Chapter 12

A Lipidomics View of Circadian Biology

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Abstract

Lipidomics approaches provide quantitative characterization of hundreds of lipid species from biological samples. Recent studies highlight the value of these methods in studying circadian biology, and their potential goes far beyond studying lipid metabolism per se. For example, lipidomics analyses of subcellular compartments can be used to determine daily rhythmicity of different organelles and their intracellular dynamics. In this chapter we describe in detail the procedure for *around the clock* shotgun lipidomics, from sample preparation to bioinformatics analyses. Sample preparation includes biochemical fractionation of nuclei and mitochondria from mouse liver harvested throughout the day. Lipid content is determined and quantified, in unbiased manner and with wide coverage, using multidimensional mass spectrometry shotgun lipidomics (MDMS-SL). Circadian parameters are then determined with nonparametric statistical tests. Overall, the approach described herein is applicable for various animal models, tissues, and organelles, and is expected to yield new insight on various aspects of circadian biology and lipid metabolism.

Key words Shotgun lipidomics, Mass spectrometry, Circadian, Mouse, Liver, Nuclei, Mitochondria, Lipid metabolism

1 Introduction

Lipids play essential roles in the structure/function of biological membranes of cells and organelles, and thus measurably define their identity [1]. Therefore, comprehension of lipid composition is essential in understanding the related functionality. Alongside increasing evidence tying circadian clocks with lipid homeostasis [2–4], technological advances evinced lipidomics to be a powerful tool that can be used as a reliable "time-teller," and readout for circadian biology [5–7]. Specifically, lipidomics analyses of subcellular compartments throughout the day can be used to determine spatial and temporal dynamics in lipid composition and uncover daily rhythmicity in intracellular organelles.

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We commence with a detailed description of the protocol used for biochemical isolation of nuclei and mitochondria from mouse tissue around the clock. These intracellular organelles were selected in view of their critical role in circadian biology and metabolism. The sample isolation procedure is based on multiple centrifugation steps, performed in different sucrose-based media.

Subsequently, samples are homogenized and spiked with appropriate internal standards. Lipids are extracted and analyzed by multidimensional mass spectrometry shotgun lipidomics (MDMS-SL). This technique provides quantitative measurements of >95% of the lipid mass, covering hundreds to thousands of lipid molecular species [8–10]. Depending on the nature of the biological sample, the technology described herein is expected to yield quantitative measurements of glycerophospholipids (e.g., cardiolipin, phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine), lysoglycerophospholipids (e.g., lysocardiolipin, lysophosphatidylcholine, lysophosphatidylethanolamine), sphingolipids (e.g., ceramide and sphingomyelin) and glycerolipids such as triacylglycerols. These molecules are characterized according to class and biochemical structure: acyl-chain length and linkage type (if relevant), and degree of saturation (i.e., number of double bonds).

Finally, the circadian analysis of the daily lipid profiles is performed using statistical tests such as the one offered in JTK_CYCLE algorithm [11]. Additional commonly used statistical analyses can be applied to dissect the lipid composition and dynamics of the different organelles.

The method described herein provides the lipid landscape of nuclei and mitochondria isolated from mouse liver throughout the day, and therefore can be used to monitor their "internal" time. This approach can be applied on different animal models, tissues, and organelles and thereby unravel various novel facets of circadian biology and lipid metabolism.

2 Materials

2.1 Sample Preparation

∗ All instruments and reagents are to be cold during use (4 °C). Since storing in cold may cause rust, preferably cool only prior to use.

2.1.1 Nuclei Isolation from Mouse Liver

Supplement all buffers with 0.5 mM DTT, 100 μM NaF, 100 μM Na_2VO_4, 0.5 μM PMSF, and 1:500 Protease Inhibitor cocktail (we use CALBIOCHEM).

