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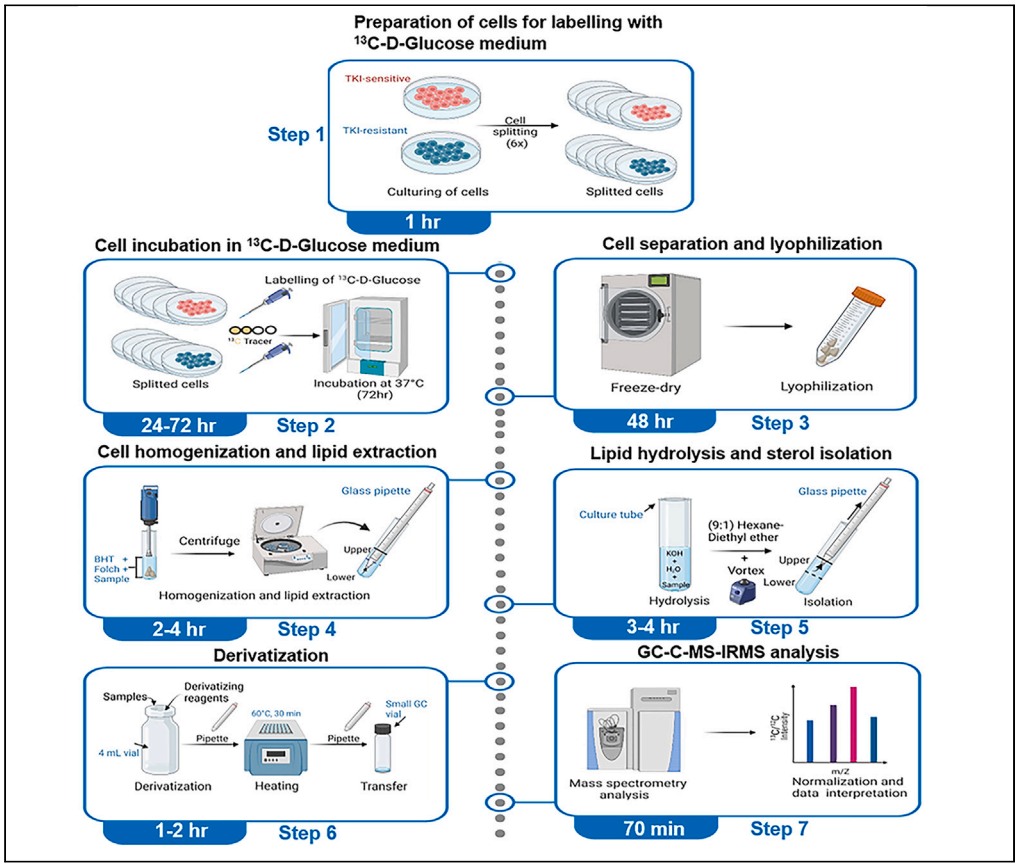
Protocol to track the biosynthesis of cholesterol in cultured HCC cells using ^{13}C compound-specific stable isotopic tracers



Cholesterol biosynthesis supports proliferation and drives resistance to tyrosine kinase inhibitor (TKI) therapy in hepatocellular carcinoma (HCC). Here, we present a protocol for using stable isotopic tracers to track the biosynthesis of cholesterol in cultured HCC cells. We describe steps for cell preparation, incubation, separation, and homogenization. We then detail lipid extraction and compound-specific isotope analysis for comparing and quantifying cholesterol synthesis between TKI-resistant HCC cells and their mock counterparts. This protocol can be expanded for use with other shorter-chained lipids.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Track fatty acid and sterol synthesis during cell cultivation using ^{13}C stable isotopes

Steps for the identification and relative quantification of six fatty acids and sterols

Lipid extraction and compound-specific isotope analysis to quantify cholesterol synthesis

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Protocol to track the biosynthesis of cholesterol in cultured HCC cells using ^{13}C compound-specific stable isotopic tracers

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SUMMARY

Cholesterol biosynthesis supports proliferation and drives resistance to tyrosine kinase inhibitor (TKI) therapy in hepatocellular carcinoma (HCC). Here, we present a protocol for using stable isotopic tracers to track the biosynthesis of cholesterol in cultured HCC cells. We describe steps for cell preparation, incubation, separation, and homogenization. We then detail lipid extraction and compound-specific isotope analysis for comparing and quantifying cholesterol synthesis between TKI-resistant HCC cells and their mock counterparts. This protocol can be expanded for use with other shorter-chained lipids.

BEFORE YOU BEGIN

The experimental procedures described in this protocol include steps necessary to culture HCC cells and label them with a ^{13}Carbon stable-isotope tracer. The significance of the isotopic-tracer is to track the assimilation of a certain compound (here, we use D-Glucose) and its biosynthesis into metabolically important molecules (here, cholesterol).

This protocol can be broadly applied to the analysis of other lipids, such as shorter carbon-chained fatty acids (See Table 1) which are pre-cursor molecules in cholesterol biosynthesis. These lipids are also isolated by our protocol and identified by a Thermo GC IsoLink II with ConFlo IV interface coupled to a Thermo DeltaV Advantage isotope ratio mass spectrometer and a Thermo triple quadrupole mass spectrometer (GC-C-MS-IRMS).

The use of ^{13}C-D-Glucose was chosen since ^{12}C-Glucose represents the major carbon (C) component of the original cell culture media. However, other C compounds of interest could be used if it is artificially enriched with ^{13}C atoms and compatible with the growth media chosen (for example, ^{13}C-bicarbonte if inorganic carbon assimilation is of interest).

Before implementing this protocol, users need to ensure that the use of any human cancer cell lines comply with their institutional guidelines.

