2nSILAC for Quantitative Proteomics of Prototrophic Baker's Yeast

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Abstract

Stable isotope labeling by amino acids in cell culture (SILAC) combined with high-resolution mass spectrometry is a quantitative strategy for the comparative analysis of (sub)proteomes. It is based on the metabolic incorporation of stable isotope-coded amino acids during growth of cells or organisms. Here, complete labeling of proteins with the amino acid(s) selected for incorporation needs to be guaranteed to enable accurate quantification on a proteomic scale. Wild-type strains of baker's yeast (*Saccharomyces cerevisiae*), which is a widely accepted and well-studied eukaryotic model organism, are generally able to synthesize all amino acids on their own (i.e., prototrophic). To render them amenable to SILAC, auxotrophies are introduced by genetic manipulations. We addressed this limitation by developing a generic strategy for complete “native” labeling of prototrophic *S. cerevisiae* with isotope-coded arginine and lysine, referred to as “2nSILAC”. It allows for directly using and screening several genome-wide yeast mutant collections that are easily accessible to the scientific community for functional proteomic studies but are based on prototrophic variants of *S. cerevisiae*.

Key words SILAC, Native SILAC, 2nSILAC, Quantitative proteome analysis, Mass spectrometry, Yeast, Prototroph, Mitochondria

1 Introduction

Stable isotope labeling by amino acids in cell culture (SILAC) [1] (see also Chaps. 8, 13) combined with high-resolution mass spectrometry (MS) is a well-established technique in functional proteomic research that is widely employed to address a variety of biological questions. SILAC relies on the metabolic incorporation of stable isotope-coded “heavy” amino acids (^2H , ^{13}C , and/or ^{15}N) into proteins during cell growth, resulting in the introduction of a distinct mass difference into peptides that contain the amino acid(s) used for labeling. Consequently, differentially labeled cells can be mixed directly after harvesting, prior to subsequent sample

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preparation and liquid chromatography-tandem MS (LC-MS/MS) analysis. Thus, experimental variations resulting from separate sample handling are reduced to a minimum, which makes SILAC an accurate technique for quantitative proteome analysis using MS [2]. This is typically highly beneficial for quantitative proteomic studies that build on multistep protocols for, e.g., the preparation of organellar or other subcellular fractions, the purification of protein complexes, or the enrichment of peptides carrying post-translational modifications.

SILAC is usually performed using heavy arginine (e.g., $^{13}\text{C}_6^{13}\text{N}_4$, Arg10) and lysine (e.g., $^{13}\text{C}_6^{13}\text{N}_2$, Lys8) for labeling. When combined with the standard protease trypsin for the proteolytic digest, all peptides contain at least one labeled amino acid (except for the C-terminus of a protein), thereby maximizing the number of SILAC peptide pairs available for accurate protein quantification. However, heavy arginine is often metabolically converted into heavy proline ($^{13}\text{C}_5^{13}\text{N}_1$, Pro6, in case Arg10 was used for labeling) [3–8], which results in satellite peaks originating from peptides containing heavy proline that are generally not considered for quantification. Depending on the extent of arginine-to-proline conversion, this may significantly impair the accuracy of quantitative data. A frequently used strategy to counteract the generation of heavy proline is the addition of unlabeled proline to the culture medium [4, 8, 9]. A further requirement for accurate quantification in generic SILAC experiments is the complete incorporation of heavy arginine and lysine into the proteome, which may be compromised in cells or organisms that are prototrophic for these amino acids.

The baker's yeast *Saccharomyces cerevisiae* is widely accepted as eukaryotic model organism, and since many vital biological processes are conserved during evolution, knowledge gained in research using yeast is often transferable to the human system [10, 11]. Numerous SILAC-based proteomic studies employing *S. cerevisiae* greatly contributed to a better understanding of many fundamental cellular functions in eukaryotes. However, commonly used *S. cerevisiae* strains such as BY4741 [12] and its derivatives are prototrophic. Thus, SILAC studies usually rely on yeast in which arginine and lysine auxotrophies have been introduced through deletion of genes coding for enzymes of the arginine and lysine biosynthetic pathways to render the strains amenable for complete SILAC labeling.

The ease of working with *S. cerevisiae*, which includes a short generation time, easy cultivation in defined media under controlled conditions, and simplicity of genetic manipulation, has fueled the generation of several yeast mutant strain collections such as gene deletion strains [13, 14] and strains expressing genes fused to an epitope tag for tandem affinity purification [15, 16], a library of GFP fusion strains [17], and the SWAp-Tag libraries

[18, 19]. These strain collections are readily available to the scientific community and, thus, provide valuable resources of yeast mutants for global and targeted functional proteomic studies, but they are based on the strain BY4741 lacking the auxotrophies for arginine and lysine. Previous studies, however, showed that prototrophic yeast can efficiently be labeled with heavy lysine [20–23], a concept referred to as “native SILAC” (nSILAC) [21].

