Cryoultramicrotomy: Electrostatic Transfer of Dry Ultrathin Frozen Sections on Grids Applied to the Central Nervous System

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Summary. Due to the extreme fragility of ultrathin frozen sections of brain tissue, the cryoultramicrotomy of non-embedded tissue has not been sufficiently used for immunocytochemical studies of the central nervous system. Sections are easily disrupted by the liquid surface tension when a droplet of sucrose is used (method by TOKUYASU, 1973) for their transfer on the grids. Use of silicotungstic acid (TSUJI, 1986) in place of sucrose improved the preservation of the ultrastructure but still could not resolve the difficulty.

This report describes a new procedure for transferring dry ultrathin frozen sections by means of electrostatic attraction induced on the membrane covering the grids. Once attached electrostatically to the membrane, the sections were retained by van der Waals' forces. The dry ultrathin frozen sections obtained from both fresh and fixed brains displayed good preservation of their ultrastructures over a large surface.

This new method which electrostatically transfers dry ultrathin frozen sections, avoiding the use of any liquid, is expected to serve for the immunocytochemical identification of neuronal cell bodies and terminals as well as their neurotransmitters and enzymes in both fresh and fixed brains.

In the last two decades the ultrastructural localization of the neurotransmitters in the central nervous system (CNS) was performed with conventional techniques of immunocytochemistry. Chemical reactions were performed either on slices of fixed and non-embedded tissues ("pre-embedding") or on ultrathin sections of fixed tissues after embedding in a plastic resin ("post-embedding"). In spite of valuable results, these techniques encountered some difficulties such as a lack of deep penetration of antibodies into the tissues (in the case of "pre-embedding") and denatura-

tion of numerous antigens after inclusion in the plastic resin (in the case of "post-embedding").

In order to avoid these difficulties, immunocytochemistry of the CNS was performed also on the ultrathin frozen sections (CELIO et al., 1986; MERIGHI et al., 1989). However, due to the extreme fragility of this type of tissue, the preservation of the sections over large surfaces was unsatisfactory. The frequently used sucrose droplet technique (TOKUYASU, 1973), including some modifications for reducing the surface tension of the liquid (TOKUYASU, 1986), considered as the main cause of the disruption of the sections, has not provided a satisfactory result. When silicotungstic acid was used for the transfer and fixation of ultrathin frozen sections of fresh tissue (TSUJI, 1986), their disruption was reduced but the preservation of the ultrastructure over a large surface of tissue was not obtained.

The present experiment was performed in order to compare the well known procedures of cryoultramicrotomy which could satisfactorily be applied to the CNS. Special attention has been given to the obtainment of ultrathin frozen sections as well as to their transfer on the grids.

In our experimental conditions we succeeded in obtaining dry ultrathin frozen sections from both fresh and fixed pieces of brain, including the diagonal band of Broca (DBB), and transferred nearly all of them on the supporting membrane of the grids by means of simple electrostatic attraction. Once attached to the membrane, the sections remained there due to van der Waals' forces. In general, the ultrastructure of the sections was satisfactorily preserved.

The results presented in this paper show that dry

ultrathin frozen sections prepared from both fresh and fixed brains can be satisfactorily transferred on grids by means of an electrostatic attraction. This method allows neurotransmitters and enzymes to be identified immunocytochemically in neuronal cell bodies and terminals on dry ultrathin frozen sections of the CNS.

MATERIALS AND METHODS

Adult wistar rats (males and females of 200 g) were anesthetized with pentobarbital (60 mg/kg) and perfused with a fixative solution of 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2–7.4 during 30 min. The fixed brains were kept in the same fixative solution at 4°C at least for 24 h before being used.

Fig. 1. Schematic drawings of the transfer steps of dry ultrathin frozen sections by means of electrostatic attraction. **a.** Dry ultrathin frozen sections on the glass knife after cutting the nervous tissue in the cold chamber. **b.** Electrostatic charging of a small plastic stick by friction with a piece of synthetic fabric. **c.** Approach of the charged small stick toward the grid (side of the supporting membrane). **d.** The small stick making contact with the supporting membrane and inducing an electrostatic charge on the membrane. **e.** Separation of the grid by means of electrically isolated forceps. **f.** Approach of the grid toward the sections on the knife, supporting membrane toward them. **g.** Electrostatic picking up of the tissue sections. **h.** The tissue sections being held on the membrane by van der Waals' forces.

Frontal sections (100 μ m) including the DBB were performed with a Vibratome (Oxford) and maintained 12 h in 17% sucrose solution in the PBS (physiological buffered saline at pH 7.2).

Small squared pieces of tissue containing the horizontal DBB (DINOPOULOS et al., 1986) were then cut off from these sections and frozen on the copper blocks in liquid nitrogen. In addition to the fixed material, small pieces of slices (500 μ m) of fresh brain, including the horizontal DBB, were frozen on the copper blocks in isopentane at -100°C .

The frozen tissue blocks were trimmed in the cold chamber of the cryoultramicrotome (Sorval MT2, USA). The original metallic cutting arm (cantilever) was replaced by a synthetic one which contracts less when exposed to low temperatures. The dry ultrathin frozen sections were obtained on a glass knife at temperatures ranging from -60°C to -90°C (Fig. 1a).

Several attempts were made to transfer the ultrathin frozen sections of fixed brain by means of a droplet of sucrose or by 5% aqueous solution of non-neutralized silicotungstic acid. Similarly to the sucrose droplet procedure, the ultrathin frozen sections were attached to the frozen droplet of silicotungstic acid in the cold chamber. At thawing, the tissue was fixed instantaneously by silicotungstic acid (TSUJI, 1986).

