bio-protocol

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Immunoisolation of Endosomal Recycling Vesicles from *Saccharomyces cerevisiae*

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Abstract

Endosomal recycling is essential for the appropriate function of the endosome. During this process, endosomal coat complexes (*i.e.*, retromer, and Mvp1) are recruited to the endosome, and deform its membrane to form recycling vesicles. To further analyze this, we developed a protocol for the immunoisolation of recycling vesicles from budding yeast. This method is a powerful way to characterize endosomal recycling pathways.

Keywords: Endosome, Recycling, Vesicles, Immunoisolation

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Background

Endosomes are specialized compartments for protein sorting. They downregulate membrane proteins through degradation. Endocytosed receptors or transporters are internalized into the intraluminal vesicles through the endosomal sorting complexes required for transport (ESCRT) pathways (Henne *et al.*, 2011). Since endosomes fuse with vacuoles (lysosomes in humans), these membrane proteins are degraded. On the other hand, some membrane proteins [*i.e.*, endosomal SNAREs (SNAP REceptors), sorting receptors, *etc.*] are recycled back to the Golgi apparatus through the recycling pathways, before fusion with vacuoles. These recycling pathways are mediated by retromer, sorting nexin 4 (Snx4), or multi-copy suppressor of vps 1 (Mvp1) coat complexes (Seaman *et al.*, 1998; Hettema *et al.*, 2003; Suzuki *et al.*, 2021). Each coat complex recognizes specific sets of membrane proteins (cargos). For example, retromer and Mvp1 sort vacuolar protein sorting 10 (Vps10) and vacuolar protein sorting 55 (Vps55), respectively (Figure 1). Thus, endosomes utilize distinct molecular machinery to determine the fate of membrane proteins, either degradation, or recycling.

In the endosomal recycling pathway, endosomal coat complexes are recruited to the endosomal surface where they recognize cargos. They sort cargo and deform the membrane into recycling tubules, which bud from the endosome, and target the Golgi. To further characterize this process, we developed an immunoisolation procedure for recycling vesicles. In this method, we immunoisolated cargo containing recycling vesicles from temperature-sensitive *sec18* mutants, which have a defect in recycling vesicle fusion with the Golgi, and accumulate these vesicles. As far as we know, this is a first method to isolate endosomal recycling vesicles from yeast. This method allows us to characterize endosomal recycling pathways biochemically (via western blotting, electron microscopy, and mass spectrometry).

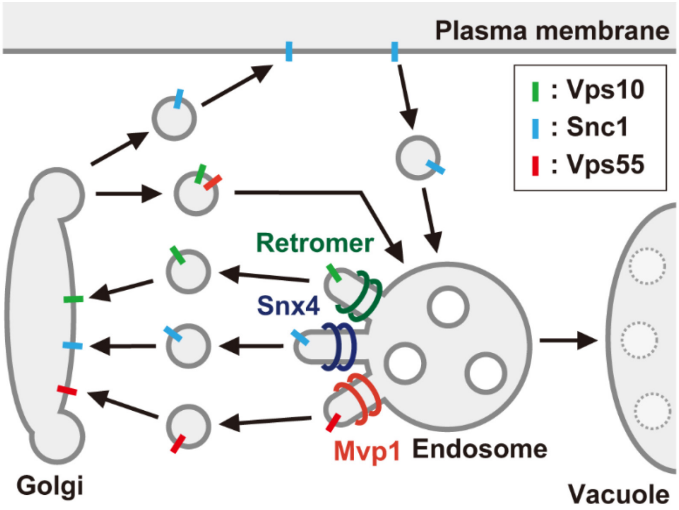

Figure 1. Endosomal recycling pathways in yeast.

Materials and Reagents

1. 1.5 mL tube
2. 250 mL flask
3. NHS beads (TAMAGAWA SEIKI, catalog number: TAS8848N1141)
4. Anti-DYKDDDDK antibodies (WAKO, catalog number: 1E6)
5. Methanol (Fisher Scientific, catalog number: A454-4)
6. 250 mM MES-NaOH (pH 6.0) (Sigma, catalog number: 69892)
7. 1 M Ethanolamine (Fisher Scientific, catalog number: AC149582500)
8. 0.1 M Glycine-HCl (pH 2.5) (VWR, catalog number: 0617-5KG)

