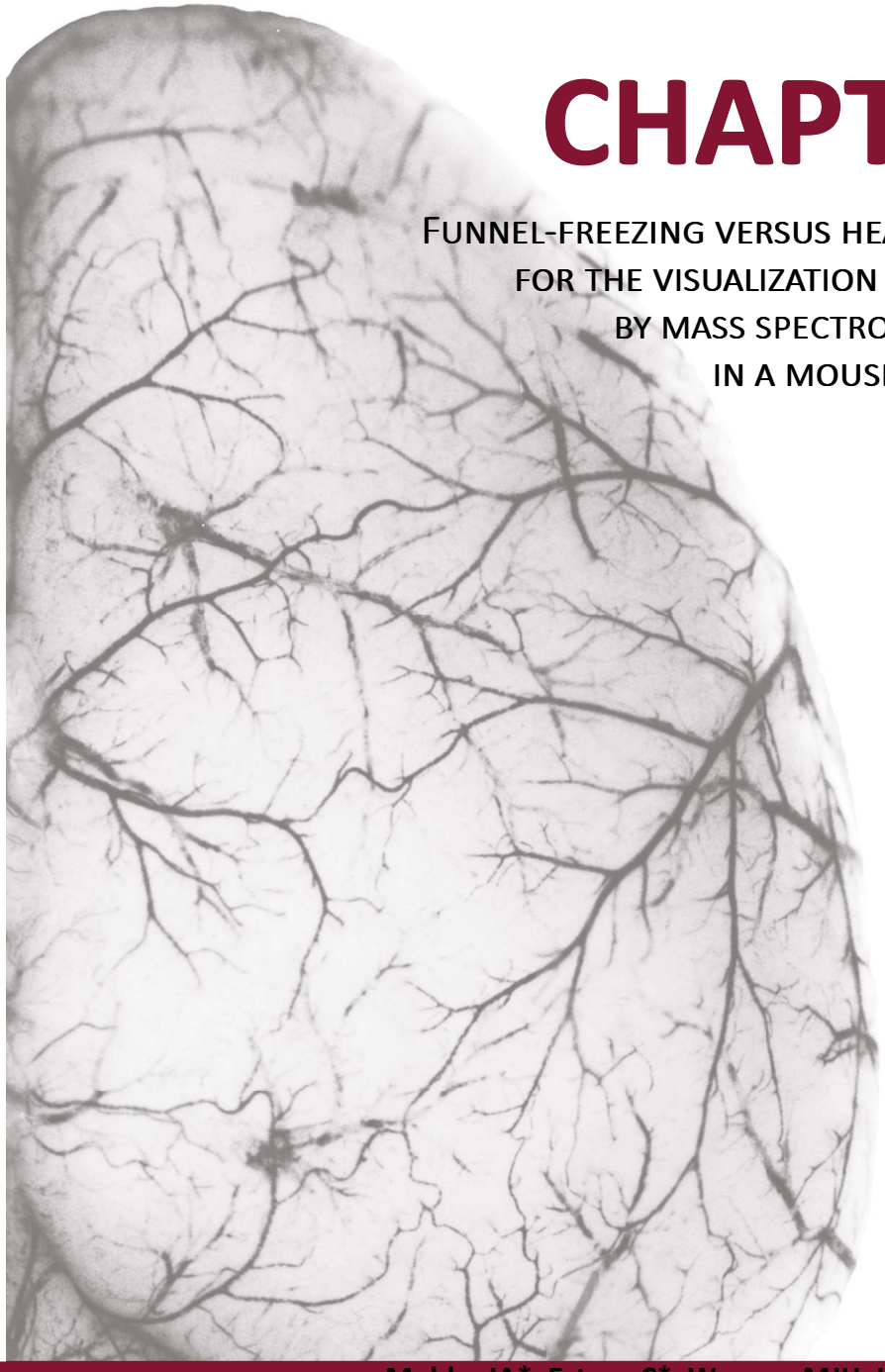
Stroke and migraine: Translational studies into a complex relationship

Mulder, I.A.



CHAPTER 2

Funnel-freezing versus heat-stabilization for the visualization of metabolites by mass spectrometry imaging in a mouse stroke model

Mulder IA*, Esteve C*, Wermer MJH, Hoehn M, Tolner EA, van den Maagdenberg AMJM and McDonnell L

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**Authors contributed equally*

ABSTRACT

Tissue preparation is the key to a successful matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging (MSI) experiment. Rapid *post mortem* changes contribute a significant challenge to the use of MSI approaches for the analysis of peptides and metabolites.

