Secondary Ion Mass Spectrometry Imaging of Dictyostelium discoideum Aggregation Streams

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Abstract

High resolution imaging mass spectrometry could become a valuable tool for cell and developmental biology, but both, high spatial and mass spectral resolution are needed to enable this. In this report, we employed Bi³ bombardment time-of-flight (Bi³ ToF-SIMS) and C₆₀ bombardment Fourier transform ion cyclotron resonance secondary ion mass spectrometry (C₆₀ FTICR-SIMS) to image Dictyostelium discoideum aggregation streams. Nearly 300 lipid species were identified from the aggregation streams. High resolution mass spectrometry imaging (FTICR-SIMS) enabled the generation of multiple molecular ion maps at the nominal mass level and provided good coverage for fatty acyls, prenol lipids, and sterol lipids. The comparison of Bi³ ToF-SIMS and C₆₀ FTICR-SIMS suggested that while the first provides fast, high spatial resolution molecular ion images, the chemical complexity of biological samples warrants the use of high resolution analyzers for accurate ion identification.

Introduction

The interrogation of biological systems with secondary ion mass spectrometry (SIMS) has seen significant growth over the last decade. [1,2,3,4,5,6,7] This relatively newfound application of a surface technique traditionally limited to the study of inorganic and small molecule analytes is largely derived from the advent of larger, cluster primary ion probes (e.g., C₆₀ [8,9,10,11] Ar clusters, [12,13] and Au nanoparticles [14,15,16,17,18,19]) which provide enhanced secondary ion yields of molecular and fragment ions from biological samples. While the use of traditional time of flight (TOF-SIMS) and magnetic sector based methodologies have intrinsic advantages for the in situ analysis of surfaces (e.g., speed, sensitivity, dynamic range, depth profiling), the complexity and number of components usually encountered in the analysis of biological systems warrant the coupling of these new sources to high mass accuracy and resolution analytical devices for direct identification of the molecules of interest. [20,21,22,23,24] In particular, this requirement grows out of the need for improved identification certainty for molecular ions generated from biological samples, which are substantially more complex relative to semiconductor and polymer-based applications, where the number of sample components is limited and the analyte of interest is typically predetermined.

Previous mass spectrometry imaging studies have shown the advantages of correlating spatial information with molecular composition for the study of a variety of biological systems. [25] A common drive has been the search for biological models and better interrogation probes with higher spatial resolution and improved molecular identification. To this end, we used Dictyostelium discoideum as a biological model for evaluating the performance of two different mass spectrometry imaging approaches. D. discoideum cells are eukaryotic cells that normally live on soil surfaces and eat bacteria. [26,27] An interesting feature of their biological cycle is that when the cells overgrow their food supply and starve, they aggregate together in dendritic streams to form groups of ~20,000 cells. The aggregated cells eventually form a fruiting body consisting of a 1–2 mm tall stalk supporting a mass of spore cells which can then be dispersed by the wind to start new colonies. Because soil surfaces are exposed to rain water, the cells can survive and undergo development in water. This feature makes D. discoideum a good model for in situ mass spectrometry imaging since it does not require the use of cleaning protocols that can potentially compromise the spatial information (e.g., removal of buffer salts and/or media components). In addition, this cell averages 10 μm in size, which is at the frontier of various surface interrogation techniques (e.g., SIMS, DESI and MALDI). [5,25,28] Although the lipid composition of D. discoideum has been studied at different developmental stages using traditional chromatographic techniques and mass spectrometry, [26,29,30,31,32] nothing is known about their distribution during chemotaxis and the aggregation process. In this article, we explore the potential for
SIMS imaging of unknown biological samples by employing traditional TOF-SIMS and accurate mass determination via FTICR-SIMS for direct molecular ion identification of biological components in D. discoideum during aggregation.

