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Stable Isotope Tracing and Metabolomics to Study In Vivo Brown Adipose Tissue Metabolic Fluxes

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Abstract

Brown adipose tissue (BAT) demonstrates extraordinary metabolic capacity. Previous research using conventional radio tracers reveals that BAT can act as a sink for a diverse menu of nutrients; still, the question of how BAT utilizes these nutrients remains unclear. Recent advances in mass spectrometry (MS) coupled to stable isotope tracing methods have greatly improved our understanding of metabolism in biology. Here, we have developed a BAT-tailored metabolomics and stable isotope tracing protocol using, as an example, the universally labeled ^{13}C-glucose, a key nutrient heavily utilized by BAT. This method enables metabolic roadmaps to be drawn and pathway fluxes to be inferred for each nutrient tracer within BAT and its application could uncover new metabolic pathways not previously appreciated for BAT physiology.

Su Myung Jung, Johnny Le, Will G. Doxsey, and John A. Haley were considered as co-first authors.

Keywords

Stable isotope tracing; Liquid chromatography-mass spectrometry (LC-MS); Metabolomics; Brown adipose tissue; Brown fat; Metabolism; Glucose; Gavage; Temperature acclimation

1 Introduction

Although intra-tissue lipolyzed fatty acids coupled with β-oxidation is a major fuel source for brown adipose tissue (BAT) non-shivering thermogenesis [1] accumulating evidence indicates BAT utilizes a variety of nutrients in vivo depending upon the conditions. For example, cold-stimulated BAT takes up copious amounts of glucose, fatty acids and branched chain amino acids (BCAA) as demonstrated by experiments utilizing fluorine-based radioactive tracers like ^{18}F-FDG (Fluoro-Deoxy Glucose), ^{18}F-FTHA (Fluoro-Thia-Heptadecanoic Acid), ^{18}F-Fluciclovine (Fluoro-lucioclovine) [2-9] and ^{123}I-BMIPP (Beta-Methyl-p-Iodo-Phenyl-Pentadecanoic acid) [10]. Although these radioactive tracers are highly sensitive and provide information on organ uptake, they are non-metabolizable and cannot inform on how nutrients are used within the organ. Chromatography coupled-mass spectrometry-based metabolomics combined with stable isotope tracing can overcome this roadblock. This technique enables quantitation of metabolite levels and provides information about metabolic flux (pathway activity).

The most common metabolomics tools are nuclear magnetic resonance (NMR), gas (GC)- and liquid chromatography (LC)-mass spectrometry (MS). NMR and GC-MS have been the gold standard in detecting specific metabolites since the 1960s because they can distinguish isomers and provide predictable electron ionization fragmentation patterns [11]. However, the sensitivity of NMR is low and GC-MS requires samples to be in the gas phase after extra steps of chemical derivatization, which limits the number of metabolites detected [11]. LC-MS has simpler sample preparation and can detect significantly more metabolites, enabling comprehensive metabolite identification and pathway analysis [12].

Isotope tracing coupled to GC-MS or LC-MS metabolomics has been widely used to understand altered metabolic fluxes in cancer [13] and is beginning to be applied to better understand brown adipocyte metabolism mainly in in vitro [14-16]. However, in vitro culture conditions do not always recapitulate the physiologically relevant and complex in vivo environment.

In vivo isotope tracing on a multicellular organism level is an advanced technique to assess organ-specific and/or whole-body metabolism. Successful in vivo tracing requires two main considerations: (1) the specific question informing the tracer delivery method and (2) the specific isotopic tracer chosen to study the metabolic pathway of interest. First, it is important to carefully consider the appropriate delivery method, which will depend upon the question being addressed and the nutrient tracer being used (Table 1). For example, if the question seeks to understand how glucose is physiologically being utilized, oral feeding with acute time course (<60 min) would be most ideal as glucose is absorbed through gastro-intestinal tract and is rapidly metabolized. In contrast, to examine glutamine utilization, intravenous delivery may be more ideal as glutamine is mainly produced and

released by peripheral tissues more than derived from the diet. Alternatively, if the question is to quantitatively understand metabolism at steady state without metabolic perturbation, a slow, non-perturbative isotope infusion method will be more adequate [12, 25]. Secondly, it is important to consider which isotope tracer(s) to use when interrogating a particular pathway. For example, to assess the pentose phosphate pathway, an ideal tracer may be a positionally labeled tracer such as [1,2-^{13}C] glucose rather than a universally labeled glucose tracer. [1,2-^{13}C] glucose generates M + 1 lactate when it goes through the pentose phosphate pathway, and [1,2-^{13}C] glucose generates M + 2 lactate when it goes through traditional glycolysis. Please refer to [12] for more details on the list of tracers and their applicable metabolic pathways and result interpretation.