In this technical note we aimed to compare the tissue fixation method *ex vivo* heat-stabilization with *in situ* funnel-freezing in a middle cerebral artery occlusion (MCAO) mouse model of stroke, which causes profound alterations in metabolite concentrations. The influence of the duration of the thaw-mounting of the tissue sections on metabolite stability was also determined. We demonstrate improved stability and biomolecule visualization when funnel-freezing was used to sacrifice the mouse compared with heat-stabilization. Results were further improved when funnel-freezing was combined with fast thaw-mounting of the brain sections.

Introduction

MALDI mass spectrometry imaging (MALDI MSI) can simultaneously record the distributions of hundreds of molecules directly from tissue samples[1] and within their histological context.[2] MSI is used to analyse metabolites, drugs, peptides, proteins, lipids and glycans, and is applied to diverse biomedical and biological applications. Tissue preparation is arguably the single most important factor that determines the success of a MALDI MSI experiment, and this is especially true for metabolites on account of their high susceptibility to *post mortem* changes.[1-3] For example, for the analysis of mouse brain tissue obtained by post-euthanasia freezing, the procedure of decapitation, brain excision and snap-freezing takes one to several minutes, during which time the remaining activity of endogenous enzymes is known to lead to post-mortem degradation.[2] Similar results have also been reported for neuropeptides.[4]

Several strategies have been reported for the reduction of post-mortem changes of metabolites in brain tissue:

I. Heat-stabilization of *ex vivo* tissues (HS) - enzymes are inactivated by heating the tissue to 95°C using high power heating blocks.[2-6] Blatherwick *et al.*[3] have demonstrated that *ex vivo* heat-stabilization is able to halt the rapid *post mortem* degradation of adenine nucleotides that otherwise occurs in *ex vivo* snap-frozen tissue.

II. *In situ* freezing (ISF) under anaesthesia is based on freezing the tissue using liquid nitrogen while maintaining blood flow and oxygenation.[2,7,8] Hattori *et al.*[7] have demonstrated that ISF is superior to *ex vivo* snapfrozen tissue for maintaining metabolite integrity, including adenine nucleotides that are prone to rapid *post mortem* change.

III. *In situ* focused microwave irradiation (FMW) - uses focused microwaves to very rapidly, <2 s, heat the tissue to deactivate enzymes.[2] Sugiura *et al.*[2] have compared FMW with ISF and *ex vivo* snap-freezing and reported that, while ISF and FMW provide similar results for most metabolites, there are several metabolites that are best analyzed using FMW on account of their very rapid *post mortem* changes.[2] However, the high expense and negative aesthetics of animal sacrifice via focused microwave irradiation has severely limited its use.

ISF and *ex vivo* heat-stabilization have been reported to be superior than *ex vivo* snap-freezing for preserving metabolic integrity but have not yet been compared. With *in situ* funnel-freezing under anaesthesia[9] blood flow is still present until the tissue is frozen. It has previously been demonstrated that warm ischemia times lead to greater *post mortem* changes than cold ischemia times.[10] Accordingly, it may be reasoned that *in situ* funnel-freezing may better preserve metabolites in their pre-sacrifice state than *ex vivo* heat-stabilization, which takes 1–2 min to excise the brain and another 1–2 min (of warm ischemia) to stabilize the tissue, time which is critical for metabolite stability. A caveat is that with *in situ* funnel-freezing enzymes are not inactivated. Accordingly, preparation of the tissue sections for MSI analysis must be very carefully controlled after *in situ* funnel-freezing as the enzymes can reactivate, continuing metabolite degradation, as soon as the tissue is thawed.[11]

In this paper we have systematically compared *in situ* funnel-freezing, *ex vivo* heat-stabilization and subsequent thaw-mounting methods for the analysis of metabolites by MALDI-MSI in a mouse model for ischemic stroke. Ischemic stroke is an often disabling event caused by interruption of blood supply to part of the brain.[12] Understanding the biomolecular profiles in the infarct core and penumbra[13] may help explain differential vulnerability and recovery of brain regions to metabolic stress and to search for potential neuroprotective or neurorestorative therapies.[14] In this context, a discriminating factor between core and

penumbra is the level of ATP;[15] previous investigations utilizing both bioluminescence and MALDI MSI (using *in situ* freezing) have reported a localized increase in ATP. As ATP is very quickly degraded post-mortem, it also represents an excellent model system to assess how well the tissue's metabolic status has been preserved.[7]

