Metabolomic Profiling to Understand Chondrocyte Metabolism

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i. Chapter Title

Metabolomic Profiling to Understand Chondrocyte Metabolism

ii. Summary/Abstract

Metabolism has long been recognized as a critical physiological process necessary to maintain homeostasis in all types of cells including the chondrocytes of articular cartilage. Alterations in metabolism in disease and metabolic adaptation to physiological stimuli such as mechanical loading are increasingly recognized as important for understanding musculoskeletal systems such as synovial joints. Metabolomics is an emerging technique that allows quantitative measurement of thousands of small molecule metabolites that serve as both products and reactants to myriad reactions of cellular biochemistry. This protocol describes procedures to perform metabolomic profiling on chondrocytes and other tissues and fluids within the synovial joint.

iii. Key Words

metabolomics; metabolomic profiling; metabolite extraction; cartilage; synovial fluid; osteoarthritis.

1. Introduction

Articular cartilage (AC) is a highly specialized form of cartilage located at joint interfaces. The main role of AC is to provide near-frictionless articulation in synovial joints. Unlike most cartilage and tissues, AC is avascular and aneural. Therefore, repair and healing are limited. AC is composed of extracellular matrix (ECM) and chondrocytes which are the sole resident cell type of AC. Chondrocytes are metabolically active and highly specialized cells that help generate, maintain, and restore the ECM [1]. ECM is primarily composed of water, collagen, and proteoglycans [2]. There are many interactions between the ECM and chondrocytes, and

chondrocytes create a specialized local microenvironment termed the pericellular matrix (PCM). Therefore, depending on loads and pressure exerted on the joint, chondrocytes respond and take action to maintain the ECM and AC homeostasis.

Chondrocytes can have spatially heterogeneous behavior. AC can be divided into four zones from the surface to the bone termed: the superficial, middle, deep, and calcified zone [1]. In each zone, chondrocyte shape, size, and frequency differ. For example, there are fewer and flatter chondrocytes located in the superficial zone but deeper into AC, chondrocytes are rounder, and more are present in comparison to those located directly at the joint interface. Together, chondrocytes and ECM provide AC with the ability to provide smooth joint articulation.

Chondrocytes must be metabolically active to maintain homeostasis. Yet several unanswered questions regarding the metabolism of chondrocytes and AC remain. Glucose is the most studied form of energy for chondrocytes. Therefore there is great interest in analyzing metabolic pathways associated with glucose metabolism including glycolysis, the pentose phosphate pathway (PPP), and the tricarboxylic acid cycle (TCA cycle) [3]. One method to study chondrocyte metabolism is metabolomic profiling.

Metabolomics is the study of small molecule intermediates, which are called metabolites. Metabolomic profiling involves analyzing patterns of metabolite expression to better understand cell physiology and pathophysiology. Metabolites “act as a spoken language, broadcasting signals from the genetic architecture and microenvironment [4].” A metabolomic profile provides information that encapsulates various factors including genetics, environment, stress, toxic exposure. Chondrocytes respond to physiological compression with large-scale changes in metabolomic profiles [5-7]. Metabolomic profiles of joints capture large-scale changes in response to injury [8,9], and metabolomic profiles of synovial fluid provide insight into pathophysiology [10,11].

The field of metabolomics is the newest edition to the "omics" field, and it has been pivotal to many fields of research. For example, metabolomics has been applied to study *Escherichia coli* under stress conditions such as pH, heat, toxic exposure, and genetic manipulation to better understand key metabolic information such as involved biological pathways and associated metabolites. Beyond *E. coli*, metabolomics has been applied to many fields including musculoskeletal research to study various systems including chondrocytes and synovial fluid.

2. Materials

2.1 Chondrocyte Harvest

1. Conical Centrifuge Tubes, 50 mL, sterile.
2. #22 scalpel blades.
3. #4 scalpel handles.
4. 100 mm surface treated tissue culture petridish, sterile.
5. Hemostats-Rochester-Ochsner Forceps.
6. Long drawn-out capillary tube glass pasteur pipettes, sterile.
7. Millipore Sigma™ Steriflip™ sterile disposable vacuum filter unit.
8. Sterile Cell Strainers, 70 μm.
9. Pen-Strep Solution: Penicillin-Streptomycin (10,000 U / mL) solution.
10. Cartilage Wash: 1x Phosphate Buffered Saline (pH 7.4), sterile, 1% Pen-Strep Solution.
11. Harvest Media: Gibco™ DMEM, high glucose, 1% Pen-Strep Solution, sterile.
12. Complete Media: Gibco™ DMEM, high glucose, 1% Pen-Strep Solution, 10% Fetal Bovine serum, sterile.
13. Gibco™ Collagenase, Type I powder.

2.2 Alginate Encapsulation and Culture

1. Sodium Alginate: Alginic acid sodium salt, from brown algae, low viscosity, Sigma-Aldrich.
2. PBS: 1x Phosphate Buffered Saline (pH 7.4), sterile.
3. 10 mL syringe with Luer-Lok tips, sterile.
4. Hypodermic needle, 22 x 1 1/2 gauge, sterile.
5. 100mm and 60mm polystrene petridishes, sterile.
6. 0.22-micron syringe filters.
7. Conical Centrifuge tubes: 15 mL, 50 mL, sterile.
8. 10 mL, 25 mL pipettes, sterile.
9. 150mM NaCl: Weigh 8.76 g of NaCl and transfer to a beaker. Add 900 mL of deionized water. Mix and make up the volume to 1lt. Autoclave at 121ºC for 20 min for a sterile solution.
10. 102mM $CaCl_2$: Weigh 11.31 g of $CaCl_2$ and transfer to a beaker. Add 900 mL of deionized water. Mix and make up the volume to 1 L. Autoclave at 121ºC for 20 min for a sterile solution.
11. Trypsin-EDTA (0.05%), phenol red.
12. Complete Media: Gibco^{TM} DMEM, high glucose, 1% Pen-Strep Solution, 10% Fetal Bovine serum, sterile.

