Proteomic characterization of Golgi membranes enriched from Arabidopsis suspension cell cultures

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Summary

The plant Golgi apparatus has a central role in the secretory pathway and is the principal site within the cell for the assembly and processing of macromolecules. The stacked membrane structures of the Golgi apparatus along with its interactions with the cytoskeleton and endoplasmic reticulum have historically made the isolation and purification of this organelle difficult. Density centrifugation has typically been used to enrich Golgi membranes from plant microsomal preparations, and aside from minor adaptations, the approach is still widely employed. Here we outline the enrichment of Golgi membranes from an Arabidopsis cell suspension culture that can be used to investigate the proteome of this organelle. We also provide a useful workflow for the examination of proteomic data as the result of multiple analyses. Finally, we highlight a simple technique to validate the subcellular localization of proteins by fluorescent tags after their identification by tandem mass spectrometry.

Key Words

Golgi apparatus, density gradient centrifugation, subcellular localization, fluorescent protein

1. Introduction

The Golgi apparatus is a unique and complex structure within the eukaryotic cell. The organelle is composed of flat membranes called cisternae that interlink to form stacks [1]. The plant Golgi apparatus is a major junction of the secretory system where proteins, lipids, carbohydrates are processed and biosynthesized prior to their distribution to all parts of the cell. This includes the synthesis of matrix polysaccharides destined for the cell wall [2,3], the production of complex *N*-glycans [4], the synthesis of glycolipids [5], sequestration of protein complexes [6], intracellular signalling [7] and trafficking of macromolecules [8].

The Golgi apparatus is an integral component of the plant endomembrane [9], and along with the endoplasmic reticulum (ER) is one of the more difficult structures to purify from plant tissue [10]. In fact it is this very proximity of the Golgi to the ER [11] and the intimate interactions with the cytoskeleton [12] that has historically made the enrichment and purification of this structure problematic. These difficulties in purifying Golgi bodies from plant material have resulted in the development of more advanced purification techniques such as LOPIT [13,14] and the application of free-flow electrophoresis [15-17]. Nonetheless, it is still suitable to enrich Golgi membranes from plant material for a variety of downstream applications, including proteomic surveys, using traditional density centrifugation procedures. In fact, the utilization of density centrifugation is still the most widely used approach for the enrichment of Golgi membranes from microbes [18], animals [19] and plants [20,21]. While Golgi membrane fractions isolated from plant material by density centrifugation alone are usually not of sufficient purity to conduct a thorough proteomic characterization of this organelle [22], the recent high purity surveys conducted on the *Arabidopsis* Golgi proteome [14,16] in conjunction with our functional knowledge of this

organelle means that a comparative or quantitative survey conducted on enriched material should still be considered a viable approach.

Here we outline a detailed protocol for the enrichment of Golgi membranes from an *Arabidopsis* suspension cell culture using a discontinuous sucrose gradient that would be suitable for proteomic investigations. A proteomic data analysis workflow is also provided to assist in the identification of high confidence candidates after mass spectrometry. Finally, an approach employing transient expression in *Nicotiana benthamiana* is outlined for the subcellular validation of candidate proteins by fluorescent tags.

2. Materials

Prepare solutions with ultrapure water (18 M Ω cm at 25°C) and analytical grade reagents. Utilize higher grade reagents (LC-MS grade) for solutions and buffers used in conjunction with mass spectrometry. Prepare reagents at room temperature. Unless otherwise stated, prepare buffers the day before and store at 4°C. Perform all centrifugation steps at 4°C.

2.1. Arabidopsis suspension cell cultures

1. Arabidopsis suspension cell cultures (*see Note 1*).
2. Temperature controlled shaking incubator (22°C, 120 rpm) with constant light (100 μ E).
3. Arabidopsis Cell Culture Medium: 2% (w/v) sucrose, α -naphthalenacetic acid (0.5 mg/L), kinetin (0.05 mg/L), 1 x Murashige and Skoog basal salt mixture [23]. Prepare media and adjust to pH 5.7 with potassium hydroxide (KOH), autoclave for 20 min at 121°C and store at 4°C (*see Note 2*).
4. 250 mL glass Erlenmeyer flasks.

2.2. Protoplast preparation of Arabidopsis suspension cell cultures

1. Digestion Buffer: 500 mM Mannitol, 5 mM 2-(N-morpholino) ethanesulfonic acid (MES), adjust to pH 5.7 with KOH. Store at 4°C. Just prior to use, add 0.4% (w/v) Cellulase “Onozuka” R-10 and 0.05% (w/v) pectolyase Y-23 (Yakult Pharmaceutical).
2. Protoplast Wash Buffer: 500 mM Mannitol, 5 mM 2-(N-morpholino) ethanesulfonic acid (MES), adjust to pH 5.7 with KOH. Store at 4°C.
3. Miracloth (Merck Millipore).
4. Variable speed bench top orbital shaker (*see Note 3*).
5. Preparative centrifuge for 250 mL tubes and capacity to $800 \times g$, such as an Avanti J25 centrifuge (Beckman Coulter) with a JLA-16.250 rotor (Beckman Coulter).

