The 3D OrbiSIMS – Label-Free Metabolic Imaging with Sub-cellular Lateral Resolution and High Mass Resolving Power

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Abstract: We report the development of a 3D OrbiSIMS instrument for label-free biomedical imaging. It combines the high spatial resolution of secondary ion mass spectrometry (SIMS; under 200 nm for inorganics and under 2 µm for biomolecules) with the high mass resolving power of an Orbitrap (> 240,000 at *m/z* 200). This allows exogenous and endogenous metabolites to be visualized in 3D with sub-cellular resolution. We imaged the distribution of neurotransmitters—gamma-aminobutyric acid (GABA), dopamine and serotonin with high spectroscopic confidence in the mouse hippocampus. We also putatively annotated and mapped the sub-cellular localisation of 29 sulfoglycosphingolipids and 45 glycerophospholipids, and confirmed lipid identities with tandem mass spectrometry. We demonstrated single-cell metabolomic profiling using rat alveolar macrophage cells incubated with different concentrations of the drug amiodarone, observing that he up-regulation of phospholipid species and cholesterol is correlated with the accumulation of amiodarone.

Aberrant regulation of biomolecules can lead to devastating pathologies. Therefore, methods able to detect and identify metabolites with sub-cellular spatial resolution would be very valuable for studying diseases as well as provide fundamental biological insights into metabolism heterogeneity at the single-cell scale . The metabolites expressed by a cell are indicative of its physiological state at a given time. The growing appreciation of the heterogeneous nature of metabolism motivates the need to measure and understand metabolite and phenotype variations at a single-cell level.[1-4] In particular, in the pharmaceutical industry, there is a pressing need to improve drug efficacy and to reduce attrition (failure)[5], especially during late-stage drug development. Metabolic imaging with sub-cellular lateral resolution would permit study of the effects of pharmaceuticals on cellular processes and identify potential toxicological effects. A label-free method is needed that can identify where drugs go to establish whether drug concentrations are sufficiently high in the right places to have a therapeutic effect, or if the medicine is concentrating within organelles and causing toxicity.[6]

As highlighted recently there are numerous analytical challenges associated with measuring the metabolome at the single-cell level.[2] These include high dynamic fluctuations, chemical diversity spanning a large dynamic range, the inability to amplify or tag metabolites, and the limited amount of material in a single cell. Single-cell measurements require a sensitive, label-free method with high

chemical specificity and dynamic range. Mass spectrometry imaging offers label-free imaging capabilities and for certain analytes has sub-femtomole sensitivity.[4]

The quest for sub-cellular resolution metabolic imaging using mass spectrometry has led to significant advances in instrumentation. Recent developments in high-resolution mass spectrometers, for example the Orbitrap™ mass analyser[7] with a mass resolving power in the range of 10^5-10^6, a mass accuracy of < 1 ppm and MS/MS capability allow direct identification of metabolites from databases such as LIPID MAPS.[8] An elegant atmospheric pressure matrix assisted laser-desorption ionisation (AP-MALDI) mass spectrometer (MS) source that combines an Orbitrap MS with a tightly focused laser beam was recently reported[9]; the authors demonstrated sampling regions down to 1.44 µm in diameter and a lateral resolution for tissue classification of 2.9 µm. The highest reported MALDI MS spatial resolution was generated using a transmission geometry approach, allowing imaging of tissue proteins with a laser spot diameter of 1 µm and a 2.5 µm step size.[10]

Secondary ion mass spectrometry (SIMS) is an increasingly popular method in the life-sciences[11,12] since it provides high spatial resolution using a focused ion beam as the probe and image in 3D with ~5 nm depth resolution.[13,14] There have been significant developments to improve its application for biological studies, in particular to include MS/MS capability for identification. Such developments include a quadrupole-orthogonal time-of-flight (ToF) spectrometer[15], the J105 (IONOPTIKA, UK) using a novel ToF-ToF design for continuous (non-pulsed) large cluster ion beams more suited to the detection of large biomolecules[12] and recently the addition of a linear ToF to a TRIFT (Physical Electronics, MN, U.S.A.) spectrometer allowing parallel MS/MS imaging.[16]

Fourier Transform mass spectrometers[17] are the most accurate for biomolecule identification since they have the highest mass resolving power, mass accuracy and are capable of high-resolution MS/MS. The first attempt to couple high spatial resolution SIMS with high mass resolving power spectrometry combined a liquid metal ion gun with a Bruker Daltonics 7.0 T APEX II (Bruker, Germany) Fourier Transform Ion Cyclotron Resonance (FT-ICR) MS[18]; the authors demonstrated the ability to acquire spectra from a 50 µm x 50 µm pixel with 30,000 mass resolving power. Another group[19] combined a C_{60} primary ion beam with a FT-ICR instrument and achieved a spatial resolution of ~40 µm and 100,000 resolving power when imaging cholesterol in a rat brain section. However, the signal-to-noise ratio, even for the abundant cholesterol molecule, was low despite the large pixel size and very long pixel acquisition times of 15 s.

The principal practical limitation of FT-ICR MS is that, although this instrument has the necessary mass resolution and accuracy, it lacks the speed for capturing 3D or large 2D images; conversely axial-ToF analysers have the speed, but lack the necessary accuracy to obtain chemical formulae. To overcome this dichotomy, we have developed the 3D OrbiSIMS which uses a hybrid mass analyser design. The 3D OrbiSIMS combines the strengths of high-speed ToF imaging with the high mass resolving power, high mass accuracy and MS/MS capability of the Q Exactive™ HF Orbitrap (Thermo Fisher Scientific, Germany).[20,21] To demonstrate the potential of the method, we examine the distribution of lipids and neurotransmitters in brain tissue and amiodarone-induced lysosomal dysfunction in macrophages.

RESULTS

The 3D OrbiSIMS instrument

We first describe the utility of the 3D OrbiSIMS from the perspective of a method for metabolic imaging and then provide technical instrument details for interested readers. A schematic of the instrument is given in Figure 1. The dual ion beam and dual analyser configuration (Fig. 1a) provides ten operational modes for spectrometry, depth profiling, 2D and 3D imaging (Fig. 1b). For 2D imaging, a typical approach is to use the ToF analyser with the bismuth liquid metal ion gun (Bi LMIG) to acquire a fast (~30 minutes) survey image and identify a region of interest (mode 6). Subsequently, an image can be acquired with the Orbitrap analyser (mode 7) giving high mass accuracy, high mass resolution and MS/MS capability. In this mode, the argon gas cluster ion beam (GCIB) is used, which creates mass spectra richer in intact biomolecules (e.g. lipids) and with significantly less fragmentation compared with a Bi LMIG primary ion beam.[22] Acquiring an image at the highest mass resolution with the Orbitrap is slow (a 256 x 256 pixel image with a mass resolving power of 240,000 at *m/z* 200 would take 9 hours). Consequently, the ToF analyser is needed.

