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TITLE:

Preparation of Chloroplast Sub-compartments from Arabidopsis for the Analysis of Protein Localization by Immunoblotting or Proteomics

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KEYWORDS:

Plant, Arabidopsis, chloroplast, organelle, plastid, envelope, stroma, thylakoid, cell fractionation, Percoll, localization, immunoblotting

SUMMARY:

Here, we describe a method to purify intact chloroplasts from Arabidopsis leaves and their three main sub-compartments (envelope, stroma, and thylakoids), using a combination of differential centrifugations, continuous Percoll gradients, and discontinuous sucrose gradients. The method is valuable for subplastidial and subcellular localization of proteins by immunoblotting and proteomics.

ABSTRACT:

Chloroplasts are major components of plant cells. Such plastids fulfill many crucial functions, such as assimilation of carbon, sulfur and nitrogen as well as synthesis of essential metabolites. These organelles consist of the following three key sub-compartments. The envelope, characterized by two membranes, surrounds the organelle and controls the communication of the plastid with other cell compartments. The stroma is the soluble phase of the chloroplast and the main site where carbon dioxide is converted into carbohydrates. The thylakoid membrane is the internal membrane network consisting of grana (flat compressed sacs) and lamellae (less dense structures), where oxygenic photosynthesis takes place. The present protocol describes step by step procedures required for the purification, using differential centrifugations and Percoll gradients, of intact chloroplasts from Arabidopsis, and their fractionation, using sucrose gradients, in three sub-compartments (*i.e.,* envelope, stroma, and thylakoids). This protocol also provides instructions on how to assess the purity of these fractions using markers associated to the various chloroplast sub-compartments. The method described here is valuable for

subplastidial localization of proteins using immunoblotting, but also for subcellular and subplastidial proteomics and other studies.

INTRODUCTION:

Chloroplasts are major components of plant cells. They derive from a cyanobacterial ancestor that has undergone an endosymbiosis and eventually evolved as an organelle during evolution[1,2]. Such organelles contain three main compartments (**Figure 1**). The envelope system is made of an inner and an outer membranes surrounding the organelle. This double membrane system contains various enzymes involved in the metabolism of lipids and pigments and is mostly devoted to the control of the communication between plastids and the cytosol. It contains various transport systems that allow the import of nuclear-encoded proteins, and the exchange of ions and metabolites between the cytosol and the chloroplast thus regulating essential metabolic functions of the plant cell[3,4]. The stroma, the soluble phase of the chloroplast, contains enzymes of the Calvin cycle (CO_2 assimilation), the synthesis of various metabolites including amino acids and vitamins, and the transcription and translation machineries of the plastid. The thylakoid membrane is a widely organized internal membrane network where the light phase of photosynthesis takes place. Thereby, chloroplasts are the place where essential metabolic pathways occur[5].

In order to decipher new regulatory mechanisms that control the chloroplast dynamics and physiology, defining the sub-plastidial localization of chloroplast proteins is thus critical to support targeted studies aiming to better understand proteins functions in model organisms[6]. In order to get access to the genuine subplastidial localization of these proteins, it is thus essential to start from highly pure subplastidial fractions (envelope membranes, stroma, and thylakoids). In this context, the aim of the present protocol is to purify intact chloroplasts from Arabidopsis leaves using differential centrifugations and continuous Percoll gradients, and to fractionate them using discontinuous sucrose gradients, in three sub-compartments (*i.e.,* envelope, stroma, and thylakoids). The method described here also provides instructions to assess the purity of purified sub-organellar fractions using markers associated to the various chloroplast sub-compartments. This protocol is valuable for subplastidial localization of proteins using immunoblotting and for further analysis of purified fractions using mass spectrometry (MS)-based proteomic studies.

PROTOCOL:

1. Preparation of Buffers, Stock Solutions, and Gradients

1.1. Prepare the following stock solutions that can be stored up to 6 months at 4 °C.

1.1.1. Prepare 1 L of Tricine buffer (1 M, pH 8.4) and Tricine buffer (1 M, pH 7.6). Adjust pH by adding KOH pellets.

1.1.2. Prepare 1 L of ethylenediaminetetraacetic acid (EDTA, 0.5 M, pH 8) and 3-(N-morpholino) propane sulfonic acid (MOPS) buffer (1 M, pH 7.8). Adjust pH by adding NaOH pellets.

1.1.3. Prepare 50 mL of $MgCl_2$ (1 M).

1.1.4. Prepare 50 mL of protease inhibitors solutions: phenylmethylsulfonyl fluoride (PMSF set up in isopropanol, 100 mM), benzamidine hydrochloride hydrate (100 mM), and ε-amino caproic acid (50 mM).

Note: While PMSF and amino caproic acid are stable in solution for months at 4 °C, benzamidine solution should be stored at -20 °C.

1.2. Prepare the following solutions the day before the experiment and store all solutions at 4 °C.

1.2.1. Prepare 4 L of grinding medium pH 8.4 containing Tricine-KOH (20 mM, pH 8.4), sorbitol (0.4 M), EDTA (10 mM, pH 8), and $NaHCO_3$ (10 mM). Adjust pH by adding NaOH pellets. Add bovine serum albumin (BSA) at 0.1% (w/v) just before use and mix well.

1.2.2. Prepare 500 mL of washing medium (2 x) pH 7.6 containing Tricine-KOH (20 mM, pH 7.6), sorbitol (0.8 M), $MgCl_2$ (5 mM), and EDTA (2.5 mM). Adjust pH by adding NaOH pellets. Dilute such solution after preparation of Percoll gradient solution to obtain washing medium (1 x).

1.2.3. Prepare 200 mL of Percoll gradient solution for chloroplast purification by mixing Percoll with washing medium (2 x) at an equal volume to get a final solution at 50% (v/v) Percoll / 0.4 M sorbitol.

1.2.4. Prepare 50 mL of sucrose solutions for chloroplast fractionation by mixing MOPS (10 mM, pH 7.8), $MgCl_2$ (4 mM), and different concentrations of sucrose (0.3 M, 0.6 M, and 0.93 M).

1.3. Prepare the following gradients and buffers prior to starting the experiment.

1.3.1. Prepare six tubes of Percoll gradients (each containing 30 mL of a 50% Percoll / 0.4 M sorbitol) by centrifugation at 38,700 x g for 55 min at 4 °C. Keep the brake off to prevent blending of the gradients. After centrifugation, store the tubes containing the preformed gradients in a cold room until use.

1.3.2. Prepare four tubes of sucrose gradients, with each gradient formed of three following sucrose layers: 3 mL of 0.93 M, 2.5 mL of 0.6 M, and 2 mL of 0.3 M sucrose. Carefully overlay each layer, using a peristaltic pump starting with 0.93 M at the bottom and finishing with 0.3 M at the top.

1.3.3. Prepare 50 mL of hypotonic medium for chloroplast lysis containing MOPS (10 mM, pH 7.8), $MgCl_2$ (4 mM), PMSF (1 mM, set up in isopropanol), benzamidine hydrochloride hydrate (1 mM), and ε-amino caproic acid (0.5 mM). Store the buffer on ice until use.

1.3.4. Prepare 50 mL of membrane washing buffer containing MOPS (10 mM, pH 7.8), PMSF (1 mM), benzamidine hydrochloride hydrate (1 mM), and ε-amino caproic acid (0.5 mM). Store the buffer on ice until use.

