Imaging venom peptides and proteins at high mass resolution, high lateral resolution and high speed using the timsTOF fleX

Understanding how animals produce and store these venoms provides new insight into the ecology and evolution of proteins and protein-secreting tissues, and can guide the development of toxins as therapeutic and agrochemical leads.

Abstract

Venoms are extremely diverse, both on a molecular and pharmacological level, but also at a functional level, i.e. how and why they are deployed. Several studies in recent years have highlighted that there is a relationship between how the venoms are stored and produced and their intended biological function. Spatial information on the distribution of toxins throughout venom-producing tissues, therefore, provides important insight into the biological roles of venoms and on the functions and potential targets of toxins, which again improves our understanding of their evolutionary histories and provides a guide for bio-discovery efforts. MALDI Imaging is ideal for investigating the spatial

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distribution of venom toxins, and the timsTOF fleX offers a unique combination of sensitivity, mass accuracy, mass resolving power, and speed for analysis of these compounds.

Introduction

The advent of MALDI Imaging (MALDI-MSI) [1] has greatly changed the way that we study venoms from various organisms. Fundamentally, MALDI-MSI enables the identification of the spatial distribution of the venom components in the context of the structure of the venom gland itself. Most venoms studied to date include a high abundance of peptides and proteins with masses between 1000 and 12,000 Da, typically with a large representation between 2000 and 8000 Da [2, 3]. Generally, this mass range requires analyses to be undertaken using linear MALDI-TOF experiments. In this report, we show that the timsTOF fleX instrument has exceptional mass resolution and mass accuracy, while still maintaining a high lateral resolution of 20 µm and high acquisition rates. As exemplified by our data from venom glands, this combination of mass resolution at high *m/z*, spatial resolution, and speed represents a massive step forward for the MALDI-MSI analysis of larger peptides / smaller proteins, specifically enabling the identification of more analytes, which could otherwise not be resolved. On the basis of mass resolution and acquisition speed, the timsTOF fleX is ideal detecting analytes between 2000 and 8000 Da.

Methods

Centipedes of the species *Scolopendra morsitans* were collected from the Darling Downs region, Queensland, Australia. The forcipules, which are modified venom-bearing legs and unique to centipedes [4], were removed from an euthanized animal and fixed overnight using 50% RCL2/ethanol, dehydrated through 70%, 90% and 100% ethanol, cleared with xylene, and impregnated with paraffin [3]. The paraffin blocks were sectioned at 7 µm using a microtome, placed onto a conductive ITO glass slide, which was heated on a heat block for 30 min, and then deparaffinised by careful washing with xylene. Once dry and free of paraffin, the sections were photographed and prepared for matrix application. 2,5-dihydroxybenzoic acid (DHB) was sublimed onto the slide in the following manner: 50 µL acetone saturated DHB plus 100 µL acetone was added to the bottom half of the sublimation device. The slide was mounted to the top half of the sublimation device; when assembled, the slide is positioned approximately 0.5 cm from the matrix material. The assembled sublimation device was placed onto a bed of stainless steel metal shot, which was at a surface temperature of 140°C. A vacuum was applied to the assembled device, and once the vacuum reached 0.9 mbar, the DHB had sublimed from the sublimation device to the slide, and the chamber was removed from the heat source. The time required was approximately 10 minutes, including the time required to gently bring the device back to atmospheric pressure. In order to observe signals for peptides and proteins a recrystallization step is required. To achieve this, we utilised a Bruker ImagePrep, with a customised program using 50% methanol with 0.2% trifluoroacetic acid. 20 cycles

