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Time Series Proteome Profiling

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Abstract

This chapter provides a detailed description of a method used to study temporal changes in the endoplasmic reticulum (ER) proteome of fibroblast cells exposed to ER stress agents (thapsigargin and thapsigargin). Differential stable isotope labeling by amino acids in cell culture (SILAC) is used in combination with crude ER fractionation, SDS–PAGE and LC-MS/MS to define altered protein expression in thapsigargin or thapsigargin treated cells versus untreated cells. Treated and untreated cells are harvested at different time points, mixed at a 1:1 ratio and processed for ER fractionation. Samples containing labeled and unlabeled proteins are separated by SDS–PAGE, bands are digested with trypsin and the resulting peptides analyzed by LC-MS/MS. Proteins are identified using Bioworks software and the Swiss-Prot data-base, whereas ratios of protein expression between treated and untreated cells are quantified using ZoomQuant software. Data visualization is facilitated by GeneSpring software. proteomics

Keywords

Time series; Proteome profiling; SILAC; LC-MS/MS; ER stress response; Subcellular

1. Introduction

Time series proteome profiling is a powerful approach for deciphering the molecular mechanisms of biological processes because this method allows for the tracking of both the quantitative and the dynamic aspects of complex protein networks. Although the changes in protein expression and trafficking that occur over time can be assessed via a proteomic approach, it is almost impossible to increase throughput and proteome coverage without losing quantitative accuracy. For instance, the extensive subcellular fractionation and separation techniques that must be used to increase proteome coverage for an organelle can introduce large variations in results from sample to sample during preparation. To circumvent such obstacles, samples to be compared can be paired for analysis and processed under the same conditions using differential stable isotope labeling techniques. In this approach, proteins or peptides in control and experimental pools are labeled with light and heavy stable isotope tags and then mixed together for a single liquid chromatography tandem mass spectrometry (LC-MS/MS) run. The light and heavy peptide pairs coelute from the chromatographic column while their masses are resolved by the mass spectrometer. Therefore, their respective intensities allow for relative quantitation between the control and

experimental samples. Though a variety of differential labeling techniques are available (1–3) we and others have found that stable isotope labeling by amino acids in cell culture (SILAC) is ideal for subcellular proteome profiling (4–6) because:

1. Cells to be analyzed are mixed before subcellular fractionation and protein extraction, greatly reducing any variation caused by experimental handling and sample processing.
2. It is the most comprehensive way to uniformly label all cellular proteins, thereby ensuring more accurate quantitative analysis.
3. Relative quantities are obtained for each tryptic peptide pair allowing for better assessment of differential protein expression.
4. It allows accurate temporal proteome profiling and monitoring of protein translocation.

Time series proteome profiling using the SILAC strategy can be implemented for any subcellular organelle (Fig. 1). Because the samples to be compared are mixed and processed in parallel, any organelle cross contamination will affect both samples equally, thus distinguishing true biological variations from technical variations. We recently implemented this strategy to examine temporal changes in the endoplasmic reticulum (ER) proteome of human fibroblast cells exposed to the ER stress inducers tunicamycin and thapsigargin (5). Our ability to quantify expression changes at six time points was made possible by pairing each time point with the same control. This control then acted as a reference point against which all data could easily be cross-correlated. Quantitative data was obtained with the use of ZoomQuant software and visualization was facilitated using the GeneSpring GX analysis platform, originally designed to process Affymetrix microarray data.

2. Materials

Unless otherwise noted, all reagents are made using distilled, deionized water (ddH₂O).

2.1. Cell Culture and Reagents

1. *Human primary fibroblasts* established from a punch skin biopsy explant from a 5-year-old donor (gift from Dr. Raphael Schiffmann, NINDS/NIH).
2. T-25 and T-75 tissue culture flasks.
3. *Low glucose Dulbecco's Modified Eagle Medium (DMEM)* containing 1 g/L D-glucose, 110 mg/L sodium pyruvate, 0.4 mg/mL pyridoxine HCl without Arg and Lys (Atlanta Biologicals, Lawrenceville, GA).
4. *Fetal bovine serum* (Invitrogen Corporation, Carlsbad, CA).
5. *Penicillin* (10,000 U/mL)/*streptomycin* (10,000 µg/mL) (100×) (Invitrogen Corporation, Carlsbad, CA).
6. *Stable isotope labeled (heavy) amino acids*: ¹³C₆-L-Arginine:HCl (¹³C₆-Arg) and ¹³C₆, ¹⁵N₂-L-Lysine:2HCl (¹³C₆, ¹⁵N₂-Lys) (Cambridge Isotopes Laboratories, Inc., Andover, MA).

