Supporting Information (ESI) for:

Atmospheric-pressure infrared laser-ablation plasma-post-ionisation mass spectrometry imaging of formalin-fixed paraffin embedded (FFPE) and fresh-frozen tissue sections with no sample preparation

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Methods

Materials

Formalin fixed paraffin embedded control mouse organs (liver, kidney) were supplied sectioned at 5 µm thickness by collaborators at AstraZeneca (Cambridge, UK). Organs were collected from the mice following termination and then fixed in 10% neutral buffered formalin overnight. Fixed organs were then trimmed, placed in labelled cassettes and processed on Leica tissue processors (ASP300S). Tissues were dehydrated through graded alcohols, cleared and infiltrated with paraffin wax overnight. Tissues were then removed from the tissue processor and embedded into paraffin wax blocks ready for sectioning. Fresh frozen (FF) control mouse liver tissue was supplied by collaborators at the Francis Crick Institute (London, UK) and sectioned in house at 10 µm thickness using a CryoStar MX70 cryo-microtome (ThermoFisher Scientific, USA). All tissues were mounted on standard glass slides (Thermo Menzel-Glaser Superfrost) and analysed without further processing. All animals and tissue were managed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. The organs used within this study are additionally used within the 3Rs principles, as they comprise control material surplus to the requirements of the original study for which they were intended.

Ion source configuration

A basic schematic of the ion source is shown in supporting information (SI) Figure S1. The ion source employed here is configured as in our previous work first introducing the Sicrit (15kHz, 1.5 kV, Plasmion, DE)\textsuperscript{1, 2} for MSI but here with the introduction of an infrared laser (NT377, Ekspla, LT) for sample desorption / ablation set to 3 µm wavelength, with 5ns pulse width, 20Hz repetition rate and operated at approximately 1 mJ resulting in an approximate tissue ablation diameter of 150 µm. Caution: lasers must be operated in a controlled environment with the appropriate mechanical, methodological and personal protective equipment (PPE) processes, and interventions in place. Pulse energy was modified by altering the laser pump power. This was coupled with standard optical components (Thorlabs, DE; Edmund Optics, UK) consisting of three mirrors and one plano-convex lens (f = 35 mm). The laser was focussed onto the sample slide in transmission geometry, held on a microscope stage (Märzhäuser Wetzlar, DE) affixed to the optical breadboard. The laser focus was visually aligned via the location of ablated region with the outer inlet capillary orifice. The outer inlet capillary was constructed from stainless steel tubing ~20 cm length (inner diameter of 0.76 mm, outer diameter of 1.59 mm, 90° bend achieved from two of 45°). The outer inlet capillary was aligned 3 mm directly above the ablation spot. Resistive wire with a standard laboratory power supply (Pro Bench, RS Components, UK) controlled and monitored by PID controller and k-type
thermocouple (5SRTC-TT-KI-30-2M, Omega Engineering Ltd.) was used to heat the capillary. The resistive wire (NI80, Omega Engineering Ltd.) was electrically insulated with heat-resistant cement (Omegabond 600, Omega Engineering Ltd., Manchester, UK). The outer capillary was coupled to the Sicrit device by standard Swagelok components and the Sicrit device was coupled to the Orbitrap Elite (Thermo, DE) ion block with the supplied inner capillary adapter. Triggering of MS acquisition was achieved using an Arduino microcontroller.

**Experimental details**

For all experiments the Orbitrap method was set to 500 ms maximum inject time, AGC off, software set mass resolving power of 120,000 (for m/z 400) in both positive and negative ion mode. Data were collected over two m/z ranges (low mass (LM): m/z 80-305 and high mass (HM): m/z 300-1000) due to very low ion intensity in the higher m/z region where single full m/z range data were collected.

