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Human Ganglia and Spinal Cord Tissue Procurement from Organ Donors and Tissue Quality Assessment

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We use this protocol and it's working

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Abstract

Purpose

Describe the human nervous tissue removal, preservation, storage, and transportation procedures during tissue procurement from organ donors.

Responsibility

The Human Tissue Procurement and Processing core at the University of Texas at Dallas is responsible for maintaining this protocol, ensuring it is updated and controlled, and ensuring that users receive proper training prior to its implementation.

Definitions and Abbreviations**aCSF** = artificial cerebral spinal fluid**BD** = Brain Dead**COD** = Cause of Death**CTOD** = Called Time of Death**DCD** = Donation upon cardiac death**Donor ID** = UNOS identification number assigned to each donor; provided by the donor network**DRG** = dorsal root ganglion**HSG** = human sensory ganglia**NMDG** = N-Methyl-D-glucamine**OCT** = optimal cutting temperature**OPO** = organ procurement organization**ORC** = operating room coordinator**OR** = operating room**PPE** = personal protective equipment**SCG** = sympathetic chain ganglion**UTD** = The University of Texas at Dallas**Tissue Recovery Sites**

OPO headquarters

Local hospitals

Tissue Recovery Teams

OPO Surgical Staff

UTD Recovery Teams: 2 members (graduate students and post-doctoral scientists) per team

Materials

Materials provided by OPO or hospital:

1. PPE including surgical scrubs, gloves, hair nets, and shoe covers
2. Waste disposal bins

Materials provided by the OPO:

1. Biohazard Packaging - Box and Bag for Tissue Transport

Materials provided by UTD (Price lab):

1. Ice-cold, carbogen-bubbled aCSF (see below)
2. Powdered dry ice (see below)
3. Wet ice
4. Containers for dry and wet ice (Globe Scientific 2.5L Rectangular Ice buckets with Lid)
5. Transportation cart
6. Recovery toolbox
7. Sterile dissection tools (large and small scissors, forceps)
8. Sterile storage tubes (1.5mL epitubes, 5mL epitubes, and 15mL conical tubes)
9. Sharpies
10. Sterile dissection dishes (Pyrex Petri Dish 150 mm x 15 mm, or stainless steel dissection tray 8.6 in x 4.3 in)
11. Large Ziploc bags

aCSF Stock Preparation

- The following reagents should be purchased and stored per manufacturer's instructions in the laboratory:

1. N-Methyl-D-glucamine - (Sigma, cat no. M2004-500G)
2. HCl - 12.1 N (Fisher, cat. no. A144-212)
3. KCl - (Sigma, cat. no. P5405-250G)
4. NaH₂PO₄ - (Sigma, cat. no. S5011-100G)
5. NaHCO₃ - (Sigma, cat. no. S5761-1KG)
6. HEPES - (Sigma, cat. no. H4034-500G)
7. D-(+)-Glucose (Sigma, cat. no. G8270-1KG)
8. L-Ascorbic acid (Sigma, cat. no. A5960-25G)
9. Thiourea (Sigma, cat. no. T8656-50G)
10. Na⁺ pyruvate (Sigma, cat. no. P2256-25G)
11. MgSO₄ (2 M, Fisher, cat. no. BP213-1)
12. CaCl₂ dihydrate (Sigma, cat. no. C7902-500G)
13. N-acetylcysteine (Sigma, cat. no. A7250-50G)

- **Stock solutions of the following reagents can be prepared:**

A	B	C	D
Reagent	Final Concentration (M)	Amount	Volume
HEPES	2	23.83g	50mL ultra-purified water
KCl	2.5	9.3188g	50mL ultra-purified water
MgSO ₄	2	24.647g	50mL ultra-purified water
CaCl ₂	1	7.35g	50mL ultra-purified water
NaH ₂ PO ₄	1.25	749.875mg	50mL ultra-purified water

- The aCSF stock consists of reagents that are stable together at high concentrations and stored at room temperature. To make the aCSF stock, add the following ingredients to 3000mL of ultra-purified water (Milli-Q-H₂O).

A	B	C	D
Reagent	Final Concentration (mM)	MW or Concentration of Stock	For 3600 mL
NMDG	93	195.2	72.7 g
HCl		12.1 N	28 mL
KCl	2.5	2.5M	4 mL
NaH ₂ PO ₄	1.25	1.25M	4 mL
NaHCO ₃	30	84.0	10.1 g
HEPES	20	2M	40 mL

- Once dissolved, bring to final volume of 3600 mL with ultrapurified water (Milli-Q-H₂O). 3600mL of stock solution is enough for 8 batches of 500mL of aCSF working solution.

aCSF Working Solution Preparation

- The aCSF working solution can be prepared by adding the following ingredients to exactly 450mL of aCSF stock solution.

A	B	C	D
Reagent	Final Concentration (mM)	MW or Concentration of Stock	For 500 mL

A	B	C	D
Glucose	25	2.5M	2.25 g
Ascorbic Acid	5	176.1	0.44 g
Thiourea	2	76.1	0.08 g
Na ⁺ pyruvate	3	110.0	0.17 g
MgSO ₄	10	2M	2.5 mL
CaCl ₂	0.5	1M	250 µL
N-acetylcysteine	12	163.2	0.98 g

- Once dissolved, adjust the pH to 7.4 with NMDG or HCl.
- Bring to final volume of 500mL with ultrapurified water (Milli-Q-H₂O).
- Check the osmolarity using an osmometer, it should be between 300-310. If necessary, adjust with ultrapurified water (Milli-Q-H₂O) or sucrose.
- aCSF working solution can be stored at 4°C for up to two weeks. Prior to leaving for a donor, the aCSF working solution should be sterile filtered and then be bubbled with carbogen gas (95% O₂, 5% CO₂) for at least 15 minutes.

