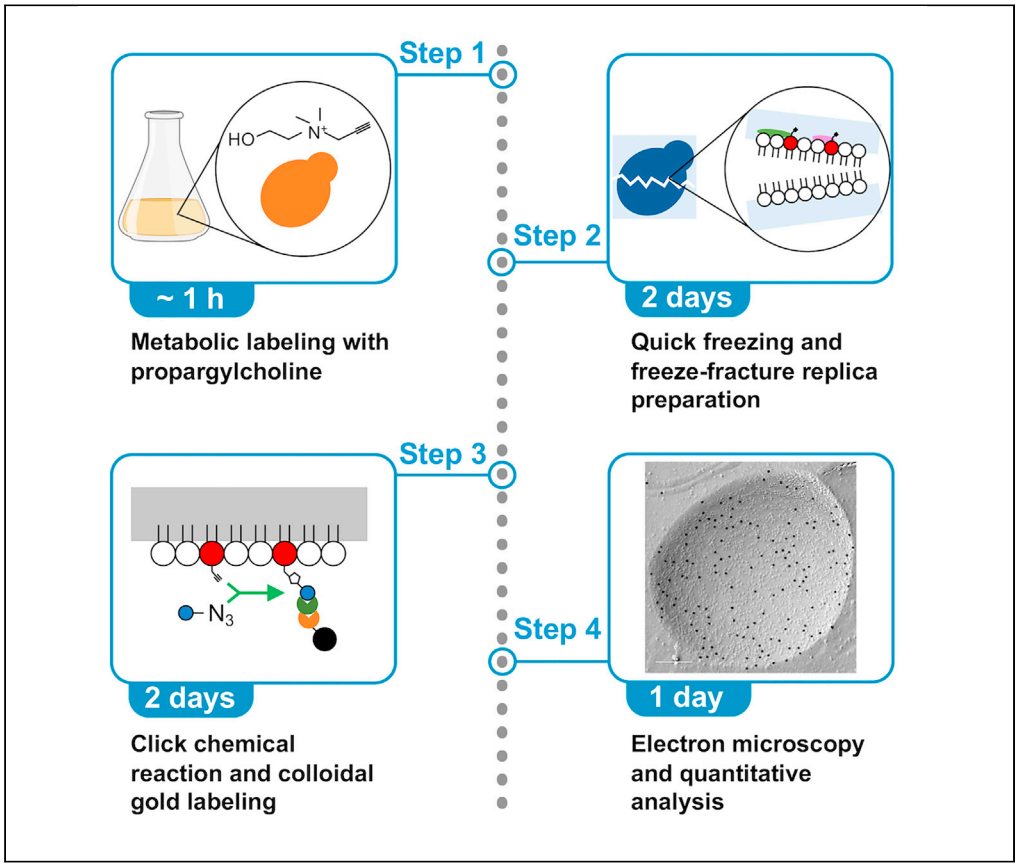
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Ultrastructural localization of *de novo* synthesized phosphatidylcholine in yeast cells by freeze-fracture electron microscopy



Phosphatidylcholine (PtdCho) is a major membrane phospholipid synthesized in the endoplasmic reticulum. Here, we provide a protocol using electron microscopy to localize PtdCho that is newly synthesized by the Kennedy pathway in yeast cells. The protocol consists of the administration of a clickable alkyne-containing choline analog to cells, quick-freezing, freeze-fracture replica preparation, conjugation of biotin-azide by click chemical reaction, and immunogold labeling. This protocol can be used to determine quantitatively to which membrane leaflets newly synthesized PtdCho is incorporated.

Takuma Tsuji, Toyoshi Fujimoto

t.tsuji.zh@juntendo.ac.jp (T.T.)
t.fujimoto.xl@juntendo.ac.jp (T.F.)

Highlights

Analysis of newly synthesized phosphatidylcholine distribution by electron microscopy

Two membrane leaflets of each organelle can be distinguished

Results can be quantified to determine membrane incorporation of phosphatidylcholine

Tsuji & Fujimoto, STAR Protocols *2*, 100990 December 17, 2021
https://doi.org/10.1016/j.xpro.2021.100990

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Ultrastructural localization of *de novo* synthesized phosphatidylcholine in yeast cells by freeze-fracture electron microscopy

Takuma Tsuji[1,2,*] and Toyoshi Fujimoto[1,3,*]

[1]Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan

[2]Technical contact

[3]Lead contact

*Correspondence: t.tsuji.zh@juntendo.ac.jp (T.T.), t.fujimoto.xl@juntendo.ac.jp (T.F.)
https://doi.org/10.1016/j.xpro.2021.100990

SUMMARY

Phosphatidylcholine (PtdCho) is a major membrane phospholipid synthesized in the endoplasmic reticulum. Here, we provide a protocol using electron microscopy to localize PtdCho that is newly synthesized by the Kennedy pathway in yeast cells. The protocol consists of the administration of a clickable alkyne-containing choline analog to cells, quick-freezing, freeze-fracture replica preparation, conjugation of biotin-azide by click chemical reaction, and immunogold labeling. This protocol can be used to determine quantitatively to which membrane leaflets newly synthesized PtdCho is incorporated.

