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ISOLATION AND PURITY ASSESSMENT OF MEMBRANES FROM NORWAY SPRUCE

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Abstract

Gaining membrane vesicles from different plant species and tissue types is crucial for membrane studies. Membrane vesicles can be used for further purification of individual membrane types, and, for example, in studies of membrane enzyme activities, transport assays, and in proteomic analysis. Membrane isolation from some species, such as conifers, has proved to be more difficult than that of angiosperm species. In this paper, we describe steps for isolating cellular membranes from developing xylem, phloem and lignin-forming tissue-cultured cells of Norway spruce, followed by partial enrichment of plasma membranes by aqueous polymer two-phase partitioning and purity analyses. The methods used are partially similar to the ones used for mono- and dicotyledonous plants, but some steps require discreet optimization, probably due to a high content of phenolic compounds present in the tissues and cultured cells of Norway spruce.

Key Words Aqueous Polymer Two-Phase Partitioning, Developing Xylem, Lignin-Forming Tissue Culture, Membrane Vesicles, Norway Spruce, Phenol-Binding Agent, Phloem, Plasma Membrane, Purity Assessment

Abbreviations

BSA	bovine serum albumin
DTT	dithiothreitol
PEG	polyethylene glycol
PVPP	insoluble polyvinylpolypyrrolidone
PVP	polyvinylpyrrolidone
SB	suspension and storage buffer
UDP-Glc	UDP-glucose

1. Introduction

Cellular organization and function depend on compartmentalization of biochemical reactions by membranes. Each membrane has a characteristic composition of proteins and lipids that alter in dependence on plant development and external factors. The outer cellular membrane (plasma membrane) is involved in ion transport, signal transduction, cell wall synthesis and other functions. Gaining membrane vesicles from different plant species and tissue types is crucial for membrane studies. Crude membrane vesicles can be used

for example, for further purification of individual membrane types (*1*), and in studies of membrane enzyme activities and transport assays (*2, 3*) and in proteomic analysis (*4*). At present, the most commonly used method for plasma membrane isolation from plant materials is partitioning in an aqueous dextran / polyethylene glycol (PEG) two-phase system (*5, 6*). By this method, membrane vesicles are separated according to their surface charge rather than according to their size and density. Right-side-out (apoplastic side out) plasma membranes preferentially migrate into the PEG-rich upper phase, whereas inside-out plasma membrane vesicles and intracellular membranes partition at the interface and into the dextran-rich lower phase. The method is relatively rapid and, in case of angiosperm species, gives plasma membrane vesicles of high purity (~90-95%; *5, 6*). Membrane isolation from conifers, however, is more demanding than from angiosperms (*7*), and extra modifications and further optimization are required to gain any membranes for further studies (*7, 8*). The plasma membranes obtained by aqueous polymer two-phase partitioning from developing xylem and lignin-forming tissue-cultured cells (*9*) of Norway spruce (*Picea abies* Karst.) were partially enriched, since vacuolar membranes co-migrated with plasma membranes into the upper phase, whereas other intracellular membranes were preferentially distributed into the lower phase (*8*).

The optimum polymer concentration of the two phase systems differs depending on plant species and the tissues used (*4, 5*). The reason for this is related to hydrophobic / hydrophilic properties of the membrane surfaces. The concentration of dextran and PEG needs to be optimized for every material.

In this paper, we present protocol modifications that are essential to obtain cellular membranes from Norway spruce tissues and cells rich in phenolic compounds. The type of a phenol-binding agent in the homogenization buffer has a clear effect on the yield of cellular membranes from lignin-forming tissues (*8*). Without addition of soluble polyvinylpyrrolidone (PVP) K-30 we were unable to get any cellular membranes from tissue-cultured spruce cells and phloem tissues, whereas insoluble polyvinylpolypyrrolidone (PVPP, Polyclar AT) was the preferred phenol-binding agent with developing xylem. We also present methods to enrich plasma membranes using aqueous polymer two-phase partitioning. The distribution of the obtained membranes is determined with specific antibodies against marker proteins of several membrane types in western blots. Also enzyme activity assays for marker enzymes of each membrane type are described, as well as chlorophyll *a* measurement to detect thylakoid membranes. The methods used are partially similar to those for mono- and dicotyledonous plants, but some steps require optimization, probably due to the high content of phenolic compounds present in developing xylem, phloem, and lignin-forming tissue-cultured cells of Norway spruce.

2. Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Store the reagents at +4 °C (unless indicated otherwise).

2.1. Cell extraction

1. 50 mM EDTA, pH 7.5. Adjust pH of EDTA solution to 7.5 with NaOH.

2. Homogenization buffer: 50 mM MOPS-KOH, pH 7.5, (s*ee* **Note 1**), 5 mM EDTA, 500 mM sucrose and freshly-added 5 mM dithiothreitol (DTT), 5 mM ascorbic acid, 4 mM cysteine and protease inhibitors.

3. Phenol-binding agent: 1.5% (w/v) polyvinylpyrrolidone (PVP) K-30 or 1.5% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). Make a pre-test to assay which one is better for your material (*see* Sub-chapter 3.2.1). Dissolve PVP K-30 in the homogenization buffer (*see* **Note 2**). If you use tissue powder ground in liquid nitrogen, add PVPP directly with the sample to the extraction mixture.

4. Basic mill.

5. Nylon cloth for filtering.

6. Centrifuge (+4 °C).

2.2. Aqueous polymer two-phase partitioning components

1. 20% (w/w) Dextran 500. Since dextran is very hygroscopic, dissolve all powder when opening a new lot. Aliquot and store at -20 °C (*see* **Note 3**).

2. 40% (w/w) polyethylene glycol (PEG) 3350 . Aliquot and store at -20 °C (*see* **Note 3**).

3. 0.2 M K-phosphate buffer, pH 7.8. Prepare 0.2 M *ortho*-phosphoric acid, adjust pH with KOH to 7.8. Alternatively, prepare 0.2 M K_2HPO_4 and 0.2 M KH_2PO_4 solutions, and adjust pH of the K_2HPO_4 solution to 7.8 with the KH_2PO_4 solution. Store at room temperature.

4. 0.2 M KCl.

5. K-phosphate-sucrose buffer: 5 mM K-phosphate buffer, pH 7.8, supplemented with 330 mM sucrose and 3 mM KCl. Dilute from 0.2 M buffer, and add sucrose and KCl. Check pH, fill to the final volume, and store at -20 °C in 50 ml tubes.

6. Suspension and storage buffer (SB): 10 mM MOPS-KOH, pH 7.4, supplemented with 330 mM sucrose.

7. 500 mM DTT, freshly-made in 50 mM EDTA.

8. Centrifuge with a swinging bucket rotor (+4 °C).

9. Ultracentrifuge (+4 °C).

10. Glass homogenizer

2.3. Membrane washing

1. Washing buffer: 10 mM MOPS-KOH buffer, pH 7.5, supplemented with 150 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA and 0.01% (w/v) Triton X-100 (*see* **Note 4**). Aliquot in 50 ml portions and store at -20 °C.