1. Homogenization buffer A, *0.3 M sucrose*: 10 mM Hepes pH 7.6, 15 mM KCl, 2 mM EDTA, 0.15 mM spermine,

0.5 mM spermidine, 10% glycerol (prepare ~4 mL per liver sample).[1]

2. Homogenization buffer B, *2.2 M sucrose*: 10 mM Hepes pH 7.6, 15 mM KCl, 2 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 10% glycerol (prepare 25 mL per liver sample: 7 for homogenization, 18 for homogenate dilution).[2]
3. Cushion buffer *2.05 M sucrose*: 10 mM Hepes pH 7.6, 15 mM KCl, 2 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 10% glycerol (prepare ~11 mL per liver sample).[3]
4. Isotonic buffer: 10 mM Hepes pH 7.6, 100 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 10% glycerol. Store in 4 °C for up to 6 months.
5. Cold phosphate-buffered saline (PBS).
6. Swinging-bucket ultracentrifuge (4 °C), we use Beckman, SW28 rotor.
7. Beckman ultracentrifuge polycarbonate[4] tubes, suitable for the above rotor (40 mL volume).
8. Electric homogenizer (we use IKA 3720001 T-18 Ultra-Turrax Digital Homogenizer).
9. Dounce homogenizer: consisting of a glass tube with a tight-fitting Teflon pestle (we use KONTES glass 23, 15 mL capacity at least).
10. Vacuum pump.
11. Syringe (of any kind, suitable to wash a tube as described in Subheading 3.2.1).
12. Spatula.
13. Microtubes (1.5 mL).
14. Delicate paper wipes (e.g., Kimwipes).

2.1.2 Mitochondria Isolation from Mouse Liver

1. Mitochondrial Isolation Buffer (MIB): 70 mM sucrose, 200 mM mannitol, 10 mM HEPES, 1 mM EDTA, pH 7.5.[5] Buffer can be stored in 4 °C for up to 6 months (visually check for contaminations before use, filtration is recommended).
2. MIB supplemented with 0.2% bovine serum albumin (BSA) fatty-acid free.[6]

[1] Melt sucrose on a slightly warm plate, prior to addition of other ingredients. Prepare buffer in advance, at least 1 day prior to fractionation. This ensures hardening which will sustain the biological sample. This buffer can be stored in 4 °C for up to 1 month. Titer to pH with either KOH or NaOH.

[2] *See* footnote 1.

[3] *See* footnote 1.

[4] Polycarbonate is important for reuse of tubes, from our experience other materials might damage when reusing.

[5] Titer with KOH to improve extraction.

[6] Its purpose is to remove fat tissue from the sample.

3. Dounce homogenizer, consisting of a glass tube with a tight-fitting Teflon pestle (we use KONTES glass 23), use 15 mL capacity at least.
4. Refrigerated centrifuge (4 °C) with SS34 rotor.
5. Round bottom tubes suitable for the above rotor.
6. Glass rod.

2.2 Shotgun Lipidomics

Quantity of biological samples for analysis: >0.2 mg protein of nuclei or >0.4 mg protein of mitochondria.[7]

1. Nano-ESI source device (TriVersa NanoMate, Advion Bioscience Ltd., Ithaca, NY, USA).
2. Chipsoft 8.3.1 software (TriVersa NanoMate, Advion Bioscience Ltd., Ithaca, NY, USA).
3. Mass spectrometer (Thermo TSQ Quantiva™ Triple Quadrupole Mass Spectrometer, San Jose, CA, USA).
4. Xcalibur™ software (Thermo Fisher Scientific, San Jose, CA, USA).
5. Kontes Microtube Pellet Pestle Rods with Motor (we use Daigger Scientific Inc., Vernon Hills, IL, USA).
6. 6 mL and 10 mL reusable culture tube with PTFE lined cap.
7. 5.75″ disposable borosilicate glass Pasteur pipets (we use Thermo Fisher Scientific).
8. Vortex mixer.
9. Tabletop centrifuge.
10. 24 position N-EVAP nitrogen evaporator (we use Organomation Associates, Inc., Berlin, MA).
11. Chemical resistance 96-well microplates.
12. Calibrated micropipettes.
13. Chloroform.
14. Methanol.
15. Methyl-*tert*-butyl ether (MTBE).
16. Millipore deionized water.
17. Hydrochloric acid.
18. Glacial acetic acid.
19. Trimethylsilyl diazomethane solution 2.0 M in hexanes.
20. Lithium chloride.
21. Isopropanol.
22. Lithium hydroxide.