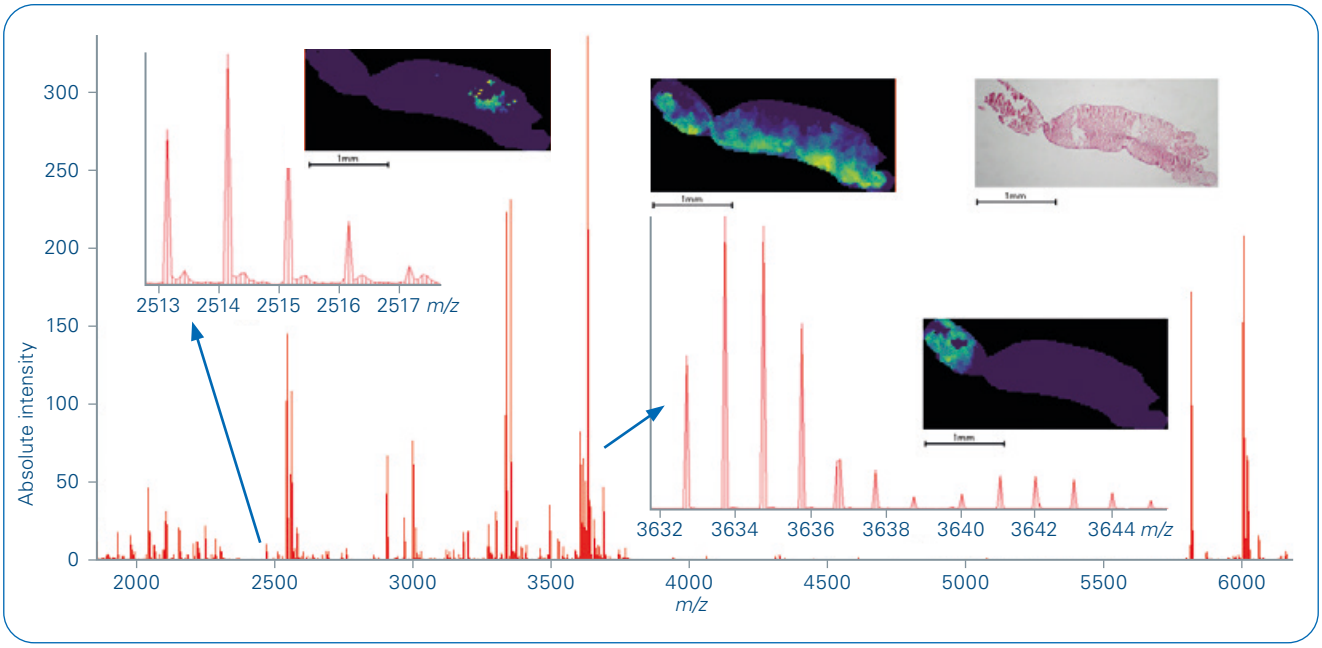
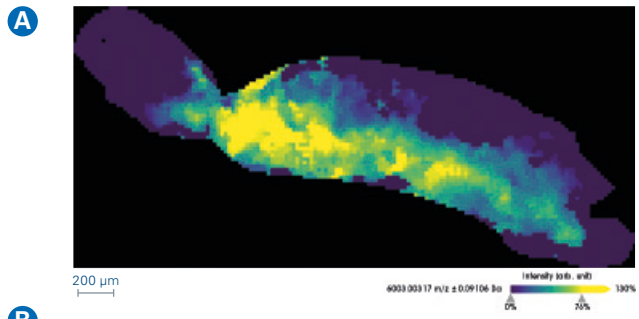
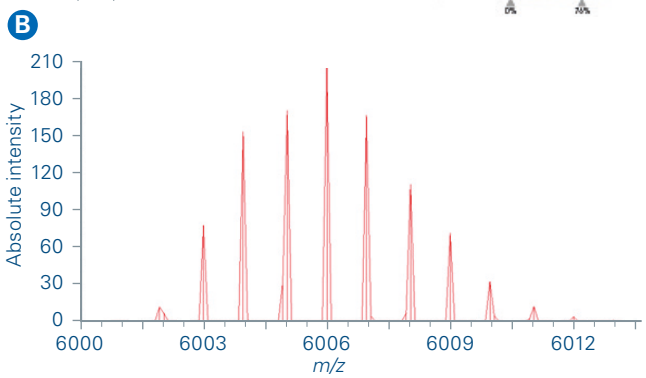
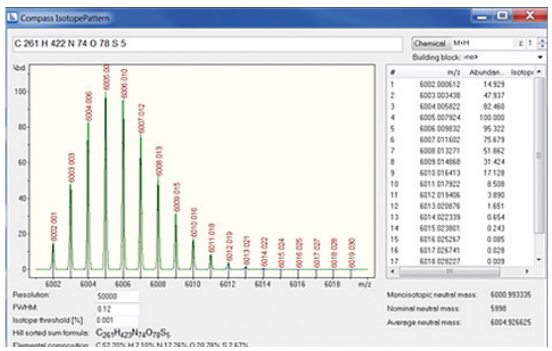


Figure 1: Averaged mass spectrum collected from the timsTOF fleX for a centipede venom gland, highlighting the signals obtained for high and low abundance peptides, also showing the post MSI H&E stained section.







