Video Article

Quantitative Analysis of Chromatin Proteomes in Disease

Emma Monte[1], Haodong Chen[1], Maria Kolmakova[1], Michelle Parvatiyar[1], Thomas M. Vondriska[1,2,3], Sarah Franklin[1,4]

[1]Department of Anesthesiology, David Geffen School of Medicine at UCLA

[2]Department of Medicine, David Geffen School of Medicine at UCLA

[3]Department of Physiology, David Geffen School of Medicine at UCLA

[4]Department of Internal Medicine, Nora Eccles Harrison Cardiovascular Research and Training Institute, University of Utah

Correspondence to: Sarah Franklin at franklin@cvrti.utah.edu

URL: http://www.jove.com/video/4294
DOI: doi:10.3791/4294

Keywords: Medicine, Issue 70, Molecular Biology, Immunology, Genetics, Genomics, Physiology, Protein, DNA, Chromatin, cardiovascular disease, proteomics, mass spectrometry

Date Published: 12/28/2012

Citation: Monte, E., Chen, H., Kolmakova, M., Parvatiyar, M., Vondriska, T.M., Franklin, S. Quantitative Analysis of Chromatin Proteomes in Disease. *J. Vis. Exp.* (70), e4294, doi:10.3791/4294 (2012).

Abstract

In the nucleus reside the proteomes whose functions are most intimately linked with gene regulation. Adult mammalian cardiomyocyte nuclei are unique due to the high percentage of binucleated cells,[1] the predominantly heterochromatic state of the DNA, and the non-dividing nature of the cardiomyocyte which renders adult nuclei in a permanent state of interphase.[2] Transcriptional regulation during development and disease have been well studied in this organ,[3-5] but what remains relatively unexplored is the role played by the nuclear proteins responsible for DNA packaging and expression, and how these proteins control changes in transcriptional programs that occur during disease.[6] In the developed world, heart disease is the number one cause of mortality for both men and women.[7] Insight on how nuclear proteins cooperate to regulate the progression of this disease is critical for advancing the current treatment options.

Mass spectrometry is the ideal tool for addressing these questions as it allows for an unbiased annotation of the nuclear proteome and relative quantification for how the abundance of these proteins changes with disease. While there have been several proteomic studies for mammalian nuclear protein complexes,[8-13] until recently[14] there has been only one study examining the cardiac nuclear proteome, and it considered the entire nucleus, rather than exploring the proteome at the level of nuclear sub compartments.[15] In large part, this shortage of work is due to the difficulty of isolating cardiac nuclei. Cardiac nuclei occur within a rigid and dense actin-myosin apparatus to which they are connected via multiple extensions from the endoplasmic reticulum, to the extent that myocyte contraction alters their overall shape.[16] Additionally, cardiomyocytes are 40% mitochondria by volume[17] which necessitates enrichment of the nucleus apart from the other organelles. Here we describe a protocol for cardiac nuclear enrichment and further fractionation into biologically-relevant compartments. Furthermore, we detail methods for label-free quantitative mass spectrometric dissection of these fractions-techniques amenable to *in vivo* experimentation in various animal models and organ systems where metabolic labeling is not feasible.

Video Link

The video component of this article can be found at http://www.jove.com/video/4294/

Protocol

The experimental workflow contains seven major steps (**Figure 1**). For any work involving samples that will be run on the mass spectrometer, the experimenter should wear a lab coat, gloves and hair net and take care to avoid contamination from dust and personal shedding of keratin.

1. Heart Homogenization and Nuclear Isolation

Mouse hearts are homogenized and an intact nuclei pellet is isolated (**Figure 2**).

1. Sacrifice adult mouse, excise the heart, rinse in ice-cold PBS, and homogenize on ice in glass dounce (we prefer the Wheaton Tissue Grinder from Fisher, #08-414-13A, but other methods may work equally well) containing 2 ml of lysis buffer (a hypotonic solution which differentially lyses the cell membrane over the organelles including the nucleus) (10 mM Tris pH 7.5, 15 mM NaCl, and 0.15% v/v Nonidet P-40 [NP-40] in deionized water plus protease and phosphatase inhibitor mixture: 10 mM sodium butyrate, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 0.2 mM Na_3VO_4, 0.1 mM NaF and 1 Roche protease pellet/10 ml - lysis buffer can be stored for up to one week at -20 °C). (**Note:** We do not find it necessary to perfuse the hearts with PBS, as our mass spectrometry data and GO analysis identify the proteins as predominately cardiomyocyte [p-value 3.8E-22] in origin as opposed to blood contamination [p value 5.0E-2].)

Copyright © 2012 Journal of Visualized Experiments

2. Pour lysate through 100 μm strainer and collect flow through in 1.5 ml centrifuge tube. At this point, unless specified, sample should be kept on ice.
3. Centrifuge at 4,000 rpm for 5 min at 4 °C.
4. Remove supernatant. (This is the cytosol, and should be stored at -80 °C. It contains the lysed mitochondria.) Resuspend pellet in 200 μl lysis buffer by triturating. (This is the crude nuclear pellet.)
5. Fill 1.5 ml centrifuge tube with 1 ml of sucrose buffer (24% sucrose weight/volume, 10 mM Tris pH 7.5, and 15 mM NaCl in deionized water with protease/phosphatase inhibitor mixture - sucrose buffer should be made fresh on the day of use). Gently layer the resuspended pellet on top of the sucrose pad and centrifuge at 5,000 rpm for 10 min at 4 °C.
6. Remove the thin film on top as well as the sucrose pad (which contain membrane). Rinse the remaining pellet with 200 μl ice-cold PBS/EDTA (1x PBS with 1 mM EDTA). (This is the nuclei pellet and can be solubilized or fixed for use in western blotting or electron microscopy [See steps 4.1 and 4.2] to quantify enrichment.)

2. Nucleoplasm and Detergent-extracted Chromatin Fractionation

The crude nuclei pellet is separated into a nucleoplasm and detergent-extracted chromatin fraction, containing proteins loosely associated with the DNA.