2.3. Protoplast homogenization

1. Homogenization Buffer: 1% (w/v) dextran (Mw 200,000), 0.4 M sucrose, 10 mM disodium hydrogen phosphate (Na_2HPO_4), 3 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (w/v) bovine serum albumin (BSA), 5 mM dithiothreitol (DTT) (*see Note 4*), pH to 7.1 with sodium hydroxide (NaOH).
2. Glass-Teflon Potter-Elvehjem Tissue Grinder with a 20 to 40 mL capacity (*see Note 5*).
3. Preparative centrifuge for 50 mL tubes and capacity to $5,000 \times g$, such as an Avanti J25 centrifuge (Beckman Coulter) with a JA-25.50 rotor (Beckman Coulter).
4. Light microscope capable of visualizing plant cells and large subcellular structures, minimum 40X objective.

2.4. Enrichment of Golgi membranes by differential centrifugation

1. Gradient Buffer 1: 1.4 M Sucrose, 10 mM Na₂HPO₄, 3 mM EDTA, pH 7.1 with NaOH, can store at -20°C.
2. Gradient Buffer 2: 1.0 M sucrose, 10 mM Na₂HPO₄, 3 mM EDTA, dextran Mw 200,000 (1% w/v), 5 mM DTT (*see Note 4*), pH 7.1 with NaOH, can store at -20°C.
3. Gradient Buffer 3: 0.2 M sucrose, 10 mM Na₂HPO₄, 3 mM EDTA, dextran Mw 200,000 (1% w/v), 5 mM DTT (*see Note 4*), pH 7.1 with NaOH, can store at -20°C.
4. Gradient Buffer 4: 0.1 M sucrose, 10 mM Na₂HPO₄, 3 mM EDTA, dextran Mw 200,000 (1% w/v), 5 mM DTT (*see Note 4*), pH 7.1 with NaOH, can store at -20°C.
5. Ultracentrifuge with swing-out rotor for 40 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).
6. Disposable plastic pipettes (1 mL).
7. 5 M KCl
8. 10 mM Tris-HCl, pH 8.5
9. Ultracentrifuge with fixed angle rotor for 2 mL tubes and capable of 100,000 × g, such as an Optima™ MAX-TL (Beckman Coulter) with a TLA-100 rotor (Beckman Coulter).
10. Protein Quantification Assay, such as Pierce™ BCA Protein Assay Kit.

2.5. Analysis of Golgi enriched membranes by mass spectrometry (LC-MS/MS)

1. Tandem mass spectrometer (MS/MS) with liquid chromatography (LC) delivery system capable of data dependent acquisition (DDA) / independent data acquisition (IDA) such as the Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer

- (Thermo Scientific) with a Proxeon Easy-nLC II HPLC (Thermo Scientific) (*see Note 6*).
2. Digestion Buffer: 1 M urea and 10 mM tris(hydroxymethyl)aminomethane (Tris-HCl), pH 8.5 solution (*see Note 7*).
 3. 1 M dithiothreitol (DTT) (*see Note 8*).
 4. 1 M iodoacetamide (IAA). (*see Note 9*).
 5. High grade trypsin, such as Trypsin, from Porcine pancreas (Sigma-Aldrich).
 6. Solid phase extraction (SPE) for peptides, such as Micro SpinColumns with C₁₈ (Harvard Apparatus, MA, USA).
 7. SPE Buffer 1: 80% acetonitrile (v/v) with 0.1% trifluoroacetic acid (v/v).
 8. SPE Buffer 2: 2% acetonitrile (v/v) with 0.1% trifluoroacetic acid (v/v).
 9. MS Buffer A: 2 % acetonitrile, 0.1 % formic acid.
 10. SpeedVac concentrator.
 11. Access to search engine to identify proteins from tandem mass spectrometry data, such as Mascot (Matrix Science) (*see Note 10*).
 12. Proteome integration, profiling and quantitation software, such as Scaffold 3 (Proteome Software) (*see Note 11*).

2.6 Validation of Golgi localization by fluorescent protein fusions

2.6.1 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 12*).

2.6.2 Plasmid preparation and *Agrobacterium tumefaciens* transformation

1. cDNA from plant material (*see Note 13*).
2. Thermocycler (PCR machine).
3. Gene specific primers (*see Note 14*).
4. DNA Polymerase *e.g.* Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific), dNTPs (*see Note 15*).
5. Agarose gel electrophoresis equipment.
6. Agarose *e.g.* UltraPure™ Agarose (Life Technologies).
7. 1 x TAE buffer *e.g.* TAE Buffer (Tris-acetate-EDTA) (50X) (Life Technologies).
8. DNA loading buffer *e.g.* Gel Loading Solution (Life Technologies).
9. DNA ladder *e.g.* 1 Kb Plus DNA Ladder (Life Technologies).
10. UV light box.
11. Gel and PCR clean-up kit *e.g.* NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel).
12. Plasmid preparation kit *e.g.* 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 *e.g.* pEarleyGate [24] (*see Note 16*).
16. Competent *Escherichia coli* (*E. coli*) *e.g.* One Shot® TOP10 Chemically Competent *E. coli* (Life Technologies).
17. Spectinomycin, kanamycin and gentamycin (Sigma-Aldrich).
18. Temperature mixer, such as the Thermomixer compact (Eppendorf).
19. Electro-competent *Agrobacterium* *e.g.* strain GV3101::pMP90 (*see Note 17*).

20. Disposable electroporation cuvettes with 1 or 2 mm gap sizes.
21. Electroporation system, such as a Gene Pulser Xcell™ Electroporation Systems (Bio-Rad).
22. Luria-Bertani (LB) media (10 g tryptone, 10 g NaCl, 5 g yeast extract, 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).

