

Optimized Protocol of Methanol Treatment for Immunofluorescent Staining in Fixed Brain Slices

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Abstract: We optimized methanol treatment in paraformaldehyde-fixed slices for immunofluorescent staining of ependymal basal bodies in brain ventricles. As 100% methanol induced severe deformations to the slices (including rolling and folding over), we tried to decrease methanol concentration. We found that 33.3% to 75% methanol could result in ideal immunostaining of basal bodies without inducing obvious deformations. Instead of treating slices at -20°C (without proper cryoprotection measurements) as suggested in previous studies, we carried out methanol treatment at room temperature. Our modified protocol can not only raise immunostaining efficiency in tissue slices, it may also prevent potential freezing damages to the samples.

Key Words: brain ventricle, cilia, γ -tubulin, β -tubulin, immunohistochemistry

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Immunohistochemical staining efficiency largely depends on tissue processing.^{1–4} As a tissue processing reagent, methanol has been widely used to fix or permeabilize cultured cells.^{5–8} It was also used to treat tissue blocks and slices.^{9,10} To achieve the best immunostaining, cells need to be treated with 100% methanol at -20°C for 10 to 20 minutes.^{10–12} We noticed that none of these studies or protocols adopted proper cryoprotection measurements. Without effective cryoprotection, treatment at -20°C might bring freezing damages to biochemical identities and/or morphologic features of the cells.³ These morphologic and biochemical alterations may result in

inconsistent immunostaining results,¹³ as suggested in another study.¹⁴

In the present study, we checked the effects of methanol treatment on paraformaldehyde-fixed brain slices before immunofluorescent staining. To our surprise, 100% methanol would result in severe deformations to the slices including rolling and folding over. We then tested various concentrations of methanol to the slices in different conditions.

MATERIALS AND METHODS

A total of 12 C57/Bl6 male mice (Jackson Laboratory, Bar Harbor, ME) at 6 to 8 weeks were used. The procedures and protocols for all animal studies were approved by Institutional Animal Care and Use Committees of Tongji University, Children's Hospital of Philadelphia, and University of Pennsylvania, in accordance with international guidelines on the ethical use of animals (National Research Council, 1996). After the mice were deeply anesthetized and perfused, the brains were removed and postfixed, and then vibratome slices were cut at $50\ \mu\text{m}$ and collected in 6 serials in PBS as reported previously.¹⁵

The slices were treated with different concentrations of methanol (0%, 33.3%, 50%, 75%, 95%, and 100%) at room temperature (RT) for 30 minutes or at -20°C for 10 minutes. Before methanol treatment at -20°C , slices were immersed in 20% sucrose overnight and then 30% sucrose for 2 days at 4°C , except for control ones. The slices were then permeabilized with 0.3% triton X-100 and blocked with a mixture of 5% normal goat serum and 1% bovine serum albumin before free-floating immunostaining.

For double immunofluorescent staining, we incubated the slices with a mouse monoclonal antibody against type IV β -tubulin and rabbit polyclonal to γ -tubulin (Both at 1:500 in PBS; AbCam USA, Cambridge, MA). To visualize the above primary antibodies, we used Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Both at 1:250 in PBS; Life Technology, Grand Island, NY), respectively. The nuclear dye Hoechst (Life Technology) was added to the secondary antibody mixture to counterstain the samples. For immunostaining and confocal imaging, we followed the previous protocol.¹⁵

RESULTS

We first checked the effects of 100% methanol at -20°C for 10 minutes on sucrose-protected brain slices.

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To our surprise, this original methanol treatment (Cell Signaling Technology Protocol, Sigma-Aldrich Protocol, Synaptic Systems Protocol) would bring severe deformations to the slices, which was clearly visible to the naked eye. The slices were seen rolling or folding over after the treatment (Fig. 1A). Under higher magnification, the nearby structures in an optical field could not be focused evenly (Fig. 1C). The deformations also occurred in slices that were not immersed in sucrose solutions before treatment at -20°C with pure methanol.

