A complete workflow for high-resolution spectral-stitching nanoelectrospray direct-infusion mass-spectrometry-based metabolomics and lipidomics

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Metabolomic and lipidomic studies measure and discover metabolic and lipid profiles in biological samples, enabling a better understanding of the metabolism of specific biological phenotypes. Accurate biological interpretations require high analytical reproducibility and sensitivity, and standardized and transparent data processing. Here we describe a complete workflow for nanoelectrospray ionization (nESI) direct-infusion mass spectrometry (DIMS) metabolomics and lipidomics. After metabolite and lipid extraction from tissues and biofluids, samples are directly infused into a high-resolution mass spectrometer (e.g., Orbitrap) using a chip-based nESI sample delivery system. nESI functions to minimize ionization suppression or enhancement effects as compared with standard electrospray ionization (ESI). Our analytical technique—named spectral stitching—measures data as several overlapping mass-to-charge (*m/z*) windows that are subsequently 'stitched' together, creating a complete mass spectrum. This considerably increases the dynamic range and detection sensitivity—about a fivefold increase in peak detection—as compared with the collection of DIMS data as a single wide mass-to-charge (*m/z* ratio) window. Data processing, statistical analysis and metabolite annotation are executed as a workflow within the user-friendly, transparent and freely available Galaxy platform (galaxyproject.org). Generated data have high mass accuracy that enables molecular formulae peak annotations. The workflow is compatible with any sample-extraction method; in this protocol, the examples are extracted using a biphasic method, with methanol, chloroform and water as the solvents. The complete workflow is reproducible, rapid and automated, which enables cost-effective analysis of >10,000 samples per year, making it ideal for high-throughput metabolomics and lipidomics screening—e.g., for clinical phenotyping, drug screening and toxicity testing.

INTRODUCTION

Nontargeted metabolomics and lipidomics techniques comprise the measurement and analysis of the steady-state levels of the multiple metabolites or lipids (termed the metabolome or lipidome) within biological samples[1–4]. Comparison of the metabolome or lipidome across different phenotypes represents a powerful and unbiased approach for discovering molecular perturbations that can in turn be used to generate biological hypotheses for more detailed investigation. This approach is applicable to diverse sample types, including biofluids[5,6], mammalian cells[7] and spent cell media[8], tissues[9] and whole organisms[10], as well as to a wide range of biological questions such as the investigation of disease[9], drug action[7] and toxicology[11]. Metabolites and lipids have many diverse functions in biological systems. They are typically the end products of complex cellular regulation networks[1] (at the genetic, epigenetic, transcriptional, translational and post-translational levels), and they can also influence and alter this regulation via feedback loops[12], protein modifications[13] and epigenetic changes[14]. They are both building blocks of complex cellular macromolecules and sources and intermediates in energy metabolism. Thus, metabolomics and lipidomics analyses can give insightful knowledge of the functional molecular status of biological systems (e.g., energetic status and the balance of anabolic and catabolic processes) and can complement other 'omic technologies as well as traditional molecular biological investigations.

To accurately and reliably interpret data derived from metabolomics and lipidomics studies, the entire workflow, including the experimental design, sample collection and extraction, as well as data acquisition, processing, metabolite identification and statistical analysis, should be robust and reproducible. High detection sensitivity of the chosen analytical approach is also desirable. The most common analytical techniques used in metabolomics include NMR spectroscopy and mass spectrometry (MS)[15]. Although NMR is highly quantitative and reproducible, MS methods are increasingly favored for metabolomics and lipidomics because of their higher sensitivity[15,16]. MS approaches often include chromatographic separation, which serves to resolve the complex mixture of metabolites or lipids before ion detection, thereby helping to distinguish between isobaric compounds and to minimize ionization suppression (or enhancement) effects. It is because of these attributes that gas chromatography MS[17] and liquid chromatography (LC) MS[18], and to a lesser extent capillary electrophoresis MS[19] and ion chromatography MS[20], are popular and widely used for metabolomics and lipidomics investigations. DIMS is an alternative approach involving the direct introduction of biological extracts into MS systems without any prior chromatographic separation[21]. Nominal-mass, flow-injection ESI DIMS approaches have previously been developed and successfully applied to high-throughput metabolomics[22]. However, substantially higher-quality DIMS data can be acquired by using automated nESI sample delivery platforms in combination with ultra-high mass resolution and accuracy MS detectors (e.g., Fourier transform ion cyclotron resonance (FT-ICR) MS, Orbitrap MS)[10,23,24]. These

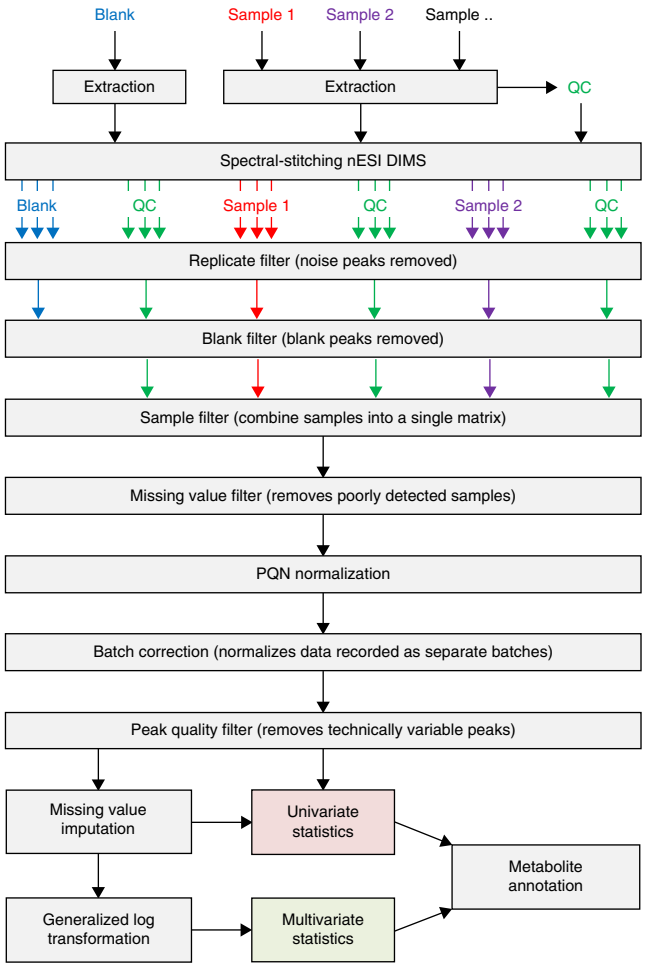

Figure 1 | Flowchart showing the full spectral-stitching nESI DIMS workflow. Blank, extraction preparation done in the absence of sample; PQN, probabilistic quotient normalization; QC, quality control sample used to measure analytical reproducibility.

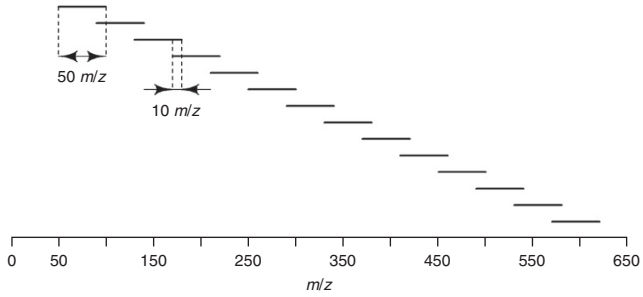

Figure 2 | Schematic of the spectral-stitching nESI DIMS method for application on the Q Exactive Hybrid Quadrupole Orbitrap mass spectrometer. Data are collected as a series of 50 *m/z* wide SIM windows that overlap by 10 *m/z*. Windows are 'stitched' together to create a full mass spectrum. The optimized parameters for application of this method on the LTQ FT, LTQ FT Ultra, Q Exactive and Orbitrap Elite mass spectrometers are shown in **Table 1**.

platforms increase the number of detectable peaks by minimizing ionization suppression and enhancement effects (relative to ESI)[25]. They also maximize the discrimination of peaks with similar accurate masses, thereby facilitating more accurate molecular formula(e) peak annotations of the data[26].

The protocol described here is a complete workflow for conducting high-resolution nESI DIMS metabolomics and lipidomics investigations (**Fig. 1**), guided by the Metabolomics Standards Initiative framework[27]. The optimized nESI DIMS method, termed 'spectral-stitching nESI DIMS', collects MS data as a series of overlapping *m/z* windows that are subsequently stitched together into a complete spectrum (**Fig. 2**; **Table 1**). This approach maximizes both the quantity and mass accuracy of detectable peaks[23,24]. The automated data processing and analysis steps are collated into an open-source workflow within Galaxy[28]. This offers a standardized, transparent and user-friendly approach without the requirement for bioinformatics expertise and knowledge of multiple programming languages and/or environments. Galaxy is an open-source workflow platform that is used for next-generation sequencing data analysis. It has many standard processing tools (accessible from its web-based user interface) that improve both the speed and reproducibility of data processing and analysis. Our recent development of the Galaxy-M spectral-stitching nESI DIMS workflow considerably increases the accessibility of the spectral-stitching nESI DIMS metabolomics approach[28].

Advantages and limitations of the spectral-stitching nESI DIMS workflow

A major advantage of the spectral-stitching nESI DIMS workflow is the generation of high-quality metabolomics or lipidomics data, with high spectral resolution, mass accuracy and dynamic range, in a short time frame and in a cost-effective manner. The high resolution and mass accuracy allow for the accurate annotation of peaks with molecular formula(e) and for compounds with similar masses to be resolved[23,26], which is particularly advantageous when analyzing complex mixtures of chemicals. Spectral-stitching nESI DIMS increases detection sensitivity fivefold as compared with standard wide-scan DIMS (for a constant number of ions entering the detector at each scan)[23] and is highly reproducible: the median relative standard deviation (RSD) of peak intensities in 80 repeated injections of the same biological sample was 8.2% (ref. 29). The intensity measurements are robust and correlate well with peak intensities measured using quantitative NMR methods for selected metabolites[11,30], demonstrating the high relative-quantification capability of the method (see ANTICIPATED RESULTS). In addition, DIMS has been suggested to have metabolomic classification and prediction capabilities comparable to those of LC–MS[31]. DIMS is more applicable to lipidomics than is NMR because of the highly congested lipid region in ^{1}H NMR spectra, and the higher sensitivity of DIMS as compared with NMR is a clear advantage for both metabolomics and lipidomics. As compared with chromatography-based MS, DIMS approaches—including the spectral-stitching method—have considerably shorter acquisition times (enabling higher throughput); no chromatographic drift (as observed for LC–MS as the column ages), enabling more robust alignment of multiple spectra; no carry-over between biological samples; and lower consumables costs.

The spectral-stitching nESI DIMS workflow is a global profiling method, and therefore it cannot detect all metabolites or lipids in a single experiment. For the analysis of specific metabolites or lipids, extraction procedures that are designed to maximize these compound classes should be used before DIMS data collection. A limitation of DIMS is ion suppression or ion enhancement,

TABLE 1 | The optimized parameters for the spectral-stitching nESI DIMS metabolomics and lipidomics methods.

