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Analysis of Neutral Lipid Synthesis in Saccharomyces cerevisiae by Metabolic Labeling and Thin Layer Chromatography

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TITLE:

Analysis of Neutral Lipid Synthesis in *Saccharomyces cerevisiae* by Metabolic Labeling and Thin Layer Chromatography

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KEYWORDS:

neutral lipid, radio-labeling, thin layer chromatography, ^{14}C-acetic acid, *S. cerevisiae*

SUMMARY:

Here, a protocol is presented for the metabolic labeling of yeast with ^{14}C-acetic acid, which is coupled with thin layer chromatography for the separation of neutral lipids.

ABSTRACT:

Neutral lipids (NLs) are a class of hydrophobic, chargeless biomolecules that play key roles in energy and lipid homeostasis. NLs are synthesized de novo from acetyl-CoA and are primarily present in eukaryotes in the form of triglycerides (TGs) and sterol-esters (SEs). The enzymes responsible for the synthesis of NLs are highly conserved from *Saccharomyces cerevisiae* (yeast) to humans, making yeast a useful model organism to dissect the function and regulation of NL metabolism enzymes. While much is known about how acetyl-CoA is converted into a diverse set of NL species, mechanisms for regulating NL metabolism enzymes, and how mis-regulation can contribute to cellular pathologies, are still being discovered. Numerous methods for the isolation and characterization of NL species have been developed and used over decades of research; however, a quantitative and simple protocol for the comprehensive characterization of major NL species has not been discussed. Here, a simple and adaptable method to quantify the de novo synthesis of major NL species in yeast is presented. We apply ^{14}C-acetic acid metabolic labeling coupled with thin layer chromatography to separate and quantify a diverse range of physiologically important NLs. Additionally, this method can be easily applied to study in vivo reaction rates of NL enzymes or degradation of NL species over time.

INTRODUCTION:

Acetyl-CoA is the fundamental building block of diverse biomolecules including neutral lipids (NLs), which serve as a versatile biomolecular currency for building membranes, generating ATP, and regulating cell signaling[1,2]. The availability of NLs to be shunted into any of these respective pathways is, in part, regulated by their storage. Lipid droplets (LDs), cytoplasmic organelles

composed of hydrophobic cores of triglycerides (TGs) and sterol-esters (SEs), are the main storage compartments of most cellular NLs. As such, LDs sequester and regulate NLs, which can be degraded and subsequently utilized for biochemical and metabolic processes[3,4]. It is known that the mis-regulation of NL and LD-associated proteins is correlated with the onset of pathologies including lipodystrophy and metabolic syndromes[5,6]. Because of this, current LD research is intensely focused on how NL synthesis is regulated spatially, temporally, and across distinct tissues of multi-cellular organisms. Due to the ubiquitous cellular roles for NLs, many enzymes responsible for the synthesis and regulation of NLs are conserved throughout eukaryotes[7]. Indeed, even some prokaryotes store NLs in LDs[8]. Therefore, genetically tractable model organisms such as *Saccharomyces cerevisiae* (budding yeast) have been useful for the study of NL synthesis and regulation.

The separation and quantification of NLs from cell extracts can be accomplished in a myriad of ways, including gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS)[9–11]. Perhaps the simplest method for separating NLs is via thin layer chromatography (TLC), which allows for subsequent densitometric quantification from a standard curve[12,13]. Although TLC provides only a course-grained separation of NLs, it remains a powerful technique because it is inexpensive, and it allows for the rapid separation of NLs from several samples simultaneously. Two of the most considerable challenges facing the study of NLs via TLC are: 1) the broad range of cellular abundances of NL species and their intermediates, and 2) the range of hydrophilicity/hydrophobicity of lipid intermediates within NL synthesis pathways. Consequently, the quantification of NL species via TLC is typically restricted to the most abundant species; however, introduction of a ^{14}C-acetic acid radiolabel can significantly enhance the detection of low abundance intermediates within NL pathways. Acetic acid is rapidly converted into acetyl-CoA by the acetyl-CoA synthetase ACS2[14], which makes ^{14}C-acetic acid a suitable radiolabeling substrate in yeast[15]. Additionally, separation of both hydrophobic NLs and hydrophilic intermediates of NLs can be achieved by TLC through the use of multiple solvent systems[16]. Here, a method is presented for the separation of NLs using ^{14}C-acetic acid metabolic labeling in yeast. Lipids labeled during the pulse period are subsequently isolated by a well-established total lipid isolation protocol[17], followed by the separation of NL species by TLC. Developing of TLC plates by both autoradiography to visualize labeled lipids, and a chemical spray to visualize total lipids, permits for multiple methods of quantification. Individual lipid bands can also be easily extracted from the TLC plate using a razor blade, and scintillation counting can be used to quantify amount of radiolabeled material within the band.

PROTOCOL:

1. Growth and labeling of yeast cells with ^{14}C-acetic acid

1.1. Inoculate a yeast culture by picking a colony from a plate and dispensing it into 20 mL of synthetic complete (SC) media containing 2% dextrose (see **Supplementary File** for the recipe of SC media). Incubate at 30 ˚C for overnight with shaking at 200 rpm.

NOTE: Growth condition, sample volume, and treatment will differ based on the lipid(s) of interest. Prior to running full experiments, optimal growth conditions and culture volumes should be empirically determined. This protocol discusses radiolabeling of yeast cultures grown to stationary phase, a growth phase when bio-membrane and cell growth slows, and NL synthesis is very active.

