Chapter 13

Preparation of Enriched Plant Clathrin-Coated Vesicles by Differential and Density Gradient Centrifugation

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Abstract

Methods for the subcellular fractionation and enrichment of specific intracellular compartments are essential tools in the analysis of compartment composition and function. In vitro characterization of isolated cell organelles and other endomembrane intermediates, including exploration of the compartment protein ensemble, offers strong clues of in vivo function identity. Here, we describe methodology for the isolation of clathrin-coated vesicles from *Arabidopsis thaliana* suspension-cultured cells on the basis of differential and density centrifugation.

Key words Clathrin, Fractionation, Negative stain electron microscopy, Plant, Vesicles

1 Introduction

Clathrin-coated vesicles (CCVs) are membranous organelles that function in the biosynthetic and endocytic trafficking of soluble and membrane proteins in all eukaryotes. Clathrin is a coat protein composed of three heavy chains (CHC) and three light chains (CLC) assembled into a three-legged structure called a clathrin triskelion. With the aid of distinct adaptor protein (AP) complexes, including AP1 and AP2, which selectively package protein and lipid cargos at the *trans*-Golgi network (TGN) and the plasma membrane, respectively, CCVs provide a route in which cellular materials are internalized by endocytosis and are transported within the late secretory pathway. The AP complexes along with numerous other accessory proteins also help direct the clathrin triskelia to form curved polygonal lattices around budding membrane invaginations, which eventually form CCVs.

In plant and mammalian cells, clathrin-mediated endocytosis is important for the uptake of nutrients and signaling receptors, intracellular communication, and signal transduction. In addition, clathrin-mediated trafficking is critical for the formation of the cell plate during cytokinesis and for the delivery of membrane and cell

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wall materials necessary for cell expansion in plants (for review *see* ref. 1). Our understanding of the mechanism of clathrin-mediated trafficking in plants is underdeveloped relative to animal and yeast. One approach to gaining a better understanding of the formation and trafficking of plant CCVs and their cargo is through the analysis of enriched preparations of isolated CCVs.

Methodology for the isolation of CCVs from mammalian microsomal membrane fractions by differential centrifugation was first described by Pearse [2] and subsequently improved upon by the use of a density gradient comprised of Ficoll and D_2O [3]. Here we describe a protocol for the isolation of CCVs from Arabidopsis suspension-cultured cells. This procedure was adapted from previously established protocols for the isolation of plant CCVs [4, 5]. In brief, the protocol describes enrichment of CCVs by differential centrifugation, velocity sedimentation, and equilibrium density gradient centrifugation from Arabidopsis suspension-cultured cell microsomal membrane extracts (*see* Fig. 1). In addition, the use of negative stain electron microscopy for the assessment of the purity and enrichment of CCV subcellular fractions is described.

2 Materials

Prepare all solutions using purified, deionized water (resistivity ≥18 at 25 °C), and reagents of analytical grade or higher (unless otherwise noted). Follow all appropriate disposal requirements with special attention directed to discarding the (mildly radioactive) D_2O and (highly toxic) OsO_4 solutions used in this experiment.

2.1 Isolation Buffers, Reagents, and Solutions (See Note 1)

1. Ethylenediaminetetraacetic acid (EDTA) Stock: To 80 mL of water in a clean beaker, add 18.61 g of $Na_2{\cdot}EDTA{\cdot}2H_2O$ (m. mass 372.20 g/mol). While stirring, add 1.8 g solid NaOH. Upon NaOH dissolution, pH to 8.0 with 10 N NaOH. When EDTA has completely dissolved, adjust final volume to 100 mL with water. Store in a plastic container at room temperature.
2. Ethylene glycol tetraacetic acid (EGTA) Stock: To 80 mL of water in a clean beaker, add 19.02 g of EGTA (m.mass 380.35 g/mol). While stirring, add 3.5 g solid NaOH. Upon NaOH dissolution, adjust pH to 8.0 with 10 N NaOH. When EGTA has completely dissolved, adjust final volume to 100 mL with water. Store in a plastic container at room temperature.
3. Magnesium Chloride ($MgCl_2$) Stock: To 80 mL of water, add 9.52 g of anhydrous $MgCl_2$ (m.mass 95.211 g/mol). Upon dissolution, adjust volume to 100 mL with water. Store at room temperature.

