Video Article

Nitrogen Cavitation and Differential Centrifugation Allows for Monitoring the Distribution of Peripheral Membrane Proteins in Cultured Cells

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Abstract

Cultured cells are useful for studying the subcellular distribution of proteins, including peripheral membrane proteins. Genetically encoded fluorescently tagged proteins have revolutionized the study of subcellular protein distribution. However, it is difficult to quantify the distribution with fluorescent microscopy, especially when proteins are partially cytosolic. Moreover, it is often important to study endogenous proteins. Biochemical assays such as immunoblots remain the gold standard for quantification of protein distribution after subcellular fractionation. Although there are commercial kits that aim to isolate cytosolic or certain membrane fractions, most of these kits are based on extraction with detergents, which may be unsuitable for studying peripheral membrane proteins that are easily extracted from membranes. Here we present a detergent-free protocol for cellular homogenization by nitrogen cavitation and subsequent separation of cytosolic and membrane-bound proteins by ultracentrifugation. We confirm the separation of subcellular organelles in soluble and pellet fractions across different cell types, and compare protein extraction among several common non-detergent-based mechanical homogenization methods. Among several advantages of nitrogen cavitation is the superior efficiency of cellular disruption with minimal physical and chemical damage to delicate organelles. Combined with ultracentrifugation, nitrogen cavitation is an excellent method to examine the shift of peripheral membrane proteins between cytosolic and membrane fractions.

Video Link

The video component of this article can be found at https://www.jove.com/video/56037/

Introduction

Cellular proteins can be divided into two classes: those that are associated with membranes and those that are not. Non-membrane associated proteins are found in the cytosol, nucleoplasm and lumina of organelles such as the endoplasmic reticulum (ER). There are two classes of membrane-associated proteins, integral and peripheral. Integral membrane proteins are also known as transmembrane proteins because one or more segments of the polypeptide chain spans the membrane, typically as an α-helix composed of hydrophobic amino acids. Transmembrane proteins are co-translationally inserted into membranes in the course of their biosynthesis and remain so configured until they are catabolized. Peripheral membrane proteins are secondarily driven to membranes, usually as a consequence of post-translational modification with hydrophobic molecules such as lipids. In contrast to integral membrane proteins, the association of peripheral membrane proteins with cellular membranes is reversible and can be regulated. Many peripheral membrane proteins function in signaling pathways, and regulated association with membranes is one mechanism for activating or inhibiting a pathway. One example of a signaling molecule that is a peripheral membrane protein is the small GTPase, RAS. After a series of post-translational modifications that include modification with a farnesyl lipid, the modified C-terminus of a mature RAS protein inserts into the cytoplasmic leaflet of the cellular membrane. Specifically, the plasma membrane is where the RAS engages its downstream effector RAF1. To prevent constitutive activation of mitogen-activated protein kinase (MAPK) pathway, multiple levels of control of RAS are in place. Besides rendering RAS inactive by hydrolyzing GTP into GDP, active RAS also can be released from the plasma membrane by modifications or interactions with solubilizing factors to inhibit signaling. Although fluorescent live imaging affords cell biologists the opportunity to observe the subcellular localization of fluorescent protein-tagged peripheral membrane proteins[1], there remains a critical need to evaluate membrane association of endogenous proteins semi-quantitatively with simple biochemical approaches.

The proper biochemical evaluation of protein partitioning between membrane and soluble fractions is critically dependent on two factors: cellular homogenization and efficient separation of membrane and soluble fractions. Although some protocols, including the most widely used commercialized kits, depend on detergent-based cell homogenization, these methods can obfuscate analysis by extracting membrane proteins into the soluble phase[2]. Accordingly, non-detergent based, mechanical methods of cell disruption provide cleaner results. There are several methods of mechanical disruption of cells grown in culture or harvested from blood or organs. These include Dounce homogenization, fine needle disruption, ball-bearing homogenization, sonication and nitrogen cavitation. Here we evaluate nitrogen cavitation and compare it to other methods. Nitrogen cavitation relies on nitrogen that is dissolved in the cytoplasm of the cells under high pressure. After equilibration, the cell suspension is abruptly exposed to atmospheric pressure such that nitrogen bubbles are formed in the cytoplasm that tear open the cell as a

consequence of their effervescence. If the pressure is sufficiently high, nitrogen effervescence can disrupt the nucleus[3] and membrane bound organelles like lysosomes[4]. However, if the pressure is kept low enough, the decompression will disrupt the plasma membrane and ER but not other organelles, thereby spilling both cytosol and intact cytoplasmic organelles into the homogenate that is designated the cavitate[5]. For this reason, nitrogen cavitation is the method of choice for isolating organelles like lysosomes and mitochondria.

However, it is also an excellent way of preparing a homogenate that can be easily separated into membrane and soluble fractions. The pressure vessel (henceforth called "the bomb") used during cavitation consists of a thick stainless steel casing that withstands high pressure, with an inlet for delivery of the nitrogen gas from a tank and an outlet port with an adjustable discharge valve.

Nitrogen cavitation has been used for cell homogenization since the 1960s[6]. In 1961, Hunter and Commerfold[7] established nitrogen cavitation as a viable option for mammalian tissue disruption. Since then, researchers have adapted the technique to various cells and tissues with success, and nitrogen cavitation has become a staple in multiple applications, including membrane preparation[8,9], nuclei and organelle preparation[10,11], and labile biochemical extraction. Currently, cell biologists more often employ other methods of cell homogenization because the benefits of nitrogen homogenization have not been widely advertised, nitrogen bombs are expensive and there is a misconception that a relatively large number of cells is required. Protocols for nitrogen cavitation to achieve cell-free homogenates with intact nuclei have not been published, and in most published evaluations volumes of 20 mL of cell suspension were used. To adapt this classic technique to suit current requirements of working with small-scale samples, we present a modified protocol of nitrogen cavitation specifically designed for cultured cells. After nitrogen cavitation, the homogenate is separated into soluble (S) and membrane (P) fractions by differential centrifugation, first with a low-speed spin to remove nuclei and unbroken cells, and then with a high-speed spin (>100,000 x g) to separate membranes from the soluble fraction. We analyze the efficiency of the separation with immunoblots and compare nitrogen cavitation with other mechanical disruption techniques. We also investigate the osmotic effect of homogenization buffer during nitrogen cavitation.

