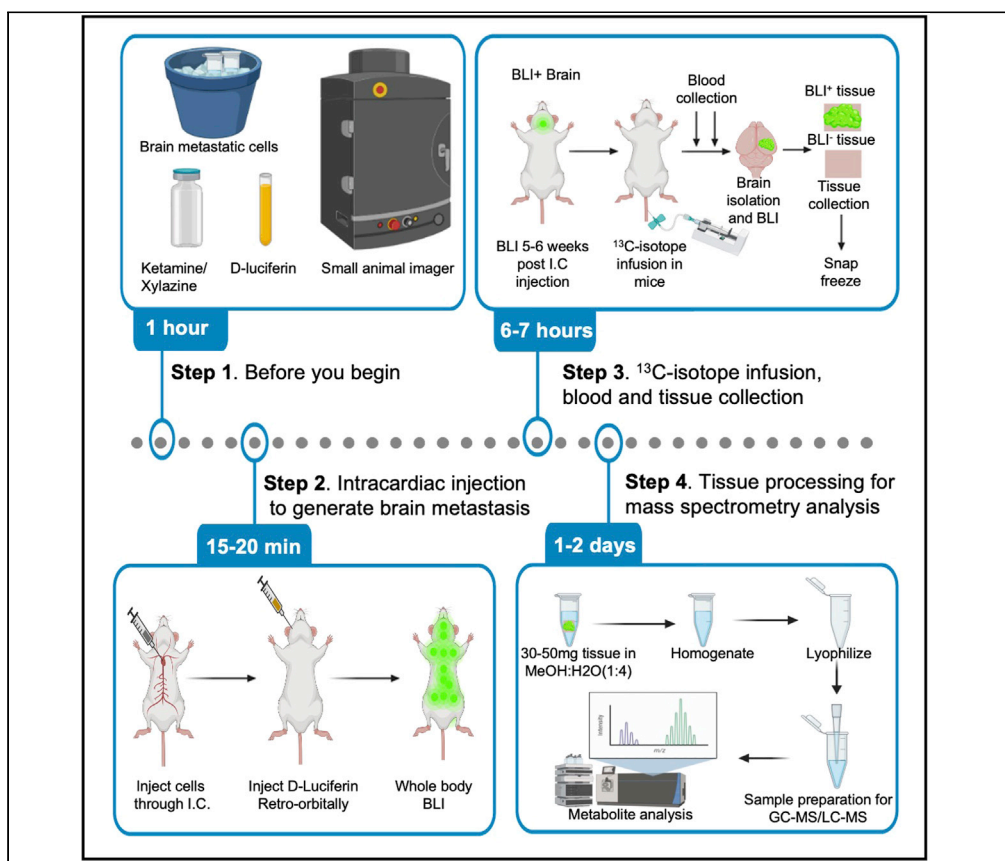## Protocol

Optimized protocol for stable isotope tracing and steady-state metabolomics in mouse HER2+ breast cancer brain metastasis

Pravat Kumar Parida, Mauricio Marquez-Palencia, Akash K. Kaushik, ..., Lauren Zacharias, Ralph DeBerardinis, Srinivas Malladi

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Highlights

Intracardiac injection of tumor cells to generate brain metastasis in mice

[13]C-glucose and glutamine infusion in mice with brain metastasis

Collect and process brain metastatic lesions for metabolite extraction

Mass spectrometry analysis, data processing, and interpretation

Analyzing the metabolic dependencies of tumor cells is vital for cancer diagnosis and treatment. Here, we describe a protocol for ^{13}C-stable glucose and glutamine isotope tracing in mice HER2+ breast cancer brain metastatic lesions. We describe how to inject cancer cells intracardially to generate brain metastatic lesions in mice. We then detail how to perform ^{13}C-stable isotope infusion in mice with established brain metastasis. Finally, we outline steps for sample collection, processing for metabolite extraction, and analyzing mass spectrometry data.

Protocol

Optimized protocol for stable isotope tracing and steady-state metabolomics in mouse HER2+ breast cancer brain metastasis

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SUMMARY

Analyzing the metabolic dependencies of tumor cells is vital for cancer diagnosis and treatment. Here, we describe a protocol for ^{13}C-stable glucose and glutamine isotope tracing in mice HER2+ breast cancer brain metastatic lesions. We describe how to inject cancer cells intracardially to generate brain metastatic lesions in mice. We then detail how to perform ^{13}C-stable isotope infusion in mice with established brain metastasis. Finally, we outline steps for sample collection, processing for metabolite extraction, and analyzing mass spectrometry data.
For complete details on the use and execution of this protocol, please refer to Parida et al. (2022).

BEFORE YOU BEGIN

During metastatic progression, tumor cells undergo metabolic adaptations to survive in circulation and distal organs (Faubert et al., 2020; Hanahan and Weinberg, 2011; Parida et al., 2022; Pavlova and Thompson, 2016). Understanding metabolic adaptations of metastatic cells in distal organs may provide insights that inform clinical management of disease and help in developing new diagnostic and therapeutic strategies.

