

Video Article

Cell Fractionation of U937 Cells in the Absence of High-speed Centrifugation

William D. McCaig¹, Matthew A. Deragon¹, Robert J. Haluska Jr.¹, Alexa L. Hodges¹, Payal S. Patel¹, Timothy J. LaRocca¹

¹Department of Basic and Clinical Sciences, Albany College of Pharmacy and Health Sciences

Correspondence to: Timothy J. LaRocca at Timothy.LaRocca@acphs.edu

URL: <https://www.jove.com/video/59022>

DOI: [doi:10.3791/59022](https://doi.org/10.3791/59022)

Keywords: Cellular fractionation, Mitochondria, Membrane, Cytoplasm, Organelles, Centrifugation, U937

Date Published: 12/13/2018

Citation: McCaig, W.D., Deragon, M.A., Haluska Jr., R.J., Hodges, A.L., Patel, P.S., LaRocca, T.J. Cell Fractionation of U937 Cells in the Absence of High-speed Centrifugation. *J. Vis. Exp.* (), e59022, doi:10.3791/59022 (2018).

Abstract

In this protocol we detail a method to obtain subcellular fractions of U937 cells without the use of ultracentrifugation or indiscriminate detergents. This method utilizes hypotonic buffers, digitonin, mechanical lysis and differential centrifugation to isolate the cytoplasm, mitochondria and plasma membrane. The process can be scaled to accommodate the needs of researchers, is inexpensive and straightforward. This method will allow researchers to determine protein localization in cells without specialized centrifuges and without the use of commercial kits, both of which can be prohibitively expensive. We have successfully used this method to separate cytosolic, plasma membrane and mitochondrial proteins in the human monocyte cell line U937.

Video Link

The video component of this article can be found at <https://www.jove.com/video/59022/>

Introduction

Reliable identification of protein localization is often necessary when examining molecular pathways in eukaryotic cells. Methods to obtain subcellular fractions are utilized by researchers to more closely examine cellular components of interest.

The majority of existing cell fractionation methods generally fall into two broad categories, detergent-based^{1,2} and ultracentrifugation-based^{3,4,5}, which can be differentiated by speed, precision and cost. Detergent based protocols rely on the use of buffers with increasing detergent strength to solubilize distinct components of the cell. This is a rapid and convenient method for processing samples and can be cost effective if the number and size of samples are small. Detergent-based kits can be purchased to isolate cytoplasmic, membrane/organelle (mixed fraction), and nuclear fractions from cells. However, several drawbacks associated with these kits limit their usefulness to researchers. They are designed to easily isolate one or two components of the cell, but are incapable of isolating all fractions from a sample concurrently. The use of detergents means that the plasma membrane and membrane-enclosed organelles will be equally solubilized and, therefore, unable to be separated from one another. An additional complication arises from the proprietary components in these kits which prevents researchers from altering conditions for specific applications. Lastly, they are limited in number of uses and may be prohibitively expensive for larger scale experiments. Non-detergent based kits exist for the isolation of mitochondria, however, they are not designed to isolate plasma membrane and the sample yield is significantly less than that from density centrifugation based isolation protocols^{6,7}.

Methods that utilize ultracentrifugation to obtain fractions are more time consuming, but often result in purer fractions than detergent-based kits. To isolate plasma membranes from cells without first solubilizing them (resulting in contamination with membrane organelles) requires them to be lysed by a non-detergent method followed by separation of cellular components via differential centrifugation—with plasma membrane isolation requiring speeds of 100,000 × *g* to accomplish. In many cases, differential centrifugation must be followed by isopycnic density gradient centrifugation for further separation of cellular fractions or removal of contaminants. While these methods are thorough and modifiable, drawbacks include cost, time consumption, and the need for an ultracentrifuge for separation of fractions and further purification via density gradient centrifugation. Most high-speed centrifuges are at a cost that is prohibitive for individual investigators and are often shared, core equipment at academic institutions. Thus, ultracentrifuge availability becomes prohibitive in these situations.

In this fractionation protocol we demonstrate the isolation of subcellular fractions without the use of solubilizing detergents and without high speed centrifugation. This method will allow researchers to isolate the plasma membrane, mitochondria and cytoplasmic components of a eukaryotic cell with minimal contamination between fractions.

Protocol

1. Prepare Buffers and Reagents

NOTE: See **Table 1**.

