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# Stable Isotope Resolved Metabolomics Studies *in Ex Vivo* TIssue Slices

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**[Abstract]** An important component of this methodology is to assess the role of the tumor microenvironment on tumor growth and survival. To tackle this problem, we have adapted the original approach of Warburg [1], by combining thin tissue slices with Stable Isotope Resolved Metabolomics (SIRM) to determine detailed metabolic activity of human tissues. SIRM enables the tracing of metabolic transformations of source molecules such as glucose or glutamine over defined time periods, and is a requirement for detailed pathway tracing and flux analysis. In our approach, we maintain freshly resected tissue slices (both cancerous and non-cancerous from the same organ of the same subject) in cell culture media, and treat with appropriate stable isotope-enriched nutrients, *e.g.* $^{13}C_6$-glucose or $^{13}C_5$, $^{15}N_2$-glutamine. These slices are viable for at least 24 h, and make it possible to eliminate systemic influence on the target tissue metabolism while maintaining the original 3D cellular architecture. It is therefore an excellent pre-clinical platform for assessing the effect of therapeutic agents on target tissue metabolism and their therapeutic efficacy on individual patients [2,3].

## Materials and Reagents

1. Dialyzed, sterile filtered fetal bovine serum (FBS) (free of serum metabolites) 10-12 kDa (Atlanta Biochemical, catalog number: S12650)
2. Tracer examples: $^{13}C_6$-glucose, $^{13}C_2$-1, 2-glucose, $^{13}C_5$,$^{15}N_2$-glutamine
3. Sources: $^{13}C_6$-glucose/D-glucose ([U-$^{13}$C], 99%) (Cambridge Isotope Laboratories, catalog number: CLM-1396-CTM), $^{13}C_2$-1, 2 glucose/D-glucose (1, 2-$^{13}C_2$, 99%) (Cambridge Isotope Laboratories, catalog number: CLM-504), $^{13}C_5$, $^{15}N_2$-glutamine/ L-glutamine ($^{13}C_5$, 99%; $^{15}N_2$, 99%) (Cambridge Isotope Laboratories, catalog number: CNLM-1275) OR
   Isotec: D-$^{13}C_6$-glucose (Sigma-Aldrich, catalog number: 660663); $^{13}C_2$-1, 2 glucose (Sigma-Aldrich, catalog number: 661422); L-Glutamine-$^{13}C_5$, $^{15}N_2$ (Sigma-Aldrich, catalog number: 607983)
4. Penicillin + Streptomycin: GE Healthcare PEN/STREP/FUNGIZONE 100 ml (Thermo Fisher Scientific, Fisher Scientific, catalog number: SV3007901)
5. Protocol™ 10% Neutral buffered formalin (Thermo Fisher Scientific, Fisher Scientific, catalog number: 032-059)

6. 25% (w/v) sterile filtered $^{13}C$ glucose (0.2 µm) in PBS (Stock solution-can be frozen, aliquoted, and stored at 4 °C)
7. Liquid nitrogen
8. 70% ethanol (v/v)
9. 60% acetonitrile in water (v/v) (Sigma Aldrich L010400 )
10. Sodium Chloride (NaCl) (Thermo Fisher Scientific, Fisher Scientific, catalog number: S271-1)
11. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9541)
12. Sodium phosphate dibasic ($Na_2HPO_4$) (Sigma-Aldrich, catalog number: S0876)
13. Potassium phosphate monobasic ($KH_2PO_4$) (Sigma-Aldrich, catalog number: P9791)
14. Relevant medium (*e.g.* DMEM, RPMI, other defined medium) which lacks the tracer of interest:
    a. Dulbecco's Modified Eagle's Medium (DMEM) is a powder formula, free of glucose, glutamine, pyruvate bicarbonate, and phenol red, giving considerable flexibility in formulation for SIRM studies (Sigma-Aldrich, catalog number: D5030) (see Recipes)
    b. RPMI 1640 is a liquid medium free of glucose and glutamine, but contains bicarbonate and phenol red (MP Biomedicals, catalog number: 091646854) (see Recipes)
15. 0.2 µm sterile filtered Phosphate Buffered Saline (PBS) (see Recipes)
16. Medium composition for 0.2% $^{13}C_6$-glucose, 2 mM $^{12}C$-Gln (100 ml) (see Recipes)
17. Medium composition for 0.2% $^{12}C$ glucose, 2 mM $^{13}C_5$, $^{15}N_2$-Gln (100 ml) (see Recipes)