2.3 Alginate Release and Agarose Encapsulation

1. Conical Centrifuge tubes: 15 mL, 50mL, sterile.
2. 2 mL, 10 mL pipettes, sterile.
3. Hemocytometer.
4. Trypan Blue, 0.4%.

5. Anodized aluminum mold with multiple holes (i.e., one per agarose construct) of diameter of 7mm and height of 12.7 mm, sterile.
6. Glass rod with a diameter of 2-3 mm, sterile.
7. Surgical blade, sterile.
8. 24 well flat bottom cell culture plates, sterile.
9. Water bath set at 40ºC.
10. PBS: 1x Phosphate Buffered Saline (pH 7.4), sterile.
11. Alginate Lysis Buffer: 150 mM NaCl, 55 mM Sodium citrate, 50 mM EDTA made in 1x PBS. Weigh 0.87 g of NaCl, 1.61 g of sodium citrate, and 1.46 g of EDTA. Add 90 mL of 1x PBS. Mix and make up the volume to 100 mL. Adjust the pH of the solution to 7. Autoclave the solution at 121ºC for 20 min.
12. Agarose, low gelling temperature, Sigma-Aldrich.
13. Harvest Media: Gibco^{TM} DMEM, high glucose, 1% Pen-Strep Solution, sterile.
14. Complete Media: Gibco^{TM} DMEM, high glucose, 1% Pen-Strep Solution, 10% Fetal Bovine serum, sterile.

2.4 Metabolite Extraction

1. Sterile heat sink (cold block) to pulverize samples
2. Steel pounding rods for pulverization, forceps, narrow spatula, all sterile.
3. Hammer.
4. Aluminum foil squares, cut in 5 inches x 5 inches, sterile.
5. 1.5 mL microcentrifuge tubes.
6. 2 pounds of liquid nitrogen.
7. Insulated bucket filled with wet ice.
8. Microcentrifuge Tubes (1.5 mL, *see* **Note 1**).

9. Precipitation solution: Prepare 10 mL of 8:2 (v/v) solution composed of 8 mL of methanol and 2 mL of HPLC grade water.
10. Resuspension solution: Prepare 10 mL of a 1:1 (v/v) solution composed of 5 mL of acetonitrile and 5 mL of HPLC grade water.
11. Vacuum concentrator capable of centrifugation under a vacuum of ~20-50 mbar.
12. MS snap cap SureStop Screw Vials & AVCS Caps.
13. Extraction solvent: Prepare 10 mL of 70:30 (v/v) methanol:acetone. Keep at -80ºC till further use.

2.5 Liquid Chromatography-Mass Spectrometry

1. Depending on accessibility and availability, extracted metabolites can be analyzed using HPLC-MS or MS/MS. Specific to this work, an Agilent 6538 Q-TOF mass spectrometer was utilized.

2.6 Data Analysis

1. Access to XCMS, MetaboAnalyst, and analysis platforms such as R and MATLAB.

3. Methods

3.1 Chondrocyte Harvest

1. Add 30 mL cartilage wash solution to a 50 mL sterile conical centrifuge tube (*see* **Note 2**).
2. Attach the scalpel blade to the scalpel handle. Using the Hemostats-Rochester-Ochsner Forceps, pick the donor joint sample and place on a sterile petridish. Holding the joint in place with the forceps, score cartilage on the donor joint with the blade using a criss-cross pattern. Scrape, harvest the small pieces of cartilage into the conical tube containing the cartilage wash solution (*see* **Note 3**).

3. Centrifuge the tube at 28 x g for 2 min. Carefully aspirate the supernatant using a pasteur pipette connected to a vacuum flask containing 10% liquid bleach. Be careful not to suck up the small pieces of cartilage. Add another 30 mL cartilage wash solution to the harvested cartilage and repeat the centrifugation process. Perform a final wash with 30 mL of sterile harvest media. Aspirate the supernatant.
4. Aliquot 30 mL of harvest media to a fresh tube and add collagenase, Type I at 2 mg/mL. Filter sterilize the solution using sterile disposable vacuum filter unit. Add the filter sterilized harvest media containing collagenase to the washed cartilage.
5. Tape the conical tube to a rotary shaker and place the shaker in an incubator. Digest the cartilage at 37ºC for 14-16 hours (*see* **Note 4**).
6. After 14-16 hours, take the tube out from the shaker. Working in a sterile hood, place a sterile 70 μm cell strainer on a new sterile 50 mL conical tube. Slowly, add the contents of the digested cartilage solution onto the cell strainer and collect the filtrate. This step removes the undigested cartilage and matrix debris (*see* **Note 5**).
7. Discard the cell strainer. Centrifuge the collected filtrate at 1000 x g for 5 min. (Place a counter tube on the opposite side of the centrifuge to balance it).
8. Gently aspirate the supernatant, taking care not to disturb the cell pellet at the bottom of the tube.
9. Resuspend the cells in 30 mL sterile 1X PBS. Centrifuge at 800 x g for 5 mins. Aspirate the supernatant and repeat the wash with 30 mL sterile 1X PBS, followed by centrifugation.
10. Aspirate the supernatant after the final wash with PBS and resuspend the cell pellet in 20 mL Complete media (Gibco^{TM} DMEM, high glucose, 1% Pen-Strep Solution, 10% Fetal Bovine Serum).

11. Place 10 mL each of the resuspended media containing the chondrocytes in two 100 mm sterile surface treated tissue culture petri dishes. Place the petri dishes in a humidified incubator at 37ºC with 5% CO_2 and let the cells grow.
12. Harvest the chondrocytes once they reach 70-80% confluency for alginate encapsulation.