SIMS enables high-resolution 3D molecular imaging and provides complementary information to other MSI methods such as AP-MALDI. In SIMS, a 3D image is created from a stack of 2D images acquired at different depths. The depth of the image is precisely controlled using an argon GCIB to gently sputter away material. Our hybrid approach (mode 10) allows high-speed 3D imaging (Fig. 1c) using the Bi LMIG with ToF acquisition for high spatial resolution images and high resolution mass spectra using the Orbitrap analyser from the argon GCIB sputtering cycle between images.

An example of this new 3D imaging mode is demonstrated for a single alveolar macrophage cell incubated in media with the drug, amiodarone, at a concentration of 6.25ug/mL for 72 hours (Figure 2). A sequence of 2D ToF MS images at successive 400 nm depths (Fig. 2a,b) are reconstructed (see Methods) to produce a 3D image showing the distribution of amiodarone (Fig. 2c). The nucleus has an unusual shape as found in our previous ToF-SIMS imaging study.[23] The high spatial resolution (~300 nm) ToF MS data but with consequently poor mass resolution are complemented by the Orbitrap spectra (Fig. 2d,e) allowing cell membrane (*m/z* 184.0732, $C_5H_{15}NO_4P^+$) and nuclear marker ions (*m/z* 156.8852, $K_2PO_4^+$; *m/z* 156.9745, $C_2H_8O_2P_3^+$) to be putatively annotated. A weaker interference ion is also evident at *m/z* 156.9759 putatively annotated as $C_{10}H_9N_2^+$.

Instrument details

The 3D OrbiSIMS is based on the TOF.SIMS 5 platform (ION-TOF GmbH, Germany) which we hybridize with a Q Exactive™ HF Orbitrap (Thermo Fisher Scientific, Germany) MS (Fig. 1a). The Orbitrap analyser provides high mass accuracy (sub-ppm), a high mass resolving power (from 15,000 at *m/z* 200 with an acquisition rate >20 spectra/s up to 480,000 at *m/z* 200 with 1 spectrum/s), high sensitivity and a high in-spectrum dynamic range (10^5 for ratio of total ion counts to noise). This is demonstrated by resolving the fine isotope structure of the mass spectrum of crystal violet (Supplementary Fig. 1 and Supplementary Table 1).

Both analysers share the same secondary ion extraction optics (2000 V potential) and consequently analyse the same point on the sample using either the 30 keV Bi LMIG or a micro-focussed 5-20 keV Ar_n GCIB with clusters in the range $1000 < n < 10,000$ (see Methods). At the heart of the 3D OrbiSIMS

is an electrostatic 90 degree deflector that selects either the ToF MS or by 90 degree deflection the Orbitrap MS. The deflector can be pulsed with a frequency of up to 30 kHz. With the Orbitrap selected, the secondary ions pass into the transfer system where they are decelerated to kinetic energies of several tens of eV and are transferred via RF ion guides to the Q Exactive. To reduce the energy spread of the secondary ions, the transfer system comprises a multi-stage collisional damping cell. This replaces the standard ESI source and atmospheric pressure to vacuum interface of the Q Exactive HF. All other components remain unaffected, allowing for acquisition of either MS or tandem MS/MS experiments with high mass resolution and mass accuracy for structural-based identification (Supplementary Fig. 2).

The micro-focused GCIB in combination with the Orbitrap MS enables simultaneous high-mass resolving power and sub-cellular resolution. The resolution of the ion beam is measured (see Methods) by imaging an electroformed mesh grid from the ion induced secondary electron image (Supplementary Figs. 3,4). The secondary ion signal intensity is a further important parameter for the achievable practical resolution. In the absence of a suitable biologically relevant lateral-resolution reference device, we use two nuclei that exhibit clear chemical contrast to make *x*-axis and *y*-axis resolution measurements using the summed intensity of 8 nuclear markers (see Methods). The average resolution is determined to be 1.34 µm ± 0.24 µm (*n*=5) (Supplementary Fig. 5). It can be seen from Supplementary Fig. 5 that the intensity of selected intact lipids is similar to the intensity for the selected nuclear markers and therefore the spatial resolution will be similar. For inorganic materials, the Bi LMIG can be operated in a quasi-continuous mode in combination with the Orbitrap MS to give the highest simultaneous spatial resolution and mass resolving power. We demonstrate (see Methods) a spatial resolution of 172 nm ± 61 nm (*n*=95) simultaneously with a mass resolving power of 355 k for ZrO^+ (Supplementary Fig. 6) using Bi_3^{2+}.

The performance for 3D imaging and depth profiling is shown in more detail using a nanostructured reference material (Supplementary Fig. 7). The *m/z* 564 profiles show sharp peaks at the Irganox 3114 delta-layers where the signal rises over four orders of magnitude. A depth resolution (see Methods) of 11-13 nm is measured across all three modes. In modes 3 and 10, the secondary ions sputtered by the GCIB are analyzed with the Orbitrap MS. This is an advantage over conventional dual-beam depth profiling (mode 9) where the secondary ions sputtered by the GCIB, typically >95% of the sampled volume, are not utilized.

Molecular pathology

The structure of the hippocampus directs neural connectivity and influences cognitive abilities.[24-26] We used the 3D OrbiSIMS to molecularly image the hippocampal region of a coronal section of mouse brain at tissue scale, cellular scale and sub-cellular scale (Figure 3). The ion image (Fig. 3a) was aligned and overlaid onto the optical images of adjacent H & E stained tissue section (Fig. 3b) to provide complementary molecular pathology. The boundaries of anatomical regions of the brain (Fig. 3c) in the ion image were defined using the histological features in the H & E section and the Allen Brain Atlas.[27]

At the tissue scale, a fast large field of view ToF-MS ion image (mode 6, mass resolving power 5,000 at *m/z* 200, acquisition time 36 mins) shows the distribution of cholesterol ($C_{27}H_{45}O$, $[M-H]^-$, *m/z* 385.3,

green), the small molecular fragment $[CNO]^-$ (*m/z* 42.0, red) and lipid fragments (the summed contribution of *m/z* 153.0 $[C_3H_6PO_5]^-$, *m/z* 255.2 $[C_{16}H_{31}O_2]^-$ and *m/z* 281.2 $[C_{18}H_{33}O_2]^-$), blue) (Fig. 3a).