Protocol

1. Buffer and Equipment Preparations

1. Chill 45 mL cell disruption bombs, 15 mL tubes, and ultracentrifugation tubes at 4 °C.
2. Prepare and chill 25 mL of homogenization buffer per 2 x 10^7 cells at 4 °C. Add one protease inhibitor tablet just before use.
   NOTE: Homogenization buffers typically contain KCl rather than NaCl to better reflect intracellular salt composition. Homogenization buffer used in this protocol consists of 10 mM HEPES at pH 7.4, 10 mM KCl and 1.5 mM $MgCl_2$ (hereinafter referred to as hypotonic homogenization buffer). Most buffers can be adapted for nitrogen cavitation (see **Discussion**).
3. Prepare and chill 6 mL of 1x Phosphate-Buffered Saline (PBS) buffer per sample at 4 °C. Add protease inhibitor tablets fresh before use.
   NOTE: PBS buffer used in this protocol consists of 10 mM Na_2HPO_4 at pH 7.4, 1.8 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl.
4. Prepare and chill 4 mL of solubilization buffer per sample at 4 °C. Add one protease inhibitor tablet just before use.
   NOTE: Solubilization buffer used in this protocol is 1x Radioimmunoprecipitation assay (RIPA) buffer, which consists of 25 mM Tris at pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% NP-40. See 4.6 NOTE.

2. Cell Harvesting

1. Grow 2 x 10^7-10 x10^9 tissue culture cells with the recommended culture media for the cell type. Typically, one 15-cm dish yields 2 x 10^7 HEK-293 cells cultured in DMEM at 90% confluency (**Figure 1**).
2. Remove the growth medium by vacuum.
3. For adherent cells, place the culture dishes on ice, wash the cells directly on the culture dishes gently with chilled homogenization buffer twice (10 mL buffer per 15 cm dish per wash) and harvest the cells with a large cell scraper in an appropriate volume of homogenization buffer; For suspension cells, collect and wash the cell pellet in chilled homogenization buffer twice (10 mL buffer per 50-mL culture per wash) with 500 x g spin at 4 °C for 5 min, and resuspend the washed cell pellet in an appropriate volume of homogenization buffer on ice.
   NOTE: The volume should be based on the requirements for protein concentration in the intended experiments as well as the minimal/maximal volume allowed in the cell disruption bomb. A general guideline is 2-10 x 10^7 cells/mL, or about 10 volumes of the cell pellet. This protocol is optimized for three 15-cm dishes of HEK-293 cells in 2 mL of homogenization buffer.

3. Nitrogen Cavitation

1. Transfer the cell suspension to a clean and chilled bomb in an ice bath on a stir plate.
   CAUTION: The bomb has high pressure, low temperature, nitrogen gas – wear appropriate personal protection.
2. Place a micro magnetic stir bar inside the bomb and turn on the stir plate to maintain suspension homogeneity.
3. Add one protease inhibitor tablet to the suspension and close the bomb per manufacturer's instruction.
4. Gradually pressurize the bomb with a nitrogen gas tank per manufacturer's instruction till the bomb pressure gauge reads 300 to 600 psi. Close all valves and disconnect the nitrogen tank.
   NOTE: The pressure required may vary with the cell type. Here we performed cavitation at 350 to 400 psi for HEK-293, NIH-3T3 and Jurkat cells.
5. Wait for 20 min to allow the nitrogen to dissolve and reach equilibrium within the cells.
6. Remove excess water around the discharge valve using a cloth towel. Open the discharge valve gently to achieve a dropwise release of homogenate and collect in a pre-chilled 15 mL tube.
   NOTE: Near the end of collection there will be a spurt of homogenate and gas will emerge with a hissing sound. Make sure that the gas does not cause previously collected cavitate to shoot out of the tube (hence the use of 15 mL tubes instead of 1.5 mL tubes). Once the spurt starts, close the discharge valve and open the nitrogen inlet valve abruptly to depressurize the bomb and achieve cavitation of the remaining cells in the bomb. Open the bomb for cavitate recovery and thorough cleaning.

NOTE: The final cavitate should have a milky appearance with foam on top. Gently stir with a pipette tip to allow the foam to subside before centrifugation.
NOTE: Examine the cavitate by phase-contrast microscopy to determine the homogenization efficiency. Add a 15 µL drop of cavitate to the surface of a microscopy slide and cover with a coverslip. Repeat step 3.4-3.6 only if too many unbroken cells are detected with a 20X objective.
NOTE: If homogenization buffer does not contain EDTA or EGTA, add it to the collected cavitate at a final concentration of 1 mM within 5 min after discharge.

