Total Triglyceride Quantification in *Caenorhabditis elegans*

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[Abstract] Several studies suggest an important role of lipid metabolism in regulating longevity of *Caenorhabditis elegans*. Therefore, assays to quantify lipids have enormous value in understanding aging and pathologies associated with it. Approximately 70% of lipid metabolism genes in the nematode have orthologs in humans. Amenability of *C. elegans* to genetic manipulations has allowed investigations into the role of specific genetic factors in lipid metabolism. Here, we describe a protocol to quantify total triglycerides in *C. elegans*, which can be extended to studies of the effects of altered environmental and genetic factors on stored fats. This protocol quantifies the picomoles of the triglycerides, in whole worm lysate. Due to the sensitivity of the assay, it could help in identifying subtle changes in the total stored fat which are not discernible with microscopy techniques.

Keywords: *Caenorhabditis elegans*, Triglycerides, Fluorometry, Fat, Metabolism

[Background] Excess calories are often stored in the form of triacylglycerol or glycogen (Cohen, 2018). Abnormal levels of triglycerides (TAGs) have been implicated in heart diseases, pancreatitis, and atherosclerosis in humans (Cameron *et al.*, 1974; Lee *et al.*, 2003; Walther and Farese, 2012; Onal *et al.*, 2017). TAGs are packed into lipid droplets in the cytosol. Lipid droplets are present in eukaryotic cells and vary in size from 20 nm to 100 μ m (Stobart *et al.*, 1986; Suzuki *et al.*, 2011). Lipid droplets consist of a hydrophobic core surrounded by a phospholipid monolayer on the outer side. These organelles undergo active biogenesis, maturation, and turnover. At the time of need, such as during caloric restriction or starvation, TAGs are hydrolyzed to generate energy (Walther and Farese, 2012). Like other eukaryotes, *C. elegans* also stores excess energy in the form of TAGs packed into lipid droplets (Ashrafi, 2007; Mak, 2012). The droplets are present predominantly in the intestine and hypodermis and can be easily visualized by staining with Sudan black, Oil-Red-O, BODIPY (Ex/Em=493/503), or Nile Red (Kimura *et al.*, 1997; Ashrafi *et al.*, 2003; O'Rourke *et al.*, 2009; Yen *et al.*, 2010; Dixit *et al.*, 2020). However, these techniques do not provide an estimate of the absolute amounts of triglycerides. Here, we describe a biochemical method for the quantification of absolute levels of triglycerides in *C. elegans*, with sensitivity in picomolar concentration. In this assay, lipases hydrolyze TAGs to release free fatty acids and glycerol. Glycerol is further oxidized to generate a product that reacts with a probe to generate fluorescence at Ex/Em of 535/587. Outline of the protocol is shown in Figure 1. Biochemical quantification is useful for the detection of the glycerol moiety of the TAG, but it does not provide information on the fatty acid composition of stored fats in terms of saturated and

desaturated fatty acids. This method can be utilized to study changes in total triglyceride content due to genetic perturbations, change in diet or pharmacological perturbations.

Figure 1. Triglyceride quantification procedure overview

Materials and Reagents

- 1 L and 2 L Conical flasks (ThermoFisher Scientific, FisherBrand)
- 60 mm Petri dish with triple vent (Tarsons, catalog number: 460061)
- 96-well flat bottom black fluorescent reading plates (SPL Life sciences, catalog number: 30496)
- 50 ml graduated centrifuge tube (Tarsons, catalog number: 546021)
- 15 ml graduated centrifuge tube (Tarsons, catalog number: 566041)
- 0.45 μ m filters (GE Healthcare, Whatman™, catalog number: 9913-2504)
- 0.010-inch diameter Platinum wire
- Aluminum foil (PRS associates, MYFOIL)
- 1.5 ml microfuge tube (Trasons)
- Caenorhabditis elegans*
- Escherichia coli* OP50 (Glycerol stock)
- NaCl (ThermoFisher Scientific, Qualigens, catalog number: 7647)
- MgSO₄ (ThermoFisher Scientific, SQ, catalog number: 10034)
- CaCl₂ (Merk Life Science, EMPLURA, catalog number: QE5Q651429)
- K₂HPO₄ (Avantor™, RANKEM, catalog number: P2607)
- KH₂PO₄ (ThermoFisher Scientific, Qualigens, catalog number: 7778)
- Na₂HPO₄ (ThermoFisher Scientific, Qualigens, catalog number: 7558)
- NaOH (SDFCL, catalog number: 20252 K05)
- Sodium hypochlorite solution (NaOCl) (Sigma-Aldrich, catalog number: 239305)
- Absolute ethanol (Merck Life Science, EMSURE, catalog number: 64-17-5)
- Molecular grade water (SIGMA, catalog number: W4502)
- Agar powder (HiMedia, catalog number: RM301)

