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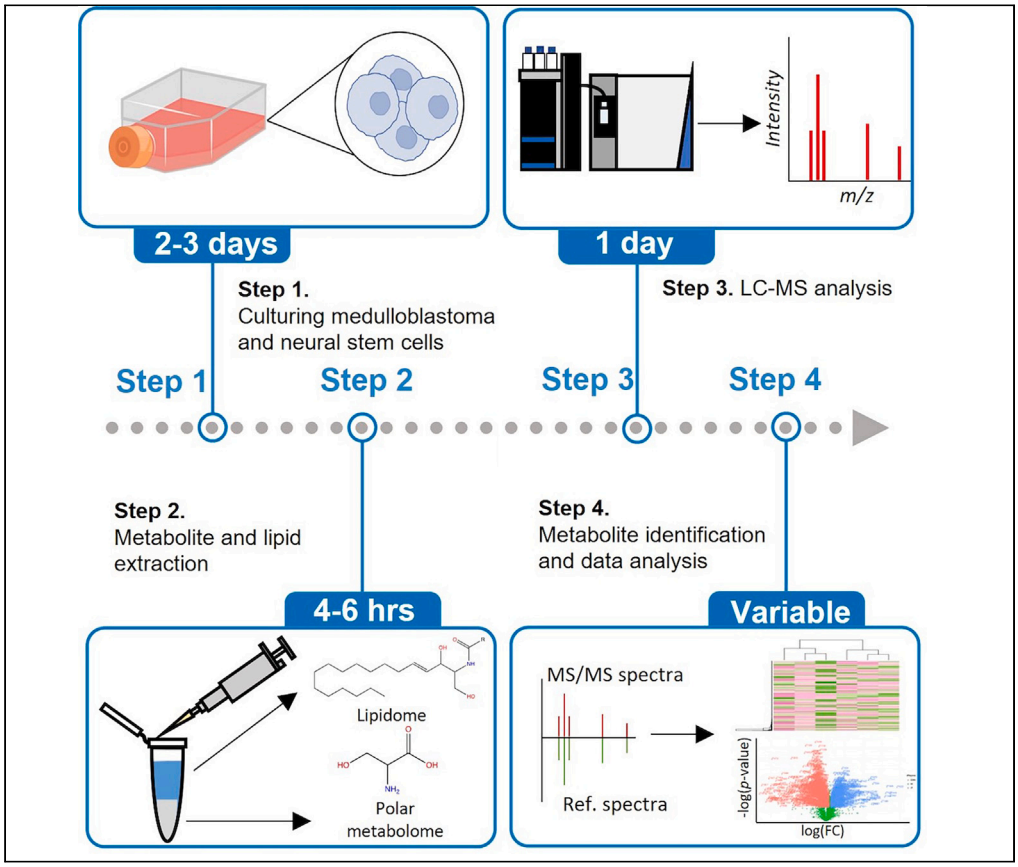
Protocol for mapping the metabolome and lipidome of medulloblastoma cells using liquid chromatography-mass spectrometry



Liquid chromatography-mass spectrometry (LC-MS)-based metabolomics and lipidomics have recently been used to show that *MYC*-amplified group 3 medulloblastoma tumors are driven by metabolic reprogramming. Here, we present a protocol to extract metabolites and lipids from human medulloblastoma brain tumor-initiating cells and normal neural stem cells. We describe untargeted LC-MS methods that can be used to achieve extensive coverage of the polar metabolome and lipidome. Finally, we detail strategies for metabolite identification and data analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Mapping the metabolome and lipidome of medulloblastoma and neural stem cells with LC-MS

Multiple LC-MS approaches to maximize metabolome and lipidome coverage

Metabolite/lipid identification approaches for untargeted data

Strategies and tools to visualize metabolomics/lipidomics data

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Protocol for mapping the metabolome and lipidome of medulloblastoma cells using liquid chromatography-mass spectrometry

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SUMMARY

Liquid chromatography-mass spectrometry (LC-MS)-based metabolomics and lipidomics have recently been used to show that *MYC*-amplified group 3 medulloblastoma tumors are driven by metabolic reprogramming. Here, we present a protocol to extract metabolites and lipids from human medulloblastoma brain tumor-initiating cells and normal neural stem cells. We describe untargeted LC-MS methods that can be used to achieve extensive coverage of the polar metabolome and lipidome. Finally, we detail strategies for metabolite identification and data analysis.

For complete details on the use and execution of this protocol, please refer to Gwynne et al.[1]

BEFORE YOU BEGIN

It is recommended that appropriate maintenance and calibration of the LC-MS instrument be performed before starting this protocol. Regular maintenance and calibration are critical for ensuring the generation of high-quality data. Prior to starting any LC-MS analysis, the ionization source should be cleaned to minimize levels of contaminants. Mass calibration should be conducted with the appropriate calibration mixes as recommended by the manufacturer. The LC system and column should also be equilibrated at starting solvent conditions before any analysis.