D

Calculated	Observed	mDa	ppm
6002.000612	6001.93307	-67.54	-11.25
6003.003438	6003.00317	-0.27	-0.04
6004.005822	6003.96635	-39.47	-6.57
6005.007924	6005.03663	28.71	4.78
6006.009832	6005.99995	-9.88	-1.65
6007.011602	6006.96336	-48.24	-8.03
6008.013271	6008.03389	20.62	3.43

Figure 2: (A) Spatial distribution and mass resolution observed for the oxidised venom peptide U-SLPTX15-Sm1a with sequence GKPEKEVNFPAPGKKPT-REDCKKACANKYTNGVMSKVIVAKLTGKNCYCKYQEN, as observed on the timsTOF fleX, (B) Zoomed in region from the averaged mass spectrum, (C) Simulated mass spectrum for U-SLPTX15-Sm1a, and (D) Mass accuracy calculations for U-SLPTX15-Sm1a as observed for the timsTOF fleX instrument.

including 2 seconds spray time, 30 seconds incubation, 20 seconds drying time was undertaken.

The prepared tissue sample was analysed using a timsTOF fleX operating in QTOF mode. The instrument was externally calibrated using the Bruker Peptide Calibration Standard II spiked with insulin ($M+H^+$ 5730.61). MALDI-MSI was performed in positive mode over a mass range of *m/z* 800-10,000 at 20 µm spatial resolution with beam scan on. Spectra were accumulated from 2000 laser shots at 10 kHz frequency.

Image visualisation and data analysis was performed using SCiLS Lab MVS and normalised using the root mean squared approach. pLSA analysis was undertaken using root mean squared normalisation, weak denoising and 9 components. H&E stains were performed on the tissue samples after the imaging analysis.

Results and discussion

Figure 1 shows the averaged mass spectrum, with some zoomed in regions for analytes of different abundance showing the spatial distributions of these analytes, for centipede venom gland samples measured on the timsTOF fleX. The spectrum shows a high number of peaks with excellent signal to noise ratio – approximately 4643 potential features were a part of the imported peak list, of which well over 1000 were relevant signals, producing *m/z* images. The spectrum features some signal from matrix clusters, however, the enhanced mass resolution makes these signals easy to identify and exclude from subsequent analyses. There is a large number of identified features at widely varying signal to noise, many producing useful imaging data, indicating that the tims TOF fleX offers excellent dynamic range for imaging mass spectrometry experiments.

Data were collected at 20 µm pixel size; the acquisition speed achieved was approximately 5 pixels per second for the timsTOF fleX (2000 shots at 10,000 Hz), with a total of 4657 pixels in the dataset. Figure 2 shows the spatial distribution and mass resolution obtained for a peptide of interest which has a mono-isotopic neutral mass of 6000.99 Da. The abundance of the mono-isotope at *m/z* 6000 is relatively low and the better ion statistics for the A+1, A+2, A+3 peaks lead to more accurate measurement of the mass for these peaks than the mono-isotope, even so, the mass accuracy as shown in Figure 2D is quite exceptional for all peaks in the isotopic envelope at this mass range. For these larger peptides, care must be taken to determine the mono-isotopic peak vs the A+1. One key factor which has a great effect for timsTOF fleX analyses of small proteins / large peptides is decoupling of the measurement from the ionisation process by virtue

