ANIMAL MODELS OF DIABETIC COMPLICATIONS CONSORTIUM
University of Michigan (U01 DK60994)

UPDATE REPORT
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Eva L. Feldman, M.D., Ph.D.
Neuropathy Phenotyping Core

Principal Investigator:
Eva L. Feldman, M.D., Ph.D.
University of Michigan
Department of Neurology
4414 Kresge III, 200 Zina Pitcher Place
Ann Arbor, MI 48109-0676
Phone: (734) 763-7274 Fax: (734)763-7275
Email: efeldman@umich.edu

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Part A:

Principal Investigator's Summary

1. Project Accomplishments:

Although several animal models of diabetic neuropathy exist, the pathological abnormalities in the rodent peripheral nerve do not duplicate those encountered in human neuropathy (1; 2). **The goal of the Neuropathy Core is to develop animal models of diabetic neuropathy that more closely resemble the human condition.** The Neuropathy Core was established at the onset of the AMDCC to determine if new mouse models of diabetic neuropathy had a phenotype that resembled the neuropathic condition in man. The Neuropathy Core was also mandated to assist in the development of these new mouse models of diabetic neuropathy.

Our main strategy is to use phenotyping criteria in animals that parallel the clinical criteria used in man. We developed a standard operating procedures manual entitled AMDCC Neuropathy Phenotyping Manual. This Manual, appended to the current report, outlines in detail our methodology for both initial and more advanced phenotyping.

A mouse model of diabetic neuropathy requires the key features present in the human condition (1; 3). These include:

1. *Evidence of clinical loss of sensory function*
2. *Electrophysiological evidence of nerve impairment*
3. *Anatomical evidence of nerve fiber loss*
4. *Advanced measures of neuropathy*

In man, diabetic neuropathy is a progressive disorder, with signs and symptoms that parallel the loss of nerve fibers that occurs over time with poor glycemic control (1-3). 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 and correlated with glycemic control. Diabetic control is evaluated in 2 ways: tail blood glucose measured following a 6 hour fast and glycated hemoglobin levels done at end of study. The initial degree of neuropathy is screened using the 3 methods discussed below. Detailed measures of neuropathy are employed when the initial screening instruments indicate a profound or unique phenotypic difference.

1. Evidence of clinical loss of sensory function

The progressive distal to proximal loss of nerve fiber function in animals with diabetes leads to decreased sensation in the animal's paws and tail. Quantitative assessment of sensory function reflects the loss of nerve function. The first component of mouse phenotyping for the presence of diabetic neuropathy is a quantitative assessment of paw and tail sensation. The time of withdrawal from a heat stimulus applied separately to the paw or to the tail is measured on a monthly basis.

2. Electrophysiological evidence of nerve impairment

Electrophysiology is considered the "gold standard" for evaluation of neuropathic deficits in man. Nerve conduction studies provide a quantitative measurement of sensory and motor nerve function. The second component of mouse phenotyping for the presence of diabetic neuropathy is assessment of motor and sensory nerve conductions.

3. Anatomical evidence of nerve fiber loss

Deficits in motor or sensory function may be caused by disruptions of axonal function or axonal loss. Analysis of myelinated fiber density, peripheral nerve structure and intraepidermal fiber density (IEFD) all lend insight into the function of the peripheral nervous system. The third component of mouse phenotyping is assessment of small myelinated and unmyelinated fibers using footpad biopsies.

4. Advanced measures of neuropathy

When the initial screening of a mouse model indicates the presence of a profound or unique neuropathy, a more detailed analysis is performed. These analyses, outlined in the Manual, include transmission electron microscopy (TEM), poly TdT mediated dUTP-biotin nick end labeling (TUNEL), immunohistochemistry (IHC) for cleaved caspase-3, and oxidative stress adducts and biochemical measures of oxidative stress adducts and enzyme activity.

Our major achievements are presented in Tables 1 and 2 and discussed in detail by individual mouse model. Table 1 represents animal models that have completed our neuropathy phenotyping protocol. Table 2 represents animals currently being phenotyped by our Core.

Table 1 Animal Models: Complete Neuropathy Phenotyping

Animal Model	Background Strain	Current Status	Phenotyping (begin-end)
db/db	C57B.KLS	phenotyping completed; significant neuropathy	Completed 12/04
GCLC +/- db/db	C57BL/6J	phenotyping complete; modest neuropathy	Completed 12/04
Glut4-/- and +/- STZ	C57BL/6J	phenotyping complete; no neuropathy	9-03 to 9-04
fyn -/- and +/- STZ	129SvJ	phenotyping complete; no neuropathy	9-03 to 9-04

db/db C57BKS mice. AMDCC data over the past 2 years have now amply demonstrated that C57Bl/6J mice are relatively resistant to developing nephropathy and neuropathy. Although db/db C7BL/6J mice are more susceptible to microvascular complications than the STZ C57Bl/6J animals, this model remained generally resistant to progressive nephropathy and neuropathy despite genetic modifications. Moreover, this model demonstrated a return to normoglycemia in several, though not all, of the AMDCC centers. In continued efforts to find the best genetically modified mouse for further neuropathy studies, we phenotyped the BKSLdb/db animals. Tail flick and hind paw analyses (weeks 12 and 24) and sciatic nerve conduction studies (weeks 12 and 24) confirm significant neuropathy in these animals (Fig. 1).

GCLC+/- db/db mice. The γ-glutamate cysteine ligase (GCLC) heavy chain was targeted for disruption to produce a glutathione knockout mouse by 2 independent groups. This enzyme is an essential enzyme in glutathione synthesis. Homozygous disruption of the heavy subunit of

GCLC is embryonically lethal. In contrast, the GCLC +/- mice are viable but show substantial decreases in GCLC protein and activity, and an approximately 20% decrease in glutathione levels. Thus, the GCLC +/- mouse should be a useful genetic model for mild endogenous oxidative stress, which could be substantially increased in diabetes. Given the resistance of normal C57Bl/6J mice to STZ diabetes, we bred these mice into the db/db C57BL/6J background resulting in 4 experimental groups: GCLC+/- db/db, GCLC+/+ db/db, GCLC+/- db/+ and GCLC+/+ db/+. At 24 weeks, there is an increase in both tail and hind paw sensory latencies between the diabetic and control mice of each genotype but no significant difference between the GCLC+/+ dbdb and GCLC+/- dbdb mice ($P = 0.4$) (Fig. 2). Tail distal motor latency was the same for all groups (Fig. 2). Tail sensory nerve conduction velocities were decreased by diabetes in both genotypes but not significantly different between the GCLC+/+ dbdb and GCLC+/- dbdb mice ($P < 0.3$). Sciatic motor nerve conduction velocities (Fig. 2) were not affected by diabetes in the GCLC+/+ mice.

GLUT4 -/- C57BL/6J mice. GLUT4 is the major insulin sensitive glucose transporter and was recently linked to cause insulin resistance (4). Following induction of diabetes by STZ injection, these mice were examined for sensory deficits and changes in nerve conduction velocity as described for the previous mouse models. Changes in tail flick or hind paw sensory function were not detected between the GLUT4-/- and GLUT4+/+ animals. Minor differences were detected between the diabetic and control animals but failed to reach significance (Fig. 3A, B).

Fyn -/- mice 129SvJ. Fyn is a src family protein kinase with multiple known cellular functions. Deletion of Fyn in mice is associated with foot process effacement and is consistent with emerging evidence that the Nephrin protein (slit diaphragm) complex functions in part to regulate the podocyte cytoskeletal architecture. The hypothesis was that decreased Fyn dosage would predispose the podocyte to injury in the diabetic environment. The effects of fyn deletion on the peripheral nervous system, whether direct or indirect, were unknown. Therefore, following induction of diabetes by STZ injection, these mice were also phenotyped for neuropathy. Similar to the GLUT4-/- mice the fyn -/- mice did not display any signs of neuropathy related to their genotype (data not shown). For both the GLUT4-/- and fyn mice, tissues were harvested and are currently stored at -80^{o}C. Further analyses have not been undertaken due to lack of a relevant phenotype; however, DRG, sciatic nerves and foot pads are available should new insights deem these analyses necessary.

2. <u>Collaboration Within Your Group</u>:

The Neuropathy Phenotyping Core has been an integral part in developing new mouse models of diabetic neuropathy as part of the grant to Dr. Brosius. Rodent models of diabetes fail to develop changes that closely resemble human diabetic nephropathy or neuropathy. While the reasons for the resistance of rodents to full-blown complications are likely multiple, they may include an increased resistance to oxidative stress or the absence of important genetic susceptibility genes (5; 6). Our general strategic approach to this dilemma is to accelerate the injury of diabetes by predisposing critical cells within the peripheral nervous system, including sensory neurons and their supporting Schwann cells, to glucose-mediated oxidative injury by 1) increasing glucose uptake via increased expression of facilitative glucose transporters or 2) by reduction of enzymes that reduce oxidative stress. This section will discuss the models we are currently phenotyping in collaborations with Dr. Brosius.

Phenotyping has begun on the initial available GLUT1tgC57BLKSdb/db mice on high fat chow. The small numbers of animals do not allow for valid statistical comparisons between the groups. These comparisons will be completed as more animals become available for phenotyping. As expected, by 16 weeks of diabetes, both tail flick and hind paw withdrawal latencies are prolonged in both the GLUT1tgC57BLKSdb/db mice and the diabetic mice without

the GLUT transgene, the C57BLKSdb/db. These same trends occur in the nerve conduction studies. Tail motor distal latency is prolonged while sciatic motor nerve and tail sensory nerve conduction velocities are decreased in animals with the BKLSdb/db genotype, regardless of transgene expression. Interestingly, there is a suggestion of a transgene effect when examining the tail sensory nerve conduction velocities. More animals are needed to determine if this is a real effect of the transgene (Fig. 4). When examining the control animals, GLUT1tgC57BLKSdb/+ and C57BLKSdb/+ have mildly prolonged latencies when compared to db/+ animals not on the BKLS background and fed normal chow. Nerve conduction velocities are however comparable.

We have begun phenotyping the Nestin Cre//SOD2loxP/loxP mice. While only 4 control and 3 experimental animals have reached the age of 28 weeks with STZ diabetes, the initial results are encouraging. The Nestin Cre//SOD2loxP/loxP animals all have poorer measures of nerve function suggesting a more neuropathic phenotype. Hind paw and tail flick latencies are prolonged (Fig. 5). Tail motor distal latencies are prolonged while sciatic motor nerve and tail sensory nerve conduction velocities are decreased when compared to the Nestin Cre//SOD2loxP/+ animals. These animals remain alive and will undergo repeat phenotyping in 4 weeks.

Finally, as described in Dr. Brosius' report, interactions between the nephrology and neuropathy investigators at Michigan occur almost daily. Coordination of nephropathy and neuropathy phenotyping begins with the birth of the animals and continues through their assignment to an experimental group to final tissue collection.

Table 3. Animal Models: Ongoing Neuropathy Phenotyping for AMDCC Investigators inside of U of M

Glut1tgC57BLKSdb/db on high fat chow	C57BKLSdb/db	Phenotyping in progress	1/05 to current
nestin Cre//SOD2 loxP/loxP	C57BL/6J	Phenotyping in progress	1/05 to current
synapsin Cre//SOD2 loxP/loxP	C57BL/6J	breeding currently	4/04 to current
Glut4-/- Akita	C57BL/6J	breeding currently	1/05 to current

3. Collaborations With Other AMDCC Groups:

Three AMDCC groups are actively using the Neuropathy Phenotyping Core: the Rockefeller University (Dr. Breslow), University of North Carolina, Chapel Hill (Drs. Clemmons and Nichol) and Cleveland Clinic (Dr. Danshgari). These collaborations are presented in Table 4.

Table 4. Animal Models: Ongoing Neuropathy Phenotyping for AMDCC Investigators outside of U of M

LDLRO mice	C57BL/6J	Phenotyping in progress	10/04 to current
LDL/Pdx-/- and Pdx -/+ mice	C57BL/6J	Phenotyping in progress	1/05 to current
Pig (hypercholesterolemic)		Phenotyping in progress	10/04 to current

Dr. Breslow's group from the Rockefeller University sent over 30 samples for IEFD analysis from the ob/ob LDLR0 and control LDLR0 mice. The feet were shipped in 4% paraformaldehyde and upon arrival were dissected, cryoprotected, embedded and sectioned. Immunohistochemistry for PGP9.5 confirms the presence of epidermal fibers. The total analysis is scheduled for completion by the end of March 2005. In addition to the foot pads, 2 year old pdx+/+ and pdx+/- mice were sent for neuropathy phenotyping. These animals were examined for nerve function according to our established protocols prior to tissue harvest. Sciatic nerves and DRG were collected and analyzed for evidence of oxidative stress. Foot pads are currently being assessed for IEFD. The eyes were placed in 10% formalin and shipped to Dr. Kern and the bladders were cryoembedded for sectioning for Dr. Danshgari. Kidneys were given to the Brosius laboratory here at Michigan.

We also received the LdL/Pdx mice from Dr. Breslow. The LdL+/+ Pdx +/+ and LdL+/+ Pdx +/- completed sensory and electrophysiological phenotyping. There were no differences in hind paw withdrawal latencies but tail flick latencies reached statistical difference ($p < .05$) with an increased withdrawal time observed in the LdL+/+ Pdx +/+. There were no statistically significant differences in electrophysiological measurements. IEFD analysis is pending on these mice.

Minor difficulties have been encountered in the analysis of IEFD in the pig skin sent by Drs. Clemmons and Nichol. Our first attempts at sectioning and staining failed. Due to the amount of subdermal fat, the sections did not stay on the slides through the entire immunohistochemistry procedure. We are currently examining the skin under two different protocols. The first was sent to us by Dr. Andrew Mizisin at the University of California San Diego (UCSD) (via the helpful intervention and guidance of Dr. Calcutt, UCSD). Biopsy punches of the skin were taken and 80% of the fat was removed. These samples were sent to the Cancer Center Histology Core for paraffin embedding and sectioning (4 μm). Initial immunohistochemistry of these samples was unsuccessful. It may be that paraffin embedding is not compatible with our current PGP9.5 antibody. We have attained new PGP9.5 antisera and will try antigen retrieval techniques with the current antibody. Examination of 20 μm cryosections of re-biopsied, fat trimmed pig skin is also underway.

In the past year, Dr. Firouz Danshgari brought his staff to the Neuropathy Phenotyping Core to measure bladder elasticity of selected animal models. Dr. Danshgari and his staff have established the feasibility of performing this test on a recovery basis and that this technique is an important addition to overall model characterization. Future experiments will include testing of bladder function prior to euthanasia and tissue harvest.

4. <u>Pertinent non-AMDCC Collaborations</u>:

Listed below are the major collaborative projects related to the consortium goals but independent of AMDCC:

1) We have obtained IRS -/- mice from the Joslin Clinic. These mice are 1 year old. Our plan is to maintain these animals for an additional 6 mo prior to phenotyping.

2) The JDRF Center for the Study of Complications in Diabetes. This Center encompasses a number of collaborative projects exploring the role of glucose transporters, oxidative stress, and growth factors in diabetic complications. It also includes several clinical projects testing antioxidants and other agents in the treatment of diabetic complications. The Center Director is Dr. Feldman. Drs. Brosius, Russell and Stevens have projects in this Center.

3) Dr. Feldman is the Principal Investigator for several collaborative NIH grants investigating the etiology, pathogenesis and treatment of diabetic polyneuropathy. Dr. Russell is a Co-Investigator on several of these grants.

4) Dr. Feldman is an investigator in neuropathy aspects of the multi-institutional Epidemiology of Diabetes Interventions and Complications (EDIC) study.

5) Dr. Russell is the Principal Investigator on a NIH project investigating the role of IGF-I in oxidative stress and apoptosis in diabetic neuropathy; collaborators on this project include Drs. Feldman and Michael Brownlee (Albert Einstein College of Medicine). Dr. Russell is also the Principal Investigator on a VA grant studying IGF-I and Schwann cells in neuropathy.

5. Address Previous EAC Comments:

Please see Dr. Coffman's report.