The protocol below describes the specific steps for LC-MS analysis of neural stem cells (NSCs)[2] or stem-like brain tumor initiating cells (BTICs)[3,4] propagated as spheres in serum-free, chemically-defined stem cell enrichment media (neurospheres and tumorspheres, respectively). However, the same metabolite extraction method and LC-MS methods can be/have been applied to other cell lines and biological samples.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Water (LC-MS grade)	Fisher Chemical	Cat. No. W6-4
Acetonitrile (LC-MS grade)	Fisher Chemical	Cat. No. A955-4
2-Propanol (LC-MS grade)	Fisher Chemical	Cat. No. A461-4
Formic acid (LC-MS grade)	Fisher Chemical	Cat. No. A117-50
Ammonium formate (LC-MS grade)	Sigma-Aldrich	Cat. No. 70221
Ammonium carbonate	Sigma-Aldrich	Cat. No. 379999-10G
Ammonium acetate	Sigma-Aldrich	Cat. No. A7262-500G
InfinityLab deactivator additive (medronic acid)	Agilent	Cat. No. 5191-4506
Ammonia solution 25% for HPLC	Sigma-Aldrich	Cat. No. 543830
Methanol (LC-MS grade)	Fisher Chemical	Cat. No. A456-4
Methyl tert-butyl ether (MTBE)	Honeywell	Cat. No. 34498
Quant-iT 1× dsDNA assay, high sensitivity (HS) and broad range (BR)	Invitrogen	Cat. No. Q33267
UltimateSPLASH ONE internal standard for lipidomic analysis	Avanti Polar Lipids	Cat. No. 330820
Software and algorithms		
Xcalibur	Thermo Fisher Scientific	Cat. No. OPTON-30965
Compound Discoverer (v3.3)	Thermo Fisher Scientific	Cat. No. OPTON-31061
R (v4.3.0)		https://www.r-project.org/
ggplot2 (v3.4.3)	CRAN	https://cran.r-project.org/web/packages/ggplot2/
ggfortify (v0.4.16)	CRAN	https://cran.r-project.org/web/packages/ggfortify/
Python (v3.11.5)		https://www.python.org
Other		
Orbitrap IQ-X Tribrid mass spectrometer	Thermo Fisher Scientific	Cat. No. FSN05-10001
Vanquish Horizon UHPLC system	Thermo Fisher Scientific	Cat. No. IQLAAAGABHFAPUMZZZ
ACQUITY UPLC CSH C18 column 1.7 µm, 100 mm × 2.1 mm	Waters Corporation	Cat. No. 186005297
SeQuant ZIC-pHILIC column 5 µm polymer, 150 × 2.1 mm	Sigma-Aldrich	Cat. No. 150460
SeQuant ZIC-pHILIC guard 20 × 2.1 mm	Sigma-Aldrich	Cat. No. 150437
Thermomixer R	Eppendorf	Cat. No. 05-400-203
Refrigerated CentriVap vacuum concentrator	Labconco	Cat. No. 7310020
TruView pH control LCMS certified clear glass, 12 × 32 mm screw neck vial, with cap and PTFE/silicone septum, 2 mL volume, 100/pk	Waters Corporation	Cat. No. 186005660CV
250 µL pulled point conical glass insert	Agilent	Cat. No. 5183-2085

MATERIALS AND EQUIPMENT

⚠ CRITICAL: Many of the reagents/solvents used for liquid chromatography are toxic, flammable, and/or caustic; see MSDS. Mobile phases should be prepared in a fume hood. All relevant personal protective equipment (PPE) (i.e., laboratory coats, gloves, and eye protection) should be worn during preparation.

Mobile phase A for LC-MS lipidomics positive-mode

Reagent	Final concentration	Amount
Acetonitrile	60.0%	600 mL
Water	38.9%	389 mL
Ammonium formate in water (1 M)	10 mM	10 mL
Formic acid	0.1%	1 mL

Mobile phase B for LC-MS lipidomics positive-mode		
Reagent	Final concentration	Amount
2-Propanol	88.9%	889 mL
Acetonitrile	10.0%	100 mL
Ammonium formate in water (1 M)	10 mM	10 mL
Formic acid	0.1%	1 mL

⚠ CRITICAL: 2-propanol and acetonitrile are toxic and flammable. Safety goggles, gloves, and lab coats are to be worn. Ensure adequate ventilation is in place prior to handling.

⚠ CRITICAL: Ammonium formate is a skin and respiratory irritant. Safety goggles, gloves, and lab coats are to be worn. Ensure adequate ventilation is in place prior to handling.

⚠ CRITICAL: Formic acid is toxic, flammable, and caustic. Ensure goggles, gloves, and lab coats are worn prior to handling.

Mobile phase A for LC-MS lipidomics negative-mode		
Reagent	Final concentration	Amount
Acetonitrile	60.0%	600 mL
Water	39.0%	390 mL
Ammonium acetate in water (1 M)	10 mM	10 mL

Mobile phase B for LC-MS lipidomics negative-mode		
Reagent	Final concentration	Amount
2-Propanol	89.0%	890 mL
Acetonitrile	10.0%	100 mL
Ammonium acetate in water (1 M)	10 mM	10 mL

Mobile phase A for LC-MS metabolomics (HILIC) negative-mode		
Reagent	Final concentration	Amount
1 M Ammonium carbonate in water	10 mM	10 mL
5 mM medronic acid in water	5 μM	1 mL
Water	N/A	To 1 L
Dilute ammonia hydroxide (~2.5% NH_3 content)	N/A	To pH 9 (2–4 mL)

Mobile phase B for LC-MS metabolomics (HILIC) negative-mode		
Reagent	Final concentration	Amount
Acetonitrile	100%	1 L

⚠ CRITICAL: Medronic acid and ammonium hydroxide are toxic and caustic. Safety goggles, gloves, and lab coats are to be worn. Ensure adequate ventilation is in place prior to handling.

LC-MS parameters for untargeted lipidomics

The instrumentation for untargeted lipidomics consists of a Orbitrap IQ-X Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Vanquish Horizon UHPLC system (Thermo Fisher Scientific). Chromatographic separation is achieved using an ACQUITY UPLC CSH

Table 1. MS source and acquisition parameters for untargeted lipidomics positive mode

Source parameter	Value
Ionization mode	Positive electrospray ionization
Spray voltage	+3.5 kV
Sheath gas flow rate	50 Arb
Auxiliary gas flow rate	10 Arb
Sweep gas flow rate	1 Arb
Ion transfer tube temperature	300°C
Vaporizer temperature	350°C
Acquisition parameter	**Value**
MS1 resolution	120,000
Scan range (*m/z*)	250–1500
AGC target (MS1)	4e5
Injection time (MS1)	50 ms
Intensity threshold	5.0e4
Dynamic exclusion	5.0 s
MS^n Acquisition mode	Data Dependent
Data Dependent Mode	Cycle Time
Time Between Master Scans (sec)	0.6
MS/MS resolution	30,000
Collision Energy Type	HCD and CID
AGC target (MS/MS)	7.5e4
Injection time (MS/MS)	Dynamic
Isolation window	1 *m/z*
Normalized collision energy (NCE)	Stepped NCE; 20, 30, 40