23. LB broth (BD Biosciences, Difco™ LB Broth Miller (Luria-Bertani), catalog number: 244620)
24. LB agar (BD Biosciences, Bacto™ Agar, catalog number: 214010)
25. Peptone (BD Biosciences, Bacto™ Peptone, catalog number: 211677)
26. Cholesterol (SIGMA, catalog number: C8667)
27. Streptomycin (Alfa Aesar, catalog number: J61299)
28. NP-40 solution (SIGMA, catalog number: 74385)
29. BioVision triglyceride quantification colorimetric/fluorometric kit (BioVision, catalog number: K622)
Reagents provided in the BioVision kit
Triglyceride Assay Buffer (TAB)
Triglyceride Probe
Lipase
Triglyceride Enzyme Mix
Triglyceride Standard (1 mM)
30. Nematode growth media (see Recipes)
31. 1 M KPO₄ (see Recipes)
32. 1 M CaCl₂ (see Recipes)
33. 1 M MgSO₄ (see Recipes)
34. 5 mg/ml Cholesterol (see Recipes)
35. 50 mg/ml Streptomycin (see Recipes)
36. 5 N NaOH (see Recipes)
37. LB media (see Recipes)

Equipment

1. Water bath
2. Autoclave (JSR, model: JSAX-80)
3. Laminar flow (JSR, model: JSCB-1200SB)
4. BOD incubator (JSR, model: JSBI-840C)
5. Rocker (SHALOM, GEL ROCKER, model: SLM-GR-100)
6. Centrifuge (Hettich, Universal 320 R, 1406)
7. Centrifuge (Eppendorf, Centrifuge 5424 R)
8. Sonicator (BRANSON, DIGITAL SONIFIER 450)
9. Spectrophotometer (TECAN, model: Infinite M200 pro)

Software

1. i-control™ (TECAN, https://lifesciences.tecan.com/plate_readers/infinite_200_pro?p=Software)
2. GraphPad Prism5 (PRISM, <https://www.graphpad.com/scientific-software/prism/>)

Procedure

A. *C. elegans* maintenance

1. Streak *E. coli* OP50 on LB agar plates without any antibiotic (see Recipes) and incubate the plate for 12 h at 37 °C.
2. Inoculate a single OP50 colony into 10 ml of LB media in a 50 ml graduated centrifuge tube and let it grow overnight (10-12 h) at 37 °C.
3. Spot ~150 µl of overnight grown OP50 culture to 60 mm NGM plates and dry in laminar flow for ~30 min. These plates can be stored at room temperature (RT).
4. Using a heat sterilized platinum wire pick, transfer 5-6 gravid worms (Figure 2A) to an OP50 seeded NGM plate and propagate them at 20 °C till they reach the adult stage and start laying eggs.
5. Adults worms can again be transferred on to a new NGM OP50 plate for maintenance.