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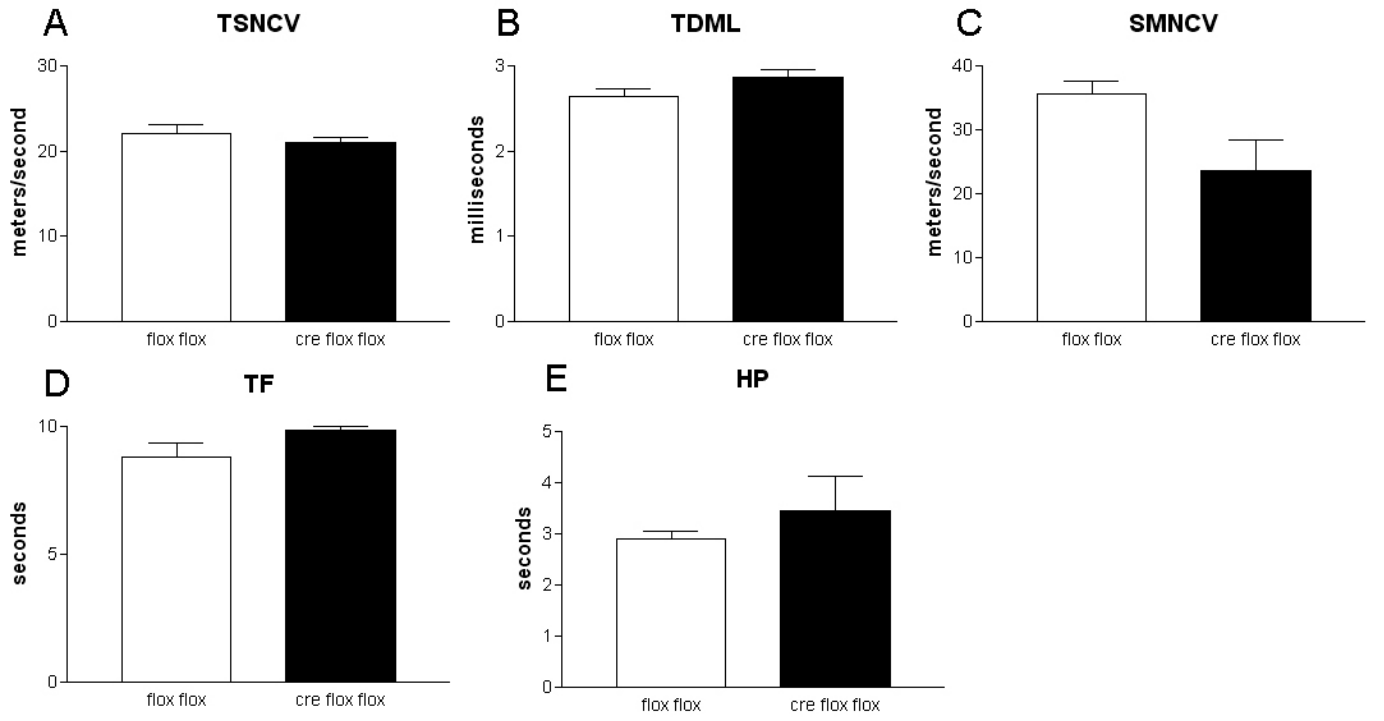

Fig. 5. Nerve conduction velocities and sensory measures in Nestin Cre//SOD2loxP/loxP. Panels A-E illustrate changes in nerve function and physiology in the Nestin Cre//SOD2loxP/loxP and loxP/- mice following 28 weeks of diabetes. A) Tail sensory nerve conduction velocity, TSNCV, B) Tail distal motor latency, TDML, C) Sciatic motor nerve conduction velocity, SMNCV, D) Tail Flick TF, E) Hind Paw, HP.

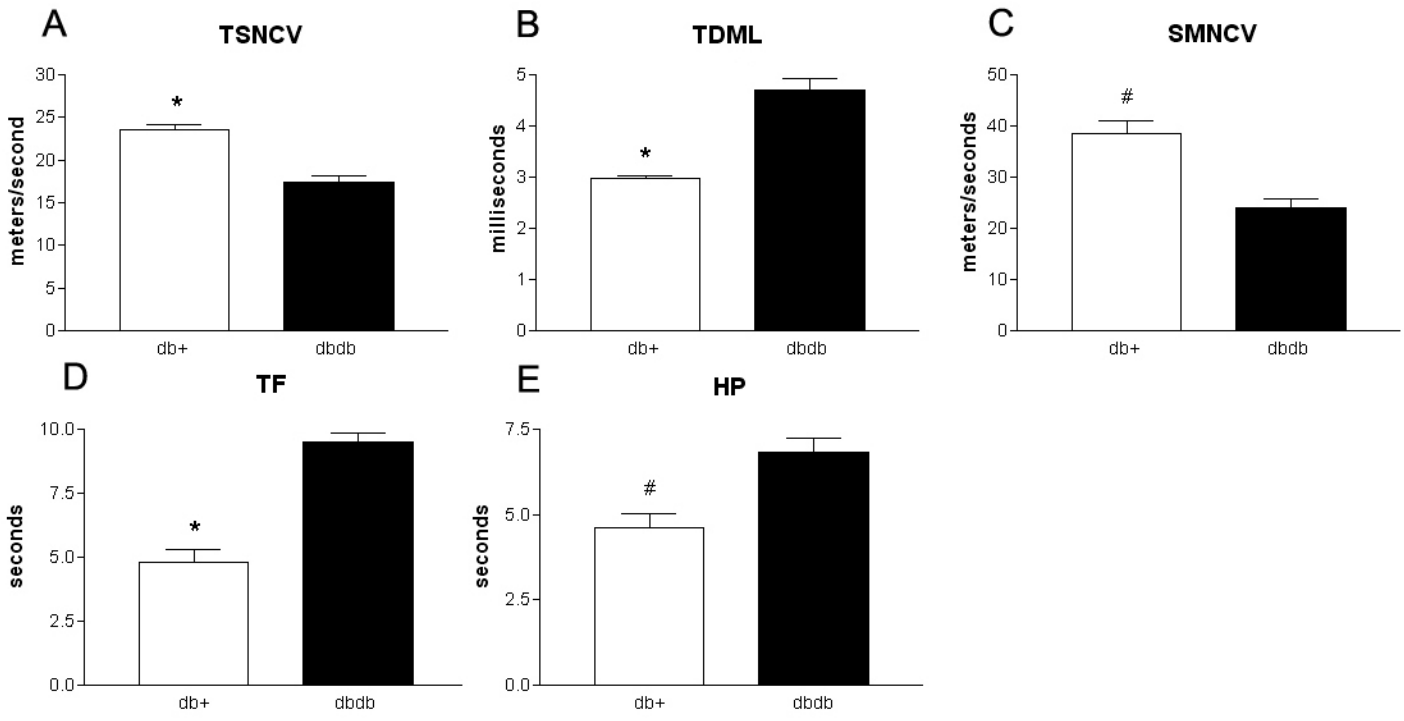

Fig. 1. Nerve conduction velocities and sensory measures in BKLS Db mice. Panels A-E illustrate changes in nerve function and physiology in the BKLS dbdb and db+ mice following 24 weeks of diabetes. A) Tail sensory nerve conduction velocity, TSNCV, B) Tail distal motor latency,TDML, C) Sciatic motor nerve conduction velocity,SMNCV, D) Tail Flick,TF, E) Hind Paw, HP.

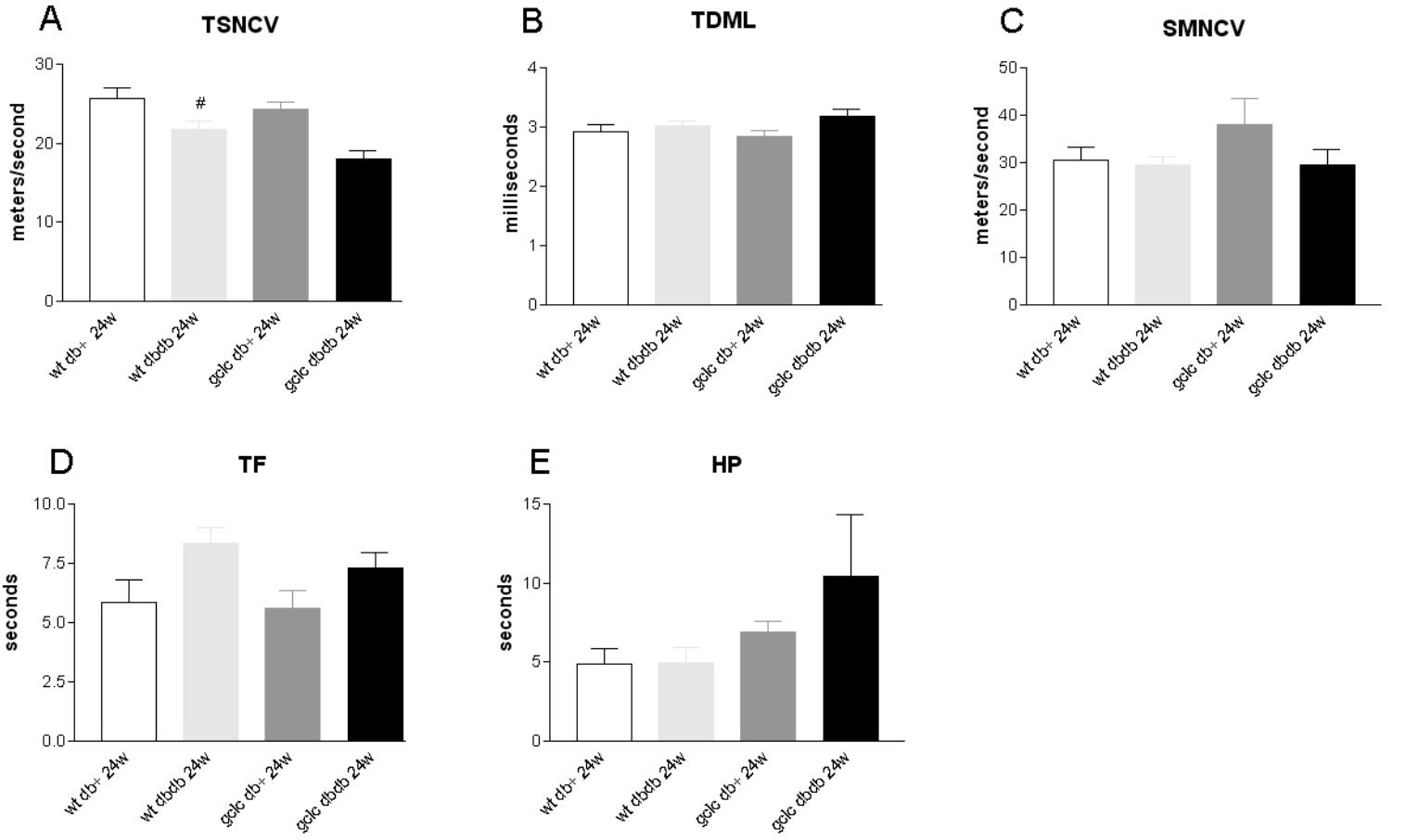

Fig. 2. Nerve conduction velocities and sensory measures in GCLC+/+ and +/- Db mice. Panels A-E illustrate changes in nerve function and physiology in the GCLC+/+ and +/- dbdb and db+ mice following 24 weeks of diabetes. A) Tail sensory nerve conduction velocity, TSNCV, B) Tail distal motor latency, TDML, C) Sciatic motor nerve conduction velocity, SMNCV, D) Tail Flick, TF, E) Hind Paw, HP.

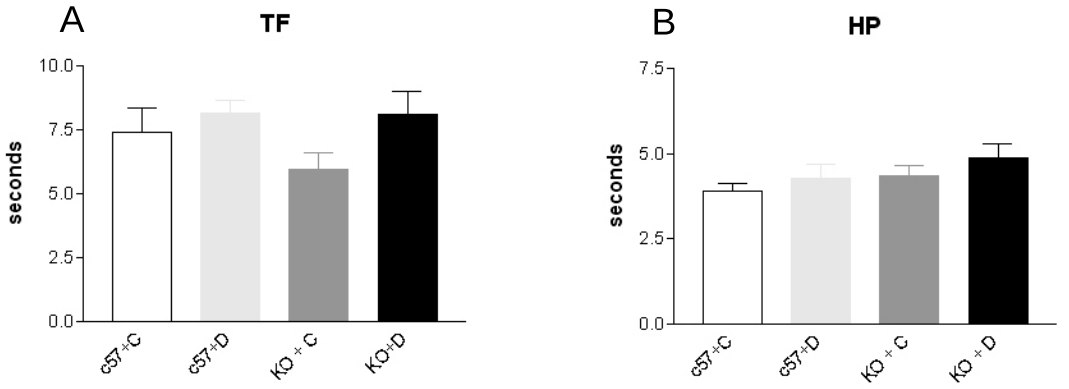

Fig. 3. Tail flick and hind paw latencies measured in GLUT4 -/- ,+/+C57BL/6J mice. A) diabetic and control tail flick (TF) and B) hind paw (HP) latencies.

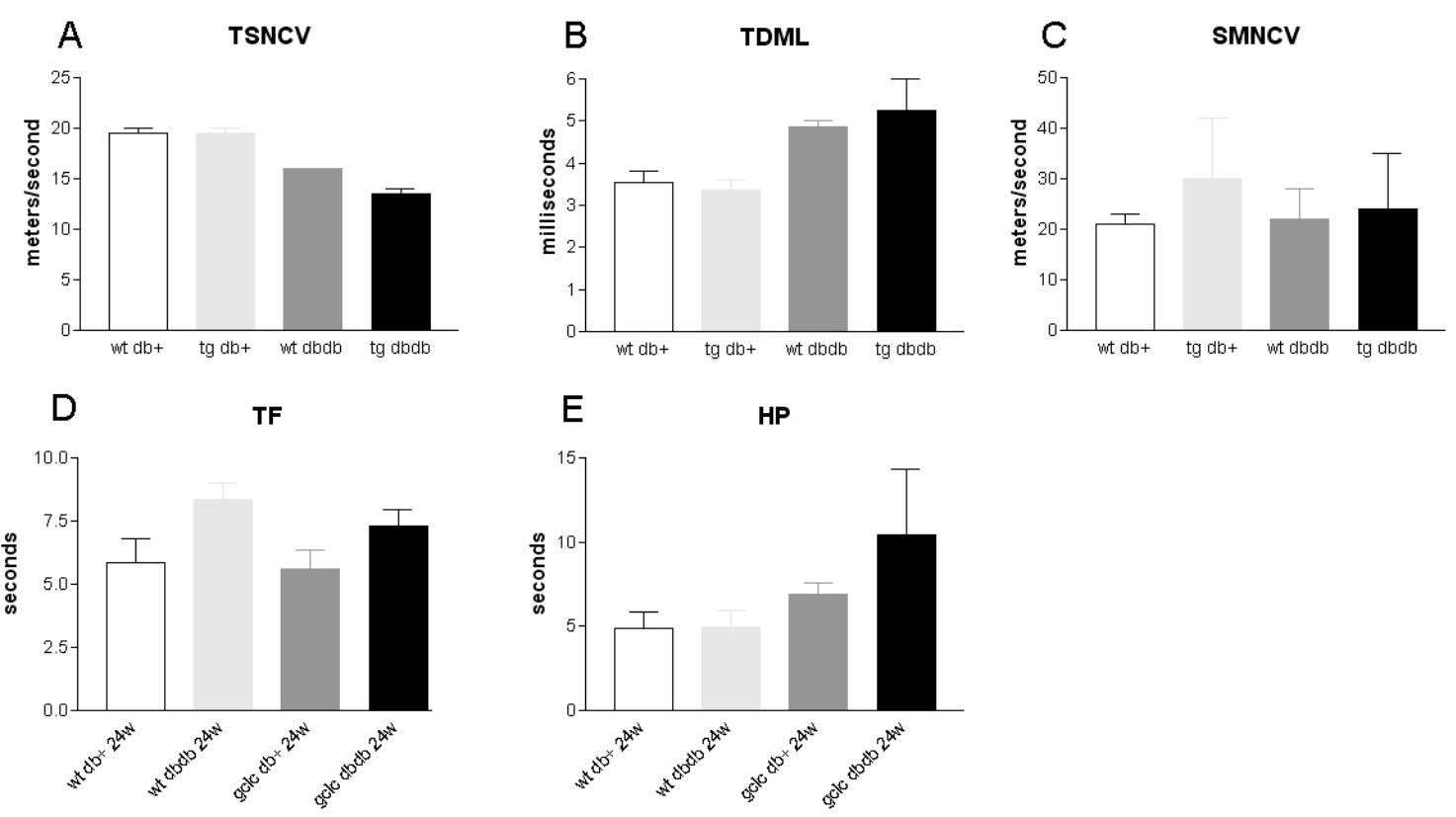

Fig. 4. Nerve conduction velocities and sensory measures in GLUT1tgC57BLKSdb/db. Panels A-E illustrate changes in nerve function and physiology in the GLUT1tgC57BLKS dbdb and db+ mice following 16 weeks of diabetes. A) Tail sensory nerve conduction velocity, TSNCV, B) Tail distal motor latency, TDML, C) Sciatic **motor nerve conduction velocity, SMNCV, D) Tail Flick, TF, E) Hind Paw, HP**

