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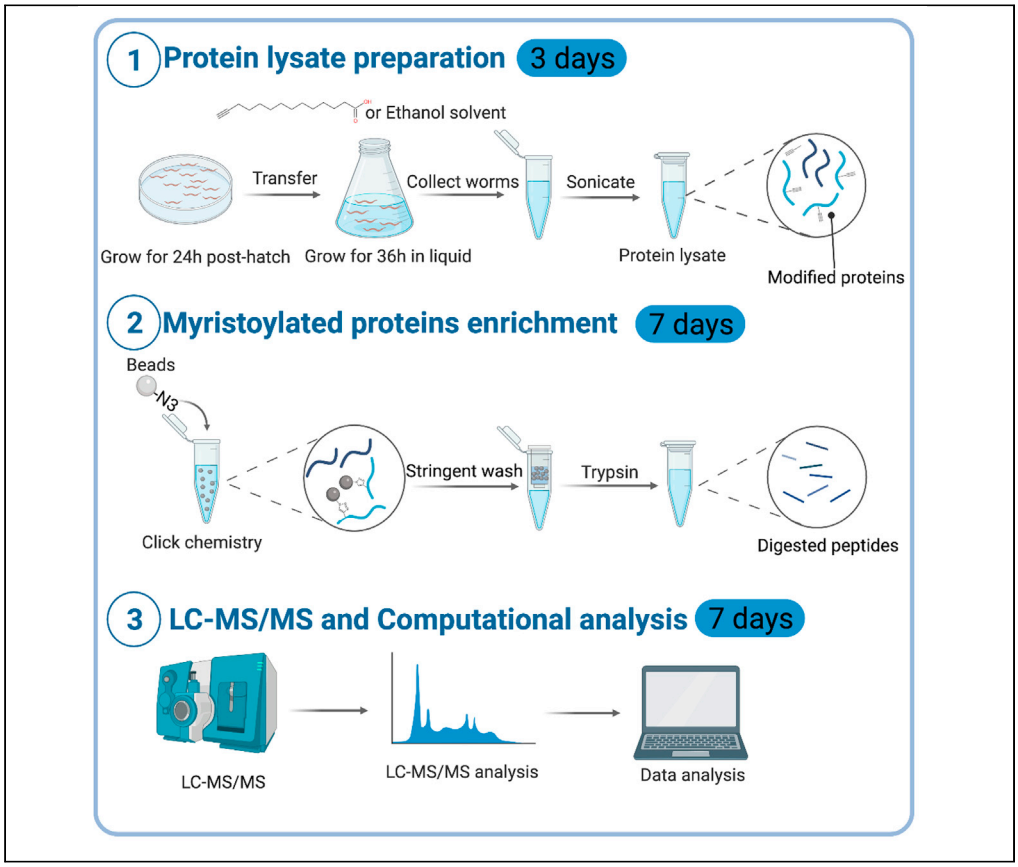
A metabolic labeling protocol to enrich myristoylated proteins from *Caenorhabditis elegans*



Myristoylation is a type of lipidation with important functions. Owing to the lack of high-quality antibodies against myristoylation, developing alternative methods for profiling myristoylated proteins is important. Here, we provide a protocol for metabolic labeling using click chemistry to profile myristoylated proteins in *C. elegans*. Our approach improves the signal/noise ratio by covalently linking the myristoylated proteins to the beads. This protocol provides a highly specific and reproducible way for enriching myristoylated proteins, which could be modified to analyze other types of lipidations.

Xin Gong, Yanwen Feng, Hongyun Tang

tanghongyun@westlake.edu.cn

Highlights

Enriching myristoylated proteins in *C. elegans*

Enriching myristoylated proteins via covalently linking them to the beads

Preparing peptides for mass spectrometry analysis through on-bead digestion

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A metabolic labeling protocol to enrich myristoylated proteins from *Caenorhabditis elegans*

Xin Gong,[1,2,3,4] Yanwen Feng,[1,2,3,4] and Hongyun Tang[1,2,3,4,5,*]

[1]Key Laboratory of Growth Regulation and Translational Research of Zhejiang Province, School of Life Sciences, Westlake University, Hangzhou 310024, China

[2]Institute of Biology, Westlake Institute for Advanced Study, Hangzhou 310024, China

[3]Westlake Laboratory of Life Sciences and Biomedicine, Hangzhou 310024, China

[4]Technical contact

[5]Lead contact

*Correspondence: tanghongyun@westlake.edu.cn
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SUMMARY

Myristoylation is a type of lipidation with important functions. Owing to the lack of high-quality antibodies against myristoylation, developing alternative methods for profiling myristoylated proteins is important. Here, we provide a protocol for metabolic labeling using click chemistry to profile myristoylated proteins in *C. elegans*. Our approach improves the signal/noise ratio by covalently linking the myristoylated proteins to the beads. This protocol provides a highly specific and reproducible way for enriching myristoylated proteins, which could be modified to analyze other types of lipidations.

For complete details on the use and execution of this protocol, please refer to Tang et al. (2021).