The next experiment was the electrostatic transfer of dry ultrathin frozen sections. An electric charge was obtained from friction of a small plastic stick on a piece of synthetic fabric (Fig. 1b). The side of the supporting membrane ("pioloform", Agar, U.K.) of the nickel grid was put in contact with the charged stick (Fig. 1c). The grid remained attached to the head of the stick, and an electric charge was induced on the supporting membrane of the grid (Fig. 1d). The grid was then separated from the head of the stick with electrically isolated forceps (Fig. 1e). The grid (the side of the supporting membrane) was directed toward the sections and attracted them electrostatically on the membrane (Fig. 1f). The sections remained attached on the membrane due to van der Waals' forces, even after the disappearance of the electrostatic forces (Fig. 1g, h). All the grids were maintained in the chamber for 30 min–1 h until freeze-drying was completely achieved (FREDERIK, 1982). The grids were then introduced one by one in an airtight box (previously cooled in the cold chamber); then the box was closed and transferred into a dessicator (containing P_2O_5) for 1 h. Finally, the sections were rehydrated in PBS during 5 min, and negatively stained with 5% silicotungstic acid (neutralized with 1 M NaOH) for 10 min.

Fig. 2. Synaptic ultrastructure of a neuropile area of the DBB. Dry ultrathin frozen sections of fixed brain have been transferred by a droplet of 5% silicotungstic acid and stained negatively by it. *SV* synaptic vesicles, *PRE* presynaptic membrane, *POST* postsynaptic membrane. $\times 200,000$

RESULTS

The transfer of the dry ultrathin frozen sections by a droplet of non-neutralized silicotungstic acid did reduce significantly their disruption but failed to provide a large undamaged surface. Neuropile areas of the DBB including nerve terminals with synaptic vesicles and both pre- and postsynaptic membranes could be observed (Fig. 2).

The electrostatic procedure allowed us to transfer onto the grids all the dry ultrathin frozen sections obtained from both fresh and fixed brains. During the transfer, though the compressed part was not spread, the majority of the sections were not disrupted.

When the dry ultrathin frozen sections of brain were rehydrated with PBS and stained negatively with silicotungstic acid, good preservation of their ultrastructure, especially the cellular organelles, was obtained (Fig. 3a).

The preservation of large surfaces of tissue allowed the cell bodies of neurons and terminals to be

observed on the same sections (Fig. 3b). Some axosomatic synapses were also seen.

The dry ultrathin frozen sections of fresh brain were transferred by electrostatic forces in the same way as those of fixed brain tissue. Though the contrast of the ultrastructures was less distinct, the myelin sheath and the mitochondria were distinctly visible (Fig. 4a, b).

DISCUSSION

The ultrathin frozen sections of the CNS are extremely fragile, probably due to the absence of collagen. It is not surprising that the surface tension of the liquid of spreading such as dimethyl sulfoxide (BERNHARD and VIRON, 1971) and droplets of sucrose (TOKUYASU, 1973) or serum (TSUJI, 1981) should disrupt the sections during their transfer on the grids, particularly in the case of fresh tissue (IGLESIAS et al., 1971).

Silicotungstic acid introduced by us (TSUJI, 1986)

Fig. 3 a. Ultrathin frozen section of the rat DBB showing the state of tissue preservation. *N* nucleus of neuron, *Nu* nucleolus, *ER* endoplasmic reticulum, *Mi* mitochondria, *T* nerve terminal, *My* myelinated axon. $\times 20,000$. **b.** Delimited area of Figure 3a, showing a nerve terminal in contact with a neuronal cell body. *T* nerve terminal, *V* synaptic vesicles, *Mi* mitochondria. $\times 66,000$

Fig. 4 a. Dry ultrathin frozen section of fresh DBB showing a zone of myelinated fibers. $\times 20,000$.
b. Delimited area of Figure 4a, showing some myelinated axons (*My*). *Mi* mitochondria. $\times 100,000$

for transfer and fixation of fresh tissue sections was re-examined in the present experiment using paraformaldehyde-fixed brains. Though the disruption of sections was considerably decreased by silicotungstic acid, as compared with the use of sucrose, the preservation of the integral surface was not obtained.

Except for the transfer by a droplet of silicotungstic acid, the electrostatic transfer of ultrathin frozen sections seems the only feasible way. The present procedure of electrostatic transfer is simpler than those previously used for non-embedded tissues (KOLLER and BERNHARD, 1964; HODSON and MARSHALL, 1970) which were not applied to dry ultrathin frozen sections, probably due to the complexity of the method used for electrostatic induction.

In comparison to our electrostatic transfer, previously employed mechanical transfers of the sections by an eyelash followed by direct pressure on the sections with a cold copper stick (CHRISTENSEN, 1971) or by aspiration of air followed by pressure on the sections from opposite side of the grid (APPLETON, 1974) are technically very demanding.

In our experimental conditions using the electrostatic forces induced on the supporting membrane, the ultrastructure of both fresh and fixed brains was preserved on large surfaces of tissue, including the neuronal cell bodies and terminals.

The weak contrast obtained on the sections of fresh tissue could be explained by the presence of soluble proteins attached on the membrane and limiting the penetration of the stain into the membrane ultrastructure.

The electrostatic transfer method of dry ultrathin frozen sections introduced in the present paper is proving useful in our laboratory in immunocytochemical identification of afferent nerve terminals on cholinergic cell bodies located in the DBB of the rat.

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