**Equipment**

1. Class II Biosafety Hood
2. Trigas incubator with oxygen sensor and $CO_2$ sensor (Thermo Fisher Scientific, model: Hera cell 150i)
3. Sterilized rocker (Rotoshake Genie) (Scientific Industries, model: SI-1100)
4. Liquid nitrogen freezer for storage
5. $K_2$-EDTA vacutainers ("purple top") (BD, catalog number: 366643)
6. Refrigerated centrifuge with swing out rotor that can accept vacutainers (*e.g.* Thermo Fisher Scientific, model: Sorvall Legend X1R and Thermo Fisher Scientific, catalog number: 75-004-261; rotor: 75003181)
7. Pipettors (variable size ranges) (USA Scientific ErgoOne)
8. Weck Knife/Dermatome (George Tiemann & Co., catalog number: 222-5-523)
9. Weigh boats (Thermo Fisher Scientific, Fisher Scientific, catalog number: 08732113 and 08732115)
10. 4-place balance (Mettler-Toledo, Thermo Fisher catalog number: 0133525)
11. Ice bucket (Thermo Fisher Scientific, Fisher Scientific, catalog number: 02-591-44)

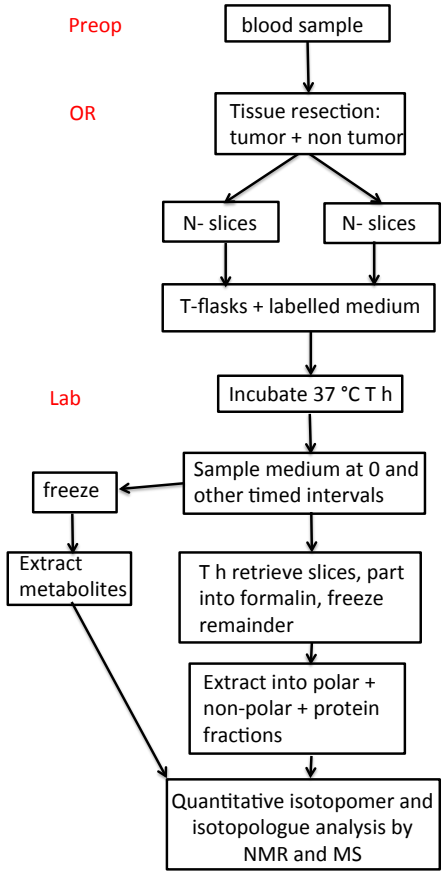
12. Sharp dissecting scissors (Thermo Fisher Scientific, Fisher Scientific, catalog number: 08940)
13. Excelta™ Plastic Tweezers (Thermo Fisher Scientific, Fisher Scientific, catalog number: 17-456-066)
14. Digital camera
15. 25 ml T Flasks NC vent cap (SARSTEDT AG & Co, catalog number: 83.1810.002)
16. Portable container for liquid nitrogen (Nalgene plastic dewar) (Thermo Fisher Scientific, Fisher Scientific, model: S34074C)
17. Sterile syringes and needles (Thermo Fisher Scientific, Fisher Scientific, catalog number: 10142534)
18. Disposable transfer pipets (Samco fine tip, 1 ml) (VWR International, catalog number: 16001192)
19. Aerosol barrier tips for 1 ml and 1-200 µl (Thermo Fisher Scientific, Fisher Scientific, catalog number: 02-707-42)
20. Screw cap plastic vials (2 ml) color coded caps (yellow, blue, green and red) (USA Scientific, catalog number: 1420-8706, 1420-8701, 1420-8702 and 1420-9704)
21. Snap top plastic vials (1.5 ml) (USA Scientific, catalog number: 1615-5510)
22. 15 ml Falcon tubes (SARSTEDT AG & Co, catalog number: 62.554.205)

**Procedure**

A. Tissue procurement

All tissue must be procured under an IRB approved protocol. As live human tissue is handled, all personnel must undergo and maintain biosafety, HIPAA and CITI certifications.

An overview of the whole process is given in Scheme 1.




**Scheme 1: FlowChart**

Preop: preoperative room

OR: Operating Room.