2.4. Protein quantitation

1. 10 mM MOPS-KOH, pH 7.4, supplemented with 330 mM sucrose and 0.01% (w/v) Triton X-100 (*see* **Note 4**).

2. Make a stock solution (1 mg/ml) of bovine serum albumin (BSA). Dilute BSA standard solutions (2, 5, 10, 15, 20, 25 µg/ml) in SB (use serial dilutions). Store in aliquots at -20 °C.

3. Bradford protein assay (*10*).

4. Multichannel pipette.

5. Microtiter plate reader.

2.5. SDS-PAGE gel electrophoresis

1. 10% (w/v) SDS stock solution.

2. Membrane protein solubilization and loading buffer (4x): 100 mM Tris–HCl, pH 6.8, supplemented with 20% (v/v) glycerol, 0.008% (w/v) bromophenol blue, and 100 mM DTT (freshly added). While dissolving membrane proteins, add SDS to final concentration of 4% (*see* Sub-chapter 5.1.).

3. Ready-made SDS-PAGE gels.

4. Running buffer (10x): 250 mM Tris base, 1.9 M glycine, 1% (w/v) SDS. For run, dilute to 1x with water.

5. SDS-PAGE apparatus.

2.6. Immunoblotting

1. Transfer buffer (Towbin): 25 mM Tris base, 200 mM glycine, 20% (v/v) methanol (*see* **Note 5**).

2. tTBS buffer: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% (w/v) Tween 20. Dilute from 10x TBS stock solution, and add Tween 20. Prepare 1 litre per gel.

3. Nitrocellulose membrane.

4. Blocking buffer: 5% (w/v) nonfat milk powder in tTBS. Prepare 50 ml per gel and mix in a magnetic stirrer for 30 minutes. Prepare fresh every time.

5. Antibody buffer: 2.5% (w/v) nonfat milk powder in tTBS. Prepare fresh every time.

6. Primary antibodies (rabbit polyclonal serum; Agrisera, Vännäs, Sweden) against 1) H^+-ATPase (AS07 260), a marker for plasma membrane; 2) cytochrome *c* oxidase subunit II (COX II; AS04 053A) for inner mitochondrial membrane, 3) vacuolar H^+-pyrophosphatase (V-PPase, AS12 1849) for tonoplast, and 4) binding immunoglobulin protein (BiP; AS09 481) for endoplasmic reticulum (ER) (*see* **Note 6**).

7. Secondary antibody goat anti-rabbit IgG (H&L) horseradish peroxidase conjugate.

8. ECL Western Blotting Detection reagent.

9. Hyperfilm ECL.

10. Immunoblotting apparatus.

11. Film developer, or solutions and tools to develop films manually.

2.7. Chlorophyll *a* measurement

1. 100% acetone

2. 80% (v/v) acetone supplemented with 0.01% (w/v) $MgCO_3$. Magnesium protects the central atom of chlorophyll.

3. Glass cyvettes (semimicro; *see* **Note 7**).

4. Spectrophotometer.

2.8. Enzyme assays

2.8.1. Glucan synthase II (a plasma membrane marker)

1. 50 mM MOPS-KOH, pH 7.2, supplemented with 330 mM sucrose. Aliquot and store at -20 °C.

2. Buffer mixture: Add 5 mM spermine, 50 mM cellobiose and 0.5 mM $CaCl_2$ to 50 mM MOPS-KOH, pH 7.2, supplemented with 330 mM sucrose. Aliquot (e.g. 1 ml aliquots), and store at -20 °C. Thaw the volume you need for the assay, and add DTT (5 mM) just before use.

3. 0.1% (w/v) Digitonin in 50 mM MOPS-KOH, pH 7.2, supplemented with 330 mM sucrose. This is a 10x stock solution, final concentration of digitonin will be 0.01% (w/v).

4. 10 mM UDP-glucose (UDP-Glc) in 50 mM MOPS-KOH, pH 7.2, supplemented with 330 mM sucrose. This is a 5x stock solution, final concentration will be 2 mM.

5. [^{14}C]UDP-Glc . Use 0.5 kBq / reaction.

6. Formic acid (98%).

7. 0.5 M ammonium acetate, pH 3.6. Adjust pH of 0.5 M acetic acid solution to 3.6 with ammonia (in the fume hood). Just before use, mix ammonium acetate with ethanol in a mixing ratio 7:3 (v/v).

8. Cellulose filter discs, 25 mm diameter.

9. Glass fiber discs, 25 mm diameter.

10. Scintillation liquid.

11. Scintillation vials (20 ml).

12. Water bath at +25 °C.

13. Scintillation counter.

2.8.2. Latent uridine diphosphatase (UDPase, a Golgi marker)

1. 34.6 mM MES-KOH, pH 6.5, supplemented with 3.46 mM $MgSO_4$ and 381 mM sucrose.

2. 34.6 mM MES-KOH, pH 6.5, supplemented with 3.46 mM $MgSO_4$, 381 mM sucrose and 0.035 % (w/v) Triton X-100 (*see* **Note 4**).

3. 90 mM Uridine 5'-diphosphate (UDP, disodium salt) in water. Prepare fresh each time.

4. 10% (w/v) SDS (phosphate free).

5. Ames reagent (*11*):

a) Ammonium molybdate in 1 N H_2SO_4. Add 28.6 ml concentrated H_2SO_4 and 4.2 g ammonium molybdate × 4 H_2O to make 1 litre (s*ee* **Note 8**). This solution is stable at room temperature.

b) 10% (w/v) ascorbic acid. Prepare ascorbic acid solution fresh every time, keep on ice.

Combine ascorbic acid and ammonium molybdate at a mixing ratio of 1:6 (v/v) just before the assay. Keep the Ames reagent on ice.

6. To make a standard curve with a known amount of inorganic phosphate, prepare 5 mM stock solution of KH_2PO_4 in SB. Prepare the following dilutions: 0, 50, 100, 250, 500, 1000 nmol KH_2PO_4 / ml. Aliquot and store at -20 °C for further use.

2.8.3. Cytochrome *c* oxidase (a marker for mitochondrial inner membrane)

1. 50 mM MOPS-KOH buffer, pH 7.4, supplemented with 0.025% (w/v) Triton X-100 (*see* **Note 4**).

2. Cytochrome *c* .

3. 400 mM Ascorbic acid in water (prepare fresh each time).

4. Gel filtration column .

5. 50 mM MOPS-KOH buffer, pH 7.4.

2.8.4. Antimycin resistant NADH : cytochrome c reductase (a marker for ER)

1. 54.3 mM MOPS-KOH, pH 7.5, supplemented with 360 mM sucrose and 0.016% (w/v) Triton X-100 (*see* **Note 4**).

2. 5 mM oxidized cytochrome *c* solution in water. Make larger volume and store in aliquots at -20 °C.