We also tried different concentrations of methanol by diluting it in PBS at -20°C . At 95% of methanol, treated slices exhibited severe deformations similar to that

caused by 100% methanol. When methanol concentration decreased to 75%, one out of a well of slices might show slight rolling. At concentrations of 50% and 33.3%, methanol would not result in any identifiable deformations to the slices.

We then tested whether methanol treatment at RT for 30 minutes could bring deformations to slices. Similarly, methanol under 75% would not result in obvious deformations to the slices (Figs. 1B, D). When methanol concentration reached 95% or 100%, the slices again exhibited severe rolling and folding over.

Finally, we compared immunofluorescent staining efficiency after methanol treatment in different conditions.

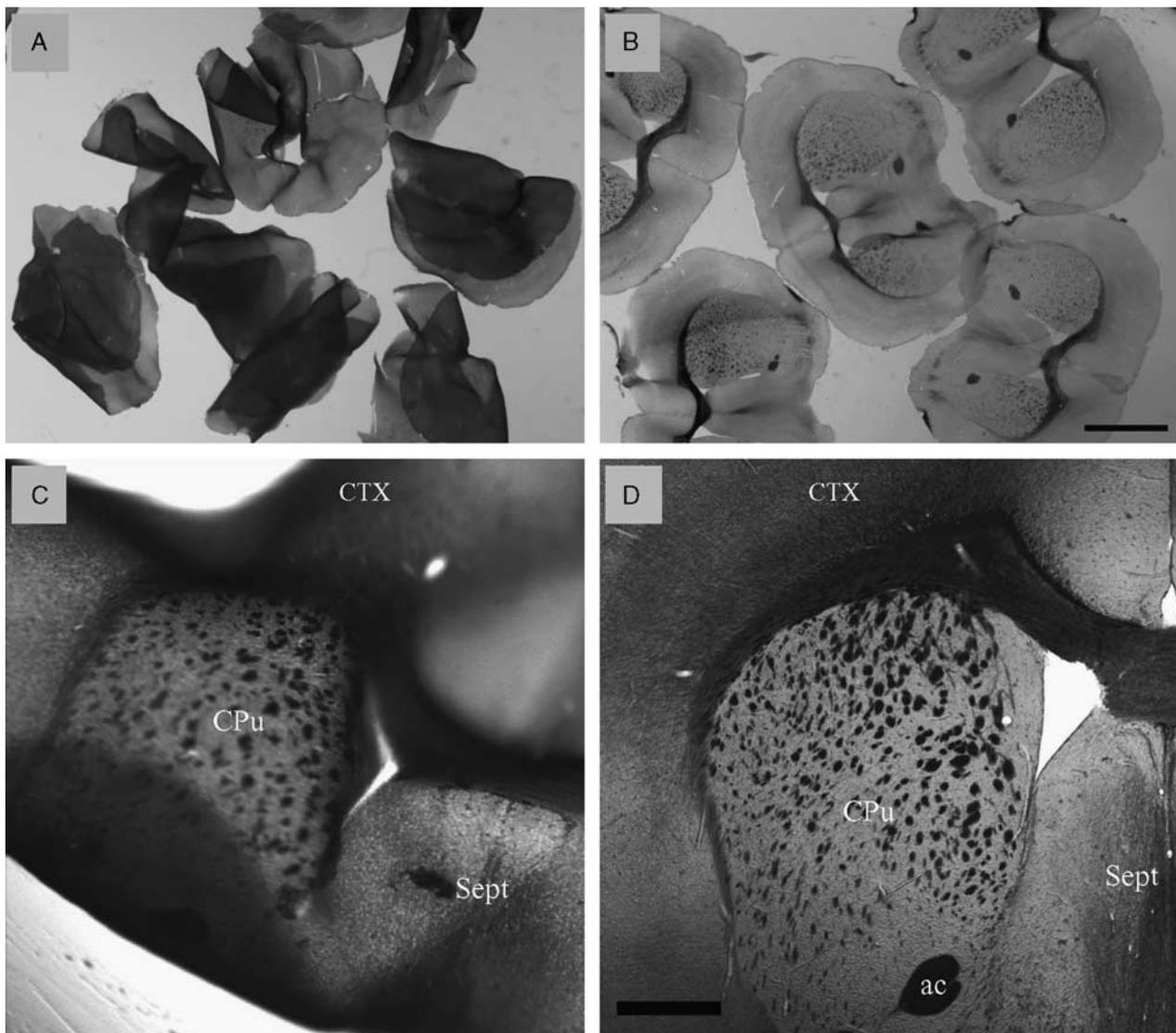


FIGURE 1. Pure methanol induces severe deformations to brain slices. Photographs taken from the slices in methanol solutions at the end of the treatment. (A) Brain slices treated with 100% methanol at -20°C . They were seen obviously folding over, resulting in uneven focus in nearby structures under higher magnification (C). (B) Seventy-five percent of methanol did not cause obvious deformations to the slices. In contrast to (C), nearby structures could be clearly seen at an identical focus in these brain slices (D). ac indicates anterior commissure; CPu, caudatus-putamen; CTX, cortex; Sept, septal region of the cerebrum. Scale bar: A and B, 2.5 mm; C and D, 1 mm.