The number of thin slices to be taken depends on the size of the tumor. A piece of tissue is also flash frozen in the OR, and additional tissue is placed in formalin for pathological analysis

1. Blood samples provide overall information about the metabolic status of the individual subjects, and the buffy coat can be used for extracting DNA or RNA for sequence analysis.
2. A 10 ml sample of blood should be drawn preoperatively into a purple top vacutainer ($K_2$-EDTA) preoperatively. Other anticoagulants such as citrate or heparin should not be used as they interfere with metabolic assays. A blood sample should also be drawn perioperatively after resection. The blood is inverted twice to ensure dissolution of the EDTA, and kept on ice immediately after blood draw. The blood should be separated into packed red cells, buffy coat and plasma within 30 min by centrifuging at 3,500 g for 15 min at 4 °C in a swing out rotor.
   Subsequent operations should be carried out in a BSL2+ biosafety cabinet.
   *Note: We use the following color codes for storage: Red = whole blood, yellow = plasma, green = buffy coat, blue = urine.*
3. Plasma is aspirated into prechilled sterile 2 ml screw cap vials at 1 ml aliquots and flash frozen in liquid $N_2$. Buffy coat is aspirated using a wide mouth plastic pipette into a 2 ml screwcap vial and flash frozen in liquid $N_2$.
4. These experiments have been carried out on fresh slices of paired cancerous (CA) and non-cancerous (NC) lung tissues resected from non-small cell lung cancer [2] and pancreatic cancer patients. Upon resection, thin slices (0.5-1 mm thick) of tissue are excised from the surface of visually non-necrotic or fibrotic tumor regions using a Weck microtome in the Operating Room (OR), within approximately 5-10 min of resection. Roughly 1 $cm^2$ tissue is targeted. (See Figure 1 A). Control non-cancerous tissue from a distant (>10 cm) region is obtained similarly. A pathologist on-site inspects the CA and NC tissue specimens. Highly necrotic tissue is discarded.
5. At the same time, a small piece of bulk CA tissue should be placed in DMEM or other appropriate medium kept room temperature for implantation into a recipient NSG mouse as patient-derived xenograft or PDX. A small piece each of CA and NC tissues is soaked in formalin for pathological examination or flash frozen in liquid $N_2$ for image-based metabolic analysis.
6. Where tissue acquisition in the OR is impractical (such as colorectal or breast cancer resections), the slices can be prepared in the pathology laboratory located close to the OR. For comparison with freshly resected tissue, speed is essential as metabolism is rapidly changing. Whenever feasible, tissue freezing should be performed in the OR.
7. The slices are placed into a drop of sterile PBS on two sterilized weigh boats to prevent sticking and for spreading slices evenly. Each weight boat is pre-numbered for CA or NC slices. The tissues on weight boats are then photographed. Each slice is rinsed briefly with sterile PBS, blotted (twice) and then carefully placed into pre-numbered (using ethanol-resistant marker pen) pre-tared (tare weight recorded) T25-flasks containing 10 ml DMEM (or other relevant medium) with the appropriate tracer (*e.g.* 10 mM $^{13}C_6$-glucose or 2 mM $^{13}C_5$, $^{15}N_2$-glutamine), 10% dialyzed FBS (as

needed), and 1x penicillin + streptomycin. The flasks with slices are brought to the culture room as soon as possible and sprayed with 70% ethanol, and wiped dry before placing them in the Biosafety hood.

8. Pipet 200 µl culture media from each flask into 1.5 ml snap-cap tubes ($t_0$-time zero media samples). Centrifuge for 10 min at 10,000 *x g* at 4 °C to remove tissue debris. Transfer 100 µl to tared 1.5 µl snap-cap tube for metabolite extraction and weigh the media transferred. Transfer the remaining media to a 1.5 ml screw-cap tube for long-term storage at -80 °C.
9. Weigh flasks in a 2-place balance inside the Biosafety hood and record weight on the flask.
10. Transfer flasks to a $CO_2$ incubator containing a rocker set to low amplitude rocking (sufficient to ensure that the liquid moves over the slices without non-laminar flow) Figure 1B) set to 37 °C and 5 % $CO_2$, with oxygen set to the desired level (*e.g.* 20%, 1%).
11. The flasks are continuously and gently rocked for 24 to 48 h for gas exchange and to maintain constant nutrient supplies at the tissue surface, while avoiding local buildup of waste products such as acids.
12. As needed, the medium can be refreshed every 12 h, and sampled at 0, 6, 12, 24…. h for analysis of nutrient uptake and waste production. The flasks are weighed before and after each medium sampling point and flask weights are recorded on the flasks.