	LTQ FT	LTQ FT Ultra		Q Exactive[a]		Orbitrap Elite[a]	
Extract type	**Polar**	**Polar**	**Non-polar**	**Polar**	**Non-polar**	**Polar**	**Non-polar**
m/z range covered	70–500 *m/z*	70–590 *m/z*	70–1010 *m/z*	50–620 *m/z*	50–1020 *m/z*	50–620 *m/z*	190–1,200 *m/z*
Total number of *m/z* windows	21	7	13	14	24	10	18
SIM window width	30 *m/z*	100 *m/z*	100 *m/z*	50 *m/z*	50 *m/z*	75 *m/z*	75 *m/z*
m/z window acquisition time	15 s (~10 transients)	15 s (~10 transients)	15 s (~10 transients)	~5.5 s (minimum of 10 microscans)	~5.5 s (minimum of 10 microscans)	~7.8 s (minimum of 10 microscans)	~7.8 s (minimum of 10 microscans)
Overlap between *m/z* windows	10 *m/z*	30 *m/z*	30 *m/z*	10 *m/z*	10 *m/z*	20 *m/z*	20 *m/z*
AGC target	1×10^5	1×10^6	1×10^6	5×10^5	5×10^5	5×10^5	5×10^5
Peak resolution	100K at 400 *m/z*	100K at 400 *m/z*	100K at 400 *m/z*	140K at 200 *m/z*	140K at 200 *m/z*	240K at 400 *m/z*	240K at 400 *m/z*
Acquisition time per injection	5 min 45 s[b]	2 min 15 s[b]	3 min 45 s[b]	2 min 30 s[b]	3 min 30 s[b]	1 min 50 s[b]	2 min 50 s[b]

Selected ion monitoring (SIM) mode is used throughout. For a schematic of the spectral-stitching nESI DIMS method, see **Figure 2**.
[a]See **Supplementary Methods 1** and **2** for Q Exactive and Orbitrap Elite instrument method files, respectively. [b]Including an initial 30-s start delay of blank or dummy scans to allow the nESI to stabilize.

which are caused by multiple different compounds entering the ionization source at the same time[32]. However, the low flow rate associated with nESI, as used here, minimizes these artifacts[25,33]. Sample lipid concentration can vary between sample types. To prevent highly abundant lipids from dominating the DIMS analysis, a dilution study is required to find the optimal sample dilution (see 'Resuspension of biological sample extracts in DIMS solvents' in the protocol below)—a sample lipid concentration of <10 pmol/µl is recommended[34]. The limitations of DIMS as compared with chromatography-based MS or NMR techniques include the inability to resolve isobaric compounds and the inability to provide any structural information on compounds unless further MS/MS analyses are conducted. However, it is typically very difficult to isolate just the ion of interest for DIMS/MS, given the complexity of the mass spectra[11]. A further limitation of DIMS is the relative-quantitative nature of the measurement—i.e., as for LC–MS metabolomics, it measures relative fold-changes between biological samples. In comparison, NMR can be regarded as fully quantitative, and LC–MS/MS using internal standards is also quantitative, albeit just for selected peaks of interest. If desired, these approaches can be used to fully quantify metabolites that are discovered to be important by the spectral-stitching nESI DIMS method workflow. Therefore, DIMS, chromatography-based MS and NMR each possess different advantages and limitations, and act as complementary approaches for metabolomics and lipidomics, depending upon the analyst's requirements.

Applications of the spectral-stitching nESI DIMS workflow

The spectral-stitching nESI DIMS workflow is ideal for large-scale discovery studies[29]. It has successfully been applied to a diverse range of biological sample types, including tissues[11], mammalian cells[7], whole-organism homogenates[10] and hemolymph[35], and is also applicable to urine[36], serum[31] and plasma analysis[37]. Furthermore, it has been used to investigate a broad range of biological questions, including studies of xenobiotic toxicity in whole organisms[10] and in specific organs[11,38]; identification of metabolic changes associated with disease[39]; elucidation of the action of therapeutic drugs on the lipidome of human leukemia cells[7]; and metabolic footprinting analysis to understand the interactions of algae with their environment[40]. The high resolution of the MS detector means that this workflow is also compatible with ^{13}C-labeling 'pulse-chase' or 'flux' approaches, wherein the metabolism and fate of a ^{13}C-labeled substrate such as ^{13}C-glucose or ^{13}C-glutamine is measured as a function of time—e.g., measurement of ^{13}C-glucose incorporation into fatty acids and phospholipids in response to drug treatments[7]. A further example of the application of this DIMS workflow is that of the classification of compounds as being of endogenous, exogenous or metabolized-exogenous origin, following exposure of an organism to an undefined chemical mixture, by analyzing both the organism's tissue and the exposome[41]. Given that metabolomics has now matured to a widely used, relatively stable analytical and computational approach, with a rapidly growing community supported by a wide range of training opportunities for researchers, the field is now poised to tackle very-large-scale challenges in the biomedical and regulatory sciences. As one example, regulatory toxicology is now opening the door to new testing strategies for determining the impacts of chemicals on human health. Given that there are approaching 100,000 chemicals used in industry and consumer products, the need for very-high-throughput screening approaches, incorporating 'omics' data generation, are paramount. The protocol reported here is an important step toward translating metabolomics into regulatory toxicology[42,43].

Experimental design

A well-designed experiment is essential to ensuring a meaningful and robust outcome to a DIMS study. Biological sample collection, preparation and nESI DIMS data generation must be randomized across sample classes (typically different biological phenotypes) to prevent user-induced bias. The amount of biological replication required to provide adequate statistical power to the study will be influenced by the type of experiment being conducted. On the basis of a decade of expertise, our recommended levels of biological replication vary from $n = 6$ (for well-controlled laboratory-based studies with relatively little intersample biological variance, such as nESI DIMS of mammalian cell culture extracts) to $n = 10$ (controlled laboratory studies but with greater biological variance, such as model organism studies) to larger n values for cases in which biological variance is not controlled (clinical and environmental biological samples). With pilot data, this estimation can be improved by applying sample size and power analyses (MetaboAnalyst 3.0, http://www.metaboanalyst.ca). Technical replication should also be included in the nESI DIMS workflow as a method for distinguishing peaks of biological origin from background noise, increasing the accuracy of the intensity measurements and allowing for an estimation of the reproducibility of peak intensity measurements[44] (see the 'Data processing' section, below). To distinguish peaks of biological origin from genuine but non-biological peaks, such as those that may arise from the sample preparation method (i.e., from solvents and/or contaminants), an 'extract blank' (sample preparation procedure conducted in the absence of a biological sample) should also be prepared and analyzed. Quality control (QC) samples are a critical component of both the nESI DIMS experimental design and data QC (see 'Data QC' section, below). Internal QC samples should be derived by pooling a small volume of ideally all biological samples within one metabolomics study, and then analyzing them at regular intervals throughout the analytical run—e.g., every fifth or sixth DIMS analysis is of a QC sample. Internal QC samples are used to monitor and correct for slight analytical variance between samples acquired through one or more analytical batches within that one study (see the signal correction algorithm, 'Data processing' section). External QC samples (i.e., using a surrogate sample) can be used to monitor and correct for analytical variance between different metabolomics studies.

Biological sample collection, storage and preparation

The time between biological sample collection and freezing should be as short as possible in order to rapidly halt enzymatic activity and cease changes to the metabolome or lipidome. For mammalian cell culture, a quenching approach is used, in which cells are sampled directly into a quenching solution (such as −40 °C aqueous methanol)[7,45]. For the collection of small model organisms (e.g., *Daphnia* and *Drosophila*)[46], tissue from larger organisms[47] and biofluids (e.g., urine[48], serum[49] or plasma[49]), we refer the reader to the appropriate specialist literature. Biological samples must remain frozen at −80 °C until extraction of the metabolites and/or lipids.

Preparation of solid biological samples typically involves their physical disruption (by a homogenization probe—e.g., Polytron—or a bead-based homogenizer—e.g., Precellys 24 (ref. 50)) in the presence of solvents to simultaneously extract the polar metabolites and/or lipids into a liquid phase while denaturing the metabolic enzymes to halt any further changes to the metabolome or lipidome[50,51]. For serum, plasma[49] and mammalian cell suspensions[7], vortex-mixing in the presence of the extraction solvent is sufficient to denature the enzymes and to extract the metabolites and/or lipids. Urine can be more simply prepared by centrifugation and dilution with water because of its very low protein (and therefore very low enzyme) content[48]. Extraction techniques should be reproducible—i.e., introduce minimal technical variation to the study—and should achieve as high a yield of metabolites and/or lipids as possible. Several solvent systems can be used[50,51]. Fine adjustments to the extraction conditions, e.g., pH and solvent polarity, can be used to extract specific classes of compounds according to their solubility characteristics[52]. Biphasic extraction methods use immiscible solvents to simultaneously extract polar metabolites and lipids into separate phases[50,51,53,54]. This reduces biological sample complexity, which is advantageous for (non-chromatography-based) nESI DIMS. Our optimized biphasic extraction—based on a procedure developed by Bligh and Dyer[53]—uses a methanol:chloroform:water ratio of 2:2:1.8 to maximize metabolite yield, metabolic sample stability and reproducibility[50]. Alternatively, the use of methyl tert-butyl ether, methanol and water is an increasingly popular biphasic approach for lipidomics that improves the extraction of some lipid classes (e.g., ceramides) as compared with the Bligh and Dyer method[54].

After extraction, extracts should be dried to maintain stability and, subsequently, to enable resuspension in appropriate solvents for DIMS; note that lipids must be dried and stored under nitrogen to prevent oxidative lipid damage, whereas polar metabolites in an aqueous methanol solution are more typically dried using a vacuum centrifugation system such as the SpeedVac concentrator.

Direct-infusion mass spectrometry

Sample ionization and analytical detection methods can strongly influence the quality of DIMS metabolomics and lipidomics data. ESI, a soft-ionization technique, is particularly appropriate for DIMS, as it can predominantly ionize the metabolites and/or lipids as intact compounds[16], aiding compound molecular formula(e) annotations. A very low flow rate through the electrospray ionization source is essential for minimizing both ionization suppression and enhancement effects[25,33]; our nanoflow ESI methods typically use a flow rate of 200–300 nl/min, as compared with the several-microliters-per-minute rates used for standard ESI sources. nESI also benefits from lower sample volume requirements and considerably higher sensitivity[55]. To aid nESI compound ionization, a modifying agent is typically added to the solubilized biological sample (e.g., formic acid or ammonium acetate), which will also influence the types of ion forms that are created (see the 'Peak annotation' section, below). High lipid concentrations in samples can lead to aggregate formation (e.g., dimers) during DIMS analysis[34]. This can be prevented by keeping lipid concentrations <10 pmol/µl when using a 2:1 methanol:chloroform ratio[34]. For high-throughput metabolomics and lipidomics, an automated chip-based nESI source (e.g., Advion TriVersa NanoMate) enables rapid, reproducible and automated acquisition of multiple biological samples without sample-to-sample cross-contamination[25,55].