1.2. Measure the OD_{600} of the overnight culture using a spectrophotometer and dilute the yeast cell culture to a final OD_{600} of 0.2 in 50 mL of fresh SC media containing 2% dextrose. Grow the cells for 24 h, or until they have reached the stationary phase (which is commonly defined by a flat lining of the cell doubling OD_{600} measurement).

1.3. Before collecting the cells, make the quenching buffer (see **Supplementary File** for details). Make two 20 mL aliquots of quenching buffer for each sample (i.e., 40 mL quenching buffer for each sample) and split evenly into two 50 mL conical tubes). Store the quenching buffer aliquots at -80 ˚C for future use.

1.4. Once the cultures have reached the desired OD or growth phase, collect the cells by centrifuging at 4,100 x *g* for 10 min. While samples are in the centrifuge, prepare radiolabeling media by adding [1-^{14}C] acetic acid sodium salt to dextrose-free SC media at a final concentration of 10 μCi/mL.

CAUTION: Proper personal protective equipment (PPE) should be worn all times when working with radioactive materials. Always follow local guidelines for the proper storage, usage, and disposal of radioactive materials.

NOTE: Both the concentration of ^{14}C-acetic acid in the labeling media and the radiolabeling incubation time should be adjusted according to the metabolite(s) of interest. Here, a 20 min radiolabeling pulse incubation is used, which is sufficient to label NL species with a range of abundance.

1.5. Remove the supernatant from the pelleted cells, and wash cell the pellet once with 20 mL of dextrose-free SC media by resuspending the pellet with a pipette. Collect the cells again by centrifuging at 4,100 x *g* for 5 min.

1.6. Resuspend the cells in 1 mL of dextrose-free SC media, and transfer the cells to a labeled 2 mL microcentrifuge tube. Collect the cells again by centrifuging at 4,100 x *g* for 2 min.

1.7. Resuspend the cells once more in 500 μL of dextrose-free SC media. Pre-cool a centrifuge equipped for 50 mL conical tubes to -10 ˚C or on the lowest temperature setting.

1.8. Begin the radiolabeling period by quickly adding 500 μL of radiolabeling media to each 500 μL of cell suspension (final ^{14}C-acetic acid concentration = 5 μCi/mL). Incubate the tubes in a rotating incubator at 30 ˚C for 20 min. 2 min before the end of the labeling period, transfer one 20 mL aliquot of quenching buffer for each sample from the -80 ˚C freezer to a bucket of ice.

1.9. Once the radiolabeling period has ended, use a pipette to plunge the entire 1 mL sample into 20 mL of cold quenching buffer. Vortex the conical tubes for 5-10 s to ensure that the sample has been thoroughly mixed with the quenching buffer. Incubate the samples in quenching buffer for 2 min on ice.

1.10. Collect the cell pellet by spinning in a centrifuge at 5,000 x *g* for 3 min set to -10 ˚C or on the lowest temperature setting. While sample tubes are spinning, transfer another set of quenching buffer aliquot from the -80 ˚C freezer to a bucket filled with ice (i.e., one 20 mL tube of quenching buffer per sample).

1.11. Remove the quenching buffer supernatant from cell pellets and replace it with 20 mL fresh, cold, quenching buffer. Vortex and shake the samples until the pellet has been dislodged from the bottom of the conical tube and resuspended fully in quenching buffer. Centrifuge the samples again at 5,000 x *g* for 3 min at -10 ˚C to collect the cells.

1.12. Once the cells are pelleted, thoroughly remove all quenching buffer from the samples by pouring off the supernatant and removing the excess with a pipette. Store tubes at -80 ˚C for further processing.

2. Isolation of total lipids from yeast

NOTE: The following protocol for lipid isolation is based on a well-established and frequently used method that efficiently extracts most neutral lipid species[17,18].

CAUTION: When using organic solvent, always wear appropriate PPE and work inside of a fume hood when possible. During lipid extraction, avoid using plastics that are incompatible with organic solvents. Polypropylene tubes are suitable for the following protocol.

2.1. Weigh 0.3 g of acid-washed glass beads for each sample and store them in 2 mL microcentrifuge tubes on ice. Remove the cell pellets from the -80 ˚C freezer and keep them on ice. Add 350 µL methanol and 700 µL chloroform to each sample, resuspend, and transfer to microcentrifuge tubes containing pre-weighed glass beads.

2.2. Lyse cells by agitating tubes on a vortex 3x for 1 min, with 30 s incubations on ice between agitations. Alternatively, cells can be lysed using a mini bead-beater for three 1-min cycles. Save 25-30 µL of whole cell lysate in a separate tube for scintillation counting.

NOTE: The saved lysate will be used to determine the relative amount of radioisotope taken up by each sample during the pulse period, which will influence the amount of each sample loaded onto the TLC plate. This is discussed further in step 3.2.

2.3. Pour the entire contents of the 2 mL microcentrifuge tube into a 15 mL glass centrifuge tube [Tube A]. Wash the 2 mL microcentrifuge tubes by adding 1 mL of methanol and vortexing for 10-

15 s. Transfer the 1 mL methanol wash to tube A and add 2 mL of chloroform to tube A followed by 400 μL of water for a final sample volume of 4.45 mL.

2.4. Vortex samples for 1 min followed by a 5 min centrifugation at 1,000 x *g*. After centrifugation, the aqueous (upper) and organic (lower) phases should be clearly visually separated with cell debris lying at the interface.

2.5. Using a glass Pasteur pipette, collect the organic phase from tube A and move to a new 15 mL glass centrifuge tube (Tube B). Add 1 mL of 1M KCl to tube B. To tube A, add 1 mL of methanol and 2 mL of chloroform. Repeat the vortexing and centrifugation steps on tube A.

