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TECH NOTE

Proteomic analysis from formalin-fixed paraffin embedded tumour samples using an Aurora Ultimate column.

Vo Van Anh Pham, Matthew J. McKay, Mark P. Molloy
Bowel Cancer and Biomarker Laboratory, Kolling Institute, The University of Sydney

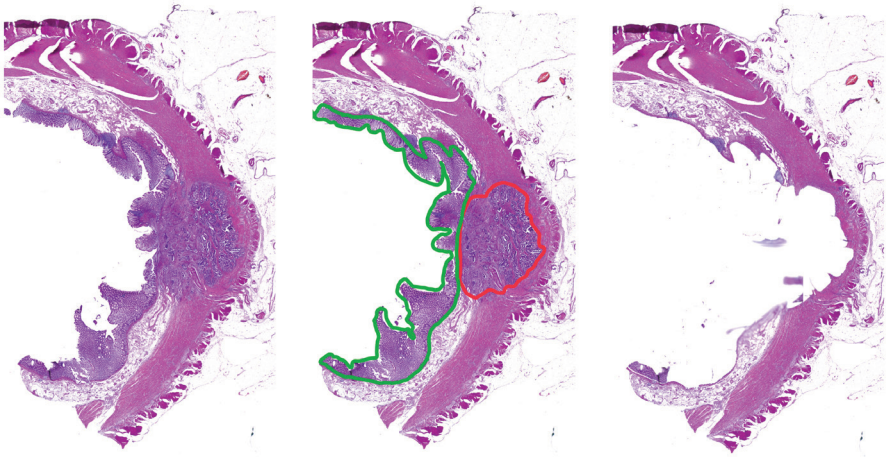
INTRODUCTION

Clinical tissues are often processed into formalin-fixed paraffin embedded blocks which can be sectioned for histopathology. These archived samples are very stable and when matched with mature clinical data represent an opportunity for biomarker discovery. Proteomic methods have been developed to extract peptides from FFPE blocks and utilise LC-MS/MS for analysis. We coupled laser microdissection to specifically excise tumour regions and normal mucosa of FFPE colorectal cancer specimens prior to LC-MS/MS analysis.

We assessed the performance of IonOpticks Aurora Ultimate column using FFPE extracted cancer samples and HeLa cell lysates. Laser microdissected samples were isolated, processed and digested with trypsin. Samples were acquired using Data-Independent Acquisition (DIA) mode on a HF-X Orbitrap mass spectrometer. The data was processed using DIA-NN 1.9.2. From this analysis, 6,000 unique proteins and 74,000 peptides were identified from a single 1µg HeLa injection. This was achieved whilst retaining low CVs, 7-second FWHM peak widths and stable retention times.

The combination of the IonOpticks Aurora Ultimate column with the Q-Exactive HF-X mass spectrometer was able to identify a broad array of peptides and proteins from a variety of different tissue, with consistent results to allow for a confident identification of markers associated with cancer biology.

Representative images of H&E stained colorectal tumour showing spatial enrichment using Laser Microdissection (LMD).



(1) Prior to dissection (2) Areas of interest annotated (3) Tissues dissected

Figure 1: (1) Patient sample prior to dissection under the LMD. (2) Patient sample with tumour regions (red) and adjacent normal tissues (green) annotated to be microdissected. (3) Patient sample after all tissues of interest were dissected and collected into a 0.6 mL tube cap, with muscular/ adipose tissues remaining in the section. Magnification 2.5x.

METHODS

Laser microdissected samples were captured with cut areas of approximately 100 × 10^6 µm2 for ~ 20µg of protein from rectal cancer and normal adjacent samples (Figure 1). Digested samples were purified using StageTips, then reconstituted in 0.1% FA.

Samples were injected using a Thermo Fisher Scientific Dionex Ultimate 3000, with a flow rate of 400 nL/ min. The LC was connected to an IonOpticks Aurora Ultimate column (25 cm x 75 µm). Acquisition was performed using a Thermo Fisher Q-Exactive HF-X hybrid quadrupole-Orbitrap MS with a 140-minute LC run time. The nanospray source was in positive mode.

