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Profiling the tumor microenvironment proteome in prostate cancer using laser capture microdissection coupled to LC–MS—A technical report

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ABSTRACT

Laser capture microdissection (LCM) allows microscopic procurement of specific cell types from tissue sections. Here, we present an optimized workflow for coupling LCM to LC–MS/MS including: sectioning of tissue, a standard LCM workflow, protein digestion and advanced LC–MS/MS. Soluble proteins extracted from benign epithelial cells, their associated stroma, tumor epithelial cells and their associated stromal cells from a single patient tissue sample were digested and profiled using advanced LC–MS/MS. The correlation between technical replicates was $R^2 = 0.99$ with a mean % CV of $9.55\% \pm 8.73$. The correlation between sample replicates was $R^2 = 0.97$ with a mean % CV of $13.83\% \pm 10.17$. This represents a robust, systematic approach for profiling of the tumor microenvironment using LCM coupled to label-free LC–MS/MS.

1. Introduction

A workflow using laser capture microdissection (LCM) that would allow for both targeted and unbiased proteomic profiling of specific target cells in tissue (that may also include, for example, immuno-MRM) could be invaluable to several experimental and clinical fields. Since its establishment, LCM has predominantly been coupled with genomic and transcriptomic analysis for large-scale studies, whereas proteomic analysis has largely lagged behind in this area due to the limited amount of sample routinely acquired using LCM. Today, while some may still argue that LCM is too challenging and labor intensive for the resulting low protein yields, the sensitivity of mass spectrometers has increased exponentially in the last number of years allowing analysis of scarce protein samples and even single cell analysis [1] as well as global proteome mapping [2]. Therefore it is now reasonable to perform large-scale LCM using limited sample amounts for global proteome analysis to complement those that are routinely performed using genomics and transcriptomic technologies. Several laboratories have studied differential protein expression in microdissected tumor tissue specimens in an effort to discover novel tumor markers [3–5]. However, the semi-quantitative approaches used in these studies may have limited the number of potential markers identified as well as the reliability of protein quantification. In order to minimize technical variations and improve reliability of protein quantification, a variety of sophisticated stable isotope labeling techniques have been developed for MS-based proteomics including chemical, metabolic, and enzymatic labeling techniques. Isotope-coded affinity tags (ICAT), isobaric tags for relative and absolute quantification (iTRAQ) and O^{18} labeling coupled with mass spectrometry provide a means of post-harvest protein labeling for protein quantification whereby relative protein expression levels are determined by the ratio of the ion intensities of the isotopically labeled peptide pairs and have successfully been applied to LCM material [6–10]. However, such labeling strategies require a relatively large amount of sample (100 μg), which requires enormous amounts of sectioned tissue for LCM not to mention the vast amount of LCM time. In addition such strategies require extensive sample handling and manipulation

Abbreviations: LCM, Laser capture microdissection; LC–MS/MS, Liquid chromatography tandem mass spectrometry.

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that can increase sample loss and contamination. Similarly, for label-free approaches in particular where peptide abundance information is critical for comparative proteome analysis, it is imperative that sample handling and manipulation be kept to a minimum. Moreover, while these efforts demonstrate significant promise, their scale is modest and undertaking larger scale analysis of individual patient tissue samples remains a formidable challenge [11].

This paper describes a robust systematic approach to coupling LCM with advanced LC–MS/MS using a telepathology approach for the proteomic profiling of the tumor microenvironment (Fig. 1). LCM requires accurate identification of the cells to be targeted and hence the pathologist has a central role in LCM-based experiments. As such, the limiting factor in LCM is generally the availability of an expert pathologist to guide the tissue micro-dissection. The telepathology approach ensures that pathological evaluation is central to the identification and annotation of the correct target cells for downstream proteomic analysis as well as recording any morphological changes as sequential sections are cut through the tissue (Fig. 1). The use of short-range separation allows for the concentration of low protein quantities into a single gel plug for digestion, helps minimize protein loss by minimal sample handling and manipulation and facilitates the removal of SDS for subsequent MS analysis.

