Measuring *in vivo* tissue metabolism using ^{13}C glucose infusions in mice

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Title: In vivo ^{13}C tracer analysis

i Abstract

Metabolic alterations are a hallmark of cancer. While determining metabolic changes *in vitro* has delivered valuable insight into the metabolism of cancer cells, it emerges that determining the *in vivo* metabolism adds an additional layer of information. Here, we therefore describe how to measure the *in vivo* metabolism of cancer tissue using ^{13}C glucose infusions in mice.

ii Keywords: *in vivo* metabolism, ^{13}C tracer analysis, metabolomics, ^{13}C glucose infusions, cancer metabolism

1 Introduction

Cancer cells alter their metabolism in support of prolonged survival and uncontrolled proliferation ***(1-5)***. These changes in cancer cells constitute metabolic vulnerabilities that can be exploited for cancer therapy ***(6)***. It emerges that, besides the *in vitro* analysis, *in vivo* analysis of metabolic changes is important to further increase our understanding, precision and efficacy of targeting metabolism for cancer treatment ***(7)***.

^{13}C tracer analysis is an established method to determine the metabolism of cancer cells in vitro. The basis of this method is that a stable isotope labelled nutrient is fed to cells. Subsequently, the labelled atoms are incorporated into downstream metabolites. Analysing with mass spectrometry which metabolites and how they are labelled allows subsequently to determine the activity of metabolic pathways and the importance of nutrients to fuel certain metabolic requirements ***(8)***. Using this powerful method *in vivo* is challenging, but delivers novel information that allows to further boost our abilities to tackle cancer. For example, *in vivo* ^{13}C tracer analysis in humans and mice has identified intra tumor heterogeneity, a strong microenvironment dependence of cancers, and metabolic changes that support metastasis formation ***(7,9-12)***.

Here, we therefore describe how to determine the metabolism of mouse tumors and patient-derived xenografts (see Chapter 15) using ^{13}C glucose infusions. In particular, we provide a protocol for the catheterization, infusion, and harvest of (cancer) tissue from mice for consecutive mass spectrometry analysis.

2 Materials

Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow waste disposal regulations. Use a lab coat, gloves, and if required safety glasses at all

times. Work if necessary under the chemical fume hood. Familiarize yourself with chemical/biological safety and ethical regulations before conducting the experiment.

2.1 Catheterization surgery

1. Tubing for jugular vein: Cut 6 cm of Cannula tubing (silicone tubing 0.012" ID x 0.025" OD). Cut a 1 mm ring out of silicone tubing (0.020" ID x 0.037" OD), and place the ring over the 6 cm cannula tubing by holding the ring open with forceps. Make a diagonal cut 11 mm away from the ring to guarantee an optimal insertion into the vein. Close the tubing at the other end with a 25 gauge catheter plug (Fig 1) (*see* **Note 1**).

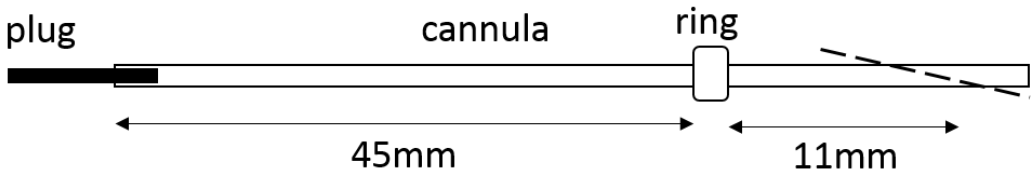

Figure 1

Cannula with ring and plug. The dotted line indicates the diagonal cut required for vein insertion.

2. Antenna: Cut 1.3 cm of metal tubing (304 S/S Hypodermic Tubing 25 gauge: 0.02 – 0.0205" OD x 0.0095 – 0.0110" ID regular wall) and smoothen the edges carefully with a file, without closing the hole in the tubing (*see* **Note 2**). Bend the metal tubing at a 90° angle, dividing it in two portions of 1/3 and 2/3 the total length. Cut 3 cm of Intramedic™ Polyethylene Tubing (0.015" ID, 0.043" OD, PE# 20, Non-Sterile) with a blade, and connect to the short (1/3) portion of the bent metal tubing. Place this construct carefully and upright in a dab of waterproof silicone glue (Fig 2.). Let it dry overnight at room temperature. When dry, cut off the sharp edges of the glue dab.