1. Triturate pellet from step 1.6 in 200 μl detergent extraction buffer (20 mM HEPES pH 7.6, 7.5 mM $MgCl_2$. 0.2 mM EDTA, 30 mM NaCl, 1 M Urea, 1% NP-40 in deionized water with protease/phosphatase inhibitor mixture - detergent extraction buffer can be stored for up to one week at -20 °C).
2. Vortex sample 2 times, 10 sec each. Place on ice 10 min.
3. Centrifuge at 13,000 rpm for 5 min at 4 °C.
4. Remove supernatant. (This is the nucleoplasm and should be stored at -80 °C). Rinse pellet with ice-cold PBS/EDTA. (This is the chromatin pellet.)
5. Triturate pellet in 300 μl Tris, SDS, EDTA buffer (50 mM Tris pH 7.4, 10 mM EDTA, 1% SDS in deionized water with protease/phosphatase inhibitor mixture - Tris, SDS, EDTA buffer can be kept for up to one week at -20 °C).
6. Sonicate 3-6 times for 10 sec each to break up DNA. Keep sample on ice between sonications.
7. Centrifuge at 13,000 rpm for 5 min at 4 °C. Keep the supernatant. (Supernatant is the detergent-extracted chromatin protein fraction and should be kept at -80 °C). The remaining pellet should be small and can be discarded.
8. If using isolated myocytes instead of the whole heart: Isolate neonatal rat ventricular myocytes from rat pups one day after birth using enzymatic digestion, followed by culture in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), 1% insulin-transferrin-sodium selenite (ITS), and 1% penicillin. After 24 hr, transfer to serum-free media (same as above, but lacking FBS). Harvest cells in lysis buffer (listed in 1.1), and begin nuclear fractionation at step 1.3.

3. Acid-extraction Fractionation - DNA-bound Protein Enrichment

A separate fractionation enriches for proteins tightly bound to the DNA, including histones.

1. Repeat steps 1.1-2.4.
2. Triturate the chromatin pellet in 400 μl of 0.4 N sulfuric acid. Vortex to remove clumps.
3. Incubate at 4 °C for 30 min or overnight while rotating.
4. Centrifuge at 16,000 x g for 10 min at 4 °C to remove nuclear debris.
5. Transfer supernatant to a fresh tube.
6. Add 132 μl of trichloroacetic acid drop-wise to the supernatant. Invert several times. Incubate on ice for 30 min.
7. Centrifuge at 16,000 x g for 10 min at 4 °C.
8. Discard supernatant. Gently rinse pellet with ice-cold acetone. (This is the histone pellet.)
9. Centrifuge at 16,000 x g for 10 min at 4 °C.
10. Repeat wash, steps 3.8-3.9.
11. Air-dry pellet.
12. Resuspend the pellet in 100 μl of Tris, SDS, EDTA buffer. Set pH to 8 by adding 1 M Tris. (Use a 1 M Tris stock that is not pH-adjusted.)
13. Sonicate in water bath for 15 min. Prevent overheating by adding ice to water bath. (This is the acid-extracted fraction and should be stored at -80 °C.)

4. Purity Check

Western blots and electron microscopy confirm successful enrichment of nuclei and depletion of other organelles. To see typical results for all of the following quality control assays, please see our previous publication.[18]

1. Perform Western blot analysis. Probe membrane containing each fraction for histone H2A or nucleoporin p62 (as nuclear markers to verify enrichment), and for adenine nucleotide transporter, BiP and tubulin (as a mitochondrial marker, endoplasmic reticulum marker, and cytoskeletal marker respectively to verify purity). To verify successful subfractionation of the nuclei, probe for histone H3 or fibrillarin (detergent and acid-extracted chromatin and intact nuclei) and SNRP70 or E2F (nucleoplasm). Probe for retinoblastoma or hypoxia inducible factor-1 (enriched in detergent-extracted chromatin over acid-extracted fraction). Additional control samples of HeLa cell lysate and whole heart lysate can also be run in the same gel: Prepare HeLa cell lysate control by adding Tris, SDS, EDTA buffer to culture dish and using a cell scraper to collect sample. Sonicate and centrifuge sample as in steps 2.6-2.7. Prepare whole heart lysate control by homogenizing the heart in 2 ml buffer (20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate in deionized water with protease and phosphatase inhibitor mixture). Sonicate and centrifuge as in steps 2.6-2.7. See step 5 for preparing protein samples for SDS-PAGE.

Copyright © 2012 Journal of Visualized Experiments

2. Electron Microscopy: Resuspend the nuclei pellet from step 1.6 in lysis buffer containing 2% gluteraldehyde and fix sample at 4 °C. Rinse samples in osmic acid, dehydrate, and embed in epoxy resin. Cut 70 nm slices using a Reichert Ultracut ultramicrotome. Stain samples in uranyl acetate and then lead and image using a JEOL 100CX Transmission Electron Microscope. Quantify enrichment by measuring the area of intact nuclei versus total area of material imaged. *We find 60-80% of nuclei to be intact.*[14]

Important considerations for enriching versus purifying nuclei. This protocol was developed to enable proteomic analyses of cardiac chromatin for the purpose of studying global gene regulation processes during disease. A major component of designing whole-proteome mass spectrometry experiments is the issue of dynamic range and being able to identify and characterize low-abundance proteins. In addition to the primary goal of providing information on nuclear and chromatin-specific biology, enriching for the nuclear proteome, increases the likeliness of detecting these low-abundance proteins, as does further subfractionation of the nuclei. However, as discussed, the adult cardiomyocyte has a dense cytoskeletal component connected to the nuclear membrane. We do not believe that we have completely purified the nuclei from these other cellular components. However, by enriching only for the nuclei, we have obtained an acceptable threshold to enable the types of analyses described.

Specifically, we have used EM to assess the purity of our crude nuclei pellet and found 60-80% of the fraction to be intact nuclei, roughly 10% mitochondria, and the rest debris. Additionally, we know from our previous study on the intact nuclei population, that while many myofilaments are not enriched by this protocol (including tubulin and actin as measured by Western) certain proteins (calsarcin-1) are enriched, suggesting a true biological population of these proteins in the cardiac nuclei.[19]

Additionally, we compared the proteins we found to be present in the acid-extracted chromatin fraction, to the predicted roles of these proteins by gene ontology. Importantly, this gene ontology analysis does not take into account the relative abundance of the different proteins (as does our Western blot data) but rather counts all proteins identified (enriched or not) as equal when identifying common pathways and cellular compartments. Analysis of these pathways and cellular compartments can be found in our previous publications.[18,20] Most importantly, the success of the enrichment in the acid-extracted chromatin fraction allowed us to identify the presence of 54 histone variants in the adult mouse myocyte,[18] many of which relied on one unique peptide for identification, and thus would likely not have been detectable without this enrichment protocol given the enormous complexity of the total cardiomyocyte proteome. Of the 1,048 proteins we identified from the cardiac nuclei, 56 of them (5.3%) were annotated by GO analysis to be part of the nucleosome (one component of the nucleus of interest). Another study looking at the whole heart, identified 6,180 proteins, of which only 11 proteins (0.18%) were annotated to be part of the nucleosome[21]. This further illustrates the strength of our protocol to meaningfully enrich for nuclear proteins.