7. *Unlabeled (light) amino acids*: L-Arginine:HCl (Arg) and L-Lysine:2HCl (Lys) (Sigma-Aldrich Corp., St. Louis, MO).
8. *SILAC “labeled” medium*: Dissolve 84 mg of $^{13}\text{C}_6$ -Arg and 146 mg of $^{13}\text{C}_6$, $^{15}\text{N}_2$ -Lys in 890 mL of DMEM. Add 10 mL of penicillin/streptomycin and 100 mL of FBS. Sterilize by passing through a 0.22- μm filter.
9. *SILAC “unlabeled” medium*: Prepare as above, but using “unlabeled” Arg and Lys.
10. *ER stress stock reagents*: 5 mg/mL tunicamycin in DMSO (1,000 $\times$), 1 mM thapsigargin in DMSO (1,000 $\times$) (Sigma- Aldrich Corp., St. Louis, MO).
11. *Phosphate buffered saline (PBS)*: 1 mM KH_2PO_4 , 155 mM NaCl, 3 mM Na_2HPO_4 . Adjust to pH 7.4.
12. *Cell lysis buffer*: 10 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA) and 2.5 M sucrose. One complete, Mini protease inhibitor cocktail tablet is added fresh for every 10 mL of buffer used (Roche Pharmaceuticals, Nutley, NJ).
13. *Protein extraction buffer*: 7 M urea, 2 M thiourea, 2% CHAPS (w/v) and fresh 50 mM DTT.

2.2. SDS-PAGE

1. *Protein concentration assay*: Bio-Rad protein assay kit II (Bio- Rad Laboratories, Inc., Hercules, CA).
2. *Sample desalting and clean up*: Bio-Spin 6 columns with Bio- Gel P-6 in Tris buffer (Bio-Rad Laboratories, Inc., Hercules, CA); vacuum centrifuge.
3. *Pre-cast polyacrylamide gel*: 10–20% Criterion Tris-HCl gel (Bio-Rad Laboratories, Inc., Hercules, CA).
4. *Laemmli sample buffer*: 2% SDS, 25% glycerol, 0.01% bromophenol blue, 62.5 mM Tris-HCl, pH 6.8 and 50 mM DL dithiothreitol (DTT) added just before use.
5. *Tris/Glycine/SDS (TGS) running buffer (10 $\times$)*: 25 mM Tris- Base, 192 mM glycine, 0.1% SDS, pH 8.3 (Bio-Rad Laboratories, Inc., Hercules, CA).
6. *Gel fixing solution*: 45% methanol, 5% acetic acid (Prepare one liter and store at room temperature).
7. *Gel staining solution*: Ready to use Bio-Safe Coomassie stain (Bio-Rad Laboratories, Inc., Hercules, CA).

2.3. In-Gel Digestion and Peptide Extraction

Except for digestion buffer, prepare 100 mL of each solution and store at room temperature. Solutions are stable at room temperature for up to 2 months.)

1. 100% acetonitrile (ACN).
2. 50% ACN.
3. 50% ACN, 5% formic acid (FA) (v/v).

4. 100 mM NH₄HCO₃.
5. 50 mM NH₄HCO₃.
6. 25 mM NH₄HCO₃.
7. 0.1% trifluoroacetic acid (TFA).
8. *Digestion buffer*: 12.5 ng/μL of mass spectrometry grade Trypsin Gold (Promega Corp, Madison, WI) in 50 mM NH₄HCO₃. Dissolve one vial containing 100 μg of lyophilized trypsin in 8 mL of ice cold 50 mM NH₄HCO₃ solution. Prepare 50–100 μL aliquots in ice chilled Eppendorf tubes and store immediately at –80°C. The solution is stable at this temperature for up to a year.

2.4. Mass Spectrometry Instruments and Bioinformatics Tools

2.4.1. Buffers

1. *Aqueous mobile phase*: 0.1% formic acid (A).
2. *Organic mobile phase*: 95% acetonitrile with 0.1% formic acid (B).

