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Research Communication

RAPID PROCESSING OF ARCHIVAL TISSUE SAMPLES FOR PROTEOMIC ANALYSIS USING PRESSURE-CYCLING TECHNOLOGY

Vinuth N. Puttamallesh[1,2], Krishna Patel[1,2] and Harsha Gowda[1,3]*

[1] *Institute of Bioinformatics, International Tech Park, Bangalore 560066, India*
[2] *Amrita School of Biotechnology, Amrita Vishwa Vidyapeetham, Kollam 690525, India*
[3] *Center for Systems Biology and Molecular Medicine, Yenepoya University, Mangalore 575018, India.*

Abstract: Advent of mass spectrometry based proteomics has revolutionized our ability to study proteins from biological specimen in a high-throughput manner. Unlike cell line based studies, biomedical research involving tissue specimen is often challenging due to limited sample availability. In addition, investigation of clinically relevant research questions often requires enormous amount of time for sample collection prospectively. Formalin fixed paraffin embedded (FFPE) archived tissue samples are a rich source of tissue specimen for biomedical research. However, there are several challenges associated with analysing FFPE samples. Protein cross-linking and degradation of proteins particularly affects proteomic analysis. We demonstrate that barocycler that uses pressure-cycling technology enables efficient protein extraction and processing of small amounts of FFPE tissue samples for proteomic analysis. We identified 3,525 proteins from six 10µm esophageal squamous cell carcinoma (ESCC) tissue sections. Barocycler allows efficient protein extraction and proteolytic digestion of proteins from FFPE tissue sections at par with conventional methods.

Key Words: Pressure cycling technology; Barocycler; Mass spectrometry.

Note: Coloured Figures and Supplementary Information available on Journal Website in "Archives" Section

Introduction

Tissue specimens are being used for more than two centuries to study physiological and pathological state of organisms. Technical advances in the field of mass spectrometry (MS) have enabled us to gain insights into protein and metabolite composition of various tissue specimens. Mass spectrometry based proteomics studies are being extensively carried out to identify potential biomarkers and therapeutic targets for various diseases including cancers. Sample availability is a limiting factor in most biomedical research studies. Sample collection itself could take a long time in biomedical research projects where they have to be prospectively collected. Formalin fixed paraffin embedded (FFPE) tissue samples serve as a rich resource considering they are archived by most hospitals. These can be goldmine for proteomics research (Longuespee *et al.*, 2014).

FFPE tissues offer advantages for researchers due to availability of large cohorts of samples with information on clinical course of the disease. However, FFPE tissue sections pose significant challenge for protein extraction due to formation of formaldehyde induced protein adducts and cross links.Several studies have tried to use heat and detergents for protein extraction from FFPE tissue samples. However, recovery of proteins and protein quality is sub-optimal. Successful use of elevated pressure or pressure cycling technology to reverse

Corresponding Author: **Harsha Gowda**
E-mail: harsha@ibioinformatics.org
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the formaldehyde induced cross linkages to improve protein recovery has been demonstrated before (Fowler *et al.*, 2010).

Pressure cycling technology (PCT) uses alternating cycles of hydrostatic high pressure and ambient pressure to rupture cells and enhances protein recovery compared to conventional methods. PCT accelerates proteolytic digestion leading to identification of higher number of peptides (Fu *et al.*, 2013). Though earlier studies have successfully extracted proteins using PCT, number of proteins identified using MS analysis remains relatively less compared to conventional methods. Hence, we set out to evaluate the utility of pressure cycling technology on FFPE tissues from esophageal squamous cell carcinoma patients coupled with ziptip fractionation to enhance proteome coverage and reduce time required for sample preparation. By using the combination of PCT, ziptip fractionation and high resolution mass spectrometry, we demonstrate a rapid sample processing work flow for archived tissue samples.

Materials and methods

Sample collection

Six FFPE tissue sections of 10µm thickness (esophageal squamous cell carcinoma) were obtained from Kidwai Memorial Institute of Oncology (KMIO), Bangalore, India.

Protein extraction using pressure cycling technology

FFPE sections were deparaffinized using 3 cycles of xylene treatment for 2 min each. FFPE sections were rehydrated by consecutive incubations in 90% and 70% ethanol followed by water for 1 minute. Rehydrated tissue sections were scraped off the glass slide and minced into small pieces using sterile blade. Around 5mg wet weight of the minced tissue was mixed with 150µl of tissue lysis buffer (4% SDS, 100mM DTT and 50mM TEABC) and transferred to PCT microtubes. Protein extraction was carried out using barocycler at 95ºC and 60 cycles of alternating pressure (1cycle= 40000psi for 50sec and 5000psi for 10sec). Protein lysate was clarified at 12000rpm for 20min and supernatant was separated. Protein concentration was estimated using bicinchoninic acid (BCA) assay.

