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## Immunopurification of Intact Endosomal Compartments for Lipid Analyses in Arabidopsis

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**Abstract** Endosomes play a major role in various cellular processes including cell–cell signaling, development and cellular responses to environment. Endosomes are dynamically organized into a complex set of endomembrane compartments themselves subcompartmentalized in distinct pools or subpopulations. It is increasingly evident that endosomes dynamics and maturation is driven by local modification of lipid composition. The diversity of membrane lipids is impressive and their homeostasis often involves crosstalk between distinct lipid classes. Hence, biochemical characterization of endosomal membrane lipidome would clarify the maturation steps of endocytic routes. Immunopurification of intact endomembrane compartments has been employed in recent years to isolate early and late endosomal compartments and can even be used to separate subpopulations of early endosomes. In this section, we will describe the immunoprecipitation protocol to isolate endosomes with the aim to analyze the lipid content. We will detail a procedure to identify the total fatty acid and sterol content of isolated endosomes as a first line of lipid identification. Advantages and limitations of the method will be discussed as well as potential pitfalls and critical steps.

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<b>Keywords</b> (separated by '-')	Organelles - Immunopurification - Golgi - Endosomes - Lipids - Fatty acids - Mass spectrometry - Plants
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## Immunopurification of Intact Endosomal Compartments for Lipid Analyses in *Arabidopsis* 2 3

Yoko Ito, Magali Grison, Nicolas Esnay, Laetitia Fouillen, and Yohann Boutté 4 5

### Abstract 6

Endosomes play a major role in various cellular processes including cell–cell signaling, development and cellular responses to environment. Endosomes are dynamically organized into a complex set of endomembrane compartments themselves subcompartmentalized in distinct pools or subpopulations. It is increasingly evident that endosomes dynamics and maturation is driven by local modification of lipid composition. The diversity of membrane lipids is impressive and their homeostasis often involves crosstalk between distinct lipid classes. Hence, biochemical characterization of endosomal membrane lipidome would clarify the maturation steps of endocytic routes. Immunopurification of intact endomembrane compartments has been employed in recent years to isolate early and late endosomal compartments and can even be used to separate subpopulations of early endosomes. In this section, we will describe the immunoprecipitation protocol to isolate endosomes with the aim to analyze the lipid content. We will detail a procedure to identify the total fatty acid and sterol content of isolated endosomes as a first line of lipid identification. Advantages and limitations of the method will be discussed as well as potential pitfalls and critical steps. 7 AU1 8 9 10 11 12 13 14 15 16 17 18

**Key words** Organelles, Immunopurification, Golgi, Endosomes, Lipids, Fatty acids, Mass spectrometry, Plants 19 20 21

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## 1 Introduction 22

The endosomal network is a highly coordinated set of endomembrane compartments which host materials coming from early endocytic events at the plasma membrane through early endosomes (EEs), which are then dispatched over recycling pathways back to the plasma membrane or degradation pathways through late endosomes (LEs). However, plants have no EEs as described in animals, and endocytic vesicles converge directly to the *trans*-Golgi Network (TGN) where the endocytic tracer FM4-64 was found to accumulate relatively fast (couple of minutes) during endocytosis 23 24 25 26 27 28 29 30 31

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Yoko Ito and Magali Grison contributed equally to this work.

before reaching LEs [1–3]. TGN is a tubulovesicular membrane network that originates from the *trans*-most cisternae of the Golgi apparatus, acting as a main central station in endosomal trafficking. In plants, the TGN can undergo homotypic fusion and can associate transiently with Golgi apparatus, similarly to what is found for EEs and TGN in mammalian cells [2, 4]. Moreover, plant TGN can detach from the Golgi apparatus to form a highly dynamic Golgi-independent structure (Golgi-Independent TGN; GI-TGN) [2, 4]. From electron microscopy (EM) ultrastructural studies, at least three populations of TGN-associated vesicles could be identified, the secretory vesicles (TGN-SVs) that do not bear any apparent coat and which size vary between ~50 and 100 nm in diameter, the clathrin vesicles (TGN-CCVs) that possess a clathrin coat and measure ~35 nm, and the COPIb-type of vesicles that carry a COPI coat and measure ~45 nm in diameter [5–7]. From confocal colocalization studies we know that TGN-SVs are marked with the V-ATPase VHA-a1, the syntaxin proteins SYP61 and SYP43 and the protein ECHIDNA while TGN-CCVs are marked with the RAB-GTPase RAB-A2a [1, 6, 8–14]. It is not completely clear if TGN-SVs and TGN-CCVs can both contain endocytic materials; however, EM and live-cell observations suggest that endocytosis tracers are found at GI-TGN within a few minutes after endocytosis, and both the CCV marker clathrin and the SV marker VHA-a1 localize on GI-TGN more than on Golgi-associated TGN [2, 3, 6]. Hence, to completely understand the time frame and maturation steps of endocytic routes, there is a stumbling block to overcome. Cell biology approaches indicate that TGN and endosomes are made of subpopulations that might not have the same lipid and protein content, reflecting the maturation and dynamics of these endomembrane compartments. This view should now be scaled up with multidisciplinary approaches to understand the chemistry and biochemistry of membranes including proteins and lipids.

In recent years, the development of methods allowing for the purification of organelles using different specific baits opened the door to the biochemical characterization of distinct endocytic compartments. Isolation of intracellular organelles was done previously by classical density gradient centrifugation but these methods have several limitations due to similar densities of distinct intracellular compartments and the diversity of small compartments found in one fraction. Localization of organelle proteins by isotope tagging (LOPIT) has been used on iodixanol gradients to group proteins using principal component analysis and allowed to clearly separate Golgi proteins from endoplasmic reticulum (ER) proteins [15]. The Golgi apparatus was also isolated by combination of density gradient and the surface charge separation technique called free-flow electrophoresis (FFE) [16]. Very recently, a subtle combination of LOPIT and FFE was used to determine the proteins and glycan content of Golgi cisternae from the ER to the *trans*-most

side of the Golgi apparatus [17]. However, these methods do not allow for separation of subpopulations of endosomes, and compartments such as TGN-SVs and TGN-CCVs would be hard to isolate independently. Nonetheless, EM studies have identified the presence of proteins to specific subpopulations of TGN or endosomes [6, 9, 13]. Hence, these hallmarks can be used as tags to isolate compartments in a target specific way with high specificity using immunoaffinity approaches. The first study used SYP61 fused to GFP as a bait for TGN using anti-GFP antibodies coupled to agarose-beads as affinity ligand [18]. This method was also adapted to magnetic beads using VHA-a1 fused to YFP as TGN bait and coupled to LOPIT technique [19]. Immunoisolation was further used on a range of markers belonging to the Wave collection [20] labeling the Golgi, TGN, EEs, or LEs [21].