Ultra-high-resolution, high-mass-accuracy detectors (e.g., the Orbitrap series of mass spectrometers and FT-ICR, both with a resolution capable of exceeding 100K at a *m*/*z* value of 200) are ideal for DIMS as they resolve peaks with similar accurate masses and allow for the annotation of peaks with molecular formula(e). This increases the coverage of the metabolome or lipidome and enhances annotation confidence as compared with lower-resolution, lower-mass-accuracy instruments. For Thermo Scientific Orbitrap series and FT-ICR mass spectrometers, the number of ions entering the detector is controlled by the Automatic Gain Control (AGC). The number of ions within the detector is approximately proportional to detection sensitivity; however, if the number of ions is too high, then the mass accuracy decreases (due to space-charge effects[56]), ultimately making unique molecular formula(e) annotation impossible. This is particularly problematic when collecting DIMS data over a single large *m*/*z* range (e.g., 100–1,000 *m*/*z*), for which a higher AGC setting is required to capture and then detect the lower-abundance compounds. A proven solution to this is provided by the spectral-stitching nESI method, in which data are collected as a series of overlapping *m*/*z* windows—by using selected ion monitoring (SIM) scan mode for the Thermo Scientific FT-ICR and Orbitrap series—and then 'stitching' these windows together to create a complete mass spectrum. The AGC target value does not need to be set as high for narrower *m*/*z* windows (with fewer detectable features) as compared with a full-scan spectrum to achieve similar peak detection sensitivity. Overall, this approach increases the dynamic range and detection sensitivity while retaining high mass accuracy by minimizing space-charge effects that would arise from high AGC settings (**Fig. 2**; **Table 1**)[23,24].

The spectral-stitching method was optimized for the FT-ICR, Q Exactive and Orbitrap Elite DIMS by determining the highest AGC setting (i.e., highest sensitivity) that retains high mass accuracy; by establishing the *m*/*z* window width for maximal peak detection; and by calculating the minimum number of scans for reliable and reproducible peak detection. Detector sensitivity at each end of the individual narrow *m*/z windows was found to be lower than across the majority of each *m*/*z* range, and thus it was characterized to establish the usable high-sensitivity region of each *m*/*z* window that was retained (and hence determine the overlap of the *m*/z windows that was required in the method to prevent signal loss). Before applying this method, optimization of the biological sample concentration is strongly advised, in order to prevent poor nESI stability, poor RSD values of the peak intensities from samples that are too concentrated and poor detection sensitivity for those samples that are not sufficiently concentrated.

Data processing

Before data processing, DIMS spectra should be viewed using the vendor's software to identify any analyses for which the electrospray failed during acquisition; such analyses should be removed. DIMS data on both the FT-ICR and Orbitrap platforms are recorded as transient (time domain) files and as triplicate technical analyses[23,24] (**Fig. 1**); for some mass spectrometers, time domain data are available to the analyst only as a single preprocessed vendor-encoded file (a .raw file, in the case of Thermo Scientific), whereas for other spectrometers the data are available in their rawest form as multiple transient data files. For this latter case (for Bruker and Thermo Scientific FT-ICR MS instruments), data processing includes averaging of the transient data, Hanning apodization and zero-filling, followed by Fourier transformation to convert time into the frequency domain using custom-written Matlab code[23,24,57]. From this point, data processing is consistent across all DIMS approaches described in this protocol (**Fig. 1**). To remove obvious noise features from the data, any peaks with a signal-to-noise ratio (SNR) threshold of less than (typically) 3:1 are removed. Data are then mass calibrated to convert from the frequency domain into *m*/*z* values, using a calibration equation (for the FT-ICR, Thermo Scientific instruments use $m/z = (A/f) + (B/f2)$[23,56]; *f* is the frequency (KhZ); A and B are calibration parameters from the instrument). External calibration uses the calibration parameters derived from the periodic calibration of the mass spectrometer using a defined sample of known standards. Postacquisition internal calibration is used to increase the mass accuracy in each window by using the accurate masses of metabolites known to be present in the mass spectra of the biological samples. Finally, the multiple narrow *m*/*z* windows are stitched together into one continuous *m*/*z* spectrum using custom-written Matlab code.

To identify genuine peaks and to remove noise features within the mass spectrum of each biological sample, only peaks that are present in at least two out of three of the technical replicate analyses of the sample are retained (termed 'replicate filtering'). Contaminate peaks arising from the extraction method are flagged by comparing the biological sample spectra with the extract blank, and are then removed if their intensity in the sample is less than three times that of the extract blank (termed 'blank filtering'). The *m*/*z* data from multiple biological samples are combined into a single data matrix with samples as rows and *m*/*z* features as columns. Peaks are retained if they are present in a given percentage of samples defined by the user (termed 'sample filtering')—e.g., 100% for the calculation of normalization quotients (see below); >80% as a default setting to achieve robust sample filtering[57]; and lower-percentage filtering in the case in which multiple classes exist in the data set and peaks within a single class need to be retained. Note that the lower the sample filter target percentage, the greater the number of missing values that will be created in the data set. At this point, the number of peaks within each biological sample is assessed, and those with abnormally high numbers of missing values are deemed technically poor and removed using 'missing-value filtering' (see 'Data QC' section, below).

Before statistical analysis, several data preprocessing steps are required. As a first step, data matrices are normalized by the probabilistic quotient normalization (PQN) approach to remove the variation caused by unequal amounts of detectable metabolites in each of the biological samples[58]. For studies in which the analytical measurements are made across several batches, the intensity measurements of QC peaks in different batches are normalized to correct the technical variation arising from inter-batch measurements using a signal correction method[59]. This approach can also be used to correct signal-intensity technical variation that arises within a single batch. The signal correction method requires QC samples to be acquired at regular intervals throughout the analytical run. Next, the RSD values of the intensities of each spectral peak are assessed within the QC samples[44], and any peaks with unacceptably high RSD values are deemed unreliable measurements and removed using the 'peak quality filter' (see 'Data QC' section, below). Missing values are now imputed into the

normalized DIMS data matrices. Missing values generally occur because (i) the metabolite is not present or (ii) the instrument fails to detect a metabolite that was present. As missing values can be problematic for statistical analyses, they are typically imputed using the K-nearest-neighbor method (KNN), which has been demonstrated to be the most suitable for DIMS data[60]. However, in cases in which the user deems missing values to arise from genuinely missing metabolites, they may choose to omit this step. Before analysis of multivariate statistics, a generalized log transformation is optimized (for each specific data set using the QC sample data) and applied to stabilize the technical variance and reduce the dominance of the highly intense and variable peaks in the multivariate statistics[61,62].

Data quality control

In summary, seven filtering steps are applied in the pipeline to ensure high data quality:

(i) if the nESI spray current fails during data collection on the mass spectrometer, the affected analyses are manually discarded; (ii) for peaks to be considered actual detected features, they must be observed in at least two DIMS measurements of the biological sample (termed 'replicate filtering', see above and Step 20); (iii) only peaks occurring at more than three times higher intensity in the biological samples relative to the blank samples are retained in the data set (termed 'blank filtering', see above and Step 23); (iv) all peaks retained in the data set occur in the majority of biological samples measured in the study (termed 'sample filtering', see above and Step 24); (v) all biological samples have a relatively consistent number of peaks (termed 'missing-value filtering', see above and Step 25); (vi) any batch effects or temporal drifts in peak intensity, assessed on a peak-by-peak basis using the QC samples, are corrected for (termed 'signal correction', see above and Step 28); (vii) the reproducibility of peak intensities is calculated within the QC samples across the data set on a peak-by-peak basis. Nonreproducibly measured peaks are excluded (termed 'peak quality filter', see Step 28).

Univariate and multivariate statistical analysis

Nontargeted nESI DIMS metabolomics and lipidomics studies aim to measure the variation and covariation of metabolite or lipid abundances between or across biological sample groups in order to generate new hypotheses for subsequent targeted investigation. Explorative analysis using unsupervised multivariate statistical methods (e.g., principal components analysis, PCA) is carried out first to assess the data reproducibility, detect possible outliers and visualize possible groupings in the data set. Subsequently, a univariate statistical test is applied to each peak in the nESI DIMS data to identify specific peaks whose intensities significantly change across different biological sample groups. These tests can be parametric (assuming a normal distribution—e.g., *t*-test or ANOVA) or nonparametric (e.g., Wilcoxon signed-rank or Kruskal–Wallis test). Because of the large number of peaks that are often analyzed (and statistically tested), a multiple testing procedure is carried out to reduce the occurrence of peaks incorrectly being identified as significant (i.e., to reduce false positives). The optimal approach for DIMS data is to apply the Benjamini–Hochberg false-discovery rate procedure[63]. A disadvantage of univariate tests is that possible correlations between peaks are not taken into account. Therefore, supervised multivariate approaches (e.g., partial least squares discriminant analysis, PLS-DA) are used as a complementary approach to discover individual metabolites or groups of metabolites that discriminate between different biological sample groups. Note that these models can be used to predict the class (i.e., healthy or diseased) of any subsequent biological samples. The prediction accuracy, based on internal cross-validation of the data or an independent test set, is used as a measure of the group separation. Statistical significance of the observed group separation is evaluated with permutation testing. In a permutation test, the labels of the biological samples are randomly permuted and a new PLS-DA model is constructed. This process is repeated a thousand (or more) times. Statistical significance of the original PLS-DA model is then assessed by relating the values of the group separation of the permutated data to those of the nonpermuted data. Although the permutation test is a powerful statistical procedure, its results should be approached cautiously for very small sample sizes ('Experimental design')[64,65]. Peaks that contribute to the group separation are identified based on PLS-DA variable importance measures such as the variable importance in the projection (VIP) or the selectivity ratio (SR). To reduce the influence of irrelevant peaks on the model, forward (variable) selection that is based on the VIP or SR can be carried out before permutation testing.

Metabolite annotation

Automated and robust annotation and identification of hundreds to thousands of peaks in MS-based metabolomics is currently a difficult, complex process and is widely regarded as a bottleneck of data interpretation[66]. Analytical and computational developments are, however, continuing to improve this procedure. Metabolite annotation in spectral-stitching DIMS studies starts by assigning elemental compositions to all (or as many as possible) *m*/*z* measurements, followed by mapping each elemental composition to a single or multiple chemical name(s). This is done using in-house-developed MI-Pack software situated within Galaxy that interacts with public databases (e.g., Human Metabolome Database (HMDB), Kyoto Encyclopedia of Genes and Genomes (KEGG), LIPID MAPS and PubChem)[67]. Because of the finite mass accuracy of mass spectrometers and the complexity of chemical space, this process often results in multiple annotations per peak; however, the ultra-high mass accuracy used here minimizes the number of annotations. To further reduce the number of false-positive assignments, we use several approaches[67,68]: a single metabolite is often detected as multiple metabolic features, including adducts (e.g., $[M+H]^+$ and $[M+Na]^+$) and naturally occurring isotopes. The latter are often used to reduce the number of incorrect elemental compositions using relative isotopic abundance measurements to calculate the number of atoms (e.g., carbon, sulfur and nitrogen) within the metabolite[24]. To categorize the confidence of metabolite identification and to make metabolite assignments within a study or across multiple studies comparable, different levels of metabolite annotation and identification have been reported and described by the Metabolomics Standards Initiative (MSI)[27]. Metabolite assignments in nESI DIMS experiments are based upon *m*/*z* values and therefore are reported as putatively annotated (defined as level 2 by the MSI); however, the ultra-high mass accuracy and additional steps used in our DIMS workflow (e.g., the use of adduct and isotope patterns) result in a high degree of confidence in the

molecular formula(e) annotations. Additional targeted experiments, such as MS^n fragmentation and spectral library matching against an authentic standard, are required to definitively identify the compound(s) of interest (defined as level 1 by MSI).