4. Separation of Cytosolic and Membrane Fractions

1. Centrifuge the cavitate at 500 x g for 10 min at 4 °C to remove unbroken cells and nuclei.
   NOTE: Repeat the centrifugation step until no visible pellet is produced and collect the Post Nuclear Supernatant (PNS) while avoiding the foam floating on top. Re-centrifuge the foam to further collect and combine PNS, if necessary (**Figure 1**).
2. Process the PNS as desired to obtain fractions of interest. For the purpose of separating cytosolic and membrane fractions, transfer the PNS to an ultracentrifuge tube and perform ultracentrifugation as desired. This protocol is optimized for a <3.5 mL sample in a polycarbonate ultracentrifuge tube and for ultracentrifugation at 350,000 x g for 1 h at 4 °C.
3. Collect the supernatant (the S fraction) using a 1 mL pipette.
4. Carefully rinse the pellet with 3 mL of cold PBS without disturbing it. Remove the PBS by vacuum.
   NOTE: If contamination of the membrane fraction by cytosolic proteins is a greater concern than the loss of sample, resuspend the pellet in 3 mL of cold PBS and re-ultracentrifuge as in step 4.2. Remove the PBS by vacuum.
5. Resuspend the pellet fully in an appropriate volume of detergent-containing solubilization buffer of choice. To achieve cell equivalence, use the same volume of solubilization buffer as the cytosolic fraction.
   NOTE: We suggest using 1x RIPA buffer as solubilization buffer for efficient membrane protein extraction. If no downstream assay is required for membrane fraction, use 1x laemmli sample buffer for maximal membrane protein extraction.
   NOTE: We suggest dislodging and transferring the pellet in solubilization buffer to a clean tube on a tube rotator at 4 °C for maximal membrane protein extraction.
   NOTE: If the pellet is too sticky (too many lipids) to be efficiently removed from the ultracentrifuge tube in solubilization buffer, we suggest snap freezing the pellet in liquid nitrogen and quickly dislodging the pellet from the ultracentrifuge tube with a mini metal spatula before the pellet thaws.
   NOTE: Alternatively, membrane pellets can be just resuspended and not solubilized in a non-detergent-containing buffer to produce a P fraction of membrane vesicle suspension, in which case the centrifugation step in 4.6 is not required. Such P fractions are useful when investigating functions such as enzyme activities that are dependent on membrane association.
6. Centrifuge the fully solubilized pellet suspension from step 4.5 at 20,000 x g in a tabletop centrifuge for 10 min at 4 °C. Collect the supernatant using a 1 mL pipette (the P fraction) and discard the pellet (insoluble lipids).
7. Perform desired assays such as western blotting with the cytosolic and/or membrane fractions, or save them at -80 °C for future use.

Representative Results

Figure 2 shows the partitioning of cellular proteins from PNS into either the soluble cytosolic fraction (S) or membrane pellet fraction (P). We examined three representative cell lines from different cell types: HEK-293 (epithelial), NIH-3T3 (fibroblast), and Jurkat (lymphocyte). Rho Guanine Dissociation Inhibitor (RhoGDI) and cation-independent mannose-6-phosphate receptor (CIMPR) were used as positive controls for cytosolic and membrane fractions, respectively. We confirmed the efficient separation of the cytosol and membrane after nitrogen cavitation at ~ 350 psi in hypotonic buffer followed by ultracentrifugation at 350,000 x g for 1 h. The total protein recovered from the S and P fractions were then analyzed with markers for the ER, endosomes and lysosomes, mitochondria, Golgi, and other organelles. All transmembrane proteins are present in the membrane fraction exclusively, including Na^+/K^+ ATPase and epidermal growth factor receptor (EGFR) from the plasma membrane, calnexin from ER, lysosomal-associated membrane protein 1 (LAMP1) from lysosomes, F_0-ATPase from mitochondria, and Golgin 97 from the *trans* Golgi network. As expected, many peripheral membrane proteins that are loosely associated with the membrane, are present both in the cytosolic and membrane fractions to varying degrees. Notable examples include early endosome antigen 1 (EEA1), Rab7/9 (late endosomes) and hexokinase 1 (outer mitochondria membrane). This demonstrates the utility of this technique in evaluation of the membrane association strength of peripheral proteins. Our laboratory has taken advantage of this cavitation technique to examine the membrane association status of several signaling molecules[5,12,13,14]. Interestingly, we found calregulin almost exclusively in the soluble fraction, suggesting that the microsomes generated from ER membranes have also been disrupted during nitrogen cavitation and the proteins in the ER lumen are released to the soluble fraction. In contrast, the integrity of mitochondria after cavitation is dependent on the cell type. We observed F_1-ATPase, an inner mitochondria peripheral protein, partially in the cytosolic fraction ranging from 2% for NIH-3T3 cells to ~35% for HEK-293 and Jurkat cells. This indicates that nitrogen cavitation at 350 psi with hypotonic buffer does not ensure mitochondrial integrity. Despite three cycles of 500 x g spins to generate the PNS from cavitate, not all nuclei were removed. Histone deacetylase 1 (HDAC1) from nucleoplasm and fibrillarin from the nucleolus, were found both in the PNS and the pellet. The presence of HDAC1 in the pellet and not in the supernatant suggests that the PNS is contaminated with nuclei, rather than nuclear disruption during cavitation. This is consistent with reports that pressurizing the bomb to 350 psi leaves nuclei intact[10], although our use of hypotonic buffer may render the nuclei fragile, as discussed in the later section.

We compared the homogenization efficiency of nitrogen cavitation with that of other common physical disruption methods. Equal amounts of Jurkat cells were suspended in identical hypotonic homogenization buffer and subjected to different disruption methods. There was detectable loss of samples in Dounce homogenization and nitrogen cavitation (in both cases about 5-10% less volume is collected). However, nitrogen cavitation gave the highest protein extraction efficiency; needle passage and Dounce yielded only 60% as much protein (**Figure 3**). Curiously, recovery of some proteins as determined by immunoblot did not show a significant difference between the different mechanical disruption methods. However, the yield of certain peripheral membrane proteins such as hexokinase 1 and RAS was higher in samples prepared with nitrogen cavitation. This supports that nitrogen cavitation may be optimal for homogenization when investigating peripheral membrane proteins. Recovery of HDAC1 proteins by nitrogen cavitation is comparable to other methods, suggesting that the nuclei remain intact in all conditions. Thus, the data in **Figure 3** proves that nitrogen cavitation offers superior homogenization results.