Powdered Dry Ice

- Dry ice (pellets or blocks) must be pulverized to a fine powder to ensure proper freezing of human tissues. Using a hammer, crush the dry ice in an ice bucket (Globe Scientific 2.5L Rectangular Ice buckets with Lid) or styrofoam container.
- To ensure that the dry ice particulates are small enough, run the pulverized dry ice through a mesh grate/netting.

OCT Embedding Materials

1. metal cryomold - Ted Pella, 27276-5
2. OCT - Fisher, 23-730-571

Hematoxylin and Eosin Staining

1. Hematoxylin (Agilent Tech, S330930-2)
2. Eosin (Sigma, HT110216)
3. Tris base (Thermo Fisher Scientific, BP152-500)
4. Nuclease-free water (Invitrogen, AM9937).
5. 100% Acetic Acid (Millipore Sigma, A6283).
6. 0.2 µm Corning 250mL Vacuum System (Corning, 430771)
7. Bluing Buffer (Agilent Tech, CS70230-2)

8. Prolong Gold Antifade Reagent (Fisher, P36930)

RNA Quality Assessment in Extracted RNA

1. RNeasy Plus Universal Mini Kit (Qiagen, 73404)
2. RNase Zap (ThermoFisher, AM9782)
3. Precellys 2 ml Tissue Homogenizing Mixed Beads tube (Bertin Corp, P000918-LYSK0-A).
4. Qiazol Lysis Reagent (Qiagen, 79306)
5. Precellys Minilys Personal Homogenizer (Bertin Corp, P000673-MLYS0-A)
6. Chloroform (Fisher Scientific, C298-500)
7. Ethanol (Fisher Scientific, 04-355-223)
8. Buffer RWT (Qiagen, 1067933)
9. Buffer RPE (Qiagen, 1018013)

RNA Quality Assessment in Intact Tissue Sections (RNAscope)

1. 10% formalin (Thermo Fisher Scientific, 23-245684)
2. PAP pen (Vector Labs, H-4000)
3. RNAscope® Multiplex Fluorescent Reagent Kit v2 (Advanced Cell Diagnostics, 323100)
4. RNAscope™ 3-plex Positive Control Probe-Hs (Advanced Cell Diagnostics, 320861)
5. RNAscope™ 3-plex Negative Control Probe (Advanced Cell Diagnostics, 320871)

Safety warnings

- ! Safety training courses on Bloodborne Pathogens, Biological Safety, Chemical Hygiene, Compressed Gases, Biological Waste Management, Personal Protective Equipment, Autoclave and Media Kitchens, General Laboratory Safety, and Cryogen Safety are required by UTD and can be taken through its BioRAFT system.

Safety Training related to Biomedical Research with Human Subjects is required by UTD and can be taken through its CITI program.

HSG Recovery Notification

- 1 OPO staff notifies the UTD Recovery Teams of a donor for HSG Study Enrollment via email and provides the following information:
 - 1) Donor ID
 - 2) Seros report
 - 3) Medication history
 - 4) Travel history
 - 5) COVID-19 test results
 - 6) Age
 - 7) Sex
 - 8) COD
 - 9) Tissue recovery site
 - 10) OR time
 - 11) Expected arrival time for UTD Recovery Team
 - 12) ORC contact information
 - 13) OPO surgical staff assigned to recovery
- 2 UTD Recovery Team review the information and accept or decline the donor for HSG Study Enrollment.
- 2.1 Exclusion criteria includes: distance to recovery site is >60 min, serology report indicates donor is positive for Hepatitis B, Toxoplasmosis, and/or Prior/Prion-like diseases.

Tissue Procurement

- 3 The UTD Recovery Team prepares the materials/equipment (ref step 9) at UTD and drives to the designated tissue recovery site for their arrival time.
- 4 The UTD Recovery Team dons PPE, enters the OR, and sets up their materials/equipment on a table within the OR.
- 5 The OPO Surgical Staff use an orthopedic saw (Stryker) to remove the ventral portion of the vertebral column from the thoracic to L5 level (ref Fig 1).

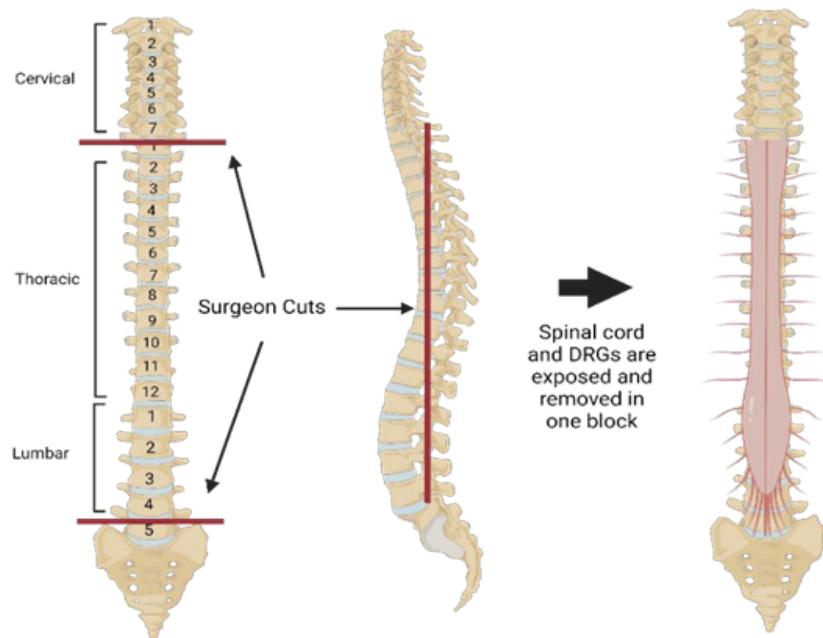


Figure 1. OPO surgical staff remove the ventral portion of the vertebral column to reveal the spinal cord and dorsal root ganglia.