C18 column (1.7 μm, 100 mm × 2.1 mm; Waters Corporation, Milford, MA, USA). During separation, the column compartment is maintained at a temperature of 65°C. The MS parameters and LC method used are shown in Tables 1, 2, and 3. The MS methods and parameters described for lipidomics are specific to the Orbitrap IQ-X Tribrid mass spectrometer. Use of these lipidomics methods

Table 2. MS source and acquisition parameters for untargeted lipidomics negative mode

Source parameter	Value
Ionization mode	Negative electrospray ionization
Spray voltage	-3.5 kV
Sheath gas flow rate	29 Arb
Auxiliary gas flow rate	11 Arb
Sweep gas flow rate	1 Arb
Ion transfer tube temperature	325°C
Vaporizer temperature	350°C
Acquisition parameter	**Value**
MS1 resolution	120,000
Scan range (*m/z*)	250–1500
AGC target (MS1)	4e5
Injection time (MS1)	50 ms
Intensity threshold	5.0e4
Dynamic exclusion	5.0 s
MS^n Acquisition mode	Data Dependent
Data Dependent Mode	Cycle Time
Time Between Master Scans (sec)	0.6
MS/MS resolution	30,000
Collision Energy Type	HCD and CID
AGC target (MS/MS)	7.5e4
Injection time (MS/MS)	Dynamic
Isolation window	1 *m/z*
Normalized collision energy (NCE)	Stepped NCE; 20, 30, 40

Table 3. LC gradient for untargeted lipidomics (17 min/sample)

Time (min)	Flow (mL/min)	%B
0.00	0.25	15.0
1.00	0.25	15.0
3.00	0.25	30.0
3.50	0.25	48.0
12.00	0.25	82.0
12.50	0.25	99.0
13.00	0.25	99.0
13.10	0.25	15.0
15.20	0.25	15.0
17.00	0.25	15.0

on other high-resolution MS instruments may require additional optimization to achieve similar results.

Note: Higher-energy collisional dissociation (HCD) and collision-induced dissociation (CID) are fragmentation techniques that can be used to acquire diagnostic fragment ions. The

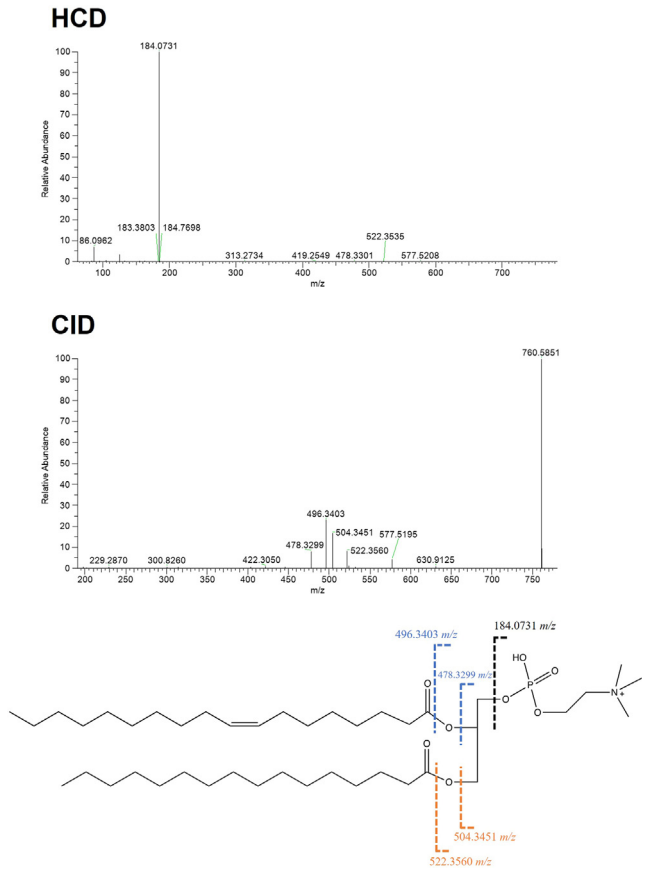

Figure 1. Comparison of MS/MS spectra of phosphatidylcholine (PC) 16:0_18:1 (760.585 *m/z*) under HCD and CID fragmentation

For PC 16:0_18:1, HCD fragmentation results in a 184.0731 *m/z* fragment, which corresponds to the phosphocholine head group (top). Complimentarily, CID fragmentation results in two fragments (496.3403 *m/z* & 522.3560 *m/z*), which corresponds to a neutral loss of acyl chains 18:1 and 16:0, respectively (middle). The two additional fragments (478.3299 *m/z* and 504.3451 *m/z*) correspond to additional water losses following the loss of the respective acyl chain. The different fragmentation methods produce complimentary MS/MS spectra that enhance the identification of PC 16:0_18:1 (bottom).

two fragmentation techniques generate distinct and complimentary fragmentation patterns, which can be used for metabolite/lipid identification (Figure 1). During lipidomics analysis, this protocol recommends the application of both HCD and CID on all samples to maximize lipid identifications.

Note: The same LC gradient is used for both positive and negative mode untargeted lipidomics.

LC-MS parameters and methods for untargeted metabolomics (HILIC)

The untargeted metabolomics workflow uses the same LC-MS instrumentation, as described above. Chromatographic separation is achieved using a SeQuant ZIC-pHILIC Guard column (20 × 2.1 mm; Sigma-Aldrich, St. Louis, MO, USA) coupled to a SeQuant ZIC-pHILIC column (5 μm polymer, 150 × 2.1 mm; Sigma-Aldrich). During separation, the column compartment is maintained at a temperature of 45°C. The MS parameters and LC method used are shown in Tables 4 and 5, respectively. The MS methods and parameters described for metabolomics are specific to the Orbitrap IQ-X Tribrid mass spectrometer. Other high-resolution MS instruments may require additional methods optimization to achieve similar results.