Table 1. Shows the GC-C-MS-IRMS method for target analytes

Original compound	Classification	NIST library identifier of analyte	Approximate retention time in min, MS (sec, IRMS)
Myristic acid /^{13}C-Myristic acid	saturated long-chain fatty acid	Methyl tetradecanoate	27 min (1612s)
Methyl palmitate /^{13}C- Methyl palmitate	fatty acid methyl ester	methyl hexadec-9-enoate / 9-Hexadecenoic acid, methyl ester	31.7min (1896s)
Palmitoleic acid /^{13}C-Palmitoleic acid	omega-7 monounsaturated fatty acid	Hexadecanoic acid, methyl ester	32.0 min (1925s)
Oleic acid /^{13}C- Oleic acid	unsaturated fatty acid	9-Octadecenoic acid (Z)-, methyl ester	36.4 min (2180s)
Stearic acid /^{13}C- Stearic acid	saturated long-chain fatty acid	Methyl stearate	37 min (2210s)
Cholesterol /^{13}C- Cholesterol	sterol	Cholesterol, TMS derivative	65 min (3895s)

⚠ CRITICAL: It is important to note that ^{13}C-D-Glucose has isotope values drastically different from natural environmental ^{12}C-D-Glucose. Thus, care should be taken with every step of this protocol to not cross-contaminate samples. Therefore, controls should be processed alongside the treatment samples. It is strongly recommended that separate sets of labware are used for ^{13}C-D-Glucose treatments and natural ^{12}C-D-Glucose treatments.

⚠ CRITICAL: All steps involving chemical reagents should be conducted in a properly working fume hood, wearing proper lab personal protective equipment (PPE).

Note: After proper cleaning, all glassware used throughout the protocol should be pre-combusted in a muffle-furnace at 500°C for at least five hours to ensure the combustion of any organic material which could potentially contaminate samples.

Institutional permissions

For the experiment involving the culturing of human cancer cell lines, the authors have obtained Biological Safety approval from the Hong Kong Polytechnic University.

Preparation of cells for labeling with ^{13}C-D-Glucose medium

Timing: 1 h

1. Sorafenib-resistant HCC and their corresponding mock cells derived from MHCC-97L are cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose and L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 mg/mL penicillin G, and 50 μg/mL streptomycin at 37°C in 10 cm dish.
2. Rinse cells with 7 mL of PBS (-).
3. Aspirate PBS (-).
4. Add 5 mL of 0.05% Trypsin.
5. Incubate cells at 37°C for 3–5 min.
6. Tap the side of the culture dish to detach cells from the plate completely.
7. Collect cells into a 15 mL collection tube using PBS supplemented with 10% FBS.
8. Centrifuge the collection tube at 1500 × *g* for 5min.
9. Discard the supernatant.
10. Suspend cells with 2 mL of complete medium.
11. Take 10 μL of cell suspension into a new 1.5 mL tube and mix with 10 μL of 0.4% Trypan blue solution.
12. Transfer 10 μL of the mixture onto a cell counting hemocytometer.
13. Count cells with cell counting hemocytometer.
14. Seed 2 × 10^6 live cells to six 10 cm culture dishes.

Figure 1. – Freeze-dried samples of MHCC-97L
The pellet of MHCC-97L cells (Mock) in six 10 cm culture dishes was freeze-dried for 48 h.

Cell incubation in ^{13}C-D-Glucose medium

Timing: 24–72 h

15. Remove the cultured medium after seeding the cells for approximately 12 h.
16. Dissolve 36 mg D-Glucose (U-^{13}C6, 99%) in 500 mL of Dulbecco's Modified Eagle Medium (DMEM) with high glucose and L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 mg/mL penicillin G, and 50 μg/mL streptomycin.
17. Add 10 mL per 10 cm culture dish.
18. Incubate for 24–72 h.

⚠ CRITICAL: It is important that a minimum of 5^10^5 cells are harvested per treatment.

⚠ CRITICAL: It is important that minimal incubation time of U-^{13}C6 is 24 h.

Cell separation and lyophilization

Timing: ~2 days

19. Aspirate the cultured medium.
20. Rinse cells with 7 mL of PBS (-) for twice.
21. Aspirate PBS (-).
22. Add 5 mL of 0.05% Trypsin.
23. Incubate cells at 37°C for 3–5 min.
24. Tap the side of the culture dish to detach cells from the plate completely.
25. Collect cells into a 50 mL collection tube using PBS (-) supplemented with 10% FBS.
26. Centrifuge the collection tube at 1500 × *g* for 5 min.
27. Discard the supernatant.
28. Wash the cell pellets with PBS (-) for two times.
29. Aspirate PBS (-).
30. Place pellet in 5 mL collection tube to freeze dryer.
31. Incubate them for 2 days.
32. Weight the dry mass of the pellet.
33. Freeze the pellet at −20°C.
34. Store freeze-dried samples in plastic 5 mL collection tubes (Figure 1).