In this chapter, we describe a generic nSILAC strategy that allows for metabolic labeling of prototrophic yeast using both heavy lysine and arginine, thus exploiting the full potential of SILAC for accurate proteome quantification. We refer to this strategy as “complete native SILAC” or “2nSILAC” [8]. It allows for the utilization of yeast from the strain collections without further genetic manipulations for the introduction of auxotrophies. 2nSILAC works efficiently for cells grown on different carbon sources and is therefore well-suited to study a large variety of biological questions. It is compatible with protocols for the purification of organelles, subcellular fractions, protein complexes, or peptide fractions, making it a universal tool for the study of protein functions. We exemplarily describe the application of 2nSILAC for the analysis of mitochondrial gene deletion effects on the mitochondrial proteome. Thus, we here provide a protocol for the cultivation of yeast cells under respiratory growth conditions, which is generally used for the study of mitochondria in *S. cerevisiae*, and for the preparation of subcellular fractions enriched in mitochondria (Fig. 1).

2 Materials

Buffers and solutions described in this chapter should be prepared with ultrapure water ($\geq 18.2 \text{ M}\Omega \times \text{cm}$ resistivity, Milli-Q quality); reagents and solvents for LC-MS sample preparation and subsequent LC-MS analysis should be HPLC grade.

2.1 Metabolic Labeling of Prototrophic Yeast Using 2nSILAC

Isogenic *S. cerevisiae* strains that are prototrophic for arginine and lysine, such as BY4741 [12] and a BY4741 deletion strain [13] that lacks the gene encoding the mitochondrial protein of your interest (see Note 1).

2.1.1 Yeast Strains

2.1.2 Culture Media

1. YPD agar plates: 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar containing appropriate selection markers (if applicable).
2. Amino acid mix (12.5 $\times$): 125 mg/L adenine, 1000 mg/L L-aspartic acid, 250 mg/L L-histidine, 625 mg/L L-isoleucine, 1250 mg/L L-leucine, 250 mg/L L-methionine, 625 mg/L L-

Fig. 1 Workflow for the proteomic analysis of mitochondrial fractions by 2nSILAC. Pre-cultures of cells, inoculated at an OD₆₀₀ of 0.1, are cultivated overnight in medium containing light arginine (Arg0) and lysine (Lys0) or the corresponding isotope-coded heavy variants (Arg10, Lys8). Main cultures are inoculated at an OD₆₀₀ of 0.025 and grown until they reach exponential growth phase. Differentially SILAC-labeled cells are mixed in equal ratio and treated with DTT and Zymolyase to digest the cell walls. The resulting spheroplasts are homogenized followed by differential centrifugation to generate a crude mitochondrial fraction. Proteins are digested in solution using LysC and trypsin and analyzed by LC-MS/MS

phenylalanine, 1250 mg/L L-threonine, 625 mg/L L-tryptophan, 625 mg/L L-tyrosine, 250 mg/L uracil, 1750 mg/L L-valine.

3. Amino acids for stable isotope labeling: $^{12}\text{C}_6^{14}\text{N}_4$ -L-arginine (Arg0), $^{13}\text{C}_6^{15}\text{N}_4$ -L-arginine (Arg10), $^{12}\text{C}_6^{14}\text{N}_2$ -L-lysine (Lys0) and $^{12}\text{C}_6^{14}\text{N}_2$ -L-lysine (Lys8).
4. Synthetic complete (SC) medium: 0.17% (w/v) YNB without amino acids, 0.5% (w/v) ammonium sulfate, 3% (v/v) glycerol/0.02% (w/v) glucose (see **Notes 2** and **3**), 1× amino acid mix, pH adjusted to 5.5 with KOH; “light” medium is supplemented with 50 mg/L Arg0 and Lys0; “heavy” medium is supplemented with 50 mg/L Arg10 and Lys8 (see **Notes 4** and **5**).
5. Sterile water and sterile pipette tips.

6. Spectrophotometer to measure optical density at 600 nm (OD_{600}).

2.2 Preparation of Whole Cell Lysates

1. Lysis buffer: 8 M urea, 75 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA.
2. Screw cap microcentrifuge tubes (2 mL).
3. Glass beads (0.4–0.6 mm in diameter).
4. Bradford reagent, bovine serum albumin (BSA), and a spectrophotometer (or any alternative method) to determine protein concentrations.
5. Minilys[®] benchtop homogenizer (Peachlab/VWR).

2.3 Small-Scale Preparation of Mitochondria-Enriched Fractions

1. Dithiothreitol (DTT) buffer: 100 mM Tris-H₂SO₄ (pH 9.4), 10 mM DTT.
2. Zymolyase buffer: 20 mM potassium phosphate (pH 7.4), 1.2 M sorbitol.
3. Zymolyase[®] 20-T (MP Biomedicals Life Sciences).
4. Homogenization buffer: 10 mM Tris-HCl (pH 7.4), 0.6 M sorbitol, 1 mM EDTA, 1 mM PMSF.
5. SEM buffer: 250 mM sucrose, 1 mM EDTA, 10 mM MOPS (pH 7.2).
6. Thermomixer.
7. 3 mL syringe (e.g., Omnifix[®], Braun).
8. 0.8 × 22 mm blunt end needle (e.g., Sterican[®], Braun).
9. BSA and Bradford reagent to determine protein concentrations.
10. Liquid nitrogen.