Here, we introduce an experimental protocol for systems-level isotope tracing coupled to LC-MS based metabolomics. We use universally labeled glucose as an example and a protocol optimized for BAT showing measurements of universally labeled [U^{13}C] glucose metabolic flux and the fate of glucose provided by oral gavage, in BAT under different temperature acclimation (Fig. 1). This strategy can be applied to examining other tracers depending upon which metabolic pathway is being interrogated and in combination with a variety of delivery methods (Table 1) and thermogenic stimuli including a range of cold temperatures and diets, beta-adrenergic agonists, and genetic or pathological conditions.

2 Materials

Tracing experiments can be either perturbative (e.g., bolus administration that increases circulating concentrations) or non-perturbative (e.g., slow steady-state infusion that minimally affects circulating concentrations). Only perturbative experiments are described in this protocol. Extraction and LC-MS solutions should be prepared with HPLC grade reagents. Wear appropriate eye protection, lab coat and gloves when working with solutions and liquid nitrogen.

2.1 Mice

1. Wild type C57BL6/J mice or genetically engineered mice along with litter, age-, sex- and strain-matched controls.
2. House mice in an Institutional Animal Care and Use Committee (IACUC)-approved animal facility in a clean room set at 22 °C and 45% humidity on a daily 12 h light/dark cycle and kept in ventilated racks, fed ad libitum, a standard chow diet, and bedding and nesting changed every 2 weeks.
3. Use a 20G × 1.5″ disposable animal feeding tube to gavage mice with tracer solutions.

2.2 Isotope Tracer

1. [U^{13}C]-glucose from adequate companies such as Cambridge Isotope Laboratories, Omicron Biochemicals, and Sigma.

2.3 Tissue Harvesting/Processing

1. Pre-chilled metal Wollenberger clamp or other tissue freeze clamping device.
2. 1 mL Syringe with a 26G × 5/8 needle.
3. 1.5 mL Microcentrifuge tubes or capillary collection tubes.
4. Tabletop centrifuge.
5. Heparin-coated blood collection tubes for plasma collection or non-coated tube for serum collection.
6. Dissection tools (forceps, large scissors, small scissors, etc.).
7. Heavy duty aluminum foil.
8. Liquid nitrogen.
9. Laboratory spatula.
10. Stainless steel tissue lysing beads, 5 mm.
11. Safe-lock microcentrifuge tubes (Eppendorf, Cat# 22363352) (*see* Note 11).
12. Waterproof cryogenic gloves.
13. Lab coat.
14. Protective face shield.

2.4 Extraction and LC-MS

1. Grinding equipment: Cryomill and 5 mm metal ball (Retsch) (*see* Note 1).
2. Extraction solution: Acetonitrile: Methanol: Water (40:40:20) mixture kept at 4 °C (*see* Note 2).
3. Chromatography solvents: (solvent A) 20 mM ammonium acetate, 20 mM ammonium hydroxide in 95:5 acetonitrile: water, pH 9.45. (Solvent B) acetonitrile.
4. LC-MS equipment: Vanquish autosampler (Thermo Fisher, San Jose, CA), Xbridge BEH amide column (2.1 mm × 150 mm, 2.5 μm particle size, 130A° pore size) (Waters, Milford, MA), Quadrupole-orbitrap mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific, San Jose, CA). Either GC-MS or LC-MS from other companies can also be used.

3 Methods

In order to acquire thermogenically inactive vs. cold-stimulated brown adipocytes in this example, mice are first acclimated to the appropriate temperatures. Thermoneutrality is a good baseline warm temperature (28–30 °C) as C57Bl6/J mice do not have to expend extra energy to maintain euthermia at this temperature. These mice are then matched with mice that have been acutely exposed for a matter of hours or subjected to prolonged cold exposure for days to weeks, prior to tracer delivery. To achieve the most physiological administration

of isotope labeled glucose, deliver the tracer (e.g., [U^{13}C]-glucose in this example) via oral gavage.