5. Protein Gel and Enzymatic Protein Digest

Proteins are separated by one dimensional SDS-PAGE and trypsin digested to be run on the mass spectrometer.

1. Thaw samples on ice and determine protein concentration for each sample using the bicinchoninic acid (BCA) protein assay.
2. Dilute samples to a known concentration using 5x Laemmli buffer, boil for 10 min and store at -20 °C for one-dimensional SDS-PAGE. Dilute samples to a known concentration using urea extraction buffer and store at -20 °C for two-dimensional gels.
3. Run gel of choice loading equal amount of protein in each lane. Protein can be transferred to a nitrocellulose membrane to be used for Western blotting (as with controls in step 4.1) or bands can be cut for mass spectrometry analysis; see following steps.
4. Remove gel from apparatus using deionized water to transfer it to a clean container. Cover gel in Oriole stain, and wrap container in aluminum foil to block light. Allow gel to incubate at room temperature on a shaker for at least 90 min.
5. Image Oriole-stained gel (**Figure 3**) using UV light, and mark where bands will be cut on the image. *We cut each lane into approximately 25 2-mm bands for studies measuring the total proteome.*
6. Place gel onto a clean surface. Use only materials that have been kept sealed or are sprayed with ethanol to prevent keratin contamination. Cut out each band, and then further slice it into 3 equal pieces. Place all three pieces of each band together into its own labeled 1.5 ml tube. Gel pieces can be stored at -20 °C for several months.
7. Prepare gel plugs for enzymatic digestion. Digest gel pieces using trypsin at 37 °C overnight. For detailed gel sample protocol see our previous publication.[22] You can digest low molecular weight bands with chymotrypsin in lieu of trypsin, to eliminate trypsin's extensive cleavage of histone tails.

6. Mass Spectrometry and Data Analysis

Samples are separated on an LC and analyzed by MS/MS. The spectra are searched against a protein database for protein identification.

1. Run 10 µl of each sample through LC/MS/MS. We use a nano-flow Eskigent LC with a 75 µm reverse phase column. Use an LC run optimized for a range of protein and peptides. Employ a linear gradient from mobile phase A (0.1%formic acid, 2%acetonitrile [ACN] in water) to mobile phase B (0.1%formic acid, 20%water in ACN): 60 min from 5% mobile phase B to 50% B, then 15 min from 50% B to 95% B and finally 10 min at constant 95% B. Use a flow rate of 200 nl/min. Acquire mass spectrometry data in a data-dependent mode. *We use a Thermo Orbitrap that fragments the top 6 most abundant parent ions.*
2. Repeat runs for at least three biological and two technical replicates. (Recommended)
3. Use software (commercially available options include BioWorks and Xcalibur; publically available options include PROWL, X!Tandem, SpectraST) to search spectra against the Uniprot database of choice via a search algorithm (such as SEQUEST or MASCOT).
4. Consider modifying search parameters to allow for cysteine carbamidomethylation and methionine oxidation, two common modifications created during the sample processing.
5. Calculate a false positive rate using reverse database searching.

Copyright © 2012 Journal of Visualized Experiments

6. Filter protein identifications to only accept matches of a threshold confidence. We recommend the following parameters to start with: Xcorr >3 (+2), >4 (+3), >5 (+4); deltaCN >0.1, consensus score ≥20, mass tolerance 2 Da for parent ion, mass tolerance of 0.5 Da for product ion, at least 2 unique peptides per protein and no more than 3 missed cleavages.

7. Label-free Quantitation

Determine the relative abundance of proteins using label-free quantitation (**Figure 4**).

1. A number of software programs are publically or commercially available for label-free quantitation of mass spectrometry data including Census (Prof. Yates' group),[23] Elucidator (Microsoft),[24] SIEVE (Thermo Scientific),[25] Scaffold (Proteome Software).[26] These programs aim to correlate the mass spectrometric signal of intact peptides or the number of peptide sequencing events with relative protein quantities between two or more states.
2. While each program incorporates a similar analysis pipeline, some programs are limited to the types of specific analysis that can be performed on the data. Initially, data from different runs is aligned, signal intensity is normalized and peptide peaks are selected for analysis.
3. The two most common methods are quantification based on spectral counting or LC-MS peak area. Abundance ratios are calculated to determine changes in peptide abundance between different groups.
4. These programs can be interfaced with proteomic search algorithms (Mascot, Sequest, X!Tandem) to correlate quantification information to protein identification.
5. To ensure accuracy and reproducibility of the data it is crucial to incorporate both biological (different experimental samples) and technical (running the same sample on the mass spectrometer multiple times) replicates of mass spec data.
6. Variation of peptide abundance in and between experimental conditions can be assessed by ANOVA and plotted via PCA (**Figure 5**).

Important considerations for label-free quantitation. When performing label-free quantitation, specific attention must be given to ensure consistent sample preparation, digestion time and LC-MS/MS conditions as each sample must be processed and analyzed separately. In contrast to metabolic labeling approaches, comparisons in label-free experiments are made on data from distinct mass spectrometry runs (because the lack of labeling obviates the possibility of running them together), thereby necessitating high reproducibility in all aspects of sample analysis (*i.e.* of sample prep, LC and MS steps) and use of high mass accuracy mass spectrometers.

To account for slight changes in sample preparation and analysis a known standard may be added to each sample to assist in normalization of data. Additionally, most software programs allow normalization of signal intensities (*e.g.* by adjusting to background noise or a known abundant analyte) after data acquisition to account for differences in injection, ionization and fragmentation. Alignment algorithms exist in most of the above-referenced software programs that assist in correcting differences in peptide elution profiles. The use of biological and technical replicates is essential in this type of study, as it allows statistical analyses to confirm the reproducibility and consistency of any observed changes in protein abundance.