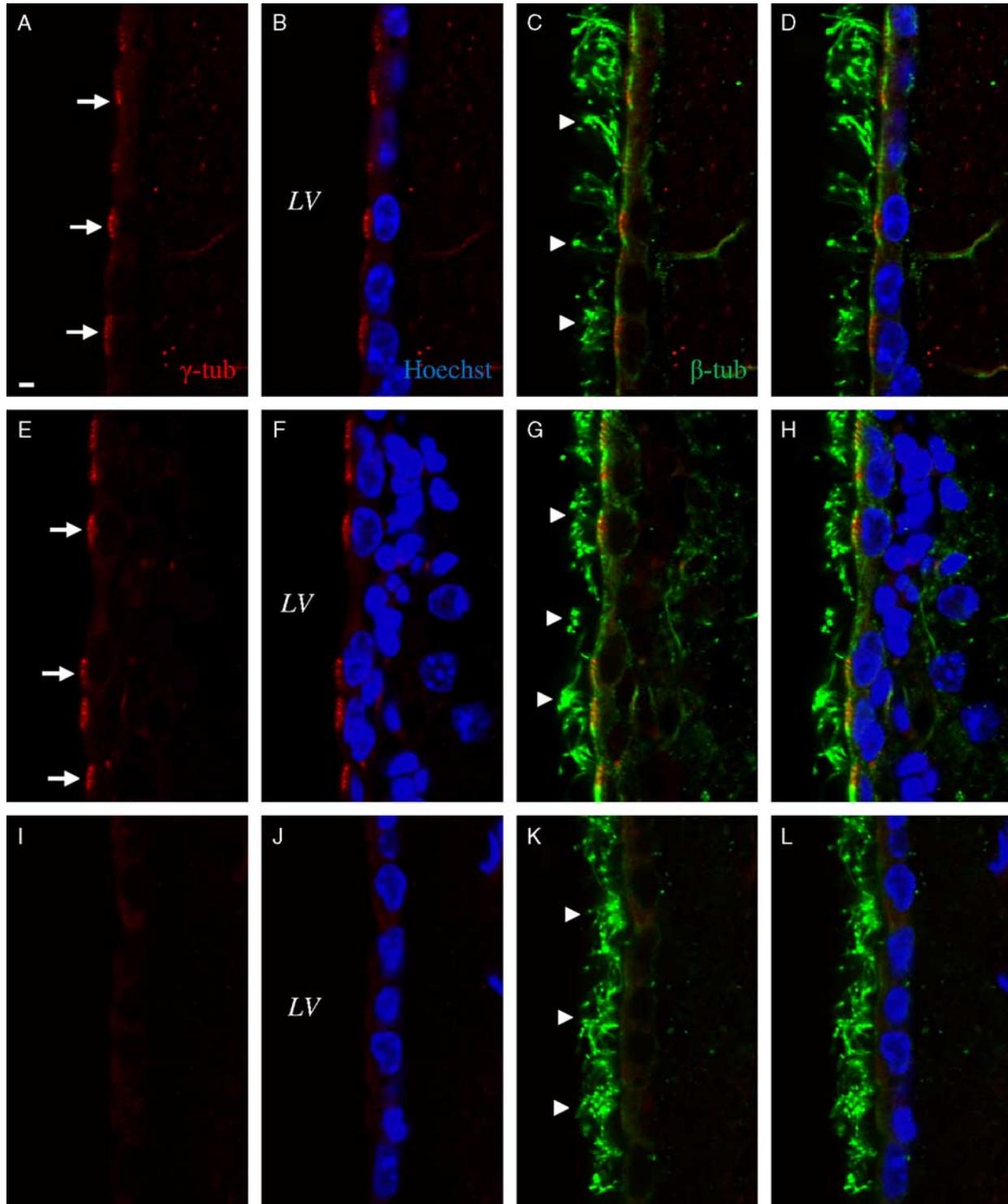


FIGURE 2. Basal body staining could be achieved only when the brain slices were treated with methanol. Basal bodies (red, arrows) were intensively stained by γ -tubulin (γ -tub) antibody at the apical surface of ependymal cells. They were localized exclusively between β -tubulin (β -tub) antibody-stained cilia (green, arrowheads) and Hoechst-counterstained nuclei (blue) of ependymal cells, which are arranged along banks of the lateral ventricle. A–D, Photomicrographs taken from a slice treated with 100% methanol at -20°C . E–H, Photomicrographs from an adjacent slice treated with 50% methanol at room temperature. I–L, Photomicrographs from a slice without methanol treatment. LV indicates lateral ventricle. Scale bar: A–L, 2 μm .

The rabbit polyclonal antibody against γ -tubulin was used to stain out the basal bodies of ependymal cells, as shown previously.¹⁶ Before immunostaining, we applied different concentration of methanol to regular vibratome slices at RT for 30 minutes or to sucrose-protected slices at -20°C for 10 minutes. From the concentration of 33.3% to 100%, methanol treatment at both temperature conditions resulted in a similar immunofluorescent staining pattern of basal bodies in these brain slices (Figs. 2A–H). As shown in Figure 2, clusters of basal bodies (red, arrows) were intensively stained. They were exclusively localized to the apical surface of the ependymal cells, between cilia (green, arrowheads) stained by type IV β -tubulin and Hoechst-counterstained nuclei (blue). Without methanol treatment, γ -tubulin antibody would not stain out any basal bodies (Figs. 2I–L).

DISCUSSION