BEFORE YOU BEGIN

Timing: 3 days

In recent years, click-chemistry-enabled probes have been optimized and applied to investigate a variety of proteomics (Parker and Pratt, 2020; Gao et al., 2018; Chuh and Pratt, 2015; Xiao et al., 2017; Qin et al., 2019; Zhang et al., 2020). The protocol below describes the detailed steps for enriching and identifying myristoylated proteins from *C. elegans* by using a myristate analog, myristic acid alkyne, which has been shown to mimic endogenous myristate to modify proteins (Burnaevskiy et al., 2015; Tang et al., 2021; Tang and Han, 2017; Thinon et al., 2014). We have also modified this protocol to determine other types of lipidations in HEK293 cells and HepG2 cells. Our protocol is capable of efficiently removing non-specific bound proteins through covalently attaching target proteins to the beads, and thus presents a highly effective strategy to pull down modified proteins.

1. Establish the *C. elegans* strains you need and grow enough gravid adults.

   Note: prepare worms from >200 6-cm nematode growth medium (NGM) plates (200 gravid adults/plate).

2. Prepare bacteria (OP50 *E. coli*) food by concentrating bacteria from 8 L of 14 h culture to 200 mL for liquid culture of *C. elegans*.

Note: Recommend preparing a large amount of bacteria food and add into worm culture when needed.

3. Make sure that all the required reagents and equipment are ready to use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
NaCl	Sigma-Aldrich	Cat# S5886
KH_2PO_4	Sigma-Aldrich	Cat# P5655
K_2HPO_4	Sigma-Aldrich	Cat# P3786
Tri-potassium citrate monohydrate	Sigma-Aldrich	Cat# C3029
Disodium EDTA	Sigma-Aldrich	Cat# E6758
$FeSO_4 \cdot 7H_2O$	Sigma-Aldrich	Cat# F8633
$MnCl_2 \cdot 4H_2O$	Sigma-Aldrich	Cat# M5005
$CuSO_4 \cdot 5H_2O$	Sigma-Aldrich	Cat# C8027
$ZnSO_4 \cdot 7H_2O$	Sigma-Aldrich	Cat# Z0251
$CaCl_2$	Sigma-Aldrich	Cat# C5670
$MgSO_4 \cdot 7H_2O$	Sigma-Aldrich	Cat# M1880
Tryptone	Sigma-Aldrich	Cat# T9410
Yeast	Sigma-Aldrich	Cat# V900886
Na_2HPO_4	Sigma-Aldrich	Cat# S5136
NH_4Cl	Sigma-Aldrich	Cat# A9434
NaOH	Sigma-Aldrich	Cat# 795429
Agar	Sigma-Aldrich	Cat# A7002
Proteose peptone	BD	Cat# 211684
Cholesterol	Sigma-Aldrich	Cat# C3045
Ethanol	Sangon Biotech	Cat# A500737-0500
Tri-potassium citrate monohydrate	Sigma-Aldrich	Cat# C3029
myristic acid alkyne	Cayman Chemical	Cat# 13267
C-18 desalting cartridges	Waters	Cat# WAT094225
SDS	Sigma-Aldrich	Cat# V-900859
DTT	Sigma-Aldrich	Cat# 10197777001
Urea	Sigma-Aldrich	Cat# U1250
1M Tris-HCL PH6.8	Beyotime	Cat# ST768
1M Tris-HCL PH8.8	Beyotime	Cat# ST788
Iodoacetamide	Sigma-Aldrich	Cat# I6125
Acetonitrile	Sigma-Aldrich	Cat# 1.00029
Trifluoroacetic acid	Sigma-Aldrich	Cat# 302031
NH_4HCO_3	Sigma-Aldrich	Cat# 09830
Cocktail	Sigma-Aldrich	Cat# 8340
Methanol	Sigma-Aldrich	Cat# 34860-1L-R
Trypsin	Promega	Cat# V5280
CHAPS	VWR	Cat# VWRC0465
NaClO	Sigma-Aldrich	Cat# 239305
BSA	GENVIEW	Cat# FA016
ECL	GenStar	Cat# E171-04
DMSO	Sigma-Aldrich	Cat# 4540
$CuSO_4 \cdot 5H_2O$	Sigma-Aldrich	Cat# 209198
Tris(3-hydroxypropyltriazolylmethyl) amine	Sigma-Aldrich	Cat# 762342
(+)-Sodium L-ascorbate	Sigma-Aldrich	Cat# A7631
ExpressPlus™ PAGE Gels	GenScript	Cat# M42010C

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli OP50	Caenorhabditis Genetics Center (CGC)	N/A
Critical commercial assays		
Click-&-Go™ Click Chemistry Reaction Buffer Kit	Click Chemistry Tools	Cat# 1001
Azide Agarose	Click Chemistry Tools	Cat# 1038-2
Click-&-Go™ Plus Protein Enrichment Kit	Click Chemistry Tools	Cat# 1235
Coomassie Blue G-250 Staining Buffer	GenStar	Cat# E154-01
Experimental models: Organisms/strains		
C. elegans: N2 (Bristol), Wild type	Caenorhabditis Genetics Center (CGC)	N/A
Software and algorithms		
BioRender	N/A	https://app.biorender.com
Antibodies		
Streptavidin-HRP (a dilution of 1:2000)	GenScript	Cat# M00091
β-Actin Rabbit Ab (a dilution of 1:3000)	ABclonal	Cat# AC026

