Subcellular protein fractionation to enhance proteomic coverage of cultured cells

Reduce complexity of cell lysates while optimizing proteomic coverage

Protein localization is traditionally studied microscopically using immunofluorescence or fluorescent fusion-proteins. Biochemical isolation of various cellular compartments or organelles typically utilizes density gradients or differential centrifugation, followed by western blotting of 1D- or 2D-polyacrylamide gels. These approaches are limited by their need for detection antibodies against specific proteins of interest and marker proteins. Alternatively, subcellular proteomic analysis by mass spectrometry not only provides information about the subcellular location of a specific protein, but also reveals global patterns of protein expression in each specific subcellular compartment. Here, we demonstrate a biochemical, detergent-based protein fractionation approach combined with high-resolution mass spectrometry to examine a broader spectrum of proteins in cytoplasmic, membrane, nuclear, chromatin-bound, and cytoskeleton fractions derived from HeLa cells.

Separation of proteins by subcelluar localization is one of the methods to enrich for proteins while maintaining some biological context. Differential detergent extraction is a classic biochemical technique used to separate proteins based on sequential solubilization of cellular compartments using different detergents [1] (Figure 1). The Thermo Scientific™ Subcellular Protein Fractionation Kit provides a combination of reagents for stepwise lysis of cells into cytoplasmic, membrane, nuclear-soluble, chromatin-bound, and cytoskeletal protein fractions that are functional. As this fractionation method does not require preparation of density gradients or ultracentrifugation, fractionation is more easily accomplished using low-speed microcentrifugation in less time and with high reproducibility. Extracts from each subcellular compartment generally have less than 15% contamination between fractions, which provides sufficient purity for most experiments studying protein localization and redistribution.

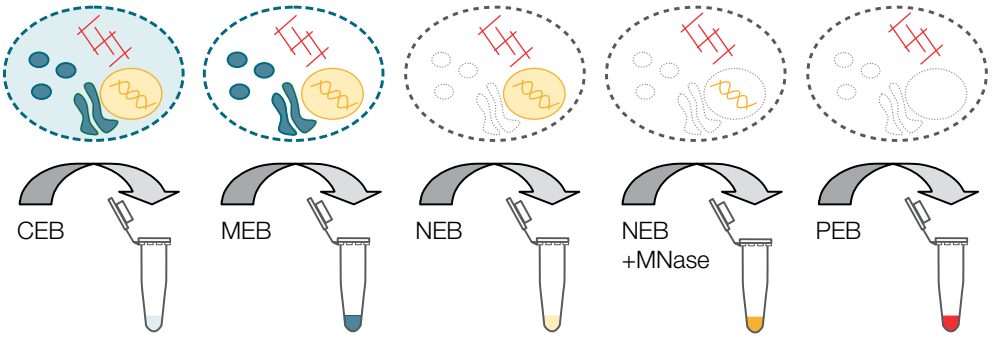

Figure 1. Schematic overview of the subcellular fractionation procedure. Cellular compartments in incubating cells are sequentially extracted with cytoplasmic extraction buffer (CEB) followed by membrane extraction buffer (MEB) and nuclear extraction buffer (NEB). Adding micrococcal nuclease (MNase) to NEB extracts chromatin-bound proteins from the cell pellet before adding the pellet extraction buffer (PEB) to solubilize cytoskeletal proteins.

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Results and discussion

Characterization of subcellular protein fractions by western blot analyses

The Subcellular Protein Fractionation Kit for Cultured Cells contains four extraction buffers, a stabilized nuclease, and Thermo Scientific™ Halt™ Protease Inhibitor Cocktail. Each kit has enough reagents to fractionate 50 cell pellets of 20 µL each, equivalent to approximately 2 g of cell paste.

Using the Subcellular Protein Fractionation Kit for Cultured Cells, HeLa cells were separated into five distinct protein fractions. Western blotting for various marker proteins in the fractions confirms localization of target proteins associated with different cellular compartments (Figure 2). Notably, the membrane extract was found to contain proteins associated with plasma membranes (EGFR), mitochondrial lumen (VDAC), and endoplasmic reticulum lumen (calreticulin). In addition, pellet fractions showed enrichment for cytoskeletal proteins (vimentin and cytokeratin 18), but tubulin was observed most abundantly in the cytoplasmic fraction (data not shown).

