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Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics

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ABBREVIATIONS

The abbreviations used are: SILAC: Stable isotope labeling by amino acids in cell culture, 2DE: two dimensional (isoelectric focusing/SDS-PAGE) gel electrophoresis: ICAT^{TM}: isotope-coded affinity tag; MS: mass spectrometry; MALDI-TOF: matrix assisted laser desorption ionization-time of flight; PMF: peptide mass fingerprinting; LC-MS: liquid chromatography-MS;

Quantitative proteomics has traditionally been performed by 2D gel electrophoresis but recently, mass spectrometric methods based on stable isotope quantitation have shown great promise for the simultaneous and automated identification and quantitation of complex protein mixtures. Here we describe a method, termed SILAC for Stable Isotope Labeling by Amino acids in Cell culture, for the *in vivo* incorporation of specific amino acids into all mammalian proteins. Mammalian cell lines are grown in media lacking a standard essential amino acid but supplemented with a non-radioactive, isotopically labeled form of that amino acid, in this case deuterated leucine (Leu-d3). We find that growth of cells maintained in these media is no different from growth in normal media as evidenced by cell morphology, doubling time and ability to differentiate. Complete incorporation of Leu-d3 occurred after five doublings in the cell lines and proteins studied. Protein populations from experimental and control samples are mixed directly after harvesting and mass spectrometric identification is straightforward as every leucine-containing peptide incorporates either all normal leucine or all Leu-d3. We have applied this technique to the relative quantitation of changes in protein expression during the process of muscle cell differentiation. Proteins that were found to be upregulated during this process include glyceraldehyde-3-phosphate dehydrogenase, fibronectin and pyruvate kinase M2. SILAC is a simple, inexpensive and accurate procedure that can be used as a quantitative proteomic approach in any cell culture system.

Proteomics, the large-scale study of the protein complement of a cell or tissue, has its origins in the technology of two-dimensional (2D) gel electrophoresis invented more than 25 years ago (1,2). In 2D gel electrophoresis, quantitation is achieved by recording differences in the staining pattern of proteins derived from two states of cell populations or tissues. Therefore, in addition to obtaining increasingly higher resolution, technology improvements in the 2D gel community have been directed towards the image analysis of 2D gels and the relative quantitation of protein spots by their intensity of staining (3-6).

Mass spectrometry has long been used in a quantitative manner in the small molecule field (7). Pharmacological researchers, for example, use isotopically labeled analogs of the compound of interest, and add a known amount to the sample for analysis. This is because mass spectrometry is not quantitative *per se*, due to varying detector response, differential ionization yields for different substances, and other factors. Observed peak ratios for isotopic analogs, however, are highly accurate since there are no chemical differences between the species and they are analyzed in the same experiment.

One of the first uses of isotopic labels in proteomics was for improved sequence assignment in peptide sequencing by tandem mass spectrometry by incorporating ^{18}O atoms at the C-terminus of a peptide (8-10). The ^{18}O technique had previously already been used in protein chemistry and was subsequently shown to have interesting uses in quantitation as well (11-14).

Structural biologists often employ ^{15}N media, in which all ^{14}N atoms are

replaced by ^{15}N, to determine phase shifts in NMR studies. The Langen and subsequently the Chait groups used this ^{15}N-substituted media for the purpose of quantifying differences between states of microorganisms (15,16). The former group used MALDI and 2D gel electrophoresis to quantify the abundance of mixed spots in 2D gels of bacterial proteins, whereas the latter group quantified relative differences in phosphopeptide abundance in yeast. While clearly showing the power of stable isotope labeling, the particular method employed was limited in its wider applications: ^{15}N-substituted media are difficult and expensive to make for mammalian systems, so the method has generally been limited to microorganisms that can be grown in these media. Additionally, the degree of incorporation is not necessarily 100% and since there are varying numbers of nitrogen atoms in the different amino acids, automated interpretation of the resulting spectra has proven difficult. Smith and co-workers have used FTICR measurements of intact proteins from micro-organisms which were labeled with deuterated leucine containing media. In this way the number of leucines could be estimated which helped in the assignment of protein identity to a measured molecular weight (17).

In 1999 Aebersold and colleagues introduced the isotope coded affinity tag (ICAT) method for relative quantitation of protein abundance (18). In this approach, an isotopically labeled affinity reagent is attached to particular amino acids in all proteins in the population. After digestion of the protein to peptides, as a necessary step in all mainstream proteomic protocols, the labeled peptides are affinity purified using the newly incorporated affinity tag, thereby achieving a simplification of the peptide mixture at the same time as incorporating the isotopic label. The method has

been applied to a range of problems such as the quantification of microsomal proteins in differentiated versus undifferentiated HL-60 cells (19). Limitations of the first iteration of the ICAT principle, which uses biotin as the affinity tag and cysteine as the reactive amino acid, include nonspecific binding to the streptavidin affinity matrix and multiple subsequent reactions at the same site. In recent improvements to the ICAT methodology the cysteines are reacted to solid beads and a labeled amino acid is attached to the cysteine(20). This method addresses many of the above limitations, and leads to a larger number of identifications of cysteine-containing peptides. However, the method is performed by cross-linking peptides to beads via their cysteine groups and photo-releasing them afterwards, which may compromise low-level analysis.

A number of similar isotopic labeling techniques have recently been proposed, which share the requirement of chemical modification of the peptides or proteins (21-23). Some of these strategies couple the labeling and peptide selection step as in the ICAT method, whereas others decouple these two steps or do not include the affinity step (24). For quantitation of phosphorylated proteins, labeling and affinity procedures targeting the phosphogroup directly have also been proposed (25-27).

In this report, we describe a stable isotope labeling strategy that we term SILAC (stable isotope labeling by amino acids in cell culture). Labeled, essential amino acids are added to amino acid deficient cell culture media and are therefore incorporated into all proteins as they are synthesized – ‘encoded into the proteome’. No chemical labeling or affinity purification steps are performed and the method is compatible with virtually all cell culture conditions, including primary cells. We show that incorporation is complete and that cells remain normal in the presence of labeled

media. The method is convenient and inexpensive and is used widely in our laboratory. As an example, we applied SILAC to the study of mouse C2C12 cells as they differentiate from myoblasts into myotubes. This process of muscle differentiation necessarily involves broad changes in the expression levels of proteins as the cells differentiate from one cell type to another. Several proteins were found to be upregulated during this process – most of these have not previously been described as upregulated proteins in this model of muscle differentiation. SILAC requires living cells but may be advantageous over other quantitative proteomics techniques whenever cell culture is used.

