Chapter 20

Proteomic Profiling of Cell Death: Stable Isotope Labeling and Mass Spectrometry Analysis

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Abstract

Proteins directly control almost all cellular processes and researchers in many biological areas routinely use mass spectrometry for the characterization of proteins. Amongst a growing list of available quantitative proteomic techniques, *S*table *I*sotope *L*abeling by *A*mino acids in *C*ulture (SILAC) remains one of the most simple, accurate, and robust techniques for cultured cellular systems. SILAC enables strict quantitative peptide measurements, thus removing false positives and facilitates large-scale kinetics of entire proteomes. In this, chapter we describe an optimized labeling strategy and experimental design for SILAC workflows for characterizing the components downstream of cell death stimuli.

Key words Mass spectrometry, Proteomics, SILAC, Proteome quantitation

1 Introduction

Programmed cell death is an essential cellular mechanism for regulating normal physiological processes and is crucial during development and in the maintenance of a healthy immune system [1]. In addition to its role in the controlled removal of cells, the selective induction of apoptosis in diseases such as cancer has become an important focus. Thus it is imperative to identify the pathways and components that are involved in the cell death pathways and to characterize their role under different conditions and stimuli. The family of Cys-dependant Asp-specific proteases called caspases drive the key mechanisms involved in programmed cell death. These caspases cleave C-terminally of aspartate and are made up of 11 distinct functional genes in the human proteome. Upon activation, these caspases initiate a downstream cascade of activation, deactivating, and translocation events on substrate proteins [2].

Several methods have been introduced to identify substrates cleaved in a caspases-dependant manner, including those that can identify the exact location of cleavage sites. Mass spectrometry based methods can be divided into those that aim to determine the

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peptides at around the specific cleavage site, and those that are applied at the global proteome level for the identification of substrates and related biological effects (both up- and downstream of caspase activation). The former methods mostly entail blocking or modifying all preexisting N-termini and depleting the subsequent de novo generated N-termini by the protease (which acts a handle for covalent attachment). Both positive and negative selection methods have been developed and have proven highly effective at mapping substrate cleavage sites [3–10]. A significant advantage of these peptide-based methods is that they identify the exact site of protease cleavage. However, as it is limited to a single peptide for protein identification, they are generally limited in the number of identifications possible (i.e., peptide parameters may not be optimal for MS identification). Additionally, contextual information about the substrate protein is not readily detectable (abundance changes and posttranslational modifications).

In contrast, global approaches applied at the whole proteome level aim to quantitate as many proteins as possible (without necessarily determining the site of cleavage) and do not bias MS analysis toward caspase substrates. Historically, two-dimensional gel electrophoresis has been used to differentiate proteome differences during cell death [11–13]; however, these techniques are limited in throughput, sensitivity, and reproducibility. Recent developments in Ultra-high Pressure nanoflow Liquid Chromatography (UPLC), mass spectrometry, and experimental workflows have dramatically improved the depth of sequencing and run-to-run reproducibility and have circumvented the methods mentioned above. In particular, *S*table *I*sotope *L*abeling with *A*mino acids in *C*ulture (SILAC) facilitates highly accurate peptide-based ratio information for every peptide identified. Comprehensive proteome coverage and accurate quantitation allows for subtle (>1.5-fold) quantitative measurements of individual proteins following triggers of cell death from the entire global proteome of a cell. This extremely high level of specificity in such comprehensive data sets is crucial for teasing out subtle nuances of death cellular signaling.

SILAC introduces a mass difference between two proteomes facilitating a reference for relative quantitation. As the two proteomes are only distinguishable by the isotope used (12C/13C, 14N/15N, and 1H/2H), they are not subject to variations in both sample processing and between LC/MS runs and are generally considered much more accurate than label-free strategies. Stable isotope methods can be subdivided into two classes: (1) Metabolic—that utilize biological incorporation of the isotopes into cells (typically the essential amino acids Arginine and Lysine are used). (2) Chemical—that utilize covalent attachment of a reagent to introduce a mass tag. This protocol will be limited to metabolic incorporation and any culture system where the amino acid source is defined can be labeled with SILAC.

