Quantitative Proteomic Profiling of Formalin Fixed Tissue by LC-MS/MS

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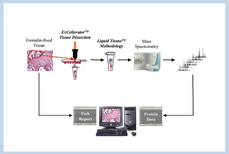
Abstract

The ability to conduct proteomic experiments for the analysis of formalin-fixed, paraffin-embedded (FFPE) archival tissue would provide the ability to examine entire collections of well-documented human and animal tissue; however, because of the inaccessible nature of proteins in FFPE tissue, conventional proteomic methodologies do not possess the means to successfully undertake such measurements. The Liquid Tissue™ methodology was developed to procure and solubilize proteins directly from FFPE tissue. Liquid Tissue™ proteins have been shown to be immunoreactive in protein dot-blot assays, and multiple studies have shown tight correlation between Liquid Tissue™ array results and immunohistochemical analysis of the same protein and tissue. The Liquid Tissue™ digestates were analyzed by microcapillary reversed-phase liquid chromatography coupled online with tandem mass spectrometry (MS/MS). Proteins were successfully identified by searching the amino acid sequence information derived from the MS/MS data against the human proteome database. The results demonstrate the ability to conduct proteomic experiments utilizing FFPE-archived tissues processed utilizing the Liquid Tissue™ methodology. Additionally, the ability to conduct quantitative proteomic measurements of the FFPE tissue digestates has been demonstrated utilizing trypsin-mediated incorporation of ^{18}O.

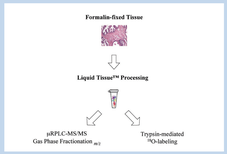
Introduction

Most pathological samples are not prepared as frozen tissues, but are routinely formalin-fixed and paraffin-embedded (FFPE) to allow for histological analysis and archival storage. FFPE is the standard method of preservation at most, if not all, medical institutions throughout the world. These samples are a vast source of human clinical material; however, standard processing methodologies limit the use of such tissue in high-throughput protein analysis applications. The fixing process required for the preservation of these samples naturally hampers the ability to extract proteins from the paraffin matrix. This fixation in turn prohibits the analysis of these proteins that may reveal potentially significant relationships between diseased state and protein presence. To overcome this limitation, we have developed a simple, efficient methodology for extraction and analysis of proteins directly from FFPE tissue. This methodology is based on a proprietary sample preparation method, which enables collection of specific histological features in FFPE tissue and preservation of proteins from that tissue for direct identification and quantitation.

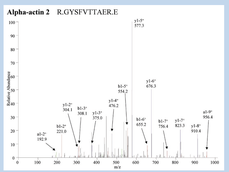
For mass spectrometric (MS) analysis, liberated proteins from the Liquid Tissue™ process are digested and the peptides analyzed by microcapillary reversed-phase liquid chromatography (μRPLC) coupled online with tandem MS (MS/MS) using either a full m/z range for precursor ion selection or fractionated in the gas phase using selective m/z ranges. In addition, quantitation can be achieved through labeling of peptides with ^{18}O. Briefly, trypsin-digested peptides from different samples are lyophilized and resuspended in either ^{16}O or ^{18}O buffer and further incubated with trypsin. As a result of product binding and two turnovers by trypsin, two mole equivalents of ^{18}O are incorporated from ^{18}O enriched solvent at the lysine/arginine carboxy-terminus of each tryptic peptide, resulting in an increase in mass by ~4 amu, allowing quantitation between two different samples by MS. These results demonstrate that the Liquid Tissue™ system not only enables the ability to identify proteins extracted from formalin-fixed tissue but also allows relative quantitation of proteins across different FFPE tissue samples.



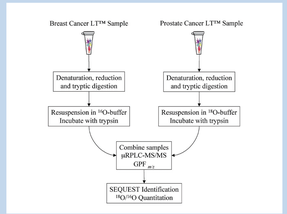
Liquid Tissue™ Technology. Isolation of proteins from formalin-fixed, paraffin-embedded tissue using the Liquid Tissue™ technology followed by μRPLC-MS/MS analysis.