2. Growth and Harvesting of Arabidopsis Leaves

2.1. For growth of Arabidopsis plants, prepare 4 large plastic pans (for a total surface of 0.5 to 1 m^2) of Arabidopsis plants by sowing 30 mg of seeds in each pan. Grow Arabidopsis plants for 5 weeks at 12-h light cycle at 23 °C (day) / 18 °C (night) with a light intensity of 150 µM m^{-2} s^{-1}.

2.2. Incubate plants in a dark and cold room (4 °C) overnight prior to the experiment (to reduce the amount of starch granules in chloroplasts).

2.3. Pre-weigh a 1 L beaker and then place it on ice before starting harvesting of leaf material.

2.4. Harvest Arabidopsis leaves by avoiding soil (compost). Re-weigh the beaker and record the tissue weight.

Note: 400 to 500 g of leaf material are expected from four pans.

2.5. Homogenize leaves in a cold room with 2 L of grinding buffer (add BSA before use) three times/2 s each time, in a blender at high speed.

2.6. Filter the homogenate in a cold room using 4 layers of muslin and one layer of nylon blutex. Gently squeeze the homogenate leaves inside the muslin/nylon blutex to extract all the liquid.

2.7. Recover the remaining tissue in the blender cup for a second extraction. Repeat steps 2.5 and 2.6 using 2 L of grinding medium and new 4-5 layers of muslin (in a cold room).

3. Purification of Crude Chloroplasts Using Differential Centrifugation

3.1. Equally distribute the crude cell extract into six 500 mL bottles and place the bottles on ice before centrifugation. Centrifuge for 2 min as soon as the maximum speed (2,070 x g) is reached (maximum acceleration and brake on, 4 °C).

3.2. Gently discard the supernatant.

3.3. Aspirate the remaining supernatant using a water pump and keep the pellets containing concentrated crude chloroplasts on ice.

3.4. Gently resuspend pellets by adding a minimal volume of washing medium (1 x) (final volume of the combined chloroplast suspensions = 36 mL) using a paintbrush or a curved plastic spatula. Use a 10 mL pipet to add 3 mL of washing medium in each bottle.

Note: Do not use pipet with very fine tips to avoid breakage of chloroplasts. Alternatively, cut the blue tip of a pipet with a razor blade to generate a larger hole.

3.5. Collect the resuspended chloroplasts in one tube by using a 10 mL pipet. Gently mix by inverting the tube to obtain a homogenous suspension prior to loading on Percoll gradients.

4. Purification of Intact Chloroplasts on Continuous Percoll Gradient

4.1. Slowly load 6 mL of the chloroplast suspension on top of each of the six Percoll gradients using a 10 mL pipet to avoid breakage of chloroplasts.

4.2. Centrifuge the gradients for 10 min at 13,300 x g, 4 °C using a swinging-bucket rotor.

Note: The acceleration should be slow, and the brake should be disconnected (brake off or slow deceleration) to prevent blending of the Percoll gradients.

4.3. Aspirate the upper phase that contains broken chloroplasts and intact mitochondria using a water pump, and then retrieve intact chloroplasts present in the lower phase (the broad dark-green band) with a 10 mL pipet. Be careful not to aspirate nuclei and cell debris (found at the bottom of the tube) with the intact chloroplasts (**Figure 2A**).

4.4. Dilute 3-4-fold the intact chloroplast suspension with washing buffer (1 x). Centrifuge for 2 min as soon as the maximum speed (2,070 x g, 4 °C) is reached (maximum acceleration and brake on).

4.5. Carefully discard the supernatant.

4.6. Completely aspirate the remaining supernatant with a water pump and keep the pellet of concentrated intact chloroplasts on ice.

4.7. Before chloroplast lysis, keep an aliquot of intact chloroplast fraction in approximately 1 mL of washing medium (1 x) for further analyses using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Keep a small aliquot of these intact chloroplasts for determination of protein concentration. Store the intact chloroplast fraction in liquid nitrogen for further experiments.

5. Lysis of Intact Chloroplasts Using a Hypotonic Buffer and Purification of Chloroplast Sub-Compartments on Discontinuous Sucrose Gradients

5.1. Lyse the purified intact chloroplasts by resuspending the pellet in hypotonic medium that contains protease inhibitors (the final volume should not exceed 12 mL).

Note: From this step, the use of pipet with fine tips (blue tips) is possible since intactness of chloroplasts is no more essential (pipetting chloroplasts up and down as long as pellet is not

entirely resuspended). Arabidopsis chloroplasts are very fragile (when compared to pea chloroplasts, for example) and their lysis is almost immediate after incubation in hypotonic medium.

5.2. Slowly load 3 mL of the lysed chloroplasts on the top of each preformed sucrose gradients using a peristaltic pump.

5.3. Ultracentrifuge the gradients for 1 h at (70,000 x g, 4 °C). Balance pairs of tubes using hypotonic medium buffer prior to centrifugation.

5.4. Carefully recover the soluble stromal proteins by pipetting the upper phase of the gradient (3 mL from each gradient) (**Figure 2B**). Take an aliquot for determination of protein concentration[7]. Store the stroma in liquid nitrogen for further experiments.

5.5. Aspirate the remaining upper phase of each gradient up to the yellow band using a water pump.

5.6. Retrieve the yellow band (the envelope) with a pipet (approximately 1 mL from each gradient). Pool the envelopes in one tube.

5.7. Remove the remaining phase of each gradient up to the thylakoid pellet using a water pump.

6. Washing and Concentration of Thylakoid and Envelope Membrane Systems

6.1. Resuspend the thylakoid pellets (green pellets) in a minimum volume (2 mL) of membrane washing buffer (1 x) (with protease inhibitors).

6.2. Dilute the envelope and thylakoid suspensions 3-4-fold in membrane washing medium (adjust volume to 10 mL) and ultracentrifuge for 1 h at (110,000 x g, 4 °C). Balance pairs of tubes using membrane washing buffer prior to centrifugation.

6.3. Carefully aspirate the supernatants using a water pump.

6.4. Add approximately 100 µL of membrane washing buffer (with protease inhibitors) to the envelope pellet. Take an aliquot for determination of protein concentration[7]. Store the purified envelope membrane preparation in liquid nitrogen.

6.5. Resuspend thylakoids pellet in 3 mL of membrane washing buffer (with protease inhibitors). Take an aliquot for determination of protein concentration[7]. Store thylakoid membrane fraction in liquid nitrogen.