In this protocol, we describe the adaption and testing of cells in SILAC media for efficient incorporation (which is particularly important when working with new cell lines for the first time). SILAC and conventional sample analysis differ only in the preparation of the media, adaption of cells, and mixing of the protein lysates prior to sample processing and MS.

Here, we also describe the use of UPLC coupled to nano-electrospray ionization mass spectrometry (nano-LCMS) for SILAC sample data acquisition (*see* **Note 1**). As researchers will have access to a variety of nano-LCMS systems from different vendors, we will describe the general principles and minimum requirements for SILAC sample analysis. For an introduction to MS peptide sequencing and proteomics, please refer to refs. 14–16. For data analysis, we will briefly detail the important steps using MaxQuant as an example data analysis workflow (that can be used with Thermo Orbitraps, Bruker QTOFs, and Sciex TripleTOFs).

Experimental design—SILAC involves incorporating stable isotope containing amino acids during cellular protein synthesis and typically involves Arginine and Lysine containing a combination of substituted 13C and 15N atoms in the amino acid molecule. Two populations of cells are grown in separate medium formulations in (1) in light media (containing the natural isotope abundance) and (2) heavy medium containing the SILAC amino acids chosen (*see* Fig. 1). The aim is to completely replace the labeled amino acid in the proteome (typically take five to eight cell passages labeled media). As SILAC depends on MS for the readout, even a small percentage of unlabelled amino acid in the labeled population can contribute to the unlabelled signal, thereby introducing quantification errors into the data. In practice, through the process of cell division and protein degradation, proteomes are generally rapidly labeled. At least 97 % incorporation should be seen before beginning an experiment (and confirmed for each new cell line before an experiment is attempted). When lysates from light and heavy labeled samples are mixed together, processed, and analyzed with MS, they are differentiated by the residue specific mass corresponding to the labeled amino acid residues in the peptide. As the quantitative information is encoded in the SILAC residue, they must be selected specifically for the experiment (i.e., for trypsin it is recommended to label Arg and Lys, as it cleaves at these basic residues, leaving charged C-termini that helps facilitate MS sequencing of all digested peptides). The area under the curve of the light and heavy labeled peptides provides the quantitative information for comparison of there relative abundance.

The following protocols describe the steps required for efficient and robust generation of samples for highly accurate quantitative comparison. The labeling conditions and samples preparation has been optimized over a wide range of human and murine cell lines and provides significant quantitative coverage of the proteome

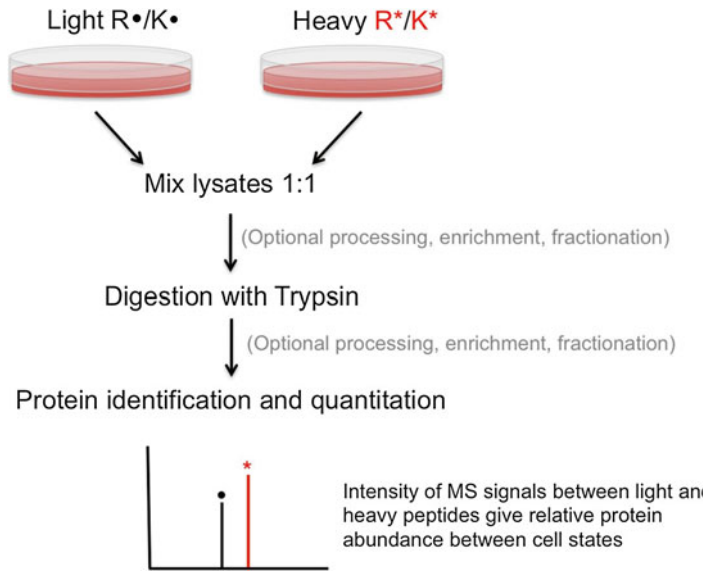

Fig. 1 Experimental design flow chart for SILAC. Cells are prepared in natural (light) amino acids and "heavy" SILAC amino acids. Cells incorporate the heavy amino acids after five to eight cell doublings and generally have no effect on morphology or growth rates. When light and heavy cell populations are mixed, they remain distinguishable by MS by the encoded isotopic mass differences. Protein abundances are determined from median relative MS peptide signal intensities. SILAC provides highly accurate relative quantification without any chemical derivatization or manipulation