Protein digestion and peptide fractionation

Around 250ug of protein was reduced using 10mM dithiothreitol (DTT) at 60ºC for 30 min followed by alkylation using 20mM iodoacetamide in dark for 10min. Buffer exchange of protein was carried out to remove SDS using 30kDa filters followed by proteolytic digestion using barocycler. Briefly, Lys-C was added to the protein lysate at 1:100 enzyme to substrate ratio and transferred to PCT microtubes. Total volume of lysate after addition of enzyme was maintained at 100µl and digestion was carried out at 32ºC for 45 cycles with alternating pressure (1cycle= 20000psi for 50sec and 5000psi for 10sec). Following Lys-C digestion, trypsin was added at 1:50 enzyme to substrate ratio and digestion step was repeated using barocycler. Peptides were fractionated to 6 fractions each using stage-tip based strong cation exchange (SCX) and reverse phase (SDB-RPS) method to reduce the complexity of the sample (Zhao *et al.*, 2016).

LC-MS/MS analysis

Mass spectrometry (MS) analysis was done on an Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Scientific, Bremen, Germany) interfaced with Easy-nLC II nanoflow liquid chromatography system (Thermo Scientific, Odense, Denmark). Peptides were loaded onto a trap column (75 µm x 2 cm) packed in-house with Magic C_{18} AQ (Michrom Bioresources Inc., Auburn, CA, USA). Peptides were resolved on an analytical column (75 µm x 25 cm) at a flow rate of 300nL/min using a linear gradient of 3-30% solvent B (0.1% formic acid in 95% acetonitrile) over 105 minutes and a total run time of 120 minutes. MS data was acquired at mass range of 400-1600 m/z using Orbitrap mass analyzer at a resolution of 120,000 at 400 m/z. The AGC target was set to 200,000 with ion injection time of 100ms and dynamic exclusion was set to 30 seconds. Precursor ions acquired with top speed mode were fragmented using HCD fragmentation with 35% normalized collision energy. Fragment ions were detected from 100-2000 m/z range at a resolution of 30,000 at 400 m/z. The AGC target for MS/MS scan was set to 50,000 with ion injection time of 200 ms. Internal calibration was carried out using lock mass option (m/z 445.1200025) from ambient air.

Data analysis

Mass spectrometry data was analyzed using Mascot and Sequest search algorithms against Human RefSeq75 protein database supplemented with frequently observed contaminants through the Proteome Discoverer platform (v1.4, Thermo Scientific, Bremen, Germany). Search parameters

included trypsin as the protease, precursor mass tolerance was set at 20ppm and fragment mass tolerance was set at 0.1Da. Carbamidomethylation at cysteine was set as a fixed modification and oxidation at methionine along with deamidation at asparagine and glutamine residues were set as variable modifications. MS data was simultaneously searched against a decoy database and the results were filtered using 1% FDR threshold for peptide identifications.

Data availability

Mass spectrometry data has been deposited to the Proteome Xchange Consortium via the PRIDE (Vizcaino *et al.*, 2016) partner repository with the dataset identifier PXD006255.

Results and Discussion

We evaluated the utility of pressure cycling technology (Barocycler) to extract and digest proteins from FFPE tissue sections for mass spectrometry based proteomic profiling studies. Proteins were extracted from six 10 µm tissue sections and digested using Lys-C and trypsin in a barocycler. The peptide digest was split in two halves and subjected to stage-tip based strong cation exchange (SCX) and styrene divinyl benzene-reverse phase sulfonated (SDB-RPS) chromatographic separation to make 6 fractions each. In total, 12 fractions were analyzed on Orbitrap fusion tribrid mass spectrometer. Workflow for rapid processing of FFPE tissue samples using barocycler is shown in Figure 1.

We identified 3,525 proteins out of which 2,731 proteins were identified using SDB-RPS fractionation and 2,782 proteins were identified using SCX fractionation. Representative MS/MS spectra show the quality of mass spectrometry data obtained from FFPE tissues (Figure 2). Two different fractionation methods used in this study increased the number of protein identifications by 22%. We identified 743 proteins exclusively in SDB-RPS based fractionation and 794 proteins exclusively in SCX based fractionation (Figure 3).

