¹³C tracer analysis and metabolomics in 3D cultured cancer cells

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i. Abstract

Metabolomics and ^{13}C tracer analysis are state-of-the-art techniques that allow determining the concentration of metabolites and the activity of metabolic pathways, respectively. Three dimensional (3D) cultures of cancer cells constitute an enriched *in vitro* environment that can be used to assay anchorage-independent growth, spheroid formation, and extracellular matrix production by (cancer) cells. Here, we describe how to perform metabolomics and ^{13}C tracer analysis in 3D cultures of cancer cells.

ii. Key Words

Spheroids, 3D cell culture, metabolomics, ^{13}C tracing, GC-MS, LC-MS, cancer metabolism.

1. Introduction

Cell culture is one of the cornerstone techniques of molecular biological research. Ever since its establishment in the 1950's, many researchers have devoted their time to continue to improve culturing techniques (1). In addition to 2D monolayer cultures, which recapitulate a proliferative phenotype, cells can also be cultured in 3D to recapitulate other cancer phenotypes (2-6), such as anchorage-independent growth, spheroid formation, and extracellular matrix production by (cancer) cells (4, 7, 8).

Metabolic reprogramming has been extensively studied in cancer cells (9-11) and the identified changes can be exploited for therapy (12). Cancer cells need to rewire their metabolism to meet their proliferation and survival requirements (13). While metabolomics allows to quantify metabolite concentrations, stable isotope tracer analysis provides information about the fate of metabolites and therefore activity of metabolic pathways (14). By culturing cancer cells in the presence of a stable isotope such as a ^{13}C labelled nutrient, cells will incorporate these isotopically labelled atoms in their metabolites. With mass spectrometry the concentration and labeling patterns of metabolites can be detected. Using these

techniques *in vitro* and *in vivo* (see also Chapters 3, 5, 6, 8, 10) can identify metabolic reprogramming in cancer cells of different phenotype (e.g. proliferation, survival, colonization) (8, 15-17), different genotype (18), and in different environments (19-21). Moreover, integrating these data with computational models has identified flux differences in cancer cells (22).

In this protocol, we describe an *in vitro* 3D culture method that allows cells to form spheroids on soft-agar coated 6-well plates. Additionally, we highlight how these spheroids can be used for metabolomics and ^{13}C tracer analysis. Specifically, we provide a protocol for rapidly stopping (quenching) the metabolism of cancer cell spheroids as well as for subsequent metabolite extraction and sample preparation for gas or liquid chromatography mass spectrometry (GC-MS, LC-MS) analysis.

2. Materials

Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow waste disposal regulations. Use a lab coat, gloves, and if required safety glasses at all times. Work if necessary under the chemical fume hood (for example when preparing/handling quenching, extraction and derivatization solutions) or under the biosafety cabinet (for example when handling cells or cell culture material). Familiarize yourself with chemical/biological safety regulations before preparing the materials.

2.1 Agar

1. 1% [weight/volume] agar: weigh agar in a glass bottle and add the appropriate amount of autoclaved Milli-Q. Autoclave the solution.
2. Autoclave
3. Microwave
4. Cell culture media of choice
5. Sterile plastic bottle to mix the 1% agar with the cell culture media
6. Cell culture tested 6-well plates

2.2 Media containing ^{13}C tracer

1. Cell culture media deprived of the ^{13}C labelled metabolite (for example DMEM/F12 without glucose)
2. Dialyzed (if the ^{13}C labelled metabolite is polar) or charcoal stripped (if the ^{13}C labelled metabolite is a fatty acid) serum
3. Pen-Strep
4. ^{13}C labelled metabolite (for example $^{13}\text{C}_6$ labeled glucose)
5. Weighing paper and spatula
6. Analytical balance (range: 0.0001-50 g)
7. Filter bottle (0.22 μm pore size)

2.3 Cell culture

1. Cells of choice
2. Sterile Trypsin
3. Sterile phosphate-buffered saline (PBS)
4. Sterile cell culture media (for example DMEM/F12)
5. Sterile tracing media (for example DMEM/F12 with $^{13}\text{C}_6$ labeled glucose)
6. Cell counter
7. Biosafety cabinet

2.4 Quenching

Work under the chemical fume hood when preparing quenching solutions.