MATERIALS AND EQUIPMENT

S Basal		
Reagent	Final concentration	Amount
NaCl	5.85 mg/mL	5.85 g
KH_2PO_4	6 mg/mL	6 g
K_2HPO_4	1 mg/mL	1 g
Cholesterol (10 mg/mL in ethanol)	0.05% (v/v)	0.5 mL
ddH_2O	n/a	999.5 mL
Total	**n/a**	**1000 mL**
Sterilization with 0.22 μm membrane filter except cholesterol. Add cholesterol before use at 20°C–25°C.		

1 M potassium citrate (pH=6.0)		
Reagent	Final concentration	Amount
Citric acid monohydrate	20 mg/mL	20 g
Tri-potassium citrate monohydrate	293.5 mg/mL	293.5 g
ddH_2O	n/a	1000 mL
Total	**n/a**	**1000 mL**
Sterilization with 0.22 μm membrane filter. Store at 20°C–25°C for 6 months.		

Trace metals solution		
Reagent	Final concentration	Amount
Disodium EDTA	1.86 mg/mL	1.86 g
$FeSO_4 \cdot 7H_2O$	0.69 mg/mL	0.69 g
$MnCl_2 \cdot 4H_2O$	0.2 mg/mL	0.2 g
$CuSO_4 \cdot 5H_2O$	0.025 mg/mL	0.025 g
$ZnSO_4 \cdot 7H_2O$	0.29 mg/mL	0.29 g
ddH_2O	n/a	1000 mL
Total	**n/a**	**1000 mL**
Sterilization with 0.22 μm membrane filter. Store in the dark (wrapped with aluminum foil) at 20°C–25°C for 6 months.		

S Medium		
Reagent	Final concentration	Amount
1 M potassium citrate (pH=6.0)	1%(v/v)	10 mL
Trace metals solution	1%(v/v)	10 mL
1 M $CaCl_2$ (in ddH_2O)	0.3% (v/v)	3 mL
1 M $MgSO_4$(in ddH_2O)	0.3% (v/v)	3 mL
S Basal	n/a	974 mL
Total	**n/a**	**1000 mL**
Prepare before use at 20°C–25°C in a sterile environment. No autoclaving. Filtering sterilization is optional.		

Bleach buffer		
Reagent	Final concentration	Amount
5 M NaOH (in ddH_2O)	0.5 M	1 mL
NaClO	20% (v/v)	2 mL
ddH_2O	n/a	7 mL
Total	**n/a**	**10 mL**
Prepare before use at 20°C–25°C. Sterilization is not required.		

Lysis buffer		
Reagent	Final concentration	Amount
1 M Tris (pH=6.8)	200 mM	20 mL
CHAPS	4% (w/v)	4 g
NaCl	1 M	5.844 g
Urea	8 M	48.048 g
ddH_2O	n/a	to 100 mL
Total	**n/a**	**100 mL**
Prepare before use at 20°C–25°C. Sterilization is not required.		

LB broth		
Reagent	Final concentration	Amount
Tryptone	10 mg/mL	10 g
Yeast	5 mg/mL	5 g
NaCl	10 mg/mL	10 g
ddH_2O	n/a	1000 mL
Total	**n/a**	**1000 mL**
Sterilize by autoclave after solids are completely dissolved. Store at 20°C–25°C for 1 month.		

1M PPB (Potassium Buffered Phosphate) dry mix		
Reagent	Final concentration	Amount
KH_2PO_4	108.3 mg/mL	108.3 g
K_2HPO_4	71.2 mg/mL	71.2 g
ddH_2O	n/a	1000 mL
Total	**n/a**	**1000 mL**
Sterilize by autoclave after solids are completely dissolved. Store at 20°C–25°C for 1 month.		

NGM dry mix		
Reagent	Final concentration	Amount
Agar	17 mg/mL	17 g
Proteose peptone	3 mg/mL	3 g
NaCl	3 mg/mL	3 g
ddH_2O	n/a	972.5 mL
Total	**n/a**	**1000 mL**
Sterilize by autoclave. Wait until the drops to 55°C–60°C. Add the following reagents.		
1M PPB	2.5% (v/v)	25 mL
1M $MgSO_4$	0.1% (v/v)	1 mL
1M $CaCl_2$	0.1% (v/v)	1 mL
Cholesterol (10 mg/mL in ethanol)	0.05% (v/v)	0.5 mL
Prepare before use at 20°C–25°C.		

M9 solution		
Reagent	Final concentration	Amount
Na_2HPO_4	6 mg/mL	6 g
KH_2PO_4	3 mg/mL	3 g
NaCl	5 mg/mL	5 g
NH_4Cl	1 mg/mL	1 g
ddH_2O	n/a	1000 mL
$MgSO_4 \cdot 7H_2O$	0.25 mg/mL	0.25 g
Total	**n/a**	**1000 mL**
Sterilize by autoclave after solids are completely dissolved. Store at 20°C–25°C for 1 month.		

