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SILACtor: software to enable dynamic SILAC studies

Michael R. Hoopmann¹, Juan D. Chavez¹, and James E. Bruce^{1,2}

¹Department of Genome Sciences, University of Washington, Seattle, WA

Abstract

Stable isotope labeling by amino acids in cell culture (SILAC) is a versatile tool in proteomics that has been used to explore protein turnover on a large-scale. However, these studies pose a significant undertaking that can be greatly simplified through the use of computational tools that automate the data analysis. While SILAC technology has enjoyed rapid adoption through the availability of several software tools, algorithms do not exist for the automated analysis of protein turnover data generated using SILAC technology. Presented here is a software tool, SILACtor, designed to trace and compare SILAC-labeled peptides across multiple time points. SILACtor is used to profile protein turnover rates for more than 500 HeLa cell proteins using a SILAC label-chase approach. Additionally, SILACtor contains a method for the automated generation of accurate mass and retention time inclusion lists that target peptides of interest showing fast or slow turnover rates relative to the other peptides observed in the samples. SILACtor enables improved protein turnover studies using SILAC technology and also provides a framework for features extensible to comparative SILAC analyses and targeted methods.

Introduction

Stable isotope labeling by amino acids in cell culture (SILAC)^{1, 2} has become a highly useful tool for quantitative analysis of complex samples by mass spectrometry. Key to the adoption and widespread use of SILAC has been the development of several computational tools to facilitate data analysis, namely feature detection, improved peptide sequence identification, and improved quantification^{3–7}. These software tools punctuate the necessity of computational approaches to support the efforts of SILAC method development.

The versatility of SILAC is seen in its application to a large number of biological studies^{8–11} as well as its flexibility in design and implementation^{2, 12, 13}. In particular, recent studies have shown SILAC to be a useful tool for the analysis of protein turnover^{14–16}. In these studies, cells are fixed at one SILAC labeling stage and protein turnover is observed, in pulse-chase fashion, after switching to the complementary SILAC-labeled media. To fit accurate turnover rates, the protein SILAC ratios must be observed and compared across multiple time points, thus adding an additional dimension of complexity to the analysis. Each time point can be considered an independent sample, and thus performing a time point analysis is similar to performing a comparative analysis of multiple SILAC samples. Although computational tools exist for analysis of individual SILAC samples^{3, 17}, tools are not available for comparative analysis of multiple samples in an automated fashion. SILACtor was developed to fulfill this need and provides unique capabilities for any time course studies based on SILAC experimental design.

²Corresponding Author: James E. Bruce, Department of Genome Sciences, University of Washington, 815 Mercer St., Seattle, WA 98109 jimbruce@u.washington.edu.

Many of the computational challenges encountered with shotgun proteomics data analysis must also be addressed in SILAC data analysis. This is especially true for SILAC samples that contain effectively double the complexity over non-labeled peptide samples. Studies have shown that existing shotgun analysis methods are incapable of sampling all ions in a complex mixture, as evidenced by large variation that occurs with repeat injections of the same sample in shotgun analysis¹⁸. To help overcome this issue in shotgun proteomics, numerous strategies have been developed for sample fractionation^{19–22} or the computational targeting of peptides by accurate mass and retention time lists^{23–25}. These approaches are logically also suitable to improve SILAC sample analysis; however, the use of mass and retention time lists in the context of SILAC analysis has not been explored. For example, generation of mass and retention time target lists for only those ions that show SILAC pair intensity ratios that significantly deviate from the mean could be used to target the most experimentally relevant peptides in subsequent sample injections.

Presented here is a new software tool, SILACtor, for the automated comparative analysis of SILAC data across multiple samples. This manuscript gives a step-by-step, detailed summary of the functions of software, which has previously been used in the analysis of multidrug resistant cancer cell lines²⁶. SILACtor is used to perform comparative protein turnover profiling of HeLa cells. HeLa cell samples used for this manuscript were chosen to demonstrate the capabilities of SILACtor and not for any specific biological conclusion. Additionally, SILACtor contains features that identify potential peptides of interest for which no sequence identification was made. Mass and retention time inclusion lists are generated from these data to target the peptides of interest during subsequent injections. Although we perform a multiple sample comparative analysis, the ability to target peptides missed by repeated sample injection and data-dependent acquisition is immediately applicable to any SILAC experiment to improve identification of key proteins of interest. No published software tools currently exist to allow time-dependence of SILAC ratios to be extracted from arrays of datasets or development of lists for targeted identification of peptides with SILAC ratios altered from the mean. These capabilities can provide useful features for many labs involved in SILAC-based research and SILACtor is made available for download at: <http://brucelab.gs.washington.edu/SILACtor.php>.

