Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62119R1
Full Title:	Cell Fractionation of U937 Cells by Isopycnic Density Gradient Purification
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biochemistry
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Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Albany, NY, USA
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TITLE:

Cell Fractionation of U937 Cells by Isopycnic Density Gradient Purification

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KEYWORDS:

Cellular fractionation, Nuclear, Mitochondria, Membrane, Cytoplasm, Organelles, Ultracentrifugation, Density Gradient, U937

SUMMARY:

This fractionation protocol will allow researchers to isolate cytoplasmic, nuclear, mitochondrial, and membrane proteins from mammalian cells. The latter two subcellular fractions are further purified via isopycnic density gradient.

ABSTRACT:

This protocol describes a method to obtain subcellular protein fractions from mammalian cells using a combination of detergents, mechanical lysis, and isopycnic density gradient centrifugation. The major advantage of this procedure is that it does not rely on the sole use of solubilizing detergents to obtain subcellular fractions. This makes it possible to separate the plasma membrane from other membrane-bound organelles of the cell. This procedure will facilitate the determination of protein localization in cells with a reproducible, scalable, and selective method. This method has been successfully used to isolate cytosolic, nuclear, mitochondrial, and plasma membrane proteins from the human monocyte cell line, U937. Although optimized for this cell line, this procedure may serve as a suitable starting point for the subcellular fractionation of other cell lines. Potential pitfalls of the procedure and how to avoid them are discussed as are alterations that may need to be considered for other cell lines.

INTRODUCTION:

Subcellular fractionation is a procedure in which cells are lysed and separated into their constituent components through several methods. This technique can be used by researchers to determine protein localization in mammalian cells or for enrichment of low-abundance proteins that would otherwise be undetectable. While methods for subcellular fractionation currently exist, as do commercial kits that can be purchased, they suffer from several limitations that this procedure attempts to overcome. Most cell fractionation methods are exclusively detergent-based[1,2], relying on the use of buffers containing increasing amounts of detergent to solubilize

different cellular components. While this method is rapid and convenient, it results in impure fractions. These are designed to allow researchers to easily isolate one or two components of the cell, but are not complex enough to isolate multiple subcellular fractions from a sample at the same time. Relying solely on detergents usually results in membrane-enclosed organelles and the plasma membrane being indiscriminately solubilized, making separation of these components difficult. An additional complication from the use of these kits is the inability of researchers to alter/optimize them for specific applications, as most of the components are proprietary formulations. Finally, these kits can be prohibitively expensive, with limitations in the number of uses that make them less than ideal for larger samples.

Despite the availability of kits for the isolation of mitochondria that do not rely on detergents, they are not designed to isolate the plasma membrane and yield significantly lower amounts of sample than standard isolation protocols[3,4]. While differential centrifugation methods are more time-consuming, they often result in distinct fractions that cannot be obtained with exclusively detergent-based kits[1]. Separation without the sole use of solubilizing detergents also allows further purification using ultracentrifugation and isopycnic density gradients, resulting in less cross-contamination. This fractionation protocol demonstrates the isolation of subcellular fractions from U937 monocytes using a combination of detergent- and high-speed centrifugation-based approaches. This method will facilitate the isolation of the nuclear, cytoplasmic, mitochondrial, and plasma membrane components of a mammalian cell with minimal contamination between the fractions.

PROTOCOL:

1. **Prepare buffers and reagents**

1.1. Prepare fresh solutions of phosphatase and protease inhibitors.

1.1.1. Add 17.4 mg of phenylmethanesulfonyl fluoride (PMSF)to 1 mL of 100% ethanol to prepare a 100 mM stock.

NOTE: Wear protective equipment when handling PMSF as it is hazardous when ingested or inhaled and upon contact with skin or eyes. It is corrosive to eyes and skin.

1.1.2. According to the manufacturer's instructions, prepare a commercially available protease inhibitor cocktail (100x).

1.1.3. Add 91.9 mg of sodium orthovanadate (SOV) to 1 mL of deionized water to prepare a 500 mM stock.

NOTE: Wear protective equipment when handling SOV as it is hazardous if ingested, inhaled, or upon contact with eyes. Severe overexposure may result in death.

1.2. Prepare lysis buffer A, cytoplasmic isolation (CI) buffer, cell solubilization (CS) buffer, nuclear

lysis (NL) buffer, lysis buffer B, cell homogenization (CH) buffer, iodixanol diluent, and detergents.

1.2.1. Add 8.7 g of sodium chloride (NaCl) and 50 mL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 1 M, pH 7.4) to 950 mL of deionized water to prepare lysis buffer A (final concentrations of 150 mM NaCl and 50 mM HEPES).

1.2.2. Prepare stock solutions of detergents as follows: add 0.1 g of sodium dodecyl sulfate (SDS) to 10 mL of lysis buffer A (final concentration of 1% SDS), add 0.1 g of sodium deoxycholate to 10 mL of lysis buffer A (final concentration of 1%), add 2.5 mg of digitonin to 10 mL of lysis buffer A (final concentration of 250 µg/mL), and add 1 mL of non-ionic, non-denaturing detergent (see the **Table of Materials**) to 9 mL of lysis buffer A (final concentration of 10% (v/v)).

1.2.3. Prepare CI buffer by adding 10 µL of PMSF stock (100 mM), 10 µL of protease inhibitor (100x), 2 µL of SOV stock (500 mM), and 100 µL of stock digitonin (250 µg/mL) to 878 µL of lysis buffer A (final concentrations of 1 mM PMSF, 1x protease inhibitor, 1 mM SOV, and 25 µg/mL digitonin). Keep the solution on ice until addition to cell pellet.

1.2.4. Prepare CS buffer by adding 10 µL of PMSF stock (100 mM), 10 µL of protease inhibitor (100x), 2 µL of SOV stock (500 mM), 100 µL of non-ionic, non-denaturing detergent stock (see the **Table of Materials**) (10%), and 118 µL of hexylene glycol stock (8.44 M) to 760 µL of lysis buffer A (final concentrations of 1 mM PMSF, 1x protease inhibitor, 1 mM SOV, 1% non-ionic, non-denaturing detergent, and 1 M hexylene glycol). Keep the solution on ice until addition to cell pellet.

1.2.5. Prepare NL buffer by adding 10 µL of PMSF stock (100 mM), 10 µL of protease inhibitor (100x), 2 µL of SOV stock (500 mM), 50 µL of sodium deoxycholate stock (10%), 100 µL of SDS stock (1%), and 118 µL of hexylene glycol stock (8.44 M) to 710 µL of lysis buffer A (final concentrations of 1 mM PMSF, 1x protease inhibitor, 1 mM SOV, 0.5% sodium deoxycholate (v/v), 0.1% SDS (w/v), and 1 M hexylene glycol). Keep the solution on ice until addition to nuclear pellet.

1.2.6. Add 20 mL of HEPES (1 M, pH 7.4), 0.74 g of potassium chloride (KCl), 0.19 g of magnesium chloride ($MgCl_2$), 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), 38.3 g of mannitol, and 23.9 g of sucrose to 980 mL of deionized water to prepare lysis buffer B (final concentrations of 20 mM HEPES, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose).

1.2.7. Prepare CH buffer by adding 10 µL of PMSF stock (100 mM) and 2 µL of SOV stock (500 mM) to 988 µL of lysis buffer B (final concentrations of 1 mM PMSF and 1 mM SOV; adjust the final volume to accommodate the number of cells being lysed). Keep the solution on ice until addition to cell pellet.

