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Title: Profiling of Permethylated Mucin O-Glycans Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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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? **Yes**

   If **Yes**, how far apart are the locations? 200 yrd

Current Protocol Length
Number of Steps: 25
Number of Shots: 58 (26 SC)

Introduction

Videographer's NOTE: The interviews were done 3 times, in different locations. This was because the talent took a bit of warming up, and there was one quiet room but was looking quite bland, so I wanted to give different options. These are all slated accordingly.

REQUIRED:

1.1. **Dimitris Latousakis:** We are studying the glycobiology of the gastrointestinal tract, with a focus on the role of the mucus layer, and in particular mucins and their glycans, in the interaction between the host and gut microbes in health and disease.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.1.4*

What are the most recent developments in your field of research?

1.2. **Dimitris Latousakis:** Mucin glycans are increasingly recognized as important factors in host-microbe interactions, as they provide nutrients and binding sites for bacteria. An alteration in the glycosylation profile can lead to a disturbance in the microbiota composition and vice versa, phenotypes that are commonly observed in disease states.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.1.1*

What technologies are currently used to advance research in your field?

1.3. **Dimitris Latousakis:** Innovations in sampling techniques can take the field forward, as well as new sample processing approaches to increase the throughput and reduce the time from sample collection to results.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.1.1*

What advantage does your protocol offer compared to other techniques?

1.4. **Dimitris Latousakis:** Other analytical techniques allow more detailed structural characterization of glycans but require long running and analysis time. The techniques described here, using MALDI-TOF mass spectrometry has the advantage of speed, which makes it high-throughput and suitable for screening and profiling.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.5.1*

Videographer: Obtain headshots for all authors.

Testimonial Questions:

How does the research community benefit from video publications as compared to standard text publications?

1.5. **Dimitris Latousakis:** In many cases, how the scientist executes a protocol is as important as what the protocol includes, and this is not easy information to convey in a written publication, as it takes up too much space and disrupts the flow of this publication.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.1.1*

Protocol

2. Desalting the Mucin O-Glycans Before Permethylation

Demonstrator: Dimitris Latousakis

2.1. To begin, mix purified mucins with 250 microliters of the beta-elimination buffer in a 4-milliliter glass vial **[1-TXT].** Seal the vial tightly with a polytetrafluoroethylene lined cap **[2]** and incubate the reaction at 45 degrees Celsius for 16 hours **[3]**. On ice, add 1 milliliter of the 5 percent acetic acid solution to the reaction mix in a dropwise manner **[4-TXT].**

2.1.1. WIDE: Talent adding beta-elimination buffer to the vial containing dissolves mucins. **TXT: Mucins: 100 µg of purified mucins in 250 µL of H_2O**

2.1.2. Talent securing the cap tightly on the vial.

2.1.3. Talent placing the vial in an incubator set to 45 degrees Celsius.

2.1.4. Talent adding acetic acid dropwise to the reaction vial while it is on ice. **TXT: Observe the effervescence caused due to hydrogen release**

2.2. For desalting the sample, plug a glass pipette with a small amount of glass wool **[1]**. Add 1.5 milliliters of the resin suspension to the glass pipette from the top, then allow it to settle and drain **[2]**.

2.2.1. Talent inserting glass wool into the pipette.

2.2.2. Talent adding resin suspension into the pipette and keeping it aside.

2.3. Now, wash the resin with 2 milliliters of 5 percent acetic acid **[1]** and discard the flowthrough **[2]**. Then, add the mucin sample to the desalting column **[3]** and collect the flowthrough in a 5-mill iliter tube **[4-TXT]**.

2.3.1. Talent pouring 5 percent acetic acid into the pipette with resin.

2.3.2. Talent discarding the flowthrough. (not nessesary)

2.3.3. Talent adding the mucin sample to the desalting column.

2.3.4. Talent collecting flowthrough in a 5-milliliter tube. **TXT: Rinse the vial with acetic acid and pass it through the column**

2.4. Wash the column two times with 1 milliliter of 5 percent acetic acid to collect the flowthrough **[1]**. Then, using a centrifugal evaporator, remove the acetic acid and concentrate the sample at 5 millibar and 30 degrees Celsius for 2 hours **[2-TXT]**. Dissolve the sample in 0.5 milliliters of methanolic acetic acid **[3]** and dry it under a stream of nitrogen **[4-TXT]**.