MATERIALS AND METHODS

Animal protocol

Male 2- to 4-month-old C57BL/6J mice were used. Experimental stroke was induced using a slightly modified middle cerebral artery occlusion model (MCAO) first described by Longa *et al.*[16] Mice were anesthetized using isoflurane (3% induction, 1.5% maintenance) in 70% pressurized air and 30% O_2. Carprofen 5mg/kg, *s.c.* (Carporal, 50 mg/mL, AST farma B.V., Oudewater, the Netherlands) was given before surgery. During surgery the mouse body temperature was maintained at 37°C using a feedback system. Briefly, the surgical procedure; a silicone-coated nylon monofilament (7017PK5Re, Doccol cooperation, Sharon, MA, USA) was introduced into the internal carotid artery, via a small incision in the right common carotid artery, to block the middle cerebral artery (MCA) at its origin, for 30 min. During the occlusion period, the mouse was allowed to wake up in a temperature-controlled incubator (V1200, Peco Services Ltd, Brough, United Kingdom) maintained at 33°C. After surgery, the animal was placed in the incubator again for 2 hours with easy access to food and water.

On a subset of animals, used for the preliminary experiments, SHAM surgery was performed using the same protocol, only without blocking the MCA. At 24 hours after MCAO mice were scanned *in vivo* using a 7T small animal MRI system (Bruker Pharmascan; Bruker, Ettlingen, Germany), under Paravision 5.1 software (Bruker). A Multi SliceMulti Echo (MSME) sequence protocol was run with TR/TE of 4.000 ms/9 ms, 20 echoes, two averages, matrix 128×128 mm, FOV of 2.50 cm, bandwidth 59523.8Hz, slice thickness of 0.5 mm and 16 slices (no gap) and quantitative T2 maps were calculated from the multi-echo trains.

For *ex vivo* heat-stabilization the mouse was sacrificed by decapitation and the brain was quickly isolated (<1.5 min). The brain was immediately stabilized using a tissue heat-stabilizer device (StabilizorTM, Denator AB, Göteborg, Sweden) at 95°C in 60–90 seconds depending on brain volume. Thereafter, brains were frozen on dry-ice and stored at -80°C.

In situ funnel-freezing was based on previous reported studies.[9,17-19] Briefly, the mouse was anesthetized using isoflurane (3% induction) and 1.5–2% isoflurane in 30% O_2 and 70% pressurized air was used to maintain deep anaesthesia. A skin incision was made from the level of the eyes to the occiput exposing the skull. A funnel was placed onto the skull, with the posterior rim of the funnel at the lambdoidal suture. The skin was pulled up around the funnel and secured with four sutures to prevent leaking. Liquid nitrogen was continuously poured into the funnel for 3 minutes. Thereafter, for easier removal of the brain, the whole animal was frozen in liquid nitrogen. Next the animal was put into dry-ice and the brain dissected using a scalpel and a dental drill. The excised and frozen brain was stored at -80°C. All animal experiments were approved by the Animal Experiment Ethics Committee of Leiden University Medical Center.

MALDI MSI

Coronal sections (12 µm, between –0.10 and +0.40 from Begma) were cut using a cryostat microtome (Leica Microsystems, Wetzlar, Germany) at -21°C. The brain sections were thaw-

mounted onto ITO glass slides (Delta Technologies, Stillwater, MN, USA) coated with 0.05% poly-L-lysine (poly-L-lysine coating used for greater adherence of tissue sections, protocol used as reported in Aichler *et al.*[20]). Sections were thaw-mounted onto the slides by localized warming of the reverse side of the MALDI target using a finger for max 3 seconds (fast) or for 1 minute (slow).[1,10] The slide-mounted tissues were stored at -80°C. For analysis the slide-mounted tissue sections were first brought to room temperature in a desiccator for 5 minutes. For matrix application a uniform coating of 9-AA (2 mg/Ml in 70% MeOH) was added using a SunCollect automated deposition system (SunChrom, Napa, CA, USA). Brain sections from three animals per group were analyzed in technical duplicate on a 9.4-Tesla SolariX MALDI-FTICR (Bruker Daltonics, Bremen, Germany), equipped with a SmartBeam II laser system that consists of a frequency tripled Nd:YAG laser operating at 355 nm, at repetition rates up to 1 kHz, and using a spatially modulated laser profile. MS data were acquired in negative mode by first accumulating the ions from 500 laser shots in an external hexapole ion trap before transferring them to the ICR cell for detection. Ions were detected in the range 50–1000 m/z and MSI was performed with a spatial resolution of 125 µm. Data acquisition, processing, and data visualization were performed using the Flex software suite (FlexControl 3.4, ftmsControl 2.0, FlexImaging 4.1 and DataAnalysis 4.2) from Bruker Daltonics.
After MSI data acquisition the matrix was washed off with 70% ethanol and the tissue samples stained with cresyl violet (Nissl stain).[21,22] High-resolution histological images were obtained with a digital slide scanner (3D Histech MIDI) and were registered to the MSI datasets using FlexImaging. A scheme of the work flow is presented in Figure 1.

