Further uses and methods of processing of fresh frozen sections of peripheral nerve

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With 4 plates (figs. 1 to 4)

Summary

Advantageous modifications of a fresh frozen sectioning technique for peripheral nerve (Williams, 1959) include reduction in size of the block of supporting tissue, the use of human hair as a ligature, the multiple embedding of specimens and the use of liver sandwiches for irregular specimens. Observation with oblique incident illumination indicates that fresh sectioned fibres remain approximately cylindrical. Fresh sections treated for 5 min with common fixatives such as 10% formal saline, Bouin's fluid, and Flemming's fluid show widespread changes in the outline, texture, and dimensions of the myelin sheaths. A modified Flemming fixation/haematoxylin staining method may be used with fresh sections; this gives reasonable preservation of the external sheath boundary but is unsuitable for measurements of internal diameter. Luft's buffered potassium permanganate solution provides an easily standardized, effective method of producing permanent preparations for quantitative studies. Holmes's silver impregnation technique may be adapted for use with fresh frozen sections. Interferometric estimations may be made upon axoplasm using 5 μ fresh frozen sections in saline. Care must be taken to determine the axoplasmic thickness present. Adequate sampling of an extended section, when using shearing system objectives, may be accomplished by serial stripping of the section under the dissecting microscope.

Introduction

In a previous report (Williams, 1959) a technique was described which allowed the production of regular, stable sections 5 μ in thickness of fresh mammalian peripheral nerve. A consideration of the appearance of such sections when immersed in normal saline and viewed using orthodox transmitted illumination (fig. 1, A), phase contrast, dark field, and polarized light (fig. 1, B), led to the selection of polarized light as the most suitable form of illumination when attempts were made to measure the internal and external diameters of the myelin sheath. Later, it was shown (Wendell-Smith and Williams, 1959) that the size relations of the myelin sheath in these sections did not differ significantly from those present in fresh nerve-fibres which had been carefully teased in normal saline. During the subsequent use of the technique in investigations on nerve-fibre anatomy, certain modifications have proved advantageous. In addition, the frequent need for processing methods which led to permanent preparations has been met, the effects of
certain common fixatives on fresh sections has been noted and the usefulness of the technique in interferometric investigations has been established.

The purpose of the present communication is to present the various modifications and further uses of the technique.

**Materials and methods**

Although these techniques have been used on a wide variety of nerves, the illustrations in the present paper are taken from sections of the nerve to the medial head of the gastrocnemius muscle (NGM) and of the tibial nerve of the adult rabbit, and from sections of the tibial nerve of the adult rat.

The details of microscopy and photography used to produce the monochrome prints may be found in Wendell-Smith and Williams (1959) and Williams and Wendell-Smith (1960).

Sections of nerve were studied in interference contrast using a Baker interference microscope provided with shearing system objectives.

**Modifications**

Originally, a block of fresh liver $2 \times 1 \times 1$ cm was recommended as a supporting tissue for the nerve. Later, it was found that the freezing process was improved and the number of deformed fibres decreased if the mass of supporting tissue was considerably reduced in size (e.g. $0.5 \times 0.5 \times 1$ cm or less). When introducing the nerve into a reception channel within the block by means of a ligature, difficulty was sometimes encountered because of a bulky knot. Dr. R. Kashef (of the Department of Anatomy, Guy's Hospital, London) suggested the use of human hair as a ligature and, when fine nerves are to be cut, this has proved satisfactory. Where a number of specimens have to be cut as expeditiously as possible, 2 or 3 reception channels may be made in the same block and the specimens sectioned simultaneously. When this is being done, grooves may be cut at appropriate points on the surface of the block to facilitate subsequent identification of the specimens.

Large or irregular specimens may be handled by suitably modifying the supporting procedure. Thus, specimens such as an optic chiasma or dorsal root ganglion with attached nerve-rootlets may be supported within a sandwich of liver the adjoining surfaces of which have been slightly hollowed out to receive the specimen. Successful sections of spinal cord may be cut after placing a segment of fresh cord in a large reception channel prepared in a block of liver with a cork borer of appropriate size.

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**Fig. 1 (plate).**

A, fresh, 5 µ transverse section of the nerve to the medial head of gastrocnemius of the rabbit (NGM), mounted in saline, using orthodox transmitted illumination. Note annuli of compact myelin, clear axonal areas, and occasional distorted fibres.