Metastasis can be initiated in mice by injecting GFP-Luciferase labeled tumor cells intracardially and tracked by bioluminescent (BLI) imaging. We share an optimized protocol to examine steady state metabolomics, perform ^{13}C-stable glucose and glutamine isotope infusion and tracing in brain metastatic lesions. Here we use established HER2+ breast cancer brain metastatic models, however one can develop or use other established cancer metastatic models and perform similar analyses in brain or other metastatic lesions.

The protocol below describes how to prepare and inject cells to establish brain metastasis, perform ^{13}C-stable glucose and glutamine isotope infusion and tracing, collect and process samples for mass spectrometry analysis and analyze and interpret data.

Institutional permissions

All animal studies need to be done in accordance with Institutional Animal Care and Use Committee guidelines. Statement identifying the committee approving the experiments and confirming that all experiments conform to the relevant regulatory standards must be obtained. Approval from institutional IACUC committee must be acquired before you begin. 4–5 weeks old female Athymic mice (Hsd: Athymic nude mice-Foxn1^{nu}) or NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) housed in animal facility room maintained in a 12/12-h light/dark cycle at a temperature of 20°C–22°C with 30%–50% humidity and fed with standard chow.

Cell preparation

⏱ Timing: Approximately 30–40 min

1. Culture GFP-Luciferase positive human breast cancer brain metastatic cells in 10 cm dish up to 70%–80% confluency.

    Note: Don't grow cells to 100% confluency as it results in reduced viability and alters proliferation rate.

2. Detach cells by adding 1 mL trypsin-EDTA solution (0.25%) to the plate. Keep plates at 37°C for 2–3 min.
3. Stop trypsinization by adding 4 mL of 10% FBS containing ice-cold culture media.
4. Collect media with detached cells in 15 mL conical tubes.
5. Centrifuge tubes at 600 g for 3 min at 4°C and discard supernatant.
6. Resuspend cells in 5 mL ice-cold 1× PBS and centrifuge at 600 g for 3 min at 4°C.
7. Aspirate PBS and resuspend cells in 2–3 mL of ice-cold PBS. Count viable cells using trypan blue exclusion method and resuspend 0.5–1.0 × 10^6 viable cells in 1 mL ice-cold PBS and keep on ice. See troubleshooting 1.

    ⚠ CRITICAL: Resuspending cells in ice-cold PBS prevents cell clumping. Getting single cell suspension prior to injection is important, cell clumping could result in embolism and mouse death. Plan number of cells needed depending on the number of mice used for injections. For each mice inject 0.5–1.0 × 10^5 cells in 100 μL. Optimization of cell number might be needed depending on the aggressiveness of the metastatic models.

Preparation of biosafety hood and animal imager

⏱ Timing: Approximately 10–15 min

8. Clean biosafety hood, heat pad, small animal imager and nose cones with 70% ethanol.
9. Turn heat pad on and set temperature to 37°C.
10. Following aseptic practices, place Webcol (70% isopropyl prep pads), 1 mL pipette, 1 mL tips, one or 2 solid plastic platform, syringes with needle, cells on ice, terpene pen and cut pieces of paper tape into the hood.

KEY RESOURCES TABLE

REAGENTS or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
RPMI-1640	Sigma-Aldrich	Cat# R8758
Penicillin-Streptomycin	Sigma-Aldrich	Cat# P0781
L-Glutamine	Sigma-Aldrich	Cat# G7513

(Continued on next page)