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

BEFORE YOU BEGIN

PtdCho can be synthesized by three different pathways: the Kennedy pathway (cytidine diphosphate [CDP]-choline pathway), the Land's cycle, and the phosphatidylethanolamine methyltransferase pathway. The Kennedy pathway exists ubiquitously in yeast and mammalian cells and can be utilized to metabolically label PtdCho using a choline analog propargylcholine (Jao et al., 2009). While prolonged incubation with propargylcholine (e.g., 1 day) labels PtdCho in all cellular membranes, a short pulse of propargylcholine incubation enables the selective labeling of *de novo* synthesized PtdCho. This method can be used for fluorescence microscopy, but combined with freeze-fracture electron microscopy, the ultrastructural distribution of *de novo* synthesized PtdCho, namely, in which leaflet (i.e., cytoplasmic or non-cytoplasmic) of which organelle membrane it is present, can be clearly determined (Figure 1). Using this methodology, we recently showed that PtdCho synthesized during autophagy is incorporated to autophagosomal membranes preferentially (Orii et al., 2021). The protocol provided below can be used to study the ultrastructural localization of *de novo* synthesized PtdCho in budding yeast, *Saccharomyces cerevisiae*, but this method can be adapted to mammalian cells (Ogasawara et al., 2020).

Preparation of solutions

Timing: 60 min

1. Prepare propargylcholine stock solution.
   a. Dissolve 10 mg of propargylcholine bromide in 48 μL of distilled water (DW) to make 1 M solution.
   b. Sterilize the solution using a 0.22 μm pore filter.

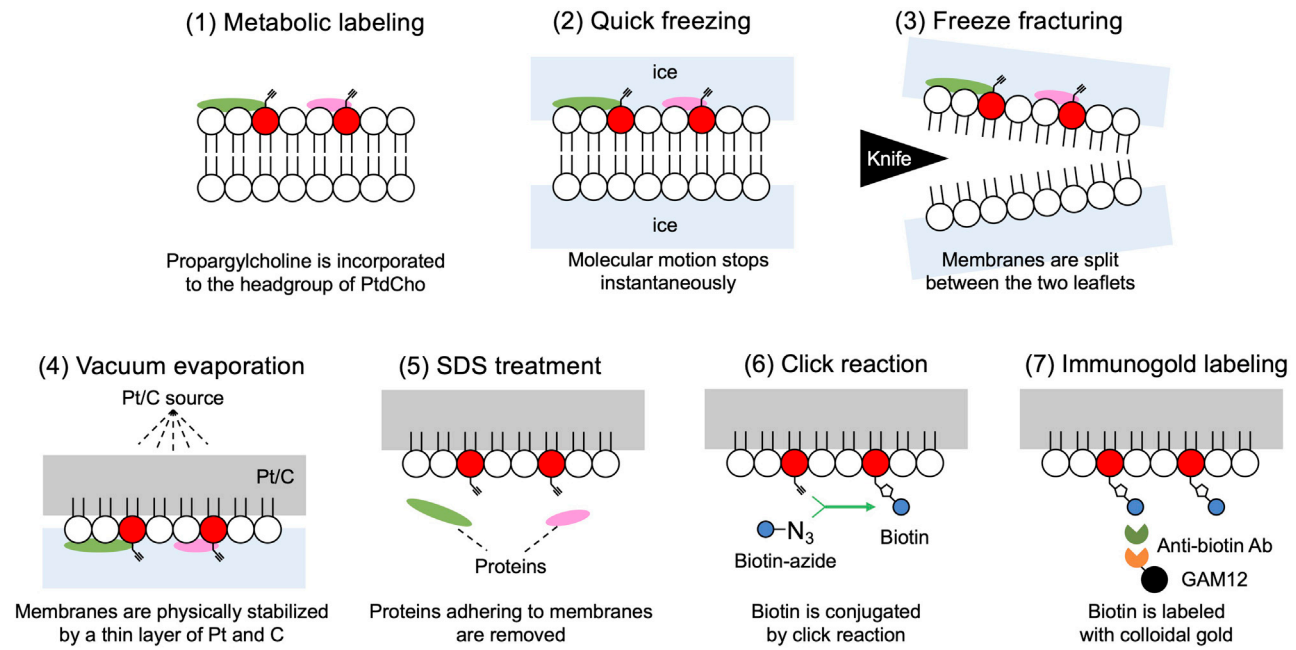


Figure 1. Outline of the procedure for electron microscopy

c. Aliquot (5–10 μL) and store at –20°C up to 1 year.

Note: Propargylcholine bromide (FW 208.1) is commercially available, but it can be synthesized as reported by Jao et al. (2009).

2. Prepare yeast culture medium according to the "materials and equipment" section.

Note: We use synthetic defined medium supplemented with casamino acid, adenine, tryptophan, and uracil (SD + CA + ATU), but other appropriate media can be used as well.

3. Prepare solutions for the click chemical reaction according to the "materials and equipment" section.
   a. 1 M $CuSO_4$ in DW
   b. 10 mM Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) in DMSO
   c. 500 mM ascorbic acid in DW
   d. 0.5 mM AFDye546 picolyl-azide in DMSO
   e. 10 mM biotin-azide in DMSO
   f. 2% gelatin from cold water fish skin (cold fish gelatin) in 0.1 M Tris-HCl (pH 7.5)
4. Prepare other solutions according to the "materials and equipment" section.
   a. 10% formaldehyde in DW
   b. 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4)
   c. 0.1% Triton X-100 in PBS
   d. 0.001% Triton X-100 in 0.1 M Tris-HCl (pH 7.5)
   e. 2% cold fish gelatin and 0.001% Triton X-100 in 0.1 M Tris-HCl (pH 7.5)
   f. 2% cold fish gelatin in PBS
   g. 1% bovine serum albumin (BSA) in PBS

⚠ CRITICAL: Sodium azide should not be included in any solution that is used before or during the click reaction, because it will compete with biotin-azide in conjugation to the alkyne residue.