[7] Else, could be performed on 10^6 cells, 100 μL body fluids

23. BCA protein assay kit (we use Fisher Scientific).
24. Lipid internal standards[8]:
    (a) 1,2-Dimyristoleoyl-*sn*-glycero-3-phosphocholine (di14:1 PC).
    (b) 1,2-Dipalmitoleoyl-*sn*-glycero-3-phosphoethanolamine (di16:1 PE).
    (c) 1,2-Dipentadecanoyl-*sn*-glycero-3-phosphoglycerol (sodium salt) (di15:0 PG).
    (d) 1,2-Dimyristoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (di14:0 PS).
    (e) 1,2-Dimyristoyl-*sn*-glycero-3-phosphate (sodium salt) (di14:0 PA).
    (f) 1,1′,2,2′-Tetramyristoyl cardiolipin (T14:0 CL).
    (g) 7,7,8,8-d_4-Palmitic acid (d_4-16:0 NEFA) (Cambridge Isotope Laboratories, Andover, MA, USA).
    (h) *N*-Lauroyl sphingomyelin (N12:0 SM).
    (i) *N*-Heptadecanoyl ceramide (N17:0 Cer).
    (j) 1-Heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (17:0 lysoPC).
    (k) 1-Myristoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (17:0 lysoPE).
    (l) 1,2,3,4-$^{13}C_4$-Palmitoyl-L-carnitine hydrochloride ($^{13}C_4$-16:0 CN) (Sigma-Aldrich, St. Louis, MO, USA).
    (m) Triheptadecenoin (T17:1 TAG) (Nu Chek, Inc. Elysian, MN).

3 Methods

3.1 Mice Handling

Use three-month-old C57BL male mice. Mice are housed under routine light schedule of 12-h light/dark regimen for at least 2 weeks to allow accommodation. Mice are sacrificed by cervical dislocation or carbon dioxide exposure at the desired light regimen, namely, under light/dark or dark/dark, according to the experimental aims.

3.2 Liver-Organelle Isolation

Livers should be harvested immediately upon sacrifice.[9] Perform all stages on ice *see* Fig. 1.

[8] We purchase all of the lipid internal standards from Avanti Polar Lipids, Inc., Alabaster, AL, USA except if otherwise noted.

[9] In case of liver analysis without fractionation rapidly freeze the tissue in liquid nitrogen and keep frozen until lipidomics.

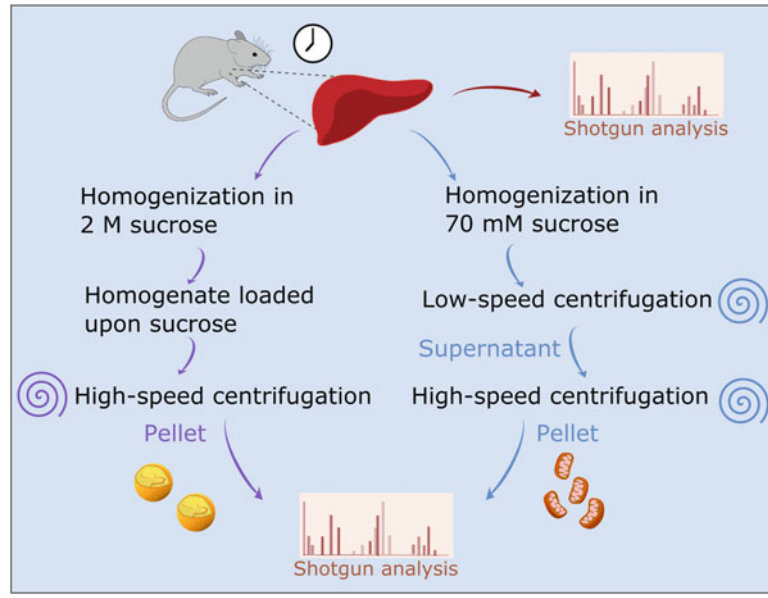

Fig. 1 General scheme of nuclear and mitochondrial fraction isolation from mouse liver. The isolation procedure is based on multiple centrifugation steps, performed in different sucrose-based media. Nuclei isolation can be done with a liver which has been frozen in −80 °C, while for mitochondria only fresh liver is to be used. For whole liver analysis without fractionation rapidly freeze the tissue in liquid nitrogen and keep frozen until lipidomics

