

Is optimal cutting temperature compound essential embedding solution treatment to cryo-sectioning of brain tissue?

Hye Kyung Baek, Ji Ae Song, Sun Shin Yi*

Department of Biomedical Laboratory Science, College of Medical Sciences, Soonchunhyang University, Asan 31538, Korea

(Received: March 14, 2016; Revised: April 13, 2016; Accepted: April 29, 2016)

Abstract : We tested a set of conditions for obtaining optimal tissue quality in preparation for histology in samples of mouse brain. C57BL/6J mice were sacrificed and perfused with 4% paraformaldehyde, after which the brains were removed and dehydrated in 30% sucrose solution. The brains were then divided into four groups according to freezing temperature and usage of optimal cutting temperature (OCT) compound. Next, we stained the sectioned brain tissues with Harris hematoxylin and eosin Y and immunohistochemistry was performed for doublecortin. The best quality tissue was obtained at -25°C and by not embedding with the OCT compound. When frozen at -25°C , the embedded tissue was significantly damaged by crystals, while at -80°C there were no meaningful differences between qualities of embedded- and non-embedded tissues. Overall, we identified a set of conditions to obtain quality frozen brain sections. Our developed protocol will help resolve matters associated with damage caused to sectioned brain tissue by crystal formation during freezing.

Keywords : brain tissue, cryosection, freezing temperature, optimal cutting temperature compound, tissue quality

Introduction

To analyze the experiment results correctly and not to waste the time and efforts, it is very important to collect high quality tissue samples from animal experiment which was performed for several months. Because the number of tissue sample obtained from an animal may be limiting, and if once there is anything wrong in tissue processing, it could affect to subsequent experiments including tissue staining, western blotting, immunohistochemistry (IHC) or polymerase chain reaction (PCR). Therefore all experimenters are required to be prudent and concentrate to all processes of perfusion, fixation and cryo-microsection of tissues.

Especially in case of IHC for brain tissue, use of floating tissues from a frozen section is the practiced method. However if the tissue condition is inferior, the experiments depending on the tissue samples will suffer as obtaining a proper IHC result that would be impractical. Any data obtained from such poor samples would also be suspect and could lead to improper interpretation of the data. For example, poor quality brain tissue staining with a doublecortin (DCX) antibody which on a good sample looks like small spot with thin branches would be impossible to analyze if there are, for example, gaps or holes in the sample. Thus, a successful IHC experiment depends on quality preserved tissue samples.

Most laboratories follow this general protocol to obtain frozen section: perfuse with 4% paraformaldehyde (PFA) in phosphate buffer (PB) after sacrificing animals, then remove the brain and fix for 12 to 24 h; dehydrate the whole brain in hypertonic solution for several days and freeze as embedded with optimal cutting temperature (OCT) compound; finally, immerse sectioned tissues in cryoprotectant solution at -20°C [1, 6]. Prudence and caution are needed for every steps. Particularly, there are optimal ranges in fixation, pH levels of dehydration or preservation solution, solution concentration, immersing period and temperature, and there are small differences that every experimenters may have in the protocol [2, 3].

Mishandling a brain tissue may lead to damaging holes in the tissue which were maybe incurred by crystals, minute ice particles, making it difficult to obtain clear, reliable staining results. To resolve the matter and collect better quality of tissue samples, we considered various conditions including freezing temperature and OCT compound using or not for cryosection of brain tissue. This research will show how each factors affect to tissue quality, contrary to common belief.

Materials and Methods

Grouping and condition establishment

Twelve mouse brains were divided into 4 groups accord-

*Corresponding author

Tel: +82-41-530-4873, Fax: +82-41-530-3085

E-mail: admiral96@sch.ac.kr

Table 1. Conditions for cryosection of brain tissues

Group	Freezing temperature	OCT compound
1	-25°C	embedded
2		non-embedded
3	-80°C	embedded
4		non-embedded

Two different conditions were applied for temperature and embedding. Other conditions were equally applied; this included perfusion flow rate, immersion period in fixative and hypertonic solution, and storage temperature. All the brain samples were sectioned and preserved in cryoprotectant solution at -25°C.

ing to 2 conditions: freezing temperature and OCT compound use or not. All brain tissues were frozen for 1 h, just before cryocutting. Conditions for each group are demonstrated in Table 1. Twelve C57BL/6 male mice were sacrificed. The mice were 8 weeks old and had an average weight of 22.0 g (approval No. SCH15-0001).