In order to establish the effects of protein concentration for sufficient protein identifications, increasing protein yields were concentrated using short range SDS-PAGE gel electrophoresis and subjected to LC–MS/MS. Fig. 2 shows the separation of 0.5 μg-15 μg of crude prostate tissue protein lysate separated based on molecular weight using a 6% SDS-PAGE gel (Fig. 2A) followed by Coomassie Blue staining. As shown by the graph in Fig. 2B, shorter separation resulted in no significant increase in the number of proteins identified from 2 μg as to 4 μg. Furthermore, loading greater than 2 μg runs the risk of causing blockages in the column. Therefore, it is preferable, and indeed feasible, to aim to obtain good protein identifications with only 2 μg total protein. In order to demonstrate the feasibility of the approach LCM analysis of discrete regions within prostate tissue was conducted. For LCM 12 tissue sections from a single patient specimen were used in order to harvest benign epithelium, its associated stroma and tumor epithelium and its associated stroma using our systematic workflow. Each step upstream and downstream of the LCM procedure, from tissue preservation to the planning of LCM sessions, is crucial to ensure accurate cell population accrual. Using digital annotation software with a rigorous annotation system allows for pre-planned LCM sessions as well as real-time viewing of annotated images ensuring that the correct cells (and regions) are acquired for downstream analysis. For this reason the "telepathology" approach was chosen; whereby top, middle and tail sections were brought forward for pathological review as shown in Fig. 1, thus ensuring documentation of changes in tissue morphology as the tissue was sectioned through, and also allowing digital pathological annotation through Spectrum software. Online viewing of annotated slides allows planning of LCM sessions as well as real time viewing of annotated images while performing LCM.

The overall aim of this work was to assess the optimsed LCM-proteomics workflow for the proteomic profiling of laser captured microdissected material. To achieve this, three technical replicates and four sample replicates were profiled using label-free nLC–MS/