REPRESENTATIVE RESULTS:

Successive steps of the procedure resulting in purified chloroplast and their sub-compartments are resumed in **Figure 2**. The Percoll gradient (**Figure 2A**) allows distinguishing intact chloroplasts from broken chloroplast and mitochondria (top of the gradient) or nuclei and cell debris (bottom of the gradient). After rupture of the Percoll-purified organelles thanks to an osmotic shock, the resulting fractions are separated on a sucrose gradient (**Figure 2B**). The stroma (soluble part of the chloroplast) is floating at the surface of the sucrose gradient. The light envelope membrane vesicles are recovered as a discrete yellow band at the 0.6/0.93 M sucrose interface. The heaviest thylakoid membranes vesicles are concentrated at the bottom of the tube. After recovery, washing and concentration of the two membrane fractions, proteins are quantified and the composition of all four fractions is analyzed on an SDS-PAGE (**Figure 2C**). The lanes are loaded on an equal protein basis (20 µg of each purified fraction). Knowing that chloroplasts contain only 1% of envelope proteins and 50% of proteins from the stroma or from the thylakoids, this tends to overestimate cross-contamination of purified envelope preparations with other chloroplast sub-compartments. However, this method allows to detect minute amounts of proteins cross-contaminating the envelope fraction. Markers from each compartment (*i.e.,* abundant proteins) are very helpful in evaluating the cross contamination of the fractions. Indeed, the thylakoid and envelope membrane fractions are expected to contain very low amounts of the large subunit of RuBisCO (RBCL), the most abundant protein from the stroma (50 kDa). Broken chloroplasts can easily be distinguished from intact chloroplast due to the loss of this stromal protein[8]. The light harvesting complex proteins (LHCP) are 25-kDa abundant thylakoid components that should barely (less than 3%) contaminate envelope membranes[9]. Finally, the phosphate-triose-phosphate translocator (TPT) is a 30-kDa protein that is only visible in the purified envelope fraction due to its strong enrichment (*i.e.,* 50 to 100 x) in the envelope fraction when compared to whole chloroplast extracts. Using the method described here, chloroplast sub-compartments are generally poorly cross-contaminated as confirmed using western-blot analyses (**Figure 2D**) relying on antibodies directed against known markers of all three sub-compartments: the soluble ketol-acid reductoisomerase (KARI) from the stroma, the chloroplast envelope copper ATPase (HMA1), and the light harvesting complex proteins (LHCP) from the thylakoid membranes. Cross-contamination of the three sub-compartments can be quantified using both immunoblotting and mass spectrometry analyses[9]. While the stroma is usually not contaminated by envelope or thylakoid fractions, the purified envelope fractions contain 3% of thylakoid proteins and up to 10% of proteins from the stroma. Proteins from the stroma poorly contaminate the thylakoid membranes (less than 1%) but thylakoids contain up to 3% of envelope membrane proteins. More than having a crucial role in identifying the genuine subplastidial location of chloroplast proteins, the present method thus also limits erroneous conclusions about subplastidial localization of proteins resulting from cross contaminations.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative scheme of chloroplast sub-compartments.

Figure 2: Purification of intact chloroplasts and their three main sub-compartments using Percoll and sucrose gradients. A. Percoll gradient allowing separation of broken and intact chloroplasts. **B**. Sucrose gradient allowing separation of stroma, envelope, and thylakoid

fractions. **C**. Representative SDS-PAGE of proteins from intact chloroplasts and their three main sub-compartments allowing to visualize abundant markers from each sub-compartment**.** Each lane contains 10 µg of proteins. Molecular weight markers: RBCL, large subunit of RuBisCO (marker for the stroma); TPT, Phosphate/triose-phosphate translocator (marker for the envelope); LHCP, light harvesting complex proteins (marker for the thylakoid). **D**. Western-blot experiments allowing to detect specific markers (using specific antibodies) from each sub-compartment: the chloroplast envelope copper ATPase HMA1[10], the light harvesting complex proteins LHCP from the thylakoid membranes[11], and the ketol-acid reductoisomerase KARI from the stroma[9].

DISCUSSION:

The present article aims to detail the step by step protocol used to purify chloroplasts (and their sub-compartments) from *Arabidopsis thaliana*. Since the availability of its complete genome sequence almost two decades ago, and of large collections of insertion mutants made available to the community, Arabidopsis is now widely accepted as a model plant. However, while this plant was perfectly adapted for genetic approaches, plant scientists needed to adapt biochemical and physiological tools to this emerging model. Protocols allowing to purify photosynthetically active chloroplasts from leaves of well-established biochemical models like spinach[12] or pea[13] thus had to be adapted. The first method describing purification of Arabidopsis chloroplasts was published in 1998[14], just before the release of the Arabidopsis genome sequence. Several years later, simple methods for isolating Arabidopsis chloroplasts compatible with studies aiming to analyze *in vitro* import of proteins in purified organelles were made available[15,16]. However, these methods did not allow to combine high level of purity and preservation of photosynthetic activity of the purified chloroplasts. More recently[17], a rapid method was established, which relies on the use of Percoll gradients, and allows to retain almost 90% of the photosynthesis rate measured in the starting leaves of Arabidopsis.

The protocol described here allows to purify Arabidopsis chloroplasts at an excellent level of purity. Indeed, immunological detection of contaminants from other cell compartments demonstrated that the purified organelles are devoid of mitochondrial and plasma membrane markers[9, 10]. This protocol was also efficient to purify chloroplasts from several Arabidopsis ecotypes[18], like Columbia (Col) or Wassilewskija (WS), *i.e.,* the ecotypes that were used for genome or expressed sequence tags (ESTs) sequencing projects but also to generate T-DNA insertion mutants in Arabidopsis. In other words, when proteomics studies have to be performed, the present protocol is compatible with these two reference ecotypes from Arabidopsis. Finally, the yield of chloroplasts using the present protocol is similar to the one obtained when starting from spinach or pea leaves (*i.e.,* 3%, as measured from the chlorophyll content in the Percoll-purified chloroplast when compared to the total chlorophyll amount present in starting leaves). In terms of proteins, the yield is close to 50 mg of chloroplast proteins, when organelles are purified from 500 g of 5-week-old Arabidopsis leaves.

To reach such a good yield (and chloroplast integrity), one should however pay special attention to several steps when using the present protocol. The chloroplast in Arabidopsis is an extremely fragile structure (this is not the case for pea chloroplasts, for example). Specific attention is thus

required in order to avoid large-scale rupture of the organelles during purification. The number and size of starch granules present in chloroplasts are critical for the preparation of intact chloroplasts. Indeed, chloroplasts containing large starch grain will generally be broken during initial differential centrifugations steps aiming to concentrate the crude chloroplast fractions[12]. Therefore, the plants should be kept overnight in a dark and cold room (4 °C) prior to the experiment, to reduce the amount of starch.

New users of the present protocol could be tempted to start from larger amounts of leaf material (huge rosettes from old Arabidopsis plants with larger leaves) trying to enhance the recovery of purified chloroplasts. However, in our hands, starting from young leaves (5-week-old) is the best compromise to combine yield, purity, and integrity of the purified organelles. Indeed, too old leaves are highly enriched in phenolic compounds that were shown to have a negative impact on chloroplast integrity[19].

Finally, the initial extraction step (grinding of the tissue) is another critical step. The blending process must be limited to few seconds. As stated above, new users might be tempted to use longer blending, thus expecting to strongly improve the yield of purified organelles. However, if longer blending effectively releases more material from leaves, it appears that the proportion of broken chloroplasts rapidly increases in the crude cell extract. Due to this high ratio of broken to intact chloroplasts in the medium, further purification steps (separation on Percoll gradients) are strongly affected and the yield of the purification is unexpectedly lower.

