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Density Gradient Ultracentrifugation for Investigating Endocytic Recycling in Mammalian Cells

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TITLE:

Density Gradient Ultracentrifugation for Investigating Endocytic Recycling in Mammalian Cells

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KEYWORDS:

ARF6, density gradient ultracentrifugation, fractionation, Rab11, recycling endosome, sucrose

SUMMARY:

This paper aims to present a protocol for preparing recycling endosomes from mammalian cells using sucrose density gradient ultracentrifugation.

ABSTRACT:

Endosomal trafficking is an essential cellular process that regulates a broad range of biological events. Proteins are internalized from the plasma membrane and then transported to the early endosomes. The internalized proteins could be transited to the lysosome for degradation or recycled back to the plasma membrane. A robust endocytic recycling pathway is required to balance the removal of membrane materials from endocytosis. Various proteins are reported to regulate the pathway, including ADP-ribosylation factor 6 (ARF6). Density gradient ultracentrifugation is a classical method for cell fractionation. After the centrifugation, organelles are sedimented at their isopycnic surface. The fractions are collected and used for other downstream applications. Described here is a protocol to obtain a recycling endosome-containing fraction from transfected mammalian cells using density gradient ultracentrifugation. The isolated fractions were subjected to standard Western blotting for analyzing their protein contents. By employing this method, we identified that the plasma membrane targeting of engulfment and cell motility 1 (ELMO1), a Ras-related C3 botulinum toxin substrate 1 (Rac1) guanine nucleotide exchange factor, is through ARF6-mediated endocytic recycling.

INTRODUCTION:

Endosomal trafficking is an essential physiological process that implicates various biological

events[1], for example, the transportation of signaling receptors, ion channels, and adhesion molecules. Proteins localized at the plasma membrane are internalized by endocytosis[2]. The internalized proteins are then sorted by the early endosome[3]. Some of the proteins are targeted to lysosomes for degradation[4]. However, a significant amount of proteins are recycled back to the cell surface by fast recycling and slow recycling processes. In fast recycling, proteins leave the early endosomes and directly return to the plasma membrane. Conversely, in slow recycling, proteins are first sorted to the endocytic recycling compartment and then transported back to the plasma membrane. Various cargo proteins, for example, clathrin, retromer complex, retriever complex and Wiskott-Aldrich syndrome protein, and SCAR Homologue (WASH) complex, participate in such membrane recycling processes[4–9]. The balance of the endocytosis and recycling event is crucial for cell survival and contributes to various cellular events[10], for instance, cell adhesion, cell migration, cell polarity, and signal transduction.

ARF6, a small GTPase, is a reported regulator of endocytic trafficking[7,11,12]. Of interest, various research groups have illustrated the importance of ARF6 in endocytic recycling[13–17]. The study aims to investigate the relationship between ARF6-mediated neurite outgrowth and endocytic recycling. The previous report suggests that the activation of ARF6 is upstream to Rac1 activity through acting on ELMO1-dedicator of cytokinesis 1 (DOCK180) complex[18]. However, how ARF6 triggers ELMO1-DOCK180 mediated Rac1 signaling remains unclear. Density gradient ultracentrifugation was employed to investigate the role of ARF6-mediated endocytic recycling in such a process. By using that, the recycling endosome-containing fraction was obtained from cell lysates[19]. The fraction was subjected to Western blotting for protein content analysis. The immunoblot results revealed that under the presence of FE65, a brain-enriched adaptor protein, active ARF6 substantially increased the level of ELMO1 in the recycling endosome-containing fraction. The following protocol includes the procedures for (1) transfecting mammalian cells; (2) preparing the samples and density gradient columns; and (3) obtaining the recycling endosome-containing fraction.

PROTOCOL:

1. Mammalian cell culture and transfection

1.1. Plate 2 x 10^6 cells in a 100 mm culture dish. Use four dishes for each transfection.

NOTE: The number of cells required may vary for different cell lines. Optimization may be necessary before proceeding to the isolation step.

1.2. The next day, transfect the cells with Lipofectamine according to the manufacturer's instructions.

2. Cell harvest

2.1. Discard the culture medium 48 h post-transfection.

2.2. Wash the cells with ice-cold PBS (10 mM sodium phosphates, 2.68 mM potassium chloride, 140 mM sodium chloride) twice.

2.3. Add 1 mL of ice-cold PBS^{+} (PBS supplemented with 0.5x protease inhibitor cocktail and 0.5x phosphatase inhibitor cocktail) to each dish.

2.4. Collect the cells with a cell scraper and transfer the cell suspension to a 15 mL centrifuge tube.

2.5. Pellet the cells by centrifugation using a swing bucket rotor at 400 x *g* for 5 min.

2.6. Discard the supernatant and resuspend the cell pellet gently in 5 mL of homogenization buffer (HB; 250 mM sucrose, 3 mM imidazole at pH 7.4, 1 mM EDTA supplemented with 0.03 mM cycloheximide, 1x protease inhibitor cocktail, and 1x phosphatase inhibitor cocktail).

