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APPLICATION NOTE

Spatial tissue proteomics of FFPE specimen using the Bioruptor® for sample preparation

INTRODUCTION

Thanks to the excellent preservation of morphological features, formalin fixed and paraffin embedded (FFPE) tissues are an invaluable resource for spatial tissue proteomics. At the same time, due to the chemical crosslinking, it is extremely difficult to lyse such samples and solubilize proteins for deep proteomic analysis. A team of researchers from the EMBL in Heidelberg led by Martin Beck, Stephan Singer (University Hospital Heidelberg and University of Greifswald) and Alessandro Ori (Leibniz Institute on Aging – Fritz Lipmann Institute) has developed a strategy that utilizes the Bioruptor Plus in combination with heat-induced reversing of formalin fixation to enhance the efficient protein extraction from FFPE tissues [1]. This enables the ultrasensitive and highly reproducible proteomic analysis of microdissected FFPE specimen with high spatial resolution.

MATERIAL REQUIRED

- 5-10 µm thick FFPE tissue sections mounted on PEN-membrane coated slides (Carl Zeiss MicroImaging GmbH, Bernried, Germany)
- Bioruptor Plus (Diagenode)
- Laser capture microdissection (LCM) system (e.g., Carl Zeiss MicroImaging GmbH)
- PCR machine (e.g., BioRad C1000 Touch™ Thermal Cycler)
- Bench top centrifuge (e.g Eppendorf Centrifuge 5804R)
- Thermomixer (e.g., Eppendorf ThermoMixer C)
- Sera-Mag Speed Beads (#45152105050250 and #65152105050250, Thermo Scientific)
- Magnetic rack
- Buffers:
    * Solubilization buffer: 80mM HEPES (#H3375-100G, Sigma) pH 8.0, 80µM DTT (#6908.3, Roth), 4% (w/v) SDS (#75746-250G, Sigma)
    * 200mM iodoacetamide (# I1149-5G, Sigma) in water, made just before use, protect from light
    * Digestion buffer for SP3: 4M Urea in 100mM HEPES pH 8.5
    * Digestion buffer for acetone precipitation: 3M Urea in 100mM HEPES pH 8.0
- LysC (#125-05061, Wako)

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- Trypsin (#V5111, Promega)
- Cresyl violet acetate
- DMSO
- Xylenes (#534056-4L, Sigma)
- Ethanol (#1.00983.2500, Merck)
- Acetonitrile (#0001204102BS, Biosolve)
- Formic acid (#00069141A8BS, Biosolve)
- Acetone (#0001037801BS, Biosolve)
- TFA (#0020234131BS, Biosolve)
- MilliQ water
- Peptide desalting material (e.g., Waters Oasis HLB µElution Plate 30µm, #186001828BA)

PROCEDURE

1. Deparaffinization and tissue staining for LCM samples:

- Incubate 2x in xylenes for 2 min
- Incubate 2x in 100% ethanol
- Wash in 90% (v/v) ethanol, 70% (v/v) ethanol, 50% (v/v) ethanol
- Stain for 15 seconds in cresyl violet acetate 1% (w/v) in ethanol
- Wash in 50% (v/v) ethanol, 70% (v/v) ethanol, 100% (v/v) ethanol
- Incubate in xylenes for 5 minutes

2. Laser Microdissection and tissue solubilization

- Use the LCM system to extract the tissue region of interest (see results section below for guidance on choosing the right area to obtain optimal amounts of peptide material)
- Place the tissue in a PCR tube containing 100 µl of Solubilization buffer
- Sonicate sample using Bioruptor Plus for 22.5 minutes (15 cycles: 1 min on, 30 sec off) at the highest settings and temperature set at 20°C
- Boil sample for 1h at 99°C in PCR machine with heated lid
- Repeat the sonication and boiling step for a total of two times

***Note**: The same solubilization procedure can also be applied to FFPE samples without LCM. See for example [2]*

3. Cysteine reduction and alkylation:

- Add 200 mM iodoacetamide to a final concentration of 15 mM and incubate for 30 min at room temperature in the dark
- Quench the reaction by adding 10 µl of 200 mM DTT

4. Detergent removal and protein digestion:

a. Using the SP3 approach [1]:

- Add 4 µl of magnetic beads stock (1:1, 40µg/µl). For magnetic beads stock preparation, see [1]
- Add 100% acetonitrile to the final concentration of 50% and incubate for 8 min at RT to allow proteins binding to the beads
- Place sample on the magnetic rack, remove the supernatant and wash the beads 2x with 70% (v/v) ethanol and once with 100% acetonitrile
- Carefully remove supernatant and air-dry beads for 1 minute
- Resuspend beads in 6µl of digestion buffer and add 1µl of LysC stock solution (0.1µg/µl in milliQ water)
- Sonicate samples using Bioruptor Plus for 4.5 minutes (3 cycles: 1 min on, 30 sec off) at the highest settings and temperature set at 20°C, incubate 5 min at 37°C, mix by pipetting and allow the reaction to proceed for 4h at 37°C
- Dilute the urea to the final concentration of 1.5M and add 1µl trypsin stock solution (1µg/µl in trypsin reconstitution buffer) and incubate for 16h at 37°C
- Add 100% acetonitrile to the final concentration of 95% (v/v) and incubate for 8 minutes at RT to allow peptide binding to the beads
- Place sample on the magnetic rack, remove the supernatant and wash the beads twice with 100% acetonitrile
- Carefully remove supernatant and air-dry beads for 1 minute
- Resuspend the beads in 9µl of 2% DMSO and sonicate using Bioruptor Plus for 4.5 minutes (3 cycles: 1 min on, 30 sec off) at the highest settings and temperature set at 20°C
- Place sample on magnetic rack, recover supernatant (containing digested peptides) and transfer it to a new tube containing 1µl of 1% (v/v) formic acid, mix by pipetting (some precipitation may occur)
- Spin for 5 min at max speed and collect the supernatant directly for LC-MS/MS analysis or for further processing (e.g., TMT labeling). For TMT labeling procedure see [1]

b. Using cold acetone precipitation (developed by Dr. Joanna Kirkpatrick, Core Facility Proteomics, Leibniz Institute on Aging – Fritz Lipmann Institute [2]):

- Add 4x volume of ice cold Acetone and freeze overnight at -20°C
- Centrifuge at 20800x g, at 4°C for 30 min
- Remove and discard supernatant. Wash with 500µl of ice cold 80% (v/v) acetone
- Centrifuge at 20800x g, at 4°C for 10 min
- Remove and discard supernatant. Wash with 500µl of ice cold 80% (v/v) acetone
- Centrifuge at 20800x g, at 4 °C for 2 min
- Air-dry protein pellet by keeping the tube open on the bench for a few minutes
- Resuspend the pellet in 40µl of Digestion buffer
- Add 1µl LysC (0.5µg/µl in milliQ water) and incubate for 4h at 37°C in a thermomixer with shaking at 600rpm
- Add 40µl milliQ water, add 1µl of trypsin (0.5µg/µl in trypsin buffer) and incubate for 16h at 37°C
- Acidify samples with 7.4µl of 10% TFA
- Desalt digested peptide using standard kits following manufacturer instructions, and analyse directly by LC-MS/MS or for further processing (e.g., TMT labeling)

RESULTS

The above-described procedure enables efficient extraction of proteins for LC-MS/MS analysis from FFPE tissues (Figure 1A). In our experience, approx. 40mm^2 of a 10µm thick tissue slide (approx. volume of 400nl) typically yield >=10µg of final digested peptides. However, it should be kept in mind that extraction efficiency and protein yield might vary between tissue types, and even among samples from the same tissue, due to, e.g. different fixation conditions. The described protocol is compatible with both label-free quantification by Data Independent Acquisition (DIA, also known as SWATH-MS), as well as quantification based on isobaric tagging with Tandem Mass Tags (TMT). In our experience, TMT-based quantification typically coupled to off-line peptide fractionation enables deeper analysis in terms of quantified protein groups [1] (Figure 1B), while DIA requires less starting material (down to 1-2µg) and is applicable to larger sample cohorts [2]. The procedure yields reproducible quantitative proteomics data, as demonstrated by the independent analysis of samples microdissected from consecutive slides of the same tissue block (Figure 1C).

