Evaluating Heat Stabilization During Sample Preparation For Mass Spectrometry Imaging

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Introduction

Frequently in science, maintaining a tissue sample as close to its *in vivo* state as possible is a challenge. When tissue is excised from its native environment changes such as alterations to protein post translational modifications, protein degradation (particularly post-mortem) and variations in metabolic pathways can occur within minutes of excision.[1-4] These changes may continue until enzymatic activity has ceased. Current sample preparation techniques include cocktails of enzyme inhibitors, snap-freezing in liquid nitrogen (SF), formaldehyde fixation, and focused microwave irradiation.[3-4] These techniques may not entirely halt all enzymatic activity and each posses their own limitations.[2,3,5,6] The process of heat stabilization (HS) renders enzymes permanently inactivated and **Figure 1** depicts the mechanism by which AB Denator's Stabilizor T1^{TM} functions. The sample preparation of HS was evaluated using sections of mice brain that were treated with HS, SF, or left at room temperature for 30 minutes (RT). Differences in sample treatments were assessed using IR-MALDESI Mass Spectrometry Imaging (MSI)[7,8] and a SRM assay.

How Heat Stabilization Works

The tissue is inserted into a Maintainor Card made of thin Teflon foil that allows for rapid conduction of heat. The card is evacuated to 5-10mbar and a class 1 laser measures the size of the tissue. Two aluminum blocks come into contact above and below the tissue and the sample is heated up to 95°C and an algorithm determines the length of time necessary for complete inactivation based on sample size.

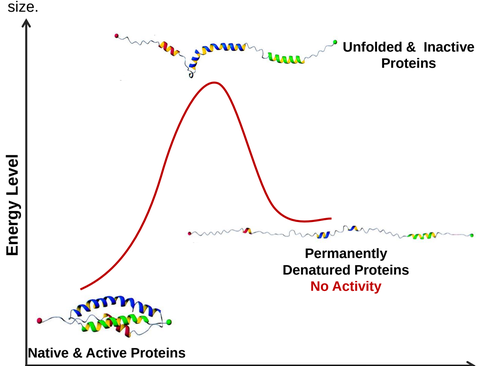

Figure 1: Enzymes are active in their native confirmations. If enough heat is applied for a sufficient length of time, the enzymes are thermally denatured, resulting in enzymes being permanently unfolded and rendered completely inactive. Figure adapted from www.denator.com and *PNAS*, **2000. 97**(12): p. 6521-6526.

MSI Experimental Design

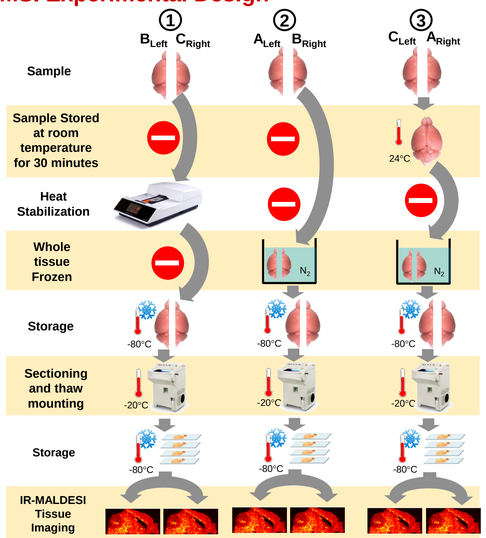

Figure 2 summarizes the MSI workflow: 3 mice were individually euthanized, brain was immediately excised and split evenly down the center. Each hemisphere received 1 of 3 treatments of HS, immediately SF or (RT) (n=6). Adjacent sections were cut at 50 µm and stored at -80°C until MSI was performed using an IR-MALDESI MSI source coupled to a Q-Exactive mass spectrometer (Thermo Scientific).

Preservation of Structures

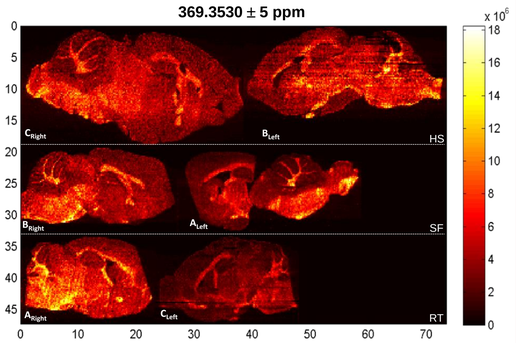

Figure 3: Putative *m/z* of cholesterol at 369.3530 ± 5 ppm [M - H_2O + H^+]$^+$, illustrates the preservation of distinct morphological features of tissues. Images generated in MSiReader and *m/z* species identified based on exact mass search in the Metlin Metabolite Mass Spectral Database.[9,10]

Lipids & Endogenous Nucleotides

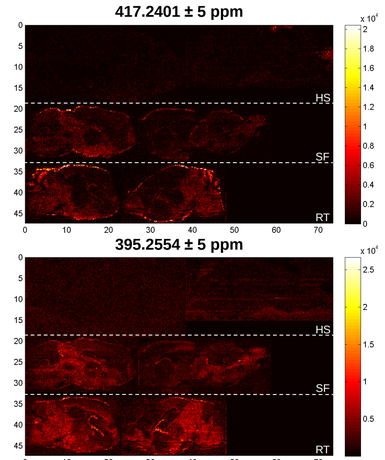

Figure 4: *m/z* 417.2401 ± 5 ppm [M+H^+]$^+$, potentially cyclic phosphatidic acid (CPA), *m/z* 395.2554 ± 5 ppm [M+H^+]$^+$ is possibly lysophosphatidic acid (LPA) or phosphatidic acid (PA). Images generated in MSiReader and *m/z* species identified based on exact mass search in the Metlin Metabolite Mass Spectral Database.[9,10]