Desalted peptides were loaded onto the column with buffers concentration at stepped gradients, started with buffer B (80% ACN, 0.1% FA) at 2% and buffer A (0.1% FA) at 98%, and ended with buffer B at 95% and buffer A at 5%.

The wide window data-independent acquisition (DIA) data was acquired with one full scan spectrum, followed by 50×25 m/z DIA windows followed by another full scan then 50×25 m/z windows with each m/z window overlapping by 50% with the first cycle resulting in a total of 101 m/z windows. Full scan spectra were acquired at 30 000 resolution, with a scan range of 395-1005 m/z. The automatic gain control (AGC) target was at 3×106 with a maximum injection time of 55ms.

All full scan spectra were acquired in centroid mode. Each DIA window was acquired using a resolution of 15 000 with an isolation width of 12 m/z, scanning through the mass range 395-1005 m/z. The AGC target was at 1×106 with a maximum injection time of 25ms. The normalised collision energy (NCE) was at to 27, and the default charge state was set to +2. All DIA spectra were acquired in centroid mode.

Data was analysed by DIA-NN 1.9.2, which uses neural networks to identify and quantify peptides to 1% FDR using a decoy database. The database used to predict peptides and generate a spectral library was the human protein FASTA database generated in October 2023 from UniProt which contained 20 434 entries.

RESULTS

Unique peptide identifications across three sample types

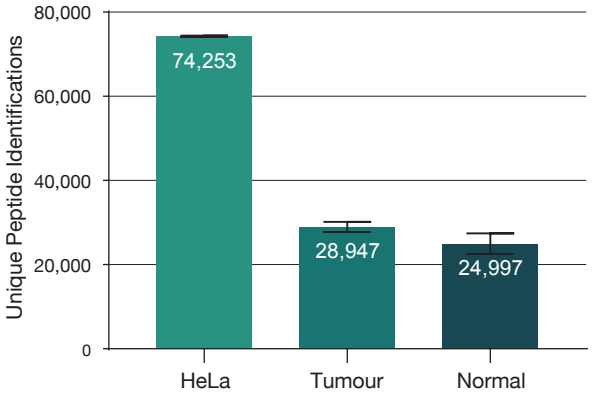


Figure 2: 1µg of HeLa tryptic digest was used (n = 4). For laser microdissected samples, both primary colorectal tumour and adjacent normal mucosa were collected (n = 3 specimens). Average Full Width at Half Maximum (FWHM) for all identified peptides was 7 seconds. Samples were run on a Thermo Fisher Scientific Dionex Ultimate 3000 and a Thermo Fisher Q-Exactive HF-X hybrid quadrupole-Orbitrap MS with the IonOpticks Aurora Ultimate (25 cm x 75 µm), on a 140-minute LC run time and a flow rate of 400 nL/ min. Data analysed using DIA-NN 1.9.2.

Unique protein identifications across three sample types

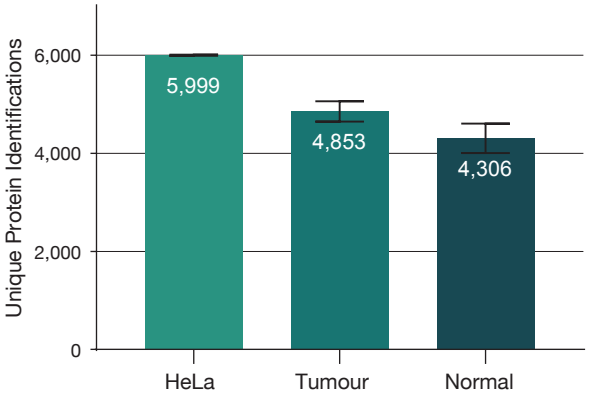


Figure 3: 1µg of HeLa tryptic digest was used (n = 4). For laser microdissected samples, both primary colorectal tumour (n = 3) and adjacent normal tissues (n = 3) were collected. Samples were run on a Thermo Fisher Scientific Dionex Ultimate 3000 and a Thermo Fisher Q-Exactive HF-X hybrid quadrupole-Orbitrap MS with the IonOpticks Aurora Ultimate (25 cm x 75 µm), on a 140-minute LC run time and a flow rate of 400 nL/ min. Data analysed using DIA-NN 1.9.2.