Table 4. MS source and acquisition parameters for untargeted metabolomics (HILIC) negative mode

Source parameter	Value
Ionization mode	Negative electrospray ionization
Spray voltage	-2.5 kV
Sheath gas flow rate	35
Auxiliary gas flow rate	7
Sweep gas flow rate	0
Ion transfer tube temperature	300°C
Vaporizer temperature	275°C
Acquisition parameter	**Value**
MS1 resolution	120,000
Scan range (*m/z*)	67–900
AGC target (MS1)	4e5
Injection time (MS1)	50 ms
Intensity threshold	5.0e4
Dynamic exclusion	5.0 s
MS^n Acquisition mode	Data Dependent
Data Dependent Mode	Cycle Time
Time Between Master Scans (sec)	0.6
MS/MS resolution	15,000
Collision Energy Type	HCD
AGC target (MS/MS)	7.5e4
Injection time (MS/MS)	Dynamic
Isolation window	1 *m/z*
Normalized collision energy (NCE)	Stepped NCE; 20, 30, 40

Table 5. LC gradient for untargeted metabolomics (HILIC) (21 min/sample)

Time (min)	Flow (mL/min)	%B
0.00	0.18	90.0
2.00	0.18	90.0
14.00	0.18	22.0
15.80	0.18	22.0
16.20	0.18	90.0
17.50	0.18	90.0
18.00	0.25	90.0
20.50	0.25	90.0
21.00	0.18	90.0

STEP-BY-STEP METHOD DETAILS

Sample procurement

This section describes the isolation of lipidome and metabolome fractions from patient-derived medulloblastoma (MB) tumor samples and donor-derived NSCs. Procurement of these samples is performed under approval of a human integrated research ethics board, and in collaboration with a clinical team that will provide surgically resected tumor tissue(s), or donor-derived fetal tissue(s). Collection and establishment of MB tumorspheres and NSC neurospheres should be performed as described in Chokshi et al. (2020) and Suk et al. (2022), respectively.[5,6]

Note: Prepare process blanks at the same time as sample procurement. A process blank is comprised of all sample preparation components (excluding the sample itself) and is subjected to the same sample preparation steps. In this case, the process blank should not have any cells, but should have all the reagents used in sample procurement and should be subjected to the same sample preparation steps as described in the following sections. Process blanks ensure that detected metabolites are derived from the biological sample itself.

Sample preparation for LC-MS analysis of metabolome and lipidome

Timing: approx. 1–2 h depending on number of samples, plus 12–16 h sample drying time.

This section describes the extraction of metabolites and lipids from donor-derived human NSCs, and patient-derived MB stem-like tumor cells. This extraction method was adapted from the methyl tert-butyl ether-methanol (MTBE) method described in Matyash et al. (2008) and Ding et al. (2021).[7,8]

⚠ CRITICAL: The solvents (i.e., acetonitrile, methanol, MTBE) used in this section are flammable and volatile. Safety goggles, gloves and lab coats are to be worn while handling solvents. Ensure there is adequate ventilation place prior to starting this section of the protocol.

⚠ CRITICAL: Extraction solvents, samples, and pipette tips should be kept chilled throughout the extraction process.

Note: The sample preparation protocol has been optimized for the extraction of metabolites and lipids from a cell pellet of $\sim 1 \times 10^6$. The volumes in this protocol can be scaled according to the number of cells harvested.

1. Re-suspend cell pellets in 255 μL of ice-cold H_2O/methanol (30:225, v/v).
2. Add 10 μL of UltimateSPLASH ONE (Avanti Polar Lipids, Alabaster, AL, USA) to each sample.

⚠ CRITICAL: UltimateSPLASH ONE is provided in volatile solvents (i.e., dichloromethane and methanol). Appropriate PPE (i.e., laboratory coats, goggles etc.) are to be worn while handling. UltimateSPLASH ONE internal standard will drip when added to samples. Care should be taken to prevent loss of the internal standard.

Note: It is recommended to use isotopically labeled internal standards (like UltimateSPLASH ONE) to achieve the highest confidence level of identification. No analogous comprehensive internal standard mix is available for polar metabolites; however, internal standards should be added for known metabolites of interest. Alternatively, the TruQuant Yeast Extract Semi-targeted QC Workflow Kit (IROA Technologies, Sea Girt, NJ, USA) have been used as internal standards for the polar metabolome.[9,10]

3. Vortex samples for 30 s.
4. Sonicate samples for 5 min in a cold-water bath.
5. Add 750 μL of ice-cold MTBE to each sample.
6. Vortex samples for 30 s.
7. Shake tubes samples for 10 min at 4°C at 650 RPM in the Thermomixer R (Eppendorf, Hamburg, Germany) temperature block.
8. Add 188 μL of LC-MS water to each tube to induce phase separation.
9. Vortex samples for 30 s.
10. Centrifuge samples for 20 min at 16,000 × *g* at 4°C.
11. Transfer 700 μL of the top (non-polar) component of each sample to a new microcentrifuge tube. This component constitutes the extracted lipidome of the NSC/MB cells.

⚠ CRITICAL: Transfer the organic layer to a new microcentrifuge tube without aspirating the aqueous (bottom) fraction.

12. Transfer 250 μL of the bottom (aqueous) component of each sample to a new microcentrifuge tube. This component constitutes the extracted polar metabolome of the NSC/MB cells.

⚠ CRITICAL: Cell debris should form as pellets following centrifugation. Transfer the aqueous fraction without disturbing the cell debris pellet. Ensure no cell debris is transferred with the extracted lipidome/metabolome layers.

13. Evaporate extracted lipidome/metabolome fractions with the vacuum concentrator (Labconco, Kansas City, MO, USA) at 10°C until fully dried.
14. Once dry, store samples at −80°C until LC-MS analysis.

