



Published in final edited form as:

Methods Mol Biol. 2019 ; 1928: 1–27. doi:10.1007/978-1-4939-9027-6_1.

Metabolic Labeling of Cultured Mammalian Cells for Stable Isotope-Resolved Metabolomics: Practical Aspects of Tissue Culture and Sample Extraction

Daniel R. Crooks¹, Teresa W.-M. Fan², W. Marston Linehan¹

¹Urologic Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, USA

²Department of Toxicology and Cancer Biology, Center for Environmental and Systems Biochemistry, Markey Cancer Center, and University of Kentucky, Lexington, USA

Abstract

Stable isotope-resolved metabolomics (SIRM) methods are used increasingly by cancer researchers to probe metabolic pathways and identify vulnerabilities in cancer cells. Analytical and computational advances are being made constantly, but tissue culture and sample extraction procedures are often variable and not elaborated in the literature. This chapter discusses basic aspects of tissue culture practices as they relate to the use of stable isotope tracers and provides a detailed metabolic labeling and metabolite extraction procedure designed to maximize the amount of information that can be obtained from a single tracer experiment.

Keywords

Tissue culture; Stable isotopes; Metabolomics; Metabolite extraction; Glutamine; Glucose

1 Introduction

At present, numerous publications and protocols exist in the literature detailing the theoretical and practical aspects of conducting stable isotope tracer experiments in mammalian cells, 3D organoid cultures, tumor tissue slices, animal models, and even in human patients [1–5]. Recent work has demonstrated that in vivo tumor metabolism can differ significantly from that observed in tumor cell monoculture [5–9]. Nevertheless, two-dimensional cell monoculture stable isotope tracer experiments are still heavily relied upon to draw conclusions about the nature of metabolic pathways in mammalian cells and tissues [10–14]. The continued reliance of researchers on 2D monoculture cell culture systems for stable isotope tracer experiments is due to a variety of factors, including the experimental power conferred by the ready control of the extracellular environment, the feasibility of extensive genetic manipulations, the lower cost and simple infrastructure needed for

³⁷Centrifugal ultrafiltration membranes contain significant amounts of glycerol that will end up in the sample. If this is an issue, then prerinse and spin the membranes with ddH₂O prior to addition of your medium samples to the membranes.

performing tissue culture experiments relative to animal and/or human patient work, and the ability to rapidly test a large variety of experimental conditions [1].

On a practical basis, access to analytical platforms, data analysis, and biological interpretation typically comprise the bottleneck in metabolomics workflows. Given the enormous amount of time that must be invested for data analysis and interpretation, careful and thorough consideration should be applied to the practical aspects of design and execution of stable isotope tracer experiments. Otherwise, subsequent in-depth analyses and interpretation efforts will be compromised by avoidable flaws in experimental design and execution. The purpose of this chapter is to provide a simple and practical laboratory protocol for stable isotope labeling of cultured mammalian cells in 2D culture in the typical cancer research laboratory setting. The growing number of cancer researchers wishing to conduct targeted stable isotope tracer experiments with their cells often do not possess the analytical instrumentation and expertise to perform mass spectrometry and NMR analyses of metabolites in their own labs. Thus, it is becoming increasingly common for researchers to perform the tracer labeling experiments in their own lab and send the resulting samples or quenched metabolite extracts to a core facility or company to perform the subsequent analyses. Data acquisition and targeted analysis in stable isotope experiments will not be discussed here, but these topics are well covered by other comprehensive literature, e.g., [3, 15–17].

The protocol detailed in Subheading 3.1 covers the practical aspects of the stable isotope labeling procedure for adherent mammalian cells. The subsequent metabolite quenching and extraction procedures tend to vary significantly between different laboratories, academic core facilities, and commercial metabolomics companies. For reference, a robust metabolite extraction protocol is detailed in Subheading 3.2; this procedure was developed and refined by Teresa W.-M. Fan, Andrew N. Lane, and Richard Higashi and represents a careful and comprehensive approach to the quantitative recovery of polar and nonpolar metabolites as well as total cellular protein from mammalian cells. This extraction procedure has several attractive features, including:

1. Initial rapid and complete inactivation of intracellular enzymes using cold 100% acetonitrile.
2. Quantitative and reproducible recovery of a broad complement of polar and nonpolar intracellular metabolites from a single sample using a sequential combination of acetonitrile, water, chloroform, and methanol.
3. Safeguards for minimizing degradation of labile polar and nonpolar metabolites during the extraction and sample reduction steps,
4. Versatile use of the resulting polar and nonpolar metabolite extracts and protein residues, e.g., analysis by both NMR and multiple modalities of mass spectrometry, as well as Western blotting and other proteomic analyses.

Although this metabolite extraction procedure is more laborious than most metabolite extraction protocols, the above features maximize the amount of information that can be obtained from a single stable isotope tracer experiment. Finally, Subheading 3.3 outlines two

simple extraction procedures for deproteinization and recovery of culture medium extracts for subsequent metabolomics analysis.

1.1 Experimental Design

Excellent summaries of the theoretical and analytical considerations involved in the design and interpretation of SIRM experiments can be found in the following references [2, 3, 18]. A discussion of preclinical cancer models that extend beyond 2D monoculture of tumor cell lines has also been published recently [1]. Briefly, experimental design considerations include consideration of which metabolites are of greatest interest for measurement, selection of a tracer molecule that is best suited to answer the questions at hand [18], selection of a sample preparation and extraction method best suited to observe the desired metabolites [19], and choice of duration of the labeling period [2, 20].

Many researchers aim to obtain a broad complement of information about the metabolic transformations of a common fuel molecule such as d-glucose or L-glutamine in their cancer cells, which can be achieved by using fully ^{13}C -labeled glucose or glutamine tracers. Doubly labeled $^{13}\text{C}_5$ -, $^{15}\text{N}_2$ -glutamine can also be utilized to gain even more information about the transformed products of glutamine; however, use of doubly labeled tracers necessitates the use of advanced analytical methods that can distinguish the two isotope labels, such as NMR and ultrahigh-resolution mass spectrometry, e.g., [21, 22]. Other tracers are particularly useful for evaluating specific pathways such as the pentose phosphate pathway ($^{13}\text{C}_2$ -1,2-glucose; [18, 23]), fatty acid biosynthesis ($^{13}\text{C}_3$ -glycerol, $^{13}\text{C}_8$ -octanoic acid; [12]), and reductive carboxylation of glutamine ($^{13}\text{C}_1$ -1-glutamine; [24]). In a sophisticated study on the compartmentalization of NAD(P)H-dependent pathways in different cellular compartments, several deuterated (^2H) forms of glucose, serine, and glycine were employed to explore the directionality of the pyridine nucleotide redox cycles [25]. A more comprehensive listing of various isotope tracers and their utility in tracer experiments has recently been published [17].

In addition to evaluating cells in the basal metabolic setting, experiments can be designed to assess perturbations in metabolic pathways caused by addition of a drug, a genetic manipulation, nutrient availability, or altered physiological parameters such as hypoxia and acidosis. In most cases, such questions can be addressed by aiming for pseudo-steady-state isotopic labeling of the metabolites of interest [2, 17, 20]. If subsequent steady-state metabolic flux modeling analysis is desired, a general rule of thumb of the tracer studies is to allow for cells to be grown in the presence of the tracer molecule for sufficient time for at least one population doubling to occur. This allows for many of the central metabolic pathways of interest to approach pseudo-steady-state isotope enrichment [2, 26]. For example, uridine-diphosphate N-acetyl glucosamine (UDP-GlcNAc), an activated form of N-acetyl glucosamine used for N- and O-linked protein glycosylation, derives carbon from glycolysis, the pentose phosphate pathway, hexosamine and pyrimidine biosynthetic pathways, and the Krebs cycle [26]. In a study utilizing a prostate cancer cell line with a doubling time of ~40 h, it was determined that ^{13}C incorporation in UDP-GlcNAc did not approach steady state until 30 h of growth in the presence of $^{13}\text{C}_6$ -glucose [26]. However, macromolecules such as proteins and cellular lipids require many population doublings to

approach steady-state labeling patterns [20], and this must be taken into account if these macromolecules are evaluated for stable isotope incorporation. In contrast, intermediates of central metabolism such as glycolysis, the pentose phosphate, and most components of the Krebs cycle reach isotopic steady state much more quickly, and for dynamic studies, rather short exposure times can be used [17].

The choice of number of treatment groups and number of experimental replicates per group must be balanced between statistical considerations and practical workflow considerations. In general, a minimum of three replicate samples should be included per treatment group. More biological replicates may be needed to detect more subtle differences in metabolite labeling patterns. Before drawing scientific conclusions, it is always important to repeat the experiment to ensure that the results are independent of cell passage numbers, density, or other subtle differences in growth conditions. It can also be very helpful to include an unlabeled sample for each treatment group, which is grown in the presence of the natural abundance form of the tracer molecule. Analysis of this additional sample allows for qualitative comparisons of the fate of the ^{13}C tracer carbons, e.g., in overlays of ^1H - $\{^{13}\text{C}\}$ HSQC NMR spectra [14], and as a technical control to discern incorrectly assigned isotope-labeled peaks during curation of mass spectra. This additional unlabeled sample can also be included as a replicate in measurement of total abundance of metabolites in the treatment group. However, the unlabeled sample should not be used to manually correct for the presence of natural abundance isotopologues of the metabolites of interest in mass spectrometry data [2], as formal algorithms must be applied for such corrections [2, 27].