2.6. Once again collect the organic phase from tube A and add it to tube B. Dispose of tube A in an appropriate container. Vortex tube B for 1 min followed by a 5 min centrifugation at 1,000 x *g*.

2.7. Remove the upper aqueous layer from tube B and dispose. Add 1 mL of fresh 1 M KCl back to tube B and repeat the vortexing/centrifugation step. Once layers are separated, carefully collect the entire bottom organic layer into a labeled 4 mL glass vial.

NOTE: At this step, lipid extracts can be stored at -80 ˚C, or the protocol can be continued for TLC separation of lipids.

3. Separation and quantification of radioisotope-labeled NLs by thin layer chromatography

3.1. If lipid extracts were placed at -80 ˚C, slowly bring to room temperature by incubating on ice and subsequently on a benchtop. Completely evaporate solvent from lipid extracts by vacuum drying or using a gentle stream of inert gas (e.g., argon or nitrogen). Meanwhile, pre-heat an oven to 145 ˚C for heating the TLC plate.

3.2. Before samples can be loaded onto the TLC plate, determine relative amounts of radiolabel taken up by the cells. Pipette 10 μL of the whole cell lysate from step 2.2 into a 6 mL glass scintillation vial, add 6 mL of scintillation fluid and place vials in a rack. Use a scintillation counter to measure the cpm or dpm of each sample using the **count single rack** option set to a 1 min counting time. Measure each whole cell lysate in duplicate to obtain an average for each sample. Adjust the loading amount according to a wild type or reference sample

NOTE: The amount of each sample to load onto the TLC plate can be determined using the following equation: (average sample counts)/(average reference counts) x desired loading volume. For example, if 20 μL of the reference sample is to be loaded onto the TLC plate, and has an average count of 1,000, then an experimental sample with an average count of 2,000 will have 10 μL loaded onto the TLC plate.

3.3. Reconstitute the sample lipids in 40-50 μL of 1:1 (v/v%) chloroform:methanol by vortexing for 5 min. Prepare 101 mL of the mobile phase solvent in a glass graduated cylinder (see the

Representative Results section for an example of major NL species separation by a 50:40:10:1 (v/v/v/v%) Hexane:Petroleum ether:Diethyl ether:Acetic acid solvent).

3.4. Pour the solvent into a glass TLC chamber containing a 20 x 20 TLC saturation pad and a tight-fitting lid. Prepare a channeled 20 x 20 silica gel 60 G plate by gently marking a line 1.5 cm above the bottom of the plate using a pencil. The line designates the origin and where the lipids will be loaded. Below the line, gently label the sample that will be loaded in each lane. Once the TLC plate has been prepped, incubate the plate in a 145 ˚C oven for at least 30 min to pre-heat the plate and remove any excess moisture.

3.5. Once the plate has been sufficiently heated, and the TLC saturation pad is saturated with solvent, remove the TLC plate from the oven and immediately proceed to loading the TLC plate. Loading the plate while it is warm ensures rapid solvent evaporation. For each lipid species of interest, load 5-20 μg of a purified lipid standard onto a lane of the TLC plate to track separation and expected migration distance. Using a pipette, spot 5μL of sample onto the origin of each lane located 1.5 cm above the bottom the of TLC plate. Repeat loading of 5 μL spots until 20-40 μL of sample has been loaded into each lane.

NOTE: 5-20 μg of unlabeled purified lipids can be added to each sample lane as tracers that can be stained and visualized following TLC separation of lipids. The presence of a stained standard allows for easy tracking and excision of radiolabeled lipid bands for subsequent scintillation counting. Which purified standards are loaded onto the plate will be determined by the NL species of interest. See the **Representative Result** section for examples of separating oleic acid (FFA), 1,2 dioleoyl-glycerol (DG), triolein (TG), cholesterol (Chol), cholesteryl-linoleate (SE), and squalene in lanes adjacent to the sample lanes.

3.6. Once the standard and the experimental samples have been loaded, place the plate in the developing chamber and wait until the solvent has reached the top of the plate (40-60 min). Once the plate is fully developed, remove it from the chamber and allow it to dry in the fume hood for 20 minutes.

3.5 After the plate is dried, cover it with plastic film and place it in a developing cassette with an autoradiography screen. Allow the plate to develop with the screen for 24-48 h.

4. Visualization and quantification of TLC separated lipids

4.1. Remove the screen from the developing cassette and place inside of a phosphor imager. Select the **Phosphor Imaging** option and develop at 800-1000 V.

NOTE: Phosphor imaging gives a qualitative view of radiolabeled lipids on the TLC plate. However, quantification of radiolabeled lipids is best accomplished by scintillation counting, which is described subsequently.

4.2. Mix 100 mL of p-anisaldehyde reagent (see **Supplementary File**) and deposit in a glass spray bottle. Spray the TLC plate with p-anisaldehyde reagent until the silica is saturated. Bake the plate in a 145 ˚C oven for 5 min, or until bands have appeared.

4.3. To quantify individual lipid species using radiolabel scintillation counting, use a razor blade to scrape the silica gel from the glass TLC plate. Transfer each silica gel band corresponding to a single radiolabeled lipid species to a glass scintillation vial and add 6 mL scintillation fluid. Vortex vigorously until the silica band has been reduced to small pieces.

4.3.1. Alternatively, lipids can be extracted from the silica gel band using the lipid extraction protocol in section 3. If lipids are extracted from the silica gel, evaporate the solvent entirely as in step 3.1, and add 6 mL scintillation fluid to the dried lipids. Place the rack containing scintillation vials into a scintillation counter. Select the **Count single rack** option and adjust the counting time to 2 min per vial. Results from the scintillation counter will be printed and can be visualized as a bar graph.