- 6 Using forceps and scissors, the OPO Surgical Staff remove the spinal cord and attached DRGs within the spinal meninges and hand it off to the UTD Recovery Team.
- 7 The UTD Recovery Team places the tissue into a sterile dissection dish (Pyrex Petri Dish 150 mm x 15 mm or stainless steel dissection tray 8.6 in x 4.3 in) and rinses the tissue with sterile saline to remove blood and other fluids. The contaminated saline is discarded in a surgical waste bin in the OR.
- 8 Ice-cold, carbogen-bubbled aCSF is added to the dissection dish.
- 9 A UTD team member takes a picture of the intact spinal cord and DRG specimens for records.
- 10 The ventral side of the spinal meninges are hemisected using large scissors, and the meningeal sleeves containing the dorsal rootlets are exposed, and exiting the meningeal sleeves, the DRGs can be visualized.
- 11 The level and extent of tissue we receive can vary from donor-to-donor. As a result, the DRG level is determined on a case-by-case basis. Some DRGs may be damaged by the saw or left inside the body cavity before being handed to the UTD team. However, the surgical dissection

approach is preferable as it expedites the procurement process for the surgical staff and preserves tissue integrity and viability for laboratory experiments.

- 11.1 As such, we use visual landmarks to identify DRG level such as the location of the L1 DRG which is located immediately adjacent to the sacral portion of the spinal cord. The more caudal DRGs (L2-L5) are located within the cauda equina. (ref Fig 2)

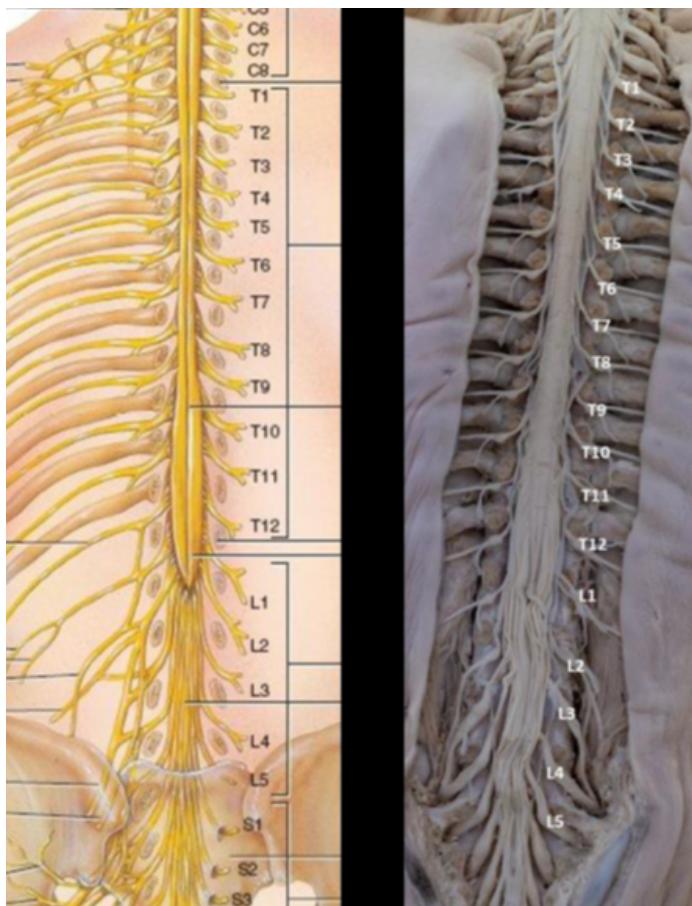


Figure 2. Organization of DRGs and anatomical landmarks.

- 11.2 DRG size is also a good indicator of level. The size of L1-L3 DRGs are visibly smaller than L4 and L5 (roughly $\frac{1}{2}$ the size). Thereby, if the 2 most caudal DRGs are large, we start our count from the bottom at L5, or if we visualize only 1, we start at L4. In some cases, the S1 DRG is extracted which is visibly much larger than the lumbar DRGs.
- 12 Each DRG is removed and briefly trimmed of any fat/connective tissue using small scissors and forceps.

- 12.1 The storage tubes, 1.5mL epitube for small DRGs, or 5mL epitubes for larger DRGs, are labeled with the donor ID and biospecimen information (e.g. AAA111 – L4 DRG).
- 12.2 Tubes used for fresh-frozen samples are prechilled on powdered dry ice and tubes used for live-tissue are filled with aCSF and placed on wet ice.
- 13 DRGs are placed into tubes filled with aCSF (live tissue preparation) or buried directly in powdered dry ice (fresh-frozen preparation).
- 14 After 2-3 minutes, the fresh-frozen DRGs are uncovered, and placed into their designated tubes over dry ice.
- 15 The spinal cord is trimmed transversely into 8-10cm segments (e.g. sacral, lumbo-sacral, lumbar, thoraco-lumbar, thoracic).
- 15.1 The storage tubes, 5mL epitubes, are labeled with the donor ID and biospecimen information (e.g. AAA111 – sacral spinal cord).
- 15.2 Tubes used for fresh-frozen samples are prechilled on powdered dry ice and tubes used for live-tissue are filled with aCSF and placed on wet ice.
- 16 The spinal cord segments are placed into tubes filled with aCSF (live tissue preparation) or buried directly in powdered dry ice (fresh-frozen preparation).
- 17 After 2-3 minutes, the spinal cord segments are uncovered, and placed into their associated tube in dry ice.
- 18 Ventral portion of the vertebral column roughly spanning the lumbar region is obtained from the OPO surgical staff. As previously stated, the ventral portion of the vertebral column is removed to gain access to the spinal cord and DRGs.
- 19 Sympathetic chain ganglia (SCG) extend along the lumbar segment of the vertebral column (ref Fig 3).