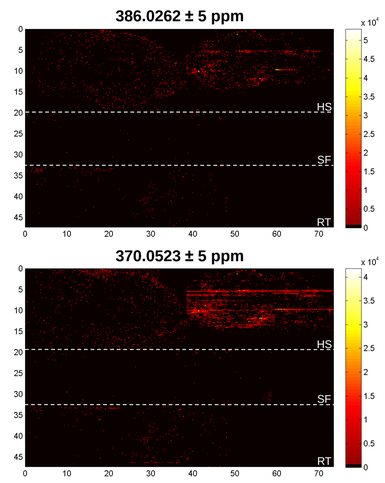

Figure 5: *m/z* of 386.0262 ± 5 ppm [M+K^+]$^+$ and 370.0523 ± 5 ppm [M+Na^+]$^+$ are predicted values for 2'-deoxyguanylate 5'-monophosphate (dGMP) or its isomer, Adenylate 5'-monophosphate (AMP). Theoretical *m/z* values calculated with Xcalibur (Thermo Scientific) software and MSiReader generated images.

Preservation of Phosphorylation States

Tenofovir diphosphate (TFVdp) and emtricitabine triphosphate (FTCtp) are the phosphorylated and active forms of the antiretroviral drugs of tenofovir (TFV) and emtricitabine (FTC). Difficulties working with these drugs arise as phosphatase enzymes in tissues can quickly remove the phosphate groups from TFVdp and FTCtp. Cervical and rectal tissues were heat stabilized at various temperatures, homogenized, and spiked with 5 ppm of TFVdp and FTCtp. The stability of TFVdp and FTCtp were monitored using LC-MS/MS and the results are shown below in **Figure 6.**

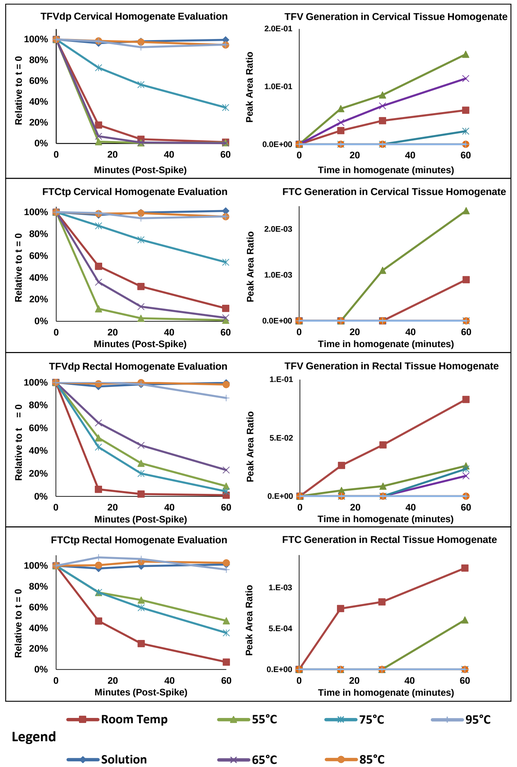

Figure 6: In the left column, the disappearance of spiked TFVdp and FTCtp for tissues heat stabilized at different temperatures was monitored. Similarly, the reformation of the parent drugs of TFV and FTC through loss of the di or tri-phosphate groups was measured in right column. Tissues heat stabilized at 85°C or 95°C preserved the phosphorylation states of TFVdp and FTCtp while lower temperatures resulted in degradation phosphorylated drugs into their parent drugs (TFV and FTC, respectively). The preservation of TFVdp and FTCtp in heat stabilized tissues at 85°C and 95°C suggested that enzyme activity had ceased. However, the drugs leaked out of the tissue and into the Maintainor card during the HS process. Because of this, HS was not a suitable sample preparation for the quantification of these small molecules.

Conclusions

- HS maintained morphological features comparable to SF or no treatment (RT group) as shown in **Figure 3**.
- CPA posses multiple cellular functions such as (but not limited to): halting cell proliferation, stopping cancer growth and metathesis, aids in forming neurons and stimulates actin stress fibers.[10-14] Comparatively, LPA also holds several functions like cell stimulation, cell signaling, platelet accumulation, smooth-muscle contraction and alterations to blood pressure. LPA has been linked to cancer development in different roles.[15-17] PA, is an important intermediate for lipid synthesis and also plays various roles through cellular signaling.[18] The absence of these biologically active molecules in the HS group and their presence in the SF and RT groups in **Figure 4** suggests enzymatic activity was halted from thermal stabilization.
- Endogenous dGMP and/or AMP were putatively observed. The phosphorylation states of the antiretroviral drugs (TFVdp and FTCtp) were preserved in tissues HS'ed at 85°C and 95°C. Lower temperature treatments resulted in a loss of phosphorylated groups, converting the active TFVdp and FTCtp drugs into the de-phosphorylated parent forms of TFV and FTC (respectively). The HS at 85°C and 95°C indicated enzymatic machinery (such as phosphatase enzymes) was de-activated.
- HS may prove to be a valuable sample preparation technique to maintain tissues close to their *in vivo* states for improved analysis.

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