Immunoisolation procedures have proved to be very useful to determine the proteome of intact and full Golgi cisternae, TGN, EEs, or LEs and get a clearer view of the identity of these compartments. It has also become increasingly evident that lipids are key determinants of membrane identity, sorting mechanisms and maturation processes. A well-known process accompanying endocytosis and maturation of endosomes in mammalian cells is the conversion of phosphoinositides (PIPs), which are negatively charged phosphorylated forms of phospholipids [22]. This change in lipid composition drives the recruitment of distinct RAB GTPases and defines a new identity to the compartment. Another example of lipid involvement in membrane maturation is the accumulation of phosphatidic acid (PA), a lipid which favor vesicle fission, at the neck of constricted COPI vesicles through the action of phospholipase D (PLD) that converts phosphatidylcholine (PC) into PA [23]. In plants, immunoisolation of distinct subdomains of TGN using magnetic beads coupled to anti-GFP antibodies has successfully reached the separation of TGN-associated SVs (using SYP61-CFP as a bait) and TGN-associated CCVs (using RAB-A2a-YFP as a bait) to analyze lipid composition [14]. Strikingly, an enrichment of  $\alpha$ -hydroxylated very-long-chain fatty acids (hVLCFAs), a specific signature of sphingolipids (SLs), was observed together with an enrichment of sterols at SVs as compared to CCVs [14]. Characterizing the complete fatty acids (FAs) and sterols profile of immunisolated compartments by gas chromatography–mass spectrometry (GC-MS) is the first step to the in-depth characterization of lipid composition. Certainly, lipidomic profiling using liquid chromatography mass spectrometry (LC-MS) would definitely be the most powerful approach to identify several classes and species of lipids in one sample. However, the diversity of lipids is estimated to include more than forty thousand molecular species as referenced in LIPID MAPS online database (<https://www.lipidmaps.org/>). A general definition of lipids would be a hydrophobic or amphiphilic molecular assembly soluble in organic

solvents. This definition includes a huge structural diversity ranging from but not limited to (1) glycerolipids composed from a glycerol backbone on which one, two or three FAs are esterified, (2) glycerophospholipids composed from a glycerol backbone on which one or two FAs, a phosphate group and amino alcohols are esterified, (3) sphingolipids composed of a ceramide backbone (made of a sphingoid base amidified with a FA) which can be further modified by glycosylation, phosphorylation, or addition of an inositol phosphate residue which can be glycosylated, (4) sterols constituted from a steroid backbone which can be modified by glycosylation and/or addition of a FA chain in the case of the sterylesters, glycosylesters, or acetylglucosylesters. The acyl chains of lipids can be hydroxylated and unsaturated to different degree and position resulting in even more diverse structures. The polar head grafted to the hydrophobic chains confers diverse polarity index to the molecular assembly. Hence, depending on the polarity of the mix of solvents used to extract lipids, some classes or species of lipids might be more efficiently extracted as compared to others, introducing a bias in their quantification. Some lipids such as the phosphorylated lipids, are more unstable than others, also skewing lipid quantification. Hence, using only one extraction method to quantify all lipids at once is not feasible. In the future, improved extraction and LC-MS methodologies will need to address these pitfalls to identify and compare the different lipid classes found in immunopurified compartments. LC-MS clearly has the potential to identify major and minor lipid species without potential biases from the separation and desorption of lipids from high-performance thin layer chromatography (HPTLC) plates.

For this purpose, performing a complete FA and sterol profile is a useful first-line of identification and characterization. Here, we describe the immunoisolation procedure used for lipid analyses. We also explain the procedure to perform a complete FA and sterol profiling of immunoisolated compartments, both at the preparative and analytical level.

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## 2 Materials

Because this protocol is for immunoprecipitation of intact membrane compartments, the GFP tag has to be exposed to the cytosolic side of the membrane so that the anti-GFP antibody can bind to it. We recommend using transmembrane proteins (e.g., SNAREs [18] or V-ATPases [19]) as baits. However, some membrane associated proteins (e.g., RABs) work as well [14, 21]. However, as RAB proteins can potentially dissociate from the membranes during purification and therefore not yield enough purified organelles, this protocol also describes and illustrates how to evaluate the efficiency of IP and validation of the extraction. The amount of

the plant material is one of the most crucial points of this experiment. At least 10 g (fresh weight) of seedling materials should be obtained for one IP. Seedlings can be collected from MS plates (12 × 12 cm) or from liquid culture. However, to reach 10 g of fresh materials 200 μL of seeds (120–160 mg of dry seeds) need to be sowed. In this protocol we will describe a liquid culture method allowing for easy production of fresh material in a relatively small amount of time.

### 2.1 Plant Material and Growth

1. *Arabidopsis thaliana* seeds expressing a GFP-tagged organelle marker has been chosen as a bait.
2. Sterilization solution: 1% (w/v) sodium dichloroisocyanurate in sterile deionized water.
3. MS medium: 4.4 g/L Murashige and Skoog medium including vitamins, 10 g/L sucrose, and 0.5 g/L 2-(*N*-morpholino) ethanesulfonic acid (MES). Adjust the pH to 5.8 with KOH and autoclaved at 110 °C for 30 min.
4. 70% (v/v) ethanol in H<sub>2</sub>O.
5. 500 mL baffled flasks.

### 2.2 Membrane Isolation and Protein Quantification

1. 50 mM HEPES in H<sub>2</sub>O. Adjust the pH to 7.5 with KOH and keep at 4 °C.
2. Vesicle isolation buffer: 0.45 M sucrose, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (DTT), and 0.5% Polyvinylpyrrolidone (PVP, *see Note 1*) dissolved in 50 mM HEPES pH 7.5. Store at –20 °C and keep at 4 °C for use. Just before use, add phenylmethylsulfonyl fluoride (PMSF) at the final concentration of 1 mM.
3. Wash buffer: 0.25 M sucrose, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA (pH 8), and 150 mM NaCl dissolved in 50 mM HEPES pH 7.5. Store at –20 °C and keep at 4 °C for use.
4. Resuspension buffer: 1 mM PMSF and 1% (v/v) protease inhibitor cocktail in wash buffer. Prepare immediately before use and keep at 4 °C.
5. Sucrose solutions: 38%, 33%, and 8% sucrose (w/v) in 50 mM HEPES, pH 7.5.
6. Bicinchoninic Acid Protein Assay Kit.
7. Mortar (with the diameter about 20 cm) and pestle.
8. Funnel.
9. Gauze or Miracloth (Millipore).

### 2.3 Immuno-precipitation

1. Anti-GFP, rabbit IgG, polyclonal (e.g., Thermo Fisher Scientific, A-11122).
2. PBS-T: 150 mM NaCl, 2.7 mM KCl, 11.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.76 mM KH<sub>2</sub>PO<sub>4</sub>, 0.02% (v/v), Tween 20. Keep at 4 °C.