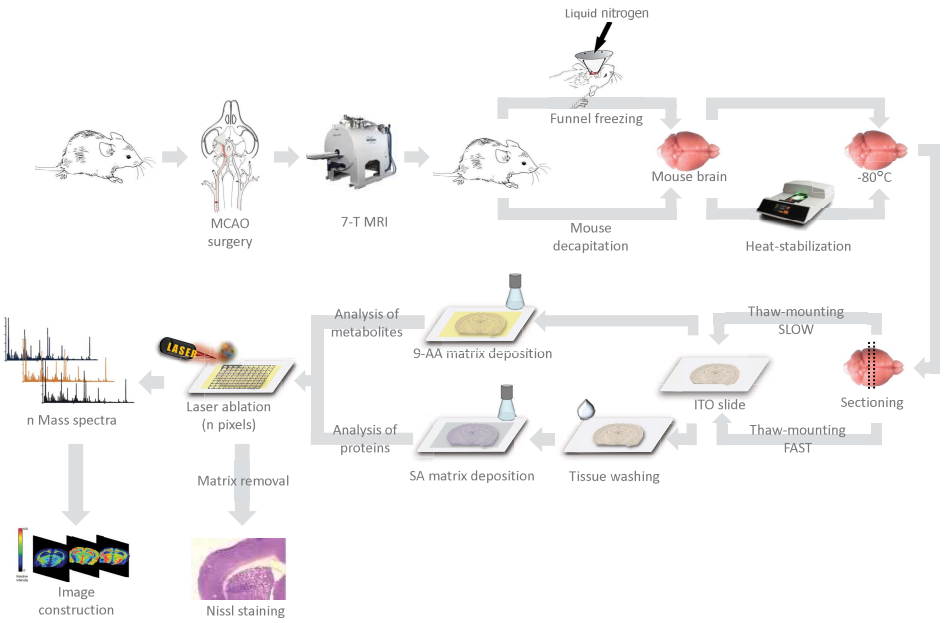

Figure 1. Schematic of the workflow used to analyze the effect of brain tissue sampling protocol. Mice first underwent MCAO surgery, were scanned using a 7T MRI at 24 hours and directly thereafter sacrificed by either ***in vivo*** funnel-freezing, or by decapitation followed by *ex vivo* heat-stabilization. Coronal tissue sections were then cut and mounted onto poly-lysine coated slides using slow (1 min) or fast (3 sec) thaw-mounting. Metabolites were analyzed by MALDI-FTICR-MSI using 9-AA as the matrix. Each section was stained with Nissl reagent after matrix removal.

Data analysis

The RMS normalized intensities of six mouse brain sections were measured for each group (two technical duplicates of three biological replicates). MS data were extracted from each MSI dataset for statistical analysis: (I) a non-paired Student's t-test (one tailed) was used for comparisons between *ex vivo* heat-stabilized and *in situ* funnel-freezing brains and (II) a paired Student's t-test (one-tailed) was used for comparisons (fast versus slow thaw-mounting) within each mouse brain. Statistical analysis was performed in Microsoft Excel 2010. Metabolite identities were assigned on the basis of the very high-mass accuracy of the high-field MALDI FTICR mass spectrometer used for the experiments (<1 ppm), in conjunction with the results of previous metabolite MALDI MSI experiments (it is now broadly established that MALDI MSI samples a consistent set of molecules) and the isotope profiles.[2,3,7,8,23,24] For selected metabolites, in which the ion intensity was sufficient for MS/MS, the ID's were confirmed by MS/MS.