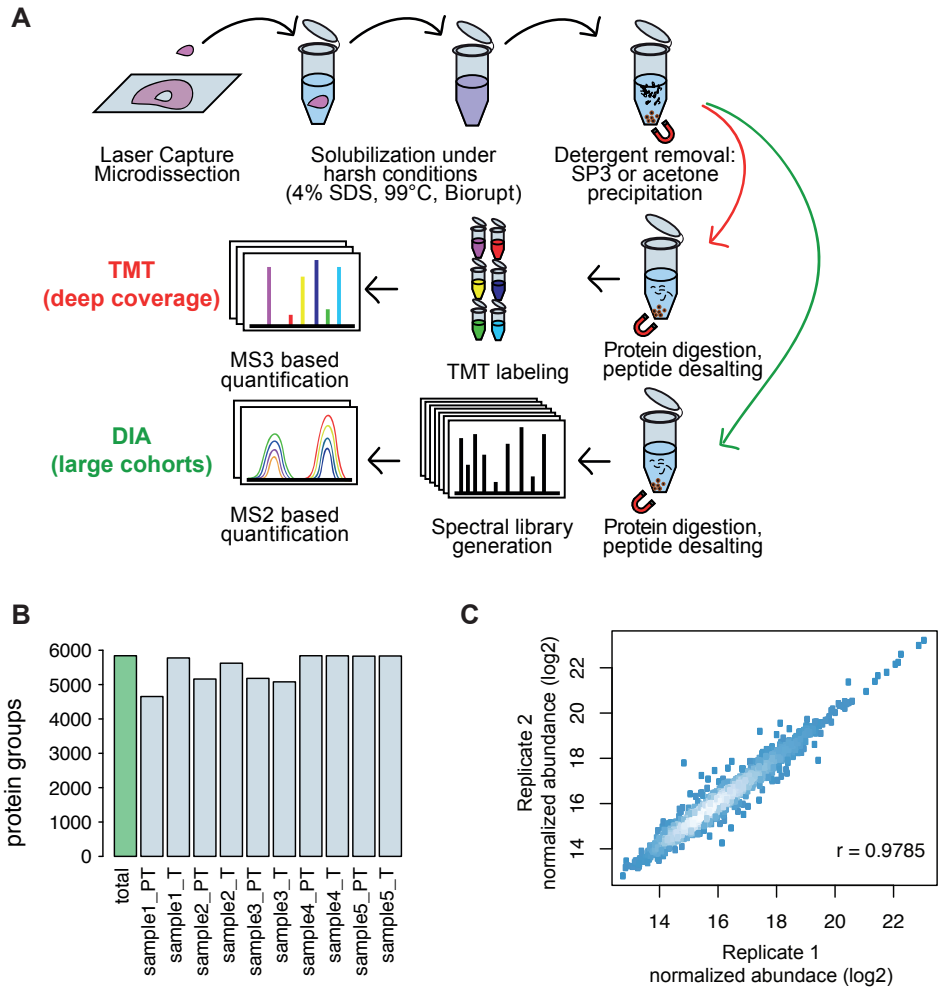

Figure 1

A. *Workflow for the proteomic analysis of microdissected FFPE specimens by quantitative mass spectrometry. (**B.**) Statistics of identified protein groups in a typical TMT10plex experiment. Tumoral (T) and peritumoral non-neoplastic tissue (PT) were microdissected from 5 hepatocellular carcinoma specimens, processed according to the TMT workflow illustrated in (**A.**), and analysed by tandem mass spectrometry following off-line high-pH reverse chromatography (16 fractions). The number of protein groups that were quantified by at least two proteotypic peptides is shown for individual samples. The green bar indicates the total number of quantified protein groups. (**C.**) Reproducible protein quantification in two samples that were independently microdissected from consecutive slices of the same tumor specimen. Data shown in (**B.**) and (**C.**) are from Buczak et al. 2018.*

REFERENCES

[1.] Buczak K, Ori A, Kirkpatrick JM, Holzer K, Dauch D, Roessler S, et al. Spatial tissue proteomics quantifies inter- and intra-tumor heterogeneity in hepatocellular carcinoma. Mol. Cell. Proteomics. 2018;mcp.RA117.000189.

[2.] Heinze I, Bens M, Calzia E, Holtze S, Dakhovnik O, Sahm A, et al. Species comparison of liver proteomes reveals links to naked mole-rat longevity and human aging. bioRxiv. 2017;1–36.