Data storage and sharing

Metabolomic studies continue to increase in size and produce ever-increasing amounts of experimental data. Open access to research data and knowledge, in a standardized and reproducible way, is important for maximizing the value of metabolomics (or any other) data sets. As the requirements of journal publishers (including data journals such as *Scientific Data* and *Gigascience*) and funding bodies to share data and results continue to grow, it is likely and indeed highly preferable that open access to data will become standard practice in metabolomics. This is supported by the increasing number of publicly available repositories for experimental data (e.g. MetaboLights and Metabolomics Workbench[69,70]), as well as the number of metabolomics data sets within these public repositories. Recently, a data descriptor for a spectral-stitching nESI DIMS metabolomics study—in which the efficacy of a signal-correction algorithm and the reproducibility of a multibatch study were evaluated—was published in such a data journal, and the data are openly available via the MetaboLights repository (MTBLS79; ref. 69). This data set serves as a benchmark for the metabolomics community and complements the current publication[29].

MATERIALS

REAGENTS

- Precollected and frozen (at −80 °C) biological samples: urine, serum, plasma and tissue **! CAUTION** Ethical regulations must be followed and patient consent must be obtained when working with human samples. If applicable, a license to undertake animal work should be obtained from the relevant local and/or national authorities. **! CAUTION** Appropriate institutional ethical approval, licensing and training must be sought before working with animals. **! CAUTION** Biological samples pose an infection risk and should be handled with adequate personal protective equipment.
- Laboratory-collected biological samples: adherent or suspension mammalian cell cultures, spent media, small whole organisms (e.g., *Daphnia magna* and *Drosophila*) **! CAUTION** Biological samples pose an infection risk and should be handled with adequate personal protective equipment.**! CAUTION** Cell lines should be regularly checked to ensure that they are authentic and that they are not infected with mycoplasma.
- Crushed wet ice
- Dry ice **! CAUTION** Dry ice causes burns and poses an asphyxiation risk. It should be handled with adequate personal protective equipment in a well-ventilated space.
- Liquid nitrogen **! CAUTION** Liquid nitrogen causes burns and poses an asphyxiation risk. It should be handled with adequate personal protective equipment in a well-ventilated space.
- Nitrogen gas, oxygen free, 30 p.s.i. (BOC, cat. no. 44-X) **! CAUTION** Nitrogen gas poses an asphyxiation risk. It should be handled in a well-ventilated space.
- HPLC-grade chloroform (J.T. Baker; SciChem, cat. no. 9174) **! CAUTION** Chloroform is flammable and toxic and should be handled in a fume hood.
- HPLC-grade methanol (J.T. Baker; SciChem, cat. no. 9822) **! CAUTION** Methanol is flammable and toxic and should be handled in a fume hood.
- HPLC-grade water (J.T. Baker; SciChem, cat. no. 9823)
- Ammonium acetate (99.999% (wt/wt); Sigma-Aldrich, cat. no. 372331)
- Formic acid (LC-MS Ultra; Sigma-Aldrich, cat. no. 14265) **! CAUTION** Formic acid is flammable and corrosive; it should be handled in a fume hood.
- Pierce LTQ Velos ESI Positive Ion Calibration Solution (Thermo Scientific, cat. no. 88323) **! CAUTION** This solution is flammable and toxic; it should be handled in a fume hood.
- Pierce LTQ Velos ESI Negative Ion Calibration Solution (Thermo Scientific, cat. no. 88324) **! CAUTION** This solution is flammable and toxic; it should be handled in a fume hood.

EQUIPMENT

- High-resolution, high-mass-accuracy Fourier transform mass spectrometer (Thermo Scientific LTQ FT Ultra Fourier transform ion cyclotron resonance mass spectrometer, Thermo Scientific Q Exactive Hybrid Quadrupole Orbitrap mass spectrometer, or Thermo Scientific Orbitrap Elite Hybrid Ion Trap-Orbitrap mass spectrometer). All instruments are controlled by a dedicated personal computer running Xcalibur (v2.0 or greater) software (Thermo Scientific)
- Automated multisample chip-based nESI sample ionization platform (Advion TriVersa NanoMate) coupled to the mass spectrometer. This is controlled by ChipSoft software (Advion, v8 or higher)
- nESI 400-nozzle electrospray chip (Advion, cat. no. 1003446)
- nESI electrospray 400 tip rack (Advion, cat. no. 1004763)
- 50 and 100 ml, glass Duran bottles (Fisher Scientific, cat. nos. 218011753 and 218012458)
- Two Hamilton syringes (750SNR 500 µl; Fisher Scientific, cat. no. 80865)
- High-quality solvent-resistant pipette tips (Fisherbrand, 200 µl and 1,000 µl; Fisher Scientific, cat. nos. 11587442 and 10787524)
- High-quality solvent-resistant plastic microcentrifuge tubes (Eppendorf, 1.5 ml and 2 ml; Fisher Scientific, cat. nos. 10509691 and 10038760)
- 1.75-ml Tall-form glass vials with aluminum-lined screw caps (Wheaton, cat. no. 151061)
- Volac glass Pasteur pipettes, 150 mm (Fisher Scientific, cat. no. D810)
- Precellys 24 bead-based homogenizer (Bertin Instruments, cat. no. EQ03119-200-RD000.0)
- Homogenization tubes for Precellys 24 homogenizer (2-ml tubes containing 1.4-mm-diameter ceramic beads (Bertin Instruments, cat. no. CK14)
- Vortex mixer (Fisher Scientific, cat. no. 13214789)
- Refrigerated centrifuge (Microcentrifuge Biofuge Primo R (Thermo Scientific, cat. no. 75005440) with bucket rotor (Thermo Scientific, cat. no. 75007591) and fixed-angle rotor (Thermo Scientific, cat. no. 75007593))
- Nitrogen sample concentrator, including sample concentrator gas reservoir and stand (Techne, cat. no. FSC400D), block heater (Techne, cat. no. DB100/3), 3× heating block inserts (Techne, cat. no. F3505), 76-mm-long needles (100 pack; Techne, cat. no. F7209)
- SpeedVac sample concentrator, including ultra low temperature vapor trap (Thermo Scientific Savant, cat. no. RVT5105230), SpeedVac (Thermo Scientific Savant, cat. no. SPD111V230) and pump (KNF Laboport, cat. no. N 820.3 FT.18)
- Carbon tips for loading nonpolar (lipid) samples into well plates (Advion, Biosciences, cat. no. 1004763)
- Plastic well plates (96 well: Fisher Scientific, cat. no. AB-0800; 384 well: Fisher Scientific, cat. no. TF-0384 or Eppendorf Twin Tec 384 colorless PCR plate, Fisher Scientific cat. no. 0030128508)
- Self-adhesive aluminum sealing tape (Corning, cat. no. 6570)
- Easy-pierce heat-sealing foil covers for well plates (Thermo Scientific, cat. no. AB-1720)
- Well plate heat sealer (Thermo Scientific, cat. no. ALPS 50 V)
- Refrigerator (4 °C)
- Freezers (−20 °C and −80 °C)
- Xcalibur software (Thermo Fisher, v≥2.0)
- An instance of Galaxy, Galaxy-M tools, workflows and associated software (see https://github.com/Viant-Metabolomics/Galaxy-M for a detailed installation guide)
- Personal computer (with Microsoft Windows 7, Intel Core i7, 2.8 GHz, 4 GB RAM and Internet access)

REAGENT SETUP

Biological sample collection and handling To halt enzymatic activity and to ensure that the biological sample is a true representation of the phenotype

being tested, samples (including tissue or biofluids—e.g., plasma, serum and urine) should be frozen as soon as possible after sampling. Small whole organisms—e.g., *Daphnia* and *Drosophila*—should be placed in a microcentrifuge tube and flash-frozen in liquid nitrogen. Adherent or nonadherent cultured mammalian cells should be quenched using −40 °C 60% (vol/vol) HPLC-grade methanol (on dry ice) as the quenching solution, following existing protocols[45,71]. To enable the calculation of cell biomass, the quenching tube should be weighed at the start (before the addition of any quenching solvents) and at the end (when only frozen cells are present).

! CAUTION Ethical approval must be sought and ethical protocols followed when working with human samples. **▲ CRITICAL** All biological samples must remain frozen at −80 °C until analysis. They can be stored for up to 12 months. The minimum recommended sample sizes for this high-sensitivity method are 1 mg biomass of tissue, mammalian cells or whole organisms, or 5 µl of biofluids.

Solvent preparations Use only high-quality plasticware (tips and tubes as recommended above) to minimize the leaching of plasticizers into the extraction solutions. Ensure that each set of consumables (tips and tubes) and solvents is from the same lot number. Extraction solvents for tissue and mammalian cells (methanol, chloroform and water, each on their own) and biofluids (methanol:chloroform in a 1:1 solution and water on its own) should be prepared and decanted into solvent-rinsed and dried 50- or 100-ml Duran bottles. Methanol:chloroform 1:1 solution should be precooled to −20 °C for >2 h. All other solvents should be prechilled on wet ice for at least 30 min. Separate vials of chloroform and methanol should be prepared to wash the Hamilton syringe that is used to remove metabolic or lipid extracts (Steps 1A(xi–xii) and 1B(v)). DIMS analysis solvents should be prepared as follows: (i) positive ion polar solvent—4:1 (vol/vol) methanol:water with a total of 0.25% (vol/vol) formic acid; (ii) negative ion polar solvent—4:1 (vol/vol) methanol:100 mM aqueous ammonium acetate; and (iii) nonpolar solvent (suitable for positive and negative ionization analyses)—2:1 (vol/vol) 7.5 mM methanolic ammonium acetate:chloroform (dissolve the ammonium acetate in the methanol before chloroform addition). All solvent mixtures are stable at room temperature (20 °C) for at least 1 week.

EQUIPMENT SETUP

Equipment used during extraction Precool both the swinging-bucket and fixed-angle microtube centrifuges to 4 °C. Turn on the Precellys 24 homogenizer to allow it to initiate. Turn on the cold trap that is connected to the SpeedVac concentrator before starting the extraction procedure (requires ~2 h to achieve operating temperature).

Establishing Galaxy-M workflows The data processing and analysis steps described here are collated into Galaxy-specific tools and workflows, referred to as Galaxy-M[28]. An extensive guide for how to install Galaxy-M is available from GitHub (https://github.com/Viant-Metabolomics/Galaxy-M). Here, tools and workflows within Galaxy-M are well documented, including default parameters, and they have self-explanatory names to guide the user through the data processing and analysis steps. Additional reading and tutorials on how to use a Galaxy instance are available online (https://wiki.galaxyproject.org/Learn).

Most DIMS studies include a large number of files and a considerable amount of data. Galaxy-M assumes that the user will store the data (e.g., .raw files with or without transient data) on a file system that is directly accessible by the Galaxy instance (i.e., no Galaxy-upload required).

PROCEDURE

Metabolite and lipid extraction from biological samples ● TIMING 2–4 h per batch

▲ CRITICAL To minimize inconsistencies across the handling of biological samples and therefore to reduce sample-to-sample variation, extract no more than 20 samples in a single batch. Repeat this section until all samples are extracted.

▲ CRITICAL Randomize the extraction order to ensure that biological sample classes are randomized within and across extraction batches.

▲ CRITICAL An 'extract blank' sample must be prepared. Here, the extraction procedure is carried out in the absence of a biological sample, and the resulting extract blank is used to identify compounds that arise solely from sample preparation procedures.

1| Perform the steps in option A if you are extracting from tissue, whole organisms or mammalian cells. Perform the steps in option B if you are extracting from biological fluids.

(A) Extraction from tissue, whole organisms or mammalian cells

(i) Weigh the biological samples, ensuring that they do not thaw by keeping them on dry ice. Mammalian cell biomass should be estimated during the quenching step (see Reagent Setup—Biological sample collection and handling, above).