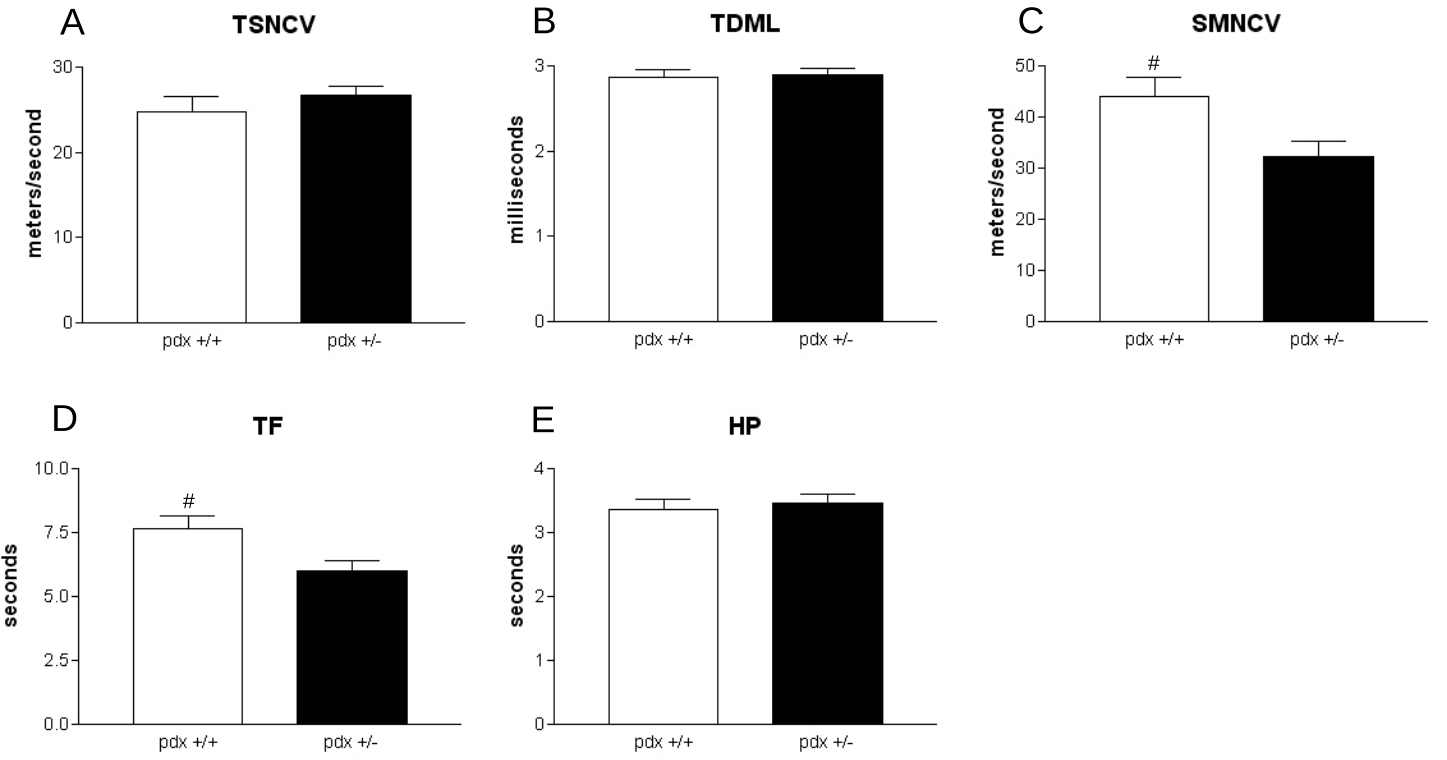

Fig. 6. Nerve conduction velocities and sensory measures in LdL+/+ Pdx +/+ Panels A-E illustrate changes in nerve function and physiology in the LdL+/+ Pdx **+/+** and Pdx+/-- mice following 104 weeks of diabetes. A) Tail sensory nerve conduction velocity, TSNCV, B) Tail distal motor latency, TDML, C) Sciatic motor nerve conduction velocity, SMNCV, D) Tail Flick, TF, E) Hind Paw, HP.

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(In Alphabetical Order)

AMDCC

NEUROPATHY

PHENOTYPING

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 AMDCC staff to examine and measure diabetic neuropathy at the whole animal, tissue and cellular levels.

APOPTAG (PEROXIDASE) SECTIONS ON SLIDES

Reagents: DNase
DN buffer (30 mM Tris base, pH 7.2, 4 mM $MgCl_2$, 0.1 mM dithiothreitol)
4% paraformaldehyde
Phosphate buffered saline (0.1 M pH 7.2, 150mM NaCl)
30% H_2O_2
Proteinase K 3 ug/ml
DAB or AEC
Counterstain
50, 70, 95, 100% ETOH
Hemo-De
Cover glass
Permount

Supplies Slotted Wheaton dishes or Coplin jars
Square Wheaton dishes or Coplin jars
Slide racks and handles
Humid chamber
Oven
Ice and ice bucket
Pap pen
Pipetmen, pipet tips, microfuge tubes etc

Kit (Intergen) Equilibration buffer aliquots, -20° C
TdT enzyme, aliquots, -20° C
Reaction buffer, aliquots, -20° C
Stopwash buffer, aliquots,-20° C
POD tagged anti-digoxygenin antibody 4° C

Preparation:

1. Thaw equilibration buffer, TdT, reaction buffer and stopwash on ice.
2. Prepare working strength TdT enzyme.
3. Thaw enough reaction buffer and TdT to cover 30 ul/well TdT: reaction buffer, 33 µl: 77 µl.
4. Prepare stopwash buffer, add 1 ml buffer to 34 ml of ddH_20.
5. Prepare a humidified chamber.
6. Prepare anti-digoxygenin antibody.
7. Set oven to 37° C.
8. Prepare DNase

Procedure:

1. Thaw and label sections, bench top or warm plate.
2. Ring sections with Pap Pen.
3. Specify 1 slide as positive and 1 slide as negative control.

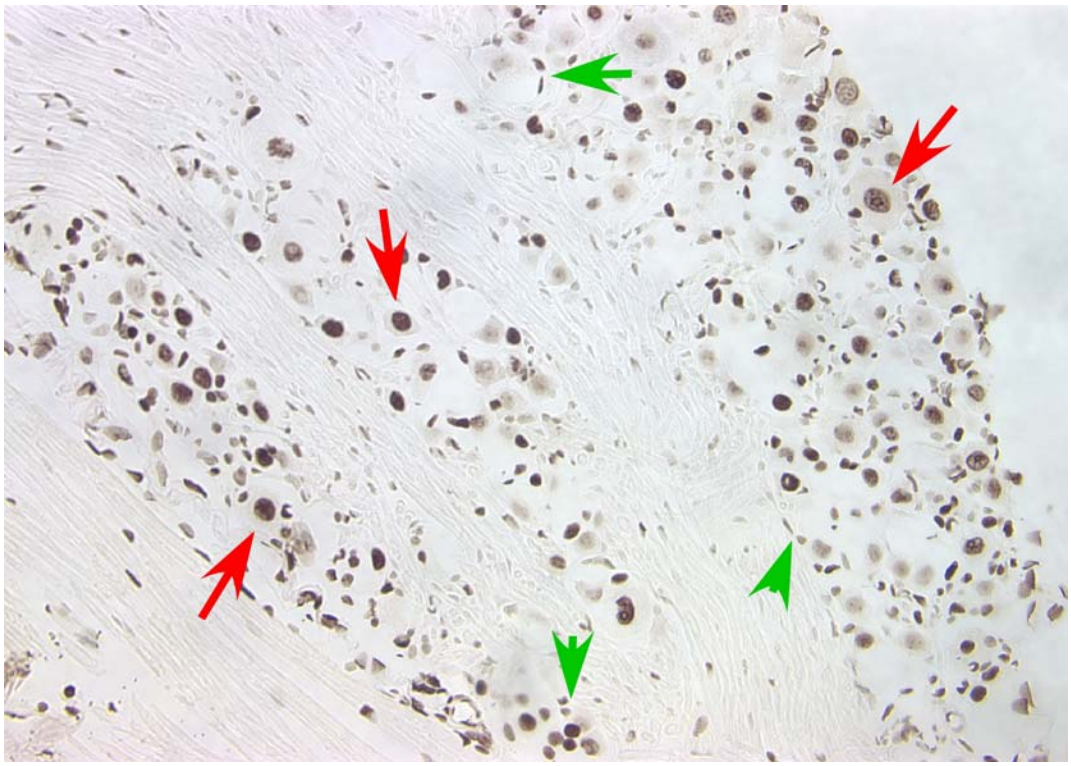
This is a must for every set of TUNEL slides.

4. If sections or cells have not been fixed, fix with 4% paraformaldehyde for 10 min followed by 3 X 10 min rinses in PB.
5. If sections or cells already fixed, hydrate in PBS for 5 minutes.
6. Quench in 0.5% H_2O_2 for 5 minutes. 5 ml 30% H_2O_2 in 300 ml PBS. If using a fluorescence kit, skip this step.

7. Rinse twice for 5 minutes in PBS.
8. Place Positive and Negative control slides in DN buffer for 5 min.
9. Apply DNase (1.0/0.1 ug/ml DN buffer) to POSITIVE and NEGATIVE CONTROL SLIDES ONLY for 10 minutes at 22^{o} C.
10. Treat the rest of slides with Proteinase K, 3 ug/ml 10 min.
11. Rinse all slides in PBS for 3 X 5 min keep DNase treated slides separate until the end of this set of rinses.
12. Apply 30 µl of equilibration buffer to each well.
13. Incubate at 22°C for 15 minutes.
14. Blot off excess equilibration buffer and add 30 µL/well TdT in reaction buffer. **Negative control does not get TDT solution, incubate with PBS.**
15. Incubate for 1 in humid chamber @ 37°C.
16. Place slides in stop wash buffer and incubate for 10 min at 22^{o} C.
17. Rinse 2 X 5 min in PBS.
18. Remove from PBS and gently touch corner of well with filter paper wedge to remove excess fluid.
19. Add Anti-Dig antibody (30 µL) for 1 hour at 22^{o} C.
20. (For peroxidase method, take DAB and glucose oxidase out of freezer at this point, follow DAB Protocol. For fluorescence method, prepare Prolong, see Prolong Protocol).
21. Wash in PBS 3 X 10 min.

Notes: Never let slides dry out ever!!!

Figure 1



DNAse treatement followed by TdT endlabeling serves as a positive control for the TUNEL reaction. All nuclei are labeled including neurons (red arrows) and satellite cells (green arrows). Experimental sections will demonstrate a range of reactivity and number of positive cells.

ANIMAL PERFUSION/TISSUE FIXATION

Reagents:

Fixatives
- 4% paraformaldehyde
- 2% paraformaldehyde
- Trumps 4% para and 0.5% glutaraldehyde
- Zamboni's
- Bouin's

Buffers
- Phosphate buffer pH 7.2, 0.1 M
- Phosphate buffered saline same as above with 150 mM NaCl
- Tris pH 7.2-7.6, 0.1 M
- Cacodylate buffer 0.15 M

Supplies:
- 21 X 3/4 butterfly needle (rat)
- 25 X 3/4 butterfly needle (mouse)
- 2 liter aspirator bottle
- tubing
- clamps
- sample jars/vials
- Dissecting tools scissors, large and small forceps, large and small hemostats

Intracardiac perfusion: For intracardiac perfusion, the fixative may be administered by peristaltic pump or by gravity. The Morphology Core uses gravity.

1. Fix bottles are placed at a height of 1 meter above the animal.
2. The animal is placed on an open metal grid over a large tray to catch the fixative. NO FIXATIVE MAY GO DOWN THE DRAIN!!!.
3. The animal should be deeply anesthetized, not responding to very firm toe pinch.
4. Rinse the chest and abdomen with 70% ethanol to reduce static and make the hair easier to cut.
5. Make an incision in the upper abdomen and cut laterally.
6. Find the xyphoid process, lift it up and cut through the diaphragm.
7. Cut the ribs laterally then cranially, avoiding the mammilary arteries do not puncture the lungs.
8. Free the heart from the pericardium.
9. Insert the fixative needle into the left ventricle, start the flow of fixative, clip the right atrium.
10. Allow fix to flow until the animal is quite stiff or at least 300 ml (rat) or 30 ml (mouse) of fix has gone through.
11. Following perfusion, dissect areas of interest and immerse in the same fixative for several hours or overnight in a mix of 50% fixative and 50% buffer solution (phosphate buffer, cacodylate buffer or Tris).

Immersion: Small animals, i.e., embryos or dissected tissue (pieces should be small, fix will not penetrate more than 3 mm) of larger animals.

1. Place tissue in fixative for 6-8 hours or overnight, change fixative after 3 hours, may fix longer in fixative and buffer if the first immersion isn't adequate.
2. Stirring or rocking increase mixing and result in better fixation. When stirring, place tissue in autopsy basket to protect it from stir bar. 5% DMSO may be added to aid in penetration of the fixative; however this also disrupts plasma membranes, and shouldn't be used for EM unless the EM protocol specifies its use.

BASIC BREEDING TO HARVEST

1. Basic breeding scheme is harem breeding: 2 females, 1 male per cage.
2. At least 3 breeding trios are active and are replaced every 6 months.
3. Tail biopsies are collect at 3-4 weeks of age for genotyping.
4. Fasting blood glucose tests are performed every 4 weeks.
5. Body weights are recorded every 4 weeks at same time as glucose test.
6. In animals not spontaneously diabetic, diabetes is induced by STZ injections at 8 weeks. 40mg/kg for 5 consecutive days.
7. Urine is collected at 12 and 24 weeks. (24 week time point is a 24 hour collection)

CRYOEMBEDDING

Supplies: Ice bucket
Dry Ice (stored in –20° C walk-in freezer)
2-methyl butane (stored in acid cabinet)
Aluminum foil
Small glass vial
Weigh boats
Forceps
OCT (Optimal Cutting Temperature, Electron Microscopy Sciences)
Alcohol-resistant marker (very important)

Procedure: Fixed Tissue

1. Following fixation (immersion or perfusion), rinse tissue in phosphate buffer (PB, 0.1M, pH 7.3) containing 5, 10, and 20% sucrose for 24 hours per step or until the tissue "sinks" to the bottom of the container or tube.

The final sucrose concentration in the tissue may vary from 5-30% depending on what conditions work best for the tissue in question.

2. Cool 2 methyl-butane in a dry ice bath until dry ice no longer boils when added.
3. Make embedding molds out of aluminum foil. Cut small squares, about 1" square or less. Use the bottom of a small glass vial or appropriately sized mold, form foil squares around bottom.
4. While foil is still on the mold, write all information concerning the tissue going in the mold.
5. Remove foil from mold, add OCT.
6. Take tissue out of 20% sucrose and place in a small dish or weigh boat containing OCT to remove any excess sucrose.
7. Place tissue in foil mold. Make sure all tissue is flat on the bottom of the mold. Cover tissue with OCT.
8. Using forceps, hold mold in 2-methyl butane until OCT freezes. Try not to drop the mold in the 2-methyl butane, as the block will freeze too fast and crack.
9. Keep in bucket on dry ice until all blocks are ready to be put in –20° C or –80° C freezer. Fold excess foil over the top of the block to prevent freeze-drying.

Procedure: Fresh Tissue

Blocks of fresh tissue may be snap frozen at approximately -40° C and immediately sectioned on the cryostat or covered in OCT, snap frozen and stored at for later sectioning. The freezing temperature is critical, the slower the tissue freezes, the more ice crystals form. If the piece of tissue is very large there is a risk of it shattering if frozen too fast.

Dosages of Tranquilizers and Anesthetics for Mice, Rats, Hamsters/Gerbils

Tranquilizer and Anesthetic	Mouse mg/kg (route)	Rat mg/kg (route)	Hamster/Gerbil mg/kg (route)
Promazine (Sparine)	4 (IM,SC)	0.5-1 (IM)	0.5-1 (IM)
Acepromazine (PromAce)	0.75 (IM,SC)	0.75 (IM)	
Chlorpromazine (Thorazine)	5-10 (IM)	1-2 (IM)	0.5 (IM)
Xylazine (Rompun)	2.5 (IM)	2.5 (IM)	10 (IM)
Diazepam (Valium)	5.0 (IP)	2.5 (IP)	5.0 (IP)
Droperidol and Fentanyl (Innovar-Vet)	0.2-0.5 ml/kg (IM)	0.1-0.5 ml/kg (IM)	contraindicated
Ketamine (Ketastet)	50-80 (IM)a	20-60 (IM)a	40-150 (IM)a 100-200 (IP)a
Pentobarbital (Diabutal, Nembutal)	35-70 (IV) 60-90 (IP)	25-40 (IV) 35-50 (IP)	30 (IV) 50-90 (IP)
Thiopental (Pentothal)	25-50 (IV) 50 (IP)	20 (IV) 20-40 (IP)	20 (IV) 40 (IP)
Thiamylal (Surital, Bio-tal)	25-50 (IV)	20 (IV) 20-25 (IP)	

a - Recommend to combine with Xylazine or Acepromazine.

DOSAGES OF ANALGESICS FOR RATS AND MICE

Analgesic	Mouse mg/kg/freq (route)	Rat mg/kg/freq (route)
Morphine	10 / 2-4 hr (SC)	10 / 2-4 hr (SC)
Meperidine (Demerol)	20 / 2-3 hr (SC, IM) 20-40 (IP)	20 / 2-3 hr (SC, IM)
Pentazocine (Talwin)	10 / 3-4 hr (SC)	10 / 4 hr (SC, IM)
Butorphanol (Turbugesic)	0.05-5.0 (SC)	0.05-2.0 (SC)
Buprenorphine (Buprenex)	2.0 / 12 hr (SC)	0.1-0.5 / 12 hr (SC)
Aspirin	120-130 (PO)	100 (PO)
Ibuprofen	7.5 (PO)	10-30 (PO)
Acetaminophen (Tylenol)	300 (IP)	110-300 (PO)

HEMATOXYLIN AND EOSIN STAINING

Solutions:

Mayer's Hematoxylin:

50 g Ammonium alum
1000.0 ml Distilled water
1.0 g Hematoxylin
0.2 g Sodium iodate
1.0 g Citric acid
50.0 g Chloral hydrate

1. Dissolve the alum in distilled water using magnetic stirrer.
2. When alum is dissolved, add hematoxylin. Dissolve completely.
3. Add sodium iodate and let stir for approximately 10 minutes.
4. Add citric acid and stir for another 10 minutes.
5. Add chloral hydrate and stir until completely dissolved.