2.4.2. Instrumentation

1. *Sample loading*: Autosampler (Dionex LC Packings, Sunnyvale, CA).
2. *Reverse-phase high pressure liquid chromatography (HPLC) system*: Dionex LC Packings nano-HPLC (Dionex-LC Packings, Sunnyvale, CA).
3. *Mass spectrometer*: LTQ (Thermo Fisher Scientific, Inc., Waltham, MA).
4. *Sample washing*: C18 trap column (5 μm, 300 μm i.d. × 5 mm), (LC Packings, Sunnyvale, CA).
5. *Sample fractionation (stationary phase)*: Zorbax C18 (3.5 μm, 100 μm × 15 cm) reverse-phase nanocolumn (Agilent Technologies, Palo Alto, CA).
6. *Sample injection*: 10-μm silica tip (New Objective Inc., Ringoes, NJ).

2.4.3. Bioinformatics

1. *Raw data collection*: Xcalibur 2.0.7 (Thermo Fisher Scientific, Inc., Waltham, MA).
2. *Protein identification*: Bioworks 3.1 (Thermo Fisher Scientific, Inc., Waltham, MA); UniProt/Swiss-Prot database (<ftp://ftp.ncbi.nih.gov/blast/db/FASTA/>) (see Note 1).
3. *Protein quantification*: ZoomQuant software (<http://proteomics.mcw.edu/ZoomQuant>).

¹It is recommended to download the most updated FASTA format protein database. The UniProt knowledgebase consists of two sections: a section containing manually curated FASTA format protein sequences referred to as the “Swiss-Prot database,” and a section with computationally analyzed records that await full manual annotation referred to as the “TrEMBL database.” Most proteomics users prefer the Swiss-Prot database as opposed to the TrEMBL database since it contains a less redundant protein list.

4. *Data normalization and visualization*: GeneSpring software (Agilent Technologies, Palo Alto, CA).

3. Methods

3.1. Stable Isotope Labeling by Amino Acids in Cell Culture

1. Thaw and seed one vial of cells into a T-25 tissue culture flask with SILAC labeled medium. Similarly thaw and seed one vial of cells into a T-25 tissue culture flask with unlabeled medium (see Note 2).
2. Culture cells at 37°C, 5% CO₂, and replace with corresponding labeled or unlabeled medium every 2–3 days, until they have reached 70–80% confluence.
3. Passage cells into a T-75 flask using their respective labeled or unlabeled medium. Continue splitting cells 1:3 each time they reach 70–80% confluence until the cells have been fully labeled with the stable isotopes (see Notes 3 and ⁴). Labeled and unlabeled cells are cultured in parallel with the same number of passages and subcultures.
4. Continue to culture the cells until they have reached 100% confluence, then proceed with ER stress experiment as follows.

3.2. ER Stress Induction

1. Add 100 µL of tunicamycin stock solution to 100 mL of the unlabeled medium (for a final concentration of 5 µg/mL) and 100 µL of thapsigargin to an additional 100 mL of unlabeled medium (for a final concentration of 1 µM).
2. To six flasks of unlabeled cells, add 12 mL of the unlabeled medium containing tunicamycin. To the remaining six flasks of unlabeled cells, add 12 mL of the unlabeled medium containing thapsigargin.
3. To the labeled cells (12 culture flasks), add 12 mL of labeled medium.
4. Incubate two dishes of the labeled cells, one dish of the tunicamycin treated cells and one dish of the thapsigargin treated cells for each of the following amounts of time: 0 min, 1, 6, 12, and 24 h (see Note 5).

²In our experience the use of 10% serum in the culture medium does not interfere with the incorporation of the exogenous stable isotope labeled amino acids and thus the conventional 3 kDa cut off dialyzed serum is omitted. We found that the use of dialyzed serum is not adequate for primary cell cultures because it lacks several necessary low mass growth factors.

³For most cell lines, approximately 97% incorporation of the stable isotope labeled amino acids is achieved after five cell doublings. However, each cell line may behave differently and full labeling should be verified by mass spectrometry before the experiment proceeds. Usually, soluble proteins are extracted from an aliquot of cells by whole cell lysis and prepared as described in Subheadings 3.4–3.7. A Sequest search for labeled and unlabeled peptides is performed to determine the level of isotope incorporation.

⁴Though twelve T-75 flasks of cells are needed for the final stage, it is wise to maintain additional flasks of labeled cells to be frozen down and stored for future use. Once the cells are fully labeled, they will remain so as long as they are always cultured in labeled medium. Therefore, cells seeded from frozen stocks are “ready to use” and do not have to go through the extensive passaging required for the initial labeling process.