1. Prepare solutions of buffer A, lysis buffer B, sample buffer and digitonin.
 1. Prepare buffer A by adding 8.77 g of NaCl and 50 mL of HEPES (1 M, pH 7.4) to 900 mL deionized water, adjust final volume to 1 L with deionized water.
NOTE: Final concentrations are 150 mM NaCl and 50 mM HEPES.
 2. Prepare lysis buffer B by adding 20 mL of HEPES (1 M, pH 7.4), 0.75 g of KCl, 0.19 g of MgCl₂, 2 mL of Ethylenediaminetetraacetic acid (0.5 M EDTA), 2 mL of ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (0.5 M EGTA), 38.26 g of mannitol and 23.96 g of sucrose to 900 mL of deionized water, adjust final volume to 1 L with deionized water.
NOTE: Final concentrations are 20 mM HEPES, 10 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 210 mM mannitol and 70 mM sucrose.
 3. Prepare sample buffer by adding 0.01 g of sodium dodecyl sulfate (SDS) to 10 mL of tris-buffered saline (TBS) for a final concentration of 0.1% SDS.
 4. Prepare a stock solution of digitonin by adding 25 mg of digitonin to 100 mL of deionized water (final concentration is 250 µg/mL).
 5. Store all buffer solutions at 4 °C and digitonin at -20 °C until the start of experiment.
2. Prepare fresh solutions of protease and phosphatase inhibitors to be added to buffer solutions prior to addition to cells.
 1. Prepare a stock solution of phenylmethanesulfonyl fluoride (PMSF) by adding 17.4 mg of PMSF to 1 mL of 100% ethanol (final concentration is 100 mM).
CAUTION: Wear appropriate protective equipment and exercise caution when handling PMSF. PMSF is hazardous if ingested and slightly hazardous in case of skin contact (irritant), eye contact (irritant) or inhalation; it is corrosive to eyes and skin.
 2. Prepare a commercially available protease inhibitor cocktail (100×) according to the manufacturer's instructions (see the **Table of Materials**).
 3. Prepare a stock solution of sodium orthovanadate (SOV) by adding 92 mg of SOV to 1 mL of deionized water (final concentration is 500 mM).
CAUTION: Wear appropriate protective equipment and use caution when handling. SOV is hazardous in case of eye contact (irritant), ingestion or inhalation. Severe over-exposure can result in death.

2. PBS Wash

1. Concentrate and wash cells in phosphate-buffered saline (PBS) prior to fractionation.
 1. Centrifuge cell suspension at an appropriate speed to create a pellet. For example, centrifuge a suspension of U937 cells at 400 × g for 10 min.
 2. Remove the supernatant, resuspend cell pellet in room-temperature PBS at a final concentration of 4 × 10⁶ cells/mL and pipette gently to break up clumps.
 3. Centrifuge the cell suspension at 400 × g for 10 min to pellet cells.
 4. Remove the supernatant and resuspend cell pellet in **ice cold** buffer A at a final concentration of 2 × 10⁷ cells/mL.
NOTE: All subsequent steps should be carried out at 4 °C or on ice and all buffers should be **pre-chilled**.

3. Cytosolic Protein Isolation

1. Extract cytosolic proteins by incubation with the detergent digitonin.
 1. Immediately prior to resuspension of cells (step 3.1.3) add 10 µL of stock PMSF (100 mM), 10 µL of protease inhibitor (100×), 2 µL of stock SOV (500 mM) and 100 µL of stock digitonin (250 µg/mL) to 878 µL of buffer A (final concentrations are 1 mM PMSF, 1× Protease Inhibitor, 1 mM SOV and 25 µg/mL digitonin; adjust the final volume as per the number of cells being used). Keep the solution on **ice** until addition to cell pellet.
 2. Centrifuge the cell suspension at 400 × g for 10 min and remove the supernatant.
 3. Resuspend the cell pellet in buffer A solution containing inhibitors and digitonin (prepared in step 3.1.1) at a final concentration of 2 × 10⁷ cells/mL, pipette gently to break up clumps.
 4. Incubate the cell suspension on an end-over-end rotator at 4 °C for 20 min.
 5. Centrifuge the cell suspension at 400 × g for 10 min. Collect the supernatant and place it in a clean centrifuge tube.
 6. Centrifuge the collected supernatant at 18,000 × g for 20 min to pellet cellular debris.
 7. Transfer the supernatant to a clean centrifuge tube.
 8. Repeat steps 3.1.5 and 3.1.6 until no pellet is obtained following centrifugation.
 9. Collect the supernatant containing the **cytosolic proteins** and store it at 4 °C (short term) or -20 °C (long term).
2. Remove excess digitonin and cytosolic proteins by centrifugation.
 1. Resuspend the digitonin-permeabilized cell pellet (from step 3.1.5) in buffer A at a final concentration of 4 × 10⁶ cells/mL and pipette gently to break up clumps.
 2. Centrifuge the digitonin-permeabilized cell suspension at 400 × g for 10 min and remove the supernatant.
NOTE: Repeated washes in buffer A can be performed to remove excess cytosolic contaminants.