2.4 LC-MS Sample Preparation

2.4.1 Acetone Precipitation

2.4.2 Proteolytic In-Solution Digest

1. Acetone (100%, ice-cold).
2. Urea buffer: 8 M urea in 50 mM NH₄HCO₃ (ABC).
1. 50 mM Tris(2-carboxy-ethyl)-phosphine (TCEP) in H₂O.
2. 500 mM iodoacetamide (IAA) or 2-chloroacetamide (CAA) dissolved in 50 mM ABC.
3. 100 mM DTT in H₂O.
4. LysC endoproteinase (Promega), 40 ng/μL dissolved in 50 mM ABC (*see Note 6*).
5. Sequencing grade modified trypsin (Promega), 20 ng/μL dissolved in 50 mM ABC.
6. 1% (v/v) trifluoroacetic acid (TFA).
7. 0.1% (v/v) TFA.

2.4.3 Desalting of Peptides

1. 100% methanol (MeOH).
2. Buffer A: 0.5% (v/v) acetic acid (AcOH).
3. Buffer B: 80% (v/v) acetonitrile (ACN)/0.5% (v/v) AcOH.
4. 200 μ L pipette tips.
5. Luer lock needle (gauge, G16; outer diameter, 1.7 mm).
6. C18 material (3 M Empore or Affinisep).
7. Glass vials with cap (CS-Chromatographie Service GmbH, Germany; article number 300 101), septa (CS-Chromatographie Service GmbH, Germany; article number 300 351), and inserts for LC analysis (Macherey-Nagel GmbH & Co. KG, Germany; article number 702 968).
8. Vacuum concentrator.

2.5 LC-MS/MS and Data Analysis

1. Solvent A: 0.1% (v/v) formic acid (FA).
2. Solvent B: 86% (v/v) ACN/0.1% (v/v) FA.
3. Nano UHPLC system (e.g., an UltiMate™ 3000 RSLCnano system; Thermo Fisher Scientific, Dreieich, Germany) equipped with a C18 pre-column (e.g., PepMap™ C18 pre-column; length, 5 mm; inner diameter, 0.3 mm; Thermo Scientific) for washing and preconcentration of peptides and an analytic C18 reversed-phase nano LC column (e.g., Acclaim™ PepMap™ RSLC column; length, 50 cm; inner diameter, 75 μ m; particle size, 2 μ m; Thermo Scientific) for peptide separation.
4. ESI-MS instrument (e.g., Q Exactive Plus mass spectrometer; Thermo Fisher Scientific, Bremen, Germany).
5. Software for protein identification and quantification (e.g., MaxQuant/Andromeda, www.maxquant.org) [24–26].
6. Protein sequence database for *S. cerevisiae* (see **Note 7**).
7. Software tools for further data processing (e.g., statistics, filtering, clustering, GO term enrichment, etc.) and data visualization such as Perseus [26], MATLAB (<https://www.mathworks.com/products/matlab.html>), or R (<https://www.r-project.org/>).

3 Methods

We here describe a sample preparation and analysis pipeline for the study of mitochondrial gene deletion effects on the mitochondrial proteome using 2nSILAC. Individual protocols include the cultivation of yeast cells under respiratory conditions, which are generally used for the study of mitochondria in *S. cerevisiae* (see **Note 8**); the preparation of mitochondria-enriched fractions; LC-MS sample

preparation, analysis, and data processing; the assessment of heavy arginine and lysine incorporation as well as arginine-to-proline conversion; and the analysis and visualization of SILAC-MS data obtained in replicate experiments.

3.1 Cultivation and Metabolic Labeling of Yeast Cells

1. Plate yeast strains (BY4741 control and gene deletion strain) on YPD agar plates (with or without selection markers, depending on the requirements of the yeast) and incubate for 2–3 days at 30 °C.
2. Prepare light and heavy SC medium and sterilize by autoclaving.
3. Use a sterile pipette tip to inoculate 1 mL of sterile water with cells from the agar plates and determine the OD₆₀₀ of the cell suspensions.
4. Use cell suspensions to prepare a pre-culture for each strain by inoculating 10 mL of light or heavy SC medium at an initial OD₆₀₀ of 0.1 (*see Note 9*).
5. Incubate pre-cultures overnight at 160 rpm and 30 °C.
6. Determine the OD₆₀₀ of the pre-cultures (*see Note 10*) and dilute them into 150 mL of fresh SC medium at an initial OD₆₀₀ of 0.025.
7. When the cells reach the exponential growth phase (OD₆₀₀ 0.5–1.5), take a 20 mL aliquot of the heavy-labeled cells to analyze the incorporation of Arg10 and Lys8 into the proteins and the extent of heavy arginine-to-proline conversion using whole cell lysates (*see Subheading 3.2*). Collect the cells by centrifugation for 5 min at 5000 × *g* and 4 °C. Remove the supernatant and proceed as described in Subheading 3.2.
8. Mix the remaining differentially labeled cells in equal ratio based on their OD₆₀₀.
9. Harvest cells by centrifugation for 5 min at 5000 × *g* and 4 °C. Remove the supernatant, store cells at –20 °C (if necessary), and proceed with the preparation of a mitochondrial fraction (Subheading 3.3).