Representative Results

Figure 4 highlights the utility of this form of relative quantification. Shown in the left panel are the individual monoisotopic peptide peaks (overlaid from different mice), which have been designated as belonging to the protein HMGB1 (identified via database search). Each peak, essentially an extracted ion chromatograph for the given peptide, comes from a different mouse. The groups represent three different physiological states: basal, cardiac hypertrophy, and heart failure, with three biological replicates for each group. By integrating the area under the curve, a relative abundance can be calculated. In the middle panel the average of these abundances is shown. It is clear from this type of analysis that the abundance of the HMGB1 protein is changing during the course of the disease. In contrast, the bottom panels show the example of histone H4 that does not change with disease. In both cases, peptides coming from animals of the same phenotypic state show similar elution profiles and relative abundance, demonstrating the reproducibility of the sample preparation and LC/MS/MS.

The reproducibility of this technique is further confirmed using principal components analysis (PCA). As shown in **Figure 5**, application of ANOVA to the total proteome results of each animal analyzed reveals a distinct clustering by cardiac phenotype (biological replicates) with the tightest association between technical replicates (different mass spec runs of the same sample) from the same animal. This provides confidence that the abundance changes observed for individual proteins of interest are indeed attributable to the difference in the physiological state.

Of equal importance as the quantification and reproducibility of the results is the increased coverage of the nuclear proteome enabled by nuclear subfractionation. **Figure 6** illustrates that analysis of the whole nucleus alone fails to uncover even a majority of the nuclear proteins identified when combining individual analyses from the separate compartments (1,048 unique proteins identified in total across the fractions versus 428 identified from the intact nuclei). Furthermore, the moderate overlap between the different fractions demonstrates the biological relevance of considering these compartments individually for added insight into nuclear function and gene regulation. Finally, the ability to specifically fractionate acid-extracted or detergent-extracted chromatin enriches for key chromatin structural proteins, such as histones, thereby enabling more focused mass spectrometric analyses (such as those that target post-translational modifications) on a group of proteins without requiring specific antibodies for each variant and isoform.

Copyright © 2012 Journal of Visualized Experiments

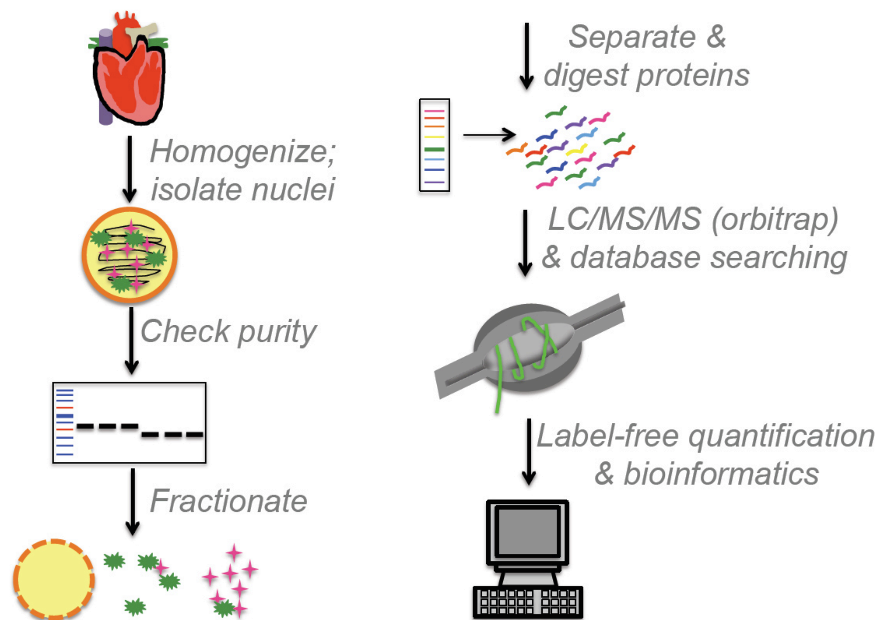

Figure 1. Flow chart for characterization of proteins in cardiac nuclear sub compartments. Whole mouse hearts are homogenized and the lysate enriched for nuclei. Nuclei are fractionated into nucleoplasm, detergent-extracted chromatin, and acid-extracted chromatin fractions. Proteins from each are isolated and separated by one-dimensional SDS-PAGE, and the bands trypsin digested and run by LC/MS/MS. After database searching, label-free quantification is used to identify abundance trends for proteins in the different fractions.

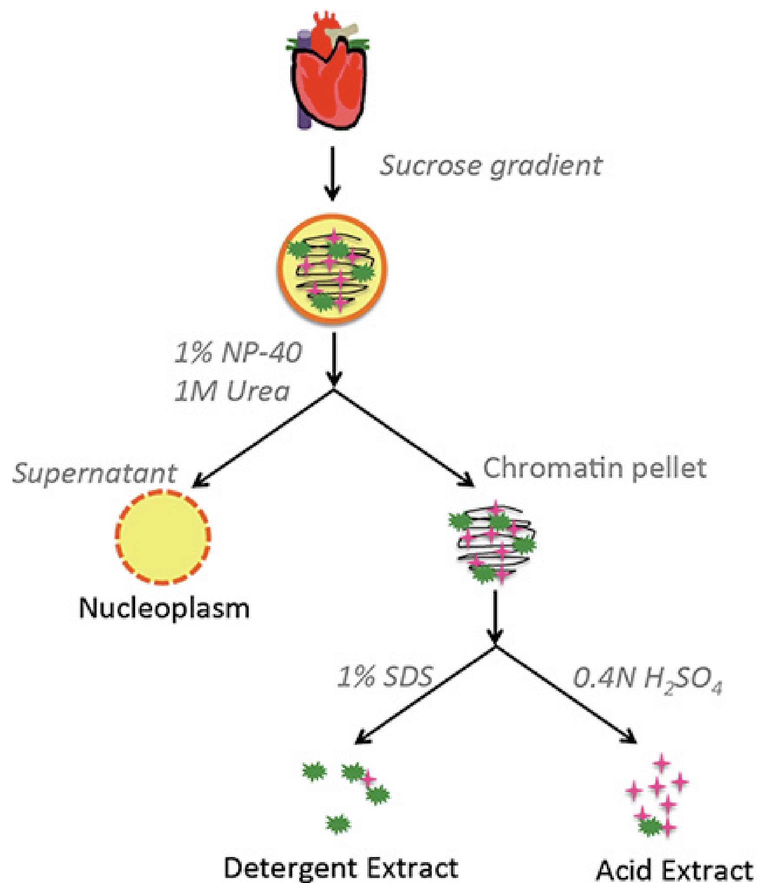

Figure 2. Nuclear fractionation schematic. Nuclei are enriched from whole heart homogenate via a sucrose gradient. Each nuclei sample can either be separated into nucleoplasm and detergent-extracted fractions or into an acid-extracted fraction that enriches for histones.