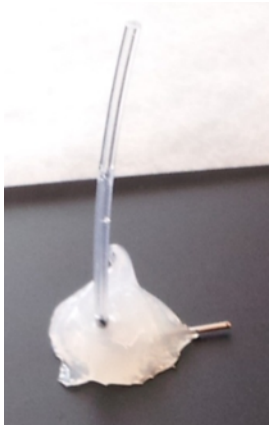

Figure 2

Antenna for connecting the cannula in the mouse right jugular vein to the syringe pump during infusions. The metal tubing is connected to the Polyethylene tubing, placed in a dab of waterproof silicone glue, and dried overnight.

3. 200 U/ml heparin- 5 mg/ml ampicillin solution: Mix 6 ml of 0.9% blood bank saline, 6 mg of 200 U/mg heparin stock, and 30 mg of ampicillin in a 15 ml conical tube. Filter the solution through a 0.2 μm syringe filter. Keep the solution sterile and at 4°C.

4. 2.5 mg/ml carprofen solution: Add 0.5 ml of 50mg/ml carprofen stock to a 15 ml conical tube containing 9.5 ml of 0.9 % blood bank saline. Filter the solution through a 0.2 μm syringe filter. Keep the solution sterile and at 4°C (*see* **Note 3**).

5. Scale (range: 0.01 – 500 g)

6. 1000 mg/g Isoflurane Iso-Vet (*see* **Note 4**)

7. Syringes (0.3 ml) with 30 gauge needles

8. Mouse shaver

9. Disinfectant with povidone iodine (e.g. Iso-Betadine)

10. 70% ethanol solution

11. Hair removal cream

12. Surgery tools: standard forceps, micro dissection forceps, surgery scissors, iris scissors, fine curved haemostat, micro dissection scissors, needle holder, ring forceps (Fine Science Tools)

13. Sterilized silk suture (5.0) for ligation

14. Silk suture with needle (16 mm needle – 75 cm – 4/0 – FW501)

15. Surgical microscope

16. Sterile cotton swabs

17. Sterile compresses

18. Heating plate containing temperature controller and rectal probe

19. Hot beads sterilizer

20. Tissue adhesive (e.g. Histoacryl)

2.2 ^{13}C glucose infusion

1. 25 gauge blunt needles (0.500" length)

2. Heparin-ampicillin solution (see item 3 in section 2.1)

3. BD Luer-Lok™ 1 ml syringe, with concentric tip and PC barrel

4. Tools: Haemostat, needle holder, surgery scissors, standard forceps, scalpel blades and handle

5. Glucometer

6. Syringe pump

7. Microdialysis swivels

8. MRE-033 Micro-Renathane Tubing. Cut 15-18 cm pieces of tubing.

9. Metal tubing for the connection between the Micro-Renathane tubing and the antenna of the mouse during infusion: Cut 1.3 cm of metal tubing (304 S/S Hypodermic Tubing 25 gauge: 0.02 – 0.0205" OD x 0.0095 – 0.0110" ID regular wall) and smoothen the edges carefully with a file, without closing the hole in the tubing.

10. Scale (range: 0.01 – 500 g)

11. Labelling solution: 500 mg/mL $^{13}C_6$ labelled glucose in deionized H_2O (*see* **Note 5**)

2.3 Collection of samples

1. Liquid Nitrogen

2. Dry ice

3. Ice

4. Labelled polyzip bags

5. Biosqueezer (e.g. Lab Services ref.1210)

6. 0.9% blood bank saline

7. 1 ml Single-use tuberculin syringe with ml graduation, Luer tip

8. Microvette for capillary blood collection

2.4 Metabolite extraction

Work under the chemical fume hood when preparing extraction solutions.