3.2 Preparation of Whole Cell Lysates

When performing SILAC experiments, we routinely check the incorporation of the isotopically labeled amino acids and the extent of arginine-to-proline conversion. To this end, we prepare whole cell lysates of heavy labeled cells and analyze the proteins by LC-MS.

1. Resuspend the cell pellet (*see Subheading 3.1, step 7*) in 500 µL of lysis buffer, transfer the sample to a 2-mL screw cap microcentrifuge tube, and add 300 mg of glass beads.
2. Disrupt and lyse the cells by bead beating using a Minilys[®] homogenizer (*see Note 11*) for 4 min at 5000 rpm. Perform

this step twice with at least 2 min cooling on ice between the cycles.

3. Remove cell debris and glass beads by centrifugation for 5 min at $15,000 \times g$ and 4°C .
4. Determine the protein concentration, e.g., by using the Bradford assay [27] with BSA as protein standard according to the standard protocol.
5. Adjust the final protein concentration to $1\text{ }\mu\text{g}/\mu\text{L}$ using lysis buffer for subsequent proteolytic in-solution digest (Subheading 3.4.2).

3.3 Small-Scale Preparation of Mitochondria-Enriched Fractions

We here describe a protocol for a small-scale preparation of mitochondria-enriched fractions [8], which is based on a medium-scale protocol published recently [28]. Briefly, cell walls are removed by treating cells with DTT and Zymolyase, a mixture of lytic enzymes. The resulting spheroplasts are homogenized using a syringe and mitochondria are enriched via differential centrifugation (*see* **Notes 12** and **13**).

1. Resuspend cells (*see* Subheading 3.1, **step 9**) in 1.5 mL of DTT buffer and transfer them to a 2 mL microcentrifuge tube.
2. Incubate cells for 10 min at 1000 rpm and 30°C using a thermomixer.
3. Pellet cells by centrifugation for 5 min at $1500 \times g$, discard supernatant, and wash cells with 1.5 mL of Zymolyase buffer.
4. Centrifuge for 5 min at $1500 \times g$, discard supernatant, and resuspend the pellet in 1.5 mL of Zymolyase buffer containing 4 mg/mL Zymolyase 20-T.
5. Incubate for 30 min at 1000 rpm and 30°C in a thermomixer.
6. Harvest spheroplasts by centrifugation for 5 min at $900 \times g$ and 4°C (*see* **Note 14**).
7. Resuspend pellet in 1.5 mL of ice-cold homogenization buffer and homogenize the spheroplasts by drawing the sample 20 times up and down through a 0.8×22 mm blunt end needle attached to a 3-mL syringe.
8. Remove cell debris by centrifugation for 10 min at $900 \times g$ and 4°C .
9. Transfer supernatant to a new microcentrifuge tube.
10. Take a small aliquot (5%) of the supernatant (S0.9) for immunoblot analysis (Fig. 3a).
11. Resuspend the pellet of **step 8** in 1.5 mL of ice-cold homogenization buffer and take an equal aliquot of 5% of the pellet fraction (P0.9) for immunoblot analysis (Fig. 3a).
12. Proceed with the supernatant from **step 9** and repeat **step 8**.

13. Transfer the supernatant to a new microcentrifuge tube and pellet mitochondria by centrifugation for 5 min at $16,800 \times g$ and 4°C . Take an aliquot (5%) of the supernatant (S16.8) for immunoblot blot analysis (Fig. 3a).
14. Wash the mitochondria-containing pellet with 2 mL of ice-cold SEM buffer and centrifuge again for 10 min at $900 \times g$ and 4°C .
15. Transfer the supernatant to a new microcentrifuge tube and centrifuge for 5 min at $16,800 \times g$ and 4°C .
16. Resuspend the mitochondrial pellet in 200 μL of ice-cold SEM buffer and determine the protein concentration, e.g., by using the Bradford assay. Take an aliquot (5%) of the mitochondria-enriched fraction (Mito) for immunoblot analysis (Fig. 3a).
17. Mitochondria-enriched fractions can either directly be used for LC-MS analysis or snap-frozen in liquid nitrogen and stored at -80°C until further use.

3.4 LC-MS Sample Preparation

3.4.1 Acetone Precipitation

1. Precipitate proteins of mitochondria-enriched fractions by adding the fivefold volume of ice-cold acetone.
2. Incubate samples at -20°C for at least 2 h.
3. Pellet precipitated proteins by centrifugation for 15 min at $15,000 \times g$ and 4°C .
4. Remove acetone and dry samples for 5 min under the laminar flow hood.
5. Resuspend proteins in urea buffer (1 $\mu\text{g}/\mu\text{L}$ final concentration) for subsequent proteolytic in-solution digest.

3.4.2 Proteolytic In-Solution Digest

The protocol for proteolytic in-solution digestion of whole cell lysates or mitochondria-enriched fractions is described for 10 μg of protein (*see* **Note 15**).