1.2.8. Prepare iodixanol diluent by addition of 12 mL of HEPES (1 M, pH 7.4), 447 mg of KCl, 114 g of $MgCl_2$, 1.2 mL of 0.5 M EDTA, 1.2 mL of 0.5 M EGTA, 21.3 g of mannitol, and 14.4 g of sucrose

to 88 mL of deionized water (final concentrations of 120 mM HEPES, 60 mM KCl, 12 mM $MgCl_2$, 6 mM EDTA, 6 mM EGTA, 1200 mM mannitol, and 420 mM sucrose).

1.2.9. Store buffers at 4 °C and digitonin at -20 °C.

2. **Cytosolic protein isolation**

NOTE: The following steps will allow for the growth and expansion of U937 cells followed by extraction of cytosolic proteins. At the concentration used, digitonin will permeabilize the plasma membrane without disrupting it, allowing for the release of cytosolic proteins and retention of other cellular proteins.

2.1. Culture the cells in RPMI 1640 with 10% fetal bovine serum at 37 °C and 5% CO_2. Ensure that the cells are grown to a final total of 6 x 10^8 cells.

NOTE: In this protocol, cells were counted using a standard hemocytometer and microscope.

2.2. Centrifuge the cultured cells at 400 × *g* for 10 min. After discarding the supernatant, resuspend the cell pellet in room temperature phosphate-buffered saline (PBS) at a final concentration of 4 × 10^6 cells/mL, and pipette gently to break up clumps.

2.3. Centrifuge the cell suspension at 400 × *g* for 10 min to pellet the cells. Discard the supernatant, and resuspend the cell pellet in ice-cold lysis buffer A (prepared in step 1.2.1) at a final concentration of 2 × 10^7 cells/mL.

2.4. Remove 7.5 mL of the cells suspended in lysis buffer A (3 × 10^7 cells), and keep on ice for nuclear protein extraction (section 6). Centrifuge the cell suspension at 4 °C, 400 × *g* for 10 min to pellet the cells.

NOTE: Perform all subsequent steps at 4 °C or on ice, and prechill all buffers.

2.5. Discard the supernatant, resuspend the cell pellet in CI buffer at a final concentration of 2 × 10^7 cells/mL, and pipette gently to break up clumps. Rotate the cell suspension end-over-end at 4 °C for 20 min.

2.6. Centrifuge the cell suspension at 4 °C, 400 × *g* for 10 min, and transfer the supernatant to a clean centrifuge tube. Save the cell pellet, and store on ice. Centrifuge the collected supernatant at 4 °C, 18,000 × *g* for 20 min to pellet cellular debris.

NOTE: This high-speed centrifugation step is critical for preventing contamination of the cytosolic fraction with organelle and membrane-bound proteins.

2.7. Discard the pellet after transferring the supernatant to a clean centrifuge tube. Repeat both centrifugation steps in step 2.6 until no pellet is obtained following centrifugation.

2.8. Collect the supernatant, which is the **cytosolic fraction.** For short-term storage (1 month), ensure that the supernatant is stored at 4 °C. For long-term storage (>1 month), combine the supernatant with 1x Laemmli buffer containing 1x reducing agent, heat at 95 °C for 7 min, and store at -20 or -80°C.

2.9. Resuspend the cell pellet (from step 2.6) in lysis buffer A at a final concentration of 4×10^6 cells/mL; pipette gently to break up clumps.

3. **Cell homogenization**

NOTE: The following steps will allow for the mechanical homogenization of digitonin-treated cells (from step 2.9), which is necessary for the isolation of the mitochondrial and membrane protein fractions.

3.1. Centrifuge the cell suspension in lysis buffer A (prepared in step 2.9), save the pellet, and discard the supernatant to remove excess digitonin and cytosolic contaminants from the cell pellet.

NOTE: Repeated washes in lysis buffer A may be performed to remove excess cytosolic contaminants.

3.2. Resuspend the cell pellet in ice-cold CH buffer (prepared in step 1.2.7) at a final concentration of 4×10^6 cells/mL. Incubate the cell suspension on ice for 30 min.

3.3. If using a bead-based method for mechanical lysis, place 30 g of prewashed stainless steel 3.2 mm beads into a 50 mL skirted tube, fill with 15 mL of lysis buffer B, and place on ice to chill. If using a Dounce homogenizer (with a tight-fitting B pestle), fill with an appropriate volume of lysis buffer B, insert the pestle, and place on ice to chill.

3.4. Following incubation (step 3.2), discard lysis buffer B from the bead tubes (or Dounce homogenizer), and transfer 15 mL of the cell suspension to the bead tube (or an appropriate volume to the homogenizer).

3.5. If using the bead-based method, place the skirted bead tubes containing the cell suspension in the blender device, and configure to run for 5 min at speed 8. If using a Dounce homogenizer, keep it on ice, and perform 40 passes with the pestle using slow, even strokes (or utilize an alternative method of mechanical cell lysis as detailed in the discussion section).

Note: If using a blender device different from the one used here, speed and time may need to be determined empirically.

3.6. Transfer the homogenate to a clean centrifuge tube, and centrifuge it at 400 × *g* for 10 min at 4 °C. Transfer the supernatant to a clean centrifuge tube and save; discard the pellet.

NOTE: The protocol may be paused here and the homogenate stored at 4 °C for the short term (24 h).

4. **Debris removal and isolation of crude mitochondrial and membrane fractions**

NOTE: The following steps will allow for the removal of cellular debris by centrifuging the homogenate at increasing speeds. This is followed by differential centrifugation for the isolation of crude mitochondrial and membrane fractions.

4.1. Centrifuge the homogenate (from step 3.6) at 500 × *g* for 10 min at 4 °C. Transfer the supernatant to a clean centrifuge tube, and discard any pellet.

4.2. Centrifuge the supernatant (from step 4.1) at 1,000 × *g* for 10 min at 4 °C. Transfer the supernatant to a clean centrifuge tube, and discard any pellet.

4.3. Centrifuge the supernatant (from step 4.2) at 2,000 × *g* for 10 min at 4 °C. Transfer the supernatant to a clean centrifuge tube, and discard any pellet.

4.4. Centrifuge the supernatant (from step 4.3) at 4,000 × *g* for 20 min at 4 °C. Transfer the supernatant to a clean centrifuge tube, and save the pellet, which is the **crude mitochondrial fraction**.

4.5. Centrifuge the supernatant (from step 4.4) at 18,000 × *g* for 1 h at 4 °C. Discard the supernatant, and save the pellet, which is the **crude membrane fraction**.

NOTE: The protocol may be paused here, and the pelleted samples stored at 4 °C for the short-term (24 h).

5. **Isopycnic density gradient purification**

NOTE: The following steps utilize isopycnic density gradient centrifugation to purify the crude mitochondrial and membrane fractions.

5.1. Prepare a 50% (v/v) **working solution** of iodixanol by mixing 1 part diluent (prepared in step 1.1.3) to 5 parts iodixanol (60% stock solution (w/v)). Prepare 10%, 15%, 20%, 25%, 30%, and 35% iodixanol solutions (v/v) by mixing 50% working solution (v/v) with lysis buffer B in appropriate quantities.

5.2. Resuspend the crude mitochondrial and membrane pellets (from steps 4.4 and 4.5) each in 200 µL of lysis buffer B. Adjust to 45% iodixanol (v/v) by adding 1800 µL of working iodixanol solution (50% (v/v)) to 200 µL of the resuspended pellet.

