High Pressure-Assisted Extraction for the Improved Proteomic Analysis of FFPE Tissue

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OVERVIEW

- Formaldehyde-induced crosslinks hampers the analysis of Formalin-fixed, paraffin-embedded (FFPE) tissue by MS
- When FFPE mouse liver was extracted using heat and elevated pressure (40,000 psi), there was a **4-fold increase in protein extraction** efficiency and up to a **30-fold increase in the number of non-redundant proteins** identified by mass spectrometry
- Heat augmented with high hydrostatic pressure (40,000 psi) improves the quality and yield of proteins extracted from archival tissue

Introduction

- Mass Spec-based proteomics hold great promise for developing knowledge of the molecular characteristics of disease. Formaldehyde-fixed, paraffin-embedded (FFPE) tissue repositories represent an invaluable resource for the retrospective study of disease progression and response to therapy.
- However, the analysis of FFPE tissues by proteomic methods has been hampered by formaldehyde-induced protein adducts and cross-links.
- Here, we demonstrate the use of heat augmented with high hydrostatic pressure (40,000 psi) as a novel method for the recovery of intact proteins from FFPE mouse liver.

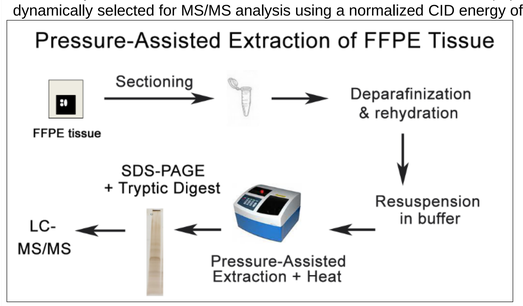
- **Advantages of FFPE Tissue for Disease Biomarker Discovery:**
  - Fresh human tissues are not readily available for proteomic analyses.
  - Results from fresh tissue cannot be directly related to the clinical course of diseases (over time).
  - **Tissue archives** contain *millions* of FFPE specimens for the analysis of large numbers of cases for which the clinical course of disease and response to treatment have been established.
- **However, analysis of archival FFPE tissues by high-throughput proteomic methods has been hampered by the adverse effects of formalin fixation.**
- **Why Use Elevated Hydrostatic Pressure to Recover Proteins from FFPE Tissue?**
  - The inability to rehydrate FFPE tissue is a **major** obstacle to the reversal of formaldehyde modifications (1).
  - Under elevated pressure, cavities in proteins become filled with water molecules, which leads to the hydration of the protein. Hydration of the buried hydrophobic residues induces protein unfolding. Our studies indicate the elevated pressure improves the recovery of proteins from FFPE tissue by hydrating and promoting solubilization of highly aggregated proteins, allowing for the subsequent reversal (by hydrolysis) of formaldehyde-induced protein adducts and cross-links (2-4).

METHODS

Preparation of FFPE Tissue: The liver from a female BALB/c mouse was given as a gift under the secondary use provision by the Department of Veterinary Pathology, Armed Forces Institute of Pathology. The liver was bifurcated with a sterile surgical scalpel and one half was immediately snap-frozen in Tissue-Tek O.C.T. compound (Sakura Finetek). The other half was fixed for 48 h at 4 °C in 10% phosphate-buffered formalin (Thermo Fisher Scientific). The formalin fixed tissue was washed for 30 minutes with distilled water and then dehydrated through a series of graded alcohols and xylenes for 1 hour each: (70%, 85%, 100%, and 100%) ethanol, and two changes of xylene. The tissue was incubated overnight at 65°C in Paraplast Plus paraffin (Thermo Fisher Scientific) before embedding.

Deparaffinization and Recovery of Proteins: 20 micron sections of the FFPE mouse liver were deparaffinized by incubating the surrogate through two changes of xylene for 10 minutes each. The surrogates were rehydrated through a series of graded alcohols for 10 minutes each: 2 changes of 100% ethanol, 85% ethanol, and 70% ethanol. The cleared surrogates were incubated in distilled water for a minimum of 30 minutes.
The rehydrated sections were resuspended in 50 mM Tris-HCl at pH 4, 7, 8, or 9 with 2% (w/v) SDS (EB1) (5) or 100 mM Tris-HCl, pH 8, 100 mM dithiothreitol (DTT), 4% SDS, (EB2) (6). The suspensions were homogenized by two 10 second cycles of sonication on ice using a Sonic Dismembrator, model 550, fitted with a 0.125 inch tapered microtip (Fisher Scientific). The homogenized tissue surrogates or FFPE liver were incubated at 100 °C for 30 minutes followed by 80°C for 2 hours under atmospheric pressure (14.7 psi) or 40,000-45,000 psi as previously described (2).