3.1 Temperature Challenge

1. Use adult male or female mice whose ages are at least more than 9-weeks old (*see* Note 3).
2. For chronic temperature acclimation, put in 1–2 mice per cage without nesting (*see* Note 4) and place them either in thermoneutrality (30 °C) [26, 27] or severe cold (6 °C) using rodent incubator equipped with air ventilation, temperature, and humidity control (RIT330SD, Power Scientific).
3. While the thermoneutrality group will be consistently housed at 30 °C during the entire 4 weeks of challenge, severe cold group should be started from 18 °C, with a decrease in temperature of 4 °C weekly until it reaches 6 °C on the fourth week [28-31] (*see* Note 5): 18 °C → 14 °C → 10 °C → 6 °C.
4. Place room temperature group (20–22 °C) mice with exact conditions as mice in rodent incubators (e.g., food, water, humidity, number of mice per cage, w/o nesting).
5. For acute cold challenge, put in 1–2 mice per cage without nesting and place in a rodent incubator set at 6 °C for up to 8 h.

3.2 Tracer Delivery

1. Prepare [U^{13}C]-glucose at 1 g/kg in 0.9% NaCl to achieve good blood enrichment (10–20%) and to mimic physiological-level increases in blood glucose level when administered at 10 μL/g bodyweight (*see* Note 6). When using a different tracer, the appropriate tracer concentrations and delivery method must be found through pilot experiments and literature searches.
2. On the day of the experiment, transfer mice to new cages for physiological fasting in the morning to early afternoon (5–6 h) to ensure synchronization of post-gastrointestinal absorption state. Circadian rhythm affects glucose metabolism. Thus, the investigators should consider this factor when they design the experiment.
3. If, however, either cold-acclimated or acutely cold exposed group is included in the experiment, SKIP **step 2** (*see* Note 7).
4. For the oral gavage, use a 20G × 1.5″ disposable animal feeding tube. Doses and volumes of tracer solution made in Subheading 2.2 will need to be optimized based on the body weight of mice. Monitor mice at least 10 min after oral gavage to make sure mice are not excessively struggling or having difficulties with breathing.

3.3 Tissue Harvest

1. Precool Wollenberger clamp [32] with liquid nitrogen for ~5 min.

2. Collect blood via cardiac puncture for terminal experiments or via tail snip for non-terminal experiments into microcentrifuge tubes (or capillary blood collection tubes) and place on ice for 20 min to allow clotting for serum collection. Then, spin at 16,000 rcf for 10 min at 4 °C. Collect and store supernatant (serum) at −80 °C. (*see* Note 8) If the supernatant is jelly, spin additional 10 min. For plasma collection, use heparin-coated tubes but not EDTA-coated tubes because EDTA can affect mass spectrometry signals.

3. Place tissue between a folded-in-half heavy-aluminum foil sheet, smash with Wollenberger clamp and freeze in liquid nitrogen. (*see* Note 9).

3.4 Tissue/Serum Processing and LC-MS

1. All tools for weighing and grinding including spatulas, forceps, tubes, metal ball etc. should be on dry ice when not using. Once tissue is frozen and processing begins, keep tissue on dry ice to avoid thawing.

2. Clearly label safe-lock microcentrifuge tubes (Eppendorf, Cat# 22363352) both on top of the cap and side of the tube since grinding can deface topside labeling. When handling liquid nitrogen temperature equipment, wear cryogenic gloves, lab coat, and face shield.

3. With folded-in-half aluminum foil containing sample sitting on dry ice, open and gently tap with a spatula to break tissue into evenly sized chips no greater than 5 mm in length (*see* Note 10).

4. Scoop/pick up chips with forceps into 2 mL safe-lock microcentrifuge tubes and add one dry ice-cold metal ball (*see* Note 11).

5. Grind tissue in Cryomill at 25 Hz for 30 s and check powder for any large chunks (*see* Note 12).

6. After ensuring no lumps/chunks remain, scoop 20–25 mg into a 1.5 mL microcentrifuge tube and record accurate weight. If tissues are not enough, 5–10 mg can be used but data variation can be bigger due to inaccurate weighing (*see* Note 13).

7. Add 40x the weight of tissue (mg) to make 25 mg tissue per 1 mL ofice-cold acetonitrile/methanol/water extraction solution, vortex for 10 s, and place on ice (*see* Note 14).

