**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*](http://www.jove.com/files/JoVE.ens)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 g 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: “g” 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