3. 100 mM Na-azide in water (very toxic, take care!).

4. Antimycin A from *Streptomyces* sp. (very toxic, take care!). Prepare 10 mM antimycin A stock solution in absolute ethanol. Store at -20 °C in a firmly closed vial. For measurements, dilute with ethanol to 1 mM.

5. 30 mM β-NADH in water. Make fresh each time, keep on ice.

2.8.5. Cl^--stimulated, NO_3^--sensitive Mg^{2+}-ATPase: a marker for tonoplast

1. 37.5 mM Tris-HCl buffer, pH 8.0, supplemented with 413 mM sucrose. Use Tris-base to make 37.5 mM solution, adjust pH with HCl to 8.0. Add sucrose and fill to the final volume. Store in aliquots at -20 °C.

2. 1.5 M KCl.

3. 1.5 M KNO_3.

4. 90 mM Adenosine 5′-triphosphate (ATP, magnesium salt) in water. Adjust pH to 8 with KOH.

5. Ames reagent (*11*):

a) Ammonium molybdate in 1 N H_2SO_4. Add 28.6 ml concentrated H_2SO_4 and 4.2 g ammonium molybdate × 4 H_2O to make 1 litre (s*ee* **Note 8**). This solution is stable at room temperature.

b) 10% (w/v) ascorbic acid. Prepare ascorbic acid solution fresh every time, keep on ice.

Combine ascorbic acid and ammonium molybdate at a mixing ratio of 1:6 (v/v) just before the assay. Keep the Ames reagent on ice.

6. To make a standard curve with a known amount of inorganic phosphate, prepare 5 mM stock solution of KH_2PO_4 in SB. Prepare the following dilutions: 0, 50, 100, 250, 500, 1000 nmol KH_2PO_4 / ml. Aliquot and store at -20 °C for further use.

3. Methods

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Plant material preparation, cell homogenization and membrane preparation have been described for developing xylem, phloem and lignin-forming, tissue-cultured cells of Norway spruce (*8*,*9*), but the methods can also be applied to other plant tissues containing a high content of phenolic compounds. Slight modifications may be needed for membrane preparation for each species and tissues. Perform all procedures for preparation of membranes on ice or at +4 °C unless mentioned otherwise. Before the work, cool down solutions, beakers, tubes, and centrifuge rotors. Especially, while working with aqueous polymer two-phase partitioning, avoid changes of temperature during the work, since separation of membranes to upper and lower phases is temperature-dependent (*12*).

3.1. Plant material

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You might start with introductore considerations, but then number the actual working steps.
Further Comments could be outlined in notes

3.1.1. Developing xylem

Harvest a Norway spruce tree with active secondary growth with the owner's permission. Peel the bark with phloem off from the trunk (**Fig. 1A**). This is easy as cells separate along the cambial layer during secondary growth. Collect developing xylem using a sharp knife or a razor blade (**Fig. 1B**). Freeze the scrapings immediately in liquid nitrogen, and wrap in aluminum foil on dry ice. Transfer to the laboratory on dry ice, and store at -80 °C. Pre-cool the mill with liquid nitrogen. Grind the material 3 times for 20 seconds. Mix the material with a cold spoon, and keep cooling the mill and the xylem material with liquid nitrogen in between. Weigh the ground powder in pre-weighed 50 ml plastic tubes. Store at -80 °C before use (*see* **Note 9**).

3.1.2. Phloem

Phloem and bark stay together when the bark is peeled off from the trunk at the time of active secondary growth. Scrape thin layer of phloem from the inner surface of the bark using sharp surgeon's knife (**Fig. 1C**). Freeze, store and grind similarly to developing xylem material (*see* **Note 9**).

3.1.3. Lignin-forming cell culture

An extracellular lignin-forming cell culture of Norway spruce (*9*) was used as a source for cellular membranes. Collect tissue-cultured cells from the liquid culture with active lignin formation. Wash the cells briefly in a Büchner funnel with cold water, wrap in pre-weighed pieces of aluminum foil. Weigh, freeze in liquid nitrogen and store at -80 °C. Grind the cells in a big mortar with pestle. Precool them with liquid nitrogen, and add liquid nitrogen several times during grinding. Cells (20-30 g) require grinding for 30 minutes to make a homogenous powder. Store the powder in 50 ml plastic tubes at -80 °C (*see* **Note 9**).

3.2. Membrane preparation (*see* **Note 10**)

3.2.1. Testing the suitability of the phenol-binding agent

To assay the preference of the phenol-binding agent, prepare cellular membranes (microsomal vesicles) i) without any phenol-binding agent; ii) with 1.5% (w/v) soluble PVP K-30; and iii) with 1.5% (w/v) insoluble PVPP in the homogenization buffer. Use 15 g of the ground tissue / cell powder. Proceed as described in Sub-chapter 3.2.2., and evaluate the membrane yield after pelleting the microsomal vesicles in ultracentrifugation. In spruce, the preferential phenol-binding agent was easily determined by judging from the size of the membrane pellet after ultracentrifugation.

3.2.2. Preparation of microsomal vesicles

To get reasonable amounts of microsomal membranes for further work, it is recommended to start with ca. 130-160 g of xylem and phloem powder, and 200 g of powdered cultured cells.

Prepare homogenization buffer ready by adding the freshly-added ingredients (*see* **Notes 10 and 11**). If you start from the amounts of material described above, 500 ml buffer is required. Mix the plant powder gradually with the homogenization buffer in a big beaker, since the plant powder should thaw in the buffer. In case of xylem that prefers insoluble PVPP as the phenol-binding agent, add the plant powder and the buffer gradually into a beaker containing PVPP. Mix with a glass rod until the plant powder is properly thawed (takes ca. 20 minutes). Then transfer the beaker on ice, do not let the mixture warm up. Save a small volume of the homogenization buffer for balancing the tubes. Filter the homogenate through a nylon cloth into a big beaker on ice. Use clean nitrile gloves, press and wring the package with hands until the plant residue is as dry as possible. Transfer the filtrate into pre-cooled centrifuge tubes, equilibrate, and centrifuge for 10 min at 10,000 *g* at +4 °C. Cell walls and intact cell organelles are pelleted, and membranes remain in the supernatant. Transfer the supernatant into new tubes by pipetting, and pellet the membranes in an ultracentrifuge for 45 min at 100,000 *g* at +4 °C (*see* **Note 12**).

In case of phloem and cultured cells, do an extra washing step for microsomal membranes, because PVP used in the homogenization buffer interferes with the following aqueous polymer two-phase partitioning (*8*). Suspend the pellets into K-phosphate-sucrose buffer. Use a glass homogenizer to resuspend the pellets into the buffer so that PVP K-30 that remained in the pellet dilutes out (*see* **Note 13**). Transfer the sample into ultracentrifuge tubes. Fill the tubes with the buffer and mix so that the washing procedure is as efficient as possible. Repeat centrifugation (100,000 *g*, 45 min, +4 °C).

