



Muscle & Nerve Biopsy Procedures Manual

ANN diagnostic vision

To coordinate a national collaborative diagnostic service and research network for neuromuscular disorders that is cost-effective, maximises availability and minimises duplication of laboratory services for muscle and nerve specimens.

The following current procedures come from the Victorian Neuromuscular Laboratory Service (VNLS) procedures manual and can be used as a guide to muscle and nerve specimen collection and processing. The VNLS is based at the Alfred Hospital, Melbourne. Direct reference to the VNLS has been deleted to make the procedures as generic as possible. Also included is skin biopsy specimen processing for establishing cell lines.

Specimen collection

Surgery is usually preplanned and hospitals are instructed to inform the laboratory 24 hours prior to surgery. The laboratory staff in turn sends the hospital a muscle or nerve biopsy kit and informs theatre staff that the specimen must be delivered back to the laboratory by 4pm on the same day of the surgery.

Muscle Biopsy Kit

- A plastic bucket, containing the following:
- Three 70mL plastic screw top specimen containers labeled as follows:
 1. **“Dry”**
 2. **“NBF”** – A 70mL specimen pot containing 10% neutral buffered formalin.
 3. **“2.5% Glut”** – Into a 70mL specimen pot decant 15mL of 2.5% glutaraldehyde on the day of dispatching to the hospital or surgery, and marked with the date of issue.
- **Two** biopsy clamps (refer to construction procedure below).
- A plastic specimen bag.
- A specimen request form.
- Muscle biopsy collection instruction.
- Place completed muscle biopsy kit(s) into an esky and dispatch to the laboratory.

Muscle biopsy clamps

Two utilux alligator clips (Middendorp Eletrical P/L, Melbourne, Victoria) are screwed onto a piece of 1.5mm NEMA GRADE GPO-3 EPOXY GLASS 18 x 27mm (Insulcut Services P/L

Thomastown, Victoria) backing board with two predrilled holes 20mm apart with a flat head screw driver.

Nerve Biopsy Kit

- A plastic drinking cup, containing the following:
- A glass tube containing **2.5% glut** and labeled with the date of issue and “2.5% glut”.
- A plastic tube containing one **nerve hook** (refer to construction procedure below).
- 1 plastic specimen bag.
- A specimen request form.
- A nerve biopsy collection instruction pamphlet.

Construction of nerve hook:

One steel sewing pin (25mm in length) is threaded through a ball type sinker (size 000, weighing approximately 1 gram) (Complete Angler, Melbourne, Victoria). With a pair of pliers crush the sinker around the head of the pin, apply a pair of surgical forceps to the pin immediately above the sinker and bend the pin to a 90 degree angle thus forming a hook which will allow the sinker to be suspended from the nerve biopsy.

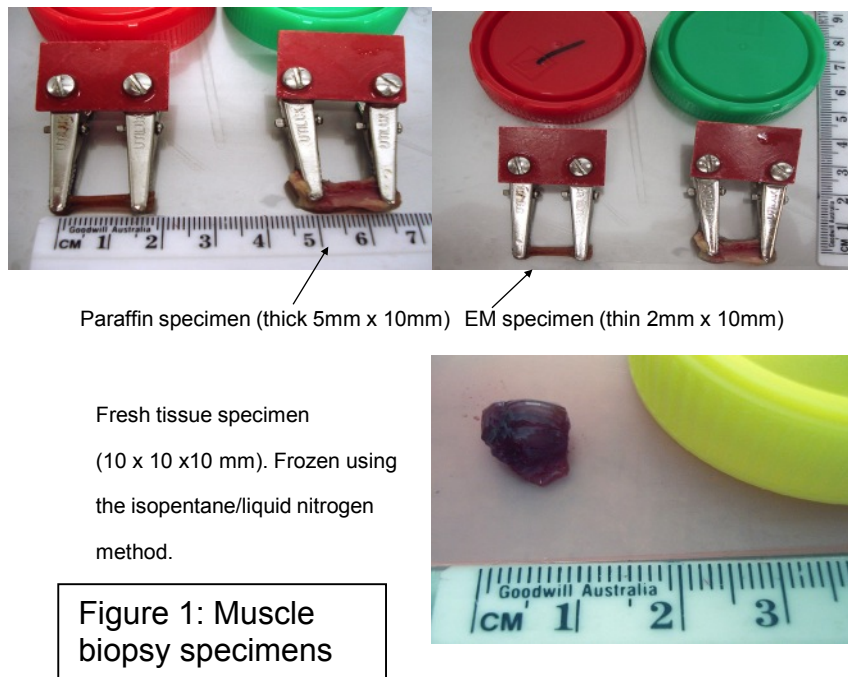
Processing

Muscle biopsy

Required specimens

The laboratory requires three specimens in order to undertake a complete range of tests on each biopsy (Figure 1). The electron microscopy specimen may be optional.