Preparation of cells

Timing: 2–3 days

5. Streak cells from a glycerol stock onto an agar plate and incubate at 30°C for 2–3 days.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-biotin (1/150 dilution)	Jackson ImmunoResearch Laboratories	CAT# 200-002-211 RRID:AB 2339006
12 nm Colloidal Gold AffiniPure Goat Anti-Mouse IgG (H+L) (EM Grade) (1/20 dilution)	Jackson ImmunoResearch Laboratories	CAT# 115-205-146 RRID:AB 2338733
Chemicals, peptides, and recombinant proteins		
Propargylcholine bromide	Cayman Chemicals	Item# 25870
$CuSO_4$ $5H_2O$	Wako	CAT#031-04411
Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine	AnaSpec	CAT# AS-63360-50
L(+)-Ascorbic acid	Wako	CAT# 014-04801
AFDye 546 picolyl-azide	Click Chemistry Tools	CAT# 1284-1
Biotin-azide	Cayman Chemicals	Item# 13040
Gelatin from cold-water fish skin	Sigma-Aldrich	CAT# G7041
Paraformaldehyde	Merck	CAT# 1.04005.1000
Triton X-100 (Polyoxyethlene(10) Octylphenyl Ether)	Wako	CAT# 169-21105
Bovine serum albumin	Nacalai Tesque	CAT# 01281-26
Zymolyase	Nacalai Tesque	CAT# 07665-55
Yeast nitrogen base without amino acids and ammonium sulfate	BD	CAT# 233520
Ammonium sulfate	Wako	CAT# 019-03435
Casamino acid	BD	CAT# 223050
D(+)-Glucose	Wako	CAT# 049-31165
Adenine sulfate	Wako	CAT# 010-19612
L-Tryptophan	Wako	CAT# 204-03382
Uracil	Sigma-Aldrich	CAT# U0750
Tris(hydroxymethyl)aminomethane	Nacalai Tesque	CAT# 35434-21
(±)-Dithiothreitol	Wako	CAT# 045-08974
NaCl	Wako	CAT#191-01665
KCl	Nacalai Tesque	CAT#28538-75
KH_2PO_4	Nacalai Tesque	CAT#28736-75
Na_2HPO_4	Wako	CAT#193-02862
NaH_2PO_4 2H2O	Wako	CAT#198-02812
Yeast extract	BD	CAT#212750
Hipolypepton	Nihon Pharmaceutical	CAT#392-02115
Agar	Wako	CAT#010-15815
Experimental models: Organisms/strains		
S. cerevisiae: Strain background: SEY6210	ATCC	ATCC: 201392
Other		
0.22 μm pore filter	Whatman	CAT#9913-2502
Spectrophotometer	DeNovix	DS-11+
Glass slide	Matsunami	CAT#S2445
Coverslip	Matsunami	C022321
VECTASHIELD	Vector	H-1000-10
Fluorescence microscope	ZEISS	Axio Observer
Transmission electron microscope	JEOL	JEM1011

MATERIALS AND EQUIPMENT

SD + CA + ATU medium		
Reagent	Final concentration	Amount
Yeast nitrogen base without amino acids and ammonium sulfate	0.17%	170 mg
Ammonium sulfate	0.5%	500 mg
Casamino acid	0.5%	500 mg
Glucose	2%	2 g
Adenine sulfate	0.004%	4 mg
Tryptophan	0.004%	4 mg
Uracil	0.002%	2 mg
Distilled water (DW)	n/a	100 mL
Total	**n/a**	**100 mL**
After autoclave sterilization, store at room temperature (RT: 15°C–25°C) for up to 1 month.		

Agar plate		
Reagent	Final concentration	Amount
Yeast extract	1%	1 g
Hipolypepton	2%	2 g
Glucose	2%	2 g
Agar	2%	2 g
DW	n/a	100 mL
Total	**n/a**	**100 mL**
Store at 4°C up to 1 month.		

10% formaldehyde		
Reagent	Final concentration	Amount
paraformaldehyde	10%	1 g
NaOH	n/a	n/a
DW	n/a	10 mL
Total	**n/a**	**10 mL**
Add the paraformaldehyde powder and a few drops of 1 N NaOH to DW and warm it to 60°C with continuous stirring until paraformaldehyde dissolves completely. Avoid storing the solution at 4°C for more than a week. The solution can be aliquoted and stored at –20°C or lower at least for a month. Formaldehyde solutions that are available commercially cannot be used for fixation of electron microscopy samples because they contain methanol, which is damaging to the cellular ultrastructure.		

Note: Heating in an alkaline pH depolymerizes paraformaldehyde to form formaldehyde, which is effective as a fixative. Avoid heating above 70°C because formaldehyde may change to formate. Preparation of the formaldehyde solution should be done under a chemical fume hood to prevent inhalation.

0.5 M Sodium phosphate buffer (pH 7.4)		
Reagent	Final concentration	Amount
Na_2HPO_4	57.8 g/L	28.9 g
NaH_2PO_4 2H2O	14.3 g/L	7.15 g
DW	n/a	500 mL
Total	**n/a**	**500 mL**
Store at RT up to 1 year.		

4% formaldehyde		
Reagent	Final concentration	Amount
10% formaldehyde	4%	0.4 mL
0.5 M sodium phosphate buffer (pH 7.4)	0.1 M	0.2 mL
DW	n/a	0.4 mL
Total	**n/a**	**1 mL**
Prepare just before use.		

PBS (pH7.4)		
Reagent	Final concentration	Amount
NaCl	8 g/L	4 g
KCl	0.2 g/L	0.1 g
KH_2PO_4	0.2 g/L	0.1 g
Na_2HPO_4	1.14 g/L	0.57 g
DW	n/a	500 mL
Total	**n/a**	**500 mL**
After autoclave sterilization, store at 4°C up to 1 month.		