Continued

REAGENTS or RESOURCE	SOURCE	IDENTIFIER
D- (+) Glucose	Sigma-Aldrich	Cat# G8270
Trypsin-EDTA solution (0.25%)	Sigma-Aldrich	Cat#T4049
Phosphate buffer saline (PBS)	Sigma-Aldrich	Cat# D8537
Sodium chloride	Sigma-Aldrich	Cat#S9888
Molecular biology grade water	Sigma-Aldrich	Cat#W4502
Ketamine	MilliporeSigma	Cat#1356009
Xylazine	MilliporeSigma	Cat#1720429
Isoflurane	Covetrus	Cat#029405
D-Luciferin potassium salt	Gold Biotechnology	Cat#LUCK-5G
Acetonitrile	Sigma-Aldrich	Cat# 1000291000
Formic acid	Sigma-Aldrich	Cat#00940
Anhydrous pyridine	Sigma-Aldrich	Cat# 270970
N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide	Sigma-Aldrich	Cat# 394882
Methanol, Optima™ LC/MS Grade	Thermo Fisher scientific	Cat#A456
Methoxyamine	Sigma-Aldrich	Cat#226904
Trypan blue 0.4% stain	Thermo Fisher scientific	Cat#T10282
D-Glucose ($^{13}C_6$)	Cambridge Isotope lab.	Cat# CLM-1396
Glutamine ($^{13}C_5$)	Cambridge Isotope lab.	Cat# CLM-184161-19
Experimental models: Organisms/strains		
Female Mouse: Hsd Athymic nude mice-Foxn1^{nu} (4–5 weeks)	Envigo	Cat#069
Female Mouse: NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (4–5 weeks)	The Jackson Laboratory	Cat#005557
Software and algorithms		
Aura	Spectral instrument imaging	https://spectralinvivo.com
Biorender	BioRender	https://biorender.com
Profinder software	Agilent Technologies	https://www.agilent.com
Maven	Maven	https://resources.elucidata.io/elmaven
Other		
Programmable Syringe Pump	Southpointe surgical supply	Cat#NE1800
Gas chromatography System	Agilent technologies	Cat#7890B
Small animal imager	Spectral Instrument imaging	Cat#AMI-HTX
iFunnel Q-TOF mass spectrometer	Agilent technologies	Cat#6550
Tissueruptor II	QIAGEN	Cat#9002755
Speed vac	Thermo Fisher scientific	Cat#SPD120
Tuberculin syringe	BD Biosciences	Cat#309625
Insulin syringe	BD Biosciences	Cat#329461
10 mL syringe	BD Biosciences	Cat#302995
20 mL syringe	BD Biosciences	Cat#302830
Scalp vein set 20G	Exelint international	Cat#26703
Scalp vein set 23G	Exelint international	Cat#26706
Webcol alcohol prep	Covidien	Cat#5110
EDTA tube	Thermo Fisher scientific	Cat#NC9414041
0.22-micron syringe filter	Thermo Fisher scientific	Cat#723-2520

MATERIALS AND EQUIPMENT

Ketamine (25 mg/mL)/Xylazine (2.5 mg/mL) cocktail		
Reagent	Final concentration	Amount
Ketamine 100 mg/mL in water	25 mg/mL	2.5 mL
Xylazine 10 mg/mL in water	2.5 mg/mL	2.5 mL
1.8% saline	0.9%	5 mL
Total	n/a	10 mL

Note: Approval from institute animal ethics committee is needed to prepare this solution. Do not store for more than 1 month at 20°C–22°C.

D-luciferin potassium salt solution (50 mM)
Dissolve 1 g of D-luciferin potassium salt in 62.81 mL of 1× PBS to get a final concentration of 50 mM.

Note: Make aliquots and store at −20°C. Can be used for months, avoid multiple freeze-thaws.

D-Glucose ($^{13}C_6$) solution (100 mg/mL)

Reagent	Final concentration	Amount
D-Glucose ($^{13}C_6$)	100 mg/mL	1 g
Molecular Biology grade water	n/a	5 mL
1.8% saline	0.9%	5 mL
Total	n/a	10 mL

Note: Prepare glucose solution using molecular biology grade water and 1.8% saline in 1:1 ratio. Filter it using 0.22-micron syringe filter. Make it fresh, do not store.

L-Glutamine ($^{13}C_5$) solution (35 mg/mL)

Reagent	Final concentration	Amount
L-Glutamine ($^{13}C_5$)	35 mg/mL	1 g
Mol. Bio. grade water	n/a	5 mL
1.8% saline	0.9%	5 mL
Total	n/a	10 mL

Note: Prepare L-Glutamine ($^{13}C_5$) solution using molecular biology grade water and 1.8% saline in 1:1 ratio. Filter it using 0.22-micron syringe filter. Make it fresh, do not store.

80:20 methanol: Water solution
Prepare 100 mL of 80:20 Methanol: Water by adding 20 mL water to 80 mL methanol.

Note: Solution can be stored for 1 month at −80°C.

STEP-BY-STEP METHOD DETAILS
### Intracardiac injections to generate brain metastasis in mice

⊙ Timing: Approximately 15–20 min per mouse

1. Anesthetize 5–6 weeks old female athymic Nude Foxn1[nu] or NSG mice by injecting intraperitoneally 50 μL of Ketamine (25 mg/mL)/Xylazine (2.5 mg/mL) cocktail. See troubleshooting 1.

   Note: In our experience, 50 μL of Ketamine/Xylazine cocktail is well tolerated by mice during intraperitoneal injection. If needed 100 μL of Ketamine/Xylazine cocktail can be administered to induce sleep and loss of toe pinch response.

2. For mice with fur, shave hair in the chest area using hair trimmer or remove hair using hair removal cream to identify injection point and obtain better bioluminescent images.

   Note: In our hands, compared to shaving, hair removal cream is more effective and faster.