Next, we compared the homogenization efficiency between hypotonic buffer and the same buffer but made isotonic with either sucrose or sodium chloride (**Figure 4**). Similar amounts of protein were recovered from Jurkat cells homogenized in hypotonic buffer versus NaCl isotonic buffer after cavitation at 350 psi. In contrast, less protein was recovered in the PNS of cells disrupted in buffer made isotonic with sucrose. Most individual proteins examined by immunoblot fit this pattern. One exception was fibrillarin that was relatively enriched in the PNS generated in isotonic buffer.

To determine if our protocol can be employed to investigate partitioning of peripheral membrane proteins between soluble and membrane fractions, we compared partitioning patterns between FLAG-tagged NRAS wild type and its prenylation-deficient mutant, C186S (**Figure 5**). At steady state, the wild type NRAS was present in both cytosol and membranes, similar to most peripheral membrane proteins, although the portion recovered in the soluble fraction is greater than for other RAS proteins[14]. When NRAS is deprived of its prenyl modification, it loses the ability to associate with membranes and becomes entirely cytosolic. The expected partitioning patterns of NRAS confirmed that our protocol is a reliable option to study the dynamics of partitioning of peripheral membrane proteins between cytosol and membranes.

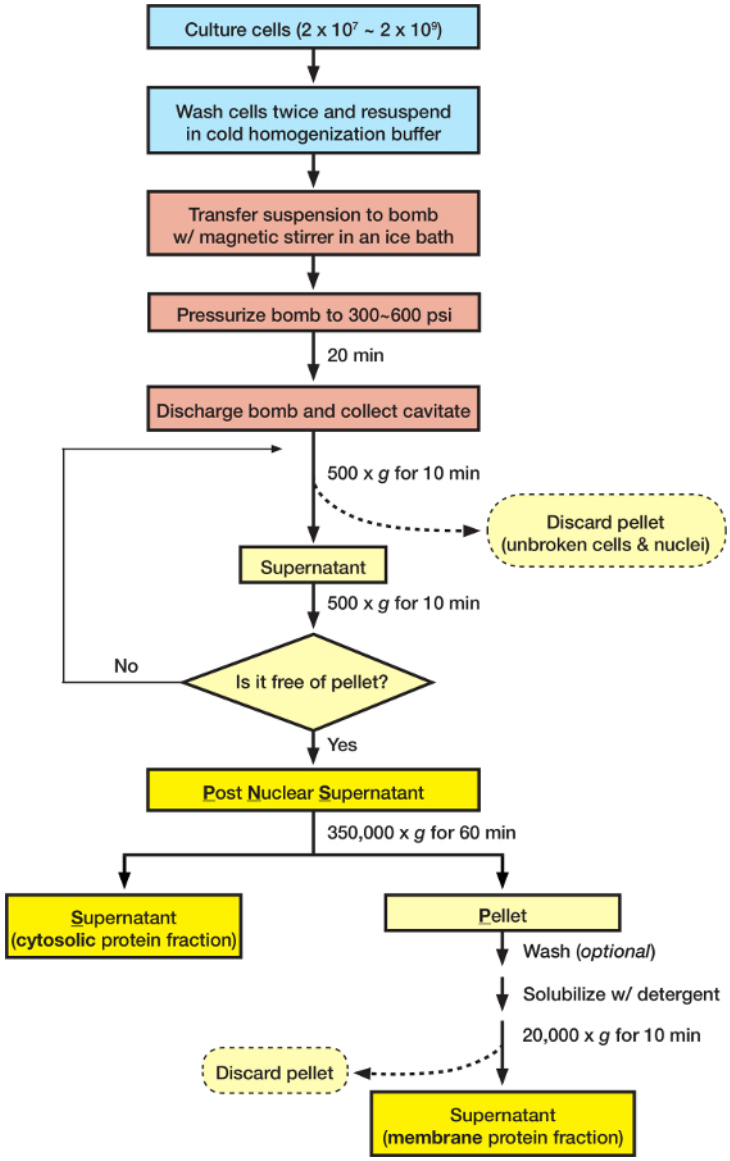

Figure 1: Flowchart summarizing step 2 (blue), step 3 (red) and step 4 (yellow) of the protocol. Please click here to view a larger version of this figure.