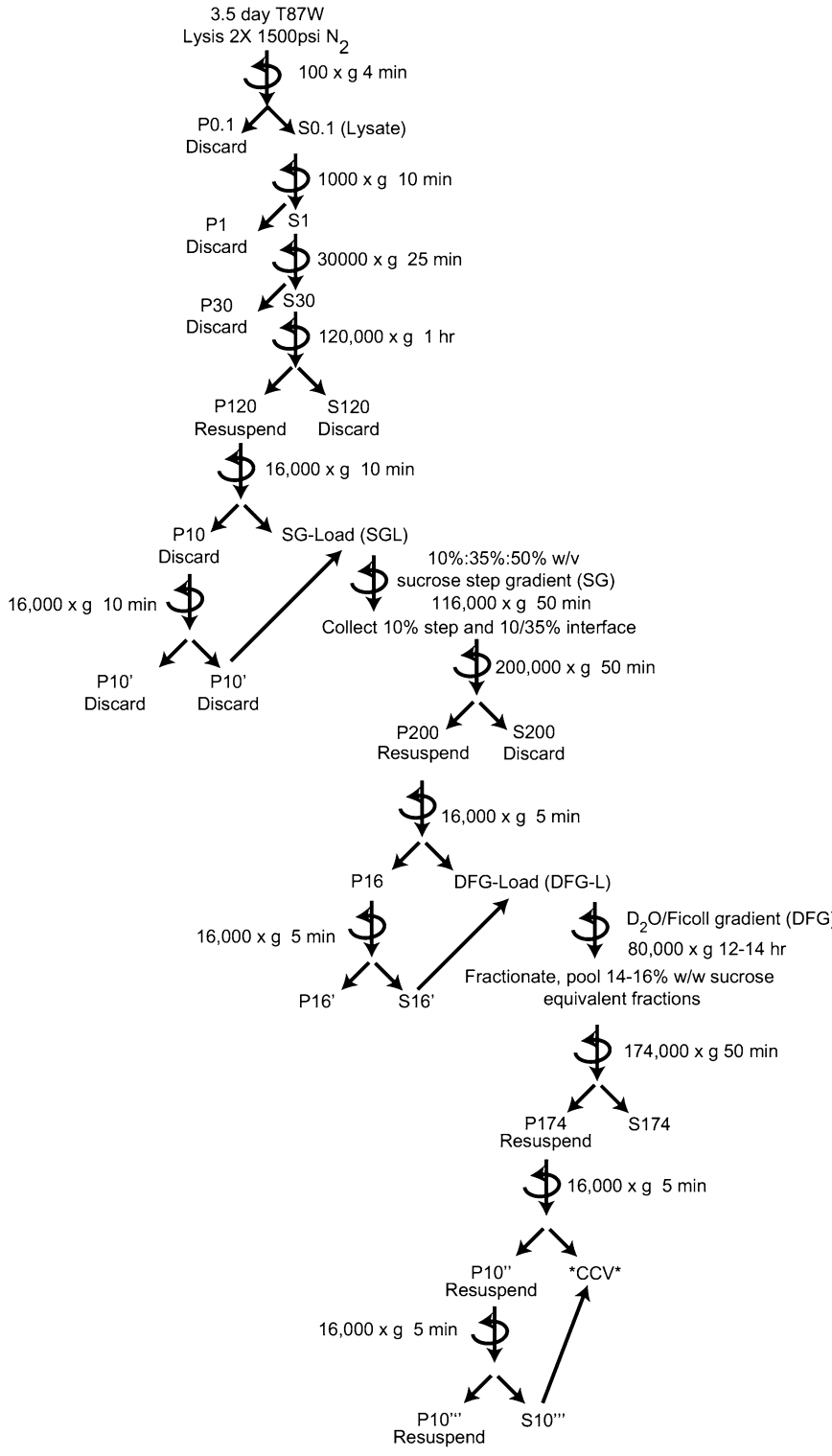

Fig. 1 Flowchart summarizing fractionation and centrifugation steps involved in the preparation of the final enriched CCV fraction