Resulting solution is a deep wine color.
One ml of solution dropped into tepid water will turn blue.
This progressive stain stains nuclei only.

Eosin:

Eosin Stock:

1.0 g Eosin Y, water soluble
100.0 ml Distilled water

Phloxine Stock:

1.0 g Phloxine B
100.0 ml Distilled water

Eosin-Phloxine Working:

100.0 ml Eosin stock
10.0 ml Phloxine stock
780.0 ml 95% EtOH
4.0 ml Acetic acid, glacial

H&E Procedure:

1. For paraffin sections and fixed cryosections, deparaffinize and hydrate to water.
2. For fresh cryosections, place in alcoholic formalin for 45 seconds and rinse in water.
3. Stain in Mayer's hematoxylin for 15 min.
4. Wash in lukewarm running water for10 minutes.
5. Rinse in distilled water.
6. 95% EtOH for one minute, agitating.
7. Counterstain in eosin-phloxine solution for 2 minutes.
8. Dehydrate and clear.
9. Coverslip and mount.

Reference: AFIP Laboratory Methods in Histotechnology, 1994, p. 16, 53-55.

IMMUNO-ELECTRON MICROSCOPY

Immunogold Electron Microscopy
Reference: Kumagai et al., Brain Research 1996, 706:313-317

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. #SlGV 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 H20*

Reynolds lead citrate

See attached protocols
Uranyl Acetate Reynolds Lead Citrate

Protocol:

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).

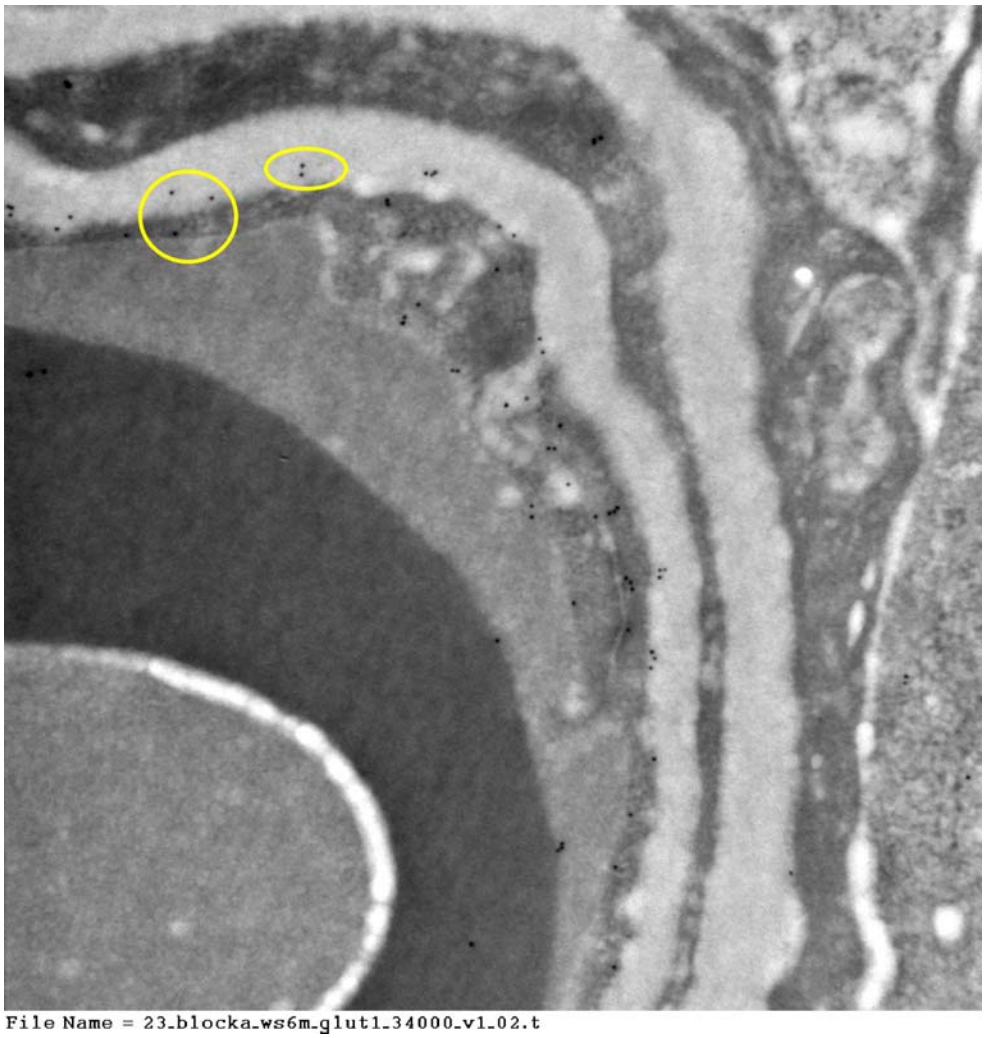
Ultrathin sections are cut at the CBL (Cell Biology Lab). Use formvar coated nickel slot grids.

2. Grids are hydrated for 10 minutes at 22^{o}C in Buffer A and then transferred to blocking Buffer for 30 min. at 22^{o}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 places 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^{o}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^{o}C. **Light protect:**
    In certain tissue, Reynold's lead citrate is used as an additional counterstain. See the attached 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



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.

IMMUNOFLUORESCENCE METHOD FOR 8DG LOCALIZATION

Yarborough et al, Cancer Res. 56:683-688, 1996

Reagents: RNase
Proteinase K
HCl
Ethos, 50, 70, 95, 100%
Hemo-De
PBS
Tris (10 mM, pH 7.5, 1 mM EDTA, 0.4 M NaCl)

Counterstains: Bis-benzimide

Other Chemicals: Normal serum
Non-fat dry milk
Bleach
H_2O_2

Mounting Media: Prolong
Gelmount

Glassware: Square Wheaton dishes
Slide racks and handles
Coplin jars
Beakers
Microfuge tubes

Procedure:

1. Deparaffinize slides by soaking in Hemo-D overnight in rocker oven. If using cryosections, thaw on warm plate 10 min, ring with PAP pen.
2. Re-hydrate through EtOH 100% -50% dH_2O.
3. Equilibrate sections in Tris. (10 mM, pH 7.5, 1 mM EDTA, 0.4 M NaCl)
4. Incubate with RNase in Tris, 100 µg/ml, 37^o C, 1 hour.
5. Rinse 2 X 10 min Tris.
6. Incubate with Proteinase K 10 µg/ml Tris 22^o C, 7 min.
7. Rinse 2 X 10 min Tris.
8. Incubate in 4 N HCl 22^o C, 7 min.
9. Rinse 2 X 10 min Tris.
10. Rinse 2 X 10 min PBS.
11. Begin normal IHC procedure (see attached) with the 8DG antibody.

IMMUNOHISTOCHEMISTRY FOR TISSUE SECTIONS

Reagent List:

Buffers
- Phosphate buffer (PB) pH 7.2, 0.1 M
- Phosphate buffered Saline (PBS) pH 7.2, 0.1 M, 150 mM NaCl
- Tris, pH 7.2- 7.6, 0.1 M
- 0.1% TX100 in PB

Fixatives:
- 4% paraformaldehyde
- 2% paraformaldehyde
- 4% paraformaldehyde with 0.5% glutaraldehyde (for TEM)
- 1:1 acetone: methanol

Primary Antibodies: Working dilutions vary

Secondary Antibodies: 1:200 to 1:500
- Biotinylated
- Avidin HRP kit
- Fluorochrome labeled

Counterstains:
- Hematoxylin
- Eosin
- Methyl Green
- Bis-benzimide (DAPI)

Other Chemicals:
- 3'3-diaminobenzidine
- glucose oxidase
- ammonium chloride
- β-D Glucose
- Ethanols 50, 70, 95, 100%
- Hemo-D
- Normal serum
- Non-fat dry milk
- **Bleach**
- H_2O_2

Mounting Media:
- Permount
- Prolong
- Gelmount

Glassware:
- Square Wheaton dishes
- Slide racks and handles
- Coplin jars
- Beakers
- Microfuge tubes

Procedure:

General Concepts:

- Keep buffers consistent through fix, rinses, antibody dilutions and chromogen development.
- Antibodies are sticky, always use plastic tips and tubes for storing, diluting and pipeting antibodies.
- Check slides after each major step in the protocol to make sure sections are still stuck to the slide. There is no point in continuing if the sections have all been removed due to over zealous washes.

Cryosections:

1. Remove slides from freezer, air dry. (warm plate)
2. Ring sections with PAP pen, if tissue was not fixed prior to embedding and sectioning, fix sections for 10-30 min, 4°C.
    - 4% paraformaldehyde
    - 2% paraformaldehyde
    - 1:1 acetone: methanol, ice cold

Paraffin Sections:

1. Deparaffinize in Hemo-D.
2. Hydrate through ethanol 100, 100, 95, 95, 70, 50, distilled water.
3. Ring sections with PAP pen, carefully, do not let sections dry.

Prepare Humid Chamber:

1. Hydrate in buffer 5 min.
2. Rinse in 0.5% H_2O_2 in buffer (5 ml 30% in 300 ml buffer), 10 min (for immunofluorescence skip and go to blocking)
3. Rinse 3 X 10 min in buffer.
4. Permeabilize and block 10 minutes:
    - 0.1% TX100 in buffer
    - 2% non-fat dry milk
    - 1% serum (serum from the animal in which the secondary antibody is raised e.g. 1° is rabbit, 2° **goat** anti-rabbit, block with normal goat)
5. Diluted primary antibody in buffer containing 0.1% TX100
6. Remove slides from blocking solution, do not rinse, dab away excess fluid with filter paper, place 1° (20-40 ul) in PAP pen ring. Dilutions are antibody specific, check antibody book for suggested dilutions.
7. Always wrap parafilm around the edge of the incubation chamber to seal:
    - Incubation times: 1°'s overnight 22° C, parafilm,
      36-48 hours 4° C, parafilm
      2-4 hours 37° C, lean toward 50-60 µl
8. Rinse in buffer 3 X 10 minutes.

Immunofluorescence:

1. Turn off overhead fluorescent lights to decrease quenching.
2. 2° antibody labeled with red or green fluorochrome.
3. Incubate 1 hour 22° C.
4. Rinse 3 X 10 min buffer.
5. Counterstain with Bis-benzimide 1ug/ml buffer, 5 min.
6. Rinse in buffer.
7. Cover with Prolong (see Prolong SOP)

Immunoperoxidase:

1. 2^{o} antibody labeled with biotin.
2. Incubate 1 hour 22^{o} C.
3. Rinse 3 X 10 min buffer.
4. Incubate in Avidin HRP I hour 22^{o} C **Prepare this solution 1 hour prior to use, it needs to equilibrate**
5. Rinse 3 X 10 min buffer.
6. DAB
7. Rinse 3 X 10 min in buffer, collect first rinse and add bleach to denature DAB (DO NOT PUT BLEACH ON THE SLIDES)
8. Dehydrate through graded ethanol 50, 70, 95, 95, 100, 100% and 2 changes of Hemo-D Counterstain.
9. Mount coverslips to slides with Permount.

INTRA-EPIDERMAL FIBER DENSITY DETERMINATION OF RODENT FOOT PAD BIOPSIES

Reagents: Paraformaldehyde
Phosphate buffer (PB, 0.1 M, pH 7.2)
Sucrose

Equipment: Razor blades

Solutions: 4% paraformaldehyde in phosphate buffer (PB, 0.1 M, pH 7.2)
5, 10, and 20% sucrose in PB

Procedure:

Perfused mouse or rat:

1. Following perfusion, remove entire foot pad with a sharp razor blade. Begin by cutting skin between the plantar surface of the foot and the toes, then start at the heel to gently remove the skin and superficial fascia of the foot. Be careful to only handle the tissue by the heel.
2. Post-fix overnight in 4% paraformaldehyde.
3. Rinse in graded sucrose 5 – 20%, minimum 12 hours in each

Fresh mouse or rat:

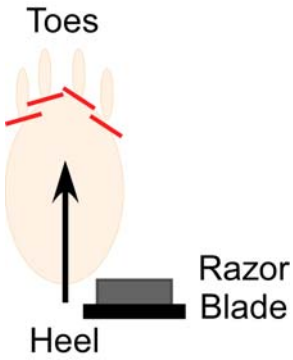
1. Remove foot and fix overnight (12-18 hours) in 4% paraformaldehyde
2. Remove skin and process as described above.

3. Follow IHC protocol using rabbit anti-PGP9.5, 1:1000 followed by AlexaFluor 598, 1:200 (Molecular Probes). Apply coverslips with ProLong antifade kit (Molecular Probes)

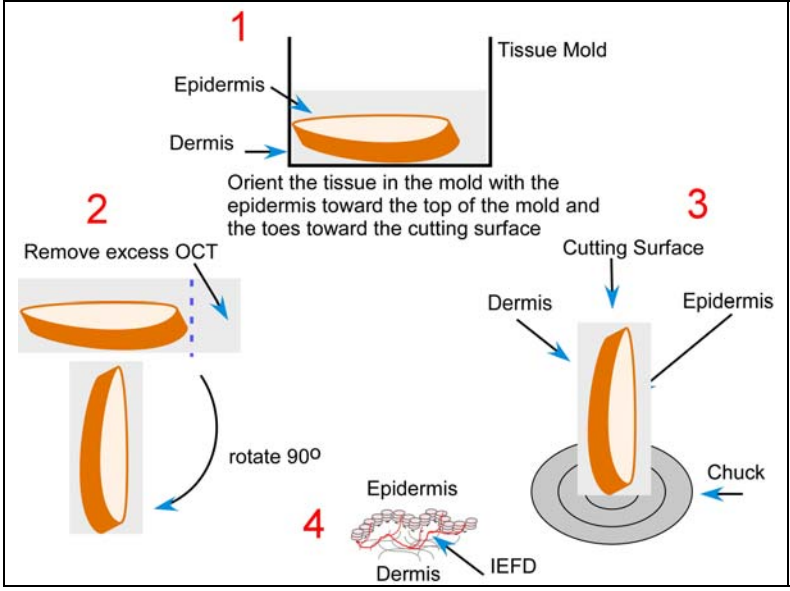
Imaging:

1. Three images per sample are collected on an Olympus FluoView 500 confocal microscope using a 60 X 1.2 water immersion objective at a resolution of 800 X 600 pixels. The optical section thickness is 0.5 um. Forty images per stack are flattened using MetaMorph (version 6.14) arithmetic option. Integrated morphometry analysis is used to exclude extraneous signals. The data are presented as the percent area of PGP9.5 positive fibers per area of epidermis.

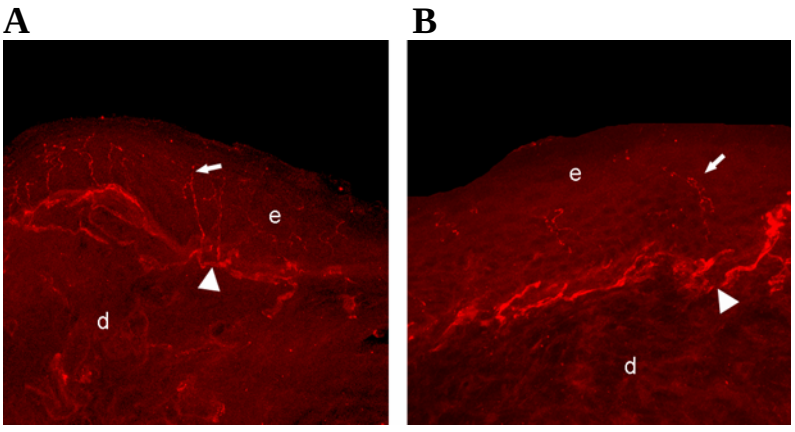
Figure 3



To dissect the foot pad, cut the skin at the toe joints with a sharp blade. Begin at the heel and move forward to remove the entire plantar surface of the foot. Handle the tissue by the heel only so as not to disrupt structures within the dermis and epidermis.



Embedding schema for all tissues in general and foot pads in particular.



IEFD in a control (A) and diabetic (B) mouse

IN SITU HYBRIDIZATION

From Dr. T.L. Wood

These reagents and solutions are used for riboprobes both S^{35} and Digoxygenin labeled

Reagents:
2X prehyb and hyb buffer (see hyb/prehyb protocol)
RNase free 4% paraformaldehyde
RNase free ETOH 50, 70, 95%
RNase free 1X PBS
RNase free 20X SSC .2X, .5X, 1X, 2X, 5X
RNase free 20% SDS
(Sodium dodecyl sulfate, a.k.a., N-lauroylsarcosine)
DepC water
Triethanolamine (TEA)
Glacial acetic acid
Acetic anhydride (must be dry, store with parafilm around the cap in a desiccant filled container, and replace every 3 months)
Formamide
1 M dithiothreitol
Rubber cement
RNAse A
Tris buffered saline (TBS)

Supplies:
RNase free tips, tubes, cylinders, slide racks and staining dishes
Filter paper
Hyb boxes
21gauge needles
3 ml syringe
Parafilm
Hybriwells

General Considerations: plastic-staining dishes should be labeled with the solutions they will always contain and should be labeled Day 1 or Day 2. Used this way, they only need to be rinsed with DepC water air-dried and put away. Graduate cylinders should be treated the same way

Day 1: All solutions, reagents, tips, tubes etc must be RNase FREE!!!