2.7. Collect the cells by centrifugation at 1,300 x *g* for 10 min.

2.8. Resuspend the cell pellet in 1 mL of HB.

2.9. Homogenize the cells with a Dounce homogenizer for 15–20 strokes.

NOTE: Other homogenization methods, for example, passing the sample through a syringe, could be used. To reveal the efficiency of homogenization observe the homogenate under a phase-contrast microscope.

2.10. Transfer the homogenate to a 2 mL centrifugation tube.

NOTE: Harvest 50 µL of homogenate with 12.5 µL of 5x sample buffer and label it as total lysate.

2.11. Add 0.7 mL of HB to the homogenate.

2.12. Spin the diluted homogenate at 2,000 x *g* for 10 min at 4 °C.

NOTE: The pellet contains nuclei and unbroken cells.

2.13. Collect 1.5 mL of the supernatant and repeat step 2.12 once.

2.14. Collect 1.4 mL of the supernatant and label it as post-nuclear supernatant (PNS).

3. Density gradient column preparation

3.1. Transfer 1.2 mL of PNS to an ultracentrifuge tube.

3.2. Add 1 mL of 62% sucrose solution (2.351 M sucrose, 3 mM imidazole at pH 7.4) to the

sample and mix well by gentle pipetting.

NOTE: The resultant solution is a 40.6% sucrose solution.

3.3. Add 3.3 mL of 35% sucrose solution (1.177 M sucrose, 3 mM imidazole at pH 7.4) carefully on top of the sample.

3.4. Add 2.2 mL of 25% sucrose solution (0.806 M sucrose, 3 mM imidazole at pH 7.4) carefully on top of the 35% sucrose solution.

NOTE: The refractive index of the 62%, 40.6%, 35%, and 25% sucrose solutions at room temperature are 1.44, 1.40, 1.39, and 1.37, respectively. Check the refractive indexes of the sucrose solutions with a refractometer to ensure the precision and consistency of the experiment.

3.5. Fill up the ultracentrifugation tube with HB.

NOTE: Temporarily store the prepared density gradient column at 4 °C.

4. Fractionation and recovery of recycling endosome-containing fraction

4.1. Centrifuge the column at 210,000 x *g* for 3 h at 4 °C.

4.2. Collect 12 fractions (1 mL each) carefully, starting from the top of the gradient.

NOTE: The recycling endosomes should be found at the interface between 35% and 25% sucrose solutions. The collected fractions can be snap-frozen in liquid nitrogen and stored at -80 °C.

4.3. Dilute all the fractions with 1 mL of dilution buffer (3 mM imidazole at pH 7.4, 1 mM EDTA).

4.4. Centrifuge the diluted sample at 100,000 x *g* for 1 h at 4 °C.

4.5. Aspirate the supernatant and add 50 µL of 1x sample buffer to harvest the fractions.

4.6. Analyze the protein contents in the fractions by western blotting.

REPRESENTATIVE RESULTS:

After fractionating the untransfected HEK293 cells by density gradient ultracentrifugation, 12 fractions were collected starting from the top of the gradient. The harvested fractions were diluted with the dilution buffer in a 1:1 ratio and subjected to a second round of centrifugation. The samples were then subjected to western blotting for analyzing their protein contents. As shown in **Figure 1**, the recycling endosome marker Rab11 is detected in fraction 7[20]. Other subcellular markers, including β-COP, COX IV, GAPDH, EEA1, Rab7, and Lamp1, were also probed.

A positive EEA1 signal is also detected in fraction 7 as well. The band intensities of GAPDH, COX IV, and Rab11 in both fraction 7 and PNS were measured with ImageLab software (Bio-Rad). The intensity ratios of the markers in fraction 7 to PNS were calculated and expressed in a bar chart ± SD.