⁵Depending on the experiment and the subcellular organelle to be studied one can use alternate time points or drug doses. A pilot study should be performed to determine the optimal dose of ER stress agent to be used. In our experiments, the concentration of thapsigargin and tunicamycin were determined by using cell viability or cytotoxicity assays, such as the MTT assay (Promega) and/or LDH releasing assay (Sigma).

3.3. Cell Harvesting and Subcellular Fractionation

3.3.1. Cell Harvesting

1. Dissolve one protease inhibitor cocktail tablet in 10 mL of lysis buffer and keep on ice during use.
2. After each time point discard the conditioned medium from each paired control and treated culture flask and add 10–15 mL of PBS to each flask to wash the cells. Repeat the washing twice to remove any serum protein contaminants from the cell surface.
3. Add 2 mL of ice-cold lysis buffer to each flask and harvest the cells with a cell scraper while keeping the flask on ice. Transfer the cell suspensions to preweighed 10-mL polypropylene conical tubes and pellet the cells by gentle centrifugation for 5 min at $300 \times g$ and 4°C .
4. Discard the supernatant and weigh the cell pellets using a precision balance. Mix equal amounts of labeled and unlabeled cells (w/w), add 1 mL of lysis buffer and process for subcellular fractionation (see Note 6).

3.3.2. ER Fractionation

1. Homogenize the cells by passing them 15 times through a 1-mL syringe with a 23 gauge needle and centrifuge for 10 min at $4,000 \times g$ and 4°C .
2. Transfer the supernatant containing the microsomal (ER) fraction to a clean Eppendorf tube and further centrifuge at $13,000 \times g$ for 20 min and 4°C to obtain the microsomal pellet.
3. Resuspend the pellet in a small volume of protein extraction buffer and vortex vigorously. Determine the protein concentration of each sample using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) and store samples at -80°C until analysis.

3.4. Prefractionation of Proteins by SDS-PAGE

1. Take aliquots containing 100 μg of total protein from each time point sample and reduce the volume to about 75 μL each by vacuum centrifugation. Desalt samples using Bio-spin 6 columns following the manufacturer's instructions.
2. Dry samples completely by vacuum centrifugation, then resuspend in 20 μL of Laemmli buffer with freshly added DTT (50 mM).
3. Boil samples for 5 min at 95°C .
4. Load samples and molecular weight marker into individual wells of a 10–20% Criterion Tris–HCl pre-cast gel. Run the gel with TGS buffer at 200 V (constant) until just after the dye front runs off the gel (45 min to 1 h).

⁶Equal amounts of wet cell material are established in each tube by removing cells from the tube containing the higher amount with a fine spatula.

5. Remove the gel from the cassette and cover with fixing solution. Incubate for 30 min at room temperature with gentle agitation.
6. Wash the gel three times for 5 min each in ddH₂O with gentle agitation.
7. Cover the gel with Bio-Safe Coomassie and stain for 1 h (this can be done at room temperature with gentle agitation, or overnight at 4°C).
8. Cover the gel with ddH₂O and destain for 1 h, replacing with clean water every 15 min.
9. With a razor blade, slice the gel on either side of the lane containing each sample. Then make horizontal slices to produce 30–40 gel bands per lane (see Note 7).

3.5. In-Gel Digestion

1. Wash gel slices twice by incubation in 50 µL of 50% ACN at room temperature, with vortexing, for 15 min each time.
2. Remove the 50% ACN and add 50 µL of 100% ACN. Wait for the gel pieces to shrink and turn white (this will happen almost immediately; some blue color may remain from the Coomassie).
3. Remove ACN and rehydrate gel pieces with 50 µL of 100 mM NH₄HCO₃.
4. Incubate at room temperature for 5 min.
5. Add 50 µL of 100% ACN (maintaining a 1:1 ratio with NH₄HCO₃).
6. Incubate at room temperature for 15 min, with vortexing.
7. Remove any liquids that did not absorb into the gel.
8. Add 50 µL of 100% ACN and wait for the gel pieces to shrink and turn white.
9. Remove all ACN.
10. Rehydrate gel pieces with 10–20 µL of digestion buffer and incubate on ice for 45 min.
11. Remove any excess digestion buffer.
12. Add 5 µL of 50 mM NH₄HCO₃.
13. Incubate overnight at 37°C (an incubator is preferable to a water bath).