List all information concerning slides used, label slides as appropriate

1. Set dry bath at 70^{o} C and thaw formamide for step 12.
2. Retrieve slides from -80^{o} C and place directly into a slide rack in a dish containing 4% paraformaldehyde for 2 min (perfusion fixed tissue) or 10 min (fresh frozen tissue)
3. Rinse slides in 1X PBS for 2 min.
4. Rinse in DepC water for 5 min; gently lift rack 1 or 2 times.
5. Dehydrate through EtOH (1 staining dish/dilution, keep for step 10)
   50% 2 min
   70% 2 min
   95% 2 min
   Air-dry for 10 min
6. Rinse slides in fresh DepC, 2 min.

7. Make up fresh TEA: 5.4 ml of TEA + 1.2 ml glacial acetic acid + 400 ml DepC water
   Mix in a cylinder designated for this solution and this solution only, rinse with DepC water and let dry until next time.
8. Rinse slides in 200 ml of TEA solution, 2 min, remove slide rack, tap on paper towels to remove excess fluid but do not allow slides to dry completely.
9. Place 1 ml of acetic anhydride to a dry staining dish in the hood, place the slide rack in the staining dish and immediately add the other 200 ml of TEA solution.
10. Agitate the rack in the dish vigorously to mix the acetic anhydride and TEA.
11. Incubate 10 min at 22^{o} C.
12. Rinse in 0.2X SSC for 10 min.
13. Dehydrate through EtOH (1 staining dish/dilution, from step 10)
    50% 2 min
    70% 2 min
    95% 2 min
    Remove slides from rack, lay on paper towel
    Air-dry for 10 min.
14. If more than one probe is to be used per slide, ring sections with rubber cement. If only one probe is to be used, use hybriwells.
15. Heat denature 2X prehyb buffer at 70^{o} C for 5 min (dry block) then quickly chill on ice.
16. Set dry block for 85-90^{o} C for step 14.
17. Mix prehyb 1:1 with formamide. Completely cover each section with enough of this mix to let stand for 1 h at 22^{o} C.
18. Calculate how much hyb buffer is necessary to cover all of the sections at 50-100 ul per section. Make up the appropriate amount of hyb buffer:
    Formamide: 2X hyb, 1:1
    1M DTT diluted to 10mM
    20% SDS diluted to 0.1%
19. Thaw RNA probe on ice.
20. Calculate volume to add to hyb mix for a specific activity of 4.0 X 10^{4} cpm/µl (radioactive probes), or 400 ng/ml (Dig labeled cRNA probes).
21. Heat denature probe at 85-90^{o} C for no more than 5 min then quickly chill on ice. Do not refreeze any heat denatured probe. Probes may be thawed and refrozen but this should be kept to a minimum. Denatured RNA probe in hyb buffer may be stored at 22^{o} C until used.
22. Remove prehyb by tilting slides and pipeting off as much as possible. Immediately add appropriate hyb mix to sections.
23. Place sides in a humid box containing 3 MM filter paper soaked in 5X SSC: formamide, 1:1, wrap with plastic (Saran wrap, if probe is fluorescent, wrap with aluminum foil) to prevent evaporation.
24. Hybridize overnight at 60-65^{o} C with DIG/cRNA. If hyb is radioactive only, 50^{o} C is ideal.

Day 2 Get these solutions warming before removing slides from oven.
200+ ml of 50% fomamide/1X SSC/10mM DTT, in a staining dish 45-50^{o} C
2 L of 0.2X SSC 50-60^{o} C, large beaker on warm plate with stir bar.

1. Note any slides on which wells have dried or probes have crossed rubber cement boundaries.
2. Place 2X SSC in a 50 ml conical tube, dip slides then place in rack.
3. Place rack immediately in 50% formamide/1 X SSC/10 mM DTT, cover with parafilm incubate, 30 min.(Store this solution at 4^{o} C for up to 6 months, refresh DTT every 2 months)
4. Rinse slides in 0.5X SSC for 30 min at 22^{o} C.
5. Make up 500 ml of RNAse buffer. Volume of RNAse solution, 100 µl X number of sections RNAse solution 0.1 mg/ml RNAse A in RNAse buffer.
6. Remove sections from rack, tap away excess fluid, apply 100 µl RNAse.
7. Incubate for 30 min, @ 22^{o}C.
8. Tap away RNAse, rinse slides in RNAse buffer 2 X 10 min.

9. Wash slides in 2 L of 0.2X SSC at 50-60^{o} C for 2 h with gentle stirring.
10. Radioactive in situ only: Dehydrate through ethanol diluted with 300 mM ammonium acetate 2 min, 50, 70, and 95%. Remove rubber cement rings, air dry slides, put on film overnight.
11. For Dig label or double Dig/radioactive skip step 10 and quick dip in TBS.
12. Block for 15 min in Blocking solution. 10% Blocking stock/TBS 1:1 Boehringer.
13. Dilute alkaline phosphatase conjugated anti-Dig 1:500 in the blocking solution.
14. Tap off blocking, add 50-75 µl of anti-Dig.
15. Incubate 1h, 22^{o} C.
16. Rinse 3 X 3 min in TBS.
17. Place slides in Buffer 3 and add levamisole 1:1000, 10 min.
18. Tap off buffer, add DAKO NBT/BCIP to sections, cover with plastic and incubate 22^{o} C overnight in the dark.

Day 3:

1. Stop color reaction by placing slides in distilled water for 1 min.
2. Rinse 2 X 10 min in 10 mM EDTA/10 mM Tris.
3. Mount coverslips with Permount, for doubles lay down on film prior to dipping.

INSULIN IMPLANTATION PROTOCOL

Omorodola Abatan

IMPLANT PRODUCT INFORMATION:

Trade name: LINPLANT (A sustained released insulin implant)
Composition: bovine insulin, palmitic acid.
Weight: About 26mg/ implant.
Dimension: 2 mm in diameter, 7mm long.
Release rate: ~2U/24hr/implant for >40days, subcutaneously.
Caution: Implant is not sterile. Implant should be immersed in diluted 2% povidine iodine or exposed to UV. Do not put in alcohol.
Species: For rats use Linplant
For mice use Ultra-low dose linBit implant.
Recommended Sites for Insertion: Subcutaneously in dorsal skin (back), neck-region.

Anesthesia:
Rat Intraperitonial injection of .3ml undiluted ketamine- HCl (Ketastet) and xylazine mix (3parts of ketamine to 1 part of xylazine) for an average 350g rat.
Mice: Dilute .1ml of ketamine and xylazine mix (3 parts of ketamine to 1part of xylazine) to .9ml of .9% saline and inject .3ml i/p to an average 30g mouse.

Prep: The designated insertion site is shaved, cleansed and drenched with Betadine. The trocar and canula are immersed in 2 % Betadine solution. The skin is pierced with a 16G disposable needle while lifting up the skin between the thumb and index finger and withdraws the needle. The implant to be inserted is briefly immersed (about 10 sec) in 2% Betadine solution. The implant is then inserted into the proximal end of the trocar and the trocar pushed through the skin orifice/opening already created. The stylet is then used to push the implant until it exits from the distal end of the inserted trocar. The inserted implant is properly milked subcutaneously away from skin orifice. The insertion is soaked with Betadine solution. The skin defect should contract soon so no suture is required.

Dose: Two implants would render a diabetic rat weighing about 350 g non-diabetic. One (or one-half) implant would maintain a diabetic as diabetic.
A dose of sustained release insulin implant would last about 45 days.
Indication for use: For severely diabetic, poor-conditioned and cachexic rat whose blood glucose > 450mg/dl or when the glucometer reading is high.
This is required especially when it is too early to sacrifice the rat for tissues.
Source: The Linplant comes in a research pack of 40 implants marketed by:

Linshin Canada Inc.
47 Marblemount Crescent North,
Ontario, Canada MIT 2H5.
Telephone: (416) 490-6634.
Fax: (416) 490-1535.
www.linshincacada.com
e-mail: linshincacada@hotmail.com

NERVE CONDUCTION VELOCITY

Equipment:

Nicolet VikingQuest Portable System with Nerve Conduction Studies VikingQuest software run on Windows NT.
HP laser printer.
Nicolet 12mm .4mm diameter disposable platinum EEG subdermal needles.
Nicolet disposable ground.
Heating lamp.
Heating pad.
Flexible tape measure.
8"x 8" Styrofoam .
Ketastet 100mg/ml (3 parts) and Rompun 20 mg/ml (1 part) --- stock solution should be diluted 1/10 for mice and as is for rats.

Settings:

Motor tests:

- Duration .02 ms.
- Range 25 Ma.
- Low frequency filter 1 Hz.
- High frequency filter 10 kHz.
- Sensitivity 1 mV.
- Time 2 ms/div.

Sensory test:

- Duration .02 ms.
- Range 25 mA.
- Low frequency filter 1 Hz.
- High frequency filter 10 kHz.
- Sensitivity 50 μV.
- Time 2 ms/div.

Procedure:

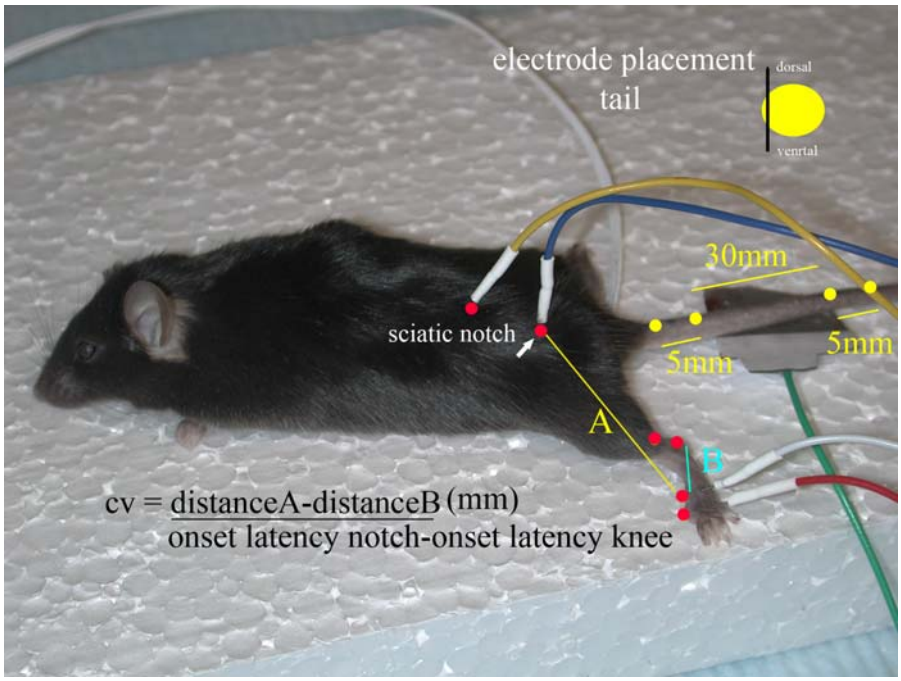
- To confirm the presence of diabetic neuropathy, nerve conduction velocity (NCV) studies will be performed. The animals will be anesthetized with 30/2.5 mg/kg ketamine/xylazine to prevent discomfort. Body temperature will be monitored with a dermal temperature probe and maintained at 32º C with a warming lamp during NCV. Body temperature will be maintained at 37ºC after NCV using a warming pad to ease animal stress from anesthetic. The nerve studies will last less than 30 min per rat or mouse. The electrodes will be cleaned with 70% alcohol between animals to maintain pathogen-free status.

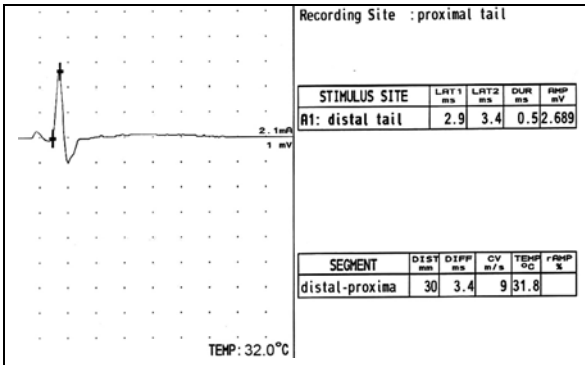
- Sciatic-tibial motor NCV will be determined by stimulating distally at the sciatic notch and distally at the knee via bipolar electrodes with supramaximal stimulation. The Sciatic-tibial motor NCV is calculated using two points of stimulation along the nerve and measuring the resultant latency. Latency is measured from initial onset to maximum negative peak.

- Tail motor latency will be determined by stimulating distally along the tail at a recorded distance of 3 cm. Latency is measured from initial onset to maximum negative peak.

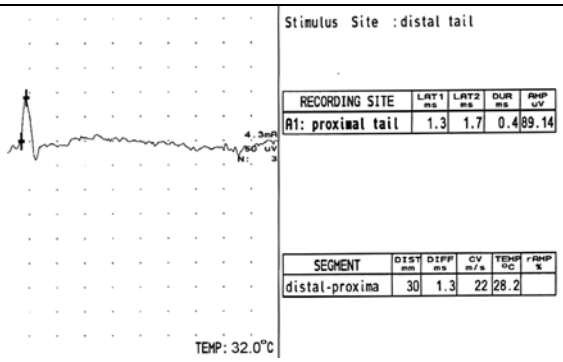
- Tail sensory NCV will be determined by stimulating proximally along the tail at a recorded distance of 3 cm. Latency is measured from initial onset to maximum negative peak.

Figure 4

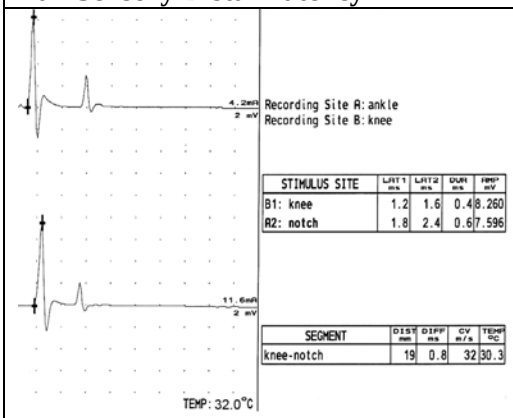




A
Tail Sensory Distal Latency



B
Tail Motor Nerve Conduction Velocity



C
Sciatic Motor Nerve Conduction Velocity

The upper panel demonstrates electrode placement for electrophysiology measures in mice.

Panels A-C are representative nerve traces. Nerve conduction measures include motor (orthdromic, proximal to distal) and sensory (antedromic, distal to proximal).

STREPTOZOTOCIN TREATMENT FOR RATS

Reagents:

Sodium citrate MW = 296.10
Citric Acid MW = 210.1
Streptozotocin (Sigma S-0130)
Sucrose

Solutions:

10 mM Sodium citrate
10 mM Citric acid
Citrate buffer; Sodium citrate + Citric acid pH 5.5
50% Sucrose water (regular tap water)

Supplies and Equipment:

ULAM Water bottles, sippers, cage clips, "Do Not Feed" and "Do Not Water" tape
1 ml syringes
5 ml tubes, plastic or glass
Aluminum foil
25 gauge 0.5 inch needles
Glucometer
Glucose test strips
Pipeter and tips
Graduate cylinder

Bring to animal room:

Bottles, Bottle Clips and Stoppers
Syringes and Needles
Tubes of Streptozotocin covered with parafilm, wrapped in foil
Citrate buffer
Pipeter for measuring citrate buffer
Sugar water
Graduate cylinder for diluting sugar water

Procedure:

1. Order animals to arrive several days before the planned injection date. This allows them to acclimatize to their new surroundings.
2. The day before STZ injection, remove the food from the feeders. Leave a clear note saying, **"Do Not Feed"** (specify appropriate rats) from (time, date) to (time, date). **INFORM ULAM**; be prepared to give them your animal protocol number. If there is evidence that the rats have been fed, contact ULAM to clarify and postpone STZ injection to the next day.
3. Order water bottles stopper/sippers, and clips the morning before STZ injection. Following STZ injection, be sure to put **"Do Not Water"** tape on the cages, or a sign to that effect on the rack.
4. Make Citrate Buffer.
5. Make 50 or 100% sugar water either the day before or the morning of the STZ injection.
6. Weigh rats. Rats must be weighed on the day of injection.
7. Calculate dose and weigh out streptozotocin. (see chart at the end of this document). 4 rats/ml, no more than 2ml/tube. Wrap the tube in foil to protect from light.
8. In the animal room, add citrate buffer to a tube, mix quickly and thoroughly.
9. Inject appropriate dose based on weight (see chart) using a 1 ml syringe and 25 gauge 0.5" needle, intraperitoneally, i.p. Use all of the STZ in the tube prior to mixing the next dose.

10. Following STZ injection, the rats are given 10% sucrose water to drink to protect them hypoglycemia. Alert the animal facility caretakers to the fact that the animals have been STZ injected so they can monitor for signs of distress. Hypoglycemia can be expected to occur anywhere from 8 to 24 hours after injection. If the 10% sucrose water is not sufficient to cover the hypoglycemia, any animal with signs of hypoglycemia can be given a 1ml bolus of 10% sucrose by gavage as a further effort to protect them. The animals are also given back their rat chow following STZ injection.
11. After 24 hours, the animals are placed on regular water. The next morning, the animals are checked for the induction of diabetes by measuring tail blood glucose. Using a standard glucometer and glucose test strips. Blood glucoses are recorded at onset and once every week or two weeks according to experimental design. Time line example, Monday fast, Tuesday inject, Wednesday evening put on regular water, Thursday check blood glucose.