0.1% Triton X-100 in PBS (PBST)		
Reagent	Final concentration	Amount
Triton X-100	0.1%	1 mL
PBS	n/a	999 mL
Total	**n/a**	**1 L**
Store at RT up to 1 year.		

2% cold fish gelatin in PBS		
Reagent	Final concentration	Amount
cold fish gelatin	2%	200 mg
PBS	n/a	10 mL
Total	**n/a**	**10 mL**
Prepare just before use.		

1% BSA in PBS		
Reagent	Final concentration	Amount
BSA	1%	100 mg
PBS	n/a	10 mL
Total	**n/a**	**10 mL**
Prepare just before use.		

0.1 M Tris-HCl (pH7.5)		
Reagent	Final concentration	Amount
Tris	0.1 M	1.21 mg
1 M HCl	n/a	n/a
DW	n/a	100 mL
Total	**n/a**	**100 mL**
Adjust pH by adding 1 M HCl. After autoclave sterilization, store at RT up to 1 month.		

2% cold fish gelatin in 0.1 M Tris-HCl (pH 7.5)		
Reagent	Final concentration	Amount
cold fish gelatin	2%	200 mg
0.1 M Tris-HCl (pH 7.5)	n/a	10 mL
Total	**n/a**	**10 mL**
Prepare just before use.		

0.001% Triton X-100 in 0.1 M Tris-HCl (pH 7.5)		
Reagent	Final concentration	Amount
Triton X-100	0.001%	10 µL
0.1 M Tris-HCl (pH 7.5)	n/a	1 L
Total	**n/a**	**1 L**
Store at RT up to 1 month.		

2% cold fish gelatin and 0.001% Triton X-100 in 0.1 M Tris-HCl (pH 7.5)		
Reagent	Final concentration	Amount
cold fish gelatin	2%	200 mg
0.001% Triton X-100 in 0.1 M Tris-HCl (pH 7.5)	n/a	10 mL
Total	**n/a**	**10 mL**
Prepare just before use.		

1 M copper sulfate ($CuSO_4$)		
Reagent	Final concentration	Amount
$CuSO_4$ $5H_2O$	1 M	249.69 mg
DW	n/a	1 mL
Total	**n/a**	**1 mL**
Store at RT up to 1 year.		

10 mM TBTA		
Reagent	Final concentration	Amount
TBTA	10 mM	0.53 mg
DMSO	n/a	100 µL
Total	**n/a**	**100 µL**
Aliquot and store at –20°C up to 1 year. Avoid repeated freeze-thawing.		

500 mM ascorbic acid		
Reagent	Final concentration	Amount
ascorbic acid	500 mM	8.8 mg
DW	n/a	100 µL
Total	**n/a**	**100 µL**
Aliquot and store at –20°C up to 1 year. Avoid repeated freeze-thawing.		

Note: Ascorbic acid solution must be prepared immediately before use or aliquoted and stored frozen at –20°C or lower. If kept in solution, ascorbic acid gradually loses the reducing power that is necessary to convert Cu^{2+} to Cu^{+} in the click reaction.

0.5 mM AFDye546 picolyl-azide		
Reagent	Final concentration	Amount
AFDye546 picolyl-azide	0.5 mM	0.1 mg
DMSO	n/a	190.4 μL
Total	**n/a**	**190.4 μL**
Aliquot and store at –20°C up to 1 year. Avoid repeated freeze-thawing.		

10 mM biotin-azide		
Reagent	Final concentration	Amount
biotin-azide	10 mM	0.33 mg
DMSO	n/a	100 μL
Total	**n/a**	**100 μL**
Aliquot and store at –20°C up to 1 year. Avoid repeated freeze-thawing.		

STEP-BY-STEP METHOD DETAILS

Yeast cell culture and metabolic labeling with propargylcholine

Timing: 5 min to inoculate cells; 48 h to culture yeast; 30 min to label yeast with propargylcholine.

Yeast is cultured in a liquid medium to a density appropriate for experimental purposes and incubated with propargylcholine for a short duration to label only *de novo* synthesized PtdCho.

1. Inoculate yeast cells on an agar plate in 2 mL of SD + CA + ATU medium in a culture tube (approx. 16 mL in capacity) and grow at 30°C while shaking at 200 rpm for 12–16 h.
2. Measure optical density at 600 nm (OD600) using a spectrophotometer and dilute the yeast culture with 10 mL of SD + CA + ATU medium to an OD600 of 0.001 in a glass conical flask and grow at 30°C with shaking at 200 rpm.
3. Grow yeast cells until they reach an appropriate density.
4. Transfer 3 mL of the yeast culture to a new culture tube and add 3 μL of 1 M propargylcholine to the final concentration of 1 mM.
5. Culture yeast for an additional 30 min at 30°C while shaking at 200 rpm.

Optional: To examine the distribution of *de novo* synthesized PtdCho in autophagic cells, cells at the density of OD600 = 0.8–1.0 are used to ensure robust induction of autophagy. They are rinsed once with DW and cultured in an autophagy-inducing medium (e.g., synthetic defined medium without nitrogen) for an appropriate duration (e.g., 2 h) before the administration of propargylcholine.

Note: Negative controls can be prepared by culturing cells without propargylcholine, or by culturing cells with a mixture of 1 mM propargylcholine and an excessive amount of choline (e.g., 5 mM).

⚠ CRITICAL: The duration of incubation with propargylcholine can be changed, but as it becomes longer, PtdCho synthesized for a longer period of time will be labeled, thus decreasing the temporal resolution of the method. Conversely, if the duration of incubation is too short, the signal may be too weak to be detected.

Click labeling for fluorescence microscopy

Timing: 30 min for fixation and permeabilization of cells; 40 min for the click chemical reaction.