**Capillary temperature study:** Capillary temperature studies were carried out by acquiring control (laser off) and tissue (laser on, rastering for 2 mm) data for each mass range at each temperature setting. The PID was set to the desired temperature and data acquired after the measured temperature had settled for approximately 5 minutes at each temperature value. Data were collected at temperatures in the following order for each tissue and polarity: room temp., 100, 200, 300, 400, 500, 600, 650, 550, 450, 350, 250, 150, 50 °C.

**Image acquisition:** For image acquisition the stage was set to flyback raster with fixed velocity or 0.085 mm/s such that the Orbitrap scan time would result in a pixel size of approximately 100 µm in the x-dimension. The stage line to line raster spacing was set to 100 µm thus creating 100 × 100 µm pixels. Each new experiment and so resulting .raw file therefore correspond to a single image raster line. As previously the imaging procedure was controlled using an Arduino Uno microcontroller (Arduino.cc) using the start/stop signals of the stage controller (TANGO Desktop) programmed by the Switchboard software (Märzhäuser Wetzlar) to trigger laser shutters and the Orbitrap data acquisition for each new raster line.

**MS/MS CID study:** MS/MS data were acquired by manually moving the stage to ablate the desired tissue whilst observing the live collision induced dissociation (CID) spectra and adjusting the collision energy corresponding to the observed precursor and fragment ion intensities. Data were then recorded at these approximately optimal collision energy settings manually rastering over tissue manually until for approximately >10 seconds.
**Data processing**

Data were converted from .raw to .mzML format by ProteoWizard\(^3\) then to .imzML\(^4\) by MSConvert\(^5\) and analysed in Spectral Analysis\(^6\) with MATLAB (R2019b, MathWorks Inc., Natick, MA, USA). Data were lock-mass corrected using peak identities supported by MS/MS data from our own studies and literature evidenced identities. Data were peak picked using the gradient method and intensity values were summed across each identified peak to create new centroid datacubes. These datacubes were saved as imzML files and uploaded to Metaspace\(^7\) (project name: Steven et al. (2022) AP-IR-LA-PPI - FFPE and Fresh Frozen tissue) for analysis and are available to view as supporting information via the following link (https://metaspace2020.eu/project/steven-2022). Ion images for low \(m/z\) range positive ion data were rotated 180 degrees to match orientation of other tissues. All displayed ion identities were annotated as either \([M+H]^+\) or \([M-H]^−\). Annotations to the HMDB v4.0 database were downloaded using METASPACE\(^8\) python client and analysed in R version 4.0.5 and RStudio 1.4. Annotations were filtered at a 10% false discovery rate (FDR) and on-tissue images selected (offSample filter set to FALSE). MS/MS data were manually submitted for analysis and comparison to theoretical fragmentation data via the MyCompoundID web interface\(^9\). The protonated or deprotonated adduct form was selected in all examples displayed (SI Table S1).

**SI Section S1**

![Figure S1. Ion source schematic and photograph of inlet-plasma assembly.](image)
Figure S2. Mean negative ion mode spectra from FF kidney tissue.

Figure S3. Mean on-tissue positive ion mode spectra from FFPE and FF kidney MSI datasets with example tentative peak assignment labels from Metaspace. A, C: low and high mass FFPE data respectively. B, D: low and high mass FF data respectively.
Figure S4. Mean off-tissue spectra from negative ion mode FFPE and FF kidney MSI datasets. A, C: low and high mass FFPE data respectively. B, D: low and high mass FF data respectively.

SI Figure S5. Ion images for prominent peaks showing background distributions, these ions are labelled in Figure 1 or Figure S4.
Figure S6. Fresh frozen tissue ion intensity vs. capillary temperature trends for glutamine and PE (38:5) both on and off tissue (blue and red respectively).

