High quality sections and molecular distribution images of neuropeptides from heat-stabilized tissue

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Background

Mass spectrometric imaging of tissue sections are used to investigate molecular distribution in tissue. Due to residual enzymatic activity in the tissue sections, changes can occur during processing prior to analysis that will change the molecular composition. Such changes can obscure original composition and lead to misinterpretations and erroneous conclusions. It has been previously shown that rapid heat stabilization using the Stabilizor system (Denator AB) can prevent such changes and reveal a molecular composition closer to the in vivo state (1, 2).
During previous investigations, it was discovered that sections of heat-stabilized tissue may become more fragile and morphology can be negatively affected. In the work presented here we show that using special precautions and techniques, good quality sections can routinely be obtained from heat-stabilized tissue. This enables confident analysis of molecular distributions in tissue sections with mass spectrometric imaging with preserved quality.

Method

Method for preservation of endogenous peptides:
Peptide extracts from snap-frozen and heat-stabilized mouse brain tissue (striatum) were extracted in 0.1% TCA in water and 10 kDa Mw cut off filters used to isolate peptides. Peptides were desalted using C18 µ ZipTips (Millipore) and eluted in 2 µL of 50% ACN/0.1% TFA and mixed 1:1 with R-cyano-4-hydroxycinnamic acid, 10 mg/mL in 50% ACN/0.1%TFA, and a dried droplet was spotted onto the MALDI target prior to MALDI analysis (Autoflex III, Bruker). Spectra were collected in reflectron mode and analyzed with FlexAnalysis, ver. 3.0 (Bruker).

Method for molecular distribution images of heat-stabilized tissue:
1. **Heat stabilization**. Fresh rat brain tissue was either heat-stabilized using the "Structure Preserve mode" setting in the Stabilizor T1 instrument or snap frozen on dry ice. The cavity of the Maintainor Tissue card was opened directly after heat stabilization to avoid compression due to steam condensation.
2. **Cryosectioning.** Samples were embedded in 2.5% CMC (Carboxy Methyl Cellulose, Sigma #C4888) before freezing in CO2 (s). 14 µm thick sections were cut using a standard cryostat. Sections were cut with one swift motion and transferred using a fine artist brush to pre-chilled slides. For difficult to cut tissue, a tape transfer system, eg. CryoJane, or slow motorized cryosectioning has been shown to produce better quality sections. Sections were thaw-mounted from one side and continuously across.
3. **Drying the mounted sections.** Mounted sections were kept frozen and dried while in the deep freezer use desiccant silica gel. Glass slides with dried mounted sections were equilibrated to room temperature in the presence of
desiccant to avoid condensation.
4. **MALDI imaging procedure.** Sections were washed in 70 and 100% EtOH. CHCA or DHB were used as matrix and applied using a TLC sprayer (Sigma Aldrich). MALDI imaging was acquired in positive ion linear mode and reflectron modes using an Ultraflex II TOF/TOF (Bruker Daltonics). Typically 300 laser shots per raster point with ~50 µm spot diameter were used. Data was analyzed using FlexImaging 2.0-3.0.

Results

The post-sampling degradation effect on endogenous peptides in snap-frozen samples is evident in the MALDI MS analysis of mouse brain extracts. In snap-frozen striatum, only one endogenous peptide was identified while in heat-stabilized samples the majority of peaks can be attributed to known endogenous peptides (fig 1).

Procedures for producing sections with well preserved morphological features from heat-stabilized brain tissue have been optimized. The key parameters contributing to sections with good morphological preservation are:
Heat stabilization of fresh tissue, embedding in CMC, rapid freezing, tape transfer system or slow motorized sectioning, artist brush transfer followed by unidirectional thaw mounting, and keeping the section frozen while desiccating (fig. 2).

The neuropeptide Pep-19 and two of its ex-vivo degradation products have been imaged in rat brain sections. In heat-stabilized samples, full length Pep-19 can be identified at higher levels than in sections from comparative snap-frozen samples. In addition to the full length Pep-19 neuropeptide, two fragments of Pep-19 have been exclusively detected in snap-frozen sections (fig. 3).

Conclusion

The work shown here demonstrate that the Stabilizor system can be introduced into the MALDI imaging workflow without the loss of tissue section quality as well as preserved molecular profiles (fig. 2).

The combination of MALDI imaging with heat-stabilized tissue prevents changes during sample handling and works up and gives more biologically relevant results. This will increase the relevance of MALDI imaging results for the study of molecular distribution of ex vivo labile molecules such as drugs, metabolites and neuropeptides.