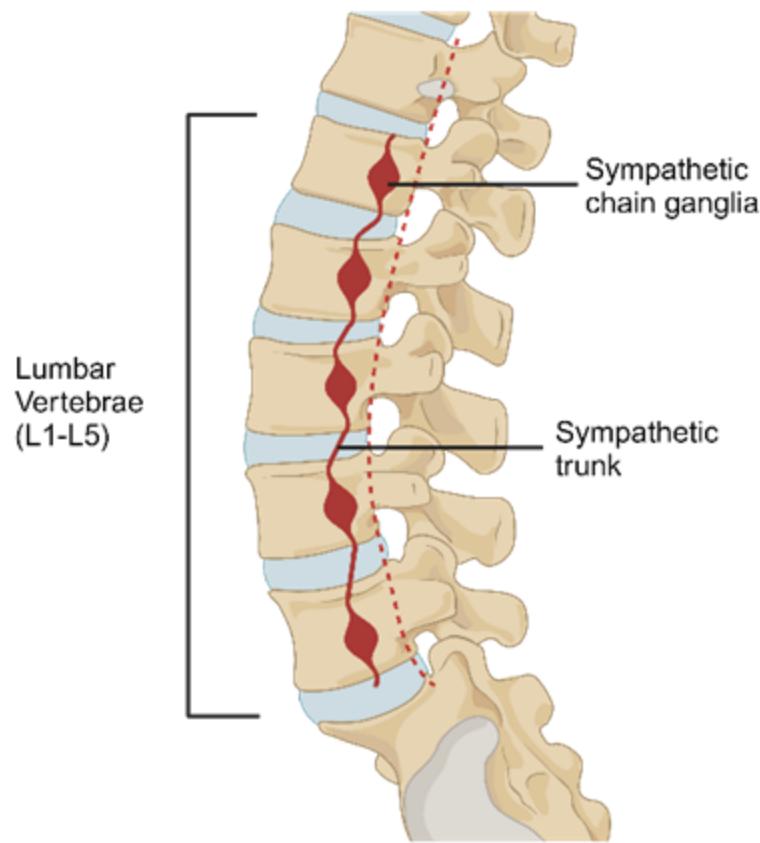


Figure 3. Sympathetic chain ganglia (SCG) are located on the ventral vertebral column. Lumbar SCGs are harvested as part of this protocol. The dotted line indicates the point at which the OPO staff saws the vertebral column.

- 19.1 Using scissors and forceps, muscle, fat, and connective tissue is trimmed from the lateral portion of the ventral vertebral column. This exposes the SCG and interganglionic nerves (also known as the sympathetic trunk) that connect two SCGs and run along the length of the vertebral column.
- 19.2 The level of the vertebra is noted by the OPO surgeon and allows for accurate recovery of lumbar SCGs.
- 19.3 SCGs are removed from the vertebral column and trimmed of excess fat and connective tissue in ice-cold aCSF.
- 19.4 The SCG tissue is then fresh-frozen in powdered dry-ice for 2-3 minutes and stored in prechilled 1.5 ml epi tubes labeled with the donor ID and biospecimen information including the vertebral level (e.g. AAA111 – L2 SCG).

- 20 While preparing the spinal cord and DRGs, the OPO Surgical Staff extracts the left and right sciatic nerves from the sacral plexus inside the pelvic cavity.
- 20.1 UTD Recovery Team places the sciatic nerves in another sterile dissection dish (Pyrex Petri Dish 150 mm x 15 mm) containing ice-cold, carbogen-bubbled aCSF.
- 21 Upon completion of DRG and spinal cord preparation, the UTD Recovery Team trims the left and right sciatic nerves into 7-8cm segments.
- 21.1 The storage tubes, 15mL conical tubes, are labeled with the donor ID and biospecimen information (e.g. AAA111 – left sciatic nerve caudal segment).
- 22 The sciatic nerve segments are buried directly in powdered dry ice (fresh-frozen preparation).
- 23 After 2-3 minutes, the sciatic nerve segments are uncovered, and placed into their associated tube in dry ice.

Packaging, Clean-up, and Transport

- 24 Excess tissue, biofluids, and dissection aCSF are disposed of in OR waste bins.
- 25 Used dissection tools, Sharpies, and dishes are placed into a large Ziploc bag and sealed.
- 26 The dry ice and wet ice buckets containing the biospecimen tubes, along with the used dissection tools, Sharpies, and dishes in the Ziploc bag are packaged inside a large biohazard bag and box provided by the OPO.
- 27 The box and toolbox are placed into the transport cart.
- 28 Upon exiting the OR, PPE is removed, and the team drives back to UTD.

Biospecimen Labeling, Logging, and Storage

- 29 A biospecimen number is assigned to each biospecimen and is denoted on the tube along with the previously labeled donor ID and biospecimen (e.g. AAA111 - L4 DRG, 1).

- 30 All frozen biospecimen tubes collected from a donor are placed into a cardboard cryo freezer box that is labeled on all sides with the donor ID and a box number. The biospecimen boxes are stored in a secure -80°C freezer.
- 31 All biospecimen tubes are logged on a tissue storage log stored on a protected data server. The following information is designated in the tissue log:
- 1) Box number
 - 2) Donor ID
 - 3) Biospecimen numbers
 - 4) Biospecimens associated with the number (e.g. 1 = Lumbar 4 DRG)
 - 5) Preservation condition (aCSF, fresh frozen in dry ice, fixed)
 - 6) Tissue status (e.g. embedded in OCT, cut in half for RNA extraction)
 - 7) Inventory (currently in freezer or if it has been used)
 - 8) Tissue notes (e.g. bloody, freezing artifact)
 - 9) Time the UTD team left the OR (subtract ~45 minutes to estimate collection time)
- 32 When a biospecimen is embedded in OCT, the biospecimen is placed into a small (5x7.5cm) zipper storage bag with a small index card denoting the donor ID, sample number, and date of embedding.
- 33 Serology reports and images of the tissue within the OR (prior to dissection) are also placed into designated folders on the protected data server for each donor.