9. 1.0 M Tris-HCl (pH 8.0) (VWR, catalog number: 95029-578)
10. Protease inhibitor (Complete Mini EDTA-free) (Roche, catalog number: 04693159001)
11. 0.5 mm Zirconia/Silica beads (Biospec Products, catalog number: 11079105Z)
12. HEPES-NaOH (pH 7.4) (VWR, catalog number: EM-5330)
13. KCl (Fisher Scientific, catalog number: P330-500)
14. EDTA (Sigma, catalog number: E9884)
15. Glycerol (Fisher Scientific, catalog number: G33-1)
16. Triton X-100 (Sigma, catalog number: 93443)
17. BSA (Sigma, catalog number: A9418)
18. Sodium azide (Sigma, catalog number: S2002)
19. Sorbitol (VWR, catalog number: 97062-204)
20. SDS (Sigma, catalog number: L3771)
21. DTT (Santa Cruz Biotechnology)
22. Bromophenol blue (Sigma, catalog number: 115-39-9)
23. Yeast extract (BD, catalog number: 210929)
24. Bacto peptone (BD, catalog number: 211677)
25. Glucose (VWR, catalog number: BDH9230)
26. Agar (VWR, catalog number: 244510)
27. Protein conjugation buffer (see Recipes)
28. Protein conjugation buffer (+0.5% Triton X-100) (see Recipes)
29. Protein storage buffer (see Recipes)
30. YEPD plate (see Recipes)
31. YEPD liquid culture (see Recipes)
32. $H_{25}S_{75}E_5$ buffer (see Recipes)
33. $H_{25}S_{75}E_5$ buffer (+PIC) (see Recipes)
34. SDS-PAGE sample buffer (see Recipes)

Strains

1. SEY 6210 *sec18*^{ts} yeast cells expressing *VPS10-3xFLAG* (Suzuki *et al.*, 2021)
2. SEY 6210 *sec18*^{ts} yeast cells expressing *VPS10-3xFLAG* (Suzuki *et al.*, 2021)

Equipment

1. Microcentrifuge (Fisher Scientific, model: AccuSpin Micro 17/17R)
2. Centrifuge (Beckman Coulter, model: Allegra X-15R)
3. Ultrasonic Cleaner (Fisher Scientific, model: FS30)
4. Micro Tube Mixer (Tomy, model: MT-360)
5. Labquake™ Tube Shaker/Rotators (Thermo Scientific)
6. Magnetic stand (TAMAGAWA SEIKI, model: TAB4899N12)
7. Labquake Tube Shaker/Rotators (Thermo Scientific, model: 415110Q)
8. SmartSpec Plus Spectrophotometer (BIO-RAD)
9. Mini Vortexer (VWR)
10. Dry Block Incubator (Fisher Scientific)
11. 1545 VWR Digital Incubator (VWR)
12. New Brunswick™ Innova® 44/44R Stackable Incubator Shakers (Eppendorf)

Procedure

A. Immobilization of anti-DYKDDDDK antibody on NHS beads

1. Transfer 5.0 mg of NHS beads into a 1.5-mL tube.
2. Centrifuge the beads at 15,000 × *g* and 4°C for 5 min, and remove the supernatant.
3. Resuspend the beads in 250 µL of methanol, and disperse them by sonication (less than 1 min).
4. Centrifuge the beads at 15,000 × *g* and 4°C for 5 min, and remove the supernatant.
5. Resuspend the beads in 160 µL of 25 mM MES-NaOH (pH 6.0), and disperse them by sonication.
6. Add 160 µL of anti-DYKDDDDK antibodies in 25 mM MES-NaOH (pH 6.0).
   Note: Resuspend 0.5 mg of anti-DYKDDDDK antibodies in 160 µL of 25 mM MES-NaOH (pH 6.0).
7. Incubate the beads with gentle vortexing, using a microtube mixer at 4°C for 30 min.
   Note: Set the dial of the microtube mixer to 6.5.
8. Collect the beads using a magnetic stand, and remove the supernatant.
   Note: Spin the tube down before collecting the beads, to collect liquid at the lip of tube.
9. Resuspend the beads in 625 µL of 1 M ethanolamine, and disperse the beads by pipetting.
10. Incubate the beads using the microtube mixer at 4°C overnight.
11. Collect the beads using a magnetic stand, and remove the supernatant.
12. Resuspend the beads in 1 mL of protein conjugation buffer, and disperse the beads by pipetting.
13. Collect the beads using a magnetic stand, and remove the supernatant.
14. Repeat steps A12 and A13 three times.
15. Resuspend the beads in 1 mL of 0.1 M Glycine-HCl (pH 2.5).
16. Collect the beads using a magnetic stand, and remove the supernatant.
17. Resuspend the beads 1 mL of 1.0 M Tris-HCl (pH 8.0).
18. Collect the beads using a magnetic stand, and remove the supernatant.
19. Resuspend the beads in 1 mL of protein conjugation buffer (+0.5% Triton X-100).
20. Incubate the beads with gentle rotation using a tube shaker/rotator at 4°C for 15 min.
21. Collect the beads using a magnetic stand, and remove the supernatant.
22. Resuspend the beads in 1 mL of protein conjugation buffer.
23. Collect the beads using a magnetic stand, and remove the supernatant.
24. Repeat steps A22 and A23 three times.
25. Resuspend the beads in 1 mL of protein storage buffer, and store at 4°C.
    Note: A volume of 1 mL of beads can be used for 100 experiments. You can store the beads at 4 °C for at least one year.