The pellet contains the cellular membranes and is called “microsomal fraction” or “microsomal vesicles”. If you continue to enrich plasma membranes, suspend the pellet into K-phosphate-sucrose buffer using a glass homogenizer. Estimate the volume of the buffer needed, keeping in mind that the pellet will increase the final volume substantially (*see* **Note 14**). If you need microsomal vesicles in your experiments, suspend the pellet into SB (or the buffer of your own choice).

3.3. Enrichment of spruce plasma membranes with aqueous polymer two-phase partitioning

3.3.1. Combining two-phase systems

For each material, you need to optimize the polymer and KCl concentration. This is done with a small-scale phase partitioning system. Combine 3.0-g phase mixture and 1.0 g of the sample in a 15 ml tube to make a 4-g phase system (Table 1). Thaw dextran and PEG solutions well before preparing the phases (overnight at +4 °C), mix all stock solutions (especially dextran and PEG) well (e.g. in a rotating shaker for at least 0.5 hour) before pipetting. Weigh the components by using a balance (use wide-tip Pasteur pipettes for dextran and PEG as they are very viscous). Start from sucrose, and then add stock solutions starting from the densest one: 1) dextran, 2) PEG, 3) K-phosphate buffer, 4) KCl, and 5) water (*see* **Note 15**). Do not tare the balance while weighing. Mix properly, and make sure that sucrose is well dissolved. Remember to make extra tubes for preparation of fresh lower phases (*see* Sub-chapter 3.3.2.). Let the phase mixtures cool to +4 °C in a fridge and make the last mixing (*see* **Note 16)**. Store at +4 °C overnight. Continue as described below for the large-scale purification, except that start with 5 g of material for each phase concentration, and use 1.0 g sample and 3.0-g phase mixture (Table 1). Assay the markers for each membrane type (*see* Sub-chapter 5), and select the concentration of the polymers for large-scale purification. You may also need to optimize the KCl concentration for optimal plasma membrane enrichment.

For large-scale purification, combine the phase mixtures in 50 ml plastic tubes (Table 2). After adding all components except the sample, add water to sum up the weight exactly to 27.00 g (*see* **Note 15**). Mix properly until sucrose is well dissolved. Let cool to +4 °C in the fridge and make the last mixing (*see* **Note 16)**. Store at +4 °C overnight.

3.3.2. Aqueous polymer two-phase partitioning (*see* **Note 17**)

Use freshly prepared microsomal vesicles for aqueous polymer two-phase partitioning. Work on ice all the time. Pipette 9.0 g of microsomal fraction on top of a 27-g phase mixture (now called tube A) resulting to a 36-g phase system (*see* **Note 18**). Remember to keep a small volume of the microsomal fraction for purity assessment. Prepare fresh lower phases for further purification by pipetting 9.0 g of K-phosphate-sucrose buffer on top of two other 27-g phase mixtures (tubes B and C; *see* **Note 19**). Close the lids, and mix the phases properly by inverting the tubes 30 times. Centrifuge in a swinging bucket rotor for 10 min (1,000 *g*, +4 °C, with no brake; *see* **Note 20**). Dextran and PEG phases are now well separated (**Fig. 2A**), make a note of their volumes. Remove as much as possible the upper phases from tubes B and C without disturbing the lower phase; these are the fresh lower phases that are used in the following plasma membrane enrichment steps. From the tube A, pipette as much upper phase as you can without disturbing the intermediate phase (~90% of upper phase) on top of the fresh lower phase in tube B. Close and invert the tube 30 times, and centrifuge as described above (**Fig. 2B**). Repeat the pipetting, inverting and centrifugation using the lower

phase of tube C (**Fig. 2C**; *see* **Note 19**). Transfer the last upper phase to an ultracentrifuge tube without disturbing the intermediate phase. Fill with SB and mix properly e.g. with a glass rod or by inverting. Dilution of the upper phase that contains PEG should be at least 2-fold.

Mark down the combined volume of the lower and the intermediate phase (if any) in tube A. Take a sample for purity assessment: mix the lower and the interphase well, and pipette e.g. 1 ml of the mixture into an ultracentrifuge tube. Add SB and pipette up and down several times to collect all sample from the pipette tip. Mix well. Dilution of the lower phase containing dextran should be at least 10-fold. Also, take a certain volume of the sample from the original microsomal fraction, and pipette this to an ultracentrifuge tube. Fill with SB and mix properly. Pellet the membranes at 100,000 *g* for 45 min at +4 °C (**Fig. 2D**).

Add a small volume of SB on top of the pellet (*see* **Note 21**). Suspend the pellet into the solution with a glass rod, transfer into a 1.5 ml tube by pipetting with a cut tip (wider tip hole). You can rinse the centrifuge tube with a small volume of the buffer, and combine it to the sample. Record the exact volume of the sample at this point. Use a small, plastic pestle to suspend the membranes properly. Aliquot the membranes into 1.5 ml tubes (e.g. 100 µl / tube), and add DTT to 1 mM final concentration from 500 mM stock solution. Do not add DTT to aliquots that you plan to use in redox reactions (e.g. cytochrome *c* oxidase, antimycin-resistant cytochrome *c* reductase). Freeze the aliquots in liquid nitrogen and store at -80 °C.

3.3. Washing membranes

Washing of cellular membranes is needed to remove the loosely attached membrane proteins and cytoplasmic proteins that were trapped inside the vesicles during membrane isolation (*4*, *13*; *see* **Note 22**). You need to wash the membranes also for marker enzyme analyses.

Thaw the membrane samples in a warm water bath (ca. +45 °C) with low motion. Transfer on ice immediately when the sample is thoroughly melted. Resuspend the membranes into cold washing buffer (10 mM MOPS-KOH buffer, pH 7.5, supplemented with 150 mM KCl, 2mM $MgCl_2$, 1 mM EDTA and 0.01% Triton X-100). Use ca. 12 ml of the buffer for maximum of 5 mg membrane protein. Incubate the mixture on ice for 30 minutes and mix regularly by inverting the tube. Pellet the membranes by centrifugation (100,000 *g*, 45 min, +4 °C) and suspend the pellet in SB using a glass rod and a small, plastic pestle. Note down the exact volume of the sample after resuspending the pellet. Use immediately for the assays.

3.4. Protein quantitation

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Dilute the membrane samples with 10 mM MOPS-KOH, pH 7.4, supplemented with 330 mM sucrose and 0.01% (w/v) Triton X-100 (*see* **Note 23**). Pipette 160 µl of samples or BSA standard solutions into microtiter plate wells. Add 40 µl of Bradford dye concentrate by using a multichannel pipette. Incubate for 10 minutes at room temperature, and measure absorbance at 595 nm. Subtract blank (buffer-only) values from the

sample and BSA standard values. Compare sample absorbance values with those of the BSA standard curve, and calculate the protein concentration (*see* **Note 24**).