A reasonable number of 10 cm dishes with cells to process in 1 day using the extraction method detailed here range from 12 to 18, and the procedure runs much more efficiently with two people working together. More dishes can be processed if desired; however, staggering of the tracer additions may be needed so that the tracer labeling period is consistent across all dishes. Example experimental setups are provided in Fig. 1, which include several configurations for different kinds of experiments, including blank “t = 0” plates with no cells but containing tracer-labeled or unlabeled medium (see Fig. 1).

1.2 Nutrient Requirement, Growth Kinetics, and Tracer Medium Choice

Cultured tumor cells exhibit different nutritional requirements depending on cell type, genotype, tissue of origin, and numerous other factors. For assessing pseudo-steady-state labeling patterns of intracellular metabolites, it is important to design the experiment such that nutrient supplies remain adequate throughout the course of the experiment. For example, if the culture medium is depleted of $^{13}\text{C}_6$ -glucose by the cells before the end of the tracing period, the resulting intracellular ^{13}C metabolite labeling patterns will likely reflect a state of altered growth and/or metabolic adaptation to alternative energy sources rather than glucose-driven labeling patterns observed during pseudo-steady-state growth. Similarly, premature depletion of glutamine tracer by cells during a glutamine tracer experiment might result in the diversion of glucose-derived carbon into pathways normally fueled by glutamine in the cultured cells. Significant unintended changes in nutrient levels and the resulting compensatory changes in cellular metabolism will make biological interpretation of metabolite labeling patterns exceedingly difficult.

Population doubling times and metabolic rates can vary greatly between different cell lines even of the same nominal origin (e.g., MDAMB231 cells double in 18 h, whereas ZR75–1 double in 80 h in rich media) [12]. Mouse cells typically double faster than analogous human cells, and the rate of metabolism reflects this difference [28]. In order to minimize the confounding effects of changing nutrient availability during the tracer experiment, preliminary experiments should be conducted in order to assess the growth rate (doubling time) and bulk utilization of the major fuels of cultured cancer cells, i.e., glucose and glutamine. These measurements can be performed using cell counting and relatively inexpensive enzymatic methods, ready-made kits, mass spectrometry, or NMR. We routinely measure the concentrations of glucose, lactate, and glutamine in both freshly prepared culture medium and in spent culture medium supernatants from experiments using immobilized enzyme electrode amperometry (YSI 2950 Bioanalyzer), which allows direct measurement of metabolites in culture medium without the need for extraction/deproteinization. The results of these simple analyses help to determine optimal cell seeding densities for stable isotope tracer experiments and can also aid in the determination of whether initial cell extracts from a stable isotope tracer experiment should be further processed and submitted for NMR and mass spectrometry analyses or discarded due to loss of adequate nutrient supplies. It is important to keep in mind that true steady-state maintenance of the extracellular environment can never be achieved in a standard culture dish [29]. Quantitative metabolite flux experiments require complex control of medium components to maintain steady-state conditions using a chemostat, sampling of multiple time points, and in-depth computational modeling of metabolic network kinetics [2, 30–32].

Another factor to take into consideration is whether a genetic manipulation or drug treatment might result in reduced cell growth rate. For instance, treatment with a cytostatic drug will result in reduced cell numbers at the end of the experiment. If this is the case, cell dishes in the “treated” group may need to be seeded at a higher density than the control group in order to obtain sufficient cell biomass for metabolite extraction at the end of the experiment. Careful consideration of the effects of decreased cellular proliferation on isotope labeling patterns will need to be included during subsequent data analysis [2].

Although the majority of routine mammalian tumor cell culture work is performed using either DMEM or RPMI 1640 culture medium with the desired tracer nutrient replaced by the corresponding stable isotope-enriched counterpart at the identical concentration, recent studies have underscored the influence of the nonphysiological nutrient concentrations and lack of certain metabolites in these culture media on cellular metabolism in culture [33]. In addition to containing nonphysiological concentrations of vitamins and amino acids [28], culture media including DMEM and RPMI 1640 also lack metabolites that are found in human plasma [34], which have been shown to influence cell metabolism and drug susceptibility [33]. These findings led to the development of a new medium formulation, human plasma-like medium (HPLM), which better recapitulates the *in vivo* human setting [33]. However, preparation of customized media is both tedious and costly, and to date HPLM is not commercially available. In most cases, cancer researchers aim to incorporate the data obtained from SIRM experiments into a larger dataset encompassing other biological outcomes such as gene and protein expression, drug susceptibility studies, analysis of invasion potential, protein subcellular localization studies, gene knockdown or

overexpression, functional analysis of gene mutations, hypoxic response, respiration studies, etc. Thus, if all possible, the stable isotope tracer experiment should be performed under identical culture conditions as those employed for other biological experiments.

1.3 Dialyzed FBS vs Non-dialyzed FBS

Dialyzed FBS is a preferred additive for stable isotope tracer experiments with mammalian cells because it retains most of the growth factors and lipids present in FBS but lacks the small molecule metabolites present in non-dialyzed serum. Depending on the stable isotope tracer and associated pathways being evaluated, the natural abundance metabolites present in non-dialyzed FBS could confound the interpretation of both extracellular and intracellular metabolite labeling patterns. Table 1 lists natural abundance concentrations of some commonly evaluated metabolites found in two separate lots of FBS obtained from two different commercial sources, measured amperometrically using a YSI 2950 Bioanalyzer. When used at the standard supplementation rate of 10%, these non-dialyzed FBS reagents would contribute ~0.4–0.8 mM natural abundance glucose and ~0.8–1.8 mM natural abundance lactate to the tracer medium. The presence of these unlabeled substrates and products of glycolysis would need to be considered in the interpretation of pseudo-steady-state labeling patterns of both intracellular and extracellular metabolites. This issue would be avoided with dialyzed FBS, as the concentrations of natural abundance glucose and lactate in two separate lots were 50–100-fold lower than the non-dialyzed FBS (Table 1). Also notable is the persistence of small amounts of carbohydrate and lactate in the municipal water supply, underscoring the need to utilize double-deionized water for experiments and to avoid the contamination of dishes and sample tubes by stray pieces of ice from the ice tray.

In our own experience with tumor cell lines that are deficient in Krebs cycle enzymes and/or respiratory chain components, we have found that some cell lines exhibit attenuated growth in dialyzed FBS, an effect that is likely due to auxotrophy for one or more small molecule metabolites present in FBS such as uridine, uric acid, etc. Many cell lines require an adaptation period of several days in the presence of dialyzed FBS, presumably to allow for transcriptional changes and metabolic adaptation, before the growth rate is restored to suitable levels. For these reasons, it is important to perform pilot experiments to test the growth characteristics of the cell line of interest in dialyzed FBS. It may also be important to test whether gene expression in the pathway(s) of interest is modulated by growth of the cells in dialyzed FBS, as metabolic compensation might occur in the absence of small molecule nutrients supplied in non-dialyzed FBS. As a rule of thumb, the same batch of dialyzed and non-dialyzed FBS should be utilized for all experiments in a project to maintain consistency of experimental results [28]. Finally, in tracer studies where extracellular vesicles such as exosomes are to be isolated or their influence on cell metabolism studied, exosome-free FBS (either non-dialyzed or dialyzed) should be adopted. Exosome-free FBS can be prepared using ultra-centrifugation or purchased commercially.

1.4 Other Considerations of Tissue Culture Experiments

Other practical factors are also important to consider in minimizing variations in tissue culture conditions. Adequate humidity in the incubator should be maintained by ensuring that ample water is present in the incubator water tray or by acquiring an incubator that has

an active humidification system. If possible, the stable isotope tracer experiment should be conducted in a dedicated incubator that is free of vibrations and will not be opened and closed excessively during the experiment. Excessive opening and closing of the incubator door leads to transient decreases in CO₂ concentration, resulting in fluctuations in culture medium pH due to changes in bicarbonate concentration and drying of the internal environment due to injection of dry CO₂ gas to restore CO₂ levels. Fluctuation in humidity is especially pertinent in tri-gas incubators operating at O₂ concentrations that are far below the ambient (~21% O₂), as large quantities of dry nitrogen gas are injected into the incubator to decrease the O₂ concentration. Table 2 shows the results of an experiment in which triplicate 10 cm dishes without cells were filled with 15 mL of DMEM/10% non-dialyzed FBS medium and allowed to incubate at 37 °C unperturbed in a 95% room air/5% CO₂ atmosphere, in either a well-humidified incubator or in an incubator in which the water tray was allowed to become dry. Plates incubated in the non-humidified incubator for 40 h showed an approx. 5% decrease in total mass, indicative of significant culture medium evaporation. In contrast, the dishes incubated in the humidified incubator lost only 1.3% of their mass over the same incubation period. The evaporative losses in the inadequately humidified incubator showed significant effects on the observed concentrations of glucose, lactate, and glutamine in the spent culture medium samples. Namely, glucose and lactate concentrations increased by 16–17%, while glutamine concentrations decreased by 1.3% in samples incubated in the non-humidified incubator. In contrast, glutamine concentration decreased significantly by 9% in the well-humidified incubator, likely reflecting the conversion of glutamine to NH₄⁺ and pyroglutamate [35], an effect that was masked in the non-humidified incubator due to evaporative loss of water in the dish. Differences in medium metabolite fluxes in non-humidified versus humidified growth conditions can also be attributed to altered cell physiology (e.g., reduced growth rate; Fan, unpublished data) and are likely to involve increases in culture medium osmolality. Thus, a poorly controlled incubator environment can considerably influence the interpretation of apparent metabolite uptake and secretion rates (Table 2).