Characterization of subcellular protein fractions by mass spectrometry

In order to identify additional proteins that fractionate with known marker proteins, subcellular protein fractions were analyzed separately using label-free mass spectrometry (MS) and also labeled with Thermo Scientific™ Tandem Mass Tag™ 6-plex (TMTsixplex™) reagents for MS/MS quantitation (Figure 3). MS results from label-free quantitation (Figure 4) and TMTsixplex reagents (Figure 5) correlated well with western blot analysis. Overall, more than 40,000 unique peptides corresponding to 5,337 proteins were identified from the combined subcellular protein fractions, which were approximately 2.5-fold greater than unfractionated whole-cell lysate (Figure 6).

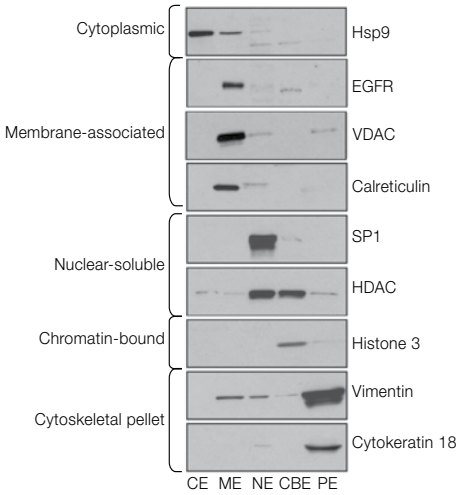

Figure 2. Western blot analysis of protein fractions using compartment-specific antibody markers. CE = cytoplasmic extract, ME = membrane extract, NE = nuclear extract, CBE = chromatin-bound extract, PE = pellet extract.