3.5. Purity assessment

3.5.1. Western blotting

Washed membranes are used to test the membrane distribution and relative abundance in the different phases by western blotting. First, determine the optimum amount of protein to be added onto the gel by using pre-tests (see Table 3 for spruce protein amounts). Equal amounts of protein (based on protein quantification) are used for different membrane fractions to create a valid comparison (*see* **Notes 25 and 26**). Solubilize membrane proteins by adding membrane protein solubilization and loading buffer with freshly added DTT onto the pellet (1/4 of the final volume of the reaction). Add SDS drop-wise from the 10% stock solution to 4% final concentration [other final concentrations: 25 mM Tris-HCl, pH 6.8, 5% glycerol, 0.002% bromophenol blue and 25 mM DTT]. To solubilize proteins, incubate for 10 minutes at +70 °C. Pellet the membranes by centrifugation (17,000 *g*, 40 min, +15 °C; *see* **Notes 27 and 28**).

Separate equal amounts of solubilized proteins from the microsomal fraction, upper and lower phases in a SDS-PAGE gel electrophoresis . Optimized amounts of protein for each spruce material are shown in Table 3. Transfer the proteins to nitrocellulose membranes with a transfer system according to manufacturer's instructions. Incubate the nitrocellulose membranes in boxes (*see* **Note 29**) containing tTBS for 5 min at room temperature. Block the membranes for 1 h at room temperature with the blocking buffer (25 ml / membrane) with agitation. Wash the membranes with tTBS (100 ml, 10 min), and incubate for 1 h at room temperature with the primary antibody diluted in the antibody buffer (10 ml / membrane; *see* **Note 30**). Optimized dilutions for each primary antibody for spruce proteins are shown in Table 3 (*see* **Note 6**). Wash the nitrocellulose membranes three times for 10 min with tTBS (á 100 ml), and incubate with the secondary antibody (dilution 1:10,000 in the antibody buffer, 10 ml / membrane; *see* **Note 30**) for 1 h at room temperature with agitation. Wash the membranes four times for 10 min with 100 ml tTBS. Detect the signals by Western Blotting Detection reagent according to manufacturer's instructions by variating the exposure times (1 sec – several minutes depending on the signal). Develop, and photograph or scan the films, and evaluate the band intensities from the images (16 bit TIFF) with ImageJ. Alternatively, measure the band intensities by taking an image with ChemiDoc Touch Imaging System, or similar equipment.

3.5.2. Chlorophyll *a* measurement

To determine the amount of thylakoid membranes, a spectrophotometric assay for chlorophyll *a* is used (*14*).

Zero the spectophotometer with 100% acetone (*see* **Note 7**). Dilute the membrane sample (in SB) with 80 % (v/v) acetone supplemented with $MgCO_3$. Test the volume of the sample needed (e.g. 30 µl; final volume

needed for semimicro cuvettes is 900 µl). Measure absorbance at 663 nm and 645 nm. Calculate the amount of chlorophyll *a* by using the following equation:

*mg chl a / g fresh weight = (12.7 * A663 – 2.69 * A645) * (V/ (1000 * W)) * dilution factor in the cuvette * dilution factors during sample preparation*

Where:

V = sample volume (ml)

W= cell fresh weight (g)

In calculations, take into account all dilutions that you made during membrane isolation.

3.5.3. Glucan synthase II activity assay

Glucan synthase II (callose synthase) is used as a marker for plasma membranes (*15*, *16*). The enzyme catalyzes formation of β-1,3-glucan from UDP-glucose. The assay, however, is not specific for plasma membrane, as also intracellular enzymes may contribute to the measured activity (*17*). For each reaction, use 15 µl of membrane sample containing 1-13 µg protein (*see* **Note 31**). First, make a [^{14}C]UDP-Glc mixture. Calculate the volume of substrate needed (10 µl / reaction) and make one extra. Add [^{14}C]UDP-Glc to a mixture containing 10 mM UDP-Glc in 50 mM MOPS-KOH, pH 7.2, 330 mM sucrose so that one reaction contains 0.5 kBq. Non-labelled UDP-Glc (at 10 mM) is included in the mixture to make sure that the substrate amount does not limit the reaction.

Prepare four different reactions in 1.5 ml tubes (final volume 50 µl) with three replicates. Add all the components except [^{14}C]UDP-Glc mixture, as that will start the reaction. Keep the tubes still on ice at this point.

- **A)** 20 µl Buffer mixture, 5 µl digitonin, 15 µl sample, 10 µl [^{14}C]UDP-Glc mixture
- **B)** 20 µl Buffer mixture, 5 µl MOPS-KOH + 330 mM sucrose, 15 µl sample, 10 µl [^{14}C]UDP-Glc mixture
- **C)** 20 µl Buffer mixture, 5 µl digitonin, 15 µl MOPS-KOH + 330 mM sucrose, 10 µl [^{14}C]UDP-Glc mixture
- **D)** 20 µl Buffer mixture, 5 µl digitonin, 15 µl sample, 5 µl MOPS-KOH + 330 mM sucrose

Start the reaction with the addition of [^{14}C]UDP-Glc mixture, incubate for 30 min at +25 °C water bath (exact time). Stop the reaction by adding 5.5 µl 98% formic acid (to 10% final concentration), mix properly, and spin down. Pipette 53 µl of the reaction mixtures **A-C** onto cellulose filter discs on aluminium foil. Let the filter papers dry properly. Transfer the filters into a 1 L flask, and wash on a platform shaker two times 45 min in a solution containing ethanol - ammonium acetate, pH 3.6 (60 disks / 500 ml). The sugar polymers

remain attached to the filter paper due to hydrogen bonding to cellulose, whereas unused UDP-Glc is washed out. Transfer the filter papers onto the foil on a tray. Detach the filter papers from each other otherwise they will stick together, and let them dry at room temperature or in an oven (at +45 °C).

Incubate the reaction vials **D** at +25 °C similarly to reactions **A-C**. After stopping the reaction, add 5 µl [^{14}C]UDP-Glc mixture to the reaction vials **D**. Mix. Pipette 53 µl of the reaction mixtures on top of glass fiber discs pre-wetted with 150 µl water, and let to dry. Reactions **D** show the total radioactivity used in each reaction (remember to multiply the values by two as half of the volume of [^{14}C]UDP-Glc mixture is used in these samples). Use glass fiber filters for these samples, since in cellulose filters UDP-Glc would absorb in between the cellulose fibers leading to quenching of the radioactivity. There are no sugar polymers in samples **D**, so do not wash the glass fiber discs.

Insert the cellulose and glass fiber discs into scintillation vials (*see* **Note 32**). Add scintillation liquid (6 ml): the discs need to be fully soaked. Scintillation count two times for 5 minutes to detect the radioactivity in the samples.

Subtract the background radioactivity (sample **C**) from sample values before calculating the results. Take into account all dilutions that you made during membrane isolation.

