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Neuropathy Phentotyping Protocols - Immuno-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

Reference:

Kumagai et al., Brain Research 1996, 706:313-317



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)
- 0.05% Tween 20 (a.k.a.polyoxyethylenesorbitan monolaurate (Sigma P 7949)
- UltraPure bovine serum albumin, electron microscopy grade

Equipment:

- Formvar-coated *nickel* slot grids, or mesh grids
- Parafilm
- Jeweler's forceps
- Porcelain EM grid dishes
- 1-, 3- and 12- cc syringes
- Millex Gv filters, .22μ
 - Large (25mm): Cat. #SLGV R25LS
 - Med. (13mm): Cat. #SLGV 013 SL
 - Small (4mm): Cat. #SIGV 004 SL
- Grid storage boxes

Solutions: NOTE, All solutions (except antibody) 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 can be denatured with cooking oil.

Solutions: All Solutions should be made fresh (it may be stored at 4°C overnight if necessary)

Buffer A:

0.1 M PBS
0.05% Tween-20
0.1% Fish gelatin
1% BSA

Blocking Buffer:

2.5% UltraPure BSA,
2.5% normal goat serum in Buffer A
PBS 0.1M



1% EM grade Glutaraldehyde in 0.1M PBS*

2% Osmium tetroxide in 0.1M PBS*

2% Uranyl Acetate in dd H₂O*

Reynolds lead citrate

See protocols

Uranyl Acetate Reynolds Lead Citrate

Troubleshooting

Before start

Formvar is extremely fragile and is punctured very easily. Handle grids with care at all times to avoid touching the formvar with the tips of the forceps. **Never** set the grids down on countertops or tissue— they should be suspended on a couple of drops buffer or stored in grid boxes—and **never** attempt to handle the grids with anything other than jewelers' forceps.

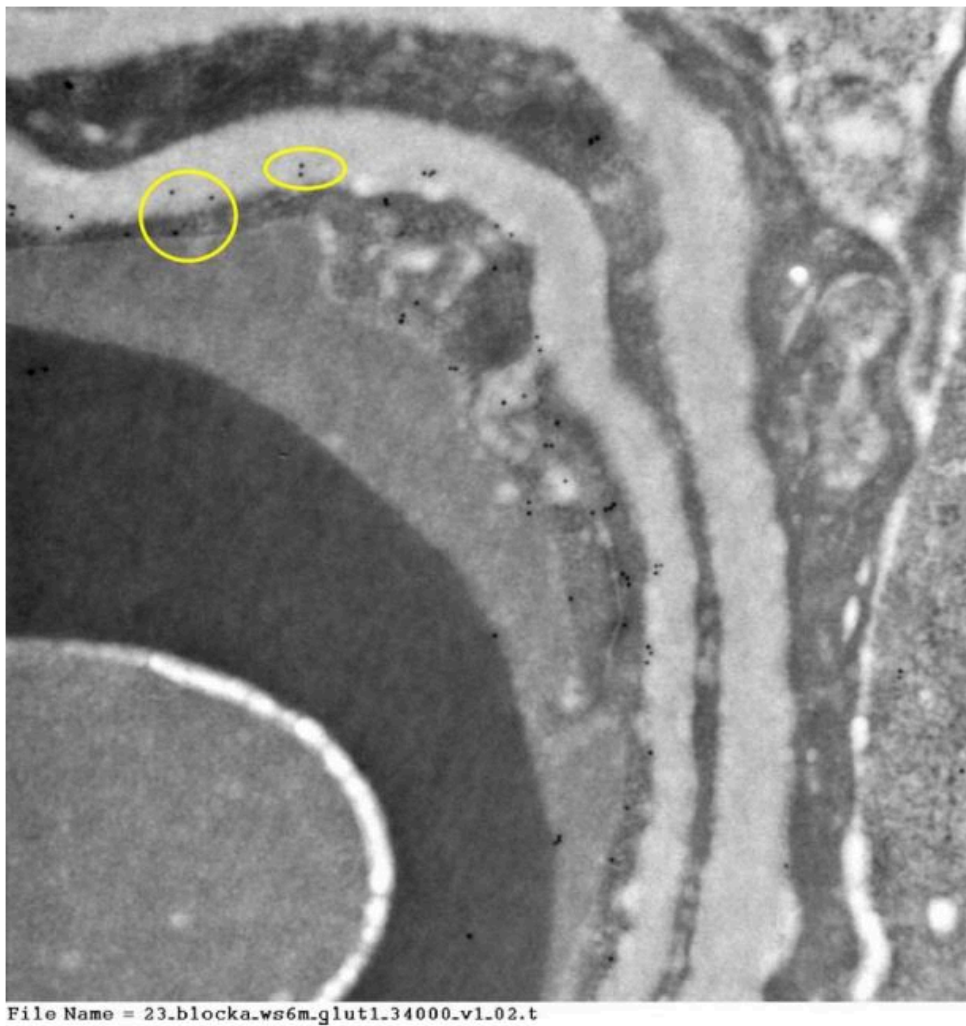
- 1 To perform immunohistochemical staining of the ultrathin sections, float the grid on 1-2 drops of buffer. Some of the grids may sink to the bottom of the drops—this is OK as long as there are no holes in the formvar itself (in this case the grid is worthless).
- 2 *Ultrathin sections are cut at the CBL (Cell Biology Lab). Use formvar coated nickel slot grids.*