Coefficient of Variation across sample types

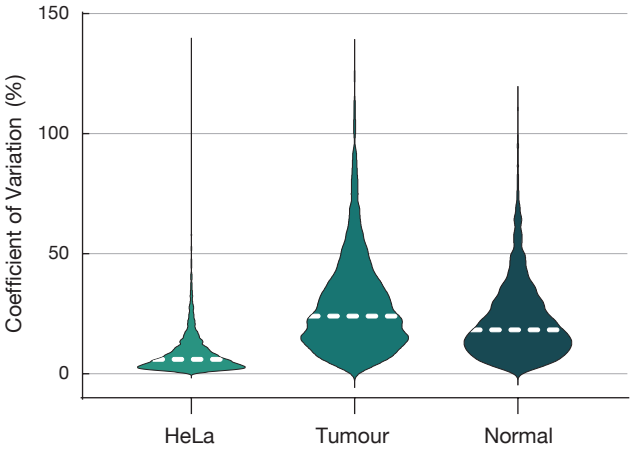


Figure 4: Violin plot of Coefficient of Variation (CV) from HeLa and colorectal tumour samples, of proteins identified in all samples. 1µg of HeLa tryptic digest was used (n = 4) (median CV = 8.06%). For laser microdissected samples, both primary rectal tumour (n = 3 specimens) (median CV = 24.1%) and their corresponding adjacent normal tissues (n = 3 specimens) (median CV = 19.25%) were collected. Samples were run on a Thermo Fisher Scientific Dionex Ultimate 3000 and a Thermo Fisher Q-Exactive HF-X hybrid quadrupole-Orbitrap MS with the IonOpticks Aurora Ultimate (25 cm x 75 µm), on a 140-minute LC run time. Dashed lines represent median value for each sample. Data analysed using DIA-NN 1.9.2.

Differentially expressed proteins

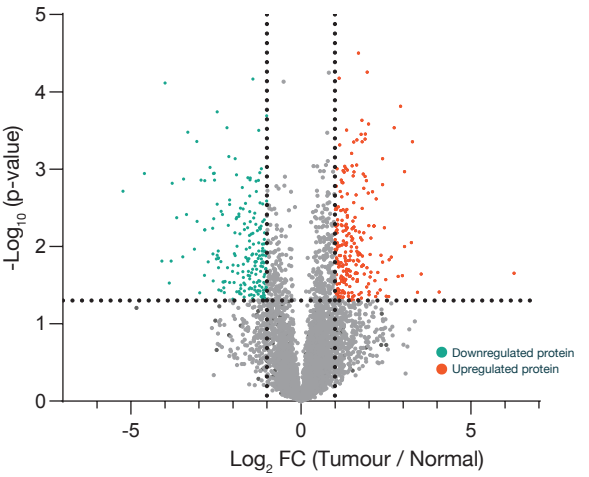


Figure 5: Volcano plot showing differentially expressed proteins between tumour and adjacent normal tissue samples of early-stage colorectal cancer. Differentially expressed proteins, indicated by green and orange dots, were identified as proteins with a p-value <0.05 and Log2 FC >1. Vertical dotted lines indicate the Log2 FC cut-off at ±1. Horizontal dotted line indicates the -Log10(p-value) cut-off at 1.3. Statistics conducted using Student's t-test, p-value < 0.05 considered significant.

CONCLUSION

The deep proteome coverage and high sensitivity of IonOpticks' Aurora Ultimate column with high resolution LC-MS/MS in data-independent acquisition mode was successfully demonstrated using proteins extracted from FFPE archival colorectal cancer biospecimens.

FEATURED PRODUCT

Aurora Ultimate 25x75 packed emitter column
Part No. AUR3-25075C18A (25 cm x 75µm ID, 1.7µm C18)