4. Cell Homogenization

1. Incubate the cells on ice in lysis buffer B and lyse them by mechanical means.
 1. Immediately prior to resuspension of cells (step 4.1.2) add 10 µL of stock PMSF (100 mM) and 2 µL of stock SOV (500 mM) to 988 µL of lysis buffer B (final concentrations are 1 mM PMSF and 1 mM SOV; adjust final volume to accommodate number of cells being lysed) and keep solution on **ice** until addition to cell pellet.

2. Resuspend the cell pellet (from step 3.2.2) in **ice cold** lysis buffer B solution containing PMSF and SOV (prepared in step 4.1.1) at a final concentration of 4×10^6 cells/mL.
3. Incubate the cell suspension on ice for 30 min.
4. Transfer the cell suspension to a pre-chilled Dounce homogenizer (with a tight-fitting B pestle) on ice and perform 40 passes with the homogenizer pestle using slow, even strokes.
NOTE: Alternatively utilize other means of mechanical cell lysis as detailed in discussion section.
5. Collect the homogenate and transfer it to a clean centrifuge tube.
6. Wash the homogenizer pestle and tube with a small volume (1 to 2 mL) of lysis buffer B and add it to the homogenate.
7. Centrifuge the homogenate at $400 \times g$ (or the minimum speed required to pellet unbroken cells) for 10 min.
8. Transfer the supernatant to a clean centrifuge tube.
Note: If a significant pellet remains repeat steps 4.1.4 through 4.1.6 to increase the yield of fractions as detailed in the discussion section. The protocol can be paused here, and the homogenate stored at 4°C for short term (24 h).

5. Differential Centrifugation

1. Centrifuge the homogenate at increasing speeds to remove cellular debris, isolate mitochondria and membrane fractions.
 1. Centrifuge the supernatant (from step 4.1.8) at $500 \times g$ for 10 min. Transfer supernatant to a clean centrifuge tube, and discard any pellet.
 2. Centrifuge supernatant (from step 5.1.1) at $1,000 \times g$ for 10 min. Transfer the supernatant to a clean centrifuge tube, and discard any pellet.
 3. Centrifuge the supernatant (from step 5.1.2) at $2,000 \times g$ for 10 min. Transfer the supernatant to a clean centrifuge tube, and discard any pellet.
 4. Centrifuge the supernatant (from step 5.1.3) at $4,000 \times g$ for 15 min. Transfer supernatant to a clean centrifuge tube, **keep** pellet containing mitochondria.
 5. Resuspend the mitochondria pellet in a small volume (0.5–1 mL) of lysis buffer B.
 6. Centrifuge the suspended pellet at $4,000 \times g$ for 15 min. Remove the supernatant and resuspend the **mitochondrial** pellet in the desired final volume of sample buffer (e.g., 250 to 500 μL , depending on the size of the pellet and desired concentration).
 7. Centrifuge the supernatant (from step 5.1.4) at $4,000 \times g$ for 15 min. Transfer the supernatant to a clean centrifuge tube. Repeat this step until no pellet is obtained following centrifugation.
 8. Spin the supernatant at $18,000 \times g$ for 3 h.
 9. Remove the supernatant, and keep the pellet containing membrane proteins. Resuspend the membrane pellet in a small volume (0.5–1 mL) of lysis buffer B.
 10. Centrifuge the suspended pellet at $18,000 \times g$ for 1 h.
 11. Remove the supernatant and resuspend the **membrane** pellet in the desired final volume of sample buffer (250 to 500 μL , depending on the size of the pellet and desired concentration).
2. Sonicate the sample pellets for 3 s in an ice bath at a power setting of 5 (50% of 125 W maximum power at 20 kHz, see **the Table of Materials**).
3. Store the samples at 4°C (short term) or -20°C (long term).
4. Examine the samples for purity of fractionation by performing a western blot utilizing antibodies against protein markers found in the cytoplasm, mitochondria and membrane compartments of the cell (refer to the representative results section).