REPRESENTATIVE RESULTS:

In this protocol, we have demonstrated that the labeling, detection, and quantification of NL species can be accomplished by ^{14}C-acetic acid metabolic labeling. Major NL species can be separated in a solvent system of 50:40:10:1 (v/v/v/v%) Hexane:Petroleum ether:Diethyl ether:Acetic acid (**Figure 1A,B**). Phosphor imaging allows for visualization of labeled free fatty acid (FFA), triacylglycerol (TG), diacylglycerol (DG), cholesterol (Chol), and squalene (SQ) (**Figure 1A**). Although SEs can be separated from other NL species in this solvent, none are detected in the autoradiogram following a 20-minute pulse. This may be attributed to slow SE synthesis during the stationary phase of growth in yeast. It is also demonstrated that purified lipid species can be separated in this method and subsequently visualized by spraying of the TLC plate with p-anisaldehyde reagent (**Figure 1B**). While NL species are well separated in this solvent, polar species like phosphatidylcholine (PC) stay at the origin (**Figure 1B**). By applying a chase period in radiolabel-free media following the pulse, relative flux through NL pathways can be measured (**Figure 1C**). After a 10-min chase, the major pool of SQ has disappeared, and total Chol is elevated. Similarly, the appearance of DG in the chase period correlates with a decrease in the FFA signal.

FIGURE AND TABLE LEGENDS:

Figure 1: ^{14}C-Acetic acid radiolabeling allows for detection of multiple NL species. (**A**) Autoradiogram of lipids separated by TLC isolated from yeast radiolabeled with ^{14}C-acetic acid in stationary phase. Clearly detectable species include free fatty acid (FFA), triglyceride (TG), diacylglycerol (DG), cholesterol (Chol), and squalene (SQ). Unlabeled bands are unidentified NL species. (**B**) Purified lipid species separated by TLC and visualized by p-anisaldehyde staining. Visualized species include all lipids mentioned in (A) in addition to sterol-esters (SE) and phosphatidylcholine (PC). (**C**) Autoradiogram of lipids separated by TLC isolated from yeast pulsed with ^{14}-acetic acid in stationary phase followed by a 10 min chase period in radiolabel-free media.

Disappearance of SQ is met with increase in Chol. Rise in DG in the chase period is accompanied by decrease in FFA species.

Supplementary File: Recipes for buffers, media, and solutions.

DISCUSSION:

Here, a versatile radiolabeling protocol to quantitatively monitor the synthesis of NL species in yeast is presented. This protocol is very modular, which allows for the procedure to be finished within 3-6 days. Additionally, a wealth of literature exists on the use of TLC to separate lipid species and metabolites, which should permit the user to detect several lipid species of interest with a simple change of TLC solvent systems[16,19].This protocol is conducive to the separation, detection, and quantification of radiolabeled lipids. It can also be coupled with a chase period in un-labeled media to detect the turnover time of labeled NLs. Collectively, this procedure gives a useful structure to begin exploring the radiolabeling of NL species.

Other methods, such as HPLC, GC-MS, and UPLC-MS provide higher resolution of lipid separation and quantification; however, it is typically not optimal to run radiolabeled samples through MS, although this can be overcome by using stable-isotopes. Nevertheless, this radiolabel method provides high detection sensitivity and versatility for many lipid species. Another advantage of this protocol compared to MS is its affordability. TLC separation of lipids is relatively simple, requires no extravagant equipment, and relies on common laboratory materials. Regarding limitations: certain low-abundance species, like lyso-lipids, may not be detectable even following incorporation of a ^{14}C label. Additionally, most TLC approaches are not suitable for 'lipidomic' characterizations, due to the course-grained separation of lipid species within a given solvent.

Yeast offer a convenient, genetically tractable model system for the study of lipids via radiolabel biochemical approaches. However, it should be noted that in specific genetic backgrounds, or during a particular metabolic growth conditions, the cellular uptake of radiolabeled-acetic acid or other radiolabels may be reduced. Labeling of cells with ^{14}C-acetic acid in the absence of glucose robustly increases the uptake of the radiolabel. Long incubations in the absence of glucose will proportionally increase radiolabel uptake; however, this may also influence the pathways in question. Therefore, labeling efficiency for a particular growth condition should be established prior to following the ^{14}C-acetic acid radiolabeling protocol in full. In particular, pay attention to the length of the radiolabeling period. The labeling time should be kept as short as possible to detect the lipid species-of-interest. Altogether, this procedure allows for the study of important lipid synthesis reactions and should permit the investigation of NL regulation in intact cells.

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DISCLOSURES:

The authors declare there are no competing interests in the preparation of this manuscript.