1. A fresh specimen received in a dry sterile container to be snap frozen for cryostat sectioning and enzyme histochemistry.
2. A clamped specimen received in neutral buffered formalin to be processed for paraffin sections.
3. A clamped specimen received in 2.5% glutaraldehyde to be processed for electron microscopic examination (optional).



These specimens are received packed in ice and the fresh tissue must be frozen as soon as possible.

1. Frozen muscle

Freezing a muscle biopsy

Equipment:

- Biological Safety Cabinet Class II (optional)
- 2 scalpel blades: No. 22 and No. 11
- Dental wax
- Wooden applicator
- 2 pairs of fine point forceps
- 1 pair of coarse forceps
- 4 plastic drinking cups
- 1 x 50 ml plastic beaker
- 50 mL 2-methylbutane (isopentane)
- 1 or more cryotubes - Greiner (2 ml)
- Cardboard labels
- Lead pencil
- Pen
- Liquid nitrogen

- Vacuum flask containing dry ice
- Muscle specimen
- Permanent Marker (Sharpie Ultra Fine Point)
- PPE - Laboratory coat, nitrile gloves

Method for freezing muscle specimens:

Note: this process can be completed on the bench top within the laboratory or theatre.

1. In a Class II Biological Safety Cabinet (optional), label a cryotube (Greiner) using a Sharpie marker with the accession number, the patient's surname and given name.
2. Label a cardboard ticket with the accession number and the patient's surname and place in the cryotube.
3. Place the cryotube in the dry ice to pre-cool.
4. Fill plastic cup carefully with liquid nitrogen whilst wearing a face mask and low temperature protective gloves.
5. Add 50mL of 2-methylbutane (isopentane) to a plastic beaker (refer to figure 2 below).
6. Carefully place beaker into the liquid nitrogen.
7. Allow the isopentane to cool (approximately 5 minutes). View the three stages of the isopentane cooling process (Figure 2):
 - ⇒ **The first stage** - growth of a few crystals of isopentane on the bottom of the beaker (approx -120°C). Muscle tissue **CANNOT** be frozen at this point.
 - ⇒ **The second stage** - the bottom and portions of the sides of the beaker contain crystals. Muscle tissue **CAN** be frozen without forming microscopic ice crystals (approx -132°C).
 - ⇒ **The third stage** - a solid mass of isopentane is obtained on the sides and bottom of the beaker together with much opacity and free crystal formation in the remainder of the liquid (approx -154.5°C). Muscle tissue **CAN** be frozen without forming microscopic ice crystals.

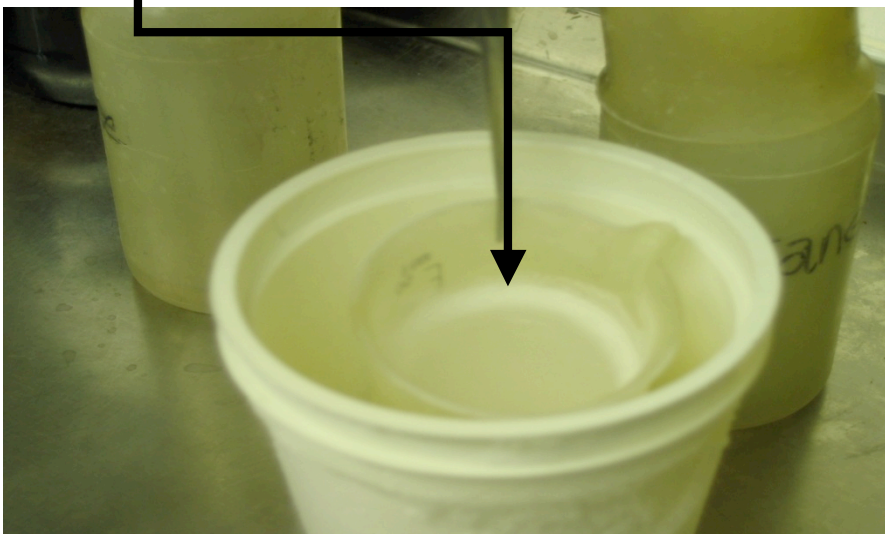


Figure 2: Isopentane/liquid nitrogen bath at stage 2.