Figure 2. Image of a (A) gravid adult, (B) young adult, and (C) eggs of *C. elegans*

B. Lipid quantification

1. Preparation of synchronized population by taking ~10 gravid adults (60-65 h old worms at 20 °C, see Figure 2A) and allowing them to lay eggs for 6-8 h on NGM plates seeded with OP50. This yields about 500 eggs per plate. Incubate these plates at 20 °C till worms reach the gravid adult stage.
2. Collect the gravid worms from 3 dishes into 6 ml M9 buffer in a 15 ml graduated centrifuge tube.
3. Let the worms settle down for 10 min.
4. Remove excess M9 buffer without disturbing the pellet of worms and add 10 ml autoclaved water.
5. Let the worms settle down and then remove water up to 2 ml mark in the 15 ml graduated centrifuge tube.
6. Prepare a 2x bleach mix by adding 5 N NaOH and NaOCl (bleach) in water (see Recipes).
7. Add 2 ml of 2x bleach mix to the suspension of worms in the water.
8. Vortex vigorously for 30 s to break open the worms. Check under a microscope to ascertain the release of eggs in the solution (Figure 2).
Note: The duration of vortexing can vary with the strength of the bleach. Vortex vigorously till worms break open and eggs are released in the solution.
9. Add 15 ml M9 buffer immediately after eggs are released.

10. Centrifuge at 850 x g for 3 min for eggs to settle down at the bottom. Remove excess M9 buffer without disturbing the pellet.
11. Repeat Steps B9 and B10 twice.
12. Resuspend the eggs in 1 ml of M9 buffer in the graduated centrifuge tube. Incubate on a tube rotator at 20 °C for 18-20 h.
13. Examine the egg suspension for hatching after 18-20 h.
14. Shake the tubes properly and spot three 5 µl drops on a plain NGM plate. Count the number of worms in each spot. Take the average of three reads to estimate total numbers of worms present.
15. Spot hatched L1 larvae on five NGM OP50 plates with an average of 300-400 worms on each and propagate the worms at 20 °C till the young adult stage (45-48 h after spotting L1, see Figure 2).
16. Collect the worms in a 15 ml graduated centrifuge tube using M9 buffer, wash twice with 10 ml M9 buffer (samples to be on ice till Step B23).
17. Let the worms settle and remove excess M9 buffer.
18. Resuspend worms in 15 ml of 5% NP-40 solution in water and let worms settle again.
19. Remove excess NP-40 solution and resuspend worms in 2 ml of 5% NP-40 solution.
20. Spot 50 µl of the suspension of worms and count the number of worms. Dilute, if necessary, to keep the same average number (~40-50 worms per 50 µl) of worms across different samples.
21. Let the worms settle again on ice and resuspend in 500 µl of 5% NP-40 solution.
22. Sonicate the worms in 5% NP-40 solution on ice for 30 min with 15 s ON and 10 s OFF at 60% amplitude. Change the ice, if required, every 10 min.
23. Transfer the sonicated lysate to a fresh 1.5 ml microfuge tube.
24. Heat the sample to 100 °C in a water bath for 2-5 min and then let it cool at RT temperature.
This is important to completely solubilize all the triglycerides in the samples.
25. Repeat Step B24 once.
26. Centrifuge at 4 °C for 2 min at 12,130 x g.
27. Transfer the supernatant to a fresh 1.5 ml microfuge tube without disturbing the pellet. Keep the supernatant on ice and discard the pellet.
28. Make 1:100 dilution of the sample in water for measurement.

C. Generation of a standard curve for triglycerides

1. Heat the triglyceride standard, provided in the BioVision kit, to 100 °C in a water bath for 1 min and vortex for 1 min.
2. Repeat Step C1 twice and let it cool. Make sure the solution is clear.
3. Dilute the 1 mM triglyceride standard to 0.4 µM (0.4 pmol/µl) with triglyceride assay buffer (TAB).
4. Prepare buffer control (TAB alone) along with 1:5, 2:5, and 4:1 dilutions of 0.4 µM triglyceride standard with TAB to generate 0, 4, 8, and 16 picomoles triglyceride per well for fluorometric quantification of triglycerides in a 96-well microtiter plate. Adjust volume to 50 µl with TAB.

Note: For creating "0, 4, 8 and 16 pmol triglyceride", we added 0, 10, 20, and 40 µl of 0.4 µM

standard to 50, 40, 30, and 10 μ l of TAB buffer respectively. For technical duplicates, a total reaction volume of 100 μ l should be prepared for each standard in 1.5 ml microcentrifuge tubes.