Methods

Sample Preparation

HeLa cells were initially grown in complete DMEM media including 10% fetal bovine serum and 1% penicillin/streptomycin. For protein turnover analysis, the media was exchanged for isotopically heavy DMEM including $^{13}\text{C}_6$ -L-arginine and $^{13}\text{C}_6$ $^{15}\text{N}_2$ -L-lysine and 10% dialyzed fetal bovine serum. Cells were harvested by scraping each hour after media exchange for the first 8 hours, then again at 12, 24, and 48 hours. Harvested cells were washed three times with ice cold PBS and re-suspended in 100 mM ammonium bicarbonate buffer prior to freeze-thaw lysis using liquid nitrogen and sonication. The samples were then stored at -80°C . The protein yield of each time point sample was measured using Coomassie Plus Bradford Assay (Thermo). 100 μg of each sample was then digested to peptides as follows. The samples were reduced in 5 mM *tris*(2-carboxyethyl)phosphine (TCEP) and alkylated in 10 mM iodoacetamide (IAA). The proteins were then digested using trypsin (Promega), at 37°C for two hours, at a 1:200 enzyme to protein ratio.

Data Acquisition

Each time point sample digest was loaded onto a 75 micron inner diameter, 35 cm length fused-silica capillary column packed with Magic Beads (Michrom Bioresources, Inc.), using

a Waters NanoAcquity system. The column was mounted on an in-house constructed nanospray source and the peptides were analyzed using high pressure liquid chromatography (HPLC) with a binary mobile phase gradient with a flow rate of 0.25 $\mu\text{L}/\text{min}$. Mobile phase component A consisted of water with 0.1% formic acid. Mobile phase component B contained acetonitrile and 0.1% formic acid. The gradient program consisted of three steps: 1) Peptide elution from 5% to 35% solvent B for 120 minutes, 2) Column wash at 80% B for 15 minutes, and 3) Column re-equilibration at 5% B for 25 minutes. Mass spectra were acquired on a novel Velos-FT hybrid mass spectrometer developed in this lab (submitted to JASMS) with an eight scan cycle: one high resolution scan in the ICR at 50,000 resolution (at 400 m/z) followed by seven MS/MS scans in the Velos ion trap. Each sample was initially analyzed using data-dependent acquisition with dynamic exclusion²⁷. Subsequent injections targeting specific peptides of interest (described later) were performed using accurate mass and retention time inclusion lists.

Software Analysis

Precursor MS spectra were analyzed using Hardklör²⁸ with standard settings that included a maximum charge state of 5+, a maximum overlapping signal depth of 3, and correlation threshold of 0.9. MS/MS spectra were analyzed using Mascot²⁹, with variable modifications of 6.020129 Da for arginine and 8.014199 Da for lysine. The UniProt human database was searched for tryptic peptides and the search included allowance for a single missed cleavage. An expect score cut-off of 0.05 was used to filter the peptide sequence identifications prior to exporting them for use in the software analysis tools described herein.

Hardklör and Mascot results were imported into SILACTor, which was developed in-house to trace SILAC-labeled proteins across multiple time points and allow targeted analysis of peptides that appear altered in abundance but for which no sequence identification was yet made. The Hardklör results were processed in SILACTor to obtain extracted ion chromatograms (XICs) for each precursor where a persistent signal was observed over at least three consecutive spectra (allowing for a single gap) with a mass tolerance of 10 ppm. Each XIC was assigned a mass value corresponding to the monoisotopic mass of the peptide at the apex of the XIC, a retention time window corresponding to the scan time of the first and last scan events in the XIC, and an XIC area that is the sum of the peptide isotope distribution intensities in each scan contributing to the XIC. Once XICs are compiled for all the data, the operation of SILACTor can be divided into five major functions: 1) Build a database of peptide sequence identifications by accurate mass and retention time, 2) combine technical replicates of the same samples to get more accurate SILAC ratios, 3) target unidentified peptide isotope distributions with interesting SILAC ratios, 4) create protein analyses from the SILAC peptide pairs with sequence identification, and 5) profile changes in protein turnover across multiple time points for each sample. Each of these functions is described in detail below and shown schematically in Figure 1. SILACTor was written in C++ and was operated on a single desktop computer of modest specifications. SILACTor is available for both the Windows (Microsoft) and GNU/Linux operating systems at <http://brucelab.gs.washington.edu/SILACTor.php>