8. Remove the fresh specimen carefully with the tip of the needle point forceps and place on to a clean piece of dental wax. Dab specimen on filter paper if excess moisture remains from specimen pot.
9. Measure the dimensions of the biopsy and record the size and any unusual observations (e.g. pale, fatty etc.).
10. The pieces to be frozen should be no larger than $5 \times 3 \times 3 \text{ mm}^3$. If they are larger the specimen needs to be cut to size.
11. If the orientation is not apparent from the specimen it can be viewed under a low power stereo dissection microscope.
12. Place the cut-down specimen into the cooled isopentane for approximately 15 - 30 seconds. Depending on the size of the muscle biopsy. For larger biopsies a greater freezing time can be used.
13. The specimen is then placed into the pre-cooled cryotube which is in the container of dry ice. The cryotube is then placed in the -80°C freezer where it is stored indefinitely.

Storage of frozen muscle

Plastic cryotube(s) are placed into a freezer box (9 x 9) and stored at -80°C indefinitely without further loss of enzyme activity after long term storage.

2. Fixed muscle for paraffin embedding:

Clamped muscle is fixed for a minimum of 12 hours in 10% neutral buffered formalin.

The muscle is cut from the inside of the clamps and dissected into two portions (for longitudinal L/S and transverse T/S orientation).

These pieces are placed into a processing cassette and processed to paraffin wax over night.

Once processed the muscle is embedded in paraffin wax in an L/S & T/S orientation, cut and stained with Haematoxylin and Eosin (+/- Congo Red and Masson trichrome).

The paraffin blocks can be stored indefinitely at room temperature.

3. Glutaraldehyde fixed muscle for resin embedding

Clamped muscle is fixed for 48 hours at 4°C in EM fixative.

The muscle is cut from the inside of the clamps and dissected into 1 mm^3 blocks ready for processing.

Nerve biopsy

Equipment:

Biological Safety Cabinet Class II (optional)

3 x 5mL labeled tubes with lids

0.133M Sodium Cacodylate Buffer pH7.3

1 x scalpel blade: (No. 22)



Figure 3: Nerve for processing

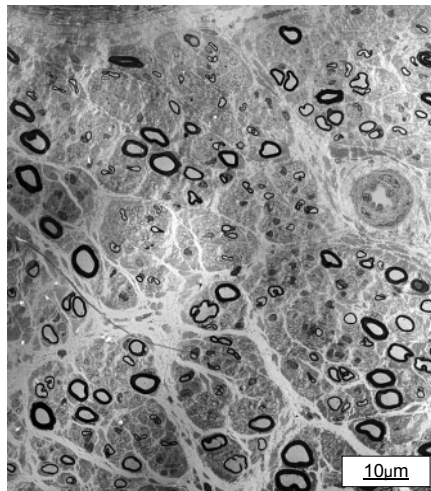
Method:

Nerve for paraffin processing:

After 48 hours in 2.5% glutaraldehyde Electron Microscopy (EM) fixative the nerve biopsy is cut into 3 x 2mm pieces and transferred into Sodium Cacodylate Buffer. Two of the pieces (L/S & T/S) remain in Sodium Cacodylate Buffer. The remaining piece is treated for two hours with Osmium Tetroxide. Four microns paraffin sections are cut and stained with Haematoxylin and Eosin, Masson Trichrome and Congo Red and unstained section.

Nerve for Electron Microscopy processing:

At the completion of the dissection for paraffin nerve processing the remaining length of nerve approximately 15mm in length is placed in Sodium Cacodylate Buffer and sent to the EM laboratory. This portion is processed for EM montage, histogram (Figure 4) and teased nerve preparation (Figure 5).

NERVE MONTAGE

Transmission Electron Micrograph of a nerve fasciculus stained with Lead Citrate/uranyl acetate – A population of large and small myelinated fibres is evident.

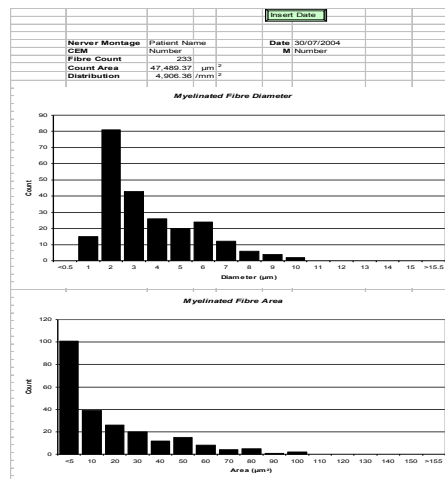


Figure 4: Montage & Histogram

Histogram - indicating the distribution of myelinated nerve fibres from the montage preparation



Figure 5: Teased nerve fibres