1. Glass bottle for waste disposal
2. 100 mM ammonium acetate: weigh 3.854 g in a Falcon tube and dissolve in 500 mL autoclaved Milli-Q (see **Note 1**).

3. Quenching buffer: 60% methanol (MS-grade), 10% ammonium acetate (100 mM), 30% autoclaved Milli-Q, store at -20 °C until quenching to ensure low temperature. Prepare in a glass bottle under the chemical fume hood.
4. One small and one bigger Styrofoam box
5. Dry ice
6. Thermometer (-40 to 0°C)
7. Denatured ethanol (96%)
8. Labelled 50 mL Falcon tubes (one per sample)
9. Labelled 15 mL Falcon tubes (one per sample)
10. Labelled Eppendorf tubes (one per sample)
11. Centrifuge (for 15 and 50 mL Falcon tubes)
12. Plastic holder for 50 mL Falcon tubes
13. Chemical fume hood

2.5 Extraction GC and LC-MS

Work under the chemical fume hood when preparing extraction solutions.

1. Safe lock Eppendorf tubes (three per sample), labelled for polar metabolites, non-polar metabolites and protein.
2. Two small and one big Styrofoam box
3. Dry ice
4. Ice
5. Thermometer (-40 to 0°C)
6. Denatured ethanol (96%)

7. Tissue lyser
8. Floating Eppendorf tube holder
9. Internal standard 1: 1 mg/mL norvaline and glutarate in autoclaved Milli-Q (400x), prepare in Eppendorf tube, can be stored at -20 °C up to one year (see **Note 2**).
10. Internal standard 2: 1 mg/mL heptadecanoic acid (C₁₇) in chloroform (MS-grade) (100x), prepare in cleaned glassware, can be stored at -80 °C for 3 to 4 weeks (see **Note 2** and 3).
11. Clean glass bottles for the extraction buffers: wash 3 times with water and 3 times with isopropanol, let dry (see **Note 4**).
12. Extraction buffer 1: 37.5% autoclaved Milli-Q, 62.5% methanol (MS-grade), account for 800 µL per sample. Add appropriate amount of 400x Norvaline/Glutarate, prepare in cleaned glassware, store at -20 °C until extraction (see **Note 2** and 5 – 8).
13. Extraction buffer 2: add 1% internal standard 2 (C₁₇) in chloroform (MS-grade), account for 500 µL per sample, prepare in cleaned glassware, store at -80 °C until extraction (see **Note 2** and 5).
14. Chemical fume hood

2.6 Derivatization for GC-MS

Work under the chemical fume hood when handling derivatization solutions.

2.6.1 Polar metabolites

1. Weighing spatula
2. Analytical balance (range: 0.0001-50 g)
3. Syringe with needle
4. 20 mg/mL O-Methoxyamine-HCl in anhydrous pyridine: Weigh 20 mg methoxyamine in a safe lock Eppendorf tube. Wash a needle with pyridine using a syringe, work under the fume hood and discard the waste appropriately. Aliquot more than 1 mL of pyridine in a safe lock Eppendorf tube using the

washed needle. Add 1 mL of pyridine to the methoxyamine. Vortex until methoxyamine is entirely dissolved. Count for at least 10-20 μL per sample (see **Note 2, 9 and 10**).

5. N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide with 1% tert-Butyldimethylchlorosilane (TBDMS). Store at -20 °C, warm up to room temperature before use. Make sure the bottle is dry before opening it under the chemical fume hood, since humidification impairs derivatization.