⚠ CRITICAL: It is important that the pellet should be freeze dried for 2 days.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Milli-Q or similar quality water	-	-
Cholesterol	Sigma-Aldrich	Cat# C8667
Methyl-β-cyclodextrin	Sigma-Aldrich	Cat# C4555
L-glutamine	Thermo Fisher Scientific	Cat# 25030081
Butylated hydroxytoluene (2,6-Di-tert-butyl-4-methyl-phenol	Sigma-Aldrich	Cat# B1378
Dichloromethane	RCI Labscan LTD	Cat# LC1040A
Isooctane (2,2,4-trimethylpentane) (HPLC Grade)	AQA	Cat# TP-1452-4000
N,O-Bis(trimethylsilyl) trifluoroacetamide	Sigma-Aldrich	Cat# 25561-30-2
Methanol (HPLC grade)	AQA	Cat# MA-1292-4000
Chloroform (HPLC grade)	VWR PROLABO	Cat# 83626.320
Ethanol, 95%	UNI-Chem	Cat# 64-17-5
Potassium hydroxide	Sigma-Aldrich	Cat# 484016
n-Hexane (HPLC grade)	Arcos Organics	Cat# 232100010
Diethyl ether	ACS	Cat# EE-1211-4000
D-Glucose (U-^{13}C6, 99%)	Cambridge Isotope Laboratories, Inc.	Cat# CLM-1396-10
Phosphate-buffered saline (PBS)	Sigma-Aldrich	Cat# P4417
Penicillin–Streptomycin (P/S) (5,000 U/mL)	Gibco	Cat# 15070063
DMEM medium, high glucose (DMEM)	Gibco	Cat# 11965092
Fetal bovine serum (FBS)	Gibco	Cat# 10270106
Trypan blue solution, 0.4%	Thermo Fisher Scientific	Cat# 15250061
Experimental models: Cell lines		
PLC/PRF/5	Japan Cancer Research Bank	JCRB0406
MHCC-97L	Mok et al.[1]	N/A
Software and algorithms		
Rstudio	Posit Software, PBC formerly RStudio, PBC	Any version
IsoDat Acquisition and Workspace	Thermo Fischer Scientific	Version 3.0
Chromeleon Console	Thermo Fischer Scientific	Version 7
Other		
Glass Pasteur pipettes	Kimble	Cat# M4150NO250SP4
Borosilicate culture tubes (16 × 100 mm) with PTFE liner caps	Pyrex	Cat# ref. 9826-16
Glass GC vial, 2 mL, 12 × 32 mm	ALWSCI Corp.	Cat# ref. 2ML-9-V1002
GC vial caps, 9 mm with PTFE liner	Agilent Technologies	Cat# 5182-0717
5 × 29 mm Conical glass insert	ALWSCI Corp.	Cat# C0000074
Borosilicate 4 mL vials, 14.75 × 45 mm	Machery-Nagel	Cat# 702962
Borosilicate 4 mL vials caps with PTFE liner, 13 mm	SUPELCO	Cat# 27141
5-mL tubes	SPL Life Sciences	Cat# 51105
15-mL tubes	SPL Life Sciences	Cat# 50015
50-mL tubes	SPL Life Sciences	Cat# 50050
10-cm culture dish	TPP	Cat# Z707651
Hemocytometer	Fisher Scientific	Cat# PHCC20040
18-mm round glass coverslips	Fisher Scientific	Cat#12-546-P
Freeze dryer	Labconco	Cat# 7670530
CO_2 incubator	Thermo Scientific	Cat# 51030303
Biological Safety Cabinet	Thermo Scientific	Cat# 1336
Refrigerated centrifuge	Thermo Scientific	Cat# 75004532
Tissue-Tearor	BIOSPEC	Cat# 985370-04
REACTI-THERM Heating Block and Nitrogen Blower	Thermo Scientific	Cat# TS-18824

MATERIALS AND EQUIPMENT

Recipes to make

- BHT solution: Combine 2 mM Butylated hydroxytoluene solution with MQ water at a 1:50 ratio.
  - Store in 4°C.
- FOLCH solution: Mix chloroform and methanol at a 2:1 ratio by volume.
  - Store in 4°C. Should be used within one month of making.
- 0.5 M methanolic potassium hydroxide KOH: Dissolve 14.03 g of KOH in 500 mL of MeOH. This recipe can be expanded as needed if the ratio remains consistent.
  - Store in 4°C.
- Hexane-diethyl ether (9:1): mix hexane with diethyl ether in a 9:1 ratio by volume.
  - Store in 4°C.
- Cholesterol Standard: dissolve 2.5 nM cholesterol in methyl-β-cyclodextrin.
  - Store in 4°C.

⚠ CRITICAL: Chloroform and diethyl-ether are chemicals known to be harmful when inhaled or exposed. Chloroform may be carcinogenic. All reagents should be made in a properly working fume hood, wearing proper lab PPE.

C-MS-IRMS, GC-C-MS-IRMS (Thermo Fischer Scientific Trace 1310 Gas Chromatograph with GC IsoLink II, coupled to a Mass Spectrometer [model. No. TSQ9000] and an Isotope-ratio Mass Spectrometer [model no. Delta V Advantage]).

Alternatives: Any similar GC-IRMS model can be used. Additional standards using known reference materials for all analytes of interest will need to be developed and analyzed under the same chromatographic conditions to identify lipid peaks. If the system does not have the coupled MS function, an aliquot of the sample could be injected separately on an GC-MS system under the same chromatographic conditions as the IRMS to aid with peak identification.

STEP-BY-STEP METHOD DETAILS

Cell homogenization and lipid extraction

Timing: 2–4 h

This section describes the process once you have collected your samples, 5^10^5 freeze-dried cells (^{13}C labeled treatment/s and control), in a 15 mL Eppendorf tube. The weight of dried cell material will depend on the cell culture efficiency but should be on the order of 1–10 mg.

1. Add 4 mL 0.01% BHT solution to all samples in the 15 mL tube.
2. Tear the sample using the Tissue-Tearor.
   a. Place 15 mL tube with sample and BHT reagent in a larger tube (50 mL or similar) filled with ice. This is to keep the sample cold while tissue tearing.
   b. Insert the Tearor probe into the liquid in the 15 mL tube until the metal reaches near the bottom.
      i. Slowly turn on the Tissue-Tearor on until the speed of 6000–8000 RPM (6–8 on the motor switch) is reached.
      ii. Tear the sample for 2 × 20 s pulses, pausing in between each pulse for 10 s to allow the sample to cool.
3. Rinse the Tissue-Tearor.
   a. Have three separate beakers (RO water, EtOH, and RO water), filled with enough liquid to cover the tearing probe.

b. Pulse the probe for 20 s in each of the three breakers, RO, EtOH, and RO to clean the probe between each sample.
c. Replace the first RO water rinse after every new sample. The EtOH and second RO wash can be replaced at will when they appear dirty.