Experimental Method

Sample Preparation

D. discoideum Ax2 cells were grown in shaking culture at 21°C in Formedium HL-5 as previously described. [33] Mid-log cells (1–2×10⁶ cells/ml) were collected by centrifugation at 1,500 g for 4 minutes, resuspended in PBM (20 mM KH₂PO₄, 10 μM CaCl₂, 1 mM MgCl₂, pH 6.1), and collected by centrifugation. The resuspension and centrifugation were repeated two more times. The cells were resuspended in PBM to 5×10⁶ cells/ml, and 10 ml of cells was placed in a 125 ml Erlenmeyer flask and shaken at room temperature for 4 hours. The cells were then diluted 1:6 with PBM, collected by centrifugation, and resuspended in deionized water. The collection and resuspension in deionized water were repeated twice, and the cells were diluted to 9×10⁵ cells/ml. 80 μl droplets of the cells were then spotted onto gold-coated silicon chips (Sigma Aldrich). After allowing cells to settle for 30 minutes, 40 μl of the overlaying water was removed and the chips were placed in a humid box at 21°C. 17 hours later, the chips with aggregating cells were gently drained by touching to a kimwipe, and placed cell-side down on a piece of dry ice. This was covered by a piece of aluminum foil, inverted, and placed in a vacuum chamber. After 12 hours, the dry ice had evaporated and the sample was desiccated. The chips with cells were then stored over a CaCl₂ desiccant at room temperature.

Instrumentation

Duplicate D. discoideum samples were analyzed in positive ion mode using a ToF SIMS² instrument (ION-TOF, Münster, Germany) and a custom C₆₀ FTICR-SIMS. The custom C₆₀ FTICR-SIMS instrument (more details in refs [21,23]) utilizes a 40 keV C₆₀ primary ion gun (Ionoptika Ltd., Hampshire, England) that is coupled to a Solarix 9.4T FTICR mass spectrometer (Bruker Daltonics Inc, Billerica, MA). The vacuum pumping scheme of the Solarix cart was modified so that the pressure in the source chamber was reduced to 3×10⁻⁷ mbar instead of the ~3 mbar at which it typically operates. The C₆₀ FTICR-SIMS images were acquired using 40 keV C₆₀ projectiles over a field of view of approximately 4 mm×6 mm with a pixel size of 125 μm and a total primary ion dose of 2.78×10¹⁴ ions/cm². Spectra were acquired using a broadband excitation over the 0–1,500 range, with 1.0 s transients collected for each pixel. Transients were zero-filled and Sine-Bell apodized prior to the maximum peak height. The C₆₀ FTICR-SIMS spectrum also shows numerous lipid-specific fragments, with the most abundant being the phosphatidylcholine head group (C₅H₁₅NPO₄SO₃⁻) at m/z 184. A total of 293 peaks in the C₆₀ FTICR-SIMS spectrum can be attributed to lipid species. When comparing the Bi₃⁺ ToF-SIMS and C₆₀ FTICR-SIMS spectra, there are some key differences that become apparent. (1) The radio-frequency ion guides and quadrupole (set to transmit m/z 160 and above) used to transfer ions from the source to the ICR cell induce a low mass cutoff as seen by the significant reduction in ion signal below m/z>200 (relative to the ToF-SIMS spectrum). [36] (2) The greater number of lipid signals detected in the 650<m/z<1200 range for C₆₀ show that this large cluster projectile is more efficient for generating intact lipid molecular ions than smaller primary ions such as Bi₃⁺ as previously noted in ref [37]. It is important to note that the ion fluences used were 2.78×10¹³ ions/cm² and 8.16×10¹² ions/cm² for the C₆₀ and Bi₃⁺ analysis, respectively. These values are at or slightly above the static limit, meaning that erosion of the sample is expected. According to the reported sputter yields for these projectiles in organic matrices at the similar fluences and kinetic energies, [38,39,40] the sampled depths are estimated to be approximately 30 nm and 15 nm for the C₆₀ and Bi₃⁺ analyses, respectively.

Figure 2 shows optical and selected ion images from the Bi₃⁺ ToF-SIMS (Figure 2: B–F) and C₆₀ FTICR-SIMS (Figure 2: H–L) spectra. The optical microscopy images (Figure 2: A, G) clearly show the cellular aggregation streams which form branched structures <200 μm in width and a few millimeters in length. The ToF-SIMS total secondary ion image (Figure 2B) shows higher intensity for ions originating from the aggregation streams and lower overall intensity from the gold substrate. C₃H₅NPO₄⁺ (m/z 37,000)
The C5H13NPO3+ and C5H15NPO4+ species are head group fragment ions from glycerophosphatidylcholines, which make up 25% of all lipids present in D. discoideum. [31] The signal at m/z = 760.6 appears to be a lipid molecular ion due to its co-localization with the aggregations streams, the observed isotopic pattern which contains significant 13C contributions, and the presence of another peak at m/z = 788.6 corresponding to the same molecule with a fatty acyl chain two carbons longer. [41,42] However, due to the limited mass accuracy afforded by ToF analysis and their absence from the C60 FTICR-SIMS spectrum, the precise identities of these supposed lipids was not determined.