Table S1. Number of assigned species in Metaspace for each dataset and molecular database. 10% FDR, on tissue filtering.

| Dataset            | HMDB v4.0 | Core met. | LipidMaps |
|--------------------|-----------|-----------|-----------|
| Neg. FFPE m/z 80-305 | 450       | 227       | 115       |
| Neg. FFPE m/z 300-1000 | 222       | 113       | 207       |
| Neg. FR m/z 80-305   | 538       | 252       | 132       |
| Neg. FR m/z 300-1000 | 338       | 244       | 440       |
| Pos. FFPE m/z 80-305 | 218       | 58        | 15        |
| Pos. FFPE m/z 300-1000 | 279       | 158       | 308       |
| Pos. FR m/z 80-305   | 243       | 74        | 18        |
| Pos. FR m/z 300-1000 | 279       | 200       | 365       |

Table S2. Summary of on-tissue MSMS data

| m/z    | Supported Identity | Tissue preservation | Adduct | Normalised collision energy | Peaks matched to database | Initial Score | Fit Score |
|--------|--------------------|---------------------|--------|----------------------------|----------------------------|---------------|-----------|
| 885.5488 | PI (18:0/20:4) | FFPE | [M-H] | 27 | 283.26, 303.23, 419.26, 437.27, 439.23, 581.31, 599.32, 601.28, 619.29, 723.50 | 0.986 | 0.986 |
| 810.5285 | PS (18:1/20:3), PS (18:0/20:4) | FFPE | [M-H] | 25 | 255.23, 279.23, 281.24, 283.26, 303.23, 419.26, 480.31, 723.50 | 1 | 0.587 |
| 806.5737 | PC (18:1/20:5) | FFPE | [M-H] | 24 | 341.30, 625.52, 627.53 | 1 | 0.354 |
| 764.5238 | PE (16:0/22:5), PE (18:0/20:5), PE (20:4/18:1) | FFPE | [M-H] | 25 | 255.23, 259.24, 281.25, 283.26, 301.22, 303.23, 327.23, 328.23, 329.25, 419.25, 452.27, 478.292 | 1 | 0.967 |
| 750.5081 | PC (16:1/18:4), PE (15:0/22:5) | FFPE | [M-H] | 25 | 255.23, 279.23, 283.26, 303.23, 391.23, 419.25, 437.27, 464.28, 695.465 | 1 | 0.942 |
| m/z      | Assignment                                      | Absolute ppm error |
|----------|-------------------------------------------------|--------------------|
| 85.0106  | thiophene                                       | < 0.1              |
| 87.0089  | pyruvate                                        | 2.3                |
| 89.0245  | lactate                                         | 1.1                |
| 94.038   | 2-hydroxypyridine                               | 1.1                |
| 101.0244 | ketobutyrate                                     | < 0.1              |
| 111.0087 | furoic acid                                      | < 0.1              |
| 115.00   | maleic acid                                      | 0.9                |