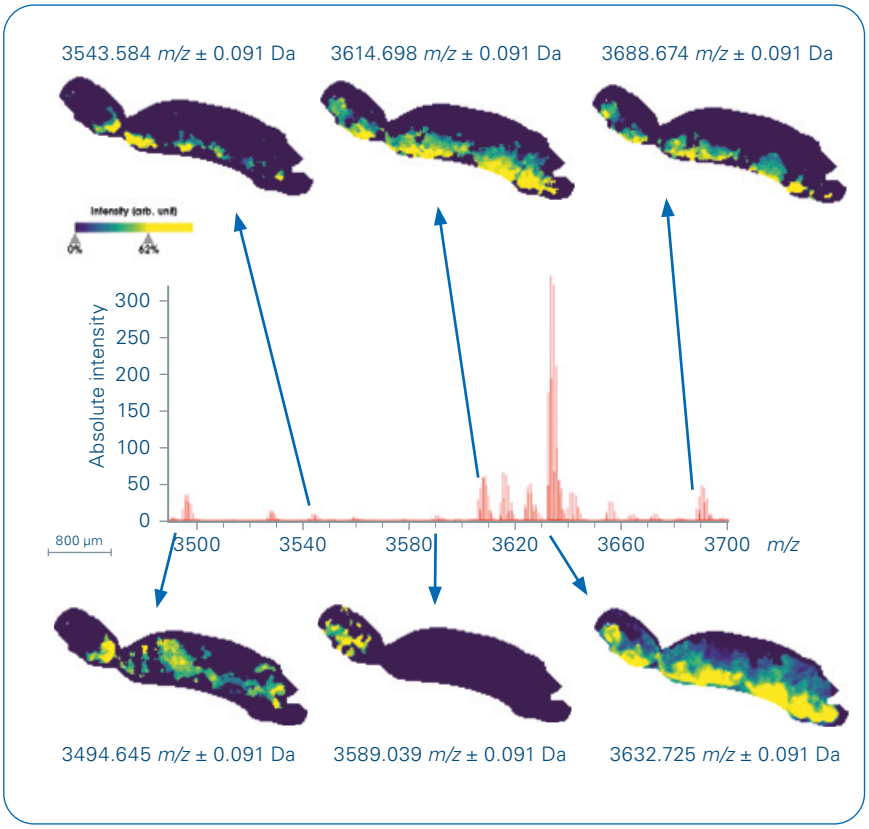


Figure 3: Highlights of the dynamic range of the timsTOF fleX instrument, showing the distributions of six representative venom components, which are present at different abundances in the tissue section.

using an orthogonal TOF rather than an axial TOF. Decoupling of the ionisation from the TOF reduces desorption and topographic effects from mass resolving power, by minimizing the impact of ion energy distribution on time focusing of analyte ions. A case in point is shown in Figure 2, where we identified the mature peptide of U-SLPTX15-Sm1a (NCBI TSA accession number GASH01000148), which is a 54-residue peptide with two intramolecular disulfide bonds and a mono-isotopic neutral mass of 6000.99 Da. Figure 2B is a zoomed in region of the total averaged mass spectrum (4657 pixels), and indicates also how stable the mass measurement is using the timsTOF fleX, this level of mass accuracy and stability is a feature of the orthogonal TOF implementation in the timsTOF fleX.

When we calculate the mass accuracy for each of the peaks in the isotopic envelope, the ppm mass accuracy is between 0 and 11 ppm (Figure 2). This is a remarkable result for a measurement of a small protein of 6 kDa directly from tissue. Similar high mass resolved and high mass accuracy data was observed across the entire mass range of *m/z* 1000 – 6500. The timsTOF fleX instrument also displays excellent dynamic range, and is capable of providing spatial distribution of high and low abundance peptides simultaneously. The observed signal to noise (Figures 1 and 3) is also excellent, and the resolving power of the instrument can easily differentiate lower signals from baseline noise.

Performing component analysis using pLSA in SCiLS Lab MVS (Figure 4), revealed the compartmentalisation of the various peptides and proteins observed in the centipede venom gland, which is consistent with our previously published analyses of this species [2, 3]. Although the biological significance of this toxin spatial heterogeneity remains unclear, it has been hypothesised to reflect physiological constraints on toxin mass-production by a relatively small number of secretory cells [2]. Similar uneven distributions of toxins have also been observed in the otherwise morphologically largely undifferentiated venom systems of other venomous animal lineages, such as snakes [5] and sea anemones [6]. However, in these cases, the spatial heterogeneity of toxins is thought to enable biochemical modulation, or regulation, of the secreted venom [5]. This has been found to be the case for scorpions, assassin bugs [7], and venomous marine cone snails [8], which possess specialised predatory and defensive parts of their venom systems. However, regardless of the biological implications of these findings, the capacity to investigate venom distributions and gland compartmentalisation demonstrates the benefit of MALDI-MSI over other protein detection methods such as Western Blot, in which spatial information is lost. Likewise, the ability to detect simultaneously such a high number of different signals from one tissue section is in striking contrast to probe-based technologies such as immunohistochemistry or in situ hybridisation.