1. *Accurate mass and retention time database:* An accurate mass and retention time database was made from the Mascot database searches of the MS/MS spectra obtained at all time points. To build the database, the peptide sequences identified with Mascot from each injection were stored with their mass and a range of retention times that included the lowest and highest retention times over which the peptide was identified. To reduce the propagation of false sequence identifications, SILACTor requires that peptides be identified in 25% or more injections. Peptides whose retention times drifted for more than 10 minutes were also removed from the database. Additionally, peptides identified from the wash and equilibration phases

of the chromatography were excluded because these phases were the least consistent from run to run. Optionally, an AMT peptide database generated by other means at the user's disposal can be used instead by importation through a simple text format.

2. *Technical replicate analysis:* SILACTor was used to perform technical replicate analyses for each time point sample. Each SILAC pair was matched by accurate mass (within 10 ppm) and retention time (within 5 minutes) across the replicate injections at each time point. The relative isotope abundance (RIA) for each pair was averaged across the number of injections over which they were observed; this reduced the effect of measurement artifacts for each peptide ratio. RIA is defined as the ratio of light peptide to the total amount of observed peptide (light plus heavy).
3. *Targeting peptides of interest:* To identify potential peptides of interest that were not identified by data dependent acquisition (DDA), i.e. those peptides that show unexpected RIAs, the mean and standard deviation for the set of SILAC pairs was computed from the technical replicate analyses for each time point sample. A mass and time target inclusion list was generated for the SILAC pairs for which no peptide sequence was yet obtained and had RIA greater than a standard deviation from the mean. The samples were re-analyzed in the mass spectrometer using the mass and retention time target inclusion lists in which an MS/MS scan event is only triggered if a precursor ion matches the same m/z and retention time as an entry in the target list.
4. *Protein analysis:* Protein inferences were made from peptide sequence identifications. If multiple peptides were observed for the same protein, their RIAs were averaged together to obtain an overall protein RIA. Peptides found with RIAs that exceeded twice the standard deviation derived from measurements on other peptides from the same protein were removed from the protein analysis and the average protein RIA was re-calculated.
5. *Turnover analysis over multiple time points:* Protein RIAs were obtained for each time point analysis after the technical replicate analyses were performed. Changes in protein RIAs were then profiled across all time points. An exponential trend line was fit to the protein profiles to obtain a rate of loss of light proteins using published methods¹⁵, with a few notable differences. In brief, the average SILAC protein RIA at each time point was fit to an exponential loss function to obtain k_{loss} :

$$RIA = e^{(-k_{loss} * t)} \quad (\text{eq. 1})$$

where t is time in hours. Because we are measuring the incorporation of heavy label from a starting time point that contains no heavy labeling, the curve fitted to the function was forced through 1 at time point 0 hours. It should be noted that SILACTor can be applied to heavy-to-light SILAC transitions and to light-to-heavy SILAC conversion. In the case of heavy-to-light SILAC conversion complete enrichment at time $t=0$ is generally not possible, but the approximation of fitting points through unity at $t=0$ was successfully applied by Doherty et al.¹⁴ SILACTor yields k_{loss} values from SILAC datasets and, with measurement of k_{dil} (the dilution rate resultant from measured cellular growth rate), the relative degradation rates k_{deg} for each protein can be determined. However, because this value is a constant, it effects all proteins equally, and thus all further data analysis is presented in terms of exponential loss (k_{loss}) of light protein.

Results

SILAC analysis, protein turnover profiles, and peptide targeting was performed using in-house developed software named SILACTor. SILACTor accepts Hardklör analysis results of MS spectra to identify SILAC pairs of peptide isotope distributions and assigns peptide sequence identifications from MS/MS spectra analyzed using database searching algorithms. For these data, Mascot was used for peptide identification; however, peptide identification results from the use of any algorithm may be converted to a simple tab-delimited text format and imported into SILACTor. SILACTor contains features that track peptide and protein RIAs across multiple samples, and output the results in tab-delimited text format. For this experiment, the different samples are time point harvests of HeLa cells. SILACTor can be used with technical replicates for each sample to build statistical significance into the results. Additionally, SILACTor is used to produce mass and time inclusion lists for follow-up targeted analysis of SILAC peptide pairs of interest for which no sequence identification was made.

