Chapter 11

Enrichment of Golgi membranes from *Triticum aestivum* (wheat) seedlings

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Abstract

The Golgi apparatus is an essential component in the plant secretory pathway. The enrichment of Golgi membranes from plant tissue is fundamental to the study of this structurally complex organelle. The utilization of density centrifugation for the enrichment of Golgi membranes is still the most widely employed isolation technique. Generally, the procedure requires optimization depending on the plant tissue being employed. Here we provide a detailed enrichment procedure that has previously been used to characterize cell wall biosynthetic complexes from wheat seedlings. We also outline several downstream analyses procedures, including a nucleoside diphosphatase assays, immunoblotting and finally localization of putative Golgi proteins by fluorescent tags.

Key words Golgi apparatus, Density gradient centrifugation, Subcellular localization, Nucleoside diphosphatase

1 Introduction

The Golgi apparatus has an essential role in the eukaryotic secretory system. It is the major site of glycan biosynthesis within the cell, including *N*-linked glycans [1], *O*-linked glycans [2], proteoglycans [3] and complex polysaccharide structures [4]. The Golgi is also involved in a range of other processes including glycolipid biosynthesis [5], vesicular transport [6], assists in the formation of the lysozyme [7] and is intimately connected to intracellular signalling pathways [8].

The Golgi apparatus was initially described by Camillo Golgi towards the end of the 19th century when analysing nerve tissue by light microscopy [9,10]. The development of electron microscopy in the mid-

20th century provided the first real insight into the complexity and heterogeneity of the Golgi apparatus in eukaryotic cells [11]. The Golgi apparatus is now commonly regarded as an integrated component of the endomembrane [12], and in conjunction with the endoplasmic reticulum (ER) represents one of the more difficult organelles to purify from the cell [13].

The development of approaches to enrich the Golgi apparatus was largely driven by the desire to assign biochemical functions to the organelle. Initial approaches to isolate the Golgi employed morphological features to enrich the structure from the cell [11,13]. However, biochemical analysis of Golgi was not undertaken until the development of discontinuous sucrose gradients that could be used to isolate sufficient quantities of the intact organelle [14,15]. The utilization of sucrose gradients and centrifugation is still the most widely used approach for the reproducible enrichment of Golgi membranes from animal [16], microbial [17] and plant [18] material.

In plants, the close physical proximity between the Golgi and the ER [19] makes the purification of this organelle even more problematic, often necessitating more advanced purification strategies such as LOPIT [20,21] or free-flow electrophoresis [22-24]. However, the approach has been widely used to generate enriched fractions to attribute biochemical functions, such as mannan synthase [25], UDP-glucose transport [26], nucleotide diphosphatase [27], cell wall biosynthetic complexes [18], *N*-linked glycan processing [28] and membrane trafficking [29]. Here we outline the enrichment of Golgi membranes from wheat seedlings using a continuous sucrose gradient that would be suitable for variety of biochemical investigations. The downstream characterization of enriched Golgi membranes by nucleotide diphosphatase assays, immunoblotting and localization of proteins by fluorescently tagged proteins is also detailed.

2 Materials

Prepare solutions with ultrapure water (18 M Ω cm at 25 °C) and analytical grade reagents. Prepare all reagents at room temperature.

2.1 *Plant growth and sample preparation*

1. *Triticum aestivum* (wheat) seeds, ca. 50 g (*see Note 1*).
2. Plant growth incubator or chamber (*see Note 2*).
3. Vermiculite, grade 2 (granule size 2 to 4mm).
4. Plastic tray *e.g.* plant growth tray (50 x 25 cm).

2.2 *Isolation of plant Golgi membranes*

1. Extraction Buffer: 50 mM HEPES-KOH, pH 6.8, 0.4 M sucrose, 1 mM dithiothreitol (DTT), 5 mM MnCl₂ and 5mM MgCl₂ (*see Note 3*).
2. Proteinase inhibitor cocktail, such as cOmplete EDTA-free proteinase inhibitor cocktail tablet (Roche) (*see Note 4*).
3. Kitchen blender, such as Magic Bullet® (*see Note 5*).
4. Miracloth (Merck Millipore).
5. Preparative centrifuge for 50 mL tubes and capacity to 5,000 × g, such as an Avanti J25 centrifuge (Beckman Coulter) with a JA-25.50 rotor (Beckman Coulter).
6. Cushion Buffer: 1.8 M sucrose in 50 mM HEPES-KOH, pH 6.8.
7. Disposable plastic pipettes (1 mL).
8. A two chamber Gradient Maker (20 mL).
9. Magnetic mixer and magnetic stir bar.
10. Peristaltic pump and tubing (1 mm internal diameter).
11. Gradient Buffer 25: 25 % (w/v) sucrose stock solution in 50 mM HEPES-KOH, pH 6.8 and 1 mM EDTA. Can be made ahead of time and stored at -20 °C.
12. Gradient Buffer 40: 40 % (w/v) sucrose stock solution in 50 mM HEPES-KOH, pH 6.8 and 1 mM EDTA. Can be made ahead of time and stored at -20 °C.
13. Ultracentrifuge, swing-out rotor for 30 mL tubes and capable of 100,000 × g for gradients, such as an Optima™ XE (Beckman Coulter) with a SW 30 Ti rotor (Beckman Coulter) and a fixed angle rotor for 12 mL tubes capable of 100,000 × g for pelleting membranes, such as a Type 70.1 Ti rotor (Beckman Coulter).
14. 18-gauge needle (*see Note 6*).
15. Fraction collector (*see Note 7*).
16. Dilution Buffer: 50 mM HEPES-KOH, pH 6.8.
17. Protein Quantification Assay, such as Pierce™ BCA Protein Assay Kit.