LC/MS of FFPE Mouse Liver: 40 µg of each fresh and FFPE liver extract was separated by SDS-PAGE on precast NuPAGE Bis-Tris 4–12% polyacrylamide gels using MES-SDS running buffer at pH 7.3 (Invitrogen). The gels were stained using SilverQuest silver staining (Invitrogen) Each gel lane was divided into approximately 10 bands per lane, and each band was digested overnight at 37°C using a standard in-gel tryptic digestion protocol.
The tryptic peptides were separated on an Agilent 1100 nanoflow LC system coupled on-line to a linear ion trap mass spectrometer (LTQ, ThermoElectron). The LTQ-MS was operated in a data-dependent mode where each full MS scan was followed by seven MS/MS scans in which the seven most abundant peptide molecular ions detected from the MS scan were dynamically selected for MS/MS analysis using a normalized CID energy of 35%.



The MS/MS spectra were analyzed using SEQUEST (ThermoElectron). The data was analyzed against a combined UniProt non-redundant mouse proteome database containing 36,799 protein sequences downloaded January 2010 (www.expasy.org). Only peptides with conventional tryptic termini (allowing for up to two internal missed cleavages), possessing delta-correlation scores (Cn) >0.08 and charge state-dependent cross-correlation (Xcorr) criteria as follows were considered as legitimate identifications: >1.9 for +1 charged peptides, >2.2 for +2 charged peptides, and >3.1 for +3 charged peptides. A reverse-database search was performed using the above database resulting in a calculated false-positive rate of <2%.

RESULTS

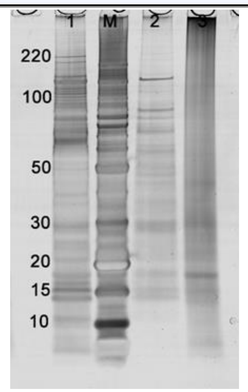


Figure 1. 1D SDS–PAGE of fresh-frozen and FFPE mouse liver extracts. Lane 1: Fresh-frozen tissue; Lane M: molecular weight marker; Lane 2: FFPE tissue extracted with heat at 40,000 psi; Lane 3: FFPE tissue extracted with heat alone. FFPE liver sections were heated in EB1.

Table 1. MS analysis for FFPE and matched fresh mouse liver

Tissue	Pressure (psi)	Extraction condition/ Buffer [a]	%Protein Extraction [c]	Unique Peptide IDs	Unique Protein IDs
Frozen,30 d	14.7	100 °C + 80 °C/ EB1	100%	9964	4581
FFPE, 30 d	14.7	100 °C + 80 °C / EB1	17%	5565	3449
FFPE, 30 d	40,000	100 °C + 80 °C/ EB1	77%	9621	5192
Frozen, 1 y	14.7	95 °C, 3 min/ EB2	100%	5872	3415
FFPE, 1 y	14.7	95 °C, 1h/ EB2	18%	107	107
FFPE, 1 y	40,000	95 °C, 1h/ EB2	79%	5180	3492

FFPE mouse liver was homogenized in extraction buffer and heated with or without elevated pressure. Fresh-frozen tissue was extracted either at atmospheric pressure using the indicated extraction condition, or on ice for 2.5 h. [a] Protocol used for protein extraction (see Material and Methods). [c]The amount of protein extracted from fresh frozen tissue was set to 100%.

RESULTS

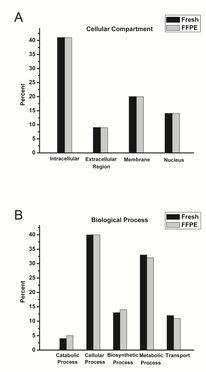


Figure 2. Gene ontology analysis of proteins identified by LC-MS/MS. Proteins identified using fresh-frozen mouse liver (black barsl) or FFPE liver extracted with heat and elevated pressure (gray bars) were categorized by subcellular localization (A) or biological process (B), using GoMiner gene ontology software.

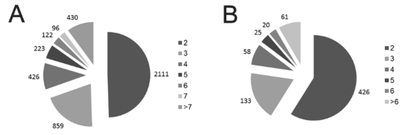


Figure 3. Total number of unique proteins identified using LC-MS/MS by two or more unique, fully tryptic peptides in FFPE mouse tissue extracted with heat and elevated pressure (40,000 psi). A) 30 day-old FFPE mouse liver. B) 1 year-old FFPE mouse liver

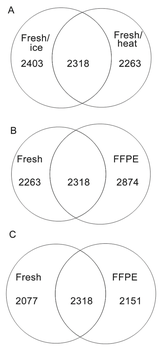


Figure 4. Venn diagrams showing the number of unique and common proteins identified using LC MS/MS analysis. Panel **A**) fresh-frozen tissue extracted on ice or with heat. Panel **B**) fresh-frozen tissue extracted with heat and FFPE mouse liver extracted in EB1 with elevated pressure and heat (40,000 psi) Panel **C)** fresh-frozen tissue extracted with heat and FFPE mouse liver extracted in EB2 with elevated pressure.

CONCLUSIONS

Recovering Stored Pathology Specimens with High Pressure + Heat:

- Improves total protein recovery by **4-fold.**
- Increases the number of unique proteins identified over samples recovered with heat alone.
- Most methods for FFPE tissue extract tryptic peptides only. Extraction of whole proteins with elevated pressure allows for validation by ELISA, Western blot, etc.

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Acknowledgement: NCI Grant R21- CA134359-01