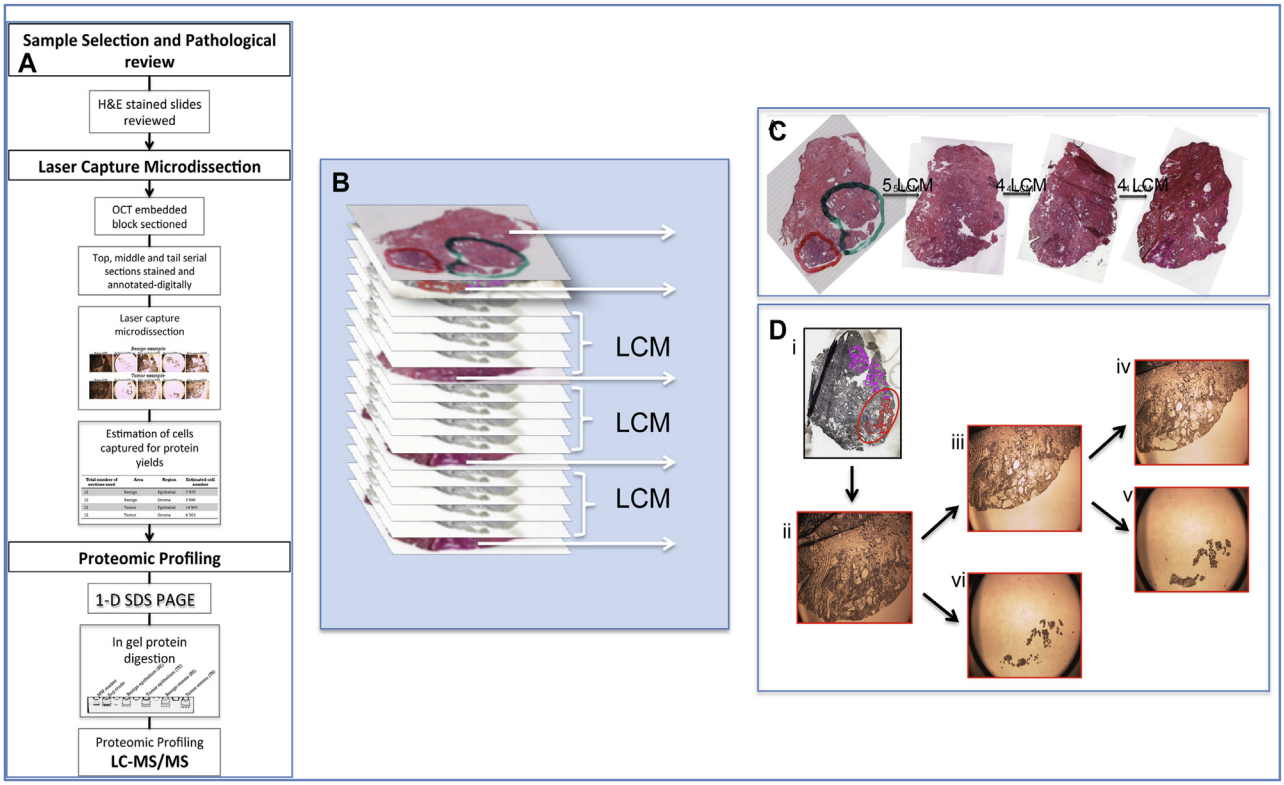

Fig. 1. Systematic workflow for the coupling of LCM to advanced LC–MS. Fig.1(A) shows a schematic illustration of the optimized workflow from sample selection and pathology review, using annotated images for correct cellular accrual to proteomic profiling using short range SDS-PAGE and LC–MS. Fig. 1B, C and D illustrate the telapathology approach implemented as part of the optimized workflow. Fig. 1(C) shows serial H&E stained sections taken from a patient sample. Panel A shows the first H&E section taken at the Dana Faber and posted to UCD. Panel B shows the sixth H&E cut section taken at St. James' Hospital. Panel C and D show the eleventh and sixteenth sections, respectively. Fig. 1(D) depicts the LCM of tumor epithelium and associated stroma from one cut section. The annotated cresyl violet-stained section is shown in D(i), before LCM is shown in D(ii), tumor epithelial cells after LCM are shown in D(iii) and associated stroma are shown in D(iv). Laser captured tumor epithelial cells are shown in D (v) and captured associated stroma are shown in D(vi).