HeLa cell harvests were obtained after growth in heavy media at each hour for the first eight hours, and again at 12, 24, and 48 hours. The samples were digested with trypsin and analyzed using standard data-dependent shotgun analysis on a Thermo FT hybrid containing LTQ-Velos on the front. Two injections and acquisitions were made for each time point sample. MS/MS spectra were analyzed with Mascot to obtain peptide sequence identifications. Because the column and chromatography conditions remained constant for the entire analysis, the Mascot search results were pooled to create an accurate mass and time database to increase the sample coverage for all time points in the analysis, as described in the methods. Peptide isotope distributions (PIDs) were identified from the MS spectra using Hardklör and requiring the PIDs to persist across three or more consecutive spectra, as described in the methods. The Hardklör software is highly customizable to analyze data acquired on virtually any high-resolution instrument²⁸ so that use of SILACTor is not limited to data acquired on the in-house developed Velos-FT used in this experiment. Peptide sequence assignments were made for the PIDs using the pooled Mascot results and SILAC peptide pairs were assigned where 1) two PID masses differed by 6.020129 Da or 8.014199 Da, for arginine- or lysine-terminated peptides, or by 12.0402648 Da, 14.034336 Da, or 16.0284072 Da to allow for a single missed cleavage, and 2) the PID extracted ion chromatograms (XICs) correlated by intensity with $r > 0.90$. The requirement for chromatographic persistence and correlation of XICs reduced the likelihood of spurious SILAC pairings, and the parameters are adjustable in SILACTor. The SILAC RIAs were determined from the chromatographic peak areas of the PIDs, as described in the methods. Peptide pairs with sequence identifications were then grouped by protein to obtain average protein RIAs at each time point (Figure 2). Protein RIAs were then plotted across each time point and an exponential decay curve fit to obtain a k_{loss} value for each protein as described in the methods.

The number of SILAC peptide isotope distribution pairs identified at each time point ranged from 60 to 3904. The fewest pairs were identified in the one hour time point where heavy labeled proteins are at their lowest quantities, and the most pairs were detected at 24 hours, or the approximate time of cell doubling. A total of 5243 non-redundant SILAC pairs were observed across all time points. However, only SILAC pairs with peptide sequence identifications were used to infer protein turnover rates, which are summarized in Supplemental Table 1. At each time point, the average RIA was used for proteins with multiple peptide identifications, as described in the methods and shown in Figure 2. If a peptide's RIA exceeded twice the standard deviation from the mean of all RIAs in the protein, it was removed and the RIA average was re-calculated. This step was performed because a single peptide with an inconsistent or incorrect RIA calculation could significantly

skew the calculated protein RIA. Such inconsistent peptide RIAs occasionally occur due to pairing with another peptide at the labeled mass or due to natural phenomena of peptides shared by multiple proteins and protein isoforms. Although removed from the protein RIA average calculation, these outlying peptide RIAs are reported to the user for manual inspection. All output is provided in tab-delimited text files that can be easily incorporated into spreadsheet or other software for easy reading and sorting (Supplemental Table 2).

Protein k_{loss} values were computed by fitting an exponential loss function to the average RIA obtained for a protein at each time point, as described in the methods. This technique for computing the k_{loss} differs in two ways from a previous approach.¹⁵ First, turnover was measured by incorporation of a label, and thus the k_{loss} function can be fit to 1 at time point 0 because the proteins are known to contain no heavy label. This is far simpler than starting from a labeled sample in which 100% labeling is generally not possible and the actual starting value must be experimentally determined. The second difference is that SILACtor was used to compute an average protein RIA for each time point, rather than compute k_{loss} for each peptide and average together to produce the protein k_{loss} . Different peptides from the same protein have been shown to exhibit the same degradation curve¹⁴ and are used here to yield protein-level statistics for measured protein degradation rates. For comparison, the set of peptide k_{loss} values and the protein k_{loss} value as computed from the methods are shown in Figure 3 for ribosomal protein RLA0. The peptide k_{loss} values ranged from 0.0297 h⁻¹ to 0.0353 h⁻¹, with an average k_{loss} of 0.0326 h⁻¹. Using our method, a similar k_{loss} of 0.0332 h⁻¹ was obtained for RLA0. Protein k_{loss} values were obtained for 512 proteins, ranging from 0.0145 h⁻¹ to 0.552 h⁻¹ (Supplemental Table 1). The k_{loss} values showed a non-normal distribution (Supplemental figures 1 and 2). Others have demonstrated a similar lack of normality of the distribution of k_{deg} values¹⁴. Thus, despite the differences in our software design, protein k_{loss} values were obtained for a substantial number of proteins and corroborate existing findings.