3.2 Alginate Encapsulation

1. Sodium alginate solution, 1.2%: Weigh 1.2 g of sodium alginate on a square piece of Aluminum foil. Wrap the foil and autoclave at 121ºC for 20 min.
2. Place a magnetic stirrer into an autoclavable 250 mL glass bottle and sterilize at 121ºC for 20 min.
3. Working under sterile conditions in a laminar flow cabinet, pipette 100 mL of sterile 1X PBS into the autoclaved glass bottle. Slowly add the sterile sodium alginate to the PBS solution in the bottle.
4. Place the bottle on a magnetic stir plate and leave the solution stirring at a low speed until the solids dissolve. For best results, allow the solution to sit overnight with gentle stirring for a homogenous solution (*see* **Note 6**).
5. Resuspend the harvested cells at a density of $4x10^6$ cells/mL in sodium alginate solution in a 50 mL conical tube.
6. Transfer the cell-alginate solution to a 10 mL syringe and slowly express through a 22-gauge needle in a dropwise fashion into a petri dish containing 102 mM $CaCl_2$ (20 mL).
7. Let the polymerization take place at room temperature for 10 mins.
8. Using a 25 mL pipette, collect the beads into a 50 mL conical tube and centrifuge at 500 x g for 5 min. Aspirate the supernatant. Be careful not to disturb the alginate beads at the bottom of the tube.

9. Resuspend the beads in 10 mL of 150mM NaCl and centrifuge at 500 x g for 5 min. Aspirate the supernatant. Repeat the NaCl wash thrice.
10. Wash the alginate beads in 10 mL of complete media after the last NaCl wash. Aspirate the supernatant.
11. Using a 10 mL pipette, resuspend the alginate encapsulated cells in 10 mL of complete media.
12. Place 5 mL each of the complete media containing alginate encapsulated cells in two sterile 60mm petri dishes and culture the beads in a humidified incubator at 37ºC with 5% CO_2. Cell encapsulation must be carried out under sterile conditions to avoid contamination to the encapsulated cells. If culturing the alginate encapsulated beads for a longer period, replace the media every 2-3 days. Collect the alginate beads in a 15 mL conical tube and centrifuge at 500 x g for 5 min. Carefully aspirate the supernatant and resuspend the alginate beads in 5 mL of complete media and plate in 60mm petri dish and incubate at 37ºC in a humidified incubator with 5% CO_2.

3.3. Alginate Release, Agarose Encapsulation, and Tissue Culture

1. Under aseptic conditions, pipette the alginate beads with the culture media into a 15 mL conical tube. Centrifuge at 500 x g for 5 min. Carefully aspirate the supernatant and resuspend the alginate beads in 10 mL of sterile 1x PBS. Centrifuge at 500x g for 5 min. Aspirate the supernatant.
2. Resuspend the alginate beads containing the chondrocytes in 5 mL of sterile alginate lysis buffer. Place the tube at 37ºC in a humidified incubator for 3-4 mins, till the solution turns clear, indicating degradation of alginate gel.
3. Centrifuge at 800 x g for 10 min. Aspirate the supernatant.
4. Resuspend the cells in sterile 1x PBS and repeat the centrifugation step.

5. Aspirate the supernatant and resuspend the cells in 1 mL of harvest media.
6. Determine the cell density using a hemocytometer and trypan blue before proceeding with agarose encapsulation.
7. Sterilize the aluminum gel mold and glass rod at 121ºC for 20 min. Unwrap and let cool in the laminar flow hood before using to make the agarose gels. Keep 10 mL and 2 mL sterile pipettes in a 37ºC incubator to keep warm for use with agarose.
8. Make a 4.95% agarose (w/v) in 1x PBS. Sterilize the solution by autoclaving at 121ºC for 20 min. Keep in a 40ºC water bath to ensure the agarose does not solidify.
9. Transfer 9 mL of 4.95% w/v sterile agarose to a 50 mL falcon tube. Working quickly add 1 mL of cells to the agarose to give a final concentration of $1x10^6$ cells/mL in 4.5% agarose. Mix the solution by swirling and vortexing at a low speed for 5 sec, taking care not to introduce bubbles in the agarose mixture.
10. Using a 2 mL sterile warm pipette, aspirate the agarose cell mixture and add to a well of the aluminum mold. Let the agarose overflow slightly over the top of the well. Repeat the process to fill up the wells with the agarose-cell mixture. Let the gels solidify. Once the gelling is complete, slide the sterile surgical blade horizontally across the top of the gel to remove excess agarose gel.
11. Use the sterile glass rod to move over the surface of the gel, trying to release it from the mold. Once the gel is loose, push through with the glass rod into a single well of a 24 well flat bottom cell culture plate, taking care not to break the gel.
12. Fill the well with 2 mL of complete media, ensuring that the gel is completely immersed in the media. Place the plate in a humidified incubator at 37ºC with 5% CO_2.

3.4. Metabolite Extraction (Figures 1-2)

3.4.1 Alginate Released Agarose Embedded Chondrocytes

1. Autoclave the tombstone, steel pounding rods and the aluminum foil squares at 121ºC for 20 min.
2. Place the tombstone and pounding rods at 4ºC until use.
3. Fill the ice bucket with ice and place the tombstone in ice.
4. Wash chondrocyte agarose gels with 1x PBS and then wrap a single gel in a sterile square of aluminum foil. Place in liquid nitrogen for approximately 1 min.
5. Remove the flash frozen gel using forceps. Unwrap and place into the well of the tombstone, making sure the gel is on its side.
6. Place the pounding rod on top of the gel and smash it with the hammer.
7. Carefully remove the smashed gel pieces with a spatula and place in a 1.5 mL microcentrifuge tube. (The gel pieces can be stored at -80ºC till further analysis).
8. For metabolite extraction from agarose gels, add 1 mL of cold 70:30 methanol:acetone solution to each gel sample.
9. Vortex vigorously every 5 min for a total of 20 min. When not vortexing, place the tubes at -20ºC.
10. Place the samples at -20°C overnight.
11. Centrifuge the samples at 19,200 x g at 4ºC for 10 min.
12. Carefully transfer the supernatant to a fresh 1.5 mL microcentrifuge tube, taking care not to aspirate the agarose chunks at the bottom of the tube.
13. Dry the supernatant in a vacuum concentrator for approximately 3 hours.
14. Resuspend the dried pellet in 100 µl of mass spectrometry grade water and acetonitrile (50:50 v/v).