Copyright © 2012 Journal of Visualized Experiments

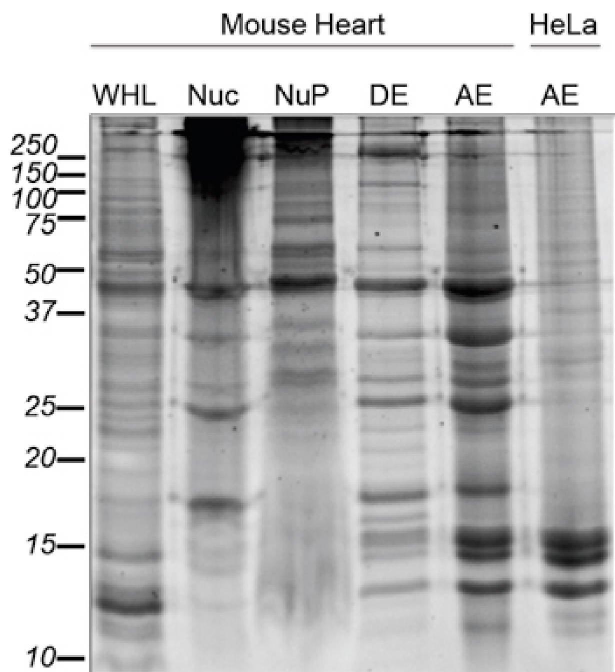

Figure 3. One-dimensional gel of the different fractions. 20 µg of whole heart lysate (WHL), intact nuclei (Nuc), nucleoplasm (NuP), detergent-extracted chromatin (DE), and acid-extracted chromatin (AE) fraction from mouse heart were run on a one-dimensional SDS-PAGE gel (12% acrylamide) along with acid-extracted chromatin fraction from HeLa cells and stained with Oriole. The different patterning of abundance of total protein at the various molecular weights demonstrate the existence of unique proteomes for the different sub compartments, including enrichment for the lower-molecular weight histones in the detergent-extracted chromatin which are further enriched in the acid-extracted fractions. Also of note is the substantially less-complex nature of the AE fraction from HeLa cells as compared to that from the heart.

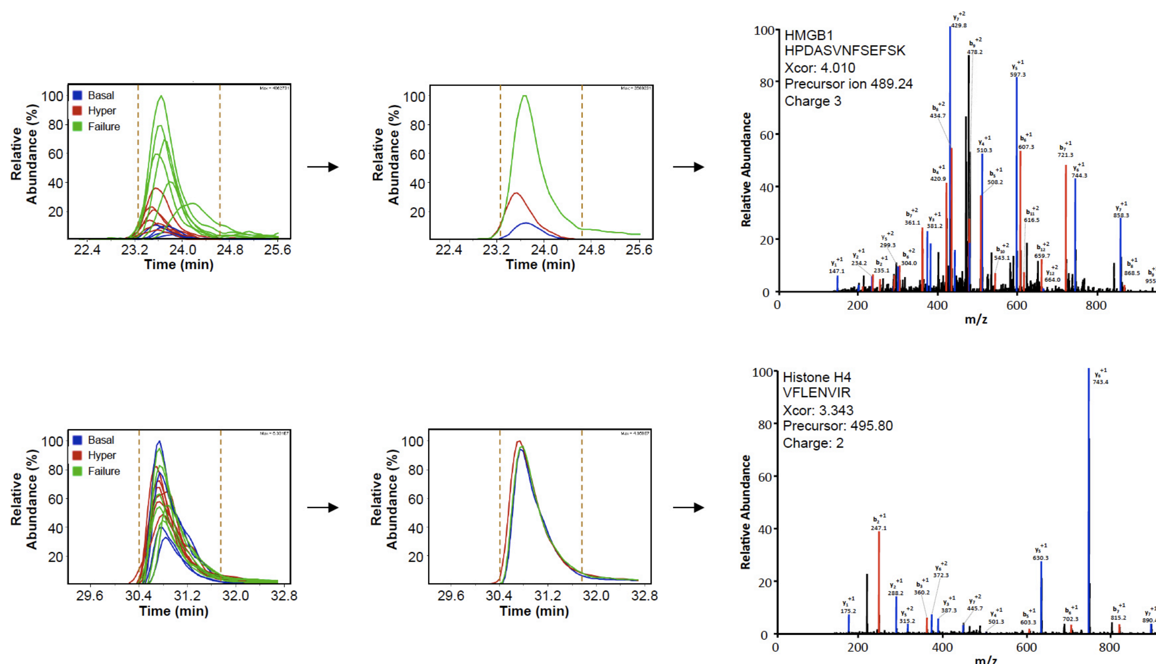

Figure 4. Relative abundance quantification. Mice from three different cardiac states: basal (blue) hypertrophy (red) and failure (green) were collected in triplicate and their protein samples ran in two technical replicates by LC/MS/MS. Shown on the left are the monoisotopic peaks for a specific peptide (whose spectra is shown on the right). Relative abundance was calculated by integration and averaged between the replicates of each phenotypic state (middle panel) to compare the relative abundance of the peptide (and the protein from which it came) in the different states of heart failure. The top panel shows a peptide from HMGB1, whose abundance is altered in disease, while the bottom panel (Histone H4) does not change. Click here to view larger figure.