Analogously, molecular ion images can also be obtained from the C60 FTICR-SIMS spectra. Two of the mass spectral features (Figure 2: H, I) which display spatial distributions corresponding to the aggregation streams are C5H15NPO4+ (m/z = 184.0737, δ = 0.2 ppm) and C12H16O5Na+ (m/z = 263.0889, δ = 0.2 ppm). As mentioned above, C5H15NPO4+ corresponds to the phosphatidylcholine head group, while according to the LIPID MAPS database, the C12H16O5Na+ species corresponds to the heterocyclic fatty acyl 3-carboxy-4-methyl-propyl-2-furanpropionic acid (LIPID MAPS ID: LMFA01150004), which has previously been detected from human uremic serum as a sodiated ion using SIMS. [43] Ion images for two unidentified peaks from the FTICR spectrum are shown in panels K and L. The image of m/z 202.0769 shows a distribution consistent with the aggregation streams.
streams with lower level concentrations between the aggregation streams. The m/z 442.2986 ion is located on the surface in proximity to, but not within the aggregation streams. Such an arrangement may mean this ion corresponds to a metabolite which is secreted from the \textit{D. discoideum} cells. The m/z 202.0769 and 442.2986 ions did not return lipid matches within the 5 ppm mass accuracy threshold, suggesting these lipids are not contained in the database, these compounds are not lipids, or the mass errors for these peaks fall outside the applied threshold range. As such, identities for these ions can not be determined from this analysis.

As in the ToF-SIMS analysis, a Au-related ion, Au$_2^+$ (m/z 393.9326, $\delta = 0.1$ ppm), can be used to visualize the substrate and not the aggregation streams.

The molecular ion images shown in Figure 2 demonstrate that ions throughout the mass range can be used to display meaningful spatial distributions. Moreover, the mass resolving power of the FTICR-SIMS instrument is most apparent when the true complexity of the sample is revealed. The excerpted mass spectrum (from the sum of all spectra) shown in Figure 3D shows that within the spectrum, there can be upwards of 10 ions within a given nominal mass, and that each of these ions may arise from different regions within the sample. Assuming a composition of carbon, hydrogen, nitrogen, oxygen, and phosphorus and a 5 ppm

Figure 3. Secondary ion images from within the m/z = 277 nominal mass. (A) Bi$_3$ ToF-SIMS and (D) C$_{60}$ FTICR-SIMS spectra excerpts showing multiple peaks within the 277 nominal mass. (B,C) Bi$_3$ ToF-SIMS ion images obtained from the first “peak” and second “peak” within the 277 nominal mass. (E–I) C$_{60}$ FTICR-SIMS ion images generated for the corresponding peaks in D with a m/z bin size of $+/-0.001$. doi:10.1371/journal.pone.0099319.g003
The peak at 277.252 has the formula [C$_{19}$H$_{32}$O$_{2}$P].

It is apparent that two of the ions (277.072 and 277.252) originate from two of the ion images generated by integrating the left and right halves of the peak cluster (3B, 3C) show some differences in mass. The selected ion images revealed at least two unresolved peaks within the m/z 277 nominal mass. The search for lipid IDs from the high resolution FTICR-SIMS spectrum against the LIPID MAPS database resulted in 293 hits throughout the spectrum within 5 ppm mass measurement accuracy. Depending on the uniqueness of each detected m/z, peaks can be assigned to a single lipid, any of multiple isomers within a given class, or to any of multiple isomers from multiple lipid classes. A summary plot showing the 512 lipid class assignments for the 293 peaks is provided in Figure 4A, with the detailed list of peak assignments included as Table S1. Figure 4B shows the number of compounds from each lipid class in the LIPID MAPS database binned every 10 Da. The three most commonly detected classes of lipids, the fatty acyls, prenol lipids, and sterol lipids, feature mass distributions which reside almost entirely within the 200–500 Da range. This happens to be the range over which most of the C$_{60}$ FTICR-SIMS signal is observed. As a general trend, as the mass of the compound increases, the probability of ion formation/survival decreases in SIMS analyses.