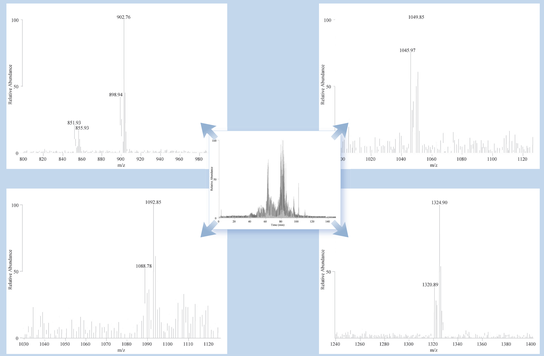
Experimental Design. Schematic of experimental design for identification of proteins in FFPE samples prepared using proprietary Liquid Tissue™ sample protocol. Ten μg each of breast and prostate cancer Liquid Tissue™ samples were desalted using C-18 ZipTip microcolumns, lyophilized to dryness, and resuspended in 0.1 % TFA.



Tandem mass spectra of identified peptides. Selected tandem mass spectra of peptides resulting from Liquid Tissue™ processed breast and prostate cancer FFPE samples.



18O Tryptic Labeling of Liquid Tissue™ Samples. Fifty μg of breast and prostate cancer Liquid Tissue™ processed FFPE samples were lyophilized to dryness. Each sample was resuspended in either ^{16}O (breast cancer) or ^{18}O (prostate cancer) water in 20% methanol. Trypsin, resuspended in the appropriately labeled water, was added to each sample at an enzyme:protein ratio of 1:50 and digested for 16 hours at 37°C. Concentrated trifluoroacetic acid was added to a final concentration of 0.4%, and the mixtures were boiled for 10 minutes. The samples were pooled and desalted using strong cation exchange chromatography. The pooled sample was lyophilized and re-suspended in 0.1% trifluoroacetic acid.



Selected mass spectra of ^{16}O / ^{18}O peptide isotopomers. Total ion chromatogram from a μRPLC-MS/MS analysis (center) and selected peptide molecular ion isotopomer pairs resulting from the ^{18}O-labeling experiment. A 4 amu mass shift (4 m/z for the $[M+H]^{+}$ peptide molecular ions) from complete incorporation of the heavy oxygen isotope at the C-terminus of the peptides is apparent.

Protein	Accession
Actin, aortic smooth muscle (Alpha-actin 2)	P03996
Alpha-actinin 1 (Alpha-actinin cytoskeletal isoform)	P12814
ALS2CR4 protein	Q96Q45
Biglycan precursor (Bone/cartilage proteoglycan I)	P21810
Collagen alpha 1(I) chain	P02452
Collagen alpha 1(V) chain	P20908
Collagen alpha 2(I) chain	P08123
Collagen alpha 3(VI) chain	P12111
Creatine kinase, B chain	P12277
Cysteine-rich protein 1	P21291
Desmin	P17661
Filamin A (Alpha-filamin)	P21333
FKSG30	Q9BYX7
Heterogeneous nuclear ribonucleoproteins A2/B1	P22626
Histone H2A	P28001
Histone H2B	P02278
Histone H4	P02304
Hypothetical protein	Q9H073
Hypothetical protein	Q9NTC3
Hypothetical protein	Q9Y427
Hypothetical protein FLJ12578 (Preprotein translocase secY subunit)	Q9H953
Hypothetical protein FLJ31053	Q96ND1
Hypothetical protein FLJ39207	Q8N8M5
Hypothetical protein FLJ40479	Q8N7Q3
JM5 protein	Q9Y484
Kinesin-like protein KIF1A (Axonal transporter of synaptic vesicles)	Q12756
Kininogen precursor (Alpha-2-thiol proteinase inhibitor)	P01042
Myosin heavy chain, nonmuscle type A	P35579
Myosin heavy chain, smooth muscle isoform	P35749
Neurolysin, mitochondrial precursor (Neurotensin endopeptidase)	Q9BYT8
Periostin precursor (PN) (Osteoblast specific factor 2)	Q15063
Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4	Q9Y3Q4
Serum albumin	P02768
Similar to KIAA0266 gene product	Q9BVJ6
Slp homolog lacking C2 domains-b (Exophilin 5)	Q8NEV8
Timeless homolog	Q9UNS1
Transgelin (Smooth muscle protein 22-alpha)	Q01995
Treacle protein (Treacher Collins syndrome protein)	Q13428
Tropomyosin 1 - alpha chain	P09493
Tubulin beta-1 chain	P07437
Type II membrane protein	Q9Y2B1
Zinc-finger protein	Q8IWR0

Proteins identified from Liquid Tissue™ processed samples. List of unique proteins and their SwissProt accession numbers identified in breast and prostate cancer FFPE tissue samples using μRPLC-MS/MS.

