**Editorial comments:**  
  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you. We have proofread the entire manuscript.  
2. Figures 3 and 4: These two figures are missing from the submission. Please upload these two figures individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

Thank you for pointing this out. We have now uploaded these figures.  
3. Please provide an email address for each author.

We have now provided email addresses for all authors in the text.  
4. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

We have now rephrased the summary according to this suggestion.

5. Abstract: Please include an overview of the method and a summary of its advantages, limitations, and applications.

We have now rephrased the Abstract according to this suggestion.

6. 1.3: The Protocol should contain only action items that direct the reader to do something. Please either write the text in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.), or move the solutions, materials and equipment information to the Materials Table.

Thank you for this suggestion. We have now moved the solutions to Table 1. Our equipment information is in the Materials and Methods.

7. 2.1.3, 3.1.2, 3.1.5, 3.2.2, etc.: Please specify centrifugation parameters throughout the protocol.

We have now added the centrifugation parameters to the protocol.

8. 4.1.6: Please specify the volume (range) of Lysis Buffer B used.

We have now added the volume of lysis buffer B.

9. 5.1.3: Does the homogenate here refer to the supernatant obtained in step 5.1.2? Please specify.

Yes it does. Homogenate has now been renamed to supernatant (for clarity) and a reference to the originating step added.

10. 5.1.19: What is the desired final volume in this experiment? Please specify.

A suggested final volume range has now been added.

11. Please revise the Acknowledgements section to include any acknowledgments and all funding sources for this work.

Thank you for pointing this out. We have now added our funding source to the Acknowledgements  
12. Please include a Disclosures section, providing information regarding the authors’ competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

Thank you for pointing this out. We now include a disclosures section and declare no conflict of interest.  
  
**Reviewers' comments:**  
  
  
  
Reviewer #1:  
  
Manuscript Summary:  
This manuscript reports the use of a method for cell fractionation in the absence of high speed centrifugations or commercially available kits. This was accomplished by performing lysis, homogenization and centrifugations of U937 cells. Although this manuscript requires some minor revisions, it was well written. This study is important and can be a very useful method for those who lack funding for commercial kits or access to a high speed centrifuge.  
  
Major Concerns:  
None.  
  
Minor Concerns:  
Comment 1. I would recommend going through the protocol itself and modifying some of the numerical values to better represent significant figures. For example, in line 69, the authors call for the addition of 8.766 g of NaCl. Additionally, in line 73, the authors call for 0.746 g of KCl. These values would be hard to measure out. Thus, I would recommend rounding up and using less digits.

Values have been rounded for clarity.

Comment 2. There is an extra space between 100 and % in line 92.

Thank you. We have now corrected this.

Comment 3. There is also a period missing on the last sentence of line 243.

This has been corrected.

Comment 4. The authors seem to provide evidence of successful fractionation using this technique. I am just curious as to whether they have tried this method with any other cell lines and whether they had success. If so, I would recommend possibly adding this to the discussion and citing data not shown.

This fractionation procedure has only been attempted in the U937 cell line (in wild type and knockout lines). The text has been updated to clarify this.

Reviewer #2:  
  
Manuscript Summary:  
This paper describes a simple and reproducible method to isolate subcellular fractions, in particular the plasma membrane, mitochondria and cytosol. This method is of potential interest for a very large number of scientists using U937 cells and many other cell types.  
  
Major Concerns:  
Although this is my general opinion, I should mention that Authors normally perform an accurate revision of the manuscript, prior to its submission for publication. I therefore ask the Authors to include all the figures before submission of the revised paper. I could not find Figs 3 and 4 in the present version of the manuscript. Please also make sure to check on the Material and Methods section, in which I could find parts still highlighted in yellow.

Thank you for pointing this out. We do not know why there was an error in uploading these figures. Accordingly, figures 3 and 4 have been uploaded to the submission website. We included the yellow highlighting in our submission as it was our understanding that this was required by the publisher to indicate filmable content for the journal.