Representative Results

Successful fractionation of undifferentiated U937⁸ cells grown in suspension was accomplished using the protocol detailed above and illustrated in **Figure 1**. The samples obtained with this method were subjected to western blotting⁹ utilizing a wet transfer method to a polyvinylidene fluoride (PVDF) membrane. The membrane was subsequently probed with antibodies against cytoplasmic, mitochondrial and membrane localized protein markers (**Figure 2**, **Figure 3**, **Figure 4**). The successful extraction of cytoplasmic proteins can be verified by probing the blot with antibodies against the cytosolic protein glyceraldehyde-3-phosphate dehydrogenase¹⁰ (GAPDH), normally localized to the cytoplasm of the cell. As shown by the immunoblot (**Figure 2**; bottom panel, lanes 1 and 2), GAPDH is found only in the digitonin extracted samples and no contamination is observed in the $4,000 \times g$ pellets (**Figure 4**; lanes 3 and 4 on the bottom panel) or $18,000 \times g$ pellets (**Figure 4**; lanes 5 and 6 on the bottom panel). Probing for the voltage-dependent anion channel (VDAC), a protein localized to the outer mitochondrial membrane¹¹, shows the successful isolation of mitochondria in the $4,000 \times g$ pellets (**Figure 4**; lanes 3 and 4 on the middle panel), while the absence of this protein in other fractions shows the lack of mitochondrial contamination in the $18,000 \times g$ pellets or digitonin-extracted samples. Probing for the Na/K-ATPase $\alpha 1$ subunit, part of an integral membrane heterodimer found primarily in the plasma membrane¹², shows the majority of this protein located in the $18,000 \times g$ pellets (**Figure 4**; lanes 5 and 6 on the top panel). This protein is also detected in the $4,000 \times g$ pellets (**Figure 4**; lanes 3 and 4 of the top panel), suggesting possible contamination with plasma membrane, though this possibility is unlikely at the low speed with which this pellet was obtained. A more plausible explanation is the presence of endoplasmic reticulum (ER) in the $4,000 \times g$ pellets sample, as transport of Na,K-ATPase subunits from the ER to the plasma membrane has been demonstrated by researchers¹³. The lack of Na,K-ATPase $\alpha 1$ protein in the digitonin extracted samples (**Figure 4**; lanes 1 and 2 on the top panel) demonstrates the purity of this fraction.

In contrast to the outcome observed during a successful fractionation (**Figure 2**), improper execution of the protocol can result in cross contamination of cellular components (**Figure 3**, **Figure 4**). A high concentration of Na,K-ATPase $\alpha 1$ protein in the $4,000 \times g$ pellets, when compared to the $18,000 \times g$ pellets (**Figure 3** [top panel, lane 2-3] and **Figure 4B** [top panel, lanes 5-12]), indicates that the organelle fraction has been contaminated with plasma membrane proteins. The presence of GAPDH in any fraction other than the digitonin-extracted cytoplasmic sample (**Figure 3** [bottom panel, lane 4] and **Figure 4A** [bottom panel, lanes 5-12]) is an indicator of failure to remove cytoplasmic proteins in subsequent steps.

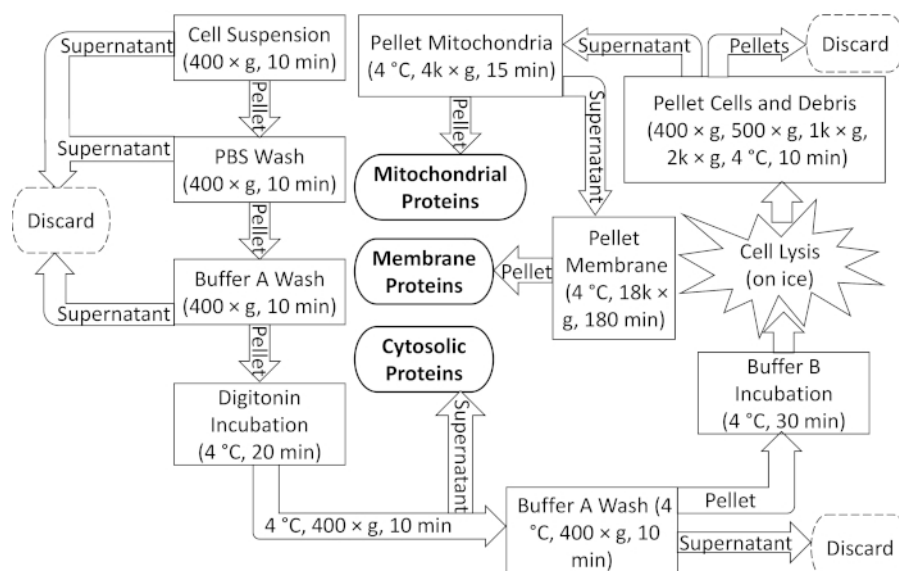


Figure 1: Diagram of the Cell Fractionation Protocol. An overview of the cell fractionation protocol represented as a flow chart. [Please click here to view a larger version of this figure.](#)