2.3 Analysis of enriched Golgi membranes by immunoblotting

1. Electrophoresis chamber for protein separation, such as XCell SureLock® Mini-Cell (Life Technologies).
2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) precast gels compatible with the electrophoresis chamber, such as NuPAGE® Novex® 12% Bis-Tris Protein Gels (Life Technologies).
3. Electrophoresis Buffer, such as. NuPAGE® MOPS SDS Running Buffer (Life Technologies).
4. Sample Buffer, such as NuPAGE® LDS Sample Buffer (Life Technologies).
5. Reducing agent, such as NuPAGE® Sample Reducing Agent (Life Technologies).
6. Protein standard, such as Precision Plus Protein Kaleidoscope® Standards (Bio-Rad).
7. Western apparatus, such as XCell II™ Blot Module (Life Technologies).
8. Western Transfer Buffer, such as NuPAGE® Transfer Buffer (Life Technologies).
9. 100% methanol.
10. Filter paper for immunoblotting.
11. PVDF membrane.
12. TBST Buffer: 50 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1 % (v/v) Tween-20.
13. Skim milk powder.
14. Golgi marker antibody, such as Arf1 - ADP-ribosylation factor 1 (Agrisera AB, *see Note 8*).
15. Secondary antibody, such as. Anti-Rabbit IgG Peroxidase (Sigma-Aldrich)
16. Chemiluminescent substrates, such as. WesternBright ECL HRP substrate (Advansta).
17. Chemiluminescent imaging system, such as MyECL Imager (Life Technologies).

2.4 Analysis of enriched Golgi membranes by nucleoside diphosphatase (NDPase) activity

1. NDP: uridine 5'-diphosphate (UDP) or inosine 5'-diphosphate (IDP).
2. NDPase Reaction Buffer: 3 mM UDP, 3 mM MnSO₄, 30 mM MES-KOH, pH 5.6, 0.03% (v/v) Triton X-100 (*see Note 9*).
3. Malachite Green Phosphate Assay kit (ScienCell Research Laboratories).
4. Absorbance microplate reader capable of measuring at 630 nm.

2.5 Validation of Golgi localization of fluorescent protein fusions

2.5.1 Cultivation of *Nicotiana benthamiana*

1. Plastic plant pots (80 x 80 mm).
2. Germination tray (280 x 540 mm) with a transparent plastic lid.
3. *Nicotiana benthamiana* 'Domin' seeds.
4. Soil, such as PRO-MIX (Premier Horticulture).

5. Plant growth chamber (*see* **Note 10**).

2.5.2 Plasmid Preparation and *Agrobacterium tumefaciens* transformation

1. cDNA from plant material (*see* **Note 11**).
2. Thermocycler (PCR machine).
3. Gene specific primers.
4. DNA Polymerase, such as Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific), dNTPs (*see* **Note 12**).
5. Agarose gel electrophoresis equipment.
6. Agarose, such as UltraPure™ Agarose (Life Technologies).
7. 1 x TAE buffer, such as TAE Buffer (Tris-acetate-EDTA, 50X) (Life Technologies).
8. DNA loading buffer, such as Gel Loading Solution (Life Technologies).
9. DNA ladder, such as 1 Kb Plus DNA Ladder (Life Technologies).
10. UV light box.
11. Gel and PCR clean-up kit, such as NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel).
12. Plasmid preparation kit such as QIAprep Spin Miniprep Kit (Qiagen).
13. pCR®8/GW/TOPO® TA Cloning Kit (Life Technologies).
14. Gateway® LR Clonase® II Enzyme mix (Life Technologies).
15. Gateway compatible vector containing a fluorescent protein, such as pEarleyGate [30] (*see* **Note 13**).
16. Competent *Escherichia coli* (*E. coli*), such as One Shot® TOP10 Chemically Competent *E. coli* (Life Technologies).
17. Spectinomycin, kanamycin and gentamycin.
18. Temperature mixer, such as Thermomixer compact (Eppendorf).
19. Competent *Agrobacterium*, such as strain GV3101::pMP90 (*see* **Note 14**).
20. Disposable electroporation cuvettes with 1 or 2 mm gap sizes.
21. Electroporation system, such as Gene Pulser Xcell™ Electroporation Systems (Bio-Rad).
22. Luria-Bertani (LB) media: 10 g tryptone, 10 g NaCl, 5 g yeast extract in 1 L water, adjust to pH 7 with NaOH and sterilize by autoclaving.
23. Luria-Bertani (LB) media supplemented with agar: 10 g tryptone, 10 g NaCl, 5 g yeast extract, 7.5 g agar in 1 L water, adjust to pH 7 with NaOH and sterilize by autoclaving.

