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LABEL FREE MOLECULAR IMAGING OF TUMOUR SECTIONS FOR TWO AND THREE DIMENSIONAL TISSUE CLASSIFICATION AND PATHWAY MAPPING

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SUMMARY

- **Mass spectrometry imaging can provide in-depth chemical information directly from tissue sections.**
- **Here, desorption electrospray ionisation (DESI) is used to map endogenous metabolites and phospholipids all within a single experiment without the need for any labelling apporaches.**
- **We use the information to study the heterogeneity in the metabolism pathways and can also use the cell specific lipid signatures to classify the various regions of tissue to aid research in this area.**

INTRODUCTION

Desorption electrospray ionisation mass spectrometry is a surface analysis technique that can rapidly analyse the lipid and metabolite composition of a tissue section. By moving the tissue under the analysis probe (Figure 1), a chemical map of the sample can be generated. As has been shown[1,2], each tissue type or disease state has its own characteristic molecular fingerprint, therefore, using histologically annotated databases of the molecular profiles, these maps can be classified using machine learning approaches. Alternatively, targeted analyses can be carried out to study metabolic pathways, where the response from molecules of interest are visualised [3,4].

This label free method requires no sample preparation other than the sectioning of the tissue. With increased throughput due to increases in the number of pixels that can be acquired per second and automation, analysing multiple sections within a single run is now possible, opening up the study of these systems in three dimensions through serial sections.

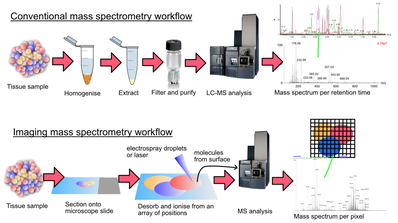


Figure 2. In imaging mass spectrometry molecules are desorbed and ionised directly from the sample surface, yielding a mass spectrum at each point. By preserving the spatial information it can provide complimentary data to the more traditional mass spectrometry approaches.

DESORPTION ELECTROSPRY IONISATION MASS SPECTROMETRY IMAGING

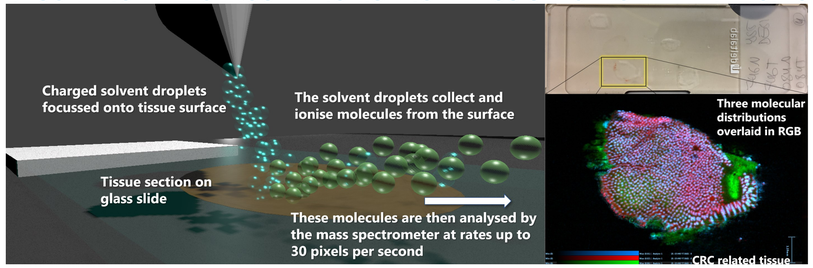


Figure 1: The DESI-MS technique requires no sample preparation other than sectioning of the tissue onto a glass slide in the typical manner. A gas flow driven stream of charged droplets impact upon the surface, collecting and ionising molecules from the surface to be analysed by the mass spectrometer. By moving the sample under the droplet stream a chemical image of the tissue can be created.

COREGISTERING H&E AND MASS SPECTROMETRY DATA

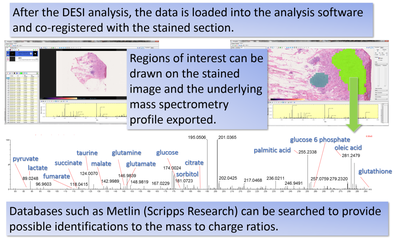


Figure 3: One method of analysing the data is to draw regions of interest on a co-registered stained section to obtain chemical profiles specific to that cell type. Online databases allow for accurate mass searching to provide identities to the ion signals.

METHODS

Analysis: Experiments were performed on a Xevo-G2-XS Q-TOF (Waters, UK) fitted with a 2D DESI stage (Prosolia, Indianapolis,USA). Snap frozen tissue, collected as part of ongoing studies, were sectioned at 20µm onto conventional glass slides. The slides were stored at –80°C until required for analysis.

