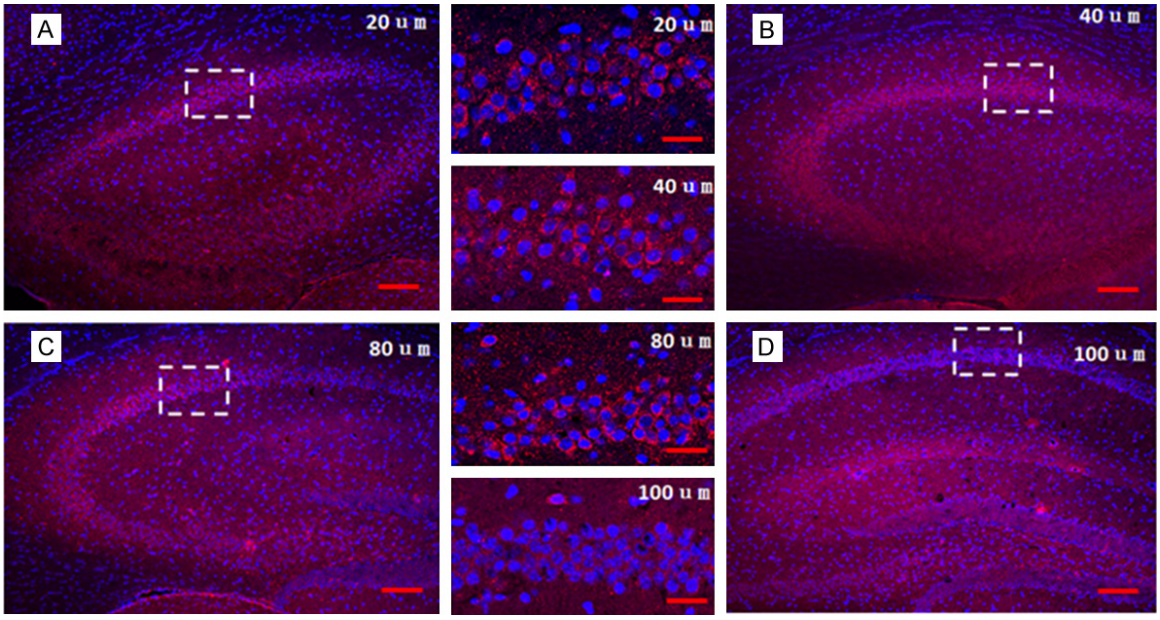

Figure 1. Fluorescence in situ hybridization for c-fos mRNA in hippocampus. (A-D) show the c-fos mRNA expression of 20 μm, 40 μm, 80 μm, and 100 μm, respectively. The middle is the enlargement of the white box. Blue represents the cell nucleus stained by DIPA and red represents c-fos mRNA. Scale bar = 100 μm.

Materials and methods

General

All animal procedures were approved by the Animal Care and Use Committee of Southern Medical University and applicable guidelines for the care and use of laboratory animals were followed. Female C57BL/6J mice aged 5-7 weeks were purchased from the Experimental Animal Center of Southern Medical University, Guangzhou, China. RNase-free or DEPC-treated water was adopted for all the buffers used for the ISH procedures and after ISH, the autoclaved double-distilled water sterilized by filtering is suggested.

Tissue preparation

The process is as follows: 1) sacrifice mice by anesthesia with an overdose intraperitoneal injection of pentobarbital sodium (15 mg/kg) and the mice were transcardially perfused with normal saline and fixative (4% paraformaldehyde in 0.1M PB, pH7.4) in the mice brains; 2) take out the brains and post-fix in 4% paraformaldehyde for 24 h at 4°C; 3) after fixation, wash samples three times in PBS; 4) brains were cut to 2-3 mm thickness in a coronal plane in cold DEPC-treated PBS; 5) wash with 50% methyl alcohol in PBST (PBS with 0.1% Tween 20) for 5 min; 6) wash with 100% methyl alcohol in PBST for 5 min; 6) fix in fresh 100% methyl alcohol and store at -20°C.

WMISH for mouse brain

The process is as follows: 1) rehydrate the brains by successive washes in a series of methanol/PBST solutions (75% methanol in PBST, 50% methanol in PBST and then 25% methanol in PBST) for 5-10 minutes in each concentration; 2) wash with PBST three times, each time for 5 min; 3) replace the last PBST wash solution with 10 μg/mL proteinase K (E00491, Fermentas) in PBST for 20-30 min. Incubation time needs to be optimized by each researcher; 4) rinse briefly in PBST; 5) rinse the samples with 0.1% Tween 20 in 4% PFA-DEPC-treated PBS solution briefly; 6) re-fix the samples 20 min with 0.1% Tween 20 in 4% PFA-DEPC-treated PBS solution at RT; 7) wash with PBST twice, each time for 10 min; 8) incubate with hybridization mix at 60°C for 1 h; 9) replace the hybridization mix with 300-500 μL digoxigenin labeled RNA probe (MK1055, Boster); 10) incubate at 60°C overnight.