6. Glass GC-MS vials with insert and magnetic caps with septum

7. Crimper

8. Heating block (60 °C)

9. Safe lock Eppendorf tubes

10. Chemical fume hood

2.6.2 Non-polar metabolites

1. 2% sulfuric acid in methanol (MS-grade): Add H_2SO_4 dropwise to the methanol. Prepare in cleaned glassware and under the chemical fume hood. Sulfuric acid has to be of high purity, since water will inhibit derivatization. Use clean glass pipets (see **Note 11**). Buffer can be stored up to 14 days at room temperature (see **Note 12**).

2. Hexane (MS-grade). Aliquot in a cleaned glass bottle under the fume hood. The aliquot should not be older than 2 weeks.

3. Saturated NaCl: Pour autoclaved Milli-Q in a cleaned glass bottle. Add as much NaCl to saturate the Milli-Q and a layer of NaCl is on the bottom.

4. Safe lock Eppendorf tubes

5. Glass GC-MS vials with insert and non-magnetic caps with septum

6. Vial Crimper

7. Heating block (60 °C)

8. Chemical fume hood

2.7 Preparation polar metabolites LC-MS

1. 60% Acetonitrile (MS-grade) (see **Note 2**)
2. Plastic vials with insert and plastic caps with septum
3. Chemical fume hood

2.8 Protein quantification for normalization

1. PierceTM BCA protein assay kit
2. 0.2 M NaOH: Weigh 0.8 g NaOH and transfer to a glass bottle. Add autoclaved Milli-Q water to a volume of 100 ml and mix (see **Note 13**).
3. Heating block (up to 90 °C)
4. Autoclaved (PBS)

3. Methods

This protocol is based on the use of 6-well plates. If another format is used, adjust volumes according to surface factor. Pool 4 or more wells per sample to have enough metabolite yield.

Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow waste disposal regulations. Use a lab coat, gloves, and if required safety glasses at all times. Work if necessary under the chemical fume hood and in the biosafety cabinet. Familiarize yourself with chemical/biological safety regulations before conducting the experiment.

3.1 Preparing the soft-agar plates

Work in sterile conditions under the biosafety cabinet.

1. Each 6-well plates will be filled with 3 mL of a 1:1 media and 1% agar solution per well. Calculate the amount of media needed, add up to 10% extra and aliquot in a sterile plastic bottle. Use sterile cell culture media, equivalent to your tracing media (see **Note 14**).
2. Heat up the autoclaved 1% agar solution in the microwave until it is liquid and boiling. Before heating, release the cap of the bottle enough to let the steam escape. Swirl from time to time during heating (see **Note 15**).
4. Prepare a (1:1) mixture of agar and media. Mix well and pipet 3 mL in each well of a 6-well plate (see **Note 16**). Let the plates cool down to room temperature for 2 hours (see **Note 17**).

3.2 Tracing media

Work in sterile conditions under the biosafety cabinet.

1. Take cell culture media without the metabolite you want to trace. Add 1% Pen-Strep and 10% dialyzed serum (when the tracer is a polar metabolite) or 10% charcoal stripped serum (when the tracer is a fatty acid) .
2. Weigh the labelled metabolite in a Falcon tube and add to the culture media at the regular media concentration. Filter the media using a 0.22 μm filter (see **Note 18**).

3.3 Seeding cells

Work in sterile conditions under the biosafety cabinet.

1. Trypsinize your cells.
2. Count the cells and make an appropriate dilution in the tracing media to seed in the agar plates. For fast proliferating cells, take 4 000 cells/mL, for moderately proliferating cells 5 000 cells/mL and for slow proliferating cells 6 000 cells/mL. Adapt cell number if necessary to, ideally, have at least 60 μg of protein at the end of the experiment for a broad coverage of metabolites.

3. Seed the cells in 3 mL tracing media per well on top of the soft-agar. Add 3 mL tracing media without cells to 1 well to measure the initial state. Shake the plates by hand to evenly distribute the single cells through the well (see **Note 19**). Place the plates with cells in a 37 °C 5% CO₂ incubator for 5 days.