Donor Demographic Information

- 34 Following recovery (within 1 month), the OPO staff provides a donor demographic table with the following information:
- 1) Donor ID
 - 2) Age
 - 3) Sex
 - 4) Ethnicity
 - 5) COD
 - 6) If they had COVID-19 recently
 - 7) Results of recent COVID-19 PCR test
 - 8) If donor received the COVID-19 vaccine
 - 9) Date and time of CTOD or BD
 - 10) Date and time of cross-clamp for organ recovery
 - 11) Diabetes Diagnosis (Yes or No)
 - 12) A1C
 - 13) Medical history (summary of hospital records and information provided by family)

- 35 Postmortem interval can be approximated using the time between cross clamp and time collected.

Tissue Quality Check

- 36 DRGs can be assessed for Tissue Integrity (ref steps 53-77.1) and RNA Quality (ref steps 78-106) using ~160 µm of sections from a single DRG (ref steps 37-52).

OCT Embedding

- 37 For donor tissues that require quality checking before experimentation, a single DRG from every donor is removed from the -80°C freezer and placed on powdered dry ice.

- 38 A metal cryomold (Ted Pella, 27276-5) is pre-chilled on powdered dry ice.

- 39 OCT (Fisher, 23-730-571) is applied to the entire bottom of the metal cryomold, creating a base, and is allowed to freeze.

- 40 2-3 drops of OCT are placed on the frozen OCT base and the DRG is placed there.

- 41 Once frozen, a thin ring of OCT is applied around the DRG and allowed to freeze. This step is repeated until the DRG is immersed in OCT. It is important to not freeze-thaw the DRG. The use of thin OCT rings around the sample allows for quick freezing of OCT without tissue thawing.

- 42 The OCT-embedded sample is stored in a -80°C freezer (ref steps 31-32).

Tissue Cryosectioning

- 43 The OCT-embedded DRGs are transferred on dry ice to a -20°C cryostat.

- 44 The samples are allowed to acclimate to -20°C for at least 10 minutes.

- 45 Charged slides are labeled with the donor ID and biospecimen and prechilled in the -20°C cryostat.

- 46 1.5mL eppendorf tubes are labeled with the donor ID and biospecimen and prechilled on dry ice.
- 47 The OCT-embedded tissue block is frozen to the cryostat chuck with OCT.
- 48 A single 20 µm section is collected on a spare slide and visualized under a light microscope for the presence of neurons (using lipofuscin as a visual aid). This step is repeated until neurons are found within the DRG.
- 49 20 µm sections are collected for each slide (2 sections for the RNAscope slide, 1 section for Hematoxylin and Eosin slide), and placed onto the slides with a paint brush.
- 50 The back of the slide is pressed against the back of the sectioner's glove as to warm the slide, so the sections briefly thaw and adhere to the slide. Once the sections are adhered which is visualized by the OCT-melting, the slide is immediately returned to the -20°C cryostat chamber. The sections will visibly re-freeze by turning white.
- 51 A 100 µm section is collected for RNA extractions and placed into the prechilled tube over dry ice.
- 52 If not being utilized immediately, the slides (in a slide box) and tubes can be temporarily stored at - 80°C.

Hematoxylin and Eosin Staining

- 53 Remove one of the prepared slide(s) from the -80°C freezer or the -20°C cryostat and transfer to the lab bench over dry ice.
- 54 Place slides in a 37°C incubator for 1 minute to thaw, and then immediately transfer the slides to a dish containing 10% formalin for 15 minutes at room temperature. The formalin incubation should be performed in a fume hood.
- 55 Dehydrate sections in ethanol by placing the slides in 50% ethanol for 1 minute, 70% ethanol for 1 minute, 100% ethanol for 1 minute, and another 100% ethanol for 1 minute.
- 56 Air dry slides briefly. Ethanol should evaporate within a few minutes.
- 57 Cover sections on each slide with 500 µL of isopropanol and incubate for 1 minute at room temperature.

- 58 Discard reagent by holding the slide at an angle with the bottom edge touching a laboratory wipe.
- 59 Air dry the slide for 2-3 minutes.
- 60 Cover sections on each slide with 1 mL of Hematoxylin (Agilent Tech, S330930-2) and incubate for 7 minutes at room temperature.
- 61 During the incubation, prepare the eosin mix which consists of 100 µL of Eosin (Sigma, HT110216) and 900 µL Tris-Acetic Acid Buffer (0.45M, pH 6.0). 1mL of eosin mix is needed per slide.
 - 61.1 Tris Acetic Acid Buffer (0.45M, pH 6.0) can be prepared as a stock and stored at room temperature.
To prepare, dissolve 11g Tris base (Thermo Fisher Scientific, BP152-500) in 100mL nuclease-free water (Invitrogen, AM9937). Adjust pH to 6.0 using 100% Acetic Acid (Millipore Sigma, A6283). Bring volume to 200mL with nuclease-free water. Filter through 0.2 µm Corning 250mL Vacuum System (Corning, 430771)
- 62 Discard reagent by holding the slide at an angle with the bottom edge touching a laboratory wipe.
- 63 Use a squirt bottle containing Milli-Q water and dispense a stream of water across the slide while holding it to remove any residual hematoxylin from the slide and sections.
- 64 Remove the excess water from the slide by holding the slide at an angle with the bottom edge touching a laboratory wipe.
- 65 Cover sections on each slide with 1 mL of Bluing Buffer (Agilent Tech, CS70230-2) and incubate for 2 minutes at room temperature.
- 66 Discard reagent by holding the slide at an angle with the bottom edge touching a laboratory wipe.
- 67 Use a squirt bottle containing Milli-Q water and dispense a stream of water across the slide while holding it to remove any residual Bluing Buffer from the slide and sections.
- 68 Remove the excess water from the slide by holding the slide at an angle with the bottom edge touching a laboratory wipe.