Post-hybridization washes

The process is as follows: 1) replace the hybridization buffer with warmed washing buffer

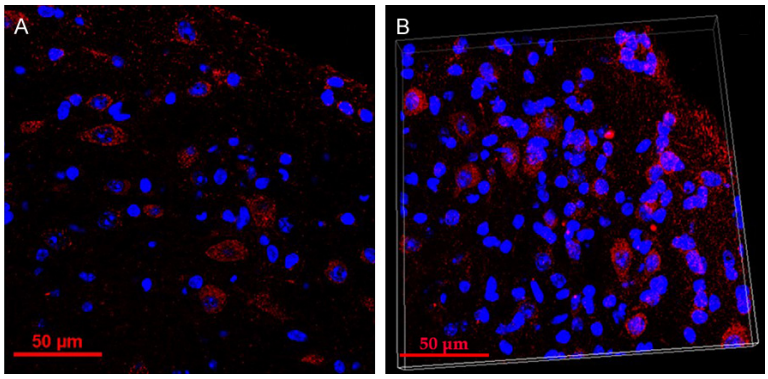

Figure 2. Representative fluorescence in situ hybridization results. (A) Representative confocal image from 20 μm sections showing c-fos mRNA labeling in auditory cortex, Scale bar = 50 μm. (B) Three-D rendering of a fluorescence ISH-processed whole-mouse brain imaged by light-sheet fluorescence microscopy. Width: 210.27 μm, height: 210.27 μm, depth: 18.00 μm.

(50% formamide, 2X SSC buffer) at 60°C and wash with the washing buffer at 60°C twice, each time for 30 min; 2) wash with 2X SSC buffer at 60°C twice, each time for 20 min; 3) wash with 0.2X SSC buffer at 60°C twice, each time for 20 min; 4) wash with PBST twice at room temperature, each time for 50 min; 5) coronal sections (20 μm thickness) were cut by freezing microtome after overnight immersed in 30% sucrose for cryoprotection.

Detection and visualization

The process is as follows: 1) sections were washed with 0.01M PBST twice, 5 min each rinse; 2) incubate sections with blocking buffer, at least 1 h; 3) add biotin peroxidase conjugated mouse anti-digoxigenin Fab fragments (100 μL per section), and incubate for 1 days at 4°C; 5) wash sections in 1X PBST at room temperature 3 times, each time for 10 min; 5) add 100-200 μL of the appropriate SABC-cy3 secondary antibody to the sections, and incubate 2 h at room temperature or 4°C overnight; 6) wash sections in 1x PBST at room temperature 3 times, each time for 10 min.

Immunohistochemistry (IHC) after ISH detection

The process is as follows: 1) to block nonspecific binding, add 5% normal goat serum on each slide and incubate for 2 h at room temperature; 2) incubate sections with the fos antibody (rabbit anti-fos polyclonal IgG, SC-52, Santa Cruz, 500X in normal goat serum) overnight at 4°C; 3) wash sections in 1X PBS at room temperature 3 times, each time for 10 min; 4) incubate with the goat anti- rabbit antibody (500X) for 2 h at room temperature; 5) after three rounds of washing in 0.01M PBS, the sections were mounted and stored at 4°C for further analysis.

Results

Effects of fluorescence ISH for c-fos mRNA in hippocampus with different the section thickness

As shown in **Figure 1**, different thickness of froze sections (20 μm, 40 μm, 80 μm, 100 μm) were performed fluorescence ISH for c-fos mRNA in hippocampus, and the result showed that 20 μm section is better for detecting hybridization signals as the cell nuclei was clear and the fluorescence intensity of c-fos mRNA was proper with lowest background signal.

The representative fluorescence in situ hybridization results

Representative confocal image from 20 μm sections showing c-fos mRNA labeling in auditory cortex and three-D rendering of a fluorescence ISH-processed whole-mouse brain imaged by light-sheet fluorescence microscopy were shown in **Figure 2**.