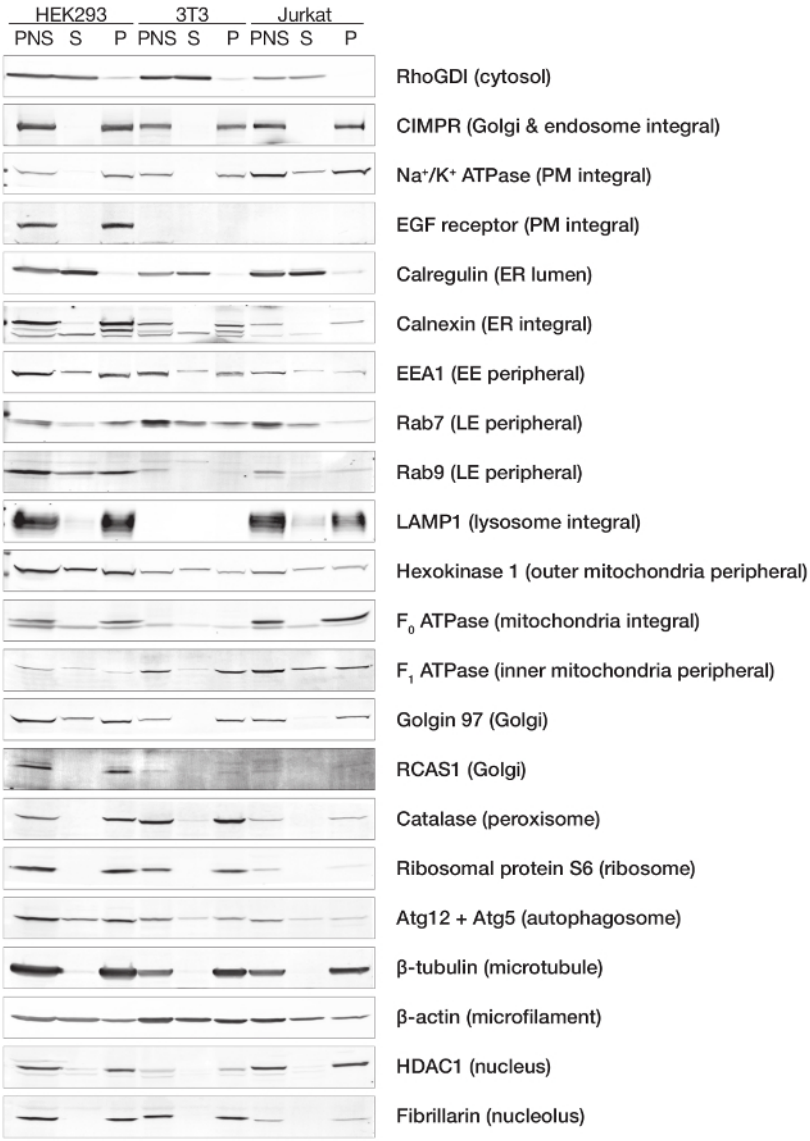

Figure 2: Protein partitioning between the cytosolic and membrane fractions. HEK-293, NIH-3T3 and Jurkat cells were subjected to nitrogen cavitation (~350 psi for 20 min, suspended in hypotonic homogenization buffer) and ultracentrifugation (~350,000 x g for 1 h) as described in the protocol. Endogenous protein levels in different fractions were analyzed by immunoblot. PNS: Receptor-binding cancer antigen expressed on SiSo Cells (RCAS), nuclear supernatant, S: supernatant, P: pellet. Please click here to view a larger version of this figure.

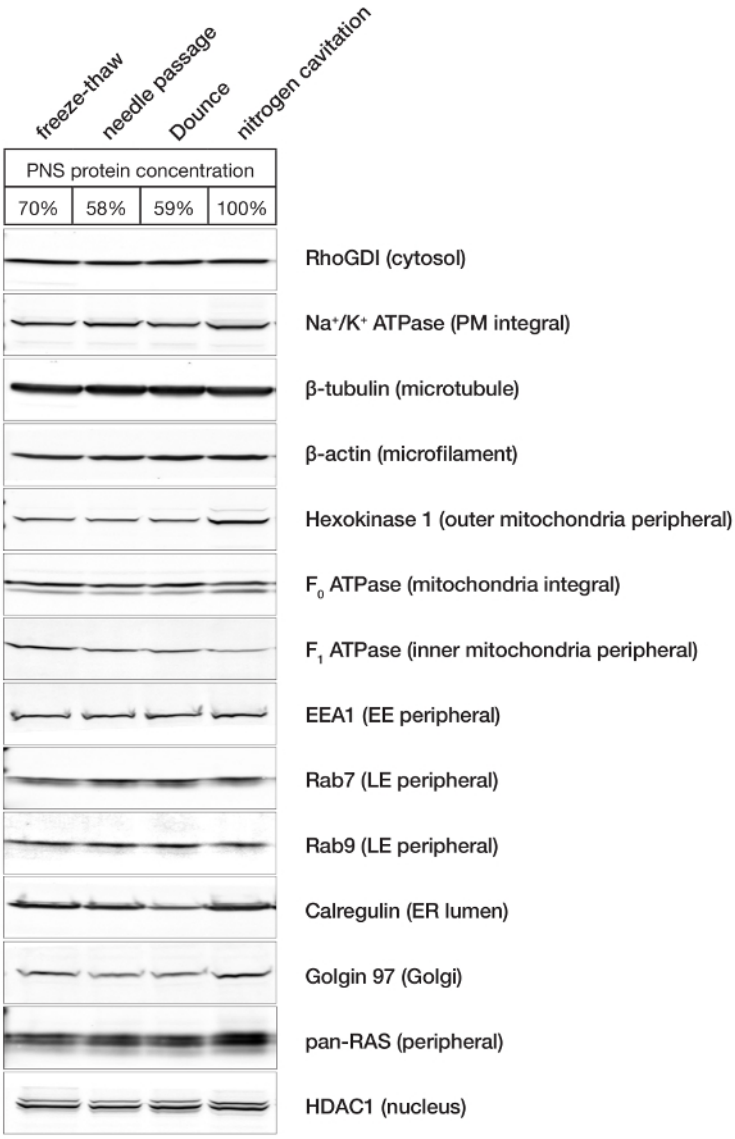

Figure 3: Comparison of different mechanical disruption techniques. Equivalent amount of Jurkat cells were suspended in hypotonic buffer and subjected to freeze-thaw (3 cycles), needle passage (5 passes, through 28G½ needle), Dounce homogenization (15 passes, Kontes 2 mL Dounce tube with a tissue grind pestle, clearance of 0.01-0.06 mm) or nitrogen cavitation (~350 psi for 20 min). Relative levels of endogenous proteins in the PNS were analyzed by immunoblot for each method. Total protein concentrations of PNS were quantified by BCA assay and normalized to the value of the PNS prepared by nitrogen cavitation. Please click here to view a larger version of this figure.