Fluorescence microscopy is useful for checking the specificity of the click reaction, overall distribution of labeling, cell-to-cell variation, etc.

6. Collect cells by centrifugation at 1,500 × g for 1 min and discard the supernatant carefully.
7. Suspend cells in 4% formaldehyde in 0.1 M sodium phosphate buffer by pipetting and incubate for 15 min at RT with gentle agitation.

   Note: Use freshly prepared 4% formaldehyde.

8. Collect cells by centrifugation at 1,500 × *g* for 1 min and rinse them once with an ample volume of PBS.
9. Resuspend cells in 0.1% Triton X-100 in PBS and incubate for 10 min with gentle agitation (e.g., 50 rpm) on a shaker or a rotator.

   Note: Triton X-100 is used to permeabilize membranes and enable penetration of the click chemical reaction solution throughout cells.

10. Collect cells by centrifugation at 1,500 × *g* for 1 min and rinse them twice with an ample volume of 0.1 M Tris-HCl (pH 7.5) by centrifugation at 1,500 × *g* for 1 min.
11. Prepare the click reaction solution as below. The final solution contains 0.1 mM $CuSO_4$, 0.1 mM TBTA, 2.5 mM ascorbic acid, and 500 nM AFDye546 picolyl-azide in 0.1 M Tris-HCl (pH 7.5).
    a. Mix 1 μL of 1 M CuSO4, 10 μL of 10 mM TBTA in DMSO, and 5 μL of 0.5 M ascorbic acid in DW in a 1.5 mL microtube.
    b. Leave the solution for 10 min at RT.
    c. Add 983 μL of 0.1 M Tris-HCl (pH 7.5) and 1 μL of 0.5 mM AFDye546 picolyl-azide to the tube and mix well.
12. Add the click reaction solution to pelleted cells, suspend them by pipetting, and incubate for 20 min at RT.
13. Collect cells by centrifugation at 1,500 × *g* for 1 min and rinse them three times with an ample volume of 0.1 M Tris-HCl (pH 7.5).
14. Put the cell pellet between the glass slide and the coverslip using an appropriate mounting medium (e.g., VECTASHIELD) and observe under a fluorescence microscope (Excitation 554 nm/Emission 570 nm).

    Note: As controls, solutions without either $CuSO_4$ or AFDye546 picolyl-azide can be used to check the specificity of the reaction.

    ⚠ CRITICAL: The click reaction solution must be prepared immediately before use.

Preparation and treatment of replicas and click reaction

Timing: 3 h for quick freezing and preparation of freeze-fracture replicas; 24 h for cleaning the replicas; 1 h for click reaction.

Freeze-fracture replicas are reacted in the click chemical reaction solution, and biotin conjugated to the alkyne residue of the PtdCho headgroup is labeled with colloidal gold particles.

15. Perform quick-freezing and prepare freeze-fracture replicas as described in Fujita et al. (2010) and Tsuji et al. (2019).

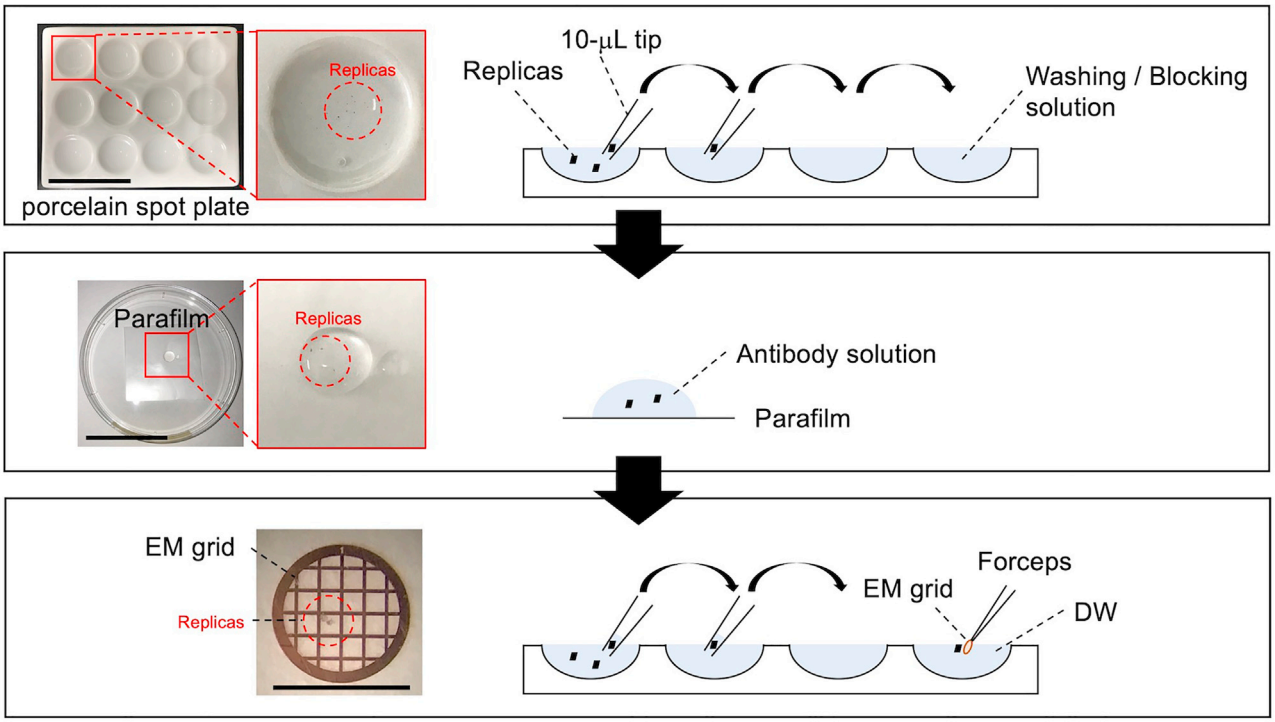


Figure 2. Illustration of washing/blocking and labeling steps
Bars = 50 mm (upper, middle); 3 mm (lower).