The influence of different times digested with proteinase K to the results of double labeling

As shown in **Figure 3**, the whole-mouse brain tissues were treated with proteinase K for 15, 30, 45 and 60 min, respectively, only the section which was treated for 30 min showed c-fos protein + neurons, c-fos mRNA + neurons and representative neurons co-expressing both transcripts of interest simultaneously. In the section that treated for 15 min, the fluorescence intensity is not strong enough, especially for the c-fos mRNA labeling, while in the section that treated for 15 min, the cell nuclei were damaged meanwhile, the fluorescence intensity of c-fos protein was degraded to pieces and fluorescence intensity of c-fos mRNA was too

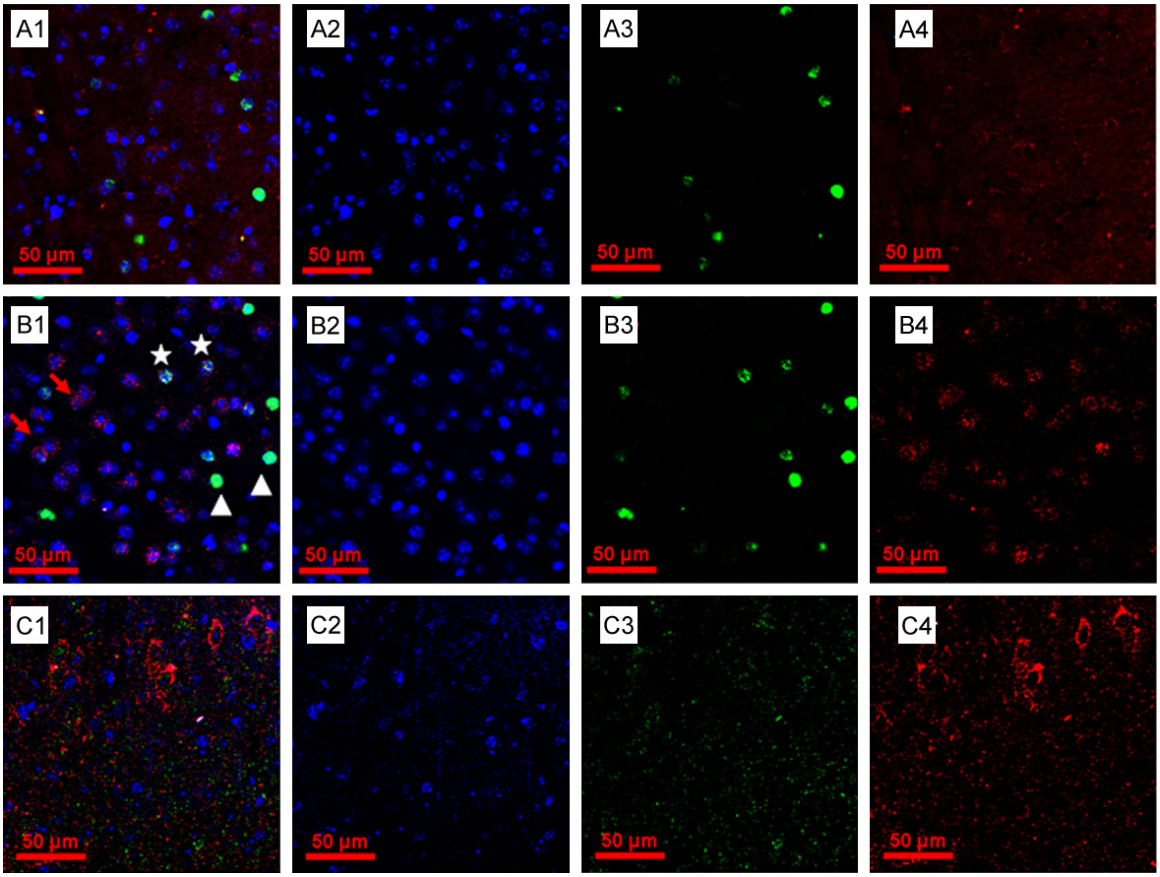

Figure 3. The influence of different times digested with proteinase K to the results of double labeling for c-fos protein (green) and mRNA (red) in mouse auditory cortex. (A1-A4) show the whole-mouse brain digested with proteinase K for 15 min; (B1-B4) show the whole-mouse brain digested with proteinase K for 30 min; (C1-C4) show the whole-mouse brain digested with proteinase K for 45 min; (A2, B2, C2) cells stained with DAPI to identify healthy nuclei (blue); (A3, B3, C3) and (A4, B4, C4) representative images showing green (c-fos protein), red (c-fos mRNA); (A1, B1, C1) the merge images of the (A1-A4, B1-B4, C1-C4), respectively. The arrowheads point to c-fos protein + neurons; the arrows point to c-fos mRNA + neurons; and asterisks show representative neurons co-expressing both transcripts of interest. Scale bar = 50 μm.

strong. Additionally, the 10 μg/Ml is the most proper treatment concentration.