2.5.3 Infiltration

1. Luria-Bertani (LB) media supplemented with agar: 10 g tryptone, 10 g NaCl, 5 g yeast extract, 7.5 g agar in 1 L water, adjust to pH 7 with NaOH and sterilize by autoclaving.
2. Sterile pipette tips or toothpicks.

3. Centrifuge, such as Allegra 25R Benchtop Centrifuge with a TA-14-50 rotor (Beckman Coulter).
4. Infiltration Buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES-KOH, pH 5.6, 10 mM MgCl₂, 100 μM acetosyringone) (*see Note 15*).
5. 1 mL disposable polypropylene syringes.

2.5.4 *Confocal Microscopy*

1. Laser Scanning Confocal Microscope (*see Note 16*).
2. Coverslip holder, such as Attotfluor® cell chamber for microscopy (Life Technologies).
3. 25 mm round glass coverslips.
4. 10% (v/v) glycerol.

3 Methods

3.1 Cultivation of wheat seedlings

1. Soak the wheat seeds (50 g) overnight in water in the dark at room temperature.
2. The following day, soak the vermiculite (2 to 3 L) with water and drain.
3. Spread the wet vermiculite into the plastic tray to a depth of around 50 mm.
4. Evenly distribute the hydrated wheat seeds over the vermiculite.
5. Place the tray in a growth chamber set at about 22 °C in the dark (*see Note 17*).
6. Leave wheat to germinate and elongate for approximately 5 days or until the seedlings are about 80 mm in length (*see Note 18*).

3.2 Enrichment of Golgi membranes from wheat

1. Harvest 5-day wheat seedlings (approximately 50 g FW) with scissors by cutting at the base. Be careful to avoid vermiculite contamination (Figure 1A). Place into a 500 mL beaker.
2. Rinse harvested seedlings in distilled water and dry on paper towel.
3. Place cut seedlings into the Magic Bullet® cup, add precooled (4 °C) Extraction Buffer (2 mL per g tissue FW) and attach the base (Figure 1B).
4. Blend seedlings using the Magic Bullet® for 10 seconds and then wait 30 seconds. Repeat two more times for a total of 30 seconds blending (*see Note 19*).
5. Filter the homogenate through two layers of Miracloth placed in a funnel into a 250 mL conical flask on ice. Gently squeeze the Miracloth to extract as much homogenate as possible.
6. Divide the homogenate evenly into two 50 mL preparative centrifuge tubes and centrifuge at 3000 x g for 20 minutes at 4 °C (*see Note 20*).
7. Carefully pour or pipette the supernatant into a 100 mL beaker on ice.
8. Add 6 mL of Cushion Buffer to each of the four 30 mL ultracentrifuge tubes. Transfer the approximately 20 mL supernatant on to Cushion Buffer (Figure 1C). Centrifuge at 100,000 x g for one hour at 4 °C.
9. Carefully remove the crude microsomal membranes with a disposable plastic pipette and place in a 15 mL plastic tube on ice (Figure 1D) (*see Note 21*).
10. Place 6 mL of Cushion Buffer into four new 30 mL ultracentrifuge tubes.
11. Add 10 mL of Gradient Buffer 40 to the Gradient Maker chamber with the outlet. Add 10 mL of Gradient Buffer 25 to the second chamber (*see Note 22*).
12. Place a magnetic stir bar into the chamber with the Gradient Buffer 40 (closest to outlet) and place Gradient Maker onto a magnetic mixer and activate.
13. Attach the tubing to the Gradient Chamber outlet and fit to the peristaltic pump.
14. Place one of the ultracentrifuge tubes with the Cushion Buffer onto ice at a 45° angle and tape the end of the tube (after the peristaltic pump) to the top of the ultracentrifuge tube.