4. 1× Clathrin Isolation Buffer (CIB) (*see* **Note 2**): 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), 0.5 mM $MgCl_2$, 3 mM EDTA, 1 mM EGTA, pH 6.4. To 750 mL water in a 2 L beaker, add 19.523 g of MES hydrate (m.mass 195.24 g/mol), 500 µL of 1 M $MgCl_2$ stock, 6 mL of EDTA stock pH 8.0, and 2 mL of EGTA stock pH 8.0. Adjust volume to 900 mL and adjust pH to 6.4 using 5 N KOH. Dilute to 1 L final volume with water. Filter sterilize (pore size ≤0.2um). Store at 4 °C.
5. 1,000× Protease Inhibitor Cocktail Dimethyl Sulfoxide (DMSO)-soluble (PIC-D) (*see* **Note 3**): 500 mM Phenylmethylsulfonyl fluoride (PMSF), 5 mg/mL pepstatin A, 1 mg/mL chymostatin. To 1 mL DMSO in a sterile 15 mL screw-top conical tube, add 176 mg PMSF, 10 mg pepstatin A, and 2 mg chymostatin. Dilute to 2 mL with DMSO and mix thoroughly. Distribute into 500 µL aliquots. Store at −80 °C [6].
6. 1,000× Protease Inhibitor Cocktail Water-soluble (PIC-W). 1 M *p*-Aminobenzamidine, 1 M e-Aminocaproic acid, 5 mg/mL aprotinin, 1 mg/mL leupeptin. To 1 mL water in a sterile 15 mL conical tube, add 416 mg *p*-Aminobenzamidine, 262 mg *ε*-Aminocaproic acid, 10 mg aprotinin, and 2 mg leupeptin. Dilute to 2 mL with water and mix thoroughly. Distribute into 500 µL aliquots. Store at −80 °C [6].
7. 100× E-64: 1 mg/mL epoxysuccinyl-L-Leucylamido (4-guanidino) butane (E-64). To 8 mL 1× CIB (see above), add 10 mg E-64. Dilute to 10 mL final volume with 1× CIB. Distribute into 1 mL aliquots. Store at −80 °C.
8. 1,000× DTT: 1 M dithiothreitol (DTT). To 1 mL water, add 308.5 mg DTT, dissolve and adjust to a final volume of 2 mL with water. Store in 500 µL aliquots at −80 °C.
9. 1× CIB* solution: 1× CIB containing 1× PIC-W, PIC-D, DTT, and E-64.
10. 50, 35, and 10 % w/v Sucrose Density Gradient solutions: To three individual, immaculately clean 100 mL graduated cylinders, add 50, 35, or 10 g of molecular biology grade sucrose (for 50 %, 35 %, and 10 % w/v solutions, respectively). Dilute to 90 mL with 1× CIB, seal the cylinder with parafilm, and mix by inversion until complete dissolution. Dilute to 100 mL total volume, seal, and mix thoroughly by inversion. Filter sterilize (pore size ≤0.2 µm). Store at 4 °C (*see* **Note 4**).
11. 90 %(w/v) D_2O/30 % Ficoll Density Gradient Solution: Into each of two clean 50 mL conical flasks, add: 22.5 g D_2O, 7.5 g Ficoll, 0.488 g MES hydrate (m.mass 195.24 g/mol), 12.5 µl of 1 M $MgCl_2$ stock, 150 µL of 500 mM EDTA stock pH 8.0, and 50 µL of 500 mM EGTA stock pH 8.0. Incubate at 60 °C while mixing (e.g., using a rotary mixer), for 1 h. Combine the two flasks and adjust pH to 6.4 using 10 N NaOH. Store at 4 °C or for long-term storage at −20 °C (*see* **Note 5**).

12. 9 %(w/v) D_2O/2 % Ficoll Density Gradient Solution: Into a clean 50 mL conical flask, add: 4.5 g D_2O, 1.0 g Ficoll (GE Healthcare), 0.976 g MES hydrate (mol mass 195.24 g/mol), 25 μL of 1 M $MgCl_2$ stock, 300 μL of 500 mM EDTA stock pH 8.0, and 100 μL of 500 mM EGTA stock pH 8.0. Incubate at 60 °C while mixing (e.g., using a rotary mixer), for 1 h. Adjust pH to 6.4 using 10 N NaOH and bring final volume to 50 mL with water. Store at 4 °C or for long-term storage at –20 °C.

2.2 Centrifugation Equipment (See Note 6)

1. Low speed/floor model centrifuge (e.g., *Beckman J2-21*) and accompanying rotor (e.g., *Beckman JA-20*) capable of accommodating 200 mL of sample at 30,000 × *g* with appropriate sample holders (e.g., *Beckman 29 × 104 mm polypropylene thick-wall tube ×8*).
2. Low speed/tabletop centrifuge and accompanying rotor (e.g., *Jouan CR 3 22 with 50 mL screw-top conical tube compatible inserts*) capable of accommodating 500 mL of sample at 10,000 × *g* with appropriate sample holders *(50 mL conical screw-top tubes ×10)*.
3. High speed ultracentrifuge (e.g., *Beckman L-60*) and accompanying rotors (e.g., Beckman *50.2Ti, SW40Ti*) capable of accommodating 200 mL of sample at 200,000 × *g* and three 10 mL gradients at 120,000 × *g* with appropriate tubes (e.g., *Beckman 26.3 mL polycarbonate capped bottles ×8, Beckman Ultraclear 14 × 95 mm tubes ×6*).
4. High speed tabletop ultracentrifuge (e.g., *Beckman TL-100*) and accompanying rotor (e.g., *Beckman TLA 100.3*) capable of accommodating 20 mL of sample at 174,000 × *g* with appropriate sample holders (*Beckman Ultraclear 13 × 51 mm tubes ×2*).
5. Microcentrifuge (e.g., *Eppendorf 5415 D*) with appropriate sample holders (*1.5 mL microfuge tubes with O-ring sealed screw-top*).