2.6.3 Infiltration

1. Luria-Bertani (LB) media (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. Preparative centrifuge, such as Allegra 25R Benchtop Centrifuge (Beckman Coulter).
4. Infiltration Buffer (10 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.6, 10 mM MgSO₄, 100 μM acetosyringone) (*see Note 18*).
5. 1 mL disposable polypropylene syringes.

2.6.4 Confocal Microscopy

1. Confocal Laser Scanning Microscope (*see Note 19*).
2. Coverslip holder, such as Attofluor® cell chamber for microscopy (Life Technologies).
3. 25 mm round glass coverslips.
4. 10 % glycerol.

3. Methods

3.1. Growth of Arabidopsis suspension cell cultures

1. The Arabidopsis suspension cell cultures are cultivated in 120 mL aliquots.
2. To maintain healthy cultures, the cells are subcultured weekly into a flask containing 100 mL of Arabidopsis Cell Culture Medium.
3. Cells are subcultured at a ratio of 1:5 to 1:10, depending on density of 7-day old cultures (*see Note 20*)
4. Typically, 15 to 20 mL from a 7-day culture are added to a new flask containing 100 mL growth media.

3.2. Preparation of protoplasts

1. Filter 7-day old cells from approximately 4 to 5 flasks through Miracloth and squeeze out the remaining culture medium to yield around 50 g of cells fresh weight.
2. Add 250 mL Digestion Buffer to a 500 mL beaker and then add 0.4% (w/v) cellulase and 0.05% (w/v) pectolyase and resuspend the enzymes (*see Note 21*). Add the 50 g of cells and gently mix, then gently transfer to a 4 L wide-bottomed conical flask (*see Note 22*)
3. Place on an orbital shaker and rotate slowly in the dark (wrapped in foil) for 3 h (*see Note 23*).
4. After 3 h, protoplasts are harvested by centrifugation at 800 x g for 5 min.
5. Gently resuspended the cell pellet in about 100 mL Digestion Buffer (no added enzymes), centrifuge at 800 x g and discard the buffer. Repeat this step two more times to ensure complete removal of cellulase and pectolyase from the protoplasts (*see Note 24*).

6. After the final wash, discard the buffer and place the protoplasts on ice.

3.3. Disruption of Arabidopsis protoplasts

1. Resuspend the pellet/protoplasts in Homogenization Buffer using a minimum ratio of 1:1 (w/v) and keep on ice. This volume is determined using the original fresh weight of the cells.
2. Transfer about 10 to 20 mL of the protoplast- buffer solution into the Potter-Elvehjem homogenizer.
3. Disrupt the protoplasts using 4 to 5 strokes at even pressure of the pestle in the Potter-Elvehjem homogenizer (*see Note 25*). Transfer disrupted protoplasts to a 250 mL beaker on ice.
4. Prior to processing / rupturing the rest of the protoplasts, check to ensure disruption was effective by examining homogenate with a light microscope.
5. If over 75 % of cells have been ruptured, process the rest of the protoplast homogenate, otherwise process the initial sample again with further strokes in the Potter-Elvehjem homogenizer.
6. Centrifuge the homogenate at 5000 x g for 15 min.
7. Carefully decant supernatant into a 250 mL beaker on ice.

3.4. Enrichment of Golgi membranes

1. Add 5 mL of Gradient Buffer 1 to two 40 mL ultracentrifuge tubes. With a disposable plastic pipette, gently layer the homogenate on to the Gradient Buffer 1 until all tubes are at least two thirds full (**Fig. 1**).
2. Ultracentrifuge homogenate at 100,000 x g for 1 h (*see Note 26*)

3. Remove the supernatant without disturbing the membrane rich lysate that has formed on the sucrose cushion (Gradient Buffer 1) (*see Note 27*).
4. Once the supernatant has been removed, carefully layer 15 to 20 mL of Gradient Buffer 2 on top of the membrane rich lysate. Follow this with 8 to 10 mL of Gradient Buffer 3 and then 5 to 8 mL of Gradient Buffer 4 (*see Note 28*).
5. Ultracentrifuge samples at 100,000 x g for 90 min.
6. After ultracentrifugation, remove tubes and inspect the banding pattern. Two distinct bands should be present above the 1.0 M Gradient Buffer (**Fig. 1**). Gently remove the upper band between the 0.1 M and 0.2 M Gradient Buffers with a disposable plastic pipette and discard. Then carefully remove the remaining upper band containing the enriched Golgi membranes situated on top of the 1.0 M Gradient Buffer and place in a 15 mL tube on ice (*see Note 29*).
7. Estimate the protein concentration of the extract using a Protein Quantification Assay and transfer the sample into 10 µg aliquots and place on ice.
8. Add 5M KCl to achieve an estimated final concentration of about 50 mM to each 10 µg aliquot of enriched Golgi membrane protein fractions and incubate for 15 min at 4°C, shake gently (*see Note 30*).
9. Ultracentrifuge samples at 100,000 x g for 60 min and discard the supernatant.
10. Wash the pellet twice with 1 mL 10 mM Tris-HCl pH 8.5.
11. At this stage, any 10 µg salt washed Golgi membrane aliquots not being processed for analysis by tandem mass spectrometry can be stored at -80°C.