Previous proteomics studies on FFPE tissues have reported identificationof 500 to 3,000 proteins (Azimzadeh *et al.*, 2012; Byrum *et al.*, 2011; Takadate *et al.*, 2013; Weisser *et al.*, 2015). Our effort using PCT for protein extraction and digestion resulted in identification of 3,474 proteins which is at par with most proteomics studies done on fresh tissues or cell lines. Barocycler system that we have used in this study allows protein extraction and digestion of 12 samples in parallel. This can result in rapid processing of large number of samples while minimising variability in protein extraction across samples. PCT also helps in bringing down the time required to do proteolytic digestion to 2 hours and 15 minutes from the conventional incubation time of 12-16 hours. Using PCT for protein extraction as well as proteolytic digestion and stage tip method

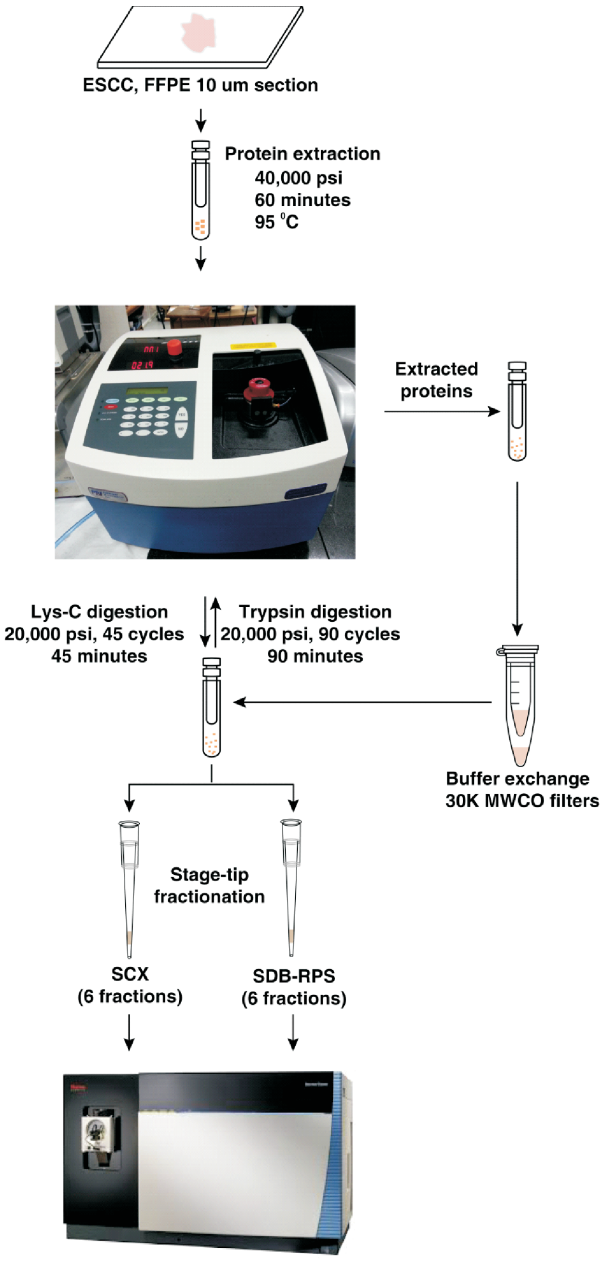

Figure 1: **Workflow for rapid processing of archival tissue samples using pressure cycling technology**