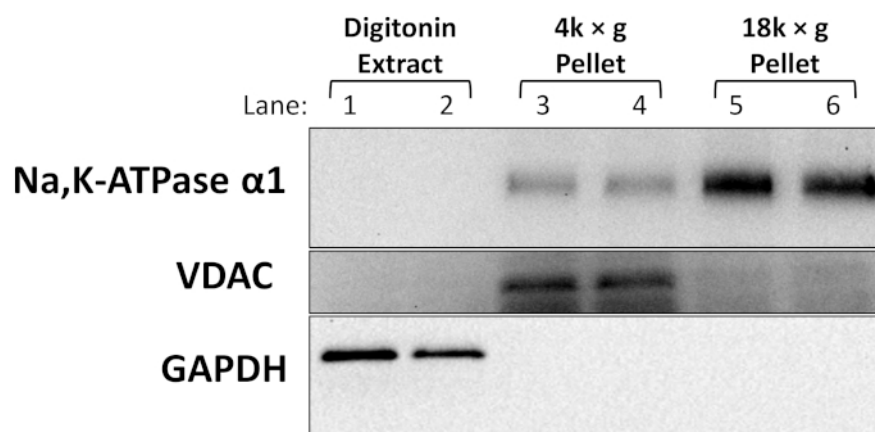


Figure 2: Successful Isolation of U937 Cell Cytoplasm, Organelle, and Membrane Fractions. Western blots of cell fractions isolated from two U937 cell cultures and probed for markers of membrane (Na, K-ATPase α1, top panel), mitochondria (VDAC, middle panel) and cytoplasm (GAPDH, bottom panel). [Please click here to view a larger version of this figure.](#)

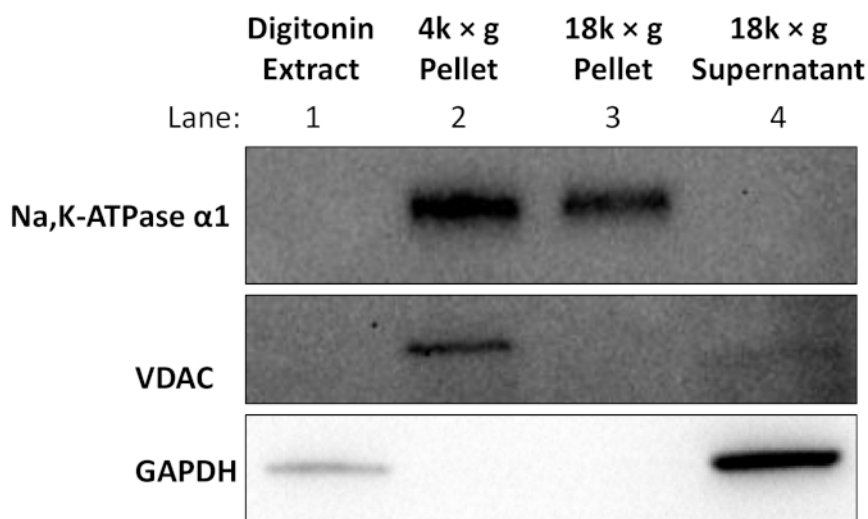


Figure 3: Failure to Fractionate U937 Cell Components. Western blots of cell fractions isolated from a single U937 cell culture showing contamination of the organelle fraction (lane 2) with Na, K-ATPase α1 (top panel) and loss of cytoplasmic components into the supernatant of the membrane pellet (lane 4). [Please click here to view a larger version of this figure.](#)