Weight (g)	mls STZ	For Dose, use	mg/ml STZ in citrate buffer
173-186	0.12		
187-201	0.13	55 mg/kg	82.5
201-217	0.14	50 mg/kg	70
218-232	0.15	45 mg/kg	67.5
233-247	0.16	40 mg/kg	60
248-262	0.17		
263-277	0.18		
278-292	0.19		
293-307	0.2		
308-322	0.21		
323-337	0.22		
338-352	0.23		
353-367	0.24		
368-382	0.25		
383-397	0.26		
398-412	0.27		
413-427	0.28		

TAIL FLICK AND HIND PAW PROTOCOL

Equipment:

- IITC Model 336 Plantar/Tail Analgesic Meter with True Tail Temp and Heated Glass
- HP laser printer
- Mouse restraint
- #2 Phillips screwdriver
- Foot switch
- Temperature probe with stand and guide (Needed for tail flick only)
- Plastic mouse chamber
- Black Sharpie

Setup:

- Must clean equipment with Sporeklenz before entering the animal room
- All restrainers need to be exclusively used in same room. If restrainer has been in another animal room, it CANNOT be used!
- Never remove cable from test head or back of tail flick machine when it is on. Failure to pay attention may damage electrical circuits in machine.

Instrument Instructions:

- Keyboard commands ///There are 7 commands/// \\\number plus E shows the setting of the function\\\
  1) #1 – Current Temperature (1E shows temp
  2) #2 – Animal number e.g. 2011E for animal 11
  3) #3 – Active Intensity (During Test) e.g. 3050E for 50% intensity
  4) #4 – Idle Intensity (Temp during the ready state) e.g. 4050E for 50%
  5) #5 – Cutoff timer (Harm prevention) e.g. 5025E for 25 seconds
  6) #6 – Trigger Temperature (Pre-warming) e.g., 6025E for 25˚C.
  7) #7 – Time of day (24 hr format{ 5 digit sequence}) e.g. 00100E for 1am
  8) #8 – Date (6 digits must be used) eg.020501 for Feb 5th, 2001

Pre-Operating Instructions:

Mice need to be acclimated to the machine. This is accomplished by placing mouse in restrainer 2-3 times briefly before test occurs.

Operating Instructions: **Tail Flick**

1. Measure and mark tail 3 cm from tip.
2. Place temp sensor on the groove in ready state (if needed).
3. For first animal type 6000E(this disables pre-warm feature), 4001E, 3025E, 5010E, and set time and date.
4. Place mouse in restrainer and align tail mark over pinhole opening (Head should be towards keypad)
5. There are two ways to start test; footswitch or the red button. Hit either one to arm the system, hit it again to start the test. The temp will ramp up to desired temp (if needed) and the test will begin and end without any user input. When the mouse moves tail, machine will shut off.
   - Make sure to keep tail as close to the groove as possible.
   - When the test ends, there will be three numbers that flash.
   - - P = Finish temp
     - B = Start temp
     - Blank = elapsed time

     The time should be recorded only.
     Test is over

Operating Instructions: Hind Paw

1. Place animals in chambers on glass warmer plate (32°C) and allow them to come to rest.
2. Place temp sensor under the animal, use the mirror to guide the beam under the hind paw.
3. For first animal type 6000E(this disables pre-warm feature), 4001E, 3025E, 5010E, and set time and date.
4. There are two ways to start test; the footswitch or the red button. Hit either one to arm the system, hit it again to start the test. The temp will ramp up to desired temp (if needed) and the test will begin and end without any user input. When the mouse moves tail, the machine will shut off.
5. When the test ends, there will be three numbers that flash.
   - P = Finish temp
   - B = Start temp
   - Blank = elapsed time

   The time should be recorded only.

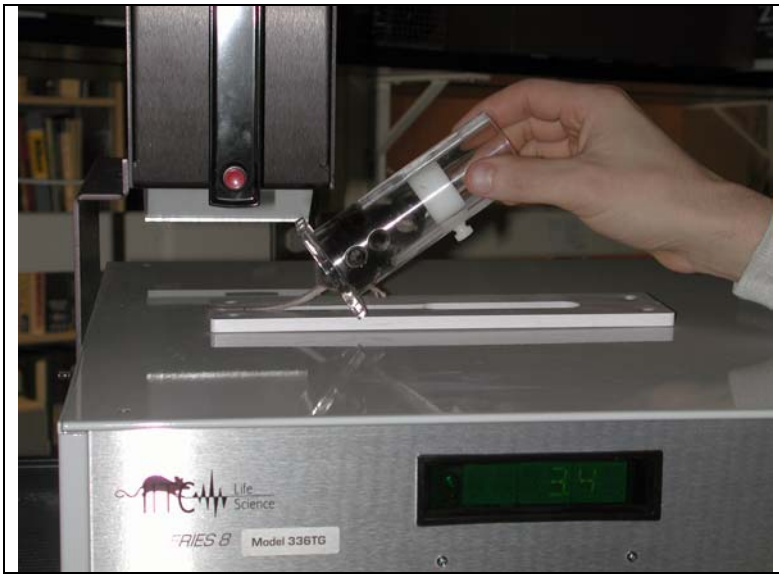
   Test is over

Additional Notes:

- Data is collected in two ways. After each test, the data written in a log book or a printer may be set attached. Manual data collection is recommended at this point.
-
- Settings to be used
  - Active Intensity is 25%
  - Idle Intensity is 1%
  - Trigger Temperature is 25 C°
  - Cutoff time is 10 seconds

- If the machine becomes confused, consult manual page 8, Troubleshooting.
- If climate of test area is controlled, then temperature ramping does not need to be used.

Figure 5

	Plantar analgesia is measured by testing the animal's sensitivity to a heat stimulus applied to the hind paw.
	A more distal measure of analgesia is measured by testing the animal's sensitivity to a heat stimulus applied to the tail.

TRANSMISSION ELECTRON MICROSCOPY

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*

Procedure:

Fixation

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

Tissue (fresh) Quickly dissect tissue of interest into 3 mm cubes and fix by immersion in 4% para, 2.5% glutaraldehyde at 4^{o}C, 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 4^{o}C for 4-8 hours, place cells on rocker.

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 ddH20 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 60^{o}C for 24 hours

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

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 4^{o}C

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.

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.

SORENSEN'S PHOSPHATE BUFFER

Solution A: 0.2M Dibasic Sodium Phosphate
26.8 g Na_2HPO_4 7H2O
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.

BIOCHEMICAL MEASURES OF NEUROPATHY

Biochemical Measures of Neuropathy: Oxidative stress is highly correlated with the metabolic changes caused by hyperglycemia. Increased levels of glucose overload mitochondria and result in the production of reactive oxygen species (ROS). In addition, the flow of excess glucose through cellular pathways decreases the cell's normal ability to detoxify ROS. As a result, the neurons and axons of the peripheral nervous system contain increased levels of ROS and decreased antioxidant capacity. The following assays are used to measure these changes in rodent models of diabetic neuropathy.

ACONITASE

Kit: OxisResearch, Bioxytech Aconitase-340, Catalog No. 21041 (store at 4°C)

Reagents & Supplies (not provided in kit)

Reagent	Supplier	Catalog No.
Sodium Citrate	Sigma	S-4641
Tris-HCl	Fisher	

Reagent Preparation:

0.2mM Sodium Citrate in 50 mM Tris-HCl pH 7.4: 3.15g in 300mL deionized H_2O, add 23.5mg Sodium Citrate. pH to 7.4 then bring up to 400mL.

NADP Reagent: Just prior to use, reconstitute the NADP with 5.5 mL deionized H_2O.

Enzyme: Reconstitute with 10 mL deionized water. Store at 4°C.

Sample Preparation — Tissue

1. Weigh tissue sample.
2. Mince tissue.
3. Homogenize at 1% (w/v) in ice cold 0.2mM sodium citrate for 15-20 seconds. (new protocol calls for sodium citrate in 50mM Tris-HCl, pH 7.4).
4. Centrifuge at 800 x g for 10 minutes at 4°C.
5. Remove 25µl supernatant for protein assay.
6. Remove supernatant and store on ice or freeze –80°C until use.

Performing Assay:

1. Thaw homogenate on ice.
2. Fill in layout on computer and save as acxxxxxx.sed where xxxxxx is the date in yyddmm format.
3. Sonicate for 20 seconds.
4. Dilute the tissue extract 10µl extract into 190µl (500µg/ml) assay buffer.
5. Add 50µl of sample to each well in triplicate. (Do 1:1 and 1:20 dilutions.)
6. Add 50µl assay buffer to blank well in triplicate.
7. Add 50µl of Substrate to each well.
8. Add 50µl of Enzyme to each well.
9. Add 50µl of NADP to each well.
10. Mix using a pipet

Reading the Plate:

1. Record absorbance change at 340nm for 5 minutes at 37°C.
2. Turn on Multiskan and open your saved file from above.
3. Place plate onto Multiskan holder and click START.
4. Save raw data as an Excel file into the acx data folder. Use the naming convention acXXXX.xls, where XXXX is the date in mmdd format.
5. Select Process>Organize. Choose the appropriate data to organize (usually Measure1), then click **OK**. This rearranges the data into columns.
6. Save organized data as an Excel file into the acx data folder. Use the naming convention acXXXXor.xls, where XXXX is the date in mmdd format.

CATALASE ASSAY

Kit: Amplex Red Catalase Assay Kit, Catalog No. A-22180.

Reagent Preparation:

Amplex Red reagent: Prepare a 10 mM stock solution. (Enough for 2 plates). Bring DMSO and Amplex Red reagent to room temp. Just prior to use dissolve 1 vial (.26mg) of 20mM Amplex Red reagent in 100µL of DMSO. Store stock solution an -20^0C, protected from light.

Reaction Buffer (5X) (0.25M sodium phosphate, pH 7.4): Dilute 4mL of Reaction buffer in 16mL of deionized water.

HRP (Horseradish peroxidase) 100 U/mL**:** Combine 15 µL of 200 U/mL HRP stock solution with 15 µL of 1X Reaction Buffer. Store frozen at -20^0C. **Reagent supplied with kit is 20U. Dissolve content with 200 µL 1X Reaction buffer and divide into 22 µL aliquots.**

20 mM H_2O_2: (**Make fresh each time.)** Dilute (check bottle for %) 17.9µL H_2O_2 (3.8%) in 982.1µL dH_2O. (Check label for exact concentration) (23µL 3% H_2O_2 into 977µL dH_2O) Use promptly.

Catalase: Prepare a 1000 U/mL stock. Reagent supplied with kit is 100U. Dissolve vial in 100 µL dH_2O. Aliquot and store at –20. Make **10 U/mL** with 1µL 1000 U/mL stock into 99µL dH_2O. Make **1 U/ml** with 10 µL 10U/ml into 90 µL dH_2O.

Performing the assay:

1. Prepare stock solution of Catalase then prepare standard curve as follows:

Volume of Catalase stock	Volume of 1X Buffer	Catalase Concentration
0 µL	75 µL	0 mU/mL
18.75 µL of 1 U/mL	56.25 µL	62.5 mU/mL
37.50 µL of 1 U/mL	37.5 µL	125 mU/mL
7.5 µL of 10 U/mL	67.5 µL	250 mU/mL
15 µL of 10 U/mL	60 µL	500 mU/mL
30 µL of 10 U/mL	45 µL	1000 mU/mL

(Final concentration will be fourfold lower, 0 to 10µM)

Tissue:

1. Homogenize tissue in 1X Reaction Buffer **on ice**.
2. Using a black plate, pipette 25 µL of diluted standards, controls (if any) and samples into wells. *(Final concentration will be fourfold lower, 0 to 10 µM)*
3. Prepare stock solution of 20mM H_2O_2 then prepare a 40 µM H_2O_2 dilution by adding 10 µl of 20 mM H_2O_2 to 4.99 mL 1X Reaction Buffer.
4. Pipet 25 µL of 40 µM H_2O_2 solution into each well.
5. Incubate for 30 minutes at 22^{o}C.
6. Prepare stock solution of 10 mM Amplex Red reagent and divide into 50 µL aliquots and freeze immediately.
7. Prepare stock solution of 100 U/ml HRP and divide into 20 mL aliquots.
8. Prepare 100 µM Amplex Red reagent containing 0.4 U/mL HRP by adding 50 µL of 10 mM Amplex Red stock solution and 20 µL of 100 U/ml HRP stock solution to 4.93 mL 1X Reaction Buffer.
9. Begin 2nd phase of reaction by adding 50 µL of the above to each well.
10. Place plate into Fluroskan holder and click START.
11. Take 4 readings @ 15 minute intervals using 544/590 filter pairs. (Generally take 3 reading which would be after 30 min. incubation as recommended.)
12. Save raw data as an Excel file into the RCx data folder. Use the naming convention RCXXXX.xls, where XXXX is the date in mmdd format.
13. Select Process>Organize. Choose the appropriate data to organize (usually Measure1), then click **OK**. This rearranges the data into columns.
14. Save organized data as an Excel file into the RHP data folder. Use the naming convention rcXXXXor.xls, where XXXX is the date in mmdd format.

(H_2) DCFDA

Reagents & Supplies:

Reagent	Supplier	Catalog No.
DCFDA (50 µg, m.wt. 535.76) DMSO (10 mg/mL) HBSS	Molecular Probes	

Reagent Preparation:

DCFDA: Dissolve 1 vial of DCFDA in 10 µL DMSO. (10mg/mL).

Assay Preparation:

1. Set up plate layout in AscentFL software. Choose DCFDA.sed and fill in your layout. Save your file as DCxxxxxx.sed with xxxxxx being the date in yy/mm/dd format.
2. Treat cells per experimental paradigm.
3. 15-30 minutes prior to reading, add 3.23 µL of DCFDA stock to 15 mL media without serum.
4. Rinse cells with HBSS.
5. Place plate into Fluroskan holder and click START.
6. Take readings using 485 nm ex, 520 nm em filter pair.
7. Save sheet with .xls extension into the DCFDA data folder or your own folder. Use the naming convention DCXXXXXX.xls, where XXXX is the date in yymmdd format.

DHE

Reagents & Supplies:

Reagent	Supplier	Catalog No.
10 mM HEPES 150 mM NaCl 5 mM KCl 1 mM $MgCl_2$ 1.8 mM $CaCl_2$ DHE DMSO (10 mg/mL)	Molecular Probes	

Reagent Preparation:

HBSS (pH 7.4): Combine HEPES, NaCl, KCl, $MgCl_2$, and $CaCl_2$. pH to 7.4 then filter to sterilize. (may be stored at 22°C for several months)

DHE: Dissolve 1 vial in 100 µL DMSO (10 mg/mL). Remove 10 µL, aliquot and freeze remainder. Protect from light and discard after thawing 3 times. Add the 10 µL to 1mL HBSS (0.1 mg/mL), then for the working dilution put 100 µL of diluted solution into 10 mL HBSS. (1 µg/mL=3µM)

Assay Preparation:

1. Open Fluoroskan and choose open under the file menu. Scroll down and select DHE.sed. Set up your plate layout. Save your layout as DHxxxxxx.sed with xxxxxx being yy/mm/dd.
2. *Treat cells per experimental paradigm.
3. 15 minutes prior to reading, gently rinse cells once with HBSS.
4. Apply 3 µMDHE in HBSS and leave on for 15 minutes.
5. Rinse cells and add HBSS.
6. Place plate into Fluroskan holder and click START.
7. Take readings using 485 nm ex, 612 nm em filter pairs for ethidium and 355 nm ex, 430 nm em for DHE.
8. Save **both** sheets with .xls extension into the DHE data folder or your own folder. Use the naming convention DHXXXX.xls, where XXXX is the date in mmdd format and add a **r** for the red reading and **b** for the blue reading.

*As an alternative, cells can be pre-loaded with DHE- do steps 3, 4, 5, 2, then 5.

GENOTYPING

Reagents & Supplies:

Reagent	Supplier	Catalog #
10X PCR Buffer	Life Technologies	10966-034
50 mM $MgCl_2$	Life Technologies	10966-034
10 mM dNTPs	Life Technologies	10297-018
Taq DNA Polymerase (5 U/µL)	Life Technologies	
0.5 mL thin-walled tubes	Life Science Products	LS-9350-X
Forward primer (10 µM)	5′–???–3′	
Reverse primer (10 µM)	5′–???–3′	
DNA Template (200 ng/µL)		
Sterile, deionized H_2O		

PCR Amplification:

1. Prepare a Master Mix containing the following volumes of reagents:

Reagent	µL
10X PCR Buffer	2.5
50 mM $MgCl_2$	1.25
dNTPs	2.5
M13 Forward Primer	1.0
M13 Reverse Primer	1.0
Taq DNA Polymerase	0.2
DNA Template	2.0
Sterile dd-H2O	14.55
Total Volume =	25.00

2. Into each 0.5 mL thin-walled tube, pipette 23.0-µL of Master Mix.
3. Pipet 2.0 µL of DNA template (200 ng/µL) into thin-walled tube. Mix gently, and spin down.
4. Amplify using the following program:

   94°C, 5 min. → 94°C, 1 min. → 64°C, 1 min. → 72°C, 1.5 min. → 72°C, 10 min. → 4°C, soak
   Repeat 35 times

5. Verify PCR product by separation on 2% agarose.
6. Store tubes at –20°C until needed. If plates will be used within a few days they may be stored at 4°C.