- 69 Cover sections on each slide with 1 mL of pre-made eosin mix (ref 61) and incubate for 1 minute at room temperature.
- 70 Discard reagent by holding the slide at an angle with the bottom edge touching a laboratory wipe.
- 71 Use a squirt bottle containing Milli-Q water and dispense a stream of water across the slide while holding it to remove any residual hematoxylin from the slide and sections.
- 72 Remove the excess water from the slide by holding the slide at an angle with the bottom edge touching a laboratory wipe.
- 73 Air dry the slides and then coverslip with Prolong Gold Antifade Reagent (Fisher, P36930).
- 74 Image slides as soon as possible (typically we image immediately following cover slipping as the eosin will leech from the sample over time). Slides are imaged mosaically on a vs120 or vs200 slide scanner (Evident Scientific) at 20X magnification (numerical aperture = 0.75) using the Brightfield imaging feature in the VS-ASW acquisition software (Evident Scientific, v3.4.1).
- 75 The raw image files (vsi) files are saved to a protected data server.

Tissue Integrity Scoring

- 76 Due to improper tissue freezing (slow freezing), nuclear and cytoplasmic vacuolization can be observed in hematoxylin and eosin stained tissue sections. The aqueous cellular compartments of cells need to be frozen appropriately to keep water molecules in a vitreous state, otherwise ice crystals can form and damage the cytoplasm and nucleus of cells upon tissue thawing. The loss of the nucleus and cytoplasm will prevent detection of molecules in these regions as their contents are removed from the tissue upon thawing. We have found that with our powdered dry ice freezing approach, we achieve freezing scores of 0-2 in 91% of our assessed samples (n=66), 9% with a score of 3, and 0% with a score of 4.
- 77 Raw image files (vsi) can be opened in Olympus CellSens (v1.18) and scored for nuclear and cytoplasmic vacuolization using the following parameters (ref Fig 4):

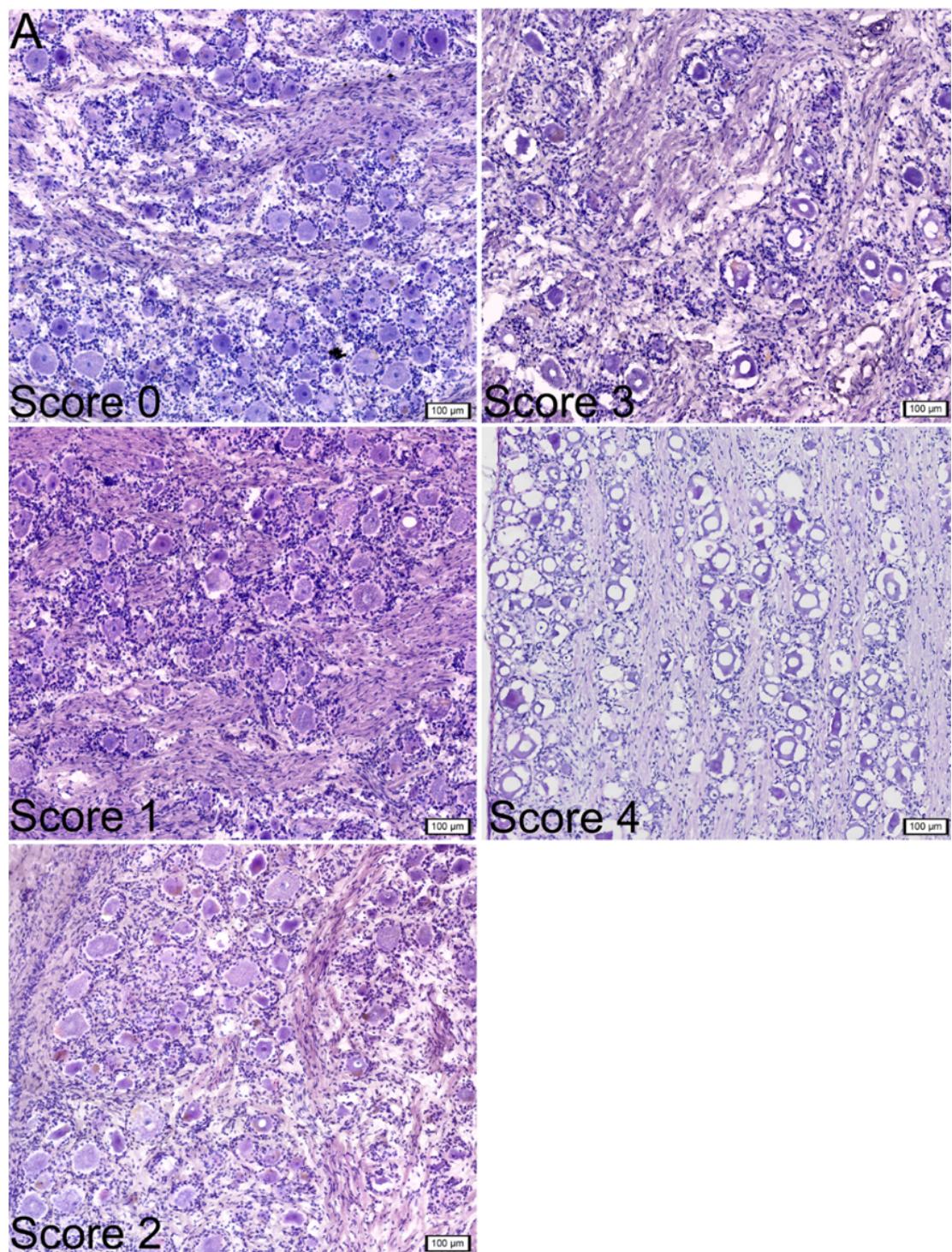


Figure 4. Freezing damage scoring system example images.

77.1 Score 0 = none

Little-to-no cytoplasmic vacuolization and/or nuclear loss in <10% of neurons

Score 1 = very low

Minor cytoplasmic vacuolization and/or nuclear loss in 10-20% of the neurons. Minor vacuolization may be visualized in the space around the neurons, but the majority of the neurons should look intact.

Score 2 = low Cytoplasmic vacuolization and/or nuclear loss in 20-50% of all the neurons. Vacuolization may also be visualized in the space around the neurons.