5.3. Create an iodixanol discontinuous gradient as follows (adjust the volumes as appropriate for the capacity of the ultracentrifuge tube): Add 1 mL of 15% iodixanol (v/v) to the bottom of an 8 mL, open-top, thin-walled ultracentrifuge tube, place 1 mL of 20% iodixanol (v/v) below the first layer (by the underlaying technique), followed by underlaying 1 mL of 25%, 1 mL of 30%, and 1 mL of 35% iodixanol (v/v).

NOTE: There should be 2 gradients created at this step, 1 for the mitochondrial fraction and 1 for the membrane fraction.

5.4. In 1 gradient, add the 2 mL of the crude mitochondrial pellet (suspended in 45% iodixanol (v/v)) using the underlay technique to the bottom of the tube below the 35% iodixanol (v/v). In the other gradient, add the 2 mL of the crude membrane pellet (suspended in 45% iodixanol (v/v)) using the underlay technique to the bottom of the tube below the 35% iodixanol (v/v).

5.5 Add 1 mL of 10% iodixanol (v/v) to the top of each gradient tube by overlaying it on top of the 15% layer. Balance the tubes within 0.1 g of each other by the addition of 10% iodixanol (v/v).

5.6. Spin the density gradient tubes at 4 °C for 18 h at 100,000 × *g*. Be sure to set acceleration and deceleration to the minimum values for the ultracentrifuge being used.

NOTE: In the mitochondrial gradient, there will be a visible band at the interface between 25% and 30% iodixanol (v/v). This is the **pure mitochondrial fraction**. In the membrane gradient, there will be a visible band in the 15% iodixanol (v/v) fraction. This is the **pure membrane fraction**. There may be additional bands in the 25% and 30% iodixanol (v/v) fractions in the membrane gradient. This is mitochondrial contamination.

5.7. Collect fractions from the top of the tube in 1 mL aliquots, being careful to minimize the volume of the fraction containing the visible bands and changing the pipette tip after each layer is collected. Alternatively, puncture the side of the thin-walled tube with a needle, and collect the visible bands.

5.8. Store the samples as described in step 2.8 until verification by protein assay and western blot.

6. Nuclear protein isolation

NOTE: Using ionic and non-ionic detergents as well as techniques such as sonication and centrifugation, the following steps will solubilize all cellular membranes and allow for the isolation of nuclear proteins.

6.1. Centrifuge the 7.5 mL aliquot of cells suspended in lysis buffer A (from step 2.4), and discard the supernatant. Add 800 µL of ice-cold CS buffer (prepared in step 1.2.4), and resuspend the pellet by pipetting and vortexing. Incubate the samples on ice (or at 4 °C) for 30 min to disrupt the plasma and organelle membranes while protecting the nuclear proteins.

6.2. Centrifuge the cell suspension at 7,000 × *g* for 10 min at 4 °C, and discard the supernatant. Add 800 µL of ice-cold NL buffer (prepared in step 1.2.5) to the nuclear pellet after inclusion of 1U/µL benzonase. Resuspend the pellet by gently pipetting. Incubate on an end-over-end rotator for 30 min at 4 °C to disrupt the nuclear membrane.

6.3. Sonicate for 5 s at 20% power with sample tubes cooled in an ice bath (3x, with 5 s pauses in between pulses), which along with benzonase (added in step 6.2), will shear the nucleic acids.

6.4. Centrifuge the sonicate at 7,800 × *g* for 10 min at 4 °C. Collect and save the supernatant, which is the **nuclear fraction**. Be sure to not disturb the insoluble pellet while collecting the supernatant. For storage, samples can be prepared as described in step 2.8.

7. Protein quantification and western blot analysis

NOTE: The following steps will quantify total protein in each fraction and confirm the purity of the subcellular fractions.

7.1. Perform a standard Bradford assay to quantify total protein in each fraction. Refer to **Table 1** for expected protein yields.

Note: Other protein assays such as bicinchoninic acid (BCA) assay or absorbance at 280 nm may be used to measure total protein in place of a Bradford assay.

7.2. Combine fractions with 1x Laemmli buffer containing 1x reducing agent, and heat at 95 °C for 7 min. Load 10 µg of each fraction into a standard SDS-polyacrylamide gel electrophoresis (PAGE) gel, and run the gel at 20 mA for 1 h.

7.3. Transfer the resolved proteins to a polyvinyldifluoride (PVDF) membrane by performing a standard immunoblot transfer at 100 V for 30 min. Block the PVDF membrane with 5% milk in 1x Tris-buffered saline (TBS) containing 0.1% of a non-ionic detergent (v/v) for 30 min at room temperature.

7.4. Add the appropriate primary antibodies to detect housekeeping proteins specific to each subcellular fraction, and incubate overnight at 4 °C. Wash the membrane with 5% milk in 1x TBS containing 0.1% of the non-ionic detergent (v/v) for 10 min at room temperature.

NOTE: For this protocol, antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:10000), histone H3 (1:2000), voltage-dependent anion channel (VDAC, 1:1000), and Na,K^{+}-ATPase were used for cytosolic, nuclear, mitochondrial, and membrane fractions, respectively.

7.5. Add the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies to the membrane (dilute according to the manufacturer's instructions), and incubate at room temperature for 1 h. Wash the membrane with 5% milk in 1x TBS containing 0.1% of the non-

ionic detergent (v/v) for 10 min at room temperature. Develop the blot using standard chemiluminescence.

REPRESENTATIVE RESULTS:

A schematic flow chart of this procedure (**Figure 1**) visually summarizes the steps that were taken to successfully fractionate U937[5] cells grown in suspension. Fractions collected from the top of the isopycnic density gradient in equal volumes (1 mL) show the purification of the mitochondrial and membrane fractions (**Figure 2**). Utilizing an antibody against VDAC, a protein localized to the outer mitochondrial membrane[6], shows that the mitochondrial fraction migrated to the 25% and 30% iodixanol (v/v) fractions (**Figure 2A**). Using an antibody against the Na,K^+-ATPase α1 subunit, part of an integral membrane heterodimer found primarily in the plasma membrane[7], shows the separation of membrane contamination from the pure mitochondrial fraction (**Figure 2A**). The pure membrane fraction migrated to the least dense fractions, 10% and 15% iodixanol (v/v) (**Figure 2B**). Mitochondrial contamination of the membrane fraction was separated by the gradient.

A western blot[8] performed with the additional localization markers (referenced in step 7.4) shows the purity of the cytosolic and nuclear fractions, while additionally verifying that the mitochondrial and membrane samples are free from contamination by proteins from other parts of the cell (**Figure 3**). Using an antibody against GAPDH, normally localized to the cytoplasm of the cell[9], shows that this protein is only found in the cytosolic fraction (**Figure 3A**, Lane 1, first panel), and that no contamination is observed in the extracted nuclear proteins, the density-purified mitochondria, or membrane fractions (**Figure 3A**; Lanes 2, 3, and 4; first panel). Probing for histone H3, a protein found in the nucleus and involved in chromatin structure[10], shows a successful nuclear extraction (**Figure 3A**, Lane 2, second panel), with some minimal detection in the cytoplasmic fraction and no cross contamination in the mitochondrial or membrane fractions.

