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Neuropathy Phentoyping Protocols - Transmission Electron Microscopy

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Protocol status: Working
We use this protocol and it's working

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Abstract

Summary:

Phenotyping of Rodents for the Presence of Diabetic Neuropathy
In man, the development of diabetic neuropathy is dependent on both the degree of glycemic control and the duration of diabetes. Diabetic neuropathy is a progressive disorder, with signs and symptoms that parallel the loss of nerve fibers over time. Consequently, assessments of neuropathy in mice are not performed at one time point, but are characterized at multiple time points during a 6 month period of diabetes. The degree of diabetes is evaluated in 2 ways: tail blood glucose measured following a 6 hour fast and glycated hemoglobin levels. The initial degree of neuropathy is screened using the methods discussed below. Detailed measures of neuropathy are employed when the initial screening instruments indicate a profound or unique phenotypic difference. This document contains protocols used by the DiaComp staff to examine and measure diabetic neuropathy at the whole animal, tissue and cellular levels.

Diabetic Complication:

Neuropathy

Materials

Reagents:

Glutaraldehyde, electron microscopy grade
Osmium tetroxide, electron microscopy grade
Uranyl Acetate, electron microscopy grade
Lead Citrate, electron microscopy grade
0.1 M Phosphate buffered saline (pH 7.2, 150 mM NaCl)

Equipment:

Specimen vials
Embedding molds
Formvar-coated copper slot grids or mesh grids
Parafilm
Jeweler's forceps
1, 3, 5, 12, 30, 60 cc syringes
Grid storage boxes

Solutions: NOTE All solutions should be filtered through the appropriate Millex-GV4 .22µm filters prior to use. NOTE, Glutaraldehyde, Osmium tetroxide, Uranyl Acetate and Reynolds Lead Citrate are harmful and should be used with proper protection and ventilation. Osmium tetroxide is particularly bad. It will oxidize any thing that it touches and turn it jet black! (including your corneas) It is denatured with cooking oil.

Solutions:

2.5% EM grade Glutaraldehyde in 0.1M PBS*
2% Osmium tetroxide in 0.1M PBS*
2% Uranyl Acetate in dd H20*
Reynolds lead citrate*

1 **Fixation**

Tissue (fixed) Following intracardiac perfusion, dissect tissue of interest into 3 mm cubes, postfix in 4% para, 2.5% glutaraldehyde 4-12 hours, 4ºC

Tissue (fresh) Quickly dissect tissue of interest into 3 mm cubes and fix by immersion in 4% para, 2.5% glutaraldehyde at 4ºC, at least 12 hours, replace fix with fresh at least once, place tissue on a rocker.

Cells Remove media, rinse gently in HBSS, fix in 4% para, and 2.5% glutaraldehyde at 4ºC for 4-8 hours, place cells on rocker.

2 **Embedding**

2-10 minute rinses in Sorensen's buffer

Post fix in 1% osmium tetroxide in Sorensen's buffer for 1 hour.

Rinse for 10 minutes in Sorensen's buffer.

Rinse 2 X 5 minutes in dd$H_2$0 to remove phosphate.

En bloc stain in aqueous 3 % uranyl acetate for 1 hour.

Dehydrate 10 minutes each in 30, 50, 70, 95, 100, 100 % EtOH.

Infiltrate with EPON resin using this schedule:

3:1 100 % EtOH: EPON	1 hour
1:1	1 hour
1:3	overnight
Full strength	2 hours
Fresh full strength	2 hours

Place in embedding mold with label WRITTEN IN PENCIL
Polymerize 60ºC for 24 hours

Ultrathin sections are cut at the CBL (Cell Biology Lab).

3 **Reynold's Lead Citrate Stain for TEM**

0.44 g Lead Nitrate
0.55 g Sodium Citrate
10 ml freshly boiled, double distilled water

Combine above ingredients in a 15 ml tube. Shake well. A white, flocculent precipitate will form. Agitate on rocking platform for at least 30 minutes.

Add 1.6 ml 1.0 N NaOH which has been freshly prepared in boiled double distilled water. (0.4 g NaOH in 10 ml boiled dd H_2O).

Mix by inverting a few times. To avoid introducing air, **DO NOT SHAKE!**

Cap tightly.

Store at 4ºC

Staining Procedure

Prepare a staining chamber by placing NaOH pellets beside a strip of dental wax inside of a petri dish. Place a few drops of water on the pellets. Cover and let it sit for a few minutes before using.

Avoid breathing into staining chamber to reduce exposure to CO_2

Place on the wax one drop of stain for each grid to be stained. The first drop should be discarded off to the side. Discard any left-over stain remaining in the pipette.

Float grids section side down on drops of stain.

Stain for 5 minutes.

Quickly rinse by dipping 10 times in each of four changes of freshly boiled, cooled double distilled water.

Blot dry on clean filter paper.

4 **Uranyl Acetate Stain for TEM**

0.06 g uranyl acetate
20ml 1:1 methanol: 70% EtOH

Mix well. Cover with foil to block out light.

Consider UAc to be radioactive.

Place clean dental wax or parafilm inside of a clean Petri dish.

Place one drop of stain for each grid on the wax, discarding the first drop off to the side.

Float one grid, specimen side down, on each drop.

Stain for 10 minutes.

Rinse by dipping each grid 10 times in each of two changes of 1:1 methanol:70% EtOH then two changes of freshly boiled, then cooled, double distilled water.

Blot dry on clean filter paper and protect grids from dust by covering with a clean Petri dish lid.

Let grids dry before post staining with lead citrate.

5 **SORENSEN'S PHOSPHATE BUFFER**

Solution A: 0.2M Dibasic Sodium Phosphate
26.8 g Na_2HPO_4 $7H_2O$
Distilled water to a final volume of 500 ml

Solution B: 0.2M Monobasic Sodium Phosphate
13.8 g NaH_2PO_4. H_2O
Distilled water to a final volume of 500 ml

Take a portion (about half) of Solution A, place it on a slow magnetic stirrer, and measure its pH. Add Solution B drop wise to bring its pH down to pH 7.4. Then dilute this final volume with an equal volume of distilled water. This is the working buffer solution. (0.1M Sorensen's buffer)
Store Solution A, Solution B, and the working solution in the refrigerator.