	3. Dynabeads Protein A for immunoprecipitation and magnetic tube holder.	216
		217
	4. Bis[sulfosuccinimidyl]suberate (BS <sup>3</sup> ).	218
	5. BS <sup>3</sup> conjugation buffer: 20 mM Na <sub>2</sub> HPO <sub>4</sub> , 0.15 M NaCl.	219
	6. BS <sup>3</sup> working solution: 5 mM in BS <sup>3</sup> conjugation buffer. Prepare before use.	220
		221
	7. BS <sup>3</sup> quenching buffer: 1 M Tris-HCl pH 7.5.	222
		223
<b>2.4 SDS-PAGE and Western Blotting</b>	1. Primary antibodies: mouse anti-CFP/GFP/YFP for IP (e.g., Roche 118144600001 at 1/1000); rabbit anti-SYP61 at 1/1000 [9] or, rabbit anti-ECHIDNA at 1/1000 [11] for TGN detection; antibodies to detect contamination from other compartments (e.g., rabbit anti-Memb11 at 1/1000 for Golgi [24], rabbit anti-V-ATPase-E [Agrisera, AS07 213] at 1/2000 for vacuole, rabbit anti-PMA2 at 1/1000 for plasma membrane [25], rabbit anti-PM-ATPase at 1/1000 for plasma membrane [Agrisera, AS07 260]).	224
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	2. Secondary antibodies: goat anti-mouse IgG-HRP conjugate, goat anti-rabbit IgG-HRP conjugate.	233
		234
	3. 5× loading buffer: 200 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 5% (w/v) sodium dodecyl sulfate (SDS), 250 mM DTT, and 0.05% (w/v) bromophenol blue.	235
		236
		237
	4. 10% TGX Stain-Free FastCast Acrylamide Kit.	238
	5. <i>N,N,N'</i> -tetramethylethylenediamine (TEMED).	239
	6. 10% (w/v) ammonium peroxodisulfate (APS).	240
	7. Running buffer: 25 mM Tris-HCl, 192 mM glycine, 0.1% SDS.	241
		242
	8. SDS-PAGE molecular weight standards.	243
	9. Transfer buffer: 25 mM Tris-HCl, 192 mM glycine, 20% (v/v) ethanol.	244
		245
	10. TBS-T: 20 mM Tris-HCl, 140 mM NaCl, 2.5 mM KCl, 0.1% (v/v) Tween 20.	246
		247
	11. Blocking buffer: 5% (w/v) nonfat dried milk in TBS-T.	248
	12. Enhanced chemiluminescence (ECL) reagents.	249
	13. Polyvinylidene difluoride (PVDF) membrane.	250
	14. Blotting paper.	251
		252
<b>2.5 Fatty Acid Profiling (Fatty Acid Methyl Esters: FAMES)</b>	1. Hydrolysis solution: 5% H <sub>2</sub> SO <sub>4</sub> in methanol.	253
	2. Hexane 99%.	254
	3. 2.5% NaCl in H <sub>2</sub> O.	255
	4. 100 mM Tris-HCl, 0.09% NaCl, pH 8.	256
	5. Distillated H <sub>2</sub> O.	257

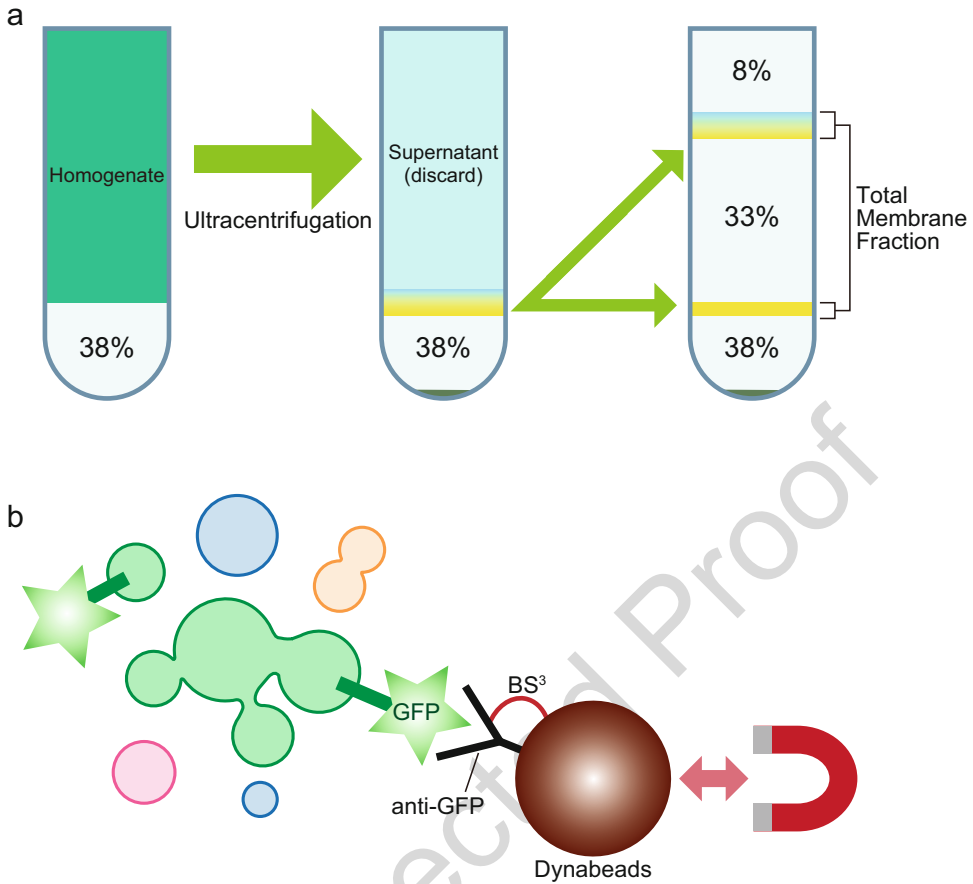
	6. 100 mM Tris-HCl.	258
	7. BSTFA-TMCS <i>N,O</i> -bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane.	259 260 261
<b>2.6 Sterol Profiling</b>	1. Ethanol.	262
	2. 11 N KOH.	263
	3. Hexane 99%.	264
	4. Distillated H <sub>2</sub> O.	265
	5. BSTFA-TMCS <i>N,O</i> -bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane.	266 267 268
<b>2.7 Lipid Standards</b>	1. Heptadecanoic acid (17:0).	269
	2. 2-hydroxytetradecanoic acid (h14:0).	270
	3. Cholestan-2-ol (also known as $\alpha$ -cholestanol). Optional:	271 272
	4. 1-Pentadecanol (OH-15:0).	273
	5. 15-hydroxypentadecanoic acid ( $\omega$ OH-15:0).	274 275
<b>2.8 Consumables</b>	1. Ice.	276
	2. 50 mL Falcon tubes.	277
	3. 10 mL and 25 mL disposable pipettes.	278
	4. 1.5 mL microcentrifuge tubes.	279
	5. 38 mL thin wall open-top ultracentrifugation tubes.	280
	6. Glass Pasteur pipettes.	281
	7. Screw glass tubes of 8 mL.	282
	8. Screw caps with Teflon seal.	283 284
<b>2.9 Equipment</b>	1. Shaker for flasks installed in plant incubator/growing facility.	285
	2. 1.5 mL tube rotator.	286
	3. Refrigerated centrifuge for centrifugation of 50 mL tubes in a swinging-bucket rotor.	287 288
	4. Ultraspeed refrigerated centrifuge for centrifugation of 38 mL tubes in a swinging—bucket rotor at $150,000 \times g$ (r max).	289 290
	5. Centrifuge for centrifugation of 8 mL glass tubes in a fixed-angle bucket rotor.	291 292
	6. Water bath.	293
	7. Dry bath.	294
	8. Spectrophotometer and cuvettes.	295
	9. Protein electrophoresis device.	296
	10. Power supply.	297

