HIGH THROUGHPUT MULTI-OMIC ANALYSES AT THE SINGLE CELL LEVEL USING ANALYTICAL SCALE CHROMATOGRAPHY WITH A MULTI-REFLECTING HIGH RESOLUTION MASS SPECTROMETER

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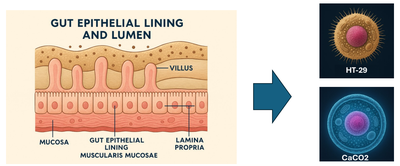
INTRODUCTION

Single cell lipidomics is becoming increasingly important and a popular approach within the clinical and biomedical fields, enabling the discovery of cell-to-cell lipidome heterogeneity that are often masked in bulk analyses. However, the low level of lipids and polar metabolites present in an individual cell, require an optimised analytical workflow with high levels of sensitivity, robustness and reproducibility. HT-29 and CaCO2 cell lines are commonly used cell lines for gastrointestinal, cancer and drug research (e.g., adsorption studies). Here, we use an LC-MS approach, configuring standard flow LC (2.1 mm column geometry) with a highly sensitive multi-reflecting time-of-flight (TOF) instrument for discovery lipidomic and metabolomic profiling of the HT-29 and CaCO2 cell lines.



CONCLUSION

- **An LC-MS workflow using conventional analytical chromatography (2.1 mm) scale has been demonstrated for single cell multi-OMIC analyses. This provides a high throughput solution for single cell analysis (~6 mins injection-injection).**
- **The high sensitivity and mass accuracy of the Xevo MRT P10, provides >400 lipid identifications at high confidence.**
- **Utilizing the CSH Phenyl-Hexyl column provides multiple benefits:**
  - **Elimination of IPA from the mobile phase eliminates contamination peaks and thereby significantly reduces the background intensity.**
  - **Configuring the LC with the Phenyl-Hexyl column and corresponding mobile phases, allows for a multi-OMIC workflow (i.e.,lipidomics and polar metabolomics) using the same mobile phases.**
- **The Lipostar data processing pipeline provides high confident identifications/curation with the rules-based approach used for database searching. Data is readily exported during data acquisition into mzML formats, providing ultimate flexibility for third party informatics.**

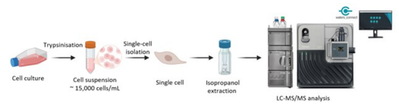
References

1. SWATH-MS/MS and DIA-MS: MS-DIAL data independent MS/MS deconvolution for comprehensive metabolome analysis. Nature Methods, 2015, 12, 523-526.
2. Development of a single mobile phase for LC-IM-MS-based discovery lipidomics and metabolic phenotyping: Application to methapyrilene hepatoxicity in the rat. J Chromatogr A., 2024, 1714:464552.

RESULTS

HT-29 and Caco-2 cells were prepared as described below and individual single cells picked using the isoPick platform. Following the LC-MS data acquisition, mzML-based datasets (ESI+/-) were processed using LipoStar2 informatics for peak picking, normalization and feature identification. Resulting raw data clearly showed that no contamination was present prior to analysis and that no carry over was observed during subsequent analyses (Figure 1). Data relating to the polar metabolite extracts were acquired using the same LC-MS configuration and the data processed using MS-Dial [1] with identifications derived from a .msp consisting of authentic standards (Figure 2). Many single cell lipidomic studies have involved the use of capillary scale chromatography, which involve longer gradients and issues can arise from the associated backpressure, particularly with isopropanol (IPA). The implementation of 2.1 mm chromatography allows for high throughput analyses, whilst making the adoption of the methodology more feasible for many more labs. Utilization of the phenyl-hexyl chemistry allows for multi-OMIC analyses to be conducted without the need for changing mobile phases [2], providing a single workflow solution for lipidomics and polar metabolomics. Statistical analysis and data curation were performed for both datasets (Figure 3). Multi-variate statistical analysis allowed for differential features to be established, prior to database searching using a combination of LipidMaps and an in-house database for comprehensive coverage. Exceptional dynamic range of the MS allowed over 5-orders of in-sample dynamic range to be achieved for a single cell extract (Figure 4). This combined with high levels of sensitivity provided over 400 lipid identifications and > 300 metabolites from a single cell using analytical flow LC (Figure 5). The stringency applied to the data curation is exemplified with the extracted ion chromatograms (XIC's) and quality of the MS/MS spectra. In general, the primary lipid class present in abundance were triglycerides (TAG's) and phospholipids (Figure 6).

1. Experimental Workflow



- *Cultured cell lines consisting of HT-29 and CaCO2 were prepared. In short, cells were cultured in flasks using DMEM at 37°C with 5% CO_2 and passaged every 2-3 days or reaching 80% confluency. Culturing lasted for a total of 21 days. Prior to sampling, the media was replaced with serum-free DMEM and washed with DPBS. Cells were then incubated with TryLE at 37°C for 5-10 mins until detachment was observed. Tryspinization was quenched and further diluted to a concentration of 15,000 cells/mL.*
- *Single cells were picked using the isoPick platform (IotaSciences, UK) and placed into LC-MS vials prior to extraction with IPA.*
- *The extracts were injected on-column and separated using a 6.0 min active gradient. MS data were collected using the Xevo MRT P10 mass spectrometer using DDA and DIA approaches.*
- *Data were automatically exported during data acquisition as mzML for subsequent data processing.*