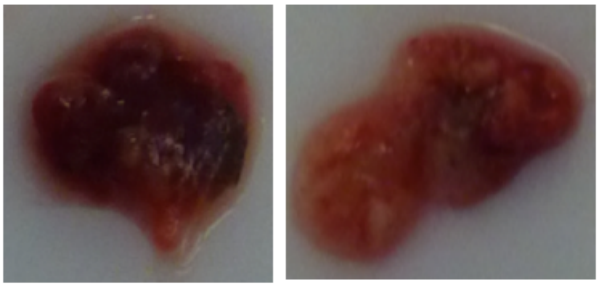
B. Tissue Harvesting

1. After 24 h incubation, weigh flasks.
2. Place flasks on ice immediately after removing from the incubator to minimize further metabolism. Up to 6 flasks can be harvested at a time. Keep tissue slices on ice as much as possible during harvest.
3. Using a transfer pipet, aspirate and transfer the conditioned media into 15-ml conical centrifuge tubes.
4. Centrifuge media for 15 min at 3,500 to 4,690 *x g*, 4 °C to remove any particulates and debris.
5. Pipet 100 µl $T_{24}$ media supernatant into 1.5-ml snap-cap tubes for metabolite extraction.
6. Pipet 1 ml media aliquot into 2-ml screw-cap tubes for long-term storage at -80 °C.
7. The remaining medium is stored separately in a 7 ml vial at -80 °C for purposes such as exosome isolation.
8. Invert and tap the flask to move the tissue slices into the cap or neck region of the flask for retrieval. Keep flask inverted on ice.
9. Wash tissue slices 3x consecutively in ice-cold 10 ml cold PBS each in a 50 ml beaker.

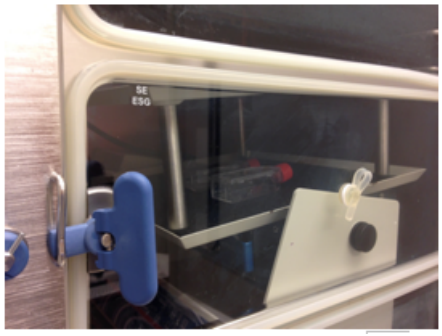
10. Blot dry the tissue slices on Kimwipe and photograph the flattened slice on a small weigh boat.
11. Weigh the whole tissue slice on small weigh boats and record the weight.
12. Split a very small piece for preservation in 1 ml buffered formalin in a 1.5 ml snap-cap tube for histology. The remaining tissue slice is split evenly and each piece should weigh no more than 20-30 mg by wet weight to facilitate tissue homogenization and extraction efficiency. Immediately after weighing, each piece is flash-frozen in liq. $N_2$ and placed in a pre-liq. $N_2$ chilled 1.5-ml snap-cap tube for long-term storage at -80 °C.
13. After 6-8 h in formalin, replace the formalin with 70% ethanol for the tissue pieces prepared for histology.
14. Homogenize tissues in cold 60% acetonitrile (v/v) and extract tissue homogenates for metabolite analyses according to standardized protocols [4,5] before analyses using stable isotope-resolving analytical techniques (*e.g.* NMR and MS) [4] (cf. Figure 1C) [6].
15. This Protocol describes the procedure for stable isotope labeling of thin tissue slices. SIRM analysis involves the quantification of isotopomers (by NMR) and isotopologues (MS) that result from metabolic transformations of source molecules (e.g. $^{13}C$ glucose or $^{13}C$,$^{15}N$ Glutamine) in cells or tissue (cf Figure 1C). The techniques of SIRM analysis by NMR and mass spectrometry are described in detail in [3,6,7].