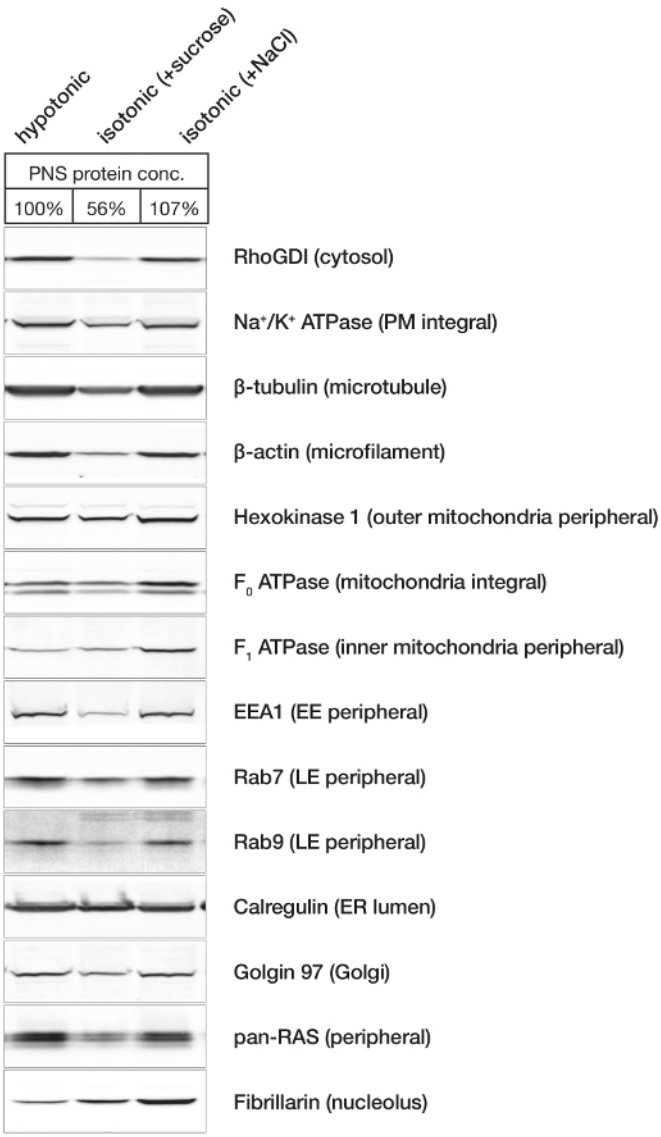

Figure 4: Comparison of hypotonic and isotonic buffers used during nitrogen cavitation. Equivalent amounts of Jurkat cells were suspended in either hypotonic buffer, or hypotonic buffer supplemented with 8.5% sucrose or 150 mM NaCl, and subjected to nitrogen cavitation (~350 psi for 20 min). Relative levels of endogenous proteins in the PNS were analyzed by immunoblot for each buffer. Total protein concentrations of PNS were quantified by BCA assay and normalized to the value of the PNS prepared in hypotonic buffer. Please click here to view a larger version of this figure.

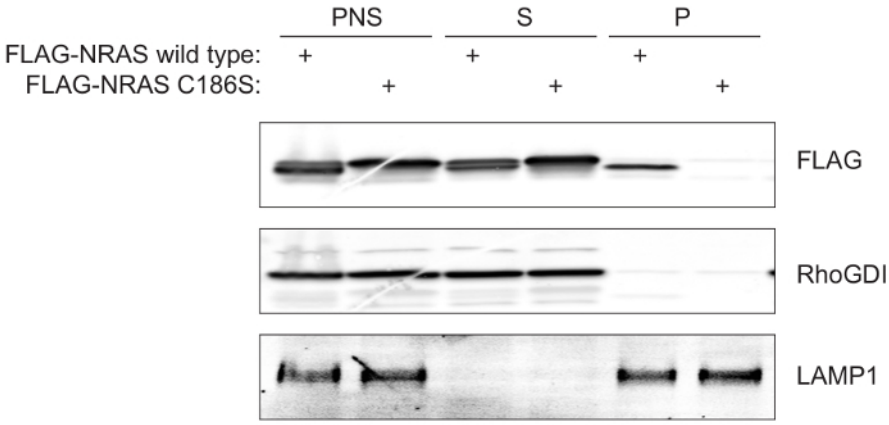

Figure 5: Farnesylated NRAS partitions into both P and S fractions. HEK-293 cells were transiently transfected with plasmids directing the expression of FLAG-tagged NRAS wild type or C186S mutant. Protein partitioning was performed as described in **Figure 2** and the indicated protein levels were analyzed by immunoblot. Reproduced with permission (Zhou, M *et al.*, 2016. Originally published in *Journal of Cell Biology*. https://doi.org/10.1083/jcb.201604061). Please click here to view a larger version of this figure.

Discussion

The advantages of nitrogen cavitation over other methods of mechanical disruption are manifold. Perhaps the most significant benefit is its ability to gently yet efficiently homogenize specimens. The physical principles of decompression cools samples instead of generating local heating damage like ultrasonic and friction/shearing based techniques. Cavitation is also extremely efficient at disrupting the plasma membrane. Because nitrogen bubbles are generated within each individual cell upon decompression, the cavitation process is limited less by the cell size, sample size or sample concentration. It therefore offers additional advantages over clearance-based Dounce or needle passage homogenization. Nitrogen cavitation also offers more consistent results as the same disruptive force that is applied uniformly throughout the sample can be reproduced with identical pressures. Moreover, each cell only experiences the disruption process for one single time and subcellular components are, therefore, not constantly exposed to variable disruptive forces. This limits the artefactual fragmentation of organelles. Concern for oxidation of labile cellular components caused by disruption is also mitigated, as nitrogen is used to saturate the cell suspension and is free of supplemental oxygen. The restricted physical and chemical stresses imposed by nitrogen cavitation makes it an ideal technique to study labile enzymes and fragile organelles, and the reproducibility of this homogenization technique is well suited for quantification.