Perfusion, removal, fixation and dehydration of brain

Using a 24-G needle, the animals were perfused (Miniplus 3; Gilson, USA) with saline followed by 4% PFA in 0.1 M PB (pH 7.35). All groups were perfused at 40 rpm (flow rate: 3.5 mL/min; 2-Stop Tygon E-Lab Tubing 1.02 mm; United States Plastic, USA). The brains were then removed carefully and post-fixed for 18 h. Finally, the whole brains were immersed in 30% sucrose in 0.1 M PB (pH 7.35) and dehydrated for 3 days at 4°C.

Embedding and cryosection

Half of dehydrated brains were completely embedded in OCT compound; the others were not embedded, just affixed one facet. All brains were frozen at -20°C or -80°C for 1 h, immediately sectioned at 30 µm and preserved in cryoprotectant at -25°C.

Hematoxylin and eosin (H&E) staining

Floating brain tissues were mounted on MAS-GP type A coated slides (Matsumani Glass, Japan) and dried sufficiently. Hydrated with descending series of ethanol (100%, 95%, 90%, 80% and 70% v/v) and washed briefly, tissue slides were stained with hematoxylin (Harris hematoxylin solution modified; Sigma, USA) for 1 min, followed by eosin Y staining (Sigma) for 7 min. In succession, dehydrated with ascending series of ethanol (70%, 80%, 90%, 95% and 100% v/v), finally the slides were cleared with xylene and mounted with Canada Balsam (Yakuri Pure Chemicals, Japan).

Immunohistochemistry

Frozen sections were washed with PBS and treated with 0.3% hydrogen peroxide (H₂O₂) in PBS for 15 min and subsequently with 5% normal rabbit serum or normal goat serum in PBS for 1 h. After that they were incubated with diluted goat anti-DCX (1 : 1,000; Santa Cruz Biotechnology, USA)

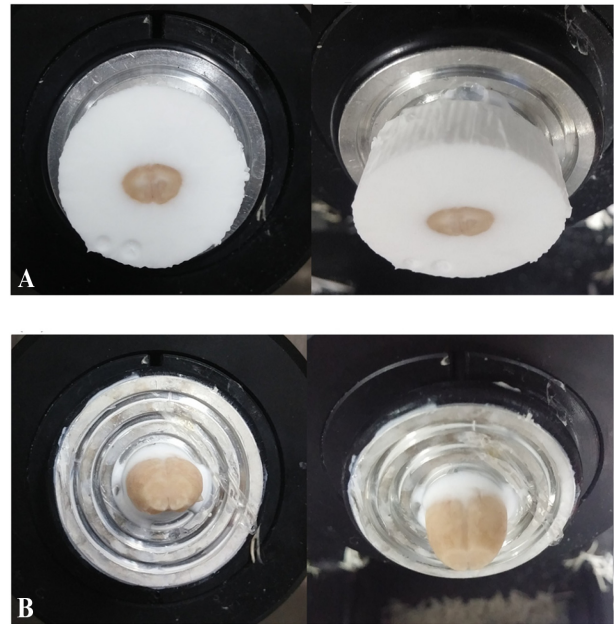


Fig. 1. Brain samples embedded with optimal cutting temperature (OCT) compound or not. (A) The whole brain was embedded with OCT compound and attached on disc after being frozen at either -25°C or -80°C. The embedded brain was fixed and sectioned with OCT compound. The other brain sample was exposed to atmosphere and frozen at -25°C or -80°C and sectioned alone. (B) A small quantity of OCT compound was used for the purpose of fixing the brain on the disc.

in 0.1% (v/v) Triton X-100 (Promega, USA) in PBS (PBST) for overnight at 4°C. After being washed with PBS, the sections were treated with biotinylated anti-goat IgG (1 : 200; Vector Laboratories, USA) in PBST for 2 h, then sequentially with ABC Solution (Vector Laboratories) according to the manufacturer's instructions. The sections were then visualized using 3, 3'-diaminobenzidine tetrachloride (Sigma), in 0.02% H₂O₂ in PBS and were mounted on MAS-GP type A coated slides (Matsumani Glass) and were dried overnight. Finally the sections were dehydrated, then mounted using Canada Balsam (Yakuri Pure Chemicals).