Finally, other general good practices in tissue culture are of the utmost importance in metabolomics experiments, in order to justify the significant cost, time, and effort involved in acquisition, analysis, and interpretation of metabolomics data. Cell lines should be regularly tested for mycoplasma, as this cryptic and antibiotic-resistant contaminant microbe can go unnoticed indefinitely in tissue cultures [28], and by nature of its physical attachment to cells in tissue culture, mycoplasma is very likely to significantly alter cellular metabolism [36]. Unless specifically desired for differentiation or other physiological strategies, cells should be seeded evenly across the dishes and should not be allowed to become 100% confluent before quenching and extraction, as some cell lines undergo contact inhibition and thus will not be in log-phase growth at the time of harvest. In some cases, cells may also show dramatic differences in intracellular metabolite levels depending on their state of confluence [37]. Cell size can vary greatly across different cell lines; thus, initial cell seeding numbers and the number of cells present at 100% confluence can be very different. In general, it is a good practice to perform initial experiments to determine the number of cells required for seeding of culture dishes such that the cells are at 70–90% confluence at the time of harvest.

1.5 Sample Preparation for Stable Isotope-Resolved Metabolomics

Numerous protocols exist for metabolite quenching/extraction from mammalian cells [3, 19, 25, 38, 39]. There is no single sample preparation technique that can quantitatively recover all intracellular metabolites, as some metabolites are inherently labile and prone to degradation, and some others are less extractable using a universal solvent system. Notable examples of metabolites that can be degraded during extraction procedures include NADPH and to a lesser extent NADH, both of which show decreased stability in acidic conditions [40]. Glutamine is inherently unstable in culture medium [35], and metabolite extraction procedures can cause marked conversion of glutamine to pyroglutamate [41]. Issues with glutamine degradation can also arise as a direct result of ionization in the mass spectrometer [42]. Direct, in-cell derivatization of labile metabolites has been shown to be useful for detection of unstable carbonyl- and aldehyde-containing metabolites including oxaloacetate, dihydroxyacetone phosphate, glyceraldehyde 3-phosphate, and acetoacetate [21, 43]. Derivatization has also been shown to be useful for resolving multiple labeled amino acids by direct infusion and ultrahigh-resolution mass spectrometry [22].

Careful studies in *E. coli* have demonstrated that significant metabolite decomposition can occur as an artifact of the extraction procedure [44] and that acetonitrile is a superior solvent for metabolite extraction in *E. coli*, especially in the case of the preservation of nucleoside triphosphates [45]. Such improvements in extraction techniques were employed in the quantitative evaluation of total metabolite concentrations and metabolite enzyme occupancy in *E. coli* [46]. In mammalian cells, acetonitrile-water was also found to be a superior polar metabolite extraction solvent [19]. When acetonitrile-water extraction is combined with a second chloroform-methanol extraction, this single procedure can yield extracts suitable for analysis of polar metabolites, total cellular lipids, and cellular proteins all from one sample, e.g., [7, 14]. This metabolite extraction procedure, termed the Fan extraction method by the first author, is outlined below in Subheading 3.2.

2 Materials

1. Laminar flow biosafety cabinet for tissue culture work.
2. Dual-gas (CO₂) or tri-gas (CO₂, N₂) humidified, temperature-regulated incubator.
3. 37 °C heated water bath
4. 10 cm tissue culture treated culture dishes (*see* Note 1)
5. Electric pipettor and serological pipettes (5, 10, 25 mL).
6. Vacuum aspirator fitted with tubing and disposable 2 mL plastic aspirator pipettes.
7. 15 mL polypropylene conical vials (Sarstedt)
8. 50 mL polypropylene conical vials (Sarstedt)

¹Poly-D-lysine or collagen-coated plates can be used for cells that are poorly adherent.

9. 1.5 mL polypropylene Eppendorf tubes
10. 2.0 mL polypropylene screw-top vials
11. 0.5 mL polypropylene screw-top vials
12. 2.0 mL screw-top glass vials
13. Basal DMEM: DMEM without glucose, glutamine, pyruvate, and phenol red (*see* Note 2).
14. Dialyzed FBS (*see* Note 3) [or exosome-depleted FBS (e.g., Gibco# A2720801) if you are studying exosomes].
15. 0.5% phenol red solution (*see* Note 4)
16. Stable isotope tracer (e.g., $^{13}\text{C}_6\text{-D-glucose}$, $^{13}\text{C}_5\text{-L-glutamine}$, $^{13}\text{C}_5$, $^{15}\text{N}_2\text{-L-glutamine}$, etc.) (*see* Notes 5 and 6).
17. DMEM tracer media (*see* Notes 7 and 8).

²Some cell lines, including mtDNA-deficient rho-0 cells and patient-derived fumarate hydratase-deficient (FH^{-/-}) tumor cell lines, grow poorly or not at all in culture medium made with dialyzed FBS without supplementation of 1 mM pyruvate. The necessity for the presence of pyruvate in the culture medium should be evaluated prior to the tracer experiment; we typically do not supplement with exogenous pyruvate unless it is necessary for cell proliferation.

³The part number given here is for pre-dialyzed FBS; however, dialyzed FBS can be prepared in-house with substantial savings in cost.

⁴While not required for tissue culture, phenol red is an invaluable tool that allows the researcher to continuously monitor the pH of the culture medium. An excessively pink color indicates greater alkalinity, usually due to loss of bicarbonate buffering by prolonged exposure of the medium bottle to room air atmosphere. Yellowing of the culture medium is frequent in highly glycolytic tumor cell cultures and is indicative of substantial acidification due to lactate secretion and possible depletion of glucose in the culture medium. Yellowing of the culture medium can also be an early indicator of bacterial contamination. Finally, changes in the color of phenol red sometimes occur after additions of drugs or other chemicals, immediately indicating the need for pH adjustment of the agent before addition to the culture medium. Caution must be taken with cells that are estrogen-sensitive, as phenol red has been reported to be a weak estrogen mimetic [49], though this may be due to lipophilic impurities in certain phenol red preparations rather than the pH indicator itself [50].

⁵The choice of the tracer to be used depends on the goals of the project. Some strategies for tracer selection in mammalian cell tracer experiments are presented in the following references [3, 12, 18, 22, 25].

⁶Prepare all tracer solutions using double-deionized water (ddH₂O, 18.2 M Ω). Sterile water for injection can be used if ddH₂O water is not available.

- a. Prepare 46.5% (w/v) $^{13}\text{C}_6\text{-glucose}$ (2.498 M) volumetrically using a volumetric flask. For example, for a 10 mL solution, weigh 4.65 g of $^{13}\text{C}_6\text{-glucose}$ powder into the flask, and bring up to 10 mL volume line with ddH₂O. 45% (w/v) $^{12}\text{C}\text{-glucose}$ solution (2.498 M) is prepared in the same way using 4.5 g of natural abundance D-glucose. The solutions should be sterile filtered using a 0.22 μm syringe filter and stored in a Parafilm-wrapped vial at 4 °C for several months or in aliquots in tightly sealed vials at -80 °C for years.
- b. Prepare 200 mM L-glutamine solutions using the appropriate molecular weight of the tracer compound in ddH₂O (e.g., $^{12}\text{C}\text{-L-glutamine}$ MW = 146.14 g/Mol, $^{13}\text{C}_5\text{-L-glutamine}$ MW = 151.11 g/Mol). The solution should be sterile filtered using a 0.22 μm syringe filter and immediately aliquoted in vials and stored at -80 °C for at least 1 year. Repeated freeze and thawing of the stock solution should be avoided.
- c. Dialyzed FBS can be stored in aliquots of 10–45 mL in sealed polypropylene conical vials at -20 °C or -80 °C.

⁷For a typical tracer experiment such as the schemes shown in Fig. 1, prepare 300 mL basal tracer medium by combining 30 mL dialyzed (or exosome-depleted) FBS with 270 mL basal DMEM and optional 300 μL of 0.5% phenol red solution. Sterile filter using a disposable 500 mL 0.2 μm filter unit. Dialyzed FBS tends to show flocculence following freeze-thaw cycles; sterile filtration of the final solution ensures that any particulate matter is removed from the tracer medium. Prepare tracer media as required by the experimental design. We typically supplement basal DMEM media with 11 mM D-glucose (0.2%) and 2 mM L-glutamine (e.g., 11 mM $^{13}\text{C}_6\text{-D-glucose}$ plus 2 mM $^{12}\text{C}\text{-L-glutamine}$ or 11 mM $^{12}\text{C}\text{-D-glucose}$ plus 2 mM $^{13}\text{C}_5\text{-L-glutamine}$).

⁸Unless it is explicitly part of your downstream analysis strategy (e.g., [22]), never include more than one tracer molecule in a given tracer medium preparation. Also, doubly labeled tracers such as $^{13}\text{C}_5\text{-}$ and $^{15}\text{N}_2\text{-L-glutamine}$ should not be utilized unless downstream analyses will include platforms that are able to distinguish the two heavy isotopes (e.g., $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ NMR or ultrahigh-resolution Orbitrap or ion cyclotron Fourier-transform mass spectrometry). Be sure to prepare ~3–5 mL excess of each type of tracer medium for sufficient distribution into each plate, and remember to include enough medium for the blank plates with no cells.