Note: For quick-freezing and freeze-fracture replica preparation, refer to instrument manuals for concrete operation.

Note: Quick-freezing stops molecular motion instantaneously and freeze-fracture physically stabilizes membranes and preserves distribution of lipids and proteins in the membrane.

16. Incubate replicas in PBS containing 20 μg/mL Zymolyase, 0.1% Triton X-100, 1 mM dithiothreitol, and 1% BSA for 2 h at 37°C.
17. Incubate replicas in 0.1 M Tris-HCl (pH 8.0) containing 2.5% SDS at 60°C for 12–16 h.

Note: Freeze-fracture replicas of yeast need to be digested with Zymolyase to remove the cell wall and then treated with SDS to remove adhering proteins (Cheng et al., 2014). For mammalian cells, SDS treatment suffices.

Pause point: Replicas before or after the Zymolyase–SDS treatment can be stored in PBS containing 50% glycerol at –20°C for months.

18. Rinse the freeze-fracture replicas three times with 0.001% Triton X-100 in 0.1 M Tris-HCl (pH 7.5).

Note: This step and most of the following steps are done in the wells of a porcelain spot plate (Figure 2), in which replicas are observed more clearly than in transparent plastic vessels. Each well can contain approx. 0.5 mL of the solution. Replicas can be transferred to a new well using a micropipette with a 10 μL tip. For each step, solutions need to be agitated thoroughly by repeated pipetting. We repeat pipetting more than 20 times for each step.

19. Treat the replicas with 2% cold fish gelatin and 0.001% Triton X-100 in 0.1 M Tris-HCl (pH 7.5) for 10 min at RT.

Note: This blocking is effective for reducing non-specific labeling in freeze-fracture replicas (Iyoshi et al., 2014).

20. Prepare the click reaction solution as below. The final solution contains 1 mM $CuSO_4$, 2.5 mM ascorbic acid, 0.1 mM TBTA, 2% cold fish gelatin, and 5 μM biotin-azide in 0.1 M Tris-HCl (pH 7.5).
    a. Mix 1 μL of 1 M $CuSO_4$, 10 μL of 10 mM TBTA, and 5 μL of 0.5 M ascorbic acid in a 1.5 mL microtube.
    b. Leave the solution for 10 min at RT.
    c. Add 983 μL of 2% cold fish gelatin in 0.1 M Tris-HCl (pH 7.5) and 0.5 μL of 10 mM biotin-azide to the tube and mix well.

⚠ CRITICAL: Click reaction solution must be prepared immediately before use.

Note: As controls, solutions without either $CuSO_4$ or biotin-azide can be used to confirm the specificity of the reaction.

21. Incubate the replicas in a click reaction solution for 30 min at RT.
22. Rinse the replicas five times with 0.001% Triton X-100 in 0.1 M Tris-HCl (pH 7.5) and once with PBST.

Immunogold labeling

Timing: 16 h

23. Incubate the replicas with 2% cold fish gelatin in PBS for 30 min at RT.
24. Rinse the replicas with PBS once.
25. Incubate the replicas with mouse anti-biotin antibody (5–10 μg/mL) in PBS containing 1% cold fish gelatin for 12–16 h at 4°C.

Note: Centrifuge the diluted antibody solution at 21,000 × *g* at 4°C for 5 min and use the supernatant. The incubation with antibodies is performed on a sheet of Parafilm using 50–100 μl of solution in a sealed container with a wet sheet of filter paper (Figure 2). The concentration of antibodies may need to be adjusted by trial and error. Once blocked with 2% cold fish gelatin, 1% is sufficient to prevent non-specific reaction during the antibody reaction.

26. Rinse the replicas five times with PBST.
27. Incubate the replicas with goat anti-mouse antibody conjugated with 12 nm colloidal gold (GAM12) in 50–100 μL of PBS containing 1% BSA for 30 min at 37°C.

Note: We generally dilute the colloidal gold-conjugated antibody in 1:20, but an appropriate dilution may vary for each lot. Centrifuge the GAM12 diluted solution at 200 × *g* at 4°C for 5 min and use the supernatant.

28. Rinse the replicas five times with 0.1% BSA in PBS.
29. Rinse the replicas twice with DW.
30. Pick up the replicas on Formvar-coated EM grids (50 mesh, copper) and dry on filter paper (Figure 2).

⚠ CRITICAL: Do not let replicas dry until step 30. Inclusion of BSA or detergent in solutions prevents replicas from sticking to the porcelain spot plate and pipette tip.

Pause point: Replicas that are labeled and picked up on EM grids can be stored in a grid box or other appropriate container for months to years.