Figure 1: Schematic overview of the workflow for harvesting a ¹³C tracer and/or metabolomics experiment of 3D cultured (cancer) cells. Collection of one set of triplicates is depicted, each sample is indicated by a color. The content of 4 6-wells containing 3D cultured (cancer) cells are pooled together in a 15 mL Falcon tube. After spinning the spheroids down, a media sample can be collected. Then the spheroids are quenched in a 50 mL Falcon tube. Extraction of one sample results in 3 phases: polar metabolites, protein and non-polar metabolites. Abbreviations: PM = polar metabolites, PROT = protein, NP = non-polar metabolites.

3.4 Quenching

Work under the chemical fume hood when handling the quenching solution.

1. Fill the big Styrofoam box with a layer of dry ice. Prepare the cold ethanol bath by pouring ethanol in the small Styrofoam box and cool it down using dry ice to -40 °C (measure with thermometer). Place a rack in the cold ethanol bath to hold 50 mL Falcon tubes. Additionally, place the bottle with quenching solution in the cold ethanol bath.

2. Collect the spheroids from at least 4 wells in 1 labelled 15 mL Falcon tubes using a P1000 pipet, this is one sample (Figure 1). Tilt the plate so the volume comes to one side of the 6-well plate. Wash the

well with media from the well by pipetting 1 mL, and squirting it out over the exposed agar to wash down all spheroids. Transfer the media with the spheroids to the 15 mL Falcon tube (see **Note 20** and **21**).

3. When 3 or 4 samples are collected, spin down the spheroids (1.5 min at 300 g). In the meantime, place the correspondingly labelled 50 mL Falcon tubes in the cold ethanol bath, remove the cap and pipet 5 mL of quenching solution in each tube (see **Note 22** and **23**). After spinning down the spheroids, collect 1 mL of media for metabolite measurement (Figure 1) in correspondingly labelled Eppendorf tubes and place in dry ice. Aspirate the remaining media from the 15 mL Falcon tube (see **Note 20** and **24**).

4. Check the temperature of the cold ethanol bath and add dry ice if necessary. Resuspend the spheroids in the remaining media with a P1000 pipet and squirt the spheroids in the cold quenching solution in the 50 mL Falcon tubes (Figure 1), without directly touching the quenching solution. After transferring all 3 or 4 samples, spin them down 1 min at 1000 g. After spinning down, place the 50 mL tubes back in the cold ethanol bath (see **Note 23**). Aspirate the quenching solution and trash it in a waste bottle (see **Note 25**). Wash the spheroids with 10 mL of cold quenching solution and spin again 1 minute at 1000 g. Place them back in the cold ethanol bath and aspirate all quenching solution. Place the samples on dry ice. When all samples are quenched, store them at -80 °C or proceed with the extraction (see **Note 20**).

3.5 Extraction

Work under the chemical fume hood when handling the extraction solutions.

3.5.1 GC-MS

1. Prepare a cold ethanol bath as described in section 3.4. Keep your samples on dry ice until extraction. Additionally, take another box with a mixture of dry ice and wet ice to keep the extraction buffers cold. Take 4 safe lock Eppendorf tubes labelled for the protein fraction and place them in the floating holder in the cold ethanol bath (see **Note 26**). Take the corresponding 50 mL Falcon tubes containing the samples and add 800 µL of extraction buffer 1 to each sample. Resuspend each sample, and transfer each sample to its corresponding Eppendorf tube in the cold ethanol bath (see **Note 2**).

2. Place the tissue lyser in the Eppendorf tube and lyse every sample with the tissue lyser until the spheroids are disintegrated (see **Note 27**). After lysing, add 500 μ L of extraction buffer 2 to each sample and vortex for 10 minutes at 4 °C. In the meantime, start extraction of the next 4 samples. To extract media samples, transfer 20 or 200 μ L media for polar or non-polar metabolites respectively from the media collected before quenching to a labelled safe lock Eppendorf on dry/wet ice. Add 800 μ L of extraction buffer 1 and 500 μ L of buffer 2 and proceed with vortexing for 10 minutes at 4 °C (see **Note 2**).