Minor Concerns:  
Please also address the following issues:  
-The title of the paper should be changed, since the method is employed for the isolation of three subcellular fractions.

The title has been altered, with the word “Complete” removed.

-Lysis Buffer A should be defined as Buffer A. It is not a lysis buffer.

We have changed this as suggested.

-Digitonin is used to "permeabilize" the cells and "isolate" cytosolic proteins. 3.2.1 and 3.2.2: please provide a more appropriate definition of cell pellet obtained after digitonin treatment.

The descriptor “digitonin-permeabilized” has been added to differentiate the cell pellets obtained after cytosolic extraction.

-Figure 1: Please provide more details on the scheme summarizing the steps of the protocol employed.

Figure 1 has been redesigned with more details, as suggested.

-A table with the composition of the buffers and solutions employed should be included. Consider also the possibility of including a table summarizing the critical steps of the procedure (i.e., the use of an appropriate digitonin concentration, assessment of the efficiency of homogenization, ecc..).

These tables have been added as suggested.

-Lines 317-318. The Authors state that the omission of SDS in the final buffer followed by density gradient centrifugation, leads to the isolation of intact mitochondria. Are these mitochondrial functional and suitable for their use in experiments measuring oxygen consumption, ROS formation, or other biological responses?

This statement has been removed. While the mitochondria should be intact and functional (based on similar isolation protocols), we have not tested this and removed this suggestion to avoid misleading readers.

- Please introduce more details on Western Blot procedure utilized.

Further details have been added to the Representative Results section indicating transfer method and membrane type.

Reviewer #3:  
  
Manuscript Summary:  
The submission " Complete Cell Fractionation in U937 Cells in the Absence of High Speed Centrifugation" by McCaig, Patel and LaRocca describes a protocol to isolate soluble, mitochondrial and membrane fractions from human cell culture. The protocol is well described, well detailed and seems to be suitable for publication in JOVE. I have a few specific comments that might improve the manuscript and that the authors should address in preparing their final manuscript.  
  
Major Concerns:  
In particular, I can't assess some of the claims of purity because figures 3 and 4 were omitted from the review copy of the manuscript I received. I think these panels would help because I can't tell how troubleshooting would work here in a case where the protocol wasn't properly fractionated. To this end, it would help if the authors described what steps are most critical for the separation to be successful. It is strongly implied (although not explicitly stated) that the total number of spins at a lower speed (e.g. 5.1.1 to 5.1.8) is that factor to ensure that the sample being worked with has contaminating fractions removed from it. Is that true? It would be worth highlighting if the authors know or suspect that this is where substantial tweaks might have to be done by someone working with different samples.

We do not know why there was an error in uploading these figures. Figures 3 and 4 have been uploaded to the submission website. A table with critical steps for purification has been added for troubleshooting purposes and adaptation to other cell lines.

Minor Concerns:  
I'd also like to see some discussion of cells types/tissues that this protocol isn't suited for. The authors describe it as working in U937 cells, but there isn't a great description of this cell line in the manuscript nor is it referenced. Have the authors ever tried this on other cells types or on intact tissues? Does it work when there is significant levels of connective tissue present? I don't think they need to test every possibility, but it would be good to explicitly state that this is really for in vitro passaged cells if that is the case.

We have not attempted this protocol with tissues or cell lines other than U937 cells. We have provided suggestions in the Discussion for the processing of adherent cells and tissues, however, these are only suggestions and we have not performed these experiments.

Line 44 - define "standard" here

This has now been defined in the text.

Line 313 - do the authors have a list of cell types they have used? Are there tissue/cell specific information they can offer here on successfully carrying out the protocol?

We have performed this protocol in U937 cells only. We have added text to reflect that we have only obtained results in the U937 cell line. We have also added suggestions for additional cell lines and tissues in the Discussion.

Line 319 - it seems a bit of an oversight to not probe for nuclear markers and nuclear marker purity here

This statement has been removed, as we have not probed for nuclear markers. The suggestion was based on similar published protocols, but we have no definitive evidence of this. The work for which this protocol was developed did not require analysis of the nuclear fraction.