The previous studies suggest that ARF6 and ELMO1 interact with FE65 to promote Rac1-mediated neurite outgrowth[21,22]. Since ARF6 is a regulator of endocytic recycling and ELMO1 plasma membrane targeting is critical for the subsequent Rac1 activation, it is hypothesized that FE65 connects ARF6 and ELMO1 to mediate the trafficking of ELMO1 to the plasma membrane. Therefore, this method was employed to investigate the ELMO1 level in the isolated recycling endosomes. HEK293 cells were transfected with either ELMO1, ELMO1 + ARF6 Q67L, or ELMO1 + ARF6 Q67L + FE65. No changes were found in ELMO1 distribution with ARF6 Q67L and FE65 overexpression (**Figure 2A**). Next, the ELMO1 level in fraction 7 (Rab11-positive) were compared between different transfections. The amount of ELMO1 in the fraction is found to elevate after the co-transfection of ARF6 Q67L. Further increase in ELMO1 level is observed when both ARF6 Q67L and FE65 are co-expressed (**Figure 2B**). Conversely, knockout of FE65 diminished the ARF6-mediated ELMO1 enrichment in fraction 7 (**Figure 2C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Recycling endosomes are found in fraction 7 after density gradient ultracentrifugation. Untransfected HEK293 cells were fractionated and the protein contents in the obtained fractions were analyzed with western blotting. Rab11 is detected in fraction 7 with an anti-Rab11 antibody (1:500). Other subcellular markers were detected with their specific antibodies, including β-COP (1:1000), COX IV (1:1000), GAPDH (1:10,000), EEA1 (1:1000), Rab7 (1:500), and Lamp1 (1:1000). Fraction 1 is the less dense top fraction, while fraction 12 is the denser bottom fraction. The bar chart shows the ratio of GAPDH, COX IV, and Rab11 in fraction 7 to PNS ± SD. This figure has been modified from Chan, W. W. R. et al.[22].

Figure 2: Expression of FE65 promotes ARF6-mediated endocytic recycling of ELMO1. (**A**) The cells transfected with either ELMO1, ELMO1 + ARF6 Q67L, or ELMO1 + ARF6 Q67L + FE65 were fractionated using density gradient ultracentrifugation. All the fractions were subjected to western blotting for analyzing the distributions of ELMO1, ARF6 Q67L, and FE65. Fraction 1 is the less dense top fraction, while fraction 12 is the denser bottom fraction. (**B**) The Rab11-positive fraction 7 from different transfections was analyzed with Western blotting to evaluate the levels of ELMO1 and ARF6 Q67L. The amount of ELMO1 in fraction 7 was elevated when the cells were co-transfecting with ARF6 Q67L. Co-expression of ARF6 Q67L and FE65 further increases the amount of ELMO1 in the fraction. (**C**) Wildtype HEK293 was transfected with ELMO1 or ELMO1 + ARF6 Q67L, whereas FE65 KO HEK293 was transfected with ELMO1 + ARF6 Q67L. The ARF6-mediated ELMO1 enrichment in fraction 7 significantly diminished in FE65 KO cells. (**B–C**) Recycling endosome marker Rab11 (1:500) and cytosol marker GAPDH (1:10,000) were probed. ELMO1, FE65, and ARF6 Q67L were detected with anti-ELMO1 B-7 (1:1000), anti-FE65 E-20 (1:1000), and anti-myc 9B11 (1:5000), respectively. The relative level of ELMO1 in fraction 7 was expressed as the densitometric ratio of the ELMO1 in fraction 7/total ELMO1. Data for the bar chart were obtained from three independent experiments. One-way ANOVA with Bonferroni

post hoc test was employed for statistical analysis. *p < 0.001. Results are mean fold change ± SD[22]. This figure has been modified from Chan, W. W. R. et al.[22].

DISCUSSION:

The above protocol outlines the procedures for isolating recycling endosomes from cultured cells by ultracentrifugation. The reliability of this method has been demonstrated by the latest publication[22], proving that recycling endosomes are successfully isolated from other organelles (**Figure 1**), such as the Golgi apparatus and mitochondria. Some critical steps need to be paid attention to for obtaining a good separation result. While preparing the sucrose solutions, it is recommended to validate the refractive indexes of the solutions with a refractometer. The refractive index of the 62%, 35%, and 25% sucrose solutions at room temperature are 1.44, 1.39, and 1.37, respectively. Also, air bubbles should be avoided from the gradient. The presence of bubbles in the column may disrupt the continuity of the gradient. Detergents should be avoided in the homogenization process since they damage the membrane of organelles. This leads to the releasing of proteins from membrane-bound organelles and could result in severe contamination. Also, all homogenizing tools should be pre-cooled before use to avoid protein degradation during homogenization. Once the gradient is prepared, it should be used as soon as possible. Although the prepared gradient could temporarily be stored at 4 °C (1–2 h), prolonged storage may interfere with the density gradient due to diffusion.

Sucrose is a widely used gradient medium because of its easy availability. In fact, there are many other alternatives, including Percoll and Ficoll-400[23,24]. These media have different physical properties when compared with sucrose. For instance, Percoll has lower osmolarity and viscosity than sucrose. These allow rapid banding of particles using lower centrifugal forces. Ficoll-400 has a lower permeability toward membranes than sucrose because of its high molecular weight and low content of dialyzable material. Therefore, changing the gradient medium may achieve a higher endosome isolation efficiency.