B, fresh, 5 µ transverse section of the NGM using plane polarized light. Note the highly illuminated, birefringent sheath of compact myelin—each sheath exhibits four points of extinction.
FIG. 1

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FIG. 2

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Observations on unfixed material

It was previously shown (Wendell-Smith and Williams, 1959) using micromanipulation methods that the individual fibres in a fresh frozen section of a nerve-trunk largely retain their cylindrical form. This view is supported by the appearance of the surface of such a section using oblique incident illumination (fig. 2, A). Here, the near circular profiles of the compact myelin sheaths may be seen. Each fibre appears to have a central crater. This is possibly an optical effect due to the oblique illumination or it may be due to a slight retraction of the column of axoplasm within the myelin sheath. This point is significant when interferometric measurements are contemplated (see below). Within a few hours fresh frozen sections begin to break up and it is thus desirable that fresh sections may be converted into an adequate form of permanent preparation. While investigating this possibility the dramatic effect of certain common fixatives on the appearance of the myelin sheath was observed.

The effect of fixatives

In each case a fresh frozen section immersed in normal saline was observed under the microscope. The saline was then drained away and the slide flooded with the fixative solution, the process again being observed under the microscope. After 5 min excess fixative was drained away, the slide briefly washed in normal saline and finally the section flooded with fresh saline and photographed.

Formaldehyde-saline (fig. 3, B). Solution used:

Commercial formaldehyde solution—1 part
Normal saline (sodium chloride 0.9% aq. w/w)—3 parts

In a fresh frozen section, the uncomplicated internodal myelin sheath appears as an almost circular profile limited internally and externally by narrow dark lines due to light scatter at the tissue interfaces (fig. 1, A). The myelin appears smooth, homogeneous, and an almost translucent pearly-grey colour. Within a few seconds of flooding with the fixative gross changes appear. The myelin sheath thickens—mainly by encroaching upon the axon region. The internal and external boundaries become irregular, developing rounded or angular surface projections. Sometimes the sheath breaks across completely. The surface of the myelin develops patchy increases in density and becomes covered with fissures and intervening elevations. After a period of approximately 1 min further changes were not observed.

Fig. 2 (plate). A, fresh, 5 µ transverse section of the NGM using oblique incident illumination. Note the preservation of cylindrical form of the compact myelin and the apparent 'cratering' of the axoplasm.

B, fresh, 5 µ transverse section of the NGM processed using a modified Flemming fixation/Wolter's haematoxylin staining technique. The external boundary of the sheath is often well preserved, the internal boundary highly variable and the axon area reduced.
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**Bouin's fixative** (fig. 3, c). Solution used:

- Picric acid (saturated solution in water) 75 cc
- 40% formaldehyde in water 25 cc
- Glacial acetic acid 5 cc

Here, the changes are much less marked. The fibres retain their smooth contour and circular outline; the axonal region is not markedly reduced. Some of the sheaths develop a faint radial striation giving a spokewheel effect. However, this fixative seems to promote the formation of isolated myelin spheres.

**Permanent preparations**

Many quantitative investigations into nerve-fibre anatomy are still carried out using techniques which involve fixation of the whole nerve-trunk in some variation of Flemming's fluid followed by paraffin wax embedding and haematoxylin staining. Such techniques are difficult to standardize (Williams and Wendell-Smith, 1960). Nevertheless, in order to emphasize the lability of preparations produced by these methods and to allow a closer comparison with previous results, a modified Flemming fixation/Wölter's haematoxylin staining method, which may be used with frozen sections, is given. It must be emphasized that for satisfactory comparative studies involving measurement, the various stages of each technique must be rigorously adhered to. This applies in particular to the composition of reagents, the times for fixation, staining, differentiation, and dehydration of the specimen and the exposure and development times of both negatives and positives.

**A modified Flemming/Wölter technique.** Fixative solution:

- 1% chromic acid in distilled water 15 ml
- 2% osmium tetroxide in distilled water 4 ml
- Glacial acetic acid 1 drop (25 mg)

The quantity of glacial acetic acid is critical; pipettes should be tested concerning the drop size delivered.

**Staining solution** (Wölter's haematoxylin):

- Haematoxylin, 1 g, dissolved in 10 to 20 ml absolute alcohol.
- Distilled water to make 100 ml
- Glacial acetic acid 2 ml
- Filter before use.

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**Fig. 3** (plate). A, fresh, 5 µ transverse section of the NGM for comparison with B, C, and D using orthodox transmitted illumination.

- B, fresh section treated for 5 min with 10% formaldehyde-saline. The sheath dimensions are altered, its boundaries are irregular and its surface finely fissured.
- C, fresh section treated for 5 min with Bouin's fixative. Good general preservation of form, fine radial striation of the myelin sheath and the occurrence of isolated myelin spheres.
- D, fresh section treated for 5 min with Flemming's fixative. Reasonable preservation of external sheath boundary, gross reduction of axon space.
FIG. 3

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FIG. 4

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The procedure is as follows:
Drain saline from slide, and flood with fixative for 5 min.
Wash in 3 changes of distilled water.
Mordant in 3% potassium dichromate for 12 h at 37°C.
Rinse in distilled water.
Stain in Wölter's haematoxylin for 15 h at 37°C.
3% potassium bichromate for 5 min at room temperature.
Differentiate in 0·25% potassium permanganate for 5 sec.
Pal's bleach for 5 min.
Wash, dehydrate (95% and absolute alcohol, 1 min in each).
Clear in 2 changes of xylene, 1 min in each.
Mount in Canada balsam.