Normalization of metabolite and lipid extracts

Timing: 2 h

This section describes the use of DNA quantification for the normalization of lipid and metabolite extracts for LC-MS analysis. Sample normalization is crucial for minimizing non-biological variation. Normalization of metabolite/lipid extracts to DNA levels is recommended as it correlates well with cell number.[11] The DNA content is quantified from the residual cell pellets that remain after sample preparation. Other common normalization methods include the use of total protein levels or cell count. Normalization to protein content has been reported to have large variability in metabolite-extracted samples.[11]

⚠ CRITICAL: Cancer cells are often aneuploid. The ploidy of the cell line or cells needs to be taken into account when normalizing to DNA levels.

15. From the remaining cell pellets, remove as much excess solvent as possible.
16. Allow the cell pellets to air-dry the excess solvent at 20°C–22°C until dry.
17. Resuspend the cell pellets in 200 μL of 1 M urea and 0.2% SDS.
18. Solubilize the cell pellets at 85°C for 30 min at 650 rpm in the Thermomixer R.
19. Once the cell pellets are dissolved, quantify the DNA content in the solubilized samples in triplicate according to the manufacturer's instructions for the Quant-iT 1× dsDNA BR Assay Kits, high sensitivity (HS) and broad range (BR) (Invitrogen, Waltham, MA, USA).
20. Using the quantified DNA values, adjust dried sample reconstitution volumes accordingly.

⚠ CRITICAL: Dried lipid fractions are typically resuspended in 200 μL of isopropanol:acetonitrile (50:50, v/v) for every 6 μg of DNA for the lipidomics method. Dried aqueous

fractions are typically resuspended in 100 μL of water:acetonitrile (30:70, v/v) for every 6 μg of DNA.

LC-MS analysis

Timing: 17 min/sample for lipidomics method; 21 min/sample for HILIC method

21. Prepare mobile phase A and B for the corresponding LC-MS method according to the "materials and equipment" section. All LC-MS mobile phases should be prepared fresh prior to each experiment. LC-MS mobile phases can be stored at 20°C–22°C just prior to use.
22. Purge the LC lines with the mobile phases.
23. Equilibrate the LC system by running the mobile phase A and B at starting conditions.
24. Allow source parameters to reach desired conditions.
25. Conduct the necessary LC-MS pre-run checks according to the manufacturer's guidelines before running any analysis.

Note: Pre-run checks of the instrumentation include minimizing background signal by cleaning the ionization source, performing mass calibration, and monitoring pump pressures and spray stability. These pre-run checks help to ensure the acquisition of high-quality data.

⚠ CRITICAL: Dried samples should be resuspended just prior to LC-MS analysis to avoid sample degradation. Reconstitution solvents and reconstituted samples should be maintained at 4°C.

26. Resuspend the dried fractions in DNA-normalized volumes isopropanol:acetonitrile (50:50, v/v) for the lipidomics method or water:acetonitrile (30:70, v/v) for the HILIC method.
27. Prepare LC-MS vials with glass inserts or conical vials for maximum recovery and transfer the resuspended samples to them.
28. In a separate sample vial, transfer an aliquot (~5 μL) from each sample to make a pooled quality control (QC) samples for column conditioning and analysis stability monitoring.
29. Reconstitute the process blank samples (See sample procurement section) with the same solvents as the samples.
30. Prepare a simple blank vial with the same solvent as the samples.

Note: Blank and QC injections are needed to ensure reproducibility and stability of the instrument. Blank and QC samples are to be monitored through the course of the experiment for carry-over. Signal intensities, retention time, and pump pressure should be monitored. During acquisition, blank and QC samples can be monitored in real-time in FreeStyle (Xcalibur) or other vendor software.

31. Transfer all vials to the autosampler, noting the position of each sample in the autosampler.
32. In Xcalibur, click on "Sequence Setup View".
33. Build the sample worklist by filling in the fields according to the headers for each sample. This includes Sample ID, File Name, Path, Instrument Method, Position, Injection Volume, etc.
34. Randomize the injection order of the samples to avoid order-dependent biases.

Note: An analysis should begin with a minimum of three simple blank injections. Blank vials are needed to check for the presence of contaminants and carry-over. This is followed by 8–10 QC loads for LC column conditioning. Following the injection of column conditioning samples, several simple blanks (2–3) and process blank samples (2–3) should be analyzed. Blank and process blank samples should also be periodically injected to monitor sample carry-over. Once complete, a final QC sample should be injected prior to sample analysis. Pooled QCs are injected regularly throughout the run and at the end of a run to monitor reproducibility

and stability of the instrument. A blank injection should also be conducted immediately following any QC injection.

35. Submit the sequence worklist.

LC-MS analysis using AcquireX

Timing: 17 min/injection for lipidomics method; 21 min/injection for HILIC method

AcquireX (Thermo Fisher Scientific) is a generalized data acquisition workflow that generates inclusion and exclusion lists from precursor *m/z* values and enables acquisition of MS/MS spectra through iterative injections in an automated manner.[12] This results in more MS/MS spectra than standard data-dependent methods, which improves compound identification. The AcquireX acquisition can be used in both the untargeted lipidomics and metabolomics workflows to maximize MS/MS spectra acquisition.

36. Click on "AcquireX View" to build the worklist for an AcquireX analysis.
37. Select the "Deep Scan" workflow.

Note: Deep Scan is one of four data acquisition workflows found in AcquireX. It allows for automated generation of both inclusion and exclusion lists. Using these inclusion and exclusion lists, the workflow determines whether MS/MS is acquired based on user-defined parameters like signal intensity.

38. Define the "Experiment Folder, "Experiment Name", "Full Scan Method, and "MS^n Template Method".

Note: The "Full Scan Method" in AcquireX is defined as the method that encompasses the entire mass range and focuses on data acquisition at the MS1 level. The parameters for the "Full Scan Method" are identical to the method described previously in this protocol except for the exclusion of the MS/MS fragmentation parameters. The MS^n Template is identical to the full data-dependent MS/MS method described earlier in the protocol.