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Figure

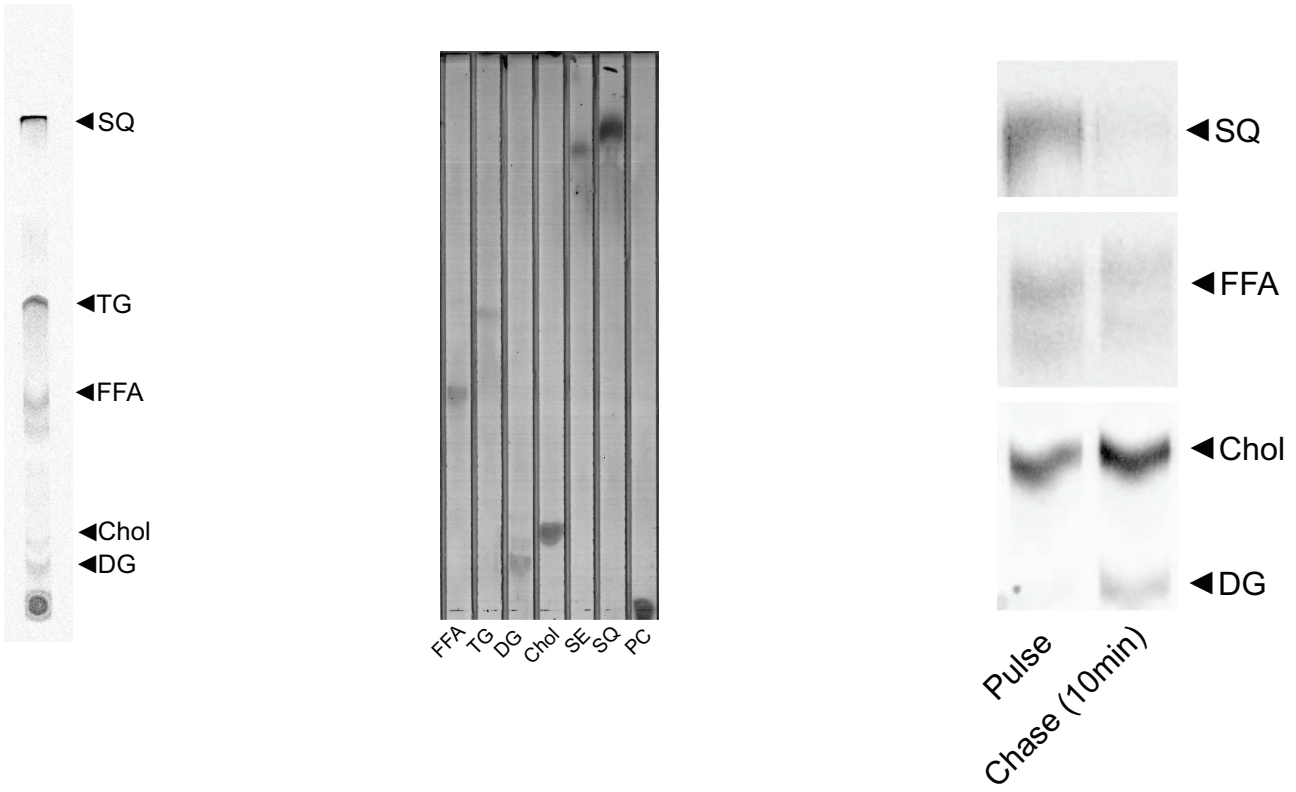

Figure 1

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number
[1-C14] Acetic acid sodium salt specific activity: 45-60mCi	PerkinElmer	NEC084H001MC
18:1 1,2 dioleoyl-sn-glycerol	Avanti	800811O
200 proof absolute ethanol	Sigma	459836
Acid washed glass beads 425-600um	Sigma	G8772
Amber bulbs for Pastuer pipettes	Fisher	03-448-24
Ammonium Sulfate >99%	Sigma	A4418
Beckman LS6500 scintillation counter	PerkinElmer	A481000
Chloroform (HPLC grade)	Fisher	C607SK
Cholesterol >99%	Sigma	C8667
Cholesteryl-linoleate >98%	Sigma	C0289
Concentrated sulfuric acid	Sigma	339741
Corning 50mL conical tubes, polypropylene with centristar cap	Sigma	CLS430829
Dextrose, anhydrous grade	Sigma	D9434
Diethyl ether anhydrous grade	Sigma	296082
Drying oven	Fisher	11-475-155
EcoLume scintillation liquid	VWR	IC88247001
Eppendorf 5424R centrifuge	Fisher	05-401-205
GE Storage phosphor screen	Sigma	GE28-9564-75
GE Typhoon FLA9500 imager		
Glacial acetic acid, ACS grade	Sigma	695092
Glass 6mL scintillation vials	Sigma	M1901
Glass centrifuge tube caps	Fisher	14-595-36A
Glass centrifuge tubes	Fisher	14-595-35A
Glass Pasteur pipette	Fisher	13-678-20C
Hexane, anhydrous grade	Sigma	296090
L-Adenine >99%	Sigma	A8626
L-Alanine >98%	Sigma	A7627
L-Arginine >99%	Sigma	A1270000
L-Asparagine >98%	Sigma	A0884
L-Aspartate >98%	Sigma	A9256
L-Cysteine >97%	Sigma	W326305
L-Glutamic acid monosodium salt monohydrate >98%	Sigma	49621

L-Glutamine >99%	Sigma	G3126
L-Glycine >99%	Sigma	G8898
L-Histidine >99%	Sigma	H8000
L-Isoleucine >98%	Sigma	I2752
L-Leucine >98%	Sigma	L8000
L-Lysine >98%	Sigma	L5501
L-Methionine, HPLC grade	Sigma	M9625
L-Phenylalanine, reagent grade	Sigma	P2126
L-Proline >99%	Sigma	P0380
L-Serine >99%	Sigma	S4500
L-Theronine, reagent grade	Sigma	T8625
L-Tryptophan >98%	Sigma	T0254
L-Tyrosine >98%	Sigma	T3754
L-Uracil >99%	Sigma	U0750
L-Valine >98%	Sigma	V0500
Methanol, ACS grade	Fisher	A412
Oleic acid >99%	Sigma	O1008
p-anisaldehyde	Sigma	A88107
Petroleum ether, ACS grade	Sigma	184519
Phosphatidylcholine, dipalmitoyl >99%	Sigma	P1652
Pipettes	Eppendorf	2231000713
Potassium chloride, ACS grade	Sigma	P3911
Sodium Hydroxide pellets, certified ACS	Fisher	S318-100
Squalene >98%	Sigma	S3626
Succinic Acid crystalline/certified	Fisher	110-15-6
TLC saturation pad	Sigma	Z265225
TLC silica gel 60G glass channeled plate	Fisher	NC9825743
Transparency plastic film	Apollo	829903
Tricine	Sigma	T0377
Triolein >99%	Sigma	T7140
Vortex mixer	Fisher	02-215-414
Whatman exposure cassette	Sigma	WHA29175523
Yeast nitrogen base without ammonium sulfate and amino acids	Sigma	Y1251