Availability of specific protocols to purify organelles have allowed a series of high throughput proteomics-based experiments to be conducted on chloroplast samples. These data were made available in several public databases[6], thus providing to biologists in the field an accurate subcellular (and subplastidial) localization for many chloroplast proteins. This was especially true for envelope proteins whose identity and location remained mostly unknown before these analyses, since envelope membranes represent a minor chloroplast component (1–2% of the chloroplast proteins) while playing a key role in chloroplast metabolism and biogenesis[5,20]. Using the protocol described here, we recently analyzed the composition of the three main chloroplast compartments from Arabidopsis (*i.e.,* the stroma, the thylakoids, and the envelope membrane system)[9]. Based on a semi-quantitative proteomics approach (spectral counting), we were able to assess the partitioning of hundreds of proteins in these three chloroplast compartments.

While the present protocol allows to purify the three main compartments of the chloroplast from Arabidopsis, it is also possible to distinguish additional sub-compartments in the chloroplast. Indeed, the envelope membrane system is made of the inner and the outer envelope membranes (**Figure 1**). However, to the best of our knowledge, a method to purify inner and outer envelope membranes from Arabidopsis chloroplasts remains to be established. Inner and outer envelope membranes can be purified from spinach[21] or pea[22] chloroplasts. The main limitation of Arabidopsis mostly results from the limiting amounts of starting material. Starting from 500 g of Arabidopsis leaves (which already requires 1 m^2 surface in a growth chamber) allows purifying only 100 µg of envelope proteins. On the other hand, it is easy to start with 5-10 kg of spinach

leaves from the market, to purify large amount of chloroplasts[8] and to end with a yield of 3 to 10 mg of envelope proteins from this material.

The same is true for thylakoid sub-compartments. Indeed, thylakoids are made of light membrane vesicles (lamellae) and dense structures (grana) (**Figure 1**). Specific protocols are available to distinguish these two compartments in Arabidopsis[23,24]. Again, based on a quantitative proteomics analysis, we recently inventoried the proteins present in these two sub-compartments[24]. These approaches, together with an in-depth investigation of the literature, allowed validating, or proposing hypotheses for, the subplastidial location of hundreds of thylakoid proteins. However, it is important to note that additional membrane microdomains are present at the curved margins of thylakoids. These lipoprotein sub-compartments, or plastoglobules, are permanently coupled to thylakoid membranes and contain a specific set of proteins[25]. Using the present protocol, it is thus not possible to distinguish these specific proteins from other thylakoid components.

Some genuine (well-known) envelope, stroma, or thylakoid components are still lacking from the lists of detected proteins. Together with targeted biochemical and immunological analyses, the continual improvement of MS sensitivity will be of great help to revisit the chloroplast content towards a complete repertoire of the composition of its various sub-compartments.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to download Figure Figure-1-Bouchnak-12-July-2018.tiff

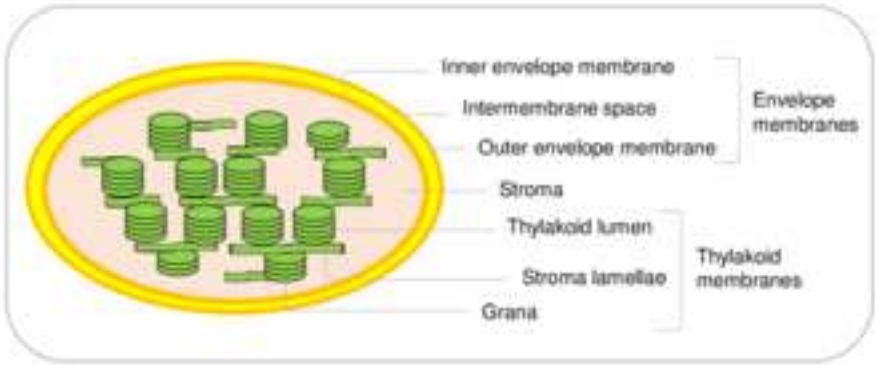

Figure 2

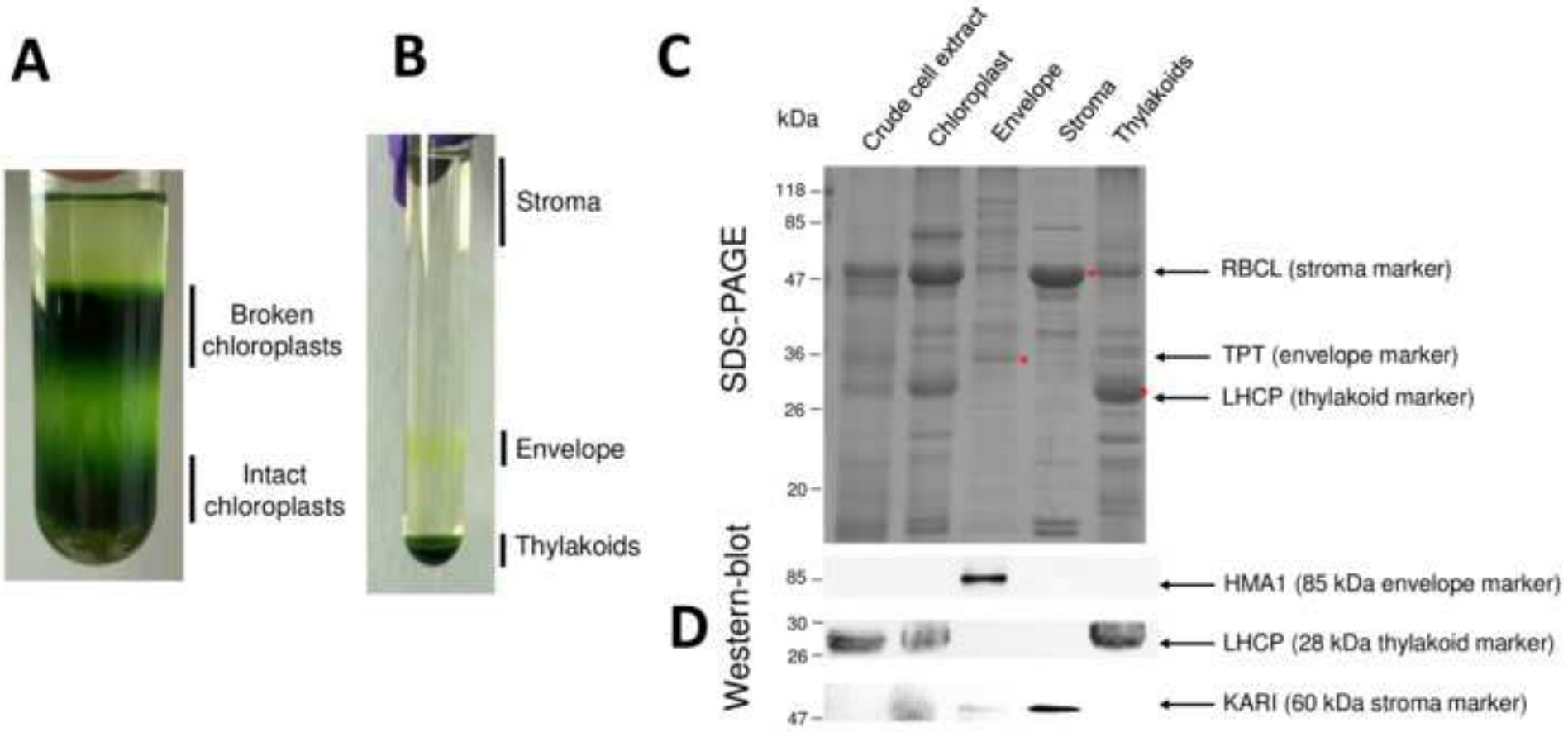

Table of Materials

Click here to download Table of Materials Bouchnak et al_JoVE_Materials_12 July 2018.xls

