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Analysis of lipid contents in human trabecular meshwork cells by multiple reaction monitoring (MRM) profiling lipidomics

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Abstract

Lipids are among the major constituents of cells and play many important cellular functions. Lipid levels in the trabecular meshwork plays an important role in the maintenance of aqueous humor drainage and intraocular pressure (IOP) homeostasis. Therefore, it is important to characterize the changes in the lipid contents in the aqueous humor outflow pathway tissues to better understand their functional significance in the maintenance of IOP. The multiple reaction monitoring (MRM)-based profiling aids in the analysis of metabolome as a collection of functional groups and is utilized as an exploratory metabolomics and lipidomics approach. The MRM-based profiling utilizes tandem mass spectrometry experiments carried out on a commercial triple quadrupole mass spectrometer with three aligned quadrupole mass filters (Q1, q2, and Q3). This screening methodology can be utilized for targeted lipidomics screen. This chapter focuses on the methodology for isolation and culturing of the TM cells, lipid extraction, and MRM-based lipidomics approach with data analysis.

1 Introduction

The intraocular pressure is regulated by the aqueous humor (AH) drainage system inside the eyes, which includes trabecular meshwork (TM), juxtacanalicular tissue (JCT), and Schlemm's canal (SC).[1] The TM AH outflow pathway consists of porous beams of endothelial-like cells encapsulated in a collagen matrix.[2] The TM cells are highly contractile and mechanosensitive and can sense pressure changes.[3–6] Cellular lipid including phospholipids and cholesterol plays an important role in tissue biomechanics by modulating the -plasma membrane remodeling; signal transduction cascades; and cell-ECM and actin cytoskeletal interactions.[7] These properties help TM sense the pressure. Mass spectrometry-based lipidomics approaches have been used to analyze the lipids in TM as well as from the AH. However, the discovery and characterization of these biomolecules has been a challenging task for years. Current conventional lipid analyses are based on liquid or gas chromatography (LC or GC) coupled with high resolution mass spectrometry (HRMS), which involve extensive sample preparation and complex protocols for the characterization

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of potential lipid biomarkers.[8–11] To improve the speed and efficiency of exploratory metabolomics and lipidomics, Multiple reaction monitoring (MRM) profiling has been developed by examining the metabolome as a collection of functional groups.[10–12] The instrumentation most commonly used for MRM profiling is the triple quadrupole mass spectrometer (QqQ). MRM-Profiling starts with a Discovery Stage, intended to interrogate the sample for many functional groups by precursor scans (Prec) and neutral loss scans (NL) usually using a triple quadrupole mass spectrometer but without chromatography. For the second stage of MRM-Profiling, Screening Stage, the information from the Prec and NL scans is organized into lists of transitions (also called MRMs) that are used for the fast, specific, and sensitive interrogation of individual samples.[10, 12–15] The data acquired undergoes statistical analysis to distinguish the features responsible for the variance between groups. Univariate and multivariate statistics can be applied, such as Student's t-test, analysis of variance (ANOVA), principal component analysis (PCA), and linear discriminant analysis (LDA), depending on the purposes of study.[10] The outcome, a panel of MRMs, contains information of potential molecules that can be used as diagnostic factors or for potential as a biomarker.[10] This review is intended to demonstrate the methodology to isolate and culture the HTM cells from human corneal rims obtained from the OR post-transplantation surgery, and method to collect lipids from HTM cells using Bligh and Dyer method[16] and the basic steps of the MRM profiling lipidomics.

2 Materials

2.1 Dissection of TM from human tissue.

1. 2% Gelatin Solution: 2% gelatin in milliQ water and autoclave it, store at 4 °C.
2. Fetal bovine serum (FBS).
3. 70% alcohol: dilute from 100% alcohol.
4. Six-well tissue culture plate.
5. Glass coverslips.
6. 20% FBS OptiMEM: Adding 5ml penicillin-streptomycin-glutamine solution (Gibco, #10378–016) into 500ml OptiMEM (Gibco, #31985–070), then using this media to make 20% FBS OptiMEM.
7. The leftover human donor corneal rims from OR used for corneal transplantation at the Indiana University Clinical Service, Indianapolis
8. Dissection microscope
9. Scalpel
10. Phosphate-buffered saline (PBS)
11. 0.5mm curette
12. Fine-tipped forceps (e.g., 5 or 6 Dumont)