	11. Stain-Free enabled imager (e.g., Bio-Rad ChemiDoc MP imaging system).	298 299
	12. Protein transfer device.	300
	13. Air flow evaporator system for lipids.	301
	14. GC-MS simple Quadrupole system equipped with an autosampler, a mass detector, and a data processing station. An HP-5MS capillary column (5% phenyl-methyl-siloxane, 30 m, 250 mm, and 0.25 mm film thickness; Agilent) is used in our laboratory.	302 303 304 305 306 307
<b>2.10 Software</b>	1. MassHunter from Agilent.	308
	2. NIST database.	309
	3. Image J ( <a href="https://imagej.net/Fiji">https://imagej.net/Fiji</a> ).	310 311
<hr/>		
<b>3 Methods</b>		312
<b>3.1 Growing Arabidopsis Seedlings in Liquid Culture</b>	1. Prepare one 1.5 mL tube for each culture flask (usually 4 tubes for 4 flasks for one IP), and place approximately 50 $\mu$ L of seeds (30–40 mg of seeds) per tube.	313 314 315
	2. Sterilize the seed surface with 70% ethanol for 1 min.	316
	3. Remove the ethanol, add 1 mL of sterilization solution, and incubate for 20–30 min with continuous mixing in a tube rotator.	317 318 319
	4. Wash 3–4 times with sterile distilled H <sub>2</sub> O ( <i>see Note 2</i> ).	320
	5. Keep seeds in the dark, at 4 °C for 2 days in sterile distilled H <sub>2</sub> O.	321 322
	6. Pour 250 mL of MS liquid medium into each 500 mL baffled flask and sterilize at 110 °C for 30 min.	323 324
	7. After the medium has cooled down at room temperature, place the seeds into each flask. Incubate for 8–9 days under 120 rpm and long-day condition (16 h light/8 h darkness) at 22 °C ( <i>see Note 3</i> ).	325 326 327 328 329
<b>3.2 Membrane Extraction and Fractionation</b>	To obtain intact membrane compartments with high purity, we disrupt the seedlings in a detergent-free buffer, remove the debris, and perform a two-step process including a sucrose cushion and a sucrose step-gradient fractionation by ultracentrifugation. The samples should be kept at 4 °C or on ice during all the manipulations; precool buffers and tubes.	330 331 332 333 334 335
	1. Precool the vesicle isolation buffer, 50 mL Falcon tubes (for collecting the homogenate), mortar, and pestle in ice. Set the swing-rotor centrifuge at 4 °C.	336 337 338
	2. Collect and weigh the seedlings ( <i>see Note 4</i> ).	339

3. Transfer the seedlings into the mortar on ice, and grind them in 2–3 volumes (e.g., 20 g of seedlings in 60 mL) vesicle isolation buffer (*see Note 5*). 340–342
4. Set the funnel and gauze/Miracloth on the precooled 50 mL Falcon tube on ice. Harvest the homogenate with a disposable pipette and filter it through the gauze/Miracloth. Squeeze the waste to get as much material as possible. 343–346
5. Centrifuge the filtered homogenate at  $1600 \times g$  for 20 min at  $4^\circ\text{C}$ . Meanwhile, precool new 50 mL Falcon tubes in ice. 347–348
6. Transfer the supernatant to the precooled 50 mL Falcon tubes by decantation, and centrifuge at  $1600 \times g$  for 15 min at  $4^\circ\text{C}$ . In total, **step 5** should be performed 3–4 times. 349–351
7. Precool the 38% sucrose solution and 38 mL centrifuge tubes in ice. 352–353
8. Transfer 8 mL 38% sucrose solution to 38 mL centrifuge tubes, and load the last supernatant of **step 6** on it (*see Note 6*, Fig. 1). Ultracentrifuge at  $150,000 \times g$  for 3 h at  $4^\circ\text{C}$  with a swinging-bucket rotor. Precool the 33% and 8% sucrose solutions in ice. 354–357
9. The membrane fraction appears at the interface between the sucrose cushion and the supernatant as a yellow layer (Fig. 1). Remove the supernatant using a 10 mL pipette (*see Note 7*). On top of the membrane fraction, carefully load 15 mL of 33% sucrose solution, followed by 10 mL of 8% sucrose solution (*see Note 8*, Fig. 1). 358–363
10. Ultracentrifuge at  $150,000 \times g$  overnight (16 h) at  $4^\circ\text{C}$ . 364
11. Precool 50 mL Falcon tubes and new 38 mL centrifuge tubes in ice. Harvest separately the membrane fractions at the 38%/33% and 33%/8% interfaces using a Pasteur pipette in precooled 50 mL Falcon tubes (*see Note 9*). 365–368
12. Dilute the collected membrane fractions into 1/3 (v/v) of 50 mM HEPES, pH 7.5. Mix gently by inverting the tubes. Transfer it into the precooled 38 mL centrifuge tubes and ultracentrifuge at  $150,000 \times g$  for 3 h at  $4^\circ\text{C}$ . Meanwhile, prepare resuspension buffer and keep it on ice. 369–373
13. Discard the supernatant by decantation. Wipe carefully the inside of the centrifuge tubes with a paper towel without touching the pellet. Resuspend the pellet (microsomal fraction) with 0.5–1 mL resuspension buffer depending on the size of the pellet. 374–379

### 3.3 Protein Quantification in the Total Membrane Fraction

If there are multiple microsomal fractions (e.g., when comparing the membranes of drug-treated and nontreated plants or mutants versus wild-type), the concentration of membrane material should be equilibrated among samples. The membrane concentration is assumed to be proportional to the protein concentration. We use



**Fig. 1** Immunoprecipitation of intact endomembrane compartments. **(a)** Two-step membrane fractionation. Left: load the homogenate of *Arabidopsis* seedlings on a 38% sucrose cushion and perform ultracentrifugation. Middle: After ultracentrifugation, the membrane fraction appears at the interface as a yellow layer. Discard the supernatant above the membrane fraction using a glass pipette. Right: By stepwise loading of 33% and 8% sucrose solutions, the membrane fraction is divided into two layers at the 8%/33% and 33%/38% interfaces. After an additional ultracentrifugation step, harvest those layers separately or together (*see Note 9*). **(b)** Schematic representation of membrane immunoprecipitation principle. Compartments which harbor the protein bait fused to GFP are recognized by the anti-GFP antibody. The antibody is conjugated to magnetic beads (Dynabeads) and cross-linked by BS<sup>3</sup>. GFP-labeled compartments will be pulled down together with beads using magnetism

the bicinchoninic acid (BCA) protein assay kit to reliably quantify protein concentration and adjust samples accordingly. 385 386