(ii) For tissues or whole organisms, place the biological material into a labeled Precellys 24 tube and set it on dry ice. For mammalian cells, place the biological material into a labeled high-quality 2-ml microcentrifuge tube and set it on dry ice.

(iii) Add 8 µl of ice-cold methanol for each milligram of frozen biological sample mass (8 µl/mg; using high-quality solvent-resistant plastic pipette tips).

(iv) For tissues or whole organisms, homogenize the samples using a Precellys 24 system (2 × 10-s bursts of 6,400 r.p.m. separated by a 5-s gap) before returning the tubes to dry ice. For mammalian cells, vortex the samples (30 s) before returning the tubes to dry ice.

(v) Label a 1.75-ml glass vial per sample and add 8 µl of ice-cold chloroform for each milligram of frozen biological sample mass (using a clean glass Hamilton syringe) to each vial and set it on wet ice.

(vi) Using a glass Pasteur pipette, transfer the homogenate from either the Precellys 24 tube (tissue or whole-organism samples) or the microcentrifuge tube (cultured cells samples) to the 1.75-ml glass vial containing chloroform. Vortex each vial (15 s) and then return the vials to the wet ice.

(vii) Add HPLC-grade water (7.2 µl/mg of frozen biological sample mass) to each vial, vortex (30 s) and then return the samples to the ice (samples should be cloudy in appearance).

(viii) Leave the samples on wet ice for 10 min to allow metabolites and lipids to partition between the polar and nonpolar solvents.

(ix) Centrifuge the samples (2,500*g*, 4 °C, 10 min) using a swinging-bucket centrifuge to induce phase separation.
▲ **CRITICAL STEP** Remove the samples from the centrifuge bucket extremely carefully to avoid disturbing the protein interface and biphasic separation of solvents.
(x) Set the samples on the bench at room temperature and allow them to sit for 5 min to allow completion of the phase separation. The sample is now biphasic, with the polar (upper) and nonpolar (lower) layers separated by an interface of denatured proteins and cell debris.
(xi) Remove a fixed volume (typically 300 µl, with the option to collect multiple aliquots) of the polar phase and add it to a high-quality 1.5-ml microcentrifuge tube using a clean Hamilton syringe. Rinse the syringe twice with methanol wash solvent (2 × 500 µl) between biological samples.
(xii) Remove a fixed volume (typically 150 µl, with the option to collect multiple aliquots) of the nonpolar phase and add it to a new 1.75-ml glass vial tube using a clean Hamilton syringe. Rinse the syringe twice with chloroform wash solvent (2 × 500 µl) between biological samples. To obtain the nonpolar phase, slide the Hamilton syringe down the side of the glass vial and gently move the protein interface out of the way with the syringe.
▲ **CRITICAL STEP** Removing the nonpolar layer requires care so as to avoid attachment of debris to the Hamilton syringe.
(xiii) Dry the polar extracts in a SpeedVac concentrator using no heat (1–2 h).
! **CAUTION** Ensure that the associated cold trap has reached operating temperature (<–100 °C) in order to prevent solvents from being vented into the atmosphere.
(xiv) Dry nonpolar biological samples under a stream of nitrogen (using the nitrogen sample concentrator; 15 min), and cap the vials quickly to ensure that a nitrogen-rich atmosphere remains inside.
! **CAUTION** This must be done in a fume hood to prevent the escape of chloroform into the laboratory.
▲ **CRITICAL STEP** This removes oxygen from the sample to prevent lipid oxidation and peroxidation.
(xv) Store dried extracts frozen at –80 °C or proceed to Step 2.
■ **PAUSE POINT** Extracted biological samples can be stored at –80 °C for up to a few months.

(B) Extraction from biofluids (serum, plasma and urine)

(i) Thaw biofluid on wet ice for 30–60 min, and then vortex (15 s) and remove a fixed volume (e.g.,200 µl) and add it to a 2-ml high-quality solvent-resistant plastic microcentrifuge tube. Keep the samples on wet ice.
(ii) Add 600 µl of methanol:chloroform (1:1, –20 °C), vortex (15 s) and return the sample to the ice.
(iii) Add 300 µl of water, vortex (15 s) and return the samples to the ice.
(iv) Centrifuge the samples (20,000*g*, 4 °C, 5 min) to induce phase separation.
(v) Follow Step 1A(x–xiv), except take a 350-µl aliquot from the polar phase and a 100-µl aliquot from the nonpolar phase.
(vi) Store dried extracts frozen at –80 °C or proceed to Step 2.
■ **PAUSE POINT** Extracted biological samples can be stored at –80 °C for up to a few months.

Resuspension of biological sample extracts in DIMS solvent ● **TIMING 1–2 h**

▲ **CRITICAL** Before biological sample resuspension, it is advisable to create and analyze a serial dilution of resuspended extracts to establish the optimal concentration, which maximizes peak counts (of biological origin) and stability of the nESI current for the specific biological samples being analyzed. Recommended starting points for the resuspension solvents (which include sample modifiers, formic acid and ammonium acetate, to enhance electrospray efficiency) and volumes are detailed in **Table 2**. This is carried out using a spare biological extract, which will be used up and not form part of the subsequent metabolomics analyses.

2| Add the appropriate type and volume of DIMS solvent to the extracts as described in **Table 2** (or the volume determined in the method optimization experiment) and vortex (30 s) to dissolve.

▲ **CRITICAL STEP** Lipid concentrations should not exceed 10 pmol/µl in order to prevent the formation of lipid aggregates[34].

3| Create a QC sample by taking a fixed volume from each biological sample and pooling, to a minimum total volume of 180 µl (enough for six QC analyses). As the number of biological samples analyzed increases, so should the minimum QC volume, such that a QC sample can be analyzed every 4–6 biological samples throughout an analytical run.

▲ **CRITICAL STEP** If data will be collected in several batches, an identical QC sample must be used across all batches. This will allow successful application of the signal correction algorithm (Step 28).

4| Centrifuge the samples (polar extracts in microcentrifuge tubes: fixed-angle rotor, 20,000*g*, 4 °C, 10 min; nonpolar extracts in glass vials: bucket rotor, 2,500*g*, 4 °C, 10 min) to remove particulates that can adversely affect nESI stability.

▲ **CRITICAL STEP** Particulates in the sample can fully or partially block the nESI nozzle, leading to loss or instability of electrospray.

5| When loading samples, place a 96-well or 384-well plate on wet ice (to prevent sample evaporation) and pipette 3 × 10 µl of each sample into triplicate (consecutive) wells along a row, using either Fisherbrand tips (polar)

TABLE 2 | Directions for the resuspension of polar and nonpolar extracts for nESI DIMS analysis.

Sample	Ion mode	Resuspension solvent	Recommended resuspension concentration
Tissue, whole organism or mammalian cell *polar* extract	Positive ion	4:1 (vol/vol) methanol:H_2O. 0.25% (vol/vol) formic acid added. Final formic acid concentration of 0.25% (vol/vol)	Half of the original extract volume
Tissue, whole organism or mammalian cell *polar* extract	Negative ion	4:1 (vol/vol) methanol:100 mM aqueous ammonium acetate. Final ammonium acetate concentration of 20 mM	Half of the original extract volume
Tissue, whole organism or mammalian cell *nonpolar* extract	Positive or negative ion	2:1 (vol/vol) 7.5 mM methanolic ammonium acetate:chloroform. Final ammonium acetate concentration of 5 mM	Double the original extract volume
Biofluid *polar* extract	Positive ion	4:1 (vol/vol) methanol:H_2O. 0.25% (vol/vol) formic acid added. Final formic acid concentration of 0.25% (vol/vol)	5–10× the original extract volume
Biofluid *polar* extract	Negative ion	4:1 (vol/vol) methanol:100 mM aqueous ammonium acetate. Final ammonium acetate concentration of 20 mM	5–10× the original extract volume
Biofluid *nonpolar* extract	Positive or negative ion	2:1 (vol/vol) 7.5 mM methanolic ammonium acetate:chloroform. Final ammonium acetate concentration of 5 mM	5–10× the original extract volume

Formic acid and ammonium acetate are sample modifiers that enhance electrospray efficiency.

or carbon tips (nonpolar). Upon completion of each well-plate row, cover with self-adhesive aluminum sealing tape to prevent solvent evaporation.

▲ **CRITICAL STEP** To avoid particulates in the sample wells, keep the samples at the same fixed angle as they were in the centrifuge and avoid disrupting the pellet (probably invisible to human eye) when pipetting.

▲ **CRITICAL STEP** Ensure that the biological sample phenotypes are randomized in the well plate (facilitating a randomization of the analytical run order). Ideally, the sample order should start with a series of trial samples, typically MS diluent (×3 technical replicates) and QC samples (×3 technical replicates), which allows the user to assess the quality of the electrospray and DIMS detection. This is followed by the extract blank sample (×3 technical replicates) and by a QC sample (×3), and then by the biological samples (×3 each) with a QC sample (×3) every 4–6 samples, ending with a QC sample (×3; see **Fig. 3** for an example). The analysis of QC samples at regular intervals throughout the sample sequence run is essential to enable signal correction data processing (Step 28).

6| Once completed, remove the self-adhesive aluminum sealing tape and heat-seal the entire well plate with the appropriate foil (170 °C for 2 s).

7| Open the ChipSoft software (controlling the TriVersa NanoMate) and in 'Interface Settings' set the plate cooler to 10 °C. Put the well plate on to the TriVersa NanoMate and allow at least 30 min for the samples to equilibrate to 10 °C.

▲ **CRITICAL STEP** Variations in sample temperature can alter the electrospray process, leading to poor technical reproducibility.

DIMS analysis ● TIMING ~9 min per polar biological sample, comprising triplicate technical replicate analysis on the Q Exactive, Orbitrap Elite or LTQ FT Ultra, and including sample loading and equilibration

8| Ensure that the well plate is located on the TriVersa NanoMate and has been cooled to 10 °C. Load a new electrospray chip and 384-tip tray into the TriVersa NanoMate and empty the tip waste tray. Align the TriVersa NanoMate with the MS source (**Box 1**). Within the ChipSoft software (in 'Sequence View'), create a sample sequence using the optimal TriVersa NanoMate method (**Box 1**).

▲ **CRITICAL STEP** Ensure that the TriVersa NanoMate contact closure setting is set to 'Trigger acquisition when Input Signal received', which enables the TriVersa NanoMate to initiate MS data collection when the nESI begins.

9| Within the MS vendor software (Xcalibur, for Thermo Scientific instruments), create a sample run order using the method created in **Box 1** (see **Supplementary Methods 1** and **2** for recommended mass spectrometry methods).

▲ **CRITICAL STEP** For FT-ICR instruments, ensure that the collection of time domain (transient) data is enabled (within the Tune program: 'Diagnostics' → 'Toggles' → 'Include FT transient').

10| Initiate the TriVersa NanoMate sample run and when the 'ChipSoft Virtual Device' window states that it is awaiting input from the MS system, initiate the MS sample run in Xcalibur. If the electrospray or mass spectrometry detection is unstable, fails to initiate, or stops, please consult the advice in the TROUBLESHOOTING section.

? TROUBLESHOOTING

■ **PAUSE POINT** DIMS data can be stored for unlimited duration until data processing is initiated.

Data processing: spectral-stitching and signal filtering ● TIMING 6–12 h, depending on the number of biological samples

▲ **CRITICAL** At any point in the data analysis process, the Galaxy history can be downloaded, archived and shared with others.