18. HPLC-grade acetonitrile (*see* Note 9).
19. HPLC-grade methanol (*see* Note 9).
20. HPLC-grade chloroform (*see* Note 9).
21. 2:1 chloroform/methanol-BHT solution (*see* Note 9)
22. Disposable plastic cell lifters (corning costar #3008) (*see* Note 10).
23. Ice buckets.
24. Rectangular anodized metal plate of at least 14 × 28 cm (e.g., Biocision, Inc. #BCS-123).
25. Phosphate-buffered saline (PBS), pH 7.4: 1.06 mM KH₂PO₄, 2.97 mM Na₂HPO₄, 155 mM NaCl.
26. Fine-tipped transfer pipettes (e.g., Samco scientific #235).
27. Freezer lyophilizer equipped for handling organic solvents (*see* Note 11).
28. Vacuum centrifuge (Eppendorf Vacufuge plus, fitted with rotors for both 1.5 mL tubes and for 2.0 mL glass vials) (*see* Note 12).

3 Methods

3.1 Metabolic Labeling of Adherent-Cultured Mammalian Cells

1. Perform preliminary experiments using tracer medium prepared with the desired natural abundance ¹²C tracer counterpart to ensure that the tracer medium composition is suitable to support cell growth and that nutrient levels remain adequate throughout the duration of the experiment. Considerations include (1) assessment of adequate cell growth rate in tracer medium, (2) determination of

⁹.Store all solvents in a flammable-certified –20° freezer in the dark.

- a. Decant 100 mL of HPLC-grade acetonitrile into an amber bottle and store at –20 °C.
- b. Decant 100 mL of HPLC-grade methanol into an amber bottle and store at –20 °C.
- c. Decant 100 mL of HPLC-grade chloroform into an amber bottle and store at –20 °C.
- d. Prepare 100 mL of chloroform/methanol-BHT solution, prepare a 100 mM butylated hydroxytoluene (BHT) stock solution in HPLC-grade methanol, and then prepare 100 mL of 2:1 chloroform/methanol-1 mM BHT in an amber bottle by combining 66 mL chloroform with 32 mL methanol and 1 mL of 100 mM BHT stock solution in an amber bottle. Seal tightly, and store at –20 °C.

¹⁰.The cell lifter is superior to hinged cell scrapers because more force can be applied at a 45° angle to scrape the solvent and quenched cells.

¹¹.This metabolite extraction procedure calls for freeze-drying/lyophilization of the resulting polar fraction aliquots. Freeze-drying allows for the removal of solvents and water from the polar fraction while maintaining the sample at below freezing temperature and minimizing exposure of the sample to oxygen during the drying process by the maintenance of high vacuum [51] and is far superior to vacuum centrifugation for the quantitative preservation of the maximum number of metabolites. However, depending on the stability of the metabolite(s) of interest, other evaporative techniques could be utilized for sample concentration, including vacuum centrifugation or drying under a flow of nitrogen gas.

The liquid nitrogen pre-trap is important for lyophilizers fitted with oil-containing pumps, as the solvents with very low melting points (e.g., methanol, acetone) derived from the sample will degrade the pump oil over time and lead to premature failure of the pump. An alternative currently being evaluated by D.R.C. is to utilize a – 86 °C lyophilizer with a Teflon-coated condenser chamber and coils, attached to an oil-free Edwards scroll pump with silencer kit, with the open ballast venting into a chemical fume hood.

¹².A vacuum centrifuge fitted with an oil-free diaphragm pump (e.g., Eppendorf Vacufuge plus™) is desirable for the evaporation of solvents such as methanol and chloroform, as oil-containing vacuum pumps are subject to the same solvent issues as discussed above.

the target cell seeding density such that cells will reach 70–90% confluence at the time of quenching/harvest, (3) selection of a tracer incubation time that equals or exceeds the cell population doubling time in order to approach pseudo-steady-state labeling of many intracellular metabolites, and (4) determination of bulk metabolite uptake and secretion at the end of the labeling period to ensure that the major nutrients (e.g., glucose and glutamine) are not depleted at the end of the labeling period.

2. Seed cells in 10 cm dishes in 10–25 mL tracer medium (*see* Note 13) containing natural abundance tracer compound (i.e., ^{12}C -glucose and ^{12}C -L-glutamine) 1 or 2 days prior to the labeling experiment, and allow them to attach and proliferate (*see* Notes 14 and 15) (*see* Note 19 **for alternative protocol for non-adherent cells**).
3. Prepare tracer media freshly and equilibrate to 37 °C several hours prior to addition to the culture dishes. Also, prewarm a sufficient quantity of basal DMEM (without FBS or any other added compounds) to allow for washing each plate once with 10 mL basal DMEM (*see* Note 16).
4. Remove the culture dishes from the incubator, and aspirate the ^{12}C tracer medium using vacuum aspiration. Gently add 10 mL of warm DMEM basal medium, rock the plate by the hand several times, and then aspirate. Finally, add 10–25 mL of prewarmed ^{13}C tracer medium to each plate, remove 0.2 mL of medium ($T = 0$), and weigh plate on a three-place balance before returning plates to the incubator. For each type of tracer medium used, include one blank plate in which no cells are seeded but tracer medium is added. This plate is incubated

¹³The standard quantity of 10 mL of tracer medium per 10 cm dish is used for routine experiments. However, for very rapidly growing cells such as HEK293 or cells exhibiting extraordinarily high levels of aerobic glycolysis (e.g., mtDNA-depleted cells or Krebs cycle enzyme-deficient tumor cells), greater amounts of medium may be needed to sustain adequate nutrient levels during routine culture and during the tracer labeling period.

¹⁴The metabolite extraction procedure outlined in this protocol allows for obtaining a protein residue for subsequent quantification and normalization of metabolite levels. If you choose a different metabolite extraction protocol that does not allow for recovery of cellular protein, then additional dishes can be included to allow for cell counting and/or cellular protein determination for inferred normalization of metabolite concentrations [3]. However, this practice is imprecise, as each plate will be seeded somewhat differently and should not formally be used for normalization of a separate plate.

¹⁵Many cell lines require 24 h or more to adhere, stabilize, and enter into log-phase growth. Do not seed the cells, and initiate the tracer labeling period simultaneously.

¹⁹For non-adherent cells or cells that are very poorly adherent, an alternative harvesting procedure is as follows:

- a. Collect cells and medium from each dish in a conical tube, and centrifuge at $350 \times g$, 5 min, 4 °C. Weakly adherent cells can be collected by gentle trituration of the tracer medium over the plate until cells are detached.
- b. Collect 1 mL of culture medium supernatant, and set aside for subsequent processing and extraction.
- c. Vacuum aspirate the remainder of the tracer medium with a fine pipette tip, add 10 mL of ice-cold PBS, and gently triturate the cell pellet. Centrifuge at $350 \times g$, 5 min, 4 °C, and then vacuum aspirate the supernatant.
- d. Repeat **step 3** once, taking care to vacuum aspirate as much PBS as possible during the second aspiration.
- e. Immediately add 1 mL cold (–20 °C) acetonitrile, and vortex the mixture in pulses to resuspend the cells. If clumps of cells are still visible, then triturate up and down with the 1 mL pipette tip to break up the clumps.
- f. Add 0.75 mL ice-cold ddH₂O and vortex, then add an additional 1 mL of acetonitrile and 0.75 mL ddH₂O, and proceed to **step 11** in Subheading 3.1.

¹⁶If the medium is excessively pink (alkaline) due to loss of bicarbonate to the air as CO₂, the media can be left in the 5% CO₂ incubator for several hours to overnight with the lid loosened to allow for re-equilibration of the bicarbonate buffering system.

alongside the plates with cells and serves as the blank plate (*see* Fig. 1, Subheading 1.2, and Notes 17 and 18).

5. On the next day, set up the lab bench for medium harvest and cell quenching by obtaining several trays of ice and pre-labeling all of the tubes that will be needed. In one ice tray, place a slab of rectangular metal of at least 14 × 28 cm (e.g., Biocision, Inc. #BCS-123) horizontally, tilted toward the front of the bench by approximately 35°. Precool a bottle of HPLC-grade acetonitrile to –20 °C.
6. At the end of the incubation period, weigh plates using the same balance, and remove the plates from the incubator in groups of three at a time. Place the dishes on the metal slab, with the pen mark on the side of the dish facing you. Transfer 1 mL of medium from each plate to a snap-cap microcentrifuge tube, and set the tube aside on ice for subsequent processing and extraction. Aspirate the remainder of the tracer medium using a fine-tipped transfer pipette into a 15 mL conical centrifuge tube if the medium is to be harvested or using the vacuum aspirator fitted with a disposable 2 mL plastic aspirator pipette if the medium is to be discarded (**for non-adherent cells**, *see* Note 19).
7. Wash the dishes three times with 10 mL ice-cold PBS by gently adding the PBS to the side of the dish where the mark is and aspirating from the dish wall slightly above the same spot each time to minimize cell loss. Make sure that the entire circumference of the dish wall and the entire surface of the plate are rinsed by each of the three washes by gentle rocking of the dish during each wash. After the third wash, allow the dish to remain tilted toward you at ~35° on the metal block with the pen mark facing toward you. Carefully observe the walls of each dish, and aspirate any residual liquid that might remain on the walls of the circumference of the plate while residual liquid on the plate is collecting near the pen mark. Finally, aspirate again any additional residual fluid from the bottom of the plate (*see* Notes 20 and 21).
8. Immediately add 1 mL cold (–20 °C) acetonitrile to quench cell metabolism. Ensure that the solvent covers the entire surface of the dish by gently but rapidly shaking the plate horizontally. Place the culture dishes on a shelf in a –20 °C freezer, and incubate for 5–20 min.