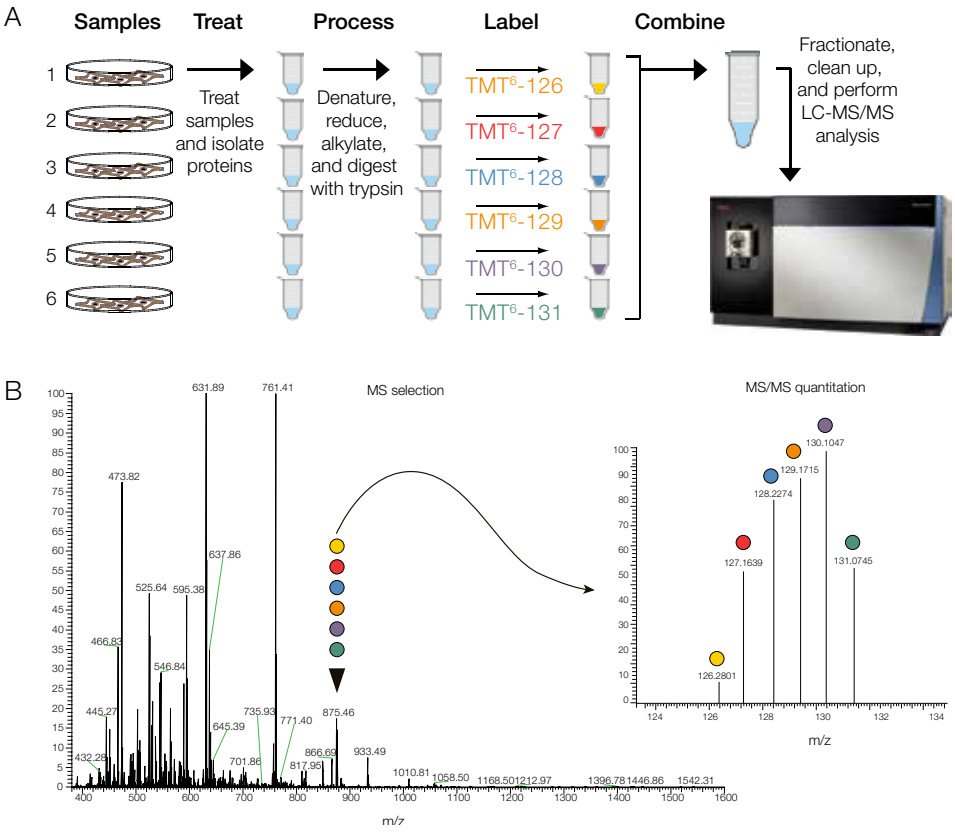

Figure 3. Schematic sample preparation with TMTsixplex reagent and LC/MS analysis. **(A)** Whole-cell extracts and subcelluar protein fractions from a HeLa cell line are reduced, alkylated, and digested before being labeled with TMTsixplex reagents. Labeled peptides from each treatment condition are combined, fractionated by high-pH, reversed-phase liquid chromatography (LC) and analyzed by LC/MS. **(B)** Peptides labeled with TMTsixplex reagents coelute during MS acquisition but generate unique reporter ion masses during MS^n for relative quantitation.

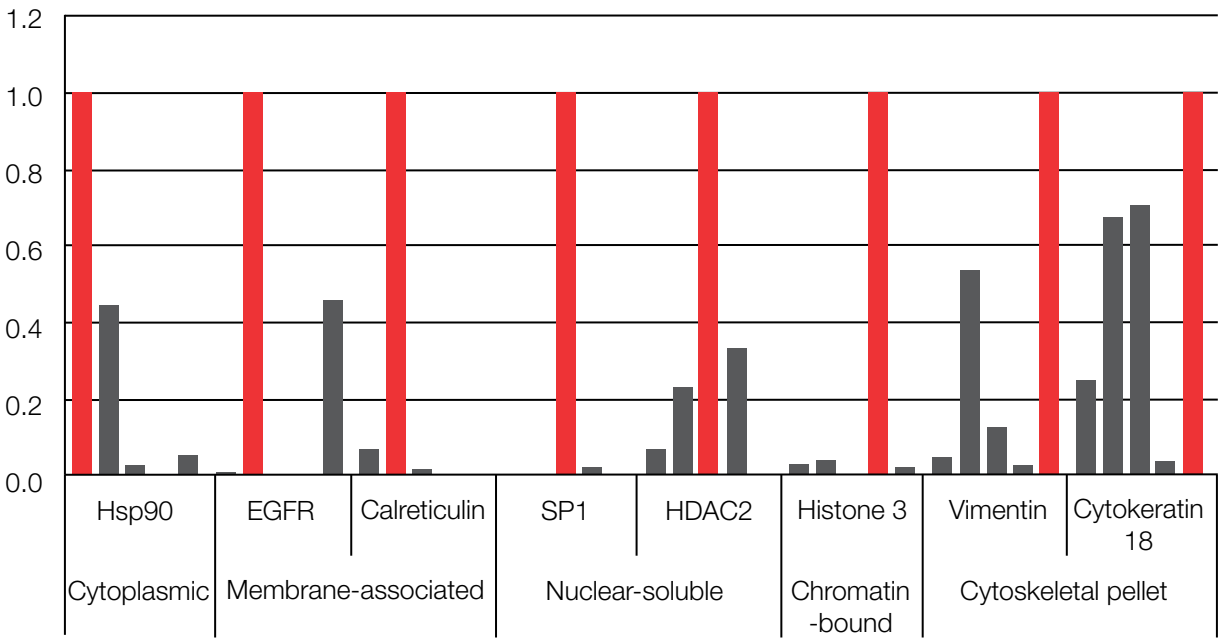

Figure 4. Analysis of protein fractions using label-free quantitation of compartment-specific marker proteins. Normalized, integrated average peak area for each fraction is shown. The red bars show normalized intensity of peptide peak area for each marker protein in different subcellular compartments. The gray bars indicate the relative intensity of marker proteins found in other fractions.

