FINAL SCRIPT: APPROVED FOR FILMING

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Title: Mitochondrial Preparation from Microglia for Glycan Analysis

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Filming location:** Will the filming need to take place in multiple locations? **NO**

Current Protocol Length
Number of Steps: 19
Number of Shots: 40

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Juhi Samal:** Broadly, our research aims at understanding the mechanistic roles of sugars or glycans in neuroinflammation and how the cellular/sub-cellular glycosylation pathways can be therapeutically targeted in aging and brain disorders.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.2*

What research gap are you addressing with your protocol?

1.2. **Juhi Samal:** Currently, there is a gap in knowledge about subcellular glycans, their role and modulation in different disease pathophysiologies including the acute and chronic brain diseases like stroke and Alzheimer's.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What advantage does your protocol offer compared to other techniques?

~~1.3. **Juhi Samal/Dan Tyrrell:** This protocol provides a platform for quantitative estimation and compositional information about mitochondrial glycans compared to the few previous studies on sub-cellular glycosylation that rely on lectins for detection.~~

~~1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.3*~~

AUTHOR'S NOTE: 1.3 was not filmed

How will your findings advance research in your field?

1.4. **Juhi Samal:** This protocol and our research aim at advancing the knowledge about the role of glycans in neuro-immune interactions and how that can be leveraged to design therapies for CNS disorders.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Isolation of Mitochondria from Microglial Cells

Demonstrator(s): Meghana Madabhushi

2.1. To begin, obtain BV-2 *(B-V-Two)* microglial cells derived from C57BL/6 *(C-Fifty-Seven-B-L-Bar-Six)* mice **[1].** Maintain them in DMEM *(D-M-E-M)* low glucose medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% non-essential amino acids **[2]**. Grow the cells in T-175 *(T-One-Seventy-Five)* flasks until they reach 70 to 80% confluency **[3]**.

2.1.1. WIDE: Talent holding a dish of BV-2 cell culture.

2.1.2. Talent adding media to the BV-2 cell culture flasks.

2.1.3. Shot of the confluent cultures in T-175 flasks in the incubator.

2.2. Aspirate the media from the flask **[1]**. Resuspend the cell pellet in 1 milliliter of growth medium **[2]**. Using trypan blue, count the cells **[3-TXT]**.

2.2.1. Talent removing media from the flask with an aspirator.

2.2.2. Talent resuspending the pellet in fresh media with a pipette.

2.2.3. Talent mixing cells with trypan blue and counting under a microscope or automated cell counter. **TXT: Proceed with mitochondrial isolation if cell pellet contains 2 x 10^7 cells**

2.3. Centrifuge the cells in a 2-milliliter microcentrifuge tube at 500 *g* for 5 minutes **[1]**. Carefully aspirate and discard the supernatant **[2]**.

2.3.1. Talent placing tubes in the centrifuge and setting parameters.

2.3.2. Talent aspirating the supernatant with a pipette, leaving the pellet intact.

2.4. Add 800 microliters of mitochondrial isolation reagent A **[1]** and vortex at medium speed for 5 seconds **[2]**. Then incubate the tube on ice for exactly 2 minutes **[3]**.
Videographer's Note: 2.4.1-2.4.3 were filmed together

2.4.1. Talent pipetting reagent A into the tube.

2.4.2. Talent vortexing the tube.

2.4.3. Talent placing the tube on ice and starting the timer.

2.5. Add 10 microliters of mitochondrial isolation reagent B **[1]**, and vortex at maximum speed for 5 seconds **[2]**. Incubate on ice for 5 minutes, vortexing at maximum speed every minute **[3]**.

Videographer's Note: 2.5.1-2.5.3 were filmed together

2.5.1. Talent adding reagent B.

2.5.2. Talent placing the tube on a vortex.

2.5.3. Talent intermittently vortexing the tube on ice every minute.

2.6. Now, add 800 microliters of mitochondrial isolation reagent C and invert the tube to mix **[1]**, and centrifuge at 700 *g* for 10 minutes at 4 degrees Celsius **[2]**.

