

^{13}C Tracer Studies of Metabolism in Mouse Tumor Xenografts

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[Abstract] Mice are widely used for human tumor xenograft studies of cancer development and drug efficacy and toxicity. Stable isotope tracing coupled with metabolomic analysis is an emerging approach for assaying metabolic network activity. In mouse models there are several routes of tracer introduction, which have particular advantages and disadvantages that depend on the model and the questions addressed. This protocol describes the bolus i.v. route via repeated tail vein injections of solutions of stable isotope enriched tracers including $^{13}\text{C}_6$ -glucose and $^{13}\text{C}_5$, $^{15}\text{N}_2$ -glutamine. Repeated injections give higher enrichments and over longer labeling periods than a single bolus. Multiple injections of glutamine are necessary to achieve adequate enrichment in engrafted tumors.

Materials and Reagents

1. 0.5 ml K₂-EDTA collection tubes (BD, catalog number: 363706)
2. Sterile syringes and gauge 30 needles (Thermo Fisher Scientific, catalog number: 10142534)
3. 5-inch squares Al foil
4. Indelible marker pens (black)-Sharpie fine tip
5. Pasteur pipet
6. Lancets (MEDpoint, Inc., model: Goldenrod 5 mm)
7. Mice bearing tumor xenograft

Note: The strain of immunocompromised recipient mouse depends on type of implant. Young NOD/SCID Gamma (NSG) mice are especially favorable for the patient derived xenograft (PDX) model.
8. Ketamine for anesthesia if needed, Schedule III controlled substance (Sigma-Aldrich, catalog number: K2753)
9. Tracer [e.g., $^{13}\text{C}_6$ -glucose, $^{13}\text{C}_2$ -1, 2-glucose (60 mg/mouse), or $^{13}\text{C}_5$, $^{15}\text{N}_2$ -glutamine (22 mg/mouse)]
10. Sources
 - a. D-Glucose- $^{13}\text{C}_6$ (Cambridge Isotope Laboratories, Inc., catalog number: CLM-1396-CTM)

- b. D-Glucose-1, 2- $^{13}\text{C}_2$ (Cambridge Isotope Laboratories, Inc., catalog number: CLM-504)
 - c. L-Glutamine- $^{13}\text{C}_5$, $^{15}\text{N}_2$ (Cambridge Isotope Laboratories, Inc., catalog number: CNLM-1275)
 - Or
 - d. D-Glucose- $^{13}\text{C}_6$ (Sigma-Aldrich, catalog number: 660663)
 - e. D-Glucose-1, 2- $^{13}\text{C}_2$ (Sigma-Aldrich, catalog number: 661422)
 - f. L-Glutamine- $^{13}\text{C}_5$, $^{15}\text{N}_2$ (Sigma-Aldrich, catalog number: 607983)
11. Liquid nitrogen
 12. 70% ethanol
 13. 10% Neutral buffered formalin (Thermo Fisher Scientific, ProtocolTM, catalog number: 032-059)
 14. 25% w/v sterile filtered (0.2 μm) ^{13}C glucose in PBS
Note: Stock solution can be frozen and stored at -25 °C.
 15. Sterile wipes (Uline, catalog number: S-16183)
 16. Sodium Chloride (NaCl) (Thermo Fisher Scientific, catalog number: S271-1)
 17. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9541)
 18. Sodium phosphate dibasic (Na_2HPO_4) anhydrous (Sigma-Aldrich, catalog number: S-0876)
 19. Potassium phosphate monobasic (KH_2PO_4) anhydrous (Sigma-Aldrich, catalog number: P9791)
 20. 10x Phosphate Buffered Saline (PBS) (see Recipes)
 21. Stock solution for 0.2% $^{13}\text{C}_6$ -glucose (see Recipes)
 22. Stock solution for $^{13}\text{C}_5$, $^{15}\text{N}_2$ -Gln (10 ml) (see Recipes)