15. Start the peristaltic pump (*see Note 23*). Wait around 30 seconds before opening the valve between the two chambers on the Gradient Maker (*see Note 24*).
16. After about 20 minutes a continuous 25 to 40 % sucrose gradient will be produced.
17. Using the same approach, pour the other three sucrose gradients required for ultracentrifugation of microsomal membranes (*see Note 25*).
18. Carefully pipette the crude microsomal membranes onto the top of the 25 to 40% sucrose gradients dividing evenly (Figure 1E).
19. Ensure paired tubes are balanced and ultracentrifuge at 100,000 x g for 16 hours at 4 °C.
20. After ultracentrifugation, collect the fractions (approximately 1 mL each) using a fraction collector or by manually pipetting (Figure 1G) (*see Note 7*).
21. Dilute fractions 1:10 in Dilution Buffer (*see Note 26*).
22. Ultracentrifuge fractions at 100,000 x g for 1 hour at 4 °C in a fixed angle rotor.
23. Discard supernatant and resuspend membrane pellets in 50 µL Dilution Buffer.
24. Estimate the protein concentration in each fraction using a Protein Quantification Assay and then store fractions at -80 °C.

3.3 Assessment of Golgi enrichment by immunoblotting

1. The analysis of samples during Golgi membrane enrichment and fractionation can be accomplished using organelles marker antibodies (Figure 2) (*see Note 8*).
2. If using a mini-gel system, around 5 to 10 µg of protein is suitable from each fraction. Add Sample Buffer (1 X) and reducing agent, then incubate at 70 °C for 5 minutes.
3. Load samples and protein standards onto a preassembled precast gel (*see Note 27*).
4. Separate the protein samples on the gel at 200 V for 45 to 60 minutes or until the dye front reaches the bottom of the gel.
5. Remove the gel from the plates and briefly rinse in distilled water.
6. Cut PVDF membrane to the size of the gel and briefly rinse in 100% (v/v) methanol, followed by distilled water then place in Western Transfer Buffer (*see Note 28*).
7. Cut two pieces of filter paper to the size of the gel and soak in Western Transfer Buffer for 2 minutes.
8. Assemble transfer stack according to the manufactures instructions, normally in the following order from the cathode, blotting pad (2), filter paper, gel, PVDF membrane, filter paper and blotting pad (2).
9. Place transfer stack into the Western apparatus, assemble, add Western Transfer Buffer and connect to electrophoretic power source.
10. Transfer for 30 V for 1 hour (*see Note 29*).
11. Disassemble transfer stack, remove PVDF membrane and place in TBST Buffer.

12. Incubate PVDF membrane with gentle shaking in TBST with 5 % (w/v) skim milk powder for 1 hour at room temperature.
13. Dilute primary antibody in TBST with 5 % (w/v) skim milk powder (*see Note 30*).
14. Incubate PVDF membrane with primary antibody diluted in TBST with 5 % (w/v) skim milk powder with gentle shaking at room temperature for 1 hour (*see Note 31*).
15. Wash PVDF membrane with TBST Buffer for 10 minutes with shaking. Repeat wash in TBST Buffer two more times.
16. Dilute HRP conjugated secondary antibody in TBST with 5 % (w/v) skim milk powder (usually around 10,000 fold dilution) and incubate with washed PVDF membrane with gentle shaking at room temperature for 1 hour (*see Note 32*).
17. Wash PVDF membrane with TBST Buffer for 10 minutes with shaking. Repeat wash in TBST Buffer two more times.
18. Prepare the HRP substrate (approximately 1 mL) by mixing the two reagents. In the case of the WesternBright ECL HRP substrate a 1:1 ratio is used (*see Note 33*).
19. Drain excess liquid from the PVDF membrane and add HRP substrate and incubate for 1 minute.
20. Image using a chemiluminescence imager.

3.4 Assessing Golgi enrichment by nucleoside diphosphatase (NDPase) assay

1. The enrichment of Golgi membranes can be assessed by enzymatic assays, specifically the Golgi specific nucleoside diphosphatase (NDPase) [31]. The assay can be undertaken on preliminary fractions such as the homogenate and crude microsome, but ultimately can be used to analyse the fractions from the sucrose gradient (Figure 2).
2. Add 1 mL of NDPase Reaction Buffer to 20 µg protein and incubate at room temperature for 30 minutes (*see Note 34*).
3. Remove 20 µL and add 40 µL of Malachite Green Reagent A, mix and incubate at room temperature for 5 minutes.
4. Add 40 µL of Malachite Green Reagent B, mix and incubate at room temperature for 10 minutes (*see Note 35*).
5. Read the absorbance at 630 nm using an Absorbance microplate reader.
6. Prepare a phosphate standard curve by diluting the supplied Phosphate Standard (10 mM) in distilled water to concentrations of 5 to 100 µM.
7. Remove 20 µL from each dilution of the standard and add 40 µL of Malachite Green Reagent A, mix and incubate at room temperature for 5 minutes.
8. Add 40 µL of Malachite Green Reagent B to the standards, mix and incubate at room temperature for 10 minutes.