Nitrogen cavitation also offers significant flexibility with regard to sample size, the composition of homogenization buffers, and the degree of organelle integrity. To scale up the homogenization, one simply needs to deploy pressure vessels of larger capacities, without sacrificing the speed, convenience or efficiency of decompression. Any homogenization buffer can be used for nitrogen cavitation; buffer choice can be tailored for compatibility with the requirements of each experiment. One can design a homogenization buffer that matches the intracellular fluid (*e.g.*, high potassium and low sodium) to minimize alterations in organelles. For example, we developed a "relaxation" buffer that minimizes *ex vivo* actin polymerization and thereby assists in collecting intact cytoplasmic vesicles from granulocytes[5]. The osmotic and ionic features of the buffer can also be modified to control the degree of cell homogenization. Additionally, the aggressiveness of cellular disruption can be controlled by adjusting the nitrogen pressure. Moderate pressure reduces the disruptive force to preserve nuclei, mitochondria and other organelles in an intact state, while high pressure disrupts these organelles. Brock *et al.* reported success with disruption of Rat Basophilic Leukemia cells while maintaining most nuclei intact at 350 psi with hypotonic buffer[10], which forms the basis of nitrogen pressure used in our current protocol.

Limitations of nitrogen cavitation include the fact that homogenization is uniform throughout the samples and different membrane structures may produce vesicles of similar sizes; this can complicate further separation of the plasma membrane, smooth microsomes, endosomes and Golgi membranes by rate-zonal density gradient centrifugation. Another concern is that the organelles become relatively fragile after homogenization due to the rupture from within. Thus, this method requires careful optimization to prevent organelle breakage in subsequent manipulations. We aimed to address some of these issues in this study.

Separation of cytosolic and membrane fractions by centrifugation has been explored in limited reports detailing the partitioning of protein fractions with a handful of protein markers[15,16]. Our method generates a crude separation of soluble and membrane-bound proteins, and does not involve isolation of particular organelles. Yet, it is still a valid concern that the homogenous vesicles of different membranes produced by cavitation may affect the efficiency of separation by ultracentrifugation. We presented a comprehensive survey by immunoblots of the isolation and partitioning of common subcellular organelles after fractionation with our protocol. As expected, purely cytosolic proteins like RhoGDI are exclusively in the soluble fraction, and transmembrane proteins on the plasma membrane (PM) such as EGFR and Na^+/K^+ ATPase, were recovered only in the pellet. Transmembrane proteins on organelles such as ER, mitochondria, Golgi and lysosomes were also recovered in the pellet, confirming that integral membrane proteins from different membranes and organelles can be successfully segregated from soluble proteins with our method. This fundamental validation is crucial for the further analysis of peripheral membrane proteins, which is the main purpose of this protocol.

Most of the peripheral membrane proteins exist in both the cytosolic and membrane fractions. Although this is often a true representation of their localization, it may in some instances represent artefactual redistribution of these proteins from the cytoplasmic surface of membranes to the soluble fraction during the homogenization process, since the strength of their association with membranes is not as strong as that of integral membrane proteins. Though nitrogen cavitation minimizes potential redistribution by exposing cells to a one-time, gentle disruption, one should be mindful of such artefacts of biochemical fractionation, and focus on the changes in the partitioning of proteins between cytosolic

and membrane fractions across experimental conditions. Nonetheless, the examination of peripheral membrane proteins at various stages of endosomal maturation is possible with this method. EEA1, a Rab5 effector that mediates docking of clathrin-coated vesicles to the early endosomes, localizes to the cytoplasmic surface of early endosomes and was recovered primarily in the pellet fraction. Late-endosome-related Rab7 and Rab9 proteins are also in the pellet fraction. However, there remains an appreciable amount of Rab7/9 in the cytosol. Because RabGDI is able to interact with prenylated Rabs and render these otherwise insoluble proteins cytosolic, Rab proteins tend to be more cytosolic than other endosome-related peripheral membrane proteins.

To characterize the nuclear and organelle integrity with homogenization by nitrogen cavitation, we immunoblotted the isolated fractions for both luminal and cytoplasmic compartments. In this analysis, calregulin is fully released into the soluble fraction, demonstrating that ER is disrupted to form microsomes and thereby leaks luminal content. Given that one half of the total area of membrane in a eukaryotic cell encloses the labyrinthine spaces of the ER, it is unsurprising that its vast network of sac-like or tubular structure fails to remain intact during the expansion of nitrogen bubbles. On the other hand, our analysis of mitochondrial integrity after cavitation shows clear dependence on the cell type, as we observed F_1-ATPase, a protein that is peripherally associated with the inner membrane of mitochondria, in the cytosolic fraction to varying degrees (~35% for HEK-293 and Jurkat, 2% for NIH-3T3). This indicates that nitrogen cavitation at 350 psi with hypotonic buffer does not guarantee mitochondrial integrity. Indeed, others have reported that to preserve mitochondria, cavitation should be performed at pressures lower than 150 psi[17]. By contrast, we found that catalase remains in the lumen of peroxisomes. Intriguingly, nuclear proteins are observed in the PNS and pellet fractions. Specifically, HDAC1, a histone deacetylase present throughout the nucleoplasm, is absent in the soluble fraction, indicating that the nuclear integrity is not compromised. This suggests that the nuclear envelope is not ruptured by cavitation, as one would expect the release of HDAC1 into the soluble fraction in that scenario. It remains probable that nuclei are not completely removed during the process of PNS preparation, and the protein remains in the pellet after ultracentrifugation. This likelihood is further supported by the fact that no DNA leakage is detected in the soluble fraction. We conclude, therefore, that using hypotonic homogenization buffer during cavitation at ~350 psi with the addition of Mg^{2+} is able to preserve nuclear integrity in multiple cell lines.