Score 3 = moderate

Cytoplasmic vacuolization and/or nuclear loss in 50-90% of all the neurons. Vacuolization may also be visualized in the space around the neurons.

Score 4 = severe

Severe cytoplasmic vacuolization in almost all neurons (>90%) resulting in almost the complete loss of the neuronal cytoplasm and/or complete loss of the nucleus in almost all neurons (>90%). Vacuolization may also be visualized in the space around the neurons.

RNA Quality Assessment in Extracted RNA (RNeasy Plus Universal Mini Kit)

- 78 The tube containing the 100 µm DRG section is removed from the -80°C freezer and transferred to the lab on dry ice.
- 79 Transfer the frozen tissue section using a pair of forceps precleaned with RNase Zap (ThermoFisher, AM9782) to a Precellys 2 ml Tissue Homogenizing Mixed Beads tube (Bertin Corp, P000918-LYSK0-A).
- 80 Add 500 µL Qiazol Lysis Reagent (Qiagen, 79306) to each tube. Ensure that tissue is completely immersed in the Qiazol.
- 81 Homogenize the tissue in a cold room (4°C) using a Precellys Minilys Personal Homogenizer (Bertin Corp, P000673-MLYS0-A) at the lowest speed (3000 rpm) for 30s.
- 82 Place the homogenate on ice for 30s-1min and then homogenize again (lowest speed, 3000rpm, 30s).
- 83 Repeat the homogenization step for a total of 3-5 times or until the tissue is homogeneous.
- 84 Transfer the lysates to a separate 1.5mL centrifuge tube and keep on ice for 10 min.

- 85 Add 100 µL of gDNA eliminator solution (mat No. 1062831 within RNeasy Plus Universal Mini kit, Qiagen, 73404) and shake vigorously for 15s.
- 86 Add 180 µL of Chloroform (Fisher Scientific, C298-500) and shake vigorously for 15s.
- 87 Keep the tubes at room temperature for 2-3 min. The solution will begin to separate into the aqueous phase (RNA), the interface (DNA), and the organic phase (protein), from top to bottom.
- 88 Centrifuge at 12,000g for 15 min at 4°C and transfer the aqueous supernatant (~500 µL) to a new tube.
- 89 Add 1 volume (~500 µL) of 70% Ethanol (Fisher Scientific, 04-355-223). Pipette to mix thoroughly.
- 90 Transfer 700 µL of the solution to RNeasy Mini Column (material within RNeasy Plus Universal Mini kit, Qiagen, 73404) placed in a 2 ml collection tube.
- 91 Centrifuge at full speed for 15s at room temperature and discard the flowthrough.
- 92 Repeat Step 90-91 with remaining lysate.
- 93 Add 700 µL of Buffer RWT (Qiagen, 1067933) to the RNeasy Mini Column, centrifuge at 8,000g for 15s at room temperature, and discard the flow-through.
- 94 Add 500 µL of Buffer RPE (Qiagen, 1018013) to the RNeasy Mini Column, centrifuge at 8,000g for 15s at room temperature, and discard the flow-through.
- 95 Repeat Step 94 once more.
- 96 Add 500 µL of Buffer RPE to the RNeasy Mini Column, centrifuge at 8,000g for 2 min at room temperature, and discard the flow-through.
- 97 Repeat Step 96 once more.

- 98 Transfer the RNeasy Mini Column in a new 2 ml collection tube and centrifuge for 1 min at full speed. Discard flow-through.
- 99 Transfer the RNeasy Mini Column to a 1.5 mL collection tube (material within RNeasy Plus Universal Mini kit, Qiagen, 73404).
- 100 Elute RNA in 30 μ L of RNase Free H₂O (reagent within RNeasy Plus Universal Mini kit, Qiagen, 73404) via centrifugation at 8,000g at room temperature for 1 minute. RNase Free H₂O should be added directly to the membrane without touching it or the sides of the column..
- 101 Place the eluent on ice and store at -80°C for long-term storage.
- 102 Assess RNA concentration and quality using NanoDrop, Fragment Analyzer, and Qubit.
- 102.1 We considered intact RNA to have RNA quality number (RQN) on the Fragment Analyzer as greater than or equal to 7. (ref Fig 5)

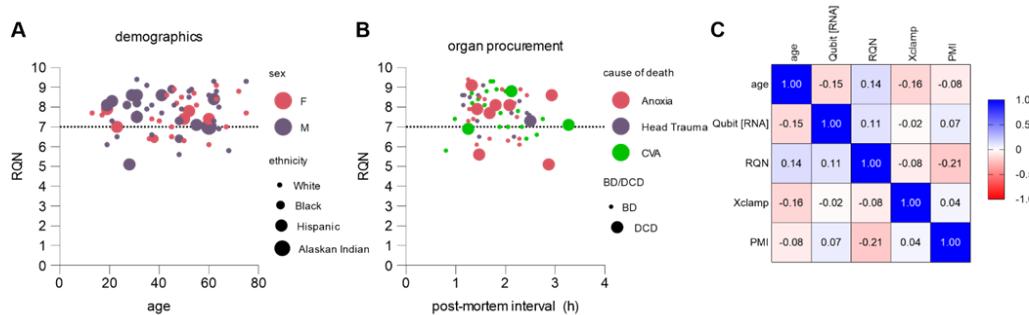


Figure 5. RNA quality number (RQN) is not associated with donor demographics (A) and factors related to organ procurement (B) in our donor dorsal root ganglia samples (n=66). C) Spearman correlation matrix of multiple variables. Of note, RNA quantity, as measured by Qubit, and RNA quality (RQN), as measured by Fragment Analyzer, are not significantly correlated with the age of the donor, time from cross-clamp (Xclamp), and post-mortem interval (PMI).

RNA Quality Assessment in Intact Tissue Sections (RNAscope)

- 103 Remove the remaining prepared slide(s) from the -80°C freezer and transfer to the lab bench over dry ice.