4. Repeat steps 2–3 for all samples.
   a. Store prepared samples, capped, on ice while preparing the remaining.
5. Lipid extract the samples.
   a. Pour 5 mL of cold Folch solution into the 15 mL tube which already contains the sample and BHT solution. Pour another 5 mL of Folch solution into a separate, clean 15 mL tube – this will be used for lipid recovery.
   b. Place 15 mL tube with sample and BHT reagent in a larger tube (50 mL or similar) filled with ice. This is to keep the sample cold while tissue tearing.
   c. Insert the Tearor probe into the sample tube until the metal reaches near the bottom.
      i. Slowly turn on the Tissue-Tearor until the speed of 6000–8000 RPM (6–8 on the motor) is reached.
      ii. Tear the sample for 2 × 20 s pulses, pausing in between each pulse to allow the sample to cool. Set aside on ice.
   d. Using the additional 15 mL tube containing 5 mL of Folch solution, insert the Tearor probe into the liquid until the metal reaches near the bottom.
      i. Slowly turn on the Tissue-Tearor until the speed of 6000–8000 RPM (6–8 on the motor) is reached.
      ii. Tear the liquid for 1 × 20 s. This is to remove and collect any sample or lipid adhered to the Tissue-Tearor.
   e. Combine both tubes into one, which should now contain the sample, 4 mL of BHT solution, and 10 mL of Folch.
6. Rinse the Tissue-Tearor.
   a. Have three separate beakers (RO water, methanol, and RO water), filled with enough liquid to cover the tearing probe.
   b. Pulse the probe for 20 s in each of the three beakers, RO, methanol, and RO to clean the probe between each sample.
   c. Replace the first RO water rinse after every new sample. The methanol and second RO wash can be replaced when they appear dirty.
7. Repeat steps 5–6 for all samples.
   a. Store prepared samples, capped, on ice while preparing the remaining.
8. Vortex all samples for 30 s.
9. Flush each tube with N_2 gas. This is to prevent lipid oxidation during extraction.
10. Store samples diagonally on ice. Place on a shaker table for 30 min at 100–150 rpm.
11. Vortex all samples for 30 s.
12. Centrifuge all samples for 10 min at 3000 × *g*.
    a. This will separate the sample into two phases.
13. Collect the lower organic phase with lipids.
    a. Using a pre-combusted glass Pasteur pipette, slowly lower the tip down the side of the tube and into the lower phase. There may be a white precipitate layer in between the two phases – push the pipette through.
    b. Once the pipette is in the lower phase, slowly draw-up the solution until the pipette is full.
       i. Quickly remove the pipette (without drawing in any upper phase) out of the tube and transfer the liquid into a pre-combusted borosilicate culture tube.
       ii. Cap the culture tube with plastic caps with PTFE liner.
    c. Repeat until all the lower phase is collected.
    d. Repeat for all samples.
14. At approximately 20°C–22°C, dry-down lower phase now in culture tubes under a constant stream of N_2 gas, to avoid any potential oxidation of the lipids. Remove when completely dry, and store in −20°C freezer.

⚠ CRITICAL: The vials must be sealed with N_2 gas during step 9. This is to prevent lipid oxidation during the extraction.

⚠ CRITICAL: Do not cross contaminate ^{12}C and ^{13}C treatments. New reagents for rinsing and new lab materials shall be used for each treatment group. The Tissue Tearor should be thoroughly cleaned between each groups processing. ^{12}C treatment controls should be processed alongside samples and analyzed to ensure no ^{13}C contamination throughout the laboratory processesing.

Note: For the Pasteur pipetting, any pipette bulb or an autopipette can be used to preference.

Pause point: If pausing for several hours, add 250 μL of DCM to the glass culture tubes and store in a −20°C freezer.

Lipid hydrolysis and isolation

Timing: 3–4 h

This section describes the process to hydrolyze the lipids and then isolate and extract the sterols and fatty acids (neutral lipids) from other lipids.

15. Add 1.5 mL of 0.5 M metholated KOH + 1.5 mL of MQ Water (1:1 ratio) to the culture tubes.
16. Flush tube with N_2 gas and vortex for 30 s.
17. Allow the sample to saponify for 2 h at approximately 20°C–22°C in the capped glass culture tube.
    a. Samples should be placed on a shaker table at 70 rpm for this duration.
18. Add 2 mL of the hexane-diethyl ether (9:1) solution to the glass culture tube.
19. Vortex for 30 s and allow to settle.
    a. A two-phase separation will occur. The upper phase of the supernatant contains the sterols and fatty acids (neutral lipids).
20. Collect the upper phase.
    a. Using a pre-combusted glass Pasteur pipette, slowly draw-up the upper phase until the pipette is full.
       i. Transfer the liquid into a new pre-combusted borosilicate culture tube. Cap tube with plastic caps with PTFE liner.
    b. Repeat until all the upper phase is collected.
21. Repeat the hexane-diethyl ether (9:1) solution extraction (steps 18–20) 2× more for each sample, resulting in 3 total extractions. This will result in 6 mL of sample liquid in the final culture tube.
22. Repeat for each sample.
    a. Store prepared samples, capped, on ice while preparing the remaining.
23. At 50°C dry-down each sample under a constant stream of N_2 gas. Remove when completely dry.

Derivatization

Timing: 1–2 h

This section derivatizes the sterols and fatty acids to make them volatile for analysis on the GC-C-MS-IRMS.