2.2 Culturing HTM cells

1. Tissue culture dish or plate.
2. 10% FBS Dulbecco's modified Eagle's medium: Adding 50ml FBS and 5ml penicillin-streptomycin-glutamine solution (Gibco, #10378–016) into 500ml Dulbecco's modified Eagle's medium to make 10% FBS Dulbecco's modified Eagle's medium.
3. Cell culture incubator (37°C humidified and 5% CO2-controlled).
4. 0.25% trypsin

2.3 Lipid extraction from HTM cells

1. 0.25% trypsin
2. 10% FBS Dulbecco's modified Eagle's medium
3. 1.7mL Eppendorf Tube
4. Laboratory centrifuge
5. PBS
6. Ultrapure water
7. Methanol
8. Chloroform
9. Vortex laboratory shaker
10. 1 mL 20-gauge needle syringe
11. Speed vac

2.4 MRM-based lipidomics analysis

1. Micro-autosampler (G1377A)
2. triple quadrupole mass spectrometer
3. Electrospray ionization (ESI) source
4. Injection solvent: acetonitrile/ methanol/ammonium acetate 300mM 3:6.65:0.35 [v/v]

3 Methods

3.1 Dissection of TM from human tissue

Add 2% gelatin into the desired well in six-well tissue culture plate (see Note 1).

Soak coverslips in 70% alcohol for 10min.

[1]For one human donor corneal ring, two wells in six-well culture plate is enough.

Take out the coverslips and lean them against the wall of the well next to the desired wells in six-well plate (see Note 2).

Take out the leftover human donor corneal ring from the transport vial.

Use PBS to wash the corneal ring four to five times.

Put the corneal ring on a glass petri plate under the microscope.

Under the microscope, use a scalpel to remove the pigment from the corneal ring, and then use a 0.5mm curette to “scoop” the TM tissue from the corneal ring. After all TM tissue separates from the corneal ring, use fine-tipped forceps (e.g., 5 or 6 Dumont) to lift out the TM tissue and put it into a 10cm plate (see Note 3).

Add one drop of FBS to the TM tissue, and then use scalpel to “chop” the TM tissue into small pieces.

Remove 2% gelatin from the desired wells in six-well plate.

Use pipette to get the TM tissue pieces with FBS and add them into desired wells in sex-well plate, then put the coverslip onto the tissue (see Note 4).

Gently press the coverslip using pipette tip to remove the bubbles between the coverslip and the dish (see Note 5).

Add 1ml 20% FBS OptiMEM into the well (see Note 6).

Put the plate into the incubator.

Next day use pipette tip presses the coverslip gently to remove the bubbles between the coverslip and the plate (see Note 7).

3.2 Culturing HTM cells

TM tissue pieces can be left undisturbed inside the incubator for 4–8 weeks until the cells migrate out from the tissue and become confluent, the 20% FBS OptiMEM should be changed every 3–5 days without disturbing the TM tissue pieces.

Once cells migrate out from the tissue and become confluent, the cells inside the well and on the coverslips can be collected using 0.25% trypsin and then plate on a 10cm tissue culture dish.

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2. Gelatin can help the TM tissue adhere to the plate and help encourage attachment and migration of TM cells out of the tissue. Make sure the alcohol is completely evaporated.
3. Using scalpel to remove the iris pigment around the TM tissue, do not damage the TM. Make sure to only “scoop” the TM tissue. After TM dissection, pieces of TM appear semi-transparent with a white/opaque hue and some pigment, while the cornea is transparent and reflective.
4. To avoid the TM tissue pieces stuck the pipette tip, using 1ml pipette to collect the TM tissue pieces.
5. Do not break the coverslip when pressing it, and make sure there is no bubble between coverslip and the plate.
6. Do not disturb the TM tissue pieces when adding the media.
7. Make sure press the coverslip without disturbing the TM tissue, and make sure remove the bubbles completely.

Use 10% FBS Dulbecco's modified Eagle's medium to culture the human TM cells (see Note 8).

When HTM cells become 70–90% confluence, cells can be trypsinized using 0.25% trypsin and split into next passage (see Note 9, 10).

3.3 Lipid extraction from HTM cells (Bligh and Dyer method).

Wash the cell culture plate with autoclaved PBS to eliminate bacteria or artifacts in cell suspension (see Note 11).