EXPERIMENTAL PROCEDURES

Leu-d0 and d3 labeled media composition - The base media, Minimum Essential Medium (MEM) Eagle's Deficient (with Earle's salts and deficient in L-Leucine, L-Lysine and L-Methionine) was obtained from Sigma-Aldrich (Catalog number M7270). The liquid medium was reconstituted according to the manufacturer's instructions. Briefly, the powdered medium was dissolved in water together with 2.2 g/L sodium bicarbonate, and the pH was adjusted to 7.4. The amino acids L-Lysine and L-Methionine were prepared as 1000x stock solutions in PBS, and added to the dissolved media to give a final concentration 72.5 mg/L and 15 mg/L, respectively. The media was filtered through a 0.22 μm filter to obtain sterile, complete medium deficient only in L-Leucine. For labeling experiments, L-Leucine or deuterium labeled L-Leucine-5,5,5-D3, 99 atom % D (Isotec Inc., Miamisburg, USA) were prepared as 250x stock solutions in PBS, sterile filtered and added to the media for a

final concentration of 52 mg/L.

Cell culture and differentiation - NIH 3T3 and C2C12 cells were grown in MEM Eagle's Deficient media supplemented with 2 mM L-glutamine and 10% dialyzed fetal bovine serum plus antibiotics in a humidified atmosphere with 5% CO_2 in air. Cell lines were grown for six cell divisions in labeling media containing either normal leucine or Leu-d3 before the start of differentiation.

Undifferentiated C2C12 cells (day 0) were grown to confluence in normal leucine ('Leu-d0') media. C2C12 cells that were used for myotube differentiation were grown in Leu-d3 media and were harvested over the course of differentiation (days 0, 2 and 5). To induce differentiation, the amount of dialyzed serum in the Leu-d3 containing media was decreased to 2%. Growth media was replaced with fresh media every two days over a period of 5 days.

Preparation of protein samples - For mixing experiments, NIH 3T3 fibroblasts were washed twice with PBS to remove serum proteins, then scraped in a lysis buffer containing 1% SDS, 1% NP-40, 50 mM Tris, pH 7.5, 150 mM NaCl and protease inhibitors (Complete^{TM} tablets, Roche Diagnostics, Mannheim, Germany). The lysate was sonicated for two cycles of 30 seconds each and centrifuged to pellet cellular debris. Protein quantitation was performed using the Bradford protein assay and mixtures of lysates were combined in protein concentration ratios of 1:1, 1:3, 1:10 (Leu-d0: Leu-d3).

For the relative quantitation of protein expression during muscle

differentiation of C2C12 myoblasts, cell lysates from different stages (days 0, 2, and 5) were prepared as described above. After determination of protein concentration with the Bradford assay, mixtures of Leu-d0 and d3-labeled samples were prepared in the following manner: an undifferentiated Leu-d0 Day 0 was mixed with an equal amount of protein from Leu-d3-labeled samples at Days 0, 2 and 5. Protein mixtures were resolved on a 10% SDS-PAGE gel and silver stained to visualize the gel bands.

Mass Spectrometric Analysis - Gel bands were excised and subjected to in-gel reduction, alkylation, and tryptic digestion as previously described(28,29). MALDI data was obtained with a Bruker Reflex III (Bruker-Daltonics Germany) and a Voyager DE-STR (Applied Biosystems) with alpha-cyanohydroxycinnamic acid as the matrix. For nanoelectrospray experiments(30), digests were desalted and concentrated on a microcolumn packed into GELoader tips(31). Peptides were eluted with 50% methanol in 5% formic acid directly into a nanospray needle and the eluate subjected to MS and MS/MS analysis on a QSTAR Pulsar quadrupole time-of-flight tandem mass spectrometer (ABI/MDS-Sciex, Toronto, Canada) and equipped with a nanoelectrospray ion source (Protana Engineering A/S, Odense, Denmark). Proteins were identified by searching peptide sequence tags(32), derived from fragment ion spectra of selected peptides, against the non-redundant protein database maintained and updated regularly at the European Bioinformatics Institute (EBI, Hinxton, UK) using the PepSea software package (MDS Proteomics A/S, Odense Denmark).
For determining quantitative ratios in cases where the Leu-d0 and Leu-d3 isotope distributions overlapped an isotopic correction factor was applied as follows: After peptide identification, the peptide sequence was submitted to the web-based tool

MS-Isotope which is part of the ProteinProspector package (http://prospector.ucsf.edu). The isotope pattern of the lower mass in the isotope pair was then subtracted from the full isotope pattern to obtain the correct peak heights of the higher mass peptide.

RESULTS AND DISCUSSION

The SILAC strategy - Mammalian cells cannot synthesize a number of amino acids, therefore these 'essential' amino acids must be supplied in cell culture medium as free amino acids in order to for the medium to support cell growth. Isotopically labeled analogs of these amino acids can be synthesized and are available commercially. If the labeled analog of an amino acid is supplied instead of the natural abundance amino acid, it will be incorporated into each newly synthesized protein chain. After a certain number of cell doublings, each instance of this particular amino acid will have been replaced by its isotopically labeled analog. If there is no chemical difference between the labeled amino acid and the natural amino acid, the cells should behave exactly like a control cell population grown with the normal amino acid. This is illustrated in Fig. 1. The experimental cell population can then be treated in a specific way, such as differentiation induction or cytokine stimulation, for example. Protein populations from both samples are then harvested and since the label is encoded directly into the amino acid sequence of every protein, the extracts can be mixed directly. Purified proteins or peptides will preserve the exact ratio of the labeled to unlabeled protein, as no more synthesis is taking place and therefore no scrambling take can take place at the amino acid level. The proteins and peptides can be then

analyzed in any of the ways in which they are analyzed in non-quantitative proteomics. Quantitation takes place at the level of the peptide mass spectrum or peptide fragment mass spectrum, exactly the same as in any other stable isotope method (such as ICAT). It is important to note that the absence of chemical steps implies the same sensitivity and throughput for SILAC as for non-quantitative methods.

Fig. 1 also contrasts SILAC with ICAT labeling, which is perhaps the most well established and representative method in quantitative proteomics by mass spectrometry. As can be seen from the figure, proteins need to be reduced and alkylated before mixing, steps that can make it difficult to maintain the samples in directly comparable states during multiple fractionation steps. Furthermore, the chemical modification and affinity purification step can be difficult to perform with very small amounts of sample and non-cysteine containing peptides are also sometimes bound to the avidin column. Finally, in cases of extensive fractionation, a large number of affinity purifications needs to be performed for a single experiment.