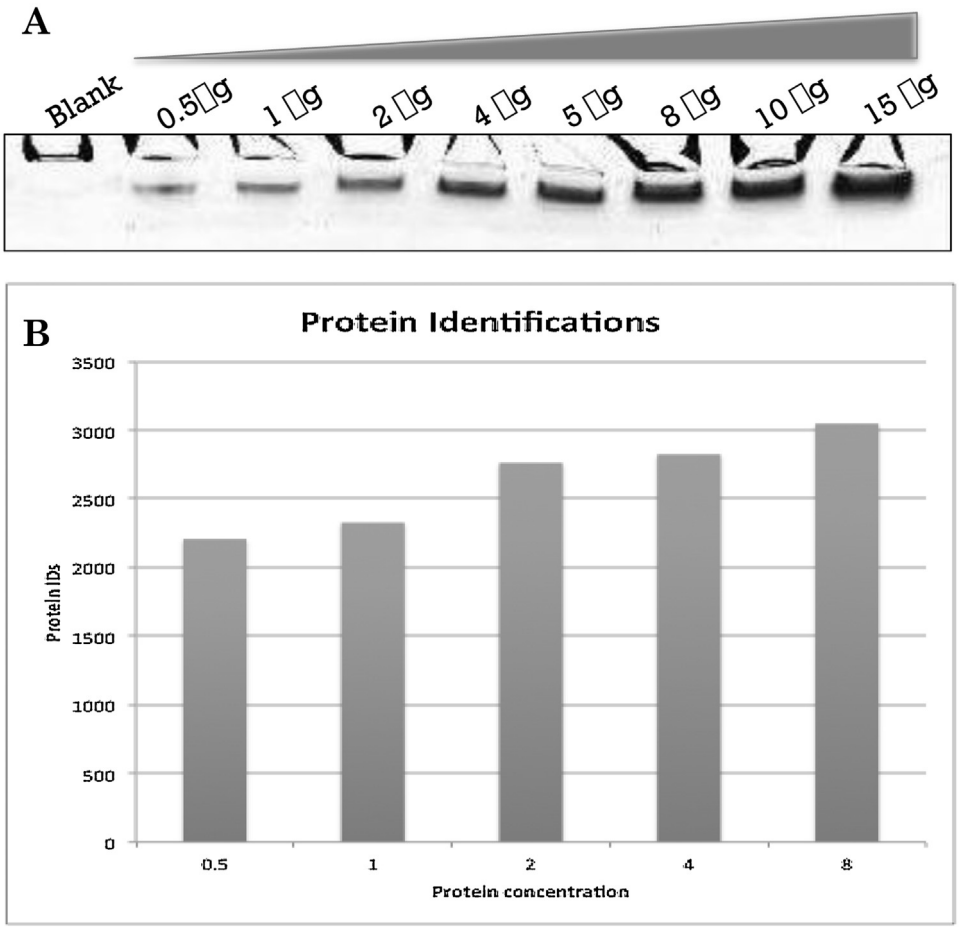

Fig. 2. SDS-PAGE approach for sample concentration and protein identifications. Panel (A) shows polyacrylamide gel (6%) was used to separate 0.5 μg–15 μg of crude prostate lysate. Panel (B) represents bar chart of total protein digested as compared to number of proteins identified using LC–MS.

MS alongside microdissected samples. Samples were normalized by the densitometry of the Coomassie-stained concentrated protein bands prior to tryptic digestion, Technical replicates were analysed at the start, middle and end of the label-free experiment and sample replicates were prepared individually and analysed throughout the experiment. The current high resolution MS instrumentation allows accurate and in depth analysis of the proteome, where careful experimental design as well as attention to detail at every stage from sample preparation, protein digestion and mass spectrometric analysis plays a role in the total number of proteins identified reproducibly and accurately during label-free proteomic profiling. In total over 2000 proteins were identified from LCM material (FDR <1%). Technical replicates ($n = 3$) showed an average Pearson correlation of 0.99% in the proteins identified (Fig. 3B). Moreover sample replicate ($n = 4$) correlation showed strong technical reproducibility with an average Pearson correlation of 0.97 (Fig. 3A). The technical and sample variance, as measured by % CV ± standard deviation across three technical replicates and four sample replicates are plotted against their average abundance on the log 10 scale for all identified peptides (FDR > 0.01%). Fig. 3C and 3D represents the % CV versus peptide abundance for technical and sample replicates respectively. The graphs show excellent reproducibility with % CV <15% clearly demonstrating the reproducibility of the methodology. This demonstrates the overall feasibility of proteomic profiling of limited quantities of protein, routinely acquired using LCM, from multiple patient samples. In conclusion, the rigorous approach described here which includes a telepathology approach as well as standardized protocols for protein digestion, MS experimental design and data acquisition provides a platform for reproducible protein identification and quantification of laser captured microdissected material.

2. Materials and methods

2.1. Tissue samples

Tissue specimens were collected from patients who had consented to have clinical data collected prospectively and to provide all prostate tissue obtained during biopsy and surgery. A single prostate tissue specimen was obtained from the Irish Prostate Cancer Research Consortium Tissue Bank and selected based upon pathological review. Snap frozen tissue was mounted on a tissue holder with the assistance of Tissue-Tek O.C.T (Sakura, SAK 4583) and fresh frozen tissue sections were cut onto individual glass slides; a single tissue section (5 μm thick) were stained with hematoxylin and eosin (H and E) for pathological review.