Probing for VDAC in all fractions shows the presence of this protein in the pure mitochondrial fraction (**Figure 3A**, Lane 3, third panel), and that no cross contamination exists in the other fractions (**Figure 3A**; Lanes 1, 2, and 4; third panel). Probing for the Na/K-ATPase α1 subunit similarly shows that this protein is located only in the pure membrane fraction (**Figure 3A**, Lane 4, fourth panel). These fractions were analyzed by densitometry to confirm reproducibility and statistical significance (**Figure 3B–E**). In contrast to the results from the successful fractionation (**Figure 3**), improper execution of this method (or failure to adhere to all recommended steps) can result in cross-contamination of cellular components (**Figure 4**). A high concentration of histone H3 in the cytosolic fraction (**Figure 4**, Lane 1, second panel) can result from a failure to properly clarify the cytosolic fraction (referenced in step 2.6). This can occur if not enough clarification centrifuge spins are performed, or if the cytosolic fraction is not clarified quickly. If the cytosolic fraction is not clarified quickly enough, it may result in lysis of cell fragments, leading to contamination of the cytosolic fraction.

Failure to perform the isopycnic density purification step will result in contamination of the membrane fraction (**Figure 4**, Lane 4, all panels), depending on how heterogenous the sample is prior to density purification. Proper adherence to all steps of the protocol is critical to obtaining

the desired separation of the subcellular fractions. When quantified using a Bradford assay, protein yield for each fraction can be determined. Expected protein yield per fraction is reported in **Table 1**. It is useful to perform a protein quantification assay prior to performing western blots for several reasons. First, it confirms that fractions indeed contain protein; second, it allows loading of SDS-PAGE gels based on protein quantity; and finally, assuming protein yields are similar to those expected (**Table 1**), it confirms proper execution of the procedure.

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of the cell fractionation procedure. An overview of the cell fractionation protocol represented as a flow chart. Abbreviations: PBS = phosphate-buffered saline; CS = cell solubilization; NL = nuclear lysis.

Figure 2: Isopycnic density purification of crude mitochondrial and membrane fractions. (**A**) Representative western blot of all fractions collected after density gradient purification of the crude mitochondrial fraction. (**B**) Representative western blot of all fractions collected after density gradient purification of the crude membrane fraction. Both density gradient purifications show migration of the mitochondrial marker, VDAC, for the 25% and 30% iodixanol (v/v) fractions and migration of the membrane marker, Na,K^+ ATPase, for the 10% and 15% iodixanol (v/v) fractions. Abbreviation: VDAC = voltage-dependent anion channel.

Figure 3: Successful isolation of U937 cytosolic, nuclear, mitochondrial, and membrane fractions. (**A**) Representative western blots of cell fractions isolated from a U937 cell culture with this technique and probed for markers of cytoplasm (GAPDH, first panel), nucleus (Histone H3, second panel), mitochondria (VDAC, third panel), and membrane (Na,K^+ ATPase α1, fourth panel). (**B–E**) Densitometry of western blots of cell fractions isolated from U937 cell cultures with this technique. Results are from 3 independent experiments. Error bars represent standard deviation. One-way ANOVA. ***$p<0.001$. Abbreviations: GAPDH = glyceraldehyde-3-phosphate dehydrogenase; VDAC = voltage-dependent anion channel; cyto = cytosolic; nuc = nuclear; mito = mitochondrial; mem = membrane.

Figure 4: Incomplete fractionation of U937 cell components. Representative western blots of cell fractions isolated from a U937 cell culture showing contamination of the cytosolic fraction with histone H3 (Lane 1, second panel) due to improper clarification of this fraction and a crude membrane fraction that was not subjected to isopycnic density gradient purification (Lane 4, all panels). Abbreviations: cyto = cytosolic; nuc = nuclear; mito = mitochondrial; mem = membrane.

Table 1: Protein yield for each subcellular fraction.

DISCUSSION:

This method is a modified version of a previously published approach to subcellular fractionation without the use of high-speed centrifugation[11]. This modified method requires more specialized equipment to achieve the best results, but is more comprehensive and consistently reproducible. The development of the initial protocol was necessary due to an inability to separate

mitochondrial and membrane samples for the analysis of protein localization during necroptosis[12]. Attempts to use the exclusively detergent-based methods found in most commercially available kits resulted in a homogeneous mixture containing the plasma membrane and all membrane-enclosed organelles in the cell. Other limitations of these kits include an inability to make alterations to the procedure, cost per sample, volume restrictions, and number of samples that can be processed. The procedure presented here may be altered to any scale, changed to isolate fewer fractions, and can be performed without the use of expensive reagents. Fraction yields can be increased by utilizing more cells; steps can be tailored to the research being performed; and execution of the method is flexible. For example, if researchers are not examining a particular subcellular fraction, they do not need to isolate that sample in the course of the procedure. Likewise, the addition of particular inhibitors or reagents can be omitted if the researcher does not plan on studying the phosphorylation state of proteins (sodium orthovanadate) or is not concerned about denaturing proteins (hexylene glycol).

The use of iodixanol as the density gradient solution is optional; however, this reagent does not interfere with subsequent examination of samples via western blotting. It is also possible to remove the iodaxanol from samples by dilution and centrifugation to recover the mitochondria or membrane, although this will affect the final yield. Other alternative density gradient solutions can be used, including sucrose. To obtain optimal results and pure fractions, there are several factors to consider, and particular critical steps in the protocol that need careful attention. This protocol is optimized for the fractionation of U937 cells, and the concentration of cells recommended at particular points in the protocol are specific to this cell line. These values were determined empirically and will most likely need to be adjusted for different types of cells, particularly, if suboptimal results are obtained when executing the protocol.

Clarification of the cytoplasmic sample should occur as soon as possible to remove unbroken cells and debris that might result in cross-contamination by proteins from other subcellular fractions (**Figure 4**, Lane 1, second panel). Homogenization can be accomplished with any form of mechanical lysis, although results presented here were obtained using a bead-based method and blender device. Alternative manual forms of homogenization (a Dounce homogenizer or passage through a small gauge needle) can also be utilized, but may result in reproducibility issues due to variability of technique by the individual performing the procedure. This group's interest is primarily in circulating leukocytes, which is why U937 cells are used in this procedure. However, this procedure may be applied to other suspension cell lines with likely little need for alteration. Portions that may need to be adjusted to accommodate another suspension cell line include cell concentrations used throughout the procedure as well as the concentration of digitonin used to extract the cytosolic fraction.

While not optimized for adherent cell lines, this procedure may serve as a starting point to which adjustments can be made to accommodate adherent cells. These adjustments include cell concentration, concentration of digitonin, and homogenization time. In addition, adherent cells are limited by surface area while suspension cells are limited by volume; this makes scaling up of suspension cells simpler. To scale up adherent cells, tissue culture plates with a large surface area (>500 cm^2) must be utilized. This procedure is a cost-effective, reproducible subcellular

fractionation with the ability to separate cytosolic, nuclear, mitochondrial, and membrane fractions with great purity. One of the biggest advantages of this procedure is the separation of mitochondrial from the membrane fraction. This is not possible in exclusively detergent-based procedures. Although detergents are used in this procedure, they are used to permeabilize, but not disrupt the plasma membrane (digitonin) and to obtain the nuclear fraction after the isolation of the mitochondrial and membrane fractions.

ACKNOWLEDGMENTS: This work was supported by NIH R15-HL135675-01 and NIH 2 R15-HL135675-02 to T.J.L.