**Representative data**

**A**



**B**



**C** ***Ex vivo* tissue slices**

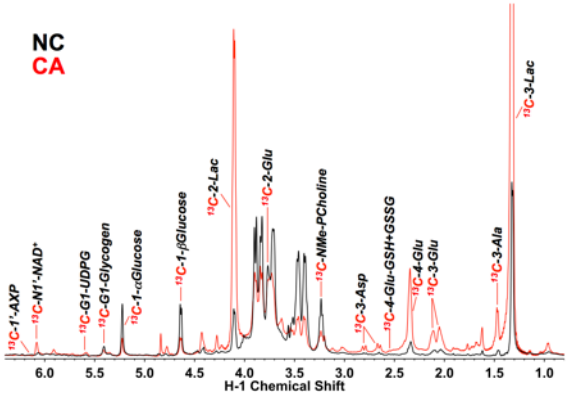


**Figure 1 Example *ex vivo* tissue slice experiment.** A. Example thin slices of non-cancerous lung tissue (NC, left) adjacent to a lung adenocarcinoma (CA, right); B. T25-flasks on a rocker inside a $CO_2$ incubator; C. Representative 1D $^{1}H\{^{13}C\}$ HSQC NMR spectra (recorded at 14.1 T, 15°C) of extracts of CA versus NC lung slices from an non small cell lung cancer (NSCLC) patient incubated for 24 h in the presence of 10 mM $^{13}C_6$-glucose. The tissue slices were pulverized and extracted as described [3,4] which produces three phases- an upper aqueous phase containing polar metabolites, a lower organic phase containing non-polar metabolites (mainly lipids) and an interfacial phase that contains protein. Here the upper phase was lyophilized and redissolved in a phosphate buffer containing 50% $D_2O$ and 25 nmol DSS-$d_6$ that serves both as a chemical shift reference and a concentration standard [8]. The HSQC spectrum detects protons attached directly to $^{13}C$, and thus gives a readout of the metabolites that have incorporated $^{13}C$ from the source molecule (glucose in this instance). The spectra of cancer and non-cancerous tissues are recorded under identical conditions, and the absolute intensities are normalized to the tissue protein weight. Peak areas were determined using peak fitting functions in MNOVA (Mestrelab Research, Santiago de Compostela, Spain) Enhanced production of various $^{13}C$ labeled metabolites in the CA tissue slice is evident, including $^{13}C$-lactate (Lac), which is consistent with the Warburg effect or accelerated glycolysis in tumor tissues [9].

**Notes**

1. These procedures have been tested on freshly resected NSCLC and pancreatic cancer, as well as in patient derived mouse xenografts. Other tissues may need experimentation with the composition of the medium and length of incubation period for metabolic viability.
2. Larger or inflammatory tumors may have substantial areas of necrosis or fibrosis that need to be avoided.
3. A Weck microtome is hand held, but with practice the surgeons can reproducibly produce slices < 1 mm thick. Slice thickness is easily estimated by measuring the area of the slice from a photograph and from the wet weigh as the average thickness h = weight/area.
4. Some tumors are highly mucilaginous and are more difficult to slice reproducibly.
5. An alternative to a Weck microtome is a vibrating microtome, which can reproducibly generate thinner slices from firm but not soft tissues (cf [10]), but is much slower. Very thin slices (100-200 µm) may show a proportionately larger wounding response and are more fragile.
6. As many tumors are heterogeneous not only in the cancer/stromal content in different regions of the tumor but also in terms of genetics, it is advisable to obtain multiple

slices from the tumor to cover this heterogeneity. This also makes histopathological examination of each slice critically important.

7. The margins of some tumors are not obvious without pathological examination. Tissue proximal to the tumor as well as distal from the tumor should be sampled for comparison.

**Recipes**

1. RPMI Medium 1640 and DMEM

   For SIRM studies, the glutamine or glucose free version of the medium should be used, with supplementation of the appropriate concentration of $^{13}$C-enriched precursors in the base medium.