11| Create a text file (as comma-separated value (.csv) format, e.g. 'samples.csv') to function as a sample identifier for the samples being processed. This contains four columns separated with a comma: first column—spectral file names; second column—class identification (e.g. control, QC); third column—batch number; and fourth column—run order.

12| Open an Internet browser and go to the Galaxy-M homepage (e.g., http://localhost:8080 for a local Galaxy instance; see 'Establishing Galaxy-M-workflows' in Equipment Setup, above).

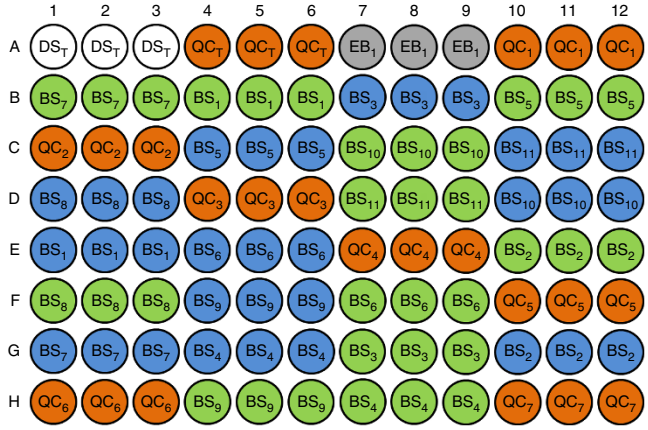

Figure 3 | Recommended arrangement of samples in a 96-well plate for a spectral-stitching nESI DIMS experiment. Samples are each loaded as triplicates and are analyzed in the order they appear in the plate (e.g., position A1, A2, A3, …., H12). The plate begins with six 'trial' samples (A1–A6, typically 3× DIMS solvent (DS_T) and 3× QC samples (QC_T)) that are used to check the nanoelectrospray stability of the DIMS system and the QC samples. This is followed by 3× extract blank (EB) samples. QC samples are injected after the EB, and then after every four to six biological samples, and at the end of the plate. Biological samples (BSs) across different sample classes (typically different biological phenotypes; shaded green and blue) are placed in a random order across the plate to minimize possible biases resulting from analysis order.

13| Select 'Tools' → 'Get Data' → 'Upload Data' from the left panel. This brings up the upload data form. Upload the .csv file created in Step 11 (ensure '.csv' is selected as the file type).

14| Use 'Tools' → 'Get Data' → 'File List Manager' (left panel) to create a file list that specifies the location of the files on the user's personal computer.

15| If the DIMS data include transient files, proceed to Step 16. If no transient data are available, then proceed to Step 18.

▲ **CRITICAL STEP** Transient data are the free induction decay MS data, which require processing as in Steps 16 and 17. For data in the processed vendor format (*.raw), these processing steps have already been completed by the vendor instrument software.

16| Run 'Tools' → 'SIM-stitching' → 'Sum Transients'. This sums the transient data for the mass spectral SIM windows within each technical replicate. To avoid the inclusion of low-quality transients (caused by poor or unstable nESI), a total ion current threshold can be set (optional). Transients are removed according this threshold.

17| Run 'Tools' → 'SIM-stitching' → 'Process Transients'. This applies Hanning apodization, zero-filling, fast Fourier transform and baseline correction to the summed transients.

18| (Optional) Create a tab-separated text file (.txt format; e.g., 'calibrants.txt') that will enable internal mass calibration of the spectra. This file contains the names (in the first column) and the exact mass (in column 2) of compounds known to be present in the sample. If this information is not known, this step can be omitted and the data will be externally calibrated (almost certainly resulting in lower mass accuracy).

▲ **CRITICAL STEP** To create a true calibrant list, additional MS experiments (e.g., fragmentation) are often needed to annotate (i.e., elemental composition) and/or identify (i.e., structure) the *m/z* values. Compounds detected in the extract blank sample can be used for calibration across different MS studies when identical extraction protocols and instruments have been used.

19| Run 'Tools' → 'SIM-stitching' → 'Mass Calibration and SIM-stitching'. This stitches the multiple *m/z* windows together (removing any *m/z* overlap in the process) and internally calibrates the *m/z* axis of the spectra (optional). The required noise threshold, as a SNR, must be stipulated (typically 3:1; data with intensities below this threshold will be discarded).

Box 1 | TriVersa NanoMate and mass spectrometer setup. ● TIMING 1 h

(A) Initial system setup

1. If the mass spectrometer has been in standby mode, allow at least 2 h to equilibrate once it is fully switched on.
2. Open the MS acquisition viewer on the PC (MS Tune for Thermo systems). Calibrate the MS according to the manufacturer's instructions using the calibration mixture.
3. Attach the TriVersa NanoMate onto the bracket on the front of the MS.
4. Load the 400-nozzle nESI chip, 400 tip set and a 96-well plate containing a test biological sample (e.g., biological extract in MS solvent, **Table 2**).
5. Ensure that the nitrogen cylinder (or generator) supplying the TriVersa NanoMate has sufficient gas remaining (recommended to have >1,000 p.s.i. for a 24 h run) and has sufficient back-pressure.
6. Position the TriVersa NanoMate so that it is central to the MS source and 3–5 mm away from the source by adjusting the positional settings in the ChipSoft software (*Method Manager/Spray Optimisation*).

(B) TriVersa NanoMate method creation, electrospray optimization and mass spectrometry tuning to enhance detection sensitivity

1. Create a method file with the recommended settings:

Sample volume	7 µl (polar), 10 µl (nonpolar)
Vent headspace	ON
Aspirate air after sample	ON
Volume of air to aspirate after sample	0.2 µl (polar), 2.0 µl (nonpolar)
Trigger acquisition when Input Signal received	ON
Spray sensing	OFF
Gas pressure	0.3 p.s.i.
Voltage to apply	1.5 kV
Positive ion or negative ion	as desired

2. To optimize the nanoelectrospray and DIMS detection for each specific biological sample, type inject a test sample by using the 'Spray Optimization' tool (within the 'Method Manager' tab). The TriVersa NanoMate electrospray current should be stable and between 100 and 300 nA (for problems see **Table 3**).

▲ **CRITICAL** A stable electrospray current is required to generate reproducible data. Electrospray stability is partially dependent on the voltage and gas pressure settings on the TriVersa NanoMate (recommended settings above). These settings are sample type dependent and therefore may require optimization to improve electrospray stability.

3. Optimize the TriVersa NanoMate voltage: set the gas pressure to 0.3 p.s.i. and the voltage to 1.5 kV, and then—while monitoring the spray stability on the mass spectrometer—decrease the voltage in steps of 0.1 kV until the spray is lost. Switch the polarity mode on the TriVersa NanoMate and then switch back to the polarity that was originally being used. If the spray does not return, add 0.2 kV to the current voltage setting and this is now the optimal voltage setting that should be used.
4. Optimize the TriVersa NanoMate pressure: set the voltage to its optimized setting and the pressure to 0.3 p.s.i. Then alter the pressure in steps of 0.05 p.s.i. and observe any improvement or deterioration of the electrospray current.
5. Optimize the position of the TriVersa NanoMate in relation to the MS source: observe the ion intensity and reproducibility in the mass spectrometer using the mass spectrometer detector program (MS Tune). Move the TriVersa NanoMate closer or further away from the mass spectrometry source (by changing settings in *Method Manager/Spray Optimisation)*. To increase the sensitivity, move the TriVersa NanoMate closer to the MS, and to increase reproducibility move it further away (the optimal distance should be in the range 3–5 mm). For problems with DIMS detection, see **Table 3**.
6. Where available, it is recommended to optimize peak detection sensitivity by using the automated tuning function (within the mass spectrometer detector program, MS Tune) on ions within the mass ranges of interest—e.g., ions ~75 *m/z* (*'low tune'*), 150 *m/z* (*'medium tune'*) and 400 *m/z* (*'high tune'*) for polar extracts. Each *m/z* range optimization is carried out independently by repeating the tuning at that *m/z* until no further increase to signal intensity is achieved, and then saving the optimal tune parameters as separate tune files (i.e., *'low tune'*, *'medium tune'* and *'high tune'*).

(C) Creation of the spectral-stitching method

1. Using the *Instrument Setup* tab in Xcalibur software, create a method with multiple segments (for cases in which transient scans are collected—LTQ FT and LTQ FT Ultra) or multiple scan events (for cases in which transient scans are not available—Q Exactive and Orbitrap Elite) that represent the *m/z* windows for the spectral-stitching method (widths, acquisition length, resolution and AGC detailed in **Fig. 2** and **Table 1**; see **Supplementary Methods 1** and **2** for Q Exactive and Orbitrap Elite instrument method files). When using scan events, set the number of acquired microscans to 10—this averages ten transient scans.
2. If available, assign a tune file to each window: *'low tune'* for windows with a center <100 *m/z*; *'medium tune'* for windows with a center 100–250 *m/z*; and *'high tune'* for windows with a center >250 *m/z*. Otherwise, assign an appropriate tune file for the entire mass range (e.g., optimize at the mid-*m/z* range being detected or within the specific *m/z* range of interest).
3. At the beginning of the method, apply a 30-s delay before MS data are collected in order to allow stabilization of the nESI spray current.

If internal calibration is required, provide 'calibrants.txt' (Step 18) and specify the minimum intensity (typically >50 SNR) and the maximum mass tolerance (typically 2.0 ppm) of peaks to be used for internal calibration.

20| Run 'Tools' → 'SIM-stitching' → 'Replicate Filter'. This step retains peaks present within at least 2 of the triplicate measurements of each biological sample (and averages their intensities) and thus functions to remove noise features.
The maximum *m/z* ppm range a peak must fall within (across the triplicate measurements) for it to be counted as the same peak must be stipulated (typically 1.5 ppm).
▲ **CRITICAL STEP** If the mass accuracy of the data is less than optimal, this *m/z* tolerance can be increased to ensure that real peaks are retained).

21| Run 'Tools' → 'SIM-stitching' → 'Align Samples'. This aligns the peaks across all the 'Replicate Filtered' biological samples. The maximum *m/z* ppm range a peak must fall within (across the biological samples) for it to be counted as the same peak must be stipulated (typically 2.5 ppm).
▲ **CRITICAL STEP** If the mass accuracy of the data is less than optimal, this *m/z* tolerance can be increased to ensure that sample-related peaks are retained.

22| Run 'Tools' → 'SIM-stitching' → 'Create DSO'. This converts the DIMS data into a DataSet Object (DSO—a construct of Eigenvector Research to facilitate data set handling and sharing (http://www.eigenvector.com/software/dataset.htm)) .xml file, which includes a peak intensity data matrix, row labels (sample classes), column labels (*m/z* values) and information provided in the text file in Step 11. The data within the DSO are updated with each additional processing step.

23| Run 'Tools' → 'SIM-stitching' → 'Blank filter'. This compares biological sample peaks with those appearing in the 'extract blank sample'. Peaks that appear in both are discarded according to the defined biological:blank peak intensity ratio (typically, peaks are retained if this ratio is >3).

24| Run 'Tools' → 'SIM-stitching' → 'Sample Filter'. This retains biological peaks that are present within a defined minimum percentage of all biological samples (80% for initial biological interpretation; lower if the experimental design suggests this; see the 'Data processing' section in the INTRODUCTION).

25| Run 'Tools' → 'SIM-stitching' → 'Missing Value Sample Filter'. This calculates the number of missing values contained in each sample within the DSO data matrix. Samples with unacceptably high numbers of missing values can be automatically excluded.

