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A high-efficiency cellular extraction system for biological proteomics

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Abstract

Recent developments in quantitative high-resolution mass spectrometry have led to significant improvements in the sensitivity and specificity of biochemical analyses of cellular reactions, protein-protein interactions, and small molecule drug discovery. These approaches depend on cellular proteome extraction that preserves native protein activities. Here, we systematically analyzed mechanical methods of cell lysis and physical protein extraction to identify those that maximize the extraction of cellular proteins while minimizing their denaturation. Cells were mechanically disrupted using Potter-Elvehjem homogenization, probe or adaptive focused acoustic sonication, and in the presence of various detergents, including polyoxyethylene ethers and esters, glycosides, and zwitterions. Using fluorescence spectroscopy, biochemical assays, and mass spectrometry proteomics, we identified the combination of adaptive focused acoustic (AFA) sonication in the presence of binary poloxamer-based mixture of octyl-β-glucoside and Pluronic F-127 to maximize the depth and yield of proteome extraction while maintaining native protein activity. This binary poloxamer extraction system allowed native proteome extraction, comparable in coverage to proteomes extracted using denaturing SDS or guanidine containing buffers, including efficient extraction of all major cellular organelles. This high-efficiency cellular extraction system should prove useful for a variety of cell biochemical studies, including structural and functional proteomics.

Keywords

cell lysis; protein extraction; binary detergent; adaptive focused acoustics

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information:

Table 1: Protein extraction efficiency and activity of Jurkat-GFP cells lysed in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5 supplemented with 1% detergents and binary 1% detergents.

Table 2: List of peptides identified in all proteomic analysis.

Table 3: List of proteins identified in all proteomic analysis.

Supp. Fig. 1: Comparison of unique peptides identified using two-step versus one-step trypsin protein digestion.

Introduction

Current high-resolution mass spectrometry enables nearly comprehensive mapping of cellular proteomes[1–3], including analysis of post-translational modifications that mediate a variety of biological processes.[4–5] Recently, mass spectrometry proteomics has also been used to improve the sensitivity of drug target discovery[6–7] and analysis of protein-protein interactions.[8] Due to the requirement of purified molecular species for analysis, all of these methods depend on the efficiency of cell disruption and protein extraction techniques that maintain native protein activity and structure.

A variety of mechanical and physical methods have been used for cell lysis and protein extraction. Traditional mechanical lysis methods include milling, cryogenic lysis, sonication and hydrodynamic shearing.[9] While these methods are robust in implementation, they require extended sample preparation time, and can suffer from potential contamination from non-disposable instruments and limited reproducibility due to requirements for manual operation.[10–11] In addition, these techniques are difficult to implement for specimens with small volumes, and some techniques such as probe sonication can create localized heating within the sample, which can lead to variable protein denaturation and aggregation.[12] Recently, adaptive focused acoustic sonication (AFA) has been developed to allow precise, non-contact, isothermal delivery of acoustic energy. AFA utilizes a concave transducer to focus emitted acoustic energy inside a processing vessel without direct contact.[13] Given a sufficiently large water bath, this technology maintains isothermal conditions, which is particularly advantageous for biological marcomolecules.[14]

To improve the efficiency of protein extraction, mechanical cell disruption is often augmented by the inclusion of detergents which disrupt cellular and organellar lipid membranes.[15] Commonly used approaches have included ionic, zwitterionic, and non-ionic species such as polyoxyethylene ethers, polyoxyethylene esters, and glycosides.[16] Efficiencies of these protein extraction systems often depend on the cell type and extraction scale, and consequently the choice of detergent for solubilization of a particular protein of interest usually requires empirical testing.[17] Most commonly, non-ionic detergents such as t-octylphenoxypolyethoxyethanol (Triton X-100) and octylphenoxypolyethoxyethanol (NP-40) are used to lyse cells and extract proteins. Although these detergents minimize protein denaturation, they are less efficient than ionic detergents such as sodium dodecyl sulfate (SDS).[18] Finally, all of these buffer systems tend to be biased towards cytosolic proteins, and their use for comprehensive proteome analysis requires formulation of heterogeneous buffer systems and specialized extraction methods.[19] There is some evidence for superior performance of binary detergent systems, but they have not been systematically studied for cell lysis and extraction.[20–22]

Here, we sought to determine the mechanical and physical cellular disruption methods that maximize the extraction of cellular proteins while minimizing their denaturation. We reasoned that a high efficiency mechanical disruption method, combined with an optimized detergent system, will permit efficient native proteome extraction.