Our results also demonstrate that ribosomes collect entirely in the membrane pellet fraction, suggesting that even free ribosomes in the cytoplasm that are unbound to the ER membranes can be completely sedimented in a non-sucrose-cushion buffer after centrifugation at 350,000 x g for 1 h. Likewise, identification of the autophagy-related protein 12 (Atg12)-Atg5 conjugate as a marker of autophagosomes, reveals strict segregation to the pellet fractions. Its presence in the cytosol may be caused by the fact that the covalent-attachment of Atg12 and Atg5 precedes autophagosomes formation. Cytoskeletal proteins are difficult to assess because of *ex vivo* polymerization. This phenomenon can be minimized with a relaxation buffer but in the protocol described here, tubulin is likely polymerized *ex vivo* upon chelation of calcium 18. In our preparations tubulin is found in the pellet suggesting polymerization whereas actin is found in both the P and S fractions.

To characterize potential advantages of nitrogen cavitation in homogenization efficiency, we reviewed some common mechanical disruption techniques. Methods optimized for tissue samples such as blender or mortar/pestle were not evaluated. Sonication was also excluded as it is difficult to disrupt the surface membrane while keeping the internal organelles intact. Our analysis focused on the following methods: freeze-thaw, needle passage and Dounce homogenization, as they are easy to perform and do not require special equipment such as a ball-bearing homogenizer. Jurkat cells were used in this analysis because of their small size, which renders them difficult to homogenize efficiently with traditional liquid shearing techniques that are dependent on clearance, such as needle passage and Dounce. Judged by the concentration of the total protein recovered, we found that cell-size independent disruption methods such as nitrogen cavitation and freeze-thaw indeed show superior efficiency of protein extraction. Similar analysis with cells of larger sizes revealed smaller differences in homogenization efficiencies, though nitrogen cavitation is still superior to alternative physical methods tested (data not shown).

Many cytosolic, endosomal and cytoskeleton proteins were efficiently recovered with all tested methods. In contrast, proteins on the ER and Golgi were optimally recovered using nitrogen cavitation. The slightly lower yield of F_1-ATPase in nitrogen cavitation relative to the other methods may reflect the fact that mitochondria are more likely to remain intact during cavitation and therefore more efficiently removed in the slow speed spin used to generate the PNS. The observation that hexokinase 1, a peripheral membrane protein found both in the cytosol and associated with the outer mitochondrial membrane, behaved in the opposite fashion relative to the F_1-ATPase likely reflects the lability of the association of hexokinase 1 relative to F_1-ATPase, which is sequestered inside the mitochondria. Importantly, although capable of disrupting cells with comparable efficiencies, alternative mechanical disruption methods gave relatively poor recovery of certain peripheral membrane proteins (hexokinase 1 and RAS). Thus, cavitation is our homogenization technique of choice for the purpose of investigating the partition of peripheral membrane proteins. Because Dounce homogenization is widely used to prepare intact nuclei, we used Dounce as a benchmark to evaluate nuclear integrity across other mechanical disruption methods. In our analysis, similar levels of recovery of HDAC1 proteins confirm that nuclei remain intact in Jurkat homogenates prepared by nitrogen cavitation at ~350 psi with hypotonic buffer.

It was apparent that the homogenization buffer used during nitrogen cavitation can significantly affect the disruption efficiency and organelle integrity. While the composition of homogenization buffer should be optimized to the requirements of the intended application, it is worth noting that researchers performing nitrogen decompression of animal tissues use buffer with a low osmotic pressure to extract nuclear contents. Hunter and Commerford showed nuclei swelling and rupture when the solutions were dilute, and nuclei preservation when using isotonic solutions[7] (which can be achieved by either adding inorganic salts such as sodium chloride or organic solutes such as sucrose or glycerol). The standard isotonic buffer used for isolating subcellular organelles other than nuclei from mammalian cells is 0.25 M sucrose containing 1 mM EDTA and buffered with Tris, HEPES, or Tricine at pH 7.0-7.6. But not all culture cells can be efficiently homogenized in one of the isotonic buffers. Hypotonic buffer (usually 10 mM Tris, HEPES, *etc.*) is often used to provide necessary osmotic stress to certain cell types to achieve complete homogenization. However, as mentioned before, hypotonic buffers tend to render the nuclei fragile and prone to leakage of DNA when EDTA is included. To promote nuclear integrity, we replace EDTA with KCl and low concentrations of divalent cations such as $MgCl_2$ or $MgSO_4$. Mg^{2+} is usually preferred over Ca^{2+} because the latter can activate certain phospholipases and proteases and inhibit RNA polymerase. Once cavitation is finished, EDTA or EGTA can be added back to the homogenates to chelate the cation, if required to inhibit metalloproteases.

Jurkat cells display a relatively large nucleus-to-cytoplasm ratio, making them an ideal cell line to study nuclear integrity. The increase of homogenization efficiency by using hypotonic buffer is minimal when comparing to isotonic buffer supplemented with NaCl. However, cavitation in an isotonic buffer supplemented with sucrose recovers approximately half the proteins extracted by using a hypotonic buffer counterpart. This is unlikely to be a direct result of isotonicity, and the discrepancy between using NaCl and using sucrose to achieve isotonicity requires further investigation. One possible explanation is that salt disrupts electrostatic interactions between proteins whereas sucrose does not. Thus, even

at the same tonicity, NaCl has an activity relevant to protein extraction that sucrose lacks. With regard to preserving nuclear integrity, we found increased fibrillarin proteins recovered with isotonic buffers, despite isotonic buffers theoretically being more protective of the nuclei. Curiously, the mitochondrial fraction was more efficiently extracted with isotonic buffer supplemented with NaCl, suggesting that low salt conditions may be suboptimal for mitochondria extraction. Considering cavitation with hypotonic buffer achieves the best balance between homogenization efficiency and nuclear integrity, the hypotonic buffer becomes the buffer of choice in our protocol of cavitation. However, we caution the readers that no buffer is a guarantee of optimal homogenization results. Our protocol should serve as a template for optimization, and pilot testing with desired buffers, cell lines of interest, and other variables (pressure, buffer composition, *etc.*) must be established for best results.

Disclosures

The authors declare that they have no competing financial interests.

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