1. Labelled 2 ml Eppendorf tubes (3 tubes per sample)

2. Grinding balls, stainless steel, 5 mm

3. Grinding balls, stainless steel, 3 mm

4. Analytical balance (range: 0.0001-50 g)

5. Forceps

6. Liquid nitrogen

7. Dry ice

8. Ice

9. MS-grade Chloroform

10. Chromasolv grade Methanol

11. Cryomill

12. Milli-Q water

13. ^{13}C yeast internal standard solution: Inoculate a 3 ml yeast preculture (prototrophic strain, such as CEN.PK JT4) in YNB medium without amino acids + 1% w/v $^{13}C_6$-glucose (add 75 µl

from a 40% w/v $^{13}C_6$-glucose stock), and shake for 12 hours. Prepare a flask with 150 ml YNB without amino acids + 2% w/v $^{13}C_6$-glucose (7.5 ml of a 40% w/v $^{13}C_6$-glucose stock), transfer the preculture to this flask, and shake for 24-26 hours. Quench the culture with a 10 mM ammonium acetate solution in 60 % methanol (pH 7.5) and extract with a 10mM ammonium acetate solution in 75% ethanol (pH 7.5). Store the extracted ^{13}C yeast internal standard solution in aliquots at -80°C. Prior to use, dry down 50, 100 and 200 µl ^{13}C yeast internal standard solution in a vacuum centrifuge, and assess the abundance of ^{13}C-labeled metabolites via liquid chromatography–mass spectrometry (LC–MS).

14. 400x Norvaline:glutarate internal standard stock solution for analysis of polar metabolites: Dissolve 1 mg each of both norvaline and glutarate in 1 ml Milli-Q water (*see* **Note 6**). Aliquots can be stored for up to one year at -20°C.

15. Extraction solution 1 (water + norvaline:glutarate + methanol): Prepare per sample 0.75 µl of 400x norvaline:glutarate stock (i.e. 0.75 µg of each norvaline and glutarate per sample) in 300 µl milli-Q water. Add 500 µl methanol per sample and mix (final ratio water:methanol = 3:5). Store extraction solution 1 at -20°C until extraction (*see* **Note 7, 8**).

16. 100xC17 internal standard stock solution for analysis of fatty acids: Dissolve 1 mg of heptadecanoic acid (C17) in 1 ml chloroform (see **Note 7**). The C17 internal standard stock can be stored at -80°C for 3-4 weeks.

17. Extraction solution 2: Prepare per sample 5 µl of 100x C17 stock (i.e. 5 µg C17 per sample) in 500 µl chloroform. Store this solution at -80°C until extraction.

18. Chemical fume hood

2.5 Derivatization of polar metabolites for gas chromatography–mass spectrometry (GC–MS) analysis

Work under the chemical fume hood when preparing derivatization solutions.

1. Methoxyamine derivatization solution: 20 mg/ml solution of O-Methoxyamine–HCl in anhydrous pyridine, counting for at least 20 µl of solution per sample. Weigh 20 mg methoxyamine in a safe lock Eppendorf tube. Wash a needle with pyridine using a syringe and discard the waste appropriately. Aliquot more than 1 ml of pyridine in a safe lock Eppendorf tube using the washed needle. Add 1 ml of pyridine to the methoxyamine. Vortex until methoxyamine is entirely dissolved (*see* **Note 9**).

2. Glass GC-MS vials with insert and magnetic caps with septum

3. Crimper

4. *N*-(tert-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide with 1% tert-Butyldimethylchlorosilane (TBDMS) derivatization agent (*see* **Note 10**).

5. Heating block

6. Safe-Lock Eppendorf tubes

7. Analytical scale (range: 0.0001-50 g)

8. Syringe with needle

9. Chemical fume hood

2.6 Derivatization of non-polar metabolites (fatty acids) for GC-MS analysis

Work under the chemical fume hood when preparing derivatization solutions.

1. 2% sulfuric acid in MS-grade methanol (*see* **Note 11**).

2. Hexane (≥97.0%, GC grade)

3. Saturated NaCl: Dissolve sodium chloride in autoclaved Milli-Q water until solution is saturated. From then on, work only with the saturated (top) fraction.