GLUTATHIONE ASSAY (GSH)

Reagents & Supplies: (not provided in kit.)

Reagent	Supplier	Catalog No.
Perchloric Acid (Lot # 227044)	Fisher	A469-500
KOH Potassium hydroxide	Fisher	P250-1
Imidazole Base	Sigma	I 2399
Potassium Chloride	Fisher	P217-500
Tris Base	Fisher	604204
Tris Hydro	Fisher	812854
EDTA (Lot 0M1037)	Fisher	16-004Y
Glutathione (Lot 070K0888)	Sigma	G-6529
o-Phthaldialdehyde (Lot 080K2519)	Sigma	P-0657
Methanol	Fisher	A 4524

Reagent Preparation:

3 *M* $HClO_4$ – 42 ml of 72% stock Perchloric acid into 58 ml ddH_2O

2*N* KOH, 0.4 *M* imidazole base, and 0.4 *M* KCl – 56.11 g KOH, 13.62 g Imidazole base and 14.91 g KCl. Add ddH_2O to bring up to 500 ml.

1.0 *M* Tris-HCl buffer pH 8.1 with 20mM EDTA – 50 mL 2M TRIS-HCL pH 8.1, 4mL 0.5 M EDTA and 46 mL ddH_2O.

0.1M Glutathione – 30.7 mg into 1 ml H_2O.

0-Phthaldialdehyde – 10 mg in 1 ml of methanol.

Sample Preparation: **Do not let samples set at 0^0 for long periods of time.**

Prior to dissection, perfuse tissue with PBS pH7.4 with 0.16 mg/ml heparin

1. Label 6 sets of micro centrifuge tubes. (If only doing GSH only need 3 sets)
2. Prepare 3 M $HClO_4$ and freeze.
3. Cut tissue into segments and weigh. (~10 mg)
4. Keeping labeled tubes on dry ice, add 3 times the weight of 3 M $HClO_4$.
5. Sonicate on ~5.
6. Add 300 µl of ddH_2O **for each 100 µl** $HClO_4$ (3.3 per µl) and vortex. (May include 1 mM EDTA in ddH_2O)
7. Centrifuged at 5000 x g for 10 min.
8. Transfer supernatant to a new labeled tube. Discard protein precipitate.
9. Immediately neutralize with 290 µl/ml of a mixture of 2 *N* KOH, 0.4 *M* imidazole base, and 0.4 *M* KCl and vortex. (Leaving a solution at pH 7.)
10. Centrifuge samples @ 5000 x g for 10 min @ 4^0 to precipitate insoluble $KClO_4$

Performing the assay:

1. Set up Fluroskan layout and area. Read using 345nm excitation and 425nm emission.
2. Prepare standards as follows: (0-1000uM)

Concentration µM/mL	H_2O	GSH
0	500	NO GSH
15.63	500	Take 500 µL from tube 3
31.25	500	Take 500 µL from tube 4
62.5	500	Take 500 µL from tube 5
125	500	Take 500 µL from tube 6
250	500	Take 500 µL from tube 7
500	500	Take 500 µL from tube 8
1000	990	10 µL 0.1M stock

3. On a clear 96 well plate add 20 µl standards to each well in duplicate
4. Add 20 µl sample extract to each sample well in duplicate or triplicate.
5. Add 180 µl Tris-HCl buffer to each well.
6. Begin reaction by adding 2 µl methanol solution of *o*-phthaldialdehyde (OPD) (10 mg/ml methanol)
7. Place plate into Fluroskan holder and click START.
8. From the drop down menu under sheet select curve fit. Choose the appropriate data to organize (usually Measure1), then click **OK**. This calculates your standard curve.
9. Save the curve fit sheet as an Excel file into your data folder. Use the naming convention GSXXXXXX.xls, where XXXXXX is the date in yymmdd.xls.

GSSG (Non-Enzymatic)

Reagents & Supplies:

Reagent	Supplier	Catalog No.
Perchloric Acid (Lot # 227044)	Fisher	A469-500
N-ethylmaleimide	EMD Bioscience	34115-5GM
KOH Potassium hydroxide	Fisher	
Imidazole Base	Fisher	
Potassium Chloride		
Tris		
HCL		
EDTA	BioWhittaker	16-004Y
O-phthaldialdehyde (Lot 80K25190	Sigma	P-0657
GSSG (Glutathione (Oxidized)) (Lot 93148120)	Roche	105 635

Reagent Preparation:

3 *M* $HClO_4$ – 42 ml of 72% stock Perchloric acid into 58 ml ddH_2O

0.04M *N*- ethylmaleimide – 5mg in 1 mL ethanol *(N*- ethylmaleimide complexes with GSH (Hissin and Hilf, 1976) thus preventing interference from GSH oxidation Per Dr. Obrosova)

2*N* KOH, 0.4 *M* imidazole base, and 0.4 *M* KCl – 56.11 g KOH, 13.62 g Imidazole base and 14.91 g KCl. Add ddH_2O to bring up to 500 ml.

***0.1* 0.1 *M* imidazole-HCl buffer pH 7.6 with 20 mM EDTA –** 68 mg in 50 ml ddH2O. Add 400 µL 0.5 M EDTA and pH to 7.6 with HCl then bring volume up to 10 mL.

GSSG Standard 100mM – 61.26 mg in 1 mL ddH_2O.

***0*-Phthaldialdehyde** – 10 mg in 1 ml of methanol.

Sample Preparation:

Prior to dissection, perfuse tissue with PBS pH7.4 with 0.16 mg/ml heparin.

1. If using extract from GSH add 100 µl 0.04 M *N*-ethylmaleimide to 200 µl homogenate then skip to Performing the Assay or
2. Label 3 sets 1.5 ml and 1 set of 0.6 ml micro centrifuge tubes.
3. Prepare 3 M $HClO_4$ and freeze.
4. Keeping labeled 1.5ml tubes on dry ice, add 3 volumes of 3 *M* $HClO_4$.
5. Cut tissue into segments and weigh. (~10 mg)
6. Place weighed segment in tube on top of $HClO_4$.
7. Place tubes in an alcohol bath maintained at -8^0 to -10^0 C. and agitate 15 minutes or until the acid completely penetrates the tissue fragments. (Make sure the temperature does not go below -10^0 to completely extract the ice. (2 M $HClO_4$ will freeze at -12^0 C.)

8. Add 333 µl of ddH_2O for each 100 µl $HClO_4$ and shake at 4^0 C for 10 min. (May include 1 mM EDTA in ddH_2O)
9. Pull off 25 µl and place in a 0.6 ml micro-centrifuge tube for protein analysis.
10. Centrifuge samples at 5000 x g for 10 min at 4^0.
11. Transfer supernatant to a new labeled tube. Discard protein precipitate.
12. Immediately neutralize with 290 µl/ml of a mixture of 2 *N* KOH, 0.4 *M* imidazole base, and 0.4 *M* KCl . (Leaving a solution at pH 7.)
13. Centrifuge at 4000 x g for 5 min to precipitate insoluble $KClO_4$.

Performing the assay:

Prepare standards as follows: (0-1000uM)

Concentration µM/mL	**H_2O**	**GSSG**
0	500	NO GSSG
15.63	500	Take 500 µL from tube 3
31.25	500	Take 500 µL from tube 4
62.5	500	Take 500 µL from tube 5
125	500	Take 500 µL from tube 6
250	500	Take 500 µL from tube 7
500	500	Take 500 µL from tube 8
1000	990	10 µL 0.1M stock

1. On a clear 96 well plate add 40 µl standards to each well in duplicate.
2. Add 40 µl sample extract to each sample well in duplicate or triplicate.
3. Add 160 µl imidazole-HCl buffer to each well. Add 0.2-10 µM NADPH
4. Begin reaction by adding 0.3 U of glutathione reductase.
5. Set up Fluroskan layout and area..
6. Read using 340 nm excitation and 460 nm emission.
7. Place plate into Fluroskan holder and click START.
8. From the drop down menu under sheet select curve fit. Choose the appropriate data to organize (usually Measure1), then click **OK**. This calculates your standard curve.
9. Save the curve fit sheet as an Excel file into your data folder. Use the naming convention GSXXXXXX.xls, where XXXXXX is the date in yymmdd.xls.

GLUTATHIONE PEROXIDASE

Kit: Cayman Glutathione Peroxidase Assay Kit, Catalog No. 703102.

Reagents & Supplies: HPLC-grade water

Reagent Preparation:

Assay Buffer (10X): Dilute 2 mL of Assay buffer concentrate with 18 mL of HPLC-grade water. Store at 4°C. Stable for 2 months.

Sample Buffer (10X): Dilute 2 mL of Sample buffer concentrate with 18 mL of HPLC-grade water. Store at 4°C. Stable for 1 month.

Glutathione Peroxidase (Control): Dilute 10 µL of supplied enzyme with 490 µL of diluted sample buffer. Aliquot 70 µL into 0.5 mL centrifuge tubes and store at –20°C.

Co-Substrate Mixture: Reconstitute the number of vials required by adding 2 mL of HPLC-grade water to each vial and vortex. Each vial will have enough reagent for 40 wells.

Cumen Hydroperoxide: Ready to use as supplied. Store at –20°C.

Sample Preparation — Tissue:

1. Homogenize the tissue in 5–10 mL of cold buffer (i.e., 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1 mM DTT) per gram of tissue. ***General Equation:*** **µL Buffer = mg Tissue X 10**
2. Centrifuge at 10,000 x g for 15 minutes at 4°C.
3. Remove supernatant for assay and store on ice. Sample can be stored at –80°C for at least one month.

Performing Assay:

1. Turn on Multiskan and open file gpx.sed.
2. **Background Wells*:*** add 120 µL of Assay Buffer and 50µL of co-substrate mixture to three wells.
3. **Positive Control Wells:** add 100 µL of Assay Buffer, 50 µL of co-substrate mixture, and 20 µL of diluted GPx (control) to three wells.
4. **Sample Wells:** add 100 µL of Assay Buffer, 50 µL of co-substrate mixture, and 20 µL of sample to three wells.
5. Initiate reactions by adding 20 µL of cumen hydroperoxide to all wells being used as quickly as possible. Note precise time the reaction is initiated.
6. Place plate onto Multiskan holder and click START.
7. Save raw data as an Excel file into the GPx data folder. Use the naming convention gxXXXX.xls, where XXXX is the date in mmdd format.
8. Select Process>Organize. Choose the appropriate data to organize (usually Measure1), then click **OK**. This rearranges the data into columns.
9. Save organized data as an Excel file into the GPx data folder. Use the naming convention gxXXXXor.xls, where XXXX is the date in mmdd format.

GLUTATHIONE REDUCTASE

Kit: Cayman Glutathione Reductase Assay Kit Catalog No. 703202.
Reagents & Supplies: (not provided in kit.)

Reagent	Supplier	Catalog No.
HPLC-grade water	Fisher	W5-4

Reagent Preparation:

Assay Buffer (10X): Dilute 2 mL of Assay buffer concentrate with 18 mL of HPLC-grade water. Store at 4°C. Stable for 2 months.

Sample Buffer (10X): Dilute 2 mL of Sample buffer concentrate with 18 mL of HPLC-grade water. Store at 4°C. Stable for 1 month.

Glutathione Reductase (Control): Dilute 10 µL of supplied enzyme with 990 µL of diluted sample buffer. Keep on ice. Aliquot 70 µL into 0.5 mL centrifuge tubes and store at –20°C.

GSSG: Ready to use. Store at $^{-}20^{o}$C.

NADPH: Reconstitute the number of vials required by adding 2 mL of HPLC-grade water to each vial and vortex. Each vial is enough reagent for 40 wells. Keep at 25^{o}C for assay. Store at 4^{o}C. Stable for 2 days.

Sample Preparation — Tissue:

1. Homogenize the tissue in 5–10 mL of cold assay buffer (i.e., 50 mM potassium phosphate, pH 7.5, 1 mM EDTA) per gram of tissue. (***General Equation:*** **µL Buffer = mg Tissue X 10**)
2. Centrifuge at 10,000 x g for 15 minutes at 4°C.
3. Remove supernatant for assay and store on ice. Sample can be stored at –80°C for at least one month.

Performing Assay:

1. Turn on Multiskan and open file GRx.sed.
2. **Background Wells:** add 120 µL of Assay Buffer and 20 µL of GSSG to three wells.
3. **Positive Control Wells:** add 100 µL of Assay Buffer, 2 0µL of GSSG, and 20 µL of diluted GR (control) to three wells.
4. **Sample Wells:** add 100 µL of Assay Buffer, 20µL of GSSG, and 2 0µL of sample to three wells.
5. Initiate reactions by adding 50 µL of NADPH to all wells as quickly as possible. Note precise time the reaction is initiated.
6. Place plate onto Multiskan holder and click START.
7. Save raw data as an Excel file into the GRx data folder. Use the naming convention gxXXXX.xls, where XXXX is the date in mmdd format.
8. Select Process>Organize. Choose the appropriate data to organize (usually Measure1), then click **OK**. This rearranges the data into columns.
9. Save organized data as an Excel file into the GRx data folder. Use the naming convention grXXXXXX.xls, where XXXXXX is the date in mmddyy format.

GLUTATHIONE S-TRANSFERASE

Reagents & Supplies:

Reagent	Supplier	Catalog No.
HPLC-grade water	Fisher	W5-4
1-Chloro-2, 4-Dinitrobenze CDNB	Sigma	237329-10G
Potassium phosphate Monobasic	Sigma	
Potassium phosphate Dibasic	Sigma	
EDTA	Fisher	16 004Y
Reduced Glutathione	Sigma	G6529-1G
GST Control		

Reagent Preparation:

Homogenizing Buffer: To 10 mL 100 mM potassium phosphate **pH to 6.5, add 400 µL** 50 mM EDTA. (Final concentration EDTA is 2mM)

Assay Buffer: Add 20 µL Triton X-100 (0.1%) to 20 mL of 100 mM potassium phosphate buffer pH 6.5. Equilibrate to 25^0C before using. .

GST (Control): Solution of rat liver.

Glutathione: Add 6.15 mg to 2 mL HPLC H_2O. (10 mM)**(Final concentration in well 1 mM)** (Habig, The first enzymatic step in mercapturic acid formation. J.Biol. Chem. 249, 7130-7139 (1974))

CDNB: (Upstairs in fume hood.) Add 4 mg CDNB to 1mL ethanol. (20 mM) (Final concentration in well 1 mM)

Sample Preparation: Tissue

1. Label 2 sets of 1.5 ml micro-centrifuge tubes and 1 set of 0.5 ml tubes.
2. If not perfused at dissection, rinse tissue with ice cold homogenization buffer to remove RBC's and clots.
3. Sonicate the tissue on ~5 in 0.5–1 mL of cold homogenizing buffer per mg of tissue.

   General Equation: **µL Buffer = mg Tissue x 10**

4. Centrifuge at 10,000 x g for 15 minutes at 4°C.
5. Remove 15 µL supernatant and place in a 0.5 ml tube for protein analysis.
6. Transfer supernatant to a new labeled tube for assay and store on ice. Sample may be stored at –80°C for at least one month.

Sample Preparation: Plasma

1. Collect blood in a tube containing an anticoagulant.
2. Centrifuge at 700-1000 x g for 10 min. at4^{0}C.
3. Place the top yellow plasma layer in a labeled 1.5ml micro-centrifuge tube.
4. Remove 15 µL supernatant and place in a 0.5 ml tube for protein analysis.

Erythrocyte Lysate:

1. Remove the white buffy layer (leukocytes) and discard.
2. Lyse the erthrocytes (RBC) in 4 times its volume of ice-cold **HPLC-grade water**.
3. Centrifuge at 10,000 x g for 15 min. at 4^{0}C.
4. Place supernatant (erythrocyte lysate) in labeled 1. 5ml micro-centrifuge tube. **Dilute prior to assay.**
5. Remove 15 µL supernatant and place in a 0.5 ml tube for protein analysis.

Performing Assay:

Final volume of the assay is 20 0µL in all wells

1. Turn on Multiskan and open file gst.sed and make sure the machine is set to **25°C.**

2. Add 20 µL of Assay Buffer to background wells, 20 µL GST control to the control wells and 20 µL sample to sample wells.

3. Add 150 µL Assay Buffer to all wells.

4. Add 20 µL of GSH to each well.

5. Initiate reactions by adding 10 µL of CDNB to all wells as quickly as possible. **Note precise time the reaction is initiated.**

6. Place plate onto Multiskan holder and click START.

7. Read absorbance once every minute at 340 nm at least 5 minutes. ***Absorbance increase should be between 0.012 & 0.064/min. If not, dilute or concentrate sample. If initial reading is >0.7, dilute sample.***

8. From the sheet menu, select Process>Organize. Choose the appropriate data to organize (usually Measure1), and click **OK**. This re-arranges the data into columns.
9. Save organized data as an Excel file into the GTx data folder. Use the naming convention GTXXXX.xls, where XXXXXX is the date in yymmdd format.

HYDROGEN PEROXIDE ASSAY

Kit: Amplex Red Hydrogen Peroxide Assay Kit, Catalog No. A-12212.