15. Store samples at -80ºC.

3.4.2 Cartilage and other soft tissues

1. Cut and dice cartilage, weight 100 mg of cartilage, and transfer to fast spin tubes.
2. Add 1 mL of 3:1 (v/v) methanol:water and 2 ceramic beads. Homogenize tissue using a tissue grinder for 2 hours (*see* **Note** 7).
3. Transfer homogenized solution to a new microcentrifuge tube to isolate sample from ceramic beads and cartilage that did not break down.
4. Centrifuge at 16,100 x g at 4ºC for 10 minutes, transfer supernatant, and store pellet at -80ºC.
5. Add 100 µL of 80:20 methanol:water, vortex samples for 1 minute, and incubate at -20ºC for 30 minutes.
6. Centrifuge at 16,100 x g for 10 minutes, transfer supernatant, and store pellet at -80ºC.
7. Dry down supernatant via vacuum concentrator. (~3 hours) (*see* **Note 9**).
8. Resuspend dried metabolites with 250 µL of 1:1 (v/v) acetonitrile:water and incubate for 1 hour at -20ºC to precipitate macromolecules (*see* **Note 10**).
9. Remove samples from -20ºC, let thaw, and vortex samples for 1 minute.
10. Centrifuge samples at 16,100 x g at 4ºC for 10 minutes, transfer supernatant, and store pellet at -80ºC.
11. Dry supernatant down via vacuum concentrator, resuspend dried metabolites with 100 µL of 1:1 acetonitrile:water and store samples in -80ºC till LC-MS analysis.
12. Transfer 50 µL of sample to mass spectrometry caps (*see* **Note 8**).

3.4.3 Synovial Fluid

1. Thaw synovial fluid samples on ice for 10-20 min, aliquot 50 µL of SF to individual microcentrifuge tubes and centrifuge at 500 x g at 4°C for 5 minutes to remove cells and debris.
2. Transfer supernatant and add 1 mL of 80:20 methanol:water, vortex for 1 minute, and incubate at -20ºC for 30 minutes. Store isolated pellet at -80ºC.
3. Centrifuge samples at 16,100 x g at 4°C for 10 min, transfer supernatant, and store pellet at -80ºC.
4. Dry down supernatant via vacuum concentrator (~3 hours) (*see* **Note 9**).
5. Resuspend dried metabolites with 250 µL of 1:1 acetonitrile:water and incubate for 1 hour at -20ºC to precipitate macromolecules (*see* **Note 10**).
6. Remove samples from -20ºC, let thaw, and vortex samples for 1 minute.
7. Centrifuge samples at 16,100 x g at 4ºC for 10 minutes, transfer supernatant, and store pellet at -80ºC.
8. Dry supernatant down via vacuum concentrator, resuspend dried metabolites with 100 µL of 1:1 acetonitrile:water and store samples in -80ºC till LC-MS analysis.
9. Transfer 50 µL of sample to mass spectrometry caps (*see* **Note 8**).

3.5 Liquid chromatography-mass spectrometry (LC-MS)

1. Extracted metabolites were analyzed using HPLC-MS (Agilent 6538 Q-TOF mass spectrometer) in positive mode (resolution: ~20 ppm, accuracy: ~5 ppm, possible ionization adducts: H^+, Na^+) (*see* **Note 11**).
2. Peak intensity values for m/z values in the experimental sample set were identified and exported using Agilent MassHunter Qualitative Analysis software.

3.6 Metabolomics: Statistical Analysis (Figure 3)

1. Following mass spectrometry analysis, XCMS is used to convert spectral data to excel format.
2. In excel, format file to include row ID, mass-to-charge ratios (m/z values), and individual samples. Information to exclude includes mzmax, maxint, npeaks, etc. (*see* **Note 12**).
3. Sort individual samples within their cohort and assign group labels. Prior to submission to MetaboAnalyst, excel file must include m/z values, individual sample names, as well as group identifiers (**Figure 4**).
4. Save excel file as a .csv or .txt file.
5. When uploading metabolite data to MetaboAnalyst, select 'statistical analysis', select 'peak intensity table' for data file type, select correct format, and upload file.
6. Steps when performing statistical analyses in MetaboAnalyst include data check, missing value, data filter, data editor, normalization, and statistics.
    a. Data check + missing value: select proceed
    b. Data filter + editor: Depending on the # of variables, select appropriate option. (i.e., if < 5000 features are present, select 'None (less than 5000 features)'). Next, select 'submit' and 'proceed'
    c. Normalization: (*see* **Note 15**)
        i. Sample normalization = none
        ii. Data transformation = log transform
        iii. Data scaling = auto scaling
        iv. Select normalize, view result, and proceed

7. Following data filtering, editing, and normalization, view statistical results (*see* **Notes 13** and **14**).
8. To download results, select ‘Download’ and download zip file.

3.7 Metabolomics: Pathway Analysis (Volcano Plot, Figure 5)

1. Open volcano.csv downloaded in the MetaboAnalyst zipfile from your initial analysis and copy to a new excel sheet (*see* **Note 12**).
2. Custom Sort the rows by the log2(FC) from high to low. Positive fold change values are now at the top of the excel file, these metabolites are considered ‘upregulated’. Fold change values at the bottom of the excel file are metabolites that are ‘downregulated.’
3. Copy the column with the m/z values, log2(FC), and raw.pval to a new sheet.
4. To identify upregulated metabolic pathways and associated metabolites, create a new column titled ‘p.value’ and assign a p-value < 0.05 to upregulated metabolites based on fold change values. Label the column with m/z values as ‘m.z’.
5. Copy ‘m.z’ column and the ‘p.value’ column with the newly assigned p-values to a new excel file and save file as a .txt file.
6. Alternatively, to identify downregulated metabolic pathways and associated metabolites, sort rows by the log2(FC) from low to high. Create a new column titled ‘p.value’ and assign a p-value < 0.05 to downregulated metabolites. Similarly, copy and paste ‘m.z’ and ‘p.value’ columns to a new excel file and save as a .txt file.
7. When uploading .txt file to MetaboAnalyst, select ‘Functional Analysis’.
8. Within the ‘A peak table’ tap select the appropriate parameters including ion mode, mass tolerance (ppm), retention time, rank (p-value or t-score), and enforcement of primary ions. Upload .txt file of interest and select ‘submit’.