9. Read the absorbance at 630 nm using an Absorbance microplate reader.
10. Generate a standard curve by linear regression and estimate the concentration of phosphate in each microsomal fraction (*see Note 36*).
11. Calculate specific activity for each fraction by converting to $\mu\text{mol Pi h}^{-1} \mu\text{g}^{-1}$ protein.

3.5 **Transient Protein Expression for Subcellular Localization**

Enriched Golgi membrane samples can be further characterized by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify proteins associated with these fractions. The subcellular localization of proteins identified by LC-MS/MS can be further confirmed using the following approach.

3.5.1 *Cultivation of Nicotiana benthamiana*

1. Fill pots (80 x 80 mm) with soil (PRO-MIX HP MYCORRHIZAE™), add tap water allow the soil to hydrate and then drain the tray when the soil is completely soaked with water.
2. Sow *N. benthamiana* ‘Domin’ seeds in the soil, place the pots in a plastic tray and cover the tray with a transparent plastic lid.
3. Allow the seeds to germinate in a 25 °C chamber with 60% humidity and 16 hr light / 8 hr dark cycles.
4. After one week remove the plastic lid and continue to grow the plants in the same growth conditions for one more week.
5. Transfer two-week old seedlings into fresh pots (prepared as before) only one plant per pot (*see Note 37*) and grow the plants under the same growth conditions until they are ready to be infiltrated (*see Note 38*).

3.5.2 *Plasmid preparation and transformation of Agrobacterium*

1. Prepare a PCR mix with PCR reaction buffer (included with polymerase), gene specific primers, dNTPs, DNA polymerase and plant cDNA. Place the reaction into a thermocycler and amplify the gene of interest (*see Note 39*).
2. Mix the PCR reaction with DNA loading buffer and load the reaction onto an agarose gel in 1 x TAE buffer with a DNA ladder. Run the gel at 80 to 120 V until the dye front is about three quarters down the gel (*see Note 40*).
3. Visualize the DNA fragments using a UV light box and confirm the expected size of the amplicon by comparing to the DNA ladder, excise the DNA fragment and extract the DNA from the agarose gel using a Gel clean-up kit.
4. Use the purified PCR product with the pCR®8/GW/TOPO® TA Cloning Kit to clone the fragment into the pCR®8 donor vector to create the entry clone.

5. Transform the reaction into competent *E.coli* and select for a positive transformant on LB plates containing 100 µg/mL spectinomycin. Purify the plasmid from an overnight culture (LB with 100 µg/mL spectinomycin) using a Plasmid preparation kit and confirm the identity of the entry clone by sequencing.
6. Use the confirmed entry clone for a LR Clonase II Enzyme reaction and introduce it into the destination vector (e.g. pEarleyGate) containing an in-frame fluorescent protein.
7. Transform the reaction into chemically competent *E.coli* and select a positive transformant containing the construct of interest from the LB plate containing kanamycin (50 µg/mL). Use toothpick to transfer the colony to a sterile culture tube containing 5 mL LB containing kanamycin (50 µg/mL) and incubate at 37 °C overnight with shaking. Purify the plasmid from an overnight culture using a Plasmid preparation kit and confirm the identity of the expression clone by sequencing.
8. Take a frozen 50 µL aliquot of competent *Agrobacterium* (see **Note 41**) and let the cells slowly thaw on ice.
9. Add 1 µL (see **Note 42**) of the expression vector, such as pEarleyGate containing cDNA of interest to the cells, mix the plasmid and the cells by tapping the tube and let the mix sit on ice for 15 minutes.
10. Place a disposable electroporation cuvette on ice and transfer the plasmid-cell mixture into the pre-chilled cuvette. Keep the cuvette with the plasmid-cell mixture on ice until the electroporation step.
11. Set up electroporation device according to the manufacturer's instructions (see **Note 43**).
12. Place the cuvette into the cuvette holder of the electroporation device (see **Note 44**), make sure that the cuvette is placed tightly between the electrodes and press the pulse button to apply voltage to the sample.
13. Immediately after the electroporation add 1 mL of LB media to the plasmid-cell mixture in the cuvette (see **Note 45**) and mix by gently pipetting up and down.
14. Transfer the contents of the cuvette into a sterile 1.5 mL tube and let the mixture incubate for at least 1 hour at 30 °C with constant agitation.
15. Plate 35 to 200 µL (see **Note 46**) of the mix onto LB agar plates supplemented with the appropriate selective antibiotics (see **Note 47**) and incubate for 48 hours at 30 °C until colonies become visible.

3.5.3 Infiltration of *N. benthamiana*

1. Pick a fresh colony of the *Agrobacterium* containing the construct of interest with a sterile pipette tip. Inoculate a 10 mL liquid LB media (containing the appropriate selective antibiotics)

with the *Agrobacterium* colony and grow the culture overnight at 30 °C under constant shaking at a rotation rate of 200 rpm to log phase.