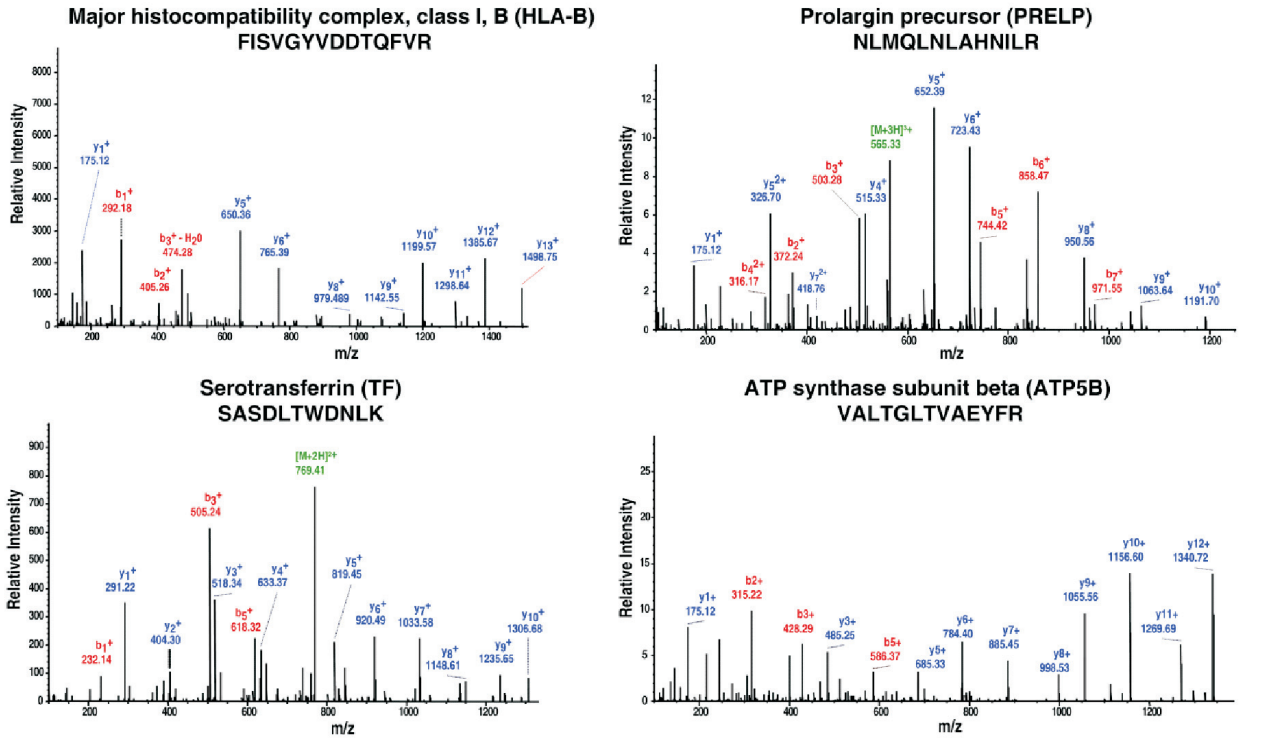

Figure 2: **Representative MS/MS spectra showing quality of mass spectrometry data from FFPE tissue samples**

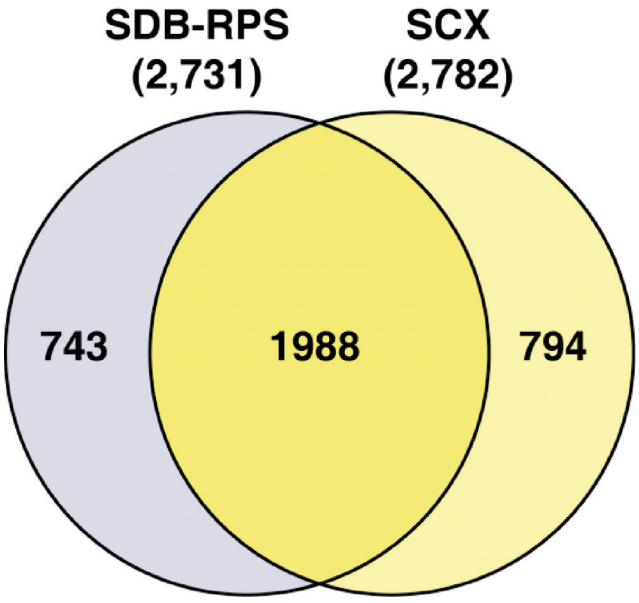

Figure 3: **Venn diagram showing proteins identified from SCX and SDB-RPS based stage-tip fractionation**

for fractionation, entire sample processing for mass spectrometry analysis can be completed in less than 6 hours.

We have demonstrated that pressure cycling technology can be successfully employed to extract proteins from small amounts of FFPE tissue specimen. As protein extraction and digestion happens in a single vial, sample loss is significantly reduced. Although we have demonstrated utility of this method to extract proteins from FFPE samples, it can also be used for protein recovery from fresh biopsy specimen where sample amounts are limited. Pressure cycling technology enables generating meaningful proteomics data from archived FFPE tissue samples that are available in most hospitals. This could serve as a huge advantage for biomarker studies where prospective sample collection can be time consuming. Coupling protein extraction and digestion with post-translational modification (PTM) enrichment methods should also enable investigation of aberrant signalling from archived tissue samples.

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Abbreviation

FFPE, Formalin fixed paraffin embedded; ESCC, Esophageal squamous cell carcinoma; MS, Mass spectrometry; PCT, Pressure cycling technology; DTT, dithiothretol; BCA, Bicinchonic acid; SCX, Strong cation exchange; SDB-RPS, styrene divinylbenzene reversed-Phase Sulfonate; FDR, False discovery rate; PRIDE, PRoteomicsIDEntifications; PTM, Post translational identification

Conflict of Interest

The authors declare no conflict of interest

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