3.2.1 Nuclei Isolation from Mouse Liver[10]

1. Rinse liver in cold PBS.
2. Place liver sample in glass homogenizer tube, complete volume to 5 mL with 0.3 M sucrose Homogenization buffer A (i.e., add ~4 mL).
3. Add 7 mL of 2.2 M sucrose Homogenization buffer B.
4. Homogenize liver using tight fitting pestle at 1150–1200 rpm (according to IKA motor setup). Run through the sample with three vertical motions. Avoid air bubbles.
5. Following homogenization, add 18 mL of 2.2 M Homogenization buffer B.
6. Shake vigorously by hand.
7. Prepare Beckman ultracentrifugation tube with 10.5 mL Cushion buffer 2.05 M sucrose.
8. Softly place the sample on top of the Cushion.[11]
9. Centrifuge for 1 h, at 48,490 × *g*, at 4 °C.

[10] Nuclei isolation can be done with a liver which has been frozen in −80 °C.

[11] This is a crucial step: materials must be very cold to avoid turbulence. Transfer the sample in slope, gently on the wall of the tube. The two phases must not mix, this would destroy the separation. If mixture occurs cease immediately and resume with the rest of the sample on top of a new Cushion. The mixed samples cannot be used.

10. Remove the upper fat layer (using spatula or other instrument of choice).
11. Remove the red fraction with vacuum.[12,13]
12. Keep tube inverted to drain the remaining liquids. While inverted, wash the walls of the tubes with DDW (it is easy done using a syringe[14]) and dry the water with delicate paper.
13. Resuspend pellet in ~0.2 mL Isotonic buffer and transfer to Microtubes tubes (1.5 mL).[15]
14. Freeze samples in −80 °C and store for lipidomics analyses.

3.2.2 Mitochondria Isolation from Mouse Liver[16]

1. Rinse liver in cold Mitochondrial Isolation Buffer (MIB) several times to remove blood.
2. Mince liver using razor or scissors in 3 mL MIB supplemented with 0.2% BSA, and transfer the sample to the glass tube.
3. Use Teflon Dounce to homogenize the sample, stroke up and down two times.[17]
4. Add 15 mL MIB and transfer[18] homogenate to centrifuge tubes.
5. Centrifuge at 600 × *g*, for 10 min, at 4 °C.
6. Following centrifugation use a pipet to extract the middle fraction.[19] Move this desired fraction to a clean centrifuge tube.
7. Centrifuge at 7000 × *g*, for 15 min, at 4 °C.
8. Remove supernatant and resuspend pellet in 10 mL MIB.
9. Centrifuge at 7000 × *g*, for 15 min, at 4 °C.[20]
10. Remove sup and resuspend pellet in 100 μL MIB.
11. Freeze samples in −80 °C, and store for lipidomics analyses.

3.3 Lipid Extraction

Perform stages 1 and 2 on ice.

1. Homogenize sample[21] in 500 μL cold PBS for 1 min using the pellet pestle rods with cordless motor at 2000–3000 RPM.

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[12] This contains crude cytosolic fraction, which, if desired, can be used for western blot. In that case, collect with a pipette and store at −80 °C until use.

[13] Vacuum far from the pellet, tilt tube to distance the liquid from the pellet.

[14] Avoid scratching the tube for further use

[15] For maximal yield first resuspend in half the volume, transfer it, and thoroughly collect the remaining pellet with the rest.

[16] Only fresh liver is to be used, mitochondria do not fractionate well from frozen liver.

[17] Manual homogenization; Avoid creating vacuum force and/or air bubbles!

[18] For maximal yield first resuspend in half the volume, transfer it, and thoroughly collect the remaining pellet with the rest.

[19] Avoid the fat layer on top and the lower layer at the bottom. Aim for ~10–13 mL to be on the safe side.