References

[1] Goodwin R.J.A., et al., "Conductive carbon tape used for support and mounting of both whole animal and fragile heat-treated tissue sections for MALDI MS imaging and quantification", Journal of Proteomics 2012, 4912-20
[2] Blatherwick, Eleanor Q., et al. "Localisation of adenine nucleotides in heat-stabilised mouse brains using ion mobility enabled MALDI imaging." International Journal of Mass Spectrometry, 2013.

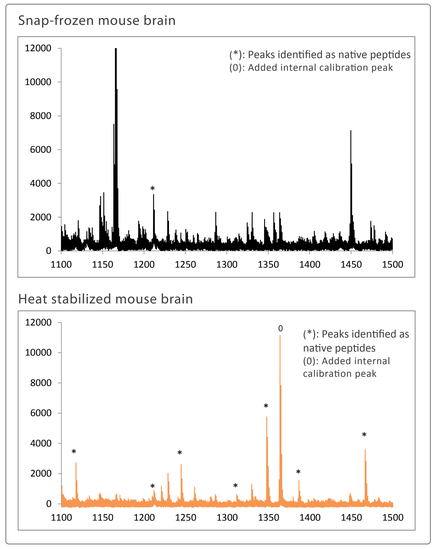

Figure 1. MALDI ion traces of heat-stabilized and snap-frozen mouse brain tissue samples. The peptide peaks identified in the heat-stabilized samples consist of known neuropeptides, endogenous peptides and novel, potentially biologically active, neuropeptides (*) that can not be found in snap-frozen samples.

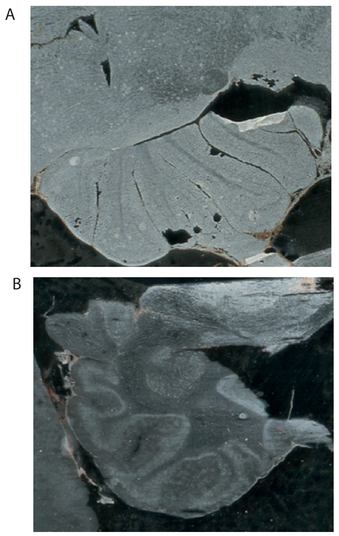

Figure 2. Cryotome sectioned sagital sections of whole rat brain (cerebellum shown), obtained using a microtome sectioning at 12 µm at -18°C. Samples and sections in the various panels were treated as follows:
A: Rat brain was heat stabilized whole and embedded directly in cold 2.5% CMC and frozen. Sections transferred to ITO coated slides using an artist brush, attached by thaw mounting by local thawing and then dried while frozen.
B: Rat brain was snap frozen on dry ice and embedded frozen in cold 2.5% CMC and frozen. Sections transferred to ITO coated slides using an artist brush, attached by thaw mounting by local thawing and then dried while frozen.

This methodology allows for preservation of the tissue morphology during sectioning, and does not affect ionization or quantitation of the sample.

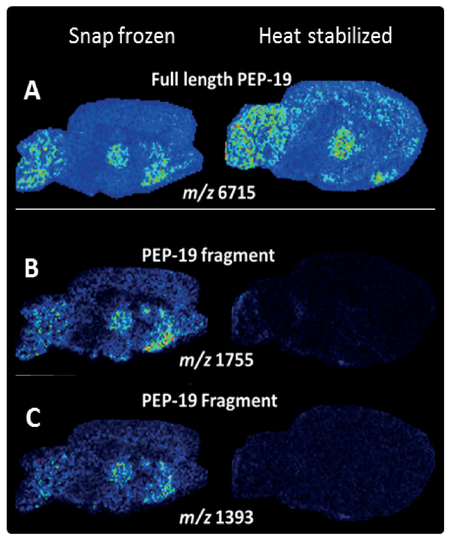

Figure 3. MALDI Imaging of full-length PEP-19 and metabolites in snap-frozen vs. heat-stabilized brain tissue samples.

The neuropeptide PEP-19 (m/z 6715) is detected at higher levels in the heat-stabilized brain than in snap-frozen brain (figure 2A) indicating preservation. Further proof of prevention of port-mortem degradation is the two well-known fragments of PEP-19 (m/z 1755 and m/z 1393) that are only found in snap-frozen tissue (figure 2B and C). This suggests extensive degradation of the peptide during sample preparation and emphasizes the need for heat stabilization of the tissue in order to avoid such changes.

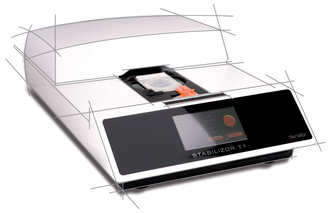

Figure 4. About Stabilizor system
- Eliminates enzyme activity from the moment of sampling
- Stops degradation permanently - without using additives
- Standardizes sample handling - to improve reproducibility of analytical workflows
- Links upstream processing to downstream results - traceable treatment parameters
- Facilitates accurate sample comparison and data interpretation downstream