Apart from density gradient ultracentrifugation, other methods can be used for cell fractionation, including free-flow electrophoresis (FFE)[25], fluorescence-activated organelle sorting (FAOS)[26], and immunoisolation[27]. FFE is a liquid phase separation method. The sample flows through the separation buffer under the influence of an electric field perpendicular to the flow direction. Deflection levels of different organelles vary based on their surface charges[25]. FAOS usually uses a fluorescent tag or antibody to label specific organelle and then followed by flow cytometry for the isolation[28,29]. Immunoisolation relies on detecting specific antigens on the surface of the targeted organelle and subsequent precipitation by antibodies[30].

When comparing with these alternatives, density gradient ultracentrifugation has its own advantages. First of all, a distribution profile of the interested protein can be obtained by performing Western blotting with the isolated fractions (**Figure 2A**). Any changes in protein subcellular localization could be easily detected. Also, ultracentrifuge is a standard instrument in most institutes, and the technical requirement for operating the centrifuge is low. In contrast, a flow cytometer and a specific electrophoresis system are required for the isolation process of FAOS and FFE, respectively. There is no specific equipment required for immunoisolation.

However, it is mainly used for isolating endosomes from a small number of cells. The preparation scale of ultracentrifugation is larger than that of immunoisolation. Besides, an antibody with high specificity is necessary for immunoisolation[31]. Furthermore, detergent- and high salt-containing buffer cannot be used in the washing steps to ensure the integrity of the endosomes. This may lead to high background and reduce the purity of the isolated organelle[31].

Since density gradient ultracentrifugation separates organelles based on density, its most significant limitation is that of resolving power toward organelles with similar density. As shown in **Figure 1**, both Rab11 and EEA1 are detected in fraction 7 because of the similar physical properties of recycling and early endosomes. Further assays are needed to confirm the changes in the level of the targeted protein in the recycling endosome. In the previous study, co-immunostaining on ELMO1 and Rab11 was performed in cells[22]. In addition to performing other assays, some measures can be adopted to overcome this problem. A continuous density gradient can resolve organelles with minor density differences[32]. However, the yield in a continuous gradient is significantly lower than that of a discontinuous gradient. It is possible to manipulate the density of the endosome by pre-treating the cells with latex beads[33]. The beads are internalized by the cells via endocytosis. The density of the beads containing endosomes is significantly reduced and can be separated from other organelles. Usually, adding another level of isolation could significantly improve the isolation of organelles with similar densities. For example, perform immunoisolation from the isolated fraction[34]. By using a specific antibody, recycling endosomes can be separated from the contaminants. Fluorescent-labeled antibodies and probes, combined with flow cytometry analysis, could also be used for precise endosome isolation[28]. FFE is also applicable for separating organelles with similar densities based on their surface charge[25].

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors declare that they have no conflicts of interest with the contents of this article.