[20] **Steps 8** and **9** are an optional wash, we perform it.

[21] Equivalent to 10–20 mg wet weight of tissue or ~1 × 10^6 cells.

2. Perform protein assay with 5–10 μL aliquot of homogenate for each sample.
3. Prepare a mixture of internal standards: di14:1 PC, di16:1 PE, di15:0 PG, di14:0 PS, di14:0 PA, T14:0 CL, d4-16:0 NEFA, N12:0 SM, N17:0 Cer, 14:0 lysoPE, ^{13}C4-16:0 carnitine, 17:0 lysoPC, and T17:1 TAG.[22]
4. Transfer a precise volume of homogenate of each sample into a disposable culture borosilicate glass tube (10 mL). Add the premixture of internal standards, based on the protein content of the transferred homogenate sample.[23]
5. Prepare extraction solvent using: chloroform/methanol (1/1, v/v) (solvent A), chloroform (solvent B), and 10 and 50 mM lithium chloride solutions.
6. For extraction add 4 mL solvent A to the 10 mL glass tube from **step 3**, and 2 mL 50 mM LiCl. Cap the tubes and vortex for 20 s.
7. Centrifuge at 4000 × *g*, for 10 min, at 4 °C.
8. Collect the bottom layer to a new borosilicate glass tube,[24] and add 2 mL solvent B to the residual top layer. Cap the tubes and vortex them for 20 s.
9. Centrifuge at 4000 × *g*, for 10 min, at 4 °C.
10. Collect the bottom layer and combine it with that collected in **step 7**.
11. Evaporate the combined chloroform layers under a nitrogen stream.
12. Resuspend individual residue in **step 10** with 4 mL of solvent A, and add 2 mL of 10 mM LiCl. Cap the tubes and vortex them for 20 s.
13. Repeat **steps 8–12**.
14. Resuspend individual lipid extract residue from **step 12** with solvent A in a volume of 200 μL/mg protein in original sample.
15. Store the samples at −20 °C until MS analysis.

[22] The stock solution of each single internal standard is prepared in chloroform/methanol (1:1, v/v) with a concentration approximately 1 mg/mL. The amount of each single lipid species in the premixture is based on the abundance of the corresponding lipid class in the samples. The molecular species of internal standards are selected because they represent <0.1% of the endogenous cellular lipid mass levels as predetermined by ESI-MS lipid analysis.

[23] *See* footnote 22.

[24] In order to eliminate the contamination from the top layer (aqueous phase) to the bottom phase, insert the glass Pasteur pipet into the upper layer with slowly air bubbling until the pipet inserts into the bottom layer, which could avoid the upper layer liquid going into the pipet. When taking the glass pipet out from the upper layer, touch the outside of the pipet on the edge of the glass tube, and quickly transfer the bottom layer to a new glass tube.

3.4 Mass Spectrometric Analysis of Lipids

1. Dilute each lipid extract solution prepared to <50 μM of total lipids with chloroform/methanol/isopropanol (1:2:4, v/v/v) with or without LiOH (2–6%) in a Teflon-coated 96-well microplate.[25]
2. Set the ionization voltage of the nanospray ionization source at 1.15 kV in the positive-ion mode, −1.15 kV in the negative-ion mode, and gas pressure at 0.55 psi. Nanospray ionization for each sample is performed by a customized sequence subroutine operated under the Chipsoft software.[26]
3. For mass spectrometric analysis, collect 2-min duration of signal averaging in the profile mode for each survey MS scan.[27] For tandem mass spectrometric analysis, set collision gas pressure at 1.0 mTorr, vary collision energy with the class of lipids, and collect a 3–5 min period of signal averaging in the profile mode for each tandem MS spectrum, including PIS and NLS, which are sensitive and specific to the lipid class or the category of lipid classes of interest [10]. All of the mass spectra are automatically acquired by a customized sequence subroutine operated under Xcalibur software.
4. Processing of MS analysis data including ion peak selection, data transferring, baseline correction, peak intensity comparison, and quantification is conducted by self-programmed Microsoft Excel macros [14].

3.5 Mass Spectro<!–[spi]break--?>metric Data Processing and Analysis

See Fig. 2.