Name of Material/ Equipment	Company	Catalog Number
Percoll	GE Healthcare	17089101
Tricine	Roth	6977.2
Sorbitol	Roth	6213.1
Ethylenediaminetetraacetic acid (EDTA)	Promega	H5032
$NaHCO_3$	Roth	8551.1
Bovine serum albumin (BSA)	Roth	8076.5
$MgCl_2$	Roth	2189.1
Phenylmethylsulfonyl fluoride (PMSF)	Sigma	P7626
Benzamidine	Sigma	B6506
ε-amino caproic acid	Fluka	21530
3-(N-morpholino) propane sulfonic acid (MOPS)	Roth	6979.3
Sucrose	Roth	9286.2
Acrylamide stock: 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide	Roth	3029.1
Tris	Fisher	BP152-5
Sodium dodecyl sulfate (SDS)	Roth	1057.1
Tetramethylethylenediamine (TEMED)	Sigma	T-8133
Ammonium persulfate (APS)	Roth	9592.1
Glycerol	Roth	3783.1
Bromophenol blue	USB	US12370
Glycin	Roth	3908.3
Gel staining medium	Clini-sciences	GEN-QC-STAIN
Ethanol	CARLO ERBA	528151
NaCl	Euromedex	1112-A
Triton X-100	Promega	H5141
Fat-free milk powder	Régilait	
HCl	Fisher	H/1150/PB15
KOH pellets	Sigma	1.05012
NaOH pellets	CARLO ERBA	480507
Anti-HMA1 antibody		Seigneurin-Berny et *al*, 2006
Anti-KARI antibody		Ferro et *al* , 2010
Anti-LHCP antibody		Vallon et *al* , 1991
P-coumaric acid	Sigma	C-9008

Luminol (3-aminophalhydrazin)	Fluka	9253
Dimethyl sulfoxide (DMSO).	Sigma	D5879
Large (30 cm × 45 cm) plastic cases	Puteaux	162135
A. thaliana seeds		
Compost "Floragard"	Puteaux	16311770
Growth rooms		
Muslin or cheesecloth	Raffin	70116
Nylon blutex 50 µm aperture	Tripette et Renaud, Sailly Saillisel	
Motor-driven blender, three speeds, 1 gallon (4 L) capacity	Waring Blender	
Fixed-angle rotors JLA-10.500 (6 × 500-mL plastic bottles)	Beckman Coulter	
Beckman JA-20 rotor	Beckman Coulter	
JA-20 (6 × 50 mL polypropylene tubes)	Sorvall instruments	
Swinging-bucket rotor JS-13.1 (6 × 50 mL polycarbonate tubes)	Beckman Coulter	
SW 41 Ti rotor (6 × 13.2 mL ultraclear tubes)	Beckman Coulter	
SW 41 Ti rotor tubes (13.2 mL ultraclear tubes)	Beckman Coulter	
Ultracentrifuge (Beckman L7)	Beckman Coulter	
Centrifuge (Beckman JSE-06D18)	Beckman Coulter	
Microcentrifuge	Eppendorf 5415D or equivalent	
Water pump connected to a Pasteur pipette via a plastic tube.		
Nitrocellulose membranes	BA85, Schleicher and Schuell	
Filter paper	3MM, Whatman, Maidstone	
Liquid nitrogen		
Peristaltic pump	Gilson	
Gel electrophoresis apparatus with the various accessories needed for protein separation by electrophoresis (combs, plates and casting apparatus).	Bio-Rad Protean 3 or equivalent	
System for protein transfer to nitrocellulose membranes	Bio-Rad Protean 3 or equivalent	

Comments/Description
Used at a 1:1000 dilution
Used at a 1:1000 dilution
Used at a 1:25,000 dilution

Around 30 mg of seeds for a whole case
12-h light cycle, set at 23°C (day) / 18°C (night) with a light intensity of 150 µmol/m^2/s.
80-cm-large
50 µm aperture

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Author(s): I. Bouchnak, L. Moyet, D. Salvi, M. Kuntz, N. Rolland

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CORRESPONDING AUTHOR:

Name: Norbert ROLLAND

Department: Laboratoire de Physiologie Cellulaire et Végétale

Institution: Univ. Grenoble Alpes, INRA, CNRS, CEA, BIG-LPCV

Article Title: preparation of chloroplast sub-compartments for the analysis of protein localization by immunoblotting or proteomics and other studies

Signature:

Date: April 5, 2018

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Dear Editor,

Please find below the text that addresses each of the editorial and peer review comments individually.
Please note that we did not understand Editorial comment number 10 (Please spell out journal titles) since we used the JoVE EndNote style file to generate the reference list as mentioned in the Instructions for authors.
Finally, one of the five reviewers requested that all products required to perform SDS-PAGE and western blots should be removed from the Materials table. As this was requested by only one reviewer, we labelled (in yellow) corresponding materials in the table so that the Editorial team might easily identify and remove (or keep) this information if required.

Yours sincerely,

Norbert Rolland

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Authors: Done
2. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Authors: Done.
3. What volumes of buffers are prepared? Authors: Done
4. 1.1.1: What concentration of KOH? Authors: Done. We use KOH pellets (included in the Table of Material list).
5. 1.1.2: What concentration of NaOH? Authors: Done. We use NaOH pellets (included in the Table of Material list).
6. 1.3.1: What happens after centrifugation? Authors: Done. We included "After centrifugation, the tubes containing the preformed gradients are stored in a cold room until use".
7. 6.1: What volume is used? Authors: Done.
8. Please specify all volumes and concentrations used throughout. We need these details to film. Authors: Done.
9. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Please note that the highlighting is only for the protocol part of the video. Authors: Done.
10. Please spell out journal titles. Authors: We do not understand this request. We used the ***JoVE EndNote style file*** to generate the reference list as mentioned in the Instructions for authors.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This is the first time I have had to review this type of editorial product and I will be very pleased to see its final version. I found that the manuscript, which will be transformed into a video available on the web, is very detailed. This product will allow, I guess, the high reproducibility and accessibility to such a complex analytical method. One of the greatest difficulties in applying a protocol is, in fact, is skill behind and knowing what is not written on a sheet, but consolidated by the best practice

Authors: Thank you for this positive evaluation of the present manuscript.

Major Concerns:
none

Minor Concerns:
the large amount of leaves needed could discourage researchers from applying the method to non-model plant systems

Authors: We agree, even if most non-model plants have larger leaves than Arabidopsis. The present method is however restricted to the purification of chloroplasts from Arabidopsis. To cite few examples, for spinach chloroplasts, a step gradient (Percoll) is used to purify intact chloroplasts. For Pea chloroplasts, a freeze-thaw step is required to break the chloroplasts before fractionation on sucrose gradient.