24. Resolubilize the dried lipid extract in 100 μL aliquots of isooctane, and transfer to a clean borosilicate 4 mL vial.

a. Repeat this step twice, resulting in 200 μL of liquid sample in the 4 mL vial.
b. After each injection of 100 μL aliquots of isooctane, briefly roll the solvent around the glass to ensure the lipids dissolve. Then pipette the liquid to 4 mL vial.

25. Pipette 100 μL of the cholesterol standard (dissolved in EtOH or DCM) into a separate clean glass GC vial. This will be used as an analytical standard during analysis.
26. At 50°C dry-down each sample under a constant stream of N_2 gas. Be careful not to over-dry the samples as the derivatization process makes compounds more volatile. Remove when completely dry.
27. Dissolve lipids in 50 μL of DCM.
28. Add 100 μL of BSTFA.
29. Flush vials with N_2 gas. Shake gently.
30. Heat vials for 30 min at 60°C in a heating block.
31. After heating, cool the vials in a fridge for 5 min.
32. At approximately 20°C–22°C, dry-down each sample under a constant stream of N_2 gas. Be careful not to over-dry the samples as the derivatization process makes compounds more volatile.
    a. Remove when completely dry.
33. Rinse samples by adding 50 μL of DCM, and drying down under a constant stream of N_2 gas. Be careful not to over-dry the samples. Remove when completely dry.
34. Add 500 μL DCM, shake gently, and using a pipette, transfer to a clean GC vial.
    a. Repeat this step 2 x more, resulting in 1.5 mL of DCM and sample in the GC vial.
35. Analyze samples immediately on GC-C-MS-IRMS for best results.

Note: n-heptane or a similar organic solvent can be used in place of isooctane.

Note: In step 34, ethyl-acetate can be used in place of DCM.

Note: For pipetting, glass pipettes or plastic pipette tips can be used.

Note: Samples can also be analyzed on a standard GC-C-IRMS. However, as this does not allow for molecular identification through the additional MS function as the GC-C-MS-IRMS does, an internal standard containing the additional fatty-acids must be analyzed as well.

Compound-specific isotope analysis of sterols and fatty acids on GC-C-MS-IRMS

Timing: 70 min per replicate injection

This section describes analysis of the derivative (previous section) via Isotope ratio gas chromatography. This protocol uses Thermo GC IsoLink II with ConFlo IV interface coupled to a Thermo DeltaV Advantage isotope ratio mass spectrometer (IRMS) and a Thermo triple quadrupole mass spectrometer (TSQ-MS). This system allows for simultaneous identification of a sample's constituents (MS) and their stable isotope ratio (IRMS).

36. After warming up the instrument, checking the backgrounds for the day and conditioning of the analytical column for the day, it is possible to start the analysis.
37. Analyze 1–2 blanks before analysis (inject DCM or Ethyl-Acetate), to ensure the instrument is working properly.
38. The cholesterol standard should be analyzed in duplicate at the beginning. Each unknown should be analyzed at least in duplicate, although triplicate is best to constrain variation.
39. A typical analytical analysis would be set up like:
    a. Blank, Blank, Cholesterol Standard, Cholesterol Standard, Unknown 1_a, Unknown 1_b, Unknown 1_c, Blank, Cholesterol Standard, Unknown 2_a, Unknown 2_b, Unknown 2_c, ..., Blank, Cholesterol Standard.

Note: The GC was fitted with a 60 m × 0.32 mm x 1 μm ID non-polar fused silica DB-5MS column and run in spitless mode. Helium was used as the carrier gas with flow rate of 2.0 mL min^{-1}. The GC oven programme was 60°C for 1 min, 60°C–140°C at 15°C/min, 140°C–300°C at 4°C/min and then held isothermally at 300°C for 23 min.[2] The backflush was left on for the first 20 min to flush any solvent. The combustion furnace was set to 1000°C. 1 μL of each sample was injected using a autosampler. Samples were analysed in duplicate for the original publication. Future studies could modify the oven method to increase the initial oven temperature ramp until the first peak elution, during the solvent backflush, to decrease overall analysis time. However, for full peak separation of analytes, it is important to keep the oven temperature ramping slow after the solvent is burnt off.

Note: Lipid peaks were identified using the National Institute of Standards and Technology library. Additionally, cholesterol peaks were identified by comparing structure and peak retention times with the in-house cholesterol standard. Derivatisation induced carbon offsets were corrected for each sample based on the known isotope value of the cholesterol standard, which was measured in powder form by a Eurovector Euro EA 3000 coupled to an IRMS (Nu perspective IRMS). Blanks followed by a cholesterol standard were run after every three unknowns in the sequence to eliminate carry-over effects.

Note: This protocol can also be applied to a standard GC-C-IRMS if proper standards are run along with the sample, to allow for chromatography peak identification and consistent analyte retention times (see previous Notes).

Note: These analytes come through in derivative form, with additional carbon atoms from the derivatization process.

EXPECTED OUTCOMES

The underlying premise of this "isotope tracer" experiment is routed in physics, where ^{12}C and ^{13}C behave the same physically and biologically.[3] The ^{13}C, here in artificially increased abundance, can be used to trace metabolic processes and their resulting metabolites – that is, which molecules incorporated the available ^{13}C during biosynthesis. This protocol describes a way to add a form of ^{13}C (here as D-Glucose) to growth media which is then assimilated by cells that are being cultured.

