**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, MgCl2 orprotease 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.

1. **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.

1. **Harvesting of Arabidopsis leaves**

-Arabidopsis plants are pregrown for 5 weeks, in large plastic pans (for a total surface of 0.5 to 1 m2) by sowing 30 mg of seeds in each 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.

-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.

1. **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.

1. **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!*

1. **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 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.

1. **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 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.