9. When specifying analysis parameters, algorithm options include Mummichog and GSEA. Input a p-value cutoff of 0.05 or less. Visual analytic options include scatter plot or heatmap.
10. Select the appropriate pathway library and select 'submit' (*see* **Note 16**).
11. MetaboAnalyst will display an enrichment factor graph and pathways. Information provided include total metabolites in pathway, number of metabolites in pathway detected in dataset, significant metabolites in pathway detected in dataset, p-value, and gamma P values (*see* **Note 17**).
12. To download metabolic results, select 'download'.

3.8 Targeted Metabolite Analysis

1. Compile a list of metabolite features of interest (e.g., metabolites of glycolysis).
2. Spike isotopically labelled standards into samples to normalize metabolite concentrations across all samples and individual groups.
3. Set parameters for metabolites of interest on mass spectrometry instrument of choice.
4. Following LC-MS analysis, begin analyzing data via MetaboAnalyst as previously discussed.
5. Utilization of XCMS and other metabolomics databases are recommended to identify and detect metabolite features of interest within dataset.

4. Notes

1. All consumables and reagents should be either HPLC- or mass spectrometry-grade.
2. Work in sterile Biosafety laminar flow hood for steps requiring sterile conditions.
3. Smaller cartilage pieces are better for digestion.

4. Do not digest the cartilage for more than 16 h.
5. All steps involving cell culture work are to be performed in the sterile Biosafety laminar flow hood.
6. Make sure the sodium alginate is completely dissolved before proceeding with encapsulation.
7. When homogenizing solid and hydrogel samples, the goal is to produce a milky-looking solution. Once this stage is reached, transfer the top layer of solution to a new microcentrifuge tube. cartilage won't break down completely, therefore, some chunks will remain at the end of this step.
8. When preparing for LC-MS analysis, utilize 50 µL for analysis and save 50 µL at - 80ºC n the case of error or needing to repeat a run.
9. When drying samples via vacuum concentration, drying time may vary but we recommend checking on samples every 15-30 minutes to prevent overdrying of the sample.
10. When extracting metabolites, if after the first round of centrifugation and vacuum concentration large amount of protein is still present, consider leaving overnight.
11. To fully analyze and investigate metabolite features in samples, consider running samples in negative mode and in reverse mode.
12. We recommend to always save the original excel file outputted from XCMS prior to major editing.
13. Statistical analyses commonly performed to generate metabolomic profiles and to gain a better understanding of the metabolism at hand includes hierarchical clustering analysis (HCA), principal component analysis (PCA), partial least squares-discriminant analysis

(PLS-DA), fold change, t-tests, volcano plot, and heatmap analysis. MetaboAnalyst will provide all statistical tests that are applicable to the data.

14. Depending on sample and objective of study, evaluate what statistical tests best fit the scope of interest. For example, consider using volcano plot analysis to determine metabolite features that are distinct and statistically significant to each individual cohort/experimental group.
15. When normalizing data via MetaboAnalyst, consider various options like pareto scaling and others. Additionally, log transformation is not always necessary.
16. When selecting a pathway library, we recommend utilizing KEGG first. To further investigate metabolic interactions, consider using Biocyc.
17. To view and search significant metabolites that were involved in a pathway and detected in dataset, click ‘view’ and KEGG IDs will be listed. IDs in red are significant and can be found on KEGG.