39. Set the AcquireX Experiment Parameters to the appropriate values (Table 6).
40. Add three header blank injections of the solvent blank and define the second header blank as the "Exclusion Reference".
41. Add three deep scan injections of the pooled QC sample to the end of the header blank injections.

Note: Three deep scan injections provide deep coverage of the metabolome/lipidome of a particular sample. Additional deep scan injections provide only minimal gain in MS/MS spectra as low intensity precursor ions generate low quality and intensity spectra.

Table 6. AcquireX experiment parameters

Parameter	Value
Exclusion override factor	3
Exclusion List Peak Window Extension (s)	3
Inclusion List Peak Window Extension (s)	3
Inclusion List Peak Fragmentation Threshold (%)	50
Preferred ions	[M + H]+1; [M-H]-1
Exclusion Duration (s)	10

42. Save and submit the AcquireX worklist.
43. Monitor the sample files throughout the entire analysis to ensure instrument stability.

Note: Monitoring samples and pooled QCs involve checking pressures to ensure stability. The base peak chromatograms of pooled QCs should also be monitored for retention shifts and changes in signal intensity. Internal standards spiked into the pooled QCs can be used as marker compounds to ensure retention time and signal reproducibility. For a given internal standard, retention time shifts should be within 10 s for the lipidomics method and 30 s for the HILIC method. It is recommended that signal intensities of a given internal standard should have coefficient of variations (CV) <15% when comparing repeated injections. CV values are dependent on the abundance of a given metabolite/lipid. As a result, metabolite/lipid levels near the limit of detection may have CVs>15%. Matrix effects are contributors to retention time shifts. They can be identified by comparing the retention time of spiked internal standard in a sample with the retention time of the internal standard alone. Matrix effects should be noted if retention times are used for metabolite/lipid identification.

Data processing using Compound Discoverer

Timing: 2–6 h depending on the number of files and type of analyses being conducted

Compound Discoverer (v3.3) is a mass spectrometry data processing and analysis software used for the investigation of metabolites and lipids. It performs peak picking, retention time alignment, MS/MS matching, and local/online database searching to identify metabolites and lipids. It also provides data visualization tools and statistical analysis capabilities to aid in data interpretation.

44. Open Compound Discoverer (v3.3) and click on "New Study and Analysis".
45. Define the "Study Name" and the "Studies Folder".
46. Select the desired template workflow to be used in the analysis.

Note: Compound Discoverer provides template metabolomics and lipidomics workflows that can be customized based on various parameters. Detailed information about the individual nodes and parameters can be accessed on the Thermo Fisher Scientific website for the product.

47. Click on "Add Files" to input the sample files to be used with the Compound Discoverer analysis.
48. Under "Input File Characterization", define the sample types and groupings for the analysis.
49. Once complete, under "Sample Groups and Ratios", define the desired comparisons for statistical analyses.
50. Click "Finish" to create the "Study" file.
51. In the "Study" file, check that all desired files are inputted, all groupings are defined, and the workflow is correct.
52. Modify data processing nodes and parameters accordingly (Table 7).
53. Click "Run" to begin data processing.

Statistical analyses

Timing: 3 h

This section describes common statistical methods and strategies used to understand metabolomics/lipidomics data.

54. From the Compound Discoverer results file, apply the appropriate statistical tests and multiple test corrections to calculate p-values.

Table 7. Compound Discoverer workflow parameters

Parameter	Value (lipidomics method)	Value (HILIC method)
Mass tolerance (Detect Compounds) [ppm]	5	5
Minimum peak intensity	100000	50000
Min. # Scans per Peak	5	5
Use Most Intense Isotope Only	True	True
Chromatographic S/N threshold	1.5	1.5
Max. Peak Width [min]	0.5	0.5
Use Peak Quality for Isotope Grouping	True	True
Filter out Features with Bad Peaks Only	True	True
Remove Potentially False Positive Isotopes	True	True
Ions	[M + H]+1, [M-H]-1, [M + NH_4]+1, [M-H+HAc]-1, [M + H-H_2O]+1, [M-H-H_2O]-1, [M+Na]+1	[M-H]-1, [M-H+HAc]-1, [M-H-H_2O]-1
Base Ions	[M + H]+1, [M-H]+1	[M + H]+1, [M-H]+1
Detect Persistent Background Ions	True	True
Area Integration	Most Common Ion	Most Common Ion
RT Tolerance [min]	0.2	0.2
Search Algorithm/Identity Search	HighChem HighRes	HighChem HighRes
Match Factor Threshold	60	60
Databases search	LipidBlast (v6.8), mzCloud	FiehnHILIC database, mzCloud

Note: The statistical tests and methods of multiple test correction will depend on the study. Common statistical tests include *Student's t-test* and *ANOVA* with post hoc Tukey's HSD. A common multiple test correction method is Benjamini-Hochberg correction. In datasets where normality or homoscedasticity assumptions are violated, non-parametric statistical tests such as the *Mann-Whitney U test* or the *Kruskal-Wallis test* can be considered.

55. Calculate the fold-changes (FCs) between the groups being compared.
56. Using p-values and FCs, select significantly different (e.g., $p < 0.05$ with multiple test correction) metabolic features with large FCs (e.g., FC > 1.5) for identification.

Alternatives: The process of identifying metabolites and lipids is very time-consuming. Thus, it is recommended to have an organized approach to checking identifications. One method is to specifically identify only significantly changing metabolic features. Alternatively, metabolites/lipids in specific metabolic pathways of interest (i.e., glycolysis, TCA cycle) can be selected for identification.

Identification of metabolites and lipids

Timing: varies

This section describes strategies for identifying metabolites and lipids from an untargeted metabolomics or lipidomics experiment. Compound Discoverer searches acquired MS/MS spectra against local and online spectral libraries (e.g., mzCloud) to make identifications. Spectral libraries contain reference MS/MS spectra, which can be matched against acquired MS/MS data. Metabolites/lipids identified using this method cannot be completely relied upon due to the presence of in-source fragments, adducts, and co-isolating ions. Because of this, manual curation/annotation of MS/MS data is often required using mirror plots for high confidence identifications.