8. For serum, dilute 5 μL of serum to 150 μL with ice-cold acetonitrile/methanol/water extraction mixture, vortex for 10 s, and place on ice (*see* Note 15).

9. Centrifuge at 16,000 rcf for 10 min at 4 °C and collect minimal amounts of supernatant (to avoid touching pellets). ~100 μL, for tissue extraction, and ~50 μL, for serum extraction are sufficient. Transfer the supernatant into an ice-cold, pre-labeled mass spectrometer vial (Thermo Scientific, Cat# 200046) for LC-MS injection. If tissue weights are not even across the samples, balance centrifuge well (*see* Note 16).

10. Inject 3 μL of sample with temperature of 4 °C set on autosampler (*see* Note 17).

11. Run chromatography gradient of solvent A and solvent B at flow rate of 150 μL/min: 0 min, 90% B; 2 min, 90% B; 3 min, 75% B; 5 min, 75% B; 7 min, 75% B; 8 min, 70% B; 9 min, 70% B; 10 min, 50% B; 12 min, 50% B; 13 min, 25% B; 14 min, 20% B; 15 min, 20% B; 16 min, 0% B; 20.5 min, 0% B; 21 min, 90% B; 25 min, 90% B.

12. Operate mass spectrometer in negative ion mode with a scan range of 70–830 m/z, and resolution of 140,000.

3.5 LC-MS Data Analysis

1. Raw LC-MS data files can be analyzed by the software provided by the corresponding LC-MS company. Alternatively, use Proteowizard software (http://proteowizard.sourceforge.net/) to convert raw data files to mzXML files and use freely available EI-Maven software (Fig. 2). (https://resources.elucidata.io/elmaven).

4 Notes

1. Before grinding, run device with adapter for 5 min at 5 Hz to precool system to liquid nitrogen temperatures.

2. This mixture is suitable for stable metabolites. If analysis of unstable metabolites like NADPH or ATP is desired, then extraction mixture with 0.5% formic acid should be used followed by immediate neutralization with 15% (w/v) ammonium bicarbonate (87.5 μL for 1 mL of 40:40:20 extraction mix) [34].

3. Using younger mice (<8-week old) for chronic cold challenge (6 °C for weeks) will impact growth and size of mice as they are still in development.

4. Co-housing with more than two or nesting will facilitate extra heat production and confound results.

5. A graduated temperature decrease will minimize distress for mice.

6. Tracer solutions should be made fresh to avoid solution contamination and consequent infection.

7. Hypothermia can occur when cold adapted- or acutely cold challenged- mice are fasted.

8. Red hue indicates hemolysis. While most metabolites are not affected by hemolysis, certain metabolites that are abundant in red blood cells (e.g., lactate, glutamate) can affect data significantly. To avoid hemolysis, do not use thin needles for blood collection or vortex before clotting is completed. Plasma and serum can have different metabolite profiles [35, 36].

9. Tissue collection to freezing should occur within 10 s to minimize metabolite turnover. Samples may be stored for a few days at −80 °C until processing.

10. It is important to mix the chips to homogenize tissue, especially for heterogenous tissues such as brain, intestines, and kidneys. Chips too large may impede

grinding. Take care to not puncture aluminum foil which can lead to sample loss.

11. Must use safe-lock tubes as normal tube caps can break during grinding losing sample. Metal ball and forceps should be dry ice cold or tissue will thaw and stick, preventing adequate grinding.

12. Caution should be taken by wearing eye protection as there is potential for liquid nitrogen to seep into microcentrifuge tube, popping off the cap at high velocity. Check that tissue is thoroughly powdered without any large chunks by gently shaking the tube and/or sifting through with a spatula. Regrind if needed and use scissors to break up large chunks.

13. When weighing tissue, ensure no condensate builds up around tubes which may drastically affect weight. Do not handle tube from the bottom as heat from fingers may transfer over and melt tissue. While scooping, ensure both tubes remain on dry ice at all times. One can achieve this by using a metal rack on top of dry ice, placing powder tube and weighing tube adjacent, then transfer powder from one to the other using a spatula all the while keeping tubes on metal rack. Do not leave powder in tube on analytical mass for too long as it may begin to thaw. In between samples, polish spatula against dry ice to removal any specks/powder from previous sample. Metal beads can be reused after undergoing cleaning procedure: rinse with deionized water several times to remove tissue debris, sonicate in deionized water for 15 min, transfer into 100% methanol for 1–2 days, and then dry and store.