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Figure 1

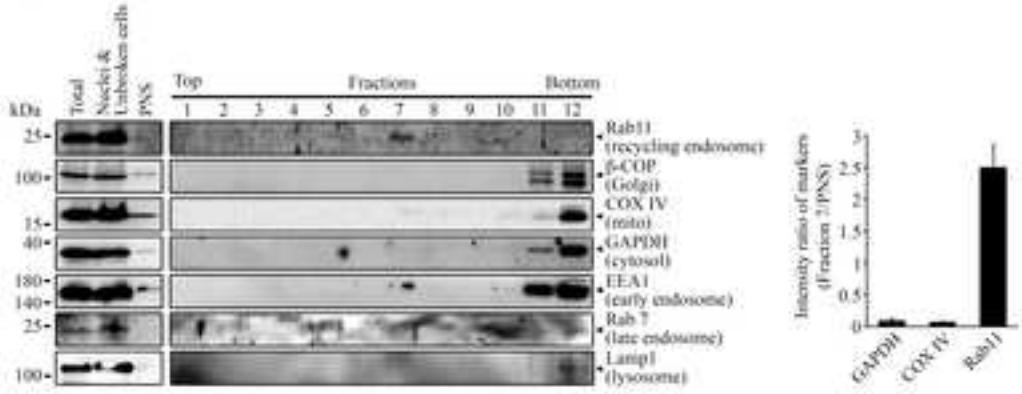
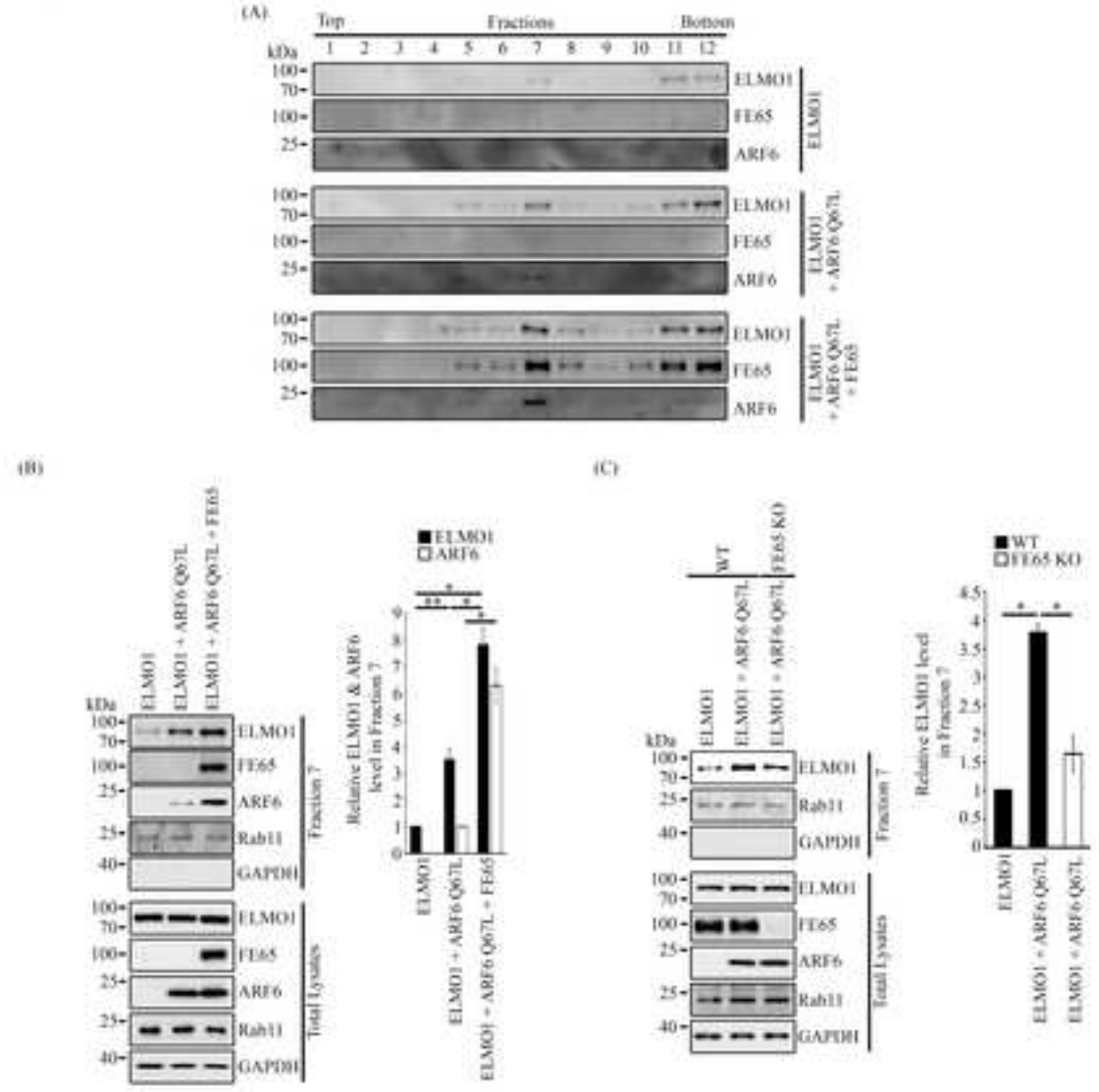


Figure 2



Name of Material/ Equipment	Company
1 mL, Open-Top Thickwall Polypropylene Tube, 11 x 34 mm	Beckman Coulter
100 mm tissue culture dish	SPL
13.2 mL, Certified Free Open-Top Thinwall Ultra-Clear Tube, 14 x 89 mm	Beckman Coulter
5x Sample Buffer	GenScript
cOmplete, EDTA-free Protease Inhibitor Cocktail	Roche
COX IV (3E11) Rabbit mAb	Cell Signaling Technology
Cycloheximide	Sigma-Aldrich
Dounce Tissue Grinder, 7 mL	DWK Life Sciences
Dulbecco's Modified Eagle Medium (DMEM) with low glucose	HyClone
ELMO1 antibody (B-7)	Santa Cruz Biotechnology
EndoFree Plasmid Maxi Kit	QIAGEN
FE65 antibody (E-20)	Santa Cruz Biotechnology
Fetal Bovine Serum, Research Grade	HyClone
GAPDH Monoclonal Antibody (6C5)	Ambion
ImageLab Software	Bio-Rad
Imidazole	Sigma-Aldrich
Lipofectamine 2000 Transfection Reagent	Invitrogen
Monoclonal Anti-β-COP antibody	Sigma
Myc-tag (9B11) mouse mAb	Cell Signaling Technology
OmniPur EDTA, Disodium Salt, Dihydrate	Calbiochem
Optima L-100 XP	Beckman Coulter
Optima MAX-TL	Beckman Coulter
Opti-MEM I Reduced Serum Media	Gibco
PBS Tablets	Gibco
PhosSTOP	Roche
RAB11A-Specific Polyclonal antibody	Proteintech
Sucrose	Affymetrix
SW 41 Ti Swinging-Bucket Rotor	Beckman Coulter
TLA-120.2 Fixed-Angle Rotor	Beckman Coulter
Trypsin-EDTA (0.05%), phenol red	Gibco