1. Add 1 μL of 50 mM TCEP and incubate for 30 min at room temperature to reduce cysteine residues (*see* **Note 16**).
2. Add 1 μL of 500 mM IAA or CAA and incubate for 30 min at room temperature to alkylate free thiol groups. When IAA is used, incubate samples in the dark.
3. Quench the alkylation reaction by adding 3 μL of 100 mM DTT.
4. For protein digestion with LysC, dilute samples to 4 M urea using 50 mM ABC, add 100 ng of LysC, and incubate for 4 h at 37°C (*see* **Note 17**).
5. For digestion with trypsin, dilute the samples further to 1.8 M urea using 50 mM ABC, add 200 ng of trypsin, and incubate overnight at 37°C .

6. To inactivate the proteases, acidify the samples by adding 45 μL of 1% TFA. Use pH paper to make sure that the samples are acidified ($\text{pH} < 3.0$).
7. Remove insoluble material by centrifugation at $13,800 \times g$ for 5 min and transfer supernatant to a new microcentrifuge tube.

3.4.3 Desalting of Peptides

StageTips can be used to remove urea, salts, and other contaminants prior to MS analysis. The method we describe is adapted from Rappsilber and colleagues [29]. In brief, monolithic C18 material is inserted into 200 μL pipette tips and is used to capture and wash proteolytic peptides. Afterward, peptides are eluted, dried in vacuo and are ready for LC-MS/MS analysis.

1. To prepare StageTips, cut out three disks of C18 matrix with a Luer lock needle and insert them into a 200 μL pipette tip (*see Note 18*). Gently push the disks toward the end of the tip without applying too much pressure.
2. Insert the StageTips into microcentrifuge tubes with a hole in the lid or use appropriate adapters to fix the StageTips in the microcentrifuge tubes.
3. Activate StageTips by adding 100 μL of MeOH and centrifuge at $800 \times g$ until StageTips are empty.
4. Wash StageTips by adding 100 μL of buffer B and centrifuge at $800 \times g$ until empty.
5. Equilibrate StageTips by adding 100 μL of buffer A and centrifuge at $800 \times g$ until empty.
6. Load the acidified samples onto the StageTips and centrifuge at $800 \times g$ until empty.
7. Add 100 μL of buffer A for washing and centrifuge at $800 \times g$ until empty. Perform this step twice (*see Note 19*).
8. Place glass inserts into microcentrifuge tubes and insert StageTips into the glass vials.
9. Elute peptides by adding 30 μL of buffer B and centrifuge at $800 \times g$ until empty.
10. Repeat **step 9**, combine eluates, and dry peptides in vacuo using a vacuum concentrator.

3.5 LC-MS/MS and Data Analysis

Peptide mixtures are analyzed by nanoflow LC-MS/MS using an UltiMate™ 3000 RSLCnano system coupled to a Q Exactive Plus mass spectrometer.

1. Place the glass inserts containing the peptide mixtures into glass vials and seal the vial with septa and caps.

2. Load the peptide mixtures onto the pre-column of the RSLCnano system and wash and preconcentrate peptides with solvent A for 5 min at a flow rate of 30 $\mu\text{L}/\text{min}$.
3. Switch pre-column and analytical column in line and elute peptides by applying the following gradient at a flow rate of 0.3 $\mu\text{L}/\text{min}$: 4% solvent B for 25 min, 4–39% B in 25 min, 39–54% B in 175 min, 54–95% B in 15 min, and 5 min at 95% B (*see Note 20*).
4. Use the following parameters to operate the Q Exactive Plus: MS scans, m/z 375–1700 at a resolution of 70,000 at m/z 200; automatic gain control, 3×10^6 ; maximum ion time, 60 ms.
5. Apply a TOP15 method for fragmentation of precursor ions ($z \geq +2$) by higher-energy collisional dissociation. Set the normalized collision energy to 28%. Acquire fragment ion spectra at a resolution of 35,000. Set automatic gain control for MS/MS scans to 10^5 ions and the maximum ion time to 120 ms. Use a dynamic exclusion time for previously selected precursor ions of 45 s.
6. Download the latest version of the MaxQuant/Andromeda software package for protein identification and quantification (*see Note 21*).
7. To evaluate labeling efficiencies for Arg10 and Lys8 as well as the extent of heavy arginine-to-proline conversion in whole cell lysates of cells grown in heavy SC medium (*see Subheading 3.2*), use the default settings in MaxQuant (refers to version 1.6.5.0) for protein identification and the required settings for SILAC-based quantification with the following exceptions: disable the option “requantify” (*see Note 22*) and add Pro6 as variable modification.