The mean RIA was calculated for all SILAC peptide pairs for each time point sample. However, at any time point many SILAC peptide pairs were observed for which no sequence identification was made. Mass and retention time target lists were generated for the SILAC pairs that had a RIA value that was significantly greater or less than the sample mean. Although analysis of all protein turnover rates indicated a non-normal distribution, a normal distribution was assumed here as an approximation of proteins that show a fast or slow rate of turnover. These unusual pairs were targeted specifically because they represent potentially the most interesting proteins; however, this targeted follow-up approach could be applied to all the SILAC pairs to improve sample coverage. The targeted method was tested using the 24 hour time point sample. First, two replicate injections of the sample were made using shotgun data dependent acquisition, and peptide sequences were identified using Mascot. A mass and time targeted list was generated from the SILAC pairs that showed a RIA at least one standard deviation from the sample mean and for which no sequence identification was made. Two replicate injections were then performed using the mass and time targeted list, and peptide sequences were identified using Mascot. The peptide IDs from the mass and time targeted run were then used to obtain peptide identifications for the SILAC pairs observed in the original shotgun-analyzed replicates.

The results of the use of mass and time lists show the benefit of specifically targeting peptides of interest. The initial shotgun analysis at 24 hours showed a mean RIA of 0.417 with a standard deviation of 0.056. Therefore, the mass and time list specifically targeted peptides with a RIA less than 0.361 and greater than 0.473 for which no peptide sequence had been obtained. Figure 4 illustrates the benefit of using mass and time targeted lists. Because of its low abundance, the CD2A1 peptide was missed by data dependent shotgun analysis. However, its RIA of 0.187 is very different from the sample mean and therefore

the peptide is of interest. A mass and time list was then used to target this peptide of interest and identification of the peptide sequence was obtained. Using the target list, 215 unique peptide spectrum matches (PSMs) were matched back to SILAC pairs. 83 (38.6%) of these PSMs were novel peptide identifications, indicating that the mass and time lists successfully targeted a subset of peptides missed by the shotgun analysis. Figure 5 illustrates the unique ability to SILACtor to extend peptide identification to lower intensity species that are observed with SILAC ratios significantly altered from the mean. When the distribution of RIAs for these novel PSMs is compared to the other PSMs identified without the targeted list, the distribution of RIAs is much wider for the peptides identified via targeting than those selected for identification without targeting. These results clearly illustrate the benefit of a targeted approach to identify peptides of interest. This is a generally useful feature for any SILAC experiment since it can increase the number of identified proteins that are found with altered SILAC pair ratios.

Discussion

Here we presented SILACtor, a software tool for the automated analysis of protein turnover using SILAC technology. These studies involve the comparison of many proteins over multiple time points, requiring several sample preparation and data acquisition iterations. Protein turnover analyses are often performed manually, limiting the expediency and desirability of performing such experiments. Therefore, tools that can automate the analysis of protein turnover are a valuable resource to advance our understanding of cellular processes. Although time point analysis was chosen to illustrate the utility of SILACtor for comparative analyses across multiple samples, it is possible to extend this approach to other types of analysis, such as comparison of an array of conditions where more than two samples are to be compared. Thus the concepts and capabilities of SILACtor presented here have immediate application in time course studies, but have potential in many other areas. In general, many time-dependent processes can be effectively analyzed with SILAC samples in this way to greatly accelerate the types of studies that can be performed with SILAC.

The use of high-resolution precursor information increases the power to identify peptides and proteins when performing SILAC time point analysis. Analysis of data-dependent MS/MS acquisition shows that complete sample coverage is not possible for complex samples, and the variability is as much as 30% from run to run¹⁸. Limiting the peptide identifications for any given injection to only those for which an MS/MS event was obtained reduces the ability to consistently trace peptides and proteins across time points, especially if the peptides are of low abundance relative to the rest of the sample. Because the chromatography conditions remained identical throughout the experiment, successful identification of a peptide sequence from one time point sample was used to identify a peptide at the same accurate mass and retention time for which no MS/MS event occurred in another time point sample^{30, 31}. This accurate mass and time (AMT) database need not be generated prior to data collection³² because the nature of time point analysis requires many sample injections into the mass spectrometer, and thus a well populated database can be achieved for a single experiment. Using the AMT database greatly increased the number of peptides and proteins that were identified in all the samples, creating a much more comprehensive analysis of the data.