2.4.1. Talent adding the column with 5 percent acetic acid.

2.4.2. Talent placing the sample in the centrifugal evaporator **TXT: Freeze the sample at -80 °C; Dry the sample overnight in a freeze dryer**

2.4.3. Talent adding 0.5 milliliters of methanolic acetic acid to the samples.
2.4.4. Shot of sample placed under a stream of nitrogen. **TXT: Repeat the dissolve/dry cycles 3 – 5x; Store the dried sample at 4 °C**

3. Extended Permethylation of Mucin O-Glycans for Better Spectral Quality

3.1. Using a glass syringe, add 4 milliliters of DMSO to the vial containing sodium hydroxide and methanol mixture **[1-TXT]**. After vortexing, centrifuge the solution at 2,000 *g* for 2 minutes **[2]**.
3.1.1. Talent using a glass syringe to add anhydrous DMSO to the vial containing sodium hydroxide and methanol mixture. **TXT: 50% NaOH: 0.2 mL; Methanol: 0.4 mL**
3.1.2. Talent placing the vial in a centrifuge.

3.2. Carefully decant the supernatant and remove the salts without disturbing the gel **[1-TXT]**. After ensuring no more salts are formed, resuspend the gel in 4 milliliters of anhydrous DMSO to prepare the permethylation base suspension **[2]**.
3.2.1. Talent gently decanting the supernatant and any salts from the vial. **TXT: Repeat the steps 5x; If required, remove the salts with a glass Pasteur pipette**
3.2.2. Talent adding 4 milliliters of anhydrous DMSO to the vial to resuspend the gel.

3.3. Next, to the dried mucin sample, add anhydrous DMSO and permethylation base, immediately followed by iodomethane **[1-TXT]**. Incubate the permethylation reaction for 30 minutes at room temperature with vigorous shaking **[2]**.
3.3.1. Talent adding anhydrous DMSO and permethylation base to the dried mucin. **TXT: DMSO: 100 µL; Permethylation base: 150 µL; Iodomethane: 80 µL (avoids moisture absorption)**
3.3.2. Talent placing the reaction samples on a shaker set to vigorous shaking.

3.4. Then, add 0.5 milliliters of water to terminate the reaction **[1]**. After the sample turns cloudy, flush it with nitrogen until it turns clear **[2]**.
3.4.1. Talent adding water to the samples to halt the reaction.
3.4.2. Close-up of flushing nitrogen stream over the cloudy sample.

3.5. Load the sample onto a Hydrophilic-Lipophilic-Balanced 96-well extraction plate **[1]**. Apply positive pressure to push the samples through the solid phase at a flow rate of approximately 1 milliliter per minute **[2]**.
3.5.1. Talent loading samples onto the HLB plate.

3.5.2. Talent applying positive pressure to ensure sample flow through the solid phase.

3.6. After discarding the flowthrough, wash the wells two times with 1 milliliter of water **[1-TXT]**.

3.6.1. Talent adding each well with water and applying positive pressure. **TXT: Apply positive pressure; Discard the flowthrough**

3.7. Elute the glycans from the plate 2 times with 1 milliliter of methanol **[1]**. Apply pressure only until methanol starts exiting the plate, then allow gravity flow to maintain the required rate **[2]**.

3.7.1. Talent adding methanol to the HLB plate for glycan elution. Videographer's NOTE: Talent said best to do in 2 shots. Part 1 was wide shot (this is 3.7.1). Part 2 was a close up (this is 3.7.2) So this slateboard that the editor sees in the shot can be IGNORED.

3.7.2. Close-up of methanol beginning to flow and collecting the flowthrough in a separate container.

3.8. Dry the sample using a centrifugal evaporator **[1]** and dissolve it in 10 microliters of TA-50 **[2]**. After mixing 1 microliter sample and the matrix, load it onto a steel MALDI *(mal-dee)* target plate and allow it to dry **[3-TXT]**.

3.8.1. Talent placing sample in a centrifugal evaporator to dry them. Videographer's NOTE: SLATED as 3.7.2. IGNORE CURRENT SLATEBOARD…. This shot is 3.8.1.