Table S3. Mass accuracy for example tentative assignments included in main article Figures, 1, 2 and 3.
| Mass        | Compound                | Amount |
|-------------|-------------------------|--------|
| 117.0192    | succinate               | 0.8    |
| 121.0509    | purine                  | 0.8    |
| 122.0358    | pyrazinamide            | 0.5    |
| 123.0553    | niacinamide             | < 0.1  |
| 125.0356    | thymine                 | < 0.1  |
| 127.0502    | thymine                 | < 0.1  |
| 134.0470    | adenine                 | 1.5    |
| 141.0481    | pyrazinemethanethiol    | < 0.1  |
| 145.0619    | glutamine               | 0.7    |
| 146.0458    | glutamate               | < 0.1  |
| 165.1022    | kynuramine              | < 0.1  |
| 171.0775    | glycylylproline         | < 0.1  |
| 179.0560    | glucose                 | 0.6    |
| 196.0616    | L-Dopa                  | 0.5    |
| 203.0828    | tryptophan              | 1.0    |
| 255.2329    | FA (16:0)               | 1.2    |
| 279.2335    | FA (18:2)               | 2.1    |
| 281.2489    | FA (18:1)               | 1.1    |
| 295.2284    | HODE                    | 2.0    |
| 303.2332    | FA (20:4)               | 1.0    |
| 327.2327    | DHA                     | 0.6    |
| 351.2175    | PGD2                    | 0.6    |
| MRM | Compound | Charge | Abundance |
|-----|----------|--------|-----------|
| 524.2788 | lysoPE (22:6) | 1.1 |
| 536.5049 | cer (34:1) | 0.2 |
| 576.3306 | LPS (22:2) | 0.2 |
| 673.4813 | PA (34:1) | 0.2 |
| 676.6612 | cer (44:1) | 0.2 |
| 669.5459 | DG (40:6) | 1.1 |
| 701.5128 | PA (36:1) | 0.3 |
| 723.4972 | PA (38:4) | 0.3 |
| 725.5115 | PA (38:4) | < 0.1 |
| 750.5079 | PC (34:5) | < 0.1 |
| 752.5222 | PC (34:5) | 0.3 |
| 768.5545 | PE (38:4) | 1.0 |
| 776.5603 | PE (p-40:5) | 0.5 |
| 778.5397 | PE (39:5) | 0.6 |
| 778.5759 | PE (p-40:5) | 1.8 |
| 790.5034 | PE (p-38:4) | 0.8 |
| 795.6293 | coenzyme Q9 | 1.0 |
| 810.5283 | PS (38:4) | 0.9 |
| 853.7286 | TG (52:5) | 0.8 |
| 885.5496 | PI (38:4) | 0.2 |
On background signal in AP-IR-LA-PPI-MSI

In addition to the data being rich in tissue endogenous ions, there are notable background ions visible in the FFPE spectra (Fig. 1 B – starred peaks) which are also prominent in the off-tissue spectra (SI Figure S4 and associated images Figure S5). These background ions likely derived from three primary sources: plasma ionisation of laboratory air, ions originating from the paraffin of FFPE tissue and carryover within the inlet capillary from tissue regions manifesting in off-tissue signal. In most datasets in this study, they are clearly distinguishable from tissue endogenous ions where image data is viewed and the on/off tissue filtering in Metaspace also appears to separate many of these. Despite this, there do appear to be confounding peaks resulting from these background species which are assigned as, for example, fatty acids. As manifest in image data some of these features can be seen in Figures 2 and 3. Their appearance within these datasets can be broadly described as hot-spot carryover, diffuse carryover and lab air / plasma background. The succinate image (Figure 2) shows these hot-spot like carryover features. These are assumed to be a result of carryover that is somewhat specific to FFPE tissue with this heated inlet configuration. The second and related phenomenon termed here as diffuse carryover is also present in this succinate image but is clearer in the images for thymine and glucose (Figure 2) where a more diffuse off-tissue signal is observed, particularly to the right or after the first few rows of the on-tissue image region. This form of carryover is present in both the FFPE and FF datasets, unlike the hotspots, and so may represent a more universal and continuous manifestation of inlet capillary contamination and carryover. Thirdly, non-tissue background is present within these datasets and examples corresponding to these, in common with background peaks labelled in Figure 1, are shown in SI Figure S4. It is known that different metabolites will degrade at different temperatures, resulting in lower detected ion intensities from equivalent initial concentration samples\(^\text{64}\). Within the cited study\(^\text{64}\) it was shown that lipids exhibited a higher temperature for degradation than glutamate, a trend that is perhaps qualitatively in agreement with the data presented within this manuscript, although in a rather different context. Therefore, in addition to plasma or paraffin derived background ion signal, it is possible that the additional heating present in this ion source will further fragment ions beyond what may be expected in other laser sampling MSI modalities. Though it is known that ablation plumes in MALDI likely reach similar temperatures.

Author contributions

RTS – project administration, conceptualization, investigation, data curation, formal analysis, methodology, visualization, writing – original draft, writing – review and editing; MN – conceptualization, investigation; AJT – formal analysis, visualization, writing – original draft; AN – formal analysis; EE – conceptualization, methodology; RG – resources, writing – review & editing; ZT
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