5. Figures

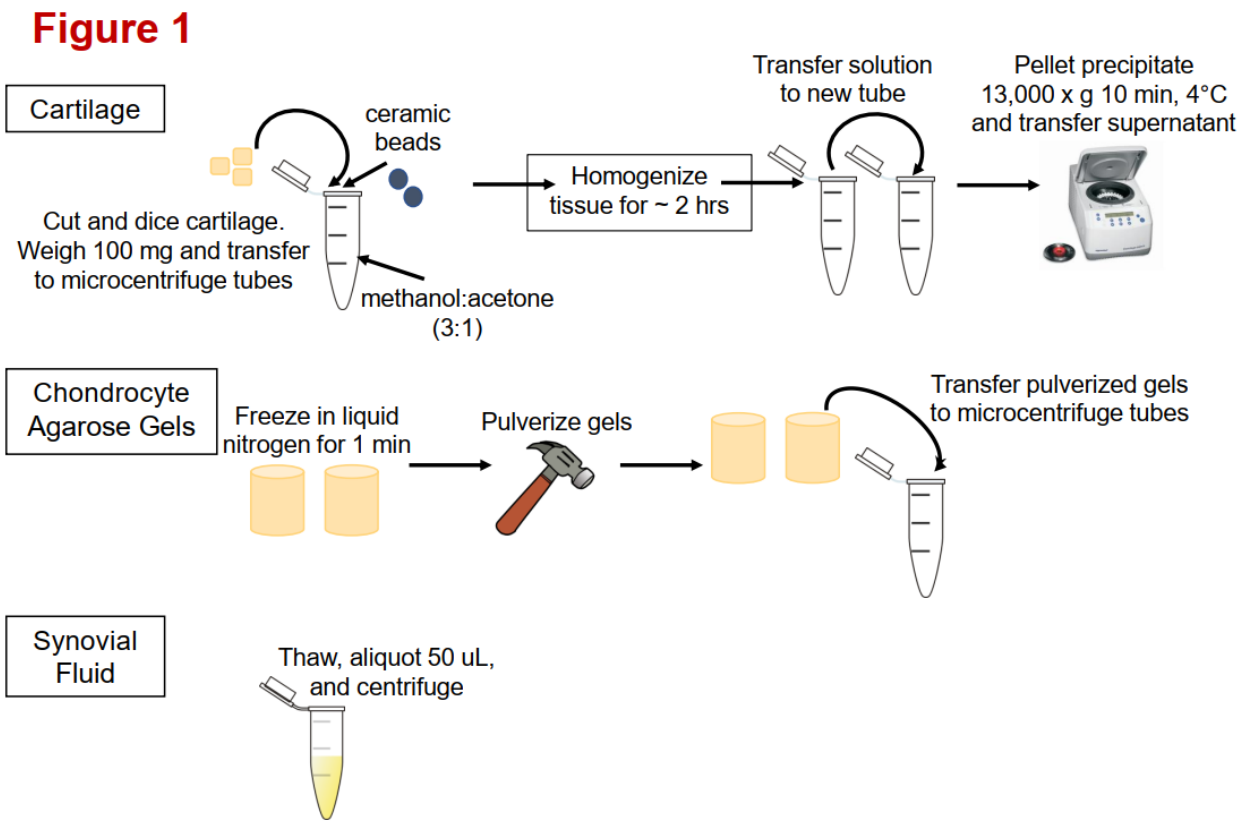


Figure 1. Metabolite extraction preparation for various types of samples. Cartilage: Begin by cutting and dicing cartilage. Weigh 100 mg of cartilage and transfer to microcentrifuge tubes. Suspend cartilage with 3:1 methanol acetone and add ceramic beads. Homogenize tissue for 2 hours. Transfer milky solution to a new microcentrifuge tubes and centrifuge sample. Chondrocyte agarose gels: Flash free gels for 1 minute, then pulverize gels. Transfer pulverized gels to microcentrifuge tubes. Synovial fluid: Thaw fluid and aliquot 50 uL to microcentrifuge tubes. Centrifuge samples to remove cells and debris.

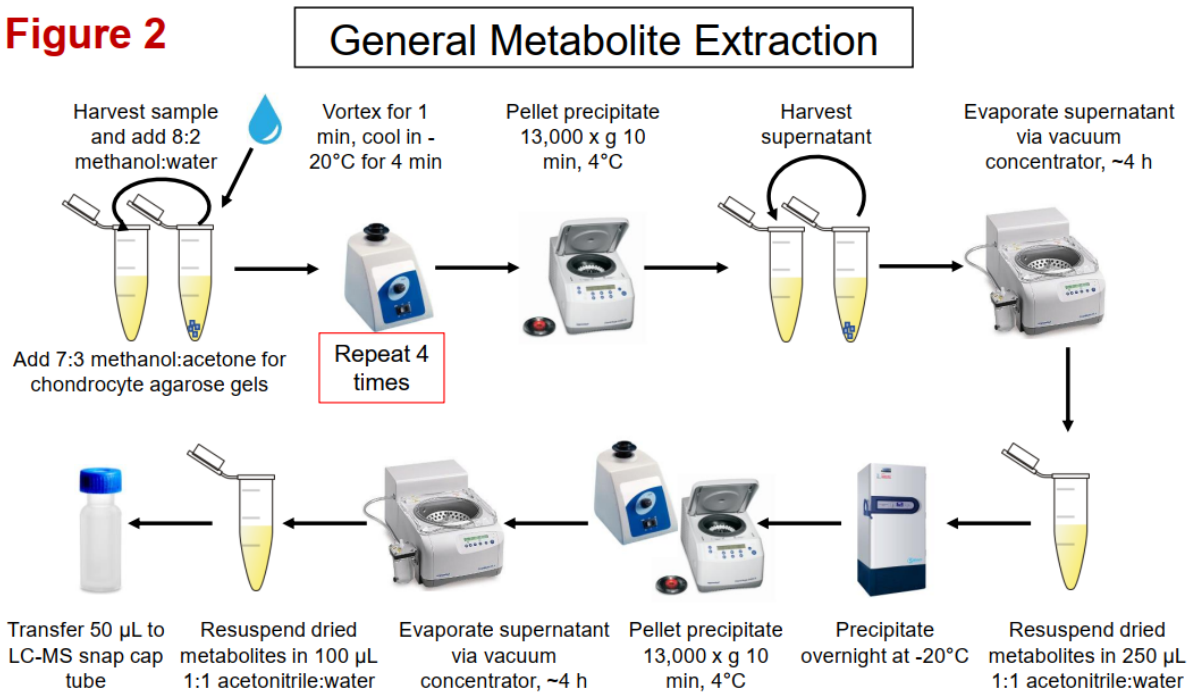


Figure 2: Experimental procedure for extracting metabolites from various tissue types.

After specific extraction protocols for individual sample types (Fig. 1), the protocol to extract metabolites is uniform. The protocol displayed applies to all three sample types discussed and could be extended to other sample types. Protocol may need alteration for sample types not discussed in this chapter.