2. Spin down the overnight culture for 10 minutes at 4000 x g at 20 °C.
3. Remove supernatant and wash the pellet with 10 mL Infiltration Buffer (*see Note 48*) and repeat the centrifugation step.
4. Resuspend the *Agrobacterium* in Infiltration Buffer to an optical density (OD) of 0.01 to 0.3 at 600 nm (*see Note 49*).
5. Infiltrate the abaxial surfaces of leaves from three to four week old *N. benthamiana* plants using a 1 mL plastic syringe without a needle (*see Note 50*). Hold the leaf firm from the adaxial side and press the syringe from abaxial side against your finger. Infiltrated areas, where the *Agrobacterium* mixture has entered the intercellular space, will turn darker green (*see Note 51*).
6. Grow plants for two additional days before monitoring expression of the fluorescent protein by confocal microscopy (*see Note 52*).

3.5.4 Confocal microscopy

1. Use a razor blade to cut out a 12 x 12 mm piece from the infiltrated leaf (*see Note 53*).
2. Place the leaf piece onto a coverslip and add a drop of 10% (v/v) glycerol on its surface.
3. Place a second coverslip on top and mount it into the coverslip holder.
4. Place the slide onto the confocal stage and focus using brightfield imaging at low magnification.
5. At this magnification use an appropriate filter or setting, depending on the fluorescent protein employed, to find cells with a fluorescent signal. Once a cell is located, a higher magnification can be employed.
6. Configure the confocal to enable sequential or simultaneous acquisitions (*see Note 54*).
7. Perform a live scan using the confocal, in this example we employed a simultaneous acquisition for yellow fluorescent protein (YFP) and mCherry (Figure 3).
8. Images can be processed with the microscope software such as ImageJ [32].

4 Notes

1. A variety of plant material can be employed for this protocol. However dark grown seedlings, such as rice, maize, pea, Arabidopsis up to 10-days provide a good source plant material and minimize contamination from chloroplasts.
2. The type of incubator or chamber will depend on the species or plant material being used. In the case of wheat seedlings an incubator that can maintain 22 °C is required with no necessity for light or humidity control.
3. The Extraction Buffer can be premade and kept at -20 °C. The day before the buffer is required, place at 4 °C to defrost overnight.
4. The cOmplete EDTA-free proteinase inhibitor cocktail tablet is added to the Extraction Buffer just prior to use. One tablet is required per 50 mL Extraction Buffer.
5. The Magic Bullet® kitchen blender is a small commercial product that can be used to process intermediate amounts (up to 50 g FW) of plant material. It is readily available from most major department stores or online and is an efficient method for processing plant material for microsomal membrane extractions.
6. The simplest way to collect the microsomal fractions after ultracentrifugation is to pierce the bottom of the tube with a needle and draw fractions using the peristaltic pump. If employing this approach (rather than removing fractions from the top), ensure that the ultracentrifuge tubes used during the 100,000 x g are thinwall ultracentrifuge tubes.
7. An automatic fraction collector with a peristaltic pump is a convenient and precise way to collect fractions (0.5 to 1 mL) after separation of microsomes. However, it is also possible to manually hand collect fractions using just a peristaltic pump and a set of Eppendorf tubes. Both these approaches can be applied to the top or the bottom of the tube. It is also possible to collect fractions by hand by carefully manually pipetting aliquots (1 mL) from the top of the gradient.
8. There are currently a limited number of commercially available antibodies for plant research. A good source of reliable plant antibodies is Agrisera AB. The company produces a collection of compartment marker antibodies against plant proteins that can be used to assess the purity of organelle enrichments. Unfortunately, both the available Golgi marker antibodies (Arf1 and Sec21p) are not exclusive for the Golgi apparatus, with both products also found in soluble plant lysate preparations. Another source of antibodies against plant proteins is Abcam. We have successfully used an antibody raised against the ER protein calreticulin (CRT1: ab2907) and an antibody against the nuclear marker histone H3 (ab1791).
9. Historically duplicate activities are undertaken; one involves detergent (Triton X-100) in the buffer and the second without detergent (latent activity). Subsequently, NDPase activity is calculated by subtracting latent activity (no detergent) from the triton-activated NDPase activity. In our experience, latent activity is inconsistent and highly variable; therefore we

generally always include detergent in the NDPase Reaction Buffer. A background signal is determined by using the extraction buffer with the NDPase Reaction Buffer.