Results and Discussion

When seeking to investigate the metabolite/peptide content of tissues it is vital that the tissue collection protocol limits the sometimes rapid *post mortem* changes that follow animal sacrifice. In a preliminary experiment, we compared the metabolite MSI signatures from brain tissue obtained using *ex vivo* heat-stabilization and *in situ* funnel-freezing with *ex vivo* snap-freezing, to check if the results we obtained were consistent with those previously reported;[3,7] it was indeed found that both methods were more effective at retaining labile metabolites but *in situ* funnel-freezing appeared to lead to more intense labile metabolite signals (Supplemental Figure 1). The results described herein describe an experiment designed to compare *ex vivo* heat-stabilization and *in situ* funnel-freezing. The comparison included both tissue stabilization methods as well as the time used for thaw mounting because *in situ* funnel-freezing does not deactivate the tissue enzymes, and thus *post mortem* changes may still occur during any subsequent tissue processing step (Figure 1). Here we used the MCAO model for ischemic stroke because the localized increase of ATP previously reported by Hattori *et al.*,[7] a molecule highly sensitive to *post mortem* degradation,[2] provides an excellent *in situ* measurement of metabolite stability and the unaffected contralateral hemisphere provides an internal control. Mouse brain tissue samples were obtained by *in situ* funnel-freezing and *ex vivo* heat-stabilization, from which 12 µm thick coronal tissue sections were placed onto the ITO-coated glass slides using fast (<3 sec) and slow (1 min) thaw-mounting. All experiments were performed in technical duplicate and biological triplicate. Figure 2A shows example MSI images recorded for AMP, ADP, and ATP together with their corresponding T2-weighted MRI and histological images. The localized increase of ATP in the ischemic penumbra was consistently detected in the MSI datasets of the tissues obtained via *in situ* funnel-freezing (Figure 2A) but not from those obtained via *ex vivo* heat-stabilization. Furthermore, it can be seen that when using *ex vivo* heat-stabilization the tissue is deformed during the process (Figure 2A and B, bottom rows) because of the pressure applied to the tissue by the thermal blocks to ensure a high thermal contact. When comparing the MS images with the T2-weighted MR image and the histological section, comparisons are more straightforward using the *in situ* funnel-freezing technique.