Grids are hydrated for 10 minutes at 22°C in Buffer A and then transferred to blocking Buffer for 30 min. at 22°C
- 3 Excess blocking serum is blotted off with filter paper (do not touch the formvar with the filter paper) and each grid is transferred to a 12-well porcelain dish containing 200-300µl of Primary.
 - When using the filter paper, just barely touch the edge of the grid.
- 4 The grids are placed in a plastic box containing moist filter papers (or c fold towels) and incubated overnight. Make sure to wet the filter paper enough so that there is no chance the water will evaporate!
- 5 The next day, fresh buffer is made and each grid is “jet washed” with 5 drops of Buffer A.
Note: “Jet washing” = rinsing the grids with drops of buffer by dripping the buffer on the tip of forceps holding the grid. **DO NOT** drip the buffer directly on the formvar, it will puncture the film coating.
- 6 Wash the grid by incubation on 2 drops of Buffer A for 10 minutes.
- 7 Repeat the wash 5 more times.
- 8 Incubate grids on Secondary Antibody for 10 (10 nm colloid gold-IgG at 1:60) dilution in Buffer A for 2 hours.
- 9 Cover box.
- 10 A Jetwash grid with 5 drops of .1 M PBS (pH 7.4) and washed by incubation on 2 drops of .1M PBS for 10 minutes.
- 11 Repeat wash step 5 times. (6X total)
- 12 Fix grids in 1% glutaraldehyde in .1M PBS(7.4) for 5 minutes.

- 13 Jetwash 5x with DDW.
- 14 Treat specimens with 2% Osmium Tetroxide in .1M PBS at 22°C for 10 minutes.
- 15 Immediately rinse in filtered DDW for minutes. **Light protect**
- 16 Specimens are counterstained in 2% aqueous urinal acetate for 30 minutes at 22°C.
Light protect:
In certain tissue, Reynold's lead citrate is used as an additional counterstain. See the protocols for lead citrate and uranyl acetate.
- 17 Jetwash specimens with 7 drops of filtered DDW and incubate on 2 drops of filtered DDW for 3 minutes x 4 times.
- 18 Air dry and place in grid box: **Very important!!!** Mark grid boxes clearly. Individual grids cannot be marked.

Figure 2

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On grid immuno-electron microscopy confirms the subcellular localization of proteins. In this image, 40 nm gold particles mark the location of GLUT1. Double labeling is performed by sequential labeling of proteins with different size gold particles.