3.6. Peptide Extraction (see Note 8)

1. Spin down the tubes to collect any condensation.
2. Add 25 µL of 25 mM NH₄HCO₃ and incubate at room temperature for 15 min.

⁷Before cutting the gel, it is a good idea to scan an image of the gel. Then, as you cut the gel, number each band and mark their location on a print out of the image. This will allow you to match identified proteins with their approximate molecular weight on the gel. Also, dicing each band into smaller pieces before placing it into its corresponding numbered tube will increase the efficiency of tryptic digestion and peptide extraction in later steps.

⁸In-gel digestion and peptide extraction can be stopped at any time and samples kept at –20°C for up to a few days.

3. Add 25 μ L of 100% ACN and incubate for 15 min at room temperature, with vortexing.
4. Recover and save the supernatant containing extracted peptides.
5. Extract additional peptides from the gel piece by adding 30 μ L of buffer comprising 50% ACN, 5% FA.
6. Incubate 10 min at room temperature, with vortexing.
7. Pool supernatant with that from the same gel piece in step 4.
8. Repeat steps 5–7.
9. Dry supernatants by vacuum centrifugation.
10. Resuspend peptides in 6 μ L of 0.1% TFA in an autosampler vial, store at -80°C .

3.7. Mass Spectrometry Analysis

1. Externally calibrate and tune the LTQ mass spectrometer using the manufacturer's tune mixture and protocol.
2. Load the sample vials onto the autosampler and inject 6 μ L into the LC-MS system using the Dionex-LC-Packings autosampler and loading pump.
3. Load peptide samples first onto a C18 trap connected in series with the C18 column and wash for 6 min using 0.1% TFA (A) before introducing them onto the C18 column.
4. Desalted peptides are turned in-line to the gradient column and eluted using a 100 min linear gradient from 5 to 60% B.
5. Introduce peptides to the mass spectrometer through a 10- μ m silica tip at 1.7 kV and the heated capillary set to 160°C .
6. Operate the LTQ mass spectrometer continuously during the chromatographic elution.
7. Acquire a survey MS scan to determine the mass and intensity of eluting peptides.
8. Acquire data dependent MS/MS scans for the top five most intense peptides in the survey scan, which will be used for protein identification searches.
9. Acquire zoom scans (14 Da window) for each precursor mass to provide higher resolution data of the unlabeled and labeled peptide pairs for quantitation (non-zoom data on the LTQ is low resolution and not ideal for quantitation).

3.8. Protein Identification and Quantification

Unfortunately there is no universal software that can perform both identification and quantification of proteins generated by the different mass spectrometry instruments and the different proteomics strategies currently in use. In our study we used the SILAC strategy together with LC-MS/MS to generate raw data followed by analysis using a combination of Bioworks and ZoomQuant software for protein identification and quantification (see Fig. 2).

1. To streamline search time, use Bioworks software to index the Swiss-Prot database for proteins rising from the human species with fully enzymatic tryptic digestion and allowing up to two missed cleavages.
2. Search the raw mass spectral data using the Sequest algorithm within Bioworks and the indexed database. Set the search parameters as follows: signal threshold 1,000, peptide mass tolerance ± 1.5 Da, fragment ion tolerance ± 0.35 Da and differential modifications of 15.99492 Da for Met oxidation, 8.01420 Da for the $^{13}\text{C}_6$, $^{15}\text{N}_2$ -Lys isotope, 6.02040 Da for the $^{13}\text{C}_6$ -Arg isotope.
3. Download and install the ZoomQuant software (see Note 9). The ZoomQuant package has three components: RawBitZ, Epiwrapper, and the ZoomQuant application.
4. Extract zoom scans by uploading the raw mass spectrometry data files to RawBitZ and saving the generated .zcn files in a new folder.
5. Upload the corresponding Sequest search files to Epiwrapper to filter identified peptides based on X-corr (1.9 for $z = 1$, 2.5 for $z = 2$, 3.5 for $z = 3$), initial rank (50), ion match (0.2) and TIC or signal to noise 2 = 1,000. Save the new .colon files in the same folder as the zoom scan files from step
6. Upload corresponding .zcn and .colon files to the ZoomQuant application as well as the label shift profile configured for differential SILAC labeling (this .lsp file will be found in the ZoomQuant program folder). The ZoomQuant application will generate a list of identified proteins with their corresponding peptides and ratios of labeled to unlabeled peptide pairs. One can select a peptide to view the quality of the corresponding zoom scan and choose valid labeled and unlabeled pairs to include in the analysis. After viewing and selecting data, save the report as HTML and Excel files.
7. Combine all Excel data files rising from one sample set (i.e., all the bands cut from one gel lane). Create a file that contains protein identifiers (e.g., accession number and/or name) in the first column and the corresponding peptide ratios in the second column. Use the sample name (e.g., the time point and ER stressor used) as the first cell in the second column. Discard any proteins that are not represented by two or more unique peptides. Save this as a .txt file for GeneSpring analysis.