- 104 Place slides in a 37°C incubator for 1 minute to thaw, and then immediately transfer the slides to a dish containing 10% formalin (Thermo Fisher Scientific, 23-245684) for 15 minutes at room temperature. The formalin incubation should be performed in a fume hood.
- 105 Dehydrate sections in Ethanol (Fisher Scientific, 04-355-223) by placing the slides in 50% ethanol for 5 minutes, 70% ethanol for 5 minutes, 100% ethanol for 5 minutes, and another 100% ethanol for 5 minutes.
- 106 Air dry slides briefly. Ethanol should evaporate within a few minutes.
- 107 Draw hydrophobic boundaries around each tissue section using a PAP pen (Vector Labs, H-4000).
- 108 Perform RNAscope multiplex v2 (Advanced Cell Diagnostics, 323100) as instructed by Advanced Cell Diagnostics (protocol 323100-USM on their website). The Protease digestion has been optimized for human DRG (10 seconds, Protease III) but this continuously needs to be optimized based on new lots of fixation and protease reagent.
 - 108.1 Positive Control (Advanced Cell Diagnostics, 320861) against housekeeping mRNAs (ubiquitin C > Peptidyl-prolyl cis-trans isomerase B > DNA-directed RNA polymerase II subunit RPB1) is tested on one section and a Negative control (Advanced Cell Diagnostics, 320871) against the bacterial DapB gene is tested on the second section on each slide.
- 109 Stained sections are imaged on an Olympus FV3000 confocal microscope at 20X magnification using standard acquisition parameters (laser power <5%, offset = 4, HV < 600, gain = 1).
- 110 The raw image files (.oir) are saved to a secure data server.
- 111 The files are converted to tif in Olympus CellSens (v8.1).
- 112 mRNA signal for the positive control should be very robust, and present in neurons and non-neuronal cells throughout the biospecimen. The sample should be relatively devoid of any freezing artifact which is visualized as cytoplasmic vacuolization in the neurons. (ref Fig 6)

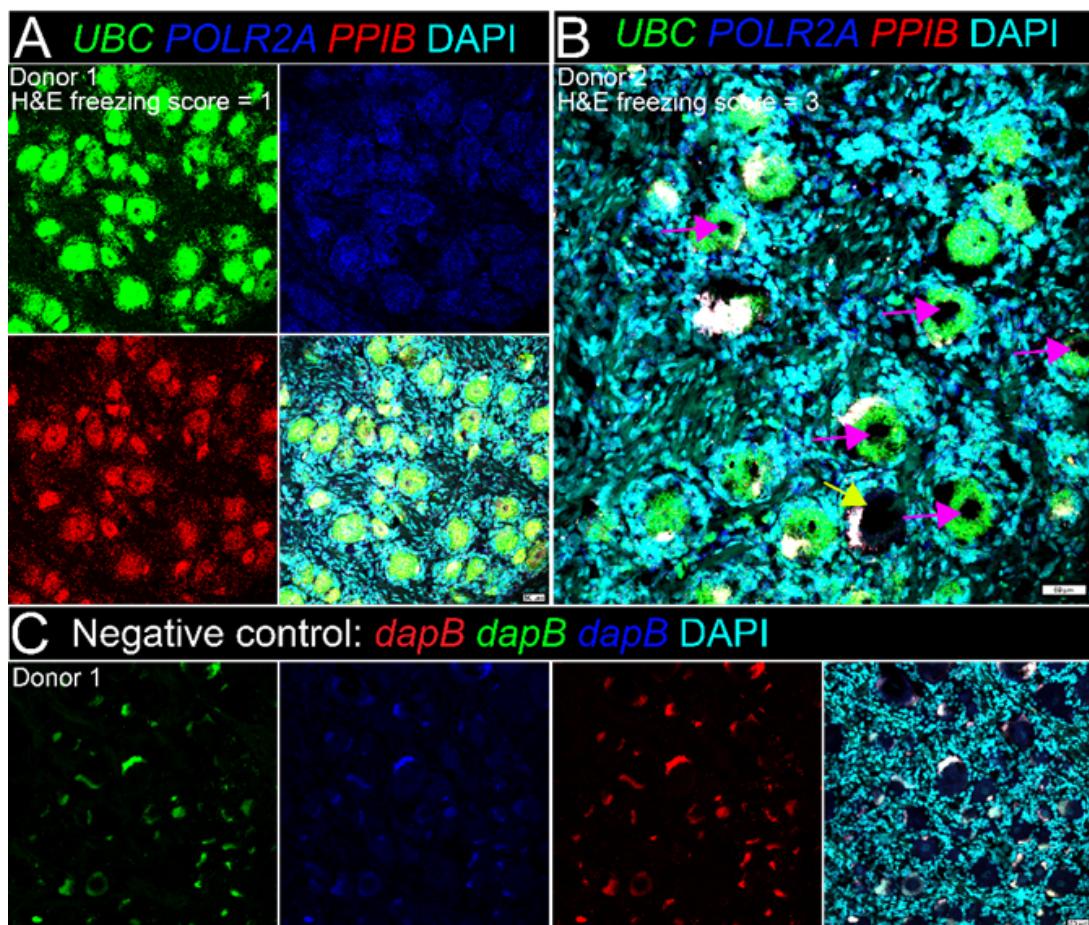


Figure 6. RNA quality assessment in intact dorsal root ganglia tissue sections. A) Robust signal was detected with all three positive control probes throughout the cytoplasm and nucleus of most neurons in Donor 1. This donor had very low-freezing damage which was predetermined using hematoxylin and eosin staining. B) Donor 2 was pre-determined to have a freezing damage score of 3, and as such, a loss of RNAscope positive control signal was observed in the nucleus of many neurons (magenta arrow) and the cytoplasm of some neurons (yellow arrow). C) Standard lipofuscin autofluorescence was detected using the negative control probe.