B. Immunoisolation of recycling vesicles

1. Streak Vps55-3xFLAG or Vps10-3xFLAG expressing *sec18*^{ts} yeast strains to the YEPD plate from glycerol stocks (yeasts in 15% glycerol and YEPD, store at -80°C).
   Note: To purify retromer-recycling vesicles, Vps10-3xFLAG sec18^{ts} *cells are used. For Mvp1-recycling vesicles, Vps55-3xFLAG sec18ts cells are used.*
2. Incubate the plate in a VWR digital incubator at 26°C for three days.
3. Inoculate the yeast cells in 5 mL of YEPD liquid medium and cultivate them in incubator shakers at 250 rpm and 26°C overnight.
4. Inoculate 0.5–2.5 µL of the preculture to 5 mL of YEPD liquid culture. Inoculate this to 100 mL of YEPD liquid media in a 250-mL flask and grow the cells in incubator shakers at 250 rpm and 26°C overnight.
5. When the cells reach mid-log phase (OD_{600} = 0.4–0.8), incubate them at in incubator shakers at 250 rpm and 37°C for 60 min.
   Note: Measure OD_{600} *of the undiluted yeast culture by pipetting 100 µL of the yeast culture into 900 µL of water.*
6. Cool the cells on ice for 5 min.
7. Harvest 80 OD_{600} unit yeast cells into a 50-mL tube, and centrifuge it using the centrifuge (Beckman Coulter, Allegra X-15R) at 4,000 × *g* and 4°C for 2 min.

8. Remove the supernatant by pipetting, and resuspend the pellet in 5 mL of cooled $H_{25}S_{75}E_5$ buffer.
9. Centrifuge the cells at 4,000 × *g* and 4°C for 2 min, and remove the supernatant by pipetting.
10. Resuspend the pellet in 1 mL of cooled $H_{25}S_{75}E_5$ buffer into a 1.5-mL micro-tube.
11. Centrifuge the cells at 10,000 × *g* for 1 min, and remove the supernatant.
12. Resuspend the cells in 1 mL of cooled $H_{25}S_{75}E_5$ buffer (+PIC), and add 500 µL of 0.5 mm Zirconia/Silica beads.
    Note: Buffer should not contain detergent, but does contain 750 mM sorbitol to maintain osmolarity.
13. Vortex the cells to lyse them at 4°C for 1 min.
14. Centrifuge the lysate in a 1.5-mL micro-tube at 500 × *g* and 4°C for 5 min, and remove the supernatant.
15. Centrifuge the supernatant at 15,000 × *g* and 4°C for 10 min, and remove the supernatant.
16. Incubate the cleared supernatant with 10 µL of pre-equilibrated anti-DYKDDDDK NHS beads (prepared in "A") with gentle rotation using tube shaker/rotators at 4°C for 60 min.
    Note: Before use, suspend anti-DYKDDDDK NHS beads in cooled $H_{25}S_{75}E_5$ buffer.
17. Collect the beads using a magnetic stand, and remove the supernatant by pipetting.
18. Resuspend the beads in 1 mL of cooled $H_{25}S_{75}E_5$ buffer.
19. Repeat steps B18 and B19 three times.
20. Transfer the beads to a new 1.5-mL tube.
21. Collect the beads using a magnetic stand, and remove the supernatant by pipetting.
22. Add 100 µL of SDS-PAGE sample buffer.
23. Incubate the beads at 98°C for 5 min.
24. Collect the beads using a magnetic stand, and remove the supernatant.
25. Analyze the sample by SDS-PAGE and western blotting.