One further difference between SILAC (using leucine) and ICAT methods is that SILAC differentially labels more than half of the tryptic peptides whereas ICAT only labels somewhat more than 20 percent. (This calculation is based on the 2 % and 10 % relative abundance of cysteine and leucine, respectively, and an average length of 14 amino acids for tryptic peptides that can be sequenced by mass spectrometry.) Conversely, ICAT achieves some decrease in complexity of the peptide mixture while SILAC does not change the peptide abundances resulting from a digest. Because the reduction in complexity in the case of ICAT is based on the ability to label cysteine residues, one is unable to detect non-cysteine containing proteins at all.

Fragmentation patterns in ICAT are influenced by the functional group attached to the cysteine whereas in SILAC they are the same as for the unlabeled peptide (see below).

Use of specialty media for stable isotope labeling - We made use of a commercially available labeling media deficient in certain amino acids, specifically, methionine, lysine and most importantly for our purposes, leucine. Our goal was to replenish the normal amino acids with the exception of the leucine which would be labeled with deuterium (L-leucine 5,5,5 – D3, or Leu-d3). We chose leucine in these experiments because it is the most abundant amino acid, allows distinction between isoleucine and leucine, and is readily available. Other essential amino acids could have been used as well. Because mammalian cells require serum-containing media for their optimal growth, free amino acids present in the serum can be taken up by the cells. To circumvent this issue, we used commercially available dialyzed serum instead of normal serum as it does not contain detectable amounts of free amino acids. To illustrate the importance of this, we grew cells in our deficient media with Leu-d3 but supplemented with normal fetal calf serum in place of the dialyzed serum. Fig. 2 clearly shows that proteins incorporate normal leucine whose only source can be the undialyzed serum. Without complete incorporation of Leu-d3 in proteins, accurate quantitation of labeled and unlabeled cells will not be possible.

In the experiments we present here, we have used a commercially available powdered Minimal Essential Medium Eagle's media formulation supplemented with the essential amino acids methionine, lysine and leucine. Extra costs incurred by SILAC compared to non-isotopic methods are related mainly to the costs of the amino acid used but are generally not a large fraction of the cost of the experiment.

The SILAC method does not require specialized handling in cell culture beyond the preparation of media that we then find generally applicable to a variety of cell lines and systems that we have tested in our laboratory. For example, we have successfully grown several other cell lines including a human cervical carcinoma cell line (HeLa), Chinese Hamster Ovary epitheloid cells (CHO-K1), African Green Monkey kidney fibroblastic cells (COS-7) and a rat pheochromocytoma suspension cell line (PC12) in d3-labeled culture media (data not shown), demonstrating the general applicability of this method to any cell culture based system.

Time course for incorporation of Leu-d3 - We performed a time course experiment to establish the minimum time required for cells to incorporate Leu-d3 fully in all proteins. The cells were grown in Leu-d3 containing medium for different lengths of time. As shown in Fig. 3, incorporation of Leu-d3 was detectable in peptides after 12 hours of growth. A larger incorporation of Leu-d3 was observed at later time points with full incorporation by day 5.

This corresponds to approximately five doublings for NIH 3T3 fibroblasts used in this experiment indicating that cell lines can be rapidly adapted for use in similar experiments to quantitate protein levels. It should be noted that in the time for the cells to reach five doublings, even those proteins with very long half-lives would still show approximately 97% ($1- 0.5^5$) incorporation of the label as the growing cells synthesize new protein to fill their required complement.

Identification of proteins from d3 labeled samples - We were able to identify proteins by both MALDI-TOF peptide mass fingerprinting (PMF) as well as through

directed peptide sequencing experiments with MS/MS. In instances where mixtures of Leu-d0 and d3-labeled samples were analyzed, the identification of leucine containing peptides was facilitated by the characteristic doublets of peak clusters present in the mass spectra. From the MS spectra, we were able to confirm these doublets were actual Leu-d0 and d3-peak clusters by comparing the spectra containing both Leu-d0 and -d3 peptides to a sample containing a single species. Using MS/MS, as seen in Fig. 4B, the similar fragmentation patterns from Leu-d0 and -d3 peptides can also help to confirm the identity of matched quantitation pairs.

In peptide mass fingerprinting, the presence of Leu-d3 in peptides gave increased confidence when matching peptides (i.e peptides putatively containing a single leucine residue should have their peptide masses shifted by 3 Da, those with more labeled residues would have their mass shifted by the corresponding amount). It was possible to identify and quantitate protein levels based on matched Leu-d0 and -d3 peptides in MALDI. However, the mixtures of proteins present in a one-dimensional SDS-PAGE, as well as the additional peaks arising from the two cell states complicated the process of protein identification by PMF. As such, we did the majority of our mass spectrometric analyses by nanoelectrospray mass spectrometry.

Fragmentation spectra of Leu-d0 and Leu-d3-containing peptides were largely identical except for the characteristic mass shift of fragments containing the leucine residue. These shifts in fragment masses would lend additional specificity to the assignment of peptide sequence tags. This is similar in principle to previous work(8) (10) where incorporation of ^{18}O in tryptic peptides led to a characteristic doublet that greatly helps in obtaining sequence tag information.

Quantitation of proteins levels using Leu-d3 - A mixing experiment was performed using known volumes of cell lysate from NIH 3T3 cells. Lysates were mixed in ratios of 1:1, 1:3 and 1:10 Leu-d0: Leu-d3. The ratios of peak heights of different leucine containing peptides were found to be consistent in the proteins analyzed (beta actin A-X and alpha enolase). Fig. 4A gives examples of two peptides mixed in the ratio 1:3; in both cases observed ratios are similar to expected ratios. We also performed the reverse mixing experiment (data not shown). With correction for the isotopic overlap in peptides containing one leucine only (see Experimental Procedures), the expected ratios were obtained again. In some instances, we observe some consistent errors in quantitating higher fold differences (i.e. greater than 6x). We believe this to be a function of the complexity of the peptide mixture present in a 1D gel band and to be further complicated by nanoelectrospray analyses which did not provide an additional step of peptide separation. Peptide separation by chromatography would remove this problem. To address this problem using NanoES, we tried to compare the relative intensities of fragment ions in the MS/MS spectra obtained from Leu-d0 and Leu-d3 samples. As shown in Fig. 4B, the observed ratios from the relative intensities from all the fragment peaks compare well with the expected ratio of 1:10. (The ratios for a selection of peaks are shown in Table 1.) It is important to note that the fragmentation patterns in labeled and unlabeled peptides are identical and no complicating features are introduced due to the presence of a label.