1. Dilute 5  $\mu$ L of the microsomal fraction with 100  $\mu$ L of resuspension buffer (1/20 dilution). Prepare 2 tubes for each sample. 387 388 389
2. Measure the protein concentration of the diluted microsomal fractions using a BCA protein assay kit. Average the results of 390 391

	the 2 tubes for each sample and calculate the original concentration ( <i>see</i> <b>Note 10</b> ).	392 393
	3. Equilibrate the protein concentration of the microsomal fraction with the resuspension buffer to the one with the lowest concentration.	394 395 396 397
<b>3.4 Immunoprecipitation</b>	Conjugate the GFP antibody to Dynabeads by BS <sup>3</sup> , and immunoprecipitate the GFP-tagged compartments from the microsomal fraction. All the steps other than BS <sup>3</sup> conjugation should be performed at 4 °C or on ice.	398 399 400 401
	1. Prepare 150 µL of Dynabeads in a 1.5 mL tube for each IP ( <i>see</i> <b>Note 11</b> ).	402 403
	2. Wash the beads with cold 1 mL PBS-T by pipetting. Set the tubes on the magnetic tube holder, wait until the beads are pulled by the magnet, and remove the PBS-T.	404 405 406
	3. Add 500 µL PBS-T and cool the tubes on ice.	407
	4. Add 15 µL of anti-GFP and incubate for at least 1 h at 4 °C under continuous rotation by the tube rotator ( <i>see</i> <b>Note 12</b> ).	408 409
	5. Remove the buffer containing the antibodies and wash the beads with 500 µL of BS <sup>3</sup> conjugation buffer ( <i>see</i> <b>Note 13</b> ).	410 411
	6. Dissolve BS <sup>3</sup> into BS <sup>3</sup> conjugation buffer (BS <sup>3</sup> working solution, <i>see</i> <b>Note 14</b> ).	412 413
	7. Remove the buffer from the beads, and resuspend the beads with the BS <sup>3</sup> working solution. Incubate for 30 min at room temperature with rotation.	414 415 416
	8. Quench the reaction by adding 25 µL BS <sup>3</sup> quenching buffer. Incubate for 15 min at room temperature with rotation.	417 418
	9. Transfer the tubes to ice. Wash the beads with 500 µL of cold PBS-T three times.	419 420
	10. Resuspend the beads with 500–1000 µL of cold resuspension buffer, and cool them in ice for at least 10 min.	421 422
	11. Remove the buffer from the beads, and add 900 µL of the equilibrated microsomal fraction from Subheading <b>3.2</b> , <b>step 13</b> . Incubate for 1 h at 4 °C with continuous rotation ( <i>see</i> <b>Note 15</b> ).	423 424 425 426
	12. Remove the supernatant. Wash the beads with 1 mL of cold wash buffer four times, and finally resuspend in 70 µL of wash buffer; this is the IP output fraction ( <i>see</i> <b>Note 16</b> ). Store at –80 °C if you do not use the samples for further analysis in a short term.	427 428 429 430 431 432
<b>3.5 Controls for IPs</b>	To check the level of nonspecific background in immunoprecipitated samples, we advise to perform an IP from the microsomal fraction using beads without GFP antibody as negative control (skipping the steps Subheading <b>3.4</b> , <b>steps 4–10</b> for the preparation	433 434 435 436

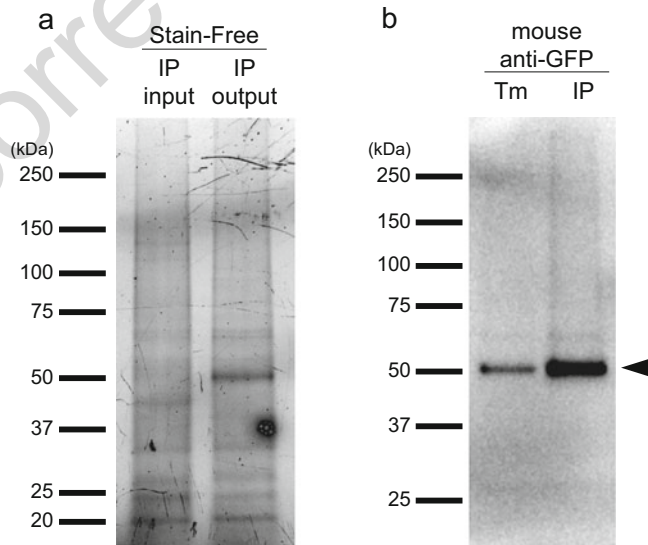
**3.6 Enrichment and Purity Assessment of the IP Fraction**

of beads). An alternative negative control can be obtained by doing an IP with GFP antibody-conjugated beads from a microsomal fraction extracted from wild-type plants that do not express any GFP-labeled protein bait.

The IP fraction should be checked by Western blotting to determine the enrichment level (the IP output fraction is compared with the microsomal IP input fraction) of the GFP-tagged compartment and contamination from other membranes. Primary antibodies appropriate for detection of SYP61 and other organelles are listed at Subheading 2.4, item 1 (see Note 17).

We used the TGX Stain-Free system from Bio-Rad, which enables rapid visualization of proteins by the fluorescence of tryptophan excited by UV light, to quantify the total amount of proteins. If the system is not available, Coomassie brilliant blue (CBB) staining is the standard alternative.

1. Add 5  $\mu$ L of 5 $\times$  loading buffer to 10  $\mu$ L of the IP output fraction or 5  $\mu$ L of the microsomal IP input fraction, adjust with 1 $\times$  loading buffer to get a final volume of 25  $\mu$ L in every tube (see Note 18).
2. Perform electrophoresis using TGX Stain-Free polyacrylamide gel.
3. Visualize the total protein profile using Stain-Free system (Fig. 2). Using the ImageJ software, define equally sized



**Fig. 2** Analysis of IP output fraction. (a) Stain-Free fluorescence image of a SDS-PAGE gel loaded with equal protein amounts of the IP input microsomal fraction (IP input) and IP output fraction (after beads incubation) using GFP-SYP61 as the IP bait. (b) Western blotting image displaying enrichment of TGN-SV in the IP output fraction as revealed by GFP-SYP61 detection by mouse anti-GFP antibody



boxes around each lane, analyze the signal intensity, and calculate the ratio of the total protein amount between samples. Dilute samples with  $1 \times$  loading buffer to equalize the protein concentration to that of the lowest one.

4. Perform a new SDS-PAGE of the equilibrated samples and transfer the proteins to PVDF membranes using standard protocols.
5. Perform immunoblotting using appropriate antibodies by standard protocols (Fig. 2). Use mouse anti-GFP antibodies to know whether the bait has been pulled-down (Fig. 2). We advise to use several other antibodies recognizing endogenous proteins of the targeted compartments (like ECHIDNA for SYP61/SVs-TGN) and antibodies against other organelle marker proteins to check for potential contaminations from other membranes. A list of appropriate antibodies is given in Subheading 2.4, item 1.

### 3.7 Lipid Extraction

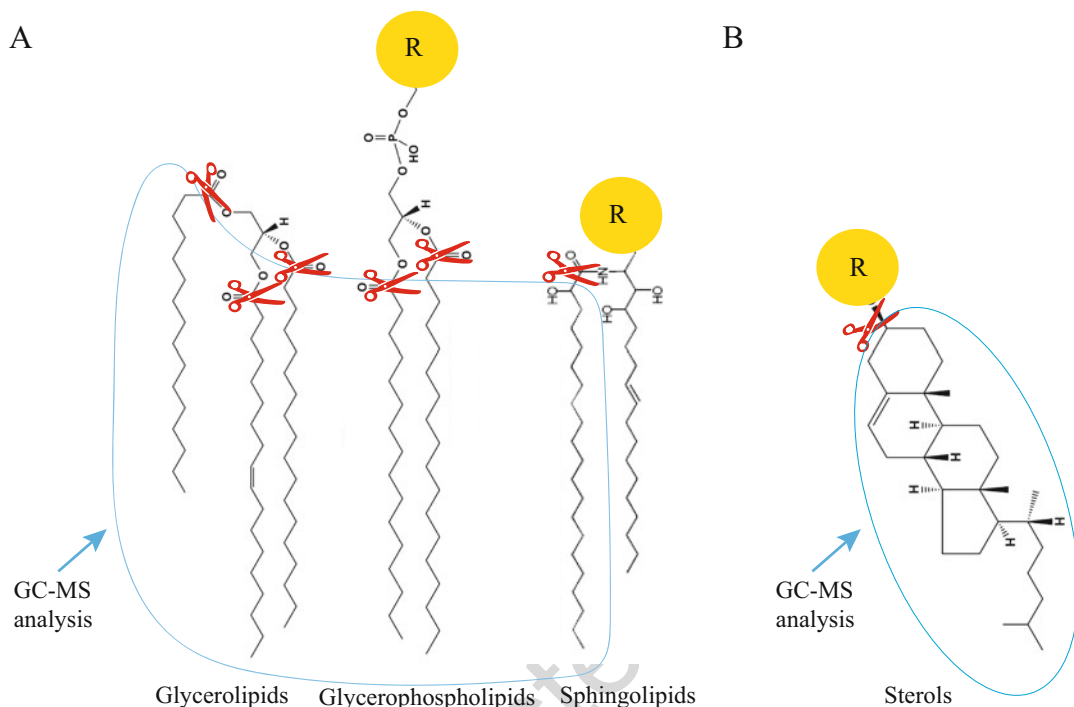
In each experiment, a negative control should be prepared following the protocols described below replacing the IP output fraction by IP wash buffer in **step 1**. In the negative control, only the standards should be detected by GC-MS.