Equipment

1. Mouse restraining system (Plas-Labs, Inc., model: 541-RR)
2. FirstHand Surgical Instrument Kits for Mice and Rats (Kent Scientific Corporation, model: INSMOUSEKIT)
3. Blue wax dissection tray (VWR International, catalog number: 10060-188)
4. Warming blanket (Kent Scientific Corporation, model: DCT-15)
5. Water bottle with DI water (Thermo Fisher Scientific, catalog number: 02-897-11)
6. Polystyrene Weigh boats (USA Scientific Inc., catalog number: 2347-1427)
7. Calipers for measuring tumor size (Thermo Fisher Scientific, catalog number: 15-077-957)
8. 2-place Balance for weighing tissues (VWR International, Adventurer Ohaus[®], catalog number: 10153-744)
9. Refrigerated Microfuge (Eppendorf, model: 5417R with rotor)

10. Dewar/bucket (Nalgene plastic dewar) for IN_2 (Thermo Fisher Scientific, catalog number: [S34074C](#))
11. Large Styrofoam box

Procedure

All experiments must be performed under an IACUC approved protocol. For human tissue xenografts, the appropriate IRB approvals must also be met.

A. Schedule

1. Allow tumor in mice to develop to at least 0.5 cm and up to 1.5 cm according to specific protocols for ectopic or orthotopic xenografts of cells or fresh tissue (PDX model) as well as other tumor models (e.g., transgenic tumor model).
2. Preparations

The day before Stable Isotope Resolved Metabolomics (SIRM) experiment:

- a. Pre-label AI squares with date, mouse number and tissue (e.g., tumor), and blood collection tubes (2 per mouse for collection immediately after tracer injection, and at the time of necropsy) with date, mouse number, collection time.
- b. Label storage box for $-80\text{ }^{\circ}\text{C}$ freezer.
- c. Prepare tracer solutions e.g., 25% $^{13}\text{C}_6$ -glucose (1.344 M) or 36.2 mg/ml $^{13}\text{C}_5$, $^{15}\text{N}_2$ -Gln (0.2 M) in sterile PBS, sterile filter, and store in aliquots at $< -20\text{ }^{\circ}\text{C}$.
- d. Label formalin bottles with date, mouse number and tumor source.
- e. Assemble all components in sealable boxes for transport.
- f. Prepare schedule sheet (see example below, Table 1).

3. Tracer Injection

When mice are ready, restrain the mouse in the mouse restrainer, sterilize the injection area of the tail vein using a sterile wipe. Inject stable isotope tracers through the tail vein (or other convenient vein, such as submandibular vein) into individual immobilized mice*. Placing the mouse on a warming blanket or gently heating the tail visibly dilates the vein to make injection easier.

- a. Record time of injection.
- b. Immediately take blood sample (see step 5 below).
- c. For $^{13}\text{C}_6$ -glucose (can be purchased from Sigma Isotec or Cambridge Isotopes Laboratories, see above): Inject 80 μl (20 mg) each 25% (w/v) stock solution (in PBS and 0.2 μm sterile filtered) at 15 min intervals 3 times (total = 322 μmol).
- d. For $^{13}\text{C}_5$, $^{15}\text{N}_2$ -Gln: Same as above except for injecting 200 μl (7.2 mg) each 36.2 mg/ml stock solution (in PBS and 0.2 μm sterile filtered) at 15 min intervals 3 times (total = 142 μmol).
- e. The bolus injections take a few seconds. The extremely high heart rate of mice (500-600 beats per minute for resting adult mice) ensures that the tracer is systemically distributed very rapidly, therefore approximating a pulse.

*Note: *We have tried injection into ketamine-anesthetized mice. Since anesthesia slows metabolism, the timing has to be lengthened. Anesthesia can also alter metabolism. With the physical restraint method, it is important to minimize stress to the restrained mice during injection. Black mice are more difficult to inject via tail vein. The ability to do timed injections reproducibly takes considerable practice.*

4. Tissue harvest

- a. At 45 min after the first injection (15 min after the last injection), take photographs of the mouse and the tumor.
- b. Take blood sample before killing mice by cervical dislocation (do not use CO₂ asphyxiation or lethal injection of barbiturates as they interfere with metabolism)
- c. Measure tumor size using calipers (Figure 1).
- d. Dissect relevant tissues, e.g., tumor, lung, liver, heart, kidney, brain, skeletal muscle, adipose, lower bowel and flash freeze tissues immediately (within 5 min of necropsy) in liquid N₂, followed by placing frozen tissues in screw-cap 2 ml microfuge tubes pre-equilibrated in liquid N₂ or in aluminum foil (made into a boat shape) floating on liquid N₂, followed by wrapping**.