The lipid extraction and sterol isolation protocols were adapted from a previous oxysterol protocol.[4] Our protocol was originally developed for the comparison of two treatments (both which received ^{13}C-D-Glucose) – one that received treatment from the Hepatocellular carcinoma molecular-targeted drug sorafenib, and one treatment that did not – to show that sorafenib upregulated cholesterol biosynthesis in patients. The control in our protocol (Table 2) is a treatment that received normal media with no additional ^{13}C, which was used to assess contamination against the cholesterol standard and to calculate the APE values. None of the treatments showed a significant difference in lipid profiles (Figure 2), indicating that there were no unexplained metabolic effects between the different treatments. Based on the hypothesis that sorafenib increased cholesterol biosynthesis, we expected the treatment that received sorafenib to have higher sorafenib ^{13}C APE values. This can be seen in Figure 3.

The precursors to cholesterol synthesis (shorter-chained fatty-acids, Table 1) can also be analyzed with this method. As is seen in Figure 3, most of the precursor fatty-acids exhibited the same conclusion as cholesterol – higher ^{13}C APE values in the sorafenib treatment than the mock, indicating increased biosynthesis. Although not the goal of the original manuscript,[1] this protocol outlined here has further applications which can be broadened to other lipid synthesis pathways, different ^{13}C sources outside of D-Glucose, and potentially different cell cultures.

Table 2. Example of raw data from GC-C-IRMS, for a selection of analytes

Incubation	Compound	Group	Corrected $\delta^{13}C/^{12}C$	Ampl 44 (mV)	Ampl 45 (mV)	Area all	At% $^{13}C/^{12}C$	RT min
72hr	Cholesterol	Standard	−23.441 ± 0.7	641	753	15.109	1.08002	64.9
72hr	Cholesterol	Control	−25.565	2647	3094	46.68	1.08409	65.0
72hr	^{13}C-Palmitoleic acid	Control	−19.258	1586	3064	7.307	1.831003	32.1
72hr	^{13}C-Cholesterol	Mock	54.993	327	415	4.967	1.169522	64.8
72hr	^{13}C-Cholesterol	Mock	55.030	313	396	4.81	1.168665	64.8
72hr	^{13}C-Cholesterol	Sora	59.453	420	532	6.562	1.174446	64.8
72hr	^{13}C-Cholesterol	Sora	59.378	474	601	7.19	1.174362	64.9
72hr	^{13}C-Palmitoleic acid	Mock	623.755	1033	1968	4.729	1.780959	32.1
72hr	^{13}C-Palmitoleic acid	Mock	624.019	1035	1971	4.76	1.782016	32.1
72hr	^{13}C-Palmitoleic acid	Sora	654.574	1563	3023	7.205	1.833092	32.1
72hr	^{13}C-Palmitoleic acid	Sora	654.093	1586	3064	7.307	1.831003	32.1

The standard deviation for the Cholesterol standard was 0.7‰ throughout the analyses. Control groups were cells grown with ^{12}C glucose and processed in the lab alongside enriched samples to track contamination. Mock and Sora groups were experimental groups grown with ^{13}C-enriched glucose.

QUANTIFICATION AND STATISTICAL ANALYSIS

^{13}C enrichment of analytes should be converted and expressed as Atom Percent Excess (APE).[5] APE is calculated using the following formulas[5]:

$$APE\ ^{13}C = atom\ \%\ ^{13}C_{expirimental\ sample} - atom\ \%^{13}C_{initial\ sample}$$

$$AP^{13}C = \frac{100}{\frac{1}{\left(\left(\frac{\delta}{1000}\right)+1\right)R_{PDB}}+1}$$

where R_x is the isotope ratio of the known international standard (here the Peedee Belemnite carbon standard), sample refers to lipid analyte isotope values in delta (δ) notation, and initial refers to the cholesterol standard isotope values in delta notation.

Note: Some GC-C-IRMS software can export the Atom % values for you. Then, to calculate APE, simply subtract the Atom % value of the standard from the analytes value.

Note: Cholesterol standard replicates had a within analysis standard deviation of ± 0.7 ‰. All analytes isotope values were equivalent or better than this analytical precision.