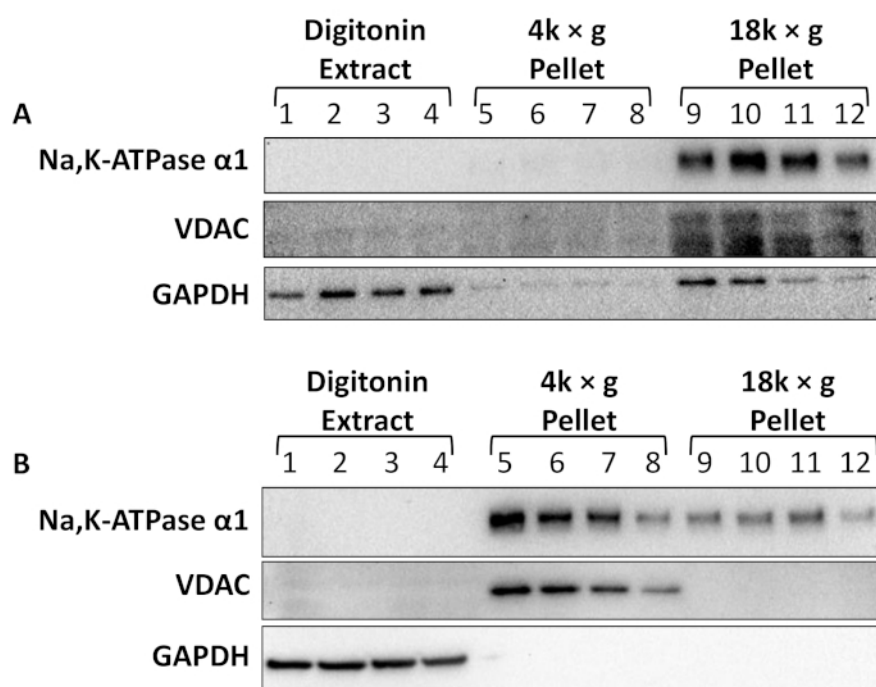


Figure 4: Cross Contamination of Cytoplasmic and Membrane Components During Fractionation. (A) Fractionation attempt with cytoplasmic cross contamination of GAPDH in organelle (bottom panel, lanes 5-8) and membrane fractions (bottom panel, lanes 9-12). (B) Fractionation attempt with plasma membrane contamination of Na, K-ATPase in mitochondrial fractions (top panel, lanes 5-8). [Please click here to view a larger version of this figure.](#)

Name	Composition (stock concentration)	Final Concentration
Buffer A	NaCl (150 mM) and HEPES (50 mM)	1X (same as stock)
Lysis Buffer B	HEPES (20 mM), KCl (10 mM), MgCl ₂ (2 mM), EDTA (1 mM), EGTA (1 mM), Mannitol (210 mM) and Sucrose (70 mM)	1X (same as stock)
Sample Buffer	Sodium dodecyl sulfate (0.1%) in Tris-buffered Saline	1X (same as stock)
Digitonin	Digitonin (250 µg/ml) in deionized water	25 µg/ml
Phenylmethanesulfonyl fluoride	Phenylmethanesulfonyl fluoride (100 mM) in 100% ethanol	1 mM
Protease Inhibitor Cocktail	Varies (See manufacturer product sheet)	1X
Sodium Orthovanadate	Sodium Orthovanadate (500 mM) in deionized water	1 mM

Table 1: Buffers and Solutions. Composition of buffers and required solutions for the procedure.

Protocol Step	Critical Factor	Explanation	Potential Issues	Possible Solutions
Cell Preparation	Cell concentration	The optimal concentration of cells that this method can process must be determined empirically for the cell type being worked with in order to obtain the best results.	Highly concentrated cell suspensions may result in inefficient lysis, leading to low yields of mitochondria and membrane fractions.	Perform initial procedure with a range of cell concentrations to determine best results.
	Pre-processing of cells	Cells must be in suspension for the fractionation procedure, this requires detaching adherent cells from culture surfaces or homogenizing tissues.	Inefficient processing may result in low fraction yields, cross contamination between subcellular fractions or other unexpected results.	Ensure that the method employed results in sufficient cells for the procedure. Count cells in suspension to determine concentration after processing and adjust accordingly.
			If the method employed compromises the plasma membrane, premature lysis may occur, resulting in cross contamination of fractions.	Ensure that plasma membrane integrity is maintained during cell harvest by using methods that avoid damaging the cell. Verify membrane integrity by examination under a microscope with the inclusion of a membrane impermeable dye (such as Trypan blue).
Cytosolic Protein Isolation	Digitonin Concentration	The optimal concentration of digitonin must be determined to avoid lysis of cells while still allowing cytosolic protein extraction through pore formation.	High concentrations of digitonin may lead to membrane rupture, cell lysis and contamination of the cytosolic fraction. Suboptimal concentrations will result in inefficient extraction of cytosolic proteins and possible cross contamination of subsequent fractions.	The concentration of digitonin should be decreased if excessive cell lysis is observed. A small cell pellet obtained following digitonin incubation may indicate membrane rupture and cell lysis.
	Post-Extraction Wash	Removal of digitonin and cytosolic proteins from permeabilized cells must be performed to avoid cross contamination.	Failure to wash cells sufficiently after cytosolic extraction may result in carry over of cytosolic proteins to other fractions.	Additional washes with Buffer A following digitonin incubation will dilute digitonin and remaining cytosolic proteins.
Mitochondrial Isolation	Cell Lysis	Lysis methods must be thorough to release cellular contents, while maintaining mitochondrial integrity for subsequent isolation.	Mechanical lysis of cells may be inefficient, leaving cells intact and resulting in low yields of mitochondrial proteins.	The amount of force needed to lyse cells and release mitochondria must be determined empirically for different cell types. Large pellets obtained after the lysis step (as well as small mitochondrial and membrane pellets) may indicate suboptimal lysis. Increase the amount of force (pestle strokes, needle passages, etc.) to minimize the post lysis pellet.
			Too much mechanical force may lyse mitochondria, contaminating the plasma membrane fraction with mitochondrial membrane proteins.	Decrease the amount of force if mitochondrial protein markers are found in membrane samples.