Catalog Number	Comments/Description
347287	
20100	
C14277	
MB01015	
11873580001	
4850S	Rabbit monoclonal antibody for detecting COX IV.
C1988	
357542	
SH30021.01	
SC-271519	Mouse monoclonal antibody for detecting ELMO1.
12362	
SC-19751	Goat polyclonal antibody for detecting FE65.
SV30160.03	
AM4300	Mouse monoclonal antibody for detecting GAPDH. Measurement of band intensity
I2399	
11668019	
G6160	Mouse monoclonal antibody for detecting β-COP.
2276S	Mouse monoclonal antibody for detecting myc tagged proteins.
4010-OP	
392050	
A95761	
31985070	
18912014	
4906845001	
20229-1-AP	Rabbit polyclonal antibody for detecting Rab11.
AAJ21931A4	
331362	
362046	
25300062	

Dear editor,

We have revised our manuscript to address the concerns of the reviewers. The changes have been marked as red in the revised manuscript. We hope that our protocol is now suitable for publishing in the JOVE.

Our responses towards the reviewers' comments are as follow:

Reviewer #1:

Major Concerns:

1) While authors show markers of recycling endosomes (Rab11), Golgi (b-COP), mitochondria (COXIV) and cytosol (GAPDH), they also need to show markers of other intracellular vesicles to confirm efficient fractionation, e.g., early, late endosomes, lysosomes.

Thank you for your comment. We have updated Fig 1 to include EEA1, Rab7 and Lamp1 marker as well. The EEA1 could be detected in both fraction 11 & 12, as well as fraction7. Weak signal of Rab7 and Lamp1 could be observed in fraction 12.

2) It is uncertain if the transfection protocol is necessary. Would this protocol of isolating recycling endosome still work if the cells are not transfected? That is, does transfection trigger the endocytic pathway?

In Fig 1, untransfected HEK293 cells were used for the fractionation to demonstrate that a Rab11-containing fraction could be obtained after the ultracentrifugation. Therefore, our protocol works well in untransfected cells and the obtaining of Rab11-positive fraction is not trigger by the transfection. We apologize for the typo in the result session, which may lead to the confusion of the cells used in Fig 1.

3) In Fig 1, the authors also need to show control lanes: total lysates as collected in step 2.10, nuclei and unbroken cells as collected in step 2.12 and PNS before ultracentrifugation as collected in step 2.14. To address the question above, this should be done for both transfected and non-transfected cells.

Thank you for the comment. The Fig 1 has been updated accordingly to include total lysates, nuclei and unbroken cells, and PNS.

4) In several places in the text, the authors mention the fraction 7 with Rab11 marker contains recycling endosomes at high purity. However, this is not truly demonstrated. Western blots data cannot be used to demonstrate purity of a fraction. The authors need to use alternative methods to demonstrate purity. For example, immunogold labeling of Rab11 and other markers followed by transmission electron microscopy can be used to demonstrate all endosomes are in fact recycling. Alternatively, discovery or targeted proteomics methods can be used to demonstrate purity of the fractions.

We apologize for our choice of wordings in the previous submission. We have amended the text such that no claiming on the purity of the obtained fraction is made.

Reviewer #2:

Major Concerns:

1) Essentially the same protocol has been published 25 years ago for the preparation of endosome fractions by floatation in a step sucrose gradient (e.g. Aniento et al. 1995. J Cell Biol 133: 29-41; reference not mentioned in the paper). It is not clear what is new in the protocol that is proposed here.

Thank you for your comment. We believe that the main objective of the JOVE is to present a visualized protocol for the researcher. Although density gradient ultracentrifugation is a traditional method for cell fractionation, we still think that it is suitable for publishing in the JOVE as a video protocol.

2) The protocol describes the preparation of a light membrane fraction, recovered at the interface between 35% and 25% sucrose. I see no evidence that recycling endosomes are enriched in this fraction (enrichment and yield are not indicated). What is the distribution of early endosomal markers (e.g. Rab5 or EEA1)?

We apologize that we could not calculate the exact yield of the isolation. Therefore, we have amended our protocol and no claim has been made on the enrichment level.

Also, we have updated Fig 1 and the early endosomal marker EEA1 is also included in the figure as well. The EEA1 could be detected in both fraction 11 & 12, as well as fraction7.