Figure 3

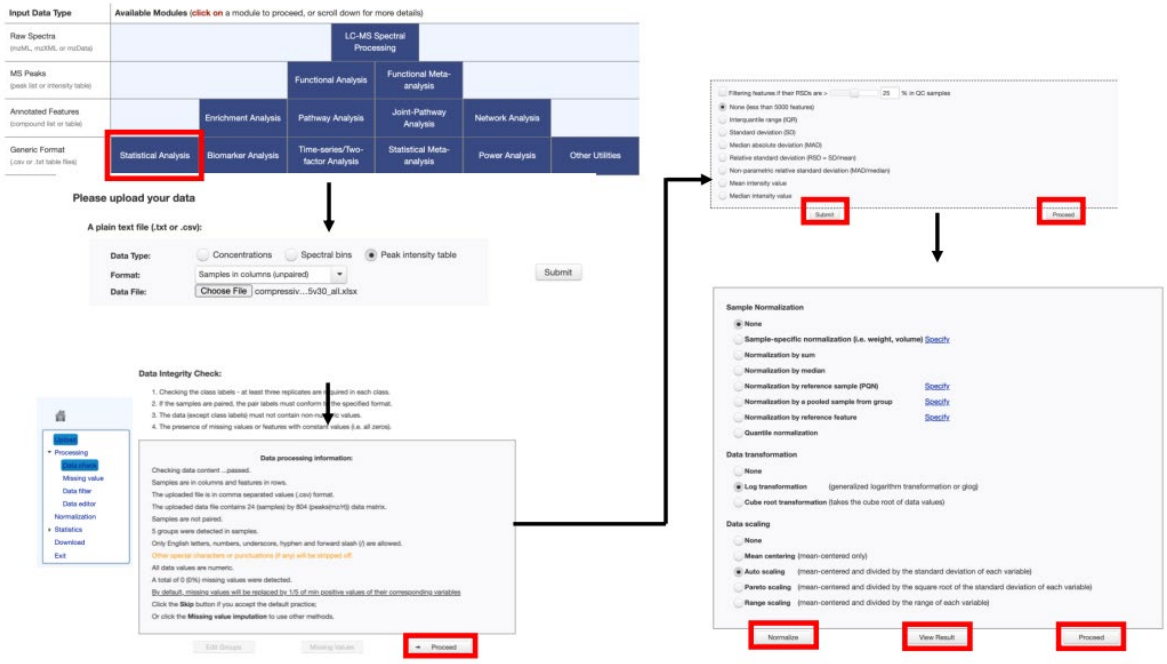
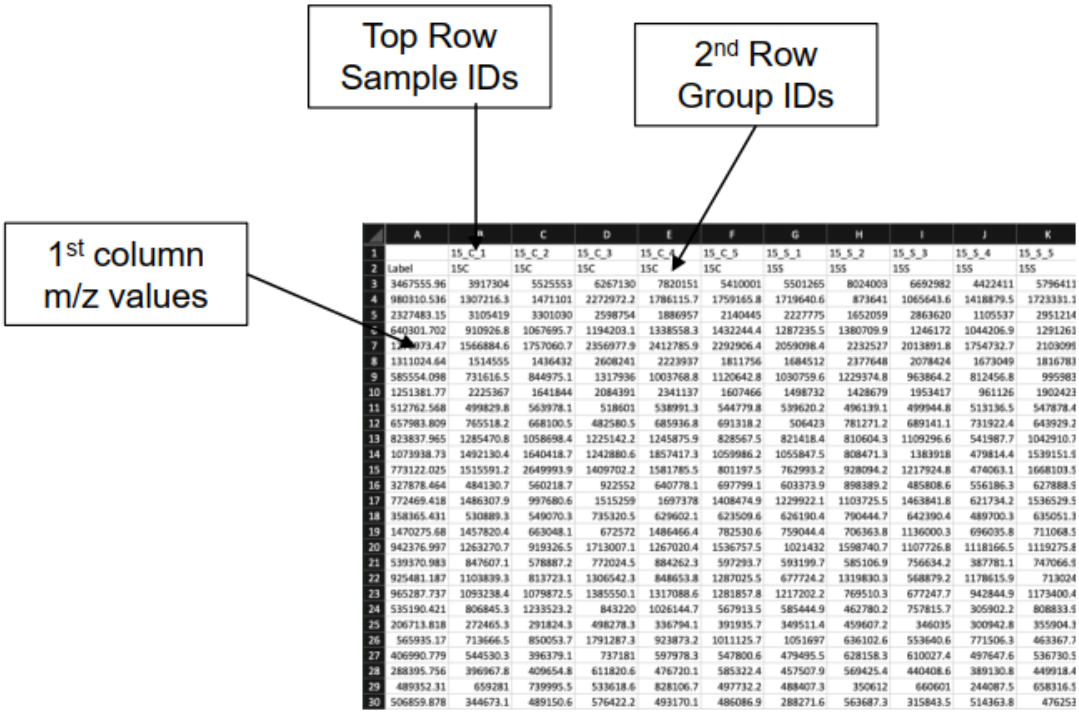


Figure 3. Workflow to perform statistical analyses on metabolic data via MetaboAnalyst.