¹⁷At the beginning of the experiment, use a permanent marker to mark the lower outer wall of the culture dish indicating where aspiration and additions will occur throughout the subsequent steps of culture and quenching. This, together with aspiration slightly above the mark, will help to minimize cell loss and keep the loss more consistent across the dishes and treatment groups during the course of experiment.

¹⁸If an inverted phase-contrast microscope with a camera is available, then take a picture of one or more regions of the dish from at least one replicate of each treatment group before and after the tracer incubation period. This will reveal any morphological or cell number changes in the tracer experiment.

²⁰Work quickly, and minimize the time between which the culture dishes leave the incubator and the cells are quenched with solvent, thus minimizing the time that elapses between removal of the dish from the relative equilibrium of the incubator to the time when cellular enzymatic activities are eliminated by the 100% acetonitrile quench.

²¹Prior to metabolite quenching and extraction, it is important to minimize the salt contribution to the final extracts, which can interfere with metabolomics analysis, particularly by mass spectrometry. Residual sodium, chloride, and phosphate ions can interfere with MS analyses via ion suppression and decreased signal-noise ratios in NMR analyses [52]. Be sure to aspirate as much liquid as possible after the last wash.

Greatly reduced salt interference can be achieved by a brief rinse of the dish with Nanopure water (30 s to 1 min) right after removing the medium components with PBS. We have found that this water rinse significantly improved Fourier-transform mass spectrometry performance without loss of cellular metabolites. The water rinse is aspirated rapidly, and the quenching solvent is added immediately.

9. Remove the dishes from the freezer, and return to the ice-cold metal block. Add 0.75 mL ice-cold ddH₂O, and gently rock the dish to ensure mixing of the water and acetonitrile (*see* Note 22).
10. With the plate angled at ~35° toward you resting on the ice-cold metal, scrape the cells and solution toward the bottom of the dish using a cell lifter with firm circular motions until all material has been scraped to the lower end of the dish. Next, rotate the dish by 90° and scrape the remaining cells and fluid to the bottom of the dish (*see* Note 23).
11. Transfer the quenched cells and solvent to a 15 mL conical centrifuge tube, and then repeat **steps 8 and 9** but omit the –20 °C incubation step. The resulting volume in the 15 mL conical vial is typically ~3.2 mL. The final solvent composition is 2:1.5 (acetonitrile-water). This solution can be kept on ice for several hours before proceeding to chloroform extraction or stored at –80 °C for up to 1 week pending chloroform extraction.
12. After all dishes of cells have been quenched and scraped, centrifuge the 1 mL culture medium samples at 3000 × *g* for 10 min at 4 °C in a microcentrifuge to remove any floating cells and large debris.
13. Transfer 900 µL of the resulting culture medium supernatant to a new pre-labeled tube for storage at –80 °C until metabolite extraction. A 100 µL of this culture medium sample can be stored separately for direct acetone extraction. Culture medium metabolite extraction procedures are outlined in Subheading 3.3.

3.2 Cell Metabolite Extraction Using the Fan Extraction Method

1. Pre-label all tubes to be used during the phase separation and extraction:
 - a. Aqueous/polar fraction: 5 mL snap-cap Eppendorf tube, pre-weighed to 0.1 mg precision. Label simply and clearly with sample number; it will be discarded after aliquoting of the polar phase.
 - b. Nonpolar/lipid fraction: 2 mL screw-cap glass vial with Teflon-faced liner, wrap with clear scotch tape around the side of the vial to protect labeling.
 - c. Protein fraction: 1.5 mL snap-cap Eppendorf tube (pre-weighed to 0.01 mg precision if protein dry mass is desired), taped around the side to protect labeling (*see* Note 24).
2. Add 1 mL ice-cold chloroform to the acetonitrile-water cell extracts in their 15 mL conical tubes, using a pre-wetted 1 mL polypropylene micropipette tip. Seal

²².An internal standard can be included in the ddH₂O at this step. 53.3 µM tris, pH 8.0 can be included in the first 0.75 mL ddH₂O addition, as a convenient internal standard in ¹H NMR analyses.

²³.Think of the cell lifter as a squeegee being used to move all liquid and cell material to the bottom of the plate, reminiscent of squeegeeing a window or windshield after washing it.

²⁴.High-quality gloss-finish scotch tape is used to protect the writing on the tube from being smeared by solvents during pipetting, centrifugal evaporation, and lyophilization.

the tubes tightly, and shake the extracts vigorously up and down at least 60 times manually or on a vortexer (*see Note 25*).

3. Centrifuge the extracts in a refrigerated swinging-bucket centrifuge at maximum speed (typically 3000–4650 × *g*, depending on the model of the centrifuge), 4 °C, 30 min. The extracts will separate into three phases: An upper aqueous phase containing polar metabolites, a lower nonpolar chloroform phase containing lipids, and a middle phase containing denatured proteins and other macromolecules. Gently transfer the tubes to an ice bucket, taking care not to disrupt the phase separation (*see Note 26*).
4. Transfer ~90% of the aqueous phase to pre-tared (± 0.1 mg) 5 mL Eppendorf tube using a fine-tipped transfer pipette, taking care not to disrupt or transfer any of the denatured protein layer along with the aqueous phase. The remaining aqueous phase will be recovered during the second extraction. Save the transfer pipette for the second extraction.
5. Transfer ~90% of the lower chloroform phase to a 2 mL glass vial until the vial is filled to the neck, using a gel-loading 200 μ L pipette tip; save the tip for a second transfer (*see Note 27*).
6. Place the 2 mL glass vial in a vacuum centrifuge, and reduce the volume of the chloroform fraction by vacuum centrifugation for 30 min without heating.
7. Centrifuge the 15 mL conical vial containing the remaining polar and nonpolar fractions as well as the protein fraction for 5 min at maximum speed at 4 °C. If more than ~400 μ L of nonpolar fraction remains in the 15 mL vial, transfer more nonpolar fraction to the respective 2 mL glass vial after the volume has been reduced sufficiently by vacuum centrifugation, until <100 μ L of nonpolar fraction remains.
8. Using a 1 mL pipettor set to 500 μ L, mix the protein fraction along with the polar and nonpolar fractions until the protein fraction is sufficiently dispersed to allow for the transfer of the remaining contents of the 15 mL tube to the 1.5 mL protein fraction tube. Set aside this pipettor with the tip still attached. Using a separate 1 mL micropipette, add 350–500 μ L ice-cold chloroform-methanol/1 mM BHT to the 15 mL vial. Then use the first pipettor and tip to pipette the solution up and down, washing the residual protein residue from both the walls of the tube and

²⁵This is the commitment step in the metabolite extraction procedure. Once chloroform is added, the fractionation procedure must be completed in the same day, as the chloroform will slowly degrade the polypropylene tubes. Never use polystyrene tubes for metabolite extractions.

²⁶If the protein interphase looks excessively thick, fuzzy, or uneven, then centrifuge for an additional 20 min.

²⁷If the protein interphase layer is sufficiently abundant and forms a solid white layer, a 1 mL pipette set to 900 μ L with a non-filtered polypropylene tip can be carefully teased along the side of the tube, past the protein layer, and down into the chloroform phase. If the protein layer is less abundant or more fluffy, a 200 μ L micropipette fitted with a gel-loading tip must be used, with the tip carefully inserted through the protein layer along the side of the tube. In either case, take care not to pipette the aqueous layer by first depressing the pipette plunger down to its first resistance point before inserting it into the tube, then slowly insert the tip past the aqueous and protein phases into the chloroform phase, and then depress the plunger slightly further to expel any aqueous phase until a small air bubble is passed. Then begin to draw the chloroform phase into the tip. Take care to minimize the amount of protein that sticks to the outer wall of the pipette tip.

the inner surfaces of the pipette tip. Transfer the protein and chloroform-methanol/BHT wash to the protein fraction tube (*see* Note 28).