2.1.1. Protein preparation and short range SDS-PAGE

For the preparation of protein extracts, the tissue specimen was ground, in the presence of liquid nitrogen, into a fine powder using pestle and mortar. Protein powder was suspended in 1 ML of lysis buffer (20 mM Tris, 7 M Urea, 2 M Tiourea, 10 mg/ml DTT). In addition, buffer was supplemented with a protease inhibitor cocktail (0.2 mM pefabloc, 1.4 mM pepstatin, 0.15 mM aprotinin, 0.3 mM E-64, 1 mM leupetin, 0.5 mM soybean trypsin inhibitor and 1 mM EDTA) to avoid proteolytic degradation of proteins. Soluable middle layer proteins were extracted following centrifugation at

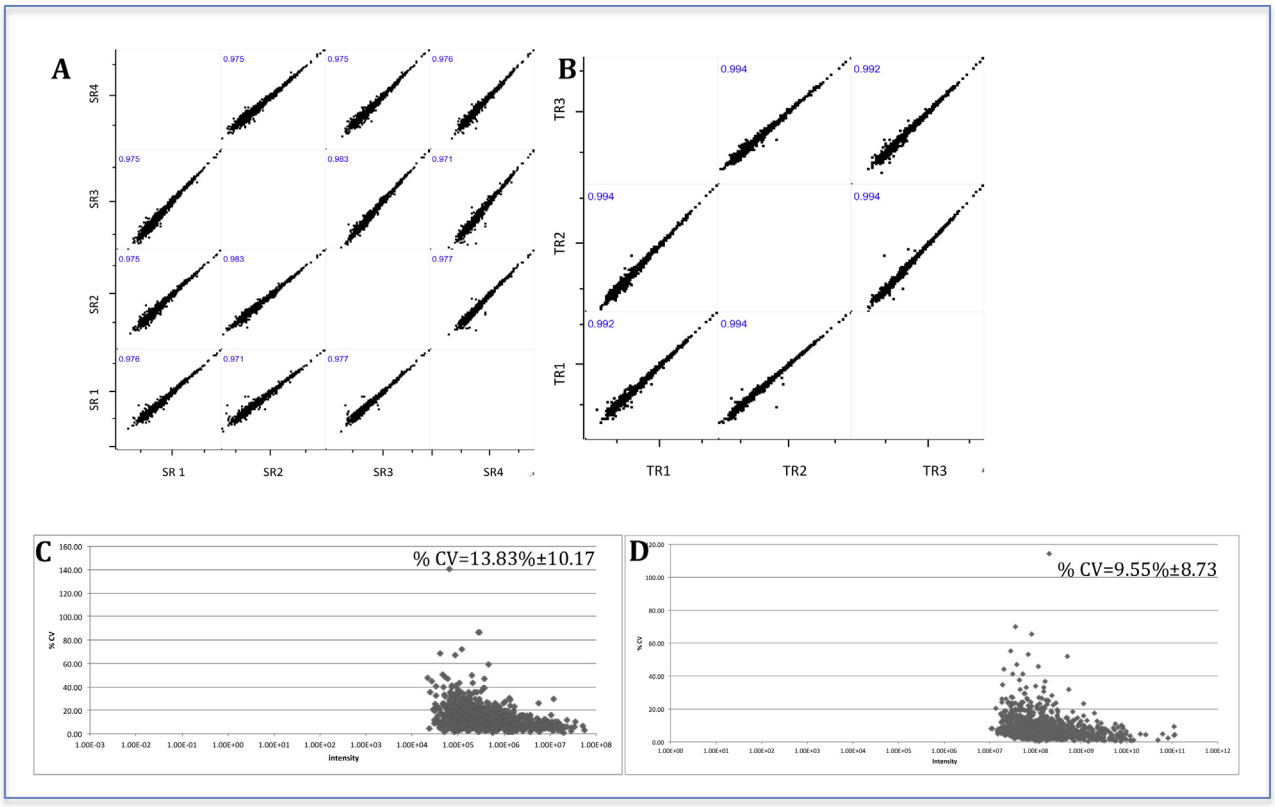

Fig. 3. Technical and sample reproducibility of protein identifications and peptide abundance by label-free LC–MS.Pearson correlation plot of protein identifications for sample (A) and (B) technical replicates. Variance as measured by% CV ± standard deviation across four sample replicates (C) and three technical replicates (D), are plotted against their average peptide abundance on the log 10 scale.

4 °C for 20 min at 20,000 × *g*. The resulting protein extract was placed in Lo-Bind 0.5 ml tubes (Sigma, Z666491) for subsequent short range SDS-PAGE on 6% polyacrylamide gels [12] and electrophoresis was performed at 80 V for 20 min or until the tracking dye fully entered the top of the resolving gel.

2.1.2. Protein digestion and LC–MS/MS

Concentrated protein bands were excised, washed and digested according to an optimized method [13]. Trypsin-generated peptides were dried by vacuum centrifugation and the peptide fractions were resuspended and prepared for LC–MS/MS analysis using C18 Stage tips according to Rappsilber et al. [14]. Digested samples were analysed on a Thermo Scientific Q-Exactive mass spectrometer coupled to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Each sample was loaded onto a Biobasic Picotip Emitter (120 mm length, 75 μm ID) column packed with Reprocil Pur C18 (1.9 μm) reverse phase media and peptides separated by an increasing acetonitrile gradient over 120 min at a flow rate of 250 nL/min using buffer A (97% H_2O, 2.5% acetonitrile, 