MS imaging data was viewed and analysed with High Definition Imaging 1.4 (Waters, UK) and SCiLS Lab Software (SCiLS, Bremen, Germany). Further processing and rendering was performed using MATLAB (The MathsWorks).

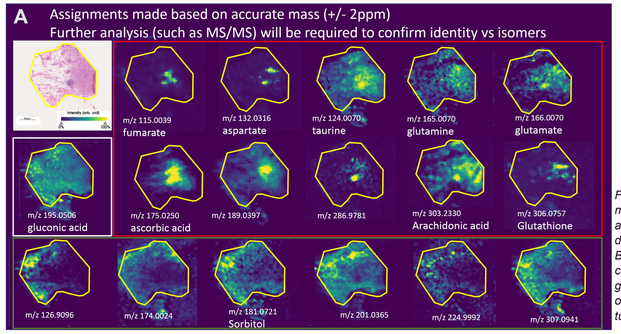
Glioma xenograft: $5x10^6$ U87 cells were injected intradermally into the flank of a mouse, and grown for 4 weeks. Animals were culled and tumours flash frozen in isopentane. Animal experiments were conducted in accordance with the U.K. Animals (Scientific Procedures) Act 1986 and were approved by local ethical review committees.

Spheroids: Wells of a 96-well plate were coated with 50 µl of warm agar (Sigma-Aldrich, UK) (1.5% in sterile water) and left to set. 4000 HCT-116 cells were plated in each well. Cells were grown for 14 days in RPMI media (supplemented with 10% (v/v) FCS, 1% (v/v) L-glutamine and 1% penicillin-streptomycin), and incubated at 37˚C and 5% CO_2.

MAPPING HETEROGENOUS METABOLISM IN BREAST TUMOURS

Understanding the metabolic differences that occur within cancer cells, and between cells in different states, is a central part of understanding the functioning and progression of tumours. With imaging mass spectrometry many of the molecules of interest can be measured in parallel, allowing a snapshot into the processes on-going in different parts of the same tissue.

Here, a section from an invasive ductal carcinoma has been imaged by DESI-MS and the data aligned with the H&E image (Figure 3). The distribution of known molecules of interest such as lactate and pyruvate can now be mapped (Figure 4B) or alternatively regions of interest can be drawn and statistical approaches applied to determine which molecules are responsible for any differentiation in chemistry. Figure 6 shows the use of SCiLS lab software to compare the sensitivity and specificity of each molecular species as a differentiator of the (in this case) two different tissue types. These Receiver Operating Characteristic (ROC) scores highlight which ion species are responsible for the differentiation of the tissue regions (see Figure 4A), whilst accurate mass searches on metabolite databases such as Metlin allow tentative assignments to be made.



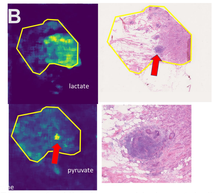


Figure 4: Histology guided metabolite mapping within breast tissue, in 4B lactate and pyruvate are shown to have very different distributions in this tumour tissue. By identifying the differentiating compounds molecule maps can be generated (4A) that show the complexity of the metabolic activity in heterogenous tumours.

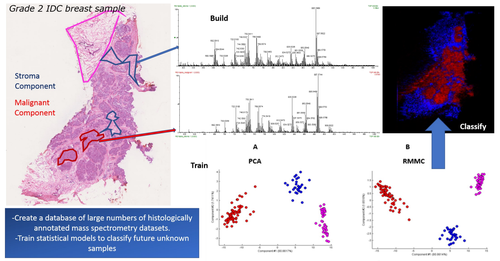


Figure 5: The workflow for creating an automated tissue identification platform. Annotations of tissue types are made on the stained sample and the 'ass spectrometry 'fingerprint' of that tissue exported to a database. Once the statistical models are built, they can be used to classify the pixels of unknown tissues, providing an automated method of tissue identification.