9. Vigorously shake and vortex the protein fraction tube for >1 min and then centrifuge the tube at maximum speed in a refrigerated microcentrifuge for 20 min at 4 °C, with the hinges of the tubes facing away from the center of the rotor.
10. Transfer as much of the upper aqueous layer in the protein fraction tube as possible to the 5 mL polar phase Eppendorf tube using the transfer pipette from **step 4** while avoiding disruption or transfer of the middle protein layer. Weigh the resulting polar fraction, and subtract the initial tube weight to determine the total weight of the polar metabolite fraction (*see* Note 29).
11. Transfer all but ~10–20 µL of the lower nonpolar lipid fraction to the 2 mL glass lipids vial, and return the lipid vials to the vacuum centrifuge, and evaporate to dryness. As soon as the lipid fractions are dry, add 300 µL of chloroform-methanol/BHT solution to them, cap the glass tube tightly, vortex gently, and store at –80 °C until analysis (*see* Note 30).
12. Add 500 µL of cold methanol to the protein fraction, vortex briefly, and then centrifuge the tube at maximum speed in a refrigerated microcentrifuge for 10 min at 4 °C with the hinges of the tubes facing away from the center of the rotor. Carefully aspirate the supernatant using a gel-loading tip via vacuum suction or a 1 mL pipette, taking care not to disrupt the protein pellet (*see* Note 31).
13. Evaporate the protein residues to dryness by vacuum centrifugation (~25–30 min). Subsequently, on the same day, measure and record the mass of the protein fraction tube with the dried protein residue to 0.01 mg and subtract the tare weight of the same tube to obtain the mass of the dry protein residue, if desired. The expected residue mass is 0.5–2 mg (*see* Note 32).
14. The polar fraction can be sub-aliquoted into many fractions and stored in polypropylene screw cap or Eppendorf tubes for different analyses. Half or more of the polar fraction in the 5 mL tube is suitable for analysis by high-field NMR spectroscopy [14, 15]. One eighth of the polar fraction in a 2 mL glass vial is suitable for MTBSTFA derivatization for analysis by GC-MS [14, 16] or ion chromatography (IC)-FTMS analysis [7, 14, 47]. One 16th of the polar fraction

²⁸Loss of some of the protein fraction due to adherence to the walls of the 15 mL conical vial as well as adherence of the protein to the inner and outer walls of the pipette tip is inevitable. The chloroform/methanol-BHT helps to reduce this loss; however, protein recovery is never 100%. Work hard to minimize this fractional loss of protein residue, but most importantly, try to keep the degree of loss consistent across all of the samples.

²⁹The net mass yield of the polar fraction can be used to assess consistency between samples and to determine split ratios for polar metabolite fraction aliquots. After weighing, the entire polar metabolite fraction can be stored at –80 °C for several days before thawing, sub-aliquoting, and lyophilization.

³⁰Avoid excessive drying time and exposure of the lipid fraction to heat/air. BHT is a preservative used in the food industry to stabilize fats; it is added here to slow the peroxidation of unsaturated lipids and fatty acids.

³¹Make sure that the hinges of the microfuge tubes face outward from the center of the microcentrifuge rotor, so that the resulting protein residue pellet is located at the same position at the bottom of every tube.

³²Loss of protein residue during vacuum centrifugation can be minimized by attaching a separate cap cut from additional Eppendorf tubes with 4–8 perforations made using an 18-gauge needle or other perforation tool. Proteins can subsequently be redissolved in lysis buffer and their concentration measured by BCA or Bradford assay.

in a 2 mL screw-cap tube is suitable for multiple direct infusion FT-MS analyses [16, 22, 48] (*see* Note 33).

15. Cap all aliquot tubes with screw caps or snap caps containing 3–6 small perforations on top, and freeze all tubes upright in flexible 9 × 9 cardboard racks by placing them in ~1 cm of liquid nitrogen in a Styrofoam or other cold-resistant tray. After all aliquots reach liquid nitrogen temperature, rapidly transfer the racks to a suitably sized lyophilization can and apply vacuum as quickly as possible. Lyophilize to dryness for 18–24 h; the polar aliquots should remain frozen under the vacuum for the duration of the lyophilization period (*see* Note 11). After lyophilization, replace the perforated caps with tightly sealed pre-labeled caps and store at –80 °C until analysis.

3.3 Acetone Extraction of Culture Medium

Proteins in culture medium are chemically precipitated using cold 80% acetone and removed by centrifugation, and the supernatant is collected for downstream analyses.

1. Add 400 µL of ice-cold acetone to a 100 µL aliquot of culture medium in a microcentrifuge tube.
2. Vortex vigorously and then incubate the samples in a –80 °C freezer for >30 min.
3. Remove the precipitated samples from the freezer, and centrifuge them at maximum speed (15–20 K × *g*) for 10 min at 4 °C.
4. Transfer the clarified supernatant to a new tube(s), and remove the solvent using either a vacuum microcentrifuge, or freeze-dry using a lyophilizer fitted with a liquid nitrogen pre-trap (*see* Notes 34 and 35).

3.4 Culture Medium Protein Removal Using Ultrafiltration

Proteins are removed by centrifugal ultrafiltration using 10,000 molecular-weight-cutoff dialysis membranes, and the flowthrough is collected for downstream analyses.

1. 300–400 µL of culture medium is added to the top of an ultrafiltration spin column (e.g., Millipore Ultracel YM-10) after extensive wash with ddH₂O.
2. Centrifuge the spin column at 5000 × *g* for 30 min at 4 °C in a microcentrifuge.
3. Collect 100 µL of the filtrate flowthrough, and remove the solvent using either a vacuum microcentrifuge, or freeze-dry using a lyophilizer fitted with a liquid nitrogen cold trap (*see* Notes 35 and 36).

³³Record the mass of each polar aliquot as it is pipetted in order to determine the exact fraction represented by the aliquot, by dividing the fraction mass by the total mass of the polar fraction. Aliquoting into the polar fraction tubes can be performed directly on an analytical balance and the polar fraction masses recorded as they are being aliquoted.

³⁴Approximately 50–75% of the dried culture medium extract is sufficient for analysis by ¹H NMR, and 1/8–1/16 quantity of the extract is suitable for mass spectrometry applications.

³⁵The choice of solvent removal method for culture medium samples depends on the lability of the metabolite(s) of interest. For example, while lactate and glucose are relatively stable to temperature and pH, other metabolites of potential interest such as glutamine and pyruvate will degrade during vacuum centrifugation.

³⁶Approximately 50–75% of the dried extract is sufficient for analysis by ¹H NMR.

Acknowledgments

This work was supported by the Intramural Research Program of the National Cancer Institute, NIH, NIH 1U24DK097215-01A1, 1P01CA163223-01A1, and 3R01ES022191-04S1.

References

1. Lane AN, Higashi RM, Fan TW (2016) Preclinical models for interrogating drug action in human cancers using stable isotope resolved metabolomics (SIRM). *Metabolomics* 12(7). 10.1007/s11306-016-1065-y
2. Buescher JM, Antoniewicz MR, Boros LG, Burgess SC, Brunengraber H, Clish CB, DeBerardinis RJ, Feron O, Frezza C, Ghesquiere B, Gottlieb E, Hiller K, Jones RG, Kamphorst JJ, Kibbey RG, Kimmelman AC, Locasale JW, Lunt SY, Maddocks OD, Malloy C, Metallo CM, Meuillet EJ, Munger J, Noh K, Rabinowitz JD, Ralser M, Sauer U, Stephanopoulos G, St-Pierre J, Tennant DA, Wittmann C, Vander Heiden MG, Vazquez A, Vousden K, Young JD, Zamboni N, Fendt SM (2015) A roadmap for interpreting (13)C metabolite labeling patterns from cells. *Curr Opin Biotechnol* 34:189–201. 10.1016/j.copbio.2015.02.003 [PubMed: 25731751]
3. Mackay GM, Zheng L, van den Broek NJ, Gottlieb E (2015) Analysis of cell metabolism using LC-MS and isotope tracers. *Methods Enzymol* 561:171–196. 10.1016/bs.mie.2015.05.016 [PubMed: 26358905]
4. Sellers K, Fox MP, Bousamra M 2nd, Slone SP, Higashi RM, Miller DM, Wang Y, Yan J, Yuneva MO, Deshpande R, Lane AN, Fan TW (2015) Pyruvate carboxylase is critical for non-small-cell lung cancer proliferation. *J Clin Invest* 125(2):687–698. 10.1172/JCI72873 [PubMed: 25607840]
5. Hensley CT, Faubert B, Yuan Q, Lev-Cohain N, Jin E, Kim J, Jiang L, Ko B, Skelton R, Loudat L, Wodzak M, Klimko C, McMillan E, Butt Y, Ni M, Oliver D, Torrealba J, Malloy CR, Kernstine K, Lenkinski RE, DeBerardinis RJ (2016) Metabolic heterogeneity in human lung tumors. *Cell* 164(4):681–694. 10.1016/j.cell.2015.12.034 [PubMed: 26853473]
6. Hui S, Ghergurovich JM, Morscher RJ, Jang C, Teng X, Lu W, Esparza LA, Reya T, Le Z, Yanxiang Guo J, White E, Rabinowitz JD (2017) Glucose feeds the TCA cycle via circulating lactate. *Nature* 551(7678):115–118. 10.1038/nature24057 [PubMed: 29045397]
7. Sun RC, Fan TW, Deng P, Higashi RM, Lane AN, Le AT, Scott TL, Sun Q, Warmoes MO, Yang Y (2017) Noninvasive liquid diet delivery of stable isotopes into mouse models for deep metabolic network tracing. *Nat Commun* 8(1):1646. 10.1038/s41467-017-01518-z [PubMed: 29158483]
8. Faubert B, Li KY, Cai L, Hensley CT, Kim J, Zacharias LG, Yang C, Do QN, Doucette S, Burguete D, Li H, Huot G, Yuan Q, Wigal T, Butt Y, Ni M, Torrealba J, Oliver D, Lenkinski RE, Malloy CR, Wachsmann JW, Young JD, Kernstine K, DeBerardinis RJ (2017) Lactate metabolism in human lung tumors. *Cell* 171(2):358–371 e359. 10.1016/j.cell.2017.09.019 [PubMed: 28985563]
9. Davidson SM, Papagiannakopoulos T, Olenchock BA, Heyman JE, Keibler MA, Luengo A, Bauer MR, Jha AK, O'Brien JP, Pierce KA, Gui DY, Sullivan LB, Wasylenko TM, Subbaraj L, Chin CR, Stephanopoulos G, Mott BT, Jacks T, Clish CB, Vander Heiden MG (2016) Environment impacts the metabolic dependencies of Ras-driven non-small cell lung cancer. *Cell Metab* 23(3):517–528. 10.1016/j.cmet.2016.01.007 [PubMed: 26853747]
10. Keibler MA, Wasylenko TM, Kelleher JK, Iliopoulos O, Vander Heiden MG, Stephanopoulos G (2016) Metabolic requirements for cancer cell proliferation. *Cancer Metab* 4:16. 10.1186/s40170-016-0156-6 [PubMed: 27540483]
11. Kim J, Hu Z, Cai L, Li K, Choi E, Faubert B, Bezwada D, Rodriguez-Canales J, Villalobos P, Lin YF, Ni M, Huffman KE, Girard L, Byers LA, Unsal-Kacmaz K, Pena CG, Heymach JV, Wauters E, Vansteenkiste J, Castrillon DH, Chen BPC, Wistuba I, Lambrechts D, Xu J, Minna JD, DeBerardinis RJ (2017) CPS1 maintains pyrimidine pools and DNA synthesis in KRAS/LKB1-mutant lung cancer cells. *Nature* 546(7656):168–172. 10.1038/nature22359 [PubMed: 28538732]
12. Lane AN, Tan J, Wang Y, Yan J, Higashi RM, Fan TW (2017) Probing the metabolic phenotype of breast cancer cells by multiple tracer stable isotope resolved metabolomics. *Metab Eng* 43(Pt B):125–136. 10.1016/j.ymben.2017.01.010 [PubMed: 28163219]