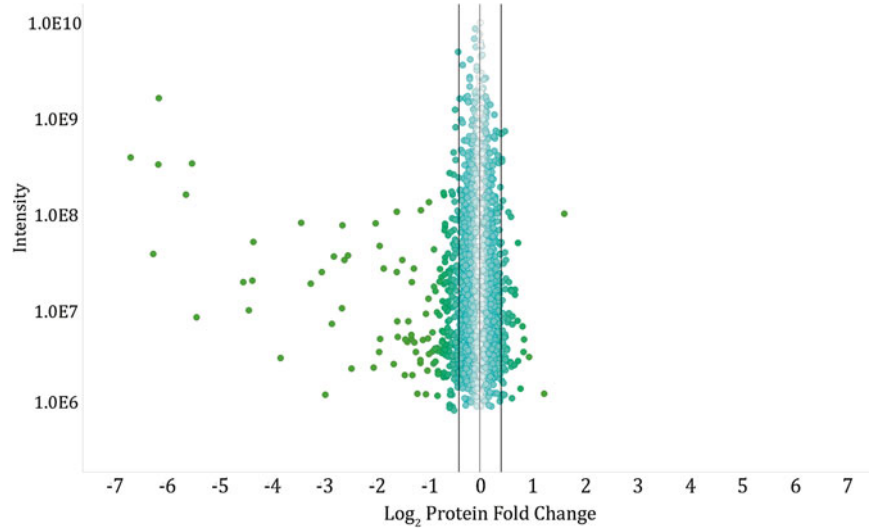

Fig. 2 A typical dataset from a SILAC experiment. Changes in MEF cell protein levels during expression of a necroptosis-inducing mutant of MLKL. Log2 protein expression ratios (WT-MLKL versus Mutant-MLKL) for >4200 mouse proteins (*x* axis) plotted against the protein intensity (summed peptide intensities per protein) (*y* axis)

(>5000 proteins quantifiable with optimized recent high-end UPLC and MS instruments). An example of the expected results can be seen in Fig. 2. Induced expression of activating mutant of MLKL was used to stimulate programmed necrosis (necroptosis) in a murine embryonic fibroblasts (MEFs) [17] and SILAC experiment was designed to identify downstream effects in this relatively uncharacterized death signaling pathway. In this example, 61 proteins (negative fold change from >4200 proteins) were significantly unregulated after a 3-h induction of the mutant MLKL protein.

2 Materials

Organic solvents are HPLC grade and reagents of the highest grade available are recommended.

2.1 Reagents

1. Cell line of choice (U937 cells used in the example shown).
2. Cell culture medium (DMEM or RPMI SILAC media—i.e., commercial cell culture medium without arginine, lysine in this example).
3. Dialyzed fetal bovine serum (FBS).
4. Glutamine.
5. L-Arginine monohydrochloride (l-Arg).
6. L-Lysine hydrochloride (L-Lys).
7. SILAC amino acids: L-arginine-13C6 monohydrochloride or L-arginine-13C615N4 hydrochloride and L-lysine-13C615N2 hydrochloride or L-Lysine-4,4,5,5-D4 hydrochloride (*see* **Note 2**).
8. Proteomics grade modified trypsin.
9. Urea.
10. SDS.
11. Tris–HCl.
12. Dithiothreitol (DTT).
13. Iodoacetamide.
14. Ammonium bicarbonate.
15. Trifluoroacetic acid (TFA).
16. Formic acid (FA).
17. Acetonitrile (ACN).
18. Sartorius Vivacon 500 30k MWCO filter units.