Quantitation of protein abundance in a cell culture model system of muscle differentiation – To test whether we could identify proteins involved in cellular processes based on quantitative changes in their abundance, we used murine C2C12

cells that have been widely used as an *in vitro* model system for muscle differentiation(33-35). Although extensively studied, the process of myogenesis is not completely understood. The conversion of myoblasts to myotubes can be characterized by three major steps – withdrawing the progenitor cells from the cell cycle, expression of muscle specific genes and fusion of these cells leading to formation of multinuclear myotubes(36). Fig. 5A shows light microscopy pictures of the dramatic morphologic changes that these cells undergo as they differentiate, when cultured in a medium low in mitogens. In order to quantitate changes in protein levels, we grew the uninduced set of cells in normal medium and allowed the cells growing in Leu-d3 containing medium to differentiate. Cell lysates were harvested at different time points and analyzed to determine the identity and change in abundance of the differentially expressed proteins.

As noted above, the process of myotube formation is accompanied by substantial alterations in cell shape, morphology and function due to combined changes in expression levels of extracellular matrix (ECM) components, intracellular proteins and nuclear factors (36,37). To demonstrate the SILAC concept we tested whether differential protein expression could be measured in this system. For analysis, we chose a combination of 1D gel electrophoresis with nanoelectrospray mass spectrometry. Lysates from different time points were separated by gel electrophoresis and Coomassie- or silver stained (see Fig. 5B). As expected, the 1D gel traces of total lysates contained few distinct features. Five bands were excised in regions of the lane containing mixed Leu-d0/Leu-d3 sample, which in separate lanes with unmixed samples had shown differential staining between day 0 and other time points. A total of nine proteins were quantified in these bands. It was possible to

obtain consistent ratios in different leucine containing peptides for the same protein. The process of quantitation was sometimes complicated by the complexity of the MS spectrum due to the large number of protein species found in the protein mixture used for nanoelectrospray analysis. In such cases, we strove to base our quantitation on peptide sets that were well separated and free from interfering peaks. A correction factor for isotopic overlap was applied if necessary (see Experimental Procedures).

The protein quantitation data are represented by histograms in Fig. 6. Not surprisingly, expression of several glucose metabolism-related enzymes was upregulated on days two and five of muscle differentiation relative to day 0. For example, glyceraldehyde-3-phosphate dehydrogenase level increased by approximately four-fold. The level of M2 isozyme of pyruvate kinase increased by nearly two-fold, which correlates with the observation that the M1 and M2 isozymes are more highly expressed in skeletal muscle than in other tissues (38). Protein synthesis-related factors such as ribosomal proteins were also found to be upregulated up to 2.5 fold, again in accordance with increased protein synthesis during the conversion process. Levels of fibronectin, one of the major components of ECM and essential for myogenesis, were also found to be upregulated. While fibronectin is known to be an essential factor in muscle cell differentiation (37,39), it had not previously been shown to be upregulated during this process. The relative levels of other proteins in Fig. 6 such as annexin II were seen to remain essentially constant over the course of differentiation, thus serving as an effective internal control.

CONCLUSION

Our experiments have shown that the process of quantitation of protein levels by SILAC can be performed using standard equipment and procedures available in most proteomics laboratories today and can be rapidly adopted by research groups equipped with cell culture facilities. While we have demonstrated here the compatibility of the method with gel electrophoresis and nanoelectrospray mass spectrometry, the higher throughputs in quantitative analysis and protein identification afforded by LC-MS/MS approaches are certain to enhance the utility of this method.

The use of stable isotopes to label proteins in mammalian cells has several advantages. First, in many biological systems the amount of starting material is limited, making it crucial to minimize the number of manipulations after harvesting proteins and SILAC requires no peptide labeling steps. Second, since the extent of incorporation is virtually 100%, there are no differences in labeling efficiency between one sample and the other. Third, because the proteins are uniformly labeled, several peptides from the same protein can be compared to ensure that the extent of change is the same. Fourth, as the quantitative tag arises from the stable isotope containing amino acid rather than isotopic nuclei (for e.g. ^{15}N enriched media), the labeling of peptides is specific to its sequence and the mass differential between two states can be specified more directly. Fifth, this method affords the opportunity to quantitate changes in small proteins as well as those that may not contain any cysteine residues at all. As our approach hinges on the incorporation of the label at the amino acid level, it follows that further protein purification strategies are unaffected.

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FIGURE LEGENDS

Fig. 1: **A schematic of SILAC (using leucine) and ICAT labeling strategies.** Shown are the steps involved in the two strategies. For SILAC, the cell culture has been adapted to normal leucine or Leu-d3 media at the start of the experiment and the isotopic state information is already 'encoded' into the amino acid sequences. Therefore, protein populations can be mixed directly after lysis and subjected to protein purification procedures. ICAT allows use of protein material from non-living sources but requires chemical modification and affinity steps.

Fig. 2: **Incorporation of Leu-d3 in proteins at various time points.** Leu-d3 media was introduced to cells already adapted to Leu-d0 at the initial time point, and without further passage of cells. Samples were obtained at 12 hrs, 24 hrs, 48 hrs, 3 days, and 5 days. The doubling time of the NIH 3T3 cells was 24 hrs (data not shown). The peaks annotated with d0 and d3 are the triply charged peaks of the peptide VAPEEHPVLLTEAPLNPK which contains three leucines. The panel shows the complete incorporation of Leu-d3 in the peptide at Day 5. The peak cluster marked with an (*) is an unrelated non-leucine containing peptide.

Fig. 3: **The use of dialyzed serum avoids nonspecific incorporation of nonlabeled leucine derived from serum in the Leu-d3 samples**. The left panel shows the same peptide as in Fig. 2 grown in medium with dialyzed serum and supplemented with Leu-d0. Only the Leu-d0 peptide is observed. The peak cluster marked with an asterisk (*) is an unrelated non-leucine containing peptide. When cells are grown in

media containing non-dialyzed serum, a heterogeneous population of Leu-d0 and d3-labeled peptides is observed (middle panel). This is due to scrambling of the population due to incorporation of free amino acids from non-dialyzed serum. Shown in the middle panel, the Leu-d0 form of the peptide VAPEEHPVLLTEAPLNPK can still be observed after 3 doublings in media containing Leu-d3 and non-dialyzed serum. The right hand panel shows the spectrum of the peptide grown in Leu-d3 supplemented media with dialyzed serum. Only the fully Leu-d3 substituted peak is observed.