2.6.1. Talent adding reagent C and inverting the tube gently.

2.6.2. Talent placing the tube in centrifuge and setting conditions.

2.7. Transfer the supernatant to a new 2-milliliter tube **[1]** and centrifuge at 3,000 *g* for 15 minutes at 4 degrees Celsius **[2]**.

2.7.1. Talent transferring the supernatant to a fresh tube.

2.7.2. Talent placing it in the centrifuge.

2.8. Transfer the supernatant containing the cytosolic portion to a new tube **[1]**. The pellet contains the isolated mitochondria **[2]**. Add 500 microliters of mitochondrial isolation reagent C to the pellet **[3]** and centrifuge at 12,000 *g* for 5 minutes **[4]**.

2.8.1. Talent pipetting the cytosolic supernatant into another tube.

2.8.2. Close-up of the mitochondrial pellet remaining in the tube.

2.8.3. Talent resuspending the pellet.

2.8.4. Talent places the tube in a centrifuge.

2.9. Use the pellet for protein quantification and processing, or store at minus 80 degrees Celsius until use **[1]**.

Talent preparing the pellet for analysis.

3. Protein Extraction and Mass Spectrometric Identification of N-Glycans from Mitochondria

Demonstrator: Juhi Samal, Tana V. Palomino, David C. Muddiman

3.1. Resuspend the isolated mitochondria in 50 microliters of protein isolation buffer **[1]**. Leave the suspension on ice for 20 minutes **[2]**.

Videographer's Note: 3.1.1-3.1.2 were filmed together

3.1.1. Talent adding buffer to the mitochondrial pellet.

3.1.2. Talent placing tube on ice.

3.2. Aspirate and dispense three times and leave on ice for 20 minutes, vortexing before use **[1]**. If not fully solubilized, add another 50 microliters of buffer and pool in the same tube **[2]**. Centrifuge at 13,000 *g* for 10 minutes **[3]**.

3.2.1. Talent performing repeated aspiration and dispensing.

3.2.2. Talent adding additional buffer and combining contents.

3.2.3. Talent placing tube in centrifuge and setting speed.

3.3. After recovering and freezing the supernatant, dry using a vacuum concentrator **[1]**.

3.3.1. FILE: 68179_Screenshot_3.3.2 00:00-14

3.4. For the detection of released glycans, resuspend the dried *N*-linked glycans in 50 microliters of LCMS *(L-C-M-S)* grade water **[1].** Pipette 5 microliters of resuspended mitochondrial glycans onto a sample spot on a Teflon microwell slide **[2].**

3.4.1. FILE: 68179_Screenshot_3.4.1 00:02-00:12

3.4.2. FILE: 68179_Screenshot_3.4.2 00:01-00:12

3.5. Ionize and detect N-glycans in negative ionization mode using an electrospray solvent consisting of 60% acetonitrile and 1 millimolar acetic acid at a flow rate of 2 microliters per minute and a voltage of 3.2 kilovolts **[1]**.

3.5.1. SCREEN: 68179_Screenshot_3.5 00:00:01-00:00:08.

3.6. Couple IR-MALDESI *(I-R-Mall-Desi)* to a HRAM *(H-R-A-M)* mass spectrometer set at a resolving power of 240,000 full width at half maximum at mass-to-charge ratio 200 to analyze between 500 and 2,000 mass-to-charge ratio in negative ionization mode **[1]**.

3.6.1. SCREEN: 68179_Screenshot_3.5 00:00:08-00.20 AND

SCREEN: 68179_Screenshot_3.6 00:02-00:42

3.7. Manually identify the N-linked glycans by searching for monoisotopic masses **[1]**. Confirm isotopic distributions using mass-to-charge spacing to determine doubly and triply charged ions with a minimum ion flux threshold of 1,000 ions per second **[2]**.