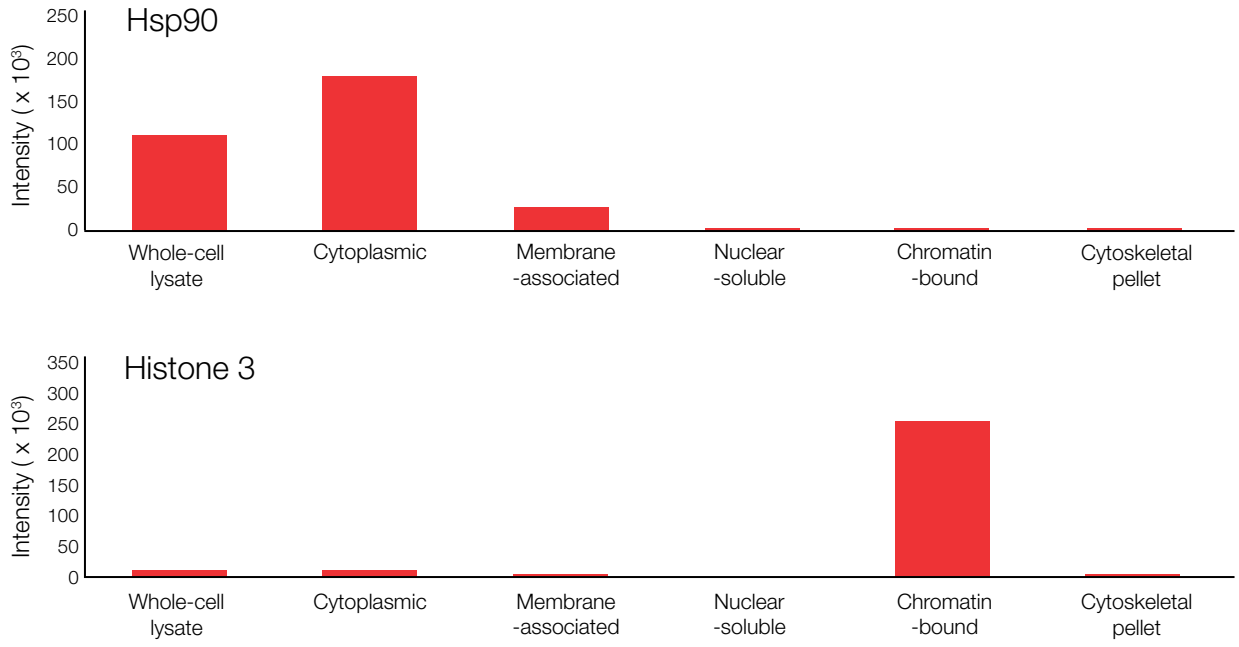

Figure 5. Representative quantitation of protein enrichment of marker proteins (Hsp90 and histone 3) using TMTsixplex reagents in different subcellular fractions relative to whole-cell lysate. The red bars show the relative intensity of TMT reagent reporter ions for unique marker proteins.