4. Glass GC-MS vials with insert and nonmagnetic caps with septum

5. Crimper

6. Heating block

7. Safe-Lock Eppendorf tubes

8. Chemical fume hood

2.7 Preparation of polar metabolite samples for LC-MS analysis

1. Prepare 60% acetonitrile (LC-MS grade) under the chemical fume hood

2. Plastic vials with insert and plastic caps with septum

3. Safe-Lock Eppendorf tubes

4. Chemical fume hood

2.8 Protein quantification

1. BCA protein assay kit

2. 0.2 M NaOH: Weigh 0.8 g NaOH and transfer to a glass recipient. Add autoclaved Milli-Q water to a volume of 100 ml and mix (*see* **Note 12**).

3. Autoclaved Phosphate Buffered Saline (PBS)

4. Heating block

5. Multichannel pipet

6. Pipetting reservoir

7. 96-well plate

8. 37°C incubator

3. Methods

Perform all procedures at room temperature unless otherwise specified.

3.1 Catheterization surgery

1. Weigh the mouse and anesthetize with 3% isoflurane mixed with 2% oxygen (*see* **Note 13**). Shave the back neck area and front chest area. Switch to 2% isoflurane mixed with 2% oxygen during surgery. Place the mouse on its back, with the head towards you, under the microscope, on a sterile heating plate in a sterile environment.

2. Apply hair removal cream on the surgery site with a sterile cotton swab. Remove it after a couple of minutes with a sterile compress. Disinfect the skin with povidone iodine (e.g. iso-Betadine) followed by a disinfection with 70% ethanol using sterile cotton swabs. Administer 50 µl of 2.5 mg/ml carprofen subcutaneously.

3. Make a small vertical incision 0.5-1 cm from cephalic to sternum on the right middle line. Blunt dissect to expose the right jugular vein using 2 standard forceps. Isolate the right jugular vein carefully. Pull a 10 cm silk suture under the vein and cut in two pieces (Fig. 3). Ligate the cephalic end of the jugular vein with one piece of suture. Loosely tie the other piece of suture at the caudal end of the exposed vein.



Figure 3

The right jugular vein (indicated with an arrow) is exposed and silk suture is placed underneath to be able to tie the vein to the cannula after insertion of the cannula.

4. Remove the plug from the cannula, flush the cannula with heparin-ampicillin solution, and carefully close it again with the plug (*see* **Note 14**). Make a small cut just below the cephalic ligature with micro dissection scissors. Insert the cannula and tie the vein to the catheter on both sides of the small ring of the cannula (*see* **Note 15**). Confirm a good connection between the

cannula and the vein by injecting 50ul heparinized saline solution in the cannula. Re-plug the cannula.

5. Turn the mouse over. Remove the small hairs between the shoulder blades with hair removal cream. Disinfect the surgery site with povidone iodine and 70% ethanol using a sterile cotton swab. Make a 1 – 1.5 cm incision between the shoulder blades. Make a bag and tunnel under the skin using a fine curved haemostat. Make a wide tunnel behind the right ear to the cannula. Gently pull back the cannula through the tunnel.

6. Flush the antenna with heparin-ampicillin solution, and close the antenna with the plug from the cannula. Cut off the part of the cannula that has air inside (*see* **Note 16**). Connect the cannula with the metal tubing of the antenna (*see* **Note 17**). Ensure that there are no holes in the connection or antenna by flushing with heparin-ampicillin solution.

7. Close the dorsal neck incision. Sew the silicon glue dab under the skin. Disinfect the wound with povidone iodine.

8. Close the ventral incision (*see* **Note 18**). Disinfect the wound with povidone iodine. Give the mouse 50 µl of 2.5 mg/ml carprofen solution subcutaneously.