13. Liu L, Shah S, Fan J, Park JO, Wellen KE, Rabinowitz JD (2016) Malic enzyme tracers reveal hypoxia-induced switch in adipocyte NADPH pathway usage. *Nat Chem Biol* 12 (5):345–352. 10.1038/nchembio.2047 [PubMed: 26999781]
14. Crooks DR, Maio N, Lane AN, Jarnik M, Higashi RM, Haller RG, Yang Y, Fan TWM, Linehan M, Rouault TA (2018) Acute loss of iron-sulfur clusters results in metabolic reprogramming and generation of lipid droplets in mammalian cells. *J Biol Chem* 293:8297. 10.1074/jbc.RA118.001885 [PubMed: 29523684]
15. Fan TW, Lane AN (2011) NMR-based stable isotope resolved metabolomics in systems biochemistry. *J Biomol NMR* 49(3–4):267–280. 10.1007/s10858-011-9484-6 [PubMed: 21350847]
16. Higashi RM, Fan TW, Lorkiewicz PK, Moseley HN, Lane AN (2014) Stable isotope-labeled tracers for metabolic pathway elucidation by GC-MS and FT-MS. *Methods Mol Biol* 1198:147–167. 10.1007/978-1-4939-1258-2_11 [PubMed: 25270929]
17. Jang C, Chen L, Rabinowitz JD (2018) Metabolomics and isotope tracing. *Cell* 173 (4):822–837. 10.1016/j.cell.2018.03.055 [PubMed: 29727671]
18. Bruntz RC, Lane AN, Higashi RM, Fan TW (2017) Exploring cancer metabolism using stable isotope-resolved metabolomics (SIRM). *J Biol Chem* 292(28):11601–11609. 10.1074/jbc.R117.776054 [PubMed: 28592486]
19. Fan TW-M (2012) *The handbook of metabolomics*. Springer, New York
20. Lane AN, Fan TW, Xie Z, Moseley HN, Higashi RM (2009) Isotopomer analysis of lipid biosynthesis by high resolution mass spectrometry and NMR. *Anal Chim Acta* 651 (2):201–208. 10.1016/j.aca.2009.08.032 [PubMed: 19782812]
21. Lane AN, Arumugam S, Lorkiewicz PK, Higashi RM, Laulhe S, Nantz MH, Moseley HN, Fan TW (2015) Chemoselective detection and discrimination of carbonyl-containing compounds in metabolite mixtures by 1H-detected 15N nuclear magnetic resonance. *Magn Reson Chem* 53(5):337–343. 10.1002/mrc.4199 [PubMed: 25616249]
22. Yang Y, Fan TW, Lane AN, Higashi RM (2017) Chloroformate derivatization for tracing the fate of amino acids in cells and tissues by multiple stable isotope resolved metabolomics (mSIRM). *Anal Chim Acta* 976:63–73. 10.1016/j.aca.2017.04.014 [PubMed: 28576319]
23. Lee WN, Boros LG, Puigjaner J, Bassilian S, Lim S, Cascante M (1998) Mass isotopomer study of the nonoxidative pathways of the pentose cycle with [1,2-¹³C₂]glucose. *Am J Phys* 274(5 Pt 1):E843–E851
24. Saxena N, Maio N, Crooks DR, Ricketts CJ, Yang Y, Wei MH, Fan TW, Lane AN, Sourbier C, Singh A, Killian JK, Meltzer PS, Vocke CD, Rouault TA, Linehan WM (2016) SDHB-deficient cancers: the role of mutations that impair Iron sulfur cluster delivery. *J Natl Cancer Inst* 108(1):d1v287. 10.1093/jnci/d1v287
25. Lewis CA, Parker SJ, Fiske BP, McCloskey D, Gui DY, Green CR, Vokes NI, Feist AM, Vander Heiden MG, Metallo CM (2014) Tracing compartmentalized NADPH metabolism in the cytosol and mitochondria of mammalian cells. *Mol Cell* 55(2):253–263. 10.1016/j.molcel.2014.05.008 [PubMed: 24882210]
26. Moseley HN, Lane AN, Belshoff AC, Higashi RM, Fan TW (2011) A novel deconvolution method for modeling UDP-N-acetyl-D-glucosamine biosynthetic pathways based on (¹³C mass isotopologue profiles under nonsteady-state conditions. *BMC Biol* 9:37. 10.1186/1741-7007-9-37 [PubMed: 21627825]
27. Moseley HN (2010) Correcting for the effects of natural abundance in stable isotope resolved metabolomics experiments involving ultrahigh resolution mass spectrometry. *BMC Bioinformatics* 11:139. 10.1186/1471-2105-11-139 [PubMed: 20236542]
28. Freshney RI (2005) *Culture of animal cells: a manual of basic techniques*, 5th edn. John Wiley & Sons, Inc., Hoboken, NJ
29. Lane AN, Fan TW, Bousamra M 2nd, Higashi RM, Yan J, Miller DM (2011) Stable isotope-resolved metabolomics (SIRM) in cancer research with clinical application to non-small cell lung cancer. *OMICS* 15(3):173–182. 10.1089/omi.2010.0088 [PubMed: 21329461]
30. Theobald U, Mailinger W, Baltes M, Rizzi M, Reuss M (1997) In vivo analysis of metabolic dynamics in *Saccharomyces cerevisiae*: I. experimental observations. *Biotechnol Bioeng*