1. Add 200 μL trypsin to cell culture plate to suspend the cells.
2. Incubate for 3–5 minutes after adding trypsin to cut all the peptide bonds.
3. Add 1mL of 10% FBS Dulbecco's modified Eagle's medium to stop the trypsin.
4. Collect cells into a 1.7mL Eppendorf Tube.
5. Centrifuge the Eppendorf tube to create a pellet of around 50,000 cells at 3000 rpm.
6. Remove supernatant from the cell pellet.
7. Wash pellet with 1 mL of autoclaved PBS.
8. Discard supernatant.
9. Repeat steps (7–8).
10. Add 200 μL of ultrapure water to the cell pellet and repeat pipetting for one minute.
11. Add 550 μL of methanol and 250 μL of chloroform, and incubate the sample for 15 minutes at 4°C.
12. Add 250 μL of ultrapure water and 250 μL of chloroform to the sample, homogenize for 30 seconds and vortex for 15–20 seconds, causing the solution to become biphasic.
13. Phase separation was improved by centrifugation at 4°C 10,000 rpm for 5 minutes (see Note 12).
14. Once separation is observed, if any bubbles are observed on top of the aqueous layer, remove them prior to removing the lipids.

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8. HTM cells have a finite number of doubling and begin to change in appearance after 6–8 passages. By passage 9 or 10, they can no longer be described as TM cells since they start to develop senescence features such as vacuoles, increased size, and reduced doubling time. For this reason, it is generally not recommended to use TM cells from human eyes at greater than 7 passages.[17]
9. When passaging, HTM cells require a much longer incubation (3–5 min) compared to other cell types.
10. One 10cm tissue culture dish can be split 1:3 to maintain appropriate cell densities. HTM cells appear to require paracrine factors from surrounding cells for growth, so splitting cells at lower cell densities may result in slow or no growth.[17]
11. One well in six-well plate for each condition is sufficient.
12. you will now have upper (aqueous) and lower (lipid) fraction. If you cannot see separation, vortex for 1 minute and repeat step 13. If this does not work, add 100 μL of DD H2O, vortex, and centrifuge.

15. Remove the bottom organic layer (contains lipids) with a 1 mL 20-gauge needle syringe (only go in with syringe once and draw up all lipids) and place in a clean 1.7mL Eppendorf Tube (see Note 13).

16. Put the sample into speed vac, make sure hear setting is off, and speed vac for ~90 minutes. If solvent still present, speed vac until samples are bone dry.

17. Sample can be stored in −80 °C until ready for MRM based lipidomics analysis.

3.4 MRM-based lipidomics analysis

1. Dilute the dried lipid extracts in 5 μL of chloroform and 45 μL of injection solvent to obtain a stock solution.

2. Dilute the stock solution 10X into injection solvent spiked with 0.1 ng/μL of Equisplash Lipidomics (Avanti Polar Lipids # 330731) for sample injection.

3. Get a few representative samples: usually one pooled sample for each pre-defined experimental group or for a full mixture if no groups are pre-defined, which contain the common features of the chosen sample subset.

4. Discovery step: Flow-inject representative sample to the ESI source of a triple quadrupole mass spectrometer using a micro-autosampler. Compile a set of methods by combining *m/z* for the molecular ion based on the LipidMAPS online database (http://www.lipidmaps.org/) with the expected product ion resulting from the precursor (Prec) or neutral loss (NL) scans (see Note 14).

5. Screening step: Organize the information from the Prec and NL scans into lists of MRM transitions (MRMs) that are used for the fast, specific, and sensitive interrogation of individual samples. Combine all potential MRMs into an appropriate number of MRM methods (no more than 200 MRM per method). For each scan method, flow-inject samples (8 μL) to the ESI source of a triple quadrupole mass spectrometer using a micro-autosampler. A capillary pump is connected to the autosampler and operated at a flow rate of 7 μL/min and pressure of 100 bar. Capillary voltage on the instrument is 5 kV and the gas flow 5.1 L/min at 300 °C. (see Note 15).

6. Process raw MS data using an in-house script and lists containing MRM transitions and export the respective ion intensity values to Microsoft Excel.

7. Normalize the absolute ion intensity of lipids against the total ion current (TIC) of the method for each sample individually.

8. Select any lipid that has a sample ion intensity higher than the blank.

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13. If accidently went in with syringe twice, there may be protein in your organic phase (lipids), so centrifuge again to get separation again, then use new syringe and repeat process.