Comments/Description

No fluorescent indicators

UT Southwestern Medical Center

W. Mike Henne, Ph.D.
W.W. Caruth, Jr. Endowed Scholar in Biomedical Research
Assistant Professor, Depts. of Cell Biology & Biophysics

December XX, 2020

Dr. Nguyen
Editor, *JOVE*

Dear Dr. Nguyen,

Thank you for your interest in our manuscript. We were happy to see that the reviewers found the study of interest, and suggested revisions that would strengthen the work. We have now addressed these reviewer concerns, which we discuss point-by-point below.

Thank you for your consideration. We anxiously await your reply. Please feel free to contact us by email at mike.henne@utsouthwestern.edu, or by phone at 214-648-6778.

Sincerely,

W. Mike Henne, Ph.D.
Assistant Professor
W.W. Caruth Jr., Endowed Scholar

Point-by-Point discussion for reviewers (author's responses in blue):

Reviewer #1:
Overall I think that 14C-acetate labeling of TAG biosynthesis in yeast is a useful method for evaluating mechanisms of yeast TAG biosynthesis as well as using yeast as a platform for characterizing TAG biosynthetic genes from other organisms. The line specific comments below are to both clarify the method, and clarify some ambiguity in the writing.

Introduction, because most acetyl-CoA for fatty acid synthesis comes from pyruvate dehydrongenase the introduction should indicate how 14C-acetate is incorporated into acetyl-CoA for fatty acid biosynthesis.

Line 54, here it may be useful to indicate what "specialized instrumentation" you are referring to. For example those new to 14C metabolic labeling may be not familiar with alternatives. Recent applications such as radio-HPLC for lipid analysis (Kotapati 2020, J Chrom B, https://doi.org/10.1016/j.jchromb.2020.122099) would be an alternative for 14C analysis, as well as 13C and MS based analysis, but each has its advantage and disadvantage which would be

worthwhile to discuss the merits and disadvantages of each. The great advantage of TLC is that it is cheap to set up, but still requires specialized equipment such as phosphor imager or other specialized audioradiographic imaging type equipment. Informing the reader on various alternatives increases the value of the manuscript through helping the reader choose the technique most suable to their needs or find the available equipment at their institution.
We have inserted several new references into the text, including references to GC-MS, HPLC, and UPLC-MS so that readers can easily assess the most advantageous method for their questions.

Line 73, give media composition or reference for the media.
We have included a 'recipe' section that contains compositions of buffers and reagents used throughout the protocol. The media composition can be found in the 'recipe' section.
Line 92. Here the specific activity and the uM amount of the acetate should be indicated, and the exact substrate composition used should be somewhere in this document. For example, is this 1-14C-acetate, or 2-14C-acetate? Is it as the acetic acid version, or as the sodium salt? Is this 14C-acetate in water or ethanol. If in ethanol, is the ethanol mostly evaporated and substrate dissolved in media? If not, does ethanol alone affect the growth of the yeast? Note, evaporation of the ethanol only works with the sodium salt version not the glacial acetic acid version.
To keep the protocol reasonably concise, we have addressed reviewer's concerns about materials/reagents by adding additional details in the materials table.
Line 92. give the components or citation for the dextrose free SC media.
See 'recipe' section
Line 96. 14C as a weak beta emitter does not require shielding as stronger isotopes do, such as 32P. For novices this comment could add unnecessary fear to the experiment.
We appreciate reviewer's concern about the use of radioisotopes; however, our local guidelines for the use of radioisotopes enforces shielding. In the interest of safety, we have kept the caution in our protocol.
Line 102. What does "short" mean? A few minutes, or a few hours? It would be best to just give the time used for the example data.
Done
Line 106. Wouldn't it be better to wash the cells with dextrose-free media without the 14C substrate so you don't affect salt and other nutrient concentrations?
Protocol has been adjusted accordingly
Lines 110-112. By resuspending the pellets in the radiolabeled media, the time it takes to resuspend your pellet will affect your time course. The cells will not label equally until they are fully resuspended, and if you are doing replicates or comparing multiple lines with replicates the time to resuspend will affect how quickly you start the actual labeling in each sample. It would be better to resuspend the cells in a smaller volume, for example 0.8 ml, then once all replicates are resuspended add 0.2 ml of the labeled substrate at a higher concentration such that the final concentration will be your 5uCi/ml.
Done
Line 118 pre-chilled to what temperature
Revision noted in the protocol
Line 137. What size beads? Define glass, are these zirconia?
Addressed in the materials table
Line 142. Why three 1 min cycles instead of a 3 min cycle? Do you re-chill the sample in between? If so this should be mentioned.
Point clarified in the revised protocol
Line 143. scintillation counting at this stage is interesting because some of the 14C-acetate substrate may be remaining. Wouldn't it be better to count the lipid extract after phase partitioning to make sure your value is not possibly skewed by remaining substrate? You could still count both the aqueous and organic phases. A higher variability in the aqueous phase than the organic phase of replicates is likely due to remaining 14C acetate.