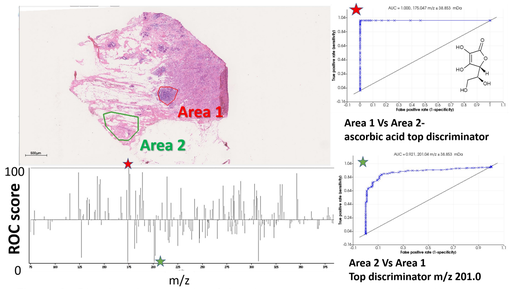


Figure 6: Directing the analysis of imaging mass spectrometry data with histology. Mass spectrometry data is extracted and compared from different regions of the tissue. The ions identified as significant can then be mapped as shown below in Figure 4.

TISSUE CLASSIFICATION

Within the same experiment as the lower molecular weight metabolites from the previous section, the DESI-MS experiment also provides strong signal within the phospholipid region of the spectrum. The pattern of these phospholipids can be used as a molecular fingerprint for different tissue types (see Figure 5). With sufficient data from well annotated tissues, a statistically robust model can be built.

Once the model has been built and cross validated, this can then potentially be applied as a classification tool for unknown tissues. There are a large selection of machine learning approaches that can be applied, and it is on going work to determine which is most suitable or whether each disease state may have an optimum data pipeline.

In Figure 5 we see that the different tissue regions yield similar but significantly different profiles in the lipid region, which are clearly separated by both the principal component analysis (PCA) and recursive maximum margin criterion (RMMC).

EXTENDING THE APPROACH INTO THREE DIMENSIONS

To fully understand the metabolic relationship between the various regions of a heterogenous tumour, it would be beneficial to create a three dimensional map. This would also allow the *ex vivo* mass spectrometry studies to better align with the *in vivo* imaging modalities.

Whilst 3D mass spectrometry imaging has been reported for over a decade[5], it is only with the combination of increased acquisition speeds and automation with regards to slide loading that such experiments have become feasible.

Figure 7 demonstrates this approach on a mouse xenograft glioma model, whilst in Figure 8 a single 1.5 mm spheroid is imaged at a voxel size of 40x40x40µm.

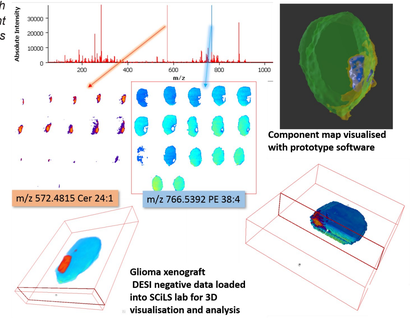


Figure 7: Studying tumours in three dimensions, here seventeen serial sections were analysed through a glioma mouse xenograft and the chemically different regions identified and plotted. An area of hypoxia is chemically distinguished within the reconstructions.

DISCUSSION

- **Imaging MS for label free mapping of metabolism and lipid synthesis in tumours is a promising approach.**
- **For a full understanding, a wider molecular coverage may be needed, work is on going to understand how a greater depth of information can be obtained.**
- **Currently, the technique can only provide semi-quantitative information, though methodologies to provide a greater degree of molecular quantitation are being developed.**

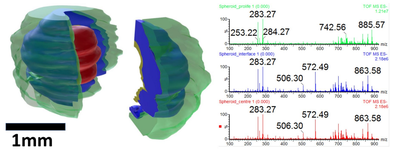


Figure 8. A three dimensional chemical reconstruction of a HCT-116 spheroid, consisting of 45 individual MS images at a voxel size of 40 x 40 x 40 micron, the colours in the map represent to the chemically distinct regions that were discovered. The average spectrum for these regions is shown to the right.

CONCLUSIONS

- **Imaging mass spectrometry can provide insights into the metabolic states of distinct regions within tumours and models of tumours to aid in the research of these systems.**
- **Automation and improvement of the imaging process allows greater numbers of samples to be processed, with minimal user input.**
- **Analysing and reconstructing serial sections from the same tissue allows 3D chemical representations to be created.**
- **The utility of this approach will be assessed for a range of research applications including drug distributions and complementarity to *in vivo* imaging techniques.**

References

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