Efficient extraction of nucleolar proteins for interactome analyses

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The efficient extraction of proteins from purified cellular organelles is critical for in vitro analyses, including identification of protein complex members by affinity purification-based quantitative proteomic approaches. When applied to purified nucleoli, classic nuclear protein extraction methods inefficiently and selectively release only \( \frac{1}{2} \)–50% of proteins. Here, we present a method that can extract up to 90% of nucleolar proteins, and apply it in a quantitative interactomic approach to identify nucleolar interaction partners for a mammalian protein phosphatase.

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Most biological processes involve the action and regulation of multiprotein complexes, and thus a key goal in cell biology is the characterization of these complexes through the reliable identification of protein interaction partners. The development of quantitative MS-based approaches coupled with efficient immunopurification of protein complexes has enabled this type of “interactome” study to be carried out for a wide range of proteins [1–5]. Importantly, when coupled with cellular fractionation, spatial information such as organelle-specific interactions can be determined [1, 2, 6].

The nucleolus, the best studied of the subnuclear organelles, is a dynamic structure that disassembles and reforms during each cell cycle around the ribosomal RNA gene clusters [7]. Its inherent density facilitates its isolation in large quantities and with high purity from cultured cells, using a combination of sonication and sucrose density centrifugation (Fig. 1A; [8]; for review, see [9]). In addition to the pivotal role of the nucleolus in ribosome biogenesis, numerous lines of evidence suggest additional roles in biogenesis of other ribonucleoprotein machines, stress sensing and regulation of the cell cycle [10, 11]. Consistent with this plurifunctionality, a wide range of proteins have been mapped to the nucleolus by proteomic analyses [6, 8, 12–14]. A key approach to identifying and characterizing the molecular complexes involved in these diverse functions is to map nucleolar protein interactomes.

The first step in an interactome experiment is the efficient release of proteins from the cells/purified organelles, preferably under the lowest stringency conditions possible to avoid disruption of protein–protein interactions. Although nucleoli are readily purified, the denaturing buffers used to solubilize them for whole proteome analysis [8, 12] are not compatible with the preservation of protein–protein interactions and immunoprecipitation (IP) of intact nucleolar complexes. We initially employed a method similar to that used routinely for purified whole nuclei [1, 2], which involves sonication in ice-cold RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, COMPLETE protease inhibitors). The buffer...
conditions are compatible with IP experiments and we have successfully used this approach to identify nuclear interaction partners for two isoforms of protein phosphatase 1 (PP1; 2) and for the survival of motor neuron protein [1]. In the case of the survival of motor neuron protein, we identified all 7 core members of the heteroprotein complex, an additional 15 known interaction partners and 1 novel interaction partner [1], confirming the potential to maintain multiprotein complexes under these extraction conditions.

When applied to purified nucleoli, sonication in RIPA buffer visibly disrupts nucleolar structure, as visualized by phase contrast microscopy, however, only half of the total protein content is extracted (Fig. 1B). This was determined by subjecting cell equivalent amounts of soluble (extracted in 200 μL of RIPA buffer) and insoluble (pelleted after extraction and solubilized in 200 μL of 8 M urea for analysis) nuclear fractions to 1-D SDS/PAGE. For each lane, 30 μL of the original 200 μL volume of extracted or nonextracted protein was added to 10 μL of 4 × NuPAGE LDS sample buffer (Invitrogen) and the samples vortexed and boiled prior to loading on precast 4–12% NuPAGE Bis-Tris gradient gels (Invitrogen). Gels were stained for 1 h with SimplyBlue Safestain (Invitrogen) and destained overnight in distilled water. Relative amounts of total Coomassie-stained protein in each lane were determined using a Fuji LAS 4000 mini imaging system and MultiGauge quantitation software (Fujifilm). Extraction efficiency was also found to be highly variable between subnucleolar domains and from protein to protein, as demonstrated by quantitative Western blot analysis using antibodies raised against proteins known to accumulate either in the fibrillar center, dense fibrillar center or in the granular component.