9. Put the mouse in a clean cage in a warm space (Fig 4.). Monitor the mouse while waking up (*see* **Note 19**).

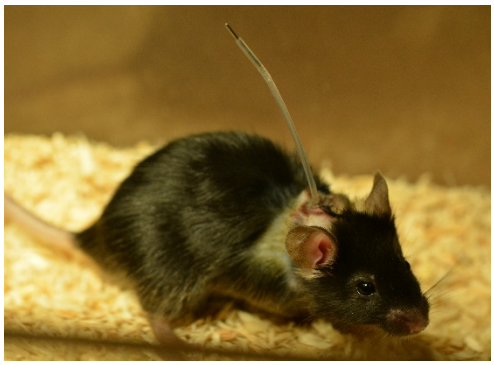

Figure 4

A C57BL6 mouse in a clean cage after surgery. The antenna is placed in the neck of the mouse, which makes it easy to handle the mouse and to connect the mouse for infusion.

3.2 $^{13}C_6$ glucose infusion

1. Fast the 6 mice for 6 hours prior to the infusion (*see* **Note 20**).

2. Prepare the syringe pump (Fig.5). Connect two 15-18 cm pieces of tubing into the two ends of the microdialysis swivel (*see* **Note 21**). One of these pieces of tubing will be connected to the syringe in the pump via a blunt needle. The other piece of tubing will be connected to the antenna of the mouse via a small hollow metal tube that you may already connect to the 15-18 cm tubing. Prior to connecting to the antenna, fill the pump syringe with the heparin-ampicillin solution, connect it to the swivel via the 15-18 cm tubing, and flush to get rid of all the air in the different parts of the line. Make sure all connections are tight.

3. Weigh the mice and place them one hour prior to infusion into a new "infusion environment", prepared with samples of old bedding (Fig.5) (*see* **Note 22**).

4. Connect the antenna of the first mouse with the heparin-ampicillin filled syringe. To do so, clamp the antenna with a needle holder and remove the plug of the antenna with another needle holder. Connect the antenna to the metal end of the 15-18 cm tubing and release the needle holder (*see* **Note 23**). Infuse for approximately 30 minutes with 0.2 µl /min heparin-ampicillin solution.

5. Infuse the mice after 6 hours of fasting with 30 mg/kg weight/min $^{13}C_6$ glucose for a total of 6 hours. To do so, first remove the heparin-ampicillin filled syringe by screwing off the blunt needle, and replace it with the syringe containing the 500 mg/ml $^{13}C_6$ glucose solution. Then, set the infusion pump to the appropriate flow rate, and start the infusion (*see* **Note 24**).

6. Start the infusion of the next mouse with an interval of 10-15 minutes.

7. Monitor the mice during the infusion (*see* **Note 25**).



Figure 5

Infusion Set-up. The mouse is placed in the "infusion environment" and is connected via his antenna to the swivel (indicated by the dotted arrow) which is in turn connected to the syringe in the infusion pump (indicated by the full arrow).

3.3 Collection of samples

1. Sacrifice the mouse with an overdose of Nembutal (3 µL per gram of animal weight of a 50 mg/mL solution). Connect the Nembutal syringe to the antenna and inject 50 µl of 60 mg/ml Nembutal (*see* **Note 26**).

2. Open the mouse and collect the blood by heart puncture. Put the blood on ice in a microvette for capillary blood collection, and spin down right after for 10 minutes at 10,000 g. Transfer the plasma to an Eppendorf tube and store at -80°C.

3. Collect the organs of interest as fast as possible. Wash the tissue in ice cold blood bank saline, remove the saline with a sterile compress, put the tissue in a labeled polyzip bag, squeeze it with the pre cooled biosqueezer, and put it into liquid nitrogen (*see* **Note 12**). Store the tissues at -80°C.

3.4 Metabolite extraction

Work under the chemical fume hood when handling the extraction solutions.

1. Weigh a piece of tissue (approximately 10 mg) while keeping it as cold as possible (*see* **Note 27**).

2. For grinding the tissue, add one 5 mm pre-cooled grinding ball and one 3 mm pre-cooled grinding ball to every tube with tissue. Cool down the Cryomill machine by opening the connection to the liquid nitrogen tank and grind the tissue for 30-40 seconds (frequency: 25/s). Place the sample tubes on dry ice immediately after grinding (*see* **Note 12**). Ground tissue can be stored at -80°C (*see* **Note 28**).

