Supporting Information for:

Towards Resolving the Spatial Metabolome with Unambiguous Molecular Annotations in Complex Biological Systems by Coupling Mass Spectrometry Imaging with Structures for

Lossless Ion Manipulations

Gabe Nagy^{1#}, Dusan Veličković^{2#}, Rosalie K. Chu², Alyssa A. Carrell³, David J. Weston³, Yehia

M. Ibrahim¹, Christopher R. Anderton^{2*}, Richard D. Smith^{1*}

¹ Biological Sciences Division and ² Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, Washington 99352
³Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

[#] Authors contributed equally

* Corresponding authors: christopher.anderton@pnnl.gov and rds@pnnl.gov

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Experimental

Materials. All four disaccharide standards, trehalose, sucrose, maltose, and cellobiose, were purchased from Sigma Aldrich (Milwaukee, WI USA) and were prepared to a final concentration of 10 μ M in 50/50 HPLC grade water/methanol (v/v) with 0.5% acetic acid (v/v).

Tripartite culturing. The tripartite sample was prepared as previously described ¹. *Nostoc muscorum* UTEX 1037 was cultivated in a 250 mL flask 100 mL (final volume) at 24°C in BG-11₀ medium at pH 8.2 at 125 rpm for a 16 hr/8 hr (day/night) cycle at 150 PAR for 21 days. *Trizodia acrobia* M277 was cultured on potato dextrose agar (PDA) (Sigma-Aldrich, Milwaukee, WI USA) plates for 21 days at 24°C in a light-free environment. Axenic *Sphagnum fallax* (peatmoss) cultures were maintained on Knop's medium at pH 5.7 with a 16 hr/8 hr (day/night) cycle at 150 PAR. For MSI experiments, 100 mg of *Nostoc muscroum* UTEX 1037, 2 mm plug of Trizodia m277 with agar removed, and 2 cm of the upper most portion of an axenic *Sphagnum fallax* individuals, were used to inoculate each plate to induce individual or symbiotic tripartite interactions (2 cm positioning from each other). Samples were grown on 1.5 mm thick BG-11₀ (pH 5.5) 1.5% agar plates at 24°C with a 16 hr/8 hr (day/night) cycle at 150 PAR.

MALDI-MSI. Following incubation, tripartite interaction areas were excised and mounted onto an ITO (indium tin oxide)-coated glass slide. Universal MALDI matrix (1:1 DHB:CHCA) was applied using an HTX TM-Sprayer (HTX Technologies, Chapel Hill, NC, USA). MALDI MSI imaging was performed on a 15 Tesla MALDI-FTICR-MS (Bruker Daltonics) with mass resolution of ~ 130,000 at 400 m/z.

LESA-SLIM SUPER IM-MS. The Advion TriVersa NanoMate (Ithaca, NY USA) was used for both direct infusion of the disaccharide standards as well as all LESA imaging experiments. For direct infusion, the flow rate was kept at 1 μ L/min with an electrospray voltage of 2 kV. For the LESA-SLIM SUPER IM-MS experiments, the LESA sampling conditions were as follows: 8.0 μ L of 70% MeOH was aspirated in the pipette tip, then 0.3 μ L of this volume was dispensed at a high of 0.2 mm above the agar. After a 1s post-dispense delay (extraction) time, the volume was re-aspirated into the pipette tip and then infused into the SLIM via nano-ESI. LESA sampling occurred serially with a 3 mm step size between probing areas. Each sample extraction location was subjected to electrospray ionization (ESI) before entering the SLIM SUPER module, where IM-MS data was collected for 2 minutes per sample. Images were created using SCiLS Lab.

This SLIM SUPER IM module used here has been previously described in detail elsewhere ²⁻⁶. Briefly, the capillary temperature was kept constant at 110 °C, the traveling wave speed was kept constant at 320 m/s, and the traveling wave amplitude was kept constant at 20 V. The SLIM chamber was kept at 4.06 tor of helium gas, while the ion funnel trap was kept at 4.00 torr of helium gas. Data was acquired with homebuilt software. 50 total SLIM SUPER IM separations were summed for each pixel and disaccharide standard to produce each IM trace. Ions were accumulated for 1 second 'in-SLIM' by halting the traveling wave in the second region (green) so that the first region (blue) acts as a greatly extended (up to 9 meter), ion accumulation region, in lieu of using the ion funnel trap for ion introduction. A previously described ion switch ⁴ enables long-pass separations, where these isomeric disaccharides were subjected to 85.5 meters of SLIM SUPER IM separation.



Figure S1. Depiction of SLIM board used in all of these experiments.



Figure S2. Comparison of signal intensities for ion introduction via the ion funnel trap (A) and in-SLIM ion accumulation (B). All conditions were kept the same as described in the experimental section. It is observed that the sensitivity was ~20 times greater when ions were introduced with in-SLIM ion accumulation. Additionally, the separation time here varied due to the different path lengths (13.5 m in A vs. 4.5 m in B) due to use of the 9 m first section for ion accumulation, and also resulting in somewhat greater IM resolution for the longer path length. However, as stated in the manuscript, a total of 85.5 m of path length was used in this work to baseline resolve all disaccharide isomers of interest.

1	2	3	4	5
6	7	8	9	10
11	12	13	14	15
16	17	18	19	20
and the second second		21		

Figure S3. Locations of all 21 pixels sampled with LESA SLIM SUPER IM-MS MSI.



Figure S4. Raw IM traces for pixels 1–21 of disaccharide species as $[M+Na]^+$ (365.1 m/z) after 85.5 meters of SLIM SUPER IM separation. Peak assignments are based on the arrival times from Figure 1 in the manuscript.

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