To demonstrate the improvement provided by the 3D OrbiSIMS, a section of the *cornu ammonis 1* (*CA1*) region of the hippocampus was imaged with sub-cellular resolution (~2 µm) and high mass resolving power (240,000 at *m/z* 200, (mode 7)). The striated tissue layers in the hippocampus, stratum radiatum (*SR*), pyramidal layer (*SP*), stratum oriens (*SO*), corpus callosum (*CC*), and the cortex are chemically diverse. The cellular distribution of *m/z* 888.6245 putatively annotated as C24:1 Sulfatide [(3'-sulfo)Gal β-Cer(d18:1/24:1)] in the *CC* layer is mapped in green (Fig. 3d). An advantage of argon GCIB is that there is little fragmentation of sulfatides as demonstrated for a reference sample of the same lipid (Supplementary Fig. 8). For phospholipids, such as a phosphoethanolamine, however, there is significantly more fragmentation (Supplementary Fig. 9). The high density of cell bodies in the *SP* layer, stained purple/blue in the H & E correlated to the distribution of nuclear markers, adenine and guanine. Their summed contributions are shown in red in the ion image. This contrasts with the low density of neuronal cells in the *SO* and *SR* layers.

The instrument's mass resolving power simultaneous with sub-cellular spatial resolution allows us to probe the chemical composition of a single nucleus. The nucleus of a single interneuron in the *SO* regions of the CA1 was selected and the mass spectra for all the pixels in the region of interest (ROI) were summed (Fig. 3e). Purine based nucleobases adenine and guanine, as well as phosphorylated species from the DNA, were detected.[28] The high mass resolving power at low molecular weight and high mass accuracy enabled putative annotation of the chemical formula of the signals.

These features also allowed the use of the LIPID MAPS database to putatively annotate 127 lipid species including fatty acids, sterols, glycerophospholipids and sphingolipids (Supplementary Figs. 10, 11 and Supplementary Tables 2-8). The localization of 29 glycosphingolipid sulfatides were putatively annotated and mapped. Both hydroxylated and non-hydroxylated sulfatides with chain lengths ranging from 14 to 26 carbons with varying degrees of fatty acid saturation were identified. The presence and assignment of C24(OH) sulfatide [(3'-sulfo)Gal β-Cer(d18:1/24:0(2OH))] was confirmed with *in situ* tandem MS (Fig. 4a). Sulfatide lipids are abundant in the extracellular membrane of myelin and are responsible for the efficient conduction of electrical signals in the nervous system.[29] These molecules play an important role in neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, and the ability to detect and identify sulfatides and other lipids at sub-cellular lateral resolution will be beneficial to neuroscientists.

In many cases, the mass spectra, in the lipid mass range, showed multiple molecular ion signatures at the same nominal mass (Fig. 4b) from a region of the hippocampus (Fig. 4c). As shown in the ion image of the CA3 region (Fig. 4d) a peak at *m/z* 904.62012, putatively annotated as C24:1 (OH) sulfatide [(3'-sulfo)Gal-Cer(d18:1/24:1(2OH))] with a mass accuracy of 1.1 ppm and a peak at *m/z* 904.65669 were chemically and spatially resolved. A minimum mass resolving power of 25,000 is needed to resolve the two peaks (Supplementary Fig. 12).

Mapping molecules in neuronal communication

Neurons employ signalling molecules, neurotransmitters, to communicate. These include glutamate, gamma-aminobutyric acid (GABA), dopamine, norepinephrine, epinephrine, histamine, serotonin and

acetylcholine. The 3D OrbiSIMS enables mapping of multiple neurotransmitter distributions in neural tissue (Fig. 5a) in a single experiment. The putatively annotated neurotransmitters, GABA or isomer ($[C_4H_8NO_2]^-$ at *m/z* 102.0560), dopamine (Fig. 5b) ($[C_8H_{10}NO_2]^-$ at *m/z* 152.0718) and serotonin ($[C_{10}H_{11}N_2O]^-$ at *m/z* 175.0877) were mapped in the CA3 (Fig. 5c,d) and CA1 (Fig. 5e) region of the hippocampus. Tandem mass spectra of reference samples confirmed these peaks are observed in the SIMS mass spectrum (Supplementary Fig. 13). The sub-ppm mass accuracy of these peaks gives confidence in their assignment. In addition, we mapped the MS^1-derived gas-phase fragment ions, identified from tandem MS reference spectra of the neurotransmitters, and found their presence and distribution to be consistent with the parent ions (Supplementary Fig. 13).

GABA was found to be most abundant in the *SO* region of the hippocampus and was below detection in the *SP* regions in both the CA3 and CA1 ion images (Fig. 5). This finding is consistent with observations made with immunocytochemistry and electrophysiology measurements.[30] The monoamine neurotransmitters, dopamine and serotonin, were homogeneously distributed throughout the hippocampal formation. The ability to detect alterations in the balance of tonic-based neurotransmitters with microscopic accuracy has the potential to elucidate the effects of neuroactive substances, such as antidepressants[31] or drugs such as cocaine which elevates dopamine levels in the hippocampus.[32]

Single-cell metabolic profiling

We evaluated the metabolomic profile of individual cells treated with the drug amiodarone, a cationic amphiphilic drug. At high doses and long-term usage, amiodarone [KEGG drug: D02910], a Vaughan-Williams class III antiarrhythmic medication, is known to cause drug-induced phospholipidosis (PL).[33-35] PL is characterized by the prevalence of 'foamy' or phospholipid-laden macrophages. The exact mechanism of phospholipidosis remains the subject of debate; a number of potential contributors have been proposed. It is believed that the hydrophobic domain of amiodarone allows the molecule to cross lipid membranes, including that of the lysosome. Inside the acidic environment of the lysosome, amiodarone is hydrolyzed.[36] The drug then accumulates in the lysosome, as this form is unable to re-cross the membrane. The excess drug has then been proposed to inhibit the function of the lysosomal phospholipase A2 (LPLA2, phospholipase A2 group XV)[37], a protein responsible for phospholipid catabolism leading to the observed phospholipid accumulation. Previously, it has been shown that ToF SIMS is able to detect the $[M+H]^+$ ion in a single rat alveolar macrophage cell[23]. Recently, in a correlative NanoSIMS and electron microscopy study, the use of I^- as a chemically specific label for amiodarone and P^- as a general lipid marker illustrated amiodarone internalisation in lysosomes in the same cell type and a correlated increase in lipid.[38]