Reviewer #2:

Manuscript Summary:
This manuscript describes a protocol for isolating intact chloroplasts and preparing highly pure stroma, thylakoid and envelope fractions from Arabidopsis leaves. The protocol is clear and easy to follow for the most part, with the exception of a few places, as listed in the specific comments (below). The protocol is accompanied by some helpful comments about critical steps. It will be a very valuable resource for researchers who are new to organelle and membrane fractionation, or for those who want to work with Arabidopsis after working with more tractable systems like pea or spinach.

Authors: Thank you for this positive evaluation of the protocol.

Major Concerns:
See comments #4, 10, 12, 13 below

Minor Concerns:
I only have a few specific comments, listed below:
1. Line 36: rewrite as "organelles consist of three…" Authors: Done
2. Line 40, 370: the term "vesicles" usually refers to small (< 100 nm diameter) structures. Use "sacs" or "lamellae" or some other term. Authors: Done
3. Line 40-41, 369: Change "more light" to "less dense" Authors: Done
4. Line 103-104: It is not clear whether the concentrations listed are for the final 1x solution or

the 2x solution. Please clarify. (Given the information in step 1.2.3, I'm guessing the 2x solution should be 0.8M sorbitol.) Authors: thank you. The 2x solution is indeed containing 0.8M sorbitol. This was corrected in the paragraph 1.2.2.
5. Line 108: Listing an approximate volume to prepare would be helpful. Authors: Done
6. Line 111: Listing an approximate volume to prepare would be helpful. Authors: Done
7. Line 127: Listing an approximate volume to prepare would be helpful. Authors: Done
8. Line 131: Listing an approximate volume to prepare would be helpful. Authors: Done
9. Line 136: I'm not sure what a "case" is. I think these are usually referred to as 'flats" or "pans". Authors: we used "plastic pans"
10. Line 139: Given the importance of the overnight cold and dark incubation of plants, this should be listed as a separate step in the protocol (2.2). Authors: Done. Step 2.2 included.
11. Line 140: What size beaker do you recommend for 400-500g of leaf tissue? Authors: Done. 1 L beaker.
12. Line 214: I'm not sure what is involved in "equilibrating" the tubes. Please clarify. Did the authors mean "balance" instead? Authors: Done. We used "balance"
13. Line 225: it appears that a step is missing. To expose the thylakoid pellet, I presume that the intervening layer between the envelope and thylakoid fractions needs to be removed. Authors: Done. We inserted a new step "5.7. Remove the remaining phase of each gradient up to the thylakoid pellet, using a water pump."
14. Line 255: In Figure 2C, are the lanes loaded on an equal protein basis or some other basis? Given the different protein content of the different fractions, how does this factor into the estimates of contamination? Authors: We inserted the following sentences "The lanes are loaded on an equal protein basis (20 µg of each purified fraction). Knowing that chloroplasts contain only 1% of envelope proteins and 50% of proteins from the stroma or from the thylakoids, this tends to overestimate cross-contamination of purified envelope preparations with other chloroplast subcompartments. However, this method allows to detect minute amounts of proteins cross-contaminating the envelope fraction".
15. Line 291. In Figure 2D, why do the LHCP antibodies recognize something in the lane with the MW ladder? Authors: thank you. We originally mixed images of the SDS-PAGE and western blots. The gel corresponding to the western blots present in Figure 2 was thus replaced by the correct one. In this SDS-PAGE, a crude cell extract was present. This explains detection of LHCP in the first lane while more minor components (HMA1/KARI) were not detected here.
16. There are a few more spots where the phrasing is just a little bit awkward, and those are not noted here. I presume these will be fixed during the editorial process. Authors: The JoVE editor will not copy-edit our manuscript. We thus tried to correct few last errors.

Reviewer #3:

Manuscript Summary:
The protocol submitted by Rolland et al. describes a fast and easy way to purify intact chloroplasts from Arabidopsis plants by using differential centrifugations and continuous Percoll gradients. The further fractionation in chloroplast sub-compartments (i.e. envelope, stroma and thylakoids) is then performed with discontinuous sucrose gradients and analysed by SDS-PAGE and Western blot.
The overall protocol is clear, well comprehensible and should be published by JoVE.

Authors: Thank you for this positive evaluation of the protocol.

Several minor points are as follows:

Minor Concerns:
· The title should contain the experimental organism, i.e. Arabidopsis. Authors: Done. We inserted “from Arabidopsis” in the Title.
· "Prepare" can be omitted in the following lines: 86,89, 94, 99, 103, 108, 111, 118, 122, 127, 131. Authors: Done.
· Approximate volumes for all buffers, gradients, and stock solutions to be set up should be given (line 86-112). Authors: Done.
· lines 94-95: It is said that the inhibitor solutions should be stored for up to 6 months at 4°C (line 84). To my knowledge, PMSF should be stable, but benzamidine and amino caproic acid should be stored at least at -20°C. Authors: Thank you for this remark. Indeed, according to manufacturer’s instructions, PMSF and amino caproic acid are stable in solution for months at 4°C, while benzamidine should be stored at -20°C. This information was inserted in the section 1.1.4.
And isn't PMSF set up in isopropanol? Authors: Done.
"benzamidine" should be replaced by "benzamidine hydrochloride hydrate" Authors: Done.
line 100: "3" in NaHCO3 should be subscripted Authors: Done.
· lines 109 + 118/119: For me "50% Percoll/0.4 M sorbitol" is a little confusing as this is a solution obtained by mixing Percoll and 2x washing medium at equal volume. The final solution should accordingly contain 50% Percoll and 0.2 M sorbitol. Or should it be 0.8 M sorbitol in line 104? Authors: Corrected, thank you. The 2x solution is indeed containing 0.8 M sorbitol.
· lines 90, 111, 127: same names for this buffer should be used everywhere ("MOPS buffer" or "MOPS-NaOH"). Authors: Corrected, thank you.
· Lines 136- 138: For how long are these plants grown? Age should be indicated. And these plants are most likely kept in the dark before harvest to reduce starch formation, right? Authors: This important step was already discussed in the “Discussion” section. We however inserted a 2.2 step in the protocol “2.2. Incubate plants in a dark and cold room (4°C) overnight prior to the experiment (to reduce the amount of starch granules in chloroplasts)” to be sure users won’t forget it.
· Lines 148-152: Is the filtrate collected on ice or in the cold room? Authors: Corrected (in a cold room), thank you.
· Line 152: It should be "2.4 and 2.5" not "2.5 and 2.6". Authors: Corrected, thank you.
· Line 156: Are these six 1 L bottles? Authors: These are six 500mL bottles. Corrected.
· Line 157+ 178 + 213 + 232: Is the centrifugation performed at 4°C? Is the centrifuge pre-cooled? Authors: Information is now provided in paragraphs 3.1, 4.2, 4.4, 5.3 and 6.2.
· Line 165+ 187 + 196 + 228: please add 1x washing medium. Authors: corrected in paragraphs 3.4, 4.4, 4.7 and 6.1.
· Lines 165/166: for clarity please add "final volume of the combined chloroplast suspensions =36 ml". Authors: Done.
· Line 171: Is the suspension mixed prior to loading on the gradient to obtain a homogenous suspension? Authors: Done. We inserted the following sentence “Gently mix (by inverting the tube) to obtain a homogenous suspension prior to loading on Percoll gradients” in paragraph 3.5.
Is the suspension filled up with 1 x washing medium to 36 ml? Authors: Done. We inserted the following sentence “final volume of the combined chloroplast suspensions = 36 mL” in