*Note: **Use non-erasable (solvent-resistant) permanent ink Sharpie pens to label sample tubes or aluminum foil to avoid label washout.*

- e. The order of dissection is dictated mainly by the major organ of interest, which should be removed first if possible. Dissected tissue is rinsed briefly in cold PBS, blotted and flash-frozen in liquid N₂ within 2-6 min of euthanasia.
- f. Tumor should be rinsed in cold PBS, blotted and weighed before flash freezing in IN₂ (Figure 1) and photographed.
- g. Typically at least three people are needed for this work.
- h. With multiple injections, it is practical to do three mice as a group, timing injections 5 min apart.

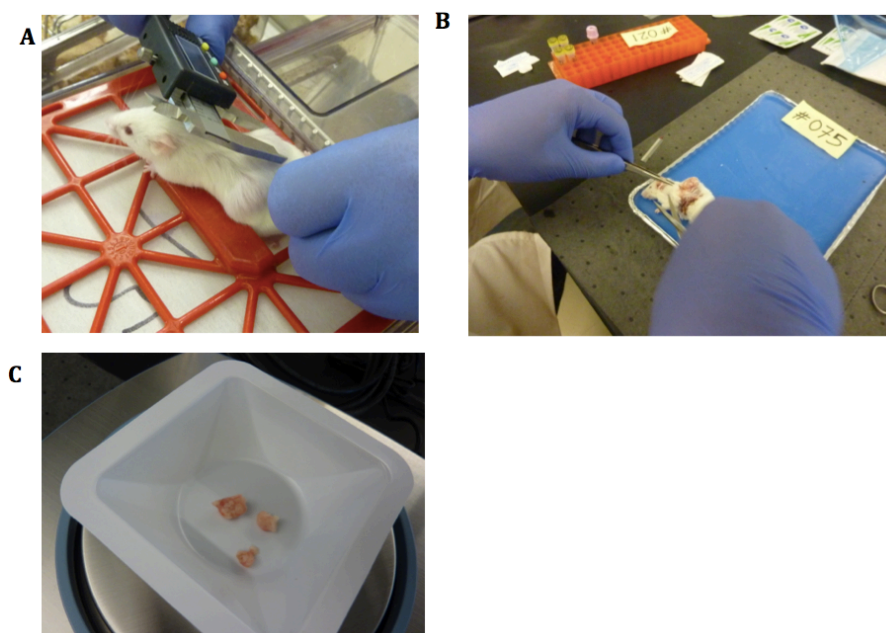


Figure 1. Mouse necropsy. A. NSG mouse with implanted tumors. Measuring size using electronic calipers; B. Dissection to reveal ectopic tumor. Blood tubes in the orange rack; C. Tumor pieces being weighed after blotting.

5. Blood sample processing

- Blood should be collected***immediately into K₂-EDTA microtubes ['purple top'] (designed for mice) after tracer injection (1st) and just prior to necropsy (2nd).
- Initial blood collection should be no more than 150 μl ; collect as much blood as possible for the 2nd collection.
- Let blood stand at room temperature for 5 min before storage on ice****.
- Centrifuge blood at 3,500 x g for 15 min at 4 °C.
- Separate plasma from blood cells and flash freeze the separated components in liquid N₂.

Notes:

- EDTA anticoagulant is preferred over citrate, heparin etc. as EDTA interferes least with metabolism
- ***We collect blood intraorbitally or via the submandibular vein using a lancet which is generally preferred (Golde et al., 2005); other methods may be applicable (e.g., from other vein) and at sacrifice, cardiac punch for maximal blood collection.
- ****It is important to keep blood at RT for 5 min to reduce hemolysis but store blood on ice thereafter until centrifugation.
- All samples are stored at -80 °C or colder.
- If necessary, samples for analysis should be shipped overnight on dry ice.
- Metabolites are extracted from tissues and blood according to established protocols, and then analyzed by high resolution NMR, GC-MS, and FT-ICR-MS to establish not only the content of metabolites, but also their labeled isotopomer and isotopologue

distributions, which represents metabolic transformation from the source tracer to the observed metabolites in the interval between injection and necropsy (Fan *et al.*, 2011; Fan, 2012a; Fan *et al.*, 2012; Lane *et al.*, 2011).