Fig. 4: **A. Observed ratios are similar to expected ratios in mixing experiments.** Volumes of cell lysates were mixed in known proportions prior to resolution on SDS-PAGE. A prominent gel band was excised, proteins were digested in gel and peptides analyzed by nanoelectrospray. As can be seen in panel A for two different peptides, the observed peptide ratios were similar to expected ratios. The two examples shown here are peptides from alpha enolase (left) and beta-actin (right). **B. Comparison of MS/MS spectra from Leu-d0 and –d3 labeled peptide, VAPEEHPVLLTEAPLNPK, from mixing experiments**. The same gel position as in panel A was excised for a mixing ratio of 1:10 (Leu-d0:Leu-d3). The left panel shows the MS/MS spectrum from the Leu-d0 labeled peptide and the right panel shows the spectrum of the Leu-d3 labeled form. Note that the fragmentation patterns are identical as expected. The fragment ions are indicated with ions from the full-length peptide in normal font. Fragment ions from the molecular species generated by the internal cleavage of the first two amino acids (PEEHPVLLTEAPLNPK) is labeled in bold oblique font. Doubly charged ions are indicated with (‘’). The masses of three prominent fragment

ions are indicated in the figure to demonstrate the shifts in the masses of fragment ions. The asterisks in both panels (*) point out the y_3 ions which bear the same mass, 358.2, in both spectra. The y ion fragments from y_4 to y_8 are shifted by +3 Da (+ 1 Leu-d3), y_9 is shifted +6 Da (+ 2 Leu-d3) and y_{10} to y_{17} fragments are shifted +9 Da (corresponding to 3 Leu-d3). The observed ratios of peak intensities between the left and the right panel closely matches the expected ratio of 1:10 in mixing experiments (see Table 1).

Fig. 5: **SILAC applied to C2C12 myoblast differentiation.** C2C12 myoblasts were grown and passaged in Leu-d0 and Leu-d3 labeled media to allow full incorporation of Leu-d3. Cells were grown to confluence and muscle differentiation induced by a five-fold reduction in serum. The micrographs shown are of C2C12 cells in the process of differentiation in Leu-d3 media (Panel A). **B. 1D gel electrophoresis of C2C12 cell lysates at day 0, day 2, and day 5.** Gels were silver stained and seven positions whose intensity changed were marked (see arrows.) Bands were then excised from other 1D gels that contained a 1:1 mixture of lysates from Leu-d0 from day 0 and Leu-d3 from days 0, 2 and 5. Gel bands were digested and analyzed by nanoelectrospray on a quadrupole time-of-flight instrument (QSTAR, ABI/MDS-SCIEX) equipped with a nanoelectrospray source (Protana Engineering A/S).

Fig. 6: **Quantitation of 9 proteins during C2C12 cell differentiation by SILAC.** Data was obtained from the experiment described in Fig. 5 and the text. The histograms represent the quantitative data obtained for nine proteins identified from the seven

bands marked in Fig. 5B, at Day 0 (white), Day 2 (gray) and Day 5 (black). For the top three panels three peptides were used in quantitation and for the other panels two peptides were selected for each protein. The error bars represent the variation between the ratios for the different peptides.

TABLES

Table 1: **Quantitation of peptides by their fragments**

Ion	Peptide Label	m/z	Intensity	Observed Ratio
b_2	Leu-d0	171.1	127	10.0
	Leu-d3	171.1	1264	
y_5	Leu-d0	568.3	243	9.1
	Leu-d3	571.3	2221	
y_6	Leu-d0	639.4	117	11.1
	Leu-d3	642.4	1297	
y_{10}	Leu-d0	1095.6	19	10.8
	Leu-d3	1104.6	206	

The peptide at m/z 652.0 (M+3H)3+ in Fig. 4B was fragmented in the Leu-d0 and Leu-d3 form. Intensities and ratios are listed for a number of fragment ions.

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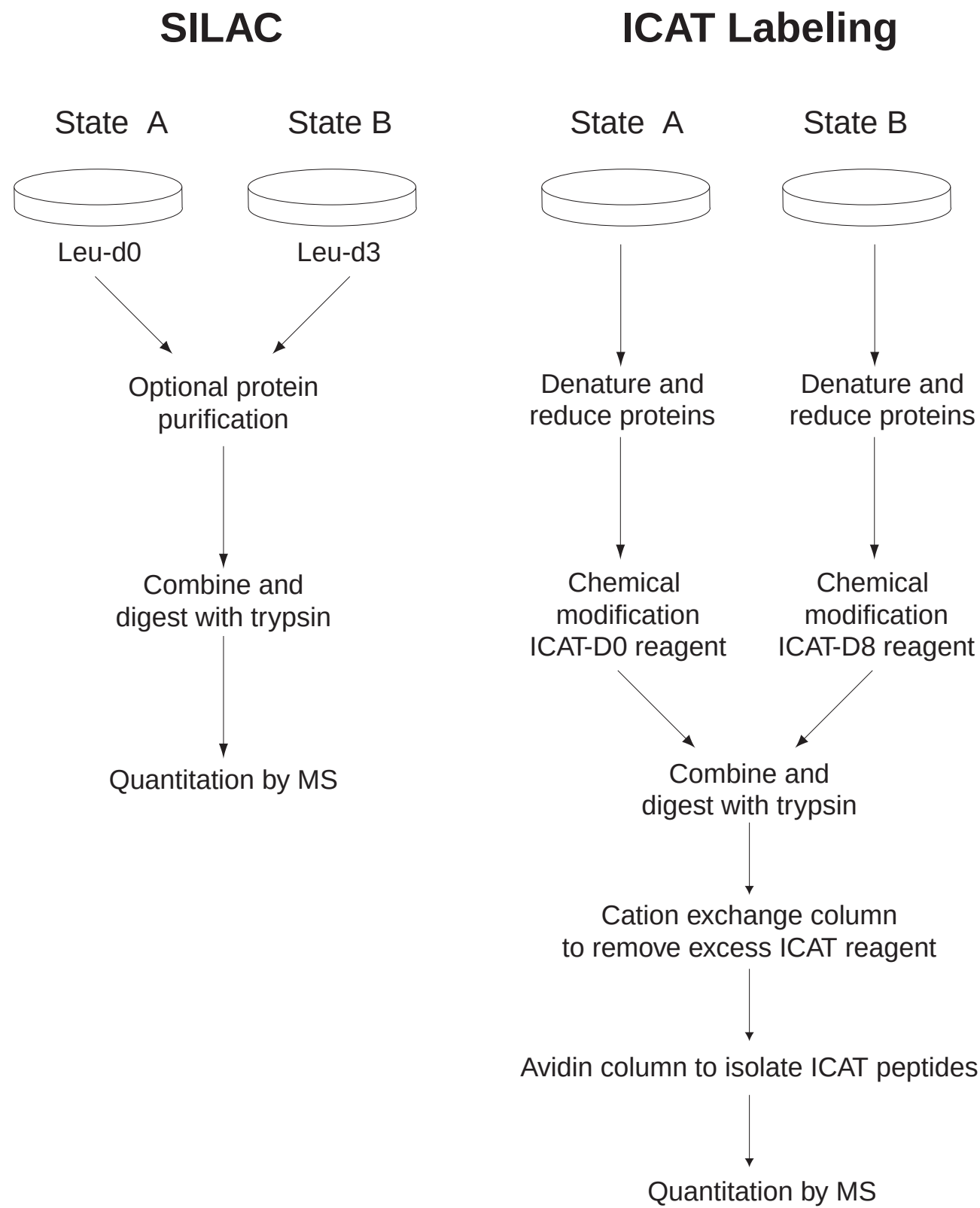
Figure 1