⚠ CRITICAL: The Metabolomics Standards Initiative (MSI) categorizes the identification of metabolites into five confidence levels.[13] The highest confidence level of identification (MSI level 1) requires a reference standard to confirm the structure. Reference standards are not available for every single metabolite/lipid; thus, MS/MS data can be sufficient for putative identification if it matches spectra from literature or spectral libraries (MSI level

2). Typically, identified metabolites and lipids should have at least MSI level 2 and the level of identification should be reported.

57. From the completed Compound Discoverer Result file, check the MS/MS spectra of individual metabolites and lipids with reference MS/MS spectra.

Note: The performance of these computational tools for identification can vary depending on the metabolite and its fragmentation pattern. Thus, there is no single search tool or algorithm that can reliably match experimental MS/MS data to those found in databases. Generally, an ideal MS/MS match has the correct diagnostic fragments within a small error (<5 ppm) and limited co-isolating/contaminating ions at similar collision energies.

58. If isotopically labeled/reference standards are available, analyzing them using identical LC-MS methods to confirm retention time and MS/MS provides the highest confidence level of metabolite identification (MSI Level 1). If the resulting retention time the reference standard does not match, then additional analyses may be needed to determine the metabolite identity as matrix effects can contribute to retention time shifts.

Note: Isotopically labeled standards have the same retention times, but *m/z* shifts corresponding to the incorporated isotopic atoms.

Note: The combinatorial nature of lipids makes it impossible for authentic reference standards to be available for every single lipid. For species that do not have standards, natural lipid mixes could be used instead. Natural lipid mixes consist of lipids belonging to the same class extracted from a specific source (i.e., Ceramide (Brain) Cat. No. 860052, Avanti Polar Lipids). Lipid species of the same class fragment in a predictable manner. Rules for lipid fragmentation have been previously established and should be used to enhance lipid identifications.[14]

59. Validate the fragmentation spectra of lipids using reference standards or lipid mixes.
60. Plot mass-to-charge vs. retention time plots (*m/z* vs. RT) to ensure identified lipids belong to the same class.

Note: In reversed-phase chromatography, lipid species within the same class will elute in a linear manner. Retention time will increase as the length of acyl chains increases. Lipids that violate this trend likely do not belong in the given lipid class. The use of both fragmentation patterns and retention time trends strengthens the lipid identifications when authentic reference standards are unavailable.

61. Correct any incorrect or ambiguous annotations provided by Compound Discoverer.

Data visualization and interpretation

Timing: 2 h

This section describes data visualization methods used to understand and depict metabolomics/lipidomics data. Clear and visually appealing figures simplifies data interpretation. Data plots described here can be generated using programming languages like R and Python.

62. Once metabolite/lipid identifications are complete, export the Compound Discoverer Result file with peak areas as a CSV file.
63. Generate boxplots using peak areas to compare the relative levels of metabolites of interests between groups. This can be done using the ggplot2 package.

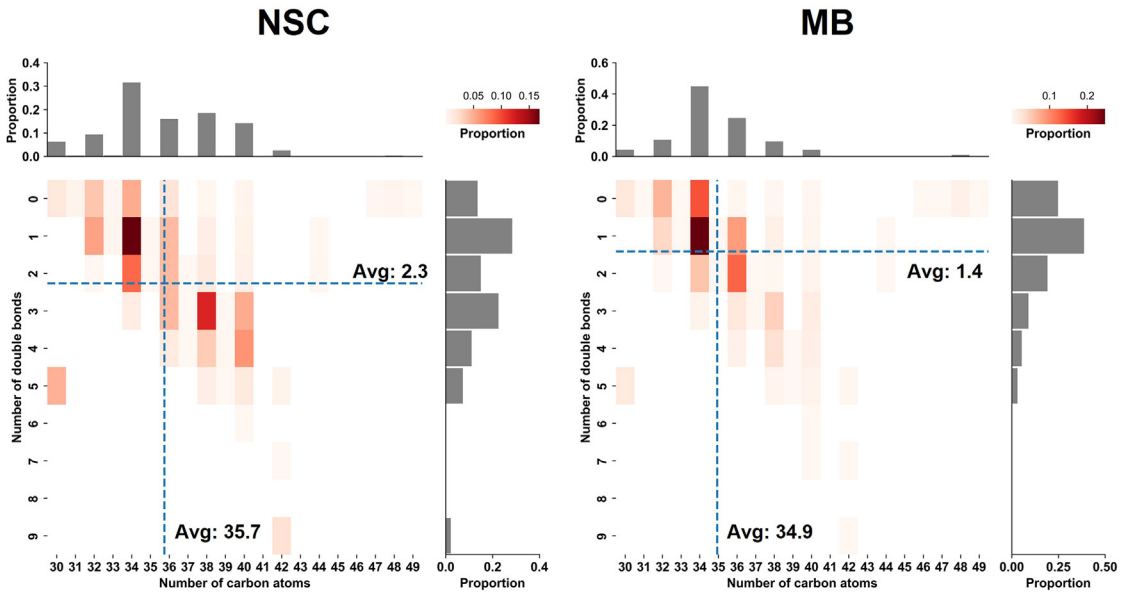

Figure 2. Fatty Acid Composition Heatmaps (FACHs) reveal a decrease in saturation levels in the diglycerides of MB cells compared to NSCs

The x-axis of the heatmap depicts the total number of carbons while the y-axis depicts the total number of double bonds on the fatty acids of a particular lipid class. The color scale corresponds to the proportion by which a lipid species makes up the total signal intensity of the lipid class. The gray bars outlining each FACH depict histograms representing the proportional abundance of total carbons or total double bonds for any given lipid species within a class. The lines on the FACHs show the mean fatty acid composition (number of carbons and number of double bonds) of a given lipid class, considering the relative abundance of each individual lipid.