Point of I.C. injection
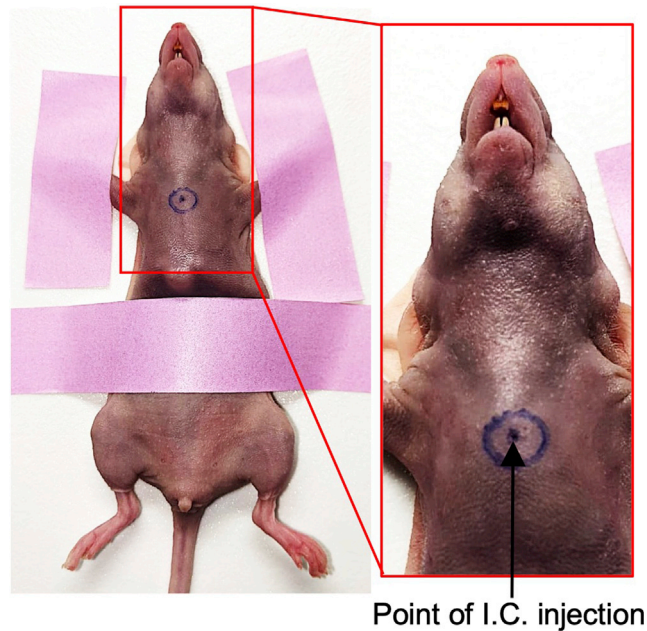
Figure 1. Image showing mice positioning and intracardiac injection site

3. Post anesthesia, put the mouse on its back with chest facing up on a flat surface and stretch fore-limbs and hind limbs and restrict any movement using paper tape (Figure 1).

   ⚠ CRITICAL: Be careful not to over stretch the animal as it could physically injure the animal.

4. Wipe the chest using Webcol and mark the injection spot targeting left ventricle (the front and middle of the chest, slightly left of the sternum between sternal notch and top of xiphoid process, (Figure 1).
5. Draw 100 μL of cells into 26G tuberculin syringe slip tip with intradermal bevel needle. Create an air space between plunger and meniscus to see cardiac pulse.

   Note: Mix cells in Eppendorf tube using 1 mL pipette before drawing cells into syringe. This process helps uniform distribution of cells and prevents clumping.

6. Hold the syringe upright and insert needle in the marked spot and check for the blood flow into the syringe. Press the plunge slowly and inject cells into the heart. See troubleshooting 2.

   Note: To avoid heart puncture, remove the needle in straight position.

7. Remove the tape and let the mouse recover on heat pad for 5 min.
8. Retro-orbitally inject 100 μL of 50 mM D-luciferin.
9. Check the distribution of cells by BLI imaging with exposure time of 30–60 s.

   Note: Uniform BLI signal should be detected throughout the mice body (Figure 2).

Weekly bioluminescence imaging (BLI) analysis to assess metastatic progression

   ⏱ Timing: Approximately 5–10 min per mouse

Measure metastatic incidence using BLI imaging every week or every two weeks.
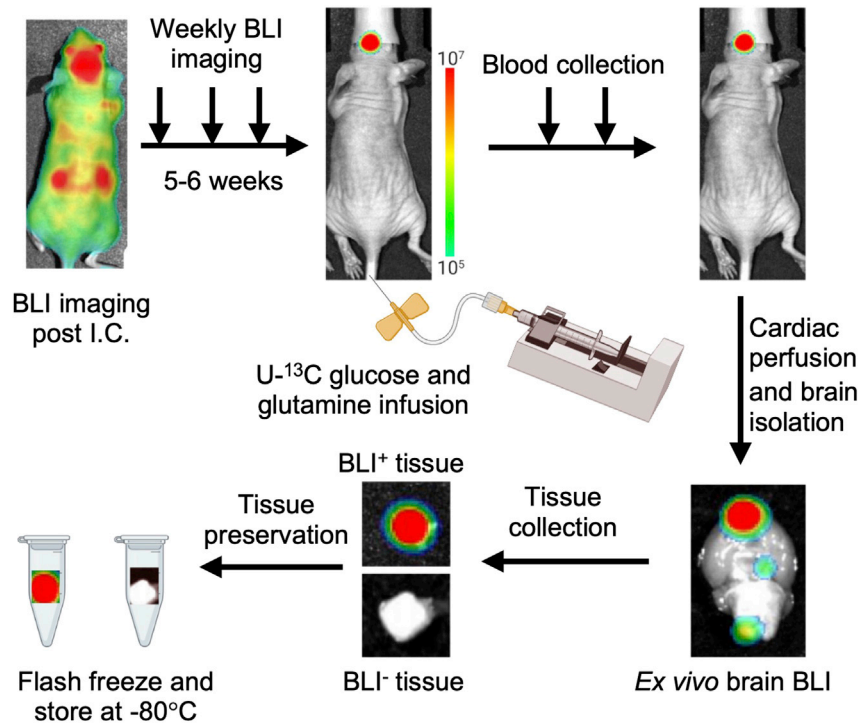
Figure 2. Schematic diagram illustrating steps to establish and collect brain metastatic lesions for metabolite analysis

10. Anesthetize animals using isoflurane in anesthesia induction chamber. Plan number of animals to anesthetize depending on the capacity of the imaging system and the anesthesia chamber.