The incorporation efficiency of Arg10 and Lys8 is calculated for individual peptides using the following equation [30]:

$$\text{Incorporation (\%)} = \frac{\text{ratio (H/L)}}{\text{ratio (H/L)} + 1} * 100$$

The degree of incorporation needs to be calculated separately for Arg10- and Lys8-containing peptides since the yeast strain(s) used may exhibit differences in incorporation for heavy arginine and lysine. Use the MaxQuant output file “evidence.txt”. Remove entries derived from the reverse and contaminant databases. Select only peptides that contain either lysine or arginine and use the non-normalized H/L ratios. For peptides showing no or complete incorporation of Arg10 or Lys8, H/L ratios cannot be computed since the corresponding heavy or light counterpart is missing. In these cases, the incorporation is manually set to 0% for no

incorporation or 100% for complete incorporation. To obtain relative quantitative data of high accuracy, the mean incorporation across all peptides should be >95% for both arginine- and lysine-containing peptides. To assess arginine-to-proline conversion, calculate (i) the percentage of Pro6-containing peptides in relation to all identified peptides or (ii) the relative intensity of Pro6-containing peptides (in %) in relation to the summed MS intensity of all peptides per dataset (*see Note 23*).

8. To analyze MS data of 2nSILAC experiments, use MaxQuant default settings for protein identification and SILAC-based quantification. Enable the options “requantify” (*see Note 22*) and “match between runs” when analyzing replicates.
9. Use the MaxQuant “proteinGroups.txt” output file for the quantitative analysis of 2nSILAC data. Remove the hits derived from contaminant and reverse database and discard entries that were only identified by site. In case the experimental setup included label switches, adjust the protein ratio H/L by inverting the respective values. Logarithmize the protein abundance ratio ($\log_2$) for each replicate to obtain normally distributed data for statistical tests. The reproducibility between individual replicates can be assessed by ratio-versus-ratio plots and calculation of the Pearson correlation. To analyze which protein groups show significant changes in abundance between the two populations of cells/mitochondria, perform a two-sided one-sample *t*-test with a hypothetical mean value of 0. Data can be visualized by plotting the mean log ratios against the $\log_{10}$ of the *p*-values (Fig. 3b). In addition, intensity-dependent and independent outlier tests can be performed to identify significant outliers relative to the overall population (e.g., significance A or B [24]).

4 Notes

1. BY4741 and derivatives thereof are available from Euroscarf (<http://www.euroscarf.de>). When using different strains than BY4741, make sure that the genotype is compatible with arginine and lysine labeling [8].
2. Growth on respiratory carbon sources such as glycerol induces mitochondrial proliferation and results in higher yields of mitochondria. Addition of 0.02% (w/v) glucose allows for reproducible cell cultivation without affecting the yield.
3. Depending on the experiment, alternative carbon sources such as galactose or glucose may be used. Utilization of galactose results in a slightly lower yield of mitochondria. When cells are grown under respiratory growth conditions using glycerol/

glucose or galactose, we recommend to use the larger set of amino acids described in this section to ensure complete incorporation of heavy arginine and lysine. For the growth of cells under fermentative conditions using glucose, a minimal set of essential amino acids is sufficient (20× amino acid mix: adenine, L-histidine, L-leucine, L-methionine, L-tryptophan, and uracil, 400 mg/L each).

4. Unlabeled proline (Pro0) may need to be added to the medium to prevent arginine-to-proline conversion. The appropriate concentration of Pro0 should be determined experimentally. Add the same amount of Pro0 to both light and heavy SC medium.
5. In case a different growth medium is used, make sure to omit external sources of light arginine and lysine (e.g., yeast extract) from the medium.
6. LysC is proteolytically active in buffer containing high concentrations of urea and is often used in combination with trypsin to increase the efficiency of protein digestion.
7. A sequence database specific for *S. cerevisiae* is included in the MaxQuant software package; alternatively, it can be downloaded from the *Saccharomyces* Genome Database (<https://downloads.yeastgenome.org/sequence/>).
8. When adapting the 2nSLAC strategy to different growth conditions, pilot experiments need to be performed, and culture conditions may need to be adjusted to ensure high incorporation efficiencies for heavy arginine and lysine as well as minimum extents of arginine-to-proline conversion.
9. To obtain quantitative data of high reliability, we strongly recommend to perform at least three biological replicates including a light/heavy label switch experiment to prevent artifacts introduced by the labeling scheme.
10. For 2nSILAC experiments, it is crucial that the OD₆₀₀ of pre-cultures does not exceed a value of 4. Higher cell densities may result in insufficient incorporation of the heavy amino acids (Fig. 2) and an increase in arginine-to-proline conversion [8].
11. Alternatively, cells can be disrupted using a vortex mixer.
12. Please note that mitochondria-enriched fractions prepared according to the protocol we describe here still contain small amounts of other organelles such as the ER and Golgi (Fig. 3a). To obtain mitochondrial fractions of higher purity (>90%), mitochondria-enriched fractions can be further purified by sucrose density gradient centrifugation as described elsewhere [28, 31].