3. After vortexing, centrifuge for 10 minutes at maximum speed at 4 °C. Centrifugation will result in the separation of 3 phases: the upper phase contains the polar metabolites, the lower phase the non-polar metabolites and in between is a white protein layer (see **Note 28**). Transfer the upper phase to the correspondingly labelled safe lock Eppendorf. If there is an interest in the non-polar phase, blow through the protein layer by pressing the pipet down to 70%, get close to the protein layer and press the remaining 30% while going through the protein layer. Then, pipet the non-polar layer and transfer to the labelled safe lock Eppendorf (see **Note 29**). Once separated, keep all 3 fractions of each sample on dry ice (Figure 1) (see **Note 2** and 30).

4. Dry the samples using a vacuum centrifuge. Polar metabolites should be dried at 4 °C for approximately 8 hours, non-polar metabolites or proteins can be dried for approximately 1 hour at 20 °C. When dried down, store at -80 °C or proceed with derivatization.

3.5.2 LC-MS

Extract the samples in the exact same procedure as for GC-MS extraction (3.5.1). Store at -80 °C or proceed with the preparation for LC-MS analysis.

3.6 Derivatization

Work under the chemical fume hood during derivatization.

3.6.1 GC-MS: Polar metabolites

1. Take the samples from the vacuum centrifuge or dry them down for 20 minutes at room temperature when taken from -80 °C storage (see **Note 31**). Add between 10 to 20 µL of 20 mg/mL methoxyamine in pyridine to each sample (see **Note 9**). Pipet up and down to wash the walls of the Eppendorf tubes. Vortex and spin down briefly, then incubate for 90 min at 37 °C (see **Note 2**).
2. Take TBDMS from -20 °C, and warm and dry to room temperature before using (see **Note 32**). Centrifuge the samples for 3-5 minutes at maximum speed. Transfer 7.5 µL of supernatant to a labelled GC-MS glass vial (see **Note 33**). Seal the glass vials with a magnetic cap using the crimper. Prepare one or more separate glass vials with TBDMS, accounts for 15 µL per sample with an extra 25 µL per vial (see **Note 34**). Seal with a non-magnetic cap. Proceed with GC-MS method and analysis as described by Elia et al. and Christen et al (8, 19) (see **Note 2 and 35**).

3.6.2 GC-MS: non-polar metabolites

1. Dry the samples under vacuum if they were stored at -80 °C. Add 500 µL of 2% sulfuric acid in methanol buffer per sample and incubate 3 hours at 60 °C or overnight at 50 °C (see **Note 2**).
2. After incubation, add 600 µL of MS-grade hexane and 100 µL of saturated NaCl and vortex for 10 minutes. Centrifuge for 5 minutes at maximum speed to separate the phases. Take 300 µL or more from the upper hexane phase, without touching the lower phase, and transfer it to a new Eppendorf tube (see **Note 36**). Dry the samples for 5 to 10 minutes at room temperature under vacuum concentration (see **Note 2**).
3. Resuspend in 50 to 100 µL MS-grade hexane, vortex for 1 minute, transfer to a glass vial and cap with a non-magnetic cap. Store at room temperature. GC-MS method and analysis according to Lorendeau et al. (22) (see **Note 2**).

3.6.3 LC-MS: Polar metabolites

Take the dried polar metabolite fractions and resuspend in 20 µL 60% acetonitrile. Vortex briefly and spin down for 3 minutes at maximum speed. Transfer 17 µL of supernatant to a plastic vial with insert

and seal with a cap with a septum. Store at 4 °C before running the samples on the LC-MS. LC-MS method and analysis as described in Elia et al. (8) (see **Note 2**).