In order to evaluate the effects on the metabolic signature at the single-cell level, 3D OrbiSIMS metabolic profiles (mode 2) were acquired for cultured macrophages incubated with various concentrations of amiodarone (0, 1.56, 6.25 and 9.38 µg/mL). The average mass spectral signature (Supplementary Fig. 14 and Supplementary Table 9) reveals variations in the lipid profiles among cells in the treatment groups. Metabolic changes for the intact lipid at *m/z* 760.5848 and putatively annotated as PC (34:1) $[C_{42}H_{83}NO_8P]^+$ (1.1 ppm) and cholesterol $[M-H_2O+H]^+$ at *m/z* 369.3514 (1.9 ppm) for individual cells from four amiodarone treatment groups, control 0 µg/ml (*n*=8, blue), 1.56 µg/ml (*n*=6, magenta), 6.25 µg/ml (*n*=3, green) and 9.38 µg/ml (*n*=7, red) are revealed in Fig. 6a,b. As expected the amiodarone intensity per cell ($[M+H]^+$ at *m/z* 646.0307 $[C_{25}H_{30}I_2NO_3]^+$ (1.3 ppm)) varies

approximately linearly with incubation concentration and also demonstrates a wide range in uptake behaviour at the single-cell level (Fig. 6c). Amiodarone is confirmed from an MS/MS analysis (Fig. 6e and Supplementary Fig. 15). The average PC (34:1) intensity increases over 10 fold and the cholesterol $[M-H_2O+H]^+$ peak increases approximately exponentially with amiodarone concentration (Fig. 6b). This finding is consistent with previous studies following amiodarone exposure.[39] Whilst there is a high degree of variability for individual cells in drug uptake and PC (34:1) upregulation we see that, when intensities are shown in a correlation plot for individual cells, that there is a linear correlation (Pearson's correlation coefficient = 0.91895, R-square = 0.83739) (Fig. 6d). Such detail can only be revealed by single-cell analysis.

Discussion

The 3D OrbiSIMS enables metabolic imaging with sub-cellular resolution. It combines the proven power of the high-field Orbitrap for biological MS with the high-speed and high-spatial resolution 3D imaging ToF-SIMS platform. We demonstrate a spatial resolution of under 200 nm (172 nm ± 61 nm) (n=95) simultaneously with a mass resolving power of 355,000 for ZrO_2 nanostructures using the Bi_3 LMIG. The micro-focused argon GCIB permits imaging of intact sulfatide and phosphoinositol lipids with a spatial resolution of under 2 µm simultaneously with a mass resolving power of >240 k at *m/z* 200. A resolution of under 1.5 µm (1.34 µm ± 0.24 µm) (n=5) is possible for a highly skilled operator. Furthermore, a mass resolving power of >480,000 at *m/z* 200 can be achieved allowing the fine structure of isotopic patterns to be resolved. The ability to resolve sub-cellular features including the nucleus of an interneuron is also demonstrated.

To the best of our knowledge, this represents the highest reported simultaneous spatial and mass resolutions. This provides complementary analysis to recently reported AP-MALDI imaging with 1.4 µm sampling (lateral resolution for tissue classification 2.9 µm).[9] Indeed, imaging of biomolecules at sub-cellular resolution requires a multi-technique strategy to bridge the length-scales and molecular-scales with the NanoSIMS 50L (CAMECA, France) allowing 50 nm resolution of elemental or stable isotope labels[40-42] through to MALDI techniques that are able to image peptides and small proteins.[9,10] The 3D OrbiSIMS is suited to metabolites and small molecules (<1000 Da) and fits between these methods, enabling label-free analysis of lipid biochemistry and the role lipids play in disease pathology at sub-cellular resolution. Sample preparation varies significantly across these methods with NanoSIMS requiring special care to prevent delocalisation of molecules by solvents.[38] AP-MALDI requires the least sample preparation though similarly, care is required not to delocalise molecules with matrix application. For 3D OrbiSIMS, care needs to be taken to freeze dry samples for vacuum compatibility. The instrument is also configured for cryo analysis (see Methods).

The application of the 3D OrbiSIMS to the single-cell and its subcellular milieu will improve understanding of the location in which a drug resides and the complex responses from on-target and off-target engagement. The 3D OrbiSIMS provides rich multiplexed data sets, capturing hundreds of compounds in a single measurement, with sub-cellular resolution. It has the potential to provide critical insight on fundamental biological processes.

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AUTHOR CONTRIBUTIONS:

MKP, AP, RH performed experiments. MKP analyzed data. PM, CN, AW prepared tissue and cell experiments and H & E pathology. FK designed continuous mode Bi LMIG. RM, AM, DG, EN designed interface to hybridize ToF and Orbitrap spectrometers. AP, MKP, RM, AM, EN, RH optimized performance of 3D OrbiSIMS. HA developed computer interfacing and computational methods. RM and EN designed cryo sample handling. AW, MRA, PM and CTD direction of pharmaceutical studies. SH and EN gave technical leadership at Thermo Fisher Scientific and ION-TOF, respectively. ISG original design concept and supervised the project. MKP and ISG wrote the paper. All authors read and commented on the paper.

COMPETING FINANCIAL INTERESTS:

EN is a director and shareholder of ION-TOF GmbH Muenster, Germany. AP, RM, FK, and HA are employees of ION-TOF GmbH.

DG, SH and AM are employees of Thermo Fisher Scientific, the corporation that produces Orbitrap mass spectrometers.

CN, PM, AW and CD (at the time of this study) are employees of GlaxoSmithKline.

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Figure 1. The 3D OrbiSIMS spectrometer and modes of operation. A) Schematic of the 3D OrbiSIMS. B) Table of methodologies and data types achievable given the ion beam and spectrometer selections. C) Hierarchy summary of 3D imaging modalities (mode 8 -10).