In addition, a vial containing 100  $\mu\text{L}$  of hexane should be prepared as negative control for hexane contaminants. Use the same hexane as used in Subheading 3.7, **steps 1** and **2**. No components should be detected. In the case of contamination by plastics, alkanes will be detected, in that case, use new hexane and proceed with new lipid extraction.

#### 3.7.1 Total Fatty Acid Extraction (Fatty Acid Methyl Esters: FAMES)

1. Transfer 25–50  $\mu\text{L}$  of the IP output fraction in a screw glass tube, named tube A (*see Note 19*).
2. Add 1 mL of the hydrolysis solution containing 5  $\mu\text{g}/\text{mL}$  of internal standard 17:0 and h14:0 (*see Note 20*).
3. Incubate overnight at 85  $^{\circ}\text{C}$  in a dry bath, be sure that the contents of tubes are not evaporating by retightening the screw caps every 5–10 min during the first 30 min. This step aims at releasing the FAs of different lipid classes from their respective backbone (Fig. 3, *see Note 21*).
4. Remove the tubes from the dry bath and let them cool down.
5. Add 1 mL of 2.5% NaCl and then 1 mL of hexane.
6. Mix vigorously (*see Note 22*).
7. Centrifuge for 5 min at  $800 \times g$  at room temperature to separate the phases.
8. Prepare new screw glass tubes containing 1 mL of 100 mM Tris-HCl, 0.09%NaCl, pH 8, named tube B.



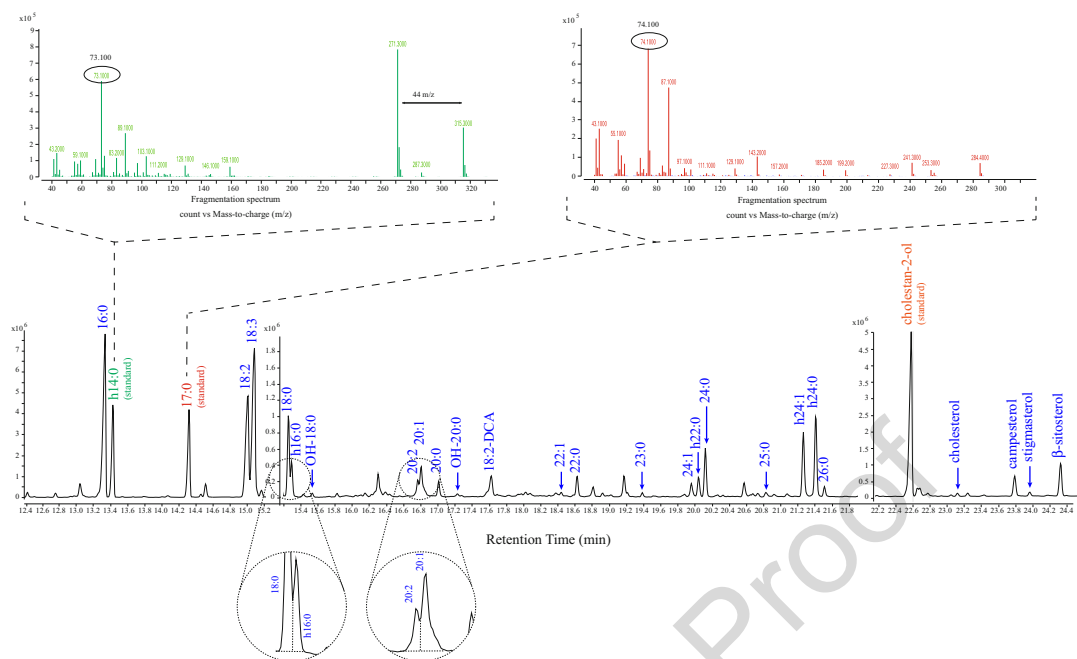


**Fig. 3** Schematic representation of the hydrolysis of fatty acids (FAs) from the lipid backbone. **(a)** FAs esterified on the glycerol backbone in glycerolipids and glycerophospholipids or amidified on the long chain base of sphingolipids are cleaved through acidic hydrolysis (Methanol + sulfuric acid, *see* Subheading 3.7.1, **step 2**). **(b)** Basic hydrolysis (Ethanol + KOH, *see* Subheading 3.7.2, **step 2**) cleaves esterified FAs chains grafted on a steroid backbone in the case of sterylesters, glycosylsterylesters, or acylated glycosylesters. Yellow circles are the radical groups esterified on glycerol backbone of glycerolipids and glycerophospholipids or esterified on the steroid backbone of sterols or amidified on the sphingoid backbone of sphingolipids; red scissors represent the cleavage of the ester or amid bonds; blue lines encircled the lipid part recovered after extraction and analyzed by GC-MS.

9. Collect the hexane upper phase (*see* **Note 23**) and transfer it to the tube B prepared in previous step. 505
10. Add 1 mL of hexane to the initial tube A. 506
11. Mix vigorously. 507
12. Centrifuge for 5 min at  $800 \times g$  at room temperature to separate the phases. 508
13. Collect the hexane upper phase and add it to tube B. 509
14. Mix vigorously the content of tube B. 510
15. Centrifuge for 5 min at  $800 \times g$  at room temperature to separate the phases. 511

