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A Simple Technique For Flat Osmicating And Flat Embedding Of Immunolabelled Vibratome Sections Of The Rat Spinal Cord For Light And Electron Microscopy

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Abstract

We describe here a simple technique for flat-osmicating and flat-embedding of immunolabelled vibratome sections. The technique is particularly useful for large specimens such as whole cross-sections of rat spinal cord. After the vibratome section has been flat-osmicated on a flat surface under a glass cover slip, it is dehydrated and then embedded by placing it in a small drop of epoxy resin on a flat base of polypropylene plastic and overlaying a small square piece of cellulose acetate cut from heat-resistant overhead projector transparency film for photocopying. The thin layer of resin containing the flat-embedded vibratome section is then separated from the base and glued onto the flat end of a pre-polymerised blank block of resin. The method produces flat-embedded vibratome sections and thus allows serial large uniformly labelled semithin and ultrathin sections to be obtained of the whole cross-section of the rat spinal cord. This facilitates the observation and quantification of labelled cells in the specimen. Because of its simplicity the technique also allows one worker to process more than 100 vibratome sections at the one time.

Keywords: flat-osmicating; flat-embedding; vibratome section; immunolabeling

1. Introduction

The pre-embedding immunolabelling technique has provided a useful method to study various tissues and, in particular, the diverse cellular populations of the nervous system. It enables the labelling of glial and neuronal cells as well as infiltrating inflammatory cells in the central nervous system in pathological conditions such as experimental autoimmune encephalomyelitis (Pender et al., 1992). In contrast to immunolabelling techniques using frozen or paraffin sections, the pre-embedding immunolabelling technique gives good preservation of tissue structure and allows high-resolution light microscopy. It also allows further detailed examination at electron microscopy. However, problems have been encountered with this technique. Because poor antibody penetration limits the reaction product to a thin layer at the surface of the vibratome maximal number of uniformly labelled semithin sections can be obtained from the shallow depth of useful tissue. Vibratome sections, which are usually 80-150 μm thick, tend to become undulated as a result of the constant stirring that is required during the immunolabelling procedure. Unless the section is kept flat during osmication, any undulation will become permanent as the tissue hardens. Even if the section is successfully flat-osmicated, it may become curved during resin polymerisation unless a flat-embedding technique is used. Quantitative studies of semithin sections cut from curved embedded vibratome sections are limited by the small area that is labelled in the plane of section.

In our studies involving the identification and quantification of apoptotic cells in the spinal cord of rats with experimental autoimmune encephalomyelitis (Pender et al., 1992), we require completely flat vibratome sections with a diameter sometimes greater than 3 mm. A number of techniques employing different means to obtain flat-embedded sections have been described (Grimley, 1965; Holländer, 1970; Schwartz, 1982; Priestley, 1984; Yu and Schwartz, 1989: Valentijn et al., 1989; DeFelipe and Fairén, 1993).

However, generally these techniques require special devices and/or involve many procedural steps and still do not produce completely satisfactory results. Most have either not mentioned or not described in detail how the fixation with OsO_4, is performed. Post-fixing with this fixative is an essential step in the processing of nervous tissue and is also a crucial step that determines the flatness of the embedded vibratome section. Schwartz (1982) has described a technique for flat-osmicating and flat-embedding of vibratome sections but this technique is difficult to perform. We have developed a simple technique that produces large flat-embedded vibratome sections and that allows one worker to process many sections at the same time.

2. Materials and methods

Vibratome sections (80-100 μm thick) were obtained from spinal cord tissue of rats that had been perfused through the ascending aorta with 4% paraformaldehyde, 0.05% glutaraldehyde in phosphate-buffered saline, pH 7.4. The vibratome sections were immunolabelled using various procedures and the efficacy of the labelling procedure was assessed by examining buffer-soaked sections under the light microscope. The sections are then washed with 0.9% saline in 5 ml vials, post-fixed with 2% paraformaldehyde, 2.5% glutaraldehyde/0.1 M sodium cacodylate buffer for 1 h and washed again with 4 changes of saline, each for 10 min. The sections are still flexible at this stage. Groups of 5-10 floating sections are transferred with about 0.5 ml saline into the compartments of a white plastic slide tray (Bel-Art, USA) or into small plastic Petri dishes. Saline is carefully removed through a fine tip of a plastic transfer pipette or using the capillary action of filter paper. Still under the protection of a fumehood, a few drops of 1% OsO_4, fixative (Dalton's solution) (Dalton, 1955) are placed on the sections that are lying flat on the bottom of the slide tray. Glass coverslips are gently placed over each group of sections to keep the sections flat during osmication.