3. Transfer the tubes to a mixture of dry ice and ice. Add to each tube 800 µl of ice cold extraction solution 1, and 500 µl of ice cold extraction solution 2 (*see* **Note 29**). Vortex the samples for 10 minutes at 4°C, and then centrifuge them for 10 minutes at 4°C and maximum speed. The contents of the tube will now have separated into 3 distinct phases (Fig 6).

4. Pipet the polar top phase (consisting of extraction solution 1) into a new Eppendorf tube, and place the tube on dry ice. This layer will contain polar metabolites such as amino acids and organic acids. The middle layer consists of protein, DNA and RNA. With a new pipet tip, cross through the middle layer while releasing some air, so that proteins, DNA, and RNA do not enter the pipet tip. Then, collect the non-polar bottom phase (consisting of extraction solution 2), transfer into a new Eppendorf tube (trying not to disturb the middle layer while removing the pipet tip), and place the tube on dry ice (*see* **Note 30**). This lower phase will contain fatty acids and other non-polar metabolites (Fig.6). Bring also the tube with the remaining protein/DNA/RNA layer into dry ice.

5. Dry down the collected samples in a vacuum centrifuge. The samples containing polar metabolites should be dried at 4°C for 8 hours, while the protein and fatty acid containing samples can be dried down at 20°C for 1 hour. When dry, store the samples at -80°C.

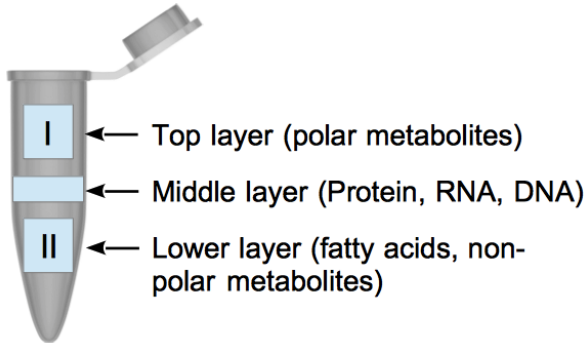

Figure 6

Eppendorf tube with the three layers after metabolite extraction. The top layer consists of extraction solution 1 containing polar metabolites. The middle layer consists of protein, DNA and RNA. The lower layer consists of extraction solution 2 containing fatty acids and non-polar metabolites.

3.5 Derivatization of polar metabolites for GC-MS analysis

Work under the chemical fume hood when handling the derivatization solutions.

1. Add 20 µl of 20 mg/ml O-Methoxyamine–HCl dissolved in pyridine to each sample, vortex briefly, and incubate for 90 minutes in a heating block at 37°C. Subsequently, centrifuge the samples for 3-5 minutes at maximum speed, transfer 7.5 µl of the supernatant into a glass GC-MS vial with insert, and seal with a magnetic cap using a crimper (*see* **Note 31**).

2. Fill a glass vial with insert with *N*-(tert-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide (TBDMS, 15 µl/sample + 25 µl extra per vial) and seal with a nonmagnetic cap (*see* **Note 32**). Fill a 2 ml glass vial without insert with pyridine, and seal with a nonmagnetic cap. The autosampler of the GC-MS will add to each sample 15 µl TBDMS, incubate for 1 hour at 60°C and resuspend the sample and load 1 µl on the column. In between loading the samples the autosampler will wash the column with pyridine (*see* **Note 33**).

3. Measure the samples using a GC-MS as described before ***(2,9)***.

3.6 Derivatization of non-polar metabolites (fatty acids) for GC-MS analysis

Work under the chemical fume hood when handling the derivatization solutions.

1. Add 500 µl of 2% sulfuric acid in methanol to each sample and incubate for 3 hours at 60°C or overnight 50°C (*see* **Note 12, 31**).