paragraph 3.4.
· Line 175: please change to "slowly load 6 ml of the chloroplast suspension on top of each of the six Percoll gradients". Authors: Done.
· Line 184: It should be referred to Fig. 2A. Authors: Done in paragraph 4.3.
· Line 193: You might add "Completely aspirate…." As this step is important and remaining sorbitol might inhibit hypotonic lysis. Authors: Done in paragraph 4.6.
· Line 217: Please refer to Fig. 2B. Authors: Done in paragraph 5.4.
· Line 228/229: This step seems to be redundant with step 6.2 in line 231. Authors: Corrected, thank you, in paragraphs 6.1 and 6.2. We distinguish resuspension of thylakoid pellet in a minimum volume (2 mL) and dilution, in up to 10 mL, of both envelope and thylakoids before ultracentrifugation.
· Line 258: please add "…low amounts of the large subunit of RuBisCO (RBCL),..." Authors: Corrected, thank you.
· Line 267: As you do so above, please also explain abbreviations for marker proteins used in Fig. 2D. Authors: Corrected, thank you.
· Line 366: µg (I only see a weird character). Authors: "µg" lost during pdf conversion? Corrected, thank you.
· Fig 2D.: Please indicate the size of detected proteins. Authors: Figure 2 was corrected accordingly and now includes sizes of detected proteins.
· Buffers etc. used for Western blotting and SDS PAGE shown in Fig. 2C+D are given in the material list. However, as these chemicals and corresponding methods are not further mentioned in the describing text one might consider to omit these from the list. Authors: As this was requested by only one of the five reviewers, we labelled (in yellow) corresponding materials in the table so that the Editor team might easily identify and remove (or keep) this information if required.
References for antibodies could also be given in the legend. Authors: The legend of Figure 2 now includes references for all three antibodies used.

Reviewer #4:

Manuscript Summary:
This manuscripts provides a protocol for fractionating chloroplasts from Arabidopsis. It goes well beyond previous protocols (e.g. Kunst; Arabidopsis Protocols, 43-48) in providing more detail on treatment of fractions and their subsequent analysis. The protocol is exhaustive and easy to follow. It incorporates advances in chloroplast biology and updates protocols that have been around for two decades.

Authors: Thank you for this positive evaluation of the manuscript.

Major Concerns:
none

Minor Concerns:
Line 154 3.1
The centrifugation speed indicated here seems a little high. In our hands, more than 1000g leads to clumping of chloroplasts and makes them more difficult to resuspend later. We use only 500g

for 2-3 min. Authors: we checked values. We centrifuge our chloroplast suspensions at 3,500 rpm using the fixed angle JLA-10.500 rotor. The calculated G-force at Max Radius (16 cm, bottom of the tube) for this rotor is 2270 *g*. The calculated G-force at Min Radius (5.4 cm, top of the tube) for this rotor is 740 g. We originally provided rpm values (3,500 in this case) in the manuscript, but had to convert these values in G-force, and agree that this is less precise when using fixed-angle rotors.
We have never had problems of chloroplast clumping using such a step. We however add 10 mM EDTA in the extraction buffer (please see paragraph 1.2.1) that might help limiting organelle aggregation.

Line 205 5.1
Please indicate how long you incubate the chloroplasts in lysis buffer and whether you support lysis by pipetting the chloroplasts up and down (and if so, how many times and what kind of pipet). Authors: Thank you for this comment. We added these sentences to paragraph 5.1: "From this step, the use of Pipetman with fine tips (blue tips) is possible since intactness of chloroplasts is no more essential (pipetting chloroplasts up and down as long as pellet is not entirely resuspended). Arabidopsis chloroplasts are very fragile (when compared to pea chloroplasts, for example) and their lysis is almost immediate after incubation in hypotonic medium".

Reviewer #5:

Manuscript Summary:
This manuscript describes a protocol for the isolation of chloroplast subcompartments. The description and illustrations are excellent. I believe that "anybody" who tries this protocol would actually succeed.

Authors: Thank you for this positive evaluation of the manuscript.

Major Concerns:
While I do think this is a very good protocol, it does not address the possibility of contamination of certain fractions by plastoglobules. Plastoglobules are fairly abundant lipoprotein particles attached to thylakoids. They contain a specific set of proteins that could interfere with subsequent proteomics studies on the purified fractions that the authors mention. The point should be addressed.

Authors: Thank you for this important comment. We added a sentence referring to plastoglobules subcompartments in the discussion section: "However, it is important to note that additional membrane microdomains are present at the curved margins of thylakoids. These lipoprotein subcompartments, or plastoglobules, are permanently coupled to thylakoid membranes and contain a specific set of proteins [25]. Using the present protocol, it is thus not possible to distinguish these specific proteins from other thylakoid components". Accordingly, we also cited a new reference (ref 25) to a recent review article from colleagues in the field.

Minor Concerns:
none

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Short introduction

Chloroplasts are major components of plant cells. Such plastids fulfill many crucial functions, such as assimilation of carbon, sulfur and nitrogen as well as synthesis of essential metabolites. In order to decipher new regulatory mechanisms that control the chloroplast dynamics and physiology, defining the sub-plastidial localization of chloroplast proteins is thus critical to support targeted studies aiming to better understand proteins functions. In order to get access to the genuine subplastidial localization of these proteins, it is thus essential to start from highly pure subplastidial fractions (envelope membranes, stroma, and thylakoids, see Figure 1). In this context, the aim of the present protocol is to purify intact chloroplasts from Arabidopsis leaves using differential centrifugations and continuous Percoll gradients, and to fractionate them using discontinuous sucrose gradients, in three sub-compartments (i.e. envelope, stroma and thylakoids). The method described here also provides instructions to assess the purity of purified sub-organellar fractions using markers associated to the various chloroplast subcompartments. This protocol is valuable for subplastidial localization of proteins using immunoblotting and for further analysis of purified fractions using MS-based proteomic studies.

PROTOCOL:

1. Prepare buffers, stock solutions and gradients before the experiment

-Some of the stock solutions can be prepared and stored up to 6 months at 4°C like Tricine and MOPS buffers, EDTA, $MgCl_2$ or protease inhibitors solutions. Other solutions should be prepared the day before the experiment. This is the case for grinding medium, washing medium and Percoll gradient solution which contain sorbitol. This is also the case for solutions used for gradients containing different concentrations of sucrose. All above cited solutions should be stored at 4°C.

2. Prepare gradients before the experiment

-Prior to starting the experiment, prepare six tubes of Percoll gradients and store the tubes in cold room until use. Then, prepare four tubes of sucrose gradients, formed of three sucrose layers containing 0.93 M, 0.6 M, and 0.3 M sucrose. Carefully overlaid each layer, using a peristaltic pump starting with 0.93 M, at the bottom and finishing with 0.3 M at the top.

-Then prepare hypotonic medium for chloroplast lysis and membrane washing buffer and store these solutions on ice until use.

3. Harvesting of Arabidopsis leaves

-Arabidopsis plants are pregrown for ~~3 to 4~~5 weeks, in large ~~cases~~ plastic pans (for a total surface of 0.5 to 1 m^2) by sowing 30 mg of seeds in each ~~case~~pan. *Do not start from too old plants! Starting from young leaves is the best compromise to combine yield, purity, and integrity of the purified organelles. It is also important to store plants overnight in a dark and cold room (4°C) prior to the experiment, to reduce the amount of starch and chloroplast breakage!*

-When starting the experiment, pre-weigh a beaker and then place it on ice before starting harvesting of leaf material.