After 30 min, a few drops of OsO_4, fixative are added to the edges of the coverslips so that the coverslips float above the sections and can be removed with fine forceps. After being soaked for a further 5 min to enable the fixative to penetrate completely into the centres of the sections, the sections are gently collected with a fine brush into large vials with a flat bottom (e.g., 20 ml scintillation glass vials). The flat-osmicated sections are then washed with 5 - 10 changes of saline over at least 2 h, with gentle and only occasional stirring. They are stained en bloc with 1% uranyl acetate in saline for 1 h, washed with saline and dehydrated in 70% ethanol, 90% ethanol and 90% acetone followed by two changes of absolute acetone before being infiltrated sequentially with mixtures of acetone/epoxy resin in the proportions 3 : 1, 1: 1, and 1: 3 and finally with full resin in which they remain overnight at 4°C.

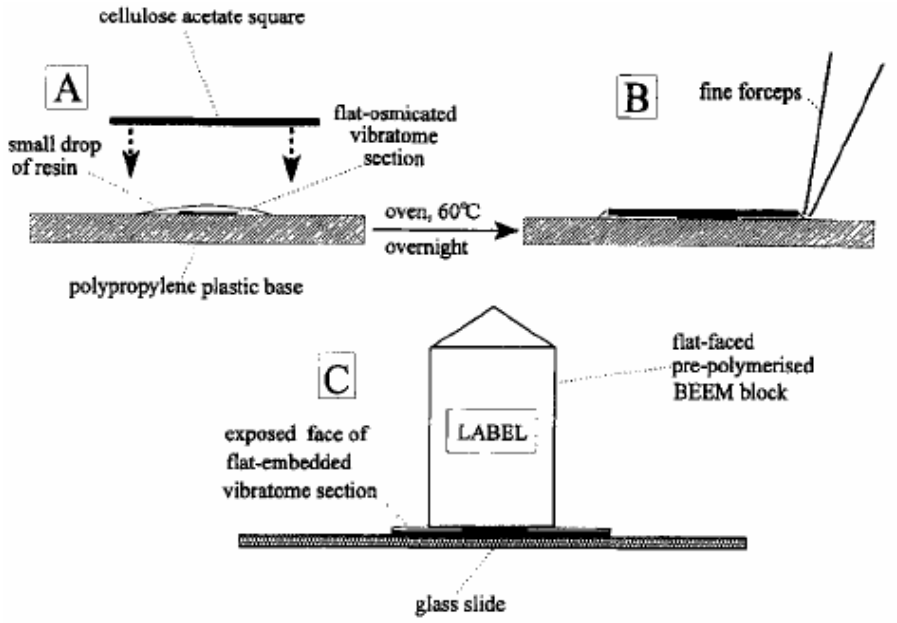

Fig. 1.
A: A square piece of cellulose acetate (cut from overhead projector transparency film for photocopying) is placed over the resin-covered flat-osmicated vibratome section. The resin spreads out to form a thin layer.
B: After polymerisation in an oven at 60°C overnight the thin layer of resin that contains the vibratome section and that is still attached to the cellulose acetate square is lifted off the polypropylene base with fine forceps.
C: The flat-embedded vibratome section is placed on a glass slide with the exposed face directed upwards. The exposed face is then glued to the flat end of a blank BEEM block. Finally the cellulose acetate square is peeled off.

Epoxy resin is prepared by mixing 20 ml of 'Epon', 16 ml of DDSA (dodecenylsuccinic anhydride), 8 ml of MNA (methyl Nadic anhydride) and 1.3 ml of BDMA (bencyldimethylamine) (Glauert, 1991). The still flat infiltrated sections are individually transferred using the thinly flattened tip of a wooden stick, with minimum resin attached, to a small drop of fresh resin placed on a flat base of polypropylene, such as the bottom of a freezer storage box. A square (1 x 1 cm) piece of cellulose acetate (cut from heat-resistant overhead projector transparency film for photocopying) is placed over each resin-covered section, as illustrated in Fig. 1. To prevent the cellulose acetate square from curling, the temperature for polymerisation is not allowed to go above 60°C. The square is held down by the surface tension of the resin between the square and the polypropylene base. The vibratome section is thereby kept flat during polymerisation. As the resin binds only loosely to the polypropylene base, the thin layer of polymerised resin containing the section can be readily separated from the base while remaining attached to the cellulose acetate square.

At this stage the embedded section can be examined light microscopically on a glass slide with the exposed face directed upwards, or the section can be trimmed to a small area of interest, if required. The exposed face is then glued with cyanoacrylate or epoxy adhesive to a blank block that has been prepared by polymerisation in a BEEM capsule in an inverted position with the cap removed and with the open end lying on a flat polypropylene surface. The cellulose acetate square is then simply peeled off and the block is ready for semithin and ultrathin sectioning. 3. Results and discussion Our new technique consistently produces large flat-embedded vibratome sections from which we can cut serial uniformly labelled semithin whole cross-sections of the rat spinal cord (Fig. 2A-C). We have successfully used this technique on vibratome sections with

diameters of up to 3.5 mm. The production of large uniformly labelled sections facilitates the observation and quantification of labelled structures within the specimens. The technique is simple and allows one worker to process more than 100 sections at the same time. For electron microscopy, we generally cut ultrathin sections from the whole cross-section of spinal cord.