10. A growth chamber capable of maintaining 25 °C, 60% humidity and a day/night cycle is optimal. However, plants can also be grown in a glasshouse with temperature control.
11. To obtain cDNA from the plant tissue of interest, extract RNA with a plant RNA extraction kit, such as the RNeasy Plant Mini Kit (Qiagen) and subsequently reverse transcribe the resultant RNA into cDNA using a Reverse Transcriptase, *such as* Superscript III Reverse Transcriptase (Life Technologies).
12. To amplify the gene of interest, any proofreading DNA polymerase can be used in accordance with the manufacturer's instructions.
13. The pEarleygate vector collection works well for our purposes, however a plethora of other vectors are available that contain strong constitutional promoters and fluorescent tags.
14. *Agrobacterium tumefaciens* strain GV3101::pMP90 is a commonly used strain, which works well to transform *N. benthamiana* and can be used to stably transform *Arabidopsis thaliana*. However other *Agrobacterium* strains are available that also work for transient expression analysis in *N. benthamiana* such as C58C1, EHA105, LBA4404, AGL1.
15. The infiltration buffer is best prepared fresh for each infiltration. Stock solutions of 0.5 M MES-KOH pH 5.5 and 1 M MgCl₂ can be prepared, autoclaved and stored at room temperature until use. For the 0.5 M MES stock solution prepare MES first and adjust the pH with KOH to 5.5. Acetosyringone should be added separately to the infiltration buffer each time. Prepare a 100 mM acetosyringone stock solution in either DMSO or 96% (v/v) ethanol (it does not dissolve in water), and keep it in aliquots at -20 °C or for shorter periods at 4 °C until use. Do not autoclave the infiltration buffer with acetosyringone.
16. The Laser Scanning Confocal Microscope (LSCM) must contain the capability to excite at the appropriate wavelengths. For example for the pEarleyGate 101 vector 514 nm for yellow fluorescent protein (YFP).
17. Since the wheat are germinated and grown at room temperature in the dark, it is also possible to grow the plant material necessary for the microsomal preparation in a cupboard in the lab covered in foil.
18. The number of days required to obtain approximately 8 cm seedlings will be dependent on various conditions including age of seed, temperature and humidity. Consequently, check the seedlings every other day to monitor their progress.
19. Once plant material is processed, it is important to maintain the lysate at around 4 °C. This is achieved by precooling buffers and apparatus where required. These steps can also be conducted in the cold room. However it is also possible to keep the extract on ice, for example,

between each blending step, the Magic Bullet® container can be removed from the instrument and placed on ice.

20. The amount of material outlined in this method results in about 100 mL of homogenate.
21. Ensure the pipette is squeezed before placing into the buffer so no air bubbles to not disrupt the band. Carefully extract the microsomal band using a gentle swirling motion with the tip of the pipette. Avoid taking excess buffer so as not to dilute the sample.
22. Ensure the valve is closed between the two chambers prior to adding the Gradient Buffers.
23. Prior to pouring the sucrose gradient, ensure that a flow rate has been selected on the peristaltic pump to provide a flow of about 1 mL/minute.
24. Ensure a suitable volume of 40% sucrose is delivered to the bottom of the gradient. At 1 mL/minute a wait of 30 to 60 seconds is probably suitable.
25. It is possible to add a Y-union to the tubing to split the flow after the peristaltic pump to enable the simultaneous production of two sucrose gradients. Using such an approach would require twice (20 mL) as much Gradient Buffer in each chamber.
26. The sucrose from the gradient needs to be diluted or high concentrations will prevent pelleting of membranes.
27. Using a precast gel system is the simplest and most convenient way to analyse multiple fractions. Assemble the apparatus with precast gel with compatible Electrophoresis Buffer and following the manufacturer's instructions.
28. Ensure methanol has been added to commercially sourced transfer buffers.
29. A range of conditions can be used including overnight transfers. Check the instructions with the specific transfer apparatus for advice on specific conditions.
30. The dilution of a primary antibody varies depending on the specificity. A range will be provided, however some testing may be required for optimal signal. A dilution range between 500 and 5000 is common for polyclonal antibodies.
31. To minimize the amount of primary antibody employed, only use enough to cover the membrane and employ a small container.
32. A variety of conjugations are available for the detection of secondary antibodies, however HRP (horse radish peroxidase) is one of the most common as it is a robust enzyme that enables detection by a variety of techniques.
33. When mixed, the HRP substrate is light sensitive. Therefore keep away from light (wrapped) and use immediately.
34. We have found that a total of around 20 µg protein and a reaction volume of 1 mL is required to produce a reliable result.
35. We have found that higher proportions of reagent are required with plant protein samples.