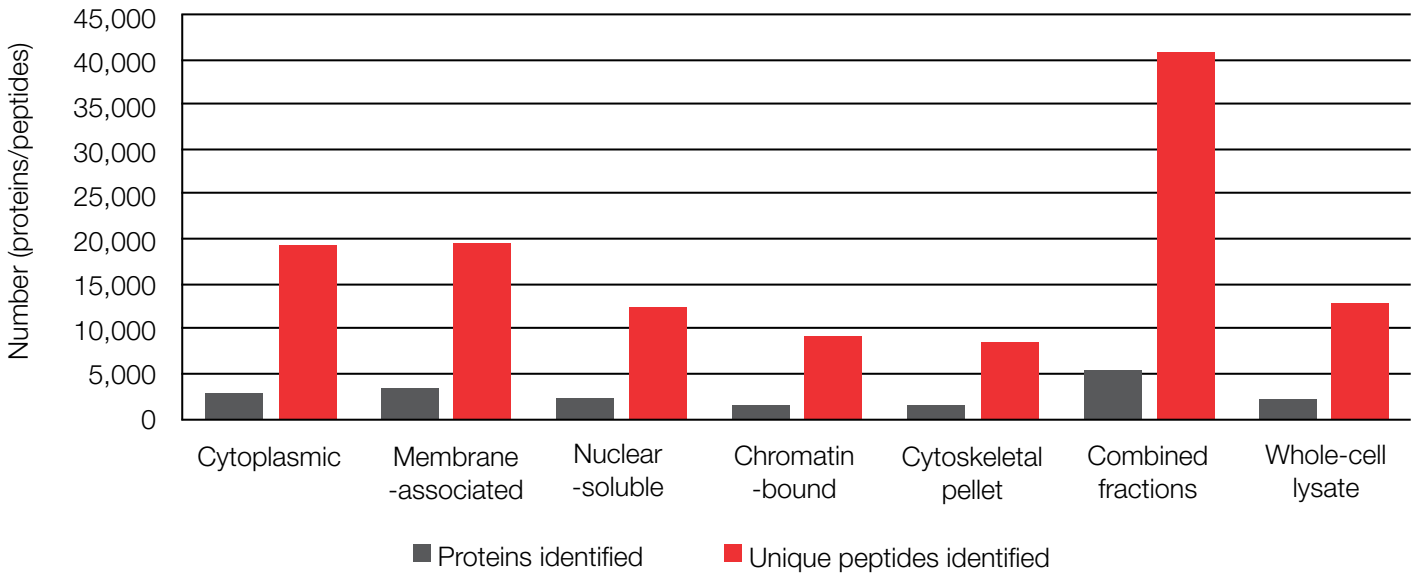

Figure 6. Number of proteins (indicated by gray bars) and peptides (indicated by red bars) identified from in-gel digests of subcellular protein fractions and whole-cell lysate.

Gene ontology analysis of subcellular protein fractions

Annotation of proteins by cellular compartment revealed significant enrichment for canonical gene ontology (GO) terms (Table 1). However, as most proteins are annotated for multiple subcellular locations, some terms did not show as much enrichment as others. In particular, proteins found in the chromatin-bound and cytoskeletal pellet fractions correlated less with the subcelluar GO terms.

Further analysis of proteins by function revealed significant increases in additional protein groups for different fractions (Table 2). Notably, the membrane-associated fraction showed enrichment for mitochondrial proteins and the pellet fraction showed enrichment of lipid raft–associated proteins.

Table 1. Gene ontology (GO) annotation enrichment of subcellular protein fractions. Average integrated peak area and standard deviation for each identified protein was calculated for individual fractions and compared to whole-cell lysate. Standard scores [(fraction area–average total area) / standard deviation] and fold change [(natural log (area for fraction / average total area)] were calculated for each fraction. Proteins with a standard score of >0.6 and fold change >0.5 were considered enriched. The enriched proteins for each fraction were annotated for cellular compartments using a UniProt database and canonical GO terms.

Fraction	No. of protein enriched	GO term	Proteins annotated	GO term matched (%)
Cytoplasmic	1,291	GO:0005737 (cytoplasm)	1,089	84.4
Membrane-associated	2,088	GO:0016020 (membrane)	1,374	65.8
Nuclear-soluble	882	GO:0005634 (nucleus)	718	81.4
Chromatin-bound	329	GO:0003682 (chromatin binding)	38	11.6
Cytoskeletal pellet	257	GO:0005856 (cytoskeleton)	62	24.1

Table 2. Additional GO annotation terms enriched in subcellular protein fractions compared to whole-cell lysate.

Fraction	GO term	Fold change
Cytoplasmic	COP9 signalosome	4.1
	BRISC complex	3.2
	SNARE complex	3.2
	BRCA1-A complex	3.2
	Prefoldin complex	3.2
Membrane-associated	Mitochondrial ribosome	7.8
	Mitochondrial respiratory chain complex 1	6.2
	SNARE complex	5.9
	Mitochondrial small ribosomal unit	5.4
	Mitochondrial large ribosomal unit	4.9
	Mitochondrial respiratory chain complex I	5.1
Nuclear-soluble	Histone methyltransferase complex	4.3
	Core transcription factor II human (TFIIH) complex	3.6
	TFIIH complex	3.6
	Transcriptional repressor complex	3.3
Chromatin-bound	Smc5–Smc6 complex	7.1
	Chromocenter	7.1
	MIS12/MIND type complex	6.0
	U2 snRNP	4.8
	Condensed nuclear chromosome, centromeric region	4.8
	Chromocenter	6.9
Cytoskeletal pellet	Ragulator complex	4.7
	Vacular protein-transporting V-type ATPase complex	4.7
	Mitochondrial inner membrane presequence translocase complex	4.7
	Heterotrimeric G-protein complex	4.5

Conclusions

We have developed an optimized protocol and kit of reagents that provide reproducible separation of proteins by subcellular localization. This simple, convenient protocol reduces sample complexity and generates protein fractions compatible with downstream techniques such as protein assays, western blotting, electrophoretic mobility shift assays, reporter-gene and enzyme-activity assays, and as demonstrated here, mass spectrometry. Although the protein fractions are not directly MS compatible due to the use of various buffer components, a short, gel-based purification enables removal of interfering compounds for digestion and labeling with TMTsixplex reagents. Reproducible fractionation at the protein and peptide level enables deeper proteomic coverage and more precise assignment of protein localization—increasing protein identifications while maintaining biological context.