| COMPONENTS | Molecular Weight | Concentration (mg/L) | Molarity (mM) RPMI | Molarity (mM) DMEM |
|---|---|---|---|---|
| **Amino Acids** | | | | |
| Glycine | 75 | 10 | 0.133 | 0.40 |
| L-Arginine | 174 | 200 | 1.15 | 0.483 |
| L-Asparagine | 132 | 50 | 0.379 | - |
| L-Aspartic acid | 133 | 20 | 0.150 | - |
| L-Cystine 2HCl | 313 | 65 | 0.208 | 0/0.2 |
| L-Glutamic Acid | 147 | 20 | 0.136 | - |
| L-Glutamine | 146 | 300 | 2.05 | 2 |
| L-Histidine | 155 | 15 | 0.0968 | 0.27 |
| L-Hydroxyproline | 131 | 20 | 0.153 | - |
| L-Isoleucine | 131 | 50 | 0.382 | 0.8 |
| L-Leucine | 131 | 50 | 0.382 | 0.8 |
| L-Lysine hydrochloride | 146 | 40 | 0.274 | 1.0 |
| L-Methionine | 149 | 15 | 0.101 | 0.2 |
| L-Phenylalanine | 165 | 15 | 0.0909 | 0.4 |
| L-Proline | 115 | 20 | 0.174 | - |
| L-Serine | 105 | 30 | 0.286 | 0.4 |
| L-Threonine | 119 | 20 | 0.168 | 0.8 |
| L-Tryptophan | 204 | 5 | 0.0245 | 0.078 |

| | | | | |
|---|---|---|---|---|
| L-Tyrosine disodium salt dihydrate | 261 | 29 | 0.111 | 0.4 |
| L-Valine | 117 | 20 | 0.171 | 0.8 |
| **Vitamins** | | | | |
| Biotin | 244 | 0.2 | 0.000820 | |
| Choline chloride | 140 | 3 | 0.0214 | 0.0285 |
| D-Calcium pantothenate | 477 | 0.25 | 0.000524 | 0.008 |
| Folic Acid | 441 | 1 | 0.00227 | 0.009 |
| i-Inositol | 180 | 35 | 0.194 | .04 |
| Niacinamide | 122 | 1 | 0.00820 | 0.033 |
| Para-Aminobenzoic Acid | 137 | 1 | 0.00730 | |
| Pyridoxine hydrochloride | 206 | 1 | 0.00485 | .019 |
| Riboflavin | 376 | 0.2 | 0.000532 | .001 |
| Thiamine hydrochloride | 337 | 1 | 0.00297 | .012 |
| Vitamin B12 | 1,355 | 0.005 | 0.0000037 | - |
| **Inorganic Salts** | | | | |
| Calcium nitrate ($Ca(NO_3)_2$ -$4H_2O$) | 236 | 100 | 0.424 | |
| Magnesium Sulfate ($MgSO_4$) (anhyd.) | 120 | 48.84 | 0.407 | |
| Potassium Chloride (KCl) | 75 | 400 | 5.33 | |
| Sodium Bicarbonate ($NaHCO_3$) | 84 | 2,000 | 23.81 | 44 |
| Sodium Chloride (NaCl) | 58 | 6,000 | 103.45 | |
| Sodium Phosphate dibasic ($Na_2HPO_4$-$7H_2O$) | 268 | 1,512 | 5.64 | |
| **Other Components** | | | | |
| Glutathione (reduced) | 307 | 1 | 0.00326 | |
| Phenol Red | 376.4 | 5 | 0.0133 | |

2. 10x PBS

Sources of reagents are given in the Materials Section

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)

3. Medium composition for 0.2% $^{13}C_6$-glucose, 2 mM $^{12}$C-Gln (100 ml)
   89.2 ml base medium minus tracer (*e.g.* glucose-free version) (89% concentration of nutrients)
   10 ml sterile filtered dialyzed FBS (10% FBS)
   0.8 ml 25% sterile filtered $^{13}C_6$ glucose (0.2 µm) in PBS (10.75 mM glucose final)
   1 ml 100x streptomycin/penicillin stock
4. Medium composition for 0.2% $^{12}$C glucose, 2 mM $^{13}C_5$, $^{15}N_2$-Gln (100 ml)
   88 ml base medium minus tracer (glutamine-free version) (88% concentration of all nutrients)
   10 ml sterile filtered dialyzed FBS (10% FBS)
   1 ml 0.2 M sterile filtered $^{13}C_5$, $^{15}N_2$-glutamine (0.2 µm) in PBS (2 mM final)
   1 ml 100x streptomycin/penicillin stock
   For hormone sensitive tissues, activated carbon-stripped FBS may be used.
   For other concentrations of FBS, adjust the volumes of the FBS and base medium accordingly.
   *Note: Glutamine stock should be made fresh or stored at -20 °C in small aliquots to avoid repeated freeze and thawing. It forms pyroglutamate on storage in solution even at neutral pH at higher temperatures.*

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The authors declare no conflicts of interest.

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