1. Loading the data: Tabular raw data from the mass spectra are transferred by self-programmed software directly from the Xcalibur-operated system.
2. Baseline correction: Mass spectrometry data usually show a varying baseline caused by the chemical noise in the matrix. The baseline levels of the tabular raw data from the mass spectra are the sum of baseline drift and noise level. The baseline drift is determined by averaging a number of lowest ion intensities. The noise level is determined based on the fact that an accelerated intensity change exists from the noise to signal [15]. The

[25] The total lipid concentration of a lipid extract can be estimated through the protein content or on the basis of the lipid analysis results from previous experiments [12]. This knowledge is useful for estimation of the concentrations of total lipids from different lipid extracts to prevent lipid aggregation during analysis through mass spectrometry. The lithium hydroxide is made of 80-time dilution of a saturated methanol solution.

[26] Since sample ionization (ChipSoft) and spectral collection (Xcalibur) are operated with two separate software programs, the ionization polarity and time controlled by the ChipSoft should be matched to those of the mass spectrometer. The mass spectrometer is triggered to start collecting spectra with the start of the nanospray.

[27] For the triple-quadrupole mass spectrometer, the first and third quadrupoles are used as independent mass analyzer with a mass resolution of 0.7 Th, and the second quadrupole serves as a collision cell for tandem mass spectrometry [13]. For the analysis of cardiolipin, a high-resolution mass spectrometer (at least Δm 10,000 full width at half maximum, FWHM) is applied to detect and differentiate the doubly charged ions.

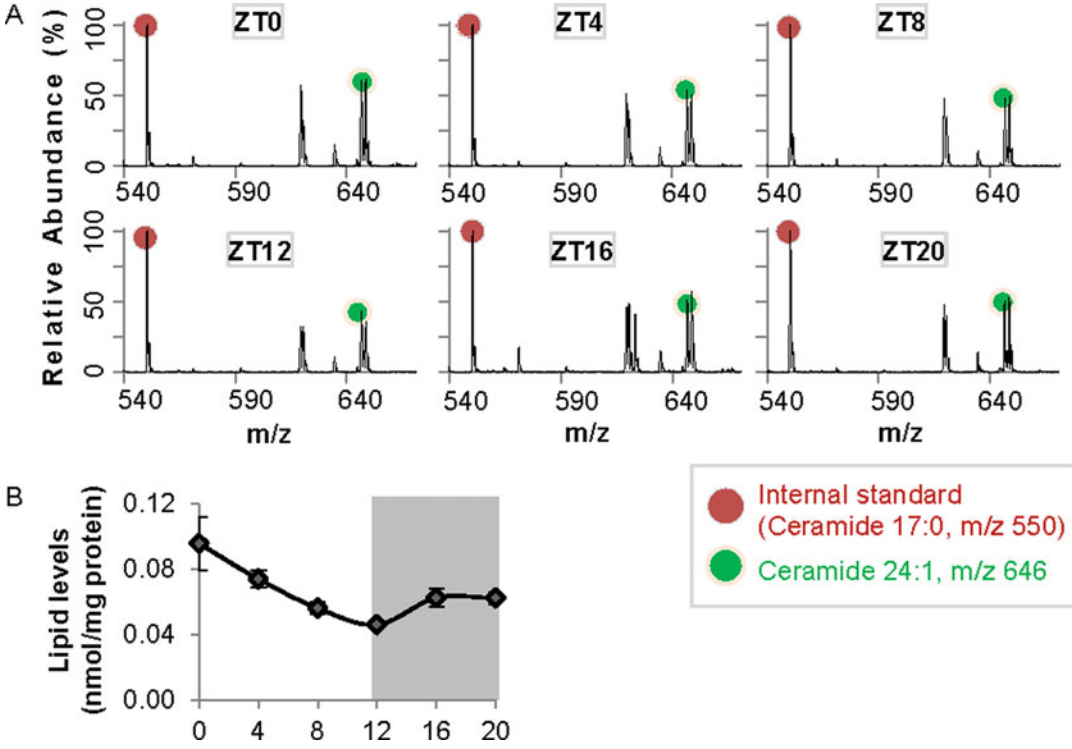

Fig. 2 Representative lipid measurements from ZT0 to ZT20, (*ZT,* Zeitgeber Time). (**a**) The mass spectrometry peaks of internal standard 17:0 ceramide (*m/z* 550, red circles) and of 24:1 ceramide (*m/z* 646, green circles) can represent their relative amounts. (**b**) The daily accumulation profile of 24:1 Ceramide as determined from the mass spectrometry data

precisely determined baseline level is deduced from the raw data for identification and quantification of lipid molecules.