3.5. Characterization of enriched Golgi fractions by tandem mass spectrometry

1. Resuspend the 10 µg salt washed Golgi membrane pellet in about 50 µL Digestion Buffer.

2. Add DTT to the diluted protein extract to a final concentration of 25 mM and incubate 30 min (room temperature).
3. Then add IAA to a final concentration of 50 mM and incubate 30 min (room temperature) in the dark.
4. Add trypsin at a 1:10 trypsin:protein ratio (*see Note 31*) and incubate overnight (37°C) (*see Note 32*).
5. Remove urea and concentrate samples with a Micro SpinColumn (25 to 75 µL capacity). Initially hydrate the C₁₈ matrix with ultrapure water (75 µL) for 10 min and centrifuge (1000 x g, 2 minutes) as per manufacturer's instructions.
6. Wash the SpinColumn with the 50 µL SPE Buffer 1 and centrifuge (1000 x g, 2 min) and prime twice with 50 µL SPE Buffer 2, centrifuging (1000 x g, 2 min) after each step.
7. Add digested peptides in urea solution to the SpinColumn and centrifuge (1000 x g, 2 min), wash twice with 50 µL SPE Buffer 2, centrifuging (1000 x g, 2 min) after each step.
8. Finally elute into a new tube with 25 to 50 µL SPE Buffer 1 by centrifuging 1000 x g for 2 min. Concentrate and remove acetonitrile with a SpeedVac concentrator until 1 to 5 µL of peptide solution remains in the tube.
9. Dilute around 4 µg of peptide in MS Buffer A to a volume of about 16 µL (*see Note 33*).
10. Analyze about 1 µg (4 µL) of the peptide sample by nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) using an automated data dependent acquisition method optimized for the analysis of complex proteomes (*see Note 34*).

11. Data produced after LC-MS/MS analysis can be interrogated with search algorithms, such as the software package Mascot (**Fig. 2A**) to identify proteins found in each sample (*see Note 35*).
12. The integration and relative quantitation of proteins identified in samples by LC-MS/MS can be achieved using software such as Scaffold, which can be used to merge multiple results from Mascot. The software enables further filters to be applied to both identified peptides and proteins (**Fig. 2B**), further ensuring that only high confidence protein matches are identified (*see Note 36*).
13. When analyzing well-characterized proteomes from systems like Arabidopsis, resources can be readily employed to analyze results. Useful approaches involve functional categorizations using Gene Ontologies [25] or the subcellular distribution of a proteome using resources like the SUBcellular Arabidopsis database [26] (**Fig. 3**).

3.5. Transient Protein Expression for Subcellular Localization

To validate proteins identified in enriched Golgi membrane preparations it is possible to easily verify their location to this organelle using the following procedure.

3.5.1. Cultivation of *Nicotiana benthamiana*

1. Plastic pots of around 80 x 80 mm containing high quality soil, such as PRO-MIX HP MYCORRHIZAE™ should be thoroughly watered and then drained.
2. About 30 seeds of *N. benthamiana* ‘Domin’ are spread onto the moist soil, and pots put onto a plastic tray and covered with a transparent plastic lid.
3. Place the trays in a 25°C chamber with 60 % humidity and 16 hr light / 8 hr dark cycle.

4. After about a week, remove the plastic lid and propagate the plants under the same growth conditions for another week.
5. Transfer the two-week old seedlings into fresh pots (as previously described).
Transplant one seedling per pot (*see Note 37*) and continue to propagate plants under identical growth conditions until ready to be infiltrated with *Agrobacterium* (*see Note 38*).

3.5.2. Plasmid preparation and transformation of *Agrobacterium*

1. Amplify the gene of interest by PCR employing gene specific primers and appropriate plant cDNA (*see Note 39*).
2. Analyse the resultant PCR product using a 1 % agarose gel with 1 x TAE buffer.
3. Visualize the PCR product using a UV light box and confirm the expected size.
4. Excise the DNA band and extract the DNA from the agarose gel using a Gel clean-up kit.
5. Clone the purified PCR product with the pCR®8/GW/TOPO® TA Cloning Kit into the pCR®8 donor vector to create the entry clone.
6. Transform competent *E.coli* with the cloning reaction and select for positive transformant on LB plates with 100 µg/mL spectinomycin.
7. Purify the plasmid DNA from an overnight *E.coli* culture (LB with 100 µg/mL spectinomycin) using a Plasmid preparation kit and confirm the identity of the entry clone by sequencing.
8. Use the confirmed entry clone in a LR Clonase II Enzyme reaction and clone it into a destination vector (*e.g.* pEarleyGate) containing an in-frame fluorescent protein.
9. Transform this reaction into *E.coli* and select on LB plate containing kanamycin (50 µg/mL). Using a sterile toothpick, transfer a colony to 5 mL LB with kanamycin (50

µg/mL) and grow at 37°C overnight with shaking. Purify the plasmid using a plasmid preparation kit and confirm the integrity of the clone by sequencing.

