

Supplementary Protocol: Label-free Imaging of Biomolecules in Murine Brain Sections Using the 3D OrbiSIMS

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Abstract

A step-by-step protocol for label-free imaging of biomolecules, including lipids and neurotransmitters, in murine brain sections using the 3D OrbiSIMS is provided. The protocol describes requirements for mounting of murine brain sections, the calibration of the Orbitrap™ mass spectrometer, the set-up of the imaging acquisition, and the analysis of the hyperspectral imaging dataset to generate biomolecule distribution maps. Following the protocol, the distribution of biomolecules with molecular weights in the range from 80 to 1200 Da can be imaged at the microscale with spatial resolution $< 2 \mu\text{m}$ ($< 1.5 \mu\text{m}$ can be achieved), high mass resolving power ($m/\Delta m$) $> 200,000$ at m/z 200 and mass accuracy < 2 ppm. The protocol may be completed within 5 hours.

Introduction

Secondary ion mass spectrometry (SIMS) is increasingly used for biomolecular imaging of tissue¹ owing to the high-spatial resolution afforded by a focussed ion beam probe. In recent years, the development of gas cluster ion beams (GCIB) has dramatically improved the ability to detect larger molecules such as lipids with a significant reduction in fragmentation² of the molecules. Fragmentation is unwanted and leads to uncertainty in the identification of an ion in the mass spectrum. The uptake of SIMS into the wider biological community has been hampered by the mass spectrometers used in SIMS instruments, which have insufficient mass resolving power to separate out mass peaks in the complex biological spectrum and insufficient mass accuracy for effective use of databases.³ The 3D OrbiSIMS combines mass spectra using a GCIB with sub-cellular resolution with Orbitrap mass spectrometry.

Reagents and Equipment

- Silver (Ag) foil (the exact type is not critical)
- ITO coated glass slide (Bruker, Germany). These are typically used for MALDI MS.
- Leica CM3050S cryostat (Leica, Germany) or similar
- 3D OrbiSIMS (Hybrid SIMS, IONTOF GmbH, Germany)

Procedure

1) Calibrate Orbitrap MS

- Use the 3D OrbiSIMS control software, SurfaceLab 6.7, to set up gas cluster ion beam (GCIB) to provide a pulsed beam of Ar_{3000}^+ clusters with an energy of 20 keV and a time-averaged current above 20 pA.
- Set the He gas pressure in the secondary ion transfer optics to the “High” setting for optimal transmission of small inorganic cluster ions to the Q Exactive™ HF.
- Navigate to the Ag sample and adjust working distance (sample-to-extractor) and target potential scheme to optimise secondary ion transmission to the Q Exactive™ HF. For a standard instrument configuration these are 1.5 mm and 57 V, respectively.
- Use the Q Exactive Tune software to calibrate mass spectrometer based on positive and then negative ion spectra obtained from the Ag film using the argon GCIB. Table 1 lists recommended secondary Ag cluster ions for the calibration.

Table 1: Positive and negative secondary ions used for Orbitrap MS calibration.

Cluster	m/z [Ag_n^+]	m/z [Ag_n^-]
^{107}Ag	106.9045	106.9056
^{109}Ag	108.9042	108.9053
$^{107}\text{Ag}^{109}\text{Ag}$	215.8093	215.8104
$^{107}\text{Ag}_2^{109}\text{Ag}$	322.7144	322.7155
$^{107}\text{Ag}_3^{109}\text{Ag}_2$	538.5242	538.5253
$^{107}\text{Ag}_4^{109}\text{Ag}_3$	754.3341	754.3352
$^{107}\text{Ag}_5^{109}\text{Ag}_4$	970.1439	970.1450
$^{107}\text{Ag}_6^{109}\text{Ag}_5$	1185.9538	1185.9549
$^{107}\text{Ag}_7^{109}\text{Ag}_6$	1401.7636	1401.7647

- 2) Mount tissue section and load sample
 - a. Section tissue in the cryo-microtome with 10 μm thickness at $-22\text{ }^{\circ}\text{C}$.
 - b. Thaw mount section onto the conductive side of a ITO coated conductive microscope slide for OrbiSIMS analysis.
 - c. Optionally cryo-microtome an adjacent tissue section for subsequent H & E staining and imaging (protocol not given here).
 - d. If OrbiSIMS analysis is not done immediately after sectioning, store the microscope slide in a suitable container at $-80\text{ }^{\circ}\text{C}$ until analysis. Care should be taken to avoid condensation when taking the microscope slides out of the freezer, e.g. by vacuum packing them before freezing, and thawing under vacuum.
 - e. Introduce the slide with the dried tissue section into the hybrid instrument's vacuum system.