Figure 2**.** 3D MS imaging using dual beam and dual spectrometer (mode 10) of single rat alveolar macrophage cell incubated in media with the drug, amiodarone. A) Sequence of 30 keV Bi_3^+ (~300 nm resolution) ToF-SIMS images of total ion counts at ~400 nm depths as the cell is sputtered away. B) As A) colour overlay of phosphocholine marker (*m/z* 184), grey, nuclear marker (*m/z* 157), magenta and amiodarone $[M+H]^+$ (*m/z* 646), green C) 3D rendering of the cell with phosphocholine marker, (*m/z* 184, grey, opacity 0.40), nuclear marker (*m/z* 157, magenta) and amiodarone $[M+H]^+$ (*m/z* 646), green. D) High mass resolution spectrum from sputtered material using Orbitrap MS (blue) and low mass resolution spectra obtained during imaging with the ToF MS (black) for phosphocholine marker. E) as D) for nuclear marker. Results presented are from a single measurement.

Figure 3. 3D OrbiSIMS MS images of the hippocampal region of a coronal section of mouse brain at tissue scale, cellular scale and sub-cellular scale. A) 30 keV Bi_3^+ (lateral resolution ~ 2 μm, pixel size 2 μm) ToF MS image (mode 6) of the hippocampus region of a coronal mouse brain section, cholesterol (*m/z* 385.3, $[C_{27}H_{45}O]^-$, green), the small molecule (*m/z* 42.0, $[CNO]^-$, red) and glycerophospholipid fragments (the summed contribution of *m/z* 153.0 $[C_3H_6PO_5]^-$, *m/z* 255.2 $[C_{16}H_{31}O_2]^-$ and *m/z* 281.2 $[C_{18}H_{33}O_2]^-$), blue). B) The distribution of cholesterol and $[CNO]^-$ in the ion image are overlaid with the histological features in the adjacent H & E stained brain section. C) Schematic outlining the anatomical regions of the brain tissue. D) 20 keV Ar_{3000} (lateral resolution ~ 2 μm, pixel size 1.95 μm) Orbitrap MS image (mode 7) of the CA1 region, the phosphoinositol headgroup (*m/z* 241.0121, $[C_6H_{10}PO_8]^-$, blue), C24:1 Sulfatide [*m/z* 888.6245, (3'-sulfo)Gal β-Cer(d18:1/24:1)] ($[C_{48}H_{90}NO_{11}S]^-$, green) and adenine (*m/z* 134.0473, $[C_5H_4N_5]^-$, red). Image is overlaid on the adjacent H & E stained brain section for orientation. Boundaries between anatomical regions shown with white dashed lines. E) Detail of D) for adenine (*m/z* 134.0473, $[C_5H_4N_5]^-$ showing individual nuclei in the *SO* region with the background subtracted mass spectrum of a single nuclei from a region of interest (black line), 15 pixels with intensity > 2.7×10^3 threshold). Results presented are from a single measurement.

Figure 4. Identification of lipids and mapping their spatial distribution requires simultaneous high spatial resolution and high mass resolution as well as tandem MS. A) 10 keV Ar_{3000} Orbitrap Tandem MS (mode 2) of mass peak *m/z* 906.63 was used to identify the lipid species to be C24 (OH) Sulfatide [(3'-sulfo)Gal β-Cer(d18:1/24:0(2OH))]. The chemical formula of each fragment peak assignment is provided. B) 20 keV Ar_{2500} (lateral resolution ~ 2 μm, pixel size 1.95 μm) Orbitrap mass spectrum (mode 7) showing a lipid molecule putatively annotated as C24:1 (OH) sulfatide [(3'-sulfo)Gal-Cer(d18:1/24:1(2OH)), blue)] and a peak at *m/z* 904.65669. C) Optical image of H & E stained adjacent tissue section showing the region of analysis (white dotted line) and D) Orbitrap MS images (parameters as A) normalized to the total ion image of C24:1 (OH) sulfatide (blue), *m/z* 904.65669 (green) and *m/z* 124.0075 shown separately and as an overlay. Results presented are from a single measurement.

Figure 5. Mapping of neurotransmitters in the hippocampus. A) The analysed regions in the CA1 and CA3 regions of the hippocampus are shown on the H & E stained tissue section. B) Negative ion mass spectrum (mode 7) between *m/z* 151.89 and *m/z* 152.10 showing a mass peak at *m/z* 152.0717 that

is consistent with the deprotonated molecular ion of dopamine ($[C_8H_{10}NO_2]^-$) with mass accuracy (0.257 ppm). C) Molecular maps in the CA3 region of, clockwise, GABA or isomer, dopamine and serotonin using a gray scale overlaid with the sulfatide head-group ($[C_6H_9O_8S]^-$, green) and adenine ($[C_5H_4N_5]^-$, red). D) Single ion mapping of the same neurotransmitters in a polychromatic scale showing the relative ion intensity in the anatomical regions. E) Similar ion images of the CA1 region (orientated to A) show the distribution of GABA or isomer. All data acquired with 20 keV Ar_{3000} (lateral resolution ~ 2 μm, pixel size 1.95 μm) Orbitrap MS (mode 7). Results presented are from a single measurement.

Figure 6. Single-cell metabolomics for macrophages incubated with different concentrations of amiodarone. Dose response curves for cells from four treatment groups, control 0 µg/ml (*n*=8, blue), 1.56 µg/ml (*n*=6, magenta), 6.25 µg/ml (*n*=3, green) and 9.38 µg/ml (*n*=7, red) incubation culture concentrations for A) PC (34:1) at *m/z* 760.5848 $[C_{42}H_{83}NO_8P]^+$ (1.1 ppm), B) cholesterol $[M\text{-}H_2O+H]^+$ at *m/z* 369.3514 $[C_{27}H_{45}]^+$ (1.9 ppm) and C) the protonated molecular ion of the drug compound, amiodarone, at *m/z* 646.0307 $[C_{25}H_{30}I_2NO_3]^+$ (1.3 ppm). For each plot the data points (spheres) are overlaid on a box plot (25th quartile, median and 75th quartile) and the average (open circle) for each treatment group is connected with dotted line. D) Scatter plot of amiodarone $[M+H]^+$ intensity with PC (34:1) $[M+H]^+$ intensity for each cell. There is a positive linear correlation (Pearson's correlation coefficient = 0.91895). E) In situ tandem MS of amiodarone in a single cell. All data acquired with 20 keV Ar_{3000}^+ Orbitrap MS (mode 2).

METHODS:

Sample preparation.

We prepared mouse brain tissue sections using the following procedure. All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals. Female CD-1 mice, typically 6-8 weeks old, were sacrificed by Schedule 1 method. The brains were quickly extracted and frozen in dry ice. The intact brain was stored at -80 °C until sectioning. Coronal tissue sections were cut with a thickness of 10 µm using a Leica CM3050S cryostat (Leica, Germany) at -22 °C. The brain sections were thaw mounted on conductive indium tin oxide (ITO) glass slides (Bruker, Germany), and stored at -80 °C until further use.