3.7 Protein quantification for normalization

1. Dissolve the dried protein layer from the extraction in 100 µL 0.2 M NaOH and incubate for 20 minutes at 90 °C (see **Note 13**, 37). Let the solution cool down to room temperature and centrifuge shortly.
2. Perform the protein quantification as described by the PierceTM BCA protein assay kit. Use the protein amount to normalize the measured metabolite levels for the amount of cells present in your sample.

4. Notes

1: ammonium acetate is composed of crystals, which will melt during weighing.

2: work under the chemical fume hood.

3: use glass pipets or inert plastic.

4: washing will remove the detergents, which will otherwise result in background peaks on the mass spectrometer.

5: use a glass pipet.

6: always prepare a bit more buffer than needed.

7: include extraction mocks (extraction buffers without a sample) to correct for metabolites present in the extraction buffers.

8: if the absolute concentration of a metabolite has to be determined, extract a metabolite standard curve at the same time.

9: this depends on the yield of your sample, usually for 3D samples 12 µL is recommended.

10: keep methoxyamine and pyridine sealed in parafilm stored in a desiccator to prevent humidification, which impairs derivatization.

11: if the glass pipet for H_2SO_4 will be reused, rinse the glass pipet 3 times with water and 3 times with isopropanol.

12: work under the chemical fume hood and wear safety glasses.

13: work with safety glasses.

14: it is not necessary to use media containing the ^{13}C tracer. The media components will not diffuse out of the agar and dilute your tracing media.

15: agar is a gel and will get very hot.

16: the hotter the agar the better, because this gives an even coating of the well.

17: plates can be stored at 4 °C for 1 week.

18: the amount of tracing media that is prepared needs to be sufficient to seed cells in 3 mL per well, to seed media without cells for the initial state, plus an additional error due to filtering the media.

19: this is very important to prevent clump formation, which occurs when the cells are too close together.

20: work as fast as possible to perturb the metabolism as little as possible.

21: collecting and quenching the spheroids does not have to be done sterilely.

22: label 50 mL Falcons on the cap since any mark on the tube will be washed out by the cold ethanol bath.

23: place the caps in a pattern on the side corresponding to the place of the tubes in the bath.

24: leave enough volume to resuspend the spheroids before putting them into cold quenching solution. An appropriate amount would be half of the conical part of a 15 mL Falcon tube, i.e. about 300-400 μL .

25: be careful not to drip any ethanol from the outside of the tube in the other tubes while aspirating.

26: take 2 mL Eppendorfs, this will give you more space to use the tissue lyser.

- 27: usually 1-2 minutes of lysing is enough to disintegrate the spheroids.
- 28: the protein layer will be small in 3D samples.
- 29: be careful with the protein layer, since it is small and delicate. If there is no interest in the non-polar layer, leave it there to avoid disturbing the protein layer.
- 30: do not take the complete volume of each phase to prevent disturbing the protein layer. Any loss of volume will be corrected by the internal standards.
- 31: derivatization is extremely sensitive to water contamination, which will inhibit the reaction..
- 32: avoid water contamination.
- 33: do not touch the pellet, since the pellet will contain particles that can damage the GC-MS column.
- 34: the extra volume is needed to correct for evaporation of TBDMS.
- 35: this procedure is based on automated derivatization done by the GC-MS. If derivatization by hand is preferred, proceed after 3.6.1.1 by adding twice the volume of methoxyamine in pyridine of TBDMS to each sample, vortex and spin down briefly and incubate for 60 minutes at 60 °C. Centrifuge at maximum speed for 2 minutes and transfer the supernatant to a labelled GC-MS glass vial. Seal with a non-magnetic cap using a crimper.
- 36: do not take anything from the lower phase, since this will destroy the GC-MS column. To test if the lower phase contaminated the upper phase, flick the tube and check for bubbles.
- 37: if the solution is not clear after incubation, incubate longer. If you expect a high yield based on the protein layer, you can add up to 200 μ L of 0.2 M NaOH.

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