Representative data

Qualitatively similar ^{13}C metabolite profiles of individual organs have been obtained from these experiments at comparable time points, as estimated from 1-D $^1\text{H}\{^{13}\text{C}\}$ -HSQC spectra (e.g., Figure 2) (Fan *et al.*, 2011; Yuneva *et al.*, 2012; Sellers *et al.*, 2015; Xie *et al.*, 2014). The amounts of ^{13}C in each metabolite per mg can be calculated from the intensity of the lactate methyl resonance compared with its concentration independently determined by either ^1H NMR or GC-MS. These methods also provide the fractional enrichments of each observable metabolite, as described in Lane *et al.* (2008). Detailed descriptions of data reduction and analysis are provided in Fan *et al.* (2011) and Lane *et al.* (2008).

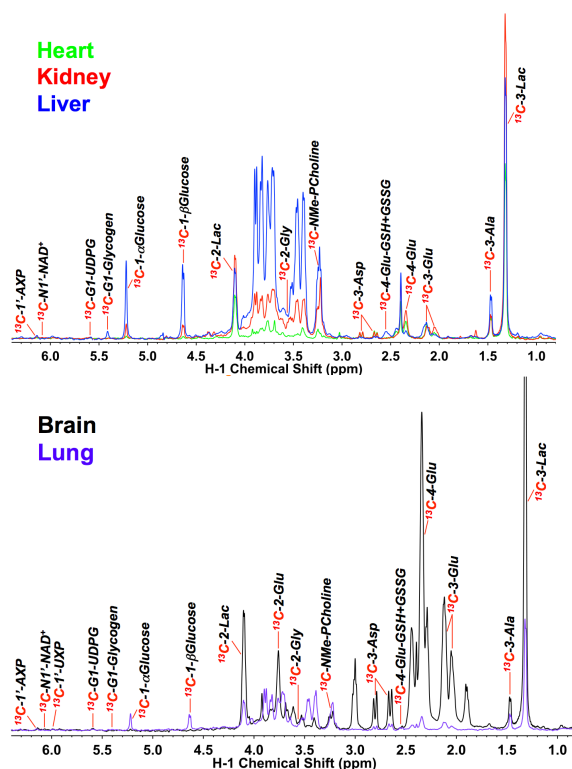


Figure 2. 1D $^1\text{H}\{^{13}\text{C}\}$ HSQC spectra of different organs of an NSG mouse infused with $^{13}\text{C}_6$ -glucose. The NSG mouse received three boluses of 20 mg each $^{13}\text{C}_6$ -glucose via tail vein injection at 15 min interval. Heart, kidney, liver, brain and lung tissues were dissected within 5 min of necropsy, flash-frozen in liq. N_2 , pulverized in liq. N_2 , and extracted with acetonitrile: H_2O : CHCl_3 (4:3:2 v/v) for polar metabolites as described in (10). The NMR spectra were normalized to the residual dry residual weight of the extract. Extracts are lyophilized prior to preparation of the NMR sample, as described (Fan, 2012b).

Table 1. Example record sheet

Title:

Date:

Experiment: Tumor from patient # (Date) implanted into 2 NSG mice (*name of researcher*).

2 mice, each piece subQ implanted on both flanks

mouse 1 F tumor sizes= ear tag #

mouse 2 M tumor sizes= ear tag #

SIRM/harvesting Date

Treatment: 3 x 80 μl 25% $^{13}\text{C}_6$ -glucose tail vein 15 min intervals (M1) or 3 x 200 μl , 36 mg/ml $^{13}\text{C}_5$, $^{15}\text{N}_2$ -Gln (M2).