SIMS analysis and histological Haematoxylin and Eosin (H & E) staining were performed on the adjacent tissue sections. The tissue was stained using a Leica ST5020 autostainer (Leica, Germany) using standard H & E protocol at GlaxoSmithKline. The stained sections were cover slipped with a xylene based mountant using a Leica CV5030 coverslipper (Leica, Germany). Optical images of the H & E stained tissue sections were obtained with a Hamamatsu Nanozoomer 2.0 HT microscope with 20x magnification (Hamamatsu Photonics, Japan). Haematoxylin (Mayer) and Eosin (1% aqueous) were obtained from Pioneer Research Chemicals Ltd (Essex, UK). The solvents, xylenes (ACS reagent >98.5%, xylenes + ethylbenzene basis) and denatured ethanol (UK IDA Standard) were obtained from Sigma Aldrich (Saint Louis, USA).

Reference samples of lipid and neurotransmitters were prepared as follows. C24:1 Mono-Sulfo Galactosyl(ß) Ceramide (d18:1/24:1) (Avanti Polar Lipids, USA, CAS 1246355-69-0, purity >99%) was drop deposited onto a clean silicon wafer from a 1:1 by volume methanol/chloroform solvent mixture. Reference samples of gamma-aminobutyric acid (GABA) (Sigma-Aldrich, USA, CAS 56-12-2, purity ≥99%), dopamine (Sigma-Aldrich, USA, CAS 62-31-7) and serotonin ((Sigma-Aldrich, USA, CAS 50-67-9, purity ≥98%), were prepared similarly. A reference sample of 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids, USA, CAS 59752-57-7, purity >99%) was prepared by pressing sample powder directly onto a previously cleaned silicon wafer using a clean metal spatula.

Single-cell studies used NR8383 cells (source is ATCC CRL-2192, Manassas, VA, USA), an immortalized cell line derived from a lung macrophage (Sprague-Dawley rat). The cells have been tested for mycoplasma within GlaxoSmithKline and are not listed in the database of commonly misidentified cell lines. These were grown on indium tin oxide (ITO) glass slides (Bruker, Germany) with Ham's F-12 Nutrient Mixture media containing GlutaMax and Phenol Red (Gibco, USA) and 15 % dialyzed, heat-inactivated fetal bovine serum (Gibco, USA). 5 mg/mL stock solutions of amiodarone hydrochloride (Sigma-Aldrich, CAS 19774-82-4, purity 98 %) were prepared in 50:50 water/ MeOH. The stock solutions were added to the growth media for a final media concentrations of 1.56 µg/mL, 6.25ug/mL and 9.38ug/mL. The cells were incubated (5 % CO2 and 37 °C) in the drug containing media for 72 hrs. Control cells were incubated alongside the dosed cells. To prepare the cells for analysis in the 3D OrbiSIMS, the media was removed and the cells were washed three times with 150 mM ammonium formate solution (Sigma-Aldrich, USA). This removes salts that cause unwanted signal suppression in SIMS. The cells were frozen and dried under vacuum (Genevac, USA) and stored at -80 °C until analysis. Before SIMS analysis, the samples were warmed to room temperature in a vacuum chamber. The cells for Figure 2 were prepared using the same method, in the same laboratory by the same person but at a later date.

To demonstrate nanoscale resolution using the Bi LMIG simultaneously with high mass resolution using the Orbitrap MS, a sample of Zirconium dioxide (ZrO_2) nanostructures was prepared. ZrO_2 nanoparticles were dispersed in aqueous solution and spotted onto a clean Al foil. During evaporation, crystals formed that were heterogeneous in size and exhibiting nanoscale features. For evaluation of the 3D imaging capability the NPL Organic Multilayer (OML) reference sample was used (NPL, UK). The reference sample consists of layers of Irganox 1010 and Irganox 3114 deposited onto a Si substrate to form four 1 nm thick layers of Irganox 3114 placed at depths of 50 nm, 100 nm, 200 nm, and 300 nm in a 400 nm Irganox 1010 film.

3D OrbiSIMS instrumentation

The instrument uses a Q Exactive™ HF for Orbitrap MS. In the Orbitrap MS modes (Figure 1) a quasi-continuous primary ion beam is used for secondary ion generation without affecting the mass resolution. This is due to the intermediate trapping capabilities of the Orbitrap for the secondary ion beam. Tandem MS, also known as MS/MS, operates in the same way as a Q Exactive™ HF. In this mode, the quadrupole mass filter isolates the precursor ions and the ions are fragmented in the higher-energy collisional dissociation (HCD) collision cell (with nitrogen gas, 0-100 eV collision energy). The fragment ions are transferred to the C-trap and orthogonally injected into the Orbitrap cell to produce MS/MS spectra with full mass resolution and mass accuracy. The MS/MS capability and the high-mass resolving power of the Orbitrap analysis improve signal to noise and the ability to detect low-abundance ions that may be obscured by nearby ions of higher intensity.

In ToF MS modes short (< ns) primary ion pulses generate secondary ions which are accelerated from the sample surface into the ToF analyser. In all SIMS experiments (both ToF MS and Orbitrap MS) an extraction potential of 2000 V is used (opposite polarity to the secondary ion). The measurement principle of the ToF requires either short primary ion pulses or in case longer primary ion pulses (< 200 ns) are applied a pulsed and delayed extraction of the mass analyser extraction electrode to obtain good mass resolution. Due to the very different ion detection systems (ToF MS uses single ion counting and Orbitrap MS uses image current detection and Fourier Transformation) the numerical values of the resulting signal intensities cannot be compared one-to-one. For the Orbitrap MS results a conversion factor needs to be determined in order to calculate the number of ions detected.

The 3D OrbiSIMS is equipped with a newly developed 30 keV bismuth cluster Bi LMIG (Bi Nanoprobe, ION-TOF GmbH) with a lateral resolution of < 200 nm (Supplementary Fig. 6). The ion gun is able to operate with selected cluster species (e.g Bi_3^+) in a quasi-continuous (DC) mode using a Wien filter. In combination with the ToF mass analyser usually 0.6 ns long ion pulses are achieved if bunching is applied. Longer pulse durations of 1.5 -150 ns can be obtained in un-bunched operation. In addition, a new 20 keV argon GCIB provides a focused beam of Ar clusters with <2 µm lateral resolution imaging (Supplementary Fig. 5). For the GCIB the size of the virtual source is larger compared to the Bi LMIG, rendering it technically more demanding to get to a small focal spot size. In order to improve the focal spot size of the gas cluster ion source the ion optical demagnification of the ion column was increased by optimization of the electrostatic lenses. Additionally, an aperture was introduced in order to block larger beam angles, thereby reducing aberration effects. The loss of beam current is more than compensated by operating the ion beam in a quasi-continuous mode for combination with the Orbitrap MS. By taking these measures it was possible to decrease the spot size to below 2 µm with a current of more than 30 pA and Orbitrap MS images with a resolution of <2 µm could be achieved.