14. Check if there are any large chunks floating in solution after vortexing as these indicate prior thawing in the process and will lead to suboptimal extraction. Sample should be tossed and a new one reweighed and extracted. Additionally, samples can be kept at −20 °C in extraction mixture for 1 h to aid in protein precipitation.

15. 5 μL should first be pipetted to the bottom of an empty eppendorf tube then diluted with extraction mix 30x to 150 μL. Pipetting serum volume first before adding organic solvent reduces pipetting error caused by liquid adhering to pipette tip.

16. Extracted samples should be loaded for injection into LC-MS same day for the best quality of data but can be stored at −80 °C for up to 1 week (signals drop, however). Take care to not disturb protein pellet which can release proteins that clog the LC system.

17. Appropriate blank (extraction solution only), procedure blank (follow all the above steps but without biological specimen), and quality control samples will be required. Serial dilutions of chemical standards can be loaded after samples to measure accurate metabolite concentrations. Randomize sample order.

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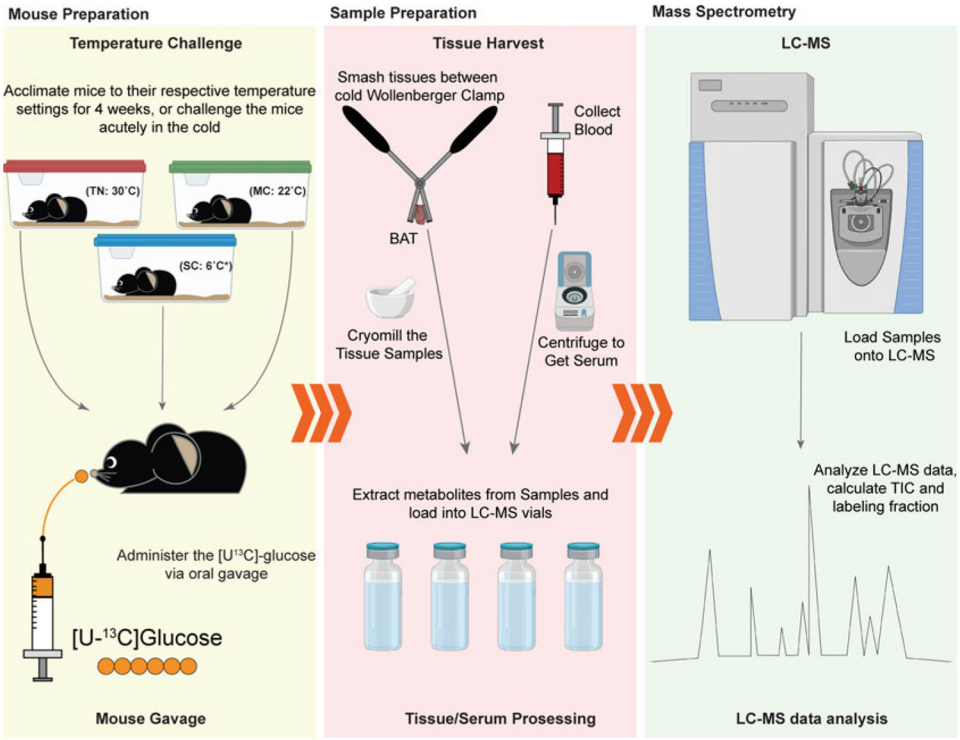

Fig. 1.

Schematic of an experimental protocol for isotope tracing in BAT from mice acclimated to different temperatures. (Made using BioRender) In order to achieve different levels of brown fat activity, the mice were acclimated to their different environments over a 4-week period. Then after administration of [U-^{13}C]glucose via gavage, their serum and tissues are harvested, with their tissues smashed in a liquid nitrogen cold Wollenberger Clamp to immediately quench the metabolism. Then the metabolites are extracted from the samples and loaded onto the LC-MS. After running the LC-MS, the data are analyzed with software. The total abundance and labeling fractions of each metabolite of interest are calculated in order to estimate flux. (The pale yellow section includes Subheading 3, **steps 1** and **2** from the methods section, the pink section includes Subheading 3, **steps 3** and **4** from the methods section, and the green section includes Subheading 3, **step 5** from the methods section.)