SILAC
State A
State B
Leu-d0
Leu-d3
Optional protein purification
Combine and digest with trypsin
Quantitation by MS
ICAT Labeling
State A
State B
Denature and reduce proteins
Denature and reduce proteins
Chemical modification ICAT-D0 reagent
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Combine and digest with trypsin
Cation exchange column to remove excess ICAT reagent
Avidin column to isolate ICAT peptides
Quantitation by MS

Figure 2

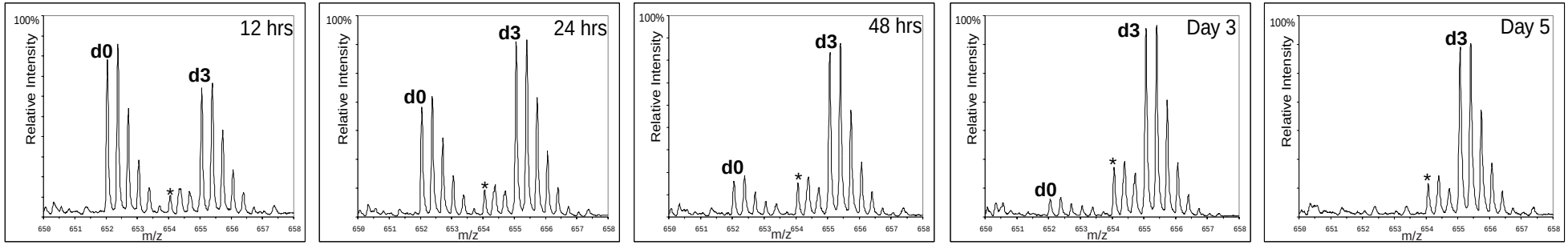
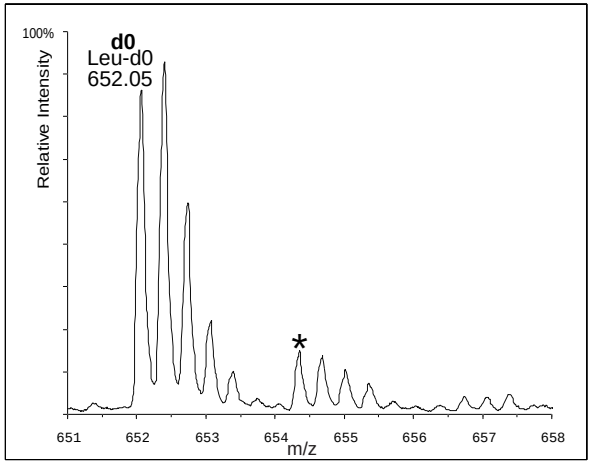
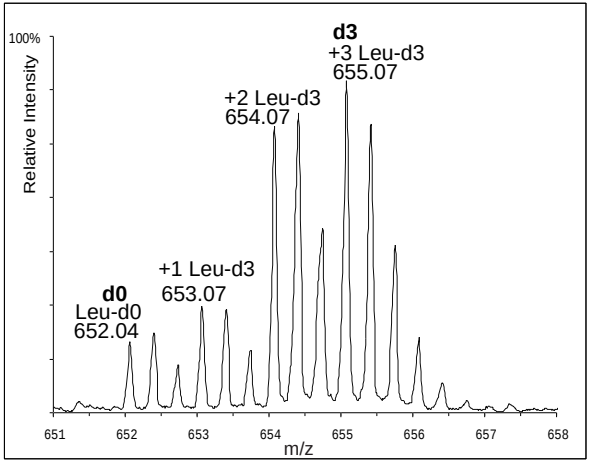

12 hrs
24 hrs
48 hrs
Day 3
Day 5
d0
d3
*
100%
Relative Intensity
650 651 652 653 654 655 656 657 658
m/z