2.3 Other Purification Equipment

1. 2× 45 mL Parr Cell Disruption Vessels (Parr Instrument Company, Moline, IL) with accompanying gas regulator and pressurized N_2 storage tank.
2. 15, 5, 2 mL Dounce tissue homogenizers.
3. Linear Gradient Pouring Apparatus (e.g., *Labconco AUTODENSIFLOW Density Gradient Fractionator*).
4. Fractionator.

2.4 Negative Stain Materials

1. Pioloform-coated 200 mesh nickel grids.
2. 4 % OsO_4 in water, 2 mL ampoule.
3. Nano-W® methylamine tungstate (Nanoprobes, Yaphank, NY).
4. Transmission electron microscope.
5. Nonmagnetic steel forceps.

6. Filter paper, 10 cm diameter, cut into eight wedges.
7. ImageJ software (http://rsb.info.nih.gov/ij/, NIH) with "Cell Counter" plug-in (http://rsbweb.nih.gov/ij/plugins/cell-counter.html, Kurt De Vos).

3 Methods

Keep all solutions and carry out all procedures on ice unless otherwise noted. Prechill cell disruption vessel, centrifuge rotors, and centrifuges to 4 °C before steps in which they are utilized. Allow premade sucrose and D_2O/Ficoll solutions to equilibrate to room temperature (20 °C) prior to forming density gradients.

3.1 Cell Collection and Lysis

1. Collect 500 mL of 3.5-day-old *Arabidopsis thaliana* T87 [7] suspension culture (*see* **Note 5**) and distribute into 50 mL conical vials or other appropriate centrifugation container. Collect cells by centrifugation for 4 min, at 100 × *g*, 4 °C. Decant and discard supernatant (*see* **Note 7**).
2. Resuspend and combine all pellets to a total volume of 150 mL with 1× CIB in a 250 mL beaker. Distribute into 50 mL conical or other appropriate containers and collect cells by centrifugation: 4 min at 100 × *g*, 4 °C.
3. Measure total cell volume and decant supernatant. Resuspend all pellets to a total volume of 100 mL with 1× CIB* and collect cells via centrifugation: 4 min at 100 × *g*, 4 °C.
4. Resuspend and pool both pellets to 70 mL total with 1× CIB* (*see* **Note 8**). Load 35 mL of the suspension into the disruption vessel(s) prechilled on ice; rinse conical with 5–10 mL of 1× CIB* and add to bomb(s). Ensure a complete seal (including the exit valve). Pressurize with N_2 to 1,500 psi and hold on ice for 20 min.
5. With constant pressure from the N_2 tank (an open line into the disruption vessel), slowly discharge the disruption vessel(s) via the exit valve into 50 mL conical flasks on ice. CAUTION: This procedure is extremely sensitive and great care must be taken to avoid sample loss (*see* **Note 9**). Analyze approximately 15 μL of the lysate by bright field microscopy to assess and verify cell breakage efficiency (*see* **Note 10**).
6. Centrifugate the lysate for 4 min at 100 × *g*, 4 °C. Collect and combine all 100 × *g* lysate supernatants in a 250 mL beaker on ice (lysate pool). Resuspend cell debris with 25 mL of 1× CIB*; spin 4 min at 100 × *g*, 4 °C. Add supernatant to the lysate pool.
7. Repeat **steps 5**–7 with the cell debris pellets. Thus, cells are subjected to two passes through the disruption vessel(s). Total volume of lysate should be approximately 200 mL. Save 500 μL labeled as "Lysate" for future analysis.