	Debris Removal	Intact cells and larger fragments must be removed from the homogenate following lysis to avoid contaminating mitochondrial samples.	Mitochondrial samples may become contaminated with cytoplasmic components or plasma membrane proteins if debris and intact cells are not removed prior to pelleting mitochondria.	Increase the number of low speed centrifugation steps prior to the mitochondrial isolation spin. If mitochondrial yield is low, it may be necessary to save pellets from low speed spins and check via western blot for mitochondrial markers.
	Post-Isolation Wash	Mitochondrial samples should be washed thoroughly to remove contaminating debris that may pellet with them.	Cell fragments may aggregate and associate with mitochondria, leading to cross contamination of cytoplasmic or membrane proteins.	Ensure that pellets are sufficiently washed with buffer to remove contaminants.
Membrane Isolation	Centrifugation Time	Centrifugation times may need to be extended to increase yield of membrane fraction sample.	Depending on the total number of processed cells and the efficiency of cell lysis, membrane fraction sample yield may be low.	Increasing the centrifugation time may improve yield of plasma membrane fraction. While the suggested time is sufficient for a large starting quantity of cells, longer times may be necessary for smaller quantities.

Table 2: Critical Steps. Summary table of protocol steps, potential issues and possible solutions for troubleshooting the protocol.

Discussion

The development of this protocol arose from an inability to separate mitochondrial and membrane samples, using commercially available kits, for analysis of protein localization during necroptosis¹⁴. The primary limitations of premade kits are their inability to be adapted to the needs of individual researchers, their cost per sample and limited number of samples able to be processed. The method presented here can be performed without the use of expensive reagents and without the necessity for expensive equipment. This method can be scaled to accommodate any number of cells and is capable of being altered to suit the needs of researchers. This allows the yield of each fraction to be increased, tailoring of steps to the research being performed and flexibility in execution of the method. The addition of sodium orthovanadate in the buffers can be omitted if researchers are not examining phosphorylation state of proteins in the final samples. The inclusion of SDS in the final buffer can likewise be omitted if researchers wish to further purify the samples *via* density gradient centrifugation. While we have only used this method to successfully fractionate U937 cells, a non-adherent monocyte cell line, the procedure should work with multiple cell lines and tissues, with minor alterations. Cells grown in suspension can be pelleted similarly to the U937 cells detailed here, while adherent cell lines require dissociation from the growth surface prior to beginning this protocol. Similarly, tissues must be thoroughly homogenized prior to fractionation of containing cells. This can be accomplished by utilizing a loose fitting Dounce homogenizer, a Potter-Elvehjem glass-polytetrafluoroethene homogenizer or by other (commercial) methods¹⁵.

There are a number of critical steps in the protocol that need careful attention to obtain optimal results and pure fractions (**Table 2**). The concentration of cells recommended here (steps 2.1.2, 2.1.4, 3.1.3, 3.2.1 and 4.1.2) are specific to U937 cells and were determined through trial and error. These values may need to be adjusted to accommodate different types of cells if suboptimal results are obtained when executing the protocol. During the cytoplasmic extraction step (step 3.1), the concentration of digitonin must be sufficient to permeabilize the plasma membrane without completely lysing the cells. Following this incubation, cells must be thoroughly washed to remove cytosolic proteins from subsequent steps or cross contamination will occur in downstream samples (**Figure 4A**). The homogenization step (step 4.1.4) can be accomplished with any form of mechanical lysis, although results presented here were obtained with a Dounce homogenizer. An alternative to using a Dounce homogenizer is to repeatedly pass cells through a narrow gauge needle (27 G recommended, 20-40 passes) until sufficient cell lysis is achieved. It may be necessary to increase the number of strokes (or syringe passes) if a large pellet of unbroken cells is obtained after homogenization (step 4.1.7). Researchers will likely need to determine the optimal amount of homogenization for the type of cell being fractionated. Removal of cellular debris following the homogenization must be thorough to avoid contamination of the organelle fraction with plasma membrane components (**Figure 4B**). If this occurs, additional low speed centrifugation steps should be performed to remove cellular debris. During development of this method up to 18 h of centrifugation at 18,000 × g was tested, with minimal increase of plasma membrane yield over the 3 h spins (**Figure 2**, lanes 5-6). Researchers may find it necessary to increase centrifugation times to obtain better yields of the plasma membrane sample.