Results

Embedding with OCT compound or not

The processed brains of a group were embedded with OCT compound and other brains were not embedded. Only one cross section of completely embedded brains was exposed at atmosphere, whereas entire surfaces of non-embedded brains were exposed. Embedding solution was only used to attach the brain on disc (Fig. 1A and B).

Tissues embedded with OCT compound or those not embedded

There was a remarkable difference between embedded and non-embedded tissues stained as visualized by H&E stain-

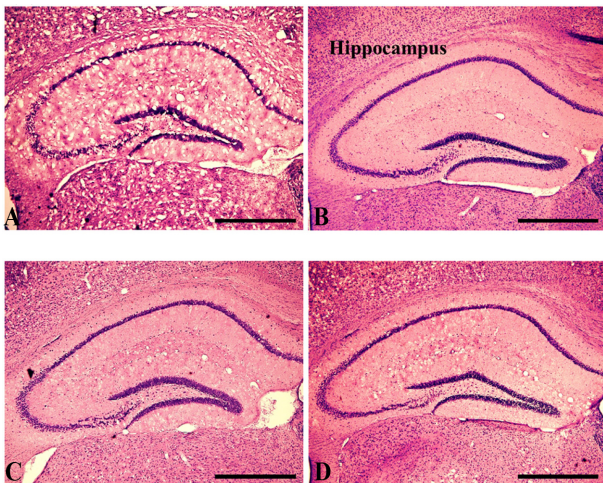


Fig. 2. (A) Hematoxylin and eosin (H&E) staining was performed to assess the overall condition of the sectioned brain tissues. There were severe damaging holes left by ice crystals in embedded and frozen tissue at -25°C and the chromatic figure was motley. (B) Meanwhile, tissue not embedded but frozen at -25°C showed clear, distinct and intact staining. (C and D) In case of being frozen at -80°C , there were no significant differences between embedded or non-embedded tissue samples in terms of staining quality. H&E stain. Scale bars = $100\mu\text{m}$.

ing. Especially at -25°C , OCT embedded tissues were riddled with gaps and holes (probably from crystal formation). In contrast, there were no holes or artifacts observed in non-embedded tissues and they were stained very clearly and vividly (Fig. 2A and B). Tissue quality according to whether the tissue was embedded in OCT or not was different at -25°C . On the contrary, at -80°C the tissue quality and staining results were similar between embedded and non-embedded tissues (Fig. 2C and D).

Difference according to freezing temperature

There was a great difference between -25°C frozen and -80°C frozen brain samples. In case of embedded brains, more clear and intact tissues were obtained from -80°C samples than from the -25°C ones. In comparing the H&E results, the damage was significantly reduced in -80°C frozen brain (Fig. 2A and C). For non-embedded brain, -25°C frozen tissue was more intact than -80°C frozen tissue. Holes and similar damage were reduced in -25°C frozen tissue, and the staining was uniform (Fig. 2B and D).

Application for IHC

Dentate gyrus of hippocampus was observed after IHC with anti-DCX antibody. In embedded -25°C frozen tissue, it was difficult to detect the length and branching of DCX because the damaged holes disrupted the branches of anti-DCX positive areas. As a result, it was problematic to confirm whether neurogenesis was increased or not in the brain areas (Fig. 3A). Meanwhile, non-embedded -25°C frozen tis-