3.2 Differential and Density Gradient Centrifugation

1. Apportion lysate into 6 × 50 mL conical tubes (or other appropriate containers) and centrifuge 10 min at 1,000 × *g*, 4 °C.
2. Transfer supernatant to Beckman JA-20 rotor-compatible tubes, or equivalent, and centrifuge 25 min at 30,000 × *g*, 4 °C.
3. Save 500 μL of supernatant labeled "S_{30}" for subsequent analysis. Transfer supernatant to 6 Beckman 50.2Ti rotor-compatible tubes, or equivalent, and centrifuge 1 h at 120,000 × *g*, 4 °C.
4. During the 1 h 120,000 × *g* centrifugation step (**step 3**, above), prepare the sucrose step gradients as follows: add 10 μL of 1,000× PIC-D, PIC-W, and DTT, and 100 μL of 100× E64 stocks to 9.87 mL of the 10 % (w/v)sucrose in 1× CIB solution; add 16 μL of PIC-D, PIC-W, and DTT, and 160 μL of E64 stocks to 15.8 mL of the 35 % (w/v) sucrose in 1× CIB solution; add 4 μL of PIC-D, PIC-W, and DTT, and 40 μL of E64 to 3.95 mL of the 50 % (w/v)sucrose in 1× CIB solution. In a Beckman 14 × 95 mm Ultra-Clear centrifuge tube, layer 1 mL of the 50 % (w/v) sucrose solution + protease inhibitors, 5 mL of 35 % (w/v), and 3 mL of 10 % (w/v) (*see* **Note 11**). Make three gradients and chill on ice once completed.
5. Save 500 μL of supernatant from the 120,000 × *g* spin, labeled "S_{120}" for subsequent analysis. Discard remaining supernatant. Resuspend 120,000 × *g* pellets into 5 mL total volume of 1× CIB* (*see* **Note 10**). Homogenize in a 15 mL dounce homogenizer; save 200 μL of suspension labeled "P_{120}" for subsequent analysis (*see* **Note 12**). Clear homogenate by centrifugation: 10 min at 16,000 × *g* and 4 °C.
6. Transfer supernatant to a 15 mL conical tube and determine volume. Calculate 11.3 mL minus the volume of the supernatant (XmL). Resuspend the loose pellet with XmL 1× CIB*; homogenize. Clear once more by centrifugation 10 min at 16,000 × *g*, 4 °C.
7. Pool supernatant with that from **step 6** in a 15 mL conical. Mix well by pipetting. Save 200 μL of supernatant labeled "Sucrose Gradient Load (SGL)" for subsequent analysis. Total volume should be as close to 11.1 mL as possible without exceeding this volume.
8. Load 3.7 mL of the SGL onto each of the three sucrose step gradients prepared earlier. When programming the centrifuge, select slow deceleration, if available. Load gradients into the Beckman SW40Ti rotor, or equivalent, and centrifuge 50 min at 116,000 × *g*, 4 °C (*see* **Note 13**).
9. Inspect gradients and compare to Fig. 2. Draw off and discard the top (0 % (w/v) sucrose) layer from the gradient (Fig. 2, A).
10. Collect the 10 % (w/v) layer (Fig. 2, B) and the 10/35 % interface (Fig. 2, C), the latter which is easily visualized as a

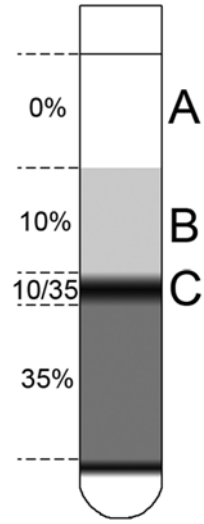

Fig. 2 Schematic of sucrose step gradient after 116,000 × *g* spin (*see* **step 8**). Layer *A* (0 % layer) is discarded whereas layers *B* and *C* (10 % layer and 10 %/35 % interface) are collected as described

thick green/yellow pad, from two of the gradients into each of two 26.3 mL capacity, 50Ti/70Ti rotor-compatible tubes. Evenly distribute the 10 % fraction and 10/35 % (w/v) interface of the remaining gradient equally between the two tubes (i.e., half of a 10 % fraction and half of a 10/35 % (w/v) interface into each tube (*see* **Note 14**).

11. Dilute the contents of each tube with 1× CIB* to ≤5 % (w/w) sucrose as measured by a refractometer (*see* **Note 15**). Centrifuge 50 min at 200,000 × *g*, 4 °C.
12. During the 200,000 × *g* spin, prepare the D_2O/Ficoll gradients (DFGs) as follows: assemble the gradient making rig composed of a GE Lifesciences SG-15 Hoefer gradient former, or equivalent, in tandem with a peristaltic pump and a deposition apparatus of choice, such as the (discontinued) Labconco Auto Densiflow Density Gradient Fractionator (*see* **Note 16**). Prepare the gradients in 14 × 95 mm Beckman Ultra-Clear™ centrifuge tubes.
13. In each of two clean 15 mL conical tubes, add protease inhibitors (11 μL of 1,000× PIC-D, PIC-W, DTT and 110 μL of 100× E-64) to 11 mL of each D_2O/Ficoll solution. Follow the gradient making procedure specific to the device in use. For the apparatus described above (SG15 + Auto Densiflow), ensure the connector and delivery stopcocks are closed and load 5 mL of the 9 %/2 % (w/v)D_2O/Ficoll (light) solution + protease inhibitors in the reservoir (back) chamber. Briefly open the stopcock to fill the channel between the back

and front chambers with the light solution and transfer any excess in the mixing chamber to the reservoir. Add 5 mL of the 90 %/30 % (w/v) (heavy) solution + protease inhibitors to the mixing (front) chamber.