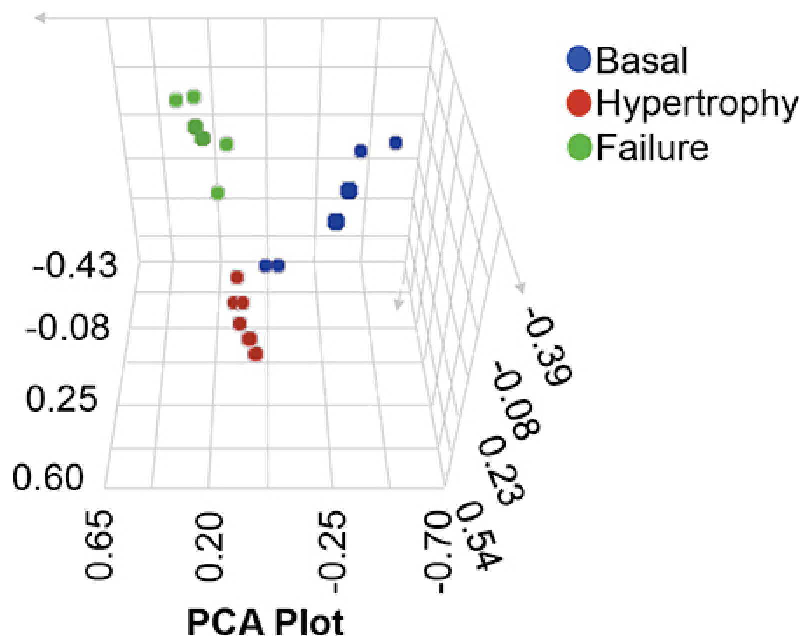

Figure 5. Principal component analysis reveals reproducibility. ANOVA analysis for each mouse (3 biological replicates/cardiac state x 2 technical replicates) plotted for peptide intensity (axes) shows clustering by disease state (basal in blue, hypertrophy in red, failure in green), which indicates successful reproducibility of the mass spec data and significant physiological differences between the different disease states.

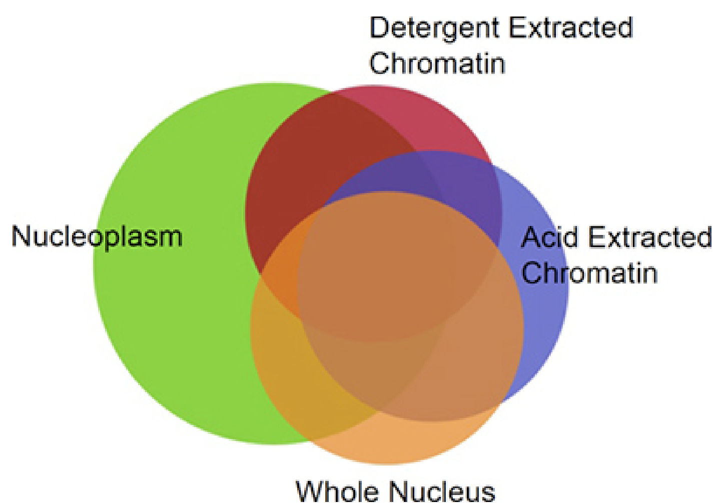

Figure 6. Global characterization of distinct nuclear sub compartments. We identified 1,048 total unique proteins across all 4 fractions. 146 proteins were shared by all 4 fractions, compared to 749 proteins identified in the nucleoplasm, 380 in the detergent-extracted chromatin fraction, 426 in the acid-extracted chromatin fraction, and 428 in the intact nuclei. The Venn diagram depicts the moderate overlap in identified proteins between the fractions, highlighting the existence of distinct regions *in vivo* and the ability of this fractionation to isolate them. Additionally, the markedly incomplete characterization of the total nuclear proteome achievable by only analyzing unfractionated nuclei (orange) justifies further fractionation.

Discussion

Two main methods for nuclear isolation have been reviewed previously:[27] one is the Behrens technique of homogenizing lyophilized tissue in a non-aqueous solvent and the second, a modification of which we use here, of homogenizing tissue in an aqueous sucrose/salt solution followed by differential or density-gradient centrifugation.

Subfractionation of the nuclei by acid extraction on tissue samples is an important tool for studying chromatin which has been used since 1960,[28] with the original goal being to analyze histones. Acid-extraction protocols were developed for this goal of purifying core nucleosomal histones components.[29] A limitation of these previous studies is an absence of information on the non-histone proteins constituting, and modifying, chromatin; we have addressed this challenge with the current protocol for characterizing distinct biological subfractions of the nucleus and

Copyright © 2012 Journal of Visualized Experiments

chromatin. This approach, which uses detergent extraction or acid extraction of chromatin in parallel, reveals the diversity of chromatin regulatory molecules and distinguishes between levels of endogenous regulation, thereby serving as a robust approach for hypothesis generation.

Applying the established acid-extraction methodology to the adult myocyte poses certain obstacles including the dense cytoskeleton and large mitochondrial population. While versions of acid-extraction have been used on whole heart tissue[30] the experiments were conducted in a targeted manner to study known proteins; the current protocol is designed and has been executed to enable unbiased proteome analysis. Additionally, we found it beneficial to examine the detergent-extracted and acid-extracted chromatin separately (only 37% overlap was observed between the proteomes of these two fractions) to better understand the dynamics through which these populations of chromatin proteins interact with DNA. Other protocols using urea-based buffers have also been described,[31,32] which, performed prior to acid extraction, enrich proteins less tightly bound to the genome. In addition to chromatin fractions, others have subfractionated the nucleus to isolate the nucleolus,[33] but subfractionation for mass spectrometry analysis of myocardial nuclei isolated from whole hearts has not been reported.

Shotgun proteomic studies on intact nuclei-that is, unfractionated organelles-have been carried out on myocardial tissue using a MudPIT approach.[15] This study identified 1,044 nuclear cardiac proteins, on the same scale as our protocol, however they relied on using an average of 8.5 replicates, with each run lasting 20 hr to detect low-abundance proteins. In contrast, we employed subfractionation, to both increase the number of identifiable proteins (without needing lengthy chromatography-our LC runs are 85 min) while importantly also offering additional biological information on the sub-nuclear localization and relative abundance of the proteins.