64. Generate principal component analysis (PCA) plots. This can be generated using the ggplot2 and ggfortify packages.
65. Perform log-transformation and z-score standardization (auto-scaling) on the exported peak areas.

Note: The scaling and transformation of metabolomics data is dependent on the data acquired. It is important to note that no single scaling and transformation method is suitable for all datasets.

66. From the analyzed data, perform hierarchal clustering to represent the data.

Alternatives: Pathway analysis (i.e., MetPA, MSEA etc.) can also be done to determine the metabolite classes and metabolic pathways enriched in a particular sample group.[15,16] It is important to note that the method in which the data is shown depends on the content being conveyed.

Alternatives: Information about individual lipid classes' selectivity for specific fatty acids (chain length and saturation) can be gained by creating Fatty Acid Composition Heatmaps (FACHs). FACHs depict all lipid species within a class based on the number of carbons and double bonds within its acyl chains (Figure 2). The colors on the heatmap represent the proportion (percentage) of lipid species containing a specific number of carbons and double bonds within a specific lipid class. Using FACHs, shifts in fatty acid elongation and saturation levels can be visualized and interpreted. This analysis is independent of the amount of sample injected and the absolute concentration levels of lipids within each class, as only the proportions of the FA composition within the same lipid class are considered and compared. This ultimately allows comparisons between different types of cells (size and morphology). Source code for the generation of FACHs is available in our Zenodo data repository.

EXPECTED OUTCOMES

This protocol describes how the metabolome and lipidome of MB cells can be profiled using LC-MS-based approaches. The metabolite extraction process described here allows for coverage of both the polar metabolome and lipidome in a single extraction method. The LC-MS methods described in this protocol regularly allows for the confident identification of >300 metabolites and >900 lipid species. The individual metabolites and lipids encompass a wide range of metabolic pathways (i.e., central carbon metabolism, TCA cycle, nucleotides, amino acids etc.) and lipid classes (glycerophospholipids, sphingolipids, glycerolipids, sterols etc.). LC-MS-based metabolomics and lipidomics approaches have become increasingly popular in cancer research, complementing other approaches such as genomics, transcriptomics, and proteomics. Metabolomics is a hypotheses-generating technique in which biologically relevant metabolic pathways can be identified and further investigated. In the context of MB, the identification of cancer-specific metabolic pathways using metabolomics serves as a starting point for novel and targeted cancer therapeutics.

LIMITATIONS

In this protocol, we describe a workflow that profiles the metabolome and lipidome of NSCs and MB cell lines. With the use of cell lines, cell culture artifacts can arise. When possible, *in vivo* models should be used to complement findings derived from cell lines. Furthermore, the chemical diversity of the metabolome and lipidome makes the detection of all compounds in a cell difficult. No single method can optimally extract all metabolites and lipids for analysis. This protocol describes a method that provides good coverage of many metabolites and lipids that are critical to cellular metabolism. In situations with specific metabolites of interest, alternative extraction and enrichment processes may be needed. Lastly, not all known metabolites have MS/MS spectra in databases. In an untargeted metabolomics experiment, only 2%–10% of compounds are typically identified.[17] The majority of the signal remains unidentified and is referred to as the dark metabolome.

TROUBLESHOOTING

Problem 1

Cell debris was transferred with the extracted polar metabolome/lipid layers (step 11–12).

Potential solution

- Re-centrifuge the sample with the cell debris and transfer the supernatant to a new tube.

Problem 2

Pump pressure is not stable and is increasing or decreasing as the run progresses (step 43).

Potential solution

- Increases in pump pressure beyond what is known to be normal are a sign of a blockage in the LC system.
  - Identify the location of the blockage and subsequently flush out the system with different solvents (i.e., water, acetonitrile, isopropanol) to clear the blockage or replace hardware (tubing, valve, etc.).
- Decreases in pump pressure can be a sign of leakage in the LC system.
  - Identify the location of the leak by looking for signs of dried solvent.
  - Tighten connections between lines and the chromatographic column systematically.

Problem 3

High background signal is present in blank samples (step 43).

Potential solution

- High background signal is a sign of contaminants within the system.
- Thoroughly flush the column and the LC system with the appropriate mobile phases.
- Clean the MS source based on the manufacturer's guidelines.
- If the background signal remains, prepare new mobile phases and change guard and chromatographic column.

Problem 4

Chimeric MS/MS spectra complicate lipid identification (step 61).

Potential solution

- Chimeric MS/MS spectra can be observed when multiple precursor ions co-isolate during the isolation step for MS/MS fragmentation.
- Using the MS1 scans, identify the potential co-isolating ions.
- If the co-isolating ion is a lipid and belongs to the same lipid class as the precursor ion, summed composition notation can be used.
- Summed composition notation can be used to describe lipid species as a sum of all carbons and double bonds within its fatty acyl chains.
  - Summed composition does not describe the exact acyl chain composition of a given lipid.

Problem 5

There are duplicate annotations for individual metabolites/lipids (step 61).

Potential solution

- Run a reference standard to determine if structural information matches the initial sample.
- If no reference standard is available, check the MS/MS spectra with literature and other databases to determine the correct identity of the metabolite.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for MS instrument methods, resources, and reagents should be directed to and will be fulfilled by the lead contact, J. Rafael Montenegro-Burke (rafa.montenegro@utoronto.ca).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Source code for the generation of FACHs can be found on Zenodo at https://doi.org/10.5281/zenodo.8364217.

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AUTHOR CONTRIBUTIONS

J.K.C., W.D.G., C.V., S.K.S., and J.R.M.-B. conceptualized the protocol. J.K.C. and W.D.G. wrote the manuscript. J.K.C., W.D.G., B.Y.L., A.T.Q., and J.R.M.-B. developed the methodology used in this protocol. C.V., S.K.S., and J.R.M.-B. reviewed and edited the manuscript.

DECLARATION OF INTERESTS

C.V. is a member of the *STAR Protocols* advisory board.

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