40 mM iodoacetamide solution		
Reagent	Final concentration	Amount
Iodoacetamide	40 mM	74 mg
Agarose Wash Buffer	n/a	10 mL
Total	**n/a**	**10 mL**
Dissolve in fresh Agarose Wash Buffer before use at 20°C–25°C. Sterilization is not required.		

Agarose Wash Buffer (pH=8.0)		
Reagent	Final concentration	Amount
1 M Tris-HCl (pH=6.8)	100 mM	10 mL
10% SDS	1% (v/v)	10 mL
NaCl	250 mM	1.461 g
0.5 M EDTA	5 mM	1 mL
ddH_2O	n/a	79 mL
Total	**n/a**	**100 mL**
Prepare before use at 20°C–25°C. Sterilization is not required.		

1 x SDS-PAGE gel loading buffer		
Reagent	Final concentration	Amount
1M Tris-HCl (pH=6.8)	62.5 mM	6.25 mL
SDS	2 % (w/v)	2 g
Bromophenol blue	0.01% (w/v)	0.01 g
Glycerol	10 % (v/v)	10 mL
DTT	200 mM	3.085 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
ddH_2O	n/a	83.75 mL
Total	**n/a**	**100 mL**

Store at −20°C for 1 year.

8 M Urea / 100mM Tris (pH=8.0)		
Reagent	Final concentration	Amount
Urea	8 M	48.048 g
1 M Tris-HCl (pH=6.8)	100 mM	10 mL
ddH_2O	n/a	to 100 mL
Total	**n/a**	**100 mL**

Prepare before use. Store in the dark (wrapped with aluminum foil) at 20°C–25°C.

STEP-BY-STEP METHOD DETAILS

C. elegans culture and lysate preparation

Timing: 3 days

1. Synchronized L1-staged *C. elegans* were grown on NGM plates spotted with 300 μL of OP50 *E. coli* at 20°C for 24 h (Figure 1). >200 6-cm NGM plates (200 worms/plate) were prepared.

   Note: >40,000 worms are needed for each treatment.

   Note: Synchronize L1 worms: suspend gravid adult worms by pouring 5 mL M9 buffer into each NGM plate, transfer the worms into 15 mL tube, pellet by centrifugation at 2000×*g* for 1 min and rinse with M9 buffer for three times by inverting the tube. Then, freshly prepare 10 mL of bleach solution, add into the 15 mL tube with pelleted worms, invert the tube for 5 mins on a rocker, pellet the released eggs by centrifugation at 1000×*g* for 1 min, discard the supernatant, and add M9 buffer to wash the pelleted eggs for three times. Lastly, allow these eggs hatch for 16 hours in 5 mL of M9 to generate synchronized L1-staged worms by placing them on a rocker at 20°C.

   Note: The worm counting process: pellet the synchronized L1-staged worms by centrifugation at 2000×*g* for 2 min and remove the supernatant to make the final volume to 3 mL.Then, mix the worms on vortex for 5 seconds, aspirate 2.5 μL of the liquid from the tube, drop the liquid on a slide and count the worms' number under a dissection microscope. Repeat the process three times to get the average number of worms. Based on the number of worms needed, the corresponding volume of liquid containing L1-staged worms is added to the NGM plates.

2. Suspend these worms grown on NGM with a 5 mL M9 buffer per plate, transfer to a 50 mL tube, pellet by centrifugation at 2000×*g* for 5 min. Resuspend the pelleted worms with 5 mL S-medium, transfer them by pipetting to a 500 mL conical flask, add 40 mL concentrated bacteria, add more S-medium to make the final volume 400 mL containing either ethanol or 60 μM myristic acid alkyne, and culture the worms by placing the flask on a shaker (150 rpm/min) at 20°C for 36 h (Figure 1).

   ⚠ CRITICAL: Culture *C. elegans* with 1000–3000 worms per 1 mL of liquid medium. High worm density will lead to uneven growth of *C. elegans* or dauer formation.

   ⚠ CRITICAL: Add about 100 μL concentrated OP50 *E. coli* per 1 mL S medium. *C. elegans* would be starved if there are not enough bacteria. Culture with too much OP50 *E. coli* causes worm death. Monitor food consumption and add food when needed.

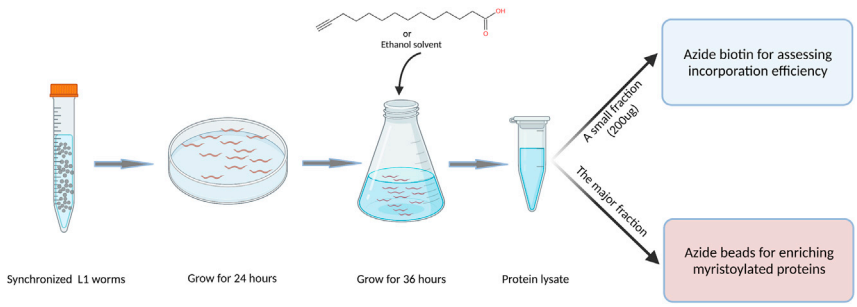

Figure 1. Schematic overview of *C. elegans* culture and sample preparation

⚠ CRITICAL: Change the medium containing bacteria food and either 60 μM myristic acid alkyne or ethanol solvent every 12 h.