Figure 1. Variable efficiencies of classic nucleolar extraction methods. Nucleoli are readily observed as phase-dense structures (arrows) in live cells (A, top panel) and can be isolated in large amounts and with high purity (A, bottom panel). Sonication of purified U2OS cell-derived nucleoli in RIPA buffer released only ~50% of the total protein, as evaluated by SDS-PAGE (B, left panel). Quantitative Western blot analyses, using protein markers for the fibrillar center, dense fibrillar component and granular component, revealed unequal release of proteins from these subnucleolar domains (B, right panel). A similar extraction of ~50% of total protein was observed for high salt (C) and DNase (D) extraction methods. Although DNase extraction demonstrated an unequal release of compartment markers (D) similar to that observed with the RIPA sonication extraction method (B), more efficient extraction was observed for most of these subnucleolar compartment markers with high salt extraction (C). For each quantitative Western blot experiment, mean ± SD is plotted (n = 3).
Chemiluminescent signals generated by HRP-conjugated secondary antibodies using the Lumi-light substrate (Roche) were also detected and quantified using the Fuji imaging system and Multigauge quantitation software. For most of these proteins, extraction efficiency was also highly variable between individual experiments, and we thus considered alternate methods.

High salt solutions are routinely used to precipitate proteins in the preparation of nuclear extracts, with the resulting extract dialyzed back into low salt solution for further analysis [15]. DNase digestion has also been employed in nuclear extraction protocols for the release of undissociated protein complexes from DNA, and in the case of purified nucleoli we hypothesized that it might also help disrupt the heterochromatin shell that has been shown to coat this organelle [11]. A caveat is that, as with detergent- and physical disruption-based methods, these conditions have the potential to disrupt lower affinity protein–protein interactions.

Previous studies have applied these techniques to the extraction of nucleolar proteins from other cell types for various assays, and we tested two published methods: high salt extraction alone [16] and a combination of DNase treatment with high salt extraction [17]. The complete protocols can be found in Supporting Information Methods. Although there was no significant improvement in the amount of total protein extracted from U2OS-derived nucleoli compared with the RIPA sonication extraction method (Figs. 1C and D versus Fig. 1B), there were differences in the extraction efficiencies of some of the subnuclear marker proteins. High salt extraction improved the efficiency of extraction for some of these markers; however, variability between experiments remained high for many of them (Fig. 1C). For the DNase experiment, extraction efficiency was improved for two of the subnuclear marker proteins compared with the RIPA sonication method (Fig. 1D).

These protein-specific extraction efficiencies suggested that a combination of techniques might result in a cumulative increase in overall efficiency and decrease in variability between experiments, as certain proteins appear to be more amenable to particular extraction methods. Indeed, DNase treatment (30 min at room temperature) followed by sonication in standard RIPA buffer did result in an increase in the amount of total protein extracted (Fig. 2A, left panel), and this efficiency was further improved by adjusting the salt concentration in the RIPA buffer from 150 to 500 mM for the sonication step (Fig. 2A, right panel). Using this DNase/high salt/sonication protocol, we compared the efficiency of extraction of a panel of nucleolar proteins from purified U2OS and HeLa cell nucleoli (Figs. 2B and C). Extraction efficiencies ranged from 87 to

![Figure 2](image-url)