    Note: Animals can be anesthetized using ketamine/xylazine solution as described earlier. However, frequent use of ketamine/xylazine can impact animal health.

11. Post anesthesia, inject 100 μL of D-luciferin through retro-orbital sinus.
12. Place mice nose into the nose cone of the imaging system platform.
13. Obtain BLI image with exposure time of 30–60 s. Look for signal in brain or organ of interest. If signal is saturated, reduce exposure time.

    Note: For tracking weekly metastatic progression using BLI signal, keep exposure time constant.

14. Determine whole body average photon flux and brain only photon flux using image analysis software. Mice are ready for isotope tracing once the average brain only or whole-body radiance is more than 10^6 p/sec/cm^2/sr.

    Note: Brain metastatic lesions are usually observed 5–6 weeks post-intracardiac injection.

Isotope infusion in mice with brain metastatic lesions

⏱ **Timing: Approximately 6–7 h per mouse**

15. Preparing mice for ^{13}C-glucose and glutamine infusion.

a. Fast mice 12–16 h for ^{13}C-glucose and ^{13}C-glutamine infusion. Fasting mice results in higher fractional enrichment of ^{13}C-glucose in plasma. However, ^{13}C-glutamine infusion can be done in mice without fasting.

Note: We observed no significant differences in fractional enrichment of glutamine in plasma upon fasting.

b. Anesthetize mice by injecting intraperitoneally 30 mg/mL of ketamine/xylazine (50 μL) and later with 20 mg/mL of ketamine hydrochloride (50 μL).
c. Place a 20-gauge catheter in the lateral tail vein of each anesthetized mouse.

16. $^{13}C_6$-glucose infusion in mice.
    a. For glucose tracing, infuse $^{13}C_6$-glucose intravenously as a bolus of 0.6 mg/g body mass over 1 min in 150 μL of saline using a programmable multi syringe holding pump system.
    b. Next, infuse bolus of 0.0138 mg/g body mass per minute for 3–4 h (in a volume of 150–200 μL/h) using same procedure (Figure 2). See troubleshooting 3.
17. $^{13}C_5$-glutamine infusion in mice.
    a. For glutamine tracing, infuse $^{13}C_5$-glutamine as a bolus of 0.2125 mg/g body mass over 1 min in 150 μL of saline.
    b. Next, infuse bolus of 0.004 mg/g body mass per minute for 5–6 h (in a volume of 150–200 μL/h) using a programmable multi syringe holding pump system. See troubleshooting 3.

Note: Monitor mice continuously throughout the infusion experiment (3 h for glucose infusion, and 5 h for glutamine infusion).

⚠ CRITICAL: Fasted mice should be hydrated enough and must be kept on warm pad throughout the time of experiment.

18. To determine fractional enrichment of ^{13}C-labeled metabolite in plasma over the course of infusion, collect blood every hour by retro-orbital puncture into an EDTA tube. Label and keep the tubes on ice.

⚠ CRITICAL: If mice appear weak, blood can be collected once every two hours.

Note: Sodium heparin or Sodium citrate tubes can be used for this process.

19. Post infusion, inject 100 μL of D-luciferin through retro-orbital sinus and obtain bioluminescent image to locate brain metastatic lesion.

Note: Prior to intracardial perfusion, D-luciferin injection is critical to detect brain metastatic lesion *ex vivo*.

20. Perform intracardial perfusion using 15–20 mL of 1× PBS in syringe connected to 23G butterfly needle.
21. Post perfusion, isolate the brain carefully by removing the skull and obtain *ex vivo* brain bioluminescent image using IVIS machine. Identify metastatic lesion using BLI signal (Figure 2).

Collect and process brain metastatic lesions for metabolite analysis

⏱ Timing: Approximately 1–2 h

Note: Metastatic lesions from several mice (n ≅ 5) need to be analyzed to increase robustness and account for variability.
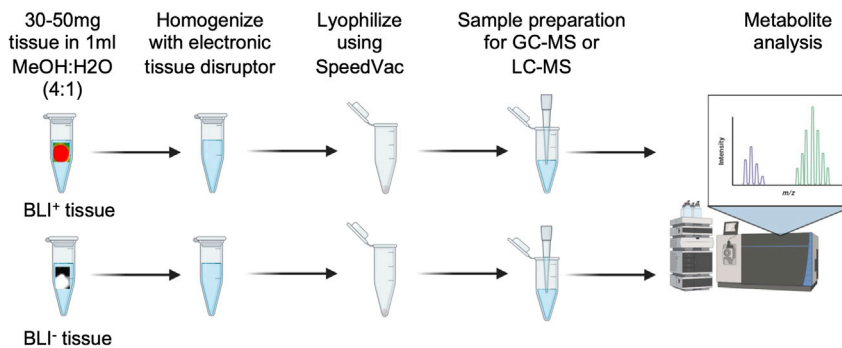
Figure 3. Schematic illustration showing metabolite extraction and analysis procedures

22. Based on the observed signal approximate the region of metastatic lesion in the brain. Resect BLI+ brain metastatic lesion using surgical blades and verify BLI signal in the resected metastatic lesion using IVIS imager. See troubleshooting 4.