⚠ CRITICAL: 60–100 μM myristic acid alkyne works the best in our hands. Culture with high concentration (>120 μM) of myristic acid alkyne causes worm death.

3. *C. elegans* were pelleted and washed with M9 for 3 times (Figure 1).

Note: C. elegans were directly poured into a 50 mL centrifuge tube, centrifuged at 2000×*g* for 5 min, and the supernatant was discarded. 20 mL M9 was added for washing, centrifuged at 2000×*g* for 5 min, and repeated for 3 times.

Pause point: The worm pellet can be stored at −80°C for a month. The frozen worm pellet is thawed on ice when proceeding to the next step.

4. Suspend the pelleted worms with 2 mL lysis buffer (pH=8.0) (200 mM Tris, 4% CHAPS, 1 M NaCl, 8 M Urea and 1% (v/v) protease inhibitor), transfer them to a 15 mL tube and then sonicate at 4°C until the worms are completely broken and dissolved. Centrifugate the worm lysate at 10,000×*g* for 5 min at 4°C and collect the supernatant. Aspirate a fraction of the supernatant (200 μg proteins) to a 2-mL tube for detecting the alkyne myristic acid incorporation efficiency (Figure 1) and use the rest of the supernatant for enriching myristoylated proteins.

Note: Sonicate (Sonics, VCX150) the worms at 35% amplitude for 3 seconds, followed by a rest period for 4 seconds. Usually, the worms would be completely broken after 4 minutes of sonication. The bottom of the 15-mL tube containing worms was inserted into a water bath filled with ice to prevent the sample from over-heat.

⚠ CRITICAL: Make sure there is no EDTA contamination in the solution, which inactivates click reaction.

Evaluation of incorporation efficiency of myristic acid alkyne

Note: Before proceeding to attach myristoylated proteins to the beads by click chemistry, a small amount of worm lysate was analyzed to determine if myristate alkyne was incorporated to modify proteins successfully. Click-&-Go^{TM} Click Chemistry Reaction Buffer Kit (Cat# 1001, Click Chemistry Tools) was used to detect proteins modified by myristate alkyne through western blot.

Optional: All the reagents can be prepared manually.

Timing: 2 days

5. Click reaction
   a. Mix 110 μL Reaction Buffer containing 40 μM azide biotin with 50 μL worm lysate (about 200 μg proteins) at 20°C–25°C.
   b. Add 20 μL Additive 1 (82mM Tris-amine in H_2O) and mix thoroughly on the vortex for 30s.
   c. Add 20 μL Copper (II) Sulfate Solution (20mM Copper (II) Sulfate in H_2O) and mix thoroughly on the vortex for 30s.
   d. Add 10 μL Reducing Agent (0.2 g/L Sodium L-ascorbate in H_2O) to make the final reaction volume to 210 μL, mix thoroughly on the vortex for 30s. The reaction was carried out in darkness and samples were rotated end-over-end for 30 min at 20°C–25°C.

6. Protein precipitation
   a. After clicking chemistry, add 600 μL 100% methanol and mix on vortex for 5 s.
   b. Add 150 μL 100% chloroform and mix on vortex for 30 s.
   c. Add 400 μL ddH_2O and mix on the vortex for 30 s.
   d. Centrifuge at 15,000×*g* for 5 min at 20°C–25°C, carefully discard the upper water phase using a pipette and keep the middle protein layer and the bottom chloroform.

   ⚠ CRITICAL: Do not disturb the interface layer containing proteins.

   Note: When adding chloroform, it should be performed in the fume hood to avoid inhalation or skin contact.

   e. Add 450 μL 100% methanol, mix thoroughly with vortex, centrifuge at 15,000×*g* for 10 min at 20°C–25°C, remove the chloroform and methanol supernatant and keep the precipitated proteins. Repeat this step 2 times.
   f. Open the lid of the tubes and let them dry at 20°C–25°C in a chemical hood for at least 15 min. The precipitated proteins can be immediately used for the next step or stored at −20°C for at least one month or at −80°C for at least three months.

7. Western blot analysis
   a. Add 50 μL 1 x SDS-PAGE gel loading buffer to the protein precipitate, and mix on vortex for 30 min.
   b. Heat the sample in a metal bath at 70°C for 10 min or in a water bath at 100°C for 5 min, centrifuge at 10,000×*g* for 1 min and load 5 μL (5–20 μg proteins) of the samples into the SDS-PAGE gel (GenScript, Cat# M42010C).
   c. Run the SDS-PAGE gel and analyze it with western blot by using streptavidin-HRP (a dilution of 1:2000) to detect the myristic acid alkyne signals from the proteins. Actin antibody (a dilution of 1:3000) was used to determine the loading amount. The expected result was shown in Figure 2.