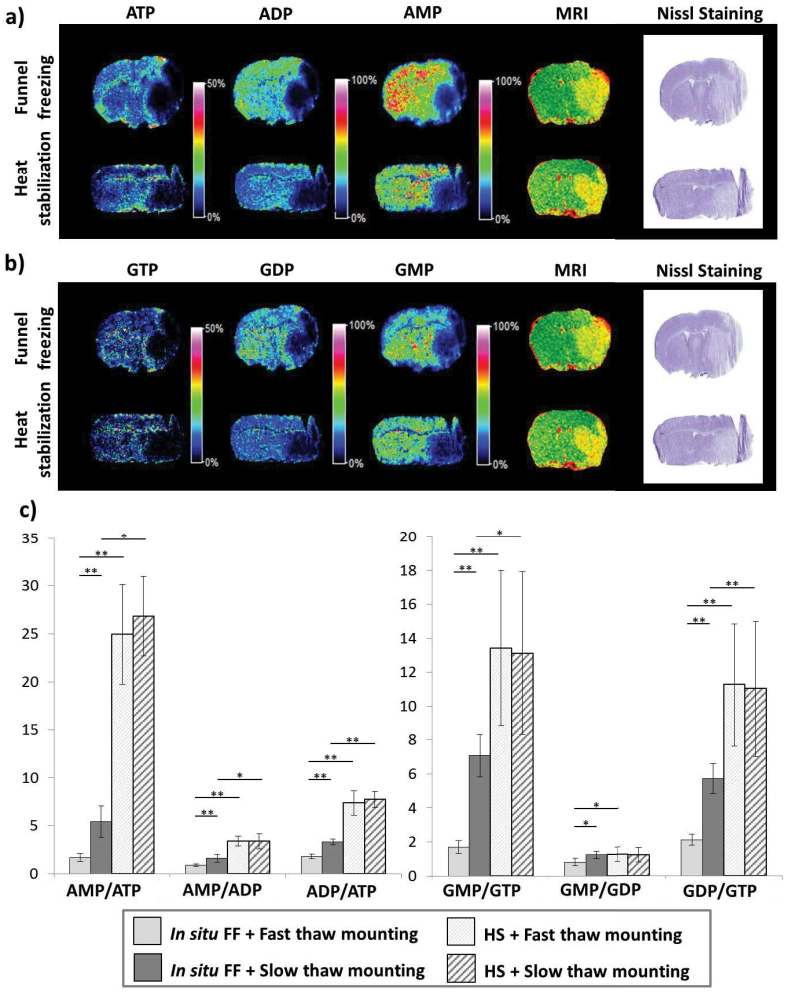

Figure 2. Metabolite MSI dataset showing the effect of the brain tissue sampling protocol on the visualization of adenosine and guanosine metabolites in mouse brain by MALDI-FTICR. (A) Images showing the visualization of the average distribution of AMP (m/z 346.055), ADP (m/z 426.022) and ATP (m/z 505.988) for mouse brain obtained via *ex vivo* heat-stabilization and *in vivo* funnel-freezing. (B) Images showing the visualization of the average distribution of GMP (m/z 362.050), GDP (m/z 442.016) and GTP (m/z 521.982) for mouse brain obtained via *ex vivo* heat-stabilization and *in vivo* funnel-freezing. (C) Average intensity ratios of AMP:ATP, ADP:AMP, ADP:ATP (left) and GMP:GTP, GDP:GMP, GDP:GTP (right) from the control hemisphere, for *ex vivo* heat-stabilization (HS) and *in vivo* funnel-freezing (FF) with the two thaw-mounting times (slow and fast). Each group consists of three biological replicates each analyzed with two technical replicates. Error bars represent standard deviation across technical replicates. (** = $p < 0.01$ and * = $p < 0.05$)

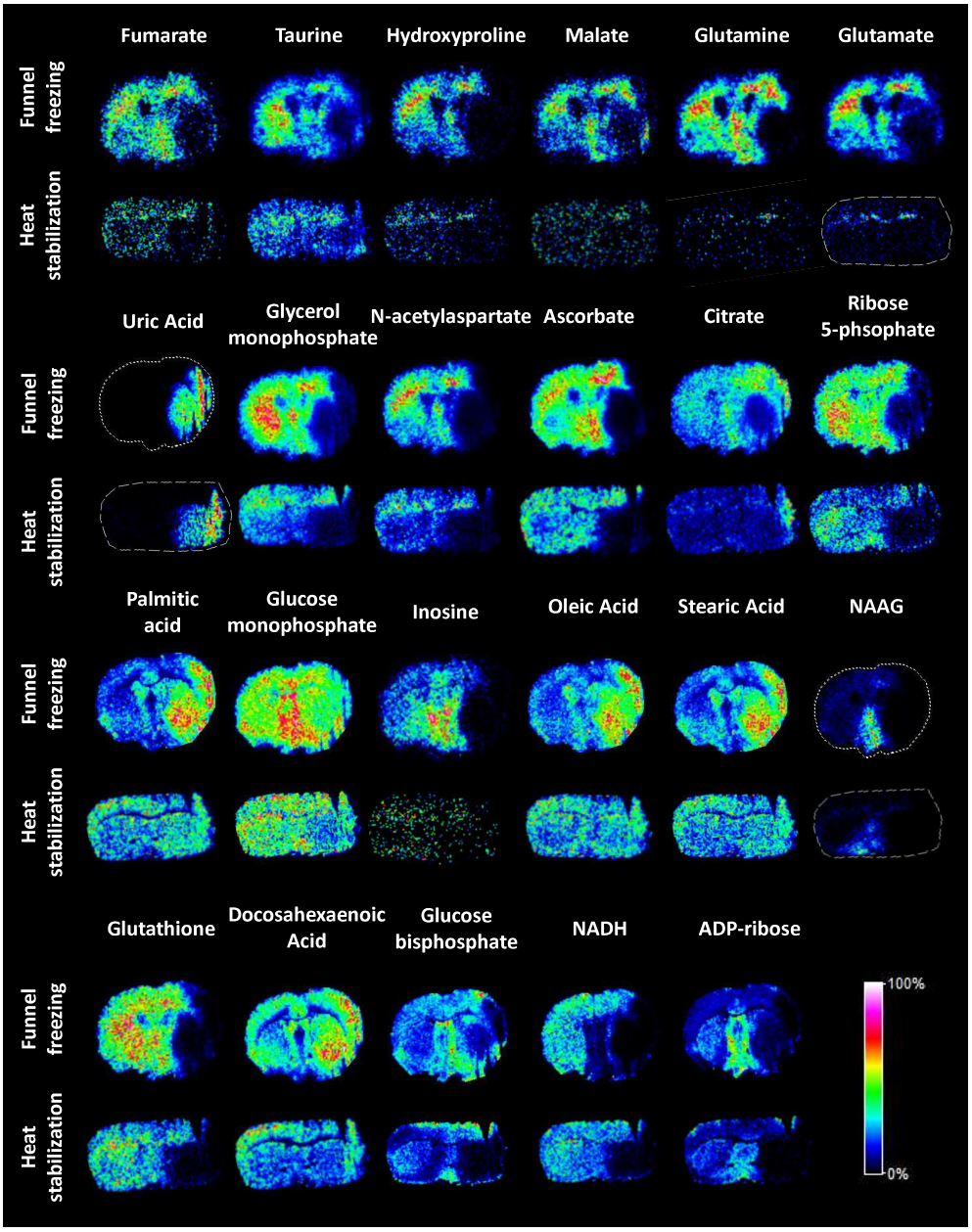

Figure 3. Metabolite MSI images of mouse brain metabolites found in the range m/z 50–1000 and analyzed by MALDI-FTICR.