**Figure 2.** Combined methods result in more efficient extraction of nucleolar proteins. The combination of DNase pre-treatment (30 min at room temperature) with sonication in standard low salt (150 mM NaCl) RIPA buffer increased extraction efficiency for U2OS-derived nucleoli from 48 to 62% (n = 2), as judged by Coomassie staining of equivalent volumes of extracted and nonextracted proteins separated by SDS-PAGE (A, left panel). Sonication in high salt (500 mM NaCl) RIPA buffer increased this efficiency even further (A, right panel, 83% total protein extracted, n = 2). When tested with a panel of subnuclear compartment markers, quantitative Western blot analyses confirm that this combined DNase/high salt/sonication method improves the efficiency of extraction for most proteins, from both U2OS- (B) and HeLa- (C) derived nucleoli. Although DNase treatment at 4°C prior to sonication did not increase the fraction of total protein extracted for either U2OS (D) or HeLa (E) cells, for several of the subnuclear marker proteins there was less variability observed between experiments. For each quantitative Western blot experiment, mean ± SD is plotted (n = 3).
97% for U2OS nucleolar proteins (with standard deviations between 1 and 11%), and from 76 to 96% for HeLa nucleolar proteins (with standard deviations between 2 and 20%), which is a significant improvement over the classical extraction methods.

A major concern with this optimized extraction protocol, however, is the inclusion of a room temperature DNase treatment step. Despite the presence of protease inhibitors in the treatment buffer, we did see degradation of some of our specific nucleolar proteins of interest (data not shown). In order to retain this step yet minimize proteolysis, we reduced the incubation temperature to 4°C. For both HeLa and U2OS nucleoli, there is no significant increase in total protein extracted or in the efficiency of extraction of our subnucleolar marker proteins; however, we did note a decrease in the variability of extraction between experiments (Figs. 2D and E). Thus, our recommended protocol for the extraction of nucleolar proteins from purified nucleoli is a combination of DNase treatment at 4°C with sonication in high salt (500 mM NaCl) RIPA buffer, followed immediately by adjustment of the salt concentration in the buffer to 150 mM prior to IP of nucleolar proteins (Supporting Information Methods). If purified nucleoli from cell types other than HeLa or U2OS cells are to be used, the fractionation/extraction protocols presented here and in Supporting Information Methods can be used as a starting point and efficiency of total protein extraction monitored by SDS-PAGE and Coomassie staining. It is also recommended that cytoplasmic, nucleoplasmic and nucleolar extracts be routinely monitored for purity by Western blot analysis with appropriate subcellular compartment markers (Supporting Information Fig. 1).

The dramatic improvement in both the efficiency and the reproducibility of protein extraction from purified nucleoli provided by this new extraction protocol offers the opportunity to characterize protein–protein interactions throughout this organelle, bearing in mind that certain lower affinity interactions may not be preserved under these conditions. An original attempt to identify nucleolar protein interaction partners for the ubiquitous serine/threonine PP1 was clearly hindered by our inability to efficiently extract nucleolar proteins using our original RIPA sonication method. PP1, which shows an accumulation in nucleoli (Fig. 3A), accounts for a large fraction of the nucleolar serine/threonine phosphatase activity [18] and is therefore likely to be involved in a wide range of nucleolar functions, including regulation of ribosome biogenesis at more than one stage. Thus, it was surprising at the time that we only identified/...
quantified 72 proteins in an initial quantitative proteomic screen of nucleolar GFP-PP1γ, which represented only a small fraction of total nucleolar protein and did not highlight any obvious functional multiprotein complexes. Subsequent SDS-PAGE analysis of soluble versus insoluble fractions revealed the overall poor extraction efficiency of the technique employed (Fig. 1B), whereas quantitative Western blot analysis of both endogenous PP1γ and GFP-tagged PP1γ stably expressed in HeLa cells confirmed that we only extracted a fraction (~40%) of the total nucleolar PP1γ (Fig. 3B). Thus, the original data set did not represent the full spectrum of PP1 complexes throughout the nucleolus. We are thus pleased to note that our new optimized extraction method, which releases >75% of total HeLa cell nucleolar protein (Fig. 2E), also releases nearly all of the nucleolar PP1γ (Fig. 3B), which can be efficiently depleted for interactome analyses (Fig. 3C).