3.7.1. SCREEN: 68179_Screenshot_3.7 00:18-00:25 .

3.7.2. SCREEN: 68179_Screenshot_3.7 00:30-00:47

3.8. Convert the raw mass spectra from mass-to-charge ratios to neutral monoisotopic masses **[1]**.

3.8.1. SCREEN: 68179_Screenshot_3.7 00:50-01:03

3.9. Then upload the monoisotopic masses to an online oligosaccharide structure prediction tool to determine potential glycan compositions **[1]**. Confirm annotations using an experimentally curated glycomic database **[2]**.

3.9.1. SCREEN: 68179_Screenshot_3.9 00:03-00:27 .

3.9.2. SCREEN: 68179_Screenshot_3.9 01:15-01:34 .

3.10. Ensure each identification is within 2.5 parts per million mass measurement accuracy margin, contains the core N-linked glycan structure and excludes pentose, 3-deoxy-D-manno-octulosonic acid, or uronic acid monosaccharides **[1]**.

3.10.1. SCREEN: 68179_Screenshot_3.10 00:03-00:40

Results

4. Results

4.1. Mitochondrial protein concentrations obtained from six independent preparations showed no significant variation, confirming high reproducibility **[1].**

4.1.1. LAB MEDIA: Figure 2B.

4.2. Western blot analysis showed COX IV *(Cox-four)* expression only in the mitochondrial fractions **[1],** and GAPDH *(G-ap--D-H)* only in cytoplasmic fractions, confirming the purity of mitochondrial isolations and the absence of non-mitochondrial contamination **[2].**

4.2.1. LAB MEDIA: Figure 3. *Video editor: Highlight the bands in COX IV row*

4.2.2. LAB MEDIA: Figure 3. *Video editor: Highlight the bands in GAPDH row*

4.3. Distinct sialylated, phosphorylated, and sulfated N-glycan structures were detected in mitochondrial extracts using IR-MALDESI **[1].**

4.3.1. LAB MEDIA: Figure 4. *Video editor: Please highlight the six peaks with the multicolored schematic over it*

4.4. Chi-squared values testing a goodness-of-fit confirm the detection of *N*-linked glycans with one and two chlorine adducts, confirming the detection of these glycan compositions using IR-MALDESI **[1].**

4.4.1. LAB MEDIA: Figure 5B. *Video editor: Please sequentially highlight A to D*

Pronunciation Guide:
1. Glycosylation
Pronunciation link:
https://www.merriam-webster.com/dictionary/glycosylation forvo.com+5howtopronounce.com+5justpronounce.com+5merriam-webster.com+13merriam-webster.com+13collinsdictionary.com+13
IPA (American): /ˌglaɪˌkoʊsəˈleɪʃən/
Phonetic Spelling: gly-koh-suh-LAY-shuhn

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2. Microglia
Pronunciation link:
https://www.merriam-webster.com/medical/microglia merriam-webster.com+12merriam-webster.com+12openmd.com+12
IPA (American): /ˌmaɪkroʊˈgliə/
Phonetic Spelling: my-kro-GLEE-uh

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3. Glycan

Pronunciation link:
https://www.merriam-webster.com/dictionary/glia youtube.com+15merriam-webster.com+15merriam-webster.com+15 (Note: “glycan” follows the same “gly-” pattern as “glia”)
IPA (American): /ˈglaɪkæn/
Phonetic Spelling: GLYE-can

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4. Glia
Pronunciation link:
https://www.merriam-webster.com/dictionary/glia
IPA (American): /ˈgli.ə/ or /ˈglaɪ.ə/
Phonetic Spelling: GLEE-uh or GLYE-uh

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5. IR-MALDESI
This acronym doesn’t have a dedicated pronunciation guide. However:
Link search: No confirmed link found
IPA (approximate American pronunciation): /aɪ ɑr mælˈdɛsi/
Phonetic Spelling: eye-ar mal-DEH-see