The method presented here is limited in comparison to the more thorough purification methods involving ultracentrifugation. Without the use of isopycnic density centrifugation it is not possible to separate individual organelles obtained after cell homogenization. While the 4,000 × g fractions contain mitochondria (as evidenced by the presence of VDAC, **Figure 2**), they likely also contain ER, golgi and additional intracellular organelles. The organelle fraction should be verified by the use of additional antibodies to protein markers of other organelles if this is of importance to the research being performed.

Disclosures

The authors declare no conflict of interest

Acknowledgements

Work was supported by NIH-1R15HL135675-01 to Timothy J. LaRocca

References

1. Baghirova, S., Hughes, B. G., Hendzel, M. J., Schulz, R. Sequential fractionation and isolation of subcellular proteins from tissue or cultured cells. *MethodsX*. **2**, e440-e445 (2015).
2. Hwang, S. I., Han, D. K. Subcellular fractionation for identification of biomarkers: Serial detergent extraction by subcellular accessibility and solubility. *Methods in Molecular Biology*. **1002**, 25-35 (2013).
3. Song, Y., Hao, Y., et al. Sample preparation project for the subcellular proteome of mouse liver. *Proteomics*. **6**, (19) 5269-5277 (2006).
4. Lenstra, J. A., Bloemendal, H. Topography of the total protein population from cultured cells upon fractionation by chemical extractions. *European Journal of Biochemistry*. **135**, (3) 413-423 (1983).
5. Michelsen, U., von Hagen, J. Chapter 19 Isolation of Subcellular Organelles and Structures. *Methods in Enzymology*. **463**, (C) 305-328 (2009).
6. Stimpson, S. E., Coorssen, J. R., Myers, S. J. Optimal isolation of mitochondria for proteomic analyses. *Analytical Biochemistry*. **475**, 1-3 (2015).
7. Williamson, C. D., Wong, D. S., Bozidis, P., Zhang, A., Colberg-Poley, A. M. Isolation of Endoplasmic Reticulum, Mitochondria, and Mitochondria-Associated Membrane and Detergent Resistant Membrane Fractions from Transfected Cells and from Human Cytomegalovirus-Infected Primary Fibroblasts. *Current protocols in cell biology*. **68**, 3.27.1-3.27.33 (2015).
8. Sundström, C., Nilsson, K. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *International Journal of Cancer*. **17**, (5) 565-77 (1976).
9. Towbin, H., Staehelin, T., Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences*. **76**, (9) 4350-4354 (1979).
10. Barber, R. D., Harmer, D. W., Coleman, R. A., Clark, B. J. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiological Genomics*. **21**, (3) 389-395 (2005).
11. Hodge, T., Colombini, M. Regulation of metabolite flux through voltage-gating of VDAC channels. *Journal of Membrane Biology*. **157**, (3) 271-279 (1997).
12. Therien, a G., Blostein, R. Mechanisms of sodium pump regulation. *American journal of physiology. Cell physiology*. **279**, (3) C541-66 (2000).
13. Devarajan, P., Stabach, P. R., De Matteis, M. A., Morrow, J. S. Na,K-ATPase transport from endoplasmic reticulum to Golgi requires the Golgi spectrin-ankyrin G119 skeleton in Madin Darby canine kidney cells. *Proceedings of the National Academy of Sciences of the United States of America*. **94**, (20) 10711-6 (1997).
14. McCaig, W. D., Patel, P. S., et al. Hyperglycemia potentiates a shift from apoptosis to RIP1-dependent necroptosis. *Cell Death Discovery*. **4**, 55 (2018).
15. Simpson, R. J. Homogenization of Mammalian Tissue. *Cold Spring Harbor Protocols*. **2010**, (7) (2010).