10. Add 1 µL of the expression vector (*e.g.* pEarleyGate vector containing gene of interest) to competent *Agrobacterium* and sit on ice for 15 min (*see Note 40*).
11. Set up the electroporation device according to the manufacturer's instructions (*see Note 41*), transfer mixture to pre-chilled cuvette and apply voltage to transform *Agrobacterium*.
12. Immediately after the electroporation add 1 mL of LB media to the cuvette (*see Note 42*) and mix by gently pipetting.
13. Transfer the contents of the cuvette into a sterile 1.5 mL microfuge tube and incubate for at least 1 hr at 30°C with constant agitation.
14. Pipette 25 to 50 µL (*see Note 43*) onto LB plates containing appropriate selective antibiotics (*see Note 44*) and incubate for 48 hrs at 30°C until colonies are visible.

3.5.3. Infiltration of *N. benthamiana*

1. Inoculate 10 mL LB media (containing the appropriate selective antibiotics) with an *Agrobacterium* colony and grow overnight at 30°C with constant shaking to log phase.
2. Centrifuge the overnight culture for 10 min at 4000 x g at 20°C.
3. Remove the supernatant, wash the pellet with 10 mL of Infiltration Buffer (*see Note 45*) 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 46*).
5. Infiltrate the abaxial surface of leaves from three to four week old *N. benthamiana* plants using a 1 mL plastic syringe without a needle (*see Note 47*). Hold the adaxial

side of the leaf firmly and press the syringe from abaxial side against your finger.

Infiltrated areas will turn a darker green (*see Note 48*).

6. Grow infiltrated plants for an additional two days before examining expression of the fluorescent protein by confocal microscopy (*see Note 49*).

3.5.4. Confocal microscopy

1. Use a sterile razor blade to cut a 12 x 12 mm piece from the infiltrated area of the leaf (*see Note 50*).
2. Place the leaf section onto a coverslip and add a drop of 10% glycerol on its surface.
3. Put a second coverslip on top and mount into a coverslip holder.
4. Place the slide onto the confocal stage and focus using brightfield at low magnification.
5. At this magnification use an appropriate filter or setting, depending on the fluorescent protein employed, to find cells exhibiting 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 51*).
7. Perform a live scan using the confocal. In our example, we employed a simultaneous acquisition for the yellow fluorescent protein (YFP) and mCherry after co-infiltrating a vector containing UXT3-YFP and the Golgi marker, α -Mannosidase I-mCherry (**Fig. 4**).
8. Images can be processed with a microscope software such as ImageJ [27].

5. Notes

1. The Arabidopsis suspension cell cultures outlined here were originally created over 20 years ago from stem explants of the accession *Landsberg erecta* [28]. While any suspension cell culture should produce similar results, in our hands, this particular suspension culture contains a population of Golgi membranes which are minimally interconnected with the cytoskeleton. This results in enriched Golgi membranes with reduced contamination when compared to Arabidopsis seedlings or recently created cell lines. This particular cell line has been distributed globally and can be sourced through diligent inquiries.
2. Prior to autoclaving, media can be dispensed into 250 mL glass Erlenmeyer flasks in 100 mL aliquots and sealed with foil until required. Large batches can be pre-made (50 to 100) and stored at 4°C in the dark.
3. A variable speed bench top orbital shaker with a relatively large orbital throw is optimal (at least 2 cm). This enables a slow rotation while maintaining the cells in solution and providing efficient enzymatic digestion of the cell wall.
4. The DTT should be added immediately prior to use. A stock solution of DTT (1 M) can be stored in aliquots at -20°C if required.
5. The performance of the homogenizer is very important to gently rupture the protoplasts. Generally, negative pressure on the up-stroke should result in an air bubble between the Teflon pestle and the homogenate. This seems to be indicative of adequate restriction between the Teflon pestle and the glass mortar which constricts the protoplasts as they pass, resulting in their disruption. The performance of the homogenizer (generation of the air bubble) can be tested with water.
6. While access to high resolution tandem mass spectrometry has become somewhat common, they are expensive instruments to purchase and maintain. As a consequence

many institutions provide access via dedicated facilities. While these facilities can seem like an expensive option, current pricing makes them highly competitive when analysing up to 50 samples per year.

7. Urea is unstable and can degrade in solution when exposed to elevated temperatures ($> 25^{\circ}\text{C}$) or over time. This degradation results in the generation of isocyanic acid which subsequently reacts with the amino terminus of proteins as well as the sidechains of lysine and arginine residues. Consequently, it is recommended that urea solutions be made fresh as required. A 2 mL urea / Tris-HCl solution is relatively simple to make and usually adequate for most purposes.
8. A stock solution of DTT can be stored at -20°C in aliquots and thawed as required.
9. A stock solution of IAA can be stored at -20°C in aliquots and thawed as required.

IAA is used to alkylate the thiol group on cysteine residues after reduction with DTT. It is virtually impossible to detect cysteine containing peptides unless controlled alkylation is undertaken. However, given the low occurrence of cysteine residues in proteins, the absence of this step has little impact on the final results, namely number of proteins identified and sequence coverage. IAA is light and heat sensitive and should be stored in the dark or wrapped in foil.
10. Suppliers of most tandem mass spectrometers designed for standard proteomic workflows will usually supply software with their instruments that can be used for the interrogation of tandem spectra for the identification of proteins. There are also a number of open source software packages available as well as a range of third party software that can also be employed to identify proteins from fragmentation spectra. In general, these algorithms are relatively similar, we generally use Mascot (a third party software package from Matrix Science) and have found that it provides quality results when high confidence cut-offs are employed. Access to the software is free if

submitting less than 1200 spectra in a single submission

(<http://www.matrixscience.com/>).