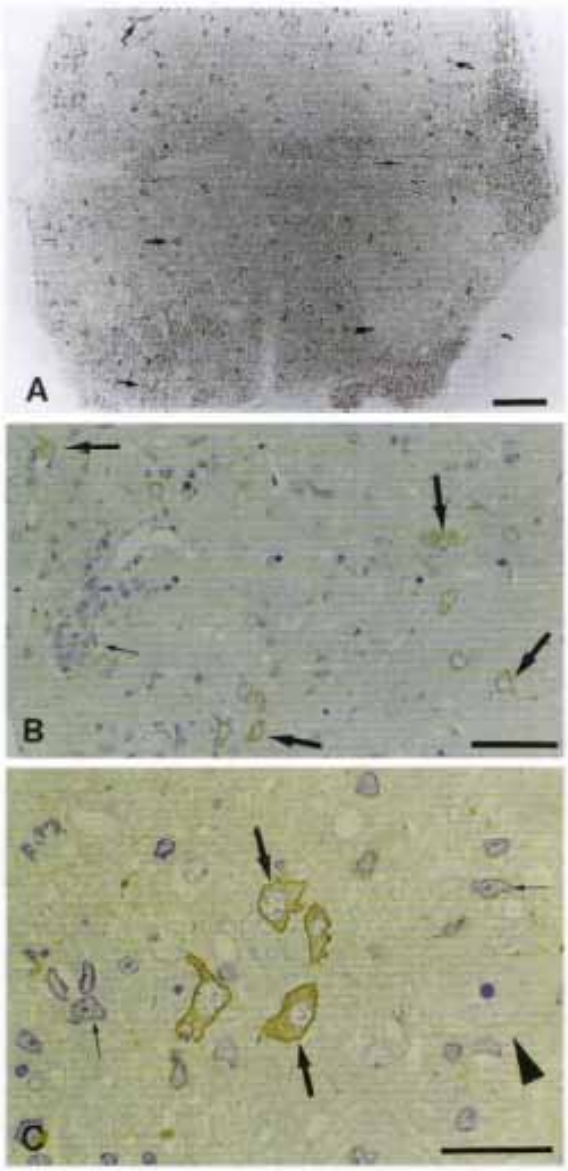

Fig. 2. Light micrographs of 1 μm semithin sections from a flat-embedded vibratome section of the sacral spinal cord of a Lewis rat with experimental autoimmune encephalomyelitis. The vibratome section was immunolabelled with Rip monoclonal antibody (Friedman et al., 1989) and the avidin-biotin-peroxidase complex technique to identify oligodendrocytes. **A**: Low power in black and white showing that the immunolabelling of oligodendrocytes (arrows) is even throughout the whole cross-section of the spinal cord (not counterstained). **B,C**: Higher powers of a section counterstained with toluidine blue and showing specific labelling of oligodendrocyte cytoplasm (large arrows). Other cells such as a neurone (arrowhead) and inflammatory cells (small arrows) am not labelled. Bars: (A) = 100μm; (B) = 50 μm; (0 = 25 μm.

In our technique the thickness of the layer of resin containing the embedded vibratome section varies automatically with the thickness of the vibratome section. The section is kept flat during polymerisation by the cellulose acetate square which is held down by the surface tension of the resin between the square and the polypropylene base. In contrast, some previously published techniques rely upon a preset thickness for the layer of resin (e.g., the thickness of a cellulose acetate sheet) (Holländer, 1970; Yu and Schwartz, 1989). If the vibratome section is thinner than the preset thickness, it is still able to curve during polymerisation; if it is thicker, it is crushed by the externally applied force. A key step in our new technique is the washing step after flat osmication. The sections need to be kept lying on the flat bottom of the washing vial for most of the time during washing. They should be stirred gently and only occasionally, as the hardening process continues for some time after osmication, and as vigorous or continuous stirring at this stage can cause distortion of the partly hardened sections. Even a slightly undulated osmicated section can not be made completely flat during embedding, as any flattening force may crack the section. Another important requirement for the production of good results is the use of a minimal amount of resin to embed the section before overlaying the cellulose acetate square. This ensures close apposition of the square and the vibratome section. In conclusion, we have described a simple new technique that consistently produces large flat-embedded vibratome sections for observation and quantification of immunolabelled structures at light and electron microscopy.

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