14. Add an appropriately sized stir bar to the mixing chamber and place the apparatus on a magnetic stir plate. Adjust stir bar speed to ensure thorough mixing in the main chamber while avoiding bubble formation. First open the delivery stopcock and then simultaneously open the reservoir stopcock and start the peristaltic pump at a low speed (≤2 mL/min). Starting at the bottom of the gradient tube, raise the output point such that solution being delivered by the pump is deposited at the rising surface of the solution. After the gradient is complete, keep on ice.
15. Decant and discard 200,000 × *g* supernatant; resuspend pellets into 2 mL total volume with 1× CIB* and homogenize in 5 mL homogenizer (*see* **Note 17**). Distribute homogenate into two 1.5 mL microfuge tubes and centrifuge 5 min at 16,000 × *g*, 4 °C.
16. Transfer supernatant to a virgin 15 mL conical tube labeled "Deuterium/Ficoll Gradient Load (DFGL)." Resuspend the large, diffuse pellets by adding 500 μL 1× CIB* (250 μL to each pellet) and homogenize. Distribute homogenate into the same two 1.5 mL microfuge tubes and spin 5 min at 16,000 × *g*, 4 °C.
17. Transfer and pool supernatants in the "DFGL" conical. Discard pellets and microfuge tubes. Adjust volume of DFGL to exactly 2.7 mL with 1× CIB*. Save 200 μL of DFGL pool for subsequent analysis.
18. Load 2.5 mL of DFGL on the D_2O/Ficoll gradient. Centrifuge overnight (12–14 h) at 80,000 × *g*, 4 °C. When programming the centrifuge, select slow deceleration, if available. Flash-freeze aliquots from previous steps with liquid nitrogen (i.e., 500 μL "lysate" sample). Store at −80 °C.
19. Compare the centrifuged D_2O/Ficoll gradient to Fig. 3; the CCVs are present in the diffuse band indicated by the asterisk. Using a fractionation method of choice, collect the entire gradient into 0.75 mL fractions. Record the density of each fraction (sucrose % w/w equivalent) as measured by a refractometer.
20. CCVs are present in fractions between 14 and 16 % (w/w) sucrose equivalent (refractive index $\eta_D^{20} = 1.33514–1.33542$). Collect and pool fractions in this range from both gradients, and dilute to 8.5 mL with 1× CIB*, or as necessary to achieve a density equivalent to ≤5 % sucrose w/w as measured by a refractometer (*see* **Note 18**). Centrifuge 1 h at 174,000 × *g*, 4 °C in the TLA100.3 rotor or equivalent.

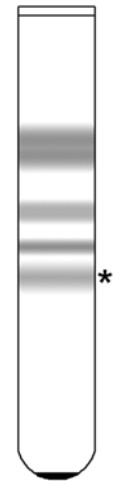

Fig. 3 Appearance of linear D_2O/Ficoll gradient after 80,000 × *g* spin (*see* **step 17**). Fractions containing CCVs (corresponding to ~14 % sucrose (w/w) equivalent) are visualized as the band indicated by the *asterisk*

21. Resuspend pellet(s) in 1× CIB* to 250 μL (*see* **Note 19**). In a microfuge tube, centrifuge resuspension 5 min at 16,000 × *g*, 4 °C.
22. Transfer supernatant to a fresh microfuge tube labeled "CCV." Resuspend pellet by adding 100 μL 1× CIB*. Centrifuge resuspension 5 min at 16,000 × *g*, 4 °C.
23. Pool supernatants. This is the final purified CCV fraction. Save 10 μL for TEM analysis. Further aliquot if desired (*see* **Note 20**). Flash-freeze and store at −80 °C.

3.3 Negative Stain TEM Analysis (See Note 21)

1. Immobilize a pioloform-coated 200-mesh nickel grid by clasping with nonmagnetic steel forceps and securing using a hinged paper binding clip (or other such method), *see* **Note 22**.
2. In an appropriate fume hood, mix one part CCV fraction and three parts 4 % (w/v) OsO_4 solution (i.e., 1 μL and 3 μL) on a bit of parafilm and mix thoroughly by pipetting (the solution should darken).
3. Gently deposit 1 μL of the CCV mixture from **step 2** on the face of the immobilized grid. Using a wedge of Whatman paper, wick away excess fluid (*see* **Note 23**). Allow to dry, approximately 2 min.
4. Place a droplet of the Nano-W solution on the parafilm piece from **step 2**. Take 1 μL from this droplet and gently pipette on the (now dry) mixture-coated grid. Wick away excess as before and allow to cure, approximately 5 min.