2. Add 600 µl hexane and 100 µl saturated NaCl to each sample, vortex 10 minutes, and centrifuge 5 minutes at maximum speed. Transfer the upper hexane phase to a new Eppendorf tube, and dry the samples for 10 minutes in a vacuum centrifuge (*see* **Note 34**).

3. Re-suspend in a defined amount of hexane, depending on the amount and type of tissue (e.g. a sample containing 5 mg normal liver was re-suspended in 440 µl hexane), vortex 10 minutes, transfer to a glass vial with insert, and seal with a nonmagnetic cap using a crimper.

4. Measure the samples using a GC-MS as described before ***(2)***.

3.7 Preparation of polar metabolites for LC-MS

Work under the chemical fume hood when handling solutions.

1. Add 60% acetonitrile to the samples (50 µl for 1-5 mg of tissue), vortex briefly, and centrifuge 3 minutes at maximum speed (*see* **Note 12, 35**). Transfer the supernatant to a plastic vial with insert, and seal with a plastic cap.

2. Measure the samples using a LC-MS instrument as described before ***(11)***.

3.8 Protein quantification

1. Re-suspend the dried protein pellet in 200 µl 0.2 M NaOH and incubate for 20 minutes in a heating block of 95°C (*see* **Note 12**).

2. For the quantification continue as described in the manual of the BCA protein assay kit.

3. Use the protein amount to normalize the measured metabolite levels to the starting material used.

4. Notes

1. Use a blade to cut the tubing to create smooth edges. Place the ring at about 1/4th of the 6 cm cannula, and make the diagonal cut on the end closest to the ring, so you keep a cannula of at least 5 cm.

2. Filing is needed to ensure that no damage is caused to the tubing when connecting the antenna.

3. Carprofen solution to be administred at 2 µl/g bodyweight subcutaneously.

4. An alternative anaesthetic is a ketamine/xylazine solution: Mix 650 µl Nimatek (100 mg/ml ketamine), 220 µl Rompun (20 mg/ml xylazine) and 9130 µl 0.9% saline in a 15 ml conical tube. Anesthetize the mouse for about half hour with a dose of 20 µl/g body weight injected intraperitoneally.

5. Heat in 37°C water bath for better dissolving, and filter (0.45 µm) to avoid clumps and to sterilize the solution.

6. Use glass bottles and glass pipets or "inert plastics" if possible. Normal plastic will get dissolved.

7. Use glass bottles and glass pipets or "inert plastics" wherever possible, since the used chemicals can dissolve some plastic materials. Work under the chemical fume hood.

8. When performing LC-MS measurements, add an appropriate amount of ^{13}C yeast internal standard solution (50-200 µl, depending on the metabolite abundances in your yeast internal standard solution determined by LC–MS (see 2.4.13)) to extraction solution 1, instead of norvaline:glutarate.

9. Work under the chemical fume hood. Seal both the methoxyamine and pyridine bottles with Parafilm and store in a dessicator, since humidification impairs the derivatization efficiency.

10. Store bottle at -20°C, warm up to room temperature and dry immediately before use, since humidification impairs the derivatization efficiency.

11. Sulfuric acid needs to be of 99.999% purity, since water will inhibit the derivatization reaction. Add sulfuric acid dropwise to the methanol. Work under the chemical fume hood, wear safety glasses, use clean glassware and clean glass pipets. Buffer can be stored up to 14 days at room temperature. If you would like to reuse the glass pipet for H_2SO_4, rinse the glass pipet 3 times with water and 3 times with isopropanol.

12. Work with safety glasses.

13. Only mice that have lost less than 10% of their pre-surgery weight can undergo the infusion procedure.

14. Make sure there are no air bubbles inside the cannula which could cause an air embolism.

15. Make sure the knot stays in place, but do not overtighten, to prevent blocking the cannula.

16. You can cut a longer portion of the cannula to make sure the cannula won't wire up in the mouse, but make sure that the cannula is still easily connectable with the antenna.

17. Prepare this connection carefully, since it will need to hold for 1-2 weeks: make a little knot with suture around the connection and glue with tissue adhesive e.g. Histoacryl.