- 55(2):305–316. 10.1002/(SICI)1097-0290(19970720)55:2<305::AID-BIT8>3.0.CO;2-M [PubMed: 18636489]
31. Zhang GF, Sadhukhan S, Tochtrop GP, Brunengraber H (2011) Metabolomics, pathway regulation, and pathway discovery. *J Biol Chem* 286(27):23631–23635. 10.1074/jbc.R110.171405 [PubMed: 21566142]
32. Vasilakou E, Machado D, Theorell A, Rocha I, Noh K, Oldiges M, Wahl SA (2016) Current state and challenges for dynamic metabolic modeling. *Curr Opin Microbiol* 33:97–104. 10.1016/j.mib.2016.07.008 [PubMed: 27472025]
33. Cantor JR, Abu-Remaileh M, Kanarek N, Freinkman E, Gao X, Louissaint A Jr, Lewis CA, Sabatini DM (2017) Physiologic medium rewires cellular metabolism and reveals uric acid as an endogenous inhibitor of UMP synthase. *Cell* 169(2):258–272 e217. 10.1016/j.cell.2017.03.023 [PubMed: 28388410]
34. Psychogios N, Hau DD, Peng J, Guo AC, Mandal R, Bouatra S, Sinelnikov I, Krishnamurthy R, Eisner R, Gautam B, Young N, Xia J, Knox C, Dong E, Huang P, Hollander Z, Pedersen TL, Smith SR, Bamforth F, Greiner R, McManus B, Newman JW, Goodfriend T, Wishart DS (2011) The human serum metabolome. *PLoS One* 6(2): e16957. 10.1371/journal.pone.0016957 [PubMed: 21359215]
35. Heeneman S, Deutz NE, Buurman WA (1993) The concentrations of glutamine and ammonia in commercially available cell culture media. *J Immunol Methods* 166(1):85–91 [PubMed: 8228290]
36. Nikfarjam L, Farzaneh P (2012) Prevention and detection of mycoplasma contamination in cell culture. *Cell J* 13(4):203–212 [PubMed: 23508237]
37. Sanchez JF, Crooks DR, Lee CT, Schoen CJ, Amable R, Zeng X, Florival-Victor T, Morales N, Truckenmiller ME, Smith DR, Freed WJ (2006) GABAergic lineage differentiation of AF5 neural progenitor cells in vitro. *Cell Tissue Res* 324(1):1–8 [PubMed: 16408195]
38. Liu X, Ser Z, Locasale JW (2014) Development and quantitative evaluation of a high-resolution metabolomics technology. *Anal Chem* 86(4):2175–2184. 10.1021/ac403845u [PubMed: 24410464]
39. Sheikh KD, Khanna S, Byers SW, Fornace A Jr, Cheema AK (2011) Small molecule metabolite extraction strategy for improving LC/MS detection of cancer cell metabolome. *J Biomol Tech* 22(1):1–4 [PubMed: 21455475]
40. Wu JT, Wu LH, Knight JA (1986) Stability of NADPH: effect of various factors on the kinetics of degradation. *Clin Chem* 32(2):314–319 [PubMed: 3943190]
41. Nagana Gowda GA, Gowda YN, Raftery D (2015) Massive glutamine cyclization to pyroglutamic acid in human serum discovered using NMR spectroscopy. *Anal Chem* 87 (7):3800–3805. 10.1021/ac504435b [PubMed: 25746059]
42. Purwaha P, Silva LP, Hawke DH, Weinstein JN, Lorenzi PL (2014) An artifact in LC-MS/MS measurement of glutamine and glutamic acid: in-source cyclization to pyroglutamic acid. *Anal Chem* 86(12):5633–5637. 10.1021/ac501451v [PubMed: 24892977]
43. Deng P, Higashi RM, Lane AN, Bruntz RC, Sun RC, Ramakrishnam Raju MV, Nantz MH, Qi Z, Fan TW (2017) Quantitative profiling of carbonyl metabolites directly in crude biological extracts using chemoselective tagging and nanoESI-FTMS. *Analyst* 143 (1):311–322. 10.1039/c7an01256j [PubMed: 29192912]
44. Kimball E, Rabinowitz JD (2006) Identifying decomposition products in extracts of cellular metabolites. *Anal Biochem* 358(2):273–280. 10.1016/j.ab.2006.07.038 [PubMed: 16962982]
45. Rabinowitz JD, Kimball E (2007) Acidic acetonitrile for cellular metabolome extraction from *Escherichia coli*. *Anal Chem* 79 (16):6167–6173. 10.1021/ac070470c [PubMed: 17630720]
46. Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD (2009) Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat Chem Biol* 5 (8):593–599. 10.1038/nchembio.186 [PubMed: 19561621]
47. Fan TW, Warmoes MO, Sun Q, Song H, Turchan-Cholewo J, Martin JT, Mahan A, Higashi RM, Lane AN (2016) Distinctly perturbed metabolic networks underlie differential tumor tissue damages induced by immune modulator beta-glucan in a two-case ex vivo non-small-cell lung cancer study. *Cold Spring Harb Mol Case Stud* 2(4):a000893. 10.1101/mcs.a000893 [PubMed: 27551682]

48. Lorkiewicz P, Higashi RM, Lane AN, Fan TW (2012) High information throughput analysis of nucleotides and their isotopically enriched isotopologues by direct-infusion FTICR-MS. *Metabolomics* 8(5):930–939. 10.1007/s11306-011-0388-y [PubMed: 23101002]
49. Welshons WV, Wolf MF, Murphy CS, Jordan VC (1988) Estrogenic activity of phenol red. *Mol Cell Endocrinol* 57(3):169–178 [PubMed: 3402660]
50. Bindal RD, Carlson KE, Katzenellenbogen BS, Katzenellenbogen JA (1988) Lipophilic impurities, not phenolsulfonphthalein, account for the estrogenic activity in commercial preparations of phenol red. *J Steroid Biochem* 31 (3):287–293 [PubMed: 3419159]
51. Villas-Boas SG (2007) *Metabolome analysis: an introduction*. Wiley-Interscience series in mass spectrometry. Wiley-Interscience, Hoboken, NJ
52. Kelly AE, Ou HD, Withers R, Dotsch V (2002) Low-conductivity buffers for high-sensitivity NMR measurements. *J Am Chem Soc* 124 (40):12013–12019 [PubMed: 12358548]

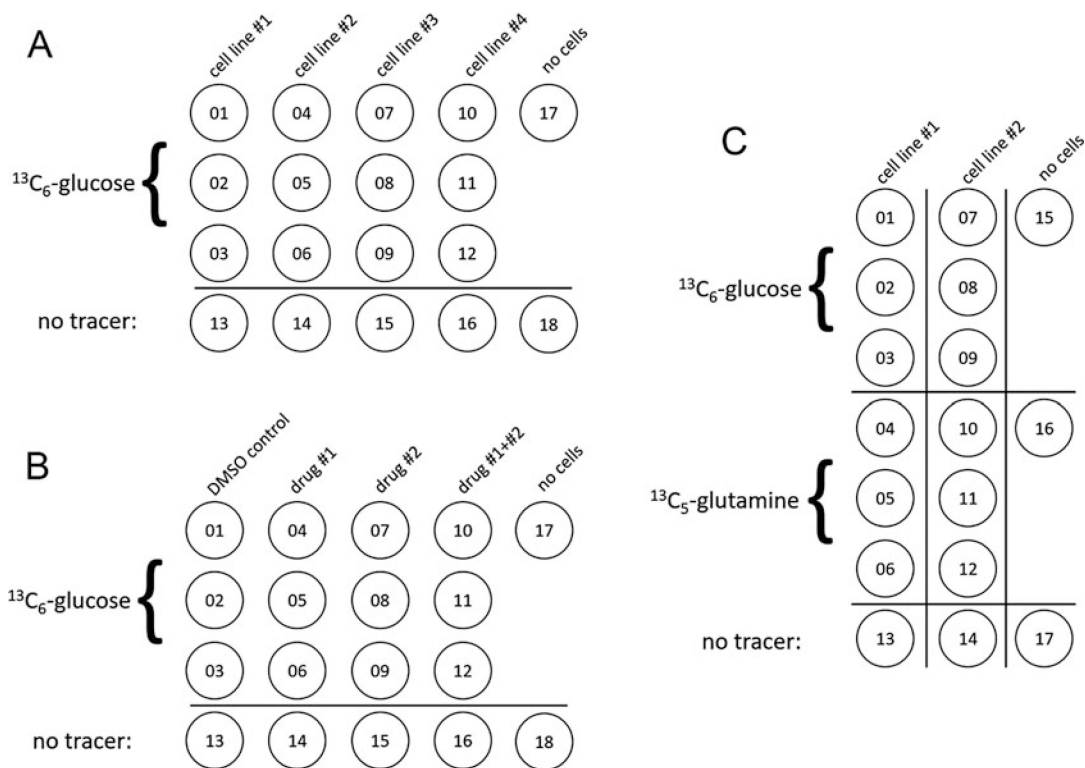


Fig. 1. Examples of experimental setups for stable isotope tracer experiments.

(a) Sample dish configuration for simultaneous $^{13}\text{C}_6\text{-glucose}$ labeling of four cell lines, including unlabeled replicate plates (#13–16) incubated with ^{12}C -glucose, as well as a blank replicate plate incubated with media but no cells. (b) Sample dish configuration for evaluation of metabolic perturbations induced by two drugs separately or in combination. Note that pilot experiments should be performed to determine the IC_{50} of each drug as well as the combination of both drugs, which may act antagonistically or synergistically. (c) Sample dish configuration for the comparative labeling of two cell lines with two different tracers, including unlabeled control replicates and the blank plate incubated with media but no cells

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Total metabolite concentrations measured amperometrically (YSI 2950 Bioanalyzer) in non-dialyzed and dialyzed FBS samples as well as laboratory water sources

	Standard heat-inactivated FBS				Dialyzed FBS			Water sources	
	Mfg #1, batch #1	Mfg #1, batch #2	Mfg #2, batch #1	Mfg #2, batch #2	Mfg #1, batch #1	Mfg #1, batch #2	Mfg #1, batch #2	Tap H ₂ O, Bethesda, MD, USA	ddH ₂ O
D-glucose, mM	7.95	4.74	3.82	2.75	0.05	0.08	0.04	0.00	0.00
L-lactate, mM	18.2	8.25	14.44	17.9	0.17	0.18	0.03	0.00	0.00
L-glutamine, mM	0.38	0.1	0.65	0.47	0.01	0.01	0.00	0.00	0.00
L-glutamate, mM	0.87	0.57	0.67	0.75	0.00	0.00	0.02	0.03	0.03
K ⁺ , mM	10.7	n/a	n/a	10.5	n/a	n/a	0.2	0.0	0.0
NH ₄ ⁺ , mM	2.9	n/a	n/a	2.2	n/a	n/a	0.01	0.0	0.0

Evaporation and changes in metabolite concentrations in culture dishes incubated in humidified versus non-humidified incubators

Table 2

	<i>t</i> = 0	Humidified	Non-humidified
Plate mass, % ctI	100%	98.7%	95.1%
[glucose], mM	11.5	12.1	13.4
[glucose], % <i>t</i> = 0	100%	104.9%	115.8%
[lactate], mM	1.76	1.86	2.06
[lactate], % <i>t</i> = 0	100%	106.0%	117.0%
[glutamine], mM	2.94	2.67	2.90
[glutamine], % <i>t</i> = 0	100%	91.0%	98.7%