11. A considerable data handling problem when dealing with results from multiple analyses of tandem spectra is data integration. While some software platforms have developed workflows to integrate multiple samples, Scaffold from Proteome Software (<http://www.proteomesoftware.com/>) provides a powerful way to combine and compare protein identifications from multiple samples. The software provides some quantitation support via spectral counting to enable limited comparative assessment between samples.
12. A growth chamber capable of maintaining 25°C and 60 % humidity with a day/night cycle is optimal. However, plants can also be grown in a glasshouse with some temperature control.
13. cDNA can be obtained from the plant tissue of interest after RNA extraction using a plant RNA extraction kit, such as the RNeasy Plant Mini Kit (Qiagen). The mRNA is then reverse transcribed into cDNA using a Reverse Transcriptase, such as Superscript III Reverse Transcriptase (Life Technologies).
14. Ensure the reverse primer does not contain a stop codon to enable a C-terminal fusion with the fluorescent protein.
15. To amplify a gene of interest and avoid errors, a proofreading DNA polymerase should be used.
16. The pEarleyGate vector collection generally works well, however many other vectors are available that contain strong promoters and fluorescent tags.
17. *Agrobacterium tumefaciens* strain GV3101::pMP90 is a commonly used and works well when transforming *N. benthamiana* and can also be used to stably transform

Arabidopsis thaliana. However other *Agrobacterium* strains are available that also work in *N. benthamiana*, including C58C1, EHA105, LBA4404, and AGL1.

18. Prepare the Infiltration Buffer fresh for each infiltration. Stock solutions of 0.5 M MES-KOH pH 5.5 and 1 M MgSO₄ can be prepared in advance but filter sterilization is recommended. For the 0.5 M MES stock solution, prepare MES first and adjust the pH to 5.5 with KOH. Acetosyringone should be added separately to the Infiltration Buffer on the day of use. Prepare a 100 mM acetosyringone stock solution in either DMSO or 96% (v/v) ethanol, and store in aliquots at -20 °C or for shorter periods at 4°C. Do not autoclave the Infiltration Buffer containing acetosyringone.
19. The Confocal Laser Scanning Microscope (CLSM) must have the capability to excite at the appropriate wavelengths. For example for the pEarleyGate 101 vector 514 nm for yellow fluorescent protein (YFP) is necessary.
20. After 7 days, a 120 mL culture should produce around 8 to 10 g of cells, fresh weight. The amount of biomass should be checked regularly and the volume of cells (usually 10 to 20 mL) used to subculture into a new flask adjusted accordingly.
21. Enzymes should be added to the Digestion Buffer just prior to use. Enzymes are easily solubilized by vigorous shaking in a 50 mL aliquot of Digestion Buffer.
22. Maintain a cell to Digestion Buffer ratio of around 1:5 (w/v) for the digestion of cells. The amount of enzyme may need to be optimized if different plant material / cell cultures are employed. This combination and ratio of enzymes seems to be optimal for primary cell walls.
23. Rotate at the cells in the Digestion Buffer at the lowest possible speed; however the cells must remain suspended.
24. The pellet comprising intact protoplasts is delicate and thus the supernatant should be carefully removed to avoid breaking of the protoplasts.

25. For the first few attempts at protoplast disruption, check the results using a light microscope with a 40 x objective. This should reveal rupturing of at least 75% of the protoplasts. The key to homogenization is ensuring enough mechanical stress to disrupt the protoplasts without destroying subcellular integrity. The number of strokes of the homogenizer should be calibrated using light microscopy.
26. This step should result in a yellow band sitting on the cushion (Gradient Buffer 1) of about 1 to 2 mm thick.
27. The removal of the supernatant to the top of the yellow membrane band will be a compromise between the quality of the resultant gradient and the disturbance of the cushion. Attempts to remove too much supernatant will likely result in a reduction in yield.
28. For the successful enrichment of Golgi membranes from other contaminating membranes on the cushion, such as mitochondria, it is not recommended to use a sucrose concentration less than about 1.0 M in Gradient Buffer 2.
29. The membrane band (discarded) found between Gradient Buffer 4 (0.1 M) and Gradient Buffer 3 (0.2 M) should contain less material and be visibly thinner than the band representing the enriched Golgi membranes directly above the 1.0 M Gradient Buffer.
30. The incubation with KCl is used to remove electrostatic interactions of non-specific peripheral proteins from the enriched Golgi membrane preparations.
31. The recommended ratio of trypsin to protein (w/w) is generally 1:20 or 50, however this will depend on the type of trypsin being utilized. If using modified trypsin (not subject to autolysis), then recommended ratios can be used, however if unmodified trypsin is used, we recommend using it at higher ratios. The advantage of unmodified

trypsin, is that autolytic products (peptides) can act as internal controls for sample handling and mass spectrometry.