-Then, harvest Arabidopsis leaves by avoiding soil (compost). Reweigh the beaker and record the tissue weight. 400 to 500 g of leaf material are expected from the initial four pans~~cases~~.

-Homogenize leaves with 2 L of grinding buffer (do not forget to add BSA before use) three

times/2 s each time, in a Waring blender at high speed. *Note that this blending process must be limited to few seconds, since longer blending strongly affects the yield of the purification!*
-Filter the homogenate using 4 layers of muslin and one layer of nylon blutex. Gently squeeze the homogenate leaves inside the muslin/nylon blutex, to extract all the liquid.
-Recover the remaining tissue in the blender cup for a second extraction. Repeat previous steps using 2 L of grinding medium and new 4-5 layers of muslin.

4. Purification of crude chloroplasts using differential centrifugation
-Equally distribute the crude cell extract into six bottles and centrifuge for 2 min as soon as the max speed (2,070 *g*) is reached (at max acceleration and using brake on parameters). Store the bottles on ice before centrifugation.
-Gently discard the supernatant.
-Aspirate the remaining supernatant using a water pump and keep the pellets containing concentrated crude chloroplasts on ice.
-Gently resuspend pellets, by adding a minimal volume of washing medium using a paintbrush or a curved plastic spatula. Use a 10 mL pipet to add 3 ml of washing medium in each bottle. *Do not use Pipetman with very fine tips, to avoid breakage of chloroplasts!*
-Collect the resuspended chloroplasts in one tube by using a 10 mL pipet.

5. Purification of intact chloroplasts on continuous Percoll gradient
-Slowly load the chloroplast suspension on the top of Percoll gradients using a 10 mL pipet to avoid breakage of chloroplasts.
-Centrifuge the gradients for 10 min at 13,300 *g* using a Swinging-bucket rotor. *Do not forget to limit the acceleration and to disconnect the brake (brake off or slow deceleration) to prevent blending of the Percoll gradients.*
-Aspirate the upper phase that contains broken chloroplasts and intact mitochondria using a water pump, and then retrieve intact chloroplasts present in the lower phase (the broad dark-green band) with a 10 mL pipet. *Be careful to not aspirate nuclei and cell debris (found at the bottom of the tube) with the intact chloroplasts!*
-Dilute 3-4-fold the intact chloroplast suspension with washing buffer. Centrifuge for 2 min as soon as the max speed (2,070 *g*) is reached (Beckman JLA-10.500 rotor, max acceleration and brake on).
-Carefully discard the supernatant.
-Aspirate the remaining supernatant with a water pump and keep the pellet of concentrated intact chloroplasts on ice.
-Before chloroplast lysis, keep an aliquot of intact chloroplast fraction in ~1ml of washing medium for further analyses using SDS-PAGE and western blotting. *Do not forget to keep a small aliquot of these intact chloroplasts for determination of protein concentration and to store this intact chloroplast aliquot in liquid nitrogen for further experiments!*

6. Lysis of intact chloroplasts using a hypotonic buffer and purification of chloroplast sub-compartments on discontinuous sucrose gradients

-Lyse the purified intact chloroplasts by resuspending the pellet in hypotonic medium that contains protease inhibitors (the final volume should not exceed 12 mL). From this step, the use of Pipetman with fine tips is possible since intactness of chloroplasts is no more essential.
-Slowly load 3 mL of the lysed chloroplasts on the top of each preformed sucrose gradients using a peristaltic pump.
-Ultracentrifuge the gradients for 1 h at 70,000 *g*. *Do not forget to ~~equilibrate~~ balance pairs of tubes using hypotonic medium buffer prior to centrifugation!*
-Carefully recover the soluble stromal proteins by pipetting the upper phase of the gradient (3 mL from each gradient). Take an aliquot for determination of protein concentration. Store the stroma in liquid nitrogen for further experiments.
-Aspirate the remaining upper phase of each gradient up to the yellow band, using a water pump. Retrieve the yellow band (the envelope) with a Pipetman (~ 1mL from each gradient). Pool the envelopes in one tube.

7. Washing and concentration of thylakoid and envelope membrane systems
-Resuspend thylakoids (green pellets) in membrane washing buffer containing protease inhibitors).
-Dilute the envelope and thylakoid suspensions 3-4-fold in membrane washing medium and ultracentrifuge for 1 h at 110,000 *g*. *Do not forget to ~~equilibrate~~ balance pairs of tubes using membrane washing buffer prior to centrifugation!*
-Carefully aspirate the supernatants using a water pump.
-Add ~100 µL of membrane washing buffer containing protease inhibitors to the envelope pellet. Take an aliquot for determination of protein concentration and store the purified envelope membrane preparation in liquid nitrogen.
-Resuspend thylakoids pellet in 3 mL of membrane washing buffer containing protease inhibitors. Take an aliquot for determination of protein concentration and store thylakoid membrane fraction in liquid nitrogen.

REPRESENTATIVE RESULTS:
Successive steps of the procedure resulting in purified chloroplast and their subcompartments are resumed in Figure 2. The Percoll gradient allows distinguishing intact chloroplasts from broken chloroplast and mitochondria (top of the gradient) or nuclei and cell debris (bottom of the gradient). After rupture of the Percoll-purified organelles thanks to an osmotic shock, the resulting fractions are separated on a sucrose gradient. The stroma (soluble part of the chloroplast) is floating at the surface of the sucrose gradient. The light envelope membrane vesicles are recovered as a discrete yellow band at the 0.6/0.93 M sucrose interface. The heaviest thylakoid membranes vesicles are concentrated at the bottom of the tube. After recovery, washing and concentration of the two membrane fractions, proteins are quantified and the composition of all four fractions is analyzed on a SDS-PAGE. Markers from each compartments are then used in evaluating the cross contamination of the fractions.

Conclusion:
The present article aims to detail the step by step protocol used to purify chloroplasts (and their subcompartments) from *Arabidopsis thaliana*. Since the availability of its complete genome

sequence, almost two decades ago, and of large collections of insertion mutants made available to the community, Arabidopsis is now widely accepted as a model plant. However, while this plant was perfectly adapted for genetic approaches, plant scientists needed to adapt biochemical and physiological tools to this emerging model.

The protocol described here allows to purify Arabidopsis chloroplasts at an excellent level of purity. This protocol was also efficient to purify chloroplasts from several Arabidopsis ecotypes, like Columbia (Col) or Wassilewskija (WS), *i.e*. the ecotypes that were used for genome or ESTs sequencing projects but also to generate T-DNA insertion mutants in Arabidopsis.

Availability of specific protocols to purify organelles have allowed a series of high throughput proteomics-based experiments to be conducted on chloroplast samples. These data were made available in several public databases, thus providing to biologists in the field an accurate subcellular (and subplastidial) localization for many chloroplast proteins. This was especially true for envelope proteins whose identity and location remained mostly unknown before these analyses, since envelope membranes represent a minor chloroplast component (1–2% of the chloroplast proteins) while playing a key role in chloroplast metabolism and biogenesis.