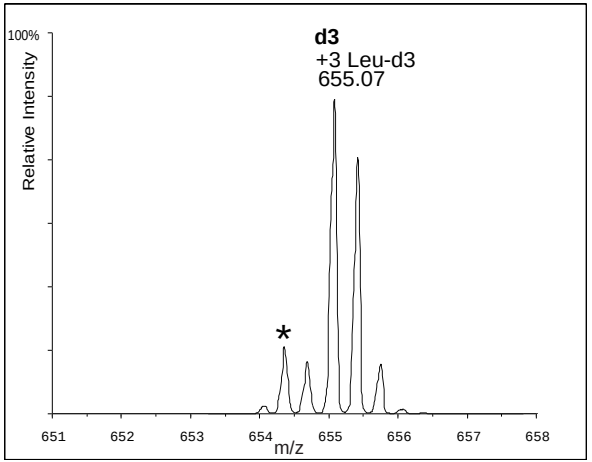
Figure 3



Leu-d0
Dialyzed Serum



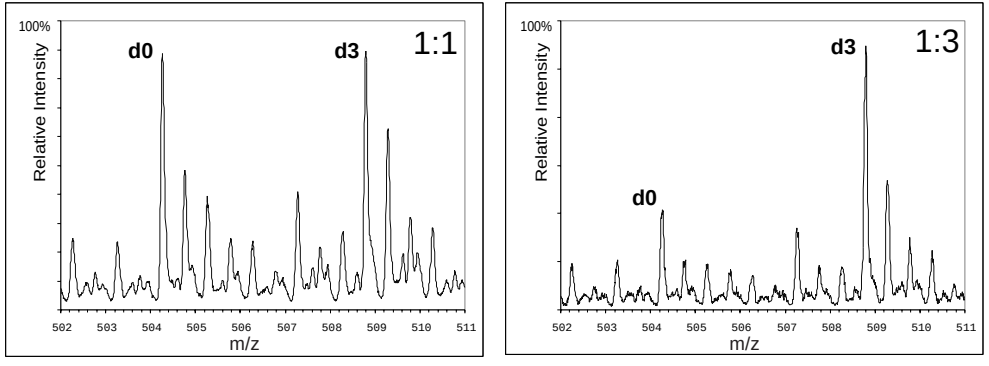
Leu-d3
Non-Dialyzed Serum



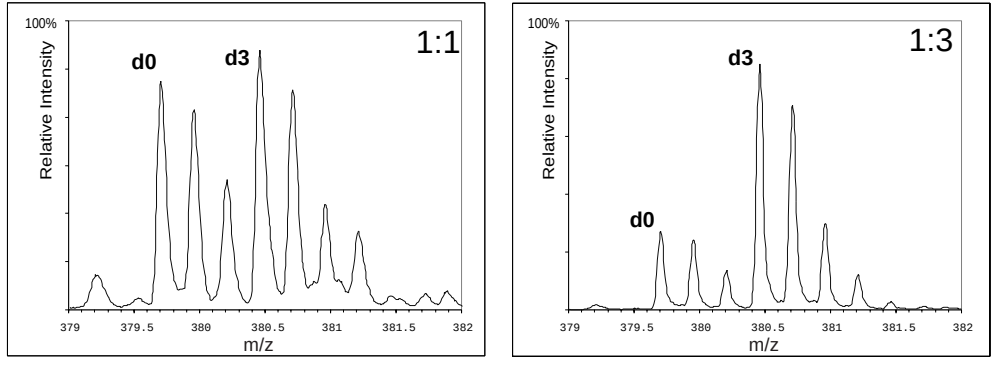
Leu-d3
Dialyzed Serum

Figure 4

A.



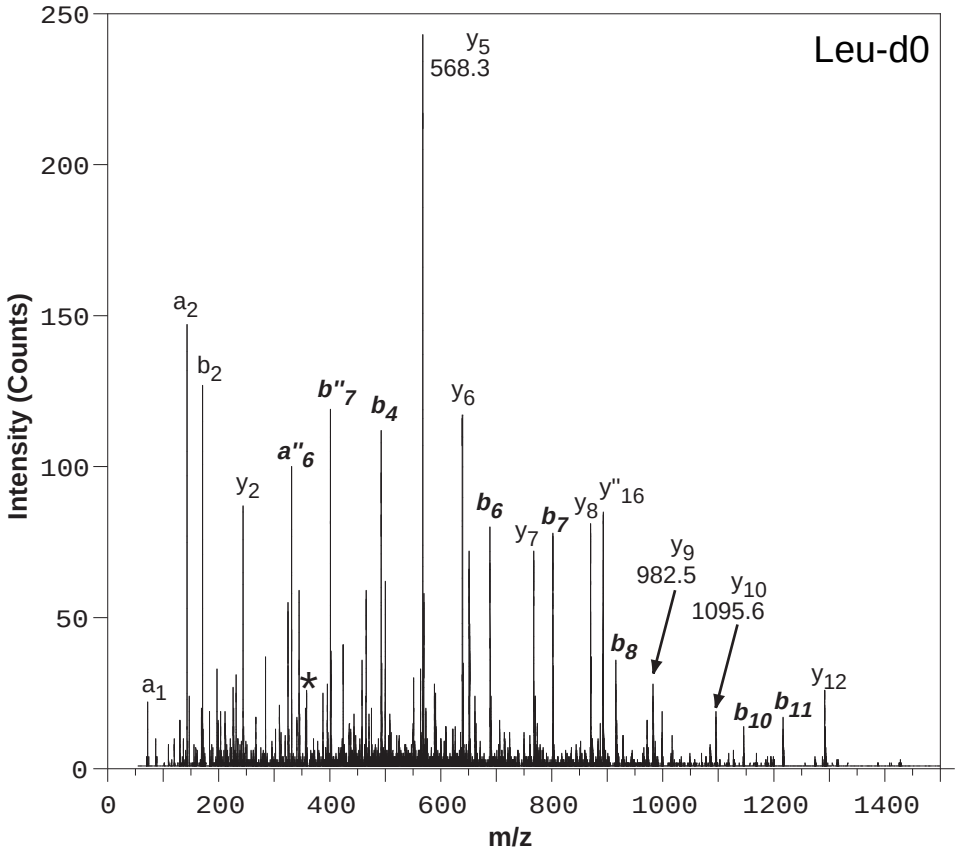
SCNC*LLL*K



IWHHTFYNE*L*R

Figure 4

B.


Leu-d0
Intensity (Counts)
m/z
y5
568.3
a2
b2
b''7
b4
a''6
y2
y6
b6
y7
b7
y8
y''16
y9
982.5
y10
1095.6
b8
b10
b11
y12
a1
*

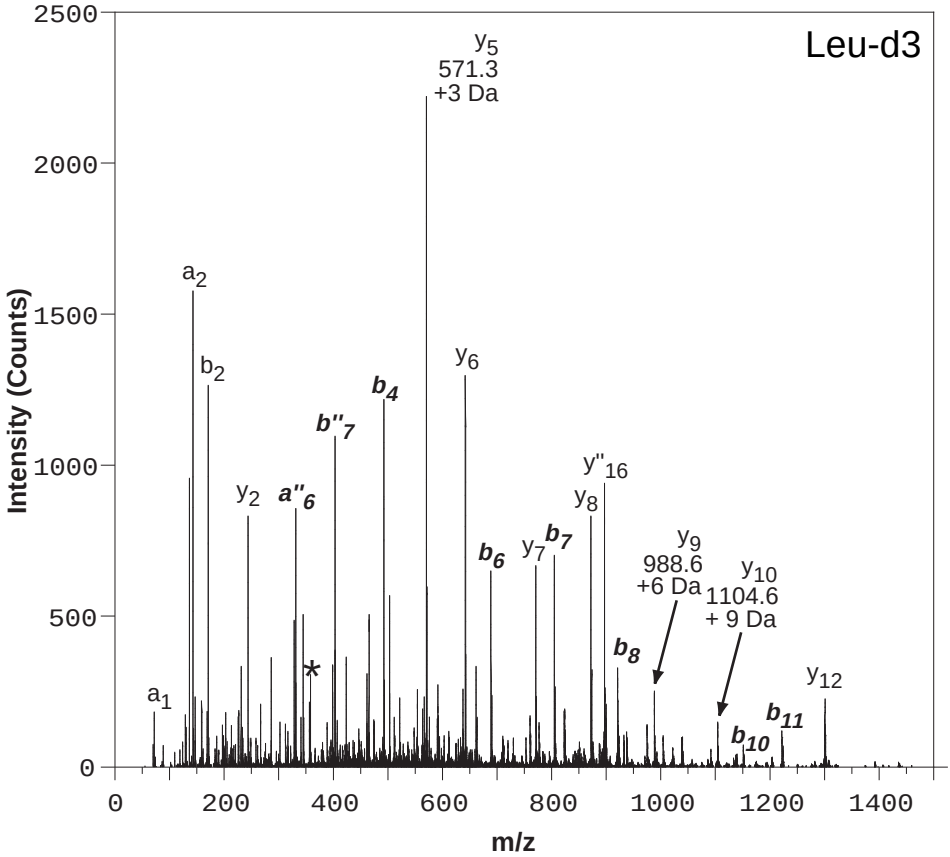
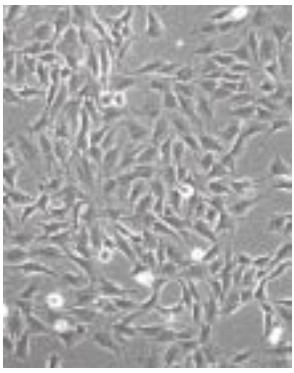
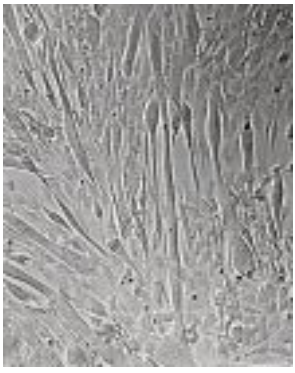