⁹There are only a few specialized software packages equipped to determine peptide ratios from a SILAC experiment. Unfortunately each instrument requires specific software. While software such as MaxQuant (7) and Census (8) are aimed at high resolution mass spectrometers (Sciex Qstar, Thermo LTQ-Orbitrap and Thermo LTQ-FTICR) fewer programs have been developed for low resolution mass spectrometers owing to the challenges in processing low resolution mass spectral data. For laboratories equipped with a low resolution LTQ there is an option to generate high resolution mass spectral data using the zoom scan capability and to analyze the data using ZoomQuant software (9). The zoom scan events allow complete resolution of labeled and unlabeled peptide pairs, especially for triply and quadruply charged ions and thus facilitate intensity ratio measurements. All these software packages are publicly available and can be installed on any standard desktop PC. (Download MSQuant at <http://msquant.sourceforge.net/>, Census at <http://fields.scripps.edu/census/download.php?menu=6> and ZoomQuant at <http://proteomics.mcw.edu/zoomquant>). Detailed instructions are provided on how to install and run each of these programs.

3.9. Data Normalization and Visualization (see Note 10)

1. Upload the generated .txt peptide ratio list from each time point to GeneSpring using accession numbers as identifiers and peptide ratios as signal intensities.
2. Use GeneSpring to normalize expression values for each time point by dividing individual peptide ratios by the median value of all ratios. This corrects for any unequal mixing of labeled and unlabeled cells that may have occurred before sample fractionation (see Note 11).
3. The GeneSpring algorithm recognizes the number of peptides per protein as it would array probe sets mapping to one gene and will determine an average ratio for each protein using the peptide count and generate *p*- or *z*-score values that can be used to filter significant data from nonsignificant data.
4. GeneSpring has several visualization options to facilitate data set comparison in a time series experiment. Set up the view depending on the type of display needed, filter for up and down-regulated proteins or show expression patterns for each single protein across different time points.

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¹⁰Software platforms for proteome profiling and data visualization are still emerging. In the meantime, we used the mature GeneSpring analysis platform that was originally designed to process Affymetrix microarray data to help filter and visualize our time series proteomics data.

¹¹Usually, mixing labeled and unlabeled cells 1:1 using a high precision balance is very accurate, but sometimes slight errors may occur and will result in the overall peptide ratios being skewed too high or too low. This can be corrected for using internal normalization by dividing the ratio of labeled and unlabeled peptide pairs in each experiment by the median value of all ratios generated in the experiment.

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Fig. 1.

Overview of the experimental design used to study temporal changes in ER stress response following treatment with tunicamycin (Tun) or thapsigargin (Thp). Control human primary fibroblasts are grown in medium in which Lys and Arg are replaced by $^{13}\text{C}_6$, $^{15}\text{N}_2$ -Lys and $^{13}\text{C}_6$ -Arg. The cells fully incorporate these amino acids after about five cell doublings. Labeled control cells remain untreated (-Tun/-Thp) while unlabeled cells are treated with an ER stress agent (+Tun or +Thp). At the indicated times, treated and untreated cells are mixed at a 1:1 ratio, then processed for subcellular fractionation. In this case, the ER fraction is prepared and proteins are extracted and further separated by SDS-PAGE. Each lane is sliced into 30–40 bands, digested by trypsin and the resulting peptides analyzed by LC-MS/MS. The base peak chromatogram is representative of the labeled and unlabeled peptide mixture obtained from one single gel band. The zoom scan image shows the mass spectrum of a pair of labeled and unlabeled peptides eluting at the retention time indicated by the arrow in the base peak chromatogram. The MS/MS window depicts the fragment ions generated from one peptide. Proteins are identified from the MS/MS data of their tryptic peptides using Bioworks software and ratios between treated and untreated samples are determined from the peak areas of labeled and unlabeled peptide pairs using the zoom scan and ZoomQuant software.

Fig. 2.

Overall workflow used to identify and quantify protein ratios in this study.