DISCLOSURES: The authors declare no conflict of interest.

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Figure 1

Click here to access/download;Figure;Fig 1 - post review.tif

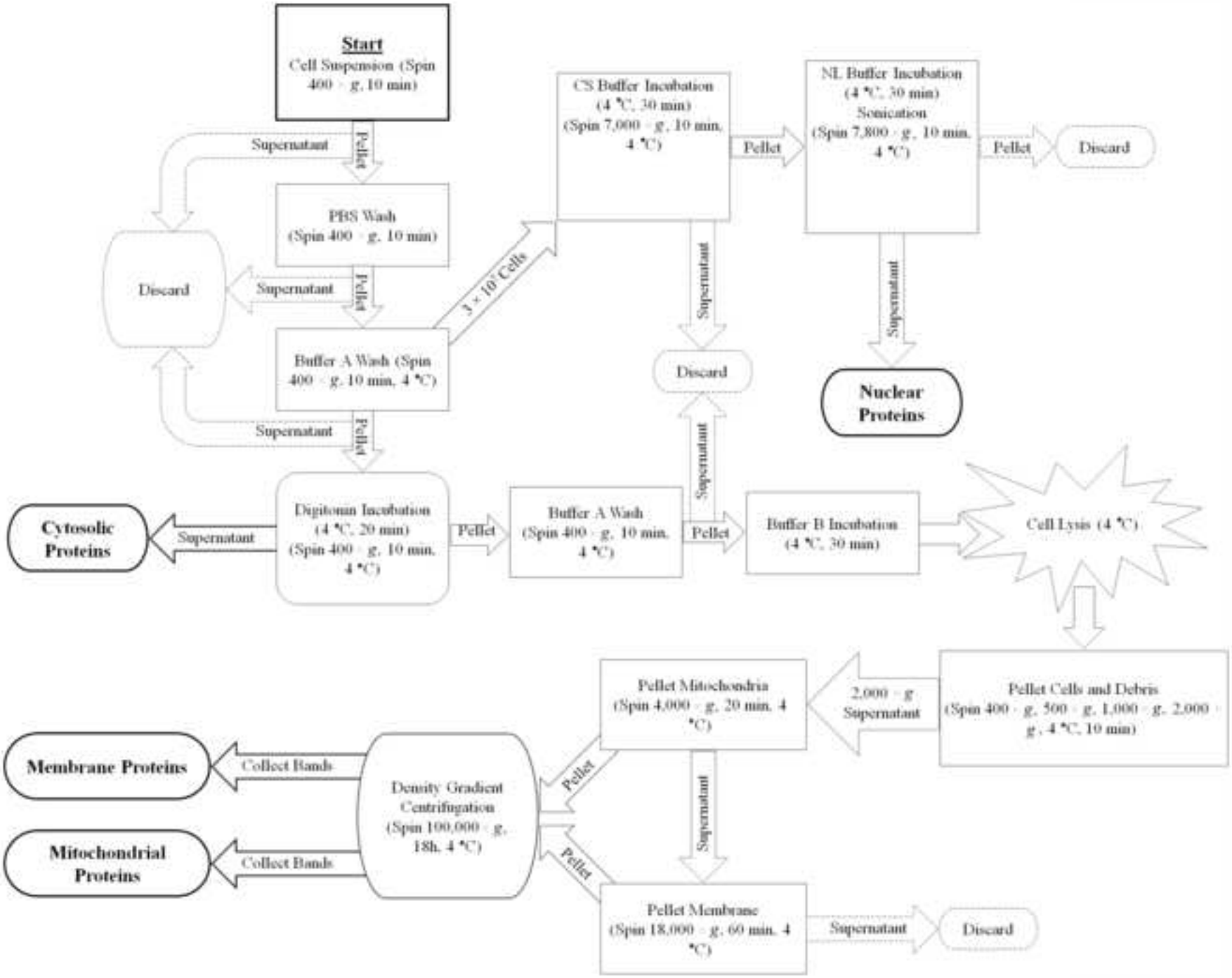


Figure 2

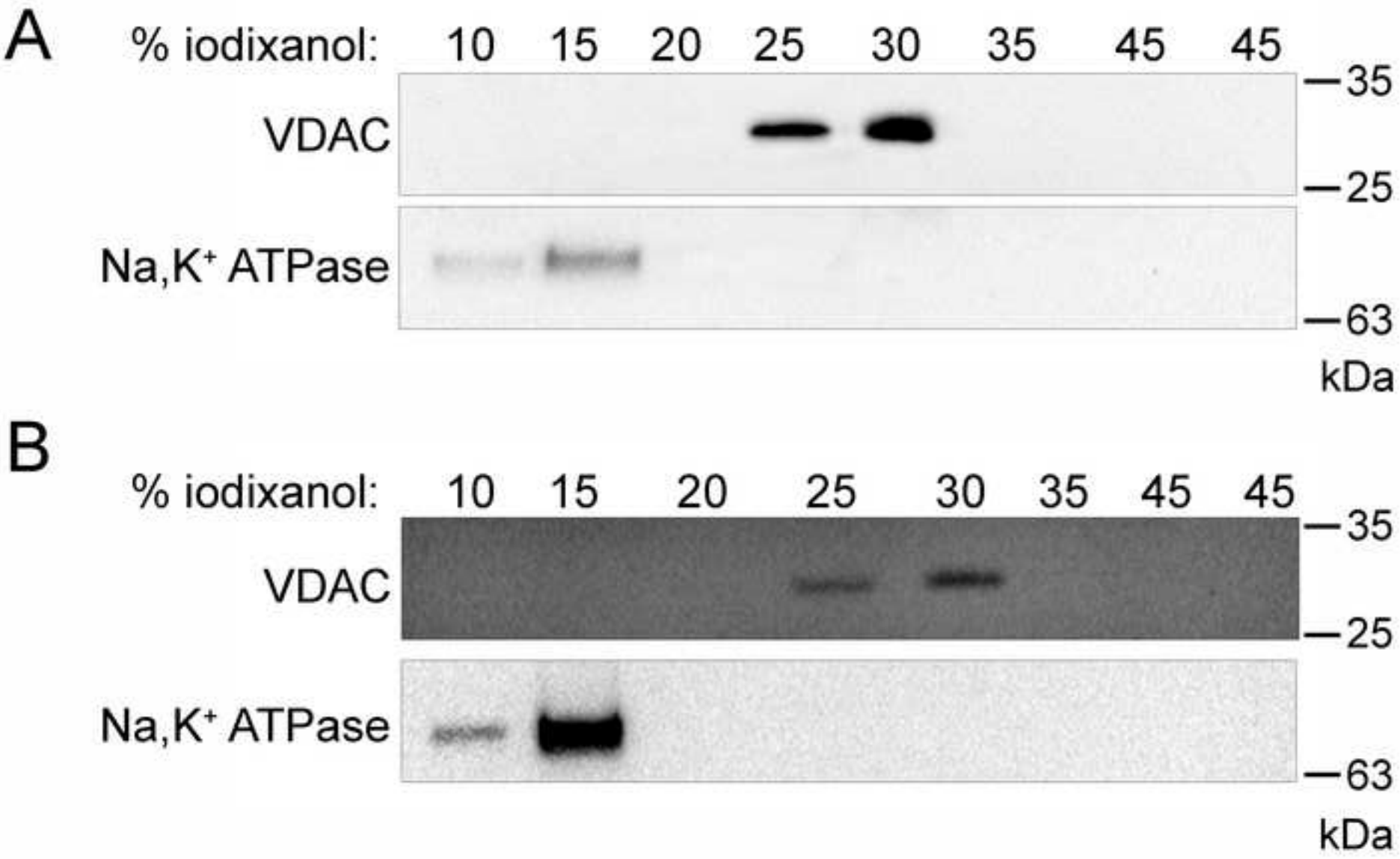


Figure 3

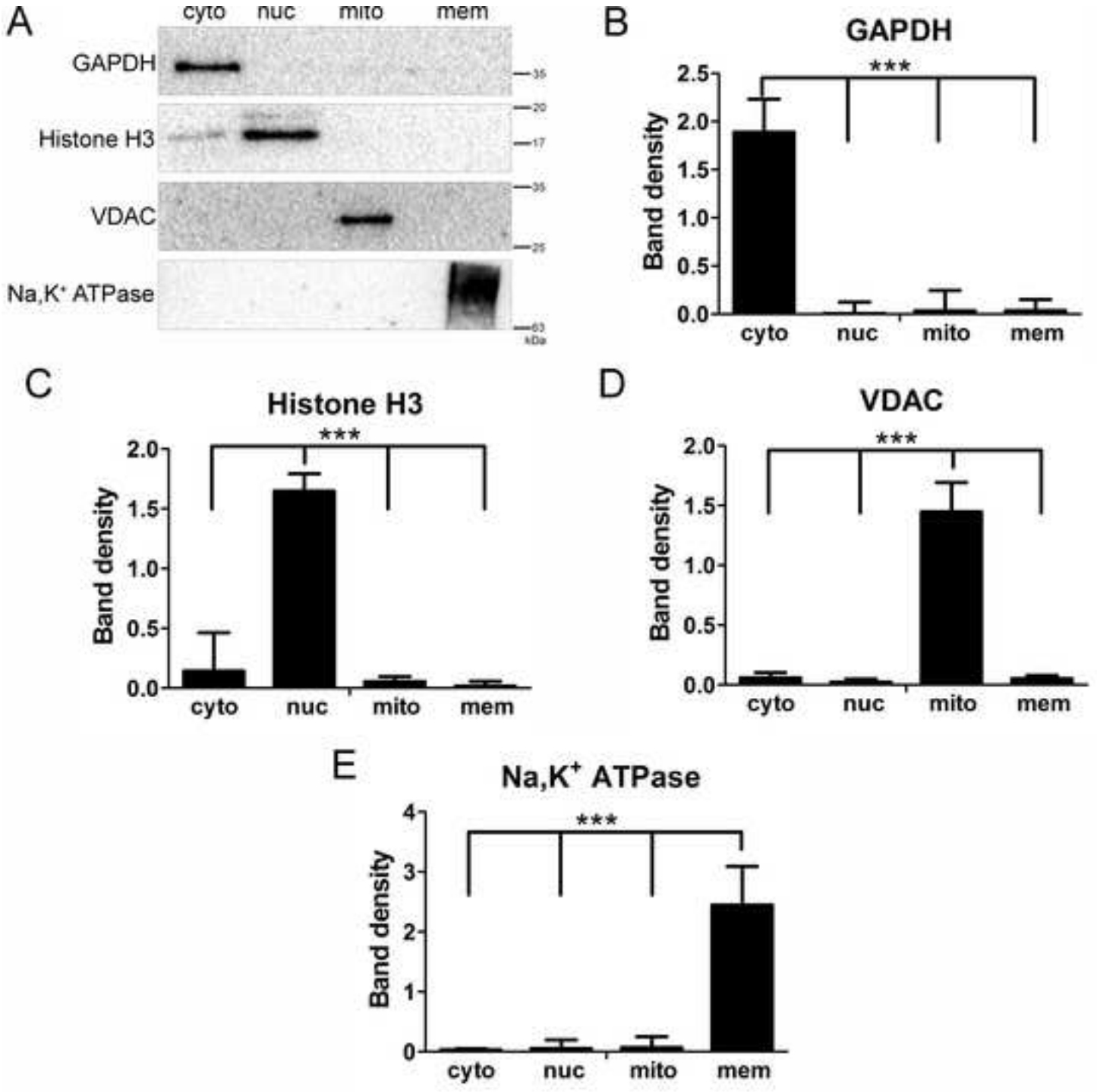
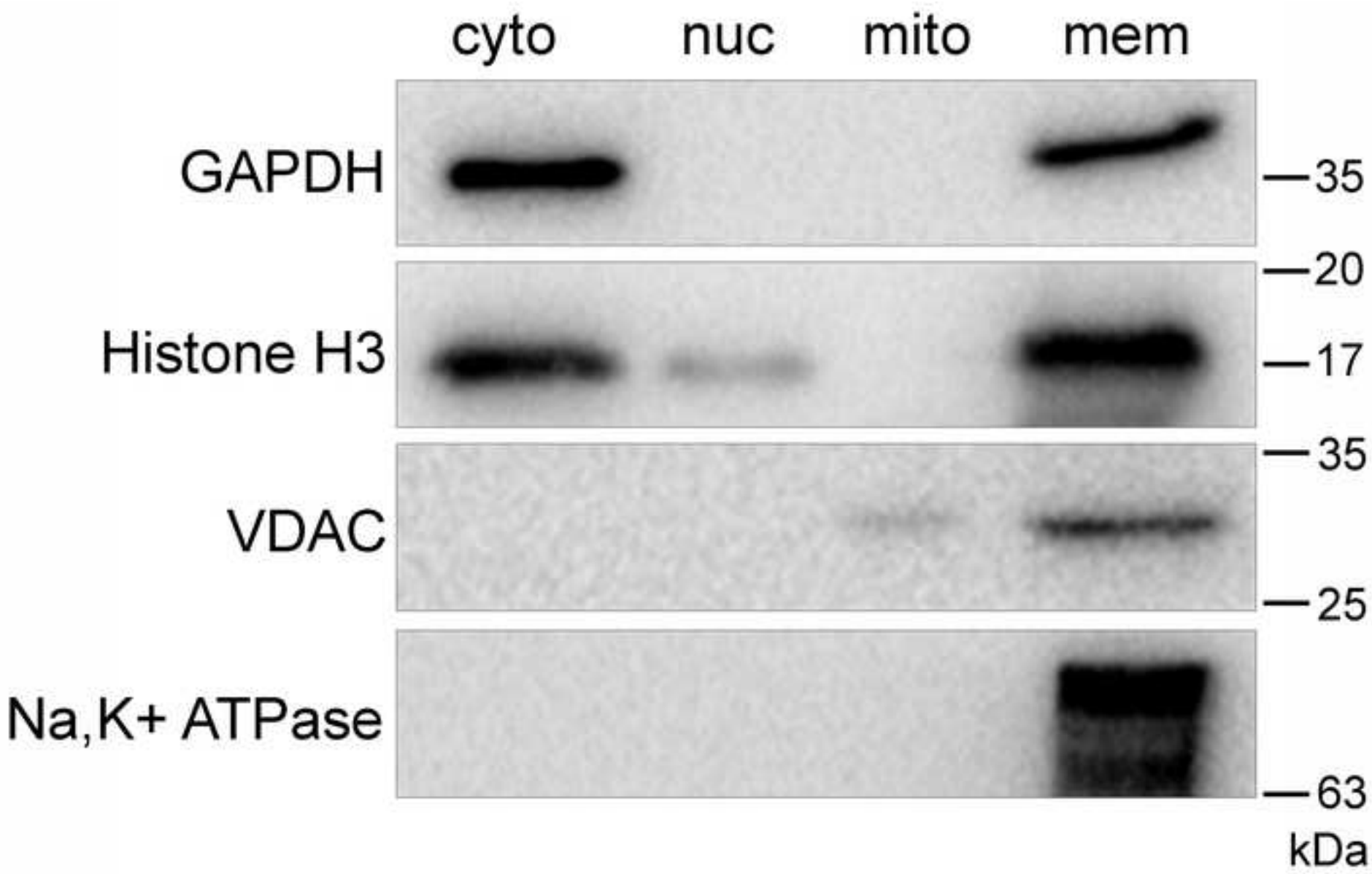