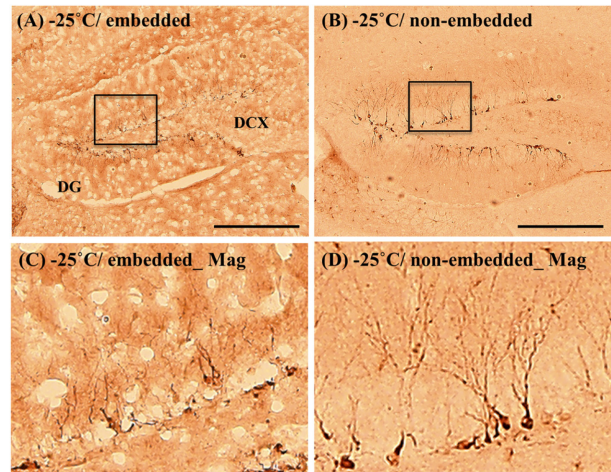


Fig. 3. Immunohistochemistry (IHC) for doublecortin (DCX). For purpose of comparing tissue quality in IHC, each sample was stained with anti-DCX antibody. All the samples were frozen at -25°C . (A) In case of the embedded tissue, analyzing the IHC result was hard because there are many damaging holes from ice crystal. (B) However, non-embedded tissue demonstrated the length, branches and neuroblast clearly a characteristic of the DCX expression. (C and D) The magnified figures of the square parts on A and B. Scale bars = $100\mu\text{m}$. DG, dentate gyrus.

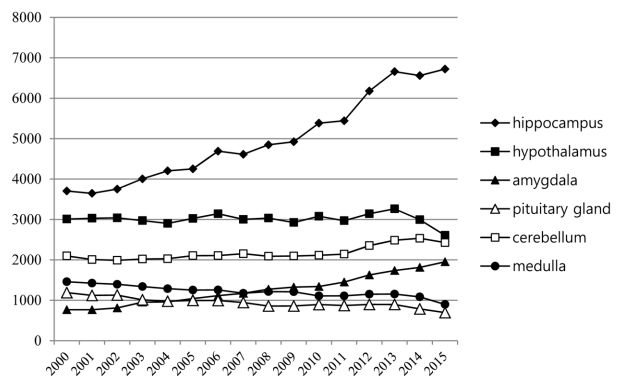


Fig. 4. The number of researches about diverse brain regions. The research rate of hippocampus among various brain regions is increasing every year. Using each name of brain region as keyword, we separately searched the number of researches on National Center for Biotechnology Information (NCBI; USA), and investigated the tendency of brain study by quoting “Result by year” provided from NCBI.

sue showed a very clear background and no damaging holes were visible. Branches of DCX were not impaired and exhibited apparent, therefore, it was possible to examine the length and the number of branches (Fig. 3B).

Discussion

Each laboratory has their own protocols and informal skills and tips, and such all the knowledge and efforts aim to obtain

the best quality of the result. Therefore, obtaining good quality tissue sample is very important in that credibility and value of the research may be increased, moreover efforts and time devoted in the experiment will be saved. Especially, gaining clear and intact tissue sample of brain is basically crucial for observing the various changes by post-experiments including IHC or immunofluorescence.

Damaging holes are generally formed because of crystals occurring in the freezing phase if the dehydration process was not performed properly [3, 7]. Moisture in the tissues should be eliminated after fixation, and if this dehydration process was not adequate, ice particles and crystals will form in the tissue upon freezing. The crystals are removed in subsequent staining steps or conservation in cryoprotectant solution after cryosectioning, leaving only damaging holes. It is sometimes hard to perfectly remove the moisture in core of a whole brain. Therefore it is important to as much as possible prevent the remaining water from having adverse effects on the tissue.

We studied to obtain the best quality of tissue samples by reducing crystals and damaging holes and to develop a protocol for optimal freezing temperature and using OCT compound more effectively. These were under conditions of completing the full dehydration steps.

Primary purpose of using embedding solution is to hold tissues on disc and to make them hard, and most products are mainly considered to satisfy this purpose. Therefore, we did not compare many kinds of OCT compounds because the solutions are thought to consist of almost same ingredients and also show similar effect as working.

All areas of brain showed same aspect with hippocampus region and damage rate was demonstrated focusing on hippocampus. Various regions of brains have studied by many scientists, however, the research rate of hippocampus is dominant and it is rapidly increasing every year. This tendency is indicated in Figure 4. We hope this study to be helpful for more many researchers. In this study, we selected hippocampus and showed it intensively. Also, the region is easily damaged due to its separability at mount process in staining and especially DCX antibody resembling branch needs very clear background for analysis. Therefore, we thought that hippocampus would be proper instance.