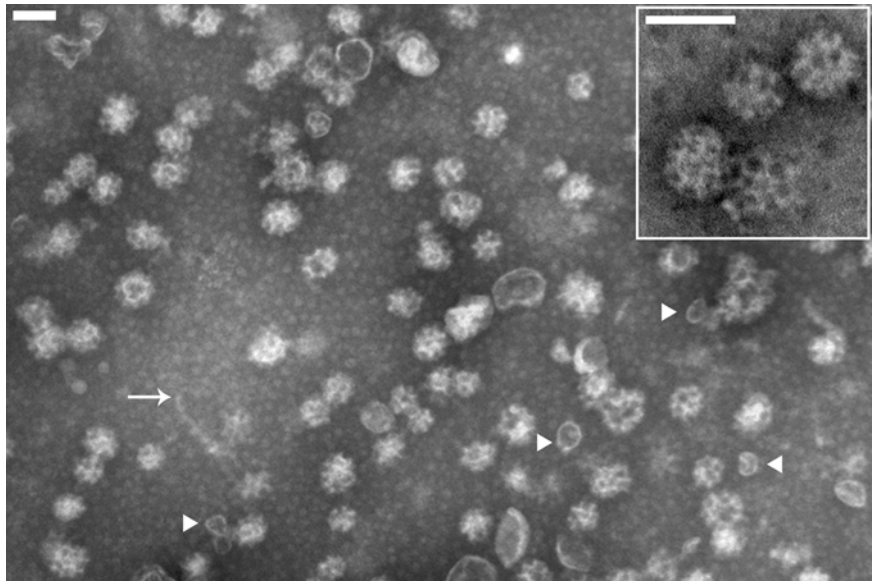

Fig. 4 Negative TEM micrograph of the final CCV fraction, prepared as described. The geometric arrangement characteristic of assembled clathrin triskelia is apparent on coated vesicles. A small proportion of uncoated vesicles (*arrowheads*) and negligible fibrous contamination (*arrow*) are also present. *Inset*: increased magnification of coated vesicles showing striking clathrin lattice organization. Scale bars 100 nm

5. Image the grid using a transmission electron microscope of choice (*see* **Note 24**). Depending on success of the wicking procedures in **steps 3** and **4**, one will observe a nonuniform distribution of both biological material and negative stain. If the enrichment is successful, the striking geometry of the clathrin triskelia (appearing as miniature soccer balls or buckyballs) should be immediately apparent on coated vesicles (Fig. 4).
6. The number of images collected should reflect the desired statistical significance. 10–20 fields are often sufficient to determine a reasonably accurate mean.
7. Open the first image with ImageJ and initialize the Cell Counter plug-in.
8. Select a counter type from the Cell Counter GUI and begin manually designating each coated vesicle by clicking on it. Once all CCVs have been labeled, select a second counter type from the Cell Counter GUI and begin to designate all apparent uncoated vesicles (*see* **Note 25**).
9. Repeat for each image and compile data (manually) to determine a mean coated/uncoated ratio with accompanying standard deviation (*see* **Note 26**).

4 Notes

1. To process the amount of cells described in this experiment, approximately 1.5 L of 1× CIB, 1 mL each of PIC-D, PIC-W, and DTT, and 8 mL of E-64 are required. Therefore, it is advisable to prepare 2 L of 1× CIB, 2 mL of PIC-D, PIC-W, and DTT, and 10 mL of E-64.
2. Early attempts to purify CCVs from plant tissue relied on a lengthy RNAse treatment (1 h at 30 °C) to address extensive ribosome contamination of the final CCV fraction [4]. Due to potential proteolytic side effects, as well as CCV uncoating, this step is omitted in this protocol. Rather, as suggested by Demmer [8], the presence of 3 mM EDTA in the isolation buffer serves both as a general inhibitor of metal-dependent proteases, as well as to promote ribosome instability by chelating structural magnesium ions which serves to reduce their levels in the final CCV fraction.
3. During the experiment, PIC-D must be kept out at room temperature (e.g., on the bench) as DMSO solidifies on ice.
4. 50 % w/v is approaching the saturation of sucrose in water. Avoid the temptation to add additional CIB to speed the dissolution process, lest one find himself with 102 mL of a 49 % sucrose solution. Confirm the sucrose concentrations using a refractometer. *Note*: refractive indices of 10 %, 35 %, and 50 % w/v sucrose are $\eta_D^{20} = 1.3473$, 1.3827, and 1.4039, respectively, at 20 °C
5. With the components listed, the 90 %/30 % D_2O/Ficoll (w/v) solution will be very nearly 50 mL. Thus it is essential to use a concentrated NaOH solution to adjust the pH of the stock solution to pH 6.4 to minimize volume changes. Take care to use lower concentrations in series (i.e., 10, 5, 1 N) as the pH approaches 6.4 to avoid overshooting the target.
6. Although we have specifically defined the centrifugation equipment and conditions employed in our lab for the preparation of CCVs the general centrifugation guidelines provided (e.g., volumes, and centrifugation times/g force) are compatible with other centrifuges, rotors, and tubes .
7. Arabidopsis suspension-cultured cells (T87) were maintained in Murashige and Skoog media supplemented 0.2 mg/L 2,4-D (MS0.2) with shaking at 140 rpm, 22 °C, under constant light. Four days before the fractionation date, dilute a saturated 1-week-old T87W culture 1:10 into fresh MS0.2. The dilution can be modified to accommodate different growth rates between cell lines or media compositions. In the end, aim for 35–45 mL collected cell volume from 500 mL of 3.5-day-old cultures.