Digitonin indicates whether the membrane vesicles are right-side out or inside-out, i.e. enzyme latency (*17*). In aqueous polymer two-phase partitioning, the right-side out plasma membranes preferentially partition into the upper PEG-phase. As the active site of the glucan synthase II enzyme is considered to be on the cytoplasmic side (*17*), a detergent, digitonin, induces increase in the enzyme activity as the substrate has an easier access to the active site. If the plasma membrane vesicles were inside-out, they preferentially partition into the lower dextran phase. The active site is already available for the substrates, and hence, digitonin does not induce activation in the enzymic activity (non-latent activity; *17*).

3.5.4. Latent uridine diphosphatase (UDPase)

Latent uridine diphosphatase (UDPase) is used as a marker for Golgi membranes (*18*).

Let the buffer warm up to the reaction temperature (+25 °C), keep UDP and the samples on ice (*see* **Note 31**). Blank contains SB instead of sample/standard. Prepare also a boiled sample control (10 min boiling) for each material.

Pipette into 1.5 ml tubes:

200 µl buffer (34.6 mM MES-KOH, pH 6.5, 3.46 mM $MgSO_4$, 381 mM sucrose, with or without 0.035% Triton X-100; final concentrations in the reaction mixture 30 mM, 3 mM, 330 mM and 0.03% or 0%, respectively). You need to prepare two lots for the assay: one with and another without Triton X-100. Make three replicates for each sample/standard/blank reaction.

Add 7.5 µl 90 mM UDP (3 mM final concentration), mix.

Add 22 µl sample (4-9 µg protein) / KH_2PO_4 standard dilutions in SB. Mix properly and incubate for 30 min at +25 °C (e.g. in a water-bath; exact time). Stop the reaction by adding 40 µl 10% SDS (1.5% final concentration). Detect the liberated phosphate by adding immediately 690 µl Ames reagent (*11*). Mix and incubate for 60 min at room temperature (+20 °C). Measure absorbance at 820 nm.

Use the standard curve to calculate the amount of liberated phosphate in your samples (*see* **Note 24**). Subtract the boiled sample value from the sample values as that shows the non-enzymatic release of inorganic phosphate due to the plant material. Take into account all dilutions that you made during membrane isolation.

Latent UDPase activity represents the difference in activity with or without Triton X-100.

3.5.5. Cytochrome *c* oxidase assay

Cytochrome *c* oxidase assay is used as a marker for mitochondrial inner membrane (*19*). Oxidation of reduced cytochrome *c* is followed at 550 nm by using a kinetics program in the spectrophotometer. Do not add DTT to samples to be analyzed for cytochrome *c* oxidase assay.

For the assay, cytochrome *c* is reduced with ascorbic acid (*20*).

1) Prepare 250 µl 10 mM cytochrome *c* solution in water.

2) Prepare 250 µl 400 mM ascorbic acid solution in water. Prepare fresh each time.

Add solutions 1) and 2) to 2 ml water so that the final volume of cytochrome-ascorbic acid mixture is 2.5 ml (final concentrations 1 mM and 40 mM, respectively). Incubate for 10 min at room temperature to reduce all cytochrome *c*. Remove ascorbic acid in a gel filtration column (PD-10, Sephadex G-25, GE Healthcare). For this, equilibrate the column at +4 °C with 25 ml 50 mM MOPS-KOH-buffer, pH 7.4, according to the manufacturer's instructions. Pipette cytochrome-ascorbic acid mixture (2.5 ml) on top of the column, and let it go into the column. Then elute the high-molecular-weight cytochrome *c* with 3.5 ml buffer. Cytochrome *c* is diluted 1.4-fold (3.5 ml / 2.5 ml), i.e. the final concentration is 714 µM. Store the reduced cytochrome *c* in aliquots at -20 °C. Wash the column with 25 ml of buffer. Store the column at +4 °C for the next use as it can be used for 5-6 times.

For the enzyme activity determination, thaw the reduced cytochrome *c*. Make sure that cytochrome *c* is totally reduced by adding a few crystals of Na-dithionite ($Na_2S_2O_4$) into the tube with a spatula. Follow whether any color change occurs. The oxidized cytochrome *c* is orange, and the reduced one is pink. Remove the extra dithionite by aeration (blow some air through the solution with a Pasteur pipette for a couple of minutes). This will lead to oxidation of the remaining dithionite.

To assay the enzyme activity (*see* **Notes 31 and 33**), pipette directly into the semimicro cuvette:

890 µl 50 mM MOPS-KOH buffer, pH 7.4, with 0.025% (w/v) Triton X-100. Place the cuvette into the cuvette chamber, and zero the spectrophotometer by pressing auto zero.

Add 70 µl reduced cytochrome *c* (final concentration 50 µM in the final volume of 1 ml). Close the cuvette with a piece of parafilm, mix by inverting. Measure the change in A550 nm for 1 min (background oxidation).

Add 40 µl of the membrane sample containing ca. 10-50 µg protein (dilute with SB if needed; *see* **Note 34**). Mix by inverting the cuvette, and immediately follow the change in A550 nm for at least 1 minute.

Subtract the background values from the sample values before calculating the final results. Calculate the activity as katals (mol/sec). Use equation

$\varepsilon = A / (c * l)$

Where:

ε550 (reduced cytochrome *c*) = 18.5 mM^{-1} cm^{-1} (*19*), *A*= change in A550 nm / min, *c* = concentration (mol/l), l= length of the light path (1 cm).

In calculations, take into account all dilutions that you made during membrane isolation.

3.5.6. Antimycin resistant NADH : cytochrome *c* reductase (a marker for ER)

Antimycin resistant NADH:cytochrome *c* reductase is used to assay the presence of ER membranes (modified from *21*). With Norway spruce samples, the concentration of antimycin A commonly used in this assay (1 µM; *21*) did not fully inhibit the antimycin sensitive part of the activity, hence, a concentration showing maximum inhibition (30 µM) was chosen (*8*). Ethanol concentration in the reaction (3%, v/v, final) did not affect the enzyme activity.

Make replicates for each sample. Do not have DTT in the samples for this assay (*see* **Notes 31 and 33**).

Pipette into the semimicro cuvette:

920 µl 54.3 mM MOPS-KOH, pH 7.5, supplemented with 360 mM sucrose and 0.016% (w/v) Triton X-100 (final concentrations: 50 mM, 330 mM and 0.015%, respectively).

Place the cuvette into the cuvette chamber, and zero the spectrophotometer by pressing auto zero. Then add

10 µl 5 mM oxidized cytochrome *c* (50 µM final)

10 µl 100 mM Na-azide (1 mM final)

30 µl 1 mM antimycin A (30 µM final)

Close the cuvette with a piece of parafilm, and mix by inverting. Add 20 µl sample (ca. 5-15 µg protein).

Mix and follow cytochrome *c* reduction at 550 nm for two minutes. This shows the non-enzymatic reaction, and the inhibitors have time to bind to corresponding sites.