3) The fractionation data in Fig 1 are not really convincing; i) cytosol and mitochondria are found both at the bottom and at the top (fractions 1-2 and 11-12), which does not make much sense; ii) the Golgi marker betaCOP co-purifies with the cytosol; iii) the only marker of another membrane compartment is RAB11 itself. In Fig 2, endogenous ARF6 is not visible on the gradient. In Fig 1 and Fig 2A, where are the tops and bottoms of these gradients (fraction 1 or 12)?

We apologize for the quality of our previous figure and we updated our Fig 1 accordingly.

The cells in Fig 2 were transfected with either ELMO1, ELMO1 + ARF6-Q67L and ELMO1 + ARF6-Q67L + FE65. The ARF6 Q67L mutant used in our experiment contains a C terminal myc tag. In our Western blot, we used an anti-myc antibody to detect the ARF6 Q67L mutant. Therefore, endogenous ARF6 is not visible on the gradient.

We apologize for not indicating the top and bottom fraction in our figures. In fact, we collected the fraction starting from the top of the gradient. Therefore, the fraction 1 is the top fraction (less dense) while the fraction 12 is the bottom fraction (denser). The top and bottom fractions have been marked on the revised Fig 1 and Fig 2A.

Minor Concerns:

1) Authors mention that fast recycling occurs from early endosomes and involves clathrin, while slow recycling occurs from recycling endosomes and is clathrin-independent, referring to a review of Maxfield and McGraw from 2004. Since then, the field has moved forward quite a bit, and I am not

sure that this is the most prominent characteristic of recycling vs. early endosomes (e.g. review by Weeratunga et al. 2020. Current Opinion in Cell Biology 65:17-27).

Thank you for your comment. We have updated the text as follow (Line 45-53):

Proteins localized at the plasma membrane are internalized by endocytosis[2]. The internalized proteins are then sorted by the early endosome[3]. Some of the proteins are targeted to lysosomes for degradation[4]. However, a significant amount of proteins are recycled back to the cell surface by fast recycling and slow recycling processes. In fast recycling, proteins leave the early endosomes and directly return to the plasma membrane. Conversely, in slow recycling, proteins are first sorted to the endocytic recycling compartment and then transported back to the plasma membrane. Various cargo proteins, for examples clathrin, retromer complex, retriever complex and Wiskott-Aldrich syndrome protein and SCAR Homologue (WASH) complex, participates in such membrane recycling process[4-9].

The newly included references are listed as follow:

4 Cullen, P. J. & Steinberg, F. To degrade or not to degrade: mechanisms and significance of endocytic recycling. Nature Reviews: Molecular Cell Biology 19, 679-696, doi:10.1038/s41580-018-0053-7 (2018).

5 Weeratunga, S., Paul, B. & Collins, B. M. Recognising the signals for endosomal trafficking. Current Opinion in Cell Biology 65, 17-27, doi:10.1016/j.ceb.2020.02.005 (2020).

6 Khan, I. & Steeg, P. S. Endocytosis: a pivotal pathway for regulating metastasis. British Journal of Cancer 124, 66-75, doi:10.1038/s41416-020-01179-8 (2021).

7 Grant, B. D. & Donaldson, J. G. Pathways and mechanisms of endocytic recycling. Nature Reviews: Molecular Cell Biology 10, 597-608, doi:10.1038/nrm2755 (2009).

8 Maxfield, F. R. & McGraw, T. E. Endocytic recycling. Nature Reviews: Molecular Cell Biology 5, 121-132, doi:10.1038/nrm1315 (2004).

9 McDonald, F. J. Explosion in the complexity of membrane protein recycling. American Journal of Physiology Cell Physiology 320, C483-C494, doi:10.1152/ajpcell.00171.2020 (2021).

2) It is a bit awkward to state that emerging evidence suggests that ARF6 plays a role in endocytic membrane trafficking, when quoting references that are 10 years old. Besides the original paper on ARF6 in endocytosis is 25 years old and should be mentioned (D'Souza-Schorey et al. 1995. Science. 267:1175-8).

Thank you for your comment. We have included more recent works to stress on the importance of ARF6 in endocytic recycling. The text has been amended as follow (Line 57-61):

ARF6, a small GTPase, is a reported regulator of endocytic trafficking [7,11,12]. Of interest, various research groups have illustrated the importance of ARF6 in endocytic recycling[13-17]. Our group aims to investigate the relationship between ARF6-mediated neurite outgrowth and endocytic recycling. Although previous report suggests that the activation of ARF6 is upstream to Rac1 activity through acting on ELMO1-dedicator of cytokinesis 1 (DOCK180) complex[18].

The newly included references are listed as follow:

13 Finicle, B. T. et al. Sphingolipids inhibit endosomal recycling of nutrient transporters by inactivating ARF6. Journal of Cell Science 131, doi:10.1242/jcs.213314 (2018).