   Note: Block with 5% BSA at 20°C–25°C.

Preparation of azide agarose beads

Timing: 30 min

Note: These steps were performed according to the Click-&-Go™ Plus Protein Enrichment Kit (Cat#1235, Click Chemistry Tools) with minor modifications.

8. Resuspend the azide agarose beads and then quickly transfer 200 μL of them into a clean 2-mL tube.

   Alternatives: Appropriate magnetic beads attached with azide (Cat# 1036, Click Chemistry Tools) can also be used used.

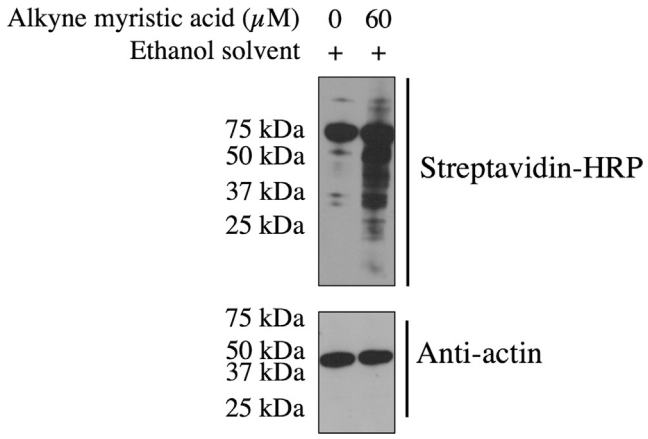

Figure 2. Metabolic labeling of proteins with myristic acid alkyne in *C. elegans*

Western blot indicating that proteins modified by myristic acid alkyne are detected. *C. elegans* were harvested and analyzed as depicted in Figure 1. Specifically, protein lysates were obtained from *C. elegans* supplemented with myristic acid alkyne or ethanol control, and then azide biotin was attached to proteins modified by myristic acid alkyne after click chemistry. Streptavidin-HRP was used to probe the labeling efficiency. Actin showing that total proteins loading amount is equal between different treatments.

9. Add 1.3 mL ddH_2O and wash the beads by manually inverting the tube 10 times, and pellet by centrifugation at 1000×*g* for 2 min at 20°C–25°C. Repeat the step twice.

Note: Be careful not to aspirate the precipitated beads.

Preparing copper (II) catalyst solution

Note: The solution was prepared as described in the Click-&-Go™ Plus Protein Enrichment Kit (Cat#1235, Click Chemistry Tools).

Timing: 20 min

10. 1 mL of copper (II) catalyst solution was used for each enrichment reaction, and was prepared as follows:
    a. 860 μL sterilized ddH_2O
    b. 100 μL Additive 1 (82mM Tris-amine in H_2O) (Component D)
    c. 20 μL Copper (II) Sulfate solution (100mM Copper (II) Sulfate in H_2O) (Component B)
    d. 20 μL Reducing Agent (80g/L Sodium L-ascorbate in H_2O) (Component C)

Alternatives: The kit is not necessary, and the solutions can be manually made.

⚠ CRITICAL: Make sure the reducing agent is excessive.

11. Invert to mix copper catalyst solution briefly

Linking alkyne-myristate modified proteins to the azide beads via click reaction

Timing: 1 day

12. Mix 200 μL washed azide agarose beads, 800 μL *C. elegans* protein lysate (about 3 mg protein) and 1000 μL copper (II) catalyst solution in a 2 mL tube. For each treatment, three such 2-mL reactions were needed. The samples were set on a rotator for thorough mixing and the click reaction was carried out at 20°C–25°C for 20 h.

Note: Seal the tube with parafilm to prevent solution leak.

Proteins bound to agarose beads were reduced and alkylated

Timing: 4 h

Note: The experiment was performed based on the manufacturer's manual with minor changes. https://clickchemistrytools.com/wp-content/uploads/2019/02/info-sheet-1235.pdf

Optional: the reagents also can be manually prepared.

13. Pellet agarose beads by centrifugation at 1000×*g* for 2 min, discard the supernatant, add 1.8 mL sterilized ddH_2O and invert the tube to clean the agarose beads at 20°C–25°C. Repeat this for 3 times. Be careful not to aspirate the beads.

⚠ CRITICAL: the Lysis Buffer needs to be removed to prevent it from interacting with the SDS in the Agarose Wash Buffer.

14. Combine the beads from these three reactions for each treatment as indicated in step #12, re-suspend the beads in 3 mL Agarose Wash Buffer and then add 30 μL of 1 M DTT. Vortex briefly.

Note: Make sure the DTT is not oxidized.

Note: The frozen agarose wash buffer needs to warm up to 20°C–25°C and be uniformly transparent before use.

15. The beads were incubated at 70°C for 15 min and then set at 20°C–25°C for 30 min. Centrifuge at 1000×*g* for 5 min at 20°C–25°C, discard supernatant and keep the beads.
16. Add 3 mL Agarose Wash Buffer containing 40 mM iodoacetamide solution to re-suspend the beads and incubate the alkylation reaction on the rotator in darkness for 30 min at 20°C–25°C.