3. Peak finding: An ion peak list of the molecular species in a lipid class of interest present in the analyzed lipid extract is generated by matching the *m/z* values of the detected ion peaks after baseline correction in the specific scan mode (i.e., PIS or NLS) with those of the candidate species in the established database of lipid molecules. This peak list represents all the detectable lipid species of each specific lipid class, containing the information about the total number of carbon atoms and the total number of double bonds in the aliphatic chain(s).
4. Acyl chain identification: Identification of acyl chain moieties is achieved by loading all PIS or NLS data represent different length and saturation of fatty acyl chains. The restriction of the total number of carbon atoms and the total number of double bonds present in the acyl chains for each individual species are identified by combination of the determined paired aliphatic chains.

5. Deisotoping of electrospray mass spectra: Before quantification of lipid molecular species of interest, two types of ^{13}C isotope effects need to be considered [8, 16]. The first type of effect comes from the different carbon number between a given molecular species and the selected internal standard used. The second type of effect is from the overlapping of the ion peak of the species of interest ($m/z = M$) with the ^{13}C isotope peak of another species containing an additional double bond ($m/z = M\text{-}2$).[28]
6. Quantification of the identified individual molecular species is performed either in a single-step or two-step procedure. An algorithm first determines whether or not overlapping or low-abundance peaks are found in the peak list of interest. If the answer is no, then the quantification is performed for the abundant and nonoverlapping peaks by direct ratiometric comparison to the ion peak intensity of the selected internal standard of the class in the survey MS scan after baseline correction and removal of ^{13}C isotope effects [10].
7. If either overlapping or low-abundance peaks are found in the peak list of interest, the two-step quantification is performed for quantification of lipid molecules. The determined nonoverlapping and abundant species plus the exogenously added internal standard are the candidate standards for the second step of quantification. The corrected ion peak intensities of the overlapping and/or low-abundance species from the class-specific PIS or NLS are used to quantify these species by ratiometric comparison with the ion-peak intensities of the candidate standards [10][29] (Fig. 2).

3.6 Lipidomics and Circadian Analyses

So far, a method for obtaining the daily lipid profiles of two subcellular fractions has been described. Assessing the biological implications should be performed on a case specific basis. The circadian rhythmicity of the lipids can be evaluated using different nonparametric statistical tests that are widely used in studying rhythmicity of mRNA transcript levels for example. Specifically, for determining the circadian periodicity we apply the JTK_CYCLE algorithm [11] with the following parameters; a window of 24 h, p value <0.05 for statistical significance. This methodology enabled us to uncover

[28] The isotope effects from other atoms (such as hydrogen, nitrogen, or phosphorus) are usually neglected due to extremely low abundance of its isotope or very small differences between the analyzed species and the selected internal standard.

[29] The differences in the number of total carbon atoms and the number of total double bonds present in fatty acyl chains of each individual species from those of the selected standards can be considered as two important variables with multivariate least-square regression to determine the correction factors for each individual molecular species for the second-step quantification. The linear dynamic range of quantification is extended dramatically by using this second step to quantify the overlapping and/or low-abundance species with one or more MS/MS scans through reduce background noise, increase S/N ratios of low-abundance species, and filter the overlapping molecules with class-specific PIS or NLS.

daily oscillation in lipid composition of different intracellular organelles and dissect their feeding and circadian clock dependency [7]. Moreover, the comprehensive temporal and spatial nature of the data facilitated the identification of broad principles related to lipid organization. For example, we screened for lipids that show similar daily profiles in each organelle with the presumption that they might be coregulated.

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