A significant reduction in AMP, ADP, and ATP signals in the ischemic region was observed for all preparations, in agreement with previous reported results.[23] The ATP signals from the control hemispheres obtained from the *ex vivo* heat-stabilized tissues were lower than those obtained using *in situ* funnel-freezing; conversely the AMP signals from the control hemispheres were greater from the *ex vivo* heat-stabilized tissues. Similar results were obtained for the nucleotides GTP, GDP, and GMP as shown in Figure 2B. However, it is known that MCAO (stroke hemisphere) and heat-stabilization (control hemisphere) can lead to increased salt levels, which can cause a global increase in ionization bias and thus lower MSI signal intensities. To circumvent differences in global ionization bias the AMP:ATP, ADP:ATP, and AMP:ADP intensity ratios were calculated (Figure 2C) from the control hemisphere. The bar chart shows that the ratio of AMP to ATP was 5-fold higher for *ex vivo* heat-stabilized tissue than for those obtained using *in situ* funnel-freezing when slow thaw-mounting was used, and around 15-fold higher when fast thaw-mounting was used. For both thaw-mounting methods (compared with heat-stabilization) the difference in AMP to ATP ratios were statistically significant. Accordingly, the results demonstrate that irrespective of the mounting method used, the *in situ* funnel-freezing method leads to less *post mortem* changes of the adenine nucleotide metabolites, which is also supported by the observation that the increase in ATP seen in the penumbra was only observed with tissues obtained using *in situ* funnel-freezing.

While the *in situ* funnel-freezing method was found to lead to reduced *post mortem* changes, it is more sensitive to subsequent tissue processing methods. A comparison of the fast and slow thaw-mounting methods on the adenine nucleotide metabolite ratios within the *in situ* funnel-freezing brain group revealed a statistically significant increase in metabolite degradation that was not present in the *ex vivo* heat-stabilized group, Figure 2C. This result indicates that for *ex vivo* heat-stabilization all ATP degradation takes place during brain excision and during heat treatment; after heat-induced denaturation the enzymes are no longer active and further metabolite degradation is limited. For *in situ* funnel-freezing the enzymes are not deactivated and so once the tissue is thawed *post mortem* metabolite degradation may continue. Nevertheless, it should be noted that ATP degradation was always lower when using *in situ* funnel-freezing. This is consistent with previous reports that *post mortem* changes occur more rapidly when the tissue is still at physiological temperatures.[10,25] Similar results were obtained for GTP, GDP and GMP, except for the GMP:GDP ratio (Figure 2C).
Sample treatment not only influences the stability of adenine nucleotide metabolites. Figure 3 shows the distribution of several metabolites that were detected by MALDI-FTICR-MSI using 9-AA as matrix. In several cases metabolites showed similar distribution for both methods. However, some of them could only be detected using *in situ* funnel-freezing or had very weak signal for *ex vivo* heat-stabilized brains, including fumarate (m/z 115.003), hydroxyproline (m/z 130.050), malate (m/z 133.014), glutamine (m/z 145.061), glutamate (m/z 146.045), citrate (m/z 191.019), and inosine (m/z 267.073). Furthermore, the intensity of metabolite peaks was a lot higher when *in situ* funnel-freezing was used, and also exhibited greater contrast with the stroke region, e.g. taurine (m/z 124.007), glycerol monophosphate (m/z 171.006), N-acetylaspartate (m/z 174.040), palmitic acid (m/z 255.232), or glutathione (m/z 306.076).
In their comparison of *in situ* freezing, focused microwave irradiation and *ex vivo* snap-freezing (no heat-stabilization) Sugiura *et al.*[2] used capillary electrophoresis to compare *post mortem* metabolite stability. One group, primarily consisting of amino acids, was stable to *post mortem* changes. Using the on-tissue derivatization methods recently reported by Shariatgorji *et al.*[26] we also compared the amino-metabolites obtained from tissues processed via *in situ* funnel-

freezing with those obtained via *ex vivo* heat-stabilization, Figure 4. It can be seen that the distributions are more comparable than those of the less stable metabolites shown in Figures 2 and 3. Again indicating that the changes are due to *post mortem* degradation.