From a whole mouse heart (approximately 0.14 g) we recover approximately 1.2 mg (0.85% of total) of protein in the intact nuclei. From the intact nuclei we achieve the following recovery for each of the subfractions (Note: the total values exceed 100% as acid-extraction and detergent-extraction are done on separate hearts, not in series): Nucleoplasm (15%), detergent-extracted chromatin (85%), and acid-extracted chromatin (54%). By comparison, Thermo Scientific has a kit (NE-PER Nuclear and Cytoplasmic Reagents) which their data sheet says yields 1.6mg cytosolic protein and 0.6 mg nuclear protein (1.5%) from 40 mg of mouse heart with a 10% cross-contamination between the two fractions. Sigma has a kit (NXTRACT, CellLytic NuCLEAR Extraction Kit) which they have tested on rabbit muscle, but not heart, and recovered from 100 mg tissue 0.46 mg of nuclear protein (0.46%) in what they term their "crude nuclear pellet" with a "few percent" of cytoplasmic contamination. Other companies, such as Epigentek also offer kits, however we were unable to find specific details on the enrichment and purity on their websites for comparison.

We have described a novel technique to addresses the current need for successful enrichment of cardiac nuclei for proteomic studies. We further describe methodology for sub compartment fractionation, which both increases the ability to detect less abundant proteins as well as allows for higher resolution, quantification and determination of protein localization. To this end, the protocol for mass spectrometry analysis offers recommended guidelines, however case-by-case considerations will most likely be necessary. For example, we found that the large number of cardiac histone variant isoforms with sequence similarity required manual inspection of the spectra to assign major peaks and confirm parent ion mass before being able to confidently assign the spectra.[14] Search parameters can also be customized for post-translational modification (PTM) identification and excellent reviews exist detailing methods and concerns with data interpretation for PTM analysis (including PTM enrichment,[34] software for PTMs,[35] analysis of combinatorial PTMs,[36] alternative fragmentation strategies[37] and alternative digestion and processing[38,39]).

The enrichment and fractionation described is from the whole heart (which is 50-70% cardiomyocyte by mass). While this sacrifices aspects of cell specificity which may be especially important for certain families of nuclear proteins, it allows for a much more rapid suspension of the proteins into proper buffers, which is not possible with adult myocyte cell isolation protocols. In this way, this methodology better preserves the integrity of the sample against degradation. However, also described are instructions for applying this protocol to isolated neonatal rat ventricular myocytes, since both isolated-cell and whole organism models are necessary tools for studying heart failure. In principle this method could also be applied to adult myocytes extracted by enzyme digestion from isolated hearts-with the caveat that changes in protein binding that occur during the isolation (which can last over an hour) will confound interpretation of the proteomic observations.

We developed this methodology to understand the changes in the nuclear proteome and chromatin makeup that contribute to disease.[14] However other applications include studying stimulus-specific changes in proteome composition in normal physiology. Additionally, sub-fractionation offers the ability for a new level of characterization of how the proteins operate and localize across distinct functional regions of the nucleus.

Disclosures

No conflicts of interest declared.

Acknowledgements

The Vondriska lab is supported by grants from the National Heart, Lung and Blood Institute of the NIH and the Laubisch Endowment at UCLA. EM is recipient of the Jennifer S. Buchwald Graduate Fellowship in Physiology at UCLA; HC is the recipient of an American Heart Association Pre-doctoral Fellowship; MP is the recipient of an NIH Ruth Kirschstein Post-doctoral Fellowship; and SF is the recipient of an NIH K99 Award.

References

1. Li, F., Wang, X., Capasso, J.M., & Gerdes, A.M. Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *J Mol Cell Cardiol.* **28**, 1737-1746, [pii] S0022-2828(96)90163-7 doi:10.1006/jmcc.1996.0163 (1996).
2. Rumyantsev, P.P. Interrelations of the proliferation and differentiation processes during cardiact myogenesis and regeneration. *Int Rev. Cytol.* **51**, 186-273 (1977).

