Rapid immunoperoxidase staining of axons for frozen section diagnosis of nerve biopsy specimens

G S DAVIDSON, KPC SO The Hospital for Sick Children, Toronto, The University of Toronto

SUMMARY A new method of freezing and embedding a nerve biopsy specimen and staining it with the immunoperoxidase technique for neurofilaments was developed to overcome the difficulties normally encountered in the assessment of tiny portions of nerve. The method clearly shows the architecture of the nerve, the exact number and size of all axons present, and the degree of fibrosis present. The entire procedure may be accomplished in 20 minutes.

Several factors affect the success of surgical anastomosis of traumatically or iatrogenically severed nerves or autogenous nerve grafts. These include the alignment of nerve fascicles in the adjacent nerve ends, the degree of endoneurial fibrosis, and the presence of traumatic neuroma. Where a number of proximal nerve fascicles are available for anastomosis to a lesser number of distal fascicles, the proximal ones may be chosen by the number of axons they contain. When a nerve graft is used the proximal stump may be shortened until a region is found with viable axons, little fibrosis, and a good fascicular architectural pattern. To assess these features the surgeon may request frozen section examination of the nerves.1-11

Unfortunately, this presents a dilemma for pathologists. Tiny portions of nerve are difficult to embed for a good cross section, and sections cut obliquely or from an irregularly embedded specimen can not be used for assessment of fascicle size. Freezing artefact limits the usefulness of the microscopy. On cross section, collagen may be indistinguishable from myelin. Unmyelinated and smaller myelinated axons are invisible, and Schwann cell nuclei are identical to fibroblast nuclei. In nerve grafts, if the distal end is connected at a second operation, most of the regenerated axons are very small, and few are myelinated, so frozen section becomes almost useless.

To overcome these problems we developed a method of freezing and embedding a nerve biopsy specimen and staining it with the immunoperoxidase technique for neurofilaments.

Material and methods

Biopsy specimens of peripheral nerve were trimmed of fat and connective tissue, if necessary, for easy longitudinal orientation. The nerve was then placed longitudinally in the crease of a partly folded piece of aluminium foil. A small amount of embedding compound (Tissue Tek OCT) was placed in the crease to completely enclose the nerve. The foil was then immersed in liquid nitrogen and then transferred to a cryostat at −20°C. The nerve was removed from the foil and excess embedding compound was trimmed from one end of the specimen with a pre-cooled razor blade to provide a flat base for re-embedding. A small amount of embedding compound was placed on a tissue holder on the freezing stage of the cryostat and, when it was just about to solidify, the flat trimmed end of the nerve was placed down on it. The nerve was held upright with fine pre-cooled forceps until re-embedding was complete. Seven micron sections were then cut.

Sections were air dried and fixed in acetone for 30 seconds, air dried again, and rinsed in two changes of TBS (Tris buffered saline—100 ml 0·05 M Tris-HCl buffer, pH 7·6, in 900 ml 0·85% NaCl). They were then incubated with monoclonal antibody against phosphorylated neurofilament for two minutes at room temperature (Sternberger-Meyer Immunocytochemicals, Inc, diluted 1/100). After washing in running tap water and then in two changes of TBS the sections were incubated with peroxidase conjugated rabbit anti-mouse antiserum (Dako) for two minutes at room temperature. They were then washed in water and TBS again, treated with DAB (0·1% 3,3′-diaminobenzidine-HCl in 0·05 mol Tris-HCl buffer, pH 7·6; 10 μl of 30% H₂O₂ added to every 50 ml just

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Fig 1  Difficulties in conventional embedding technique cause irregular fascicle shape, making estimation of relative sizes difficult. (Regenerating nerve graft, haematoxylin and eosin.)

Fig 2  Laying the biopsy specimen inside the crease of a folded piece of foil and then “double-embedding” provides a perfect cross section. (Regenerating nerve graft, haematoxylin and eosin.)

Fig 3  Artefact caused by slow freezing distorts the micro-architecture, making recognition of axons difficult or impossible. (Regenerating nerve graft, haematoxylin and eosin.)

Fig 4  Rapid freezing in liquid nitrogen reduces freezing artefact, permitting better interpretation of the nerve biopsy specimen. (Regenerating nerve graft, haematoxylin and eosin.)
Fig 5  With the use of liquid nitrogen, the lack of freezing artefact permits recognition of myelin rings. The amount of eosinophilic material between them helps to assess fibrosis. (Proximal nerve stump, haematoxylin and eosin.)

Fig 6  Immunoperoxidase stain shows both large and small axons dark brown. Lighter blue-gray staining myelin rings are visible around them. The absence of eosinophilic staining between myelin rings indicates that there is little or no fibrosis. (Proximal nerve stump, immunoperoxidase stain for neurofilament.)

Fig 7  Low magnification view of immunoperoxidase stain shows the number, shape, and size of the fascicles. Fascicles may be selected for anastomosis based on their size and axonal density. (Regenerating nerve graft, immunoperoxidase stain for neurofilament.)

Fig 8  Higher magnification shows the axons are nearly perpendicular to the section. This, together with the fascicular pattern seen at low magnification, rules out traumatic neuroma formation. (Regenerating nerve graft, immunoperoxidase stain for neurofilament.)
before use) for two minutes, washed well in TBS and then running water, and counterstained with filtered Harris’s haematoxylin for 10 seconds. Finally, they were washed and blued by standard techniques, dehydrated through alcohols, cleared in xylol, and mounted in synthetic resinous mounting medium.

Biopsy specimens were also examined by conventional means. They were placed in embedding compound on the freezing stage of a cryostat. Sections were cut and stained with haematoxylin and eosin.

Results

Fig 1 shows the poor orientation of a biopsy specimen embedded directly on to the freezing stage of a cryostat. Oblique section made assessment of the size of the fascicles difficult. In contrast, the use of metal foil and “double embedding” provided a perfect cross section (fig 2). Embedding biopsy specimens on the freezing stage of the cryostat without using liquid nitrogen produced slow freezing, with severe artefact making interpretation almost impossible (fig 3). Fig 4 shows the excellent preservation of structural detail provided by rapid freezing in liquid nitrogen. With the haematoxylin and eosin stain, myelin rings are visible (fig 5). In fig 6 the immunoperoxidase stain for neurofilament shows the number and size of the axons. Myelin rings can be easily visualised around them, and the lack of any other endoneurial tissue illustrates the absence of fibrosis. At low magnification the use of rapid freezing and the neurofilament stain provided a clear picture of the number, size, and shape of the fascicles (fig 7). In fig 8 higher magnification shows the exact size of the axons. Most of them are round or oval, indicating that they are running parallel in the nerve. This information, together with the architectural features seen at low magnification, allows the presence of neuroma formation to be confirmed or ruled out.

Discussion

The difficulty of interpreting frozen section specimens of nerves may be avoided by using this method of rapid freezing and immunoperoxidase stain for neurofilaments. Using it, we have had no difficulty in being able to report the lack or presence of fibrosis, the shape and size of the fascicles, and the number, size, and degree of myelination of the axons. Surgery is facilitated by returning to the surgeon with a sketch of the nerve and its fascicles, or, better still, a polaroid micrograph. It is important to obtain a good cross section of the nerve, so great care must be taken in the embedding stage of the procedure. The entire procedure can be performed in 20 minutes. We have found that the surgeon is happy to wait this long, in return for a more complex and accurate diagnosis than can be delivered by conventional frozen section examination.

References


Requests for reprints to: Dr G S Davidson, Department of Pathology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada.
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G S Davidson and K P So

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