Normalization, signal correction, data quality assessment, missing value imputation and generalized log (G-log) transformation ● TIMING 30 min–1 h

26| Run 'Tools' → 'Matrix Processing' → 'PQN Normalisation'. This normalizes the data matrix (DSO) from Step 25. The normalization quotients for this are calculated using peaks that are present in all samples.

27| (Optional) Run 'Tools' → 'Matrix Processing' → 'Peak Outlier Detection'. This tool detects outliers detrimental to the effectiveness of the signal-intensity correction in Step 28. For each peak, it fits a second- or third-order polynomial regression curve to the non-QC peaks when samples are ordered by injection. Only non-QC data points are used in this curve fitting, in order to avoid statistical bias. A confidence interval for each curve is calculated (typically 95 or 99%) and any (QC) data point lying outside the confidence interval is considered an outlier, and can be removed[59].

28| (Optional) For single- or multiple-batch studies, the signal intensity correction tool can be applied. Run 'Tools' → 'Matrix Processing' → 'Signal Correction and Peak Quality Filtering'. For each peak, the tool applies robust cubic smoothing splines to the QC data points ordered by injection. The resulting fitting curves are then used to correct for the technical variation arising from interbatch measurements[59]. This approach can also be used to correct technical variation in signal intensity that arises within a single batch. In addition, peaks that are highly variable across batches can be filtered based on different criteria (e.g., RSD >30%).

29| Run 'Tools' → 'Matrix Processing' → 'Missing Value Imputation'. This step uses the KNN method to impute any values that are missing from the normalized data matrix. K can be set by the user (default is 5).

30| (This step is required only when calculating multivariate statistics.) Run 'Tools' → 'Matrix Processing' → 'G-log Transformation'. This optimizes the lambda parameter from the QC data matrix (i.e., QC samples within the data matrix are automatically selected), and then applies the generalized log transformation to the normalized data matrix.

Statistics analyses ● TIMING 1–2 h

31| Univariate statistical analysis can be applied to the normalized data matrix immediately before missing value imputation (from Step 28) or after missing value imputation (from Step 29). Run 'Tools' → 'Statistics' → 'Univariate Analysis', or export the data for further analysis using external statistical packages ('Tools' → 'DataSet Object').

32| Multivariate statistical analysis can be applied to the generalized log-transformed normalized data matrix (from Step 30). Run 'Tools' → 'Statistics' → 'PCA and Scores Test', or export the data for further analysis using external statistical packages ('Tools' → 'DataSet Object').

Metabolite annotation ● TIMING 1 h, and substantially longer, >8 h, when 'Molecular Formulae Search' is used

33| Run 'Tools' → 'DataSet Object' → 'Get Peak List' to create a peak list from the biological sample data matrix (Step 25).

34| (Optional step; time-consuming) Run 'Tools' → 'MI-Pack' → 'Molecular Formulae Search'. This matches each peak *m/z* value to one or more elemental compositions ($C_cH_hN_nO_oP_pS_s$, including adducts, such as [M+H]+, [M+Na]+, and [M+K]+) within a mass error tolerance stipulated by the user (e.g., typically within ±1 ppm for polar or ±2 ppm for nonpolar FT-ICR MS analysis).

35| Run 'Tools' → 'MI-Pack' → 'Single-Peak Search'. This assigns a putative biological compound annotation to each *m/z* value (or elemental composition) within a mass error tolerance stipulated by the user (typically within ±1 ppm for FT-ICR MS) using database(s) specified by the user (e.g., KEGG, HMDB and LIPID MAPS).

36| Run 'Tools' → 'MI-Pack' → 'Peak-Pattern Search'. This annotates adduct/isotope patterns (e.g., ^{12}C–^{13}C and $[M+^{39}K]^+$ – $[M+^{41}K]^+$) and relative isotopic abundance measurements within the data.

37| Run 'Tools' → 'MI Pack' → 'Combine Outputs'. This produces a summary file of the outputs produced by Steps 33–36.

Depositing of data from metabolomics studies into public repositories ● TIMING variable

38| We recommend that all the DIMS data, including .raw files and data matrices, be deposited into a public repository (i.e., MetaboLights[69] (http://www.ebi.ac.uk/metabolights) and/or Metabolomics Workbench[70] (http://www.metabolomicsworkbench.org)). To do this, follow the instructions on the data repository's website. A stable identifier will be assigned to the data set, which can then be used to cite the data set in a publication.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Problem	Possible reason	Solution	Step where the problem originates	Step where the problem becomes evident
The nESI spray fails to initiate, is unstable or stops	Particulates in the sample	Centrifuge the resuspended sample at 4 °C before analysis. Take care not to disrupt the pellet during sample loading. Do not set the TriVersa NanoMate well plate temperature <10 °C, which may encourage a precipitate to form	4–5	10
	Sample too concentrated or too salty	Create (and analyse) a serial dilution of the resuspended sample to establish the most concentrated sample that gives a stable spray	2	10
	Low sample viscosity, causing the sample to drip from the tip before analysis	In ChipSoft 'Method Manager', increase the 'Volume of air to aspirate after sample' in 0.5-µl steps	8	10
	TriVersa NanoMate nESI chip too close to MS source	In the 'Spray Optimisation' window within the ChipSoft 'Method Manager', move the TriVersa NanoMate away from the MS source	8	10

(continued)

TABLE 3 | Troubleshooting table (continued).

Problem	Possible reason	Solution	Step where the problem originates	Step where the problem becomes evident
	Low nitrogen gas pressure in the supply to the TriVersa NanoMate	Check gas cylinder pressure	8	10
	nESI chip not loaded correctly	Remove and replace the chip	8	10
	Nonoptimal TriVersa NanoMate pressure and voltage settings	Optimize the TriVersa NanoMate voltage and pressure settings as described in **Box 1**	8	10
Abnormally high nESI spray current (>400 nA)	Sample too concentrated or too salty	Create (and analyze) a serial dilution of the resuspended sample to establish the most concentrated sample that gives a stable spray	2	10
Abnormally low nESI spray current (<50 nA)	Sample too dilute or insufficient modifier (formic acid or ammonium acetate) added	Create (and analyze) a serial dilution of the resuspended sample to establish the most concentrated sample that gives a stable spray. Check modifier addition (**Table 2**)	2	10
nESI is stable, but the stability of ions entering the MS is poor	TriVersa NanoMate nESI chip is too close to the mass spectrometer source	In the 'Spray Optimisation' window within the ChipSoft 'Method Manager', move the TriVersa NanoMate away from the MS source	8	10
Good nESI current (100–300 nA) but low ion intensity in MS	The TriVersa NanoMate nESI chip is too far away from the mass spectrometer source	In the 'Spray Optimisation' window within the ChipSoft 'Method Manager', move the TriVersa NanoMate closer to the MS source	8	10
TriVersa NanoMate fails to pick up tips	Glue was deposited on the mandrel from self-adhesive foil	Clean the mandrel with xylene. Use only heat-sealed foil to cover the well plates	6	10
TriVersa NanoMate is running, but MS is failing to initiate	Contact closure failure	Check the contact closure cable connection between the TriVersa NanoMate and the mass spectrometer	8	10

● TIMING

Step 1A or B, metabolite and lipid extraction from biological samples: 2–4 h per batch
Steps 2–7, resuspension of biological sample extracts in DIMS solvent: 1–2 h
Steps 8–10, DIMS analysis: ~9 min per polar biological sample, comprising triplicate technical replicate analysis on the Q Exactive, Orbitrap Elite or LTQ FT Ultra, and including sample loading and equilibration
Steps 11–25, data processing: spectral-stitching and signal filtering: 6–12 h depending on the number of biological samples
Steps 26–30, normalization, signal correction, data quality assessment, missing value imputation and generalized log (G-log) transformation: 30 min–1 h
Steps 31 and 32, statistics analyses: 1–2 h
Steps 33–37, metabolite annotation: 1 h, and substantially longer, >8 h, when 'Molecular Formulae Search' is used
Step 38, depositing of data from metabolomics studies into public repositories: variable
Box 1, TriVersa NanoMate and mass spectrometer setup: 1 h

ANTICIPATED RESULTS

The spectral-stitching nESI metabolomics/lipidomics method—in which data are collected as a series of overlapping *m/z* windows that are 'stitched' together (**Fig. 2**)—generates high-quality data in less than 10 min per biological sample (including sample loading and equilibration, and triplicate sample acquisition; **Table 1**). This approach substantially increases the dynamic range of the spectrum (**Fig. 4**)[23,24] without increasing the number of ions entering the detector, which could lead to space-charge effects and poor mass accuracy[56]. The number of detected peaks increased more than

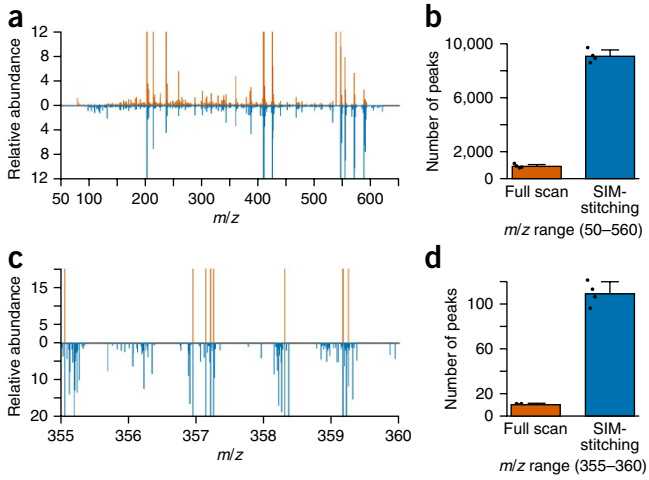

Figure 4 | Detection sensitivity of the spectral-stitching nESI DIMS method (blue) is superior to standard full-scan analysis (dark orange). (**a**–**d**) Data show the polar metabolites in a serum extract analyzed on a Q Exactive Hybrid Quadrupole Orbitrap mass spectrometer. Four QCs were each collected in triplicate, and only peaks occurring in at least two out of three technical replicates were retained. **a** shows the complete 50–620 *m/z* range; **c** is a zoomed-in version of **a** covering the 355–360 *m/z* range; **b** and **d** show the average number of peaks detected over the 50–620 *m/z* and 355–360 *m/z* ranges, respectively. Error bars represent the standard deviation of four QC samples and circles represent the peak counts from each individual spectrum.

fivefold as compared with a single wide-scan *m/z* collection (**Fig. 4**)[23,24]. The use of nESI is critical because it lowers ionization suppression as compared with standard ESI, which also enhances peak detection[25]. Detection sensitivity is further increased by applying the method on an instrument with a physically larger detector that allows more ions to enter without increasing space-charge effects: a threefold increase in peak detection was observed on the LTQ FT Ultra, with larger detector as compared with that