Previous reports of the D. discoideum lipid profile are almost exclusively limited thin-layer chromatographic measurements of the types and relative abundances of the general lipid classes without regard to the specific lipids present. As an example, Paquet et al. recently reported that neutral, phosphoethanolamine, and phosphocholine lipids constitute over 80% of a total lipid extract from D. discoideum. However, variations in ionization probability between lipid classes and a mass dependent detection probability preclude quantitative comparisons of this type from mass spectrometric data. There have also been many reports of the fatty acid profile of D. discoideum obtained from hydrolyzed lipid extracts, but these fatty acids have not been linked back to their parent lipid class. In order to obtain a more detailed lipid profile, the fatty acyls should be detected along with their corresponding head groups. This could be done either by using a solvent prefractionation method to isolate the various lipid class prior to hydrolysis and subsequent GC analysis [45] or by analysis of the original intact molecular ions [24] as was done here. This has been done for the most abundant sphingolipids from D. discoideum using liquid chromatography mass spectrometry; [32] however, this analysis was performed in negative ion mode while our MS analyses were acquired in positive ion mode. The author did propose identities for the four most abundant lipids observed in positive ion mode to be PC(36:4), PC(34:4), PC(32:2), and PS(32:1), but these ions were not observed in the C$_{60}$ FTICR-SIMS spectrum.

Despite the fact that lipid profiling using this approach is biased by the mass range and ionization probability of the desorbed molecules, it does offer a rapid tool for molecular differentiation and cell state classification. A current limitation of this approach (e.g., compared to LC-MS lipid profiling) lies in the inability to differentiate isobaric species. The identity of structural isomers is often important in lipid analysis and efforts have been made to incorporate MS/MS capabilities into SIMS analysis. [46,47]. The current FTICR-SIMS instrument is also capable of MS/MS measurements, [21] though none were performed during the course of this study. Another limitation of the current prototype...
lies in the sub-optimal focusing of the C$_{60}^+$ primary ion beam which has a diameter of $\approx$ 75 $\mu$m and the lack of ion raster optics which means mechanical stage movement must be used to generate ion images rather than the more precise method of beam rastering. Other groups have shown that C$_{60}$ beams can be focused down to 20 $\mu$m using ion rastered to create images with sub-micron spatial resolution. [48] Such improvements would be necessary for the current instrument to resolve smaller surface features such as lipid distributions within Dictyostelium aggregation streams or individual Dictyostelium cells.

**Conclusions**

Bi$_3$ ToF-SIMS and C$_{60}$ FTICR-MS offer complementary information, where the first analysis provides short analysis times and high spatial resolution while the second demonstrates the need for higher mass resolving power when interrogating biological samples. In particular, the use of high mass resolving power in SIMS (e.g., FTICR-SIMS) was shown to be effective for the analysis of a variety of chemical classes with molecular ion masses $<1,000$ Da (e.g., fatty acyls, prenol lipids, and sterol lipids). Further incorporation of high resolution mass analyzers with high spatial resolution surface probes will permit a better identification of molecular components in biological matrices, a necessary step in the progression towards single cell mass spectrometry imaging.

**Supporting Information**

**Figure S1** Bi$_3$ ToF-SIMS secondary ion images from within the m/z = 277 nominal mass. (A-D) Bi$_3$ ToF-SIMS selected ion images produced from the (E) segmented peaks within the m/z 277 nominal mass. (F) Optical image and (G) summed image of the full m/z 277 peak.

**Table S1** List of lipids with $<5$ ppm mass error identified from the LIPID MAPS database.

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**Author Contributions**

Conceived and designed the experiments: FFL, RHG. Performed the experiments: FFL, DFS, JDD, Analyzed the data: FFL, JDD, CRA. Contributed reagents/materials/analysis tools: RMAH LPT FFL RHG. Wrote the paper: JDD FFL DFS RMAH LPT RHG.

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