2.2 Equipment

1. Mass spectrometer with nano-electrospray source (best results from high resolution instrument capable of resolving >30,000 resolution—Orbitraps or QTOFs).

2. Nanoflow HPLC (best results from ultra-high pressure instruments—Waters NanoAcquity or Thermo UHPLC).
3. Protein and peptide identification software tools (*see* **Note 3**).
4. Quantitation software (*see* **Note 4**).
5. Bench-top microcentrifuge (>16,000 × *g*, with cooling).
6. Oven (for 37 °C incubation for trypsin digestion).
7. Vacuum evaporator centrifuge.
8. Waters NanoAcquity trapping column (150 μm ID 5 μm Symmetry × 20 mm)—or equivalent.
9. Waters NanoAcquity analytical column (75 μm ID 1.7 μm BEH × 250 mm)—or equivalent (*see* **Note 5**).
10. Filter-aided sample preparation microfuge tubes (FASP).

2.3 Buffers and Reagent Preparation

1. Phosphate buffered saline (PBS).
2. Amino acid stock solutions: Prepare concentrated 0.1 ml stock solutions by dissolving amino acids in PBS or FBS free culture medium. Arginine (0.798 mM), lysine (0.398 mM) are prepared as 500 times concentration stocks for use in DMEM (RPMI Arg 0.925 mM and Lys 0.274 mM). Filter amino acid solutions through a 0.22-μm syringe filter and store at –20 °C for up to 12 months.
3. Stable isotope-labeled amino acid stock solutions are prepared in the same manner but the increased molecular weight of the amino acids bearing 13C or 15N should be taken into account for equimolar amounts in both light and heavy media.

Filter Aided Sample Preparation Buffers

1. Lysis buffer: 100 mM Tris–HCl, pH 8.0, 100 mM DTT, 4 % SDS.
2. Wash buffer 1: 100 mM Tris–HCl, pH 8.0, 8 M Urea.
3. Wash buffer 2: 50 mM Ammonium bicarbonate.
4. Digestion buffer: 50 mM Ammonium bicarbonate and trypsin at 1:100 trypsin:protein ratio.

3 Methods

3.1 Preparation of SILAC Media (Triplex Labeling Optional)

1. Measure out 45 ml media in a 50 ml tube and add 5 ml dialyzed fetal calf serum.
2. Add 0.1 ml Arginine and Lysine stock solutions to tubes labeled as follows:

   Arg 0—for light label (to Light 50 ml Tube).

   Arg 6—for medium label (to Medium 50 ml Tube).

Arg 10—for heavy label (to Heavy 50 ml Tube) (for triplex only).

Lys 0—for light label (to Light 50 ml Tube).

Lys 4—medium label (to Medium 50 ml Tube).

Lys 8—for heavy label (to Heavy 50 ml Tube) (for triplex only).

(*see* **Note 6**).

3. Add Glutamine and antibiotics as required.

3.2 Adaptation of Cells from Normal to SILAC Media

1. Passage cells in a 6-well plate, growing in normal medium to 80 % confluency and seed 10–15 % (or appropriate for the specific cell line) of the original cells into two culture dishes, each containing light and heavy SILAC medium, respectively.
2. Change or subculture medium (using either light or heavy SILAC medium) every 2–3 days.
3. From the seventh passage, keep a small number of heavy labeled cells (2e5 cells) to check for SILAC incorporation (for this sample proceed to Subheading 3.3) (*see* **Note 7**).
4. For experiment samples, expand the last passage (typically at passage 8 is more than sufficient for most cell lines) into the required number of cells (5e5–2e6 for Shotgun proteomics).