2. Data

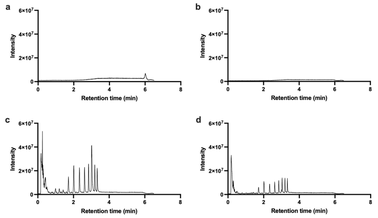
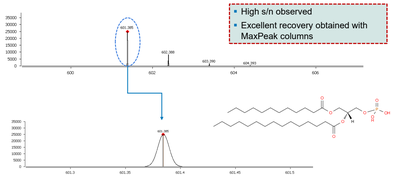


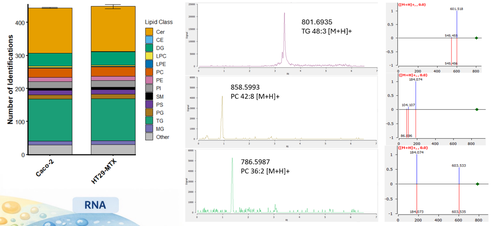
Figure 1 - Total Ion Chromatograms (TIC) covering the length of the short gradient with the phenyl-hexyl chemistry. This TIC's consist of solvent blank injections prior to single cell analyses (a) in addition to a blank injection post analyses (b), indicating no presence of carryover following the analysis of the lipid extracts. Single cell extracts for Caco-2 (c) and HT-29 (d) highlight significantly more abundant lipid intensities for the Caco-2 when compared with HT-29.



The CSH Phenyl Hexyl RP column with MaxPeak technology provides multiple benefits for lipidomic analyses:

- Non-specific binding, which is typically observed with stainless steel components is eliminated with the coating.

- This is particularly the case for phosphate and carboxylate- containing lipids. The example provided above relates to a phosphatidic acid (PA) lipid, which shows excellent recovery and high s/n.



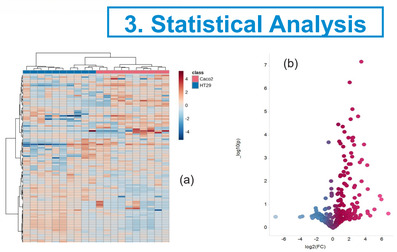


Figure 3 - Hierarchical clustering (a) of the normalized intensities (lipid-based analyses) for the single cell replicates for both cell lines. These are based on the top 100 features with Ward clustering and Euclidean Distance. A large proportion of the features which show differential expression between the cell lines are related to triglycerides, followed by a variety of phospholipids and ceramides. These data are also displayed as a volcano plot (b) based on fold change vs. significance.

3. Statistical Analysis

Figure 5 - Number of curated lipid identifications from a single cell extract presented for Caco-2 and HT29 (left), which covers a broad coverage of lipid classes. Both cell lines provide >400 lipid ID's. Extracted ion chromatograms and associated spectra (right) show the level of confidence assigned to the ID's. A minimum s/n of 3 is assigned with high quality MS/MS spectra.

4. Processed Data

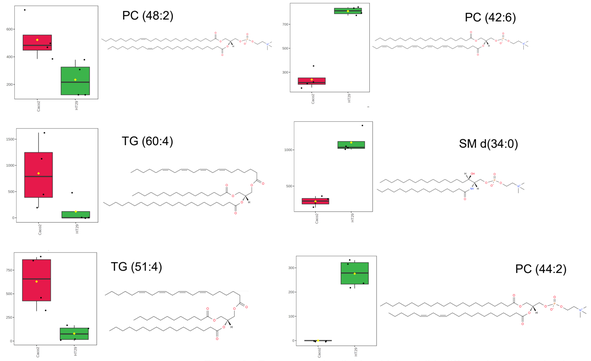
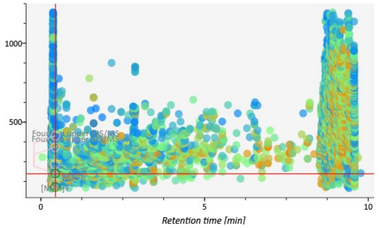
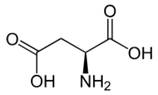


Figure 6 - Representative lipids which exhibit differential expression between both cell lines. The bulk of the lipids identified were triglycerides or phospholipid in nature. It is evident that no particular lipid class dominates in terms of expression but there are clear differences between the various sub-classes which do present themselves as dysregulated.





Aspartic acid

Figure 2 - Metabolomic extract processed via MS-Dial. The Peak Spot Viewer (above) shows all potential features peak picked and tentatively identified as metabolite compounds. These were further curated to provide >300 highly curated compounds. One example shown is aspartic acid (m/z 134). Various amino acids have been identified as being present for various biological roles in colon cancer cell lines such as CaCO2 and HT-29.

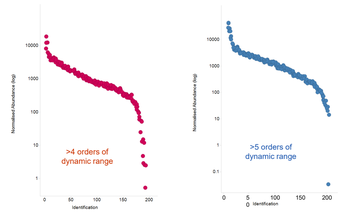


Figure 4 - In-sample dynamic range of curated lipid identifications from single cell extracts of CaCO2 (red) and HT-29 (blue) are shown, exhibiting dynamic ranges of up to more than 5-orders. Further demonstrating the exceptional dynamic range of the Xevo MRT P10 with the potential to gain >5 orders of dynamic range for experimental datasets.

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