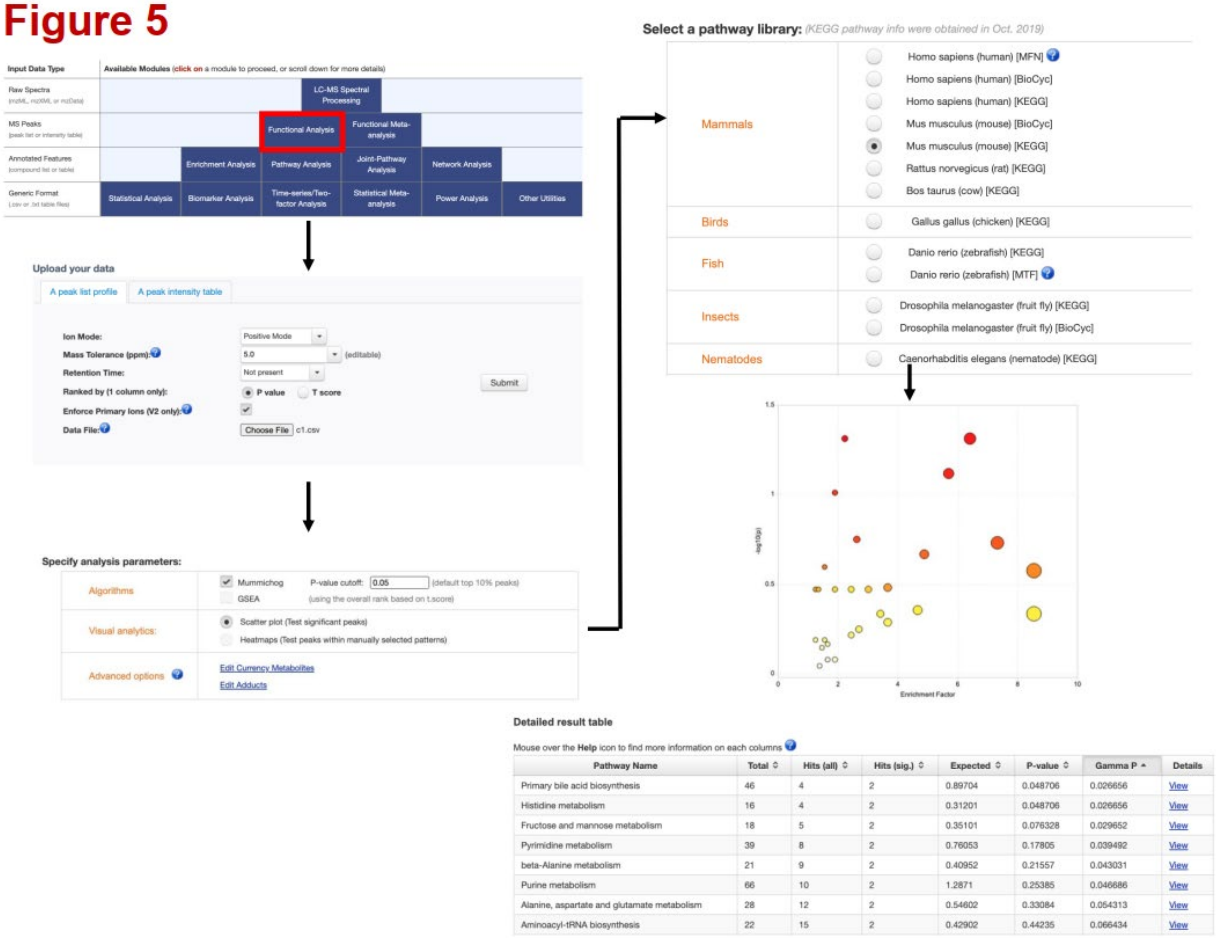
Figure 4



	A	B	C	D	E	F	G	H	I	J	K
1		15_C_1	15_C_2	15_C_3	15_C_4	15_C_5	15_S_1	15_S_2	15_S_3	15_S_4	15_S_5
2	Label	15C	15C	15C	15C	15C	15S	15S	15S	15S	15S
3	3467555.96	3917304	5525553	6267130	7820151	5410001	5501265	8024003	6692982	4422411	5796411
4	980310.536	1307216.3	1471101	2272972.2	1786115.7	1759165.8	1719640.6	873641	1065643.6	1418879.5	1723331.1
5	2327483.15	3105419	3301030	2598754	1886957	2140445	2227775	1652059	2863620	1105537	2951214
6	640301.702	910926.8	1067695.7	1194203.1	1338558.3	1432244.4	1287235.5	1380709.9	1246172	1044206.9	1291261
7	1[illegible]073.47	1566884.6	1757060.7	2356977.9	2412785.9	2292906.4	2059098.4	2232527	2013891.8	1754732.7	2103099
8	1311024.64	1514555	1436432	2608241	2223937	1811756	1684512	2377648	2078424	1673049	1816783
9	585554.098	731616.5	844975.1	1317936	1003768.8	1120642.8	1030759.6	1229374.8	963864.2	812456.8	995983
10	1251381.77	2225367	1641844	2084391	2341137	1607466	1498732	1428679	1953417	961126	1902423
11	512762.568	499829.8	563978.1	518601	538991.3	544779.8	539620.2	496139.1	499944.8	513136.5	547878.4
12	657983.809	765518.2	668100.5	482580.5	685936.8	691318.2	506423	781271.2	689141.1	731922.4	643929.2
13	823837.965	1285470.8	1058698.4	1225142.2	1245875.9	828567.5	821418.4	810604.3	1109296.6	541987.7	1042910.7
14	1073938.73	1492130.4	1640418.7	1242880.6	1857417.3	1059986.2	1055847.5	808471.3	1383918	479814.4	1539151.9
15	773122.025	1515591.2	2649993.9	1409702.2	1581785.5	801197.5	762993.2	928094.2	1217924.8	474063.1	1668103.5
16	327878.464	484130.7	560218.7	922552	640778.1	697799.1	603373.9	898389.2	485808.6	556186.3	627888.5
17	772469.418	1486307.9	997680.6	1515259	1697378	1408474.9	1229922.1	1103725.5	1463841.8	621734.2	1536529.5
18	358365.431	530889.3	549070.3	735320.5	629602.1	623509.6	626190.4	790444.7	642390.4	489700.3	635053.3
19	1470275.68	1457820.4	663048.1	672572	1486466.4	782530.6	759044.4	706363.8	1136000.3	696035.8	711068.5
20	942376.997	1263270.7	919326.5	1713007.1	1267020.4	1536757.5	1021432	1598740.7	1107726.8	1118166.5	1119275.8
21	539370.983	847607.1	578887.2	772024.5	884262.3	597293.7	593199.7	585106.9	756634.2	387781.1	747066.9
22	925481.187	1103839.3	813723.1	1306542.3	848653.8	1287025.5	677724.2	1319830.3	568879.2	1178615.9	713024
23	965287.737	1093238.4	1079872.5	1385550.1	1317088.6	1281857.8	1217202.2	769510.3	677247.7	942844.9	1173400.4
24	535190.421	806845.3	1233523.2	843220	1026144.7	567913.5	585444.9	462780.2	757815.7	305902.2	808833.9
25	206713.818	272465.3	291824.3	498278.3	336794.1	391935.7	349511.4	459607.2	346035	300942.8	355904.3
26	565935.17	713666.5	850053.7	1791287.3	923873.2	1011125.7	1051697	636102.6	553640.6	771506.3	463367.7
27	406990.779	544530.3	396379.1	737181	597978.3	547800.6	479495.5	628158.3	610027.4	497647.6	536730.5
28	288395.756	396967.8	409654.8	611820.6	476720.1	585322.4	457507.9	569425.4	440408.6	389130.8	449918.4
29	489352.31	659281	739995.5	533618.6	828106.7	497732.2	488407.3	350612	660601	244087.5	658316.5
30	506859.878	344673.1	489150.6	576422.2	493170.1	486086.9	288271.6	563687.3	315843.5	514363.8	476253

Figure 4. Example input file for MetaboAnalyst. Required formatting for input to MetaboAnalyst.

Figure 5



Pathway Name	Total	Hits (all)	Hits (sig.)	Expected	P-value	Gamma P	Details
Primary bile acid biosynthesis	46	4	2	0.89704	0.048706	0.026656	View
Histidine metabolism	16	4	2	0.31201	0.048706	0.026656	View
Fructose and mannose metabolism	18	5	2	0.35101	0.076328	0.029652	View
Pyrimidine metabolism	39	8	2	0.76053	0.17805	0.039492	View
beta-Alanine metabolism	21	9	2	0.40952	0.21557	0.043031	View
Purine metabolism	66	10	2	1.2871	0.25385	0.046686	View
Alanine, aspartate and glutamate metabolism	28	12	2	0.54602	0.33084	0.054313	View
Aminoacyl-tRNA biosynthesis	22	15	2	0.42902	0.44235	0.066434	View

Figure 5. Workflow to perform a functional pathway analysis via MetaboAnalyst.

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