Figure 4

Click here to access/download;Figure;Fig 4 - post review.tif



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Benzonase Nuclease	Sigma-Aldrich	E1014	
Bullet Blender Tissue Homogenizer	Next Advance	61-BB50-DX	
digitonin	Sigma	D141	
end-over-end rotator	ThermoFisher		
Ethylenediaminetetraacetic acid (EDTA)	Sigma	E9884	
ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)	Sigma	E3889	
GAPDH (14C10)	Cell Signalling Technologies	2118	
HEPES	VWR	97064-360	
Hexylene glycol	Sigma	68340	
Igepal	Sigma	I7771	Non-ionic, non-denaturing detergent
KCl	Sigma	P9333	
Mannitol	Sigma	M9647	
$MgCl_2$	Sigma	M8266	
NaCl	Sigma	S9888	
Na, K-ATPase a1 (D4Y7E)	Cell Signalling Technologies	23565	
Open-Top Polyclear Tubes, 16 x 52 mm	Seton Scientific	7048	
OptiPrep (Iodixanol) Density Gradient Medium	Sigma	D1556-250ML	
phenylmethanesulfonyl fluoride (PMSF)	Sigma	P7626	
Protease Inhibitor Cocktail, General Use	VWR	M221-1ML	
refrigerated centrifuge	ThermoFisher		
S50-ST Swinging Bucket Rotor	Eppendorf		
Sodium dodecyl sulfate (SDS)	Sigma	436143	
Sodium deoxycholate	Sigma	D6750	
sodium orthovanadate (SOV)	Sigma	567540	
sonicator	ThermoFisher		
Sorvall MX120 Plus Micro-Ultracentrifuge	ThermoFisher		
Stainless Steel Beads 3.2 mm	Next Advance	SSB32	
Sucrose	Sigma	S0389	

Tris-buffered Saline (TBS)	VWR	97062-370	
Tween 20			non-ionic detergent in western blotting bu
VDAC (D73D12)	Cell Signalling Technologies	4661	

ıffers

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. Please define all abbreviations at first use.

We have done this.

2. Please revise the following lines to avoid overlap with previously published work: 37-39; 52-55; 61-79; 87-104; 111-161; 173-178, 222-232, 246-264, 278-284, 288-292, 296-302, 425-428.

We have made an effort to revise these lines.

3. Please provide an email address for each author.

We now provide this.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: OptiPrep; Bullet Blender, etc

We have now corrected this.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have ensured this.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. 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. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have made sure to provide sufficient detail.

7. Please move the information in 1.3 to the Table of Materials and delete it from the protocol.

We have moved this.

8. 2.1.1: If these are the centrifugation conditions to be used for many steps, please delete the word "example" and use the actual conditions at the very first mention of these conditions. Afterwards, please say "Centrifugation was performed as in step X.Y.Z".

We have now done this.

9. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep, and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

We have done this.

10. As we are a methods journal, please add limitations of the technique to the Discussion.

We have now done this.

11. Please sort the Materials Table alphabetically by the name of the material.

We have now done this.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes a multi-step protocol to separate and isolate sub-cellular compartments (cytosol, nuclei, mitochondria, membrane) from U937 cells using regular and isopycnic density gradient centrifugation steps. The method is optimized specifically for use with U937 cells as written.

Major Concerns:

1. The limitation of the technique is that it is very complex and specifically meant and optimized for U937 cells. Thus it is not widely applicable, although on the other hand it may be a starting point to use for others to optimize.

Our laboratory works primarily with suspension cells and we mainly work with U937 cells. We intend for this to be a potential starting point for fractionations with other cell types. We have now clarified this in the text.

2. The title is somewhat unclear. What is meant by "enhanced" Enhanced in comparison to what? The authors have published a previous JoVE paper in 2019 where they outlined a cell fractionation protocol using the same cells in the absence of high speed centrifugation steps.

We have changed the title in order to clarify it.

3. The methods and reagents for measuring the purity of the isolated fractions by Western blot (Figs. 2-4) were not provided. The figure legends for the same are not informative enough. The Western blots show only a narrow band in the region of interest and there are no molecular weight markers. The results are representative of a single isolation. There are no data on the reproducibility of the procedure.

We now include the methods for western blot detection. We also include MW markers now. Regarding reproducibility, Fig 3 now contains densitometry analysis of 3 independent western blots along with one-way ANOVA for statistical analysis.

4. The paper sections and protocol in particular as written are quite difficult to read and understand. I will try to summarize some of my concerns/questions as follows:

a) Introduction: What is meant by "...it suffers from a lack of specificity (for what?) and an inability to separate fractions with 'similar biochemical properties'." As there are many diverse 'biochemical properties'...what is exactly meant here? Should all proteins extracted within a specific subcellular fraction exhibit these (which?) properties?

We have clarified the text in the Introduction.

b) please give some references for the section in lines 48-49 "Methods that utilize differential centrifugation"

We now provide this reference.

c) Line 53 says that the method will allow isolation of subcellular fractions without the use of solubilizing detergents, however, several detergents are indeed used in the protocol.
You are correct. We mean to say the sole use of solubilizing detergents. We have clarified this now.

d) Protocol. Please give full chemical/salt names with hydration status (ie. xH2O) as weights were given to measure out. This will enhance reproducibility by others.
We have now done this

e) Do not capitalize reagent names.
We have corrected this

f) Define and label all % concentrations as either w/v or v/v.
We have now done this

g) name the protease inhibitor cocktail and source you used in the protocol itself.
We would be happy to do this but the editor has instructed us to remove all commercial language from the manuscript. This information can be found in the Table of Materials, however.

h) all centrifuge speeds should be written in full and consistently. ie. 18,000g; 100,000g etc.
We have corrected this

i) A major difficulty is that the sections of the protocol do not have the best titled sections and the flow of the steps is not obvious from reading the given section headings. For example 'Cell Homogenization' appears as section 5(!). There is no clear section that describes 'digitonin permeabilized cells' per se. Improving the section names and general organization and order of sections will help. Also a brief preamble paragraph that describes how the major steps to follow fit together to yield which particular fraction (with ref. to Figure 1) will help.
We have made an effort to reorganize these steps and make the instructions clearer and more succinct. We include preamble paragraphs that describe the intent of the following steps. Thank you for this suggestion. We believe it has strengthened the manuscript.

j) Give the number of cells (or a range) to start that will result in the ability to provide all the fractions to be had from this protocol.
We now include this information.

k) 3.2.1 refers to 3.1.4...but do you mean 3.1.5?
Yes, but this has now changed after we have reorganized the manuscript.
l) Is step 5.1 done in the absence of PMSF and other protease inhibitors?
No. There is PMSF.

m) 6.1.1. There is no step 4.1.8!
It was 5.1.8 but this has now changed after we have reorganized the manuscript.

n) The number and extent of these issues make it seem that the protocol may not be useful nor reproducible by others.
We have addressed these issues. Please see above.

o) Representative Results. Please use paragraphs (also for the Discussion). Line 361 states Fractions 7-8 but there is no Fraction 8 in the figure.
We have now corrected this.

p) Figure 4 is not very useful not knowing what was done improperly to yield such a result.
We have elaborated on what was done improperly to yield this result.

q) The Discussion needs clear paragraphs and a clear concluding paragraph that wraps up the paper. It now ends on a technical detail.
We have added a concluding paragraph.