5. Add 2 μ l lipase to each 50 μ l of standard and mix well. For duplicates, add 4 μ l of lipase to each 100 μ l standard, mix well, and split 50 μ l in each well of the 96-well microtiter plate. Incubate the mix at room RT on a rocker for 20 min.

Note: Flat bottom black 96-well microtiter plate is used for this experiment.

6. Prepare the TAG reaction mix by mixing 2 μ l TAG enzyme mix, 0.4 μ l TAG probe, and 47.6 μ l of TAB for each well. Prepare a master mix for the total number of standard wells plus one buffer reaction to reduce variation. For example, for 4 standards (including buffer control) in duplicates, it will be 18 μ l TAG enzyme mix, 3.6 μ l TAG probe, and 428.4 μ l of TAB buffer. A buffer reaction is added to take care of volume loss due to pipetting errors.

Note: Protect the reaction from light.

7. Add 50 μ l of the TAG reaction mix to buffer control and each standard well of the 96-well microtiter plate. Incubate the reaction on the rocker for 60 min at RT.
8. Measure the fluorescence at Ex/Em of 535/590 nm using TECAN Infinite M200 pro spectrophotometer (see Figure 3).

Step 1: Raw data obtained at 590 nm

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1																
2																
3																
4																
5																
6																
7																
8																
9																
10																
11																
12																
13																
14																
15																
16																
17																
18																
19																
20																
21																

Standard curve generation:

Average of Standard duplicates including buffer control:

(B1+C1)/2 (B2+C2)/2 (B3+C3)/2 (B4+C4)/2

6735 14952 21678 46365.5

Subtract buffer control av. from each standard av. Reading (Corrected fluorescence)

B1-B13 C13-B13 D13-B13 E13-B13

0 8227 15243 39630.5

Generate a standard XY standard curve using GraphPad Prism (Step2-step6)

1B, 1C=Standard buffer control
2B, 2C=Standard 4 pmol/well
3B, 3C=Standard 8 pmol/well
4B, 4C=Standard 16 pmol/well
1D, 1E= WT sample 1 (duplicates)
2D, 2E= WT sample 2 (duplicates)
3D, 3E= WT sample 3 (duplicates)
4D, 4E= str-2 sample 1 (duplicates)
5D, 5E= str-2 sample 2 (duplicates)
6D, 6E= str-2 sample 3 (duplicates)
1F, 1G= -Lipase control for WT sample 1(duplicates)
2F, 2G= -Lipase control for WT sample 2(duplicates)
3F, 3G= -Lipase control for WT sample 3(duplicates)
4F, 4G= -Lipase control for str-2 sample 1(duplicates)
5F, 5G= -Lipase control for str-2 sample 2(duplicates)
6F, 6G= -Lipase control for str-2 sample 3(duplicates)

Step 2: Select XY graph in GraphPad

Step 3: Enter values in the sheet

	X	Y
1	Title	0
2	Title	4
3	Title	8
4	Title	16
5	Title	

Step 4: Curve obtained

Step 6: Curve obtained after applying linear regression

To be used in figure 6

Step 5: Apply liner regression from 'Analyze Data'

Figure 3. Steps for generation of triglyceride standard curve using Biovision kit for triglyceride quantification

- Generate standard curve by plotting fluorescence vs triglycerides concentration (pmol) using PRISM (see steps in Figure 3). Obtain a fit by applying linear regression.
- The standard curve is used to interpolate values of x (triglyceride concentrations) for known values of y (follow steps in Figure 4).