3.3 Sample Preparation of Cell Lysates

1. Wash cells 2× in PBS and lyse cells with Lysis buffer (30 µl per 1e6 cells).
2. Vortex the lysate for 1 min.
3. To the 30 µl of Lysis cell mixture add 170 µl 8 M urea wash buffer and pellet the debris by centrifuging for 10 min at 16,000–20,000 × g in a bench top centrifuge at 18 °C.
4. Collect the supernatant in a Sartorious vivcon 500 filter unit, taking care to avoid DNA or the cell pellet (*see* **Note 8**).
5. Spin filter unit at 14,000 × *g* for 15 min and add 100 µl iodoacetamide to a final concentration of 25 mM to alkylate cysteines. Gently vortex to mix and incubate in the dark for 20 min. Then spin at 14,000 × *g* for 15 min at 18 °C.
6. Aspirate and discard flow through.
7. Add 200 µl 8 M Urea wash buffer and spin at 14,000 × *g* for 15 min at 18 °C. Repeat two more times.
8. Aspirate and discard flow through.
9. Add 200 µl 50 mM ammonium bicarbonate and spin at 14,000 × *g* 15 min at 18 °C.
10. Aspirate and discard flow through.
11. Repeat **step 9** two more times.
12. On the final wash, leave the remaining flow through to prevent unwanted drying of the filter membrane.

13. Add Trypsin at an enzyme: substrate ratio of 1:100 and incubate at 37 °C overnight.
14. Transfer filter unit to a FASP microfuge tube and spin at 14,000 × *g* at 18 °C for 8 min.
15. Add 40 µl 50 mM ammonium bicarbonate and spin at 14,000 × *g* at 18 °C for 8 min. Repeat once more.
16. Acidify pooled flow through to a final concentration of 1 % Formic acid.
17. Concentrate flow through using Vacuum evaporator centrifuge until dry.
18. Proceed to nanoscale LC–MS to identify proteins and peptides in a shotgun analysis.

3.4 Mass Spectrometry, Peptide Identification, and Protein Quantitation

1. Inject approximately 1–2 µg of peptide into a column for nanoflow LC–MS analysis. Typical gradient lengths of 2–4 h per sample offer the highest yield of identifications per analysis time. By increasing the number of replicates and utilizing the "match-between runs" feature of the MaxQuant we observed significantly more identifications.
2. From the acquired data, identify peptides and proteins using MaxQuant search software [18] making sure to include the modified masses of SILAC amino acids to the search parameters (*see* **Note 9**).
3. Find the ratio of summed signal intensities (area under the curve) from the light and heavy peptide extracted ion chromatograms to give the relative peptide abundance ratio between the two cell states (found in the Peptides.txt output if using Maxquant).
4. Obtain peptide ratios for all validated peptides in a protein and average these to give the average protein ratio (found in the Poteins.txt output if using Maxquant).
5. Statistical analysis of replicate samples can be performed using a variety of software packages (R, Matlab or Perseus or equivalent). Typically a minimum of three biological replicates is performed and a *t*-test performed on the ratios of all identified peptides per protein group.

4 Notes

1. The setup and operation protocol is outside the scope of this protocol and would be left to the host instrument facility.
2. Either combination of l-Arg and L-Lys are amenable for duplex experiments or all can be used in triplex labeling experiments.

3. Maxquant used in this example—Many alternatives available including Mascot, MS+GF+, SpectrumMill, XTandem, SEQUEST, or equivalent.
4. MaxQuant is used in this example. Alternatives include MSQuant, SpectrumMill, Proteome discoverer, OpenMS, or other mass spectrometer instrument vendors' software capable of handling SILAC data.
5. Longer direct injection columns can provide increased peak capacity and identifications at the expense of long injection times and longer analysis times.
6. Can be stored at 4 °C for up to 2 months.
7. You must perform this labeling check if this is the first time SILAC is used with this cell stock to avoid incomplete incorporation and potential errors in quantification. For phagocytic cell lines, Arginase conversion of heavy Arginine to heavy Proline should also be monitored at this step (and if present it can generally be rectified by doubling the free Proline concentration in the media).
8. DNA may not pellet completely and will appear as a gel-like clump, which is easily removed when aspirating with a pipette.
9. For up to date explanations of the software refer to the MaxQuant webpage—http://141.61.102.17/maxquant_doku/doku.php?id=start and the MaxQuant summer school tutorial videos—https://www.youtube.com/channel/UCKYzYTm1cnmc0CFAMhxDO8w0

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