Reagents & Supplies: HPLC-grade water

Reagent Preparation:

Amplex Red reagent: Prepare a ~20 mM stock solution. Bring DMSO and 1 vial of Amplex Red reagent to room temp. Just prior to use dissolve the Amplex Red reagent in 200 µL DMSO. Store stock solution an -20^0C, protected from light.

Reaction Buffer (5X) (0.25M sodium phosphate, pH 7.4): Dilute 5 mL of Reaction buffer in 20 mL of deionized water.

HRP (Horseradish peroxidase): Dissolve 1 vial of HRP in 1 mL of 1X Reaction Buffer (200 U/mL). After use divide remaining stock into small aliquots and store frozen at -20^0C.

20mM H_2O_2: Dilute (check bottle for %) 17.9 µL H_2O_2 (3.8%) in 982.1 µL dH_2O. (Check label for exact concentration) (23 µL 3% H_2O_2 into 977 µL dH_2O) Use promptly.

Resorufin, sodium salt: add 1mL dH_2O to a vial of resorufin solid. (2 mM stock). Store at -20^0C, protected from light.

Sample Preparation:

1. Prepare stock solutions above for Amplex Red Reagent, Reaction Buffer, HRP, 20 mM H_2O_2 and Resorufin, sodium salt.
2. Prepare H_2O_2 standard curve. Label tubes 1-5. Add 999 µL 1X Reaction Buffer to tube 2. Add 100 µL 1X Reaction Buffer to tubes 2 – 5. Add 1 µL of 20 mM H_2O_2 working dilution prepared above to tube # 1 to produce concentrations of 20 µM. Take 100 µL from tube 1 and add to tube 2. Take 100 µl from tube 2 and add to tube 3. Take 100 µl from tube 3 and add to tube 4. Tube 5 is dH_2O only. *(Final concentration will be twofold lower, 0 to 10 µM.*
3. Dilute samples in 1X Reaction Buffer.
4. Pipette 100 µL of diluted standards, controls (if any) and samples into wells. (For DRG we used 25 µL)
5. From 20 µM stock solution of Amplex Red reagent - Prepare 400 µM dilution containing 2 U/mL HRP by adding 20 0µl of Amplex Red stock solution and 100 µL of 200 U/mL HRP stock solution to 9.7 mL of 1X Reaction Buffer.
6. Initiate reaction by adding 100 µL from above to each well.
7. Place plate into Fluroskan holder and click START.
8. Take 4 readings at 15 minute intervals using 544/590 filter pairs.
9. Save raw data as an Excel file into the RHPx data folder. Use the naming convention RHXXXX.xls, where XXXX is the date in mmdd format.
10. Select Process>Organize. Choose the appropriate data to organize (usually Measure1), then click **OK**. This rearranges the data into columns.
11. Save organized data as an Excel file into the RHP data folder. Use the naming convention rhXXXXor.xls, where XXXX is the date in mmdd format.

LOWRY PROTEIN ASSAY

Reagents: (individual catalog #'s)

Reagent	Supplier	Catalog No.
Reagent A (Store at 22°C)	Bio-Rad	500-0113
Reagent B (Store at 22°C)	Bio-Rad	500-0114
Reagent S (Store at 22°C)	Bio-Rad	500-0115
Standard (Lyophilized Bovine Plasma Gamma Globin (Store at 4°C)	Bio-Rad	500-0005

Performing the Assay:

1. Thaw samples on ice.
2. If samples contain detergent: add 20ul of reagent S to each ml for reagent A
3. Prepare standard as follows:

Standard #	Protein	Buffer
0.0	0 µl	15 µl
0.2	2.1 µl	12.9 µl
0.4	4.3 µl	10.7 µl
0.6	6.4 µl	8.6 µl
0.8	8.6 µl	6.4 µl
1.0	10.7 µl	4.3 µl
1.2	12.9 µl	2.1 µl
1.4	15µl	0 µl

4. Pipet 5 µl of standards and samples into plate.
5. Add 25 µl of reagent A to each well.
6. Add 200 µl reagent B to each well.
7. Place plate in reader and press START.

Reading the Plate – (wavelength 750 nm)

1. Wipe the bottom of plate to remove finger prints, dirt, etc.
2. Open ascent software to saved template. Place plate onto Multiskan holder and click START. Plate will shake for 5 seconds then incubate 15 min. then read plate at 750 nm.
3. Save raw data as an Excel file into the Lowry data folder. Use the naming convention LYXXXXXX.xls, where XXXXXX is the date in yymmdd format.
4. Select Process>Organize. Choose the appropriate data to organize (usually Measure1), then click **OK**. This rearranges the data into columns.
5. Save organized data as an Excel file into the LY data folder. Use the naming convention LYXXXXor.xls, where XXXX is the date in mmdd format.

NADH OXIDASE

Reagents & Supplies:

Reagent	Supplier	Catalog No.
50 mM Tris	Gibco	15504-012
50 mM MES buffer	Sigma	
150μM NADH	Sigma	N 6879

Reagent Preparation:

Tris-MES buffer (pH 7.0): Prepare 50 mM Tris buffer solution and pH to 7.0 with 50 mM MES. 50 mM Tris - 302.85 mg Tris in 50ml deionized H_2O. 50 mM MES - 319.89 mg MES in 50ml deionized H_2O.

NADH: Prepare 600 μM solution. 2.55 mg in 6mL de-ion H_2O, enough for whole plate.

Sample Preparation:

Tissue:

1. Sonicate tissue on ice in 20 mM PB pH 7.4 with PMSF inhibitor or thaw samples on ice.

Performing the Assay:

2. Turn on Multiskan and set temp to 37^0 and set up plate layout.
3. Remove 25 μL for protein analysis.
4. Prepare NADH, enough for whole plate.
5. Dilute samples 1:5 with de-ionized H_2O.
6. Using a clear plate: Add 50 μL sample to wells and 50μL diluted sample in duplicate.
7. Add 50 μL buffer to 3 wells for blanks for positive control.
8. Add 100 μL Tris-Mes to each sample and blanks.
9. For negative control add 200 μL Tris-Mes to 3 wells.
10. Place plate in Multiskan and add 50 μL 600 μM NADH to the sample and positive blanks. **Do not add NADH to the 3 negative control wells.**
11. Press start and read at 340 nm for 10 minutes at 1 minute intervals.
12. Save raw data as an Excel file into the NADHx data folder. Use the naming convention NAXXXX.xls, where XXXX is the date in mmdd format.

TBARS

Reagents & Supplies:

Reagent	Supplier	Catalog No.
Thiobarbituric Acid (TBA)	ICN	190284
Trichloroacetic Acid	Sigma	490-10
1,1,3,3-tetramethoxypropane	Acros	148611000

Reagent Preparation:

Thiobarbituric Acid (TBA): 67 mg in 1mL DMSO then add 9 mL H_2O.

10% Trichloroacetic Acid (w/v): in H_2O.

1,1,3,3-tetramethoxypropane: 4.167 µL in 1mL Ethanol then add 49 mL H_2O. (500 µM)

Sample Preparation: **- Tissue:**

1. Label 1 sets of 1.5mL micro-centrifuge tubes, 1 set screw top tubes and 1 set of 0.5 mL tubes.
2. Weighed out ~20 mg and sonicated in 200 µL RIPA buffer + inhibitors.
3. Centrifuged at 3000 for 10 min at4^0.
4. Remove 10 µL aliquot into the 0.5mL tubes for protein analysis.
5. Place 100 µL lysate into a labeled 1.5mL micro-centrifuge tube.

Plasma: Place 100 µL plasma into a labeled 1.5 mL micro-centrifuge tube.

6. Add 200 µL ice cold 10% Trichloroacetic acid to precipitate protein.
7. Incubate for 15 minutes on ice.
8. Prepare standards as follows:

CONCENTRATION (µM)	H_2O	TETRAMETHOXYPROPANE
0	500	----------
0.625	500	500 from tube 3
1.25	500	500 from tube 4
2.5	500	500 from tube 5
5.	500	500 from tube 6
10	800	200 from tube 7
50	500	500 from tube 8
100	800	200 of 500 uM stock

9. Centrifuge samples at2200 x g for 15 min. at at4^{0}C.
10. Place 200 µL supernatant and standards into new labeled 1.5 ml tube.
11. Add and equal volume of 0.67% (w/v) TBA. (Check pH)
12. Incubate in a boiling water bath for 10 min.
13. Cool. Sample is ready for assay.

Performing Assay:

1. While samples are cooling, layout on computer and save as TBxxxxxx.sed where xxxxxx is the date in yyddmm format.
2. Load 150 µL into each standard well in duplicate.
3. Load 150 µL into each samples well in duplicate.
4. Put in plate reader and press start.

Reading the Plate:

- *Record absorbance at 532 nm.*

1. Turn on Multiskan and open your saved file TBxxxxxx.sed.
2. Place plate onto Multiskan holder and click START.
3. Select Process>Curve Fit. Choose the appropriate data (usually Measure1), then click **OK**.
4. Save Curve Fit data sheet as an Excel file into the Data folder/TBARS data folder. Use the naming convention TBxxxxxx.xls, where xxxxxx is the date in yymmdd format.

TRAP* ASSAY

Reagents & Supplies:

Reagent	Supplier	Catalog No.
100 mM ABAP	Sigma	44,091-4
30 mM PB pH 7.0	————	————
Luminol	Amersham	RPN2106

Reagent Preparation:

100mM ABAP*: Add 54.24 mg to 2 mL 30 mM PB.

Luminol: Mix 2 reagent ½ & ½..

Sample Preparation - DRG:

1. Remove 3 DRG from vial, cut in half and weigh.
2. Add 25 µL 30mM PB and sonicate on 4 on ice.
3. Spin at maximum g's for 10 min at 4^0C.
4. Remove supernatant and store on ice.
5. Set up plate layout on computer. **Be sure light shield is in place.**

Performing the Assay:

1. Using a **White Solid Bottom** plate, prepare plate by loading buffer for serial dilution. No PB in 1st well, 5 µL PB in following 4 wells and 5 µL in 1 control well. (Control is PB, Luminol and ABAP) (Dilutions, 1:1, 1:2, 1:4, 1:8, 1:16)
2. Prepare ABAP just prior to running assay by dissolving 54.2 mg ABAP in 2 mL PB. (Enough for 3 columns)
3. Load 5 µL sample in wells 1 & 2. Mix the sample & PB in well 2 and remove 5 µL and place in 3rd well with PB and so on. On last dilution discard 5 µL.
4. Prepare Luminol in a 50 mL conical tube and add 200 µL per well.
5. Place plate in Fluoroskan and add 60 µL ABAP per well and press **START.**
6. Read every 30 seconds for 20 minutes.
7. When reading is done, Select Process>Organize. Choose the appropriate data to organize (usually Measure1), then click **OK**. This rearranges the data into columns.
8. Save organized data as an Excel file into the TRAP Assay data folder. Use the naming convention trXXXXXX.xls, where XXXXXX is the date in yymmdd format.

*ABAP = 2,2'-azobis(amidinopropane) dihydrochloride

*TRAP = Total Radical-trapping Antioxidant Parameter

WESTERN ANALYSIS

Reagents & Supplies:

Reagent	Supplier	Catalog No.
12.5% Acrylamide gel stock solution	Sigma	
10% Ammonium persulfate (APS)	Sigma	
TEMED	Sigma	
2-propanol	Sigma	
10X TBS	Sigma	
10x Sample Buffer	Sigma	
10X Running Buffer		
10X Transfer Buffer		
5% Fat Free Milk	Grocery Store	
Gel loading tips	Fisher	
Nitrocellulose	Schleicher & Schuell	
ECL kit	Amersham	

Reagent Preparation:

10X TBS	**10X TBS**
1 Liter	**4** Liters
12.1 g Tris Base	48.4 g Tris Base
87.7 g NaCl	350.8 g NaCl
950 mL ddH_2O	3800 mL ddH_2O
pH to 8 with conc. HCL	pH to 8 with conc. HCL
Bring final volume up to 1000 mL	Bring final volume up to 4000 mL
TBST (1L)	**Milk** (100 mL)
100 mL 10X TBS	10 mL 10X TBS
900 mL ddH_2O	90 mL ddH_2O
10 mL 10% TWEEN-20	1 mL Thimerosol 1%
	1 mL TWEEN-20 10%
	5g milk
500mL TBS	**10% TWEEN-20**
50 mL 10X TBS	10 mL TWEEN-20
450 mL ddH_2O	90 mL ddH_2O

Separating Gel Preparation:

1 gel: 10 mL 12.5% gel stock
50 µL 10% APS
7 µL TEMED

Stacking Gel Preparation:

2 gels: 5 mL stacking gel stock
30 µL 10% APS
5 µL TEMED

1. Wash & dry plates.
2. Assemble rig and fill plates with H_2O to check for leaks.
3. Pour off water and wipe dry with kimwipe.
4. Load gel to about the top of the door.
5. Add 2-propanol to cover the edge.
6. Wait ~ 40 minutes to polymerize.
7. Thaw samples on ice.
8. When gel is ready, pour off 2-propanol and rinse with H_2O.
9. Remove excess H_2O with kimwipe.
10. Prepare and load stacking gel and insert comb making sure there are no bubbles under the teeth.
11. Put a beaker of water on the hot plate to boil.
12. Prepare samples. Check with whomever requested the western as to what preparations need to be done, e.g. sonicate, dilute (**dilute plasma 1:50.)** etc. and what buffer to use. (warm buffer in 37^0C water bath)
13. Label new screw top tubes for samples, controls, and markers.
14. If not already done, do protein analysis on samples. Generally load 70 µg so divide 70 by your µg/µL protein to find out the volume to load. Divide this number by 5 to know how much 10X sample buffer to add. (Want a 2X dilution)
15. OR Dilute sample as instructed by assay requester. (24 x 70 µg/OD = µL to load) (Take the answer and divide by 5, that's the amount of 10x sample buffer)
16. Add 2 µL 10X sample buffer to 10 µl rainbow protein marker. (times 2 for 2 gels)
17. Add 2 µL 10X sample buffer to 10 µl Biotinylated protein marker.
18. Boil samples and markers for 5 minutes and cool on ice.
19. When gel is done gently remove combs.
20. Assemble rig with short plate on the inside, press down and close doors.
21. Fill inside chamber with running buffer to about ½ way between top of sm & lg plate and make sure there are no leaks.
22. Pour more running buffer into outside of rig to the bottom of the gate.
23. Load rainbow protein marker, biotinylated marker, samples and + & - controls.
24. Set volts at 200 and run for 50-60 minutes.
25. Remove gel from rig, remove wells and soak gel in transfer buffer for 15 minutes.
26. Cut and label nitrocellulose membrane to size and soak in transfer buffer with gel.
27. In another dish, soak 2 fiber pads and 2 fiber paper for each gel.

28. Assemble the sandwich with black side down in transfer buffer, making sure there are no bubbles between each layer put 1 fiber pad, 1 Whatman paper, gel, nitrocellulose, 1 Whatman paper, and 1 fiber pad.
29. Put a stir bar in the bottom of the rig and place the sandwich in the transfer unit with the black part in the back. (Protein runs from black to red, to the membrane)
30. Fill the ice pack and place behind the sandwich.
31. Fill the unit with 1X transfer buffer until the ice pack floats or the top of the lower ledge.
32. Transfer at 100V for 1 hour (100 kd-30 kd) or 69 V for very low proteins (or maybe just 15 minutes for Bcl-2)
33. Rinse the membrane in 1X TBS for 10 minutes.
34. Block overnight at 4^0 or at 22^oC for 2 hours in TBST/milk for polyclonal antibodies or TBST/BSA for mAbs.
35. Quick rinse once with TBST.
36. Incubate 2 hours at 22^oC or overnight at 4^0 in primary antibody in TBST/milk or TBST/BSA on rocker. (Primary antibody can be re-used) *For anti-phosphotyrosine immunoblotting only*: incubate 1 hour in TBST/milk
37. Wash 3 x's for 5 minutes with TBST.
38. Incubate for 1 hour in secondary antibody in TBST/milk or TBST/BSA.
39. Quick rinse once with TBST.
40. Wash 3 x's for 5 minutes each in TBST.
41. Wash 20 minutes in 1X TBS.
42. In a 15 mL conical tube, develop with small cell signaling bottles using 9 mL H_2O and 500 μL of each reagent. Expose for 1 minute.

WESTERN BLOT STRIPPING

Reagents & Supplies:

10% SDS	Gibco	15525-025
0.15 g Dithiothreitol		
2 mL 0.5 M Tris pH 6.8	Gibco	15504-012
6 mL ddH_2O		

Performing assay:

Rainbow markers do not withstand stripping, so if you don't have a biotinylated marker on your blot, be sure to mark the location of the rainbow markers with a pen or pencil before stripping

1. Incubate your blot for 10-20 min at 70^0 C. to strip. (10 min. for 20-40 μg protein and 15-20 min. for loaded protein over 40 μg or a very strong antibody)
2. Quick rinse in TBST.
3. Rinse 3 X's 10 minutes each in TBST.
4. Re-block blot.
5. Run a loading control after each stripping.

OR

0.5% Triton X-100
0.5 mL Triton X-100
95.5 mL ddH_2O

1. Incubate membrane in 0.5% Triton X-100 solution for 30 min. at room temp on a rocker.
2. Rinse 3 X's 10 minutes each in TBST.
3. Re-block blot.