- |                         |  |  |
|-------------------------|--|--|
| 16.                     | Recover gently the hexane upper phase taking care not to contaminate the sample with the lower aqueous phase ( <i>see Note 24</i> ) and transfer to a new glass tube.  | 515<br>516<br>517                      |
| 17.                     | Evaporate the hexane through air flow evaporator.  | 518                                    |
| 18.                     | Add 200 mL of BSTFA.   | 519                                    |
| 19.                     | Incubate for 15 min at 110 °C in a dry bath.   | 520                                    |
| 20.                     | Evaporate the BSTFA through air flux evaporator ( <i>see Note 25</i> ).  | 521<br>522                             |
| 21.                     | Once BSTFA is completely evaporated add 100 µL of hexane and close the lid immediately to avoid hexane evaporation.  | 523<br>524                             |
| 22.                     | Vortex.  | 525                                    |
| 23.                     | Transfer into GC vials containing a conical insert.  | 526                                    |
| 24.                     | Run the sample in a GC-MS instrument using the FAMES methods.  | 527<br>528<br>529                      |
| 3.7.2 Sterol Extraction | 1. Transfer 25–50 µL of the IP output fraction in a screw glass tube.  | 530<br>531                             |
|                         | 2. Add 1 mL of ethanol containing 5 µg/mL of cholestan-2-ol and then 100 µL of 11 N KOH; this is a saponification step which eliminates FAs. Please note that this method will not distinguish between sterols, sterylesters, sterylglycosides or acetylated sterylglycosides in which a FA chain and/or a glucose is grafted on a steroid backbone. | 532<br>533<br>534<br>535<br>536<br>537 |
|                         | 3. Incubate for 1 h at 80 °C in a dry bath.  | 538                                    |
|                         | 4. Let cool the samples.   | 539                                    |
|                         | 5. Add 1 mL of hexane.   | 540                                    |
|                         | 6. Vortex.   | 541                                    |
|                         | 7. Add 2 mL of distilled H <sub>2</sub> O.   | 542                                    |
|                         | 8. Mix vigorously.   | 543                                    |
|                         | 9. Centrifuge for 5 min at 800 × <i>g</i> at room temperature to separate the phases.  | 544<br>545                             |
|                         | 10. Recover gently the hexane upper phase taking care not to contaminate the sample with the lower aqueous phase ( <i>see Note 24</i> ).   | 546<br>547<br>548                      |
|                         | 11. Evaporate the hexane through air flow evaporator.  | 549                                    |
|                         | 12. Add 200 mL of BSTFA.   | 550                                    |
|                         | 13. Incubate for 15 min at 110 °C in a dry bath.   | 551                                    |
|                         | 14. Evaporate the BSTFA through air flux evaporator.   | 552                                    |
|                         | 15. Once BSTFA is completely evaporated add 100 µL of hexane ( <i>see Note 26</i> ).   | 553<br>554                             |
|                         | 16. Vortex.  | 555                                    |

	17. Transfer into GC vials containing a conical insert.	556
	18. Run the sample in GC-MS instrument using the sterol methods.	557 558 559
<b>3.8 GC-MS Analysis</b>		
<b>3.8.1 GC-MS Method</b>	1. Set the helium carrier gas at 2 mL/min.	560
	2. Use the splitless mode for injection.	561
	3. Set the temperatures of injector to 250 °C and the auxiliary detector to 352 °C.	562 563
	4. Set the oven temperature to be held at 50 °C for 1 min.	564
	5. Program a 25 °C/min ramp to 150 °C (2-min hold) and a 10 °C/min ramp to 320 °C (6-min hold).	565 566
	6. MS Analyzer is set in scan only with mass range 40–700 $m/z$ in positive mode with an electron emission set to 70 eV.	567 568
	7. The temperature of MS Source is set to 230 °C and the MS Quad to 50 °C.	569 570 571
<b>3.8.2 Identification of Peaks</b>	Quantification of FAMES and sterols was based upon peak areas that were derived from the total ion current. Each sample chromatogram is analyzed using the MassHunter software equipped with a NIST database. An automatic identification of the lipid species using algorithms developed by Agilent is recommended. However, manual integration could also be added to automatic integration results in order to consider lipids that are below the automatic threshold detection or lipids for which peaks would be too close to be separated by automatic detection (example in Fig. 4). An example of chromatogram resulting from a SYP61-GFP IP output fraction is given in Fig. 4, <i>see</i> also Table 1. The retention times given in Fig. 4 are only indicative as these values change depending on the column and age of the column. The major FAs 16:0, 18:2, and 18:3 are typical for glycerolipids (DAG, TAG) and glycerophospholipids (phospholipids) while the $\alpha$ -hydroxylated FAs (hFAs) are a specific feature of sphingolipids with h24:1 and h24:0 being enriched in SYP61 IP as described before [14]. Nonhydroxylated 24:0 FA is strongly present in the sphingolipid pool but could also be detected in the glycerophospholipid pool [14]. The fatty alcohols OH-18:0 and OH-20:0 as well as the dicarboxylic acid 18:2-DCA are typical from extracellular lipids such as cutin and suberin, which precursors are probably transported through the SYP61 compartment to reach the plasma membrane and be secreted in the apoplast.	572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596
<b>3.8.3 Calculation</b>	The relative quantification is obtained by normalizing the peak area of one lipid species to the appropriate internal standard area. Non-hydroxylated FAs, including dicarboxylic acids (DCAs), are normalized to 17:0; hFAs are normalized to h14:0. If other types of FAs, such as fatty alcohols or $\omega$ -hydroxyFAs ( $\omega$ -OH-FAs), are	597 598 599 600 601



**Fig. 4** Example of a typical FAMES and sterols chromatogram from a SYP61-GFP IP. Fragmentation spectrum of the  $\alpha$ -hydroxylated FA standard h14:0 is shown in green. The TMS group at  $m/z$  73.1 and the  $m/z$  44 differences between the two major peaks are specific features of the  $\alpha$ -hydroxylated FAs. Fragmentation spectrum of the nonhydroxylated FA standard 17:0 is shown in red. The TMS group is at  $m/z$  74.1, which is typical of nonhydroxylated FA. All FAs are shown in blue. The dotted circles show two peaks close to one another for which manual integration could be helpful. All other peaks were automatically integrated

found, quantify them relatively to OH-15:0 (1-pentadecanol) or 602  
 $\omega$ -OH-15:0 (15-hydroxypentadecanoic acid) standards, respec- 603  
tively. These standards will need to be added at Subheading 3.7.1, 604  
**step 2** (see Note 20). 605

After normalization to the standard, the value obtained is 606  
expressed in  $\mu\text{g}$  and then ng. This value could be further trans- 607  
formed in nmol using the specific molecular weight of the 608  
specific FA. 609

Different IP could yield different amount of material and thus 610  
different lipids quantity (in ng or nmol). We advise to always 611  
perform IPs with the same protein concentration of microsomal 612  
IP input fraction, and in the same volume. However, the yield of 613  
the IPs could still be different. Hence, we do not advise to compare 614  
directly the lipid quantity values (in ng or nmol) obtained from 615  
different IPs. Instead, these values should be normalized. A classical 616  
way is to normalize the lipid quantity values against the fresh or dry 617  
weight of starting seedling material. This is, however, not possible 618  
in this protocol as multiple steps of membrane fractionation are 619  
performed prior to the IP, potentially leading to different amounts 620  
of IP input material. Protein quantification of IP output fractions is 621  
still possible but not reliable as antibodies used for IPs and possibly 622