Start the enzymatic reaction by adding 10 µl 30 mM NADH (0.3 mM final).

Mix and follow reduction of cytochrome *c* at 550 nm. Use the first minute linear increase for calculating the results (*see* **Note 34**). Subtract the non-enzymatic background before calculating the final results. Calculate the activity as katals (mol/sec). Use equation

$\varepsilon = A / (c * l)$

Where:

ε550 (cytochrome *c*) = 18.5 mM^{-1} cm^{-1} (*19*), *A*= change in A550 nm / min, *c* = concentration (mol/l), l= length of the light path (1 cm).

In calculations, take into account all dilutions that you made during membrane isolation.

3.5.7. Cl^--stimulated, NO_3^--sensitive Mg^{2+}-ATPase: a marker enzyme for tonoplast

Cl^--stimulated, NO_3^- sensitive Mg^{2+}-ATPase activity is used as a marker for vacuolar membranes (*18*). The activity is not inhibited by *ortho*-vanadate (*22*). Release of inorganic phosphate is determined by the method of Ames (*11*).

Pipette into a 1.5 ml tube (final volume 224.5 µl; make three replicates for each reaction (*see* **Notes 31 and 33**):

180 µl buffer (37.5 mM Tris-HCl, pH 8.0, supplemented with 413 mM sucrose)

7.5 µl 1.5 M KCl **or** 1.5 M KNO_3 (50 mM final)

7.5 µl 90 mM ATP (for samples) **or** 7.5 µl H_2O (for standard reactions)

7.5 µl H_2O

Mix and start the reaction with 22 µl sample (containing 3-9 µg protein) or KH_2PO_4 standard solution (*see* **Note 35**).

Mix the components, incubate for 30 min at +25 °C in a water bath (exact time). Stop the reaction by adding 40 µl 10% SDS followed immediately by 690 µl Ames reagent. Mix, incubate for 60 min at room temperature. Measure absorbance at 820 nm.

Remember to make a blank (SB only instead of a sample) to zero the spectrophotometer. In calculations, take into account all dilutions that you made during membrane isolation. Difference in activity observed between KCl and KNO_3 is the nitrate-sensitive ATPase activity.

Notes

1. You can make a stock solution (5x) of MOPS (250 mM, pH 7.5). Before cell extraction, dilute to 1-fold and add the required components.
2. It takes ca. 30 minutes to dissolve PVP K-30 in a magnetic stirrer.
3. Freeze and store the dextran and PEG stock solutions (e.g. in 50 ml aliquots) at -20 °C. Dextran is very hygroscopic (i.e., it absorbs water easily), hence, the exact concentration can be estimated by polarimeter only (*5*). Store the powder in a dry place, and dissolve the whole lot at the same time. Aliquot and store in the freezer. This way you guarantee the reproducibility of the aqueous polymer two-phase partitioning in the following purification rounds, since small differences in the stock solutions used to prepare the two-phase systems affect the phase separation. Ideally, the dextran and PEG concentration for the aqueous polymer two-phase partitioning need to be optimized again every time new stock solutions are made. Optimization can be done in a small scale starting with 5 g ground material for each phase concentration. In aqueous two-phase partitioning, use 3.0-g phase system and 1.0 g of sample (Table 1). Optimization is time-consuming, hence, preparation of large volumes of stock solutions is justified.
4. Weigh Triton X-100 on a balance as it is a sticky solution, and rinse carefully into the final solution.
5. Collect Towbin buffer after use, as you can use this buffer for several (5-6) times.
6. If you are using a species with no experience of the antibody in question, the suitability (specificity) and dilution of the antibody needs to be tested according to manufacturer's instructions.
7. Plastic cuvettes are not suitable with acetone.
8. Addition of H_2SO_4 to water is an exothermic reaction, i.e., it generates heat. Add H_2SO_4 to water in portions, let cool to room temperature before adjusting the final volume. Use safety glasses when preparing the solution.
9. After grinding the plant material in liquid nitrogen, put the ground powder in 50 ml plastic tubes and store at -80 °C. Do not, however, close the lids very tightly, as the tubes might explode in the freezer or when you remove them from the freezer.
10. To make the day of membrane preparation as easy and fluent as possible, make all possible preparations in the previous day. For example, weigh the ingredients of the homogenization buffer, put the centrifuge rotors to a cold room, and mark the tubes.
11. Use a fume hood as DTT is smelly.
12. Equilibrate centrifuge tubes carefully on a balance, especially for the ultracentrifuge. Check that the ultracentrifuge tubes and the rotor can be used at the *g* forces that you intend to use.

13. Be gentle when re-suspending the membrane vesicles into the buffer. Only use just enough force and time to resuspend, do not grind the membranes at this point.
14. If you started with 130-160 g xylem or phloem powder, or with 200 g of cultured cells, and you continue with aqueous polymer two-phase partitioning, 19-20 ml is a good final volume to aim into when suspending the pellet.
15. By having the pipetting order from the densest solution to the least dense, you can correct small mistakes since the addition of a new component goes on top of the previous solution.
16. Combining of the phases can be done in temperature other than that where the real separation of membranes is conducted. But since the temperature affects phase separation, the final mixing and aqueous polymer two-phase partitioning need to be done at constant temperature (+4 °C).
17. If you prepare membranes in a large scale (e.g. start from 130-200 g of plant material) you need two persons working, as it is good to be as fast as possible (especially if you later intend to use the vesicles to study enzyme activities). Still, the procedure takes a long working day. Do all possible preparations beforehand (e.g. prepare homogenization buffer and two-phase mixtures, mark tubes, and plan the volumes of membrane aliquots you will store).
18. If you start with 160 g xylem powder, you will have ~19-20 ml of microsomal fraction for aqueous polymer two-phase partitioning. This means that you can conduct the whole two-phase protocol in two sets of tubes A, B, and C.
19. In pre-tests, you need to determine the optimum number of phases to be used for each material.
20. Use of a brake leads to a formation of a big intermediate phase.
21. The volume of the buffer to be added has to be estimated by the size of the pellets. In general, 0.5 ml is a good volume to start with.
22. Enzyme activities are retained better if washing of the membranes is conducted just prior to assaying the activities.
23. Membrane preparations done according to our procedure should be diluted 50-1,000 times for the protein assay. Use serial dilutions.
24. Use only sample values that are in the linear region of the standard curve.
25. For solubilization of membrane proteins, calculate the volumes for pipetting ready, and treat all samples (microsomal fraction, upper phase, lower phase) as similarly as possible since you compare the band sizes between these samples. When planning of the pipetting volumes, take into account the maximum volume that can be loaded onto the gel.
26. Protein amount to be used in western blot needs to be optimized for each plant tissue and for each antibody.
27. Pellet the membranes by centrifugation at +15 °C to avoid precipitation of SDS.
28. In solubilization with SDS, different plant membranes behave differently. For example, xylem proteins are easily solubilized, whereas phloem and cell culture membranes form a big pellet in centrifugation after incubation at +70 °C.