Labeling the subcellular fractions with TMTsixplex reagents resulted in measurment of relative protein expression ratios that correlated well with label-free quantitation by MS and western blot analyses. GO annotation analysis showed significant enrichment for identified proteins in each fraction and assignment of additional protein functional groups to subcellular protein fractions based on detergent solubility.

References

1. Walker JM, editor (2005) Differential detergent fractionation of eukaryotic cells, *The Proteomic Handbook*. pp. 37–48.
2. McAllister GC et al. (2014) MultiNotch MS3 enables accurate, sensitive, and multiplexed detection of differential expression across cancer cell line proteomes. *Anal Chem* 86(14):7150–7158.

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Methods

Sample preparation

HeLa cells were lyzed using 50 mM HEPES buffer at pH 8 and 1% SDS with sonication to generate a whole-cell extract (WCE) or processed using the Subcellular Protein Fractionation Kit for Cultured Cells (Cat. No. 78840) to generate cytoplasmic (CE), membrane (ME), nuclear-soluble (NE), chromatin-bound (CBE), and pellet (PE) extracts.

Each subcellular protein fraction was briefly separated by SDS-PAGE before in-gel reduction, alkylation, and digestion with trypsin. Peptides from each fraction were labeled with TMTsixplex reagents (Cat. No. 90064) and fractionated using the Thermo Scientific™ Pierce™ High pH Reversed-Phase Peptide Fractionation Kit (Cat. No. 84868) to generate eight fractions for LC/MS analysis. Label-free peptides from each fraction were also analyzed by LC/MS. Western blotting using antibodies against HSP90, EGFR, VDAC, calreticulin, SP1, histone 3, HDAC2, vimentin, and cytokeratin 18 was also performed.

Liquid chromatography

Samples were separated by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Thermo Scientific™ Dionex™ Ultimate 3000 system connected to a Thermo Scientific™ EASY-Spray™ column, 150 mm (L) x 0.075 mm (D) or a Thermo Scientific™ Acclaim™ PepMap™ C18 column, 150 mm (L) x 0.075 mm (D) over a 3 hr, 5–30% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at a 300 nL/min flow rate.

Mass spectrometry

Spectra were acquired using an Thermo Scientific™ Orbitrap Fusion™ mass spectrometer using top-speed Fourier transform MS (FTMS) full scan at 120,000 and $FTMS^2$ high energy collisional dissociation (HCD) at 30K resolution, or ion trap MS^2 ($ITMS^2$) collision-induced dissociation (CID) and $FTMS^3$ HCD (synchronous precursor selection (SPS) using 10 fragments from the MS^2 spectra) [2].

Data analysis

Spectral data files were analyzed using Thermo Scientific™ Proteome Discoverer™ 1.4 software using the SEQUEST™ HT search engine with a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 dalton. Carbamidomethylation (+57.021 daltons) for cysteine and TMT isobaric labeling (+229.162 daltons) for lysine and N-terminus residues were treated as static modifications with variable methionine oxidation (+15.996 daltons). Data were searched against a Swiss-Prot™ human database with a 1% false discovery rate (FDR) criteria using the Percolator algorithm. Proteome Discoverer 1.4 software was used to calculate TMT reporter ratios with mass tolerance of ±10 ppm without applying the isotopic correction factors. A protein ratio was expressed as a median value of the ratios for all quantifiable spectra of the peptides pertaining to that protein.

Editor's note

The data in this article were presented at the 2015 annual meeting of American Society for Mass Spectrometry in a poster titled: Protein Fractionation by Subcellular Location to Enhance Proteomic Coverage of Cultured Cells.

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