3. Olson, E.N. & Schneider, M.D. Sizing up the heart: development redux in disease. *Genes Dev.* **17**, 1937-1956, doi:10.1101/gad.11101031110103 [pii] (2003).
4. Molkentin, J.D. & Dorn, G.W., 2nd. Cytoplasmic signaling pathways that regulate cardiac hypertrophy. *Annu Rev. Physiol.* **63**, 391-426, doi:10.1146/annurev.physiol.63.1.39163/1/391 (2001).
5. Fishman, M.C. & Chien, K.R. Fashioning the vertebrate heart: earliest embryonic decisions. *Development.* **124**, 2099-2117 (1997).
6. Rajabi, M., Kassiotis, C., Razeghi, P., & Taegtmeyer, H. Return to the fetal gene program protects the stressed heart: a strong hypothesis. *Heart Fail Rev.* **12**, 331-343 doi:10.1007/s10741-007-9034-1 (2007).
7. Roger, V.L., *et al.* Heart disease and stroke statistics--2011 update: a report from the American Heart Association. *Circulation.* **123**, e18-e209, doi:10.1161/CIR.0b013e3182009701 (2011).
8. Schirmer, E.C., Florens, L., Guan, T., Yates, J.R., 3rd, & Gerace, L. Nuclear membrane proteins with potential disease links found by subtractive proteomics. *Science.* **301**, 1380-1382, doi:10.1126/science.1088176301/5638/1380 (2003).
9. Lambert, J.P., Mitchell, L., Rudner, A., Baetz, K., & Figeys, D. A novel proteomics approach for the discovery of chromatin-associated protein networks. *Mol. Cell Proteomics.* **8**, 870-882, [pii] M800447-MCP200 doi:10.1074/mcp.M800447-MCP200 (2009).
10. Malik, P., *et al.* Cell-specific and lamin-dependent targeting of novel transmembrane proteins in the nuclear envelope. *Cell Mol Life Sci.* **67**, 1353-1369, doi:10.1007/s00018-010-0257-2 (2010).
11. Tweedie-Cullen, R.Y., Reck, J.M., & Mansuy, I.M. Comprehensive mapping of post-translational modifications on synaptic, nuclear, and histone proteins in the adult mouse brain. *J. Proteome Res.* **8**, 4966-4982, doi:10.1021/pr9003739 (2009).
12. Chu, D.S., *et al.* Sperm chromatin proteomics identifies evolutionarily conserved fertility factors. *Nature.* **443**, 101-105, [pii] nature05050 doi:10.1038/nature05050 (2006).
13. Uchiyama, S., *et al.* Proteome analysis of human metaphase chromosomes. *J. Biol. Chem.* **280**, 16994-17004, [pii] M412774200 doi:10.1074/jbc.M412774200 (2005).
14. Franklin, S., *et al.* Specialized compartments of cardiac nuclei exhibit distinct proteomic anatomy. *Mol. Cell Proteomics.* **10**, 703, doi:M110.000703 [pii] 10.1074/mcp.M110.000703 (2011).
15. Kislinger, T., *et al.* Global survey of organ and organelle protein expression in mouse: combined proteomic and transcriptomic profiling. *Cell.* **125**, 173-186 (2006).
16. Moore, D.H. & Ruska, H. Electron microscope study of mammalian cardiac muscle cells. *J. Biophys Biochem. Cytol.* **3**, 261-268 (1957).
17. Anversa, P., Vitali-Mazza, L., Visioli, O., & Marchetti, G. Experimental cardiac hypertrophy: a quantitative ultrastructural study in the compensatory stage. *J. Mol. Cell Cardiol.* **3**, 213-227, [pii] 0022-2828(71)90042-3 (1971).
18. Franklin, S., *et al.* Specialized compartments of cardiac nuclei exhibit distinct proteomic anatomy. *Mol Cell. Proteomics* **10**, M110 000703, doi:10.1074/mcp.M110.000703 (2011).
19. Paulsson, A.K., *et al.* Post-translational regulation of calsarcin-1 during pressure overload-induced cardiac hypertrophy. *Journal of molecular and cellular cardiology.* **48**, 1206-1214, doi:10.1016/j.yjmcc.2010.02.009 (2010).
20. Franklin, S., *et al.* Quantitative analysis of the chromatin proteome in disease reveals remodeling principles and identifies high mobility group protein b2 as a regulator of hypertrophic growth. *Mol Cell Proteomics.* **11**, M111 014258, doi:10.1074/mcp.M111.014258 (2012).
21. Gramolini, A.O., *et al.* Comparative proteomics profiling of a phospholamban mutant mouse model of dilated cardiomyopathy reveals progressive intracellular stress responses. *Mol Cell Proteomics.* **7**, 519-533, doi:10.1074/mcp.M700245-MCP200 (2008).
22. Singh, H., Warburton, S., Vondriska, T.M., & Khakh, B.S. Proteomics to Identify Proteins Interacting with P2X2 Ligand-Gated Cation Channels. *J. Vis. Exp.* (27), e1178, doi:10.3791/1178 (2009).
23. Park, S.K., Venable, J.D., Xu, T., & Yates, J.R., 3rd. A quantitative analysis software tool for mass spectrometry-based proteomics. *Nat. Methods.* **5**, 319-322, doi:10.1038/nmeth.1195 (2008).
24. Lomenick, B., *et al.* Target identification using drug affinity responsive target stability (DARTS). *Proc. Natl. Acad. Sci. U. S. A.* **106**, 21984-21989, [pii] 0910040106 doi:10.1073/pnas.0910040106 (2009).
25. Kamleh, A., *et al.* Metabolomic profiling using Orbitrap Fourier transform mass spectrometry with hydrophilic interaction chromatography: a method with wide applicability to analysis of biomolecules. *Rapid Commun. Mass Spectrom.* **22**, 1912-1918, doi:10.1002/rcm.3564 (2008).
26. Searle, B.C. Scaffold: a bioinformatic tool for validating MS/MS-based proteomic studies. *Proteomics.* **10**, 1265-1269, doi:10.1002/pmic.200900437 (2010).
27. Murray, K. The Basic Proteins of Cell Nuclei. *Annu Rev. Biochem.* **34**, 209-246, doi:10.1146/annurev.bi.34.070165.001233 (1965).
28. Johns, E.W., Phillips, D.M., Simson, P., & Butler, J.A. Improved fractionations of arginine-rich histones from calf thymus. *Biochem. J.* **77**, 631-636 (1960).
29. Rodriguez-Collazo, P., Leuba, S.H., & Zlatanova, J. Robust methods for purification of histones from cultured mammalian cells with the preservation of their native modifications. *Nucleic Acids Res.* **37**, e81, doi:10.1093/nar/gkp273 (2009).
30. Kuehl, L., Salmond, B., & Tran, L. Concentrations of high-mobility-group proteins in the nucleus and cytoplasm of several rat tissues. *J. Cell Biol.* **99**, 648-654 (1984).
31. Gronow, M. & Griffiths, G. Rapid isolation and separation of the non-histone proteins of rat liver nuclei. *FEBS Lett.* **15**, 340-344 (1971).
32. Mischke, B.G. & Ward, O.G. Isolation and tissue specificity of chromatin-associated proteins in Vicia faba. *Can J. Biochem.* **53**, 91-95 (1975).
33. Andersen, J.S., *et al.* Directed proteomic analysis of the human nucleolus. *Curr. Biol.* **12**, 1-11 (2002).
34. Zhao, Y. & Jensen, O.N. Modification-specific proteomics: strategies for characterization of post-translational modifications using enrichment techniques. *Proteomics.* **9**, 4632-4641, doi:10.1002/pmic.200900398 (2009).
35. Ahrne, E., Muller, M., & Lisacek, F. Unrestricted identification of modified proteins using MS/MS. *Proteomics.* **10**, 671-686, doi:10.1002/pmic.200900502 (2010).
36. Young, N.L., Plazas-Mayorca, M.D., & Garcia, B.A. Systems-wide proteomic characterization of combinatorial post-translational modification patterns. *Expert Rev. Proteomics.* **7**, 79-92, doi:10.1586/epr.09.100 (2010).
37. Guthals, A. & Bandeira, N. Peptide identification by tandem mass spectrometry with alternate fragmentation modes. *Mol. Cell Proteomics*. doi:10.1074/mcp.R112.018556 (2012).
38. Wu, C., *et al.* A protease for 'middle-down' proteomics. *Nat. Methods*. doi:10.1038/nmeth.2074 (2012).
39. Tran, J.C., *et al.* Mapping intact protein isoforms in discovery mode using top-down proteomics. *Nature.* **480**, 254-258, doi:10.1038/nature10575 (2011).

Copyright © 2012 Journal of Visualized Experiments