Figure 4. Interpolation from the standard curve to obtain triglyceride concentration in biological samples

D. Measurement of triglycerides in *C. elegans* samples

1. Dilute worm supernatant (from Step B28) to 1:2, 1:5, and 1:10 with TAB to make up the final volume to 50 µl in a 96-well microtiter plate, in duplicates.
2. Add 2 µl lipase to each sample and mix it well. Incubate the mix at RT on a rocker for 20 min.
3. Include no lipase control for each sample to measure basal level glycerol present in the sample.
4. For each well, prepare 50 µl of triglyceride reaction mix by adding 2 µl Triglyceride enzyme mix, 0.4 µl triglyceride probe, and 47.6 µl TAB. Prepare a single master mix for all the wells.
Note: Protect the reaction from light.
5. Add 50 µl of the triglyceride reaction mix to each sample well of the 96-well microtiter plate (from Step D2) and incubate at RT for 60 min.
6. Measure the fluorescence at Ex/Em of 535/590 nm for each sample with lipase and respective no lipase control (raw values shown in the snapshot in Figure 5).

Step 1: Calculations for corrected fluorescence values for samples

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	<->	1	2	3	4	5	6							
2	B	6959	14923	21799	46175			1B, 1C=Standard buffer control						
3	C	6511	15001	22157	46556			2B, 2C= Standard 4 pmol/well						
4	D	23253	25653	18215	27439	17700	19238	3B, 3C=Standard 8 pmol/well						
5	E	22950	24996	19025	27531	17515	19320	4B, 4C=Standard 16 pmol/well						
6	F	5896	6901	6700	7353	7452	7572	1D, 1E= WT sample 1 (duplicates)						
7	G	6800	5351	7310	7099	7853	7601	2D, 2E= WT sample 2 (duplicates)						
8								3D, 3E= WT sample 3 (duplicates)						
9								4D, 4E= str-2 sample 1 (duplicates)						
10								5D, 5E= str-2 sample 2 (duplicates)						
11								6D, 6E= str-2 sample 3 (duplicates)						
12								1F, 1G= -Lipase control for WT sample 1(duplicates)						
13								2F, 2G= -Lipase control for WT sample 2(duplicates)						
14								3F, 3G= -Lipase control for WT sample 3(duplicates)						
15								4F, 4G= -Lipase control for str-2 sample 1(duplicates)						
16								5F, 5G= -Lipase control for str-2 sample 2(duplicates)						
17								6F, 6G= -Lipase control for str-2 sample 3(duplicates)						
18														

Step 2: Calculations for number of present per µl of each sample

	(n)	(r)	(w)
	Number of worms in 100 µl	Av. worms per 100 µl	Total worms in 2 ml
WT I	89, 101, 95	95	1900
WT II	96, 111, 114	107	2140
WT III	75, 84, 96	85	1700
STR-2 I	118, 126, 127	124	2480
STR-2 II	68, 76, 87	77	1540
STR-2 III	93, 99, 81	91	1820

Step 3: Calculations for TAG quantification in each samples

	(b)	(c)	(x=b-c)	(y)	(Z=y x dilution factor(a))	(Q=Z/w)
	Av. Sample read	Av. No lipase control read	Corrected fluorescence	pmol per well	pmole per µl of sample	pmol per worm nmol per worm
WT I	23101.5	7126	15975.5	7.080788	3540.394	819.88 0.82
WT II	25324.5	6976	18348.5	8.037542	4018.771	807.51 0.81
WT III	18620	8005	10615	4.919523	2459.7615	723.46 0.72
STR-2 I	27485	7226	20259	8.807824	4403.912	639.28 0.64
STR-2 II	17607.5	7652.5	9955	4.653422	2326.711	513.69 0.51
STR-2 III	19279	7586.5	11692.5	5.353953	2676.9765	603.06 0.60

Figure 5. Calculations for triglyceride quantification in *C. elegans* samples

Data analysis

1. Subtract the fluorescence value of no lipase control from fluorescence value of respective worm sample with lipase. This provides corrected fluorescence value.
2. Apply the corrected fluorescence values to the triglyceride linear regression fit line (from Step C9) to get the picomoles of TAGs present in sample per well by interpolation in GraphPad Prism5 (see Figure 4 and Figure 5).
3. Calculate the concentration of triglycerides per µl in worm samples (Figure 5).