The protocol was not adjusted on this point. We find that washing with two subsequent steps of quenching buffer nearly entirely removes exogenous radiolabel substrate. Additionally, it is important to perform scintillation counting at this step to determine total cellular uptake of the radiolabel, which may not be accurately reflected in the lipid fraction alone. Altogether, this may help users troubleshoot the protocol, if they find no observable detection of radiolabeled lipids in their TLC.

Lines 183-184. is that ratio v/v/v/v? If so it needs to be indicated. Also why not prepare 101 ml of solvent, by using your solvent ratios as volumes in ml. As written to prepare just 100 ml would require more difficult math and measuring of volumes.

Done

Line 184. What grade of solvents are used? ACS, HPLC, et.

Noted in materials table

Line 185. A filter pad is not clear, please define this.

Point clarified in the text

Line 189, do you cool the plate before loading? Also what is the purpose of this step, to remove moisture? If so it would be helpful to just indicate it.

Point clarified in the revised protocol. It is important to load samples on the TLC plate while still hot. This facilitates solvent evaporation while loading.

Line 190. Are you using plates with precut lanes? If so indicate. Are you loading the lipids as a band or a spot?

Materials section

Line 190 and 194. What is your purified standard?

Added to ‘representative results’ and ‘materials’ sections

Details of the "autoradiography screen" should be included

Done

Line 206 and others, phosphorimager is two words phosphor imager.

Done

Line 208, what image analysis software.

Densitometry quantification has been amended from this protocol for simplicity

Lines 215-216. Explain this process

Done

Line 226. As above whenever you indicate a solvent ratios you need to indicate how you determined the ratios, volume, mass, etc.

Done

Lines 249-251. You mention a wealth of literature but no citations, could you give examples or a review article such as: Fuchs B, Süß R, Teuber K, Eibisch M, Schiller J (2011) Lipid analysis by thin-layer chromatography—A review of the current state. Journal of Chromatography A 1218: 2754-2774

References added

Lines 262-264. This statement is not true. By converting the lipids (either individual or whole extract) to FAME you can separate various chain lengths and unsaturation by various TLC systems (combinations of reverse phase and argentation TLC) and then quantitate by phosphor imaging. See review mentioned above, or Christie WW (2003) Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids, Ed 3rd. The Oily Press an imprint of PJ Barnes & Associates, Bridgwater, England. An example of this type of analysis in current research is: Pollard M, Martin TM, Shachar-Hill Y (2015) Lipid analysis of developing Camelina sativa seeds and cultured embryos. Phytochemistry 118: 23-32

Point about acyl-chain length has been removed

Reviewer #2:

This work mainly described a method using 14 C-acetate metabolic labeling for NLs quantification and a simple separation of NLs from cell extracts using TLC. According to the results, a useful and

rapid separation protocol was established with clean, individual bands.
Comment 1: In the introduction, the author mentioned the quantification of NLs was studied, however, there is not any result of the quantification work. Please provide the scintillation counting results.
Scintillation counting results are printed from scintillation counters. We show here examples of autoradiograms, but scintillation counting results can be plotted using familiar graphing softwares
Comment 2: Would you please show the extraction efficiency of the "well-characterized protocol for the extraction of total lipids"
Reference added for efficiency of the Folch extraction

Reviewer #3:
Manuscript Summary:
This manuscript describes a versatile radio labeling protocol to quantitatively monitor the synthesis of neutral lipid species in yeast. The protocol is clear, well-written, and will be of use to the community. Variations of the protocol to measure total lipids or or lipid turnover during a pulse-chase experiment are included. Pros and cons to relative to other methods such as lipid-based mass spectrometry are discussed. I recommend publication in JOVE. I have several minor suggestions to improve the manuscript.

Major Concerns:
None.

Minor Concerns:
-It would be helpful to have a section on selecting materials. I personally learned the hard way that polystyrene tubes dissolve in chloroform! Polypropylene tubes are compatible with several organic solvents. In addition, some discussion of the parameters to consider when choosing TLC plates would be helpful (e.g., glass-backed vs. aluminum foil, presence of fluorescent indicator, particle size, pore size, thickness). As the variety of TLC plates available is somewhat overwhelming, a discussion of these parameters would be helpful. For example, it can be important to avoid fluorescent indicators in the plate if using fluorescent lipids in an experiment.
Please see Materials section
-Line 116: What is meant by "plunge the entire sample volume in 20 ml quenching buffer"? Is the quenching buffer added to the sample, or vice versa?
Clarification made in the text
-Line 188: How are lipids loaded on the TLC plate? Using glass capillary tubes? How far from the bottom of the plate?
Done

Reviewer #4:
Manuscript Summary:
The protocol presented by Rogers and Henne is straightforward and would benefit researchers working in the field of lipids. However, there are issues that need to be addressed before publication.