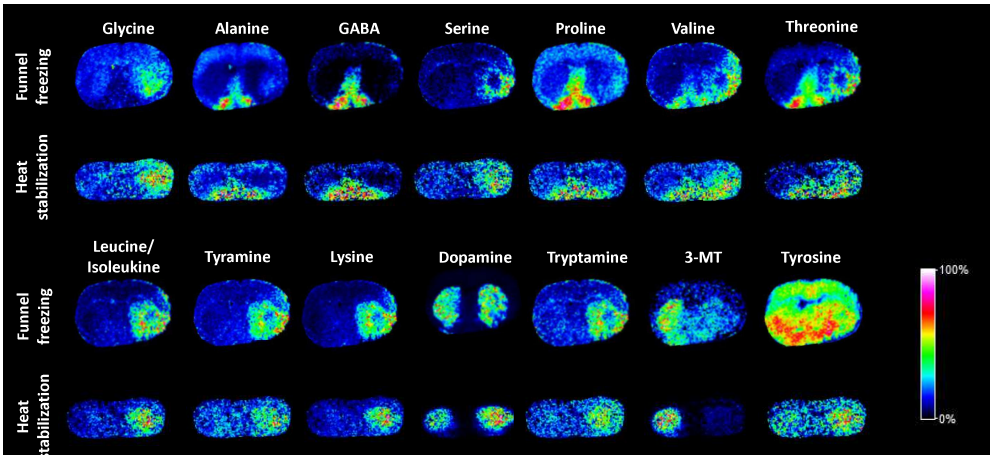

Figure 4. Amino-metabolite MSI images of mouse brain sections analyzed using MALDI-FTICR and the on-tissue derivatization agent 2,4-diphenyl-pyranylium tetrafluoro-borate.[26]

Concluding Remarks

These results again highlight the crucial role of the tissue in MSI experiments, how it was sampled and how it was mounted. Two different tissue sampling strategies, *in situ* funnel-freezing and *ex vivo* heat-stabilization, as well as fast and slow thaw-mounting, were investigated for their impact on metabolite levels and distributions using MALDI-FTICR-MSI. The results demonstrated that *in situ* funnel-freezing has lower degradation compared to heat-stabilization irrespective of the thaw-mounting method, but care should be taken to keep the thaw-mounting short (and thus reproducible) to limit metabolite degradation and variation between experiments. *In situ* funnel-freezing has the additional advantage of causing little deformation to the mouse brain, allowing alignment to other imaging datasets and reference tissue atlases.

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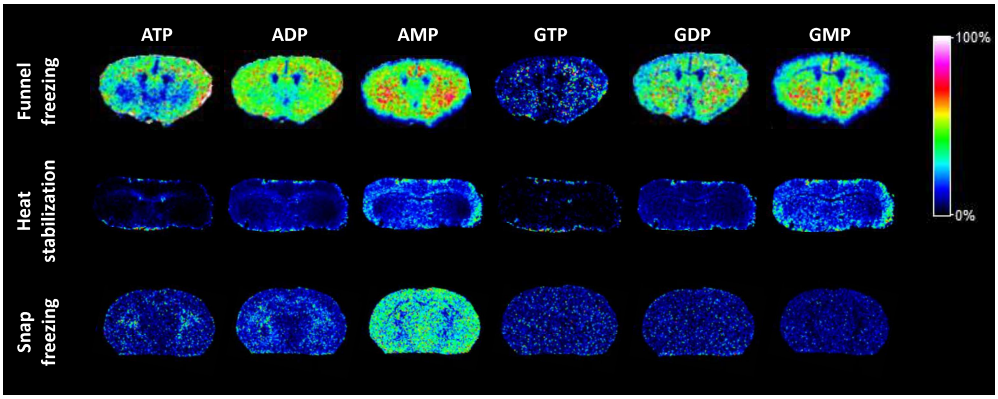
SUPPLEMENTAL MATERIAL



Supplemental Figure 1. Metabolite MSI dataset of control brain tissue after SHAM surgery showing the effect of the tissue sampling protocol on the visualization of adenosine and guanosine metabolites in mouse brain by MALDI-FTICR. Images showing the visualization of the average distribution of AMP (m/z 346.055), ADP (m/z 426.022), ATP (m/z 505.988) GMP (m/z 362.050), GDP (m/z 442.016) and GTP (m/z 521.982) for mouse brain obtained via snap-freezing, *ex vivo* heat-stabilization and *in vivo* funnel-freezing.