In a previous study¹⁶ and the present report, we showed that methanol treatment is necessary for immunofluorescent and immunogold staining of ependymal basal bodies in paraformaldehyde-fixed brain slices. With the present optimization, treatment with methanol at the concentration from 33.3% to 75% can result in ideal staining of basal bodies without inducing obvious deformations to the brain slices. Our modification to methanol treatment protocol can largely raise immunostaining efficiency in fixed brain slices. As original methanol treatment^{5–8} was usually performed in cultured cells, deformations we showed here have not been reported. Noguchi et al⁹ documented that methanol would not cause obvious morphologic changes, compared with other fixatives.⁹ In fact, the authors applied methanol to tissue blocks,⁹ not directly to tissue slices as we did. With the present findings, we suggest that pure methanol should be avoided for the treatment of tissue slices.

It has been reported that methanol alone can be used as an ideal fixative.⁹ Because of its low molecular weight and fast tissue penetration ability, methanol treatment can result in best DNA/RNA preservation with less tissue shrinkage.⁹ However, Hoetelmans et al⁵ showed an obvious loss in integrity of intracellular structure and proteins after fixation with acetone or methanol, compared with paraformaldehyde fixation. They suggested that methanol should be used in addition to formalin/paraformaldehyde fixation to get the best preservation effects,⁵ with which the present protocol is highly in consistence. As our optimized protocol was carried out at RT, there will be no freezing damages to the samples induced by treatment at -20°C . At confocal microscopic level, histochemical and/or morphologic changes due to freezing might not be obvious

enough. At electron microscopic level, however, ultrastructural alterations from freezing may become more prominent. Accordingly, methanol treatment has to be performed at RT for immunogold staining of ependymal basal bodies.¹⁶

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