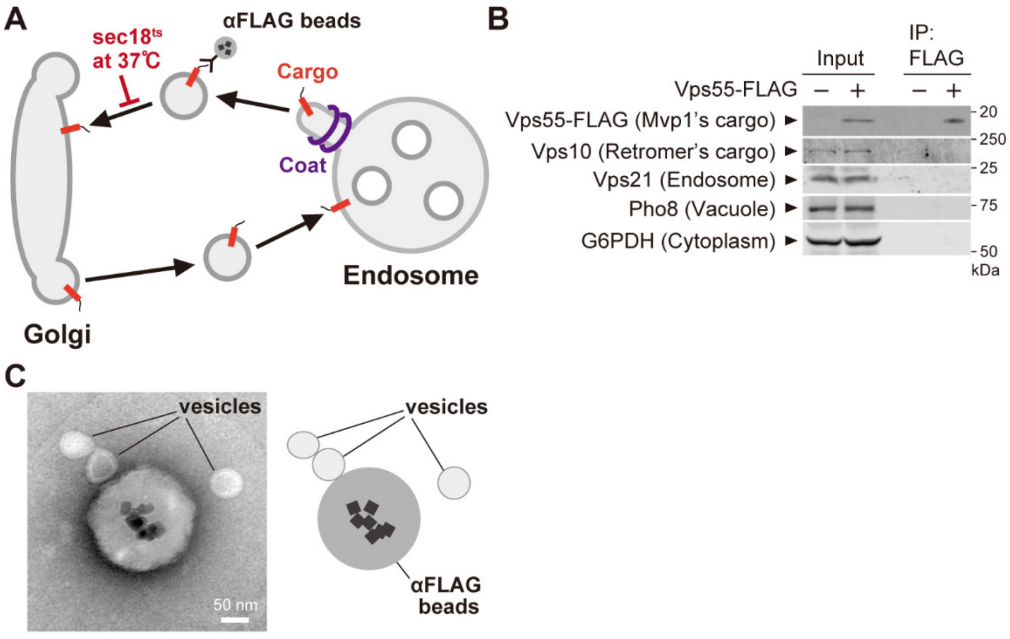

Figure 2. Immunoisolation of endosomal recycling vesicles.
A. Schematic for immunoisolation of endosomal recycling vesicles. To accumulate cargo-containing recycling vesicles, a temperature-sensitive mutant of Sec18, which is essential for the fusion of recycling vesicles with the Golgi apparatus, was used. After 60 min of incubation at the non-permissive temperature (37°C), cells expressing FLAG-fused cargo proteins (Vps10 or Vps55) were lysed. Then, cargo-containing recycling vesicles were immunoisolated by anti-FLAG magnetic beads. B. Western blotting analysis of the isolated Vps55-FLAG-containing vesicles. C. Electron microscopy (EM) analysis of the isolated Vps55-FLAG-containing vesicles. Data reproduced with modification for space limits from Suzuki *et al.* (2021).

Recipes

1. **Protein conjugation buffer**
   10 mM HEPES-NaOH (pH 7.4)
   50 mM KCl
   1 mM EDTA
   10% Glycerol
   Note: Protein conjugation buffer can be stored at room temperature (RT).

2. **Protein conjugation buffer (+0.5% Triton X-100)**
   10 mM HEPES-NaOH (pH 7.4)
   50 mM KCl
   1 mM EDTA
   10% Glycerol
   0.5% Triton X-100
   Note: Protein conjugation buffer (+0.5% Triton X-100) can be stored at RT.

3. **Protein storage buffer**
   10 mM HEPES-NaOH (pH 7.4)
   50 mM KCl
   1 mM EDTA
   10% Glycerol
   1 mg/mL BSA
   3 mM Sodium azide
   Note: Protein storage buffer can be stored at RT.

4. **$H_{25}S_{75}E_5$ buffer**
   25 mM HEPES-NaOH (pH 7.4)
   750 mM Sorbitol
   5 mM EDTA
   Note: $H_{25}S_{75}E_5$ buffer can be stored at RT.

5. **$H_{25}S_{75}E_5$ buffer (+PIC)**
   25 mM HEPES-NaOH (pH 7.4)
   750 mM Sorbitol
   5 mM EDTA
   1× Protease inhibitor cocktail
   Note: $H_{25}S_{75}E_5$ buffer (+PIC) needs to be freshly prepared.

6. **SDS-PAGE sample buffer**
   75 mM Tris-HCl (pH 7.5)
   2% (w/v) SDS
   10% Glycerol
   100 mM DTT
   Bromophenol blue
   Note: SDS-PAGE sample buffer needs to be freshly prepared.

7. **YEPD plate**
   1% (w/v) Yeast extract
   2% (w/v) Bacto peptone
   2% Glucose
   2% Agar

8. YEPD liquid culture
   1% (w/v) Yeast extract
   2% (w/v) Bacto peptone
   2% Glucose

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Competing interests

The authors declare that no competing interests exist.

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