$$Z = \frac{x}{a} * d$$

where,

Z: picomoles of triglycerides per µl of the sample

a: volume of sample added to the well

d: sample dilution factor

4. Absolute amount of triglycerides present per worm can be calculated (Figure 5)

$$Q = \frac{(Z)}{w}$$

where,

Q: picomoles of triglycerides per worm

w: number of worms per present ul of the sample

$$w = \frac{(n)}{r}$$

where,

n: total number of worms for each sample (from Step B20)

r: volume of worm suspension in Step B19

Here, we have shown an example of TAGs quantification in wild type N2 animals (denoted as WT) and *str-2 (ok3148)* animals. STR-2 chemosensory G-protein coupled receptor controls the life span of *C. elegans* at high temperatures by regulating fat metabolism. *str-2* mutants have reduced total lipid content as confirmed by Oil-Red-O staining and triglycerides quantification by BioVision kit (Figure 6). For WT and *str-2 (ok3148)* animals, we found that the triglyceride level was 0.78 ± 0.03 and 0.58 ± 0.03 nanomoles per worm respectively (Dixit *et al.*, 2020). One of the caveat of the biochemical method is that it can not provide information on the spatial distribution of stored fats in various tissues of worms. Thus, a combination of staining methods and biochemistry is desirable for the study of fat metabolism in worms.

Figure 6. Triglycerides levels in WT and *str-2 (ok3148)* mutant of *C. elegans*. A. Mean $\pm$ SEM triglycerides/worm in WT and *str-2* animals. B. Lipids droplets stained by Oil-Red-O in WT and *str-2* animals (Scale bar: 200 μ m).

Recipes

- Nematode growth media (1 L)
 - 3 g NaCl
 - 2.5 g Peptone
 - 17 g Agar
 - Water up to 1 L
 - Autoclave the medium at 121 °C and 15 psi for 30 min
 - Cool down the media to ~50 °C and add the following:

- 1 ml 1 M CaCl_2
- 1 ml 1 M MgSO_4
- 1 ml of 5 mg/ml Cholesterol
- 1 ml 50 mg/ml streptomycin
- 25 ml 1 M KPO_4
- Mix well and pour in 60 mm Petri dish
- 2. 1 M KPO_4 (200 ml)
 - 21.66 g of KH_2PO_4
 - 7.12 g of K_2HPO_4
 - Water up to 200 ml
 - Autoclave the preparation at 121 °C and 15 psi for 30 min
- 3. 1 M CaCl_2 (100 ml)
 - 14.7 g of CaCl_2
 - Water up to 100 ml
 - Autoclave the solution at 121 °C and 15 psi for 30 min
- 4. 1 M MgSO_4 (100 ml)
 - 12.037 g of MgSO_4
 - Water of 100 ml
 - Autoclave the solution at 121 °C and 15 psi for 30 min
- 5. 5 mg/ml Cholesterol
 - 0.25 g of cholesterol
 - 50 ml absolute ethanol
 - Filter sterilize the solution with 0.45 μm filter
- 6. 50 mg/ml Streptomycin
 - 0.5 g of streptomycin
 - Water up to 10 ml
 - Filter sterilize with 0.45 μm syringe filter
- 7. 5 N NaOH
 - 8 g NaOH
 - Water up to 40 ml
 - Filter sterilize with 0.45 μm syringe filter
- 8. LB media
 - 2.5 g LB broth
 - water up to 100 ml
 - Autoclave the LB broth at 121 °C and 15 psi for 30 min
- 9. LB agar
 - 2.5 g LB broth
 - 2 g agar
 - Water up to 100 ml

Autoclave the preparation at 121 °C and 15 psi for 30 min
Cool down the media to ~50 °C and pour in 60 mm Petri dishes

10. M9 buffer (500 ml)

1.5 g KH₂PO₄

3 g Na₂HPO₄

2.5 g NaCl

0.5 ml of 1 M MgSO₄

Water up to 500 ml

Autoclave the solution at 121 °C and 15 psi for 30 min

11. 2x Bleach Mix (2 ml)

400 µl 5 N NaOH

900 µl of NaOCl (bleach)

Water up to 2 ml

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Competing interests

We declare no financial or non-financial competing interests.

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