32. Overnight digestion is likely excessive, however the timing usually suits the standard sample processing workflow.
33. These volumes and concentrations are dependent on the liquid chromatography method being employed.
34. The analysis of a 1 µg complex peptide lysate by nanoflow LC-MS/MS should result in the high confidence identification of between 1000 and 2000 proteins.
35. To ensure only high confidence proteins and peptides are identified from fragmentation spectra, employ recommended protein and peptide cut-offs to ensure spectral match probabilities are < 0.05 or false discovery rates are $< 1\%$. If using Mascot, an ions score value is provided for peptide matches and needs to be entered manually in the “Ions score or expect cut-off” box. Using such an approach, only high confidence peptide matches are used to define the resultant identified proteins. This means that even proteins identified by a single peptide can be regarded as high confidence matches, although a replicate experiment will be required to independently identify and confirm its existence in the sample.
36. There are further settings or views in Scaffold that can result in a final set of identified proteins that would not be considered high confidence even with the outlined settings (**Fig. 2B**). This includes the following settings in the ‘View’ menu: ‘Show Entire Protein Clusters’ and ‘Show Lower Scoring Matches’. If requiring high confidence identifications, both should be unchecked.
37. Space the pots within the tray to ensure plants do not crowd each other as they mature.

38. Plants around three to four weeks after germination with a rosette diameter of approximately 80 to 100 mm seem to be the most suitable for infiltration.
39. Ensure the appropriate plant tissue in which the gene is most highly expressed is used. 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. Competent *Agrobacterium* can be prepared in large batches and store 50 μ L aliquots at -80°C until required. About 1 μ L (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.
41. Make sure that your settings are adjusted for the gap size of the cuvette being employed and that the setting is appropriate for *Agrobacterium*.
42. It is essential to promptly add the LB-media to the mixture to avoid a decrease in transformation efficiency.
43. The volume largely depends on the efficiency rate of the competent *Agrobacterium*. Usually 50 μ L is sufficient to produce enough colonies.
44. Ensure correct antibiotics are used for selection. Generally antibiotics for both the *Agrobacterium* host and plasmid are necessary. The *Agrobacterium* GV3101::pMP90 is resistant to gentamycin and rifampicin and the pEarleyGate vector series confer kanamycin resistance.
45. Some protocols do not include a wash step; however we have found that this improves infiltration and protein expression. This could be due to the effect of antibiotics in the culture media.
46. The OD required depends on the expression of the protein of interest. Generally we have found that a lower OD (0.01 to 0.15) gives a better result and minimizes protein

aggregates. However low expressed proteins may require higher ODs. When co-infiltrating multiple constructs, for example, a gene of interest and an organelle marker protein, mix a combination of the two *Agrobacterium* solutions to achieve the final OD.

47. Start with the top leaves as they have less vascular bundles compared to older leaves.

We have found that slightly piercing the leaf by holding the syringe at a 45° angle while holding the leaf upright can assist infiltration.

48. It is important to use as little pressure as possible while injecting the *Agrobacterium* mixture to avoid punching holes and wounding the leaf.

49. Since not all proteins express with the same efficiency, expression should be monitored at various time points. Generally we monitor expression from 48 to 72 hours.

50. Make sure the infiltrated area is chosen and avoid cutting too close to the region where the syringe was applied as this region will be wounded and will result in a high auto-fluorescence signal.

51. Most 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. This can be useful when examining the Golgi apparatus as they tend to move around in the cell. While an emission range can be narrowed to reduce crosstalk when using simultaneous scanning, it will reduce the signal intensity. Sequential scanning is often essential to ensure the signal from both fluorophores is valid, but often fixation is required to ensure overlap. Such an approach is important if using fluorophores with similar emission wavelengths such as YFP and green fluorescent protein (GFP).

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5. References

1. Morré DJ, Mollenhauer HH (2009) The Golgi apparatus: The first 100 years. Springer, New York, USA
2. Scheller HV, Ulvskov P (2010) Hemicelluloses. *Annu Rev Plant Biol* 61:263-289
3. Harholt J, Suttangkakul A, Scheller HV (2010) Biosynthesis of pectin. *Plant Physiol* 153:384-395
4. Song W, Henquet MGL, Mentink RA et al. (2011) *N*-glycoproteomics in plants: Perspectives and challenges. *J Proteomics* 74:1463-1474
5. Rennie EA, Ebert B, Miles GP et al. (2014) Identification of a sphingolipid alpha-glucuronosyltransferase that is essential for pollen function in Arabidopsis. *Plant Cell* 26:3314-3325
6. Wightman R, Turner S (2010) Trafficking of the Pplant cellulose synthase complex. *Plant Physiol* 153:427-432
7. Ordenes VR, Moreno I, Maturana D et al. (2012) *In vivo* analysis of the calcium signature in the plant Golgi apparatus reveals unique dynamics. *Cell Calcium* 52:397-404
8. McFarlane HE, Watanabe Y, Yang WL et al. (2014) Golgi- and trans-Golgi network-mediated vesicle trafficking is required for wax secretion from epidermal cells. *Plant Physiol* 164:1250-1260
9. Morré DJ, Mollenhauer HH (1974) The endomembrane concept: a functional integration of endoplasmic reticulum and Golgi apparatus. In: Robards AW (ed) *Dynamic Aspects of Plant infrastructure*. McGraw-Hill, New York, USA, pp 84-137.
10. Morré DJ, Mollenhauer HH (1964) Isolation of Golgi apparatus from plant cells. *J Cell Biol* 23:295-305
11. Boevink P, Oparka K, Cruz SS et al. (1998) Stacks on tracks: the plant Golgi apparatus traffics on an actin/ER network. *Plant J* 15:441-447