Discussion

WMISH has been widely applied in the spatial and temporal order of gene expression and the establishment of the profile of gene expression pattern [10-13]. Considering the structural features of the brain tissue limits the application of conventional WMISH protocol to some extent, we optimized WMISF for c-fos mRNA and protein detection in whole mouse brains, including the fixation and post-fixation methods, proteinase K digestion time, the section thickness, dying method, composition of hybridization buffer as well as durations of incubation and washing steps. As the conventional protocol has been confirmed by thousands of scholars, all the data in this study are intercompared without the control group.

The probe penetration can be affected by the thickness of samples and the interfere from surrounding layer, which need to pay a particular attention in the process of fixation and permeabilization [14]. In the combination of IHC and ISH, especially, it is necessary to preserve the antigens for antibody binding in detection of protein and RNA simultaneously [15]. As the WMISH analysis of brain tissue in adult mouse was characterized by the large thickness of brain tissue, the probe is not easy to enter the internal structure of the brain tissue [16]. Therefore, first of all, we optimized the fixation and post-fixation methods in the steps of per-

meabilization and high-temperature washing in order to preserve the tissue morphology. Briefly, in the fixation step, the brain tissue was fix in 4% (wt/vol) paraformal-dehyde for 2 h, which could preserve the tissue's morphology by the sufficient penetration of fixative. Subsequently, the brain tissue was treated with increasing concentrations of methanol for better and long-term storage of samples.

When we need to start latter experiment, the tissue was rehydrated following by the permeabilization step by using proteinase K which is more aggressive compared with other agencies used in most fluorescence ISH protocols, such as acetylation solution [11-12]. while some researches even omit the permeabilization step [13]. Most protocols for ISH and fluorescence ISH in brain tissues use proteinase K with the concentration ranging from 20 to 100 μg/mL and treatment time ranging from a few minutes to 1 h. The permeabilization is a vital step in WHISH which allows the probe to penetrate into tissue so as to generate a strong signal. Therefore, the incubation time of proteinase K should be precisely measured. To be specific, on the one hand, the short incubation time will decrease the ratio of probe that penetrated into the tissue (**Figure 3A**); on the other hand, proteinase K over-treatment will damage the brain tissues leading to the gradual disintegration of tissues in remaining steps (**Figure 3C**). To order to minimize the affecting factors of proteinase K digestion effect and increase the reliability of results, a proper concentration (10 μg/mL) and a suitable incubation time (30 min) were set in our protocol, followed by a 20 min post-fixation step (**Figure 3B**).

After the probe was combined with the target gene, the gradient elution was carried out, and kept in sucrose. Then, froze sections of brain tissue were prepared and hybridization signal were studied. With the comparison of the different thickness of froze sections (20 μm, 40 μm, 80 μm, 100 μm), we found that 20 μm section is better for detecting hybridization signals (**Figure 1**), and can be performed ISH and IHC double staining (**Figures 2**, **3**). Similar to immunohistochemistry, there are two methods for dyeing process in fluorescence ISH: stick section and free-floating section. The former is that the slices are pasted on the slide glass to be stained by immunohistochemistry [17]; the latter is that slices are floated in a solution container (such as 24 well plates) to be dyed [18]. In our studies, we found that the free-floating section of brain tissue in fluorescence ISH has high sensitivity and good repeatability after hybridization.

After the hybridization, the needed brain sections were selected for the next step, and the other brain sections were stored at -20°C. Therefore, to a certain extent, the use of experimental reagents is reduced, and experimental funds and experiment time was saved. For example, the 3 mm thick of brain tissues in fluorescence ISH only uses 300 μL of digoxigenin labeled oligonucleotide probe mixture, and its dosage is greatly reduced compared with conventional method [19-21]. Furthermore, the experimental steps before hybridization were shortened to avoid the pollution.

In this study, these experimental steps such as the digestion time of proteinase K, temperature control, post-hybridization washing, kept in sucrose and slice thickness adjustment were optimized. The procedure was simplified, the experimental cost was reduced, and the color rendering specificity was improved. A more practical method of WMISH technique of adult mouse brain was successfully established. Detection and analysis of signals throughout the brain can be used for maintaining morphology and histological examination.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhongju Xiao, Department of Physiology, School of Basic Medical Science, Southern Medical University, Key Laboratory of Psychiatric Disorders of Guangdong Province, No.1023-1063 Shatai South Road, Baiyun District, Guangzhou 510515, PR China. Tel: +86-18620848059; E-mail: xiaozj@fimmu.com

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