Experimental Section

Reagents

All reagents were of ACS grade, and obtained from Thermo Scientific, unless noted otherwise. Pluronic F-127 and NDSB-195 were obtained from Santa Cruz (Dallas, TX).

Cell culture

Cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Brunswick, Germany). All cells but retinal pigment epithelial (RPE) were cultured in RPMI-1640 medium (Invitrogen, Grand Island, NY) supplemented with penicillin/streptomycin and 10% fetal bovine serum. RPE cells were cultured in DMEM (Invitrogen, Grand Island, NY) medium supplemented with penicillin/streptomycin and 10% fetal bovine serum. Suspension and adherent cells were maintained at a density of 0.5–1 million cells/ml or 50–90% confluence, respectively, at 37 ºC in a humidified atmosphere with 5% CO_2. Jurkat cells engineered to express GFP were generated by retroviral transduction as described.[23–24] Cell counts were measured using the Neubauer hemocytometer (Hausser Scientific, Horsham, PA), according to manufacturer's instructions.

Cell lysis and protein extraction

Harvested cells were washed once with cold phosphate-buffered saline solution, and frozen at −80 ºC for 12 hours. Thawed cells were resuspended in cold 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 μM tris-carboxyethyl phosphine (Lysis buffer), supplemented with 1% (v/v) detergents as indicated or with 6 M guanidine hydrochloride (GdmCl). For comparison of mechanical disruption methods, suspensions of 100 million cells in 1.8 ml of lysis buffer were processed by thawing at room temperature for 30 minutes, or using 20 strokes of the 2 ml Potter-Elvehjem homogenizer on ice (Corning, Corning, NY), or disruption using 2 mm sonication probe at 10% power for 600 seconds at 4 ºC using the Misonix 3000 probe sonicator (QSonica, Newtown, CT), or disruption using the E210 adaptive focused acoustic sonicator (Covaris, Woburn, CA) and 13×65 mm tubes operating in the frequency sweep mode at 1000 Hz, 20% duty cycle, and intensity 8 for 600 seconds at 4 ºC, or incubation at 95 ºC for 5 min in the lysis buffer supplemented with 1% SDS. All lysates were clarified by centrifugation at 16,000 *g* for 10 minutes at 4 ºC. Protein concentration was measured using the bicinchoninic acid assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Protein purification

Extracted proteins were precipitated using chloroform and methanol,[25] and precipitated proteins were dissolved in 150 μl of 0.1% (w/v) RapiGest (Waters, Milford, MA) in 50 mM ammonium bicarbonate, pH 8.5 (ABC buffer) by incubating for 30 minutes at 37 ºC under continuous shaking. Proteins that were extracted using 6 M guanidine hydrochloride were diluted 6-fold with ABC buffer to final guanidine concentration of 1 M. Solubilized proteins were reduced with 5 mM dithiothreitol by incubation for 30 minutes at 56 ºC, and alkylated with 15 mM iodoacetamide by incubation at room temperature for 30 minutes in the dark. Proteins were subsequently digested with 2 μg lysyl-C endopeptidase (Wako chemicals,

Richmond, VA) by incubation at 37 ºC for 6 hours, followed by digestion with 4 μg of porcine trypsin (Promega, Madison, WI) at 37 ºC for 18 hours. RapiGest was removed by hydrolysis with 200 mM HCl at 37 ºC for 45 minutes and centrifugation at 16,000 × *g* for 20 minutes. Tryptic peptides were purified by reverse phase chromatography using C_{18} SpinTips (Nest Group, Southborough, MA) according to the manufacturer's instructions, and concentrated using vacuum centrifugation.