The accurate mass and retention time inclusion lists generated in this experiment differ from typical inclusion lists in ways beneficial to SILAC data acquisition and analysis. For example, SILAC experiments can result in larger inclusion lists due to the presence of multiple isotope distributions for each peptide. For SILAC analysis, only one entry per SILAC peptide pair is needed: either the light or heavy peptide, which significantly reduces redundancy in the inclusion lists. Careful choice of which label to target will depend on the

expected RIAs for the sample, and the list can be tailored to choose whether to use the light or heavy peptide if one is greater than the other. For example, in the early time point analysis presented here, the list would use only the light peptide masses, which are greater in abundance and would likely provide the best MS/MS spectra for identification. However, for late time points, such as 48 hours or more, the heavy peptide masses would be a better set of targets. For improved validation of SILAC pairs of peptides, both light and heavy peptides can be placed in the list to provide sequence validation for both signals in a SILAC pairing. Furthermore, all signals that could not be paired as SILAC partners could be excluded from the target peptide list. Thus, the mass and retention time inclusion list from SILACtor can target only those peptides with RIAs that suggest they are relevant to the SILAC analysis.

Accurate mass and retention time inclusion lists can be a simple tool for targeting peptides of interest. Although the inclusion lists used in this experiment specifically targeted peptides of interest based on their RIA variation from the mean, a far simpler technique for generating lists is possible for any typical SILAC experiment. For example, a standard SILAC experiment is comprised of a 1:1 mixture of cells grown in light or heavy media with the goal of identifying proteins that show different relative abundance ratios. With an expected 1:1 ratio for every observed peptide, the inclusion list can easily be devised to target all peptides that show an unequal relative abundance ratio. Thus, subsequent sample injections in the mass spectrometer can be used to target the peptides and proteins of the most biological importance to the experiment. Given that SILAC samples are by definition doubly complex over non-labeled samples in terms of potential targets for MS/MS identification, effective use of inclusion lists would dramatically improve SILAC experiment results.

Conclusion

SILACtor is a software tool for the automated analysis of protein turnover rates or virtually any dynamic measurement using SILAC technology. The software makes evident the need for specialized tools that simplify the difficult task of performing comparative analysis of SILAC data across multiple samples. Despite the direct application of SILACtor to protein turnover profiling, many of the features of the software are extensible to other SILAC applications. The generation of accurate mass and retention time inclusion lists targeting peptides that show unusual SILAC label ratios can be applied to any SILAC experiment for which identifying these peptides provides meaningful results. The automated analysis methods and tools presented here further extend the versatility of SILAC technology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic diagram of the steps incorporated in SILACtor analysis. SILAC datasets are first converted into a database of peptide sequences, accurate masses and retention times for all identified peptides. Technical replicates are analyzed, combined and an average RIA is calculated for each SILAC mass pair, identified or not. SILAC peptides with ratios that differ significantly from the mean for which previous MS/MS experiments were not performed are targeted for MS/MS analysis. All SILAC peptides that were identified to originate from a common protein are grouped and protein levels are calculated based on the average of all grouped peptides. Finally, light-labeled protein levels derived from multiple time points acquired after switching to heavy SILAC cell culture medium are used to determine loss of light-labeled proteins.

Figure 2.

Example of fitting the exponential decay curve. At each time point, measurements in relative isotope abundance (RIA) for the peptides observed for a given protein are averaged together. The mean RIA values are used to fit an exponential decay curve to obtain a k_{loss} coefficient for the protein.

Figure 3. Comparison of peptide and protein RIA calculations. The RIAs for individual peptides from the protein, RLA0, are plotted with dashed lines. The protein RIA is plotted as a solid line.

Figure 4.

Example of targeted analysis using automatically generated mass and retention time inclusion lists. The peptide represented by peaks at 450.245 and 452.252 is of interest due to the unusually low RIA value when compared to the rest of the peptides in the sample. However, the peptide was not selected for MS/MS because of its low abundance relative to other more ions eluting at the same time. The mass and time inclusion list targets this specific peptide for MS/MS analysis and sequence identification is made.

Figure 5.

Comparison of the RIAs of peptides identified at 24 hours using mass and retention time inclusion lists. The inclusion lists were used to target peptides without sequence identification that displayed RIAs that differed from the rest of the sample. The novel peptides identified using the mass and time lists (red bars) showed higher frequencies of low and high RIAs when compared to the shotgun acquired peptide sequences (blue bars).