Leu-d3
Intensity (Counts)
m/z
y5
571.3
+3 Da
a2
b2
b4
b''7
y6
y''16
y8
y2
a''6
b6
y7
b7
y9
988.6
+6 Da
y10
1104.6
+ 9 Da
b8
y12
b10
b11
a1
*

Figure 5

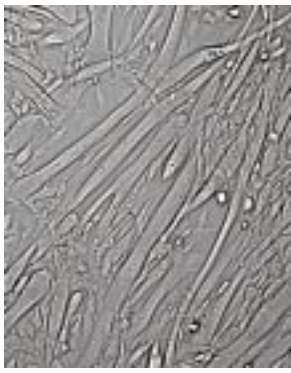
A.



Undifferentiated



Day 2 Differentiation



Day 5 Differentiation

B.

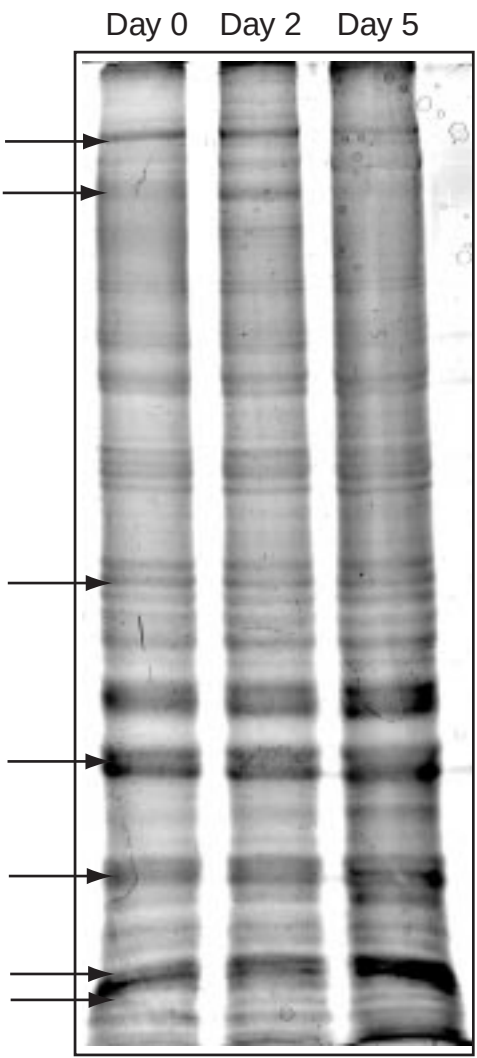
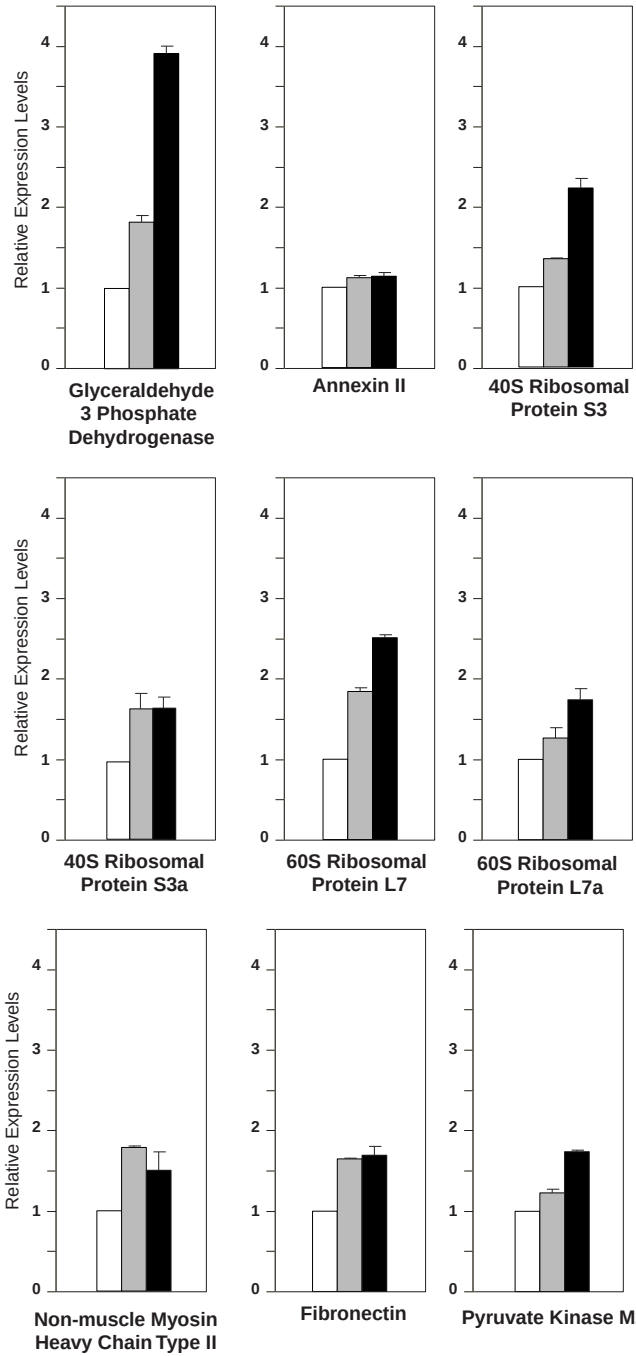


Figure 6


Relative Expression Levels
0
1
2
3
4
Glyceraldehyde 3 Phosphate Dehydrogenase
Annexin II
40S Ribosomal Protein S3
40S Ribosomal Protein S3a
60S Ribosomal Protein L7
60S Ribosomal Protein L7a
Non-muscle Myosin Heavy Chain Type II
Fibronectin
Pyruvate Kinase M2