⚠ CRITICAL: The isolated BLI positive tissue is likely to contain primarily tumor cells along with few brain resident cells. Minimize number of non-tumor cells in the collected tissue by precisely cutting only regions with high BLI signal. As controls pick BLI negative region within the brain and from the same region in mice with no brain metastasis.

23. Flash freeze collected tissues in liquid nitrogen and store at −80°C.
24. Homogenize 30–50 mg of metastatic lesion using an electronic tissue disruptor (Qiagen) in 1 mL chilled 80% methanol (LC-MS grade, Fisher Scientific). See troubleshooting 5.

   Note: A minimum of 15 mg of tissue is required for further processing.

25. Subject the homogenized solution three freeze-thaw cycles using liquid nitrogen and 37°C water bath.
26. Centrifuge samples at 13,000 g for 10 min to precipitate macromolecules.
27. Collect supernatant carefully and lyophilize using Speed-Vac (Figure 3).

Process blood samples for metabolite analysis

⏱ Timing: Approximately 1 h

28. Blood samples stored on ice were centrifuged at 2,500 g at 4°C for 10 min.

   Note: This is a time sensitive process, do not store blood samples on ice for more than an hour.

29. Collect blood plasma (top yellow layer) carefully and freeze at −80°C.
30. Add 10–15 μL of blood plasma to 80:20 methanol: water and subjected to evaporation using Speed-Vac.

***In vivo* steady-state metabolite profile in brain metastatic lesions**

⏱ Timing: Approximately 60–70 min

31. For *in vivo* steady state metabolite analysis, once the average brain only or whole-body photon flux reached 10^6–10^7 p/sec/cm^2/sr, mice were subjected to cardiac perfusion post D-luciferin injection and brain was isolated as described earlier.
32. Use *ex vivo* brain BLI signal to mark and surgically remove metastatic lesions.

Note: The isolated BLI positive tissue is likely to contain primarily tumor cells along with few non-tumor cells. Minimize number of non-tumor cells in the collected tissue by selecting BLI high signal regions. As controls for normalization pick BLI negative region within the brain and from the same region in mice with no brain metastasis.

33. Flash freeze tumors in liquid nitrogen and store at −80°C until further processing.
34. Sample preparation for Mass spectroscopy.
    a. Homogenize metastatic lesions (30–50 mg) using an electronic tissue disruptor (Qiagen) in 1 mL chilled 80% methanol (LC-MS grade, Fisher Scientific).
    b. Next, follow three freeze-thaw cycles by using liquid nitrogen and a 37°C water bath.
    c. Centrifuge samples at 13,000 g for 10 min at 4°C.
    d. Collect supernatant and lyophilize using a Speed-Vac.

Gas chromatography mass spectrometry (GC-MS)

⏲ Timing: Approximately 60–70 min

35. Resuspend lyophilized samples with 40–60 μL of anhydrous pyridine containing methoxyamine (10 mg/mL).
36. Vortex the solution and add it to a pre-ready GC-MS autoinjector vial and heat at 70°C for 10 min.
37. Next, add 80–120 μL N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) derivatization reagent to each vial, vortex to mix and heat vials at 70°C for 1 h.
38. Post incubation, inject 1 μL of sample into Agilent 7890 gas chromatograph coupled to mass selective detector Agilent 5975C. See troubleshooting 6.
39. Process the obtained raw data using maven software with in-house optimized database, and the natural abundance of ^{13}C correction. Export files as spreadsheet (.csv).

Liquid chromatography-mass spectrometry (LC-MS)

⏲ Timing: Approximately 60–70 min

40. Resuspend lyophilized samples in 100 μL 80:20 acetonitrile: water solution (HPLC grade). For steady state metabolite analysis, lyophilized samples can be resuspended in 100 μL of 0.1% formic acid in water and added to vials with pre-slit screw caps.
41. Perform data acquisition using reverse-phase chromatography on a 1290 UHPLC liquid chromatography (LC) system interfaced to a high-resolution mass spectrometry (HRMS) 6550 iFunnel Q-TOF mass spectrometer (MS) (Agilent Technologies, CA).
42. Operate MS in both positive and negative (ESI+ and ESI-) modes. Separate analytes using an Acquity UPLC® HSS T3 column (1.8 μm, 2.1 × 150 mm, Waters, MA).
43. Keep the column at 20°C–22°C. Mobile phase A composition is 0.1% formic acid in water and mobile phase B composition is 0.1% formic acid in 100% acetonitrile (ACN).
44. The LC gradient is 0 min: 1% B; 5 min: 5% B; 15 min: 99% B; 23 min: 99% B; 24 min: 1% B; 25 min: 1% B. The flow rate was 250 μL/min. Use 5 μL of sample for injection.
45. Set ESI source conditions as follows: dry gas temperature 225°C and flow 18 L/minute, fragmentor voltage 175 V, sheath gas temperature 350°C and flow 12 L/min, nozzle voltage 500 V, and capillary voltage +3,500 V in positive mode and −3,500 V in negative.
46. Set the instrument to acquire over the full m/z range of 40–1,700 in both modes, with the MS acquisition rate of 1 spectrum per sec in profile format.
47. Process raw data files (.d) using Profinder B.08.00 SP3 software (Agilent Technologies, CA) with an in-house database created using same analysis conditions and determined retention time and accurate mass information on 600 standards from Mass Spectrometry Metabolite Library (IROA Technologies, MA).