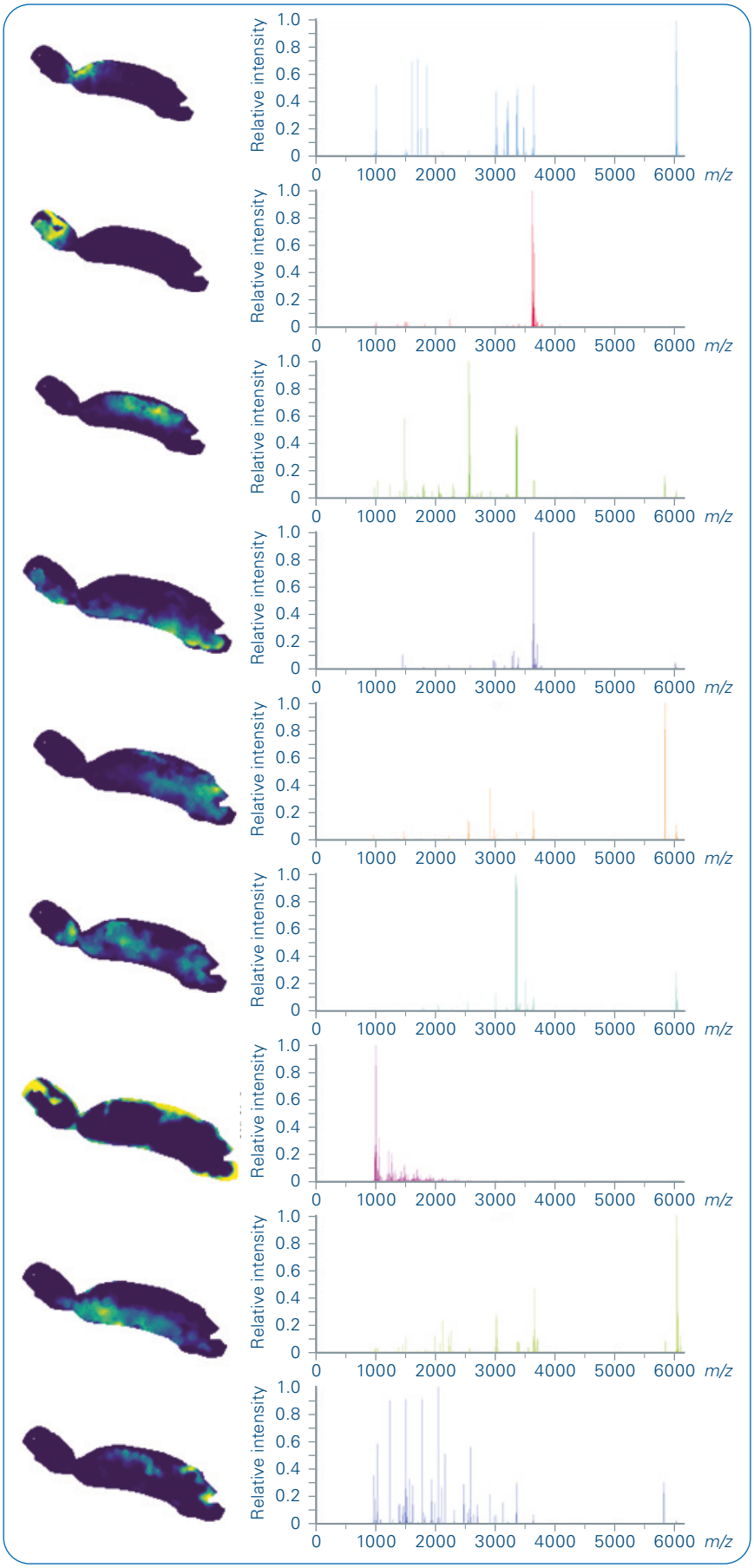


Figure 4: pLSA analysis of the timsTOF fleX data set collected for the centipede venom gland.

Conclusion

- The timsTOF fleX offers a unique combination of sensitivity, mass accuracy, mass resolving power, and speed for imaging mass spectrometric analysis of peptides and small proteins.
- On the basis of this experiment, the timsTOF fleX offers exciting new possibilities for venom biology.
- Importantly, it will open the possibility to annotate more of the peptides and proteins we observe during imaging experiments, and provide a much more intimate connection between proteomics (bottom up and top down), and genomic workflows.

Learn More

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