Beads rinse

Timing: 1 day

17. Spin columns in the kit were used to wash the beads. Transfer the beads to the spin columns, and centrifuge at 1000×*g* for 1 min at 20°C–25°C.
18. Spin columns in the kit were used to wash the beads. Transfer the beads to the spin columns, and centrifuge at 1000×*g* for 1 min at 20°C–25°C.
19. Resuspend the beads in the spin columns with 2 mL Agarose Wash Buffer with 1% SDS, and then allow the liquid flow through the column by gravity at 20°C–25°C. Repeat this step 10 times.
20. Wash the beads by adding 2 mL of 8 M urea/100 mM Tris (pH=8.0) to the spin column. Let the liquid flow through the column by gravity. Repeat this step 20 times to remove SDS.
21. Wash the beads with 2 mL of 20% acetonitrile-ddH_2O solution. Let the solution flow through the spin column by gravity. Repeat this step 20 times.

Note: Centrifugation at 1000×*g* for 1 min can be also performed to spin down the washing buffer. Given many repeated washing steps, we recommend having the washing buffer flow through by gravity, which was convenient to carry out. It takes around 2–3 minutes to have 2 mL of wash buffer flowing through by gravity.

⚠ CRITICAL: The target proteins are covalently linked to the beads. This stringent wash can effectively remove proteins non-specifically bound to the beads. Moreover, SDS causes

damages to mass spectrometry. Thus, washing the beads with 8 M urea and 20% acetonitrile to get rid of residual SDS before mass spec analysis is crucial.

Trypsin digestion of beads-bound proteins

Timing: 18 h

22. Cap the bottom of the spin column to prevent solution flowing through, resuspend the beads with 0.5 mL of digestion buffer (40 mM NH_4HCO_3, 10% acetonitrile), and transfer the beads into a clean 2 mL-tube.
23. Cap the bottom of the spin column to prevent solution flowing through, resuspend the beads with 0.5 mL of digestion buffer (40 mM NH_4HCO_3, 10% acetonitrile), and transfer the beads into a clean 2 mL-tube.
24. Wash the spin column with 0.5 mL additional digestion buffer, and then transfer it to the 2 mL-tube with transferred beads. Repeat this step 2 times.
25. Centrifuge at 1000×*g* for 5 min at 20°C–25°C to pellet the beads, discard the supernatant and leave about 0.2 mL of digestion buffer above the beads. Do not suck the beads out.
26. Add 30 μL of 0.1 μg/μL trypsin to the beads, tap to mix gently, and then incubate at 37°C for 14 h. Flick the tube 4–5 times during the incubation. Continuous shaking is not required.

Note: If specific digestion sites are preferred, other proteases, such as Pepsin and Glu-c, can be also used.

Peptides preparation for mass spectrometry analysis

Timing: 16 h

27. Centrifuge at 1000×*g* for 5 min at 20°C–25°C to pellet the beads and then carefully transfer the supernatant with digested peptides into a clean 1.5 mL-tube.
28. Resuspend the beads in additional 300 μL ddH_2O, vortex briefly, pellet the beads by centrifugation for 5 min at 1000×*g* at 20°C–25°C, and then transfer the supernatant to the clean 1.5 mL-tube with the transferred digested peptides. Repeat this step 3 times.

⚠ CRITICAL: Make sure the final acetonitrile concentration is 2%.

29. Add 2 μL of 100% Trifluoroacetic acid (TFA) to acidify the diluted digest.
30. The digested peptides were desalted with a C-18 column. Add the next solution after each solution completely flows through by gravity.
    a. Add 1 mL of 100% methanol to the C-18 column, allow it to flow through and discard the effluent.
    b. Add 2 mL of ddH_2O to the cartridge, allow it to flow through and discard the effluent.
    c. Add the acidified digest to the C-18 column, allow it to flow through by gravity, and then add the effluent back to the C-18 column.
    d. Add 1 mL of 5% methanol to the C-18 column and discard the effluent.
    e. Add 200 μL of 80% methanol to the C-18 column and collect the effluent with a new 1.5 mL tube. Repeat one time.
    f. Dry the desalted peptide solution with a vacuum centrifugal concentrator (Christ, CT02-50), centrifuging at 1300 rpm for 2 h at −60°C. Keep them at −20°C until ready for MS analysis.

EXPECTED OUTCOMES

This protocol provides an efficient method to enrich and identify the myristoylated proteins from *C. elegans*. Western blot was used to analyze the efficiency that myristic acid alkyne was used to modify proteins, as described in step #7. As shown in Figure 2, the signals from the myristic acid

alkyne treated worms are expected to be much stronger than the ones from the solvent control group. Regarding the outcome from mass spectrometry analysis, previously characterized myristoylated proteins should be identified, which indicates the method works. With this method, many proteins including previously-unknown myristoylated proteins were enriched, and the top 40 candidates from the mass spectrometry analysis were shown in our previous study (Tang et al., 2021).