We repeated our quantitative Stable isotope labeling by amino acids in cell culture (SILAC)-based IP of GFP-PP1γ from purified HeLa cell nucleoli (Fig. 3A), using parental HeLa cell nucleoli as our built-in negative control. The experiment was carried out as described previously [1], with parental HeLa cells grown in DMEM containing 13C-Arginine and Lysine ("light" media) and HeLa cells in DMEM containing 13C-Arginine and D4-Lysine ("heavy" media) for six passages. Cells were harvested, nucleoli purified and nucleolar extracts prepared as described above. Equivalent total protein amounts of nucleolar extract were used for the control and experimental IPs. Extracts were precleared by incubation with 10 μL of Sepharose beads alone for 30 min at 4°C and transferred to 50 μL of the highly efficient affinity reagent GFP-Trap_A K (Chromotek) [1, 19] for a 1 h incubation at 4°C. Beads were washed once with 1 mL of ice-cold RIPA buffer, and then control and experimental beads for each condition carefully combined and washed twice with 1 mL ice-cold RIPA buffer. Bound proteins were eluted, reduced and alkylated, and then separated by 1-D SDS-PAGE as described previously [1]. Gels were Comassie stained and destained overnight prior to excision of slices. Peptides resulting from in-gel digestion with trypsin (Promega) were extracted from the gel slices for automated LC-MS/MS analysis as described previously [1], using an LTQ-Orbitrap mass spectrometer system (ThermoElectron). A combination of the MaxQuant quantitation software [20] with the MASCOT search engine v 2.2 (Matrix Science) and the International Protein Index human protein database version 3.37 (69,290 proteins, to which 175 commonly observed contaminants and all the reversed sequences had been added) was used for protein identification/quantitation.

By comparing the ratio of heavy:light Arg and/or Lys for each protein, we can readily identify environmental contaminants (ratio <1); all peptides contain only 13C/1H forms of Arg and Lys), proteins that bind nonspecifically to the affinity matrix in both our control and PP1γ IP (ratio = 1:1) and proteins that are enriched specifically in our PP1γ IP (ratio >1), either binding PP1γ directly or associating with it indirectly as part of a larger protein complex. In this experiment, >800 proteins were identified/quantified. It should be noted that although this was due in large part to our improved protein extraction method, there has also been an improvement in the resolution and sensitivity of the mass spectrometer employed in these experiments.

When plotted as enrichment (log SILAC ratio) versus abundance (log peptide intensity), the data show that a large number of nucleolar proteins bind nonspecifically to the affinity matrix (i.e. log SILAC ratio ~0), as we have demonstrated previously for whole cell, cytoplasmic and nuclear extracts [1, 2]. Proteins that are enriched in the PP1 IP include PP1γ itself, as expected (Fig. 3D). In addition, three proteins known or believed to be targeting subunits for PP1, based on the presence of a consensus PP1 binding motif [21] were identified (Fig. 3D). This provides the first evidence for interaction of these proteins with PP1 in the nucleolus.

We also found several groups of related nucleolar proteins with similar enrichment profiles. As it is unlikely that all of these proteins bind PP1 directly (few possess putative binding motifs), we hypothesize that PP1 is targeted to these complexes, perhaps by as yet unidentified targeting subunits, and that these proteins copurify with PP1 as multiprotein complexes. As shown in Figs. 3D and E, they include protein subunits shared by the RNase P and MRP complexes (Fig. 3E), which are involved in maturation of tRNA and processing of precursor RNA, respectively [22], and the four core protein members of H/ACA ribonucleoprotein complexes [23]. PP1γ also copurified all of the core protein members of the RNA exosome complex (Fig. 3F), including those that form both the cap structure and the PHe ring [24]. Taken together, these results demonstrate the suitability of our nucleolar extraction method for the analysis of protein–protein interactions by quantitative proteomics, and provide us with a unique insight into potential nucleolar roles for an essential mammalian phosphatase.

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References