The product name of OCT compound we used to embed or attach all brains was not specified, being concerned with adverse effect for the manufacturer. Because our results implied that embedding solution is not always essential and may give tissues some damages.

From our results, good quality tissue was obtained from the -25°C freezing temperature and not having been embedded in OCT. In addition at -25°C freezing temperature, the OCT embedded tissue was significantly more damaged than non-embedded tissue. This is contrary to common belief that OCT would protect a cryopreserved tissue from freezing damage. From this damage, it was hard to analyze the branch length of DCX in the IHC images.

These results were surprising for the OCT treated samples [5]. We suppose that the moisture remaining in exterior and interior of the brain after dehydration was allowed to evaporate if OCT compound was not used, but blocked if OCT compound was present and embedded the whole brain. It was interesting that freezing damage was significantly reduced in non-embedded tissue, whereas embedded tissue was severely affected at -25°C freezing temperature.

At -80°C freezing temperature, there were no meaningful differences between qualities of embedded and non-embedded tissues. It is thought that with a lower freezing temperature, the contraction of tissue becomes stronger and with the increased contraction of OCT at -80°C , the trapped ice crystal could not grow. However, for the quality of the samples, it seems that evaporation of remaining water may be more important than protection from a lowered freezing temperature.

As obtaining good quality of tissue sample is important, so is the convenience of the protocol. And from our results, cryosectioning of non-embedded tissue at -25°C gave the best result. It is also an advantage that by not using OCT, the experimenter can perform the sectioning of the whole brain without having to adjust balance of right and left portion of the brain and non-embedded tissue may be more convenient to balance, because experimenter can observe the whole brain exposed to atmosphere. Of course, if the brain is impaired or if only partial of brain is needed, embedding with OCT compound is essential. In that case, freezing the brain at -80°C would provide a better result than -25°C . It is remarkable that the quality of tissue frozen at -25°C is superior. Generally the freezing temperature of the cryostat machine is hard to set below -30°C , and using isopentane and liquid nitrogen to reach lower temperatures is not only somewhat dangerous but also cumbersome [4].

From our results, the freezing temperature and usage of OCT compound greatly influenced tissue quality. Better results may also be shown by a combination of each of these conditions. Also in addition to the two conditions elaborated in this study, various other conditions may influence tissue quality. These may include perfusion flow rate, fixation temperature and duration, and the concentration of the hypertonic solution.

Our research clearly demonstrated that especially for IHC obtaining a quality tissue sample is required for best quality of data and increased accuracy of analysis. In conclusion, this study provides the methods to avoid tissue damage from ice formation and this improving the quality of the sectioned samples. From our results, an experimenter can also improve their own protocols by employing some of the steps elaborated here and obtain more quality images for histology.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. NRF-2015R1C1A1A02037060).

References

1. **Akagi T, Ishida K, Tohno H, Hanasaka T.** Preservation of ultrastructure and immunoreactivity in cryosections of brain tissue stored in a sucrose-gelatin solution at freezing temperatures. *J Microsc* 2008, **231**, 21-27.
2. **de Graaf IAM, Koster HJ.** Cryopreservation of precision-cut tissue slices for application in drug metabolism research. *Toxicol In Vitro* 2003, **17**, 1-17.
3. **Fischer AH, Jacobson KA, Rose J, Zeller R.** Cryosectioning tissues. *CSH Protoc* 2008, **3**, pdb.prot4991.
4. **Ishii T, Kasama K, Kondo M, Takahashi T.** Cryostat sectioning of formalin-fixed brain: further attempt to improve section quality by previous infiltration with O.C.T. compound. *Tohoku J Exp Med* 1993, **171**, 101-105.
5. **Jennes L.** Immunohistochemical detection of neuropeptides/transmitters in mammalian brain. *Methods Cell Biol* 2013, **113**, 123-147.
6. **Roos MW, Johansson A, Sperber GO.** *In situ* freezing of the rabbit and rat brain. *Cryobiology* 1997, **35**, 187-191.
7. **Slot JW, Geuze HJ.** Cryosectioning and immunolabeling. *Nat Protoc* 2007, **2**, 2480-2491.