The 3D OrbiSIMS can accommodate a large analysis area (up to the size of the sample holder, 100 mm x 70 mm). Its field of view and lateral resolution is ultimately limited by the acquisition time and the

size of the data file. Details of the GCIB with Orbitrap MS imaging mode 7 are provided in the Supplementary Protocol.[43] The instrument has cryogenic sample cooling in both the analysis and preparation chamber (sample temperature < -130 °C (143 K)) with a Leica VCT 500 (Leica, Germany) sample transfer system compatible with cryo-SEM and cryo-TEM instruments.

Pathways to reproducibility

To improve reproducibility between operators, we use mouse brain tissue samples from the same batch for training purposes and validation of the instrument performance. Images with the same spatial resolution and mass resolving power can be reproduced. To help with reproducibility, we include in the Supplementary Protocol[43] an author outside of this study. Reproducibility between instruments is not yet tested as this is currently the only instrument.

3D OrbiSIMS experimental methods

Mass calibration of the Q Exactive instrument was performed once a day using silver cluster ions. Mass calibration of the ToF was performed using the same ions. For the 3D single-cell image (mode 10) in Figure 2 the high lateral resolution images were obtained with 30 keV Bi_3^+ LMIG (approximately 300 nm resolution) from an analysis area of 100 µm x 100 µm using the ToF MS. These images are interleaved by Orbitrap MS acquired during the 20 keV Ar_{3000}^+ GCIB sputtering cycle from the same field of view, but with an additional sputter border of 20 µm width to avoid edge effects. 50 scans were accumulated in the ToF images corresponding to a total primary ion dose of 3.3×10^{12} ions/cm². The total GCIB sputtering dose was 1.1×10^{15} ions/cm². The cell height is estimated from AFM topographic images, Asylum MFP-3D (Asylum Research, USA), of 4 cells from the same sample post exposure to the 3D OrbiSIMS vacuum. The average cell height is 2.04 µm ± 0.23 µm (µ ± 1σ) $n = 4$ (2.04 µm, 2.23 µm, 2.18 µm, 1.71 µm). In Figure 3-5, the coronal tissue section was imaged with a focused 20 keV Ar_{3000}^+ ion source (in Fig. 4, 20 keV Ar_{2500}^+) with the Orbitrap MS (mode 7). A step-by-step protocol for label-free imaging of biomolecules, including lipids and neurotransmitters, in murine brain sections using the 3D OrbiSIMS is provided in the Supplementary Protocol.[43] An ion image containing 256 x 256 pixels was acquired over an area of 500 µm x 500 µm [pixel size = 1.95 µm x 1.95 µm]. Approximately, 2300 shots at 240 µs per cycle were accumulated in the C-trap per pixel. The Orbitrap analyser was operated in negative and positive ion mode at the 240,000 at *m*/*z* 200 mass resolution setting [512 ms transient time]. Mass spectra information was collected for a mass range from 60 to 1000 Da. The total ion dose was 2.0×10^{15} ions/cm^2. The total ion image acquisition time was approximately 10 hrs. Fast imaging survey image of the hippocampus formation was obtained with a pulsed 30 keV Bi_3^+ ion beam and the ToF mass analyser (mode 6). Stage and ion beam rastering were used to image the 3.5 mm x 2.3 mm analysis area [pixel size = 2 µm, ion images down binned to 8 µm by combining adjacent pixels]. The fast ToF MS image was acquired in negative ion mode and took approximately 36 mins. The total ion dose was 4.2×10^{10} ions /cm^2. The mass resolving power of the image was approximately 5000 at *m/z* 200. In Fig. 3d, the spectrum is for 15 pixels defined by an intensity threshold of > 2.7×10^3 with a background spectrum subtracted. The background spectrum was obtained by summing 15 pixels in the *SO* region of the ion image where the adenine signal is absent. The tandem MS spectrum in Fig. 4a was obtained using a 10 keV argon cluster beam. The collisional cell energy was set to 65 V. The spectra were accumulated for 60 s as the ion beam rastered in a random pattern in the *CC* region of the brain section. The tandem MS spectrum of a cholesterol (Supplementary Fig. 2) at *m/z* 369.3509 was acquired with a collision energy of 30 eV from the corpus callosum of a coronal mouse brain section, where it was found in abundance. The mass spectrum was taken at full mass resolving power and summed over 60 seconds.

We acquired metabolic profiles of amiodarone dosed cells (Fig. 6) using a 20 keV Ar_{3000}^+ ion beam and the Orbitrap MS (Mode 2). Spectra were summed across an analysis area of 20 µm x 20 µm until the entire cell was consumed. The Orbitrap analyser was operated in positive ion mode at the 240,000 at

m/z 200 mass resolution setting [512 ms transient time]. Mass spectra information was collected for a mass range from 60 to 1000 Da. The total ion dose ranged from 1.3 x 10^{14} to 7.8 x 10^{14} ions/cm^2.

To demonstrate the capability for depth profiling and 3D imaging we obtained negative ion data from the NPL organic multilayer reference material for three modes: mode 3 (single beam, 5 keV Ar_{2000}^+, Orbitrap MS); mode 9 (analysis with 30 keV Bi_3 and ToF MS, sputtering 5 keV Ar_{2000}^+) and mode 10 (5 keV Ar_{2000}^+ sputtering and Orbitrap MS analysis and 30 keV Bi_3 with ToF MS imaging). For all three modes, a 5 keV Ar_{2000}^+ beam with a current of 41 pA was used for sputtering a 250 µm × 250 µm region of the sample. The beam is defocussed to a spot size of 20 µm. Electrons with an energy of 21 eV and a current of 10 µA were used for charge compensation. In operational modes 3 and 10, secondary ions sputtered by the Ar_{2000}^+ beam from the central 130 µm × 130 µm of the crater were analysed using the Orbitrap spectrometer. In the dual beam modes, modes 9 and 10, secondary ion images with 128 x 128 pixels were obtained from the central 130 µm × 130 µm of the crater using a pulsed 30 keV Bi_3^+ with a beam current of 0.05 pA, and using the ToF analyser. These dual beam experiments were carried out in the non-interlaced mode. In mode 9, the sample was sputtered using the Ar_{2000}^+ beam for 2.0 s per cycle and imaged using the Bi_3^+ beam for 3.28 s. The ion dose ratio was 0.7% (Bi_3^+-to-Ar_{2000}^+). In mode 10, the sample was sputtered for 7.5 s per cycle, giving a dose ratio of 0.2%. The depth resolution is measured as the full width at half maximum of the *m/z* 564 delta layer peaks.