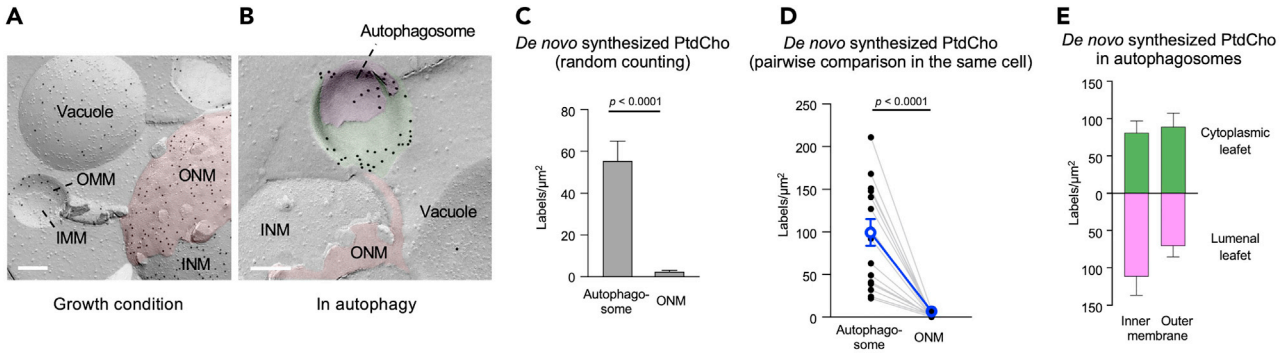


Figure 3. Electron microscopy of *de novo* synthesized PtdCho labeling

(A and B) Yeast in the exponential growth phase (A). Yeast in autophagy induced by amino acid starvation (B). The cytoplasmic and lumenal leaflets of autophagosomes are colored in green and magenta, respectively. Organelles can be identified by their characteristic morphology (e.g., nuclear pores in the nuclear membrane). Bar = 200 nm. ONM: outer nuclear membrane (colored in orange), INM: inner nuclear membrane, OMM: outer mitochondrial membrane, INM: inner mitochondrial membrane.

(C) Quantification of the label density in autophagy in photos taken randomly. Mean ± SEM. Mann-Whitney U test.

(D) Pairwise comparison of the label density in autophagosomes and ONM in the same cells. Wilcoxon signed-rank test using the statistical language R. Mean ± SEM is shown by blue circles and error bars.

(E) The labeling density in the cytoplasmic and lumenal leaflets of autophagosomal membranes. Mean ± SEM. Adapted from Orii et al. (2021). (©2021 Orii et al. Originally published in *Journal of Cell Biology*. https://doi.org/10.1083/jcb.202009194)

Data acquisition and analysis

Timing: 1–2 days

31. Take photos randomly by transmission electron microscope. Colloidal gold particles of 12 nm in diameter can be easily observed in images at a magnification of 15,000 × or higher.
32. Collect images containing membranes of interest (e.g., cytosolic leaflet of the ER).

Note: For quantification, we generally use more than 15 images for each membrane of interest.

33. Measure the area of membranes (μm^2) using ImageJ, count the number of colloidal gold labels, and calculate the density of gold labels (labels/μm^2) for each area.
34. Compare the result statistically using the Mann-Whitney U test or other appropriate methods (Figure 3).

Note: To further validate the result and refute the possibility of cell-to-cell variation, comparison of two different membranes of interest in the same cell (e.g., autophagosomal membrane and the outer nuclear membrane) is desirable (Figure 3D). The data pairs can be analyzed by the Wilcoxon signed-rank test using the statistical language R.

EXPECTED OUTCOMES

In yeast in the exponential growth phase, *de novo* synthesized PtdCho is labeled most densely in the ER membrane and the outer nuclear membrane, whereas it is much less so in other membranes, such as mitochondria, the plasma membrane, and the vacuole (lysosome in yeast). In contrast, in cells undergoing autophagy induced by amino acid starvation, the labeling is most intense in the autophagosomal membrane (Figure 3). The difference in the *de novo* synthesized PtdCho distribution between the growth phase and starvation can also be observed by fluorescence microscopy (Figure 4), but detailed distribution, for example, which membrane leaflet (i.e., cytoplasmic or non-cytoplasmic) of organelles the PtdCho label exists, can only be determined by electron microscopy (Figure 3E).

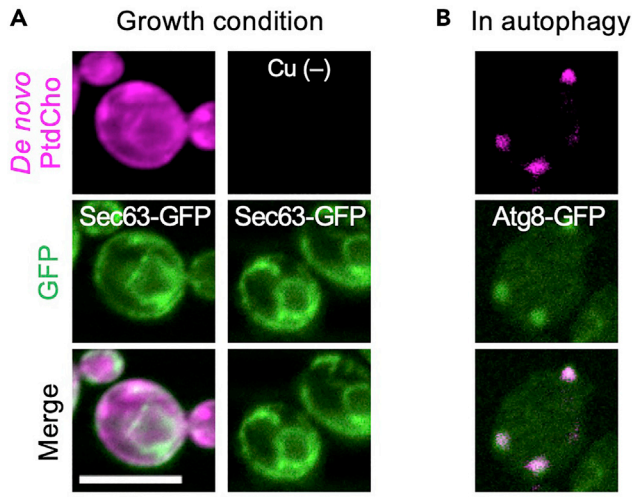


Figure 4. Fluorescence microscopy of *de novo* synthesized PtdCho labeling

Yeast in the exponential growth phase (A) and in autophagy induced by amino acid starvation (B). A negative control omitting $CuSO_4$ in the click reaction solution is also shown. Sec63-GFP (ER marker), Atg8-GFP (autophagosome marker). Bar = 5 μm. Adapted from Orii et al. (2021). (©2021 Orii et al. Originally published in *Journal of Cell Biology*. https://doi.org/10.1083/jcb.202009194)

LIMITATIONS

Although this method only detects PtdCho synthesized by the Kennedy pathway, PtdCho can also be synthesized by the Land's cycle and the phosphatidylethanolamine methyltransferase pathway. Therefore, the absence of signals in a membrane does not necessarily indicate that *de novo* synthesized PC is not incorporated there.

Propargylcholine is incorporated not only to PtdCho but also to other choline-containing phospholipids, i.e., lysophosphatidylcholine in yeast, and lysophosphatidylcholine and sphingomyelin in mammalian cells. After a short pulse of propargylcholine loading, however, incorporation to phospholipids other than PtdCho is thought to be negligible (Jao et al., 2009).