14. The output of the discovery step in MRM-profiling is represented by hundreds of precursor ion/product ion pairs, each ion pair consisting of one selected precursor ion and its related product ion,[12] the two ions being interconnected by a specified neutral loss or a specified precursor ion. For each scan, a list of precursor/product ion pairs is acquired: these are MRM transitions.[10]

15. If there is a large number of MRMs, due to the limited time of signal at flow injection (usually less than 1 min), obtain at least 10 scans for each MRM (e.g. 1,586 MRMs can be organized into 10 methods, each one involving 2 minutes of data acquisition by flow injection, and each of the ten methods including a maximum of 200 MRM transitions).[15]

9. Data analysis using relative ion abundances is performed using Metaboanalyst 5.0 (https://www.metaboanalyst.ca/) for univariate and multivariate statistics by principal component analysis (PCA).

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References

1. Sunderland DK, Sapra A. Physiology, Aqueous Humor Circulation. StatPearls. Treasure Island (FL); 2022.
2. Sacca SC, Gandolfi S, Bagnis A, et al. The Outflow Pathway: A Tissue With Morphological and Functional Unity. J Cell Physiol 2016;231:1876–1893. [PubMed: 26754581]
3. Lutjen-Drecoll E Functional morphology of the trabecular meshwork in primate eyes. Prog Retin Eye Res 1999;18:91–119. [PubMed: 9920500]
4. Wiederholt M, Schafer R, Wagner U, Lepple-Wienhues A. Contractile response of the isolated trabecular meshwork and ciliary muscle to cholinergic and adrenergic agents. Ger J Ophthalmol 1996;5:146–153. [PubMed: 8803576]
5. Wiederholt M, Bielka S, Schweig F, Lutjen-Drecoll E, Lepple-Wienhues A. Regulation of outflow rate and resistance in the perfused anterior segment of the bovine eye. Exp Eye Res 1995;61:223–234. [PubMed: 7556486]
6. Wiederholt M, Sturm A, Lepple-Wienhues A. Relaxation of trabecular meshwork and ciliary muscle by release of nitric oxide. Invest Ophthalmol Vis Sci 1994;35:2515–2520. [PubMed: 7512945]
7. Humphrey JD, Dufresne ER, Schwartz MA. Mechanotransduction and extracellular matrix homeostasis. Nature reviews Molecular cell biology 2014;15:802–812. [PubMed: 25355505]
8. Watson AD. Thematic review series: systems biology approaches to metabolic and cardiovascular disorders. Lipidomics: a global approach to lipid analysis in biological systems. J Lipid Res 2006;47:2101–2111. [PubMed: 16902246]
9. Li M, Yang L, Bai Y, Liu H. Analytical methods in lipidomics and their applications. Anal Chem 2014;86:161–175. [PubMed: 24215393]
10. Xie Z, Ferreira CR, Virequ AA, Cooks RG. Multiple reaction monitoring profiling (MRM profiling): Small molecule exploratory analysis guided by chemical functionality. Chem Phys Lipids 2021;235:105048. [PubMed: 33561466]
11. Ferreira CR, Yannell KE, Mollenhauer B, et al. Chemical profiling of cerebrospinal fluid by multiple reaction monitoring mass spectrometry. Analyst 2016;141:5252–5255. [PubMed: 27517482]
12. Cordeiro FB, Ferreira CR, Sobreira TJP, et al. Multiple reaction monitoring (MRM)-profiling for biomarker discovery applied to human polycystic ovarian syndrome. Rapid Commun Mass Spectrom 2017;31:1462–1470. [PubMed: 28656689]
13. Edwards ME, De Luca T, Ferreira CR, et al. Multiple reaction monitoring profiling as an analytical strategy to investigate lipids in extracellular vesicles. J Mass Spectrom 2021;56:e4681. [PubMed: 33210411]
14. Yannell KE, Ferreira CR, Tichy SE, Cooks RG. Multiple reaction monitoring (MRM)-profiling with biomarker identification by LC-QTOF to characterize coronary artery disease. Analyst 2018;143:5014–5022. [PubMed: 30226503]
15. de Lima CB, Ferreira CR, Milazzotto MP, Sobreira TJP, Vireque AA, Cooks RG. Comprehensive lipid profiling of early stage oocytes and embryos by MRM profiling. J Mass Spectrom 2018;53:1247–1252. [PubMed: 30325087]
16. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911–917. [PubMed: 13671378]

17. Keller KE, Bhattacharya SK, Borras T, et al. Consensus recommendations for trabecular meshwork cell isolation, characterization and culture. Exp Eye Res 2018;171:164–173. [PubMed: 29526795]