Fig. 2 Incorporation of isotope-coded arginine and lysine into proteins of prototrophic yeast. Prototrophic yeast was cultured in medium containing heavy arginine and lysine. Pre-cultures were grown up to an OD₆₀₀ of 5.5 (a) or 3.5 (b). Corresponding main cultures were cultivated until they reached an OD₆₀₀ of 4.0. As shown for the entirety of arginine- and lysine-containing peptides (a and b, top) and exemplarily for the arginine-containing peptide YVDPNVLPETESLALVIDR (a and b, bottom), peptides from cells that were pre-cultured to a high density exhibit incomplete incorporation of heavy amino acids, in particular heavy arginine, at early time points (e.g., at OD₆₀₀ of 0.5). In contrast, peptides from cells that were pre-cultured to a lower density show virtually complete incorporation (>98.5%) of both heavy arginine and lysine. L/H, isotope pattern of the light/heavy peptide species; m/z, mass-to-charge ratio. Data shown in this figure are taken from Dannenmaier et al. (2018) [8] (<https://pubs.acs.org/doi/10.1021/acs.analchem.8b02557>). Permissions related to this material need to be directed to ACS Publications

13. To evaluate the quality of the mitochondrial fraction, we recommend to monitor the enrichment of the mitochondria during individual steps of the differential centrifugation. To this end, take aliquots of samples at critical steps during the preparation (as indicated in the protocol) and analyze them by SDS-PAGE and immunoblotting using antisera against marker proteins for mitochondria and selected other subcellular compartments (Fig. 3a).
14. Suspensions of spheroplasts need to be handled gently; cut off pipette tips to prevent disrupting the organelles.
15. For deep proteome coverage, sample fractionation on protein or peptide level such as SDS-PAGE, strong cation exchange chromatography, high pH reversed-phase chromatography, or other alternatives can be integrated into the workflow.

Fig. 3 Analysis of mitochondrial gene deletion effects on the mitochondrial proteome using 2nSILAC. (a) A mitochondria-enriched fraction (Mito) was generated by differential centrifugation. Equal aliquots of samples were analyzed by SDS-PAGE and immunoblotting using antibodies against marker proteins for different organelles as indicated. The mitochondria-enriched fraction was virtually devoid of Adh1/2 and Hhf1/2, but it still contained a significant amount of the ER protein Pdi1 (see **Note 12**). P/S0.9, pellet/supernatant of the second $900 \times g$ centrifugation; S16.8, supernatant of the $16,800 \times g$ centrifugation. (b) Mitochondrial proteomes of wild-type (WT) cells and cells lacking the gene *SDH5* (*sdh5*Δ) coding for an assembly factor of the succinate dehydrogenase complex (SDH; complex II of the mitochondrial respiratory chain) are quantitatively compared following the 2nSILAC strategy as described in Fig. 1. The experiment was performed in three independent replicates, and data are visualized by plotting the mean log₂ of normalized (norm.) *sdh5*Δ/WT ratios of proteins quantified in at least two replicates against the negative log₁₀ of their *p*-values determined in a two-sided Student's *t*-test. In addition, an intensity-based outlier *t*-test (Significance B) was performed. Proteins considered to be significantly affected by *SDH5* deletion were required to have a *p*-value of <0.05 in both the Student's *t*-test and the outlier analysis (indicated by large filled circles). The 2nSILAC study shows that deletion of the assembly factor SDH5 leads to a strong reduction of the levels of all four core components of the SDH complex (i.e., Sdh1-4). Data shown in this figure are taken from Dannenmaier et al. (2018) [8] (<https://pubs.acs.org/doi/10.1021/acs.analchem.8b02557>). Permissions related to this material need to be directed to ACS Publications

16. Avoid heating the samples since this may lead to carbamylation of lysine residues by the urea present in the buffer in high concentration (i.e., 8 M).
17. Protein digestion with LysC may be omitted. However, we recommend to perform double digestion with both LysC and trypsin, as it improves proteome coverage and accurate relative quantification [8, 32].
18. The estimated binding capacity per disk is 2–4 μg. Make sure not to over- or underpack as this may lead to clogging or loss of sample.

19. After this step, StageTips can be stored at 4 °C for several weeks. In this case, peptides need to be rehydrated by adding 100 µL of buffer A before continuing with the elution.
20. Depending on the complexity of the sample and the analytical column used for peptide separation, slope and duration of the gradient may need to be adjusted to identify maximum numbers of peptides and proteins.
21. Useful information about MaxQuant including computational requirements is provided in literature [24, 25, 33–35] and on the MaxQuant documentation website. We further suggest to subscribe to the MaxQuant Google group for additional information, recommendations, advice for troubleshooting, and for posting questions.
22. The option “requantify” generally allows for the calculation of SILAC ratios in case peptides are only present in the isotope-labeled (i.e., heavy) or unlabeled (light) form by assigning a peptide intensity for the missing counterpart from signals in MS spectra at the m/z value expected for the missing counterpart [33]. This feature is of advantage when high ratios are expected, e.g., in gene deletion studies or in affinity purification MS experiments. However, “requantify” should not be applied to analyze the incorporation of heavy amino acids into peptides/proteins since this would result in incorrect H/L ratios.
23. Please note that the calculation of arginine-to-proline conversion based on identified peptides generally underestimates the occurrence of the conversion since only sequenced Pro6-containing peptides are taken into account.