48. The in-house database matching parameters were mass tolerance 10 ppm; retention time tolerance 0.5 min. Peak integration result was manually curated in Profinder for improved consistency and exported as a spreadsheet (.csv).
49. Next, to analyze the obtained data two different normalizations are necessary.
    a. Normalization to natural abundance of the isotope ^{13}C (1.109%).
    b. Normalization to infused isotope e.g., glucose or glutamine in blood plasma.

EXPECTED OUTCOMES

Glucose and glutamine are the most prominent sources of bioenergetics and biosynthesis in cancer cells (DeBerardinis and Cheng, 2010). Glucose could be directed through glycolysis into TCA cycle, the pentose phosphate pathway, and several biomolecule synthesis pathways. (DeBerardinis et al., 2007; Mullen et al., 2011). Likewise, glutamine could undergo oxidative glutaminolysis or reductive decarboxylation and helps in synthesis of key cellular metabolites.

Through a phenotypic screen in mice, we isolated isogenic HER2+ synchronous (S-BM) and metachronous (M-BM) brain metastatic cells. By investigating these phenotypically distinct brain-tropic S-BM and M-BM cells, we uncovered the metabolic differences within these metastatic population using isotope tracing and steady state metabolite analysis (Parida et al., 2022). Isotope tracing revealed key differences in glucose and glutamine catabolism between S-BM and M-BM brain metastases. In metastatic lesions of similar size and comparable BLI signal intensity, no significant differences in glucose catabolism was observed between S-BM and M-BM (Figure 4A). However, a marked increase in labeling of the TCA intermediates succinate, malate, aspartate and citrate (M+4) from glutamine was observed in M-BM metastatic lesions compared to S-BM (Figure 4B). Moreover, a significant increase in M+5/M+4 citrate ratio suggests reductive glutamine catabolism was predominant in S-BM lesions compared to M-BM lesions where oxidative carboxylation was predominant (Figure 4C).

By comparing isotope tracing data along with steady-state metabolite profile one can start defining key biosynthetic pathways within metastatic tumor. Moreover, similar approach can be utilized to understand metabolic differences between tumor cells at the primary and metastatic sites. To further validate these findings, assessing expression of metabolic enzymes responsible of these altered nutrient utilization patterns and inhibiting their function either pharmacologically or by genetic modulation is needed.

These studies are likely to highlight differences between the glucose and glutamine utilization patterns in metastasis from tumor cells with distinct oncogenomic status or phenotypic traits. This protocol can be applied to other metastatic mouse models and adapted to optimize isotope labeled tracing of other energy substrates such as lactate, pyruvate, acetate and palmitate (Bos et al., 2010; Nair and Malladi, 2019). Defining metabolic state and flux within metastatic lesions associated with genomic and phenotypic differences are likely to provide novel insights that can be further exploited to diagnose and treat metastasis (Dupuy et al., 2015; Malladi et al., 2016; Parida et al., 2022).

LIMITATIONS

^{13}C-tracing experiments described here demonstrate carbon enrichment into pathway of interest but do not provide detailed information on overall metabolic flux.

Despite high BLI signal, obtaining enough metastatic lesion (15 mg) for analysis can be a challenge. Likewise, vascularity of metastatic lesion presents a challenge, poor vascularity, less oxygen supply to tumor tissue can results in poor infusion and detection.