Protein	Average Ratio
Similar to pre-pro-megakarycyte potentiating factor	12.50
Zinc-finger helicase	7.69
Hemoglobin beta chain	2.50
Ubiquitin carboxyl-terminal hydrolase 3	2.22
T-cell lymphoma invasion and metastasis 2	2.17
Helicase B	2.13
Periostin precursor (Osteoblast specific factor 2)	2.06
Collagen alpha 1(III) chain precursor	2.00
Apolipoprotein B-100	1.85
Protocadherin gamma B3 precursor	0.42
RAS-responsive element binding protein 1	0.42
Egl nine homolog 2 (Hypoxia-inducible factor prolyl hydroxylase 1)	0.41
Transgelin (22 kDa actin-binding protein)	0.41
Calponin H1, smooth muscle	0.40
Fibronectin (Cold-insoluble globulin)	0.40
Desmin	0.40
Receptor protein-tyrosine kinase erbB-3	0.24
Taste receptor type 2 member 48	0.21
Serine-protein kinase ATM (Ataxia telangiectasia mutated)	0.10

^{16}O / ^{18}O quantitation of proteins from Liquid Tissue™ processed samples. List of select proteins identified in breast (^{16}O-labeled) and prostate (^{18}O-labeled) cancer FFPE tissue samples and their calculated isotope ratios ($^{18}O/^{16}O$).

Methods

Microcapillary LC-MS/MS Analysis. Microcapillary RPLC was performed using an Agilent 1100 capillary LC system (Agilent Technologies, Palo Alto, CA) coupled online to an ion trap (IT) MS (LCQ DecaXP, Thermo Electron, San Jose, CA) with the nanoelectrospray interface supplied by the manufacturer. Microcapillary RPLC separations of each sample were performed using 75 μm i.d. x 360 o.d. x 10 cm long fused silica capillary columns (Polymicro Technologies, Phoenix, AZ) that were slurry packed in house with 3 μm, 300 Å pore size C-18 silica-bonded stationary phase (Vydac). After injecting 1 μL of sample, the column was washed for 30 min with 98% mobile phase A (0.1% formic acid in water) at a flow rate of 0.5 μL/min. Peptides were eluted using a linear gradient of 2% mobile phase B (0.1% formic acid in acetonitrile) to 40% solvent B in 110 minutes, then to 98% B in an additional 30 minutes, all at a constant flow rate of 0.25 μL/min.

The IT mass spectrometer was operated in a data-dependent MS/MS mode in which each full MS scan was followed by three MS/MS scans, where the three most abundant peptide molecular ions were dynamically selected for collision-induced dissociation (CID) using a normalized collision energy of 38%. Dynamic exclusion was utilized to prevent redundant acquisition of peptides previously selected for MS/MS. The heated capillary temperature and electrospray voltage were set at 160 °C and 1.3 kV, respectively. Data were collected over a broad precursor ion selection scan range of 475 to 2000 m/z, followed by eight segmented precursor selection scan range fractionations in the gas phase, $GPF_{m/z}$, of increasing mass-to-charge ranges of 200 m/z intervals with overlapping m/z ranges, 400–605, 595–805, 795–1005, 995–1205, 1195–1405, 1395–1605, 1595–1805, and 1795–2000 m/z.

Bioinformatic Analysis. Tandem MS spectra from the μRPLC-MS/MS analyses were searched against the Swiss Protein human proteomic database from the European Bioinformatics Institute (http://www.ebi.ac.uk/integr8/EBI-Integr8-HomePage.do) with SEQUEST operating on an 18-node Beowulf cluster. For a peptide to be considered legitimately identified, it had to achieve stringent charge state and proteolytic cleavage-dependent cross correlation (Xcorr) scores of 1.9 for a charge state of +1, 2.2 for a charge state of +2, and 3.1 for a charge state of +3, all with delta correlation scores (ΔCn) of 0.08.

Conclusions

- Liquid Tissue™ sample preparation effectively extracts proteins from formalin-fixed, paraffin-embedded tissue, which enables μRPLC-MS/MS analysis.
- Over forty different proteins were identified by μRPLC-MS/MS using gas-phase fractionation of molecular ions in the m/z range.
- Quantitation of more than 100 peptide pairs using trypsin-mediated ^{18}O-labeling shows the ability to compare relative amounts of proteins between FFPE samples.
- These results represent significant progress in the development of a simple protocol for the extraction of proteins from formalin-fixed, paraffin-embedded tissues for proteomic analysis and eventual correlation to histological information.

Acknowledgement

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