Minor Concerns:
Please use page number and a header for all manuscript pages.
We now include this.

Reviewer #2:
Manuscript Summary:
The authors of this paper describe a protocol to obtain subcellular protein fractions from eukaryotic cells. The method described in this manuscript can help provide researchers with an alternative method to fractionation of cellular organelles in the endomembrane system since commercially available kits rely on the use of detergents which solubilize membranes. The authors were able to successfully fractionate the cell and were demonstrated by Western immunoblotting.

Major Concerns:
None.

Minor Concerns:
Comment 1. Figure 1 was a bit distorted. I am not sure if it is the resolution of the paper for review, but a higher resolution image is needed here.
We have provided a higher resolution image.
Comment 2. Manuscript may need some minor copy editing. Throughout the manuscript it seemed many words were capitalized that did not need to be.
This has now been addressed.

Reviewer #3:
Manuscript Summary:
This manuscript presents a protocol for subcellular protein fractionation from eukaryotic cells.

Major Concerns:

-This protocol has been benchmarked only with a single type of cells, U937, which are suspending cells. The protocol should be validated with different types of cells, at least with adherent cells, to demonstrate the broad applicability.
We primarily work with U937 cells which is why this cell line is used. We intend for this procedure to be a starting point for those working with adherent cell lines. We now discuss potential alterations that may need to be considered if attempting this procedure with adherent cells in lines 488-497.
-In the introduction, the manuscript should elaborate the summary, highlights, differences, and improvements of the protocol in comparison to existing other protocols. It was not clear to me the advantage of "without the use of solubilizing detergents".
We have made an effort to clarify this. The major advantage vs. kits that solely use solubilizing detergents is the separation of mitochondria from the plasma membrane and other membrane-bound organelles. This is not possible with kits that rely solely on solubilizing detergents.

Minor Concerns:
-I see some values with too many significant figures like "8.766g NaCl", "0.746g KCl", "38.26g Mannitol", "23.96g Sucrose" and "91.95 mg". I wonder if such precise specifications are necessary for this protocol. I recommend reevaluating these numbers and providing values with appropriate significant figures to reproduce the protocol.
We have now corrected this.

Reviewer #4:
Manuscript Summary:
This is a useful method for fractionation of cultured cells into mitochondrial, nuclear, cytosolic, and membrane fractions with appropriate caveats regarding protocol optimization for use with cell lines other than U937.

Minor Concerns:
3.1.1-3.1.3 Is there a way to visually determine the appropriate volume to dilute cells--Should researcher expect the suspension to be opaque, cloudy, or clear?
Unfortunately, there is not a reliable way to do this.
3.1.7 note that pellet should be reserved for subsequent processing in step 3.2.1
We have now corrected this.
4.2.7 define 'short term'-- hours or days? is long term storage feasible and at what temperature, if not, please state that long term storage is not suitable
We now define this and add parameters for long-term storage in lines 173-176.
7.1.9- suggest examples of mitochondrial or membrane marker proteins to use for western blot assessments (lipoproteins or proteins expressed in multiple subcellular locations are likely inappropriate for use as markers) or reference appropriate figures
We now include this in lines 355-359.
An additional piece of information that the authors might include would be expected protein yield as a range of protein estimates (ug/ul when suspended in x ul of volume) for the fractions produced from U937 cells fractionated as described (starting with 2 x 10^7 cells/mL as recommended in step 2.1.4) as assessed by BCA or Bradford protein assay. Given that protein estimation may be a faster preliminary check on the efficacy of the protocol and is likely a

necessary intermediate step (though not necessary to describe in detail in this manuscript) prior to western blot sample preparation. Unusually dilute or concentrated fractions identified by comparison to expected protein concentrations/yields may allow researchers to troubleshoot aspects of this protocol prior to execution of western blots, thus further conserving reagents and time necessary for optimization of this protocol using other cell lines.
Thank you for this suggestion. We now include expected protein yield for each fraction in Table 1. We believe this has strengthened the manuscript.

Reviewer #5:
Manuscript Summary:
This protocol describes how to obtain subcellular fractions from eukaryotic cells, specifically monocyte cell line U937. The protocol is an extension/modification of a previously published protocol and requires the use of high-speed centrifugation. This method may be more useful than commercial kits as it allows optimalization. However the text and the figures need careful inspection. The protocols in generally should be clear and free of errors.

Major Concerns:
1) The protocol is not in agreement with the figure 1 that shows the flowchart. Therefore it is hard to follow.
a) The centrifugation force needed after CS buffer incubation is specified as "400 x g" in the figure and "7000 x g" in the text. (see 4.2.2)
b) The centrifugation force needed after NL buffer incubation is specified as "400 x g" in the figure and "7800 x g" in the text. (see 4.2.6)
c) The references to the previous steps should be checked (for example see 6.1.1 - there is reference to step 4.1.8 that does not exist).
e) The text says that you use the fraction of the homogenate that was treated with digitonin to isolate the nuclear proteins (see 4.1.2) but the figure shows something different.
e) The text probably says that you use the fraction of the homogenate that was not treated with digitonin (see 5.1.1) for subsequent differential centrifugation and density gradients but the figure shows something different.
Regarding points A-E above, we have corrected the Flowchart.

2) The yield of the method is not specified in any way.
We now specify the yield of each fraction in Table 1.

Minor Concerns:
1) Could you comment why you chose the technique of underlaying the sample below the density gradient? What is the benefit of it in contrast to the overlaying the sample above the gradient?
Underlaying is performed when possible to minimize mixing of the different fractions. They are more prone to mixing if overlaying is performed.

2) You can check the subcellular fractions and density gradients by enzyme assays instead of performing western blots. This enables you to have an overview of their content in shorter time.

Thank you for this suggestion. Another reviewer suggested we include a Bradford assay which we now include in lines 340-341. Table 1 shows the expected yield for each fraction.

3) You can check the percentage of the OptiPrep/sacharose by tabletop refractometer.
Thank you for this suggestion

4) The method/machine used for measuring the cell concentration is not described.
A standard hemocytometer was used and cells were counted visually in a microscope. We now include this in the text in line 136.

5) The caption of Figure 2 does not inform what sample (18k x g pellet or 4k x g pellet) was analysed in this case. According to the text (part "representative results", line 357) it was the 18k x g pellet. Could you show a similar analysis of 4k x g pellet?
Originally, this figure just showed an analysis of the 18k pellet. We now include a similar analysis of the 4K pellet.
6) More comments regarding the modifications of concentrations/centrifugation speed/time etc. would be useful for the optimalization that will be required if the protocol was applied to other cell lines.
We now address considerations that may need to be taken when working with other cell lines in lines 488-497.