Nanoelectrospray ionization liquid chromatography tandem mass spectrometry

Tryptic peptides were dissolved in 0.1% (v/v) formic acid in water and were resolved using reverse phase nanoflow liquid chromatography (Ekspert nanoLC 425, Eksigent, Redwood city, CA), as coupled to the Orbitrap Fusion mass spectrometer (Thermo, San Jose, CA). We used trap-elute chromatography using a trap column fabricated from 4 cm × 150 μm internal diameter fused silica capillary (Polymicro Technologies, Phoenix, AZ) with a 2 mm silicate frit and packed with Poros R2-C18 10 μm particles (Life Technologies, Norwalk, CT), as described.[26–27] The analytical column consisted of a 25 cm × 75 μm internal diameter integrated electrospray emitter column (New Objective, Woburn, MA), packed with ReproSil-Pur C18-AQ 1.9 μm particles (Dr. Maisch, Ammerbuch-Entringen, Germany). Two micrograms of peptide mixtures were loaded onto the trap column at 5 μl/min, and washed with 10 column volumes of 0.1% formic acid in water. Peptides were resolved over 90 minutes using a 5% to 35% linear gradient of acetonitrile in 0.1% formic acid. Eluting peptides were ionized using DPV-565 PicoView ion source (New Objective, Woburn, MA) operating at 1700 V. Precursor ion scans were recorded from 400–2000 m/z in the Orbitrap at a resolution of 120,000 at m/z 200 with automatic gain control target of 1×10^5 ions and maximum injection time of 50 ms. We used data-dependent mass spectral acquisition with monoisotopic precursor selection (5 ppm tolerance), charge ion selection (2–7), dynamic exclusion (30 sec), HCD fragmentation (normalized collision energy 32, isolation window 1 Th) using the top speed algorithm with a duty cycle of 3 sec, which generated 15 fragment ion scans for every precursor scan on average.[28] Product ion spectra were recorded in the linear ion trap with an automatic gain control target of 1×10^4 ions and maximum injection time of 150 ms.

Recorded mass spectra were searched against the UniProt human reference proteome (20,300 sequences, version 04-2014) using Byonic version 2.2.9 (Protein Metrics, Palo Alto, CA).[29] Cysteine carbamidomethylation was set as fixed modification and the following variable modifications were allowed: acetylation (N-terminus, lysine and arginine residues), oxidation (methionine), methylation (arginine and lysine) and phosphorylation (tyrosine, threonine and serine residues). Searches were conducted with up to two missed cleavages, and 5 ppm and 0.5 Da mass tolerances for precursor and fragment ions respectively, with of 1% protein FDR or 20 decoy proteins.[29] Gene Ontology (GO) annotations terms from the protein data repository at NCBI were used to establish cellular locations using Scaffold Q+ version 4.4.1.1. (Proteome Software, Portland, OR).[30] The raw and processed mass spectrometry proteomics data are available from PeptideAtlas with the accession number PASS00669 (http://www.peptideatlas.org/).

Results and Discussion

To develop a general approach for native proteome extraction, we sought to identify cellular disruption methods that maximize the number and variety of extracted proteins while preserving their native functional activities. We engineered the commonly used Jurkat human leukemia cells to express green fluorescent protein (GFP), whose fluorescence can be readily measured in cellular lysates, thereby permitting rapid screening of various methods. To establish baseline native protein activity of cellular lysates, we subjected cells to freeze-thawing, and measured the total extracted protein and fluorescence of GFP (Fig. 1a & b). Lysis of cells in the presence of sodium dodecyl sulfate (SDS) led to significant increase in the total protein extracted, but expectedly caused protein denaturation, as evidenced by the loss of GFP fluorescence. Pestle homogenization and focused acoustic sonication (AFA), but not probe sonication, enhanced protein extraction while maintaining native protein activity in the absence of detergents (Fig. 1a & b).

To test the ability of detergents to enhance protein extraction while preserving native protein activity, we lysed cells using focused acoustic sonication because of its automation and high reproducibility, as well as isothermal conditions and homogeneous energy transfer that minimize local heating and protein denaturation. We selected to study fifteen detergents, which span most chemical classes (Supp. Table 1). Cells were lysed in an in isotonic neutral buffer, supplemented with detergents dissolved at 1% concentration, chosen to be uniformly above their critical micellar concentrations (Supp. Table 1). In agreement with prior studies, we found that the addition of non-ionic detergents NP-40 and octyl-β-glucoside (OBG) significantly increased protein extraction yield and activity, respectively, as compared to samples lysed with isotonic buffer using AFA alone (Supp. Table 1).