18. Ensure to not make a hole in the cannula.

19. Allow a surgery recovery of one week before performing the infusions. Flush the cannula-antenna every 3 days with heparin-ampicillin solution to get the mouse used to handling and to confirm the connection is still fine.

20. Start the fasting in the morning to not disrupt the normal circadian physiology of the mice (nocturnal animals). Overnight fasting might induce a catabolic metabolism and glycogen store

depletion. We perform in one day infusions of 6 mice simultaneously with an interval of 10-15 minutes in between each mouse.

21. After infusion, clean the swivels carefully by alternating flushes with water and air to prevent to clog the swivels with the glucose solution.

22. Only mice that have lost less than 10% weight compared to prior surgery can undergo the infusion procedure.

23. This step requires two persons: One person holds the mouse and the second person connects the mouse. Make sure the connection is air-tight. Flush the antenna of the mouse before connecting it to the syringe pump: Connect a heparin-ampicillin solution filled syringe to the antenna and release the needle holder. Flush with about 20 µl heparin-ampicillin solution. Re-clamp the antenna with the needle holder and connect to the metal part of the tubing.

24. The infusion flowrate is defined both by the weight of the mouse and by the $^{13}C_6$ glucose solution concentration. For example, for a 20 g mouse and a 500 mg/mL $^{13}C_6$ glucose solution, the required flowrate will be 1.2 µl/min for an infusion of 6 hours.

25. The tube might be tangled up due to the movement of the mouse.

26. The mouse should die immediately after the Nembutal injection.

27. Keep tissue in a box with liquid nitrogen. Pre-cool Eppendorf tube with liquid nitrogen before placing it on the balance. Tare the balance with the Eppendorf tube on it, and quickly break off a piece of tissue with a liquid nitrogen cooled tweezer. Place the tissue into the Eppendorf tube as fast as possible, read the weight, and transfer the closed Eppendorf tube with the tissue into a tube holder floating in liquid nitrogen. Label your tubes on the cap and on the side to avoid losing the label.

28. Wear face protection when using the Cryomill, as tubes could break if liquid nitrogen enters the tube. In a case where liquid nitrogen has entered a tube, open the tube as fast as possible

and let the liquid nitrogen evaporate. Regrind the tissue if it is not completely ground after one round in the Cryomill. Beads can stay in the tube for the whole extraction process.

29. When a LC-MS analysis is performed, ^{13}C yeast internal standard is added to the extraction solution 1 instead of norvaline:glutarate (see 2.4.15).

30. Work under a fume hood. Release air when going through the middle layer (protein phase) by depressing the pipet by 80% and press the rest out as bubbles while crossing the middle layer. Do not take the complete liquid from each phase to not disturb the middle (protein containing) layer, as the loss of some volume from each phase can later be corrected by the internal standards.

31. Work under the chemical fume hood. Samples must be completely dry before addition of the derivatization solution. If the samples were stored at -80°C, re-dry them for 10 minutes in the vacuum centrifuge at 20°C.

32. While adding 20µl O-Methoxyamine–HCl dissolved in pyridine solution to your sample, wash the walls of the Eppendorf tubes by pipetting up and down.

33. Work under the chemical fume hood. The 25µl extra volume of TBDMS is needed to correct for evaporation of TBDMS. The derivatization of polar metabolites for GC-MS analysis can also be performed by hand. Add 20 µl 20 mg/ml methoxyamine dissolved in pyridine to each sample, vortex briefly and incubate for 90 minutes in a heating block at 37°C. Subsequently, add 40µl TBDMS, vortex briefly and incubate for 60 minutes in a heating block at 60°C. Centrifuge the samples for 2 minutes at maximum speed, transfer the supernatant to a glass GC-MS vial with insert and seal with a non-magnetic cap using a crimper.

34. Work under the chemical fume hood. Make sure to only collect the upper phase. Any addition of the lower phase will destroy the GC-MS column. Bubbles detected when flicking the vials are indicative of liquid from the lower phase in your sample. When using hexane, prepare an aliquot in a cleaned glass bottle, the aliquot should not be older than 2 weeks.

35. Work under the chemical fume hood.

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