12. Akkerman M, Overdijk EJR, Schel JHN et al. (2011) Golgi body motility in the plant cell cortex correlates with actin cytoskeleton organization. *Plant Cell Physiol* 52:1844-1855
13. Dunkley TPJ, Watson R, Griffin JL et al. (2004) Localization of organelle proteins by isotope tagging (LOPIT). *Mol Cell Proteomics* 3:1128-1134
14. Nikolovski N, Rubtsov D, Segura MP et al. (2012) Putative glycosyltransferases and other plant Golgi apparatus proteins are revealed by LOPIT proteomics. *Plant Physiol* 160:1037-1051
15. Parsons HT, Weinberg CS, Macdonald LJ et al. (2013) Golgi enrichment and proteomic analysis of developing *Pinus radiata* xylem by free-flow electrophoresis. *PLOS ONE* 8:e84669
16. Parsons HT, Christiansen K, Knierim B et al. (2012) Isolation and proteomic characterization of the Arabidopsis Golgi defines functional and novel targets involved in plant cell wall biosynthesis. *Plant Physiol* 159:12-26
17. Parsons HT, González Fernández-Niño SM, Heazlewood JL (2014) Separation of the plant Golgi apparatus and endoplasmic reticulum by free-flow electrophoresis. In: Jorrín Novo JV, Komatsu S, Weckwerth W, Weinkoop S (eds) *Plant Proteomics: Methods and Protocols*, Second Edition, vol 1072. Humana Press, New York, pp 527-539.
18. Forsmark A, Rossi G, Wadskog I et al. (2011) Quantitative proteomics of yeast post-Golgi vesicles reveals a discriminating role for Sro7p in protein secretion. *Traffic* 12:740-753
19. Gilchrist A, Au CE, Hiding J et al. (2006) Quantitative proteomics analysis of the secretory pathway. *Cell* 127:1265-1281
20. Mast S, Peng L, Jordan TW et al. (2010) Proteomic analysis of membrane preparations from developing *Pinus radiata* compression wood. *Tree Physiology* 30:1456-1468

21. Zeng W, Jiang N, Nadella R et al. (2010) A glucurono(arabino)xylan synthase complex from wheat contains members of the GT43, GT47, and GT75 families and functions cooperatively. *Plant Physiol* 154:78-97
22. Nikolovski N, Shliaha PV, Gatto L et al. (2014) Label-free protein quantification for plant Golgi protein localization and abundance. *Plant Physiol* 166:1033-1043
23. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plantarum* 15:473-497
24. Earley KW, Haag JR, Pontes O et al. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J* 45:616-629
25. Gene Ontology Consortium (2004) The Gene Ontology (GO) database and informatics resource. *Nucleic Acids Res* 32:D258-D261
26. Heazlewood JL, Verboom RE, Tonti-Filippini J et al. (2007) SUBA: The Arabidopsis subcellular database. *Nucleic Acids Res* 35:D213-D218
27. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671-675
28. May MJ, Leaver CJ (1993) Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. *Plant Physiol* 103:621-627
29. Tanz SK, Castleden I, Hooper CM et al. (2013) SUBA3: a database for integrating experimentation and prediction to define the SUBcellular location of proteins in Arabidopsis. *Nucleic Acids Res* 41:D1185-1191
30. Zybaylov B, Mosley AL, Sardu ME et al. (2006) Statistical analysis of membrane proteome expression changes in *Saccharomyces cerevisiae*. *J Proteome Res* 5:2339-2347
31. Ebert B, Rautengarten C, Guo X et al. (2015) Identification and characterization of a Golgi-localized UDP-xylose transporter family from Arabidopsis. *Plant Cell* 27:1218-1227

Figure Captions

Fig. 1. Flowchart outlining the procedure for the enrichment of Golgi membranes from *Arabidopsis* suspension cell cultures using a discontinuous gradient.

Fig. 2. Recommended protein and peptide filters for the analysis of mass spectrometry data derived from the analysis of *Arabidopsis* samples **(A)** Mascot (Matrix Science) and **(B)** Scaffold (Proteome Software).

Fig. 3. Subcellular breakdown of a typical dataset resulting from a proteomic analysis of enriched Golgi membranes from the *Arabidopsis* suspension cell culture. The analysis outlines proteins identified from two independent enrichment experiments using the outlined enrichment and data workflow. In total, 1,102 proteins were identified after both samples were combined using Scaffold. The subcellular locations were obtained from the SUBcellular *Arabidopsis* (SUBA) database [29] and distributions are shown according to the total normalized spectral abundance factor (NSAF) [30], obtained from Scaffold, for proteins allocated to each compartment.

Fig. 4. The transient subcellular localization of the *cis*-Golgi marker α -Mannosidase I-mCherry (α ManI-mCherry) and a resident Golgi protein (UXT3-YFP) in *N. benthamiana* leaves [31] with an overlay (merge) indicating co-localization. Bar = 25 μ m.

Figure 1

A

Peptide Summary Report

Format As	Peptide Summary ▼		Help					
	Significance threshold p<	0.05	Max. number of hits	AUTO				
	Standard scoring	☐	MudPIT scoring	☒	Ions score or expect cut-off	32	Show sub-sets	0
	Show pop-ups	☒	Suppress pop-ups	☐	Sort unassigned	Decreasing Score ▼	Require bold red	☒
	Preferred taxonomy	All entries ▼						

B

Protein Threshold:	99.0% ▼	Min # Peptides:	1 ▼	Peptide Threshold:	95% ▼
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Figure 2

Figure 3

Figure 4