Since binary mixtures of detergents have been used to extract and crystallize membrane proteins,[31] we analyzed the performance of mixtures of detergents that exhibited superior protein extraction efficiency with those that demonstrated enhanced protein stabilization (Fig. 1c, Supp. Table 1). Thus, we tested the binary combinations of NP-40 and Pluronic F-127 (Pluronic) with OBG, MEGA-8 or NDSB-195 (Supp. Table 1). We found that the combination of Pluronic F-127 and OBG exhibited significantly higher protein extraction efficiency, as compared to each detergent alone (Fig. 1c). Importantly, this combination maintained high native protein activity, as measured using GFP fluorescence (Fig. 1d). We confirmed that this effect was not limited to GFP fluorescence by measuring the activity of cellular reductase enzymes, which was similarly preserved by the combination of Pluronic and OBG (Supp. Table 1).

Having established that in Jurkat cells the combination of Pluronic F-127 and OBG maximizes total protein extraction while maintaining native protein activities, we sought to confirm that this effect is generalizable to other mammalian cells. Thus, we lysed and extracted proteins from cell lines derived from cervical carcinoma (HeLa S3), retinal pigment epithelium (RPE), and acute myeloid leukemia (OCI-AML2) cells. Since many detergents, such as Pluronic F-127 and SDS are not compatible with MS analysis, we used organic phase extraction to remove detergents from purified proteins. To maximize the generation of tryptic peptides for mass spectrometric analysis, we used a two-step digestion

method using Lys-C endopeptidase followed by trypsin digestion (Supp. Fig 1). Tandem mass spectrometry and statistical database matching were then used to identify unique peptides and their corresponding proteins (Fig. 2a & b). For all cells studied, we found that similar numbers of proteins and peptides were extracted by SDS and native Pluronic F-127 and OBG (Fig. 2a & b). We confirmed that the observed depths of proteome coverage were not limited by organic phase extraction by analyzing proteomes extracted directly using 6 M guanidine hydrochloride or RapiGest without chloroform-methanol protein precipitation (Fig. 2a & b).

Similar efficiencies of extraction were observed for the post-translationally phosphorylated, acetylated, and methylated proteins between native Pluronic F-127 and OBG and denaturing extraction conditions (Fig. 2c). In addition, we found that proteins extracted by the denaturing SDS were also efficiently extracted by the native Pluronic F-127 and OBG (Fig. 2d). Importantly, native protein extraction using Pluronic F-127 and OBG captured proteins from a diverse set of subcellular structures and organelles, as assessed using Gene Ontology (GO) annotation analysis (Fig. 3). Thus, the binary combination of Pluronic F-127 and OBG maximizes the apparent depth and yield of native proteome extraction of mammalian cells as compared to conventional methods.

In summary, using a systematic analysis of cell lysis and proteome extraction methods, we optimized an extraction system that maximizes proteome yield while maintaining native protein activities. This approach is based on the combination of a non-ionic triblock copolymer (Pluronic F-127) and octyl-β-glucoside to extract proteins from cells disrupted by focused acoustic sonication. Though both the commonly used non-ionic extraction detergent NP-40 and Pluronic F-127 are polymers of ethylene oxide, the apparently superior performance of Pluronic F-127 suggests that triblock polymerization of propylene and ethylene oxide may be responsible for its enhanced miscibility of cellular structures.

Poloxamer-based detergents such as Pluronic F-127 have been observed to increase the miscibility of substances with different hydrophobicity and to suppress protein aggregation,[32–33] suggesting that its unique physical properties and micellar phase formation may be responsible for the high efficiency as a native proteome extraction system.[34–35] In addition, the use of focused acoustic sonication that minimizes inhomogeneous energy transfer and local heating likely acts to reduce protein denaturation during cell disruption.[36]