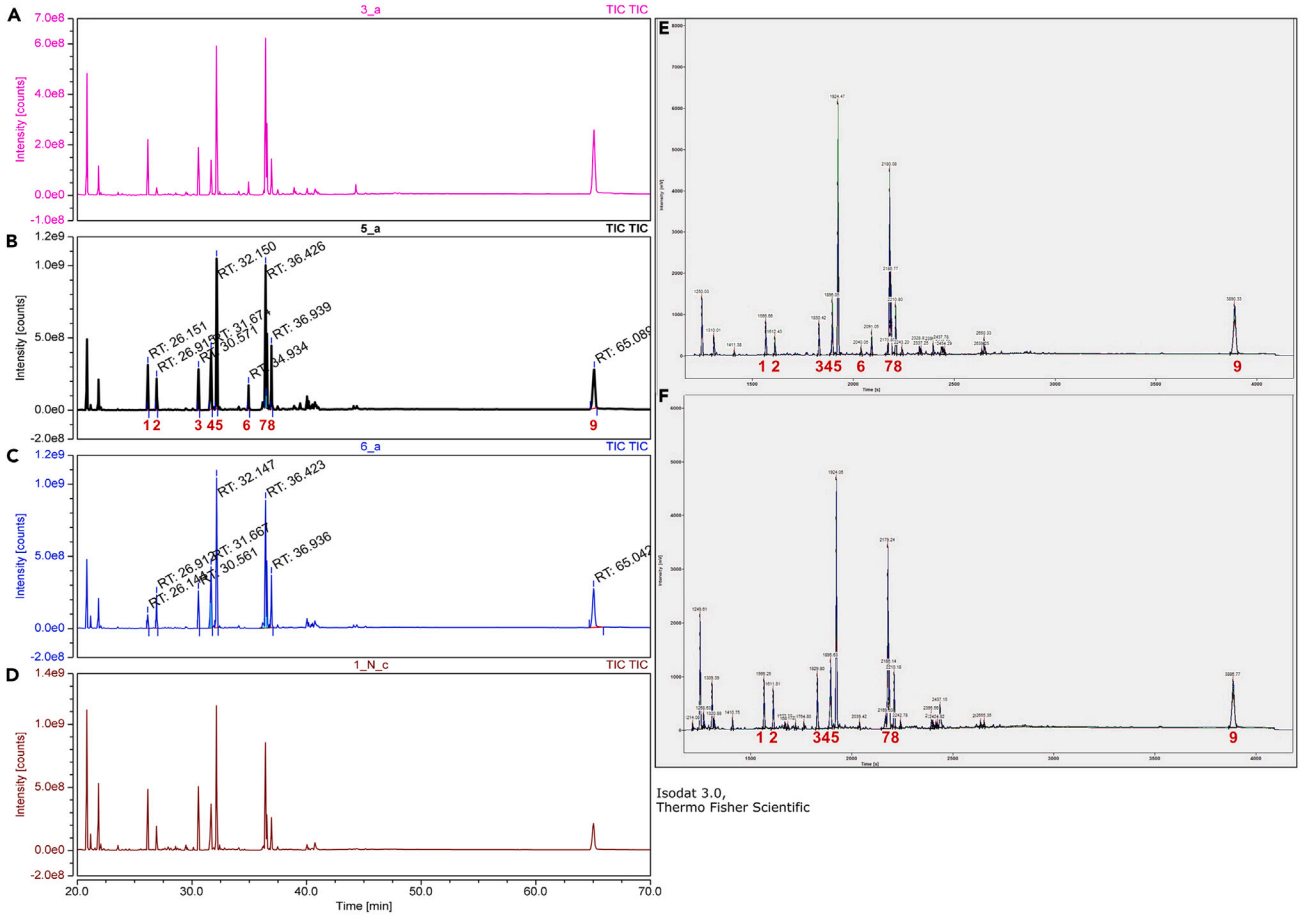
LIMITATIONS

This protocol has only been tested on cell counts of 5^10^5. The use of more cells would be acceptable and would simply require a greater dilution of the derivative (Step 34) with DCM. Culturing less cells is possible, but further testing will be needed to ensure a large enough cholesterol peak can be achieved on the GC-C-IRMS, since cholesterol is typically the lowest peak of all the lipids analyzed.

TROUBLESHOOTING

Problem 1

Certain analyte peaks elute out of the GC at very similar retention times (for example, Methyl palmitate and Palmitoleic acid; Table 1). If care is not taken when choosing the appropriate instrument method/program for analysis (see "compound-specific isotope analysis of sterols and fatty acids on GC-C-MS-IRMS" section) these peaks could co-elute, making their peak integration and isotope value determination impossible.



Peak No.	Ret.Time min	Library Compound	Library
1	26.15	Butylated Hydroxytoluene, TMS derivative	mainlib
2	26.92	Methyl tetradecanoate	mainlib
3	30.57	3,5-Di-tert-butyl-4-hydroxybenzoic acid, TMS derivative	mainlib
4	31.67	Methyl hexadec-9-enoate	mainlib
5	32.15	Hexadecanoic acid, methyl ester	mainlib
6	34.93	Palmitic Acid, TMS derivative	mainlib
7	36.43	9-Octadecenoic acid (Z)-, methyl ester	mainlib
8	36.94	Methyl stearate	mainlib
9	65.09	Cholesterol, TMS derivative	mainlib

Chromeleon 7,
Version 7.2.10.23925, Thermo Fisher Scientific

Printed by TSQ9000

Figure 2. – Typical chromatogram from MS and IRMS

Injections shown are from treatments A/E) mock-PLC/PRF/5, B) mock-MHCC-97L, C) sorafenib-MHCC-97L, D/F) control: MHCC-97L that did not receive ^{13}C-Glucose. The red numbers below Panel B and F correspond to the peak numbers given in the Library Compound Output (purple table). The first two unlabeled peaks were solvent biproducts. Note that Palmitic Acid (#6) was not measured in every sample.

Potential solution

- One possible solution is to decrease the helium flow of the GC from 2.0 mL min^{-1}, perhaps by as much as 0.5 mL min^{-1}. However, it should be noted that decreased flow rates will increase the width of the peaks, potentially affecting the calculated isotopic value. Additionally, or subsequently, you could decrease the speed of the temperature ramping for the GC oven program.