14 Lu, H. et al. APE1 Upregulates MMP-14 via Redox-Sensitive ARF6-Mediated Recycling to Promote Cell Invasion of Esophageal Adenocarcinoma. Cancer Research 79, 4426-4438, doi:10.1158/0008-5472.CAN-19-0237 (2019).

15 Qi, S. et al. Arf6-driven endocytic recycling of CD147 determines HCC malignant phenotypes. Journal of Experimental and Clinical Cancer Research 38, 471, doi:10.1186/s13046-019-1464-9 (2019).

16 Crupi, M. J. F. et al. GGA3-mediated recycling of the RET receptor tyrosine kinase contributes to cell migration and invasion. Oncogene 39, 1361-1377, doi:10.1038/s41388-019-1068-z (2020).

17 Gamara, J. et al. Assessment of Arf6 Deletion in PLB-985 Differentiated in Neutrophil-Like Cells and in Mouse Neutrophils: Impact on Adhesion and Migration. Mediators of Inflammation 2020, 2713074, doi:10.1155/2020/2713074 (2020).

3) Step 3.2. Authors should indicate what % sucrose should be obtained after mixing the PNS with 62% sucrose, presumably 40.6 %. It is also wise to recommend that % sucrose must be measure with a refractometer in order to obtain precise values and consistent results.

We apologize that we have miss out the resultant sucrose percentage of the resultant solution and a note has been added as follow (Line 131):

> Note: The resultant solution is a 40.6% sucrose solution.

Moreover, we totally agree that measuring the refractive indexes of the sucrose solutions is important for the reproducibility of the experiment. A note has been added to remind the readers as follow (Line 139-142):

> NOTE: The refractive index of the 62%, 40.6%, 35%, and 25% sucrose solutions at room temperature are 1.44, 1.40, 1.39, and 1.37, respectively. It is recommended to check the refractive indexes of the sucrose solutions with a refractometer to ensure the precision and consistency of the experiment.

Yours faithfully,

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Dear Editor,

We have revised our manuscript to address the concerns of the reviewers. The changes have been marked as red in the revised manuscript. We hope that our protocol is now suitable for publishing in the JOVE.

Our responses towards the reviewers' comments are as follow:

Reviewer #1:
Manuscript Summary:
The authors have updated Fig 1, which now includes Western blots on total lysates, nuclei and unbroken cells, and PNS before ultracentrifugation. The fact that Western blot signals for most markers are similar between Total and Nuclei/unbroken cells, the authors need to note this in the text and also include that the method described here is not an optimal method for extracting proteins from endosomes. In fact it is a very poor/inefficient method since majority of the endosomes still seem to be in the nuclei/unbroken cell fraction, which is discarded.

In our previous revision, we loaded same volume of sample for the Total and Nuclei/unbroken cells. However, due to the differences in the sample collection procedures, the total protein level in the Total and Nuclei/unbroken cells fraction could be different. A note concerning about the homogenization method has been added to the protocol (line 110 – 112) as follow:

NOTE: Other homogenization method, for example passing the sample through a syringe, could be used. The efficiency of homogenization could be checked by observing the homogenate under a phase contrast microscope.

Also, the authors actually did not address the point 3 fully. That is, they didn't show results in non-transfected cells. This is a minor point.

As mentioned in the previous letter, the immunoblots in Fig 1 is obtained from HEK293 cells without transfection, i.e., non-transfected cells.

Reviewer #2:

Major Concerns:
The authors have basically addressed my queries in this revised version of the paper. However, I still have two comments, which the authors may wish to take into consideration in order to improve the paper.

i) In the rebuttal letter, the authors write that they did not calculate yield and enrichment values. This is surprising: the principle of subcellular fractionation is to obtain fractions with a defined yield and a precise enrichment.

Thank you for the suggestion and we calculated the enrichment values by performing densitometric analysis with our immunoblot results with the ImageLab software (Bio-rad). The band intensities of GAPDH, COX IV and Rab11 in PNS and fraction 7 have been measured and the intensity ratio of the markers in the fraction 7 to the PNS are calculated. The result is expressed as a bar chart in Fig 1.

ii) The profiles of Lamp1 and Rab7 are not nice in new Fig 1. Moreover, the profiles of GAPDH ad COX IV look completely different from the previous Fig 1. How many times were these experiments done?

As shown in Fig 1, the levels of Lamp1 and Rab7 are very low in the PNS fraction. Therefore, the isolation of Lamp1- or Rab7-positive organelles, which are lysosomes and late endosomes, may not be efficient in our experimental conditions. All the experiments have been performed three times.

Reviewer #3:
No concerns have been raised by the reviewer.

Yours faithfully,

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