After 5 min fixation in Flemming's fluid widespread changes are seen in the myelin sheath (fig. 3, D). The external boundary of the sheath remains almost circular but develops slight surface irregularities. The sheath thickens enormously, the internal boundary encroaching on the axonal area which is markedly diminished and sometimes obliterated altogether. The dense surface of the myelin has a granular appearance and is traversed by fine fissures.

In the final preparation after staining and mounting, the external boundary of the sheath is still fairly smooth and approximately circular. The axonal spaces have in the main increased in size but many remain small and present extreme variability of contour. Some of the size changes following this form of processing have been assessed (Williams and Wendell-Smith, 1960). Whilst such methods may provide an adequate estimate of the external diameter of the myelin sheath, they are totally unsuited for estimations of internal diameter.

Buffered potassium permanganate. Whilst assessing the effects of the various fixative solutions used in electron microscopy on fresh frozen sections of nerve, Luft's buffered potassium permanganate was found to provide an extremely simple and effective method of producing permanent preparations. This technique has also been used by Miss V. M. Brooks in the Anatomy Department of Edinburgh University.

Solutions used:
Buffer: Sodium veronal 14·7 gm
Sodium acetate 9·7 gm
Water 500 ml

Fixative: 1·2% potassium permanganate solution 1 part
Buffer solution 1 part
Adjust pH to 7·5

Fig. 4 (plate), A, fresh 5 μ transverse section of the tibial nerve of the rat processed using Luft's buffered potassium permanganate. Good preservation of sheath boundaries which are sharply demarcated from surrounding tissues.

B, fresh 5 μ transverse section of the tibial nerve of the rabbit processed by Holmes's silver impregnation method. Axons of all sizes are impregnated but show irregularity of contour.
Keep in glass stoppered bottles in the refrigerator. Dip pipette well below surface when withdrawing fixative. Use immediately after removing from refrigerator.

The procedure is as follows:

Prepare and select fresh frozen sections mounted on slides.
Drain saline and flood slide with buffered potassium permanganate for 5 min.
Wash in distilled water.
Dehydrate (95% and absolute alcohol, 1 min in each).
Clear in xylene (2 changes, 1 min in each).
Mount in Canada balsam.

Fig. 4, A shows a section prepared in this manner. The circular, smooth internal and external contours of the sheath and the homogeneous character of the myelin have been preserved. The margins of the sheath are sufficiently sharp to allow measurements to be carried out on suitably enlarged photographs. An investigation into the magnitude of the size changes induced by this method, when compared with a fresh frozen section, will be published elsewhere.

Silver impregnation. After 5 min fixation on the slide of a fresh frozen section by either 10% formal-saline or Bouin's fluid, silver impregnation of the axons may be carried out using a standard form of Holmes's technique (Carleton and Drury, 1957). The axons of all sizes (fig. 4, B) show evidences of differential shrinkage with many angular projections from their surfaces. The smaller axons appear as irregular dense black dots whilst the large axons are dense peripherally and pale centrally.

Interferometry of axoplasm

A fresh frozen section, 5 μ in thickness, of a peripheral nerve, mounted on a slide and covered with two drops of normal saline may be examined and photographed in interference contrast, at various settings of the microscope analyser. In such a preparation, the colour tone of the axoplasm is easily distinguished from that of the 'reference area' of saline nearby and the variation of each with different analyser settings is also easily seen. It has thus proved a comparatively simple matter to make estimations of the light retardation that occurs when different zones of axoplasm are observed. It should be noted that it is an essential preliminary to estimate the thickness of axoplasm present before attempting absolute measurements of density, dry mass, &c. The axoplasmic thickness may be measured by comparing the retardation in two fluids of different refractive index (Hale, 1958).

When shearing system objectives are used it is only possible to carry out estimations on one edge of an extended section because of the superimposition of 'in focus' and 'out of focus' images elsewhere. This difficulty may be overcome by dissecting away serial strips of tissue from the edge of the section, under the dissecting microscope. Using these methods it has been found
possible to estimate axoplasmic density in different types of nerve-fibre, at different distances from the parent cell body and also under various experimental conditions.

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References


Williams, P. L., 1959. Ibid., 100, 425.