Major Concerns:
In section 2.2, cell lysis for lipid extraction is usually done in the presence of solvents to deactivate any cellular enzymes that can act on lipids (e.g., lipases). In the protocol described here, the cells are lysed first and then the solvents are added. If one is analyzing a few samples, this may not affect the lipids, but if many samples are analyzed (which is common for time course experiments), the action of enzymes may have a significant effect
Clarification made in the text
In section 3.3, it is not clear what is meant by "Adjust the loading of radiolabeled samples

according to the scintillation counting results obtained in step 2.4". Please explain. Also, what is the purified standard that is used here? Is this a pure lipid compound or a mixture of lipids?
More details were added to the protocol
In section 4.3, it seems that anisaldehyde is used to detect the non-radioactive standards that are used to determine the position of the radioactive lipids on the TLC plates. However, in the "Representative results" gives the impression that anisaldehyde is also used for quantification. Please elaborate
Notes about quantification of total lipids has been amended from the text for simplicity
It is surprising that steryl esters are not detected in the radiolabeling experiment, because the use of 14C-acetate for analyzing steryl esters is very common. Do authors have an explanation for this discrepancy?
Details added
Page 12, line 264, I disagree with the statement "Such questions should be addressed using MS based approaches". The acyl chain length and degree of saturation can be analyzed by gas chromatography which is much easier than MS
As addressed above in the last comment from reviewer 1, this was removed from the manuscript
Minor Concerns:
In the title, the notion that the lipid synthesis can be quantified may be considered incorrect. The synthesis per se cannot be quantified, what is quantified is the rate of synthesis. Authors may want to rephrase that
In section 2.1, 400ul is probably meant
Done
Page 12, line 263, "lipid or fatty acyl chain length", the "or" seems to be misplaced here.
Done

Reviewer #5:
Manuscript Summary:
In this manuscript, Rogers and Henne describe a method to quantitatively monitor the production of neutral lipids in Saccharomyces cerevisiae via metabolic labeling and thin layer chromatography.
The range of biological questions that can be addressed with the described method are clearly presented in the introduction and discussion sections. Additionally, suggestions for further applications such as monitoring enzymatic reaction rates in vivo are included. Limitations of the method are being discussed adequately throughout the manuscript. The protocols are very clear and easy to follow. The method is broken down into modules that can easily be employed independently of each other.
I support publication of the manuscript. Below, you will find minor comments and suggestions to further improve the manuscript

Major Concerns:
None

Minor Concerns:
L1-2: Title: I suggest not to mention the abbreviated term S. cerevisiae in the title, but rather Saccharomyces cerevisiae, or maybe yeast, dependent on how easily the method can be adapted for other types of yeast.
The title was kept the same for brevity
L37: "NLs (…) serve as (…) currency for (…) generating energy" is inaccurate. It should be "generating ATP", or "converting energy".
Done
L73: Consider including the exact recipe for synthetic complete media. Although different media can be used, it may be useful (e.g. for trouble shooting) to know which exact type of media

(including brand and exact composition of YNB and amino acid mix, pH, …) the authors used to achieve their results, particularly because metabolic regulation is complex and seemingly small variations can have unexpected effects.
Done
L73 versus 82: Why is the 2% dextrose only being mentioned in L82? Is the pre-culture without dextrose?
Point clarified in the text
Throughout: Please include (w/v) or (v/v) when describing compositions of buffers using %
Done
L137-139: I assume it should say 400µl of cold MilliQ water instead of 400ml. Also, although it is obvious once one reads point 2.2, it could say more clearly that glass beads and cell pellets should be combined in the same microcentrifuge tube.
Done
L277-8: "some cells may become quiescent or dead" please rephrase.
Done
References: The authors may consider suggesting further reading about TLC.
References added for TLC
Representative results: The data provided is indeed representative for the method described, but it seems a bit minimalistic. The authors could consider including something that illustrates the dynamic aspect of the method, such as a time course of metabolic labeling and/or a pulse-chase experiment.
We have added a new figure, which shows the use of a chase period to determine the relative flux within NL pathways
Material list:
Please re-check for completeness.
The source of the lipid standards used should be mentioned.
Done
Product CLS430829/Sigma is listed twice
Fixed

Recipes:

20% dextrose solution:
For one liter of 20% dextrose: Dissolve 200g of dextrose in 600mL of MilliQ water. Once all the dextrose has dissolved, bring the solution up to 1000mL with MilliQ water and filter sterilize using a 0.45μm or 0.22μm membrane.

Complete dropout powder mix: In a 500mL beaker, add together 1.25g adenine, 0.9g arginine, 3.0g aspartate, 3.0g glutamate, 0.9g lysine, 0.6g methionine, 1.5g phenylalanine, 11.25g serine, 6.0g threonine, 0.9g tyrosine, 4.5g valine, 1.2g alanine, 1.2g asparagine, 1.2g cysteine, 1.2g glutamine, 1.2g glycine, 1.2g isoleucine, 1.2g proline, 0.6g histidine, 1.8g leucine, 1.2g tryptophan, and 0.6g uracil. Once all powders have been deposited in the beaker, mix thoroughly with a spatula. In small increments, transfer portions of the mixed powder to a mortar, and gently crush with a pestle. Transfer the crushed powder to a new 500mL beaker. Once all powder has been crushed, mix again thoroughly and store in 50mL conical tubes at room temperature.

Synthetic-Complete (SC) media: For one liter of SC media, dissolve the following in 600mL of MilliQ water: 10g succinic acid, 6g sodium hydroxide, 5g ammonium sulfate, 1.7g yeast nitrogen base without ammonium sulfate and amino acids, and 1.3g complete dropout powder mix (see recipe for details). Once all components are dissolved, bring the solution up to 900mL wilh MilliQ water and autoclave to sterilize. After the media has cooled, add 100mL of filter-sterilized 20% dextrose to the media (final dextrose concentration = 2%).

p-anisaldehyde reagent: For 100mL of p-anisaldehyde spray reagent: Add 500μL of p-anisaldehyde to 10mL of glacial acetic acid. Once mixed, add 85mL of 200 proof absolute ethanol and 5mL of concentrated sulfuric acid.