Data analysis

The 3D OrbiSIMS was controlled by software provided by SurfaceLab Version 6.7 (ION-TOF, Germany), which utilized the Thermo Fisher provided application programming interface (API) for both control of the Orbitrap MS portion of the instrument as well as online retrieval of the data. Both ToF-SIMS and Orbitrap MS image analyses were performed using SurfaceLab Version 6.7 (ION-TOF, Germany), OriginPro 2016 and MATLAB 2016a. Chemical structures were drawn in ChemDraw 14.0.

All Orbitrap MS images, depth profiles and linescans were created with a tolerance between ±2 and ±15 ppm, depending on the width of the signal peak, which increases proportional to the square root of its mass. This is the reciprocal of the resolving power at a given mass x 10^6. The resolving power is plotted in Supplementary Fig. 1b.

In Figure 2, the ToF-SIMS ion images for *m/z* 184, 157 and 646 were smoothed with a smoothing filter (average 1, ION-TOF SurfaceLab). 3D renderings were constructed using an iso surface visualisation for *m/z* 184 with a 7.2% iso value, 40% opacity, *xy* binning of 16 pixels and *z* binning of 4 scans. For the nuclear marker, *m/z* 157, and amiodarone [M+H]$^+$, *m/z* 646, a so-called volume visualization using a threshold at 15% with the same binning. SurfaceLab Version 6.7 (ION-TOF, Germany) was used. The 3D image was created after the *z* position of the voxels was adjusted to take the initial sample topography into account, using the assumption that the indium tin oxide substrate is a uniform flat plane.[44]

Statistical analysis

Samples were not randomised. The biological samples are included to demonstrate the new capabilities with the OrbiSIMS method rather than specific and comprehensive biological studies. The investigators were not blinded to group allocation.

The resolution of the GCIB beam was measured by imaging an electroformed mesh grid from the ion induced secondary electron image (Supplementary Fig. 3a). To achieve a reliable measurement the lateral resolution was measured from 246 line scans across the edge of the grid in different locations spread over 4.7 µm for both the x-axis and y-axis (Supplementary Fig. 3b,c). The FWHM of the ion beam profile (lateral resolution) is determined from the distance interval between the 88% to 12% intensities of an error function fit to the linescan across the edge. The resolution measurements are

fitted to a normal distribution with a mean and standard deviation of 1.72 µm ± 0.24 µm (*n*=246) across the *x*-axis and 1.04 µm ± 0.16 µm (*n*=246) across the *y*-axis (Supplementary Fig. 3d). The difference in the *x*-axis and *y*-axis FWHM lateral resolution measurements is due to the 45° angle of incidence along the *x*-axis (Supplementary Fig. 3). The same procedure was conducted with the secondary ion extraction potential on (needed for ion detection) and resolution degrades with the ion extraction optics (Supplementary Fig. 4). The average FWHM lateral resolution was 2.49 µm ± 0.36 µm (µ ± 1σ) (*n*=243, grey bars) across the *x*-axis and 1.84 µm ± 0.36 µm (µ ± 1σ) (*n*=254, red bars) across the *y*-axis. For a practical measurement of the resolution in tissue imaging 5 measurements are made from the intensity line scan of summed intensity of nuclear markers $[C_4N_3]^-$ at *m/z* 90.0095 (2.8 ppm), $[CN_2O_2P]^-$ at *m/z* 102.9702 (0.6 ppm), $[C_4H_2N_4]^-$ at *m/z* 106.0285 (0.3 ppm), $[C_4H_3N_4]^-$ at *m/z* 107.0207 (0.2 ppm), $[C_5HN_4]^-$ at *m/z* 117.0207 (0.2 ppm), $[C_5H_3N_4]^-$ at *m/z* 119.0363 (0.3 ppm), $[C_5HN_4O]^-$ at *m/z* 133.0156 (0.1 ppm) and $[C_5H_4N_5]^-$ at *m/z* 134.0472 (-0.1 ppm). The mean and standard deviation values are 1.34 µm ± 0.24 µm (*n*=5).

The spatial resolution for the 30 kV Bi_3^{2+} LMIG was determined using the ZrO_2 nanostructures shown in the total ion image (Supplementary Fig. 6a) and the ZrO^+ image (Supplementary Fig. 6b). We acquired a high resolution secondary electron image (Supplementary Fig. 6c) and ion image (Supplementary Fig. 6d) from a 20 µm x 20 µm region with a pixel size of 78 nm. As previously, 95 *y*-axis line scans of the total ion intensity across the edge of the crystal were acquired (Supplementary Fig. 6e). Measurements were made over a length of 1.5 µm. A normal distribution is fitted to the population to give the mean and standard deviation of 172 nm ± 61 nm (µ ± 1σ) (*n*=95). The bismuth ion gun was operated at 60 µs per cycle and 1800 shots per pixel. The secondary ions were collected in the C-trap for 500 ms before being injected into the Orbitrap analyser. The trap was operated in positive ion mode, at the 240,000 at *m/z* 200 mass resolution setting. The total ion dose was 4.28 x 10^{16} ions/cm^2. The total ion image acquisition time was approximately 9 hrs.

The single-cell drug accumulation and lipid upregulation (Fig. 6d) correlation calculations were performed in OriginPro software (2015; OriginLab, MA, USA). The correlation test was performed using a Pearson's (2-tailed) correlation test at the 95% confidence level.

Data availability

The data that support the findings of this study are available in figshare with the identifier 10.6084/m9.figshare.5459680.[45] We provide the Orbitrap data in the mzML format for spectra and the centroided ImzML format for images. The ToF image in Figure 2 is in the GRD data (TOF-SIMS generic raw data) format. Data may be opened using Spectral Analysis software[46] available from Github: https://github.com/AlanRace/SpectralAnalysis.

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