We anticipate that the generation of deep native proteomes using this improved extraction system will prove useful for structural and chemical proteomics, drug target discovery, and studies of cellular biochemistry and protein organization. While we assessed native protein activity using GFP fluorescence and mitochondrial dye reduction assays, it is possible that the described extraction system may interfere with the function of specific proteins. Similarly, though we observed comparable coverage of cellular proteomes extracted using octyl-β-glucoside and Pluronic F-127 and denaturing systems such as SDS or guanidine, it is possible that certain less abundant protein classes were not extracted. Future studies will aim to determine the physical mechanisms of protein stabilization and cellular extraction mediated by binary poloxamer-based detergent mixtures, and optimize them for use in specific applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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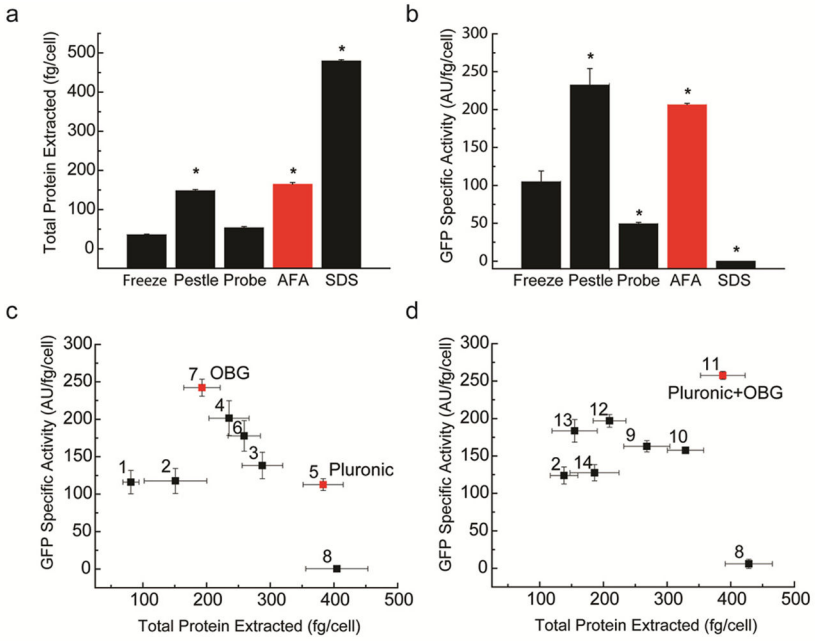

Fig. 1.

Focused acoustic sonication and pestle homogenization preserve native protein activity during cellular disruption. Total protein extracted (a) and GFP fluorescence (b) from Jurkat-GFP cells disrupted using freeze-thaw (Freeze), pestle homogenization (Pestle), probe sonication (Probe), focused acoustic sonication (AFA), or boiling in the presence of 1% sodium dodecyl sulfate (SDS). Addition of poloxamer (Pluronic F-127) and octyl-β-glucoside (OBG) is superior to conventionally used detergents and maximizes native protein. GFP fluorescence of lysates as a function of total protein extracted for Jurkat-GFP cells disrupted using focused acoustic sonication in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, and supplemented with 1% individual detergents (c), or binary mixtures of 1% detergents (d), as labeled. Complete lists of detergents are listed in Supp. Table 1. Lysis in 1% SDS (SDS) serves as the positive control for total available cellular protein, and freeze-thaw in the absence of detergents (Freeze) is the control for unperturbed GFP fluorescence activity. Error bars represent standard deviations of three biologic replicates. * $p < 0.01$ for two-tailed *t*-test as compared to AFA and SDS.