- 3) Acquire mass spectrum (optional)
 - a. Set up GCIB to provide a 20 keV Ar_{3000}^{+} beam with a current greater than 1 pA. A cluster size of 3000 is the minimum recommended. Below this, increased fragmentation of molecules occurs. Larger clusters can be used but with lower beam current and longer acquisition times.
 - b. Measure and record the beam current, this is needed to calculate the dose. It is good practice to also measure the beam current at the end of the experiment.
 - c. Set up electron gun to flood sample surface with $> 10\text{ }\mu\text{A}$ low energy (20 eV) electrons for charge compensation between GCIB ion pulses.
 - d. Set He pressure in secondary ion transfer optics to the "Low" setting for optimal transmission of organic secondary ions to the Q Exactive™ HF.
 - e. On tissue section, adjust target potential pulsing scheme to optimise secondary ion transmission to Q Exactive™ HF mass analyser for spectrum acquisition conditions, using a random raster pattern for the GCIB.
 - f. Use in-instrument video camera system to find region of interest on tissue section. Optionally, a secondary ion image of a larger area of the sample may be obtained using a Bi_3^{+} beam and the ToF MS to help identify regions of interest for imaging with the GCIB and the Q Exactive™ HF. Note that doing this before the Orbitrap acquisition can result in damage. A low ion dose is needed $< 1 \times 10^{11}$ ions / cm^2 .
 - g. Start spectrum acquisition using SurfaceLab 6.7 with a set mass resolving power of 240,000, using a random raster pattern for the GCIB.

- 4) Acquire image
 - a. Set up 20 keV Ar_{3000}^{+} focused pulsed beam with a spot size smaller than 2 μm (1.5 μm can be achieved) and a current between 1 pA and 30 pA.
 - b. Set up electron gun to flood the sample surface with $> 10\text{ }\mu\text{A}$ low energy (20 eV) electrons for charge compensation between gas cluster ion pulses.
 - c. Set He pressure in secondary ion transfer optics to the "Low" setting for optimal transmission of organic secondary ions to the Q Exactive™ HF.
 - d. On tissue section, adjust target potential pulsing scheme to optimise secondary ion transmission to Q Exactive™ HF mass analyser for imaging conditions, using a sawtooth raster pattern.
 - e. Use in-instrument video camera system to find region of interest on tissue section.
 - f. Start image acquisition using SurfaceLab 6.7 with a set mass resolving power of 240,000 and an image resolution for which the pixel-to-pixel distance corresponds to the GCIB

spot size, e.g. an image resolution of 150×150 pixels for a field of view of $250 \mu\text{m} \times 250 \mu\text{m}$. Use a saw tooth raster pattern for the GCIB.

- 5) Obtain MS/MS spectrum for secondary ions of interest (optional)
 - a. Inspect mass spectra obtained in steps 3 and 4 in SurfaceLab 6.7 and identify mass peaks of interest.
 - b. Set up the instrument for spectrum or image acquisition following step 3 or step 4.
 - c. Start spectrum acquisition from region of interest using SurfaceLab 6.7, selecting the option for tandem mass spectrometry using the higher-energy collision-induced dissociation cell in the Q Exactive™ HF. The normalised collision energy needs adapting to the analyte. Useful normalised collision energies are typically in the range from 15 to 60 eV.

- 6) Interpret data and generate biomolecule distribution images
 - a. Inspect mass spectra in SurfaceLab 6.7 and review potential putative secondary ion assignments for peaks of interest.
 - b. Select peaks for image generation, ensuring that peak integration windows encompass the full width of the mass spectral peaks.
 - c. Adjust secondary ion intensity-to-colour transformation functions for individual images to highlight relevant features of the data.
 - d. Where required, make composite multi-colour images, e.g. RGB, based on individual secondary ion images or summed images.

Troubleshooting

Step 2b. The tissue section needs mounting on the conductive side of the ITO which is easily identified using a standard multi-meter to measure resistivity.

Step 4a. Avoid high GCIB duty cycles (> 60%) that do not permit efficient charge compensation.

Step 4d. There may initially be no secondary ions going through to the Orbitrap MS. The optimal target potential setting may be more than 100 V from the default setting.

Step 4f. Insufficient charge compensation may cause secondary ion signal intensities to be low. Decreasing the GCIB duty cycle or defocussing the beam slightly may improve the signal intensity in that case.

Step 4a. If too much fragmentation of molecules is observed, increase the number of argon atoms in the cluster to 5,000 or 10,000.

Step 5c. A high precursor intensity is required for tandem MS analysis. It may be necessary to perform the analysis according to step 3 using a defocused beam with a current > 30 pA (to increase the signal).

Time Taken

The entire protocol can be completed within 5 hours. Tissue sectioning and mounting is typically done in batches and may take between 30 minutes to 120 minutes. Sample introduction and mass spectrometer calibration takes less than 30 minutes. The duration of the image acquisition depends

linearly on the number of pixels in the image; acquisition of a 128 × 128 pixels image takes less than 2.5 h. The initial data inspection and image generation may be carried out in less than 1 hour, however, a comprehensive analysis of a single mass spectrometry imaging dataset may take longer.

Table 2: Minimum time taken to acquire an image in minutes depending on mass resolving power and image resolution.

		Image resolution (pixels)			
		32×32	64×64	128×128	256×256
Mass resolving power ($\Delta m/m$)	15000	0.3	1.4	5.5	21.8
	30000	0.9	3.4	13.7	54.6
	60000	1.9	7.5	30.0	120.1
	120000	4.1	16.4	65.5	262.1
	240000	8.5	34.1	136.5	546.1

Associated Publications

This protocol was developed in conjunction with the development of the 3D OrbiSIMS instrument as described in the publication “The 3D OrbiSIMS – A new Method for Label-Free Metabolic Imaging with Sub-Cellular Lateral Resolution and High Mass Resolving Power”. The protocol was used to generate Figures 3 and 5 of that publication. See the publication and supporting information for expected results.

References

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