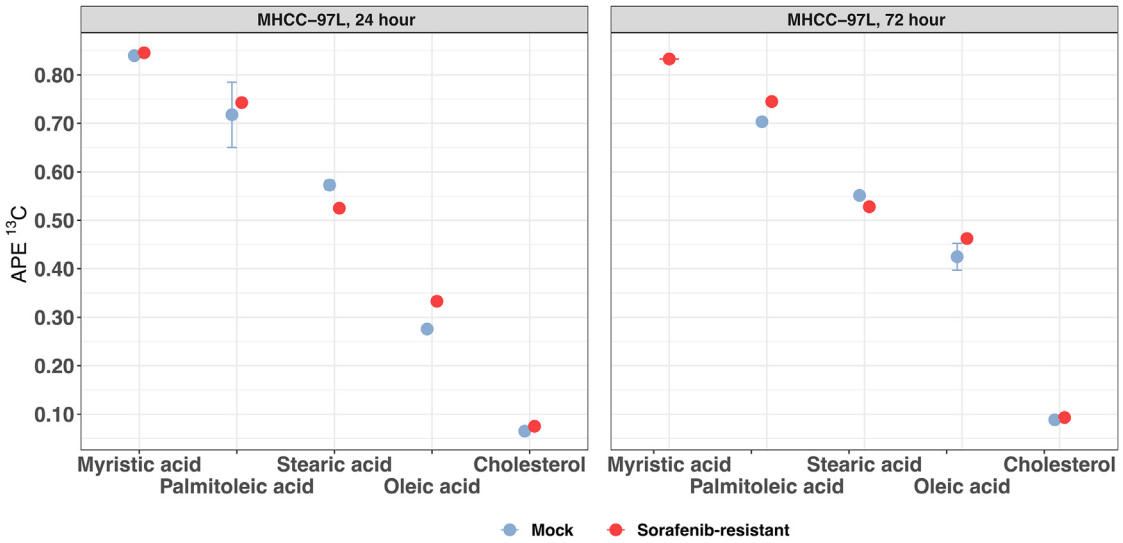


Figure 3. Expected stable isotope analysis results

Results in APE showing the mock and sorafenib treatments for the MHCC-97L cell cultures, for the 24-h (left) and 72-h (right) incubation times. Lipids are organized by carbon chain length (Myristic acid (14), Palmitoleic acid (16), Stearic acid (18), Oleic acid (18), Cholesterol (27). Values for Myristic acid from the Mock 72-h treatment were not analyzed. Error bars indicate the standard deviation from the mean value points.

For example, our current protocol is 140°C–300°C at 4 °C/min – this could be changed to 140°C–300°C at 3 °C/min or similar for increased peak separation. Note, this will increase the overall time of analysis for each injection.

Problem 2

An isotopic "carry-over" effect can result when highly enriched samples are run consecutively on an IRMS, where residual material is retained within the system (e.g., within the combustion reactor or GC column, etc.). Due to the high isotopic values of these samples, this "carry-over" can elute with the next sample, contaminating the results.

Potential solution

- This method went through rigorous pilot testing to identify the level of carry over effect, with numerous analyses testing sequences of alternating injections of highly enriched samples, normal enrichment standards, and blanks (1 μL DCM injections). For example, we ran multiple injections of a highly enriched sample (with isotope values in the 100's of permil), followed by a serious of standards (with isotope values around −20 ‰) to track how long it took for the carry-over to last before the standard values were normal. Then, we repeated this test injecting blanks in between, to see how many blanks to took to clear the carry-over away. We found that injecting one blank was sufficient to remove any carry-over from up to 6 consecutive injections of enriched samples. We therefore created a conservative analysis sequence that injected one blank every 4 enriched injections, making sure to inject a blank before each standard. Using this sequence method, the average values for our standard was −23.4 ± 0.7 achieved throughout the entire analysis.

Problem 3

As mentioned in the "Critical" section after Step 14, the use of isotopically-enriched materials well above natural levels creates a potential for contamination throughout sample processing.

Potential solution

Studies should process a "treatment control" alongside the unknown samples, to monitor any contamination. Here, we processed cultured cells that were grown without ^{13}C-Glucose. These cells

were then prepared and analyzed alongside the isotopically enriched samples. This created a control on the sample treatment itself, which could monitor any contamination (which would result in higher delta values; see Table 2), independent from our analytical standard used to correct the data.

Problem 4

Cell incubation time (Step 18) is important to determine based on your cells of interest, the target analyte, and its metabolic pathway. For example, cholesterol is a complex fatty acid, and newly assimilated carbon will be routed to its production slower than precursors of simpler, shorter chained fatty acids such as Myristic acid. Thus, the incubation time must be long enough to ensure incorporation of the tracer compound (here, ^{13}C-Glucose).

Potential solution

Pilot tests should be conducted to ensure adequate time for isotope tracer routing. Here, we tested both 24- and 72-h incubations. Although 24 h was sufficient to see the assimilated ^{13}C-Glucose in all fatty acids, the effect-size was greater in the 72-h incubation, which was ultimately used for publication.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Jonathan D. Cybulski, cybulski.j@gmail.com.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study does not report datasets and did not generate original code.

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AUTHOR CONTRIBUTIONS

J.D.C. and D.M.B. conceived the protocols. T.K.W.L. and C.O.N.L developed and implemented the cell culturing. J.D.C. developed the protocol and wrote the manuscript. J.D.C. and K.S.L. performed the CSIA analysis. J.D.C. performed the data analysis. T.K.W.L. and D.M.B. acquired or provided funding for this study. All authors reviewed manuscript drafts and approved for publication.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Mok, E.H.K., Leung, C.O.N., Zhou, L., Lei, M.M.L., Leung, H.W., Tong, M., Wong, T.L., Lau, E.Y.T., Ng, I.O.L., Ding, J., et al. (2022). Caspase 3-induced SREBP2 activation drives drug resistance through the promotion of cholesterol biosynthesis in hepatocellular carcinoma. Cancer Res. *82*, 3102–3115.

2. Crandall, J.B., Teece, M.A., Estes, B.A., Manfrino, C., and Ciesla, J.H. (2016). Nutrient acquisition strategies in mesophotic hard corals using compound specific stable isotope analysis of sterols. J. Exp. Mar. Biol. Ecol. *474*, 133–141. https://doi.org/10.1016/j.jembe.2015.10.010.

3. Fry, B. (2006). Stable Isotope Ecology (Springer).

4. Riols, F., and Bertrand-Michel, J. (2018). Analysis of Oxysterols. In Clinical Metabolomics: Methods and Protocols Methods in Molecular Biology, M. Giera, ed. (Humana Press), pp. 267–275. https://doi.org/10.1007/978-1-4939-7592-1_19.

5. Slater, C., Preston, T., and Weaver, L.T. (2001). Stable isotopes and the international system of units. Rapid Commun. Mass Spectrom. *15*, 1270–1273. https://doi.org/10.1002/rcm.328.