QUANTIFICATION AND STATISTICAL ANALYSIS

Mass spectrum was analyzed using the Thermo Xcalibur Qual Browser 4.1 and Proteome Discovery 2.4 for database searching.

LIMITATIONS

Although metabolic labeling with corresponding metabolite analog is a commonly used method for detecting protein modifications, the efficiency of metabolite analogs being utilized for modifying proteins could be low. Therefore, optimizing the supplementation concentration and incubation time is necessary for maximizing the label efficiency. Additionally, the beads used in this protocol are not cleavable, thus the whole length of myristoylated proteins cannot be released. Cleavable beads (cat. #1153, Click Chemistry Tools) can be used to collect the modified proteins for further analyses (e.g., western blot) or to specifically enrich the peptides with modification sites.

TROUBLESHOOTING

Problem 1

Low yield of enriched proteins (1, 2, 3).

Possible causes and solutions

A low yield from using animals at different developmental stages.

Protein myristoylation level varies in animals from different stages. Increase protein lysates by using more animals if worms at a developmental stage different from that described here are used.

A low yield from low incorporation efficiency.

Optimize the supplementation conditions.

A low yield of enriched proteins resulted from low click chemistry efficiency.

Make sure the reagents are effective, and ensure the reducing agent is not oxidized.

A low yield from low peptide digestion rate.

Optimize the trypsin digestion conditions.

Problem 2

No enriched proteins are found after mass spec analysis (4, 5, 6).

Possible causes and solutions

High noise from non-specific bound proteins.

Increase wash times.

High background from the failure of Click chemistry.

Ensure click reaction is effective.

No enrichment resulted from low myristic acid alkyne incorporation efficiency.

Optimize the myristic acid alkyne supplementation conditions.

Problem 3
High background signals are observed from the western blotting membrane (7).

Possible solution
Instead of using milk, use BSA to block the membrane.

Problem 4
Western blot shows no obvious signal from the myristic acid alkyne-treated animals (8).

Possible solution
Optimize the supplementation time and concentration, and make sure the click chemistry is effective.

Problem 5
Signal suppression during MS analysis (9).

Possible solution
Increase wash times with 8M urea and 20% acetonitrile to remove all traces of SDS detergent.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hongyun Tang (tanghongyun@westlake.edu.cn).

Materials availability
This study did not generate any unique reagents.

Data and code availability
This study generated a new dataset of myristoylated proteins listed in Table S1 in (Tang et al., 2021).

This study did not generate a new code for analysis.

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AUTHOR CONTRIBUTIONS
X.G. and Y.F. wrote the manuscript. H.T. designed the research, performed the experiment and edited the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES

Burnaevskiy, N., Peng, T., Reddick, L.E., Hang, H.C., and Alto, N.M. (2015). Myristoylome profiling reveals a concerted mechanism of ARF GTPase deacylation by the bacterial protease IpaJ. Mol. Cell *58*, 110–122.

Chuh, K.N., and Pratt, M.R. (2015). Chemical methods for the proteome-wide identification of posttranslationally modified proteins. Curr. Opin. Chem. Biol. *24*, 27–37.

Gao, J., Mfuh, A., Amako, Y., and Woo, C.M. (2018). Small molecule interactome mapping by photoaffinity labeling reveals binding site hotspots for the NSAIDs. J. Am. Chem. Soc. *140*, 4259–4268.

Parker, C.G., and Pratt, M.R. (2020). Click chemistry in proteomic investigations. Cell *180*, 605–632.

Qin, W., Qin, K., Zhang, Y., Jia, W., Chen, Y., Cheng, B., Peng, L., Chen, N., Liu, Y., Zhou, W., et al. (2019). S-glycosylation-based cysteine profiling reveals regulation of glycolysis by itaconate. Nat. Chem. Biol. *15*, 983–991.

Tang, H., Cui, M., and Han, M. (2021). Fatty acids impact sarcomere integrity through myristoylation and ER homeostasis. Cell Rep *36*, 109539.

Tang, H., and Han, M. (2017). Fatty acids regulate germline sex determination through ACS-4-dependent myristoylation. Cell *169*, 457–469.e13.

Thinon, E., Serwa, R.A., Broncel, M., Brannigan, J.A., Brassat, U., Wright, M.H., Heal, W.P., Wilkinson, A.J., Mann, D.J., and Tate, E.W. (2014). Global profiling of co- and post-translationally N-myristoylated proteomes in human cells. Nat. Commun. *5*, 4919.

Xiao, X., Tang, J.J., Peng, C., Wang, Y., Fu, L., Qiu, Z.P., Xiong, Y., Yang, L.F., Cui, H.W., He, X.L., et al. (2017). Cholesterol modification of smoothened is required for hedgehog signaling. Mol. Cell *66*, 154–162.e10.

Zhang, M., Zhou, L., Xu, Y., Yang, M., Xu, Y., Komaniecki, G.P., Kosciuk, T., Chen, X., Lu, X., Zou, X., et al. (2020). A STAT3 palmitoylation cycle promotes TH17 differentiation and colitis. Nature *586*, 434–439.