It is often difficult to compare the amount of newly synthesized PtdCho in two different conditions. This is because some medium contains choline (e.g., Dulbecco's modified Eagle's medium), which competes with propargylcholine for incorporation to PtdCho.

When executed properly, non-specific labeling appears very low. To ensure the specificity of labeling, especially when studying new samples and/or new conditions, it is important to run control experiments as described above.

TROUBLESHOOTING

Problem 1

Difficulty of picking up replicas onto EM grids (step 30).

Potential solution

1) Use fine forceps that can hold EM grids securely. 2) Keep replicas floating on the surface of DW. It is difficult to pick up replicas that are sinking in water. 3) Do not contaminate DW with surfactant, which prevents replicas from adhering to the formvar membrane.

Problem 2

Folded replicas (step 30).

Potential solution

1) Use smaller replica pieces. Large replicas tend to be folded tightly. 2) Hook a replica at the EM grid edge and repeat moving it up and down across the surface of DW.

Problem 3

Low signal (step 31).

Potential solution

1) Incubate cells with propargylcholine for a longer time (e.g., 60 min), although this will also decrease the time resolution of the method. 2) Use a longer incubation time for the click reaction. Non-specific labeling may also increase, but this can be checked by taking appropriate controls. 3) Prepare a fresh ascorbic acid solution. The reducing power of ascorbic acid is lost quickly in solution. 4) Check that antibodies retain proper reactivity. The shelf life of colloidal gold-conjugated antibodies is shorter than that of non-conjugated ones.

Problem 4

High background labeling (e.g., colloidal gold particles are seen in non-membrane areas) (step 31).

Potential solution

1) Check the specificity of the click reaction by fluorescence microscopy. 2) Clean replicas extensively with SDS solution before labeling. 3) Wash replicas extensively after antibody incubation. 4) Incubate replicas in blocking solution longer. 5) Use fresh antibodies. 6) Decrease the antibody concentration.

Problem 5

Highly variable labeling intensity (step 31)

Potential solution

1) Check if cell-to-cell variation also exists in fluorescence microscopy. 2) Decrease the number of replicas for an experiment to avoid undesirable dilution of antibodies and prolonged washing time. 3) Incubate cells with propargylcholine longer.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Toyoshi Fujimoto (t.fujimoto.xl@juntendo.ac.jp).

Materials availability

This study did not generate new unique reagents/any new material.

Data and code availability

This study did not generate any unique datasets or code.

ACKNOWLEDGMENTS

This study was supported by Grants-in-Aid for Scientific Research from the Japan Society of the Promotion of Science to T.T. (19K07265 and 20H05339) and to T.F. (15H05902 and 18H04023) by JST, CREST Grant Number JPMJCR20E3, Japan to T.T., and grants from the Nakatani Foundation for Advancement of Measuring Technologies in Biomedical Engineering and the Takeda Science Foundation to T.F. The authors thank Dr. Jinglei Cheng (Nagoya University) and Ms. Hiroko Osakada (Juntendo University) for technical assistance.

AUTHOR CONTRIBUTIONS

T.F. conceived, designed, and supervised the project. T.T. conducted most of the experiments and data analysis. T.T. and T.F. wrote the manuscript and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Cheng, J., Fujita, A., Yamamoto, H., Tatematsu, T., Kakuta, S., Obara, K., Ohsumi, Y., and Fujimoto, T. (2014). Yeast and mammalian autophagosomes exhibit distinct phosphatidylinositol 3-phosphate asymmetries. Nat. Commun. *5*, 3207. https://doi.org/10.1038/ncomms4207.

Fujita, A., Cheng, J., and Fujimoto, T. (2010). Quantitative electron microscopy for the nanoscale analysis of membrane lipid distribution. Nat. Protoc. *5*, 661–669. https://doi.org/10.1038/nprot.2010.20.

Iyoshi, S., Cheng, J., Tatematsu, T., Takatori, S., Taki, M., Yamamoto, Y., Salic, A., and Fujimoto, T. (2014). Asymmetrical distribution of choline phospholipids revealed by click chemistry and freeze-fracture electron microscopy. ACS Chem. Biol. *9*, 2217–2222. https://doi.org/10.1021/cb500558n.

Jao, C.Y., Roth, M., Welti, R., and Salic, A. (2009). Metabolic labeling and direct imaging of choline phospholipids in vivo. Proc. Natl. Acad. Sci. U S A *106*, 15332–15337. https://doi.org/10.1073/pnas.0907864106.

Ogasawara, Y., Cheng, J., Tatematsu, T., Uchida, M., Murase, O., Yoshikawa, S., Ohsaki, Y., and Fujimoto, T. (2020). Long-term autophagy is sustained by activation of CCTbeta3 on lipid droplets. Nat. Commun. *11*, 4480. https://doi.org/10.1038/s41467-020-18153-w.

Orii, M., Tsuji, T., Ogasawara, Y., and Fujimoto, T. (2021). Transmembrane phospholipid translocation mediated by Atg9 is involved in autophagosome formation. J. Cell Biol. *220*. https://doi.org/10.1083/jcb.202009194.

Tsuji, T., Cheng, J., Tatematsu, T., Ebata, A., Kamikawa, H., Fujita, A., Gyobu, S., Segawa, K., Arai, H., Taguchi, T., et al. (2019). Predominant localization of phosphatidylserine at the cytoplasmic leaflet of the ER, and its TMEM16K-dependent redistribution. Proc. Natl. Acad. Sci. U S A *116*, 13368–13373. https://doi.org/10.1073/pnas.1822025116.