t.1 **Table 1**  
 List of FAs typically found in SYP61 IP fraction

AU2

t.2	FA short name	FA nomenclature name
t.3	16:0	Hexadecanoic acid
t.4	h14:0	2-hydroxytetradecanoate
t.5	17:0	Heptadecanoic acid
t.6	18:2	Octadecadienoic acid
t.7	18:3	Octadecatrienoic acid
t.8	18:0	Octadecanoic acid
t.9	h16:0	2-hydroxyhexadecanoate
t.10	OH-18:0	1-octadecanol
t.11	20:2	Eicosadienoic acid
t.12	20:1	Eicosenoic acid
t.13	20:0	Eicosanoic acid
t.14	OH-20:0	1-eicosanol
t.15	18:2-DCA	Octadecadiynoic acid
t.16	22:1	Docosenoic acid
t.17	22:0	Docosanoic acid
t.18	26:1	Hexacosenoic acid
t.19	23:0	Tricosanoic acid
t.20	24:1	Tetracosenoic acid
t.21	h22:0	2-hydroxydocosanoate
t.22	24:0	Tetracosanoic acid
t.23	25:0	Pentacosanoic acid
t.24	h24:1	2-hydroxytetracosenoate
t.25	h24:0	2-hydroxytetracosanoate
t.26	26:0	Hexacosanoic acid
t.27	h26:0	2-hydroxyhexacosanoate

the magnetic beads would interfere with most protein quantifica- 623  
 tion assays (*see Note 18*). Moreover, the protein–lipid ratio in 624  
 membranes is clearly variable from one endomembrane compart- 625  
 ment to another or from one experimental condition to another. 626  
 Hence, using the protein–lipid ratio as a way of normalizing lipid 627  
 quantities is clearly not the best approach. Instead, we suggest that, 628  
 for IP samples, the quantity of one specific FA species should be 629  
 normalized to the total amount of FAs found in the profile. The 630  
 value will then be expressed as ng% or nmol%. 631  
 632

## 4 Notes

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1. There are multiple products with different molecular weight for PVP. Use the one with the average molecular weight of 10,000. 634  
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2. Because we use large amounts of seeds, the culture can easily be contaminated with bacteria or fungi. Hence, seed sterilization should be performed carefully. 637  
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3. For IPs of intact membrane compartments, it is always preferable to work with fresh material. We advise not to keep plant material at  $-80^{\circ}\text{C}$  before starting the membrane extraction, fractionation, and IP. 640  
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4. Be careful not to let the seedlings dry during manipulation. Fresh weight should be at least 10 g, ideally 15–20 g. 644  
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5. It is easier to start grinding with a small volume of buffer (e.g., 25 mL), and add the rest during grinding. Because of the toxicity of PMSF, the manipulation should be carried out under a chemical fume hood. 646  
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6. Load the supernatant slowly and gently in order not to disrupt the interface between the sucrose solution and the supernatant. It is easier to load the first several milliliters with a P1000 pipetman. The thin wall open-top centrifuge tubes must be filled up to 2–3 mm from the tube top to avoid deformation of the tubes during ultracentrifugation. If the amount of the supernatant is not enough to fill the tubes, add vesicle isolation buffer. 650  
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7. When removing the supernatant, keep a thin layer of supernatant of approximately the same thickness of the yellow layer to avoid pipetting membranes. 658  
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8. By loading 33% sucrose solution, the membrane fraction is separated into two layers beneath and above the 33% sucrose solution. The upper fraction can accidentally get into the pipette during this process. To avoid it, keep the sucrose solution continuously flowing out of the pipette while loading. The loading of the two sucrose solutions should be performed carefully; similar to the previous step, use a P1000 pipetman to load the first several milliliters. Do not disrupt the interfaces. 661  
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9. Most of the small early endosomal compartments, TGN, and Golgi will be located at the 33%/8% interface, while an important part of the late endosomal compartments will be located to both 38%/33% and 33%/8% interfaces. Hence, it is advisable to check beforehand by Western blot in which interface the targeted compartment carrying the protein-GFP bait is most enriched. In any case, it is alternatively possible to combine the two fractions together. 669  
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10. The protein concentration of the original microsomal fraction should be at least 1 mg/mL, ideally around 2 mg/mL. If the concentration is lower than 1 mg/mL, we advise to start a new membrane extraction. 677  
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11. Before taking Dynabeads from the vial, resuspend the beads by vortex; beads sink to the bottom of the vial. 681  
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12. To thoroughly mix beads and antibodies, invert the tubes several times before setting the tubes on the rotator. The incubation can be overnight. 683  
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13. Collect the beads sticking on the lid of the tubes. 686
14. Prepare this solution freshly. 687
15. The incubation should neither be longer nor shorter than 1 h. 688
16. The IP output fraction contains the immunopurified membranes bound to the magnetic beads. As the beads sediment in the tube, homogenize by pipetting before using the IP output fraction for subsequent steps. 689  
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17. Because the IP is performed by using rabbit anti-GFP IgG, rabbit primary antibodies should not be used for the detection of GFP or proteins of similar molecular weight to IgG. 693  
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18. These amounts are suggested as a reference. Alternatively, protein quantification using a BCA protein assay could be performed. However, this method might be less reliable than visualizing the total protein profile on the TGX Stain-Free system due to (1) the presence of antibodies used for the IPs which create a bias in the protein quantification while they could easily be spotted on an SDS-PAGE, (2) the beads could interfere on their own with the protein quantification assay. 696  
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19. The beads are resistant to the hydrolysis solution and do not generate any molecules detectable by GC-MS. 704  
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20. The 17:0 standard is used to quantify the most common FAs coming from the glycerolipid and glycerophospholipid pool. The h14:0 standard is used to quantify the  $\alpha$ -hydroxylated (2-hydroxy)-FAs coming exclusively from the sphingolipid pool. Dicarboxylic acids (DCA) are FAs typical of the suberin and cutin lipids, which could be found in the FAs profile; quantification of DCA compounds is made with the 17:0 standard. Other types of FAs, such as fatty alcohols or  $\omega$ -hydroxyFAs, need to be quantified relative to OH-15:0 (1-pentadecanol) or  $\omega$ -OH-15:0 (15-hydroxypentadecanoic acid) standards, respectively; add 5  $\mu$ g of each in the hydrolysis solution. 706  
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21. Depending on the type of chemical bounds between FAs and the backbone, the hydrolysis will occur at different efficiency (Fig. 3). FAs of glycerolipids and glycerophospholipids are 718  
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- esterified on the glycerol backbone while FAs of sphingolipids are amidified on the sphingoid backbone, which is more difficult to hydrolyze than an ester bound. Hence, the overnight incubation is necessary to correctly visualize the FAs coming from the sphingolipid pool. Please note that FAs esterified on a steroid backbone, such as in the case of sterylesters, glycosylesters, or acetylglycosylesters, will also be hydrolyzed and thus be part of the FAs pool detected in the profiling.
22. Shake tube for homogeneous mixing. Vortex only may not result in efficient mixing.
  23. Accidental collection of small amounts of the lower aqueous phase does not matter at this stage, which is aimed at adjusting the pH of the hexane phase.
  24. Contrary to Subheading 3.7.1, step 9, it is important not to collect any of the lower aqueous phase as this will interfere with Subheading 3.7.1, step 18 resulting in misleading chromatogram results and may destroy the GC column. If some of the aqueous phase was accidentally collected, a white residue coming from the hydrolysis of trimethylsilyl (TMS) molecules that release silicon atoms, will appear in Subheading 3.7.1, step 20.
  25. If a white residue appears in the tube, add 1 mL of hexane and then 1 mL of 100 mM Tris-HCl, 0.09% NaCl, pH 8 and resume at Subheading 3.7.1, step 14.
  26. If a white residue appears in the tube, add 1 mL of hexane and then 1 mL of distilled water and resume at Subheading 3.7.2, step 8.

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# Author Queries

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