Anesthetic Y/N.

Organs extracted sequentially:

Blood, tumor, lung, heart, liver, kidney, brain...

Time	Process	Time post injection / min	comments
M1	80 µl Glc	0	
	Blood		
	80 µl Glc		
	80 µl Glc		
	blood		
	sac		
	Tumor		Mass: g
	Lung		
	Heart		
	Liver		
	kidney		
	brain		
M2	200 µl Gln	0	
	Blood		
	200 µl Gln		
	200 µl Gln		
	blood		
	sac		
	Tumor		Mass: g
	Lung		
	Heart		
	Liver		
	kidney		
	brain		

Put sample into formalin for pathology. Place small samples into medium for further propagation in NSG mice and cell culturing.

Notes

1. The injections can be achieved with restraints, which is compromised by release of stress hormones. Alternatively the mice can be anesthetized with ketamine, which can slow general metabolism while they are unconscious and alter certain metabolic pathway(s) due to the non-intended effect of the anesthetic agent.

2. Tail vein injection of black mice can be challenging. Alternatives are intraperitoneal injection (which has a different time course), feeding or direct infusion via the jugular vein or carotid artery (de Graaf *et al.*, 2011) (<http://jaxmice.jax.org/preconditioned/surgical/pricing.html>).
3. For xenografts a variety of immune compromised strains are available, which can be used according to the study design (<http://jaxmice.jax.org/findmice/index.html>). Xenografts can be formed after injection of established cancer cells (Fan *et al.*, 2011; Lane *et al.*, 2011), primary cell lines, and fresh tumor tissue (PDX model) (Sellers *et al.*, 2015) either subcutaneously or orthotopically. The amounts tissue needed and the number of cells depends greatly on the source of the tumor or cell line characteristics.
4. Blood analysis is crucial to monitoring the amount of tracer delivered and the overall utilization over the period of the experiment. The transient nature of the experiments provides both information about pathways that are operative during the short labeling time period and the relative rates of uptake and utilization of different tissues. This can be refined by time course analysis of a group of mice harvested at different times post injection. Different numbers of injections also can be used to extend or decrease the degree of labeling in different groups of metabolites. Examples of blood metabolite time courses in mice injected with ^{13}C -glucose are in Fan *et al.* (2011).
5. With bolus injections isotopic steady state cannot be reached (de Graaf *et al.*, 2011; Maher *et al.*, 2012), and metabolites with slow turnover do not become significantly labeled. For labeling these metabolites, continuous infusion via an appropriate vein for a longer period (Marin-Valencia *et al.*, 2012) or longer-term tracer feeding can be employed.
6. Due to some variability of mice age, handling, and tumor status, it may not be practical to average metabolite values from different individuals. 5 or more mice/group are needed depending on the effect size. Preferably all the same gender should be used, and whenever possible littermates. With PDX models we usually match the mouse gender to that of the tumor donor.

Recipes

1. 10x PBS
80 g NaCl
2 g KCl
14.4 g Na_2HPO_4 anhydrous
2.4 g KH_2PO_4 anhydrous
Dissolve in 950 ml 18 MOhm water, pH to 7.4, make to 1 L, sterile filter (0.2 μm)

2. Stock solution for $^{13}\text{C}_6$ -glucose (10 ml)
2.5 g $^{13}\text{C}_6$ -glucose
1x PBS to a total of 10 ml
Sterile filter (0.2 mm) into sterile screw cap container
Stored at $-4\text{ }^{\circ}\text{C}$
3. Stock solution for $^{13}\text{C}_5$, $^{15}\text{N}_2$ -Gln (10 ml)
0.362 g $^{13}\text{C}_5$, $^{15}\text{N}_2$ -Gln
1x PBS to a total of 10 ml
Sterile filter (0.2 mm) 1 ml aliquots into sterile screw cap vials
Stored at $-80\text{ }^{\circ}\text{C}$

Note: Glutamine stock should be made fresh or stored at $-20\text{ }^{\circ}\text{C}$ in small aliquots to avoid repeated freezing and thawing. It forms pyroglutamate on storage in solution at neutral pH at higher temperatures.

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