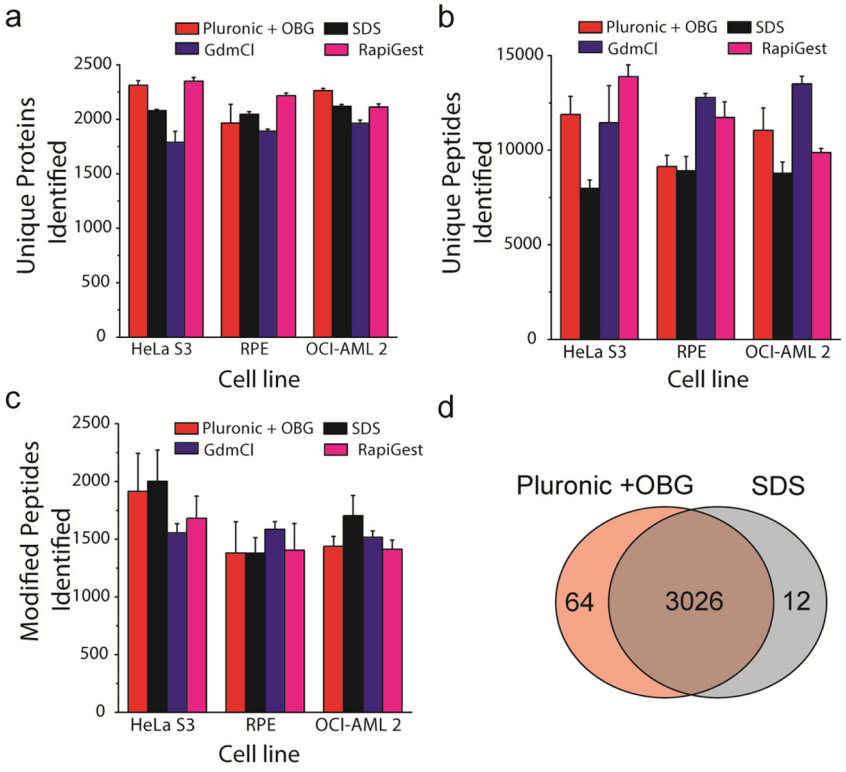

Fig. 2.

Native proteome extraction from diverse mammalian cell types using focused acoustic sonication of cells lysed in admixture of poloxamer and octyl glucoside. (a) Unique proteins and (b) unique peptides identified. (c) Modified peptides identified in unfractionated lysates of cervical carcinoma (HeLa S3) cells, retinal pigment epithelium (RPE), acute myeloid leukemia (OCI-AML2) cells, lysed in the mixture of 1% poloxamer and octyl glucoside (Pluronic + OBG, red) as compared to 1% SDS (SDS, black), 6 M guanidine hydrochloride (GdmCl, purple) and RapiGest (RapiGest, pink). (d) Venn diagram showing the overlap of unique proteins identified from cells, lysed in the mixture of 1% poloxamer and octyl glucoside (Pluronic + OBG, red) as compared to 1% SDS (SDS, black). Error bars represent standard deviations of three technical replicates.

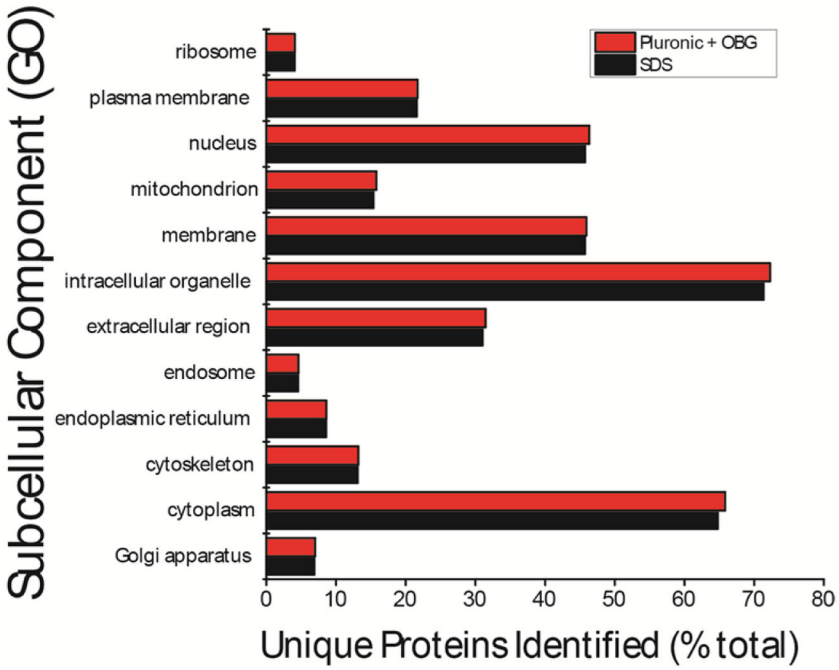

Fig. 3.

Unique proteins identified as a function of Gene Ontology (GO) annotated subcellular locations, in unfractionated lysates of cervical carcinoma (HeLa S3) cells, retinal pigment epithelium (RPE), acute myeloid leukemia (OCI-AML2) cells, lysed in the admixture of 1% poloxamer and octyl glucoside (Pluronic + OBG, red) as compared to 1% SDS (SDS, black).