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References

1. Ong S-E, Blagoev B, Kratchmarova I et al (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* 1:376–386
2. Lau H-T, Suh HW, Golkowski M et al (2014) Comparing SILAC- and stable isotope dimethyl-labeling approaches for quantitative proteomics. *J Proteome Res* 13:4164–4174
3. Ong S-E, Kratchmarova I, Mann M (2003) Properties of ^{13}C -substituted arginine in stable isotope labeling by amino acids in cell culture (SILAC). *J Proteome Res* 2:173–181

4. Bendall SC, Hughes C, Stewart MH et al (2008) Prevention of amino acid conversion in SILAC experiments with embryonic stem cells. *Mol Cell Proteomics* 7:1587–1597
5. Bicho CC, de Lima Alves F, Chen ZA et al (2010) A genetic engineering solution to the “arginine conversion problem” in stable isotope labeling by amino acids in cell culture (SILAC). *Mol Cell Proteomics* 9:1567–1577
6. Park SK, Liao L, Kim JY et al (2009) A computational approach to correct arginine-to-proline conversion in quantitative proteomics. *Nat Methods* 6:184–185
7. Lössner C, Warnken U, Pscherer A et al (2011) Preventing arginine-to-proline conversion in a cell-line-independent manner during cell cultivation under stable isotope labeling by amino acids in cell culture (SILAC) conditions. *Anal Biochem* 412:123–125
8. Dannenmaier S, Stiller SB, Morgenstern M et al (2018) Complete native stable isotope labeling by amino acids of *Saccharomyces cerevisiae* for global proteomic analysis. *Anal Chem* 90:10,501–10,509
9. Piechura H, Oeljeklaus S, Warscheid B (2012) SILAC for the study of mammalian cell lines and yeast protein complexes. *Methods Mol Biol* 893:201–221
10. Kolkman A, Slijper M, Heck AJR (2005) Development and application of proteomics technologies in *Saccharomyces cerevisiae*. *Trends Biotechnol* 23:598–604
11. Botstein D, Fink GR (2011) Yeast: an experimental organism for 21st century biology. *Genetics* 189:695–704
12. Brachmann CB, Davies A, Cost GJ et al (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14:115–132
13. Giaever G, Chu AM, Ni L et al (2002) Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* 418:387–391
14. Giaever G, Nislow C (2014) The yeast deletion collection: a decade of functional genomics. *Genetics* 197:451–465
15. Gavin AC, Bösch M, Krause R et al (2002) Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* 415:141–147
16. Ghaemmaghami S, Huh W-K, Bower K et al (2003) Global analysis of protein expression in yeast. *Nature* 425:737–741
17. Huh W-K, Falvo JV, Gerke LC et al (2003) Global analysis of protein localization in budding yeast. *Nature* 425:686–691
18. Yofe I, Weill U, Meurer M et al (2016) One library to make them all: streamlining the creation of yeast libraries via a SWAp-Tag strategy. *Nat Methods* 13:371–378
19. Weill U, Yofe I, Sass E et al (2018) Genome-wide SWAp-Tag yeast libraries for proteome exploration. *Nat Methods* 15:617–622
20. Dilworth DJ, Saleem RA, Rogers RS et al (2010) QTIPS: a novel method of unsupervised determination of isotopic amino acid distribution in SILAC experiments. *J Am Soc Mass Spectrom* 21:1417–1422
21. Fröhlich F, Christiano R, Walther TC (2013) Native SILAC: metabolic labeling of proteins in prototroph microorganisms based on lysine synthesis regulation. *Mol Cell Proteomics* 12:1995–2005
22. Kaneva IN, Longworth J, Sudbery PE et al (2018) Quantitative proteomic analysis in *Candida albicans* using SILAC-based mass spectrometry. *Proteomics* 18:1700278
23. Christiano R, Nagaraj N, Fröhlich F et al (2014) Global proteome turnover analyses of the Yeasts *S. cerevisiae* and *S. pombe*. *Cell Rep* 9:1959–1965
24. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 26:1367–1372
25. Cox J, Neuhauser N, Michalski A et al (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res* 10:1794–1805
26. Tyanova S, Temu T, Sinitcyn P et al (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* 13:731–740
27. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
28. Morgenstern M, Stiller SB, Lübbert P et al (2017) Definition of a high-confidence mitochondrial proteome at quantitative scale. *Cell Rep* 19:2836–2852
29. Rappsilber J, Ishihama Y, Mann M (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal Chem* 75:663–670
30. Oeljeklaus S, Schummer A, Suppanz I et al (2014) SILAC labeling of yeast for the study of membrane protein complexes. *Methods Mol Biol* 1188:23–46

31. Meisinger C, Sommer T, Pfanner N (2000) Purification of *Saccharomyces cerevisiae* mitochondria devoid of microsomal and cytosolic contaminations. *Anal Biochem* 287:339–342
32. Glatzer T, Ludwig C, Ahrné E et al (2012) Large-scale quantitative assessment of different in-solution protein digestion protocols reveals superior cleavage efficiency of tandem Lys-C/trypsin proteolysis over trypsin digestion. *J Proteome Res* 11:5145–5156
33. Tyanova S, Temu T, Cox J (2016) The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat Protoc* 11:2301–2319
34. Cox J, Matic I, Hilger M et al (2009) A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nat Protoc* 4:698–705
35. Tyanova S, Mann M, Cox J (2014) MaxQuant for in-depth analysis of large SILAC datasets. *Methods Mol Biol* 1188:351–364

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