Identifying and defining metabolic state of micro-metastasis or few disseminated tumor cells remains a challenge at this time.
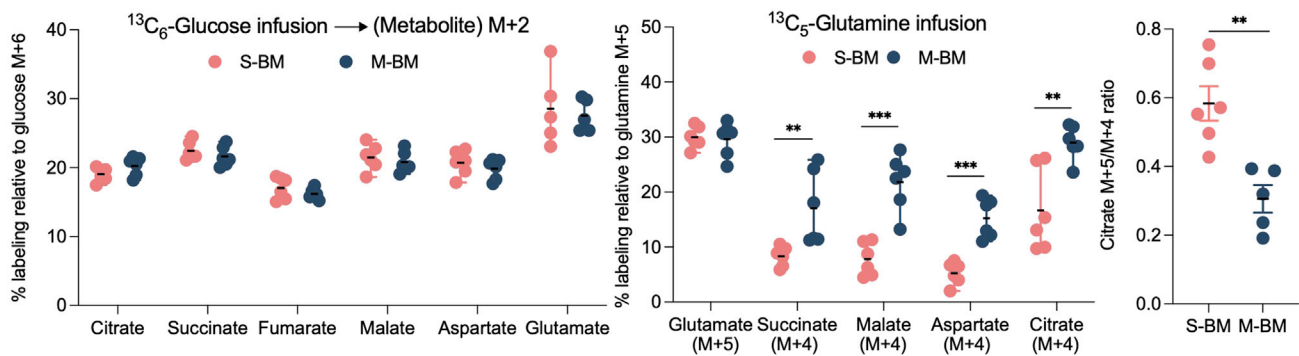
Figure 4. In vivo $^{13}C_6$-glucose and $^{13}C_5$-glutamine tracing in S-BM and M-BM metastatic lesions

(A) $^{13}C_6$-glucose tracing in brain metastatic lesions reveals enrichment of indicated metabolites.

(B) Total labeling of indicated metabolites in brain metastatic lesions analyzed by mass spectrometry and normalized to enriched $^{13}C_5$-glutamine.

(C) M+5/M+4 citrate ratios highlighting reductive carboxylation of glutamine in S-BM and oxidative carboxylation in M-BM.

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Loss of labile compounds during metabolite extraction and sample preparation for mass spectrometry analysis is a limitation.

TROUBLESHOOTING

Problem 1

Mice death post intracardiac injection. (before you begin step 7 and step-by-step method details step 1).

Potential solution

- Cell clumping during injection can cause embolism in mice, which can lead to animal mortality. Resuspending single cell suspension in ice-cold PBS and storing on ice prevents cell clumping.
- High dose of Ketamine/Xylazine can cause cardiovascular and respiratory depression, and anomaly in heartbeat patterns, which can lead to animal mortality. Thus, optimization of Ketamine/Xylazine cocktail is required.
- In our hands, 50 μL of low dose mouse Ketamine (25 mg/mL)/ Xylazine (2.5 mg/mL) cocktail produces minimal mortality in athymic and NSG mice. Mice can tolerate up to 100 μL of this combination.

Problem 2

Luciferase signal in lungs - Failure of intracardiac injection. (step-by-step method details step 6).

Potential solution

- One can use ultrasound as a guide to perform intracardiac injection.
- Don't push the needle deep into the chest area that it may pierce through the heart.

Problem 3

Low enrichment of tracer in blood plasma and tumor tissue. (step-by-step method details steps 16 and 17).

Potential solution

Increase the concentration of the metabolite by 5%–10% and/or infusion time.

Problem 4

No luciferase signal in resected metastatic lesion. (step-by-step method details step 22).

Potential solution

This might be due imprecise selection for metastatic lesion or loss of luciferase signal.

- Image resected region along with the remaining brain to confirm isolation of BLI positive metastatic lesion.
- Luciferase signal remains stable for approximately 20–25 min. So, one should aim to perform perfusion, brain isolation, BLI imaging, metastatic lesion collection and conformation within 20–25 min.

Problem 5

Not enough metastatic lesion for downstream analysis. (step-by-step method details step 24).

Potential solution

Metastatic lesions with average radiance of 10^6 p/sec/cm^2/sr should provide enough material for mass spectrometry analysis. If not, consider combining multiple lesions from an animal.

Problem 6

Poor quality GC-MS data and hard to interpret. (step-by-step method details step 38).

Potential solution

- If the GC-MS data peaks are too small to confidently interpret, inject more sample (3 μL, instead of 1 μL) into the GC-MS.
- If the GC-MS data has a large contaminating peak, it is likely to be from phosphates. It is important to never wash the tumor tissue with PBS.

RESOURCE AVAILABILITY

Lead contact

Further information and request for resources and reagents should be directed to the lead contact, Srinivas Malladi (srinivas.malladi@utsouthwestern.edu).

Materials availability

All materials are available upon reasonable request.

Data and code availability

This study did not generate or analyze any data sets.

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AUTHOR CONTRIBUTIONS

Experimental design and conceptualization, P.K.P. and S.M.; methodology, P.K.P., M.M.P., A.K.K., K.K., V.N., J.S., H.V., L.J., and S.M.; writing – original draft, P.K.P. and S.M.; writing – review & editing, P.K.P., R.J.D., and S.M.; funding acquisition, S.M.; supervision, S.M.

DECLARATION OF INTERESTS

R.J.D. is a founder of Atavistik Biosciences and an advisor for Agios Pharmaceuticals, Nirogy Therapeutics, and Vida Ventures.

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