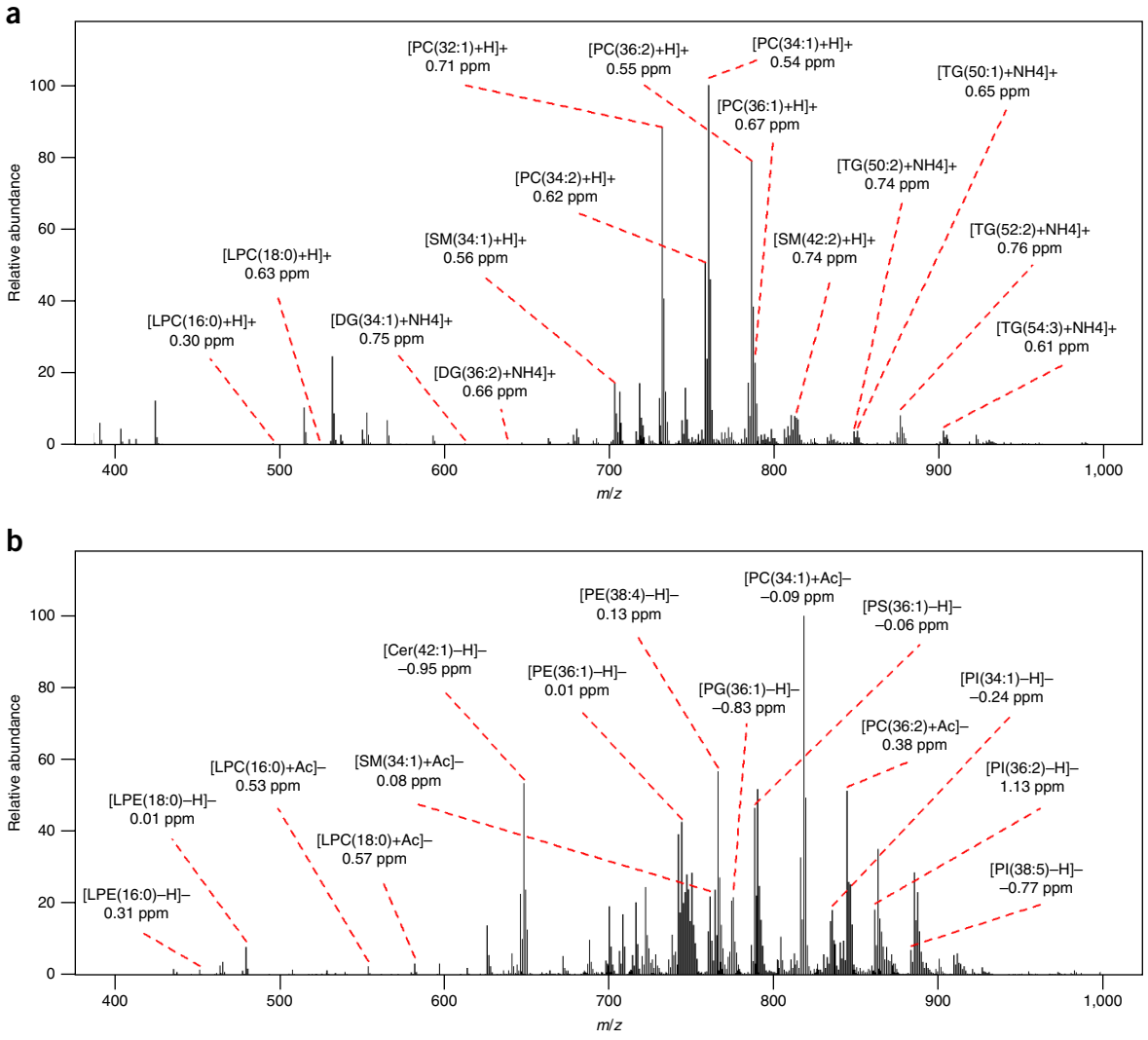

Figure 5 | Analysis of a human leukemia cell line (HL60) lipid extract by the spectral-stitching nESI DIMS method. Data were collected by FT-ICR MS in (**a**) positive ion mode and (**b**) negative ion mode. Selected annotations and ppm *m/z* errors are shown. Ac, acetate ion; Cer, ceramide; DG, diacylglycerol; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TG, triacylglycerol.

TABLE 4 | Average of the relative RSD values of peak intensity measurements, from *n* repeated acquisitions of the same QC sample, using the spectral-stitching nESI DIMS method.

Instrument	RSD values of intensity measurements	Sample type	Number of peaks assessed	Number of repeated QC acquisitions (*n*)
LTQ FT	8.1% (mean)	Polar liver extract[23]	17 Endogenous compounds	3
LTQ FT Ultra	8.2% (median)[a]	Polar liver extract[29]	All peaks in the data set	80[b]
Orbitrap Elite	13.5% (median)	Polar serum extract	All peaks in the data set	10
Q Exactive	16.0% (median)	Polar human bronchial epithelial cell line extract	All peaks in the data set	9

[a]Including batch correction[29]. [b]Data acquired as eight batches of ten samples over a 7-day period.

of the LTQ FT[24]. For each type and size of MS detector, the spectral-stitching method, including *m/z* window width, should be optimized to maximize the number of peaks detected (**Table 1**).

The mass accuracy of data generated by the spectral-stitching nESI method on the FT-ICR MS is ±1 ppm for peaks >600 *m/z*[23,24]. Collecting the data as multiple *m/z* windows aids the internal mass calibration because each window's *m/z* range can be shifted independently of the others'. The ultra-high mass accuracy achieved by this method enables molecular formula(e) annotation of peaks and allows the assignment of putative metabolite and lipid names. Expected numbers of detected and annotated metabolite and/or lipid features vary, depending on sample type. Negative ion spectral-stitching nESI metabolomics of ca. 1 mg of *Daphnia pulex-pulicaria* detected and putatively annotated 1,973 and 369 peaks (± 1.5 ppm mass tolerance), respectively, whereas negative ion lipidomics of ca. 10^7 (ca. 10 mg) human leukemia cells detected and putatively annotated 2,934 and 898 peaks (± 2 ppm tolerance), respectively. A lipid spectrum in which selected peaks have been annotated is shown in **Figure 5**.

The spectral-stitching nESI DIMS method demonstrated high analytical reproducibility: median peak intensity RSD measurements across the QC samples were ~8% for both the LTQ FT and the LTQ FT Ultra, ~13.5% for the (LTQ) Orbitrap Elite and ~16% for the Q Exactive (**Table 4**). These results suggest that nESI DIMS using a hybrid instrument with a linear ion trap (i.e., LTQ) yields more-reproducible peak intensities. These levels of reproducibility have been shown to be maintained in large-scale studies in which data were collected over several days. For example, PCA of a large *D. pulex-pulicaria* metabolomics study showed highly reproducible QC samples that were tightly clustered on the PCA scores plot (**Fig. 6**; ref. 72). Such low technical variance enabled biological variance to be visualized, with a clear distinction of the metabolomes of F0 and F1 *D. pulex-pulicaria* generations (**Fig. 6**; ref. 72). When the spectral-stitching nESI DIMS method was used for lipidomics, reliable and meaningful biological information for identifying drug-induced lipid changes in leukemia cells was generated[7]. Metabolite-intensity measurements were shown to be robust and comparable to those made by NMR spectroscopy—i.e., tyrosine-, creatine- and phosphocreatine-intensity measurements derived from the same sample by the two analytical methods were strongly correlated (**Fig. 7**; ref. 11). A more extensive comparison of the spectral-stitching nESI DIMS and NMR approaches recently showed that the DIMS approach is capable of relative quantification comparable to NMR for several metabolites[30]. NMR spectroscopy is a quantitative technique that is well established in the field of metabolomics, which in turn demonstrates the robustness of the spectral-stitching method.

To conclude, the spectral-stitching nESI DIMS method is reproducible, sensitive and of high throughput, with ultra-high mass accuracy. These attributes make it an ideal tool for rapid and large-scale metabolomics or lipidomics analyses to identify phenotype-induced perturbations to the metabolome or lipidome.

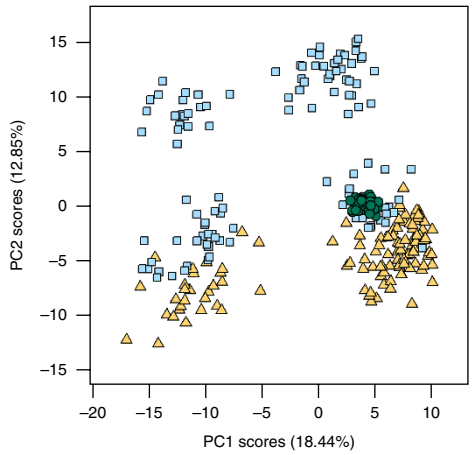

Figure 6 | PCA scores plot of polar extracts of *Daphnia pulex-pulicaria* collected by the spectral-stitching nESI DIMS method, in negative ion mode, on the LTQ FT Ultra[72]. F0-generation animals (light orange triangles) are separated from F1-generation animals (light blue squares) along PC2. Within the F0 and F1 generations, there are clusters along the PC1 and PC2 axes. These are caused by biological differences of unknown origin. Most importantly, the 28 QC samples (green circles) are tightly clustered, indicating the high analytical reproducibility of the spectral-stitching method. PC, principal component.

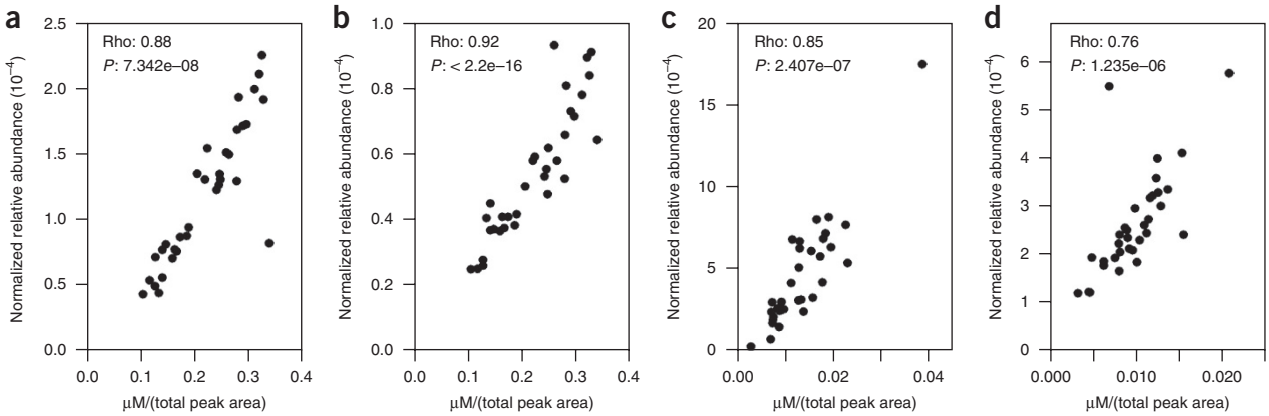

Figure 7 | Intensity measurements by the spectral-stitching nESI DIMS method are consistent with NMR spectroscopic measurements. (**a**–**d**) Comparison of LTQ FT spectral-stitching nESI DIMS data and NMR spectroscopic data measured on the same polar liver extract[11]. Probabilistic quotient normalized (PQN; ref. 58) intensity measurements showing the NMR peak areas on the *x* axis against DIMS peak intensity on the *y* axis (the normalization process makes the intensity values small (10^{-4})). (**a**) tyrosine versus $[tyrosine - H]^-$, (**b**) tyrosine versus $[tyrosine + Cl]^-$, (**c**) creatine versus $[creatine - H]^-$ and (**d**) phosphocreatine versus $[phosphocreatine - H]^-$. Spearman's rank correlation coefficients (rho) and associated *P* values are shown.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS A.D.S. and R.J.M.W. codeveloped the analytical methods and computational workflow, acquired data and wrote the manuscript. J.E. contributed to developing the computational workflow and writing of the manuscript. M.R.J. contributed to developing the analytical methods and acquired data. M.R.V. codeveloped the analytical methods and computational workflows, edited the manuscript and was the academic lead.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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