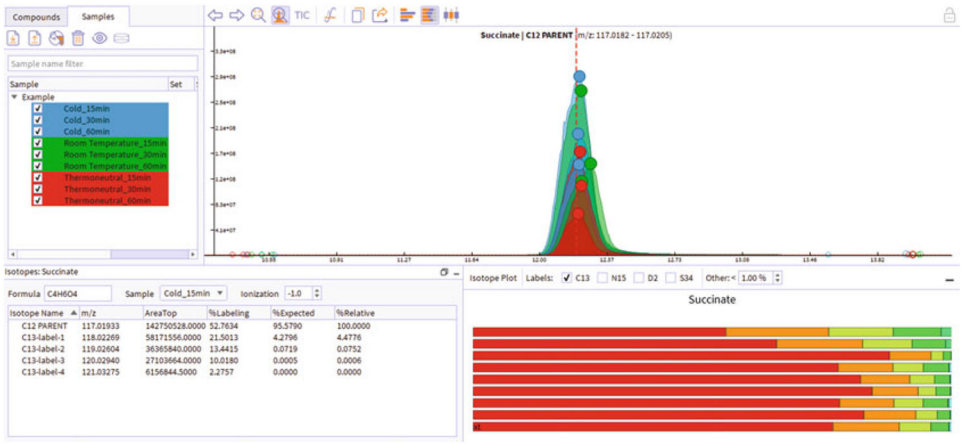

Fig. 2.

Example EI-MAVEN user interface. Top left panel displays 9 mzXML files of cold, room temperature, and thermoneutral housed mice (from top to bottom, $n = 3$ per group). Top right panel shows succinate peaks. Bottom left panel contains quantitation of isotope labeling of succinate. Bottom right panel is horizontal bar plot showing percent labeling of succinate, with colors indicating different isotopologues. For more details about using EI-MAVEN, visit https:/resources.elucidata.io/elmaven. Calculate total ion counts (reflecting metabolite concentrations) and the labeling fraction of the metabolites of interest, after performing natural isotope correction using R package, AccuCor (https:/rdrr.io/github/XiaoyangSu/AccuCor/) [33]. Note that, for perturbative experiments, the labeling fraction of metabolites is affected by the pool size (e.g., high intra-tissue concentrations of endogenous, unlabeled metabolites will dilute their labeling fractions). In this case, the total labeled carbons of each metabolite (calculated by multiplying the number of carbons for each isotopologue ion count and adding up) is the best way to estimate flux. Learn more about the basic concepts of isotope tracing, see Ref. [12]

Table 1

Tracer delivery methods for in vivo stable isotope tracer studies with their pros and cons

Delivery method	Pros	Cons
Oral Gavage	Physiological delivery for dietary metabolites passing through digestive tract and liver (e.g., glucose, most amino acids and lipids). Can trigger GI tract/digestive system-derived hormonal release. Easy delivery and can be used for bolus studies with time course [17, 18]	Acute bolus feeding could cause metabolic perturbations [19]. Hard to achieve steady-state labeling condition.
Tail vein (intravenous, IV)	Instantly delivers tracer into the blood stream allowing for acute bolus. Could be the way to physiologically deliver organ-producing metabolites (e.g., lactate and glutamine). Can be used for flux analysis with time course [20]	Less physiologically relevant for dietary metabolites because this method bypasses the digestive tract and liver. Can have low success rate with limited injectable volume. Require heating of the tail to help visualize the vein which is not optimal for temperature experiments
Retroorbital	Less stressful than tail vein injection with similar efficacy [21]. Higher success rate than tail vein injections. Larger volume can be injected compared to tail vein.	Less physiologically relevant for dietary metabolites as is tail-vein injection. Requires anesthesia.
Intraperitoneal	More physiological delivery than IV only bypassing digestive tract but still subject to first-pass metabolism by hepatic system [22]	Less physiologically relevant for dietary metabolites. Slower and less diffusion into the blood stream than IV after hepatic metabolism.
IV Infusion	Maintains constant blood tracer levels for pseudo-steady-state analysis [12, 13, 23, 24]. Minimal metabolic perturbation during the process if tracer infusion rate does not change metabolite concentrations. Multiple time points are less required.	Mice have to be pre-catheterized. Need mathematical modeling to account for metabolite interconversion [12,24]