3.8.2. Talent adding TA50 to the dried samples.av Videographer's NOTE: SLATED AS 3.8.1. IGNORE CURRENT SLATEBOARD…this shot is 3.8.2

3.8.3. Close-up spotting the mixture onto a MALDI target plate.

4. MALDI-TOF MS Data Analysis for Processed Mucin Glycans

~~4.1. Perform the MALDI-TOF analysis of the desalted and permethylated mucin samples **[1]**.~~

4.1.1. ~~Talent working at the MALDI-TOF unit.~~ Videographer's NOTE: Talent said this shot was not needed.

4.2. For data analysis, load the exported .ascII *(A-S-C-2)* file of the mass spectrum in Glyco-work-bench **[1]**. After performing baseline correction, compute the peak centroids and, in the pop-up window, select the appropriate mass range, minimum signal-to-noise ratio, and minimum mass spectrometry peak intensity **[2]**.

4.2.1. SCREEN: JoVE67751_screenfile_1. : 00:00–00:15.
4.2.2. SCREEN: JoVE67751_screenfile_1. : 00:17-00:29.

4.3. Then, use the Glyco-Peakfinder tool to identify the structure compositions that match the peak list **[1]**. Choose **perMe** *(per-me)* as the Derivatization, **redEnd** *(red-end)* as the Reducing End, and set the minimum and maximum number of expected residues **[2]**.
4.3.1. SCREEN: JoVE67751_screenfile_1. 00:34-00:40.
4.3.2. SCREEN: JoVE67751_screenfile_1. : 00:40-00:44.

4.4. For mucins, select Hexose, N-Acetyl-Hexosamine, Deoxy-Hexose, and N-Acetyl-Neuraminic acid **[1]**. For sulfated glycans, use the **other residue** option with a mass of 87.9 **[2]**. For MALDI-TOF data, set the maximum number of sodium ions and charges to 1 each, and set the accuracy to 0.5 Daltons **[3]**.
4.4.1. SCREEN: JoVE67751_screenfile_1. : 00:45-00:49.
4.4.2. SCREEN: JoVE67751_screenfile_1. : 00:49-00:53.
4.4.3. SCREEN: JoVE67751_screenfile_1. : 00:54-00:57. *Video editor: Highlight "max # Na ions = 1", "max # charges =1" and "accuracy = 0.5"*

4.5. Select all correct annotations with the control key and click on each of them **[1]**. Right-click on the selected annotations and choose **Add to the annotated peak list [2]**.
4.5.1. SCREEN: JoVE67751_screenfile_1. : 01:00-01:10.
4.5.2. SCREEN: JoVE67751_screenfile_1. : 01:40-01:47.

4.6. To generate a report comparing multiple annotated spectra, go to **Tools** followed by **Reporting** and **Create a report comparing different profiles [1]**. Print the report as a PDF to save it **[2]**.
4.6.1. SCREEN: JoVE67751_screenfile_1. : 02:44-02:51.
4.6.2. SCREEN: JoVE67751_screenfile_1. : 02:59-03:06.

4.7. For fragmentation spectra analysis, load the spectra onto Glyco-work-bench **[1]** and generate the peak list as demonstrated earlier **[2]**.
4.7.1. SCREEN: JoVE67751_screenfile_1. : 03:20-03:30.
4.7.2. SCREEN: JoVE67751_screenfile_1. : 03:30-03:40.

4.8. Draw putative glycan structures that correspond to the annotated glycan compositions **[1]**. To generate fragments for each structure,right-click to select **Copy fragments into canvas [2]** and choose **Compute fragments** under the Fragments tool **[3]**.
4.8.1. SCREEN: JoVE67751_screenfile_1. : 04:14-04:20.
4.8.2. SCREEN: JoVE67751_screenfile_1. : 05:16-05:22.
4.8.3. SCREEN: JoVE67751_screenfile_1. : 05:31-05:40.

4.9. For further fragmentation analysis, click on a peak of interest in the spectrum viewer **[1]**. Right-click and select **Find all structures matching the peaks** to query the mass-to-charge ratio of the selected peak against online databases **[2]**. Select putative structures of interest and right-click to copy them to the canvas window with the appropriate option **[3]**.

4.9.1. SCREEN: JoVE67751_screenfile_1. : 005:50-05:54.

4.9.2. SCREEN: JoVE67751_screenfile_1. 06:28-06:33.

4.9.3. SCREEN: JoVE67751_screenfile_1. 06:56-:07:03.

4.10. To compute possible fragments from each glycan structure, select the structure in the canvas **[1]**. Then, under **Tools** and **Fragments**, select **Compute fragments for the current structure [2]**. If fragments for more than one glycan structure have been computed, go to the **Fragments table view** under the Summary tab to view the comparison table **[3]**.