8. We have written this protocol under the assumption that the experimenter possesses a pressure-based cell disruption vessel(s) with a minimum capacity ≥90 mL total. Of course, this protocol can be adjusted to meet higher or lower capacities.
9. The use of protective eyewear when discharging the disruption vessel is highly advised. Care must be taken such that the rapid release of nitrogen from the exit valve immediately following complete drainage of the sample does not discharge directly into the sample, as this will result in significant splashing and loss of sample. We find the most success by bleeding approximately 40 mL of sample (our pressure vessel has a 45 mL capacity) into one conical tube and collecting the remainder in another empty tube.
10. After the first pass of the intact cells through the cell disruption vessel, we typically observe ≥60 % of all cells to be lysed as determined by visual estimation. After the second pass, this value approaches ≥90 %.
11. Layering the gradients takes a steady hand (and steadier thumb if using an adjustable micropipettor). With each successive layer, avoid disturbing that which lies below with slow but consistent addition. It might be a good idea to prepare a practice gradient with the 10/35/50 % (w/v) solutions lacking expensive protease inhibitors.
12. Starting with a small volume, roughly 500 μL, resuspend 3–4 pellets and transfer to the homogenizer; repeating for all tubes will yield a volume of approximately 2–3 mL. Subsequently, wash tubes 3–4 times with roughly 750 μL of 1× CIB* until 5 mL total volume is achieved. The S_{120} and P_{120} fractions can be used to determine the presence of soluble and membrane-bound fractions of a given protein.
13. Gently handle the loaded gradients during balancing and when placing the rotor in the ultracentrifuge. The Ultra-ClearTM tubes are very full (to prevent tube collapse) and easily spill with sufficient perturbation.
14. When collecting the 10/35 % (w/v) sucrose gradient interface, avoid taking excess amounts of the 35 % (w/v) layer which is depleted of CCVs and is rich in contaminating denser membranes.
15. If the experimenter is using Beckman polycarbonate, 26.3 mL, capped bottles, the appropriate dilution can be achieved by filling each tube to the base of the neck. Make sure to check the refractive index to confirm sucrose %; failure to dilute sufficiently (<6 %(w/v) sucrose) will impede membrane pelleting in the next step.

16. Regardless of deposition methodology (either automated or by hand using a needle), the key is that the solution being delivered by the peristaltic pump is deposited at the rising surface of the gradient (as described in **step 14**).
17. As with other resuspension steps, it is prudent to begin with approximately half the desired final resuspension volume. In this case, we recommend that pellets be resuspended with 1 mL of buffer and that the residual material that adheres to the centrifuge tube walls be collected by washing the tubes with two additional volumes of 500 μL buffer.
18. In our experience, two 0.75 mL fractions fall in this range. Thus, the pooled two fractions would be diluted to ~4.3 mL.
19. The membrane pellet is a significant contributor of volume in this resuspension step. Accordingly, resuspending in 250 μL as described might actually result in 300 μL final volume. Take this into consideration if there is a desired final volume.
20. The enrichment of CCV in the final fraction relative to contaminating uncoated membranes is determined by the following two methods: (1) immunoblotting for known CCV-associated proteins (e.g., clathrin light chain) and other organelle markers (e.g., chloroplast, ER) to determine enrichment or depletion, respectively, between collected aliquots from various steps in the procedure (i.e., "lysate," "S_{30},"), and (2) examining a sample from the final CCV fraction via negative stain transmission electron microscopy (as described in this protocol).
21. This portion of the procedure involves working with OsO_4, an extremely toxic compound. Accordingly, perform **steps 1–4** in an appropriate fume hood and carefully follow all appropriate safety measures.
22. The benefits of this arrangement are twofold: (1) the grid is secure and stable for the following steps and (2) holding the grid instead of lying it on a surface improves airflow and thus drying times.
23. Avoid touching the surface of the grid with the paper wedge. Instead, very gently touch edges of the fluid droplet to "coax" the mixture to cover all or most of the grid.
24. To determine the relative enrichment of coated vs. uncoated vesicles, we suggest capturing images at 25,000× magnification, wherein coated and uncoated vesicles can be clearly differentiated, yet a large number of vesicles are in the field of view.
25. In our experience, the TEM fields contain materials other than coated and uncoated vesicles, including long fibers and apparently fragmented membranes of small size (10–20 nm). It is up to the user to determine what constitutes an uncoated vesicle versus membrane debris. One metric might be diameter,

achieved by labeling membranes similar to the size of CCVs (50–150 nm) as "uncoated vesicles" and excluding all others.

26. Undoubtedly, this ratio will fluctuate between CCV preparations due to a number of factors (differences in biological sample, minor alterations in experiment procedure, etc.). Over many preps ($n = 11$), we observe 76 ± 10 % of all vesicles to be coated.

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