36. If the amount of phosphate produced in a fraction is outside the range of the standard curve, employ a lower amount of protein such as 1 to 5 μg .
37. To ensure that the plants have adequate space in the tray to develop healthy leaves, do not fill the entire tray with pots.
38. Plants should not be too old. Generally we found that plants that are three to four weeks old with a rosette diameter of 80 to 100 mm are suitable for infiltration.
39. To successfully amplify the gene of interest it is important to use the appropriate plant tissue, in which the gene is most highly expressed. It is also important to use a proofreading polymerase for the PCR reaction to avoid the introduction of sequence errors. For the same reason the number of PCR cycles should be kept to a minimum.
40. For most applications a 1% (w/v) agarose gel can be used. However if the separation is not optimal, the agarose concentration needs to be adjusted to concentrations ranging from 0.6% (w/v) to 2% (w/v) depending on the size of the PCR fragment.
41. Competent *Agrobacterium* can be prepared in large batches and store 50 μL aliquots at $-80\text{ }^{\circ}\text{C}$ until use.
42. Using 1 μL (25 to 100 ng) of the plasmid preparation in water or TE buffer works for most *Agrobacterium* transformations. Ensure that plasmid preparations with higher DNA concentrations are diluted or the transformation can be inhibited.
43. Make sure that your settings are adjusted for the gap size of the cuvette being employed and that the setting is appropriate for *Agrobacterium*.
44. Wipe of any residual ice particles from the cuvette since that could impede the electroporation step.
45. It is essential to add the LB-media to the mixture promptly; any delay will reduce the transformation efficiency.
46. The volume of the *Agrobacterium*-plasmid mixture to be plated largely depends on the efficiency rate of the competent *Agrobacterium*. Typically plating 35 μL is sufficient to produce enough positive colonies.
47. Make sure to use the correct antibiotics for selection. In general antibiotics for both the *Agrobacterium* host and the plasmid are necessary. The *Agrobacterium* strain GV3101::pMP90 is resistant to gentamycin and rifampicin and the pEarleyGate vector series confer kanamycin resistance.
48. Some protocols do not include a wash step of the *Agrobacterium* cells. However in our hands it seems to improve the success rate of the infiltration and the protein expression. This is potentially because the antibiotics in the culture media affect the viability of the leaves.
49. The optical density (OD) of the *Agrobacterium* Infiltration Buffer required depends on the expression of the protein of interest. Generally we find that lower optical densities (0.01 to

0.15) give better results and do not cause protein aggregates. However lowly expressed proteins may require the use of higher optical densities. Infiltration at different concentrations enables the determination of optimal concentrations. To co-infiltrate multiple constructs, for example, a gene of interest and an organelle marker protein, mix a combination of the two *Agrobacterium* solutions to the final OD.

50. Start by using the top leaves of the plant as they do not have as many veins as the older leaves and are therefore easier to infiltrate. Some researchers use a needle to make small cuts in the epidermis at the position where they want to infiltrate. We have found that piercing the leaf a little bit with the syringe at a 45° angle while holding the leaf upright also helps.
51. It is important to use as little pressure as possible while injecting the *Agrobacterium* mixture to avoid punching holes into the leaves. Strongly wounded leaves will die and cannot be used for microscopy.
52. Since not all proteins express with the same efficiency, expression should be monitored at different time points we have found that monitoring the expression after 48 and 72 hours generally gave the best results.
53. Make sure to cut a piece of the leaf from the infiltrated area, however avoid cutting a piece too close to the area where you pressed the syringe against the leaf since this area will be wounded and will produce a high auto-fluorescence signal.
54. Modern confocal microscopes are capable of performing simultaneous scans (fast, crosstalk between signals) and sequential scans (slow, but less crosstalk between signals). The use of simultaneous scanning enables the visualization of two signals without the need for fixation, which is especially useful for Golgi apparatus. While the emission range can be narrowed to reduce crosstalk, this will reduce the signal intensity. Sequential scanning is often essential to ensure the signal from both fluorophores is valid. This is especially important if using fluorophores with similar emission wavelengths such as YFP and green fluorescent protein (GFP).

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Figure Legends

Figure 1. Flowchart outlining the basic procedure for the isolation and fractionation of microsomal membranes from wheat seedlings.

Figure 2. Immunoblotting and nucleoside diphosphatase (NDPase) assays of plant protein lysates during microsomal enrichment. The immunoblotting and NDPase assays highlight the enrichment of an Arabidopsis microsomal preparation using a discontinuous sucrose gradient. However they are indicative of results that would be obtained from selected fractions of a continuous sucrose gradient. Organelle marker antibodies are: Arf1 (ADP-ribosylation factor 1, Agrisera AB, AS08 325); CRT1 (calreticulin, Abcam, ab2907); PsbA (D1 protein of PSII, Agrisera AB, AS01 016); VDAC1 (voltage-dependent anion-selective channel protein 1, Agrisera AB, AS07 212).

Fig. 3. Subcellular localization of (A) the Arabidopsis Golgi-localized nucleotide sugar transporter (URGT1-YFP) and (B) co-localization with the *cis*-Golgi marker α -Mannosidase I-mCherry construct using transient expression in *N. benthamiana*. (C) Overlay of the signal from the two constructs.

Figure 1

Figure 2

Figure 3