0.5% acetic acid) and buffer B (97% acetonitrile, 2.5% H_2O, 0.5% acetic acid). From 0–16 min the sample was loaded on to the column at 500 nl/min, from 16 to 17 min buffer B increased from 1 to 2% and the flow rate decreased from 500 to 250 nl/min, from 17 to 123 min buffer B increased from 2 to 27% at a flow rate of 250 nl/min. From 123–124 min buffer B increased from 27 to 90% and the flow rate increased from 250 to 500 nl/min. From 124 to 130 min buffer B remained at 90% and a flow rate of 500 nl/min. From 130 to 131 min buffer B decreased from 90 to 1% at a flow rate of 500 nl/min. From 131 to 132 min buffer B remained at 1% at a flow rate of 500 nl/min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 220 °C, and with a potential of 2300 V applied to the frit. All data was acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution (70,000) MS scan (300–1600 m/z) was performed to select the 12 most intense ions prior to MS/MS analysis using HCD. Raw data was de novo sequenced and searched against the Homo Sapiens subset of the Uniprot database (2014_11 version) using the search engine PEAKS Studio 6 (version 6) with the following parameters applied: enzyme: trypsin, up to two missed cleavages, fixed modifications: carbimidomethylated cysteine, variable modifications: oxidation methionine, precursor ion tolerance: 10 ppm, product ion tolerance: 0.3 Da and maximum variable post translational modifications per peptide: 3 with a false discovery rate (FDR) of ≤1%, average local confidence (ALC) of ≥65%, a total local confidence of (TLC) of ≥6, and peptide score (−10l gP) of ≥15. Subsequently, the raw data flies were processed through MaxQuant (V.1.2.7.4) software with the same parameter settings as Peaks Studio 6 and including: peptide and protein false discover rate were set to 0.1%, unique and razor peptides were set at 1 for protein identifications. The Label free Quantification (LFQ) values were generated with a minimum of 2 peptides required per protein.

Conflict of interest

The authors declare that they have no conflicts of interest to report.

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