4.10.1. SCREEN: JoVE67751_screenfile_1. 07:10-07:20.

4.10.2. SCREEN: JoVE67751_screenfile_1. 10:50-10:55.

4.10.3. SCREEN: JoVE67751_screenfile_1. 07:38-07:45.

4.11. Select the fragments matching the peak list for each potential glycan structure. Right-click and choose **Copy fragments to canvas** from the dropdown menu **[1]**.

4.11.1. SCREEN: JoVE67751_screenfile_1. 10:25-10:35.

4.12. Finally, in the **Tools** tab, go to **Profiler** followed by **Annotate peaks with all structures [1]**. Then select **Tools, Reporting** and **Create a report of the annotations [2]**.

4.12.1. SCREEN: JoVE67751_screenfile_1. 10:56-11:03.

4.12.2. SCREEN: JoVE67751_screenfile_1. 11:05-11:15 .

Results

5. Results

5.1. Desalting by ion exchange resulted in better recovery of larger glycans, with these larger structures making up 31% of total glycans **[1]**, compared to 21% for PGC extraction **[2]**. Among the glycan structures identified, one glycan was identified only through in the ion-exchange desalted sample **[3]**.

5.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the top graph in A with the value mentioned 31.4%.*

5.1.2. LAB MEDIA: Figure 2A. *Video editor: Highlight the bottom graph in A with the value mentioned 21.6%.*

5.1.3. LAB MEDIA: Figure 2A. *Video editor: Highlight the compound name "redEnd……dHex$_2$" below the "asterisk (*)" at the top of the figure*

5.2. Furthermore, desalting by ion exchange and borate removal led to spectra with increased signal-to-noise ratios **[1]** compared to those produced after desalting by solid phase extraction with PGC **[2]**.

5.2.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the pink/red graph for "ion exchange".*

5.2.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the bottom graph in A with the value mentioned 21.6%.*

5.3. Permethylation reaction times of 30 and 90 minutes led to the identification of 33 glycan peaks **[1]**, while a 5-minute reaction identified only 31 peaks **[2]**.

5.3.1. LAB MEDIA: Figure 3. *Video editor: Highlight the spectra for "30 and 90 min reaction"*.

5.3.2. LAB MEDIA: Figure 3. *Video editor: Highlight the spectra for "5 min reaction"*.

Pronunciation Guides:

peristalsis
Pronunciation link: https://www.merriam-webster.com/dictionary/peristalsis
IPA: /ˌpɛrəˈstɔlsəs/
Phonetic Spelling: per-uh-STOL-suhs

glycan
Pronunciation link: https://www.merriam-webster.com/dictionary/glycan

IPA: /ˈglaɪkæn/
Phonetic Spelling: gly-kan

effervescence
Pronunciation link: https://www.merriam-webster.com/dictionary/effervescence
IPA: /ˌɛfərˈvɛsəns/
Phonetic Spelling: ef-er-VESS-ens

hydrophilic
Pronunciation link: https://www.merriam-webster.com/dictionary/hydrophilic
IPA: /ˌhaɪdrəˈfɪlɪk/
Phonetic Spelling: hy-dro-FIL-ik

lipophilic
Pronunciation link: https://www.merriam-webster.com/dictionary/lipophilic
IPA: /ˌlɪpoʊˈfɪlɪk/
Phonetic Spelling: li-po-FIL-ik

anion exchange
Pronunciation link: https://www.merriam-webster.com/dictionary/anion%20exchange
IPA: /ˈænˌaɪən ɪksˈtʃeɪndʒ/
Phonetic Spelling: AN-ee-uhn iks-CHAYNJ

borate
Pronunciation link: https://www.merriam-webster.com/dictionary/borate
IPA: /ˈbɔːreɪt/
Phonetic Spelling: BOR-ayt

permethylation
Pronunciation link: https://www.synonyms.com/pronounce/permethylation
IPA: /pərˌmɛθɪˈleɪʃən/
Phonetic Spelling: per-meth-uh-LAY-shun

MALDI-TOF
Pronunciation link: https://www.howtopronounce.com/maldi-tof
IPA: /ˈmældi ˌtiːˈoʊˈɛf/
Phonetic Spelling: mal-dee T-oh-eff