29. Empty pipette tip boxes are useful in western blotting as membrane washing containers.
30. It is advisable to roll the nitrocellulose membrane in a loose roll and insert it into the 50 ml tube. By this way, 10 ml of antibody solution is enough for the whole membrane, when the tube is incubated on its side on a rotating shaker.
31. Thaw the samples just immediately before the assay. It is recommended to thaw enzyme samples quickly in a warm water-bath (ca. +45 °C). Keep the tubes in motion so that the ice block keeps the enzyme sample cool while melting. Transfer the tubes on ice immediately after all ice has disappeared.
32. Be careful that the sample side is always in the same way, e.g. upwards.
33. Let the buffer warm to the measuring temperature before the start of the assay. Use a temperature-controlled cuvette chamber in the spectrophotometer, or keep the buffer in a water bath to keep the temperature constant during the enzyme assay.
34. If change in absorbance per minute is too high (over 100 mA units), dilute the sample with SB and measure again.
35. Reaction supplemented with KCl is enough for the KH_2PO_4 standard measurement, as no enzyme activity is followed with the standard reactions.

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Figure captions:

Fig. 1. (A) Peeling off bark from a Norway spruce trunk at the time of active secondary growth. (B) Collecting developing xylem from the top of mature secondary xylem. (C) Collecting phloem from the inner bark.

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Fig. 2. Aqueous polymer two-phase partitioning of cellular membranes of tissue-cultured Norway spruce cells as an example. The migration of thylakoid membranes into the lower phase is easy to follow as this cell culture is green with well-developed chloroplasts. (A) Microsomal fraction was mixed with the phase system: the first phases after separation of the PEG (upper phase) and dextran phases (lower phase). (B) For further purification, the first upper phase was pipetted on top of the fresh lower phase. The second phases after phase separation: some green color is still visible in the lower phase. (C) The third phases in the consecutive purification of the second upper phase with a fresh lower phase. (D) Membranes pelleted by ultracentrifugation. LP: lower and intermediate phases from the first phase separation containing the intracellular membranes; MF: microsomal fraction containing all cellular membranes; UP: upper phase after three phase separations. Thylakoid membranes have diminished drastically, visible as absence of the green color in the upper phase membranes.

Table captions:

Table 1. Optimization of the concentration of polymers in a 4-g phase system: combining components of the phase system.

Table 2. Preparation of the 36-g aqueous polymer two-phase partitioning system. The optimum concentration of dextran, PEG and KCl needs to be determined for each material. For Norway spruce, the optimised concentration of dextran/PEG was determined to be 6.15% (w/w) and 3 mM KCl for the tissue-cultured cells, and 6.3% (w/w) and 3 mM KCl for xylem and phloem. Other incredients (final concentrations): 330 mM sucrose, 5 mM K-phosphate, 1 mM DTT and 0.1 mM EDTA.

Table 3. Optimized amounts of spruce proteins to be used in western blotting, and optimized dilutions of the primary antibodies for various membrane types (*see* **Note 6**). H^+-ATPase, a marker for plasma membranes; cytochrome *c* oxidase subunit II (COX II), a marker for inner mitochondrial membranes; vacuolar H^+-pyrophosphatase (V-PPase), a marker for tonoplast; binding immunoglobulin protein BiP, a marker for ER membranes.

Table 1. Optimization of the concentration of polymers in a 4-g phase system: combining components of the phase system.

Components	6.0/6.0%	6.1/6.1%	6.2/6.2%	6.3/6.3%	6.4%/6.4%
20% Dextran	1.20 g	1.22 g	1.24 g	1.26 g	1.28 g
40% PEG 3350	0.60 g	0.61g	0.62 g	0.63 g	0.64 g
Sucrose (powder)	0.339 g	0.339 g	0.339 g	0.339 g	0.339 g
0.2 M K-phosphate, pH 7.8*	75 µl	75 µl	75 µl	75 µl	75 µl
0.2 M KCl (3 mM final)*	45 µl	45 µl	45 µl	45 µl	45 µl
0.5 M DTT in 50 mM EDTA**	8 µl	8 µl	8 µl	8 µl	8 µl
Water to sum the weight to	3.00 g	3.00 g	3.00 g	3.00 g	3.00 g
Sample	1.0 g	1.0 g	1.0 g	1.0 g	1.0 g
Final weight of the phase system	4.0 g	4.0 g	4.0 g	4.0 g	4.0 g

* Note that the sample contains K-phosphate and KCl making the final concentration 5 mM and 3 mM, respectively.

** Use of DTT and EDTA depends on the further use of your sample. If you study redox enzymes, you should omit these.

Table 2. Preparation of the 36-g aqueous polymer two-phase partitioning system. The optimum concentration of dextran, PEG and KCl needs to be determined for each material. For Norway spruce, the optimised concentration of dextran/PEG was determined to be 6.15% (w/w) and 3 mM KCl for the tissue-cultured cells, and 6.3% (w/w) and 3 mM KCl for xylem and phloem. Other incredients (final concentrations): 330 mM sucrose, 5 mM K-phosphate, 1 mM DTT and 0.1 mM EDTA.

Components	6.15/6.15%	6.3/6.3%
20% Dextran	11.07 g	11.34 g
40% PEG 3350	5.535 g	5.670 g
Sucrose (powder)	3.05 g	3.05 g
0.2 M K-phosphate, pH 7.8*	0.675 g	0.675 g
0.2 M KCl (3 mM final)*	405 µl	405 µl
0.5 M DTT in 50 mM EDTA**	72 µl	72 µl
Water to sum the weight to	27.00 g	27.00 g
Sample	9.0 g	9.0 g
Final weight of the phase system	36.0 g	36.0 g

* Note that the sample contains K-phosphate and KCl making the final concentration 5 mM and 3 mM, respectively.

** Use of DTT and EDTA depends on the further use of your sample. If you study redox enzymes, you should omit these.

Table 3. Optimized amounts of spruce proteins to be used in western blotting, and optimized dilutions of the primary antibodies for various membrane types (*see* **Note 6**). H^+-ATPase, a marker for plasma membranes; cytochrome *c* oxidase subunit II (COX II), a marker for inner mitochondrial membranes; vacuolar H^+-pyrophosphatase (V-PPase), a marker for tonoplast; binding immunoglobulin protein BiP, a marker for ER membranes.

	Antibody against		Antibody against		Antibody against		Antibody against	
	H^+-ATPase		COX II		V-PPase		BiP	
Spruce material	µg protein	Antibody dilution	µg protein	Antibody dilution	µg protein	Antibody dilution	µg protein	Antibody dilution
Developing xylem	5	1:2,500	5	1:3,000	0.2-0.4	1:5,000	10	1:300,000
Phloem	10	1:2,500	10	1:3,000	1	1:5,000	10	1:300,000
Cultured cells	10	1:2,500	10	1:3,000	1	1:5,000	10	1:300,000